Molecular Ecology of Marine Isoprene Degradation

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2014

A thesis submitted to the School of Environmental Sciences in fulfilment of the

requirements for the degree of Doctor of Philosophy

University of East Anglia, Norwich, UK

September 2014

Declaration

I declare that the content of this thesis entitled "Molecular Ecology of Marine Isoprene Degradation" was undertaken and completed by myself under the supervision of Professor J.C.Murrell, unless otherwise acknowledged and has not been submitted in support of an application for another degree or qualification in this or any other university or institution

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Acknowledgements

I would like to thank my primary supervisor, Professor J.C.Murrell, for his help and guidance throughout my PhD. I would also like to thank past and present members of the Murrell lab at the University of Warwick and the University of East Anglia, especially Myriam El Khawand and Dr. Andrew Crombie for their help and collaboration on this project. I thank my secondary supervisor Dr. Jonathon Todd for his advice. I would especially like to thank our collaborators on this project, including Dr. Terry McGenity at the University of Essex, Professor Dan Arp at Oregon State University, Capt. Collett at the 47th Regiment Royal Horse Artillery for assistance with sampling, and Sue Slade at the University of Warwick proteomics facility. Special thanks go to my family for their continued support.

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List of Abbreviations

µM micromolar
µg microgram
µl microlitre
ATP adenosine triphosphate
BIS *N*,*N'*-methylenebisacrylamide
BLAST basic local alignment search tool
bp base pairs
BSA bovine serum albumin
CCN cloud condensation nuclei
Da Dalton
DEPC diethylpyrocarbonate
DMSO dimethylsulfoxide
DMADP dimethylallyl diphosphate
DNA deoxyribonucleic acid
DNase deoxyribonuclease
DNMS dilute nitrate mineral salts
dNTP deoxynucleotide triphosphate
dO_2 dissolved oxygen
EDTA ethylendiaminetetraacetic acid
FID flame ionisation detector
FAD flavin-adenine dinucleotide
g gram / acceleration due to gravity
GC gas chromatography
GMBA 2-glutathionyl-2-methyl-3-butenoic acid
h hour
HGMB 1-hydroxy-2-glutathionyl-2-methyl-3-butene
IMO isoprene monooxygenase
IPTG isopropyl β-D-1-thiogalactopyranoside
l litre
LacZ β-galactosidase
LB lysogeny broth
LC/ESI liquid chromatography electrospray ionisation
LDH lactate dehydrogenase
M molar
MAMS marine ammonia mineral salts medium
MEP methyl erythritol phosphate
mg milligram
min minute
ml millilitre
mM millimolar
mol mole
mRNA messenger RNA
MS/MS tandem mass spectrometry
MVA mevalonate
NAD^+ nicotinamide adenine dinucleotide (oxidised form)
NADH nicotinamide adenine dinucleotide (reduced form)
$NADP^+$ nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH nicotinamide adenine dinucleotide phosphate (reduced form)

NCBI National Centre for Biotechnology Information
ng nanogram
nm nanometre
nM nanomolar
NMHC non-methane hydrocarbon
Nmol nanomole
NMS nitrate mineral salts
NO nitrogen oxide
OD_{540} optical density at 540 nm
OH hydroxyl radical
orf open reading frame
PAGE polyacrylamide gel electrophoresis
PCR polymerase chain reaction
PEP phosphoenolpyruvate
PIPES 1,4-piperazinediethanesulfonic acid
PLFA phospolipid fatty acid
PQQ pyrroloquinoline quinone
pMMO particulate methane monooxygenase
pmol picomole
ppt parts per thousand
PrMO propane monooxygenase
Psi pounds per square inch (unit of pressure)
Psu practical salinity units
QIIME Quantitative Insights Into Microbial Ecology
RFLP restriction fragment length polymorphism
RNA ribonucleic acid
RNase ribonuclease
ROS reactive oxygen species
rRNA ribosomal ribonucleic acid
RT-PCR reverse transcriptase PCR
RubisCO ribulose 1,5-bisphosphate carboxylase-oxygenase
RuMP ribulose monophosphate
s seconds
SDIMO soluble diiron monooxygenase
SDS sodium dodecyl sulphate
SIP stable isotope probing
sMMO soluble methane monooxygenase
SOC super optimal broth with catabolite repression
TAE tris acetate EDTA
TBE tris borate EDTA
TCA trichloroacetic acid / tricarboxylic acid
TE tris EDTA
TEMED *N*,*N*,*N'*,*N'*-tetramethyl-ethane-1,2-diamine
Tg teragram
Tris tris(hydroxymethyl)aminomethane
v/v volume to volume
VOC volatile organic compound
w/v weight to volume
X-gal 5-bromo-4-chloro-3-indoyl-β-D-galactoside

Abstract

Isoprene is an atmospheric trace gas whose emissions to the atmosphere are roughly equal to that of methane. It is highly reactive and has the potential to affect climate through a variety of interactions in the atmosphere, including the formation of ozone. In the marine environment alone, algae produce up to 11 Tg y^{-1} of isoprene. To date, little is known about its degradation by microbes in the marine environment. In this project, isoprene-degrading bacteria from a range of marine sites were characterised and Illumina sequencing was used to mine the genomes of isoprene-degrading strains related to *Gordonia polyisoprenivorans* and *Mycobacterium hodleri*, isolated from the Colne Estuary, Essex. From these genomes, we retrieved novel sequences encoding isoprene monooxygenase, previously identified in a terrestrial *Rhodococcus* species. This information allowed the design of specific PCR primer sets for the *isoA* gene, encoding the alpha subunit of isoprene monooxygenase, to retrieve isoprene-specific genes from environmental samples. The primers amplify *isoA* from a wide range of marine genera. A database of *isoA* sequences from extant isoprene degraders and *isoA* sequences retrieved by PCR from DNA from a variety of different marine environments was created. The data obtained demonstrated that isoprene monooxygenase genes are widespread in the marine environment. Other work focused on the physiology of isoprene-degrading bacteria, particularly the marine isolate *Gordonia polyisoprenivorans.* SDS-PAGE, oxygen electrode assays and RT-PCR were also used to investigate the regulation of soluble diiron centre monooxygenases in this organism, and showed that two separate, inducible monooxygenase enzyme systems exist in this organism and are responsible for the oxidation of isoprene and propane. DNA-Stable Isotope Probing revealed that members of the genera *Rhodococcus, Mycobacterium, Gordonia* and *Microbacterium* are active isoprene degraders in the Colne Estuary.

Chapter 1

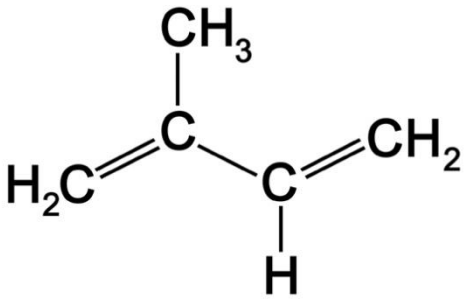
Introduction

Chapter 1: Introduction

1.1 The structure of isoprene

Isoprene (short for isoterpene), or 2-methyl-1,3-butadiene, is an organic hydrocarbon with the chemical formula $CH_2=C(CH_3)CH=CH_2$ (see figure 1.1).

Figure 1.1: The structure of isoprene



Isoprene is a colourless, highly volatile liquid at room temperature and has a boiling point of 34.07°C. As it contains two double bonds, isoprene is highly reactive. It is the monomer of polyisoprene, natural rubber, and a structural motif found in a wide range of compounds known as the isoprenoids, which are derived from isoprene. Examples include the carotenes, phytol, retinol (vitamin A), tocopherol (vitamin E) and squalene. Heme A has an isoprenoid tail, and lanosterol, the sterol precursor in animals, is derived from squalene and hence from isoprene.

1.2 Industrial uses of isoprene

Approximately 95% of industrially produced isoprene is used to manufacture cis-1,4-polyisoprene, a type of synthetic rubber (Kuzma *et al.*1995). Approximately 800,000 tonnes are produced annually as a side-product in the production of ethane by thermal cracking of oil. Applications of synthetic rubber include medical equipment, baby bottles, toys, shoe soles, tyres, and in adhesives as well as paints. Isoprenoid molecules are also used in cancer therapy, treatment of infectious diseases and crop protection (Withers & Keasling. 2007).

Current research has investigated the potential use of isoprene as a biofuel (Xue & Ahring. 2011). Isoprene is easier to separate from fermentation broth than ethanol, since it is

present in the upper gas phase of a fermentor due to its low boiling point (34°C) and low solubility in water.

1.3 The effect of isoprene on the atmosphere and global climate

Isoprene emissions to the atmosphere are roughly equivalent to that of methane. Isoprene emissions comprise ~40% of all non-methane hydrocarbons released to the atmosphere (Fan & Zang. 2004). Isoprene is one of the most abundant and reactive biogenic hydrocarbons. It can account for up to 80% of all hydrocarbons released from deciduous forests (Grosjean *et al.* 1993), although it's ambient concentration is diurnal and seasonal (Duane *et al.* 2002). Isoprene, as described previously, is highly reactive and can undergo photochemical oxidation in the atmosphere, initiated by hydroxyl radicals, ozone (O_3), nitroxyl radicals (NO_3) and halogen radicals. The chemistry of these reactions is hugely complicated and can involve thousand of reactions, leading to the formation of hundreds of highly reactive intermediate species with their own atmospheric effects. One of the most significant effects on atmospheric chemistry is the reaction of isoprene with hydroxyl radicals to form ozone, represented by the equation $C_5H_8 + OH \rightarrow \rightarrow RO_2 + NO \rightarrow \rightarrow O_3$ (Geng *et al.* 2011). This reaction also leads to the formation of carbonyls such as formaldehyde and acetaldehyde which photodissociate, leading to the formation of OH radicals. Volatile organic compounds (VOCs) react readily in the presence of nitrous oxides to form tropospheric ozone. Isoprene is known to influence atmospheric conditions leading to climate change, owing to chemical reactions in the presence of nitric oxides which remove hydroxyl radicals from the atmosphere. This can potentially elevate the formation of ozone (Fehsenfeld *et al.* 1992), and the removal of hydroxyl radicals from the atmosphere can increase the atmospheric residence time of methane, also known to be a significant contributor to global warming. OH radicals react readily with VOCs in a long cascade of reactions forming products such as organic peroxyradicals and HO_2 (Sharkey *et al.* 2007). These react with NO to form NO_2, which is photolysed to NO+O, which then react with O_2 to form O_3 (see Figure 1.2)

Figure 1.2: An example of isoprene interaction with reactive oxygen species to form ozone (taken from Sharkey *et al.* 2007)

$$RH + OH + O_2 \rightarrow RO_2 + H_2O$$
$$RO_2 + NO \rightarrow RO + NO_2$$
$$RO + O_2 \rightarrow R'CHO + HO_2$$
$$HO_2 + NO \rightarrow OH + NO_2$$
$$2(NO_2 + O_2 \rightarrow NO + O_3)$$
$$\text{Net} : RH + 4O_2 \rightarrow R'HCO + 2O_3 + H_2O$$

The upper atmospheric ozone layer is important to life on Earth as ozone filters out sunlight wavelengths from about 200 nm (UV rays) to 315 nm. However, low level tropospheric ozone is considered an atmospheric pollutant. It is a powerful oxidising agent which can react readily with chemical compounds, forming toxic oxides. Ozone itself is hazardous to human health, causing irritation and damage to the respiratory system. Tropospheric ozone is also a greenhouse gas. Formation of tropospheric ozone in this manner will likely increase due to increasing pollution by nitrogen oxides. Modelling of isoprene emissions will become increasingly important in the future due to the impacts of changing land-use created by the growing demand for food production and biofuels (Hardacre *et al.* 2013). The conversion of agricultural land to grow biofuel crops, which emit large quantities of isoprene, could lead to a rise in isoprene emissions of 1.4% by 2030 (Armstrong 2013). Conversely, the concurrent replacement of forest and grasslands with food crops could lead to a decrease in emissions.

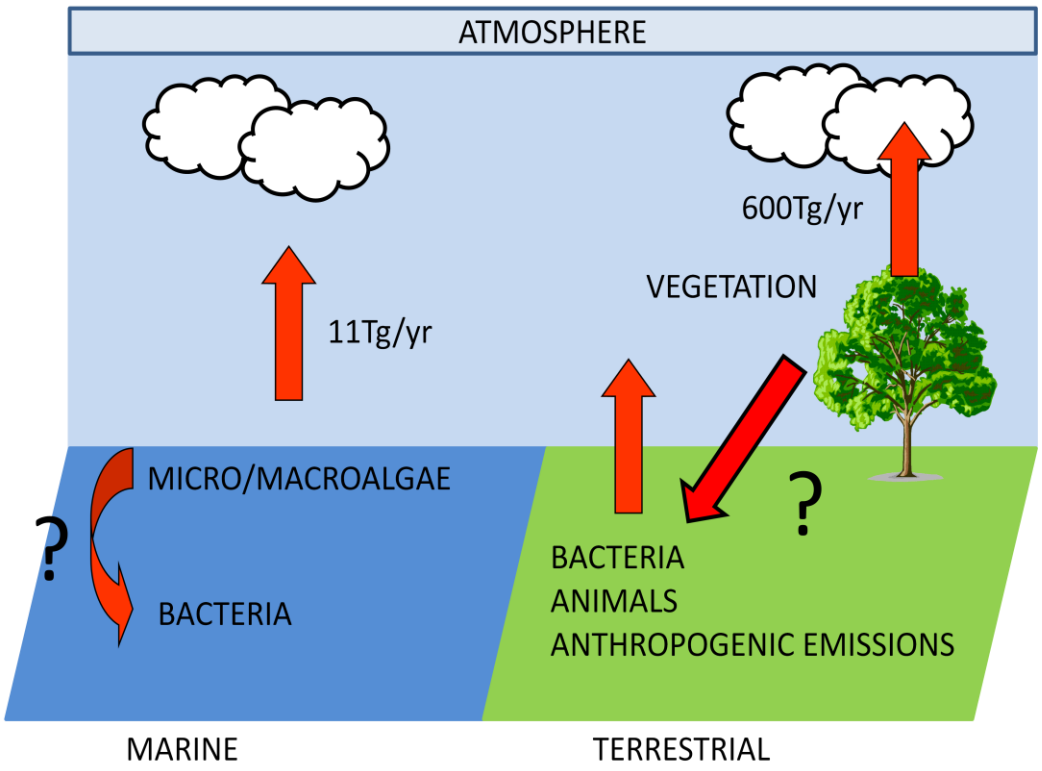
Other effects of isoprene include its impact on the formation of secondary organic aerosols, which contribute to global cooling. Epoxyisoprene contributes to the formation of aerosols and haze (Paulot *et al.* 2009*).* In the marine environment, organic aerosols contribute significantly to the Earth's radiative budget, biogeochemical cycling, and have numerous impacts on ecosystems and air quality (O'Dowd and de Leeuw. 2007). Atmospheric oxidation of isoprene can yield methylglyoxyl and formaldehyde, neither of which contain double bonds and thus reside in the atmosphere for up to 15 times longer than isoprene (Duane *et al.* 2002). The oxidation products accumulate in the atmosphere and air at a rate that is dependent on the balance between its sources and sinks. To further complicate the overall picture, in certain conditions (such as those measured over some tropical forests) isoprene is considered to act as a buffer for OH radicals, with a balance between acting as a source and sink for OH radicals (Taraborelli *et al.* 2012). There is significant interest in the

sources and sinks of the climate active gas isoprene due to the potential effects on the marine climate.

The effects of atmospheric isoprene cannot be accurately modelled without full knowledge of its cycling in terrestrial and marine environments. This has potentially increasing importance due to the positive feedback loop that could exist with isoprene production: isoprene that enters the atmosphere leads to an increase in the amount of greenhouse gases, thus leading to a rise in global temperature that will drive increased isoprene production from plants. Thus far, the only sinks studied in detail for isoprene are photochemical degradation, photolysis, partitioning to airborne particulate matter and vegetation surfaces (Duane *et al*. 2002).

1.4 Isoprene cycling and the global isoprene budget

Figure 1.3: The isoprene cycle as based on current literature



1.4.1 Isoprene sources and production

1.4.1.1 Isoprene production by humans and animals

Isoprene is formed as a metabolic product in both humans and other higher eukaryotes including mammals. Its production is estimated at roughly 17 mg/day for a 70 kg person

and is the most common volatile organic compound (VOC) in the human body (Gelmont *et al.* 1981), although its formation can be inhibited by cholesterol-reducing statins which inhibit the formation of mevalonate, thus blocking the mevalonate pathway of isoprene biosynthesis (see Figure 1.5). The majority of excreted isoprene is released by exhalation, and monitoring of VOCs in human breath can act as an indicator of metabolic disease.

1.4.1.2 Production of isoprene by terrestrial vegetation

The most significant source of isoprene emissions to the atmosphere is terrestrial vegetation. For example, the characteristic 'blue haze' that covers the Blue Mountains in Australia and Blue Ridge Mountains of Virginia is due to isoprene emissions. The annual production of isoprene by terrestrial vegetation is estimated at 600 million tonnes (Guenther *et al.* 2006)(see Figure 1.3) Approximately 50% of this comes from tropical trees, with the other 50% emitted by shrub vegetation. *De novo* isoprene emission from plants consists of approximately 2% of all carbon fixed during photosynthesis (Srikanta Dani *et al.* 2014). The major isoprene producers are oak, poplar, eucalyptus and legumes, all of which are important and widely planted genera. In particular, poplar trees are of interest as a fast growing biomass crop that can be used for renewable bioenergy. RNA interference technology was used to knock down isoprene production in *Populus canescens* (Behnke *et al.* 2011) which was found to not inhibit growth nor biomass yield of this plant, suggesting a method for engineering more environmentally sustainable poplar plants. Several broad leaf deciduous trees are known to be strong isoprene emitters, such as *Populus* sp. *Quercus* sp. and *Robinia pseudoacacia* (Duane *et al.* 2002).

Several species of plants emit isoprene, which is produced via the MEP (methyl erythritol phosphate) pathway in the chloroplast, followed by conversion of DMADP (dimethylallyl diphosphate) to isoprene via isoprene synthase. There is no method of predicting which plants emit isoprene: the mosses, ferns, gymnosperms and angiosperms all have species which emit isoprene in varied amounts, however there is no consistent phylogenetic distribution and it is thought that isoprene production in plants evolved independently several times. However, it is rare for gymnosperms to be deciduous, and almost all deciduous gymnosperms emit isoprene (Srikanta Dani *et al.* 2014). Even amongst clades there is variation, for example the North American oak is a significant isoprene emitter whereas many European oaks are not.

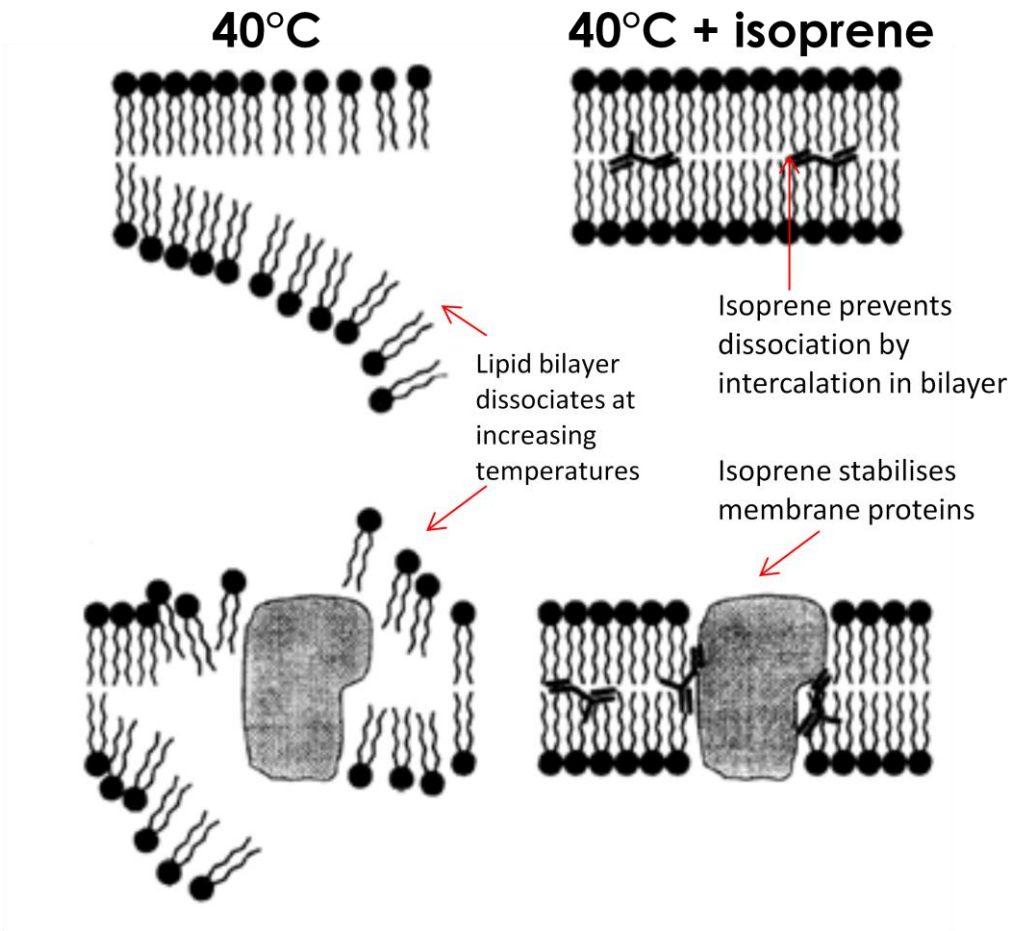
The production of isoprene has a significant energy cost, with 20 ATP molecules and 14 NADH molecules to produce a single molecule of isoprene from five molecules of CO_2. Isoprene production also leads to significant loss of carbon when plants are under carbon limitation. The production of isoprene is a process which is regulated at the transcriptional level as well as being regulated by the availability of the substrate DMADP and also the activity of isoprene synthase. It also remains unconfirmed why only some plant taxa produce isoprene, as well as huge observed differences in the magnitude of emission rates. Therefore there has been much speculation in the literature as to the exact advantage afforded by isoprene production in plants (Sharkey *et al.* 2008), and several hypotheses have been proposed:

Thermotolerance

Isoprene emission is both light and temperature dependent as well as being linked to photosynthesis (Monson and Fall. 1989). Isoprene emission increases with temperature up to a maximum of approximately 40℃. It has been shown that isoprene emission provides tolerance to short high temperature episodes (Sharkey *et al.* 2001) and feeding isoprene in the gas stream to non-isoprene emitting plants or isoprene emitters where the MEP pathway has been inhibited by fosmidomycin gives up to 16% increased tolerance to heat stress in photosynthesis. Genetically engineered poplar trees that no longer emit isoprene showed increased damage after heat spikes (Behnke *et al*. 2007). Isoprene emission in plants is not definitively correlated with increasing temperatures – desert plants such as cacti have high stomatal conductance allowing heat to be lost easily, whereas the large leaves of tropical trees are more highly insulated, increasing temperature in the leaf compared to the surrounding air, and the humidity in these environments reduces the potential for heat loss by evaporation. As might be predicted, there are very few isoprene emitting plants in hot, dry deserts whereas many tropical plants emit isoprene.

The lipid bilayer of the plant cell and the chloroplasts can be destabilised by increasing temperatures. An increase in isoprene concentration has been shown to stabilise the lipid membrane by intercalating in the lipid bilayer, as well as acting to stabilise membrane proteins (as shown in Figure 1.4). This prevents dissociation of the membrane which can disrupt proton gradients. Isoprene is also found in the cell membrane monolayer of Archaea, which are commonly found in extreme environments (Boucher *et al.* 2004).

Figure 1.4: The intercalation of isoprene in the cellular membrane lipid bilayer in increasing temperatures



(Adapted from Sharkey *et al.* 2006)

Protection against reactive oxygen species

It is interesting to note that while isoprene emissions can increase tropospheric ozone in the presence of NO_2, isoprene may also prevent damage to plants by ozone and other reactive oxygen species (Loreto *et al.* 2001) However, ozone stress has not been a significant evolutionary pressure throughout history, therefore it is unlikely that isoprene emission in plants evolved solely for this reason. Reactive oxygen species (ROS) include hydrogen peroxide, singlet oxygen, superoxide anion and hydroxyl radicals. Accumulation of these species in plant tissues can cause severe damage in the plant due to oxidation of cellular components such as nucleic acids, protein and lipids (Jardine *et al.* 2012) and eventually leads to cell death by apoptosis. Ozone, hydrogen peroxide, nitric oxide and singlet oxygen can all be quenched by isoprene (Velikova *et al.* 2004). It has been shown that under high ROS production rates, the production rate of isoprene in the leaf exceeds emissions

(Jardine *et al.* 2012) and isoprene is instead oxidised within the leaf by reacting with ROS and the products of these reactions, most commonly methyl vinyl ketone and methacrolein, are emitted to the atmosphere.

Protection against insects

As previously mentioned, RNA interference can be used to prevent isoprene emission in transgenic poplar plants. One effect of this was an increased susceptibility to herbivores such as the willow leaf beetle *Phratora vitellinae,* which subsequently attacked the plants. Loivamäki *et al.* (2008) observed that egg laying females of the wasp species Diadegma semiclausum were repelled by isoprene-emitting plants. Electrical measurements of the wasp's antennae showed that this wasp could detect and respond to isoprene at low concentrations. Another report (Laothawornkitkul *et al*. 2008) showed that transgenic tobacco plants engineered to emit isoprene repelled tobacco hornworm caterpillars. Crucially, the host larvae of the parasitic wasp *D. semiclausum* do not feed on isoprene-emitting plants. It is possible that isoprene acts as a cue to repel parasitic insects from seeking out plants that do not carry their host, however it is unlikely that isoprene emission evolved in plants for this reason, as it confers no advantage to the plant to repel the natural predators of its herbivores. It is more likely that a co-evolution of insect species occurred to exploit the natural emission of volatiles by certain plants.

1.4.1.3 Pathways of isoprene production

Isoprene production is carried out by the enzyme isoprene synthase, which catalyses the conversion of dimethylallyl diphosphate to form isoprene and diphosphate. Isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are the fundamental building blocks used to synthesize key biological isoprenoids including cholesterol and other steroids. The isoprenoids are the largest group of natural products, comprising approximately 25,000 known compounds (Odoll 2011)

There are two metabolic pathways which synthesise isopentenyl pyrophosphate and dimethylallyl pyrophosphate, each found in different organisms:

- The **mevalonate (MVA) pathway**, found in plants and insects (as well as all other higher eukaryotes and some bacteria)

- The **non-mevalonate pathway** or methyl D-erythritol 4-phosphate **(MEP)** pathway, which occurs in plant chloroplasts, protozoa, algae, cyanobacteria, and eubacteria

The MVA (Figure 1.5) and MEP (Figure 1.6) pathways do not share any common enzymes or enzymatic steps and each begins with a different precursor: the MEP pathway begins with the generation of deoxyxyluose 5-phosphate (DOXP) from pyruvate and glyceraldehyde 3-phosphate, whereas the MVA pathway begins with Acetyl-CoA. Within the bacteria, almost all Gram negative bacteria and Mycobacteria contain the MEP pathway, whereas Gram positive bacteria including clinically important species of groups such as staphylococci and streptococci contain the MVA pathway. Isoprenoid structures are essential to all tested bacteria except obligate intracellular parasitic bacteria such as *Rickettsia* spp.(Lange *et al.* 2000). Disruption of any of the enzymes in either pathway is lethal to the organism.

Figure 1.5: The Mevalonate pathway of isoprene production (taken from Schmidmaier *et al.* 2004)

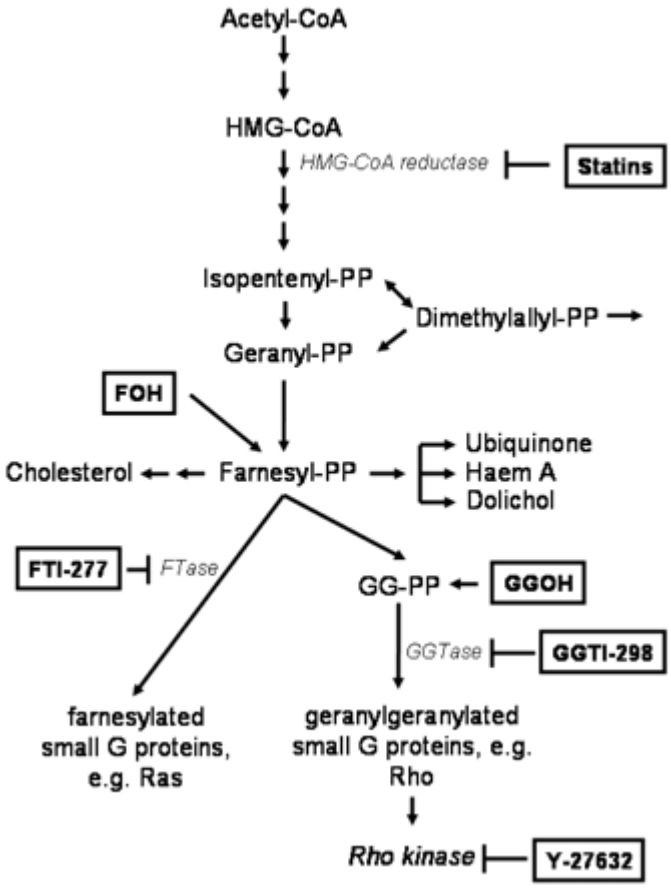
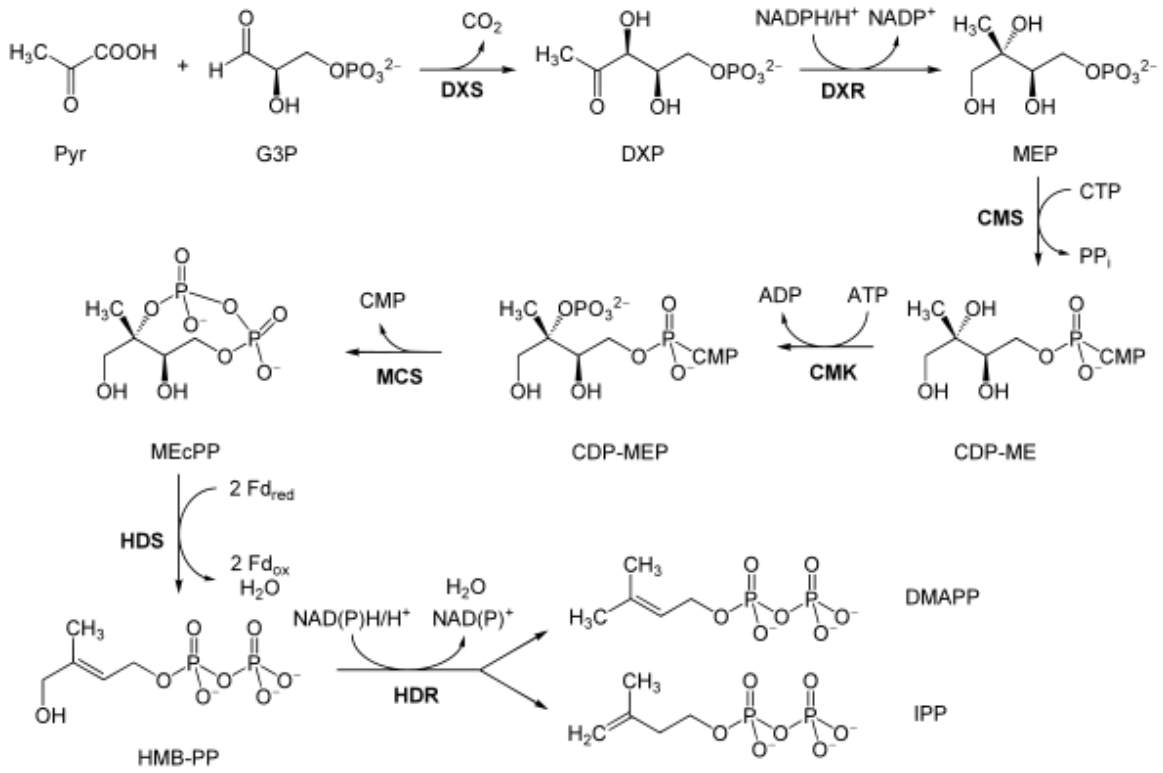


Figure 1.6: The non-mevalonate pathway for isoprene production



("Non-mevalonate pathway" by Yikrazuul - Own work. Licensed under Public domain via Wikimedia Commons)

(Pyr = Pyruvate, G3P = glyceraldehyde-3-phosphate, DXP = 1-deoxy-D-xylulose-5-phosphate, MEP = 2-C-methylerythritol-4-phosphate, CDP-ME = 4-diphosphocytidyl-2-C-methylerythritol, ME_cPP = 2-C-methyl-D-erythritol 2,4-cyclopyrophosphate, HMB-PP = (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate. DXS = DOXP synthase, DXR = DOXP reductase, CMS = 2-C-methyl-D-erythriol 4-phosphate cytidyltransferase, CMK = 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, MCS = 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, HDS = HMB-PP synthase, HDR = HMB-PP reductase)

1.4.1.4 Algal isoprene production in the marine environment

Isoprene and other non-methane hydrocarbons (NMHCs) are produced naturally in the marine environment. Emissions have been observed in open ocean and coastal areas as well as measured in algal rockpools (Broadgate *et al.* 2004), although atmospheric flux is significantly lower than that observed in terrestrial environments (Baker *et al.* 2000) Approximately 1 Tg carbon year $^{-1}$ is released from the oceans as a result of production by both macro- and microalgae (see Figure 1.3). This is potentially only a small fraction of the amount of isoprene produced by marine algae, and marine bacterial communities that

associate with algae are potentially responsible for degradation of isoprene produced, meaning that only a fraction enters the atmosphere. Macroalgae (seaweeds) such as the green macroalgae *Ulva instestinalis*, produce isoprene in a temperature- and light-dependent manner (Broadgate *et al.* 2004) and isoprene emissions are strongly seasonally dependent and correlated with chlorophyll content in the seawater (Broadgate *et al.* 1997). Experiments showed that production of isoprene increased by a factor of 4-10 when temperature was increased to 27.3°C from 7.5°C. Phytoplankton are another confirmed source of isoprene, as well as the light and temperature-dependent production of isoprene by *Prochlorococcus* spp. (Shaw *et al.* 2003). Isoprene production was shown to correlate to cell size when observing isoprene production in *Prochlorococcus, Synechococcus, Micromonas pusilla, Pelagomonas calceolate* and *Emiliana huxleyi.* Several species of diatoms, dinoflagellates, and *Cyanobacteria* from marine environments produce isoprene in pure culture (Milne *et al.* 1995; McKay *et al.* 1996) however cultures of these algal cultures contaminated with heterotrophic bacteria did not affect net isoprene measured (Shaw *et al.* 2003) suggesting that isoprene degradation is a specialised property not found in most common marine heterotrophs. Depth profiles have shown a correlation between peak isoprene concentration and bulk chlorophyll concentration (Baker *et al.* 2000). The pathway for isoprene biosynthesis has been found in several freshwater algae such as *Chlorella fusca*, as well as the cyanobacterium *Synechocystis* (Disch *et al.* 1998) although these organisms have not been confirmed to produce isoprene under laboratory observations. The temperature dependent production of isoprene follows a typical enzymatic temperature response, suggesting that the effect of temperature on isoprene production is due to the effect on the activity of key enzymes in the pathway, such as isoprene synthase. As little is known about marine isoprene production, several questions remain, such as the magnitude and spatial distribution of the isoprene flux. A better understanding of the marine microbial communities that contribute to the degradation of this compound (hence reducing net emission to the atmosphere) can help to provide an improved answer to these questions. For examples, isoprene emissions show a marked seasonality, and isoprene concentrations peak sharply in association with seasonal algal blooms such as those seen in the North Sea (Broadgate *et al.* 1997). Marine stratiform clouds have a significant influence on the ocean climate (Arnold *et al.* 2009) and these are affected greatly by aerosol particles acting as cloud condensation nuclei (CCN). Isoprene emitted even in small amounts from the oceans can perturb atmospheric chemistry due to the distance of open ocean areas from terrestrial isoprene sources. Isoprene concentrations

in marine environments are highly variable, with studies on the Colne estuary, UK, showing high values at the freshwater end of the estuary (with increased sediment) and lower values at the marine end (7 × 10-4 to 0.9
nmol l^{-1} from the marine to the freshwater end) (Alvarez *et al.* 2009) and the annual production rate for this estuary is estimated at 681 μmol m^{-2} y^{-1} (Exton *et al.* 2012) but natural seawater concentrations are estimated at around 1-50 pM (Matsunaga *et al.* 2002).

1.4.1.5 Isoprene production in bacteria

There is also some microbial production of isoprene, the best characterised examples of which are in *Escherichia coli* and *Bacillus subtilis,* which emits the highest amount of isoprene known amongst bacterial species (Kuzma *et al.* 1995). This bacterium produces isoprene via the MEP pathway at various points throughout its life cycle, for example during glucose catabolism and sporulation (Wagner *et al.* 2009) as well as during secretion and catabolism of acetoin. Several hypotheses exist as to why bacteria produce isoprene. Isoprene may act as a signalling molecule, or be released as an overflow metabolite in the process of the formation of isoprenoid structures, in which excess DMAPP is converted to isoprene. A second possibility is that isoprene acts as a signalling molecule in repelling predators and inhibiting growth of competitors. *B. subtilis* is known to use quorum intercellular signalling, and isoprene may act as a quorum sensing secondary metabolite (Fall and Copley. 2000) In *E. coli,* the genes encoding the MEP pathway enzymes have been documented in full (Eisenreich *et al.* 2004).

1.4.2 Isoprene sinks and degradation

1.4.2.1 Microbial consumption of isoprene in the terrestrial environment

Some soil bacteria can degrade isoprene as a sink (Cleveland and Yavitt. 1998). Consumption of isoprene in field chambers was rapid (672.60 ± 30.12 to 2,718.36 ± 86.40 pmol grams dry $weight^{-1}$ day^{-1}) and was confirmed to be biological in origin as autoclaved control soil did not consume isoprene. Isoprene consumption was diminished under low oxygen conditions and low moisture. In particular, soil dwelling members of the genera *Nocardia* and *Xanthobacter* oxidize isoprene as an alternative energy source (Hou *et al.* 1981). It has previously been suggested that propene-grown *Xanthobacter* spp. and methanotrophs may be able to oxidise isoprene but not grow on this substrate as a sole carbon and energy source (Cleveland and Yavitt. 1998). The estimated soil sink for

isoprene is 20 million tons/yr, around 4% of total biogenic emissions. However, it is unclear whether bacterial degradation of isoprene in forest environments will have a significant effect on forest air composition (Fall and Copley. 2000), and whether or not bacterial production of isoprene in soils may outstrip consumption, making soil a net source of isoprene. Two putative isoprene degrading strains, *Alcaligenes denitrificans* and *Rhodococcus erythropolis* JE77 were shown to co-oxidise trichloroethene (TCE) with isoprene (Ewers *et al.* 1990). van Ginkel *et al.* (1987) first demonstrated that isoprene degradation in *Nocardia* was likely catalysed by a monooxygenase enzyme that oxidised isoprene to 1,2-epoxy-2-methyl-3-butene an 1,2-3,4-diepoxybutane. *Rhodococcus erythropolis* JE77 showed glutathione-dependent activity towards 1,2-epoxy-2-methyl-3-butene in cell extract. In both *Nocardia* and *R. erythropolis* JE77, the enzymes and pathway involved in isoprene degradation remained uncharacterised.

1.4.2.2 Isoprene degradation in the marine environment

Oceanic depth profiles of isoprene concentrations first indicated that it might be consumed by marine bacteria (Moore and Wang. 2006). It has been suggested that marine algae provide a source of isoprene that can support isoprene-degrading microbial communities. Oil spill degrading microbes such as the ubiquitous hydrocarbon degrading bacterium *Alkanivorax borkumensis* can degrade isoprene (Alvarez *et al*. 2009), leading to the hypothesis that low concentrations of isoprene in seawater can help to sustain these microbes between oil spills.

Terry McGenity's group at the University of Essex demonstrated that isoprene degradation and sample enrichment occurs in a variety of marine and coastal environments, including the Colne Estuary, Essex (UK), Etang de Berre (France) and Indonesia (Alvarez *et al*. 2009). Samples were enriched with isoprene at 0.1% v/v, equivalent to a concentration of 81.9 ppm. in the headspace and 0.581 mM in solution. Their initial isoprene enrichments suggested that the rate of isoprene degradation increased at decreased isoprene concentrations. Primary Indonesian enrichments completely degraded isoprene within 300 days of incubation, with subsequent subcultures degrading 95% of added isoprene within 40 days. In the Colne estuary, the highest rate of isoprene degradation corresponded to the highest measured concentrations of isoprene in sediment samples by an order of magnitude. Isoprene concentration in the water is linked to the tides, and the productive phototrophs and diatom-rich microphytobenthos. Concentrations of isoprene in

samples were measured as 7×10^{-4} to 0.9 nM from the marine to the freshwater end of the Colne estuary, Essex. Other studies have reported concentrations in seawater of between 7×10^{-4} and 0.054 nM (Milne *et al.* 1995; Broadgate *et al.* 1997; Matsunaga *et al*, 2002).

Pyrosequencing of partial 16S rRNA genes showed that the bacterial genera which formed the isoprene degrading communities varied between sampling points, with *Rhodococcus* spp. and *Mycobacterium* spp. dominating in the Colne Estuary, compared to *Stappia* spp. being the main organisms present in the Indonesian enrichments (Alvarez *et al.* 2009). Other genera shown to potentially contribute to isoprene degradation included *Actinosynemma, Microbacterium, Lentzea* and *Ochrobacterium.* Overall, with the exception of the Indonesian enrichments, 16S rRNA gene sequences obtained from enrichments were dominated by Actinobacteria (Alvarez *et al.*2009) Enrichments of samples taken from Arctic environments showed no isoprene degradation, possibly due to the link between isoprene production and temperature or low biomass in these samples compared to the other locations. The overall conclusion was that isoprene degradation by bacteria takes place in many diverse marine environments, including both temperate and tropical marine environments, and is carried out by a wide range of bacterial genera.

Isolates obtained from the Colne Estuary and Etang de Berre enrichments were co-cultured in a mixture with algae (*Dunaliella tertiolecta* and *Phaeodactylum tricornutum*) to test for degradation of isoprene released by algal cultures (Alvarez *et al.*2009). This experiment demonstrated that communities of isoprene degrading bacteria may associate with algae in the marine environment, and can degrade isoprene at environmentally relevant concentrations as well as under laboratory enrichment conditions. The isoprene degrading strains tested, including species of *Rhodococcus, Gordonia* and *Mycobacterium,* could degrade other carbon sources including alkanes, suggesting that they may be nutritionally versatile and supplement their growth with isoprene produced by algae rather than obligate isoprene degraders.

1.5 Isoprene monooxygenase in *Rhodococcus* AD45

Isoprene metabolism has been thus far best characterised in the terrestrial organism *Rhodococcus* AD45 (Vlieg *et al.* 2000). This strain was first isolated on isoprene as its sole carbon and energy source. The km for oxidation of isoprene by intact *Rhodococcus* cells is 0.8mM (Vlieg *et al.* 1998). The gene cluster has been cloned and shown to encode a multicomponent monooxygenase with similarity to other multicomponent soluble diiron centre monooxygenases such as alkene monooxygenase from *Xanthobacte*r PY2 (Small and Ensign. 1997). The genes *isoABCDEF* encode the isoprene monooxygenase and *isoG*, *isoH*, *isoI* and *isoJ* encode four other genes involved in isoprene degradation; a glutathione *S*-transferase, IsoI; a second glutathione *S*-transferase, IsoJ*;* and a 1-hydroxy-2-glutathionyl-2-methyl-3-butene dehydrogenase, IsoH. This gene cluster is shown in Figure 1.7. The gene products of *isoABCDEF* show high sequence similarity and identical genetic organisation to monooxygenase enzymes with activity toward alkenes and aromatic compounds (Leahy *et al.* 2003). In particular, the highest sequence identity was shared with the alkene monooxygenase of *Xanthobacter* Py2 (Zhou *et al.* 1999). This similarity suggests that isoprene monooxygenase is a diiron $(\alpha\beta\gamma)_2$ structure, where the alpha subunit is encoded by *isoA*, the beta subunit by *isoE,* and the gamma subunit by *isoB.* The full list of the genes in this cluster and the molecular weight and function of their corresponding polypeptide product is shown in Table 1.1.

Table 1.1: Identification and expected sizes of polypeptides encoded by *Rhodococcus* AD45 gene cluster

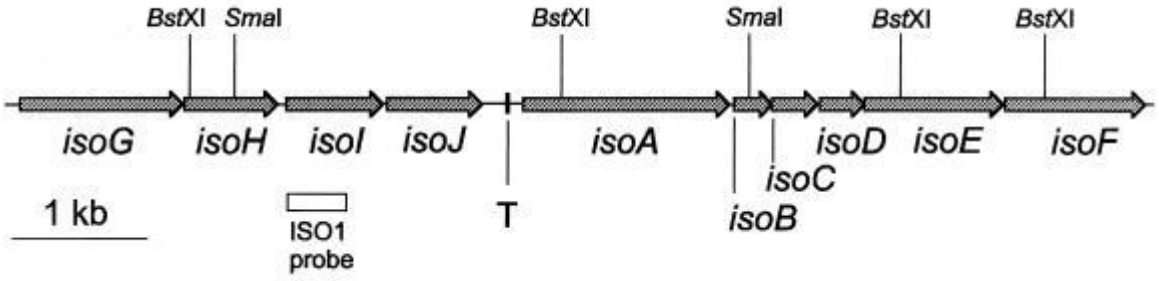
ORF	Gene product	
	Molecular mass (Da)	**Identification**
isoG	43,450	Putative racemase
isoH	24,033	NAD-dependent HGMB-dehydrogenase
isoI	27,096	Glutathione *S*-transferase active with epoxides
isoJ	26,342	Glutathione *S*-transferase active with CDNB and DCNB
isoA	58,461	Oxygenase α-subunit
isoB	10,331	Oxygenase γ-subunit
isoC	12,772	Ferredoxin
isoD	12,380	Effector or coupling protein
isoE	38,510	Oxygenase β-subunit
isoF	37,322	Reductase

(Adapted from Vlieg *et al.* 2000)

CDNB = 1-chloro-2,4-dinitrobenzene

DCNB = Dichloronitrobenzene

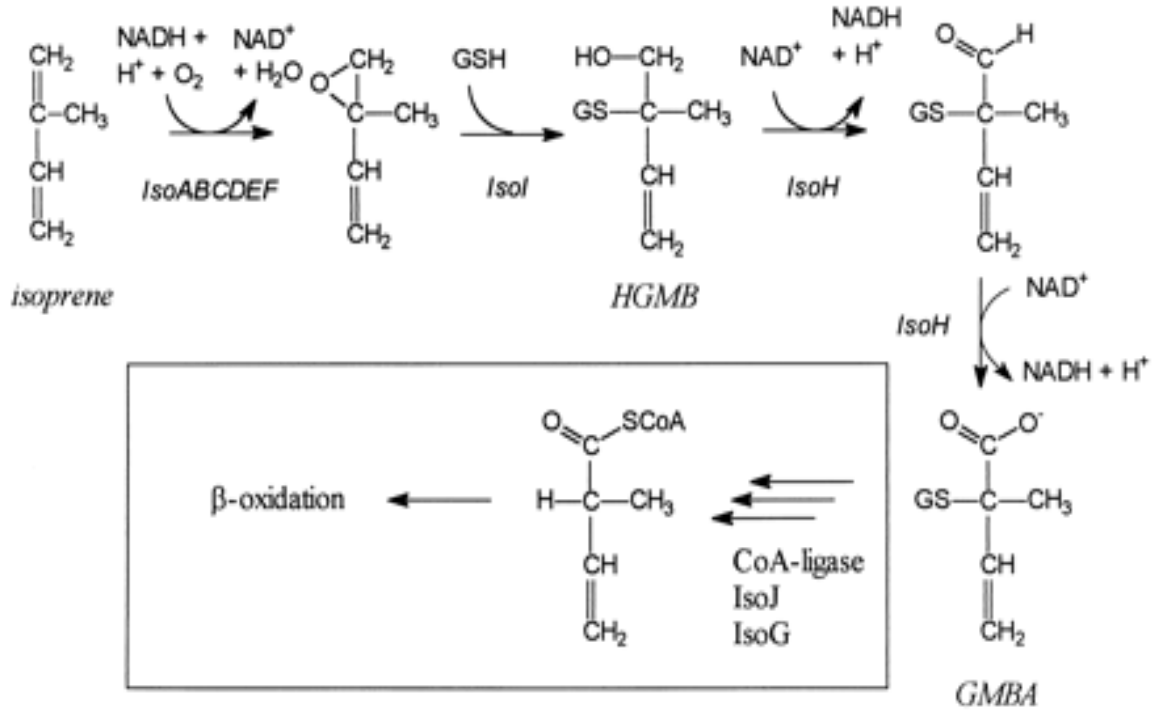
Figure 1.7: Gene cluster encoding isoprene degradation enzymes in *Rhodococcus* AD45



(Vlieg *et al*, 2000)

Isoprene degradation begins with the oxidation of the methyl-substituted double bond by the isoprene monooxygenase IsoABCDEF (abbreviation IMO), yielding 1,2-epoxy-2-methyl-3-butene, followed by the cleavage of the epoxide ring through conjugation to glutathione, catalysed by glutathione-S-transferase IsoI. This step is crucial in preventing the epoxide from alkylating DNA and cellular proteins. This enzyme has an unusually high Km for glutathione (>5 mM) (Fall and Copley. 2000). The IsoH protein, a dehydrogenase, then carries out two further oxidation steps. It catalyses oxidation of the alcohol moiety in HGMB (1-hydroxy-2-glutathionyl-2-methyl-3-butene) to the carboxylic acid, but is not active with other aliphatic primary or secondary alcohols and appears to be unique among alcohol dehydrogenases. (Vlieg et al., 1999) A schematic of the currently accepted pathway is shown in Figure 1.8. The part of the pathway highlighted by a box is currently speculative with regards to the fate of isoprene in cell metabolism. It is possible that GMBA (2-glutathionyl-2-methyl-3-butenoic acid) is converted to the corresponding CoA thioester by an unknown ligase. This would provide a role for the IsoG protein, which is homologous to α-methylacyl-CoA racemases. Glutathione could possibly then be removed by IsoJ, the second glutathione-S-transferase encoded in the gene cluster to form 2-methyl-3-butenyl CoA, which can enter β-oxidation to yield yield acetyl-CoA and propionyl-CoA, components of central carbon metabolic pathways.

Figure 1.8: Putative pathway for isoprene degradation in *Rhodococcus AD45*



(Vlieg *et al*, 2000)

(HGMB = 1-hydroxy-2-glutathionyl-2-methyl-3-butene , GMBA = 2-glutathionyl-2-methyl-3-butenoic acid)

Rhodococcus AD45 can also oxidise chlorinated ethenes to their corresponding chlorinated epoxyethanes. These epoxides are toxic and toxic effects are seen in other organisms when chlorinated ethenes are cooxidised by monooxygenase enzyme systems, which is a limiting factor in the use of monooxygenases in the removal of dichloroethene. Vlieg *et al* (1999) showed that the glutathione-S-transferase IsoI could dechlorinate *cis*-1,2-dichloroepoxyethane without the formation of toxic products, making it potentially useful in the detoxification of chlorinated ethane epoxides.

1.6 The soluble diiron centre monooxygenases

Monooxygenases catalyse the insertion of one oxygen atom into an organic substrate (Torres Pazmino *et al.* 2010). These enzymes are grouped according to cofactors. Oxygenase enzymes with activity towards aliphatic and aromatic compounds utilize a diverse array of prosthetic groups including flavin, haem, copper, binuclear iron centres and mononuclear iron centres (Arp *et al*., 2001). Heme-dependent cytochrome P450 monooxygenases are widespread amongst bacteria, where they contain a ferredoxin

reductase and a ferredoxin to transfer electrons to P450. Enzymes of the flavin containing monooxygenase family catalyse reactions via a NAPH cofactor and a bound FAD prosthetic group, and are useful in the metabolism of xenobiotics (Lawton *et al.* 1994). These enzymes catalyse the oxygenation of nucleophilic nitrogen, sulphur, phosphorus and selenium atoms in a range of structurally diverse compounds, for example the nitrogen atom in amine compounds.

There are two forms of methane monooxygenase, both of which oxidise the C-H bond in methane as well as several other substrates (Murrell *et al.* 2000). The particulate form (pMMO) is a copper containing membrane bound enzyme found in all methanotrophs with the exception of the facultative methanotroph *Methylocella silvestris* (Crombie and Murrell. 2014). Soluble methane monooxygenase (sMMO) is a soluble diiron centre monooxygenase which oxidises methane to methanol in the following reaction (Lipscomb. 1994):

$CH_4 + O_2 + NAD(P)H + H^+ \rightarrow CH_3OH + NAD(P)^+ + H_2O$

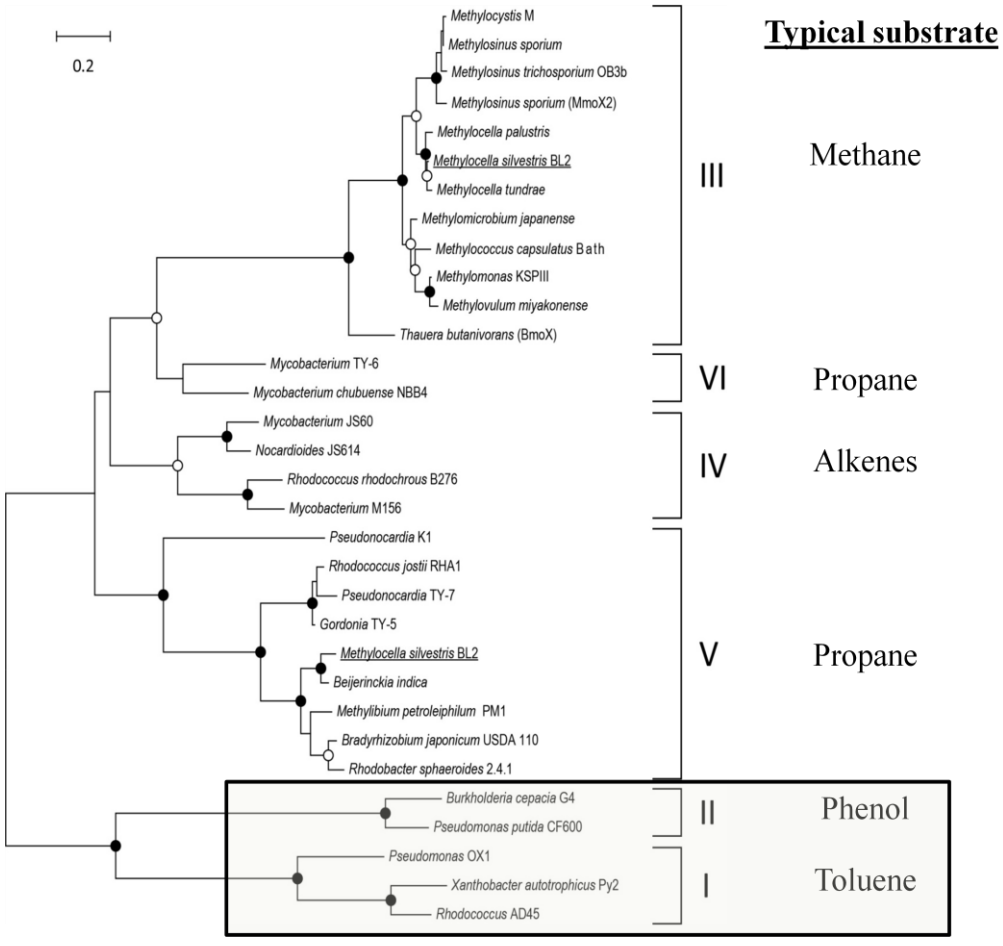
In this reaction, reducing equivalents from NADPH are used to split the O-O bond to oxidise methane to methanol. This methanol is then further metabolised to formaldehyde via methanol dehydrogenase and to formate via formaldehyde dehydrogenase. sMMO contains three protein components in an $\alpha_2\beta_2\gamma_2$ structure: an alpha subunit hydroxylase enzyme containing the dinuclear iron cluster active site of catalysis, a B protein, and a reductase.

As well as methane, monooxygenase enzyme systems have been described that catalyse the oxidation of other short chain alkanes and alkenes such as propane and butane (Sluis *et al.* 2002). Soluble toluene monooxygenases have also been described, including four-component diiron monooxygenases (Pikus *et al*. 1996), three-component diiron monooxygenases and three-component iron dioxygenases (Leahy *et al.* 2003).

There is little evidence for persistent vertical transmission of soluble diiron monooxygenases, and it can be hypothesised that these genes are likely to move between chromosome and plasmid frequently as they provide adaptive fitness in different environments. The alkene monooxygenase of *Xanthobacter* Py2 is encoded on a 320 kb megaplasmid (Zhou *et al.* 1999). It is hypothesised that the soluble diiron centre monooxygenases arose first in methanotrophic bacteria and were transmitted over time by

horizontal transfer to the pseudomonads and the Actinobacteria in response to environmental conditions and hydrocarbon substrates available (Leahy *et al.* 2003). Horizontal gene transfer can be indicated by changes in the mol % GC content of these operons in comparison to the rest of the genome. Examples of horizontally transferred monooxygenases that are plasmid encoded include toluene 4-monooxygenase, the phenol hydroxylase of *Pseudomonas putida* CF600 and the isoprene monooxygenase of *Rhodococcus* sp. AD45, all of which are closely related in amino acid sequence (Leahy *et al.* 2003).

Figure 1.9: Alignment of α-subunit of SDIMO hydroxylases (taken from Crombie and Murrell. 2014)



SDIMO enzymes are grouped based on their typical substrate (Figure 1.9), although most SDIMO enzymes can oxidise a broad range of substrates. Groups I and II are the toluene/phenol monooxygenases, which includes the *Xanthobacter* Py2 monooxygenase. The isoprene monooxygenase cloned and sequenced from *Rhodococcus* AD45 would be placed in group I based on its phylogeny, being most closely related to soluble diiron

centre toluene monooxygenases like that found in *Frankia* sp. EU1F (see Chapter 6). Group III consists of the soluble methane monooxygenases, while group V and VI SDIMOs have activity towards propane, and group IV towards alkenes.

1.6.1 SDIMOS in the Actinobacteria

Mycobacterium chubuense NBB4 is an example of a strain within the phylum Actinobacteria that possesses multiple monooxygenase enzyme systems, including soluble diiron centre monooxygenases such as propane monooxygenase (Coleman *et al.* 2010). These multiple, diverse monooxygenase enzyme systems allow this organism to grow on a wide range of substrates including short chain gaseous alkanes and alkenes, C_5-C_{10} alkanes, aromatics and alcohols, meaning *M. chubuense* NBB4 is hugely metabolically versatile and potentially of interest for biocatalysis and bioremediation.

1.6.2 Alkene monooxygenase of *Xanthobacter* Py2

Xanthobacter Py2 is a strain capable of aerobic growth on aliphatic alkenes. This bacterium contains an inducible alkene monooxygenase that catalyses the epoxidation of short chain alkenes in an O_2- and NADH dependent reaction (Small and Ensign. 2002). Strain Py2 was originally isolated on propene as a sole carbon and energy source. Its alkene monooxygenase catalyses the epoxidation of propene to form epoxypropane. The components of the alkene monooxygenase from *Xanthobacter* Py2 (designated Xamo) are homologous to aromatic monooxygenases such as toluene and benzene monooxygenases (Zhou *et al.* 1999). *Xanthobacter* Py2 can oxidise benzene, toluene, and phenol and can also grow on phenol.

Alkene monooxygenase enzymes often display a more restricted substrate range, however the alkene monooxygenase from PY2 can catalyse the epoxidation of C_2-C_6 aliphatic alkenes as well as several chlorinated alkenes which are known environmental pollutants and hazards to human health.

The alkene monooxygenase from *Xanthobacter* Py2 (Zhou *et al.* 1999) is a multicomponent enzyme consisting of an NADH reductase, a Rieske-type ferredoxin, an oxygenase which contains the active site for epoxidation, and a small protein of unknown function, but which may serve as a regulatory protein with a function analogous to that of component B from soluble methane monooxygenase (Green and Dalton. 1979).

1.7 Use of soluble diiron centre monooxygenases in bioremediation

Pollution, particularly with xenobiotics, is a massive problem in an industrialised society. Microbial enzymes can be used as a cost effective and environmentally-friendly method of degrading environmental pollutants. For example, the enzyme sMMO can epoxidate the pollutant trichloroethylene, which contaminates large amounts of soil and groundwater and is considered hazardous to human health (Gist and Berg.1995). Most soluble diiron centre monooxygenase enzyme systems appear to be induced in the presence of a particular substrate, meaning targeted proliferation of microbial populations is possible. Technologies for pollutant removal can often require high temperatures or use of further chemicals, such as base-catalyzed dechlorination (deMarini *et al.* 1992). The broad substrate range of microbial enzymes such as the soluble diiron centre monooxygenases makes them of potential interest in environmental bioremediation and also industry, however many of these enzymes are effective only under laboratory conditions and limited by bioavailability of substrates as well as temperature, pH and nutrient availability. However, bioengineering of microbial enzymes could yield novel enzymes with altered substrate specificities or improved kinetics. For this to happen, a broad understanding of the structure and function of monooxygenase enzymes is needed.

Sites contaminated by human activities are a rich source of novel microorganisms with activity towards pollutants such as aromatic compounds and toxic metals (Narancic *et al.* 2012). For example, several strains of chemoorganotrophic Gram-positive bacteria have been isolated from petrochemical contaminated river sediments, and shown to grow on a wide range of aromatic compounds in the presence of heavy metals, such as benzene, biphenyl and naphthalene. Several of these strains were members of the phylum Actinobacteria, including species of *Gordonia, Rhodococcus* and *Streptomyces.* Aromatic compounds are abundant environmental pollutants and display high recalcitrance in the environment, meaning microbial transformation of these compounds is crucial. These strains may be a rich source of novel oxygenase enzymes with potential applications in bioremedication and biocatalysis.

1.8 Microbial biodegradation of natural rubber

Natural rubber, produced by over 2,000 plant species and commercially obtained mainly from the rubber plant *Hevea brasiliensis,* is composed mainly of poly(cis-1,4-isoprene) (Rose and Steibüchel. 2005). 75% of the natural rubber produced commercially is used in

the production of rubber tyres. Bacteria that can grow on natural rubber as a sole carbon source form two distinct groups: clear-zone-forming and non-clear-zone forming. Clear zone forming strains include several members of the actinomycetes, which create halos when grown on latex overlay agar plates and do not require direct contact with the substrate, as they metabolize polyisoprene by secretion of enzymes (Linos *et al.* 2000). Members of the non-clear-zone forming group require direct contact with the substrate and do not form halos on latex plates. This group includes members of the *Corynebacterium-Nocardia-Mycobacterium* group, such as *Gordonia polyisoprenivorans* VH2, *Gordonia westfalica* Kb1 and *Mycobacterium fortuitum* NF4. Plasmid-free mutants of *G. westfalica* Kb1 could not grow on polyisoprene as a sole carbon source, indicating that the genes involved in natural rubber degradation were located on the 101-kbp megaplasmid in this strain (Linos *et al.* 2000).

1.9 Xenobiotic degradation in the genus *Gordonia*

Several members of the genus *Gordonia* have been isolated from various environments for their ability to transform and degrade environmental pollutants and xenobiotics (Arenskötter *et al.* 2004). They likely play an important environmental role in the bioremediation of these compounds as well as in industrial processes such as wastewater treatment (Bendinger *et al.* 1992). *Gordonia* species can degrade poorly accessible carbon sources such as *t* –butyl ether, methyl *t* –butyl ether, cyclic alkanes and polycyclic aromatic hydrocarbons (Drzyzga 2012). Other compounds that can be degraded or transformed include alkylpyridines, which are toxic environmental pollutants, *s*-triazine which is highly persistent in the environment, and biodesulfurization of fuels which may be an economical alternative to industrial biocatalytic sulfur removal from fossil fuels.

