Antifungals of *Acromyrmex*, *Allomerus*, and *Tetraponera* ant- and cultivar-associated bacteria

Jörg Barke

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School of Biological Sciences,
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Abstract

The central purpose of this thesis is to test the utility of ant-microbe associations for discovering antifungal compounds with novel molecular (sub-) structures. Novel antifungals displaying reduced adverse side-effects, increased water-solubilities, and/or strong fungicidal properties would be helpful in medical science for responding to the rising prevalence of human mycoses and for solving problems with adverse side-effects in currently used antifungal drugs. Host-symbiont systems may represent a source of bacterial species that, due to their co-evolved association with hosts, have evolved antifungals differing in their molecular structures from antifungals of well-explored environmental sources.

To test the suitability of ant-microbe symbioses for discovering novel antifungals, I characterise ectosymbiotic bacteria and antifungal metabolites in three ant-associated niches: *Acromyrmex octospinosus* worker ants, fungal cultivars of *Allomerus decemarticulatus* and *A. octoarticulatus*, and *Tetraponera penzigi* worker ants and their putative fungal cultivar. I find that the *A. octospinosus* and *T. penzigi* niches hosted multiple bioactive actinobacteria including *Streptomyces*, *Pseudonocardia*, *Nocardiopsis*, and *Saccharopolyspora*.

A *Streptomyces* strain from the *Acromyrmex octospinosus* niche was shown to produce the structurally unmodified antifungals candicidin and antimycin, which is suggestive of bacterial recruitment from environmental sources. Antimycin consisted of two sets of six molecules varying in number of attached CH_2 groups. Both sets differ in polarity and fragmentation pattern. Only one set of antimycins inhibited the growth of *Candida albicans*, and this set revealed tandem-LCMS analogies with an antimycin A_1-A_4 standard.

A *Pseudonocardia* strain from the same *A. octospinosus* niche was found to produce a partially novel (to science) and potentially water-soluble nystatin-like antifungal, which is suggestive of host-symbiont coevolution. These findings indicate that the host ant is able to use multiple-drug therapy in the form of antifungal ‘cocktails’ to tackle resistance evolution in fungal pathogens.

Overall, this research demonstrates that ant-microbe associations provide new opportunities for finding abundant bioactive bacteria and novel antibiotics.

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Abbreviations

%	Percent
2G	20% Glycerol
°C	Degree Celsius
ACN	Acetonitrile
AD	*Allomerus decemarticulatus*
amu	Atomic mass unit
AO	*Allomerus octoarticulatus*
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BPC	Base peak chromatogram
BuOH	Butanol
CAS	Chemical Abstracts Service
CBS	Centraalbureau voor Schimmelcultures
cm	Centimetre
CN	*Cordia nodosa*
dH_2O	Distilled water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP's	Deoxyribonucleotides
EA	*Escovopsis aspergilloides*
EB buffer	QIAGEN elution buffer
EIC	Extracted ion chromatogram
EWB	*Escovopsis weberi* from Brazil
EWC	*Escovopsis weberi* from Colombia
EtAc	Ethyl acetate
EtOH	Ethanol
g	Gram
H^+	Hydrogen ion

HC	Hydrolysed chitin
HP	*Hirtella physophora*
HPLC	High-Performance Liquid Chromatography
Hz	Hertz
IFR	Institute for Food Research
JIC	John Innes Centre
kb	Kilobase
kV	Kilovolt
LB	Lennox broth
LCMS	Liquid Chromatography Mass Spectrometry
$[M+H]^+$	Hydrogen adduct in positive ion mode
$[M-H]^-$	Hydrogen adduct in negative ion mode
mAU	Milli Absorbance Units
MeOH	Methanol
mg	Milligram
ml	Millilitre
mm	Millimetre
mM	Millimolar
m/s	Metre per second
MS	Mass Spectrometry on parent ions
MS	Mannitol soy-flour
MS^2	Mass Spectrometry on daughter ions
µm	Micrometer
µl	Microlitre
µl/min	Microlitre per minute
NCBI	National Center for Biotechnology Information
nm	Nanometre
NNSPS	Selection of actinobacteria in the genera *Nocardia*, *Nocardiopsis*, *Pseudonocardia*, *Saccharopolyspora*, and *Streptomyces*
NP	Normal-phase

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDA	Photodiode array
PDA	Potato dextrose agar
PE buffer	QIAGEN washing buffer
QG buffer	QIAGEN solubilization and binding buffer
RP	Reversed-phase
rpm	Revolutions per minute
RT	Retention time
s.e.	Standard error
TBE	Tris/Borate/EDTA buffer
TGE	Tris/Glycine/EDTA buffer
TIC	Total ion chromatogram
TLC	Thin Layer Chromatography
TSB	Tryptone soya broth
TSB/YEME	Mixture of TSB and YEME (1:1)
UEA	University of East Anglia
UTC	Universal Time Coordinated
V	Volt
YEME	Yeast extract-malt extract

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Chapter 1) Introduction

1.1 Antifungals and their application

Only a small proportion of fungal genera includes species that are pathogenic to humans, animals, and plants (De Lucca 2007, Richardson 2003). However, these species impose a large and increasing burden to society by harming the health of humans, their domestic animals, and their crops. This burden can be quantified by the prevalence of fungal infections (mycoses) in humans. For example, Slavin *et al.* (2004) studied the occurrence in Australia of life-threatening human mycoses caused by opportunistic fungi in the genera *Aspergillus* and *Candida*. In contrast to primary fungal pathogens, which attack healthy humans, opportunistic fungal pathogens attack weakened hosts, especially those that are immunocompromised (e.g. AIDS) or very ill (e.g. cancer) (Slavin *et al.* 2004). Surveying data of the National Hospital Morbidity Database, between 1995-1999, the authors found that the total cost of hospitalisation during the study period of patients diagnosed with aspergillosis was AUS$ 42.8 million (equals £ 26.9 or € 33.6 million; calculated May 30, 2012, 10:03 UTC) and of patients with candidiasis was AUS$ 520.5 million (equals £ 326.5 or € 408.6 million; calculated May 30, 2012, 10:03 UTC). The number of people affected by these diseases is on the rise; for example, over the study period the annual growth of hospitalisation due to aspergilloides and candidiases was 13.8% and 3.5%, respectively.

One important and widely used clinical intervention for lowering the economic and human burden of mycoses is the application of antifungal substances. However, the set of currently used antifungal compounds suffers from several problems: difficulty of application, pathogen resistance, and adverse side-effects in patients. Therefore, there is a need to discover new antifungal antibiotics. The goal of this thesis is to assess the utility of a novel niche, ant-microbe associations, for the discovery of novel antifungals. In the introduction, I first briefly review mechanisms of antifungal action, fungal resistance mechanisms to antifungals, and some of the problems with currently

used antifungals, which together demonstrate why we need to find new antifungal compounds. I then briefly relate some of the history of antifungal prospecting and the use of insects as novel niches for antifungal discovery. Finally, I outline the plan of my thesis.

1.1.1 Mechanisms of antifungal action

As suggested by their name, antifungals are antibiotics that disrupt vital functions in fungal cells, possessing fungistatic (growth inhibiting) and/or fungicidal (killing) properties. Such substances are used for the treatment of fungal infections in humans. Antifungal drugs can be characterised by their mechanism of action on the fungal pathogen, which describes how the antifungals inhibit fungal cells on a molecular level. The molecular targets of some medically important antifungal classes include RNA/DNA, the cell wall, and the cell membrane.

Molecular target: RNA/DNA. 5-fluorocytosine is an antifungal substance that develops its potency when entering fungal cells and being transformed into 5-fluorouracil and the follow-up metabolites 5-fluorouridine triphosphate and 5-fluorodeoxyuridine monophosphate (Waldorf and Polak 1983, Polak 1990, Georgopapadakou 1998, Odds *et al*. 2003). These processes are mediated by the enzymes cytosine permease, cytosine deaminase, and uracil phosphoribosyl transferase. While 5-fluorouridine triphosphate is incorporated into fungal RNA, where it interferes with protein synthesis, and 5-fluorodeoxyuridine monophosphate inhibits thymidylate synthase, which disrupts DNA synthesis.

Molecular target: the cell wall. Fungal cell walls, among others, consist of (1,3)-β-glucan and chitin, of which the first forms the main component and the second is thought to increase stability (Levitz 2010). Echinocandins and nikkomycins are classes of antifungals that interfere with cell wall synthesis. Echinocandins interrupt the functioning of (1,3)-β-glucan synthase that polymerises uridine diphosphate glucose molecules to β-glucan, which leads to osmotic lysis (Georgopapadakou 1998, Kontoyiannis and Lewis 2002).

Nikkomycins inhibit chitin synthase (i.e. Chs3) that polymerises UDP-N-acetylglucosamine to chitin, leading to a weakening of the fungal cell wall and death (Georgopapadakou 1998).

Molecular target: the cell membrane. The phospholipid bilayer of the fungal cell membrane contains ergosterol, which is the fungal equivalent of cholesterol in human cells. Various classes of antifungals, including allylamines, azoles, morpholines, and polyenes affect different stages of the ergosterol biosynthesis and membrane functioning. For example, the polyene antifungals amphotericin B, nystatin, and candicidin target ergosterol molecules in fungal cells. Like all phospholipids, the chemical structure of these antifungal substances has a polar 'head' and an apolar 'tail'; this allows them to bind ergosterol hydrophobically, integrate into the membrane bilayer, and form membrane-spanning pores that increase membrane permeability, and thereby reduce fungal survival (Gray *et al*. 2012). Compounds of the remaining classes interfere with various enzymes involved in ergosterol biosynthesis (e.g. squalene epoxidase, lanosterol-14α demethylase, Δ^{7-8} isomerase), processing the acetyl-coenzyme A-derived intermediate squalene to lanosterol and ultimately to ergosterol (Georgopapadakou 1998).

1.1.2 Resistance mechanisms to antifungals

Despite these multiple modes of antifungal action, and because fungal pathogens are diverse and continuously evolving, many pathogens show innate (primary) or acquired (secondary) resistance to known antifungal compounds.

Resistance to 5-fluorocytosine. Filamentous fungi often lack enzymes responsible for the uptake and processing of 5-fluorocytosine (Odds *et al*. 2003). This makes them innately resistant to this antifungal since the active metabolites 5-fluorouridine triphosphate and 5-fluorodeoxyuridine monophosphate, which disrupt protein and DNA synthesis, are not formed. The yeast *Candida albicans* belongs to a set of organisms that is susceptible to 5-fluorocytosine but can acquire resistance when developing defects in the

enzyme uracil phosphoribosyl transferase, which is involved in the processing of 5-fluorocytosine (Vanden Bossche 1997).

Resistance to echinocandins. The strength of the echinocandin antifungal caspofungin varies across fungal species; for example, it shows a strong inhibitory activity against many *Candida* species, slows down the growth of *Aspergillus* spp., and is inactive against *Cryptococcus neoformans* (Hector 2005). The resistance of certain *Candida albicans* strains towards this antifungal likely stems from a mutation in the gene encoding the (1,3)-β-D-glucan synthase (Baixench *et al*. 2007), which produces glucan, the main component of fungal cell walls.

Resistance to polyenes and azoles. Fungi with an acquired resistance to the polyene antifungal amphotericin B are uncommon, since the antifungal is potently disrupting cell functioning without having to enter fungal cells; nevertheless, polyene resistance in *Candida*, among others, derives from mutations in ergosterol biosynthesis genes resulting in an alteration of the ergosterol content in fungal membranes (reviewed in: Masia Canuto and Gutierrez Rodero 2002, Kontoyiannis and Lewis 2002), and thus a diminished potential for pore formation. Interestingly, treatment of fungal pathogens with azoles reduces the concentration of membrane-bound ergosterol, and hence the susceptibility to polyenes.

Candida spp. that are resistant to azoles have been found to exhibit multiple resistance mechanisms (reviewed in Masia Canuto and Gutierrez Rodero 2002). These include mutation and/or overexpression of the gene encoding lanosterol-14α demethylase, which is a molecular target of azoles, as well as up-regulation of multidrug efflux transporter genes and reduction of fungal membrane permeability to reduce the accumulation of antifungal molecules within fungal cells.

1.1.3 Problems with currently used antifungals

Not only can fungal pathogens evolve resistance to antifungal compounds, it is inherently difficult to treat fungal infections because many antifungals are

weakly absorbed by the host body, metabolised by the host to inactive molecules, and/or irregularly distributed over body tissues (reviewed in Hector 2005), all of which prevent antifungals from achieving minimum inhibitory concentrations at sites of fungal infection. Furthermore, because of the close phylogenetic affinity of animals to fungi, relative to bacteria, antifungals are notorious for producing damaging side-effects in animal hosts. For example, because of its affinity for ergosterol, amphotericin B also binds to cholesterol and forms pores in animal cell membranes (Kaneshiro *et al*. 2000, Baginski *et al.* 2002), which results in so many adverse side-effects that the drug's nickname is 'amphoterrible'.

Such side-effects limit the use of standard strategies that have been developed for overcoming or preventing the evolution of resistance in fungal infections, such as applying high doses, varying the antifungal used over time, or simultaneously exposing the pathogen to multiple antifungals at once. In short, even relative to antibacterials, for which there is already a shortage of effective compounds (Butler and Buss 2006), there is a great need for new antifungals, which explains strong pharmaceutical interest in finding ways to discover unknown antifungals, especially those with novel sub-structures that show improved lethality toward fungal pathogens, while exhibiting stronger affinities to fungal target molecules to reduce side-effects. To achieve this goal, antifungal drug discovery and development explores natural, semi-synthetic, and synthetic molecules. Natural-product antifungals are unmodified bioactive compounds; semi-synthetic and synthetic ones refer to structurally modified natural products and chemically synthesised bioactive molecules, respectively. This thesis concentrates on the discovery of new natural-product antibiotics.

1.2 Sources of new natural-product antifungals

Miyadoh (1993) studied the origin of antibiotics that were published in the *Journal of Antibiotics* (1984-1993) and in patent applications (1983-1992). The author found that around 70% of antibiotic compounds were produced by actinomycetes, 20% by fungi, and only 10% by other bacteria. The name

'actinomycetes' refers to bacteria in the phylum *Actinobacteria* that morphologically resemble fungal mycelia in their production of filaments. Actinobacteria are common soil organisms, characterised by DNA with a high guanine and cytosine content. Miyadoh (1993) also found that antibiotics with an actinomycete origin were mainly produced by *Streptomyces* (68%), with smaller contributions from the bacterial genera: *Nocardia*, *Saccharopolyspora*, *Nocardiopsis*, *Amycolatopsis*, and *Pseudonocardia*. The statistics for antifungal compounds are comparable to the ones for antibiotics in general in that actinobacteria represent the largest group of producers (60%); fungi (32%) and other bacteria (8%) represent smaller producer groups. This shows that (actino)bacteria represent an important source of novel metabolites with potentially favourable pharmaceutical properties. Some researchers have tried to increase the exploitation of this source by studying 'cryptic' or 'silent' pathways in known antibiotic-producers (Hertweck 2009). Bacteria often have more genes encoding antibiotic compounds than are transcribed under laboratory conditions. Some cryptic antibiotic biosynthesis genes can be switched on by growing the producing bacteria on specific media, under specific temperature regimes, or in the presence of chemical inducers. For instance, the chemical substance sodium butyrate is known to influence the (de)acetylation of histones in eukaryotic cells, around which the DNA is packed. The acetylation of histones lowers DNA binding and hence allows an increased transcription of genes (Hertweck 2009), including antibiotic biosynthesis genes. Sodium butyrate appears to influence DNA binding of histone-like proteins in bacteria as well and has been reported to increase antifungal production in certain bacteria (Martin and McDaniel 1976, Moore *et al*. 2012).

Other researchers have focused on screening novel or under-explored environmental niches to discover unknown bacteria and novel biosynthesis genes. One interesting set of novel niches is symbioses of bacteria with animals, fungi, and plants. Symbioses are long-term associations between different species, and symbioses that result in fitness benefits to both species are called 'mutualisms.' The most common form of mutual benefit occurs when

physically larger partners ('hosts') provide housing and food to smaller partners ('symbionts'), which in turn provide specialised services or compounds. For example, termites disperse to patches of dead wood, and the protozoan symbionts of termite guts produce enzymes that can digest cellulose, which feeds both the termites and its symbionts (Poinar 2009).

Symbiotic mutualisms involving microbes may be of particular pharmaceutical interest; firstly, hosts may provide living conditions to their symbionts that differ markedly from host-unassociated, potentially well-explored, and more accessible habitats that have been extensively surveyed by antibiotics researchers (Schmidt 2008, Piel 2009, Seipke *et al.* 2011c). Secondly, symbionts and hosts are likely to have coevolved, and bacterial metabolites therefore have evolved to serve hosts' needs. For instance, symbionts have been reported to provide their hosts with a large diversity of small nutritional and non-nutritional molecules, serving diverse ecological functions in the hosts' defence, predation, and health-care (Schmidt 2008, Seipke *et al*. 2011c). Both factors may increase the genetic and molecular novelty of the bacterial metabolites, compared to compounds that have been characterised to date. Additionally, it is possible that the symbiotic niche has selected for antifungals that are less harmful to the animal hosts, while still being effective against fungal pathogens. Below, I briefly survey the large diversity of pharmaceutically exploitable host-symbiont systems by providing key examples spanning three kingdoms; however, the focus is on the chemical exchange between insects and bacteria since this is the major topic of this thesis.

1.2.1 Fungus-bacteria mutualism

The mutualism between the fungal host *Rhizopus microsporus* and bacterial symbionts in the genus *Burkholderia* has been investigated by Partida-Matinez and Hertweck (2005). The fungus is known to attack rice seedlings, causing root swellings, and to kill plant tissue. Both mutualistic partners benefit from the bacterial production of rhizoxin. This secondary metabolite is able to disrupt plant cell division by binding β-tubulin; the killed plant tissue serves as

food for the fungus and also supports the growth of the bacterium. Previously, rhizoxin-production has been attributed to the fungus; however, the authors showed that the bioactive metabolites are of bacterial origin and that the microbes are hosted endosymbiotically. 16S rDNA of the *Burkholderia* symbionts and ketosynthase genes, encoding parts of the polyketide synthase that is involved in rhizoxin metabolism, could be traced in symbiont-containing *R. microsporus* strains but were absent from non-rhizoxin producing fungi. Furthermore, the bacterial symbiont could be isolated and introduced into a symbiont-free host, which thereafter re-established rhizoxin production. Due to its cytocidal effects, rhizoxin has been studied for its antitumor properties.

1.2.2 Plant-bacteria mutualism

Marine algae have been studied as a source for novel bacterial antibiotics (Armstrong *et al*. 2001, Penesyan *et al*. 2009). For example, the alga *Delisea pulchra* has been shown to host a diverse community of episymbiotic bacteria (Penesyan *et al*. 2009). These have been suggested to contribute in preventing the establishment of biofouling, describing the growth of epiphytic organisms on the plant surfaces. Penesyan *et al*. (2009) found the bacterial community of *D. pulchra* to consist of 79% Gamma-*Proteobacteria*, 13% Alpha-*Proteobacteria*, 4% *Bacteroidetes*, and 4% *Firmicutes*; most of the isolated Gamma-*Proteobacteria* belonged to the genus *Microbulbifer*. The bacterial isolates were tested positively for inhibition of diverse mammalian pathogens, including Gram-positive (*Streptococcus suis*, *Staphylococcus aureus*) and Gram-negative (*Neisseria canis*) bacteria, as well as against a fungal pathogen (*Candida albicans*). One explanation for the secretion of antibacterials and antifungals is to prevent bacterial competitors from colonising the algal surface tissue (Armstrong *et al*. 2001, Penesyan *et al*. 2009), and these compounds may be exploitable pharmaceutically.

1.2.3 Animal-bacteria mutualism

1.2.3.2 Solitary wasps: Philanthus triangulum

The European beewolf *Philanthus triangulum* hosts mutualistic *Streptomyces* bacteria (Kaltenpoth *et al*. 2005). The life-cycle of this solitary wasp includes a phase during which its larvae develop underground. Fertilised females excavate tunnel-like cavities in the soil, into which they place paralysed prey insects (i.e. honeybees), which serve as food resources for the developing beewolf larvae.

The females inoculate the brood chambers with secretions from their antennae, which contain *Streptomyces* bacteria. The bacteria are subsequently taken up by the larval offspring and applied to their cocoons when pupating. The bacteria are thought to protect the pupae from bacterial and fungal infections, originating from the soil and/or from the prey carcasses. The presence of the bacterial symbionts increases the survivorship of the beewolf offspring (Kaltenpoth *et al*. 2005), as shown in a test where only one of fifteen (6.7%) *Streptomyces*-deprived beewolf larvae survived to pupation, whereas 15 out of 18 (83.3%) control larvae that had access to the bacterial symbiont survived.

The bacterial symbionts produce antibiotics (Kroiss *et al*. 2010). In particular, the bacterial solutions applied to the brood cells contain streptochlorin ($C_{11}H_7N_2OCl$, 218.0 amu), as well as piericidin A_1 ($C_{25}H_{37}NO_4$, 415.3 amu), B_1 ($C_{26}H_{39}NO_4$, 429.3 amu), and A_5 ($C_{26}H_{39}NO_4$, 429.3 amu). Furthermore, cocoon extracts have been shown to contain these and five other piericidin derivates, including glucopiericidin A ($C_{31}H_{47}NO_9$, 577.3 amu). These antibiotics are not novel but were unknown to insect systems. Piericidin A_1 and B_1 were the most abundant substances. The authors isolated streptochlorin, piericidin A_1 and B_1, as well as glucopiericidin A; they challenged the substances against a diverse set of fungi (e.g. general and entomopathogenic fungi) and bacteria. Piericidin A_1 inhibited the growth of eight test organisms; streptochlorin and piericidin B_1 were more specific in their antibiotic action but particularly strong in inhibiting fungi that showed resistance towards piericidin A_1.

1.2.3.3 Solitary wasps: Sceliphron caementarium and *Chalybion californicum*

Another example is given by the mud dauber wasps *Sceliphron caementarium* and *Chalybion californicum* (Poulsen *et al*. 2011), which also house mutualistic *Streptomyces* bacteria. *S. caementarium* builds its nest chambers from mud. There, foundresses deposit eggs and paralysed arthropod prey (e.g. spiders) that serve as food for the wasp larvae. *C. californicum* parasitises *S. caementarium* by emptying and ovipositing into nest chambers previously occupied by *S. caementarium*. Both the soil and prey represent potential sources of harmful fungi and bacteria.

Streptomyces bacteria can be found on the integuments of both species and possess antibacterial and antifungal properties (Poulsen *et al*. 2011). To date, no integumental structures have been identified in the insects to support ectosymbiotic bacteria; hence, the bacterial isolates could be only contaminants from the soil. Nevertheless, cuticular washes from 33 wasps have been shown to contain a variety of 24 bacterial morphotypes (Poulsen *et al*. 2011).

Liquid growth cultures of 15 strains were analysed metabolomically, resulting in the discovery of 11 antibiotic substances. These metabolites were produced by 10 of the 15 bacterial strains, including the well-known compounds: streptazoline (e.g. $C_{11}H_{13}NO_3$, 207.229 amu, CAS: 80152-07-4); streptazon B (e.g. $C_{10}H_{11}NO$, 161.203 amu, Reaxys ID: 8682540); bafilomycin A_1 (e.g. $C_{35}H_{58}O_9$, 622.84 amu, CAS: 88899-55-2) and B_1 (e.g. $C_{44}H_{65}NO_{13}$, 815.999 amu, Reaxys ID: 20434788); antimycin A_1 (e.g. $C_{28}H_{40}N_2O_9$, 548.634 amu, CAS 116095-18-2), A_2 (e.g. $C_{27}H_{38}N_2O_9$, 534.607 amu, CAS 118890-43-0), A_3 (e.g. $C_{26}H_{36}N_2O_9$, 520.58 amu, CAS 116095-17-1), and A_4 (e.g. $C_{25}H_{34}N_2O_9$, 506.553 amu, CAS 117603-45-9); daunomycin (e.g. $C_{27}H_{29}NO_{10}$, 527.528 amu, Reaxys ID: 18461908); mycangimycin (e.g. $C_{20}H_{24}O_4$, 328.408 amu, CAS: 1093661-81-4); as well as the novel compound: sceliphrolactam (e.g. $C_{28}H_{35}NO_6$, 481.589 amu, CAS: 1267888-95-8). During colony bioassays, the 15 bacterial isolates were tested against a set of 16 fungal strains, including entomopathogens, and against one another. A complex pattern of bacterial and fungal inhibition was found. For example, bacteria producing different antifungals inhibited the same

fungal strain, and strains of the same fungal species showed different susceptibilities to the same bacterial strain. The secretions also inhibited bacteria; however, inhibition patterns were even more variable. Poulsen *et al*. (2011) carried out the bioassays with bacterial colonies instead of isolated antibiotics; therefore, it cannot be excluded that the bacteria produced multiple antibiotic substances on agar plates.

1.2.3.4 Southern pine beetles: Dendroctonus frontalis

Secretions from bacterial symbionts are also used for protecting food resources in insect fungicultural systems. Insect fungiculture is common, and known to occur in ants, termites, beetles, and gall midges (Kaltenpoth *et al*. 2009). For example, the southern pine beetle *Dendroctonus frontalis* cultivates mutualistic fungi of the species *Entomocorticium sp.* and *Ceratocysfiopsis ranaculosus* (Klepzig *et al*. 2001). Female beetles chew galleries in the inner bark and phloem of living pine trees into which they oviposit and inoculate their fungal cultivars. Typically, pure fungal cultures of either species are carried by the females in integumental pouches, so-called mycangia. The fungal hyphae appear to serve as food for the developing larvae (Klepzig *et al*. 2001). The activities of the adults and offspring destroy the phloem and lead to tree death. In addition, the females often vector a third fungus, *Ophiostoma minus*, which hitchhikes on their exoskeleton. The parasite has been suggested to compete with the fungal mutualists for phloem space. In vitro, this fungus is able to readily overgrow *Ceratocysfiopsis ranaculosus* but is less likely to overgrow *Entomocorticium sp.* (Klepzig *et al*. 2001). Due to this, and because of representing a richer nutritional substrate for the larvae, it has been suggested that the beetle offspring may be better off when surrounded by latter.

Bacteria of the genus *Streptomyces*, found in the females' mycangium and inside the fungal cultivar, are thought to defend the fungal cultivar against *O. minus* (Scott *et al.* 2008). In particular, red and white bacterial morphotypes have been isolated from female beetles. In agar plate bioassays, these were challenged against the fungal mutualist *Entomocorticium sp.* and the fungal

parasite *O. minus*. The red morphotype was able to inhibit the growth of the fungal parasite, whereas the white morphotype was not; both bacterial morphotypes only slightly inhibited the growth of the fungal mutualist (Scott *et al.* 2008, Oh *et al*. 2009). In consequence, the bacterial symbionts have been proposed to benefit the beetle fungiculture. In a chemical analysis, the red morphotype was shown to produce a novel antibiotic with the name mycangimycin (e.g. $C_{20}H_{24}O_4$, 328.408 amu, CAS: 1093661-81-4).

In summary, host-symbiont systems represent promising and under-explored environmental niches for finding new, potentially coevolved, bacteria with novel antibiotic biosynthesis genes of potential pharmaceutical importance.

1.3 Outline of this study

In this study, four ant species (*Hymenoptera*, *Formicidae*), *Acromyrmex octospinosus* (*Myrmicinae*), *Allomerus decemarticulatus and A. octoarticulatus* (*Myrmicinae*), and *Tetraponera penzigi* (*Pseudomyrmecinae*), were investigated regarding their associations to bioactive bacteria that could provide their (putative) hosts with potentially novel and pharmaceutically exploitable antifungal metabolites. The following is an overview of the main results.

Chapter 2, titled "***Acromyrmex octospinosus* ant-associated bacteria**," describes the composition of the bacterial microbiome on the cuticles of *A. octospinosus* worker ants. *A. octospinosus* is known to cultivate the fungus *Leucoagaricus gongylophorus* (*Agaricales*, *Agaricaceae*), which produces nutritive food bodies (gongylidia) to provide the ants with food. In particular, this association is obligate since the queen and larvae of *Acromyrmex* exclusively feed on gongylidia. The health of the fungal cultivar is endangered by diverse non-cultivar fungi. Among those, fungi in the genus *Escovopsis*, are common, virulent, and specialised. The health of the fungal cultivar has been shown to partly depend on antifungal metabolites of bacterial symbionts. To date, bioactive *Pseudonocardia*, *Streptomyces*, and *Amycolatopsis* bacteria have been isolated from the cuticles of attine workers (Currie *et al.* 1999b, Currie *et*

al. 2003, Kost *et al*. 2007, Haeder *et al.* 2009, Oh. *et al.* 2009, Sen *et al*. 2009), and bioactive *Streptomyces* and *Burkholderia* bacteria have been isolated from fungal cultivars of attine ants (Haeder *et al*. 2009, Santos *et al*. 2004). Furthermore, these bacterial symbionts have been shown to produce diverse antifungal metabolites, including candicidin, actinomycin (D and X_2), antimycin A_1-A_4, valinomycin, and the novel antifungal dentigerumycin (Haeder *et al.* 2009, Schoenian *et al*. 2011, Oh. *et al.* 2009).

In addition to the potential for bioprospecting of novel antibiotics, the attine system provides an interesting evolutionary dilemma. How does antibiotic defence, provided by the ant-associated bacteria, remain effective against *Escovopsis* and other non-cultivar fungi over ecological and evolutionary time-spans? So far, two hypotheses have been proposed: firstly, metabolites of vertically transmitted and coevolved, attine-associated *Pseudonocardia* symbionts may be in an evolutionary 'arms race' with resistance mechanisms in *Escovopsis* (Currie *et al.* 1999b, Currie *et al*. 2003, Mueller 2012). Secondly, resident attine-associated *Pseudonocardia* strains may be exchanged for or augmented by actinobacteria with effective antifungal metabolites (Zhang *et al.* 2007, Mueller *et al*. 2008, Mueller 2012), potentially recruited from the soil environment; this environmental recruitment might occur at all times or in response to exposure to resistant *Escovopsis* strains. A different version of the second hypothesis is that antifungal 'cocktails', produced by mixed communities of bacteria not only provide protection against a variety of harmful non-cultivar fungi, including *Escovopsis*, but also slow-down the evolution of resistance in single parasite strains. The first empirical support for environmental recruitment and mixed bacterial communities came from Kost *et al*. (2007), who showed that *Acromyrmex* ants, in addition to *Pseudonocardia*, also associate with *Streptomyces*. As a further step to test the hypothesis that antifungal cocktails help *A. octospinosus* prevent the rise of pathogen resistance, this chapter focuses on identifying the bioactive bacterial microbiome of worker ants in *Acromyrmex octospinosus*.

The data presented in this chapter show that diverse genera of Gram-positive bacteria can be isolated from the cuticles of *A. octospinosus* workers. Selected isolates, including *Pseudonocardia*, *Streptomyces*, and *Nocardiopsis* strains, as well as their growth culture media, were challenged in bioassays against *Candida albicans* and three *Escovopsis* strains. Representatives of all tested bacterial genera were shown to inhibit at least one of the test fungi. The data thus confirm the potential of *Pseudonocardia* and *Streptomyces* as mutualistic partners, but further studies are needed to characterise the ecological role of *Nocardiopsis* in the *A. octospinosus* symbiosis. A portion of these results was published by Barke *et al.* (2010) in *BMC Biology*. In this paper, the hypothesis is tested that the observed variety of bacterial symbionts, indeed translates into a diversity of antifungal metabolites. In particular, two antifungal metabolites are described that are metabolised by two worker ant-associated bacterial species, one *Pseudonocardia* and one *Streptomyces*, which in Chapter 2 were shown to originate from the same ant colony and to inhibit diverse test fungi. The data demonstrate that the putatively coevolved *Pseudonocardia* strain can produce a novel variant of the polyene antifungal nystatin and that the apparently environmentally recruited *Streptomyces* strain can produce the well-known compound candicidin. Hence, a diversity of bacterial symbionts indeed translates into a diversity of antifungal metabolites, which may explain the long-term fungicultural success of *A. octospinosus*.

In an invited 'auto-commentary' on Barke *et al*. (2010), Barke *et al*. (2011) place the vertical transmission and environmental recruitment hypotheses into general mutualism theory, explaining how the first invokes 'partner fidelity feedback' and the second invokes 'partner choice'; the auto-commentary forms the discussion of Chapter 2.

Chapter 3, titled "**Antifungals of an *Acromyrmex octospinosus* ant-associated *Streptomyces* strain**," demonstrates that the *Streptomyces* strain that had previously been shown to produce candicidin (Barke *et al*. 2010) can also produce antimycins, which is another class of antifungals. For the

purification and identification of the non-candicidin antifungal, the bacterium was grown in a liquid growth culture. Apolar molecules, which characterise many antifungals, were extracted from the growth supernatants with butanol. The extract was purified in bioassay-guided normal- and reversed-phase column fractionations. The compound masses of the parent ions in the double-purified extract were detected by mass spectrometry. These compounds were identified by determining their masses/formulae and the masses/formulae of their sub-structures (i.e. daughter ions), generated during MS^2 analyses.

The data, presented in this chapter, show that the double-purified extract from the growth cultures, in addition to candicidin, also contains antimycin. In particular, two sets of six antimycin molecules could be detected, corresponding to diverse antimycins that differ in polarity and mass by the attachment of CH_2 groups. A tandem-LCMS analysis confirmed analogies between the second compound set and an antimycin A_1-A_4 standard. The data illustrate that even single bacterial associates are able to provide their ant hosts with multiple antifungal metabolites, increasing the compound diversity of antifungal cocktails. This work was published as part of Seipke *et al*. (2011a).

Chapter 4, titled "***Allomerus decemarticulatus* and *A. octoarticulatus* cultivar-associated bacteria**," describes the presence of bacteria in the fungal cultivar of *Allomerus* ants. Here, the idea of investigating new environmental niches for unknown bacterial symbionts is applied to the ant genus *Allomerus*, which has never been studied this way. *A. decemarticulatus* and *A. octoarticulatus* ants are hosted by the myrmecophytic trees *Hirtella physophora* and *Cordia nodosa*, respectively, in exchange for protection against herbivores. The ants are known to maintain a fungal monoculture in the order *Chaetothyriales* (Ruiz-González *et al.* 2010). The fungus is thought to stabilise trap constructions, built out of carton and other detritus by the ants on host-tree branches, to capture insect prey. Spores of diverse, and potentially harmful, non-cultivar fungi have been isolated from the traps (Ruiz-González *et al.* 2010), that if left unattended, could render the traps useless. Here, the hypothesis is

tested that cultivar-associated bacterial symbionts may promote the health of the fungal mutualist. Recall that in the *Acromyrmex* system, cultivar-associated *Burkholderia* and *Streptomyces* bacteria have been suggested to defend the fungal mutualist against non-cultivar fungi (Santos *et al*. 2004, Haeder *et al*. 2009). The focus here is on the *Allomerus*' fungal cultivar, not on the ants themselves, because the latter are not known to possess integumental structures to house bacterial symbionts.

The data presented in this chapter show that diverse genera of Gram-positive and Gram-negative bacteria can be isolated from the cultivar traps. Selected isolates, including *Streptomyces* and *Nocardia* strains, and their growth cultures, were challenged in bioassays against *Candida albicans* and two non-cultivar fungi that also were isolated from the cultivar traps. Under laboratory conditions, none of the bacterial isolates inhibited the test fungi. Addition of the chemical inducer sodium butyrate did cause two *Streptomyces* strains to inhibit *Candida*, but only weakly. These results are, at most, only weakly consistent with the hypothesis that cultivar-associated bacteria promote the health of the fungal cultivar in this mutualism.

Chapter 5, titled "***Tetraponera penzigi* ant- and cultivar-associated bacteria**," describes microbes that were isolated from *T. penzigi* worker ants and their putative fungal cultivar. The ants are hosted by the myrmecophyte *Acacia drepanolobium* as host defence against herbivory. *T. penzigi* ant-inhabited domatia have been found to consistently contain hyphal growth of a fungus in the genus *Chaetomonium*, which might feed *T. penzigi* in some way. Here, I test the hypothesis that, analogous to the fungal cultivar of previous chapter, this putatively cultivated fungus is protected against potentially harmful non-cultivar fungi by worker ant- and cultivar-associated bacteria.

The data provided in this chapter show that diverse genera of Gram-positives and Gram-negatives can be isolated from the worker ants and their putative fungal cultivar. Selected bacteria, including *Streptomyces* and *Saccharopolyspora* strains, as well as their culture supernatants were

challenged in bioassays against *Candida albicans* and three non-cultivar fungi, isolated from the fungal cultivar. Both *Streptomyces* and *Saccharopolyspora* strains strongly inhibited the growth of test fungi. Hence, the data suggest that the metabolites of ant- and cultivar-associated bacteria in this system may indeed promote the health of the putative fungal cultivar. The *Tetraponera*-bacteria association therefore becomes a new and promising candidate for the discovery of novel antibiotics.

Chapter 2) *Acromyrmex octospinosus* ant-associated bacteria

2.1 Introduction

2.1.1 Mutualistic partners

The origin of attine ants (*Hymenoptera*, *Formicidae*, *Attini*) dates back around 50 million years ago (Schultz and Brady 2008). Since then, the tribe *Attini*, which is endemic to the New World, has diversified into more than 230 species and thirteen genera. The so-called 'lower attines' include the genera *Mycocepurus*, *Myrmicopcrypta*, *Apterostigma*, *Mycetophylax*, *Mycetarotes*, *Mycetosoritis*, *Cyphomyrmex*, and *Mycetagroicus*; the 'higher attines' include the genera *Sericomyrmex*, *Trachymyrmex*, *Acromyrmex*, *Pseudoatta*, and *Atta* (Schultz and Brady 2008, Solomon *et al*. 2011). Attine species differ enormously in their biogeography, ecology, and associations to fungi. For example, despite all being known to cultivate fungi for food – hence the common name fungus-growing ants – their cultivars differ among others in identity and growth requirements (e.g. substrate). Schultz and Brady (2008) distinguish five types of attine fungiculture, including 'lower agriculture,' 'coral fungus agriculture,' 'yeast agriculture,' 'higher agriculture,' and 'leaf-cutter agriculture.' Higher agriculture is carried out by the higher attines *Sericomyrmex* and *Trachymyrmex*; and leaf-cutter agriculture is carried out by the higher attines *Atta* and *Acromyrmex* that are commonly referred to as 'leaf-cutting ants' or 'leaf-cutter ants'. The genera *Atta*, comprised of 15 species, and *Acromyrmex*, comprised of 24 species, cultivate the basidiomycetous fungal species *Leucoagaricus gongylophorus* (*Agaricales*, *Agaricaceae*). For brevity, *L. gongylophorus* will henceforth be referred to as the 'cultivar'. *Atta* and *Acromyrmex*, and also certain *Trachymyrmex* species (Schultz and Brady 2008), collect large volumes of plant leaves and flowers, masticate them to make nutrients more available, and use them as substrate for their cultivar. *L. gongylophorus*, which seems not to occur outside of the mutualism, converts the plant material into hyphal-tip swellings, called gongylidia (Schultz and Brady 2008). These contain high concentrations of lipids and carbohydrates, and are selectively harvested by the worker ants to

feed the larvae and queen. The worker ants supplement their own diets with plant secretions. Furthermore, the ants propagate and protect their cultivar; for example, the cultivar is propagated vertically, from parent to daughter colonies, by newly emerging and dispersing queens (gynes). These carry a small inoculum of the fungal resident in their mouth pocket (infrabuccal pocket). After nest initiation, the cultivar is taken care of by the gynes; in more established nests, this responsibility is taken over by the worker ants.

2.1.2 Fungal parasites

Leaf-cutter ant colonies maintain single strains of their *L. gongylophorus* cultivar. The potential disadvantage of relying exclusively for food on a genetically depauperate and vertically transmitted fungal monoculture is that the fitness of the ants crucially depends on the health of the cultivar, but the entire cultivar could be destroyed by just a single pathogen species or genotype, if the pathogen is able to overcome the cultivar's defences, including the cultivar's own antifungal compounds (Wang *et al.* 1999). In addition, any given lineage of the mutualistic fungus has a reduced capacity to evolve novel resistance mechanisms and/or antifungal molecules, as the only source for novel genetic variation is mutation, as opposed to sex and recombination. In contrast, the genetic diversity of harmful and/or competing non-cultivar fungi, as well as the genetic variability and potential of single fungal pathogens to evolve new virulence mechanisms, are maintained at high levels by both mutations and sexual reproduction.

Specialised and unspecialised non-cultivar fungi are indeed common in attine nests. Of those, fungi in the genus *Escovopsis* (*Hypocreales*, *Hypocreaceae*) appear to be common, virulent, and specialised. For example, Currie *et al.* (1999a) analysed the presence of non-cultivar fungi in gardens of 201 colonies of eight attine genera, including *Atta* and *Acromyrmex*. 39.7% of 2,480 garden pieces were infected with non-cultivar fungi, and 26.0% of the 984 infectants belonged to the genus *Escovopsis*. In a study by Rodrigues *et al*. (2008), 37 nests of ten *Acromyrmex* species were investigated for non-cultivar

fungi. A total of 33 fungal species of 16 genera could be isolated from the nests; most prevalent were the genera *Cunninghamella*, *Escovopsis*, *Fusarium*, *Mucor*, *Penicillium*, and *Trichoderma*. *Fusarium oxysporum* and *Escovopsis weberi* were the most common species, occurring in 40.5% and 27% of the nests, respectively. However, in contrast to *Escovopsis*, fungi in the genus *Fusarium* can also be found outside of the symbiosis (Rodrigues *et al*. 2008).

Furthermore, the virulence of *Escovopsis* stems from its ability to feed on the ant-cultivar by degrading its hyphae and obtaining its nutrients without the need to penetrate the fungus, making it a 'contact necrotroph' (Reynolds and Currie 2004). Also, *Escovopsis* may overgrow cultivar gardens or form permanent infections. The latter have been shown to severely reduce garden biomass and worker production in colonies of *Atta colombica* (Currie 2001).

In addition, *Escovopsis* seems to be horizontally transmitted; *Escovopsis* is absent from fungal inocula carried by the gynes of *Atta columbica*, it is also absent from young colonies that have not started collecting leaf-material, but it is present in around 60% of 1-2 year-old nests (Currie *et al.* 1999a). Attine nests therefore likely experience a constant influx of virulent and genetically variable *Escovopsis* strains.

2.1.3 Exploitation control of non-cultivar fungi

An array of exploitation control mechanisms has been identified that reduce the likelihood of successful infection by *Escovopsis* and other pathogenic and parasitic fungi. These control mechanisms include the separation of cultivars into distinct and remote underground nest chambers, the use of garden platforms to isolate initial nests from the soil (Fernández-Marín *et al.* 2007), the cleaning of contaminants from the leaf-material (Mangone and Currie 2007), the production of antifungals by the cultivated fungus itself (Wang *et al.* 1999), and the production of antifungal compounds by cultivar-associated bacteria (Santos *et al.* 2004, Haeder *et al*. 2009). These control mechanisms filter out many susceptible strains of non-cultivar fungi, but obviously do not guarantee a full protection against all non-cultivar strains and species. In

particular, sets of cultivar-specific *Escovopsis* strains seem to be able to generate successful infections in attine gardens. For example, Gerardo *et al.* (2006) suggest that specific associations between *Escovopsis* strains and clades of the mutualistic fungus, grown by attine ant *Apterostigma*, can be explained by 'chemotaxic attraction' and 'antibiotic susceptibility'.

Attine ants that obligately depend on their fungal mutualist for food should be selected to eliminate cultivar-specific *Escovopsis* strains from the nest. Higher attines remove and kill fungal intruders by grooming spores of non-cultivar fungi, weeding infected garden material, applying bioactive metapleural gland secretions, and using antifungal secretions of cuticular bacteria. The prevalence of these control mechanisms differs among attine genera and castes. For example, the attine genera *Atta* and *Sericomyrmex*, which do not show any (visible) growth of cuticular bacteria, confront *Escovopsis* infections and general contaminations behaviourally and with secretions from their metapleural glands. Fernández-Marín *et al.* (2009) report increased grooming activities, using metapleural gland secretions, after experimental infections of their nest with *Escovopsis*. In contrast, the attine genera *Acromyrmex* and *Trachymyrmex* (but also diverse lower attines), use secretions of bacterial symbionts to target *Escovopsis*. They down-regulate metapleural gland grooming and up-regulate bacterial coverages after experimental nest infections with *Escovopsis* (Fernández-Marín *et al.* 2009).

The behavioural and chemical defence strategies of *Acromyrmex* ants are caste-specific (Poulsen *et al*. 2002). Older major workers collect plant material; minor workers and young major workers tend the garden, care for the brood, and prepare the leaf-material collected by older nestmates. The fungal gardens are structured by the degradation status of the plant material into a 'top part', containing the highest proportion of plant material and unspecialised infectants, and a 'bottom part' containing the highest proportion of cultivar biomass and *Escovopsis* parasites. With their relatively large metapleural glands, minor workers at the top limit the influx of non-cultivar fungi on the fresh leaf-material. Metapleural gland secretions of *Acromyrmex octospinosus* contain a chemical

cocktail that has broad-spectrum bioactivity (Ortius-Lechner *et al*. 2000). Interestingly, although the metapleural gland secretions do not harm *Escovopsis* hyphae, the secretions do kill its spores (Bot *et al.* 2002). Thus, minor workers can provide some defence against the specialist parasite *Escovopsis* as long as it enters nests in its spore stage. In contrast, young major workers at the bottom do not possess such large metapleural glands but do maintain a high abundance of cuticular bacteria, whose antifungal secretions kill *Escovopsis* even in hyphal state (e.g. Currie *et al*. 1999b, Currie *et al*. 2003, Kost *et al*. 2007, Haeder *et al*. 2009, Oh. *et al.* 2009, Sen *et al.* 2009, Barke *et al*. 2010).

One of the still unresolved fundamental questions regarding this three-part mutualism is what the identity and composition of the bacterial symbionts is. From 1999 onwards, numerous papers, mainly published by Cameron Currie and colleagues (e.g. Currie *et al*. 1999b), propagated the view that single attine colonies symbiotically associate with single lineages of bacteria in the genus *Pseudonocardia* (*Actinomycetales*, *Pseudonocardiaceae*) (N. B. papers of Currie *et al*. published in 1999-2003 referred to the genus *Streptomyces*; this was later corrected by the authors to *Pseudonocardia*, Currie *et al*. 2003). In return for antifungals, the bacteria benefit from nutrients via secretions from 'gland cell-associated tubercles' (Currie *et al*. 2006). In this scenario, vertical transmission leads to host-symbiont coevolution, in which the fitness of both are highest if bacterial antifungals kill current *Escovopsis* attackers; to retain antifungal efficacy and ensure long-term fungicultural successes, the bacterial metabolites provided to the host, must show structural innovations to meet reduced parasite resistances (Currie *et al*. 1999b, Currie *et al*. 2003).

More recently, attine colonies have been found to associate with bacterial communities, including diverse actinobacteria, rather than to associate with single *Pseudonocardia* lineages. In the above view, the newly discovered ectosymbionts would be described as contaminants rather than beneficial symbionts. In order to refute this assumption, it needs to be demonstrated that associations to non-*Pseudonocardia* strains are common and that their antifungals kill *Escovopsis* – also in the field. To date, Gram-positive

Pseudonocardia, *Streptomyces* (*Actinomycetales*, *Streptomycetaceae*), and *Amycolatopsis* (*Actinomycetales*, *Pseudonocardiaceae*) bacteria have been isolated from attine ants (Currie *et al.* 1999b, Currie *et al*. 2003, Kost *et al.* 2007, Haeder *et al*. 2009, Oh. *et al.* 2009, Sen *et al.* 2009, Barke *et al.* 2010). Also, Gram-positive *Streptomyces* and Gram-negative *Burkholderia* (*Burkholderiales*, *Burkholderiaceae*) bacteria have been isolated from attine cultivars (Santos *et al*. 2004, Haeder *et al*. 2009). However, non-actinobacteria have been largely neglected in favour of *Pseudonocardia*- and *Streptomyces*-like actinobacteria that are well-known for their capability to produce novel drugs (Miyadoh 1993).

Non-*Pseudonocardia* actinobacterial symbionts have been shown to produce antifungals that inhibit *Escovopsis* (Kost *et al.* 2007, Haeder *et al*. 2009, Sen *et al.* 2009, Barke *et al.* 2010). For example, Sen *et al.* (2009) tested ten *Pseudonocardia* and two *Amycolatopsis* strains, isolated from the attine genera *Trachymyrmex*, *Cyphomyrmex*, and *Mycocepurus*, against 23 fungal strains. The fungal opponents included saprotrophs (e.g. *Cyphellophora*), endophytes (e.g. *Alternaria*), entomopathogens (e.g. *Beauveria*), fungal cultivars (i.e. *Leucocoprinus*), and nest parasites (i.e. *Escovopsis*). The bacterial strains inhibited 56.3-72.7% of the test fungi, including the fungal mutualists and *Escovopsis* parasites, indicating a secretion of metabolites with broad-spectrum antifungal properties. Because the bacterial metabolites also inhibit the growth of the fungal cultivar, the bacterial secretions have to be applied carefully, not to harm the fungal mutualist. How antifungals can be applied discretely has been described for *Trachymyrmex zeteki* (Little *et al*. 2006). Gram-positive bacteria not only occur on the integuments of *T. zeteki* worker ants but also in their infrabuccal pockets. It is unknown whether the bacterial mutualists are cultivated inside the infrabuccal pocket or continuously replenished from the cuticle. Nevertheless, this is the place where the worker ants store infected garden material and spores of non-cultivar fungi that they collect during grooming and weeding. According to the authors, the treated pocket material rarely contains viable *Escovopsis* spores. Furthermore, Schoenian *et al.* (2011) have reported the presence of *Streptomyces*-antifungals on the cuticles of *A. echinatior*.

Due to the discovery of bioactive bacterial communities on ants and in attine colonies, as described above, of nest-unassociated bacteria being able to inhibit *Escovopsis* (Kost *et al.* 2007), and of nest-associated and environmentally collected *Pseudonocardia* strains being phylogenetically related (Mueller *et al.* 2010, Mueller *et al*. 2012), integumental bacteria have been proposed to be environmentally recruited rather than coevolved (Barke *et al.* 2011, Mueller *et al.* 2012). Furthermore, *Acromyrmex* ants are even able to distinguish between *Pseudonocardia* strains of closely related *Acromyrmex* species, which would allow them to exchange resident ectosymbionts (Zhang *et al.* 2007). This line of thinking suggests an alternative hypothesis of how resistance evolution in *Escovopsis* can be prevented; mixed bacterial communities of recruited bacteria may produce antifungal 'cocktails', which may not only target a range of non-cultivar fungi but also slow down the evolution of resistance in current individual fungal strains.

2.2 Aims

In Chapter 2, and a paper by Barke *et al*. (2010), experiments are described to test the hypothesis that antifungal cocktails of mixed bacterial communities allow *Acromyrmex octospinosus* to defend their fungal mutualist against non-cultivar fungi. In the chapter, the bioactive actinobacterial microbiome of *A. octospinosus* workers was investigated in a culture-dependent way. The recovered cuticular bacteria were genetically identified, and their potential as mutualists was confirmed in bioassays by challenging the bacteria and their growth cultures against various test fungi, including the medically important yeast *Candida albicans* and *Escovopsis* spp. nest parasites. In Barke *et al*. (2010), the question is examined whether the diversity of cuticular bacteria indeed translates into a diversity of antifungal compounds.

2.3 Materials and Methods

2.3.1 Sampling host ants

Worker ants of three *Acromyrmex octospinosus* colonies, A, B, and C, were obtained from a supplier of insects in Essex, United Kingdom (www.martingoss.co.uk). The ant colonies were collected near the Blanchisseuse Road, around 6.4 km north of Arima, Trinidad (Fig. 2.1). Photographs of a typical ant nest, the fungal cultivar, and a worker ant are shown in figure 2.2; the colour of the fungal garden is composed of white cultivar hyphae on a brown background of masticated leaf-material. The sampled colonies, including the queen, different worker castes (minor and major workers), and the fungal garden were transported by the supplier from Trinidad to the United Kingdom. There, the colonies remained in captivity for around 12 weeks before sub-samples of the nests were sent to the University of East Anglia (UEA). The sub-samples consisted of some garden material and 60, 86, and 68 garden-associated worker ants in the three colonies respectively (Table 2.1). The ants were placed individually into sterile 1.5 ml Eppendorf tubes.

Sampling location	Worker ants	Colony
Blanchiseusse Road, Trinidad	1-60 (60 Ants)	A
Blanchiseusse Road, Trinidad	76-161 (86 Ants)	B
Blanchiseusse Road, Trinidad	271-338 (68 Ants)	C

Table 2.1 *Acromyrmex octospinosus* worker ants. From Martin Goss (www.martingoss.co.uk) worker ants of three ant colonies were purchased originating from Trinidad.

2.3.2 Isolation of ant-associated bacteria

Ectosymbiotic bacteria were isolated from the cuticles of the worker ants in a category 2 microbiological laboratory at the University of East Anglia. The isolations were carried out using sterile technique. For example, sensitive parts of the work were done in a safety cabinet or next to a Bunsen burner, equipment was sterilised (e.g. forceps, glass spreaders, Eppendorf tubes), and isolation media and liquids were autoclaved.

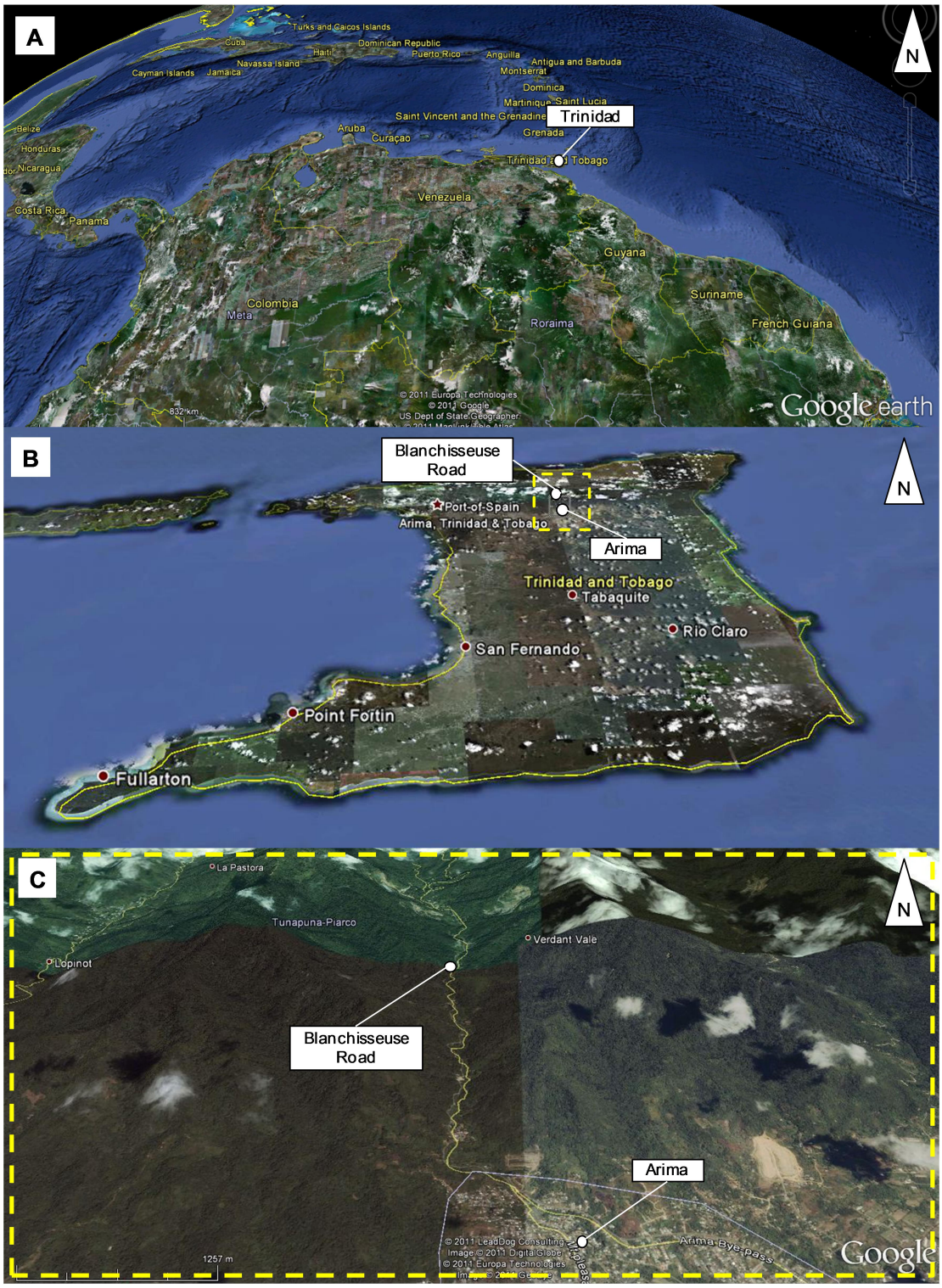


Figure 2.1 (A-C) Origin of *Acromyrmex octospinosus* worker ants. Three ant colonies (including queen, worker ants, and fungal cultivar) were collected near the Blanchisseuse Road, north of the city Arima, Trinidad. The colonies were sub-sampled in the United Kingdom for worker ants and supplied by Martin Goss (www.martingoss.co.uk) to the University of East Anglia.

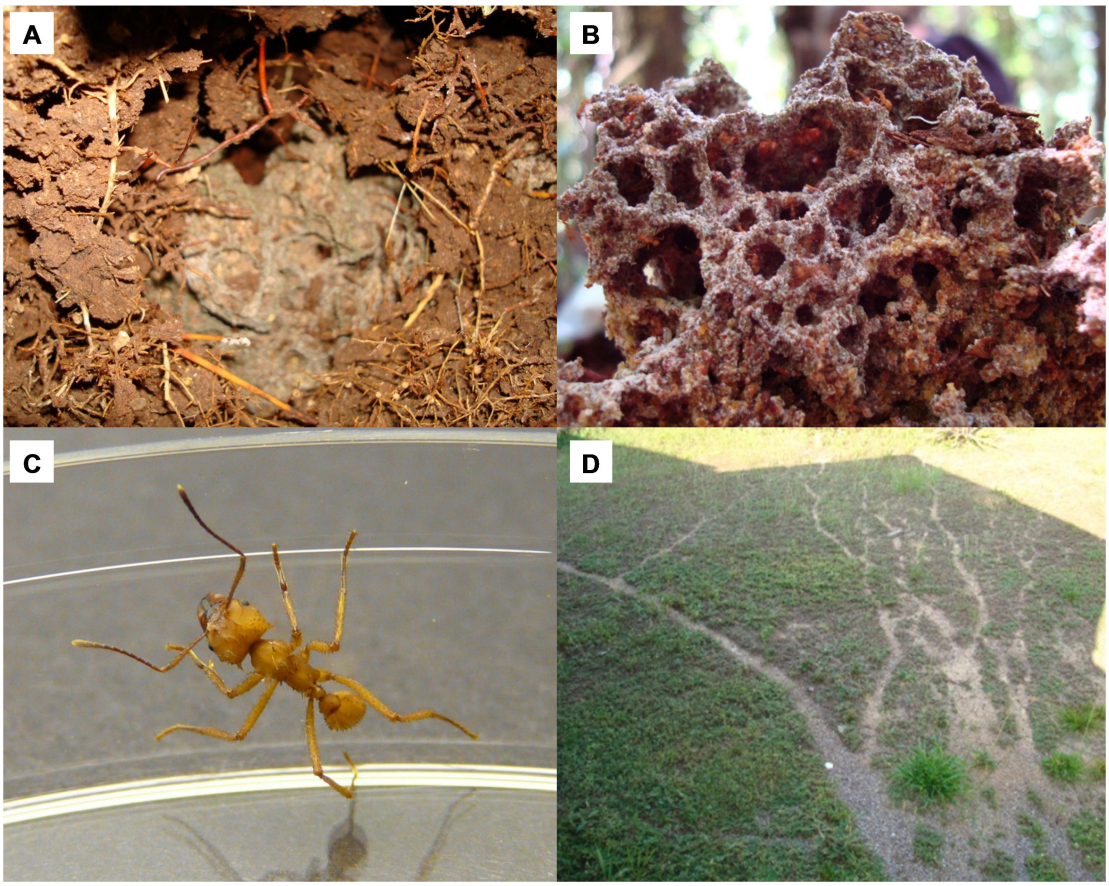


Figure 2.2 *Acromyrmex* and *Atta* leaf-cutter ants. **(A)** Nest chamber of an *Acromyrmex* sp. field colony. **(B)** Fungal cultivar *Leucoagaricus gongylophorus*. **(C)** *Acromyrmex* worker ant. The integument of this worker lacks an obvious white coating consisting of ectosymbiotic bacteria. **(D)** 'Ant highways' through a lawn. The paths here were built by *Atta*, a sister genus of *Acromyrmex*, to transport collected leaf-material back to the nest. Both ant genera use freshly cut plant-material for fungiculture.

The bacterial isolates were grown on hydrolysed chitin (HC) or mannitol soy-flour (MS) agar plates (Table 2.2). Fresh agar plates were prepared by enriching 100 ml batches of autoclaved medium (50 ^{o}C) with 1 ml of nystatin (5 mg (Sigma N3503) in 1 ml dimethyl sulfoxide) and 1 ml of cycloheximide (5 mg (Fisher 35742-0050) in 1 ml of 100% ethanol) solutions to prevent fungal spores of cuticular origin from overgrowing bacterial colonies; the medium was then spread over four Petri dishes. The bacterial ectosymbionts were isolated from workers by streaking and washing. Streaking describes the transfer of cuticular bacteria to agar plates by scraping the ventral and dorsal sides of the ants over agar plates.

Medium	Ingredients	Weight (g), volume (ml), percent (%)
Hydrolysed chitin (HC) agar	Hydrolysed crab shell chitin	3.0 g
	Agar	20.0 g
	Tap water	1000 ml
Lennox broth (LB)	Tryptone	10.0 g
	Yeast extract	5.0 g
	NaCl	5.0 g
	Distilled water	1000 ml
Lennox broth (LB) agar	Tryptone	10.0 g
	Yeast extract	5.0 g
	NaCl	5.0 g
	Agar	15.0 g
	Distilled water	1000 ml
Lennox broth (LB) soft agar	Tryptone	10.0 g
	Yeast extract	5.0 g
	NaCl	5.0 g
	Agar	5.0 g
	Distilled water	1000 ml
Mannitol soy-flour (MS) agar	Organic soy-flour	20.0 g
	Mannitol	20.0 g
	Agar	20.0 g
	(+SB, 150mM sodium butyrate)	(16.5 g)
	Tap water	1000 ml
Mannitol soy-flour (MS) broth	Organic soy-flour	20.0 g
	Mannitol	20.0 g
	(+SB, incl. 150mM sodium butyrate)	(16.5 g)
	Tap water	1000 ml
Potato dextrose agar (PDA)	Glucose	20.0 g
	Instant mashed potato (smash)	5.0 g
	Agar	20.0 g
	Distilled water	1000 ml
Sabouraud agar (pH=5.6)	Dextrose	40.0 g
	Peptone	10.0 g
	Agar	20.0 g
	Distilled water	1000 ml
Tryptone soya broth (TSB)	Tryptone soya broth (Oxoid)	30.0 g
	Distilled water	1000 ml
Yeast extract-malt extract (YEME)	Yeast extract	3.0 g
	Peptone	5.0 g
	Malt extract	3.0 g
	Glucose	10.0 g
	Distilled water	1000 ml
TSB/YEME	TSB	50 %
	YEME	50 %

Table 2.2 Media for cultivation of fungi and bacteria.

Washing describes the transfer of ectosymbiotic bacteria into 20% of aqueous glycerol (2G) by placing a worker ant in 500 µl of 2G and vortexing it for at least 10 seconds to detach cuticular bacteria. The washes of some ants were diluted ten times, and 100-200 µl of the (diluted) wash were used for inoculating agar plates. The original wash was preserved at -20 ^{o}C. In particular, all of the 60 worker ants of sub-sample A were streaked; five ants with a visible bacterial coating were additionally washed. All 86 workers of colony B were washed. The wash of all samples was additionally diluted ten times. Lastly, 54 of the 68 workers of colony C were washed, and the remainder was streaked.

Inoculated agar plates were incubated at room temperature (21-28 ^{o}C). Actinobacterial morphotypes were purified from agar plates containing mixed bacterial communities by repeatedly picking and propagating individual colonies to fresh MS agar plates. The propagation plates contained the same antifungal concentrations as described above and were incubated at 30 ^{o}C. In total, 16 actinobacterial lineages were purified, each of which received an individual ID, consisting of the prefix ‘E’ and a number (2-17). These were maintained on agar plates (4 ^{o}C) and in glycerol.

Bacterial glycerol stocks were prepared by transferring cell material of individual source colonies to equivalent MS agar plates containing nystatin and cycloheximide. The cell material was spread across the whole surface of the target plates. Then the plates were incubated at 30 ^{o}C to promote abundant growth and sometimes spore production. The spores of spore-producing bacterial lineages were collected by scrubbing the surface of the bacterial lawns with sterile cotton buds and 1.5 ml of 2G. Cell material was collected from bacterial strains that did not produce any spores. Here, the surface of the agar plates was scrubbed with sterile cotton buds and 1.5 ml of 2G. The glycerol stocks were preserved at -20 ^{o}C.

2.3.3 Procurement of fungal symbionts

The fungal mutualist *L. gongylophorus* was kindly provided by Ulrich Mueller from the University of Texas (www.sbs.utexas.edu/muelleru/). Several

Escovopsis strains were purchased from the Fungal Biodiversity Centre (www.cbs.knaw.nl), a culture collection in Utrecht, the Netherlands. Included was an *Escovopsis weberi* strain from a Brazilian ant nest (EWB, CBS 810.71); an *E. weberi* strain from a Colombian ant nest (EWC, CBS 110660) and an *E. aspergilloides* strain from a *Trachymyrmex ruthae* nest (EA, CBS 423.93). The fungi were maintained on MS agar, potato dextrose agar (PDA), and Sabouraud agar (Table 2.2). Fresh agar plates were prepared in the same way as the bacterial isolation plates, but the antifungals were replaced with antibacterials. In particular, 1 ml of filter-sterilised carbenicillin (5 mg (Fisher BPE2648-5) in 1 ml dH_2O) and 1 ml of filter-sterilised streptomycin (5 mg (Fisher BPE910-50) in 1 ml dH_2O) solutions were added to 100 ml of medium. The medium was either equally sub-divided over four Petri dishes for short-term cultivation purposes or added to Bijoux's for long-term storage of the fungal strains. Bijoux's are plastic containers enclosed by screw caps; due to their cylindrical shape, the agar can form slopes offering increased surface areas for fungal growth. Agar plates and Bijoux's were inoculated by transferring fungal hyphae from source plates; the fungal inocula were grown at room temperature or at 30 ^{o}C.

2.3.4 Light microscopy

Stereomicroscope photographs of the bacterial isolates and fungal strains were taken in the Henry Wellcome Laboratory for Cell Imaging (www.uea.ac.uk/bio/biobmi) with a Zeiss SV11 M2 Bio Quad stereomicroscope and an AxioCam HRc CCD camera. To keep the entire surface of the bacterial colonies in focus, young and thus mostly flat colonies were used. Nonetheless, magnifications were adjusted to suit the size differences between bacterial species. Photographs of the bacterial colonies and fungal hyphae were taken at magnifications of 2.0, 3.2, 4.0, 5.0, and 6.6X. Scale bars of 1-2 mm were added to the pictures with AxioVision software (Carl Zeiss, Welwyn Garden City, UK).

2.3.5 Extraction of genomic DNA

DNA was extracted from fungal and bacterial samples for genetic identification. The DNA of *L. gongylophorus* and *Escovopsis* EWB, EWC, and EA were extracted from the fungal hyphae (Table 2.3). A loop of hyphal material was added to Eppendorf tubes, containing glass beads and 800 µl of ice-cold TGE buffer (Table 2.4). Also, DNA was extracted from growth cultures of the bacterial isolates E_2-E_{17} (Table 2.3).

Sample type	Name	Sample origin	Host type	Host colony	Host ant
Fungal cultivar	*L. gongylophorus*	Ulrich Mueller (University of Texas)	-	-	-
Non-cultivar fungus	*E. weberi* EWB (CBS810.71)	CBS Fungal Biodiversity Center	-	-	-
Non-cultivar fungus	*E. weberi* EWC (CBS110660)	CBS Fungal Biodiversity Center	-	-	-
Non-cultivar fungus	*E. aspergilloides* EA (CBS423.93)	CBS Fungal Biodiversity Center	-	-	-
Bacterial isolate	E_2	Blanchiseusse Road, Trinidad	Worker ant	C	328
Bacterial isolate	E_3	Blanchiseusse Road, Trinidad	Worker ant	A	41
Bacterial isolate	E_4	Blanchiseusse Road, Trinidad	Worker ant	C	308
Bacterial isolate	E_5	Blanchiseusse Road, Trinidad	Worker ant	C	315
Bacterial isolate	E_6	Blanchiseusse Road, Trinidad	Worker ant	C	315
Bacterial isolate	E_7	Blanchiseusse Road, Trinidad	Worker ant	C	311
Bacterial isolate	E_8	Blanchiseusse Road, Trinidad	Worker ant	C	319
Bacterial isolate	E_9	Blanchiseusse Road, Trinidad	Worker ant	B	126
Bacterial isolate	E_{10}	Blanchiseusse Road, Trinidad	Worker ant	C	315
Bacterial isolate	E_{11}	Blanchiseusse Road, Trinidad	Worker ant	B	119
Bacterial isolate	E_{12}	Blanchiseusse Road, Trinidad	Worker ant	B	84
Bacterial isolate	E_{13}	Blanchiseusse Road, Trinidad	Worker ant	A	18
Bacterial isolate	E_{14}	Blanchiseusse Road, Trinidad	Worker ant	C	308
Bacterial isolate	E_{15}	Blanchiseusse Road, Trinidad	Worker ant	C	329
Bacterial isolate	E_{16}	Blanchiseusse Road, Trinidad	Worker ant	C	320
Bacterial isolate	E_{17}	Blanchiseusse Road, Trinidad	Worker ant	B	109

Table 2.3 Origin of fungal and bacterial symbionts.

The cultures were obtained by growing the bacteria in closed-up glass containers (universals) containing 10 ml liquid TSB/YEME (Table 2.2). The universals also contained springs to prevent cell clustering and to increase the oxygen concentration of the medium. The medium was either inoculated with 50 µl of the glycerol stocks or with a toothpick tip full of bacterial cells of single source colonies. The universals were grown overnight in a shaker incubator (200 rpm, 30 ^{o}C), and cells were harvested by centrifugation (4000 rpm, 5 minutes, 4 ^{o}C). After discarding the supernatants, the pellets were re-suspended in 800 µl of ice-cold TGE buffer. The solutions were placed into 2.0 ml Eppendorf tubes containing a small layer of glass beads.

Solution	Ingredients	Volume (µl), concentration (mM)
Tris/Borate/EDTA (TBE) buffer	Tris Base	90 mM
	Boric Acid	90 mM
	EDTA	2 mM
Tris/Glycine/EDTA (TGE) buffer (pH=8.0)	Tris	50 mM
	EDTA	10 mM
1 kb DNA ladder	Ladder Invitrogen (Cat.No.15615-016)	100 µl
	Dye	200 µl
	Distilled water	700 µl

Table 2.4 Solutions for DNA extractions and gel electrophoresis.

Fungal and bacterial samples were bead-beaten (6x30 seconds, 6 m/s). Before, during, and after bead-beating sessions, the samples were stored on ice for a minimum of 5 minutes, which is expected to reduce DNA degradation due to overheating and DNase activity. The solid remains in the samples were then separated from the DNA containing supernatants in a benchtop centrifuge (13,200 rpm, 1 minute). The supernatants were transferred to fresh 1.5 ml Eppendorf tubes and 0.5 ml of phenol:chloroform:isoamyl alcohol (25:24:1) were added. Then the tubes were vortexed for 1 minute and centrifuged (13,200 rpm, 5 minutes) to separate the aqueous supernatant from the phenol-chloroform. The upper (aqueous) layer was transferred to fresh microcentrifuge tubes. The washing procedure was repeated until the aqueous layer and phase boundary were clear and thus free of proteins. Depending on the expected DNA concentration, the purified supernatants were either washed one time with 0.5 ml chloroform:isoamyl alcohol (24:1), to remove phenol traces, or were ethanol precipitated. For ethanol precipitation, 1 ml of 100% ethanol was added to the cleaned supernatant and mixed by inverting the tube several times. The DNA was then pelleted in a benchtop centrifuge (13,200 rpm, 5 minutes). The DNA pellet was washed a second time with 200 µl of 70% ethanol. The ethanol was again removed by repeated centrifugation (13,200 rpm, 2 minutes), and the tube was left for several minutes in the safety cabinet for a final drying step. Then the dry DNA pellet was re-suspended in 50 µl of sterile dH_2O.

2.3.6 Amplification and purification of diagnostic genes

The fungal and bacterial DNA extracts were used for the amplification of diagnostic genes by polymerase chain reaction (PCR). The sample-specific reaction mixtures and thermocycles are presented in table 2.5. Fragments of the fungal (ITS region) and bacterial (16S SSU) rDNA genes were amplified using the primer sets ITS1-F–ITS4 and 515–1492, respectively (Table 2.6). The PCR reactions were analysed by running 5 µl sub-samples on diagnostic electrophoresis gels, containing agarose, Tris/Borate/EDTA (TBE) buffer (Table 2.4), and ethidium bromide. The PCR products were separated at 100 V; the quality (band strength) and correctness (band position) of the amplicons were compared under UV light to a 1 kb ladder (Table 2.4).

Polymerase chain reactions can result in the amplification of multiple genes, and hence reaction mixtures may contain a variety of amplicons. Amplicons with the correct sizes were selectively recovered from the reactions by separating the remainder of the reaction mixtures (35 µl) on purification agarose gels. Under low UV light conditions, amplicons with the correct lengths were cut out of the gel and placed individually into Eppendorf tubes. 500 µl of 'QG buffer' from the Qiagen Gel Extraction Kit (www.qiagen.com) were added to the tubes, which then were incubated at 50 ^{o}C for 10 minutes to completely dissolve the agarose and release the DNA. 200 µl of isopropanol was added to the mixture. Then the DNA was loaded on a QIAGEN QIAquick spin column.

A

PCR mixture	Volume
dH_2O	23.0 µl
GoTaq buffer (incl. loading buffer)	8.0 µl
GoTaq MgCl (25 mM)	2.4 µl
DMSO (100%)	2.0 µl
Bioline dNTP's (10 mM)	1.0 µl
Forward primer (25 µM)	1.0 µl
Reverse primer (25 µM)	1.0 µl
DNA	1.0 µl
GoTag polymerase	0.6 µl
Total	**40.0 µl**

B

Step	Fungi		Bacteria	
	Temperature	Minutes	Temperature	Minute
1	94 ^{o}C	4:00	94 ^{o}C	12:00
2	95 ^{o}C	0:30	94 ^{o}C	1:00
3	53 ^{o}C	1:00	45 ^{o}C	0:45
4	72 ^{o}C	1:00	72 ^{o}C	1:30
5	Go 34x to step 2		Go 29x to step 2	
6	72 ^{o}C	10:00	72 ^{o}C	20:00
7	end		end	

Table 2.5 (A-B) PCR mixture and thermocycles for the partial amplification of fungal (ITS region) and bacterial (16S SSU) rDNA genes.

Name	Sequence	Reference	Gene	Amplicon length
ITS1-F	5'-CTTGGTCATTTAGAGGAAGTAA-3'	Gardens and Bruns (1993)	18S rDNA	± 600 bp
ITS4	5'-TCCTCCGCTTATTGATATGC-3'	White *et al*. (1990)		
515	5'-GTGCCAGCMGCCGCGGTAA-3'	Turner *et al.* (1999)	16S rDNA	± 1000 bp
1492	5'-GGTTACCTTGTTACGACTT-3'	Turner et al. (1999)		

Table 2.6 Primer sets for the partial amplification of fungal (ITS region) and bacterial (16S SSU) rDNA genes.

The filter bound DNA was washed with 0.5 ml 'QG buffer' and 0.75 ml 'PE buffer' to remove traces of agarose and salts from the DNA, respectively. The PE buffer was then removed completely by repeated centrifugation and the DNA was eluted from the filter with 30 µl of 'EB buffer'.

2.3.7 Sequencing and BLAST-matching

The purified amplicons were sequenced with the sample-specific forward primers, ITS1-F and 515, under conditions presented in table 2.7. The sequenced PCR products were analysed by The Genome Analysis Centre TGAC (www.jicgenomelab.co.uk) using ABI 3730XL sequencers (Life Technologies). The sequences were quality-checked and truncated with FinchTV (www.geospiza.com/Products/finchtv.shtml). Then the reads were matched to the BLAST database of the National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). In particular, the BLASTn search compared the sample reads to database sequences in the

A

Sequencing mixture	Volume
dH_2O	5.5 µl
5x sequencing buffer	1.5 µl
Forward primer (3.2 µM)	1.0 µl
DNA	1.0 µl
Enzyme E3.1	1.0 µl
Total	**10.0 µl**

B

Step	Temperature	Minutes
1	96 °C	1:00
2	96 °C	0:10
3	50 °C	0:05
4	60 °C	4:00
5	Go 25x to step 2	
6	60 °C	0:10
7	end	

Table 2.7 (A-B) Reaction mixture and thermocycles for the sequencing of PCR amplicons.

nucleotide collection (nr/nt), optimising for highly similar sequences (megablast).

2.3.8 Colony bioassays

A selection of twelve actinobacterial isolates (E_2-E_4, E_6, E_8-E_{15}) was tested with colony bioassays against *Candida albicans* (CA_6, Table 3.2) and *Escovopsis spp.* (EWB, EWC, EA). The selected bacteria all belong to genera that are well-known for their antibiotic production, and will henceforth be referred to as 'NNSPS strains'. This acronym is based on the initial letters of the genera *Nocardia*, *Nocardiopsis*, *Streptomyces*, *Pseudonocardia*, and *Saccharopolyspora* (*Nocardia* and *Saccharopolyspora* were isolated in Chapters 4 and 5 from *Allomerus* and *Tetraponera*, respectively).

2.3.8.1 Bioassays against Candida

The NNSPS strains were challenged in colony bioassays against *Candida* CA_6, which was kindly provided by Neil Gow from the University of Aberdeen, United Kingdom. The yeast was maintained on agar plates containing carbenicillin- and streptomycin-enriched Lennox broth (LB) agar (Table 2.2), as described for the media of the filamentous fungi.

Universals containing 10 ml of TSB/YEME and a spring were inoculated with 30 µl of the bacterial glycerol stocks. The cultures were grown on a shaker incubator for the accumulation of sufficient biomass (280 rpm, 30 ^{o}C). The length of the incubation period was strain-specific since some strains (i.e. *Pseudonocardia*) grew more slowly. The cell material was harvested by centrifugation (4000 rpm, 5 min) and the supernatant was removed. To investigate the effect of sodium butyrate on the production of bioactive metabolites, the bacteria were grown on two sets of MS agar plates containing no antibiotics. The medium of one set was enriched with 150 mM of sodium butyrate; the medium of the other set did not contain the additive. A standardised inoculum of the bacterial cell pellet was point-inoculated into the centre of the bioassay plates and incubated for thirteen days at 21 ^{o}C. Uninoculated agar plates and dishes with *Streptomyces lividans* colonies, with

and without sodium butyrate, served as a negative control. One day before the plates were due to be overlaid with *Candida*, *Candida* was grown in universals containing 10 ml of Lennox broth and incubated in a shaker incubator at 37 ^{o}C (280 rpm) (Table 2.2).

In order to overlay the bioassay plates, which were pre-warmed to 30 ^{o}C, 1 ml of the *Candida* culture was added to universals containing 5 ml of Lennox broth (LB) soft agar (50 ^{o}C, Table 2.2). The soft agar was poured over the bioassay plates and contacted the bacterial colonies but was never poured over colonies to prevent the spread of bacterial cells and the growth of daughter colonies. The agar plates were incubated for 2 days at 26 ^{o}C to give *Candida* sufficient time for growing and developing a whitish appearance, which facilitated scoring the presence or absence of inhibition zones via contrasts with the brown colour of the medium. Inhibition zones are characterised by the lack of fungal growth around bacterial colonies, due to the presence of bacterial antifungals in the surrounding medium.

2.3.8.2 Bioassays against Escovopsis EA, EWB, EWC

The selected bacteria were also tested against *Escovopsis spp.* (EA, EWB, EWC). For the bioassays, 100 ml of TSB/YEME were inoculated with 50 µl from the bacterial glycerol stocks. The flasks were incubated in a shaker incubator to accumulate sufficient biomass (180 rpm, 30 ^{o}C). As mentioned above, some strains grew more slowly than others and therefore strain-specific incubation periods were used. The cells were harvested by centrifugation (5 minutes, 4000 rpm, 4 ^{o}C), and supernatants were discarded. Standardised fractions of the cell pellets were point-inoculated to the centre of MS agar plates, containing no antibiotics (and no sodium butyrate). The plates were incubated for 10-14 days at 21 ^{o}C. When the bacterial colonies grew to an average diameter of 1.3 cm (0.9-1.8 cm), *Escovopsis* was introduced at the edge of the bioassay plates; negative control plates, not containing any bacteria, were also inoculated with hyphal material. The plates were incubated at 21 ^{o}C. Under a stereomicroscope, the distance between the fungal growth front and the bacterial colony was

measured (test plates), or between the fungal opponent and the centre of control plates. The distances were measured every second day for a maximum of 22 days. Bioassays against EWB and EWC numbered 6-12 replicates and those against EA numbered 3-15 replicates.

2.3.9 Supernatant bioassays

Candida CA_6 and *Escovopsis* EWC were also challenged against culture supernatants of the NNSPS strains (E_2-E_4, E_6, E_8-E_{15}). Supernatant bioassays were conducted to discover whether the bacteria secrete their antifungals into a liquid environment.

2.3.9.1 Obtaining supernatants

Mannitol soy-flour (MS) broth (Table 2.2) was used as liquid environment to test antifungal production. The bacterial isolates were grown in flasks containing a spring and 50 ml of MS broth. The flasks were inoculated with 30 µl from the bacterial glycerol stocks. As negative controls, one flask remained uninoculated, and another flask was inoculated with spores of *S. lividans*. The experiment was carried out in duplicate with one set of growth media containing 150 mM of sodium butyrate and the other set without. Inoculated flasks were incubated in a shaker incubator for 14 days (30 ^{o}C, 250 rpm). Possibly due to the small inoculation volume (and/or low spore/cell concentrations in the glycerol stocks), a longer incubation period was needed to accumulate bacterial cells and sometimes observe a colour change. The supernatants were harvested by centrifuging out much of the cell material and insoluble medium (30 minutes, 4000 rpm, 4 ^{o}C). After this treatment, the supernatants of the coloured growth cultures retained their colouration, which can possibly be attributed to secreted (non)bioactive metabolites. For carrying out bioassays, small sub-samples of the supernatants were centrifuged a second time with a benchtop centrifuge (5 minutes, 13,200 rpm). The supernatants were then filter-sterilised with 0.2 µm filters.

2.3.9.2 Bioassays against Candida

The sterile supernatants, with and without sodium butyrate, were tested against *Candida* CA_6. Two sets of paper filter disks, with a diameter of 0.9 cm, were each inoculated with 100 µl from the supernatants and the negative control liquids. Sterile Petri dishes containing LB agar were coated with 400 µl of a one-day-old *C. albicans* culture, grown in 10 ml of Lennox broth at 37 ^{o}C. When the disks and plates were dry, the filters were attached to the *Candida* lawn with 60 µl of dH_2O. The presence or absence of inhibition zones was scored after the plates were incubated at 26 ^{o}C for two days.

2.3.9.3 Bioassays against Escovopsis EWC

The same sterile growth supernatants, lacking sodium butyrate and previously being bioassayed against *Candida*, were tested here against *Escovopsis* EWC. As a negative control, the corresponding supernatant from an uninoculated batch of MS broth was used. Paper filter disks, with a diameter of 4 cm, were loaded each with 2 ml of the supernatants. Compared to the smaller filter disks for the *Candida* bioassays, comparably large filter disks were used for the bioassays against *Escovopsis*, since size had to be adjusted to account for the length of the hyphae of the filamentous fungus. With 200 µl of dH_2O, the dry disks were attached to MS agar plates, containing no antibiotics. Thereafter, the fungal strains were introduced by transferring a standardised inoculum of hyphal material to the edge of the bioassay plates. The plates were incubated at 26 ^{o}C until the filter disks were either contacted by the fungi or clearly avoided.

2.4 Results

2.4.1 Isolation and microscopy of ant-associated bacteria

The bioactive bacterial microbiome of *Acromyrmex* worker ants was studied in a culture-dependent way by isolating ectosymbiotic bacteria from the cuticles of worker ants. The workers originated from three ant colonies (A, B, C), collected in Trinidad, and imported to the United Kingdom. Sub-samples of these colonies were sent to the UEA around 12 weeks post-collection. The sub-

samples contained 60, 86, and 68 worker ants and some garden cultivar. The reason for using workers from the cultivar garden was to increase the likelihood for young major workers with abundant cuticular bacteria. The ectosymbiotic bacteria were removed from the cuticles of the individuals by washing and streaking. The majority (65%) of the 214 worker ants was washed in 2G. Because microbial cultures were not obviously dense on the cuticle, the wash of most ants was used undiluted for inoculations of HC and MS agar plates; only a fraction of the washes (86 out of 140 washes) was additionally diluted ten times. Ectosymbiotic bacteria from the remaining 35% of the 214 workers were isolated by streaking their ventral and dorsal sides over agar plates with the same media. The nutrient content of the media was chosen to be low in order to decrease the likelihood of fast-growing bacteria out-competing and/or inhibiting the growth of slow-growing ones, thereby increasing plate biodiversity.

Most, if not all, isolation plates harboured a community of bacteria, including Gram-positive and Gram-negative morphotypes. Actinobacteria were isolated from the mixed bacterial communities by repeated propagation of single colonies to fresh MS agar plates. This medium was chosen because morphotypes in general were less distinguishable on HC than on MS agar. The focus was placed on actinobacteria due to their well-known association with attine ants and because this taxon contains many antibiotic-producing bacterial genera. Due to the qualitative character of the isolation method, morphotypes were not selected twice. Therefore, no conclusions can be made about the distribution and abundance of individual strains on the cuticles of the worker ants. Furthermore, bacterial species may be lost on isolation plates due to the culturing conditions, such as temperature, humidity, and ingredients of the isolation media. The purified bacterial lineages were preserved in 2G. Not all actinobacterial isolates produced spores, even after extended incubation times. Hence, glycerol stocks contained both spores of spore-producing lineages and cell material of strains that did not produce spores. Due to their role in the bacterial reproduction, spores are more resistant to environmental stresses than

regular cells and thus are expected to be more suitable for long-term storage in glycerol; however, cell survival was checked regularly.

Sixteen bacterial morphotypes (E_2-E_{17}) were purified from the isolation plates. Figure 2.3 shows stereomicroscope photographs of the bacterial isolates. The secondary metabolites of some bacteria accumulated as transparent or coloured droplets on the colony surface.

2.4.2 Procurement and microscopy of fungal symbionts

The fungal parasites were obtained from ant colonies other than the ones used for the bacterial isolations. The garden material of colonies A-C, obtained along with the worker ants, was plated out on agar plates containing different fungal isolation media (data not shown). The agar plates were then screened for *Escovopsis*; however, other non-cultivar fungi rapidly overgrew the fungal inocula instead. Hence, *Escovopsis* was obtained from the Fungal Biodiversity Centre. The same *Escovopsis* strains were also used by Kost *et al.* (2007), CBS 810.71, Schoenian *et al.* (2011), CBS 110660, and Haeder *et al.* (2009), CBS 110660 and CBS 423.93, to carry out fungal bioassays.

The *Escovopsis* strains originated from different attine genera and geographical distributions. In particular, *Escovopsis weberi* EWB and EWC were isolated from attine nests (genus not identified) in Brazil and Colombia, respectively; *E. aspergilloides* (EA) was isolated from a *Trachymyrmex* nest. Multiple *Escovopsis* strains were chosen to account for the possibility that attine ants may maximise their bacterial defence by continuously adjusting their symbionts to changing cultivar specific *Escovopsis* invaders. In this case, bioassay challenges between bacterial symbionts and alien *Escovopsis* strains could lead to suboptimal matches in antifungal effectiveness and fungal susceptibility. In other words, testing bacterial symbionts against various (nest-unrelated) *Escovopsis* strains may increase the likelihood of finding a susceptible strain. On MS agar, *Escovopsis* EWC and EA showed consistent and dense growth patterns; in contrast, *Escovopsis* EWB often grew patchily. The fungal cultivar *L. gongylophorus* was obtained from Ulrich Mueller.

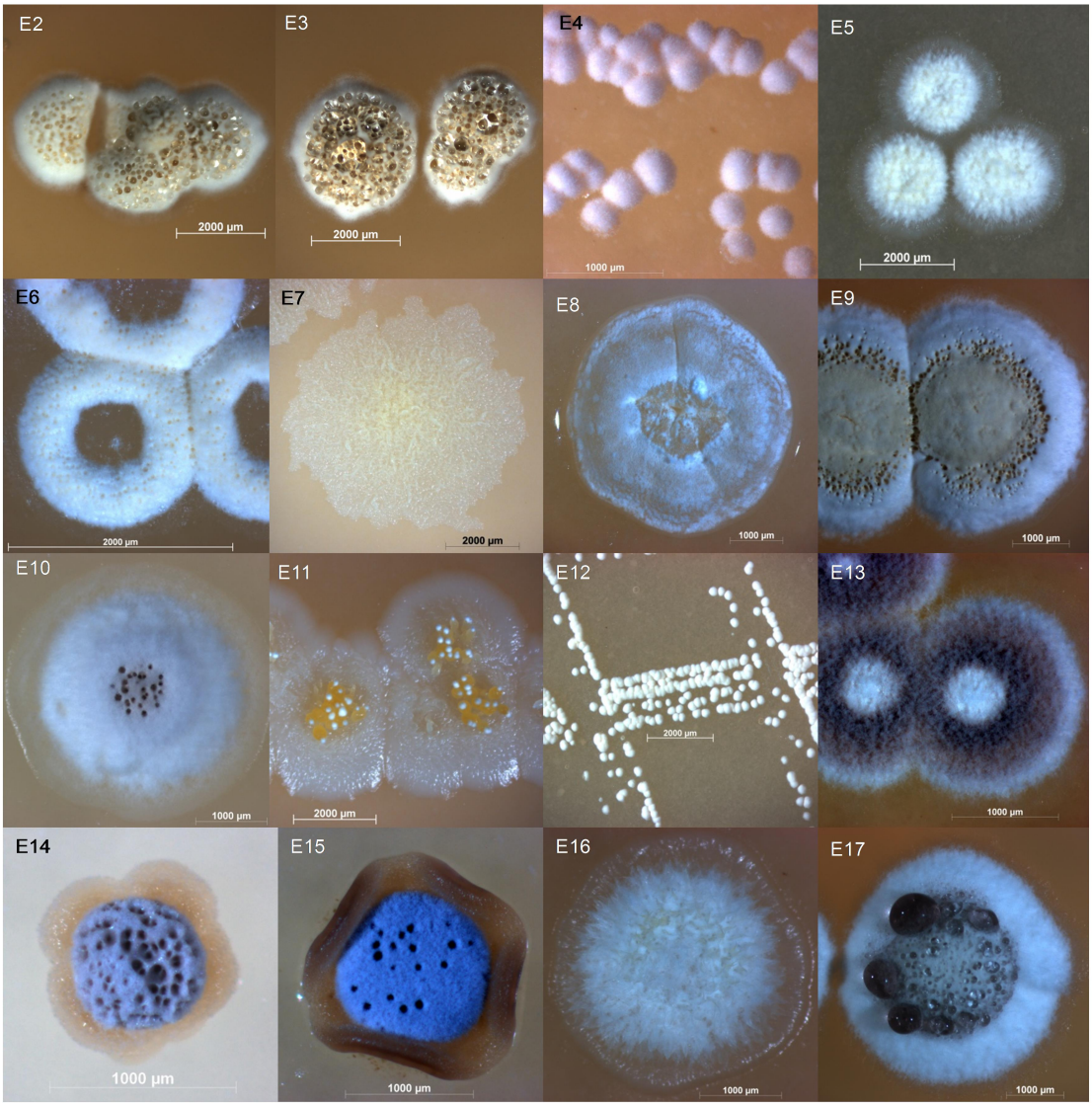


Figure 2.3 Microscopy photographs of 16 bacterial isolates (E_2-E_{17}). The bacteria were isolated from the cuticles of *Acromyrmex octospinosus* worker ants and belong to the actinobacterial genera *Streptomyces* (E_2, E_6, E_8, E_9, E_{10}, E_{13}, E_{14}, E_{15}), *Pseudonocardia* (E_4, E_{12}), *Nocardiopsis* (E_5, E_{11}, E_{16}, E_{17}), and *Tsukamurella* (E_7). Despite of morphologically resembling *Streptomyces*, strain E_3 genetically matches *Streptomyces* and *Nocardia* sequences of the NCBI database. An overview of the bacterial origin and details on their genetic identity are provided in tables 2.3 and 2.8, respectively. A selection ('NNSPS strains') of twelve bacteria (E_2-E_4, E_6, E_8-E_{15}), belonging to antibiotic-producing genera, are bioassayed against various test fungi.

Stereomicroscope pictures of *Escovopsis* and a regular photograph of the fungal cultivar are shown in figure 2.4.

2.4.3 Amplification, purification, sequencing, and BLAST-matching *of diagnostic genes from genomic DNA extracts*

Genomic DNA was extracted from the fungal mutualist (*L. gongylophorus*), the fungal parasites (*Escovopsis* EWB, EWC, EA), and the bacterial isolates (E_2-E_{17}) (Table 2.3). The ITS region of the fungal rDNA gene was sequenced

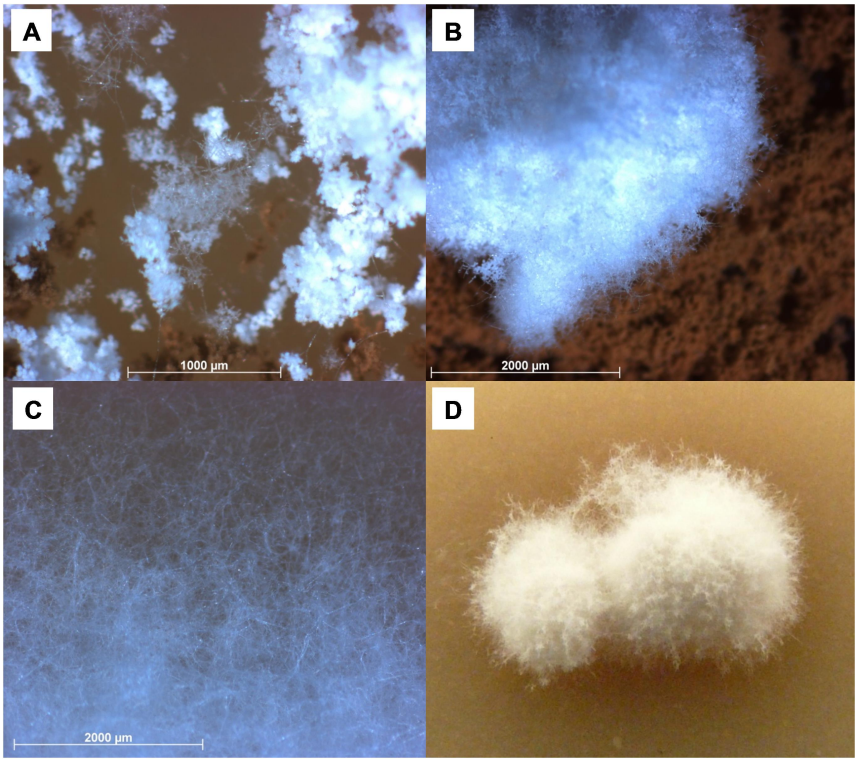


Figure 2.4 Fungal symbionts of attine ants. **(A-C)** Fungal parasites obtained from the Fungal Biodiversity Centre (www.cbs.knaw.nl). In sequence, the pictures show *Escovopsis weberi* (EWB) from a Brazilian ant nest, *Escovopsis weberi* (EWC) from a Colombian ant nests, and *Escovopsis aspergilloides* (EA) from a *Trachymyrmex ruthae* nest. *Escovopsis* is known to feed on the hyphae of the fungal cultivar. **(D)** The *Acromyrmex* ant-associated fungal cultivar *Leucoagaricus gongylophorus*, provided by Ulrich Mueller from the University of Texas. An overview on the fungal origin, and details on their genetic identity, is provided in tables 2.3 and 2.8.

partially. The ITS sequence of *L. gongylophorus* is 280 bp in length and shows BLAST analogies with *L. gongylophorus* (AY642813, Table 2.8). The ITS reads of *Escovopsis* EWB, EWC, and EA are respectively 604 bp, 593 bp, and 613 bp in length. The sequences of both *E. weberi* strains match *Escovopsis* (FJ948131, Table 2.8). In contrast, the one of *E. aspergilloides* matches a *Trametes trogii* entry (HM989941, Table 2.8), which might be explained by a limitation of corresponding sequences in the NCBI database. The Supplementary Information A_1 provides a detailed listing of the sequencing data.

In addition, a fragment of the 16S rDNA gene was sequenced from the genomes of the bacterial isolates. The bacterial sequences are on average 750 bp (608-897 bp) in length. Based on NCBI database searches, the strains can be sub-divided into four actinobacterial genera including *Nocardiopsis*, *Pseudonocardia*, *Streptomyces*, and *Tsukamurella*. Strains E_2, E_6, E_8, E_9, E_{10}, E_{13}, E_{14}, and E_{15} match *Streptomyces*; the sequence of strain E_3 relates to database entries of both *Streptomyces* and *Nocardia* (but morphologically resembles *Streptomyces*), strains E_4 and E_{12} are in the genus *Pseudonocardia*; strains E_5, E_{11}, E_{16}, and E_{17} belong to a group of *Nocardiopsis* bacteria; and E_7 is a *Tsukamurella* strain (accession nos. in Table 2.8). It is interesting to note that, in contrast with the vertically transmitted ant-associate *Pseudonocardia*, bacterial morphotypes in the genus *Streptomyces* are more numerous and also can be isolated from all of the ant colonies (A-C). A detailed listing of the sequencing data is shown in the Supplementary Information A_1.

2.4.4 Colony bioassays

Streptomyces E_2, E_3, E_6, E_8, E_9, E_{10}, E_{13}, E_{14}, and E_{15}; *Pseudonocardia* E_4 and E_{12}; and *Nocardiopsis* E_{11} ('NNSPS' strains) were tested for their ability to inhibit the medically important yeast *Candida* CA_6 and nest parasites *Escovopsis* EWB, EWC, and EA.

Sample type	Name	PCR primers	Sequencing primer	Sequence length	Genus	Closest relative (accession)	Max score	Total score	Query coverage	E value	Max ident
Fungal cultivar	*L. gongylophorus*	ITS1-F–ITS4	ITS1-F	280 bp	*Leucoagaricus*	*Leucoagaricus gongylophorus* isolate I internal transcribed spacer 1, partial sequence (AY642813.1)	496	496	100%	4.00E-137	98%
Non-cultivar fungus	*E. weberi* EWB	ITS1-F–ITS4	ITS1-F	604 bp	*Escovopsis*	*Escovopsis* sp. TF2CWG 18S ribosomal RNA gene, partial sequence (FJ948131.1)	702	702	100%	0	88%
Non-cultivar fungus	*E. weberi* EWC	ITS1-F–ITS4	ITS1-F	593 bp	*Escovopsis*	*Escovopsis* sp. TF2CWG 18S ribosomal RNA gene, partial sequence (FJ948131.1)	695	695	100%	0	88%
Non-cultivar fungus	*E. aspergilloides* EA	ITS1-F–ITS4	ITS1-F	613 bp	*Trametes*	*Trametes trogii* isolate TEM H2 18S ribosomal RNA gene, partial sequence (HM989941.1)	1127	1127	100%	0	99%
Bacterial isolate	E_2	515–1492	515	750 bp	*Streptomyces*	*Streptomyces* sp. S1(2010) 16S ribosomal RNA gene, partial sequence (HM179225.1)	1386	1386	100%	0	100%
Bacterial isolate	E_3	515–1492	515	708 bp	*Streptomyces*	*Streptomyces* sp. SA36 16S ribosomal RNA gene, partial sequence (GU294698.1)	1308	1308	100%	0	100%
					Nocardia	*Nocardia* sp. JJE-2 16S ribosomal RNA gene, partial sequence (GU132484.1)	1308	1308	100%	0	100%
Bacterial isolate	E_4	515–1492	515	707 bp	*Pseudonocardia*	*Pseudonocardia* sp. P1(2010) 16S ribosomal RNA gene, partial sequence (HM179227.1)	1306	1306	100%	0	100%
Bacterial isolate	E_5	515–1492	515	608 bp	*Nocardiopsis*	*Nocardiopsis* sp. JJF-6 16S ribosomal RNA gene, partial sequence (GU132509.1)	1118	1118	100%	0	99%
Bacterial isolate	E_6	515–1492	515	725 bp	*Streptomyces*	*Streptomyces* sp. S3(2010) 16S ribosomal RNA gene, partial sequence (HM179228.1)	1339	1339	100%	0	100%
Bacterial isolate	E_7	515–1492	515	762 bp	*Tsukamurella*	*Tsukamurella* sp. C35 16S ribosomal RNA gene, partial sequence (GU183394.1)	1408	1408	100%	0	100%
Bacterial isolate	E_8	515–1492	515	830 bp	*Streptomyces*	*Streptomyces* sp. S4(2010) 16S ribosomal RNA gene, partial sequence (HM179229.1)	1533	1533	100%	0	100%
Bacterial isolate	E_9	515–1492	515	760 bp	*Streptomyces*	*Streptomyces* sp. FXJ8.036 16S ribosomal RNA gene, partial sequence (HQ622492.1)	1404	1404	100%	0	100%
Bacterial isolate	E_{10}	515–1492	515	697 bp	*Streptomyces*	*Streptomyces* sp. FXJ8.063 16S ribosomal RNA gene, partial sequence (HQ622509.1)	1288	1288	100%	0	100%
Bacterial isolate	E_{11}	515–1492	515	701 bp	*Nocardiopsis*	*Nocardiopsis* sp. SA6 16S ribosomal RNA gene, partial sequence (GU997639.1)	1295	1295	100%	0	100%
Bacterial isolate	E_{12}	515–1492	515	825 bp	*Pseudonocardia*	*Pseudonocardia* sp. P2(2010) 16S ribosomal RNA gene, partial sequence (HM179232.1)	1524	1524	100%	0	100%
Bacterial isolate	E_{13}	515–1492	515	780 bp	*Streptomyces*	*Streptomyces* sp. S7(2010) 16S ribosomal RNA gene, partial sequence (HM179233.1)	1441	1441	100%	0	100%
Bacterial isolate	E_{14}	515–1492	515	656 bp	*Streptomyces*	*Streptomyces* sp. BK166 partial 16S rRNA gene, strain BK166 (FR692106.1)	1212	1212	100%	0	100%
Bacterial isolate	E_{15}	515–1492	515	783 bp	*Streptomyces*	*Streptomyces* sp. S9(2010) 16S ribosomal RNA gene, partial sequence (HM179235.1)	1447	1447	100%	0	100%
Bacterial isolate	E_{16}	515–1492	515	897 bp	*Nocardiopsis*	*Nocardiopsis* sp. 13634A 16S ribosomal RNA gene, partial sequence (EU741113.1)	1652	1652	100%	0	99%
Bacterial isolate	E_{17}	515–1492	515	817 bp	*Nocardiopsis*	*Nocardiopsis* sp. JJF-6 16S ribosomal RNA gene, partial sequence (GU132509.1)	1509	1509	100%	0	100%

Table 2.8 Genetic identity of fungal and bacterial symbionts. Details on their origin and an overview of the sequencing data are presented in table 2.3 and in the Supplementary Information A_1, respectively.

2.4.4.1 Bioassays against Candida

The NNSPS isolates were challenged against *Candida* CA_6 on agar plates that were or were not enriched with (150 mM) sodium butyrate; these were overlaid with soft agar containing the yeast. *Streptomyces* E_8, E_9, and E_6 produced secondary metabolites that inhibited *Candida* on agar plates without sodium butyrate (Fig. 2.5_A). *Streptomyces* E_8 and E_9, and *Pseudonocardia* E_4, inhibited *Candida* on sodium butyrate-containing agar plates (Fig. 2.5_B). The results indicate that only a minority of bacterial isolates is able to inhibit the growth of *Candida in vitro*, and confirm that sodium butyrate can stimulate antifungal production in certain bacteria (i.e. E_4). It is possible that using a lower concentration of sodium butyrate might have more effectively stimulated antifungal production by the other bacterial strains because the concentration used seemed to prevent *Streptomyces* E_6 from growing and producing bioactive metabolites.

2.4.4.2 Bioassays against Escovopsis

The NNSPS strains were also tested on agar plates against *Escovopsis* spp. (EWB, EWC, EA). The time-series graphs of figure 2.6 show the average distances (+/- s.e.) between the fungal growth front and bacterial colonies as a percentage of the starting distance on day zero. A numerical representation of the data and photographs of representative replica bioassay plates are given in table 2.9 and figure 2.7, respectively.

At the end of the experiment (day 22), one of the bacterial strains (*Nocardiopsis* E_{11}) was not overgrown by *Escovopsis* EA, relating to one of 15 replica plates; the replica plates of all other strains were all overgrown by the parasitic fungus (Fig. 2.6_A). However, the average size of the inhibition zones in bioassay plates of *Nocardiopsis* E_{11} was only 0.01 cm (Table 2.9).

In comparison, *Escovopsis* EWB was more susceptible to some of the bacterial metabolites as indicated by generally less ‘S-shaped’ curves in the graph (Fig. 2.6_B).

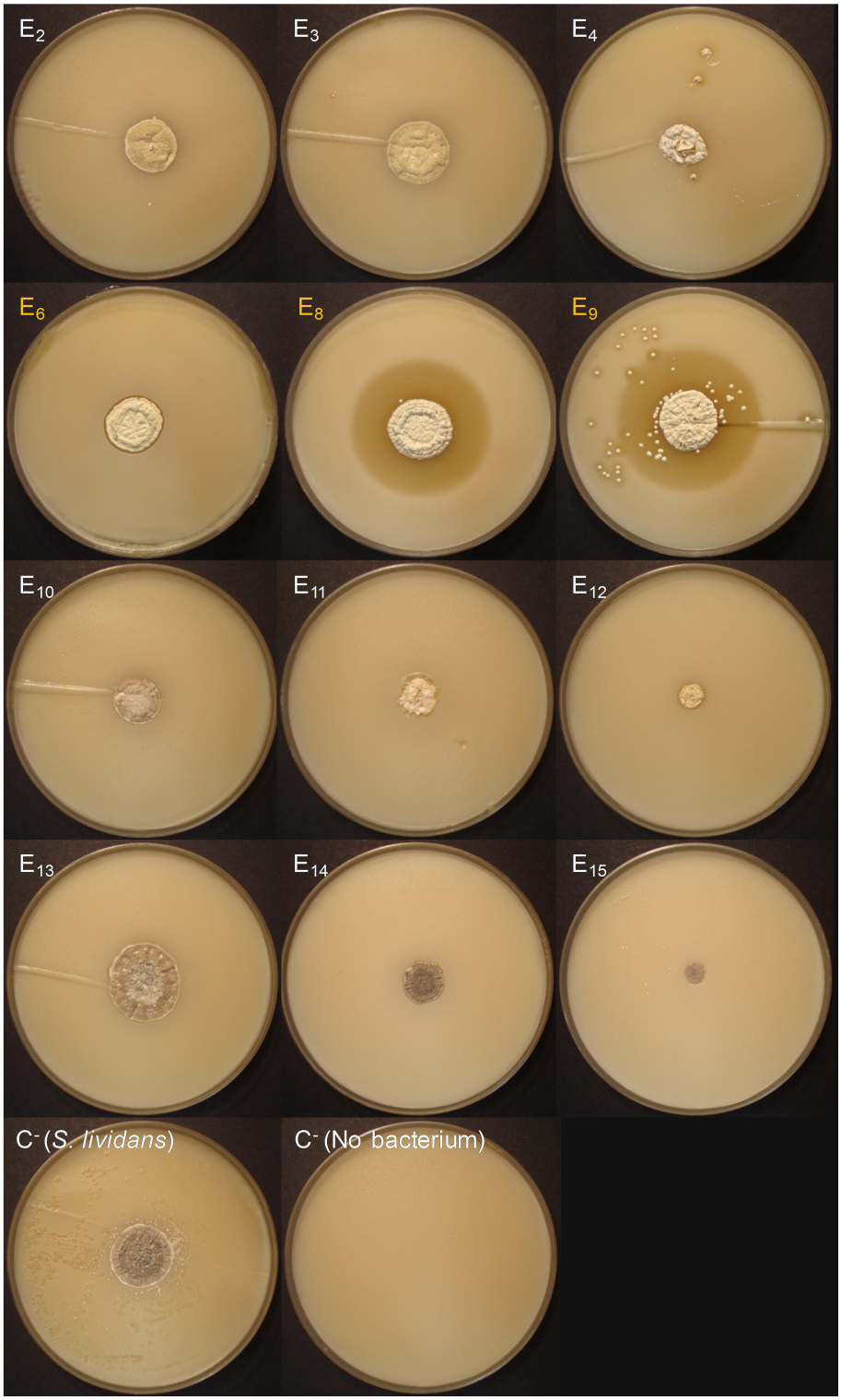


Figure 2.5 (A) *Candida* (CA_6) colony bioassays of NNSPS isolates (E_2-E_4, E_6, E_8-E_{15}). An uninoculated agar plate (C^- No bacterium) and one with a *S. lividans* colony (C^- *S. lividans*) were included as negative controls. *Streptomyces* E_8, E_9, and E_6 (orange font) are secreting effective antifungals into the medium surrounding the colony, resulting in inhibition zones with no or reduced fungal growth.

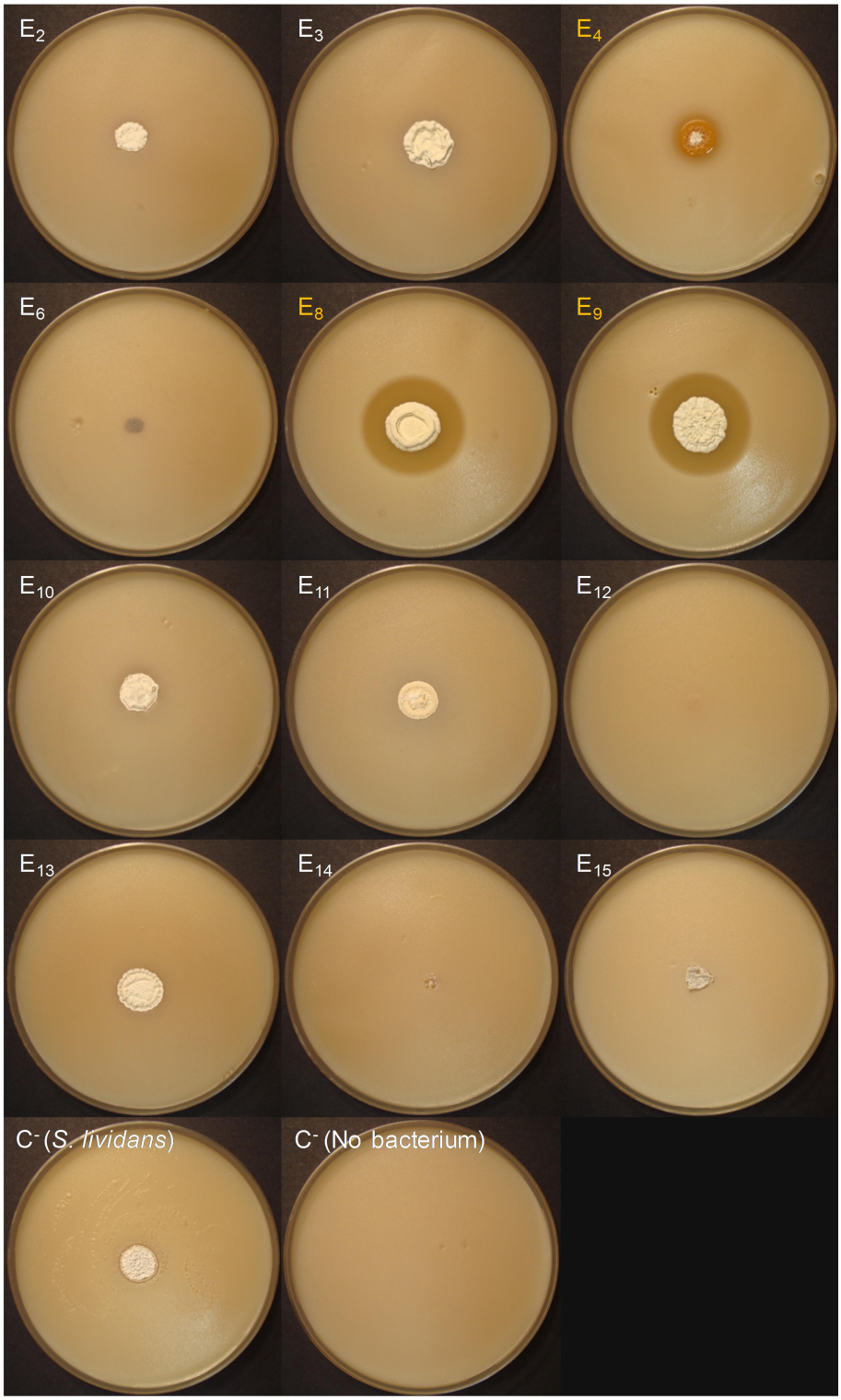


Figure 2.5 (B) *Candida* (CA_6) colony bioassays of NNSPS isolates (E_2-E_4, E_6, E_8-E_{15}). An uninoculated agar plate (C^- No bacterium) and one with a *S. lividans* colony (C^- *S. lividans*) were included as negative controls. Here, the agar plates were enriched with 150 mM of sodium butyrate. *Streptomyces* E_8 and E_9, as well as *Pseudonocardia* E_4 (orange font) are inhibiting *Candida*.

In particular, replica plates of *Streptomyces* E_6 (0.12 cm), E_9 (0.02 cm), and E_{13} (0.15 cm), *Pseudonocardia* E_4 (0.03 cm), as well as *Nocardiopsis* E_{11} (0.31 cm) were not overgrown by the fungus (Table 2.9). The most inhibitory bacterial strain was *Nocardiopsis* E_{11}, since 75% of its replica colonies remained uncontacted by *Escovopsis* EWB, leading to a final mean distance between bacterial and fungal opponents of 0.31 cm.

In contrast with EA and EWB, *Escovopsis* EWC was more susceptible to the bacterial metabolites (Fig. 2.6_C). On average, 95% of the replica colonies of *Streptomyces* E_2, E_3, E_6, E_8, E_9, E_{10}, E_{13}, and E_{14}, *Pseudonocardia* E_4, and *Nocardiopsis* E_{11}, were not contacted by fungal hyphae after 22 days (Table 2.9). The bioassay plates of the remaining strains, *Streptomyces* E_{15} and *Pseudonocardia* E_{12}, were all overgrown by the parasitic fungus. Among the successful strains, *Streptomyces* E_8, E_9, and E_{13}, as well as *Nocardiopsis* E_{11} showed the largest mean inhibition zones, spanning 1.17 cm, 1.12 cm, and 1.05 cm, as well as 1.92 cm, respectively. In contrast, *Streptomyces* E_3 produced the smallest inhibition zones of only 0.22 cm.

In summary, the metabolites of some NNSPS strains indeed possess antifungal activity against *Escovopsis* spp., but two of the fungal test strains (EWB, EA) were mostly resistant to the compounds.

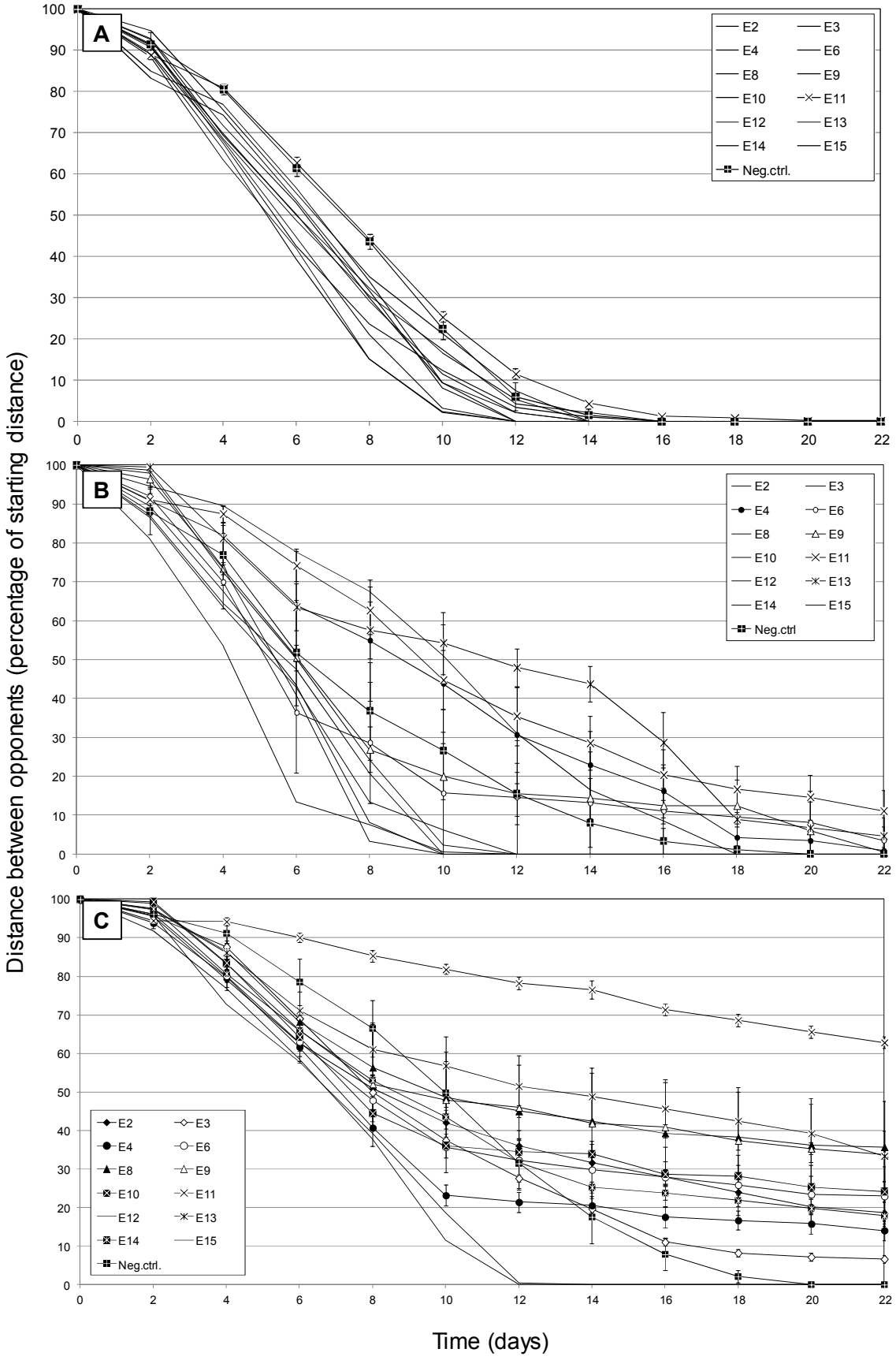

A
B
C
E2
E3
E4
E6
E8
E9
E10
E11
E12
E13
E14
E15
Neg.ctrl.
Neg.ctrl
Distance between opponents (percentage of starting distance)
Time (days)

Figure 2.6 *Escovopsis* (EA, EWB, EWC) colony bioassays of NNSPS isolates (E_2-E_4, E_6, E_8-E_{15}). The bacteria were inoculated into the centre of agar plates containing no sodium butyrate, and the fungi were grown from the edge. The X-axes show the time over a period of 22 days; the Y-axes show the distance between the fungi and bacterial colonies as a percentage of the starting distance, averaged across replica plates (± s.e.). Slopes that are shallower than the control therefore indicate fungal inhibition. However, bacterial strains are only termed successful (marker displayed), if at least one of its replica colonies is not overgrown and the mean distance value at the end of the experiment remained larger than zero. A numerical representation of these data is given in table 2.9. **(A)** Bioassays against *Escovopsis* EA. This fungus is highly resistant to any of the bacterial secretions, and exhibiting 'S-shaped' growth curves. Most successful is *Nocardiopsis* E_{11}, showing a mean final distance of 0.01 cm. **(B)** Bioassays against *Escovopsis* EWB. Various bacterial strains are successful against this fungus (E_4, E_6, E_9, E_{11}, E_{13}). Again most successful is *Nocardiopsis* E_{11}; 75% of its replica colonies are not overgrown by the fungus, resulting in a mean final distance of 0.31 cm. **(C)** Bioassays against *Escovopsis* EWC. Ten out of twelve bacterial isolates are successfully inhibiting the growth of this fungus; among the successful strains are *Nocardiopsis* E_{11} (1.92 cm) as well as *Streptomyces* E_8 (1.17 cm), E_9 (1.12 cm), and E_{13} (1.05 cm).

