Establishment of a genetic toolbox for ammonia-oxidising archaea

Timothy Matsiko Ninsiima Klein (MSc)

A thesis submitted to the School of Biological Sciences at the University of East Anglia in partial fulfilment of the requirements for the degree of Doctor of Philosophy

May 2024

Abstract

A genetic system is an essential tool to decipher the complex molecular mechanisms that dictate the physiology of an organism. The primary goal of this study was to establish a foundation for the development of a genetic system for ammonia-oxidising archaea (AOA) using '*Ca*. Nitrosocosmicus franklandus C13' as a model. To facilitate mutant selection, a novel cultivation method (Liquid-Solid method) was developed that enables the growth of ammonia-oxidising archaea on solid medium as single colonies. The growth of '*Ca*. N. franklandus C13' and *N. viennensis* EN76 on solid medium was dependent on the use of Phytagel™ (gellan gum) as the gelling agent as agar-based gels were inhibitory. The antibiotics puromycin and hygromycin B were identified as potential candidates for use as selective agents. Both antibiotics strongly inhibited nitrite production at concentrations of > 27 μg/ml. Addressing the lack of native plasmids for the AOA, three *E. coli* - '*Ca*. N. franklandus C13' shuttle vectors based on the chromosomal replication origin of '*Ca*. N. franklandus C13' were assembled. Plasmid pfrank-CRISPR-amoB, contains the necessary elements needed for CRISPR-Cas9-based genome editing. In contrast, the plasmids pfrank-mCherry-Cdc-orb and pfrank-mCherry-orb are *mCherry* reporter gene-based plasmids. In addition to containing a reporter gene, the latter plasmids differ in the presence or absence of a *cis* or *trans* Orc1/Cdc6 encoding *cdc* gene respectively. All three plasmids contain the puromycin resistance gene (*pac*). The transformation attempts of '*Ca*. N. franklandus C13' using either $CaCl_2$ heat-shock, electroporation and polyethylene glycol were unsuccessful. This work serves as a much needed reference for future efforts aiming to establish a genetic system for the AOA.

Key words: *Ammonia-oxidising archaea (AOA), genetic system, Phytagel™, solid medium, colonies, antibiotics, electroporation, polyethylene glycol, $CaCl_2$ heat-shock.*

Table of Contents

List of Figures

List of Tables

Abbreviations

2-DOS	2-deoxystreptamine
AMO	Ammonia monooxygenase
Anammox	Anaerobic ammonia oxidation
AOA	Ammonia-oxidising archaea
AOB	Ammonia-oxidising bacteria
ATP	Adenosine triphosphate
bp	Base pair
Cas	CRISPR-associated
CI	Chloroform Isoamyl alcohol
cm	Centimetre
Comammox	Complete ammonia oxidation
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
crRNA	CRISPR ribonucleic acid
CTAB	Cetyltrimethylammonium bromide
DAPI	4′,6-diamidino-2-phenylindole
DBS	Double stranded break
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FISH	Fluorescence in situ hybridization
FS	Field strength
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
GDGT	Glycerol dibiphytanyl glycerol tetraethers
HAO	Hydroxylamine oxidoreductase

HCl	Hydrochloric acid
HDR	Homology-dependent repair
Hsp90	Heat-shock protein 90
Kb	Kilo base
Km	Michaelis Menten constant
Kv	Kilo volts
LB	Lysogeny Broth
LS method	Liquid-Solid method
MEP	Methyl-ammonia permeases
MGE	Mobile Genetic Elements
MIC	Minimal inhibitory concentration
ms	Milliseconds
ng	nanograms
NHEJ	Non-homology end joining
NLS	Nuclear localising signal
nM	Nanomolar
OD	Optical Density
ORB	Origin recognition box
Pac	Puromycin N-acetyl transferase
PAM	Protospacer adjacent motif
PCI	Phenol Chloroform Isoamyl alcohol
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PES	Polyethersulfone
POE-PCR	Prolonged overlap enhanced polymerase chain reaction

R-M	Restriction modification system
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
RVD	Repeat-variable di-residue
S-layer	Surface layer protein
SEM	Standard error of the mean
sgRNA	Single guide RNA
SOC	Super optimal medium with catabolic repressor medium
ssRNA	Single stranded ribonucleic acid
TALEN	Transcription activator-like effector nucleases
TBE	Tris Borate EDTA
TE buffer	Tris-EDTA buffer
TEM	Transmission Electron Microscopy
tracrRNA	Trans-activating ribonucleic acid
TS3	Transcription factor III translocation signal
U	Units (enzyme)
UTR	Untranslated region
V	Volts
ZFN	Zinc-finger nuclease
µF	Microfaraday
µM	Micromolar

Declaration

I certify that the work contained in the thesis submitted by me for the degree of Doctor of Philosophy is my original work including the data presented in Chapter 3 which has been published in the journal *FEMS Microbiology* titled 'Cultivation of ammonia-oxidising archaea on solid medium' and is available at https://doi.org/10.1093/femsle/fnac029 and has not been previously submitted by me for a degree at this or any other university.

Acknowledgements

Firstly, I would like to thank my supervisors Prof. Laura Lehtovirta-Morley and Prof. Matt Hutchings for the opportunity to take part in this project as well as their generous support over the last four years. It was a pleasure.

A special thank you to Dr. Andrew Crombie who has been extremely patient and always took the time to discuss ideas in the lab and offering help whenever it was needed.

I would also like to thank the ARIES DTP (NERC) for funding this project.

I would also like to thank Prof. Crista Schleper and Max Dreer from the University of Vienna who were excellent collaborators on this project.

I would also like to acknowledge the excellent training and support offered by Dr. Lianna Phogosyan. Thank you!

Big thank you to Dr. Ainsley Beaton (John Innes Centre) for all the training and support.

Thanks to the ELSA lab who have supported me through this very challenging PhD experience.

I would also like to thank Dr. Vincent Mugisha without who's unconditional support and encouragement this opportunity may not have been possible.

Lastly, I would like to acknowledge the support offered by my family throughout my academic experience especially my mum without whom this opportunity would not have been possible.

Chapter 1: Literature review

1.1 General overview

Nitrification is the biological oxidation of ammonia (NH_3) to nitrate (NO_3^-) via nitrite (NO_2^-) (Li *et al.*, 2018; Norton and Ouyang, 2019). It is a key process in the biogeochemical cycling of nitrogen in the environment and is primarily mediated by microbes known as nitrifiers. In addition to its role in nitrogen cycling, nitrification is used in waste-water treatment plants to minimise the ammonia levels in the effluent. However, nitrification may also have negative environmental impacts. For example, a large proportion (50 - 70%) of nitrogen-based fertilizers are lost through nitrification which negatively impacts crop yields (Beeckman, *et al*., 2018; Wang *et al.*, 2021). Nitrification is also responsible for conversion of nitrogen-based fertilizers into nitrate which is highly mobile in soil and is readily leached into aquatic habitats leading to eutrophication (Lehtovirta-Morley, 2018). Furthermore, nitrification has been found to contribute towards the release of greenhouse gasses in the form of nitrous oxide (N_2O) thus impacting the climate (Wrage *et al.*, 2001). Consequently, there is an urgent need to develop effective management practices capable of mitigating the negative impacts of nitrification. This requires an in-depth understanding of the microbial communities that are responsible for this process.

The first and rate-limiting step of nitrification, ammonia oxidation, is performed by three phylogenetically and physiologically distinct groups of microorganisms which include ammonia-oxidising bacteria (AOB), ammonia-oxidising archaea (AOA) and the complete ammonia-oxidising bacteria also known as ‘comammox’ (You *et al*., 2009; Daims *et al*., 2015; Li *et al*., 2018). A fourth group of ammonia-oxidising nitrifiers, the anaerobic ammonia oxidisers (anammox), are responsible for ammonia-oxidation in anaerobic environments (Kartal *et al.*, 2007).

While AOB have been extensively studied for over a century, the first AOA strain was only isolated in 2005 (Könneke *et al.*, 2005). Consequently, unlike their bacterial counterparts, AOA have not been studied as extensively in the context of their physiology, biochemistry, and ecological role. Interestingly, molecular studies indicate these archaea are highly abundant and ubiquitous in the environment often outnumbering AOB, which suggests that AOA play an important role in the global nitrogen cycle. Although significant strides forward have been made in expanding our understanding of AOA physiology, biochemistry and ecological role, key research gaps still exist. For example, the mechanisms used by these archaea to outcompete AOB and adapt to diverse ecological niches are unknown. Additionally, it is evident that AOA and AOB differ in their ammonia oxidation pathways based on missing genes based on genome analysis (Lehtovirta-Morley, 2018).

Unfortunately, addressing these knowledge gaps is compounded by the high incidence of hypothetical genes/proteins in archaeal genomes (Makarova *et al*., 2019). Thus, the establishment of a genetic toolbox or system for an AOA model(s) would be highly valuable in overcoming this research bottleneck. This is because a genetic system is essential to deciphering molecular mechanisms that underpin the physiological and biochemical characteristics of an organism through the functional characterisation of key genes/proteins involved. As no such tools have been reported for any AOA, this study aims to address this research gap by laying the foundation for the development of a genetic system for the model strain '*Ca*. N. franklandus C13'.

1.2 Nitrogen cycle: A brief overview

Nitrogen constitutes ~78% of the atmosphere making it the most abundant atmospheric element (Stein and Klotz, 2016). It is essential to life and is a vital component of biomolecules such as amino acids, nucleic acids, and adenosine triphosphate (ATP). Despite its abundance, nitrogen is inert, and most organisms are incapable of breaking the strong triple bond between the two nitrogen atoms (Fields, 2004; MacLeod and Holland, 2013). The movement of nitrogen through the environment is facilitated by both abiotic and biotic factors of which the latter play a major role (Arp and Stein, 2003).

The nitrogen cycle (N-cycle) is comprised of several reactions that transform elemental nitrogen into different nitrogen oxide species through a series of redox reactions facilitating its transfer through the environment (Rodionov *et al.*, 2005). Generally, the N-cycle (*Figure 1.1*) is divided up into three main processes: (i) nitrogen fixation/ammonification (ii) nitrification and (iii) denitrification (Herbert, 1999). Additional processes may include (i) dissimilatory nitrate reduction to ammonia (DNRA) whereby nitrate and other oxidised forms of nitrogen serve as the electron acceptor, and (ii) anaerobic oxidation of ammonia (Van De Graaf *et al.*, 1996; Chutivisut *et al.*, 2018).

Nitrogen fixation involves the conversion of dinitrogen (N_2) to ammonia, an important biologically available form of nitrogen (Vicente and Dean, 2017). Nitrogen fixation is catalysed by both abiotic (e.g. lightning) and biotic (e.g. microbial activity) factors. The decomposition of organic matter through the process of ammonification and anthropogenic sources further contributes towards the ammonia pool in the environment. The microbes that mediate biological nitrogen fixation via the catalytic

action of the nitrogenase enzyme are collectively known as diazotrophs (Zhao *et al.*, 2006). Bacterial diazotrophs belong to phyla such as Actinobacteria, Cyanobacteria, Chlorobi, Chloroflexi, Proteobacteria and the Firmicutes (Solanki *et al*., 2020) whereas archaeal diazotrophs include *Methanosarcina barkeri* and *Methanococcus maripaludis* (Leigh, 2000).

In the second step, the ammonia generated from nitrogen fixation feeds into nitrification. Nitrification consists of two sequential steps: (i) oxidation of ammonia to nitrite and (ii) oxidation of nitrite to nitrate (Prosser, 1990). Four distinct microbial groups are responsible for this process including AOB, AOA, nitrite oxidising bacteria (NOB) and the comammox bacteria.

The N-cycle is concluded by denitrification, an anaerobic respiratory process that produces dinitrogen gas through the sequential reduction of nitrate → nitrite → nitric oxide (NO) → nitrous oxide and finally the production of dinitrogen (Stein and Klotz, 2016). Additionally, production of dinitrogen may also occur through: (i) nitrifier denitrification whereby ammonia oxidisers reduce nitrite (Kozlowski *et al.*, 2016) and (ii) anaerobic ammonia oxidation (Hu *et al.*, 2011).

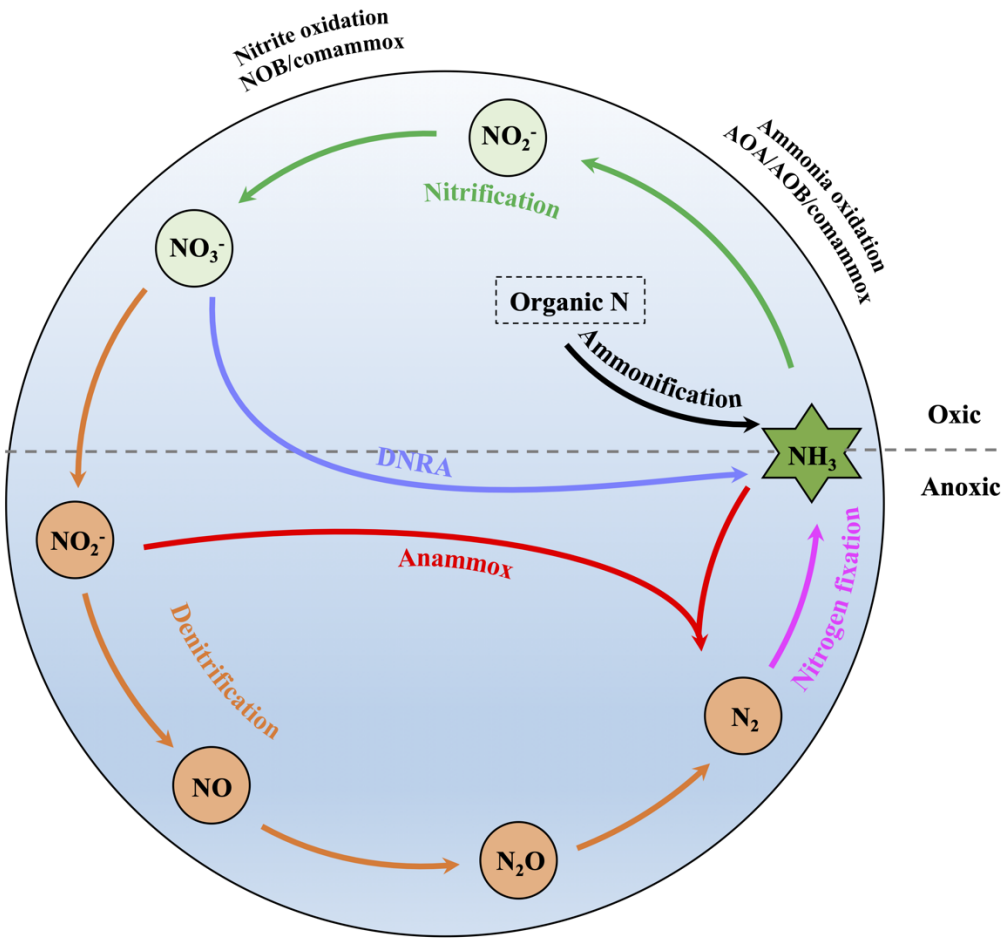


Figure 1.1: Reactions of the nitrogen cycle**.** Dinitrogen (N_2) is converted to ammonia (NH_3) via nitrogen fixation (pink). Anthropogenic ammonia and ammonification further contribute towards the ammonia pool (black). Under anoxic conditions, ammonia may also be produced via dissimilatory nitrate reduction to ammonium or DNRA (purple). The second step, nitrification, is the sequential oxidation of ammonia to nitrite (NO_2^-) and nitrate (NO_3^-) in the presence of oxygen (green). The oxidation of ammonia to nitrite is catalysed by ammonia-oxidising bacteria (AOB) or archaea (AOA). The complete ammonia oxidisers (comammox) are capable of oxidising ammonia through to nitrate. The nitrite oxidisers (NOB) oxidise nitrite to nitrate. Denitrification, the final step involves the sequential reduction of nitrate → nitrite → nitric oxide (NO) → nitrous oxide (N_2O) back into N_2 (orange). The oxidation of ammonia under anoxic conditions (anammox) together with nitrite further contributes to the production of N_2 (red). Adapted from (Rose *et al.*, 2021).

1.3 Ammonia-oxidising microorganisms

Ammonia-oxidising microorganisms are essential to nitrification as they catalyse the first and rate-limiting step, ammonia oxidation. As discussed previously, ammonia-oxidising microorganisms include AOA, AOB and the comammox. Ammonia oxidation may also occur under anoxic conditions by anammox bacteria. The following sections aim to introduce ammonia oxidisers with a primary focus on the AOA, providing a general overview of these organisms and highlighting some key aspects in which they differ from the AOB with regards to their ecophysiology, biochemistry and niche adaptation.

1.3.1 Ammonia-oxidising bacteria (AOB)

AOB are ubiquitous in the environment and inhabit diverse ecological niches including marine and terrestrial habitats (Horz *et al.*, 2004). Phylogenetically, all known AOB fall within the phylum Proteobacteria and cluster into the β and γ sub-divisions (Head *et al.*, 1993; Purkhold *et al.*, 2000; Christman *et al.*, 2011). AOB in the β-proteobacteria cluster include genera such as *Nitrosomonas* and *Nitrosospira* and are widely distributed in soil ecosystems (Campbell *et al.*, 2011). In contrast, AOB in the γ-proteobacteria cluster include *Nitrosococcus oceani*, *Nitrosococcus halophilus*, '*Candidatus* Nitrosoglobus terrae' and the novel "*Candidatus* Nitrosacidococcus tergens" sp. RJ19 (Watson, 1965; Teske *et al.*, 1994; Ward and O'Mullan, 2002; Hayatsu *et al.*, 2017; Picone *et al.*, 2021). AOB aerobically oxidise ammonia as their sole energy source and fix inorganic carbon (CO_2) using the Calvin cycle (Chain *et al.*, 2003; Hommes, *et al*., 2003). AOB are also capable of utilising organic compounds such as urea as an alternative source of ammonia (Burton and Prosser, 2001).

Research into AOB for over a century has provided significant insight into nitrification. However, the relatively recent discovery of AOA and comammox bacteria has resulted in a paradigm shift in our understanding of nitrification and the ecophysiology of the microorganisms involved.

1.3.2 Ammonia-oxidising archaea (AOA)

The existence of archaeal nitrifiers was first hypothesised following the detection of ammonia monooxygenase genes (AMO) in archaeal-associated sequences from Sargasso Sea samples (Venter *et al.*, 2004). The AMO is the enzyme responsible for the oxidation of ammonia. Treusch and colleagues (2005) further identified archaeal Group 1.1b-associated ammonia monooxygenase genes in a fosmid library constructed from grassland soil. However, the isolation of the first archaeal nitrifier, *Nitrosopumilus maritimus* SCM1 from a tropical aquarium, provided the first concrete proof of the existence of AOA (Könneke *et al.*, 2005). Since then, several archaeal ammonia-oxidisers have been isolated from a range of different ecological niches. The widespread distribution of AOA in the environment and their role in nitrification provides strong support for their importance to the global N-cycle.

Phylogeny, based on 16S rRNA sequence analysis, places AOA in the archaeal phylum Thermoproteota (formerly known as Thaumarchaeota) (Brochier-Armanet *et al.*, 2008; Rinke *et al.*, 2021). AOA can be further classed into five phylogenetic groups including *Nitrosopumilus*, *Nitrosotalea*, *Nitrosocaldus*, *Nitrosophaera* and *Nitrosocosmicus* (Nitrosophaera-sister cluster) (Pester *et al*., 2012; Lehtovirta-Morley *et al*., 2016). The seminal discovery of archaeal nitrifiers piqued the interest of numerous research groups who set out to understand their contribution to the N-cycle and their relationship with bacterial nitrifiers.

1.3.2.1 General metabolism

AOA are chemolithoautotrophs utilising ammonia and inorganic carbon as their sole energy and carbon sources respectively (Kozlowski *et al*., 2016; Könneke *et al.*, 2014). Interestingly, genomic and experimental studies show slight differences in the metabolic properties of AOA and AOB. For example, AOA fix inorganic carbon using a highly energy efficient derivative of the hydroxypropionate/hydroxybutyrate pathway (Könneke *et al.*, 2014). This pathway is believed to provide AOA with a strong physiological advantage particularly in oligotrophic environments.

As seen with AOB, certain AOA are capable of utilising urea and cyanate as alternative sources of ammonia (Palatinszky *et al*., 2015; Lehtovirta-Morley, *et al*., 2016). Urea metabolism is thought to provide a competitive advantage for AOA living at low pH. This is because acidic conditions result in substrate limiting conditions due to a shift in the $NH_3 \leftrightarrow NH_4^+$ equilibrium to the ionized NH_4^+ species which is not a substrate for the AMO (Li *et al*., 2018; Lehtovirta-Morley *et al.*, 2016). In contrast, urea hydrolysis is pH-independent and could therefore serve as an ammonia source under acidic conditions. Currently, support for this hypothesis is limited due to the absence of urease/cyanase genes in the genomes of obligate acidophilic AOA strains (Lehtovirta-Morley, *et al.*, 2016). It can, however, not be ruled out that perhaps urea/cyanate support survival at low pH in other strains but requires further investigation.

Urea and cyanate may also serve as sources of ammonia under ammonia-limiting conditions created by pH-independent factors. This has been proposed by Tolar and colleagues (2017) who performed extensive metagenomic and biochemical studies on archaea inhabiting polar regions to determine the possibility of urea as an alternative

energy source. Their results revealed urea concentrations exceeding those of ammonia within this ecosphere and an abundance of urease-associated genes. These observations led the authors to propose that urea metabolism may indeed provide a competitive advantage for AOA under ammonia-limiting conditions. Furthermore, sequences originating from *N. maritimus* dominated the polar oceans where high urea concentrations were measured, thus providing further support for urea as an alternative energy source in ammonia-limiting environments. Interestingly, the AOA strain '*Ca*. Cenarchaeum symbiosum', which forms a symbiotic relationship with a marine sponge, is thought to utilize the urea excreted by its host as an energy source (Kirchman, 2012).

1.3.2.2 The biochemistry of ammonia oxidation in archaea

The oxidation of ammonia, catalysed by the AMO enzyme, results in the production of hydroxylamine (NH_2OH). AMO is a copper-containing membrane-bound protein and is present in all organisms capable of ammonia oxidation (Tavormina *et al*., 2011; Lehtovirta-Morley, 2018). The AMO is made up of three main protein subunits encoded by the genes *amoA*, *amoB* and *amoC* (Junier *et al.*, 2009).

In AOB, these genes exhibit a conserved operon-like organisation in a specific order, *amoA*, *amoB*, *amoC* (Arp *et al*., 2007). This contrasts with the AMO gene organisation in AOA, where the subunit genes are often arranged in the order *amoA*, *amoX*, *amoC* and *amoB* (Lehtovirta-Morley, 2018). The *amoX* gene encodes a hypothetical protein of unknown function and is associated with the AMO operon of all sequenced AOA genomes (Bartossek *et al.*, 2012). It has been suggested that *amoX* may encode an additional AMO subunit (Kerou *et al*., 2016). Recently, two additional AMO subunits

unique to the AOA, AmoY and AmoZ were discovered although their function is currently unknown (Hodgskiss *et al.*, 2023).

Research into ammonia oxidation has revealed distinct differences between the mechanism of the bacteria and archaeal pathways. In AOB, it was initially thought that the oxidation of hydroxylamine by hydroxylamine oxidoreductase (HAO) directly resulted in the formation of nitrite (Arp *et al*., 2002). However, recent studies demonstrate that nitric oxide and not nitrite is the product of HAO activity (Caranto and Lancaster, 2017). The authors further proposed nitrite reductase (NirK) as the third enzyme responsible for the conversion of nitric oxide to nitrite. This newly proposed pathway retains the original electron count with three electrons produced from hydroxylamine oxidation and one from nitric oxide oxidation. Of the four electrons, two are recycled back to the AMO to facilitate ammonia oxidation and two electrons are utilized for cellular respiration. An illustration of the bacterial ammonia oxidation pathway is shown in *Figure 1.2*.

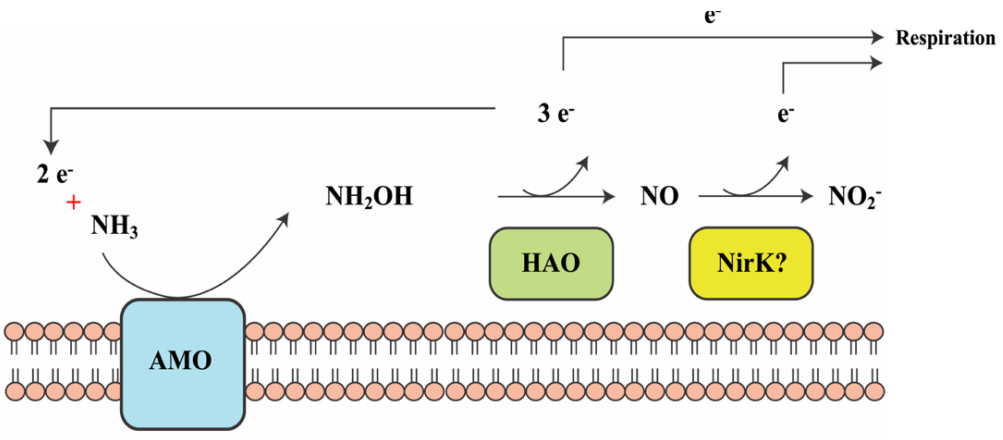
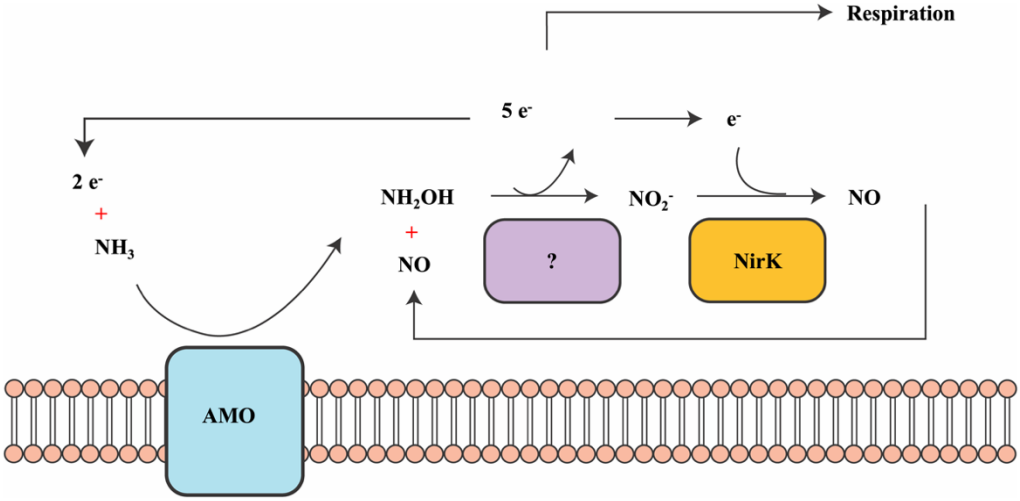


Figure 1.2: The revised bacterial ammonia oxidation pathway**.** In line with previous models of bacterial ammonia oxidation, the ammonia monooxygenase (AMO) oxidises ammonia (NH_3) to hydroxylamine (NH_2OH). However, nitric oxide (NO) rather than nitrite (NO_2^-) is the product of hydroxylamine oxidoreductase (HAO). The resulting nitric oxide is hypothesised to be further oxidised into nitrite by a nitrite reductase (NirK). Adapted from (Lehtovirta-Morley, 2018).

In contrast, the archaeal ammonia oxidation pathway is unlikely to follow a similar sequence of reactions and most likely utilizes a unique enzyme repertoire. This is supported by the consistent lack of HAO homologues encoded in the currently available AOA genomes despite hydroxylamine being an intermediate of ammonia oxidation (Vajrala *et al.*, 2013). Consequently, several distinct archaeal ammonia oxidation pathways have been proposed based on genome and biochemical data. For example, the two-step pathway proposes that hydroxylamine is converted to nitrite with the help of a multi-copper oxidase (MCO1) and nitric oxide as a co-reactant (Kozlowski *et al.*, 2016). The nitric oxide required for this reaction is produced from the reduction of nitrite by NirK. An illustration of the proposed archaeal two-step ammonia oxidation pathway is presented in *Figure 1.3A*. However, some AOA such as those from the *Nitrosotalea* lineage lack MCO1 homologues suggesting that these organisms may exploit an alternative mechanism (Kerou *et al.*, 2016; Herbold *et al.*, 2017). This led to the proposal of the three-step archaeal ammonia oxidation model by

Lehtovirta-Morley (2018). This model shares similarities with the bacterial pathway but lacks a NirK. This is based on the observation that certain AOA lack *nirK* homologues (Daebeler *et al*., 2018; Abby *et al*., 2018). The proposed three-step archaeal ammonia oxidation pathway is shown in *Figure 1.3B*.

(A) Two-step pathway (archaeal)



(B) Three-step pathway (archaeal)

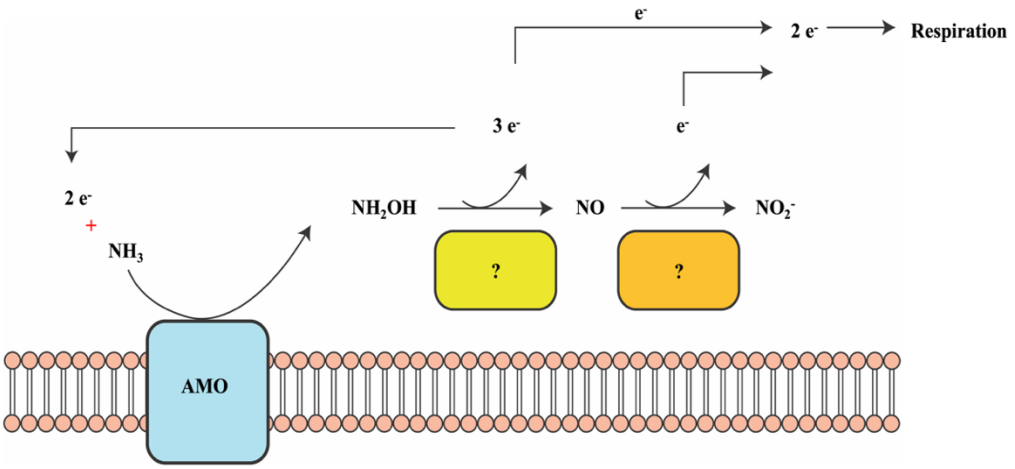


Figure 1.3: The proposed ammonia oxidation pathways in archaea. **(A)** In the two-step pathway, hydroxylamine (NH_2OH) and nitric oxide (NO) serve as co-reactants for an unknown enzyme capable of NH_2OH oxidation (possibly a multi-copper oxidase) (Kerou *et al.*, 2016). Nitrite (NO_2^-) could subsequently be reduced to nitric oxide by a Nirk, but this is yet to be validated. **(B)** The three-step model was proposed based on the absence of *nir*K homologues in some AOA whereby, hydroxylamine is directly converted into nitric oxide and nitrite by unknown enzymes. Adapted from (Lehtovirta-Morley, 2018).

1.3.2.3 Distribution of AOA in the environment

The plethora of ecological studies carried out to date have revealed a global distribution of AOA in a range of environments including oceans (Wuchter *et al.*, 2006), lakes (Bollmann, *et al*., 2014), soils (Tourna *et al*., 2011; Lehtovirta-Morley, *et al*., 2016), and even extreme ecosystems such as hot springs (de La Torre *et al.*, 2008; Hatzenpichler *et al.*, 2008). This global distribution of AOA coupled with their abundance is a strong indicator of their important role in the global N-cycle.

While the dominance of AOA is particularly evident in marine or aquatic ecosystems (Francis *et al*., 2005; Wuchter *et al*., 2006; Wang *et al*., 2017), their abundance relative to AOB in soils is not so clearcut. For example, soils receiving nitrogen-based fertilisers (e.g. agricultural soils) are usually associated with a higher abundance of AOB relative to AOA (Shen *et al.*, 2008; Jia and Conrad, 2009). AOB also tend to dominate engineered systems such as wastewater treatment plants (WWTP) that receive high amounts of ammonia (Park *et al.*, 2006). In contrast, unfertilised soils whose primary source of ammonia originates from the mineralisation of organic matter, tend to be dominated by AOA (Di *et al.*, 2009, 2010). These distribution patterns led to the suggestion that AOA are adapted to oligotrophic or ammonia limited conditions (Offre *et al*., 2009; Verhamme *et al*., 2011). Interestingly, this generalisation does not apply to AOA strains such as *N. viennensis* EN76 and '*Ca*. N. franklandus C13' that appear to be adapted to higher ammonia concentrations (Lehtovirta-Morley *et al*., 2016).

In addition to ammonia concentrations, distribution patterns of AOA are influenced by pH. A large-scale *in silico* study on AOA global distribution and abundance demonstrated the tendency for the *Nitrosotalea* group to cluster and dominate in acidic

soils (Gubry-Rangin *et al.*, 2011). Two acidophilic AOA strains from the *Nitrosotalea* group were later isolated from acidic soils (Lehtovirta-Morley *et al*., 2011, 2014). Evidently, AOA are not only widely distributed in diverse ecosystems, but they are also present in high numbers. This abundance and widespread distribution of these archaea has raised questions into how they outcompete AOB and the molecular mechanisms by which they adapt to these different ecological niches.

1.3.3 Niche specialization of AOA

The physiological adaptation to a specific set of biotic or abiotic conditions in the environment is known as 'niche specialization' which consequently results in the partitioning of resources termed 'niche differentiation' (Erguder *et al.*, 2009; Prosser and Nicol, 2012). Based on the distribution patterns of AOA (and AOB), two main drivers of niche differentiation have been proposed; (i) substrate availability and (ii) pH (Lehtovirta-Morley, 2018; Prosser *et al.*, 2020).

1.3.3.1 Substrate

Both AOA and AOB are dependent on ammonia as their sole energy source and this substrate is therefore expected to play an important role in shaping the community structure of these microorganisms. Substrate-dependent adaptations are proposed to be linked to ammonia affinity, tolerance to varying substrate concentrations and an adaptation to different sources of ammonia (Prosser and Nicol, 2012). AOA dominance at ammonia limiting conditions is attributed to these archaea having a higher affinity for ammonia than AOB. This hypothesis was supported by the low ammonia half saturation constant (K_m) of ~133 nM of the marine dwelling AOA strain *N. maritimus* SCM1 (Martens-Habbena *et al.*, 2009). Similarly, the moderately thermophilic AOA strain *N. gargensis* isolated from a hot spring, demonstrates high growth rates at

ammonium concentrations as low as 140 μM (Hatzenpichler *et al.*, 2008). In contrast, the AOB model strains *N. europaea* and *N. oceani* exhibit significantly larger Km values for ammonia in the range ~553 μM and 101 μM respectively (Martens-Habbena *et al.*, 2009). Most recently, ammonia affinities were determined for a larger pool of AOA strains representing all major lineages (Jung *et al.*, 2022). Interestingly, while the authors found that AOA generally exhibited much higher ammonia affinities than AOB, the *Nitrosocosmicus* lineage exhibited affinities similar to those of AOBs. Although the exact mechanisms by which *Nitrosocosmicus* strains adapt to higher ammonia concentrations are still unclear, evidence for the role of polyamines has recently been demonstrated in '*Ca*. Nitrosocosmicus agrestis' (Liu *et al.*, 2021).

Related to substrate affinity, an additional mechanism proposed to play a role in niche differentiation is the efficiency of substrate uptake facilitated by either passive or facilitated ammonia uptake. Facilitated (or active) ammonia uptake is expected to be important under low ammonia availability (Winkler, 2006). Ammonia transport in AOA is predicted to be facilitated by the presence of the ammonia transporters Amt-1 and Amt-2 that cluster within the methyl-ammonia permeases (MEP) protein family (Offre *et al.*, 2014). Interestingly, Amt-type transporters are absent in AOB and instead, AOB genomes encode the functionally distinct rhesus-type transporters that are distantly related to the MEP protein family (Offre *et al.*, 2014). Some AOB such as *Nitrosomonas eutropha* C91 lack genes encoding ammonia transporters in their genome (Stein *et al.*, 2007). A transcriptional analysis of the *amt* genes in *N. maritimus* SCM1 found that the Amt-2 transporter was important under ammonia limiting conditions (Nakagawa and Stahl, 2013). However, in the absence of genetic tools that could be used for *amt* knockout studies, it is challenging to determine what role these ammonia transporters play in AOA biology.

1.3.3.2 pH

pH as a driver of niche differentiation among ammonia-oxidising microorganisms is primarily related to substrate availability. For example, at acidic pH, the $NH_3 \leftrightarrow NH_4^+$ equilibrium shifts away from ammonia with a subsequent increase in the concentration of the ionized form (NH_4^+). This results in substrate limitation since ammonia as opposed to ammonium is the preferred substrate for the archaeal AMO, based on work done on *N. europaea*. (Suzuki *et al*., 1974; Hatzenpichler, 2012). A decrease in pH also affects the availability of bicarbonate (HCO_3^-) which archaeal ammonia oxidisers fix as their sole a carbon source. Additionally, at acidic pH, the $NO_2^- \leftrightarrow HNO_2$ equilibrium increases the availability of cytotoxic nitrous acid (Herbold *et al*., 2017).

As discussed previously, some AOA are capable of utilising alternative organic compounds such as urea and cyanate as a source of ammonia. These organic compounds have also been proposed to play a role in the adaptation to acidic conditions because they can be taken up into the cell where they are hydrolysed to ammonia which is not affected by the acidic environment outside of the cell (Burton and Prosser, 2001: Allison and Prosser, 1993). However, the absence of urease/cyanase genes in the genomes of the obligate acidophilic AOA strains *Nitrosotalea devanaterra* Nd1 and *Nitrosotalea sinensis* Nd2 provides poor support for this hypothesis (Lehtovirta-Morley *et al.*, 2011, 2014). The mechanisms by which AOA dominate and thrive in acidic environments are largely unknown and are currently being investigated.

An in-depth analysis of *Nitrosotalea* genomes identified a range of possible alternative mechanisms for the adaptation to acidic pH (Lehtovirta-Morley *et al.*, 2016; Herbold *et al.*, 2017). These mechanisms were based on four main characteristics including ion

transport, enhanced proton consumption, cell wall/membrane properties and carbon metabolism at low pH. The genome of *N. devanaterra* Nd1 was found to contain genes that encode for various cation transporters such as *kdp* (P-type ATPase involved in potassium transport), *mgt*A (P-type ATPase involved in magnesium transport) and CPA1-family of Na^+/H^+ exchangers that play a role in pH homeostasis. The acetolactate decarboxylase encoded in the genome of *N. devanaterra* Nd1 is proposed to enhance cytoplasmic consumption of protons through the production of acetoin from acetolactate. With regards to cell wall properties, the use of glycerol dibiphytanyl glycerol tetraether lipids (GDGT) with a larger number of cyclopentane groups (GDGT-4) may contribute to lower membrane permeability minimising entry of protons. Lastly, carbon metabolism at low pH using bicarbonate may be possible through use of a dual functional carbonic anhydrase. This enzyme could counter acidification of the cytoplasm by breaking down bicarbonate depending on the direction of the reaction (Lehtovirta-Morley *et al*., 2016). A summary of the proposed mechanisms used by acidophilic AOA for survival at low pH are illustrated in *Figure 1.4*.

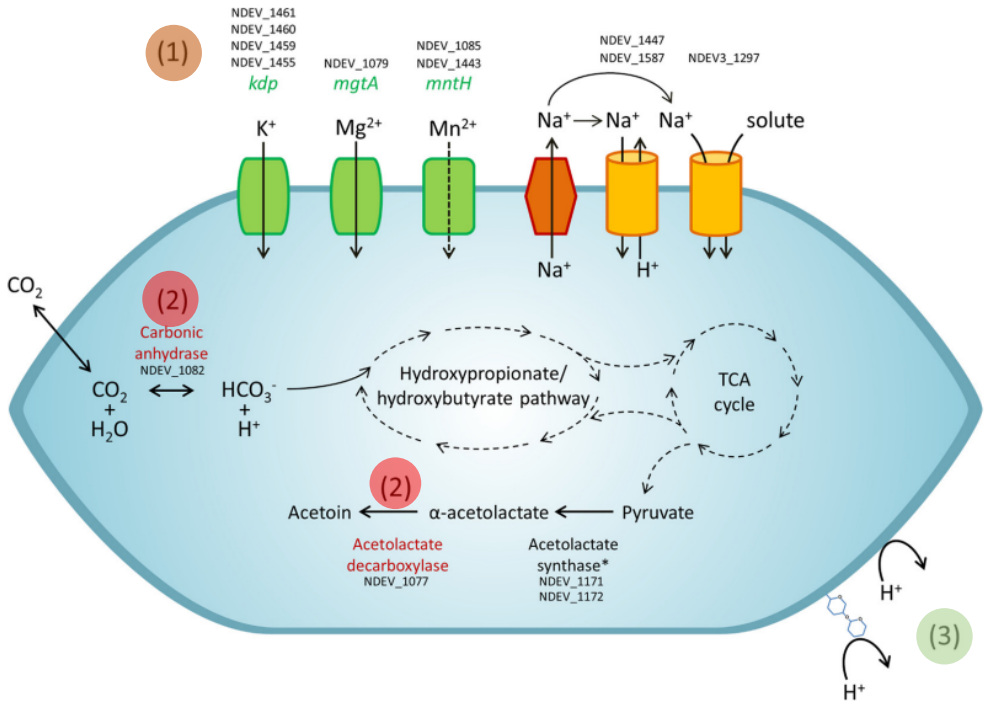


Figure 1.4: Proposed mechanisms of low pH adaptation by the acidophilic AOA strain, *N. devanaterra* Nd1. **(1)** *N. devanaterra* Nd1 contains several cationic transporters which have the capacity to play a role in pH homeostasis. **(2)** *N. devanaterra* Nd1 may rely on acetolactate decarboxylase to consume the excess protons and present at low pH. Alternatively, carbonic anhydrase may facilitate buffering of the cytoplasm. **(3)** Adaptation to low pH may also result from reduced cell permeability due to the presence of GDGT-4 lipids and/or glycosylation which prevent an influx of protons into the cell. (Lehtovirta-Morley *et al*., 2016).

In summary, AOA are major players in the nitrogen cycle where they contribute to the nitrification process. Nitrification is associated with greenhouse gas emissions (N_2O) as well as reduced ammonia fertiliser use efficiency which impacts crop yields. Therefore, a better understanding of these archaea could inform the development of better land management and agricultural practices. However, this requires a better understanding of the biology of these archaea. In the preceding sections, key knowledge gaps regarding AOA ecophysiology that are currently under investigation have been highlighted. These include **(i)** the identity of the unknown enzymes driving archaeal ammonia oxidation, **(ii)** the role of ammonia transporters in niche adaptation

strategies, and **(iii)** the unknown mechanisms by which acid tolerant AOA (e.g. the *Nitrosotalea* group) thrive in low pH environments.

These questions could benefit from genetic studies such as gene knockout experiments that have the potential to shed light on these cellular processes. Unfortunately, the lack of a functional genetic system for these archaea is currently a major bottleneck in AOA research.

1.4 Genome-editing technologies

Mutations play a key role in driving the evolution of organisms across all domains of life. Geneticists have long been interested in studying the various aspects of genetic material including inheritance and function. However, the low rate at which mutations occurred in nature hindered any significant advances in the field (Muller, 1927). This led to the search for methods capable of inducing mutations and various physical and chemical agents have proven to be useful in this regard. For example, X-rays have been used to induce genetic mutations in Drosophila flies (Muller, 1927; Würgler and Maier, 1972) while alkylating agents such as ethyl methane sulfonate have been used to study DNA repair kinetics in plants (Gichner *et al.*, 1999, 2000). Despite the success of such methods in inducing genetic mutations, the resulting mutations were random and unpredictable limiting their use.

A second group of mutagens, termed transposons or 'jumping genes', were later discovered by Barbara McClintock during her research on maize plants (Mcclintock, 1950). Transposons are mobile genetic elements (MGE) that are abundant in all domains of life (Ravindran, 2012). The mutagenic potential of transposons arises from the ability of these genetic elements to insert themselves randomly in the genome including within intragenic loci. Unfortunately, just like the physical and chemical

mutagens mentioned previously, transposon-mediated mutagenesis is random and unpredictable requiring the screening of a large number of mutants.

This sparked interest in developing methods that allow the introduction of precise user-defined mutations also known as 'targeted genome-editing'. Notable discoveries that have contributed towards the development of targeted genome-editing tools in use today include deciphering of the structure of DNA, the development of the polymerase chain reaction (PCR), discovery of restriction enzymes as well as DNA sequencing technologies (Doudna and Charpentier, 2014). Targeted gene disruption, however, began in earnest with work on homologous recombination in yeast and mammalian cells (Hinnen *et al*., 1978; Rothstein, 1983; Smithies *et al*., 1985). Although homologous recombination-mediated genome editing is still widely used today, it is limited by its poor efficiency (Capecchi, 1989; Silva *et al.*, 2011). A turning point in targeted genome-editing came about when it became evident that the efficiency of homologous recombination could be increased by introducing a double-stranded break (DSB) at a user defined genomic locus (Jasin and Berg, 1988; Hasty *et al*., 1992; Adli, 2018).

Once a DSB has been introduced, it may be repaired by two main pathways including homology-dependent repair (HDR) or non-homologous end joining repair (NHEJ) (Rouet *et al*., 1994; Adli, 2018). Repair of the DSB using the HDR pathways requires the presence of a template containing homology to the region containing the break (Xue and Greene, 2021). In comparison, NHEJ does not require homology and instead involves the direct ligation of the DNA ends and is considered to be error-prone (Rodgers and McVey, 2016). Most of the contemporary genome-editing technologies such as zinc-finger nucleases (ZFN), transcription activator-like effector nucleases

(TALEN) and the CRISPR-Cas systems rely on the creation of a DBS at a user-define genomic loci (Ran *et al.*, 2013).

1.4.1 Zinc-finger nucleases (ZFN)

Zinc-finger nucleases (ZFN) are one of the pioneering platforms for targeted genome-editing. Structurally, ZFN are comprised of a fusion between a DNA binding zinc-finger protein (C2H2-type) and a non-specific endonuclease such as *Fok*I (Smith *et al.*, 2000). Zinc-fingers are protein domains that interact with or bind to a zinc-ion for structural stability (Krishna *et al*., 2003). Each zinc-finger binds to three consecutive DNA base-pairs via essential amino-acid residues (referred to as critical residues) that facilitate contact with DNA (Smith *et al.*, 2000). It is the ability to manipulate these 'critical residues' within a zinc-finger protein that researchers have exploited to alter the specificity of each ZFN to a user-defined genomic locus.

A functional ZFN requires the binding of two ZFN monomers to opposite ends of the target region, which allows dimerization of the *Fok*I nuclease, an essential process for DSB formation (Bitinaite *et al.*, 1998; Kim and Kini, 2017). The architecture and mechanism of a ZFN is illustrated in *Figure 1.5*.

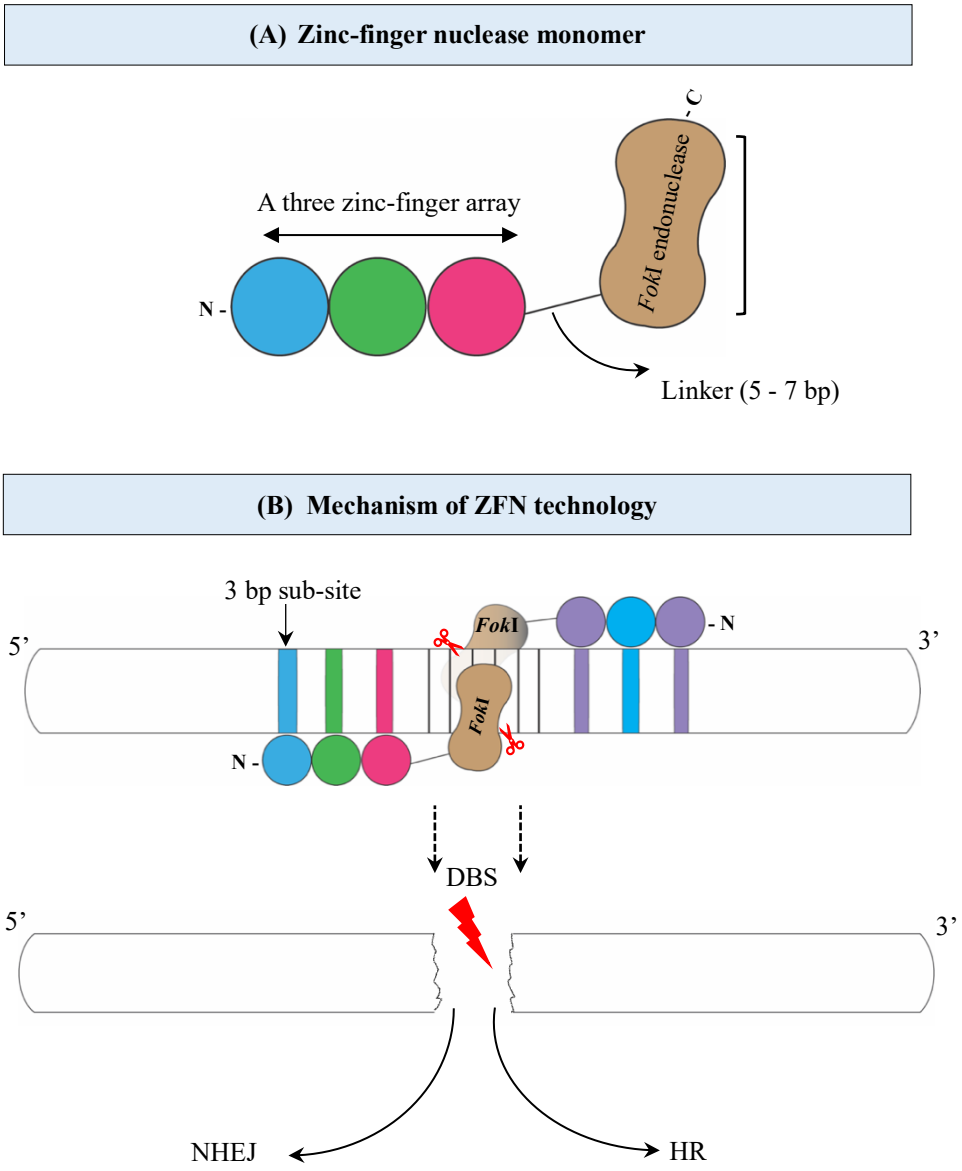


Figure 1.5: Structure and mechanism of zinc-finger nuclease (ZFN) genome-editing technology. **(A)** General architecture of a ZFN monomer. A ZFN monomer contains an array of zinc-finger proteins (ZFP) (coloured spheres), whereby each ZFP is specific to a nucleotide triplet. At the C-terminal, the ZFP array is fused to the *Fok*I endonuclease via a linker. **(B)** A functional ZFN requires binding of each monomer to opposite sides of the target sequence resulting in *Fok*I dimerization. The *Fok*I creates a DSB at the target site that may be repaired either by NHEJ or HR. Adapted from (Isalan, 2011).