1.10 Stable isotope probing (SIP)

Stable isotope probing is a cultivation independent technique in microbial ecology that allows the identification of groups of microorganisms that actively degrade a substrate in a given environment. In this method, stable-isotope-labelled carbon (^{13}C) or nitrogen (^{15}N) sources are assimilated into microbial biomass of environmental samples. The ‘heavy’ labelled carbon or nitrogen are incorporated into biomolecules such as DNA (DNA-SIP) (Dunford and Neufeld. 2010), RNA (RNA-SIP) (Dumont *et al*. 2011), proteins (protein SIP)(Taubert *et al.* 2012) or phospholipid fatty acids (PLFA-SIP) (Webster *et al.* 2006*)*. Stable isotope probing can also, often in combination with high throughput sequencing

technologies, be used to track changes in microbial communities perturbed by a given substrate, as well as to track the flow of nutrients through an ecosystem by measuring the incorporation of labelled carbon or nitrogen into biomolecules (Dumont and Murrell. 2005)(Radajewski *et al.* 2000)(Pan *et al.* 2011)

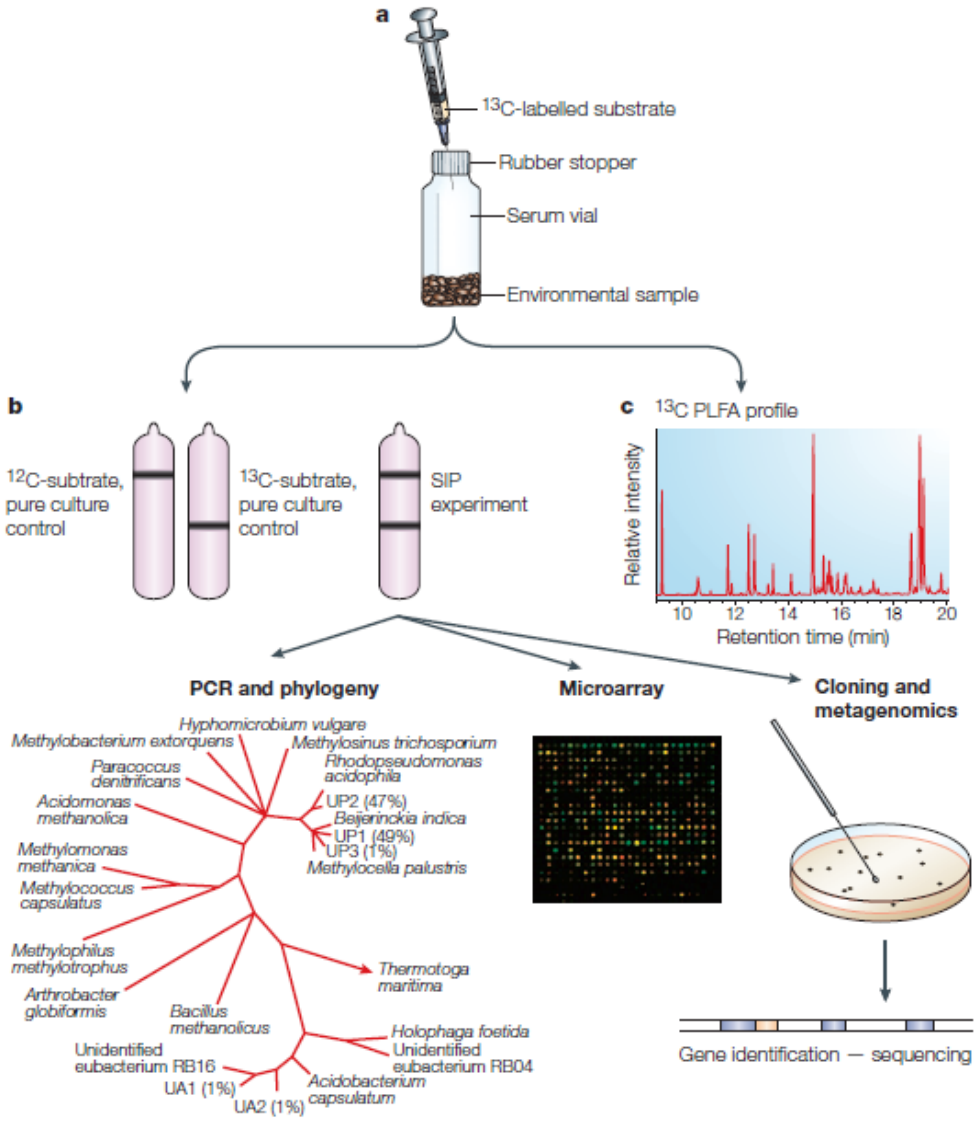
1.10.1 Phospholipid fatty acid (PLFA) and RNA SIP

There are advantages and disadvantages to each method of stable isotope probing. PLFA-SIP is the most sensitive method, requiring low incorporation of labelled substrate into fatty acids (Evershed *et al*. 2006), far lower than that of DNA-SIP. However, the phylogenetic information available is limited compared to DNA-SIP. RNA-SIP and Protein-SIP allow one to link better phylogeny to metabolic function by not only showing that genes are present in active organisms, but that they are actively transcribed and translated into functional proteins.

1.10.2 DNA Stable Isotope Probing

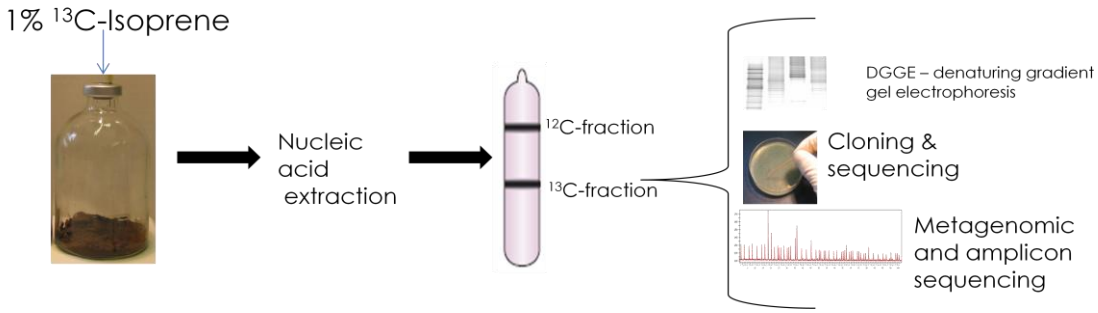
In DNA-SIP, stable isotope enriched DNA can be separated from bulk DNA by ultracentrifugation in a caesium chloride gradient (Murrell and Smith. 2009). DNA-SIP methods have been used to identify organisms that actively degrade a wide range of growth substrates. A summary of the DNA-SIP procedure is shown in Figure 1.10 which shows some of the potential downstream applications of isolating 'heavy' labelled DNA, such as amplification of 16S rRNA genes and other target genes by PCR. The 16S rRNA gene is an important taxonomic biomarker that can be used to assess the phylogeny and diversity of active labelled microorganisms within a sample. Figure 1.11 shows a schematic of a DNA-SIP experiment showing several downstream applications, including analysis of community profiles by denaturing gradient gel electrophoresis, cloning and sequencing of functional genes, and metagenomic analysis.

Figure 1.10: DNA-based stable isotope probing (DNA-SIP)



(From Dumont & Murrell. 2005)

Figure 1.11: A schematic of a ^{13}C-labelled isoprene incubation, DNA extraction and downstream analysis



1.11 Project hypotheses and aims

As described in this chapter, organisms containing soluble diiron centre monooxygenase enzymes systems play an important role in nutrient cycling in the environment. The cycling of isoprene is not fully understood as there is little understanding of the importance of microbes as a sink for this gas, and the subsequent effect on atmospheric chemistry and climate. This work aims to assess isoprene degradation in the marine environment. The hypothesis and aims for this study are therefore as follows:

Bacteria that can degrade isoprene are widespread in the marine environment. Those that degrade isoprene possess an inducible soluble diiron centre isoprene monooxygenase enzyme system.

- **Isolate and characterise bacteria capable of growth on isoprene as a sole carbon source.**
- **Investigate regulation of isoprene degradation.**
- **Design functional gene probes to assess diversity of isoprene degraders in marine environments.**
- **Identify active isoprene degraders in environmental samples using stable isotope probing.**

Chapter 2

Materials and Methods

Chapter 2 – Materials and Methods

2.1. Chemicals and reagents

Analytical grade reagents used in this research were from Fisher Scientific (Loughborough, UK), Melford Laboratories Ltd (Ipswich, UK) or Sigma-Aldrich Corporation (St Louis, USA). Molecular biology grade reagents were obtained from Bioline Reagents Ltd. (London, UK), Fermentas Molecular Biology Tools (Leon-Rot, Germany), Promega UK (Southampton, UK) and Roche Diagnostics Ltd. (Burgess Hill, UK). Acetylene and propane gases were obtained from BOC (Manchester, UK). Methane and propane used for cultures were 99.5% purity grade.

Custom oligonucleotide primers were obtained from Invitrogen (Paisley, UK)

Culture media and agar were purchased from Oxoid Ltd (Cambridge, UK). Difco^{TM} Marine Broth medium was purchased from BD Biosciences (Oxford, UK). Isoprene (99% purity) was obtained from Sigma-Aldrich (USA). Fully ^{13}C-labelled isoprene was manufactured and sent by Gregg Whited at Dupont, USA.

2.2. Bacterial strains

Table 2.1: List of organisms used in this study

Strain	Reference	Location
Gordonia polyisoprenivorans i37	Alvarez *et al.* 2009	Colne Estuary (Essex, UK)
Mycobacterium hodleri i29a2*	Alvarez *et al.* 2009	Colne Estuary (Essex, UK)
Rhodococcus erythropolis i24	Alvarez *et al.* 2009	Colne Estuary (Essex, UK)
Rhodococcus wratislaviensis i48	Alvarez *et al.* 2009	Colne Estuary (Essex, UK)
Micrococcus luteus i61b	Alvarez *et al.* 2009	Colne Estuary (Essex, UK)
Salinibacterium sp. i8b2	Alvarez *et al.* 2009	Colne Estuary (Essex, UK)
Loktanella i8bn	Alvarez *et al.* 2009	Colne Estuary (Essex, UK)
Rhodococcus globerulus i8a	Alvarez *et al.* 2009	Colne Estuary (Essex, UK)
Rhodococcus globerulus i8a1	Alvarez *et al.* 2009	Colne Estuary (Essex, UK)
Rhodococcus globerulus i8a2	Alvarez *et al.* 2009	Colne Estuary (Essex, UK)
Leifsonia sp. i49	Alvarez *et al.* 2009	Colne Estuary (Essex, UK)
Stappia sp. iL42	This study	L4 sampling station (Plymouth, UK)
Microbacterium sp. iP1	This study	Penarth coast (Wales)
Rhodococcus wratislaviensis i29b	Alvarez *et al.* 2009	Colne Estuary (Essex, UK)
Mycobacterium fortuitum i61b	Alvarez *et al.* 2009	Colne Estuary (Essex, UK)
Shinella sp. i39	Alvarez *et al.* 2009	Colne Estuary (Essex, UK)
Microbacterium sp. i39y	This study	Colne Estuary (Essex, UK)
Rhodococcus globerulus AD45	Van Hylckama Vlieg *et al.* 2000	Freshwater sediment
Xanthobacter autotrophicus Py2	Zhou *et al.* 1999	Freshwater
Rhodococcus wratislaviensis SC4	El Khawand and Murrell, Unpublished	Soil, Leamington Spa (UK)
Rhodococcus wratislaviensis LB1	El Khawand and Murrell, Unpublished	Leaf, University of Warwick (UK)
Sphingomonas B1	Zylstra & Kim. 1997	-
Pseudomonas PpF1	Finette *et al.* 1984	-
Pseudomonas ML2	Fong *et al.* 2000	-
Rhodococcus PD630	German collection of microorganisms and cell cultures DSMZ	-
Escherichia coli JM109	Chung et al. 1989	-
Escherichia coli TOP10	Invitrogen	-

Rhodococcus globerulus AD45 was provided by Professor Dick.B.Janssen (Groningen Biomolecular Sciences and Biotechnology nstitute, The Netherlands).

[2]*Xanthobacter* PY2 was provided by David Leak (University of Bath, UK).

Sphingomonas B1, *Pseudomonas* PpF1, *Pseudomonas* ML2 were kindly provided by Chris Allen (Queen's University, Belfast).

Rhodococcus wratislaviensis LB1 and *Rhodococcus wratislaviensis* SC4 were provided by Myriam El Khawand (University of East Anglia, UK).

Gordonia polyisoprenivorans, *Mycobacterium hodleri*, *Salinibacterium* sp., *Micrococcus luteus*, *Leifsonia* sp., *Rhodococcus globerulus*, *Rhodococcus wratislaviensis*, *Rhodococcus erythropolis*, *Microbacterium* sp., and *Mycobacterium fortuitum* were kindly provided by Terry McGenity (University of Essex, UK) (Alvarez *et al.* 2009).

- DNA from *Mycobacterium* NBB4 was from Andrew Holmes (University of Sydney, Australia).
- DNA from *Methylocella silvestris* BL2 was provided by Andrew Crombie (University of East Anglia, UK).
- DNA from *Rhodococcus jostii* strain 8 was provided by Jinderat Ekprasert (University of East Anglia, UK).

2.3 Culture media and growth of organisms

Cultivation and maintenance of bacterial strains

Solutions and growth media were prepared using Milli-Q water and sterilised by autoclaving at 15psi for 15 minutes at 121˚C. Solutions sensitive to autoclaving, for example vitamin solution and glucose solution, were sterilised using 0.2µM pore-size sterile filter units (Sartorius Minisart, Göttingen, Germany) and were added to cooled autoclaved media.

Marine Broth

Marine Broth was purchased in dehydrated form and prepared according to the manufacturers' instructions (BD Biosciences).

MAMS medium

MAMS medium was prepared according to Goodwin *et al.* (2001) and autoclaved. MAMS contains per litre: 20g NaCl, 10ml $(NH_4)_2SO_4$ solution (10g/100ml), 10ml $CaCl_2.2H_2O$ solution (2g/100ml), 10ml MS solution (per 100ml: 10g $MgSO_4.7H_2O$, 20mg $FeSO_4.7H_2O$, 0.2g $Na_2MoO_4.2H_2O$), 30µl $Na_2WO_4 \cdot 2H_2O$, 1ml SL10 trace element solution (Widdel *et al*., 1983), 0.5µl Na_3VO_4 + Na_2SeO_3 solution (10µg/ml each). Phosphate solution was prepared and autoclaved separately, containing (per 100ml): 3.6g KH_2PO_4, 23.4g K_2HPO_4 (anhydrous).

After autoclaving, filter sterilised vitamin solution (Kanagawa *et al.* 1982) was added at 1ml/litre after cooling, as well as phosphate solution at 10ml/litre.

Minimal medium was then supplemented with carbon sources as described below.

Growth of marine isoprene degrading strains on isoprene

Frozen glycerol stocks of isolates were obtained from Terry McGenity (University of Essex)(Table 2.1) and first streaked out on Marine Broth plates and subcultured to single colonies. Colonies were picked and used to inoculate 10ml Marine Broth cultures in 20ml plastic universal tubes, and grown at 30°C with 150rpm shaking until turbid (OD_{540} ~0.5). 5ml of these cultures were then used to inoculate 25ml of MAMS medium in 120ml serum vials. 1% (v/v) isoprene was added in gaseous form using a 100µl glass syringe. Cultures were incubated at 30°C with 150 rpm shaking. Larger cultures (50ml/100ml/300ml/500ml) were grown in conical Quickfit flasks (250ml/500ml/1 litre/2 litre respectively) at 30°C with 150rpm shaking, fitted with SubaSeal (Sigma-Aldrich) stoppers.

Growth of marine isoprene degrading strains on alternative carbon sources

50mM solutions of glucose, fructose, sucrose, pyruvate, succinate and acetate were prepared, filter sterilised/autoclaved and added to a final concentration of 5mM to 25ml MAMS medium in a 120ml serum vial inoculated with 5ml of Marine Broth grown cultures,. Cultures were incubated at 30°C with shaking at 150 rpm.

For gaseous alkane/alkene cultures, 25ml MAMS were inoculated with 5ml Marine Broth grown culture in 120ml serum vials, with gas substrates (propane, ethane, ethane, methane, butane, but-2-ene, but-1-ene) added from a gas bladder using a plastic syringe to 10% (v/v). Cultures were incubated at with 150rpm shaking at at 30°C.

Acetone, propanol, ethanol, methanol and toluene were added to a final concentration of 1mM in 25ml MAMS inoculated with 5ml grown *Gordonia* cells initially grown on propane as described above (OD_{540} 0.8) in a 120ml serum vial with 150rpm shaking at at 30°C.

Microscopy

Cells were routinely observed to assess purity and cell morphology at 1000 x magnification in phase-contrast under a Zeiss Axioskop 50 microscope, 130 VA Typ B,

and documented using the AxioCam camera system and Axiovison Rel 4.8 software (all supplied by Carl Zeiss Ltd, Cambridge UK).

Maintenance of isoprene degrading strains

Strains were maintained in 25ml MAMS with 0.5% added isoprene, and on Marine Broth agar plates.

Growth of isoprene degrading strains at different temperatures/salinities

Gordonia, Mycobacterium, Microbacterium and *Rhodococcus* strains were grown in 120ml serum vials, with 25ml MAMS medium + 1% (v/v) isoprene at 4°C, 18°C, 24°C, 30°C and 37°C, with OD_{540} measurements taken by extracting 0.8ml of sample using a plastic syringe, and measuring in a spectrophotometer in a plastic cuvette. Readings were taken at 1, 3, 5, 7, 12, 14 and 21 days to assess growth.

These strains were simultaneously grown in 25ml MAMS medium + 1% isoprene, MAMS medium modified to contain 0%, 0.1%, 0.2%, 0.5%, 0.8%, 1%, 1.5%, 2%, 3%, 4% and 5% NaCl. Cultures were incubated at 30°C with shaking. OD_{540} measurements were taken at 1, 3, 5, 7, 12, 14 and 21 days to assess growth. Strains were also tested on MAMS +2% agar plates by adding 1.5% agar to MAMS medium with salt concentrations as described above and autoclaving. Plates were incubated at 30°C and growth assessed visually after 5 days.

Antibiotics

Antibiotics were filter sterilized and added aseptically to cooled media, the final concentrations were as follows except where otherwise indicated: ampicillin 100µg ml^{-1}, kanamycin 25 µg ml^{-1}, gentamycin 5µg ml^{-1}, streptomycin 5µg ml^{-1}.

Antibiotic sensitivity of selected isoprene degrading strains

Gordonia polyisoprenivorans and *Mycobacterium hodleri* strains were tested for sensitivity to selected antibiotics. Marine Broth plates were prepared and supplemented with kanamycin, gentamycin and streptomycin at the final concentrations: 5µg ml^{-1}, 10µg ml^{-1}, 20µg ml^{-1}, 30µg ml^{-1}, 50µg ml^{-1}, 60µg ml^{-1}, 100µg ml^{-1}.

Growth of non-isoprene degrading control organisms

Pseudomonas PpF1 (Finette *et al.* 1984)(Table 2.1) was revived in nutrient broth and grown on M9 minimal salts medium (Sigma Aldrich) +1.5% Agar No.1 plates + 50µl toluene (added to cotton wool and placed in Gilson tip in the lid).

Pseudomonas ML2 (Fong *et al.* 2000)(Table 2.1) was revived in nutrient broth, checked for growth on benzene on minimal salts agar plates + 50µl benzene (cotton wool as above) and maintained on minimal salts medium agar plates + succinate.

Sphingomonas yanoikuyae B1 (Zylstra and Kim. 1997)(Table 2.1) was revived in nutrient broth, purified then grown on minimal salts medium agar plates with 0.05% added yeast extract and 100mg biphenyl.

Xanthobacter autotrophicus PY2 (Zhou *et al.* 1999)(Table 2.1) was streaked onto R2A (Oxoid) agar plates, grown to single colony and examined by microscopy to assess purity. Single colonies were used to inoculate 30ml NMS medium (Bowman and Sayler, 1994) with 5µM added isoprene in triplicate 120ml serum vials to test for growth on isoprene.

Other media

LB medium (Sambrook and Russell, 2001) contained (per litre): 10 g tryptone, 5 g yeast extract and 10 g NaCl.

SOC medium for transformations was prepared by adding 20 mM filter sterilised glucose solution to autoclaved SOB medium. SOB medium contained (per litre): 5g yeast extract, 20g tryptone, 0.5g NaCl, 2.5 mM KCl (10 ml of a 250 mM solution), adjusted to pH 7 prior to autoclaving, and 10 mM $MgCl_2$ (5 ml of a 2M solution, filter-sterilised, added after autoclaving).

R2A agar (CM0906) and Nutrient Broth (CM0001) was purchased in the form of dehydrated culture medium from Oxoid and prepared according to the manufacturer's instructions.

Monitoring isoprene depletion using gas chromatography (GC)

Isoprene in flasks was measured using the Agilent 7890B gas chromatograph fitted with a Poropak Q column (6ft by 1/8"). Run settings were as follows: Injector 250˚C, Oven 175˚C, FID (Flame Ionisation Detector) 300˚C. Helium was used as a carrier gas.

Retention time for isoprene is approximately 4 minutes using these settings. Samples were compared to a series of isoprene standards of 0.1%, 0.2%, 0.5% and 1% (v/v) in 120ml serum vials.

Effect of isoprene concentration on growth of *Escherichia coli*

E. coli cells were grown in duplicate 10ml of LB medium in 120ml serum vials with isoprene added at 0.1%, 0.2%, 0.5%, 1.0%, 1.5% and 2% (v/v) to test for potential toxic effects of isoprene on growth of *E.coli* in LB medium.

Isoprene toxicity for selected isoprene degrading strains

Gordonia polyisoprenivorans i37, *Mycobacterium hodleri* i29a2*, *Microbacterium* i49, *Stappia* iP2, *Rhodococcus erythropolis* i24, *Rhodococcus wratislaviensis* i48 and *Salinibacterium* sp. i8b2 were grown in 20ml MAMS medium in duplicate 120ml serum vials with isoprene added at 0.1%, 0.2%, 0.5%, 0.8%, 1.0%, 1.2% and 1.5% (v/v) to test for toxic effects of isoprene during growth on isoprene as a carbon source.

2.4 Acetylene inhibition assays

Acetylene inhibition of isoprene monooxygenase in *Gordonia polyisoprenivorans*

Gordonia polyisoprenivorans cells were grown in a 250ml Quickfit flask in 50ml MAMS medium +1% (v/v) isoprene to an OD_{540} of 0.9-1.0. 10ml of cells were transferred to 40ml MAMS medium (minus a carbon source) and starved for 45 min, before adding acetylene gas (C_2H_2) using a syringe to a final concentration of 100µM (done in duplicate vials), 100µM acetylene + 1% (v/v) isoprene in duplicate vials, and 1% (v/v) added isoprene alone in duplicate vials as a control. Acetylene can act as an irreversible competitive inhibitor of methane monooxygenase and therefore possibly for isoprene monooxygenase (Prior and Dalton, 2006). Vials containing acetylene + isoprene were incubated at 30°C with shaking to test for growth of *Gordonia polyisoprenivorans* on isoprene in the presence of acetylene. Vials containing acetylene only were incubated at room temperature for 1 hour to allow possible binding of acetylene to isoprene monooxygenase. Cells were then harvested, resuspended in 0.1M phosphate buffer (pH 7.4) and used for oxygen electrode assays to test for oxidation of isoprene (see Section 2.19) in comparison to cells incubated with isoprene only.

2.5 DNA extraction, processing and storage

DNA extraction using a commercial DNA Soil Kit

DNA was extracted from environmental samples and isolates for routine PCR assays using the FastSpin DNA soil kit (MP Biomedicals, Santa Ana, USA) according to the manufacturer's instructions.

DNA purification

DNA was purified using the Nucleospin column kit (Macherey-Nagel, Düren, Germany) according to the manufacturers' instructions.

Quantification of DNA/RNA

DNA and RNA concentrations and purity were estimated by agarose gel electrophoresis (Section 2.8) and comparison to a known quantity of 1kb ladder (Fermentas) or using a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, USA).

2.6 Genomic DNA extraction for genome sequencing

Genomic DNA was prepared from *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2* using the Marmur method (Marmur *et al.* 1961) with 2% SDS (w/v) to achieve more efficient lysis of cells. This DNA was sent to Professor Dan Arp at Oregon State University for *de novo* sequencing using a 454 pyrosequencing platform.

2.7 Genome sequencing and analysis

The genome of *Gordonia polyisoprenivorans* i37 was sequenced at the University of Oregon, USA. The genomes of *Mycobacterium* and *Rhodococcus* were sequenced at the University of Warwick Genomics Facility, UK. DNA was quanitifed by the Genomics Facility using a Qubit fluorometer (Life Technologies Corporation). Libraries were prepared using the Illumina TruSeq DNA kit (Illumina) and sequenced using a run type of 70bp, paired-end (PE) reads. Raw reads were assembled into contigs using CLC Bio Genomics Workbench (default settings) and automatically annotated using the RAST server (Aziz *et al.* 2008).

2.8 Agarose gel electrophoresis

Extracted DNA and PCR gene products were observed and analysed by agarose gel electrophoresis. Samples were run at 90V for 30 min on a 1% (w/v) agarose gel containing

0.5 µg ml^{-1} ethidium bromide in 1 x TBE buffer. GeneRuler^{TM} 1kb DNA (Fermentas) ladder was used as a marker for size comparison of DNA fragments.

2.9 Polymerase chain reaction (PCR)

All PCR reactions were in 50µl reaction volume using a Tetrad (Bio-Rad) thermal cycler, using DreamTaq (Fermentas) or premixed Taq mixture (Labtech). PCR reactions typically contained the following reaction mixture and cycling conditions (unless specified elsewhere):

16S rRNA (DreamTaq) (Fermentas): 1 × buffer, 1.5 mM $MgCl_2$, 0.2mM dNTPs (each), 0.4µM forward and reverse primer, 2.5 units *Taq* DNA polymerase, 0.07% (w/v) BSA.

16S rRNA (PREMIX): 1 × labTAQ Mix (including labTAQ DNA polymerase, 400nM forward primer, 400nM reverse primer, 3mM $MgCl_2$, 0.07% (w/v) BSA.

16S colony PCR: Addition of 2% (v/v) DMSO to 16S rRNA premix reaction tube

Cycling conditions: denaturation 95˚C (5 minutes), 25-35 cycles of denaturation 95˚C for 30s, annealing (primer dependent temperature) for 30s, elongation at 72˚C for 1 min/kb expected product. Colony PCR increased initial denaturation step to 10 minutes.

Table 2.2: List of PCR primers used in this study

Designation	References	Primer sequences (5'-3')	Target gene	Amplicon length (bp)	Annealing temperature (°C)	Cycles
341-GCf	Muyzer *et al.* (1993)	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG GCACGGGGGGCCTACGGAGGCAGCAG	Bacterial 16S rRNA gene	~550	55	30-35
907r	Lane *et al*. (1985)	CCGTCAATTCMTTTRAGTTT				
27f	Lane *et al.* (1990)	AGAGTTTGATC**M**TGGCTCAG	Bacterial 16S rRNA gene	~1460	55	30-35
1492r	Lane *et al.* (1990)	TACGGYTACCTTGTTAGGACTT				
M13f	Invitrogen	GTAAAACGACGGCCAG	Insert-flanking regions of pGEM®-T Easy Vector	Insert size + ~200 bp	55	30
M13r	Invitrogen	CAGGAAACAGCTATGAC				
SP6	Promega	TATTTAGGTGACACTATAG	Insert-flanking regions of pGEM®-T Easy Vector	-	-	-
T7	Promega	TAATACGACTCACTATAGGG				
F876	Invitrogen	GCTGGAGTGGATCGTTAACCA	*isoA* qRT-PCR product	70bp	-	-
R946	Invitrogen	CCCAGTACCACGGCTTCTTC			-	-
F120	Invitrogen	GCAGATCATGCGGTCGTACTT	*prmA* qRT-PCR product	70bp	-	-
R222	Invitrogen	CTGGACCTGACGGAACATGTT				

2.10 Cloning of PCR products

Cloning of *isoA* PCR amplified sequences of functional gene products was carried out using the Promega pGEM®-T Easy Vector system according to the manufacturers instructions: PCR products were ligated into the pGEM®-T Easy Vector T4 DNA ligase (Promega) according to the manufacturers' instructions in a final volume of 10µl. and transformed either into chemically competent *Escherichia coli* JM109 cells or TOP10 cells. Transformants were streaked on LB agar plates supplemented with ampicillin (100µg ml^{-1}) IPTG (50 µl of a 0.2 M solution) and X-Gal (25 µl of a 40 mg / ml solution) for white / blue selection. No IPTG was added to the plates when using TOP10 cells due to the absence of a *lac* repressor in these cells. White colonies were picked and re-amplified by colony PCR using primer set M13f / M13r. PCR products were analysed on a 1% (w/v) agarose gel and products of the correct length were submitted for sequencing analysis (Section 2.12) using the M13f and M13r primers.

2.11 RFLP analysis of cloned PCR products from enrichment samples

isoA gene clones were reamplified by PCR, purified and digested using *Hin*cII and *Rsa*I restriction enzymes (Fermentas) according to the manufacturers' recommendations, to ensure sequencing of a diverse range of clone sequences. Restriction enzymes were chosen using the online tool NEBCUTTER (Vincze *et al*. 2003). Digests were analysed by running on a 2% agarose gel to observe digestion profile using the Bio-Rad Gel Doc XR gel documentation system.

2.12 DNA sequencing and phylogenetic analysis

PCR products were sequenced using the Sanger method on a 3730A automated sequencing system (PE Applied Biosystems). 16S rRNA gene sequences were analysed with the Basic Local Alignment Search Tool (BLAST) on the NCBI GenBank database (Altschul *et al*., 1990).

Neighbour-joining nucleotide and amino acid phylogenetic trees were created for partial 16S rRNA genes and cloned *isoA* gene products using the MEGA5 program (Tamura *et al*., 2013) with bootstrap values of 500.

2.13 Extraction of protein and protein purification

Strains were grown in 400ml MAMS medium in 2 litre Kwikfit flasks, containing either glucose to 5mM, isoprene to 1% (v/v) or propane to 10% (v/v), at 30°C with shaking. Cells were harvested at OD_{540} 0.8-1.0 by centrifuging at 12,000g for 30 min, resuspending in 100ml MAMS medium, recentrifuging for 25 min at 10,000g and cells were then resuspended in 1ml PIPES PIPES (piperazine-N-N'-bis(2-ethanesulfonic acid) buffer. Resuspended cell pellets were then boiled for 15 min in buffer containing 2% (w/v) SDS followed by centrifugation at 14,000g for 5 min to pellet cellular debris. Cell extract was then prepared as described in Section 2.14

2.14 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Preparation of cell extract

Harvested cell pellets were resuspended in 1ml of 50mM buffer pH 7.0. Cells were broken by four passages through a French pressure cell (American Instrument Company, Silver Spring, MD) at 110mPa on ice. Cell debris was removed by centrifugation (1,0000g, 15 mins, 4˚C), and the supernatant removed as cell-free extract.

For *Gordonia polyisoprenivorans,* protein was prepared by lysing the cells using the French press at 137mPa for three passages, and quantified using the BioRad assay.

Protein quantification

Total protein in extract was estimated using the Bio-Rad Protein Assay (Bio-Rad laboratories Inc., Hercules, CA, USA) according to manufacturers' instructions by comparison to BSA (bovine serum albumin) standards.

Polypeptides from cell free extracts were separated using a 4% (w/v) stacking gel and a 12.5% polyacrylamide resolving gel in the X-cell II Mini-Cell apparatus (Novex). Gels were prepared as follows using 40% (w/v) acrylamide/bis (37.5:1) (Amresco, Solon, OH, USA):

	4% Stacking Gel	12.5% Resolving Gel
Acrylamide/bis	0.5ml	3.125ml
Tris 0.5M pH 6.8	1.25ml	-
Tris 3.0M pH 8.8	-	1.25ml
10% (v/v) SDS	50µl	0.1ml
10% (v/v) Ammonium persulfate (APS)	25µl	75µl
N,N,N',N'-tetramethyl-ethane-1,2-diamine (TEMED)	5µl	5µl
H_2O	3.17ml	5.41ml

Cell-free extracts were mixed with SDS-PAGE sample buffer (63mM Tris-HCl (pH6.8), 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 0.00125% (w/v) bromophenol blue) in a ratio of 4:1. 40µg of protein samples were loaded onto a 12.5% (w/v) polyacrylamide gel and electrophoresis was carried out at 90V for 30 min through the stacking gel followed by 160V for 60 min through the resolving gel. Running buffer contained 72 gl^{-1} glycine, 15 gl^{-1} Tris base, and 5 gl^{-1} SDS. Polypeptide mass was determined by comparison to a PageRuler Plus prestained protein ladder (Fermentas).

Gels were stained using Coomassie blue (50% methanol, 10% acetic acid, 0.05g Coomassie blue, dH_2O to 500ml) and destained using a destain solution of 50% methanol/10% acetic acid//40% dH_2O.

Proteomic analysis

Bands of interest were cut from the gel using a sterile razor blade, suspended in 200µl deionized water and sent to the Biological Mass Spectrometry and Proteomics Group facility (University of Warwick) for sequencing by tryptic digest and nanoLC-ESI-MS/MS (Yang *et al.* 2007). Detected peptides were identified by comparison to a database of *Rhodococcus* AD45 polypeptides obtained from Genbank.

2.15 RNA extraction and storage

For RNA extraction, all equipment including glassware, water and solutions were treated with a 0.1% v/v solution diethylpyrocarbonate (DEPC) by shaking overnight at 37 °C prior to autoclaving. All plasticware, tips etc was RNase-free. Total RNA was isolated from *Gordonia polyisoprenivorans* using the hot acid-phenol method (Gilbert et al. 2000). The quality of the RNA was checked by running on a 1% (w/v) TBE-agarose gel to observe banding patterns and smears. DNA was removed by two treatments using Qiagen RNase-

free DNase, each followed by purification using an RNeasy spin column (Quiagen, Crawley, UK) following the manufacturer's instructions. Removal of all traces of DNA was confirmed by the absence 16S rRNA gene PCR using 1 µl of RNA template and 35 PCR cycles. Absence of a 16S rRNA gene product confirmed removal of trace DNA. RNA was stored at -80℃.

2.16 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

cDNA was created using the RNA template by reverse transcription, performed using Superscript III (Invitrogen), according to the manufacturer's instructions using random hexamers. Between 50 ng and 1 µg of RNA was used for first strand cDNA synthesis with 200 ng random hexamers. Negative controls contained water in place of reverse transcriptase, controls with DNA template, and controls without template. Reverse transcription was carried out at 55 °C.

qRT-PCR assays were carried out to detect three target genes in *Gordonia polyisoprenivorans* i37: *isoA* (alpha subunit of isoprene monooxygenase), *prmA* (alpha subunit of propane monooxygenase) and *rpoB* (beta subunit of RNA polymerase, 'housekeeping' reference gene for normalization). Primers were designed using Primer Express software (Applied Biosystems). cDNA was prepared as described above from 50ml cultures of propane, isoprene and glucose-grown cells. Each qRT-PCR reaction were prepared in a final volume of 20 µl in a 96 well plate, comprising 2 µl of cDNA, 0.25 µM of forward and reverse primers, and 2 x Fast SYBR Green Master Mix (Applied Biosystems).. Each plate contained a dilution series of standards for each target gene. cDNA for standards was created using a 50/50 mix of cDNA from propane and isoprene-grown cells. 10µl of cDNA from propane-grown cells was mixed with 10µl of cDNA from isoprene-grown cells and diluted 1/10 in RNase free water. This was then diluted 5/45 in RNase free water 4 times to create a dilution series for each target.
Each 96-well plate acted as one biological replicate. *isoA*, *prmA* and *rpoB* primers were used at a final concentration of 250µM. Negative controls were set up in duplicate for each target with 2 µl of cDNA-synthesis reactions in which the reverse transcriptase was not added. cDNA from each growth condition was diluted 1/10.The setup of the 96 well plate was as follows (all in 20µl final volume):

- 5 × triplicate cDNA standards with *rpoB* primers (15 wells)
- 5 × triplicate cDNA standards with *isoA* primers (15 wells)
- 5 × triplicate cDNA standards with *prmA* primers (15 wells)
- cDNA from propane-grown cultures in triplicate with *rpoB* primers (3 wells)
- cDNA from propane-grown cultures in triplicate with *prmA* primers (3 wells)
- cDNA from propane-grown cultures in triplicate with *isoA* primers (3 wells)
- cDNA from glucose-grown cultures in triplicate with *rpoB* primers (3 wells)
- cDNA from glucose-grown cultures in triplicate with *prmA* primers (3 wells)
- cDNA from glucose-grown cultures in triplicate with *isoA* primers (3 wells)
- cDNA from isoprene-grown cultures in triplicate with *rpoB* primers (3 wells)
- cDNA from isoprene-grown cultures in triplicate with *prmA* primers (3 wells)
- cDNA from isoprene-grown cultures in triplicate with *isoA* primers (3 wells)
- Triplicate propane, isoprene and glucose negative control (no RNA transcriptase) with *isoA* primers (9 wells)
- Triplicate propane, isoprene and glucose negative control (no RNA transscriptase) with *rpoB* primers (9 wells)
- Duplicate propane, isoprene and glucose negative control (no RNA transcriptase) with *prmA* primers (6 wells)

The 96-well plates were briefly vortexed to ensure uniform mixing of samples, followed by brief centrifugation for 15min at 5000g. PCR cycling was carried out using an Applied Biosystems StepOne^{TM} Plus Real system using default settings for 2-step cycling: 95 °C, 20 s (polymerase activation), then 95 °C, 3 s; 60 °C, 30 s for 40 cycles, then 95° C, 15 s; then a melt curve 60° C to 95° C in 0.3° C increments (1 min each). StepOne software v 2.2.2 was used for data analysis

2.17 Oxygen electrode assays

Substrate-induced oxygen consumption was measured using a Clark oxygen electrode (Rank Brothers Ltd, Cambridge, UK) maintained at 25˚C using a circulating water bath (Churchill Co. Ltd, Perivale, UK) to prevent denaturing of enzymes during assays.

Gordonia and *Mycobacterium* cells were grown in 300ml MAMS medium supplemented with 5 mM glucose, 1% (v/v) isoprene, or 10% (v/v) propane. Cells were harvested and

resuspended in 0.1M phosphate buffer (pH 7.4) containing 1% NaCl, then starved for 30 min on ice. Substrate was added after 2 minutes to allow a stable rate of endogenous oxygen consumption to be established and measured. Oxygen consumption was calculated by subtracting the endogenous rate from the substrate-induced rate.

Cells were tested for oxidation of saturated aqueous solutions (prepared by bubbling a gas bladder of substrate through 10ml of dH_2O in a 120ml serum vial) of methane, ethane, propane, butane, but-1-ene, but-2-ene, ethane, propene, as well as 100mM solutions of glucose, acetate and succinate. For liquids, 10µM solutions of methanol, ethanol, propanol and isoprene were prepared in dH_2O. Propane and isoprene grown 50µl cells were added to 3ml phosphate buffer (pH 5.5) in the oxygen electrode chamber. For testing oxidation of substrates, oxygen consumption was tested by addition of 50µl-100µl of the substrate solution. Propane and isoprene-grown *Gordonia* cells were tested for oxidation of 100µl of the following substrates (0.1M): methyl acetate, acetone, acetol and propionate.

2.18 Environmental sampling

Several environmental samples were taken to be used in this study to test for isoprene degradation, enrichment and isolation of isoprene degrading microorganisms, probing with *isoA* functional gene probes and construction of *isoA* clone libraries. Samples were collected from Penarth coast (Wales), the L4 sampling station (Plymouth, UK), Hythe (Colne estuary, Essex, UK), Wivenhoe (Colne Estuary, Essex, UK), Stiffkey Salt Marsh (Norfolk, UK) and samples provided by the Centre for Environment, Fisheries and Aquaculture Science (CEFAS, UK). Futher information regarding these samples can be found in Chapter 3.

2.19 Enrichment and isolation of isoprene degrading microorganisms

100ml of marine and estuarine water samples were incubated with 0.5% (v/v) added isoprene. 5ml of MAMS medium were added to marine samples taken from L4 sampling station and Stiffkey salt marsh. Marine samples were incubated at room temperature without shaking. Estuarine samples containing sediment were incubated at 30°C with shaking. Flasks were assessed for turbidity by eye at 2 weeks and then further subcultured by inoculating 20ml of enrichment into 50ml of MAMS medium supplemented with 0.5% (v/v) isoprene, for 3 passages in total. At each subculture, 50µl of enrichment was plated onto duplicate Marine Broth and MAMS plates. Marine Broth plates were incubated at

room temperature and 30°C respectively. MAMS plates were incubated at 30°C in an atmosphere of 1% (v/v) isoprene. Single colonies were picked and restreaked onto R2A plates to purify. Single colonies were then restreaked onto Marine Broth plates and single colonies used to inoculate 20ml MAMS + 0.5% (v/v) isoprene in triplicate serum vials, to test for growth on isoprene and a sole carbon and energy source.

2.20 DNA Stable isotope probing (DNA-SIP) of estuarine samples

To identify active isoprene degraders in marine and estuarine environments, DNA-SIP incubations were set up using samples taken from two points along the Colne Estuary (Essex, UK): Hythe and Wivenhoe (see Chapter 7).

Hythe sample incubations

For Hythe samples, triplicate 120ml serum vials were set up containing 25ml of sample water, 0.5g of sample sediment, and 1ml of MAMS medium, with 1% (v/v) added ^{13}C-labelled isoprene (provided by Gregg Whited, Dumont, USA) as the sole carbon and energy source. In addition, duplicate serum vials were set up with 1% (v/v) added ^{12}C-labelled isoprene. Duplicate control vials contained 25ml of autoclaved sample water + 1% (v/v) added ^{12}C-labelled isoprene + 1ml MAMS. Vials were sealed with a butyl rubber cap and an aluminium crimp top. These samples were incubated at 25°C with 50rpm shaking in the dark. Isoprene depletion was measured using gas chromatography by comparison to gas standards of 0.1%, 0.5%, 1% and 1.5% isoprene to calculate the amount of isoprene consumed. Assuming water samples require 5 µmol ^{13}C per ml incorporation of label (Chen *et al.* 2010) and assuming 50% incorporation of label into DNA, time point 1 samples were sacrificed after approximately 50µmol of isoprene had been consumed to ensure sufficient ^{13}C-labelling of biomass. Time point 1 was sacrificed after 7 days incubation. Time points 2 and 3 were sacrificed after 14 and 21 days, respectively. Samples were centrifuged at 12,000g and sediment and cell pellets resuspended in 1ml PIPES buffer (100mM, pH 6.9). DNA was extracted from these pellets using the FastSpin DNA soil kit (MP Biomedicals, Santa Ana, USA).

Wivenhoe sample incubations

Samples taken from Wivenhoe along the Colne Estuary (Essex, UK) were used to set up a second DNA-SIP incubation (see Chapter 7 for location). In this experiment, 30ml of sample water + 1g of sample sediment were incubated with 0.2% (v/v) ^{13}C-labelled

isoprene as their sole carbon source in duplicate serum vials. Duplicate 120ml serum vials containing 30ml sample + 1g sediment were also incubated with 0.2% (v/v) unlabelled isoprene. Duplicate controls contained 30ml of autoclaved sample water + 1g autoclaved sediment incubated with 0.2% unlabelled isoprene. These samples were incubated at room temperature without shaking in the light and isoprene incorporation was measured using gas chromatography. T1 samples were sacrificed after approximately 30µmoles of isoprene had been consumed, at 12 days. Time points 2 and 3 were sacrificed at 15 and 18 days, respectively. Samples were centrifuged at 12,000g and sediment and cell pellets resuspended in PIPES buffer as before. DNA was extracted from these pellets using the FastSpin DNA soil kit.

Ultracentrifugation and fractionation of DNA

From each sample, approximately 3µg of total extracted DNA was added to caesium chloride (CsCl) solutions for isopycnic ultracentrifugation and gradient fractionation following the protocol described by Neufeld *et al.,* 2007 to separate ^{13}C-labelled DNA from unlabelled (^{12}C) DNA. Samples were fractionated into 12 fractions each of approximately 400µl and the DNA precipitated according to Neufeld *et al.*2007. The caesium chloride density gradient was quantified using a digital refractomer (Reichert AR2000, Reichert Analytical Instruments). Precipitated DNA was suspended in 30µl nuclease-free water (Ambion). DNA in these fractions was evaluated for quality by running on a 1% (w/v) agarose gel and quantified using a NanoDrop spectrophotometer.

2.21 Denaturing gradient gel electrophoresis (DGGE)

16S rRNA genes were amplified using the 341-GC(forward) and 907r PCR primer set as shown in Table 2.2. DGGE of bacterial 16S rRNA gene fragments was carried out using the DCode^{TM} Universal Mutation Detection System (Bio-Rad) according to the manufacturer's instructions. PCR products were loaded on a 1 mm thick vertical gel containing 8% (w/v) polyacrylamide (acrylamide-bisacrylamide, 37.5:1) in 1x Tris-acetate-EDTA (TAE) buffer (pH 8).. A linear gradient of 30 - 70% denaturant (with 100% denaturant corresponding to 7 M urea and 40% (v/v) de-ionised formamide) was used for the separation of 16S rRNA gene fragments. Electrophoresis was carried out for 16 hours at 75V. Gels were stained using 3 µl SYBR® Gold Nucleic Acid Gel Stain (Invitrogen) in 50 ml TAE buffer for 1 hour in the dark. Gels were rinsed with the same TAE buffer and

bands were viewed under the Bio-Rad Gel Doc XR gel documentation system using the Amber Filter 5206 (Bio-Rad).

2.22 Illumina sequencing of 16S rRNA and isoA amplicons from DNA-SIP fractions

isoA gene PCR was carried out as described in Chapter 6.

For amplicon sequencing, PCR products were generated for the 16S rRNA gene using the 27f/519r primer set, and for *isoA* using the isoAF/isoAR primer set. PCR products were generated from the light and heavy fractions of ^{12}C and ^{13}C for time point 1. Replicates were pooled. Approximately 400ng of PCR product was required for amplicon sequencing by MR DNA Molecular Research LP (Texas, USA). *isoA* products were sequences using the isoAF forward primer.

The Q25 sequence data derived from the sequencing process was processed using a proprietary analysis pipeline by MR DNA Molecular Research LP. Sequences were depleted of barcodes and primers then short sequences < 200bp removed, sequences with ambiguous base calls removed, and sequences with homopolymer runs exceeding 6bp removed. Sequences were then denoised and chimeras removed. Operational taxonomic units were defined after removal of singleton sequences, clustering at 3% divergence (97% similarity). OTUs were then taxonomically classified using BLASTn against a curated GreenGenes database (DeSantis *et al*. 2006) and compiled into each taxonomic level.

isoA sequences were analysed using QIIME (Quantitative Insights Into Microbial Ecology) software within Biolinux. Raw reads were split into separate libraries, assigned to OTUs and each representative OTU assigned to a taxonomy using BLASTn function within QIIME.

Chapter 3

Isolation and characterisation of marine isoprene degrading isolates

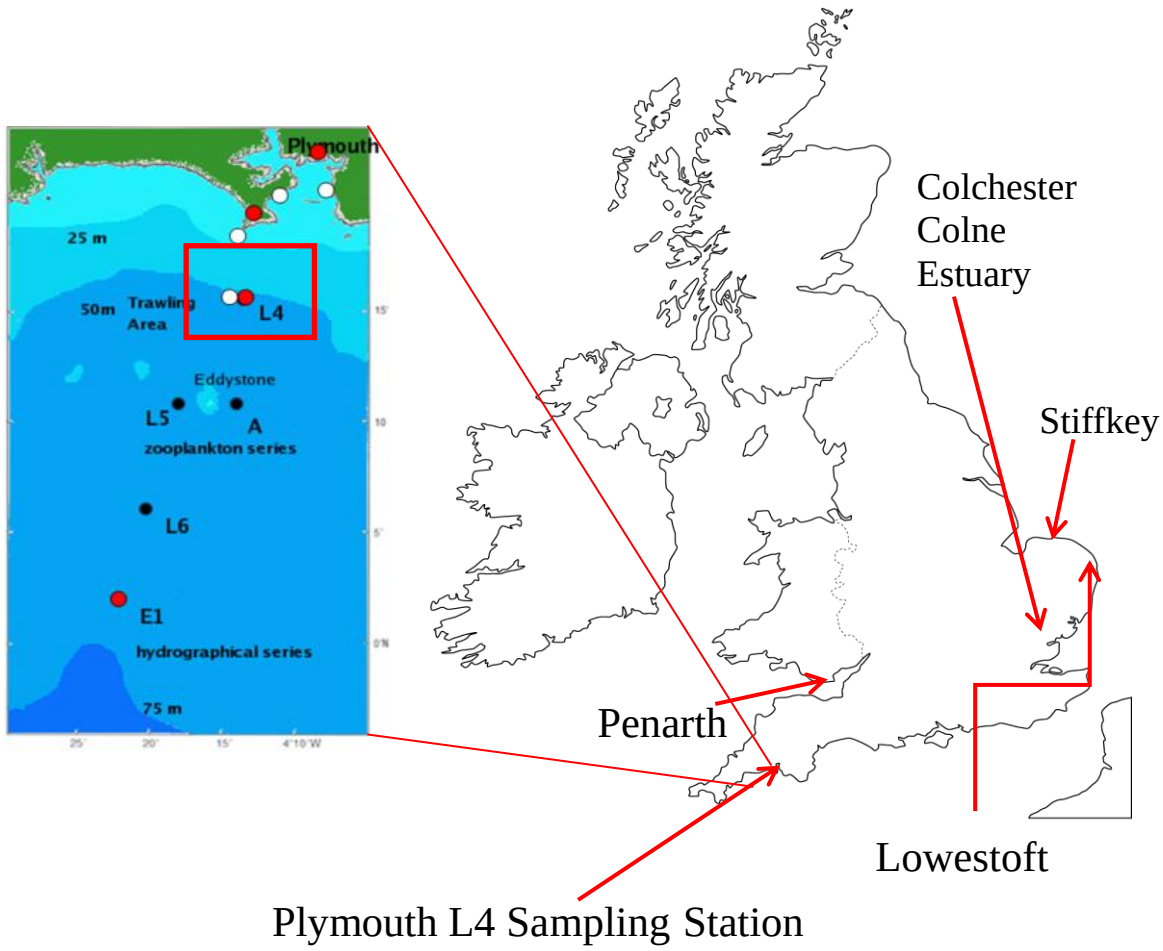
Chapter 3: Isolation and characterisation of marine isoprene degrading bacteria

3.1 Introduction

Previous research indicates that isoprene degrading bacteria representing several different genera are present in several different marine and estuarine samples (Alvarez *et al.* 2009). The primary aim of this work was to isolate and characterise bacteria from marine environments which were capable of using isoprene as their sole carbon and energy source. Previous work (Alvarez *et al.* 2009) showed that samples taken from the Colne Estuary, Essex, UK, could completely degrade isoprene at 0.01% and 0.001% (v/v). Several isoprene degrading strains were isolated in that study. The first aim of this study was to measure isoprene depletion in a range of marine, coastal and estuarine water samples, followed by isolation of novel isoprene degrading strains as well as characterisation of the physiology of previously isolated isoprene degraders based on their growth on isoprene and other carbon sources, temperature range, salt tolerance, isoprene tolerance, and polypeptide profiles when grown on alternative carbon sources.

3.2 Enrichment and isolation of isoprene degrading bacteria from marine, coastal and estuarine environments

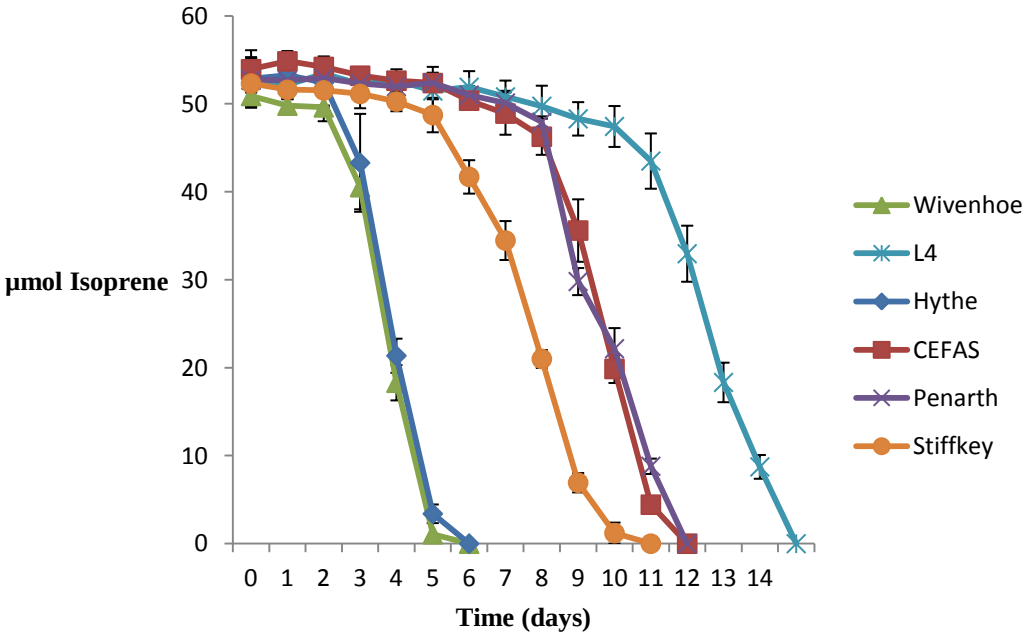
Figure 3.1: Map of United Kingdom showing environmental sampling sites



Four sampling sites were used in this study. Two sites along the Colne Estuary were chosen for collection of water and sediment samples. These were located at Hythe and Wivenhoe, Essex, which represent two different salinities in the estuary (practical salinity units are an order of magnitude greater at Wivenhoe than the Hythe). In previous studies (Alvarez *et al.* 2009), water samples collected from these sites showed complete degradation of isoprene, and pyrosequencing of these enriched samples showed that several genera, in particular *Mycobacterium* species, dominated these enrichments. In the Alvarez *et al.* (2009) study, several different isoprene degrading bacteria were isolated. Many of the isolates obtained were kindly provided by Terry McGenity, University of Essex for use in this work, as well as isoprene degraders that Alvarez *et al.* (2009) isolated

from Etang de Berre, France (see Table 3.1). Water from the L4 sampling station was provided by Jo Dixon of Plymouth Marine Laboratory, UK. This sampling station is located approximately 40 miles off the coast of Plymouth at approximately 35 psu salinity, and large amounts of data are available regarding climate, nutrient composition and conditions at the station. L4 water samples used in this study were collected during a summer algal bloom as large amounts of isoprene are thought to be produced by algae (Shaw et al. 2010) and therefore it is hypothesised that communities of isoprene degrading bacteria may be associated with these blooms. Samples were taken from Penarth pier in the Bristol Channel, which can be considered estuarine (~2% NaCl) rather than true marine samples. Water and sand samples were taken from the beach at Stiffkey salt marsh, as well as algal pools further inland at the Stiffkey salt marsh. Here vegetation cover is mainly *Spartina* grasses, which are known to produce large quantities of DMS (Pakulski & Kiene. 1992) and the salt concentration of water is high (~30ppt). Atlantic water samples were also provided by Martin Johnson of UEA/CEFAS. These samples consisted of water from the coast at Lowestoft, Norfolk which had been filtered through a sand filter, and so the exact effect of the filtration on the microbial community is not known.

Figure 3.2: Depletion of isoprene by environmental samples from a variety of marine, coastal and estuarine sites as measured by gas chromatography



Error bars represent 1 standard deviation. For all samples n=3

Samples from all sites were enriched with 0.5% (v/v) isoprene as described in Materials and Methods, section 2.21 and isoprene depletion was monitored using gas chromatography as described in Section 2.2. The rate of isoprene depletion across all sites is shown in Figure 3.2. Samples taken from the Colne Estuary degraded isoprene at the fastest rate (approximately 1.25 µmol isoprene/ml water/day) and with the shortest lag times irrespective of sampling location down the estuary. This is to be expected due to the sediment present in these samples increasing microbial biomass compared to water samples without added sediment. L4, CEFAS, Penarth and Stiffkey samples degraded isoprene at a similar rate; however open ocean and coastal samples had longer lag phases compared to Stiffkey samples, where water was taken from inland algal pools. Nutrient limitation is likely to be a key factor in isoprene degradation, as addition of minimal medium to the enrichment shortens the lag phase across all sample sites (data not shown). Starting biomass is also an important factor in determining the rate of isoprene degradation in a sample, as samples containing sediment degraded isoprene at faster rates and with shorter lag times than filtered water samples (Hythe and Wivenhoe samples on Figure 3.2).

Two new isolates were obtained from these isoprene enrichments, with the isolation procedure described in Materials and Methods section 2.21. Isolation of isoprene degraders was attempted for all enrichment samples, however no novel isoprene degrading strains were isolated from the Colne Estuary, Stiffkey salt marsh or CEFAS seawater samples. A single isolate was obtained from Penarth seawater enriched with isoprene: this was identified by 16S rRNA sequencing as belonging to the genus *Microbacterium*, and was closely related (98% nucleotide identity) to two other isoprene degrading *Microbacterium* species previously isolated from the Colne Estuary (Alvarez *et al.*2009). Several putative isolates were obtained from L4 isoprene enrichments, including a *Stappia* sp., *Marinobacter* sp. and *Vibrio* sp. These putative isolates were all transferred to liquid minimal medium with added isoprene (0.5% (v/v)) as a sole carbon source to test for growth on isoprene. Of these isolates, only the isolated *Microbacterium* and *Stappia* sp. grew in liquid in pure culture and could be considered true isoprene degrading isolates. Table 3.1 shows the isolates used in this study organised by phyla. Strains isolated from the Colne Estuary and Etang de Berre, France were kindly provided by Terry McGenity of the University of Essex and previously shown to degrade isoprene.

Table 3.1: Summary table of isoprene degrading isolates by genus and location

Isolate	Location	Phylogeny
Gordonia polyisoprenivorans i37	Colne Estuary	Actinomycetales, Corynebacterineae
Mycobacterium hodleri i29a2*	Colne Estuary	Actinomycetales, Corynebacterineae
Mycobacterium fortuitum i61a	Colne Estuary	Actinomycetales, Corynebacterineae
Rhodococcus globerulus i8a	Colne Estuary	Actinomycetales, Corynebacterineae
Rhodococcus globerulus i8a2	Colne Estuary	Actinomycetales, Corynebacterineae
Rhodococcus globerulus i29a2	Colne Estuary	Actinomycetales, Corynebacterineae
Rhodococcus globerulus i29a1	Colne Estuary	Actinomycetales, Corynebacterineae
Rhodococcus erythropolis i24	Colne Estuary	Actinomycetales, Corynebacterineae
Rhodococcus erythropolis i8a1	Colne Estuary	Actinomycetales, Corynebacterineae
Rhodococcus wratislaviensis i48	Etang de Berre	Actinomycetales, Corynebacterineae
Rhodococcus erythropolis i47	Colne Estuary	Actinomycetales, Corynebacterineae
Rhodococcus wratislaviensis i39w	Colne Estuary	Actinomycetales, Corynebacterineae
Rhodococcus wratislaviensis i29b	Colne Estuary	Actinomycetales, Corynebacterineae
Rhodococcus wratislaviensis i34	Colne Estuary	Actinomycetales, Corynebacterineae
Microbacterium sp. i39y	Colne Estuary	Actinomycetales, Microbacteriaceae
Microbacterium sp. P1*	Penarth	Actinomycetales, Microbacteriaceae
Microbacterium oxydans sp. i49	Colne Estuary	Actinomycetales, Microbacteriaceae
Salinibacterium sp. i8b2	Colne Estuary	Actinomycetales, Microbacteriaceae
Micrococcus luteus sp. i61b	Colne Estuary	Actinomycetales, Micrococcaceae
Loktanella sp. i8b1	Colne Estuary	Alphaproteobacteria, Rhodobacteraceae
Stappia sp. iL42*	L4	Alphaproteobacteria, *Rhodobacteraceae*

*Isolated in this study

The majority of isoprene degrading isolates fall within the phylum Actinobacteria, with most belonging to the suborder Corynebacterineae, a group of high-GC gram positive, acid fast bacteria. Several isolates belong to the Microbacteriaceae, which are common gram positive organisms in the environment. Two isolates are gram negative Alphaproteobacteria from the *Rhodobacteraceae*. Isolate iL42 is of the genus *Stappia*, which dominated 16S rRNA gene sequences in DNA from isoprene-enriched Indonesian water samples (Alvarez et al. 2009). The majority of isolates belong to the genus *Rhodococcus*, which dominated 16S rRNA gene sequences of DNA from isoprene-enriched water samples from Etang de Berre, France, and are closely related to the only previously confirmed isoprene degrader *Rhodococcus* AD45 (van Hycklama Vlieg *et al.*2000*)*.

Members of the genus *Rhodococcus* (Finnerty 1992) are aerobic, gram positive, acid-fast, mycolic acid-containing nocardioform actinomycetes and are common in nature. They are of significant interest for potential biotechnological applications due to their ability to degrade and transform a diverse range of substrates. Many species of *Rhodococcus* are able to metabolise gaseous and liquid hydrocarbons such as propane and butane (Shennan and Levi, 1987)

Members of the genus *Gordonia* (Arenskötter *et al.* 2004) are Gram-positive actinomycetes closly related to *Rhodococcus* spp. Like *Rhodococcus* spp., *Gordonia* species are common in the environment and can degrade substituted and nonsubstituted hydrocarbons, widespread toxic environmental pollutants and other xenobiotics (Arenskötter *et al.* 2004). In particular, *Gordonia* species are almost unique in their ability to degrade *cis*-1,4-polyisoprene, natural rubber (Linos *et al.* 2000) and almost all of the most potent polyisoprene degraders belong to this genus, including the closest relative to the isoprene degrading isolate *Gordonia polyisoprenivorans* i37 (Linos *et al.* 1999).

Members of the genus *Mycobacterium* are aerobic, acid-fast, rod shaped actinomycetes. (Hartmans *et al.* 2006) *Mycobacterium* species are divided into two groups based on growth rate: slow-growing and fast-growing. Fast-growing species are generally considered non-pathogenic and are common in the environment. Many species among the rapid growing *Mycobacterium* contain carotenoid pigments, giving colonies an orange colour. Several species of *Mycobacterium* have been isolated which can metabolise gaseous hydrocarbons, particularly the C2 compounds ethane and ethene (Hashimoto *et al.* 2000) (Brisbane & Ladd, 1972) and propane (Masuda *et al.* 2012) (Coleman & Perry, 1985). The *Mycobacterium* strain NBB4 can grow on C2 -C4 alkenes and C2 -C16 alkanes (Coleman *et al.* 2011) and contains multiple monooxygenase enzyme systems (Coleman *et al.* 2012).

Mycobacterium hodleri was first described by Kleespies *et al.* having been isolated from a fluoranthene-polluted soil (Kleespies *et al.* 1996). It has not previously been tested for growth on isoprene or other short-chain hydrocarbons such as propane.

Members of the genus *Microbacterium* are gram positive, yellow coryneform rods found in soil and other environments (Laffineur *et al.* 2003). Some strains have been previously shown to degrade polycyclic aromatic hydrocarbons (Sheng *et al.* 2009) and crude oil

(Schippers *et al.* 2005). *Microbacterium oxydans* is known to associate with marine macroalgae (Kim *et al.* 2013).

Little is known about the genus *Salinibacterium,* which consists of only two described species (Han *et al.* 2003) (Zhang *et al.* 2003). They are gram-positive, salt-tolerant, high %GC DNA Actinobacteria which form yellow colonies. None have so far been tested for their ability to grow on isoprene or other short chain alkanes and alkenes.