Strain	*E. aspergilloides* (EA)				*E. weberi* (EWB)				*E. weberi* (EWC)			
	No. replica plates	No. success-ful plates	Success rate (%)	Mean distance (cm)	No. replica plates	No. success-ful plates	Success rate (%)	Mean distance (cm)	No. replica plates	No. success-ful plates	Success rate (%)	Mean distance (cm)
E_2	3	0	0.0%	0.00	6	0	0.0%	0.00	6	6	100.0%	0.57
E_3	3	0	0.0%	0.00	6	0	0.0%	0.00	6	4	66.7%	0.22
E_4	4	0	0.0%	0.00	7	1	14.3%	0.03	7	7	100.0%	0.46
E_6	3	0	0.0%	0.00	6	1	16.7%	0.12	6	6	100.0%	0.77
E_8	3	0	0.0%	0.00	6	0	0.0%	0.00	6	5	83.3%	1.17
E_9	3	0	0.0%	0.00	6	1	16.7%	0.02	6	6	100.0%	1.12
E_{10}	3	0	0.0%	0.00	6	0	0.0%	0.00	6	6	100.0%	0.72
E_{11}	15	1	6.7%	0.01	12	9	75.0%	0.31	12	12	100.0%	1.92
E_{12}	4	0	0.0%	0.00	7	0	0.0%	0.00	7	0	0.0%	0.00
E_{13}	3	0	0.0%	0.00	6	1	16.7%	0.15	6	6	100.0%	1.05
E_{14}	3	0	0.0%	0.00	6	0	0.0%	0.00	6	6	100.0%	0.60
E_{15}	3	0	0.0%	0.00	6	0	0.0%	0.00	6	0	0.0%	0.00
C^-	6	0	0.0%	0.00	9	0	0.0%	0.00	12	0	0.0%	0.00

Table 2.9 Numerical representation of the colony bioassay results of figure 2.6. The NNSPS strains are challenged against *Escovopsis* EA, EWB, and EWC. Successful bioassay performances are highlighted with red boxes.

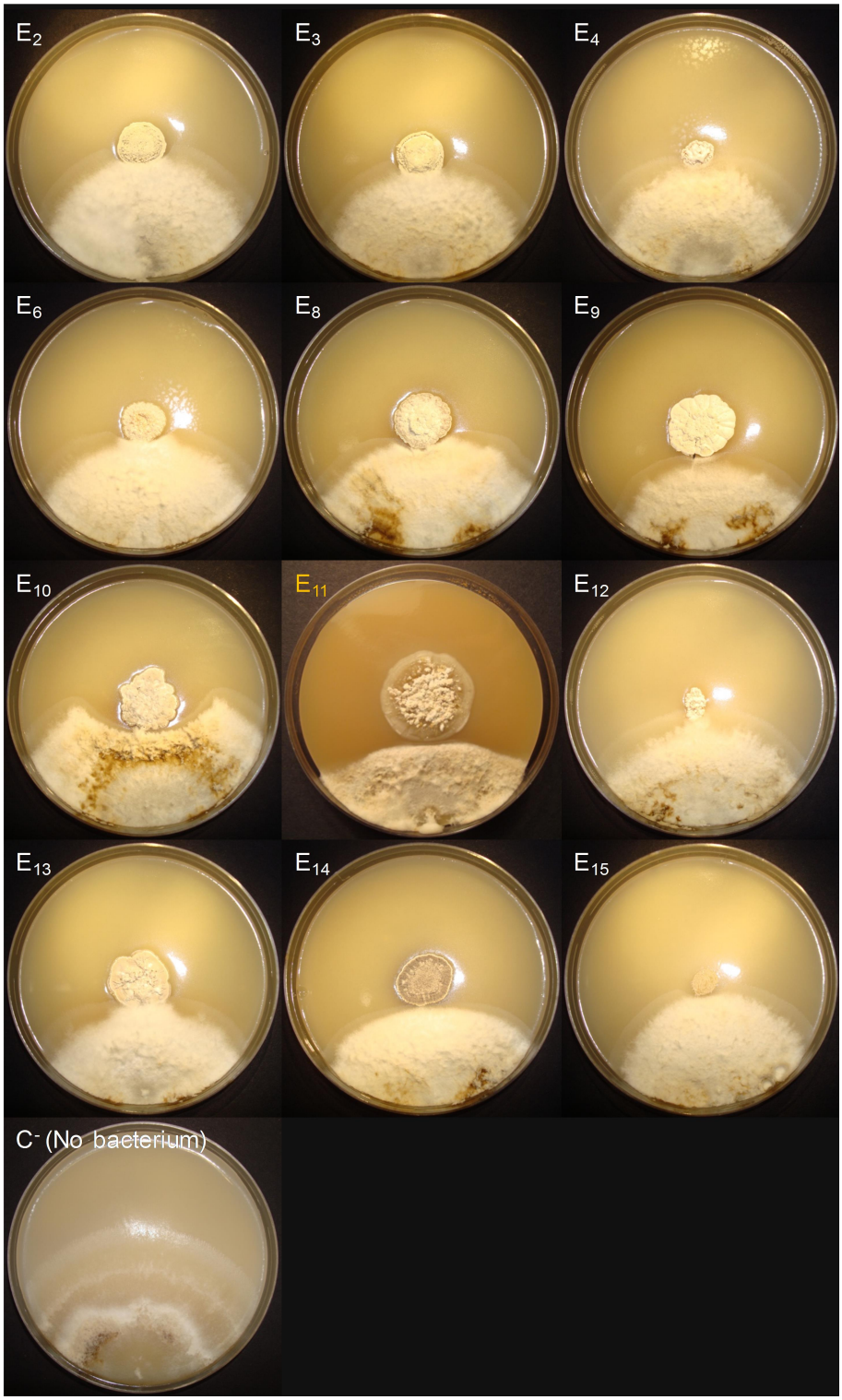


Figure 2.7 (A) *Escovopsis* (EA) colony bioassays, as described in figure 2.6. Replica colonies not overgrown by the test fungus are shown for successful bacterial strains and overgrown colonies for unsuccessful strains. Due to the microscopic size of the fungal hyphae, the fungal growth front is largely unrecognisable on the pictures, and thus successful strains are highlighted with orange font.

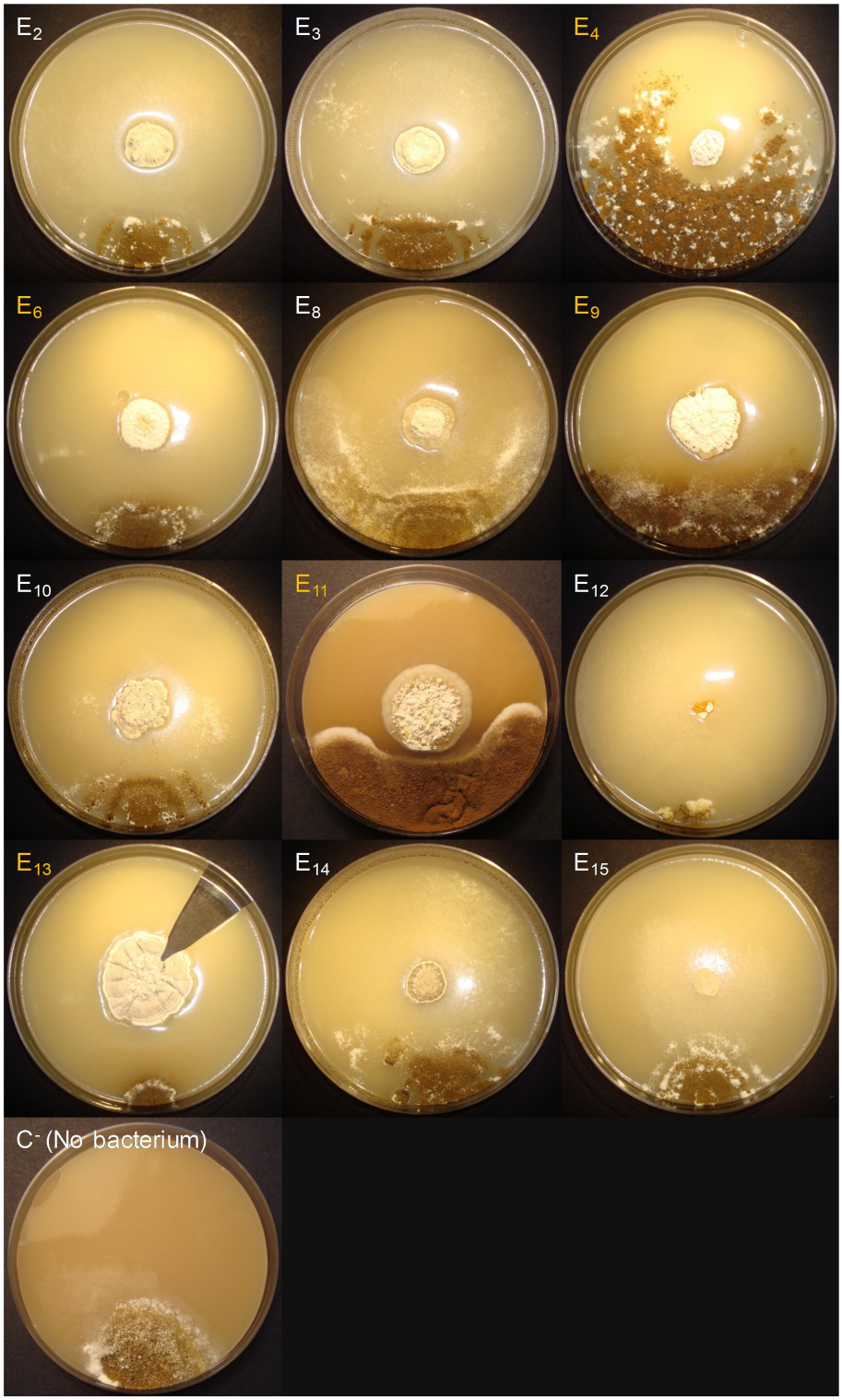


Figure 2.7 (B) *Escovopsis* (EWB) colony bioassays, as described in figure 2.6. Replica colonies not overgrown by the test fungus are shown for successful bacterial strains and overgrown colonies for unsuccessful strains. Successful strains are highlighted with orange font.

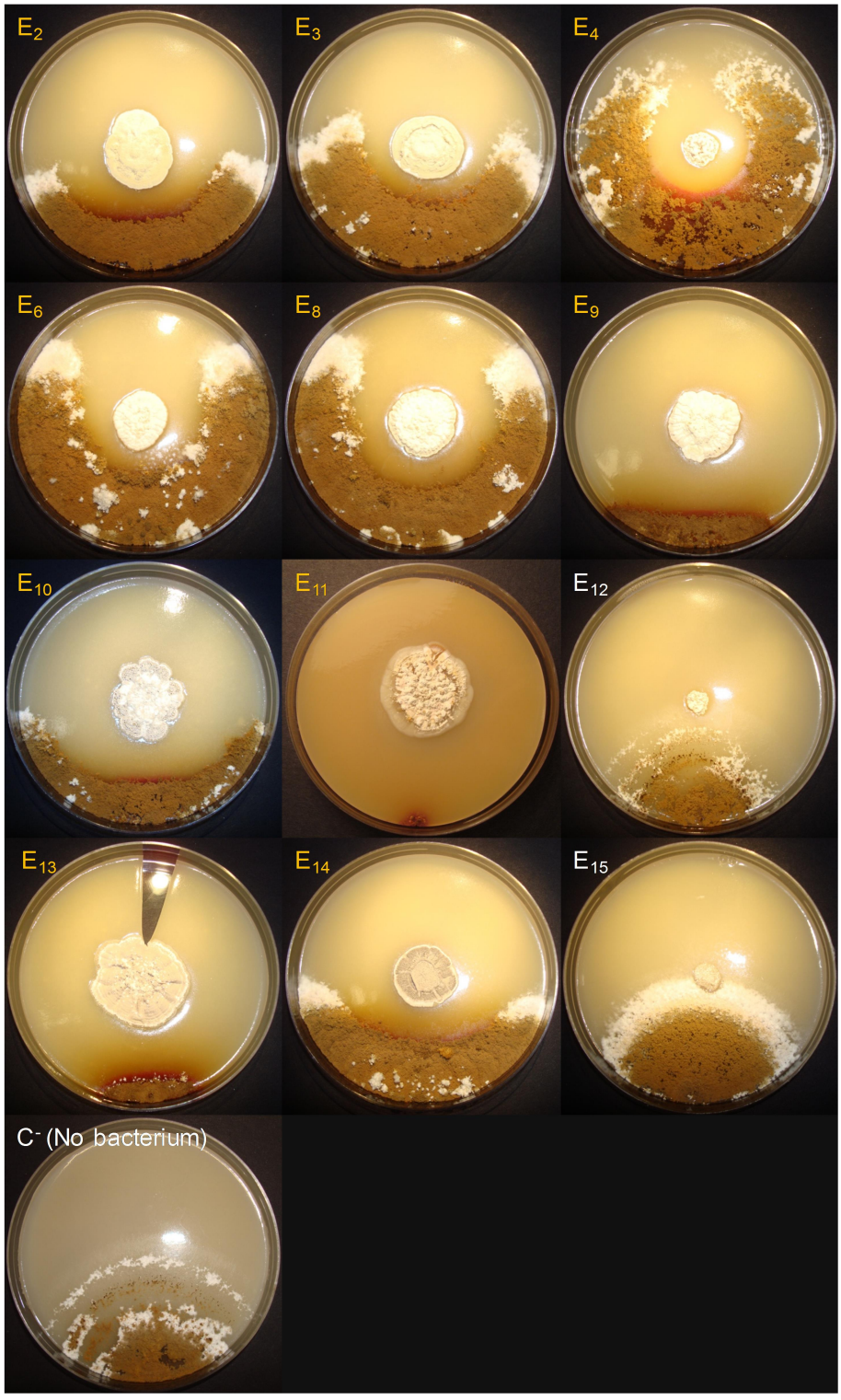


Figure 2.7 (C) *Escovopsis* (EWC) colony bioassays, as described in figure 2.6. Replica colonies not overgrown by the test fungus are shown for successful bacterial strains and overgrown colonies for unsuccessful strains. Successful strains are highlighted with orange font.

2.4.5 Supernatant bioassays

The NNSPS isolates (E_2-E_4, E_6, E_8-E_{15}) were also tested for their ability to secrete antifungal metabolites into a liquid environment, with and without enrichment by sodium butyrate. The culture media were challenged against *Candida* CA_6 and *Escovopsis* EWC, which is the most susceptible of the three *Escovopsis* strains (Fig. 2.6_C).

2.4.5.1 Bioassays against Candida

Culture media from *Streptomyces* E_8 and E_9 alone inhibited *Candida*, with and without sodium butyrate (Fig. $2.8_{A\text{-}F}$). Recall that *Streptomyces* E_8 and E_9 also inhibited the yeast during colony bioassays both in the presence and absence of sodium butyrate (Fig. $2.5_{A\text{-}B}$).

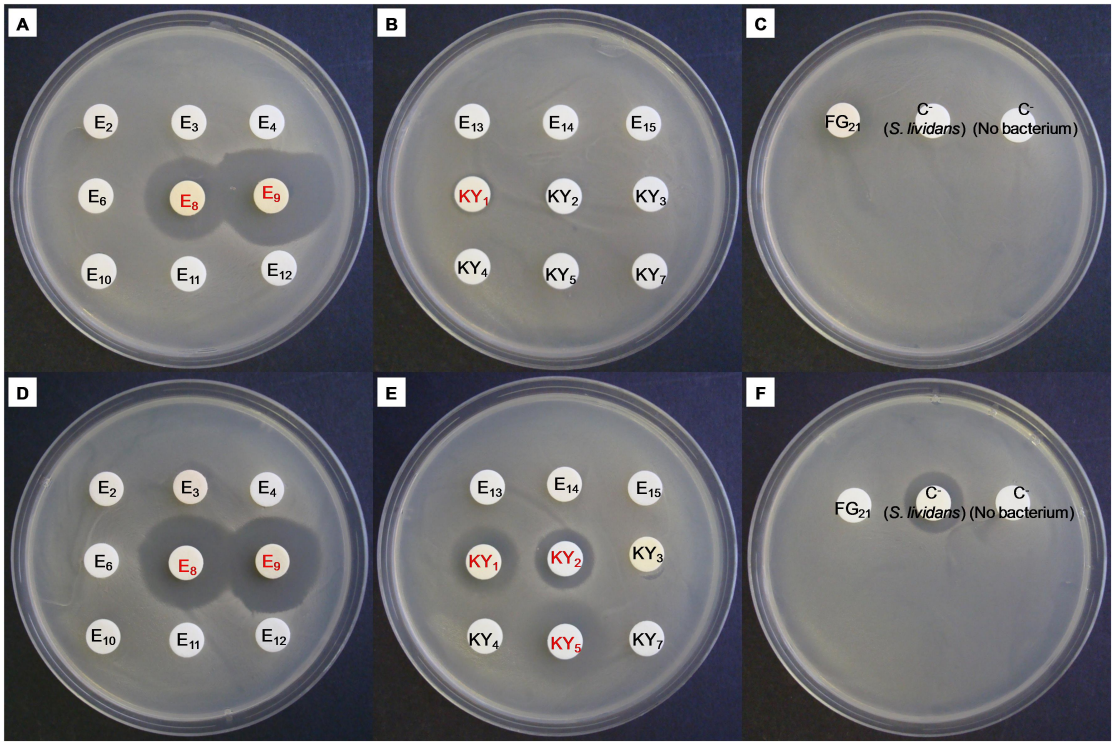


Figure 2.8 *Candida* (CA_6) supernatant bioassays testing the bioactivity of NNSPS (E_2-E_4, E_6, E_8-E_{15}) growth cultures. As negative controls, a growth culture of *S. lividans* (C^- *S. lividans*) and an uninoculated batch of medium (C^- No bacterium) were included. One set of media contained 150 mM sodium butyrate and another one did not. The supernatants were inoculated onto paper filter disks and attached to *Candida*-coated agar plates. **(A-C)** The supernatants of *Streptomyces* E_8 and E_9 (red font), grown in the absence of the inducer, are inhibiting *Candida*. **(D-F)** In the presence of sodium butyrate, the liquid growth media of *Streptomyces* E_8 and E_9 (red font) are inhibiting *Candida*.

Unlike the Petri dish bioassays, in which *Streptomyces* E_6 in the absence of sodium butyrate and *Pseudonocardia* E_4 in its presence (Fig. 2.5_{A-B}) were able to inhibit *Candida*, none of their growth supernatants inhibited *Candida* (Fig. $2.8_{A,D}$).

Surprisingly, the supernatant of *S. lividans* (negative control) also inhibited *Candida* when sodium butyrate was added to the medium (Fig. 2.8_F). Since the supernatants of most test strains, as well as the supernatant of the other negative control, which was completely uninoculated, did not show any effect on *Candida*, the bioactivity of *S. lividans* is most parsimoniously attributed to the uncharacterised products of *S. lividans*, rather than to the medium.

2.4.5.2 Bioassays against Escovopsis

Similar to the results with *Candida*, supernatants of only *Streptomyces* E_8 and E_9 successfully inhibited the growth of *Escovopsis* (Fig. 2.9).

2.5 Discussion

The data presented in this chapter confirm the recent observation that *Acromyrmex* spp. worker ants associate with a diversity of bacterial ectosymbionts (e.g. Kost *et al*. 2007, Haeder *et al.* 2009, Barke *et al*. 2010). In this study, bacteria of the genera *Pseudonocardia*, *Streptomyces*, *Tsukamurella*, and *Nocardiopsis* were isolated from *A. octospinosus* worker ants. In contrast to the genera *Pseudonocardia* and *Streptomyces*, bacteria in the genus *Nocardiopsis* have never been reported as symbionts of this ant genus before; hence, future studies will be needed to establish the ubiquity and importance of this association.

A selection of the bacterial isolates was challenged on agar plates against the medically important yeast *Candida albicans* and three *Escovopsis* strains (EA, EWB, EWC). *Streptomyces* E_8, E_9, and E_6, inhibited *Candida*. Diverse *Streptomyces*, *Pseudonocardia*, and *Nocardiopsis* strains inhibited the growth of *Escovopsis* EWB, EWC, and EA; particularly strong were combinations of *Streptomyces* E_8, E_9, and E_{13}, as well as *Nocardiopsis* E_{11} with *Escovopsis*

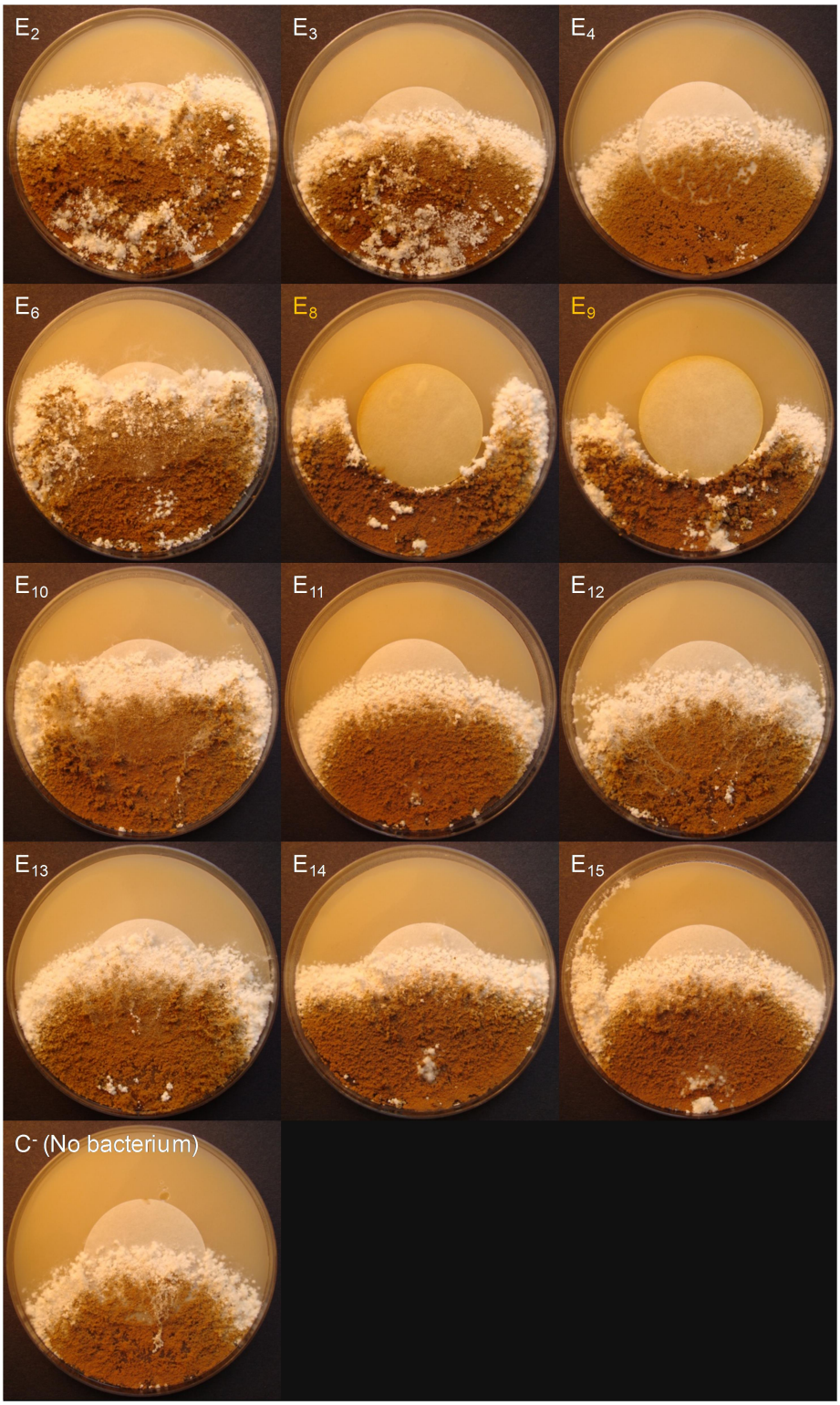


Figure 2.9 *Escovopsis* (EWC) supernatant bioassays testing the bioactivity of NNSPS (E_2-E_4, E_6, E_8-E_{15}) growth cultures. The supernatant of an uninoculated batch of medium (C^- No bacterium) was included as a negative control. Only the supernatants of *Streptomyces* E_8 and E_9 (orange font), soaked onto paper filter disks and containing no sodium butyrate, are inhibiting the test fungus.

EWC. The bioassay results of *Nocardiopsis* E_{11} (Table 2.9) illustrate that different fungal strains, even of the same species (i.e. *Escovopsis weberi*), experience different levels of stress upon being exposed to the same bacterial metabolites. In general, metabolites that have fungicidal effects on one test fungus may have only fungistatic or no inhibitory effects on another test fungus. Decreased susceptibilities in resistant strains may for example be explained by modifications in, or a reduction of, fungal target molecules. In contrast, observed differences between replica plates, in which a bacterial strain is challenged against a single test fungus, do not originate from susceptibility differences. These may rather be explained by intrinsic variabilities in the growth pattern of *Escovopsis* or by slight differences in the number of inoculated cells resulting in slight variations in the amounts of secreted antifungals. Consequently, the bioassay results in which a single *Nocardiopsis* E_{11} colony (out of 15 replica colonies) remained free of *Escovopsis* EA hyphae (Table 2.9) can be explained by a generally high fungal resistance that due to the above reasons has not resulted in pathogen contact within the period of the experiment.

Perhaps surprisingly, the best-studied attine-symbiont, *Pseudonocardia,* showed weaker inhibitory properties against the test fungi than did many of the *Streptomyces* and *Nocardiopsis* strains. The difference in inhibitory strength between *Pseudonocardia* and *Streptomyces* symbionts has previously been recognised by Haeder *et al*. (2009). Of the bacteria that successfully inhibited fungi in the agar plate bioassays, only *Streptomyces* E_8 and E_9 also produced antifungal compounds in liquid medium. This trait makes these two strains suitable candidates for antifungal isolation and identification.

To induce the bacterial isolates to produce cryptic antifungals under laboratory conditions, the microbes were additionally grown on agar plates containing the chemical inducer sodium butyrate. *Pseudonocardia* E_4 was successfully induced to secrete antifungals. In contrast, *Streptomyces* E_8 and E_9 exhibited similar levels of antifungal inhibition in both the presence and absence of sodium butyrate, although bioassays alone are not enough to detect changes in the composition of the compounds produced. There is still a need to optimise

sodium butyrate concentrations, as illustrated by the growth inhibition of *Streptomyces* E_6 when grown with a standard concentration of 150 mM of sodium butyrate.

Possible sources of errors inherent to the culture-dependent method used in this chapter are the following. In contrast to 454 sequencing approaches, culture-dependent methods may fail to detect unculturable and/or rare bacteria in microbial communities of ectosymbionts, as a result of unfavourable growth media or competition with other bacteria. Furthermore, selection of bacterial morphotypes focused on actinobacteria, which are well-known for their association to attine ants, but this resulted in neglecting other potentially mutualistic bacteria. In addition, the culture-dependent method does not allow quantitative estimates of the dominance and distribution of bacterial strains, meaning that few conclusions can be made about their importance to the mutualism.

With these caveats in mind, the data presented in this chapter show that *A. octospinosus* ants host a diverse community of bioactive bacteria that may offer a variety of pharmacologically interesting antifungal metabolites, which might provide antifungal inhibition benefits to the host ants.

2.5.1 Implications for mutualism theory

The diverse community of bacteria associated with *A. octospinosus* is shown to express itself in the ability to produce a diversity of antifungals, forming a so-called antifungal 'cocktail' (Barke *et al*. 2010). In particular, *Pseudonocardia* E_4 (renamed 'P_1' in Barke *et al.* 2010) is shown to produce a water-soluble, bioactive compound that structurally resembles, but is not identical to, the well-known antifungal nystatin, which shows a poor water-solubility (Day *et al*. 2011). We called this new compound 'nystatin P1'. *Streptomyces* E_8 (renamed 'S_4' in Barke *et al.* 2010), another bacterial isolate of the same ant colony as *Pseudonocardia* E_4, is found to produce the well-known antifungal candicidin. Both compounds belong to the same class of antifungals,

polyenes, which bind ergosterol in the fungal membranes, aggregate to pores, and increase membrane permeability, leading to fungal death.

The novelty of nystatin P1 has been discussed by Barke *et al.* (2010) in terms of host-symbiont coevolution. Previously, a *Pseudonocardia* strain hosted by the attine ant *Apterostigma dentigerum* was also found to produce a novel antifungal, called dentigerumycin (Oh *et al*. 2009). The production of novel antibiotic compounds supports the view, originating with Currie *et al.* (1999b, 2003), that *Pseudonocardia* is a long-term, vertically transmitted associate of attine ants, which is engaged in a coevolutionary arms race with fungal pathogens. Barke *et al*. (2011) place this idea within mutualism theory as a version of 'partner fidelity feedback' (Sachs *et al*. 2004). Strict vertical transmission of a *Pseudonocardia* lineage by an attine ant lineage aligns the fitness interests between host and symbionts. A host-symbiont mutualism, however, is disturbed when *Escovopsis* develops resistance to the antifungal produced by the symbiont, leading to colony death. However, variation among symbionts in their antifungal biosynthesis genes, and thus their ability to produce novel antifungal, is expected to increase the reproductive success of some hosts, forming the next generation of host-symbiont combinations. The features of the symbionts thus feed back on the fitness of the host, and in consequence on their own fitness.

In contrast, the production of the structurally unmodified candicidin molecules by *Streptomyces* E_8 has been discussed to be consistent with environmental recruitment of existing actinobacteria from the soil (Barke *et al.* 2010) because candicidin-producing *Streptomyces* are widespread in the environment (Jørgensen *et al.* 2009) and because the same compound has also been found in an *A. octospinosus* ant-associated *Streptomyces* strain isolated from a geographically distinct ant colony (Haeder *et al*. 2009). Furthermore, the well-known antifungals antimycins A_1-A_4, valinomycin, and actinomycin D and X_2 have been found to be produced by *Acromyrmex* spp. ant-associated *Streptomyces* strains (Schoenian *et al*. 2011) that also have been found in non-ant environments.

Environmental recruitment is a form of 'partner choice' (Bull and Rice 1991), which describes when there is positive assortment (matching) of partners that happen to have aligned fitness interests. In other words, by selectively recruiting bacteria that already have the capacity to produce antibiotics that kill *Escovopsis* and other parasites, attine colonies increase their own fitness and the fitness of the recruited symbionts. In addition, the recruitment of multiple antibiotic-producing bacteria can further increase antibiotic efficacy and delay the evolution of resistance (Barke *et al*. 2011).

Two mechanisms are proposed to be able to bring about partner choice in mutualisms: screening and signalling (Archetti *et al.* 2011a,b). Screening occurs when a host couples a 'demanding environment' with a high reward. The evolutionary challenge is to set up the 'demanding environment' in such a way that only beneficial symbionts are able to live on the host and benefit from the host's rewards. Scheuring and Yu (2012) use screening to show how vertically propagated *Pseudonocardia* symbiont can help attine ants to recruit other antibiotic-producing bacteria from the environment. Namely, by providing a nutrient-rich cuticular environment, attine ants enable their *Pseudonocardia* symbionts to produce abundant antibacterials, and this is the demanding environment. Antibiotic-producing bacteria are more likely to be resistant to these antibacterials, and are therefore able to colonise the nutrient-rich attine cuticle, while non-antibiotic-producing bacteria are less likely to have resistance genes and thus are less able to colonise the attine cuticle. In short, antibiotic-producers are better competitors for the attine niche. Finally, in the attine system, the antibiotic-producing bacteria are mostly actinobacteria, which are known to produce both antibacterials and antifungals, so a host that recruits antibacterial-producing actinobacteria is likely also to recruit in antifungal production capacity.

Thus, competition among bacteria for host resources does not only have to reduce the fitness of the host, competition can also help to produce successful partner choice. In this context, it would be interesting to ask which ant castes are responsible for recruiting bacteria from the environment. Three options

would be: the queen recruits diverse bacteria before or during nest initiation and passes the bacterial community on to the offspring, including the garden workers; in the case of *Acromyrmex* colonies, older major workers might collect bacteria from outside of the nest and pass these on to the young major workers in the garden; the young major workers themselves sample bacteria from the soil inside the nest. The answer to this question would provide valuable information about the overall diversity of antifungal cocktails and how flexible ant colonies are in adjusting their cocktails to current parasite attacks.

Chapter 3) Antifungals of an *Acromyrmex octospinosus* ant-associated *Streptomyces* strain

3.1 Introduction

Since the 1990s, the rate of discovery of new natural product drugs has steadily decreased (Li and Vederas 2009); this trend can be attributed to the fact that many pharmaceutical companies have closed their natural-product research programmes due to re-discovering the same compounds in soil-collected fungi and actinobacteria, and have switched to the development of synthetic drugs. Additionally, the number of antifungals suitable for human use is limited by two other factors. Firstly, fungal pathogens are diverse and constantly evolving, showing innate and acquired resistances. For example, fungi with reduced concentrations of membrane-bound ergosterol are less susceptible to polyene drugs (Young *et al*. 2003). Secondly, mammalian and fungal cells are more biochemically similar than are bacteria and human cells, so compounds inhibiting and/or killing fungal cells are likely to have adverse side-effects on the human host. For example, the polyene antifungal amphotericin B, which often is used for treating systemic mycoses, at high concentrations also shows affinities to cholesterol in mammalian cells (Kaneshiro *et al*. 2000, Baginski *et al.* 2002). Hence, research efforts now also include soil-unrelated niches to find unknown bacteria and novel antifungal metabolites, showing strong bioactivities and less side-effects, increasing the medical potential for treating human mycoses.

During recent years, a new research strategy has been to focus survey effort on underexplored niches for natural-product discovery. One increasingly important class of underexplored niches is the host-symbiont mutualism, including mutualistic associations between bacteria and European beewolf *Philanthus triangulum* (Kroiss *et al*. 2010), mud dauber wasps *Sceliphron caementarium* and *Chalybion californicum* (Poulsen *et al*. 2011), and the southern pine beetle *Dendroctonus frontalis* (Scott *et al.* 2008). Host-symbiont associations, especially those involving bacterial or fungal symbionts, are

typically characterised by the exchange of small nutritional and non-nutritional molecules (Schmidt 2008), importantly including antibiotics. These antibiotics can be used by hosts for defending themselves (Seipke *et al*. 2011c, Mueller *et al*. 2012), for example when secreted by integumental biofilms of attine ant-associated bacteria, their offspring (Kaltenpoth *et al*. 2005, Poulsen *et al*. 2011), and/or other mutualistic partners (e.g. fungal cultivars; Scott *et al.* 2008, Santos *et al*. 2004, Currie *et al*. 1999b, Currie *et al*. 2003, Kost *et al*. 2007, Haeder *et al.* 2009, Oh *et al*. 2009, Sen *et al*. 2009, Schoenian *et al*. 2011) from pathogens.

Because the fitnesses of host and symbiont are, by definition, aligned in mutualisms (Weyl *et al.* 2010, Archetti *et al.* 2011a), there is and has been selection on the host and symbiont to produce beneficial metabolites for the other partner. One of the best-studied examples is bacterial symbionts that produce antibiotics, which are effective against current pathogens of the host. Furthermore, because those antibiotics are applied on the host or on its offspring and/or other mutualistic hosts, there is also selection on host and symbiont to evolve antibiotics that do not cause harm to the host.

In consequence, we might expect that symbionts produce antibiotics that markedly differ in their metabolic nature from the antibiotics found in soil-bacteria, resulting in fewer adverse side-effects to the host. As it turns out, the structures of a number of symbiont-produced antibiotics have been published, some of which indeed possess molecular structures that are novel to science (Barke *et al*. 2010, Kroiss *et al*. 2010, Poulsen *et al*. 2011, Scott *et al.* 2008, Haeder *et al*. 2009, Oh *et al*. 2009, Schoenian *et al*. 2011).

By far the best-studied example of a host-symbiont mutualism in which the symbiont produces antibiotics is found in the attine ants (*Hymenoptera*, *Formicidae*, *Attini*). Attine ants are known for cultivating mutualistic fungi. The two most derived genera in this tribe are *Acromyrmex* and *Atta*, collectively known as ‘leaf-cutter’ ants because they cut leaves from live plants and carry them to their nests. There, the leaf-material is masticated and fed to the symbiotic basidiomycete *Leucoagaricus gongylophorus* (*Agaricales*, *Agaricaceae*). In return, the fungus produces nutritious food bodies (gongylidia)

that are used to feed the ant larvae and queen. The fitness of attine cultivars is endangered by diverse non-cultivar fungi, of which fungi in the genus *Escovopsis* are common, virulent, and specialised (Currie *et al*. 1999a, Rodrigues *et al.* 2008, Reynolds and Currie 2004, Currie 2001, Gerardo *et al.* 2006). They can overgrow cultivar gardens, form permanent infections, and feed on the cultivar hyphae. However, an array of ant- and cultivar-associated bacteria has been suggested, not only to benefit the health of the ants, but also to promote the health of the fungal cultivars. For example, from attine workers the Gram-positive genera *Pseudonocardia*, *Streptomyces*, and *Amycolatopsis* have been isolated (Currie *et al.* 1999b, Currie *et al*. 2003, Kost *et al*. 2007, Haeder *et al.* 2009, Sen *et al*. 2009, Oh *et al*. 2009). These occur on the integument of attine ants and inside their mouth pocket (infrabuccal pocket), which serves the collection of infected garden material, where they produce antifungal metabolites. Furthermore, bioactive *Streptomyces* and Gram-negative *Burkholderia* bacteria have also been isolated from the fungal cultivar (Haeder *et al.* 2009, Santos *et al*. 2004).

I now briefly review the chemically characterised antibiotics that have been isolated from attine-associated bacterial symbionts. *Acromyrmex spp.* ant- and cultivar-associated *Streptomyces* strains have been reported to produce the well-known antifungal candicidin ($C_{59}H_{84}N_2O_{18}$, 1109.57938 amu $[M+H]^+$, Haeder *et al*. 2009). This compound was challenged against the fungal mutualist *L. gongylophorus* and five non-cultivar fungi, including *Escovopsis weberi*, *E. aspergilloides*, *Beauveria bassiana*, *Metarhizium anisopliae*, and *Lecanicillium lecanii*. While candicidin inhibited *Escovopsis spp.*, the growth of other non-cultivar fungi and fungal mutualist was largely unaffected.

Furthermore, an *Apterostigma dentigerum* ant-associated *Pseudonocardia* strain has been shown to exhibit inhibitory properties towards *Escovopsis* sp. (Oh *et al.* 2009). In contrast, the metabolites of this *Pseudonocardia* strain apparently did not reduce the growth of the fungal mutualist. The bioactivity of the bacterial strain has been traced back to a novel antibiotic, named dentigerumycin ($C_{40}H_{67}N_9O_{13}$, 882.01248 amu, PubChem ID 25181359).

Nine *Streptomyces*, two *Pseudonocardia*, four *Nocardiopsis*, and one *Tsukamurella* strain have been isolated from *Acromyrmex octospinosus* workers (Barke *et al*. 2010). A selection of isolates, including nine *Streptomyces* (E_2-E_3, E_6, E_8-E_{10}, E_{13}-E_{15}), two *Pseudonocardia* (E_4, E_{12}), and one *Nocardiopsis* (E_{11}) was tested in colony bioassays against three *Escovopsis* strains (EWC, EWB, EA) and *Candida albicans*. Most susceptible against the bacterial secretions was *Escovopsis* EWC and *C. albicans*. In particular, eight *Streptomyces* (E_2-E_3, E_6, E_8-E_{10}, E_{13}-E_{14}), one *Pseudonocardia* (E_4), and the *Nocardiopsis* (E_{11}) strain inhibited *Escovopsis* EWC; three *Streptomyces* (E_6, E_8-E_9) and one *Pseudonocardia* (E_4) strain inhibited *C. albicans*.

Pseudonocardia E_4 (renamed P_1) produces a novel nystatin-like antifungal, 'nystatin P1' (Barke *et al.* 2010). The genome has been shown to contain biosynthesis genes with high amino acid similarities to proteins needed by *P. autotrophica* to produce an antifungal called 'nystatin-like *Pseudonocardia* polyene' (NPP, 1129.6 amu $[M+H]^+$), an antifungal that is related to nystatin A_1 of *S. noursei*. In an LCMS analysis, a methanol extract from a *Pseudonocardia* E_4 growth culture was analysed alongside a standard of nystatin A_1 ($C_{47}H_{75}NO_{17}$, 925.5035 amu, KEGG ID C12155). Similar retention times and polyene specific UV absorbances indicated analogies between the standard (926.5 amu $[M+H]^+$) and a sample ion with a mass of 1088.6 amu $[M+H]^+$, confirming the production of a nystatin-like compound. The mass difference between both compounds has been suggested to originate from an additional hexose molecule, potentially rendering the sample molecule more water-soluble. The NPP molecule of *P. autotrophica*, which also contains an additional sugar moiety (*N*-acetyl-glucosamine), has been shown to possess a 300-fold higher water solubility than nystatin A_1 (Lee *et al*. 2012).

In addition, *Streptomyces* E_8 (renamed S_4), which was isolated from the same ant colony, has been shown to produce the well-known antifungal candicidin (Barke *et al.* 2010). Two candicidin biosynthesis genes were discovered in the bacterial genome, and the bacterium was shown to produce candicidin (Barke *et al.* 2010). A liquid growth culture of this strain indeed

contained the candicidin mass 1109.6 amu $[M+H]^+$, showing the same MS^2 fragmentation and UV absorbance pattern as candicidin identified by Haeder *et al.* (2009).

Lastly, fourteen *Acromyrmex octospinosus*, *A. echinatior*, and *A. volcanus* ant- and cultivar-associated *Streptomyces* strains have been shown to produce the well-known antibiotics valinomycin (e.g. $C_{54}H_{90}N_6O_{18}$, 1111.34 amu, CAS: 14295-14-8); actinomycin D (e.g. $C_{62}H_{86}N_{12}O_{16}$, 1255.44 amu, Reaxys ID: 20503753); actinomycin X_2 (structurally similar to: $C_{62}H_{84}N_{12}O_{17}$, 1269.42 amu, Reaxys ID: 8386016); as well as antimycin A_1 (e.g. $C_{28}H_{40}N_2O_9$, 548.634 amu, CAS: 116095-18-2), A_2 (e.g. $C_{27}H_{38}N_2O_9$, 534.607 amu, CAS: 118890-43-0), A_3 (e.g. $C_{26}H_{36}N_2O_9$, 520.58 amu, CAS: 116095-17-1), and A_4 (e.g. $C_{25}H_{34}N_2O_9$, 506.553 amu, CAS: 117603-45-9) (Schoenian *et al*. 2011). These compounds were challenged against diverse test fungi and bacteria. Among others, fungal opponents included the fungal mutualist (i.e. *L. gongylophorus*), garden-associated non-cultivar fungi (e.g. *Escovopsis weberi*, CBS 110660), and entomopathogens (e.g. *Beauveria bassiana*). Bacterial opponents, among others, included *Acromyrmex*-isolated *Streptomyces* and *Pseudonocardia* strains as well as *Bacillus subtilis*. The authors show a complex pattern of fungal and bacterial inhibition. For example, valinomycin only inhibited *Bacillus*, while the growth of *Escovopsis* and *L. gongylophorus* were unaffected by this antibiotic. Actinomycin D inhibited diverse *Streptomyces* and *Pseudonocardia* strains; the compound did not inhibit *E. weberi* and *L. gongylophorus*. In contrast, antimycins exclusively inhibited the fungal opponents, including *Escovopsis*, and fungal mutualist.

In conclusion, actinomycete symbionts of attine ant hosts indeed have the capacity to produce multiple antibiotic metabolites. Some of these antibiotics possess (partly) novel structures (e.g. dentigerumycin and nystatin P1), which is consistent with pairwise coevolution (Oh *et al.* 2009, Barke *et al*. 2010, Barke *et al*. 2011). Other compounds (candicidin, valinomycin, actinomycin D and X_2, antimycin A_1-A_4) are well known from non-symbiotic bacteria, which is consistent with environmental recruitment of bacterial species that primarily live

in the soil (Haeder *et al*. 2009, Barke *et al*. 2010, Barke *et al*. 2011, Schoenian *et al*. 2011). Mechanisms of action, even for well-known compounds, are often unknown or established only with regard to specific and ant system-unrelated test organisms and cell lines. However, in some cases, the chemical structure of compounds allows suggestions on their molecular targets. For example, sceliphrolactam, which was isolated from mud dauber wasp-associated (*Sceliphron caementarium* and *Chalybion californicum*) *Streptomyces* (Poulsen *et al*. 2011), is suggested to function as a polyene antifungal due to its molecular structure.

3.2 Aims

The aim of this chapter is to investigate the full inhibitory potential of a single *A. octospinosus* ant-associated bacterium. Hence, the same highly bioactive *Streptomyces* strain (E_8), which has been shown to produce candicidin (Barke *et al*. 2010), was chosen here for follow-up study. Bioassay-guided column fractionations, during which bioactive bacterial metabolites become separated from non-bioactive compounds of bacterial origin and/or the medium, were used to enable mass identifications of non-candicidin antifungals. In particular, the butanol extract of a liquid growth culture was purified in bioassay-guided normal- and reversed-phase column fractionations (Fig. 3.1). This allowed a determination of the metabolic features of the bioactive compounds by low- and high-resolution LCMS, and a confirmation of mass analogies to a known antifungal by comparison to a standard.

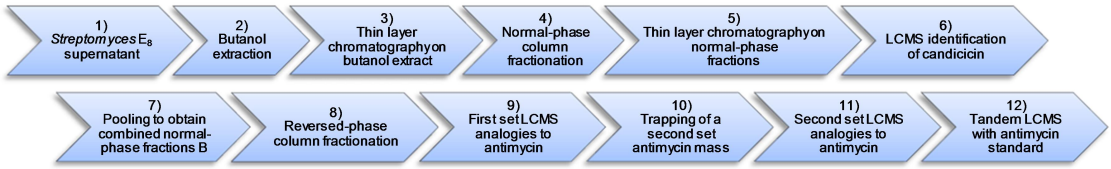


Figure 3.1 Bioassay-guided column fractionation. During multiple purification steps, the number of compounds in bioactive fractions was reduced to levels, which allow a mass identification of antifungals other than candicidin.

3.3 Materials and Methods

3.3.1 Culture supernatants

3.3.1.1 Supernatants – large-scale production

Sixteen 250 ml flasks, each containing a spring and 100 ml of a mannitol soy-flour (MS) broth (Table 3.1), were autoclaved for sterilisation. Fifteen of the flasks were inoculated with 50 µl from a *Streptomyces* E_8 glycerol stock. As a control, one flask was inoculated with an equal volume from a *S. lividans* glycerol stock. To guarantee strong bioassay performance, the same *Streptomyces* spore/cell material was used as for experiments carried out in Chapter 2. The flasks were incubated for 19 days in a shaker incubator (30 ^{o}C, 180 rpm). At day 13 of the incubation period, the flasks were heat-shocked by placing them in a 50 ^{o}C water bath for 45 minutes. The culture supernatants were separated from most of the cell material and undissolved components of the medium. To do so, the growth cultures were centrifuged twice for 30 minutes (4 ^{o}C, 4000 rpm). In total, 1250 ml of *Streptomyces* E_8 supernatant and slightly less than 100 ml of *S. lividans* supernatant were harvested; some of the liquid was lost due to evaporation and as moisture in the discarded medium. The *Streptomyces* E_8 supernatant was sub-divided. 1050 ml were preserved for a butanol extraction and a proceeding column fractionation; 200 ml were dedicated for bioassays. All of the *S. lividans* supernatant was reserved for bioassays. The supernatants were stored at -20 ^{o}C.

Medium	Ingredients	Weight (g), volume (ml)
Lennox broth (LB)	Tryptone	10.0 g
	Yeast extract	5.0 g
	NaCl	5.0 g
	Distilled water	1000 ml
Lennox broth (LB) agar	Tryptone	10.0 g
	Yeast extract	5.0 g
	NaCl	5.0 g
	Agar	15.0 g
	Distilled water	1000 ml
Mannitol soy-flour (MS) broth	Organic soy-flour	20.0 g
	Mannitol	20.0 g
	Tap water	1000 ml
Mannitol soy-flour (MS) agar	Organic soy-flour	20.0 g
	Mannitol	20.0 g
	Agar	20.0 g
	Tap water	1000 ml

Table 3.1 Media for cultivation of bacteria and fungi.

3.3.1.2 Supernatants – small-scale production

In a similar way, further batches of *Streptomyces* E_8 and *S. lividans* supernatants were obtained. Two 250 ml flasks, each containing 100 ml of a sterile MS broth, were inoculated with 50 µl from the corresponding glycerol stocks. The flasks were incubated on a shaker incubator for 12 days (30 ^{o}C, 180 rpm). At day 9 of the incubation period, the flasks were heat-shocked by placing them for 30 minutes in a 50 ^{o}C water bath. The supernatants were harvested by centrifuging the growth cultures for 30 minutes (4 ^{o}C, 4000 rpm). The supernatants were stored at -20 ^{o}C.

3.3.1.3 Bioassays against Candida

The *Streptomyces* E_8 supernatant of the large-scale production was challenged against human pathogenic yeast *Candida* spp. A small sub-sample of the 200 ml fraction, dedicated for bioassays, was filter-sterilised by passing it through a 0.45 µm filter. Then, the sterile supernatant was diluted with sterile dH_2O to obtain concentrations ranging from 90-10% of the original concentration. Additionally, 100% and 0% concentrations were included in the bioassays, consisting of the crude supernatant and pure dH_2O, respectively. These dilutions were tested against fourteen *Candida* strains, including *Candida albicans* CA_1-CA_{11}, *C. tropicalis* CA_{12}, *C. glabrata* CA_{13} and *C. dubliniensis* CA_{14} (Table 3.2). The yeasts were inoculated from source plates into glass containers (universals), containing 10 ml of Lennox broth (LB, Table 3.1). Then, the universals were incubated in a shaker incubator for one day (37 ^{o}C, 230 rpm). Fourteen LB agar

Strain	Species	Clade
CA_1	*Candida albicans*	1
CA_2	*Candida albicans*	1
CA_3	*Candida albicans*	1
CA_4	*Candida albicans*	2
CA_5	*Candida albicans*	2
CA_6	*Candida albicans*	3
CA_7	*Candida albicans*	3
CA_8	*Candida albicans*	4
CA_9	*Candida albicans*	4
CA_{10}	*Candida albicans*	11
CA_{11}	*Candida albicans*	11
CA_{12}	*Candida tropicalis*	-
CA_{13}	*Candida glabrata*	-
CA_{14}	*Candida dubliniensis*	-

Table 3.2 *Candida* spp. test fungi provided by Neil Gow from the University of Aberdeen.

plates (Table 3.1), containing no antibiotics, were coated with 0.5 ml of the yeast cultures. When dry, 50 µl of the supernatant dilutions were inoculated on the *Candida*-coated agar plates in such a way that each of the fungal pathogens was challenged with any of the eleven dilutions. The presence or absence of fungal inhibition was measured after two days of incubation at 26 ^{o}C.

3.3.1.4 Bioassays against Escovopsis

The *Streptomyces* E_8 and *S. lividans* supernatants of the large-scale production was challenged against *Escovopsis*. Small sub-samples of the 200 ml *Streptomyces* E_8 and 100 ml *S. lividans* supernatants, dedicated for bioassays, were filter-sterilised by passing them through a 0.45 µm filter. 500 µl of the sterile supernatants were inoculated into the centre of mannitol soy-flour (MS) agar plates (Table 3.1), lacking any antibiotics. When dry, '*Escovopsis* EWC' was point-inoculated to the bioassay plates at a distance of 1 cm from the supernatants. The plates were incubated at room temperature until the supernatant inocula were either clearly avoided or overgrown by the fungus.

3.3.2 Butanol-extracts

3.3.2.1 Butanol extraction – large-scale production

1050 ml of the *Streptomyces* E_8 supernatant from the large-scale production, to be used for column fractionations and antifungal identification, were extracted with 3150 ml of butanol. This was done by adding 50-100 ml batches of supernatant to a separation funnel and washing them three times with the same volumes of butanol. Butanol is an organic solvent, which is more apolar than water (polarity index: BuOH=4.0, H_2O=9.0). Due to these properties, BuOH and water are immiscible and show phase separation. Many antifungals, but also non-bioactive metabolites and ingredients of the growth medium, are less polar than the aqueous environment in which the bacteria were grown. The mixing of aqueous supernatants with BuOH allows such compounds to associate with the relatively apolar solvent, and subsequently separate out. The organic phases of the washes were harvested and subsequently vacuum

concentrated. While the dry residue was stored at -20 ^{o}C, the aqueous parts of these washes were disposed.

Similarly, another sub-sample (45 ml) of the 200 ml fraction from the *Streptomyces* E_8 large-scale production, dedicated for bioassays, was extracted with 150 ml of butanol. The organic phase was evaporated. In contrast to an earlier extraction, here the aqueous phase was preserved. Both, residue and aqueous phase were stored at -20 ^{o}C.

3.3.2.2 Butanol extraction – small-scale production

In the same way as above, sub-samples (3 ml) of the 100 ml batches of *Streptomyces* E_8 and *S. lividans* supernatants from the small-scale production were BuOH extracted. The supernatants were washed with 9 ml of butanol. The organic solvents were evaporated and the residues stored at -20 ^{o}C. The aqueous phase was disposed.

3.3.2.3 Bioassays against Candida

The butanol extract of the 45 ml sub-sample from the *Streptomyces* E_8 large-scale production, obtained above, was challenged against *Candida*. In particular, some of the original supernatant, the BuOH extract (re-dissolved in 100% MeOH) and the aqueous phase were included in the bioassays, alongside 100% MeOH and 100% BuOH that served as negative controls. Five paper filter disks, with a diameter of 0.9 cm, were inoculated with 200 µl of the test and control liquids. '*Candida* CA_6' was inoculated into a universal, containing 10 ml of Lennox broth, and was grown for 2 days in a shaker incubator (37 ^{o}C, 280 rpm). A 9 cm square Petri dish, containing LB agar and no antibiotics, was coated with 800 µl of the *Candida* culture. When dry, the disks were fixed to the *Candida* lawn with 80 µl of sterile dH_2O. The plates were incubated for 2 days at room temperature, after which the presence or absence of fungal inhibition was scored.

3.3.2.4 Bioassays against Escovopsis

The butanol extract of the 3 ml sub-samples from the *Streptomyces* E_8 and *S. lividans* small-scale productions were challenged against *Escovopsis*. The residues were re-dissolved in 400 µl of 50% aqueous MeOH. The re-dissolved extracts were used completely to inoculate two filter disks, with a diameter of 4 cm. When dry, the disks were attached to MS agar plates, lacking any antibiotics, with 200 µl of sterile dH_2O. Then, *Escovopsis* EWC was inoculated at the edge of the bioassay plates. The bioassay plates were incubated at room temperature until the fungus clearly avoided or overgrew the filters.

3.3.2.5 Thin layer chromatography of butanol extracts

The BuOH extract of the 1050 ml sub-sample from the *Streptomyces* E_8 large-scale production, was analysed by thin layer chromatography (TLC). A small fraction of the residue was re-dissolved in 50% of aqueous MeOH (HPLC grade). The extract was point-inoculated 1 cm from the base of six silica plates. Glass containers were prepared, containing six mixtures of methanol (MeOH) and ethyl acetate (EtAc), including ratios of 100:0, 90:10, 80:20, 70:30, 60:40, and 50:50 (MeOH:EtAc). Paper filters resting in the solvent mixtures saturated the internal atmosphere of the closed-up containers. When dry, the TLC plates were carefully placed into the solvent mixtures and left in the container until the solvent front reached a migration height of 9 cm. Then the TLC plates were dried and analysed under short and long wave UV light; the distribution of UV-active compounds was sketched at a scale of 1:1. Then the plates were stained with potassium permanganate. This inorganic chemical is a strongly oxidising agent and visualises various functional molecular groups; however, since the extract is expected to also contain innumerable oxidisable, but non-bioactive compounds, the staining was only used to study the suitability of specific solvent mixtures to separate compounds in general. This information has been used for the following normal-phase column fractionation.

3.3.3 Normal-phase column fractionation

3.3.3.1 Fractionation

The remaining residue of the BuOH extract, originating from the 1050 ml of *Streptomyces* E_8 large-scale production and previously analysed by TLC, was here purified by normal-phase (NP) column fractionation. The residue (2.8 g) was dissolved in a 15 ml mixture of MeOH:EtAc (4:11). A small sub-sample of this column starter material was preserved for a later TLC analysis. Approximately 50 g of silicate are needed for separating compounds per gram of extract. Hence, the column was filled with 140 g of silicate. To prevent the silicate from being washed out, the base of the glass column was loaded with a layer of cotton wool and sand. Under pressure, the column was flushed twice with 100% EtAc; this was done to support the settling of the granules. Then, the dissolved extract was loaded into a small layer of solvent that was left on top of the silicate when closing the column. The sample was carefully run into the granule layer by again opening the base of the column and carefully topping it up with 100% EtAc. Once the sample disappeared from the surface, the column was topped-up with a solvent mixture of MeOH:EtAc in a ratio of 60:40.

When the compounds of the extract migrated half-way through the column, the collection of eluent started. During the collection of fractions 1-50, the solvent condition remained unchanged; while collecting fractions 51-66, the MeOH:EtAc ratio was changed to 70:30; and during the collection of fractions 67-85, the polarity of the mobile phase was increased to 100% MeOH. The normal-phase fractions were stored in universals at -20 ^{o}C.

3.3.3.2 Bioassays against Candida

Universals with 10 ml of Lennox broth were inoculated with *Candida* CA_6. To ensure abundant fungal growth the cultures were incubated for several days in a shaker incubator (37 ^{o}C, 280 rpm). LB agar plates, containing no antibiotics, were coated with 400 µl of the yeast cultures. Single paper filter disks, with a diameter of 0.9 cm, were inoculated with 50 µl of the NP fractions (1-85). Two control disks were inoculated with 50 µl of 100% MeOH, another two with 50 µl

of 100% EtAc, and one disk remained uninoculated. When dry, the disks were attached to *Candida*-coated agar plates with 50 µl of sterile dH_2O. Then, the plates were incubated at room temperature for 2 days after which the diameter of the inhibition zones was measured. The experiment was repeated three times.

3.3.3.3 Thin layer chromatography

The NP fractions (1-85), were analysed by TLC in the same way as the crude BuOH extract previously. The column starter material was point-inoculated onto the first lane of each TLC plate, serving as reference, and the NP fractions onto any lane thereafter. As mobile phase, a mixture of MeOH (HPLC grade):EtAc was used in a ratio of 70:30. The TLC plates were not analysed under UV light, but were directly stained with potassium permanganate.

3.3.4 Identification of candicidin

Sub-samples of the crude BuOH extract and the NP fractions 24 (apolar) and 79 (polar), which previously have been shown to contain antifungals with differing polarities, were analysed here by low- and high-resolution liquid chromatography mass spectrometry (LCMS). The reversed-phase (RP) high-performance liquid chromatography (HPLC) systems were set up in the metabolomics facility at the John Innes Centre (JIC).

3.3.4.1 Low-resolution LCMS

Low-resolution LCMS data were generated by running a sub-sample of the crude BuOH extract on a surveyor HPLC system that was attached to a Synergi PolarRP column (Phenomenex). The column had a length of 150 mm, diameter of 2 mm, and particle size of 4 µm. The solvent mixture contained acetonitrile (ACN) and water (0.1% formic acid), and was run through the system with a flow rate of 260 µl/min and a temperature of 30 ^{o}C. At the beginning of the run the solvent mixture contained 2% acetonitrile (T0-2'). Within 25 minutes, the ACN

concentration was gradually increased to 95% (T2-27'). After three minutes of running the solvent mixture isocratically at 95% ACN (T27-30'), meaning that solvent ratios remained unchanged, the concentration of acetonitrile was reduced to 2% within 30 seconds (T30-30.5').

Compound masses in the eluent between 100-2000 amu were detected with a DecaXP^{plus} ion trap in positive ion mode (+ESI). Furthermore, MS^2 data were produced with 35% collision energy. The spray chamber conditions included the use of 50 units sheath gas, 5 units aux gas, 350 ºC capillary temperature, and 5.2 kV spray voltage. UV spectra were collected at 1 Hz from 200-600 nm.

3.3.4.2 High-resolution LCMS

High-resolution LCMS data were obtained by running sub-samples of the normal-phase fractions 24 and 79 on a Luna C_{18} RP column. The column had a length of 50 mm, diameter of 2 mm, and particle size of 3 µm. The solvent mixture contained MeOH and dH_2O (0.1% formic acid) and was run through the system at a flow rate of 150 µl/min and a temperature of 30 °C. At the start of the run, the solvent mixture contained 5% MeOH (T0-1'). Then, the concentration of MeOH was increased to 90% within 15 minutes (T1-15'). The proportion of MeOH was kept isocratically at 90% for two more minutes (T15-17'), before being reduced to 5% within 30 seconds (T17-17.5').

Compound masses in the eluent between 150-1500 amu were detected with an orbitrap mass spectrometer in positive ion mode (+ESI), at a resolution of 60,000. MS^2 data were produced with 35% collision energy, and were detected at low-resolution quality. The spray chamber conditions included the use of 32 units sheath gas, 6 units aux gas, 4.4 kV spray voltage, 275 °C capillary temperature, and 40 V tube lens. No UV spectra were collected during this run. The computer software Xcalibur was used to calculate molecular formulae of detected MS ions.

3.3.5 Pooling of normal-phase fractions

3.3.5.1 Pooling

Selected normal-phase fractions (1-85) were combined with one another, according to their bioactivity, exhibited in figure 3.7. In particular, four groups were formed, consisting of fractions 1-21 (A), 22-43 (B), 44-66 (C), and 67-85 (D). The combined fractions (A-D) then were vacuum-concentrated and, depending on their solubilities, re-dissolved in varying concentrations of aqueous MeOH (HPLC grade); these were stored at -20 ^{o}C.

To determine the bioactivities of the supernatants and precipitates, which apparently have formed during the storing period, the samples were centrifuged for 10 minutes (4000 rpm, 4 ^{o}C). The combined fractions A showed a transparent supernatant and a minuscule pellet; combined fractions B had a coloured supernatant and a relatively large pellet; combined fractions C contained a slightly coloured supernatant and a small pellet; and combined fractions D showed a slightly coloured supernatant and a small pellet. The supernatants and precipitates were challenged against *Candida*. In preparation for the bioassays, pellet sub-samples were re-dissolved in 100% DMSO.

3.3.5.2 Bioassays against Candida

Paper filter disks, with a diameter of 0.9 cm, were inoculated with 50 µl from the supernatants or dissolved pellets. MS agar plates, containing no antibiotics, were coated with a 400 µl lawn of a two-day-old *Candida* CA_6 culture, grown in 10 ml of Lennox broth (37 ^{o}C, 280 rpm). When dry, the disks were attached to the *Candida* lawn with 50 µl of dH_2O. The bioassay plates were incubated for two days at 26 ^{o}C.

3.3.6 Reversed-phase column fractionation

3.3.6.1 Fractionation

The supernatant and pellet material of the combined fractions B were reversed-phase (RP) column fractionated. The remainder of the precipitate was re-dissolved in 100% DMSO. The supernatant and re-dissolved pellet was

separated on a HPLC system, located at the UEA, which was connected to a C_{18} RP column (Phenomenex). The solvent mixture contained MeOH (HPLC grade) and dH_2O (0.1% formic acid), and was run through the system with a flow rate of 3 ml/min and a temperature of 30 ^oC. Within 35 minutes, the solvents were run in a gradient from 5-95% of aqueous MeOH (T0-35'). After 10 minutes of running the solvents isocratically at 95% methanol (T35-45'), the MeOH concentration was reduced to 5% within 2.2 minutes (T45-47.2').

During multiple fractionation runs, 900 µl sub-samples of the supernatant or dissolved pellet material were loaded into the system. An autosampler was set to collect 80 fractions (1.5 ml) per run for a period of 40 minutes (T10-50'). Simultaneously, the UV absorbance (200-600 nm) of compounds in the eluent was measured by a diode array detector.

3.3.6.2 Bioassays against Candida

Paper filter disks, with a diameter of 0.9 cm, were inoculated with 200 µl of the RP fractions from the first fractionation run of a supernatant sample. In addition, the eluents preceding (T0-5', T5-10') and following (T50-55', T55-60') the fraction collection, were bioassayed as well. As negative controls, two disks were inoculated with equal volumes of 100% MeOH and dH_2O. LB agar plates, containing no antibiotics, were coated with 400 µl of a two-day-old *Candida* CA_6 culture, grown in 10 ml of Lennox broth (37 ^oC, 280 rpm). When dry, the filter disks were attached to the *Candida* lawn with 80 µl of sterile dH_2O. Then, the plates were incubated for 2 days at 26 ^oC.

3.3.6.3 Bioassays of extract and waste

The double-purified (NP/RP) extract and the waste of the RP column fractionation, described above, were challenged against *Candida* CA_6. The extract and waste were both vacuum concentrated and the residues re-dissolved in 500 µl of 100% MeOH. Two paper filter disks, with a diameter of 0.9 cm, were inoculated with 50 µl of the extract and waste. The colour of both disks differed from one another; while the disk with the extract was golden brown, the

one with the waste was matte red. As a negative control, a disk was inoculated with 95% MeOH. LB agar plates, containing no antibiotics, were inoculated with 400 µl of a two-day-old *Candida* CA_6 culture, grown in 10 ml of Lennox broth (37 ^oC, 280 rpm). The paper disks were attached to the *Candida*-coated agar plates with 60 µl of dH_2O. The plates were incubated for two days at 26 ^oC.

3.3.7 Identification of antimycin

The double-purified (NP/RP) combined fractions B were analysed by low- and high-resolution LCMS. The HPLC systems, located at the John Innes Centre (JIC), were previously used for tracing candicidin.

3.3.7.1 Low-resolution LCMS

Low-resolution LCMS data of the double-purified (NP/RP) combined fractions B were obtained by running a sub-sample on a surveyor HPLC system that was attached to a Luna C_8 column (Phenomenex). The column had a length of 100 mm, diameter of 2 mm, and particle size of 3 µm. The solvent mixture contained acetonitrile and water (0.1% formic acid) and was run through the system at a flow rate of 220 µl/min and a temperature of 30 °C. At the start of the LCMS run, the solvent mixture contained 2% acetonitrile and was then increased to 95% within 15 minutes (T0-15'). The acetonitrile concentration was kept isocratically at 95% for 2 minutes (T15-17'), before being reduced to 5% within 30 seconds (T17-17.5').

Compound masses in the eluent, between 150-1500 amu, were detected with a DecaXP^{plus} ion trap in positive ion mode (+ESI). MS^2 data were produced with 35% collision energy. The spray chamber conditions included the use of 50 units sheath gas, 5 units aux gas, 3.8 kV spray voltage, and a capillary temperature of 350 ^oC. In addition to MS and MS^2 data, UV spectra were collected at 1 Hz from 200-600 nm.

3.3.7.2 High-resolution LCMS

High-resolution LCMS data of the double-purified (NP/RP) extract were obtained by running a sub-sample on a HPLC system, connected to a Luna C_{18} column. The column had a length of 50 mm, diameter of 2 mm, and particle size of 3 µm. The solvent mixture contained MeOH and water (0.1% formic acid), and was run through the system at a flow rate of 150 µl/min and a temperature of 30 °C. At the beginning, the solvent composition contained 5% MeOH (T0-1'). Within 15 minutes, the percentage of MeOH was increased to 90% (T1-15'). The proportion of MeOH remained isocratically at 90% for 2 more minutes (T15-17'), before being reduced to 5% within 30 seconds (T17-17.5').

Compound masses in the eluent, between 150-1500 amu, were detected with an orbitrap mass spectrometer in positive ion mode (+ESI) and a resolution of 60,000. MS^2 spectra were produced with 35% collision energy, and were detected at a resolution of 30,000. The spray chamber conditions included the use of 32 units sheath gas, 6 units aux gas, 4.4 kV spray voltage, 275 °C capillary temperature, and a tube-lens voltage of 40 V. The computer software Xcalibur was used to calculate molecular formulae of detected MS and MS^2 ions.

3.3.7.3 Database comparison

The molecular formulae of selected MS parent ions, detected by low- and high-resolution LCMS, were matched to the SciFinder (https://scifinder.cas.org/scifinder) and Reaxys (www.reaxys.com) databases. Whenever formulae for both hydrogen and sodium adducts were available, the former were used for compound identification. In addition of finding chemical analogies between MS parent ions and database molecules, the molecular formulae of MS^2 daughter ions were used to determine the accuracy of these analogies.

3.3.8 Trapping of antimycin

3.3.8.1 Trapping of multiple antimycin masses

The double-purified (NP/RP) combined fractions B, here, were fractionated by trapping. The trapping system, located at the Institute of Food Research (IFR) in Norwich, was connected to a Gemini C_{18} RP column (Phenomenex). The column had a length of 150 mm, diameter of 4.6 mm, and particle size of 5 µm. The solvent mixture consisted of acetonitrile and water (0.1% formic acid), and was run through the system at a flow rate of 1 ml/min. At the start of the run, the solvent mixture contained 50% acetonitrile going to a 95% concentration within 20 minutes (T0-20'). Then, the solvent mixture was kept isocratically at 95% for five minutes (T20-25'), before the acetonitrile concentration was reduced to 50% within one minute (T25-26'). The eluent was split in a ratio of 19:1; while the smaller part was sent to a Bruker microtof to be used for mass spectrometry, the compounds of the larger part were either trapped on a Spark Holland Hydrasphere Resin General Purpose filter (10-12 µm) or directed to the waste. The mass spectrometer was set to detect masses in positive ion mode (+ESI). The live detection of masses in the eluent aided the trapping of specific compounds and RT intervals. The system was loaded with a 25 µl sub-sample of the extract and nine RT intervals were trapped, including 2E7 (T7.0-7.7'), 2E8 (T8.0-8.8'), 2E9 (T9.2-10.2'), 2E10 (T10.7-11.5'), 2E12 (T11.9-13.0'), 2F1 (T13.5-14.2'), 2F2 (T14.9-15.6'), 2F3 (T16.2-16.8'), and 2F4 (T17.0-22.5'). When dry, the trapped compounds were re-diluted from the filters in 160 µl of deuterated ACN and stored at -20 ^{o}C.

Due to the small loading volume of 25 µl, the fraction material was used completely for carrying out bioassays against *Candida* CA_6. Nine paper filter disks, with a diameter of 0.9 cm, were inoculated with the trapped fractions. As a negative control, another disk was loaded with 100% deuterated ACN. LB agar plates, containing no antifungals, were coated with 400 µl of a two-day-old *Candida* growth culture, grown in 10 ml of Lennox broth (37 ^{o}C, 280 rpm). When dry, the filters were attached to the agar plates with 80 µl of distilled water. Then, the plates were incubated for 2 days at 26 ^{o}C.

3.3.8.2 Trapping of a second set antimycin mass

The trapping system at the IFR was also used for isolating a 'larger' concentration (50 µl loading volume) of a second set 535 amu $[M+H]^+$ ion from the double-purified (NP/RP) combined fractions B. This molecule was isolated for carrying out a follow-up low- and high-resolution LCMS analyses in negative ion mode (-ESI). Due to failing ionisation of second set ions in positive ion mode, no molecular formulae of these have yet been determined. The same chromatography was applied during this LCMS run as for the LCMS run described above, with the exception that the mass spectrometer was set to detect masses in negative ion mode. The LCMS system was run twice to collect compounds in 50 µl of extract. The compounds in the trapped RT area 2G4 (T14.75-15.2') were accumulated on a Hydrasphere Resin General Purpose filter. When dry, the compounds were re-diluted from the filter in 160 µl of deuterated acetonitrile and stored at -20 ^{o}C.

With a bioassay the bioactivity of fraction 2G4 was tested against *Candida* CA_6. The testing procedure was also adopted from the above trapping experiment, except that this time smaller filter disks, with a diameter of 4 mm, were used. These were inoculated with 20 µl of the eluent; in contrast, the remainder of fraction 2G4 was preserved for future low- and high-resolution LCMS analyses, described below. LB agar plates, containing no antibiotics, were coated with 400 µl of a two-day-old *Candida* culture, grown in 10 ml of Lennox broth (37 ^{o}C, 280 rpm). When dry, the inoculated filters were attached to the *Candida* lawn with 60 µl of dH_2O and incubated for two days at 26^{o}C.

3.3.9 LCMS analysis of the second set antimycin mass

3.3.9.1 Low-resolution LCMS

Low-resolution LCMS data were obtained by running a sub-sample of fraction 2G4 on a Surveyor HPLC system, located at the John Innes Centre (JIC), which previously was used for the identification of candicidin and the bioactive molecules of the combined fractions B. The system was attached to a Luna C_{18} column; the column had a length of 50 mm, diameter of 2 mm, and

particle size of 3 µm. The solvent mixture contained acetonitrile (0.1% formic acid) and water (0.1% formic acid), and was run through the system at a flow rate of 150 µl/min and a temperature of 30 ^{o}C. At the start of the run, the solvent mixture contained 50% acetonitrile increasing to a concentration of 95% within seven minutes (T0-7'). The concentration of ACN was maintained isocratically for one minute (T7-8'), before being reduced to 50% within 0.2 minutes (T8-8.2').

Compound masses in the eluent, between 150-1500 amu, were detected with a DecaXP^{plus} ion trap in negative ion mode (-ESI). MS^2 data were produced with 35% collision energy. The spray chamber conditions included the use of 50 units sheath gas, 5 units aux gas, 3.5 kV spray voltage, and a capillary temperature of 350 ^{o}C. UV spectra were collected from 200-600 nm.

3.3.9.2 High-resolution LCMS

High-resolution LCMS data of fraction 2G4 were obtained by running a sub-sample on a Surveyor HPLC system, located at the John Innes Centre (JIC), which was connected to a Luna C_{18} column. The column had a length of 50 mm, diameter of 2 mm, and particle size of 3 µm. The system was run with the same chromatography as described for the low-resolution LCMS analysis.

Compound masses in the eluent between 150-1500 amu, were detected with an orbitrap mass spectrometer in negative ion mode (-ESI), at a resolution of 60,000. MS^2 data were produced with 35% collision energy; daughter ions were detected at a resolution of 30,000. The spray chamber conditions included the use of 30 units sheath gas, spray voltage of 4.5 kV, capillary temperature of 300 °C, and a tube-lens voltage of 168 V. Molecular formulae of MS and MS^2 masses were calculated using the software Xcalibur, allowing a maximum of 35xC, 60xH, 10xN, and 15xO atoms.

3.3.10 Tandem-LCMS analysis of antimycin and an antimycin standard

Low-resolution LCMS data, of the double-purified (NP/RP) combined fractions B and an antimycin A_1-A_4 standard (Sigma-Aldrich A8674), were

obtained by running sub-samples of both in separation, and in combination, of one another. The Surveyor HPLC system, set up at the John Innes Centre, was attached to a Luna C_8 column (Phenomenex). The column had a length of 100 mm, diameter of 2 mm, and particle size of 3 µm. The solvent mixture contained acetonitrile and water (0.1% formic acid), and was run through the system at a flow rate of 220 µl/min and a temperature of 30 ^{o}C. At the beginning of the run, the solvent mixture contained 2% ACN increasing to 95% within 15 minutes (T0-15'). After 2 minutes of isocratic solvent conditions (T15-17'), the proportion of ACN was reduced to 5% within 30 seconds (T17-17.5').

Compound masses in the eluent, between 150-1500 amu, were detected with a DecaXP^{plus} ion trap in positive ion mode (+ESI). MS^2 data were produced with 35% collision energy. The spray chamber conditions included the use of 50 units sheath gas, 5 units aux gas, spray voltage of 3.8 kV, and a capillary temperature of 350 ^{o}C. UV spectra were detected from 200-600 nm.

3.4 Results

In this chapter, the hypothesis is tested that the *Acromyrmex* ant-associated bacterium *Streptomyces* E_8, in addition to candicidin (Barke *et al*. 2010), is able to produce other antifungal compounds. Bioactive compounds, extracted from a *Streptomyces* E_8 liquid growth medium, were column-purified and mass-identified. Based on the mass data, molecular formulae could be calculated that allowed the identification of antifungal compounds.

Mechanisms of antifungal action can be fungal taxon-specific; this means that each test fungus species might be inhibited by only a subset of tested antifungals. During the purification of the *Streptomyces* E_8 antifungals, initial bioassays made use of two test fungi, namely '*Escovopsis* EWC' and '*Candida* CA_6'. Later challenges made use of the faster and more consistently growing *Candida* fungus, facilitating high-volume bioassays. Despite the possibility that these test fungi are unaffected by some or many compounds, I was still able to detect the presence of multiple antifungal compounds.

3.4.1 Culture supernatants

3.4.1.1 Supernatants – large- and small-scale productions

Streptomyces E_8 and *S. lividans* supernatants were obtained from relatively nutrient-poor culture broths. From a large-scale production, 1250 ml of *Streptomyces* E_8 supernatant were obtained. Of those, a majority (1050 ml) was dedicated for a BuOH extraction with proceeding column fractionations; a minority (200 ml) was used for bioassays. Included in the large-scale production was a negative control; in particular, 100 ml of *S. lividans* supernatant were obtained and destined for bioassays. From a small-scale production, 100 ml of *Streptomyces* E_8 and *S. lividans* supernatants were obtained, both dedicated for bioassays.

3.4.1.2 Bioassays against Candida

The bioactivity of the *Streptomyces* E_8 supernatant, destined for column fractionations, was confirmed by bioassaying a small sub-sample of the 200 ml fraction from the large-scale production against various *Candida* strains. Among the test fungi were eleven *C. albicans* strains (CA_1-CA_{11}) and three other *Candida* species, including *C. tropicalis* (CA_{12}), *C. glabrata* (CA_{13}), and *C. dubliniensis* (CA_{14}). The supernatant was diluted with distilled water to obtain concentrations between 100% and 10%; the same distilled water was used as negative control (0%). The eleven dilutions were applied to *Candida* (CA_1-CA_{14}) -coated agar plates and fungal inhibition was measured qualitatively.

Inhibition zones, ranging from weak to strong, were given scores (Table 3.3). A score of three was given to zones with complete fungal inhibition (lack of fungal growth) and zero was used for zones without fungal inhibition (unaffected fungal growth). Intermediate scores (one and two) were attributed to intermediate bioassay results. In the table, the dilution factor is set out against fungal identity. The more concentrated the supernatant, and/or the less resistant a given *Candida* strain, the higher the inhibition score of a particular dilution. Since the applied supernatant concentrations are the same for all test fungi, the line totals (last column) display how resistant fungal strains are. For example,

Candida CA_3, CA_5, CA_6, and CA_{13} show the lowest resistance to the *Streptomyces* E_8 supernatant; among those, *Candida* CA_6 is chosen as test fungus for high-volume bioassays.

Strain	100%	90%	80%	70%	60%	50%	40%	30%	20%	10%	0%	Total
CA_1	3	3	3	3	3	3	3	3	2	1	0	**27**
CA_2	3	3	3	3	3	3	3	3	2	1	0	**27**
CA_3	3	3	3	3	3	3	3	3	3	2	0	**29**
CA_4	3	3	3	3	3	3	3	3	2	2	0	**28**
CA_5	3	3	3	3	3	3	3	3	3	2	0	**29**
CA_6	3	3	3	3	3	3	3	3	3	2	0	**29**
CA_7	3	3	3	3	3	3	3	3	2	1	0	**27**
CA_8	3	3	3	3	3	3	3	2	2	1	0	**26**
CA_9	3	3	3	3	3	3	3	3	2	1	0	**27**
CA_{10}	3	3	3	3	3	3	3	3	2	1	0	**27**
CA_{11}	3	3	3	3	3	3	3	2	1	1	0	**25**
CA_{12}	3	3	3	2	1	1	0	0	0	0	0	**13**
CA_{13}	3	3	3	3	3	3	3	3	3	2	0	**29**
CA_{14}	3	3	3	3	3	3	3	3	3	1	0	**28**

Table 3.3 Inhibitory effect of the *Streptomyces* (E_8) growth supernatant on fourteen *Candida* strains. Fungal inhibition is scored qualitatively by allocating credits, ranging from three (strongest bioactivity and no fungal growth) to zero (no bioactivity and unaffected fungal growth). The line totals of the inhibition scores show the strength of fungal resistance. Most susceptible to the bacterial antifungals are *Candida* CA_3, CA_5, CA_6, and CA_{13}. *Candida* CA_6 was chosen as test fungus for follow-up bioassay-guided column fractionations.

3.4.1.3 Bioassays against Escovopsis

The *Streptomyces* E_8 supernatant from the large-scale production, destined for bioassays, was also tested against *Escovopsis* EWC. In contrast to other filamentous fungi, such as *Escovopsis* EWB, this *Escovopsis* strain showed high susceptibilities and a consistent growth during previous colony bioassays (Fig. 2.6; Fig. 2.7). The supernatant of *S. lividans* from the large-scale production served as a negative control. The supernatants were added to the centre of agar plates and *Escovopsis* was inoculated in a distance of 1 cm from

the edge of the supernatant. After incubation, the presence or absence of fungal inhibition was scored.

In contrast to the supernatant of *S. lividans*, the supernatant of *Streptomyces* E_8 inhibits the growth of the nest parasite (Fig. 3.2). This effect may be attributed to the same or to different antifungals that already inhibited *Candida*.

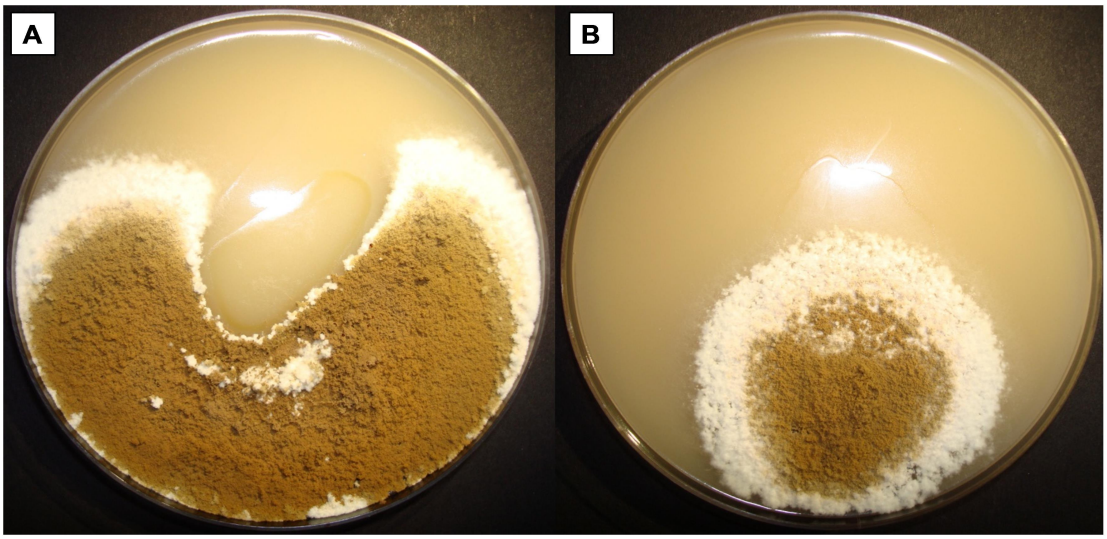


Figure 3.2 *Escovopsis* (EWC) supernatant bioassays. **(A)** *Escovopsis* EWC is challenged against the growth culture supernatant of *Streptomyces* E_8. The test fungus avoids overgrowing the centrally inoculated supernatant, showing fungal inhibition. **(B)** As a negative control, the growth culture supernatant of *S. lividans* is included. The test fungus, *Escovopsis* EWC, overgrows the centrally inoculated supernatant.

3.4.2 Butanol-extracts

3.4.2.1 Butanol extraction – large- and small-scale productions

Apolar molecules in the *Streptomyces* E_8 and *S. lividans* supernatants, from the large- and small-scale productions, were extracted with butanol. In particular, 1050 ml from the *Streptomyces* E_8 large-scale production, destined for column fractionations, were extracted. Also, 45 ml of 200 ml from the *Streptomyces* E_8 large-scale production, dedicated for bioassays, were extracted. In addition, 3 ml of 100 ml from the *Streptomyces* E_8 and *S. lividans* small-scale productions, destined for bioassays, were extracted. Typically, the

aqueous phase of the washes was disposed, except for the 45 ml extraction. The BuOH extracts and aqueous phase were challenged against different test fungi.

3.4.2.2 Bioassays against Candida

The butanol extract, originating from a 45 ml sub-sample of the *Streptomyces* E_8 large-scale production, was re-dissolved in 100% MeOH and challenged against *Candida* CA_6. In addition, the bioassays included some of the original supernatant, the aqueous phase, and two negative controls that consisted of 100% BuOH and 100% MeOH. BuOH has been used for extracting the supernatants and MeOH has been used as holding liquid for the residues. All liquids were inoculated on paper filter disks. When dry, the filters were attached with distilled water to a *Candida*-coated agar plate. After incubation, the presence or absence of fungal inhibition was scored.

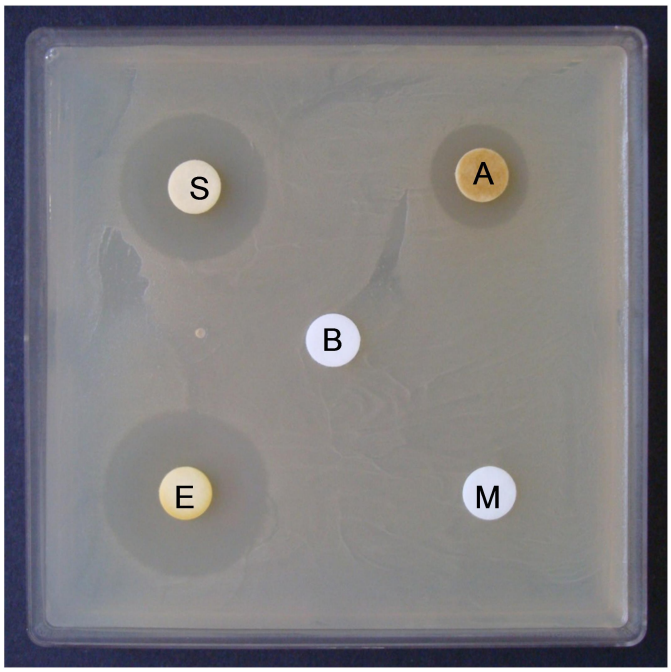


Figure 3.3 *Candida* (CA_6) bioassays testing the bioactivity of the *Streptomyces* E_8 growth culture (S), as well as the aqueous (A) and organic phase (E) of a BuOH extraction. As negative controls, 100% MeOH (M) and 100% BuOH (B) are used. The liquids were inoculated onto paper filter disks and when dry attached to a *Candida*-coated agar plate. Despite some antifungal molecules remained in the aqueous phase, sufficient compounds were trapped in the organic phase for the *Streptomyces* E_8 antifungals to be extracted by butanol. No conclusions can be drawn from the diameters of the inhibition zones, since neither the entire residue of the organic phase nor the full volume of the aqueous phase are used for the bioassays.

Not only the crude supernatant (S) and the

extract (E) inhibit the growth of *Candida*, but also the aqueous phase (A), indicating an incomplete absorption of antifungal molecules by the organic phase (Fig. 3.3). The size of the inhibition zones cannot be compared with one another, as not the entire apolar residue and aqueous phase were used for inoculating the filter disks. The results indicate that butanol is suitable for the extraction of antifungals from the *Streptomyces* E_8 growth supernatant.

3.4.2.3 Bioassays against Escovopsis

The BuOH extracts, obtained from 3 ml sub-samples of the *Streptomyces* E_8 and *S. lividans* small-scale productions, were re-dissolved in 50% of aqueous MeOH, and challenged against *Escovopsis* EWC. When dry, the filters were attached with distilled water to the centre of the agar plates. Then, the nest parasite was inoculated at the edge of the bioassay plates. After incubation, the presence or absence of fungal inhibition was scored.

While the filter with the BuOH extract of *S. lividans* is completely overgrown by the fungus, *Escovopsis* avoids contacting the disk with the BuOH extract of *Streptomyces* E_8 (Fig. 3.4).

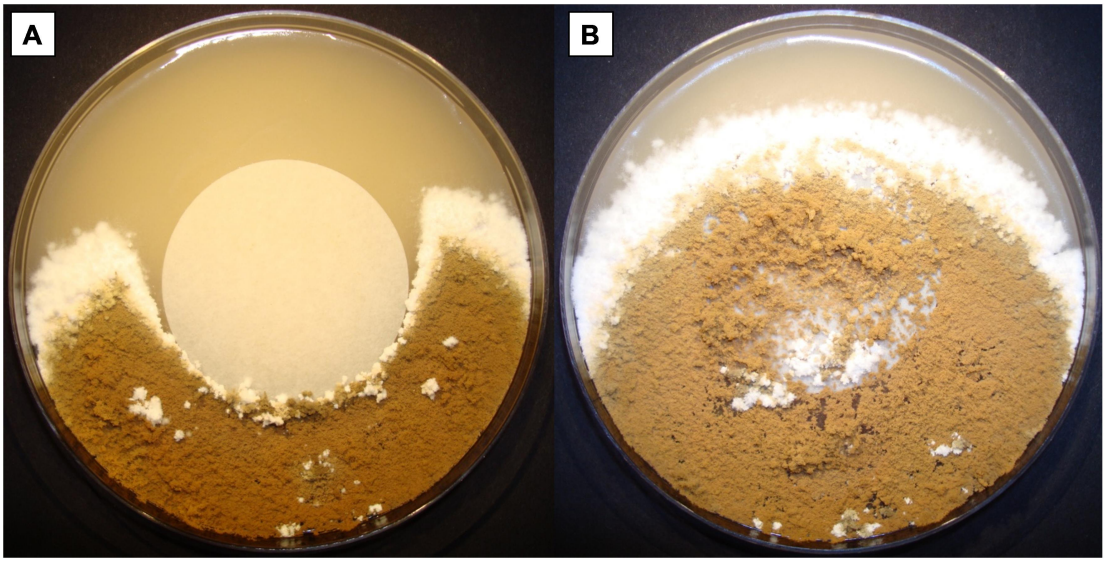


Figure 3.4 (A-B) *Escovopsis* (EWC) bioassays testing the bioactivity of the organic phases of *Streptomyces* E_8 and *S. lividans* BuOH extracts. In contrast to the filter of the negative control (B), the test fungus clearly avoids to overgrow the filter with the *Streptomyces* E_8 extract (A), showing that the extract contains antifungals that not only inhibit *Candida* (Fig. 3.3) but also *Escovopsis*.

3.4.2.4 Thin layer chromatography of butanol extracts

A sub-sample of the BuOH extract, obtained from 1050 ml of the *Streptomyces* E_8 large-scale production, was re-dissolved in 50% of aqueous MeOH and analysed by thin layer chromatography (TLC). The dissolved residue was point-inoculated to the base of six silica plates. Mixtures of methanol (MeOH) and ethyl acetate (EtAc) in ratios, ranging from 100:0 to 50:50 (MeOH:EtAc), served as the mobile phase. The migration rate of compounds across TLC plates does not only depend on the polarities of the compounds themselves, but also on the polarities of the stationary (silicate) and mobile (solvent mixture) phases. The more polar the compounds, the slower their migration rate, meaning that relatively apolar compounds will settle relatively close to the solvent front.

Figure 3.5 shows a schematic drawing of the TLC plates under short- (bottom left) and long- (bottom right) wave UV light as well as photographs of the TLC plates after being stained with the oxidising agent potassium permanganate (top). A MeOH:EtAc ratio of 70:30 shows the best separation of oxidisable compounds in the BuOH extract. In general, the TLC results may indicate that the (non-)bioactive compounds in the BuOH extract are separated well by this solvent system.

3.4.3 Normal-phase column fractionation

3.4.3.1 Fractionation

The BuOH extract from a 1050 ml *Streptomyces* E_8 large-scale production, previously analysed by TLC, was purified by normal-phase (NP) column fractionation. The remaining residue was re-dissolved in a MeOH:EtAc (4:11) mixture. A sub-sample of this column starter material was preserved for later TLC analysis. The extract was run through a column, containing silica as

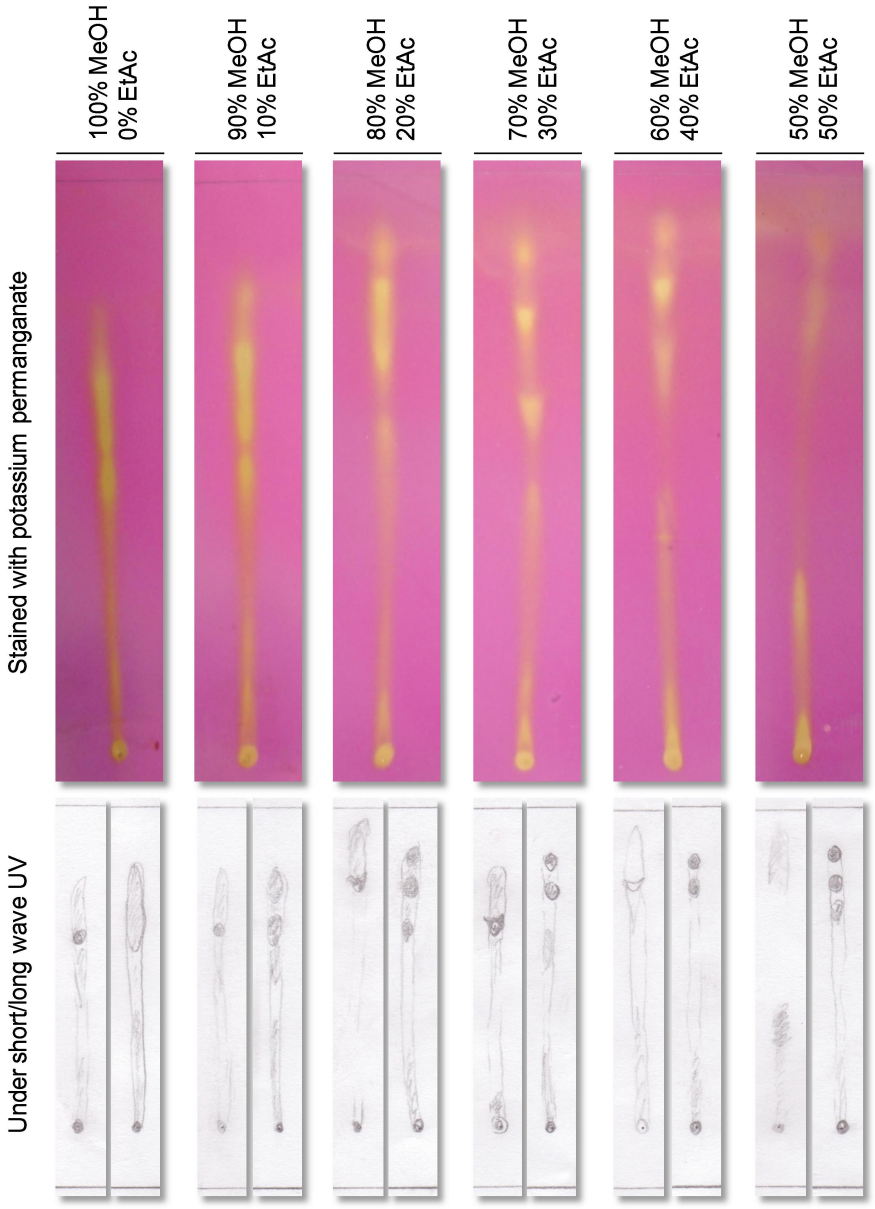


Figure 3.5 Thin layer chromatography (TLC) analysis on the residue of a *Streptomyces* E_8 BuOH extract to determine the optimal solvent conditions for a normal-phase column fractionation. Sub-samples of the residue are re-dissolved, inoculated to the base of TLC plates, and run across plates using solvent conditions ranging from relatively polar (100:0 MeOH:EtAc) to more apolar (50:50 MeOH:EtAc). In the lower panel of the figure, the TLC plates are shown under short- (left) and long- (right) wave UV light; in the upper panel the same plates are shown after being stained with the oxidising agent potassium permanganate. The figure shows that the stainable (non-)bioactive compounds separate best under solvent conditions of 70% MeOH and 30% EtAc.

stationary phase, with increasingly polar solvent mixtures (mobile phase) spanning MeOH:EtAc ratios of 0:100, 60:40, 70:30, and 100:0. These solvent ratios were guided by findings from a previous TLC analysis, described above, in which a 70:30 MeOH:EtAc ratio exhibited the best separation of oxidisable compounds. The eluent, leaving the column, was captured in a total of 85 fractions.

Some normal-phase fractions (12-19, 24-46, 78-80) show a yellow/brown colouration (Fig. 3.6), whereas the remainder is transparent. Since early fractions eluted under relatively apolar conditions (EtAc>MeOH) and later ones under relatively polar conditions (EtAc<MeOH), the fractions should contain different molecules.

3.4.3.2 Bioassays against Candida

To localise the antifungal molecules in the NP fractions (1-85), bioassays were carried out, in which the fractions were tested against *Candida* CA_6. Small amounts of each fraction were loaded onto paper filter disks. When dry, the filters were attached to *Candida*-coated agar plates with distilled water. After incubation, the diameters of inhibition zones around test filters were measured.

These bioassays (Fig. 3.7) were carried out in triplicate and the diameters of the inhibition zones, averaged across replicate plates, are presented in figure 3.8. The graph shows a baseline of 0.9 cm, which corresponds to the diameter of the filters, and indicates the absence of fungal inhibition. Two sets of compounds, including fractions 22-43 and 67-85, show averages above 0.9 cm. Due to the polarity difference of the eluents in both sets, this finding reveals that *Streptomyces* E_8 produces at least two antifungals.

3.4.3.3 Thin layer chromatography

The normal-phase fractions (1-85) were analysed by TLC. As reference, the column starter material was point-inoculated onto the first lane of each TLC plate, the NP fractions onto any lane thereafter. Since the crude BuOH extract separated best under solvent conditions, consisting of a MeOH:EtAc ratio of



Figure 3.6 Fractions (1-85) of a normal-phase column fractionation of the *Streptomyces* E_8 BuOH extract. As solvent system, changing ratios of EtAc and MeOH are used; while initial fractions are relatively apolar, later ones are more polar. Fractions 12-19, 24-46, and 78-80 are coloured and the remaining fractions are transparent.

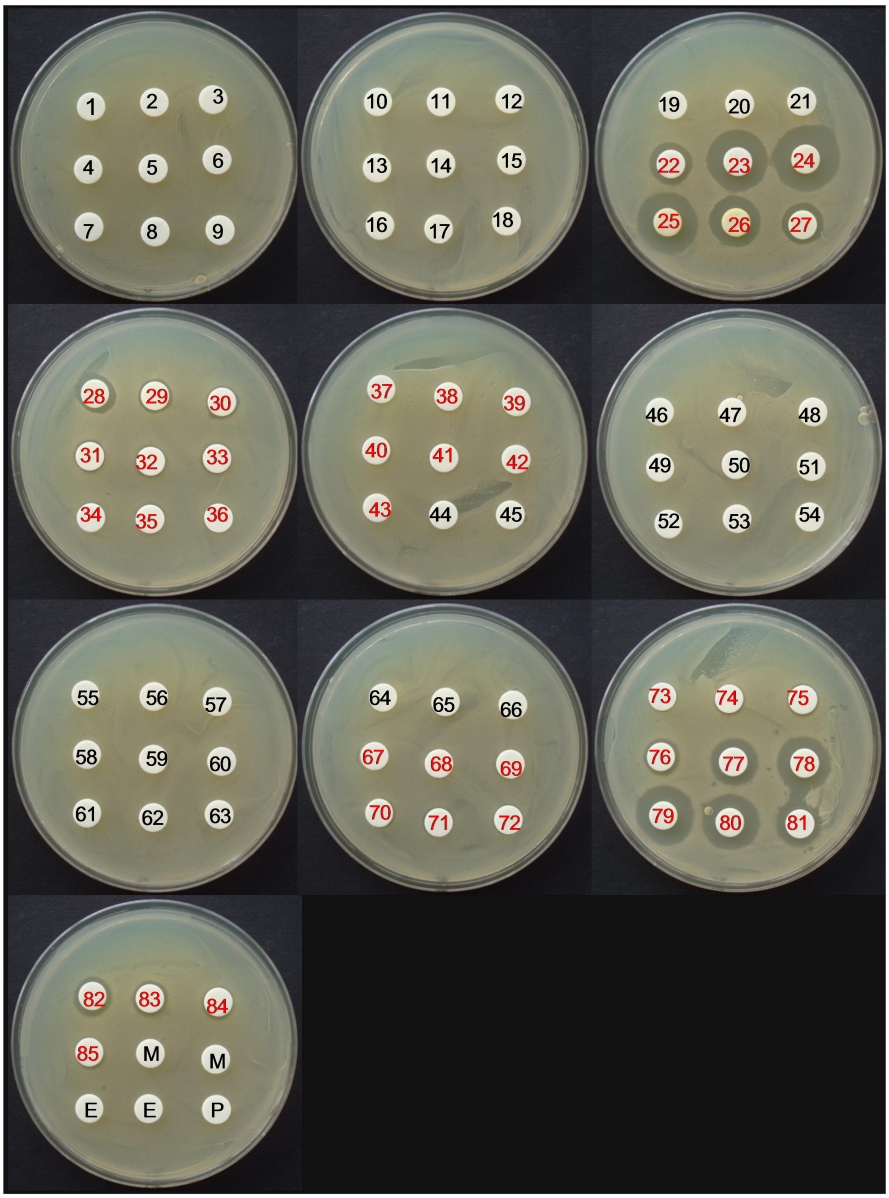


Figure 3.7 *Candida* (CA_6) bioassays testing the bioactivity of normal-phase fractions 1-85 (Fig. 3.6). Paper filter disks, inoculated with fraction sub-samples, are attached to *Candida*-coated agar plates. As negative controls, one uninoculated filter disk (P), two disks with 100% MeOH (M), and two disks with 100% EtAc (E) were included. Two sets of fractions, namely fractions 22-43 and 67-85, are inhibiting the growth of *Candida* (red font). Both sets differ too much in their polarity to contain the same antifungal compound(s). Analogies between the observed bioactivity (22-43, 67-85) and the colouration (12-19, 24-46, 78-80) of fractions may be linked or, alternatively, attributed to different molecules. This experiment is carried out in triplicate; average diameters of the inhibition zones are shown in figure 3.8.

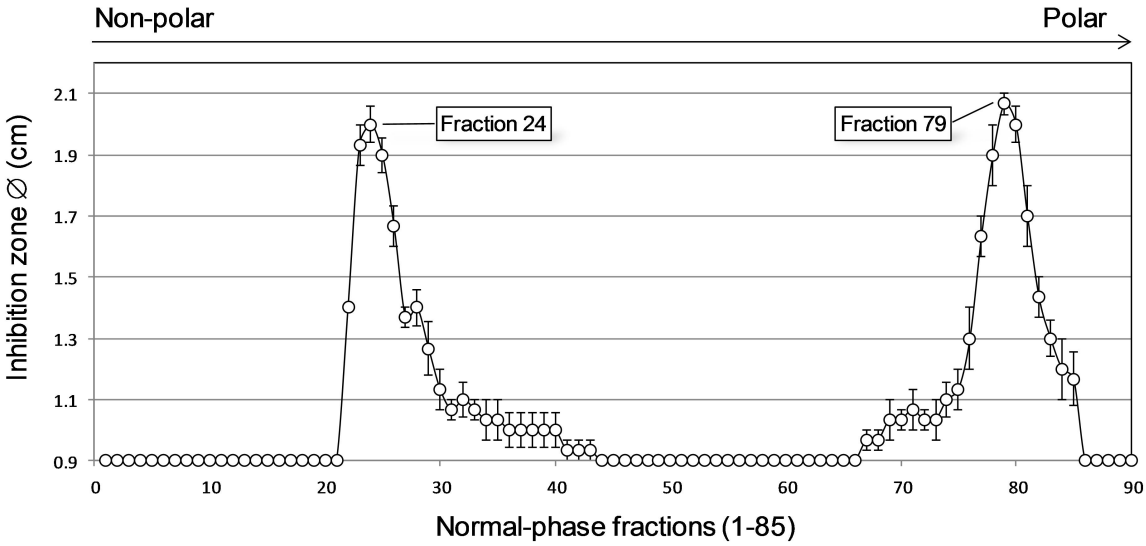


Figure 3.8 Graphical representation of bioassay results on the normal-phase fractions 1-85, presented in figure 3.7. The X-axis shows the fraction identity and the Y-axis the average diameters (± s.e.) of the inhibition zones around filter disks. The baseline of 0.9 cm is formed by the diameter of the filter disks, and values above 0.9 cm stem from fungal inhibition. Filters 85-90 are negative controls. Two sets of fractions are bioactive, namely fractions 22-43 and 67-85. Due to the differences in their polarity, both sets must contain different antifungals. In other words, *Streptomyces* E_8 must secrete a minimum of two antifungals into the liquid medium.

70:30, the same solvent conditions were applied here. The TLC plates were stained with the oxidising agent potassium permanganate.

Only photographs of selected TLC plates are presented in figure 3.9; in contrast TLC plates inoculated with non-bioactive or uncoloured fractions, or fractions that could not be stained with potassium permanganate, were excluded from this selection. Only the relatively apolar fractions 13-15 and 22-29 could be stained. The stainable fractions 13-15 have previously been shown to also possess a colouration (12-19) but no inhibitory effect on *Candida*. In contrast, the oxidisable fractions 22-29 have earlier been shown to possess both a colouration (24-46) and an inhibitory effect on the yeast (22-43). Finally, no oxidation signal could be detected from the relatively polar fractions 78-80 or 67-85, which have previously been shown to possess a colouration and an inhibitory effect on *Candida*, respectively. The results confirm the previous observation that the applied solvent ratios indeed suited the fractionation of the *Streptomyces* E_8 BuOH extract; after all, oxidisable compounds in the column starter material indeed have separated into different normal-phase fractions.

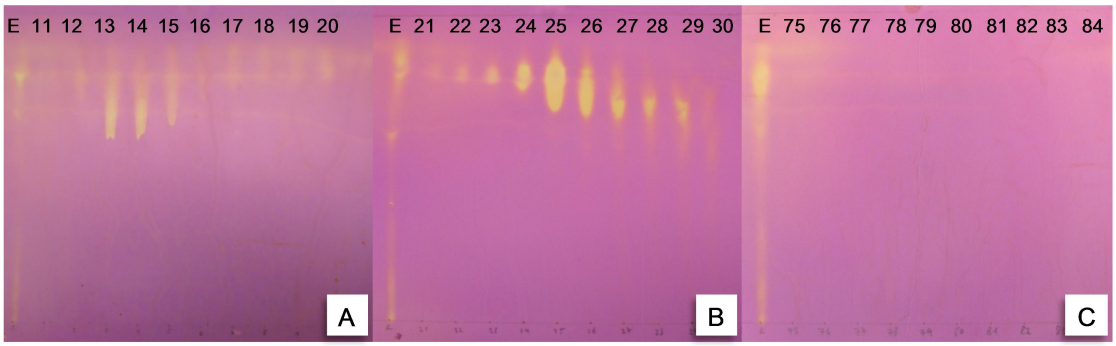


Figure 3.9 Thin layer chromatography (TLC) on the normal-phase fractions (1-85). The TLC plates are inoculated with sub-samples of the normal-phase fractions and the crude BuOH extract that was used as column starter material (E, first lane). A solvent mixture of 70% MeOH and 30% EtAc was used as mobile phase. Here, only those TLC plates are shown, which were inoculated with bioactive (22-43, 67-85) or coloured (12-19, 24-46, 78-80) fractions, or which contain fractions that are stainable with potassium permanganate. Three TLC plates meet these criteria. **(A)** Fractions 13-15 are stainable with potassium permanganate, showed a colouration (12-19), but did not inhibit *Candida*. **(B)** Fractions 22-29 and surrounding are oxidisable, showed a colouration (24-46), and inhibited *Candida* (fractions 22-43). **(C)** Fractions 78-80 showed a colouration and fractions 67-85 inhibited *Candida*; however, none of these fractions contained compounds stainable with potassium permanganate.

3.4.4 Identification of candicidin

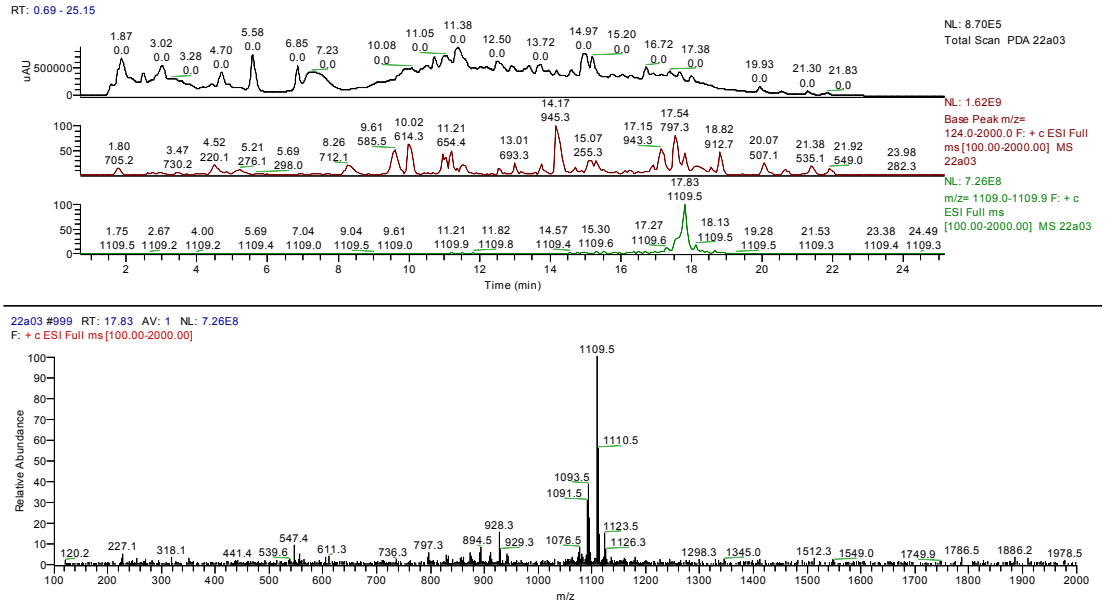
To test the hypothesis that *Streptomyces* E_8 produces other antifungals in addition to candicidin (Barke *et al*. 2010), the presence of latter was analysed in first place. In particular, the crude BuOH extract and two bioactive normal-phase fractions were examined by low- and high-resolution LCMS, respectively. Haeder *et al*. (2009) found candicidin ($C_{59}H_{84}N_2O_{18}$), produced by an *Acromyrmex* cultivar-associated *Streptomyces* bacterium, to possess an accurate ion mass of 1109.57938 amu $[M+H]^+$. The antifungal absorbed UV light at maxima of 408 nm, 384 nm, 364 nm, and 344 nm. During a MS^2 analysis, the authors also showed that the candicidin molecules fragment into daughter ions with masses of 1091.56827 amu and 928.48458 amu $[M+H]^+$. Respectively, these ions were suggested to represent sub-structures of the candicidin molecule, lacking a water molecule $[M+H-H_2O]^+$ and a water molecule plus the mycosamine subunit $[M+H\text{-mycosmine-}H_2O]^+$.

3.4.4.1 Low-resolution LCMS

During a LCMS analysis, the compounds of the BuOH extract were separated on a reversed-phase (RP) column. The masses of the compounds in the eluent, accurate to ± 0.1 amu, were detected by a mass spectrometer in low-resolution and positive ion mode (+ESI). Also UV absorbance and MS^2 data were collected.

The upper part of figure $3.10_{A\text{-}B}$ shows a photodiode array (PDA) trace, base peak chromatogram (BPC) for compound masses in between 124-2000 amu, and mass range for compounds in between 1109.0-1109.9 amu. The PDA and BPC signals reveal that the BuOH extract is loaded with innumerable molecules, most of which are expected to originate from the medium. In contrast, the mass range shows the presence of one dominant peak, eluting after 17.83 minutes. In the lower part of figure 3.10_A, the full MS of this PDA peak is given, which is dominated by an ion mass of 1109.5 amu $[M+H]^+$. To verify or falsify that this mass relates to candicidin, its UV absorbance and MS^2 pattern are analysed.

A



B

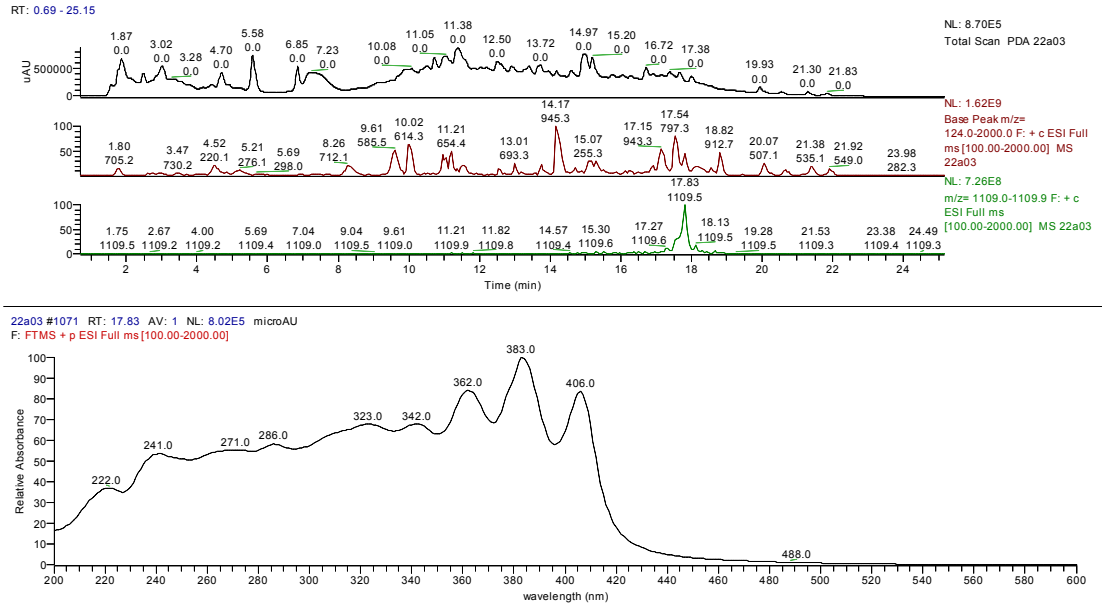
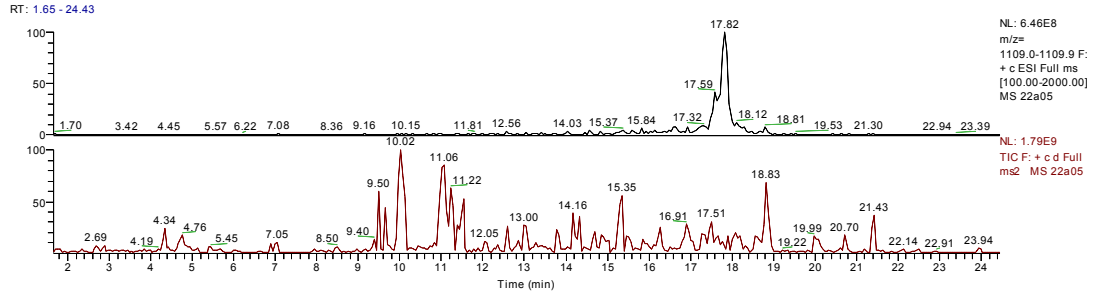
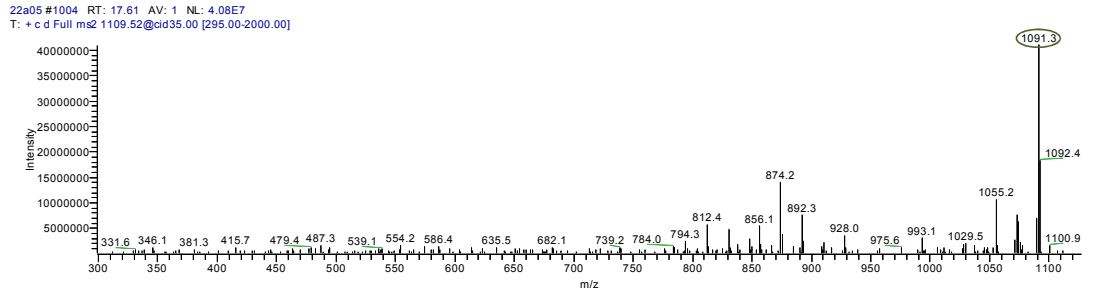


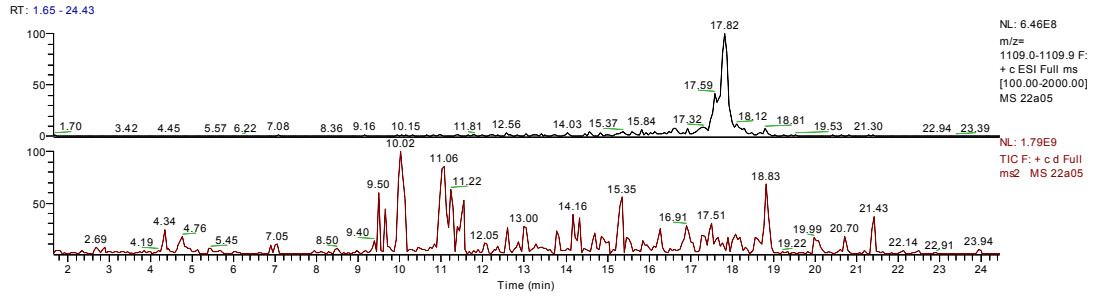
Figure 3.10 (A-B) Low-resolution reversed-phase LCMS on a *Streptomyces* E_8 BuOH extract, in positive ion mode (+ESI). The goal is to check whether the extract contains candicidin. The upper panel of both graphs shows a PDA trace, base peak chromatogram (BPC), and mass range for compounds between 1109.0-1109.9 amu. The latter clearly shows the presence of a single ion peak with a retention time of 17.83 minutes. (A) The full MS of this peak (RT=17.83') is dominated by an ion with a mass of 1109.5 amu $[M+H]^+$. (B) The UV absorbance of the peak (RT=17.83') reveals the presence of four absorbance maxima including 406 nm, 383 nm, 362 nm, and 342 nm. Both, mass and UV data correspond to the data of candicidin D, presented by Haeder *et al*. (2009).

C





D



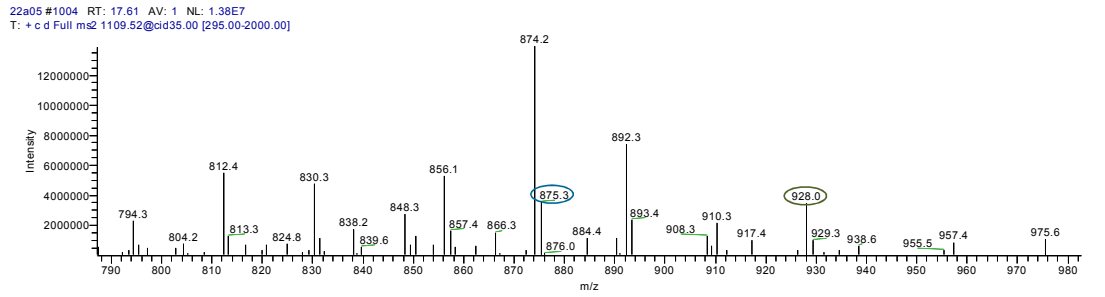


Figure 3.10 (C-D) Low-resolution reversed-phase LCMS on a *Streptomyces* E_8 BuOH extract, in positive ion mode (+ESI). The fragmentation of the 1109.5 amu $[M+H]^+$ parent ion, shown above, reveals the presence of at least three diagnostic daughter ions with masses of 1091.3 amu, 928.0 amu, and 875.3 amu $[M+H]^+$. Haeder *et al*. (2009) suggested the 1091.56827 amu and 928.48458 amu $[M+H]^+$ daughters to match the parent ion lacking a water molecule $[M+H-H_2O]^+$ or a water molecule plus the mycosamine sub-unit $[M+H-H_2O\text{-mycosamine}]^+$. In addition, the 875.3 amu $[M+H]^+$ daughter may correspond to a sub-structure of the parent molecule highlighted teal in figure 3.10_F.

E

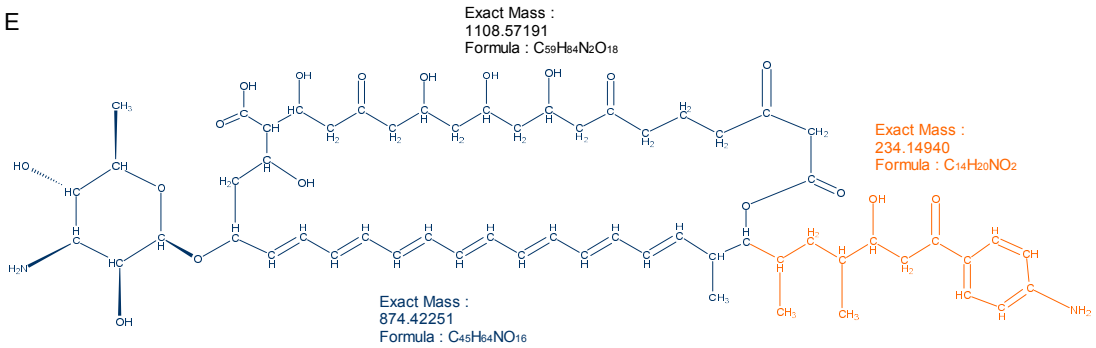


Figure 3.10 (E) Low-resolution reversed-phase LCMS on a *Streptomyces* E_8 BuOH extract, in positive ion mode (+ESI). Molecular structure of candicidin D (Reaxys ID: 6471601). Above, candicidin is suggested to be present in the BuOH extract of the bacterium. The teal colouration indicates a part of the molecule, with a mass of 874 amu, which in figure 3.10_D has been identified as diagnostic daughter ion.