1.4.2 Transcription-activator like effector nucleases (TALEN)

Transcription activator-like effectors (TALE) are proteins excreted by bacterial plant pathogens such as those in the genus *Xanthomonas* and influence the transcription of host genes (Boch and Bonas, 2010). TALE proteins consist of (i) an N-terminal located transcription factor III (TS3) translocation signal (ii) two C-terminal nuclear localization signal (NLS) domains and (iii) an acid activation domain responsible for gene transcription (Bogdanove *et al*., 2010).

The TALE proteins have a centrally located DNA-binding domain made up of monomers ~33 - 35 amino acids in length, each binding to a specific nucleotide in DNA (Bogdanove and Voytas, 2011; Nemudryi *et al.*, 2014). The amino acid residues at position '12' and '13' of each DNA-binding domain are referred to as repeat 'variable di-residues' (RVD) and dictate the DNA-binding specificity (Garg *et al.*, 2012). It is these two hypervariable residues that have made it possible to customise the TALE DNA binding specificity. A TALE protein fused to the non-specific endonuclease *Fok*I (see ZFN), is the basis for the TALEN genome-editing technology (Joung and Sander, 2013). The structure and mechanism of the TALEN genome-editing system is shown in *Figure 1.6.*

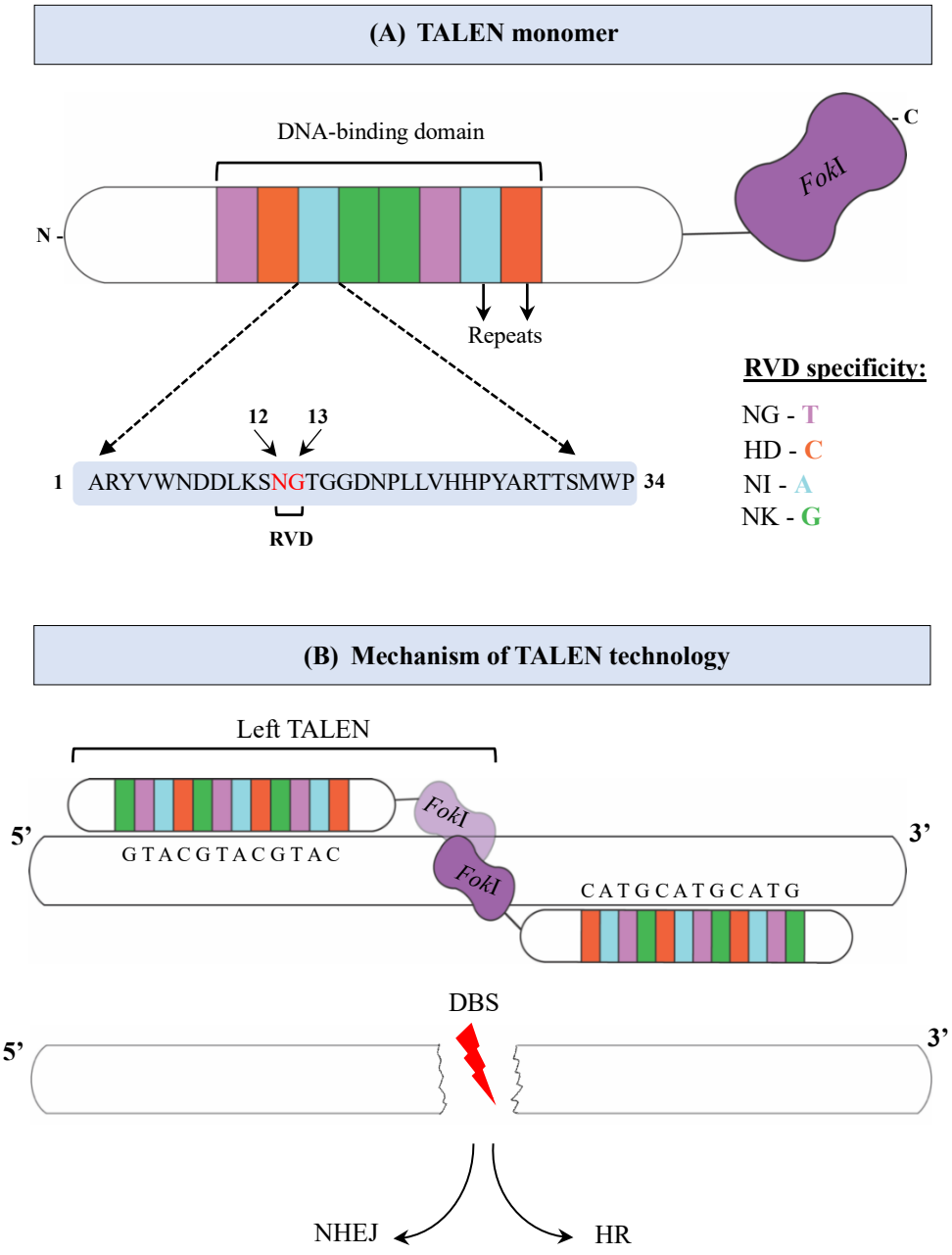


Figure 1.6: Structure and mechanism of transcription activator-like effector nuclease (TALEN) genome-editing technology. **(A)** A TALEN monomer consists of a TALE protein bound to a *Fok*I endonuclease. The DNA binding domain within each monomer contains several amino acid repeats (coloured rectangles) spanning ~34 amino acids. Within each 34 amino acid repeat are two variable residues at position 12 and 13 (highlighted in red) termed repeat variable di-residues (RVD) that specify the nucleotide specificity. **(B)** To induce a DSB, two TALEN monomers bind to opposite sides of the target site allowing the *Fok*I to dimerise and introducing a double-stranded break (DBS). The DBS may be repaired by non-homologous end joining (NHEJ) or homology repair (HR). Adapted from (Naitou *et al*., 2015).

1.4.3 CRISPR

1.4.3.1 Overview

Clustered Regularly Interspaced Short Palindromic Repeat sequences or 'CRISPR', is an adaptive immunity system used by both bacteria and archaea to fight off viruses and other mobile genetic elements (Bhaya *et al*., 2011; Vestergaard *et al*., 2014). The repeats associated with the CRISPR system were first identified in the genome of an *E. coli* K12 strain (Ishino *et al.*, 1987). However, it was not until a few years later that the function and importance of these repeats was fully investigated and understood (Doudna and Charpentier, 2014). The term 'CRISPR' was coined to avoid confusion as it specifically refers to the family of repeats that are characteristically interspaced by spacer sequences (Jansen *et al.*, 2002). CRISPR genomic loci were found to also contain CRISPR-associated genes (*cas*) whose function at the time was unclear and was loosely inferred from sequence alignments (Jansen *et al.*, 2002).

The first evidence that hinted at CRISPR being an adaptive immune mechanism was from the seminal work on *Streptococcus spp*. (Bolotin *et al.*, 2005). The authors found significant homology of the spacer sequences to *Streptococcus* spp. phages and other extrachromosomal elements. Mojica and colleagues (2005) made similar claims regarding the origin of the intervening spacers and correlated their presence to resistance of the spacer-containing strains to specific viruses. Two years on, Barrangou and colleagues (2007) provided experimental evidence for CRISPR acting as an adaptive immune system in *Streptococcus thermophilus*. Since then, CRISPR-Cas systems have been detected in ~90% of archaeal and ~40% of bacterial genomes (Adli, 2018).

1.4.3.2 Classification of CRISPR-Cas systems

The classification of CRISPR-Cas systems is rather challenging due to factors such as a high rate of evolution of the adaptation and effector modules, the lack of distinct *cas* gene signatures and *cas* sequence clustering as well as variable *cas* gene composition (Makarova *et al*., 2011, 2015, 2018). Nonetheless, the current CRISPR-Cas classification recognises two distinct groups, class 1 and class 2 (Makarova *et al*., 2017). The currently accepted CRISPR-Cas classification system utilises a multifactor approach based on the presence of a characteristic *cas* gene within a type/subtype, the overall Cas protein composition, CRISPR loci architecture, the phylogeny of genes within the subsets and experimental observations (Makarova *et al.*, 2020).

Class 1 CRISPR-Cas systems are widely distributed in both bacterial and archaeal genomes. These CRISPR-Cas systems are characterised by the use of a multi-protein effector module and are comprised of types I, III and IV (Makarova *et al.*, 2020). In addition to the three types, the latest CRISPR-Cas classification system recognises an additional 16 subtypes. In comparison, while class 1 CRISPR-Cas systems are widespread in both bacteria and archaea, class 2 systems are highly prevalent in bacterial genomes (Makarova *et al.*, 2020). This skewed distribution has been attributed to the lack of RNAse III in archaea which is needed to process the crRNAs (Makarova *et al*., 2020). Class 2 CRISPR-Cas systems utilise a single multidomain effector protein and include types II, V and most recently VI (Makarova *et al.*, 2020). Currently, 17 subtypes have been identified. A summary of the current CRISPR-Cas classification system and Cas composition is illustrated in *Figure 1.7*.

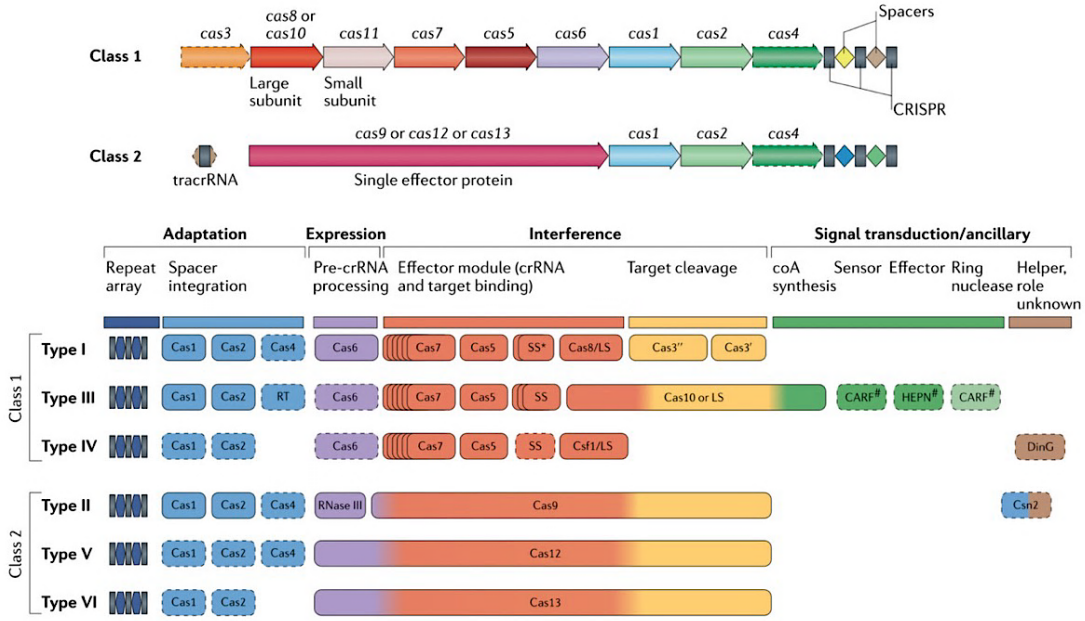


Figure 1.7: Classification of CRISPR-Cas systems. CRISPR-Cas systems can be divided into either class 1 that use multiple effector proteins or class 2 that use a single effector protein. CRISPR-Cas systems are further divided into six types (I - VI) which differ mainly based on mechanistic properties. CRISPR-Cas types and subtypes differ in the genetic components of their functional modules (adaptation, expression and interference). Genes that may or may not be present in a particular type are presented with dashed lines (e.g. *Cas6*). Proteins with multiple functions across the modules are represented as multicoloured genes. Adapted from (Makarova *et al*., 2020).

1.4.3.3 Structure of CRISPR-Cas loci

CRISPR-Cas loci are generally characterised by the presence of 'repeat-spacer-repeat' arrays. The spacer sequences are short segments of DNA that originate from previously encountered mobile genetic elements such as phages and plasmids (Mojica *et al*., 2005). The function of these spacer sequences is to serve as an infection memory to facilitate recognition of the mobile genetic elements during future encounters (Wimmer and Beisel, 2020). A CRISPR-Cas locus also contains an operon encoding *cas* genes (Sorek *et al*., 2008). Located 5' of the CRISPR-array is an AT-rich leader sequence containing the promoter elements needed to transcribe the array (Kieper *et*

al., 2019; Sorek *et al*., 2008). The general structure of a CRISPR-Cas locus is illustrated in *Figure 1.8*.

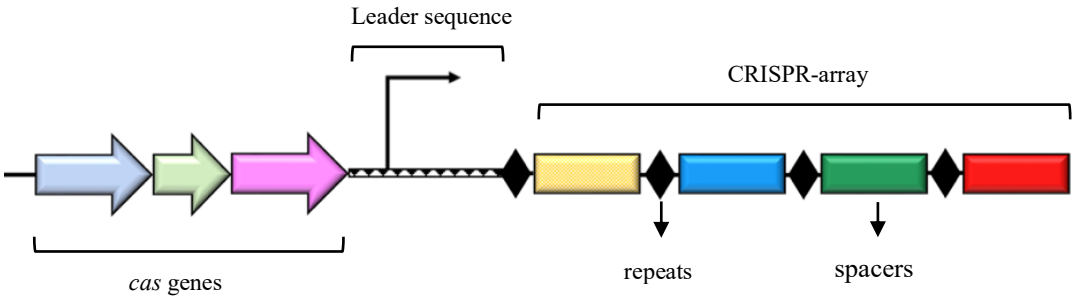


Figure 1.8: General structure of a CRISPR-Cas genomic locus. The CRISPR-array consists of spacers (coloured rectangles) and repeat sequences (black diamonds). The spacer sequences originate from foreign invading genetic material such as viruses. The leader sequence found adjacent to the CRISPR-array, is an AT- rich stretch of DNA of varying length that contains the necessary regulatory elements needed to transcribe the array. A CRISPR-Cas locus also contains varying composition of *cas* genes depending on the CRISPR-Cas system. Adapted from (Jiang and Doudna, 2015).

1.4.3.4 Stages of CRISPR-Cas adaptive immunity

Fundamentally, the CRISPR-Cas system operates as an RNA-guided defence system against invading genetic elements akin to eukaryotic interference RNA (RNAi) (Bosher and Labouesse, 2000). CRISPR-Cas adaptive immunity consists of three main stages including **(i)** the adaptation stage **(ii)** expression and maturation and **(iii)** interference (Rath *et al.*, 2015; Makarova *et al.*, 2020).

1.4.3.4.1 Adaptation

Adaptation is the first stage of CRISPR-Cas adaptive immunity and involves the acquisition of a new segment of DNA (the spacer) from an invading genetic element. The acquisition of new spacer sequences is mediated primarily by the proteins Cas1 and Cas2 (Nuñez *et al.*, 2014, 2015). Cas1 and Cas2 are present in all CRISPR-Cas systems except in the recently discovered type IV system (Makarova *et al.*, 2015,

2020). Cas1 and Cas2 form a complex (consisting of two Cas1 dimers and a single Cas2 dimer) that recognises a specific sequence in the DNA of the invader known as the protospacer-adjacent motif or PAM (Amitai and Sorek, 2016). Once the PAM is recognised by the Cas1-Cas2 complex, a short segment of DNA (adjacent to the PAM) is excised and integrated into the CRISPR-array as a newly acquired spacer.

Integration of the newly acquired spacer occurs at the junction of leader and repeat sequences with the latter simultaneously undergoing duplication (Nuñez *et al.*, 2015). Once the new spacer has been integrated into a CRISPR-Cas locus of the organism, the host can recognise the invading element during any future encounters. It is interesting to note that certain CRISPR-Cas systems are also capable of obtaining new spacers from ssRNA typically associated with type III CRISPR systems that encode a reverse transcriptase at the CRISPR-Cas locus (Silas *et al.*, 2016). An illustration of the adaptation stage of CRISPR-Cas adaptive immunity is presented in *Figure 1.9.*

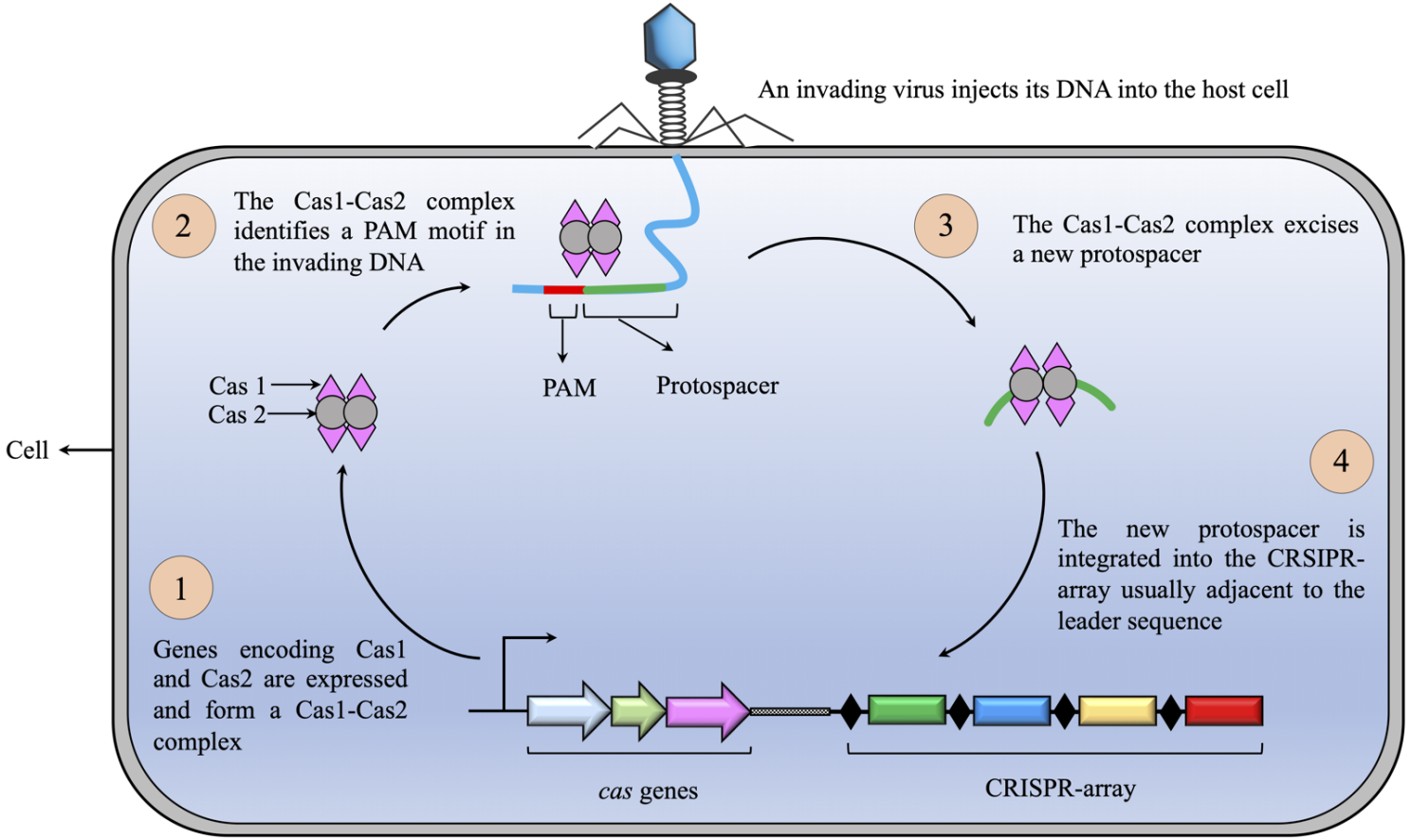


Figure 1.9: Illustration of the adaptation stage of CRISPR-Cas immunity. The adaption complex consisting of the Cas1 and Cas 2 proteins binds to the invading DNA at a protospacer adjacent motif site (PAM). A segment of DNA (protospacer) adjacent to the PAM is subsequently excised and integrated into the CRISPR-array becoming a new spacer. Newly acquired spacers are inserted in the region closest to the leader sequence. Adapted from (Amitai and Sorek, 2016).

1.4.3.4.2 Expression and maturation

During the second stage of CRISPR-Cas immunity, the CRISPR-array is transcribed as a single transcript termed precursor CRISPR RNA or pre-crRNA. The pre-crRNA is processed into individual mature crRNAs that proceed to form a ribonucleic protein complex with an effector(s) protein that attacks foreign invading elements. The processing of pre-crRNA may vary slightly depending on the CRISPR-Cas system or subtype. For example, class 1 CRISPR-Cas systems utilise the endoribonuclease Cas6 to cleave the pre-crRNA transcript into smaller spacer-containing units (Carte *et al.*, 2008; Deltcheva *et al.*, 2011). An exception to this is the type 1-C subtype that employs Cas5d to cleave pre-crRNA into small units (Nam *et al.*, 2012; Charpentier *et al.*, 2015). In the recently discovered type IV systems, processing of pre-crRNA was determined to be mediated by Csf5 (Özcan *et al.*, 2019).

In contrast, class II CRISPR-Cas systems exhibit slight variation in their mechanism of crRNA biogenesis. For example, type II CRISPR-Cas require an additional trans-activating CRISPR RNA (tracrRNA) for the processing of pre-crRNA (Charpentier, 2015). The tracrRNA together with Cas9 (formerly Csn1) and RNAse III mediate the processing and maturation of crRNA (Deltcheva *et al.*, 2011). Interestingly, a unique crRNA biogenesis mechanism was identified in the type II-C CRISPR-Cas system of *Neisseria meningitidis* whereby internal promoters, present within the CRISPR repeats, mediate transcription of individual crRNAs (Zhang *et al.*, 2013; Mir *et al.*, 2018). In the type V and VI systems, the effector proteins Cas12a (formerly cpf1) and Cas13 are responsible for crRNA maturation respectively (Fonfara *et al.*, 2016; Zheng *et al.*, 2020). A generic mechanism of crRNA biogenesis is illustrated in *Figure 1.10.*

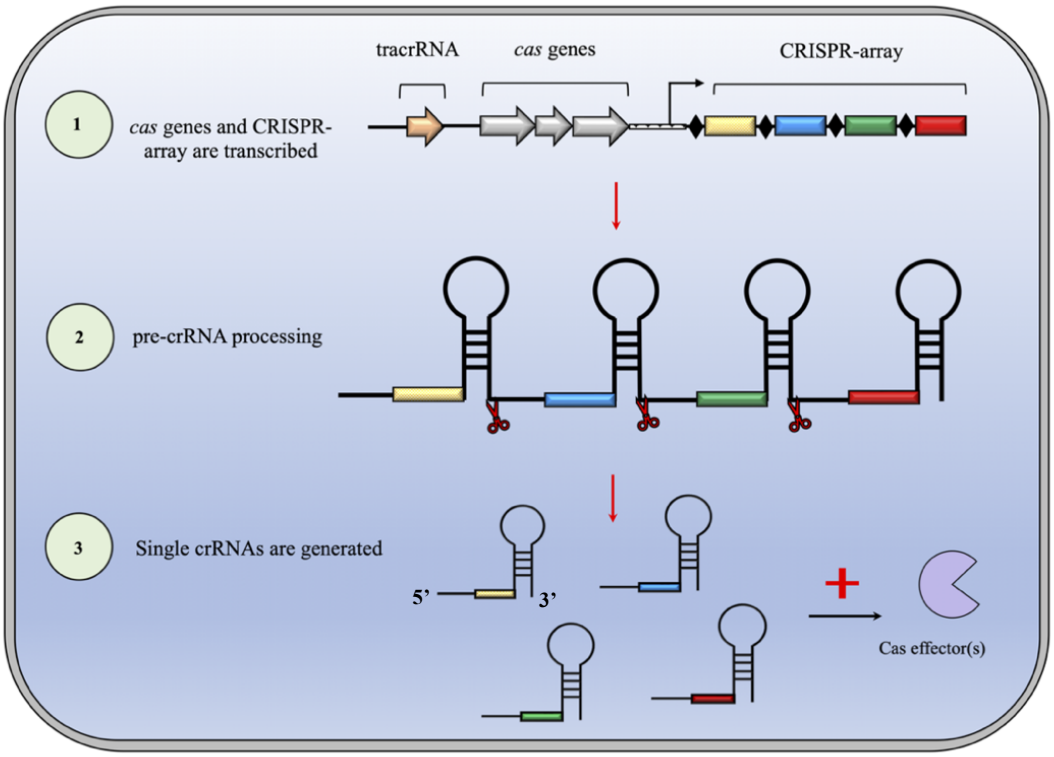


Figure 1.10: A generic illustration of the CRISPR-Cas expression and maturation stage. The CRISPR-array is transcribed as a single transcript into a pre-crRNA molecule. The processing and maturation of the pre-crRNA involves the cleaving of the transcript into individual crRNA each containing a single spacer flanked by a truncated repeat sequence. The individual crRNA forms a ribonucleoprotein with an effector(s) prior to targeting an invading genetic element. Additional elements such as the tracrRNA are required in certain CRISPR-Cas systems (e.g type II Cas9). Adapted from (Amitai and Sorek, 2016).

1.4.3.4.3 Interference

Once a spacer is integrated into the CRISPR-array, the host cell can recognise and degrade the invading virus or plasmid during any future encounters. Interference requires the formation of a ribonucleoprotein complex comprised of the crRNA and the effector endonuclease and tracrRNA in the case of type II systems. This complex recognises a specific sequence (protospacer) in the DNA of the invading element via the PAM. Complementary base-pairing between the crRNA and the protospacer induces a conformational change in the interference complex causing target cleavage

(Wang *et al*., 2022). An illustration of the CRISPR-Cas immunity interference stage is shown in *Figure 1.11*.

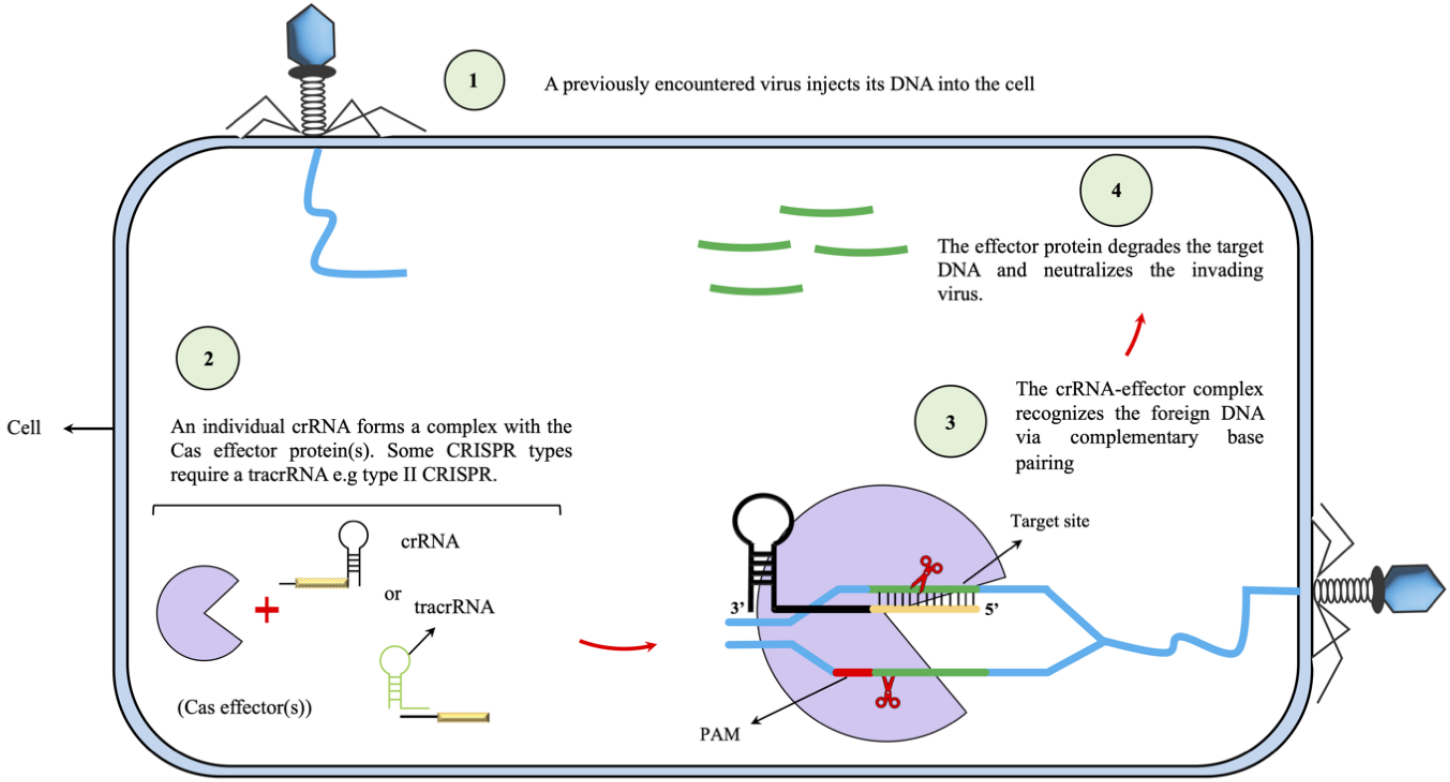


Figure 1.11: Mechanism of CRISPR-Cas interference. Upon processing of the pre-crRNA, individual crRNA units form a ribonucleic protein complex (RNP) with a Cas effector(s). In the case of type II systems, the crRNA must also associate with a tracrRNA. The RNP is guided to the target site recognising the protospacer adjacent motif (PAM) and subsequent degradation of the invading DNA by the effector protein. Adapted from (Wang *et al*., 2022).

1.4.3.5 CRISPR-Cas as a genome-editing tool

CRISPR-Cas technology has been widely adopted and favoured as a genome-editing tool in a range of models including mammals, fungi, plants and bacteria (Mali *et al*., 2013; Fauser *et al*., 2014; Li *et al*., 2015; Wenderoth *et al*., 2017). The popularity of CRISPR-Cas as a genome-editing tool can be attributed to its relative simplicity relative to pioneering platforms such as ZFN and TALEN. For example, customisation of ZFN/TALEN requires tedious and laborious protein engineering for every new target (Wright *et al*., 2006; Li *et al*., 2020; Javaid *et al*., 2022; Xiaoshuai *et al*., 2022). Furthermore, the routine use of ZFN is hindered by their high costs making them inaccessible to most research groups (Sander *et al.*, 2011). In contrast, the customisation of CRISPR-Cas systems relies on the synthesis of a ~20 bp oligonucleotide (spacer) complementary to the desired genomic locus which is relatively simple and cost-effective. Aside from its simplicity, CRISPR-Cas genome-editing technology offers remarkable mechanistic versatility. For example. depending on the CRISPR-Cas system employed, a user can target either RNA or DNA which is not possible with ZFN or TALEN technology. In addition to genome-editing, CRISPR-Cas technology may also be used to control gene expression exemplified by CRISPRi technology (Larson *et al.*, 2013). An added advantage of CRISPR-Cas is the ability to manipulate multiple targets (multiplexing) simultaneously (Cobb *et al*., 2015).

Although genome-editing of eukaryotic and bacterial models with CRISPR-Cas has seen substantial advances, the same cannot be said for the archaea. This is surprising as CRISPR-Cas systems are absent from eukaryotes and are in fact more abundant in archaea than they are in bacteria (Sorek *et al*., 2008; Pourcel *et al.*, 2020). Nonetheless, the successful use of CRISPR-Cas in archaea has been demonstrated in just a handful

of strains. For example, the RNA-targeting type IIIB CRISPR-Cas system in the archaeon *Sulfolobus solfataricus* was repurposed as a tool to target the mRNA of chromosomal genes to influence their expression (Zebec *et al.*, 2014). In the closely related *Sulfolobus islandicus*, the native type-IA and III-B CRISPR-Cas systems were exploited as genetic tools (Peng *et al.*, 2015; Li *et al.*, 2016). Among the haloarchaea, an endogenous CRISPR-Cas type I-B system found in *H. volcanii*, was used to manipulate gene expression (Stachler and Marchfelder, 2016). The exploitation of endogenous CRISPR-Cas systems is particularly important for archaeal extremophiles. This is because the Cas proteins do not require further adaptation to function under extreme conditions (Luo *et al*., 2016; Gophna *et al*., 2017).

Type II CRISPR-Cas systems (e.g. Cas9) are absent in archaea and have primarily been relevant in mesophilic bacterial models (Li *et al.*, 2016). The first description of a Cas9-based system for an archaeon was reported by Nayak and colleagues (2017) and was developed for the methanogen *Methanosarcina acetivorans*. More recently, a CRISPR-Cas12a system was developed for the archaeon *Methanococcus maripaludis* which was used to knockout and replace the operon encoding the flagella apparatus with heterologous genes (Bao *et al*., 2022). The development of genome-editing tools for the archaea based on class 2 systems is a major development for archaeal genetics. This is because non-native CRISPR-Cas systems could be important for strains that lack endogenous CRISPR-Cas systems. Furthermore, the need for a single effector protein by class 2 CRISPR-Cas systems simplifies and reduces the genetic load that needs to be introduced into the cell.

1.4.3.5.1 Restriction-modification systems

In addition to adaptive immunity, prokaryotic organisms also make use of innate immune defence mechanisms to fight off invading genetic elements such as viruses. A well-studied and ubiquitous prokaryotic innate immune system is the restriction-modification (R-M) system (Vasu and Nagaraja, 2013). In one study, R-M systems were detected in 83% of prokaryotic genomes compared to CRISPR-Cas systems found in ~39% of sequenced prokaryotic genomes in the NCBI RefSeq database (Tesson *et al*., 2022).

R-M systems consists of two enzymatic activities including a restriction endonuclease and a DNA methyltransferase (Vasu and Nagaraja, 2013; Dimitriu *et al*., 2020). The restriction endonuclease activity is responsible for cleaving DNA at specific recognition sites (Rusinov *et al*., 2018). In contrast, the DNA methyltransferase plays a role in facilitating the differentiation between the invading/foreign and host DNA. This is achieved by the incorporation of a methyl-group at adenine or cytosine residues within the restriction endonuclease recognition site of the host DNA thus protecting it from cleavage (Tock and Dryden, 2005). Interestingly, the R-M system is capable of stimulating CRISPR-Cas adaptive immunity (Maguin *et al*., 2022). This interplay between R-M and CRISPR-Cas systems has also been shown to augment resistance to phage infection with DNA methylation having no effect on the latter (Dupuis *et al*., 2013).

Four types of R-M systems are recognised and include types I, II, III and IV which may be further grouped into various subtypes (Loenen *et al*., 2014; Roberts *et al*., 2003). A major difference between the R-M systems is that while the restriction endonucleases of types I-III specifically target unmethylated DNA at their respective

recognition sites, type IV restriction endonucleases target methylated DNA at their recognition sites (Bai *et al*., 2017; Roberts *et al*., 2003). Additional differences between the different R-M systems may include subunit composition, specificity of the recognition sequence and cleavage position relative to the recognition site (Vasu and Nagaraja, 2013; Bai *et al*., 2018).

The discovery of R-M systems predates CRISPR-Cas and they have played a pivotal role in advancement of molecular biology and especially genome-editing technologies. For example, pioneering genome-editing technologies such as ZFN and TALEN, exploit the *Fok*I restriction endonuclease (originating from the R-M system of *Flavobacterium Okeanokoites*) to introduce double-stranded breaks (Kita *et al*., 1989). Most recently, a novel genome-editing technology termed R-M system-mediated genome-editing (or RMGE) based on the type IV R-M system, has been developed for bacteria and yeasts (Bai *et al*., 2018). The RGME approach makes use of an exogenous restriction endonuclease or methyltransferases as counterselection markers in strains with or without type IV R-M systems.

Despite the importance of R-M systems in recombinant DNA technology and genome-editing, they may also present as major barriers to these technologies and the genetic tractability of microorganisms (Freed *et al*., 2018; Johnston *et al*., 2019). This is due to the fact that successful genome-editing of an organism is dependent on the maintenance of heterologous DNA (e.g. plasmids) within a host cell. However, R-M systems can target and degrade this heterologous DNA resulting in poor transformation efficiencies and consequently hinder genome-editing efforts. A typical approach used to protect the recombinant DNA (e.g. plasmids) from degradation by R-M systems, entails passing the DNA through a strain containing the necessary methyltransferases

to match the methylation pattern of the target host (Johnston *et al*., 2019). A recently developed approach makes use of special vectors termed SyngenicDNA minicircle plasmids to facilitate evasion of R-M systems. SyngenicDNA minicircle plasmids are synthetic vectors specially designed to be deficient in recognition sequences making them resistant to degradation by R-M systems in the target host (Johnston *et al*., 2019). Evidently, R-M systems play an important role in genetic engineering efforts acting both as tools to introduce genetic changes and potential barriers to the introduction of such changes. Therefore, R-M systems are integral to the success of genome-editing regardless of the technology used.

1.5 Current state of archaeal genetic tools

Archaea, unlike their bacterial and eukaryotic counterparts, have not seen nearly as much success in the development of genetic tools. This is perhaps not surprising given that archaea were only classified into their own domain in the late 1970s (Woese and Fox, 1977). Initially, only two archaeal phyla were recognised, Crenarchaeota and the Euryarchaeota (Spang *et al.*, 2010). The Euryarchaeota are widely distributed in both marine and soil sediments and include representatives such as methane-producing archaea (i.e. methanogens) and extreme halophiles (Offre *et al*., 2013). The phylum Crenarchaeota is mainly comprised of hyperthermophilic organisms (e.g. Sulfolobales and Thermoproteales) that can be found in hydrothermal vents and hot springs (Perevalova *et al.*, 2008).

Over the years, a plethora of novel archaeal lineages have been described with notable examples such as the Nanoarchaeota (Huber *et al.*, 2002), Korarchaeota (Barns *et al.*, 1996), Thaumarchaeota (reclassified as Thermoproteota) (Rinke *et al.*, 2021) and the recently discovered Asgard archaea (Zaremba-Niedzwiedzka *et al.*, 2017). These new

archaeal lineages, discovered from diverse ecological niches, represent new research opportunities to understand archaeal metabolism, physiology and explore biotechnological potential. However, cultured representatives of these novel archaeal phyla are too few, which not only hinders their physiological characterisation but also the establishment of a genetic toolbox.

Genetic tools are essential to investigating the mechanisms that underpin the molecular processes of an organism. With regards to archaea, the availability of a genetic system is particularly important due to the prevalence of poorly annotated genomes or so-called 'genomic dark matter' (Makarova *et al*., 2019). 'OMICS' approaches have undoubtedly been key to inferring function of genes and proteins present in this 'dark-matter'. However, instances where the genes or proteins have ancient evolutionary histories, it becomes increasingly challenging to identify homologues and confidently assign function without the use of reverse or even forward genetics. Furthermore, novel molecular processes that may be unique to archaea, would be challenging to decipher without genetic tools as the associated proteins are unlikely to have homologues in the other phylogenetic lineages.

In this work, a genetic toolbox is defined as a set of elements that together make up a genetic system. The four main elements of a genetic toolbox are: **(i)** the ability to grow the target organism as single colonies on solid growth medium which facilitates mutant screening **(ii)** a selection system (e.g. antibiotics resistance or auxotrophy) **(iii)** a DNA vehicle which allows the transfer of genetic material (e.g. plasmids) and **(iv)** a method to introduce DNA into a host cell (e.g. transformation, transduction, transfection, conjugation) (Leigh *et al.*, 2011; Fink *et al.*, 2021). Currently, archaea for whom a genetic system has been developed belong exclusively to the Crenarchaeota and the

Euryarchaeota (Atomi *et al*., 2012). The subsequent sections will explore the current advances and factors that have contributed to the development of genetic tools for these archaea.

1.5.1 Euryarchaeota

1.5.1.1 Methanogens

Anaerobic methane-producing archaea (i.e. methanogens) were the first archaea to be discovered (Stephenson and Stickland, 1933; Buan, 2018). These archaea are the only known microorganisms capable of biological methane production. As such, methanogens have been under intense research for decades to better understand their contribution to carbon cycling as well as their biotechnological potential (e.g. renewable energy source). Furthermore, due to the role of methane as a potent greenhouse gas, mitigation efforts rely on in-depth knowledge of methanogen biology. Consequently, the development of genetic tools for the methanogens has been a major focus for decades. Of the seven recognised methanogen orders (Adam *et al.*, 2017; Buan, 2018), genetic tools are currently only available for the Methanococcales and the Methanosarcinales which include genera such as *Methanococcus* and *Methanosarcina* (Leigh *et al*., 2011; Atomi *et al*., 2012).

These strains grow well in the laboratory and readily form single colonies on solid growth media with high plating efficiencies (Jones *et al*., 1983; Sowers *et al*., 1993; Leigh *et al*., 2011). The mesophilic nature of most genetically tractable methanogens has favoured the use of antibiotics as selective agents. For example, puromycin, a translation inhibitor isolated from *Streptomyces alboniger*, has proven to be a suitable antibiotic in strains such as *Methanococcus voltae* and *Methanosarcina mazei* (Gernhardt *et al.*, 1990; Conway De Macario *et al.*, 1996). It is interesting that the

puromycin resistance gene, puromycin N-acetyltransferase (*pac*), retains its activity following codon optimisation even in distantly related microbes with a significant difference in GC content (e.g. 73% GC in *S. alboniger* vs 30% GC in *M. voltae*). Thus, the *pac* gene may also be useful in other archaeal systems as a selectable marker. The aminoglycoside antibiotic, neomycin, has also proven to be suitable for strains such as *M. mazei* and *M. maripuladis* (Argyle *et al*., 1996; Mondorf *et al*., 2012).

Genome analysis of the available methanogens has led to the discovery of several naturally occurring plasmids in both mesophilic and extremophilic methanogens (Lange and Ahring, 2001). Such plasmids are highly useful elements for establishing genetic tools as they contain the native endogenous replication and partitioning machinery. This means that they are more likely to be taken up and maintained in the host cell from which they were isolated. Unsurprisingly, several methanogen shuttle vectors that are based on these native plasmids have been constructed. For example, Metcalf and colleagues (1997) designed an *E. coli-M. acetivorans* shuttle-vector derived from the native *M. acetivorans* plasmid, pC-2A. In *Methanococcus maripaludis* C5, the native plasmid pURB500 was used to develop an *E. coli* shuttle vector (Tumbula *et al*., 1997). Other natural plasmids identified in methanogens include pME2001 from the thermophile *Methanobacterium thermoautotrophicum* (Marburg) (Bokranz *et al*., 1990) and the related plasmid, pME2200 from *Methanobacterium thermoautotrophicum* ZH3 (Stettler *et al*., 1995). Recently, the cryptid plasmid from *Methanothermobacter marburgensis* pME2001 was used as a shuttle vector in *Methanothermobacter thermoautotrophicus* ΔH (Fink *et al.*, 2021).

The majority of transformation methods used for methanogen models are either physical or chemical methods. For example, polyethylene glycol (PEG) and liposome-

mediated transformation have successfully been used for *M. acetivorans* (Metcalf *et al.*, 1997; Oelgeschläger and Rother, 2009). In strains such as *M. voltae*, electroporation is an effective transformation method (Micheletti *et al*., 1991; Patel *et al*., 1994). Interestingly, strains such as *M. voltae*, have been found to exhibit natural competency albeit at lower efficiencies than those of electroporation (Micheletti *et al*., 1991).

Although homologous recombination is still the primary approach to introducing genomic changes, CRISPR-Cas tools have recently been developed for a handful of methanogens. For example, Nayak and Metcalf (2017) developed the first archaeal type II CRISPR-Cas system in the model methanogen *M. acetivorans*. Additionally, a CRISPRi system, which is dependent on a deactivated Cas9 was developed to control expression of nitrogen fixation-associated genes in the archaeon *M. acetivorans* (Dhamad and Lessner, 2020). An additional type II CRISPR-Cas system (CRISPR-Cas12a) was recently developed for the methanogen *M. maripaludis* (Bao *et al*., 2022).

1.5.1.2 Haloarchaea

In the late 1930s, Benjamin Wilkansky (now known as Benjamin Elazari Volcani) published the first report on living microorganisms in the Dead Sea (Wilkansky, 1936; Oren and Ventosa, 1999). These were later identified as halophilic archaea or haloarchaea, a unique group of archaea that thrive in high salinity environments (Purdy *et al.*, 2004). The haloarchaea belong to the family *Halobacteriaceae* comprising the genera *Halobacterium*, *Haloarcula,* and *Haloferax* among others (Fendrihan *et al.*, 2006). The extremophilic lifestyle of haloarchaea has generated interest in exploring their biotechnological potential (Singh and Singh, 2017). Consequently, there have been great strides made in developing genetic tools for these archaea.

Genetically tractable haloarchaeal include strains such as *Haloferax volcanii* and *Halobacterium salinarium* (Allers *et al.*, 2004; Silva-Rocha *et al.*, 2015). These strains exhibit rapid growth in the laboratory (Robinson *et al.*, 2005) and readily form single colonies on agar plates which is conducive to mutant screening (Mullakhanbhai and Larsen, 1975). Development of visible colonies in a little as 4 days has been reported for *Haloferax* strains (Holmes and Dyall-Smith, 1990). Antibiotic-based selection is widely used for the haloarchaea. For example, novobiocin a DNA gyrase inhibitor is an important selective agent for strains such as *Haloferax* Aa. 2.2 at concentrations as low as 0.005 µg/ml (Holmes and Dyall-Smith, 1990). The cholesterol lowering drug mevinolin, is another important selection agent for strains such as *H. volcanii* (Lam and Doolittle, 1992). Mevinolin inhibits the 3-hydroxy-methylglutaryl coenzyme A reductase (HMG-CoA reductase) which forms part of the mevalonate pathway responsible for the production of lipid biosynthesis precursors (Goldstein and Brown, 1990; Atomi *et al*., 2012; Vinokur *et al*., 2014). In addition to antibiotics, auxotrophy-based selection has also proven useful for the haloarchaea. One such system is the orotidine-5'-monophosphate decarboxylase (ura3/pyrF) selection system that is often used as a counterselection system (Atomi *et al*., 2012). It works on the principle of sensitivity to 5-fluroorotic acid (5-FOA) by cells containing an *ura3*/*pyrF* gene, whereas as cells that have lost this gene are able to grow in the presence of uracil. This selection system was successfully developed and used in *Haloferax mediterranei* and *Haloarcula hispanica* (Liu *et al.*, 2011). Allers and colleagues (2004) further developed a selection system based on leucine and tryptophan auxotrophy.

As seen with the methanogens, efforts towards the establishment of genetic tools for the haloarchaeal have also benefitted from the discovery of native plasmids that act as templates for the design of shuttle vectors. The multicopy plasmid pHK2 from

Haloferax Aa.2.2, was repurposed into an *E. coli-Haloferax* shuttle vector (Holmes and Dyall-Smith, 1990). The plasmid pHV2 originating in *H. volcanii* was instrumental in establishing transformation protocols for this strain (Charlebois *et al.*, 1987). With regards to inducing DNA uptake in the haloarchaea, transformation is primarily limited to the use of PEG-mediated transformation (Cline *et al*., 1989a; Cline, *et al*., 1989b). Transformation using PEG can be done on either whole cells or spheroplasts (S-layer removed) (Dyall-Smith, 2009).

Similar to the methanogens, genetic manipulation of the haloarchaea has primarily been achieved using homologous recombination (Bitan-Banin *et al*., 2003). However, the endogenous type IB CRISPR-Cas system has been exploited to manipulate gene expression in *H. volcanii* (Stachler and Marchfelder, 2016).

1.5.1.3 Hyperthermophilic Euryarchaeota

Hyperthermophilic lineages within the phylum Euryarchaeota include the orders Archaeoglobales, Methanococcales and Thermococcales (Leigh *et al.*, 2011). Archaea from the order Thermococcales often represent the dominant archaeal community in both deep and shallow-sea hydrothermal vents (Holden *et al.*, 2001; Nercessian *et al.*, 2003; Zhang *et al.*, 2012). Three main archaeal genera make up the Thermococcales including *Pyrococcus*, *Palaecoccus* and *Thermoccocus* (Zillig *et al.*, 1983; Fiala and Stetter, 1986; Takai *et al.*, 2000; Lepage *et al.*, 2004; Le Guellec *et al.*, 2021). The study of these hyperthermophiles promises fascinating insights into life at temperature extremes and potential for novel biotechnological discoveries, particularly thermostable enzymes (Vieille and Zeikus, 2001). Therefore, considerable efforts have been made towards the development of a genetic system for this group of archaea.

The main genetically tractable model strains include *Thermococcus kodakarensis*, *Pyrococcus abyssi*, *Pyrococcus yayanosii* and *Pyrococcus furiosus*. These strains are reportedly relatively easy to handle and grow well in the laboratory to high cell densities (Lucas *et al.*, 2002). Relevant to the development of genetic systems, these strains also readily form colonies on growth medium solidified with Gelrite™ (gellan gum) (Sato *et al*., 2003; Waege *et al*., 2010; Li *et al*., 2015).

Due to the extremely high temperatures needed for growth, antibiotic-based selection systems are not widely used for these hyperthermophiles. Furthermore, one of the few available thermostable antibiotic markers, a thermostable hygromycin phosphotransferase (*hpt*), has limited use in strains such as *P. abyssi* due to the innate resistance to hygromycin B (Lucas *et al.*, 2002). However, an exception is simvastatin (also known as mevinolin), that has proven to be a useful selection agent in both *Pyrococcus* and *Thermococcus* strains and exhibits stability at elevated temperatures (> 85ºC) for several days (Li *et al.*, 2015; Song *et al.*, 2021). In contrast, systems based on auxotrophy including uracil or tryptophan auxotrophy are readily available and widely used for these archaea (Santangelo *et al*., 2008b). An additional selection system for *T. kodakarensis* is based on the toxic purine analogue, 6-methyl purine (6MP), and is usually used as counterselection system. Resistance to 6MP is due to the inactivation of the hypoxanthine guanine phosphoribosyltransferase gene encoded by TK0664 (Hileman and Santangelo, 2012).

Several native plasmids have been identified serving as templates for the design of shuttle vectors. The first shuttle vector for a hyperthermophilic Euryarchaeote was based on the native plasmid pGT5 (from *P. abyssi*) which was fused to pUC19 to create the shuttle vector pCSVI (Aagaard *et al.*, 1996). Interestingly, the pCSVI shuttle vector

is capable of stable replication in both *P. furiosus* and the crenarchaeote *S. acidocaldarius* (Aagaard *et al.*, 1996). Plasmid pGT5 has also been fused to the plasmid pLitmus38 (bacterial) to create the shuttle vector pYS2 (Lucas *et al.*, 2002). In contrast to the genetically tractable *Pyrococcus*, *T. kodakarensis* does not harbour its own native plasmids (Hileman and Santangelo, 2012). However, plasmids identified in the closely related *Thermococcus* species are capable of replicating in *T. kodakarensis*. For example, plasmid pTN1 from *Thermococcus nautilus* was used to create the *E. coli*-T. *kodakarensis* shuttle vector pTKO1 (Santangelo *et al*., 2008). Plasmid pTP2 from *Thermococcus prieurii* formed the basis of various *E. coli-T. kodakarensis* shuttle vectors (Catchpole *et al.*, 2018). A notable recent discovery is plasmid pT33-3, the first non-Sulfolobales archaeal conjugative plasmid discovered (Catchpole *et al.*, 2023).