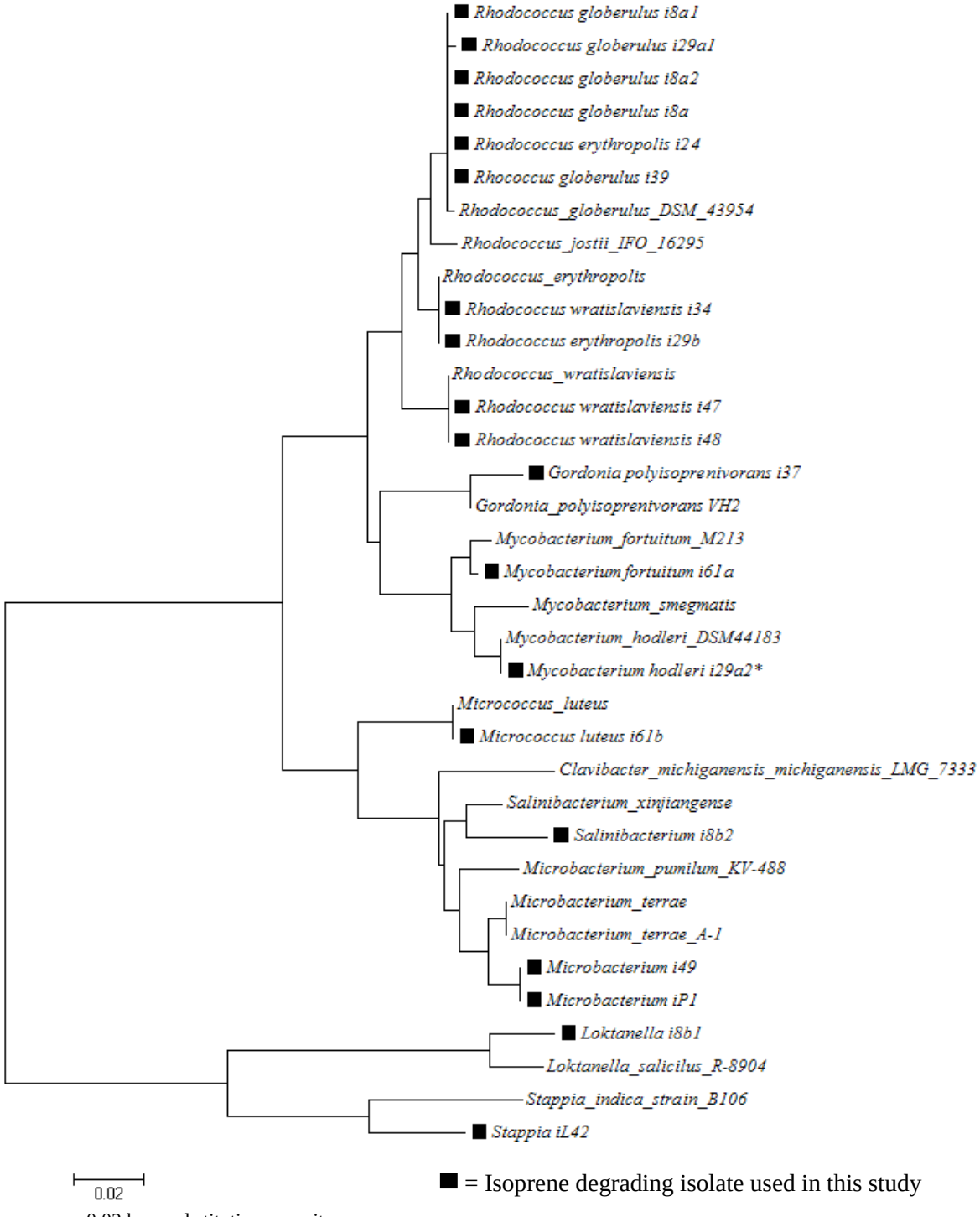
Members of the genus *Micrococcus* are high %GC DNA, tetrad forming Actinobacteria often associated with mammalian skin, however they have also been isolated from soil and marine samples, particularly in areas contaminated by humans. They are not considered to be highly significant in marine environments. *Micrococcus roseus* has been shown to degrade the side chains of cholesterol, an isoprenoid molecule. (Dogra & Khazi, 2001).

Members of the genus *Loktanella* are high GC, gram-negative chemoheterotrophs (Van Trappen *et al.* 2004). *Loktanella* and other members of the *Rhodobacteraceae* have been associated with sulphur cycling, and are noted in particular for their correlation with algal blooms (Gonzalez *et al.* 2000).

Members of the genus *Stappia* are known to play a key role in marine CO-cycling (Weber & King, 2007). They are found in a variety of marine environments such as warm temperate surface and permanently cold deep-sea waters, sediments, phytoplankton, macroalgae, and salt marshes (Boettecher *et al.* 2000)

These isolates represent several distinct genera within the Actinobacteria and *Rhodobacteraceae*. Many belong to genera known for their diverse metabolic capabilities and ability to degrade hydrocarbons, and several marine isolates are known to associate with algae. With the exception of the *Rhodococcus* isolates, all of these isolates represent the first described isoprene-degrading members of their respective genera (Vlieg *et al.* 2000). Figure 3.3 shows a neighbour joining 16S rRNA gene phylogenetic tree of the isolates used in this study.

Figure 3.3: 16S rRNA gene phylogeny of isolates



0.02 base substitutions per site

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.74286174 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al* 2011) and are in the units of the number of

base substitutions per site. The analysis involved 35 16S rRNA gene sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 489 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. The tree was completed using 16S rRNA sequences obtained from GenBank for closely related non-isoprene degrading Actinobacteria.

Table 3.2: Description of single colonies of isolates streaked on marine broth agar plates

Isolate	Colony
Gordonia polyisoprenivorans i37	Translucent peach/orange, mucoid, circular, raised, entire
Mycobacterium hodleri i29a2*	Opaque orange, smooth, flat, opaque, entire
Mycobacterium fortuitum i61a	Opaque white, smooth, circular, convex, entire
Rhodococcus globerulus i8a	Opaque peach/orange, rough, circular, convex, entire
Rhodococcus globerulus i8a2	Opague orange, smooth, circular, convex, entire
Rhodococcus globerulus i29a2	Opaque white/cream, smooth, irregular, pulvinate, entire
Rhodococcus globerulus i29a1	Translucent white, mucoid, irregular, convex
Rhodococcus erythropolis i24	Opaque cream/peach, smooth, irregular, convex, entire
Rhodococcus erythropolis i8a1	Opaque orange, rough, circular, convex, entire
Rhodococcus wratislaviensis i48	Opaque cream, smooth, raised, circular, entire
Rhodococcus erythropolis i47	Opaque cream, smooth, raised, circular, entire
Rhodococcus wratislaviensis i39w	Opaque cream/peach, smooth, pulvinate, irregular, erose
Rhodococcus wratislaviensis i29b	Opaque cream, mucoid, irregular, convex, entire
Rhodococcus wratislaviensis i34	Opaq ue cream, smooth, irregular, convex, undulate
Microbacterium sp. i39y	Bright yellow, smooth, circular, translucent, convex, entire
Microbacterium sp. P1	Bright yellow, smooth, circular, translucent, convex, entire
Leifsonia sp. i49	Yellow, smooth, circular, translucent, raised, entire
Micrococcus luteus i61b	Opaque pale yellow, smooth, circular, raised, entire
Loktanella sp. i8b1	Opaque cream to brown, smooth, circular, convex, entire
Stappia sp. L42	Opaque cream, smooth, circular, raised, entire
Salinibacterium sp. i8b2	Opaque yellow, smooth, circular, convex, undulate

Texture:

- Mucoid – thick, wet
- Smooth – glistening, smooth
- Rough – dry, matte

Shape:

- Circular
- Irregular

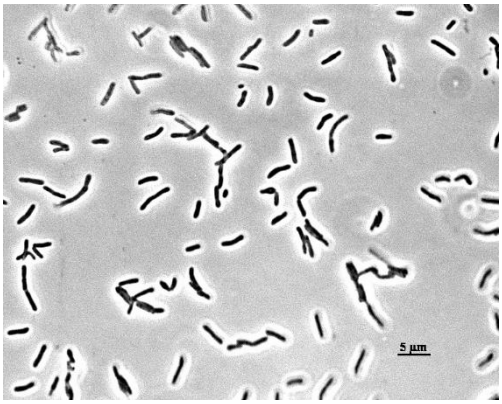
Elevation:

- Raised – slight elevation
- Convex – greater elevation, upward sloping
- Pulvinate – high elevation, sloping steeply

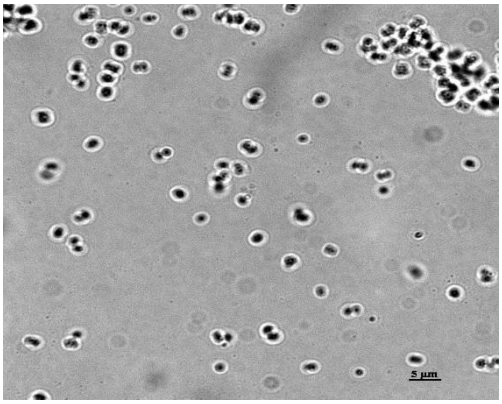
Margin:

- Entire – smooth, no projections
- Undulate – wavy
- Erose – small spiked projections

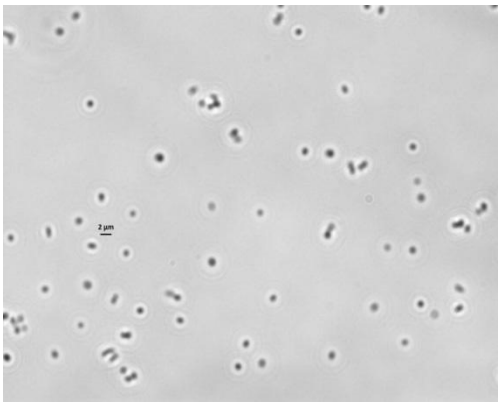
Figure 3.4: Isoprene degrading isolates as observed using light microscopy at 100×magnification



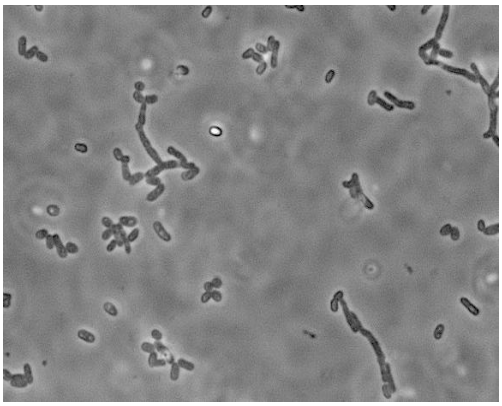
Mycobacterium hodleri i29a2*



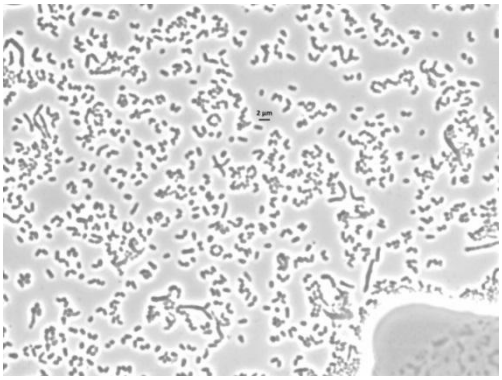
Micrococcus luteus i61b



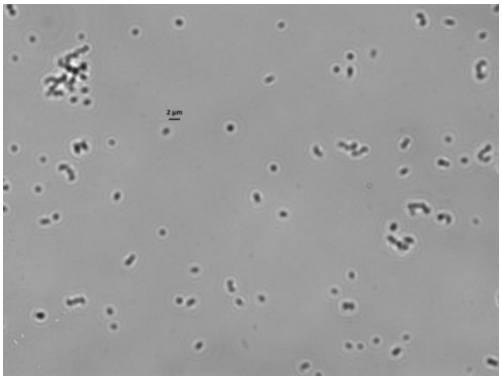
Rhodococcus wratislaviensis i47



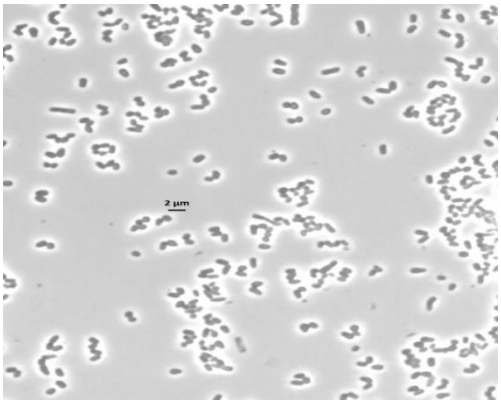
Rhodococcus wratislaviensis i48



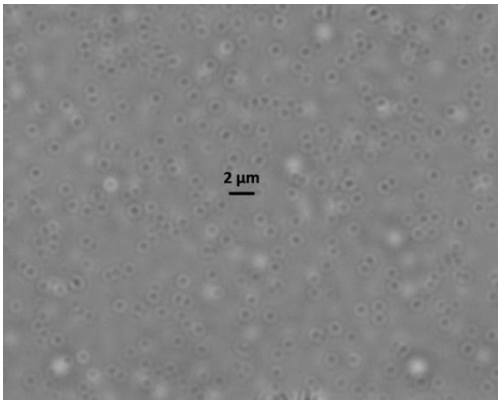
Rhodococcus erythropolis i24



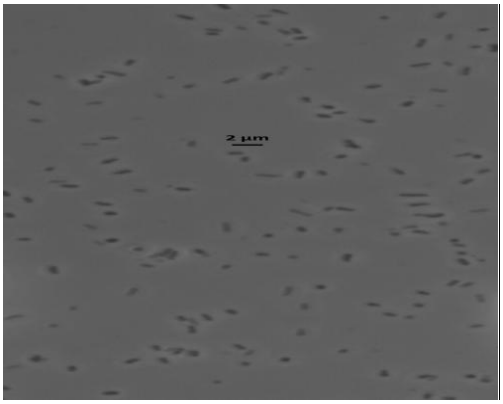
Rhodococcus erythropolis i29b



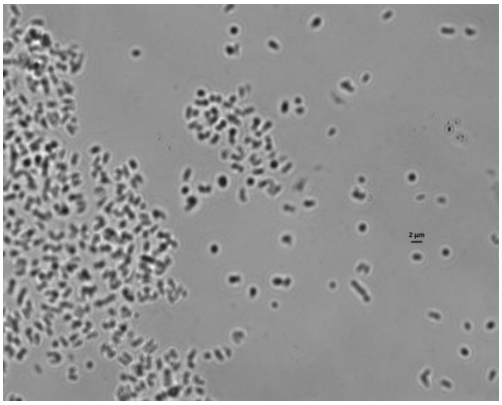
Rhodococcus erythropolis i34



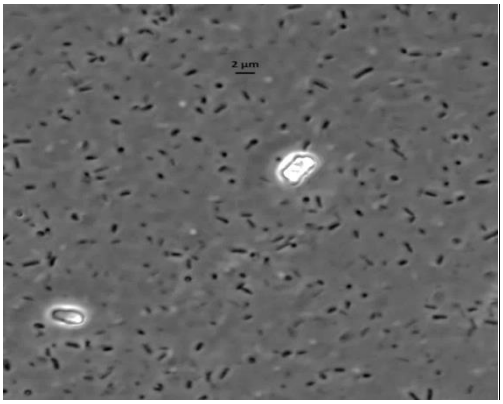
Loktanella sp. i8b1



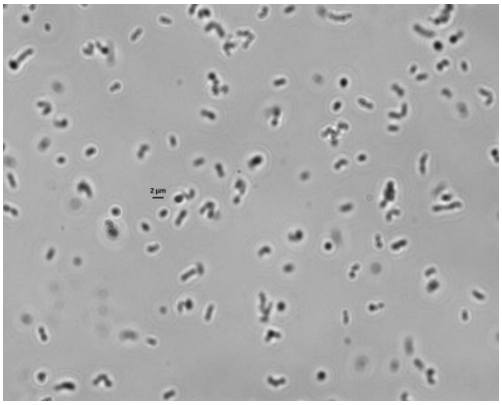
Microbacterium sp. iP1



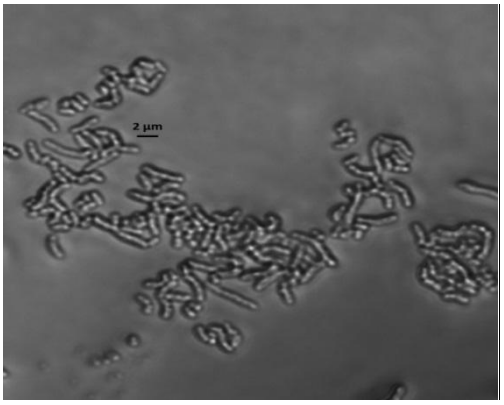
Rhodococcus globerulus i8a



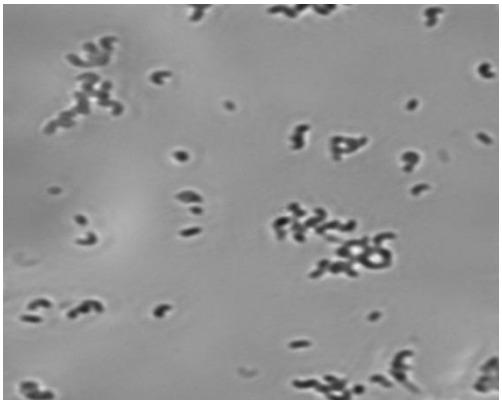
Microbacterium sp. i39w



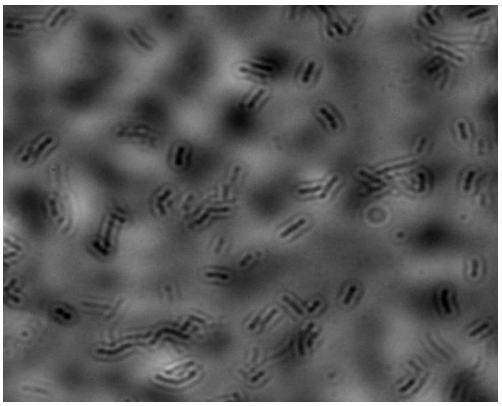
Rhocococcus globerulus i8a1



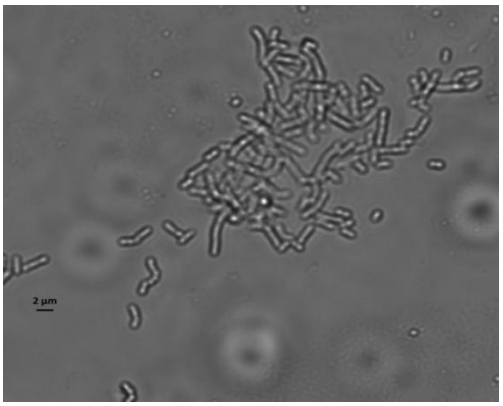
*Rhodococcus globerulus i*29a1



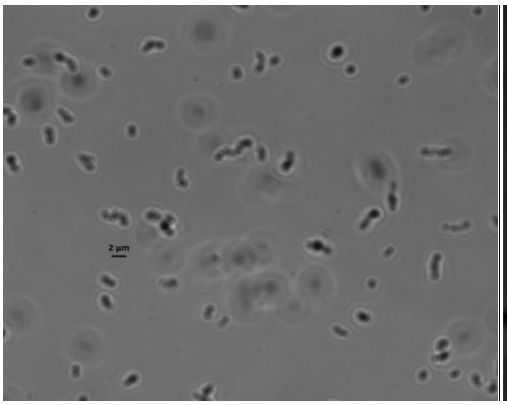
Gordonia polyisoprenivorans i37



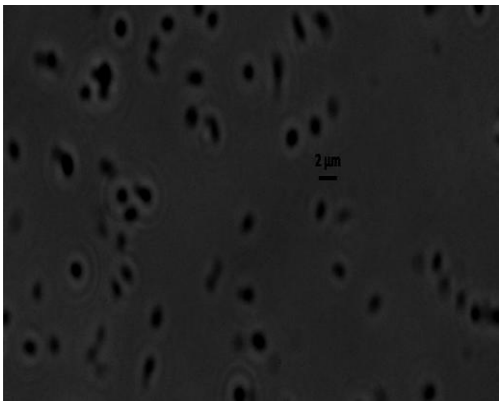
Salinibacterium sp. i8b2



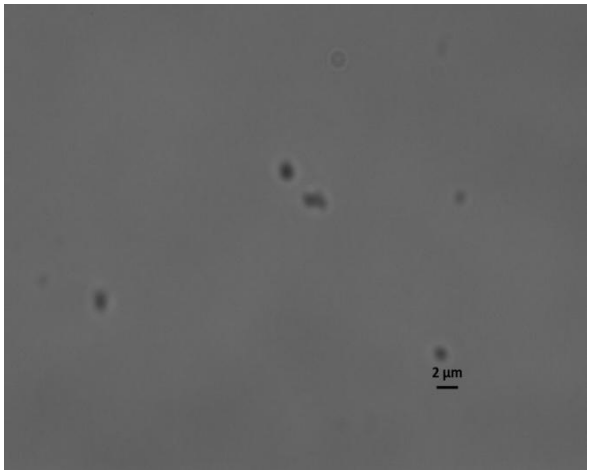
Rhodococcus globerulus i29a2



Microbacterium sp. i49

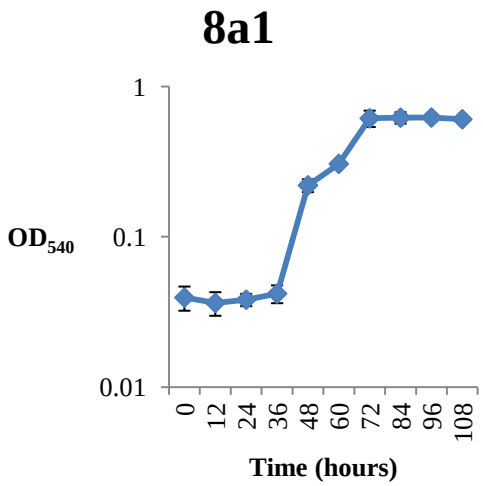


Stappia iL42



Mycobacterium fortuiutum i61a

Figure 3.5: Growth curves of isolates on isoprene in liquid culture



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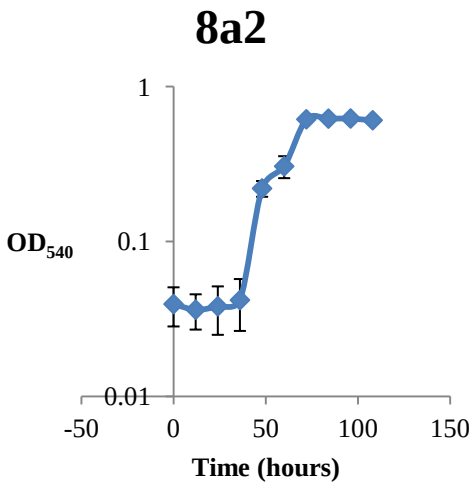
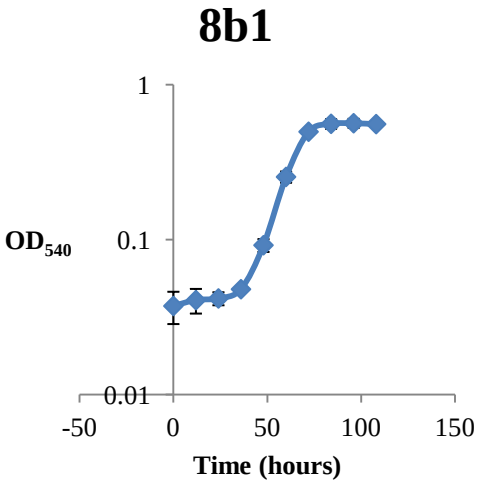
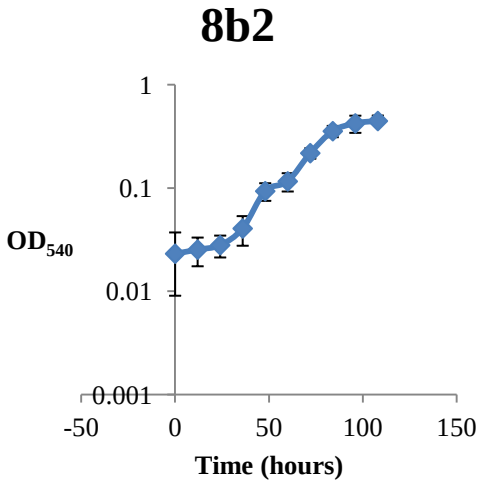


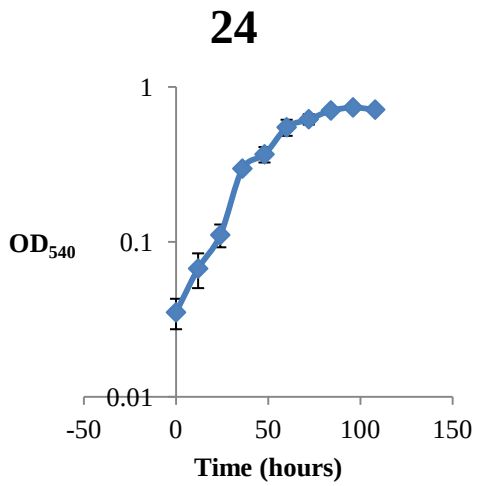
B.

C.

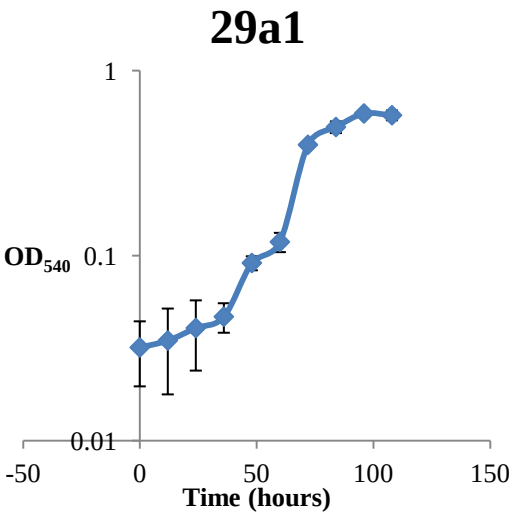




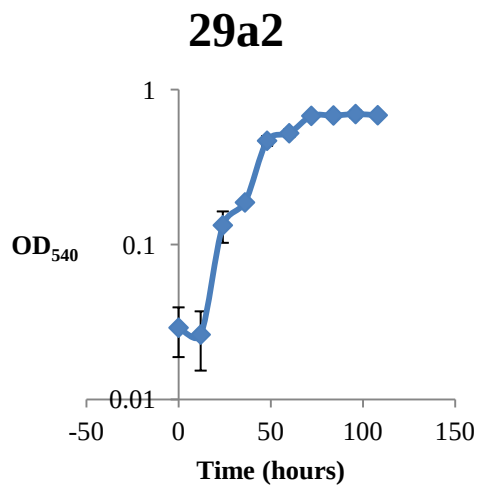
D.



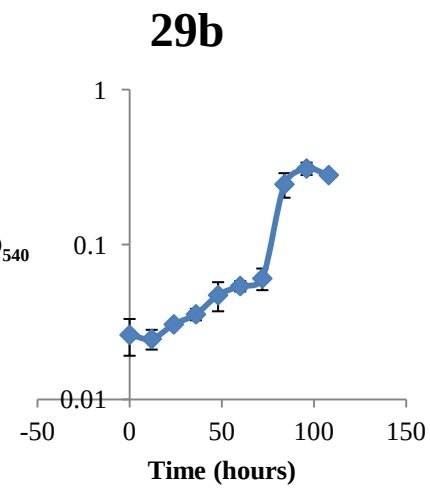
E.



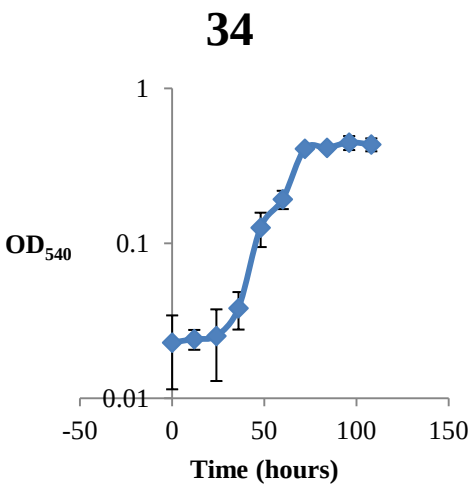
F.


29a2
1
0.1
0.01
OD540
-50
0
50
100
150
Time (hours)

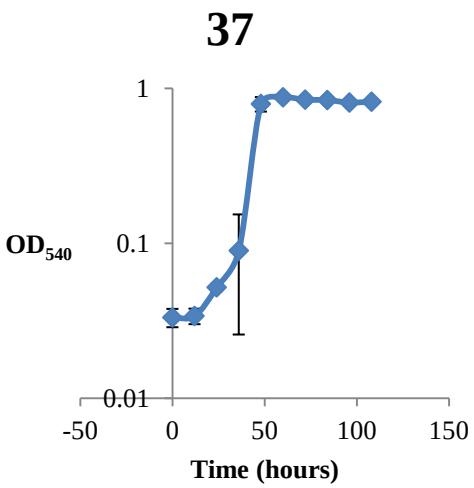
G.


29b
1
0.1
0.01
OD540
-50
0
50
100
150
Time (hours)

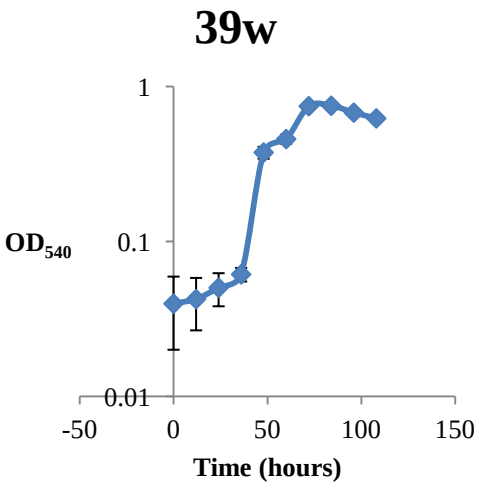
H.


34
1
0.1
0.01
OD540
-50
0
50
100
150
Time (hours)

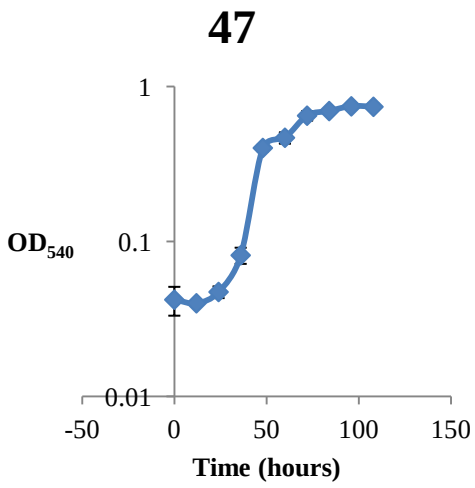
I.


37
1
0.1
0.01
OD540
-50
0
50
100
150
Time (hours)

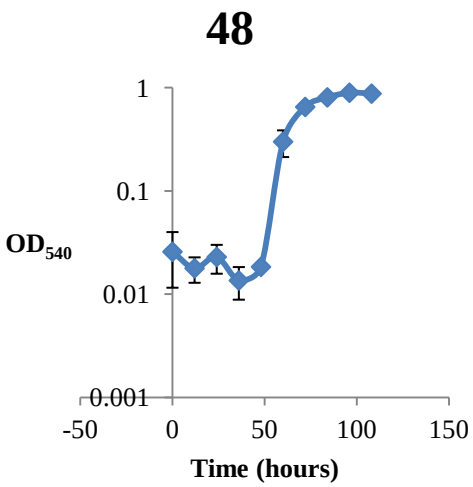
J.


39w
1
0.1
0.01
OD540
-50
0
50
100
150
Time (hours)

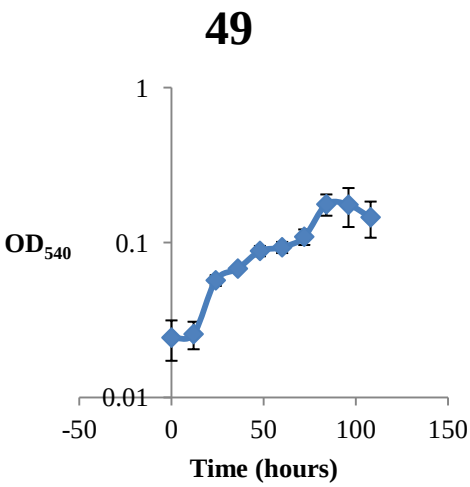
K.


47
1
0.1
0.01
OD540
-50
0
50
100
150
Time (hours)

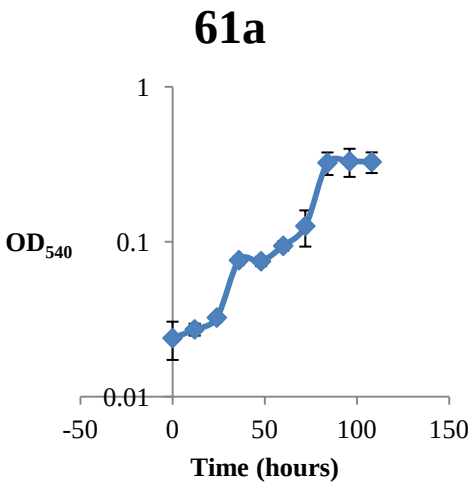
L.


48
1
0.1
0.01
0.001
OD540
-50
0
50
100
150
Time (hours)

M.

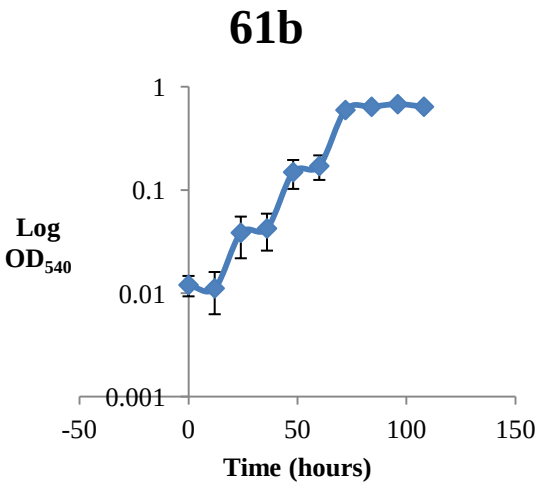

49
1
0.1
0.01
OD540
-50
0
50
100
150
Time (hours)

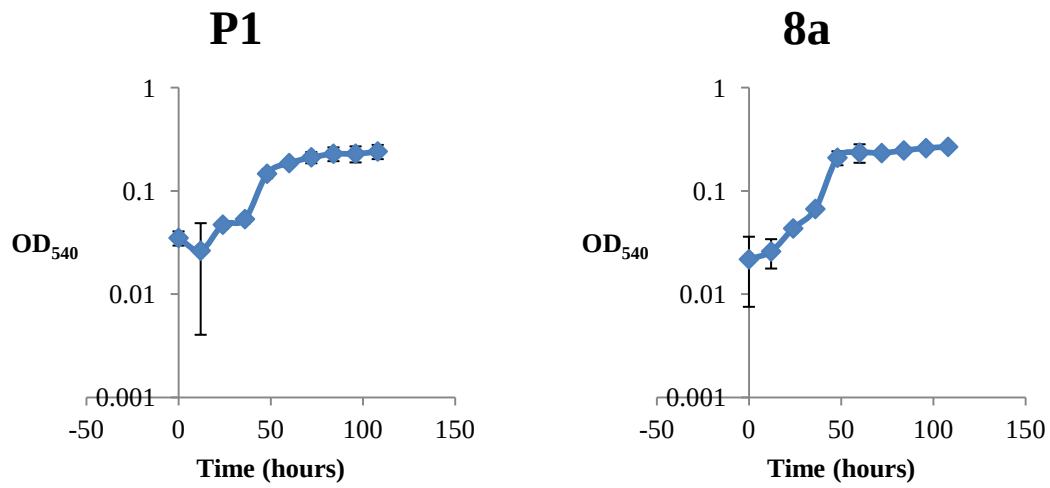
N.


61a
1
0.1
0.01
OD540
-50
0
50
100
150
Time (hours)

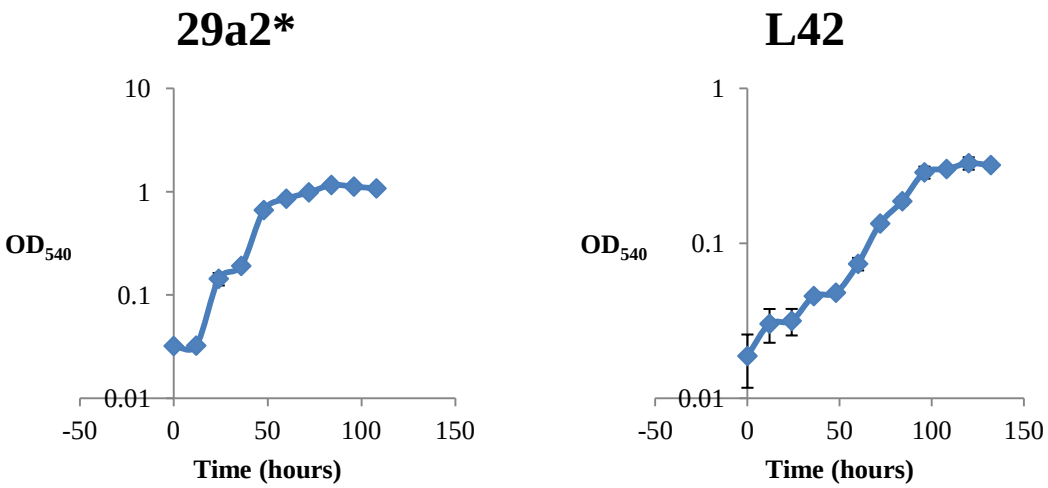
O.

P.


61b
1
0.1
0.01
0.001
Log
OD540
-50
0
50
100
150
Time (hours)



Q. R.



S. T.

A. *Rhodococcus globerulus* i8a1 **B.** *Rhodococcus globerulus* i8a2 **C.** *Loktanella* i8b2 **D.** *Salinibacterium* i8b2 **E.** *Rhodococcus erythropolis* i24 F. *Rhodococcus globerulus* i29a1 **G.** *Rhodococcus globerulus* i29a2 **H.** *Rhodococcus erythropolis* i29b **I.** *Rhodococcus erythropolis* i34 **J.** *Gordonia polyisoprenivorans* i37 **K.** *Rhodococcus erythropolis* i39w **L.** *Rhodococcus wratislaviensis* i47 **M.** *Rhodococcus wratislaviensis* i48 **N.** *Leifsonia* i49 **O.** *Mycobacterium fortuitum* i61a **P.** *Micrococcus luteus* i61b **Q.** *Microbacterium* iP1 **R.** *Rhodococcus globerulus* i8a **S.** *Mycobacterium hodleri* i29a2* **T.** *Stappia* iL42

For all isolates error bars = 1 standard deviation. n=3

Figure 3.5 shows growth curves for each isoprene degrading strain grown on 1% (v/v) isoprene as a sole carbon and energy source. Table 3.3 show the growth rates and doubling times of isolates grown on isoprene. Cells were grown in MAMS minimal medium as

described in Materials and Methods section 2.3. All strains were able to grow on isoprene under these conditions to a sufficient cell density for DNA and protein extraction, and no attempt was made to optimise growth for individual strains.

Strains of *Rhodococcus, Gordonia* and *Mycobacterium* grew to a higher cell density than other isolates. The isolates *Rhodococcus* i48 *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2* grew to the highest cell density as seen in Figure 3.5, therefore were selected as candidates for further analysis of their physiology and regulation as described in Chapter 4 and for genome sequencing as described in Chapter 5.

Previous studies on marine isoprene degradation (Alvarez *et al.* 2009) suggested that the rate of isoprene degradation decreased with increasing isoprene concentration in environmental samples. This effect was not seen in environmental samples enriched in this study (Figure 3.1).

Table 3.3: Growth rates and doubling times of isoprene degrading strains grown on isoprene as sole carbon source

For all isolates n=3

Isolate	Specific growth rate μ (hr^{-1})	Doubling time td (hours)	Maximum OD_{540} obtained*
***Rhodococcus* i8a1**	0.076	9.12	0.6111
***Gordonia* i37**	0.164	4.21	0.8832
***Rhodococcus* i8a2**	0.044	15.5	0.7348
***Loktanella* i8bn**	0.0697	9.94	0.5372
***Rhodococcus* i24**	0.027	25.3	0.7328
***Rhodococcus* i34**	0.044	15.85	0.4182
***Rhodococcus* i39**	0.051	13.63	0.7861
***Rhodococcus i*47**	0.129	5.37	0.7217
***Rhodococcus* i48**	0.063	10.99	0.8841
***Leifsonia* i49**	0.016	43.95	0.119
***Micrococcus* i61b**	0.121	5.75	0.6725
***Rhodococcus* i29a1**	0.063	11.07	0.5711
***Rhodococcus* i29a2**	0.035	19.67	0.6911
Mycobacterium* i29a2	0.063	11.04	1.1384
***Rhodococcus* i8a**	0.028	25.1	0.2631
***Salinibacterium* i8b2**	0.057	12.05	0.462
***Rhodococcus* i29b**	0.113	6.11	0.309
Mycobacterium i61a	0.079	8.13	0.2956
***Microbacterium* ip1**	0.053	13.8	0.199
***Stappia* il42**	0.0384	18.04	0.3512

*Under standard (i.e. non-optimal) growth conditions

Table 3.4: Growth of isolates in miminal medium with added carbon sources

Substrate	*Rhodococcus* i8a1	*Gordonia* i37	*Rhodococcus* i8a2	*Loktanella* i8bn	*Rhodococcus* i24	*Rhodococcus* i34	*Shinella* i39	*Rhodococcus* i47	*Rhodococcus* i48	*Leifsonia* i49	*Micrococcus* i61b	*Rhodococcus* i29a1	*Rhodococcus* i29a2	*Mycobacterium* i29a2*	*Rhodococcus* i8a	*Salinibacterium* i8b2
Methane	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethane	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Propane	+	+	+	-	+	+	-	+	+	-	-	+	+	+	+	-
Butane	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Ethene	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Propene	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
But-2-ene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Propan-1-ol	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Propan-2-ol	+	+	+	-	+	+	-	+	+	-	-	+	+	+	-	-
Polyisoprene	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acetone	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methanol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Epoxyisoprene	N/A*	+	N/A	N/A	+	N/A	N/A	+	N/A	N/A	N/A	N/A	+	+	N/A	N/A
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-
Acetate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Succinate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
Toluene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phenol	+	+	+	-	+	+	-	+	+	-	-	+	+	-	+	-
1,3-but-diene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Positive growth was determined by observable change in turbidity of the liquid culture. Negative growth was confirmed by measurement of optical density.

*N/A – not tested

Several isolates representing distinct genera were tested for their ability to grow on alternative substrates as their sole carbon and energy source. Actinobacteria, such as *Rhodococcus*, display broad metabolic diversity and are known to degrade a wide range of different hydrocarbons and xenobiotic substrates in the environment, including chlorinated phenolics, steroids, lignin, coal, and petroleum (Finnerty, 1992). Many soluble diiron centre monooxygenases such as methane, alkane and alkene monooxygenases are known to oxidise a broad range of substrates. In addition, bacteria can possess more than one SDIMO or other monooxygenase systems with different substrate specificities, such as the facultative methanotroph *Methylocella silvestris* (Crombie and Murrell, 2014) which contains both a methane monooxygenase and a propane monooxygenase, or *Mycobacterium* NBB4, which contains 6 different monooxygenase systems (Coleman *et al*, 2012).

All *Rhodococcus, Mycobacterium* and *Gordonia* isolates were able to grow on both propane and isoprene as a sole carbon source. These genera are closely related within the Actinobacteria, and are each known for their metabolic diversity and degradation of multiple pollutants in the environment (Finnerty, 1992). Several species of *Mycobacterium, Gordonia* and *Rhodococcus* are known to grow on propane as their sole carbon source. The propane-degrading *Gordonia.* TY5 metabolises propane via a novel Baeyer-Villiger acetone monooxygenase (Kotani *et al.* 2007). *Gordonia polyisoprenivorans* i37, when tested, was also able to grow on acetone as a sole carbon source. It is of interest as to whether a multifunctional form of isoprene monooxygenase exists within this isolate which can oxidise propane, or whether these isolates contain more than one soluble diiron centre monooxygenase system.

Most isolates were unable to grow on 1,3-butadiene, which is structurally the closest short-chain hydrocarbon to isoprene. *Rhodococcus* AD45 can oxidise short chain alkenes but is unable to grow (Crombie and Murrell, unpublished). Therefore it is possible that the isoprene monooxygenase can oxidise many of these substrates but lack the downstream enzymes to metabolise the oxidation products further.

Most isolates were unable to grow on any other short chain hydrocarbons, with the exception of *Mycobacterium hodleri* strain i29a2*, which grew on ethane as its sole carbon and energy source, *Rhodococcus wratislaviensis* i48, which grew on butane, and *Rhodococcus wratislaviensis* i47, which grew on ethane. It is unknown whether these isolates possess multiple monooxygenase enzyme systems or a single multifunctional enzyme.

Only one of the isolates tested could grow on polyisoprene: *Gordonia polyisoprenivorans* i37. *Gordonia polyisoprenivorans* i37 is closely related to documented polyisoprene degrading *Gordonia polyisoprenivorans* and *Gordonia westfalica* strains (Linos *et al.* 1999, 2002). However, unlike known polyisoprene degrading *Gordonia* strains, which usually require full adhesive contact with the substrate and form only partial clear zones on plates (Rose & Steinbüchel, 2005), *Gordonia polyisoprenivorans* i37 showed full clear zone formation usually associated with enzyme-secreting polyisoprene degraders.

Five strains (*Gordonia* i37, *Rhodococcus* i24, *Rhodococcus* i47, *Rhodococcus* i29a2 and *Mycobacterium* i29a2*) were tested for growth on epoxyisoprene as their sole carbon and energy source. Epoxyisoprene is produced as the product of isoprene oxidation by isoprene monooxygenase. All strains tested were able to grow on epoxyisoprene, suggesting that the downstream enzymes IsoHIJ, encoding the dehydrogenase and glutathione-S-transferases (see Chapter 1), are still produced in the absence of isoprene.

Table 3.5: Growth of isolates on MAMS minimal media plates supplemented with different concentrations of NaCl

Conc^{n} NaCl (%)	*Rhodococcus* i8a1	*Gordonia* i37	*Microbacterium* P1	*Loktanella* i8b1	*Rhodococcus* i24	*Rhodococcus* i34	*Shinella* i39	*Rhodococcus* i47	*Rhodococcus* i48	*Leifsonia* i49	*Micrococcus* i61b	*Rhodococcus* i29a1	*Rhodococcus* i29a2	*Mycobacterium* i29a2*	*Mycobacterium* i61a	*Salinibacterium* i8b2
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
4	-	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-
3	-	+	+	+	-	-	-	+	+	+	+	-	-	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
0.8	-	+	-	+	-	+	+	-	-	+	-	-	+	-	+	-
0.5	-	+	-	+	-	+	-	-	-	+	-	-	+	-	+	-
0.3	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
0.1	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
0	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-

Growth was determined by the observation of colonies after 2 weeks

Isolates from the Colne estuary were tested for NaCl tolerance. Results are shown in Table 3.5. Some of these strains were isolated from low salinity water samples at the Hythe and therefore were tested at different salinities to ensure these represent truly estuarine/marine isolates and not isoprene degraders that have been washed into the estuary from soil. Open ocean seawater has a salinity of approximately 3.5% (w/v), and salinity is often slightly lower in estuaries where the river meets the sea. All isolates tested were able to grow on isoprene at up to 2% salinity. No isolates grew significantly below 0.5% (w/v)salinity with the exception of *Loktanella* strain i8b1. Previously described salt-tolerant *Loktanella* species are known to have a wide range of salt tolerance (Van Trappen *et al.* 2004). None of the *Rhodococcus* strains were able to grow at above 2% (w/v) NaCl concentration, whereas the Actinobacteria *Gordonia* strain i37 and *Mycobacterium* strains i29a2* and i61a were able to grow at 3% (w/v) NaCl, as could *Microbacterium* P1 and *Salinibacterium* i8b2. *Micrococcus* i61b and *Loktanella* i8b1 were able to grow at 4% (w/v) NaCl, and *Leifsonia* i49 exhibited weak growth at 5% (w/v) NaCl. These results correspond to the phylogeny of the isoprene degrading strains. The *Rhodococcus* strains, though isolated from different environments, are closely related by their 16S rRNA sequences and form a distinct clade within the 16S rRNA sequences of isoprene degraders, being more closely related to terrestrial isoprene degrading *Rhodococcus* species. NaCl tolerance therefore would appear to be genus specific rather than dependent on the environment of isolation in isoprene degrading bacteria.

Table 3.6: Growth of isolates on mimimal medium + 1% isoprene at different temperatures

Temperature (˚C)	*Rhodococcus* i8a1	*Gordonia* i37	*Rhodococcus* i8a2	*Loktanella* i8bn	*Rhodococcus* i24	*Rhodococcus* i34	*Shinella* i39	*Rhodococcus* i47	*Rhodococcus* i48	*Leifsonia* i49	*Micrococcus* i61b	*Rhodococcus* i29a1	*Rhodococcus* i29a2	*Mycobacterium* i29a2*	*Rhodococcus* i8a	*Salinibacterium* i8b2
37	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-
30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Growth was assessed by change in optical density as measured using the spectrophotometer (Chapter 2).

Table 3.6 shows the temperature ranges for growth of isolates on isoprene. All isolates tested grew within the range of 4-30˚C with the exception of *Gordonia polyisoprenivorans* i37, which grew within the range of 10-37˚C, and *Micrococcus luteus* i61b, which grew within the range of 4-37˚C There are several known pathogenic species of *Gordonia*, for example *Gordonia bronchialis.* There have been 11 reported cases of opportunistic bloodstream infections with *Gordonia* species (Verma *et al.* 2006) and *Gordonia polyisoprenivorans* has been indicated in a single case of opportunistic native valve endocarditis in humans. It is therefore not surprising that this environmental isolate is able to grow at 37˚C.

Micrococcus luteus is a frequent coloniser of mammalian skin, where it can break down components of sweat. It has been implicated as an occasional opportunistic pathogen, particularly in immunocompromised patients such as HIV sufferers (Smith *et al.* 1999) Therefore; the two strains able to grow above 30˚C both have known associations with humans. However, both also grow at temperatures relevant to the environment from which they were isolated.

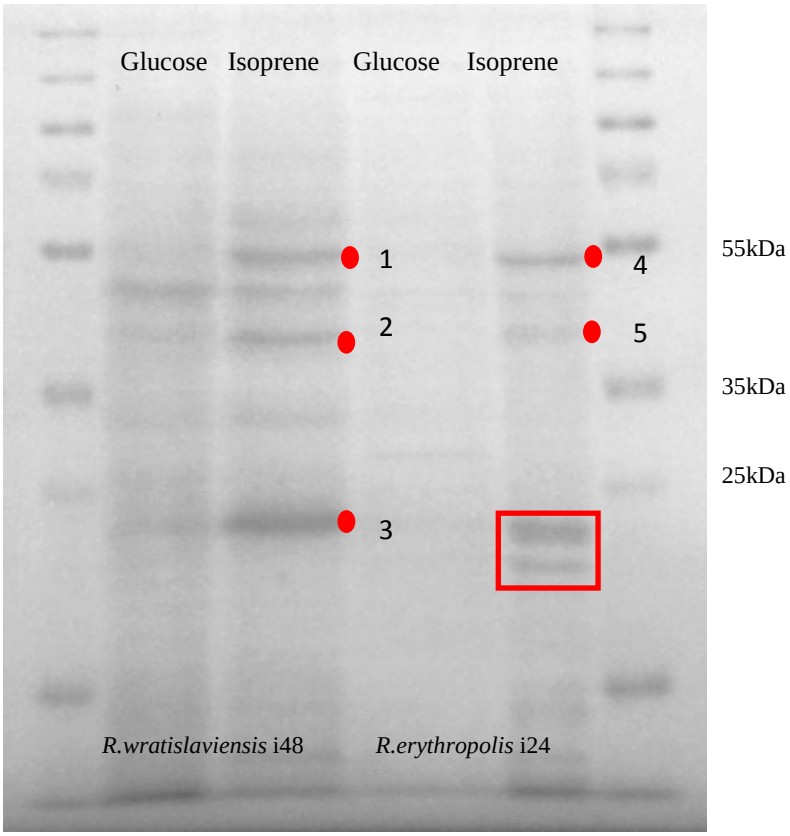
Toxicity of isoprene

The isolates *Gordonia polyisoprenivorans* i37, *Mycobacterium hodleri* i29a2*, *Microbacterium* i49, *Stappia* iP2, *Rhodococcus erythropolis* i24, *Rhodococcus wratislaviensis* i48 and *Salinibacterium* sp. i8b2 were all tested for possible toxic effects of isoprene at high concentrations. All of the isolates could grow on isoprene at 1.5% (v/v) isoprene, although *Mycobacterium hodleri* exhibited weaker growth above 1% (v/v) isoprene. None of the isolates tested could grow above 1.5% (v/v) isoprene.

3.3 SDS-PAGE of protein extracts from isolates grown on glucose vs isoprene

Several isolates representing the breadth of isoprene degrading genera were selected for polypeptide analysis. Cells were grown on isoprene and glucose as described in Materials and Methods section 2.3. Additionally, some isolates were grown on propane as a sole carbon source. Cultures were used to prepare whole cells extract as described in Materials and Methods section 2.13. These whole cell extracts were run on a 12.5% SDS-PAGE gel and bands of interest were excised from the gel for trypsin digest and LC-MS/MS analysis at the Univeristy of Warwick Proteomics Facility. The aim of this work was to compare polypeptide profiles of isolates grown on different carbon sources, and to detect known isoprene-related peptides when compared to a database of polypeptides encoded by the isoprene gene cluster of *Rhodococcus* AD45 (shown in Table 3.7). Samples were also analysed against a general database of *Rhodococcus* peptides, and if no peptide matches were found, against the UniProt database.

Figure 3.6.1 Comparative SDS-PAGE of *Rhodococcus wratislaviensis* i48 and *Rhodococcus erythropolis* i24 strains grown on glucose and isoprene as sole carbon source



Protein loaded per lane: ~20µg

Table 3.7: Expected size of polypeptides encoded by genes of the *Rhodococcus* AD45 isoprene gene cluster

ORF	Molecular mass (Da)	Identification
isoG	43,450	Putative racemase
isoH	24,033	NAD-dependent HGMB-dehydrogenase
isoI	27,096	Glutathione *S*-transferase active with epoxides
isoJ	26,342	Glutathione *S*-transferase active with CDNB and DCNB
isoA	58,461	Oxygenase α-subunit
isoB	10,331	Oxygenase γ-subunit
isoC	12,772	Ferredoxin
isoD	12,380	Effector or coupling protein
isoE	38,510	Oxygenase β-subunit
isoF	37,322	Reductase

CDNB: 1-chloro-2,4-dinitrobenzene

DCNB: 2,4-dichloro-1-nitrobenzene

Adapted from Vlieg *et al.* (2000)

Figure 3.6.1 shows a clear difference in the polypeptide profiles of *Rhodococcus wratislaviensis* i48 and *Rhodococcus erythropolis* i24 when grown on glucose and isoprene respectively. Table 3.7 shows the expected size of several of the polypeptides encoded by the isoprene gene cluster of *Rhodococcus* AD45.The dominant isoprene-induced bands corresponding to a molecular mass of ~58kDa, ~38kDa and ~25-27kDa were excised from the gel and sent for proteomic analysis as described in Materials and Methods section 2.15, and compared to a database of known isoprene-related polypeptides from *Rhodococcus* AD45. In *Rhodococcus erythropolis* i24 and *Rhodococcus wratislaviensis* i48, the ~58kDa bands contained 10 peptides that were a match for the monooxygenase alpha subunit IsoA, the ~38kDa band contained 7 peptides that were a match for the monooxygenase beta subunit IsoE and the racemase IsoG, and the bands at ~24kDa contained 4 peptides that matched the dehydrogenase IsoH and and 3 peptides matching the glutathione-S-

transferase IsoI in *R.wratislaviensis* i48. These results and examples of other detected peptides can be seen in Table 3.8. These results suggest that these polypeptides are induced in these *Rhodococcus* isolates during growth on isoprene.