Figure 3.10_B shows the UV absorbance of the eluent with a retention time (RT) of 17.83 minutes. Due to the diversity of unrelated absorbances, this and no earlier PDA peak was chosen, despite the slight delay between PDA and mass detection. The UV signal clearly shows the presence of four absorbance maxima at higher wavelengths, including 406 nm, 383 nm, 362 nm, and 342 nm, which resemble the maxima found by Haeder *et al*. (2009). In general, corresponding maxima may vary by up to 5 nm, for example, due to slight differences in the solvent mixing and/or presence of coeluting contaminants.

Since molecules tend to fragment at compound-specific locations, MS^2 data provide fingerprints of compounds. These sub-structures, as represented by diagnostic daughter ions, provide information on the structural constellation and identity of the parent molecule. The MS^2 fragmentation pattern of the 1109.5 amu $[M+H]^+$ parent ion is shown in figure $3.10_{C\text{-}D}$. Daughter ions with masses of 875.3 amu and 1091.3 amu $[M+H]^+$ could be detected matching a sub-structure of candicidin D (Reaxys ID: 6471601), highlighted teal in figure 3.10_E, and candicidin D lacking a water molecule, respectively. In addition, a daughter ion with a mass of 928.0 amu $[M+H]^+$ was detected, suggested to match candicidin D lacking a water molecule plus the mycosamine subunit (Haeder *et al.* 2009). In conclusion, both the UV and MS^2 data support the

suspicion that the 1109.5 amu $[M+H]^+$ parent ion in the *Streptomyces* E_8 BuOH extract indeed is candicidin.

3.4.4.2 High-resolution LCMS

Low-resolution LCMS has suggested candicidin to be present in the *Streptomyces* E_8 BuOH extract. In order to trace the destination of the antifungal after normal-phase column fractionation, two bioactive normal-phase fractions, including the relatively apolar fraction 24 and the more polar fraction 79 (Fig. 3.8), were analysed by high-resolution LCMS. The orbitrap mass spectrometer was set to detect MS and MS^2 masses, accurate to ± 0.00001 amu, in positive ion mode (+ESI) that served in calculating molecular formulae.

The upper panel of figure $3.11_{A\text{-}B}$ shows a BPC for compounds in between 124-1500 amu and a mass range of 1109.0-1109.9 amu, present in fraction 79. The mass range shows an accumulation of 1109 amu $[M+H]^+$ masses with a 'mean' retention time of 14.56 minutes. In the lower panel, the full MS is given for retention times, ranging from 13.81-15.16 minutes, as well as a simulation of the candicidin mass 1109.57919 amu $[M+H]^+$. With a mass difference of only 0.00131 amu, the simulation matches a sample ion with a mass of 1109.57788 amu $[M+H]^+$ most closely. Based on the accurate sample mass, a molecular formula was calculated, accepting a maximum of 100xN, 100xO, 100xC, 100xH, and 1xNa atoms, in case the detected ion is a sodium adduct. Among the calculated formulae was $C_{59}H_{85}N_2O_{18}$, which corresponds to the formula of the neutral candicidin molecule ($C_{59}H_{84}N_2O_{18}$). Unfortunately, no accurate MS^2 data of the 1109 amu $[M+H]^+$ parent ion has been generated.

The upper panel of figure $3.11_{C\text{-}D}$ provides a BPC for compound masses in between 50-1500 amu and a mass range of 1109.0-1109.9 amu, present in fraction 24. The mass range shows the absence of a dominant 1109 amu $[M+H]^+$ peak. In the lower panel, a full MS for retention times is shown, ranging from 9.90-22.08 minutes, as well as a simulation of the candicidin mass 1109.57919 amu $[M+H]^+$.

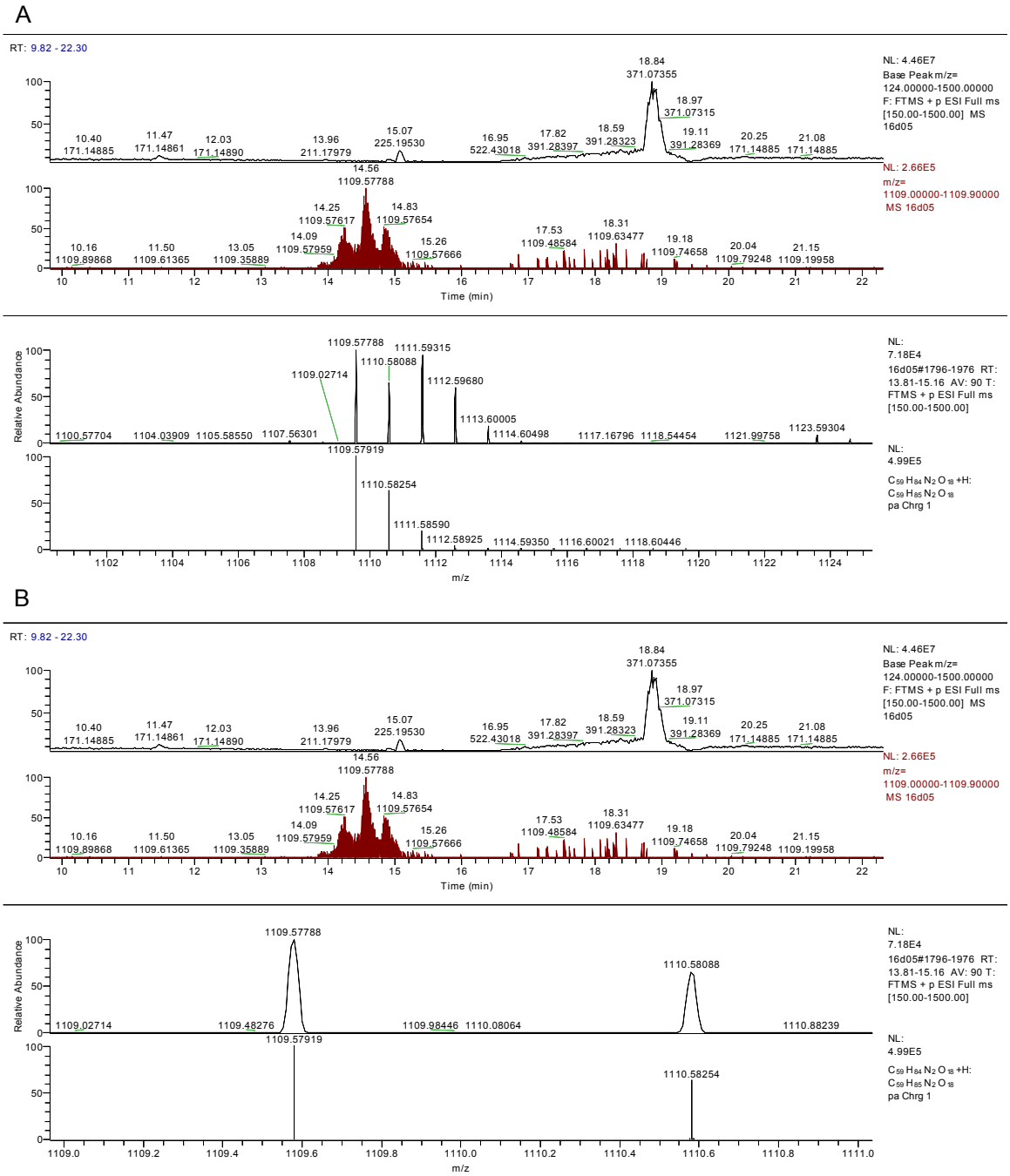
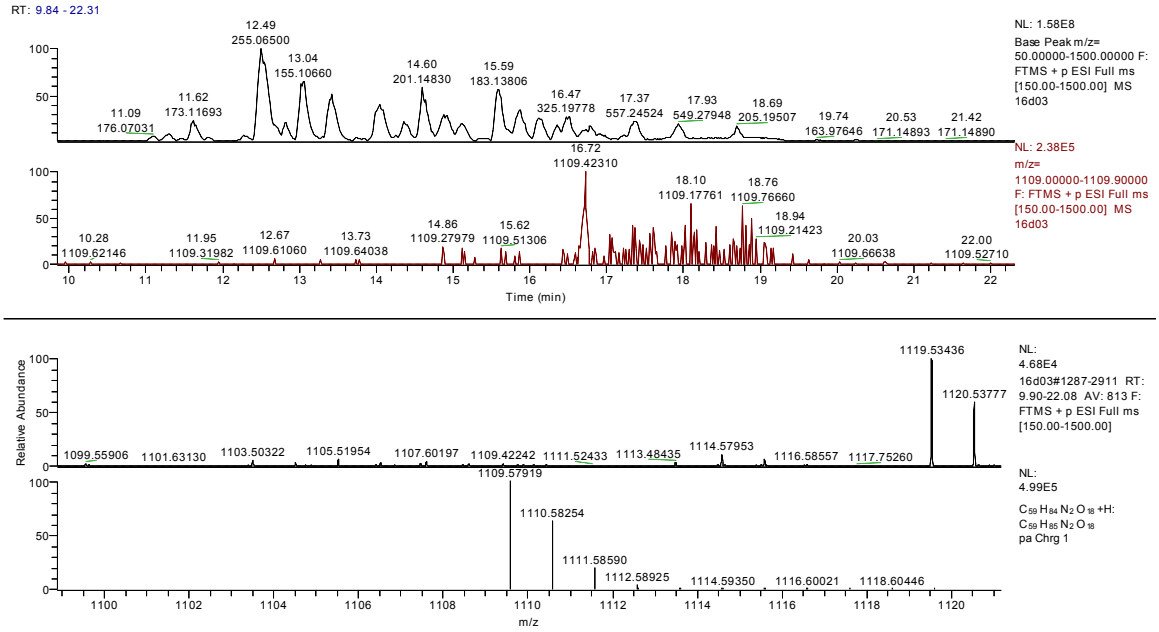


Figure 3.11 (A-B) High-resolution reversed-phase LCMS on normal-phase fraction 79 (Fig. 3.8), in positive ion mode (+ESI). The upper panel of the graphs shows a base peak chromatogram (BPC) and mass range for compounds between 1109.0-1109.9 amu. The latter reveals the presence of a 1109.57788 amu $[M+H]^+$ ion peak eluting at RT=14.56'. The lower panel of the graphs shows a simulation on the candicidin formula based on which its accurate mass is expected to be 1109.57919 amu $[M+H]^+$. The mass difference between simulation and sample mass is 0.00131 amu; however, the molecular formula of the sample ion ($C_{59}H_{85}N_2O_{18}$) matches the formula of the neutral candicidin mass ($C_{59}H_{84}N_2O_{18}$). Hence, the candicidin molecules detected by low-resolution LCMS in the *Streptomyces* E_8 BuOH extract (Fig. 3.10) likely eluted into the relatively polar set of bioactive normal-phase fractions.

C



D

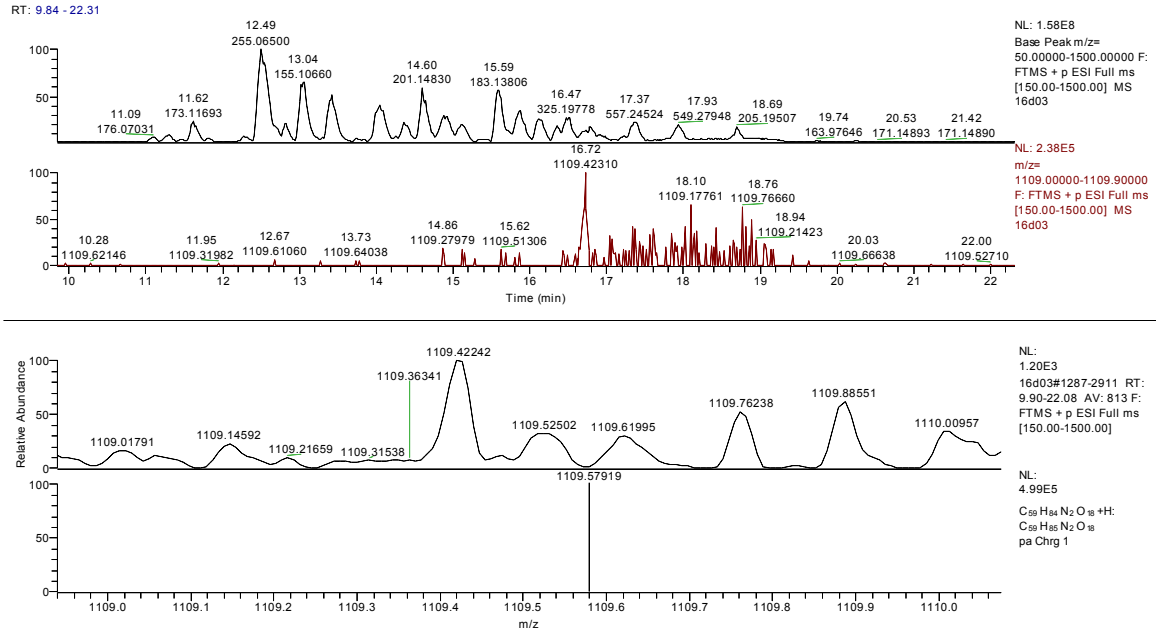


Figure 3.11 (C-D) High-resolution reversed-phase LCMS on normal-phase fraction 24 (Fig. 3.8), in positive ion mode (+ESI). The upper panel of the graphs shows a base peak chromatogram (BPC) and mass range for compounds between 1109.0-1109.9 amu. A strong 1109 amu mass signal, as observed for fraction 79, is absent from the mass range of fraction 24. The lower panel of the graphs shows that the difference between the simulated candicidin mass (1109.57919 amu $[M+H]^+$) and the closest sample masses (1109.52502 amu and 1109.61995 amu $[M+H]^+$) are 0.05417 amu and 0.04076 amu. These differences are too large to be explained by orbitrap measuring errors. For this reason, candicidin can be regarded as absent from the more apolar set of bioactive normal-phase fractions.

The simulated mass does not match any of the full MS masses; the best matching sample masses, 1109.52502 amu and 1109.61995 amu $[M+H]^+$, differ from the simulation by 0.05417 amu and 0.04076 amu, respectively. These differences are too large to be explained by orbitrap measuring errors and may hence indicate the absence of candicidin in fraction 24.

In conclusion, these results provide further evidence that the 1109 amu $[M+H]^+$ ion, which is traced in the *Streptomyces* E_8 BuOH extract, indeed corresponds to candicidin. During a normal-phase column fractionation of the crude BuOH extract, the antifungal apparently eluted into the relatively polar fraction 79; in contrast, candicidin was absent from the more apolar fraction 24.

3.4.5 Pooling of normal-phase fractions

3.4.5.1 Pooling

The normal-phase fractions 1-21 (non-bioactive), 22-43 (bioactive), 44-66 (non-bioactive), and 67-85 (bioactive) were pooled to increase antifungal concentrations. Figure 3.12 shows an overview of the pooled fractions, which in the following will be referred to as 'combined fractions A-D'. After evaporating the holding liquids, re-dissolving the residues, and temporally storing the samples, precipitation accumulated at the bottom of the flasks. Hence, the supernatants and precipitates of the combined fractions A-D were challenged against *Candida* separately.

3.4.5.2 Bioassays against Candida

For the bioassays, small sub-samples of the pellets were re-dissolved in 100% DMSO. The supernatants and dissolved pellet materials, alongside two negative controls (100% DMSO and 100% MeOH), were inoculated onto paper filter disks. Then, the filters were attached with distilled water to *Candida*-coated agar plates and fungal inhibition was recorded.

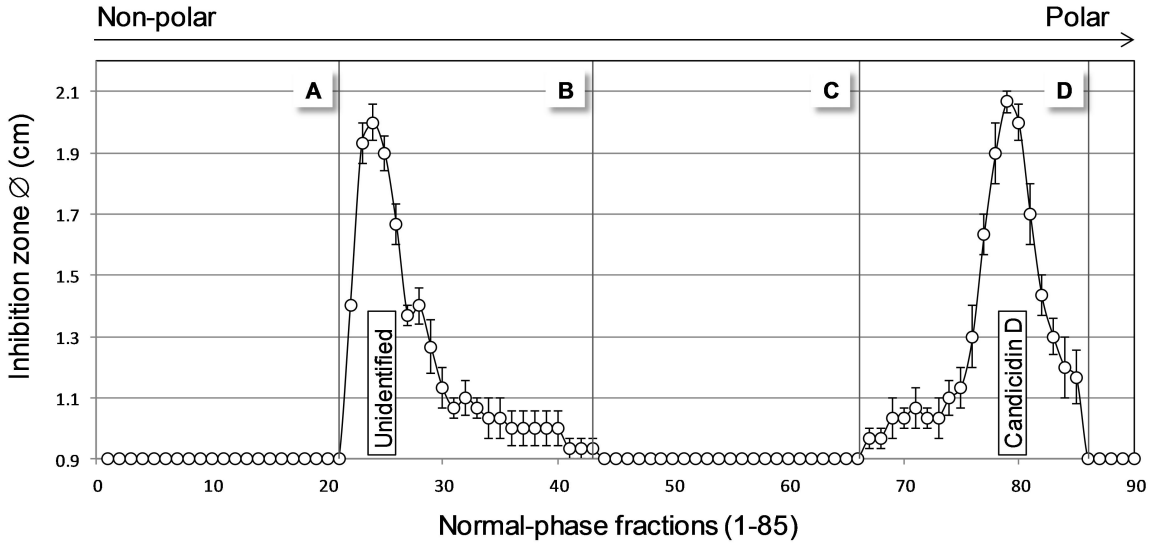


Figure 3.12 Pooling of the normal-phase fractions 1-85 into the combined fractions 1-21 (A), 22-43 (B), 44-66 (C), and 67-85 (D) according to their bioactivity. The combined fractions B and D should contain most of the bioactive molecules present in the *Streptomyces* E_8 BuOH extract. Due to indications suggesting candicidin to be present in fraction 79 (Fig. 3.11), the combined fractions B are used for a follow-up reversed-phase column purification.

The supernatants and pellets of the combined fractions B-D were bioactive (Fig. 3.13). Sub-lethal antifungal concentrations in fractions 44-66 (Fig. 3.12) are responsible for the inhibitory properties of the combined fractions C in this experiment; these antifungals must be the same compounds causing bioactivity in the combined fractions B and/or D. Also, the results indicate that some of the antifungal molecules in the supernatants of the combined fractions B and D dropped out of solution.

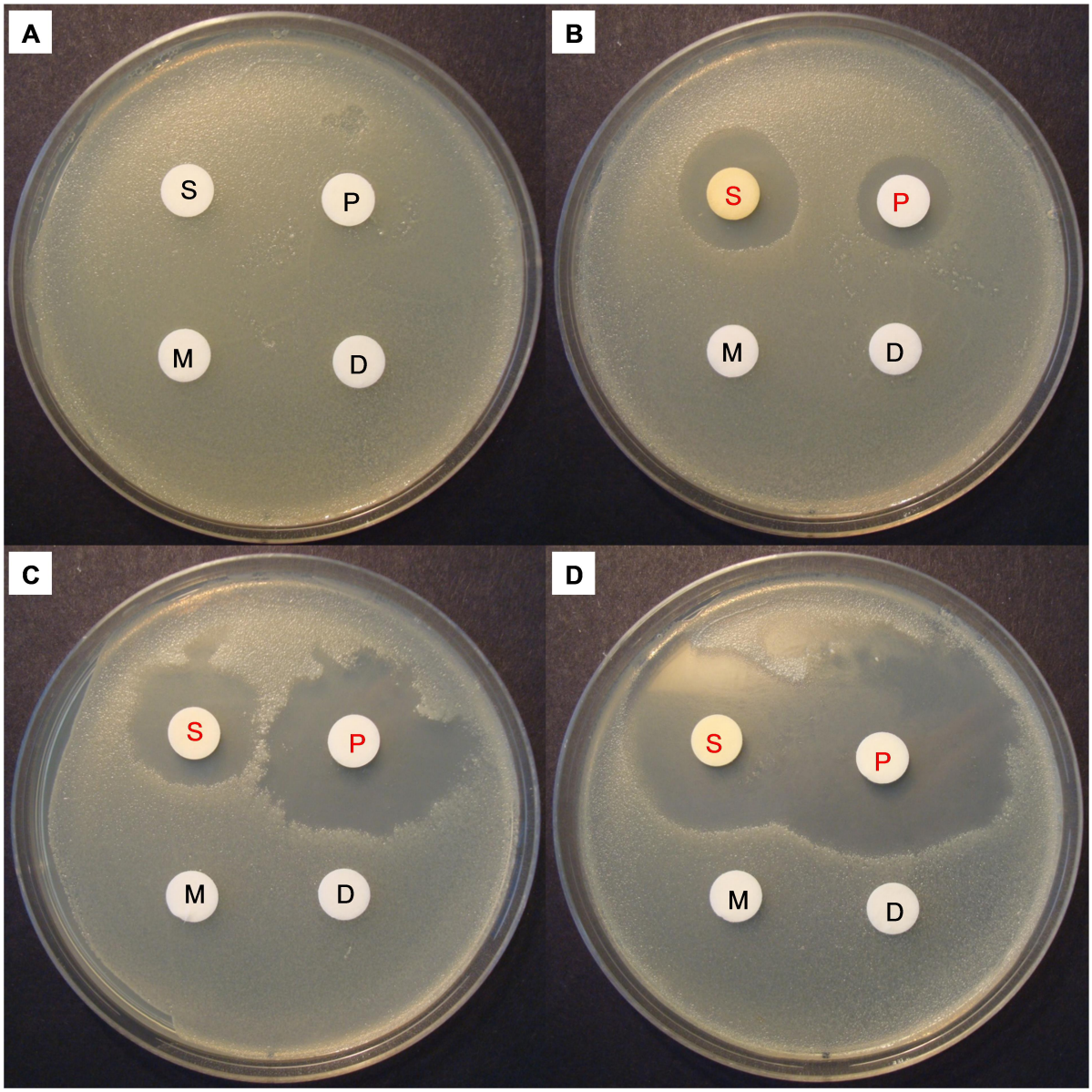


Figure 3.13 (A-D) Bioassays of supernatants and precipitates against *Candida* CA_6. Precipitation accumulated at the bottom of the vials holding the combined fractions A-D. Sub-samples of the precipitates (P), dissolved in DMSO, the remaining supernatants (S), and two negative controls, 100% MeOH (M) and 100% DMSO (D), were inoculated onto paper filter disks and attached to *Candida*-coated agar plates. The supernatants and pellets of the combined fractions B, C, and D are inhibiting the test fungus. The results indicate that some of the antifungal molecules dropped out of solution. The inhibition zones of B are relatively small with clear-cut edges and the one of D (and C) are relatively large with diffuse edges. This observation may, in addition to the polarity differences, indicate that both antifungals differ from one another.

3.4.6 Reversed-phase column fractionation

3.4.6.1 Fractionation

To determine the identity of the antifungal in the combined fractions B, the still highly contaminated sample (Fig. $3.11_{C\text{-}D}$) was column-purified a second time. During multiple HPLC runs, the supernatant and DMSO-dissolved pellet were purified by reversed-phase (RP) column fractionation. The solvent conditions changed from relatively polar (dH_2O>MeOH) to more apolar (dH_2O<MeOH) conditions. Simultaneously, the UV absorbance of eluting compounds was measured; the eluent of each run was sub-divided over 80 fractions.

Figure 3.14_A shows the UV absorbance signal of the first fractionation run; here, the X-axis presents the retention time (minutes), and the Y-axis displays the UV absorbance for wavelengths between 200-600 nm. A red colouration indicates a strong UV absorbance, whereas a blue colouration displays a weak UV absorbance. The graph shows that the chosen chromatography leads to a good separation of compounds, and thus a separation of bioactive and non-bioactive compounds.

3.4.6.2 Bioassays against Candida

Sub-samples of the RP fractions, originating from the first fractionation run, were inoculated onto filter disks. When dry, the disks were attached with distilled water to *Candida*-coated agar plates and fungal inhibition was recorded. The bioactivity clearly localises in the relatively apolar fractions 58-65 (Fig. 3.15). This result is expected since the antifungal, which during normal-phase column fractionation have eluted under relatively apolar conditions (EtAc>MeOH), also eluted under relatively apolar conditions during this reversed-phase column fractionation (dH_2O<MeOH). Interestingly, the RT area into which the bioactive molecules eluted (RT≈38.5-42.5'), generally shows a relatively poor UV absorbance. In contrast, the RT area directly preceding the bioactive area is characterised by the presence of four dominant compounds (RT≈34.0-37.5').

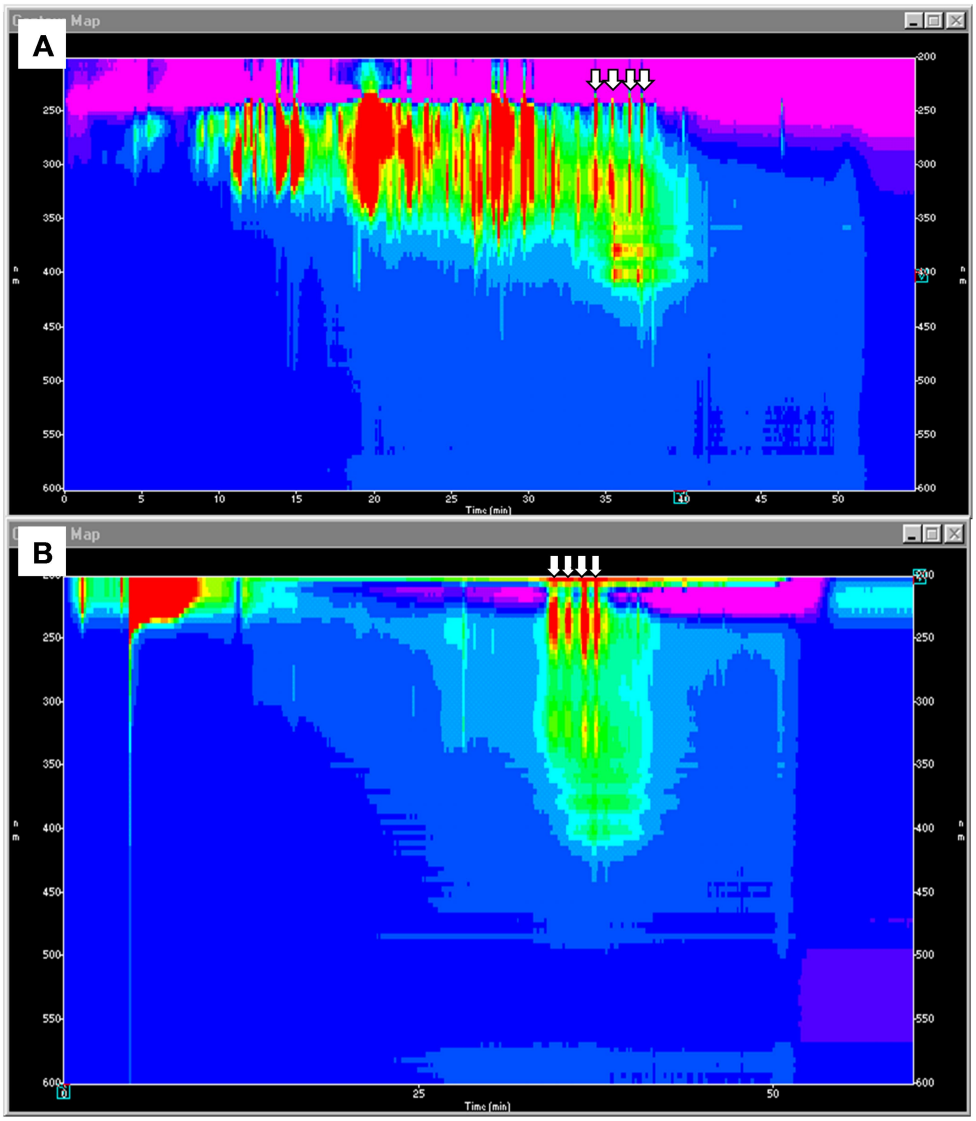


Figure 3.14 Reversed-phase column fractionation on combined fractions B. The bioactive supernatant and dissolved precipitate were fractionated. Per HPLC run, 80 fractions of eluent were collected. **(A)** UV absorbance signal from the first fractionation run. The Y-axis shows the absorbance from 200-600 nm and the X-axis the time in minutes. The strength of the signal is presented as a colour gradient from blue (relatively weak) to red (relatively strong). The graph indicates that the compounds of the extract separated well under the present solvent conditions. Challenges between the reversed-phase fractions of the first fractionation run and *Candida* CA_6 are given in figure 3.15. The bioassays show that the antifungal molecules eluted under relatively apolar conditions (dH_2O<MeOH) into fractions 58-65 (RT=~38.5-42.5'). The UV absorbance at the bioactive RT area is fairly weak not revealing the presence of any abundant molecules. In contrast, the preceding RT area (RT=~34.0-37.5') is characterised by the presence of four dominant compounds (white arrows). Due to this observation, follow-up fractionation runs combined the eluent of both RT areas. **(B)** A small sub-sample of the double-purified (NP/RP) combined fractions B was re-analysed in a final HPLC run. As expected, the majority of the pollutants is absent from the sample.



Figure 3.15 Bioassays of the reversed-phase fractions from the first HPLC run (Fig. 3.14_A) against *Candida* CA_6. Sub-samples of the fractions were inoculated onto paper filter disks and attached to *Candida*-coated agar plates. The bioassay reveals that the antifungal molecules are eluting under relatively apolar conditions (dH_2O<MeOH) into fractions 58-65 (red font). This corresponds to a retention time of ~38.5-42.5' minutes.

Due to the weak UV absorbance in the bioactive RT area and the close proximity to the dominant compounds, follow-up fractionation runs combined the eluent of both RT areas.

The purity of the selected eluent, containing the four dominant compounds and the bioactive RT area, was analysed in a final HPLC run (Fig. 3.14_B) using the same chromatography as described above. The graph shows a significant reduction of contaminating compounds in the double-purified (NP/RP) combined fractions B.

3.4.6.3 Bioassays of extract and waste

In bioassays, the double-purified (NP/RP) combined fractions B (E) were challenged against *Candida* CA_6 alongside eluents, which during the above reversed-phase column fractionation, were directed to the waste (W). This experiment was carried out to determine whether antifungal molecules had been lost to the waste. Sub-samples of both liquids were inoculated onto paper filter disks. A filter inoculated with 100% MeOH (C^-) served as negative control. When dry, the disks were attached with water to *Candida*-coated agar plates. All bioactive molecules appear to have accumulated in the extract and no bioactive molecules appear to have

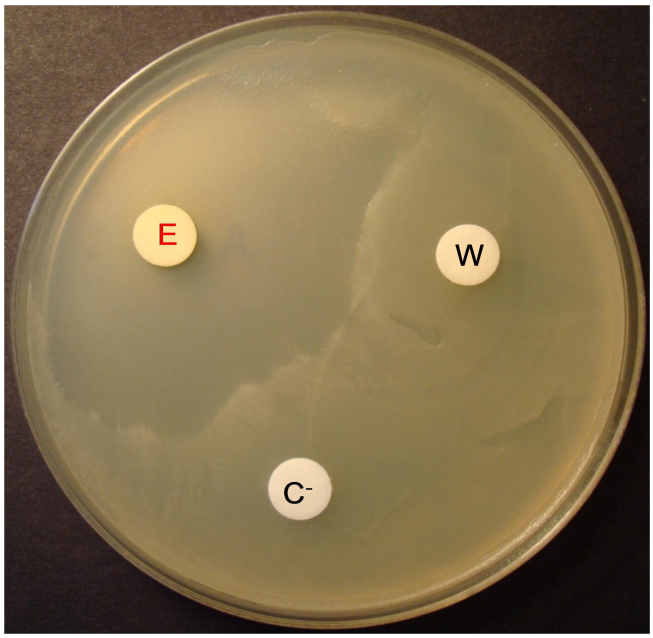


Figure 3.16 *Candida* (CA_6) bioassays testing the bioactivity of the double-purified (NP/RP) combined fractions B. The selected extract (E) and remaining waste (W) of the reversed-phase column fractionation were inoculated onto paper filter disks and attached to *Candida*-coated agar plates. As a negative control, a disk with 100% MeOH (C^-) was included. This test results confirm that no antifungal molecules are lost to the waste.

been lost to the waste (Fig. 3.16).

3.4.7 Identification of antimycin

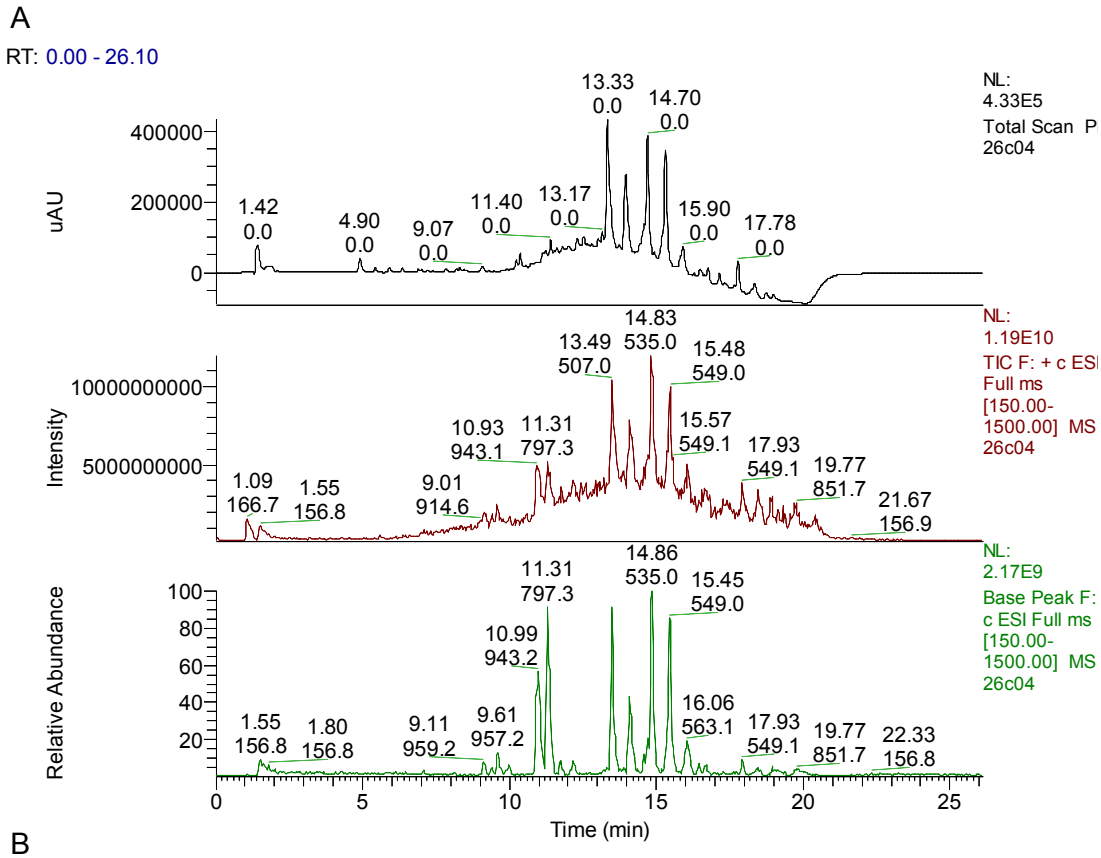
The compound masses of the double-purified (NP/RP) combined fractions B were analysed by low- and high resolution LCMS.

3.4.7.1 Low-resolution LCMS

MS, MS^2, and UV absorbance data of the double-purified (NP/RP) combined fractions B were collected during a low-resolution LCMS analysis in positive ion mode (+ESI). The detected masses are accurate to ± 0.1 amu.

Figure 3.17_{A-B} shows a PDA trace of this run, a total ion chromatogram (TIC), and a BPC for compounds in between 150-1500 amu. A numerical representation and detailed overview of the data are given in table 3.4 and the Supplementary Information B_1, respectively. The MS data show the presence of four major compounds, previously observed during the reversed-phase column fractionation, which have masses of 507 amu, 521 amu, 535 amu, and 549 amu $[M+H]^+$; these are followed by several minor peaks with masses of 563 amu, (805 amu), and 577 amu $[M+H]^+$. In the following, these are referred to as 'first-set compounds'. The same masses, excluding the 805 amu ion, elute from the column a second time under slightly more apolar solvent conditions, and are referred to as 'second-set compounds'. In contrast with the retention times of the first-set compounds, the retention times of the second-set compounds, roughly speaking, match the bioactive RT area defined above. In general, the mass signals of the second-set compounds were much weaker than those of the first-set compounds. This can be attributed to disadvantageous ionisation properties or a low molecular abundance, which also would explain the weak absorbance signal experienced during the reversed-phase column fractionation. Therefore, the compounds of both sets additionally are visualised in extracted ion chromatograms (EIC); these data are presented in table 3.4 and the Supplementary Information B_2. The masses therein have been identified as hydrogen adducts since their sodium counterparts (mass difference of

A


RT: 0.00 - 26.10
uAU
Intensity
Relative Abundance
Time (min)
NL: 4.33E5 Total Scan P 26c04
NL: 1.19E10 TIC F: + c ESI Full ms [150.00-1500.00] MS 26c04
NL: 2.17E9 Base Peak F: c ESI Full ms [150.00-1500.00] MS 26c04

B

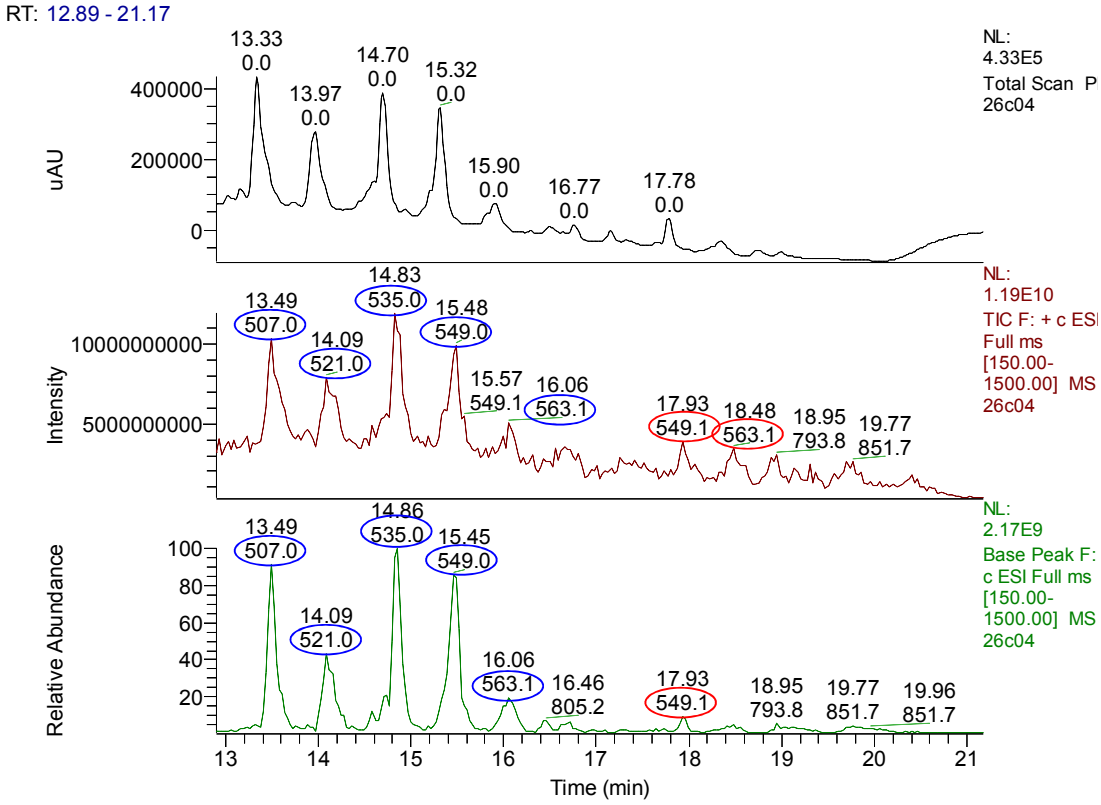

RT: 12.89 - 21.17
uAU
Intensity
Relative Abundance
Time (min)
NL: 4.33E5 Total Scan P 26c04
NL: 1.19E10 TIC F: + c ESI Full ms [150.00-1500.00] MS 26c04
NL: 2.17E9 Base Peak F: c ESI Full ms [150.00-1500.00] MS 26c04

Figure 3.17 (A-B) Low-resolution reversed-phase LCMS of the double-purified (NP/RP) combined fractions B in positive ion mode (+ESI). The graphs show a PDA, TIC, and BPC trace of the run. A detailed overview of the data is given in table 3.4 and the Supplementary Information B_1. Six compound masses, including the four dominant compounds described earlier (Fig. 3.14), are eluting from the column that in the following will be referred to as 'first-set compounds' (blue circles). The same six compound masses are eluting from the column under slightly more apolar solvent conditions, and will in the following be referred to as 'second-set compounds' (red circles). The retention times of the second-set compounds may correspond to the retention times of compounds in the bioactive RT area (Fig. 3.14). Due to a weak UV absorbance and mass signal, the UV, MS, and MS^2 data of second-set compounds are incomplete. Hence, extracted ion chromatograms (EIC) are presented in the Supplementary Information B_2 that better visualise the compounds of both compound sets, including masses of 507 amu, 521 amu, 535 amu, 549 amu, 563 amu, and 577 amu $[M+H]^+$. Not only that second-set compounds are more apolar than first-set compounds, but also within sets larger compounds are more apolar than smaller ones. The results indicate that the bioactivity against *Candida* CA_6 may stem from an antifungal complex of six second-set compounds.

		Sample set 1						
	Peak	**1**	**2**	**3**	**4**	**5**		**6**
MS masses	EIC	507-508	521-522	535-536	549-550	563-564	805-806	577-578
	RT	13.49	14.12	14.86	15.48	16.06	16.46	16.71
	Parent ion	507.0	521.0	535.0	549.0	563.1	805.2	577.2
	Adduct	H	H	H	H	H	H	H
UV data	RT	13.33	13.97	14.70	15.32	15.90	-	16.50
	UV max.	244.0	240.0	244.0	242.0	243.0	-	248.0
	UV max.	319.0	321.0	320.0	321.0	323.0	-	324.0
	UV max.	-	358.0	-	-	361.0	-	361.0
	UV max.	383.0	383.0	383.0	383.0	383.0	-	383.0
	UV max.	405.0	405.0	405.0	405.0	405.0	-	450.0
MS^2 masses	RT	13.42	14.26	14.66	15.29	15.89	16.42	16.51
	Parent ion	507.02	521.21	535.09	549.08	563.09	805.23	577.14
	Daughter ion (body)	247.0	247.0	247.0	247.0	247.0	265.0	247.0
	Daughter ion (body)	264.9	264.9	264.9	264.9	264.9	435.1	265.0
	Daughter ion (tail)	242.9	256.9	271.0	285.0	299.0	522.8	313.0
	Daughter ion (body)	219.1	219.1	219.0	219.0	219.0	787.2	219.1

		Sample set 2						
	Peak	**1**	**2**	**3**	**4**	**5**		**6**
MS masses	EIC	507-508	521-522	535-536	549-550	563-564	805-806	577-578
	RT	-	-	17.29	17.93	18.48	-	19.14
	Parent ion	-	-	535.0	549.1	563.1	-	577.2
	Adduct	-	-	H	H	H	-	H
UV data	RT	-	-	17.15	17.78	18.33	-	18.98
	UV max.	-	-	247.0	241.0	249.0	-	253.0
	UV max.	-	-	322.0	320.0	320.0	-	322.0
	UV max.	-	-	361.0	359.0	360.0	-	361.0
	UV max.	-	-	383.0	383.0	383.0	-	383.0
	UV max.	-	-	405.0	405.0	405.0	-	405.0
MS^2 masses	RT	-	-	17.31	17.89	18.40	-	19.13
	Parent ion	-	-	535.01	549.04	563.17	-	577.22
	Daughter ion (body)	-	-	265.0	265.0	265.0	-	265.0
	Daughter ion (body)	-	-	237.1	237.2	237.1	-	237.2

Table 3.4 Numerical representation of the low-resolution reversed-phase LCMS data, shown in figure 3.17. The table shows UV, MS, and MS^2 data for compounds of the double-purified (NP/RP) combined fractions B in positive ion mode (+ESI). Both compound sets contain masses of 507 amu, 521 amu, 535 amu, 549 amu, 563 amu, and 577 amu $[M+H]^+$. First-set compounds tend to fragment into daughter ions with masses of 247 amu and 265 amu; also parent-specific daughter ions are generated with masses of 243 amu (parent 507 amu), 257 amu (parent 521 amu), 271 amu (parent 535 amu), 285 amu (parent 549 amu), 299 amu (parent 563 amu), and 313 amu (parent 577 amu) $[M+H]^+$. In contrast, second-set compounds produce daughter ions with masses of 265 amu and 237 amu $[M+H]^+$. Here, no parent-specific daughter ions can be detected. The results show that, in addition to bioactivity and polarity differences, first- and second-set compounds differ in their chemical structures.

21.981945 amu) were consistently present.

The UV absorbance data of first- and second-set compounds are presented in table 3.4 and the Supplementary Information B_1. Again, due to the relatively low signal/noise ratio, the absorbance data of the second-set compounds is incomplete or less accurate. However, an assumption is that first- (240-248 nm; 319-324 nm) and second- (241-253 nm; 320-322 nm) set compounds absorb at two wavelengths.

In accordance with the differential polarities, first- and second- set compounds show differing MS^2 data (Table 3.4.; Supplementary Information B_1). For example, first-set compounds, except for the 805 amu ion, fragment into daughter ions with masses of 247 amu, 265 amu, and 219 amu $[M+H]^+$. In addition, the presence of daughter ions with parent specific masses can be observed, including 243 amu (parent 507 amu), 257 amu (parent 521 amu), 271 amu (parent 535 amu), 285 amu (parent 549 amu), 299 amu (parent 563 amu), and 313 amu (parent 577 amu) $[M+H]^+$. In contrast, second-set compounds consistently fragment into daughters with masses of 265 amu and 237 amu $[M+H]^+$; daughter ions with parent specific masses are absent. This suggests structural differences between corresponding first- and second-set parent ions possessing equal masses. Unfortunately, no MS^2 data could be obtained from the 507 amu and 521 amu $[M+H]^+$ parent ions of the second set.

In summary, two sets of compound masses are detected in the double-purified (NP/RP) combined fractions B. The retention times of the second-set compounds may correspond to the retention times of compounds in the bioactive RT area, described during reversed-phase column fragmentation (Fig. 3.14). No bioactivity is observed to originate from first-set compounds, which appear to slightly differ in structure from second-set compounds as indicated by differential polarities and MS^2 fragmentation patterns. The mass similarities between both compound sets aided the identification of the bioactive second-set compounds, as described below.

3.4.7.2 High-resolution LCMS

MS and MS^2 data of the double-purified (NP/RP) combined fractions B were collected during high-resolution LCMS in positive ion mode (+ESI). The detected masses are accurate to ± 0.00001 amu.

Figure 3.18_{A-B} shows the TIC and BPC for compounds in between 150-1500 amu. A numerical representation and detailed overview of these data are given in table 3.5 and the Supplementary Information B_3, respectively. All compounds of the first set, except for the 805 amu $[M+H]^+$ ion, could be detected; in contrast, none of the second-set compounds ionised. The accurate masses of the first-set compounds are 507.23364 amu, 521.24878 amu, 535.26483 amu, 549.28027 amu, 563.29529 amu, and 577.31219 amu $[M+H]^+$. Interestingly, the compound masses differ by 14 amu, which corresponds to a CH_2 group. Based on the accurate masses, the molecular formulae of the compounds are calculated to be $C_{25}H_{35}N_2O_9$, $C_{26}H_{37}N_2O_9$, $C_{27}H_{39}N_2O_9$, $C_{28}H_{41}N_2O_9$, $C_{29}H_{43}N_2O_9$, and $C_{30}H_{45}N_2O_9$. EIC data of the compounds are presented in table 3.5 and the Supplementary Information B_4.

Except for the largest first-set compound, with a mass of 577.31219 amu, accurate MS^2 masses were detected for all parent ions (Table 3.5; Supplementary Information B_3). The fragmentation patterns of all parent ions included the daughters 247.071 amu, 265.082 amu, and 219.076 amu $[M+H]^+$; their molecular formulae are: $C_{12}H_{11}N_2O_4$, $C_{12}H_{13}N_2O_5$, and $C_{11}H_{11}N_2O_3$, respectively. Also, daughter ions with parent specific masses were detected that have previously been observed during low-resolution LCMS (Table 3.4). Their accurate masses were 243.15884 amu (parent 507 amu), 257.17462 amu (parent 521 amu), 271.18994 amu (parent 535 amu), 285.20584 amu (parent 549 amu), and 299.22144 amu (parent 563 amu) $[M+H]^+$. The molecular formulae of these sub-structures are $C_{13}H_{23}O_4$, $C_{14}H_{25}O_4$, $C_{15}H_{27}O_4$, $C_{16}H_{29}O_4$, and $C_{17}H_{31}O_4$, respectively. In contrast with the molecular formulae of the 247 amu, 265 amu, and 219 amu daughters, the molecular formulae of the daughters with parent specific masses, do not contain any nitrogen atoms.

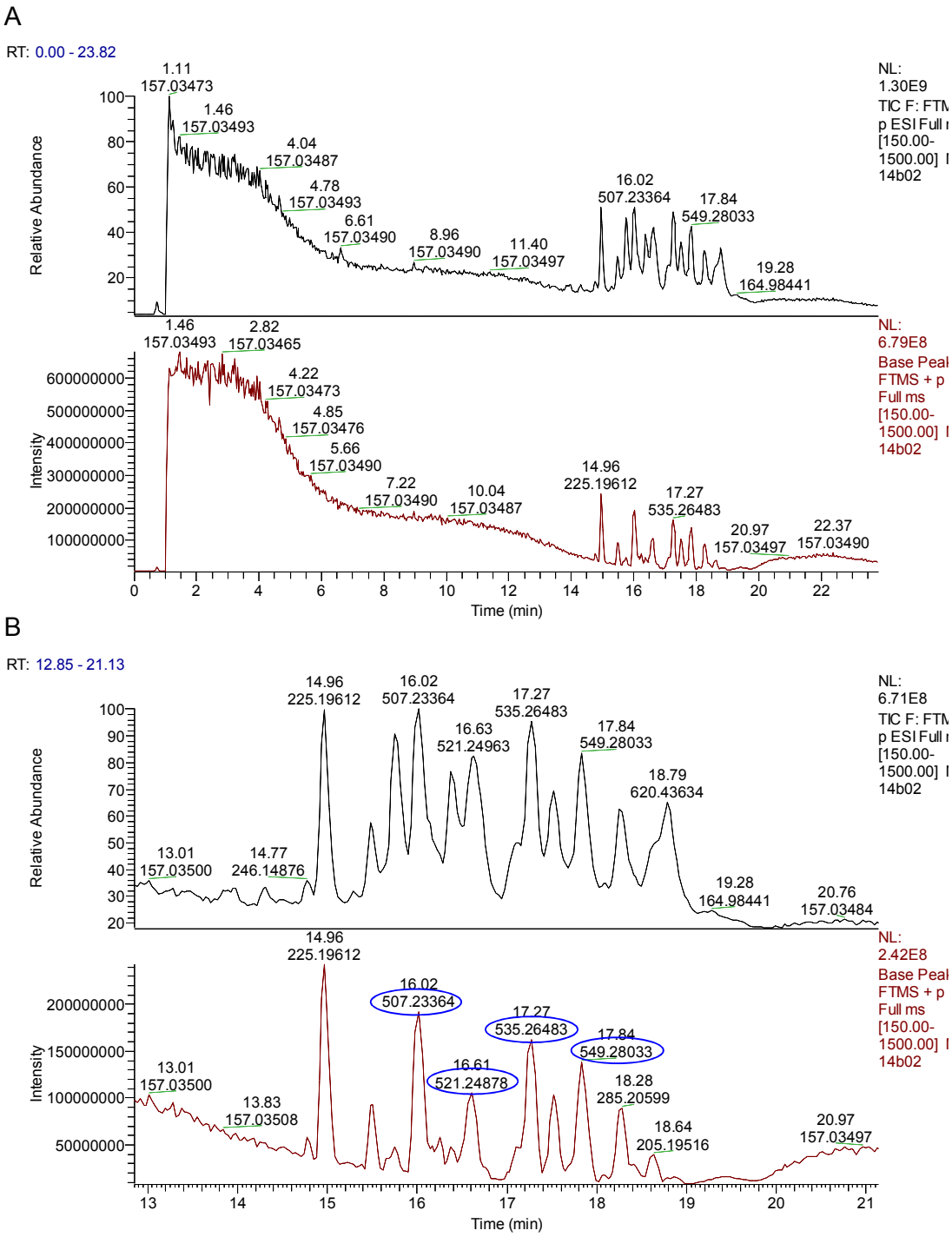


Figure 3.18 (A-B) High-resolution reversed-phase LCMS on the double-purified (NP/RP) combined fractions B in positive ion mode (+ESI). During this analysis only first-set ions could be detected (blue circles). The accurate MS and MS^2 data of these ions are shown in table 3.5.

			Sample set 1					
		Peak	**1**	**2**	**3**	**4**	**5**	**6**
MS masses		EIC	507-508	521-522	535-536	549-550	563-564	577-578
		RT	16.02	16.61	17.27	17.84	18.41	19.24
		Parent ion	507.23364	521.24878	535.26483	549.28027	563.29529	577.31219
		Formula	$C_{25}H_{35}N_2O_9$	$C_{26}H_{37}N_2O_9$	$C_{27}H_{39}N_2O_9$	$C_{28}H_{41}N_2O_9$	$C_{29}H_{43}N_2O_9$	$C_{30}H_{45}N_2O_9$
		Error	-0.132	-1.107	-0.331	-0.705	-1.806	0.403
		Adduct	H	H	H	H	H	H
MS^2 masses	**Parent ion**	RT	15.94	16.70	17.32	17.69	18.40	-
		Parent ion	507.23	521.25	535.27	549.28	563.30	-
	Daughter ion (body)	Mass	247.07104	247.07109	247.07115	247.07115	247.07112	-
		Formula	$C_{12}H_{11}N_2O_4$	$C_{12}H_{11}N_2O_4$	$C_{12}H_{11}N_2O_4$	$C_{12}H_{11}N_2O_4$	$C_{12}H_{11}N_2O_4$	-
		Error	-1.187	-0.985	-0.742	-0.742	-0.863	-
	Daughter ion (body)	Mass	265.08154	265.08163	265.08167	265.08160	265.08176	-
		Formula	$C_{12}H_{13}N_2O_5$	$C_{12}H_{13}N_2O_5$	$C_{12}H_{13}N_2O_5$	$C_{12}H_{13}N_2O_5$	$C_{12}H_{13}N_2O_5$	-
		Error	-1.237	-1.011	-0.860	-1.124	-0.521	-
	Daughter ion (tail)	Mass	243.15884	257.17462	271.18994	285.20584	299.22144	-
		Formula	$C_{13}H_{23}O_4$	$C_{14}H_{25}O_4$	$C_{15}H_{27}O_4$	$C_{16}H_{29}O_4$	$C_{17}H_{31}O_4$	-
		Error	-1.010	-0.450	-1.644	-0.687	-0.822	-
	Daughter ion (body)	Mass	219.07617	219.07640	219.07632	219.07623	-	-
		Formula	$C_{11}H_{11}N_2O_3$	$C_{11}H_{11}N_2O_3$	$C_{11}H_{11}N_2O_3$	$C_{11}H_{11}N_2O_3$	-	-
		Error	-1.135	-0.085	-0.451	-0.861	-	-

Table 3.5 Numerical representation of the high-resolution reversed-phase LCMS data, given in figure 3.18. The table shows accurate MS and MS^2 data for compounds of the double-purified (NP/RP) combined fractions B in positive ion mode (+ESI). The second-set compounds, as detected by low-resolution LCMS (Table 3.4), did not ionise. Based on the accurate MS data, molecular formulae are calculated. The formulae differ between the smallest (507.23364 amu $[M+H]^+$; $C_{25}H_{35}N_2O_9$) and the largest (577.31219 amu $[M+H]^+$; $C_{30}H_{45}N_2O_9$) molecule by the stepwise addition of five CH_2 groups (14 amu). No MS^2 data could be obtained from the 577.31219 amu $[M+H]^+$ parent ion. All other molecules fragmented into daughters with masses of 247.071 amu ($C_{12}H_{11}N_2O_4$), 265.082 amu ($C_{12}H_{13}N_2O_5$), and 219.076 amu ($C_{11}H_{11}N_2O_3$) $[M+H]^+$. Interestingly, their molecular formulae all contain two nitrogen atoms. In contrast, the parent-specific daughter ions, ranging in mass from 243.15884-299.22144 amu $[M+H]^+$ ($C_{13\text{-}17}H_{23\text{-}31}O_4$), do not contain any nitrogen atoms. While the parent formulae are used for screening databases of natural products, the daughter formulae are used for determining the accuracy of the search hits.

3.4.7.3 Database comparison

The molecular formulae of the first-set parent ions were matched to databases of natural products, including SciFinder and Reaxys. An antifungal complex with the name antimycin matches these molecular formulae most closely. While the 'body' structures of antimycin molecules are relatively conserved, the 'tail' structures are more variable. Most antimycins differ in mass and molecular structure by CH_2 groups, most commonly being attached to the tail. Due to the accumulation of CH_2 groups, larger antimycins are more apolar than smaller ones. This is in accordance with the finding that the higher-mass first-set compounds eluted at more apolar conditions, during low- and high-resolution LCMS, than the lower-mass compounds. Figure 3.19 shows representative antimycin structures, with masses (formulae) of 506.22643 amu ($C_{25}H_{34}N_2O_9$; Reaxys ID: 1277391), 520.24208 amu ($C_{26}H_{36}N_2O_9$; Reaxys ID: 1277644), 534.25773 amu ($C_{27}H_{38}N_2O_9$; Reaxys ID: 8175643), 548.27338 amu ($C_{28}H_{40}N_2O_9$; Reaxys ID: 6553177), 562.28903 amu ($C_{29}H_{42}N_2O_9$; CAS: 679395-05-2), and 576.30468 amu ($C_{30}H_{44}N_2O_9$; CAS: 679395-06-3), corresponding to the sample masses and formulae.

The relatively conserved antimycin 'body structures', highlighted in teal, have an accurate neutral mass of 264.07462 amu ($C_{12}H_{12}N_2O_5$) and correspond to sample daughter ions with a mass of 265.082 amu $[M+H]^+$ ($C_{12}H_{13}N_2O_5$), indicated as 'daughter ion (body)' in table 3.5. Due to the presence of nitrogen atoms in the molecular formulae of the other sample daughter ions, 247.071 amu ($C_{12}H_{11}N_2O_4$) and 219.076 amu ($C_{11}H_{11}N_2O_3$) $[M+H]^+$, these must also be sub-structures originating from the antimycin body.

The more variable antimycin 'tail structures', highlighted orange in figure 3.19, have accurate neutral masses of 242.15181 amu ($C_{13}H_{22}O_4$), 256.16746 amu ($C_{14}H_{24}O_4$), 270.18311 amu ($C_{15}H_{26}O_4$), 284.19876 amu ($C_{16}H_{28}O_4$), 298.21441 amu ($C_{17}H_{30}O_4$), and 312.23006 ($C_{18}H_{32}O_4$). These sub-structures correspond to sample daughter ions with masses of 243.15884 amu ($C_{13}H_{23}O_4$), 257.17462 amu ($C_{14}H_{25}O_4$), 271.18994 amu ($C_{15}H_{27}O_4$), 285.20584 amu ($C_{16}H_{29}O_4$), and 299.22144 amu ($C_{17}H_{31}O_4$) $[M+H]^+$, indicated as 'daughter ion

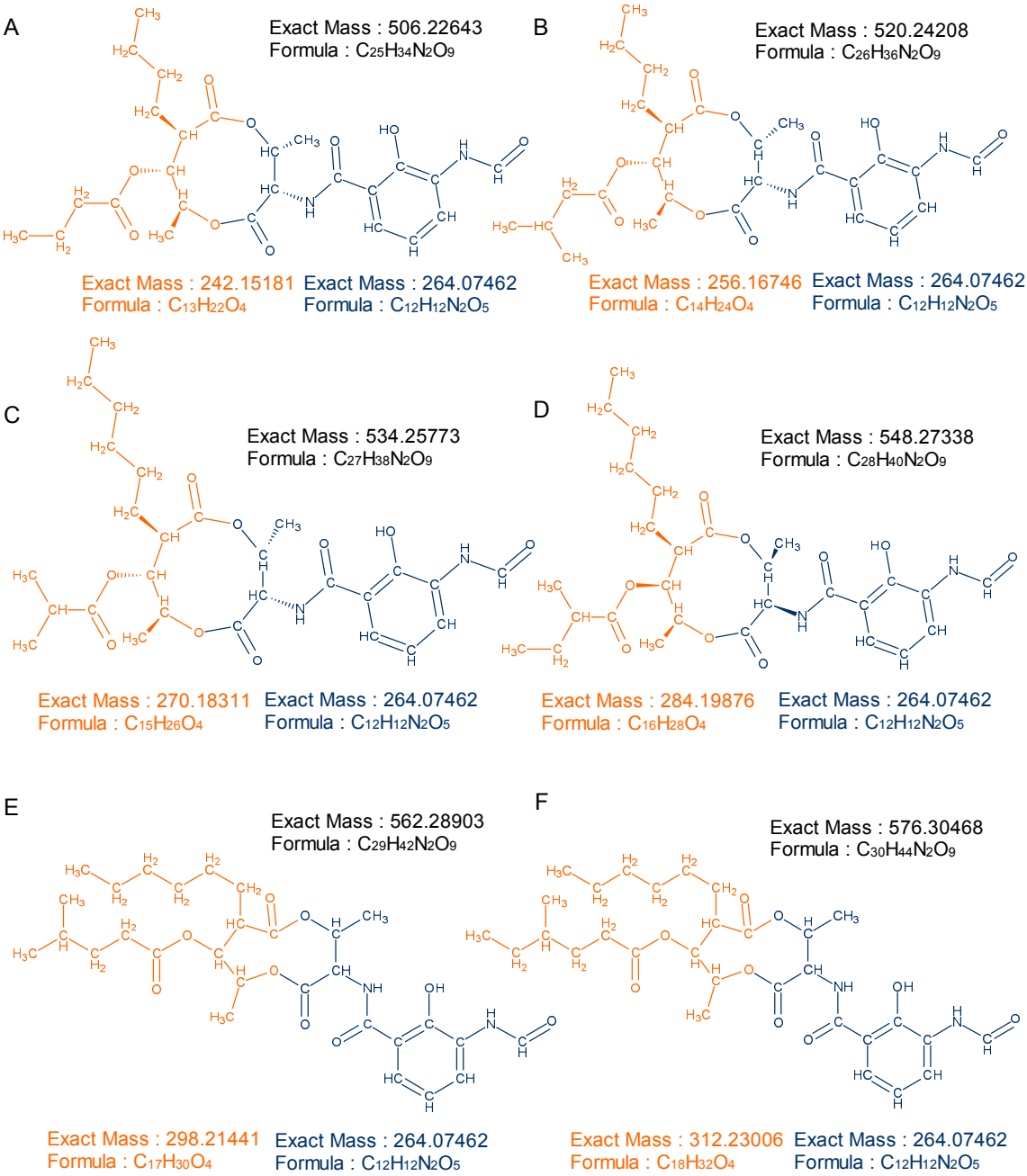


Figure 3.19 Analogies to antimycin molecules by molecular masses and formulae of first-set sample ions, as described in table 3.5. Members of this antifungal family often differ in mass and structure by the addition of CH_2 groups (14 amu). As illustrated by representative chemical structures, these moieties often are attached to the 'tail structure' (orange), turning it more variable than the relatively conserved 'body structure' (teal). This renders larger molecules also more apolar than smaller ones. In particular, the antimycins shown here, have neutral masses (formulae) of 506.22643 amu ($C_{25}H_{34}N_2O_9$; Reaxys ID: 1277391), 520.24208 amu ($C_{26}H_{36}N_2O_9$; Reaxys ID: 1277644), 534.25773 amu ($C_{27}H_{38}N_2O_9$; Reaxys ID: 8175643), 548.27338 amu ($C_{28}H_{40}N_2O_9$; Reaxys ID: 6553177), 562.28903 amu

($C_{29}H_{42}N_2O_9$; SciFinder ID: 679395-05-2), and 576.30468 amu ($C_{30}H_{44}N_2O_9$; SciFinder ID: 679395-06-3). These correspond to the masses (formulae) of the hydrogen adducts in the sample, including 507.23364 amu ($C_{25}H_{35}N_2O_9$), 521.24878 amu ($C_{26}H_{37}N_2O_9$), 535.26483 amu ($C_{27}H_{39}N_2O_9$), 549.28027 amu ($C_{28}H_{41}N_2O_9$), 563.29529 amu ($C_{29}H_{43}N_2O_9$), and 577.31219 amu ($C_{30}H_{45}N_2O_9$) $[M+H]^+$ (Table 3.5). The MS^2 data confirm these analogies. For example, a sub-structure of the 506.22643 amu antimycin parent, highlighted teal, has a neutral mass (formula) of 264.07462 amu ($C_{12}H_{12}N_2O_5$). This corresponds to a sub-structure of the 507.23364 amu $[M+H]^+$ sample ion with a mass (formula) of 265.08154 amu $[M+H]^+$ ($C_{12}H_{13}N_2O_5$) (Table 3.5). Similarly, a sub-structure of the 506.22643 amu antimycin parent, highlighted orange, has a neutral mass (formula) of 242.15181 amu ($C_{13}H_{22}O_4$). This corresponds to a sub-structure of the 507.23364 amu $[M+H]^+$ sample ion with a mass (formula) of 243.15884 amu $[M+H]^+$ ($C_{13}H_{23}O_4$) (Table 3.5). The same comparison can be made with regard to the remaining antimycin tail-structures with neutral masses (formulae) of 256.16746 amu ($C_{14}H_{24}O_4$), 270.18311 amu ($C_{15}H_{26}O_4$), 284.19876 amu ($C_{16}H_{28}O_4$), 298.21441 amu ($C_{17}H_{30}O_4$), and 312.23006 ($C_{18}H_{32}O_4$). No MS^2 data could be obtained from the 577.31219 amu $[M+H]^+$ sample parent ion and thus the presence of expected 247 amu, 265 amu, and 219 amu $[M+H]^+$ daughter ions from the body and an expected 313 amu $[M+H]^+$ ion from the tail could not be confirmed.

(tail)' in table 3.5. As mentioned earlier, no MS^2 data could be collected for the 577.31219 amu $[M+H]^+$ sample parent ion. Therefore, the presence of a 312 amu ($C_{18}H_{32}O_4$) daughter could not be confirmed by high-resolution LCMS; however, a corresponding daughter ion (313.0 amu $[M+H]^+$) was detected during low-resolution LCMS (Table 3.4).

Antimycins with the same masses may have different molecular structures (and thus varying names), depending on where CH_2 groups are attached. For example, the 507 amu $[M+H]^+$ parent ion corresponds to antimycin A_4 (506 amu); the 521 amu $[M+H]^+$ parent ion to antimycins A_3 and A_7 (520 amu); the 535 amu $[M+H]^+$ parent ion to antimycins A_2, A_8, A_{11}, and A_{17} (534 amu); the 549 amu $[M+H]^+$ parent ion to antimycins A_1, A_{12}, and A_{13} (548 amu); the 563 amu $[M+H]^+$ parent ion to antimycins A_{10}, A_{14}, and A_{15} (562 amu); and the 577 amu $[M+H]^+$ parent ion to antimycin A_{16} (576 amu) (Table 3.6).

506 amu		520 amu		534 amu		548 amu		562 amu		576 amu	
Registry Number:	1222674-31-8	Registry Number:	1058162-14-3	Registry Number:	1177731-89-3	Registry Number:	1262758-07-5	Registry Number:	1059699-16-9	Registry Number:	1060752-54-6
Formula:	C25 H34 N2 O9	Formula:	C26 H36 N2 O9	Formula:	C27 H38 N2 O9	Formula:	C28 H40 N2 O9	Formula:	C29 H42 N2 O9	Formula:	C30 H44 N2 O9
CA Index Name:	INDEX NAME NOT YET ASSIGN	CA Index Name:	Butanoic acid, 3-methyl-,(2S,3S,7	CA Index Name:	INDEX NAME NOT YET ASSIGN	CA Index Name:	INDEX NAME NOT YET ASSIGN	CA Index Name:	INDEX NAME NOT YET ASSIGN	CA Index Name:	INDEX NAME NOT YET ASSIGN
Molecular Weight	506.55	Molecular Weight	520.57	Molecular Weight	534.6	Molecular Weight	548.63	Molecular Weight	562.65	Molecular Weight	576.68
Registry Number:	1058162-25-6	Registry Number:	1058162-10-9	Registry Number:	1056986-80-1	Registry Number:	1217539-87-1	Registry Number:	959675-72-0	Registry Number:	679395-06-3
Formula:	C25 H34 N2 O9	Formula:	C26 H36 N2 O9	Formula:	C27 H38 N2 O9	Formula:	C28 H40 N2 O9	Formula:	C29 H42 N2 O9	Formula:	C30 H44 N2 O9
CA Index Name:	Butanoic acid, 3-methyl-,(3S,7R,8	CA Index Name:	Butanoic acid, 3-methyl-,(3S,6S,7	CA Index Name:	INDEX NAME NOT YET ASSIGN	CA Index Name:	INDEX NAME NOT YET ASSIGN	CA Index Name:	INDEX NAME NOT YET ASSIGN	CA Index Name:	Hexanoic acid, 4-methyl-,(2R,3S,6
Molecular Weight	506.55	Molecular Weight	520.57	Molecular Weight	534.6	Molecular Weight	548.63	Molecular Weight	562.65	Other Names:	Antimycin A16
Registry Number:	871107-10-7	Registry Number:	1058161-86-6	Registry Number:	1026419-62-4	Registry Number:	857196-81-7	Registry Number:	868072-43-9	Molecular Weight	576.68
Formula:	C25 H34 N2 O9	Formula:	C26 H36 N2 O9	Formula:	C27 H38 N2 O9	Formula:	C28 H40 N2 O9	Formula:	C29 H42 N2 O9	Registry Number:	561304-91-4
CA Index Name:	Butanoic acid, 2(or 3)-methyl-,(2R	CA Index Name:	Butanoic acid, 3-methyl-,(2R,3S,7	CA Index Name:	INDEX NAME NOT YET ASSIGN	CA Index Name:	INDEX NAME NOT YET ASSIGN	CA Index Name:	Butanoic acid, 2(or 3)-methyl-,(2R	Formula:	C30 H44 N2 O9
Registry Number:	464157-54-8	Molecular Weight	520.57	Molecular Weight	534.6	Other Names:	1,5-Dioxonane-2,6-dione,3-(3-forr	Other Names:	Antimycin A10	CA Index Name:	Pentanoic acid,(2R,3S,6S,7R,8R)
Formula:	C25 H34 N2 O9	Registry Number:	959366-91-7	Registry Number:	877061-67-1	Molecular Weight	548.63	Registry Number:	679395-08-5	Molecular Weight	576.68
CA Index Name:	Butanoic acid, 3-methyl-,(3S,6S,7	Formula:	C26 H36 N2 O9	Formula:	C27 H38 N2 O9	Registry Number:	679395-03-0	Formula:	C29 H42 N2 O9	Registry Number:	327993-46-4
Molecular Weight	506.55	CA Index Name:	INDEX NAME NOT YET ASSIGN	CA Index Name:	Butanoic acid, 2-methyl-,3-[[3-(fon	Formula:	C28 H40 N2 O9	CA Index Name:	Butanoic acid, 3-methyl-,(2R,3S,6	Formula:	C30 H44 N2 O9
Registry Number:	327993-49-7	Molecular Weight	520.57	Other Names:	Antimycin A17	CA Index Name:	Hexanoic acid, 4-methyl-,(2R,3S,6	Other Names:	SPA 10191-b	CA Index Name:	Butanoic acid, 3-methyl-,(2R,3S,6
Formula:	C25 H34 N2 O9	Registry Number:	622829-22-5	Molecular Weight	534.6	Other Names:	Antimycin A13	Molecular Weight	562.65	Molecular Weight	576.68
CA Index Name:	Butanoic acid, 3-methyl-,(2R,3S,7	Formula:	C26 H36 N2 O9	Registry Number:	868862-13-9	Molecular Weight	548.63				
Molecular Weight	506.55	CA Index Name:	Butanoic acid, 2-methyl-,(2R,3S,6	Formula:	C27 H38 N2 O9			Registry Number:	679395-07-4		
		Other Names:	(2'R)-antimycin A3a	CA Index Name:	Butanoic acid, 2(or 3)-methyl-,(2R	Registry Number:	679395-02-9	Formula:	C29 H42 N2 O9		
Registry Number:	321599-65-9	Molecular Weight	520.57	Other Names:	2-Methoxyantimycin A3	Formula:	C28 H40 N2 O9	CA Index Name:	Butanoic acid, 2-methyl-,(2R,3S,6		
Formula:	C25 H34 N2 O9					CA Index Name:	Pentanoic acid, 4-methyl-,(2R,3S,	Other Names:	SPA 10191a		
CA Index Name:	Propanoic acid, 2-methyl-,(3S,6S,	Registry Number:	503291-88-1	Registry Number:	857839-31-7	Other Names:	Antimycin A12	Molecular Weight	562.65		
Molecular Weight	506.55	Formula:	C26 H36 N2 O9	Formula:	C27 H38 N2 O9	Molecular Weight	548.63				
		CA Index Name:	2,3(1H)-Isoquinolinedicarboxylic a	CA Index Name:	INDEX NAME NOT YET ASSIGN			Registry Number:	679395-05-2		
Registry Number:	118890-47-4	Molecular Weight	520.57	Other Names:	1,5-Dioxonane-2,6-dione,3-(3-ace	Registry Number:	561304-90-3	Formula:	C29 H42 N2 O9		
Formula:	C25 H34 N2 O9			Molecular Weight	534.6	Formula:	C28 H40 N2 O9	CA Index Name:	Pentanoic acid, 4-methyl-,(2R,3S,		
CA Index Name:	Butanoic acid, 3-methyl-,(2R,3S,6	Registry Number:	197791-89-2			CA Index Name:	Heptanoic acid,(2R,3S,6S,7R,8R,	Other Names:	Antimycin A15		
Other Names:	2-O-Methylantimycin A5	Formula:	C26 H36 N2 O9	Registry Number:	679395-01-8	Molecular Weight	548.63	Molecular Weight	562.65		
Molecular Weight	506.55	CA Index Name:	Butanoic acid,3-[[3-(formylamino)-	Formula:	C27 H38 N2 O9						
		Other Names:	Antimycin A7b	CA Index Name:	Pentanoic acid, 4-methyl-,(2R,3S,	Registry Number:	327993-47-5	Registry Number:	679395-04-1		
Registry Number:	118890-46-3	Molecular Weight	520.57	Other Names:	Antimycin A11	Formula:	C28 H40 N2 O9	Formula:	C29 H42 N2 O9		
Formula:	C25 H34 N2 O9			Molecular Weight	534.6	CA Index Name:	Butanoic acid, 3-methyl-,(2R,3S,6	CA Index Name:	Hexanoic acid, 4-methyl-,(2R,3S,6		
CA Index Name:	Butanoic acid, 2-methyl-,8-ethyl-3-	Registry Number:	197791-88-1			Molecular Weight	548.63	Other Names:	Antimycin A14		
Other Names:	1,5-Dioxonane, butanoic acid deri	Formula:	C26 H36 N2 O9	Registry Number:	327993-48-6			Molecular Weight	562.65		
Molecular Weight	506.55	CA Index Name:	Propanoic acid, 2-methyl-,(2R,3S,	Formula:	C27 H38 N2 O9	Registry Number:	118890-41-8				
		Other Names:	Antimycin A7a	CA Index Name:	Butanoic acid, 3-methyl-,(2R,3S,7	Formula:	C28 H40 N2 O9	Registry Number:	561304-93-6		
Registry Number:	117603-45-9	Molecular Weight	520.57	Molecular Weight	534.6	CA Index Name:	Butanoic acid,3-[[3-(formylamino)-	Formula:	C29 H42 N2 O9		
Formula:	C25 H34 N2 O9					Other Names:	1,5-Dioxonane, butanoic acid deri	CA Index Name:	Pentanoic acid,(2R,3S,6S,7R,8R)		
CA Index Name:	Butanoic acid,8-butyl-3-[[3-(formyl	Registry Number:	139066-86-7	Registry Number:	197791-91-6	Molecular Weight	548.63	Molecular Weight	562.65		
Other Names:	Antimycin A4b	Formula:	C26 H36 N2 O9	Formula:	C27 H38 N2 O9						
Molecular Weight	506.55	CA Index Name:	Butanoic acid, 3-methyl-,(2S,3R,6	CA Index Name:	Butanoic acid, 3-methyl-,3-[[3-(fon	Registry Number:	118890-40-7	Registry Number:	561304-89-0		
		Other Names:	Butanoic acid, 3-methyl-,8-butyl-3-	Other Names:	Antimycin A8b	Formula:	C28 H40 N2 O9	Formula:	C29 H42 N2 O9		
Registry Number:	28068-12-4	Molecular Weight	520.57	Molecular Weight	534.6	CA Index Name:	Propanoic acid, 2-methyl-,3-[[3-(fo	CA Index Name:	Hexanoic acid,(2R,3S,6S,7R,8R)-		
Formula:	C25 H34 N2 O9					Other Names:	1,5-Dioxonane, propanoic acid de	Molecular Weight	562.65		
CA Index Name:	Propanoic acid, 2-methyl-,(2R,3S,	Registry Number:	118890-45-2	Registry Number:	197791-90-5	Molecular Weight	548.63				
Other Names:	Antimycin A4a	Formula:	C26 H36 N2 O9	Formula:	C27 H38 N2 O9			Registry Number:	118890-39-4		
Molecular Weight	506.55	CA Index Name:	Butanoic acid,8-butyl-3-[[3-(formyl	CA Index Name:	Butanoic acid, 2-methyl-,3-[[3-(fon	Registry Number:	116095-18-2	Formula:	C29 H42 N2 O9		
		Other Names:	1,5-Dioxonane, butanoic acid deri	Other Names:	Antimycin A8a	Formula:	C28 H40 N2 O9	CA Index Name:	Butanoic acid, 3-methyl-,(2R,3S,6		
		Molecular Weight	520.57	Molecular Weight	534.6	CA Index Name:	Butanoic acid, 3-methyl-,3-[[3-(fori	Other Names:	Threonine, N-3-formamido-o-anis		
						Other Names:	Antimycin A1b	Molecular Weight	562.65		
		Registry Number:	118890-44-1	Registry Number:	125543-39-7	Molecular Weight	548.63				
		Formula:	C26 H36 N2 O9	Formula:	C27 H38 N2 O9			Registry Number:	118890-38-3		
		CA Index Name:	Propanoic acid, 2-methyl-,8-butyl-	CA Index Name:	Threonine, N-(3-acetamidosalicyl	Registry Number:	28068-16-8	Formula:	C29 H42 N2 O9		
		Other Names:	1,5-Dioxonane, propanoic acid de			Formula:	C28 H40 N2 O9	CA Index Name:	Butanoic acid, 2-methyl-,3-[[3-(fori		
		Molecular Weight	520.57	Registry Number:	118890-43-0	CA Index Name:	Butanoic acid, 2-methyl-,(2R,3S,6	Other Names:	1,5-Dioxonane, butanoic acid deri		
				Formula:	C27 H38 N2 O9	Other Names:	Antimycin A1a	Molecular Weight	562.65		
		Registry Number:	116095-17-1	CA Index Name:	Butanoic acid, 3-methyl-,(2R,3S,6	Molecular Weight	548.63				
		Formula:	C26 H36 N2 O9	Other Names:	Butanoic acid, 3-methyl-,8-butyl-3-			Registry Number:	21788-42-1		
		CA Index Name:	Butanoic acid, 3-methyl-,(2R,3S,6	Molecular Weight	534.6	Registry Number:	642-15-9	Formula:	C29 H42 N2 O9		
		Other Names:	Antimycin A3b			Formula:	C28 H40 N2 O9	CA Index Name:	Butanoic acid, 3-methyl-,(2R,3S,6		
		Molecular Weight	520.57	Registry Number:	118890-42-9	CA Index Name:	Butanoic acid, 2(or 3)-methyl-,(2R	Other Names:	Octanoic acid, 2-(1,2-dihydroxypro		
				Formula:	C27 H38 N2 O9	Other Names:	Antimycin A1	Molecular Weight	562.65		
		Registry Number:	41955-14-0	CA Index Name:	Butanoic acid, 2-methyl-,8-butyl-3-	Class Identifier:	Incompletely Defined Substance				
		Formula:	C26 H36 N2 O9	Other Names:	1,5-Dioxonane, butanoic acid deri						
		CA Index Name:	Butanoic acid, 3-methyl-,8-butyl-3-	Molecular Weight	534.6						
		Other Names:	1,5-Dioxonane, butanoic acid deri								
		Molecular Weight	520.57	Registry Number:	117552-77-9						
				Formula:	C27 H38 N2 O9						
		Registry Number:	28068-14-6	CA Index Name:	Butanoic acid,3-[[3-(formylamino)-						
		Formula:	C26 H36 N2 O9	Other Names:	Antimycin A2b						
		CA Index Name:	Butanoic acid, 2-methyl-,(2R,3S,6	Molecular Weight	534.6						
		Other Names:	Antimycin A3a								
		Molecular Weight	520.57	Registry Number:	28068-15-7						
				Formula:	C27 H38 N2 O9						
		Registry Number:	22083-60-9	CA Index Name:	Propanoic acid, 2-methyl-,(2R,3S,						
		Formula:	C26 H36 N2 O9	Other Names:	Antimycin A2a						
		CA Index Name:	Butanoic acid, 2-methyl-,8-butyl-3-	Molecular Weight	534.6						
		Other Names:	Isovaleric acid, 8-ester with N-(7-t								
		Molecular Weight	520.57	Registry Number:	21788-41-0						
				Formula:	C27 H38 N2 O9						
		Registry Number:	522-70-3	CA Index Name:	Butanoic acid, 3-methyl-,(2R,3S,6						
		Formula:	C26 H36 N2 O9	Other Names:	Hexanoic acid, 2-(1,2-dihydroxypr						
		CA Index Name:	Butanoic acid, 2(or 3)-methyl-,(2R	Molecular Weight	534.6						
		Other Names:	Antimycin A3								

Table 3.6 Overview of antimycins matching the molecular formulae of the first-set sample ions.

Taking into account antimycins with CH_2 attachments to the 'body structures', increases the number of matching antimycins; for example, 2-O-Methylantimycin A_5 matches the 507 amu $[M+H]^+$ ion and 2-Methoxyantimycin A_3 matches the 535 amu $[M+H]^+$ ion (Table 3.6). An indication to which antimycins, sample molecules relate, may be obtained from the fact that certain bacteria are known for producing specific combinations of antimycin molecules.

3.4.8 Trapping of antimycin

The antimycin masses of the first and second compound set, which during RP column fractionation appeared to differ in bioactivity (bioactive fractions eluted after the four dominant compounds of the first compound set, Fig. 3.14), were studied by trapping and bioassaying individual ions. In addition, the second-set 535 amu $[M+H]^+$ antimycin mass was isolated for a follow-up high-resolution LCMS analysis.

3.4.8.1 Trapping of multiple antimycin masses

The double-purified (NP/RP) combined fractions B were sub-sampled by harvesting specific RT ranges from the eluent. While the LCMS system uses a minority of the eluent for live mass detection, the remainder can either be directed to the waste, or trapped on filters. The mass spectrometer was set to detect compound masses in positive ion mode (+ESI). The detected masses are accurate to ± 0.001 amu.

Figure 3.20 displays the trapped RT areas 2E7-2F4, dissection peaks of (un)trapped compounds, and the distribution of first- (blue arrows) and second- (red arrows) set 'antimycin masses', with masses of 507 amu, 521 amu, 535 amu, 549 amu, 563 amu, and 577 amu $[M+H]^+$. Due to an overlap in the retention times of compound sets, a low signal/noise ratio, or absent ionisation, not all compounds could be trapped (individually). For example, the second-set 521 amu $[M+H]^+$ ion did not ionise, the second-set 507 amu $[M+H]^+$ ion eluted at a retention time between two nearby fractions (2E12 and 2F1), and the second-set 563 amu and 577 amu $[M+H]^+$ ions were trapped on the same filter.

Nonetheless, most compounds were trapped individually, which allowed testing their bioactivity against *Candida* in separation of one another. A numerical representation and a detailed overview of the data are given in table 3.7 and the Supplementary Information $B_{5\text{-}6}$, respectively.

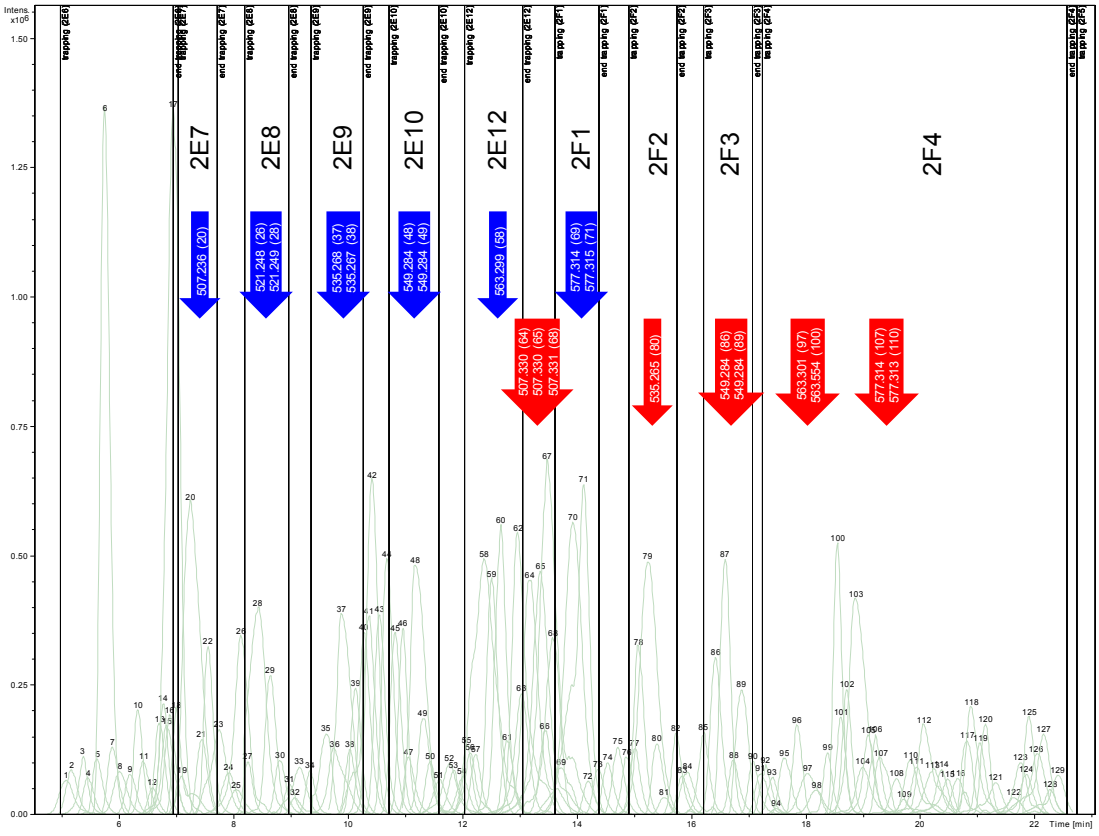


Figure 3.20 Fractionation of a 25 µl sub-sample from the double-purified (NP/RP) combined fractions B by reversed-phase LCMS in positive ion mode (+ESI). Sample masses detected in the eluent are displayed as numbered dissection peaks; trapped RT ranges (fractions 2E7-2F4) are visualised using black bars; and the placement of first- and second-set antimycin masses is shown respectively by blue and red arrows (mass, dissection peak no.). Despite of overlapping retention times and the fact that not all compounds ionised, most antimycin masses could be trapped individually making them available for follow-up bioassays. A detailed overview on the dissection peaks of fractions 2E9, 2E10, 2F2, and 2F3, and extracted ion chromatograms on parent ions with masses of 507 amu, 521 amu, 535 amu, 549 amu, 563 amu, and 577 amu $[M+H]^+$ are shown in the Supplementary Information $B_{5\text{-}6}$.

Fraction	RT	Dissection peak	Antimycin masses (dissection peak)		
2E7	7.0-7.7	18-23	507.236 (20)	-	-
2E8	8.0-8.8	26-31	521.248 (26)	521.249 (28)	-
2E9	9.2-10.2	34-40	535.268 (37)	535.267 (38)	-
2E10	10.7-11.5	44-51	549.284 (48)	549.284 (49)	-
2E12	11.9-13.0	55-63	563.299 (58)	-	-
-	-	63-68	507.330 (64)	507.330 (65)	507.331 (68)
2F1	13.5-14.2	69-73	577.314 (69)	577.315 (71)	-
2F2	14.9-15.6	76-82	535.265 (80)	-	-
2F3	16.2-16.8	85-90	549.284 (86)	549.284 (89)	-
2F4	17.0-22.5	91-129	563.301 (97)	563.554 (100)	-
2F4	17.0-22.5	91-129	577.314 (107)	577.313 (110)	-

Table 3.7 Numerical representation of the trapping data, described in figure 3.20. Trapped fractions 2E7-2F4, containing first- (blue font) and second- (red font) set antimycin masses.

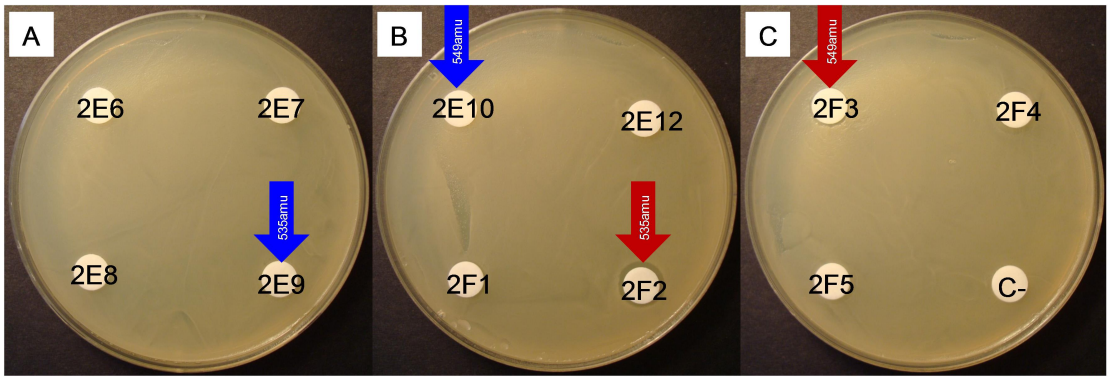


Figure 3.21 (A-C) Bioassays of trapped fractions 2E7-2F4 against *Candida* CA_6. The compounds, described in figure 3.20, were eluted from the trapping filters into fresh holding liquids, inoculated onto filter disks, and placed on *Candida*-coated agar plates. As negative control a filter was included inoculated with deuterated ACN (C^-), serving as holding liquid for the trapped compounds. Despite of their strong ionisation signal, and potentially high molecular abundance, none of the fractions holding first-set antimycin masses inhibit *Candida*. In contrast, despite their weak ionisation signal, and potentially low molecular abundance, some of the fractions holding second-set antimycin masses inhibit *Candida*. In particular, fractions with the second-set 535 amu and 549 amu $[M+H]^+$ ions (red arrows) inhibit the pathogen and fractions with the corresponding first-set antimycin masses (blue arrows) do not affect its growth. Probably, it is due to the small column loading volume of only 25 µl that the observed fungal inhibition is generally small and that some second-set fractions remain ineffective.

The trapped fractions were bioassayed against *Candida* CA_6 (Fig. 3.21). The compounds were eluted from the trapping filters and inoculated onto fresh filter disks. When dry, the disks were attached to *Candida*-coated agar plates.

Despite of their strong LCMS signal, and potentially high molecular abundance, none of the first-set antimycin masses shows any bioactivity. This confirms previous observations, obtained during the RP column fractionation, that the four major compounds of the first compound set preceded the bioactive RT area (Fig. 3.14); in contrast, despite their weak LCMS signal, and likely low molecular abundance, some fractions holding second-set antimycin masses show bioactivities. For example, the second-set fractions 2F2 (535 amu $[M+H]^+$) and 2F3 (549 amu $[M+H]^+$) are bioactive, whereas the first-set fractions 2E9 (535 amu $[M+H]^+$) and 2E10 (549 amu $[M+H]^+$) containing corresponding compound masses are not bioactive. The reason for not all second-set antimycin masses-containing fractions to be bioactive, may be attributed to inherent differences in their inhibitory properties towards *Candida*, or more likely to the low column loading volume of 25 µl. In combination with polarity differences and inconsistencies in their MS^2 fragmentation, these results may illustrate chemical differences between first- and second-set antimycin masses.

3.4.8.2 Trapping of a second set antimycin mass

To determine the molecular formula of a second-set antimycin mass, specific RT ranges were trapped containing the second-set 533 amu $[M-H]^-$ ion. This ion corresponds to the 535 amu $[M+H]^+$ ion detected above in positive ion mode (Fig. 3.20). The trapping was carried out with the same chromatography as described above; masses, accurate to ± 0.001 amu, were detected in negative ion mode (-ESI). In general, the negative ion mode showed stronger LCMS signals than the positive ion mode (data not shown). Also, the volume of the double-purified (NP/RP) combined fractions B, which was fractionated during two HPLC runs, was increased to 50 µl.

Figure 3.22 displays the trapped RT areas 2G4 (T14.8-15.2') and 2G5 (T15.2- 16.1'), sampled during one of the fractionation runs, dissection peaks of

(un)trapped compounds, and localisation of the mass of interest. The filters contain multiple, slightly differing, 533 amu $[M-H]^-$ masses. Fraction 2G5 contains ion masses of 533.252 amu, 533.253 amu, and 547.267 amu $[M-H]^-$; fraction 2G4 only contains ion masses of 533.211 amu and 533.252 amu $[M-H]^-$. A numerical representation and a detailed overview of the mass data are given in table 3.8 and the Supplementary Information $B_{7\text{-}8}$, respectively.

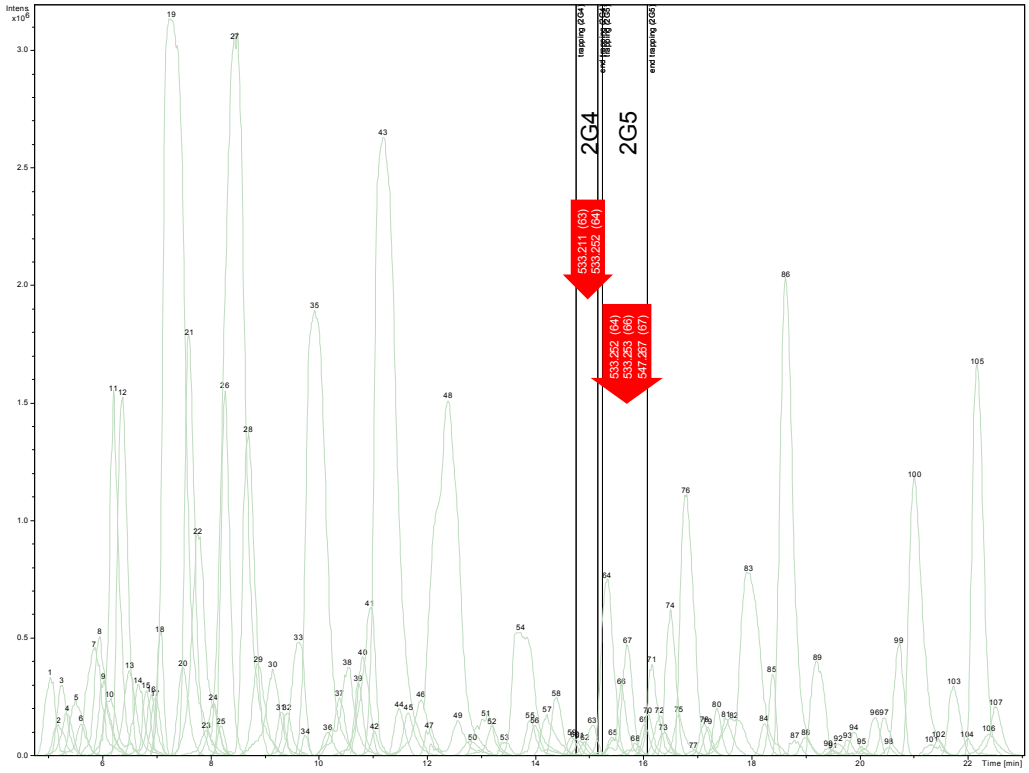


Figure 3.22 Fractionation of a 50 µl sub-sample from the double-purified (NP/RP) combined fractions B by reversed-phase LCMS in negative ion mode (-ESI). The trapping focuses on collecting molecules of the second-set 533 amu $[M-H]^-$ antimycin mass. The eluent contains multiple, slightly differing, second-set 533 amu $[M-H]^-$ masses that are trapped on two neighbouring filters (2G4 and 2G5). In addition to ion masses of 533.252 amu and 533.253 amu $[M-H]^-$, fraction 2G5 also contains an ion mass of 547.267 amu $[M-H]^-$. In contrast, fraction 2G4 only contains ion masses of 533.211 amu and 533.252 amu $[M-H]^-$. Due to its purity (Table 3.8) and strong inhibitory properties against *Candida* (Fig. 3.23) fraction 2G4 is used for a follow-up LCMS analysis. A detailed overview on the dissection peaks of fractions 2G4 and 2G5, and extracted ion chromatograms on the 505 amu, 519 amu, 533 amu, 547 amu, 561 amu, and 575 amu $[M-H]^-$ antimycin masses are shown in the Supplementary Information $B_{7\text{-}8}$.

Fractions 2G4 and 2G5 were challenged against *Candida* CA_6 in the same way as described above. The bioassays show that both fractions are bioactive (Fig. 3.23).

Fraction	RT	Dissection peak	Anitimycin masses (dissection peak)		
2G4	14.75-15.2	59-64	533.211 (63)	533.252 (64)	-
2G5	15.23-16.1	64-70	533.252 (64)	533.253 (66)	547.267 (67)

Table 3.8 Numerical representation of the trapping data, shown in figure 3.22. Masses are detected in negative ion mode. Fractions 2G4 and 2G5 both contain the requested 533 amu $[M-H]^-$ antimycin mass. In addition, fraction 2G5 also contains a larger antimycin mass.

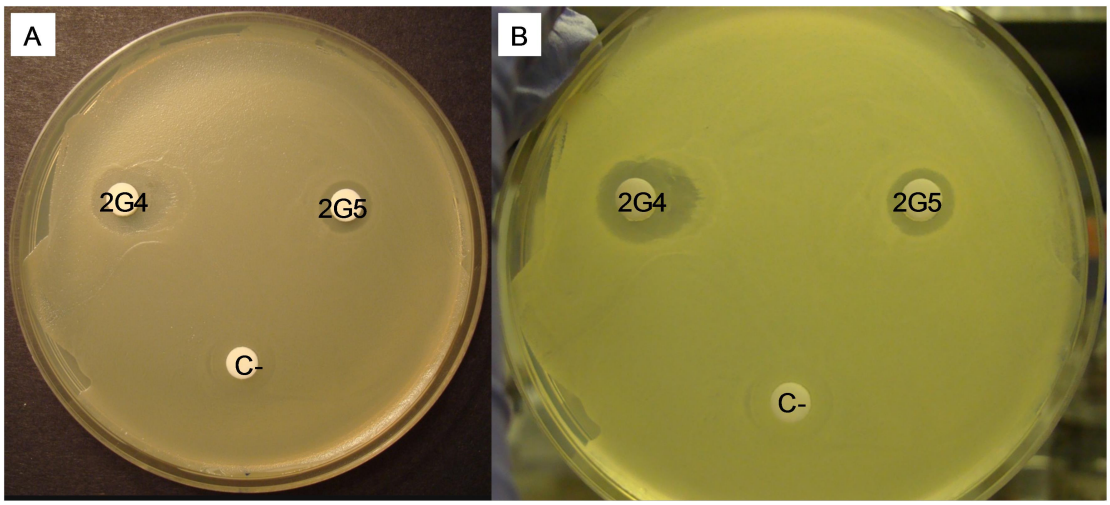


Figure 3.23 (A-B) Bioassays of trapped fractions 2G4 and 2G5 against *Candida* CA_6. The compounds, described in figure 3.22, were eluted from the trapping filters into fresh holding liquids, inoculated onto filter disks, and placed on *Candida*-coated agar plates. As negative control a filter was included inoculated with deuterated ACN (C^-), serving as holding liquid for the trapped compounds. Both fractions strongly inhibit the yeast. Since inoculation volumes are equal, the size difference of the inhibition zones may suggest that most of the putative antimycin molecules eluted into fraction 2G4.

3.4.9 LCMS analysis of the second set antimycin mass

Due to its strong bioactivity (Fig 3.23) and because of not containing any larger antimycin masses (Table 3.8), a low- and high-resolution LCMS analysis has been carried out on the second-set 533 amu $[M-H]^-$ antimycin mass in fraction 2G4. During low-resolution LCMS, UV, MS, and MS^2 data were collected; during high-resolution LCMS, accurate MS and MS^2 data were generated, serving the calculation of molecular formulae. The system was set to detect MS and MS^2 masses in negative ion mode (-ESI), accurate to ± 0.1 amu and ± 0.00001 amu.

3.4.9.1 Low-resolution LCMS

Figure 3.24_A shows the low-resolution LCMS data obtained from fraction 2G4, including a PDA, a BPC for compound masses in between 150-1500 amu, and a mass range of 532.7-533.7 amu. The mass range shows the presence of a dominant peak (red circle), eluting with a retention time of 10.52 minutes. The full MS of this peak is dominated by an ion with a mass of 533.2 amu $[M-H]^-$ (Fig. 3.24_B).

The fragmentation pattern of this second set ion exhibits the presence of daughter ions with masses of 245.0 amu and 263.0 amu $[M-H]^-$ (Fig. 3.24_C). These daughters are comparable to daughter ions, 247 amu and 265 amu $[M+H]^+$, which previously have been detected in positive ion mode only for first set ions (Table 3.4). However, the parent specific 271 amu $[M+H]^+$ analogue remained undetected. Despite positive and negative ion mode fragmentations cannot be compared to one another, the above findings suggest stronger structural analogies between first- and second-set compounds than expected based on observations in positive ion mode (Table 3.4).

The 533.2 amu $[M-H]^-$ ion absorbs light at two wavelengths, namely at 238.0 nm and 319.0 nm (Fig. 3.24_D); also antimycin A_2 has been shown to absorb at two wavelengths, namely at 239.0 nm and 318.0 nm (Supplementary Information B_9). Since fraction 2G4 contains less contaminants than the double-

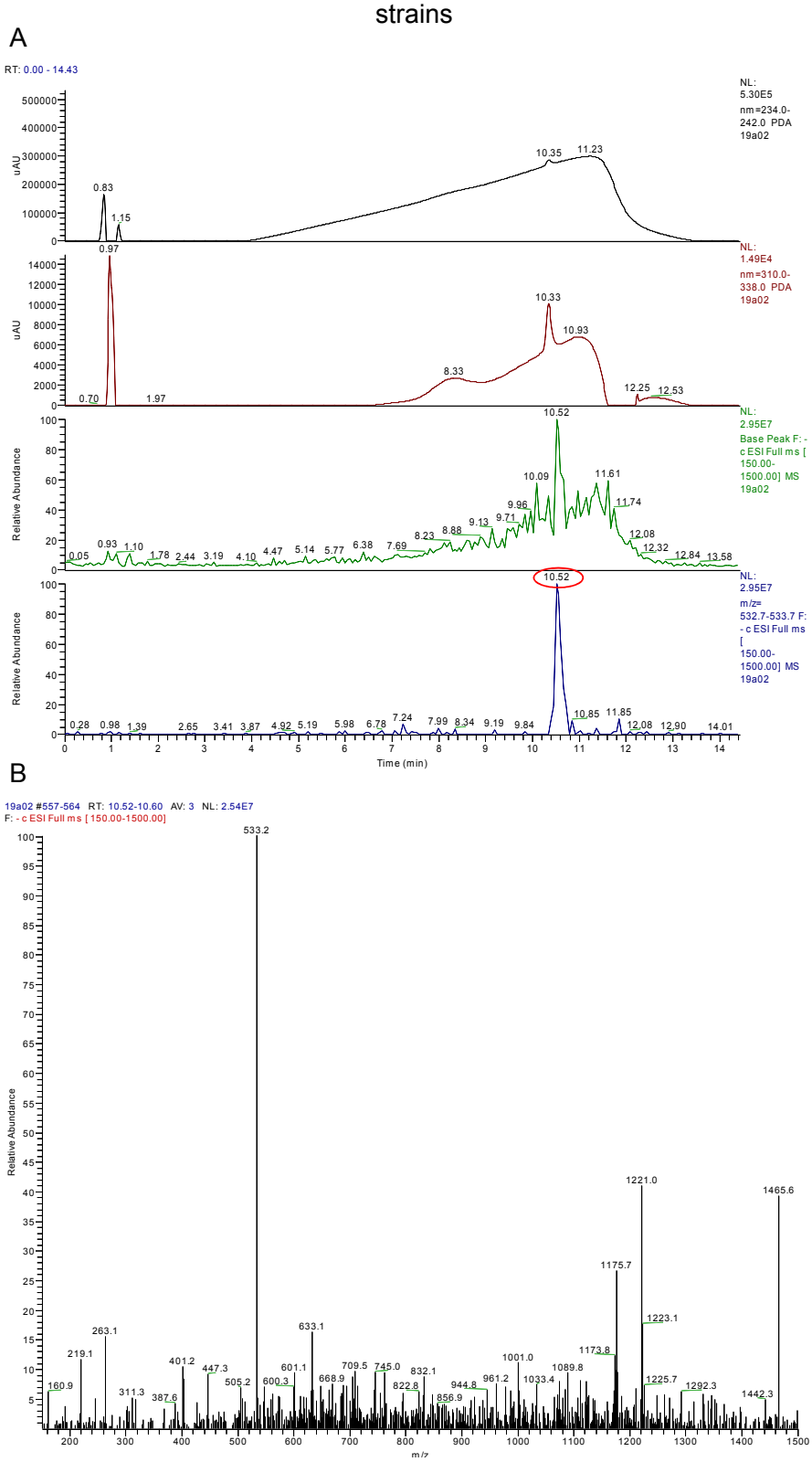
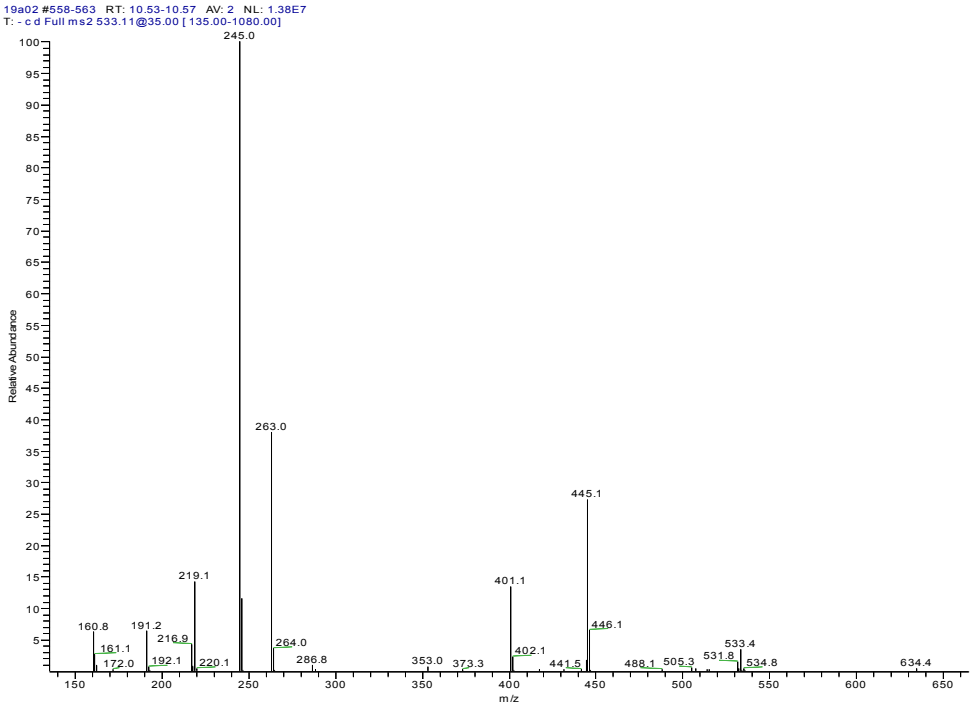


Figure 3.24 (A-B) Low-resolution reversed-phase LCMS on fraction 2G4 (Fig. 3.22), in negative ion mode (-ESI). (A) The graph shows a PDA, BPC, and mass range for compounds in between 532.7-533.7 amu $[M-H]^-$. The last clearly shows an ion peak (red circle) with the required mass at 10.52 minutes. (B) The full MS of this peak reveals the presence of a dominant 533.2 amu $[M-H]^-$ ion.

C



D

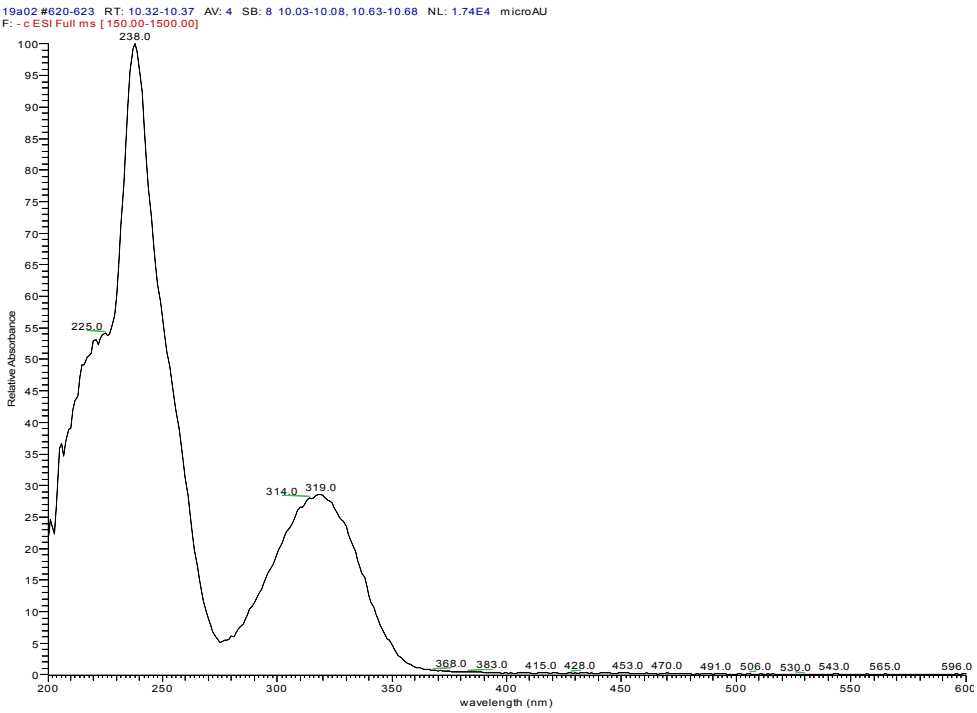


Figure 3.24 (C-D) Low-resolution reversed-phase LCMS on fraction 2G4 (Fig. 3.22), in negative ion mode (-ESI). (C) The MS^2 data obtained from the 533 amu parent, presented above, show daughter ions with masses of 245.0 amu and 263.0 amu $[M-H]^-$. As expected, the 263.0 amu daughter ion matches a sub-structure of the antimycin molecule, marked teal in figure 3.19. (D) The UV absorbance of the 533 amu $[M-H]^-$ parent ion shows two maxima at 238 nm and 319 nm, which matches the UV absorbance of antimycin.

purified (NP/RP) combined fractions B, which also exhibited absorbance maxima at higher wavelengths (Table 3.4), these may indeed have originated from coeluting background molecules.

3.4.9.2 High-resolution LCMS

High-resolution LCMS data of fraction 2G4, showing a PDA and a mass range of 532.7500-533.7500 amu are presented in figure 3.25_A. The mass range reveals the presence of a single peak (red circle) eluting with a retention time of 8.25 minutes. The full MS of this peak clearly shows the presence of a dominant 533.2501 amu $[M-H]^-$ ion (Fig. 3.25_B); based on the accurate mass, its molecular formula is found to be $C_{27}H_{37}N_2O_9$. The MS^2 data show the presence of daughter ions with masses of 245.0563 amu and 263.0667 amu $[M-H]^-$, possessing molecular formulae of $C_{12}H_9N_2O_4$ and $C_{12}H_{11}N_2O_5$, respectively (Fig. 3.25_C, Table 3.9). These daughters are analogous to daughter ions, 247.071 amu ($C_{12}H_{11}N_2O_4$) and 265.082 amu ($C_{12}H_{13}N_2O_5$) $[M+H]^+$, previously detected for first-set ions in positive ion mode (Table 3.5). Since containing nitrogen, the daughter ions must correspond to antimycin body structures, as highlighted teal in figure 3.19. In conclusion, analogies between the molecular formulae of antimycins and first-set antimycin masses, established during an LCMS analysis in positive ion mode (Table 3.5), here are shown to also exist between antimycins and a bioactive second-set sample ion (533.2501 amu $[M-H]^-$).

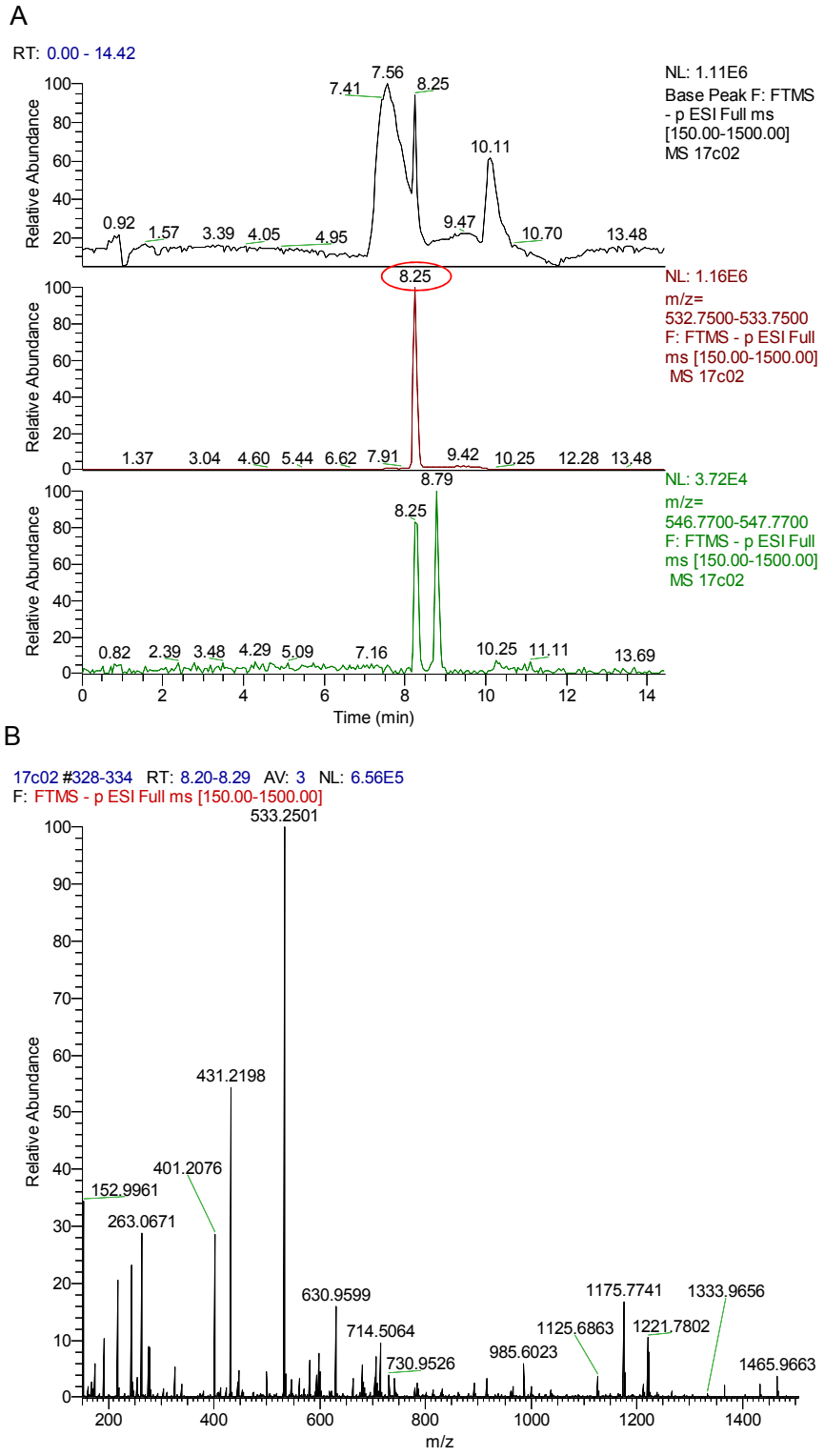


Figure 3.25 (A-B) High-resolution reversed-phase LCMS on fraction 2G4 (Fig. 3.22), in negative ion mode (-ESI). (A) The graph shows a BPC trace as well as a mass range for compounds between 532.75-533.75 amu $[M-H]^-$. The last clearly shows a dominant ion peak (red circle) at 8.25 minutes. (B) Full MS of the peak showing an accurate ion mass of 533.2501 amu $[M-H]^-$. The molecular formula of this ion is $C_{27}H_{37}O_9N_2$ and matches antimycin.

C

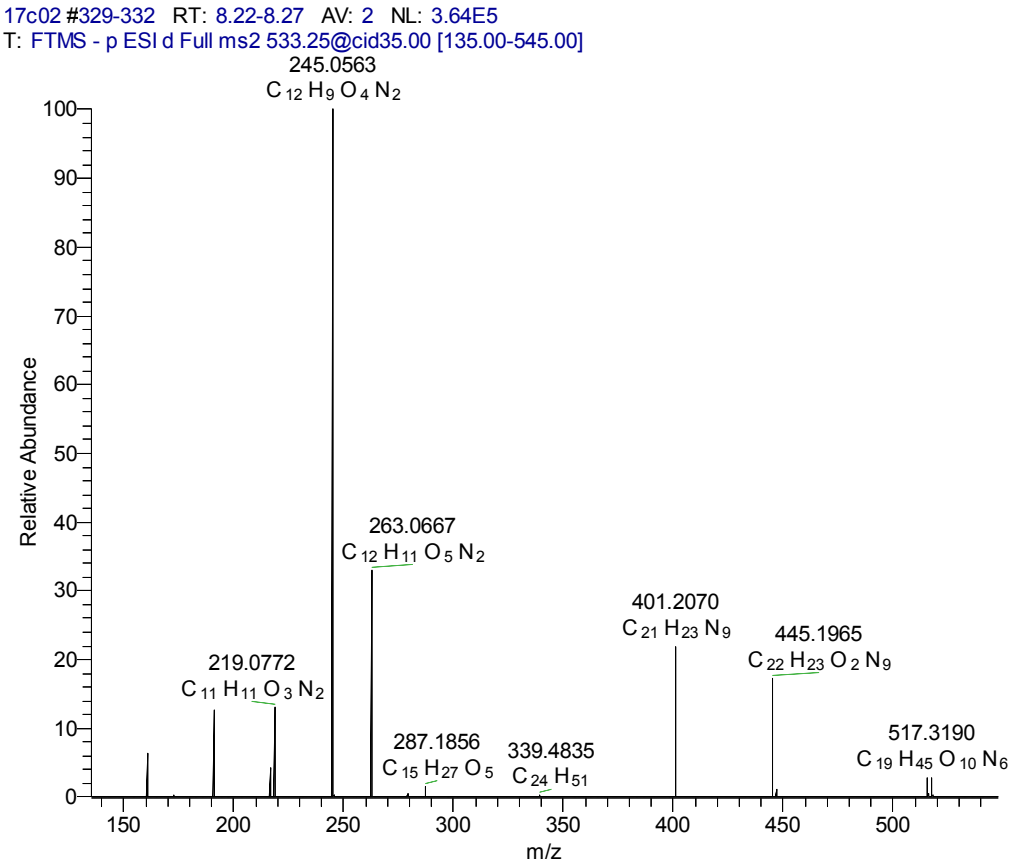


Figure 3.25 (C) High-resolution reversed-phase LCMS on fraction 2G4 (Fig. 3.22), in negative ion mode (-ESI). The fragmentation of the parent molecule, shown above, results in daughter ions with masses (formulae) of 245.0563 amu ($C_{12}H_9O_4N_2$) and 263.0667 amu ($C_{12}H_{11}O_5N_2$) $[M\text{-}H]^-$. As expected, the latter matches a sub-structure of the antimycin molecule, marked teal in figure 3.19. The data show that the negative ion mode suits well to visualise second-set antimycin masses, which failed to ionise during a high-resolution LCMS analysis in positive ion mode (Fig. 3.18). The results show that, in addition to first-set sample masses, a second-set sample mass also matches antimycin.