It has been determined that the certain *Thermococcal* strains such as *T. kodakarensis* are capable of natural transformation which is efficient enough to be exploited for genetic studies (Sato *et al.*, 2003). Similarly, *P. furiosus* COM1, also exhibits natural competency and seems to be a characteristic feature of these hyperthermophiles (Lipscomb *et al.*, 2011). In addition to natural transformation, DNA uptake may also be induced using methods such as $CaCl_2$ heat-shock treatment (Sato *et al.*, 2003, 2005; Lipscomb *et al.*, 2011).

Targeted genomic alterations among the hyperthermophilic Euryarchaeota are exclusively introduced via homologous recombination. However, reports on the characterisation of CRISPR-Cas systems from *T. kodakarensis* (Elmore *et al.*, 2013) and *P. furiousus* (Elmore *et al.*, 2015) suggests that CRISPR-Cas-based genome-editing tools could be available in the near future.

1.5.2 Crenarchaeota

1.5.2.1 Sulfolobales

The Crenarchaeota order Sulfolobales, contains genera such as *Sulfolobus*, *Saccharolobus*, *Sulfurococcus* and *Sulfodiicoccus* (Brock *et al.*, 1972; Johnson, 1998; Sakai and Kurosawa, 2017, 2018). These archaea are characterised by their hyperthermophilic and acidophilic lifestyle and have been isolated from volcanic solfataras, calderas and hot springs. As with other hyperthermophilic archaea, they have attracted the attention for the potential of novel biotechnology discoveries and a better understanding of life at extreme conditions. Despite their extremophilic nature, efforts into the development of a genetic system for the Sulfolobales have been rather successful.

Genetically tractable strains primarily belong to the genus *Sulfolobus* and include *S. solfataricus* and *S. islandicus*. These strains exhibit rapid growth and readily form individual colonies on solid growth medium (Reilly and Grogan, 2001; Wagner *et al.*, 2009; Leigh *et al.*, 2011). Consequently, mutant selection on solid medium is possible for the strains.

An interesting and unique feature of the Sulfolobales is the abundance of conjugative plasmids (Prangishvili *et al*., 1998). Examples of conjugative plasmids include pNOB8 (*Sulfolobus* sp.), pING1 (*S. islandicus*) and pM164 (*S. islandicus* strains) (Schleper *et al.*, 1995; Stedman *et al.*, 2000). Non-conjugative plasmids have also been discovered and include pRN1 (Berkner *et al.*, 2007). In addition to plasmids, the well characterised *Sulfolobus* spindle-shaped virus 1 (SSV1) from *Sulfolobus shibatae* has been a valuable tool for *Sulfolobus* genetic studies. For example, elements of the SSV1 virus have been used as the basis for construction of both integrative and multi-copy

shuttle vectors (Cannio *et al.*, 1998; Jonuscheit *et al.*, 2003). In addition to acting as DNA vectors, the high prevalence of conjugative plasmids for the Sulfolobales provides an added advantage as a transformation method via conjugative transfer (Elferink *et al*., 1996). It has also been demonstrated that the SSV1 virus particles can be packaged with DNA and used to transform cells via transduction (Schleper *et al*., 1992). Genetically tractable *Sulfolobus* sp. are also transformed using electroporation at high efficiencies (Schleper *et al*., 1992; Stedman *et al.*, 2000; Schelert *et al.*, 2004).

With regards to mutant selection, auxotrophy-based systems have proven to be superior among the Sulfolobales. For example, using β-galactosidase deficient *Sulfolobus* mutants (Δ*lacS*), transformants can be selected for on minimal media supplemented with lactose (Schelert *et al.*, 2004). The *lacS* gene (encoding β-galactosidase), further functions as a reporter gene when medium is supplemented with X-gal which facilitates blue/white screening (Jonuscheit *et al.*, 2003). In addition to the *lacS* system, uracil-auxotrophy is widely used in *Sulfolobus* genetic systems (Berkner and Lipps, 2008). Although antibiotic-based selection systems are not commonly used for the Sulfolobales, a thermostable phosphotransferase was developed facilitating hygromycin B selection at temperatures reaching 75ºC (Cannio *et al.*, 2001).

Ultimately, targeted genomic manipulation or gene knockout/in in the Sulfolobales is achieved by homologous recombination. However, as seen with the previous archaeal groups, efforts are being made into the development of CRISPR-Cas-based genetic tools. A notable example is the endogenous CRISPR-Cas system (type III-CMR) in *S. solfataricus* that was used to influence gene expression (Zebec *et al.*, 2014).

Table 1.1: Summary of genetic tools currently available for archaea.

Strain	Phylum	Transformation method	Plasmid /virus	Selection markers	Reference
Haloferax volcanii	Euryarchaeota	PEG-mediated	✓	Mevinolin 3-isopropylmalate dehydrogenase Leucine auxotrophy (l*ue*B) Tryptophan synthase (*trp*A) - Tryptophan auxotrophy	(Lam and Doolittle, 1989) (Allers *et al.*, 2004) (Charlebois *et al*., 1987)
Haloferax strain Aa 2.2	Euryarchaeota	PEG-mediated	✓	Novobiocin (*Gyr*B)	(Holmes *et al*., 1994) (Holmes and Dyall-Smith, 1990)
Haloferax mediterranei *Haloarcula hispanica*	Euryarchaeota	$CaCl_2$ heat-shock	✓/x	Orotidine-5'-monophosphate decarboxylase (*ura*3/*pyr*F) -uracil auxotrophy	(Liu *et al.*, 2011)
Methanosarcina acetivorans	Euryarchaeota	Liposome-mediated PEG-mediated Electroporation	✓	Puromycin resistance (puromycin transacetylase, *pac*)	(Nayak and Metcalf, 2017) (Metcalf *et al*., 1997)
Methanococcus voltae	Euryarchaeota	Natural competency PEG-mediated Electroporation Liposome-mediated	✓/x	Puromycin resistance (puromycin transacetylase, *pac*)	(Bertani and Baresi, 1987) (Gernhardt *et al.*, 1990) (Patel *et al.*, 1994) (Chaban *et al.*, 2009)

Table 1.1: Continued.

Methanococcus maripaludis	Euryarchaeota	Natural competency PEG-mediated	✓	Neomycin (aminoglycoside phosphotransferase genes (*APH*3'II and APH3'I)) Puromycin	(Argyle, *et al*., 1996) Tumbula *et al*., 1994,1997) (Bao *et al*., 2022)
Thermococcus kodakarensis	Euryarchaeota	$CaCl_2$ heat-shock	✓/x	*trp*E auxotrophy *pyr*F auxotrophy	(Sato *et al.*, 2005) (Aagaard *et al.*, 1996)
Sulfolobus solfataricus	Crenarchaeota	$CaCl_2$ heat-shock Electroporation Conjugation	✓	β-galactosidase (*lac*S)-blue/white selection Hygromycin B (hygromycin phosphotransferase, *hph*	(Schleper *et al*., 1992) (Cannio *et al*., 1998) (Jonuscheit *et al*., 2003) (Elferink et al., 1996)
Sulfolobus islandicus	Crenarchaeota	Shuttle vectors (*E. coli*) Electroporation Conjugation	✓	*pyr*E/F auxotrophy *lac*S β-galactosidaase-blue/white selection	(Deng *et al.*, 2009) (Stedman *et al*., 2000) (Sanchez-Nieves *et al*., 2023)

✓/x: No native plasmid present but plasmids from closely related strains or those based on *E. coli* plasmids are available.

1.5.3 Barriers to the establishment of genetic tools for the Thaumarchaeota

AOA have a relatively recent history with the first strain only isolated in 2005 (Könneke *et al.*, 2005). This is in contrast to archaea from the major phyla some of which were discovered decades ago (Stephenson and Stickland, 1933; Wilkansky, 1936). It is therefore unsurprising that the technical advancements for the AOA such as genetic tools are still lagging behind these archaea. But aside from a relatively recent history, virtually no reports on the development of genetic tools have been published.

As noted previously, the ability to grow an organism on solid medium as single colonies is essential to mutant screening. A unifying feature of the genetically tractable archaea we have discussed previously is that they all readily form single colonies on solid growth medium. This is in contrast to the AOA strains currently available in culture, of which none exhibit growth on solid growth medium. All AOA strains in culture today are maintained exclusively as liquid cultures. Presently, only a single attempt to grow an AOA on solid growth medium as distinct colonies has been reported but achieved limited success (Chu *et al.*, 2015). While it is possible to screen for mutants grown in liquid growth medium using methods such as fluorescence activated cell sorting (Abuaita and Withey, 2011), this approach is not suitable or attractive for routine use as it is costly and time consuming. Furthermore, the majority of AOA strains exhibit slow growth rates which may increase the chances of contamination as antibiotics used for selection systems may degrade before the desired mutants have been enriched. It will therefore be worthwhile to investigate this general inability of the Thaumarchaeota to grow on solid growth medium.

A robust selection system is an essential component of any genetic toolbox and is needed to ensure the isolation of a homogenous population of cells harbouring a

desired genotype for further study. The selection of bacterial and archaeal mutants relies on either antibiotic or auxotrophy-based selection mechanisms. In order to employ auxotrophy-based selection, the availability of a relevant auxotrophic mutant obtained naturally or via genetic manipulation is essential. Such mutants may also be generated using physical methods such as UV irradiation (Bertani and Baresi, 1987). In contrast, the use of antibiotics is relatively straight forward and is dependent on the sensitivity of the model strain to the antibiotic and availability a resistance gene. The latter approach therefore seems like a suitable method particularly for initial efforts.

Unfortunately, only a handful of antibiotics are currently known to inhibit AOA and include compounds such as cycloheximide and simvastatin (Vajrala *et al*., 2014; Zhao *et al*., 2020). Additionally, AOA also seem to exhibit resistance to a range of protein synthesis inhibitors that are typically used to prevent bacterial growth in culture (Abby *et al*., 2018). Therefore, it will be necessary to focus on antibiotic sensitivity testing of the Thaumarchaeota before a selection system can be established.

Genetic manipulation or genome-editing largely rely on the introduction of the necessary genetic information into a host cell. This is often achieved by using plasmids (self-replicating or even integrative) that act as molecular vehicles. As gleaned from the genetically tractable archaea, the development of genetic systems has greatly benefitted from the availability of native plasmids or other mobile genetic elements such as viruses and conjugative plasmids. This is because native plasmids can be reengineered into customised vectors carrying user-defined genetic information. Furthermore, with regards to conjugative plasmids, they have an added advantage of being used as a transformation method. More importantly, native plasmids are expected to contain all the necessary machinery needed for the

maintenance/replication and partitioning of the plasmid. One can therefore be relatively confident that the organism from which the plasmid originates has the ability to maintain vector DNA.

In the phylum Thaumarchaeota, native extrachromosomal self-replicating plasmids are yet to be discovered (Forterre *et al.*, 2014). However, recent reports have identified several MGE such as proviruses, integrative-conjugative elements and cryptic integrated elements in some AOA genomes (Krupovic *et al.*, 2011, 2019). Kim and colleagues (2019) have also isolated a novel group of viruses, the *Nitrosopumilus* spindle-shaped viruses (NSV), that infect marine-dwelling AOA in the genus *Nitrosopumilus*. The discovery of such MGE in Thaumarchaeota is significant as it sheds light on the possible mechanisms of DNA transfer/uptake in these archaea. More importantly, these elements could serve as the basis for developing natural transformation protocols based on transduction or conjugation. However, as these MGE are yet to be studied in detail and characterised, initial efforts to develop DNA uptake protocols for the AOA will likely rely primarily on artificial transformation.

Lastly, the fastidious nature of AOA is a major bottleneck in developing a genetic system. For example, long-term preservation techniques of cultures are currently lacking, and liquid cultures must be continuously maintained. This implies that successful transformants would also need to be continuously maintained indefinitely which may have unknown effects on the introduced mutations. A further major issue often faced during AOA cultivation is that these archaea are often sensitive to chemical contaminations in glassware, solvents and even plastics which complicate their handling. This may also limit the use of antibiotics to those that are water-soluble.

To reiterate, the four main components of a genetic toolbox include the **(i)** the ability to grow the target organism as single colonies on solid growth medium which facilitates mutant screening **(ii)** a selection system (e.g. antibiotics resistance or auxotrophy) **(iii)** a DNA vehicle which allows the transfer of genetic material (e.g. plasmids) and **(iv)** a method to introduce DNA into a host cell (e.g. transformation, transduction, transfection, conjugation) (Leigh *et al.*, 2011; Fink *et al.*, 2021). While these tools are available for archaea from the major phyla, it is evident from the above discussion that none have been established for the AOA. Therefore, if a genetic system is to be developed for the AOA, each of these elements will need to be addressed.

1.6 Aims

A major goal of AOA research is aimed at deciphering the molecular mechanisms underpinning various aspects of their ecophysiology. However, characterisation of these molecular mechanisms at the genetic level is dependent on the availability of a functional genetic system. The lack of such tools for the AOA has therefore created a bottleneck in their study. Therefore, the overall aim of this project is to lay the foundation for the establishment of an AOA genetic system using the soil isolate '*Ca*. N. franklandus C13' as a model strain. In addition to being relatively easy to maintain in culture, the genus *Nitrosocosmicus* is widely distributed and abundant in soil particularly those receiving high ammonia inputs and represents an important model to study.

1.7 Objectives

(i) Determine key factors that preclude the growth of AOA including the model strain '*Ca*. N. franklandus C13' on solid growth medium as single colonies.

(ii) Developing a novel cultivation method(s) to grow AOA on solid medium as single colonies.

(iii) Determine the sensitivity of '*Ca*. N. franklandus C13' to commercial antibiotics to identify an appropriate selection marker(s).

(iv) Construct artificial self-replicating plasmids for use in '*Ca*. N. franklandus C13'.

(v) Explore various artificial transformation methods as a means to induce DNA uptake in '*Ca*. N. franklandus C13'.

Chapter 2: Materials and Methods

2.1 General chemicals

All chemicals used in this study were supplied by Fisher Scientific (Loughborough, UK), Sigma-Aldrich (St. Louis, MO, USA), ThermoScientific (New Jersey, USA) and Honeywell Fluka (Germany) unless otherwise stated. All commercially available antibiotics used in this study were sourced from the following suppliers: puromycin dihydrochloride (Fisher, Leicestershire), hygromycin B (Sigma-Aldrich), novobiocin salt (Sigma-Aldrich), apramycin (Sigma-Aldrich), neomycin sulfate (Fisher Scientific) and ciprofloxacin (Sigma-Aldrich).

2.2 Bacterial strains used in this study

Table 2.1: Bacterial strains used in this study.

Strain	Genotype	Supplier
E. coli DH5α	F- φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(rK-, mk+) phoA supE44	ThermoScientific (New Jersey, USA)

2.3 Routine cultivation of ammonia-oxidising archaea

Batch cultures of '*Ca*. Nitrosocosmicus franklandus C13' and *Nitrososphaera viennensis* EN76 were routinely maintained in fresh-water medium (FWM). FWM consists of a basal salt solution containing NaCl (1 g/l), $MgCl_2.6H_2O$ (0.4 g/l), $CaCl_2.2H_2O$ (0.1 g/l), KH_2PO_4 (0.2 g/l) and KCl (0.5 g/l). The FWM basal salt solution was supplemented with sterile stocks of modified trace element solution (1 ml/l, *Appendix A*), FeNaEDTA (7.5 µM), $NaHCO_3$ (2 mM), vitamin solution (1 ml, *Appendix B*), HEPES buffer (10 mM), and the pH indicator phenol red at a final

concentration of 1.4 µM. The pH was maintained between 7 - 8. In addition, '*Ca*. N. franklandus C13' and *N. viennensis* EN76 cultures were supplied with NH_4Cl at a final concentration of 5 mM and 3 mM respectively. *N. viennensis* EN76 required sodium pyruvate at a final concentration of 0.5 mM. Cultures were incubated at 37ºC in the dark under static conditions. Note: The antibiotic streptomycin was used at a final concentration of 50 µg/ml to prevent the growth of heterotrophic bacteria.

Batch cultures of the marine AOA strain, *Nitrosopumilus maritimus* SCM1, were maintained in synthetic crenarchaeote medium composed of a basal salt solution of NaCl (26 g/l), $MgCl_2.6H_2O$ (5 g/l), $MgSO_4.7H_2O$ (5 g/l), $CaCl_2.2H_2O$ (1.5 g/l) and KBr (0.1 g/l). The basal salt solution was supplemented with modified trace element solution (1ml/l, *Appendix A*), FeNaEDTA (7.5 µM), $NaHCO_3$ (2 mM), NH_4Cl (1 mM), HEPES (10 mM) and KH_2PO_4 (2.9 mM). The pH was maintained between 7 - 8. Cultures were incubated at 28ºC in the dark under static conditions.

Batch cultures of the acid-tolerant AOA strain '*Candidatus* Nitrosotalea sinensis Nd2' were routinely maintained in modified FWM. The basal salt solution consisted of NaCl (1 g/l), $MgCl_2.6H_2O$ (0.4 g/l), $CaCl_2.2H_2O$ (0.1 g/l), KH_2PO_4 (0.2 g/l) and KCl (0.5 g/l). This basal salt solution was further supplemented with sterile stocks of modified trace element solution (1 ml/l, *Appendix A*), FeNaEDTA (7.5 µM), $NaHCO_3$ (4 mM), NH_4Cl (500 µM), 4 ml of MES hydrate (0.5 M, pH 5.3) and 2 ml of 10% (v/v) HCl. The pH was maintained in the range 4.8 - 5.6. Cultures were incubated at 37ºC in the dark under static conditions.

All glassware was acid-washed in 10% (v/v) nitric acid prior to use and cultures were regular monitored for the presence of heterotrophic bacteria by plating on lysogeny broth agar (LB) and R2A agar.

2.4 Nitrite measurements

The growth of the AOA was monitored by measuring total nitrite production in the cultures. Nitrite concentrations were measured using the Greiss colorimetric assay with sulphanilamide and N-(1-naphthyl) ethylenediamide in a 96-well plate format as previously described (Lehtovirta-Morley *et al*., 2016). Absorbances were measured at 540 nm using a VersaMax™ plate reader (Molecular Devices).

2.5 Cell counting

Total archaeal cell counts were determined microscopically using the fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI). A volume of 1 ml of cells was stained with 6 µg/ml of DAPI in the dark for a minimum of 10 min. Note: To prevent clumping, the cells were passed through a 1 ml syringe connected to a 25 gauge needle (4 - 5 times) prior to DAPI staining. The cells were subsequently transferred onto black 0.22 µM (25 mm) polycarbonate filter membranes (Cytiva Whatman™, Cyclopore™) using a vacuum manifold and allowed to dry. Filters were mounted onto a glass slide with 10 µl of AF2 anti-fadent (Citifluor, USA) and viewed using a Zeiss AXIO Scope.A1 microscope (Carl Zeiss, Germany).

2.6 Assessing the effect of solidifying agents on the growth of AOA

Various gelling agents were screened to determine their effect on growth. For the strain '*Ca*. N. franklandus C13', cells were harvested from 500 ml of a mid-late exponential culture (~700 – 1200 µM NO_2^-) onto a 0.2 µM membrane filter (PES, Millipore) using a vacuum filtration manifold. The cells were washed on the membrane with 50 - 100 ml of sterile FWM basal salts. The membrane filter was subsequently transferred into 10 ml of FWM basal salts and briefly vortexed. This cell suspension was serially

diluted (1:10) and 80 – 100 µl of this suspension was used for further experiments (10^{-1}, ~10^6 cells/ml).

For *N. viennensis* EN76 and '*Ca*. N. sinensis Nd2', 100 µl of a mid-late exponential culture (700 – 1000 µM or ~80 µM NO_2^- respectively) was used for the growth experiments.

To assess the effects of 1.5% (w/v) Agar (Formedium, UK), 1.5% (w/v) Bacto-Agar (BD Difco™), 1.4% (w/v) Noble-Agar (BD Difco™), 1.4% (w/v) Agarose (Melford, UK) and 1.7% (w/v) Phytagel™ (Sigma-Aldrich®) on the growth of AOA, a biphasic mixture of the gel and liquid medium was used (*Figure 2.1*). Briefly, to each 30 ml plastic screw-cap vial (Greiner Bio-One), 10 ml of molten gelling agent (containing all medium supplements) was added and allowed to solidify. The solid layer was subsequently overlayed with an equal volume of liquid medium and inoculated as described above. All experiments were performed in triplicate and control experiments were performed in the absence of a gelling agent (i.e. liquid medium only). Vials were incubated in the dark at 37°C under static conditions. Total nitrite production was measured to determine the effects the different gelling agents on growth.

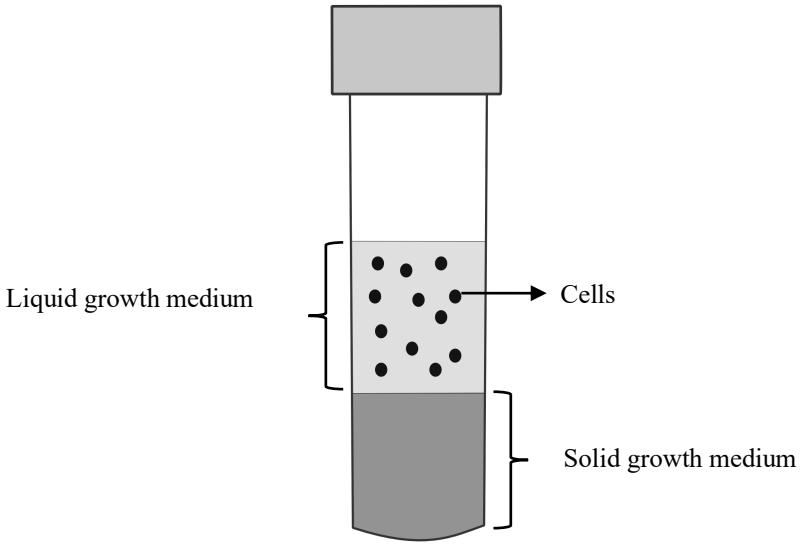


Figure 2.1: Experimental setup used to pre-screen gelling agents. Solid FWM was prepared using a range of gelling agents including Phytagel™, Agarose, Agar, Nobel-Agar and Bacto-Agar. Solid FWM was added to the vial and allowed to set. The solid layer was subsequently overlayed with liquid FWM and inoculated with the strain of choice. Total nitrite production was monitored over time to determine the effect of each gelling agent on growth.

2.7 Cultivation of AOA on solid growth media using the Liquid-Solid method (LS)

'*Ca*. N. franklandus C13' was grown on solid media using the Liquid-Solid method (LS-method). Briefly, the LS-method consists of a solid-phase (Phytagel™) in which the cells are embedded and a liquid-phase in which the gel-embedded cells are submerged. The LS-method involves four main steps **(i)** cell harvesting **(ii)** gel preparation **(iii)** inoculation and **(iv)** maintenance. Experiments were performed in triplicate in acid-washed 100 ml Duran glass bottles. The method was repeated five times and has been validated independently in our laboratory.

2.7.1 Cell preparation

Cells were aseptically harvested from 500 ml of a mid-late exponential culture onto a 0.2 µM pore size Millipore Express® PLUS PES membrane filters (Merck, USA) using a vacuum filtration manifold. The cells were resuspended in a sterile solution of FWM basal salts and diluted using a four-fold (1:10) dilution series.

2.7.2 Gel preparation

Phytagel™ (0.6% (w/v)) was dissolved in FWM basal salts (containing phosphates) and sterilized by autoclaving at 121°C for 15 min (15 psi). All media supplements including streptomycin (50 µg/ml) were added once the molten gel had cooled to ~42°C. A 20 - 25 ml aliquot of the molten Phytagel™ was transferred to each sterile 100 ml Duran bottle and allowed to set. The second Phytagel™ layer was prepared as above but inoculated with the AOA strains of choice as described below. **Note:** A single Phytagel™ layer may also be used.

2.7.3 Inoculation and monitoring

The molten Phytagel™ was inoculated with '*Ca*. N. franklandus C13' using 50 - 80 µl of the diluted cell suspension (i.e. 10^{-1} (~10^{7} cells/ml) or 10^{-4} (~10^{4} cells/ml)). The Phytagel™**:**cell mixture was gently swirled to ensure even distribution of the cells. Once the inoculated Phytagel™ layer had solidified, it was overlayed with an equal volume of liquid FWM medium containing streptomycin (50 µg/ml). The bottles were incubated in the dark at 37°C under static conditions.

Growth of the gel-embedded cells was monitored by measuring nitrite production in the top liquid layer as previously described (see *section 2.4*). In addition, the bottles were also observed for a change in colour from pink to yellow (due to the presence of

the pH indicator phenol red) which is an indicator of media acidification resulting from ammonia oxidation. Once nitrite accumulation had ceased, the cultures were maintained by regularly replacing the top liquid layer with fresh medium. An illustration of the LS-method design is presented in *Figure 2.2.*

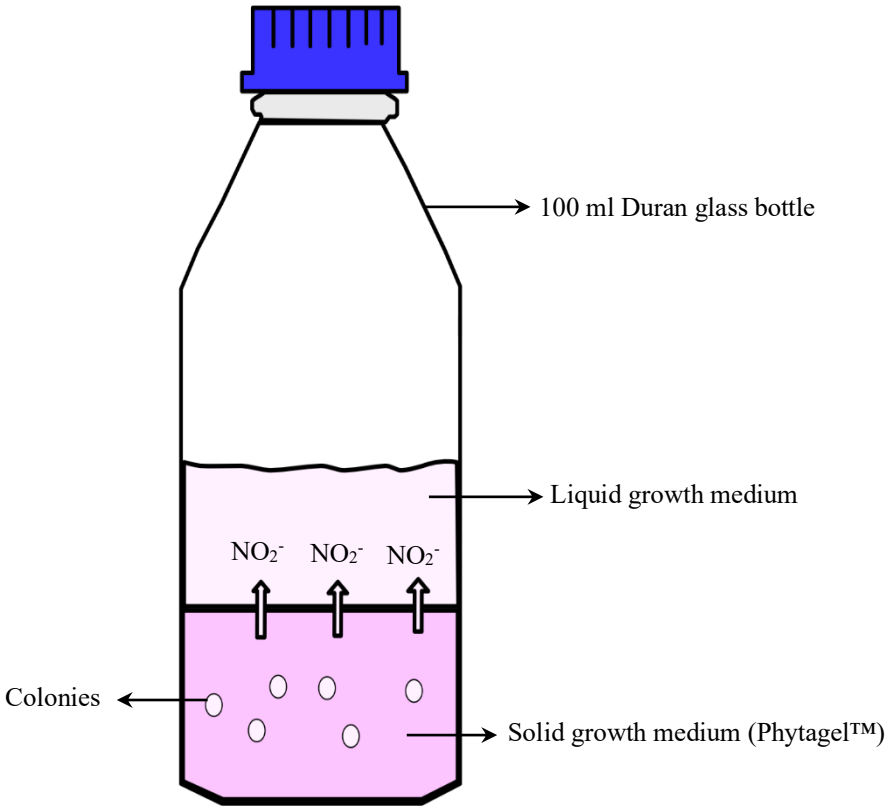


Figure 2.2: Illustration of the Liquid-Solid cultivation method. Cells are immobilised in the gel by inoculation into molten (but cool) Phytagel™. All requirements for growth are present in the solid phase. Once the gel has solidified and the cells are immobilised, a top layer of liquid growth medium equal to the volume of the solid growth medium is added. In theory, the nitrite (NO_2^-) is expected to diffuse into the top liquid layer and is subsequently discarded once the liquid phase is replenished. Regular replenishment of the liquid growth medium minimises the accumulation of NO_2^- which is toxic to AOA.

2.8 Plating efficiency

The plating efficiency for '*Ca*. N. franklandus C13' growing on solid medium was determined as a measure of the number of colonies observed to the number of cells plated i.e. number of colonies counted/ number of cells plated. This ratio was further expressed as a percentage. Cell counts were performed as described previously.

2.9 Fluorescence *in-situ* hybridisation (FISH)

Cells growing within the Phytagel™ were harvested using the tip of a sterile glass Pasteur pipette. Samples from three replicates were pooled totalling ~100 µl of the Phytagel™:cell mixture to which an equal volume of sterile FWM solution was added. Gel-embedded cells were released by vortex mixing for 10 min. Phytagel™ pieces were removed by centrifugation at 2,000 *g* for 5 min. The supernatant was transferred to a fresh tube and cells were harvested at 16,000 *g* for 40 - 60 min. The biomass was resuspended in 20 µl of 1X Phosphate buffer saline (PBS) buffer and fixed with 3 volumes of 4% (v/v) in 1X PBS) formaldehyde (ACROS organics, USA) at room temperature (RT) for 30 min. Fixed cells were harvested at 16,000 *g* for 15 min and washed once with 500 µl 1X PBS buffer. The washed and fixed cells were finally resuspended in 50 - 100 µl of a 50:50 mix of PBS:absolute ethanol and stored at -20°C.

Fixed cells (10 µl) were spotted onto Teflon coated slides (Thermo Scientific, USA) and oven dried at 46°C for 10 min. The samples were dehydrated in an increasing ethanol series of 50% → 80% → 100% for 3 min each. A 35% (v/v) formamide hybridization-buffer was prepared containing: NaCl (0.9 M), Tris (20 mM) and SDS (0.001% (v/v)). To each well, 10 µl of the hybridization-buffer and fluorescent probes were added. Probes specifically targeting the archaeal and bacterial 16S rRNA were

added at a final concentration of 3 ng/µl and 5 ng/µl, respectively. All fluorescent probes used in this study are listed in *Table 2.2*. The fluorescent probes were hybridized overnight in an air-tight humidity chamber at 46°C. Following hybridization, the slides were rinsed in washing-buffer containing NaCl (70 mM), Tris-HCl (20 mM, pH 8) and EDTA (5 mM) for 15 min at 48°C. Finally, the slides were briefly plunged in ice-cold MilliQ water and dried at 46°C for 1 min. Cells were embedded in Vectashield mounting solution containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories Inc., Burlingame, CA, USA). Images were acquired with a Zeiss AXIO Scope.A1 microscope and analysed with the Zen (2011) v. 1.0.0.0 software (Carl Zeiss, Germany).

Table 2.2: Oligonucleotide probes used for fluorescence in-situ hybridisation.

Probe	Target group	Sequence (5' → 3')	References
Arch915	Archaea	GTGCTCCCCCGCCAATTCCT	(Stahl and Amann, 1991)
EUB338 1	Most bacteria	GCTGCC TCC CGTAGGAGT	(Amann *et al.*, 1990)
EUB338 2	Planctomycetales	GCAGCCACCCGTAGGTGT	(Amann *et al.*, 1990)
EUB338 3	Verrucomicrobiales	GCTGCCACCCGTAGGTGT	(Daims *et al.*, 1999)

2.10 Transmission Electron Microscopy (TEM)

Phytagel™ plugs containing gel-embedded '*Ca*. N. franklandus C13' cells were sampled with a wide-bore tip (blue tips) and placed in a sterile solution of FWM. The gel plugs were cut into 1 mm^2 cubes and fixed overnight using a 2.5% (v/v) solution of glutaraldehyde in 0.05 M sodium cacodylate (pH 7.3) at 4°C. A stationary phase liquid-batch culture of '*Ca*. N. franklandus C13' was used as a control. Prior to fixation, the cells were embedded in agarose using glutaraldehyde at a final

concentration of 2.5% (v/v). The cells were pelleted by low-speed centrifugation and most of the supernatant discarded. The cell pellet was mixed with an equal volume of 2% (w/v) low-melting point Agarose at 37°C and briefly plunged in ice. Low-melting point Agarose containing cells was cut into cubes measuring 1 mm^2. These cubes containing concentrated cells were fixed overnight in a solution of 2.5% (v/v) glutaraldehyde in 0.05 M sodium cacodylate (pH 7.3) at 4°C.

The fixed samples were embedded using a Leica EM TP embedding machine (Leica, Milton Keynes, UK). The fixative was washed out with three successive 15 min washes in 0.05 M sodium cacodylate and post-fixed in 1% (w/v) osmium tetroxide (OsO_4) in 0.05 M sodium cacodylate at RT. The cells were washed thrice with distilled water. Ethanol dehydration was performed in an increasing ethanol series (30% → 50% → 70% → 95% and two times in 100%) for 1 hr each. Following dehydration, samples were gradually infiltrated with LR White resin (London Resin Company, Reading, Berkshire) by successive changes of resin:ethanol mixes at RT (1:1 for 1 hr, 2:1 for 1 hr, 3:1 for 1 hr, 100% resin for 1 hr then 100% resin for 16 hrs and a fresh change again for a further 8 hrs). The samples were transferred into gelatin capsules containing fresh LR White and incubated at 60°C for 16 hrs to polymerize.

Ultra-thin sections of approximately 80 nm were cut with a glass knife using a Leica UC7 ultramicrotome (Leica, Milton Keynes, UK). The sections were picked up on 200 mesh copper grids coated with Formvar and carbon film (EM Resolutions, Sheffield, UK). Sections were stained with 2% (w/v) uranyl acetate for 1 hr and 1% (w/v) lead citrate for 1 min and subsequently washed with distilled water and air dried. The grids were viewed in a FEI Talos 200C transmission electron microscope (FEI UK Ltd,

Cambridge, UK) at 200 kV and imaged using a Gatan OneView 4K x 4K digital camera (Gatan, Cambridge, UK) to record DM4.

2.11 Antibiotic sensitivity testing

All antibiotic stocks were dissolved in MilliQ water and filter sterilised using 0.22 µM Millipore Express® PLUS PES membrane filters (Merck, USA).

2.11.1 Rationale

Commercially available antibiotics were selected based on five main criteria **(i)** The antibiotic must have previously exhibited anti-archaeal activity, **(ii)** The antibiotic must have a known resistance marker, **(iii)** the stability of the antibiotics at the growth temperatures of the model strains and **(iv)** the antibiotic must be water-soluble. Additionally, antibiotics that are known to have no activity against the majority of AOA were avoided. A list of antibiotics used in this work are listed in *Table 2.3.*

Table 2.3: List of antibiotics tested in this study.

Antibiotic	Class	Mode of action	Reference
Puromycin	Aminonucleoside	Inhibition of protein synthesis	(Vara *et al.*, 1985) (Pérez-González *et al.*, 1985)
Hygromycin B	Aminoglycoside	Inhibition of protein synthesis	(Borovinskaya *et al.*, 2008) (Gritz and Davies, 1983)
Neomycin	Aminoglycoside	Inhibition of protein synthesis	(Jana and Deb, 2006)
Novobiocin	Aminocoumarin	Topoisomerase II inhibitor (DNA gyrase)	(Vos *et al.*, 2011)
Apramycin	Aminoglycoside	Inhibition of protein synthesis	(Tsai *et al.*, 2013)
Formicamycin J	Formicamycin	Inhibits ourified DNA gyrase *in vitro*	(Matt Hutchings, Personal communication)

Table 2.3: Continued.

Fasamycin E	Fasamycin	Inhibits fatty acid synthase II (FASII) *in vivo* and purified DNA gyrase *in vitro*	(Feng *et al.*, 2012) (Matt Hutchings, Personal communication)
Fasamycin G	Fasamycin	Inhibits FASII *in vivo* and purified DNA gyrase *in vitro*	(Feng *et al.*, 2012) (Matt Hutchings, Personal communication)
Formicamycin I	Formicamycin	Inhibits purified DNA gyrase *in vitro*	(Matt Hutchings, Personal communication)

2.11.2 Whole cell testing

'*Ca*. N. franklandus C13' cells were harvested from 500 ml of an exponentially growing culture (~0.7 - 1.2 mM NO_2^-) onto a 0.2 µM PES membrane filter (Millipore) under vacuum. The cells were washed once with sterile FWM salts and resuspended in 10 ml of FWM salts. This cell suspension was diluted 1:10 of which 100 µl was used to inoculate 5 ml of sterile FWM. Antibiotics were added to the desired concentration and the cultures were incubated at 37°C in the dark under static conditions.

N. maritimus SCM1 was grown to mid-late exponential phase (~200 - 500 µM NO_2^-) and diluted 1:3 in 1x SCM salt solution. Of this diluted culture, a volume of 100 µl was used to inoculate 5 ml of sterile medium. Antibiotics were added to the desired concentration and the *N. maritimus* SCM1 cultures were incubated at 28°C in the dark under static conditions.

N. viennensis EN76 cells were harvested from 400 - 500 ml of an exponentially growing culture by centrifugation at 5000 rpm for 30 - 40 mins. The cells were resuspended in 10 ml of sterile FWM salts and diluted 1:10 of which 80 - 100 µl was

used to inoculate 5 ml of sterile FWM medium. Once the antibiotics had been added to the desired concentration, *N. viennensis* EN76 cultures were incubated at 37°C in the dark under static conditions.

Total nitrite production was measured to determine the sensitivity to the antibiotics. All assays for all three strains were performed in 30 ml plastic universal vials (Bio-GreinerOne).

2.11.3 Fasamycin and formicamycin sensitivity testing

Fasamycin and formicamycin congeners were provided in kind by Matt Hutchings (John Innes Centre, Norwich). All fasamycin and formicamycin stocks were prepared in 100% DMSO. Ciprofloxacin, a known DNA gyrase inhibitor, was used as a positive control and prepared in 0.1 M HCl. These compounds were only tested against '*Ca*. N. franklandus C13' and *N. viennensis* EN76 and the assays were performed as described in *section 2.11.2* above. In addition, all antibiotic treated cultures contained DMSO and HCl at a final concentration of 0.05% (v/v) and 0.05 mM respectively. A list of fasamycin and formicamycin congeners tested in this study is provided in *Table 2.3* above.

2.12 Genetic manipulations

2.12.1 Extraction of genomic DNA from archaeal cells

2.12.1.1 Sample preparation

'*Ca*. N. franklandus C13' cells growing within Phytagel™ were harvested aseptically using a sterile glass Pasteur pipette. Samples from three replicates were pooled (approximately 100 µl) and resuspended in 100 µl of FWM basal salts. Cells growing in liquid cultures were harvested from > 1 ml by centrifugation at 16,000 *g* for 10 - 30 min depending on the cell density.

2.12.1.2 DNA extraction

Cells were resuspended in 500 µl of extraction buffer containing NaCl (8.2 g), 2.5 g ($Na_2.SO_3$), 40 ml 0.5 M Tris-HCl (pH 7.5), 20 ml 0.5 M EDTA (pH 8), 20 ml 10% (w/v) SDS and transferred to 2 ml screwcap Blue matrix Ribolyser tubes (MP Biomedicals, UK). Following the addition of a Phenol:Chloroform:Isoamyl alcohol (PCI, 25:24:1) mixture, samples were disrupted using the FastPrep-24™ 5G (MP Biomedicals, UK) at 4 m/sec for 25 sec. The mixture was centrifuged at 16,000 *g* for 5 min and RT and the aqueous layer transferred to a fresh 1.5 ml tube to which 500 µl of chloroform:Isoamyl alcohol (CI, 24:1) was added and thoroughly mixed. This mixture was centrifuged at 16,000 *g* for 5 min and the aqueous layer transferred to a fresh 1.5 ml tube. Two volumes of 30% (w/v) PEG_{6000}-1.6 M NaCl and 1.5 µl glycogen were added and mixed thoroughly. This mixture was incubated for 2 hrs at RT or overnight at 4°C. DNA was pelleted at 16,000 *g* at RT and washed once with 1 ml of ice-cold 70% ethanol. DNA resuspended in 50 µl of TE buffer (100 mM Tris-HCl (pH 8), 0.1 mM EDTA) and stored at -20°C.

2.12.2 Plasmid DNA extraction

E. coli DH5α cells were harvested from 2 - 5 ml of an overnight culture at 5000 rpm for 4 min at RT. Subsequent plasmid extractions were done using the QiaPrep® Spin Miniprep kit (Qiagen), according to the manufacturer's instructions.

2.12.3 DNA quantification

All DNA quantifications were done using the Qubit™ 2.0 fluorometer using the dsDNA high-sensitivity assay kit (Invitrogen, USA) according to the manufacturer's instructions.

2.12.4 Agarose gel electrophoresis

DNA was visualised and resolved using agarose gel electrophoresis. Agarose gels (0.8% (w/v)) were prepared in 1x TBE buffer (Tris-base (10.8 g/l), Boric acid (5.5 g/l) and EDTA (2 mM)) and stained with 0.6 µg/ml ethidium bromide prior to casting. DNA to be resolved was premixed with 1x loading dye.

2.12.5 Restriction digestion analysis

All restriction endonuclease enzymes (FastDigest) used in this work were purchased from ThermoFisher Scientific (MO, USA). Reactions were performed in a total volume of 20 µl containing: 1x FastDigest Green buffer, 1 µl of each restriction enzyme(s), plasmid DNA (1 µg) and nuclease-free Milli-Q® water.

2.12.6 DNase I digestion

Each DNase I reaction (New England BioLabs, UK) was carried out in a total volume of 101 µl containing 10 µl of DNase I buffer, 1 µl of DNase I and 90 µl of the cell

suspension. This mixture was briefly vortexed (~5 sec) and incubated at 37°C for 10 min. The DNase I enzyme was inactivated at 75°C for 10 min in the presence of 5 mM EDTA.

2.12.7 Primer design

All primers were designed using SnapGene version 6.2.1 (www.snapgene.com) and the NCBI PrimerBlast software (Ye *et al.*, 2012). Primer quality was validated using the OligoAnalyzer software (https://www.idtdna.com/SciTools). Primer stocks were resuspended and stored in TE buffer containing 100 mM Tris-HCl (pH 8) and 0.1 mM EDTA at -20°C.

2.12.8 Polymerase chain reaction (PCR)

All PCR reactions were performed in 25 - 50 µl reactions containing; 0.02 U/µl Q5 High-fidelity DNA polymerase (New England BioLabs, UK), 1x Q5 reaction buffer, 0.4 - 0.5 µM of the forward and reverse primer and 0.2 mM dNTP mix. Thermocycling reactions were performed using an automated thermal cycler (DNA Engine Tetrad® 2, BioRad).

Primers and PCR thermocycling parameters that were used to add homology arms needed for a Gibson assembly are listed in *Table 2.4*. General primers and their PCR thermocycling parameters are listed in *Table 2.5*. Where necessary, PCR generated sequences were analysed using BioEdit sequence alignment editor v.7.2.5 (Hall, 1999) and the NCBI BLAST tool (Altschul *et al.*, 1990).

Table 2.4: Primers and PCR parameters used to add homology arms needed for a Gibson assembly.

Primer pair	Sequence (5' → 3')	Description	Thermocycling parameters	Reference
Primers used for plasmid pfrank-CRISPR and pfrank-CRISPR-amoB				
Cas9-Pac13-overlap-F	tggtgtcgacgcgctgttctctcgtacgct-AGGTGTACTGCCTTCCAGACG	Primers amplify the *Cas9* gene and add homology arms to the adjacent *pac* gene (30 bp) and the pCRISPomyces-2 region spanning the *gapdh* promoter and the apramycin resistance gene (30 bp). **Product size**: 4670 bp (+60 bp)	Initial denaturation: 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 68°C for 30 sec, 72°C for 2:30 sec and a final extension at 72°C for 2 min. Hold at 10°C.	This study
Cas9-lacZ-overlap-R	tgtatcggtttatcagcttgctttcgaggt-CCATCGCCCACGCATAAC			
oriC13-lacZ-overlap-F	gatatgaaaaagcctgaactcaccgcgacg-GGTCGGCCATTCTTTAACAC	Primers amplify the '*Ca*. N. *franklandus* C13' ORB-containing fragment and add homology arms to the pCRISPomyces-2 region spanning *gapdh* promoter and the apramycin resistance gene (30 bp) and the *pac* gene (30 bp). **Product size**: 791 bp (+60 bp)	Initial denaturation: 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 64°C for 30 sec, 72°C for 30 sec and a final extension at 72°C for 2 min. Hold at 10°C.	This study
oricC13-Pac13-overlap-R	gtcagagcgctgtaactggaacgggttcta-GGATTTTCATAGTGGATAGATTGCC			

Table 2.4: Continued.

Pac13-OriC13-overlap-F	tagaaggcaatctatccactatgaaaatcc-TAGAACCCGTTCCAGTTAC	Primers amplify the *pac* gene and add a total of 60 bp homology arms to the ORB-containing fragment and the *Cas9* gene.	Initial denaturation: 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 62°C for 15 sec, 72°C for 40 sec and a final extension of 2 mins. Hold at 10°C.	This study
Pac13-Cas9-overlap-R	ctcttcgttcgtctggaaggcagtacacct-AGCGTACGAGAGAACAGC	**Product size:** 1057 bp (+60 bp)		
LacZApra-Cas9-overlap-F	tatatcggttatgcgtgggcgatgg-ACCTCGAAAGCAAGCTGATAAAC	Primers amplify the region of the pCRISPomyces-2 plasmid spanning the *gapdh* promoter to the apramycin resistance gene and adds a total of 50 bp homology arms to *Cas9* and the ORB-containing fragment.	Initial denaturation: 98°C for 30 sec, 30 cycles 98°C for 10 sec, 66°C for 30 sec, 72°C for 1:30 sec and a final extension at 72°C for 2 min. Hold at 10°C.	This study
LacZApra-OriC-overlap-R	ctgttgtgttaaagaatggccgacc-CGTCGCGGTGAGTTCAGG	**Product size:** 2825 bp (+50 bp)		
amoB-Cas9-overlap-F	tatatcggttatgcgtgggcgatgg-ACGTGATATCTGAAGATTTATAATG	Primers amplify the *amoB* promoter from '*Ca*. N. franklandus C13' and add homology arms to the *Cas9* gene (25 bp) and the pCRISPomyces-2 region spanning the *gapdh* promoter and the apramycin resistance gene (26 bp).	Initial denaturation: 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 58°C for 10 sec, 72°C for 25 sec and a final extension at 72°C for 2 mins. Hold at 10°C.	This study
amoB-lacZ-overlap-R	ctgtagcggctgagaagacttgcgta-CTAGGGAATCCACTATTTACATAG	**Product size:** 330 bp (+51 bp)		

Table 2.4: Continued.

LacZ-amoB-overlap-F	tctatgtaaatagtggattccctag-TACGCAAGTCTTCTCAGCCGC	Primers are used to linearize the pfrank-CRISPR vector and add homology arms to the *amoB* promoter sequence (53 bp).	Initial denaturation: 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 68°C for 30 sec, 72°C for 4:30 sec, and a final extension at 72°C for 2 mins. Hold at 10°C.	This study
Cas9-amoB-overlap-R	gaacattataaatcttcagatatcacgt-CCATCGCCCACGCATAAC	**Product size:** 9004 bp (+53 bp)		
Primers used for pfrank-mCherry-Cdc-orb				
LacZApra-mCherryIDT-overlap-F	ttatgcgtgggcgatggtac-ACCTCGAAAGCAAGCTGATAAAC	Primers amplify the pCRISPomyces-2 region spanning *gapdh* promoter and the apramycin resistance gene adding homology arms to the *mCherryIDT* gene (20 bp) and the *Cdc* gene and ORB-containing fragment (25 bp).	Initial denaturation: 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 66°C for 30 sec, 72°C 1:30 sec, and 72°C for 2 min. Hold at 10°C.	This study
LacZApra-cdc-ori-overlap-R	aattgtgatcgattgttctgatcga-CGTCGCGGTGAGTTCAGG	**Product size:** 2825 bp (+45 bp)		
Pac-ori-Cdc-overlap-F	atctttagaaggcaatctatccact-TAGAACCCGTTCCAGTTAC	Primers amplify the *pac* gene and add homology arms to the *mCherryIDT* gene (25 bp) and *Cdc* gene (25 bp).	Initial denaturation: 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 62°C for 15 sec, 72°C 40 sec, and 72°C for 2 min. Hold at 10°C.	This study
Pac-mCherryIDT-overlap-R	tcgtctggaaggcagtacaccttac-AGCGTACGAGAGAACAGC	**Product size:** 1057 bp (+50 bp)		

Table 2.4: Continued.

mCherryIDT-Pac-overlap-F mCherryIDT-LacZapra-overlap-R	cgcgctgttctctcgtacgct-GTAAGGTGTACTGCCTTCCAGA cggtttatcagcttgctttcgaggt-GTACCATCGCCCACGCATAA	Primers amplify the *mCherryIDT* gene adding homology arms to the *pac* gene (21 bp) and the pCRISPomyces-2 region spanning the *gapdh* promoter and apramycin resistance gene (25 bp). **Product size:** 1280 bp (+46 bp)	Initial denaturation: 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 67°C for 15 sec, 72°C 50 sec, and 72°C for 2 min. Hold at 10°C.	This study
oric-Cdc-LacZApra-overlap-F oric-Cdc-Pac-overlap-R	gaaaaagcctgaactcaccgcgacg-TCGATCAGAACAATCGATCACAA gcgctgtaactggaacgggttcta-AGTGGATAGATTGCCTTCTAAAGAT	Primers amplify the *Cdc* gene and the adjacent ORB-containing region of '*Ca*. N. franklandus C13' adding homology arms to the pCRISPomyces-2 region spanning *gapdh* promoter and the apramycin resistance gene (25 bp) and *pac* gene (24 bp). **Product size:** 1863 bp (+49 bp)	Initial denaturation: 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 64°C for 15 sec, 72°C 1 min and 72°C for 2 min. Hold at 10°C.	This study

Table 2.4: Continued.

Primers used for plasmid pfrank-mCherry-orb				
LacZApra-mCherryIDT-overlap-F LacZ-oricC13-overlap-R	ttatgcgtgggcgatggtac-ACCTCGAAAGCAAGCTGATAAAC ctgttgtgttaaagaatggccgacc-CGTCGCGGTGAGTTCAGG	Primers amplify the pCRISPomyces-2 region spanning *gapdh* promoter and apramycin resistance gene adding homology arms to the *mCherryIDT* gene (20 bp) and the adjacent ORB-containing sequence (25 bp). **Product size:** 2825 bp (+45 bp)	Initial denaturation: 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 66°C for 30 sec, 72°C 1:30 sec and a final extension of 72°C for 2 min. Hold at 10°C.	This study
OricC13-LacZ-overlap_F OricC13-pacC13-overlap-R	gatatgaaaaagcctgaactcaccgcgacg-GGTCGGCCATTCTTTAACAC gtcagagcgctgtaactggaacgggttcta-GGATTTTCATAGTGGATAGATTGCC	Amplify the predicted replication origin of '*Ca.* N. franklandus C13' and add homology arms to the pCRISPomyces-2 region spanning *gapdh* promoter and apramycin resistance gene (30 bp) and the *pac* gene (30 bp). **Product size:** 791 bp (+60 bp)	Initial denaturation: 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 64°C for 30 sec, 72°C 30 sec and a final extension of 72°C for 2 min. Hold at 10°C.	This study

Table 2.4: Continued.