Table 3.8: Overview of peptides detected by LC-MS/MS analysis of excised polypeptide bands from *Rhodococcus wratislaviensis* i48 and *Rhodococcus erythropolis* i24

Sample 1 (sample numbers correspond to Figure 3.6.1)

Accession	Entry	Description	Molecular Mass (Da)	Peptides	Coverage (%)
Q0SGP9	ATPB_RHOSR	ATP synthase subunit beta OS *Rhodococcus* sp strain RHA1 GN atpD	51984	23	59.01
Q0RXM5	Q0RXM5_RHOSR	Aldehyde dehydrogenase NAD OS *Rhodococcus* sp strain RHA1 GN thcA	55135	12	39.84
Q0S0C6	Q0S0C6_RHOSR	6 phosphogluconate dehydrogenase decarboxylating OS *Rhodococcus* sp strain RHA1 GN gnd3	51722	12	45.13
Q0S538	Q0S538_RHOSR	Succinate semialdehyde dehydrogenase NAD P OS *Rhodococcus* sp strain RHA1 GN gabD4	51248	11	30.33
IsoA	IsoA_MEK	IsoA	59303	10	15.76
Q0S2Y7	Q0S2Y7_RHOSR	Adenosylhomocysteinase OS *Rhodococcus* sp strain RHA1 GN ahcY	54077	9	19.84
Q0SGN1	SYR_RHOSR	Arginyl tRNA synthetase OS *Rhodococcus* sp strain RHA1 GN argS	59162	8	13.82
Q0S4B6	Q0S4B6_RHOSR	Probable cystathionine beta synthase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro05843	48398	8	19.96
Q0S527	Q0S527_RHOSR	Bifunctional purine biosynthesis protein purH OS *Rhodococcus* sp strain RHA1 GN purH	54339	6	16.80
Q0SI13	Q0SI13_RHOSR	Probable branched chain amino acid ABC transporter binding protein OS *Rhodococcus* sp strain RHA1	44475	5	22.05
Q0S2P7	Q0S2P7_RHOSR	Phosphoglucomutase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro06413	59904	5	16.58
Q0SHL0	AMPA_RHOSR	Probable cytosol aminopeptidase OS *Rhodococcus* sp strain RHA1 GN pepA	51776	4	12.08
Q0SAB8	Q0SAB8_RHOSR	Glutamate synthase small subunit OS *Rhodococcus* sp strain RHA1 GN RHA1 ro03718	52681	4	12.01
Q0S6P4	Q0S6P4_RHOSR	Acetyl coenzyme A carboxylase carboxyl transferase alpha and beta subunits OS *Rhodococcus* sp strai	56565	4	9.50
Q0S510	Q0S510_RHOSR	Succinate semialdehyde dehydrogenase NAD P OS *Rhodococcus* sp strain RHA1 GN gabD5	50973	3	10.54
Q0SGI1	Q0SGI1_RHOSR	Methylmalonate semialdehyde dehydrogenase Acylating OS *Rhodococcus* sp strain RHA1 GN mmsA2	53957	3	8.20
Q0SHK2	Q0SHK2_RHOSR	Glutamine synthetase OS *Rhodococcus* sp strain RHA1 GN glnA1	53450	3	9.83
Q0SHR9	MURD_RHOSR	UDP N acetylmuramoylalanine D glutamate ligase OS *Rhodococcus* sp strain RHA1 GN murD	51337	3	9.42
Q0SI05	Q0SI05_RHOSR	ABC transporter ATP binding component OS *Rhodococcus* sp strain RHA1 GN RHA1 ro01004	67160	3	4.54
Q0SKD7	Q0SKD7_RHOSR	Possible modulator of DNA gyrase protein TldD OS *Rhodococcus* sp strain RHA1 GN RHA1 ro00163	48714	3	7.28
Q0S0I8	Q0S0I8_RHOSR	Glucose 6 phosphate 1 dehydrogenase OS *Rhodococcus* sp strain RHA1 GN zwf4	57475	3	6.81
Q0S4W6	Q0S4W6_RHOSR	Glucose 6 phosphate 1 dehydrogenase OS *Rhodococcus* sp strain RHA1 GN zwf3	51704	3	10.81
Q0S345	Q0S345_RHOSR	Glycerol kinase OS *Rhodococcus* sp strain RHA1 GN glpK	54256	2	4.63
Q0S3R7	Q0S3R7_RHOSR	Putative uncharacterized protein OS *Rhodococcus* sp strain RHA1 GN RHA1 ro06042	50967	2	4.71
Q0SDT3	Q0SDT3_RHOSR	Aldehyde dehydrogenase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro02498	53039	2	5.18
Q0S0J5	Q0S0J5_RHOSR	Glyceraldehyde 3 phosphate dehydrogenase OS *Rhodococcus* sp strain RHA1 GN gap2	35642	2	10.62

Sample 2

Accession	Entry	Description	Molecular Mass (Da)	Peptides	Coverage (%)
Q0SB36	Q0SB36_RHOSR	Possible myoinositol 1 phosphate synthase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro03447	39137	12	36.57
Q0S2H3	ILVC_RHOSR	Ketol acid reductoisomerase OS *Rhodococcus* sp strain RHA1 GN ilvC	36324	9	29.08
Q0S6R3	SERC_RHOSR	Phosphoserine aminotransferase OS *Rhodococcus* sp strain RHA1 GN serC	39574	8	32.44
Q0S0J5	Q0S0J5_RHOSR	Glyceraldehyde 3 phosphate dehydrogenase OS *Rhodococcus* sp strain RHA1 GN gap2	35642	8	30.09
Q0SGE0	Q0SGE0_RHOSR	NAD P transhydrogenase alpha subunit OS *Rhodococcus* sp strain RHA1 GN pntA1	37969	7	24.59
Q0RYZ2	Q0RYZ2_RHOSR	Citrate Si synthase OS *Rhodococcus* sp strain RHA1 GN citA3	41344	7	24.02
IsoG	IsoG_MEK	IsoG	43418	7	17.04
Q0SGR3	Q0SGR3_RHOSR	Acetyl CoA C acetyltransferase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro01455	40618	6	18.39
Q0S0H3	Q0S0H3_RHOSR	Cysteine desulfurase OS *Rhodococcus* sp strain RHA1 GN sufS	44948	6	19.09
Q0S194	Q0S194_RHOSR	Putative uncharacterized protein OS *Rhodococcus* sp strain RHA1 GN RHA1 ro06926	37734	5	23.27
Q0S4Q8	Q0S4Q8_RHOSR	Probable cysteine desulfurase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro05699	41184	5	16.88
Q0SF95	Q0SF95_RHOSR	Transcription antitermination protein nusG OS *Rhodococcus* sp strain RHA1 GN RHA1 ro01980	28376	5	25.76
Q0SFN6	Q0SFN6_RHOSR	Putative uncharacterized protein OS *Rhodococcus* sp strain RHA1 GN RHA1 ro01839	50925	5	13.43
Q0SB23	Q0SB23_RHOSR	Probable acetyl CoA C acetyltransferase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro03460	42311	5	15.10
IsoE	IsoE_MEK	IsoE	38483	5	14.62
Q0S9I0	Q0S9I0_RHOSR	Superoxide dismutase OS *Rhodococcus* sp strain RHA1 GN sodA	22996	4	24.64
Q0SD47	Q0SD47_RHOSR	Probable acetyl CoA C acetyltransferase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro02734	42575	4	15.80
Q0S0J4	PGK_RHOSR	Phosphoglycerate kinase OS *Rhodococcus* sp strain RHA1 GN pgk	42075	4	12.66
Q0SGP8	ATPG_RHOSR	ATP synthase gamma chain OS *Rhodococcus* sp strain RHA1 GN atpG	34909	3	10.43
Q0S5V9	Q0S5V9_RHOSR	Cyanuric acid amidohydrolase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro05296	38896	2	6.23
Q0S8Z1	Q0S8Z1_RHOSR	Acetyl CoA C acyltransferase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro04204	42419	2	6.93
Q0SDZ3	Q0SDZ3_RHOSR	Putative uncharacterized protein OS *Rhodococcus* sp strain RHA1 GN RHA1 ro02438	11155	2	31.13
Q0SEX3	Q0SEX3_RHOSR	Possible membrane protein OS *Rhodococcus* sp strain RHA1 GN RHA1 ro02106	51418	2	4.31
Q0SF52	Q0SF52_RHOSR	Probable cysteine synthase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro02027	40213	2	6.23
Q0SFF4	EFTU_RHOSR	Elongation factor Tu OS *Rhodococcus* sp strain RHA1 GN tuf	43509	2	7.83
Q0SFG4	Q0SFG4_RHOSR	Probable acyl CoA dehydrogenase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro01911	43046	2	5.12
Q0SFG5	Q0SFG5_RHOSR	Probable acyl CoA dehydrogenase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro01910	39607	2	11.47
Q0SHF9	Q0SHF9_RHOSR	3 oxoacyl acyl carrier protein synthase OS *Rhodococcus* sp strain RHA1 GN fabF	42941	2	6.97
Q0SHL1	Q0SHL1_RHOSR	Aminomethyltransferase 1 OS *Rhodococcus* sp strain RHA1 GN gcvT1	38623	2	8.74
Q0SHM2	Q0SHM2_RHOSR	Probable cytochrome c oxidase subunit II OS *Rhodococcus* sp strain RHA1 GN RHA1 ro01137	39158	2	7.89
Q0S1G1	Q0S1G1_RHOSR	Probable thiolase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro06853	42299	2	3.98

Sample 3

Accession	Entry	Description	Molecular Mass (Da)	Peptides	Coverage (%)
Q0S9N7	Q0S9N7_RHOSR	3 hydroxyacyl CoA dehydrogenase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro03952	25701	10	55.34
Q0S8M5	Q0S8M5_RHOSR	Transcriptional regulator OS *Rhodococcus* sp strain RHA1 GN RHA1 ro04321	26959	8	39.92
Q0S1P5	Q0S1P5_RHOSR	Glutamate binding protein OS *Rhodococcus* sp strain RHA1 GN gluB1	29252	8	40.43
Q0S3F9	RS5_RHOSR	30S ribosomal protein S5 OS *Rhodococcus* sp strain RHA1 GN rpsE	22296	5	32.11
Q0S3E8	RS4_RHOSR	30S ribosomal protein S4 OS *Rhodococcus* sp strain RHA1 GN rpsD	23437	5	29.35
Q0SIF9	PSB_RHOSR	Proteasome subunit beta OS *Rhodococcus* sp strain RHA1 GN prcB	31067	4	19.39
IsoI	IsoI_MEK	IsoI	27076	4	19.33
Q0S9I0	Q0S9I0_RHOSR	Superoxide dismutase OS *Rhodococcus* sp strain RHA1 GN sodA	22996	3	20.77
Q0SDY3	Q0SDY3_RHOSR	Probable tellerium resistance protein OS *Rhodococcus* sp strain RHA1 GN RHA1 ro02448	20172	3	24.08
IsoH	IsoH_MEK	IsoH	24047	3	10.18
Q0SAN3	Q0SAN3_RHOSR	Probable oxidoreductase short chain dehydrogenase reductase family protein OS *Rhodococcus* sp str	23412	2	7.21
Q0SER7	Q0SER7_RHOSR	Probable carboxyvinyl carboxyphosphonate phosphorylmutase OS *Rhodococcus* sp strain RHA1 GN RHA1 r	26250	2	18.00
Q0SE90	Q0SE90_RHOSR	3 oxoacyl acyl carrier protein reductase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro02340	26024	2	9.13
Q0S890	ISPD_RHOSR	2 C methyl D erythritol 4 phosphate cytidylyltransferase OS *Rhodococcus* sp strain RHA1 GN ispD	23497	2	10.62

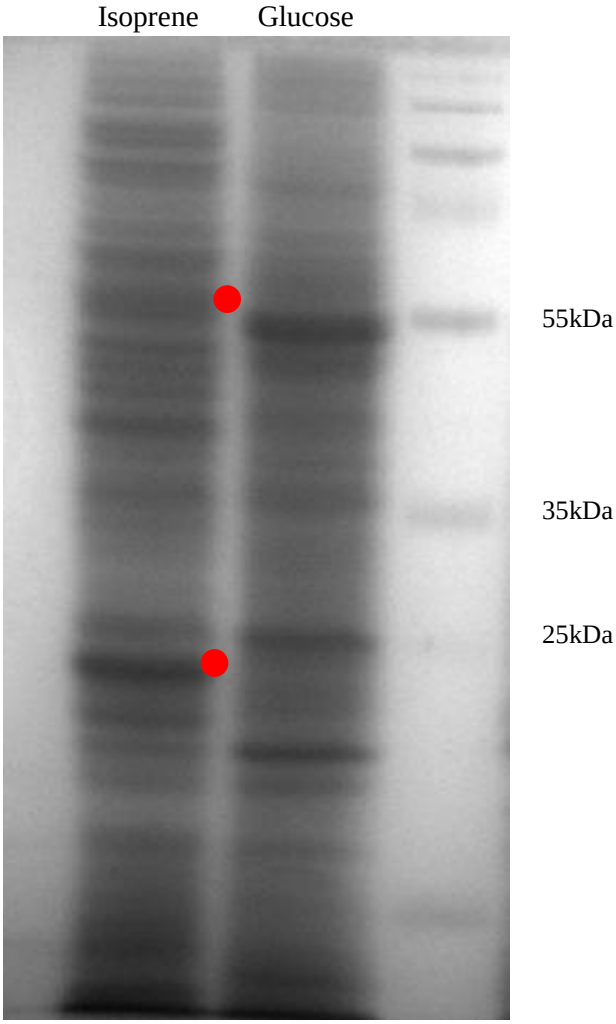
Sample 4

Accession	Entry	Description	Molecular Mass (Da)	Peptides	Coverage (%)
IsoA	IsoA_MEK	IsoA	59303	20	43.77
Q0SI02	Q0SI02_RHOSR	Pyruvate kinase OS *Rhodococcus* sp strain RHA1 GN pyk1	50782	10	24.36
Q0S2I7	Q0S2I7_RHOSR	Glutamyl tRNA Gln amidotransferase subunit A OS *Rhodococcus* sp strain RHA1 GN gatA	51253	5	13.59
Q0S2G8	SYE_RHOSR	Glutamyl tRNA synthetase OS *Rhodococcus* sp strain RHA1 GN gltX	54178	5	10.79
Q0SH62	PROA_RHOSR	Gamma glutamyl phosphate reductase OS *Rhodococcus* sp strain RHA1 GN proA	43137	4	9.93
Q0S8X3	Q0S8X3_RHOSR	Probable acetyl propionyl CoA carboxylase alpha subunit OS *Rhodococcus* sp strain RHA1 GN RHA1 ro0	198698	3	1.53
Q0S0C6	Q0S0C6_RHOSR	6 phosphogluconate dehydrogenase decarboxylating OS *Rhodococcus* sp strain RHA1 GN gnd3	51722	3	9.11
Q0S9E8	SYS_RHOSR	Seryl tRNA synthetase OS *Rhodococcus* sp strain RHA1 GN serS	45590	2	6.94
Q0SHA8	Q0SHA8_RHOSR	Sulfate adenylyltransferase large subunit OS *Rhodococcus* sp strain RHA1 GN RHA1 ro01254	46758	2	5.25
Q0S208	PNP_RHOSR	Polyribonucleotide nucleotidyltransferase OS *Rhodococcus* sp strain RHA1 GN pnp	80179	2	4.49

Sample 5

Accession	Entry	Description	Molecular Mass (Da)	Peptides	Coverage (%)
Q0SFF4	EFTU_RHOSR	Elongation factor Tu OS *Rhodococcus* sp strain RHA1 GN tuf	43509	10	38.89
IsoE	IsoE_MEK	IsoE	38483	9	31.87
Q0S2H3	ILVC_RHOSR	Ketol acid reductoisomerase OS *Rhodococcus* sp strain RHA1 GN ilvC	36324	8	23.74
Q0S533	SUCC_RHOSR	Succinyl CoA ligase ADP forming subunit beta OS *Rhodococcus* sp strain RHA1 GN sucC	40790	4	10.80
Q0SET3	CH601_RHOSR	60 kDa chaperonin 1 OS *Rhodococcus* sp strain RHA1 GN groL1	56617	3	6.47
Q0S4B8	Q0S4B8_RHOSR	Probable beta ketoadipyl CoA thiolase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro05841	42329	3	9.14
Q0SGE0	Q0SGE0_RHOSR	NAD P transhydrogenase alpha subunit OS *Rhodococcus* sp strain RHA1 GN pntA1	37969	2	7.30
Q0SGP8	ATPG_RHOSR	ATP synthase gamma chain OS *Rhodococcus* sp strain RHA1 GN atpG	34909	2	7.67
Q0SGP9	ATPB_RHOSR	ATP synthase subunit beta OS *Rhodococcus* sp strain RHA1 GN atpD	51984	2	3.93
Q0S4I1	ENO_RHOSR	Enolase OS *Rhodococcus* sp strain RHA1 GN eno	44848	2	7.71

Figure 3.6.2: Comparative SDS-PAGE of cell free extracts from *Rhodococcus globerulus* i8a2 grown on glucose and isoprene as sole carbon source



Protein loaded: 30µg

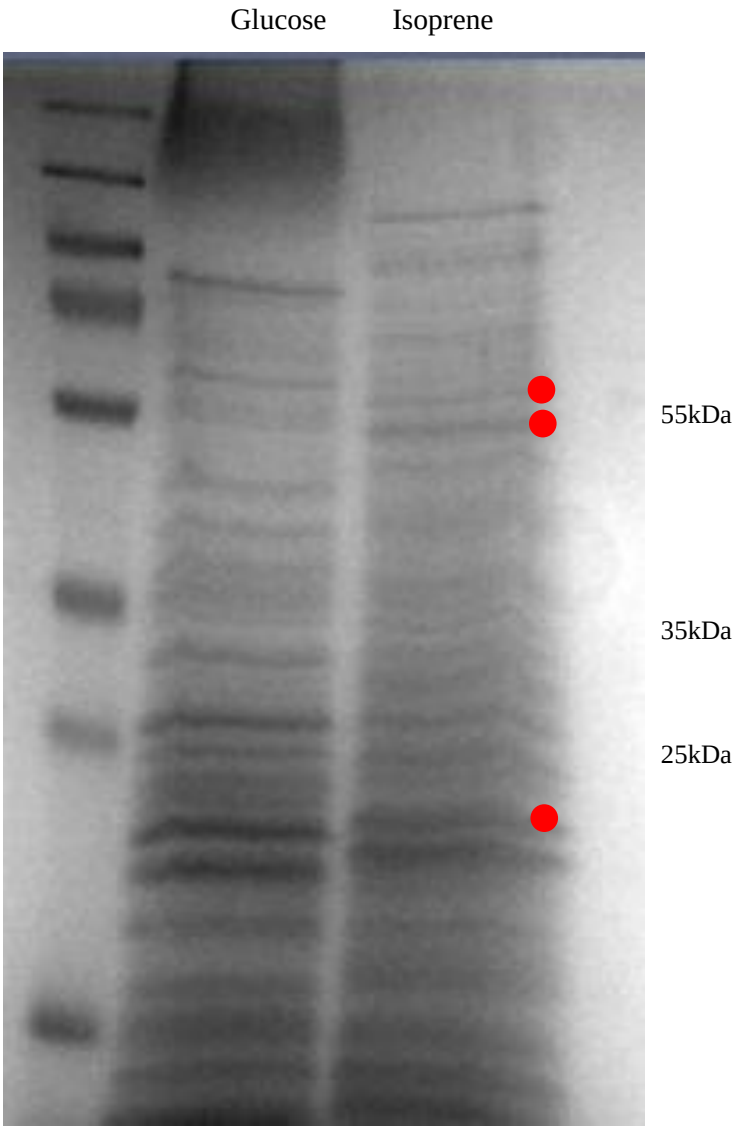
Figure 3.6.2 shows a clear difference in the polypeptide profiles of *Rhodococcus globerulus* i8a2 grown on glucose and isoprene. Bands highlighted with red dots were sent for mass spectrometry analysis, with peptide matches obtained for the monooxygenase alpha subunit, IsoA, the beta subunit, IsoE, the dehydrogenase, IsoH, and the glutathione-S-transferases, IsoI and IsoJ, as shown in Table 3.9.

Table 3.9: Overview of peptides detected by LC-MS/MS analysis of excised bands from *Rhodococcus globerulus* i8a2

Accession	Entry	Description	Molecular Mass (Da)	Peptides	Coverage (%)
Q0SFF4	EFTU_RHOSR	Elongation factor Tu OS *Rhodococcus* sp strain RHA1 GN tuf	43509	16	43.69
Q0S4I1	ENO_RHOSR	Enolase OS *Rhodococcus* sp strain RHA1 GN eno	44848	11	32.24
Q0S6Q7	Q0S6Q7_RHOSR	Citrate synthase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro04998	48097	8	14.62
Q0S4A0	Q0S4A0_RHOSR	Serine hydroxymethyltransferase 3 OS *Rhodococcus* sp strain RHA1 GN glyA4	46033	8	15.78
Q0S0L4	METK_RHOSR	S adenosylmethionine synthase OS *Rhodococcus* sp strain RHA1 GN metK	43138	4	10.64
Q0S0K2	RIBBA_RHOSR	Riboflavin biosynthesis protein ribBA OS *Rhodococcus* sp strain RHA1 GN ribBA	45140	3	8.15
Q0SET3	CH601_RHOSR	60 kDa chaperonin 1 OS *Rhodococcus* sp strain RHA1 GN groL1	56617	3	6.47
Q0SGW0	Q0SGW0_RHOSR	Glutamate dehydrogenase NADP OS *Rhodococcus* sp strain RHA1 GN gdh	48115	3	10.51
Q0SI58	ASSY_RHOSR	Argininosuccinate synthase OS *Rhodococcus* sp strain RHA1 GN argG	43998	3	8.77
IsoA	IsoA_MEK	IsoA	59303	3	6.23
Q0S655	Q0S655_RHOSR	Probable thiolase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro05200	46497	2	4.50

Accession	Entry	Description	Molecular Mass (Da)	Peptides	Coverage (%)
IsoI	IsoI_MEK	IsoI	27076	15	68.49
Q0S8M5	Q0S8M5_RHOSR	Transcriptional regulator OS *Rhodococcus* sp strain RHA1 GN RHA1 ro04321	26959	9	41.56
IsoJ	IsoJ_MEK	IsoJ	26323	7	24.46
Q0SET3	CH601_RHOSR	60 kDa chaperonin 1 OS *Rhodococcus* sp strain RHA1 GN groL1	56617	3	5.55
IsoE	IsoE_MEK	IsoE	38483	3	9.06
Q0S3F9	RS5_RHOSR	30S ribosomal protein S5 OS *Rhodococcus* sp strain RHA1 GN rpsE	22296	2	13.76
Q0S9I9	Q0S9I9_RHOSR	Short chain dehydrogenase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro04000	26506	2	7.45
Q0S9N7	Q0S9N7_RHOSR	3 hydroxyacyl CoA dehydrogenase OS *Rhodococcus* sp strain RHA1 GN RHA1 ro03952	25701	2	7.11
IsoH	IsoH_MEK	IsoH	24047	2	14.16

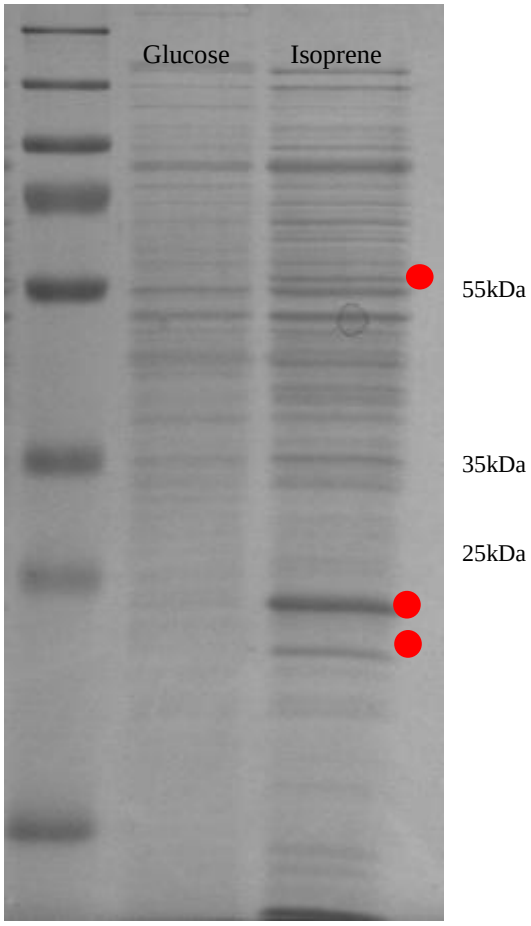
Figure 3.6.3: Comparative SDS-PAGE of *Micrococcus luteus* i61b grown on glucose and isoprene as sole carbon source



Protein loaded: ~25µg

Figure 3.6.3 shows the polypeptide profiles of *Micrococcus luteus* i61b whole cell extract from cells grown on glucose and isoprene. There are differences between the polypeptide profiles for this isolate grown on different carbon substrates. The bands highlighted with red dots on Figure 3.6.3 appear to be induced during growth on isoprene, and correspond approximately to the molecular mass of isoprene related polypeptides from *Rhodococcus* AD45 as shown in Table 3.7.

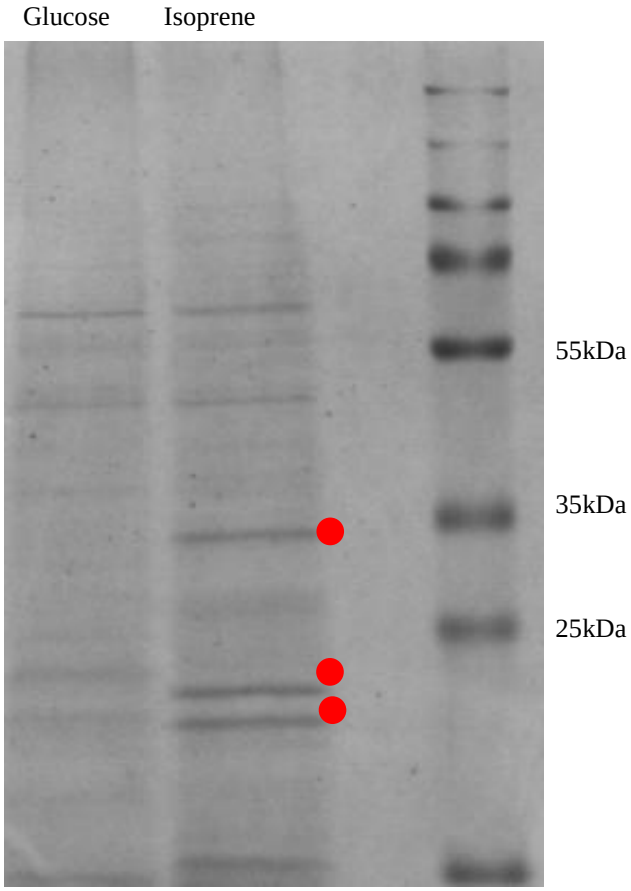
Figure 3.6.4: Comparative SDS-PAGE of cell extracts of *Loktanella* i8b1 grown on glucose and isoprene as sole carbon source



Protein loaded: ~ 25µg

Figure 3.6.4 shows clear differences in the polypeptide profile of *Loktanella* i8b1 grown on glucose and isoprene. The bands highlighted with red dots were clearly induced during growth on isoprene and correspond approximately to the expected mass of several peptides encoded by the *Rhodococcus* AD45 gene cluster.

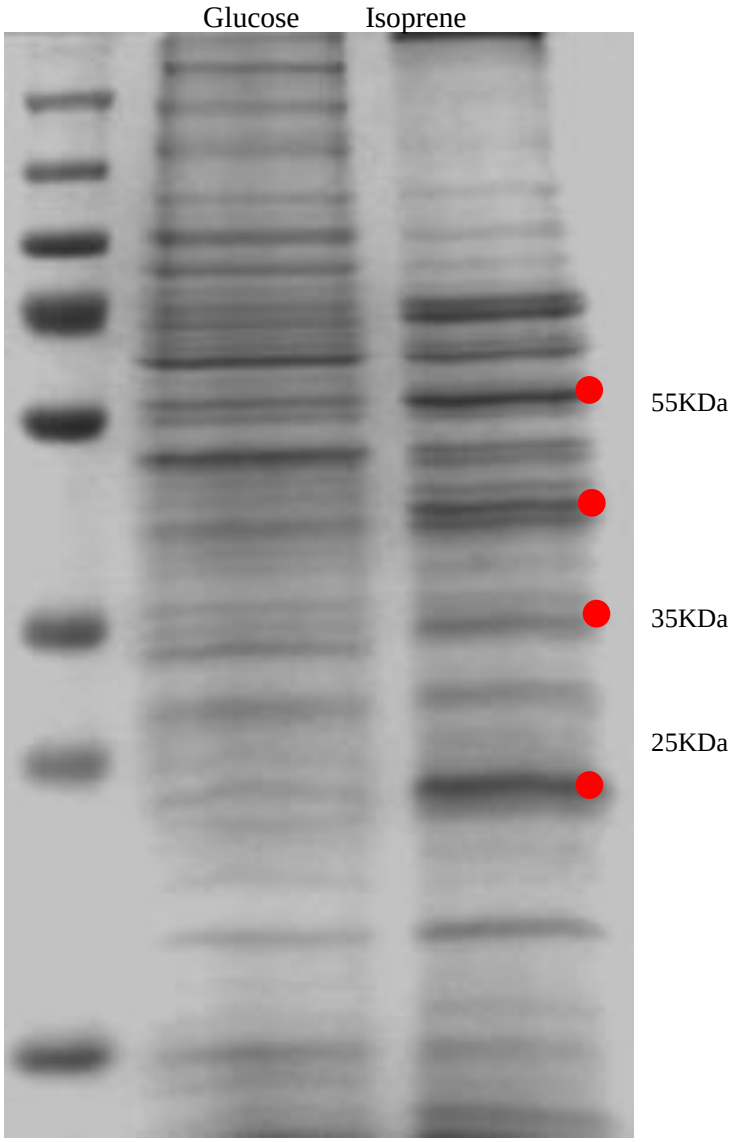
Figure 3.6.5: Comparative SDS-PAGE of cell extracts of *Salinibacterium* i8b2 grown on glucose and isoprene as sole carbon source



Protein loaded: ~20µg

Figure 3.6.5 shows some differences between the polypeptide profiles of *Salinibacterium* i8b2 cells grown on glucose and isoprene, including bands strongly induced during growth on isoprene and corresponding approximately to the expected mass of IsoH/I/J, based on previous proteomics results.

Figure 3.6.6: Comparative SDS-PAGE of cell extracts of *Leifsonia* i49 grown on glucose and isoprene as sole carbon source



Protein loaded: ~ 30µg

Figure 3.6.6 shows a clear difference in the polypeptide profiles of *Leifsonia* i49 cells grown on glucose and isoprene. The bands highlighted were excised from the gel and analysed by mass spectrometry by comparison to the database of isoprene related polypeptides from *Rhodococcus* AD45, and by comparison to the general UniProt database of peptides. 2 IsoH peptides were detected in the mass spectrometry samples with 10.18% coverage.

3.4 Summary of Results

This study showed that isoprene degradation occurs in a wide range of marine, coastal and estuarine environments. Samples were taken from two points along the Colne estuary (Essex). Samples taken from this estuary had previously been shown to completely degrade isoprene (Alvarez *et al.* 2009). Samples were also taken from the Penarth coast, Stiffkey salt marsh, the L4 sampling station and Lowestoft coast, none of which had previously been tested for isoprene degradation. In all environments tested, isoprene was completely depleted in enrichment cultures after 15 days. This rate of depletion is faster than previously reported for isoprene enrichments from the Colne Estuary (Alvarez *et al.* 2009) and suggests that isoprene degradation is widespread in the marine environment.

Two new isolates were obtained from these enrichments: a *Microbacterium* species, and a *Stappia* species. Isolate *Stappia* sp. iL42 represents the first confirmed isoprene degrading *Stappia* isolate, previously identified as a putative isoprene degrader using cultivation independent methods (Alvarez *et al.* 2009). This isolate dominated the 16S rRNA gene sequences retrieved by pyrosequencing of DNA from isoprene-enriched Indonesian water samples.

This study characterised 21 previously isolated isoprene degrading strains from the Colne estuary (Essex), as well as newly isolated strains from the Penarth coast (Wales) and the L4 sampling station (Plymouth). The majority of these isolates fell within the phylum Actinobacteria, with several related representatives of *Rhodococcus* and *Mycobacterium.* Members of these genera are known for their broad metabolic diversity and have previously been shown to degrade a diverse range of hydrocarbon substrates via soluble diiron centre monooxygenase enzyme systems. All isolates grew well on isoprene in liquid culture and several isolates were also able to grow on other short chain hydrocarbons as sole carbon and energy source. Many of the isolates grew on propane, suggesting a possible link between isoprene and propane degradation in these isolates. Soluble diiron centre monooxygenases often oxidise a number of different hydrocarbon substrates, raising the possibility of a multifunctional monooxygenase enzyme that can oxidise both isoprene and propane.

Comparative SDS-PAGE was carried out on a range of isolates grown on glucose or isoprene as their sole carbon source. Whole cell extracts from these cultures were analysed by SDS-PAGE profiling and mass spectrometry analysis of excised polypeptide bands. In

all isolates tested, a clear distinction could be seen between the polypeptide profiles of isoprene grown cells versus glucose grown cells. Bands induced only in isoprene grown cells were excised from the SDS-PAGE gel and sent for mass spectrometry analysis. Detected peptides were compared to a database of isoprene-related polypeptides from *Rhodococcus* AD45. Isoprene-related peptides were detected in all isoprene-grown *Rhodococcus* samples, including the alpha subunit IsoA, the beta subunit IsoE, the dehydrogenase IsoH, the glutathione-S-transferases IsoI and IsoJ, and the racemase IsoG. No isoprene-related peptides could be detected in samples from glucose-grown cells. This suggests that the isoprene monooxygenase gene cluster is induced during growth on isoprene. This regulation will be explored further in Chapter 4.

Chapter 4

Physiology and regulation of isoprene and propane metabolism in *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2*

Chapter 4: Physiology and regulation of isoprene and propane metabolism in *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2*

Introduction

The strains *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2* are two examples of fast-growing isoprene degrading Actinobacteria, isolated from the Colne Estuary, Essex (as described in Chapter 3). In addition to isoprene, both strains can grow on propane as an alternative sole carbon and energy source, as shown in Chapter 3 Table 3.4. *Mycobacterium* can additionally grow on ethane as a sole carbon source. Neither species has been previously tested for growth on propane, although the published genome of *Gordonia polyisoprenivorans* VH2 does contain an annotated putative phenol/propane monooxygenase (Hiessl *et al*, 2012).

SDS-PAGE, oxygen electrode assays and qRT-PCR assays were used to assess the transcription and translation of isoprene monooxygenase and other isoprene related polypeptides under different growth conditions, and to test for the ability of each strain to oxidise diverse hydrocarbon substrates when grown on different carbon sources. These data provide preliminary information regarding the regulation of the isoprene monooxygenase enzyme, indicating whether it is constitutively expressed or induced under certain growth conditions, and whether isoprene monooxygenase also acts as a multifunctional propane monooxygenase in these isolates.

4.1 Growth of *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2* on short chain hydrocarbons

Figure 4.1.1 Semi log plot showing growth of *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2* on propane as a sole carbon source

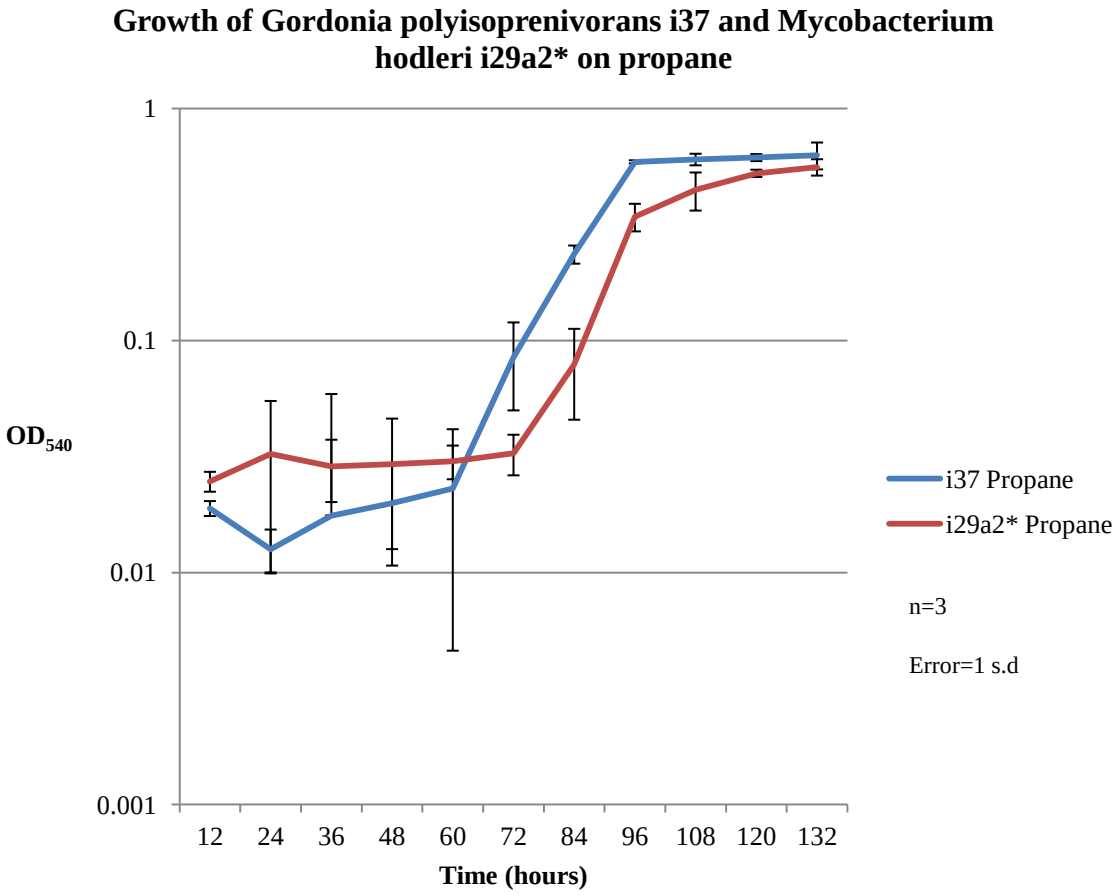


Figure 4.1.1 shows a semi-log plot of the growth of *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2* when grown on propane as a sole carbon source. Isolate *Gordonia* i37 grew on propane at a slower rate than growth on isoprene as shown in Table 4.1.1. The growth rates were approximately equal for isolate *Mycobacterium* i29a2*. Both isolates reach a lower cell density when grown on propane compared to growth on isoprene or glucose, where both isolates grew to an OD_{540} greater than 1 (as shown in Figure 3.5).

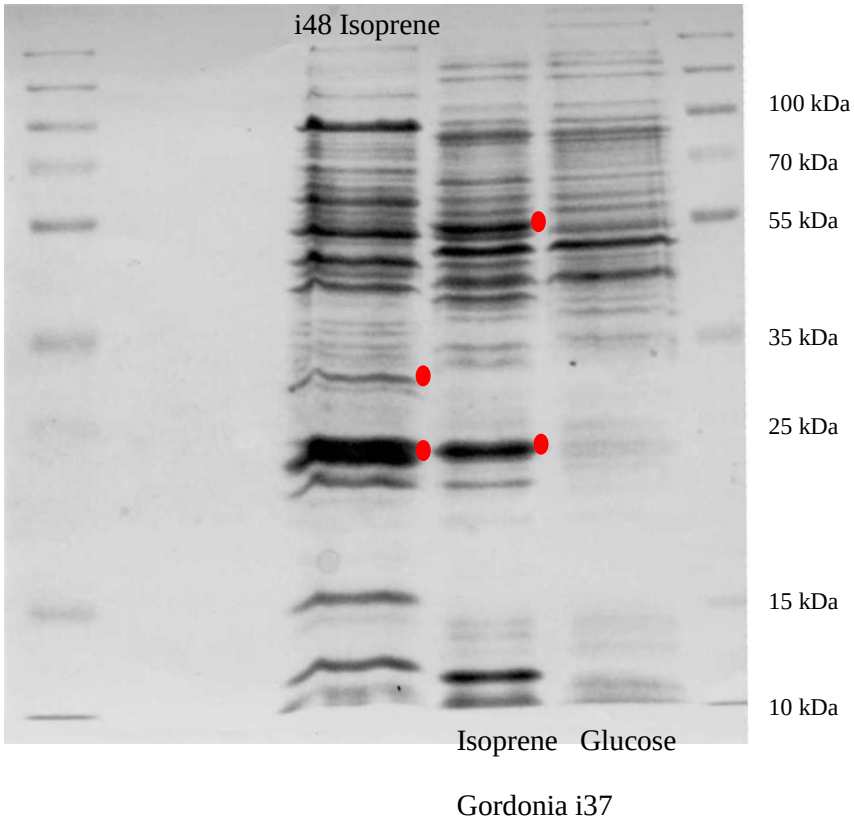
Table 4.1.1 Exponential growth rate and doubling times of *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2* grown on isoprene and propane (n=3)

	i37 Isoprene	**i37 Propane**	**i29a2* Isoprene**	**i29a2* Propane**
Growth rate (hr^{-1})	0.164	0.056	0.063	0.065
Doubling time (hr)	4.21	12.36	11.04	10.61

4.2 SDS-PAGE and polypeptide analysis of *Gordonia polyisoprenivorans* i37 and *Mycobacterium* i29a2*

SDS-PAGE was carried out on whole cell extract from glucose, isoprene and propane grown cells as described in Materials and Methods section 2.14. Whole cell extracts were run on a 12.5% polyacrylamide gel and the polypeptide profiles for each growth condition were compared to assess presence of induced bands during growth on either isoprene or propane and to compare these with isoprene-related polypeptides of a known size from *Rhodococcus* AD45.

Figure 4.2.1: Comparative SDS-PAGE showing polypeptide profiles of *Gordonia polyisoprenivorans* i37 grown on glucose and isoprene



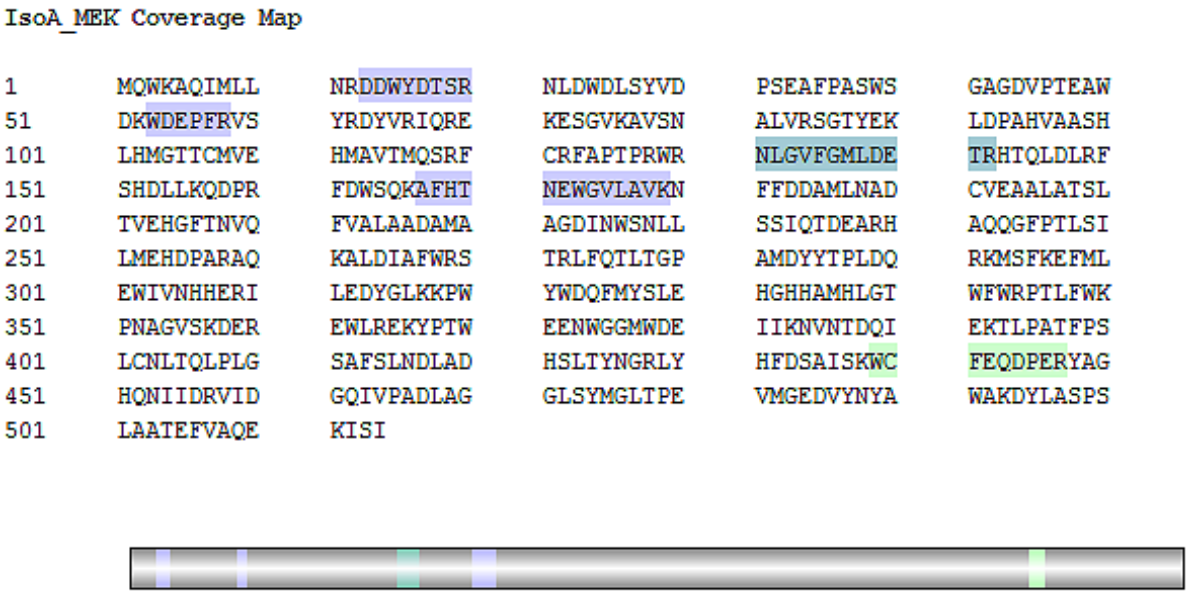
Protein loaded: 40 µg per lane

An SDS-PAGE polypeptide profile of *Gordonia* i37 grown on isoprene and glucose alongside isoprene-grown *Rhodococcus wratislaviensis* i48 for comparison is shown in Figure 4.2.1. *R.wratislaviensis* i48 was isolated by Alvarez *et al.*(2009) from the Colne Estuary. Isoprene-related polypeptides were detected in this organism in Chapter 3. The polypeptide profiles in this case appear similar, but not identical, for the two isolates, with a large induced band at approximately 25kDa, which corresponds approximately to the polypeptide mass of IsoH/I/J. The red dots highlight the bands that were sent for identification by mass spectrometry as described in Materials and Methods section 2.15. In *Rhodococcus* isolate 48, proteomics results identified peptide matches for IsoC, a 12.7kDa ferredoxin encoded by the *Rhodococcus* AD45 gene cluster. In the *Gordonia* isolate, the band at approximately 55kDa contained peptide matches to IsoA, the isoprene monooxygenase alpha subunit, as shown in Table 4.2.1. Examining the mass spectrometry data identified the location of these peptides within the IsoA polypeptide (see Figure 4.2.2). This gave insight into whether they are widespread throughout the polypeptide,

indicating a similar enzyme with sequence divergence, or whether they consist of conserved residues around the active site, potentially indicating a novel soluble diiron centre monooxygenase.

No other bands gave peptide matches when compared to a database of isoprene related polypeptides from *Rhodococcus* AD45. When compared to the *Gordonia* database obtained through genome sequencing (see Chapter 5), the large induced band of around 25kDa had peptide matches to a short chain dehydrogenase reductase from *Gordonia bronchialis* (Lucas *et al.*2009), which when analysed using a BLAST protein search showed high sequence similarity to IsoH.

Figure 4.2.2: Location of peptides detected in isoprene grown *Gordonia polyisoprenivorans* i37 within the IsoA polypeptide



IsoA peptides detected by mass spectrometry are highlighted on the coverage map shown on Figure 4.2.2. These peptides span the length of the IsoA polypeptide and do not consist of active site conserved residues.

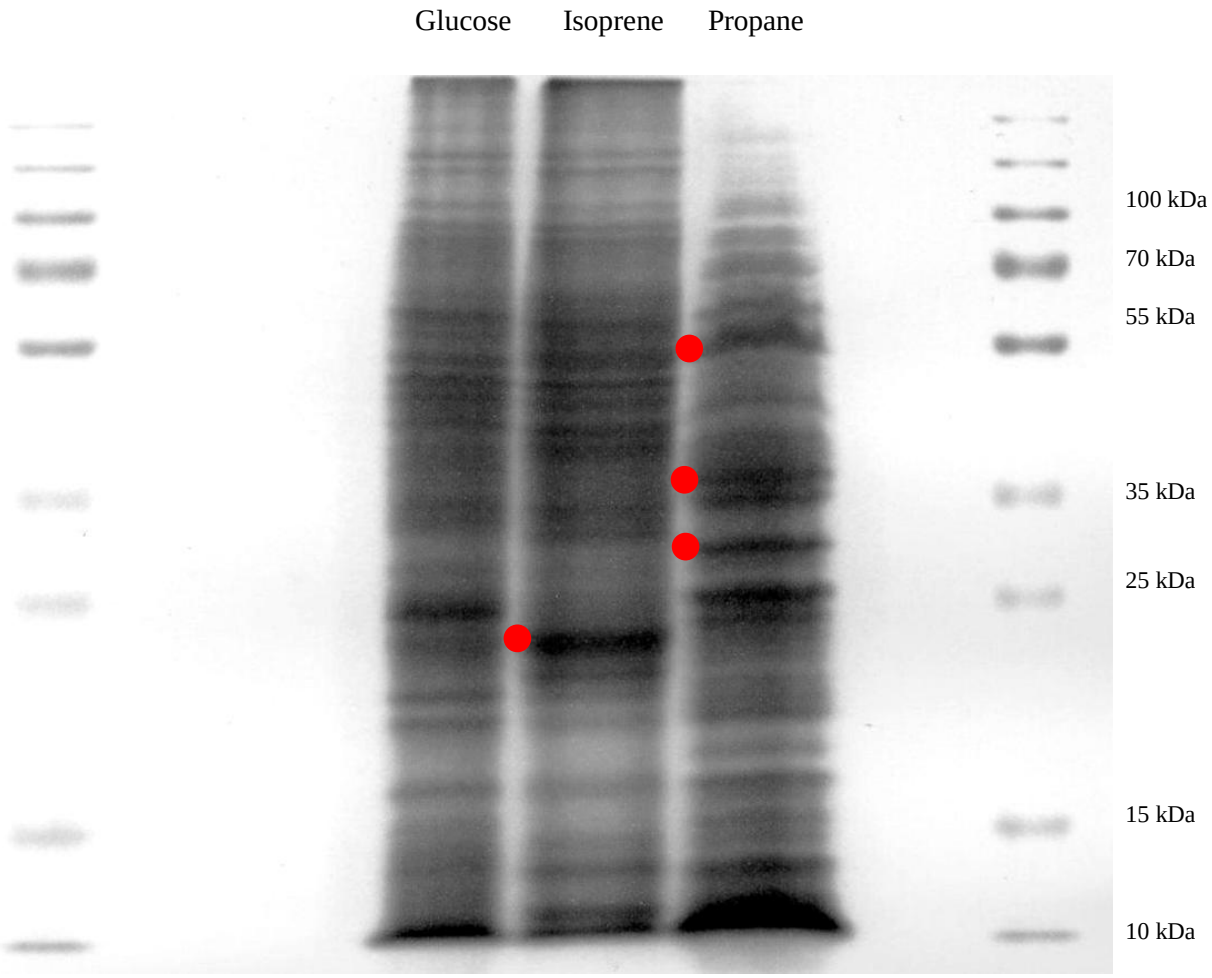
Table 4.2.1: IsoA peptides observed in isoprene grown *Gordonia polyisoprenivorans* i37 samples

Peptide molecular mass	**Start**	**End**	**Sequence**	**Retention Time (min)**
1056.4148	13	20	(R)DDWYDTSR(N)	25.95
848.3817	53	58	(K)WDEPFR(V)	29.18
1350.6602	131	142	(R)NLGVFGMLDETR(H)	35.66
1366.655	131	142	(R)NLGVFGMLDETR(H)	32.22
1470.7618	167	179	(K)AFHTNEWGVLAVK(N)	31.28
1265.5134	439	447	(K)WCFEQDPER(Y)	28.24

In mass spectrometry, polypeptides are broken into ionized fragments for detection according to their mass-to-charge ratio. The molecules are retained by the column and then elute at different times (called the retention time, shown in Table 4.2.1), and this allows the

mass spectrometer downstream to capture, ionize, accelerate, deflect, and detect the ionized molecules separately.

Figure 4.2.3: Comparative SDS-PAGE of *Gordonia polyisoprenivorans* i37 grown on glucose, propane and isoprene as sole carbon source

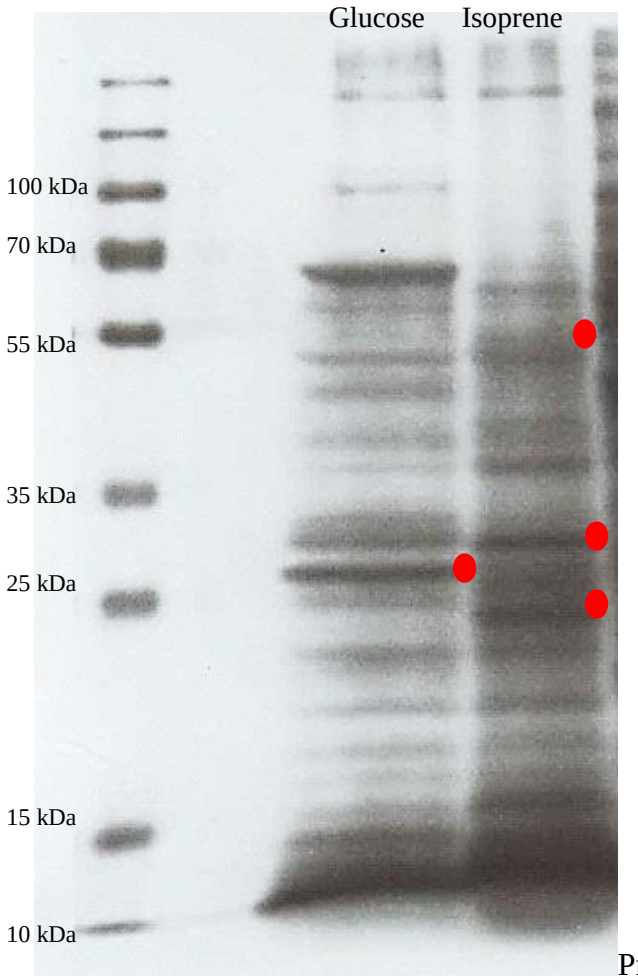


Protein loaded:~30µg per lane

Figure 4.2.3 shows the polypeptide profile of *Gordonia polyisoprenivorans* i37 grown on glucose, isoprene and propane as a sole carbon source. The polypeptide profiles differ for the three different growth conditions, with bands induced in the isoprene grown cells that are not present in glucose grown cells. Additionally, cells grown on propane display a different polypeptide profile to cells grown on isoprene, suggesting that the isoprene gene cluster is not induced during growth on propane, and that a separate enzyme system exists in this organism to oxidise propane. Induced bands (highlighted) from the isoprene and propane lanes were cut out and sent for mass spectrometry analysis. No propane monooxygenase peptides were detected in bands from propane grown cells when

compared to the UniProtKB/Swiss-Prot and NCBI databases. Bands excised from the propane grown polypeptide profile gave peptide matches to alcohol dehydrogenase and aldehyde dehydrogenase, both of which could have a potential role in the oxidation of propane. IsoH and IsoI peptides were detected in samples taken from isoprene grown cells.

Figure 4.2.4: Comparative SDS-PAGE showing polypeptide profiles of *Mycobacterium hodleri* i29a2*grown on glucose and isoprene



Protein loaded: ~30µg per lane

Figure 4.2.4 shows the polypeptide profile of *Mycobacterium hodleri* i29a2* grown on glucose and isoprene as a sole carbon source. The polypeptide profiles for this isolate differ for the two growth conditions. The bands highlighted were excised from the gel and sent for identification by mass spectrometry. There were no matches for isoprene related peptides in bands excised from the glucose-grown lane. Peptide matches for bands excised from the isoprene-grown lane are shown in Table 4.2.2. These bands contained peptide matches for the isoprene monooxygenase alpha subunit IsoA and the glutathione-S-transferases IsoI and IsoJ when compared to a database of polypeptides from *Rhodococcus* AD45. In both *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2*, isoprene-related polypeptides were induced during growth on isoprene compared to glucose.

Table 4.2.2: Peptides observed in isoprene grown *Mycobacterium hodleri* i29a2*samples

Accession	Entry	Description	Molecular Mass (Da)	Peptides	Coverage (%)
Q0S8M5	Q0S8M5_RHOSR	Transcriptional regulator OS *Rhodococcus* sp strain RHA1 GN RHA1 ro04321	26959	3	14.81
Q0S2Q3	Q0S2Q3_RHOSR	ABC superfamily ATP binding component OS *Rhodococcus* sp strain RHA1 GN RHA1 ro06407	25363	2	11.35
IsoI	IsoI_MEK	IsoI	27076	2	10.92
Q0SGP9	ATPB_RHOSR	ATP synthase subunit beta OS *Rhodococcus* sp strain RHA1 GN atpD	51984	10	22.15
Q0S2Y7	Q0S2Y7_RHOSR	Adenosyl homocysteinase OS *Rhodococcus* sp. strain RHA1 GN ahcY	54077	2	4.05
IsoA	IsoA_MEK	IsoA	59303	2	5.25
IsoI	IsoI_MEK	IsoI	27076	14	69.33
IsoJ	IsoJ_MEK	IsoJ	26323	7	43.35

4.3 Oxygen electrode assays in *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2*

Gordonia polyisoprenivorans i37 and *Mycobacterium hodleri* i29a2* were grown on glucose, propane and isoprene as described in Materials and Methods section 2.1. Cells were used to assay for oxidation of hydrocarbon substrates in a Clark oxygen electrode as described in Materials and Methods section 2.19. Cells were tested for oxidation of saturated solutions of methane, ethane, propane, butane, but-1-ene, but-2-ene, ethane, propene, methanol, ethanol, propanol and isoprene, as well as 100mM solutions of glucose, acetate and succinate. Table 4.3.1 shows the substrate oxidation profiles for each isolate grown on each of the three substrates.

Substrate	*Gordonia* i37 Glucose	*Gordonia* i37 Isoprene	*Gordonia* i37 Propane	*Mycobacterium* i29a2* Glucose	*Mycobacterium* i29a2* Isoprene	*Mycobacterium* i29a2* Propane
Isoprene	-	+	-	-	+	-
Methane	-	-	-	-	-	-
Ethane	+	+	+	+	+	+
Propane	-	-	+	-	-	+
Butane	-	+	-	-	+	-
Ethene	+	+	+	+	+	+
Propene	-	-	-	-	-	-
But-2-ene	-	+	-	-	-	-
Glucose	+	+	+	+	+	+
Fructose	+	+	+	+	+	+
Acetate	+	+	+	+	+	+
Succinate	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+
1,3-but-diene	-	+	-	-	N/A	-

Table 4.3.1: Substrates oxidised by isoprene, glucose and propane grown cells of *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2* in a Clark oxygen electrode

(+ = oxygen consumed by cells upon addition of substrate in oxygen electrode)

Gordonia polyisoprenivorans i37 and *Mycobacterium hodleri* i29a2* can both oxidise a wide range of carbon sources, including several short chain hydrocarbons such as ethane. The oxidation profiles of the isolates differ from their overall growth profiles. Therefore these isolates possess enzymes that are able to oxidise a wide range of hydrocarbons, but probably lack complete pathways to allow both organisms to grow on these substrates as a sole carbon source. Many soluble diiron centre monooxygenases can oxidise a wide range of hydrocarbon substrates (Leahy *et al*, 2003). It is possible that isoprene monooxygenase is able to oxidise short chain hydrocarbons such as ethane and butane. Isoprene-grown *Gordonia polyisoprenivorans* i37 cells could oxidise 1,3-but-diene but could not grow on this substrate. The structural similarity between isoprene and 1,3-but-diene makes it likely that this substrate can occupy the active site of isoprene monooxygenase.

Table 4.3.1 shows the substrates oxidised by *Gordonia* i37 and *Mycobacterium* i29a2* cells grown on different carbon sources when tested in an oxygen electrode. Both isolates could oxidise all polysaccharides and complex carbon sources tested under all growth conditions. These sugars would be expected to enter glycolysis either directly as glucose or be converted into glycolysis intermediates. This leads to the formation of acetyl CoA which is oxidised in the citric acid cycle. These pathways are constitutively expressed in aerobic heterotrophs such as *Gordonia* and *Mycobacterium* (Tian *et al.* 2005).

Neither isolate could oxidise methane or propene under any growth condition.

Oxygen electrode studies also suggested that growth of these isolates on propane is due to a propane monooxygenase system, which was separate from the isoprene monooxygenase. Isolates grown on isoprene and propane can oxidise a variety of substrates but crucially, cells grown on isoprene do not oxidise propane and *vice versa*. Cells grown on glucose are unable to oxidise isoprene, supporting the SDS-PAGE results indicating the induction of the polypeptides during growth on isoprene.

Table 4.3.2: Rate of oxidation of key substrates by isoprene-grown *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2*cells (n=3)

	***Gordonia* i37 Isoprene Rate (nmol 0_2/min/mg dry weight cells)**	***Mycobacterium* i29a2* Isoprene Rate (nmol 0_2/min/mg dry weight cells)**
Isoprene	5.72	5.98
Propane	0	0
Glucose	4.24	4.38
Ethane	6.12	5.81

Table 4.3.3: Rate of oxidation of key substrates by glucose-grown *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2*cells (n=3)

	***Gordonia* i37 Glucose Rate (nmol 02/min/mg dry weight)**	***Mycobacterium* i29a2* Glucose Rate (nmol 02/min/mg dry weight)**
Isoprene	0	0
Propane	0	0
Glucose	6.39	7.11
Ethane	5.66	5.28

Table 4.3.4: Rate of oxidation of key substrates by propane-grown *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2*cells (n=3)

	***Gordonia* i37 Propane Rate (nmol 0_2/min/mg dry weight cells)**	***Mycobacterium* i29a2* Propane Rate (nmol 0_2/min/mg dry weight cells)**
Isoprene	0	0
Propane	3.74	4.10
Glucose	4.91	5.16
Ethane	5.79	6.67

The rates of oxidation of propane, isoprene, glucose and ethane in *Gordonia* i37 and *Mycobacterium* i29a2* grown on different carbon sources are shown in Tables 4.3.2, 4.3.3 and 4.3.4. Rate of oxidation of propane by both isolates was lower than other substrates, while comparatively high rates of oxidation of ethane was noted for both isolates under all growth conditions, although only *Mycobacterium hodleri* i29a2* can grow on this substrate as a sole carbon source. Thus far the enzyme system responsible for oxidation of ethane in both isolates is unknown.

Table 4.3.5: Oxygen consumption data for terminal and subterminal propane oxidation pathway intermediates by *Gordonia polyisoprenivorans* i37 grown on propane, isoprene and glucose (n=3)

	Propane	**Isoprene**	**Glucose**
1-propanol	+	-	-
2-propanol	+	-	-
Propanal	+	-	-
Acetone	+	-	-
Acetate	+	+	+
Propionate	+	+	+
Methylacetate	+	+	+
Acetol	-	-	-
Pyruvaldehyde	+	+	+

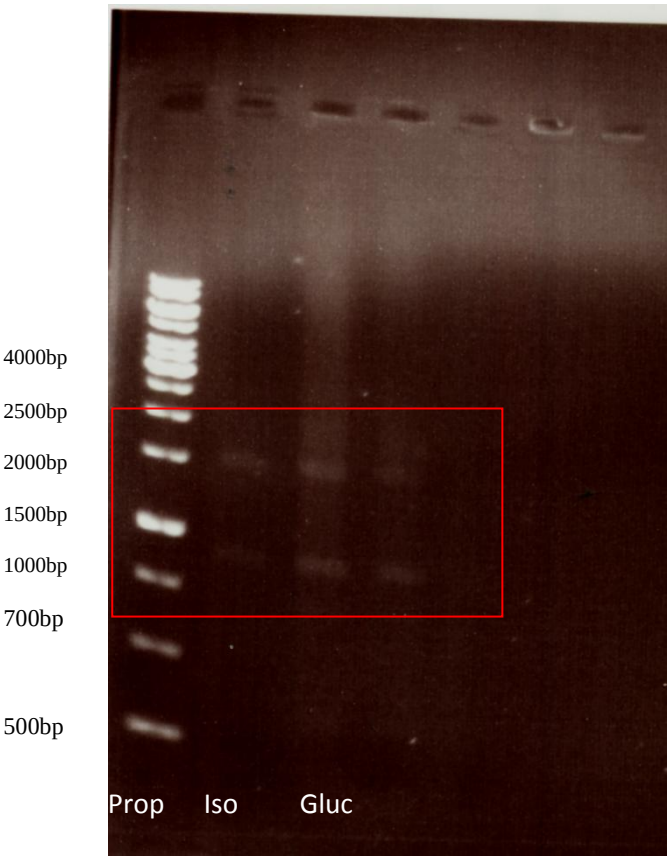
Propane, isoprene and glucose grown *Gordonia polyisoprenivorans* i37 cells were tested for oxidation of terminal and subterminal propane oxidation pathway intermediates. This isolate can grow on propane, 1-propanol, 2-propanol, acetone, acetate and propionate. Growth on both 1-propanol and 2-propanol suggests that this isolate may oxidise propane via both the terminal and subterminal propane oxidation pathways. Isoprene and glucose grown cells could oxidise acetate, propionate, methylacetate and pyruvaldehye, but not 1-propanol, 2-propanol, propanal or acetol. Acetate, propionate, methylacetate and pyruvaldehyde are ubiquitous in cells and act as intermediates in several other central metabolic pathways such as glycolysis and amino acid biosynthesis (Inoue and Kimora, 1995). Only propane grown cells could oxidise 1-propanol and 2-propanol, as well as all other intermediates in the terminal and subterminal propane oxidation pathways. This

suggests that this isolate can grow on propane via both pathways. In subterminal propane oxidation, there are two possible routes for metabolism of acetone (Ashraf *et al.* 1994). Acetone may either undergo a Baeyer-Villiger-type reaction to form methylacetate, or undergo terminal oxidation to form acetol. Propane grown *Gordonia polyisoprenivorans* i37 cells did not oxidise acetol, suggesting that in this isolate acetone is metabolised via methylacetate to methanol plus acetate. Chapter 5 will discuss the genome of this organism and cover in more detail propane metabolism in *Gordonia* i37.

4.4 qRT-PCR analysis of *Gordonia polyisoprenivorans* i37

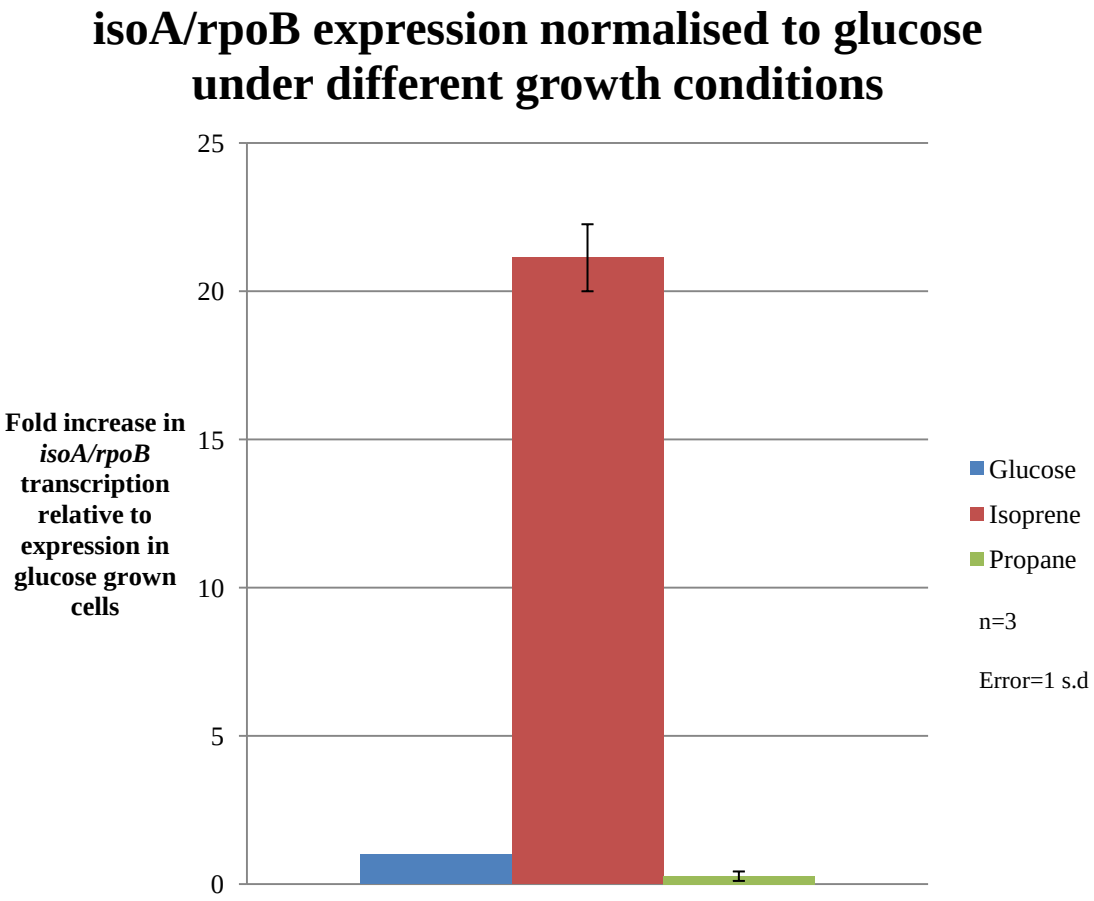
RNA was extracted from *Gordonia polyisoprenivorans* i37 grown on isoprene, glucose and isoprene as described in Materials and Methods section 2.16. The extracted RNA was used to assay for transcription of the gene encoding the isoprene monooxygenase alpha subunit, *isoA*, and *prmA*, the gene encoding the alpha subunit of the *Gordonia polyisoprenivorans* i37 propane monooxygenase (see Chapter 5). This experiment would show quantitative differences in transcription of these two genes under different growth conditions, to determine whether transcription is up or downregulated when cells are grown on alternative carbon sources.

Figure 4.4.1: Agarose gel showing RNA extracted from *Gordonia polyisoprenivorans* i37 grown on glucose, isoprene and propane



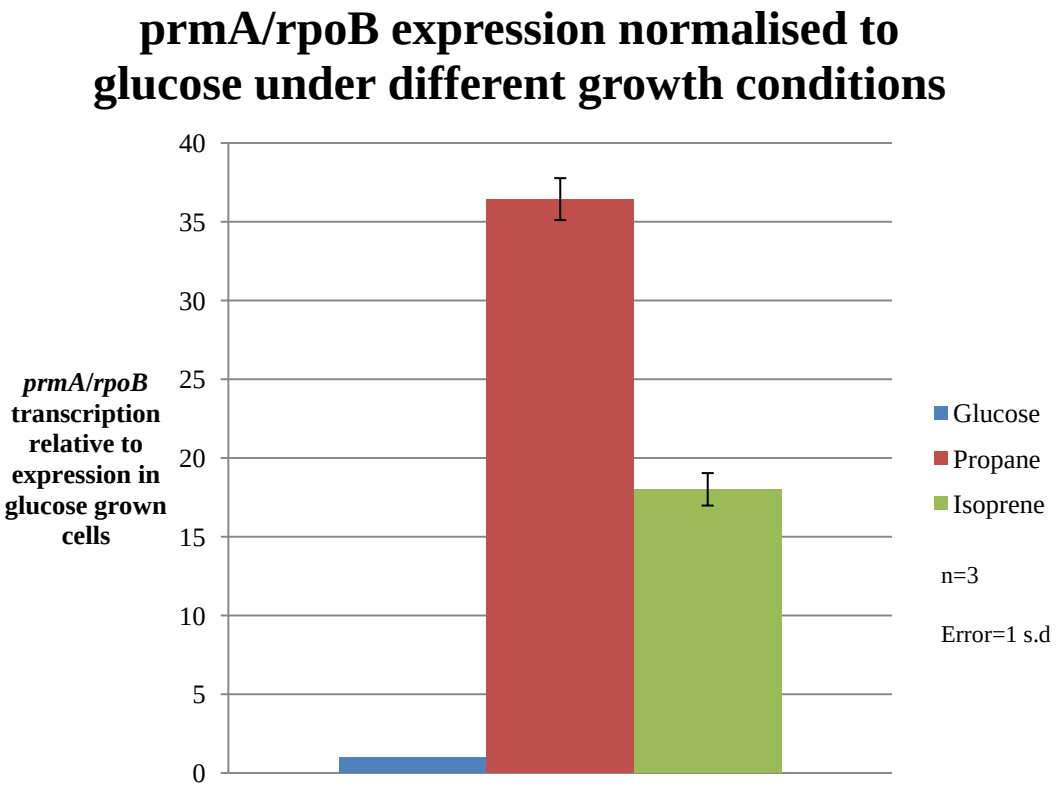
RNA was extracted from *Gordonia* i37 cells grown on isoprene, propane and glucose as described in materials and methods section 2.16. Figure 4.4.1 shows the extracted RNA loaded onto a 1% agarose gel and run alongside a DNA ladder. As this is not a denaturing gel and the size markers are DNA not RNA, it is not possible to assess whether the bands obtained are of the correct size, however two faint, distinct bands can be seen, corresponding to 23S and 16S rRNA subunits. The size of these would be confirmed using an RNA ladder and a denaturing formamide gel (RNA forms extensive secondary structures that prevent it migrating exactly according to size). However, it can be seen that the RNA remains mostly intact, and no smear of degraded RNA can be seen next to the lower molecular weight markers.