Mass detection			Low-resolution	High-resolution
Sample set			2	2
MS masses		EIC	532.7-533.7 amu	532.7500-533.7500 amu
		RT	10.52-10.60	8.20-8.29
		Parent ion	533.2	533.2501
		Formula	-	$C_{27}H_{37}N_2O_9$
		Error	-	1.43064
		Adduct	-	H
UV data		RT	10.32-10.37	-
		UV max.	238.0	-
		UV max.	319.0	-
MS^2 masses	Parent ion	RT	10.53-10.57	8.22-8.27
		Parent ion	533.11	533.25
	Daughter ion (body)	Mass	245.0	245.0563
		Formula	-	$C_{12}H_9N_2O_4$
		Error	-	-
	Daughter ion (body)	Mass	263.0	263.0667
		Formula	-	$C_{12}H_{11}N_2O_5$
		Error	-	-
	Daughter ion (body)	Mass	219.1	219.0772
		Formula	-	$C_{11}H_{11}N_2O_3$
		Error	-	-

Table 3.9 Numerical representation of low- and high-resolution LCMS data on fraction 2G4, presented in figures 3.24 and 3.25. UV, MS, and MS^2 data are obtained by low-resolution LCMS; MS and MS^2 data are generated by high-resolution LCMS. Compound masses are detected in negative ion mode (-ESI). Based on the accurate mass data, molecular formulae are calculated for parent and daughter ions. In an earlier LCMS analysis in positive ion mode (Table 3.4), first-set antimycin masses typically produced 265 amu, 247 amu, and 219 amu $[M+H]^+$ daughter ions, whereas second-set ones fragmented into daughters with masses of 265 amu and 237 amu $[M+H]^+$. Interestingly, here the fragmentation of a second-set 533 amu parent (-ESI), resulted in daughter ions with masses of 263 amu and 245 amu $[M-H]^-$, previously found in the MS^2 data of first-set compounds exclusively. However, no tail structure is detected of the 533 amu second-set ion, which is expected to be 269 amu $[M-H]^-$ in mass. Despite the fact that MS^2 data in positive and negative ion mode cannot be compared, the findings support that the chemical structures of both compounds sets are highly similar.

3.4.10 Tandem-LCMS analysis of antimycin and an antimycin standard

As a final test of the hypothesis that the antimycin masses purified from the *Streptomyces* E_8 growth culture indeed are antimycins, a tandem-LCMS analysis was carried out. In this analysis the double-purified (NP/RP) combined fractions B was compared to an antimycin A_1-A_4 standard. Analogous substances must show similar retention times, UV spectra, as well as MS and MS^2 data. The detector was set to detect masses in positive ion mode (+ESI), accurate to ± 0.1 amu. Also, UV spectra of the compounds were generated.

Table 3.10 shows UV, MS, and MS^2 data of the sample compounds and antimycin standard; a detailed overview and extracted ion chromatograms of them are presented in the Supplementary Information $B_{9\text{-}10}$. The data show that the antimycin standard contains five, instead of four, antimycin molecules. In addition to ions with masses of 507 amu, 521 amu, 535 amu, and 549 amu $[M+H]^+$ (antimycin A_1-A_4), an antimycin molecule with a mass of 563 amu $[M+H]^+$ (e.g. antimycin A_{10}, A_{14} or A_{15}) is detected in the standard. Also, the data show that the compounds of the antimycin standard clearly match in retention times the second-set compounds. This means that the standard does not correlate to the first compound set, which previously has failed to exhibit bioactivity during the RP column fractionation (Fig. 3.14) and trapping (Fig. 3.21). The compounds of the standard, fragment into daughter ions with masses of 265 amu and 237 amu $[M+H]^+$, which are characteristic for the second-set compounds (Table 3.4). Due to the low signal/noise ratio of the second-set compounds, no accurate UV absorbance data could be collected from the double-purified (NP/RP) combined fractions B during this tandem-LCMS run (Table 3.10). However, comparing the UV absorbance of the 535 amu $[M+H]^+$ (239 nm, 318 nm) ion from the antimycin standard with the one of the second-set 533 amu $[M-H]^-$ (238 nm, 319 nm) ion from fraction 2G4 (Table 3.9), it becomes obvious that both share similar maxima. In summary, the tandem-LCMS results confirm that the second-set antimycin masses indeed are antimycins; speculations on the identity of the first-set compounds are given in the discussion.

		Sample set 2					
	Peak	**1**	**2**	**3**	**4**	**5**	**6**
MS masses	EIC	507-508	521-522	535-536	549-550	563-564	577-578
	RT	-	-	17.29	17.93	18.48	19.14
	Parent ion	-	-	535.0	549.1	563.1	577.2
	Adduct	-	-	H	H	H	H
UV data	RT	-	-	17.15	17.78	18.33	18.98
	UV max.	-	-	247.0	241.0	249.0	253.0
	UV max.	-	-	322.0	320.0	320.0	322.0
	UV max.	-	-	361.0	359.0	360.0	361.0
	UV max.	-	-	383.0	383.0	383.0	383.0
	UV max.	-	-	405.0	405.0	405.0	405.0
MS^2 masses	RT	-	-	17.31	17.89	18.40	19.13
	Parent ion	-	-	535.01	549.04	563.17	577.22
	Daughter ion (body)	-	-	265.0	265.0	265.0	265.0
	Daughter ion (body)	-	-	237.1	237.2	237.1	237.2

		Antimycin standard					
	Peak	**1**	**2**	**3**	**4**	**5**	**6**
MS masses	EIC	507-508	521-522	535-536	549-550	563-564	577-578
	RT	15.91	16.61	17.29	17.96	18.58	-
	Parent ion	507.0	521.0	535.0	549.0	563.1	-
	Adduct	H	H	H	H	H	-
UV data	RT	15.82	16.48	17.17	17.78	-	-
	UV max.	238.0	240.0	239.0	240.0	-	-
	UV max.	318.0	318.0	318.0	318.0	-	-
MS^2 masses	RT	15.92	16.51	17.13	17.89	18.39	-
	Parent ion	507.02	521.12	535.22	549.04	563.14	-
	Daughter ion (body)	265.0	265.0	265.0	265.0	265.0	-
	Daughter ion (body)	237.1	237.1	237.0	237.1	237.2	-

Table 3.10 Low-resolution tandem-LCMS analysis on the double-purified (NP/RP) combined fractions B and an antimycin A_1-A_4 standard, in positive ion mode (+ESI). The table reveals that the standard consists of five, instead of four, compounds. In addition to 507 amu, 521 amu, 535 amu, and 549 amu $[M+H]^+$ ions, a 563 amu ion was detected. The table also shows that the standard matches the second, and not the first, compound set. For example, the antimycins of the standard share the same retention times as the bioactive sample molecules of the second-set. Furthermore, the MS^2 data of the standard match the fragmentation data obtained from the second-set sample molecules. For example, sample and standard both produce daughter ions with masses of 265 amu and 237 amu $[M+H]^+$; also, the standard did not produce any parent-specific daughters. In summary, the tandem-LCMS data confirm that second-set sample molecules are antimycins. In consequence, the structurally similar first-set sample molecules must either be antimycins, which due to technical reasons or resistances failed to inhibit *Candida*, or they are products in the antimycin biosynthesis that share the same masses and similar structures but need modifications to become bioactive.

3.5 Discussion

The *Streptomyces* E_8 supernatant, obtained from a liquid growth culture, was tested positive for bioactivity against diverse *Candida* strains and *Escovopsis* EWC. The antifungal molecules, responsible for fungal growth inhibition, were harvested by extracting the supernatant with butanol. In a bioassay-guided normal-phase column fractionation on the BuOH extract, two sets of normal-phase fractions turned out to inhibit *Candida* CA_6, revealing the presence of at least two antifungals. By LCMS, the more polar set of bioactive normal-phase fractions, represented by fraction 79, was shown to contain the antifungal candicidin. Its presence was confirmed by matching UV and mass (MS, MS^2) data, which also were used for generating molecular formulae, to candicidin identified by Haeder *et al*. (2009).

The more apolar set of bioactive normal-phase fractions, represented by fraction 24, did not contain candicidin and were recombined; in a follow-up bioassay-guided reversed-phase column fractionation these fractions were purified a second time. By LCMS, the bioactive reversed-phase fractions were shown to contain two sets of six compound masses. Accurate mass data (MS, MS^2) on first-set masses, which were used for generating molecular formulae, revealed analogies to the antifungal antimycin. In contrast, no accurate mass data could be collected from any of the second-set masses in this ion mode.

Bioassays, in which individually trapped first- and second-set antimycin masses were challenged against *Candida* CA_6 revealed that only second-set masses, which slightly differ in their polarities and MS^2 fractionation patterns from first-set masses, exhibited fungal inhibition. Using LCMS in a different ion mode, UV and mass data (MS, MS^2) of a second-set ion could be collected, which were used for generating molecular formulae, and these data turned out to match the antifungal antimycin as well. Finally, in a tandem-LCMS analysis, an antimycin A_1-A_4 standard was shown to match second-set antimycin masses in retention time, UV, MS, and MS^2 data confirming that second-set masses indeed are antimycins. Further investigations will be needed to reveal the full identity of the first-set antimycin masses, which in this study showed no

bioactivity against *Candida* CA_6 and had differential polarities and MS^2 fragmentation patterns from the second-set antimycin masses and the antimycin A_1-A_4 standard.

3.5.1 Two sets of antimycin molecules

The finding that both sets of masses are antimycins has been published by Seipke *et al.* (2011a). The genome of *Streptomyces* E_8 (renamed 'S_4' in Barke *et al.* 2010) has been published by Seipke *et al*. (2011b). In addition to biosynthetic gene clusters encoding non-bioactive secondary metabolites, eleven gene clusters have been identified encoding antibiotic, or as yet unknown, compounds (Seipke *et al*. 2011a). These include non-ribosomal peptide synthetase (NRPS), polyketide synthase (PKS), and hybrid NRPS/PKS gene clusters. Non-ribosomal peptide synthetases and polyketide synthases polymerise amino acids and carboxylic acids as substrate to form (antibiotic) compounds, respectively.

In particular, five gene clusters encode antibiotic compounds and show sequence analogies to gene clusters that encode candicidin (PKS), mannopeptimycin (NRPS), fredericamycin (PKS), gramicidin (NRPS), and kendomycin (PKS). Six gene clusters (three NRPS and three NRPS/PKS) show no analogies to known antibiotics and therefore potentially could encode novel compounds.

Seipke *et al*. (2011a) also report on three *Streptomyces* E_8 (deletion) knock-out mutants that, respectively, lack a gene (Δ*fscC*) in the candicidin biosynthesis pathway, lack a gene (Δ*antC*) in one of the three unknown NRPS/PKS gene clusters (scaffold06, coordinates 81953–106578) that encodes a non-ribosomal peptide synthetase, or lack both target genes.

Alongside the wild-type, the three knock-out mutants were individually challenged against *Escovopsis* EWC and *Candida* CA_6. Interestingly, the double-mutant (Δ*fscC*, Δ*antC*) lost almost all of its bioactivity against *Candida* but still strongly inhibited the growth of *Escovopsis*. This indicates that

Streptomyces E_8 must at least produce three antifungals and illustrates the value of using multiple test fungi for bioassay-guided column fractionations.

The deletion of the *antC* gene disrupts the strains' ability to inhibit *Candida*, but not its ability to inhibit *Escovopsis*. This indicates that in contrast to the gene product of the unknown NRPS/PKS gene cluster (scaffold06, coordinates 81953–106578), which is responsible for most of the inhibition of *Candida*, the third unknown antifungal must be ineffective against the yeast. Hence, the strong bioactivity of the normal-phase fractions described in this study, containing candicidin and antimycin (Fig. 3.12), cannot be attributed to the third antifungal.

Extracts of liquid growth cultures from the wild-type and Δ*antC* mutant were analysed by low-resolution LCMS alongside an antimycin A_1-A_4 standard (Seipke *et al*. 2011a). Extracted ion chromatograms show that the wild-type produces two compound sets with masses of 507 amu, 521 amu, 535 amu, and 549 amu $[M+H]^+$. The absence of larger antimycin masses, including masses of 563 amu and 577 amu $[M+H]^+$, can tentatively be attributed to the relatively short growth regimes used by the authors. Seipke *et al*. (2011a) also show that the antimycin standard shares the same retention times as the second-set compounds, which is consistent with the tandem-LCMS data, presented in this chapter (Table 3.10). One important observation is that the growth culture of the Δ*antC* mutant does not contain any second-set antimycin masses, which indicates that the unknown NRPS/PKS gene cluster (scaffold06, coordinates 81953–106578) must encode the antimycin biosynthetic pathway.

Another important finding is that the Δ*antC* mutant is also unable to produce first-set antimycin masses. This indicates that the first-set compounds are antimycins too, or inactive precursors of antimycin, which would explain why first-set compounds exhibited differential polarities and MS^2 fragmentation patterns from the second-set compounds (Table 3.4), and displayed no bioactivity against *Candida* CA_6 in this chapter (Fig. 3.21).

3.5.2 Streptomyces E_8 provides multiple antifungals

In summary, the *Acromyrmex* ant-associated *Streptomyces* strain E_8 is able to produce candicidin, plus two sets of six antimycin molecules, as revealed by biochemical analysis, plus a third, so-far unidentified antifungal, as revealed by knock-out experiments disrupting candicidin and antimycin biosynthesis in the same strain, plus, potentially, a number of other antibiotics, as revealed by sequence analysis, some of which might be novel to science (Seipke *et al*. 2011a).

The antifungals candicidin and antimycin differ in their mechanism of action by disrupting different fungal target molecules. The well-known antibiotic candicidin is a polyene antifungal, which due to its amphipathic properties, can integrate into fungal cell membranes. Its availability to *A. octospinosus* has been published by Haeder *et al.* (2009) and Barke *et al*. (2010). Also, the distribution of two candicidin biosynthesis genes (*fscM* and *fscP*) across selected ant- and cultivar-associated bacterial symbionts, described in this study, has been analysed in the Supplementary Information C. The data reveal that candicidin production may not be limited to bacterial associates of the *Acromyrmex* system in which *Streptomyces* E_8 and E_9, originating from two *A. octospinosus* colonies, both possess candicidin biosynthesis genes (Fig. S_2). These genes can also be found in *Streptomyces* KY_1, a bacterial associate of *Tetraponera penzigi* (Chapter 5).

The antimycin antibiotics were discovered over 60 years ago and their molecular structures are well-described (Dunshee *et al.* 1949, Ortiz-Gómez *et al.* 1995). Antimycins interfere with the electron transport chain and integrity of mitochondria. By binding enzyme complex III, antimycins reduce the efflux of hydrogen ions (H^+) and hence decrease membrane potential, leading to inhibition of the adenosine triphosphate (ATP) metabolism. Their availability to *Acromyrmex* spp. has been reported recently by Schoenian *et al.* (2011) and Seipke *et al*. (2011a). The Supplementary Information C describes the phylogenetic relationship of *Streptomyces* E_8 to other antimycin (and/or candicidin) producers. The data show that *Streptomyces* E_8 shares a

phylogenetic cluster (Fig. S_3) with *Streptomyces* E_9 and several candicidin- plus antimycin A_1-A_4-producers, isolated from *A. octospinosus* and *A. volcanus*. The bacterial associate of *T. penzigi*, *Streptomyces* KY_1, might possibly produce antimycin in addition to candicidin since it is not only genetically related to candicidin-producers but also to bacteria in the cluster of candicidin- plus antimycin A_1-A_4-producers.

It is unknown whether candicidin and antimycin antagonise or synergise in their effects against pathogenic fungi. Potentially, an increased permeability of fungal cells due to candicidin may increase mitochondrial exposure to antimycins. The fact that both compounds are unmodified relative to compounds produced by soil-actinobacteria, fits the environmental recruitment hypothesis (Barke *et al.* 2011) in which *Acromyrmex* ants are proposed to recruit beneficial *Streptomyces* bacteria from the environment. It would not be useful to invest in exploring insect niches if this would lead to an isolation of recently acquired environmental recruits of attine hosts, and thus to a re-discovery of the same antifungal compounds as previously found by pharmaceutical companies studying soil-actinobacteria; then, to invest in exploring cryptic pathways, for example by the application of chemical inducers or by mining bacterial genomes for antibiotic biosynthesis clusters, would be more promising. However, (1) not the complete bacterial microbiome of attine ants appears to be recruited; for example, *Pseudonocardia* symbionts seem to be coevolved and produce antifungal with novel chemical structures (i.e. nystatin P1 in Barke *et al*. 2010, dentigerumycin in Oh *et al*. 2009); (2) also, it is unknown whether attine ant-associated bacteria, other than *Pseudonocardia* and *Streptomyces*, are coevolved or environmentally recruited. For example, a bioactive *Nocardiopsis* strain was isolated from *Acromyrmex octospinosus* workers during this study, which could be coevolved with the ant host; (3) despite attine ants may recruit most of their *Streptomyces* strains from the environment, investing in the exploration of host-symbiont systems may nonetheless be profitable since attine ants preferentially associate with antibiotic-producing bacteria. This

conglomeration of selected bacteria on attine hosts allows an easy access to bioactive strains, reducing the costs of prospecting.

Chapter 4) *Allomerus decemarticulatus and A. octoarticulatus* cultivar-associated bacteria

4.1 Introduction

4.1.1 Mutualistic partners

Mutualistic relationships between plant-ants and ant-plants can be sub-divided into myrmecophilic and myrmecophytic associations. Plant-ants are well-known to protect their plant host against a diversity of herbivores. In return, ant-plants provide their ant symbionts with food rewards and with nesting space. In particular, both myrmecophilic and myrmecophytic plants provide their ant associates with extrafloral nectar, food bodies or indirectly by secreted honeydew of hemipteran trophobionts (reviewed in Heil and McKey 2003). Myrmecophytic associations, which are less abundant but more persistent, provide their ant symbionts additionally with permanent nesting sites, including hollow stems, thorns, petioles or leaf pouches ('domatia'). Some ant species enrich the plant-provided diet by making use of host-unrelated food resources, including insects, pollen grains, fungal spores and/or detritus (Davidson *et al*. 2003, Belin-Depoux and Bastien 2002).

The focal species here are the two ant species *Allomerus decemarticulatus* and *A. octoarticulatus* (*Hymenoptera*, *Formicidae*), which live symbiotically and exclusively with the ant-plants *Hirtella physophora* (*Malpighiales*, *Chrysobalanaceae*) and *Cordia nodosa* (*Lamiales*, *Boraginaceae*). The host plants supply *Allomerus* (Fig. 4.1) with nectar from extrafloral nectaries and with domatia. *Hirtella* produces pairs of leaf pouches that are attached to single leaves (Fig. 4.2), and *Cordia* produces single-chambered domatia that are interconnected to multiple leaves (Fig. 4.3). Typically, host trees are inhabited by a single ant colony that occupies most or all the domatia on a plant.

Smaller worker ants can be found inside of the domatia, which care for the brood (pupae, larvae, and eggs), the queen, and the alates; in contrast, larger workers patrol leaves, and the largest *Allomerus* workers build and maintain

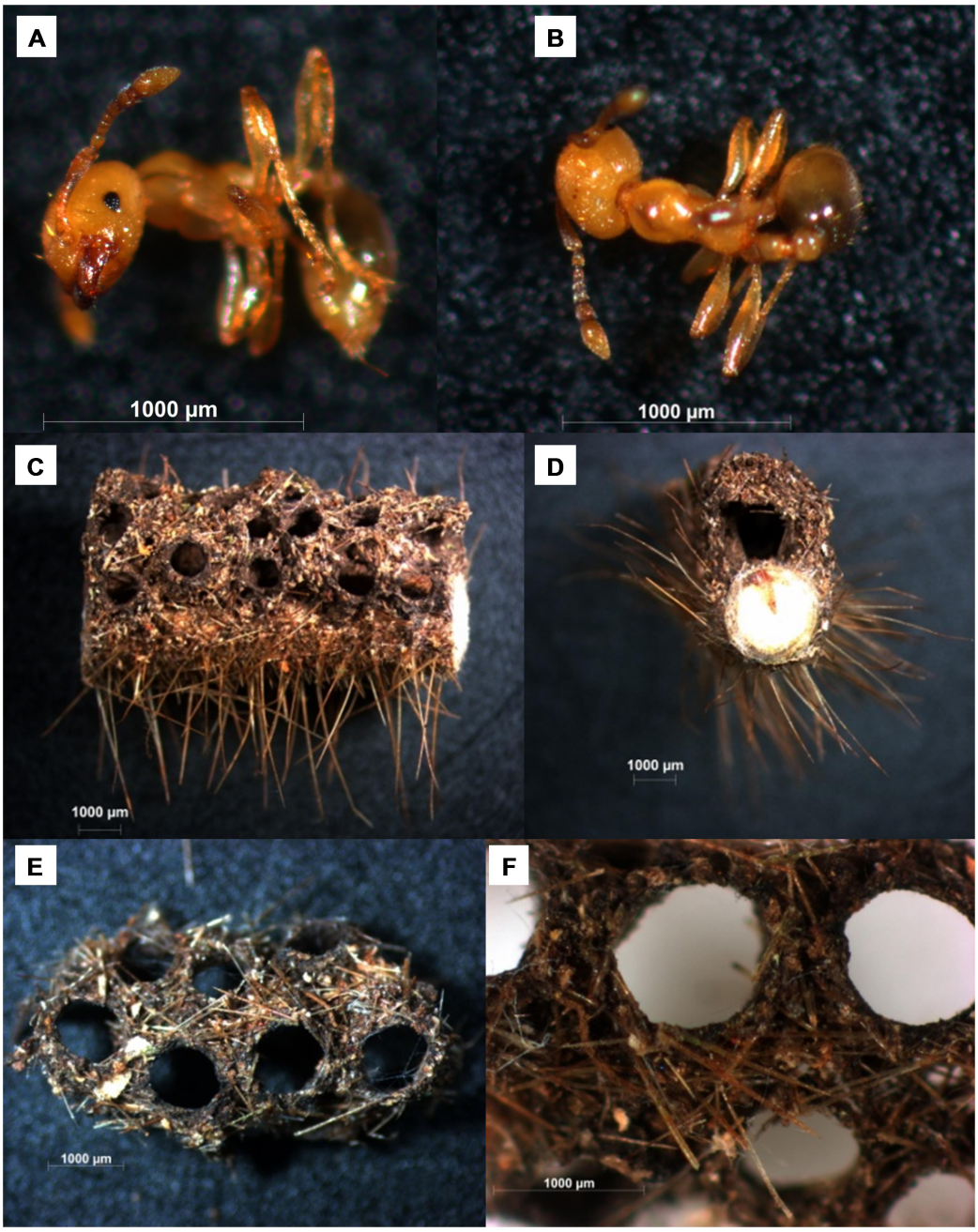


Figure 4.1 Microscopy photographs of *Allomerus* ant and fungal trap samples. **(A-B)** Ventral and dorsal side of a worker ant. **(C-D)** Part of a fungal trap gallery, which is built by *A. decemarticulatus* on the myrmecophyte *Hirtella physophora* and used to capture insects. The traps are typically built at the underside of the branches; to better view the trap construction, the branch is turned upside-down. **(E-F)** Close-up of the fungal trap, showing that it consists of cut trichomes (tree hairs) and fungal material (dark brown). The fungal cultivar *Trimmatostroma cordae* is thought to provide stability to the trap construction.

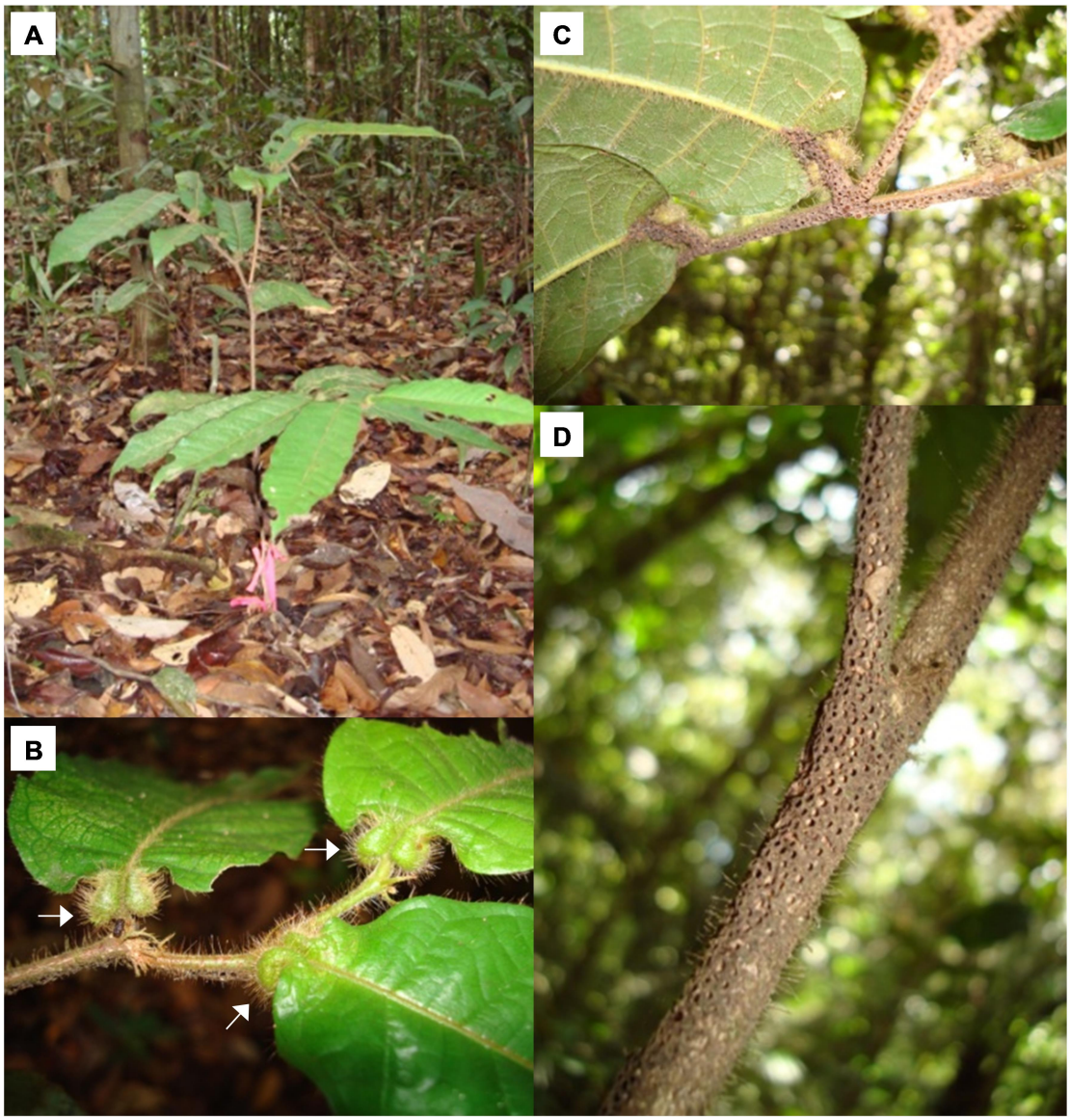


Figure 4.2 Host tree *Hirtella physophora* and ant symbiont *Allomerus decemarticulatus*. **(A)** *H. physophora* host tree in the rainforest of French Guiana. **(B)** Branch of the myrmecophytic tree with several domatia (nesting sites), each consisting of double-chambered leaf pouches (white arrows). **(C-D)** While the smallest worker ants take care of the brood and queen inside of the domatia, larger workers patrol on the leaves, and the largest workers construct and maintain trap galleries. The trap galleries are running alongside the branches and extend to the trunk; they even interconnect domatia of different leaves and branches.

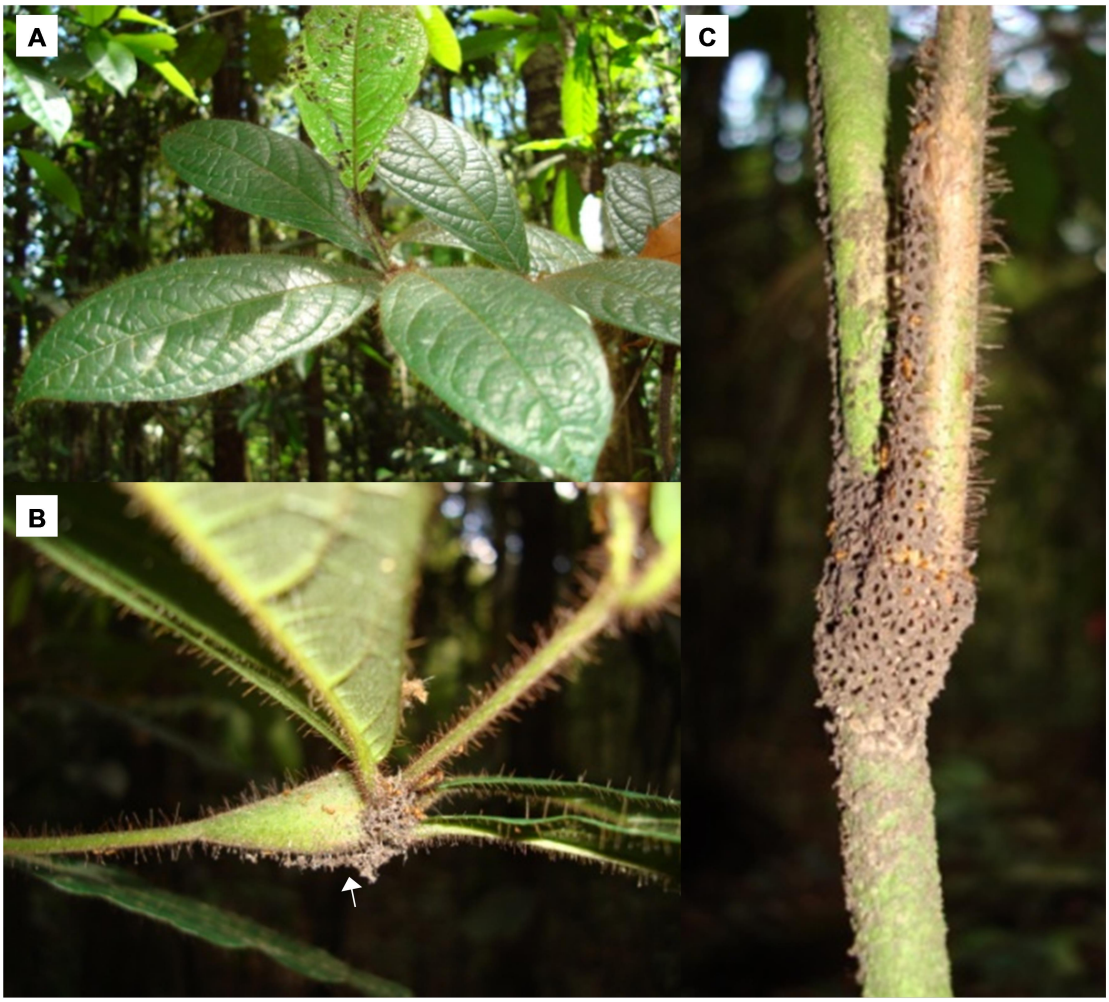


Figure 4.3 Host tree *Cordia nodosa* and ant symbiont *Allomerus octoarticulatus*. **(A-B)** *C. nodosa* host tree in the rainforest of French Guiana producing domatia with single cavities (white arrow). **(C)** The trap galleries are built near the entrance of domatia and at the trunk.

galleried traps, which are used to capture visiting arthropods (Dejean *et al.* 2005) (Fig. 4.4).

The trap galleries are built along the stem and along the undersides of branches and domatia, possibly to avoid rain damage and/or to coincide with where travelling arthropods are most likely to land on the host plants. The galleries are tunnel shaped constructions gridded with small, circular openings (Fig. 4.1) underneath which worker ants wait with opened mandibles for prey to

land, at which time the ants grab onto the legs, move in opposite directions to stretch the prey's legs, and sting the insect to death (Dejean *et al*. 2005) (Fig. 4.4). The workers then collectively move the killed prey towards the nearest leaf pouches, where the insect is dismantled and consumed.

The trap galleries are built upon hair-like structures (trichomes) that cover the branches and trunk of both *Hirtella* and *Cordia*. The ants cut a pathway through and interweave trichomes to form the trap scaffold, which is covered with carton and frass. The completed trap is then inoculated with a fungus that grows over and connects individual trichomes, thereby providing stability (Ruiz-González *et al.* 2010).



Figure 4.4 Insect caught by *Allomerus decemarticulatus*. A foreign ant queen has been caught by the ant colony. The largest workers wait inside of the trap galleries for insects to land. While some ants immobilise prey by clinging to its feet, others overwhelm and sting it to death. The insect is dismantled near a domatium. Note that the branch is turned upside-down to view the catch.

Hirtella-hosted *A. decemarticulatus* ants build extensive trap galleries that even interconnect domatia of different branches; here, the fungus covers the

whole trap gallery. In contrast, *Cordia*-hosted *A. octoarticulatus* ants build trap galleries that cover the entrances of domatia, sometimes extending to the underside of branches, and also along the stem. In this symbiosis, apparently only parts of the trap galleries are inoculated with the fungus, namely, areas near the entrance of domatia containing the queen. It is unknown whether differences in trap construction and location reflect differences in hunting strategies.

From trap galleries and foundress-occupied domatia of 108 *A. decemarticulatus* and 31 *A. octoarticulatus* colonies, Ruiz-González *et al.* (2010) isolated primarily one fungal morphotype. This fungus has been shown, based on 114 concatenated full ITS and EF1α sequences, to belong to 16 genetically related haplotypes; one of which was dominant, representing more than 75% of the sequences (Ruiz-González *et al.* 2010). The cultivar has been placed, based on 124 full ITS sequences, in the ascomycete order *Chaetothyriales*, close to *Trimmatostroma cordae* (AJ244263) and to fungal collections associated with the ant genus *Azteca* (FJ538958, FJ538959, FJ538960). Vertical transmission of the fungal cultivar by new queens is implicated (Lauth *et al.* 2011), which is consistent with the observations that the cultivar is absent from unoccupied trees and disappears when ant colonies die. *Allomerus* ants also appear to invest in their association with the cultivar, feeding the fungus with prey debris and vegetable material, and manipulating its growth (Ruiz-González *et al.* 2010, Lauth *et al.* 2011). In particular, the ants have been proposed to limit the growth of the fungal mutualist to the galleries, to use the fungal material for repairing (artificial) damages in the walls of the domatia, and to overgrow the stockades that new gynes build to enclose themselves in the domatia before the start of reproduction.

4.1.2 Exploitation control of non-cultivar fungi

Just as with the fungal cultivar of attine ants, vertical transmission may help to ensure that the trap cultivar is available to all new ant colonies and also that the fitness interests of ant and fungal cultivar are aligned. However, vertical

transmission results in reduced genetic relatedness at the population level, which can translate into increased vulnerability to disease and competition because the ability of the fungal cultivar to engage in interference competition with competitors or to defend against pathogens would be impaired by having access to only the same set of fungal metabolites as all neighbouring colonies.

Trap galleries indeed are subject to fungal and bacterial contamination, vectored by water droplets from the overstory, faeces of birds, and/or prey insects; fungal traps also receive wind-dispersed fungal spores. Spores from up to 44 fungal species have been isolated from the cultivars (Ruiz-González *et al.* 2010). In vitro, inocula of the slow-growing fungal mutualist are regularly overgrown by some of these fast-growing non-cultivar fungi. If invasive fungi consume or overgrow the *Trimmatostroma* cultivar that stabilises the trap gallery, the traps could be rendered useless, thereby reducing *Allomerus'* fitness. Infection zones might also spread to the domatia and infect the ant brood.

In parallel with attine ants, several mechanisms can hypothetically prevent non-cultivar fungi and pathogens from infesting fungal traps, and explain the widespread occurrence of the fungal cultivar. The ants may (1) physically remove non-cultivar fungi or apply metapleural gland secretions. Spores and hyphae of non-cultivar fungi indeed are present in the infrabuccal pellets of workers as indicated by unpublished data (M. X. Ruiz-Gonzalez 2009); (2) the fungal cultivar could defend itself by secreting antibiotics; (3) ant-associated bacteria could produce antibiotics that selectively target non-cultivar fungi and bacterial pathogens; and/or (4) cultivar-associated bacteria could do the same.

Seipke *et al*. (2012) recently presented evidence for the third mechanism, demonstrating that an antibiotic-producing microbiome is present on the cuticles of *A. decemarticulatus* and *A. octoarticulatus*. Six *Streptomyces* and one *Amycolatopsis* strains were isolated from worker ant cuticles. Both genera have previously been reported from attine worker ants (e.g. Haeder *et al*. 2009, Sen *et al*. 2009). The seven bacterial *Allomerus* isolates were bioassayed against *Candida* CA_6 and two non-cultivar fungi, including *Beauveria* (F_3) and

Annulohypoxylon (F_5). The *Amycolatopsis* strain was able to inhibit the growth of all fungal opponents *in vitro*, while the bioactivity of the *Streptomyces* strains was more selective. One *Streptomyces* strain inhibited *Candida* and *Beauveria* (F_3); another strain inhibited *Candida* and *Annulohypoxylon* (F_5); and one strain inhibited the growth of *Candida* only.

4.2 Aims

In this chapter, the fourth hypothesis is tested that the fungal cultivar itself hosts an antibiotic-producing microbiome that is used to attack non-cultivar fungi and pathogens. Fungal traps could offer a rich habitat for bacterial symbionts, following the findings that bioactive *Streptomyces* symbionts have been reported from the fungal cultivar of *Acromyrmex octospinosus* (Haeder *et al*. 2009) and that bioactive *Burkholderia* have been isolated from the fungal cultivar of *Atta sexdens* (Santos *et al*. 2004). From a pharmaceutical point of view, this under-explored environmental niche could represent a source of putatively coevolved bacteria and novel antifungals.

4.3 Materials and Methods

4.3.1 Sampling fungal cultivars

Worker ants and their fungal cultivar were sampled from *Allomerus decemarticulatus* and *A. octoarticulatus* colonies, hosted by *Hirtella physophora* and *Cordia nodosa*, respectively. The samples were collected in French Guiana from three inland sites and one coastal location (Fig. 4.5). From the inland sites, Basevie, Area 9, and Area 24, which all are close to the field station at Barrage de Petit Saut, 28 *Hirtella*-hosted *A. decemarticulatus* colonies were sampled as well as nine *Cordia*-hosted *A. octoarticulatus* colonies (Table 4.1). From the coastal site, la Montagne des Singes (near Kourou), two *Hirtella*-hosted *A. decemarticulatus* colonies, but no *Cordia*-hosted *A. octoarticulatus* colonies, were sampled. Instead, ants and fungal cultivar samples of 20, seemingly rare, *Hirtella*-hosted *A. octoarticulatus* colonies were sampled. This is the only known site to harbour this host symbiont combination. Typically, *Hirtella* samples

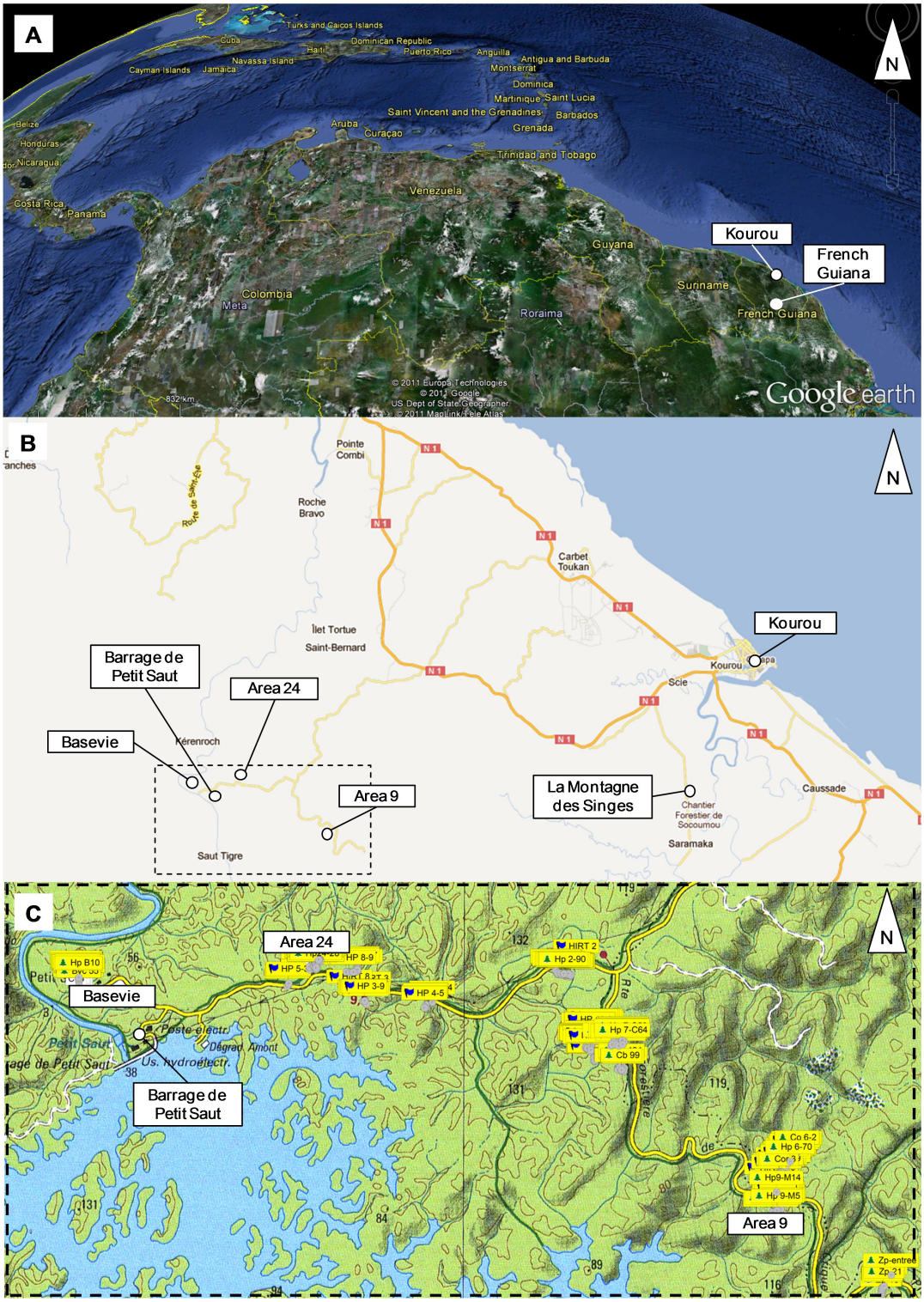


Figure 4.5 (A-B) Sampling locations in French Guiana, where *Allomerus* spp. worker ants and their fungal cultivar were sampled. The sampling sites include 'Basevie', 'Area 24', and 'Area 9', located near the field station at Barrage de Petit Saut, as well as 'la Montagne des Singes', located near Kourou.

Sampling location	Colony	Host tree	Ant species	Worker ants	Fungal cultivar	Preserved in
Area 24, French Guiana	53	CN	AO	53A,C	53B	2G
Area 24, French Guiana	54	CN	AO	54A,C	54B	2G
Area 24, French Guiana	56	CN	AO	56A,C	56B	2G
Area 24, French Guiana	51	HP	AD	51A,C	51B	2G
Area 24, French Guiana	52	HP	AD	52A,C	52B	2G
Area 24, French Guiana	55	HP	AD	55A,C	55B	2G
Area 24, French Guiana	57	HP	AD	57A,C	57B	2G
Area 24, French Guiana	58	HP	AD	58A,C	58B	2G
Area 24, French Guiana	59	HP	AD	59A,C	59B	2G
Area 9, French Guiana	42	CN	AO	42A,C	42B	PBS
Area 9, French Guiana	50	CN	AO	50A,C	50B	PBS
Area 9, French Guiana	41	HP	AD	41A,C	41B	PBS
Area 9, French Guiana	43	HP	AD	43A,C	43B	PBS
Area 9, French Guiana	39	HP	AD	39A,C	39B	2G
Area 9, French Guiana	40	HP	AD	40A,C	40B	2G
Area 9, French Guiana	44	HP	AD	44A,C	44B	2G
Area 9, French Guiana	45	HP	AD	45A,C	45B	2G
Area 9, French Guiana	46	HP	AD	46A,C	46B	2G
Area 9, French Guiana	47	HP	AD	47A,C	47B	2G
Area 9, French Guiana	48	HP	AD	48A,C	48B	2G
Area 9, French Guiana	49	HP	AD	49A,C	49B	2G
Basevie, French Guiana	6	CN	AO	6A,C	6B	PBS
Basevie, French Guiana	7	CN	AO	7A,C	7B	PBS
Basevie, French Guiana	13	CN	AO	13A,C	13B	PBS
Basevie, French Guiana	16	CN	AO	16A,C	16B	PBS
Basevie, French Guiana	1	HP	AD	1A,C	1B	PBS
Basevie, French Guiana	2	HP	AD	2A,C	2B	PBS
Basevie, French Guiana	3	HP	AD	3A,C	3B	PBS
Basevie, French Guiana	4	HP	AD	4A,C	4B	PBS
Basevie, French Guiana	5	HP	AD	5A,C	5B	PBS
Basevie, French Guiana	8	HP	AD	8A,C	8B	PBS
Basevie, French Guiana	9	HP	AD	9A,C	9B	PBS
Basevie, French Guiana	10	HP	AD	10A,C	10B	PBS
Basevie, French Guiana	11	HP	AD	11A,C	11B	PBS
Basevie, French Guiana	12	HP	AD	12A,C	12B	PBS
Basevie, French Guiana	14	HP	AD	14A,C	14B	PBS
Basevie, French Guiana	15	HP	AD	15A,C	15B	PBS
La montagne des singes, French Guiana	32	HP	AD	32A,C	32B	PBS
La montagne des singes, French Guiana	35	HP	AD	35A,C	35B	PBS
La montagne des singes, French Guiana	17	HP	AO	17A,C	17B	PBS
La montagne des singes, French Guiana	18	HP	AO	18A,C	18B	PBS
La montagne des singes, French Guiana	19	HP	AO	19A,C	19B	PBS
La montagne des singes, French Guiana	20	HP	AO	20A,C	20B	PBS
La montagne des singes, French Guiana	21	HP	AO	21A,C	21B	PBS
La montagne des singes, French Guiana	22	HP	AO	22A,C	22B	PBS
La montagne des singes, French Guiana	23	HP	AO	23A,C	23B	PBS
La montagne des singes, French Guiana	24	HP	AO	24A,C	24B	PBS
La montagne des singes, French Guiana	25	HP	AO	25A,C	25B	PBS
La montagne des singes, French Guiana	26	HP	AO	26A,C	26B	PBS
La montagne des singes, French Guiana	27	HP	AO	27A,C	27B	PBS
La montagne des singes, French Guiana	28	HP	AO	28A,C	28B	PBS
La montagne des singes, French Guiana	29	HP	AO	29A,C	29B	PBS
La montagne des singes, French Guiana	30	HP	AO	30A,C	30B	PBS
La montagne des singes, French Guiana	31	HP	AO	31A,C	31B	PBS
La montagne des singes, French Guiana	33	HP	AO	33A,C	33B	PBS
La montagne des singes, French Guiana	34	HP	AO	34A,C	34B	PBS
La montagne des singes, French Guiana	36	HP	AO	36A,C	36B	PBS
La montagne des singes, French Guiana	37	HP	AO	37A,C	37B	PBS
La montagne des singes, French Guiana	38	HP	AO	38A,C	38B	PBS

Table 4.1 Domatia, leaf/leaves, and some of the fungal traps were sampled from 28 *Hirtella* (HP)-hosted *A. decemarticulatus* (AD) and nine *Cordia* (CN)-hosted *A. octoarticulatus* (AO) colonies nearby Barrage de Petit Saut; two *Hirtella*-hosted *A. decemarticulatus* and 20 *Hirtella*-hosted *A. octoarticulatus* colonies from 'la Montagne de Singes'. Worker ants (A, C) and their fungal cultivar (B) were isolated from the samples, the latter of which was used for the bacterial isolations.

consisted of a double-chambered domatium, associated leaf, and leaf petiole with some of the fungal trap.

Cordia samples consisted of a single-chambered domatium, multiple associated leaves and leaf petiole with some of the fungal trap. The samples were bagged and brought from the collection sites to the field station, where they were stored at 4 ^{o}C, until processing. *Hirtella*-hosted *A. decemarticulatus* and *A. octoarticulatus* colonies, from la Montagne des Singes, were distinguished morphologically by counting the number of antennal segments of representative worker ants under a stereomicroscope, ten for *A. decemarticulatus* and eight for *A. octoarticulatus*.

At the field station of Barrage de Petit Saut, the worker ants were removed from the domatia, leaves, and fungal traps under a stereomicroscope. The work bench was sterilised with 70% ethanol, the isolation took place nearby an open flame, and the insects were picked-up with soft forceps that previously were sterilised. The reason for combining workers of different size classes and functions was their disrupted functional distribution across leaves, domatia, and traps after having been disturbed during collection. The corresponding fungal traps were also collected from the plant material. Ants and fungal material were preserved in Eppendorf tubes containing 1 ml of phosphate buffered saline (PBS) or 1 ml of 20% glycerol (2G). Differing storage media were used to reduce the risk of killing bacterial strains due to uncertain and potentially unfavourable transport within French Guiana and during transit to the United Kingdom. Before and after their transport, the PBS samples were stored at 4 ^{o}C and the 2G samples at -20 ^{o}C. PBS has been shown to possess good qualities as a preservative of bacterial cells (Liao and Shollenberger 2003), and also has the advantage that samples can be stored unfrozen. Some samples were stored in glycerol since the composition of bacterial species and the presence of relatively rare, but possibly important species, is likely more stable at -20 ^{o}C; the disadvantage here is, that temporarily higher temperatures during transit could be lethal to certain bacterial species.

4.3.2 Isolation of cultivar-associated bacteria

Bacterial isolations were carried out in a category 2 microbiological laboratory at the University of East Anglia, United Kingdom. Bacteria were isolated from the fungal trap sample of 59 *A. decemarticulatus* or *A. octoarticulatus* colonies (Table 4.1). The samples were vortexed for a minimum of 10 seconds. Then the solid particles of the traps were separated from the supernatants by briefly centrifuging. The 2G and PBS supernatants were then transferred to fresh Eppendorf tubes. Small sub-samples of the supernatants were serially diluted (dilution factors: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-5}, 10^{-7}, 10^{-8}) and used for inoculating mannitol soy-flour (MS) agar plates (Table 4.2). Fresh agar plates were prepared by adding 1 ml of nystatin (5 mg (Sigma N3503-25MU) in 1 ml dimethyl sulfoxide (DMSO)) and 1 ml of cycloheximide (5 mg (Fisher 35742-0050) in 1 ml 100% ethanol) solutions to 100 ml autoclaved agar (50 ^{o}C); the medium was then sub-divided over four Petri dishes.

Inoculated agar plates were incubated at 30 ^{o}C, and morphotypes of Gram-positive and Gram-negative bacteria were purified by repeatedly propagating single colonies to fresh MS agar plates. Each morphotype was selected once, regardless of colony abundance. In total, 20 bacterial lineages (FG_1-FG_{15}, FG_{17}-FG_{21}) could be isolated (Table 4.3); most of the strains (85%) were isolated from individual traps, and the rest were obtained from pilot isolation plates that had been inoculated with combinations of cultivar supernatants. The purified strains were stored in 2G, as described for the bacterial isolates of the *Acromyrmex* system (Chapter 2.3.2).

4.3.3 Aquisition of fungal symbionts

Ruiz-González *et al*. (2010) isolated various non-cultivar fungi from fungal traps. Sub-cultures of the fungal mutualist (F_1) and of four non-cultivar fungi (F_2-F_5) were provided by the authors (Table 4.3). The fungal strains were maintained on MS, potato dextrose (PDA), and Sabouraud agar (Table 4.2). Fresh agar plates were prepared by enriching 100 ml batches of autoclaved medium (50 ^{o}C) with 1 ml of filter-sterilised carbenicillin (5 mg (Fisher BPE2648-

Medium	Ingredients	Weight (g), volume (ml), percent (%)
Lennox broth (LB)	Tryptone	10.0 g
	Yeast extract	5.0 g
	NaCl	5.0 g
	Distilled water	1000 ml
Lennox broth (LB) agar	Tryptone	10.0 g
	Yeast extract	5.0 g
	NaCl	5.0 g
	Agar	15.0 g
	Distilled water	1000 ml
Lennox broth (LB) soft agar	Tryptone	10.0 g
	Yeast extract	5.0 g
	NaCl	5.0 g
	Agar	5.0 g
	Distilled water	1000 ml
Mannitol soy-flour (MS) agar	Organic soy-flour	20.0 g
	Mannitol	20.0 g
	Agar	20.0 g
	(+SB, 150mM sodium butyrate)	(16.5 g)
	Tap water	1000 ml
Mannitol soy-flour (MS) broth	Organic soy-flour	20.0 g
	Mannitol	20.0 g
	(+SB, incl. 150mM sodium butyrate)	(16.5 g)
	Tap water	1000 ml
Potato dextrose agar (PDA)	Glucose	20.0 g
	Instant mashed potato (smash)	5.0 g
	Agar	20.0 g
	Distilled water	1000 ml
Sabouraud agar (pH=5.6)	Dextrose	40.0 g
	Peptone	10.0 g
	Agar	20.0 g
	Distilled water	1000 ml
Tryptone soya broth (TSB)	Tryptone soya broth (Oxoid)	30.0 g
	Distilled water	1000 ml
Yeast extract-malt extract (YEME)	Yeast extract	3.0 g
	Peptone	5.0 g
	Malt extract	3.0 g
	Glucose	10.0 g
	Distilled water	1000 ml
TSB/YEME	TSB	50 %
	YEME	50 %

Table 4.2 Media for the cultivation of bacteria and fungi.

Sample type	Name	Sample origin	Host type	Host colony	Host tree	Ant species
Fungal cultivar	*Trimmatostroma cordae* F_1	Area 24, Area 9, Basievie, French Guiana	-	-	-	-
Non-cultivar fungus	F_2	Area 24, Area 9, Basievie, French Guiana	-	-	-	-
Non-cultivar fungus	F_3	Area 24, Area 9, Basievie, French Guiana	-	-	-	-
Non-cultivar fungus	F_4	Area 24, Area 9, Basievie, French Guiana	-	-	-	-
Non-cultivar fungus	F_5	Area 24, Area 9, Basievie, French Guiana	-	-	-	-
Bacterial isolate	FG_1	Area 24, French Guiana	Cultivar trap	54B	CN	AO
Bacterial isolate	FG_2	Area 24, French Guiana	Cultivar trap	51B,52B,55B,57B,58B,59B	HP	AD
Bacterial isolate	FG_3	Area 24, French Guiana	Cultivar trap	52B	HP	AD
Bacterial isolate	FG_4	Area 9, French Guiana	Cultivar trap	48B	HP	AD
Bacterial isolate	FG_5	Area 24, French Guiana	Cultivar trap	52B	HP	AD
Bacterial isolate	FG_6	Area 24, French Guiana	Cultivar trap	53B,54B,56B	CN	AO
Bacterial isolate	FG_7	Area 9, French Guiana	Cultivar trap	47B	HP	AD
Bacterial isolate	FG_8	La Montagne des Singes, French Guiana	Cultivar trap	34B	HP	AO
Bacterial isolate	FG_9	Area 24, French Guiana	Cultivar trap	53B	CN	AO
Bacterial isolate	FG_{10}	La Montagne des Singes, French Guiana	Cultivar trap	37B	HP	AO
Bacterial isolate	FG_{11}	Area 9, French Guiana	Cultivar trap	45B	HP	AD
Bacterial isolate	FG_{12}	La Montagne des Singes, French Guiana	Cultivar trap	17B	HP	AO
Bacterial isolate	FG_{13}	La Montagne des Singes, French Guiana	Cultivar trap	37B	HP	AO
Bacterial isolate	FG_{14}	La Montagne des Singes, French Guiana	Cultivar trap	34B	HP	AO
Bacterial isolate	FG_{15}	La Montagne des Singes, French Guiana	Cultivar trap	24B	HP	AO
Bacterial isolate	FG_{16}	La Montagne des Singes, French Guiana	Worker ants	28A,29A,19A,33A,36A	HP	AO
Bacterial isolate	FG_{17}	Area 24, French Guiana	Cultivar trap	54B	CN	AO
Bacterial isolate	FG_{18}	Area 9, French Guiana	Cultivar trap	40B	HP	AD
Bacterial isolate	FG_{19}	Basevie, French Guiana	Cultivar trap	2B	HP	AD
Bacterial isolate	FG_{20}	La Montagne des Singes, French Guiana	Cultivar trap	34B	HP	AO
Bacterial isolate	FG_{21}	Area 24, French Guiana	Cultivar trap	53B,54B,56B	CN	AO

Table 4.3 Origin of fungal and bacterial symbionts.

5) in 1 ml dH_2O) and 1 ml of filter-sterilised streptomycin (5 mg (Fisher BPE910-50) in 1 ml dH_2O) solutions. The medium was either equally sub-divided over four Petri dishes for short-term usage or added as slopes to cylindrical containers (Bijoux's) for long-term storage of strains. The fungal inocula were either grown at room temperature or at 30 ^oC.

4.3.4 Light microscopy

Stereomicroscope photographs of the bacterial isolates, a fungal trap, and a worker ant were taken in the Henry Wellcome Laboratory for Cell Imaging (www.uea.ac.uk/bio/biobmi) with a Zeiss SV11 M2 Bio Quad stereomicroscope and an AxioCam HRc CCD camera. Young and thus relatively flat bacterial colonies were used, and magnifications were adjusted to suit the size variation of bacterial species. Photographs of the bacterial colonies were taken at magnifications of 1.0, 1.6, 2.0, 2.5, 3.2, 4.0, 5.0, and 6.6X; photographs of

fungal and ant samples were taken at magnifications of 1.6, 3.2, and 6.6X. Scale bars of 1 mm were added to the pictures with AxioVision software (Carl Zeiss, Welwyn Garden City, UK).

4.3.5 Extraction of genomic DNA

Hyphal material of the fungal strains F_1, F_2, F_3, F_4, and F_5 (Table 4.3) was added to Eppendorf tubes, containing glass beads and 800 µl of ice-cold TGE buffer (Table 4.4). DNA extractions were carried out on overnight cultures of the bacterial isolates (FG_1-FG_{15}, FG_{17}-FG_{21}) (Table 4.3). Glass containers (universals), containing 10 ml of TSB/YEME (Table 4.2) and a spring, were inoculated with 50 µl from the glycerol stocks or cell material of single plate colonies. The universals were incubated overnight in a shaker incubator (200 rpm, 30 ^{o}C). The bacterial cells were harvested by centrifugation (4000 rpm, 5 minutes, 4 ^{o}C); the supernatants of the samples were discarded, whereas the cell pellets were re-suspended in 800 µl of TGE buffer and placed into Eppendorf tube containing a small layer of glass beads.

Solution	Ingredients	Concentration (mM)
Tris/Glycine/EDTA (TGE) buffer (pH=8.0)	Tris	50 mM
	EDTA	10 mM

Table 4.4 Solution for DNA extractions.

The fungal and bacterial samples were DNA extracted using a procedure, which is summarised below; for a detailed description of the method see Chapter 2.3.5. The fungal and bacterial samples were stored on ice for a minimum of 5 minutes before, during, and after bead-beating sessions (6x30 seconds, 6 m/s). Repeatedly, 0.5 ml of phenol:chloroform:isoamyl alcohol (25:24:1) was added, vortexed (1 minute), and centrifuged (13,200 rpm, 5 minutes). The purified supernatants were either washed one time with 0.5 ml chloroform:isoamyl alcohol (24:1) or precipitated with 1 ml of 100% ethanol, after which the dried DNA pellet was re-suspended in 50 µl of sterile dH_2O.

4.3.6 Amplification and purification of diagnostic genes

Diagnostic genes were amplified from the fungal (ITS region) and bacterial (16S rDNA) genomes by polymerase chain reaction (PCR), using sample-specific primers (ITS1-F-ITS4, 515-1492, Table 4.5) and PCR conditions (Table 4.6). Selected amplicons were recovered from the gel using a Qiagen Gel Extraction Kit and following the manual (www.qiagen.com).

Name	Sequence	Reference	Gene	Amplicon length
ITS1-F	5'-CTTGGTCATTTAGAGGAAGTAA-3'	Gardens and Bruns (1993)	18S rDNA	± 600 bp
ITS4	5'-TCCTCCGCTTATTGATATGC-3'	White *et al*. (1990)		
515	5'-GTGCCAGCMGCCGCGGTAA-3'	Turner *et al.* (1999)	16S rDNA	± 1000 bp
1492	5'-GGTTACCTTGTTACGACTT-3'	Turner et al. (1999)		

Table 4.5 Primer sets for the partial amplification of fungal (ITS region) and bacterial (16S SSU) rDNA genes.

A

PCR mixture	Volume
dH_2O	23.0 µl
GoTaq buffer (incl. loading buffer)	8.0 µl
GoTaq MgCl (25 mM)	2.4 µl
DMSO (100%)	2.0 µl
Bioline dNTP's (10 mM)	1.0 µl
Forward primer (25 µM)	1.0 µl
Reverse primer (25 µM)	1.0 µl
DNA	1.0 µl
GoTag polymerase	0.6 µl
Total	**40.0 µl**

B

Step	Fungi		Bacteria	
	Temperature	Minutes	Temperature	Minutes
1	94 ºC	4:00	94 ºC	12:00
2	95 ºC	0:30	94 ºC	1:00
3	53 ºC	1:00	45 ºC	0:45
4	72 ºC	1:00	72 ºC	1:30
5	Go 34x to step 2		Go 29x to step 2	
6	72 ºC	10:00	72 ºC	20:00
7	end		end	

Table 4.6 (A-B) PCR mixture and thermocycles for the partial amplification of fungal (ITS region) and bacterial (16S SSU) rDNA genes.

4.3.7 Sequencing and BLAST-matching

The cleaned amplicons were sequenced (Table 4.7) using the sample-specific forward primers (ITS1-F, 515). The sequenced PCR products were analysed by The Genome Analysis Centre TGAC (www.jicgenomelab.co.uk)

using ABI 3730XL sequencers (Life Technologies). The sequences were quality-checked and truncated with FinchTV (www.geospiza.com/Products/finchtv.shtml). Then the reads were matched to the BLAST database of the National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). A BLASTn search compared the sample reads to database sequences in the nucleotide collection (nr/nt), optimising for highly similar sequences (megablast).

A

Sequencing mixture	Volume
dH_2O	5.5 µl
5x sequencing buffer	1.5 µl
Forward primer (3.2 µM)	1.0 µl
DNA	1.0 µl
Enzyme E3.1	1.0 µl
Total	**10.0 µl**

B

Step	Temperature	Minutes
1	96 °C	1:00
2	96 °C	0:10
3	50 °C	0:05
4	60 °C	4:00
5	Go 25x to step 2	
6	60 °C	0:10
7	end	

Table 4.7 (A-B) Reaction mixture and thermocycles for the sequencing of PCR amplicons.

4.3.8 Colony bioassays

Selected bacterial isolates of antibiotic-producing genera were tested in colony bioassays against *Candida albicans* (CA_6, Table 3.2) and two non-cultivar fungi (i.e. F_3 and F_5, Table 4.3). No challenges could be carried out against other non-cultivar fungi (i.e. F_2 and F_4) nor, unfortunately, against the fungal mutualist (i.e. F_1), since the fungal colonies showed inherently patchy growth on agar plates. In total, the bacterial selection included nine of the twenty bacterial isolates FG_1-FG_8 and FG_{21}, which are hereby collectively referred to as the 'NNSPS strains', using the initial letters of the genera *Nocardia*, *Nocardiopsis*, *Streptomyces*, *Pseudonocardia*, and *Saccharopolyspora* (*Nocardiopsis* and *Pseudonocardia* have been isolated in Chapter 2 from *Acromyrmex* and *Saccharopolyspora* has been isolated in Chapter 5 from *Tetraponera*).

4.3.8.1 Bioassays against Candida

The selected bacteria were tested in colony bioassays against *Candida* CA_6. Universals with 10 ml of TSB/YEME and a spring were inoculated with 30 µl from the bacterial glycerol stocks and grown in a shaker incubator for a sufficient accumulation of biomass (280 rpm, 30 ^oC). The cell material was harvested by centrifugation (4000 rpm, 5 min), and the supernatants were disposed. The centre of MS agar plates, containing no antibiotics, was point-inoculated with standardised fractions from the cell pellets. As negative controls, one uninoculated plate and one inoculated with cells of *Streptomyces lividans* were included.

The experiment was carried out in duplicate; one set of agar plates contained 150 mM of sodium butyrate, which is shown to increase antifungal production in certain bacteria (Martin and McDaniel 1976, Moore *et al*. 2012), and one set did not. Inoculated plates were incubated for thirteen days at 21 ^oC. One day before the plates were due to be overlaid with *Candida*, the yeast was grown in 10 ml of Lennox broth (LB) (280 rpm, 37 ^oC). Then, 1 ml of the culture was added to 5 ml of LB soft agar (50 ^oC) (Table 4.2). The medium was poured over the bioassay plates, which were pre-warmed to 30 ^oC, contacting but not overlaying the bacterial colonies. The bioassay plates were incubated for 2 days at 26 ^oC for *Candida* to grow and become whitish in colour, after which the presence or absence of inhibition zones was scored.

4.3.8.2 Bioassays against non-cultivar fungi F_3 and F_5

In a second bioassay experiment by Ryan Seipke, the selected bacteria were challenged against the non-cultivar fungi F_3 and F_5, in the absence of sodium butyrate. Standardised fractions of cell material from bacterial plate colonies were point-inoculated into the centre of MS agar plates, containing no antibiotics. As a negative control, mycelia of *S. lividans* were inoculated onto an additional agar plate. The plates were incubated at 30 ^oC until mature colonies had grown. Then the hyphal material of the fungal strains was point-inoculated

at the edge of the bioassay plates. The plates were again incubated at 30 ^{o}C until the fungi clearly overgrew or clearly avoided the bacterial colonies.

4.3.9 Supernatant bioassays

Growth cultures of the selected NNSPS strains (FG_1-FG_8, FG_{21}) were challenged against *Candida* CA_6. This test was performed to see whether any of these strains secrete bioactive metabolites into a liquid medium, from which they could be recovered. No supernatant bioassays were carried out against the non-cultivar fungi F_3 and F_5 because none of the bacterial isolates had been shown to inhibit these strains in colony bioassays.

4.3.9.1 Obtaining supernatants

Growth cultures of the selected bacteria were obtained by inoculating 50 ml batches of MS broth (Table 4.2) with 30 µl from the bacterial glycerol stocks. As negative controls, one batch of medium was inoculated with spores of *S. lividans* and another batch remained uninoculated. One set of growth media contained 150 mM sodium butyrate, and another one did not. Due to the relatively low inoculation volume (and possibly a low spore/cell concentration of the glycerol stocks), the flasks had to be shaker incubated for a period of 14 days (30 ^{o}C, 250 rpm), before the accumulation of abundant cell material could be observed. The supernatants were harvested by pelleting-out and disposing much of the cell material and insoluble medium (30 minutes, 4000 rpm, 4 ^{o}C). For the bioassays, small sub-samples of the supernatants were centrifuged a second time with a benchtop centrifuge (5 minutes, 13,200 rpm), before being filter-sterilised with 0.2 µm filters.

4.3.9.2 Bioassays against Candida

The bacterial supernatants of strains, grown in the presence or absence of sodium butyrate, were challenged against *Candida* CA_6. 100 µl of the sterilised supernatants were inoculated onto paper filter disks, with a diameter of 0.9 cm. As negative controls, two filter disks were inoculated with the control liquids. LB

agar plates were coated with 400 µl of one-day-old *Candida* growth cultures, which were grown in universals containing 10 ml of Lennox broth (Table 4.2). When dry, the filters were attached to the agar with 60 µl of dH_2O. The bioassay plates were incubated for two days at 26 ^oC; then the presence or absence of inhibition zones was scored.

4.4 Results

4.4.1 Isolation and microscopy of cultivar-associated bacteria

In French Guiana, myrmecophytes *Hirtella physophora* and *Cordia nodosa* were sampled for *Allomerus decemarticulatus* and *A. octoarticulatus* worker ants and their fungal cultivars. These samples originated from 30 *Hirtella*-hosted *A. decemarticulatus*, 9 *Cordia*-hosted *A. octoarticulatus* and 20 *Hirtella*-hosted *A. octoarticulatus* ant colonies (Table 4.1). All *Hirtella* samples included an inhabited and double-chambered domatium, the associated leaf, and a part of the leaf petiole with some fungal trap; in contrast, all *Cordia* samples contained a single-chambered domatium, associated leaves, and the leaf petiole with some fungal trap. The samples were bagged and processed in the field station at Barrage de Petit Saut. The worker ants were removed from the leaves, domatia, and fungal trap, and were preserved in aliquots of sterile PBS or 2G. Thereafter, the fungal cultivars were removed from the plant material and preserved in separate aliquots of PBS and 2G. In a culture-dependent way, bacteria were isolated from the fungal cultivars in a category 2 microbiological laboratory at the University of East Anglia, United Kingdom. For the isolation, diluted PBS and 2G holding liquids were inoculated onto agar plates and unique morphotypes of Gram-positive and Gram-negative bacteria were purified.

In total, 20 unique bacterial morphotypes were isolated from the fungal traps. Each strain received an ID, consisting of the prefix ‘FG’ and an individual number. Stereomicroscope pictures of the bacterial isolates (FG_1-FG_{15}, FG_{17}-FG_{21}) are presented in figure 4.6 and their origin is shown in table 4.3.

4.4.2 Acquisition of fungal symbionts

Diverse species of non-cultivar fungi were isolated from the *Trimmatostroma cordae* 'trap' fungal cultivar. Sub-samples of the fungal cultivar (F_1) and four non-cultivar fungi (F_2-F_5) were provided by Ruiz-González *et al.* (2010) (Fig. 4.7; Table 4.3). These strains originated from the same myrmecophytes as the fungal cultivars, which are used for the bacterial isolations.

4.4.3 Amplification, purification, sequencing, and BLAST-matching *of diagnostic genes from genomic DNA extracts*

The morphologically identified fungal cultivar and four non-cultivar fungi (Table 4.3) were identified genetically by partially sequencing the ITS region of the rDNA gene. The sequences of the fungal cultivar F_1 (915 bp), and non-cultivars F_2 (540 bp), F_3 (494 bp), F_4 (519 bp), and F_5 (693 bp), show BLAST analogies to rDNA sequences of *Trimmatostroma*, *Penicillium*, *Beauveria*, *Cladosporium*, and *Annulohypoxylon/Xylariaceae/Hypoxylon*, respectively (Table 4.8). An overview of the sequencing data is given in the Supplementary Information A_1.

Furthermore, a segment of the bacterial 16S rDNA gene was sequenced to genetically identify the microbial isolates (Table 4.3). The sequences of the bacterial strains FG_1-FG_{15}, FG_{17}-FG_{21} (633-919 bp; mean 883 bp) show BLAST analogies to the 16S rDNA of diverse bacterial phyla (Table 4.8). Also, diverse actinobacteria in the genus *Streptomyces* could be isolated from the traps, which is interesting because baiting experiments (Yu and Pierce 1998) have suggested that *Allomerus* exclusively completes its life cycle within their host trees, and hence might not contact actinobacteria-rich soils. A summary of the sequencing data is presented in the Supplementary Information A_1.

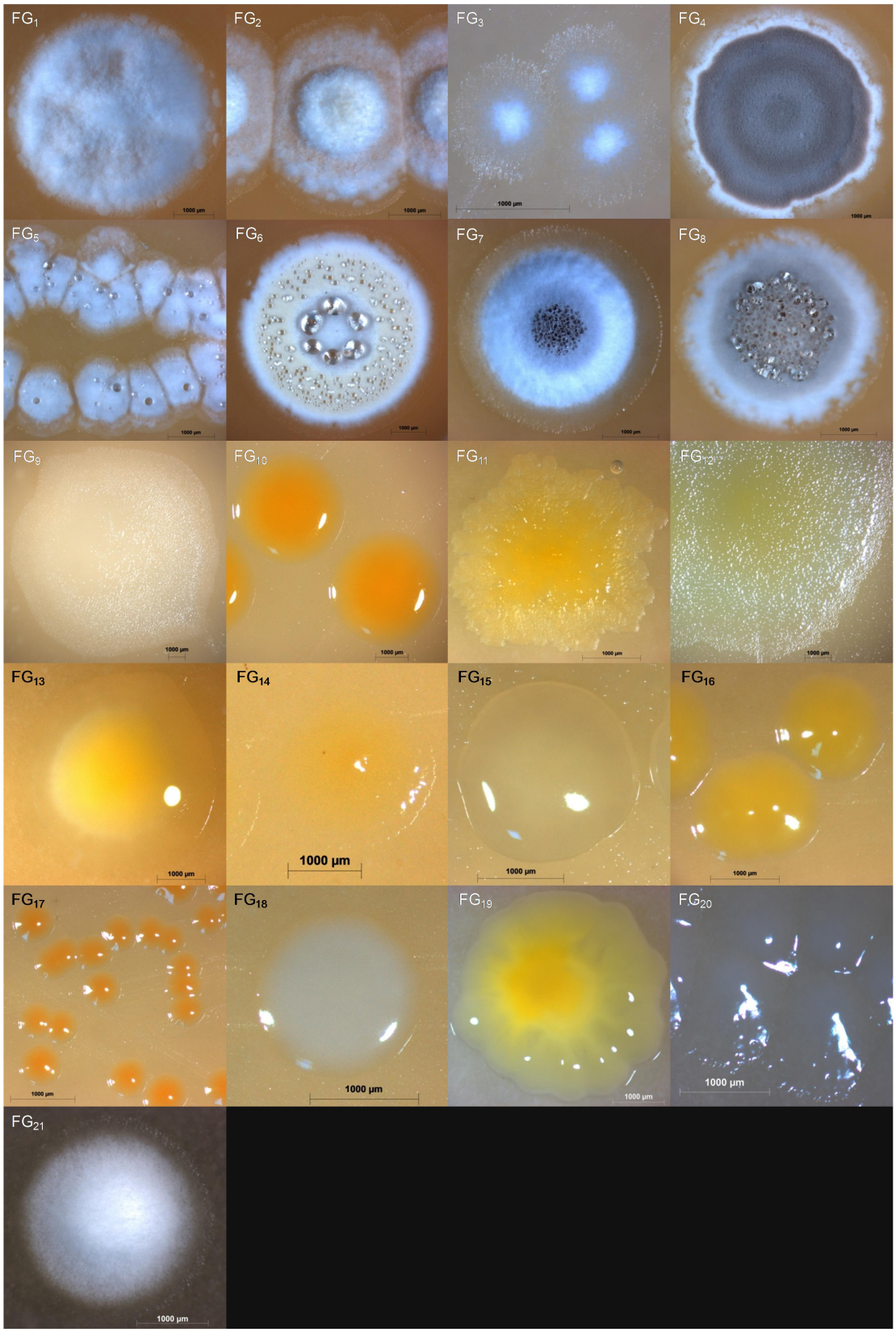


Figure 4.6 Microscopy photographs of 20 bacterial isolates (FG_1-FG_{15}, FG_{17}-

FG_{21}), originating from the fungal cultivar of *Allomerus* spp. In addition to bacteria in the phylum Actinobacteria, bacteria of other phyla were isolated, including Gram-positives and Gram-negatives of the phyla *Firmicutes*, *Proteobacteria*, and *Bacterioidetes*. An overview of the bacterial origin and details on their genetic identity are provided in the tables 4.3 and 4.8, respectively. A selection of nine bacterial isolates (FG_1-FG_8, FG_{21}), belonging to antibiotic-producing genera, are referred to as 'NNSPS strains'. Strains FG_1, FG_2, FG_4-FG_7 and FG_{21} belong to the genus *Streptomyces*, strain FG_3 to the genus *Nocardia*, and strain FG_8 BLASTS to the genera *Streptomyces* and *Nocardia*, despite morphological analogies to bacteria in the genus *Streptomyces*.

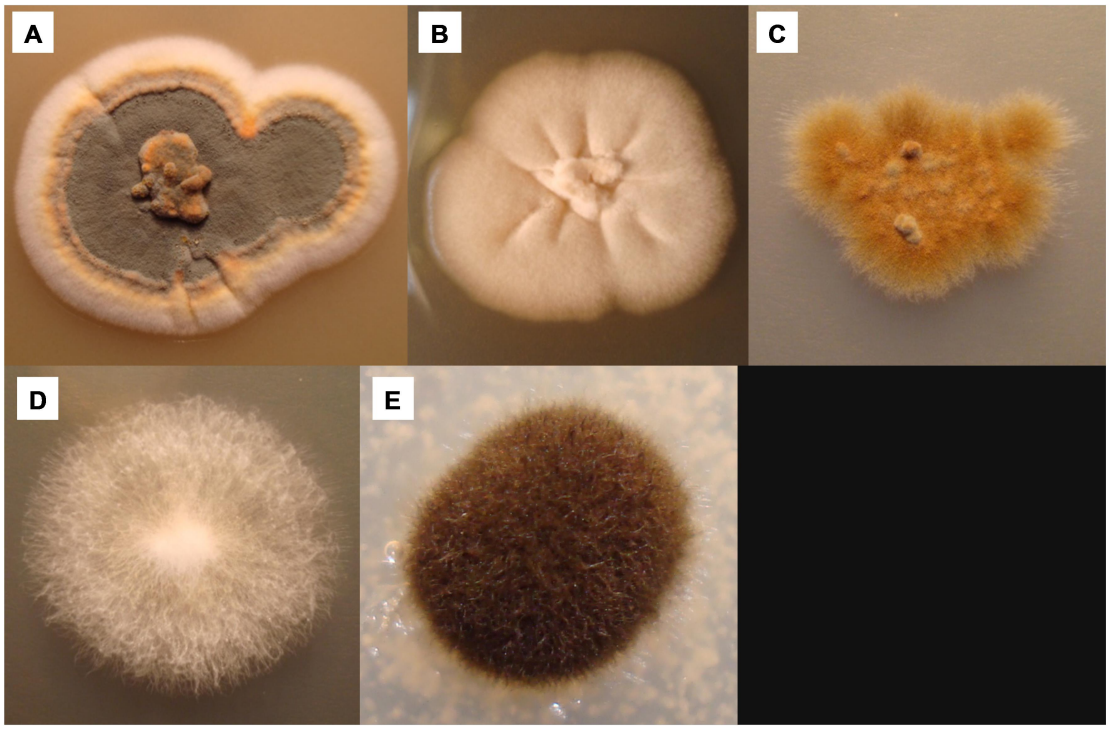


Figure 4.7 Fungal symbionts of *Allomerus* spp., provided by Jérôme Orivel and Mario Ruiz-González from the University of Toulouse. **(A-D)** Non-cultivar fungi that were isolated from the fungal cultivar of *Allomerus* spp. In sequence, they belong to the genera *Penicillium* (F_2), *Beauveria* (F_3), *Cladosporium* (F_4), and *Annulohypoxylon/Xylariaceae/Hypoxylon* (F_5). **(E)** Fungal cultivar *Trimmatostroma* (F_1). While strains B and D spread over agar plates by growing hyphae, strains A, C, and E do not spread as readily. Tables 4.3 and 4.8 provide a detailed overview on the origin and genetic identity of the strains, respectively.

Sample type	Name	PCR primers	Sequencing primer	Sequence length	Genus	Closest relative (accession)	Max score	Total score	Query coverage	E value	Max ident
Fungal cultivar	*Trimmatostroma cordae* F_1	ITS1-F – ITS4R	ITS1-F	915 bp	*Trimmatostroma*	*Trimmatostroma cordae* 5.8S rRNA gene and internal transcribed spacers 1 and 2 (ITS1, ITS2), strain CBS 418.93 (AJ244263.1)	440	545	42%	7.00E-120	93%
Non-cultivar fungus	F_2	ITS1-F – ITS4R	ITS1-F	540 bp	*Penicillium*	*Penicillium multicolor* isolate M2 internal transcribed spacer 1, partial sequence (HM595496.1)	990	990	100%	0	99%
Non-cultivar fungus	F_3	ITS1-F – ITS4R	ITS1-F	494 bp	*Beauveria*	*Beauveria* sp. LA264c 18S ribosomal RNA gene, partial sequence (HQ022490.1)	798	798	94%	0	97%
Non-cultivar fungus	F_4	ITS1-F – ITS4R	ITS1-F	519 bp	*Cladosporium*	*Cladosporium* sp. F42 18S ribosomal RNA gene, partial sequence (FJ755822.1)	953	953	100%	0	99%
Non-cultivar fungus	F_5	ITS1-F – ITS4R	ITS1-F	693 bp	*Annulohypoxylon*	*Annulohypoxylon stygium* isolate 2713 18S ribosomal RNA gene, partial sequence (EU272517.1)	1280	1280	100%	0	100%
					Xylariaceae	*Xylariaceae* sp. Vega369 internal transcribed spacer 1 (EF694657.1)	1280	1280	100%	0	100%
					Hypoxylon	*Hypoxylon stygium* 18S rRNA gene, partial sequence (AJ390409.1)	1280	1280	100%	0	100%
Bacterial isolate	FG_1	515F – 1492R	515F	908 bp	*Streptomyces*	Uncultured *Streptomyces* sp. clone OUT1 16S ribosomal RNA gene, partial sequence (EU372800.1)	1677	1677	100%	0	100%
Bacterial isolate	FG_2	515F – 1492R	515F	908 bp	*Streptomyces*	Uncultured *Streptomyces* sp. clone OUT1 16S ribosomal RNA gene, partial sequence (EU372800.1)	1677	1677	100%	0	100%
Bacterial isolate	FG_3	515F – 1492R	515F	900 bp	*Nocardia*	*Nocardia* sp. 30905 16S ribosomal RNA gene, partial sequence (FJ262980.1)	1607	1607	100%	0	98%
Bacterial isolate	FG_4	515F – 1492R	515F	908 bp	*Streptomyces*	*Streptomyces seoulensis* gene for 16S rRNA, partial sequence, strain: NBRC 16668 (AB249970.1)	1657	1657	99%	0	99%
Bacterial isolate	FG_5	515F – 1492R	515F	888 bp	*Streptomyces*	*Streptomyces setonensis* strain HBUM174896 16S ribosomal RNA gene, partial sequence (EU841611.1)	1570	1570	99%	0	98%
Bacterial isolate	FG_6	515F – 1492R	515F	905 bp	*Streptomyces*	*Streptomyces* sp. VAN21 16S ribosomal RNA gene, partial sequence (HM018120.1)	1672	1672	100%	0	100%
Bacterial isolate	FG_7	515F – 1492R	515F	905 bp	*Streptomyces*	*Streptomyces* sp. HB096 16S ribosomal RNA gene, partial sequence (GU213492.1)	1661	1661	100%	0	99%
Bacterial isolate	FG_8	515F – 1492R	515F	914 bp	*Streptomyces*	*Streptomyces* sp. HB328 16S ribosomal RNA gene, partial sequence (GQ863929.1)	1683	1683	100%	0	99%
					Nocardia	*Nocardia* sp. JJE-2 16S ribosomal RNA gene, partial sequence (GU132484.1)	1683	1683	100%	0	99%
Bacterial isolate	FG_9	515F – 1492R	515F	895 bp	*Bacillus*	*Bacillus* sp. bk_40 16S ribosomal RNA gene, partial sequence (HQ538665.1)	1653	1653	100%	0	100%
					Brevibacillus	*Brevibacillus brevis* strain H2 16S ribosomal RNA gene, partial sequence (HM449127.1)	1653	1653	100%	0	100%
Bacterial isolate	FG_{10}	515F – 1492R	515F	896 bp	*Chryseobacterium*	*Chryseobacterium taiwanense* gene for 16S rRNA, partial sequence, strain: LB-B (AB495176.1)	1639	1639	100%	0	99%
Bacterial isolate	FG_{11}	515F – 1492R	515F	899 bp	*Tsukamurella*	*Tsukamurella* sp. 5-1-47 16S ribosomal RNA gene, partial sequence (GU929204.1)	1655	1655	100%	0	99%
Bacterial isolate	FG_{12}	515F – 1492R	515F	633 bp	*Pseudomonas*	*Pseudomonas* sp. E11(2010) 16S ribosomal RNA gene, partial sequence (HQ641264.1)	1170	1170	100%	0	100%
Bacterial isolate	FG_{13}	515F – 1492R	515F	918 bp	*Pantoea*	*Pantoea agglomerans* partial 16S rRNA gene, strain WAB1925 (AM184264.1)	1652	1652	100%	0	99%
Bacterial isolate	FG_{14}	515F – 1492R	515F	859 bp	*Stenotrophomonas*	*Stenotrophomonas* sp. enrichment culture clone ECC3-1 16S ribosomal RNA gene, partial sequence (GU056297.1)	1581	1581	100%	0	99%
Bacterial isolate	FG_{15}	515F – 1492R	515F	814 bp	*Stenotrophomonas*	*Stenotrophomonas maltophilia* strain NK-ST 16S ribosomal RNA gene, partial sequence (EU056836.1)	1498	1498	100%	0	99%
Bacterial isolate	FG_{16}	515F – 1492R	515F	879 bp	*Pseudomonas*	*Pseudomonas* sp. IPC3 16S ribosomal RNA gene, partial sequence (HQ108171.1)	1624	1624	100%	0	100%
Bacterial isolate	FG_{17}	515F – 1492R	515F	894 bp	*Microbacterium*	*Microbacterium* sp. HY16(2010) 16S ribosomal RNA gene, partial sequence (HM579807.1)	1652	1652	100%	0	100%
					Cellulosimicrobium	*Cellulosimicrobium cellulans* strain ZFJ-17 16S ribosomal RNA gene, partial sequence (EU931556.1)	1652	1652	100%	0	100%
Bacterial isolate	FG_{18}	515F – 1492R	515F	897 bp	*Staphylococcus*	*Staphylococcus* sp. R9-9A 16S ribosomal RNA gene, partial sequence (HQ154574.1)	1657	1657	100%	0	100%
Bacterial isolate	FG_{19}	515F – 1492R	515F	919 bp	*Pseudomonas*	*Pseudomonas* sp. HMPB1 16S rRNA gene (AM745260.1)	1653	1653	100%	0	99%
Bacterial isolate	FG_{20}	515F – 1492R	515F	919 bp	*Pseudomonas*	Uncultured *Pseudomonas* sp. clone Filt.155 16S ribosomal RNA gene, partial sequence (HM152742.1)	1698	1698	100%	0	100%
Bacterial isolate	FG_{21}	515F – 1492R	515F	884 bp	*Streptomyces*	Uncultured *Streptomyces* sp. clone OUT1 16S ribosomal RNA gene, partial sequence (EU372800.1)	1633	1633	100%	0	100%

Table 4.8 Genetic identity of fungal and bacterial symbionts. Details on their origin and an overview of the sequencing data are presented in table 4.3 and the Supplementary Information A_1, respectively.

4.4.4 Colony bioassays

In colony bioassays, selected bacterial isolates ('NNSPS' strains) of well-known antifungal-producing genera, including *Streptomyces* (FG_1, FG_2, FG_4, FG_5, FG_6, FG_7, FG_{21}), *Nocardia* (FG_3) and *Streptomyces/Nocardia* (FG_8), were challenged against *Candida* CA_6 and the non-cultivar fungi *Beauveria* (F_3) and *Annulohypoxylon*/*Xylariaceae*/*Hypoxylon* (F_5). Due to their patchy growth on agar plates, neither the fungal cultivar *Trimmatostroma* (F_1) nor the non-cultivar fungi *Penicillium* (F_2) and *Cladosporium* (F_4) were used for the assays.

4.4.4.1 Bioassays against Candida

The NNSPS strains were tested against *C. albicans* on agar plates, some containing 150 mM of the chemical inducer sodium butyrate, and were overlaid with soft agar containing the yeast. In the absence of sodium butyrate, none of the bacteria isolates produced any bioactive metabolites that inhibit the growth of *Candida* (Fig. 4.8_A). However, the additive did stimulate *Streptomyces* FG_1 and FG_{21} to inhibit the yeast (Fig. 4.8_B), but only weakly as compared to the previously tested *Acromyrmex* ant-symbionts *Streptomyces* E_8 and E_9 (Fig. 2.5_B).

4.4.4.2 Bioassays against non-cultivar fungi F_3 and F_5

The NNSPS isolates were also challenged against the non-cultivar fungi *Beauveria* (F_3) and *Annulohypoxylon*/*Xylariaceae*/*Hypoxylon* (F_5) in the absence of sodium butyrate. The fungi were inoculated at the edge of bioassay plates containing the bacterial colonies. All bacterial colonies were overgrown by the fungal opponents, indicating the absence of effective antifungals (Fig. 4.9_{A-B}).

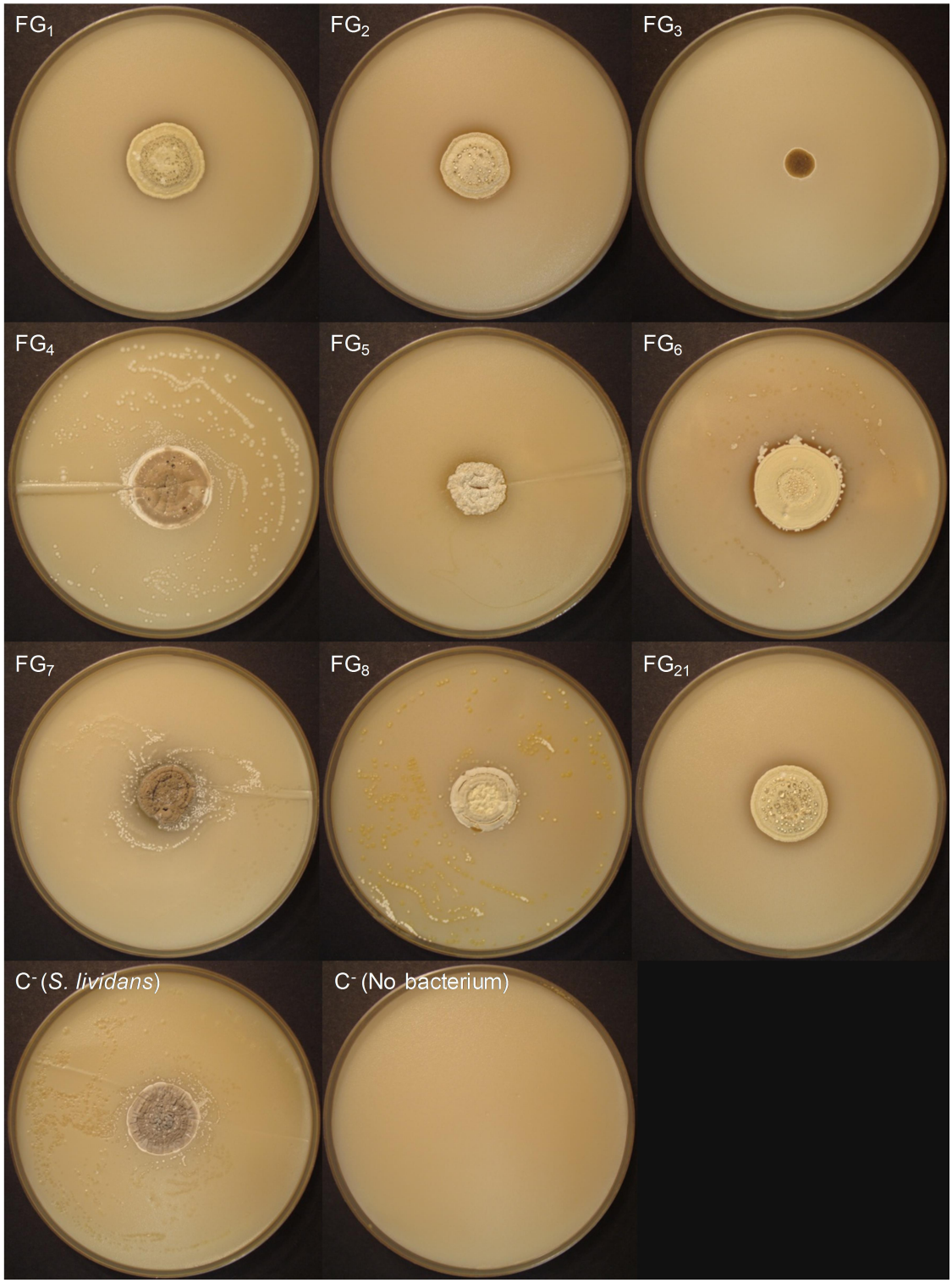


Figure 4.8 (A) *Candida* (CA_6) colony bioassays of NNSPS isolates (FG_1-FG_8, FG_{21}). As negative controls, an uninoculated agar plate (C^- No bacterium) and one with a *S. lividans* colony (C^- *S. lividans*) were included. None of the *Streptomyces* and *Nocardia* strains is inhibiting *Candida*.

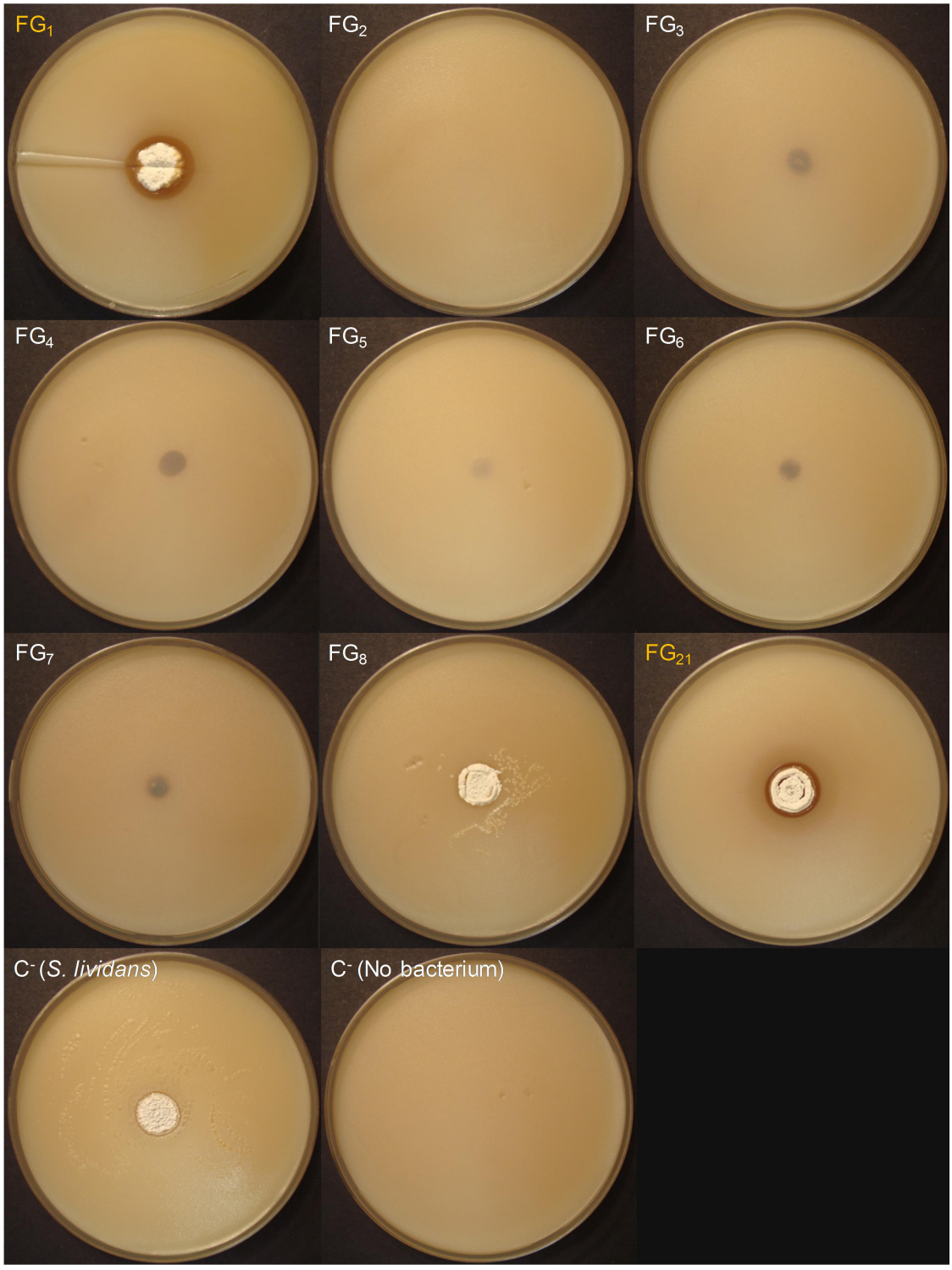


Figure 4.8 (B) *Candida* (CA_6) colony bioassays of NNSPS isolates (FG_1-FG_8, FG_{21}). As negative controls, an uninoculated agar plate (C^- No bacterium) and one with a *S. lividans* colony (C^- *S. lividans*) were included. In the presence of 150 mM sodium butyrate, added to the medium, *Streptomyces* FG_1 and FG_{21} (orange font) are secreting effective antifungals into the surrounding medium resulting in small inhibition zones with no or reduced fungal growth.

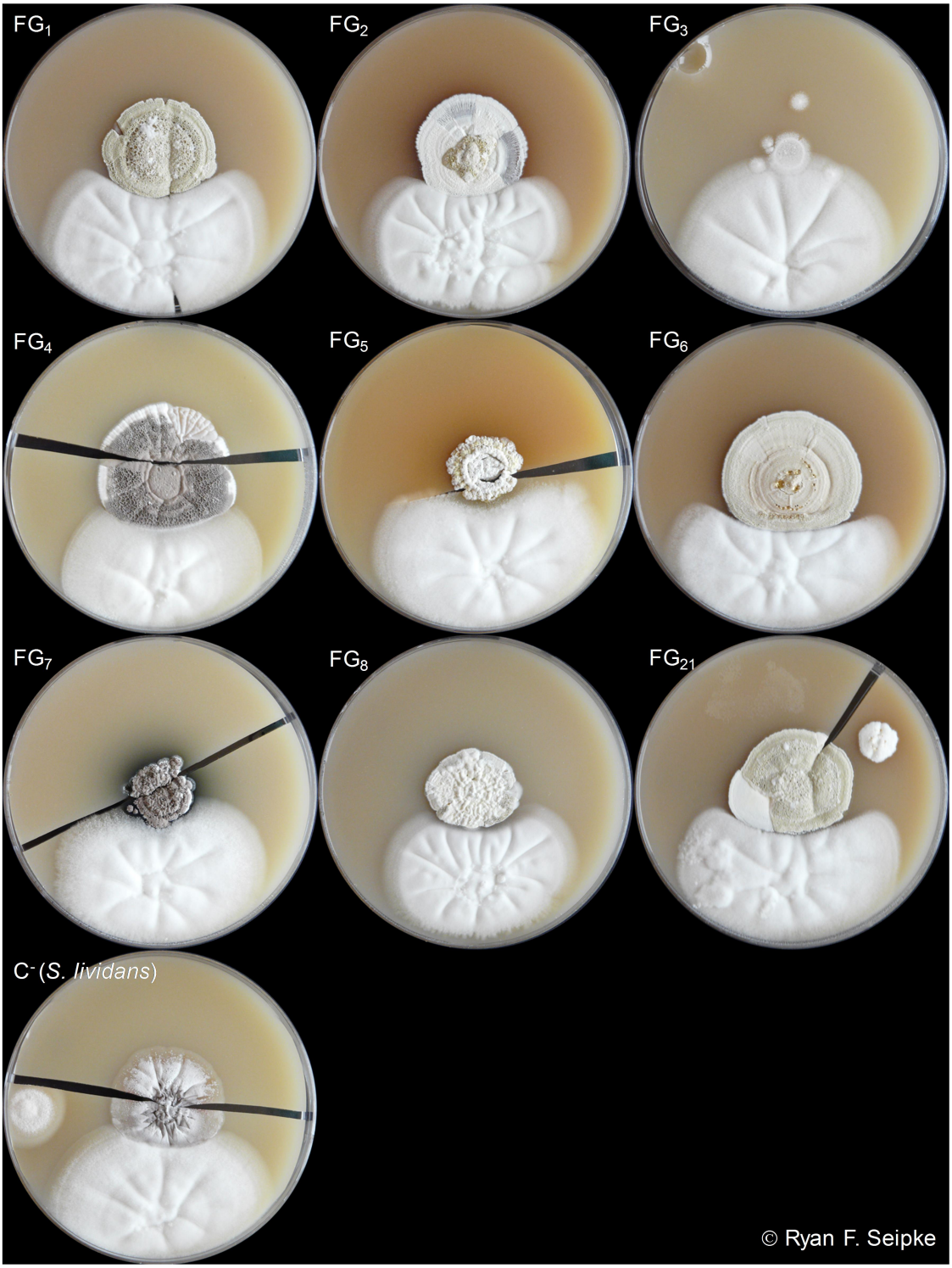


Figure 4.9 (A) *Beauveria* (F_3) colony bioassays of NNSPS isolates (FG_1-FG_8, FG_{21}). An agar plate with a *S. lividans* colony (C^- *S. lividans*) was included as a negative control. None of the bacterial isolates is inhibiting the growth of the test fungus on agar plates not containing any sodium butyrate. Ryan Seipke carried out the experiment and provided photographs of the bioassay plates.

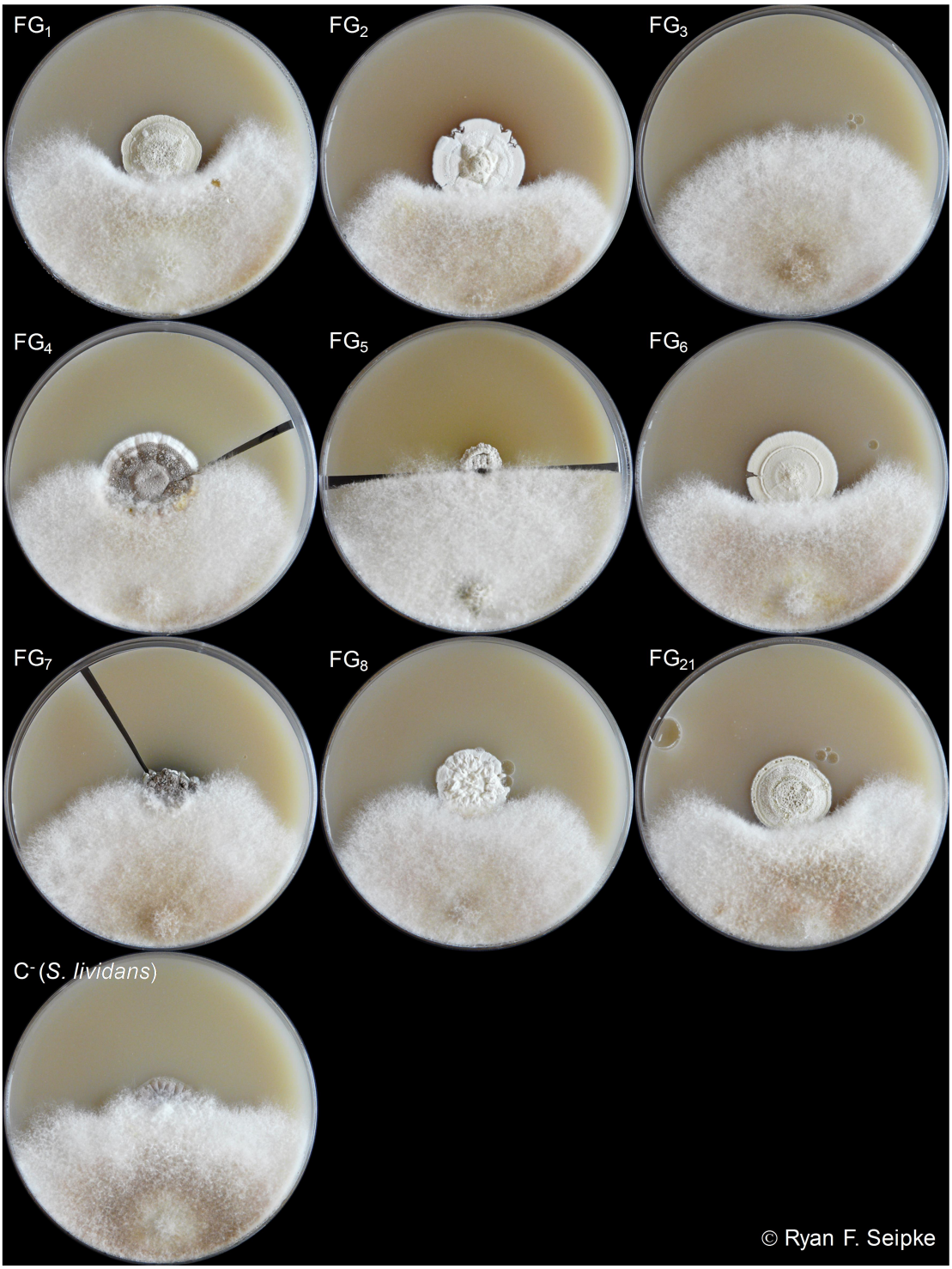


Figure 4.9 (B) *Annulohypoxylon/Xylariaceae/Hypoxylon* (F_5) colony bioassays of NNSPS isolates (FG_1-FG_8, FG_{21}). An agar plate with a *S. lividans* colony (C^- *S. lividans*) was included as a negative control. The test fungus is resistant to the bacterial secretions when grown on agar plates not containing any sodium butyrate. Ryan Seipke carried out the experiment and provided photographs of the bioassay plates.

4.4.5 Supernatant bioassays

The NNSPS isolates (FG_1-FG_8, FG_{21}) were tested for secretion of antifungals into a liquid medium via bioassays against *Candida* CA_6. Filamentous non-cultivar fungi were not tested because *Beauveria* (F_3) and *Annulohypoxylon*/*Xylariaceae*/*Hypoxylon* (F_5) were resistant to the bacterial isolates on agar plates (Fig. 4.9_{A-B}).

4.4.5.1 Bioassays against Candida

The bacterial supernatants, enriched or not enriched with sodium butyrate, were sterilised, added to paper filter disks, and attached to *Candida*-coated agar plates. None of the supernatants, lacking the additive, shows bioactivity against *Candida* (Fig. 4.10_{A-B}). This means that the bacteria not inhibiting fungal growth on agar plates, in the absence of the inducer sodium butyrate (Fig. 4.8_A), also did not secrete any effective antifungals into the liquid medium. Furthermore, *Streptomyces* FG_1 and FG_{21}, which have exhibited fungal inhibition on sodium butyrate-containing agar plates (Fig. 4.8_B), also did not secrete any effective antifungals into the liquid medium, despite the medium included sodium butyrate (Fig. 4.10_{C-D}).

4.5 Discussion

The results presented in this chapter show that diverse bacterial genera can be found in association to the fungal cultivar of *Allomerus decemarticulatus* and *A. octoarticulatus*. In particular, 20 unique bacterial morphotypes were isolated from the fungal traps of the ant species. Nine of the isolates, including the *Streptomyces* and *Nocardia* species, were tested in colony bioassays against the medically important yeast *Candida* CA_6 and two non-cultivar fungi, including *Beauveria* (F_3) and *Annulohypoxylon*/*Xylariaceae*/*Hypoxylon* (F_5). These filamentous fungi were isolated from the same myrmecophytes as some of the bacteria. Despite their taxonomic identities, none of the bacterial strains inhibited any of the test fungi. The same bacterial isolates were also challenged against *Candida* in the presence of the chemical inducer sodium butyrate, which

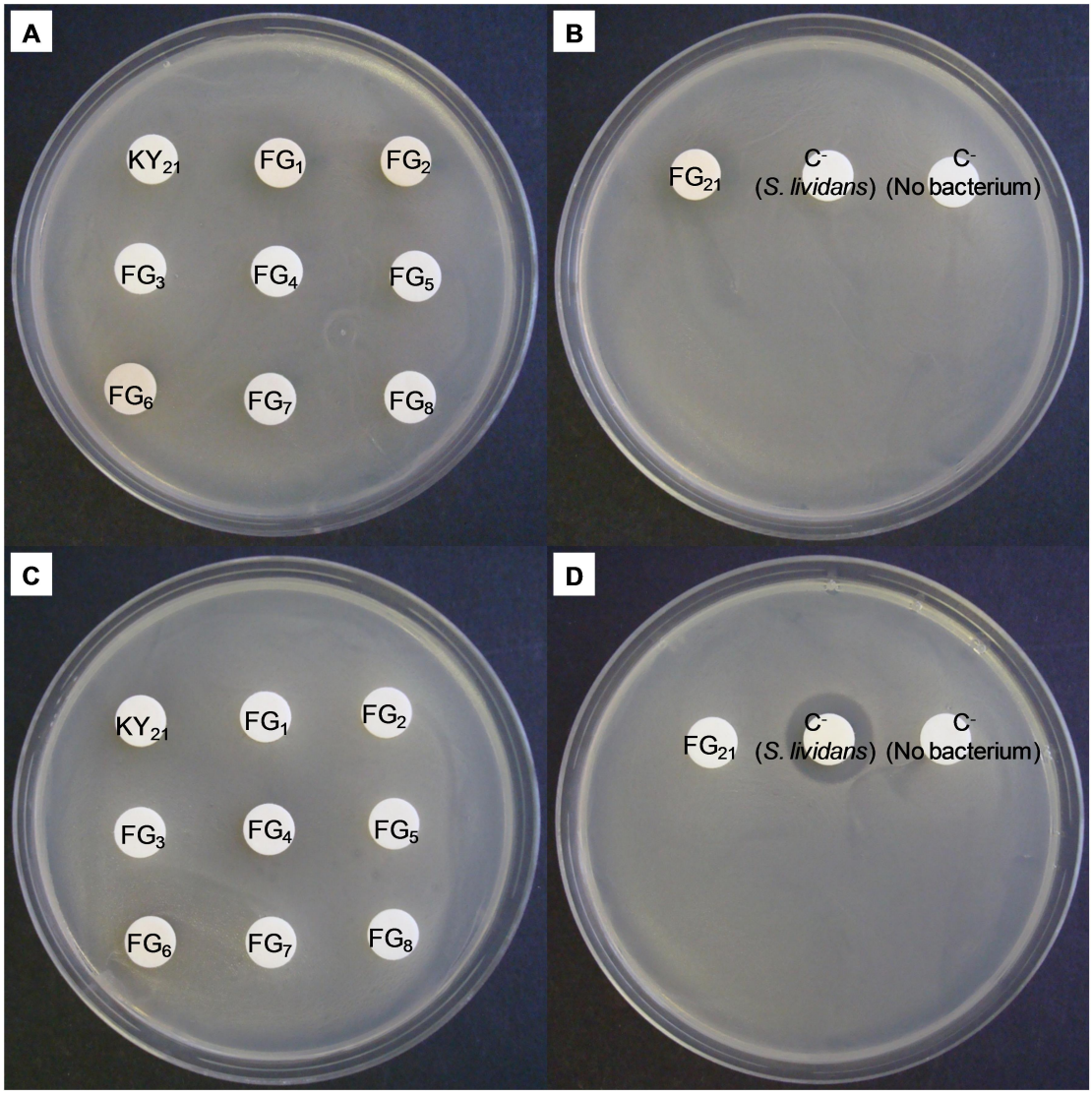


Figure 4.10 *Candida* (CA_6) supernatant bioassays of NNSPS (FG_1-FG_8, FG_{21}) growth cultures. As negative controls, a growth culture of *S. lividans* (C^- *S. lividans*) and an uninoculated batch of medium (C^- No bacterium) were included. The experiment was carried out in duplicate; one set of liquid growth cultures contained 150 mM of sodium butyrate. The supernatants were inoculated onto paper filter disks and attached to *Candida*-coated agar plates. **(A-B)** In the absence of sodium butyrate, none of the bacterial supernatants is inhibiting *Candida*. **(C-D)** In the presence of the inducer, also none of the bacterial supernatants is inhibiting the fungal growth. Recall that on agar plates, containing sodium butyrate, *Streptomyces* FG_1 and FG_{21} showed their ability to produce antifungals (Fig. 4.8_B).

is known to stimulate antibiotic production in some actinobacteria. In the presence of sodium butyrate, two of the tested *Streptomyces* isolates inhibited the yeast. This result confirms the utility of sodium butyrate as chemical inducer and also shows that under some conditions, some cultivar-associated bacteria are able to produce antifungals. Since the fungal cultivar or *Allomerus* ants itself might be able to manipulate the chemical environment of the traps in order to stimulate antifungal production in bacteria, bacterial associates like *Streptomyces* FG_1 and FG_{21} might be antibiotic-producing symbionts that, in combination with worker ant-associated bacteria (Seipke *et al.* 2012), help to defend the fungal traps against competitor and pathogenic fungi. However, the overall conclusion is that these results support the interpretation that the main source of bacterial antifungals, if bacterial antifungals play a crucial role in the defence of the *Allomerus*-associated cultivar, is more likely to be from a microbiome on *Allomerus* itself (Seipke *et al*. 2012).

Chapter 5) *Tetraponera penzigi* ant- and cultivar-associated bacteria

5.1 Introduction

5.1.1 Mutualistic partners

The overstorey vegetation (most upper canopy layer) of the Kenyan Laikipia district is dominated by the swollen-thorn acacia *Acacia drepanolobium* (*Fabales*, *Fabaceae*) that in large parts of the area forms a monoculture (Young *et al.* 1997). *A. drepanolobium* defends itself against herbivory by hosting protective ant colonies. The ant species *Crematogaster sjostedti*, *C. mimosae*, *C. nigriceps*, and *Tetraponera penzigi* (*Hymenoptera*, *Formicidae*) are particularly common (Young *et al.* 1997, Stanton *et al.* 2002). The latter three ant species only inhabit *A. drepanolobium*, while *C. sjostedti* also inhabits the co-occurring *Acacia seyal* (Young *et al.* 1997).

A. drepanolobium provides its resident ant colonies with nesting sites (domatia) and nutrition. 5-40% of the thorns exhibit swollen and hollow bases (Stanton *et al.* 2002) that are used by three of the ant species for breeding purposes and contain queen(s), workers, alates, and brood (pupae, larvae, eggs). In contrast, *C. sjostedti* primarily uses cavities in the stem of *A. drepanolobium*, which are created by cerambycid beetles (Palmer *et al.* 2008). In addition to nesting space, the ant-acacia also feeds its ants directly with carbohydrate-rich nectar of extrafloral nectaries, or, in the case of *C. sjostedti* and *C. mimosae*, also indirectly via honeydew-producing scale insects (Young *et al.* 1997). In return, the ants defend their hosts against herbivores. The ant investment in this protection service varies across species; *C. mimosae* and *C. nigriceps* are highly aggressive against herbivores, relative to *T. penzigi* and *C. sjostedti*, showing higher numbers of recruiting workers in response to simulated disturbance (Palmer and Brody 2007, Palmer *et al*. 2010).

The ant species appear to dominate different life-stages of *A. drepanolobium.* Saplings with a height up to 0.5 m are primarily associated with single *T. penzigi*, *C. mimosae*, or *C. nigriceps* colonies*;* trees taller than 1.0 m

are increasingly occupied by *C. mimosae* and *C. sjostedti* (Young *et al.* 1997). This niche diversification is likely driven by a 'competition-colonisation trade-off' (Stanton *et al*. 2002). According to the authors, *C. sjostedti* and *C. mimosae* invest in colony size, allowing their colonies to displace other ant colonies from neighbouring trees; while the gynes of *C. sjostedti* mainly remain in their parent trees, the gynes of *C. mimosae* also inhabit neighbouring saplings. In contrast, *T. penzigi* and *C. nigriceps* colonies are competitively subordinate to colonies of the above species; however, they invest in dispersal and produce many gynes that are able to disperse to and win saplings and older trees that, for example due to low-intensity fires, have lost their ant occupants.

Naomi E. Pierce and Dino Martins, from Harvard University, observed that domatia occupied by *T. penzigi* (Fig. 5.1) often contained fungal hyphae, and unoccupied and closed domatia had no observable hyphal growths (N. E. Pierce and D. Martins, unpublished data), suggesting the existence of ant fungiculture. In addition, the fungi of different ant nests consistently shared the same morphology, all belonging to the ascomycete genus *Chaetomonium* (*Sordariales, Chaetomiaceae*), suggesting that the fungal growth is a monoculture.

Some support for the hypothesis that *T. penzigi* cultivates a fungal monoculture comes from another African plant-ant that has been shown to cultivate ascomycetous fungi in domatia. One example is found in the myrmecophyte *Leonardoxa africana africana*, which can be found in the coastal rainforests of Cameroon and surrounding countries, and its ant symbiont *Petalomyrmex phylax*. The ants cultivate a fungus in the ascomycete order *Chaetothyriales* (e.g. EU856528) (Defossez *et al.* 2009, Blatrix *et al*. 2009). 95% of 961 domatia that were occupied by the ant mutualist also contained the fungus; in contrast, none of 108 domatia that were occupied by the parasitic ant *Cataulacus mckeyi*, which uses the trees' resources but fails to protect the tree against herbivory, showed any fungal growth. The authors also reported *P. phylax* worker behaviours that appear to actively maintain the fungus; hyphae were chewed, transported, and 'supplied' with detritus and faeces.

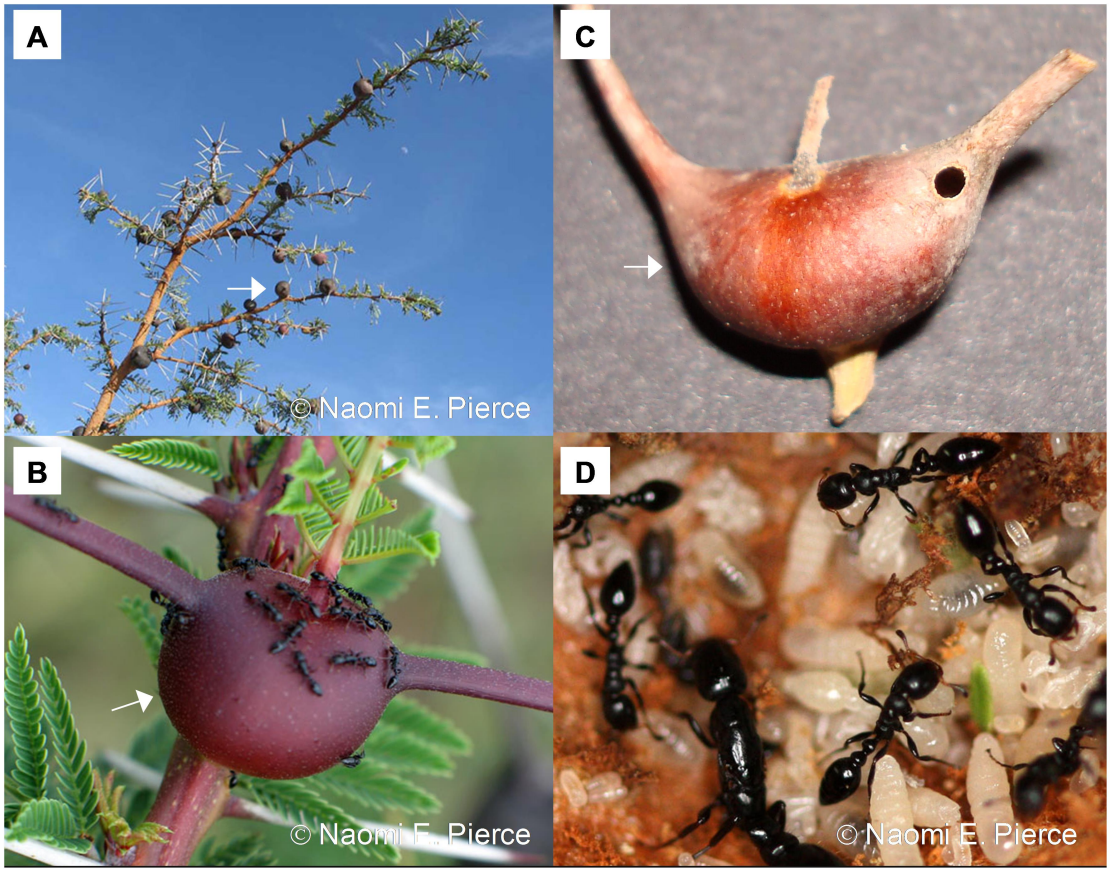


Figure 5.1 Host tree *Acacia drepanolobium* and ant symbiont *Tetraponera penzigi*. **(A)** The myrmecophyte *A. drepanolobium* defends itself from herbivory by producing thorns and housing symbiotic ant colonies. Trees of differing size ranges are occupied by four species of ants, including *Crematogaster sjostedti*, *C. mimosae*, *C. nigriceps*, and *Tetraponera penzigi.* **(B-C)** Ant symbiont *T. penzigi* is provided by the tree with housing in the form of single-chambered domatia (white arrows) and nectar from extrafloral nectaries. **(D)** The domatia of *T. penzigi* that are used for breeding purposes appear to consistently contain a single fungal morphotype, which belongs to the genus *Chaetomonium*. Photographs A, B, and D are provided by Naomi E. Pierce and Dino Martins (Harvard University).

Furthermore, Blatrix *et al*. (2012) show that the *Chaetothyriales* fungi in the domatia of *Petalomyrmex phylax*, *Tetraponera aethiops*, and *Pseudomyrmex penetrator* that are hosted by *Leonardoxa africana africana* (Cameroon), *Barteria fistulosa* (Cameroon), and *Tachigali* sp. (French Guiana), respectively, serve as food resource for the developing larvae. Calcofluor- and ^{15}N-labelled fungal material was found to be present in the larval gut of the three ant species.