PacC13-oricC13-overlap-Forward	tagaaggcaatctatccactatgaaaatcc-TAGAACCCGTTCCAGTTAC	Amplify the *pac* gene and add homology to the replication origin (30 bp) and the *mCherryIDT* gene (25 bp). **Product size:** 1057 bp (+55 bp)	Initial denaturation: 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 62°C for 15 sec, 72°C 40 sec and a final extension of 72°C for 2 min. Hold at 10°C.	This study
Pac-mCherryIDT-overlap-R	tcgtctggaaggcagtacaccttac-AGCGTACGAGAGAACAGC			
mCherryIDT-Pac-overlap-F	cgcgctgttctctcgtacgct-GTAAGGTGTACTGCCTTCCAGA	Amplify the *mCherryIDT* gene adding homology to the *Pac* gene (21 bp) and the pCRISPomyces-2 region spanning the *gapdh* promoter and apramycin resistance gene (25 bp). **Product size:** 1280 bp (+46)	Initial denaturation: 98°C for 30 sec, 30 cycles of 98°C for 10 sec, 67°C for 15 sec, 72°C 50 sec and a final extension of 72°C for 2 min. Hold at 10°C.	This study
mCherryIDT-LacZApra-overlap-R	cggtttatcagcttgctttcgaggt-GTACCATCGCCCACGCATAA			

Note: Primer sequences presented in lower case represent the sequences of the homology arms. In contrast, sequences presented in upper case represent the sequences that are specific to the desired fragment being amplified.

Table 2.5: General primers and PCR thermocycling parameters used in this study.

Primer pair	Sequence (5' → 3')	Description	Thermocycling parameters	Reference
771F 957R	ACGGTGAGGGATGAAAGCT CGGCGTTGACTCCAATTG	Primers are used to amplify the 16S rRNA gene for ammonia-oxidising archaea (AOA). **Product size:** 200 bp	95°C for 5 min, 30 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec and a final extension at 72°C for 10 min	(Ochsenreiter *et al.*, 2003)
A109F 1492R	ACKGCTCAGTAACACGT GGTTACCTTGTTACGACTT	Primers are used to amplify the archaeal 16S rRNA gene. **Product size:** 1383 bp	95°C for 5 mins, 30 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1:30 sec, 72°C for 6 min.	(Grosskopf, *et al*., 1998)
ureC1F ureC1R	AAGGAGCTGGTGGAGGTCAC AATGCGACGTCCTCTGCAAC	Primers are used to amplify the *'Ca. N. franklandus* C13'-specific urease alpha subunit (*ureC*) gene. **Product size:** 175 bp	98°C for 30 sec, 25 cycles of 98°C for 10 sec, 56°C for 30 sec, 72°C 20 sec and a final extension 72°C 2 min.	This study
27F 1492R	AGAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT	Primers are used to amplify the bacterial 16S rRNA gene. **Product size:** ~1400 bp	95°C for 3 min, 30 cycles of 95°C for 30 sec, 55°C for 45 sec, 72°C for 1:20 sec and a final extension of 72°C 5 min.	(Lane, 1991)

2.12.9 General rationale for plasmid design

All three plasmids constructed in this study were designed to replicate in *E. coli*. To facilitate replication and selection in *E. coli*, a fragment from the plasmid pCRISPomyces-2 (Cobb *et al*., 2015) containing the ColE1 origin and the apramycin resistance gene was inserted into all plasmids used in this study (see *Figure 2.3*). All three plasmids contain the puromycin resistance gene (*pac*) to facilitate puromycin selection in '*Ca*. N. franklandus C13'.

With regards to propagation in the AOA host, all three plasmids were placed under the control of the '*Ca*. N franklandus C13' chromosomal replication origin. The location of this replication origin was mapped using the web-based tool 'Ori-Finder 2' freely available at (http://tubic.tju.edu.cn/Ori-Finder2/) (Luo *et al*., 2014). The following settings were used for the analysis; motif: Common, the count of ORB motif in oriC: 3 and a FIMO P-value of 1E-04.

2.12.10 Custom gene synthesis

The *Streptococcus pyogenes Cas9* gene (*spCas9*) and the puromycin *N-acetyltransferase* gene (*pac*) were codon-optimised to reflect the codon-usage of '*Ca*. N. franklandus C13' using SnapGene software version 6.2.1. The codon-optimised *spCas9* gene fragment was placed under the control of both the 5' and 3' untranslated region (UTR) of the *amo*A gene. In comparison, the *pac* gene was placed under the control of the 5'UTR of the thermosome subunit B (*ths*B) gene and 3'UTR of urease alpha subunit (*ure*C) gene. Following *in silico* design, the DNA fragments were synthesised using the IDT custom gene synthesis service (Integrated DNA Technologies, USA). All custom genes were cloned into a pUCIDT-kanamycin vector flanked by *Sna*BI restriction endonuclease sites.

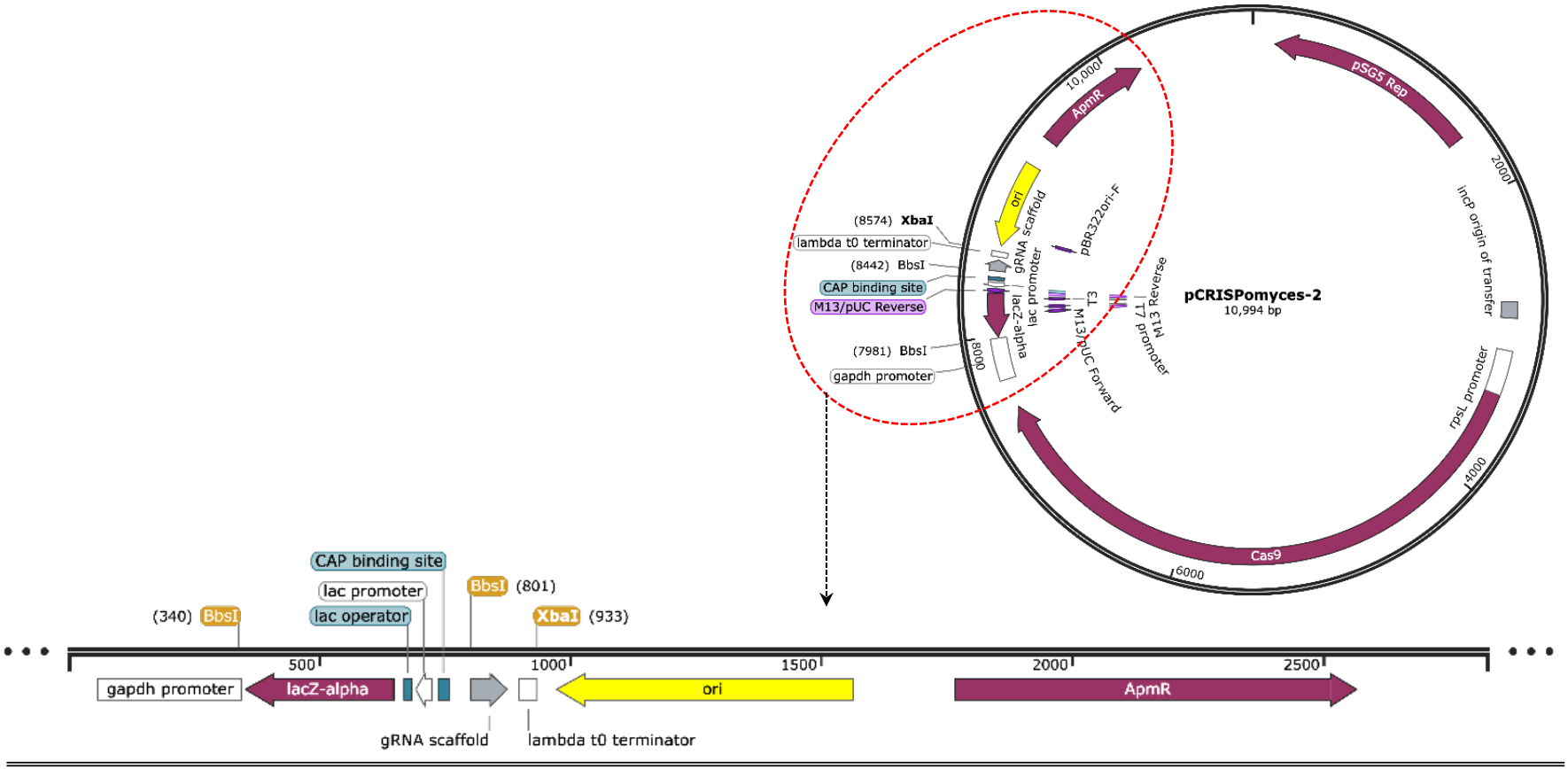


Figure 2.3: Elements of the CRISPR-Cas9-based plasmid, pCRISPomyces-2, used to construct the *E. coli-Ca*. N. franklandus C13 shuttle vectors. The region spanning the *gapdh* promoter and the apramycin resistance gene (*Apm*R) circled by a red dotted line was incorporated into each shuttle vector used in this work. This region contains the ColE1 origin of replication and the apramycin resistance gene (*Apm*R) necessary for propagation and selection in *E. coli* respectively. In addition, this fragment contains two *Bbs*I restriction sites flanking the *lacZ* gene which are used to insert a custom spacer sequence adjacent to the gRNA scaffold via golden gate cloning. The *Xba*I site facilitates insertion of a homology-repair template.

2.12.11 Gibson assembly

Gibson assembly (Gibson *et al.*, 2009) was used to assemble the desired DNA fragments into circular plasmid constructs. Sequence overhangs complementary to the adjacent fragments were added by PCR (refer to *Table 2.4*). The length of the overhangs was dependent on the number of fragments being assembled (i.e. 2 - 3 fragments: 15 - 20 bp, 4 - 6 fragments: 20 - 30 bp). All fragments to be assembled were purified prior to assembly using the High Pure PCR Template preparation Kit (Roche, USA).

Once purified, fragments were assembled using the NEBuilder HiFi DNA assembly kit (New England BioLabs, UK) according to the manufacturer's instructions. Briefly, all NEBuilder HiFi DNA assembly reactions contained: 1x NEBuilder HiFi DNA assembly master mix, recommended pmol amounts of desired DNA fragments (see below) and nuclease-free Milli-Q® water to a final volume of 20 µl. This reaction mixture was incubated at 50ºC for 60 min in an automated thermocycler (DNA Engine Tetrad® 2, BioRad). A vector:insert ratio of 1:1 was used for plasmids pfrank-mCherry-Cdc-orb and pfrank-mCherry-orb with a total of 0.2 pmol DNA. In contrast, for plasmid pfrank-CRISPR, a vector:insert ratio of 1:9 was used with a total of 0.2 pmol DNA. Following assembly, 1 - 2 µl of the assembly mixture was used to transform *E. coli* DH5α cells (refer to *section 2.12.13*).

2.12.12 Preparation of electrocompetent cells

A colony of *E. coli* DH5α was transferred to 10 ml of LB medium and incubated overnight at 37ºC shaking at 200 rpm. From the overnight culture, 4 ml was used to inoculate 400 ml of LB and incubated at 37ºC shaking at 200 rpm. The cells were grown to an OD_{600} between 0.35 - 0.4 and immediately placed on ice for 30 - 40 mins.

To ensure even cooling, flasks were regularly swirled. Once cooled, the cells were washed 5 - 6 times with sterile ice-cold Milli-Q® water (7000 *g*, 5 min at 4ºC). The washed cells were resuspended in 40 ml of sterile ice-cold 10% (v/v) glycerol and centrifuged at 10,000 *g* for 20 min at 4ºC. The cells were subsequently resuspended in 2 ml ice-cold 10% (v/v) glycerol and 70 µl transferred to fresh 1.5 ml tubes (pre-chilled). These aliquots were snap frozen in liquid nitrogen and stored at -80ºC.

2.12.13 Transformation of electrocompetent cells

Electrocompetent *E. coli* DH5α cells (70 µl) were allowed to thaw on ice. Plasmid DNA (≤ 10 ng) in a volume of 1 - 3 µl or the Gibson reaction mix (≤ 2 µl) was added just before the cells were completely thawed and gently mixed using a pipette tip. The cells and DNA were incubated on ice for 1 min and transferred to a pre-chilled 0.1 cm electroporation cuvette (BioRad, USA). The cells were electroporated using the Gene Pulser Xcell™ electroporation system (BioRad, USA) using the following settings: 1.8 kV/cm, 25 µF and a resistance of 200 ohms (Ω). The cells were immediately transferred to 930 µl of SOC medium and incubated at 37ºC for 1 hr shaking at 200 rpm. Following recovery, the cells were spread onto LB agar plates containing 50 µg/ml apramycin or 30 µg/ml kanamycin, 80 µg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-gal) and 100 µM Isopropyl β-d-1-thiogalactopyranoside (IPTG) and incubated overnight at 37ºC.

2.13 Transformation of '*Ca*. N. franklandus C13'

2.13.1 $CaCl_2$ heat-shock treatment

Cells grown at 42ºC were harvested from 500 ml of an exponential culture onto a 0.2 µm pore size membrane filter (PES, Millipore) using a vacuum filtration manifold. The cells were washed three times with ice-cold transformation buffer. The transformation buffer was modified from (Sato *et al*., 2003) containing NaCl (1 g/l), $MgCl_2.6H_2O$ (0.4 g/l), $CaCl_2.2H_2O$ (11.8 g/l) and KCl (0.5 g/l). The cells were resuspended in 6 ml of chilled transformation buffer and centrifuged at 16,500 *g* for 10 min at 4°C. Washed cells were pooled in a final volume of 2 ml of transformation buffer and 50 µl aliquots were transferred to sterile 1.5 ml tubes on ice. Varying amounts of plasmid DNA were subsequently added (250 ng, 500 ng, 2000 ng) in a final volume of 2 - 4 µl. The cell and plasmid mixtures were incubated on ice for 1 hr and heat-shocked for 45 sec at either 45°C, 55°C, 65°C or 80°C. Following heat shock, cells were kept on ice for ≥ 10 min and transferred to 10 ml of FWM and incubated at 42°C in the dark (static). Following a recovery period of 2 - 3 days, puromycin at a final concentration of 200 µM was added. Nitrite production was continuously monitored as previously described.

2.13.2 Polyethylene glycol-mediated transformation (PEG)

The PEG-mediated transformation protocol used in this study was adapted from the protocols for haloarchaeal genetics© handbook version 7.2 (Dyall-Smith, 2009). Briefly, cells grown at 42ºC were harvested from 600 ml of a mid-late exponential culture and resuspended in 6 ml of buffered spheroplasting buffer containing NaCl (17 mM), KCl (6.7 mM), Tris-HCl (50 mM, pH 8.2) and sucrose 1.4% (w/v). This cell

suspension was centrifuged at 16,500 *g* for 10 min at RT. Once pelleted, the cells were resuspended in 2.5 ml of spheroplasting buffer and 100 µl aliquoted into fresh 1.5 ml tubes. Total cell counts in each 100 µl aliquot was ~2.3 x 10^7 cells. The cells were mixed with 1 µg of pfrank-mCherry-Cdc-orb and incubated for 15 min at RT. Following incubation, an equal volume of PEG was added and briefly vortexed for 5 - 10 sec. **Note**: PEG_{600} and PEG_{6000} were used at concentrations of 35% and 60%. PEG-treated cells were incubated at RT for 40 min. Once treated with PEG, the cells were washed once with 500 µl of buffered spheroplasting buffer at 17,000 *g* for 15 mins (RT). PEG-treated cells were subsequently transferred to 10 ml FWM and incubated statically at 37°C. Puromycin at a final concentration of 200 µM was added after 68 hrs. Nitrite production was continuously monitored.

2.13.3 Electroporation

2.13.3.1 Pre-screening of different field strengths (exponential-decay)

'*Ca*. N franklandus C13' cells grown at 42ºC, were harvested from 500 ml of an exponentially growing culture onto a 0.2 µm pore size membrane filter (PES, Millipore) using a vacuum filtration manifold. The harvested cells were washed on the membrane three times with chilled 75 ml of electroporation buffer (20 mM sucrose). The cells were subsequently resuspended in 10 ml of chilled electroporation buffer and harvested at 16,500 *g* for 5 min at 4ºC. The cells were subsequently pooled into a single 1.5 ml tubes and washed four times with 1 ml of electroporation buffer at 16,500 *g* for 5 min. A volume of 50 µl of cells was transferred to a chilled 1 mm electroporation cuvette (Bio-Rad, USA). Electroporation was done using the Gene Pulser Xcell™ system (Bio-Rad, USA) in the absence of any plasmid DNA. The cells were pulsed at a field strength of either 20, 24 or 28 kV/cm (1000 ohms and 25 µF). Field strengths

were calculated using the formular voltage/cuvette gap-width. Following electroporation, they were transferred to 10 ml of FWM in plastic vials (Greiner Bio-one) and incubated at 42ºC (static). Nitrite production was monitored as described previously.

2.13.3.2 Response of '*Ca*. N. franklandus C13' to square wave pulses

'*Ca*. N franklandus C13' cells grown at 37ºC, were harvested from 500 ml of an exponentially growing culture onto a 0.2 µm pore size membrane filter (PES, Millipore) using a vacuum filtration manifold. The cells were washed on the membrane using 35 ml of chilled electroporation buffer. The cells were resuspended in 10 ml of chilled electroporation buffer and harvested at 16,500 *g* for 5 min at 4ºC. The cells were subsequently pooled into a single 1.5 ml tube and washed four times with 1 ml of electroporation buffer (chilled) at 16,500 *g* for 5 min and finally resuspended into 1 ml of electroporation buffer on ice. A volume of 50 µl of cells was transferred to a pre-chilled 1 mm electroporation cuvette (Bio-Rad, USA). Using square wave pulse settings, electroporation was done using the Gene Pulser Xcell™ system (Bio-Rad, USA) in the absence of any plasmid DNA. Immediately following electroporation, the cells were transferred to 10 ml FWM in plastic vials and incubated at 37ºC (static). Nitrite production was regularly measured as previously described. The square wave electrical parameters used are summarised in *Table 2.6*.

Table 2.6: Electrical parameters used to determine the response of '*Ca*. N. franklandus C13' to square wave pulses.

Parameters	Parameters 1 (P1)	Parameters 2 (P2)	Parameters 3 (P3)
Voltage (V)	2400	2400	2400
Pulse length (ms)	1	2	5
No. of pulses	2	2	2
Pulse interval (sec)	5	5	5

2.13.4 Electroporation of '*Ca*. N. franklandus C13' with plasmid DNA

'*Ca*. N franklandus C13' cells grown at 42ºC, were harvested from 500 ml of an exponentially growing culture onto a 0.2 µm pore size membrane filter (PES, Millipore) using a vacuum filtration manifold and washed thrice with 35 ml electroporation buffer. The cells were resuspended in 6 ml of electroporation buffer and harvested at 16,500 *g* for 10 min at 4°C and split into two 1 ml aliquots. The cells from one of these aliquots was treated with 20 µM hexadecyltrimethylammonium bromide (CTAB) for 30 min on ice. Cells from both treatment groups (i.e. CTAB vs No CTAB) were washed four times with ice-cold electroporation buffer at 16,500 *g* for 5 min at 4°C. To fresh 1.5 ml tubes, 50 µl of cells and plasmid DNA (10 ng or 50 ng) were added and incubated on ice for ≥ 2 min. '*Ca*. N franklandus C13' cells were pulsed at 25 kV/cm, 25 µF and 1000 Ω. Total cell counts ranged from 7 x 10^5 (CTAB treated) to 5 x 10^6 (untreated) cells/50 µl. Following electroporation, the cells were immediately transferred to 10 ml FWM and incubated at 42°C (static). After a 48-hr recovery period, puromycin at a final concentration 200 µM was added and nitrite

production was continuously monitored. The use of the cationic detergent CTAB to permeabilise '*Ca*. N franklandus C13' cells was based on the method reported by Rajagopal and colleagues (2014).

2.13.5 PCR screening of transformed cultures

Cells from 2 ml of selected cultures were harvested by centrifugation at 17,000 *g* for 30 min. The cells were resuspended in 90 µl of nuclease-free Milli-Q® water and carefully transferred to a fresh 1.5 ml tube ensuring minimal contact with the walls of the tube. The cell suspension was subsequently treated with DNase I (refer to *section 2.12.6)* to degrade any extracellular plasmid DNA. The DNase I treated cells were transferred to 2 ml lysing matrix E tubes (MP Biomedicals, USA) again ensuring minimal contact with the walls of the tube. Total DNA was subsequently extracted using the previously described phenol/chloroform method (see *section 2.12.1.2*).

PCR was performed as previously described using the primer pair mCherryIDT-Pac-overlap-F/mCherryIDT-LacZapra-overlap-R (refer to *Table 2.4*) targeting the plasmid-specific *mCherry* gene.

2.14 Prediction of restriction-modification (R-M) systems in '*Ca*. N. franklandus C13'

Components of the R-M system in '*Ca*. N. franklandus C13' including restriction endonucleases and methyltransferases were predicted using the publicly available REBASE database (Roberts *et al.*, 2003, 2010). A BLASTp analysis to identify homologs and predict the possible recognition sequences was done using the 'closest neighbour' option.

Chapter 3: Cultivation of ammonia-oxidising archaea on solid medium

3.1 Introduction

Ammonia-oxidising archaea (AOA) are an important group of microorganisms affiliated with the phylum Thermoproteota (NCBI phylum: Thaumarchaeota) (Brochier-Armanet *et al.*, 2008; Rinke *et al.*, 2021). AOA are involved in the oxidation of ammonia to nitrite, the first and rate-limiting step of nitrification. The role of AOA in nitrification coupled with their ubiquitous distribution in both marine and terrestrial ecosystems, highlights their pivotal role in biogeochemical cycling of nitrogen (de la Torre *et al.*, 2008; Wang *et al.*, 2017; Alves *et al.*, 2018; Beeckman *et al*., 2018). While nitrification is vital for nitrogen cycling, it is also associated with nitrogen fertilizer loss and leaching of nitrites and nitrates into aquatic systems leading to eutrophication (Raun and Johnson, 1999). Thus, there is an urgent need to further explore AOA and the molecular mechanisms underpinning their physiology.

Since the discovery and isolation of the first AOA strain *Nitrosopumilus maritimus* SCM1 in 2005 (Könneke *et al.*, 2005) our understanding of AOA has steadily expanded through culture-dependent and culture-independent approaches. Nonetheless, large gaps in our knowledge of the biochemistry, physiology and niche adaptation of AOA still exist due to limitations to the currently available methods.

Culture-independent methods, which mainly comprise of 'OMICS'-based approaches, provide valuable insights into the possible functional roles of AOA in the environment. However, the high percentage of archaeal genes of unknown function and the lack of comprehensive nucleic acid and protein databases, limit the ability to decipher molecular pathways in AOA (Makarova *et al*., 2019). This is exemplified by current efforts to elucidate the archaeal ammonia oxidation pathway which, based on genomic

and biochemical studies, differs considerably from that of ammonia oxidising bacteria (Lehtovirta-Morley, 2018).

In many microorganisms, gene function is investigated with knock-out/knock-in genetic experiments. However, this approach is currently not feasible for AOA because a genetic system has not yet been developed for this group of microbes. This is a major stumbling block in exploring and validating the hypotheses generated from metagenomics, proteomics and transcriptomics data.

A major limiting factor in the development of a genetic system for AOA is the inability to routinely grow them on solid growth medium as single colonies (Leigh *et al.*, 2011; Kohler and Metcalf, 2012). These single colonies represent a clonal population of cells and therefore enable the precise analysis of specific genotypes. Currently, AOA are exclusively grown in liquid cultures and reports on their cultivation on solid growth medium are limited to a single publication by Chu and colleagues (2015) who demonstrated the growth of *Nitrosopumilus* sp. AR in low-melting point agarose. However, the growth observed in that study was a continuous mass of cells and no distinct colonies were evident.

While the reasons for the limited success in growing AOA on solid medium are currently unclear, the role of solidifying agents on microbial growth is often overlooked (Janssen *et al.*, 2002; Tamaki *et al.*, 2009). For instance, Tanaka and colleagues (2014) demonstrated agar to be inhibitory for microbial growth due to the production of hydrogen peroxide when agar was autoclaved together with phosphates. The inhibition of colony formation of the chemolithoautotroph *Thiobacillus ferrooxidans* on agar has also been previously reported and is attributed to the release of free sugars (i.e., D-galactose) from agar at low pH (Tuovinen and Kelly, 1973).

Unsurprisingly, microbiologists have explored the use of alternative solidifying agents such as gellan gum with promising results. For example, novel and previously uncultured microorganisms of the phylum Verrucomicrobia were isolated when using gellan gum as the gelling agent (Tamaki *et al.*, 2009). Tamaki and colleagues (2005) also found that using gellan gum as the solidifying agent increased the diversity of bacterial isolates from lake sediments. It is therefore evident that the choice of solidifying agents is an important consideration for successful cultivation of microbes on solid growth medium.

It is also important to note that during ammonia oxidation, the release of two protons results in media acidification shifting the $NO_2^- \leftrightarrow HNO_2$ equilibrium towards nitrous acid (HNO_2) which is toxic to the AOA (Herbold *et al*., 2017). In addition, a decrease in pH reduces the substrate availability due to ionization of ammonia (NH_3) into ammonium ($NH4^+$) (Picone *et al*., 2021). Unlike liquid cultures (e.g. fed-batch or continuous) for whom nutrients can easily be replenished thus minimising these toxic effects, solid growth medium may be challenging to amend. Therefore, due to the slow growth rates of AOA, these archaea may be rapidly inhibited by these conditions when grown on solid medium before colonies can develop.

3.2 Chapter aims

The inability to grow AOA on solid medium as single colonies is an important limiting factor towards the establishment of a genetic toolbox. In this chapter, the main goal is to develop a novel method for the cultivation of AOA on solid growth medium as single colonies. It was hypothesised that the choice of gelling agent is a key factor to consider when growing AOA on solid growth medium.

3.3 Results

3.3.1 Effects of different solidifying agents on AOA growth

Three AOA strains were grown in the presence of different solidifying agents to determine their effect on growth. In the presence of Phytagel™, *'Ca.* N. franklandus C13' grew at comparable rates to the control cultures with growth rates of 0.47 d^{-1} and 0.40 d^{-1} respectively (*Figure 3.1A*). In comparison, agarose resulted in a mild decrease in the growth rate of *'Ca.* N. franklandus C13' cultures (0.22 d^{-1}) (*Figure 3.1A*). These observations were in sharp contrast to agar, Bacto-agar and Noble-agar that all severely inhibited nitrite accumulation in *'Ca.* N. franklandus C13' cultures (*Figure 3.1B* and *Figure 3.1C*).

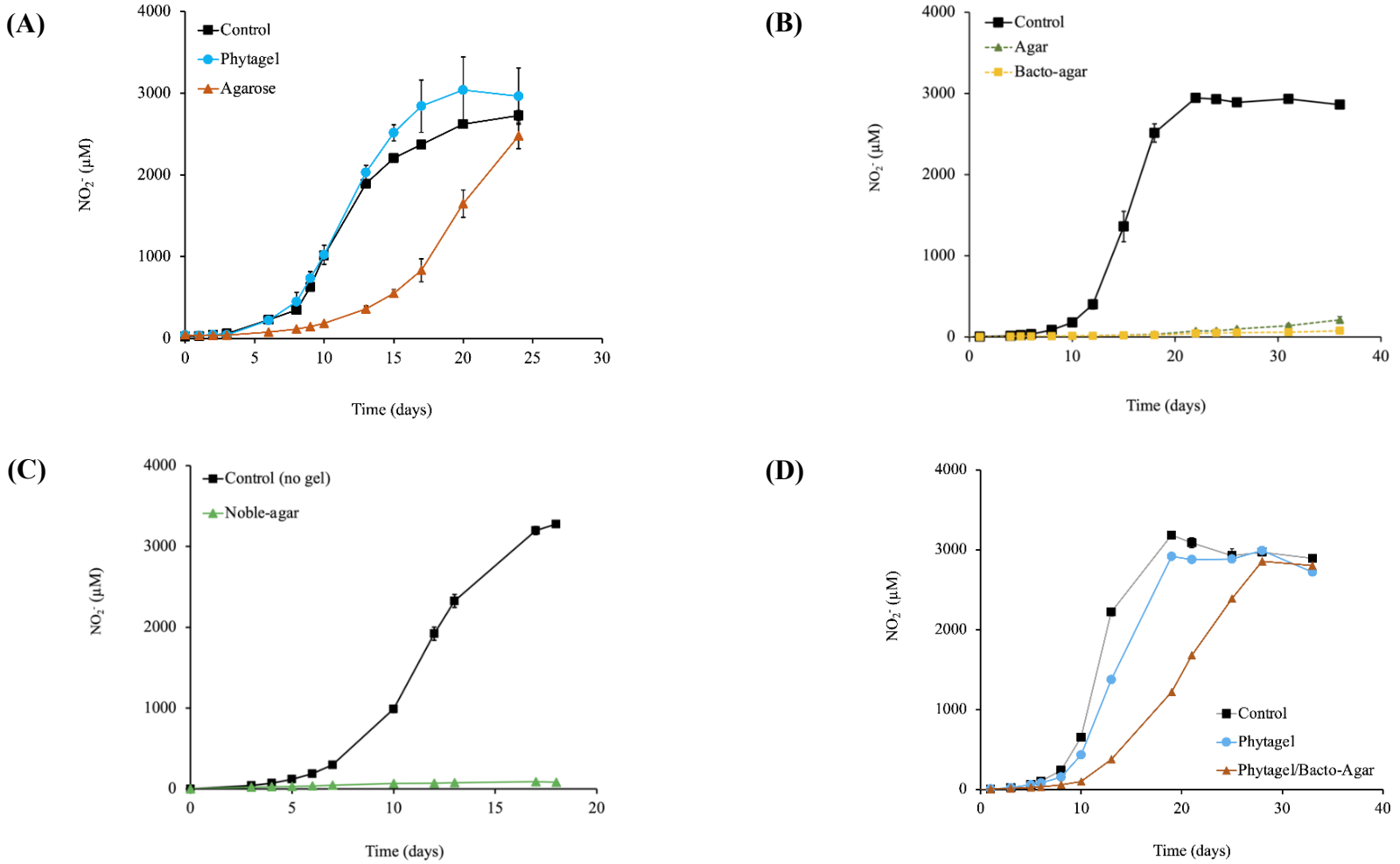


Figure 3.1: Effects of different solidifying agents on the growth of '*Ca*. N. franklandus C13'. **(A)** Phytagel™ and agarose **B)** agar and Bacto-agar **(C)** Noble-agar and **(D)** Phytagel™ and Bacto-agar (50:50) mixture. The control cultures were grown in the absence of any solidifying agents. Nitrite concentrations plotted represent the average of three replicate cultures. Error bars are standard errors of the means and may be smaller than the size of the symbol.

To further validate the toxic effects of Bacto-agar, a blended mixture (50:50) of Phytagel™**:**Bacto-agar was prepared. In this gel mixture, the growth rates of the *'Ca.* N. franklandus C13*'* cultures declined to 0.34 d^{-1} compared to 0.45 d^{-1} in pure Phytagel™ (*Figure 3.1D*). This strongly suggested the presence of a toxic component in the Bacto-agar that negatively affects growth.

The effects of Phytagel™ and Bacto-agar on growth were further tested on *N. viennensis* EN76 and the acidophilic *'Ca.* N. sinensis Nd2' (see *Appendix C*). The growth rates of *N. viennensis* EN76 in Phytagel™ (0.56 d^{-1}) and the control (0.54 d^{-1}) were comparable to the pattern observed in *'Ca.* N. franklandus C13'. In contrast, the acidophilic *'Ca.* N. sinensis' Nd2 was unable to grow in the presence of both solidifying agents. These data are summarised in *Table 3.1.*

Table 3.1: A summary of the effects of gelling agents on the growth of different AOA strains.

	Gelling agent				
Strain	**Agar**	**Bacto-Agar**	**Agarose**	**Phytagel™**	**Noble-Agar**
'*Ca*. N. franklandus C13'	**x**	**x**	✓	✓✓	**x**
N. viennensis EN76	NT	**x**	**x**	✓✓	NT
'*Ca.* N. sinensis Nd2'	NT	**x**	NT	**x**	NT

NT: Not tested
(**x**): Poor/no growth
(✓): Moderate growth
(✓✓): No effect on growth

3.3.2 Cultivation of AOA on solid growth medium

Phytagel™ was selected as a suitable solidifying agent for both *'Ca.* N. franklandus C13' and *N. viennensis* EN76 as it did not negatively affect their growth. The LS-method was subsequently developed to produce single colonies. The LS-method involves inoculating AOA cells into molten Phytagel™ and allowing this mixture to solidify. The solidified cell:Phytagel™ layer is subsequently overlayed with liquid medium. Nitrite accumulation in the top liquid layer of '*Ca*. N franklandus C13' cultures is shown in (*Figure 3.2A*). A similar analysis for *N. viennensis* EN76 is presented in *Appendix D*.

Accumulation of nitrite in the top liquid layer in cultures inoculated with *'Ca.* N. franklandus C13' or *N. viennensis* EN76 indicated that the cells were actively growing in Phytagel™. In addition, a change in colour of the liquid growth medium from pink to yellow was observed, indicating acidification of the growth medium which is consistent with ammonia oxidation (*Figure 3.2B*).

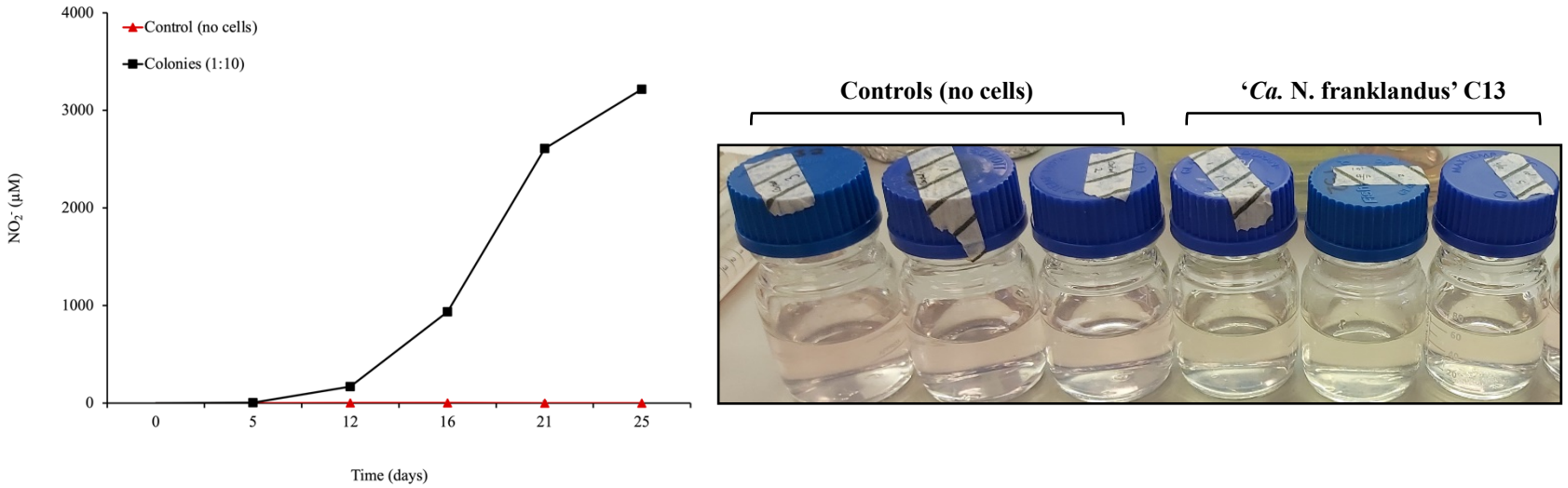


Figure 3.2: Evidence of actively growing AOA cells embedded in Phytagel™. Nitrite (NO_2^-) was measured in cultures containing the high-density inoculum. **(A)** Total nitrite accumulation by '*Ca*. N. franklandus' C13 cells growing within Phytagel™. Nitrite concentrations are the averages of three replicate cultures. Error bars represent the standard error of the means (SEM) and may be smaller than the symbol **(B)** Evidence of media acidification based on colour change of phenol red pH indicator. Control (left) and high-density inoculum of '*Ca.* N. franklandus' C13 (right).

3.3.3 Colony development

Single colonies were observed developing from *'Ca.* N. franklandus C13' cultures once the liquid phase had been replaced approximately twice (*Figure 3.3*). The high-density inoculum resulted in a lawn of colonies that took approximately 4 - 5 weeks to appear (data not shown). In comparison, colonies from the low-density inoculum took a longer time to appear at about 7 - 8 weeks. The colonies from both strains were white/off-white in appearance and gradually became more distinct with the regular replacement of the growth medium. *N. viennensis* EN76 colonies are shown in *Appendix E*.

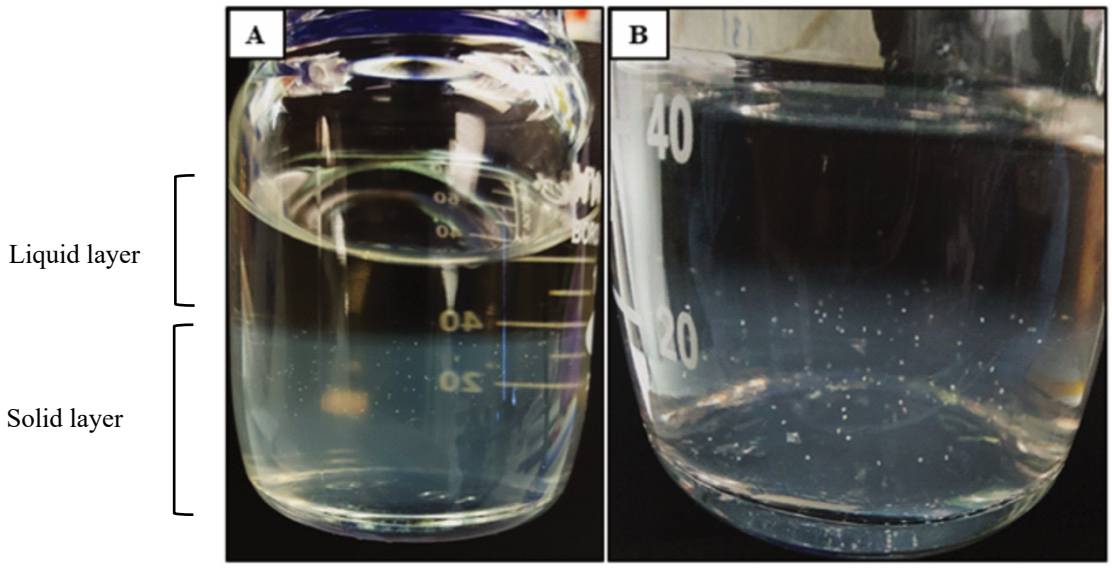


Figure 3.3: Single colonies of '*Ca*. N. franklandus C13' developing in Phytagel™. '*Ca*. N. franklandus C13' was grown on solid growth medium as single colonies using the Liquid-Solid (LS) method. The colonies were grown and maintained in 100 ml Duran glass bottles and measured < 1mm in diameter.

3.3.4 Plating efficiency

The plating efficiency was used to determine the number of colonies arising from the inoculum. The number of '*Ca*. N. franklandus C13' colonies ranged between 100 -154, which translated into a plating efficiency of > 85%. This indicated that a high proportion of input cells formed colonies supporting the gel-toxicity tests that showed Phytagel™ to have no toxic effect on the growth of '*Ca*. N. franklandus C13'. The plating efficiency for *N. viennensis* EN76 using the LS-method was not determined.

3.3.5 Sequence analysis of colonies

PCR amplification of the 16S rRNA gene from the *'Ca*. N. franklandus C13' colonies using both archaeal and AOA-specific primers generated bands of the expected size i.e. ~1383 bp and ~200 bp respectively (*Figure 3.4A* and *Figure 3.4B* respectively). A BLASTn analysis of these amplicons revealed a 100% and 99.5% nucleotide identity respectively (query cover:100%) to the 16S rRNA gene sequence in the published genome of *'Ca.* N. franklandus C13' genome with the accession number LR216287.1. The query sequences used ranged between 1247 bp (archaeal) and 190 bp (AOA-specific) in length. In addition, amplification of the urease alpha subunit gene (*ure*C) present in the genome of *'Ca.* N. franklandus C13' generated the expected 175 bp band (*Figure 3.4C*). A BLASTx analysis using a 119 bp sequence revealed 100% amino acid identity to the UreC from *'Ca.* N. franklandus C13' with the accession number WP_145988037.1. A very faint band for the bacterial primers was detected in the DNA extracted from the colony biomass (*Figure 3.4D*).

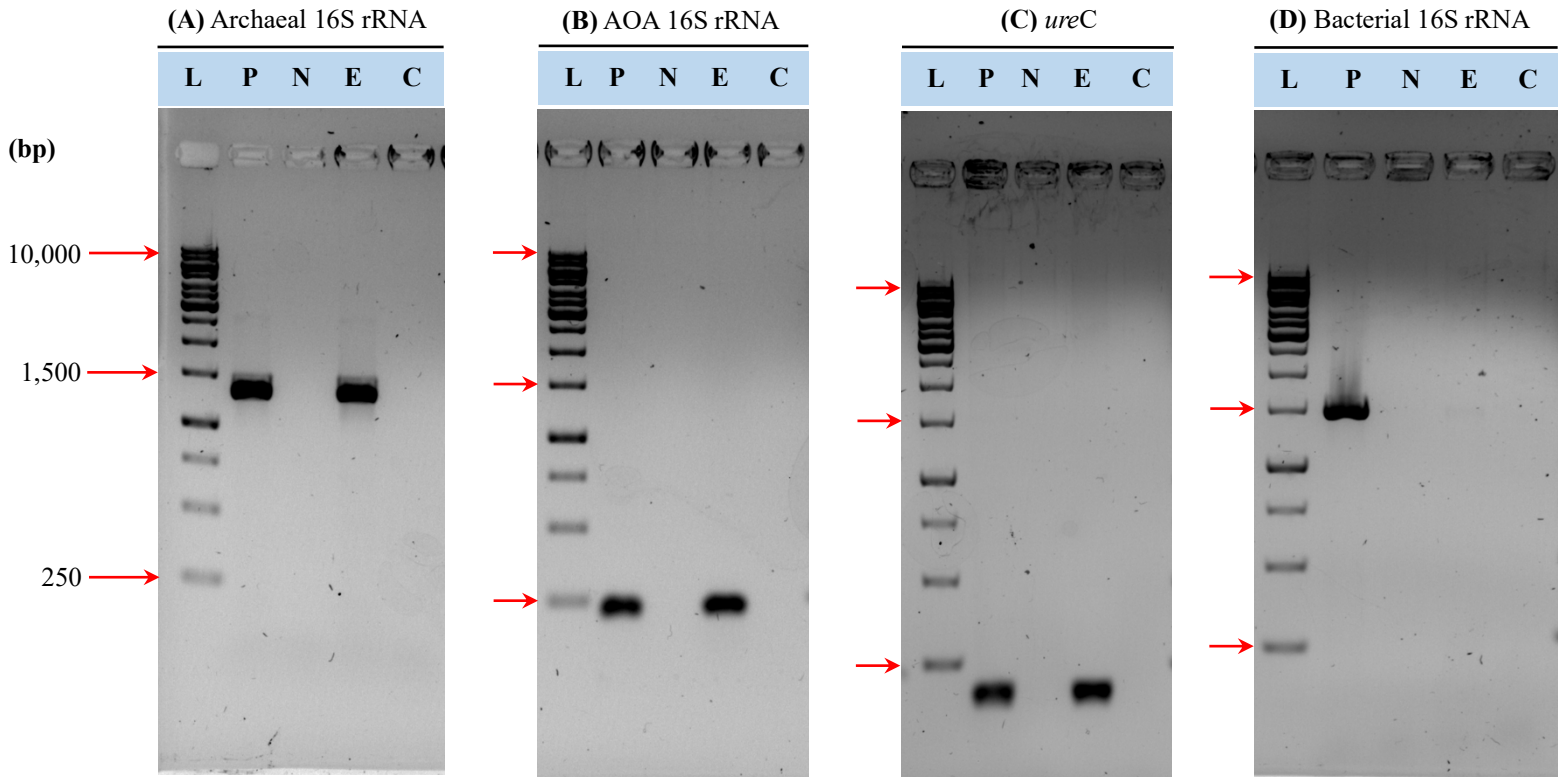


Figure 3.4: PCR verification of '*Ca*. N. franklandus C13' colonies. The following lane descriptions apply to all four agarose gels presented: **(Lane 1)**: 1 kb Generuler ladder (L), **(Lane 2)**: Positive control (P), **(Lane 3)**: Negative control (N), **(Lane 4)**: Genomic DNA extracted from colony biomass (E) and **(C)**: DNA extracted from negative control cultures (C). **Panel (A):** Amplification of the archaeal 16S rRNA gene (1383 bp). **Panel (B):** Amplification of an AOA-specific 16S rRNA gene (200 bp). **Panel (C):** Amplification of the '*Ca*. N. franklandus C13'-specific urease alpha subunit gene (*ure*C) (175 bp). **Panel (D):** Amplification of the bacterial 16S rRNA gene (~1400 bp). PCR amplicons were resolved in a 1% (w/v) TBE agarose gel.

3.3.6 Fluorescence microscopy

Fluorescence *in situ* hybridisation (FISH) using archaeal-specific fluorescent probes was used to confirm the identity of the colony-forming cells within the Phytagel™ (*Figure 3.5*). The counterstain DAPI (blue), indiscriminately labels all cells within the sample and revealed irregular cocci, a characteristic cell morphology of *'Ca.* N. franklandus C13'. A signal from the archaeal probes (violet) confirmed the presence of archaeal cells and overlapped with the DAPI signal. The lack of signal from the bacterial probes (green) suggests the absence of bacterial cells demonstrating that a homogenous population of archaeal cells was present within the Phytagel™.

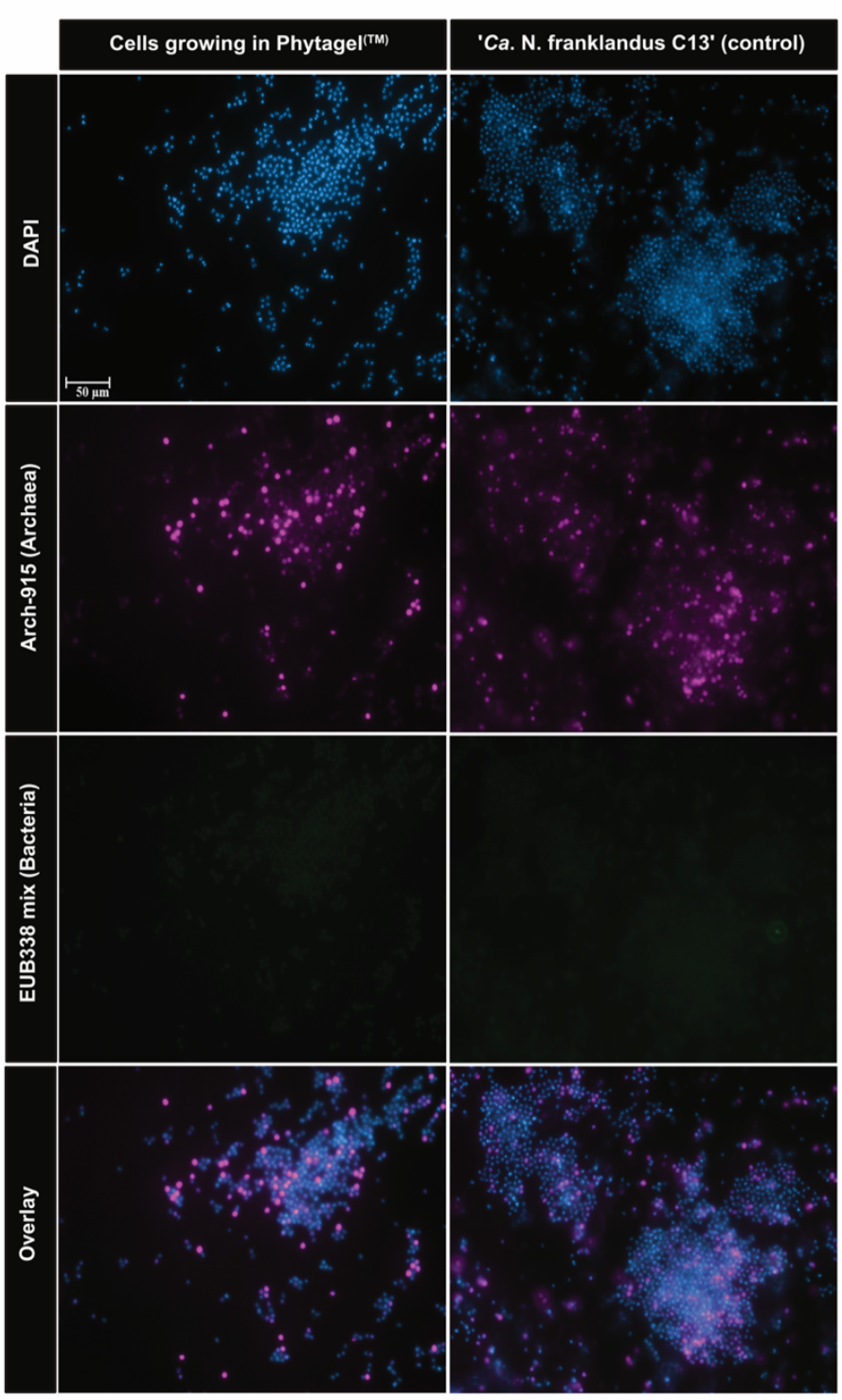


Figure 3.5: Fluorescent micrographs of '*Ca*. N. franklandus C13' cells. The left panel represents the cells growing within Phytagel™ while the right panel are cells obtained from a pure liquid culture used as a control. Cells from a pure culture of *Nitrosomonas europaea* ATCC 19178 were used as a control for the bacterial probes (data not shown). Cells are stained with FISH probes for archaea (Arch-915, violet), bacteria (EUB338 mix, green) and DAPI (blue). Images were viewed at 1000 X magnification.

3.3.7 Transmission electron microscopy (TEM)

The cells growing within the Phytagel™ were further analysed using high-resolution transmission electron microscopy (TEM). The general cell morphology was compared to cells obtained from a pure liquid batch culture of *'Ca.* N. franklandus C13' as a control (*Figure 3.6*). At low magnification (2000 X), a homogenous population of irregular cocci was observed, a morphology that is typical of this strain. This data indicates that the cells growing within Phytagel™ share general morphology to those in the reference sample.