Figure 4.4.2: qRT-PCR assay of *isoA* in *Gordonia polyisoprenivorans* i37 grown on glucose, isoprene and propane



The qRT-PCR experiment compared transcription of the *isoA* gene under three different growth conditions: minimal medium with added glucose, isoprene and propane as a sole carbon source respectively as described in Materials and Methods section 2.17. Changes in transcription levels were calculated by comparison of *isoA* Ct values to that of the housekeeping gene *rpoB,* encoding the β subunit of bacterial RNA polymerase, under the different growth conditions followed by normalising against glucose grown samples, arbitrarily set as a value of 1. As shown in Figure 4.4.2, there was a 21-fold increase in *isoA* transcription in cells grown on isoprene compared to glucose, indicating induction of isoprene monooxygenase during growth on isoprene. There was a 3.7-fold decrease in *isoA* transcription in cells grown on propane compared to glucose, indicating possible suppression of isoprene monooxygenase transcription in the presence of propane.

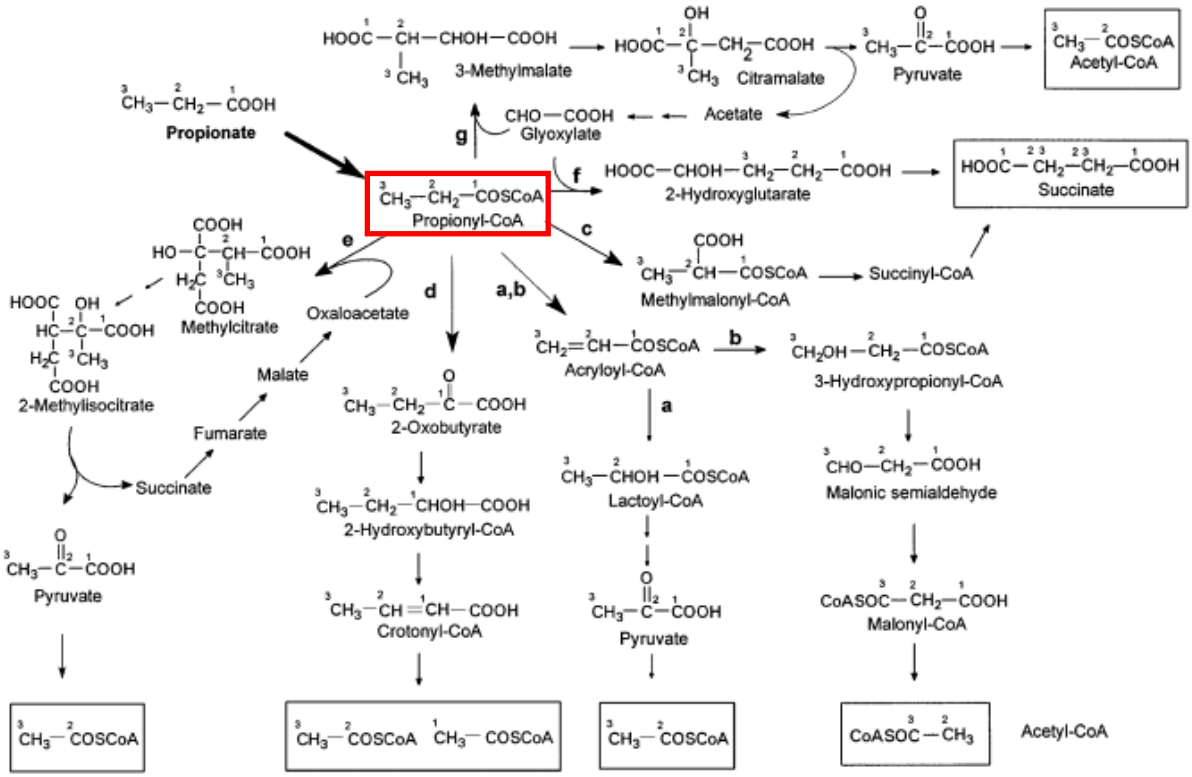
Figure 4.4.3: qRT-PCR assay of *prmA* gene in *Gordonia polyisoprenivorans* i37 grown on glucose, isoprene and propane



The qRT-PCR assays compared transcription of the *prmA* gene, encoding the alpha subunit of propane monooxygenase from the *Gordonia polyisoprenivorans* i37 genome (see chapter 5), under three different growth conditions: minimal medium with added glucose, isoprene and propane as a sole carbon source respectively as described in Materials and Methods section 2.1. Changes in transcription levels were calculated by comparison of *prmA* Ct values to that of housekeeping gene *rpoB* under the different growth conditions followed by normalising against glucose grown samples, arbitrarily set as a value of 1. There was a 36-fold increase in *prmA* transcription in cells grown on propane compared to glucose (Figure 4.4.3), indicating that transcription of propane monooxygenase is induced during growth on propane and not consitutively expressed above a basal level. There was also an 18 fold-increase in *prmA* transcription in cells grown on isoprene compared to glucose, indicating that growth on isoprene, as well as propane, can induce transcription of propane monooxygenase. However, oxygen electrode assay data shows that isoprene-grown *Gordonia* i37 cells do not oxidise propane. It is possible that a downstream product common to both isoprene and propane metabolism acts as an inducer of propane

monooxygenase, for example propionyl-CoA which is formed as a product of beta oxidation of odd numbered fatty acids, which is proposed as the fate of 2-glutathionyl-2-methyl-3-butenoic acid in isoprene degradation (Vlieg *et al*, 2000). Propionyl-CoA is an intermediate in the methylmalonyl-CoA pathway of propionate metabolism following terminal oxidation of propane (see Figure 4.4.4) which is well characterised in heterotrophic bacteria (Textor *et al.* 1997). SDS-PAGE and oxygen electrode data suggest that the propane monooxygenase polypeptide is not translated despite this increase in transcription, this might be due to further downstream regulation of propane monooxygenase gene expression such as antisense RNA binding, which are common in prokaryotes, with various genera containing antisense transcripts to a large percentage of genes (Georg and Hess. 2011).

Figure 4.4.4: Propionate metabolism in *Escherichia coli*



(Textor *et al*, 1997)

4.5 Acetylene inhibition assays of isoprene monooxygenase in *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2*

Gordonia polyisoprenivorans i37 and *Mycobacterium hodleri* i29a2* were tested for inhibition of isoprene monooxygenase by addition of acetylene. Cells were grown in minimal medium to OD_{540} 0.5 with 0.5% added isoprene as described in Materials and Methods section 2.4 and 1.5ml acetylene was added at mid exponential phase. Acetylene is a known inhibitor of methane monooxygenase (Prior and Dalton. 1985) (Lontoh *et al*, 2006). The flasks with added acetylene showed no difference in growth compared to control flasks as measured by optical density, or isoprene consumption as measured using gas chromatography. This suggests that acetylene is not an inhibitor of isoprene monooxygenase. As acetylene is a suicide substrate of methane monooxygenase, this allows us to distinguish between cooxidation of isoprene by soluble methane monooxygenase and oxidation by isoprene monooxygenase, particularly in environmental samples.

4.6 Antibiotic resistance in *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2*

In addition to investigating the regulation of isoprene monooxygenase, *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2* were screened for antibiotic resistance to gentamycin, kanamycin and streptomycin. These data are useful in determining antibiotic markers for carrying out marker exchange mutagenesis in these isolates, for example generating *isoA* and *prmA* knockout mutants. Mutations in the isoprene degradation pathway enzymes would give confirmation of the activity of these enzymes in these organisms, as well as possibly identifying any redundancy in the pathway, for example the two glutathione-S-transferases IsoI and IsoJ. Also mutations in *isoA* versus *prmA* would give conclusive proof that isoprene and propane, respectively, are oxidised by these two enzymes in *Gordonia* and *Mycobacterium.* Table 4.6.1 shows the growth profile of *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2* on selected antibiotics on solid agar as described in Materials and Methods section 2.1. Both isolates were resistant to kanamycin up to 60µg/ml, and *Gordonia* was resistant to kanamycin at 80µg/ml. *Gordonia* was resistant to gentamycin up to 40µg/ml whereas *Mycobacterium* was sensitive to gentamycin at 20µg/ml. *Gordonia* was sensitive to streptomycin at 10µg/ml and *Mycobacterium* at 20µg/ml. Table 4.6.2 shows the antibiotic resistance profile of *Gordonia polyisoprenivorans* i37 grown in minimal medium + isoprene with antibiotics added to growth medium as described in Materials and Methods section 2.1.

Table 4.6.1: Growth of *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2* on selected antibiotics on solid agar

	Gordonia	***Mycobacterium hodleri***
Gentamycin 10µg/ml	+	+
Gentamycin 20µg/ml	+	-
Gentamycin 40µg/ml	+	-
Gentamycin 60µg/ml	-	-
Gentamycin 80µg/ml	-	-
Kanamycin 20µg/ml	+	+
Kanamycin 40µg/ml	+	+
Kanamycin 60µg/ml	+	+
Kanamycin 80µg/ml	+	-
Streptomycin 5µg/ml	+	+
Streptomycin 10µg/ml	-	+
Streptomycin 20µg/ml	-	-

Growth assessed by the appearance of colonies on plate after 14 days

Table 4.6.2: Growth of *Gordonia polyisoprenivorans* i37 in liquid minimal medium + isoprene supplemented with selected antibiotics

	***Gordonia polyisoprenivorans* i37**
Gentamycin 10µg/ml	+
Gentamycin 20µg/ml	+
Gentamycin 40µg/ml	+
Gentamycin 50µg/ml	-
Kanamycin 20µg/ml	+
Kanamycin 40µg/ml	+
Kanamycin 60µg/ml	+
Kanamycin 80µg/ml	-
Streptomycin 5µg/ml	+
Streptomycin 10µg/ml	-
Streptomycin 20µg/ml	-

Growth assessed by visualisation of culture turbidity after 14 days

4.7 Summary of Results

SDS-PAGE and qRT-PCR were used to assess the transcription and translation of isoprene monooxygenase and propane monooxygenase in *Gordonia polyisoprenivorans* i37. Isoprene related peptides were detected only in cell extract from isoprene-grown *Gordonia* i37 cells, indicating induction of these enzymes by isoprene or a downstream metabolic intermediate of isoprene degradation, such as epoxyisoprene. No isoprene related peptides were detected in propane-grown *Gordonia* i37 cell extracts and the polypeptide profiles of this isolate grown on isoprene and propane clearly differed. This is strong evidence that this strain encodes a second monooxygenase enzyme that oxidises propane during growth on this substrate, which will be explored further in Chapter 5. RT-PCR primer sets were designed to target the isoprene monooxygenase alpha subunit *isoA* and the propane monooxygenase alpha subunit *prmA*. qRT-PCR assays showed an upregulation of *isoA* transcription during growth of *Gordonia* i37 on isoprene compared to growth on glucose, providing further evidence that isoprene related enzymes are inducible rather than constitutively expressed. qRT-PCR assays were also carried out to quantify RNA transcripts extracted from propane-grown *Gordonia* i37, which showed a clear upregulation in *prmA* transcription during growth on propane, as well as during growth on isoprene, potentially due to induction of this enzyme systems by a metabolic intermediate common to both propane and isoprene degradation pathways. *isoA* transcription was however not upregulated during growth of this isolate on propane, indicating a regulatory mechanism in which propane monooxygenase transcription can be induced by a downstream metabolite of isoprene metabolism, but isoprene monooxygenase transcription is likely not induced by this same metabolite, suggesting that the isoprene monooxygenase gene cluster and downstream genes are induced by a different system. Additionally, oxygen electrode assays suggest that this isolate cannot oxidise propane when cells are grown on isoprene as a carbon source, and the polypeptide profiles of isoprene and propane grown *Gordonia* cell extracts are significantly different with no common major induced polypeptide bands. This could possibly suggest that while *prmA* transcription is upregulated during growth on isoprene, the cells do not contain a functional propane monooxygenase, whether this is because the remaining genes are not transcribed or the mRNA transcripts for these genes are not translated due to further downstream regulation. Chapter 5 will discuss the genes and genomic organisation of these isolates in more detail.

Chapter 5

Analysis of the genomes of *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2*

Chapter 5: Genome mining in *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2*

5.1 Introduction

Gordonia polyisoprenivorans i37 and *Mycobacterium hodleri* i29a2* are isoprene-degrading Actinobacteria isolated from the Colne Estuary, Essex (see Chapter 4). Both are capable of growth on propane as a sole carbon source, as are several other isoprene-degrading Actinobacteria. Chapter 4 focused on the physiology and regulation of isoprene monooxygenase under different growth conditions, and the results of the SDS-PAGE, mass spectrometry and oxygen electrode analyses suggest that these isolates contain at least two different monooxygenase enzymes, a soluble diiron centre isoprene monooxygenase and a putative propane monooxygenase. Attempts to retrieve sequences for the isoprene monooxygenase alpha subunit using PCR had been previously unsuccessful (see Chapter 6), therefore genome sequencing of these isolates was used as an alternative approach to obtain sequences for *isoA.*

5.2 The genome of *Gordonia polyisoprenivorans* i37

Genomic DNA was prepared and the genome sequenced as described in Materials and Methods section 2.6

The genome consists of 6,308,839bp assembled into 458 contigs. RAST has annotated genes within 374 subsystems (Table 5.1), with subsystem coverage of 33%. 49% of gene sequences not allocated to a subsystem were hypothetical proteins. The genome contains 5584 coding sequences and 58 tRNAs.

Table 5.1: Overall numbers of genes within subsystems for *Gordonia polyisoprenivorans*

Subsystem	Number of genes
Cofactors, Vitamins, Prosthetic Groups, Pigments	372
Cell Wall and Capsule	62
Virulence, Disease and Defence	41
Potassium metabolism	15
Photosynthesis	0
Miscellaneous	47
Phages, Prophages, Transposable elements, Plasmids	0
Membrane transport	60
Iron acquisition and metabolism	18
RNA metabolism	78
Nucleosides and Nucleotides	102
Protein metabolism	186
Cell Division and Cell Cycle	21
Motility and Chemotaxis	5
Regulation and Cell signalling	47
Secondary metabolism	4
DNA metabolism	92
Regulons	0
Fatty Acids, Lipids and Isoprenoids	302
Nitrogen metabolism	31
Dormancy and Sporulation	3
Respiration	126
Stress response	111
Metabolism of Aromatic Compounds	88
Amino Acids and Derivatives	487
Sulfur Metabolism	66
Phosphorous Metabolism	32
Carbohydrates	518

5.2.1 Analysis of histidine biosynthesis pathways and aminoacyl tRNA biosynthesis in *Gordonia polyisoprenivorans*

The quality of the incomplete *Gordonia* genome was assessed using amino acid biosynthesis pathways and aminoacyl tRNAs as indicators. *Gordonia polyisoprenivorans* is a non-auxotroph, and therefore should contain genes encoding complete amino acid

biosynthesis pathways. Histidine biosynthesis was chosen due to the large number of characterised enzymes involved in this pathway (Table 5.2).

Table 5.2: Histidine biosynthesis genes in *Gordonia polyisoprenivorans* genome

Histidine biosynthesis gene in *E.coli* K12	**Present in genome?**
ATP phosphoribosyltransferase *hisG*	+
Phosphoribosyl-ATP pyrophosphatase *hisI*	+
Phosphoribosyl CMP cyclohydrolase *hisE*	+
N-(5'-phospho-L-ribosyl-formimino)-5-amino-1-(5'-phosphoribosyl)-4-imidazolecarboxamide isomerase *hisA*	+
Imidazole glycerol phosphate synthase *HisH, HisF*	+
Imidazole glycerol-phosphate dehydratase / histidinol-phosphatase *hisB*	+
Histidinol-phosphate aminotransferase *hisC*	+
Histidinol dehydrogenase *hisD*	+

The *Gordonia* genome also contained aminoacyl tRNAs for all 20 amino acids, with 58 tRNAs overall. This is identical to the number found in the genome of *Gordonia bronchialis* DSM 43247. While this cannot be taken as confirmation that the genome is 'complete' it does provide further evidence that it has sufficient coverage to mine effectively for genes of interest. It is however important to remember that this organism may still contain genes not found in this genome sequence.

5.2.2 Analysis of *Gordonia polyisoprenivorans* isoprene gene cluster sequences

The *Gordonia* genome was screened for putative isoprene degradation related gene sequences related to those found in the isoprene degradation gene cluster of *Rhodococcus globerulus* AD45. The entire isoprene gene cluster was found (Figure 5.1), with an additional unique copy of *isoG* located in a different contig and with no apparent isoprene related genomic context and sharing 72% nucleotide identity with the copy of *isoG* located in the isoprene gene cluster. Table 5.3 gives the nucleotide and amino acid identities compared to *Rhodococcus* AD45. In addition to the isoprene gene cluster described in *Rhodococcus* AD45 (van Hylckama Vlieg *et al.* 2000), the isoprene cluster in i37 is flanked 5' by a glutamate-cysteine ligase precursor, which is the first enzyme in the

biosynthesis of glutathione, and 3' by a glutathione synthetase, which is consistent with recent unpublished genome data collected for *Rhodococcus* AD45 and suggests a potential role for the synthesis of glutathione during isoprene degradation, in particular the breaking of the epoxyisoprene ring in *Rhodococcus* AD45 by conjugation to glutathione. There are two annotated aldehyde dehydrogenase genes within the cluster, the first, immediately 5' of *isoA* and the isoprene monooxygenase structural genes, shows 74% amino acid identity to an aldehyde dehydrogenase from *Rhodococcus* sp. JVH1, the second immediately 3' of *isoF* with 58% amino acid identity to JVH1.

Figure 5.1: Organisation of isoprene gene cluster in *Gordonia polyisoprenivorans* i37

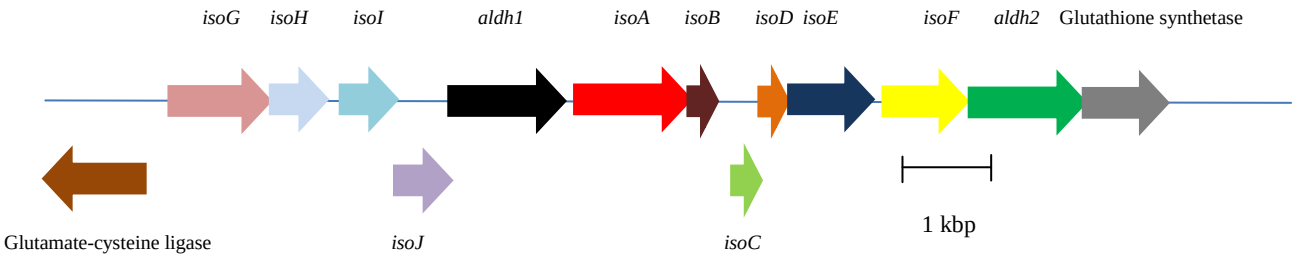


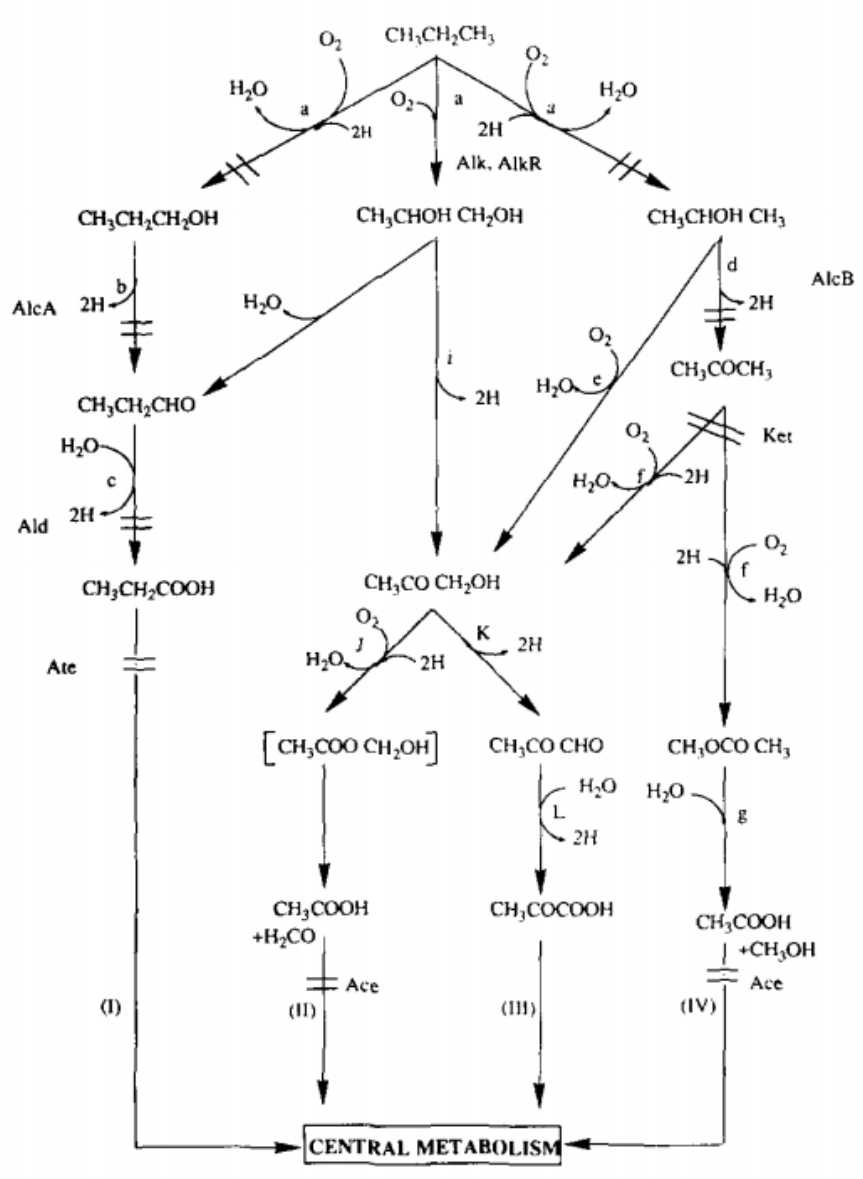
Table 5.3: Nucleotide and amino acid identities of isoprene degradation genes found in the *Gordonia polyisoprenivorans* i37 genome compared to *Rhodococcus* AD45

Gene	Gene product function	% Nucleotide identity to AD45	% Amino acid identity to AD45
isoA	Isoprene monooxygenase Alpha subunit	81	86
isoB	Gamma subunit	63	57
isoC	Ferredoxin	77	78
isoD	Effector/coupling protein	73	71
isoE	Beta subunit	72	71
isoF	Reductase	66	64
isoG1	Racemase	75	78
isoG2	Racemase	71	72
isoH	Dehydrogenase	73	79
isoI	Glutathione-S-transferase	79	80
isoJ	Glutathione-S-transferase	70	71

5.2.3 Analysis of propane monooxygenase gene sequences in *Gordonia polyisoprenivorans*

Gordonia polyisoprenivorans i37 grows well on propane and has been confirmed to oxidize both 1- and 2-propanol (see Chapter 4), suggesting an ability to oxidize propane via both the terminal and subterminal propane oxidation pathways (Figure 5.2). However, no propane monooxygenase polypeptides were identified by proteomics when *Gordonia* was grown on propane as a sole carbon source. Therefore, the genome was screened for putative propane monooxygenase genes as well as genes related to subterminal propane oxidation in the related propane degrading species *Gordonia* TY5 (Kotani *et al.* 2003)

Figure 5.2: Terminal and subterminal oxidation routes of propane metabolism in *Rhodococcus rhodochrous* PNKb1



(Ashraf and Murrell. 1990)

Table 5.4: Nucleotide and amino acid identities of putative propane related genes found in *Gordonia polyisoprenivorans* i37 genome compared to *Gordonia* TY5

Gene	Nucleotide identity to *Gordonia* TY5	Amino acid identity to *Gordonia* TY5	Contig number
prmA (propane monooxygenase hydroxylase large subunit)	99	99	127
prmB (reductase)	99	91	127
prmC (hydroxylase small subunit)	99	98	127
prmD (coupling protein)	99	100	127
adhI (alcohol dehydrogenase)	99	99	127
acma (acetone monooxygenase)	96	98	004
acmb (methyl acetate hydrolase)	96	99	004
orf1 putative alcohol dehydrogenase	97	99	004
orf2 putative LuxR transcriptional regulator	97	98	004

A putative propane monooxygenase gene cluster was found in the genome with high sequence similarity to that found in *Gordonia* TY5, as shown in Table 5.4. In *Gordonia* TY5, propane is oxidized via the subterminal pathway to form acetone. This organism also contains an acetone monooxygenase AcmA, which catalyses the oxidation of acetone to methyl acetate, and a methyl acetate hydrolase AcmB, which hydrolyzes methyl acetate to acetic acid and methanol (Ashraf and Murrell. 1990)(see Figure 5.2). The presence of these two genes in the genome of i37 shows genetic potential for similar subterminal oxidation

of propane via the pathway: Propane → 2-propanol → Acetone → Methyl acetate → Acetic acid + methanol, which supports the oxygen electrode assay data collected for this organism (Chapter 4 Table 4.3.5).

A gene encoding a putative GroEL chaperone protein is located immediately downstream of the putative alcohol dehydrogenase. This arrangement is also seen in the genome of *Gordonia* TY5 (Kotani *et al.* 2003), and has been associated with stress-induced upregulation of *prm* genes in *Rhodococcus* RHA1 (Sharp *et al.* 2007).

5.2.4 Analysis of polyisoprene degradation-related gene sequences in *Gordonia polyisoprenivorans* i37

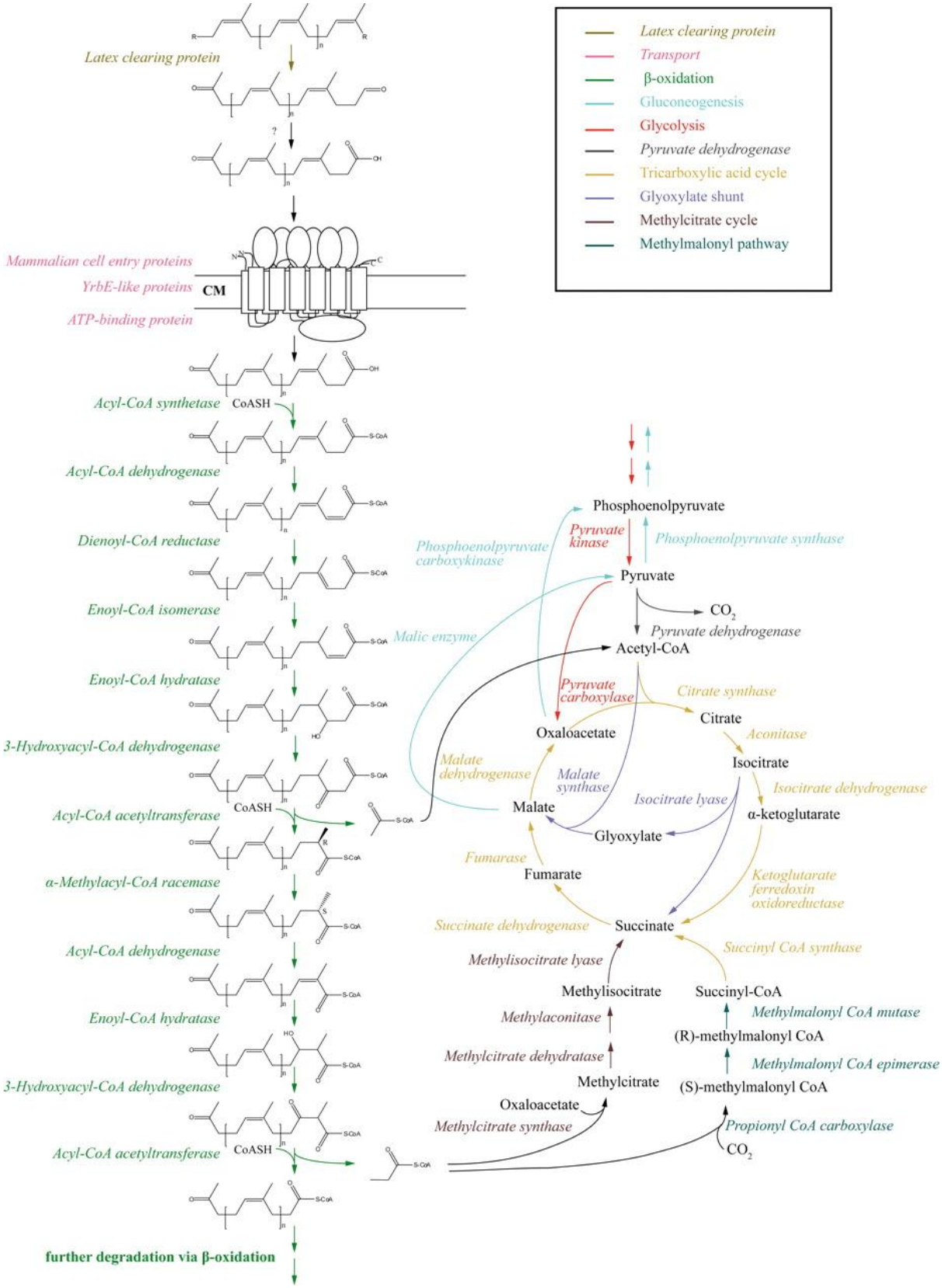
The *cis*-isomer of polyisoprene is the main component of natural rubber and has a number of important industrial applications. The trans polymer [poly(trans-1,4-isoprene)] is known as the main component of gutta percha, another industrially relevant compound used in golf balls and root canal fillings. The rubber degradation pathway of *Gordonia polyisoprenivorans* VH2 was mentioned in Chapter 1. In contrast, very little is known about the degradation of gutta percha (GP), and only six GP degrading isolates are known (Warneke *et al.* 2007). All GP-degrading isolates also decompose natural rubber, and transposon mutagenesis studies suggest the degradation pathways are similar.

The *Gordonia polyisoprenivorans* i37 genome was screened for genes encoding latex-clearing proteins that are known to be responsible for the initial step in rubber degradation in the closely related *Gordonia polyisoprenivorans* strain VH2 (Hiessl *et al.* 2012). This gene is widespread in rubber degrading actinobacteria and encodes a product which cleaves the double bonds within the polymer to form short rubber oligomers with aldehyde and keto groups at their termini. The i37 genome contains a latex clearing protein with 98% nucleotide and 99% amino acid identity to that of the latex clearing protein of *G.polyisoprenivorans* VH2. The genome was also screened for a second enzyme, RoxA (rubber oxygenase) which catalyses the initial cleavage step in a few Gram-negative bacteria (Seidel *et al.* 2013). The *roxA* gene from the rubber degrading *Xanthomonas* sp.35Y was used as a query sequence, with no matches found in the *Gordonia polyisoprenivorans* i37 genome. The *Gordonia polyisoprenivorans* i37 genome also contains a cluster of five *mce* genes in an identical arrangement to that found in *Gordonia polyisoprenivorans* VH2. These *mce* clusters are thought to encode transporters that

transport the cleavage products of rubber degradation into cells for further metabolism (Hiessl *et al.* 2012).

A putative TetR family transcriptional regulator is located immediately upstream of the *lcp* gene. Such regulators are found upstream of *lcp* in the genomes of almost all polyisoprene degrading bacteria and are thought to play an important role in the regulation of *lcp* transcription. In both *Gordonia polyisoprenivorans* VH2 and i37, a cluster of genes involved in the biosynthesis of phytoene, is located directly upstream of *lcp.* Phytoene is a 40-carbon intermediate in the synthesis of carotenoids, which are tetraterpanoids consisting of 8 isoprene units. These genes include an annotated geranylgeranyl diphosphate synthase, which catalyzes the synthesis of GGPP from farnesyl diphosphate and isopentenyl diphosphate, an annotated isopentenyl-diphosphate delta-isomerase gene, which catalyses the conversion of IPP to DMAPP in the mevalonate pathway of isoprenoid biosynthesis, and a gene encoding phytoene synthase, which catalyzes the condensation of two molecules of geranylgeranyl diphosphate (GGPP) to give prephytoene diphosphate (PPPP) and the subsequent rearrangement of the cyclopropylcarbinyl intermediate to phytoene (Iwata-Reuyl *et al.* 2003). The phytoene synthase enzyme possesses a similar structure to squalene synthase. Clear zone- forming polyisoprene degraders are known to be unable to grow on squalene (Rose and Steinbüchel, 2005). Organic acids produced by degradation of polyisoprene subsequently enter β-oxidation and central carbon metabolism (Figure 5.3).

Figure 5.3: Predicted pathway of poly(*cis*-1,4-isoprene) degradation in *Gordonia polyisoprenivorans* VH2



(Taken from Hiessl *et al.* 2012)

Additionally, the *Gordonia polyisoprenivorans* i37 genome was screened for a homologue of the extracellular superoxide dismutase SodA, which is thought to provide protection against reactive oxygen intermediates during polyisoprene degradation (Schulte *et al.* 2008). The genome contained a putative *sodA* gene with 97% nucleotide identity to that found in *Gordonia* VH2.

5.2.5 Other monooxygenases present in the *Gordonia polyisoprenivorans* i37 genome

The genome of *Gordonia polyisoprenivorans* i37 contained several annotated putative monooxygenase genes, however BLAST searches returned only similar putative genes from genomes of related organisms, therefore no potential function could be assigned.

Cytochrome p450-type monooxygenases

A cluster encoding a potential cytochrome p450 type monooxygenase system was identified, containing genes encoding a cytochrome p450 monooxygenase, cytochrome p450 hydroxylase, ferredoxin and short chain dehydrogenase. Cytochrome p450 monooxygenases are widespread among actinomycetes and can oxidize a broad range of substrates including many xenobiotics (O'Keefe and Harder. 1991) and are widely used in bioremediation (van Beilen and Funhoff. 2007).

AlkB-type alkane hydroxylases

The *Gordonia* i37 genome was also checked for alkB alkane hydroxylases, which are integral membrane non-heme diiron monooxygenases found in a wide range of Proteobacteria and Actinomycetales. These enzymes require a reduced rubredoxin. C_6-C_{22} compounds have been observed as substrates. The genome was screened using *alkB* genes from *Gordonia alkanivorans* and *Gordonia westfalica* as query BLAST sequences, with no significant hits found.

Long-chain alkane monooxygenases

The genome was screened for genes encoding the flavin-binding long chain alkane monooxygenase gene *almA* using *Acinetobacter* and *Rhodoccus qingshengii almA* genes as query BLAST sequences. No genes were found with sequence similarity to *almA.*

Phenol hydroxylase

The isolate *Gordonia polyisoprenivorans* i37 can grow on phenol as a sole carbon source. Phenol-2-monooxygenase, also known as phenol hydroxylase, is an FAD-dependent oxidoreductase that catalyses the reaction:

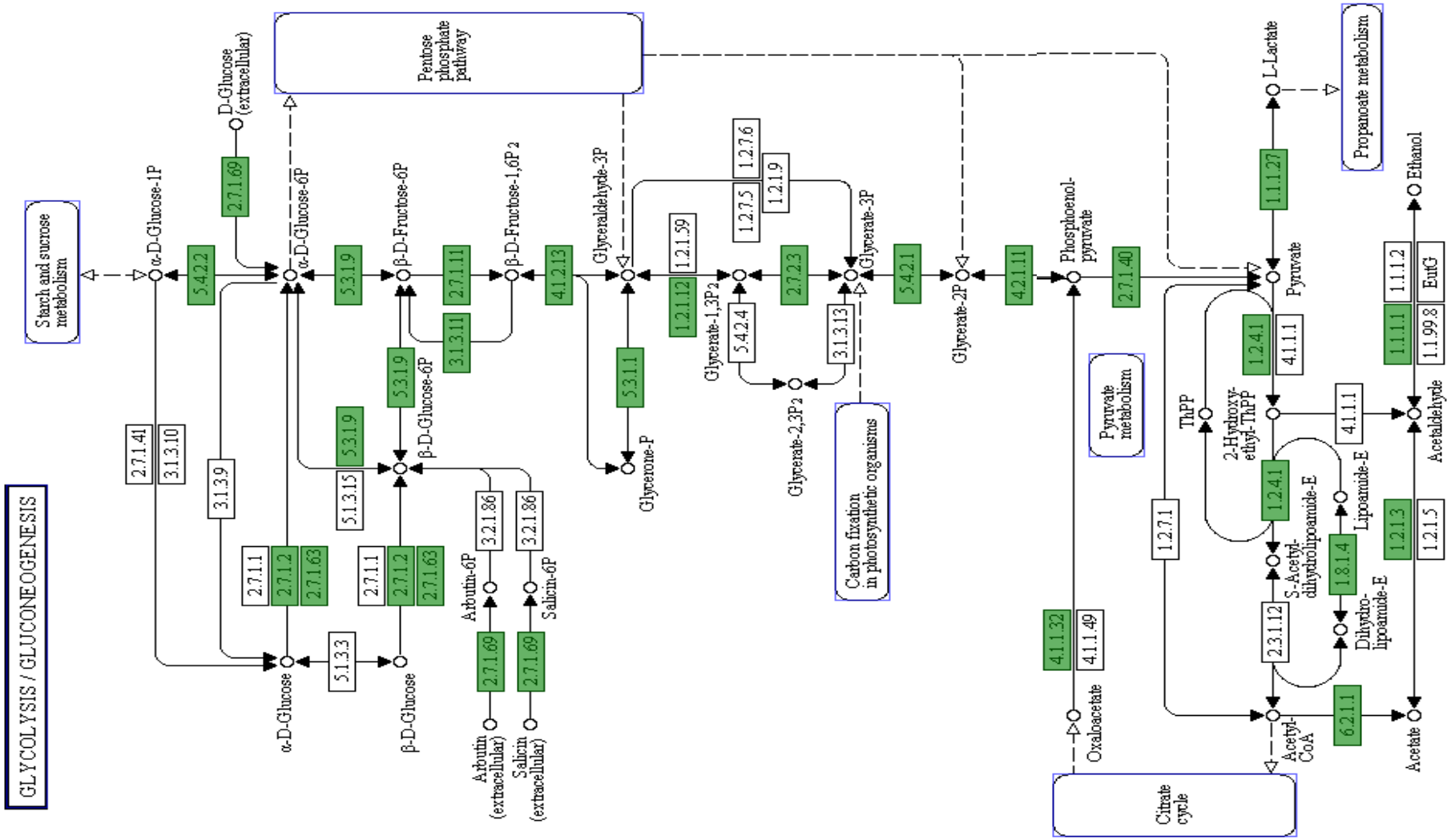
Phenol + NADPH + H^+ + O_2 ⇌ catechol + $NADP^+$ + H_2O.

This enzyme has been characterised as a two-component flavin dependent monooxygenase in *Rhodococcus erythropolis* UPV-1 (Saa *et al.* 2010), with the two proteins encoded by the genes *pheA1* and *pheA2*. *pheA1* was used as a query BLAST sequence against the *Gordonia polyisoprenivorans* i37 genome. A putative phenol hydroxylase was found with 85% nucleotide identity to *Rhodococcus erythropolis* UPV-1.

5.2.6 Kegg metabolic pathway analysis for *Gordonia polyisoprenivorans*

The metabolic potential of *Gordonia polyisoprenivorans* i37 was determined using Kegg recruitment maps generated in The Seed Viewer on the RAST website. These provide useful information regarding the central metabolism of the organism for specific pathways such as those involved in central carbon and nitrogen metabolism. Examination of these pathways also provides further evidence that the sequence data obtained has sufficient coverage for effective genome mining.

Figure 5.4: Kegg recruitment plot showing the genes involved in glycolysis in the *Gordonia* i37 genome. Genes highlighted in green indicates presence in the genome.



The *Gordonia polyisoprenivorans* i37 genome contains the complete set of genes required for glycolysis as shown in Figure 5.4. Genes are present for enzymes that are required for glucose metabolism into α-D-glucose-6-phosphate (2.7.1.2, 5.3.1.9) which is converted into D-glyceraldehyde-3-phosphate, as well as acting as the initial intermediate for the pentose phosphate pathway as an alternative to glycolysis. The D-glyceraldehyde-3-phosphate can be converted into pyruvate by the enzymes 1.2.1.12, 2.7.2.3, 5.4.2.1, 4.2.1.11 and 2.7.1.40, via the intermediates fructose-1,6-bisphosphate and glyceraldehyde-3-phosphate. Several enzymes (1.2.4.1, 2.3.1.12 and 1.8.1.4) are needed to further metabolise pyruvate to Acetyl CoA in order to enter the TCA cycle to yield more ATP and complete conversion to CO_2. Of these enzymes, the gene encoding 2.3.1.12 (dihydrolipoamide acetyltransferase) is missing, possible due to the genome being incomplete, or that in this organism Acetyl CoA is generated from oxaloacetate in the TCA cycle, and is also a product of fatty acid metabolism.

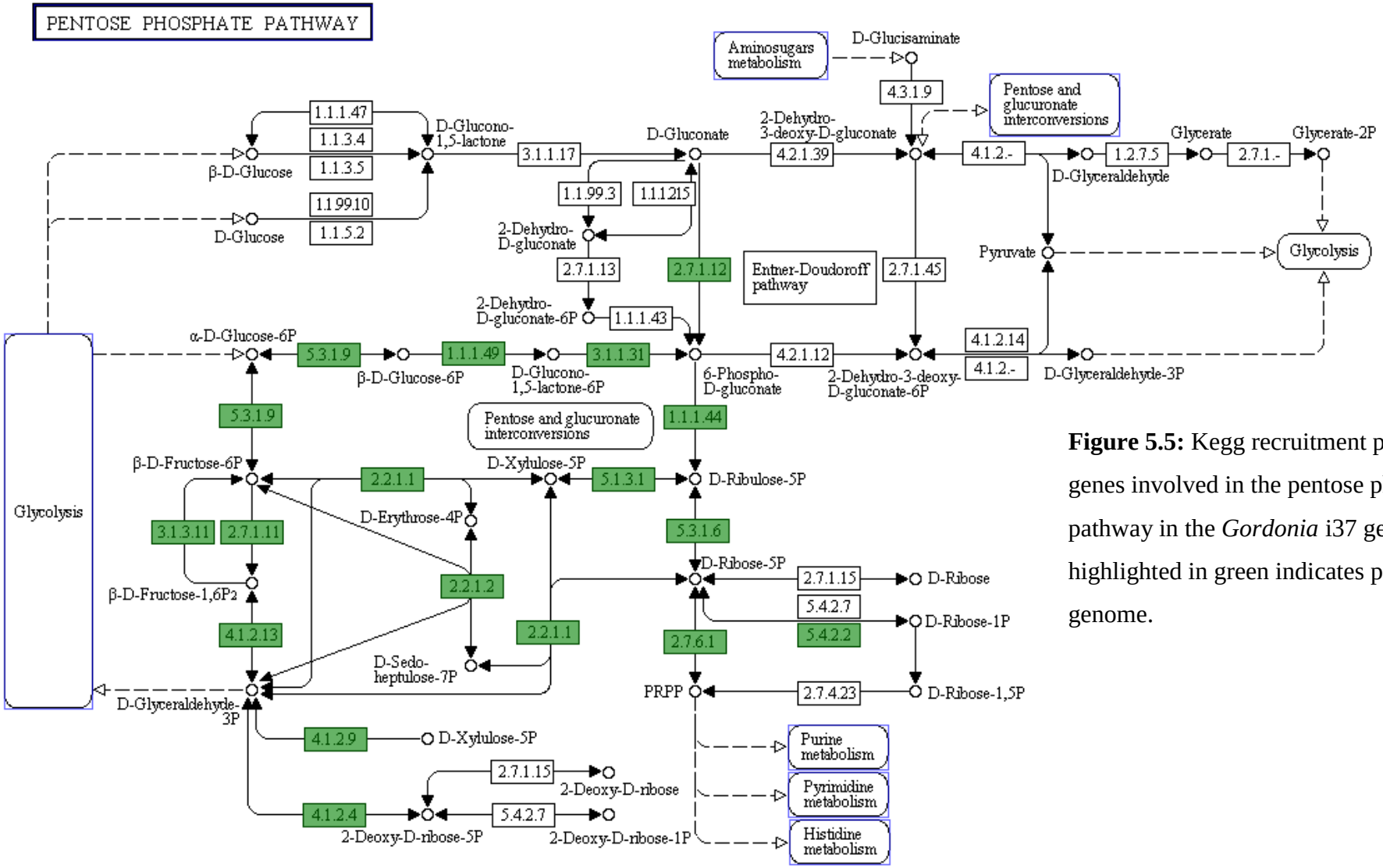


Figure 5.5: Kegg recruitment plot showing the genes involved in the pentose phosphate pathway in the *Gordonia* i37 genome. Genes highlighted in green indicates presence in the genome.

The pentose phosphate pathway is a process that generates NADPH and 5-carbon sugars, including ribose-5-phosphate (used in the synthesis of nucleotides) and erythrose-4-phosphate (used in the synthesis of aromatic amino acids). There are two phases in the pathway, the oxidative phase, in which NADPH is generated, and the non-oxidative synthesis of 5-carbon sugars. The *Gordonia* genome (see Figure 5.5) contains all of the genes (5.3.1.9 glucose-6-phosphate isomerase, 1.1.1.49 NADP-glucose-6-phosphate dehydrogenase, 3.1.1.31 6-phosphogluconolactonase, 1.1.1.44 phosphogluconate dehydrogenase, 5.3.1.6 ribose-5-phosphate isomerase, 5.1.3.1 ribulose-phosphate 3-epimerase, 5.3.1.9 glucose-6-phosphate isomerase, 2.2.1.1 transketolase, 2.2.1.2 transaldolase, 3.1.3.11 fructose-bisphosphatase, 2.7.1.11 6-phosphofructokinase, 4.1.2.13 fructose-bisphosphate aldolase, 4.1.2.9 phosphoketolase) necessary for this organism to carry out the reaction:

Glucose 6-phosphate + 2 $NADP^+$ + H_2O → ribulose 5-phosphate + 2 NADPH + 2 H^+ + CO_2 (oxidative phase)

3 ribulose-5-phosphate → 1 ribose-5-phosphate + 2 xylulose-5-phosphate → 2 fructose-6-phosphate + glyceraldehyde-3-phosphate (non oxidative).

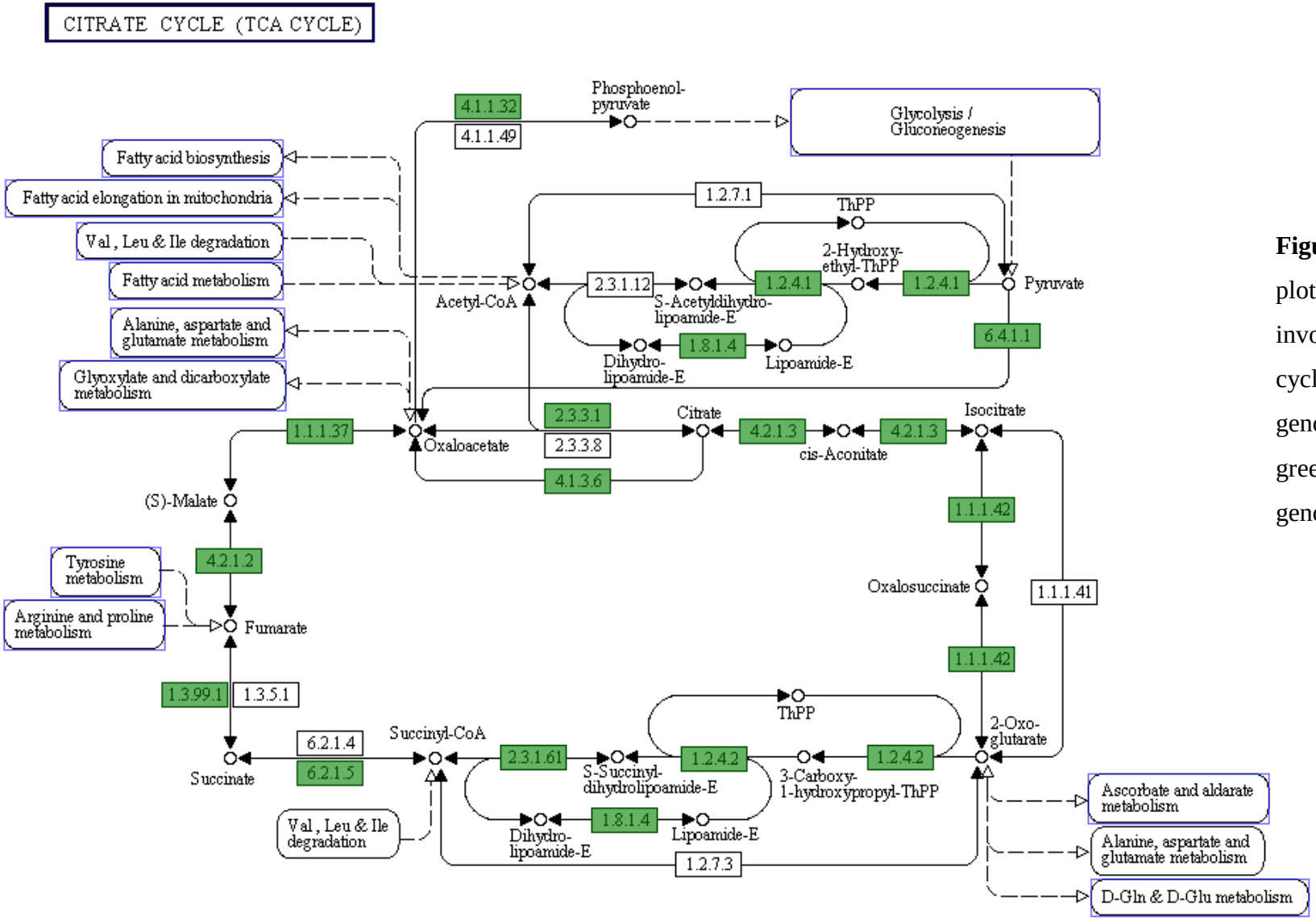


Figure 5.6 Kegg recruitment plot showing the genes involved in the citrate (TCA) cycle in the *Gordonia* i37 genome. Genes highlighted in green indicates presence in the genome.

Pyruvate produced from glycolysis feeds into the TCA (Tricarboxylic acid) cycle, also known as the Krebs or citric acid cycle (Lowenstein. 1969). The TCA cycle is a key component of the pathways by which aerobic organisms produce energy through the formation of reducing equivalents. Pyruvate is carboxylated by pyruvate carboxylase (6.4.1.1) also with the addition of malate by malate dehydrogenase (1.1.1.37), to form oxaloacetate which enters the cycle, or into acetyl-CoA as described previously for pyruvate metabolism. Genes encoding both enzymes 6.4.1.1 and 1.1.1.37 can be found in the *Gordonia polyisoprenivorans* i37 genome (see Figure 5.6). Both oxaloactate and acetyl-CoA feed into the TCA cycle to produce citrate via citrate synthase. There are a complete set of genes present in the *Gordonia* i37 genome that encode for all of the necessary enzymes (4.2.1.3 aconitate hydratase, 1.1.1.41 isocitrate dehydrogenase, 1.1.1.42 isocitrate dehydrogenase, 1.2.4.2 oxoglutarate dehydrogenase, 2.3.1.61 dihydrolipoyl lysine-residue succinyltransferase, 1.8.1.4 dihydrolipoyl dehydrogenase, 6.2.1.5 succinyl-CoA synthetase, 1.3.99.1 succinate dehydrogenase, 4.2.1.2 fumarate hydratase and 1.1.1.37 malate dehydrogenase) that are required for the TCA cycle to convert citrate into malate and oxaloacetate and to produce reducing equivalents.

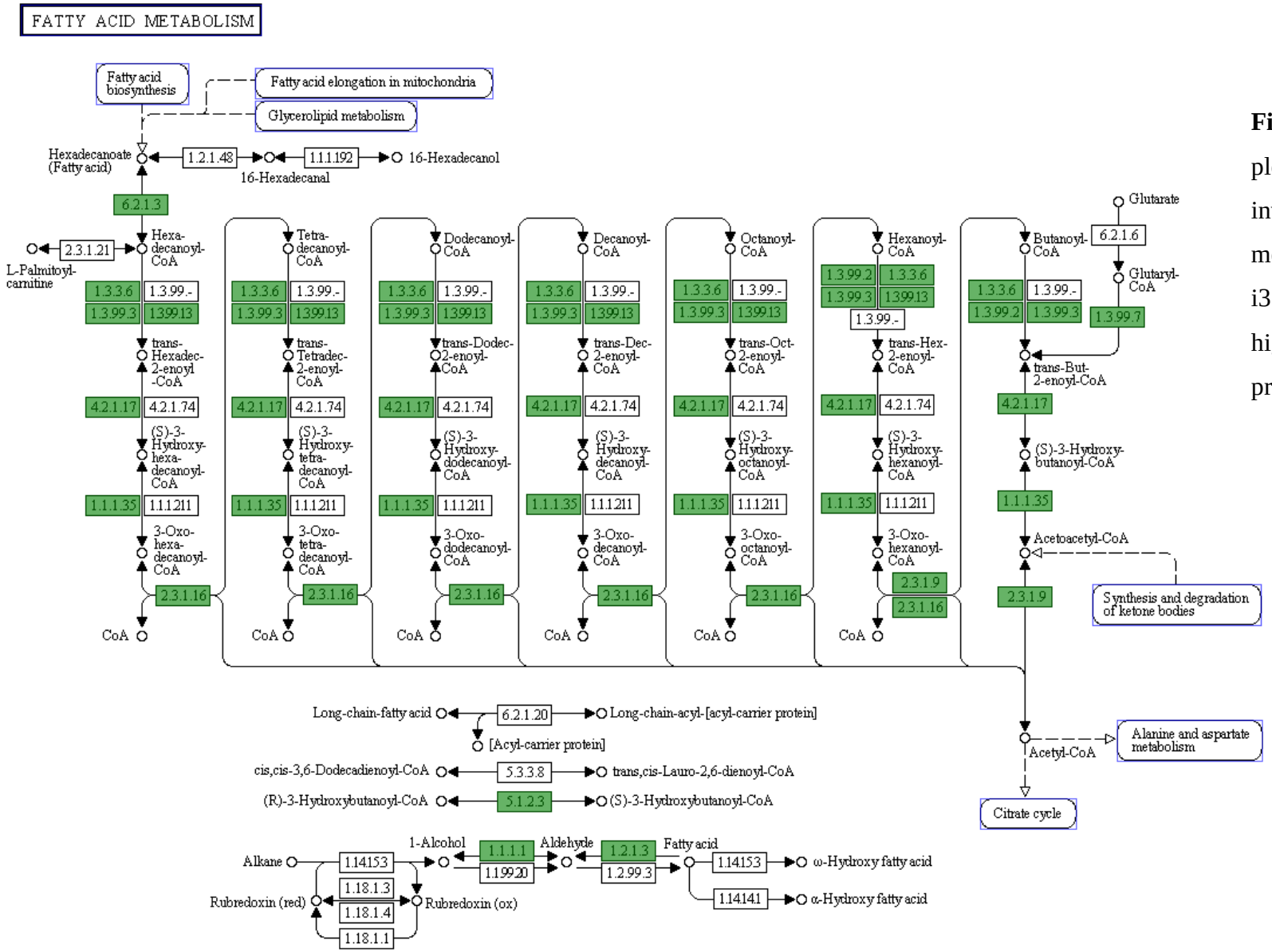


Figure 5.7: Kegg recruitment plot showing the genes involved in fatty acid metabolism in the *Gordonia* i37 genome. Genes highlighted in green indicates presence in the genome.

Figure 5.7 shows the Kegg recruitment plot for fatty acid metabolism in *Gordonia* i37. In the isoprene degradation pathway proposed for *Rhodococcus* AD45 (van Hylckama Vlieg *et al.* 2000), the product 2-glutathionyl-2-methyl-3-butenoic acid is converted to a fatty acyl-CoA which then enters β-oxidation. This proceeds via an oxidation step carried out by acyl-CoA dehydrogenase (1.3.99.3) followed by hydration to form a secondary alcohol, carried out by enoyl-CoA hydratase (4.2.1.17). Subsequently, 3-hydroxyacyl-CoA dehydrogenase (1.1.1.35) oxidizes this secondary alcohol to a ketone. Finally, acetyl-CoA is generated via Acetyl-CoA acetyltransferase (2.3.1.9) and can enter the citrate cycle.

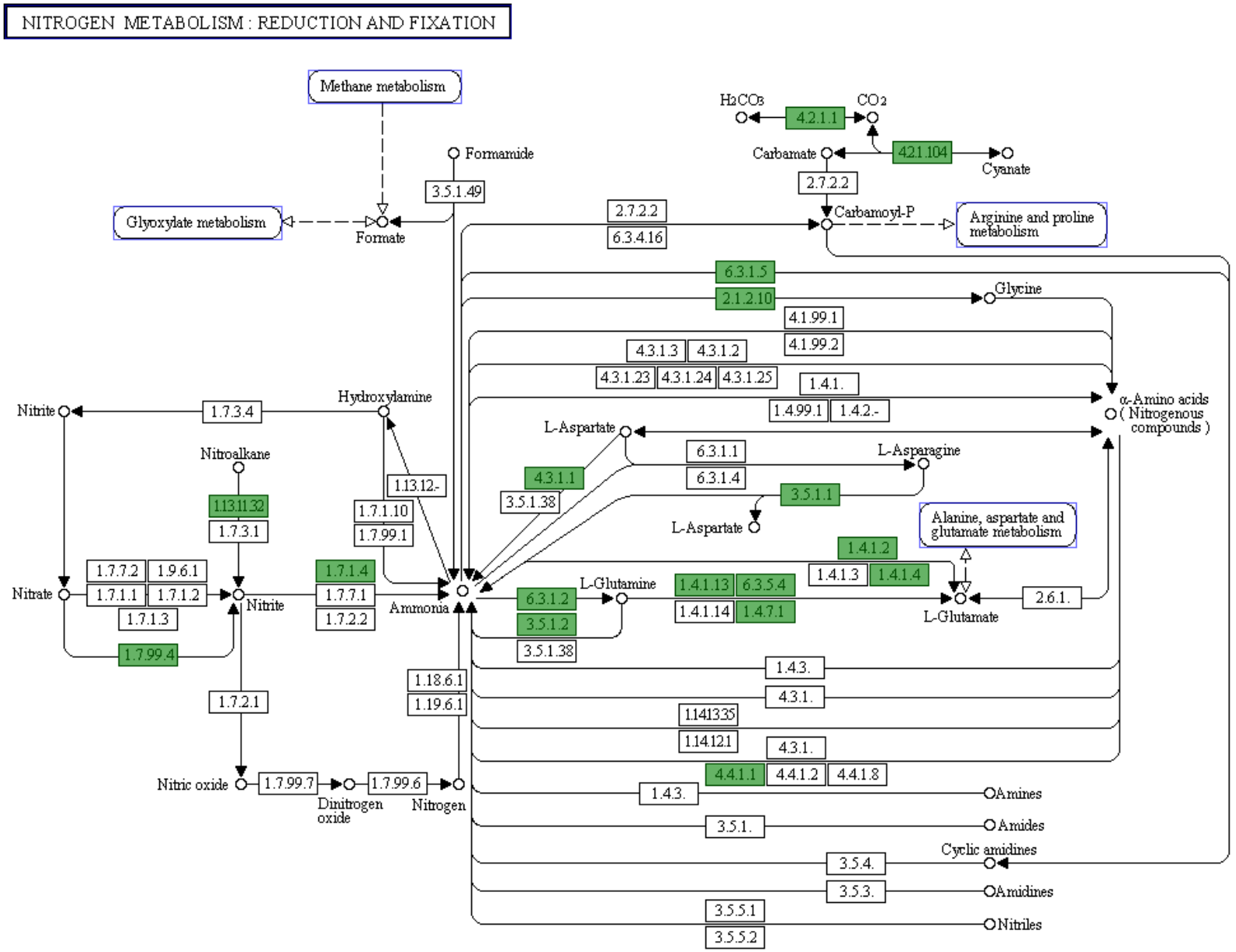


Figure 5.8 Kegg recruitment plot showing the genes involved in nitrogen metabolism in the *Gordonia* i37 genome. Genes highlighted in green indicates presence in the genome.

Gordonia also contains genes encoding the enzymes assimilatory nitrate reductase (1.7.99.4) and nitrite reductase (1.7.1.4) (Figure 5.8) which reduce nitrate to nitrite and subsequently to ammonia. The genome does not contain genes which encode the enzymes involved in nitrification, which oxidize ammonia to nitrite (ammonia monooxygenase), or nitrite to nitric oxide, and this organism lacks a nitrogenase enzyme to allow fixation of nitrogen gas to ammonia. There are several genes present in the *Gordonia* genome encoding enzymes for subsequent assimilation of ammonia, for example those involved in amino acid production via glycine (2.1.2.10 aminomethyltransferase) and the production of glutamine via ATP-dependent condensation of glutamate with ammonia by glutamine synthetase, and glutamate via an NADPH dependent glutamate synthase, in the GS-GOGAT pathway (6.3.1.2 glutamine synthetase, 1.4.1.13 glutamate synthase and 6.3.5.4 asparagine synthase). Not shown on this Kegg map is the enzyme 1.4.1.2, glutamate dehydrogenase, which catalyses the conversion of glutamate to alpha-ketoglutarate (Lightfoot *et al.* 1988).

5.3 The genome of *Mycobacterium hodleri* i29a2*

The 7,073,261 bp genome of *Mycobacterium hodleri* i29a2* assembled into 125 contigs, with 6875 coding sequences and 48 RNAs. 35% of sequences were within 424 defined subsystems, with 52% of genes not assigned to a subsystem annotated as hypothetical proteins (see Table 5.5). Of main interest within the *Mycobacterium hodleri* i29a2* were genes encoding putative isoprene and propane monooxygenase enzymes.