5.1.2 Exploitation control of non-cultivar fungi

Non-cultivar fungi were not observed in either the *P. phylax or T. penzigi* systems. However, ant species cultivating a fungal monoculture should be under selective pressure to defend their presumed mutualist against any unspecialised and/or specialised non-cultivar fungi that may prey on or compete with the cultivars. Multiple mechanisms can theoretically prevent non-cultivar fungi from successfully attacking and killing the putative fungal cultivar of *T. penzigi*. Analogous to the *Allomerus* system, (1) *T. penzigi* worker ants could physically remove fungal contaminants or exclude parasitic fungi using bioactive metapleural gland secretions; (2) cultivar-secreted antifungals could serve a role in its self-defence; (3) ant-associated symbiotic bacteria could produce antifungal metabolites that inhibit non-cultivar fungi; and/or (4) cultivar-associated bacteria could produce antifungals that target non-cultivar fungi to which the cultivar itself has evolved resistances.

5.2 Aims

In this chapter, the third and fourth hypotheses are tested that ant- and/or cultivar-associated bacteria produce antifungals capable of killing non-cultivar fungi. As in *Allomerus,* the worker ants of *T. penzigi* are not known to have specialised morphological structures to support ectosymbiotic bacteria and also exhibit no obvious bacterial coatings. Nevertheless, bioactive actinobacteria have been isolated from *Allomerus* spp. worker ants (Seipke *et al*. 2012) and may hence be present on *T. penzigi* worker ants too.

Actinobacteria were also isolated from the fungal cultivar of *Allomerus* spp. but exhibited no inhibitory properties towards non-cultivar fungi isolated from the same ant colonies (Fig. $4.9_{A\text{-}B}$). However, because bioactive *Streptomyces* and *Burkholderia* bacteria can be isolated from the fungal gardens of attine ants (Santos *et al.* 2004, Haeder *et al*. 2009), I also test for bioactive bacterial symbionts in the putative fungal cultivar of *T. penzigi*. Note that the scope of this chapter does not include testing whether the fungal cultivar in fact benefits *T. penzigi*.

5.3 Materials and Methods

5.3.1 Ant and cultivar samples

Acacia drepanolobium samples, including *T. penzigi* workers and their putative fungal cultivar, were provided by Naomi E. Pierce and Dino Martins. Sampling sites included Kitengela (S1°23'526'' E36°49'108'') and Ngong Hills (S1°26'946'' E36°38'358'') from southern Kenya (near Nairobi), as well as Mpala Ranch and Mpala Road (N0°32' E36°42') from central Kenya (Laikipia district) (Fig. 5.2). Multiple occupied domatia were collected from each of 28 ant colonies (Table 5.1). In individual containers, the domatia of single myrmecophytes were transported to the United States, where they were sub-sampled twice. The first time, on average five worker ants were collected from each colony and were preserved in 1 ml of 20% glycerol (2G); the second time, domatia of selected colonies were collected. Between sub-sampling sessions, the ants (of single colonies) were free to move in and out of their domatia and translocate their fungal cultivar. Hence, the sub-sampled domatia included varying numbers of worker ants, and in only three cases some fungal material. Bacterial isolations were carried out in a category 2 microbiological laboratory at the University of East Anglia, United Kingdom. Worker ants were recovered using sterile technique from the domatia with soft forceps, and were placed in 1 ml of 2G; also, the majority of the cultivar material, found inside the domatia, was preserved in 1 ml of 2G. The remainder of the cultivar material was used for isolating non-cultivar fungi, as described below.

5.3.2 Isolation of ant- and cultivar-associated bacteria

The ant and cultivar samples were vortexed for a minimum of 10 seconds. Then, solid particles were removed from the samples by centrifugation. Sub-samples of the 2G supernatants were serially diluted (dilution factors: 10^{-2} and 10^{-4}) and used for inoculating mannitol soy-flour (MS) agar plates (Table 5.2).



Figure 5.2 (A-C) Origin of *Tetraponera penzigi* worker ants and their putative fungal cultivar, collected by Naomi E. Pierce and Dino Martins from four locations in Kenya. The sampling sites include Kitengela and Ngong Hills from the south of the country, as well as Mpala Ranch and Mpala Road from its centre.

Sampling location	Colony	Sub-sample	Host type
Kitengela, Kenya	1	2G	± 5 Ants
Kitengela, Kenya	2	2G	± 5 Ants
Kitengela, Kenya	3	2G	± 5 Ants
Kitengela, Kenya	4	2G	± 5 Ants
Kitengela, Kenya	6	2G	± 5 Ants
Kitengela, Kenya	8	2G	± 5 Ants
Kitengela, Kenya	17	2G	± 5 Ants
Kitengela, Kenya	27	2G	± 5 Ants
Kitengela, Kenya	29	2G	± 5 Ants
Kitengela, Kenya	30	2G	± 5 Ants
Kitengela, Kenya	4	Domatium	12 Ants
Kitengela, Kenya	4	Domatium	Hyphal material
Kitengela, Kenya	17	Domatium	1 Ant
Ngong Hills, Kenya	5	2G	± 5 Ants
Ngong Hills, Kenya	14	2G	± 5 Ants
Ngong Hills, Kenya	19	2G	± 5 Ants
Ngong Hills, Kenya	20	2G	± 5 Ants
Ngong Hills, Kenya	21	2G	± 5 Ants
Ngong Hills, Kenya	25	2G	± 5 Ants
Ngong Hills, Kenya	26	2G	± 5 Ants
Ngong Hills, Kenya	14	Domatium	2 Ants
Ngong Hills, Kenya	14	Domatium	Hyphal material
Ngong Hills, Kenya	21	Domatium	1 Ant
Ngong Hills, Kenya	21	Domatium	Hyphal material
Mpala Ranch, Kenya	12	2G	± 5 Ants
Mpala Ranch, Kenya	13	2G	± 5 Ants
Mpala Ranch, Kenya	16	2G	± 5 Ants
Mpala Ranch, Kenya	18	2G	± 5 Ants
Mpala Ranch, Kenya	22	2G	± 5 Ants
Mpala Ranch, Kenya	24	2G	± 5 Ants
Mpala Ranch, Kenya	22	Domatium	5 Ants
Mpala Road, Kenya	9	2G	± 5 Ants
Mpala Road, Kenya	10	2G	± 5 Ants
Mpala Road, Kenya	11	2G	± 5 Ants
Mpala Road, Kenya	32	2G	± 5 Ants
Mpala Road, Kenya	31	Domatium	7 Ants

Table 5.1 Samples of *Tetraponera penzigi* worker ants and their putative fungal cultivar. From each of 28 *Acacia drepanolobium* trees, Naomi E. Pierce and Dino Martins collected multiple *T. penzigi*-occupied domatia. These were sub-sampled twice; first, for on average five worker ants that were provided in glycerol solutions (2G) and second, for domatia from which worker ants and some fungal material were isolated. These samples were stored in 2G as well.

Fresh agar plates were prepared by inoculating 100 ml batches of autoclaved MS agar (50 ^{o}C) with 1 ml of nystatin (5 mg (Sigma N3503-25MU) in 1 ml dimethyl sulfoxide (DMSO)) and 1 ml of cycloheximide (5 mg (Fisher 35742-0050) in 1 ml 100% ethanol) solutions, and subsequently sub-dividing the medium over four Petri dishes. Inoculated agar plates were incubated at 30 ^{o}C and successfully establishing bacterial morphotypes, both Gram-positives and Gram-negatives, were purified by repeatedly propagating single colonies to fresh agar plates. Each morphotype was selected once, regardless of its abundance.

In total, 20 bacterial lineages (KY_1-KY_5, KY_7-KY_{21}) could be isolated (Table 5.3). Glycerol stocks of the purified lineages were prepared as described for the bacterial isolates of the *Acromyrmex* system (Chapter 2.3.2).

5.3.3 Isolation of fungal symbionts

Some of the cultivar material, found inside of the domatia, was inoculated onto MS, potato dextrose (PDA), and Sabouraud agar plates (Table 5.2). Fresh agar plates, here, were prepared by supplementing 100 ml batches of autoclaved agar (50 ^{o}C) with filter-sterilised solutions of 1 ml carbenicillin (5 mg (Fisher BPE2648-5) in 1 ml dH_2O) and 1 ml streptomycin (5 mg (Fisher BPE910-50) in 1 ml dH_2O). The medium was either equally sub-divided over four Petri dishes or added as slopes to cylindrical containers (Bijoux's). Upcoming non-cultivar fungi were isolated by repeatedly transferring their hyphae onto fresh agar plates and incubating the inocula at room temperature or 30 ^{o}C. In total, five non-cultivar fungi (F_1-F_4, F_6) could be isolated (Table 5.3). In addition to worker ants and occupied domatia, Naomi E. Pierce and Dino Martins also provided an inoculum of the fungal mutualist (F_5). The fungal strains were stored in Bijoux's.

Medium	Ingredients	Weight (g), volume (ml), percent (%)
Lennox broth (LB)	Tryptone	10.0 g
	Yeast extract	5.0 g
	NaCl	5.0 g
	Distilled water	1000 ml
Lennox broth (LB) agar	Tryptone	10.0 g
	Yeast extract	5.0 g
	NaCl	5.0 g
	Agar	15.0 g
	Distilled water	1000 ml
Lennox broth (LB) soft agar	Tryptone	10.0 g
	Yeast extract	5.0 g
	NaCl	5.0 g
	Agar	5.0 g
	Distilled water	1000 ml
Mannitol soy-flour (MS) agar	Organic soy-flour	20.0 g
	Mannitol	20.0 g
	Agar	20.0 g
	(+SB, 150mM sodium butyrate)	(16.5 g)
	Tap water	1000 ml
Mannitol soy-flour (MS) broth	Organic soy-flour	20.0 g
	Mannitol	20.0 g
	(+SB, incl. 150mM sodium butyrate)	(16.5 g)
	Tap water	1000 ml
Potato dextrose agar (PDA)	Glucose	20.0 g
	Instant mashed potato (smash)	5.0 g
	Agar	20.0 g
	Distilled water	1000 ml
Sabouraud agar (pH=5.6)	Dextrose	40.0 g
	Peptone	10.0 g
	Agar	20.0 g
	Distilled water	1000 ml
Tryptone soya broth (TSB)	Tryptone soya broth (Oxoid)	30.0 g
	Distilled water	1000 ml
Yeast extract-malt extract (YEME)	Yeast extract	3.0 g
	Peptone	5.0 g
	Malt extract	3.0 g
	Glucose	10.0 g
	Distilled water	1000 ml
TSB/YEME	TSB	50 %
	YEME	50 %

Table 5.2 Media for the cultivation of bacteria and fungi.

Sample type	Name	Sample origin	Host type	Host colony
Mutualistic fungus	*Chaetomonium* F_5	Kenya	-	-
Non-mutualistic fungus	F_1	Kenya	-	-
Non-mutualistic fungus	F_2	Kenya	-	-
Non-mutualistic fungus	F_3	Kenya	-	-
Non-mutualistic fungus	F_4	Kenya	-	-
Non-mutualistic fungus	F_6	Kenya	-	-
Bacterial isolate	KY_1	Kitengela, Kenya	Worker ants	27
Bacterial isolate	KY_2	Ngong Hills, Kenya	Worker ants	26
Bacterial isolate	KY_3	Ngong Hills, Kenya	Worker ants	14
Bacterial isolate	KY_4	Ngong Hills, Kenya	Worker ants	5
Bacterial isolate	KY_5	Ngong Hills, Kenya	Cultivar	21
Bacterial isolate	KY_7	Kitengela, Kenya	Worker ants	17
Bacterial isolate	KY_8	Kitengela, Kenya	Cultivar	4
Bacterial isolate	KY_9	Ngong Hills, Kenya	Worker ants	25
Bacterial isolate	KY_{10}	Ngong Hills, Kenya	Worker ants	5
Bacterial isolate	KY_{11}	Kitengela, Kenya	Worker ants	17
Bacterial isolate	KY_{12}	Mpala Road, Kenya	Worker ants	31a
Bacterial isolate	KY_{13}	Mpala Ranch, Kenya	Worker ants	16
Bacterial isolate	KY_{14}	Ngong Hills, Kenya	Cultivar	14a
Bacterial isolate	KY_{15}	Ngong Hills, Kenya	Worker ants	21
Bacterial isolate	KY_{16}	Ngong Hills, Kenya	Cultivar	14a
Bacterial isolate	KY_{17}	Kitengla, Kenya	Worker ants	4
Bacterial isolate	KY_{18}	Ngong Hills, Kenya	Cultivar	21
Bacterial isolate	KY_{19}	Mpala Road, Kenya	Worker ants	9
Bacterial isolate	KY_{20}	Mpala Road, Kenya	Worker ants	9
Bacterial isolate	KY_{21}	Ngong Hills, Kenya	Worker ants	14

Table 5.3 Origin of fungal and bacterial symbionts.

5.3.4 Light microscopy

Stereomicroscope photographs of the bacterial isolates were taken in the Henry Wellcome Laboratory for Cell Imaging (www.uea.ac.uk/bio/biobmi) with a Zeiss SV11 M2 Bio Quad stereomicroscope and an AxioCam HRc CCD camera. Young and thus relatively flat colonies were used, and magnifications were adjusted to suit size variances between bacterial species. In particular, photographs were taken at magnifications of 2.0, 3.2, 4.0, 5.0, and 6.6X. Scale bars of 1 mm were added to the pictures with AxioVision software (Carl Zeiss, Welwyn Garden City, UK).

5.3.5 Extraction of genomic DNA

The (non-)cultivar fungi, including F_1, F_2, F_3, F_4, F_5, and F_6, were DNA extracted (Table 5.3). Hyphal material was added to Eppendorf tubes containing glass beads and 800 µl of ice-cold TGE buffer (Table 5.4). In addition, liquid cultures of the bacterial isolates, KY_1-KY_5, KY_7-KY_{21}, were DNA extracted (Table 5.3). Glass containers (universals), holding 10 ml of TSB/YEME (Table 5.2) and a spring, were inoculated with 50 µl from the glycerol stocks or cell material of single plate colonies. The cultures were grown overnight in a shaker incubator (200 rpm, 30 ^{o}C), after which they were harvested by centrifugation (4000 rpm, 5 minutes, 4 ^{o}C). The supernatants were discarded and the cell pellets were re-suspended in 800 µl of TGE buffer. The solutions were placed into Eppendorf tubes containing a small layer of glass beads, and were stored on ice.

Solution	Ingredients	Concentration (mM)
Tris/Glycine/EDTA (TGE) buffer (pH=8.0)	Tris	50 mM
	EDTA	10 mM

Table 5.4 Solution for DNA extractions.

A detailed procedure for extracting DNA from the fungi and bacteria is given in Chapter 2.3.5. In short, the fungal and bacterial samples of *T. penzigi* were DNA extracted by bead-beating (6x30 seconds, 6 m/s). Before, during, and after bead-beating sessions, the samples were stored on ice (>5 minutes). The DNA-containing supernatants were repeatedly enriched with 0.5 ml of

phenol:chloroform:isoamyl alcohol (25:24:1), vortexed (1 minute), and benchtop-centrifuged (13,200 rpm, 5 minutes). The DNA in the aqueous phase of the samples was either washed one time with 0.5 ml chloroform:isoamyl alcohol (24:1) or precipitated with 1 ml of 100% ethanol. The precipitate was harvested by centrifugation, the pellet rinsed with 70% ethanol, dried, and re-suspended in 50 µl of sterile dH_2O.

5.3.6 Amplification and purification of diagnostic genes

By polymerase chain reaction (PCR), diagnostic genes were amplified from the fungal (ITS region) and bacterial (16S rDNA) genomes using the sample-specific primers, ITS1-F-ITS4 and 515-1492 (Table 5.5), and PCR conditions listed in table 5.6. Selected amplicons were recovered from the gel, following the instructions of the Qiagen Gel Extraction Kit manual (www.qiagen.com).

Name	Sequence	Reference	Gene	Amplicon length
ITS1-F	5'-CTTGGTCATTTAGAGGAAGTAA-3'	Gardens and Bruns (1993)	18S rDNA	± 600 bp
ITS4	5'-TCCTCCGCTTATTGATATGC-3'	White *et al*. (1990)		
515	5'-GTGCCAGCMGCCGCGGTAA-3'	Turner *et al.* (1999)	16S rDNA	± 1000 bp
1492	5'-GGTTACCTTGTTACGACTT-3'	Turner et al. (1999)		

Table 5.5 Primer sets for the partial amplification of fungal (ITS region) and bacterial (16S SSU) rDNA genes.

A

PCR mixture	Volume
dH_2O	23.0 µl
GoTaq buffer (incl. loading buffer)	8.0 µl
GoTaq MgCl (25 mM)	2.4 µl
DMSO (100%)	2.0 µl
Bioline dNTP's (10 mM)	1.0 µl
Forward primer (25 µM)	1.0 µl
Reverse primer (25 µM)	1.0 µl
DNA	1.0 µl
GoTag polymerase	0.6 µl
Total	**40.0 µl**

B

Step	Fungi		Bacteria	
	Temperature	Minutes	Temperature	Minutes
1	94 °C	4:00	94 °C	12:00
2	95 °C	0:30	94 °C	1:00
3	53 °C	1:00	45 °C	0:45
4	72 °C	1:00	72 °C	1:30
5	Go 34x to step 2		Go 29x to step 2	
6	72 °C	10:00	72 °C	20:00
7	end		end	

Table 5.6 (A-B) PCR mixture and thermocycles for the partial amplification of fungal (ITS region) and bacterial (16S SSU) rDNA genes.

5.3.7 Sequencing and BLAST-matching

The cleaned amplicons were sequenced (Table 5.7) with the sample-specific forward primers (ITS1-F, 515). The sequencing reactions were analysed by The Genome Analysis Centre TGAC (www.jicgenomelab.co.uk) using ABI 3730XL sequencers (Life Technologies). The sequences were quality-checked and truncated with FinchTV (www.geospiza.com/Products/finchtv.shtml). The sequences were then matched to the BLAST database of the National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). With a BLASTn search, the fungal and bacterial sequences were compared to database reads of the nucleotide collection (nr/nt), optimising for highly similar sequences (megablast).

A

Sequencing mixture	Volume
dH_2O	5.5 µl
5x sequencing buffer	1.5 µl
Forward primer (3.2 µM)	1.0 µl
DNA	1.0 µl
Enzyme E3.1	1.0 µl
Total	**10.0 µl**

B

Step	Temperature	Minutes
1	96 °C	1:00
2	96 °C	0:10
3	50 °C	0:05
4	60 °C	4:00
5	Go 25x to step 2	
6	60 °C	0:10
7	end	

Table 5.7 (A-B) Reaction mixture and thermocycles for sequencing of PCR amplicons.

5.3.9 Colony bioassays

Selected bacterial isolates of antibiotic-producing genera were tested in colony bioassays against *Candida albicans* (CA_6, Table 3.2) and three non-cultivar fungi (F_1, F_2, F_3). No challenges were carried out against other non-cultivar fungi (F_4, F_6), nor against the fungal mutualist (F_5), since their hyphae did not spread smoothly across agar plates. The bacterial selection included seven bacteria, KY_1-KY_5, KY_7 and KY_{21}, which in the following will be referred to as 'NNSPS strains'; this acronym is based on the initial letters of the genera *Nocardia*, *Nocardiopsis*, *Streptomyces*, *Pseudonocardia*, and

Saccharopolyspora (*Pseudonocardia* and *Nocardiopsis* have been isolated from *Acromyrmex* in Chapter 2; *Nocardia* has been isolated from *Allomerus* in Chapter 4).

5.3.9.1 Bioassays against Candida

In colony bioassays, the selected bacteria were tested against *Candida* CA_6. Universals containing 10 ml of TSB/YEME and a spring were inoculated with 30 µl from the bacterial glycerol stocks and were placed in a shaker incubator for a sufficient accumulation of biomass (280 rpm, 30 ^{o}C). The spring was added to increase the oxygen concentration of the medium and to reduce cell clustering. The cell material was harvested by centrifugation (4000 rpm, 5 min); the supernatants were disposed. The bacteria were point-inoculated into the centre of MS agar plates, containing no antibiotics, by transferring standardised fractions of cell material. As negative controls, one uninoculated plate and one inoculated with cell material of *S. lividans* were included.

The experiment was carried out in duplicate; one set of agar plates was enriched with 150 mM sodium butyrate, which increases antifungal production in certain bacteria (Martin and McDaniel 1976, Moore *et al*. 2012), and the other set was not. The inoculated plates were incubated for thirteen days at 21 ^{o}C. A one-day-old *Candida* culture was prepared by growing the yeast in 10 ml Lennox broth (LB) (280 rpm, 37 ^{o}C). Then, 1 ml of the culture was added to 5 ml of LB soft agar (50 ^{o}C) (Table 5.2). The medium was poured over the bioassay plates (30 ^{o}C) contacting, but not overlaying, the bacterial colonies. The bioassay plates were incubated for 2 days at 26 ^{o}C to allow *Candida* to grow, after which the presence or absence of inhibition zones was scored.

5.3.9.2 Bioassays against non-cultivar fungi F_1, F_2, and F_3

In the absence of sodium butyrate, the selected bacteria were challenged against the non-cultivar fungi, F_1, F_2, and F_3 by Ryan Seipke. The bacterial isolates were point-inoculated into the centre of MS agar plates, containing no antibiotics, by transferring mycelium from bacterial plate colonies. Mycelium of

S. lividans was inoculated onto an additional agar plate serving as negative control. The agar plates were incubated at 30 ^{o}C to obtain maturated colonies; then hyphal material of the test fungi was point-inoculated at the edge of the plates, which thereafter were incubated (30 ^{o}C) until the fungi clearly overgrew or clearly avoided the bacterial colonies.

5.3.10 Supernatant bioassays

Growth cultures of the selected NNSPS strains (KY_1-KY_5, KY_7, KY_{21}) were challenged against *Candida* CA_6 and one of the non-cultivar fungi (F_1). This test was conducted to indicate whether the bacterial strains are secreting antifungals into a liquid medium, from which the metabolites could be extracted.

5.3.10.1 Obtaining supernatants

Batches of 50 ml MS broth (Table 5.2) were inoculated with 30 µl from the bacterial glycerol stocks. As negative controls, one batch of medium remained uninoculated and another was inoculated with *S. lividans*. Two sets of growth cultures were obtained; one set contained 150 mM sodium butyrate, and the other one did not. Inoculated flasks were incubated in a shaker incubator for 14 days (30 ^{o}C, 250 rpm). Due to the relatively low inoculation volume (and/or a low spore/cell concentration of the glycerol stocks), a relatively long incubation period was needed before observing abundant cell growth. The supernatants were harvested by pelleting-out the majority of cell material and insoluble medium (30 minutes, 4000 rpm, 4 ^{o}C). For the bioassays, small sub-samples of the supernatants were centrifuged a second time with a benchtop centrifuge (5 minutes, 13,200 rpm), which then were filter-sterilised with 0.2 µm filters.

5.3.10.2 Bioassays against Candida

Challenges were carried out in which the bacterial supernatants, containing and not containing sodium butyrate, were tested against *Candida* CA_6. Paper filter disks, 0.9 cm in diameter, were inoculated with 100 µl of the sterile supernatants and control liquids. LB agar plates were coated with 400 µl

of a one-day-old *Candida* culture, grown in 10 ml Lennox broth. The dry disks were attached to the agar plates using 60 µl of dH_2O. The presence or absence of inhibition zones was scored after incubating the plates for two days at 26 ^oC.

5.3.10.2 Bioassays against non-cultivar fungus F_1

The supernatants of the bacteria, grown in the absence of sodium butyrate, were also challenged against a non-cultivar fungus (F_1). Disks with a diameter of 4 cm were loaded with 2 ml of the filter-sterilised supernatants. As negative control, one filter was inoculated with the same volume of supernatant from the uninoculated medium. When dry, the disks were attached with 200 µl of dH_2O to MS agar plates, containing no antibiotics. Thereafter, hyphal material of the fungal strain was point-inoculated at the edge of the bioassay plates. The plates were incubated at 26 ^oC until the filter disks were either contacted by the fungi or clearly avoided.

5.4 Results

5.4.1 Isolation and microscopy of ant- and cultivar-associated bacteria

Naomi E. Pierce and Dino Martins sampled multiple domatia containing *T. penzigi* worker ants and their putative fungal cultivar from 28 Kenyan *A. drepanolobium* trees (Fig. 5.2). From the domatia, worker ants and their putative fungal cultivar (Table 5.1) were isolated, and stored in glycerol solutions (2G). Subsequently, the solutions were diluted and used for inoculating agar plates, from which unique morphotypes of Gram-positive and Gram-negative bacteria were isolated.

In total, 20 bacterial lineages were isolated from the worker ants and their putative fungal cultivar. Each strain obtained an ID, consisting of the prefix 'KY' and an individual number (KY_1-KY_5, KY_7-KY_{21}). Photographs of the bacterial isolates, and their origin, are presented in figure 5.3 and table 5.3, respectively.

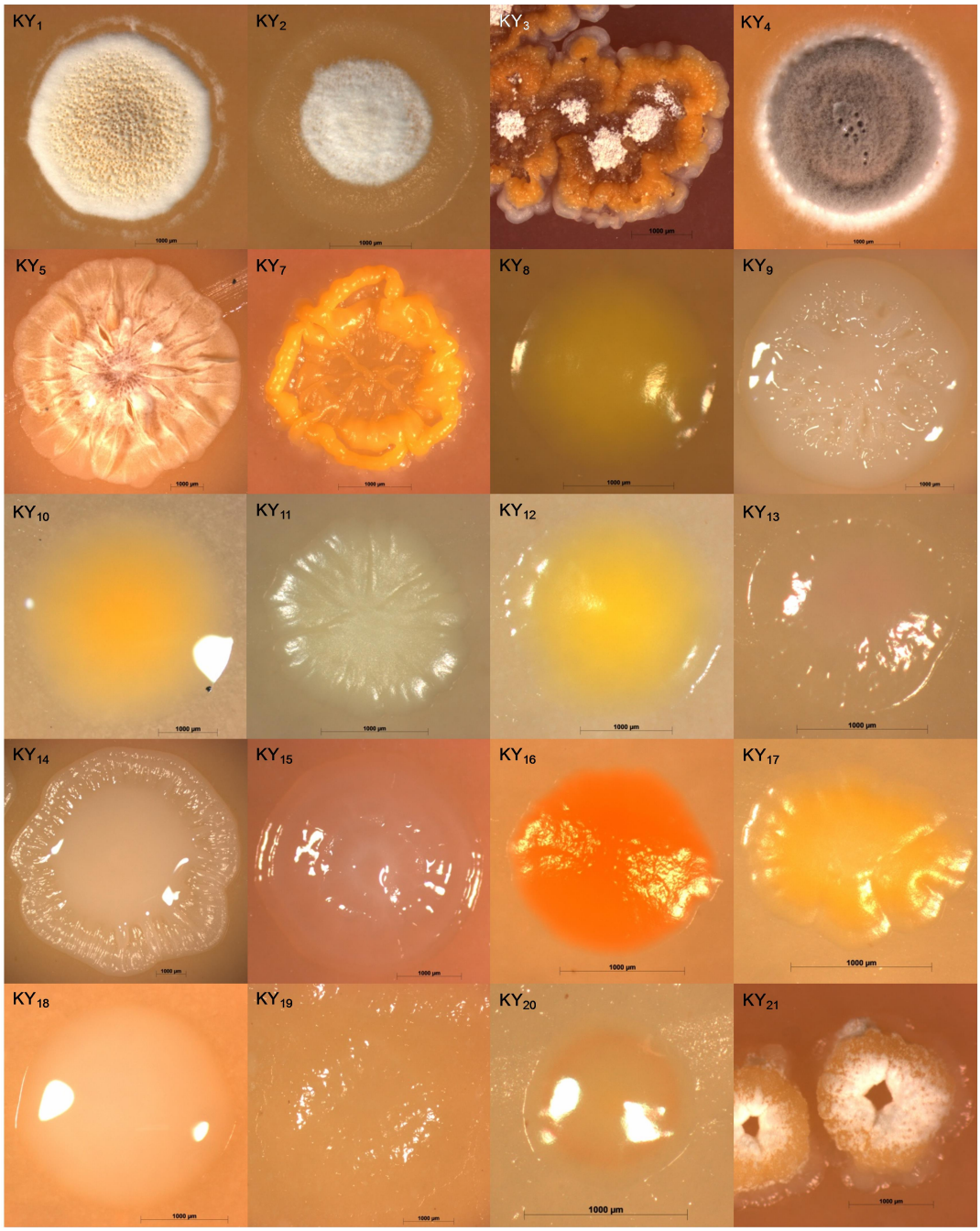


Figure 5.3 Microscopy photographs of 20 bacterial isolates (KY_1-KY_5, KY_7-KY_{21}). The bacteria were isolated from *Tetraponera* worker ants and their fungal cultivars. In addition to actinobacteria, also Gram-positives and Gram-negatives of other phyla, including *Firmicutes*, *Proteobacteria*, and *Bacterioidetes*, were isolated. An overview of the bacterial origin and details on their genetic identity are provided in the tables 5.3 and 5.8, respectively. A selection of seven bacteria, KY_1-KY_5, KY_7, and KY_{21}, belonging to antibiotic-producing genera, will be referred to as 'NNSPS strains'. In particular, strains KY_1, KY_2, KY_4, and KY_5 belong to the genus *Streptomyces* and strains KY_3, KY_7, and KY_{21} belong to the genus *Saccharopolyspora*.

5.4.2 Isolation of fungal symbionts

The remaining cultivar material found inside some domatia, which was not used for bacterial extractions, was inoculated onto agar plates for isolating non-cultivar fungi. Five lineages of non-cultivar fungi were isolated (F_1-F_4, F_6). In addition, a separate sample of the putative fungal cultivar (F_5) of *T. penzigi* was provided by Naomi E. Pierce and Dino Martins. Photographs of the fungal cultivar and non-cultivars, and their origin, are shown in figure 5.4 and table 5.3, respectively.

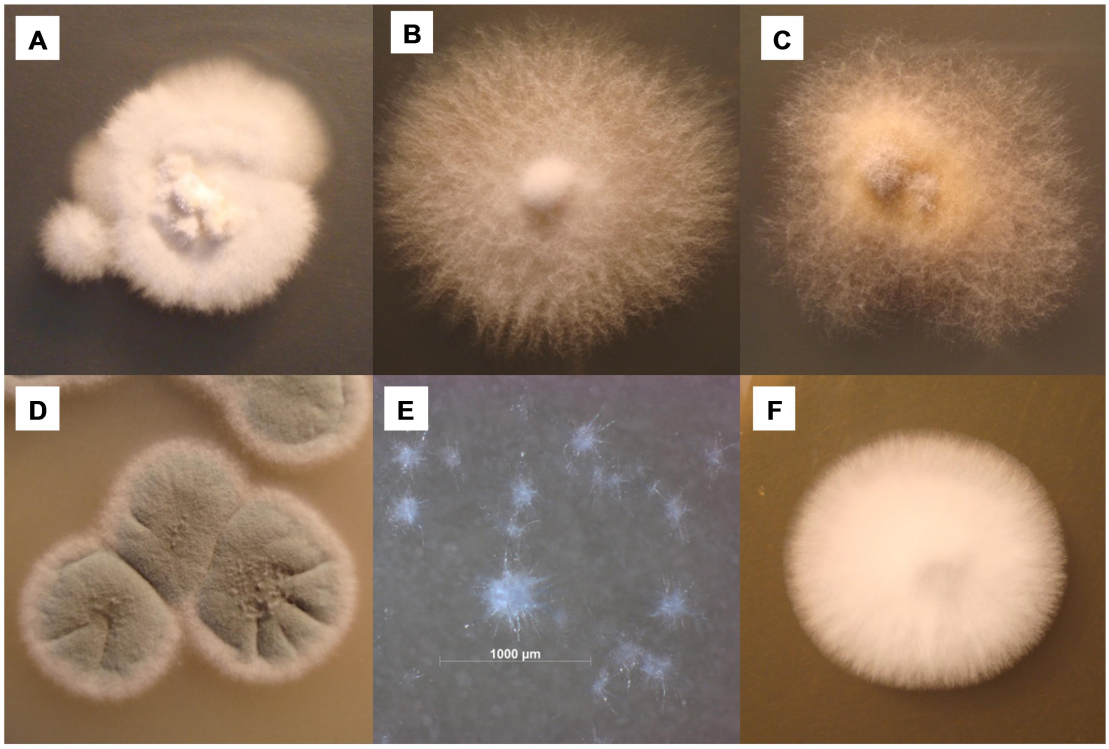


Figure 5.4 Fungal symbionts of *Tetraponera penzigi*. **(A-E)** Non-cultivar fungi isolated from the fungal cultivar. In sequence, they belong to the genera *Acremonium* (F_1), *Fusarium* (F_2), *Alternaria* (F_3), *Penicillium* (F_4), and *Claviceps* (F_6). **(F)** Fungal cultivar *Chaetomonium* (F_5). Tables 5.3 and 5.8 provide a detailed overview on the origin and genetic identity of the strains. Strains A, B, and C spread their hyphae across agar plates; whereas strains D, E, and F grow locally.

5.4.3 Amplification, purification, sequencing, and BLAST-matching of diagnostic genes from genomic DNA extracts

The morphologically identified fungal cultivar (F_5) and five non-cultivar fungi (F_1-F_4, F_6) (Table 5.3) were identified genetically. The fungi were DNA extracted and a fragment of their rDNA gene (ITS region) was sequenced with a sample-specific forward primer.

The sequences of the fungal cultivar F_5 (538 bp), as well as the fungal non-cultivars F_1 (546 bp), F_2 (505 bp), F_3 (528 bp), F_4 (552 bp), and F_6 (485 bp), show BLAST analogies to rDNA sequences of *Chaetomonium,* as well as *Acremonium*, *Fusarium*, *Alternaria*, *Penicillium*, and *Claviceps*, respectively (Table 5.8). The sequencing data are given in the Supplementary Information A_1.

The bacterial isolates, KY_1-KY_5, KY_7-KY_{21} (Table 5.3), were identified genetically by extracting their DNA and sequencing a segment of the 16S rDNA gene with a sample-specific forward primer. The sequences of the bacterial strains KY_1-KY_5, KY_7-KY_{21} (445-886 bp; mean 718 bp) show BLAST analogies to 16S rDNA sequences of diverse bacterial genera (Table 5.8). Morphotypes of the actinobacterial genera *Streptomyces* and *Saccharopolyspora* are relatively common in comparison to most other bacterial genera. The sequences are displayed in the Supplementary Information A_1.

Sample type	Name	PCR primers	Sequencing primer	Sequence length	Genus	Closest relative (accession)	Max score	Total score	Query coverage	E value	Max ident
Mutualistic fungus	Chaetomonium F_5	ITS1-F – ITS4	ITS1-F	538 bp	*Corynascus*	Corynascus kuwaitiensis 18S rRNA gene, 5.8S rRNA gene, 26S rRNA gene, ITS1 and ITS2, isolate Kw140 (AJ715483.1)	861	861	100%	0	95%
			ITS1-F		*Chaetomium*	Chaetomium atrobrunneum strain CGMCC 3.3594 18S ribosomal RNA gene, partial sequence (GU966500.1)	857	857	99%	0	95%
Non-mutualistic fungus	F_1	ITS1-F – ITS4	ITS1-F	546 bp	*Acremonium*	Acremonium implicatum 18S ribosomal RNA gene, partial sequence (AF368810.1)	1009	1009	100%	0	100%
Non-mutualistic fungus	F_2	ITS1-F – ITS4	ITS1-F	505 bp	*Fusarium*	Fusarium polyphialidicum 18S rRNA gene, partial (X94172.1)	933	933	100%	0	100%
Non-mutualistic fungus	F_3	ITS1-F – ITS4	ITS1-F	528 bp	*Alternaria*	Alternaria sp. Ponipodef 07 18S ribosomal RNA gene, partial sequence (HQ731643.1)	976	976	100%	0	100%
Non-mutualistic fungus	F_4	ITS1-F – ITS4	ITS1-F	552 bp	*Penicillium*	Penicillium sizovae strain CBS 115968 18S ribosomal RNA gene, partial sequence (GU944585.1)	1005	1005	98%	0	100%
Non-mutualistic fungus	F_6	ITS1-F – ITS4	ITS1-F	485 bp	*Claviceps*	Claviceps sp. ICMP 16980 internal transcribed spacer 1, partial sequence (EU770250.1)	566	566	84%	6.00E-158	91%
Bacterial isolate	KY_1	515 – 1492	515	847 bp	*Streptomyces*	*Streptomyces* sp. L131D 16S ribosomal RNA gene, partial sequence (HM797397.1)	1565	1565	100%	0	100%
Bacterial isolate	KY_2	515 – 1492	515	563 bp	*Streptomyces*	*Streptomyces* sp. HBUM 49432 16S ribosomal RNA gene, partial sequence (EU158328.1)	1040	1040	100%	0	100%
Bacterial isolate	KY_3	515 – 1492	515	767 bp	*Saccharopolyspora*	*Saccharopolyspora* sp. 8-15 16S ribosomal RNA gene, partial sequence (HQ622522.1)	1411	1411	100%	0	99%
Bacterial isolate	KY_4	515 – 1492	515	811 bp	*Streptomyces*	*Streptomyces* sp. XAS586 16S ribosomal RNA gene, partial sequence (GQ395241.1)	1498	1498	100%	0	100%
Bacterial isolate	KY_5	515 – 1492	515	837 bp	*Streptomyces*	*Streptomyces* sp. CS38 16S ribosomal RNA gene, partial sequence (EF494232.1)	1546	1546	100%	0	100%
Bacterial isolate	KY_7	515 – 1492	515	690 bp	*Saccharopolyspora*	*Saccharopolyspora* sp. 8-15 16S ribosomal RNA gene, partial sequence (HQ622522.1)	1269	1269	100%	0	99%
Bacterial isolate	KY_8	515 – 1492	515	445 bp	*Kocuria*	*Kocuria* sp. M1-36 16S ribosomal RNA gene, partial sequence (HQ425309.1)	822	822	100%	0	100%
Bacterial isolate	KY_9	515 – 1492	515	584 bp	*Serratia*	*Serratia* sp. PfRB002 gene for 16S rRNA, partial sequence (AB560603.1)	1079	1079	100%	0	100%
					Bacillus	*Bacillus subtilis* strain XJtks46.1 16S ribosomal RNA gene, partial sequence (HQ123474.1)	1079	1079	100%	0	100%
					Pseudomonas	*Pseudomonas* sp. bk_23 16S ribosomal RNA gene, partial sequence (HQ538682.1)	1079	1079	100%	0	100%
Bacterial isolate	KY_{10}	515 – 1492	515	719 bp	*Chryseobacterium*	*Chryseobacterium daejeonense* strain RB-18 16S ribosomal RNA gene, partial sequence (EF077177.1)	1279	1279	100%	0	98%
Bacterial isolate	KY_{11}	515 – 1492	515	711 bp	*Staphylococcus*	*Staphylococcus* sp. 2A12S8 16S ribosomal RNA gene, partial sequence (HQ246299.1)	1314	1314	100%	0	100%
Bacterial isolate	KY_{12}	515 – 1492	515	796 bp	*Pseudomonas*	*Pseudomonas* sp. sk0927 1-3 16S ribosomal RNA gene, partial sequence (FJ751243.1)	1471	1471	100%	0	100%
Bacterial isolate	KY_{13}	515 – 1492	515	628 bp	*Roseomonas*	*Roseomonas* sp. BZ44 16S ribosomal RNA gene, partial sequence (HQ588850.1)	1160	1160	100%	0	100%
Bacterial isolate	KY_{14}	515 – 1492	515	652 bp	*Bacillus*	*Bacillus* sp. SG2 16S ribosomal RNA gene, partial sequence (HM057848.1)	1205	1205	100%	0	100%
					Brevibacterium	*Brevibacterium frigoritolerans* strain DSM 8801 16S ribosomal RNA gene, partial sequence (GU252128.1)	1205	1205	100%	0	100%
Bacterial isolate	KY_{15}	515F – 1492R	515	735 bp	*Serratia*	*Serratia* sp. GmRB009 gene for 16S rRNA, partial sequence (AB560573.1)	1352	1352	100%	0	99%
					Bacillus	*Bacillus subtilis* strain XJtks46.1 16S ribosomal RNA gene, partial sequence (HQ123474.1)	1352	1352	100%	0	99%
					Pseudomonas	*Pseudomonas fluorescens* strain YR20 16S ribosomal RNA gene, partial sequence (HM224401.1)	1352	1352	100%	0	99%
Bacterial isolate	KY_{16}	515F – 1492R	515	773 bp	*Gordonia*	Uncultured *Gordonia* sp. clone T0417 16S ribosomal RNA gene, partial sequence (HQ616261.1)	1423	1423	100%	0	99%
Bacterial isolate	KY_{17}	515F – 1492R	515	886 bp	*Staphylococcus*	*Staphylococcus* sp. 2A12S8 16S ribosomal RNA gene, partial sequence (HQ246299.1)	1631	1631	100%	0	99%
					Shewanella	*Shewanella* sp. S12a partial 16S rRNA gene, strain S12 (FN994186.1)	1631	1631	100%	0	99%
Bacterial isolate	KY_{18}	515F – 1492R	515	827 bp	*Arthrobacter*	*Arthrobacter globiformis* partial 16S rRNA gene, strain MA100 (FN908766.1)	1522	1522	100%	0	99%
Bacterial isolate	KY_{19}	515F – 1492R	515	631 bp	*Stenotrophomonas*	*Stenotrophomonas* sp. TeRB010 gene for 16S rRNA, partial sequence (AB560630.1)	1166	1166	100%	0	100%
					Pseudomonas	*Pseudomonas geniculata* strain NBG2 16S ribosomal RNA gene, partial sequence (HQ256559.1)	1166	1166	100%	0	100%
					Xanthomonas	*Xanthomonas* sp. TE9 16S ribosomal RNA gene, partial sequence (GQ381284.1)	1166	1166	100%	0	100%
Bacterial isolate	KY_{20}	515F – 1492R	515	689 bp	*Microbacterium*	*Microbacterium* sp. HY16(2010) 16S ribosomal RNA gene, partial sequence (HM579807.1)	1273	1273	100%	0	100%
					Cellulosimicrobium	*Cellulosimicrobium* sp. 6A12S5 16S ribosomal RNA gene, partial sequence (HQ246240.1)	1273	1273	100%	0	100%
Bacterial isolate	KY_{21}	515F – 1492R	515	772 bp	*Saccharopolyspora*	*Saccharopolyspora* sp. 8-15 16S ribosomal RNA gene, partial sequence (HQ622522.1)	1415	1415	100%	0	99%

Table 5.8 Genetic identity of fungal and bacterial symbionts. Details on their origin and an overview of the sequencing data are presented in table 5.3 and Supplementary Information A_1, respectively.

5.4.5 Colony bioassays

In colony bioassays, selected bacterial isolates of well-known antifungal-producing genera were challenged against *Candida albicans* (CA_6) and the non-cultivar fungi *Acremonium* (F_1), *Fusarium* (F_2), and *Alternaria* (F_3). Due to their patchy growth, agar plate bioassays did not include the fungal cultivar *Chaetomonium* (F_5) nor the non-cultivar fungi *Penicillium* (F_4) or *Claviceps* (F_6). The bacterial selection (NNSPS strains) includes *Streptomyces* KY_1, KY_2, KY_4, and KY_5 as well as *Saccharopolyspora* KY_3, KY_7, and KY_{21}. In particular, the ant isolates KY_1 and KY_7 originate from Kitengela. The ant isolates KY_2, KY_3, KY_4, and KY_{21}, as well as the cultivar isolate KY_5, originate from Ngong Hills.

5.4.5.1 Bioassays against Candida

The selected bacteria were tested against *Candida* CA_6 on agar plates, some of which contained 150 mM of the chemical inducer sodium butyrate. The bacteria were grown in the centre of agar plates; as negative controls, an uninoculated and a *S. lividans*-containing agar plate, were included. The bioassay plates were overlaid with soft agar, containing the yeast. After incubation, the presence or absence of fungal inhibition was scored.

The bacterial strains KY_5, KY_1, KY_2, KY_3, KY_7, and KY_{21} successfully inhibited the growth of *Candida* in the absence of sodium butyrate (Fig. 5.5_A). In the presence of the additive, however, the bioactivity of some strains was reduced or disappeared, whereas the bioactivity of others increased (Fig. 5.5_B). *Saccharopolyspora* (KY_3, KY_7, KY_{21}) grew poorly at the applied concentrations of sodium butyrate and therefore failed to secrete bioactive metabolites; the sizes of the inhibition zones produced by *Streptomyces* KY_2 and KY_1 were unchanged; and the fungal inhibition of *Streptomyces* KY_5 increased in the presence of the inducer. This shows that multiple bacterial isolates are able to inhibit *Candida*; on the other hand, the results also show that sodium butyrate influences the bacterial secretion of antifungals. The growth-inhibiting effects of *Streptomyces* KY_1 and KY_5 on *Candida* are comparable to the bioactivities exhibited against the yeast by the *Acromyrmex* ant-associated *Streptomyces*

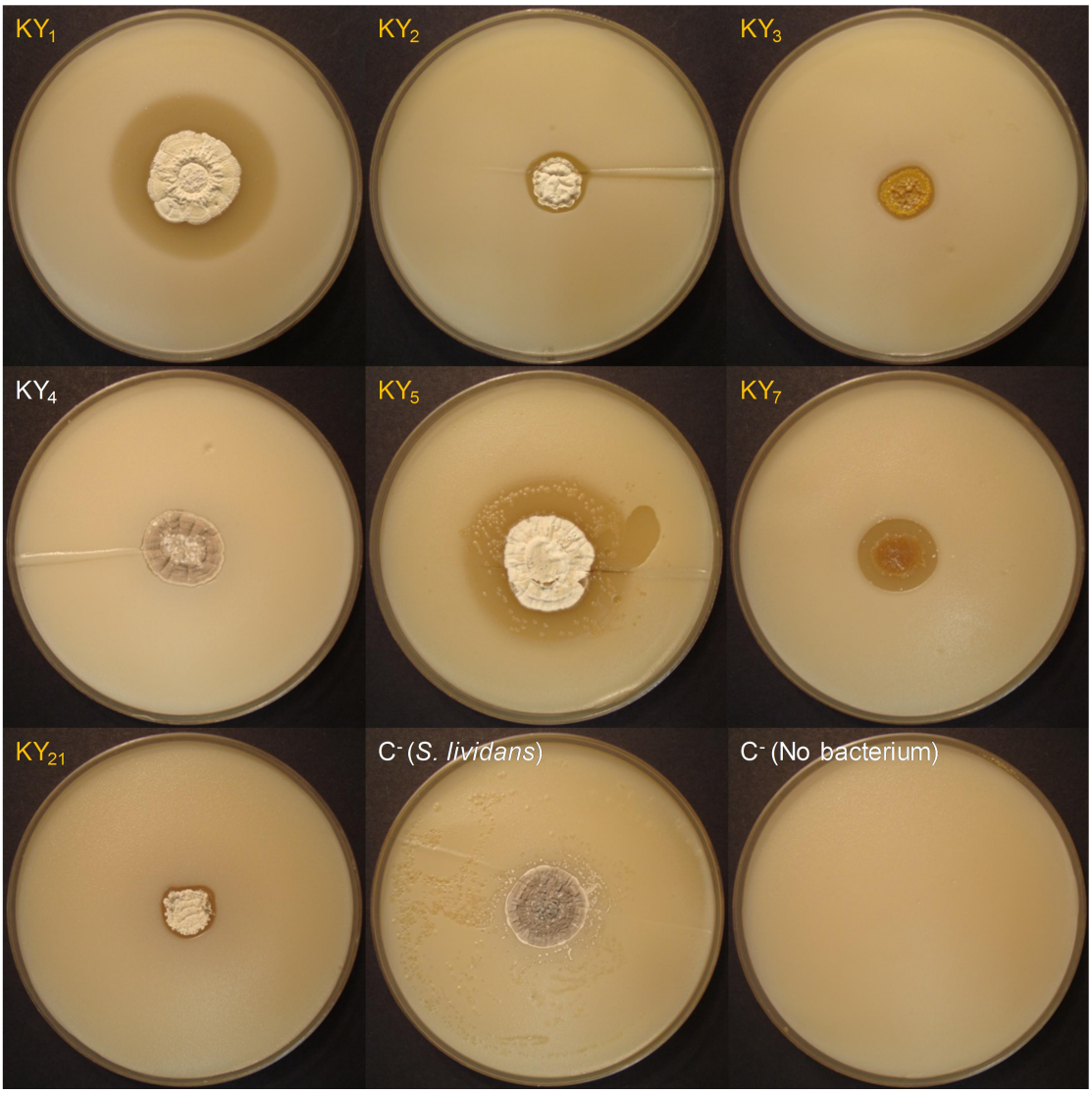


Figure 5.5 (A) *Candida* (CA_6) colony bioassays of NNSPS isolates (KY_1-KY_5, KY_7, KY_{21}). As negative controls, an uninoculated agar plate (C^- No bacterium) and one with a *S. lividans* colony (C^- *S. lividans*) were included. The bacteria were grown on agar plates, not containing sodium butyrate, which were overlaid with soft agar containing the test fungus. *Streptomyces* KY_1 and KY_5 are exhibiting a relatively strong fungal inhibition; in contrast, *Streptomyces* KY_2 and *Saccharopolyspora* KY_3, KY_7, and KY_{21} display a relatively weak inhibitory effect. Bioactive strains are highlighted by orange font.

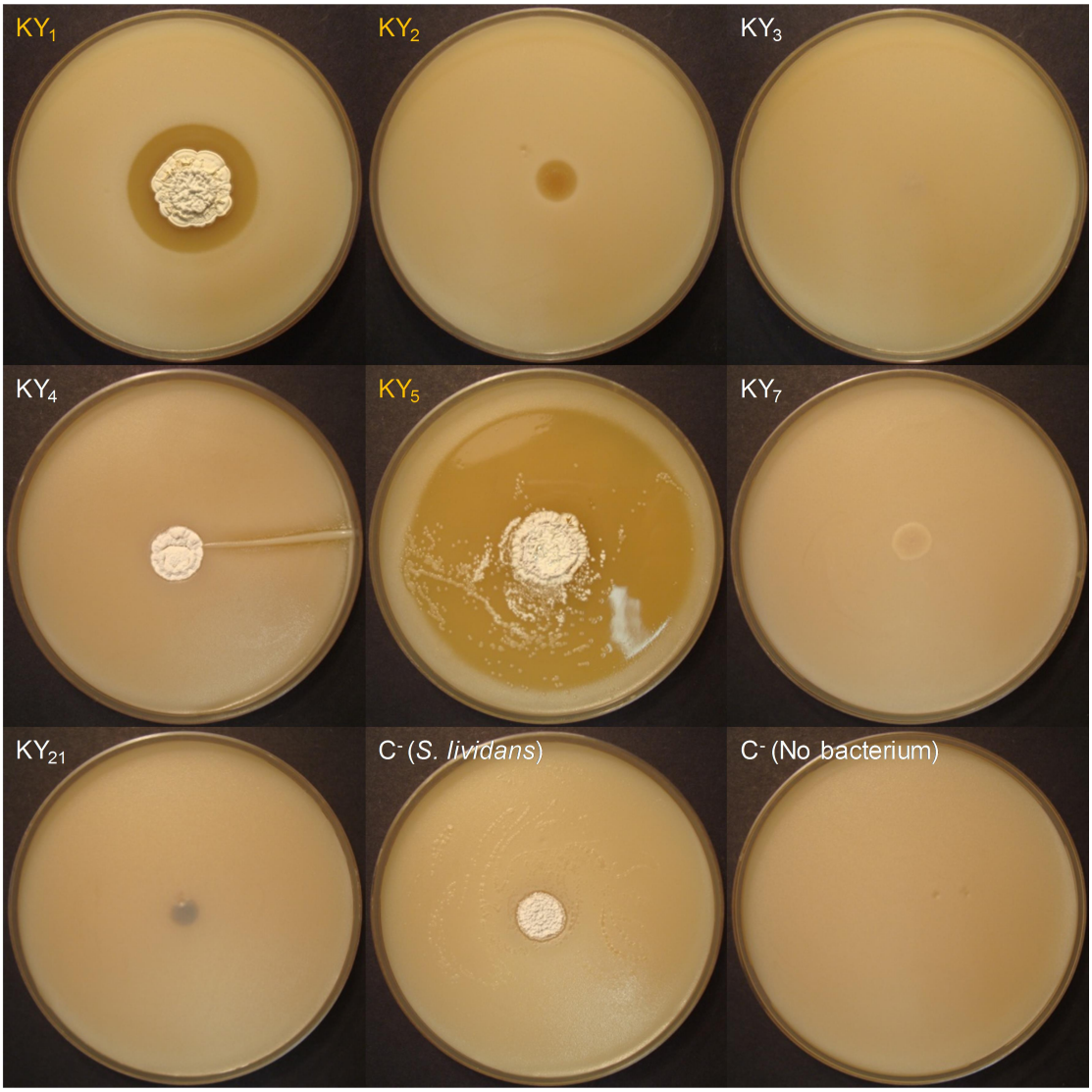


Figure 5.5 (B) *Candida* (CA_6) colony bioassays of NNSPS isolates (KY_1-KY_5, KY_7, KY_{21}). As negative controls, an uninoculated agar plate (C^- No bacterium) and one with a *S. lividans* colony (C^- *S. lividans*) were included. The bacteria were grown on agar plates, enriched with 150 mM of sodium butyrate, and overlaid with soft agar containing the test fungus. In the presence of the chemical inducer, *Saccharopolyspora* KY_3, KY_7, and KY_{21} are not growing well and do not produce antifungals. *Streptomyces* KY_5 and KY_1 are showing a relatively strong fungal inhibition; in contrast, *Streptomyces* KY_2 exhibits a relatively weak bioactivity. Bioactive strains are highlighted by orange font.

strains E_8 and E_9 (Fig. 2.5_{A-B}).

5.4.5.2 Bioassays against non-cultivar fungi F_1, F_2, and F_3

In another bioassay experiment, the selected bacteria were also challenged in the absence of sodium butyrate against the non-cultivar fungi *Acremonium* (F_1), *Fusarium* (F_2), and *Alternaria* (F_3). The bacteria were grown in the centre of agar plates; an agar plate with *S. lividans* served as negative control. The fungi were point-inoculated at the edge of the bioassay plates. After incubation, the presence or absence of fungal inhibition was scored.

The non-cultivar fungus *Acremonium* (F_1) is susceptible to the secretions of *Streptomyces* KY_5, as well as to the metabolites of *Saccharopolyspora* KY_3, KY_7, and KY_{21} (Fig. 5.6_A). In contrast, the other non-cultivar fungi, *Fusarium* (F_2) and *Alternaria* (F_3), are not inhibited by any bacteria (Fig. 5.6_{B-C}); even *Saccharopolyspora* colonies, which in the beginning of the experiment managed to establish small inhibition zones against *Fusarium* (F_2), were later overgrown.

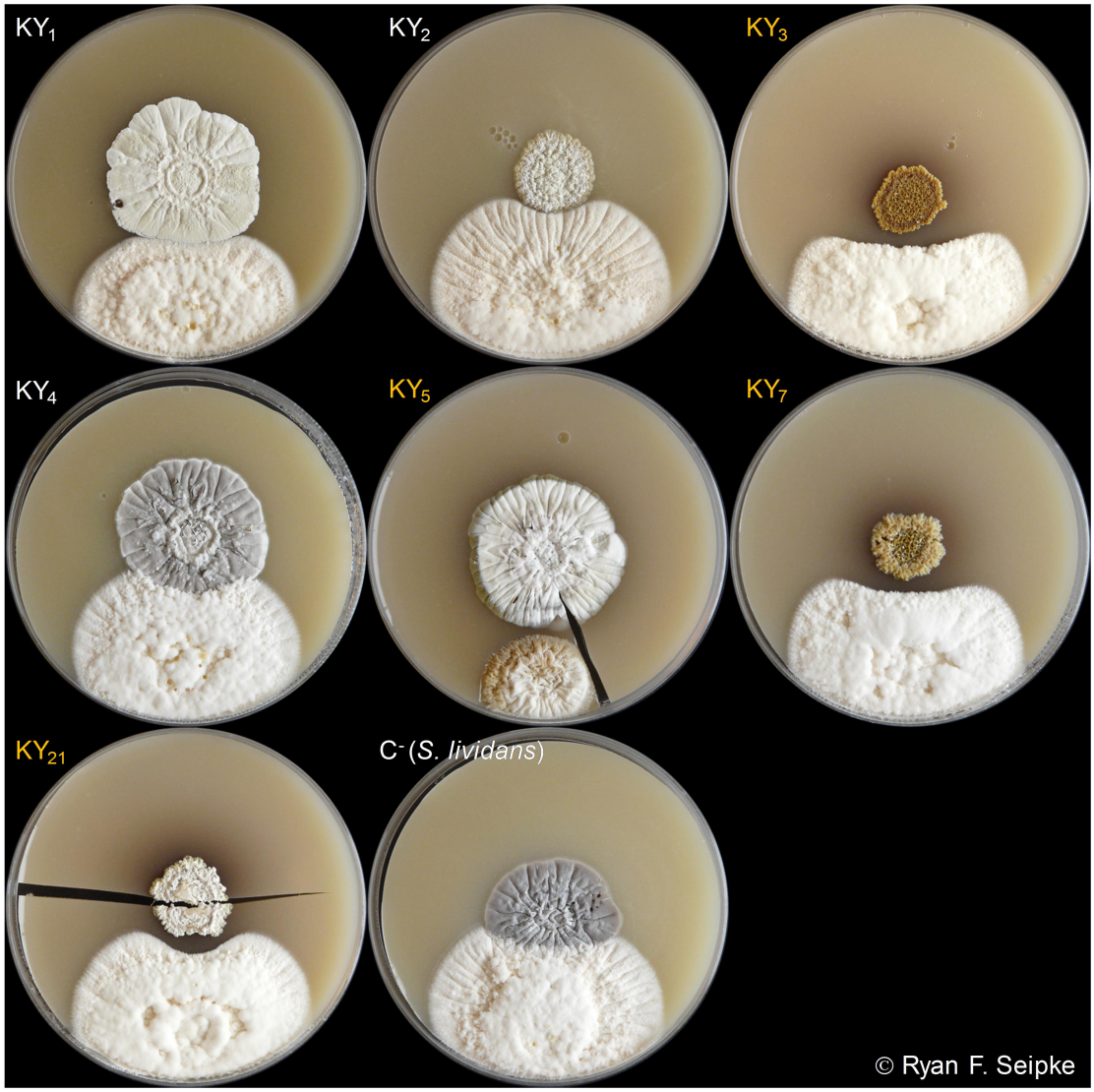


Figure 5.6 (A) *Acremonium* (F_1) colony bioassays of NNSPS isolates (KY_1-KY_5, KY_7, KY_{21}). The bacteria were grown in the centre of agar plates, not containing sodium butyrate. As a negative control, an agar plate with a *S. lividans* colony (C^- *S. lividans*) was included. The test fungi were introduced at the edge of the bioassay plates. *Acremonium* (F_1) is inhibited by *Saccharopolyspora* KY_3, KY_7, and KY_{21} as well as by *Streptomyces* KY_5. The colony of *Streptomyces* KY_5 even expanded to almost contact the fungus. Bioactive bacteria are highlighted by orange font. Ryan Seipke carried out the experiment and provided photographs of the bioassay plates.

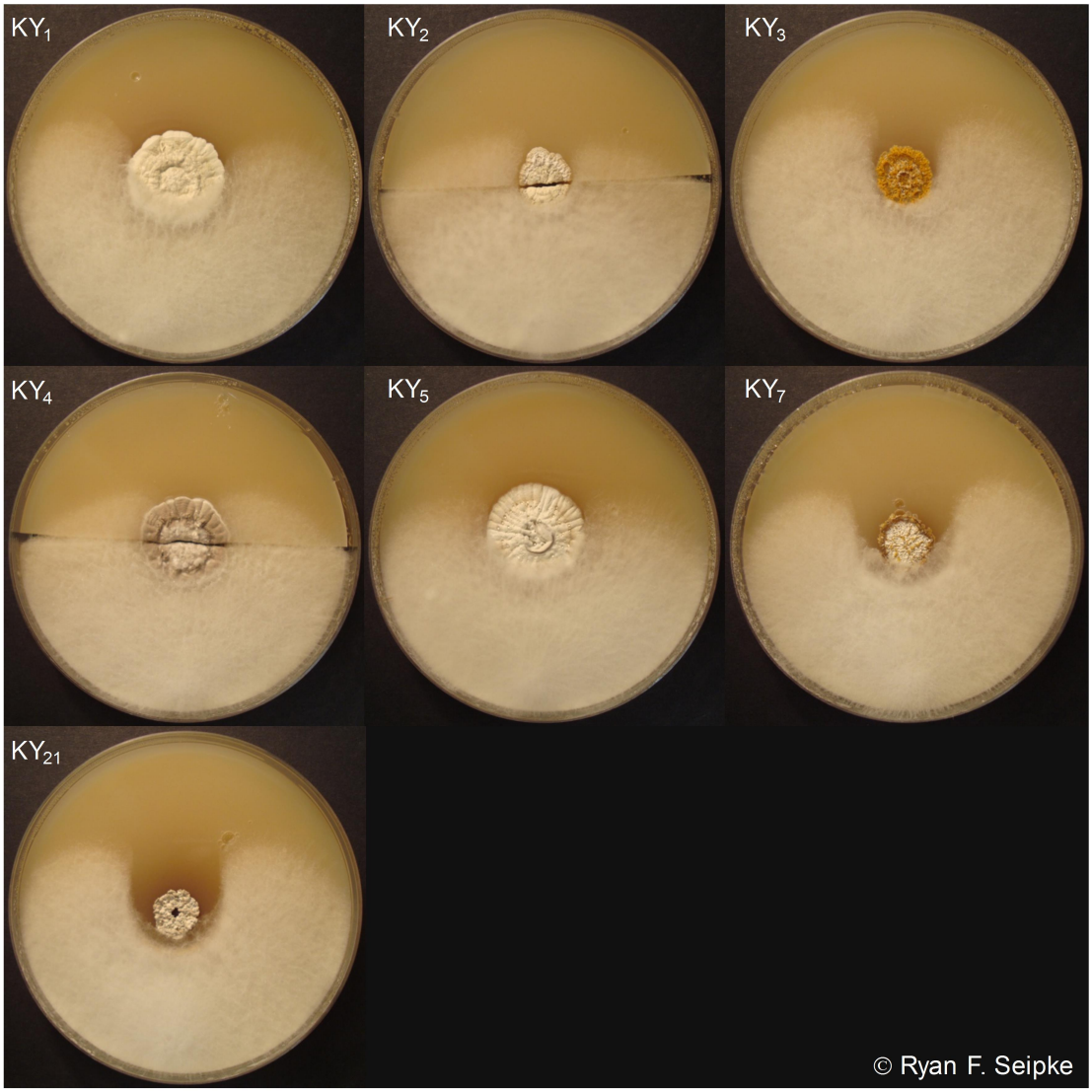


Figure 5.6 (B) *Fusarium* (F_2) colony bioassays of NNSPS isolates (KY_1-KY_5, KY_7, KY_{21}). The bacteria were grown in the centre of agar plates, not containing sodium butyrate. The test fungi were introduced at the edge of the bioassay plates. None of the bacterial isolates is able to inhibit the growth of *Fusarium* (F_2). Even the *Saccharopolyspora* strains, which initially managed to inhibit the test fungus, later became overgrown. Ryan Seipke carried out the experiment and provided photographs of the bioassay plates.

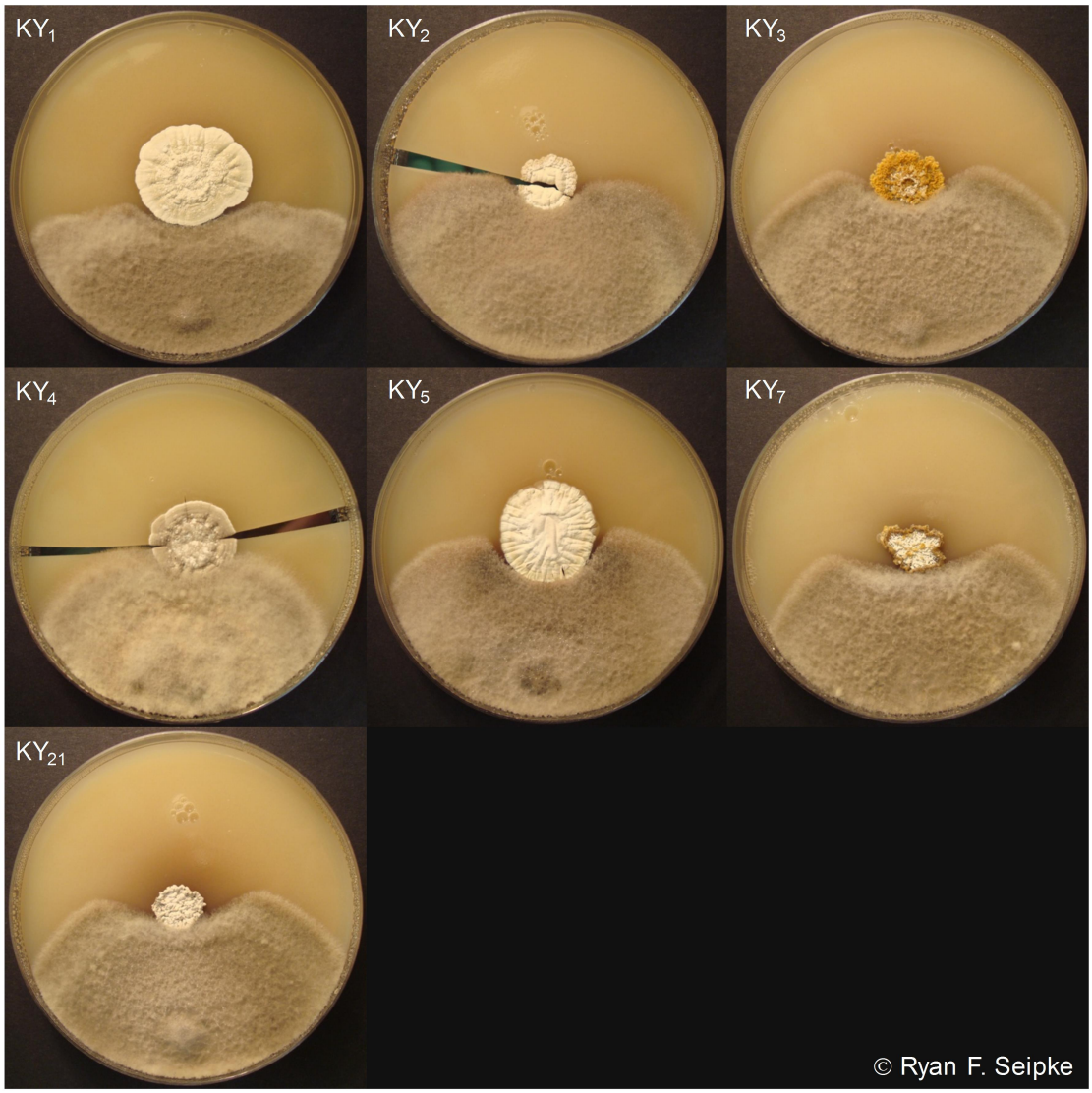


Figure 5.6 (C) *Alternaria* (F_3) colony bioassays of NNSPS isolates (KY_1-KY_5, KY_7, KY_{21}). The bacteria were grown in the centre of agar plates, not containing sodium butyrate. The test fungi were introduced at the edge of the bioassay plates. None of the bacterial isolates is able to inhibit the growth of *Alternaria* (F_3). Ryan Seipke carried out the experiment and provided photographs of the bioassay plates.

5.4.6 Supernatant bioassays

The capability of the NNSPS isolates (KY_1-KY_5, KY_7, KY_{21}) was tested to secrete antifungals into a liquid medium, some of which contained 150 mM of sodium butyrate. Due to the likelihood that a change in growth conditions may trigger antifungal production, here also those strains were included that previously failed to inhibit fungal strains on agar plates. An uninoculated batch of medium and a *S. lividans* growth culture served as negative control. The sterilised supernatants were tested against *Candida* CA_6 and *Acremonium* (F_1). No challenges were carried out against *Fusarium* (F_2) and *Alternaria* (F_3) since they were found not to be susceptible to any bacteria in colony bioassays (Fig. $5.6_{B\text{-}C}$).

5.4.6.2 Bioassays against Candida

The sterilised supernatants of the selected bacteria, grown in the presence and absence of sodium butyrate, were added to paper filter disks, alongside the negative controls. With distilled water, the filters were attached to *Candida*-coated agar plates. After incubation, the presence or absence of fungal inhibition was scored.

Only the supernatant of *Streptomyces* KY_1, grown in the absence of sodium butyrate, inhibits *Candida*; the supernatants of all other strains do not inhibit the yeast (Fig. $5.7_{A\text{-}C}$). This result means that only a small fraction of the bacterial strains, which inhibited *Candida* on sodium butyrate-lacking agar plates (KY_1, KY_5, KY_7, KY_3, KY_{21}, KY_2; Fig. 5.5_A), secretes antifungals into the liquid medium.

The supernatants of *Streptomyces* KY_5, KY_1, and KY_2, grown in the presence of sodium butyrate, inhibit *Candida*; all other supernatants proved ineffective against the yeast (Fig. $5.7_{D\text{-}F}$). *Streptomyces* KY_5, KY_1, and KY_2 also inhibited the growth of *Candida* on sodium butyrate-containing agar plates (Fig. 5.5_B).

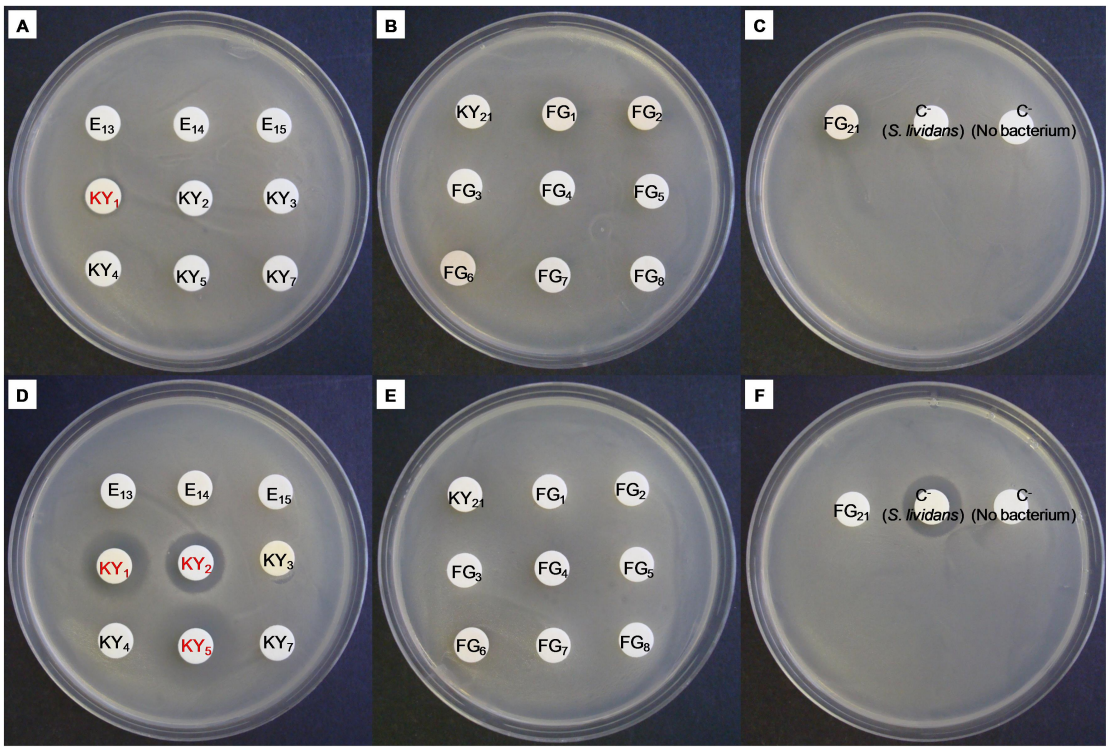


Figure 5.7 *Candida* (CA_6) supernatant bioassays of NNSPS (KY_1-KY_5, KY_7, KY_{21}) growth cultures. As negative controls, a growth culture of *S. lividans* (C^- *S. lividans*) and an uninoculated batch of medium (C^- No bacterium) were included. One set of media contained 150 mM sodium butyrate and another one did not. The supernatants were inoculated onto paper filter disks and attached to *Candida*-coated agar plates. **(A-C)** The supernatant of *Streptomyces* KY_1, containing no sodium butyrate, inhibits *Candida*. The remaining strains apparently secreted no, or insufficient amounts of, antifungal molecules into the liquid medium. **(D-F)** The supernatants of *Streptomyces* KY_1, KY_2, and KY_5, containing sodium butyrate, inhibit the yeast. Bioactive strains are highlighted by red font.

5.4.6.3 Bioassays against non-cultivar fungus F_1

The sterilised supernatants of the selected bacteria, grown in the absence of sodium butyrate, were also tested against *Acremonium* (F_1). The supernatants were inoculated onto filter disks, which with distilled water were attached to the centre of agar plates. *Acremonium* (F_1) was point-inoculated at the edge of the bioassay plates. After an incubation period, the presence or absence of fungal inhibition was scored.

None of the growth supernatants inhibits *Acremonium* (F_1) (Fig. 5.8). This

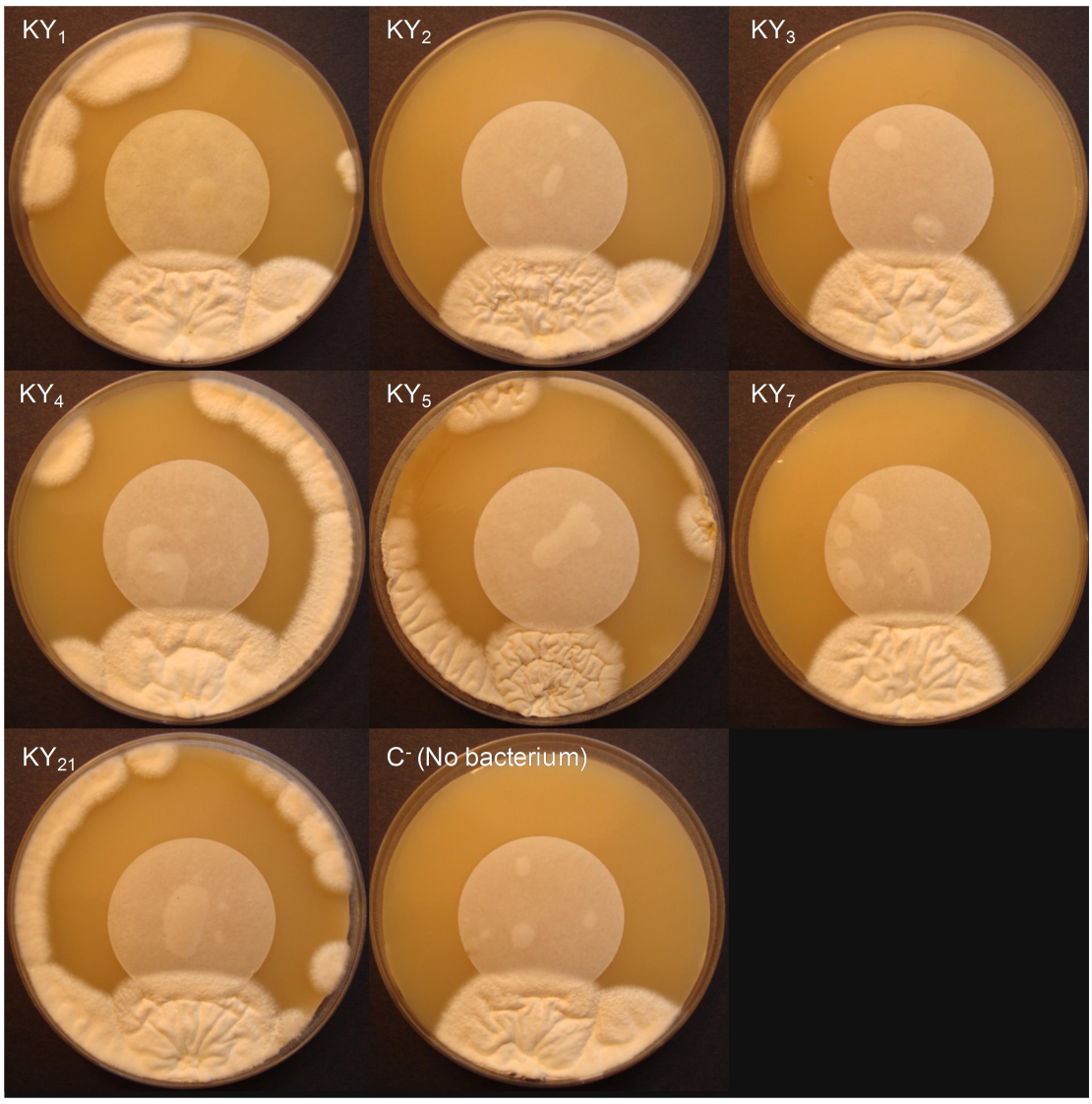


Figure 5.8 *Acremonium* (F_1) supernatant bioassays of NNSPS (KY_1-KY_5, KY_7, KY_{21}) growth cultures. In contrast to *Fusarium* (F_2) and *Alternaria* (F_3), this fungus shows susceptibilities to bacteria secretions in colony bioassays (Fig. 5.6_A). As a negative control, the supernatant of an uninoculated batch of medium (C^- No bacterium) was included. The supernatants, not containing sodium butyrate, were inoculated onto paper filter disks and attached to agar plates. The test fungus was introduced at the edge of the bioassay plates. None of the bacterial supernatants is inhibiting the fungal growth.

shows that none of the bacterial strains that inhibited the fungus on sodium butyrate-lacking agar plates (KY_5, KY_3, KY_7, KY_{21}; Fig. 5.6_A) secreted effective antifungals into the liquid medium. However, the observation that the same *Streptomyces* KY_1 supernatant, previously inhibited *Candida* (Fig. 5.7_A) and that the filamentous fungus was not susceptible to the secretions of this *Streptomyces* strain on agar plates (Fig. 5.6_A) suggest that the supernatant contains antifungals that selectively affect the yeast but not its filamentous counterpart.

5.5 Discussion

The data presented in this chapter show that *T. penzigi* worker ants and their fungal cultivars are associated with diverse genera of bioactive bacteria. In total, 20 bacterial morphotypes were isolated; seven actinobacteria, including several *Streptomyces* and *Saccharopolyspora* strains, were tested in colony bioassays against the medically important yeast *Candida albicans* and three non-cultivar, filamentous fungi: *Acremonium* (F_1), *Fusarium* (F_2), and *Alternaria* (F_3). These three fungal species were isolated from the same *Chaetomonium* fungal cultivar material as some of the cultivar-associated bacteria. Three *Streptomyces* (KY_1, KY_2, KY_5) and three *Saccharopolyspora* (KY_3, KY_7, KY_{21}) strains inhibited the growth of *Candida*. Also, one of these *Streptomyces* strains (KY_5) and all *Saccharopolyspora* strains inhibited *Acremonium* (F_1). None of the bacterial isolates inhibited the other two non-cultivar fungi. Due to the patchy growth characteristics of the *Chaetomonium* fungal cultivar, the *Streptomyces* strain (KY_5) that inhibited *Acremonium* could not be tested against *Chaetomonium*; this pairing would have been of particular interest since the bacterium was isolated from the fungal cultivar itself.

Furthermore, sodium butyrate was found to increase or decrease the bacterial antifungal production in a strain-specific way. For example, one *Streptomyces* strain (KY_5) displayed increased fungal inhibition under the influence of the chemical inducer. In contrast, the *Saccharopolyspora* strains (KY_3, KY_7, KY_{21}) were unable to grow in the presence of the substance and in

consequence did not produce antifungals. Potentially, those strains may display an increased antifungal production under a lower concentration of sodium butyrate.

Finally, three *Streptomyces* strains (KY_1, KY_2, KY_5) demonstrated the ability to secrete antifungal metabolites into a liquid environment, as shown by inhibition of *Candida*. Here, sodium butyrate clearly promoted antifungal production by increasing the number of secreting *Streptomyces* strains from one (KY_1) to three.

In conclusion, the results presented in this chapter indicate that *T. penzigi* and its putative fungal cultivar, *Chaetomonium*, indeed have the opportunity to benefit from the bioactive secretions of diverse *Streptomyces* and *Saccharopolyspora* strains. The ability of some of the isolates to secrete their antifungals into liquid medium will enable a straightforward isolation and identification of the bioactive metabolites.

Chapter 6) General Conclusions

The central purpose of this thesis was to test the utility of ant-microbe associations for discovering bioactive bacteria and antifungal compounds that have novel molecular (sub-)structures. Novel antifungals are in demand due to the rising prevalence of human mycoses (Slavin *et al.* 2004) and due to problems that accompany currently used antifungal drugs. These problems include rising fungal resistance towards specific antifungal compounds, adverse side-effects in humans in response to antifungal drugs, and difficulties with the application of antifungals, due for example, to the low water-solubility of polyenes (Day *et al*. 2011). Finding compounds with novel (sub-)structures displaying reduced side-effects, increased water-solubilities, and strong fungicidal and/or fungistatic properties would allow an application of higher and thus potentially more effective antifungal doses. In addition, a broader range of antifungal compounds *per se* allows the application of different antifungal drugs over time and the application of multiple antifungals simultaneously. All three strategies can prevent resistance evolution in fungal pathogens

Mutualistic symbioses between insects (hosts) and antifungal-producing bacteria (symbionts) are of increasing interest to research since some host-symbiont systems appear to have solved the 'antibiotic resistance problem', and this interest is indicated by the number of recently published studies (Scott *et al.* 2008, Oh. *et al.* 2009, Haeder *et al.* 2009, Kroiss *et al*. 2010, Barke *et al*. 2010, Poulsen *et al*. 2011, Schoenian *et al*. 2011). The antibiotic resistance problem is the widespread observation that fungal pathogens evolve and acquire resistance against specific antifungals. Host-symbiont systems could (1) teach us strategies for reducing resistance evolution in fungal pathogens and (2) provide us with bacterial species that, due to their co-evolved association with hosts, have evolved antifungals with novel molecular structures.

The best-studied example of a host-symbiont mutualism in which the symbiont produces antibiotics is found in attine ants (*Hymenoptera*, *Formicidae*, *Attini*). The mutualism between attine ants, their basidiomycetous fungal cultivar

Leucoagaricus gongylophorus (*Agaricales*, *Agaricaceae*), and bacterial symbionts has existed for over 50 million years (Schultz and Brady 2008). It represents a system that apparently has found a solution to successfully defend against fungal pathogens, such as *Escovopsis* spp. (*Hypocreales*, *Hypocreaceae*) that attack and kill the fungal cultivar, over evolutionary time-spans. Bacterial mutualists, which inhabit the integument of worker ants and also occur inside of their infrabuccal pocket, must adapt to a host that provides them with a specific environment and in return demands antifungal molecules that target current *Escovopsis* invaders. Hence, the bacterial symbionts of attine ants may phylogenetically differ from their free-living, and potentially well-explored, counterparts producing antifungals that are novel to science. Some antifungals of attine ant-associated bacteria that have been identified so far indeed possess novel chemical structures, while others possess unmodified chemical structures. For example, the novel antifungal compound dentigerumycin (Oh *et al*. 2009) has been isolated from an *Apterostigma* ant-associated *Pseudonocardia* symbiont. The well-known antifungals candicidin (Haeder *et al*. 2009), as well as antimycin, valinomycin, and actinomycin (Schoenian *et al.* 2011) have been isolated from *Acromyrmex* ant-associated *Streptomyces* strains.

In this study, *Acromyrmex octospinosus* worker ants from Trinidad (Chapter 2), the fungal cultivar of *Allomerus decemarticulatus* and *A. octoarticulatus* from French Guiana (Chapter 4), and *Tetraponera penzigi* worker ants and their putative fungal cultivar from Kenya (Chapter 5) were examined for associations with bioactive ectosymbiotic bacteria. The three ant systems combine the favourable characteristics of (1) being under selection pressure to evolve the use of bacterial metabolites in order to protect their (putative) fungal cultivars from fungal pathogens and competitors, and (2) differing in their ecology and biogeography. The combination of factors potentially increases the power to discover novel bacterial symbionts and novel antifungal metabolites.

As a first step to pursue our goal of finding novel antifungals, microbes were isolated from the three ant systems. As illustrated in the Supplementary Information C, these isolates belong to four bacterial phyla, including *Actinobacteria*, *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* forming well-defined phylogenetic clusters (Fig. S_1). The phylum *Actinobacteria* is dominated by strains of the genus *Streptomyces*, which belongs to the sub-order *Streptomycineae*. *Streptomyces* strains could be isolated from the *Acromyrmex*, *Allomerus*, and *Tetraponera* ant systems that were sampled across two continents, as well as from both the worker ants and their fungal cultivars. This supports the view that *Streptomyces* is no accidental contaminant but may serve a functional role in ant fungicultural systems. Previously, this bacterial genus has been shown to serve functional roles in other insect systems such as the one of the southern pine beetle *Dendroctonus frontalis* (Scott *et al*. 2008) and the one of the European beewolf *Philanthus triangulum* (Kaltenpoth *et al*. 2005). A cluster of less common bacterial morphotypes is formed by strains of the genera *Pseudonocardia* and *Saccharopolyspora*, which are members of the sub-order *Pseudonocardineae*.

Selected actinobacterial isolates and their growth supernatants were challenged against the medically important yeast *Candida albicans* and diverse nest-isolated non-cultivar fungi, in the presence and absence of the chemical inducer sodium butyrate. Multiple actinobacteria, isolated from *Acromyrmex octospinosus* worker ants, were found to inhibit *Candida* and three *Escovopsis* strains in agar plate bioassays. Only two of them, *Streptomyces* E_8 and E_9, secreted antifungals into a liquid medium that inhibited *Candida* and *Escovopsis* EWC, facilitating antifungal purification and identification. Two actinobacteria, isolated from the fungal cultivar of *Allomerus decemarticulatus* and *A. octoarticulatus*, were found to inhibit *Candida* in agar plate bioassays (in the presence of sodium butyrate). In contrast, none of them inhibited the growth of two non-cultivar fungi on agar plates and secreted antifungals into a liquid medium. Diverse actinobacteria isolated from *T. penzigi* worker ants and their putative fungal cultivar were found to inhibit *Candida* and one of three non-

cultivar fungi in agar plate bioassays. Of those, *Streptomyces* KY_1, KY_2, and KY_5 secreted their antifungals into a liquid medium, inhibiting the growth of *Candida*. These results show that ant-microbe associations indeed represent a rich source of actinobacteria, allowing the isolation of diverse *Pseudonocardia*, *Streptomyces*, *Nocardiopsis*, and *Saccharopolyspora* strains, and that many of the strains produce secondary metabolites with antifungal properties.

Two bioactive bacteria isolated from worker ants of one *Acromyrmex octospinosus* colony, *Pseudonocardia* E_4 (renamed 'P_1' in Barke *et al.* 2010) and *Streptomyces* E_8 (renamed 'S_4' in Barke *et al.* 2010), were analysed in detail to determine the identity and novelty of their antifungal metabolites. *Pseudonocardia* E_4 was shown to produce the partially novel (to science) antifungal 'nystatin P1,' suggesting a coevolutionary history with the symbiont (Barke *et al.* 2010). Due to the modified chemical structure of nystatin P1, the molecule probably exhibits an increased water-solubility compared to standard nystatin A_1. The nystatin-like *Pseudonocardia* polyene (NPP) of *P. autotrophica*, which like nystatin P1 possesses an additional sugar molecule, exhibits a strongly increased water solubility as compared to nystatin A_1 (Lee *et al*. 2012). Increasing the water-solubility of polyene antifungals, which form pores in the membranes of fungal pathogens, may benefit the treatment of systemic mycoses by facilitating their application. *Streptomyces* E_8 was shown to produce the well-known antifungals candicidin and antimycin, which is consistent with the recent recruitment of the bacterium from the environment (Barke *et al*. 2010, Seipke *et al*. 2011a). The antimycins found in this study are even more numerous than those found from a different *Streptomyces* species isolated by Schoenian *et al.* (2011) from an *A. octospinosus* ant. These results indicate that *A. octospinosus* has the capacity to use antifungal 'cocktails' to reduce resistance evolution in fungal pathogens (Barke *et al*. 2010) and show that this ant-symbiont system provides the opportunity to find novel antibiotics. In general, however, the selection pressure for novel antifungals to evolve may be reduced by the production of antifungal cocktails in vertically transmitted symbionts and/or bacterial consortia. The fitness interests between hosts and

symbionts are and, for the time being, will be aligned through the production of bioactive metabolites that in combination prevent or limit the evolution of resistant pathogens. This again reduces the need to obtain, or the benefit of being associated with, bacteria possessing novel antifungal biosynthesis genes.

A chemical analysis (Chapter 3) of the antimycin metabolites of *Streptomyces* E_8 revealed the presence of two compound sets, each consisting of six molecules, which differ in the number of attached CH_2 groups; the larger molecules within sets contain more CH_2 groups than the smaller molecules. This compound variety and the production of larger, and potentially more costly, antimycin molecules may allow *Streptomyces* E_8 to inhibit diverse fungal pathogens within the ant system and also to react to pathogen adaptations. This hypothesis could be tested by isolating individual antimycins and bioassaying them against test fungi that differ or resemble each other in their genetic constitution. Despite the fact that both compound sets share the same MS data, meaning that they contain the same six antimycin masses, both compound sets differ in their polarity, fragmentation pattern, and bioactivity. In bioassays, the antimycin molecules of only one compound set inhibited the growth of *Candida*, and revealed tandem-LCMS analogies with an antimycin A_1-A_4 standard. A future study should identify why the other set of antimycin compounds failed to inhibit *Candida*, possibly by including other test fungi is this bioassay analysis.

Future studies may also investigate how varying concentrations of sodium butyrate or other chemical inducers affect antifungal production in the bacterial isolates. For example, *Saccharopolyspora* KY_3, KY_7, and KY_{21}, which inhibited *Candida* on agar plates containing no sodium butyrate, were unable to grow mature colonies and produce antifungals on agar plates containing 150 mM of sodium butyrate. However, reducing the concentration of sodium butyrate may increase antifungal production in these strains as compared to the antifungal production in the absence of the inducer.

Overall, this study shows that ant-bacteria mutualisms represent a rich source of bioactive bacteria and in some cases novel antifungals that could be exploited pharmaceutically.

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Supplementary Information

A DVD with the Supplementary Information A-C is in the attachment of the thesis.

Supplementary Information A

A_1) Bacterial and fungal rDNA gene sequences
A_2) Candicidin gene sequences
A_3) Extended bacterial rDNA gene sequences

Supplementary Information B

B_1) LCMS on double-purified combined fractions B
B_2) LCMS on double-purified combined fractions B
B_3) LCMS on double-purified combined fractions B
B_4) LCMS on double-purified combined fractions B
B_5) LCMS on double-purified combined fractions B
B_6) LCMS on double-purified combined fractions B
B_7) LCMS on double-purified combined fractions B
B_8) LCMS on double-purified combined fractions B
B_9) LCMS on antimycin A_1-A_4
B_{10}) Tandem-LCMS

Supplementary Information C

C) Candicidin and antimycin producing bacteria

Supplementary Information A_1) Bacterial and fungal rDNA gene sequences

Partial sequences of the fungal rDNA gene (ITS region) and the bacterial 16S rDNA gene. The sequencing data, generated with the sample-specific forward primers, are categorised by ant-system (*Acromyrmex*, *Allomerus*, and *Tetraponera*). The title of each sequence describes the name of the strain, the PCR primer set used, and the length of the sequence.

Acromyrmex system

Fungi

Mutualistic fungus *Leucoagaricus gongylophorus*, ITS1-F–ITS4, 280 bp
GTGCACACCTGTCTTGGACTCTTCTTACTATTCATCCACCCTGTGCACTTCTTGTGGTCTTCTTTCAAGGTCGAAGTG
GGCTTGGTCTTTCTAGGCTCTATGAGGTCTTGAGGAGGGGCATGTTTGCTTTTGTCATAAACAAGTATAGCAGGAAT
GTTTTCTAACGGGCAACCTATGCCTGTGATTTAATATCATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGA
TGAAAAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAAAATTCA

Non-cultivar fungus *Escovopsis weberi* EWB, ITS1-F–ITS4, 604 bp
CATTACCGAGTTTTCATCAACTCCCTTACCCCATGTGAACGTTACCATACCGTTGCTTCGGCGGTGCGGCCCCGGC
GGCCCGGCTCCCTGCGAGCCGGCGCCTGCCGGGCTCAGGCCCCCGCCGGAGGACCCAAACTCCATGTTTTTTTG
CGAGTCTCTGAGGCGGCCCTGGGCCGATCAAAAAACAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCT
GGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC
GCACATTGCGCCCGCCAGCACTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCTGCCGCCCCCCGGGG
CGGCGGGCTCTTGGGGGACGGCCGGCCGCCTAGCGCGGCGGTGCCGCCCCCGAAATCCAGTGGCGACCGAGCC
GCGAACTCCTCTGCGCAGTAGACTTTGAGGCCCCTGCGGCCGAGACGGACTCGCACCGGAGAGCGGATCGGCCC
CGCCGTGAAACCCCCCAACTTCTGAAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAA
TAA

Non-cultivar fungus *Escovopsis weberi* EWC, ITS1-F–ITS4, 593 bp
TTACCGAGTTTTTATCAACTCCCTTACCCCATGTGAACGTTACCATACCGTTGCTTCGGCGGTGCGGCCCCGGCGG
CCCGGCTCCCTGCGAGCCGGCGCCTGCCGGGCTCAGGCCCCCGCCGGAGGACCCAAACTCCATGTCTTTTTGCG
AGTCTCTGAGGCGGCCCTGGGCCGATCAAAAAAACAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGG
CATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC
ACATTGCGCCCGCCAGCACTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCTGCCGCCCCCCGGGGCG
GCGGGCTCTTGGGGGACGGCCGGCCGCCTCGCGCGGCGGTGCCGCCCCCGAAATGCAGTGGCGACCGAGCCGC
GAACTCCTCTGCGCAGTAGACTTGAGGCCCCTGCGGCCGAACGAGACTCGCACCGGATAGCGGATCGGCCCCGC
CGTGAAACCCCAAACTTCTGAAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATA

Non-cultivar fungus *Escovopsis aspergilloides* EA, ITS1-F–ITS4, 613 bp
CATTATCGAGTTTTGAAACGGGTTGTAGCTGGCCTCTCCGGAGGCATGTGCACGCCCTGCTCATCCACTCTACACC
TGTGCACTTACTGTGGGTATCGGAAGGCGTCGCGTCGTTTGCGGCGAGGCGTTAACCGTGCCTACGTCTTACTACA
AACGCTTCAGTATCAGAATGTGTATTGCGATGTAACGCATCTATATACAACTTTCAGCAACGGATCTCTTGGCTCTC
GCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
CACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATGAAATTCTCAAACCCATAAGTCTTTGCG
GGCTTACGGGCTTTGGACTTGGAGGCTTTGTCGGCGACCGCGAGGTCTGTCGACTCCTCTCAAATGCATTAGCTTG
ATTCCTTGCGGATCGGCTCTCGGTGTGATAATTGTCTACGCCGTGACCGTGAAGCGTTTTGGCGAGCTTCTAATCG
TCTCTTACGAGACAGTTTACATTGACCTCTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAA

Bacteria

Bacterial isolate E_2, 515–1492, 750 bp
TTGGGCGTAAGAGCTCGTAGGCGGCTTGTCACGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCG
ATACGGGCTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGG
AACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAG
CTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATATACCGGAAAGCATC
AGAGATGGTGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGG
TTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAG
ACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACG
TGCTACAATGGCCGGTACAATGAGCTGCGATGCCGCGAGGCGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGA

Bacterial isolate E_3, 515–1492, 708 bp
AGCTCGTAGGCGGCTTGTCACGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCTAG
CTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGG
CGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAA
GTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAG
CATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATATACCGGAAAGCATCAGAGATGGTGC
CCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACTGCCGGGGT
CAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGG
CCGGTACAATGAGCTGCGATGCCGCGAGGCGGA

Bacterial isolate E_4, 515–1492, 707 bp
TTGGGCGTAAGAGCTCGTAGGCGGTGTGTCGCGTCGGCCGTGAAAACTTGGGGCTTAACTCTGAGCGTGCGGTCG
ATACGGGCATCACTTGAGTTCGGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAG
GAACACCGGTGGCGAAGGCGGGTCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGCGCTAGGTGTGGGGACCATTCCACGGTTTCTGCGCCGC
AGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATGCACAGGATCGC
GGCAGAGATGTCGTTTCCCTTGTGGCCTGTGTGCAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCTTATTCCATGTTGCCAGCACGTAGTGGTGGGGACTCATGGGAGACTG
CCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACACATGCT
ACAATGGCTCATACAGAGGGCTGCGAGACCGTGA

Bacterial isolate E_5, 515–1492, 608 bp
CGTAAGAGCTCGTAGGCGGCATGTCGCGTCTGCTGTGAAAGACCGGGGCTTAACTCCGGTTCTGCAGTGGATACG
GGCATGCTAGAGGTAGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACAC
CGGTGGCGAAGGCGGGTCTCTGGGCCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGAT
ACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGTGTGGGGACTTTCCACGGTTTCCGCGCCGTAGCTAACG
CATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCG
GCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTAGAGA
TACAGGGTCATTTAGTTGGTGGGTGACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAG
TCCCGCAACGAGCGCAACCCTTGTTCCATGTTGCCAGCACGTAATGGTGGGGACTCATGGGAGACTGCCGGGGTC
AACTCGGA

Bacterial isolate E_6, 515–1492, 725 bp
TAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGA
AATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAA
GCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATT
CCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAG
GAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCT
TGACATACACCGGAAACGGCCAGAGATGGTCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAAGCCCTTCGG
GGTGTTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCC
CTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTC
AAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTC

Bacterial isolate E_7, 515–1492, 762 bp
TTAACCTCGGGCCTGCAGGCGATACGGGCAGACTTGAGTACTGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTG
GAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAA
AGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGTACTAGGTGTGGGTTTCCT
TCCACGGGATCCGTGCCGTAGCTAACGCATTAAGTACCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAA
GGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGT
TTGACATATAGAGGATCGCCGGAGAGATTCGGTTTGCCTTGTGCCTTCTATACAGGTGGTGCATGGCTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCTCATGTTGCCAGCACGTAATGGTGG
GGACTCGTGAGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGTCC
AGGGCTTCACACATGCTACAATGGCGCGTACAGAGGGCTGCGATACCGTGAGGTGGAGCGAATCCCTTAAAGCGC
GTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTG
CGGTGAATAC

Bacterial isolate E_8, 515–1492, 830 bp
TTGGGCGTAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCG
ATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGG
AACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAG
CTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCT
GGAGACAGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGG
TTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGA
CCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGT
GCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGAT
TGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTC
CCGG

Bacterial isolate E_9, 515–1492, 760 bp
AGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAG
GCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTG
GCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCT
GGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATT
AAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGG
AGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCTGGAGACAGG
CGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC

GCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGG
GTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAAT
GGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCT
GCAACTCGACCCC

Bacterial isolate E_{10}, 515–1492, 697 bp
TAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGA
AATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAA
GCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATT
CCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAG
GAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCT
TGACATACACCGGAAACGGCCAGAGATGGTCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAAGCCCCTTCG
GGGGTGTTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGC
CCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCAAGGTGGAGCGAATCT
CAAAAAGCCGGTCTCAGTTCGGA

Bacterial isolate E_{11}, 515–1492, 701 bp
TTAACCCCGGTTCTGCAGTGGATACGGGCATGCTAGAGGTAGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTG
AAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCCTTACCTGACGCTGAGGAGCGAA
AGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGTGTGGGGACTTT
CCACGGTTTCCGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAG
GAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTT
TGACATCACCCGTGGACCTGCAGAGATGTGGGGTCATTTAGTTGGCGGGTGACAGGTGGTGCATGGCTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCCATGTTGCCAGCACGTAATGGTG
GGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTC
TTGGGCTGCAAACATGCTACAATGGCCGGTACAATGGGCGTGCGATACCGTGAGGTGGAGCGAATCCCTAAAAGC
CGGTCTCAGTTCGGATTGGGGTCTGC

Bacterial isolate E_{12}, 515–1492, 825 bp
TTGGGCGTAAGAGCTCGTAGGCGGTGTGTCGCGTCGGCCGTGAAAACTTGGGGCTTAACTCTGAGCGTGCGGTCG
ATACGGGCATCACTTGAGTTCGGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAG
GAACACCGGTGGCGAAGGCGGGTCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGCGCTAGGTGTGGGGACCATTCCACGGTTTCTGTGCCGCA
GCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGC
ACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATGCACCAGACATCC
CTAGAGATAGGGCTTCCCTTGTGGTTGGTGTGCAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCCATGTTGCCAGCACGTTATGGTGGGGACTCATGGGAGACTGC
CGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACACATGCTA
CAATGGCTCATACAGAGGGCTGCGAGACCGTGAGGTGGAGCGAATCCCTTAAAGTGAGTCTCAGTTCGGATCGGG
GTCTGCAACTCGACCCCGTGAAGTTGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCG
G

Bacterial isolate E_{13}, 515–1492, 780 bp
TTGGGCGTAAGAGCTCGTAGGCGGCTTGTCGCGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTC
GATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAG
GAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGA
TTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCA
GCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCA
CAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCA
TCAGAGATGGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGA
GACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACAC
GTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCAAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGG
ATTGGGGTCTGCAACTCGACCCCATGAAGTCG

Bacterial isolate E_{14}, 515–1492, 656 bp
GGTCTGCAGTCGATACGGGCAGGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCA
GATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGG
AGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGT
CGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACG
GGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACA
CCGGAAAGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGG
GACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTT
GGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATGCCGCGAGGCGGA

Bacterial isolate E_{15}, 515–1492, 783 bp
TTGGGCGTAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCG
ATACGGGCAGGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGG

AACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAG
CTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATC
AGAGATGGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGG
TTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAG
ACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACG
TGCTACAATGGCCGGTACAATGAGCTGCGATGCCGCGAGGCGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGA
TTGGGGTCTGCAACTCGACCCCATGAAGTCGGA

Bacterial isolate E_{16}, 515–1492, 897 bp
TTGGGCGTAAGAGCTCGTAGGCGGCATGTCGCGTCTGCTGTGAAAGACCGGGGCTTAACTCCGGTTCTGCAGTGG
ATACGGGCATGCTAGAGGTAGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGG
AACACCGGTGGCGAAGGCGGGTCTCTGGGCCTTACCTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGAT
TAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGTGTGGGGACTTTCCACGGTTTCCGCGCCGTAGC
TAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACA
AGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACCTGTA
GAGATACAGGGTCATTTAGTTGGTGGGTGACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGG
TTAAGTCCCGCAACGAGCGCAACCCTTGTTCCATGTTGCCAGCACGTAATGGTGGGGACTCATGGGAGACTGCCG
GGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCAAACATGCTACA
ATGGCCGGTACAATGGGCGTGCGATACCGTAAGGTGGAGCGAATCCCTTAAAGCCGGTCTCAGTTCGGATTGGGG
TCTGCAACTCGACCCCATGAAGGTGGAGTCGCTAGTAATCGCGGATCAGCAACGCCGCGGTGAATACGTTCCCGG
GCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGCAACACCCGAAACTTGTGGCCTAACCCTTCGGGGA

Bacterial isolate E_{17}, 515–1492, 817 bp
GTTCTGCAGTGGATACGGGCATGCTAGAGGTAGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCA
GATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCCTTACCTGACGCTGAGGAGCGAAAGCATGGGG
AGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGTGTGGGGACTTTCCACGGTTTC
CGCGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGG
GGGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACC
CGTGGACCTGTAGAGATACAGGGTCATTTAGTTGGTGGGTGACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCCATGTTGCCAGCACGTAATGGTGGGGACTCATG
GGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCA
AACATGCTACAATGGCCGGTACAATGGGCGTGCGATACCGTAAGGTGGAGCGAATCCCTTAAAGCCGGTCTCAGTT
CGGATTGGGGTCTGCAACTCGACCCCATGAAGGTGGAGTCGCTAGTAATCGCGGATCAGCAACGCCGCGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGCAACACCCGAAACTTGTG

Allomerus system

Fungi

Mutualistic fungus *Trimmatostroma cordae* F_1, ITS1-F–ITS4, 915 bp
GCGCTTGCGTCTCGGATAAGTTAATGCATCCTTCTGCAGTGACACTTATTGGAAGCCTTAGATAACCGCCAGAAATG
GTACGGTTCCCGCGACTTTAAACAAGCAGGATCCTAAGGGTTAGTGGTGCCGCCTCTGCTTAATTTATTTAAGCACG
CGGTGCTGCGACACTATCAAATTGCGGGAAGTCCCTTAGAGCCGGCAGTACCGCTCCCTCGTCGAAATGATCGGG
GCAGCACCAGGTTAATGACCTCGGGTATGGTAACAATCTCGCCGGATTGGGTTACCTGCAGCCAAGTCCTCCGCG
GAGGGGTGGTCGTCCACGGGCGGCCGTCTTTCGCGGTCGGATGCAGTTCACAGGCCAAATGGTAGTGGGTGGTC
CCCTTTTGTAAGGGCGATTGCTTAAGATATGGTCGGGCCCCCAGGGAAATCCTGGGGGGTGAGTTCACAAAAGAAT
AAGTAACAAGAACAAACAAAAACGTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTTAGGGTTCTCTCCCG
AGGCCCGACCTCCCAACCCTGTGTCTACCACCTGTGTAACGTGTTGCTTCGGCGGACCGGCAGTCCCCTTACGGG
GGTCCGTCGCCGGGTGTCGCCCCTGGAGAGCGTCCGCCGATGCCCCCAACCAAACAACGCCCAAACCCAAATTAG
CGGACCTAAAACTTCTGAATCAAACCTTTTTGATGTACCAATCAAAAACAAAACTTTCAACAACGGATCTCTTGGTTC
TGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGCGAATTGCAGAATTTCCGTGAGTCATCGAATCTTTGA
ACGCACATTGCGCCCTTTGGCATTCCGAAGGGCATGCCTGTTCGAGCGTCATTATCCTCTCTCAAACCTCGGGTTT
GGTG

Non-cultivar fungus F_2, ITS1-F–ITS4, 540 bp
TACTGAGTGAGGGCCCTCCGGGTCCAACCTCCCACCCGTGTTTATTCGTACCTTGTTGCTTCGGCAGGCCCGCCTC
ACGGCCGCCGGGGGGCTTCTGCCCCCGGGCCCGCGCCTGCCGGAGACAATTCTGAACGCTGTCTGAAGAATGCA
GTCTGAGCGATTAGCAAAATTAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCG
AAATGCGATAATTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATT
CCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGCCCTGTTCCCCCGGG
AACAGGCCCGAAAGGCAGTGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTCACCCGCTCTGTAGG
CCCGGCCGGCGCCCGTCGACCCCCCAACCTTTTTTTTTCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAA
CTTAAGCATATCA

Non-cultivar fungus F_3, ITS1-F–ITS4, 494 bp
TTGCTTCGGCGGGACCGCCCCGGCGCCCTCAGCGGCCCGGAACCAGGCGCCCGCCGGAGGACCCAAACTCTTG
CTTTGCATAGTGGCATTCTCTGAGTCTCACAAACAAAAATAAATCAAAACTTTCAACAACGGATCTCTTGGCTCTGGC

ATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCA
CATTGCGCCCGCCAGCACTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACCCCTAGGGAGCT
TGGTGTTGGGGACCGGCCCGCCCTCTGCGGCGGCCGCCGCCCCCGAAATGCAGTGGCGAACCTCGCCGCAGCCT
CCCCTGCGTAGTAGCACAACCTCGCACCGGAGCGCGGAGACGGTCACGCCGTAAAACGCCCAACTTTCCTAGAGT
TGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATAT

Non-cultivar fungus F_4, ITS1-F–ITS4, 519 bp
TACAAGTTGACCCCGGCCCCCGGGCCGGGATGTTCACAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGGGCGAC
CCTGCCTCCGGGCGGGGGCCCCGGGTGGACACTTCAAAACTCTTGCGTAACTTTGCAGTCTGAGTAAATTTAATTA
ATAAATTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGT
GAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGT
TCGAGCGTCATTTCACCACTCAAGCCTCGCTTGGTATTGGGCGACGCGGTCCGCCGCGCGCCTCAAATCGACCGG
CTGGGTCTTTCGTCCCCTCAGCGTTGTGGAAACTATTCGCTAAAGGGTGCCGCGGGAGGCCACGCCGTAAAACAA
CCCCATTTTCTAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAA

Non-cultivar fungus F_5, ITS1-F–ITS4, 693 bp
TACTGAGTTATCAAAAACTCCAACCCTTTGTGAACCTACCTATGTTTCCTCCGGCGTACCGCTTTAGCCTACCCACA
GGGCTCCCCTAAGGGGGGGTTCTGCTGGGGAGGTGCCTGAGTGCTACCTATCCTTCGGGGTACGGTTAGTGCAGT
GAAGGTGCTGACCAAGGCCTCGGCGGCGCCGAGTAGGACCGCTCCAAACTTAAGCACCTAGTGCATCCAACCCCG
CGTTGAACAACTATCGAAAATCTGCTTTTGCTTTTTTTCTTTACGCTAAAACGTCTTTCCCGGTTGGAATTATTGCTCG
AAATAATAATTTCTTTACCCTGCAGTCGTTTGTTTTCAAGCTACAATATCTGCTCGAAAATTGTTCAAAGCTCTGAGG
GGTCTGAATGAATTCATAAAATTGGCAAAAGCCACCTATAAACTACGGTTCTTAGGGGGTGATCAAACCAAGGTTTT
AAAAACCAAATACGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT
AAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGC
ATGCCTATTCGAGCGTCATTACAACCCTTAAGCCTTGTAGCTTAGCGTTGGGAATCTACCCCTCACTGAGGGGTAGT
T

Bacteria

Bacterial isolate FG_1, 515–1492, 908 bp
GTAAGAGCTCGTAGGCGGCTTGTCACGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGG
GCTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACC
GGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACG
CATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAG
CGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATATACCGGAAAGCATCAGAGAT
GGTGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCTTTCGGGGTGATGGGGACTCACAGGAGACTGCC
GGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTAC
AATGGCCGGTACAATGAGCTGCGATGCCGTGAGGCGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGG
TCTGCAACTCGACCCCATGAAGTCGGAGTTGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGG
CCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGG
AGCTGTC

Bacterial isolate FG_2, 515–1492, 908 bp
GTAAGAGCTCGTAGGCGGCTTGTCACGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGG
GCTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACC
GGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACG
CATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAG
CGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATATACCGGAAAGCATCAGAGAT
GGTGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCTTTCGGGGTGATGGGGACTCACAGGAGACTGCC
GGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTAC
AATGGCCGGTACAATGAGCTGCGATGCCGTGAGGCGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGG
TCTGCAACTCGACCCCATGAAGTCGGAGTTGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGG
CCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGG
AGCTGTC

Bacterial isolate FG_3, 515–1492, 900 bp
CGTAAGAGCTTGTAGGCGGTTCGTCGCGTCGTTCGTGAAAACTCGGGGCTCAACCCCGAGCTTGCGGGCGATACG
GGCGGACTAGAGTACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACAC
CGGTGGCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGAAGCGAAAGCGTGGGTAGCGAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGGTGGGCGCTAGGTGTGGGTTTCCTTCCACGGGATCCGTGCCGTAGCTAAC
GCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC
GGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATACACCAGAAAGCTGTAGAG
ATATGGCCCCCCTTGTGGTTGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAG
TCCCGCAACGAGCGCAACCCTTATCTTATGTTGCCAGCGCGTAATGGCGGGGACTCGTGAGAGACTGCCGGGGTC
AACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATGGC

CGGTACAGAGGGCTGCGATACCGTGAGGTGGAGCGAATCCCTTAAAGCCGGTCTCAGTTCGGATCGGGGTCTGCA
ACTCGACCCCGTGAAGTTGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTG
TACACACCGCCCGTCACGTCATGAAAGTCGGTAACACCCGAAGCCGGTGGCCTAACCCTTGTGGAGGGAGCCGTC

Bacterial isolate FG_4, 515–1492, 908 bp
GTAAGAGCTCGTAGGCGGCTTGTCACGTCGATTGTGAAAGCCCGAGGCTTAACCTCGGGTCTGCAGTCGATACGG
GCTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACC
GGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGGTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACG
CATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAG
CGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATCAGAGAT
GGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACTGCC
GGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTAC
AATGGCAGGTACAATGAGCTGCGAAACCGTGAGGTAGAGCGAATCTCAAAAAGCCTGTCTCAGTTCGGATTGGGGT
CTGCAACTCGACCCCATGAAGTCGGAGTTGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGC
CTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGA
GCTGTC

Bacterial isolate FG_5, 515–1492, 888 bp
GTAAGAGCTCGTAGGCGGCTTGTCGCGTCGGGTGTGAAAGACCGGGGCTTAACCCCGGTTCTGCATTCGATACGG
GCAGGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACC
GGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGGTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACG
CATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAG
CGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATATACCGGAAACGGCCAGAGAT
GGTCGCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGT
CCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCAACACCTTCGGGTGGTTGGGGACTCACAGGAGACCGCC
GGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTAC
AATGGCCGGTACAAAGAGCTGCGAAGCCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGG
TCTGCAACTCGACCCCATGAAGTCGGAGTTGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGG
CCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACC

Bacterial isolate FG_6, 515–1492, 905 bp
AGAGCTCGTAGGCGGCTTGTCACGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCT
AGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGT
GGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCC
TGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCAT
TAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCG
GAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATATACCGGAAAGCATCAGAGATGG
TGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCTTTCGGGGTGATGGGGACTCACAGGAGACTGCCGGG
GTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAAT
GGCCGGTACAATGAGCTGCGATGCCGTGAGGCGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCT
GCAACTCGACCCCATGAAGTCGGAGTTGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCT
TGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAG
CTGTC

Bacterial isolate FG_7, 515–1492, 905 bp
GAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGAGGCTTAACCTCGGGTCTGCAGTCGATACGGGCAG
GCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTG
GCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCT
GGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATT
AAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGG
AGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATCAGAGATGGT
GCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGG
GTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAAT
GGCCGGTACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCT
GCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCC
TTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCCTTGTGGGGAGGG
AGCTGTC

Bacterial isolate FG_8, 515–1492, 914 bp
TTGGGCGTAAGAGCTCGTAGGCGGCTTGTCACGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCG
ATACGGGCTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGG
AACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAG
CTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATATACCGGAAAGCATC

AGAGATGGTGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGG
TTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAG
ACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACG
TGCTACAATGGCCGGTACAATGAGCTGCGATGCCGCGAGGCGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGA
TTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTTGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTT
CCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTG
GGAGGGAGCTGTC

Bacterial isolate FG_9, 515–1492, 895 bp
GCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAG
ACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTG
GCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAG
CACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGC
ATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTC
TCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAG
GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAG
AGCTGCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACA
TGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC
GTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACCTTTTTGGAGCCAGCCGCCTA

Bacterial isolate FG_{10}, 515–1492, 896 bp
TTTAAGGGTCCGTAGGCGGATCTGTAAGTCAGTGGTGAAATCTCACAGCTTAACTGTGAAACTGCCATTGATACTGC
AGGTCTTGAGTAAGGTAGAAGTGGCTGGAATAAGTAGTGTAGCGGTGAAATGCATAGATATTACTTAGAACACCAAT
TGCGAAGGCAGGTCACTATGTCTTAACTGACGCTGATGGACGAAAGCGTGGGGAGCGAACAGGATTAGATACCCT
GGTAGTCCACGCTGTAAACGATGCTAACTCGTTTTTGGGTTTTCGGATTCAGAGACTAAGCGAAAGTGATAAGTTAG
CCACCTGGGGAGTACGAACGCAAGTTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGATTATGTG
GTTTAATTCGATGATACGCGAGGAACCTTACCAAGGCTTAAATGGGAATTGATCGGTTTAGAAATAGACCTTCCTTC
GGGCAATTTTCAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTTAGGTTAAGTCCTGCAACGAGCGC
AACCCCTGTTACTAGTTGCTACCATTAAGTTGAGGACTCTAGTAAGACTGCCTACGCAAGTAGAGAGGAAGGTGGG
GATGACGTCAAATCATCACGGCCCTTACGCCTTGGGCCACACACGTAATACAATGGCCGGTACAGAGGGCAGCTA
CACAGCGATGTGATGCAAATCTCGAAAGCCGGTCTCAGTTCGGATTGGAGTCTGCAACTCGACTCTATGAAGCTGG
AATCGCTAGTAATCGCGCATCAGCCATGGCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGCC
ATGGAAGTCTGGGGTACCTGAAGTCGGTGACCGTAAAAGGAGCTGCCTAGGGTAAAACAG

Bacterial isolate FG_{11}, 515–1492, 899 bp
GAGCTCGTAGGCGGTTTGTCACGTCGTCTGTGAAAACCCGAGGCTTAACCTCGGGCCTGCAGGCGATACGGGCAG
ACTTGAGTACTGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGGTG
GCGAAGGCGGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCCT
GGTAGTCCACGCCGTAAACGGTGGGTACTAGGTGTGGGTTTCCTTCCACGGGATCCGTGCCGTAGCTAACGCATT
AAGTACCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGG
AGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATATAGAGGATCGCCGCAGAGATGTG
GTTTGCCTTGTGCCTTCTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
CAACGAGCGCAACCCTTGTCTCATGTTGCCAGCACGTAATGGTGGGGACTCGTGAGAGACTGCCGGGGTCAACTC
GGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATGGCGCGTA
CAGAGGGCTGCGATACCGTGAGGTGGAGCGAATCCCTTAAAGCGCGTCTCAGTTCGGATTGGGGTCTGCAACTCG
ACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACA
CCGCCCGTCACGTCATGAAAGTCGGTAACACCCGAAGCCGGTGGCCTAACCCCTTGTGGGAGGGAGCTGTCGA

Bacterial isolate FG_{12}, 515–1492, 633 bp
GAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAG
ATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAG
CAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGT
GGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGG
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAA
CTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGA
GATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAG
ACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACG
TGCTACAATGGTCGGTACAAAGGGTTGCC

Bacterial isolate FG_{13}, 515–1492, 918 bp
AAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTCGAAACTGGC
AGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGG
TGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTT
AAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGCGAATTTGGTAGAGATACCTT
GGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACT

GGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATA
CAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCG
ACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACAC
CGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTATGTAGCTTAACCTTCGGGAGGGCGCTTACCACTTTGTG
ATTCATGACTGGGGT

Bacterial isolate FG_{14}, 515–1492, 859 bp
AACCTGGGAATTGCGATGGAAACTGGTCGACTAGAGTGTGGCAGAGGGTAGTGGAATTCCTGGTGTAGCAGTGAA
ATGCGTAGAGATCAGGAGGAACATCCGTGGCGAAGGCGACTGCCTGGGCCAACACTGACACTGAGGCACGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTT
GGCACGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAA
TTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGA
CATGCACGGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACCGTGACACAGGTGCTGCATGGCTGTCGTCAGC
TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGG
AACTCTAAGGAGACCGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCA
GGGCTACACACGTACTACAATGGTAGGGACAGAGGGCTGCAAGCCGGCGACGGTGAGCCAATCCCAGAAACCCTA
TCTCAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATTGCTGC
GGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTTGCACCAGAAGCAGGTAGCTT
AACCTTCGGGAGGGCGCTTGCCACGGTGTGG

Bacterial isolate FG_{15}, 515–1492, 814 bp
CTGGGAACTGCAGTGGAAACTGGACGACTAGAATGTGGTAGAGGGTAGCGGAATTCCTGGTGTAGCAGTGAAATG
CGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACCAACATTGACACTGAGGCACGAAAGCGTG
GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTTGGCA
CGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACATG
TCGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTC
TAAGGAGACCGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGC
TACACACGTACTACAATGGTAGGGACAGAGGGCTGCAAGCCGGCGACGGTAAGCCAATCCCAGAAACCCTATCTC
AGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAGATCAGCATTGCTGCGGTG
AATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTTGTTGCACCAGAA

Bacterial isolate FG_{16}, 515–1492, 879 bp
GCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAA
GCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTG
GCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGAATCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGTT
GACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT
GTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTG
CCTTCGGGAACTCTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAA
CGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGA
GGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAG
AGGGTTGCCAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACT
GCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACACCG
CCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTC

Bacterial isolate FG_{17}, 515–1492, 894 bp
GAGCTCGTAGGCGGTCTGTCGCGTCTGCTGTGAAAACCCGAGGCTCAACCTCGGGCCTGCAGTGGGTACGGGCA
GACTAGAGTGCGGTAGGGGAGATTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGAT
GGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGGGTGGGGAGCAAACAGGCTTAGATACCC
TGGTAGTCCACCCCGTAAACGTTGGGAACTAGTTGTGGGGTCCATTCCACGGATTCCGTGACGCAGCTAACGCATT
AAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGCGG
AGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATATAGAGGAAACGTCTGGAAACAGT
CGCCCCGCAAGGTCTCTATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCG
CAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGAACTCATGGGATACTGCCGGGGTCAACTC
GGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTAC
AAAGGGCTGCAATACCGTGAGGTGGAGCGAATCCCAAAAAGCCGGTCCCAGTTCGGATTGAGGTCTGCAACTCGA
CCTCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGTCTTGTACACAC
CGCCCGTCAAGTCATGAAAGTCGGTAACACCTGAAGCCGGTGGCCCAACCCTTGTGGAGGGAGCCGTC