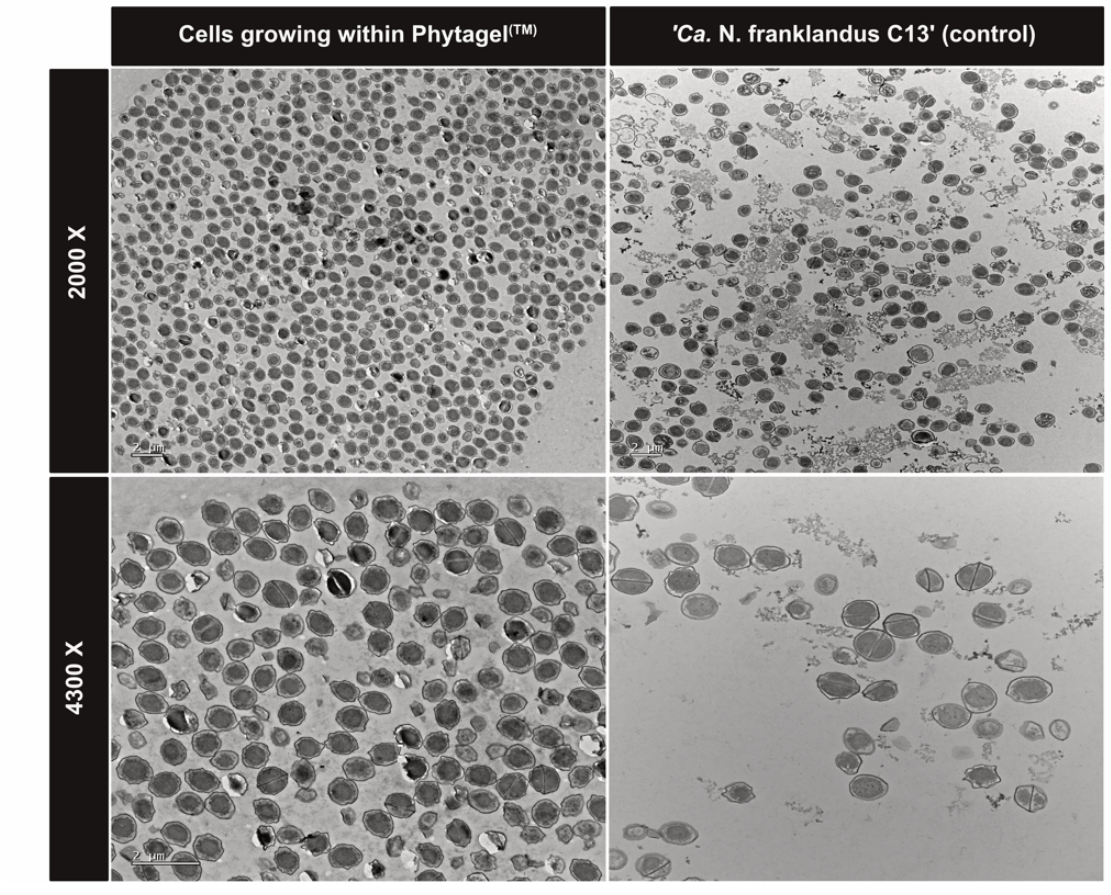
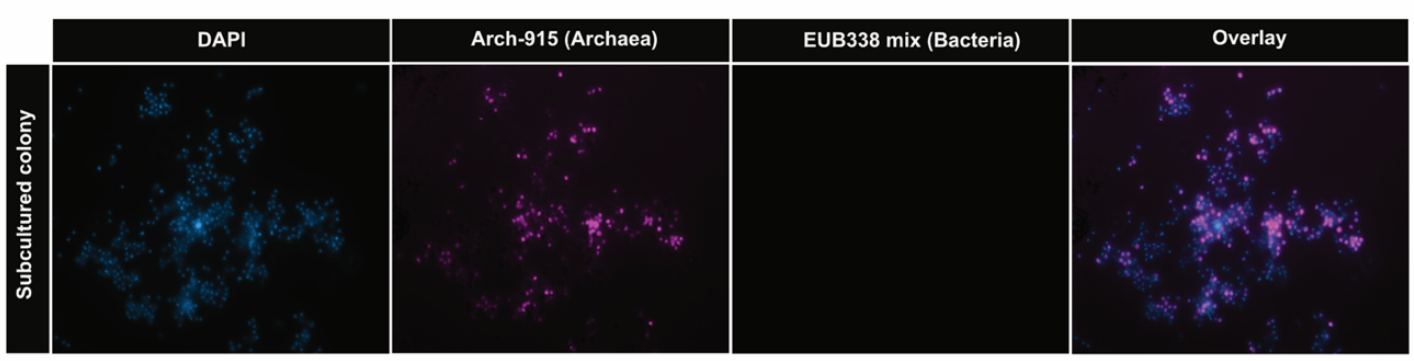


Figure 3.6: Transmission electron micrographs of '*Ca*. N. franklandus C13' cells. High-resolution microscopy was used to compare the cells growing within Phytagel™ to those obtained from a pure culture. Images are presented in increasing magnification from top to bottom. **Left panel:** Cells growing within Phytagel™. **Right panel:** Cells obtained from a pure batch grown culture of *'Ca.* N. franklandus C13'.

3.3.8 Subculturing from AOA colonies

To further confirm that the resulting colonies originate from *'Ca.* N. franklandus C13', a small sample of the gel-embedded cells were transferred to fresh liquid growth medium, and nitrite production (an indicator of growth) monitored. The accumulation of nitrite in all three sub-cultures indicated the presence of viable and actively growing ammonia-oxidising cells (*Figure 3.7B*). The sub-cultured cells were further analysed by FISH microscopy using archaeal-specific probes. The sole signal obtained from the archaeal cultures originated from the archaeal probes while no bacterial-specific fluorescence could be detected indicating a homogenous population of AOA cells in the sub-cultures (*Figure 3.7A*).

(A)



(B)

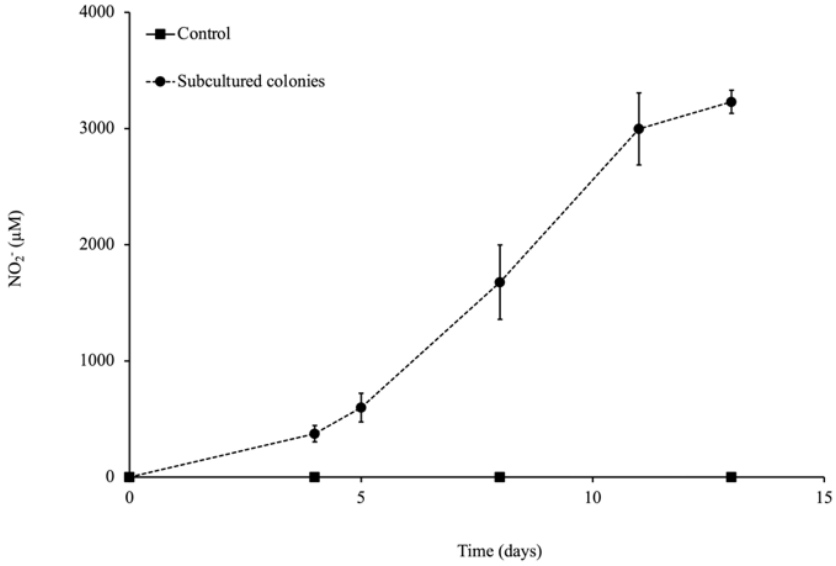


Figure 3.7: Analysis of subcultured '*Ca*. N. franklandus C13' colonies. **(A)**: Fluorescent micrographs of cells obtained from a colony of '*Ca*. N. franklandus C13'. Cells were stained with FISH probes for archaea (Arch-915, violet), bacteria (EUB338 mix, green) and DAPI (blue). Images were viewed at 1,000 X magnification. **(B):** Nitrite production from the subcultured colonies. Nitrite concentrations were measured in triplicate and error bars are standard errors of the means (SEM) and may be smaller than the size of the symbol.

3.4 Discussion

The primary goal of this work was to develop a novel cultivation method for the AOA that allows their growth on solid growth medium as distinct colonies. The ability to grow an organism on solid medium as single colonies is an important element of a genetic toolbox as it is conducive to rapid and cost-effective mutant screening following transformation. It was hypothesised that the choice of gelling agent plays an important role in the ability to grow AOA on solid growth medium. The screening of different gelling agents revealed that all AOA strains tested in this study were inhibited by agar-based gels. In contrast, the response to agarose and Phytagel™ varied between strains. This was evident for *'Ca.* N. franklandus C13' which tolerated agarose whereas *N. viennensis* EN76 did not.

Inhibition of microbial growth by agar has been well documented and is attributed to various factors including the production of hydrogen peroxide and the release of free sugars (Tuovinen and Kelly, 1973; Tanaka *et al*., 2014). The production of hydrogen peroxide is particularly important for the Thaumarchaeota as some AOA strains lack catalases which are responsible for hydrogen peroxide detoxification (Qin *et al.*, 2014; Kim *et al.*, 2016). Furthermore, Sands and Bennett (1966) discuss numerous case studies on the variable effect that agar has on microbial growth that are associated with the source of the agar as well as the brand. Other possible contributors to growth inhibition by agar is the presence of sulfated polysaccharides which exhibit a range of bioactive properties (Farias *et al.*, 2000; Ngo and Kim, 2013). The large number of possible reasons for the inhibition of the AOA strains by agar-based gels warrant further investigation but are beyond the scope of the present study.

Both *'Ca.* N. franklandus C13' and *N. viennensis* EN76 tolerated Phytagel™ well, whilst the acidophilic strain *'Ca.* N. sinensis Nd2' did not. Phytagel™ is the trade name for gellan gum, an exopolysaccharide produced by *Sphingomonas elodea* (Vartak *et al.*, 1995). It is a tetrasaccharide composed of repeating units of D-glucose (2), L-rhamnose (1) and D-glucuronic acid (1) (Kang *et al.*, 1982; Das *et al.*, 2015). Reasons for the inhibition of the acidophilic AOA strain by this polymer are unclear but may be linked to the low pH and requires further testing. It is interesting to note that gellan gum was previously used to successfully isolate an ammonia-oxidising bacterium (AOB) from soil (Takahashi *et al.*, 1992). Phytagel™ is an excellent alternative to agar as it exhibits desirable physical properties such as high optical clarity and gel strength (Jaeger *et al.*, 2015). However, it needs to be strongly emphasized that the successful growth of the AOA strains in this study on Phytagel™ was dependent on the batch and brand used (data not shown). The inability to reproduce previously published data upon changing to a new batch of Phytagel™ has recently been reported (Jacques *et al.*, 2020). The authors proposed that a variation in hardness between Phytagel™ batches was a possible factor influencing reproducibility.

In addition to gelling agents, the accumulation of toxic levels of nitrite, coupled with the slow growth rates likely contributes towards the difficulty in cultivation of AOA on solid medium. To overcome this issue, the Liquid-Solid approach (LS) was developed. The principle behind the LS-method, which consists of a biphasic growth matrix, is that the nitrite excreted by the AOA cells within the gels diffuses into the overlaying liquid phase which is subsequently aspirated off and replaced with fresh growth medium. This overcomes or in the very least minimises the toxic accumulation of nitrite, thus facilitating the continuous growth of the cells. Replenishing of the liquid

medium in the top layer may also slow acidification of the solid medium preventing substrate limitation.

In this study, it has been demonstrated that the LS-method facilitates the development of visible distinct single with a high plating efficiency. It is also possible to pick the single colonies produced from the low-density inoculum using a thin sterile glass Pasteur pipette. However, due to the three-dimensional growth of the colonies within the gel, picking single colonies is challenging as the pipette may easily come into contact with nearby colonies.

To validate that the colonies indeed originate from AOA cells, both molecular and microscopy-based methods were employed. Cells growing within the gel were labelled with FISH probes targeting archaeal and bacterial 16S rRNA. A signal was detected for the archaeal-specific probes but not for the bacterial probes indicating the presence of only archaeal cells. Additionally, the cells exhibited a cellular morphology (irregular cocci) identical to the cells from a pure culture of *'Ca.* N. franklandus C13'. However, the use of the Cy3-labeled Arch-915 probe resulted in a weak fluorescent signal from the *'Ca.* N. franklandus C13' cells (refer to *Figure 3.5*). This may result from low rRNA content and poor cell permeability (Wagner *et al*., 2003). Therefore, FISH was coupled with high-resolution TEM, PCR and sequence analysis which all indicate that the cells are *'Ca.* N. franklandus C13'. A very faint band was amplified with the bacterial primers which is most likely transient DNA as no signal from the bacterial FISH probes was detected and no bacterial cells could be discerned from the high-resolution electron micrographs. In addition, no bacterial cells were detected by FISH from the colonies that were subcultured.

Aside from mutant screening, the LS-method may have applications in the isolation of AOA which is currently restricted to time-consuming enrichment procedures. For example, if a dilute sample of a highly enriched AOA culture is grown using the LS-method and visible colonies are produced, then it may be possible to pick a desired colony expediting the purification process. This approach may also be useful for the purification of bacteria capable of the complete oxidation of ammonia to nitrate via nitrite also known as comammox. Currently a single pure culture of a comammox exists, which hinders their thorough physiological characterisation (Daims *et al.*, 2015; Sakoula *et al.*, 2021). A main caveat associated with the LS-method is that it is seemingly not applicable to acidophilic AOA. Further work is necessary to better understand the inhibition of Phytagel™ and agar-based gels on *'Ca.* N. sinensis Nd2'. Also, it is difficult to determine colony counts due to their three-dimensional arrangement. Free-floating cells in the liquid phase presumably due to the detachment from the gel-matrix were also observed which may affect picking of a homogenous population of cells following transformation. Lastly, the LS-method is limited by the slow growth rates of the AOA. Colonies from the high-density inoculum took on average 5 - 6 weeks to be visible, whereas those from the low-density inoculum took about two months to reach a size that can easily be picked.

Overall, the ability to grow an AOA strain as single colonies on solid growth medium is a major advancement towards the development of a genetic system for these archaea.

3.5 Summary and outlook

In this chapter, a novel cultivation method that allows the growth of AOA on solid growth medium as single colonies is described. The data here suggests that the growth of AOA on solid growth medium is dependent on the gelling agent used. The relative

simplicity and no requirement for specialised equipment makes the LS-method accessible to any microbiology laboratory working with this group of microbes. Unfortunately, the cause of the inhibitory effects of agar-based gelling agents has not been explored and will be useful to investigate in the future. Furthermore, the LS-method is currently not applicable to acid-tolerant AOA strains as they were inhibited by all gelling agents tested.

Chapter 4: Insights into the antibiotic sensitivity of ammonia-oxidising archaea

4.1 Introduction

Antibiotics are an important tool in archaeal research where they are used as selective agents in genetic studies and aid in the selective isolation of archaea from mixed microbial communities (Liu *et al.*, 2019). Furthermore, as not usually pathogenic and therefore not historically the target of intense antibiotic research, the innate resistance of archaea to numerous antibiotics is exploited to differentiate between the biological activity of bacteria and archaea (Elevi Bardavid and Oren, 2008; Taylor *et al.*, 2010; Vajrala *et al.*, 2014; Zhao *et al.*, 2020). Archaea represent a unique model for testing new drugs/antibiotics because in addition to uniquely archaeal elements, they also contain both eukaryotic and bacterial features and may therefore reveal novel or unexpected mechanisms and targets (Williams *et al.*, 2013). The current knowledge on archaeal antibiotic sensitivity patterns has primarily been gleaned from the Crenarchaeota and Euryarchaeota. This has revealed that archaea from these phyla are primarily sensitive to inhibitors of DNA and protein synthesis (Khelaifia and Drancourt, 2012).

A major family of protein synthesis inhibitors known to inhibit archaea include the aminoglycosides. These antibiotics inhibit protein synthesis by targeting the ribosome machinery via interactions with rRNA (Kulik *et al.*, 2015; Lin *et al.*, 2018). This interaction with rRNA (e.g. 16S rRNA) is facilitated by the polycationic nature of these compounds due to the presence of several amino groups (Wang and Tor, 1997). Structurally, aminoglycosides are large hydrophilic molecules comprised of an aminocyclitol ring (commonly 2-deoxystreptamine, 2-DOS) bound to one or more amino sugars by a glycosidic bond (Mingeot-Leclercq *et al.*, 1999; Magnet and Blanchard, 2005). Based on the functionality of the aminocyclitol ring,

aminoglycosides can further be categorised into four structural sub-classes: **(i)** streptidine-containing (streptomycin) **(ii)** mono-substituted deoxystreptamine (e.g. hygromycin B, apramycin) **(iii)** 4,5 di-substituted deoxystreptamine or 4,5 2-DOS (e.g. neomycin) and the **(iv)** 4,6 di-substituted deoxystreptamine or 4,6 2-DOS (e.g. kanamycin, gentamycin) (Krause *et al.*, 2016). This structural diversity plays an important role in the biological activity of these antibiotics (Quirke *et al.*, 2022). Aminoglycosides such as hygromycin B and neomycin play an important role in archaeal genetics where they are used as selective agents (Argyle *et al*., 1996; Cannio *et al.*, 2001). For the Thaumarchaeota, aminoglycosides such as kanamycin and streptomycin are frequently used to prevent bacterial growth in both pure and enrichment cultures (Stieglmeier *et al.*, 2014; Lehtovirta-Morley *et al.*, 2016; Abby *et al.*, 2018; Bayer *et al.*, 2019). However, whether this insensitivity of the Thaumarchaeota to aminoglycosides applies to all structural classes is currently unclear.

In addition to aminoglycosides, another important archaeal protein synthesis inhibitor is puromycin which is classified as an aminonucleoside. Structurally, puromycin shares similarities with transfer RNA (tRNA) in that it is comprised of a nucleoside linked to an amino acid. However, the bond between these two moieties in puromycin is a peptide-bond instead of an ester-bond as seen in amino acid-tRNA (aa-tRNA) (Aviner, 2020). Although the puromycin molecule via its amino group can be incorporated into the nascent polypeptide chain, its peptide-bond is resistant to cleavage by the incoming aa-tRNA causing premature termination of chain elongation (Aviner, 2020). Puromycin is an important selective agent in both eukaryotic (Iwaki and Umemura, 2011; Caputo *et al.*, 2021) and prokaryotic systems including archaea particularly the methanogens (Tumbula *et al*., 1994).

With regards to DNA synthesis inhibitors known to target archaea, major antibiotic families include the quinolones (e.g. ciprofloxacin) and coumarins such as novobiocin (Khelaifia and Drancourt, 2012). These antibiotics may also be classed as topoisomerase inhibitors as their primary targets are type II DNA topoisomerases which includes enzymes such as DNA gyrase and topoisomerase IV (Confreres and Maxwell, 1992; Drlica *et al.*, 2009). Novobiocin is an important selective agent in archaeal genetic studies particularly for halophilic systems (Holmes *et al*., 1994; Atomi *et al*., 2012).

An emerging class of compounds that is currently of interest are the fasamycins. The fasamycins were first discovered following the heterologous expression of a type II polyketide synthase biosynthetic gene cluster from environmental DNA (Feng *et al.*, 2012). Subsequent analysis of fasamycin-resistant mutants led to the identification of a mutation in the *fab*F gene which encodes an enzyme involved in the type II fatty acid biosynthesis pathway (Feng *et al.*, 2012). Interestingly, in *Streptomyces formicae*, isolated from the African fungus-growing plant-ant *Tetraponera penzigi*, the fasamycin biosynthetic pathway has expanded such that fasamycins are the biosynthetic precursors of the formicamycins, which represent a new structural class of antibiotics (Qin *et al.*, 2017). Both compounds are currently of significant interest due to their potent activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* (VRE) (Yang *et al.*, 2020). Due to this promising activity, ongoing research is currently looking to investigate the scope of their biological activity.

Currently, antibiotic sensitivity testing of the Thaumarchaeota has not received nearly as much attention as the main archaeal phyla. While a handful of reports have

investigated antibiotic sensitivity of AOA strains, this information is limited to a very few strains and even fewer compounds or compound families (Vajrala *et al*., 2014; Zhao *et al*., 2020). Additionally, in some instances the antibiotic sensitivity of AOA can only be inferred from mixed community cultures or enrichments (Schauss *et al.*, 2009; Liu *et al.*, 2019). This makes it challenging to differentiate between true antibiotic sensitivity and inhibition resulting from indirect effects (Woo *et al*., 2022). Furthermore, it is also difficult to discern lineage-dependent sensitivity patterns among AOA from mixed communities which may have some practical applications in biased enrichments. For example, is it possible to isolate a specific group of AOA from a mixed community of microorganisms using antibiotics? However, in the context of the present study, this limited knowledge on the antibiotic sensitivity of AOA is an important bottleneck in the establishment of a genetic toolbox.

4.2 Chapter aims

The primary goal of this chapter is to identify antibiotics that can be used as selective agents for the model AOA strain '*Ca.* N. franklandus C13'. In addition, this work aims to expand on the current insights into AOA antibiotic sensitivity by testing the shortlisted antibiotics against two additional phylogenetically and physiologically distinct AOA strains. Finally, in an effort to gain further understanding of the scope of their biological activity and mechanism of action, various fasamycin and formicamycin congeners will be tested against two AOA strains.

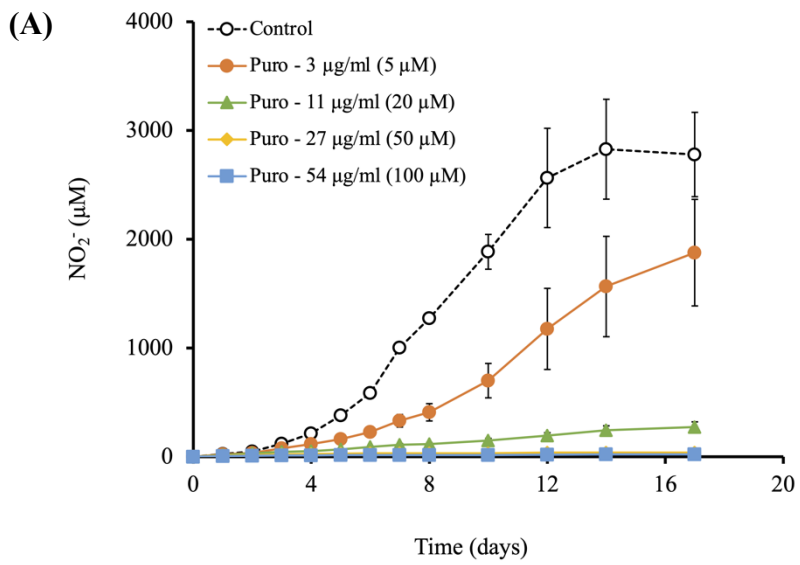
4.3 Results

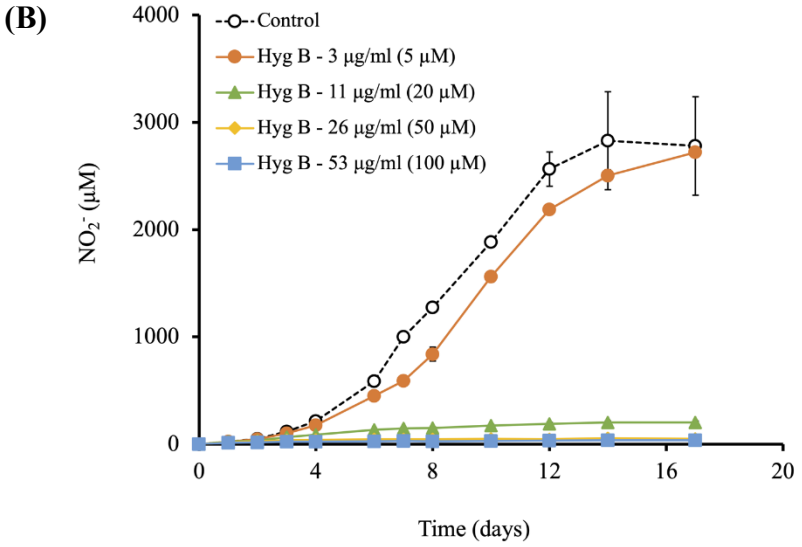
All antibiotic sensitivity tests were performed in liquid cultures and untreated cultures served as the negative control. Growth curves were generated by plotting the total nitrite concentration produced against time. The inhibition of nitrite production was used as an indicator of antibiotic sensitivity. Candidate antibiotics to be tested (*Table 2.3*) were shortlisted based four main criteria listed in *section 2.11.1* and are classified as either protein or DNA inhibitors.

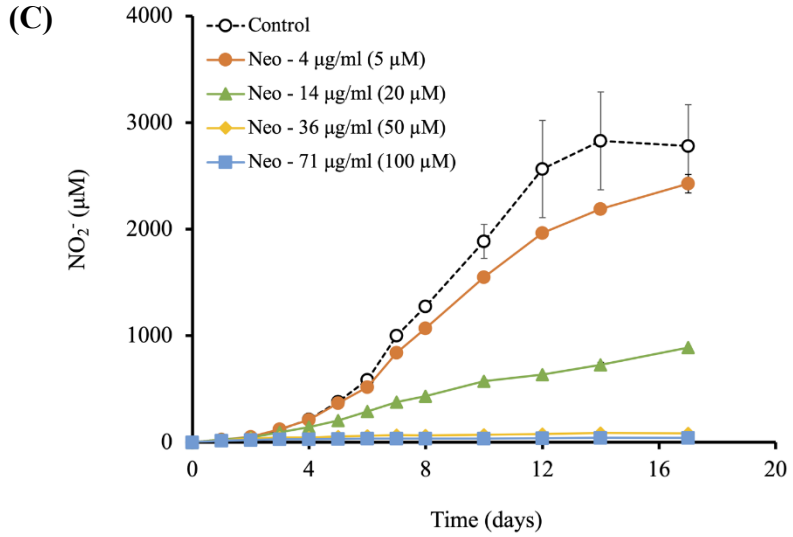
4.3.1 Antibiotic sensitivity of '*Ca*. N. franklandus C13'

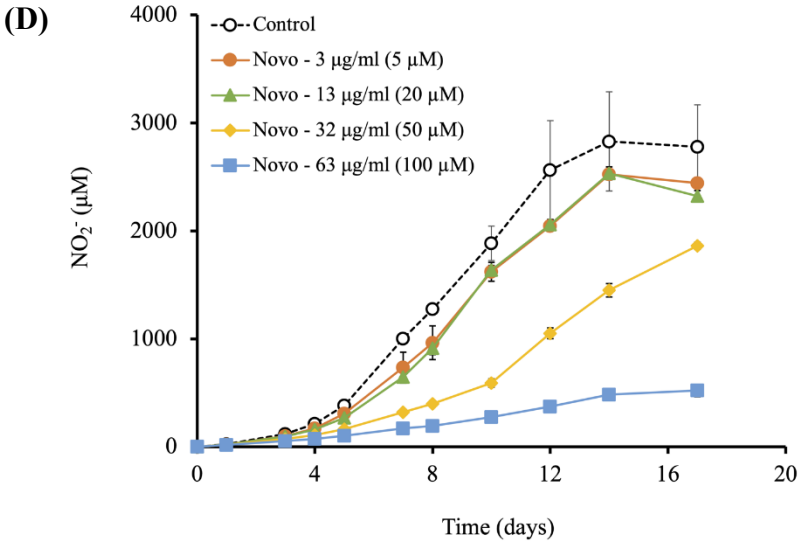
The goal of antibiotic sensitivity testing was to identify a suitable selective agent(s) and determine the appropriate concentrations at which it should be used. The antibiotics puromycin, hygromycin B and neomycin were consequently identified as potential selective agents for '*Ca*. N. franklandus C13'. Puromycin and hygromycin B were the most promising and showed potent inhibition of nitrite production at concentrations of ≥ 27 µg/ml (*Figure 4.1A* and *Figure 4.1B* respectively). Long-term monitoring of puromycin and hygromycin B treated cultures (at concentrations ≥ 27 µg/ml) for ~65 days did not indicate development of resistant mutants (*Appendix F*). In comparison, neomycin significantly inhibited nitrite production at a concentration of ≥ 36 µg/ml (*Figure 4.1C*). Novobiocin only moderately inhibited nitrite production at concentrations > 63 µg/ml (*Figure 4.1D*). Nitrite production was not inhibited by apramycin at any of the concentrations tested.

Overall, under the conditions tested, '*Ca*. N. franklandus C13' was sensitive to all the antibiotics tested (albeit to varying degrees) except for apramycin. Growth curves of antibiotic treated cultures are presented in *Figure 4.1*.


(A)
Control
Puro - 3 µg/ml (5 µM)
Puro - 11 µg/ml (20 µM)
Puro - 27 µg/ml (50 µM)
Puro - 54 µg/ml (100 µM)
NO2- (µM)
Time (days)


(B)
Control
Hyg B - 3 µg/ml (5 µM)
Hyg B - 11 µg/ml (20 µM)
Hyg B - 26 µg/ml (50 µM)
Hyg B - 53 µg/ml (100 µM)
NO2- (µM)
Time (days)


(C)
Control
Neo - 4 µg/ml (5 µM)
Neo - 14 µg/ml (20 µM)
Neo - 36 µg/ml (50 µM)
Neo - 71 µg/ml (100 µM)
NO2- (µM)
Time (days)


(D)
Control
Novo - 3 µg/ml (5 µM)
Novo - 13 µg/ml (20 µM)
Novo - 32 µg/ml (50 µM)
Novo - 63 µg/ml (100 µM)
NO2- (µM)
Time (days)

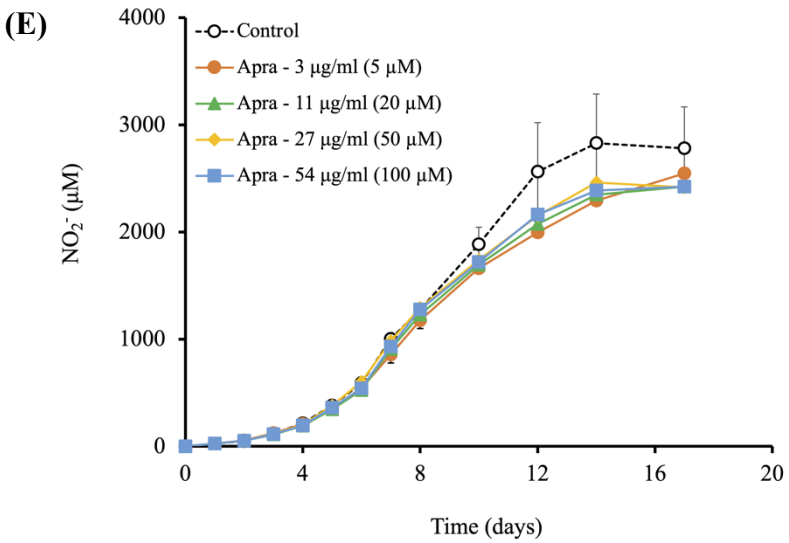


Figure 4.1: *In vivo* antibiotic sensitivity of '*Ca*. N. franklandus C13' cells in liquid cultures. Inhibition of nitrite (NO_2^-) production was assumed to indicate sensitivity. All antibiotic treated cultures were compared to untreated cultures as a control. Antibiotics tested include (**A**) puromycin, (**B**) hygromycin B, (**C**) neomycin, (**D**) novobiocin and (**E**) apramycin. The nitrite concentrations plotted represent the average of three replicate cultures. Error bars represent the standard error of mean (SEM) and may be smaller than the symbol.

4.3.2 Testing for lineage-dependent antibiotic sensitivity among three AOA strains

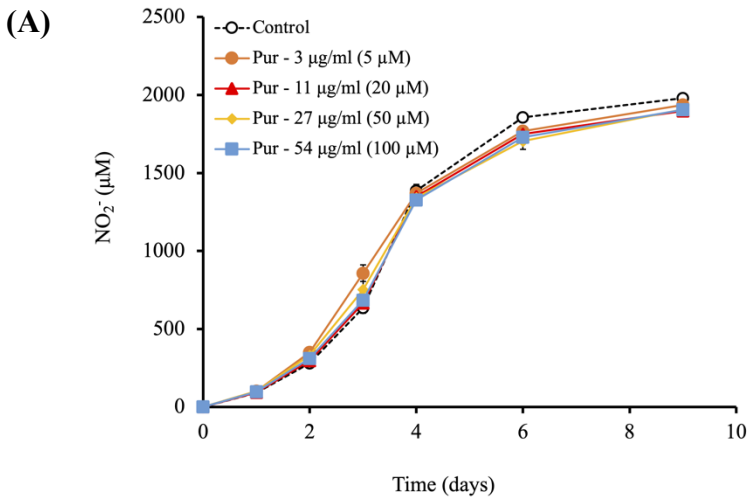
Reports on the antibiotic sensitivity patterns of archaea in the phylum Thaumarchaeota are scarce. Due to the physiological diversity exhibited by these archaea, it was interesting to determine whether a lineage-dependent sensitivity pattern was evident. Consequently, in addition to '*Ca*. N. franklandus C13', two additional AOA strains from distinct genera were screened including *N. viennensis* EN76 (soil isolate) and *N. maritimus* SCM1 (marine isolate).

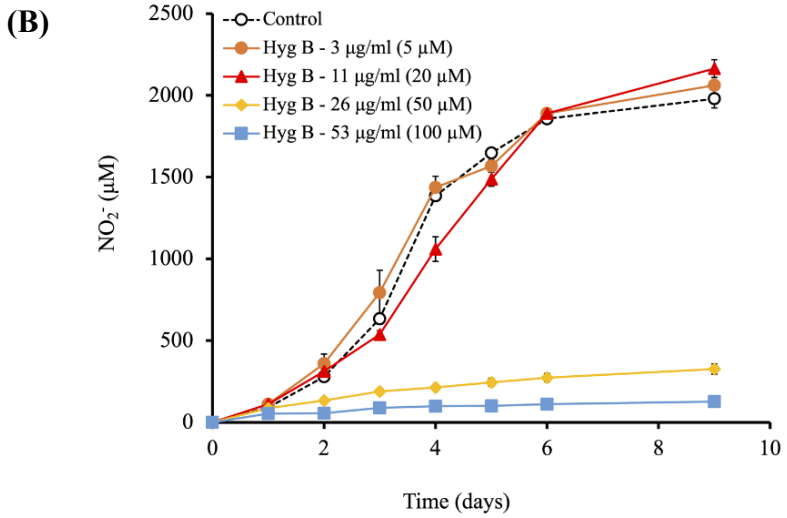
Under the conditions tested, hygromycin B strongly inhibited nitrite production in *N. viennensis* EN76 cultures at concentrations > 26 µg/ml (*Figure 4.2B*). Nitrite production in neomycin treated cultures was negligible at the highest concentration tested (71 µg/ml) (*Figure 4.2C*). In comparison, puromycin and apramycin had no effect on the nitrite production in *N. viennensis* EN76 cultures (*Figure 4.2A* and *Figure 4.2E* respectively). Although *N. viennensis* EN76 was insensitive to novobiocin, a concentration-dependent reduction in nitrite production was observed at the late stages of growth (*Figure 4.2D*).

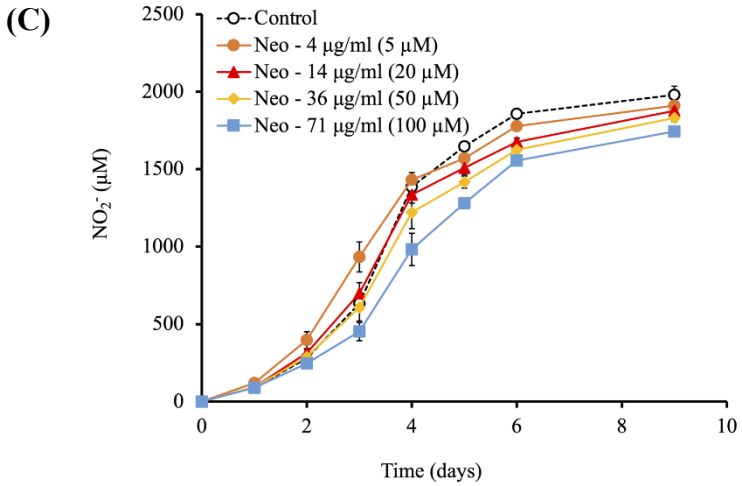
N. maritimus SCM1 was strongly inhibited by puromycin with nitrite production inhibited at concentrations ≥ 11 µg/ml (*Figure 4.3A*). Novobiocin strongly inhibited nitrite production at concentrations as low as 3 µg/ml (*Figure 4.3D*). In contrast, nitrite concentration in *N. maritimus* SCM1 cultures was unaffected by hygromycin B and apramycin (*Figure 4.3B* and *Figure 4.3E* respectively).

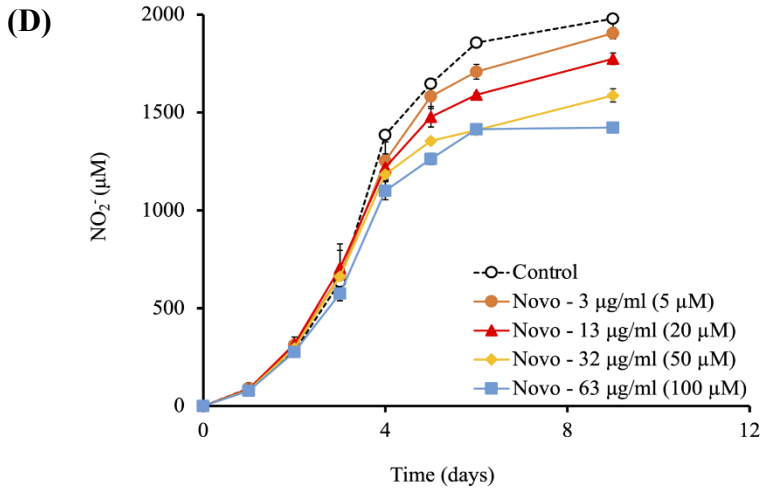
In summary, antibiotic sensitivity patterns differed between the three AOA strains. Exceptions include the resistance to apramycin and the sensitivity to neomycin and novobiocin by all three strains. Growth curves of the antibiotic treated cultures of *N.*

viennensis EN76 and *N. maritimus* SCM1 are shown in *Figure 4.2* and *Figure 4.3* respectively. A summary of the antibiotic sensitivity patterns for all three AOA strains is presented in *Table 4.1*.


(A)
Control
Pur - 3 µg/ml (5 µM)
Pur - 11 µg/ml (20 µM)
Pur - 27 µg/ml (50 µM)
Pur - 54 µg/ml (100 µM)
NO2- (µM)
Time (days)


(B)
Control
Hyg B - 3 µg/ml (5 µM)
Hyg B - 11 µg/ml (20 µM)
Hyg B - 26 µg/ml (50 µM)
Hyg B - 53 µg/ml (100 µM)
NO2- (µM)
Time (days)


(C)
Control
Neo - 4 µg/ml (5 µM)
Neo - 14 µg/ml (20 µM)
Neo - 36 µg/ml (50 µM)
Neo - 71 µg/ml (100 µM)
NO2- (µM)
Time (days)


(D)
Control
Novo - 3 µg/ml (5 µM)
Novo - 13 µg/ml (20 µM)
Novo - 32 µg/ml (50 µM)
Novo - 63 µg/ml (100 µM)
NO2- (µM)
Time (days)

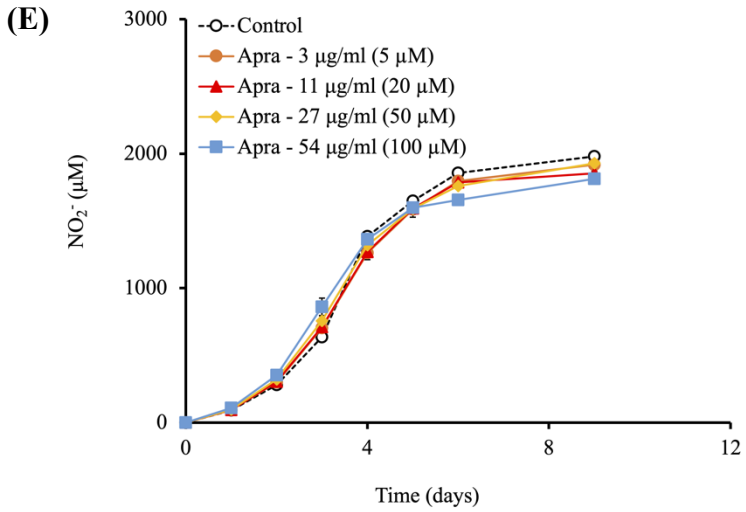
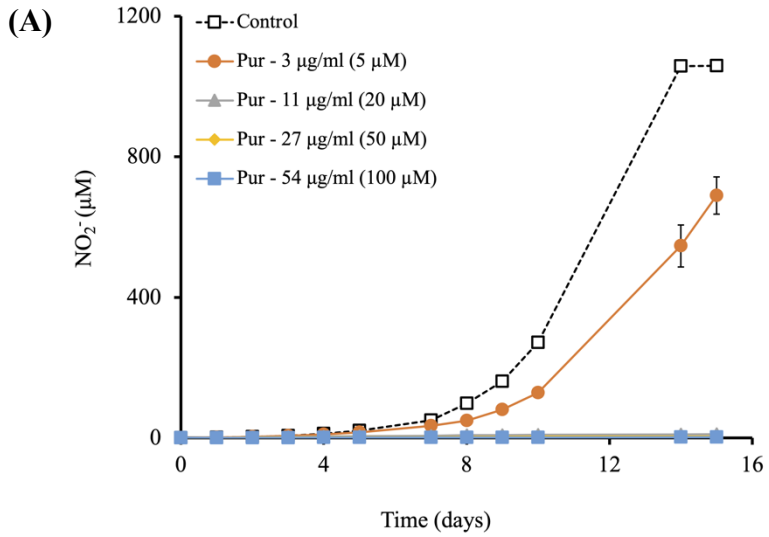
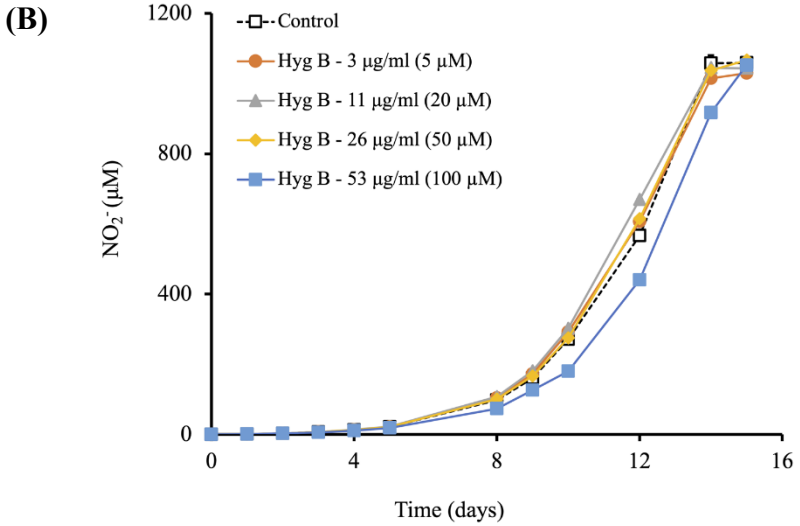
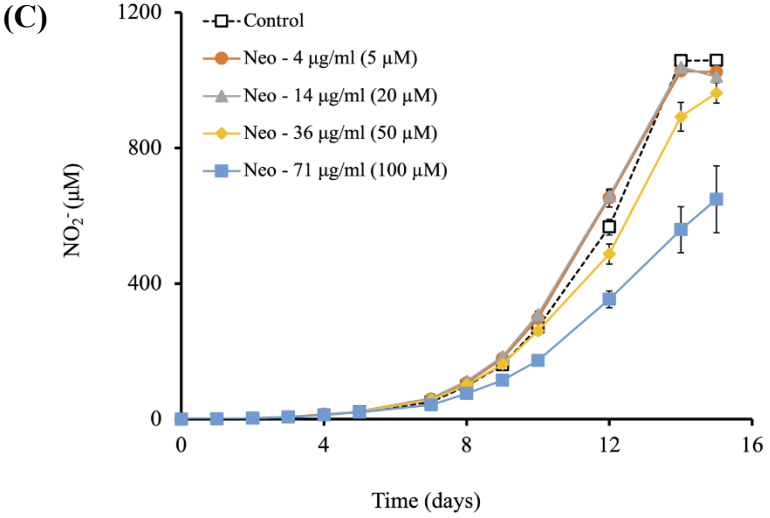
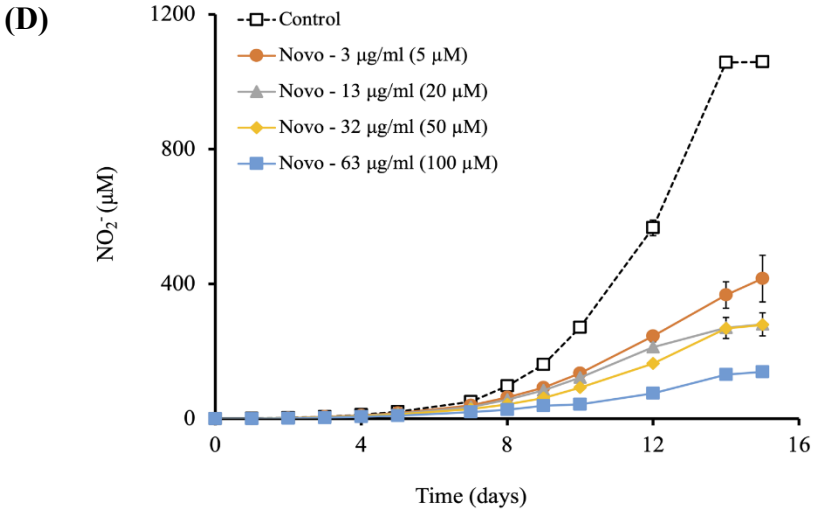


Figure 4.2: *In vivo* antibiotic sensitivity of *N. viennensis* EN76 in liquid cultures. Inhibition of nitrite production (NO_2^-) was assumed to indicate sensitivity. All antibiotic treated cultures were compared to untreated cultures as a control. Antibiotics tested include (**A**) puromycin, (**B**) hygromycin B, (**C**) neomycin, (**D**) novobiocin and (**E**) apramycin. Nitrite concentrations plotted represent the average of three replicate cultures. Error bars represent the standard error of mean (SEM) and may be smaller than the symbol.


(A)
Control
Pur - 3 µg/ml (5 µM)
Pur - 11 µg/ml (20 µM)
Pur - 27 µg/ml (50 µM)
Pur - 54 µg/ml (100 µM)
NO_2^- (µM)
Time (days)


(B)
Control
Hyg B - 3 µg/ml (5 µM)
Hyg B - 11 µg/ml (20 µM)
Hyg B - 26 µg/ml (50 µM)
Hyg B - 53 µg/ml (100 µM)
NO_2^- (µM)
Time (days)


(C)
Control
Neo - 4 µg/ml (5 µM)
Neo - 14 µg/ml (20 µM)
Neo - 36 µg/ml (50 µM)
Neo - 71 µg/ml (100 µM)
NO_2^- (µM)
Time (days)


(D)
Control
Novo - 3 µg/ml (5 µM)
Novo - 13 µg/ml (20 µM)
Novo - 32 µg/ml (50 µM)
Novo - 63 µg/ml (100 µM)
NO_2^- (µM)
Time (days)

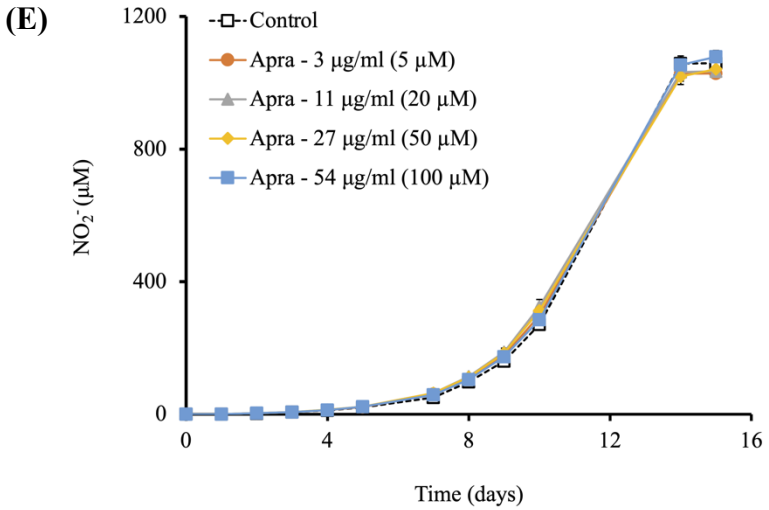


Figure 4.3: *In vivo* antibiotic sensitivity of *N. maritimus* SCM1 in liquid cultures. Inhibition of nitrite production (NO_2^-) was assumed to indicate sensitivity. All antibiotic treated cultures were compared to untreated cultures as a control. Antibiotics tested include (**A**) puromycin, (**B**) hygromycin B, (**C**) neomycin, (**D**) novobiocin and (**E**) apramycin Nitrite concentrations plotted represent the average of three replicate cultures. Error bars represent the standard error of mean (SEM) and may be smaller than the symbol.

Table 4.1: Summary of the antibiotic sensitivities of three AOA strains.

		Antibiotic				
Strain	**Habitat**	**Puromycin**	**Hygromycin B**	**Neomycin**	**Novobiocin**	**Apramycin**
'Ca. N. franklandus C13'	Soil	✓	✓	✓	✓	**x**
N. viennensis EN76	Soil	**x**	✓	✓/**x**	✓/**x**	**x**
N. maritimus SCM1	Marine	✓	**x**	✓	✓	**x**

(✓) - Sensitive

(✓/**x**) - Mildly sensitive

(**x**) - Resistant

4.3.3 AOA sensitivity to aminoglycosides

As discussed previously, aminoglycosides are an important class of protein synthesis inhibitors with potent activity against archaea in the major phyla. Aminoglycoside antibiotics are structurally diverse and can be grouped into four structural classes described previously. Three of the antibiotics shortlisted in this work are classified as aminoglycosides including hygromycin B, neomycin and apramycin. Structurally, hygromycin B and apramycin are both classed as mono-substituted deoxystreptamines whereas neomycin is a 4,5 di-substituted 2-DOS aminoglycoside.

While *N. maritimus* SCM1 was resistant to both mono-substituted aminoglycosides tested, '*Ca*. N. franklandus C13' and *N. viennensis* EN76 were sensitive to hygromycin B. All three strains were resistant to apramycin but sensitive to neomycin, the only representative of the 4,5 2-DOS tested in this study. As noted previously, AOA are

generally resistant to the 4,6 di-substituted 2-DOS (e.g. kanamycin and gentamycin) and the streptidine class (streptomycin) aminoglycosides and were therefore not tested in this study (Lehtovirta-Morley *et al.*, 2014,2016; Stieglmeier *et al.*, 2014; Abby *et al.*, 2018; Bayer *et al.*, 2019). A summary of the sensitivity of the three AOA strains to aminoglycoside antibiotics is presented in *Table 4.2*.

Table 4.2: Sensitivity of the three AOA strains to aminoglycoside antibiotics.

Aminoglycoside	Structural class	'*Ca.* N. franklandus C13'	*N. viennensis* EN76	*N. maritimus* SCM1
Hygromycin B	Mono-substituted 2-DOS	✓	✓	**x**
Apramycin	Mono-substituted 2-DOS	**x**	**x**	**x**
Neomycin	4,5 di-substituted 2-DOS	✓	✓/ **x**	✓
Kanamycin	4,6 di-substituted 2-DOS	**x**	**x**	**x**
Streptomycin	Streptidine	**x**	**x**	**x**

- Aminoglycosides tested in this study are highlighted in **bold.**
- Similar structural families are highlighted with the same colour.

4.3.4 AOA sensitivity to fasamycin and formicamycin congeners

The fasamycins and formicamycins exhibit potent activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) without the formation of drug-resistant mutants which makes them attractive drug candidates (Holmes *et al.*, 2018). With regards to their molecular targets, recent reports indicate that they are potent inhibitors of the bacterial type II topoisomerase, DNA gyrase. However, these compounds have previously not been tested in an archaeal model and may have a novel target(s) in this group of microorganisms. The

Thaumarchaeota are an interesting archaeal model to test as they lack DNA gyrase (Villain *et al*., 2022). Therefore, to better understand the scope of the biological activity of the fasamycins and formicamycins, they were tested against the strains *N. viennensis* EN76 and '*Ca*. N. franklandus C13'.