Table 5.5: Overall numbers of genes within subsystems for *Mycobacterium hodleri*

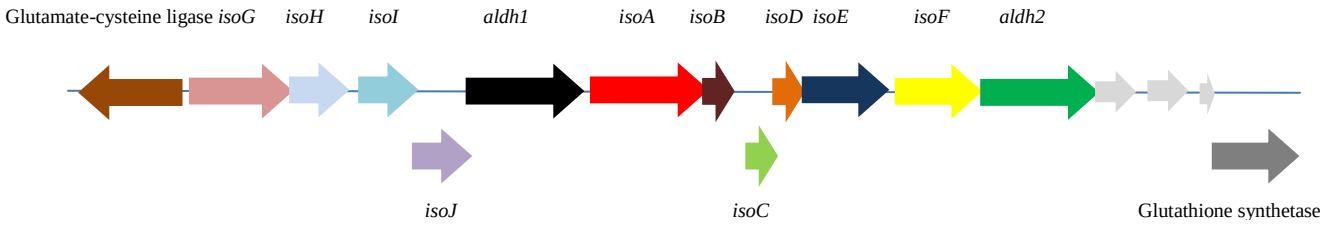
Subsystem	Number of genes
Cofactors, Vitamins, Prosthetic Groups, Pigments	479
Cell Wall and Capsule	90
Virulence, Disease and Defence	145
Potassium metabolism	19
Photosynthesis	0
Miscellanous	41
Phages, Prophages, Transposable elements, Plasmids	3
Membrane transport	84
Iron acquisition and metabolism	12
RNA metabolism	79
Nucleosides and Nucleotides	111
Protein metabolism	250
Cell Division and Cell Cycle	30
Motility and Chemotaxis	7
Regulation and Cell signalling	61
Secondary metabolism	12
DNA metabolism	140
Regulons	1
Fatty Acids, Lipids and Isoprenoids	385
Nitrogen metabolism	41
Dormancy and Sporulation	3
Respiration	183
Stress response	168
Metabolism of Aromatic Compounds	92
Amino Acids and Derivatives	716
Sulfur Metabolism	71
Phosphorous Metabolism	40
Carbohydrates	765

5.3.1 Analysis of isoprene gene cluster sequences in *Mycobacterium hodleri*

Table 5.6: Nucleotide and amino acid identities of isoprene degradation genes found in *Mycobacterium* genome compared to *Rhodococcus* AD45

Gene	Gene product function	% Nucleotide identity to AD45	% Amino acid identity to AD45
isoA	Isoprene monooxygenase alpha subunit	84	86
isoB	Gamma subunit	69	66
isoC	Ferredoxin	74	71
isoD	Effector/coupling protein	86	82
isoE	Beta subunit	74	74
isoF	Reductase	67	64
isoG	Racemase	78	83
isoH	Dehydrogenase	72	78
isoI	Glutathione-S-transferase	79	82
isoJ	Glutathione-S-transferase	73	74

Figure 5.9 Genomic arrangement of isoprene gene cluster in *Mycobacterium hodleri*



Like *Gordonia* i37, *Mycobacterium* i29a2* contains genes encoding the complete isoprene degradation cluster of enzymes, IsoA-J, in an almost identical arrangement (Figure 5.9). In both organisms, the structural monooxygenase genes *isoA-F* are flanked by two aldehyde dehydrogenases of unknown function. Both organisms also contain a glutamate-cysteine

ligase immediately upstream of *isoG*. *Mycobacterium hodleri* i29a2*also has a downstream gene encoding a glutathione synthetase, although in this organism it is separated from the main cluster by three hypothetical proteins of unknown function. Comparing the isoprene cluster for *Rhodococcus* AD45, *Gordonia* i37 and *Mycobacterium hodleri* i29a2*, the isoprene monooxygenase alpha subunit is the most highly conserved gene and protein, giving further evidence that this gene is ideal for use as an isoprene gene marker as the site of the conserved diiron ligand binding centre of the enzyme.

5.3.2 Analysis of propane monooxygenase gene sequences in *Mycobacterium hodleri*

Table 5.7: Nucleotide and amino acid identities of putative propane related genes found in *Mycobacterium hodleri* i29a2* genome compared to *Mycobacterium goodie* (Furaya *et al.* 2010).

Gene	**Nucleotide identity to *Mycobacterium goodii***	**Amino acid identity to *Mycobacterium goodie***
mimA (propane monooxygenase hydroxylase large subunit)	91	97
mimB (reductase)	86	88
mimC (hydroxylase small subunit)	87	92
mimD (coupling protein)	89	94
adhI (alcohol dehydrogenase) (**Comparison to *Gordonia* TY5**)	68	59

In oxygen electrode assays, *Mycobacterium hodleri* i29a2* has been shown to oxidize 2-propanol but not 1-propanol. This indicates subterminal propane oxidation via 2-propanol in this organism. Unlike *Gordonia polyisoprenivorans*, no TY5-like acetone monooxygenase was found in the genome of this organism. The propane monooxygenase

genes found in *Mycobacterium hodleri* show highest sequence identity to the *mimABCD* genes from *Mycobacterium smegmatis* and *Mycobacterium goodii,* which exhibit high amino acid similarities with propane monooxygenases from *Rhodococcus* sp. strain RHA1 (Sharp *et al.* 2007) and *Gordonia* sp. strain TY-5 (Kotani *et al.* 2003). *MimABCD* encode a binuclear iron monooxygenase consisting of an oxygenase large subunit, a reductase, an oxygenase small subunit, and a coupling protein, respectively. This enzyme is essential for propane and acetone metabolism in these bacteria (Furuya *et al.* 2010) as well as being able to oxidize phenol to hydroquinone in the presence of acetone (although the organisms, like *Mycobacterium hodleri,* cannot grow on phenol.) *Mycobacterium smegmatis* is capable of growth on 1-propanol; however this activity was shown to be independent of MimA in deletion mutants, whereas MimA deletion mutants lost the ability to grow on propane or acetone. It is therefore likely that the subterminal oxidation of propane and acetone in *Mycobacterium hodleri* is carried out by this Mim-like propane monooxygenase. Immediately downstream of these genes is an annotated threonine dehydrogenase, which when using BLAST analysis shows highest sequence identity to the alcohol dehydrogenase from *Gordonia* TY5, although the sequence identity is low (68%). The function cannot be determined with certainty, but it is possible that this dehydrogenase carries out a similar function in *Mycobacterium* as in *Gordonia.*

5.3.4 Analysis of monooxygenase enzyme systems in *Mycobacterium hodleri*

As mentioned in Chapter 4, *Mycobacterium* species are known to oxidize and grow on many short chain alkanes and alkenes as a carbon source. *Mycobacterium chubuense* NBB4 is particularly interesting in that it contains 6 different monooxygenase systems, including homologues of ethene MO (etnABCD), propene MO (pmoABCD), propane MO (smoABCD), butane MO (smoXYB1C1Z), cytochrome P450 (CYP153; fdx-cyp-fdr) and alkB (alkB-rubA1-rubA2) (Coleman *et al.* 2011). Table 5.8 below shows the nucleotide gene sequences from *M.chubuense* NBB4 that were used as query gene sequences in BLAST searches of the *Mycobacterium hodleri* genome:

Table 5.8: BLAST search query nucleotide sequences from *Mycobacterium chubuense* NBB4

Genbank accession number	Putative function
GU145557.1	Alkane hydroxylase gene cluster, partial sequence
DQ264727.1	Soluble di-iron monooxygenase alpha subunit (SDIMO *pmoC*) gene, partial cds
DQ264726.1	Soluble di-iron monooxygenase alpha subunit (SDIMO group 6) gene, partial cds
DQ264725.1	Soluble di-iron monooxygenase alpha subunit (SDIMO group 3) gene, partial cds
DQ264724.1	Soluble di-iron monooxygenase alpha subunit (SDIMO *etnC*) gene, partial cds
GU174750.1	Putative peroxide stress sensor protein, putative 1,3-propanediol dehydrogenase, putative nitrate/nitrite sensor protein, putative luxR-like virulence response regulator, putative propane monooxygenase alpha subunit, putative propane monooxygenase beta subunit, putative propane monooxygenase coupling protein, putative propane monooxygenase reductase, putative AdhC-like Zn- and NAD-dependent alcohol (hexanol) dehydrogenase, and putative hsp60-like chaperonin genes, complete cds; and putative peroxide stress sensor protein gene, partial cds
GU174754.1	Putative AraC family transcriptional regulator gene, partial cds; and putative ferredoxin, putative cytochrome P450, putative ferredoxin reductase, and putative TetR family transcriptional regulator genes, complete cds
GU174751.2	Fosmid insert sequence – copper containing hydrocarbon monooxygenase

No matches were found for the *M.chubuense* NBB4 SDIMO alpha subunit sequences in the *Mycobacterium hodleri* i29a2* genome using BLAST search. However, the *M.hodleri* i29a2* genome contains a putative alkane hydroxylase with 85% nucleotide and 81% amino acid identity to that of *M.chubuense* NBB4, and a putative cytochrome P450 with 82% nucleotide identity to that of NBB4. *M.chubuense* NBB4 can grow on alkanes (ethane, propane, butane, pentane, hexane, heptane, octane and hexadecane), alkenes (ethene, propene, butene), and aromatics (toluene), but does not grow on isoprene. This metabolic diversity is thought to be due to the unprecedented number and diversity of genes encoding monooxygenase enzymes, although the precise function of these monooxygenases remains unknown. *M.hodleri* i29a2* is presumed to oxidize isoprene via isoprene monooxygenase, which is absent in *M.chubuense* NBB4. A

As discussed previously, the propane monooxygenase genes found in the genome of *M.hodleri* i29a2* show highest sequence similarity to the propane monooxygenase of *M.smegmatis* and *M.goodii*. No match was found in the *M.hodleri* i29a2* genome using the propane monooxygenase gene from *M.chubuense* NBB4 as a query BLAST sequence, however an alcohol dehydrogenase with 83% nucleotide identity was found. Unfortunately, the monooxygenase genes in NBB4 have not been specifically associated with oxidation of a particular substrate, and therefore no conclusions can be drawn regarding the function of the putative cytochrome P450 and alkane hydroxylase in *M.hodleri* i29a2*. A sequence encoding a membrane-bound copper containing hydrocarbon monooxygenase in NBB4 was used as a BLAST query sequence (Coleman *et al.* 2012). A gene sequence was found in the genome of *Mycobacterium hodleri* i29a2* with 83% nucleotide identity to the NBB4 *hmo* genes. CuMMO enzymes, which form part of the ammonia monooxygenase superfamily, had previously been linked to oxidation of ethane in Actinobacteria, and evidence in NBB4 suggests that this enzyme is responsible for oxidation of C2 hydrocarbons. This enzyme is not present in the genome of *Gordonia polyisoprenivorans* i37, which cannot grow on ethane. Therefore, it is possible that *Mycobacterium hodleri* i29a2* can grow on ethane as a sole carbon source via oxidation by a copper containing hydrocarbon monooxygenase.

The genome of *Mycobacterium hodleri* i29a2* was screened for a putative phenol hydroxylase using *pheA1* from *Rhodococcus erythropolis* UPV-1 as a query BLAST sequence. No matching sequences were found.

5.3.4 Kegg metabolic pathway analysis for *Mycobacterium hodleri* i29a2*

A brief Kegg metabolic analysis was carried out for the *Mycobacterium hodleri* i29a2* genome, to assess any major differences in central carbon and nitrogen metabolism between *Mycobacterium* and *Gordonia.* A full analysis of metabolic potential was not done for either isolate, as for the purposes of this work the focus was mining for monooxygenase enzyme systems to ascertain potential for growth on alkanes/alkenes.

Like *Gordonia polyisoprenivorans* i37, *Mycobacterium hodleri* i29a2* contains a complete suite of genes required for glycolysis, the pentose phosphate pathway, the TCA cycle, fatty acid metabolism, nitrate and ammonia assimilation. No major differences were observed between the isolates. The Kegg recruitment plots showing central carbon, nitrogen and fatty acid metabolism in recruitment plots showing central carbon, nitrogen and fatty acid metabolism in *Mycobacterium hodleri* i29a2* are shown in Figures 5.10, 5.11, 5.12, 5.13 and 5.14.

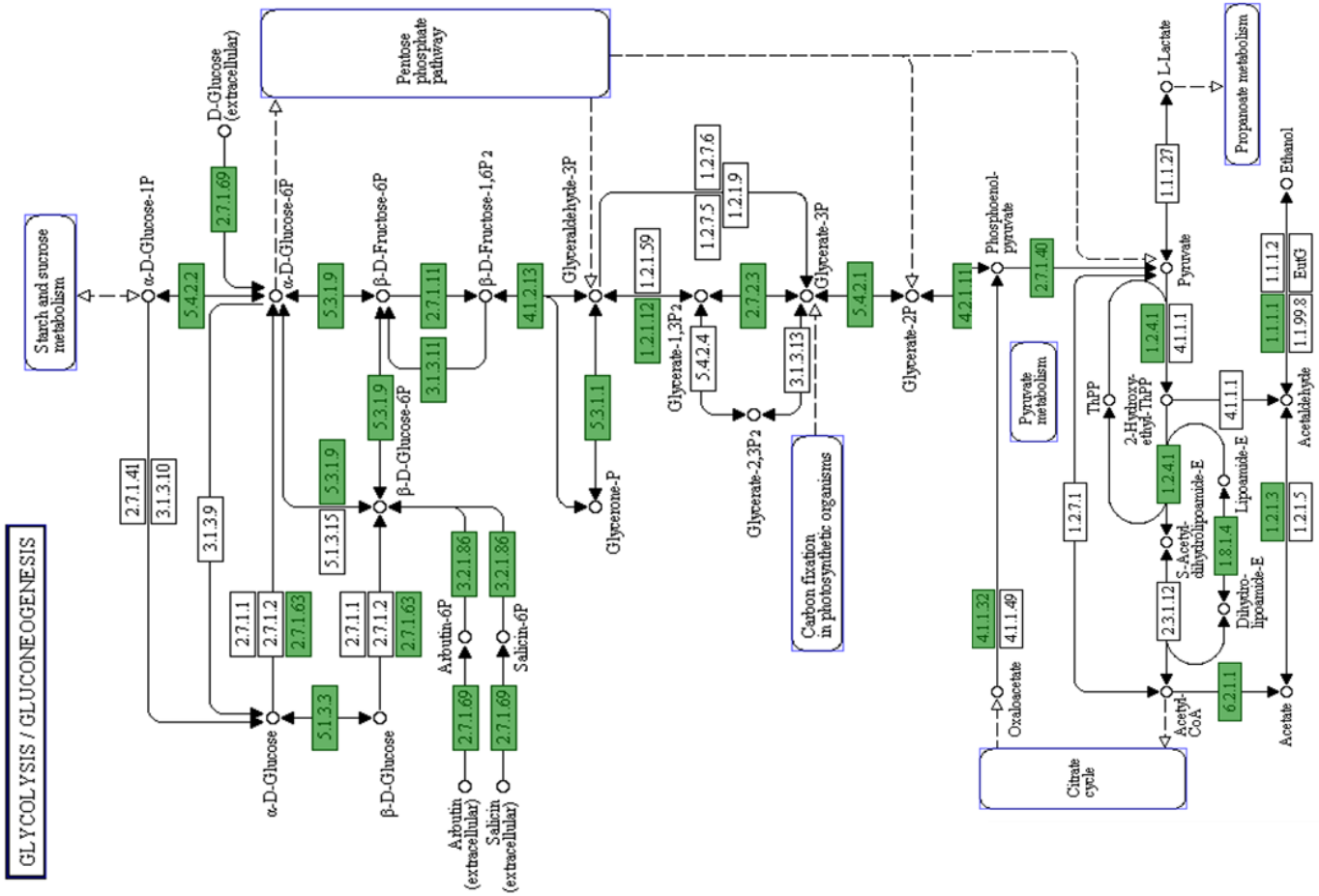


Figure 5.10: Kegg recruitment plot showing the genes involved in glycolysis in the *Mycobacterium hodleri* i29a2* genome. Genes highlighted in green indicates presence in the genome.

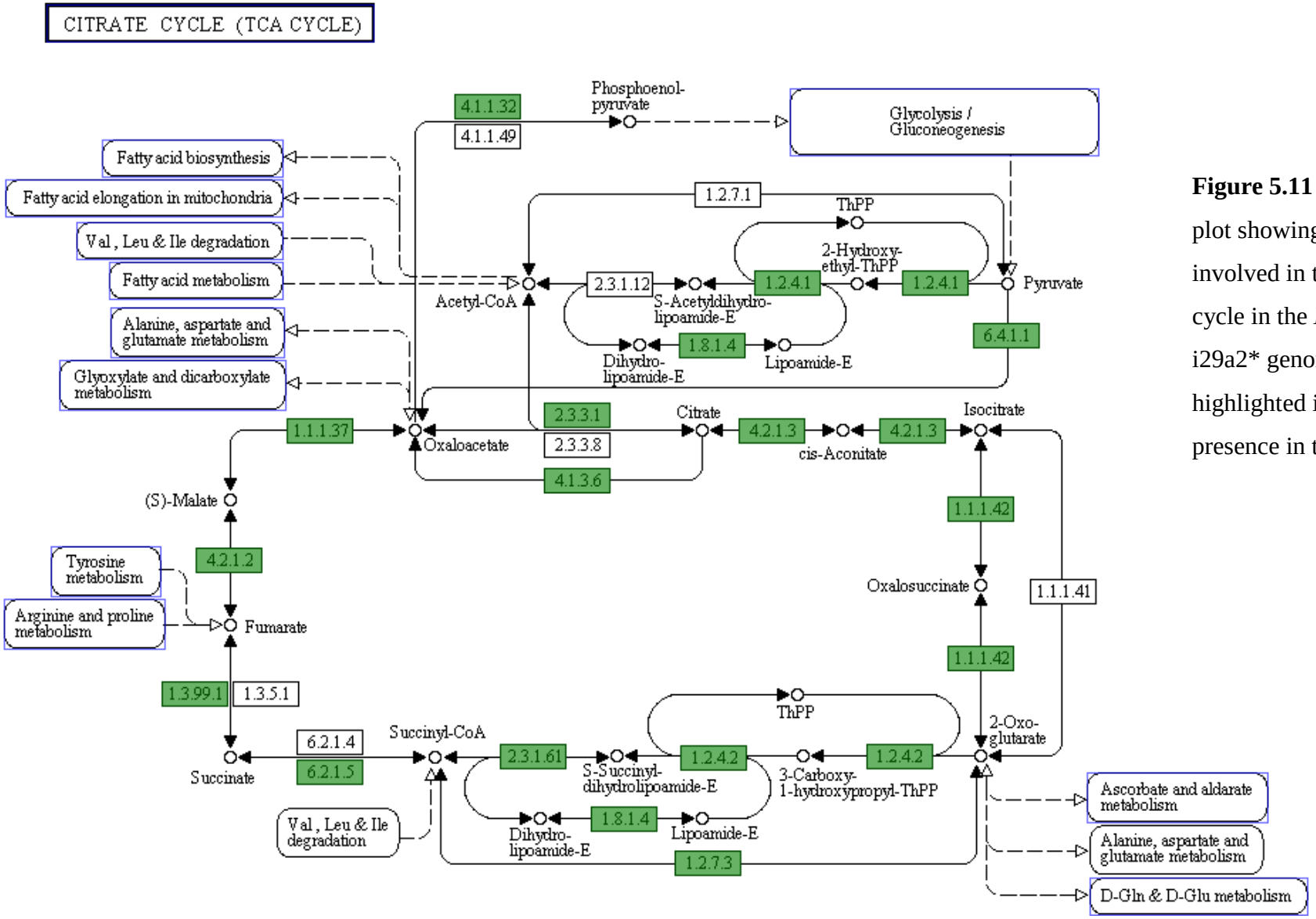


Figure 5.11 Kegg recruitment plot showing the genes involved in the citrate (TCA) cycle in the *Mycobacterium* i29a2* genome. Genes highlighted in green indicates presence in the genome.

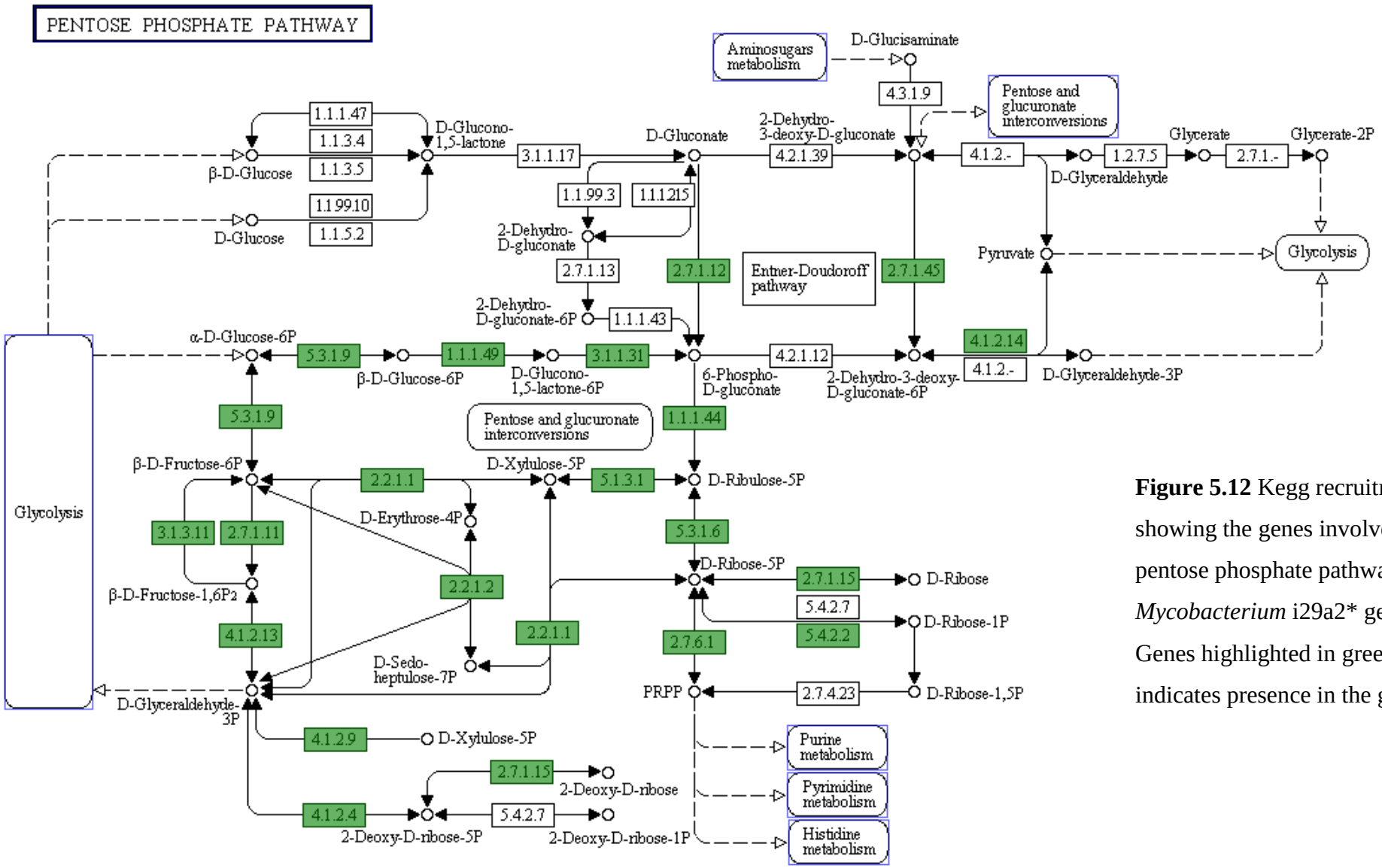


Figure 5.12 Kegg recruitment plot showing the genes involved in the pentose phosphate pathway in the *Mycobacterium* i29a2* genome. Genes highlighted in green indicates presence in the genome.

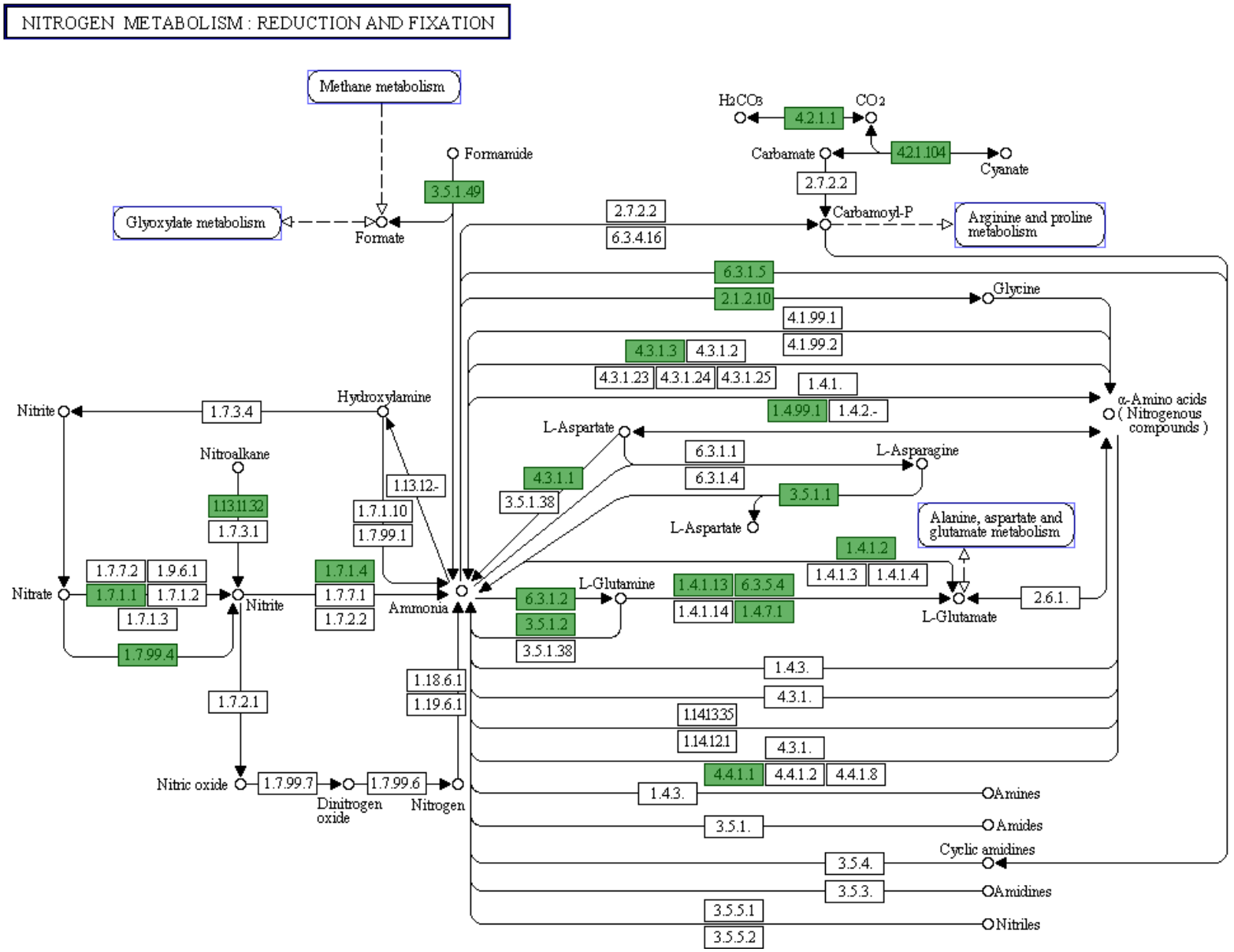


Figure 5.13: Kegg recruitment plot showing the genes involved in nitrogen metabolism in the *Mycobacterium* i29a2* genome. Genes highlighted in green indicates presence in the genome.

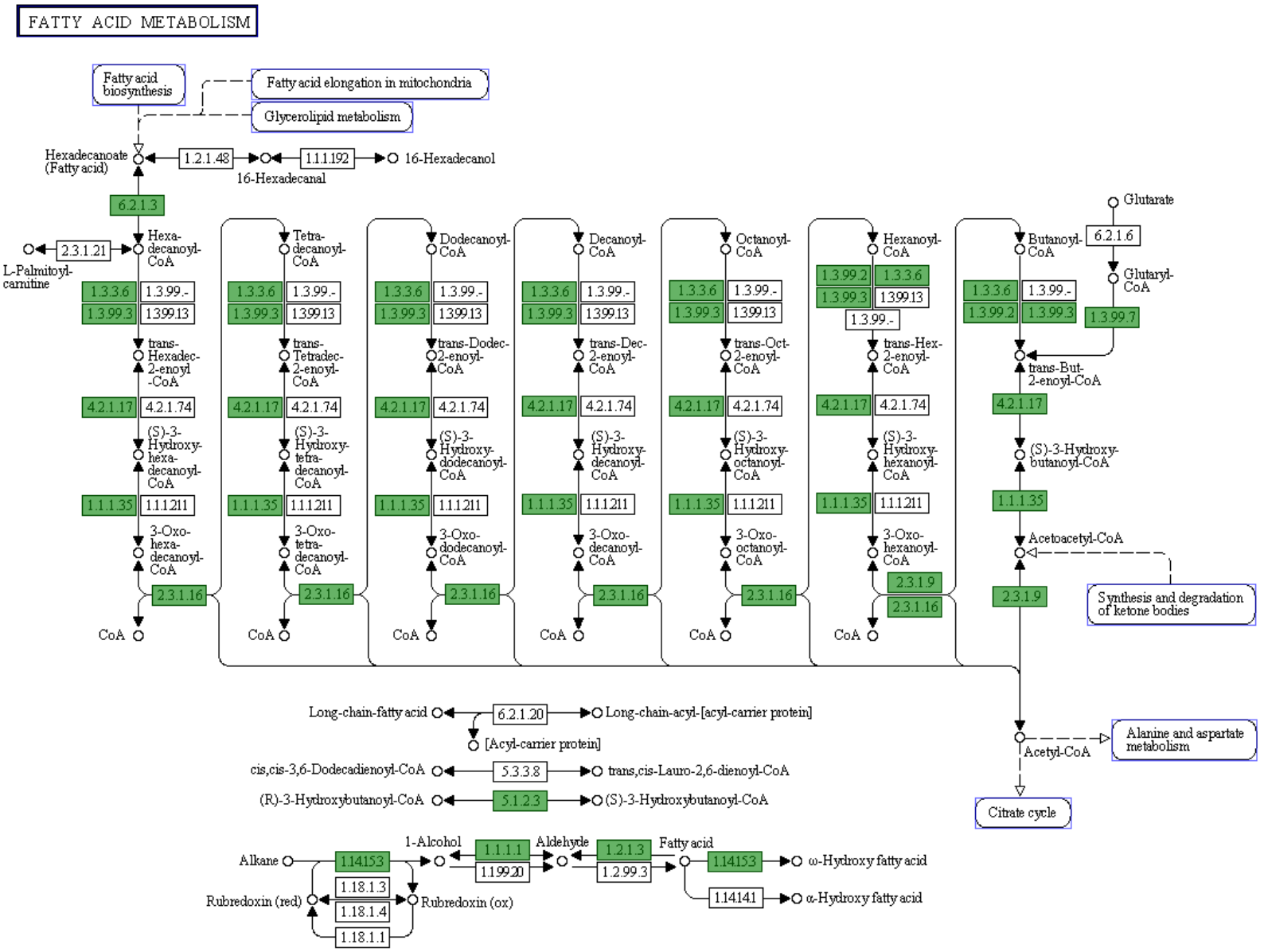


Figure 5.14: Kegg recruitment plot showing the genes involved in fatty acid metabolism in the *Mycobacterium* i29a2* genome. Genes highlighted in green indicates presence in the genome.

5. 4 Summary of Results

The primary objective of this work was to mine the genomes of *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2* to assess their metabolic potential and diversity. In particular, the analysis focused on monooxygenase enzyme systems, including soluble diiron centre monooxygenases, AlkB-type alkane hydroxylases and cytochrome P450 enzymes. This would allow further insight into the ability of these organisms to oxidize and grow on short chain alkanes, particularly propane, and would give further evidence that separate monooxygenase enzyme systems exist in these organisms, rather than a single multifunctional enzyme being responsible for oxidation of propane and isoprene. In both the *Gordonia* i37 and *Mycobacterium* i29a2* genomes, a putative propane monooxygenase was found with high sequence similarity to monooxygenases from other propane-degrading microorganisms within the Actinobacteria. This supports previous SDS-PAGE and proteomics data (see Chapter 4), which showed that the polypeptide profiles of isoprene and propane grown cells are different, as well as oxygen electrode data which showed different oxidation profiles of propane and isoprene grown cells. In *Gordonia* i37, transcription of *isoA* and *prmA* were shown to be induced during growth on isoprene and propane, respectively. Therefore, it is likely that *Gordonia* i37 and *Mycobacterium* i29a2* both oxidize propane via a soluble diiron centre propane monooxygenase enzyme.

In addition, the *Gordonia* i37 genome contained a Baeyer-Villiger type acetone monoxygenase with high sequence similarity to that found in *Gordonia* TY5. Therefore, based on oxygen electrode and growth data (see Table 4.3.1), it can be proposed that *Gordonia* i37 can oxidize propane via the subterminal pathway, via 2-propanol, acetone and methyl acetate to form acetic acid. *Gordonia* i37 can also oxidize and grow on 1-propanol as a sole carbon source, indicating that terminal propane oxidation also occurs in this isolate; however the enzymes by which it occurs remain unknown.

Overall, the suite of genes involved in central carbon and nitrogen metabolism was similar for both isolates. Many of these genes would be expected to be conserved across all bacterial species, and particularly in closely related genera such as *Gordonia* and *Mycobacterium.*

There are several key differences in the two isolates. Firstly, no Baeyer-Villiger type acetone monooxygenase was found in the genome of *Mycobacterium hodleri* i29a2*,

which would suggest that this isolate oxidizes propane via the characterised subterminal pathway, via acetone and propanoic acid (see Figure 5.2).

Secondly, *Gordonia polyisoprenivorans* i37 is able to grow on natural rubber as a sole carbon source while *Mycobacterium* i29a2* cannot (McGenity, unpublished). Several genera within the Actinobacteria are able to adhere to and grow on hydrophobic substances such as natural rubber due to the mycolic acids in their cell envelope. This difference between the isolates can be explained by the *lcp* genes being present only in the genome of *Gordonia* i37 and absent in *Mycobacterium* i29a2*.

This study confirmed that *Gordonia polyisoprenivorans* i37 can grow on phenol as a sole carbon source whereas *Mycobacterium hodleri* i29a2* cannot. *Gordonia* i37 may convert phenol to catechol via a phenol hydroxylase with high sequence identity to that found in *Rhodococcus erythropolis* UPV-1, which is absent in the genome of *Mycobacterium* i29a2*.

Finally, *Mycobacterium hodleri* i29a2* is able to grow on ethane as a sole carbon and energy source in addition to propane and isoprene. In *Mycobacterium* i29a2* and *Gordonia* i37, both isoprene and propane grown cells were able to oxidize ethane. The exact enzyme responsible remains unknown, however the genome of *Mycobacterium* i29a2* did not contain a putative soluble diiron ethane monooxygenase, suggesting that either ethane is an alternative substrate for the *Mycobacterium* i29a2* propane monooxygenase, or an alternative enzyme system exists in this isolate. The *Mycobacterium* i29a2* genome did contain a putative copper containing hydrocarbon monooxygenase with high nucleotide identity to that found in *Mycobacterium* NBB4. Growth of NBB4 in medium without copper inhibited growth of the organism on ethane, therefore a useful experiment would be to grow *Mycobacterium* i29a2* in medium with copper omitted, to test for inhibited growth on ethane.

Both genomes contained the full isoprene monooxygenase gene cluster with high sequence similarity to the isoprene degradation genes found in *Rhodococcus* AD45. In addition, both genomes were flanked 5’ by a glutamate-cysteine ligase precursor and 3’ by a glutathione synthetase. This arrangement can also be found in the genome of *Rhodococcus* AD45 (Khawand *et al.*unpublished). These enzymes may therefore be considered as part of the isoprene degradation gene cluster, with a potential role in the synthesis of glutathione during isoprene degradation.

The sequences for isoprene monooxygenase and propane monooxygenase retrieved from the genome of *Gordonia polyisoprenivorans* i37 allowed the design of PCR primers targeting isoprene monooxygenase and propane monooxygenase for qRT-PCR analysis of this isolate as described in Chapter 4.

Sequencing the genomes of these isolates allowed the retrieval of full length sequences encoding the alpha subunit of isoprene monooxygenase. This subunit contains the conserved catalytic binding site of the enzyme, and was therefore chosen as a functional gene marker for isoprene degradation. These gene sequences therefore allow the design of functional gene primers to probe environmental samples for *isoA*, to assess the abundance and diversity of isoprene degraders in marine samples as described in Chapter 6.

Chapter 6

Design and testing of a PCR primer set for the isoprene monooxygenase alpha subunit

Chapter 6: Design and testing of a PCR primer set based on isoprene monooxygenase alpha subunit

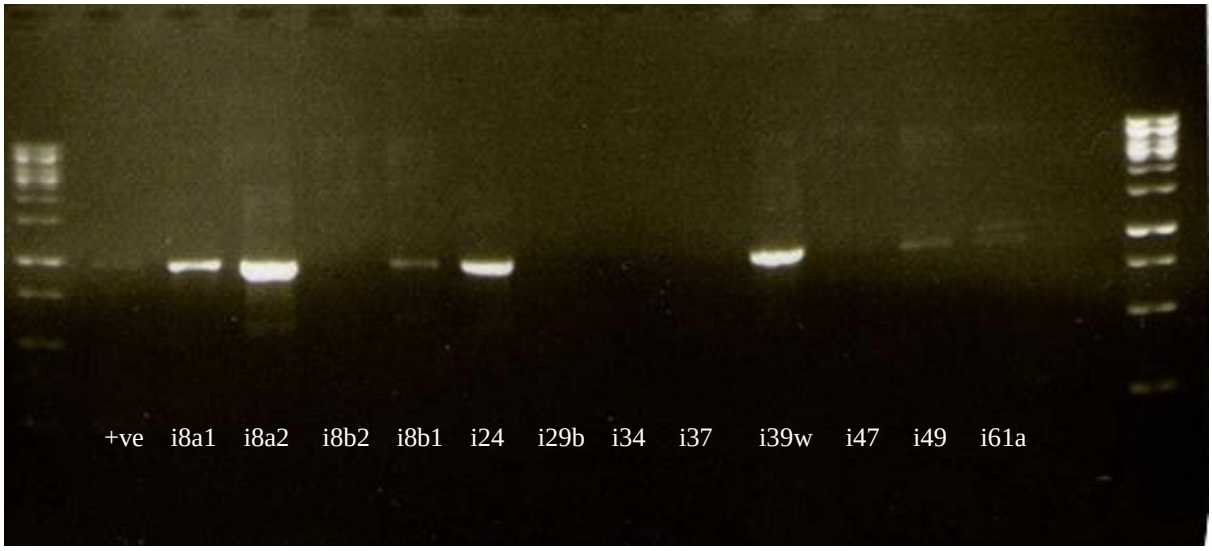
6.1 Introduction

Cultivation-independent work is vital in studying microbial diversity in environmental samples due to the difficulties in growing the vast majority of microbes under laboratory conditions. Molecular methods, independent of cultivation, allow assessment of the entire microbial community within a sample. The most traditionally used phylogenetic marker for studying microbial communities is the 16S rRNA gene (Case *et al.* 2006), which due to its ubiquity is useful in evaluating the microbial diversity of a sample but cannot convey anything regarding the metabolic functions of these species in the environment, nor can it link physiology with phylogeny. The first aim of this work was to design a PCR primer set based on the *isoA* gene sequences obtained from the genomes of the marine isoprene degrading strains *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2*(see Chapter 5) as well as three terrestrial isoprene-degrading isolates: the type strain *Rhodococcus* AD45 (van Hylckama Vlieg *et al.* 2000) and two strains of *Rhodococcus wratislaviensis* isolated by Myriam El Khawand from soil and a leaf. These primers, once designed, could be tested with the other marine isolates and used to retrieve *isoA* gene sequences from these organisms. A database of *isoA* sequences could then be built and conclusions could be drawn regarding the phylogeny of *isoA* gene sequences in marine and terrestrial isoprene degrading isolates. These 'functional gene probes' could then be used to go back into the environment and interrogate environmental samples to assess the presence and diversity of *isoA* sequences in a wide range of environments, similar to the use of the *pmoA* and *mmoX* genes encoding subunits of methane monooxygenase enzymes to detect methanotrophic bacteria in the environment (McDonald *et al.* 2008) (Luesken *et al.* 2011).

6.2 Design and testing of *isoA* primers based on *Rhodococcus* AD45 gene sequence

A primer set was first designed based on the *isoA* gene sequence from *Rhodococcus* AD45 (Vlieg *et al.* 2000), the only known *isoA* sequence available when beginning this work. Also included in the alignment was a sequence from a related monooxygenase of unknown function found in the genome of *Frankia* EUN1f (draft JGI genome) a nitrogen-fixing organism isolated from *Elaeagnus umbellata*, the autumn olive.

Figure 6.1: Testing of original *isoA* primers with DNA from marine isoprene degrading isolates



Key: i29a2*=*M.holderi,* i37= *G.polyisoprenivorans,* SC4=*R.wratislaviensis,* i47= *R.wratislaviensis,* SM= salt marsh DNA, i29a2= *R.globerulus,* i8b1= *Loktanella,* i8a1= *R.globerulus,* i49=*Leifsonia,* i39y=*Microbacterium,* i61b= *M.luteus,* i61a= *M.fortuitum,* i8a2=*R.globerulus.*

The sequence of the initial primer set was as follows:

Iso494F (forward): AGAAGGCRTTCCACACCAAC (R=T/C)

Iso1457R (reverse): GAGGTGATGGGRGAGGARGT

Figure 6.1 shows the initial testing of the primer set Iso494F/Iso1457R using 10µM of each primer in a standard PCR reaction master mix as described in Materials and Methods section 2.9. The PCR cycle used was as follows: 94°C for 30s, 55°C for 30s, 72°C for 1 minute, repeated for 30 cycles. DNA extracted from *Rhodococcus* AD45 was used as the positive control for the reaction. The expected size of the PCR product was ~1kb. A weak PCR product of the correct size was obtained from *Rhodococcus* AD45, which when sequenced was confirmed to be *isoA.* A PCR product of the correct size was also obtained for *Rhodococcus* strains i8a1, i8a2, i24, i39w, *Loktanella* i8b1, *Leifsonia* i49 and *Mycobacterium* i61a, however when sequenced these products were not correct. The primers were unsuccessful in amplifying *isoA* from any of the isolated isoprene degrading strains. The decision was subsequently taken to sequence the genomes of *Gordonia* i37, *Mycobacterium* i29a2* (see Chapter 5) as well as the genomes of two terrestrial isoprene

degrading strains, *Rhodococcus wratislaviensis* SC4 (isolated from soil) and *Rhodococcus wratislaviensis* LB1 (isolated from a leaf) in order to mine the genomes for sequences encoding the isoprene monooxygenase gene cluster.

6.3 Design of *isoA* primers based on *isoA* sequences from the genomes of *Gordonia, Mycobacterium* and terrestrial *Rhodococcus* isolates

Chapter 5 described the retrieval of full length *isoA* sequences from the genomes of *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2*. Full length *isoA* sequences were also obtained from the genomes of two terrestrial isolates by Myriam El Khawand (University of East Anglia, UK). The terrestrial isolate *Rhodococcus wratislaviensis* SC4 was isolated from isoprene spiked soil in Leamington Spa, UK. The isolate *Rhodococcus wratislaviensis* LB1 was isolated from the surface of a leaf taken from a horse chestnut tree at the University of Warwick, Coventry, UK.

Figure 6.2 Alignment of *isoA* gene sequences from marine and terrestrial isoprene degrading isolates

Figure 6.2 shows the alignment of the different *isoA* sequences and highlights the conserved amino acid regions that were used to design the forward and reverse primer for the new *isoA* primer set. This alignment includes the sequence for the *xamoA* gene encoding an alkene monooxygenase from *Xanthobacter* Py2 (Zhou *et al.* 1999) and conserved regions were chosen specifically to exclude this sequence in order to increase the specificity of the primer set binding to *isoA* sequences only. This alkene monooxygenase from *Xanthobacter* Py2 has been shown to oxidize benzene, toluene and phenol and the bacterium can grow on the latter. The primer set chosen was as follows:

isoAF309 (forward): TGCATGGTCGARCAYATG (R=A/G)

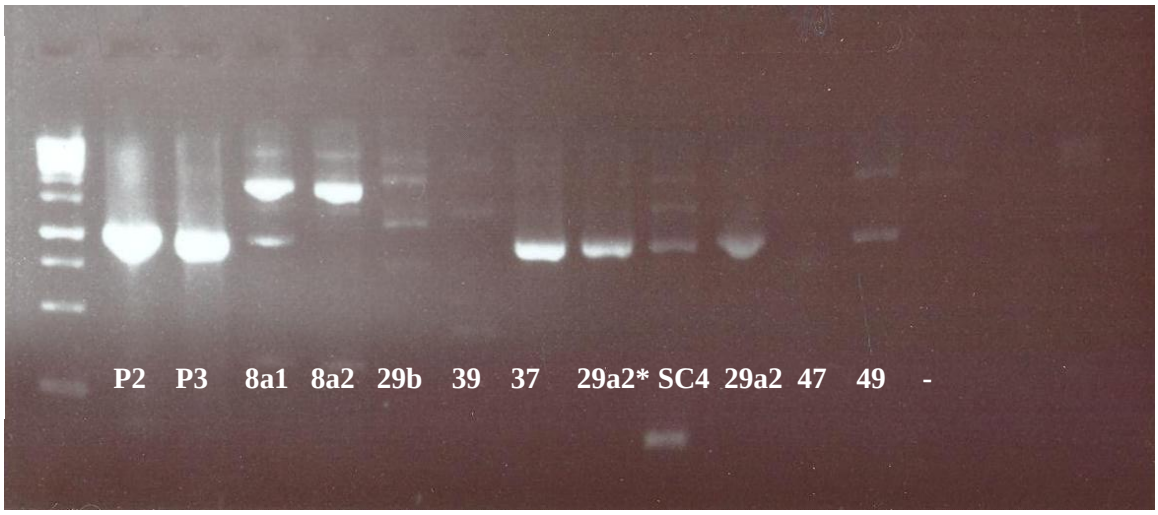
isoAR1301 (reverse): GRTCYTGYTCGAAGCACCACTT (Y=T/C)

Codon usage was also considered when designing the primers in order to minimise degeneracy in the PCR primer set.

6.3.1 Testing of *isoA* primers in marine and estuarine isolates

Isolated isoprene -degrading strains from marine, estuarine (see chapter 3) and soil environments were used to test the sensitivity and specificity of the new primer set.

Figure 6.3 Products obtained during testing of newly designed *isoA* primers with DNA from marine isolates and with DNA from enrichments with environmental samples

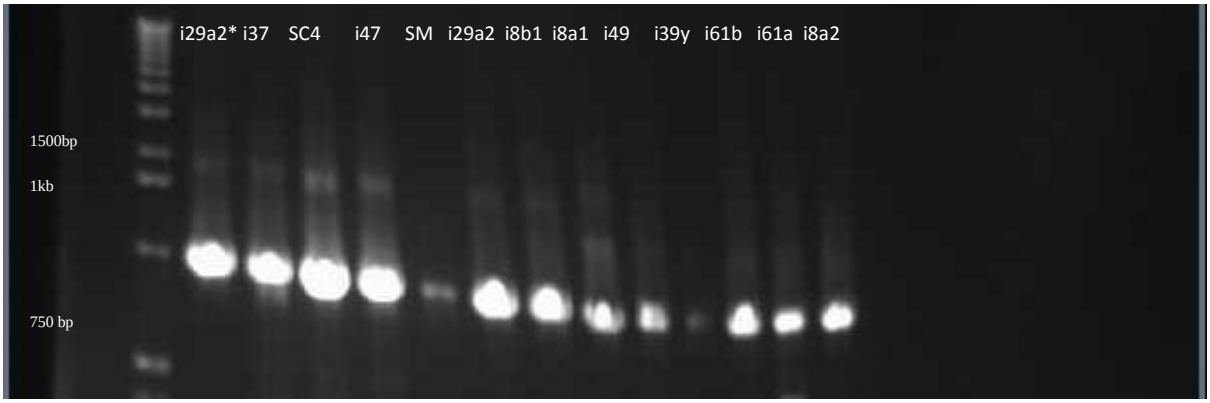


(P2/P3= DNA from Penarth water samples) Please see Figure 6.1 for key of strains

The newly developed PCR primer set was tested using DNA from isoprene degrading isolates of different genera, as well as environmental DNA from isoprene-enriched seawater samples from Penarth, Wales (see Chapter 3) in a PCR reaction using master mix and PCR cycles as described for Figure 6.1. DNA from *Gordonia polyisoprenivorans* i37, *Mycobacterium hodleri* i29a2* and *Rhodococcus wratislaviensis* SC4 was used as positive controls for this reaction. The expected size of the PCR product was ~1kb. The primers were successful in amplifying *isoA* from all positive controls and environmental DNA, and gave a product of the correct size for *Leifsonia* i49 and *Rhodococcus* i29a2. However, these PCR conditions were unsuccessful in amplifying the *isoA* gene from several

confirmed isoprene degrading strains as well as producing several non-specific bands as seen in Figure 6.3.

Figure 6.4 Products obtained from testing of *isoA* primers using optimised touchdown PCR protocol with hot start



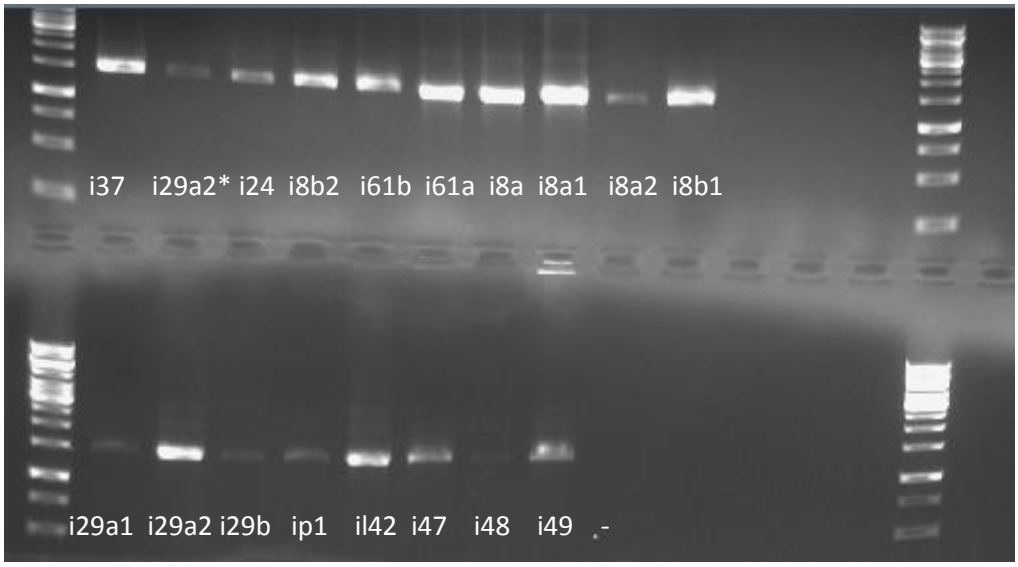
Please see Figure 6.1 for key of strains

Figure 6.4 shows the initial results of using the newly designed *isoA* PCR primers with an altered PCR protocol, with 30ng DNA in each reaction. Using this protocol, a product of the correct size (~1kb) could be amplified from a variety of estuarine isoprene-degrading strains as well as DNA extracted from Stiffkey salt marsh samples (see Chapter 1). The PCR protocol used was as follows:

1. 94°C for 10 mins,
2. 94°C for 30s
3. 94°C for 45s
4. 72°C for 45s (decrease by 1°C each cycle)
5. 72°C for 1 min
6. Cycle to step 2 18 more times
7. 94°C for 30s
8. 54°C for 45s
9. 72°C for 1 min
10. Cycle to step 7 29 more times
11. 72°C for 5 mins
12. 4°C for 15 mins

Figure 6.5 shows the products obtained using the PCR protocol with 1µl of DNA from each marine isolates in the reaction.

Figure 6.5 Amplification of *isoA* gene PCR products using DNA from marine isolates



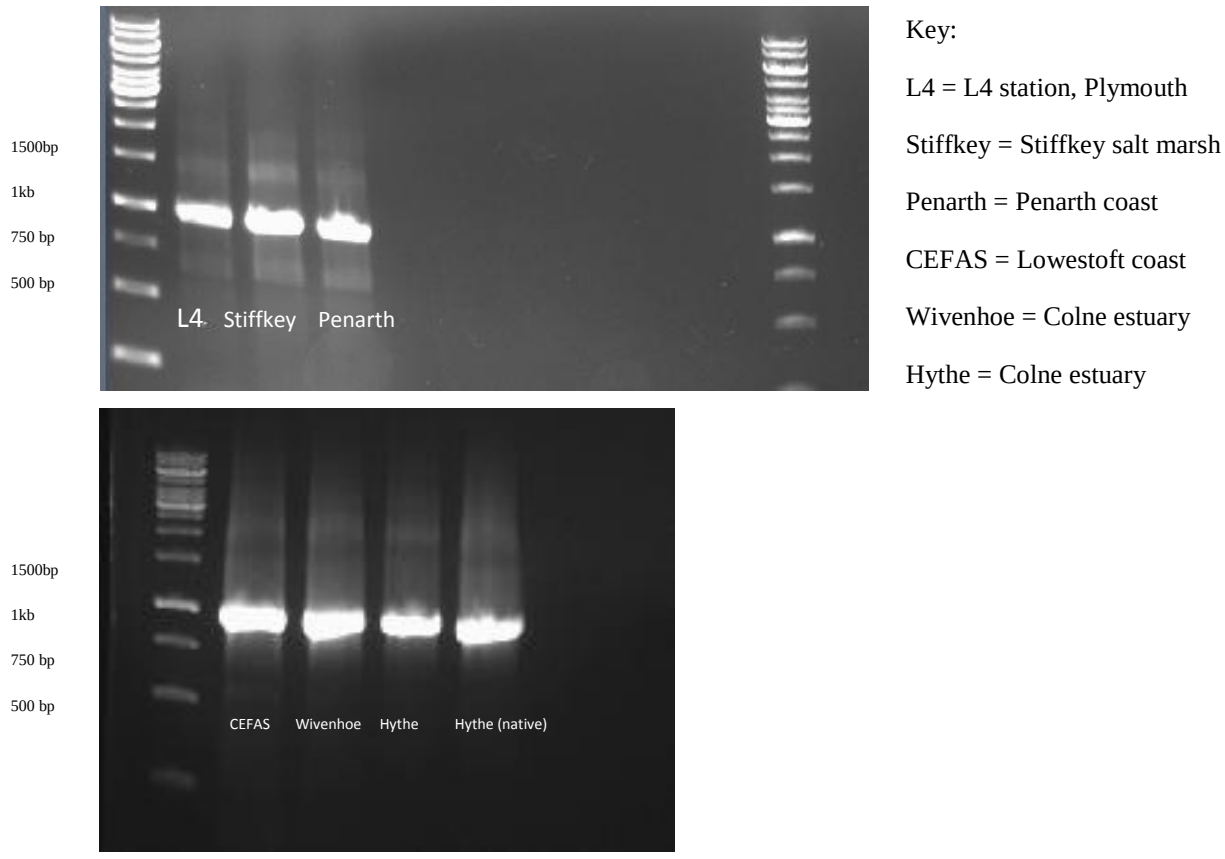
Key: i29a2*=*M.holderi,* i37= *G.polyisoprenivorans,* SC4=*R.wratislaviensis,* i47= *R.wratislaviensis,* i29a2= *R.globerulus,* i8b1= *Loktanella*, i8a1= *R.globerulus,* i49=*Leifsonia,* i61b= *M.luteus,* i61a= *M.fortuitum,* i8a2=*R.globerulus.* i24= *R.erythropolis,* iP1= *Microbacterium,* iL42=*Stappia,* i8a=*R.globerulus,* i29a1=*R.globerulus,* i29b=*R.wratislaviensis,* i48= *R.wratislaviensis,* i8b2=*Salinibacterium*

6.3.2 Testing of *isoA* primers with DNA from marine, coastal and estuarine environments and assessment of *isoA* diversity in environmental samples

Water and sediment from several marine, coastal and estuarine environments were used in this work as previously described in Chapter 2. Samples from the Colne Estuary (Essex), the marine L4 sampling station (Plymouth) (http://www.westernchannelobservatory.org.uk/), Stiffkey salt marsh (Norfolk), Penarth (South Wales) and water provided by CEFAS (Lowestoft, Suffolk) were all enriched with isoprene as their sole added carbon source, and each of these water samples completely degraded the added isoprene. Several isoprene-degrading strains were isolated from these enrichments, and the *isoA* gene was amplified and sequenced from these isolates using the new *isoA* PCR primer set. DNA was extracted from these enrichments and used as the template for a PCR reaction as described previously. Additionally, DNA was extracted from non-enriched sediment from the Hythe (Essex) and also used as a template for a PCR reaction using our newly designed *isoA* PCR

primers. The PCR reaction was successful in amplifying a strong *isoA* product of the correct size with DNA from each of these environmental enrichments as well as the native unenriched Hythe DNA, as shown in Figure 6.6. This confirms that isoprene-degrading bacteria are present in a wide range of marine, coastal and estuarine environments, and that at least some of the isoprene degrading bacteria found in these environments possess a soluble diiron centre isoprene monooxygenase enzyme system, and likely degrade isoprene via the established pathway found in *Rhodococcus* AD45 or a similar pathway. The *isoA* gene PCR products amplified were then used to create clone libraries for each of these environments, to assess the diversity of isoprene monooxygenase alpha subunit sequences found in the environment.

Figure 6.6 PCR products amplified with new *isoA* primers using DNA from enriched and unenriched environmental samples



A PCR product of the correct size was obtained from all environments tested, including enriched water samples taken from the L4, Stiffkey salt marsh, Penarth, Wivenhoe and CEFAS sampling sites, as well as Hythe estuary samples that had not been enriched with isoprene. This indicates that isoprene degrading bacteria are present in a wide range of

marine, coastal and estuarine environments, and that bacteria present in these environments possess an isoprene monooxygenase enzyme system. Following the addition of isoprene to water samples, isoprene consumption is rapid, indicating that the isoprene degraders in these environments can rapidly synthesise the necessary enzymes to degrade isoprene, and grow rapidly. Clone libraries were constructed from these *isoA* PCR products and unique clone sequences determined by RFLP analysis as described in materials and methods section 2.11. For each clone library, 20 clones were selected at random for RFLP analysis and one representative of each unique RFLP profile was sent for DNA sequencing. Table 6.1 shows the unique *isoA* sequences retrieved from these clone libraries, including several clones from terrestrial soil samples obtained by Myriam El Khawand (University of East Anglia).

Table 6.1: Unique *isoA* clone sequences obtained from environmental enrichments and their percentage nucleotide and amino acid identity to isoprene monooxygenase from *Rhodococcus* AD45

Clone	% Nucleotide identity	% AA identity
394-1	98	98
394-2	90	96
394-3	90	96
394-4	98	98
394-5	98	98
394-6	83	95
L41	82	92
SM2	83	92
B27	88	99
Marine B4	82	91
B24	82	91
B23	88	94
Marine B7	83	93
B29	82	92
B26	81	93
B30	81	94
B21	82	94
H2	99	99
H4	83	87
H6	86	91
H8	85	88
H10	87	84
H11	86	92
H14	86	91
H15	85	90
H16	99	99
H18	87	91

Key:

394-(1-6): Soil clones

L41: L4 seawater

SM2: Stiffkey salt marsh

B: Penarth seawater

H: Colne estuary, Hythe

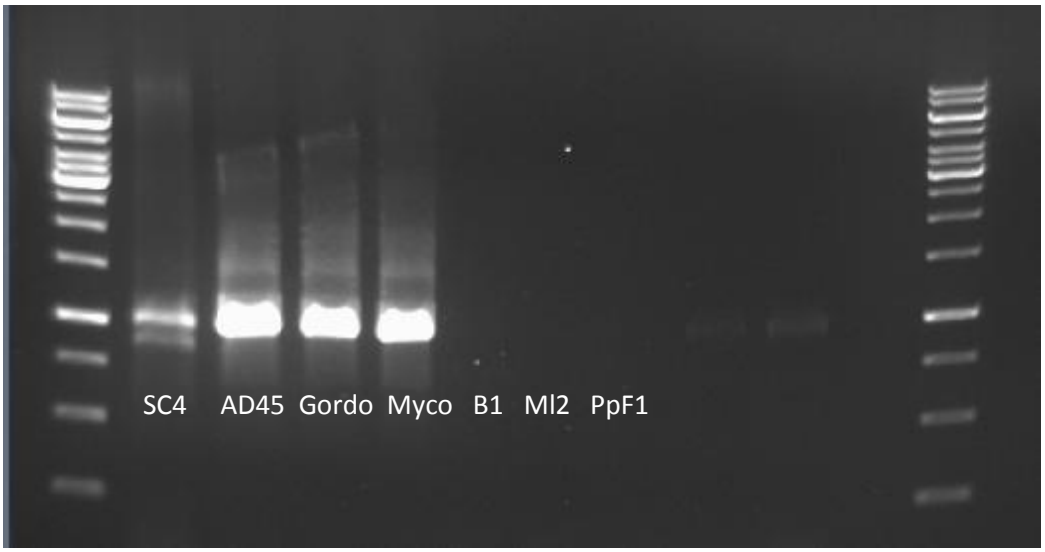
Figure 6.7 Neighbour joining tree of α-subunit of isoprene monooxygenase from marine and soil isolates and environmental clones

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nej. 1987). The optimal tree with the sum of branch length = 1.43912700 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004) and are in the units of the number of base substitutions per site. The analysis involved 76 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 193 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.* 2011).

Figure 6.7 shows the diversity of *isoA* nucleotide sequences retrieved from isolated isoprene degrading strains as well as marine, coastal, estuarine and terrestrial (soil) environmental samples. Clones are named according to their location. Clones designated 'Soil clone' were retrieved by PCR and cloning by Myriam El Khawand (University of East Anglia) using the same primer set to target the *isoA* gene. As can be seen from the phylogenetic tree of these sequences, the isolates and clones form two major groups: clone sequences from ocean and coastal environments group with marine isolates from Wivenhoe, L4, Etang de Berre (Alvarez *et al.*2009*)* and Penarth. These sequences are distinct from estuarine *Rhodococcus* isolates and *isoA* sequences retrieved from the Hythe, further inland in the Colne Estuary. These *isoA* sequences are more closely related to sequences from terrestrial *Rhodococcus* isolates and sequences from *isoA* clones retrieved from isoprene-enriched soil samples. Marine and estuarine sequences were more closely related to each other than 'terrestrial' sequences, indicating lower diversity of *isoA* in the marine environment, and several sequences shared up to 99% identity at the nucleotide level even across genus and phyla, whereas a higher diversity of *isoA* sequences was seen in *Rhodococcus* and *Mycobacterium* sequences isolated from soil and the Hythe estuary. One possible explanation for this could be horizontal gene transfer in the marine environment. In *Rhodococcus* AD45, the genes encoding isoprene monooxygenase are located on a plasmid (Crombie *et al.*unpublished) and several members of the Actinobacteria are known to possess multiple plasmids containing genes for degradation of compounds such as phenol (Sekine *et al.* 2004) (Zidkova *et al.* 2012) as well as megaplasmids such as that found in the strain *Gordonia westfalica* (Bröker *et al.* 2008). Based on these data, it is difficult to establish a solid phylogenetic framework within which retrieved *isoA* sequences can potentially be assigned to a known species or genus, however this may be possible in the future if different isoprene degrading strains are isolated from different phyla which possess a more diverse sequence for isoprene monooxygenase.

6.3.3 Testing of *isoA* primers with negative controls

Figure 6.8 Testing of *isoA* negative controls using PCR primers targeting the isoprene monooxygenase alpha subunit



SC4 – *R.wratislaviensis* SC4

Gordo-*G.polyisoprenivorans* i37

Myco-*M.hodleri* i29a2*

B1=*S.yanoikuyae* B1

ML2- *P.putida* ML2

PpF1-*P.putida* PpF1

DNA from several strains of bacteria containing different monooxygenase or dioxygenase enzymes were used as negative controls for this study. *Spingomonas yanoikuyae* B1 grows on biphenyl using a well characterised biphenyl dioxygenase (Zylstra & Kim. 1997). *Pseudomonas putida* ML2 grows on benzene via a constitutively expressed benzene dioxygenase (Fong *et al.* 2000) and *Pseudomonas putida* PpF1 oxidises toluene via a toluene diooxygenase (Finette *et al.* 1984). All of these strains were *isoA* negative after testing with the newly designed *isoA* specific primers.

Several other strains containing soluble diiron centre monooxygenase enzyme systems were also used as *isoA* negative controls. Also tested but not shown in Figure 6.8 were DNA samples from *Methylocella silvestris* (Crombie & Murrell 2014) which can oxidise methane and propane via soluble diiron centre monooxygenase enzyme systems, *Rhodococcus jostii* strain 8 (Ekprasert and Murrell, unpublished), a propane-utilising strain that does not grow on isoprene, and DNA extracted from *Mycobacterium* NBB4 (Coleman *et al.* 2011) which contains several soluble diiron centre monooxygenase genes but no gene encoding isoprene monooxygenase. All of the above DNA samples yielded no PCR product when tested using the *isoA* primer set.