Bacterial isolate FG_{18}, 515–1492, 897 bp
GCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAG
ACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTG
GCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAG
CACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGC
ATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGACCACTCTGGAGACAGAGCTTT
CCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGTTGACTGCCGGTGACAAACCGG

AGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGACAATACAA
AGGGCAGCTAAACCGCGAGGTCATGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGACTA
CATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCC
CGTCACACCACGAGAGTTTGTAACACCCGAAGCCGGTGGAGTAACCATTATGGAGCTAGCCGTCGA

Bacterial isolate FG_{19}, 515–1492, 919 bp
GCGCGCGTAGGTGGTTTGCCAAGTTGAATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAG
GCTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACATCAGTGG
CGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGG
TAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGGGTCCTTGAGACTTTAGTGGCGCAGCTAACGCATTAAGTT
GACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCAT
GTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTG
CCTTCGGGAACTCTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAA
CGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTAAGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGA
GGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTACAG
AGGGTTGCCAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACT
GCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACACCG
CCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTCGGGAGGACGGTTACCACGGTGTGA
TTCATGACTGGGGTGA

Bacterial isolate FG_{20}, 515–1492, 919 bp
AAGCGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGC
GAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAG
TGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCC
TGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGAATCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAA
GTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAG
CATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATGCAGAGAACTTTCCAGAGATGGATTG
GTGCCTTCGGGAACTCTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC
GTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTCATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACC
GGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGTCGGTA
CAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCG
ACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTACACAC
CGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTCGGGAGGACGGTTACCACGGTGT
GATTCATGACTGGGGT

Bacterial isolate FG_{21}, 515–1492, 884 bp
TTGGGCGTAAGAGCTCGTAGGCGGCTTGTCACGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCG
ATACGGGCTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGG
AACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAG
CTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATATACCGGAAAGCATC
AGAGATGGTGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGG
TTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCTTTCGGGGTGATGGGGACTCACAGGAG
ACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACG
TGCTACAATGGCCGGTACAATGAGCTGCGATGCCGTGAGGCGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGA
TTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTTGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTT
CCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGG

Tetraponera system

Fungi

Mutualistic fungus *Chaetomonium* F_5, ITS1-F–ITS4, 538 bp
TTACAGAGTTGCAAAACTCCCTAAACCATTGTGAACGTTACCCAAACCGTTGCTTCGGCGGGCGGCGCCCAGTGCG
CCCCCCGGCCCCTCGCGGGGCGCCCGCCGGAGGTACCCCAAACCATTGATACTTTATGGCCTCTCTGAGTCTTCT
GTACTGAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAA
GTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCAT
GCCTGTTCGAGCGTCATTTCAACCATCAAGCCCCCGGCTTGTGTTGGGGACCTGCGGCTGCCGCAGGCCCTGAAA
AGCAGTGGCGGGCTCGCTGTCACACCGAGCGTAGTAGATCTCATCTCGCTCCGGGCGTGCTGCGGGTTCCGGCC
GTTAAACCACCTTCATAACCCAAGGTTGACCTCGGATCAGGTAGGAAGACCCGCTGAACTTAAGCATATCAATAAGC
GGAGGAA

Non-cultivar fungus F_1, ITS1-F–ITS4, 546 bp
ATTACCAGAGTGCCTTTTGGCTCTCCAACCCACTGTGAACATACCTACGTTTCCCTCGGCGGGCTCAGCGCGTTGC
GGTTCTGCCGCCTCGCGTCCGCCGGGGGCACCCAAACTCGAATTTATATCGTGTATCTCTGAGGGGCGAAAGCCC
GTAAAACAAATGAATCAAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGAT
AAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCGGCACTCCGGCGGG
CATGCCTGTCCGAGCGTCATTTCAACCCTCGGGCCCACCCCTCGCGGGGAACGGGCCCGGCGTTGGGGACCGGA

GGCCGCCCCGGCGGCACCCGCCCCCTAAATTCAGTGGCGGTCGCGCCGCAGCCTCCCCTGCGTAGTAGCACACC
TCGCACCGGAGAGCGGCACGGCCACGCCTCGAAACCCCCCAATTTTTCAGGTTGACCTCGGATCAGGTAGGAATA
CCCGCTGAACTTAAGCATA

Non-cultivar fungus F_2, ITS1-F–ITS4, 505 bp
TTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCTATTGTTGCCTCGGCGGATCAGCCCGGCCCCGGTAAA
ACGGGACGGCCCGCCAGAGGACCCCTAAACTCTGTTTTTAGTGTAACTTCTGAGTAAAACAAACAAATAAATCAAAA
CTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAA
TTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCAT
TTCAACCCTCAAGCACAGCTTGGTGTTGGGATTCGTGAGGTAAACTCACGGTCCCCAAATTGATTGGCGGTCACGT
CGAGCTTCCATAGCGTAGTAATTACACCTCGTTACTGGTAATCGTCGCGGCCACGCCGTTAAACCCCAACTTCTGAA
TGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATAT

Non-cultivar fungus F_3, ITS1-F–ITS4, 528 bp
TTACACAAATATGAAGGCGGGCTGGAACCTCTCGGGGTTACAGCCTTGCTGAATTATTCACCCTTGTCTTTTGCGTA
CTTCTTGTTTCCTTGGTGGGTTCGCCCACCACTAGGACAAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAAC
AAATTAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGT
AGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGC
CTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTCTTGTCTCTAGCTTTGCTGGAGACTCGCC
TTAAAGTAATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAGCACAAGTCGCACTCTCTATCAGCAAAGGTCTAGCA
TCCATTAAGCCTTTTTTTCAACTTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATA

Non-cultivar fungus F_4, ITS1-F–ITS4, 552 bp
TTACCGAGTGAGGGCCCTCTGGGTCCAACCTCCCACCCGTGTTGCACGAACCTGTGTTGCTTCGGCGGGCCCGCC
GCCTAGGCCGCCGGGGGGCATCCGCCCCCGGGCCCGCGCCCGCCGAAGCCCCCCTTCTGAACGCTGTCTGAAG
TTGCAGTCTGAGAAACTAGCTAAATTAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACG
CAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCTCTG
GTATTCCGGAGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCCCCGTCCCCC
CCGCGCCGGGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTCGTCA
CCCGCTCTTGTAGGCCCGGCCGGCGCCAGCCGACCCCCAACCTTTATATTTTCTCAGGTTGACCTCGGATCAGGTA
GGGATACCCGCTGAACTTAAGCATAT

Non-cultivar fungus F_6 (KY_6), ITS1-F–ITS4, 485 bp
TGTTGCTTCGGCGCTGCGTCCCGCGCCCCCTAGGGGGACGGACCCAGGCGCGCGCCGAGGACCCCAAACTCTTG
GTTTTATTTACGAAGTCTGAGTGGCCTTTTGGCAAACAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGG
CATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC
ACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCCGGCTTGGTGTT
GGGGACCGATCGGCTAGATCCGATCGCCCCCGAAATCTAGTGGCGATCCCACCAGCGCGTCCTCTGCGTAGTAAT
ATACCCCGCTCTGGAACGTGGTGTGGTCATGCCGTTAAACCCCCTACTTTTTAAGGTTGACCTCGGATCAGGTAGG
AATACCCGCTGAACTTAAGCATATCAATA

Bacteria

Bacterial isolate KY_1, 515–1492, 847 bp
TAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGA
AATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAA
GCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATT
CCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAG
GAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCT
TGACATACACCGGAAACGTCTGGAGACAGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTG
GTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCC
TTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAA
AAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAG
CATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAG
CCGGTGGCCCAACCCCTTGTGGG

Bacterial isolate KY_2, 515–1492, 563 bp
ACCCCGGGTCTGCATTCGATACGGGCAGGCTAGAGTTCGGCAGGGGAGATTGGAATTCCTGGTGTAGCGGTGAAA
TGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGC
GTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGCACTAGGTGTGGGCGGCATTCC
ACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGA
ATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTG
ACATACACCGGAAAACTCTGGAGACAGGGTCCCCCTTTGGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTGTGTTGCCAGCATGCCTTTCGGGGT
GATGGGGACTCACGGGAGACTGCCGGGGTCAACTCGGA

Bacterial isolate KY_3, 515–1492, 767 bp
CGTAAGAGCTCGTAGGCGGTTTGTCGCGTCGGCCGTGAAAACCTGCCGCTTAACGGTGGGCGTGCGGTCGATACG
GGCAGACTTGAGTTCGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACAC
CGGTGGCGAAGGCGGGTCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGGTGGGCGCTAGGTGTGGGGACTGTTTCCACGGTTCCTGTGCCGTAGCTAA
CGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAG
CGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATGCACAGGATCGCCCCTGA
GAGGGGGTTTCCCTTGTGGCCTGTGTGCAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCACGTGATGGTGGGGACTCGCAGGAGACTGCCGGG
GTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGCCCAGGGCTTCACACATGCTACAATG
GCTGGTACAGAGGGTTGCGATACCGTGAGGTGGAGCGAATCCTTAAAGCCGGTCTCAGTTCGGATCGGGGTCTGC
AACTCGACCCCGTGAAGT

Bacterial isolate KY_4, 515–1492, 811 bp
TTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTG
AAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAA
AGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACAT
TCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAA
GGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGG
CTTGACATACACCGGAAAGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTC
AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTG
TGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCC
CCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCAAGGTGGAGCGAATCTC
AAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCA
GCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTC

Bacterial isolate KY_5, 515–1492, 837 bp
CTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGT
GAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAA
AGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGAACTAGGTGTTGGCGACAT
TCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAA
GGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGC
TTGACATATACCGGAAAGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAG
CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCCCTTCGGG
GTGATGGGGACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCC
TTATGTCTTGGGCTGCACACGTGCTACAATGGCAGGTACAATGAGCTGCGAAGCCGCGAGGCGGAGCGAATCTCA
AAAAGCCTGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTTGCTAGTAATCGCAGATCAG
CATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAG
CCGGTGGCCC

Bacterial isolate KY_7, 515–1492, 690 bp
TTGGGCGTAAGAGCTCGTAGGCGGTTTGTCGCGTCGGCCGTGAAAACCTGCCGCTTAACGGTGGGCGTGCGGTC
GATACGGGCAGACTTGAGTTCGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAG
GAACACCGGTGGCGAAGGCGGGTCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCGCTAGGTGTGGGGACTGTTTCCACGGTTCCTGTGCCG
TAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATGCACAGGATCGC
CCCTGAGAGGGGGTTTCCCTTGTGGCCTGTGTGCAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCACGTGATGGTGGGGACTCGCAGGAGACTG
CCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGCCCAGGGCTTCACACATGCT
ACAATGGCTGGTACAGA

Bacterial isolate KY_8, 515–1492, 445 bp
CCCCGGGTGTGCAGTGGGTACGGGCAGACTTGAGTGCAGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAAT
GCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCTGTTACTGACGCTGAGGAGCGAAAGCAT
GGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGAACATTCCAC
GTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT
TGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGAC
ATACACCGGACCGGGCCAGAGATGGTCTTTCCCCCTTGTGGGGCTGGTGTACAGGTGGTGCATGGTTGTC

Bacterial isolate KY_9, 515–1492, 584 bp
GGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCG
TAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGG
GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGT
GGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACG
GGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAG
AGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTG
TGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAG
GAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCC

Bacterial isolate KY_{10}, 515–1492, 719 bp
AAGGGTCCGTAGGCGGATGTGTAAGTCAGTGGTGAAATCTCACAGCTTAACTGTGAAACTGCCATTGATACTGCAT
GTCTTGAGTAAGGTAGAAGTGGCTGGAATAAGTAGTGTAGCGGTGAAATGCATAGATATTACTTAGAACACCAATTG
CGAAGGCAGGTCACTATGTCTTAACTGACGCTGATGGACGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGG
TAGTCCACGCTGTAAACGATGCTTACTCGTTTTTGGGGCTTTTGCTTCAGAGACTAAGCGAAAGTGATAAGTAAGCC
ACCTGGGGAGTACGAACGCAAGTTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGATTATGTGGT
TTAATTCGATGATACGCGAGGAACCTTACCAAGGCTTAAATGGGAATTGATCGGTTTAGAAATAGACCTTCCTTCGG
GCAATTTTCAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCCGTGAGGTGTTAGGTTAAGTCCTGCAACGAGCGCAA
CCCCTGTCACTAGTTGCCATCATTCAGTTGGGGACTCTAGTGAGACTGCCTACGCAAGTAGAGAGGAAGGTGGGG
ATGACGTCAAATCATCACGGCCCTTACGCCTTGGGCCACACACGTAATACAATGGCCGGTACAGAGGGCAGCTACA
CAGCGATGTGATGCAAATCTCGAAAGCCGGTCTCA

Bacterial isolate KY_{11}, 515–1492, 711 bp
CAACCGTGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGA
AATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAG
CGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCC
GCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAAT
TGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACA
TCCTTTGAAAACTCTAGAGATAGAGCCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCAC
TCTAGGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGC
TACACACGTGCTACAATGGACAATACAAAGGGCAGCTAAACCGCGAGGTCATGCAAATCCCATAAAGTTGTTCTCA
GTTCGGATTGTAGTCTGCAACTCGACTAC

Bacterial isolate KY_{12}, 515–1492, 796 bp
CTGGGCGTAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCA
AAACTGGCAAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGA
ACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGCCGTTGGAATCCTTGAGATTTTAGTGGCGCAGCTAAC
GCATTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCG
GTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCCTTGACATGCAGAGAACTTTCCAGAGAT
GGATTGGTGCCTTCGGGAACTCTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGAC
AAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAATGGT
CGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCA
ACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATC

Bacterial isolate KY_{13}, 515–1492, 628 bp
GGACTGCGCTTGATACTGGGTTGCTTGAGGATGGAAGAGGCTCGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAG
ATATTGGGAAGAACACCGGTGGCGAAGGCGGCGAGCTGGTCCATTACTGACGCTGAGGCGCGACAGCGTGGGGA
GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTGCGCTGGATGTTGGGGCCCATAGGGTCTCA
GTGTCGTAGCCAACGCGGTAAGCGCACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGG
GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCCCTTGACATGGTC
ACGACCGGTCCAGAGATGGACTTTCCTAGCAATAGGCGTGATGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCTCTAGTTGCCAGCATGTTCGGGTGGGCACT
CTAGAGGAACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGCTGGG
CTACACACGTGCTACAATGGCGGTGACAGA

Bacterial isolate KY_{14}, 515–1492, 652 bp
AACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCAAGTGTAGCGGTGAA
ATGCGTAGAGATTTGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGC
GTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCG
CCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATT
GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACA
TCCTCTGACAACCCTAGAGATAGGGCTTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGC
TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCA
CTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGG
GCTACACACGTGCTACAATGGATGGTACAAAGGGCTGCAAACCTGCGAA

Bacterial isolate KY_{15}, 515–1492, 735 bp
CTGGGCGTAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTG
AAACTGGCAAGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGG
AATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTA
ACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAG
CGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAGA
TGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGA

TAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGG
CGTATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTACGTCGTA

Bacterial isolate KY_{16}, 515–1492, 773 bp
CTGGGCGTAAGAGCTCGTAGGCGGTTTGTCGCGTCGTCTGTGAAATTCTGCAACTCAATTGCAGGCGTGCAGGCG
ATACGGGCAGACTTGAGTACTACAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGG
AACACCGGTGGCGAAGGCGGGTCTCTGGGTAGTAACTGACGCTGAGGAGCGAAAGCGTGGGTAGCGAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGTACTAGGTGTGGGTTCCTTTTCACGGGATCCGTGCCGTAGC
TAACGCATTAAGTACCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACA
AGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATACACCAGACGCGGCTA
GAGATAGTCGTTCCCTTGTGGTTGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTT
AAGTCCCGCAACGAGCGCAACCCTTGTCCTGTATTGCCAGCGGGTTATGCCGGGGACTTGCAGGAGACTGCCGGG
GTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATG
GCTGGTACAGAGGGCTGCGATACCGTGAGGTGGAGCGAATCCCTTAAAGCCAGTCTCAGTTCGGATTGGGGTCTG
CAACTCGACCCCATGAAGTC

Bacterial isolate KY_{17}, 515–1492, 886 bp
TTGGGCGTAAGCGCGCGTAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGG
AAACTGGGAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGG
AACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAA
CGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGC
GGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTTTGAAAACTCTAGAGAT
AGAGCCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGT
TAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGTTGCCATCATTAAGTTGGGCACTCTAGGTTGACTGCCGGTGA
CAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGA
CAATACAAAGGGCAGCTAAACCGCGAGGTCATGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAAC
TCGACTACATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTGTACA
CACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGCCGGTGGAGTAAC

Bacterial isolate KY_{18}, 515–1492, 827 bp
TTGGGCGTAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCCGTGAAAGTCCGGGGCTCAACTCCGGATCTGCGGTGG
GTACGGGCAGACTAGAGTGATGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGG
AACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATT
AGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCT
AACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAA
GCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGGACCGGATCGCCGCA
GAAATGTGGTTTCTCCTTTGGGGCCGGTTCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGG
TTAAGTCCCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCACGTAGTGGTGGGGACTCATGGGAGACTGCCG
GGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAA
TGGCCGGTACAAAGGGTTGCGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGGGGTC
TGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGG

Bacterial isolate KY_{19}, 515–1492, 631 bp
CTGGGAACTGCAGTGGATACTGGGCGACTAGAATGTGGTAGAGGGTAGCGGAATTCCTGGTGTAGCAGTGAAATG
CGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACCAACATTGACACTGAGGCACGAAAGCGTG
GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTTGGGTGCAATTTGGCA
CGCAGTATCGAAGCTAACGCGTTAAGTTCGCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACATG
TCGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCACGTAATGGTGGGAACTC
TAAGGAGACCGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGC
TACACACGTACTACAATGGTAGGGACAGA

Bacterial isolate KY_{20}, 515–1492, 689 bp
AACCTCGGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAGATTGGAATTCCTGGTGTAGCGGTGG
AATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAG
GGTGGGGAGCAAACAGGCTTAGATACCCTGGTAGTCCACCCCGTAAACGTTGGGAACTAGTTGTGGGGTCCATTC
CACGGATTCCGTGACGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGG
AATTGACGGGGACCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTT
GACATATAGAGGAAACGTCTGGAAACAGTCGCCCCGCAAGGTCTCTATACAGGTGGTGCATGGTTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTATGTTGCCAGCACGTAATGGTGGGAA
CTCATGGGATACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGG
GCTTCACGCATGCTACAATGGCCGGTACAAAGGGCTGCAATACCGTGAGGTGGAGCGAATCCCAAAAAGCCGGTC
CCAGTTCGGATTG

Bacterial isolate KY_{21}, 515–1492, 772 bp
TTGGGCGTAAGAGCTCGTAGGCGGTTTGTCGCGTCGGCCGTGAAAACCTGCCGCTTAACGGTGGGCGTGCGGTC
GATACGGGCAGACTTGAGTTCGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAG

GAACACCGGTGGCGAAGGCGGGTCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCGCTAGGTGTGGGGACTGTTTCCACGGTTCCTGTGCCG
TAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATGCACAGGATCGC
CCCTGAGAGGGGGTTTCCCTTGTGGCCTGTGTGCAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCACGTGATGGTGGGGACTCGCAGGAGACTG
CCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGCCCAGGGCTTCACACATGCT
ACAATGGCTGGTACAGAGGGTGGCGATACCGTGAGGTGGAGCGAATCCTTAAAGCCGGTCTCAGTTCGGATCGGG
GTCTGCAACTCGACCCCGTGAAGT

Reference strains

Bacteria

Reference strain *Streptomyces lividans*, 515–1492, 812 bp
TTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTG
AAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAA
AGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACAT
TCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAA
GGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGG
CTTGACATACACCGGAAAGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTC
AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAAGCCCTTC
GGGGTGTTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGC
CCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCAAGGTGGAGCGAATCT
CAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATC
AGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTC

Reference strain *Salmonella enterica*, 515–1492, 865 bp
GAACTGCATTCGAAACTGGCAGGCTTGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTA
GAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGCCCTTGAGGCGTGG
CTTCCGGAGCTAACGCGTTAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGG
GGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAG
AAGAATCCAGAGATGGATTTGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTG
AAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGATTAGGTCGGGAACTCAAAGGA
GACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACAC
GTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGG
ATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTT
CCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGA
GGGCGCTTACCACTTTGTGATTCATGACTGGGGTGA

Reference strain *Escherichia coli*, 515–1492, 813 bp
GGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCG
TAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGG
GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCG
TGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGAC
GGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCC
ACGGAAGTTTTCAGAGATGAGAATGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTG
TTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCA
AAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTA
CACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAG
TCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAG

Reference strain *Bacillus subtilis*, 515–1492, 826 bp
GGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGA
GATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAG
CGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAG
TGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGG
GCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGA
CAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTG
AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTG
ACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACG
TGCTACAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGAT
CGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTC
CCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTAG

Supplementary Information A_2) Candicidin gene sequences

Partial sequences of two bacterial candicidin biosynthesis genes *fscM* and *fscP*. The sequencing data, generated with the gene-specific forward primers, are categorised by ant-system (*Acromyrmex* and *Tetraponera*). The title of each sequence describes the name of the strain, the PCR primer set used, and the length of the sequence.

Candicidin biosynthesis gene *fscM*

Acromyrmex

Bacterial isolate E_8, fscM F–R, 898 bp

AGGTGTACCGCGAGGTCACCCAGTCCTCCCGCTTCAAGGCCCACCGCGCGGTCGTCCGGCAGTCCTACCGGCCC
GCGCTCCAGGCGCGCAAGTACCGCGAGCTGGAGCAGGTCGTCGACGAGGTCCGGCCGGCGCTGATGGTCGTCG
ACTGCGTCGCGGGCTTCGGCGTCGACCTGGCCCTGGCCCGGGGCATCCCGTACGTGCTGAACGTGCCGTTCGTC
GCCAGCAACGTGCTGACCTCGCACAACCCGTTCGGCGCCTCGTACACCCCCAAGAGCTTCCCGGTGCCCAACTCG
GGGCTGCCCGCGCGGATGTCGGTGCGGCAGAAGCTGGCCAACACGCTGTTCAAGTGGCGGACGCTCGGCATGTT
CCTGCACCCGGACATGGCGGCACTGCTGCGCGAGGACGCCGCGATACGCAAGGAGCTGGGCATCGCCCCGCCG
AACGCCATGACCCGGGTCGACGAGGCCGCCGCCGTGGTGTGCTCCTCCGTCGCGGAACTGGACTACCCCTTCGA
CATCCCGGACCGGGTGAGCCTGGTGGGCGCCGTGCTGCCGCCGCTGCCCGAGGCACCGGACGACGACGAGGTC
ACCAGGTGGCTCGACGCGCAGTCGTCGGTGGTCTACATGGGCTTCGGCACCATCACCCGCCTCACCCGCGAGGA
GGTCGCGGCGCTGGTGGAGGTGGCCCGGCGGATGTCCGGCACCCACCAGTTCCTGTGGAAGCTGCCCAAGGAG
CAGCAGCACCTGCTGCCCGAGGCCGGGTCGCTGCCGGACAACCTGCGGGTGGAGAGCTGGGTGCCCTCGCAGC
TCGACGTGCTGGCCCACCCGAACGTCTCCGTCTTCTTCTCGCACGGCGGCGGCAACGCCTACCACGAGGGCGTCT
ACTTCGGTAAGCC

Bacterial isolate E_9, fscM F–R, 901 bp

GCGGTGTACCGCGAGGTCACCCAGTCCTCCCGCTTCAAGGCCCACCGCGCGGTCGTCCGGCAGTCCTACCGGCC
CGCGCTCCAGGCGCGCAAGTACCGCGAGCTGGAGCAGGTCGTCGACGAGGTCCGGCCGGCGCTGATGGTCGTC
GACTGCGTCGCGGGCTTCGGCGTCGACCTGGCCCTGGCCCGGGGCATCCCGTACGTGCTGAACGTGCCGTTCGT
CGCCAGCAACGTCCTGACCTCGCACAACCCGTTCGGCGCCTCGTACACCCCCAAGAGCTTCCCGGTGCCCAACTC
GGGGCTGCCCCGCGCGGATGTCGGTGCGGCAGAAGCTGGCCAACACGCTGTTCAAGTGGCGGACGCTCGGCATGT
TCCTGCACCCGGACATGGCGGCACTGCTGCGCGAGGACGCCGCGATACGCAAGGAGCTGGGCATCGCCCCGCC
GAACGCCATGACCCGGGTCGACGAGGCCGCCGCCGTGGTGTGCTCCTCCGTCGCGGAACTGGACTACCCCTTCG
ACATCCCGGACCGGGTGAGCCTGGTGGGCGCCGTGCTGCCGCCGCTGCCCGAGGCACCGGACGACGACGAGGT
CACCAGGTGGCTCGACGCGCAGTCGTCGGTGGTCTACATGGGCTTCGGCACCATCACGCGCCTCACCCGCGAGG
AGGTCGCGGCGCTGGTGGAGGTGGCCCGGCGGATGTCCGGCACCCACCAGTTCCTGTGGAAGCTGCCCAAGGAG
CAGCAGCACCTGCTGCCCGAGGCCGGGTCGCTGCCGGACAACCTGCGGGTGGAGAGCTGGGTGCCCTCGCAGC
TCGACGTGCTGGCCCACCCGAACGTCTCCGTCTTCTTCTCGCACGGCGGCGGCAACGCCTACCACGAGGGCGTCT
ACTTCGGTAAGCCGC

Bacterial isolate E_{11}, fscM F–R, 737 bp

GCACGGGGCGGTTGCGTCTCATGCCCGTATTGTCGCACCTCCGGGGGGTGGGAGCCAAAAGGCGGGTGGCCGC
GGGCGTGCGCGGGGTTCCAGGGTCAAGGCCTTGACCATCGCCGGTTCGGGCCCGATGCGCGATCGAGATGCCG
GTGCTCTTGGAAATGCGGATCGGCCCGTGTTAATCGGGGTCGCCGCTGGTCGCCGGGTTTCTCCTTCCAGGGGAA
ATGCGTATTTGATTCCCGTCGCCCGGAGTGTGAGGATGTCCCGTGCCCTGCCAGCGGAAAGGCCGGGTGAACTGG
TCGGACCGGGGGCTGGACCGCCGGACGGCCCCGCAAGCGTGAGGATGAGGCCTGGTGTCGTTTCTCGACAGGG
AGAAGAGCGTCGCCCCACCGGGGTACAGCCGCTGGTTCGTGCCGCCCGCGGCTCTCGCGGTGCACTTGTCCATC
GGCCAGGTGTACGCGTTCAGTGTGTTCAAGGCGCCGTTGGTGGAGCGTTTCGATTCGTCGTTGACGGCGATCGGC
GTGGTGTTCAGCATCGCCATTGTCATGCTGGGGCTGTCGGCCGCCTTCGGCGGCAAGTGGGTGGAGGCCAACGG
GCCGCGCAAGGCCATGTTCGTGTCGGGCCTGTGCTGGACGACCGGGTTCGCGGTGGGCTCGCTGGGCGTGGCC
ACCGGGCAGCTGTGGCTGCTGTACCTGGGGTACGGGGTCATCGGCGGCATCAGCCTGACCCTGGACAAGCCG

Tetraponera

Bacterial isolate KY_1, fscM F–R, 844 bp

TCCTACCGGCCCGCGCTCCAGGCGCGCAAGTACCGCGAGCTGGAGCAGGTCGTCGACGAGGTCCGGCCGGCGC
TGATGGTCGTCGACTGCGTCGCGGGCTTCGGCGTCGACCTGGCCCTGGCCCGGGGCATCCCGTACGTGCTGAAC
GTGCCGTTCGTCGCCAGCAACGTCCTGACCTCGCACAACCCGTTCGGCGCCTCGTACACCCCCAAGAGCTTCCCG
GTGCCCAACTCGGGGCTGCCCGCGCGGATGTCGGTGCGGCAGAAGCTGGCCAACACGCTGTTCAAGTGGCGGAC
GCTCGGCATGTTCCTGCACCCGGACATGGCGGCACTGCTGCGCGAGGACGCCGCGATACGCAAGGAGCTGGGCA
TCGCCCCGCCGAACGCCATGACCCGGGTCGACGAGGCCGCCGCCGTGGTCTGCTCCTCCGTCGCGGAACTGGAC
TACCCCTTCGACATCCCGGACCGGGTGAGCCTGGTGGGCGCCGTGCTGCCGCCGCTGCCCGAGGCACCGGACGA
CGACGAGGTCACCAGGTGGCTCGACGCGCAGTCGTCGGTGGTCTACATGGGCTTCGGCACCATCACCCGCCTCA
CCCGCGAGGAGGTCGCGGCGCTGGTGGAGGTGGCCCGGCGGATGTCCGGCACCCACCAGTTCCTGTGGAAGCT
GCCCAAGGAGCAGCAGCACCTGCTGCCCGAGGCCGGGTCGCTGCCGGACAACCTGCGGGTGGAGAGCTGGGTG
CCCTCGCAGCTCGACGTGCTGGCCCATCCGAACGTCTCCGTCTTCTTCTCGCACGGCGGCGGCAACGCCTACCAC
GAGGGCGTCTACTTCGGTAAGCCGCAGGTCG

Bacterial isolate KY_5, fscM F–R, 777 bp

CCGGGCCGGGGCTTCCTGCCCGCGCCCGGCACCGTCACCGCCTTCGCGCCGCCCTCGGGCCCCGGTGTCCGCC
TGGACGCGGGCGTGGAGTCGGGCTCGGTCATCGGCCCGGCCTGGGACTCGCTCCTGGCCAAGCTGATCGTGACC
GGTGCGACGCGCGAGCAGGCGCTGCAGCGTGCGGCGCGCCCTGGACGAGTTCACCGTCGAGGGCATGGCCA
CCGCCATCCCCTTCCACCGCGCAGTGGTCAGGGACCCGGCTTTCGCGCCCGAACTGACCGGCTCCAGCGGCCCG
TTCACCATCTTCACGCGGTGGATCGAGACCGAGTTCGTCAACGAGATCCCGGCCTTCGCCGCGCCCGCGGCGGAC

GCGGAGACCGATGAGGAGCCGGGCCGCGAGACGGTCGTCGTCGAGGTCGGCGGCAAGCGCCTGGAAGTCTCCC
TGCCGTCCTCCCTCGGCATGACGATCGCGCGGACCGCGGCGGCGGGCGGCGCCCGCCCCAAGCGGCGCGCGG
CCAAGAAGTCCGGCCCCGCCGCCTCCGGCGACACCCTCGCCTCGCCCATGCAGGGCACGATCGTCAAGGTCGCG
GTCGAGGAGGGCCAGGAGGTCAACGAGGGCGACCTGATCGTGGTCCTCGAAGCGATGAAGATGGAACAGCCCCT
CAACGCCCACCGGTCGGGCACGATCAAGGGCCTCACGGCGGAGGTGGGCGCGAGCCTGACCTCCGGTGCGGTC
ATCTGTGAGATCAAGGACTGACTCGGCGACGGGGTTCCCCGG

Candicidin biosynthesis gene *fscP*

Acromyrmex

Bacterial isolate E_8, fscP F–R, 887 bp
GGCGCTCGGCGCGCTCCGGGTTGAAGGCGTGCGGGCAGCCGAACGCCTCGGTGTCGTGGTTGGCGGAGGCGAG
CAGCGGCACGATGCCCTCGCCCTTGCGGATGGTCTGGCCGGCGATCTCCACGTCGTCCACGGCGACGCGCAGCG
CCACCATGTCGGCCACCGAGTGCAGGCGCAGCAGCTCCTCCACGAGCCGGTCGTCGCCGATCCACTCCCGGTGC
GAGAGCAGCGTGACGACGCCGAGCCCGATGTTGTTCGCCGTGGTCTCGTGGCCCGCGATCAGCAGGAGCAGCAG
GACACCCGACAGCTCCTCGTCCGAGAGCTTGCCGGTCGACAGCAGGCGGCTGATGAGGTCCTCGCCGCGCCACT
TCTTCTTGATGACGATGAGCCGGTTGATGTACCGCAGGAGCTGCCGGGTGGCCTCGGCGCGCTCCTCGTCGGTCG
AGGTGCGGATGGCGACCAGCGTGCGGGTGCGGGACTCGAAGAAGTCCCGGTCCGCCGACGGCACGCCCAGCAG
CGCGGAGATGACCAGGGACGGCACGGGCAGCGCGAAGTCGTTCACCAGGTCCGCGGTGTTCCCGCCGGCCAGC
ATCGCGTCGATCCGCTCGTCCACGGTCCGTTCGATCGCCGGCCGCAGCTCACGGACGCGGCGCACGGTGAACTC
GGGGATCAGCACCTTGCGGAAACGGTCGTGCTCGGGGGAGTCCAGACCGACGAACCAGCCGGGGATCTGCTCCT
GCTTGGGCACCCCCATCGTCTCGCCGACGTTGGGGAAGCCCTCGTTGCCGGGGTTGGCGCTGATCCGGGGATGG
GTGAGGACCGCGCGCACGTCCTCGTGCCGGGTCACCAGCCAGATCGGCCGGCCGTTCGGCAGGTGCGTCTGGAC
C

Bacterial isolate E_9, fscP F–R, 887 bp
GGCGCTCGGCGCGCTCCGGGTTGAAGGCGTGCGGGCAGCCGAACGCCTCGGTGTCGTGGTTGGCGGAGGCGAG
CAGCGGCACGATGCCCTCGCCCTTGCGGATGGTCTGGCCGGCGATCTCCACGTCGTCCACGGCGACGCGCAGCG
CCACCATGTCGGCCACCGAGTGCAGGCGCAGCAGCTCCTCCACGAGCCGGTCGTCGCCGATCCACTCCCGGTGC
GAGAGCAGCGTGACGACGCCGAGCCCGATGTTGTTCGCCGTGGTCTCGTGGCCCGCGATCAGCAGGAGCAGCAG
GACACCCGACAGCTCCTCGTCCGAGAGCTTGCCGGTCGACAGCAGGCGGCTGATGAGGTCCTCGCCGCGCCACT
TCTTCTTGATGACGATGAGCCGGTTGATGTACCGCAGGAGCTGCCGGGTGGCCTCGGCGCGCTCCTCGTCGGTCG
AGGTGCGGATGGCGACCAGCGTGCGGGTACGGGACTCGAAGAAGTCCCGGTCCGCCGACGGCACGCCCAGCAG
CGCGGAGATGACCAGGGACGGCACGGGCAGCGCGAAGTCGTTCACCAGGTCCGCGGTGTTCCCGCCGGCCAGC
ATCGCGTCGATCCGCTCGTCCACGGTCCGTTCGATCGCCGGCCGCAGCTCACGGACGCGGCGCACGGTGAACTC
GGGGATCAGCACCTTGCGGAAACGGTCGTGCTCGGGGGAGTCCAGACCGACGAACCAGCCGGGGATCTGCTCCT
GCTTGGGCACTCCCATCGTCTCGCCGACGTTGGGGAAGCCCTCGTTGTCGGGGTTGGCGCTGATCCGGGGATGG
GTGAGGACCGCGCGCACGTCCTCGTGCCGGGTCACCAGCCAGATCGGCCGGCCGTTCGGCAGATGCGTCTGGAC
C

Tetraponera

Bacterial isolate KY_1, fscP F–R, 798 bp
GGAGGCGAGCAGCGGCACGATGCCCTCGCCCTTGCGGATGGTCTGGCCGGCGATTTCCACGTCGTCCACGGCCA
CTCGCAGCGCCACCATGTCGGCCACCGAGTGCAGGCGCAGCAGCTCCTCCACGAGCCGGTCGTTGCCGATCCAC
TCCCGGTGCGAGAGCAGCGTGACGACGCCGAGCCCGATGTTGTTCGCCGTGGTCTCGTGGCCCGCGATCAGCAG
GAGCAGCAGGACCCCCGACAGCTCCTCGTCCGAGAGCTGGCCCGTCGACAGCAGGCGGCTGATGAGGTCCTCGC
CGCGCCACTTCTTCTTGACGACGATGAGCCGGTTGATGTAGCGCAGGAGCTGCCGGGTGGCCTCGGCGCGCTCC
TCGTCGGTCGAGGTGCGGATGGCGACCAGCGTGCGGGTGCGGGACTCGAAGAAGTCCCGGTCCGCCGACGGCA
CGCCCAGCAGCGAGGAGATGACCAGGGACGGCACGGGCAGCGCGAAGTCGGTCACCAGGTCCGCGGTGTTCCC
CCCGGCCAGCATCGCGTCGATCCGCTCGTCCACGGTCCGCTCGATCGCCGGCCGCAGCTCACGGACGCGGCGCA
CGGTGAACTCGGGGATGAGCACCTTGCGGAAACGGTCGTGCTCGGGGGAGTCCAGACCGACGAACCAGCCGGGG
ATCTGCTCCTGCTTGGGCACCCCCATCGTCTCGCCGACGTTGGGGAAGCCCTCGTTGTCGGGATTCGCGCTGATC
CGGGGATGGGTGAGGACCGCGCGCACGTCCTCGTGCCGGGTCACCAGCCAGATCGGCCG

Supplementary Information A_3) Extended bacterial rDNA gene sequences

Partial sequences of the bacterial 16S rDNA gene. The sequencing data, generated with the gene-specific forward primers, are categorised by ant-system (*Acromyrmex* and *Tetraponera*). In particular, the sequences consist of two overlapping and merged forward reads of amplicons generated with the 515–1492 and fD2–16Sr primer sets. The title of each sequence describes the name of the strain, the PCR primer sets used, and the length of the sequence.

Acromyrmex system

Bacteria

Bacterial isolate E_2, 515–1492 merged with fD2–16Sr, 1228 bp

AGTCGACGATGAAGCCGCTTCGGTGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCAC
TCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATAACACTCTGTCCCGCATGGGACGGGGTTAAAAGCTCC
GGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGC
CGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTT
TCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTA
ATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGATGTGA
AAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTG
GTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCT
GAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTG
TTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCT
AAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACC
TTACCAAGGCTTGACATATACCGGAAAGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGG
CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCAT
GCCCTTCGGGGTGATGGGGACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTC
ATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATGCCGCGAGGCGGA
GCGAATCTCAAAAAGCCGGTCTCAGTTCGGA

Bacterial isolate E_4, 515–1492 merged with fD2–16Sr, 1189 bp

TGCAGTCGAGCGGTAAGGCCCTTTCGGGGGTACACGAGCGGCGAACGGGTGAGTAACACGTGGGTGACCTGCCC
TCCACTCTGGGATAAGCCCGGGAAACTGGGTCTAATACCGGATAGGACCTCTCAACGCATGTTGGGTGGTGGAAA
GTTTTTTCGGTGGGGGATGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGGTGACGG
GTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATATTGCGCAATGGGCGGAAGCCTGACGCAGCGACGCCGCGTGGGGGATGACGGCCTTCGGGTTGTAAA
CCTCTTTCGCCAGGGACGAAGCTTTTGTGACGGTACCTGGAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCG
CGGTAACACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTGTGTCGCGTCGGC
CGTGAAAACTTGGGGCTTAACTCTGAGCGTGCGGTCGATACGGGCATCACTTGAGTTCGGCAGGGGAGACTGGAA
TTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCCGATACT
GACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGCG
CTAGGTGTGGGGACCATTCCACGGTTTCTGCGCCGCAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCC
GCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGC
GAAGAACCTTACCTGGGTTTGACATGCACAGGATCGCGGCAGAGATGTCGTTTCCCTTGTGGCCTGTGTGCAGGTG
GTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTCCATGTT
GCCAGCACGTAGTGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGT
CATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATGGCTCATACAGAGGGCTGCGAGACCGTGA

Bacterial isolate E_6, 515–1492 merged with fD2–16Sr, 1262 bp

TGCAGTCGACGATGAACCGCTTTCGGGCGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTG
CACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATACTCACTGCCTTGGGCATCCTTGGTGGTGGAAAG
CTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGG
TAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACC
TCTTTCAGCAGGGAAGAAGCGAGAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGC
GGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTT
GTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAAT
TCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTG
ACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCAC
TAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGC
AAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGA
AGAACCTTACCAAGGCTTGACATACACCGGAAACGGCCAGAGATGGTCGCCCCCTTGTGGTCGGTGTACAGGTGG
TGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTG
CCAGCAAGCCCTTCGGGGTGTTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGAC
GTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCG
AGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTC

Bacterial isolate E_8, 515–1492 merged with fD2–16Sr, 1312 bp

TGCAGTCGAACGATGAACCGCTTTCGGGCGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCT
GCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGACTGTCCATCGCATGGTGGATGGTGTAAAG
CTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAGTGGCTCACCAAGGCGACGACGGG
TAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACC
TCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTG
TGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTC
CTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGAC

GCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTA
GGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAA
GGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAG
AACCTTACCAAGGCTTGACATACACCGGAAACGTCTGGAGACAGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTG
CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGC
CAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGT
CAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAG
GTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCT
AGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGG

Bacterial isolate E_9, 515–1492 merged with fD2–16Sr, 1249 bp
AGTCGACGATGAACCGCTTTCGGGCGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCAC
TCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGACTGTCCATCGCATGGTGGATGGTGTAAAGCTCC
GGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAGTGGCTCACCAAGGCGACGACGGGTAGC
CGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTT
TCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTA
ATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGA
AAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCT
GGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACG
CTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAG
GTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAG
GCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGA
ACCTTACCAAGGCTTGACATACACCGGAAACGTCTGGAGACAGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTGC
ATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCA
GCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCA
AGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGT
GGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCC

Bacterial isolate E_{10}, 515–1492 merged with fD2–16Sr, 1170 bp
AATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATTGACCTTCACGGGCATCTGTGA
GGTTCGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGC
GACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAG
GCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGG
GTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCC
AGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTC
ACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGA
GATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGG
CCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG
GTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGT
ACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACG
CAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGGCCAGAGATGGTCGCCCCCTTGTGGTCGGTGT
ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGT
CCCGTGTTGCCAGCAAGCCCCTTCGGGGGTGTTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGG
TGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTG
CGATACCGCAAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGA

Bacterial isolate E_{13}, 515–1492 merged with fD2–16Sr, 1198 bp
AATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATACTGACCCTCGCAGGCATCTGCG
AGGTTCGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGG
CGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA
GGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCG
GGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGC
CAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGT
CGCGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGG
AGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGG
GCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAG
TACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGAC
GCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTG
TACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTG
TCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTG
GGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGC
GATACCGCAAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAG
TCG

Bacterial isolate E_{14}, 515–1492 merged with fD2–16Sr, 1200 bp
TGCAAGTCGAACGATGAAGCCCTTCGGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTT
CACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATAATACTTTCTCCCTCCTGGGAGAAGGTTGAAAGCT
CCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTAGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTA

GCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTC
TTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTG
AAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTGTGGTAGGGGAGATCGGAATTCCT
GGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGC
TGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGT
GTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGC
TAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAAC
CTTACCAAGGCTTGACATACACCGGAAAGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCAT
GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGC
ATGCCCTTCGGGGTGATGGGGACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAG
TCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATGCCGCGAGGCG
GA

Tetraponera system

Bacteria

Bacterial isolate KY_1, 515–1492 merged with fD2–16Sr, 1349 bp
TAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAAT
ACCGGATATGACTGTCCGCCGCATGGTGGATGGTGTAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCA
GCTTGTTGGTGAGGTAGTGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGC
GACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACGGTACCT
GCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATT
GGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCG
ATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGG
AACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGCAACATTCCACGTTGTCCGTGCCGCAG
CTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCAC
AAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCT
GGAGACAGGCGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGG
TTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGA
CCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGT
GCTACAATGGCCGGTACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATT
GGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCC
CGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGG

Bacterial isolate KY_2, 515–1492 merged with fD2–16Sr, 1040 bp
GCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGACCACCGGCCGCATGGTC
TGGTGGTGGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCA
AGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACG
GGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCT
TCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGCGAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCC
TGTCGCGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCAGGCTAGAGTTCGGCAG
GGGAGATTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTC
TGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGTTGGGCACTAGGTGTGGGCGGCATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGG
GAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTC
GACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAACTCTGGAGACAGGGTCCCCCTTTGGGTCG
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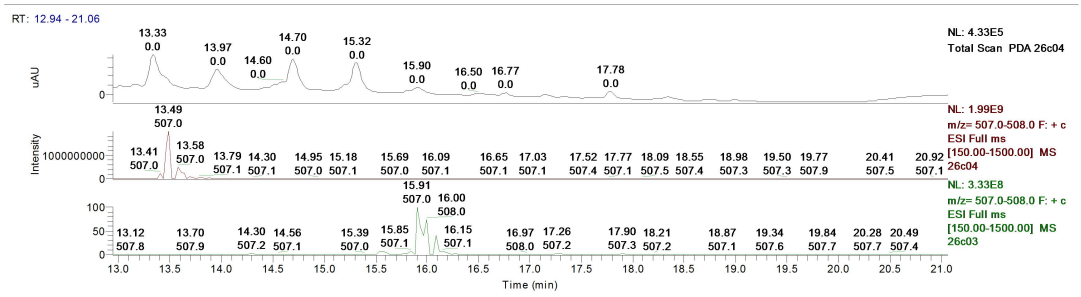
Bacterial isolate KY_5, 515–1492 merged with fD2–16Sr, 1307 bp
AATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATAATACCGTCTCTCGCATGGGAGAG
GGTTGAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAGAAGCTCACCAAGGCG
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CAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGG
TTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCA
CGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTGTGGTAGGGGAG
ATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGC
CATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGG
TGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTA
CGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGC
AACGCGAAGAACCTTACCAAGGCTTGACATATACCGGAAAGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTATAC
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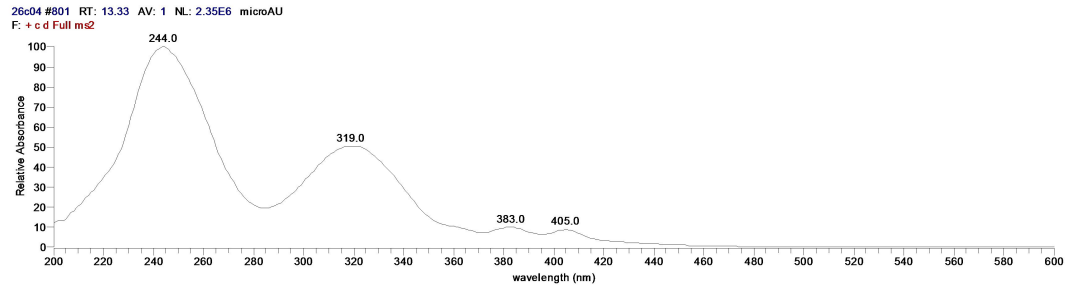
GTGTTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGG
ACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCAGGTACAATGAGCTGCGAAG
CCGCGAGGCGGAGCGAATCTCAAAAAGCCTGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGG
AGTTGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCA
CGAAAGTCGGTAACACCCGAAGCCGGTGGCCC

Supplementary Information B_1) LCMS on double-purified combined fractions B

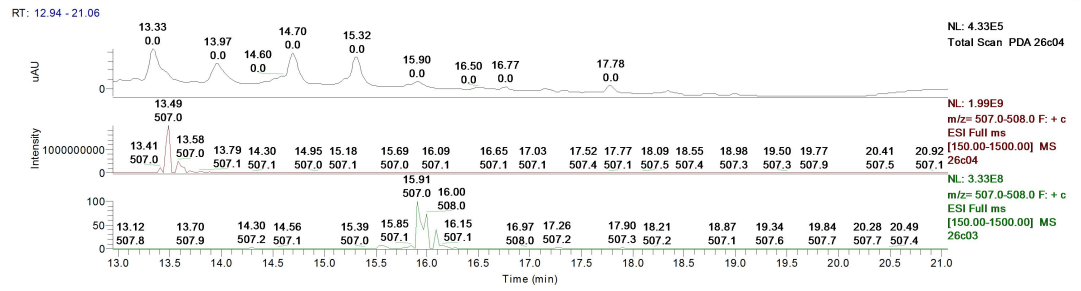
Low-resolution LCMS data on the double-purified combined fractions B of *Streptomyces* E_8. UV, MS, and MS^2 data are presented on ions with masses of 507 amu, 521 amu, 535 amu, 549 amu, 563 amu, (805 amu), and 577 amu $[M+H]^+$. The LCMS system used for generating these data has been located at the John Innes Centre and was set to detect ions in positive ion mode (+ESI).

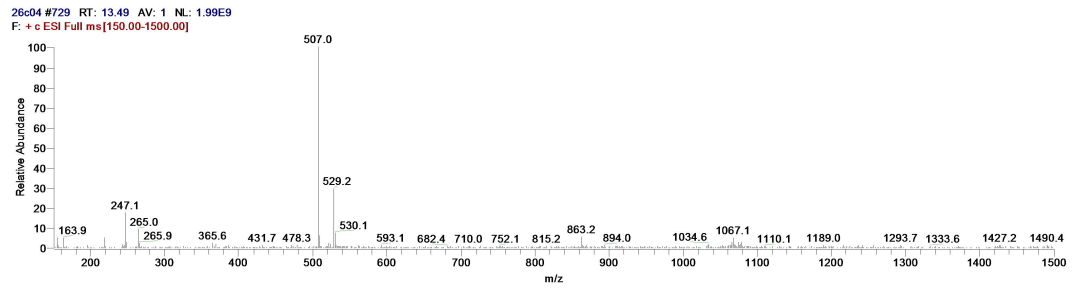
507 amu (+ESI)



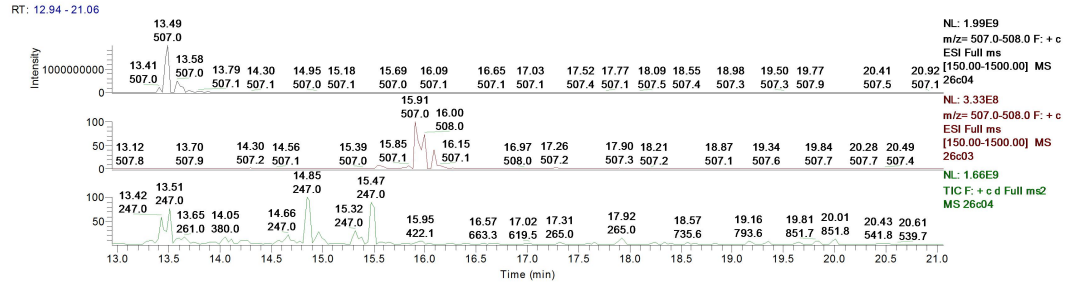


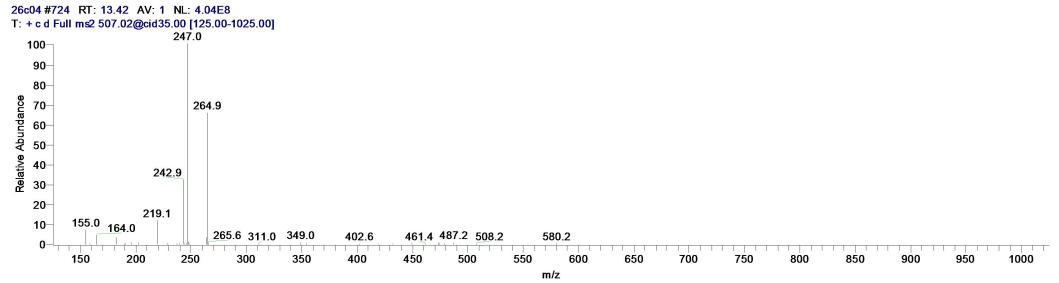
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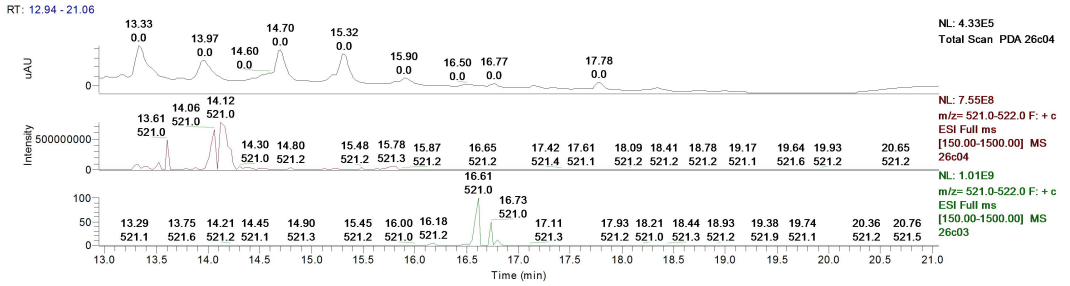


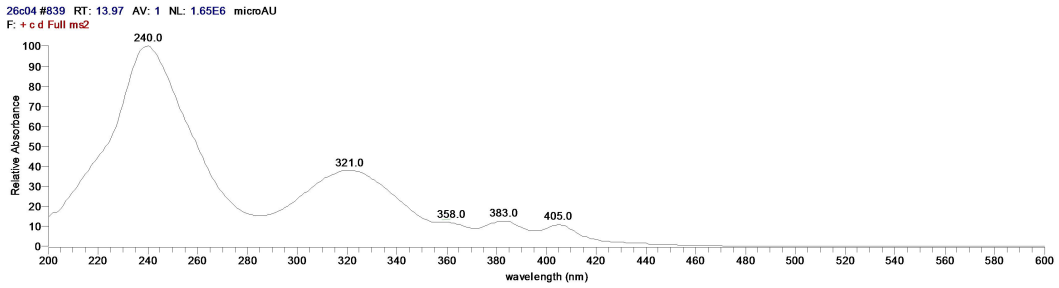
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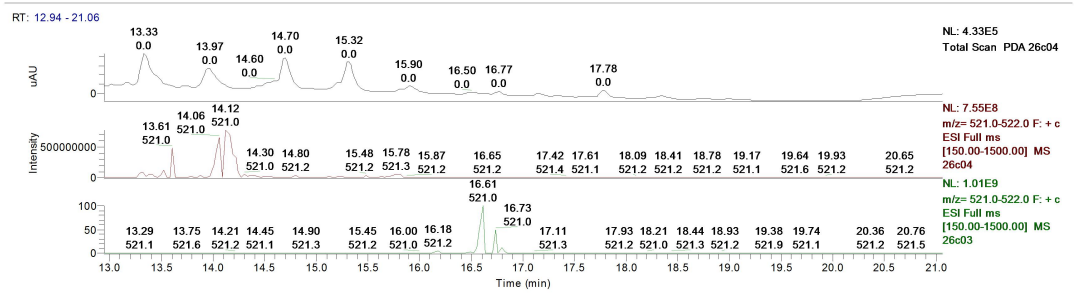


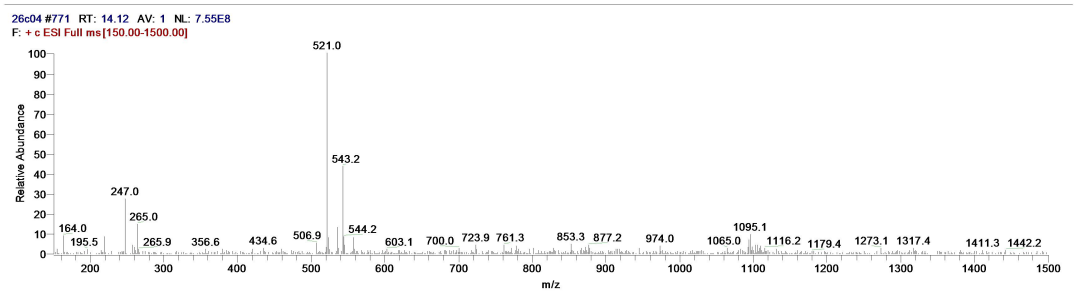
521 amu (+ESI)



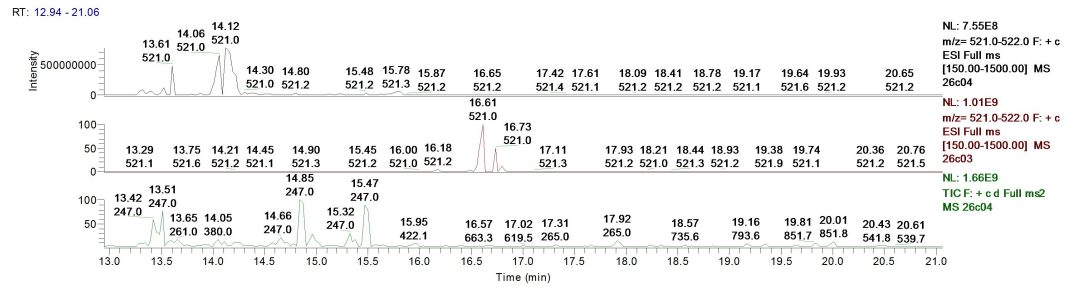


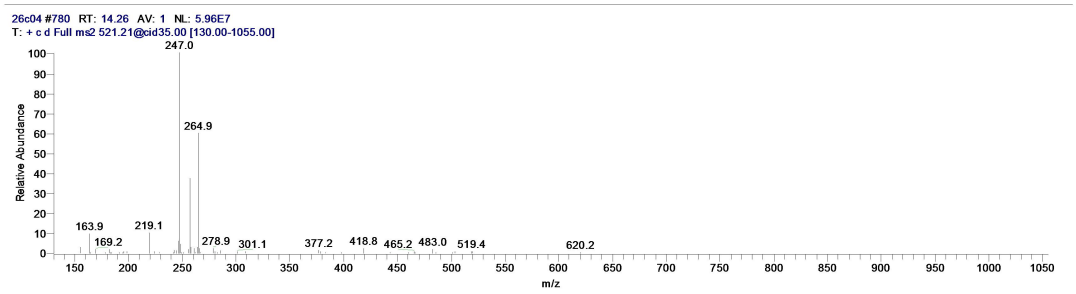
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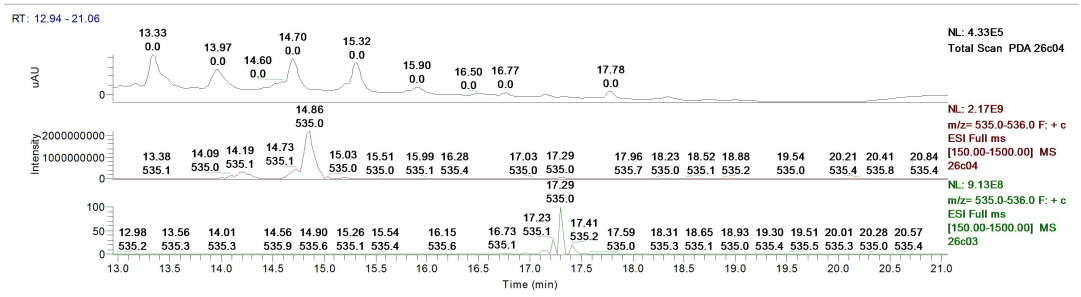


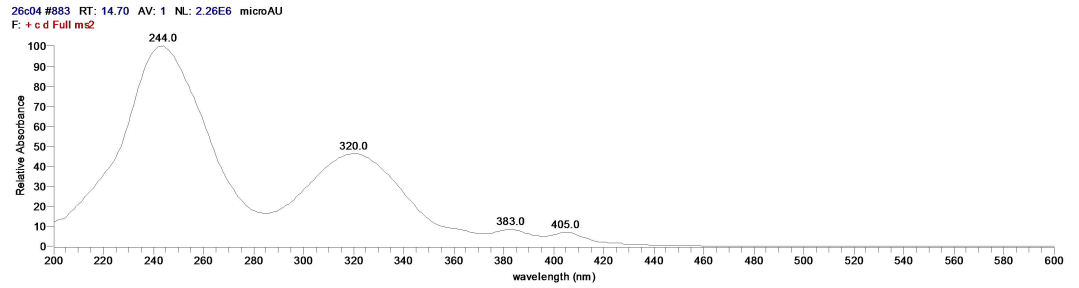
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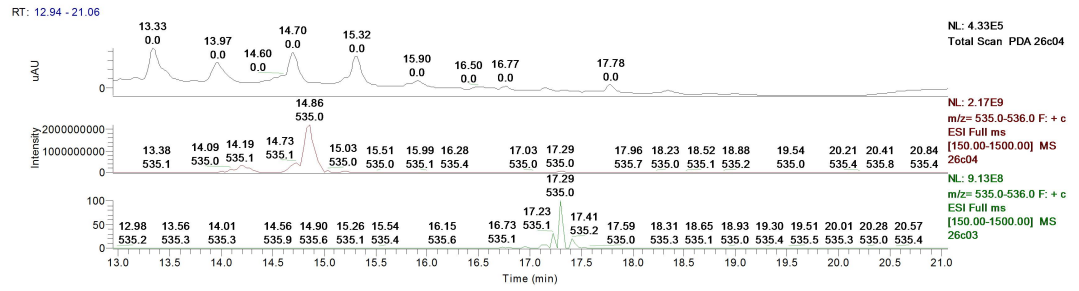


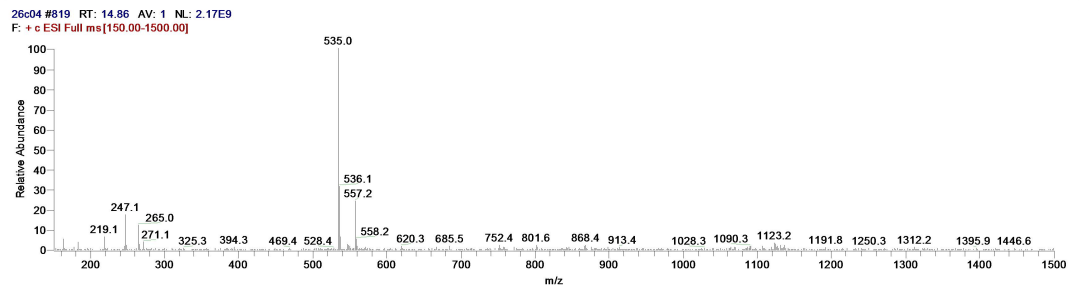
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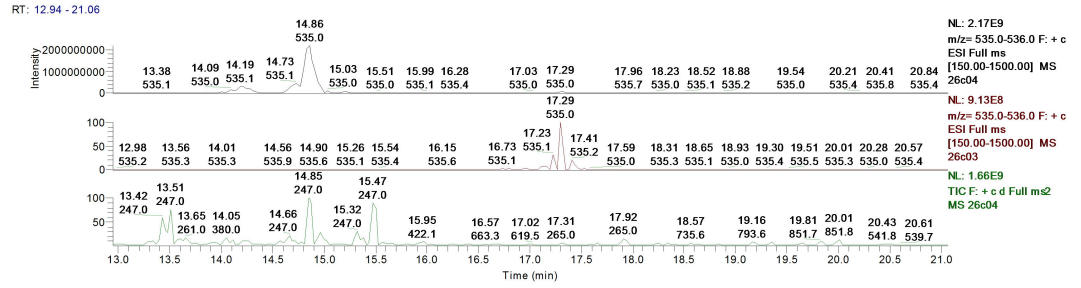


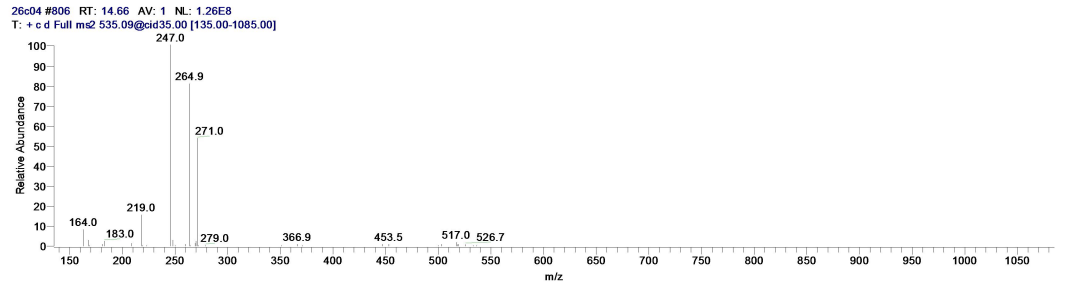
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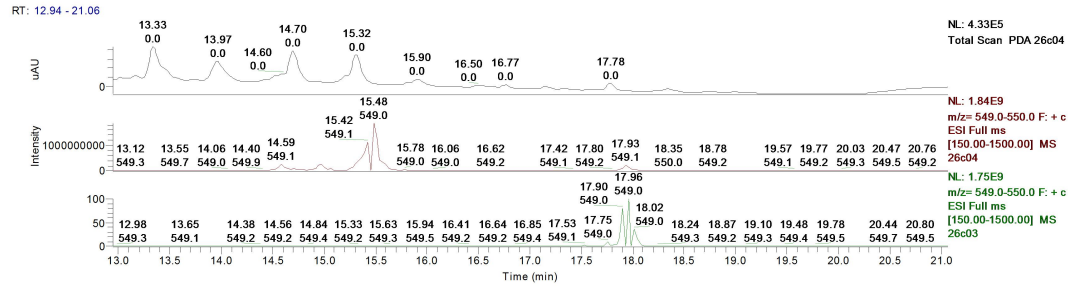


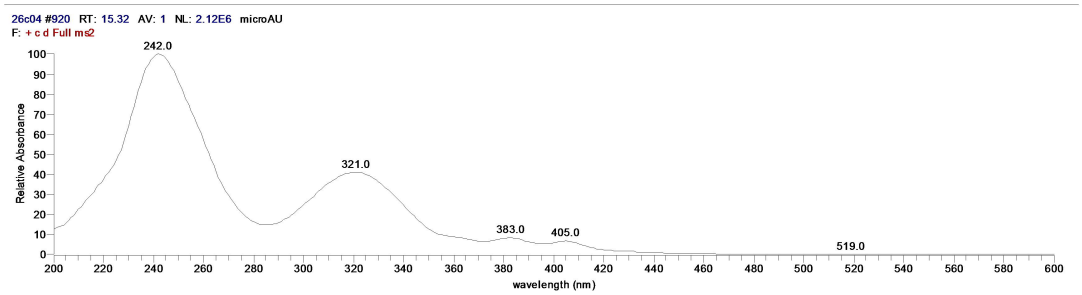
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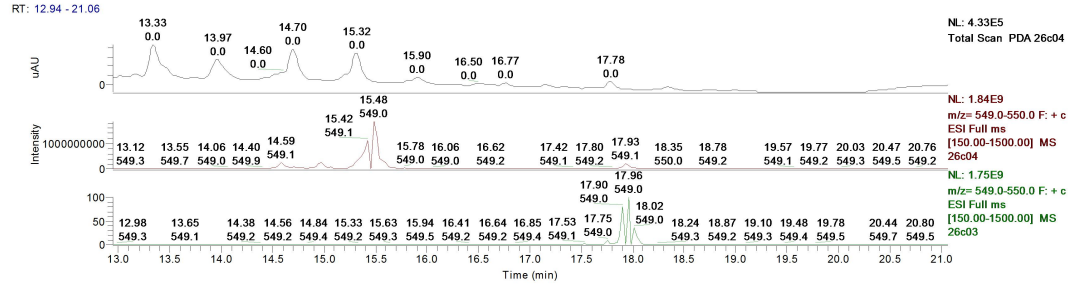


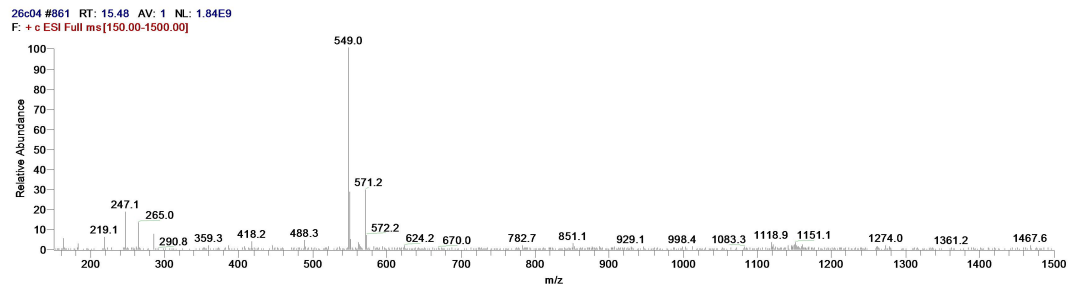
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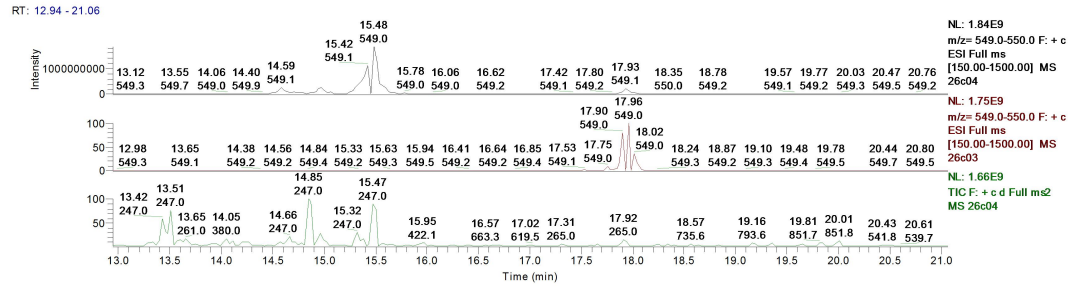


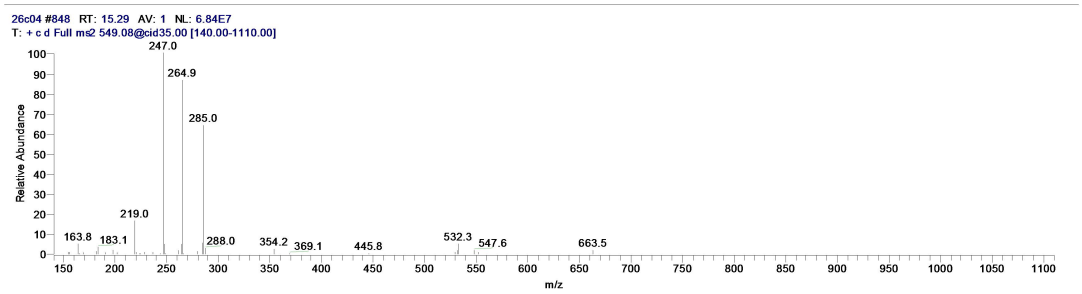
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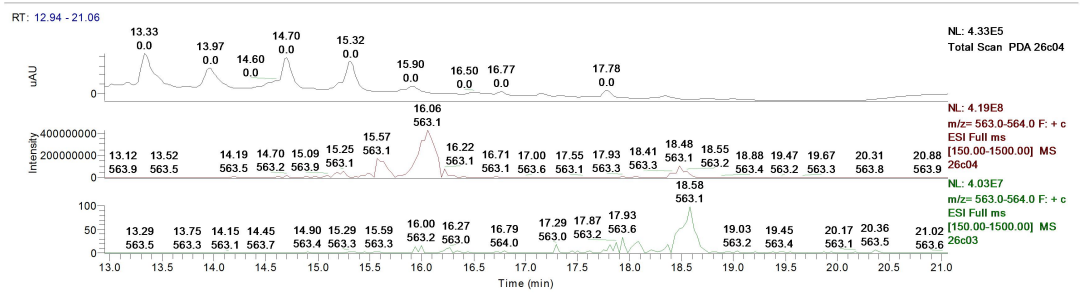


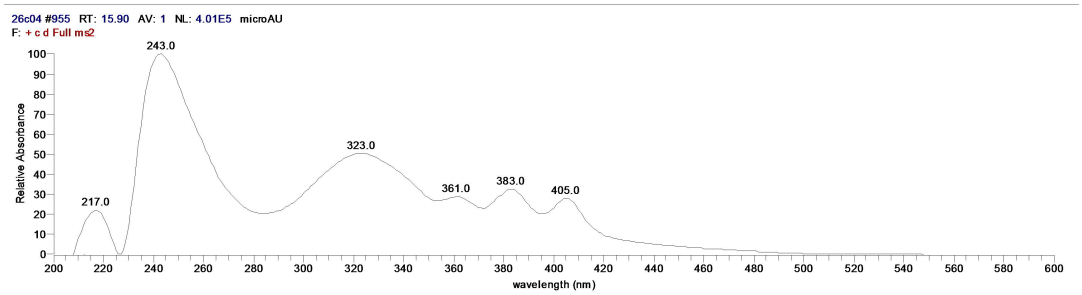
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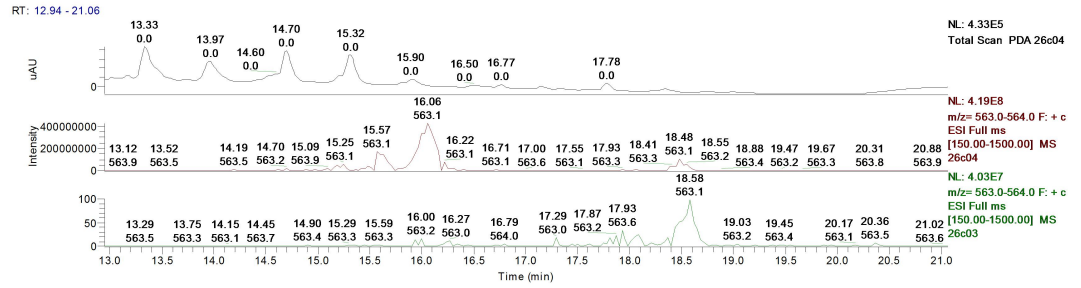


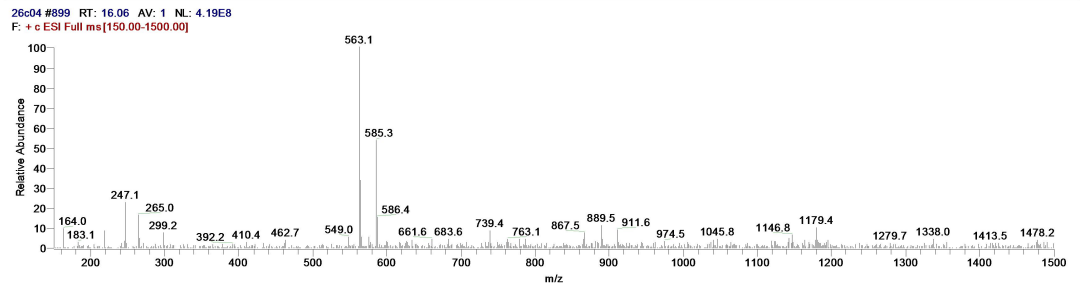
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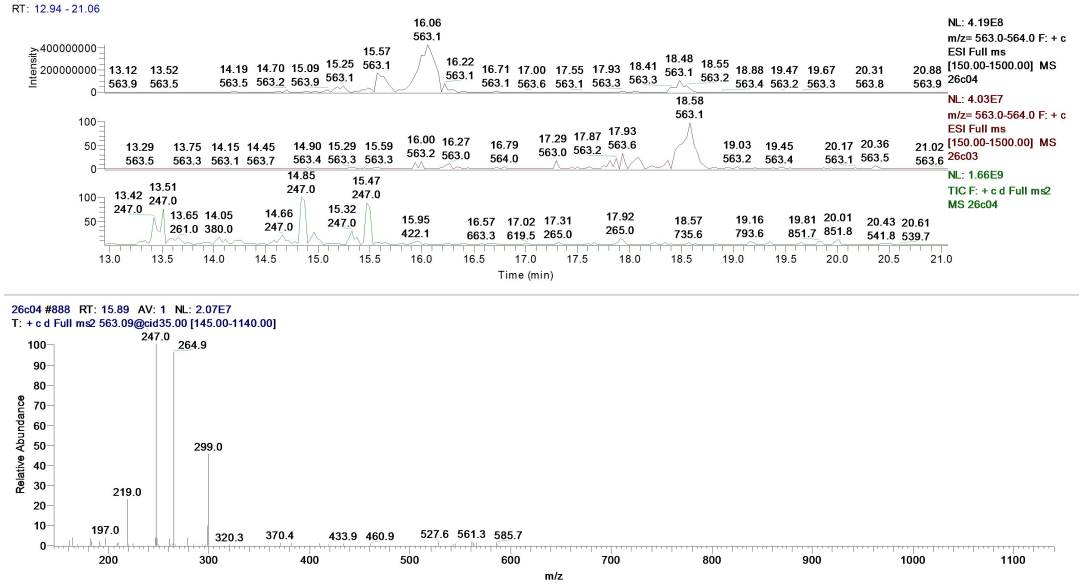


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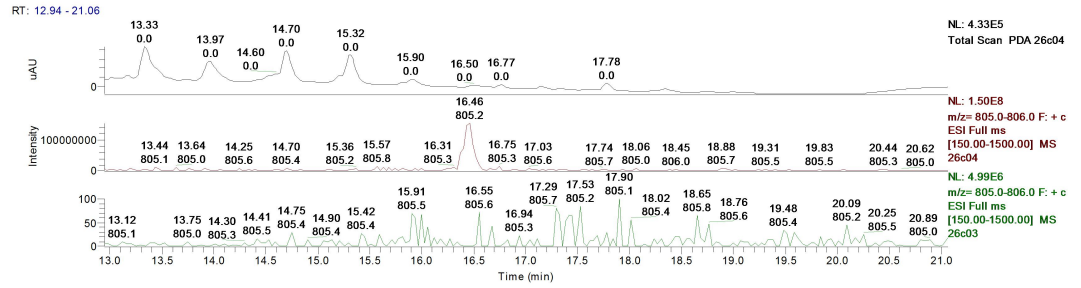


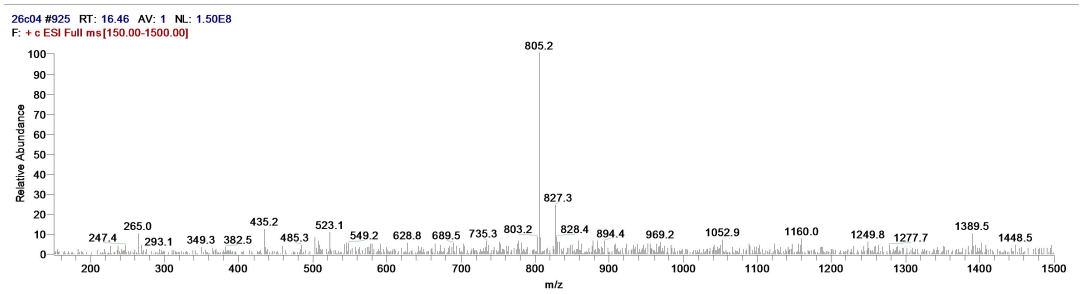


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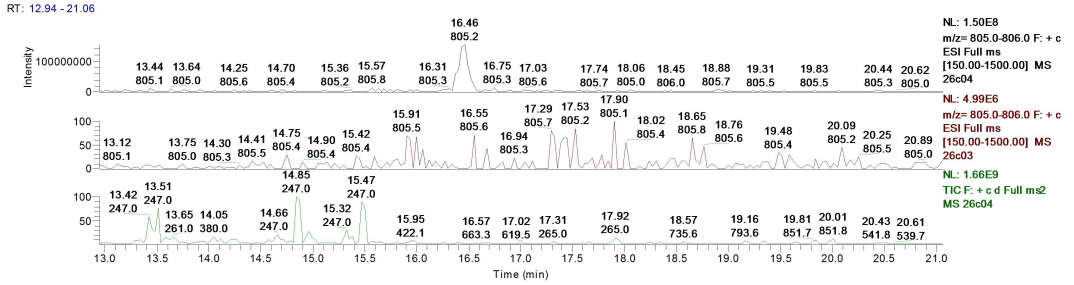


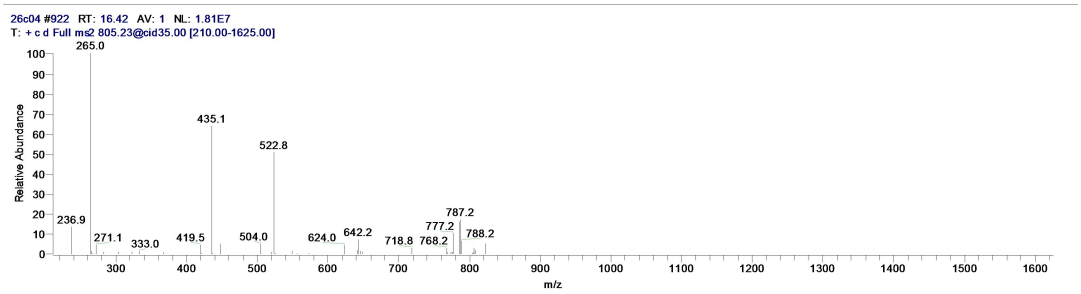
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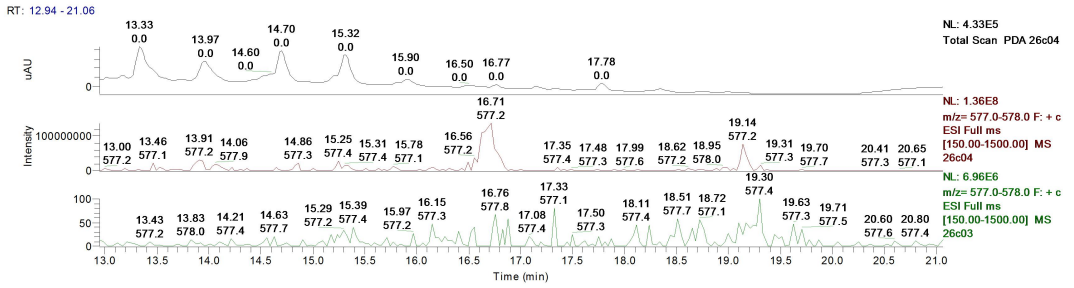


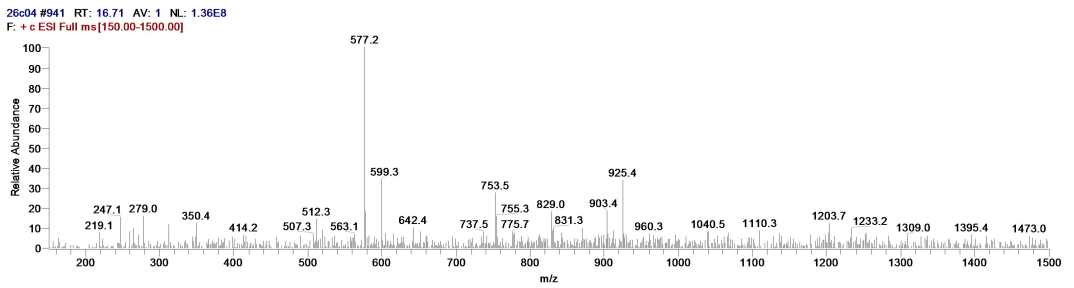
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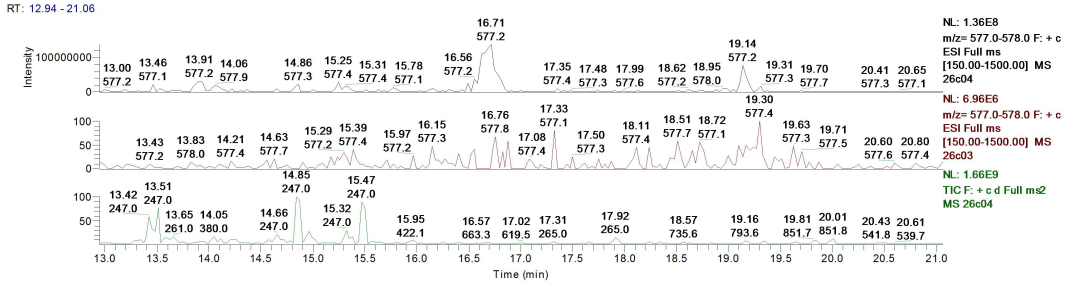


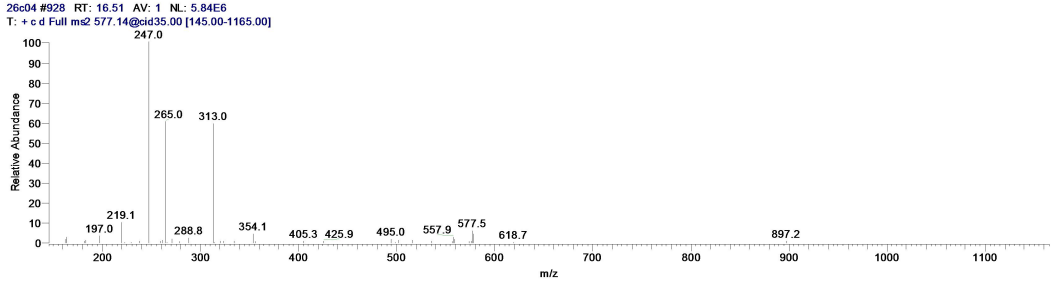
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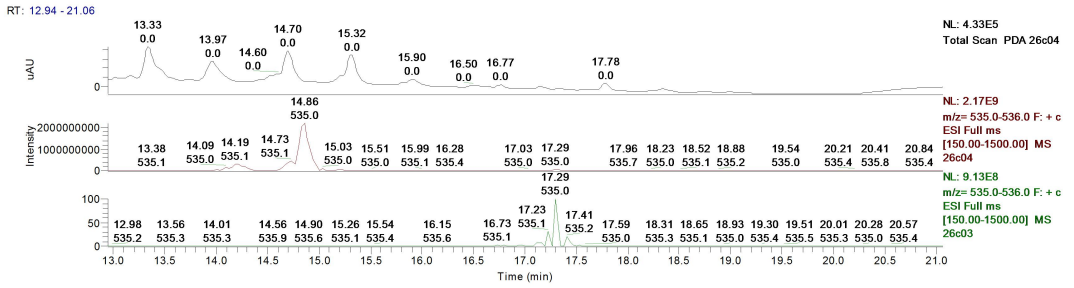


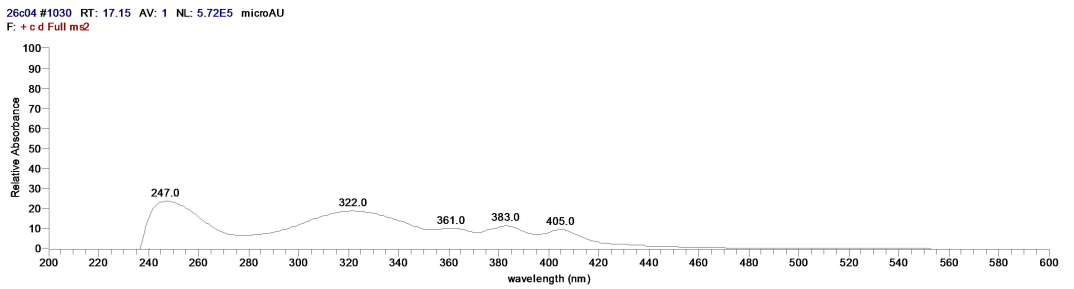
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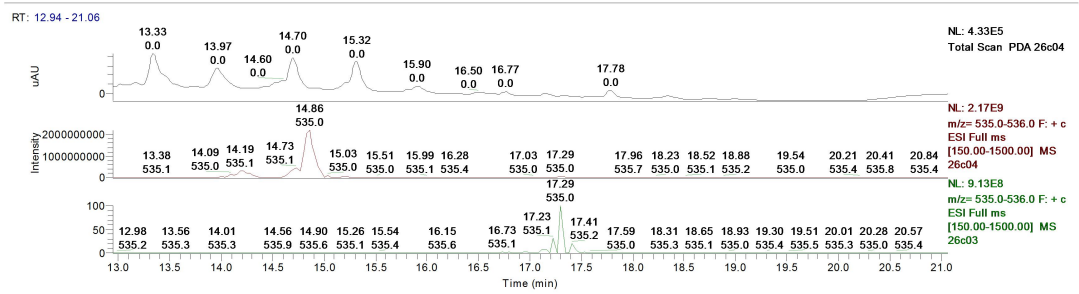


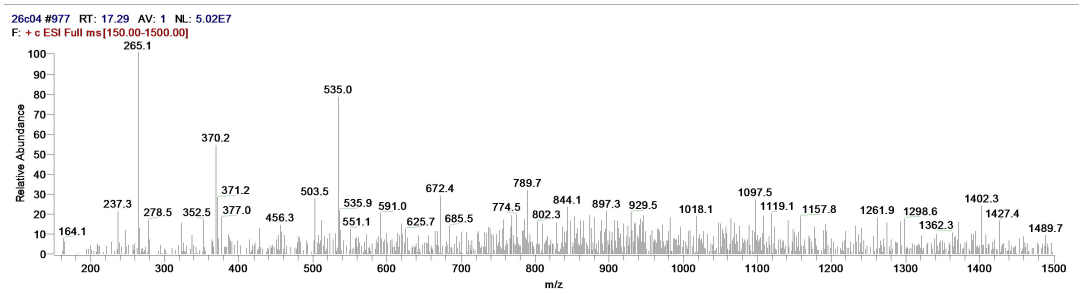
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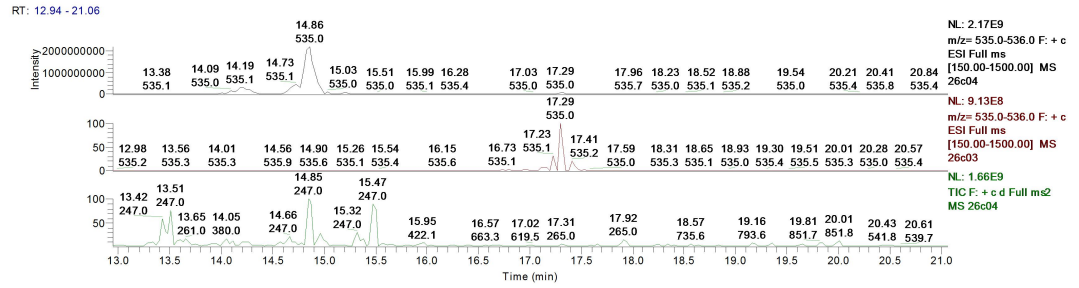


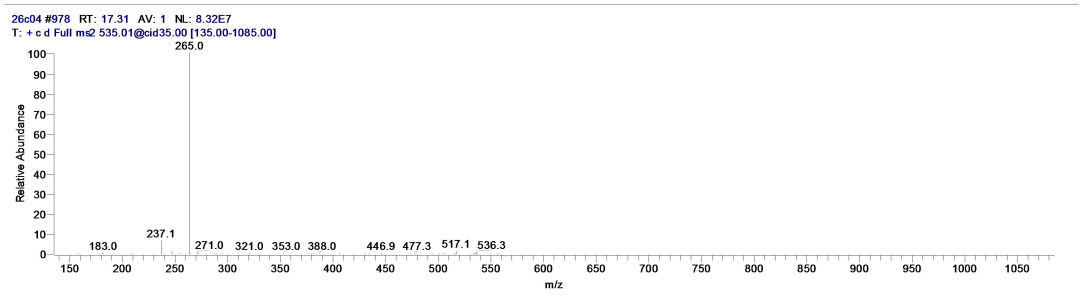
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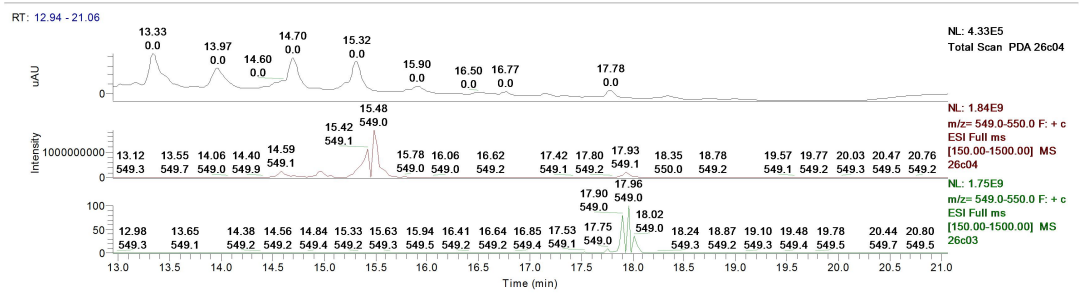


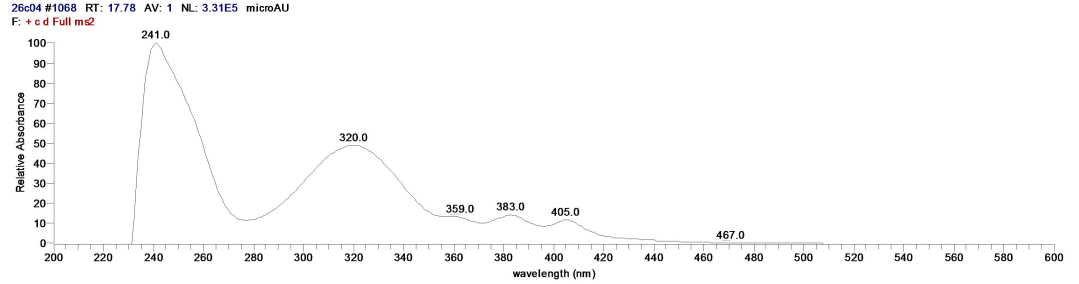
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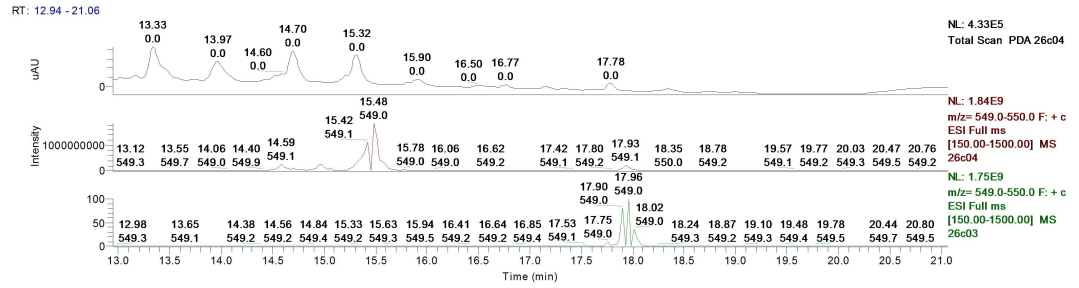


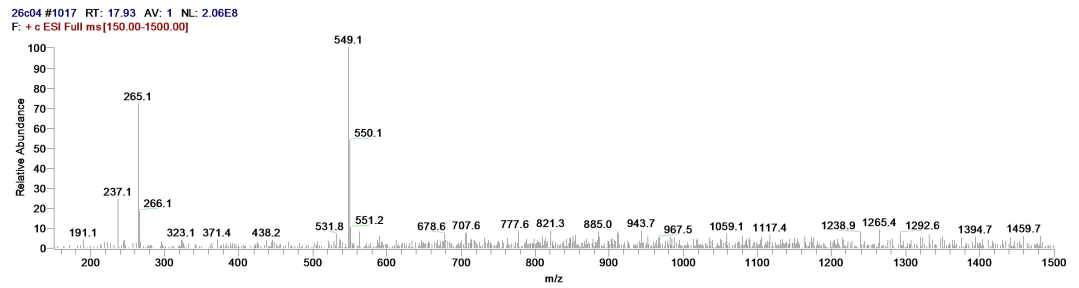
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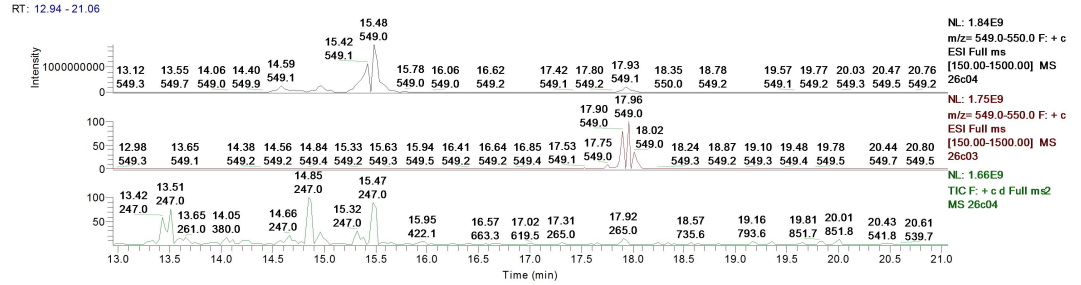


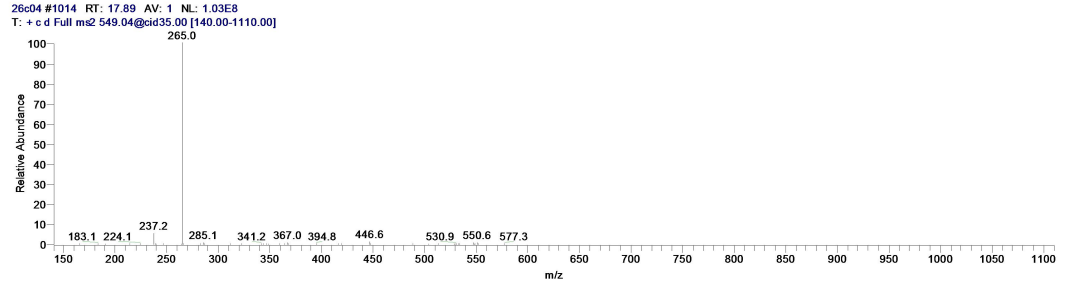
549 amu (+ESI)



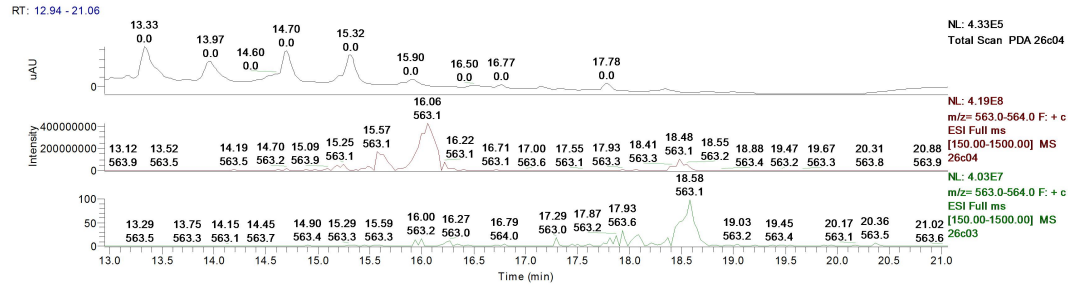


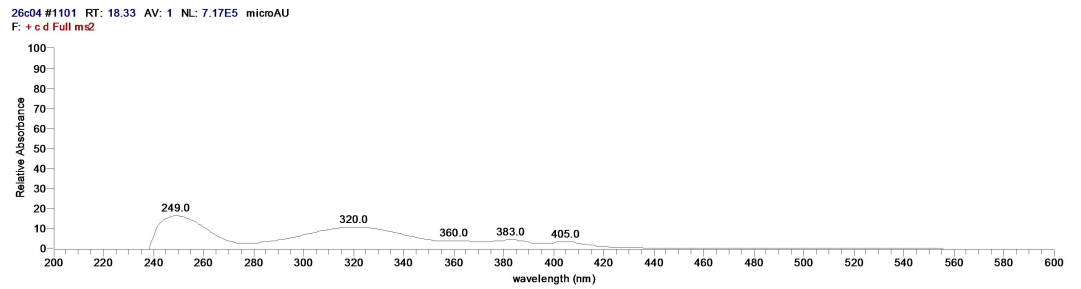
549 amu (+ESI)



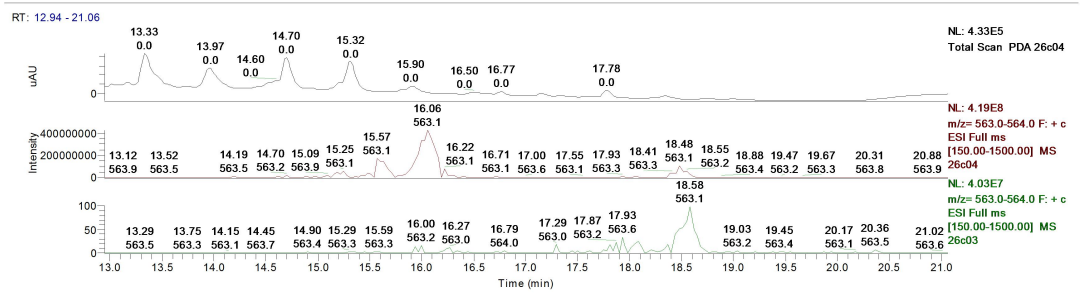


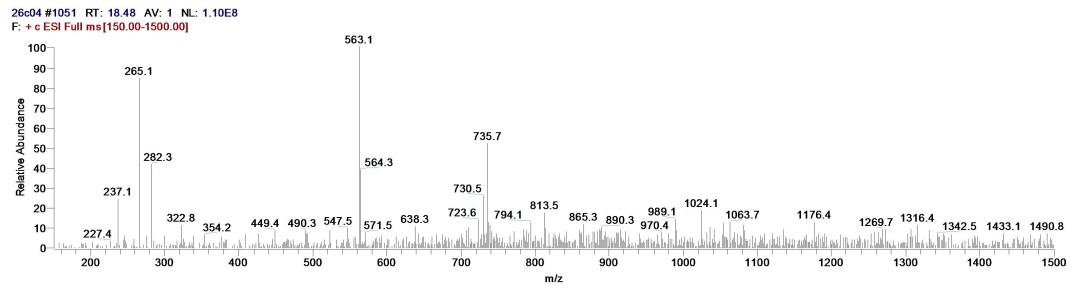
563 amu (+ESI)



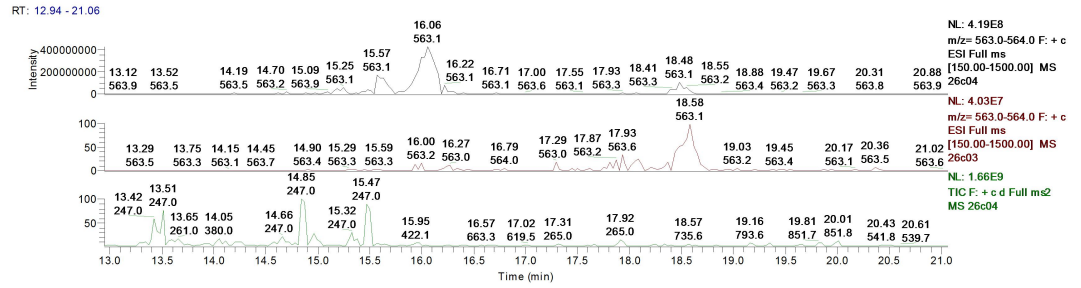


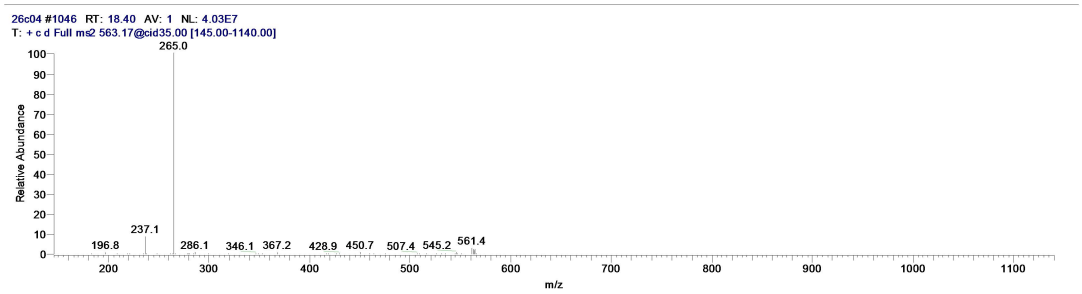
563 amu (+ESI)



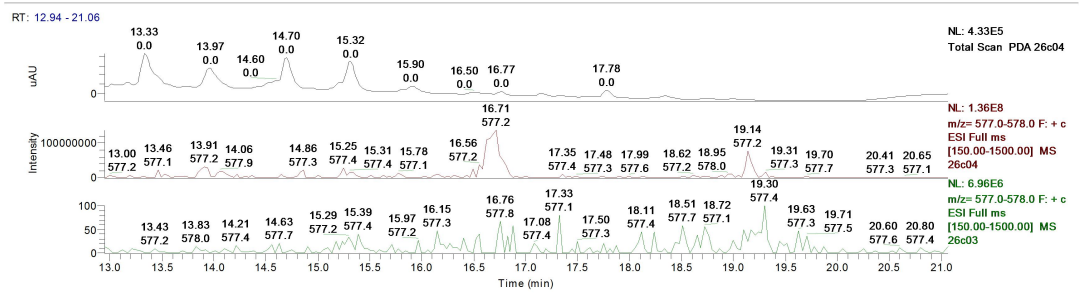


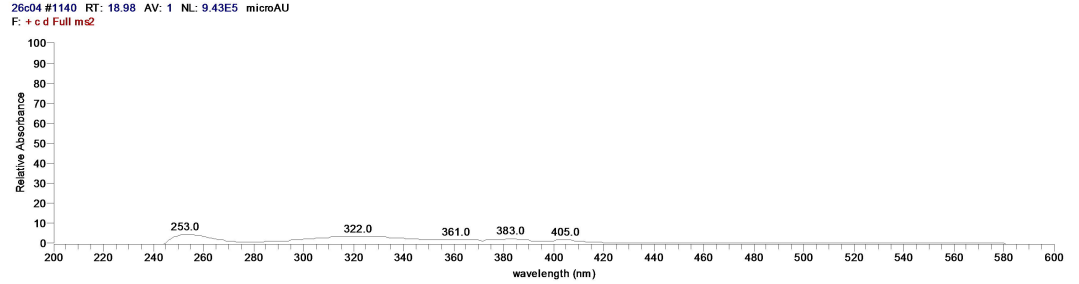
563 amu (+ESI)



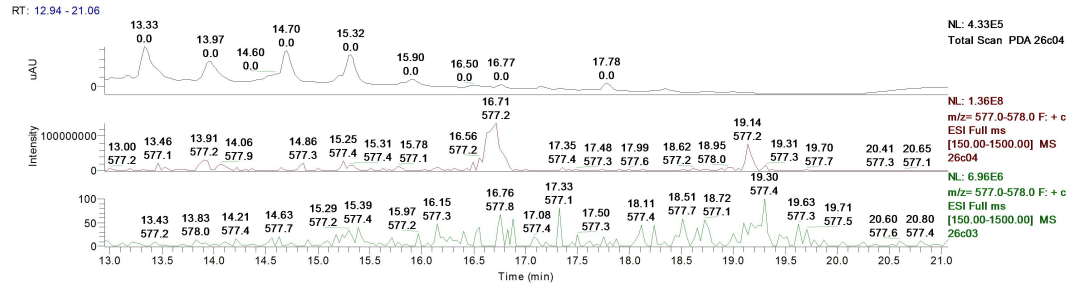


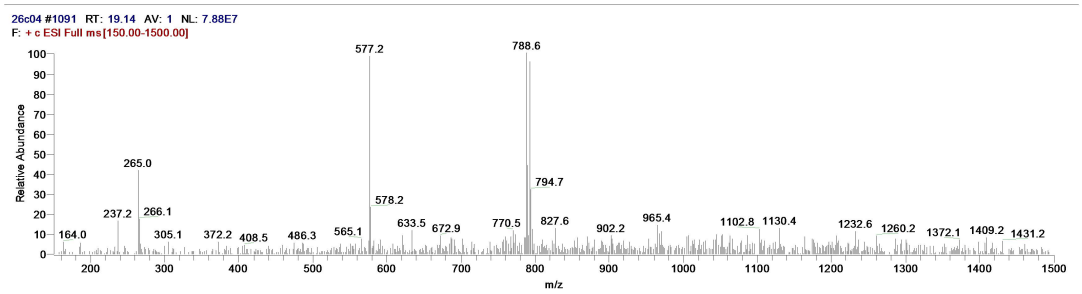
577 amu (+ESI)



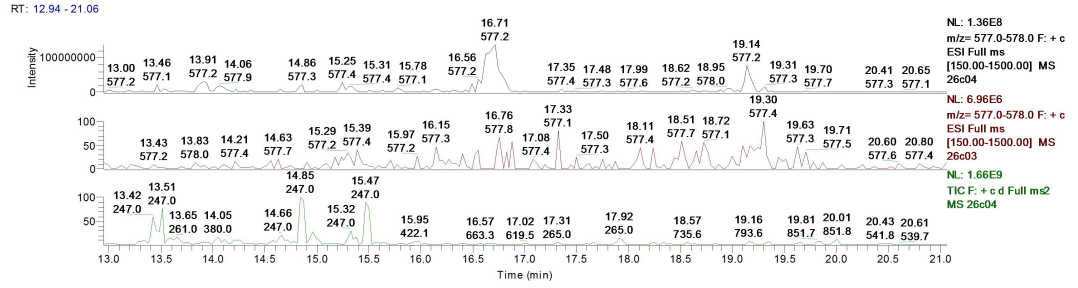


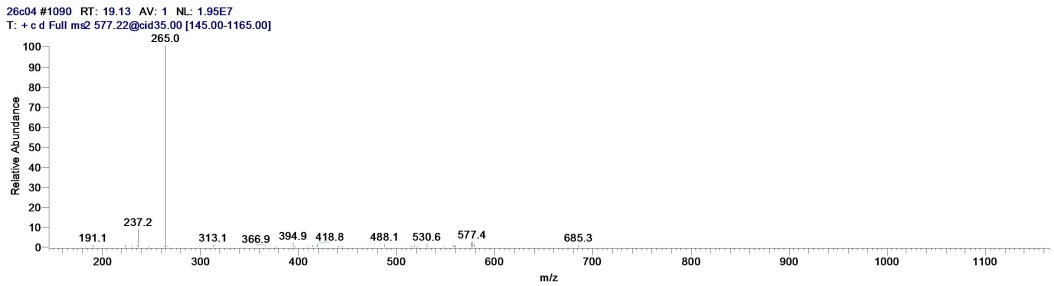
577 amu (+ESI)





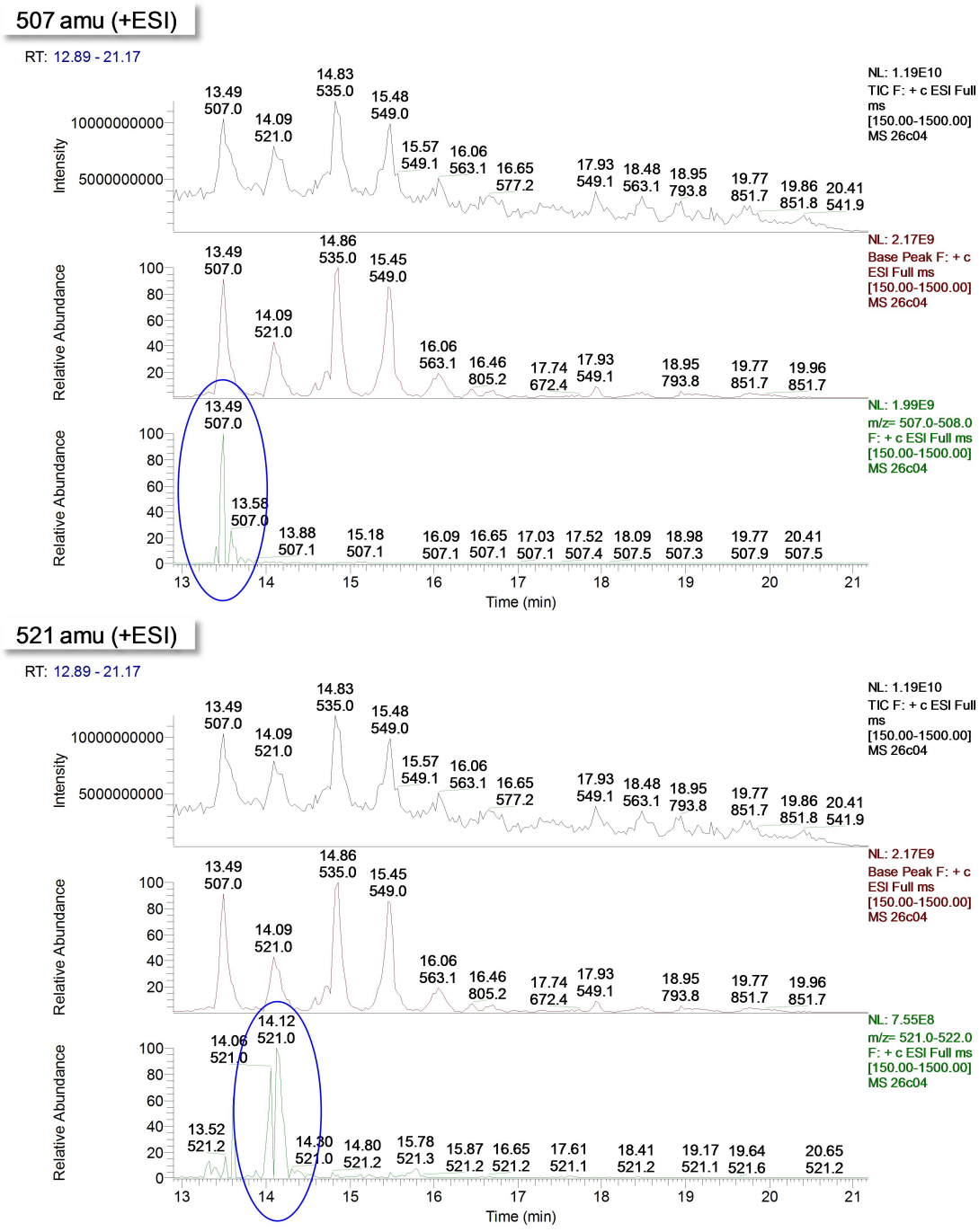
577 amu (+ESI)


RT: 12.94 - 21.06
Intensity
Time (min)
NL: 1.36E8
m/z= 577.0-578.0 F: + c ESI Full ms [150.00-1500.00] MS 26c04
NL: 6.96E6
m/z= 577.0-578.0 F: + c ESI Full ms [150.00-1500.00] MS 26c03
NL: 1.66E9
TIC F: + c d Full ms2 MS 26c04

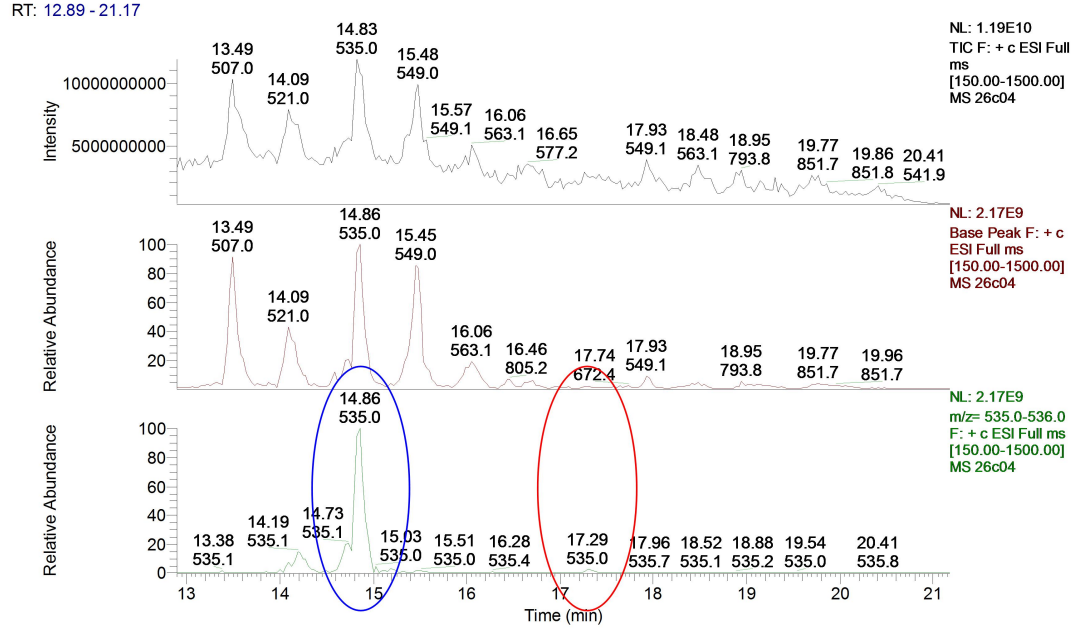

26c04 #1090 RT: 19.13 AV: 1 NL: 1.95E7
T: + c d Full ms2 577.22@cid35.00 [145.00-1165.00]
Relative Abundance
m/z

Supplementary Information B_2) LCMS on double-purified combined fractions B

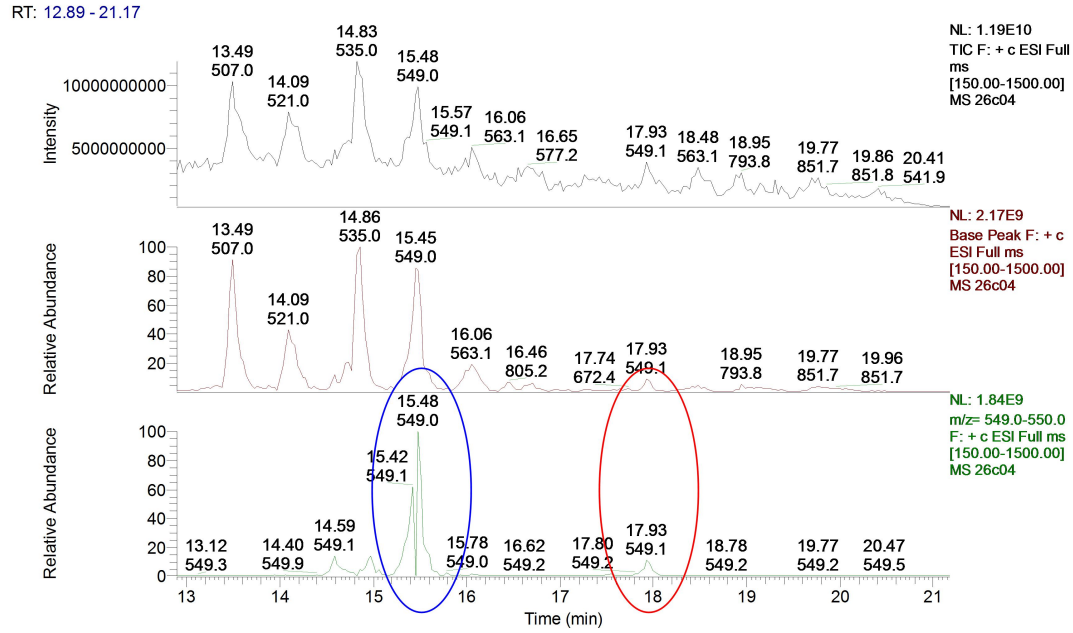
Low-resolution LCMS data on the double-purified combined fractions B of *Streptomyces* E_8. Extracted ion chromatograms are presented on ions with masses of 507 amu, 521 amu, 535 amu, 549 amu, 563 amu, (805 amu), and 577 amu $[M+H]^+$. The LCMS system used for generating these data has been located at the John Innes Centre and was set to detect ions in positive ion mode (+ESI).