The congeners fasamycin G and fasamycin E strongly inhibited *N. viennensis* EN76 with an MIC of 1 and 0.5 µg/ml respectively (*Figure 4.5*). Similarly, '*Ca*. N. franklandus C13' was strongly inhibited by fasamycin E with an MIC of 0.5 µg/ml (*Figure 4.4*). The congener fasamycin G was not tested against '*Ca*. N. franklandus C13' in this study.

With regards to the formicamycins, '*Ca*. N. franklandus C13' was sensitive to both congeners (formicamycin I and J) with an MIC of 1 µg/ml. In contrast, *N. vienniensis* EN76 was only sensitive to formicamycin J (MIC; 1 µg/ml) but was resistant to formicamycin I (under the conditions tested).

Overall, these data show that the fasamycins and formicamycins exhibit potent biological activity against '*Ca*. N. franklandus C13' and *N. vienniensis* EN76 with MIC values ≤ 1 µg/ml. A summary of the sensitivity of '*Ca*. N. franklandus C13' and *N. vienniensis* EN76 to the various fasamycin and formicamycin congeners is provided in *Table 4.3*.

The solvent controls for DMSO (0.05% (v/v)) and HCl (0.05 mM) in *N. viennensis* EN76 cultures had no effect on growth. In contrast, while HCl (0.05 mM) had no effect on '*Ca*. N. franklandus C13', 0.05% DMSO only had mild effects on growth. These data strongly suggest that the observed activity is due to the activity of the fasamycin and formicamycin antibiotics. Ciprofloxacin was used as a positive control for fasamycins and formicamycins as preliminary evidence in bacterial systems indicate

that they target DNA gyrase (Matt Hutchings, personal communication). Both AOA strains were resistant to ciprofloxacin at the concentrations tested.

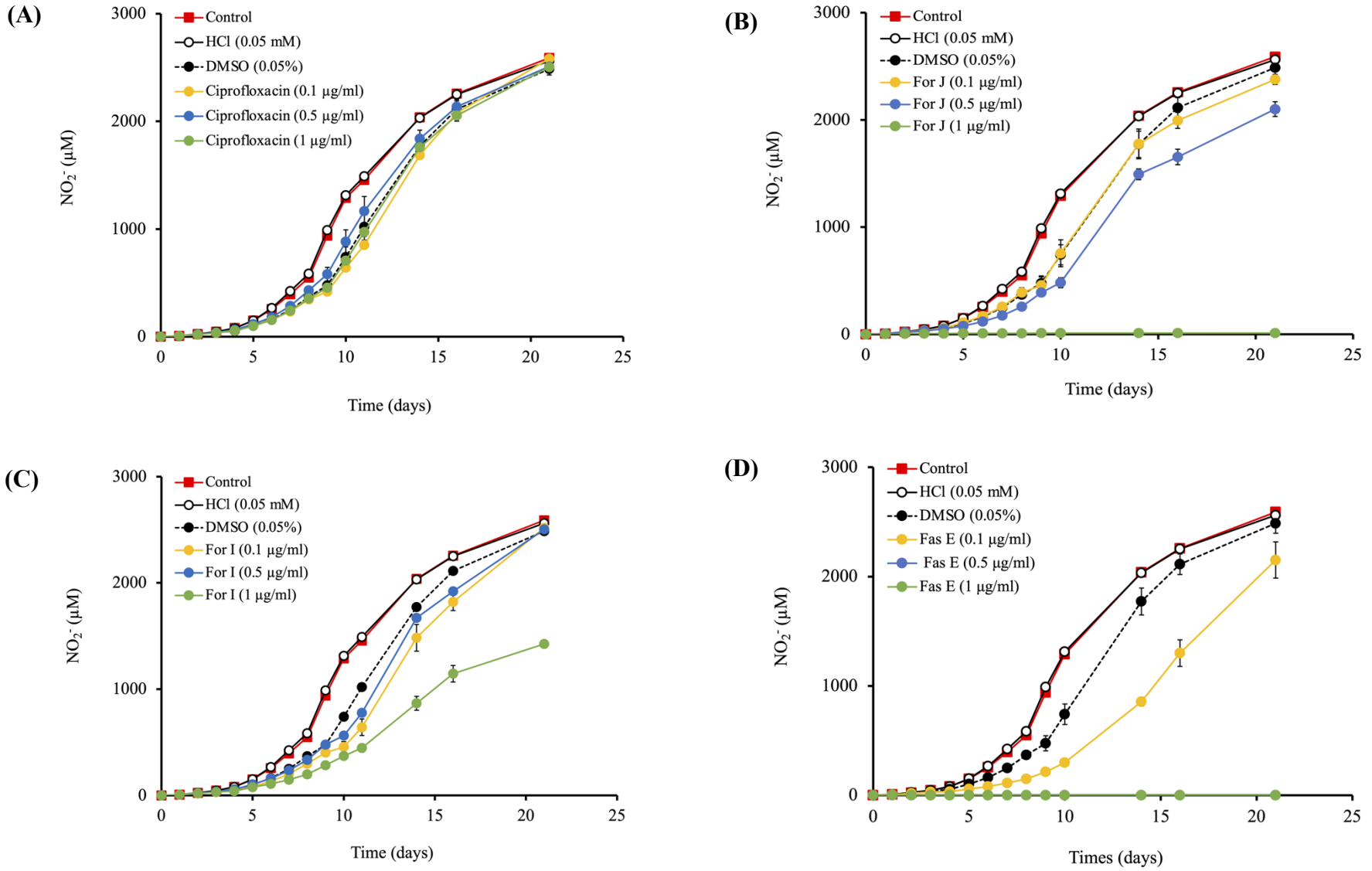
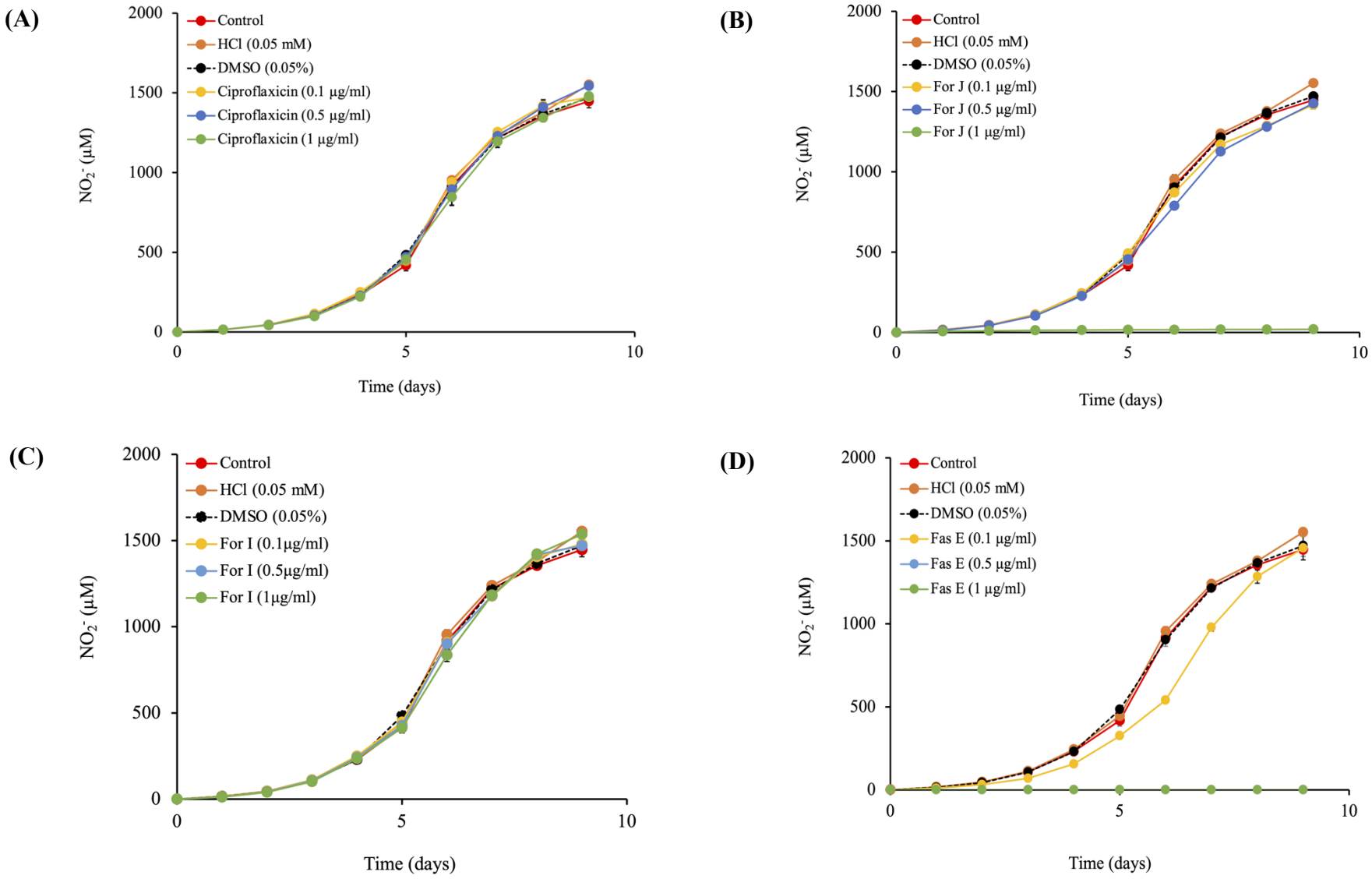

(A)
Control
HCl (0.05 mM)
DMSO (0.05%)
Ciprofloxacin (0.1 µg/ml)
Ciprofloxacin (0.5 µg/ml)
Ciprofloxacin (1 µg/ml)
NO_2^- (µM)
Time (days)
(B)
Control
HCl (0.05 mM)
DMSO (0.05%)
For J (0.1 µg/ml)
For J (0.5 µg/ml)
For J (1 µg/ml)
NO_2^- (µM)
Time (days)
(C)
Control
HCl (0.05 mM)
DMSO (0.05%)
For I (0.1 µg/ml)
For I (0.5 µg/ml)
For I (1 µg/ml)
NO_2^- (µM)
Time (days)
(D)
Control
HCl (0.05 mM)
DMSO (0.05%)
Fas E (0.1 µg/ml)
Fas E (0.5 µg/ml)
Fas E (1 µg/ml)
NO_2^- (µM)
Times (days)

Figure 4.4: Sensitivity of '*Ca*. N. franklandus C13' to various fasamycin and formicamycin congeners. Experiments included 0.05 mM HCl or 0.05% DMSO as solvent controls for ciprofloxacin and fasamycins/formicamycins respectively. Ciprofloxacin **(A)** was used as a positive control. Fasamycin and formicamycin congeners tested include **(B)** Formicamycin J, **(C)** Formicamycin I, and **(D)** Fasamycin E. All sensitivity tests were performed in liquid cultures. Nitrite concentrations (NO_2^-) plotted represent the average of three replicate cultures. Error bars represent the standard error of mean (SEM) and may be smaller than the symbol.


(A)
Control
HCl (0.05 mM)
DMSO (0.05%)
Ciproflaxicin (0.1 µg/ml)
Ciproflaxicin (0.5 µg/ml)
Ciproflaxicin (1 µg/ml)
NO_2^- (µM)
Time (days)
(B)
Control
HCl (0.05 mM)
DMSO (0.05%)
For J (0.1 µg/ml)
For J (0.5 µg/ml)
For J (1 µg/ml)
NO_2^- (µM)
Time (days)
(C)
Control
HCl (0.05 mM)
DMSO (0.05%)
For I (0.1µg/ml)
For I (0.5µg/ml)
For I (1µg/ml)
NO_2^- (µM)
Time (days)
(D)
Control
HCl (0.05 mM)
DMSO (0.05%)
Fas E (0.1 µg/ml)
Fas E (0.5 µg/ml)
Fas E (1 µg/ml)
NO_2^- (µM)
Time (days)

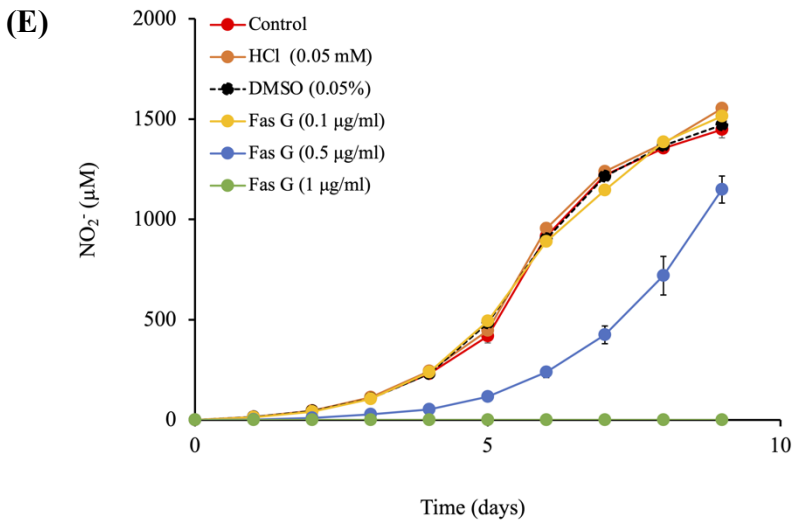


Figure 4.5: Sensitivity of *N. viennensis* EN76 to congeners of the fasamycin and formicamycin antibiotics. Experiments included 0.05 mM HCl or 0.05% DMSO as solvent controls for ciprofloxacin and fasamycins/formicamycins respectively. Ciprofloxacin **(A)** was used as a positive control. Fasamycin and formicamycin congeners tested include **(B)** Formicamycin J, **(C)** Formicamycin I, **(D)** Fasamycin E, and **(E)** Fasamycin G. All sensitivity tests were performed in liquid cultures. Nitrite concentrations (NO_2^-) plotted represent the average of three replicate cultures. Error bars represent the standard error of mean (SEM) and may be smaller than the symbol.

Table 4.3: Sensitivity of AOA to different fasamycin and formicamycin congeners.

	MIC (µg/ml)			
Strain	**For J**	**For I**	**Fas G**	**Fas E**
'*Ca*. N. franklandus C13'	**1**	**>1**	**ND**	**0.5**
N. viennensis EN76	**1**	**>1**	**1**	**0.5**

ND- Not determined.

4.4 Discussion

4.4.1 Selective agents for '*Ca*. N. franklandus C13'

The primary aim of this study was to identify a suitable selective agent(s) for '*Ca*. N. franklandus C13'. The choice to use an antibiotic-based selection system for the initial development efforts was because creating an auxotrophic mutant requires an already pre-existing genetic system (Fatma *et al*., 2020). In addition, no natural or spontaneous auxotrophic mutants of any AOA are currently known.

The potent inhibition of this strain by puromycin and hygromycin B highlights these antibiotics as potential candidates. While not as potent as puromycin and hygromycin B, the antibiotic neomycin may also be a suitable alternative should be considered in future studies. The antibiotic puromycin is widely used as a selection agent in methanogen genetic systems (Gernhardt *et al.*, 1990; Patel *et al.*, 1994; Argyle *et al*., 1996; Nayak and Metcalf, 2017). It is typically used at concentrations ranging between 2 - 10 µg/ml which is relatively low compared to those determined for '*Ca*. N. franklandus C13' in this work. Similarly, in eukaryotic systems, puromycin is used at concentrations at ranging around 10 µg/ml (De La Luna *et al.*, 1988). In practice, it

may be necessary to use higher concentrations (> 27 µg/ml) of puromycin as higher cell densities are likely to be used for the transformation experiments. Therefore, although puromycin is a strong candidate, further experimentation may be needed to determine appropriate concentrations based on factors such as cell density. In bacterial models such as *Mycoplasma* sp., the *pac* gene is capable of conferring resistance to the bacterium *M. capricolum* up to 500 µg/ml of puromycin (Algire *et al.*, 2009). Therefore, if the expression of the Pac protein is sufficient, and equally important if the protein is active, then using a higher puromycin concentration is unlikely to pose any problems.

In contrast, hygromycin B is not as widely used in archaeal selection systems but has been useful in the Sulfolobales (Atomi *et al*., 2012). An advantage of hygromycin B as a selection agent is the availability of a thermotolerant hygromycin phosphotransferase (*hpt*) (Cannio *et al*., 2001). This could be useful with thermophilic AOA strains. Typical concentrations of hygromycin B used for *S. solfataricus*, an important genetic model for the Sulfolobales, are around 300 µg/ml (Cannio *et al*., 2001). In bacterial models such as *E. coli*, hygromycin B is used at concentrations exceeding 140 µg/ml (Kalivoda *et al.*, 2011). These concentrations far exceed the concentrations needed to strongly inhibit '*Ca*. N. franklandus C13' (~11 µg/ml). More importantly, this indicates that the *hpt* resistance cassette is capable of conferring resistance to hygromycin B concentrations far exceeding those needed for '*Ca*. N. franklandus C13'. Although it remains to be investigated experimentally, the concentrations needed to inhibit '*Ca*. N. franklandus C13' by hygromycin are within a practical range.

The potent action of puromycin and hygromycin B make these two antibiotics attractive candidates for a selection system. However, it is still unclear at what rate (if at all) resistant '*Ca*. N. franklandus C13' mutants eventually develop. During incubation of '*Ca*. N. franklandus C13' for a prolonged period of time (~65 days), no evidence of mutant development was observed (*Appendix F*). However, this was done at lethal antibiotic concentrations and future work will need to monitor resistance development at sublethal concentrations which are more conducive to resistance development (Andersson and Hughes, 2014; Friman *et al.*, 2015). In methanogen models such as *M. maripaludis* and *M. voltae*, puromycin exhibited a low rate of mutant development (Possot *et al.*, 1988; Argyle *et al.*,1996). A low rate of development of resistant mutants is particularly desirable for '*Ca*. N. franklandus C13' cells as it takes approximately two months for visible colonies of '*Ca*. N. franklandus C13' to develop (see Chapter 3).

4.4.2 Antibiotic sensitivity patterns of three distinct AOA strains

Overall, the results in this study clearly indicate that antibiotic sensitivity patterns differ between phylogenetically and physiologically distinct AOA strains. Unfortunately, as only a single representative from each genus was tested, it is premature to suggest a lineage-dependent sensitivity pattern. Similarly, a structure-activity pattern particularly for the aminoglycosides was difficult to discern. For example, although all three strains were resistant to apramycin, a monosubstituted aminoglycoside, the two soil strains were sensitive to hygromycin B. Additionally, as stated previously, the 4,6 2-DOS aminoglycosides such as kanamycin and gentamycin are widely used to treat both AOA enrichments and pure cultures to inhibit bacterial growth. This widespread use of the 4,6 2-DOS aminoglycosides may suggest that the

AOA are generally resistant to this structural class. It was however recently shown that tobramycin, also a 4,6 2-DOS aminoglycoside, significantly reduced the abundance of AOA in an enrichment culture (Liu *et al.*, 2019). However, caution must be taken when interpreting this data as antibiotics may have indirect effects on AOA in mixed cultures as previously noted. It has also been observed that all three AOA strains exhibit sensitivity (to varying degrees) to neomycin, a member of the 4,5 2-DOS aminoglycosides. However, since only a single representative of the 4,5 2-DOS family was tested, a generalisation cannot conclusively be made.

Although a definitive lineage-dependent pattern could not be determined from this work, contrasting sensitivity patterns may have some important practical applications in the isolation of AOA. For example, during isolation efforts and maintenance of pure cultures, it is commonplace to use a cocktail of antibiotics to retard bacterial growth. This work has identified an additional antibiotic, apramycin, that does not inhibit any of the three AOA strains but has previously been shown to be highly effective against Gram-negative bacteria (Matt *et al.*, 2012; Yang *et al.*, 2020). Since all known bacterial nitrifiers are Gram-negative, apramycin presents a possible selective inhibitor of AOB which could outcompete the slow growing AOA in enrichment cultures. Furthermore, such selective inhibitors are highly desired when studying mixed communities (Zhao *et al*., 2020) and apramycin could be used to study AOA-specific activity in mixed communities. However, this needs to be experimentally validated. Lastly, if a lineage-dependent sensitivity pattern was eventually determined for the AOA, it could be used to deliberately introduce bias in the isolation efforts allowing the enrichment of a specific AOA lineage conducive to diversifying culture collections.

It is unsurprising that the AOA strains do not share antibiotic sensitivity patterns as they are not only phylogenetically, but also physiologically distinct. An example of a physiological distinction that may contribute towards contrasting sensitivities is the outer cell envelope. The outermost cell envelope is an important cellular structure that controls the influx/efflux of different molecules and acts as a 'molecular sieve'. While '*Ca*. N. franklandus C13' is surrounded by a yet uncharacterised outer cell membrane (Nicol *et al.*, 2019), both *N. viennensis* EN76 and *N. maritimus* SCM1 are enveloped by a proteinaceous surface layer (S-layer). However, the S-layers present in these two strains differ in their symmetry i.e. P3 and P6 symmetry respectively (Stieglmeier *et al.*, 2014; Qin *et al.*, 2017). Whether contrasting S-layer symmetry influences antibiotic sensitivity is unclear.

The P6 (or hexagonal) S-layer symmetry is common among methanogenic and halophilic archaea (Rodrigues-Oliveira *et al.*, 2017). It is therefore interesting that *N. maritimus* SCM1 shares similar antibiotic sensitivity patterns with several methanogenic and halophilic strains. For example, the methanogenic strains *Methanococcus voltae*, *Methanosarcina acetivorans* and *Methanosarcina mazei* all have P6 symmetry and are all sensitive to puromycin but only mildly or completely unaffected by hygromycin B (Possot, *et al*., 1988; Mondorf *et al*., 2012; Nayak and Metcalf, 2017). In comparison, *N. viennensis* EN76 with P3 S-layer symmetry shares antibiotic sensitivities with other archaea that contain P3 S-layer symmetry such as the Sulfolobales (Cannio *et al.*, 1998, 2001).

Whether S-layer symmetry influences antibiotic sensitivity could be investigated using various methods. For example, using cell-free systems one can determine whether in the absence of the cell wall barrier the antibiotics are able to interact with their

molecular target(s). Such an approach has previously been used to determine the sensitivity of archaeal ribosomes to protein synthesis inhibitors (Cammarano *et al.*, 1985; Londei *et al.*, 1988). Therefore, if it is determined that whole cells are resistant while the ribosomes (cell-free models) are susceptible to the antibiotic, then this may suggest that the antibiotic is not able to cross the cell wall barrier. Alternatively, the use of antibiotics conjugated to fluorophores has been an important experimental approach for bacterial pathogens to determine the mode of action and resistance mechanism (Miao *et al.*, 2020). These fluorophores could be used to visualise antibiotic uptake using fluorescent microscopy.

An additional factor to consider is the growth medium used for these strains. While '*Ca*. N. franklandus C13 and *N. viennensis* EN76 grow in virtually identical growth medium, *N. maritimus* SCM1 requires a growth medium with a higher salt content and ionic strength. These conditions have previously been reported to influence the activity of aminoglycoside antibiotics (Coronado *et al.*, 1995). The authors demonstrated in thirteen halophilic bacterial strains that when the salt concentration in the medium was lowered from 10% to 1%, the minimal inhibitory concentration of the aminoglycoside antibiotics decreased. The authors similarly demonstrated this effect in *E. coli*. It is therefore possible that the salt concentration of the growth medium may be a contributing factor to the different sensitivity patterns of the AOA strains.

As noted previously, aminoglycosides inhibit protein synthesis by interacting with the ribosome machinery such as the 16S rRNA of the small 30S subunit (Kulik *et al.*, 2015). Since 16S rRNA is a phylogenetic marker, the contrasting sensitivities of the AOA to aminoglycosides may be attributed to sequence variation in this phylogenetic marker. Indeed, structural studies of aminoglycosides bound to their ribosomal targets

in bacterial models have identified critical nucleotides that influence sensitivity of microorganisms to aminoglycosides (Alangaden *et al.*, 1998; Recht and Puglisi, 2001). Although beyond the scope of this study, it would be interesting to explore whether differences at these critical nucleotides plays a role in the sensitivity patterns of AOA to aminoglycosides. In addition to mutations in the rRNA, resistance to aminoglycosides is also mediated by aminoglycoside modifying enzymes such as phosphotransferases, acetyl-CoA dependent aminoglycoside acetyltransferases and aminoglycoside nucleotidyltransferases (Becker and Cooper, 2013). It is, however, challenging to assign these functions to particular genes in the absence of relevant genetic studies that are dependent on the availability of a genetic system.

4.4.3 Sensitivity to topoisomerase inhibitors

DNA topoisomerases can be found in all domains of life, where they are involved in altering and regulating the topological state (e.g. supercoiling) of DNA to facilitate essential processes such as replication, transcription, and recombination (Deweese *et al*.,2009; Evans-Roberts and Maxwell, 2009). These enzymes can be classified as either type I or type II depending on whether they create a transient single or double-stranded break in DNA (Champoux, 2001). Type I topoisomerases function independently of ATP hydrolysis while type II topoisomerases are dependent on ATPase activity (Baker *et al*., 2009). For reference, the general classification of prokaryotic DNA topoisomerases is provided in *Appendix G*.

An important target for antibacterial compounds is DNA gyrase due to its absence in mammalian cells (Maxwell, 1997; Khan *et al.*, 2018). DNA gyrase is classified as a type IIA topoisomerases and thus requires ATP to cut both strands of DNA to resolve DNA tangle and supercoils (Vos *et al.*, 2011; Vanden Broeck *et al.*, 2019). The

antibiotics novobiocin and ciprofloxacin are well-known inhibitors of DNA gyrase but target different sites (Serizawa *et al.*, 2010). Novobiocin targets the ATPase domain situated within a structural region known as the Bergerat fold of DNA gyrase subunit B (*Gyr*B) (Maxwell and Lawson, 2003). Aside from DNA gyrase, the Bergerat fold may also be found in other proteins such as Hsp90, Histidine Kinases, and MutL that are collectively known as 'GHKL' (**G**yrase, **H**sp90, Histidine **K**inase and Mut**L)** **(**Dutta and Inouye, 2000). In contrast, ciprofloxacin targets DNA gyrase subunit A (*Gyr*A) hindering its ability to repair the double-stranded break leaving the single-strands vulnerable to exonuclease degradation (Shariati *et al.*, 2022).

Interestingly, it has recently been established that DNA gyrase is absent from the archaeal phylum Thaumarchaeota (Villain *et al.*, 2022). Therefore, while the resistance to ciprofloxacin is to be expected, the sensitivity to novobiocin by all three AOA strains was rather surprising and may indicate the presence of an alternative target(s) in these archaea. A likely target for novobiocin in these strains is a type II topoisomerase due to the presence of a Bergerat fold in these enzymes. The only type I topoisomerase that is ATP-dependent is the reverse gyrase, but this is enzyme is exclusively present in hyperthermophilic prokaryotes (Schoeffler and Berger, 2008).

As summarised in *Table 4.4*, topoisomerase VI is the sole type II topoisomerase annotated in the genomes of the two AOA strains. Topoisomerase VI is widely distributed in the archaea and may co-exist with DNA gyrase (Garnier *et al.*, 2021). Topoisomerase VI is the only representative of the type IIB topoisomerases and forms an A_2B_2 heterotetramer (Champoux, 2001; Corbett and Berger, 2003). Since topoisomerase VI contains a Bergerat fold, it is tempting to propose topoisomerase VI as the likely target for novobiocin in these archaea. Unfortunately, previous *in vitro*

tests on topoisomerase VI from *Sulfolobus shibatae*, showed that this enzyme is insensitive to novobiocin (Bergerats *et al*., 1994). While this insensitivity could be attributed to the high temperatures at which these assays were conducted (i.e. 80ºC), the authors reported that novobiocin was stable for at least 20 minutes under these conditions. Furthermore, the topoisomerase VI from the mesophilic archaeon *Methanosarcina mazei* is also insensitive to novobiocin (Dmitry Ghilarov (JIC), personal communication). Thus, topoisomerase VI is an unlikely target for novobiocin in the AOA.

Based on these observations, it may be necessary to consider that novobiocin may be targeting other Bergerat fold-contain proteins such as those within the GHKL family mentioned previously. For example, novobiocin has been reported to bind to the bacterial histidine protein kinase EnvZ, albeit with a weaker affinity than to *Gyr*B of DNA gyrase (Plesniak *et al.*, 2008). Since several histidine kinases are annotated in the genomes of these AOA strains (data not shown), they could be potential targets for novobiocin. Another member of the GHKL family of proteins is MutL which is part of the mismatch repair system (Ishino *et al.*, 2018). Novobiocin has been shown to bind to this protein with a low affinity (Ban and Yang, 1998). It has been established that the MutL repair system is not widespread in archaea which instead may use the EndoMS endonuclease-based system (Pérez-Arnaiz *et al.*, 2020). To the best of our knowledge no literature is currently available on the interaction between novobiocin and the EndoMS endonuclease protein.

It should also be noted that the Bergerat fold-containing proteins do not all necessarily exhibit similar sensitivity patterns. For example, while the drug radicicol targets the Bergerat fold of the human Hsp90 and the archaeal topoisomerase VI (Sharma *et al*.,

1998; Gadelle *et al.*, 2005), it has no effect on DNA gyrase (Gadelle *et al.*, 2005). Additionally, geldanamycin, also a Hsp90 inhibitor, has no effect on topoisomerase VI as it is too big to interact adequately with the target site (Gadelle *et al.*, 2005). Thus, other factors play a role in the interaction of the Bergerat fold with different compounds.

Interestingly, the sensitivity to novobiocin does not seem to be a general trait among the AOA. For example, an enrichment of the thermophilic AOA strain '*Ca*. Nitrosocaldus cavascurensis' that grows optimally at 68ºC exhibited no response to treatment with 100 µg/ml of novobiocin (Abby *et al.*, 2018). Although it has previously been noted that novobiocin is stable at elevated temperatures (i.e. ~80ºC) for several minutes, it may be possible that its activity gradually diminishes following extended incubation periods under such conditions. Alternatively, novobiocin may be degraded by other microorganisms in the enrichment or have a higher affinity for the bacterial DNA gyrase present in the bacterial community of the enrichment.

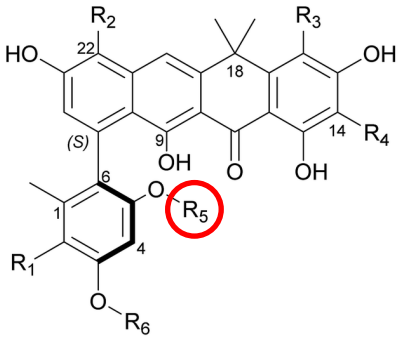
4.4.3.1 AOA sensitivity to fasamycins and formicamycins

Recent data have revealed that both the fasamycins and formicamycins are potent inhibitors of bacterial type II topoisomerase inhibitors (i.e. DNA gyrase), preventing its ability to bind DNA (Matt Hutchings, Personal communication). Their ability to target topoisomerases is particularly significant as these enzymes are essential for cellular processes such as DNA processing and are therefore attractive targets for drug development. Due to their potential as drug candidates, efforts are currently underway to determine the extent of their biological activity and whether structural congeners have alternative molecular targets or mechanisms of action. In this regard, archaea from the phylum Thaumarchaeota are an interesting model to test as they lack DNA

gyrase and contain a distinct topoisomerase repertoire to those found in bacteria (Villain *et al*., 2022). For example, in addition to the previously described topoisomerase VI, AOA also contain the unique topoisomerase IB which is scarcely found in bacteria (Brochier-Armanet *et al*., 2008).

All the fasamycins and formicamycins congeners tested in this study exhibited potent anti-archaeal activity against '*Ca*. N. franklandus C13' and *N. viennensis* EN76 with very low MIC values. This is in contrast to all the other antibiotics to which these strains were sensitive and could be attributed a higher affinity to their targets. It is also possible that these compounds are taken up into the cell efficiently based on the fact that they are dissolved in DMSO which is known to increase the permeability of the cell membrane (Notman *et al.*, 2006; Mi *et al.*, 2016). Additionally, it has been established that the activity of these compounds is generally enhanced by the increasing number of halogen atoms (usually chlorine) at positions R1 - R5 (*Figure 4.6*) and is likely linked to their ability to cross cell envelopes and enter the cells (Matt Hutchings, personal communication). Interestingly however, fasamycin E has the least number of Cl atoms (only at positions R1 and R2) but exhibits highly potent activity with an MIC of 0.5 µg/ml against both AOA strains. When exclusively considering the formicamycins, a methyl-side group at position R5, as seen in Formicamycin J, seems to be associated with stronger activity when compared to the presence of a hydrogen atom at this position (formicamycin I). Scaffolds of the fasamycins and formicamycins are illustrated in *Figure 4.6*. A focus of future work will need to test the numerous structural congeners available (with different combinations of R-side groups) to reveal the effects of these changes on their biological activity.

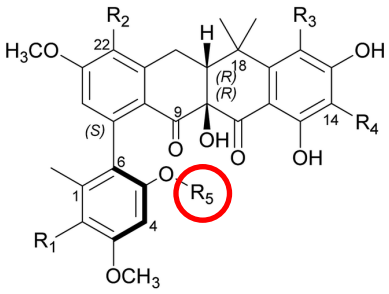
Fasamycin



Fasamycin E:

R1= Cl, **R2** = Cl, **R3** = H, **R4** = H, **R5** = H, **R6** = CH_3

Formicamycin



Formicamycin I:

R1 = Cl, **R2** = Cl, **R3** = Cl, **R4** = Cl, R5 = H

Formicamycin J:

R1 = Cl, R2 = Cl, R3 = Cl, R4 = Cl, R5 = CH_3

Figure 4.6: General chemical structure of the novel fasamycin and formicamycins antibiotics. An increasing number of halogen atoms (particularly chlorine) at positions R1-R5 have been associated with increased activity of these compounds. For comparison, different side groups present in the formicamycin congeners tested in this study are highlighted in red. The structure of fasamycin G is currently unknown. Structures were obtained from (Qin *et al*., 2017).

More interesting is the highly potent activity against the AOA strains despite lacking the type II topoisomerase DNA gyrase, a known target for these compounds. This may suggest the presence of an alternative target for the fasmaycins and formicamycins in archaea. As the sole type II topoisomerase annotated in the genomes, topoisomerase VI is a strong candidate target for these compounds. The low MIC may also indicate that the unknown target is an essential component of the cell. This is supported by the fact that topoisomerase VI is the only topoisomerase encoded in the genomes of the two AOA strains that is capable of decatenase activity (McKie *et al*., 2022). Whether topoisomerase VI is essential to the AOA can easily be determined by testing the response of these archaea to radicicol, a known inhibitor of topoisomerase VI (Gadelle

et al., 2005). However, to confidently identify the target of these compounds in archaea, it will be necessary to perform *in vitro* assays with the purified AOA topoisomerase VI and structure determination of the compound-enzyme complexes.

Table 4.4: List of genes annotated as topoisomerases in the three AOA strains.

Strain	Annotated ORFs (MicroScope)	Type	BLASTp hit	Accession no.	% Identity
'*Ca*. N. franklandus C13	DNA topoisomerase I, eukaryotic type (NFRAN_v2_0095)	**I**	• DNA topoisomerase I (Candidatus Nitrosocosmicus franklandus)	WP_232037898.1	99.83
			• DNA topoisomerase I (Thermoproteota archaeon, MAG)	MDQ2684725.1	79.48
			• DNA topoisomerase I (Candidatus Nitrosocosmicus sp., MAG)	NOJ31060.1	82.08
	DNA topoisomerase I (NFRAN_V2_0625)		• DNA topoisomerase I (topA) (Candidatus Nitrosocosmicus franklandus)	WP_269472330.1	100
		I	• DNA topoisomerase I (topA) (Candidatus Nitrosocosmicus franklandus)	VFJ12947.1	99.87
			• DNA topoisomerase I (Candidatus Nitrosocosmicus sp., MAG)	MDR4492235.1	65.34
	DNA topoisomerase VI subunit A (NFRAN_v2_1367)	**II**	• Hypothetical protein (region, DNA topoisomerase IV subunit A, Candidatus Nitrosocosmicus franklandus)	WP_134483663.1	99.73
			• Hypothetical protein region, DNA topoisomerase IV subunit A, Candidatus Nitrosocosmicus hydrocola)	WP_148685627.1	95.63

			• Hyporthetical protein MY3_04595 (MAG) Thermoproteota archaeon	MDQ2685104.1 95.63

Table 4.4: Continued.

	Type 2 DNA topoisomerase 6 subunit B (modular protein) (NFRAN_v2_1368	II	• Type 2 DNA topoisomerase 6 subunit B (Candidatus Nitrosocosmicus franklandus) • DNA topoisomerase VI subunit B (Candidatus Nitrosocosmicus franklandus) • DNA topoisomerase VI subunit B (Candidatus Nitrosocosmicus Hydrocola)	VFJ13690.1 100 WP_172602159.1 100 WP_148685626.1 66.81
N. viennensis EN76	DNA topoisomerase IB (NVIE_015110)	**I**	• DNA topoisomerase I [Nitrososphaera viennensis] • DNA topoisomerase I [Candidatus Nitrososphaera evergladensis] • DNA topoisomerase I [uncultured Nitrososphaera sp.].	WP_227717284.1 100 WP_226987022.1 95.20 WP_294804997.1 95.20

Table 4.4: Continued.

	DNA topoisomerase IA (NVIE_020340)	**I**	• DNA topoisomerase I [Nitrososphaera viennensis]. (gene: topA)	WP_075055085.1	99.86
			• DNA topoisomerase I [Candidatus Nitrososphaera evergladensis]. (gene: topA)	WP_148699231.1	88.98
			• DNA topoisomerase I, partial [uncultured Nitrososphaera sp.]	WP_294806687.1	87.95
	Topoisomerase IIB subunitA (NVIE_028330)		• hypothetical protein [Nitrososphaera viennensis]	WP_075055726.1	100
			• hypothetical protein [Candidatus Nitrososphaera evergladensis].	WP_148699972.1	98.64
			• hypothetical protein [uncultured Nitrososphaera sp.].	WP_294807652.1	98.37
	Topoisomerase IIB subunitB (NVIE_028340)		• DNA topoisomerase VI subunit B [Nitrososphaera viennensis].	WP_075055727.1	99.85
			• DNA topoisomerase VI subunit B [Candidatus Nitrososphaera evergladensis].	WP_158385118.1	94.86
			• DNA topoisomerase VI subunit B [uncultured Nitrososphaera sp.].	WP_294807649.1	94.39

4.5 Summary and outlook

In this chapter, we have identified two main antibiotics puromycin and hygromycin B as potential selective agents for '*Ca*. N. franklandus C13'. The inclusion of additional AOA strains in the antibiotic sensitivity testing efforts revealed that these archaea differ in their sensitivity patterns. However, due to insufficient data, a lineage-dependent sensitivity pattern could not be discerned. Nevertheless, contrasting sensitivity patterns may have some useful practical applications in AOA research. Overall the antibiotic sensitivity testing of AOA reveals that they are sensitive to protein and DNA synthesis inhibitors similar to archaea from the major phyla. We have also shown for the first time, the anti-archaeal activity of the fasamycins and formicamycins, a novel group of compounds with promising pharmaceutical potential.

A few outstanding questions are still unanswered and will be the focus of future studies. For example, it will be necessary to determine whether spontaneous puromycin and hygromycin B resistant mutants develop, to better gauge the long-term use of these selective agents. Additionally, more strains will need to be tested to determine whether a lineage-dependent sensitivity pattern is present among the AOA. Finally, and more importantly, structural studies will need to be conducted to determine the (presumably protein) molecular targets for the fasamycins and formicamycins antibiotics.

Chapter 5: Construction and validation of plasmids for '*Ca*. N. franklandus C13'

5.1 Introduction

Plasmids are linear or circular DNA molecules that can exist and replicate independently of the main host chromosome (Shintani *et al*., 2015). These molecules play an important role in the exchange of genetic information between microorganisms and have been discovered in all domains of life (Griffiths, 1995; Frost *et al.*, 2005; Wang *et al.*, 2015). In molecular biology, plasmids are useful as 'molecular vehicles' that can transfer genetic information into or between cells which facilitates the introduction of genetic modifications such as increased protein expression, deletions and even insertions making them an indispensable element of a genetic toolbox.

Presently, all known archaeal plasmids originate either from the Euryarchaeota (e.g. methanogens and *Thermococcus* sp.) or Crenarchaeaota (e.g. Sulfolobales). This is in contrast to the Thaumarchaeota for whom plasmids are yet to be discovered. This lack of plasmids presents as a major bottleneck for the development of genetic tools because they provide several advantages. Firstly, native plasmids are guaranteed to contain the necessary replication and maintenance machinery. Secondly, the presence of native plasmids is a promising indicator that the strain has the ability to maintain such extrachromosomal genetic material. In addition, native plasmids can be reengineered into shuttle vectors or expression vectors that are important for genetic studies.

In the absence of native plasmids, a plausible solution is to construct artificial plasmids. Shuttle vectors are a particularly attractive choice, as they facilitate the manipulation and propagation of the plasmids in a well-established model strain such as *E. coli* prior to their transfer to the desired host (Scott *et al*., 2021). Some of the basic components of a functional shuttle vector include a replication origin for each of

the hosts, a selectable marker and gene expression elements such as promoters, terminators, and ribosome-binding sites (RBS) (Yan and Fong, 2017).

Since there are no plasmids currently available for the AOA, the chromosomal replication origin would need to be used to facilitate autonomous replication in the host cells. Plasmids under the control of a chromosomal replication origin have successfully been used in both bacterial and archaeal models (Zakrzewska-Czerwińska *et al.*, 1995; Lee *et al*., 2008; Farkas *et al.*, 2011). Archaeal replication origins are AT-rich genomic loci that are flanked by unique repeated sequence motifs known as origin recognition boxes (ORB), to which the initiator protein Orc1/Cdc6 binds (Robinson *et al.*, 2004; Barry and Bell, 2006; Lestini *et al*., 2015). The Orc1/Cdc6 protein shares homology to both subunit 1 of the eukaryotic origin recognition complex (Orc1) and Cdc6 (Robinson *et al.*, 2004; Ausiannikava and Allers, 2017). The eukaryotic Orc1 promotes recognition of the replication origin whereas Cdc6 recruits the replicative helicases (Lestini *et al*., 2015). Since the archaeal Orc1/Cdc6 shares homology to both these proteins, it is expected to be capable of performing both functions (Lestini *et al*., 2015). Unlike bacteria, archaea may contain multiple replication origins within their genomes (Robinson *et al.*, 2004; Norais *et al.*, 2007; Pelve *et al.*, 2013).

Various methods are available to map the location of replication origins and may include marker-frequency analysis (Lundgren *et al*., 2004; Coker *et al*., 2009; Pelve *et al*., 2013), 2D gel electrophoresis and chromatin immunoprecipitation (Matsunaga *et al.*, 2001), Z-curve analysis (Luo *et al*., 2014; Luo *et al.*, 2019) and autonomously replicating sequence activity assays (ARS) (Stinchcomb *et al.*, 1980; Berquist and DasSarma, 2003). The experimental approaches are technically challenging and time-consuming (Gao, 2014) and are more suited to strains for whom genetic tools already

exist. For example, the ARS method was previously used to characterise a chromosomal replication origin in the haloarchaeon *Halobacterium* strain NRC-1 (Berquist and DasSarma, 2003).

In comparison, mapping of replication origins using *in silico* based methods such as the Z-curve analysis are more suited to novel strains or those for whom genetic systems are yet to be established. Additionally, *in silico* methods may also be useful in instances where experimental techniques (e.g. marker frequency analysis) fail as previously seen with the archaeon *Methanocaldococcus jannaschii* (Maisnier-Patin *et al*., 2002). The Z-curve method, developed in 1994, is a geometrical approach to the analysis of a DNA sequence and produces a graphical three-dimensional representation of any given DNA sequence (Zhang and Zhang, 1994; Zhang and Zhang, 2014). Unlike similar methods such as GC-skew analysis, the Z-curve method takes into consideration all four nucleotides (Gao, 2014).

A Z-curve is generated by plotting a series of nodes i.e. P_0, P_1, P_2....P_N (whereby '*N*' represents the length of the DNA sequence being analysed) on the coordinates *x*, *y* and *z* that are determined by previously described mathematical formulas (Zhang and Zhang, 1994; Zhang and Zhang, 2004). The resulting graphs reflect the genomic distribution (or disparity) of different nucleotide groups within a given DNA sequence. These nucleotide groups are based on the biochemical properties of the four nucleotides (A, T, G and C) and include purine/pyrimidines (R/Y), amino/keto (M/K) and weak/strong hydrogen bonds (S/W) which correspond to the *x*, *y* and *z* coordinates respectively (Zhang and Zhang, 2014). These nucleotide classifications are summarised in *Table 5.1*. The nucleotide disparity curves (R/Y, M/K, S/W) may also

be plotted individually creating two-dimensional plots. Note: curves depicting the S/W nucleotide distribution may also be plotted as individual AT and GC disparity curves.

Based on the extensive work done using the Z-curve to accurately locate the replication origins in bacterial and archaeal genomes, replication origins can typically be found in regions where a change in polarity of the nucleotide distribution visible (i.e. global maximum or minimum of the Z-curve) (Zhang *et al*., 2003: Zhang and Zhang, 2014). Additionally, DNA replication genes (e.g. the archaeal *cdc6* or bacterial *DnaA*) are frequently present in the vicinity of these regions (Zhang and Zhang, 2005). Notable examples of model archaeal strains for which the Z-curve has successfully been used to predict replication origin include *Halobacterium* NRC-1 (Zhang and Zhang, 2003), *S. acidocaldarius* (Chen *et al*., 2005), *M. jannaschii* (Zhang and Zhang, 2004) and *M. mazei* (Zhang and Zhang, 2002). Freely available web-based tools (e.g. Ori-Finder), are based on the Z-curve algorithms and are currently available to assist in the prediction and location of replication origins in bacterial and archaeal genomes (Gao and Zhang, 2008: Luo *et al*., 2014: Dong *et al*., 2022).

Table 5.1: Nucleotide classification groups used to generate Z-curves.

	Nucleotide classification					
	Purine	**Pyrimidine**	**Amino**	**Keto**	**Strong hydrogen bonds**	**Weak hydrogen bonds**
Letter code	R	Y	M	K	S	W
Nucleotide	A/G	C/T	A/C	G/T	G/C	A/T

Due to the lack of pre-existing genetic tools, functional characterisation of gene expression elements such as promoters, transcription terminators and RBS is not yet feasible for the AOA. However, since the location of such elements can usually be predicted (i.e. 5' UTR for promoters/RBS and 3'UTR for transcription terminators), the corresponding genomic loci can be amplified by PCR and fused to the desired genes on the plasmid. The core archaeal promoter elements to which transcription factors bind include a TATA-box located ~26 - 30 bp upstream of the transcription start site and the transcription factor B-responsive element (BRE) immediately upstream of the TATA-box (Reiter *et al*., 1990; Bell and Jackson, 1998; Bell *et al.*, 1999; Juven-Gershon *et al.*, 2008). These promoter motifs can be detected using *in silico* approaches (Bartossek *et al.*, 2012; Sganzerla Martinez *et al.*, 2023). However, a caveat to using an *in silico* approach for promoter detection is that recognisable motifs may not always be present (Sganzerla Martinez *et al.*, 2023).

Like bacteria, archaeal transcription termination may either be intrinsic or rely on additional protein factors also referred to as factor-dependent termination (Thomm *et al*., 1993; Santangelo *et al.*, 2009; Walker *et al*., 2017). The intrinsic termination signals in archaea consist of stretches of thymine (i.e. oligo (dT)) (Wich *et al.*, 1986; Brown *et al.*, 1989). This was further supported by the work of Thomm and colleagues (1988), who created deletion mutants of the wild-type termination signal (5'-TTTTATTTT-3') from the $tRNA^{val}$ gene of *Methanococcus vannielii* confirming the role of oligo (dT) signals in transcription termination. Interestingly, although both bacterial and archaeal transcription termination rely on oligo (dT) stretches, the preceding RNA-hairpin-forming inverted repeats were dispensable in the archaeon *Thermococcus kodakarensis* (Santangelo *et al.*, 2009). However, the ability of RNA hairpin structures that are typical in bacterial intrinsic terminators to replace oligo (dT)

terminators in methanogenic archaea suggests that these structures may still play an important role in archaea (Thomm *et al*., 1993). These studies suggest that archaeal transcription termination mechanistically resembles that employed by RNA polymerase III which is not factor-dependent but rather depends on intrinsic sequence signals (Santangelo and Reeve, 2006; Berkemer *et al.*, 2020).

CRISPR-Cas is an RNA-guided prokaryotic adaptive immune system that is highly abundant in archaea (Makarova *et al.*, 2020). A detailed description of the mechanism of the CRISPR-Cas immune system is provided in Chapter 1. CRISPR-Cas systems, particularly class 2 systems (e.g. Cas9), have been developed into a highly efficient genome-editing tool and are widely used in both bacterial and eukaryotic models (Mali *et al.*, 2013; Li *et al.*, 2015). Unlike the class 1 CRISPR-Cas systems, class 2 systems (e.g. Cas9) are particularly attractive due to their simplicity and rely on a single effector protein. In archaea however, the use of CRISPR-Cas is not nearly as prevalent and is limited to a handful of strains in the two major archaeal phyla. Furthermore, the CRISPR-Cas systems used for these archaea are often endogenous class 1 CRISPR-Cas systems (Zebec *et al.*, 2014; Stachler *et al.*, 2017). A major disadvantage of endogenous CRISPR-Cas systems is that they can vary significantly between closely related strains and therefore have limited use (Nayak and Metcalf, 2017). Furthermore, since the endogenous CRISPR-Cas systems that have been used for archaea originate from extremophilic strains they might require significant adaptation to function in a mesophilic host. It is for this reason that the recent development of class 2 CRISPR-Cas genome-editing tools for the archaea is so important (Nayak and Metcalf, 2017; Bao *et al*., 2022). The mesophilic nature of many AOA strains, the low genetic load needed for class 2 CRISPR-Cas systems, and the high-efficiency associated with this platform, makes class 2 CRISPR-Cas systems an ideal choice for the Thaumarchaeota.

5.2 Chapter aims

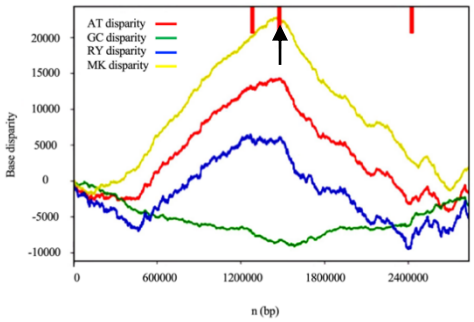
The primary goal of this study was to design and assemble artificial plasmids for '*Ca*. N. franklandus C13' which included a CRISPR-Cas9-based plasmid. In addition, we describe the design of two *mCherry* reporter gene plasmids to aid in the characterisation of transcriptional elements and detect DNA uptake.