6.4 Summary of Results

A new PCR primer set was designed that specifically targets the *isoA* gene encoding the alpha subunit of isoprene monooxygenase. This primer set was based on *isoA* sequences retrieved from the genomes of extant isoprene degraders, with a final product of ~1kb in length, covering a large length of the total 1.5kb *isoA* gene. The primers were tested and subsequently the PCR protocol optimised. These PCR primers were shown to be specific for the *isoA* gene, and did not amplify any PCR product from strains containing other soluble diiron centre monooxygenase enzyme systems. An *isoA* gene sequence was amplified, cloned and sequenced from all isolated isoprene degrading strains, allowing the construction of phylogenetic trees based on the *isoA* gene, which will in the future provide a framework to help in assessing the phylogeny of *isoA* genes retrieved from environmental samples. These *isoA* sequences showed high diversity which suggests that our PCR primer set is successful in amplifying all *isoA* sequences.

As well as testing our primer set using isolated isoprene degraders, the primers were used as probes to assess the presence and diversity of *isoA* gene sequences in environmental samples and isoprene enrichments. *isoA* sequences were amplified in all environments tested, including isoprene enriched samples from soil, coastal, estuarine and marine environments. One aquatic environment, the Hythe (Colne Estuary, Essex) gave an *isoA* product using native DNA from unenriched samples. This might be due to the higher biomass present in these samples compared to other marine seawater samples. Native soil DNA from Norfolk also gave an *isoA* PCR product (data not shown). This suggests that isoprene degraders possessing an isoprene monooxygenase are widespread in the environment, albeit in low numbers. Clone libraries constructed using these *isoA* PCR products yielded several new unique *isoA* sequences.

Sequences from isolated isoprene degraders and environmental clone libraries showed high diversity. Sequences grouped seemingly based on environment rather than genus – many isolates from the Colne Estuary had high nucleotide identity between their *isoA* sequences despite being from different genera, whereas *Rhodococcus* species from soil environments and inland, less saline estuarine environments had high identity to each other. Analysis of clone libraries from soil environments suggests that *Rhodococcus*-like *isoA* sequences are most common. The high sequence identity between marine isolates could suggest a high

rate of horizontal gene transfer between these organisms, relatives of which are known to carry plasmids encoding soluble diiron centre monooxygenase enzyme systems.

This newly designed *isoA* PCR primer set is a useful tool in probing environmental samples for indicators of isoprene degradation. Data presented here suggest that isoprene degradation may take place in a wide range of environments. Chapter 7 describes the use of these primers in combination with DNA-SIP experiments to assess the diversity of *isoA* sequences retrieved from the heavy ^{13}C labelled fraction of isoprene enriched estuarine samples.

Chapter 7

Identification of active isoprene-degrading bacteria in the Colne Estuary, Essex using DNA-Stable Isotope Probing

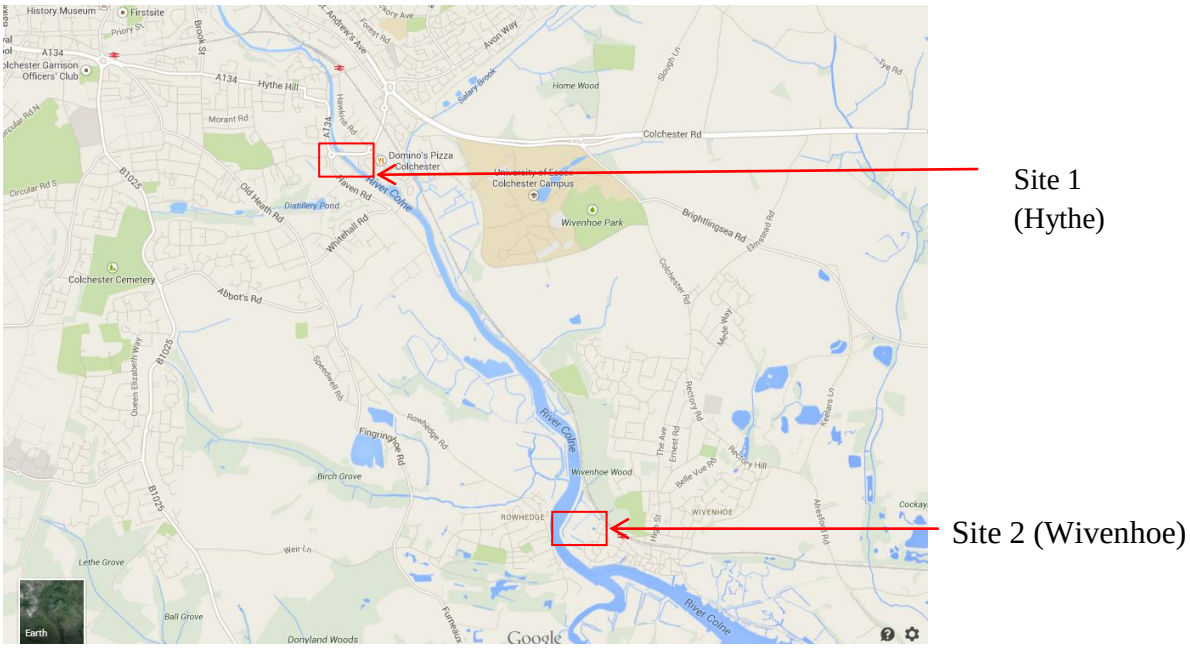
Chapter 7: Using DNA Stable Isotope Probing to identify active isoprene degraders in the Colne Estuary, Essex

7.1 Introduction

As discussed in previous chapters, several isoprene degrading bacteria have previously been isolated from the Colne Estuary, Essex. These strains include various members of the phylum Actinobacteria, including several strains of *Rhodococcus* and *Mycobacterium.* However, no studies have ever identified active isoprene degraders in environmental samples. Isoprene enriched samples taken from various points along the Colne (The Hythe, Wivenhoe, Brightlingsea) completely degraded isoprene at a concentration of 0.01% and 0.001% (v/v)(Alvarez *et al*. 2009). 16S rRNA amplicon pyrosequencing of DNA from these samples showed that they were dominated by *Mycobacterium* spp.. DNA-Stable isotope probing methods can link phylogeny to function in environmental samples, by incubating environmental samples in the presence of ^{13}C labelled isoprene as their sole carbon source, followed by the separation of the sample DNA based on density. The labelled 'heavy' DNA can be analysed using several different molecular methods as described in Chapter 1 Section 1.10.2. There were several constraints to consider when designing the DNA-SIP protocol for this study. Previous enrichment experiments showed that seawater samples taken from the Colne estuary enriched with 0.01% and 0.001% (v/v) isoprene took up to 100 days to completely degrade this isoprene (Alvarez *et al*. 2009). This would not allow sufficient labelling of DNA for downstream analysis, and it is difficult to accurately measure isoprene at these concentrations, which are still higher than those found in the environment. Therefore, in these initial studies, sediment was collected along with water samples to increase bacterial biomass, and the concentration of isoprene was increased to allow sufficient incorporation of ^{13}C into biomass. All extant isoprene degraders can degrade isoprene at the concentrations used in these experiments despite being isolated at lower concentrations.

Water and surface sediment samples were taken from the edge of the Colne Estuary at low tide, at the Hythe, Essex in April 2013, and at high tide at Wivenhoe, Essex in November 2013. The sampling sites are marked on the map shown in Figure 7.1.

Figure 7.1: Sampling sites along the Colne Estuary, Essex



7.2 Experimental Design

DNA-SIP methods used were described in Section 2.20. For Hythe samples, 120ml serum vials were set up in triplicate containing 25ml of Colne Estuary water, 0.5g of Colne Estuary sediment, and 1ml of MAMS medium, with 1% (v/v) added ^{13}C-labelled isoprene as sole carbon and energy source. Duplicate serum vials were also set up with 1% (v/v) added unlabelled isoprene. Duplicate control vials contained 25ml of autoclaved sample water + 1% (v/v) added unlabelled isoprene + 1ml MAMS. These samples were incubated at 25°C with shaking at 50rpm. Time point 1 was sacrificed after 7 days of incubation. Time points 2 and 3 were sacrificed after 14 and 21 days, respectively. Each time point corresponded to approximately 100 µmoles incorporation of fully labelled ^{13}C-isoprene as measured by gas chromatography.

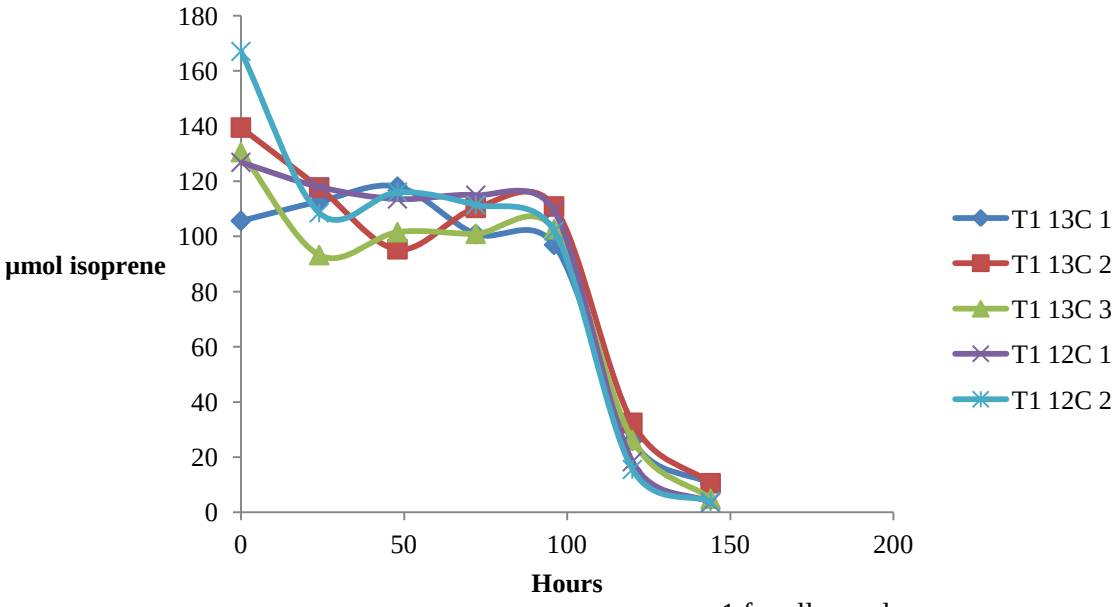
For Wivenhoe samples, 30ml of sample water + 1g of sample sediment were incubated with 0.2% (v/v) ^{13C}-labelled isoprene as their sole carbon source in duplicate serum vials. Duplicate serum vials containing 30ml sample + 1g sediment were also incubated with 0.2% ^{12}C-labelled isoprene. Duplicate controls contained 30ml of autoclaved sample water + 1g autoclaved sediment incubated with 0.2% ^{12}C-labelled isoprene. These samples were incubated at room temperature (approximately 23-25°C) without shaking because of practical constraints. T1 samples were sacrificed after approximately 30µmoles of isoprene had been consumed, at 12 days. Time points 2 and 3 were sacrificed at 15 and 18 days,

respectively. Figure 7.2 shows the depletion of isoprene in these vials as measured by gas chromatography.

DNA extracted from these samples was then added to caesium chloride solution for density gradient ultracentrifugation and fractionation. DNA from both ^{12}C and ^{13}C fractions was used as a template in16S rRNA and *isoA* gene PCR assays. The 16S rRNA gene products were analysed using denaturing gradient gel electrophoresis to assess differences in the 16S rRNA profiles between both fractions and time points. These community profiles identified the best samples for 16S rRNA and *isoA* amplicon sequencing.

7.3 Results – Hythe incubations

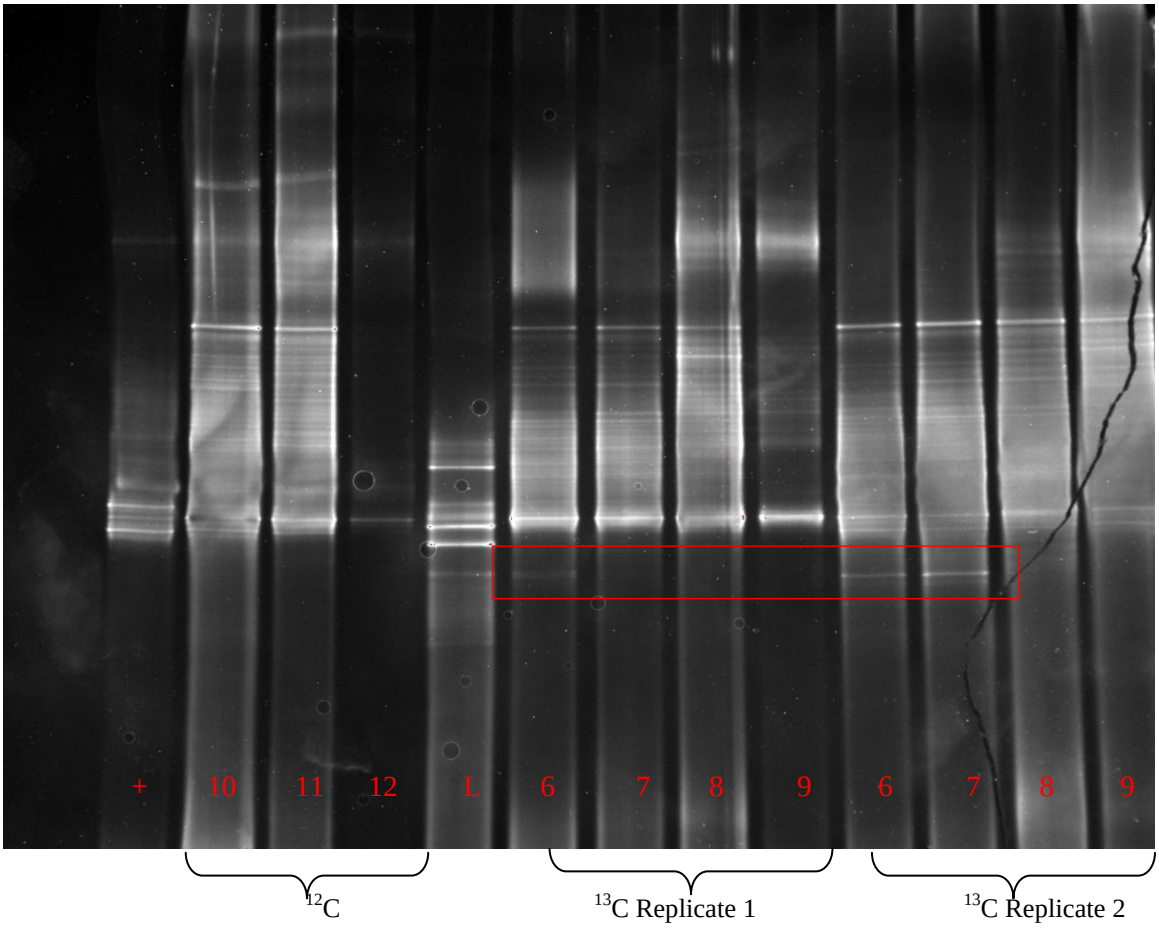
Figure 7.2: Isoprene depletion in ^{12}C and ^{13}C incubated samples of Hythe water and sediment measured using gas chromatography (Time point 1)



n=1 for all samples

Figure 7.2 shows the isoprene depletion of isoprene-incubated Hythe samples at time point 1. Control autoclaved water and sediment samples (not shown) did not consume any isoprene over the time measured. All samples had a lag phase of approximately 100 hours. All isoprene was completely consumed within 48 hours in all samples (rate ~0.156µmol/hr ml sample^{-1}).

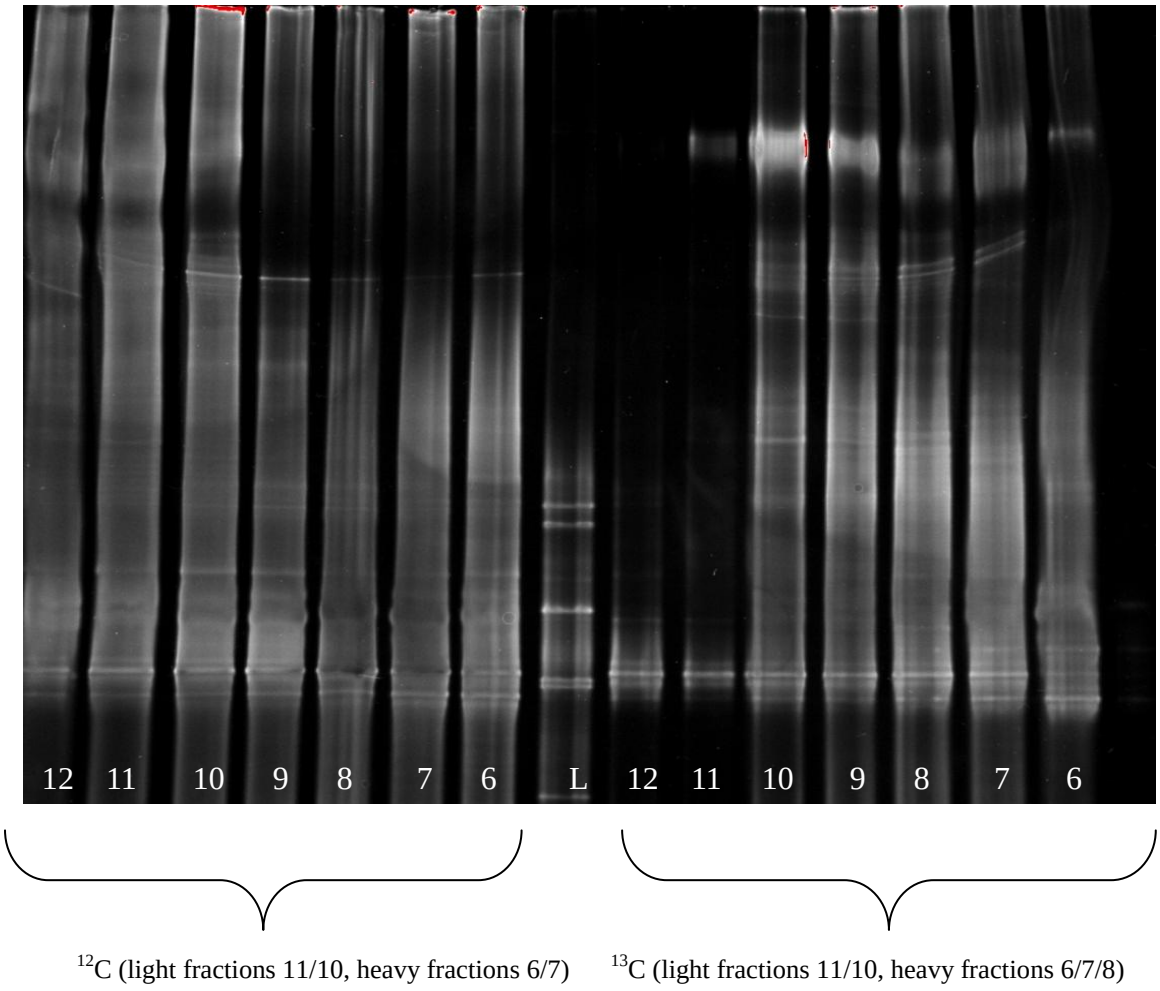
Figure 7.3: DGGE profile of 16S rRNA gene products amplified using 341GC-907R primers from DNA from CsCl gradient fractions from both ^{13}C and ^{12}C-labelled isoprene incubations with Hythe sediments and water samples at T1



N.B Fractions 6-9 contain 'heavy' DNA of density 1.72-1.715. Fractions 10-12 contain 'light' DNA, density 1.71-1.70

L= Ladder, + = Positive control (micture of 16S rRNA products from known isoprene degrading strains of *Rhodococcus* and *Gordonia* spp.)

Figure 7.4: DGGE profile of 16S rRNA gene products amplified from DNA from CsCl gradient fractions from ^{13}C and ^{12}C isoprene incubations with Hythe water and sediment samples at T2 (fractions 6-12)

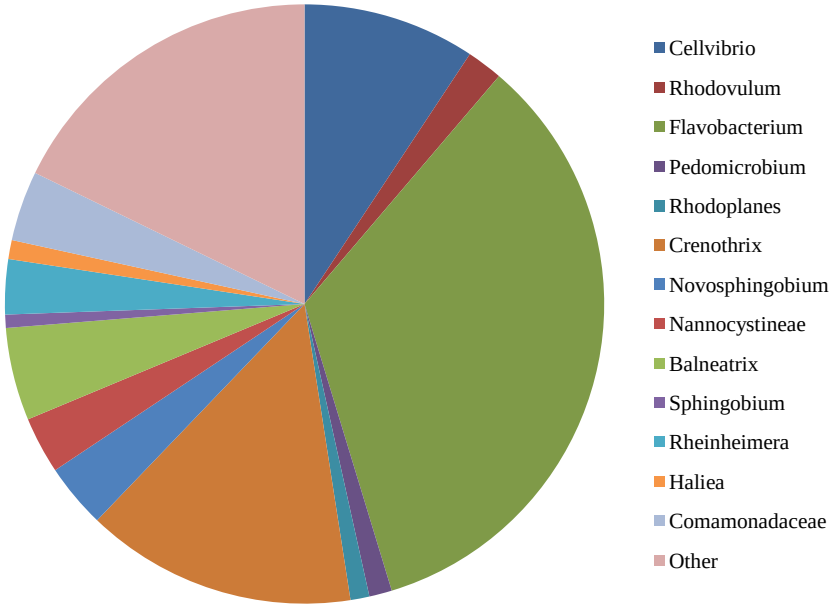


L= ladder

Figure 7.3 shows the 16S rRNA profiles for duplicates of time point one (T1) ^{13}C heavy fractions (6-9) and a single replicate of ^{12}C light fractions (10-12) for comparison. According to the density measurements for these fractions, the heavy fractions for ^{13}C replicates 1 and 2 were fraction 6 and fraction 6/7 respectively. This can be seen in the 16S rRNA profiles, where there is a clear band present only in these fractions which may represent an isoprene degrading bacterium. This band is absent from the light fractions of the ^{12}C replicate (fractions 10/11) indicating a potential difference in the enriched organisms between the ^{13}C and ^{12}C samples. There are bands present in fractions 10/11 not

seen in fraction 12. Figure 7.4 shows the 16S rRNA DGGE profile of fractions 6-12 from time point 2 ^{12}C and ^{13}C replicates. Comparing time points 1 and 2, there are no obvious differences in the DGGE profiles, and no unique bands are apparent in the fractions of time point 2 that might represent enriched isoprene degraders unique from T1. This would suggest that the enrichment of isoprene degraders under these experimental conditions is rapid (within 7 days). It was therefore decided for this preliminary work to concentrate on further analysis of T1 only, to determine the active isoprene degraders at this time point and assess the presence and diversity of the *isoA* gene within this sample. Samples were sent for 16S rRNA and *isoA* gene amplicon sequencing as described in Materials and Methods section 2.22. Figure 7.5 shows the 16S rRNA sequences obtained from unenriched native DNA extracted from the Hythe samples. The T0 (unenriched) samples were dominated by members of the genera *Flavobacterium, Cellvibrio,Crenothrix* and *Balneatrix,* a common freshwater bacterium from the Proteobacteria (Dauga *et al.* 1993)

Figure 7.5: 16S rRNA amplicon sequences obtained from unenriched T0 DNA



Figures 7.6 and 7.10 show the sequences obtained from the heavy fraction of the ^{13}C enriched samples at the genus and species level. In these samples, *Flavobacterium*, which represent 35% of the total sequences at T0, are absent. Approximately 50% of the 16S rRNA sequences obtained from the heavy fraction of the ^{13}C samples belonged to the genus *Rhodococcus*, including *Rhodococcus globerulus, Rhodococcus erythropolis, Rhodococcus wratislaviensis, Rhodococcus jostii* and *Rhodococcus koreensis.* The ^{12}C

light samples were also dominated by *Rhodococcus* species (Figures 7.8, 7.13). The ^{12}C heavy samples (Figures 7.9, 7.11) contained almost entirely *Rhodococcus* sequences, while the ^{13}C light samples (Figures 7.7, 7.12) were dominated by a mix of both *Rhodococcus* and *Flavobacterium* sequences, suggesting a possible smearing of the density gradient during the fractionation process or a PCR bias towards these organisms due to their massive overall abundance after the SIP incubation.

Figure 7.6: Sequences obtained by 16S rRNA ampliconsequencing of T1 ^{13}C heavy fraction DNA

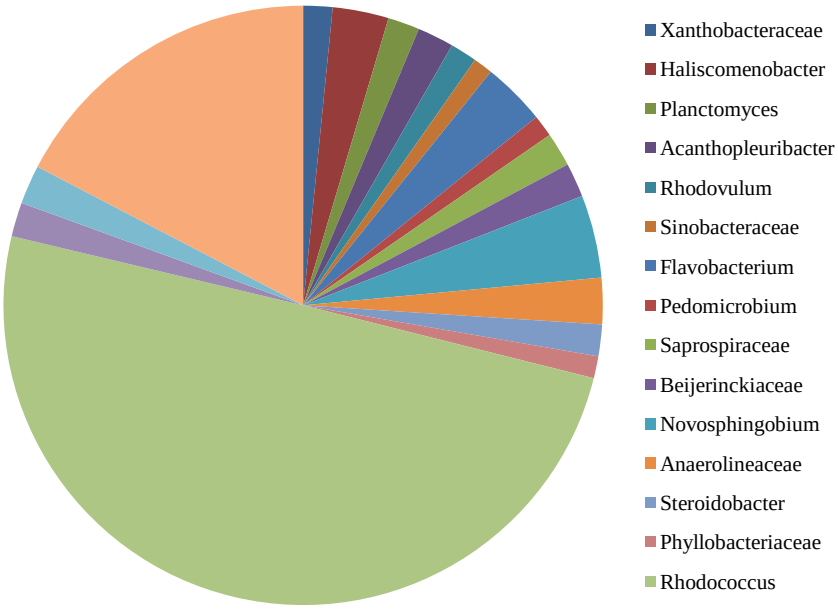


Figure 7.7: Sequences obtained by 16S rRNA amplicon sequencing of T1 ^{13}C light fraction DNA

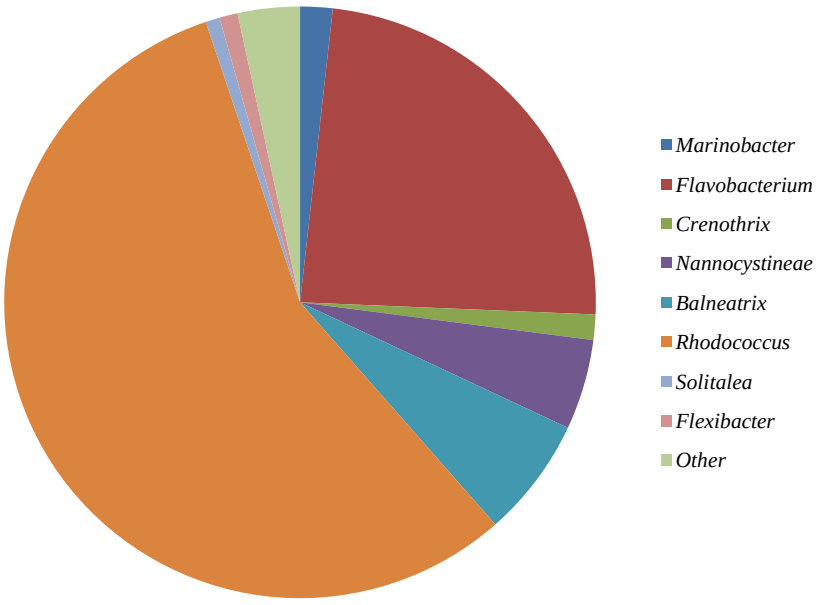


Figure 7.8: 16S rRNA sequences obtained by amplicon sequencing of T1 ^{12}C light fraction DNA

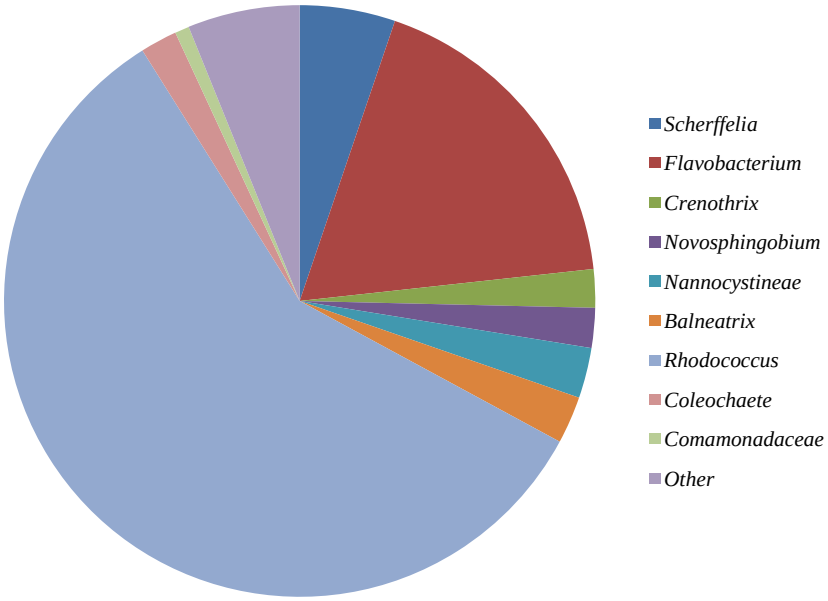


Figure 7.9: Species obtained from 16S rRNA sequencing of ^{12}C Heavy fraction DNA

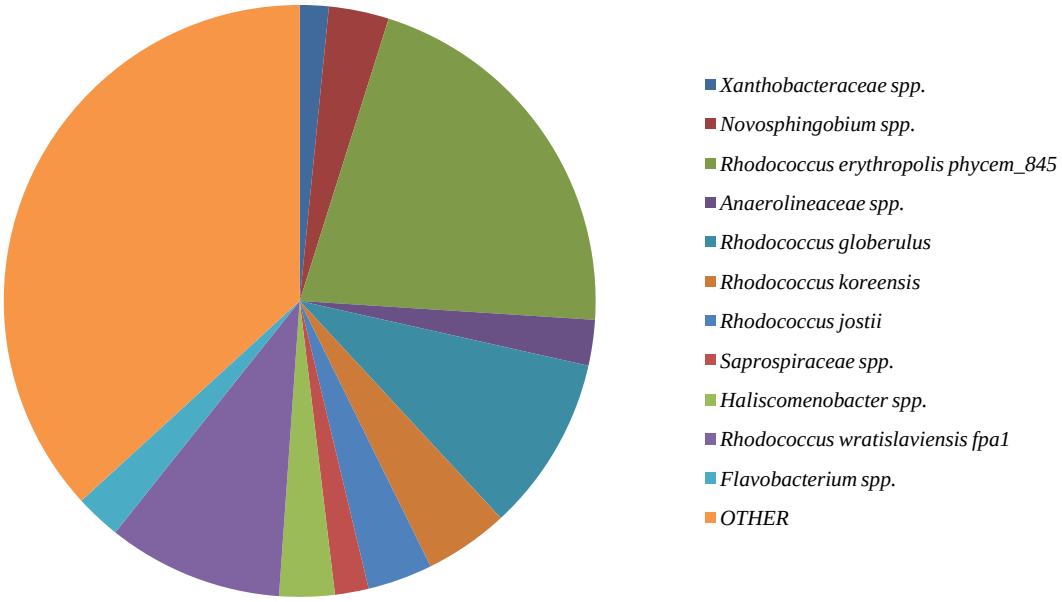


Figure 7.10: Species obtained by 16S rRNA amplicon sequencing of unfractionated T0 DNA

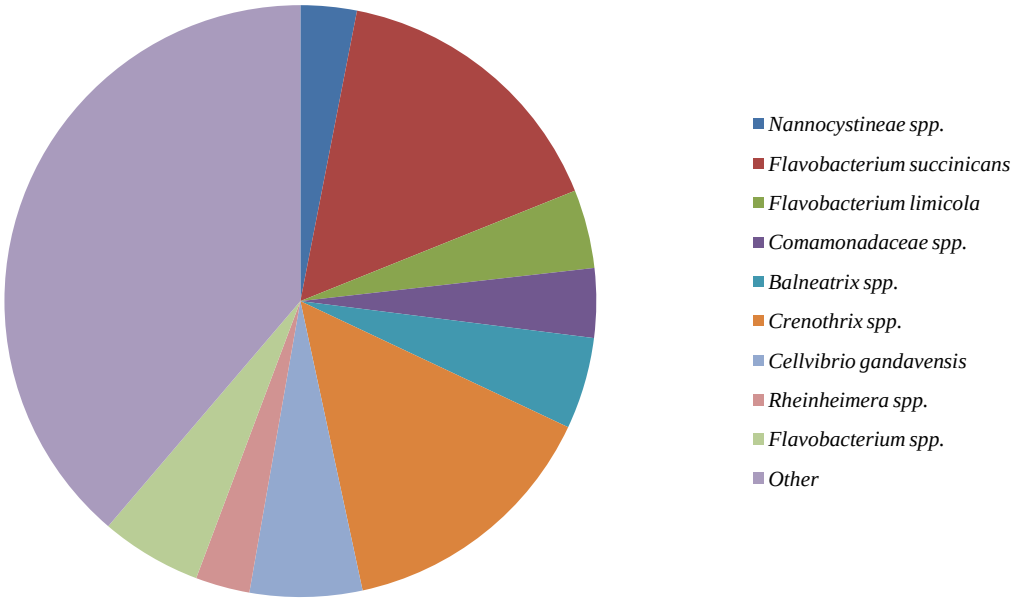


Figure 7.11: Species obtained from 16S rRNA amplicon sequencing of ^{12}C Heavy fraction DNA

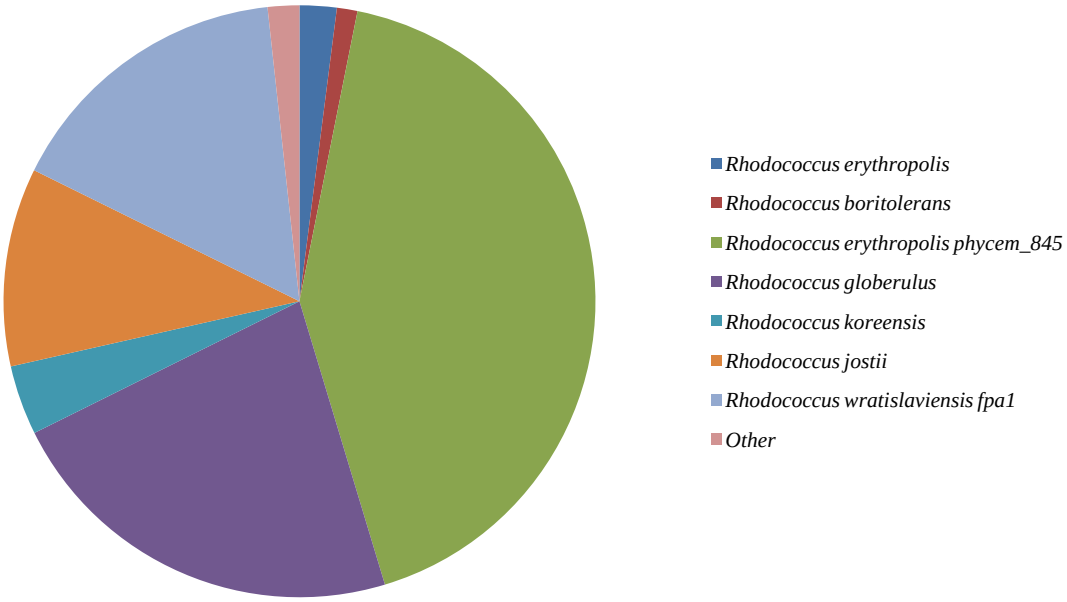


Figure 7.12: Species obtained from 16S rRNA amplicon sequencing of ^{13}C light fraction DNA

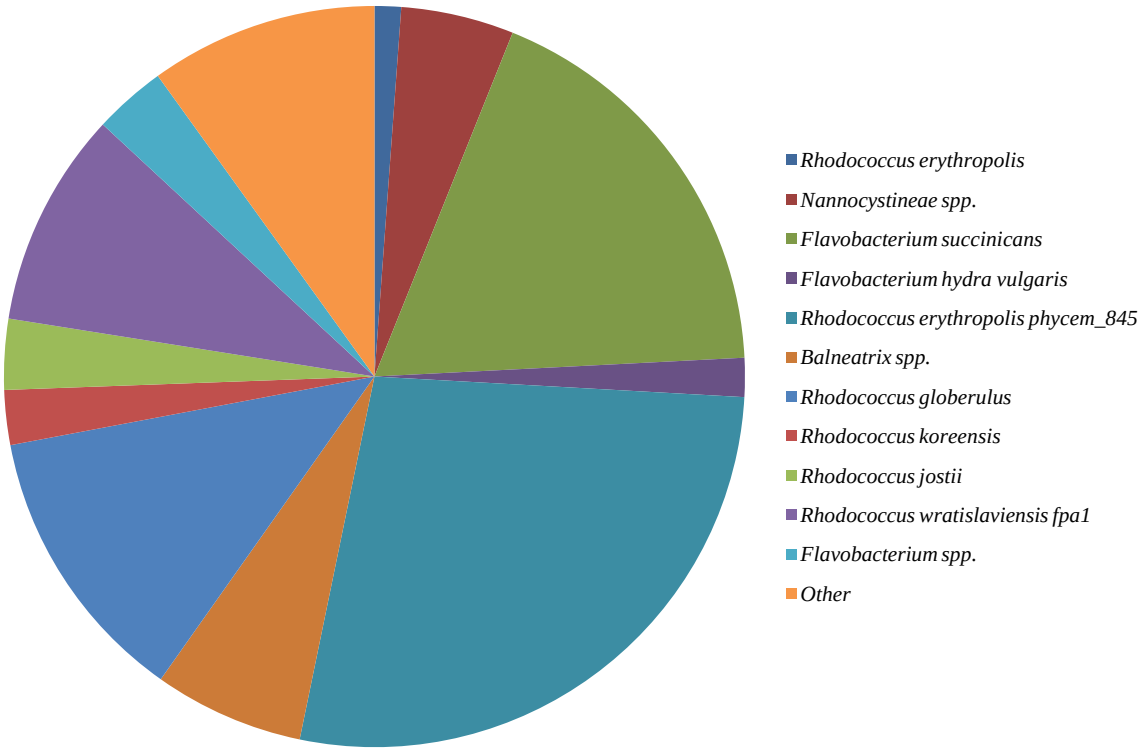


Figure 7.13: Species obtained from 16S rRNA amplicon sequencing of ^{12}C Light fraction DNA

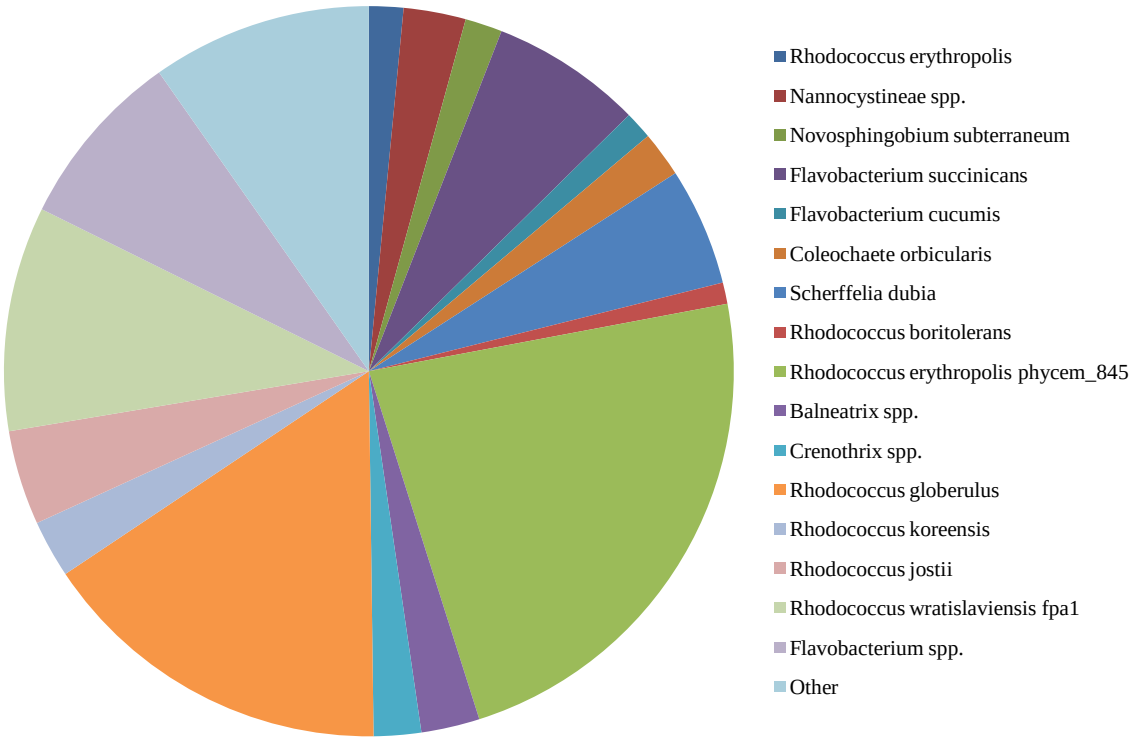


Figure 7.14: Comparison of 16S rRNA genes in ^{13}C heavy DNA at T1 and original T0 water samples

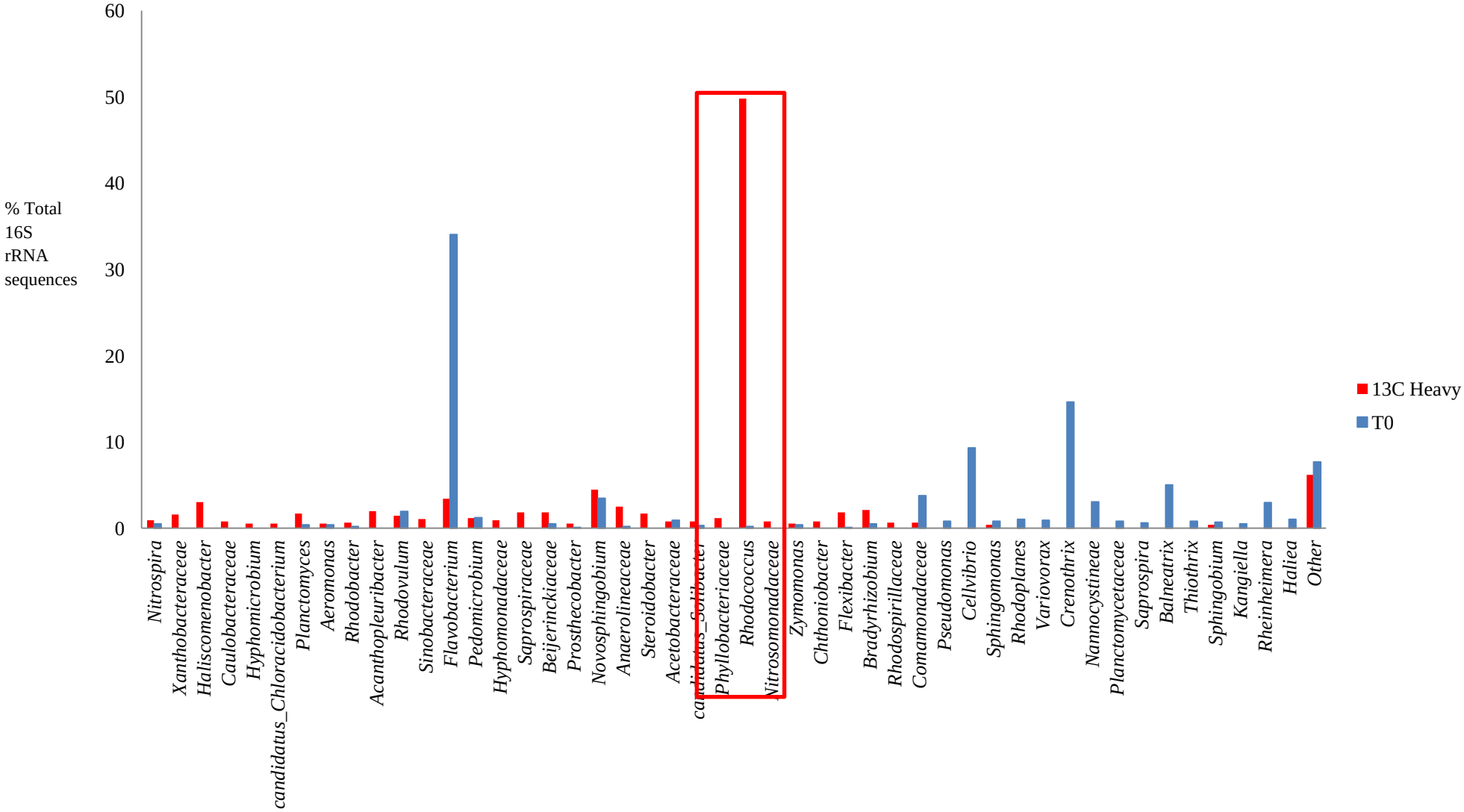


Figure 7.14 compares the percentages of 16S rRNA sequences obtained from each genus in samples from the time point one ^{13}C heavy fraction 6/7 versus native unenriched DNA (T0). This shows which genera are enriched in the labelled DNA fractions compared to the unenriched environmental sample DNA and are therefore likely isoprene degraders in the estuary environment. In this experiment, only *Rhodococcus* was significantly enriched in the ^{13}C labelled heavy DNA fractions, comprising a significant percentage of the overall 16S rRNA sequences as previously discussed. No other genera were significantly enriched, indicating that under these experimental conditions, *Rhodococcus* is an active isoprene degrader in estuarine samples taken from the Hythe. Figure 7.10 shows the diversity of *Rhodococcus* species that were detected in the labelled heavy DNA fraction. This indicates that several different members of this genus were active isoprene degraders in these samples. Table 7.1 shows the diversity of *isoA* gene sequences obtained in the ^{13}C labelled heavy DNA fraction. *isoA* amplicon sequencing was carried out using the forward *isoA* specific primer (see Chapter 6) as previous alignment data indicated that the amino acid sequence at the N-terminus of the IsoA polypeptide is more diverse and accurate in resolving the phylogeny of the IsoA polyeptide than the sequence at the carboxy terminus, which is more conserved. The average length of the *isoA* sequences obtained by amplicon sequencing was 365bp. The number of sequences obtained per amplicon sequence varied. These data included 40 unique *isoA* sequences when compared to IsoA of *Rhodococcus* AD45. Figure 7.15 shows the phylogeny of these *isoA* nucleotide sequences compared to *isoA* sequences from known isoprene degrading isolates. Almost all of the *isoA* sequences obtained appeared to be completely novel when compared to sequences from existing isolates, with a large diversity of sequences.

Table 7.1: Unique *isoA* sequences obtained from ^{13}C heavy fraction DNA

Denovo no.	Nucleotide identity to *isoA* of AD45	Sequence length (bp)	Amino acid similarity to IsoA of AD45
0	83%	360	72%
1	79%	333	71%
2	83%	266	77%
3	80%	286	70%
4	85%	444	80%
5	88%	227	82%
6	87%	375	80%
7	82%	433	75%
8	82%	434	78%
9	82%	397	72%
10	81%	349	70%
11	80%	369	70%
12	85%	362	72%
13	82%	258	71%
14	77%	314	67%
15	82%	427	70%
16	83%	300	78%
17	86%	438	80%
18	83%	249	79%
19	81%	310	74%
20	83%	361	70%
21	81%	293	71%
22	86%	282	75%
23	82%	261	70%
24	80%	385	70%
25	78%	310	70%
26	82%	316	75%
27	82%	349	71%
28	84%	443	77%
29	90%	395	84%
30	80%	382	69%
31	79%	322	68%
32	81%	423	72%
33	83%	443	72%
34	83%	455	71%
35	80%	465	71%
36	86%	435	75%
37	88%	441	78%
38	84%	431	71%
39	82%	374	70%
40	84%	452	74%

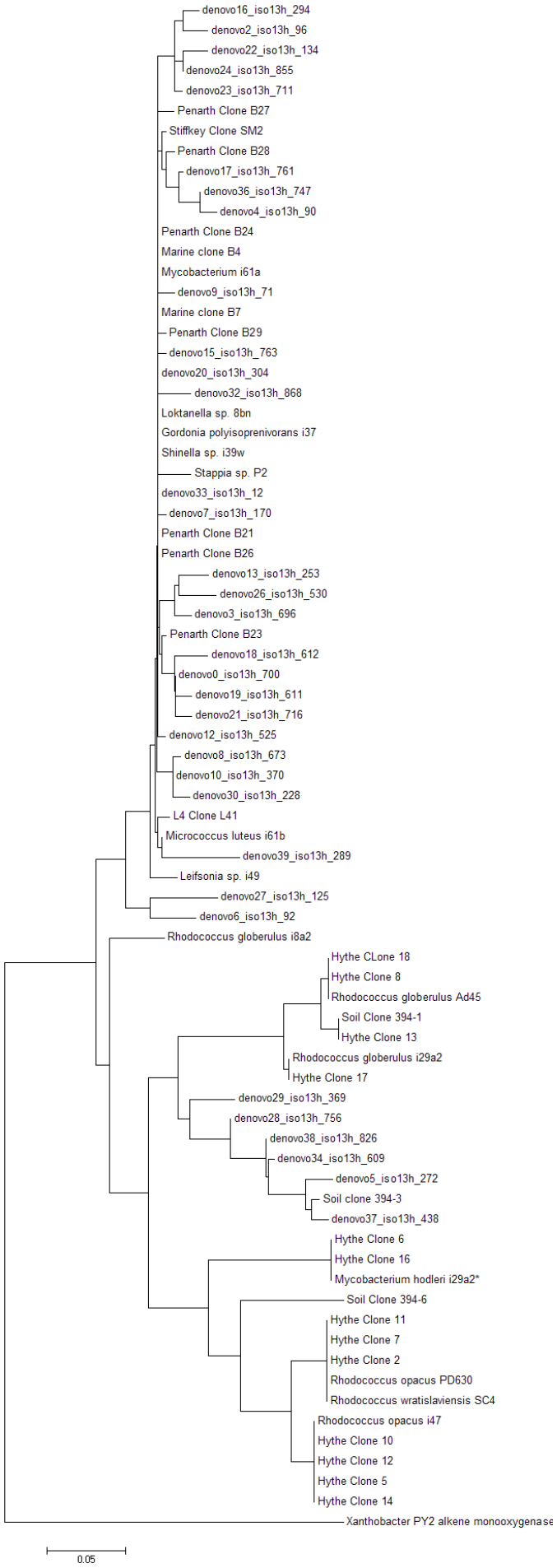


Figure 7.15: Neighbour joining tree of *isoA* nucleotide sequences derived from the heavy DNA of ^{13}C incubated samples at time point 1

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei. 1987). The optimal tree with the sum of branch length = 1.43912700 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004) and are in the units of the number of base substitutions per site. The analysis involved 76 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 193 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.* 2011). Bootstrap value:500.

Figure 7.16 Amino acid alignment of example *isoA* sequences obtained from the heavy DNA of ^{13}C incubated Hythe samples at time point 1

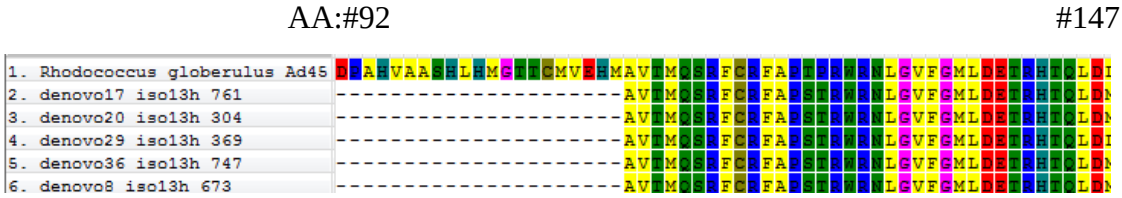


Table 7.1 shows the nucleotide and amino acid identities of the *isoA* sequences retrieved by 454 amplicon sequencing and compared to *isoA* of *Rhodococcus* AD45. An amino acid alignment of example IsoA sequences is shown in Figure 7.16.

7.4 Results – Wivenhoe incubations

As described in 7.1, a second DNA-SIP experiment was carried out using water and surface sediment samples taken from the Wivenhoe site along the Colne Estuary, Essex. The aim of this experiment was to identify the active isoprene-degrading bacteria at this site and also assess the changes in bacterial diversity between the two sampling sites.

Figure 7.17.1: Gas chromatography measurements showing isoprene depletion in Wivenhoe samples at time point 1 (n=1)

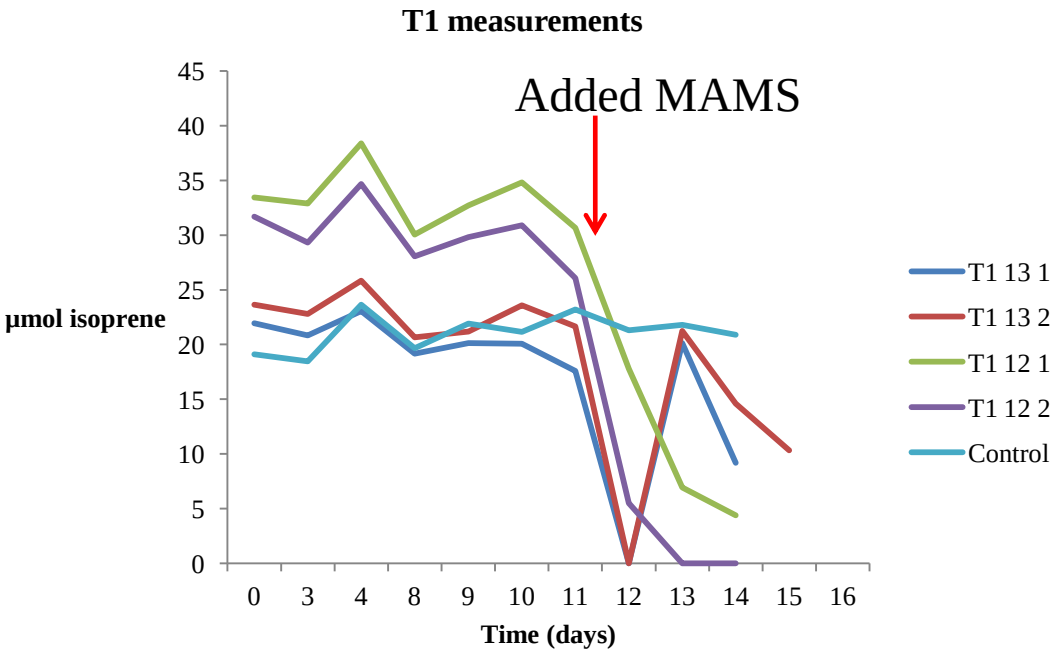


Figure 7.17.1 shows the depletion of isoprene in T1 sample incubations measured by gas chromatography. After approximately 10 days incubation, no activity was seen, contrasting with previous results obtained for the Hythe samples, which fully depleted 50µmol isoprene in 3 days. The decision was taken to add nutrients to the sample in the form of minimal MAMS medium (see Materials and Methods for composition). Samples were nutrient limited and after addition of MAMS, isoprene depletion was rapid in the incubations, while no isoprene depletion was seen in autoclaved control samples. All samples had completely depleted the added isoprene after 2-3 days additional incubation. Some replicates were respiked due to slight discrepancies in the initial volume of added isoprene and all samples were sacrificed at 14 days after 30 µmoles of isoprene had depleted.

Figure 7.17.2: Gas chromatography measurements showing isoprene depletion in Wivenhoe samples (time points 2 and 3) (n=1)

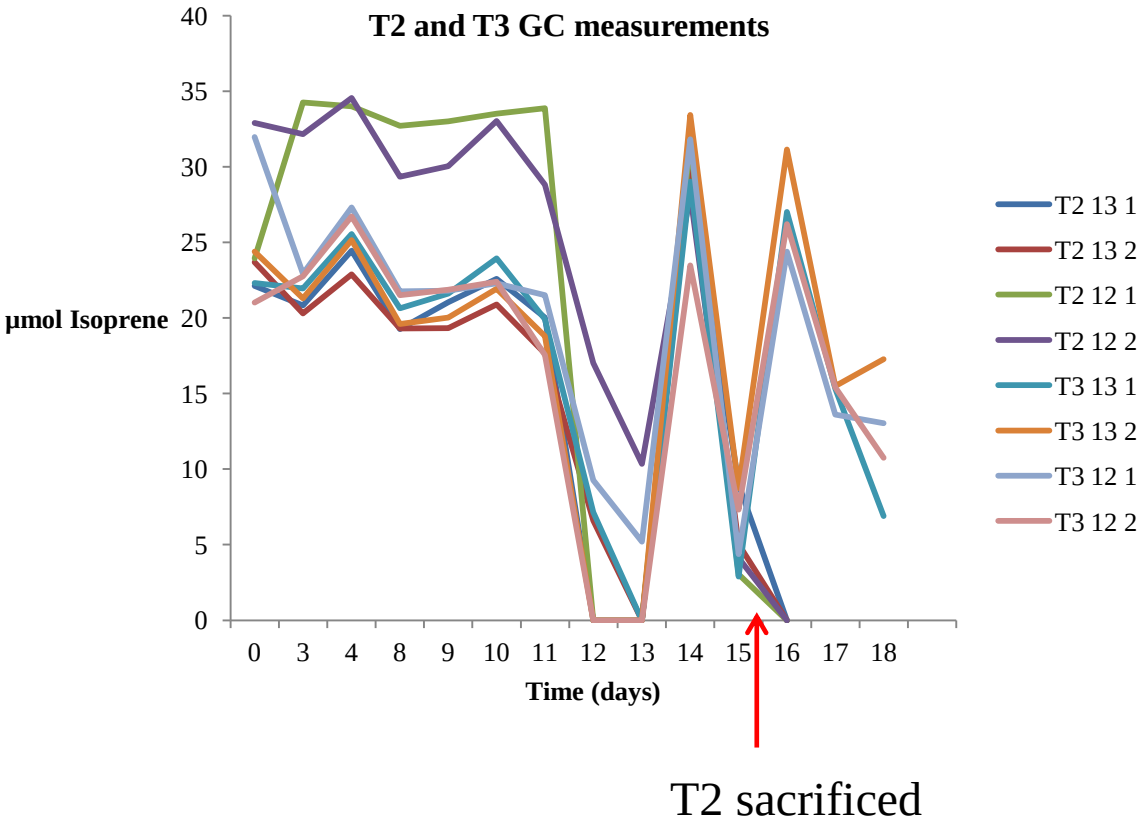


Figure 7.17.2 shows the depletion of isoprene in T2 and T3 sample incubations measured by gas chromatography. Samples were respiked with an additional 30µmol of isoprene and

depletion was rapid, with all samples having completely degraded the added isoprene within 1-2 days. T2 incubations were sacrificed at 15 days and T3 samples were respiked with an additional 30µmol of isoprene. The measured depletion of isoprene was initially rapid as seen in T2, however at 18 days the isoprene in the samples had not been further depleted. This effect was also seen in the Hythe incubations and it was likely due to oxygen depletion in the serum vials. T3 samples were therefore sacrificed at 18 days.

Figure 7.18: Refractometer density measurements of fractions obtained from T1 ^{13}C incubated Wivenhoe samples

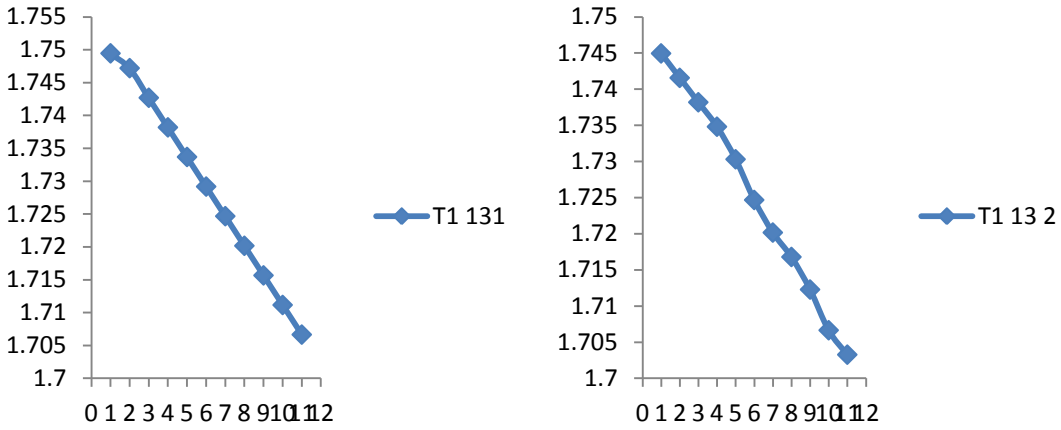
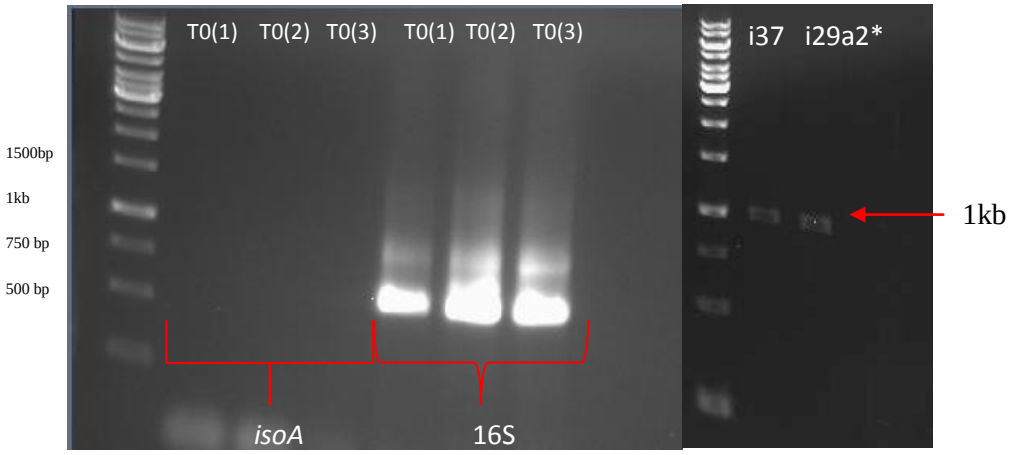


Figure 7.19: *isoA* and 16S rRNA PCR products amplified from unenriched T0 DNA replicates



16S rRNA gene PCR products were amplified using 341GC-907R primers which have an expected product size of ~550bp. The 16S rRNA gene PCR products obtained were of the correct size (Figure 7.19).