507 amu (+ESI)
RT: 12.89 - 21.17
NL: 1.19E10
TIC F: + c ESI Full ms [150.00-1500.00] MS 26c04
NL: 2.17E9
Base Peak F: + c ESI Full ms [150.00-1500.00] MS 26c04
NL: 1.99E9
m/z= 507.0-508.0 F: + c ESI Full ms [150.00-1500.00] MS 26c04
Intensity
Relative Abundance
Time (min)
521 amu (+ESI)
RT: 12.89 - 21.17
NL: 1.19E10
TIC F: + c ESI Full ms [150.00-1500.00] MS 26c04
NL: 2.17E9
Base Peak F: + c ESI Full ms [150.00-1500.00] MS 26c04
NL: 7.55E8
m/z= 521.0-522.0 F: + c ESI Full ms [150.00-1500.00] MS 26c04
Intensity
Relative Abundance
Time (min)

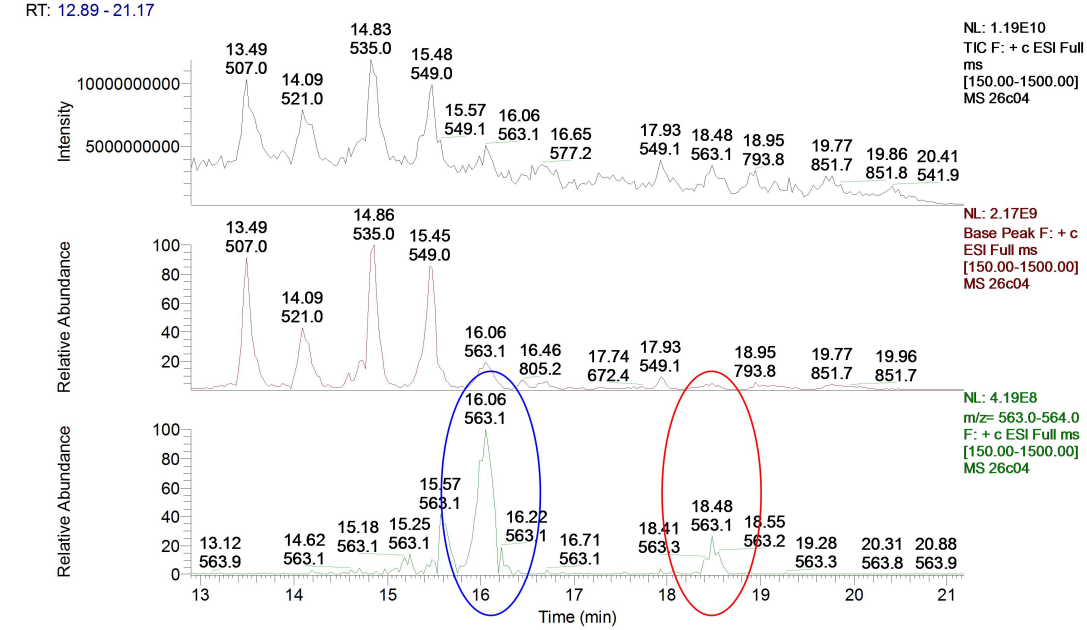
535 amu (+ESI)



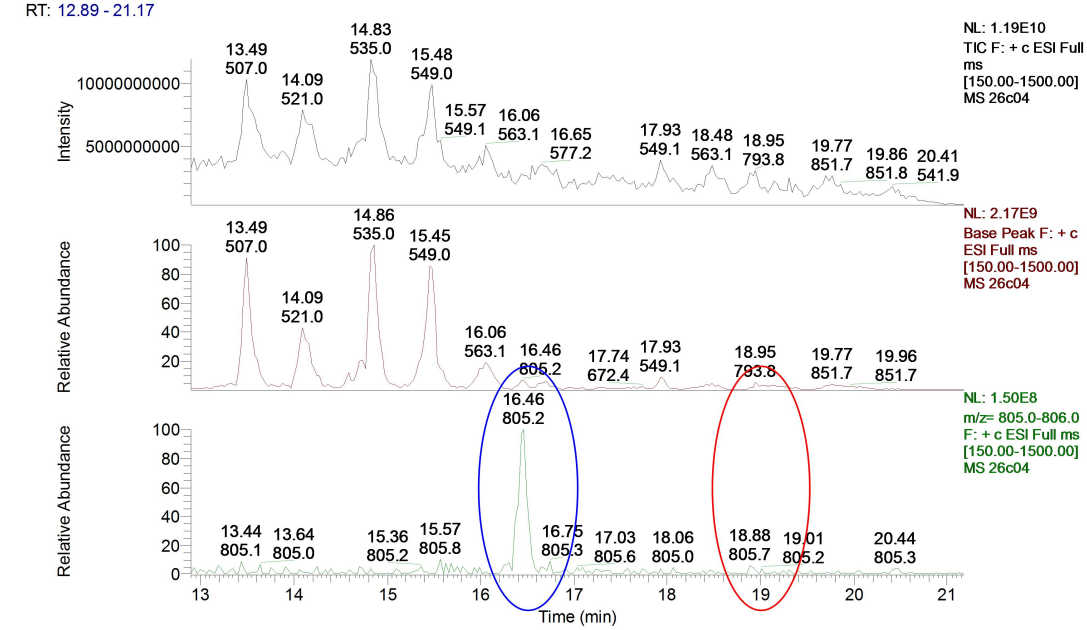
549 amu (+ESI)

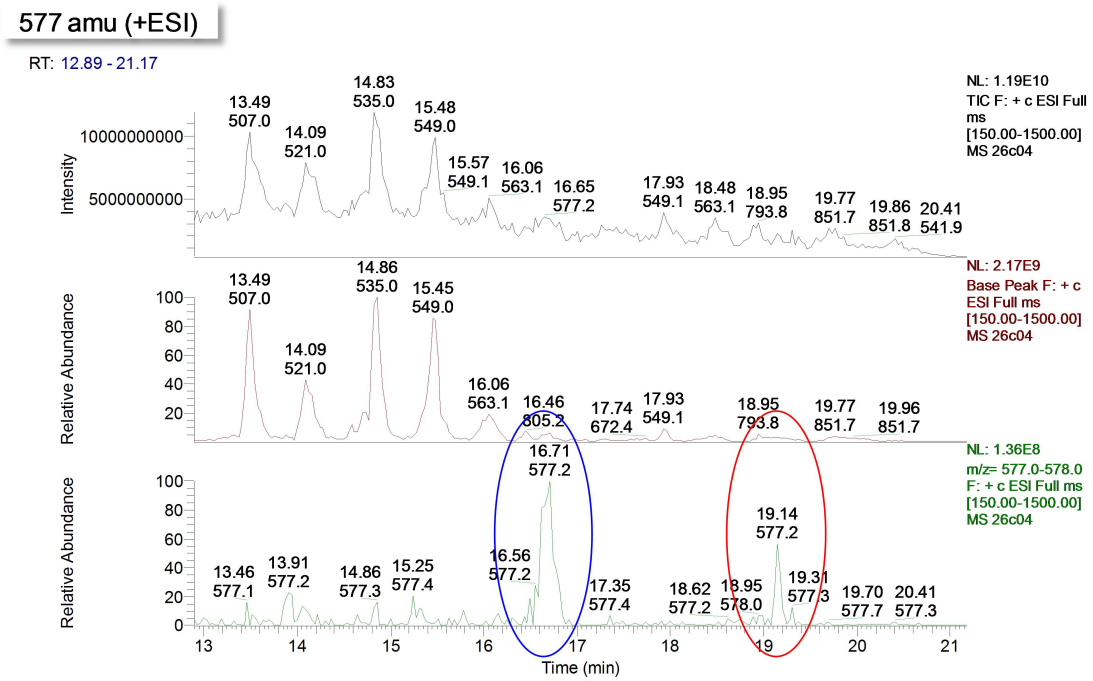


563 amu (+ESI)



805 amu (+ESI)

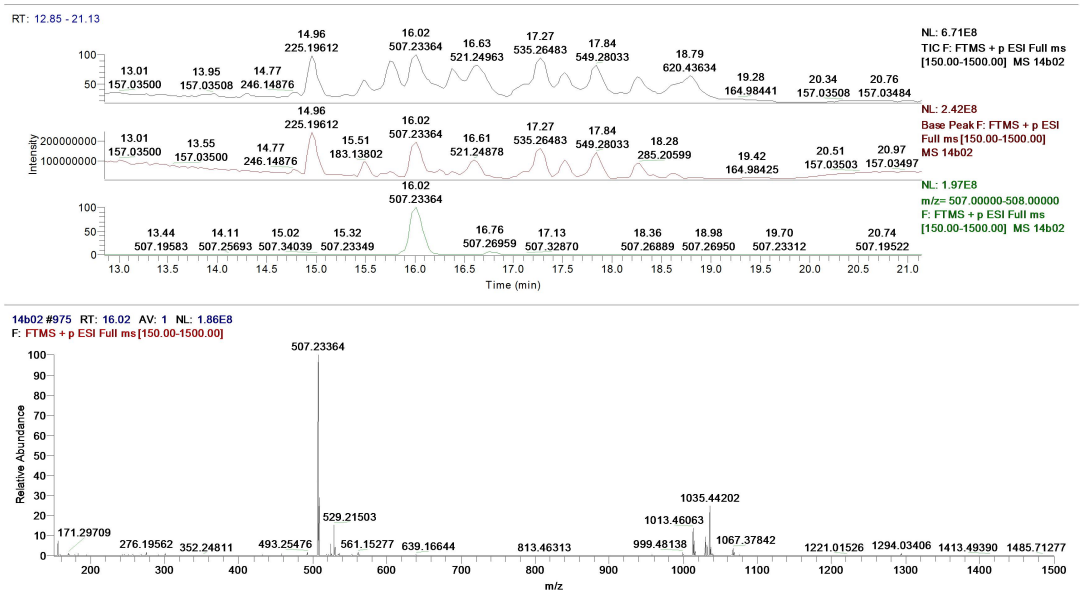



577 amu (+ESI)
RT: 12.89 - 21.17
NL: 1.19E10
TIC F: + c ESI Full ms [150.00-1500.00] MS 26c04
Intensity
NL: 2.17E9
Base Peak F: + c ESI Full ms [150.00-1500.00] MS 26c04
Relative Abundance
NL: 1.36E8
m/z= 577.0-578.0 F: + c ESI Full ms [150.00-1500.00] MS 26c04
Relative Abundance
Time (min)

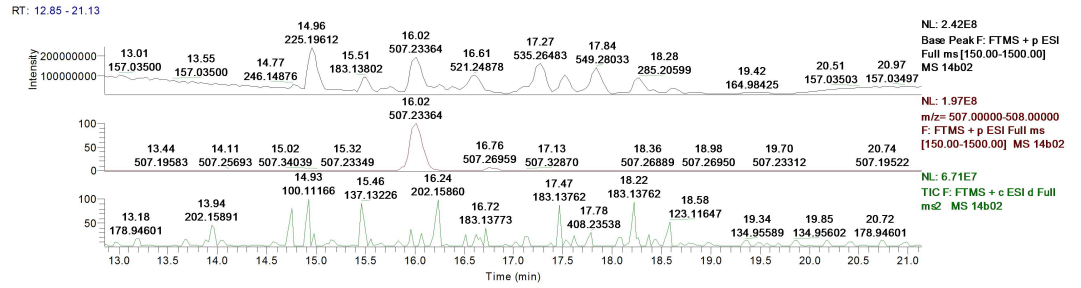
Supplementary Information B_3) LCMS on double-purified combined fractions B

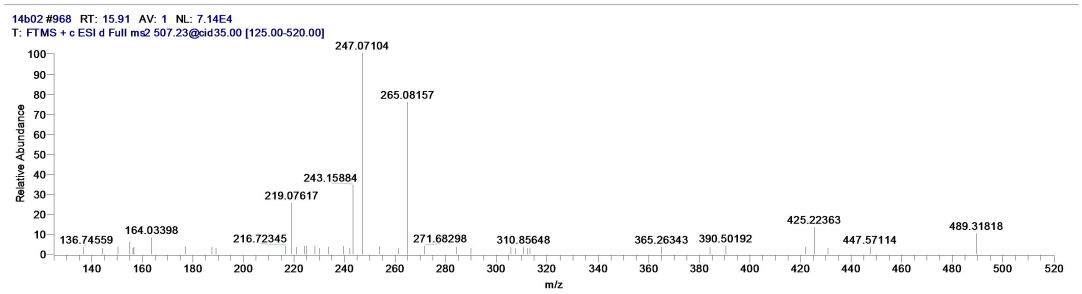
High-resolution LCMS data on the double-purified combined fractions B of *Streptomyces* E_8. MS and MS^2 data are presented on ions with masses of 507 amu, 521 amu, 535 amu, 549 amu, 563 amu, and 577 amu $[M+H]^+$. The LCMS system used for generating these data has been located at the John Innes Centre and was set to detect ions in positive ion mode (+ESI).

507 amu (+ESI)

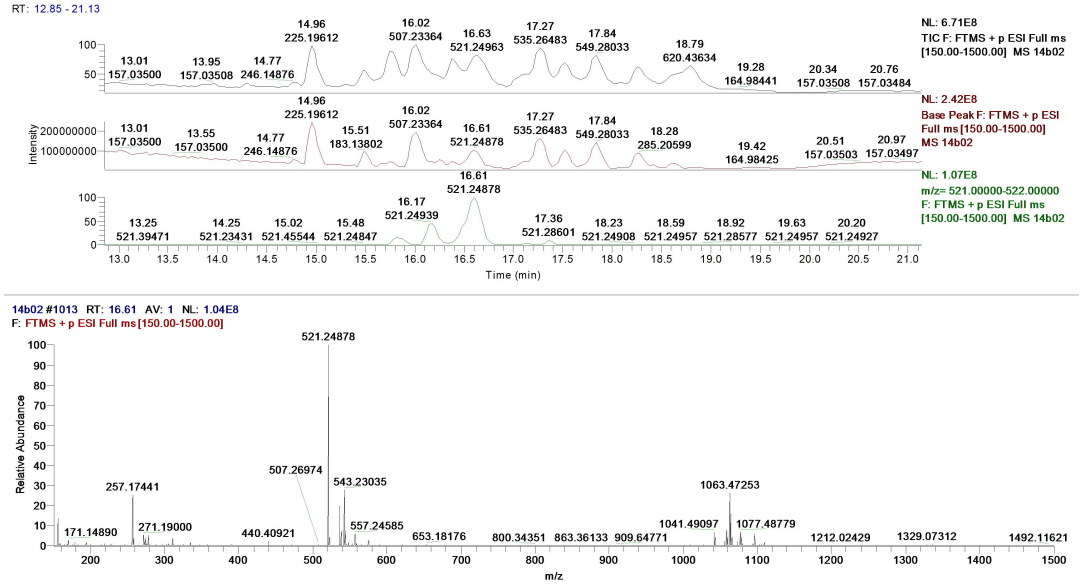


507 amu (+ESI)

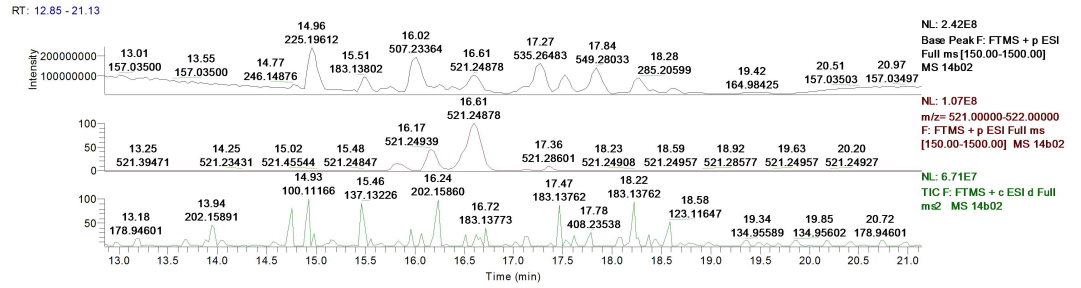


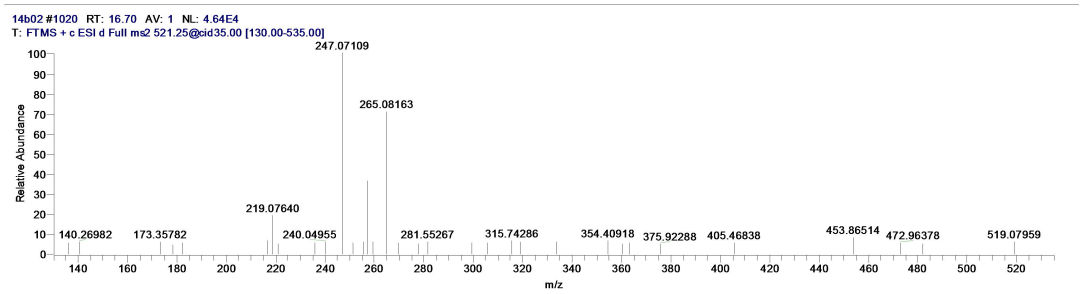


521 amu (+ESI)

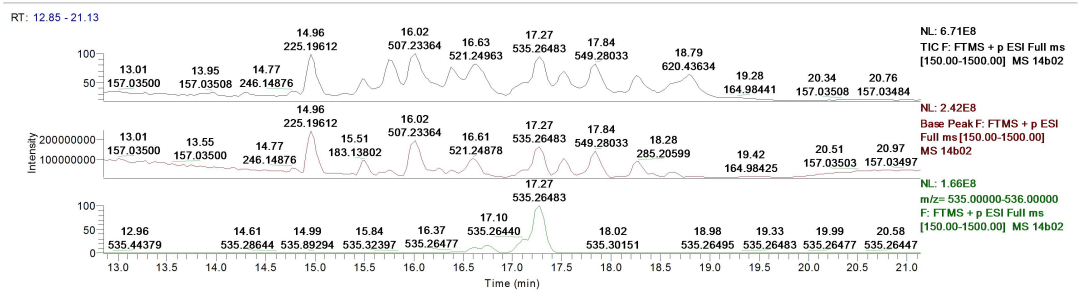


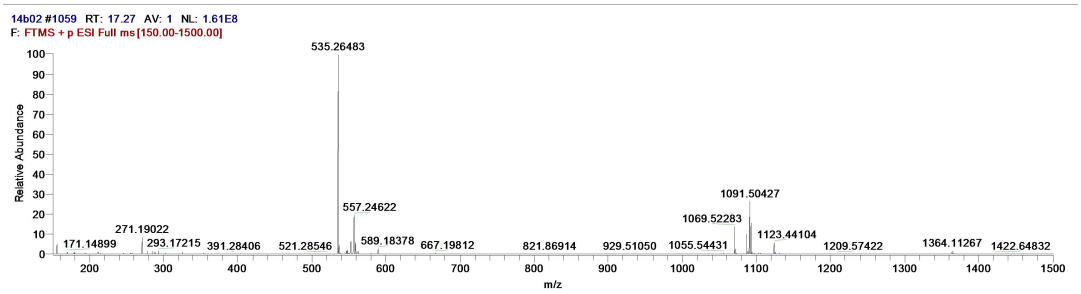
521 amu (+ESI)



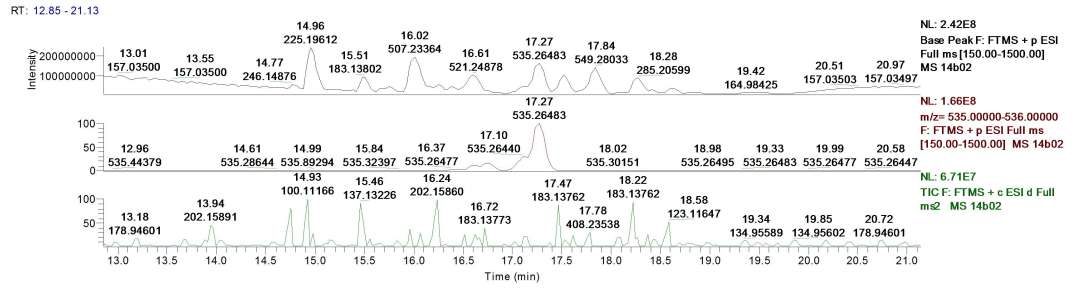


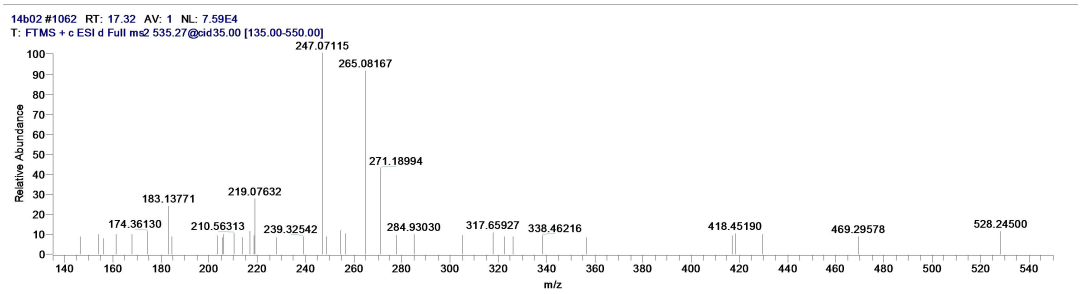
535 amu (+ESI)



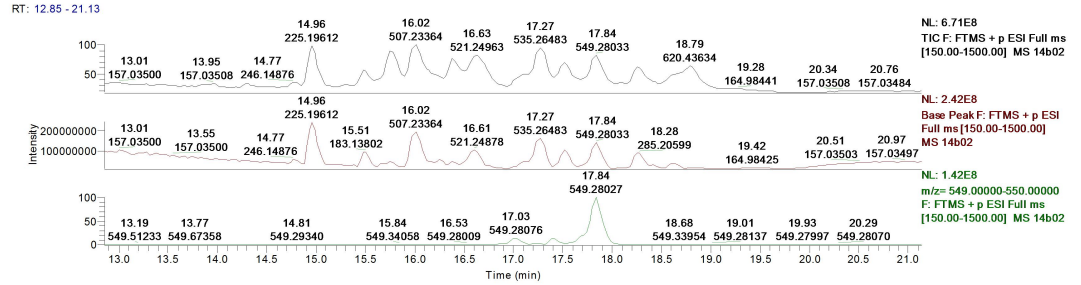


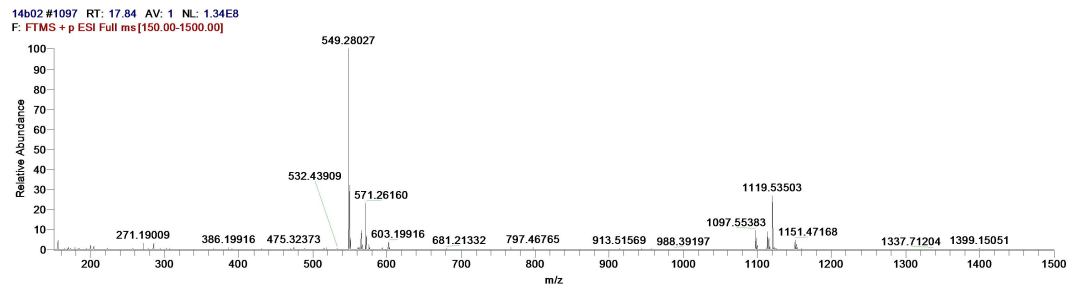
535 amu (+ESI)



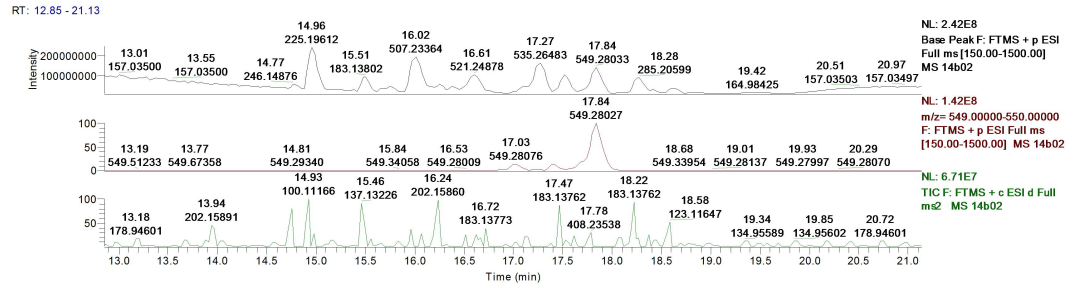


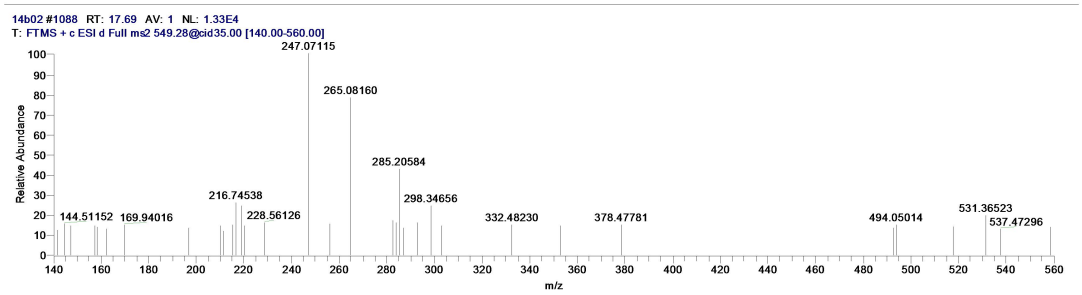
549 amu (+ESI)



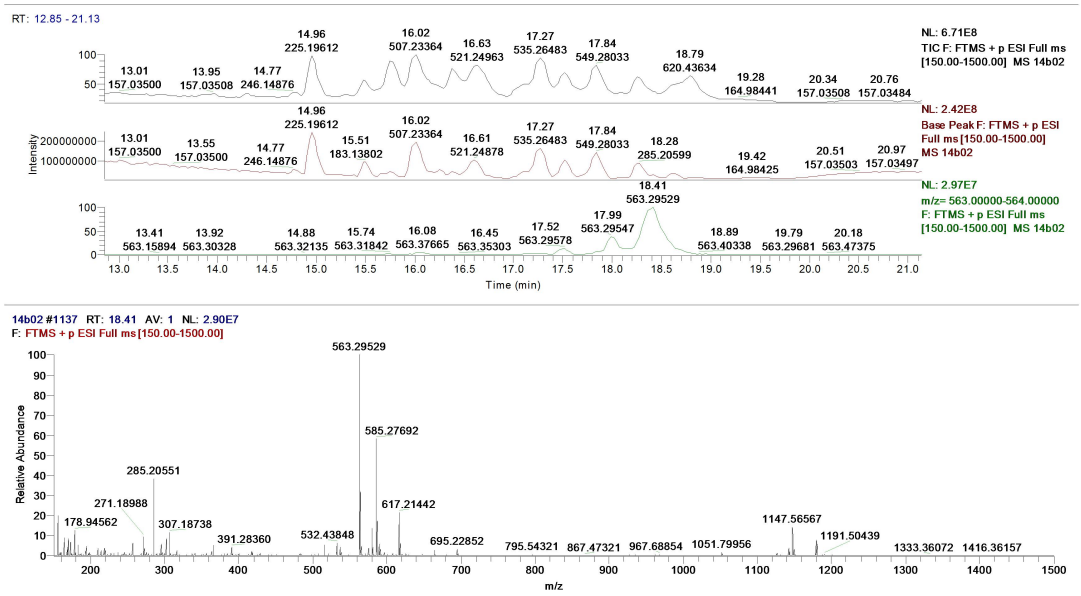


549 amu (+ESI)

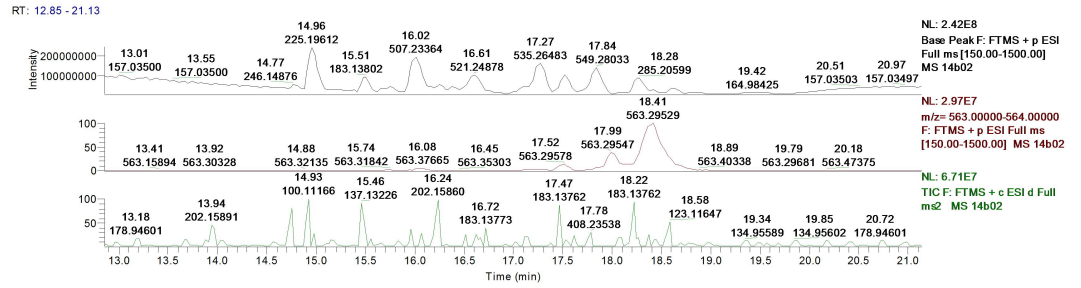


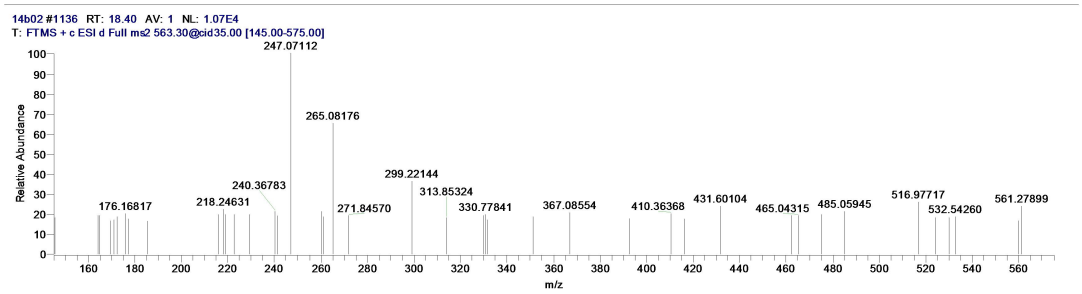


563 amu (+ESI)

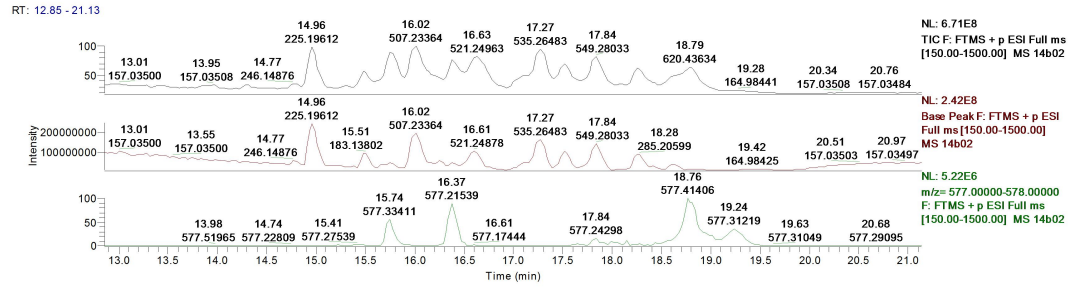


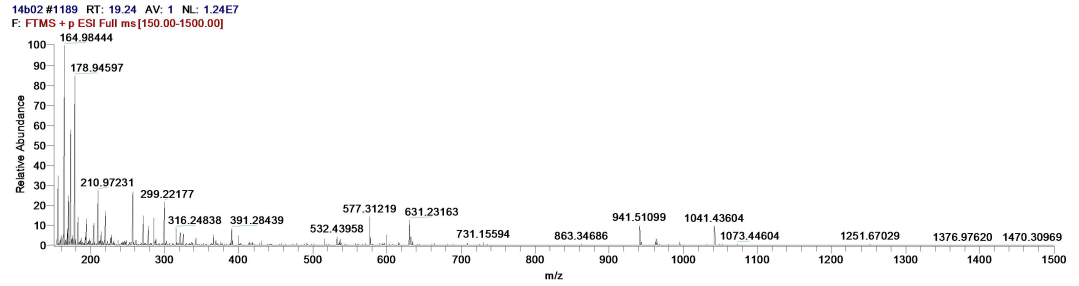
563 amu (+ESI)





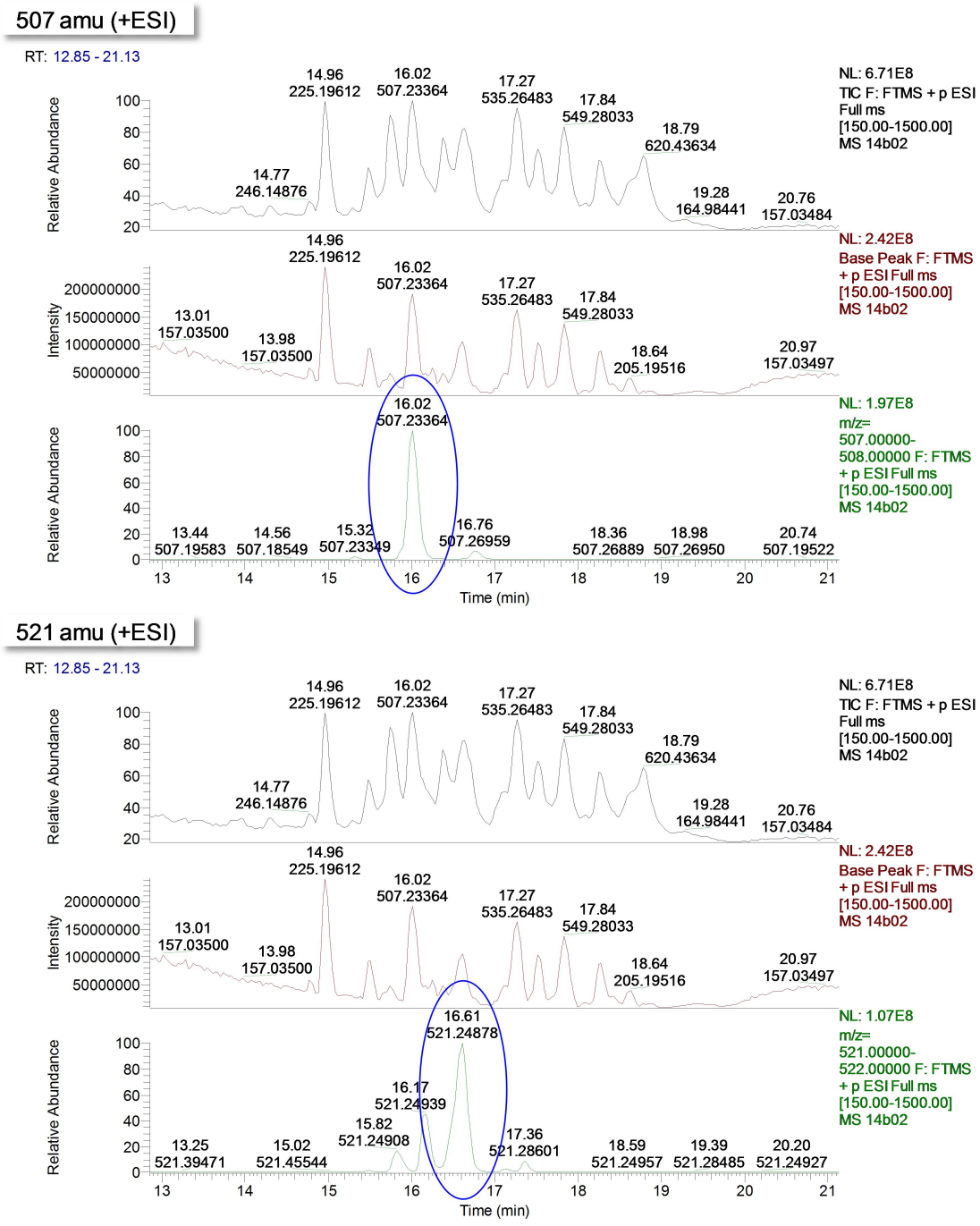
577 amu (+ESI)

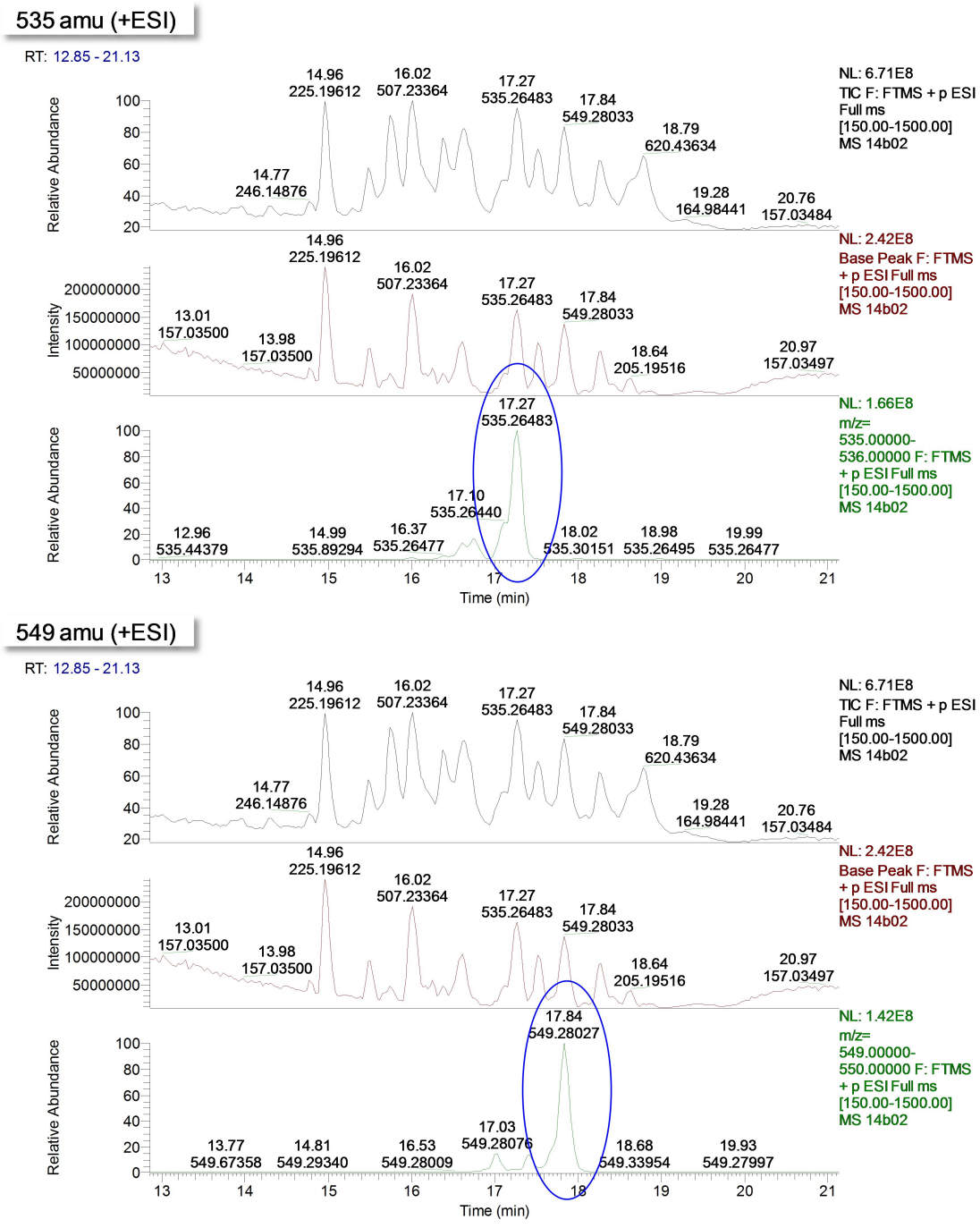

RT: 12.85 - 21.13
NL: 6.71E8
TIC F: FTMS + p ESI Full ms [150.00-1500.00] MS 14b02
NL: 2.42E8
Base Peak F: FTMS + p ESI Full ms [150.00-1500.00] MS 14b02
NL: 5.22E6
m/z= 577.00000-578.00000 F: FTMS + p ESI Full ms [150.00-1500.00] MS 14b02
Intensity
Time (min)


14b02 #1189 RT: 19.24 AV: 1 NL: 1.24E7
F: FTMS + p ESI Full ms [150.00-1500.00]
Relative Abundance
m/z

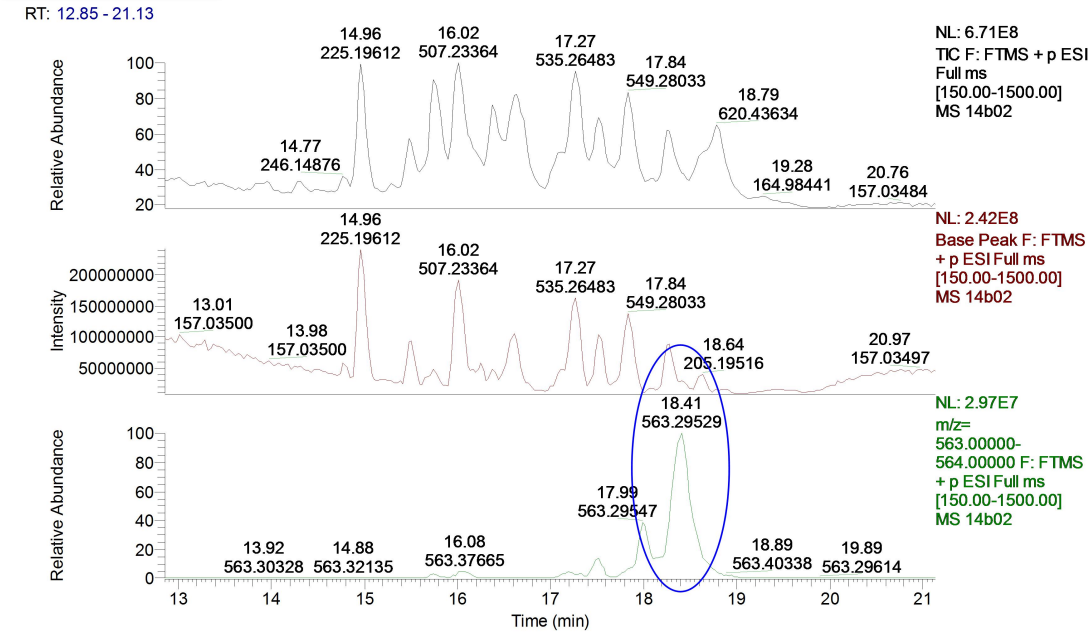
Supplementary Information B_4) LCMS on double-purified combined fractions B

High-resolution LCMS data on the double-purified combined fractions B of *Streptomyces* E_8. Extracted ion chromatograms are presented on ions with masses of 507 amu, 521 amu, 535 amu, 549 amu, 563 amu, and 577 amu $[M+H]^+$. The LCMS system used for generating these data has been located at the John Innes Centre and was set to detect ions in positive ion mode (+ESI).

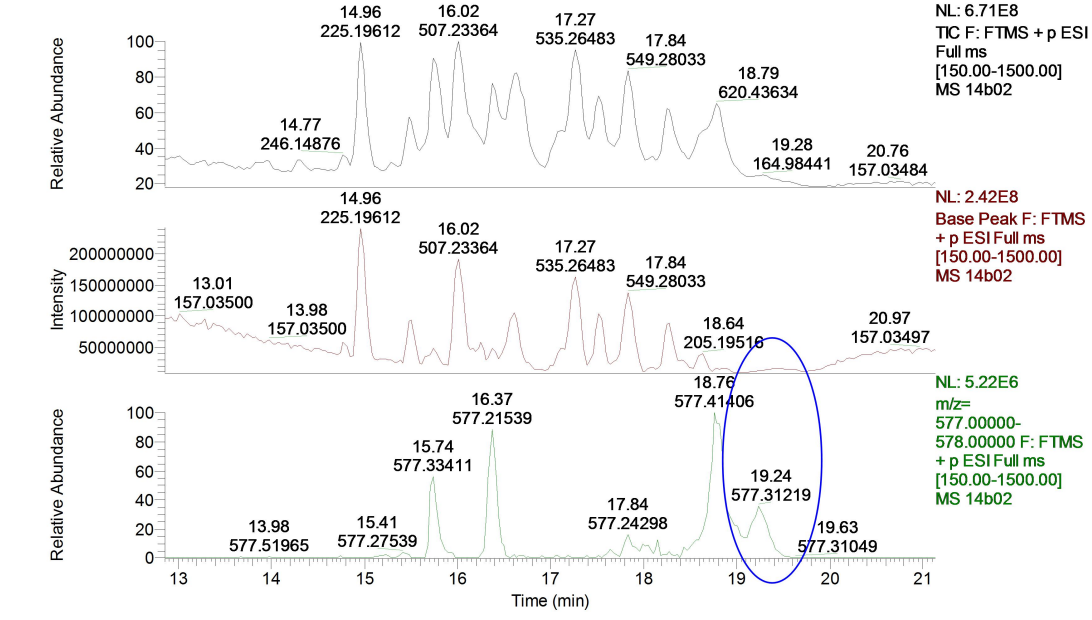

507 amu (+ESI)
RT: 12.85 - 21.13
Relative Abundance
Intensity
Time (min)
NL: 6.71E8
TIC F: FTMS + p ESI Full ms [150.00-1500.00] MS 14b02
NL: 2.42E8
Base Peak F: FTMS + p ESI Full ms [150.00-1500.00] MS 14b02
NL: 1.97E8
m/z= 507.00000-508.00000 F: FTMS + p ESI Full ms [150.00-1500.00] MS 14b02
14.96 225.19612
16.02 507.23364
17.27 535.26483
17.84 549.28033
18.79 620.43634
14.77 246.14876
19.28 164.98441
20.76 157.03484
13.01 157.03500
13.98 157.03500
18.64 205.19516
20.97 157.03497
13.44 507.19583
14.56 507.18549
15.32 507.23349
16.76 507.26959
18.36 507.26889
18.98 507.26950
20.74 507.19522
521 amu (+ESI)
RT: 12.85 - 21.13
NL: 6.71E8
TIC F: FTMS + p ESI Full ms [150.00-1500.00] MS 14b02
NL: 2.42E8
Base Peak F: FTMS + p ESI Full ms [150.00-1500.00] MS 14b02
NL: 1.07E8
m/z= 521.00000-522.00000 F: FTMS + p ESI Full ms [150.00-1500.00] MS 14b02
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16.17 521.24939
15.82 521.24908
13.25 521.39471
15.02 521.45544
17.36 521.28601
18.59 521.24957
19.39 521.28485
20.20 521.24927


535 amu (+ESI)
RT: 12.85 - 21.13
Relative Abundance
Intensity
Time (min)
NL: 6.71E8
TIC F: FTMS + p ESI Full ms [150.00-1500.00] MS 14b02
NL: 2.42E8
Base Peak F: FTMS + p ESI Full ms [150.00-1500.00] MS 14b02
NL: 1.66E8
m/z= 535.00000-536.00000 F: FTMS + p ESI Full ms [150.00-1500.00] MS 14b02
549 amu (+ESI)
RT: 12.85 - 21.13
NL: 6.71E8
TIC F: FTMS + p ESI Full ms [150.00-1500.00] MS 14b02
NL: 2.42E8
Base Peak F: FTMS + p ESI Full ms [150.00-1500.00] MS 14b02
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563 amu (+ESI)



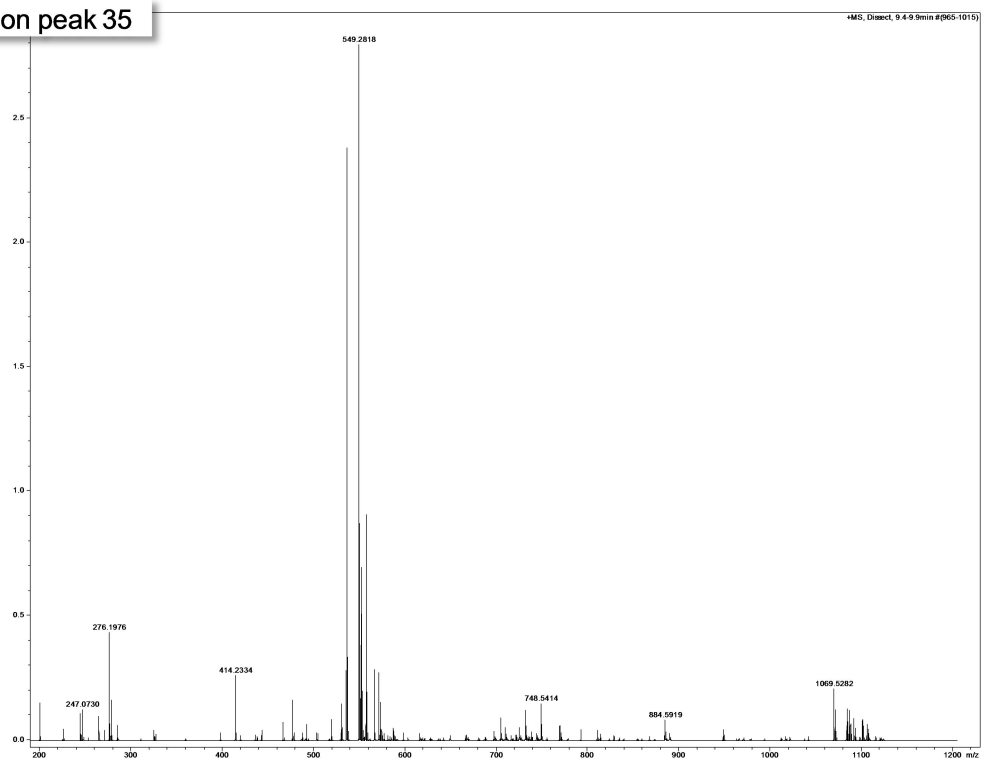
577 amu (+ESI)



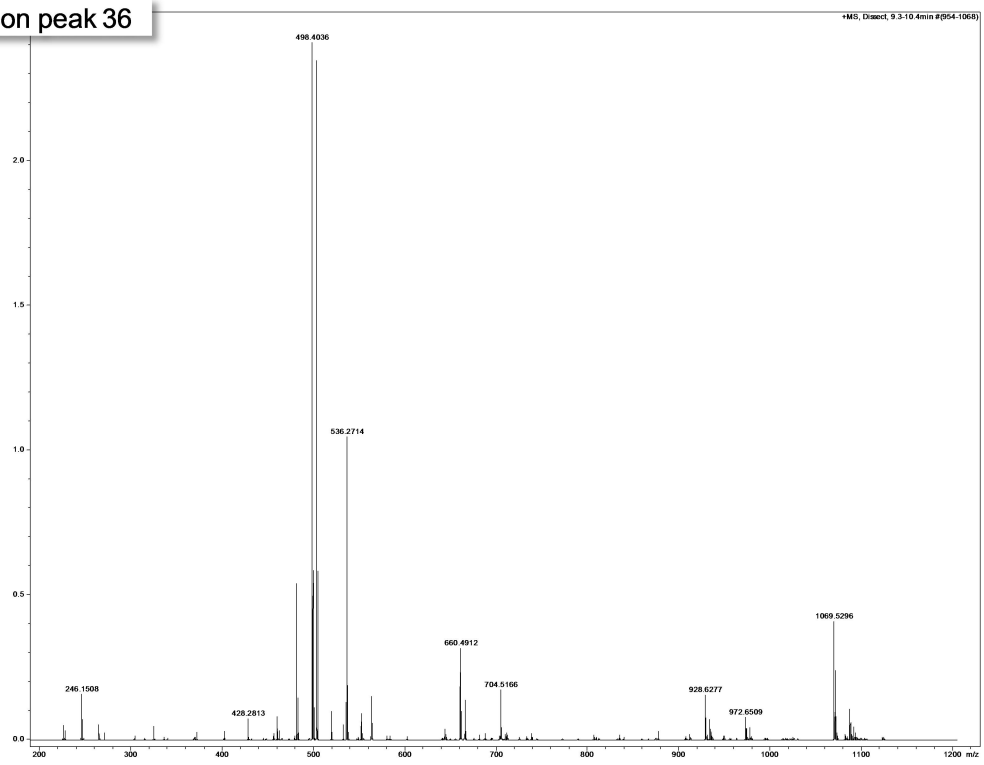
Supplementary Information B_5) LCMS on double-purified combined fractions B

High-resolution LCMS data on the double-purified combined fractions B of *Streptomyces* E_8. Dissection peaks are presented of fractions 2E9, 2E10, 2F2, and 2F3 containing ions with masses of 535 amu and 549 amu $[M+H]^+$ The LCMS system used for generating these data has been located at the Institute of Food Research and was set to detect ions in positive ion mode (+ESI).

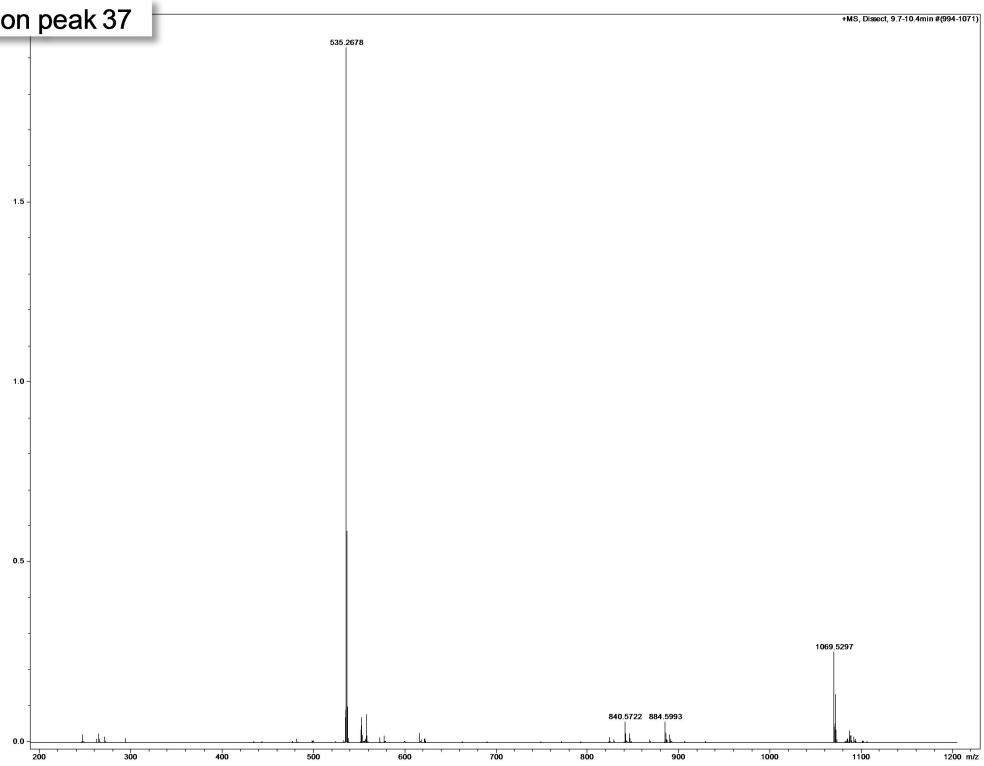
Dissection peak 35



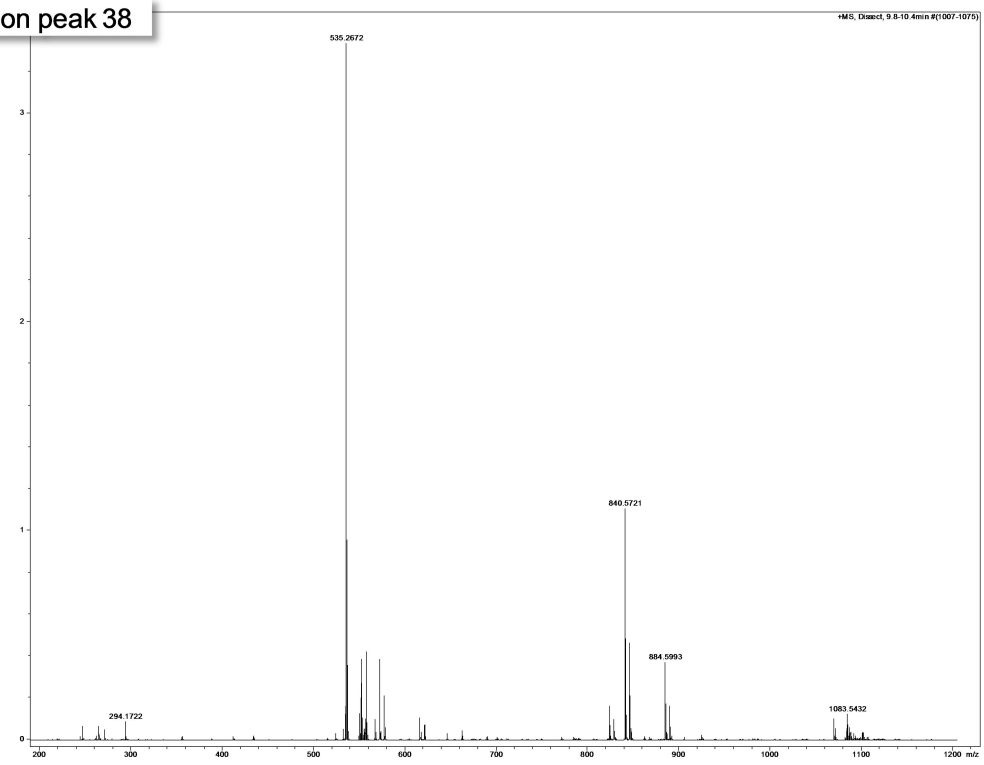
Dissection peak 36



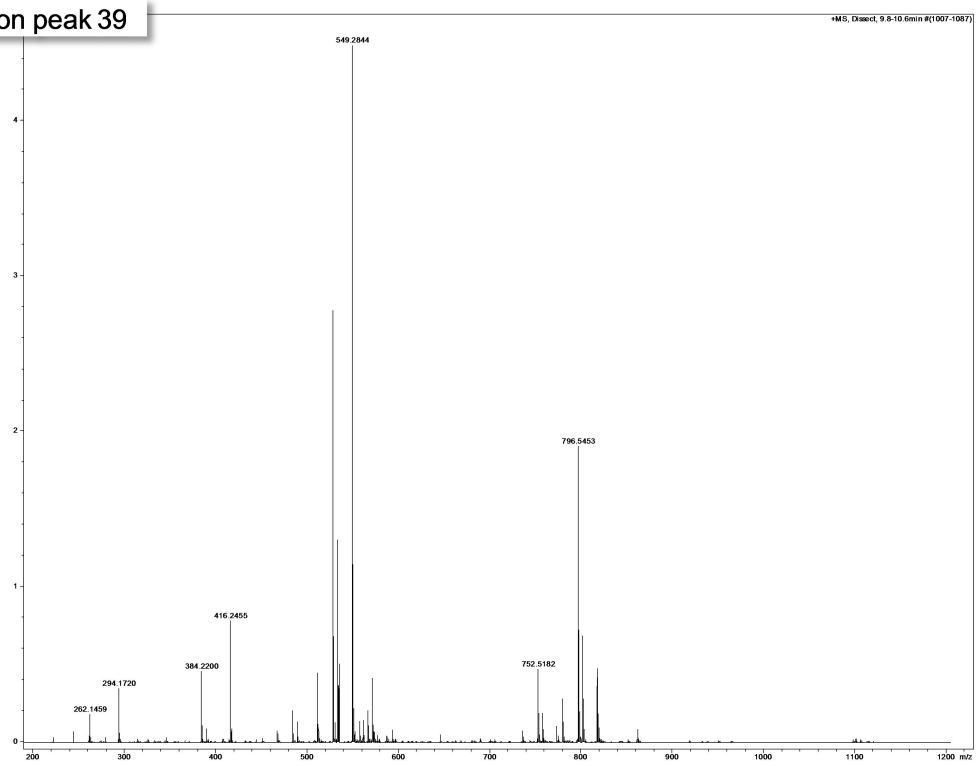
Dissection peak 37



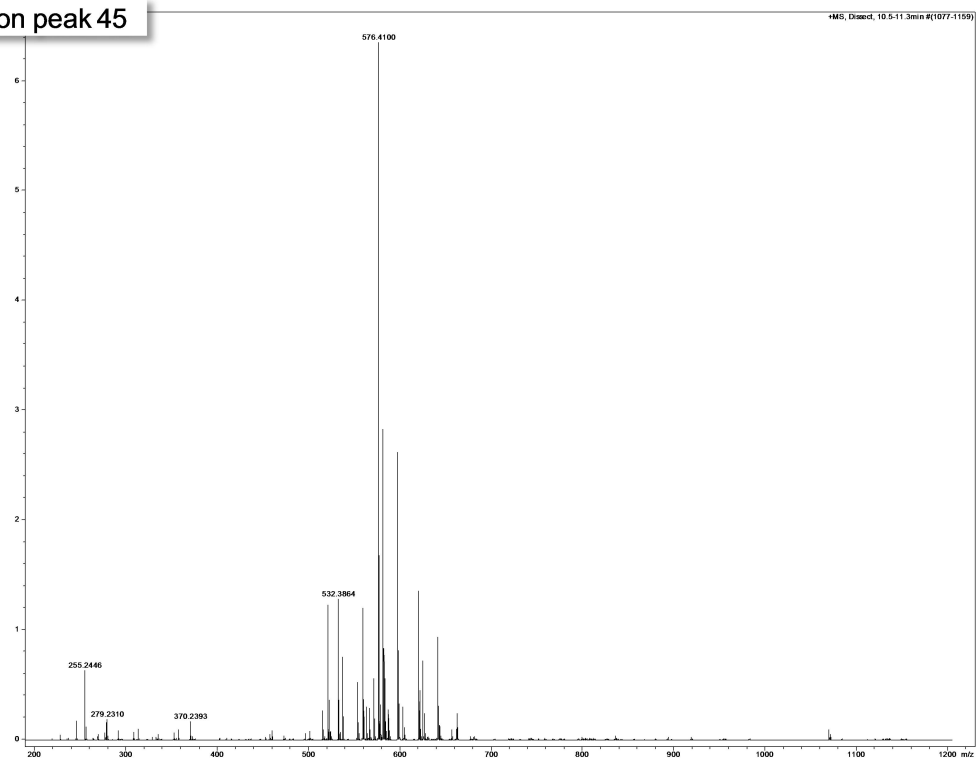
Dissection peak 38



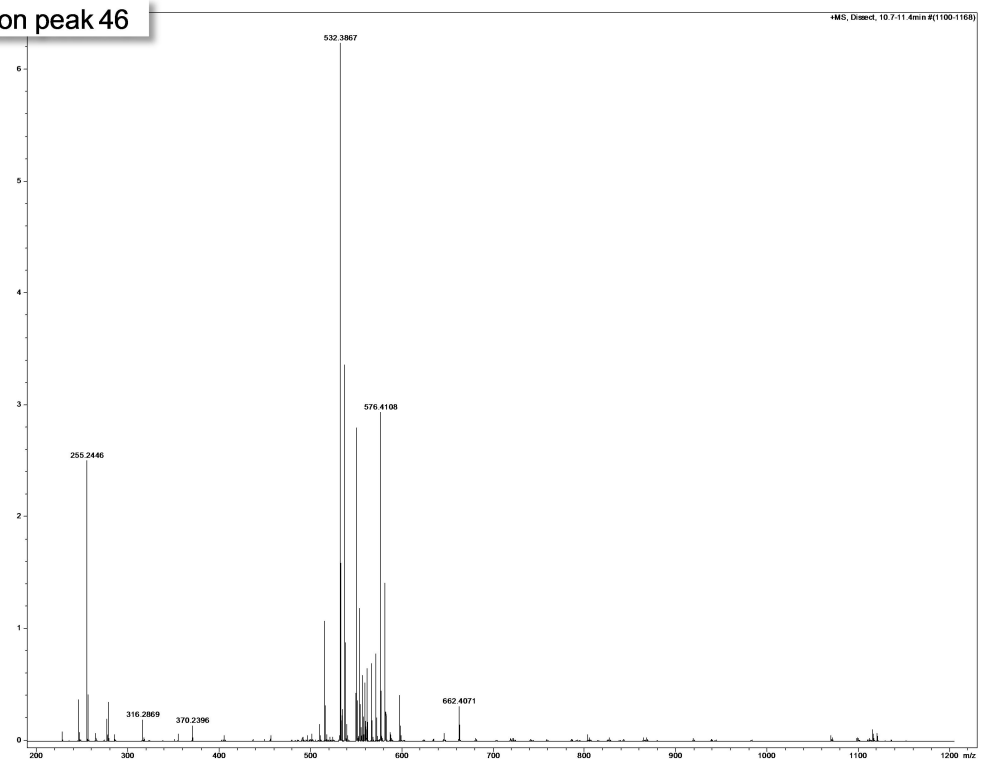
Dissection peak 39



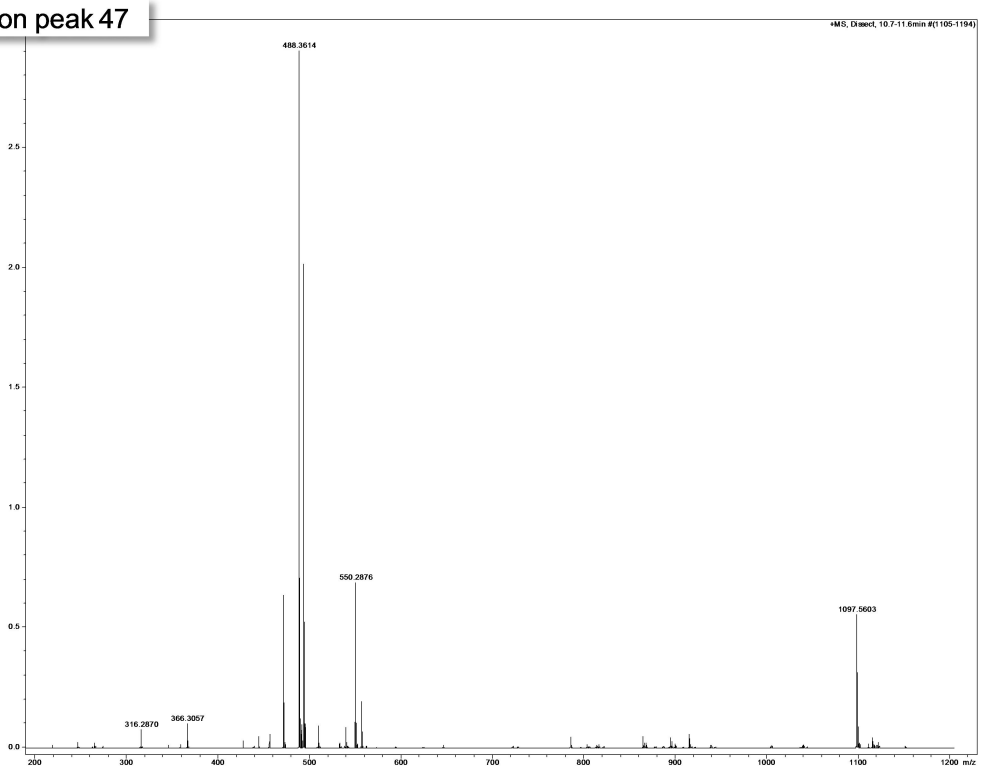
Dissection peak 45



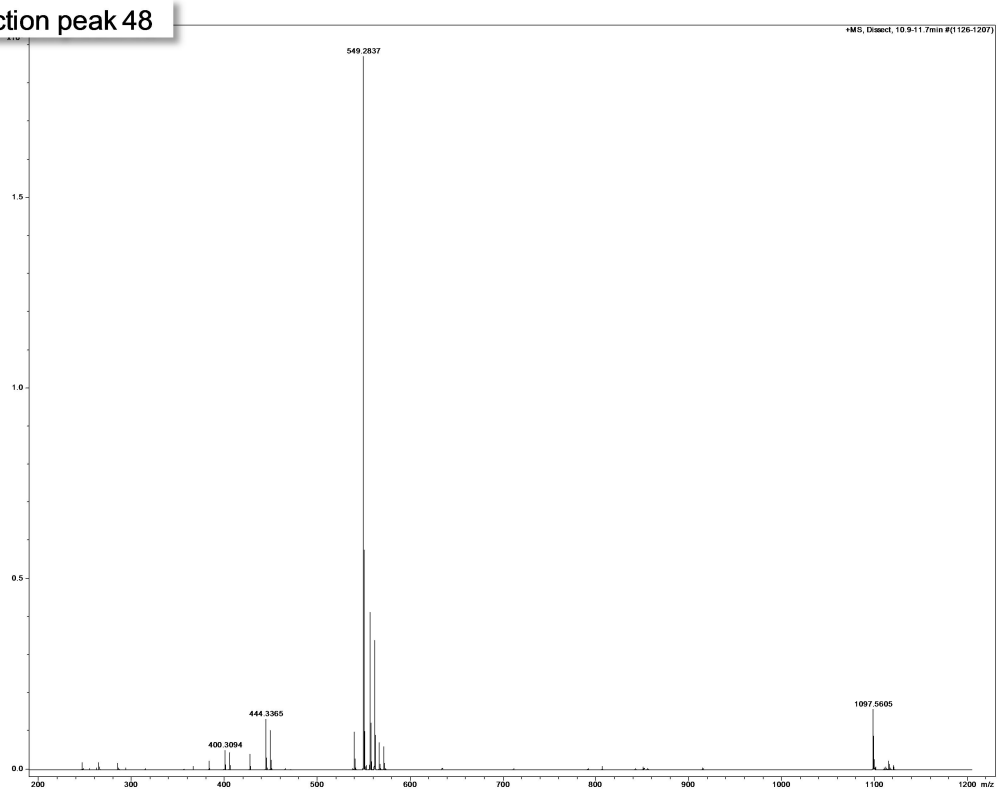
Dissection peak 46



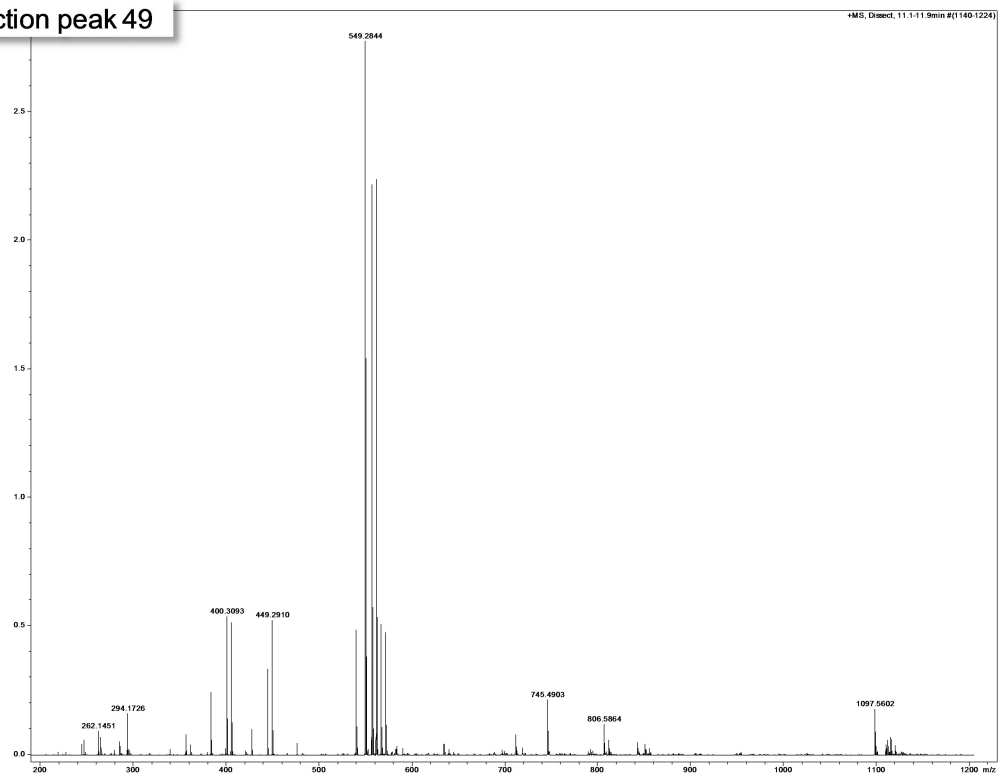
Dissection peak 47



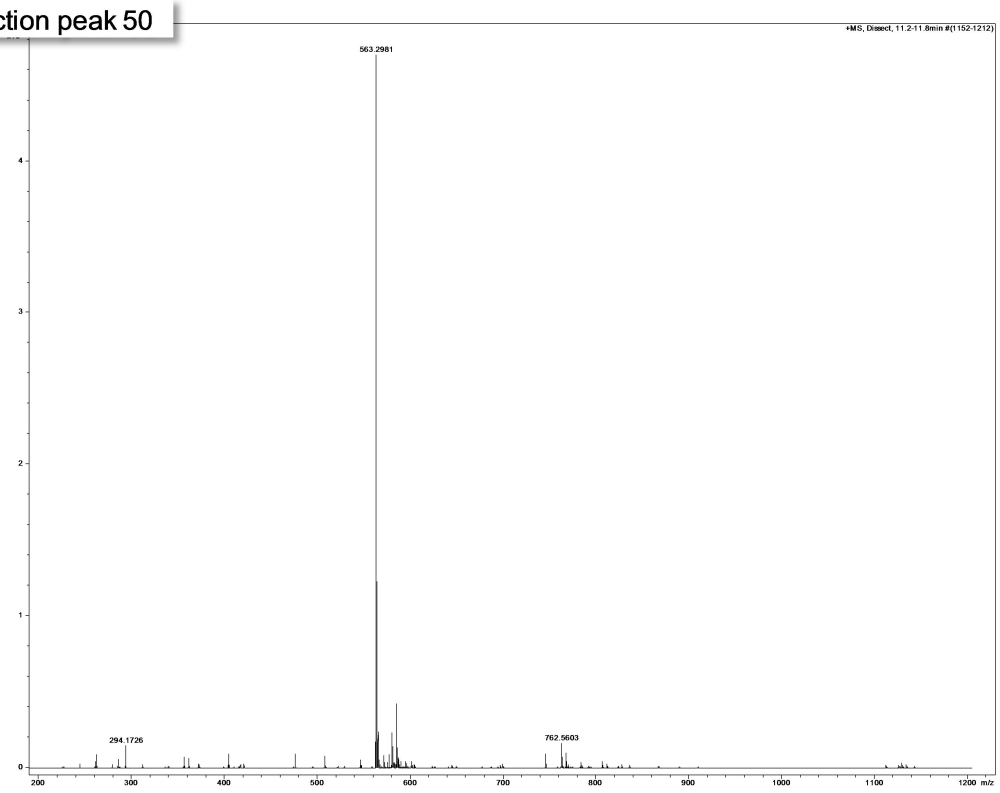
Dissection peak 48



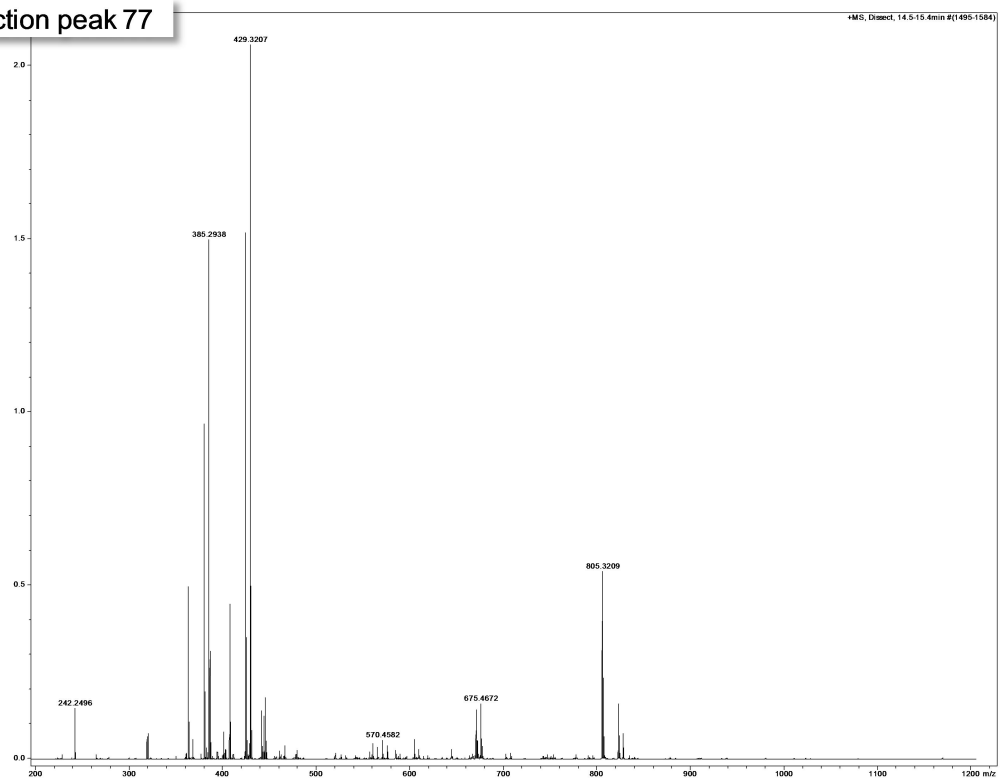
Dissection peak 49



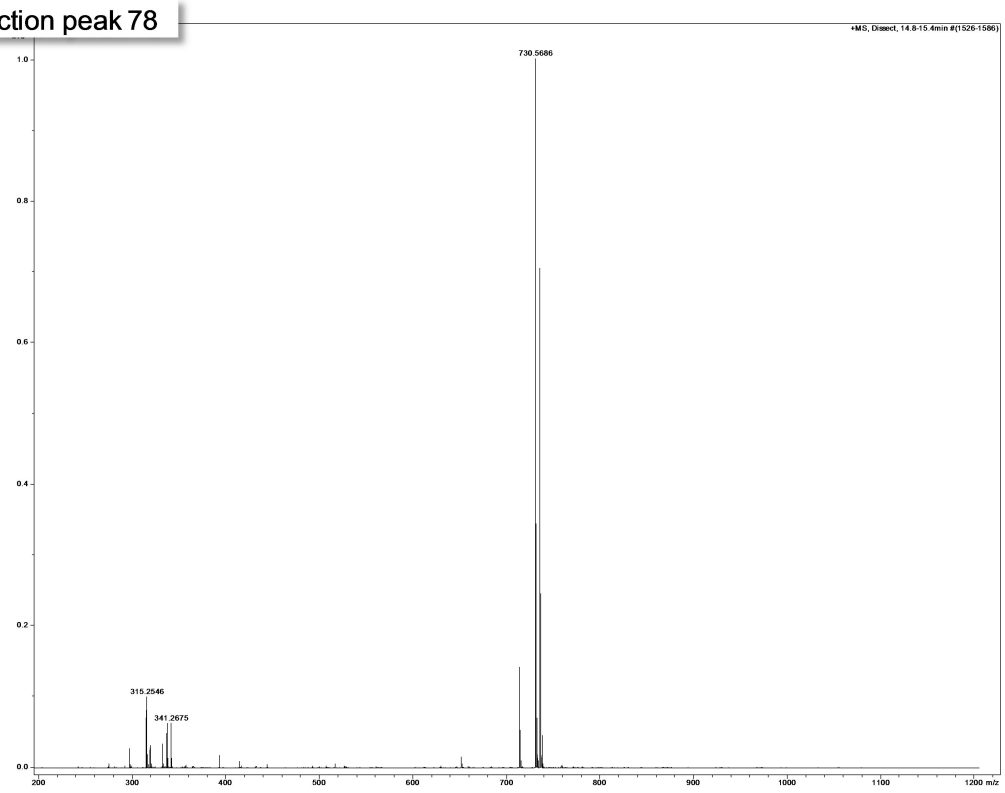
Dissection peak 50



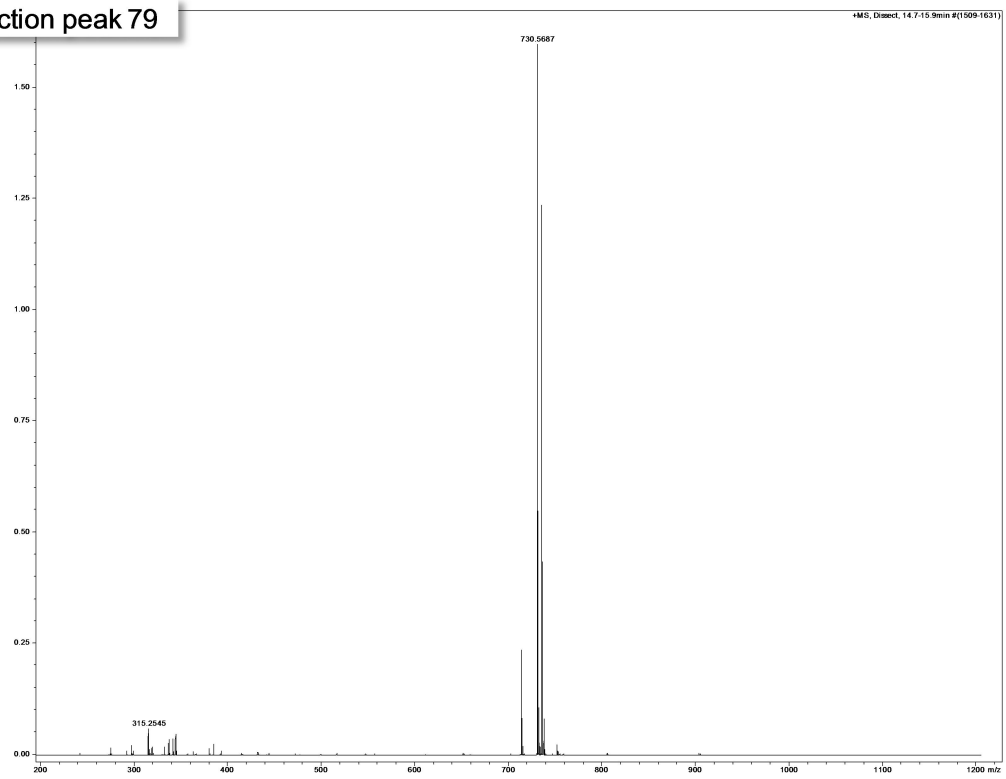
Dissection peak 77



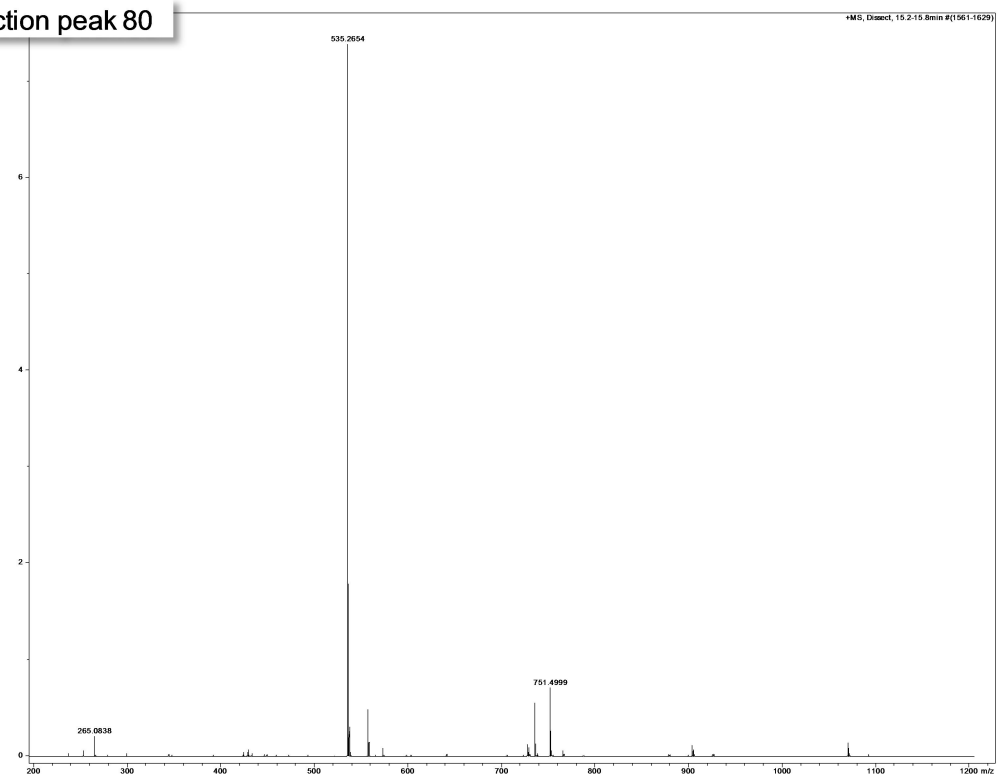
Dissection peak 78



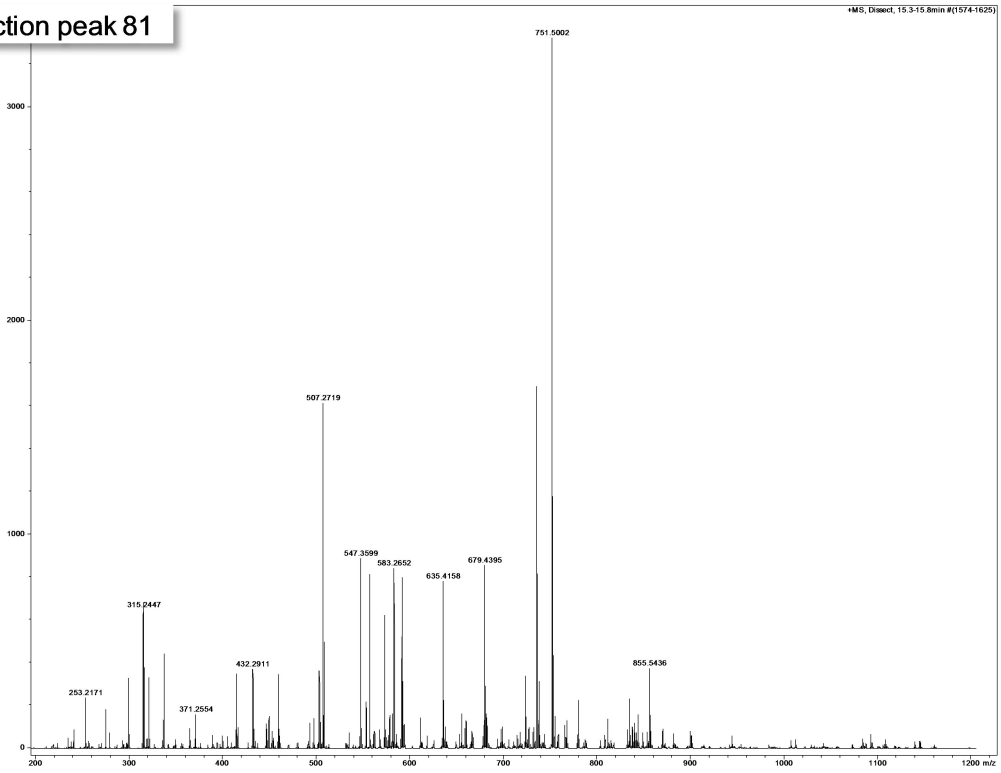
Dissection peak 79



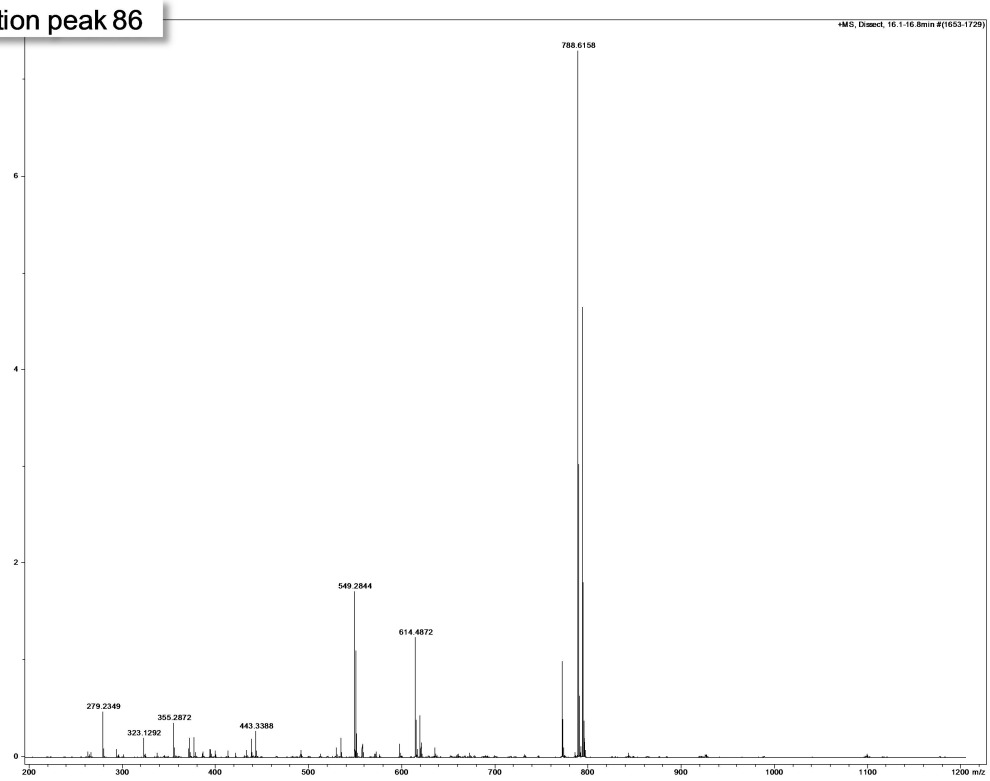
Dissection peak 80



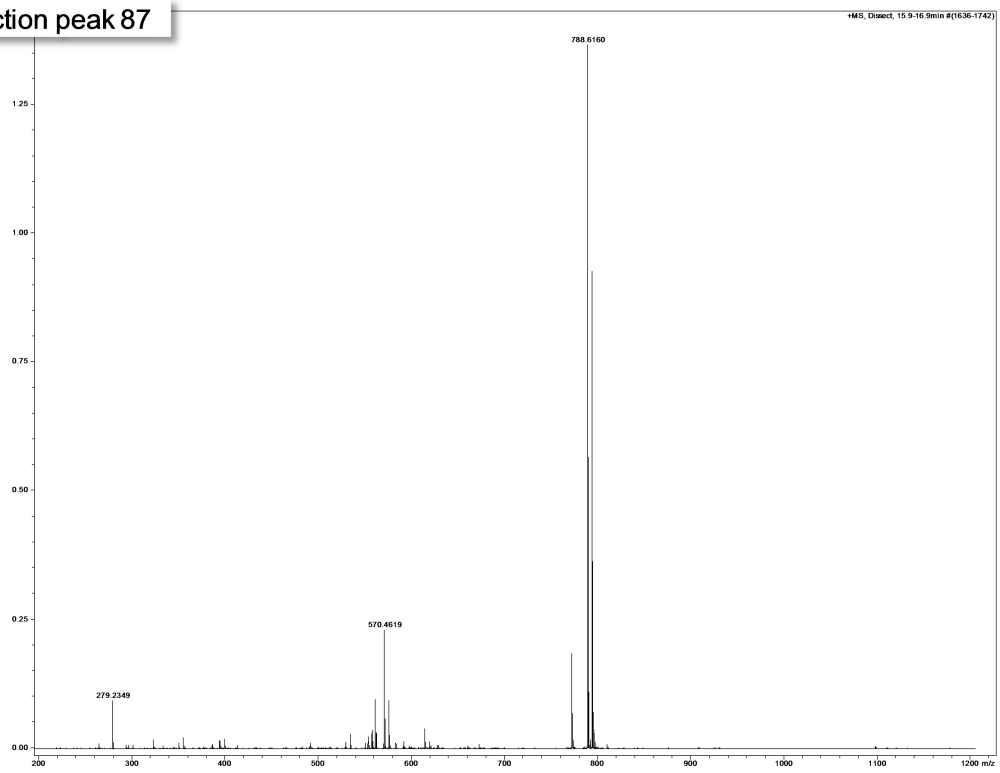
Dissection peak 81



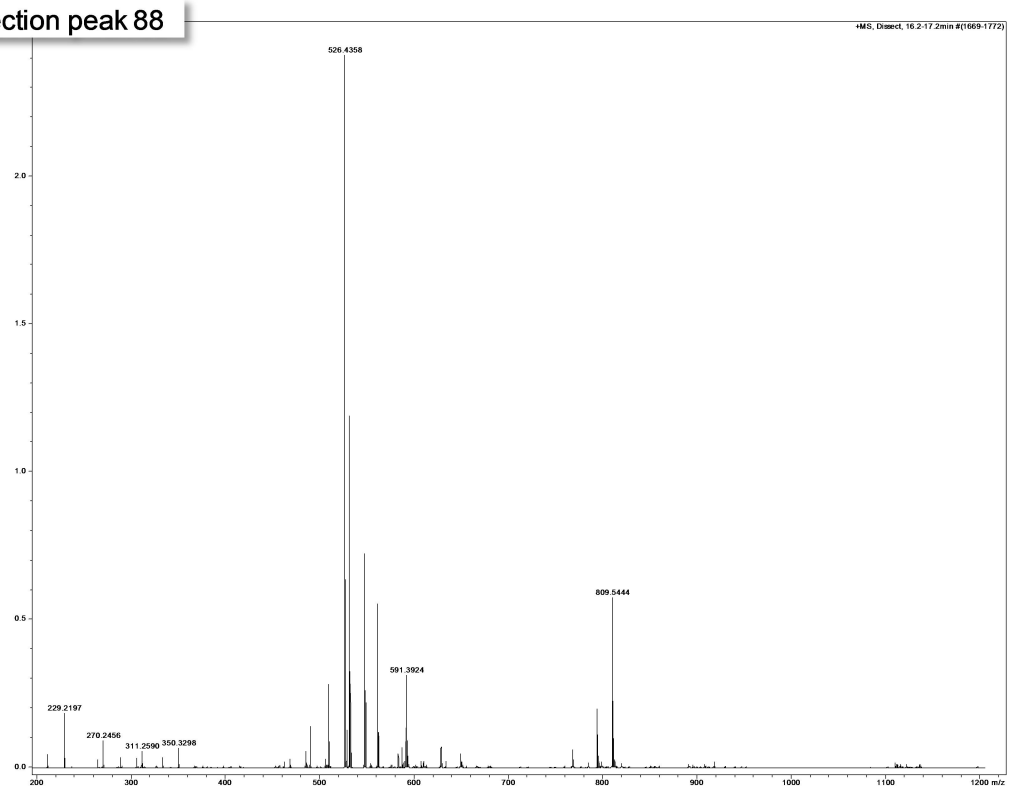
Dissection peak 86



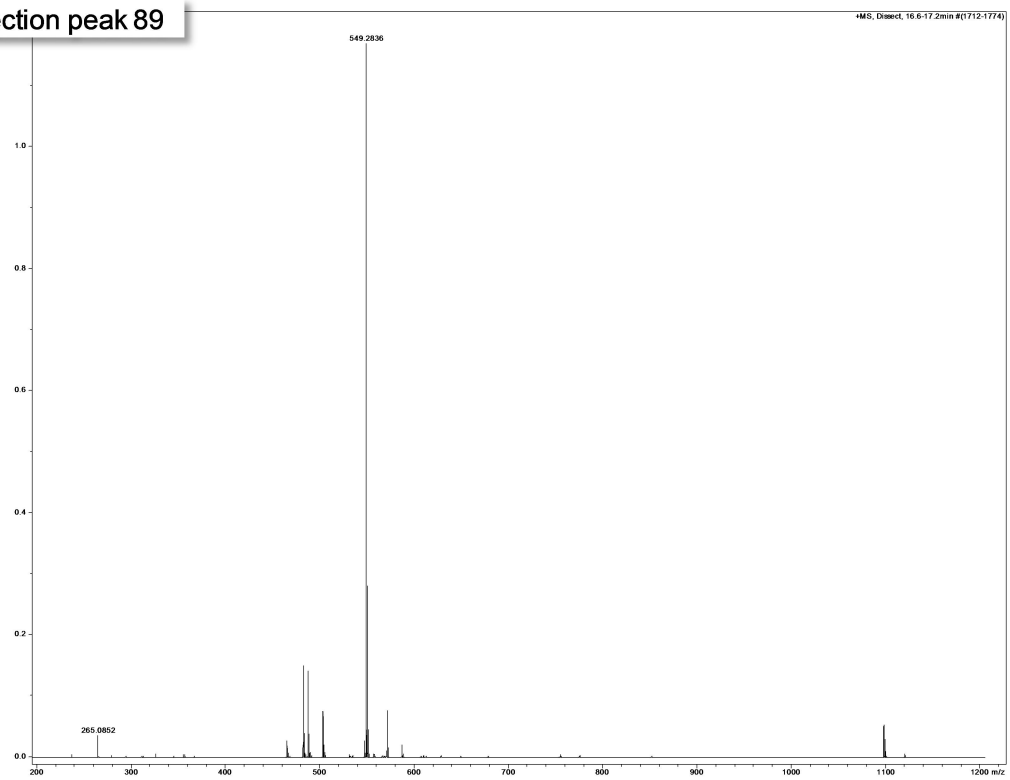
Dissection peak 87



Dissection peak 88



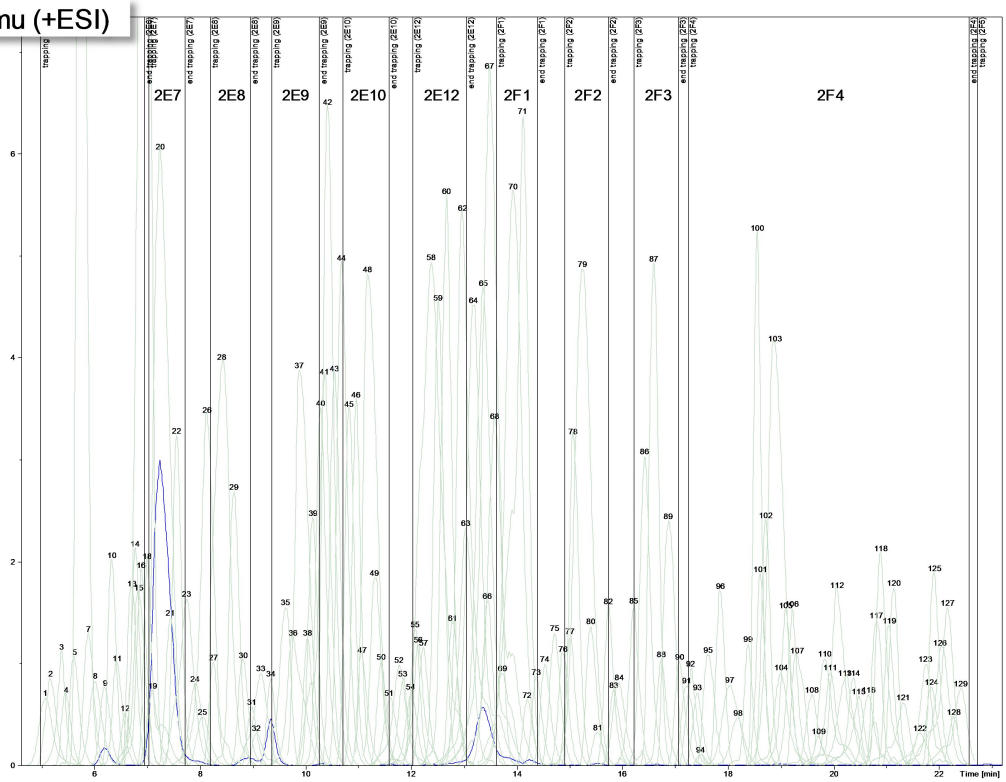
Dissection peak 89



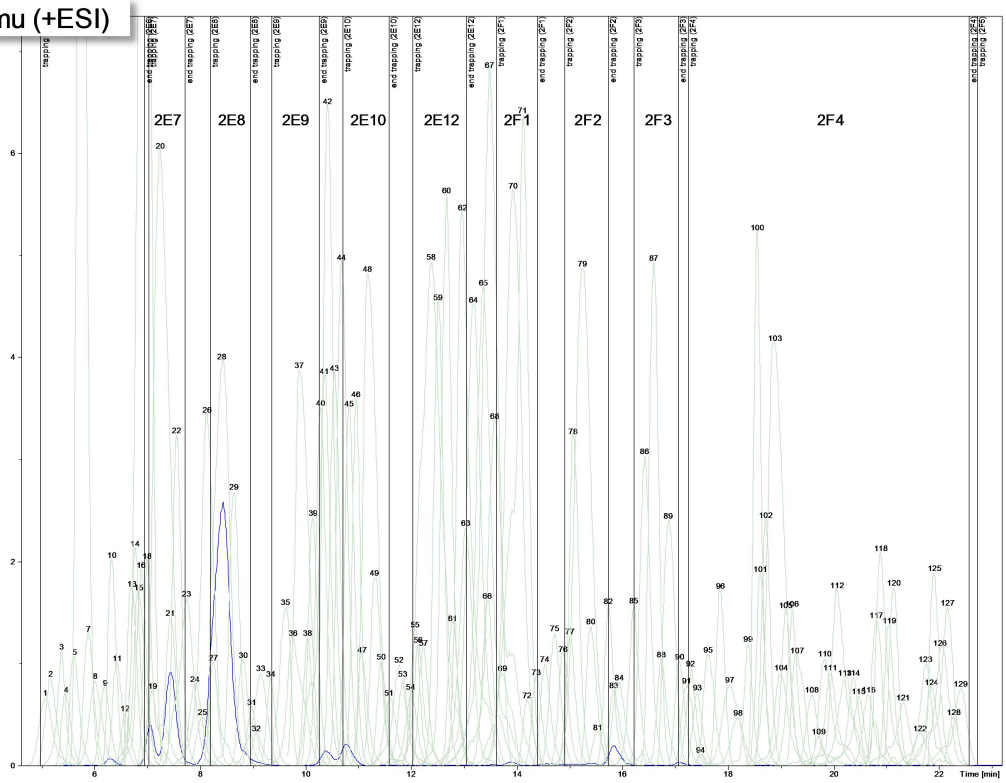
Supplementary Information B_6) LCMS on double-purified combined fractions B

High-resolution LCMS data on the double-purified combined fractions B of *Streptomyces* E_8. Extracted ion chromatograms are presented on ions with masses of 507 amu, 521 amu, 535 amu, 549 amu, 563 amu, and 577 amu $[M+H]^+$. The LCMS system used for generating these data has been located at the Institute of Food Research and was set to detect ions in positive ion mode (+ESI).

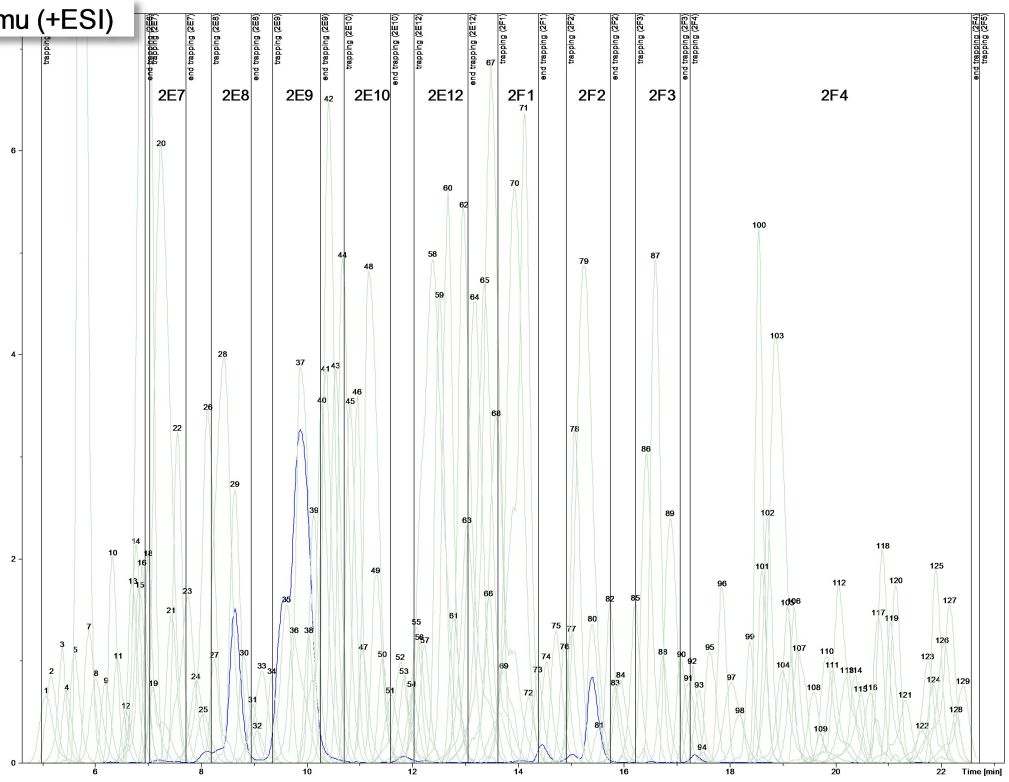
507 amu (+ESI)



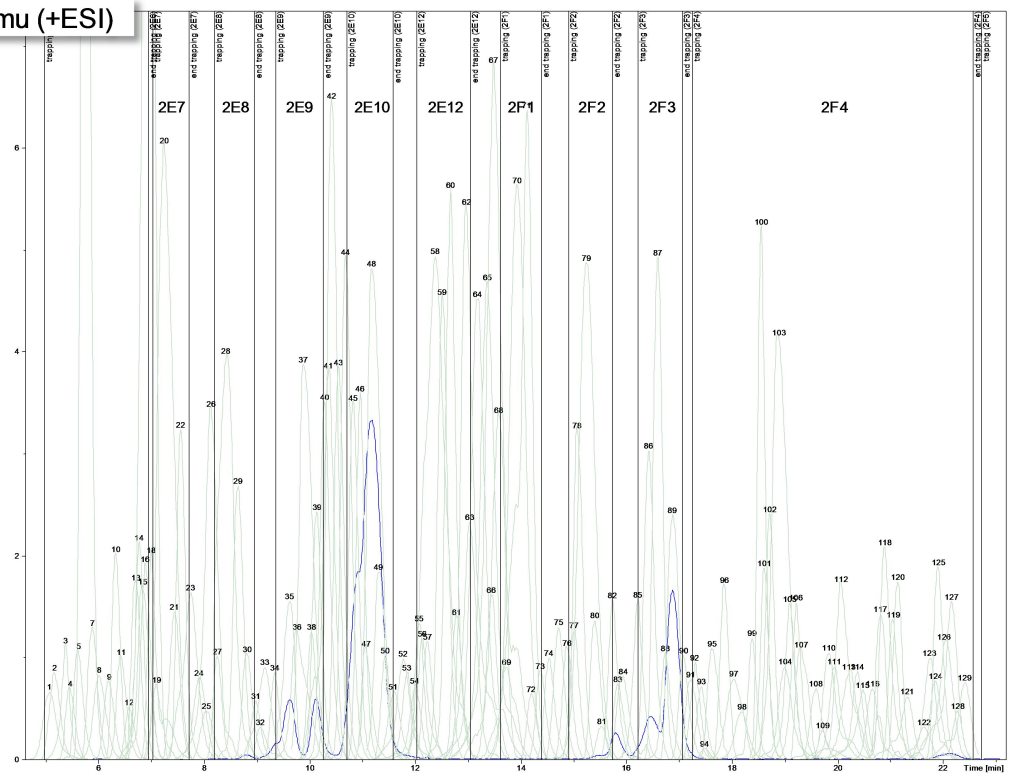
521 amu (+ESI)



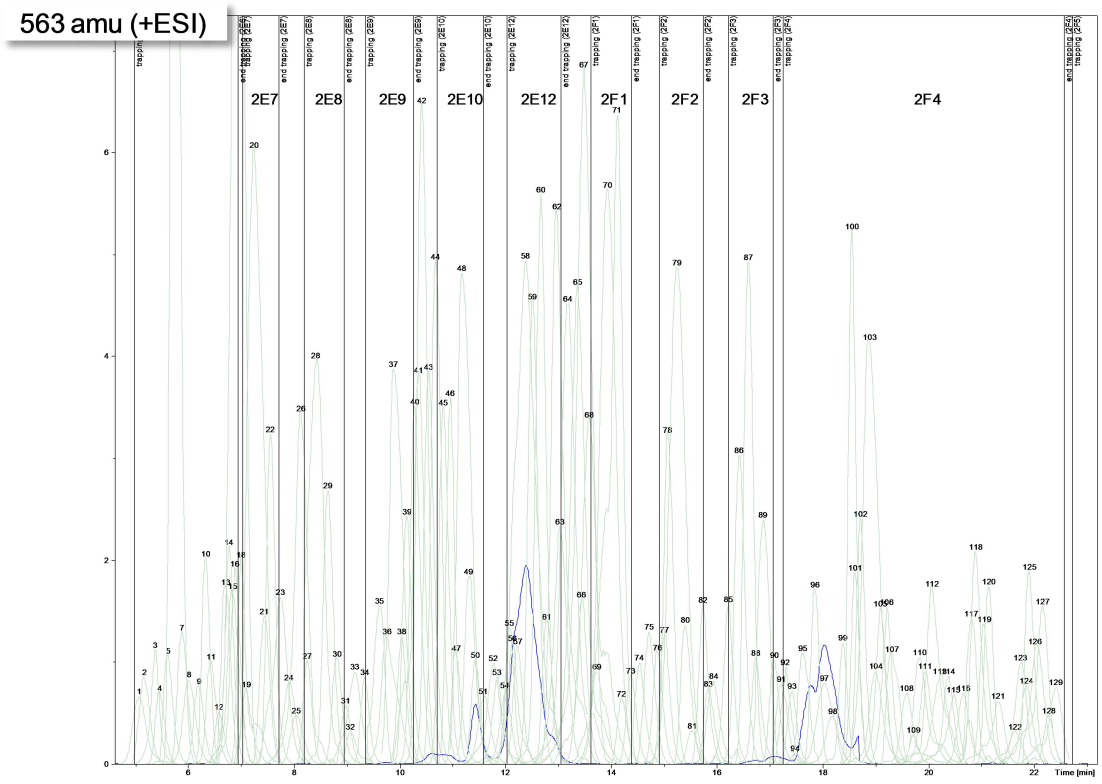
535 amu (+ESI)



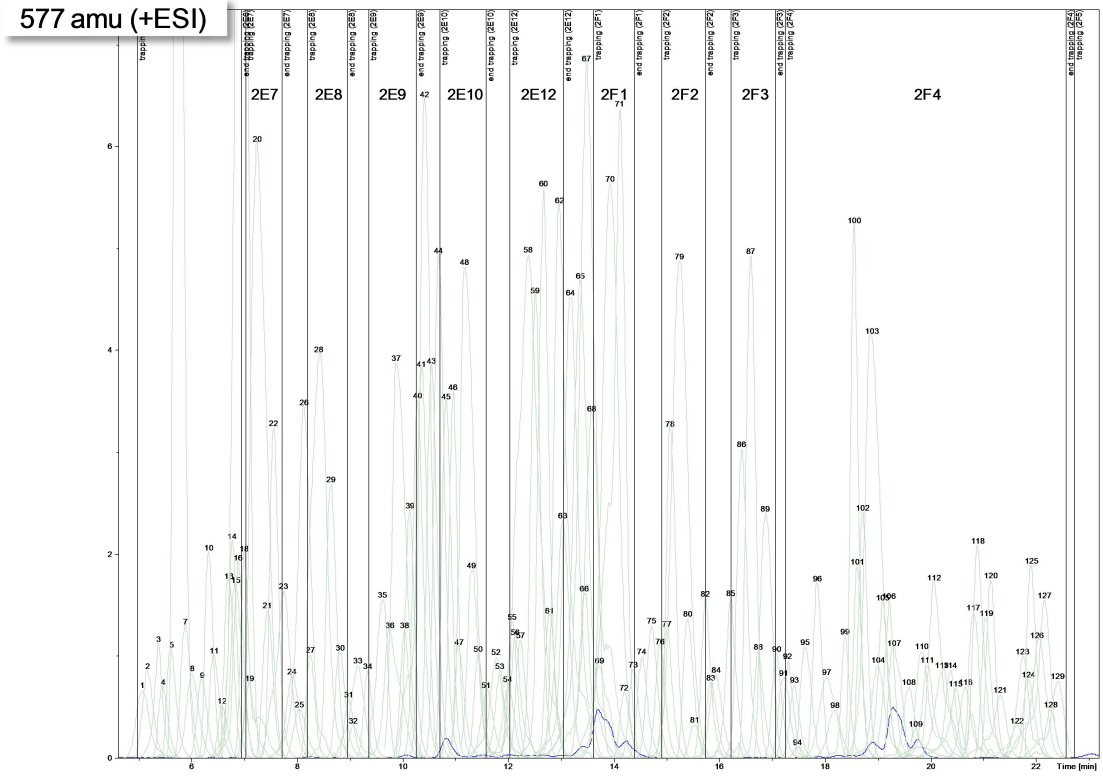
549 amu (+ESI)



563 amu (+ESI)



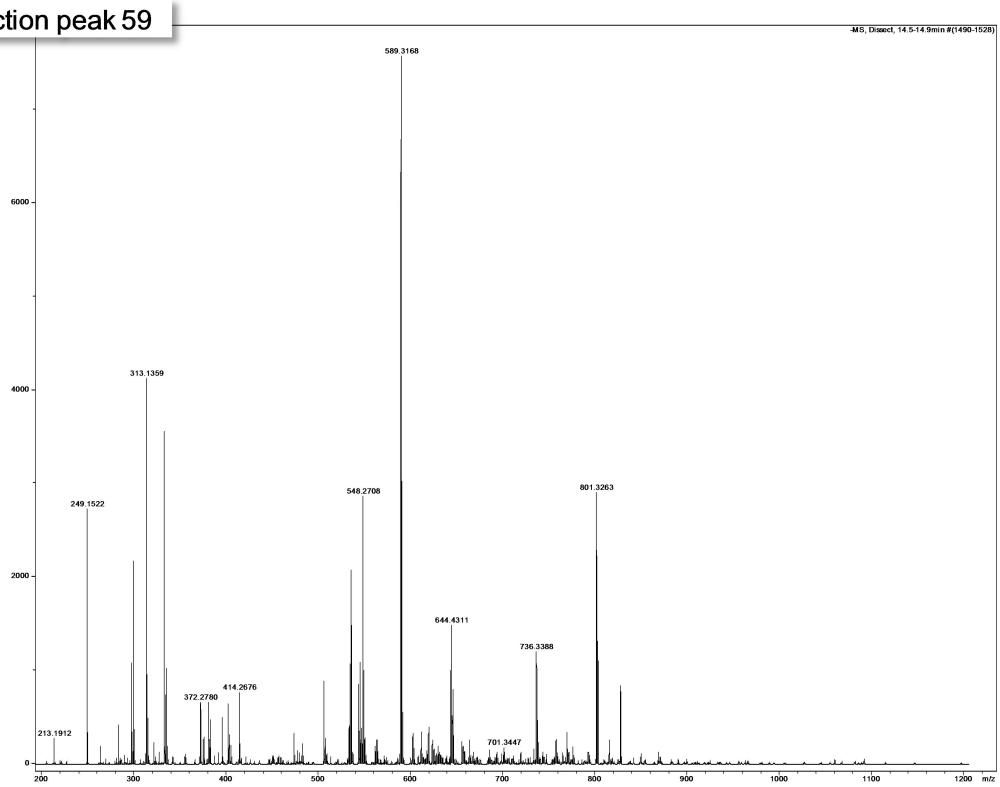
577 amu (+ESI)



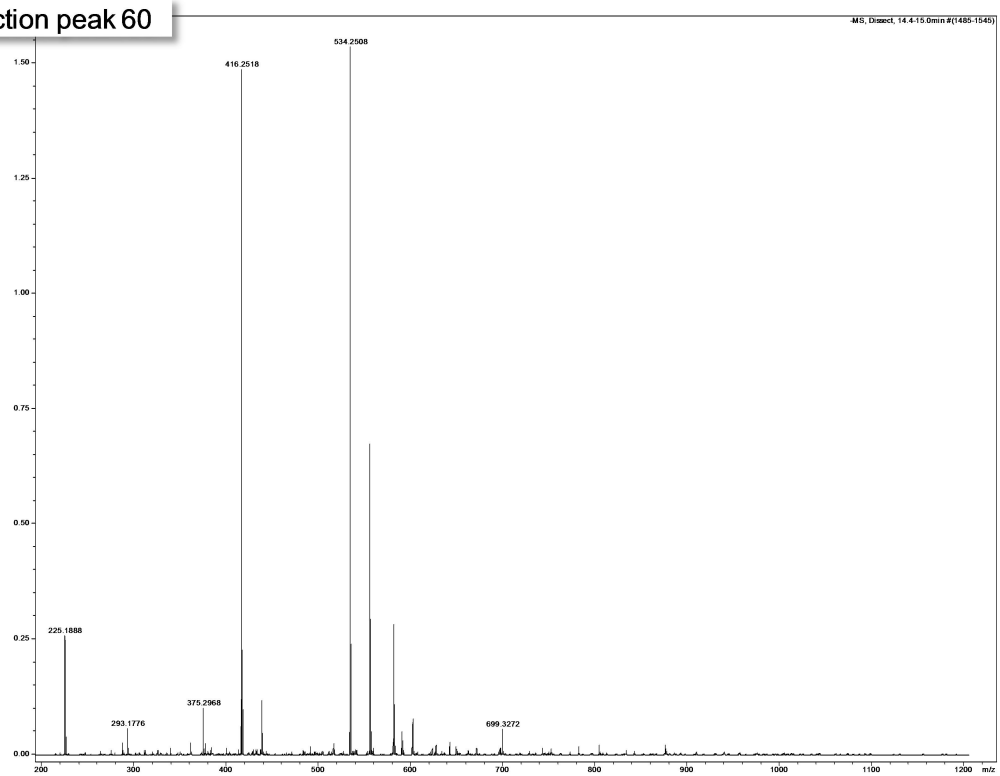
Supplementary Information B_7) LCMS on double-purified combined fractions B

High-resolution LCMS data on the double-purified combined fractions B of *Streptomyces* E_8. Dissection peaks are presented of fractions 2G4 and 2G5 containing ions with masses of 533 amu and 547 amu $[M-H]^-$. The LCMS system used for generating these data has been located at the Institute of Food Research and was set to detect ions in negative ion mode (-ESI).