5.3 Results

5.3.1 Mapping the '*Ca*. N. franklandus C13' origin of replication

The putative chromosomal replication origin of '*Ca*. N. franklandus C13' was mapped using the *in-silico* Z-curve method. The resulting (R/Y), (M/K) and (S/W) disparity plots predicted the presence of a single replication origin at the global maximum (*Figure 5.1A*). Note that S/W plots are labelled as AT and GC disparity in the Z-curve. Adjacent to the predicted replication origin was an ORF encoding a *cdc* gene (sometimes annotated as *orc1*/*cdc6*) (Zhang and Zhang, 2005; Luo *et al*., 2014). The predicted replication origin encompasses a 518 bp region that spans the first 278 bp of the adjacent *cdc* gene (ORF NFRAN_1687) and the entire downstream intergenic region between the *cdc* gene and ORF NFRAN_1688 as illustrated in *Figure 5.1B*. Three predicted ORB motifs were present in the downstream intergenic region of the *cdc* gene.

(A) Nucleotide disparity plots (Z-curve)



(B) Location of the '*Ca*. N. franklandus C13' origin

aatattctccattttaataaaaaaaaatcgagtgacacacccctttttcttctaatcgaggagcaataagataataccatgttaatttactctttgttatt
aacaatatttcttaaacactataatcaactaatttagacatatcaataaaaaataataatacaattttaatcttaattatttatttttaac**TGCATGA**
GAAATTATGGGGTgaccgtttgtaacttgtattaacaataacattaacagataaatgatatttgataataagagtgatttaa**CCCC**
CTTTATTTCCAGTGGAgagacattaaattaattcacaatgataataagagtgatttaa**CCCCTTTATTTCCAG**
TGGAacatgttaaactctttaacaataaaccaagttatcaaaagagaaaagtgaaattaaaatccaagcacaattttgttaacaaaagctcat
catttggttatcaaaatttgttctataatgttaagttaacatacacactaatattcacagataagataga

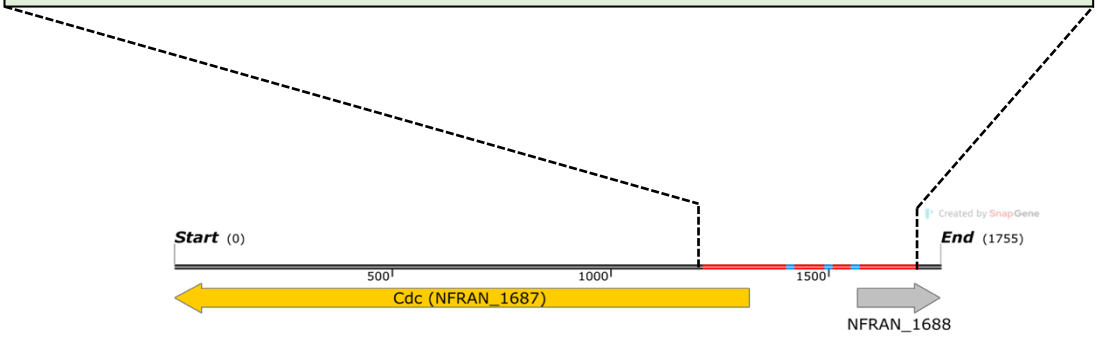


Figure 5.1: Mapping of the '*Ca*. N. franklandus C13' origin of replication using the Z-curve analysis method. **(A)** Z-curve showing the nucleotide disparity of AT, GC, RY and MK in the genome of '*Ca*. N. franklandus C13' where **AT** = A vs. T nucleotides, **GC** = G vs C nucleotides, **RY** = purines vs pyrimidines and **MK** = amino vs keto containing nucleotides. The **S/W** disparity is presented as separate AT and GC disparity plots. The red vertical lines at the top of the Z-curve indicate the location of replication associated genes within the genome while the black arrow shows the location of the origin (global maximum). The Z-curve was generated using the web-based tool ORI-Finder 2. **(B)** Genomic context and sequence of the predicted replication origin including ORB motifs. The ORB motifs are located in the upstream intergenic region of the *cdc* gene (NFRAN_1687) and are highlighted in blue or bold (uppercase). The start codon of the *cdc* gene is highlighted in red.

5.3.2 *cis*-DNA regulatory elements

5.3.2.1 Promoter regions

Plasmid encoded genes were placed under the control of the sequences corresponding to the 5' untranslated region (5' UTR) of highly expressed genes encoding ammonia monooxygenase subunit A (*amoA*), ammonia monooxygenase subunit B (*amoB*) and thermosome subunit B (*thsB*) in '*Ca*. N. franklandus C13'. The 5' UTR is expected to contain the necessary promoter elements to drive transcription including the BRE and TATA-box. The rationale behind the use of promoters from highly expressed genes is that such genes are expected to be under the control of strong promoters. Where possible, > 100 bp of the 5' UTR was used to increase the likelihood of capturing all the necessary elements (*Figure 5.2*). Sequence alignments detected the presence of BRE and TATA-box promoter elements in the 5' UTR of all three sequences (i.e. *amoA*, *amoB* and *thsB*) (*Figure 5.3*). Since the RBS are found downstream of promoters, and the sequences immediately upstream of the start codon were included, RBS were predicted to be present without any further analysis.

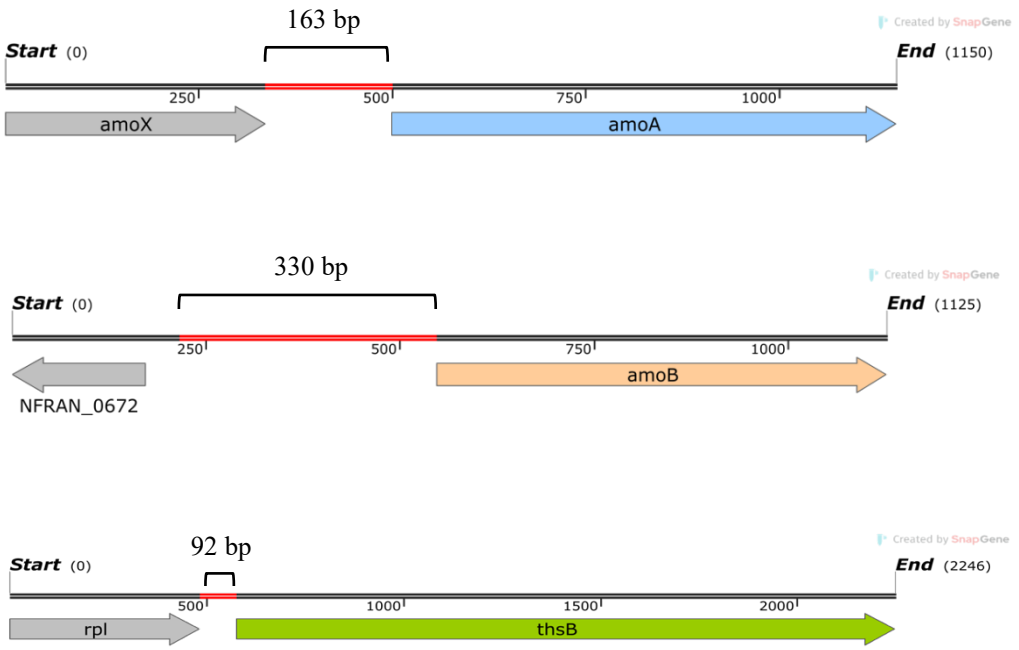
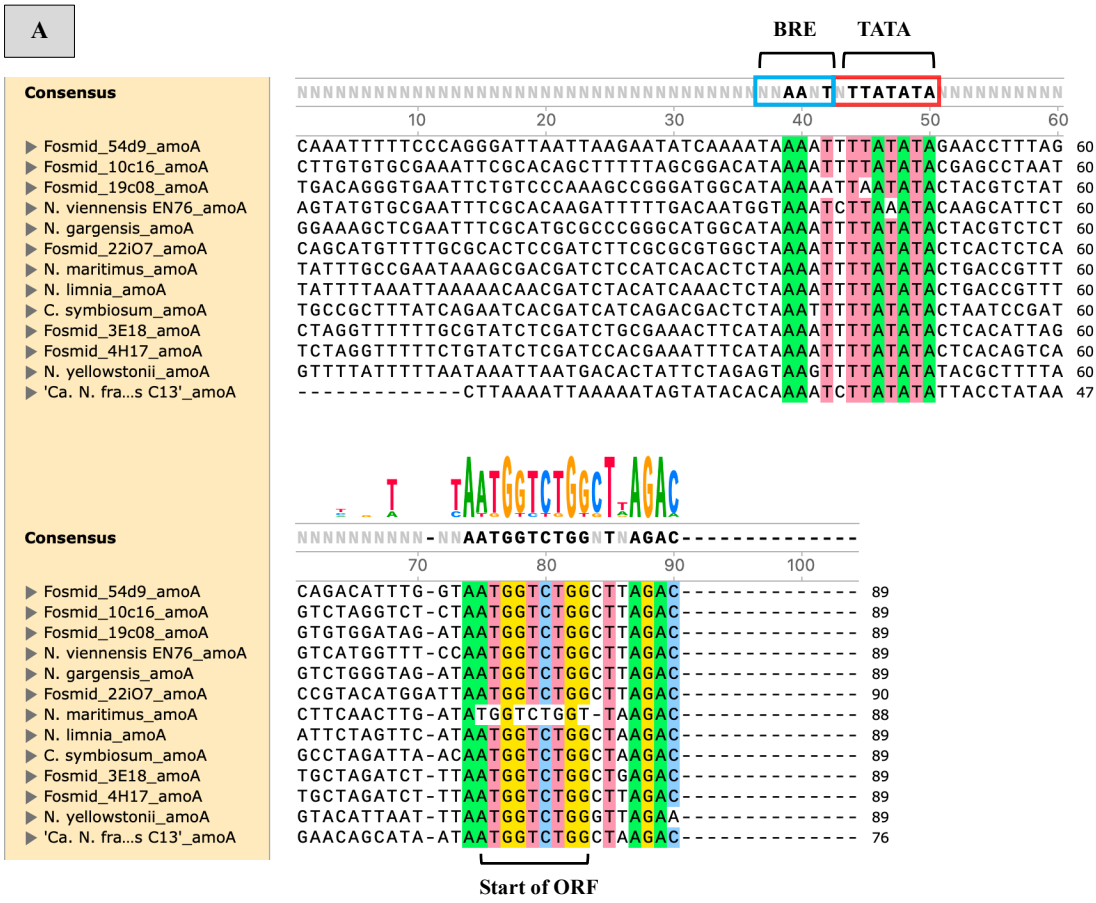


Figure 5.2: The 5' UTR regions expected to contain promoter elements used in this study. The upstream regions (> 100 bp) of highly expressed genes in '*Ca*. N. franklandus C13' were selected as promoters and are highlighted in red. Highly expressed genes include the genes for ammonia monooxygenase subunit A and B (*amo*A, *amo*B) and thermosome subunit B (*ths*B).


A
BRE
TATA
Consensus
Fosmid_54d9_amoA
Fosmid_10c16_amoA
Fosmid_19c08_amoA
N. viennensis EN76_amoA
N. gargensis_amoA
Fosmid_22i07_amoA
N. maritimus_amoA
N. limnia_amoA
C. symbiosum_amoA
Fosmid_3E18_amoA
Fosmid_4H17_amoA
N. yellowstonii_amoA
'Ca. N. fra...s C13'_amoA
Start of ORF
B
BRE
TATA
54d9_amoB
19c08_amoB
N.viennensis_amoB
22i07_amoB
N.maritimus_amoB
N.limnia_amoB
C.symbiosum_amoB
N.yellowstonii_amoB
'Ca. N. franklandus C13'_amoB

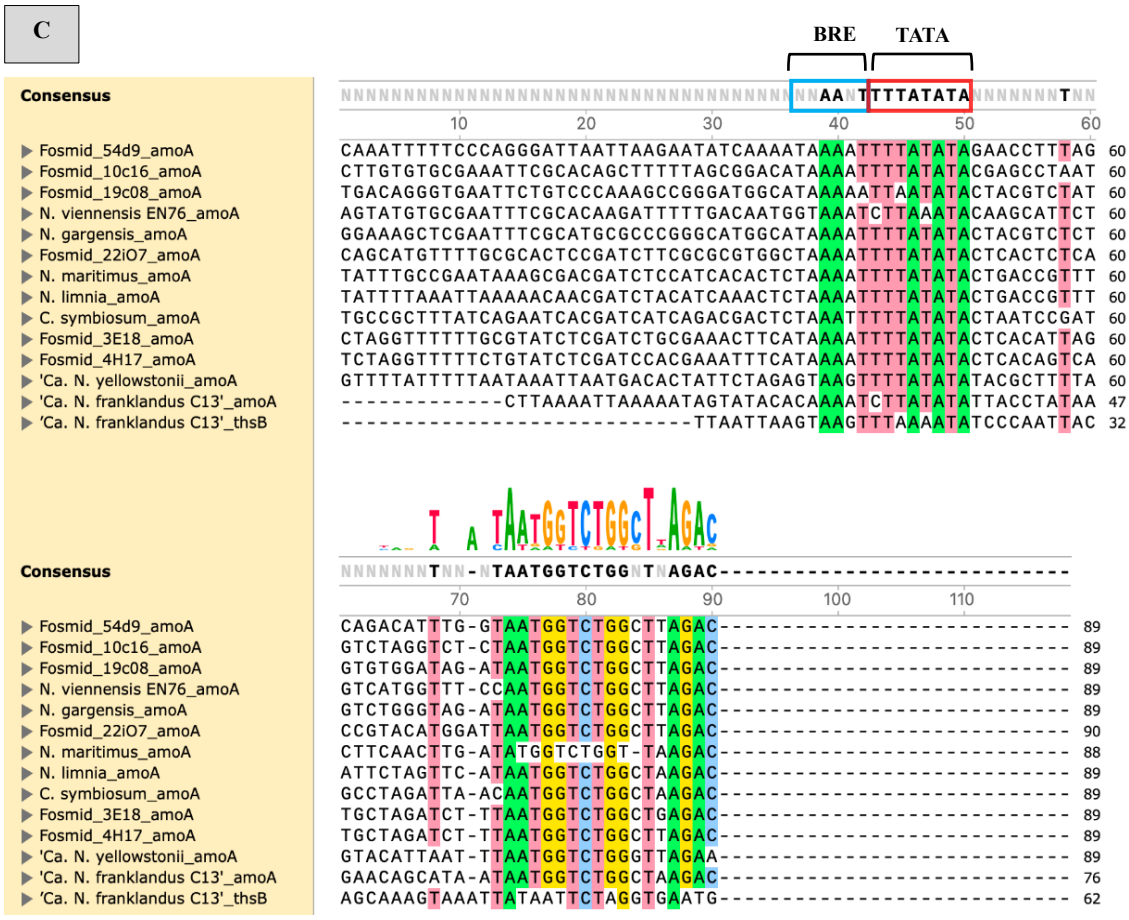
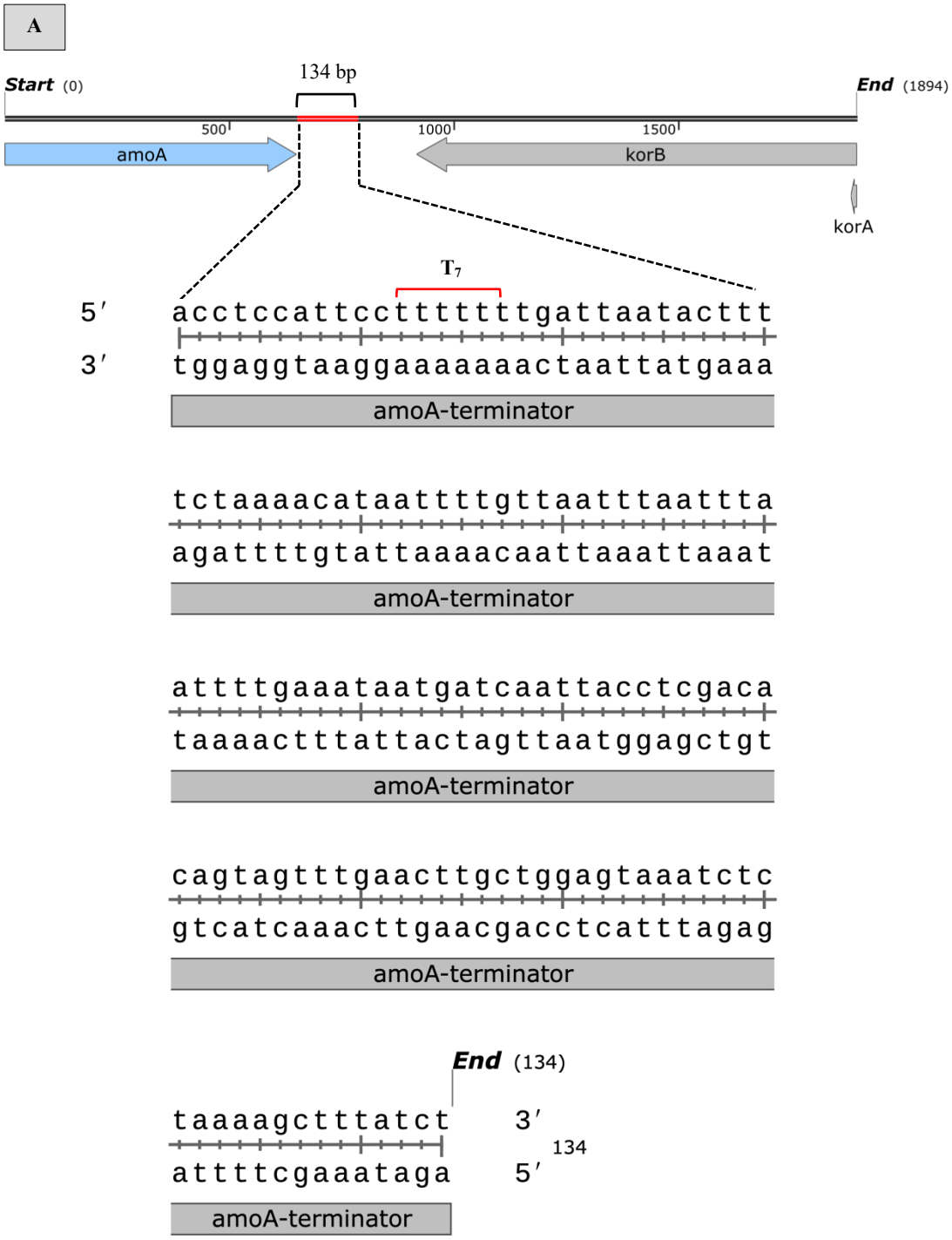


Figure 5.3: Detection of the archaeal promoter signals BRE and TATA-box in the 5' UTR of highly expressed genes in '*Ca*. N. franklandus C13'. Selected genes include the ammonia monooxygenase subunit A and B (*amo*A and *amo*B) and thermosome subunit B (*ths*B). Panels A, B and C represent multiple sequence alignments of the *amo*A, *amo*B and *ths*B promoter regions respectively with a previously published dataset known to contain promoter elements. Putative BRE and TATA-box elements are highlighted in blue and red boxes respectively. Nucleotides highlighted in colour are shared between the sequences at a threshold of 85%. The reference dataset to which these sequences were aligned were obtained from (Bartossek *et al*., 2012)

5.3.2.2 Terminator regions

The 3' UTR of the *amo*A and *ure*C genes was used as transcription terminators for *Cas9* and the *pac* gene respectively. These genes were selected as they have previously been found to be highly expressed in '*Ca*. N. franklandus C13'. Visual inspection of these sequences revealed runs of thymidine residues (oligo dT) which are reminiscent of intrinsic transcription termination signals (see *section 5.1*).


A
Start (0)
134 bp
End (1894)
500
1000
1500
amoA
korB
korA
T7
5′ acctccattcctttttttgattaatacttt
3′ tggaggtaaggaaaaaaactaattatgaaa
amoA-terminator
tctaaaacataattttgttaatttaattta
agattttgtattaaaacaattaaattaaat
amoA-terminator
attttgaaataatgatcaattacctcgaca
taaaactttattactagttaatggagctgt
amoA-terminator
cagtagtttgaacttgctggagtaaatctc
gtcatcaaacttgaacgacctcatttagag
amoA-terminator
End (134)
taaaagctttatct 3′
134
attttcgaaataga 5′
amoA-terminator

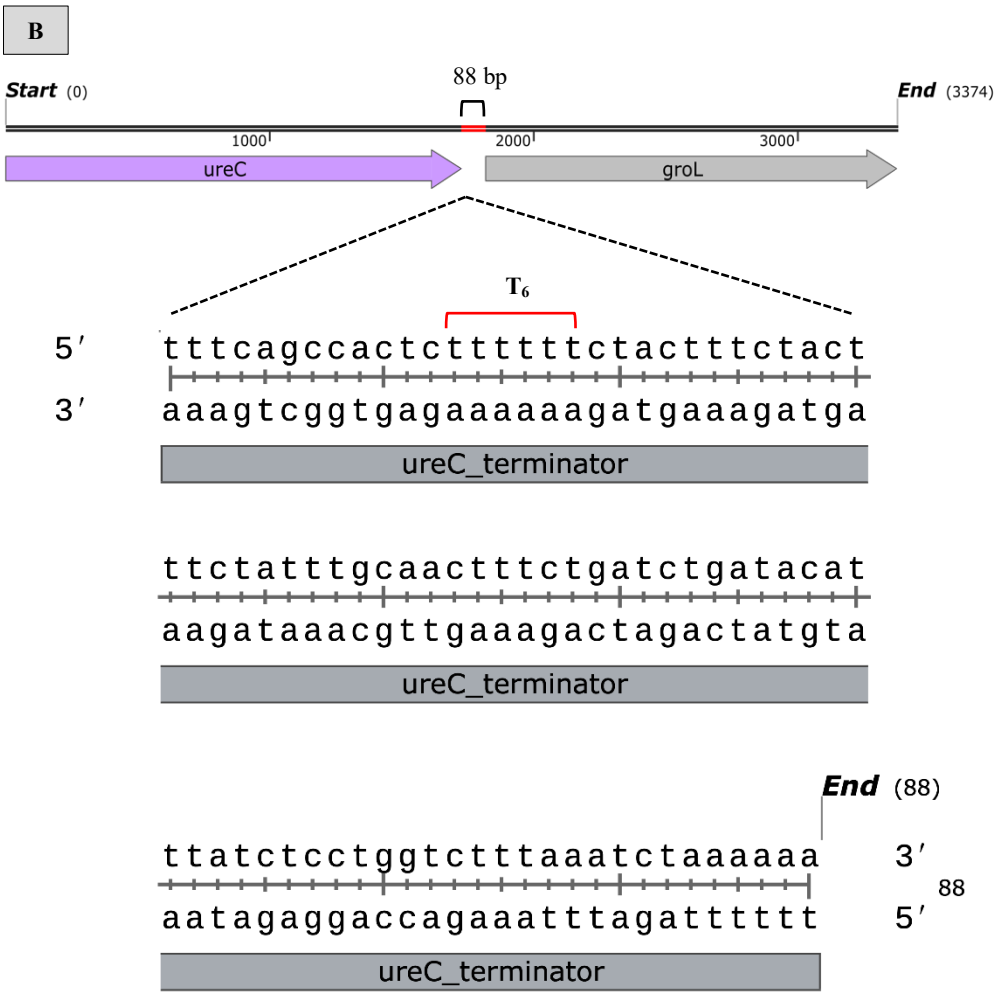


Figure 5.4: Terminator sequences used in this work. The 3' UTR immediately downstream of the stop codons of the highly expressed genes for ammonia monooxygenase subunit A (*amo*A) and urease subunit alpha (*ure*C) were used as terminator signals in the shuttle vectors. The length of the terminator regions varied between 134 bp for *amo*A and 88 bp for *ure*C. Putative intrinsic transcription termination signals (i.e. oligo (dT)) were detected (visually) in the selected sequences. **(A)** The *amo*A 3' UTR had a stretch of seven thymines (T_7). **(B)** In contrast, the *ure*C 3' UTR has a stretch of six thymines (T_6).

5.3.3 Validation of plasmids

5.3.3.1 Validation of the plasmids 'pfrank-CRISPR' and 'pfrank-CRISPR-amoB'

The pCRISPomyces- vector is used for CRISPR-Cas9 genome-editing in *Streptomyces* (Cobb *et al*., 2015). The pfrank-CRISPR plasmid constructed in this study is a circular 9.3 kb plasmid derived from the pCRISPomyces-2 plasmid. pfrank-CRISPR was assembled from the following components: **(i)** A codon-optimized *Streptococcus pyogenes Cas9* gene flanked by the entire 163 bp upstream intergenic region of the *amoA* gene as a putative promoter. The 3' UTR (134 bp) downstream of the *amoA* gene was used as a putative terminator; **(ii)** A codon-optimized puromycin N-acetyltransferase gene (*pac*). The *pac* gene was flanked by 92 bp of the upstream intergenic region of *thsB* as a putative promoter. The downstream region (88 bp) of the urease alpha subunit gene (*ureC*) was used as a terminator; **(iii)** A 791 bp fragment containing the predicted '*Ca*. N. franklandus C13' replication origin spanning 278 bp of the *cdc* gene (5' - 3') and 513 bp of the immediate upstream region of the *cdc* gene with the latter containing the ORB motifs and; **(iv)** A fragment of the pCRISPomyces-2 vector that spans the *lacZ-alpha* gene and the apramycin resistance gene (See Chapter 2). This fragment also contains a gRNA scaffold.

A unique feature of the pCRISPomyces-2 vector is that it allows one to conveniently fuse the spacer to the tracrRNA into a single guide RNA or sgRNA. This is due to the presence of two *Bbs*I sites, one immediately upstream of the *lacZ-alpha* gene and the second site immediately upstream of the gRNA scaffold (tracrRNA). The spacer can then be inserted via Golden gate cloning. The complete sgRNA is under the control of the *gapdh* promoter. Additionally, an *Xba*I site downstream of the gRNA scaffold facilitates insertion of a homology repair template.

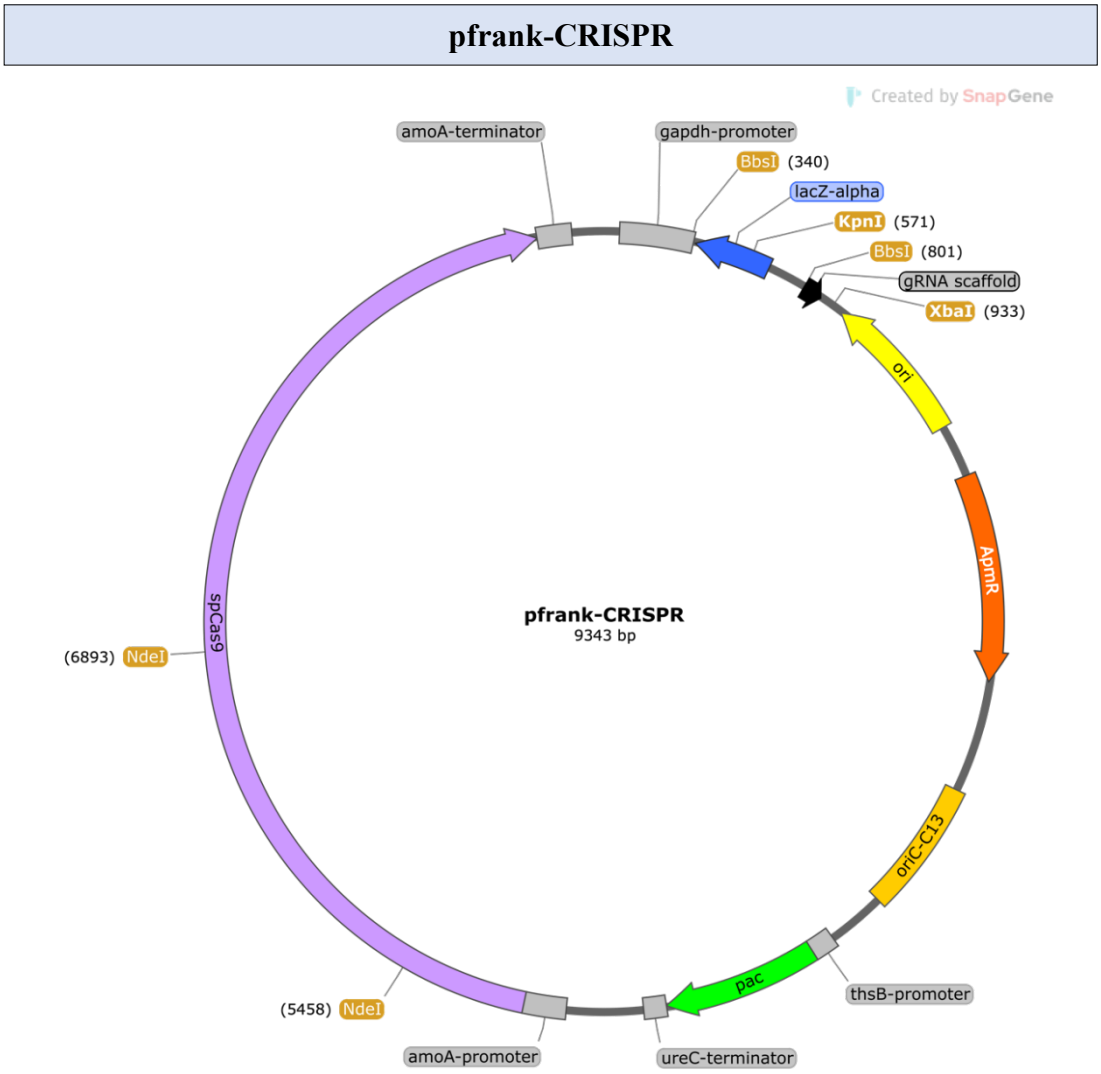


Figure 5.5: General architecture of the plasmid pfrank-CRISPR. pfrank-CRISPR is a circular 9343 bp long plasmid and contains the type II CRISPR-Cas9 elements from *Streptococcus pyogenes* Cas9 (purple) and a gRNA scaffold (black). The puromycin N-acetyltransferase gene (*pac*) allows selection using the antibiotic puromycin. The '*Ca*. N. franklandus C13' chromosomal replication origin (oriC-C13) was used to facilitate autonomous replication of this plasmid. Regulatory elements such as promoters and terminators are shown in grey.

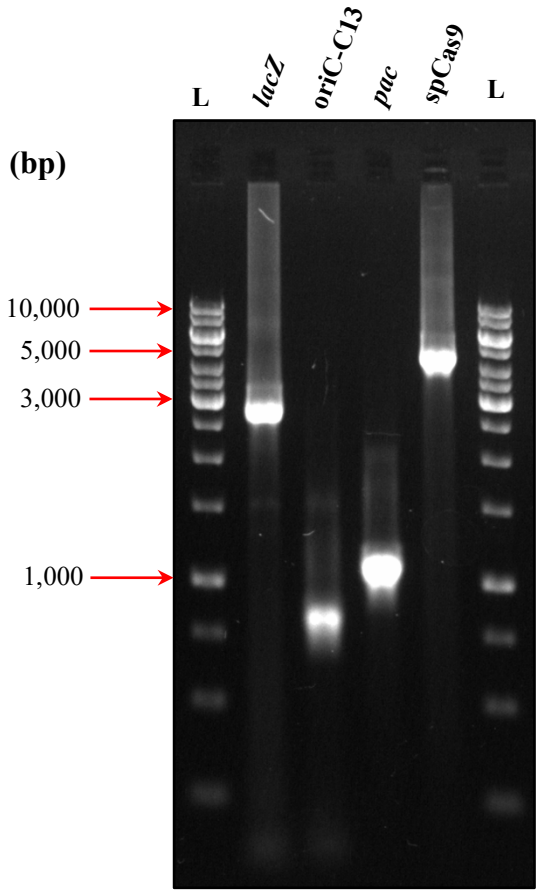


Figure 5.6: Amplification of the individual DNA fragments making up pfrank-CRISPR plasmid containing homology arms for a Gibson assembly. **Lane 1 and 6:** 1 kb Generuler ladder (L), **Lane 2:** pCRISPomyces-2 fragment spanning *lac*Z and the apramycin resistance gene (2875 bp), **Lane 3:** Fragment containing the '*Ca*. N. franklandus C13' chromosomal replication origin, oriC-C13 (851 bp), **Lane 4:** *pac* gene (1117 bp), **Lane 5:** spCas9 fragment with an expected amplicon size of 4730 bp. All PCR amplicons were resolved in a 0.8% (w/v) TBE agarose gel.

5.3.3.1.1 Agarose gel electrophoresis analysis of plasmid DNA

pfrank-CRISPR was constructed by Gibson assembly using PCR-amplified fragments containing the necessary homology arms (refer to Chapter 2). Blue colonies were selected as the most likely cells containing the desired constructs capable of replication in *E. coli*. Plasmid yields > 200 ng/µl and bright bands on an agarose gel suggested the presence of high copy plasmids (*Figure 5.7*). Of the two clones analysed, only clone 1 (C1) was closer to the expected size (~9.3 kb).

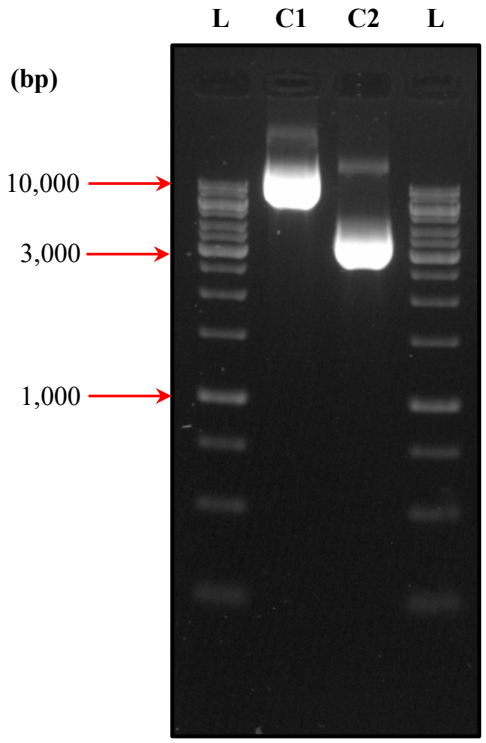


Figure 5.7: Agarose gel resolution of plasmid DNA extracted from potential pfrank-CRISPR transformants. **Lane 1:** 1 kb Generuler ladder (L), **Lane 2:** Clone 1 (C1), **Lane 3:** Clone 2 (C2) and **Lane 4:** 1 kb Generuler ladder (L). Plasmid DNA was resolved in a 0.8% (w/v) TBE agarose gel.

5.3.3.2 PCR screening of a pfrank-CRISPR transformant

Using plasmid DNA from clone 1 as a template, the four individual DNA fragments used for the assembly of pfrank-CRISPR were PCR amplified. All PCR reactions yielded amplicons of the expected size as shown in the agarose gel in *Figure 5.8*.

.

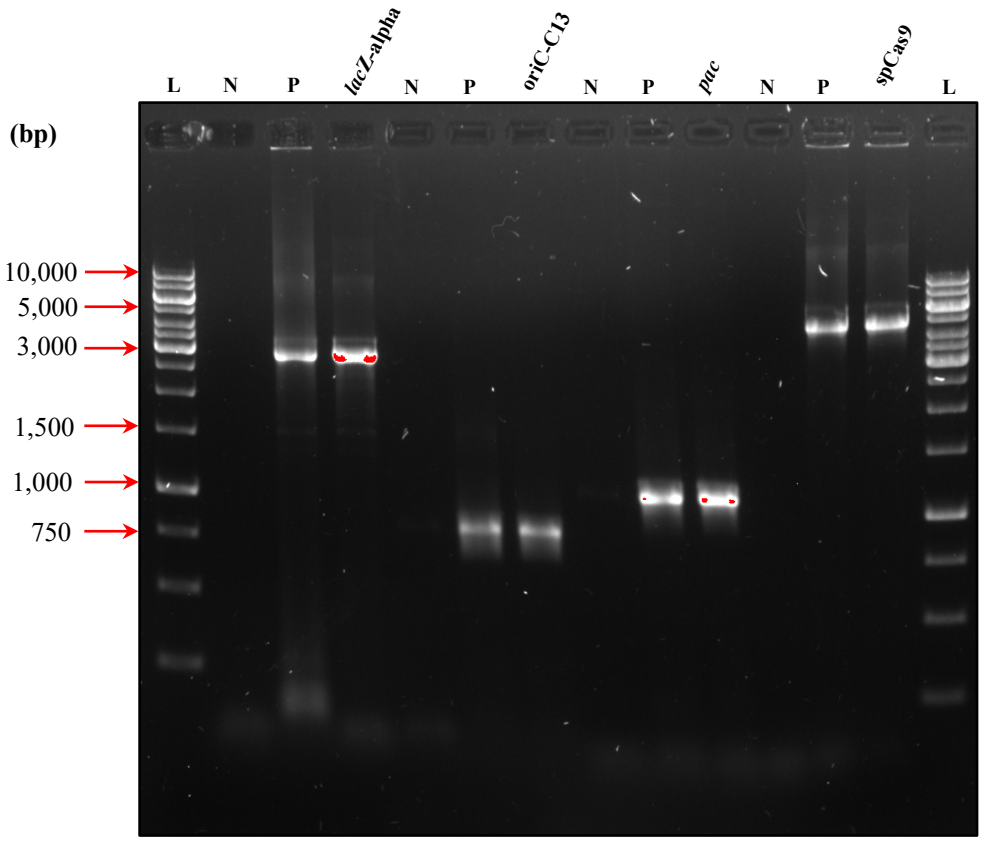


Figure 5.8: PCR screening of a potential pfrank-CRISPR transformant. Clone 1 (C1) was expected to contain the correctly assembled plasmid. Using primers to each of the four individual fragments i.e. *lacZ-alpha*-containing fragment (l*acZ*-alpha), the chromosomal replication origin of '*Ca*. N. franklandus C13' (oriC-C13), puromycin resistance gene (*pac*) and the *S. pyogenes* Cas9 (spCas9). Note: Lanes labelled **"N"** and **"P"** indicate the negative and positive controls respectively. The individual fragments used for the assembly were used as the positive controls. **Lane 1 and 14:** 1 kb Generuler ladder (L). **Lane 4:** Amplification of *lac*Z-alpha with an expected amplicon size of 2875 bp. **Lane 7:** Amplification of oriC-C13 with an expected amplicon size of 815 bp. **Lane 10:** Amplification of the *pac* gene with an expected size of 1117 bp. **Lane 13:** Amplification of the spCas9 with an expected amplicon size of 4730 bp. PCR amplicons were resolved in a 0.8% (w/v) TBE agarose gel.

5.3.3.3 Restriction digestion analysis

Plasmid DNA from clone 1 was further analysed by restriction digestion. Clone 2, which likely contains the incorrect assembly product, was included in this analysis as a negative control. As expected, restriction digestion of clone 1 resulted in the expected banding pattern whereas clone 2 did not. The resulting bands from the restriction analysis are presented in *Figure 5.9*.

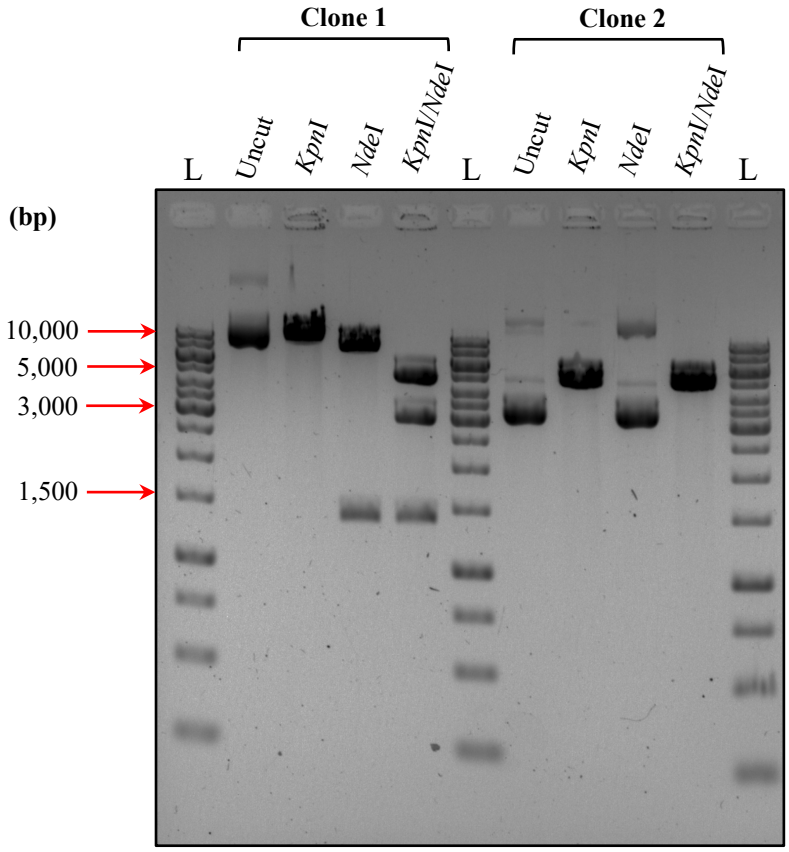


Figure 5.9: A restriction digestion analysis of potential pfrank-CRISPR plasmids. Clone 1 was expected to contain the correctly assembled pfrank-CRISPR plasmid. Single/double digests were done using *Kpn*I and *Nde*I. **Lane 1, 6 and 11:** 1 kb Generuler ladder (L), **Lane 2:** Clone 1 uncut plasmid DNA, **Lane 3 and 4:** Clone 1 *Kpn*I and *Nde*I single digests, **Lane 5:** Clone 1 *Kpn*I/*Nde*I double digest, **Lane 7:** Clone 2 uncut plasmid DNA, **Lane 8 and 9:** Clone 2 *Kpn*I and *Nde*I single digests and **Lane 10:** Clone 2 *Kpn*I/*Nde*I double digest. The expected bands from the single digests were 1435 bp and 7908 bp (*Nde*I), 9343 bp (*Kpn*I) and 4887 bp, 1435 bp and 3021 bp for the *Nde*I/*Kpn*I double digest. Plasmid DNA was resolved on a 0.8% (w/v) TBE agarose gel.

5.3.3.4 Whole-plasmid sequencing

Based on the PCR and restriction digestion analysis, clone 1 likely contained the correctly assembled pfrank-CRISPR plasmid and was subjected to whole-plasmid sequencing. In addition to sequence comparison, read-length histograms were used to further validate the presence of the correct molecular species in the samples. In a read-length histogram, a single dominant peak located around the size of the plasmid on the x-axis is an indicator that a single plasmid species is present in the sample. Based on these histograms, a single dominant peak around 9 kb was evident which corresponds to the pfrank-CRISPR plasmid (*Figure 5.10A*).

Since the *gapdh* promoter downstream of the *lac*Z-alpha gene is of bacterial origin, it was replaced with an endogenous promoter from '*Ca*. N. franklandus C13' i.e. the 5' UTR of the *amo*B subunit gene (330 bp) expected to contain a promoter. Replacement of the *gapdh* promoter with the 5'UTR of the *amo*B gene resulted in the plasmid pfrank-CRISPR-amoB plasmid. This final construct was subsequently sequenced to confirm the correct construct. A single dominant peak in read-length histogram for pfrank-CRISPR-amoB around the expected size (~9 kb) strongly suggested the presence of a single plasmid species (*Figure 5.10B*). A plasmid map of pfrank-CRISPR-amoB is shown in *Figure 5.11*.

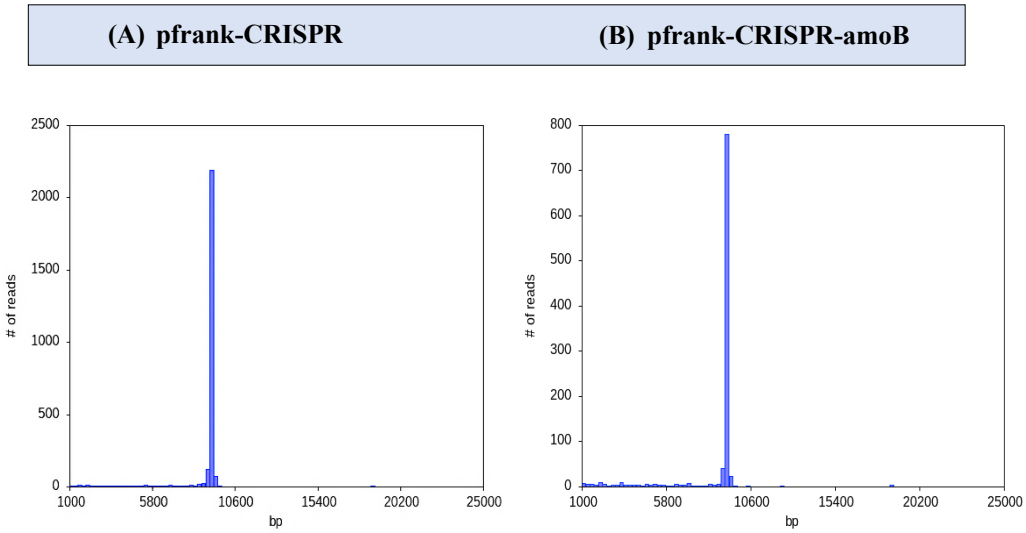


Figure 5.10: Read-length histograms of the plasmids pfrank-CRISPR and pfrank-CRISPR-amoB. These histograms are generated following whole plasmid sequencing and are used to determine whether a single plasmid species is present in the sample. In the case of a pure plasmid sample, a single dominant peak corresponding to the plasmid size on the x-axis is observed. A single dominant peak was evident for **(A)** pfrank-CRISPR and **(B)** pfrank-CRISPR-amoB at around 9 kb indicating a single plasmid species.

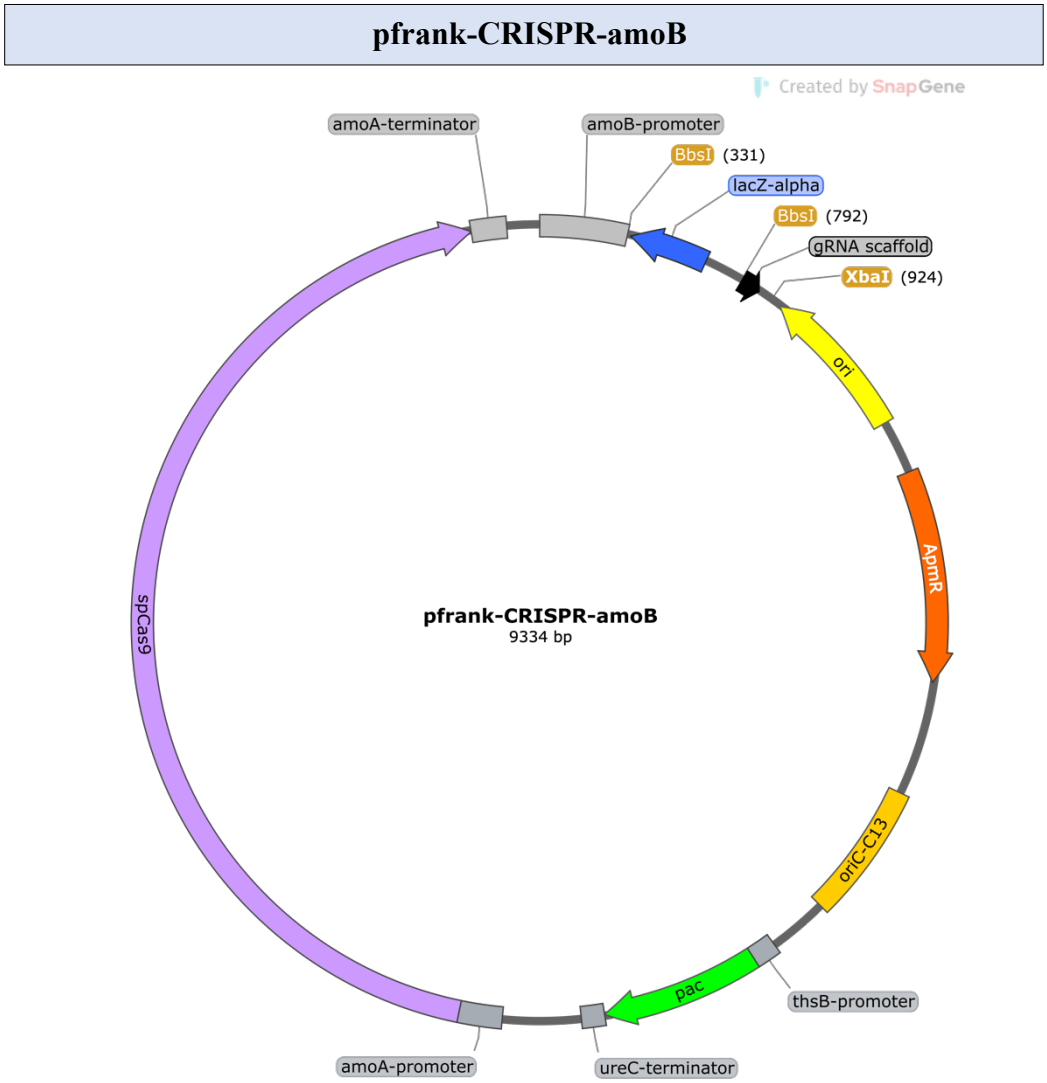


Figure 5.11: General architecture of the pfrank-CRISPR-amoB. The plasmid pfrank-CRISPR-amoB was constructed from pfrank-CRISPR by replacing the *gapdh* promoter with the 5' UTR region of the ammonia monooxygenase subunit B gene (*amoB*) from '*Ca*. N. franklandus C13'. All other features are identical to the plasmid pfrank-CRISPR previously described.

5.3.3.5 The pfrank-mCherry plasmids

To determine whether the cloned sequence was sufficient for plasmid replication, was recognised by the cell replication machinery, and if then *cdc* gene encoding the initiator protein (Orc1/Cdc6) is required in *trans* or *cis*, two distinct versions of the pfrank-mCherry were constructed including pfrank-mCherry-Cdc-orb and pfrank-mCherry-orb. Both constructs contain a fragment from pCRISPomyces-2 containing the *lac*Z-alpha gene, the apramycin resistance gene, and the high copy number *E. coli* replication origin (ColE1) (see *section 2.12.10*).

The plasmid pfrank-mCherry-Cdc-orb (7025 bp) contains a *cis*-located *cdc* gene adjacent to the predicted chromosomal replication origin while pfrank-mCherry-orb (5953 bp) lacks a *cis*-located *cdc* gene. In addition, both pfrank-mCherry constructs contain the gene encoding the red fluorescent reporter protein, *mCherry* (Shaner *et al.*, 2004). The *mCherry* gene was placed under the control of the *amoA* 5' UTR and 3' UTR as the potential promoter and terminator respectively (see *section 5.3.2*). Both plasmid versions contain the *pac* gene to confer puromycin resistance. Plasmid maps for the pfrank-mCherry constructs are shown in *Figure 5.12*.