Figure 7.20: T0/T1/T2/T3 *isoA* PCR products and 16S rRNA PCR products amplified from unfractionated DNA

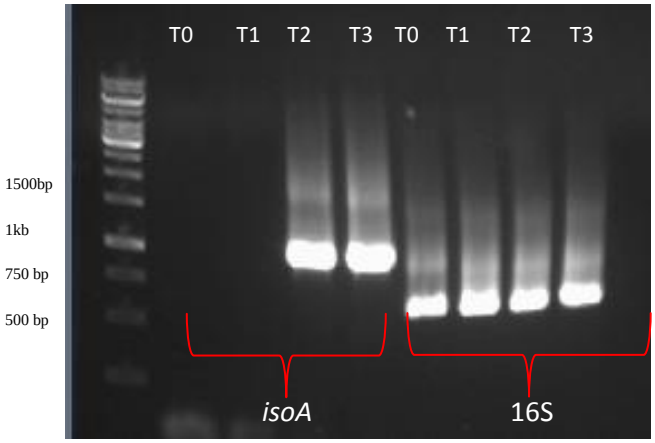
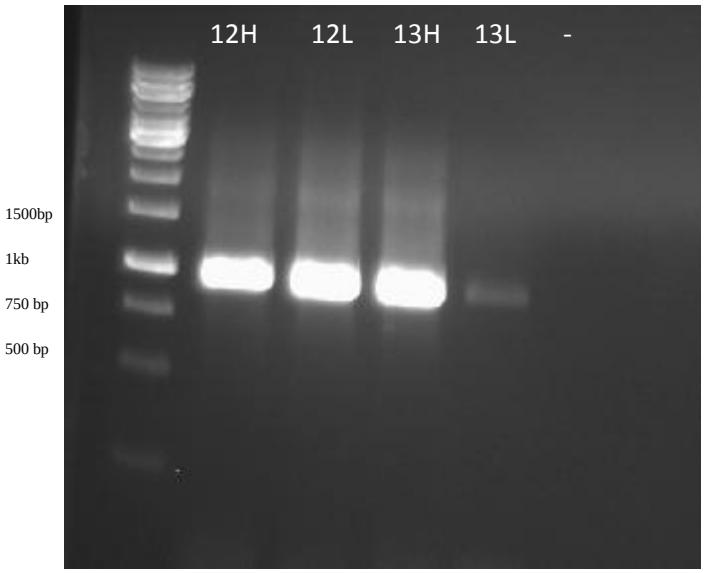


Figure 7.21: T1 *isoA* PCR products amplified from $^{13}C/^{12}C$ Light and Heavy DNA



L= light fractions

H=Heavy fractions

Figure 7.22: *isoA* PCR products amplified from T1 ^{13}C fractions

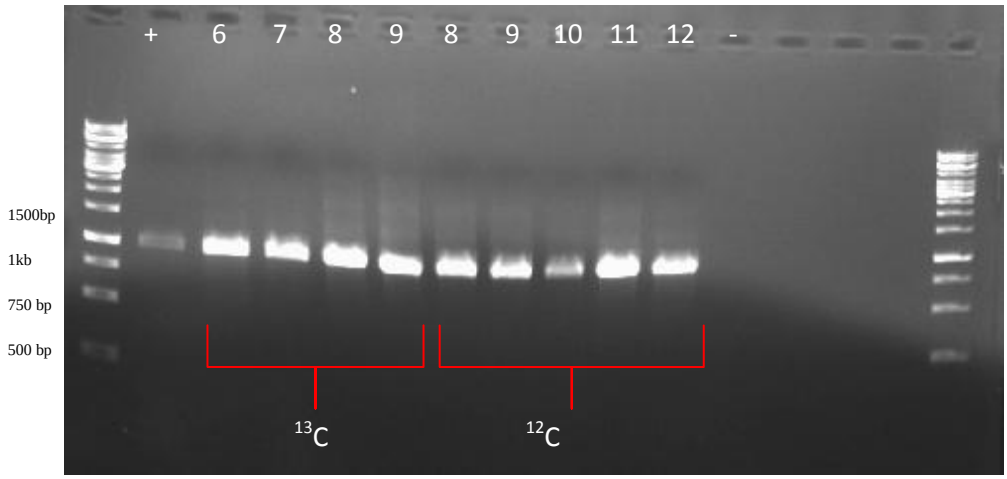


Figure 7.23: 16S rRNA PCR products amplified from DNA from ^{12}C and ^{13}C Wivenhoe water incubations using 341GC-907 primer set

Figure 7.23.1: 16S rRNA PCR products obtained from Wivenhoe T1 fractions 6-12 from ^{12}C and ^{13}C incubated samples

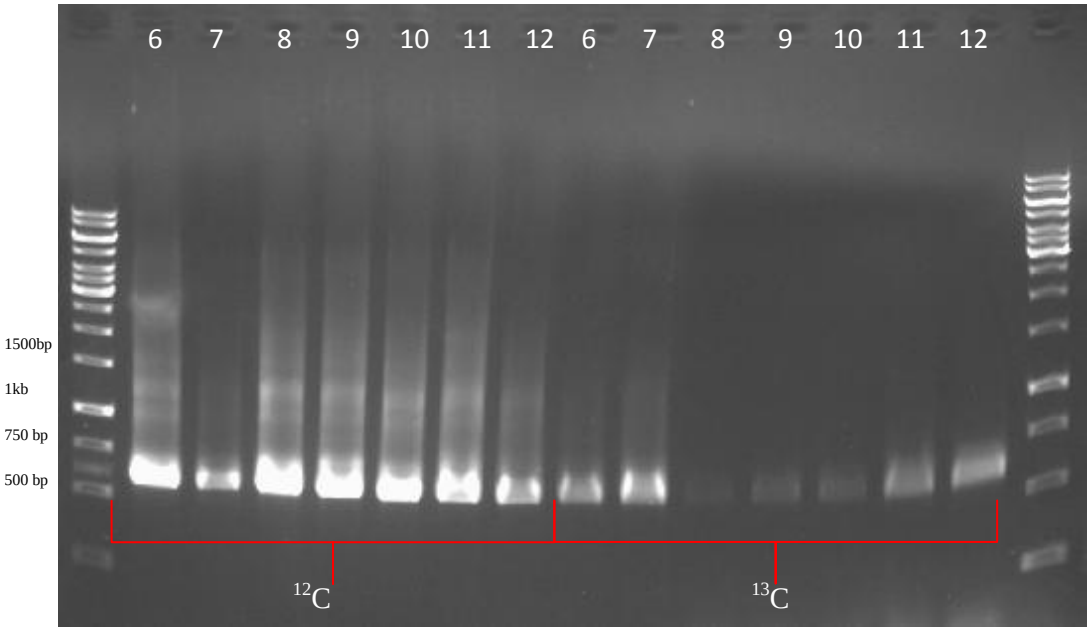
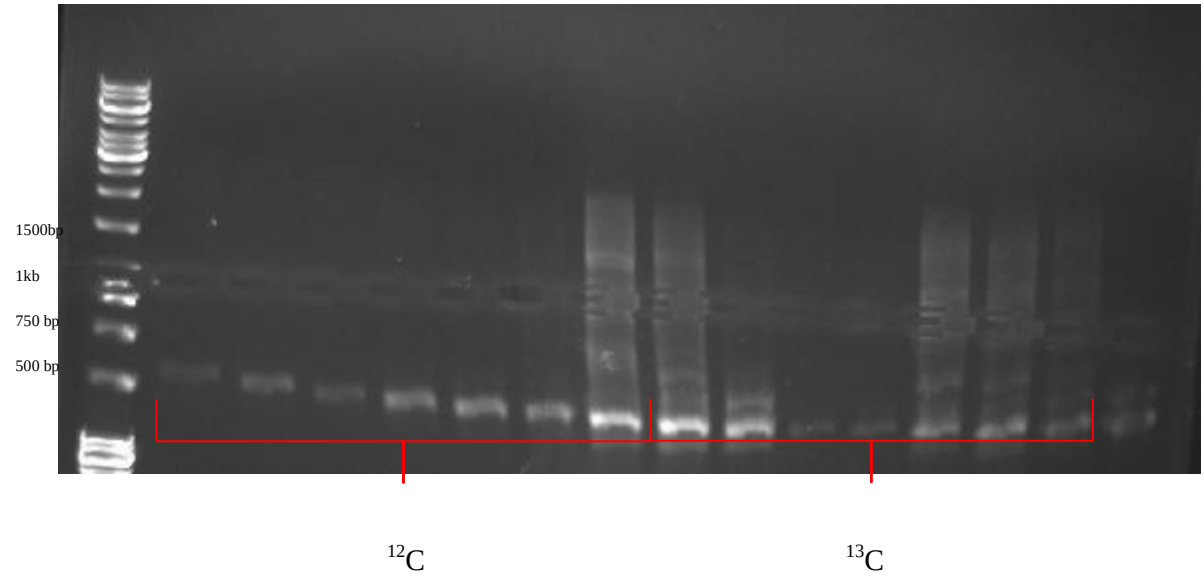


Figure 7.23.2 : 16S rRNA pCR products obtained from Wivenhoe T2 $^{12}C/^{13}C$ incubations fractions 6-12



Figures 7.19, 7.20 and 7.21 show the results of *isoA* PCR reactions using unfractionated DNA from each incubated time point as well as native T0 DNA. The *isoA* PCR primer set and conditions used in this reaction were described in Chapter 6. Initially an *isoA* product was amplified only from T2 and T3 DNA templates No product was amplified from

unfractionated T1 DNA, however a PCR product was obtained with DNA from all T1 fractions tested (see Figures 7.22 and 7.23.1) indicating that the unfractionated T1 DNA may have contained humic substances inhibiting the PCR reaction. No *isoA* PCR product could be amplified from native T0 DNA templates, indicating that isoprene degrading bacteria must be at extremely low abundance in the original environmental sample. This is unsurprising due to the low biomass in the estuarine samples.A 16S rRNA gene PCR product of the correct size was amplified from all T1 and T2 fractions (Figure 7.23.3) as well as T0/T1/T2/T3 unfractionated DNA. The primer set used was 341GC-907 as described in Materials and Methods section 2.24. These primers contain a GC clamp sequence which allows the product to be run on a denaturing gradient gel to profile the community. A PCR product of the correct size (~550bp) was amplified from all templates.

Figure 7.24: DGGE gel showing 16S rRNA profiles from T0-T3 unfractionated DNA and ^{13}C incubated T2 replicate 1 fractions 5-12

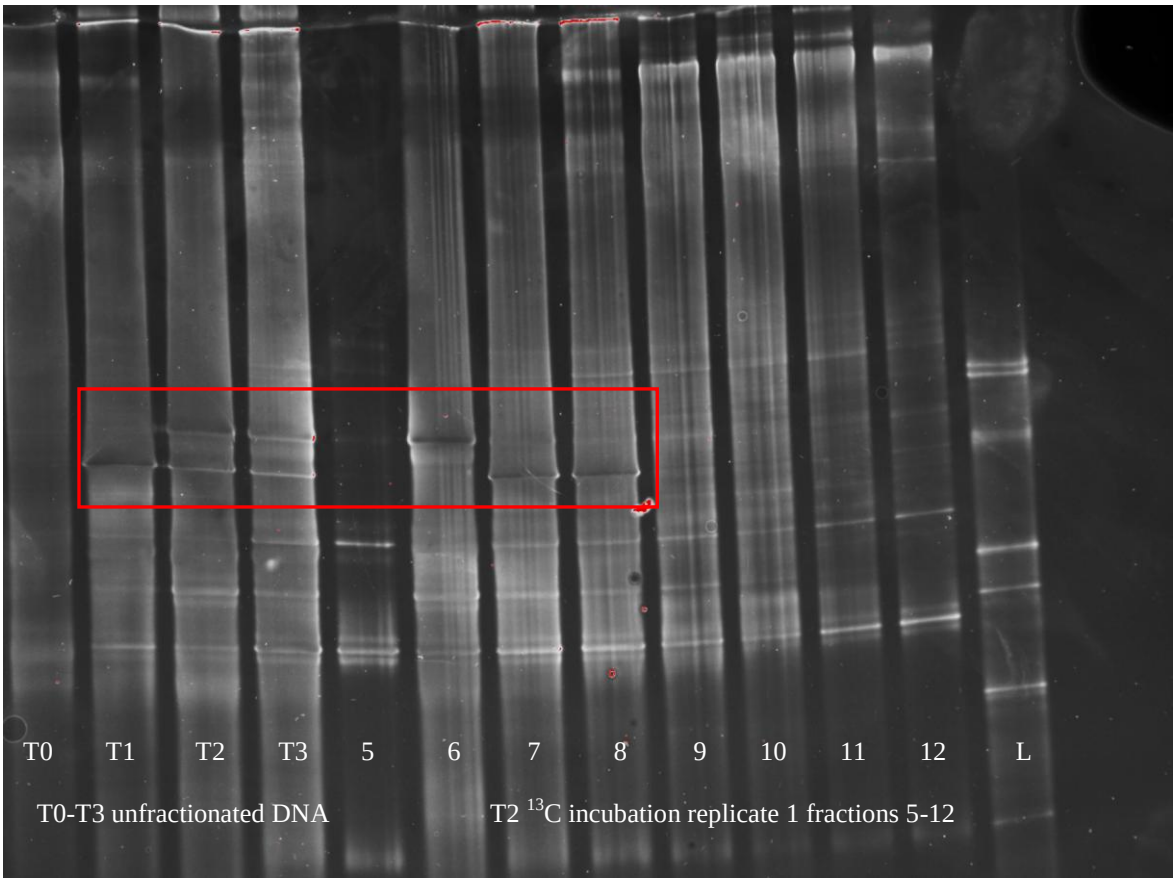


Figure 7.24 shows the DGGE profile of the 16S rRNA gene PCR products obtained from the unfractionated T0/T1/T2/T3 DNA. Additionally, Figure 7.25 shows a second DGGE profile of T0/T1/T2. As highlighted in Figure 7.24, there are clearly bands that are unique to the profiles of time points after enrichment with isoprene, with several bands that could represent isoprene degrading bacteria. There was little difference seen between the T2 and T3 profiles, whereas the T2 and T3 profiles contained bands not present in T1. Figure 7.24 also shows the disappearence of several bands over time from T0 as the samples are enriched with isoprene in T1 and T2. These profiles show that there is a clear change in the community after enrichment with isoprene, with a likely increase in isoprene degrading bacteria over time and a depletion of non isoprene degraders that are outcompeted.
Figure 7.25 also shows the DGGE profiles of the 16S rRNA sequences obtained from T1 unfractionated ^{12}C and ^{13}C isoprene enriched sample replicates. The DGGE profiles for these replicates appear identical across T1 suggesting that there is reliable replication between samples.

Figure 7.25: DGGE profiles of 16S rRNA sequences amplified from T0/T1/T2 unfractionated DNA and T1 $^{12}C/^{13}C$ replicate unfractionated DNA

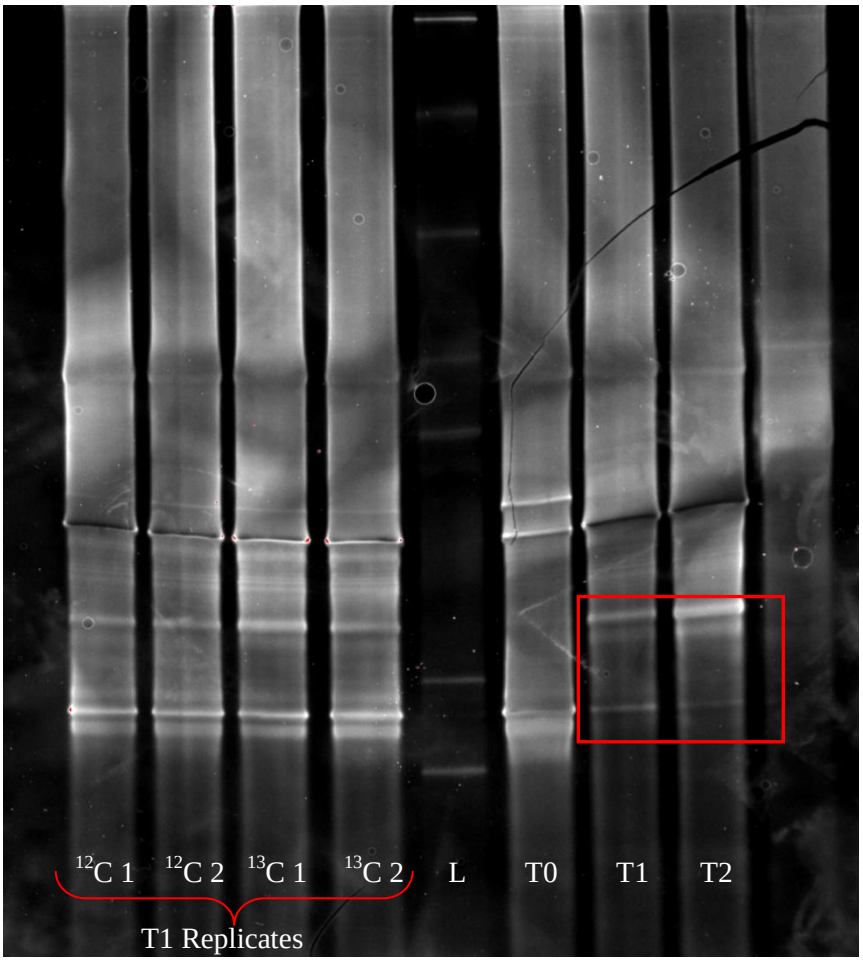


Figure 7.26: DGGE profile of 16S rRNA PCR products amplified from DNA from CsCl fractions of Wivenhoe ^{12}C and ^{13}C incubations at T1

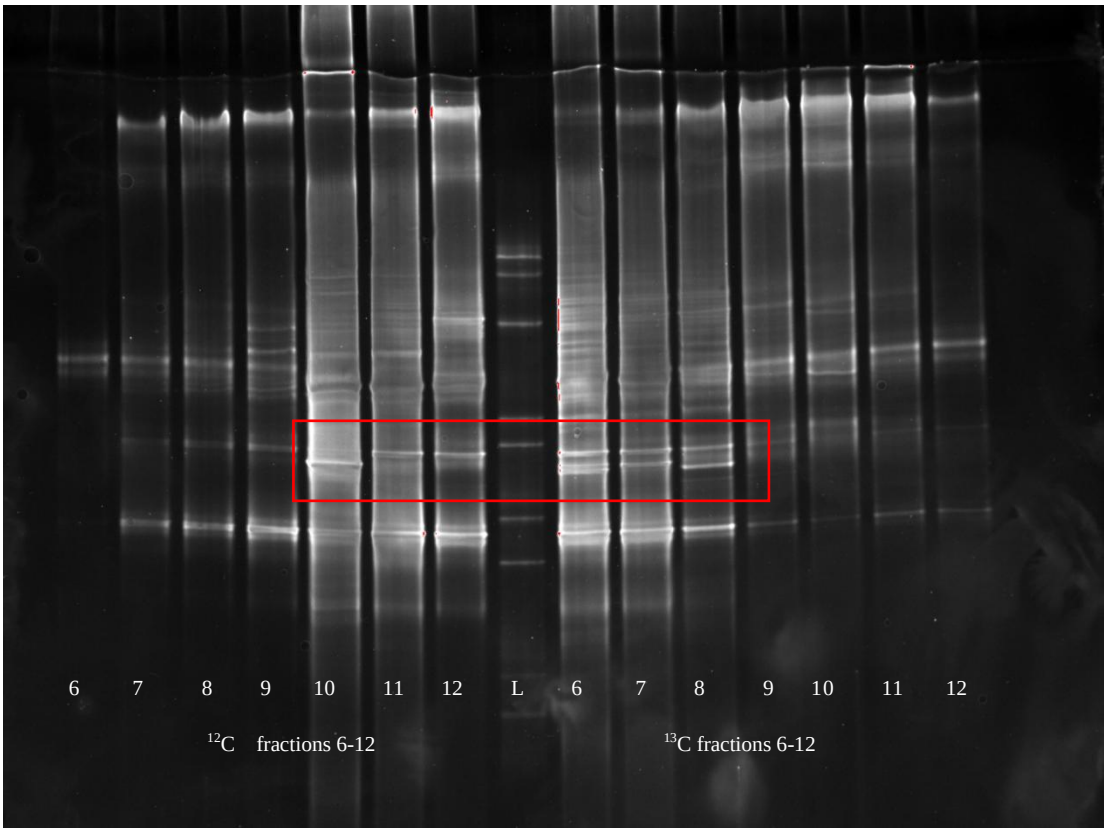
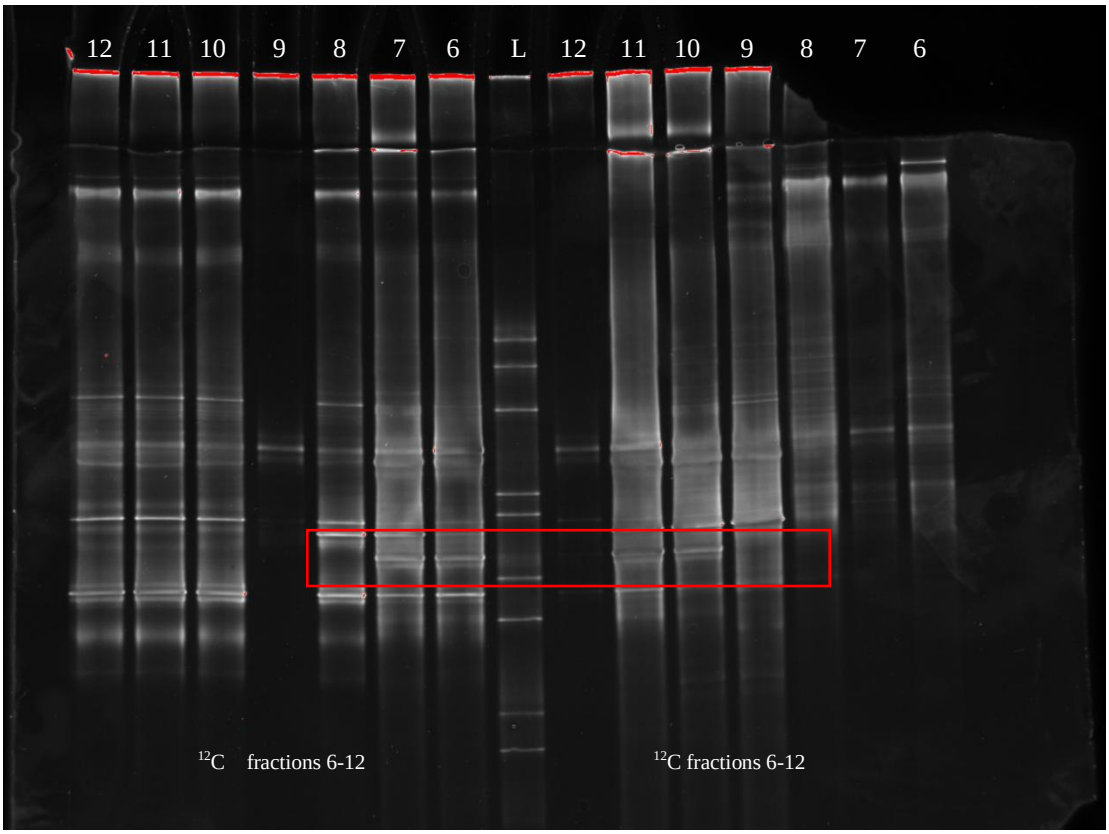


Figure 7.27: DGGE profile of 16S rRNA PCR products amplified from DNA from CsCl fractions of Wivenhoe ^{12}C and ^{13}C incubations at T2



Figures 7.26 and 7.27 show the 16S rRNA DGGE profiles of T1/T2 fractions 6-12 from both ^{12}C and ^{13}C incubated samples. As can be seen on the highlighted figure, there are bands that appear in the heavy ^{13}C fractions of these samples (fractions 6-8 for T1 as shown by refractomer readings in Figure 7.18), fractions 6 and 7 for T2 as predicted by density of the fractions). These bands correspond to those that appear in the light fractions of ^{12}C incubated samples (fractions 10/11) and likely represent the 16S rRNA gene product from an isoprene degrading organism. The profiles appear different between time points 1 and 2 indicating that different organisms have been enriched at each time point. Based on these DGGE profiles, the decision was taken to sequence representative replicates of light and heavy fractions from both ^{12}C and ^{13}C incubated samples at time points 1 and 2.These data should reveal not only the active isoprene degraders in these environmental samples, but the enrichment of the isoprene degrading community over time.

Figure 7.28: Sequence diversity obtained from 16S rRNA amplicon sequencing of DNA from T1 replicates

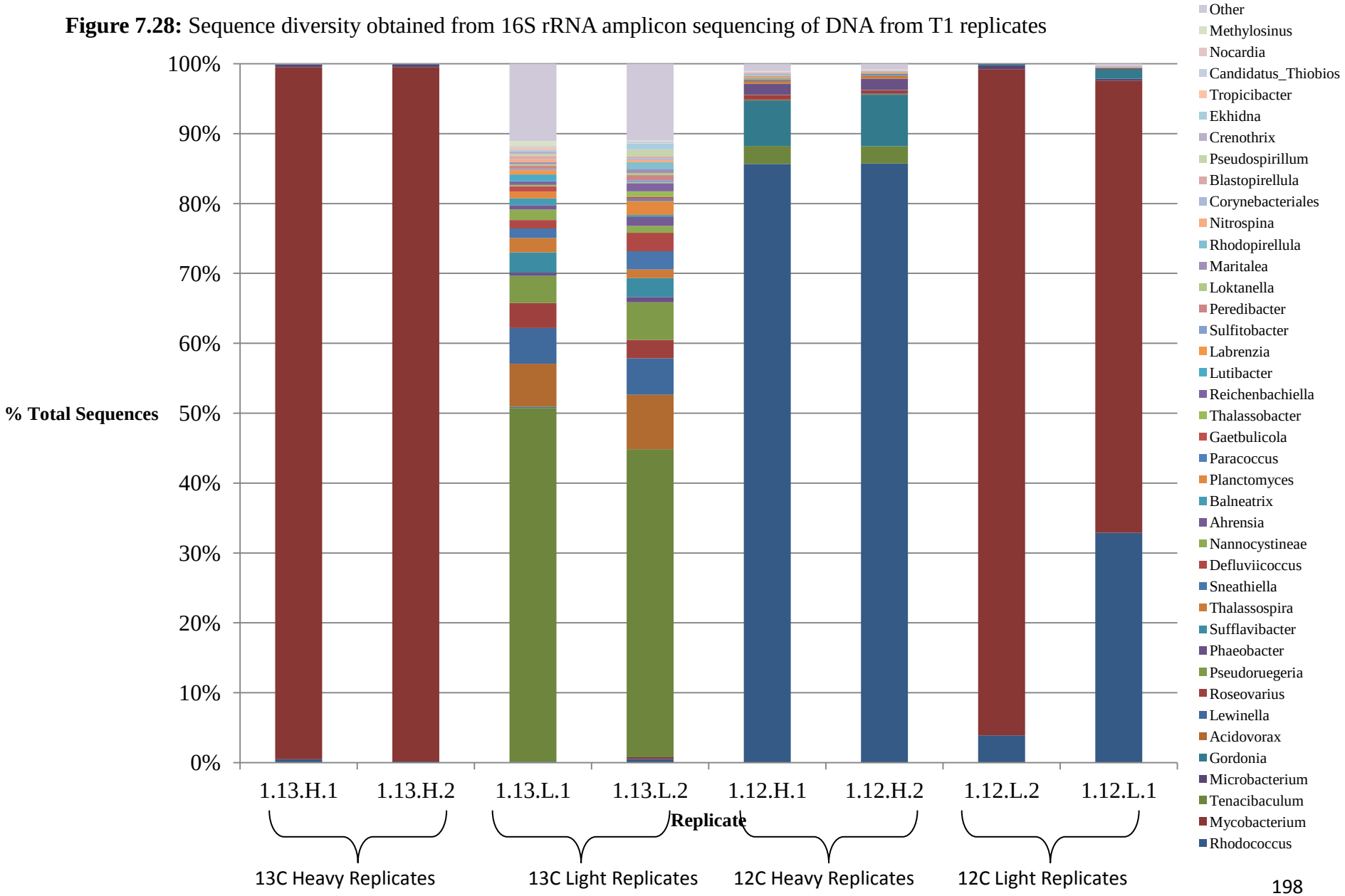


Figure 7.29 Sequence diversity obtained from 16S rRNA amplicon sequencing of DNA from T2 Replicates

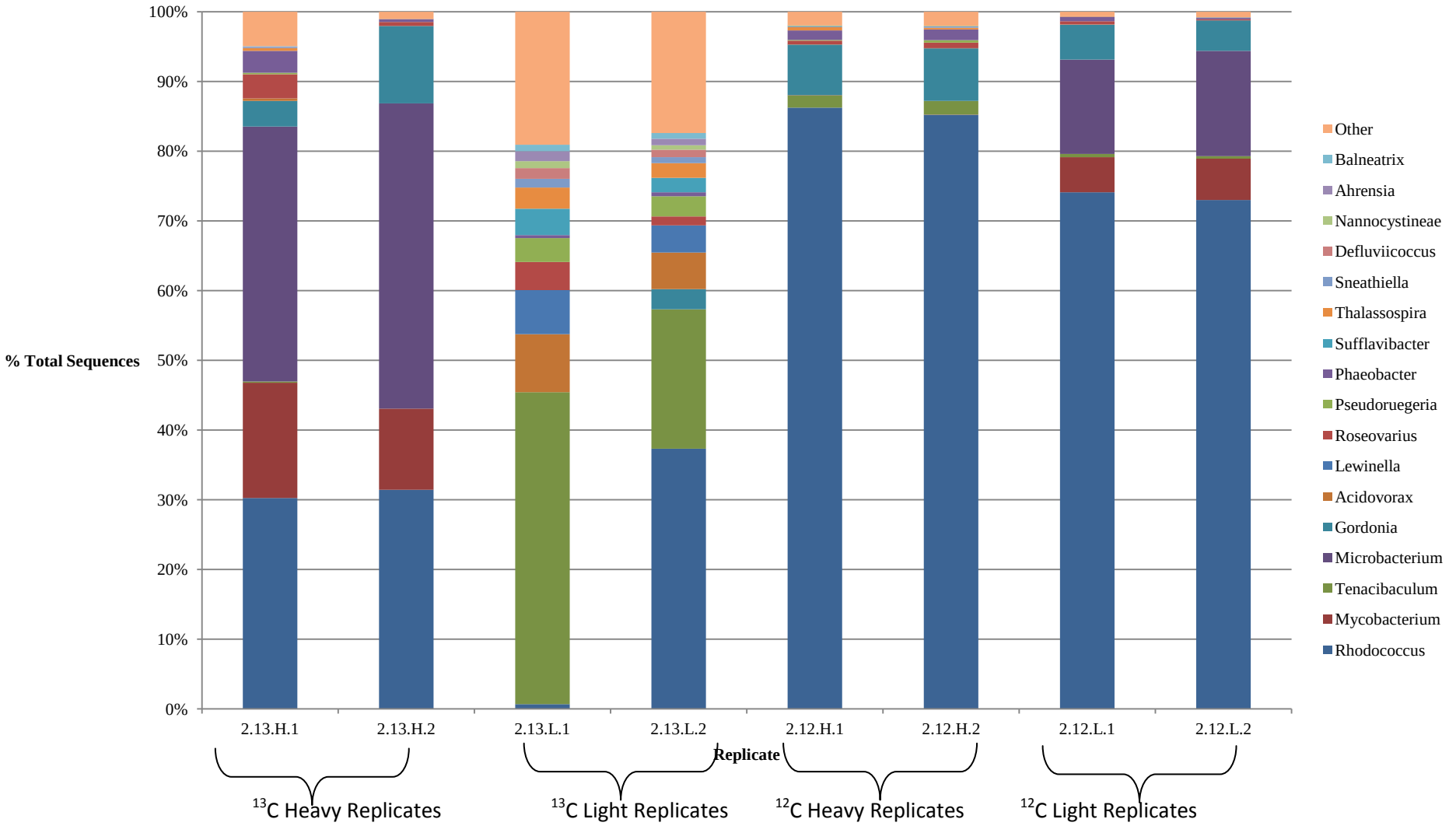


Figure 7.28 and Figure 7.29 show the 16S rRNA sequences obtained from Illumina sequencing of all samples from time points 1 and 2. The average number of sequences obtained per sample was 5386. Sequences are depleted of barcodes and primers then short sequences < 200bp and sequences with homopolymer runs exceeding 6bp removed. Sequences are then denoised and chimeras removed. Operational taxonomic units were defined after removal of singleton sequences, clustering at 3% divergence (97% similarity).There is a high similarity between biological replicates, with the main anomaly being the greater proportion of *Rhodococcus* sequences obtained from all ^{12}C isoprene incubated samples at time point 2 compared to samples incubated with ^{13}C isoprene. The difference is likely due to the natural variability of sediment added to the samples.

Figure 7.30: 16S rRNA sequences obtained from amplicon sequencing of unenriched T0 Wivenhoe DNA

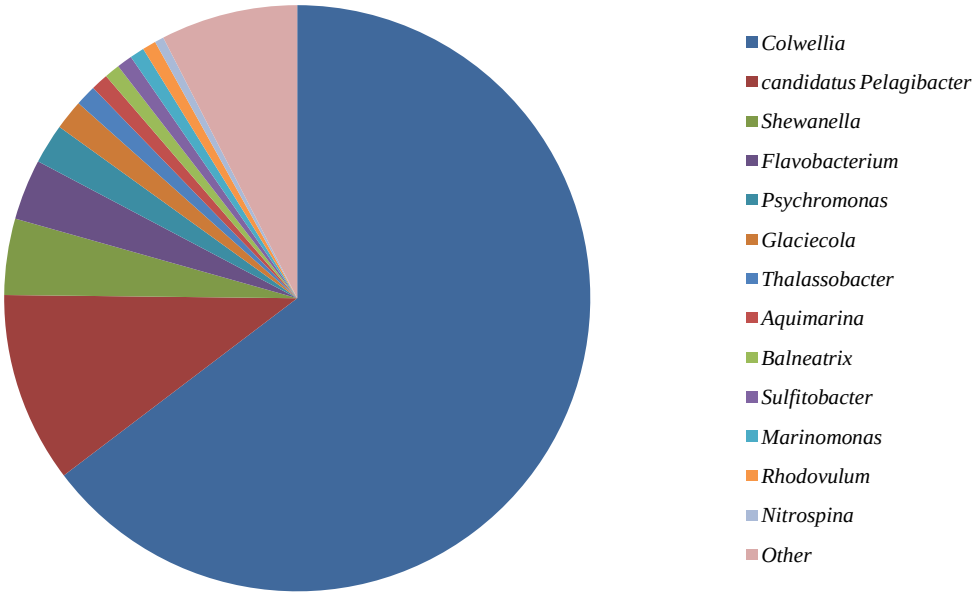


Figure 7.30 shows the diversity of 16S rRNA gene sequences obtained from T0, or native DNA of the unenriched water and sediment. The community as this site differs greatly from that found at the Hythe (Figure 7.4) with *Colwellia* sequences dominating the sample along with candidatus *Pelagibacter* sequences and *Shewanella* species. *Flavobacterium* sequences, which dominated the T0 samples taken from the Hythe, were greatly reduced in the Wivenhoe samples. No known isoprene degrading genera were represented in the 16S rRNA sequences obtained at T0, indicating that if present, these organisms exist in small numbers in this environment.

Figure 7.31: 16S rRNA sequences obtained by amplicon sequencing of T1 ^{13}C Heavy Replicate 1 DNA

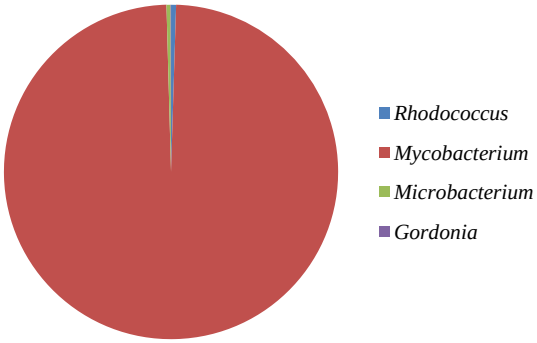


Figure 7.33: 16S rRNA sequences obtained from T1 ^{12}C Light Replicate 1 DNA

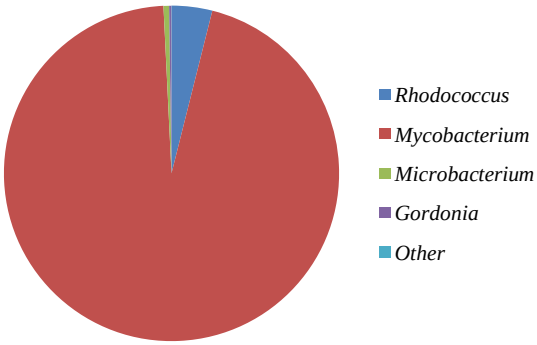


Figure 7.34: 16S rRNA sequences obtained from amplicon sequencing of T1 ^{12}C Light Replicate 2 DNA

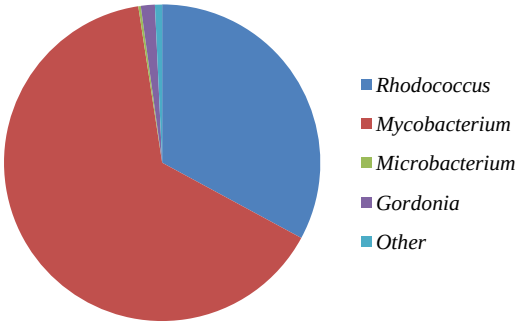


Figure 7.35: 16S rRNA sequences obtained from amplicon sequencing of T2 ^{13}C Heavy Replicate 1 DNA

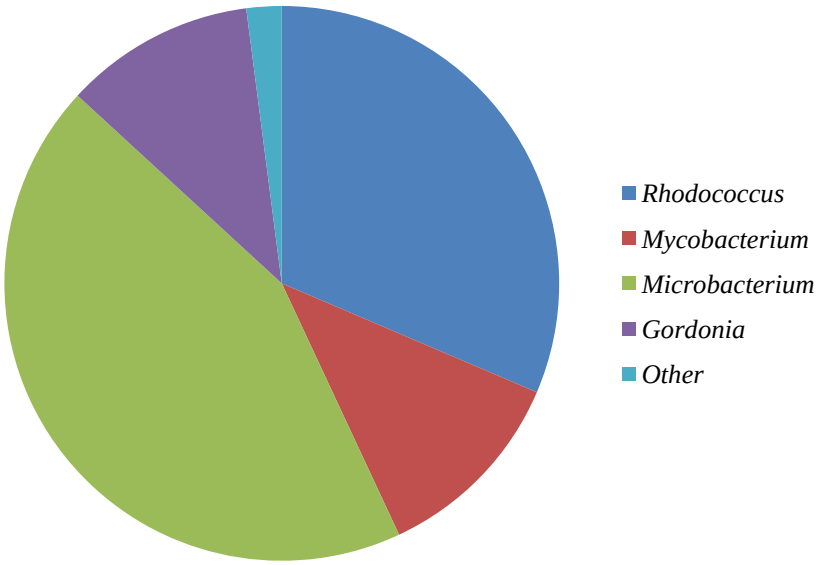


Figure 7.36: 16S rRNA sequences obtained from amplicon sequencing of T2 ^{13}C Heavy Replicate 1 DNA

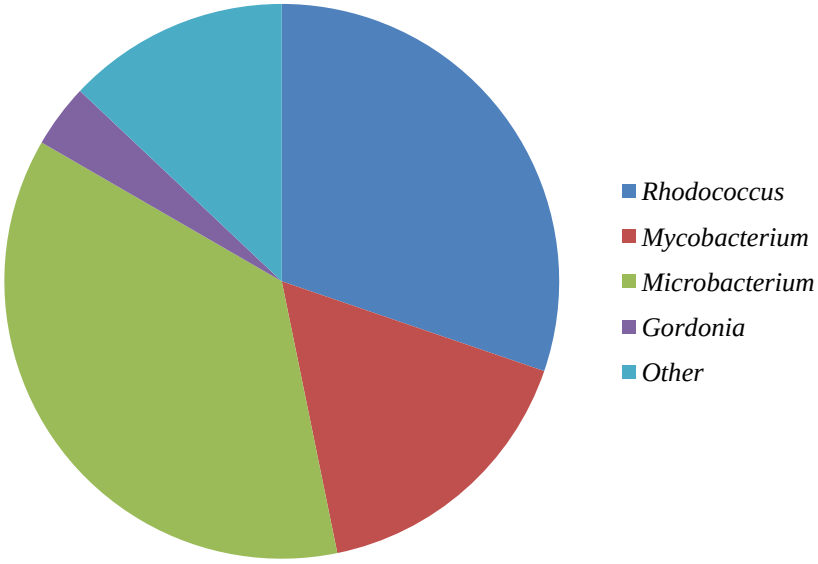


Figure 7.37: 16S rRNA sequences obtained from amplicon sequencing of T2 ^{12}C Light Replicate 1 DNA

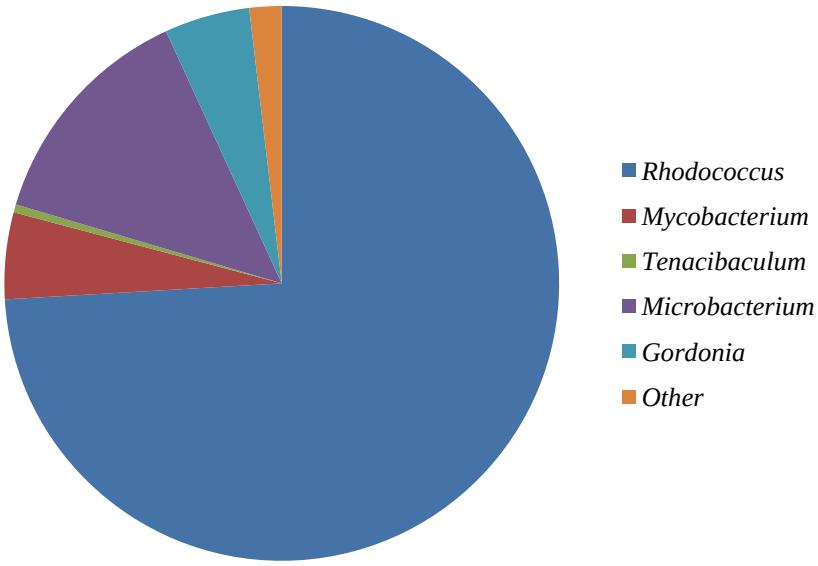


Figure 7.38: 16S rRNA sequences obtained from amplicon sequencing of T2 ^{12}C Light Replicate 2 DNA

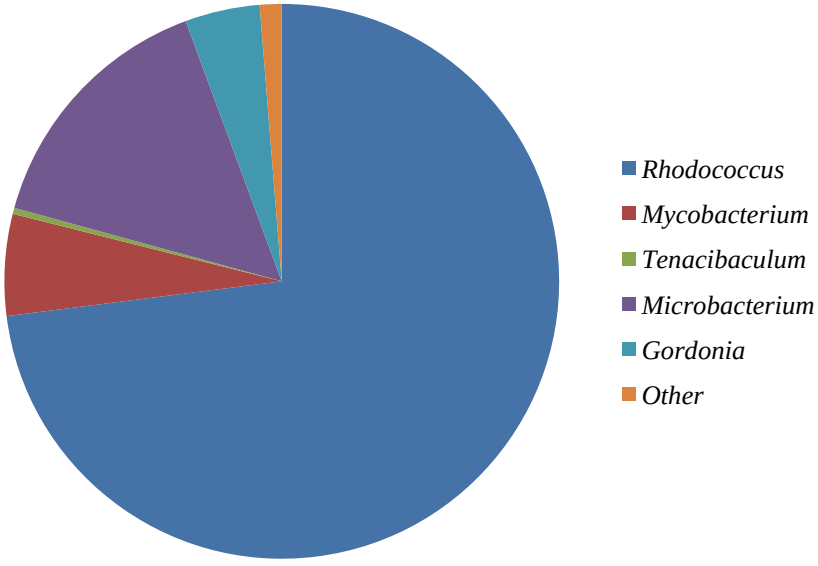


Figure 7.31 and Figure 7.32 show the 16S rRNA sequences retrieved from the heavy fractions of replicate ^{13}C incubations at time point 1. These sequences were almost entirely dominated by *Mycobacterium* species. Members of the genus *Mycobacterium* have been previously shown to degrade isoprene, with two isoprene degrading species isolated from the Colne Estuary (chapter 3). *Mycobacterium hodleri* i29a2* (Alvaerz *et al.* 2009) grows rapidly on isoprene in laboratory cultures, and it is possible that growth of this genus is also rapid in the environment in the presence of isoprene. It must be remembered that the concentration of isoprene used in these DNA-SIP experiments exceeds what would be found in the environment. *M.hodleri* can grow on isoprene at concentrations above those used in this experiment (up to 1.2%). It is possible that the experimental conditions were biased towards the growth of *Mycobacterium* due to its rapid growth rate and high tolerance for isoprene (as shown in Chapters 3 and 4).

Mycobacterium also dominated the light fractions of the ^{12}C incubated replicates Figures 7.33 and 7.34), as would be expected, however the percentage of overall 16S rRNA sequences belonging to *Mycobacterium* was reduced and these samples contained a significant number of *Rhodococcus* 16S rRNA sequences. The difference between the ^{12}C and ^{13}C replicates at time point 1 is likely due to natural variation in the numbers of different cells present in sediment used in the incubations.

Significant differences were seen between the time point 1 and time point 2 samples for both ^{13}C and ^{12}C-incubated samples. At time point 2, a more diverse community of isoprene degraders emerged. *Mycobacterium* 16S rRNA gene sequences still formed a significant percentage of the overall sequences but are no longer the dominant genus, as shown in Figures 7.35, 7.36, 7.37 and 7.38. There is an increase in the proportion of *Rhodococcus* 16S rRNA sequences, which dominate the samples. *Rhodococcus* have been shown to degrade isoprene both in the preliminary Hythe DNA-SIP experiment and also with several laboratory strains isolated from isoprene-enriched water and soil samples. Also significantly enriched in the ^{13}C heavy fraction/^{12}C light fraction are 16S rRNA sequences from the Actinobacteria *Microbacterium* and *Gordonia.* Members of these genera have been isolated and grow on isoprene as a sole carbon and energy source (Alvarez *et al.* 2009)(Chapter 3), and the genome sequence of *Gordonia polyisoprenivorans* i37 (see Chapter 5) confirms that this organism possesses a soluble diiron centre isoprene monooxygenase gene cluster, as does *Mycobacterium hodleri* i29a2*. PCR primers targeting *isoA* were successful in amplifying *isoA* gene sequences

from all *Rhodococcus* and *Microbacterium* isolates tested (see Chapter 6). Polypeptide profiles of these isolates grown on different carbon sources and qRT-PCR of the *isoA* gene in *Gordonia polyisoprenivorans* i37 shows that isoprene monooxygenase is transcribed and expressed during growth on isoprene. Therefore, it can be concluded with confidence that *Microbacterium, Rhodococcus,Gordonia* and *Mycobacterium* are active isoprene degraders in the Colne Estuary, and that these isolates degrade isoprene via an inducible soluble diiron centre isoprene monooxygenase.

7.5 Summary of Results

The purpose of this study was to identify active isoprene degraders in two different sampling sites along the Colne Estuary, Essex. The first of these sampling sites, at the Hythe, is located further inland than the second site at Wivenhoe, which is further seaward. DGGE profiling of 16S rRNA genes at different time points showed no differences in the community, and therefore only time point 1 was selected for further analysis. These samples, when incubated at 30°C with isoprene as their sole carbon source, showed a rapid enrichment in *Rhodococcus* species in the heavy ^{13}C labelled DNA, indicating that *Rhodococcus* is an active isoprene degrader in this environment. Several *Rhodococcus* species have been isolated from various points along the Colne Estuary and also from terrestrial soils. The head of the Colne estuary at Hythe had the highest recorded biomass of phytoplankton (including euglenoid algae and benthic diatoms) when measured at four points along the estuary. Primary production of algal blooms in this area of the estuary is estimated at 150g C m^{-2} yr^{-1}, far exceeding other sites at Wivenhoe, Brightlingsea and Aldboro (Kocum *et al*. 2002). These communities form biofilms on the sediment of the estuary and may provide a source of isoprene to maintain isoprene degrading communities in the estuary at low levels. *Rhodococcus* species represented only 0.2% of the sequences retrieved by high throughput sequencing of the 16S rRNA gene PCR product obtained from T0 template DNA, however *Rhodococcus* species represented 50% of the total 16S rRNA sequences retrieved from the labelled ^{13}C fraction DNA. The *isoA* gene sequences retrieved from the labelled ^{13}C fraction DNA showed high sequence similarity to known terrestrial isoprene degrading *Rhodococcus* species and *isoA* clones from soil environments. Although the Colne estuary is tidal, the Hythe sampling site is far inland, with a large proportion of the estuary basin regularly exposed by retreating water at low tide, and receives pollution from human activities in Colchester, Essex, therefore this site may be considered as similar to terrestrial sampling sites. It must however be noted that these samples were incubated at higher temperatures than those found in the environment, and at an isoprene concentrations that far exceeds natural concentrations. While this preliminary experiment confirms that *Rhodococcus* spp. can utilise isoprene in the environment, the experimental design may have been heavily biased in favour of these organisms, with slower growing species outcompeted by fast growing *Rhodococcus* spp., as well as species that utilise isoprene only at lower temperatures and at lower isoprene concentrations. For this reason, a second DNA-SIP experiment was implemented using

samples taken from further seaward along the Colne estuary, at Wivenhoe. Several changes were made to the experimental protocol to assess any differences in the diversity of isoprene degrading organisms detected. Samples were incubated with 0.2% (v/v) isoprene at 25°C. Samples required the addition of nutrients after 7 days of incubation, after which consumption of isoprene was rapid, with 30µmoles consumed in 3 days. This suggests that isoprene degradation in sediments may be nutrient limited, and it is known that the concentrations of various nutrients along the Colne estuary vary inversely with salinity. DGGE profiling of 16S rRNA genes from time points 1 and 2 showed a difference in the community between the two time points. In the time point 1 samples, 16S rRNA gene sequencing of the ^{13}C heavy fraction showed a massive enrichment in *Mycobacterium* species, which represented approximately 98% of the total sequences. Previous studies showed that *Mycobacterium* species dominated pyrosequencing of isoprene enriched samples from the Colne estuary (Alvarez *et al*. 2009), and so far two isoprene degrading *Mycobacterium* species have been isolated. There was a higher diversity of 16S rRNA sequences obtained from the ^{13}C labelled fraction from time point 2 samples, with representatives of the *Actinobacteria* dominating, including *Mycobacterium, Gordonia, Rhodococcus* and *Microbacterium.* There are currently laboratory isolates representing each of these genera including several species of *Rhodococcus* and *Microbacterium.* The majority of isolated isoprene degrading bacteria thus far fall within the phylum *Actinobacteria*, and are members of genera noted for their diverse metabolic capabilities. Soluble diiron centre monooxygenase enzymes have been characterised in several species of *Gordonia, Mycobacterium* and *Rhodococcus.* Whilst these incubations were carried out in conditions not representative of those in the environment, there is sufficient evidence that members of these genera can degrade isoprene in the environment, although it is likely that these heterotrophs utilise a number of different carbon sources and may degrade isoprene only under specific conditions, determined by nutrient availability in the estuary.

Chapter 8

Discussion

8.1 Isoprene degradation occurs in marine samples from several different environments

The first aim of this study was to investigate the distribution of isoprene degradation in the marine environment. Previous studies had indicated that marine isoprene degradation is widespread geographically (Alvarez *et al.* 2009) and occurs in estuarine, lake and open ocean environments.

For this work, samples were taken from a diverse range of environments (Chapter 3). Open ocean samples were collected from the L4 sampling station at Plymouth during a summer algal bloom, a high salinity environment where isoprene production might be expected to be at its annual peak, whereas bacterial biomass in these samples was low. Coastal samples were collected from Penarth, South Wales, which constitutes a different environment for this study due to the influence of the Bristol Channel. Two sets of samples were collected from the Norfolk coast at Stiffkey saltmarsh, another high salinity environment. Water from both the saltmarsh shore and also marsh algal pools was collected. Lastly, this study continued and extended the work of Alvarez *et al* 2009, using samples collected from two points along the Colne Estuary, Essex, which was the site of previous study and from which several isoprene-degrading genera had been isolated. All of these samples showed complete degradation of isoprene in enrichment samples, at rates higher than those previously reported for the Colne Estuary. The concentration of isoprene used for these initial enrichments was higher than that used by Alvarez *et al.*2009, and the rapid degradation of isoprene in these samples contradicts their conclusion that the rate of isoprene degradation is faster at lower isoprene concentrations.

Two isoprene-degrading strains were isolated from Penarth and L4 enrichments and are now in pure culture. They have been shown in the laboratory to utilise isoprene as their sole carbon and energy source, and in the case of *Stappia*, represent the first known isoprene-degrading members of their genus. There were no environmental samples tested that did not show consumption of isoprene. This indicates that isoprene degradation is in fact widespread in the marine environment, and that even in samples with low biomass there are representative isoprene degraders that become massively enriched in the presence of isoprene.

Further work could extend this to non-UK environments, with tropical environments of particular interest due to the high levels of isoprene production in these areas. The largest

source of isoprene for the global atmosphere is the Amazon Basin (Alves *et al.* 2014) and it is generally assumed that tropical forests provide most of the annual global isoprene emissions to the atmosphere. The Amazon drainage basin covers an area of about 6,915,000 km^2 and contains both the Amazon River, the second longest river in the world which drains into the Atlantic Ocean, and its tributaries. The Amazon River accounts for approximately 20% of the total water carried to the oceans by rivers. The river also has several tidal estuaries, large plumes that create mixing with the Atlantic and large amounts of wave energy which push sediments out to the ocean. This area could be of huge potential interest for study of isoprene degradation rates and could potentially help correlate isoprene degradation rates to emissions directly.

Also, further algal co-culture experiments could be carried out to explore the hypothesis that isoprene-degrading communities form around algal blooms. Shaw *et al* (2003) found no evidence of microbial consumption of isoprene in algal co-cultures, although the exact strains used for inoculum were not identified in the paper. Alvarez *et al.* (2009) showed that the isoprene produced by algae (*Dunaliella tertiolecta* and *Phaeodactylum tricornutum*) could support the growth of a mixture of isoprene-degrading strains isolated from the Colne Estuary. However, in these experiments, no increase in bacterial cell number was observed. This suggests that natural concentrations of isoprene in seawater can maintain isoprene-degrading bacteria in the environment but not allow rapid growth, which could explain why these strains are nutritionally versatile as opposed to obligate isoprene degraders. These experiments could be extended using molecular methods now that a functional gene primer set for isoprene monooxygenase is available (see below). This could provide revised estimates as to the amount of isoprene produced by algae that might potentially be consumed by bacteria before reaching the atmosphere. This approach could be important data in modelling future climate scenarios in which temperatures and isoprene production might increase.

Another potential experiment could be to use Raman-Fluorescence *in situ* hybridization (Ramen-FISH) to study algal samples. The coupling of fluorescence *in situ* hybridization with Raman microscopy allows cultivation-independent identification of single microbial cells that have incorporated a ^{13}C label into their biomass (Huang *et al.* 2007). In this method, the incorporation of ^{13}C stable isotope into microbial cells causes significant changes in the wavelengths of observed resonance spectra when compared with ^{12}C-based spectra, known as the ‘red shift’. This method could allow visualization of isoprene-

degrading microbial communities surrounding algal cultures and analysis of uncultivated microorganisms at the single cell level. The Raman tweezer technique combines Raman spectroscopy with optical tweezers which can isolate a single cell using a laser beam (Snook *et al.*2008) which could allow for single cell genomic analysis of isoprene-degrading bacteria that cannot be isolated in the laboratory.

8.2 The *Gordonia* genome contains a gene cluster encoding a soluble diiron centre isoprene monooxygenase. In *Gordonia polyisoprenivorans*, polypeptides associated with isoprene degradation are induced during growth on isoprene.

The hypothesis that was tested in this study stated that isoprene-degrading bacteria possess an inducible isoprene monooxygenase gene cluster like that found in *Rhodococcus* AD45. This has been confirmed by experiments done in this study (Chapter 4 and 5), which used genome sequencing to identify and retrieve the sequences for the isoprene monooxygenase gene cluster in isolated strains, qRT-PCR to assess transcription of the isoprene monooxygenase alpha subunit under different growth conditions, SDS-PAGE to view the polypeptide profiles of isolates grown on different carbon substrates, and oxygen electrode assays to assess the oxidation profiles of isolates grown on different carbon sources. While oxygen electrode assays can give some information regarding the activity of isoprene monooxygenase, such as its rate of oxygen consumption in the presence of isoprene, these data could be extended by the development of an enzyme assay to assess the kinetics of isoprene monooxygenase, as well as determining its substrate affinity and specificity.

The genomes of *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2* were sequenced and contained the full isoprene gene cluster. These genes had high sequence similarity to those found in *Rhodococcus* AD45, and with an identical genetic organisation.

Gordonia polyisoprenivorans i37 and *Mycobacterium hodleri* i29a2* both grow on other carbon sources. *G.polyisoprenivorans* i37 can grow on both propane and polyisoprene, and *M.hodleri* i29a2* can grow on propane and ethane. Several isoprene-degrading strains can grow on propane as a sole carbon and energy source, including several *Rhodococcus* species. The model isoprene-degrading organism *Rhodococcus* AD45 does not grow on propane, and based on the genetics of these organisms, it would not appear that growth on propane and isoprene is linked. The *Gordonia* i37 genome also contained genes encoding polypeptides associated with degradation of these alternative carbon sources. Genes were identified that encode latex clearing proteins associated with rubber degradation in other

Gordonia species. The *G.polyisoprenivorans* i37 genome also contained gene encoding both propane monooxygenase and acetone monooxygenase with high sequence similarity to those found in *Gordonia* TY5, which indicates genetic potential for propane metabolism via 2-propanol and acetone. This also showed that oxidation of propane in these organisms is carried out by propane monooxygenase, confirming that isoprene monooxygenase does not act as a multifunctional isoprene/propane monooxygenase. The gene clusters encoding the isoprene and propane monooxygenase enzymes are located in different contigs and do not appear to be located in similar areas of the genome, although this is not possible to determine for certain while the genome remains incomplete. While several isoprene-degrading microorganisms also grow on propane, several do not, meaning there is an unlikely to be a connection between the two. Closing the genomes of *G.polyisoprenivorans* i37 and *M.hodleri* i29a2* would also determine whether or not isoprene and/or propane related genes are located in the chromosome or on an extra-chromosomal plasmid in these bacteria.

It has been shown that the genes encoding isoprene monooxygenase are induced during growth on isoprene. Expression of monooxygenase enzyme systems in heterotrophs is usually regulated, with gene expression being induced in the presence of the substrate or a downstream metabolite. It was shown using qRT-PCR that isoprene monooxygenase is expressed in *Gordonia*. *isoA* transcription increased 18-fold during growth on isoprene compared to glucose and propane, and likewise transcription of *prmA*, the alpha subunit of propane monooxygenase, was upregulated during growth on propane. Non-quantitative RT-PCR experiments in *Rhodococcus* AD45 (El Khawand and Murrell, unpublished) suggest that there is low level transcription of *isoA* under all growth conditions. This suggests a feedback system for regulation of isoprene monooxygenase, where this enzyme (and possibly the rest of the isoprene gene cluster) is constitutively expressed at low levels, and that in the presence of higher concentrations of isoprene an increase in a downstream metabolite such as epoxyisoprene causes transcription of the cluster to be upregulated.

Investigation into the regulation of isoprene monooxygenase could have important implications in biotechnology. Due to the concern for global warming and the depletion of the current petroleum reserves, there is motivation to move away from fossil fuels for the production of natural rubber and isoprenoid medicines (Yang *et al.* 2012). Isoprene can also be used as an alternative biofuel. Studies have attempted to metabolically engineer enhanced isoprene biosynthesis pathways in *Saccharomyces cerevisiae* in order to lower

production costs (Lv *et al.* 2014),as well as *Escherichia coli* and *Bacillus subtilis* (Xue and Ahring. 2011). This study revealed the first data regarding the regulation of isoprene degradation and its induction in the presence of isoprene. This implies the existence of a regulatory system or promoter regulated by isoprene which could be used to improve isoprene production in industrial processes.

To extend this work further, *isoA* and *prmA* knockout mutations could be constructed in *Gordonia polyisoprenivorans* i37 and *Mycobacterium hodleri* i29a2*. This would confirm previous conclusions that isoprene monooxygenase and propane monooxygenase are responsible for growth on isoprene and propane, respectively, and a complete loss of phenotype in these mutants would confirm that isoprene monooxygenase does not oxidise propane, and *vice versa* for propane monooxygenase. *isoA* has been previously mutated in *Rhodococcus* AD45 (El Khawand and Murrell, unpublished) and the mutant strain was unable to grow on isoprene as a sole carbon and energy source. This could be extended to other genes in the isoprene gene cluster to determine essential genes and to confirm the entire pathway. Of particular interest would be the two glutathione-S-transferase enzymes IsoI and IsoJ, and whether or not there is redundancy in which one of these catalyses the transferral of glutathione to epoxyisoprene in isoprene degradation. Also, two copies of *isoG* exist in *Gordonia polyisoprenivorans* i37, and it would be of interest to ascertain if both encode functional IsoG proteins that have a role in isoprene degradation, or if the second copy, not located within the isoprene gene cluster, is superfluous, possibly as a result of a genetic duplication.

8.3 A PCR primer set has been designed that can amplify the *isoA* gene in environmental samples – a future gene probe for isoprene degraders.

The alpha subunit of isoprene monooxygenase can act as a biomarker for isoprene degradation, allowing cultivation independent assessment of the presence and diversity of isoprene degraders in environmental samples (Chapter 6). The alpha subunit was chosen as this is the site of the conserved diiron centre of the enzyme. Current climate models tend to use simple isoprene emission equations with fixed variables that assume that current temperatures and land use will remain constant into the future. Thus, the current isoprene emissions models and subsequent climate predictions likely include large errors (Monson *et al.*2007) Future models could likely be improved by knowledge of the distribution of isoprene degraders in the environment and a better understanding of how isoprene

emission measurements are affected by microbial consumption. Of particular importance is the influence of isoprene emissions on global tropospheric ozone production.

8,4 DNA-SIP experiments indicated that *Rhodococcus* sp., *Mycobacterium* sp. and other Actinobacteria are active isoprene degraders in the Colne estuary.

This work represents the first study to identify active isoprene degraders in the environment, and confirmation that several of the genera that have been isolated and shown to degrade isoprene in the laboratory are indeed active in the environment (Chapter 7). This work continued and expanded upon the work of Alvarez *et al.* (2009) who isolated isoprene-degrading strains from various sites along the Colne estuary, Essex. Several different members of the Actinobacteria were identified as isoprene degraders in the estuary, with the exact genera differing between the two sites examined at the Hythe and Wivenhoe. This suggests a possible effect of increasing salinity upon microbial isoprene-degrading communities in the environment. *isoA* gene sequences retrieved from the Hythe incubations group closely together with cloned *isoA* sequences obtained from terrestrial enrichments, suggesting that at the innermost points of the Colne estuary land wash-off and human activities may determine the composition of isoprene degrading communities. A more diverse range of active isoprene degraders was identified further seaward at the Wivenhoe sampling site.

Further experiments could extend this work to different marine environments. Future sampling could be carried out at a number of sites in the Colne Estuary, including Brightlingsea at the mouth of the estuary, to observe the effect of increasing salinity on isoprene-degrading microbial communities. DNA-SIP experiments could be coupled with *in situ* measurements of isoprene production during algal blooms, eventually linking seasonality, temperature, light and isoprene production by algae with development of isoprene-degrading microbial communities. Pyrosequencing of 16S rRNA genes in enriched samples from Indonesia showed that *Stappia* species dominated in these environments (Alvarez *et al.* 2009). All of the genera indicated as active isoprene degraders in the Colne Estuary have been isolated and shown to degrade isoprene in the laboratory. Further SIP experiments could possibly identify currently unknown isoprene-degrading genera.

DNA-SIP experiments at Wivenhoe showed a massive enrichment of a single species of *Mycobacterium* after 3 days. Metagenomic sequencing of DNA extracted at this time point

might allow the genome of this bacterium to be constructed, bypassing the need for isolation. This strategy is possible, however, it is generally reliant upon having a reference genome sequenced from a closely related organism (Carr *et al.* 2013). Metagenomic sequencing combined with DNA-SIP allows the identification of 'rare' low abundance members of the microbial community as well as identifying enzymes and metabolic processes, some of which might be novel. This approach was demonstrated in Dumont *et al.* (2006) in identifying the methane monooxygenase operon in ^{13}C methane stable isotope probing experiments of forest soils. Ideally, further stable isotope probing experiments would use isoprene concentrations closer to those found *in situ*, without nutrient amendment which was necessary in this study due to time constraints. The main limitation with the methods used in this work was the sensitivity of the gas chromatograph in detecting isoprene in samples. Isoprene was detectable in samples using our gas chromatograph at approximately 0.1%, which is still far higher than the concentrations found in the environment. For this study, the quantification of isoprene in incubations was necessary to ensure accurate replication in the incubations, and to follow the rate of isoprene depletion. At low concentrations of isoprene, it is also more difficult to achieve sufficient incorporation of ^{13}C label into biomass, which is a limitation of DNA-SIP, which lacks the sensitivity of RNA-SIP and in particular PFLA-SIP. The Colne estuary would be a good site to follow up this work using incubations carried out at lower concentrations, since large amounts of data are now available regarding the consumption of isoprene in samples taken from the Colne Estuary.

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