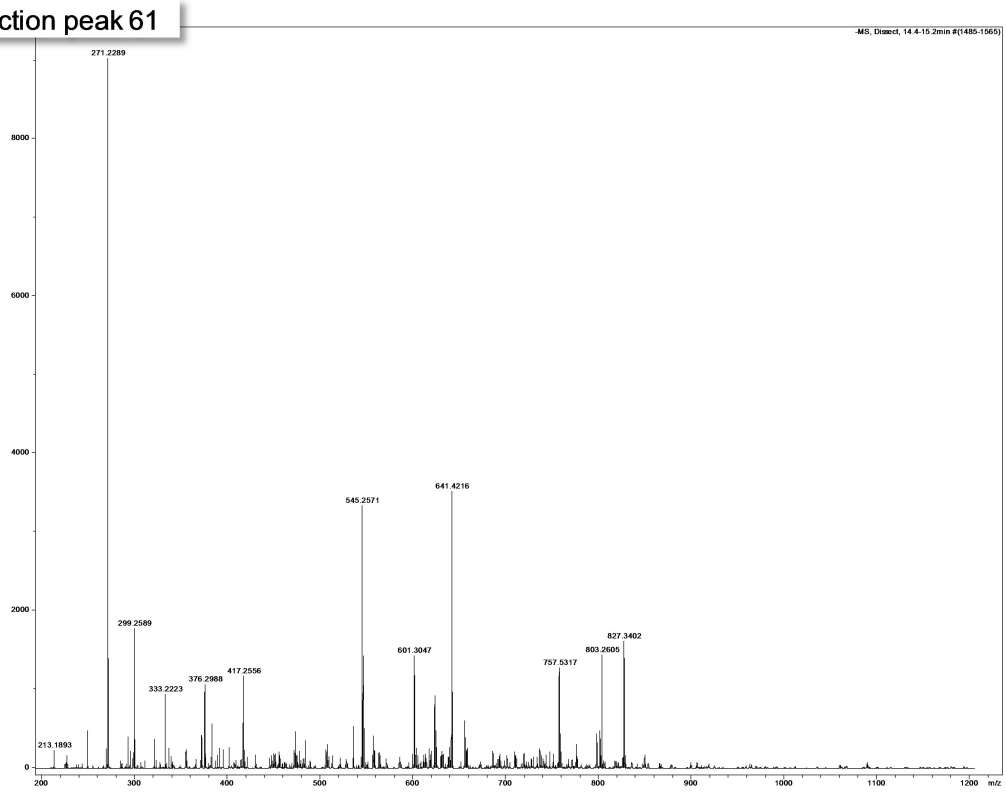
Dissection peak 59



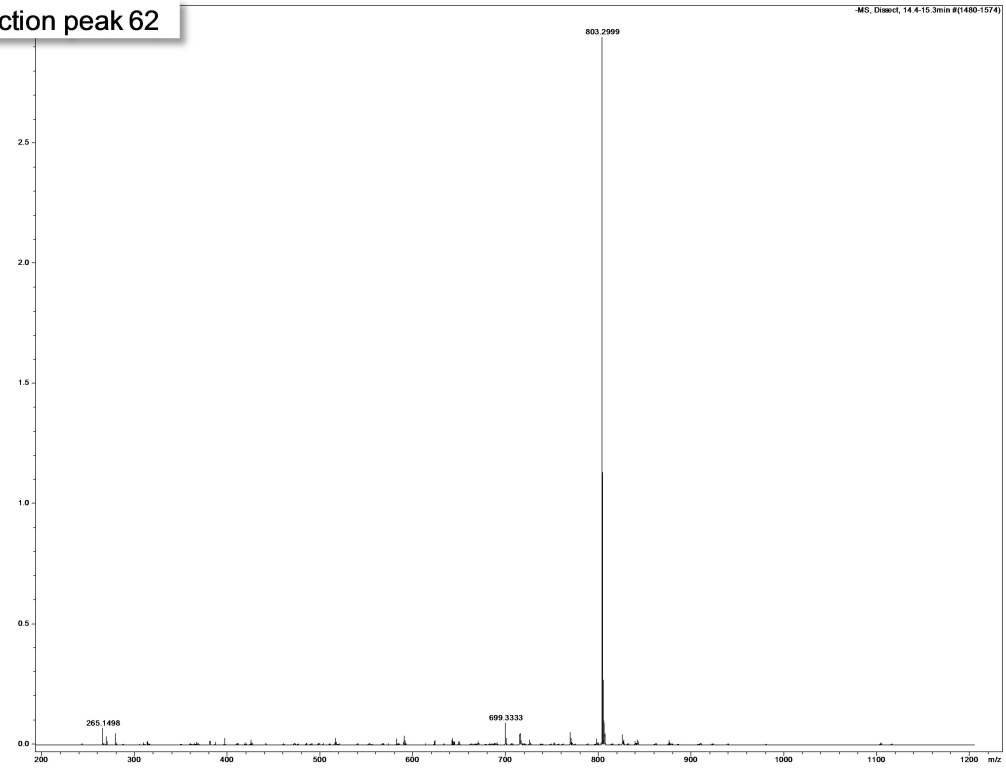
Dissection peak 60



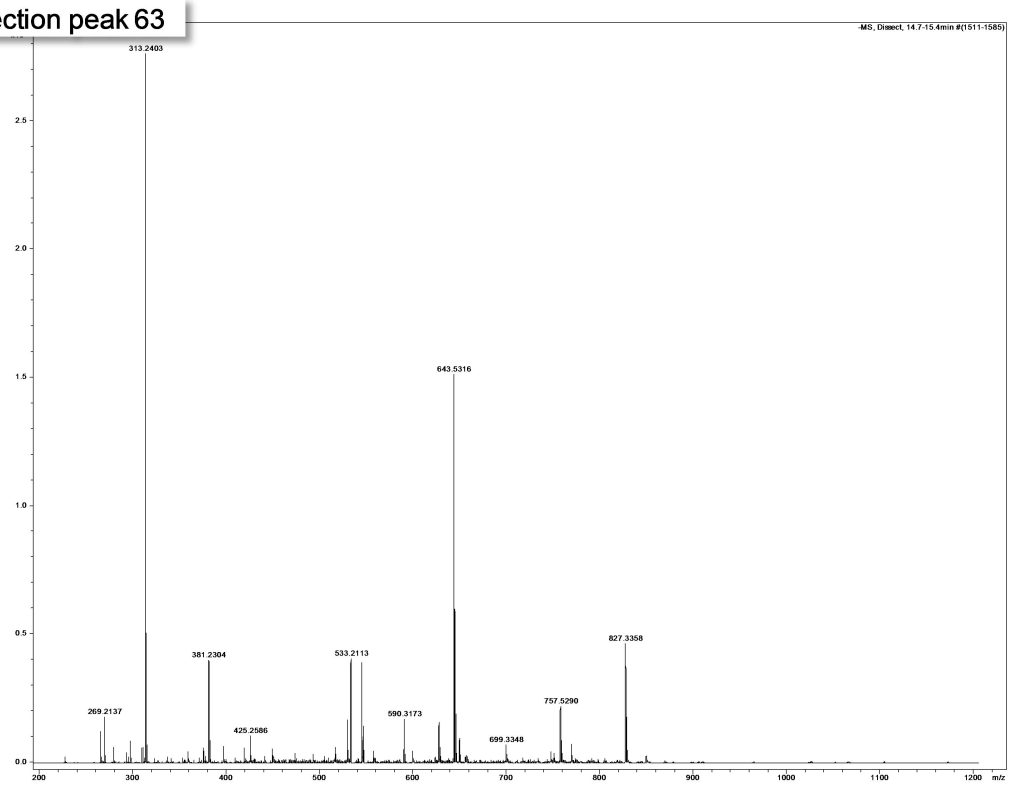
Dissection peak 61



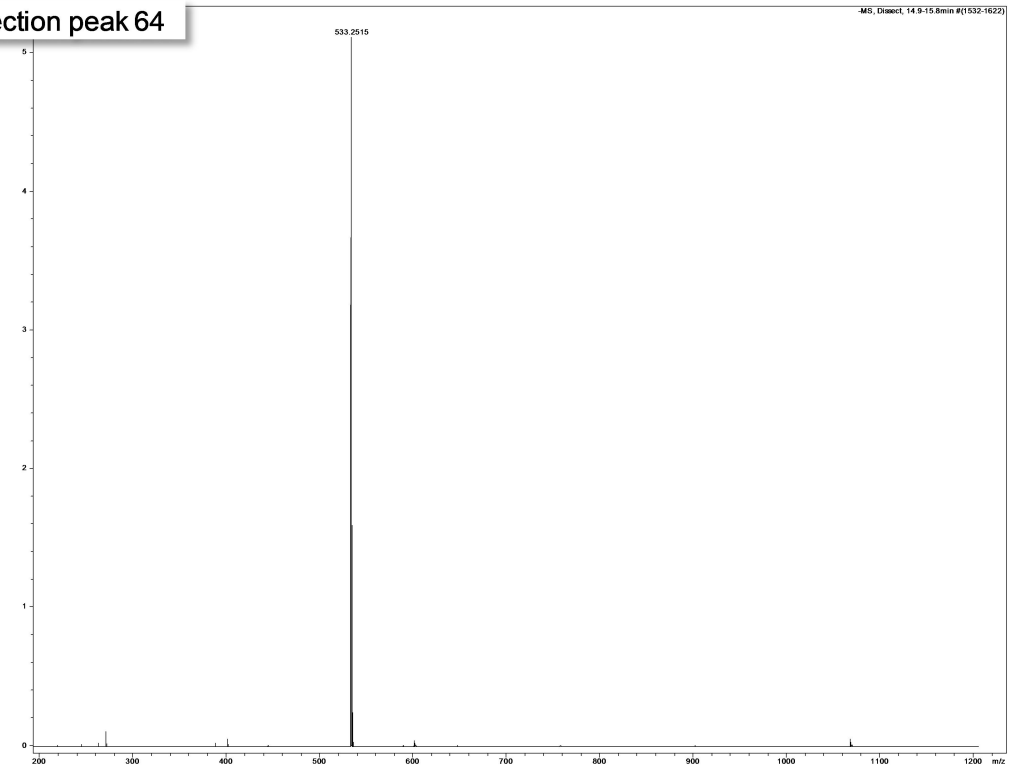
Dissection peak 62



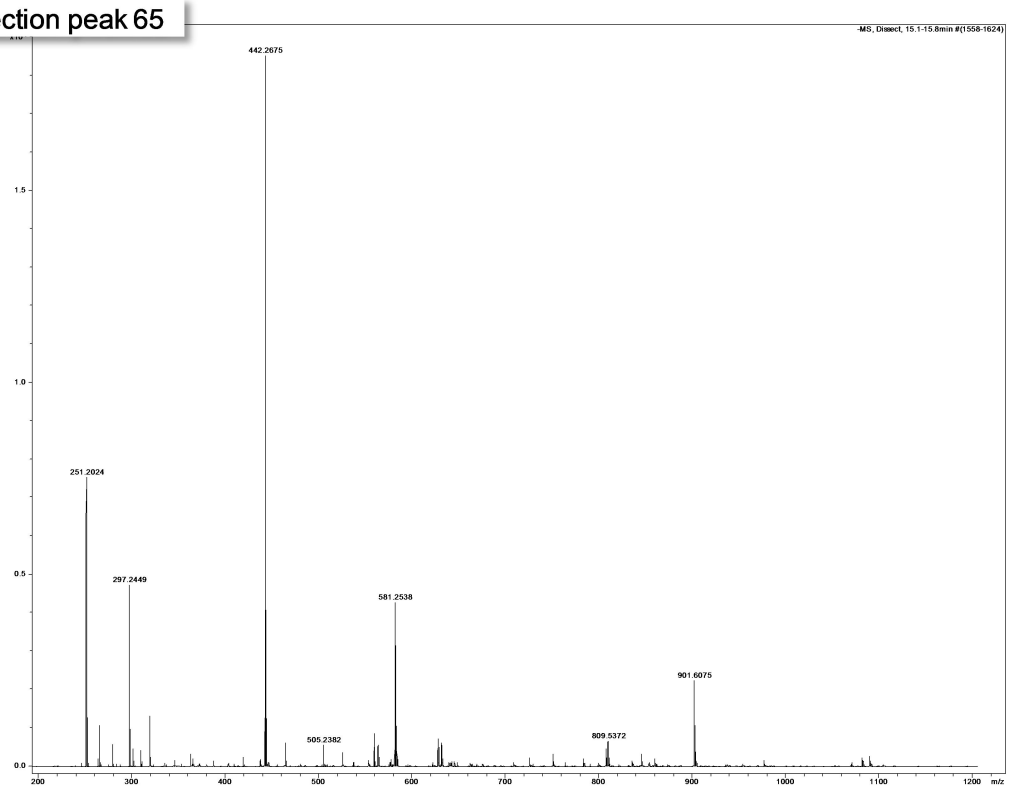
Dissection peak 63



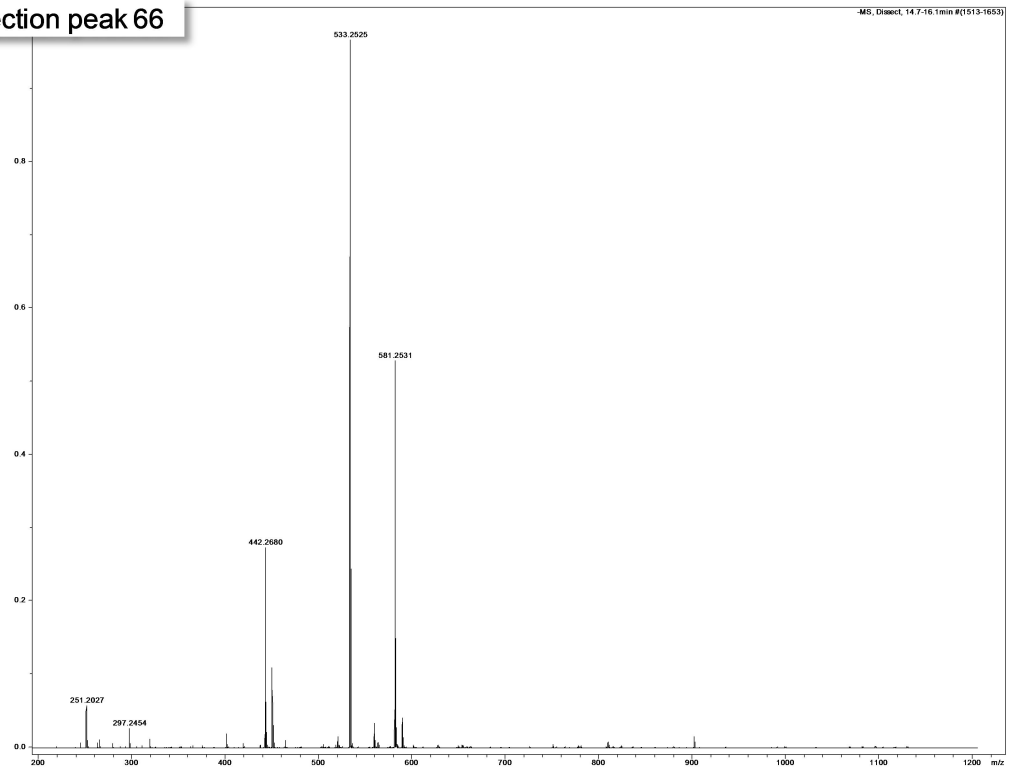
Dissection peak 64



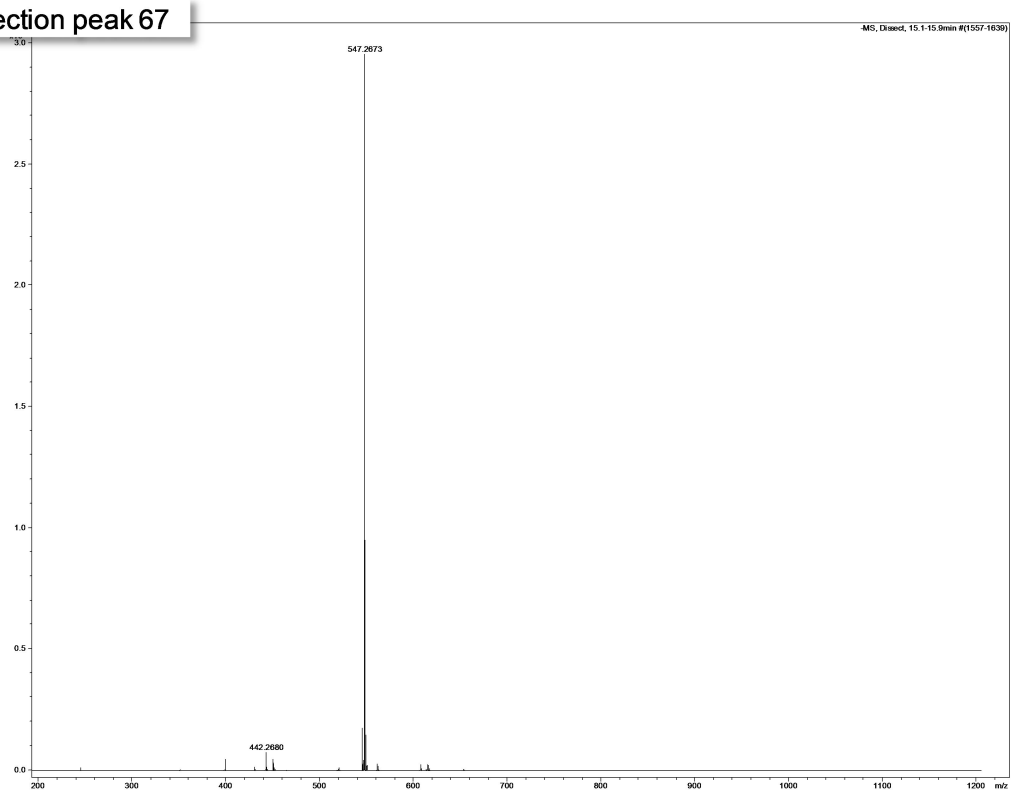
Dissection peak 65



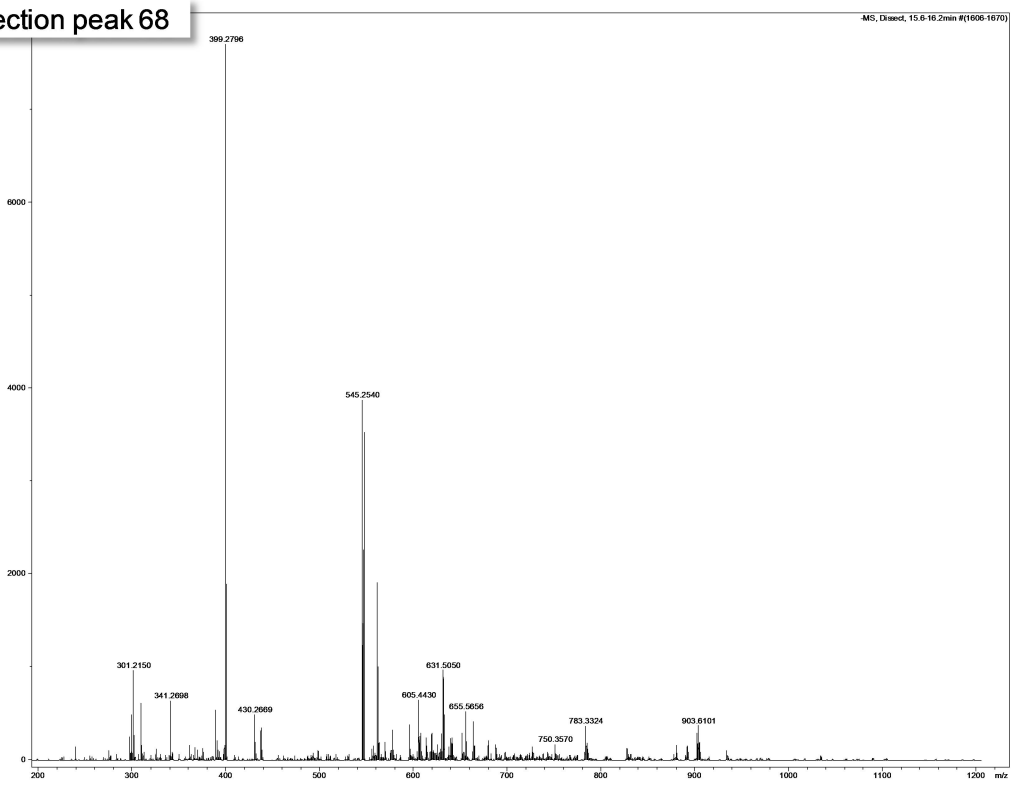
Dissection peak 66



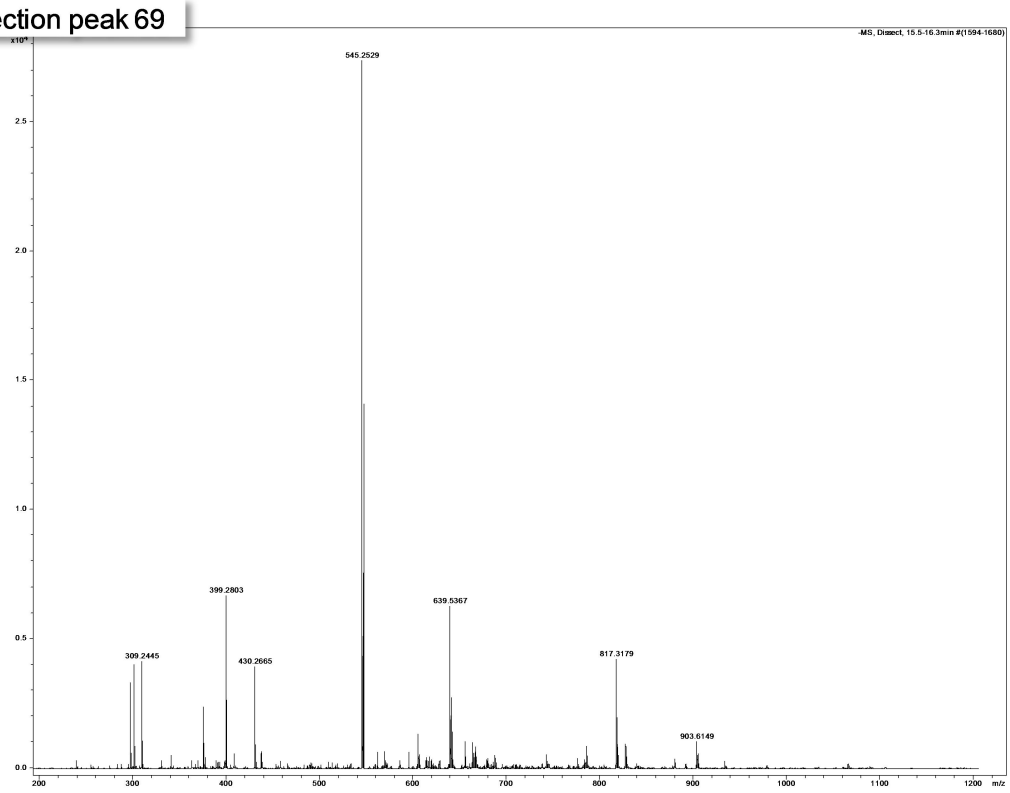
Dissection peak 67



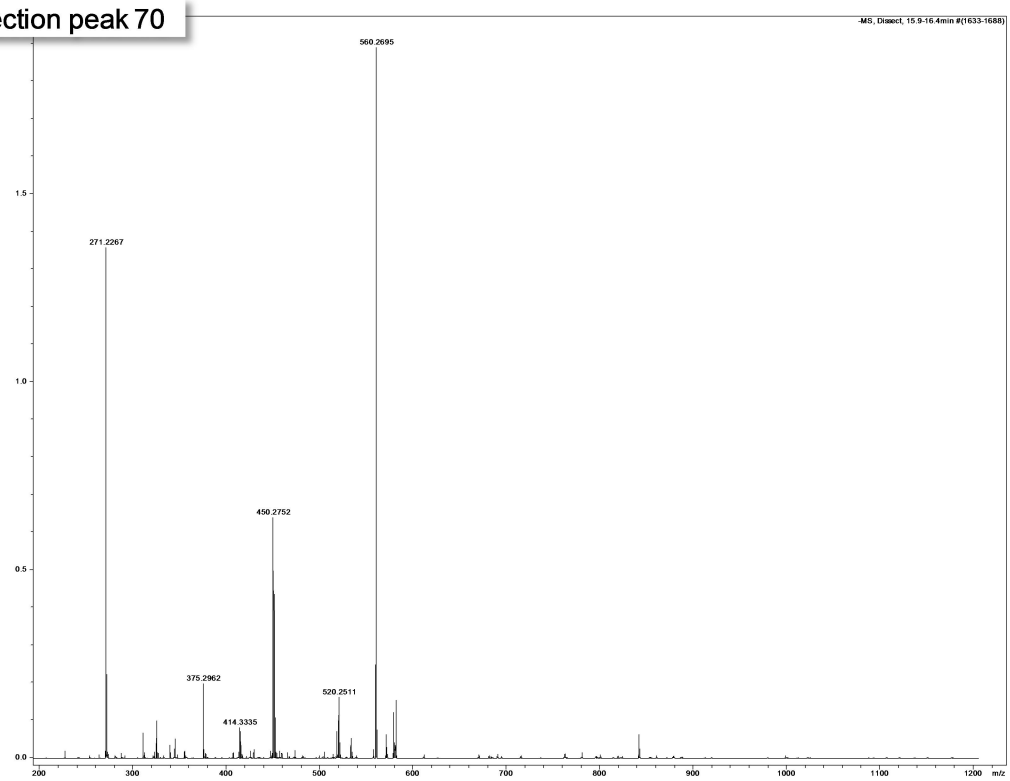
Dissection peak 68



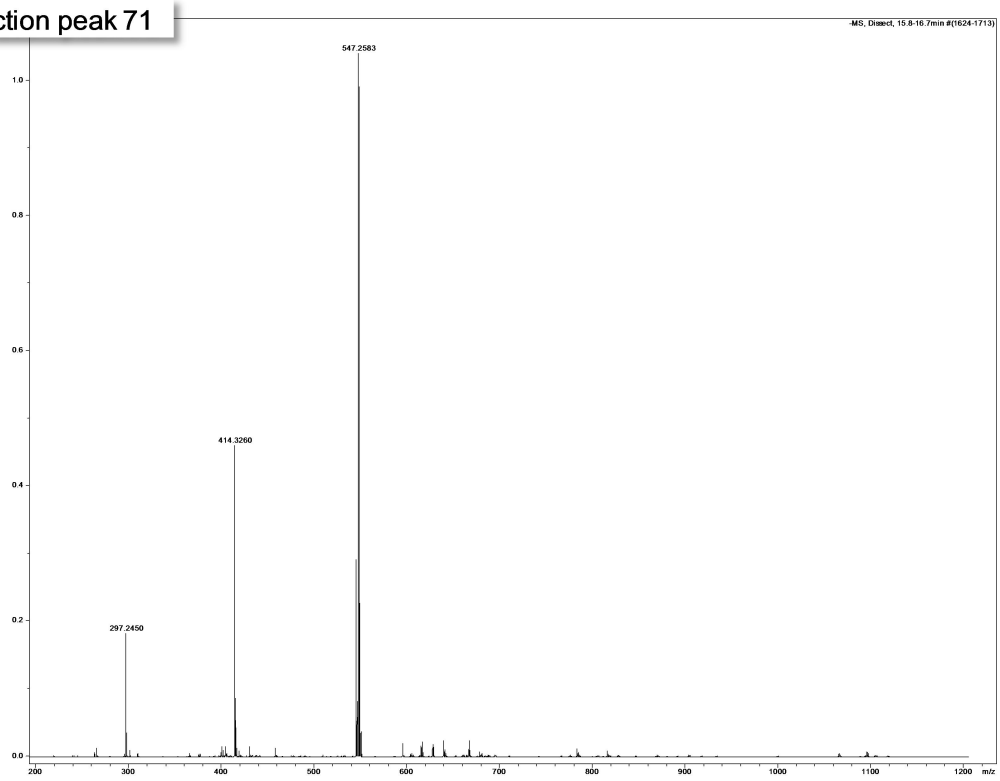
Dissection peak 69



Dissection peak 70



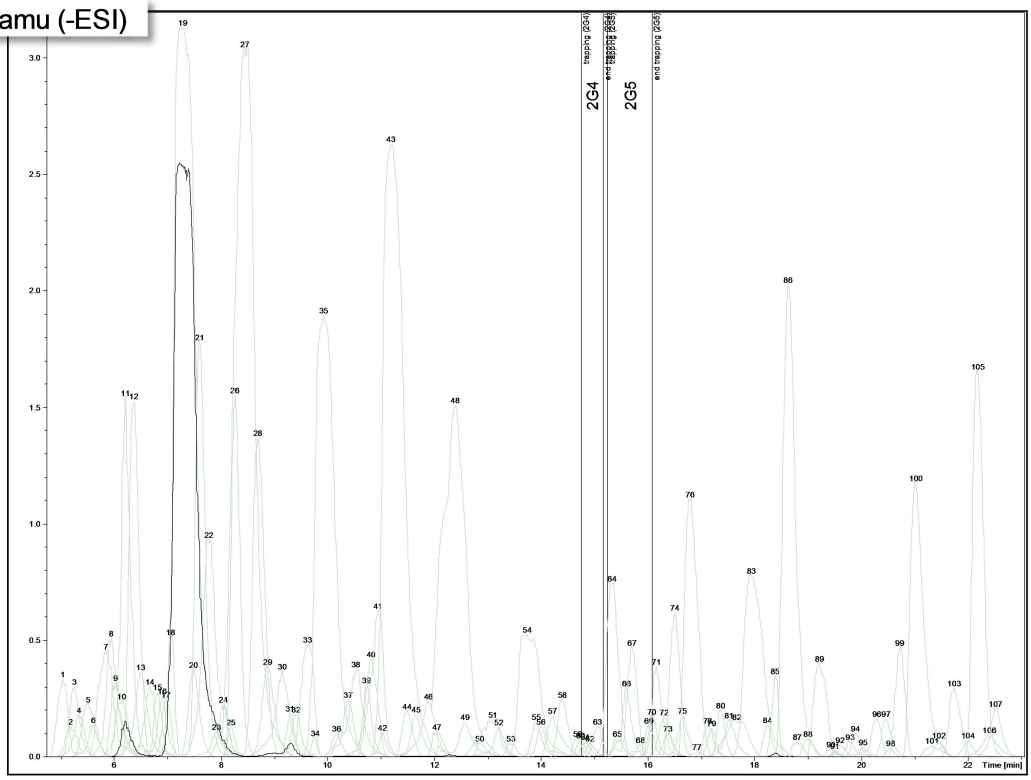
Dissection peak 71



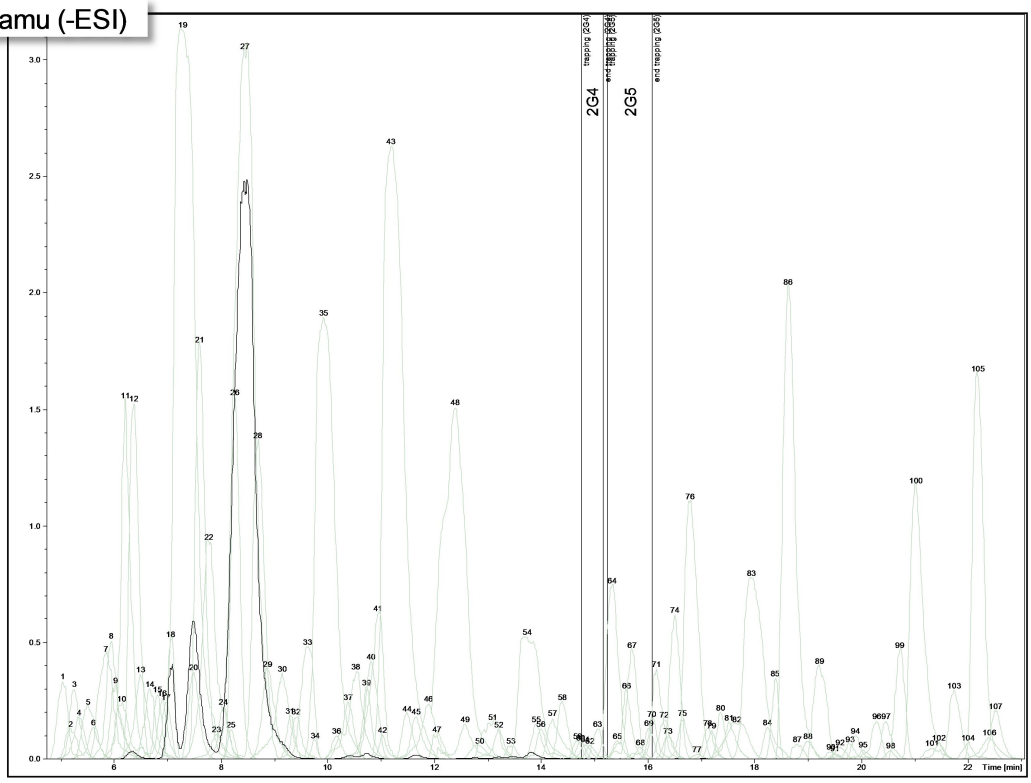
Supplementary Information B_8) LCMS on double-purified combined fractions B

High-resolution LCMS data on the double-purified combined fractions B of *Streptomyces* E_8. Extracted ions chromatograms are presented on ions with masses of 505 amu, 519 amu, 533 amu, 547 amu, 561 amu, and 575 amu $[M-H]^-$. The LCMS system used for generating these data has been located at the Institute of Food Research and was set to detect ions in negative ion mode (-ESI).

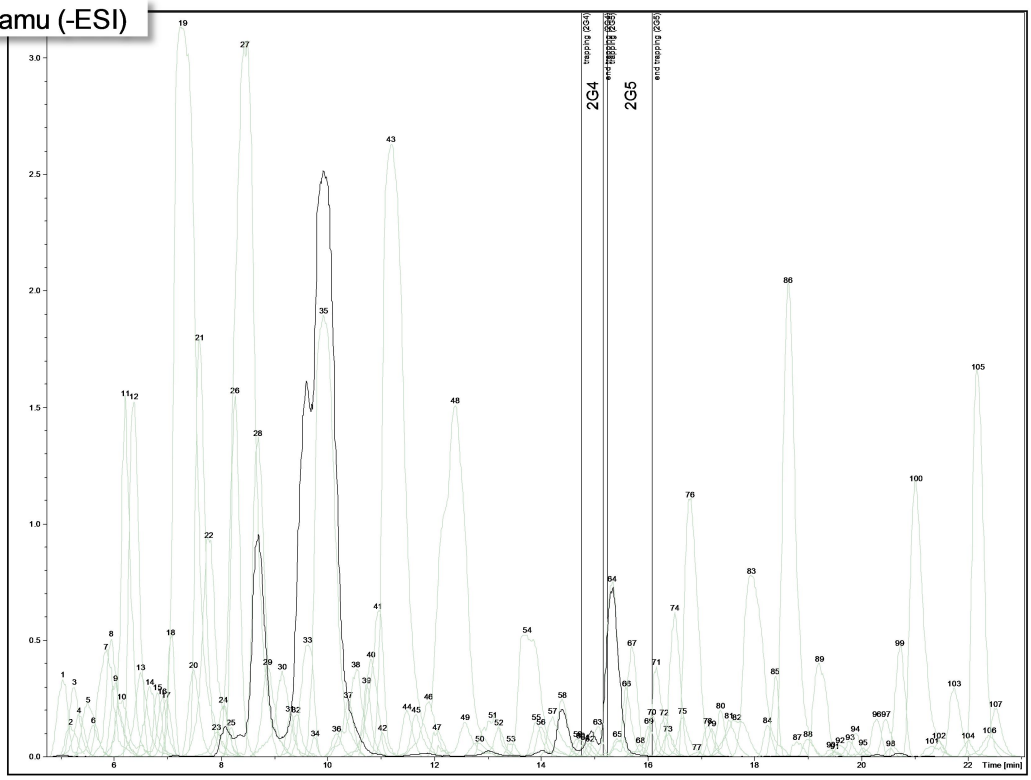
505 amu (-ESI)



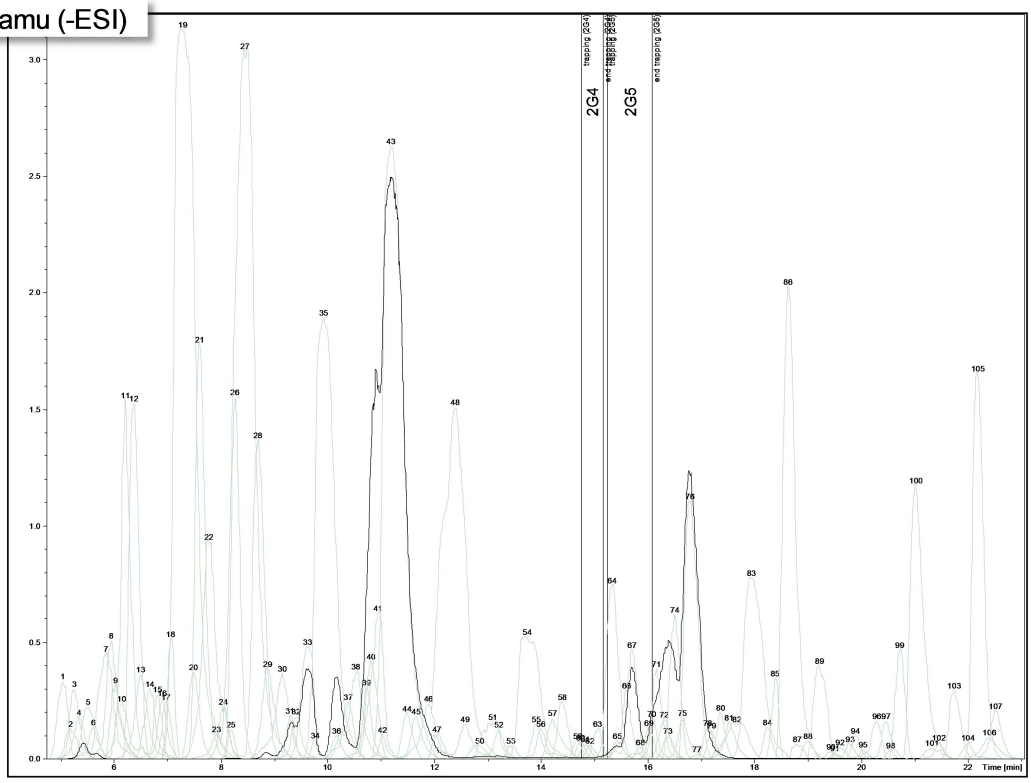
519 amu (-ESI)



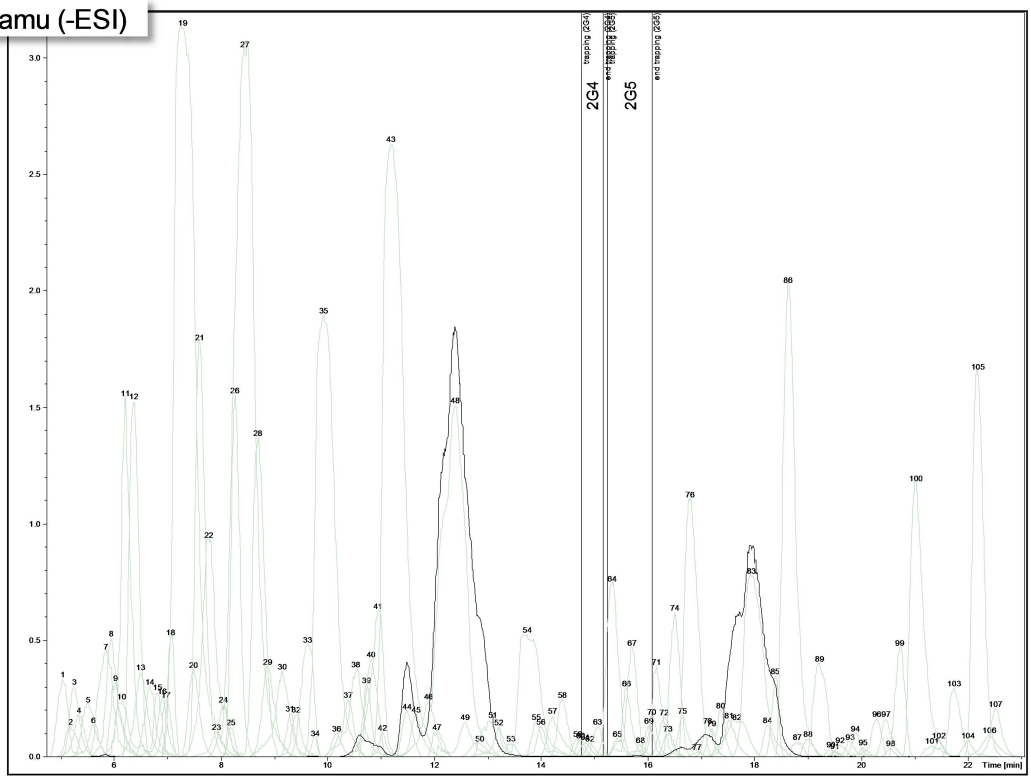
533 amu (-ESI)



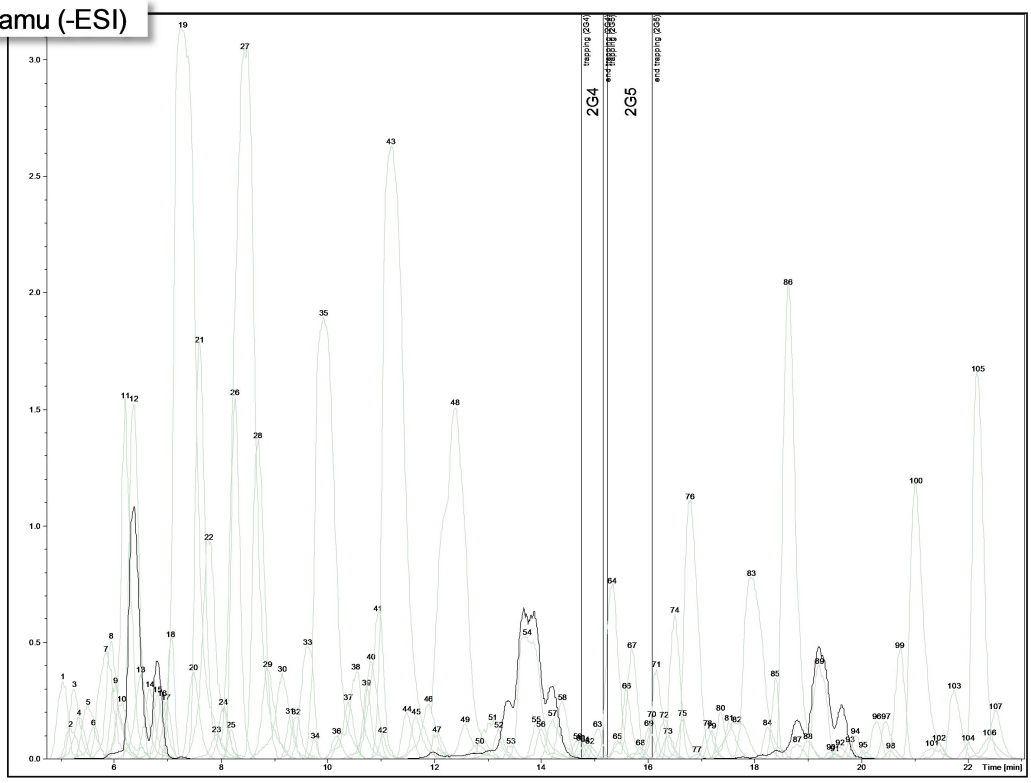
547 amu (-ESI)



561 amu (-ESI)



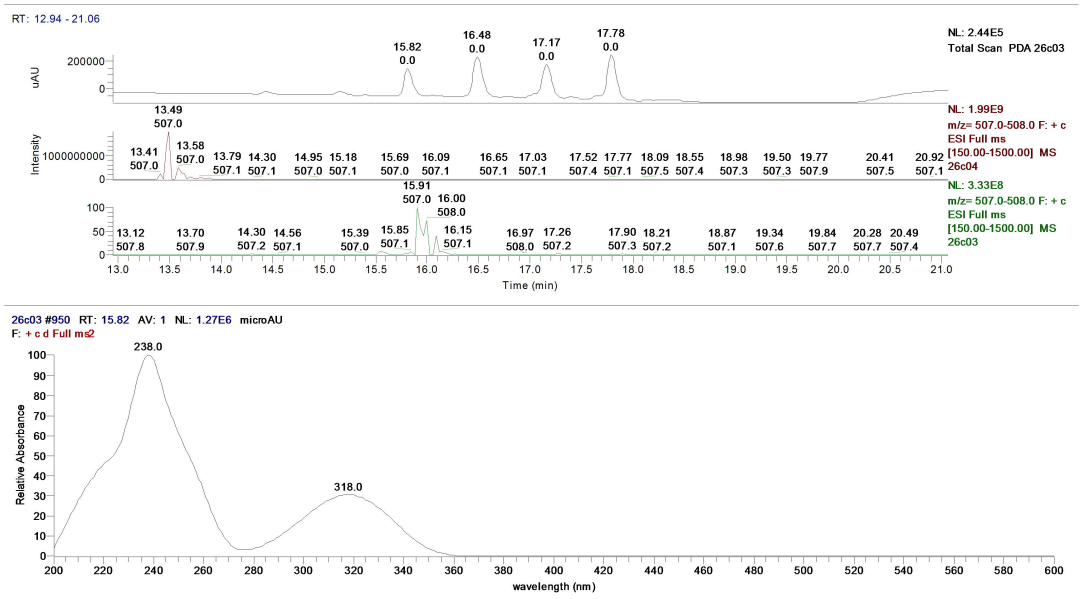
575 amu (-ESI)



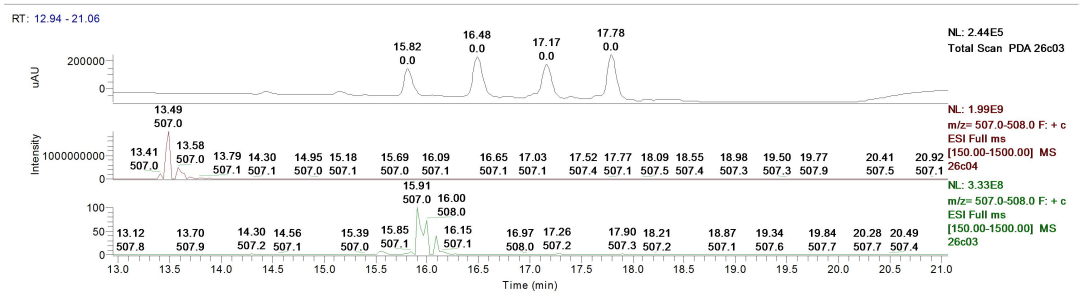
Supplementary Information B_9) LCMS on antimycin A_1-A_4

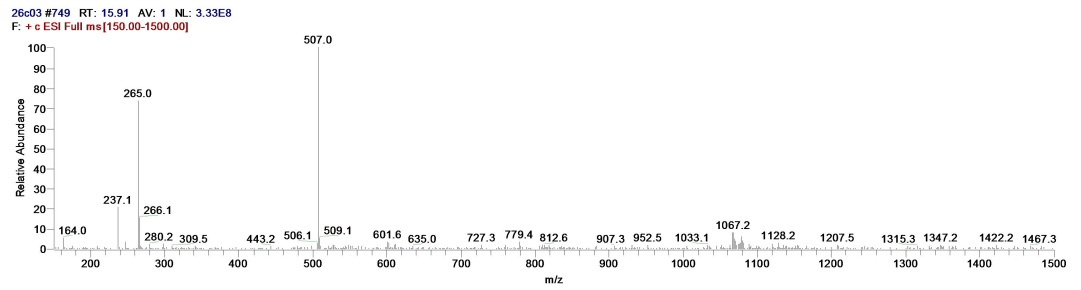
Low-resolution LCMS data on an antimycin A_1-A_4 standard. UV, MS, and MS^2 data on ions with masses of 507 amu (A_4), 521 amu (A_3), 535 amu (A_2), 549 amu (A_1) $[M+H]^+$. The same type of data is presented for an additional antimycin mass, 563 amu $[M+H]^+$, detected in the standard. The LCMS system used for generating these data has been located at the John Innes Centre and was set to detect ions in positive ion mode (+ESI).

507 amu (+ESI)

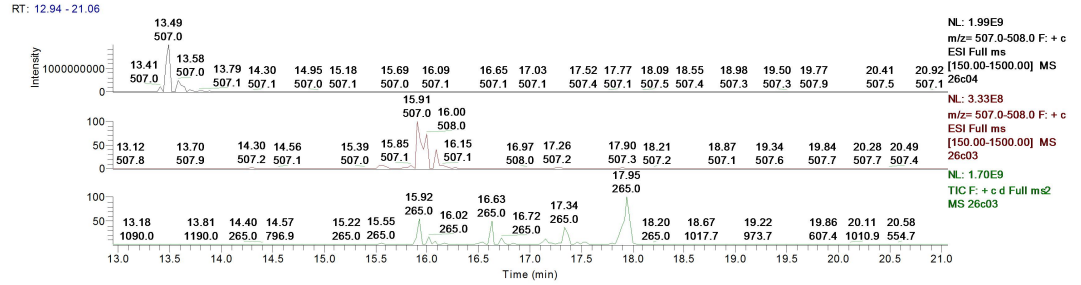


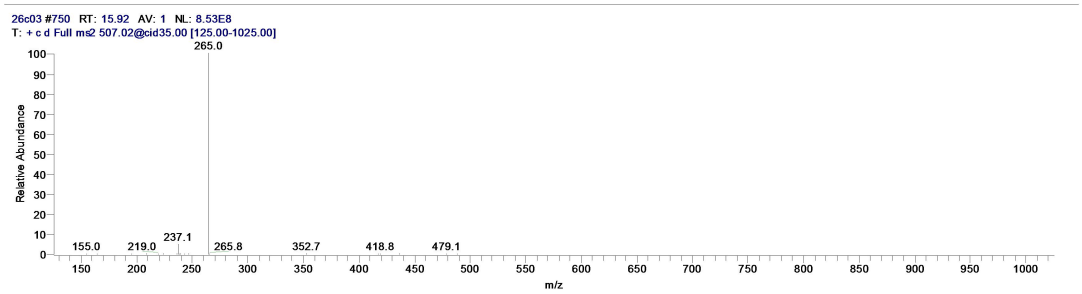
507 amu (+ESI)



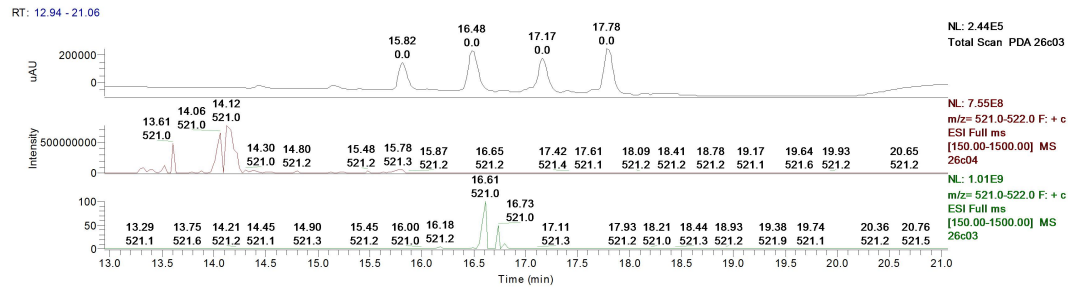


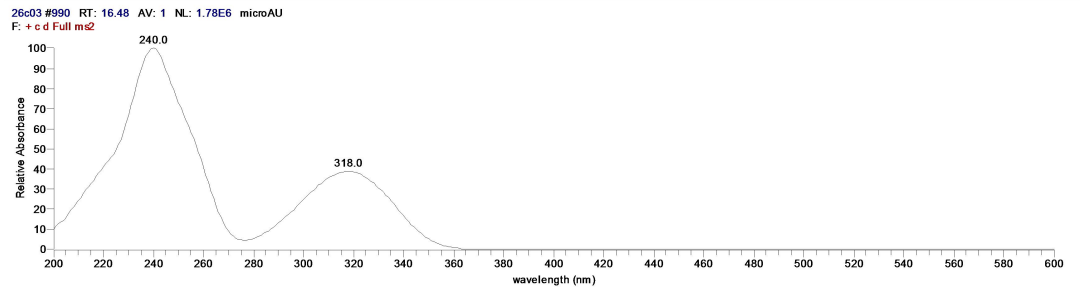
507 amu (+ESI)



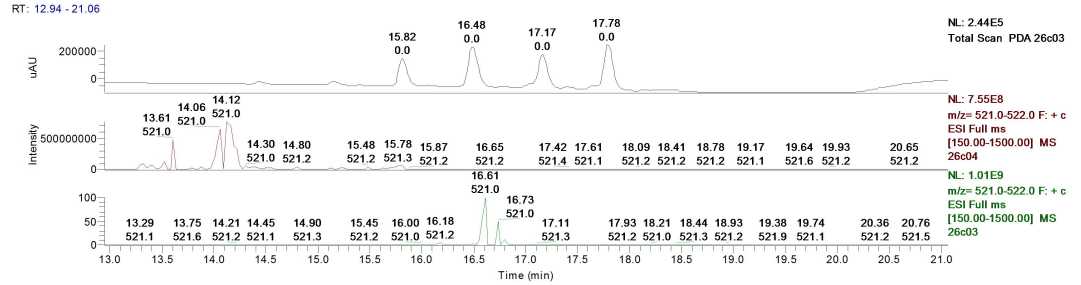


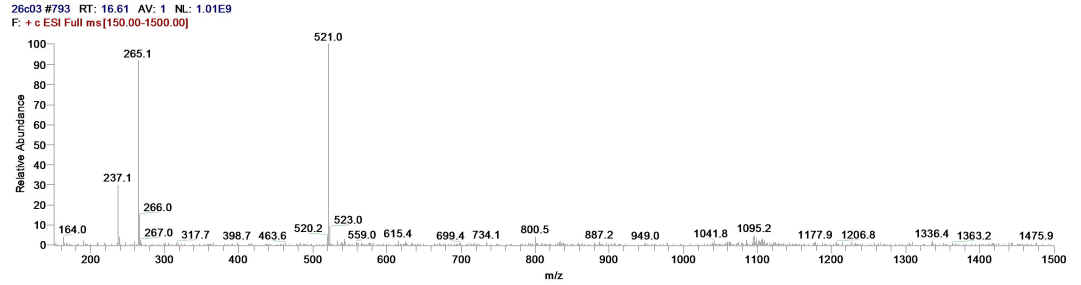
521 amu (+ESI)



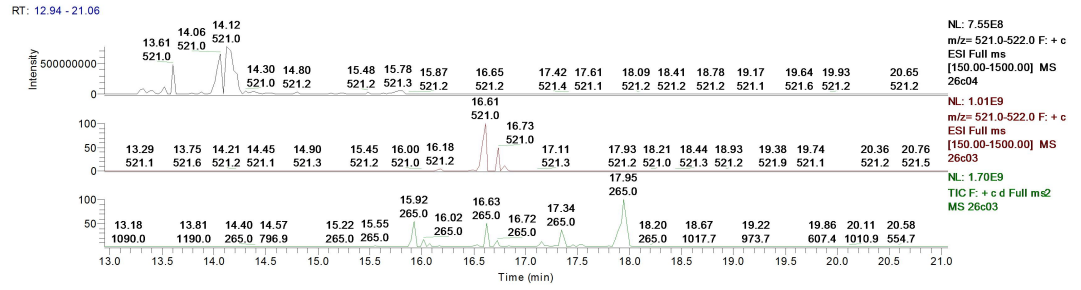


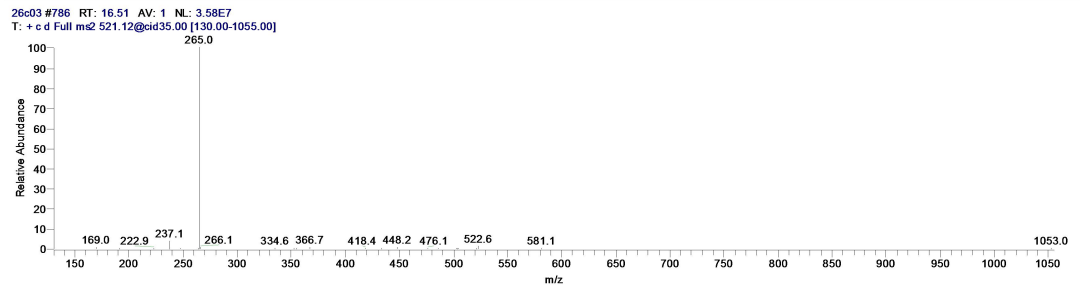
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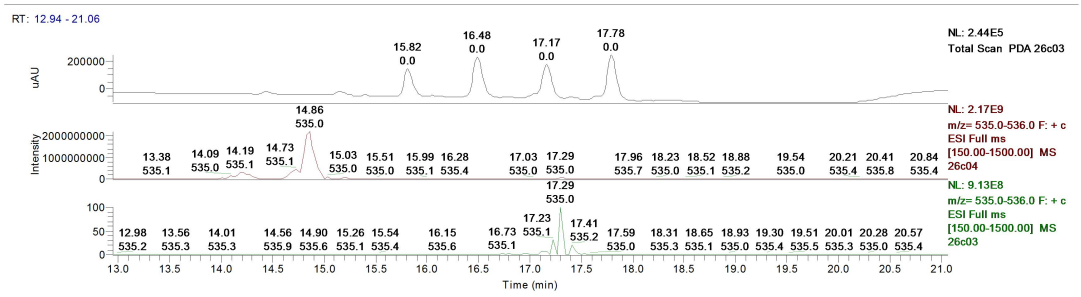


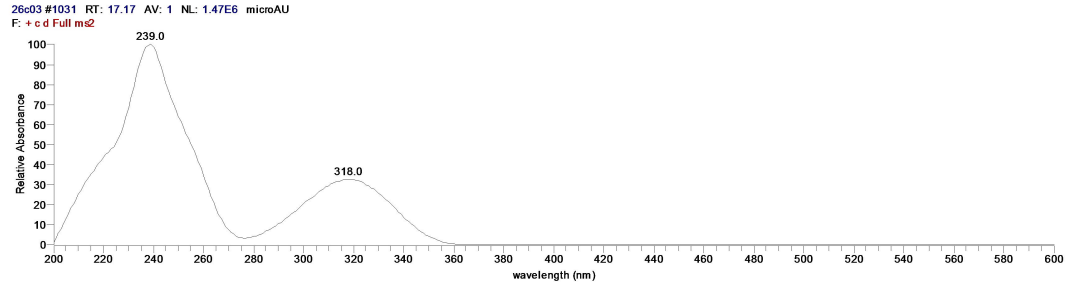
521 amu (+ESI)



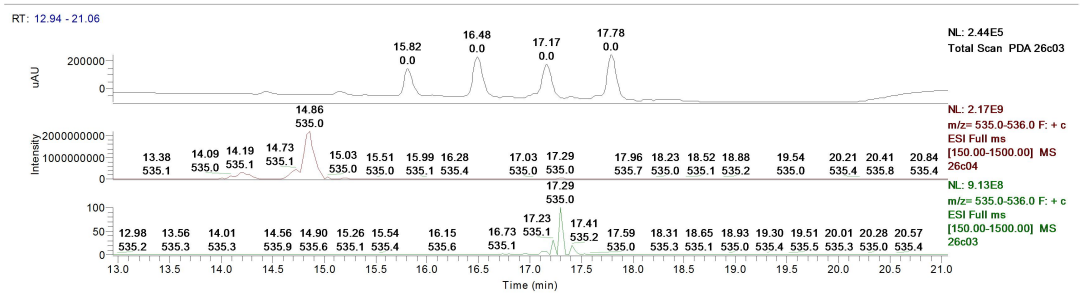


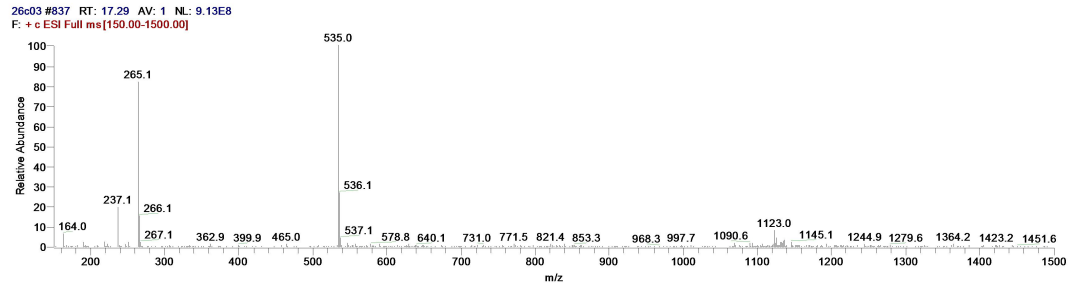
535 amu (+ESI)



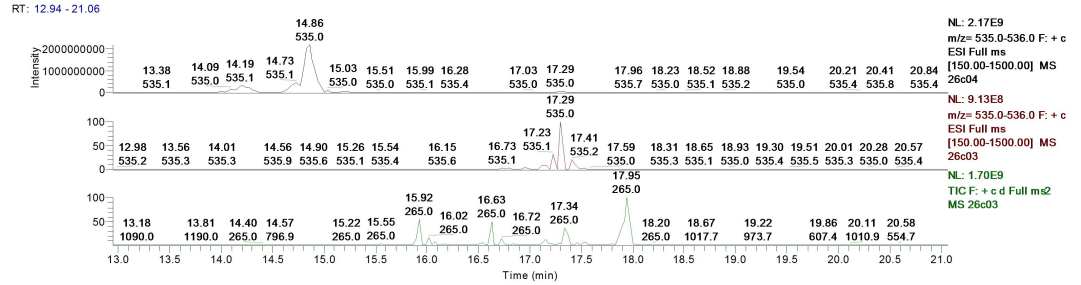


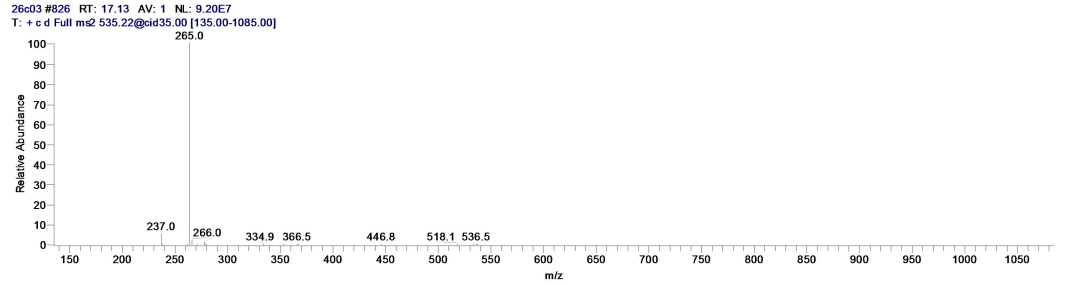
535 amu (+ESI)



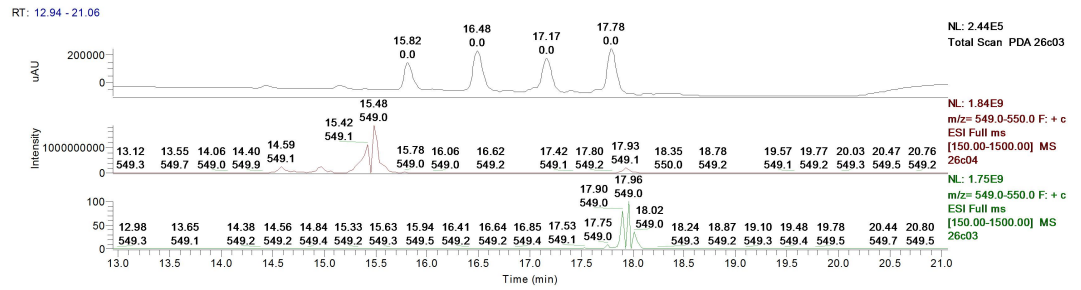


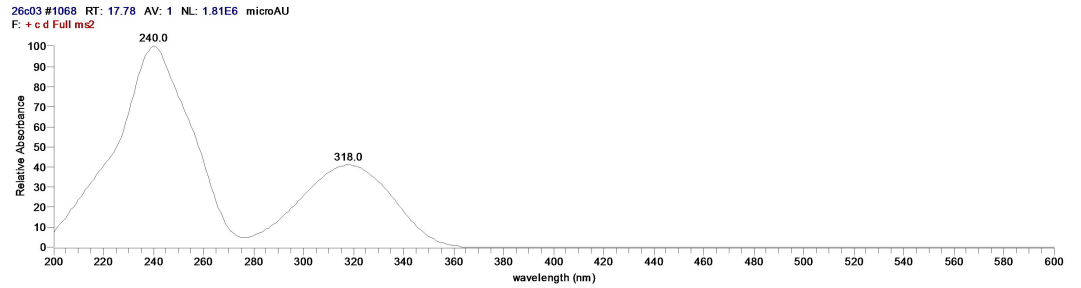
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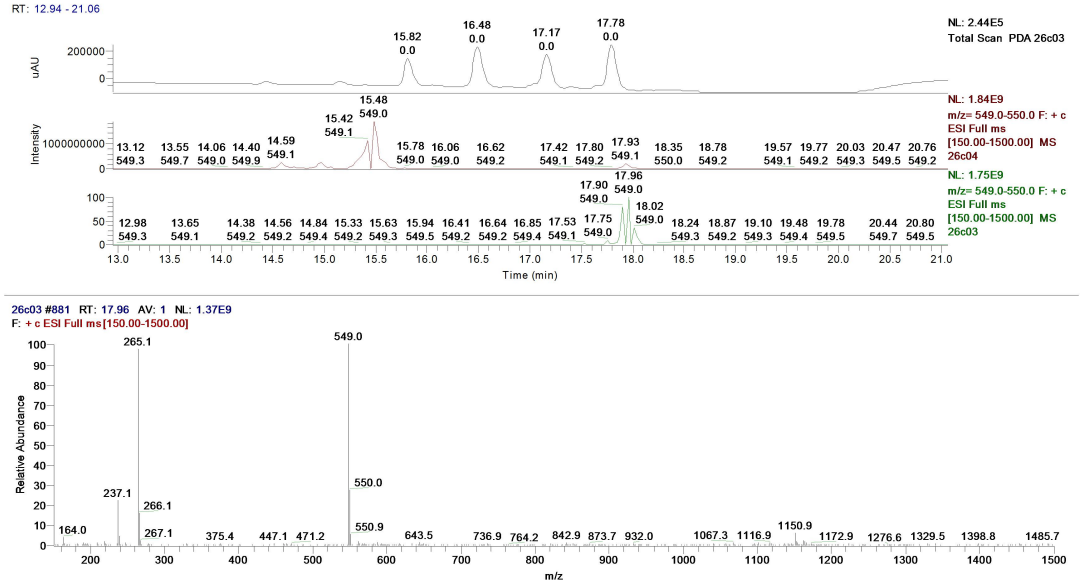


549 amu (+ESI)

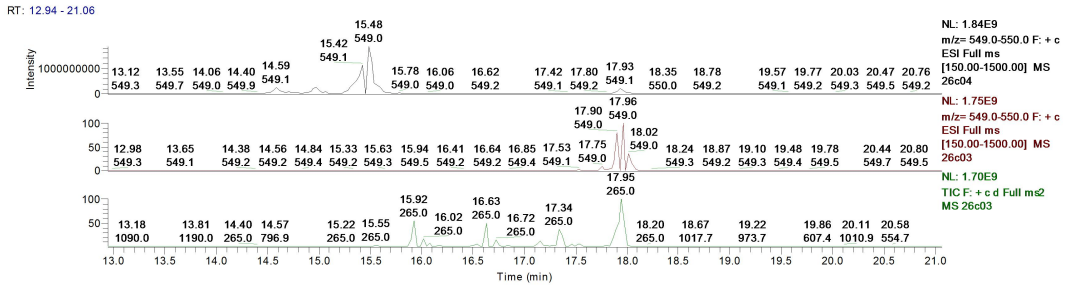


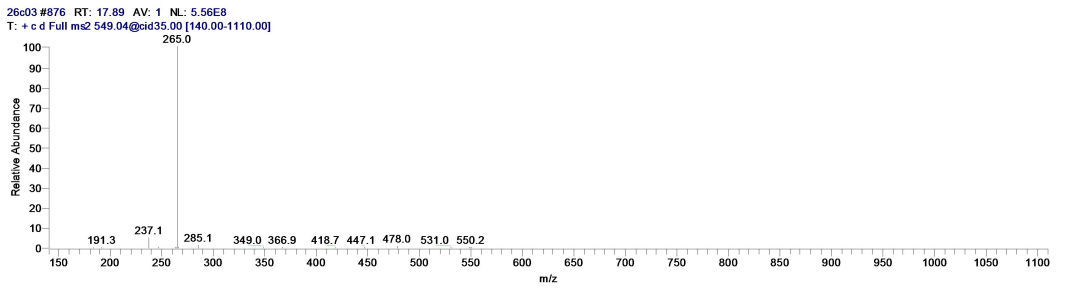


549 amu (+ESI)

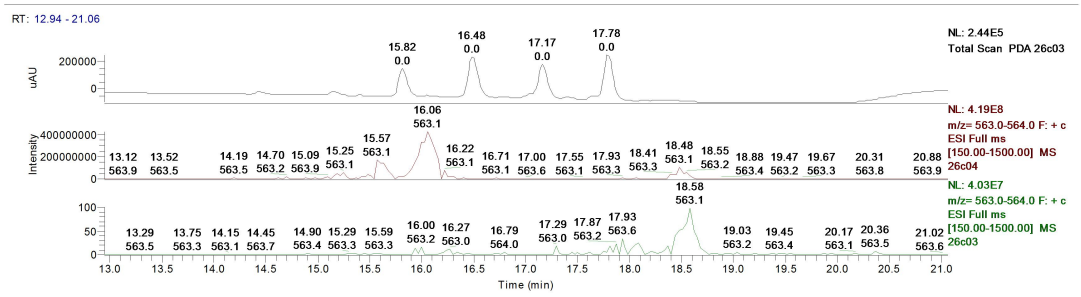


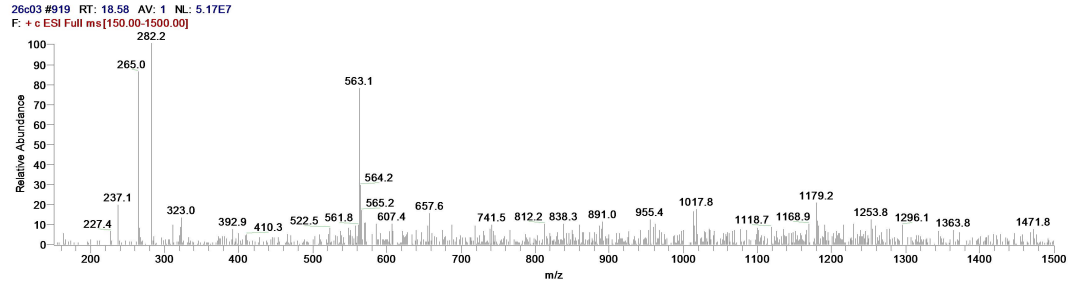
549 amu (+ESI)



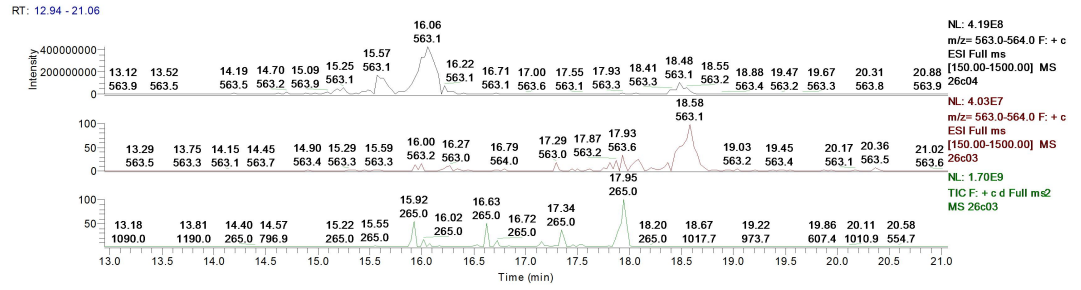


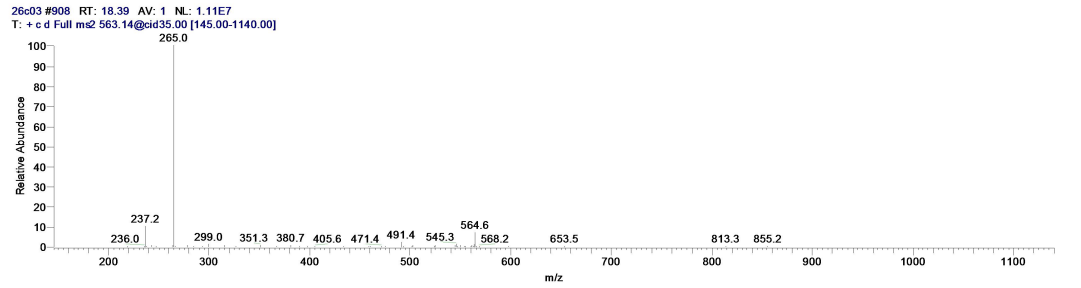
563 amu (+ESI)





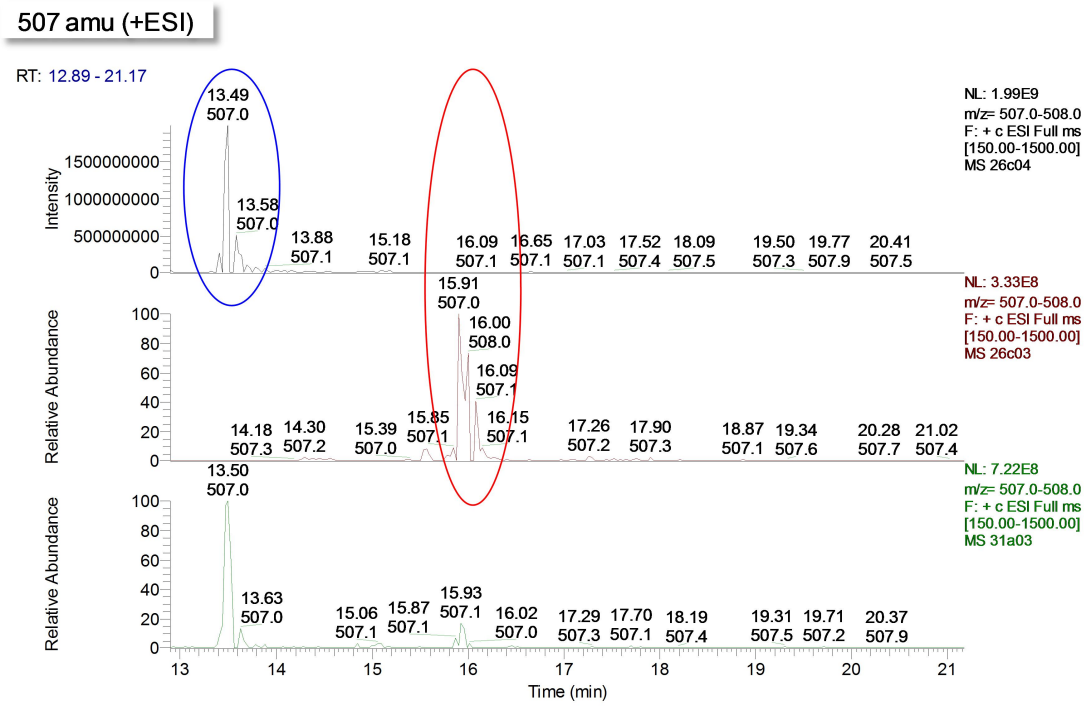
563 amu (+ESI)

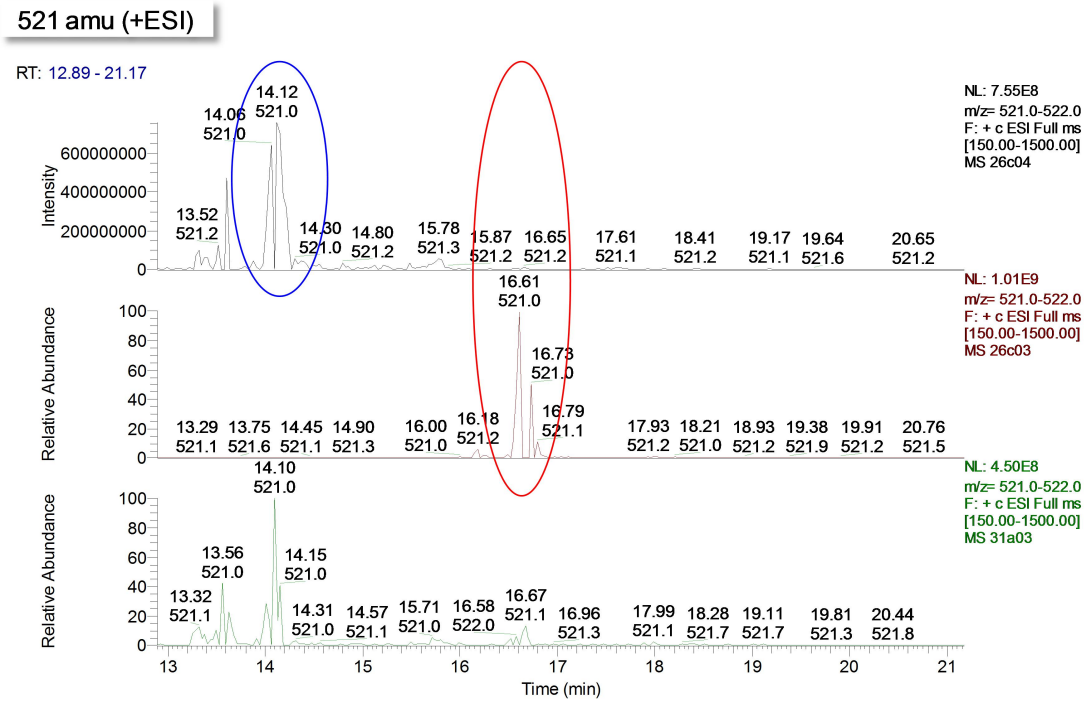




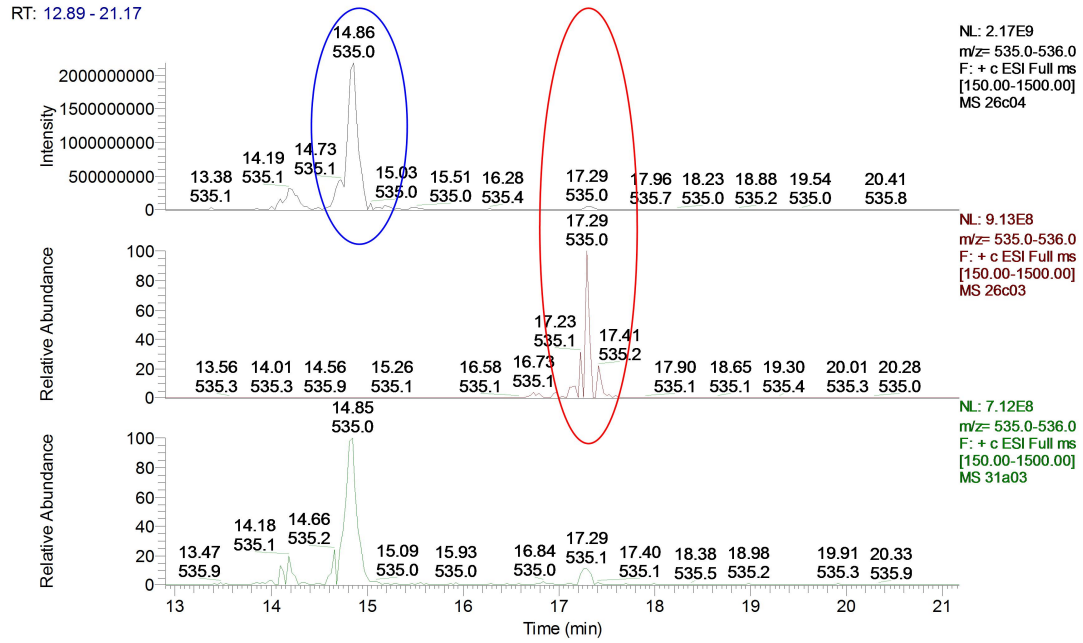
Supplementary Information B_{10}) Tandem-LCMS on antimycin A_1-A_4 and double-purified combined fractions B

Low-resolution tandem-LCMS data on an antimycin A_1-A_4 standard (507 amu, 521 amu, 535 amu, 549 amu, 563 amu $[M+H]^+$) and the double-purified combined fractions B (507 amu, 521 amu, 535 amu, 549 amu, 563 amu, 805 amu, and 577 amu $[M+H]^+$). Extracted ion chromatograms are presented on ions with masses of 507 amu, 521 amu, 535 amu, 549 amu, 563 amu, (805 amu), and 577 amu $[M+H]^+$. The LCMS system used for generating these data has been located at the John Innes Centre and was set to detect ions in positive ion mode (+ESI).

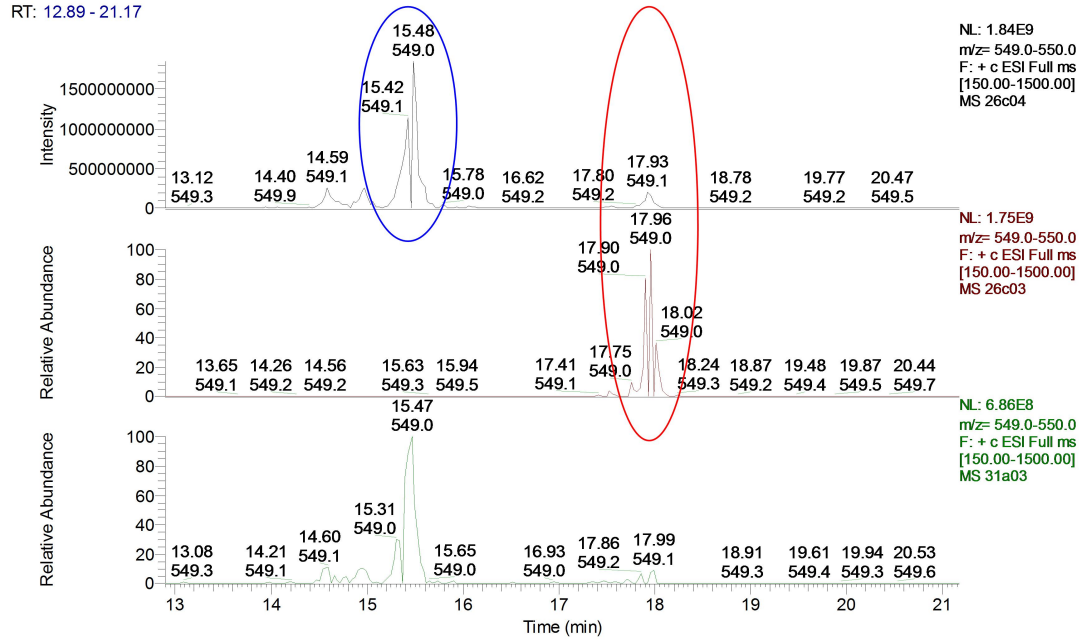

507 amu (+ESI)
RT: 12.89 - 21.17
Intensity
Relative Abundance
Relative Abundance
Time (min)
NL: 1.99E9
m/z= 507.0-508.0
F: + c ESI Full ms
[150.00-1500.00]
MS 26c04
NL: 3.33E8
m/z= 507.0-508.0
F: + c ESI Full ms
[150.00-1500.00]
MS 26c03
NL: 7.22E8
m/z= 507.0-508.0
F: + c ESI Full ms
[150.00-1500.00]
MS 31a03


521 amu (+ESI)
RT: 12.89 - 21.17
Intensity
Relative Abundance
Relative Abundance
Time (min)
NL: 7.55E8
m/z= 521.0-522.0
F: + c ESI Full ms
[150.00-1500.00]
MS 26c04
NL: 1.01E9
m/z= 521.0-522.0
F: + c ESI Full ms
[150.00-1500.00]
MS 26c03
NL: 4.50E8
m/z= 521.0-522.0
F: + c ESI Full ms
[150.00-1500.00]
MS 31a03

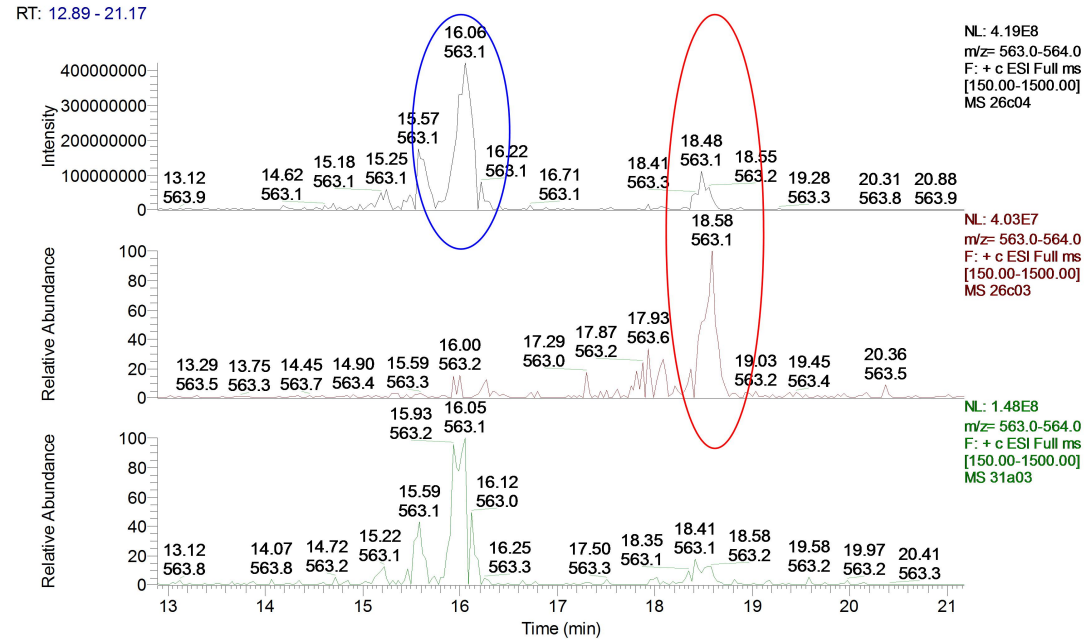
535 amu (+ESI)



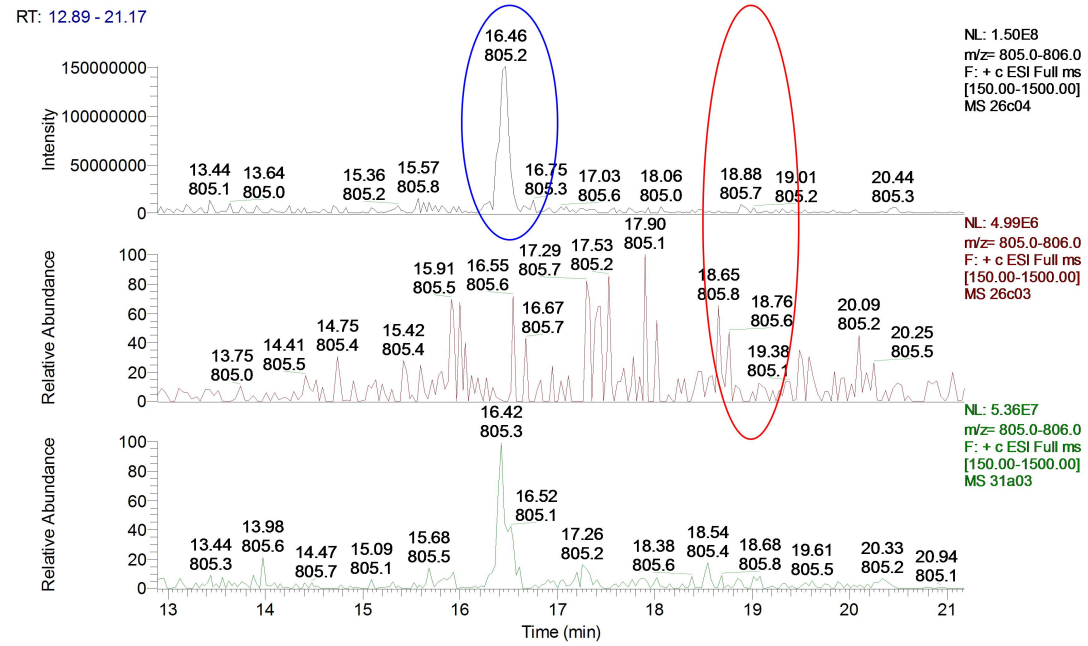
549 amu (+ESI)

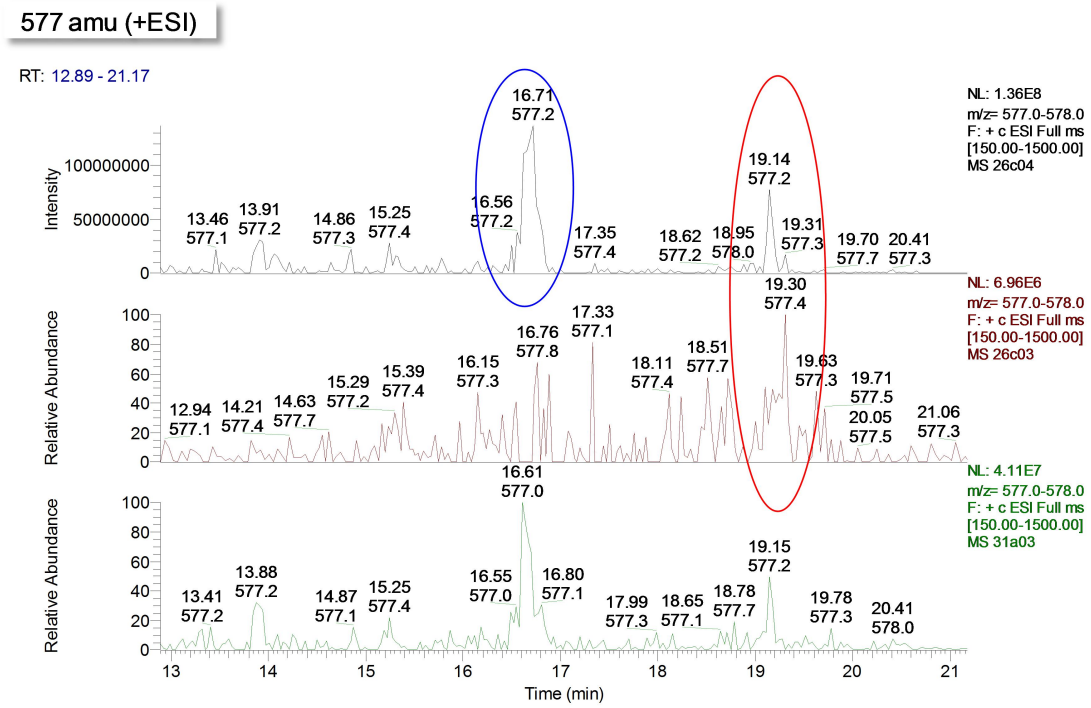


563 amu (+ESI)



805 amu (+ESI)




577 amu (+ESI)
RT: 12.89 - 21.17
Intensity
Relative Abundance
Relative Abundance
Time (min)
NL: 1.36E8
m/z= 577.0-578.0
F: + c ESI Full ms
[150.00-1500.00]
MS 26c04
NL: 6.96E6
m/z= 577.0-578.0
F: + c ESI Full ms
[150.00-1500.00]
MS 26c03
NL: 4.11E7
m/z= 577.0-578.0
F: + c ESI Full ms
[150.00-1500.00]
MS 31a03

Supplementary Information C) Candicidin and antimycin producing bacteria

Distribution of candicidin biosynthesis genes among bacterial isolates and the genetic relatedness of bacterial isolates to antimycin producers.

Materials and Methods

Plotting bioactivities

A Neighbor-Joining (NJ) tree was generated based on 16S rDNA sequences of 56 bacterial isolates. In particular, 16 bacteria originated from worker ants of *Acromyrmex octospinosus* (E_2-E_{17}), 20 bacteria originated from fungal cultivars of *Allomerus decemarticulatus* and *A. octoarticulatus* (FG_1-FG_{15}, FG_{17}-FG_{21}), and 20 bacteria originated from worker ants and their putative fungal cultivars of *Tetraponera penzigi* (KY_1-KY_5, KY_7-KY_{21}). Corresponding 16S rDNA sequences of four laboratory organisms, including *Streptomyces lividans* (1126), *Bacillus subtilis* (|A|), *Salmonella enterica* (SL1344), and *Escherichia coli* (DH5α), served as phylogenetic references. The DNA extraction, PCR amplification, and sequencing of the 16S rDNA have been described in the corresponding chapters (Chapters 2, 4, 5), where the reads were used for the identification of the bacterial isolates. The truncated sequences of the bacterial isolates and reference strains were introduced to BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned with ClustalW (Number of Bootstraps: 10000). Using MEGA5 (www.megasoftware.net/), the aligned sequences were used to calculate a Neighbor-Joining tree (Number of Bootstraps: 10000, pairwise deletion of gaps, Kimura 2-parameter substitution model). In the phylogenetic tree, the entries display information about the ant system ('Δ/▲' = *A. octospinosus*, '□/■' = *A. decemarticulatus* or *A. octoarticulatus*, '○/●' = *T. penzigi*, '◊' = laboratory strain), the name (e.g. E_2, FG_2, KY_2), the host type ('A' = worker ant, 'C' = fungal cultivar), and the genus (e.g. *Streptomyces*) of the bacteria.

The phylogenetic tree was used for plotting the bioassay results, in which all 'NNSPS' strains (filled symbols) and their culture supernatants, were tested against diverse fungi (Chapters 2, 4, 5). Positive bioassay outcomes of strains showing fungal inhibition are highlighted with red boxes. The shortages in the boxes, including 'ColEA-', 'ColEWB-', 'ColEWC-', 'ColAcre-', 'ColCan-/+', 'SupCan-/+', 'SupEWC-', reveal the nature of the bioassays. Hereby, 'Col' and 'Sup' stand for colony and supernatant bioassays; 'EWB', 'EA', and 'EWC' refer

to *Escovopsis* strains and 'Acre' refers to an *Acremonium* strain, against which *Acromyrmex*- and *Tetraponera*-associated NNSPS strains were tested, respectively; 'Can' corresponds to the yeast *Candida albicans* CA_6; and '+/-' indicates whether bacterial colonies were grown in the presence/absence of sodium butyrate.

Candicidin gene screen

The genomes of the NNSPS isolates (E_2-E_4, E_6, E_8-E_{15}; FG_1-FG_8, FG_{21}; KY_1-KY_5, KY_7, KY_{21}) were screened by polymerase chain reaction (PCR) for the presence of two candicidin biosynthesis genes, *fscM* (1035 bp) and *fscP* (1039 bp). These genes were amplified (Table S_1) using the same DNA extracts that were used above for the amplification of the 16S rDNA gene, and primers that were published by Haeder *et al.* (2009).

Amplicons within the correct size range were cleaned with a Qiagen Gel Extraction Kit, according to the instructions in the manual, and sequenced (Table S_2) with the gene-specific forward primers. The sequencing products were analysed by The Genome Analysis Centre TGAC (www.jicgenomelab.co.uk) using ABI 3730XL sequencers (Life Technologies). The sequences were analysed with FinchTV (www.geospiza.com/Products/finchtv.shtml), truncated, and matched to the NCBI BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

A

PCR mixture	Volume
dH_2O	32.00 µl
GoTaq buffer (incl. loading buffer)	10.00 µl
GoTaq MgCl (25 mM)	5.50 µl
Bioline dNTP's (10 mM)	1.50 µl
Forward primer (25 µM)	0.25 µl
Reverse primer (25 µM)	0.25 µl
DNA	0.50 µl
GoTag polymerase	0.50 µl
	50.50 µl

B

Step	Temperature	Minutes
1	96 °C	10:00
2	96 °C	1:00
3	60 °C	0:45
4	72 °C	1:30
5	Go 40x to step 2	
6	72 °C	10:00
7	end	

Table S_1 (A-B) PCR mixture and thermocycles for the amplification of candicidin biosynthesis genes, *fscM* and *fscP*.

A

Sequencing mixture	Volume
dH_2O	5.5 µl
5x sequencing buffer	1.5 µl
Forward primer (3.2 µM)	1.0 µl
DNA	1.0 µl
Enzyme E3.1	1.0 µl
Total	**10.0 µl**

B

Step	Temperature	Minutes
1	96 °C	1:00
2	96 °C	0:10
3	50 °C	0:05
4	60 °C	4:00
5	Go 25x to step 2	
6	60 °C	0:10
7	end	

Table S_2 (A-B) Reaction mixture and thermocycles for the sequencing of candicidin biosynthesis genes.

Phylogenetic comparison

Twelve bioactive *Streptomyces* isolates were placed in a phylogenetic context with 52 *Streptomyces* reference strains that are known to produce specific antifungals. In particular, 20 of the 52 reference strains were previously used in a phylogenetic analysis by Poulsen *et al*. (2011); a part of the sequences originated from Kaltenpoth *et al*. (2006) and Scott *et al*. (2008). In addition, 32 of the 52 bacteria were previously used in a phylogenetic analysis by Schoenian *et al.* (2011); the sequences partly originated from Haeder *et al*. (2009). The sequences of the reference strains were downloaded from the NCBI database (www.ncbi.nlm.nih.gov/gquery/).

The twelve selected *Streptomyces* isolates originated from the worker ants of *A. octospinosus* (E_2, E_6, E_8-E_{10}, E_{13}-E_{14}), fungal cultivars of *A. decemarticulatus* and *A. octoarticulatus* (FG_1, FG_{21}), and worker ants and their putative fungal cultivar of *T. penzigi* (KY_1-KY_2, KY_5). One *Pseudonocardia* strain (E_4), originating from the worker ants of *A. octospinosus*, served as outgroup. The phylogenetic resolution was increased by extending the fragment of the bacterial 16S rDNA gene that was sequenced. In particular, the previously described 515 forward sequences (515-1492, Table 2.6), used for the bacterial identification, were merged with newly generated and overlapping fD2 forward sequences (fD2-16Sr, Table S_3), covering the initial part of the 16S rDNA gene.

The fD2 forward primer resembles the well-known forward primers, '8F' (Turner *et al*. 1999) and '27F' (Lane *et al.* 1991), but lacks the first nucleotide; also, it shows an 'A' at the position where primer 27F has an 'M' (A+C) and where primer 8F has a 'C'. The PCR mixture and thermocycles for generating fD2-16Sr amplicons are displayed in table S_4.

Unfortunately, no fD2-16Sr amplicons could be generated for *Streptomyces* FG_1 and FG_{21}, leading to their exclusion from the analysis. Amplicons of the remaining strains were cleaned with a Qiagen Gel Extraction Kit, according to the instructions in the manual, and sequenced with the gene-specific forward primer (fD2) as described above. The sequencing products were analysed by The Genome Analysis Centre TGAC (www.jicgenomelab.co.uk), using ABI 3730XL sequencers (Life Technologies), truncated in FinchTV (www.geospiza.com/Products/finchtv.shtml), and merged with the 515 forward sequences in BioEdit. The merged sequences were matched to the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) BLAST database.

Name	Sequence	Reference	Gene	Amplicon length
fD2	5'-GAGTTTGATCATGGCTCAG-3'	Bereswill *et al*. (1995)	16S rDNA	1400 bp
16Sr	5'-TTGCGGGACTTAACCCAACAT-3'	Aittamaa *et al*. (2008)		

Table S_3 Primer sequences of fD2 and 16Sr used for the partial amplification of the bacterial 16S rDNA gene.

A

PCR mixture	Volume
dH_2O	32.00 µl
GoTaq buffer (incl. loading buffer)	10.00 µl
GoTaq MgCl (25 mM)	5.50 µl
Bioline dNTP's (10 mM)	1.50 µl
Forward primer (25 µM)	0.25 µl
Reverse primer (25 µM)	0.25 µl
DNA	0.50 µl
GoTag polymerase	0.50 µl
	50.50 µl

B

Step	Temperature	Minutes
1	94 ºC	5:00
2	94 ºC	1:00
3	45 ºC	0:45
4	72 ºC	1:30
5	Go 34x to step 2	
6	72 ºC	5:00
7	end	

Table S_4 (A-B) PCR mixture and thermocycles for the generation of fD2-16Sr amplicons.

Then, the sequences of the 52 *Streptomyces* reference strains, ten *Streptomyces* test strains, and one *Pseudonocardia* outgroup were aligned in BioEdit by ClustalW (Number of Bootstraps: 10000). In MEGA5 (www.megasoftware.net/) the aligned sequences were used to calculate a Neighbor-Joining (NJ) tree (Number of Bootstraps: 10000, pairwise deletion of gaps, Kimura 2-parameter substitution model).

Results

Plotting bioactivities

The phylogenetic relationship of 56 bacteria, which have been isolated from *Acromyrmex* (E_2-E_{17}), *Allomerus* (FG_1-FG_{15}, FG_{17}-FG_{21}), and *Tetraponera* (KY_1-KY_5, KY_7-KY_{21}) worker ants/cultivars, were analysed by generating a Neighbor-Joining tree (Fig. S_1). The phylogenetic tree is based on fragments of the bacterial 16S rDNA gene (mean 791 bp, 445-919 bp). The tree shows that the bacterial isolates fall into four overall clusters, representing the bacterial phyla *Bacterioidetes*, *Proteobacteria*, *Firmicutes*, and *Actinobacteria*. While the first two phyla contain Gram-negative bacteria, the last two phyla contain Gram-positive bacteria.

The outcomes of bioassays, in which 28 actinobacterial isolates (NNSPS strains: E_2-E_4, E_6, E_8-E_{15}; FG_1-FG_8, FG_{21}; KY_1-KY_5, KY_7, KY_{21}) were tested against diverse fungi, are plotted to the phylogenetic tree. In particular, only those bioassay performances, in which bacterial strains showed fungal inhibition, are highlighted (red boxes). In other words, bioactively successful strains obtain more boxes than bioactively unsuccessful strains of the same ant system. The results indicate that the bacterial bioactivity appears to be clustered; for example, a clade in the sub-order *Streptomycineae*, including *Streptomyces* E_8 and E_9 as well as *Streptomyces* KY_1, was particularly bioactive. Strikingly, strains of the typical attine ant-associate *Pseudonocardia*, as represented by the bacterial isolates E_4 and E_{12}, are not as successful in inhibiting the test fungi as many of the *Streptomyces* strains.

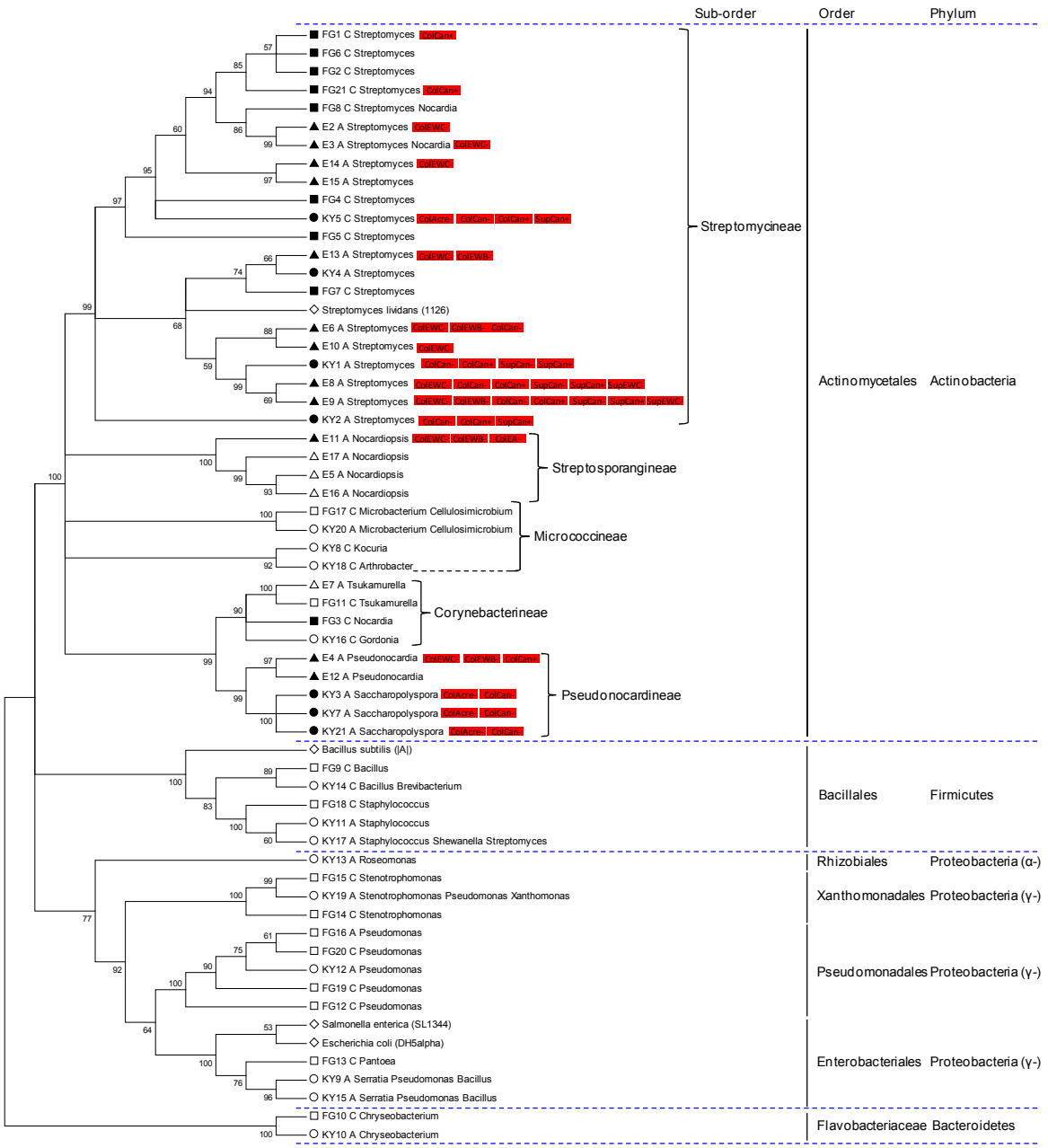


Figure S_1 Neighbor-Joining tree of 56 bacterial isolates. The phylogenetic tree is based on partial 16S rDNA sequences of bacteria, isolated from the worker ants of *A. octospinosus* ('Δ/▲'), fungal cultivar of *A. decemarticulatus* and *A. octoarticulatus* ('□/■'), and *T. penzigi* workers and their putative fungal cultivar ('○/●'). As phylogenetic references, corresponding sequences of *S. lividans*, *S. enterica*, *E. coli*, and *B. subtilis* are included ('◊'). The phylogenetic tree shows that the bacterial isolates belong to four phyla. A selection of 28 actinobacteria (filled symbols), NNSPS strains, and their cultivar supernatants are challenged against various fungal opponents. Positive bioassay outcomes are highlighted with red boxes. In general, it can be said that the more boxes the more bioactively successful strains (of the same ant system) are. For example, *Streptomyces* E_8, E_9, and KY_1 belong to the most bioactive bacterial strains.

Candicidin gene screen

The genomes of the NNSPS isolates were PCR-screened for two genes, *fscM* and *fscP*, in the candicidin biosynthesis pathway. The amplicons were analysed on diagnostic electrophoresis gels (Fig. S_2), which contain two negative controls (*S. lividans* and sterile dH_2O) and one positive control (*Streptomyces* E_8). In a chemical analysis, *Streptomyces* E_8 has been shown to produce candicidin (Chapter 3). All amplicons within the correct size range (framed) have been sequenced, and their reads were matched to the NCBI BLAST database. The sequencing information is provided in the Supplementary Information A_2.

Amplification of the *fscM* gene generated relevant amplicons in twelve bacterial isolates (E_2-E_3, E_8-E_9, E_{11}-E_{13}, FG_8, KY_1, KY_4-KY_5, KY_{21}). All amplicons, besides the one of KY_4, could be sequenced; also, the sequences of E_{12} and KY_{21} are not matching any sequences of the NCBI database. The sequences of E_8, E_9, and KY_1 show strong BLAST analogies to the candicidin genes of *Streptomyces griseus* (yellow frames, Fig. S_2; Table S_5). The sequences of E_{11} and KY_5 show 'weaker' BLAST analogies to candicidin (*Streptomyces griseus*) and oviedomycin/jadomycin (*Streptomyces antibioticus*/ *Streptomyces venezuelae*) genes, respectively (yellow frames, Fig. S_2; Table S_5). Strains E_2, E_3, E_{13}, and FG_8 show analogies to gene sequences not encoding any antifungal compounds. Strong and weak BLAST analogies are defined in terms of relatively high and low 'max scores', as shown in table S_5.

Amplifications of the *fscP* gene generated amplicons in thirteen bacteria (E_4, E_8-E_9, E_{12}, E_{15}, FG_1, FG_5-FG_7, KY_1, KY_3, KY_5, KY_7). All amplicons, besides the ones of FG_6 and KY_3, could be sequenced; the sequences of E_{12}, FG_5, KY_5, and KY_7 do not match any sequences of the NCBI database. The reads of E_4, E_{15}, FG_1, and FG_7 show BLAST analogies to sequences not encoding any antifungal compounds, and the sequences of E_8, E_9, and KY_1 show 'strong' BLAST analogies, as defined above, to the candicidin genes of *Streptomyces griseus* (yellow frames, Fig. S_2; Table S_5).

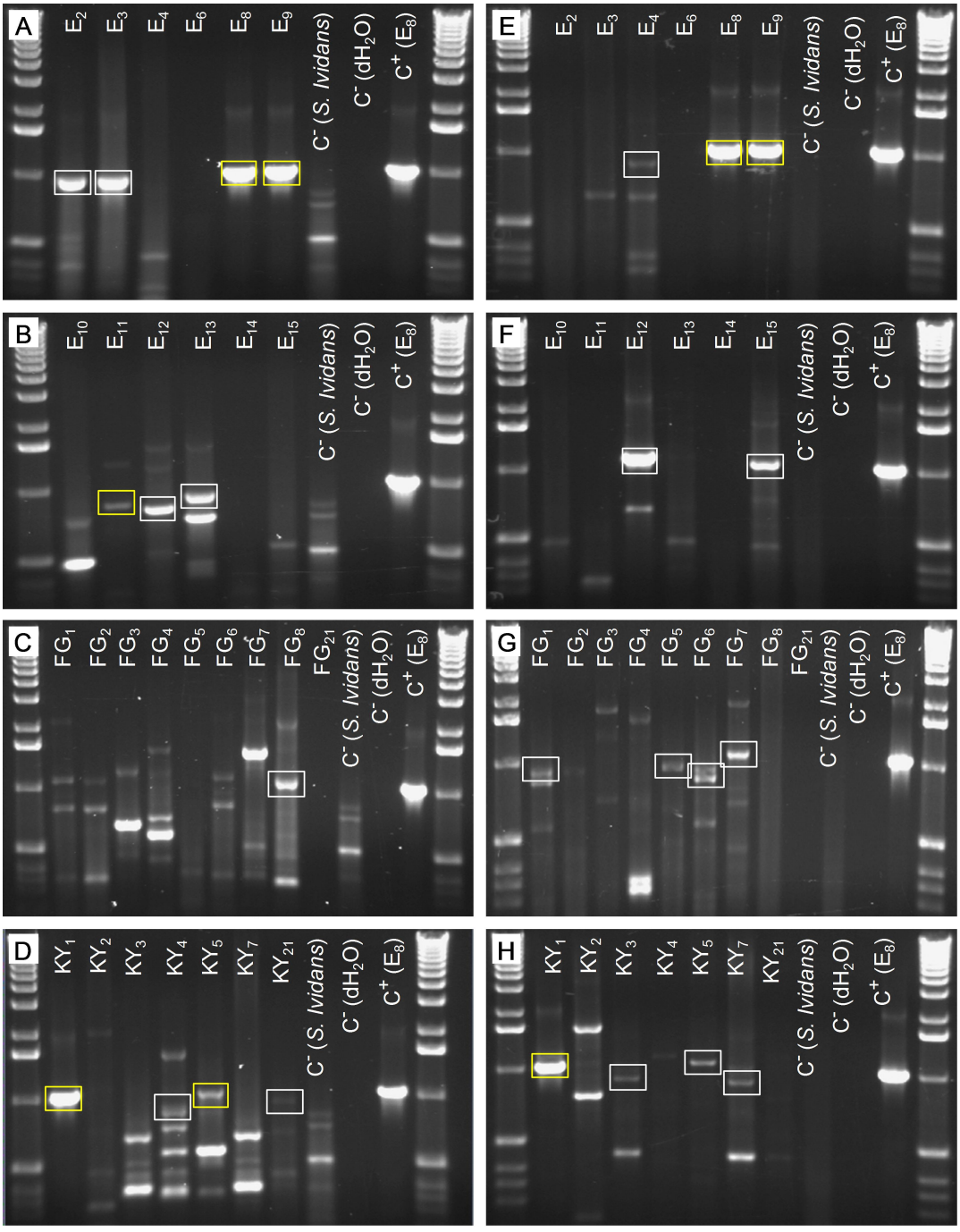


Figure S_2 Distribution of two candicidin biosynthesis genes, *fscM* and *fscP* among bacterial isolates. *FscM* (1035 bp, A-D) and *fscP* (1039 bp, E-H) amplicons of the 28 NNSPS isolates within the correct size range (framed), are sequenced. Reads with BLAST analogies to antifungal genes are highlighted with yellow frames. For example, *Streptomyces* E_8, E_9, and KY_1 likely possess both candicidin biosynthesis genes.

Despite of the weaknesses regarding this screening method (e.g. primer incompatibilities), successful amplifications confirm the presence of candicidin biosynthesis genes. The data show that the genomes of *Streptomyces* E_8, E_9, and KY_1 contain two candicidin biosynthesis genes, indicating that candicidin-producing bacteria may not only be associated to *A. octospinosus* (i.e. *Streptomyces* E_8 and E_9) but also to non-attine ants such as *T. penzigi* (i.e. *Streptomyces* KY_1). In addition, *Nocardiopsis* E_{11} and *Streptomyces* KY_5 may be able to produce candicidin- and oviedomycin/jadomycin-like metabolites, respectively.

Name	PCR primers	Sequencing primer	Sequence length	Closest analogies to antifungal genes (accession)	Max score	Total score	Query coverage	E value	Max ident
E_8	fscM F/R	fscM F	898 bp	*Streptomyces griseus* partial ORF1, canA gene, canC gene, canF gene, canT gene, canRA gene and canRB gene (AJ300302.1)	1642	1642	100%	0	99%
E_9	fscM F/R	fscM F	901 bp	*Streptomyces griseus* partial ORF1, canA gene, canC gene, canF gene, canT gene, canRA gene and canRB gene (AJ300302.1)	1655	1655	99%	0	99%
E_{11}	fscM F/R	fscM F	737 bp	*Streptomyces griseus* partial ORF1, canA gene, canC gene, canF gene, canT gene, canRA gene and canRB gene (AJ300302.1)	52.8	52.8	3%	0.003	100%
KY_1	fscM F/R	fscM F	844 bp	*Streptomyces griseus* partial ORF1, canA gene, canC gene, canF gene, canT gene, canRA gene and canRB gene (AJ300302.1)	1537	1537	100%	0	99%
KY_5	fscM F/R	fscM F	777 bp	*Streptomyces antibioticus* oviedomycin biosynthetic gene cluster, strain ATCC 11891 (AJ632203.2)	627	627	89%	4.00E-176	83%
				Streptomyces venezuelae JadJ (jadJ), jadomycin polyketide synthase cyclase (jadI) (AF126429.1)	573	573	97%	6.00E-160	81%
E_8	fscP F/R	fscP F	887 bp	*Streptomyces griseus* partial ORF1, canA gene, canC gene, canF gene, canT gene, canRA gene and canRB gene (AJ300302.1)	1605	1605	100%	0	99%
E_9	fscP F/R	fscP F	887 bp	*Streptomyces griseus* partial ORF1, canA gene, canC gene, canF gene, canT gene, canRA gene and canRB gene (AJ300302.1)	1594	1594	100%	0	99%
KY_1	fscP F/R	fscP F	798 bp	*Streptomyces griseus* partial ORF1, canA gene, canC gene, canF gene, canT gene, canRA gene and canRB gene (AJ300302.1)	1369	1369	100%	0	97%

Table S_5 NCBI BLAST analogies of two candicidin biosynthesis genes, *fscM* and *fscP*.

Phylogenetic comparison

A Neighbor-Joining (NJ) tree was generated based on the 16S rDNA sequences of 52 *Streptomyces* reference strains ('◊'), known to produce specific antifungals, ten bioactive *Streptomyces* isolates, and a *Pseudonocardia* (E_4) isolate that was used as phylogenetic outgroup. The *Streptomyces* isolates originated from worker ants of *A. octospinosus* ('▲' = E_2, E_6, E_8-E_{10}, E_{13}-E_{14}), and worker ants and their putative fungal cultivar of *T. penzigi* ('●' = KY_1-KY_2, KY_5). The sequencing data (1040-1349 bp, mean of 1228 bp) are presented in the Supplementary Information A_3 and their BLAST analogies in Table S_6.

The phylogenetic tree (Fig. S_3) shows that *Tetraponera* ant-associated *Streptomyces* strain KY_2 as well as a cluster of three *Streptomyces philanthi* bacteria (DQ375802, DQ375805, DQ375788) are phylogenetically separated from the remaining strains, which can be subdivided into two overall clusters. The 'upper overall cluster' includes the bacterial isolates E_2, E_{14}, and KY_5 as well as producers of a vast variety of antifungal compounds, such as actinomycin, antimycin, bafilomycin, candicidin, daunomycin, mycangimycin, sceliphrolactam, streptazoline, streptazon, and valinomycin. In contrast, the 'lower overall cluster' is dominated by candicidin and/or antimycin producers, but also valinomycin and mycangimycin producers are represented. This overall cluster can be sub-divided into three sub-clusters, one of which contains the *Acromyrmex* ant isolate *Streptomyces* E_6 and *Streptomyces* symbionts of the southern pine beetle *Dendroctonus frontalis*. One of the *D. frontalis* isolates has been shown to produce the antifungal mycangimycin (Scott *et al.* 2008). Both, *Streptomyces* E_6 and the mycangimycin producer are genetically related to *S. thermosacchari* (Table S_6). This finding suggests that *Acromyrmex* ants may be able to access mycangimycin.

Sample type	Name	PCR primers	Sequencing primers	Sequence length	Closest relative (accession)	Max score	Total score	Query coverage	E value	Max identity
Bacterial isolate	E_2	fD2-16Sr, 515-1492	fD2, 515	1228 bp	*Streptomyces acrimycini* strain AS 4.1673 16S ribosomal RNA gene, partial sequence (AY999889.1)	2263	2263	100%	0	99%
					Streptomyces albus subsp. *albus* gene for 16S rRNA, partial sequence (AB184741.1)	2263	2263	100%	0	99%
					Streptomyces baarnensis gene for 16S rRNA, partial sequence (AB184615.1)	2263	2263	100%	0	99%
					Streptomyces caviscabies strain ATCC51928 16S ribosomal RNA gene, partial sequence (AF112160.1)	2263	2263	100%	0	99%
					Streptomyces fimicarius gene for 16S rRNA, partial sequence (AB184269.1)	2263	2263	100%	0	99%
					Streptomyces flavofuscus strain 173911 16S ribosomal RNA gene, partial sequence (EU593623.1)	2263	2263	100%	0	99%
					Streptomyces setonii gene for 16S rRNA, partial sequence (AB184300.1)	2263	2263	100%	0	99%
Bacterial isolate	E_4	fD2-16Sr, 515-1492	fD2, 515	1189 bp	*Pseudonocardia ammonioxydans* strain H9 16S ribosomal RNA gene, partial sequence (AY500143.1)	2124	2124	100%	0	98%
Bacterial isolate	E_6	fD2-16Sr, 515-1492	fD2, 515	1262 bp	*Streptomyces thermosacchari* strain LLJ-05 16S ribosomal RNA gene, partial sequence (HQ170508.1)	2289	2289	100%	0	99%
Bacterial isolate	E_8	fD2-16Sr, 515-1492	fD2, 515	1312 bp	*Streptomyces violascens* isolate XSD-115 16S ribosomal RNA gene, partial sequence (EU273550.1)	2423	2423	100%	0	100%
Bacterial isolate	E_9	fD2-16Sr, 515-1492	fD2, 515	1249 bp	*Streptomyces flavofungini* isolate XSD-104 16S ribosomal RNA gene, partial sequence (EU273540.1)	2307	2307	100%	0	100%
Bacterial isolate	E_{10}	fD2-16Sr, 515-1492	fD2, 515	1170 bp	*Streptomyces carnosus* strain HBUM174871 16S ribosomal RNA gene, partial sequence (FJ486392.1)	2161	2161	100%	0	100%
					Streptomyces litmocidini strain RT-13 16S ribosomal RNA gene, partial sequence (HQ909754.1)	2161	2161	100%	0	100%
Bacterial isolate	E_{13}	fD2-16Sr, 515-1492	fD2, 515	1198 bp	*Streptomyces collinus* subsp. *albescens* gene for 16S rRNA, partial sequence (AB184101.2)	2213	2213	100%	0	100%
Bacterial isolate	E_{14}	fD2-16Sr, 515-1492	fD2, 515	1200 bp	*Streptomyces drozdowiczii* partial 16S rRNA gene (AM921646.1)	2211	2211	100%	0	99%
Bacterial isolate	KY_1	fD2-16Sr, 515-1492	fD2, 515	1349 bp	*Streptomyces griseus* subsp. *griseus* gene for 16S rRNA (AB045867.1)	2486	2486	100%	0	99%
Bacterial isolate	KY_2	fD2-16Sr, 515-1492	fD2, 515	1040 bp	*Streptomyces cacaoi* subsp. *cacaoi* gene for 16S rRNA, partial sequence (AB184183.1)	1921	1921	100%	0	100%
					Streptomyces violaceoruber strain HBUM49432 16S ribosomal RNA gene, partial sequence (GQ163473.1)	1921	1921	100%	0	100%
Bacterial isolate	KY_5	fD2-16Sr, 515-1492	fD2, 515	1307 bp	*Streptomyces kanamyceticus* gene for 16S rRNA, partial sequence (AB184388.1)	2405	2405	100%	0	99%

Table S_6 16S rDNA BLAST analogies of eleven bioactive NNSPS strains. The strains included *Streptomyces* E_2, E_6, E_8-E_{10}, E_{13}-E_{14}, KY_1-KY_2, KY_5, as well as *Pseudonocardia* E_4, which served as outgroup for a phylogenetic analysis. The data show that *Streptomyces* E_8 is genetically related to *Streptomyces violascens*, *Streptomyces* E_9 to *S. flavofungini*, *Streptomyces* E_6 to *S. thermosacchari*, *Streptomyces* KY_1 to *S. griseus*, and *Streptomyces* KY_5 to *S. kanamyceticus*.

The second sub-cluster includes the *Acromyrmex* ant isolates *Streptomyces* E_{10} and E_{13} along with valinomycin- and candicidin-producing reference strains. The third sub-cluster contains *Acromyrmex* ant isolates *Streptomyces* E_8 and E_9 as well as *Tetraponera* ant isolate *Streptomyces* KY_1. Various candicidin and/or antimycin producers dominate this sub-cluster. Interestingly, the distribution of candicidin plus antimycin producers appears to be limited to this sub-cluster. This finding is analogous to the chemical (Chapter 3) and genetic (Supplementary Information C) data presented earlier, showing that *Streptomyces* E_8 is able to produce candicidin and antimycin.

This phylogenetic analysis provides a straightforward and useful approach to obtain indications of what antifungals bacterial isolates may produce. Unfortunately, these data have been unavailable when the antimycin metabolites of *Streptomyces* E_8 were chemically identified.

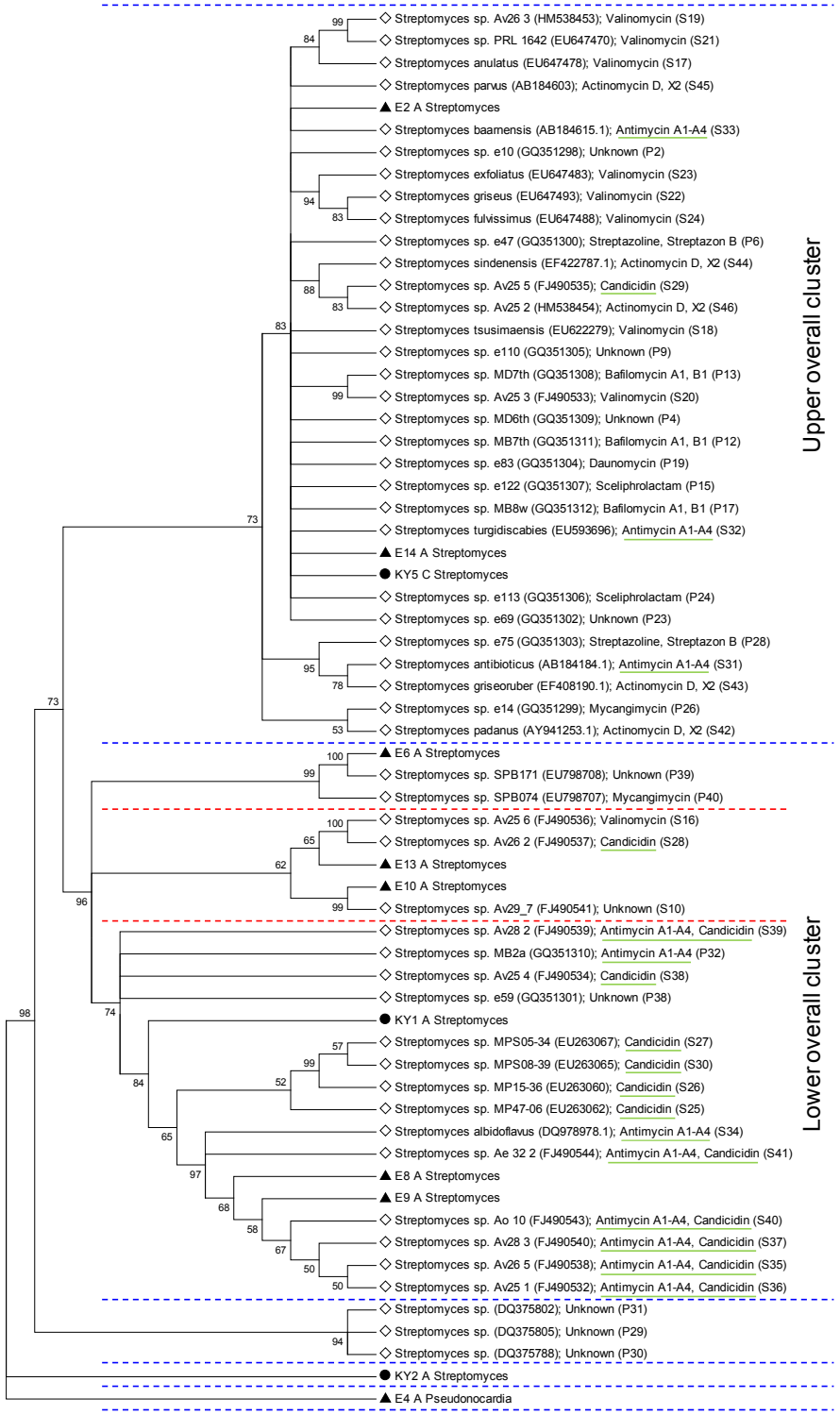


Figure S_3 Neighbor-Joining tree of 52 bacterial reference strains (◊), known for producing specific antifungals (Poulsen *et al*. 2011, Kaltenpoth *et al*. 2006, Scott *et al*. 2008, Schoenian *et al.* 2011, Haeder *et al*. 2009), and eleven bioactive NNSPS isolates. In particular, partial 16S rDNA sequences were included of *Streptomyces* E_2, E_6, E_8-E_{10}, E_{13}-E_{14} ('▲'), and *Streptomyces* KY_1-KY_2, KY_5 (●). *Pseudonocardia* E_4 served as phylogenetic outgroup. A 50% threshold for the bootstrap values is handled. The data indicate that *Streptomyces* E_8 and E_9, and possibly *Streptomyces* KY_1, are able to produce candicidin and antimycin.

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