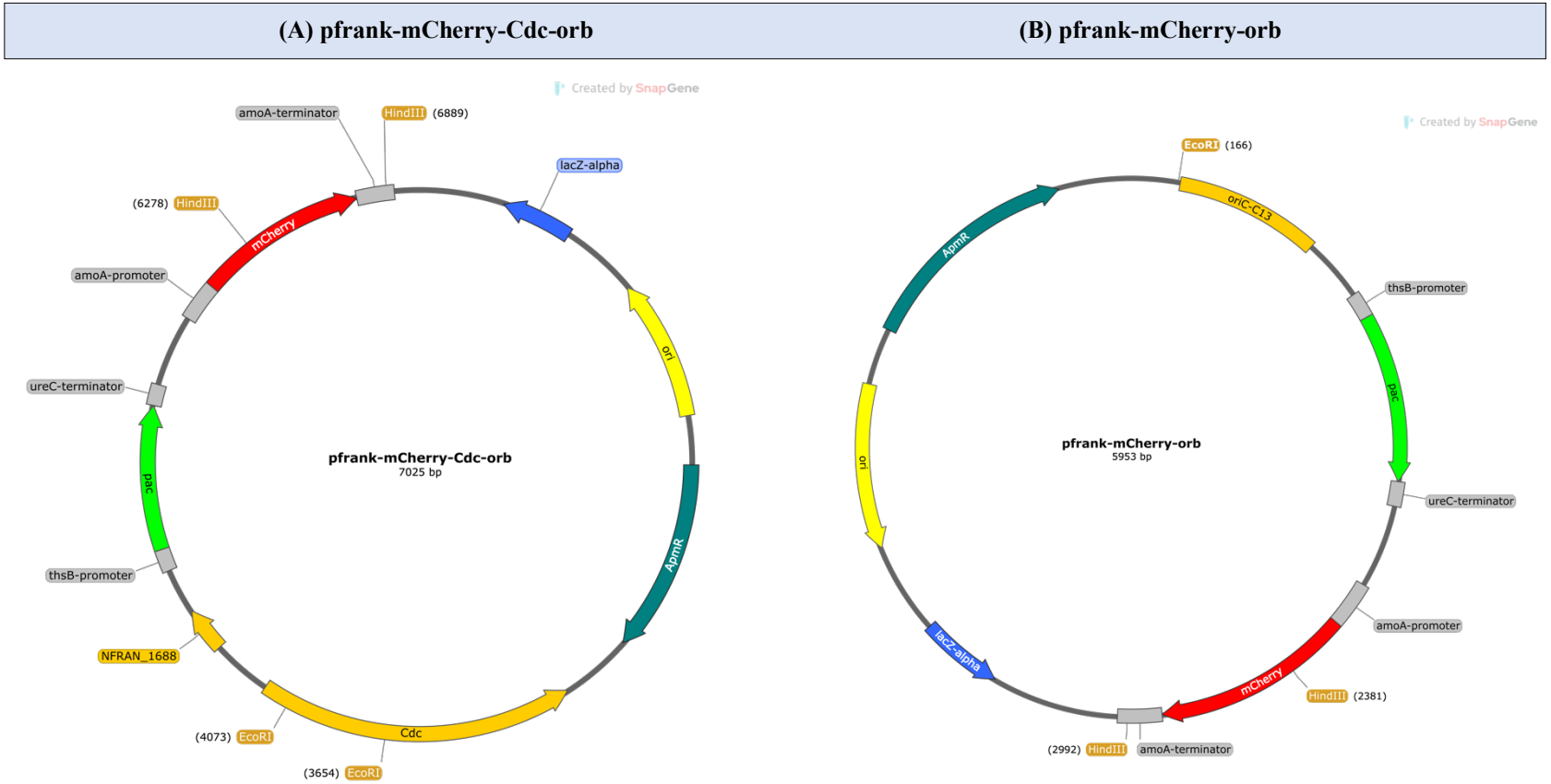


Figure 5.12: General architecture of the pfrank-mCherry plasmids. Both plasmids contain the *mCherry* reporter gene under the control of the putative *amoA* promoter and terminator. pfrank-mCherry-Cdc-orb contains a *cis* located *cdc* gene adjacent to the replication origin while pfrank-mCherry-orb only contained the replication origin (oriC-C13). Both plasmids contain the *pac* gene to facilitate puromycin selection.

5.3.3.5.1 Restriction digestion analysis

Both versions of the pfrank-mCherry plasmids were constructed by Gibson assembly using PCR-amplified fragments containing the necessary homology arms. Following transformation of *E. coli* cells with the assembly mixture, blue colonies were selected as described for the plasmid pfrank-CRISPR above. Two clones transformed with pfrank-mCherry-orb (Orb1 and Orb3) and three clones transformed with pfrank-mCherry-Cdc-orb (Cdc1.1, Cdc1.2 and Cdc1.3), were analysed by restriction digestion using *Eco*RI and *Hin*dIII. With the exception of Orb3, plasmid DNA from all other transformants resulted in the expected banding patterns suggesting assembly of the correct plasmids (*Figure 5.13*).

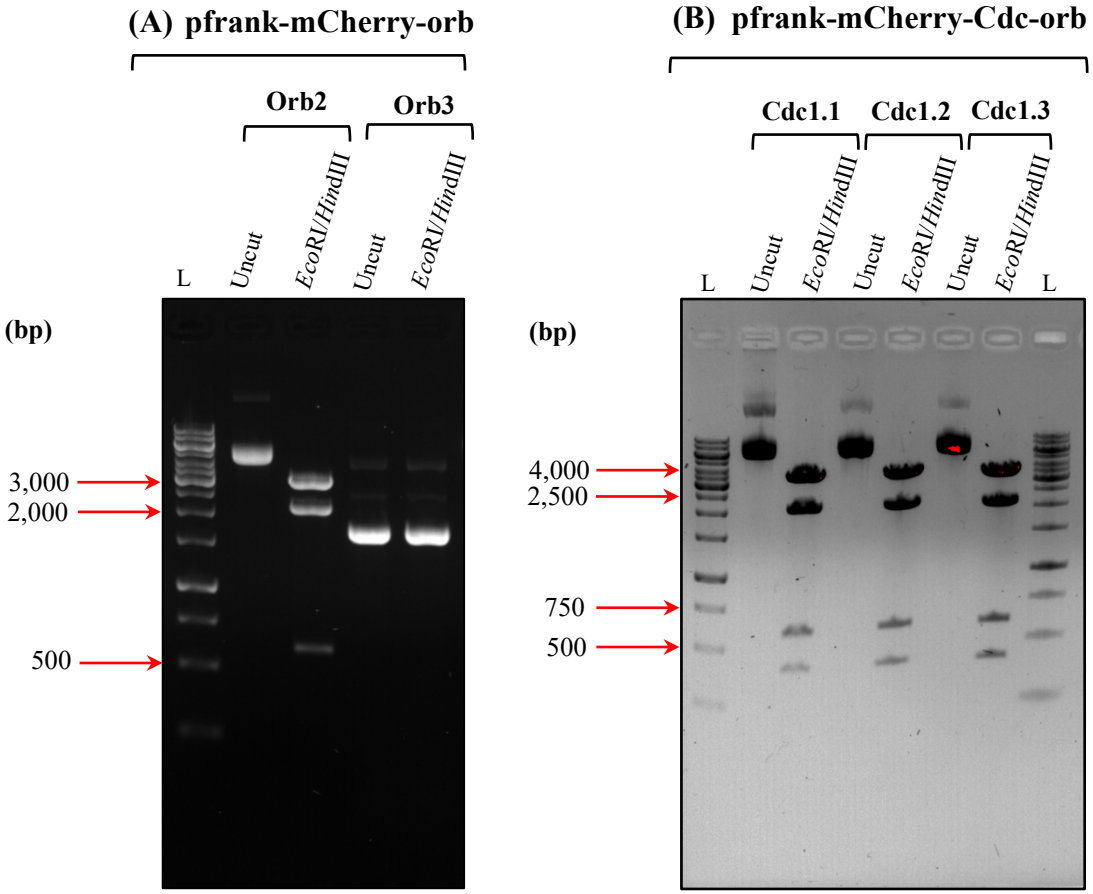


Figure 5.13: Restriction analysis of the pfrank-mCherry plasmids. **(A)** Restriction analysis of pfrank-mCherry-orb transformants digested with *Eco*RI and *Hin*dIII. **Lane 1:** 1 kb Generuler ladder (L), **Lane 2:** uncut clone 2 plasmid DNA (Orb2), **Lane 3:** *Eco*RI/*Hin*dIII digested clone 2 plasmid DNA, **Lane 4:** uncut clone 3 plasmid DNA (Orb3), **Lane 5:** *Eco*RI/*Hin*dIII digested clone 3 plasmid DNA. Clone 2 exhibited bands of the expected size corresponding to 611 bp, 2215 bp and 3127 bp. **(B)** Restriction analysis of pfrank-mCherry-Cdc-orb transformants with *Eco*RI and *Hin*dIII. **Lane 1 and 8:** 1 kb Generuler ladder (L), **Lanes 2, 4, 6:** uncut plasmid DNA, **Lanes 3:** *Eco*RI/*Hin*dIII digested clone 1 (Cdc1.1), **Lane 5:** *Eco*RI/*Hin*dIII digested clone 1 (Cdc1.2), **Lanes 7:** *Eco*RI/*Hin*dIII digested clone 1 (Cdc1.3). All three clones exhibited the expected banding patter corresponding to 419 bp, 611 bp, 2205 bp and 3790 bp. DNA was resolved on a 0.8% (w/v) TBE agarose gel.

5.3.3.5.2 Whole-plasmid sequencing

Based on the restriction analysis presented in *Figure 5.13* the clones Orb2 (pfrank-mCherry-orb) and Cdc1.2 (pfrank-mCherry-Cdc-orb) were further verified by whole-plasmid sequencing. The read-length histograms for plasmids pfrank-mCherry-Cdc-orb (*Figure 5.14A*) and pfrank-mCherry-orb (*Figure 5.14B*) revealed single dominant peaks at the approximate sizes indicating the presence of a single plasmid species.

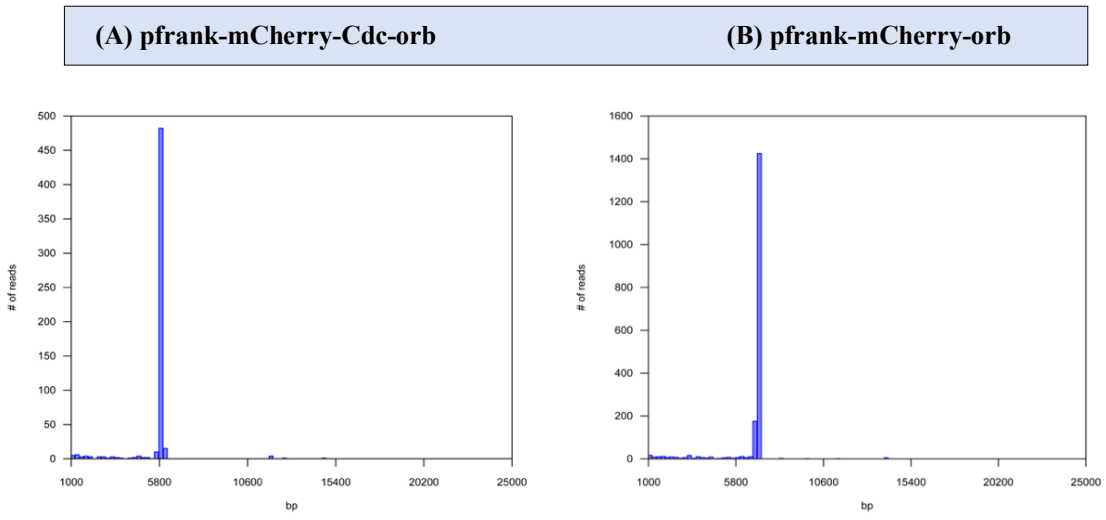


Figure 5.14: Read-length histograms generated following whole plasmid sequencing of the pfrank-mCherry plasmids. The x-axis represents the size of the reads while the y-axis corresponds to the number of reads in the sample. A single dominant peak corresponding to the expected plasmid size indicates the presence of single plasmid species. **(A)** pfrank-mCherry-Cdc-orb **(B)** pfrank-mCherry-orb.

5.4 Discussion

The main goal of this study was to construct artificial plasmids for '*Ca*. N. franklandus C13' to address the lack of native AOA plasmids. In addition to being molecular vehicles for genetic information, these plasmids will play an important role as DNA uptake indicators to facilitate the development of transformation protocols. The two main unifying features of the three plasmids are that they are all designed as *E. coli* shuttle vectors and are all under the control of the '*Ca*. N. franklandus C13' chromosomal replication origin. Being *E. coli* shuttle vectors, these plasmids can all easily be manipulated and propagated in a well-established host.

To ensure autonomous replication of these plasmids in *'Ca*. N. franklandus C13', it was necessary to locate the genomic region containing the replication origin. Using the Z-curve method, a single replication origin was identified in the genome of '*Ca*. N. franklandus C13' located adjacent to the Orc1/Cdc6 encoding gene, *cdc*. This is consistent with the theory of a single-origin mode of DNA replication among the Thaumarchaeota (Pelve *et al*., 2013). Furthermore, the co-localisation of the predicted *'Ca*. N. franklandus C13' replication origin with a *cdc* gene is a characteristic feature of previously studied archaeal replication origins (Berquist and DasSarma, 2003; Zhang and Zhang, 2003). A single replication origin in '*Ca*. N. franklandus C13' contrasts with other archaeal lineages such as Haloarchaea and the Sulfolobales whose genomes contain multiple origins (Zhang and Zhang, 2003; Lundgren *et al.*, 2004). In the context of plasmid design, the dependence on a single replication origin had important implications. For example, in *H. hispanica*, it was shown that the Orc1/Cdc6 initiation protein is specific to its co-localised replication origin (Wu *et al.*, 2014).

Therefore, since *'Ca*. N. franklandus C13' relies on a single replication origin, its Orc1/Cdc6 is likely to be specific to its adjacent origin.

An important consideration associated with the use of the chromosomal replication origin in the plasmids is whether the Orc1/Cdc6 initiator protein is required in *cis* or *trans*. In the *Halobacterium* sp. strain NRC-1, it was observed that plasmids lost the ability to self-replicate when *orc1/cdc6* (*orc7*) was deleted despite still containing the replication origin (Berquis and DasSarma, 2003). In *P. furiosus*, however, plasmids based on the chromosomal replication origin were still capable of self-replication in the absence of a *cis orc1/cdc6* gene (Farkas *et al.*, 2011). Similar observations were made in the *H. hispanica* (Wu *et al*., 2014). This formed the basis for the construction of plasmid pfrank-mCherry-Cdc-orb which unlike pfrank-mCherry-orb, contains a cis *cdc* gene.

Unfortunately, the use of a chromosomal replication origin in plasmids is associated with a few limitations. Firstly, plasmids under the control of a chromosomal replication origin are likely to be maintained at a low copy number (Moriya *et al*., 1992; Zakrzewska-Czerwińska, *et al*., 1995; Farkas *et al*., 2011). In bacterial systems using plasmids based on *oriC* (i.e. a chromosomal origin), the low copy number has been attributed to competition of the origins for the initiator protein, DnaA (Zakrzewska-Czerwińska, *et al*., 1995). Consequently, this may result in lower expression levels of the selection marker hindering effective mutant screening. For example, kanamycin resistant plasmids for *Bacillus subtilis* were found to be ineffective at low copy numbers (Moriya *et al.*, 1992). Furthermore, while high copy number plasmids depend on passive diffusion for faithful partitioning during cell division, low copy number plasmids depend on an active partitioning system (typically encoded on the plasmid)

to ensure their maintenance following cell division (Schumacher, 2012). Since the three plasmids constructed in this study are expected to exist as low-copy plasmids and none contained genes encoding a partitioning-system, they may be vulnerable to plasmid loss (i.e. plasmid instability) during replication. When using the chromosomal replication, it may not be clear (in the absence of experimental validation) how much sequence information surrounding the replication origin is sufficient to ensure self-replication. In the same study discussed above (Berquist and DasSarma, 2003), only plasmids containing greater than 500 bp of sequence upstream of the *orc1/cdc6* gene (and a *cis orc1/cdc6*) were able to self-replicate. In the present study, all three plasmids contained ~513 bp of sequence upstream of the *cdc* gene, and it remains to be determined whether this is sufficient for autonomous replication. Future work will need to test the use of plasmids containing varying lengths of this region. Another caveat associated with the use of plasmids based on the chromosomal replication origin is that there is increased homology between the plasmid and genome. The plasmid containing the additional *cdc* region (pfrank-mCherry-Cdc-orb) has an even larger region of homology to genome. This plasmid-genome homology may increase the chance of unwanted plasmid integration into the genome (Lee *et al*., 2008; Yan and Fong, 2017).

The main rationale behind the design of plasmid pfrank-CRISPR-amoB was to establish the first CRISPR-based genome-editing platform for an AOA. Specifically, this plasmid contains the necessary elements for a type II CRISPR-Cas system (i.e. Cas9 and tracrRNA). This is particularly relevant for '*Ca*. N. franklandus C13' as it lacks an endogenous CRISPR-Cas system. Furthermore, unlike endogenous CRISPR-Cas systems, a non-native type II system could be used for multiple strains. This is based on the argument by Nayak and colleagues (2017) justifying the development of

a type II CRISPR-Cas genome-editing tool for an archaeal strain. The type II CRISPR-Cas9 system is also attractive due to the minimal genetic components required. In the context of the present study, it was important to keep the size of the plasmid pfrank-CRISPR-amoB to a minimum particularly at such an early stage where it is not yet clear how plasmid size impacts the cell or the transformation efficiency.

Aside from a replication origin, construction of shuttle vectors requires the necessary gene expression elements such as promoters, terminators and RBS. In this work, promoters were selected based on their expected location i.e. upstream of highly expressed genes. Comparison of these sequences to those known to contain the expected archaeal promoter signal (TATA-box and BRE) provided support for the presence of putative promoter signals. However, in the absence of functional characterisation, several challenges arise. For example, it is unclear whether the putative promoters are constitutively active, or whether they are strong/weak promoters. Their association with essential and highly expressed genes suggests that the putative promoters used in this work are likely strong promoters (Riley and Guss, 2021). While it may seem advantageous to use a strong promoter, especially if the plasmids are expected to be maintained at low copy numbers, such elements might also result in a significant metabolic burden on the cell. An alternative approach in the absence of functionally characterised promoters would be to utilise the promoters from already established archaeal models. The use of exogenous promoters has been successfully demonstrated in *T. kodakarensis* whereby the strong promoter P*hmtb* from *Methanothermobacter thermautotrophicus* was functional (Santangelo *et al.*, 2008a).

Additionally, it may be interesting to test whether promoters predicted from AOA genomes function in already established archaeal genetic systems. The lack of

functionally characterised gene expression elements for the '*Ca*. N. franklandus C13' prompted the use of a reporter gene (*mCherry*) in the pfrank-mCherry plasmids.

Transcription terminators were selected using the same criteria as the promoters, and manual inspection indicated that an intrinsic termination signal (i.e. oligo (dT)) may be present. However, a systemic analysis of the 3' UTR of '*Ca*. N. franklandus C13' is essential to detect true transcription termination signals. To date, this has only been done for a handful of archaeal strains none of which are in the phylum Thaumarchaeota (Dar *et al.*, 2016; Berkemer *et al.*, 2020). An interesting approach would be to design synthetic terminators with varying stretches of oligo dT's which can easily be amplified by PCR or commercially synthesised. Alternatively, as suggested for the promoters, it may be possible to utilise characterised terminators from other archaea.

5.5 Summary and outlook

To our knowledge, this is the first report on the construction of artificial plasmids for use in an AOA. All three plasmids contain the '*Ca*. N. franklandus C13' chromosomal replication origin. While we have established that these vectors replicate in *E. coli*, it is yet to be determined whether they can be transferred into and maintained in '*Ca*. N. franklandus C13' and this will be explored in the subsequent chapter. This work also highlights important aspects of AOA biology that have largely been ignored but will be key to establishing a genetic toolbox. It will also be important for further work to focus on the various mobile genetic elements that have been discovered in other AOA strains (see Chapter 1), as these could replace the use of artificial constructs and thus may be more effective.

Chapter 6: Transformation of '*Ca*. N. franklandus C13'

6.1 Introduction

The final component of a genetic toolbox is the ability to introduce exogenous DNA into the host cell via transformation. In nature, the uptake of exogenous DNA is an important evolutionary process for the survival and adaptation of microorganisms to fluctuating environmental conditions (Fonseca *et al.*, 2020). In molecular biology, however, the ability of microbes to take up exogenous DNA has been exploited as a tool to introduce heterologous genetic material into a desired host strain(s) for the purpose of increased protein expression or other genetic manipulations.

Some bacteria and archaea are naturally capable of directly taking up DNA from their environment through natural transformation, a process that relies on specialised endogenous machinery (Peabody *et al.*, 2003; Panja *et al.*, 2008; Muschiol *et al.*, 2015; Van Wolferen *et al.*, 2016; Fonseca *et al.*, 2020). Alternative methods for DNA uptake and exchange among microorganisms include (i) transduction which is mediated by viruses and (ii) conjugation that requires physical contact between cells (Schleper *et al.*, 1995; Fonseca *et al.*, 2020).

Transformation may also be artificially induced using physical or chemical methods such as electroporation (Dower and Ragsdale, 1988; Potter and Heller, 2010), calcium chloride heat-shock treatment ($CaCl_2$ heat-shock), polyethylene glycol (PEG) method (Klebe *et al.*, 1983) and liposome-mediated transformation (Dickson, 1995) to name a few. However, these transformation methods are not all equally effective, and their efficacy may vary depending on the strain being used. For this reason, when working with new strains or non-model organisms, it is necessary to explore different methods.

The $CaCl_2$ heat-shock method is a popular choice for both bacterial and archaeal models and is often seen to yield high transformation efficiencies (Hanahan, 1983). This method involves the treatment of cells with a high concentration of Ca^{2+} ions at low temperatures which helps to neutralise the negative charge of DNA facilitating its interaction with the cell (Asif *et al.*, 2017). The subsequent heat-shock step is important for creating conditions conducive for DNA uptake. For example, mechanistic studies on the effects of the heat-shock step in *E. coli* revealed a decrease in the membrane potential which is needed for the entry of extracellular DNA (Panja *et al.*, 2006). Furthermore, heat-shock treatment was found to contribute to lipid loss (and consequent membrane rigidification) which is conducive for pore formation, therefore enabling crossing of DNA into the cell (Panja *et al.*, 2008).

Another widely used transformation method is electroporation which is applicable to a range of cell types including archaea (Schleper *et al*., 1992), fungi (Rehman *et al.*, 2016), plants (Furuhata *et al.*, 2019), mammalian cells (Chicaybam *et al.*, 2017), and bacteria (Dower *et al*., 1988). The general mechanism of electroporation involves the exposure of cells to a strong electrical field that results in an increase of the transmembrane potential. If this transmembrane potential is sufficiently high, structural changes to the lipid membrane occur, resulting in pore formation which is conducive to DNA entry (Kotnik *et al.*, 2019; Vižintin and Miklavčič, 2022). Two main electroporation wave forms or pulse types are typically used during transformation experiments and include exponential-decay and square wave pulses (Young and Dean, 2015). When using square wave pulses, a constant voltage is applied for a user-specified duration (Jordan *et al.*, 2008). The advantage of using square wave pulses is that it allows one to control the number of pulses as well the duration between each of these pulses. In contrast, when using exponential-decay pulses, the duration of the

pulse (time constant) is dependent on the stored charged (capacitance) and the resistance of the sample (Jordan *et al.*, 2008). Although electroporation has proven to be a highly efficient transformation method, its success is dependent on numerous factors including the pulse type used, field strength, duration of the pulses, DNA concentration and even sample buffer used among others (Dower *et al*., 1988; Sherba *et al*., 2020). In addition to these physical parameters, the efficacy of transformation by electroporation may also be influenced by the physiological state of the cell (exponentially growing vs stationary) (Yi and Kuipers, 2017) as well as the presence or absence of a cell wall (Azencott *et al*., 2007). It is therefore essential that a wide range of parameters are considered and tested when establishing an electroporation protocol for novel or non-model strains.

PEG-mediated transformation is an inexpensive and easy to use transformation method that has proven to be successful for a range of organisms including yeast, bacteria, and archaea (Klebe *et al.*, 1983; Cline *et al*., 1989a; Tumbula, *et al*., 1994; Stachler *et al.*, 2017). It involves the incubation of cells and DNA in a PEG solution and may be used to transform either whole cells (i.e. cells with an intact cell wall) or cells whose cell wall/outer membrane has been removed (i.e. spheroplasts). The exact mechanism of PEG-mediated transformation is not well described in the available literature, but it has been reported to increase adsorption of the DNA to the cell surface and cell permeability (Gietz *et al.*, 1995; Zheng *et al.*, 2005). The efficiency of PEG-mediated transformation is influenced by a plethora of factors including the molecular weight and concentration of the PEG which must be considered when working with a new strain(s) (Klebe *et al.*, 1983).

Successful transformation involves the entry of DNA into the cell, evasion of host-defences and its continuous maintenance within the cell either autonomously or via genome integration (Riley and Guss, 2021)**.** Each of these steps face their own set of challenges. For example, successful DNA entry into a cell requires the genetic material to travel across two cell barriers i.e. the cell wall and cell membrane. For this reason, it is often desirable to disrupt the cell wall using methods such as chemical or enzymatic treatment to form protoplasts (or spheroplasts) prior to transformation (Brzobohatý and Kovác, 1986; Dyall-Smith, 2009; Kawai *et al*., 2010; Wang *et al.*, 2019). The evasion of host-defences, particularly the restriction-modification system (R-M), is considered as a major limiting factor of transformation (Vasu and Nagaraja, 2013; Johnston *et al.*, 2019). The R-M defence system consists of two components, a restriction endonuclease and a methyltransferase (Johnston *et al.*, 2019). Methyltransferases are responsible for the modification of host DNA via methylation which provides protection against degradation by the restriction endonuclease. Therefore, the heterologous DNA being introduced into a cell needs to be appropriately modified (methylated) prior to transformation. Other host immune mechanisms such as CRISPR-Cas systems are not expected to pose a significant challenge to transformation because the specific sequences captured by the endogenous CRISPR-Cas system (e.g. spacers) are unlikely to be present in the heterologous DNA being introduced (Riley and Guss, 2021). In some instances, inactivation of the host DNases has also been shown to improve the efficiency of transformation. This is exemplified by the inactivation of the DNA repair system RecBCD (which also degrades linear double-stranded DNA) in *E. coli* to facilitate transformation with linear dsDNA (Datta *et al*., 2006). Similarly, in the strain *Vibrio alginolyticus*, removal of DNases had a significant increase in the transformation efficiency (Kawagishi *et al.*, 1994).

Lastly, following entry and successful evasion of the host-defence systems, the DNA needs to be maintained either as an extrachromosomal self-replicating plasmid or its subsequent integration into the genome (Riley and Guss, 2021). Self-replicating plasmids are dependent on the presence of a replication origin while in the case of integrative plasmids appropriate homology needs to be present. Furthermore, in order for cells to retain plasmids, they need to be maintained under selective conditions to prevent plasmid loss (Schmidt *et al.*, 2012).

6.2 Chapter aims

The successful transformation of an archaeon in the phylum Thaumarchaeota is yet to be reported. Therefore, in this chapter, transformation of '*Ca*. N. franklandus C13' was attempted using the plasmids described in Chapter 5. Three main transformation methods were tested including $CaCl_2$ heat-shock, electroporation and PEG-mediated transformation. Furthermore, an aim was to determine whether the chromosomal replication origin placed into these plasmids is sufficient for autonomous maintenance. Lastly, it was also desirable to determine whether the puromycin N-acetyltransferase (*pac*) is capable of sufficiently conferring puromycin resistance to '*Ca*. N. franklandus C13' transformants.

6.3 Results

Following transformation of '*Ca*. N. franklandus C13', cells were maintained in liquid cultures and nitrite production monitored (as described in Chapter 2) to determine their growth response following the different treatments. Some variation in the initial nitrite concentrations (i.e. those measured immediately following the addition of puromycin) was observed. These are expected to result from biological differences between the cultures or unequal distribution of biomass between replicates.

In theory, cultures successfully transformed with plasmid DNA were expected to exhibit exponential nitrite production while no nitrite accumulation was expected in the control cultures. However, following transformation (with all three methods) and the addition of puromycin the selective agent, nitrite production did not cease (including in the control cultures). This was despite using a significantly higher concentration of puromycin (i.e. 200 μM) than that used in the antibiotic screening tests (refer to Chapter 4). Nevertheless, this 'post-selection' nitrite production occurred at a very low rate evidenced by the plateau of the grow curves.

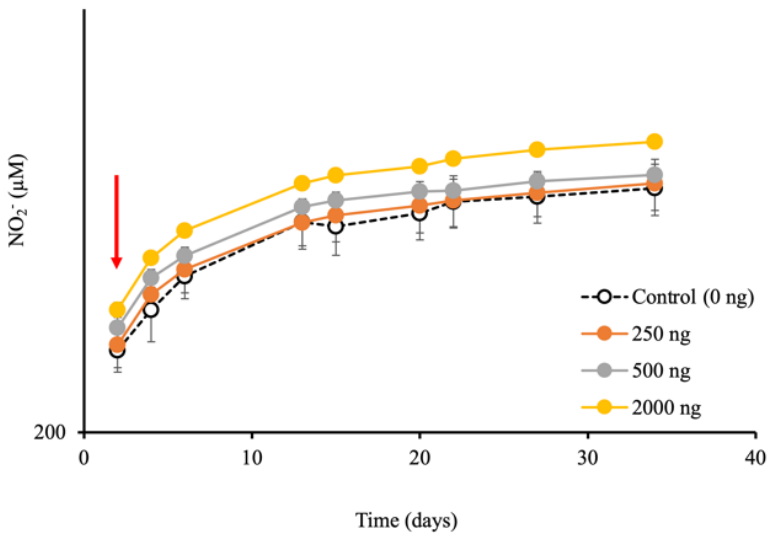
6.4 $CaCl_2$ heat-shock transformation of '*Ca*. N. franklandus C13'

Transformation of '*Ca*. N. franklandus C13' cells using the $CaCl_2$ heat-shock method was done using either 250 ng, 500 ng or 2000 ng of plasmid DNA (pfrank-mCherry-Cdc-orb and pfrank-mCherry-orb). In addition, the heat-shock temperatures used included 45ºC, 55ºC, 65ºC and 80ºC. In all treatments, regardless of the heat-shock temperature and plasmid concentration used, cultures did not exhibit exponential nitrite production (*Figure 6.1* and *Figure 6.2*). It was therefore concluded that the

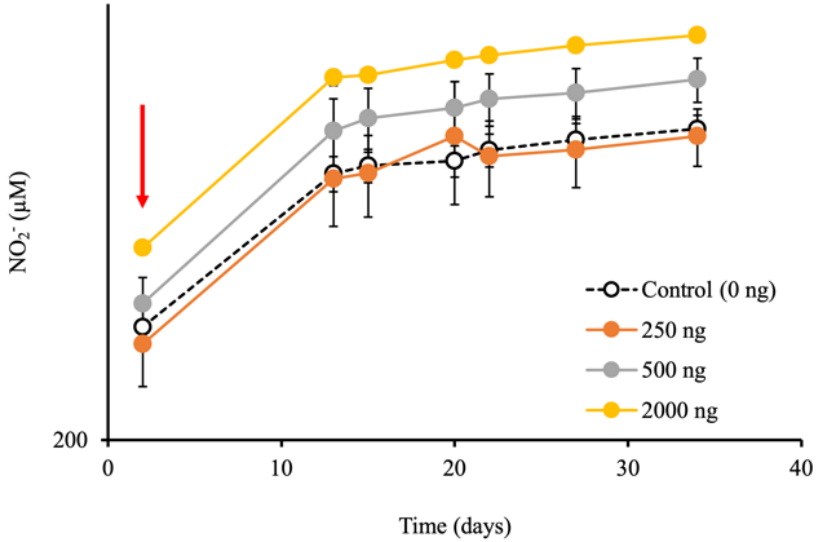
attempts to transform '*Ca*. N. franklandus C13' using the $CaCl_2$ heat-shock method were unsuccessful.

Although it has been noted that nitrite accumulation did not cease following the addition of puromycin, cultures heat-shocked at 80ºC exhibited a rapid plateau following the addition of the antibiotic when compared to the lower temperatures.

(A) Heat-shock 45°C (pfrank-mCherry-orb)



(B) Heat-shock 55°C (pfrank-mCherry-orb)



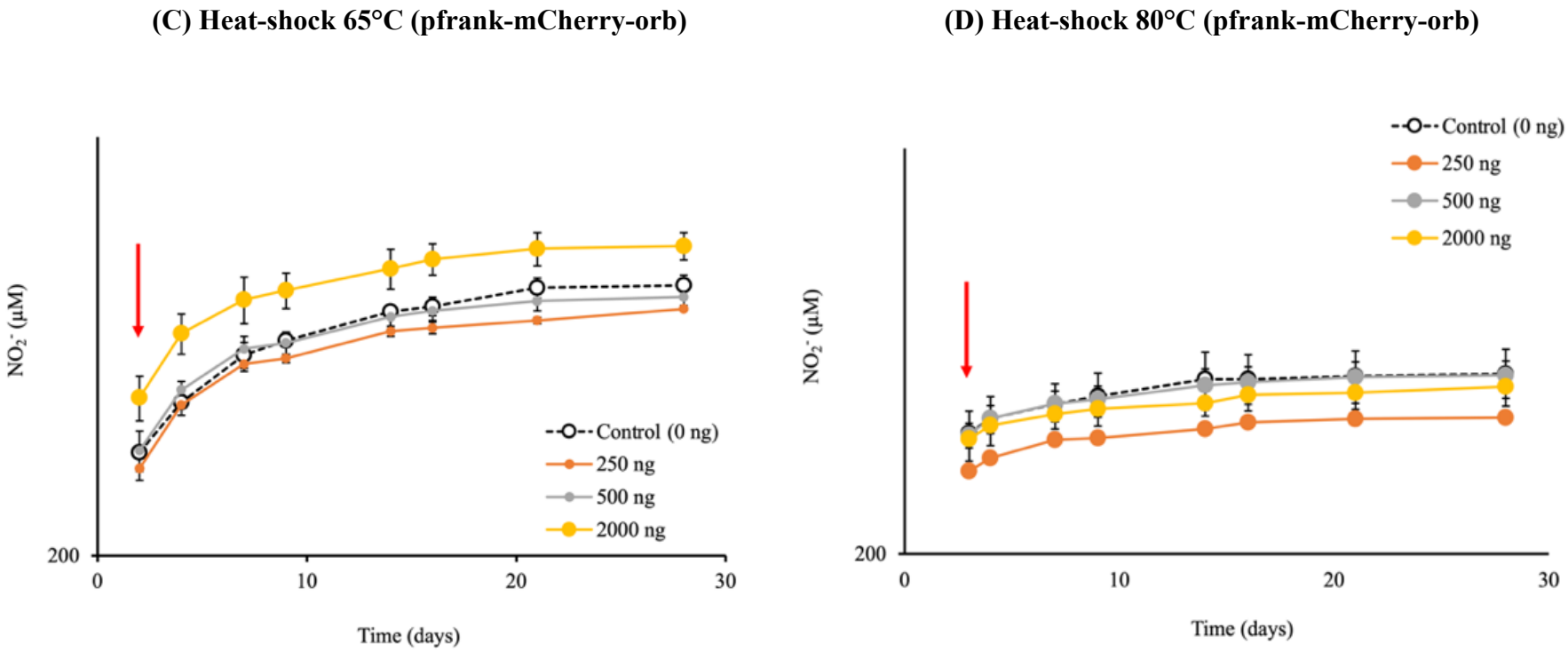
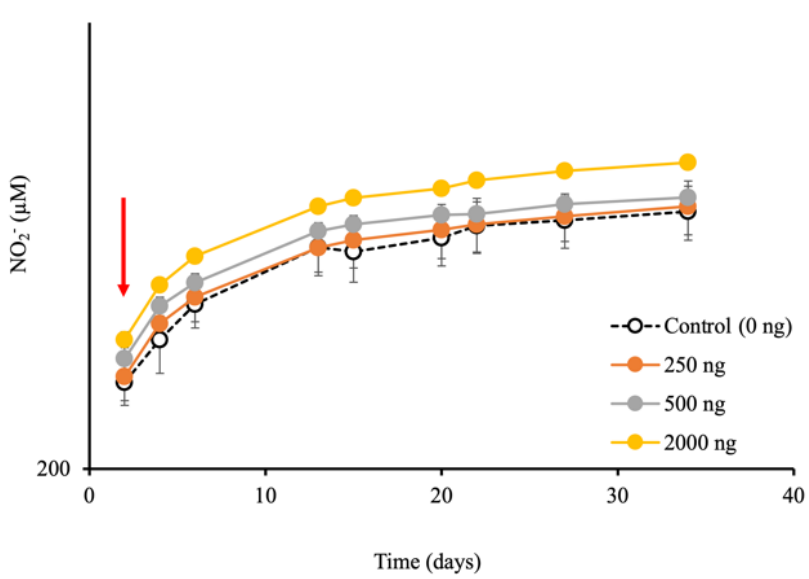
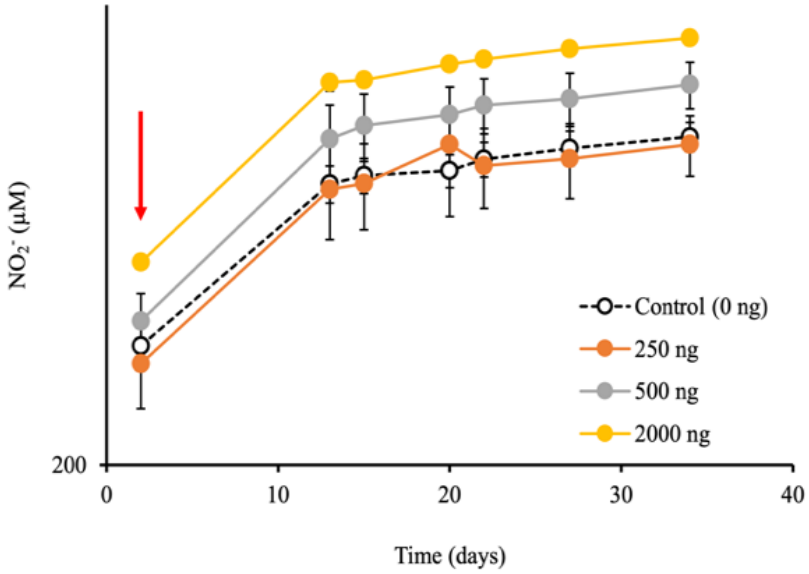


Figure 6.1: Nitrite production of '*Ca*. N. franklandus C13' cells transformed with pfrank-mCherry-orb using the $CaCl_2$ heat-shock method. A heat-shock temperature of either **(A)** 45ºC **(B)** 55ºC **(C)** 65ºC or **(D)** 80ºC was used. Control cultures were transformed in the absence of plasmid DNA. Growth curves were plotted using nitrite concentrations obtained from the average of two replicate cultures on a logarithmic scale. Error bars are standard error of the mean (SEM) and may be smaller than the size of the symbol. Initial nitrite measurements were taken following the addition of puromycin. Red arrows indicate time points when puromycin was added.

(A) Heat-shock 45°C (pfrank-mCherry-Cdc-orb)



(B) Heat-shock 55°C (pfrank-mCherry-Cdc-orb)



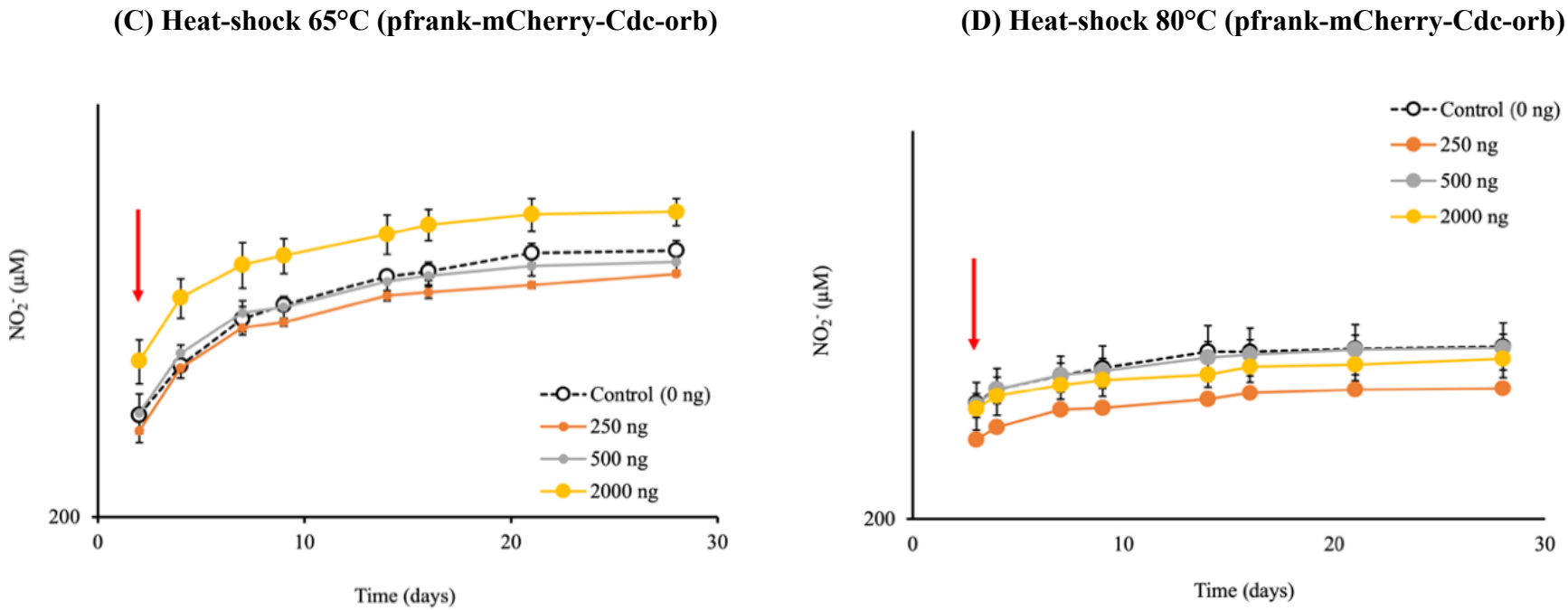


Figure 6.2: Nitrite production of '*Ca*. N. franklandus C13' cells transformed with the plasmid pfrank-mCherry-Cdc-orb using the $CaCl_2$ heat-shock method. A heat-shock temperature of either **(A)** 45ºC **(B)** 55ºC **(C)** 65ºC or **(D)** 80ºC was used. Control cultures were transformed in the absence of plasmid DNA. Growth curves were generated by plotting nitrite concentrations obtained from the average of two replicate cultures against time on a logarithmic scale. Error bars represent standard error of the mean (SEM) and may be smaller than the size of the symbol. Red arrows indicate time points when puromycin was added.

6.4.1 PCR screening of $CaCl_2$ heat-shock treated cells

Despite no evidence of successful transformation, total DNA was extracted from transformed cultures at the end of the incubation period and screened by PCR using primers targeting the plasmid-specific *mCherry* gene. Prior to the extraction of total DNA, the cells were treated with DNase I to degrade any extracellular DNA to minimise false positives (as described in *section 2.12.6*). Total DNA as opposed to plasmid DNA was extracted as the alkaline lysis method is yet to be tested on these archaea. Additionally, since the plasmids were expected to be maintained at low copy numbers, alkaline lysis would likely result in low yields (refer to Chapter 5).

Amplicons of the expected size were obtained for cultures transformed with 250 ng (both replicates) and 2000 ng (one replicate) of pfrank-mCherry-Cdc-orb using a heat-shock temperature of 45ºC. The expected amplicons were also detected in cultures transformed using 500 ng (one replicate) of pfrank-mCherry-Cdc-orb using a heat-shock temperature of 65ºC. These PCR screening results are presented in *Figure 6.3*.

PCR screening also detected the expected amplicons in cultures transformed with 2000 ng of the plasmid pfrank-mCherry-orb and heat-shocked at 45ºC (*Figure 6.4*). No amplicons were amplified in any of the control cultures i.e. cells transformed in the absence of plasmid DNA.

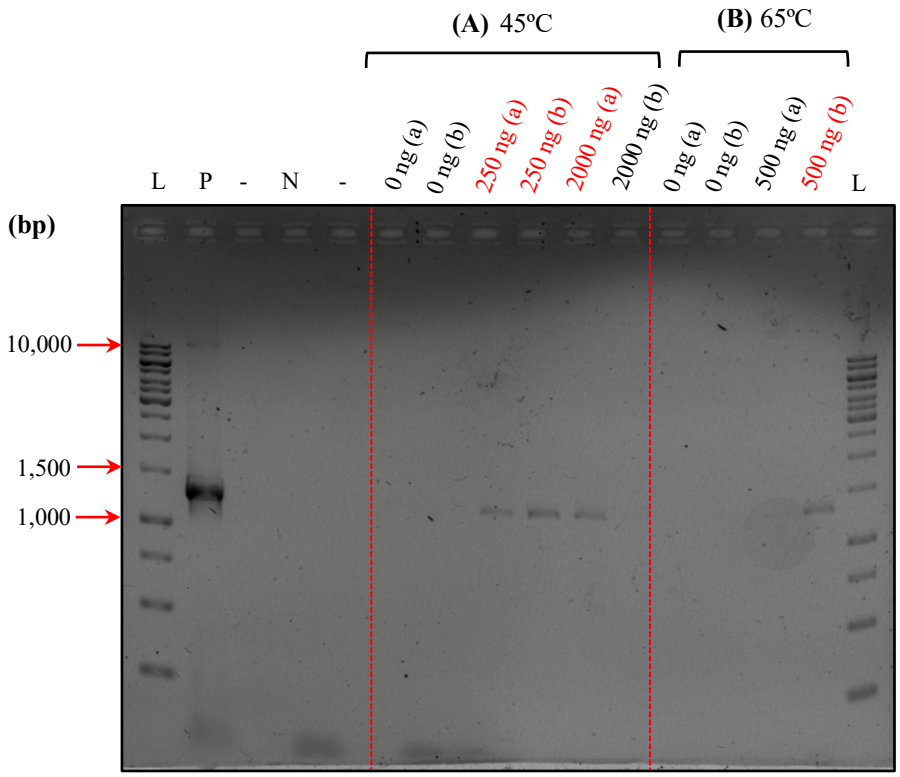


Figure 6.3: PCR screening of '*Ca*. N. franklandus C13' cells transformed with pfrank-mCherry-Cdc-orb using the $CaCl_2$ heat-shock method. Total DNA was used as a PCR template to detect the presence of the plasmid-contained *mCherry* gene with an expected amplicon size of 1326 bp. Both replicates of each culture were screened (e.g. 0 ng (a) and (b)). **(A)** Cells transformed with 250 ng or 2000 ng of plasmid DNA and heat-shocked at 45ºC. **(B)** Cells transformed with 500 ng of plasmid DNA and heat-shocked at 65ºC. **Lane 1 and 16:** 1kb Generuler ladder (L). **Lane 2:** Positive control (P), **Lane 3 and 5:** Blank wells (-) **Lane 4:** no template control (N), **Lane 6 and 7:** Cells transformed with 0 ng of plasmid DNA. **Lane 8 and 9:** Cells transformed with 250 ng plasmid DNA. **Lane 10 and 11:** Cells transformed with 2000 ng plasmid DNA. **Lane 12 and 13:** Cells transformed with 0 ng of plasmid DNA. **Lane 14 and 15:** Cells transformed with 500 ng plasmid DNA. Cultures for which an amplicon was detected are highlighted in red. PCR amplicons were resolved on a 0.8% (w/v) TBE agarose gel.

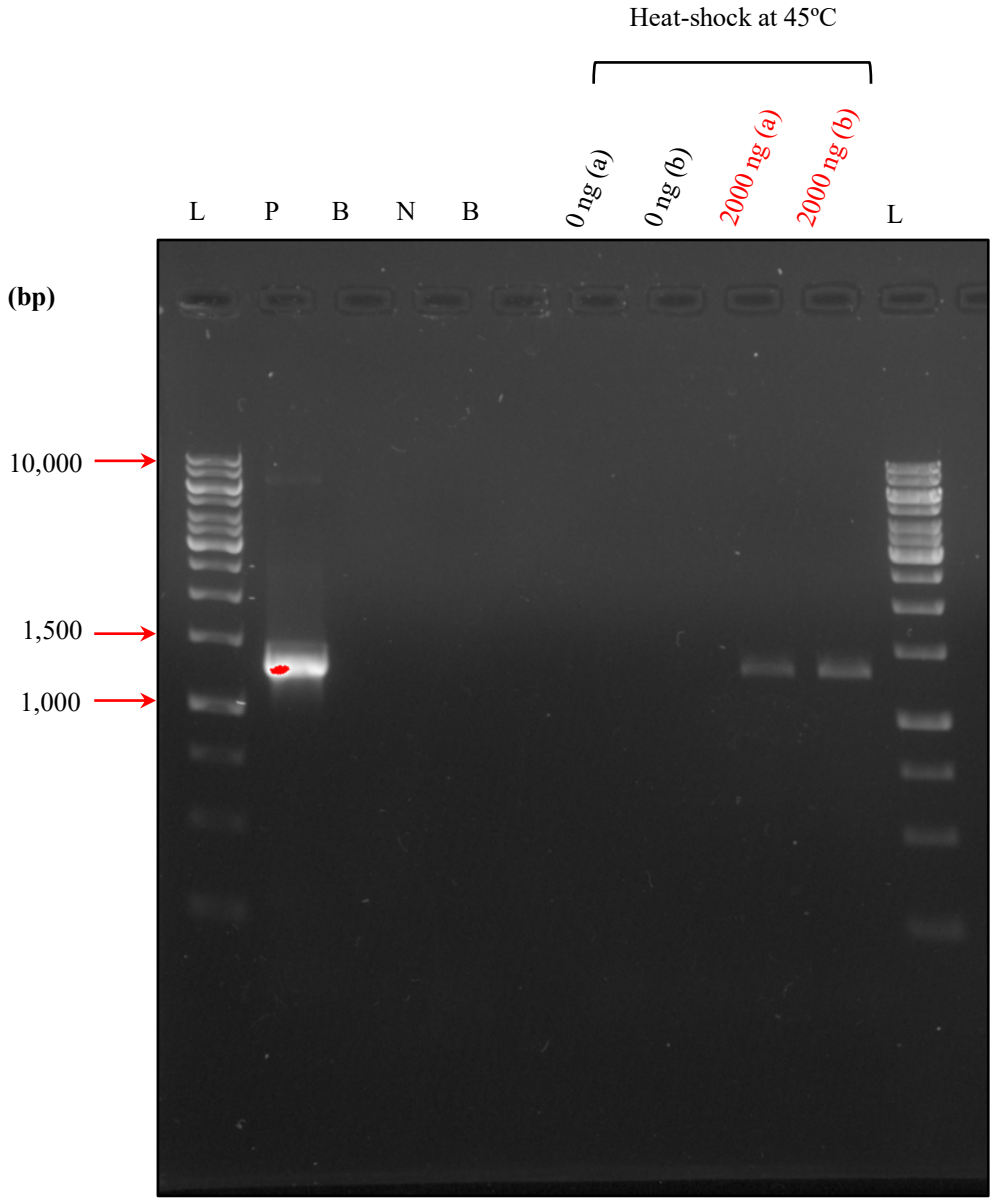


Figure 6.4: PCR screening of '*Ca*. N. franklandus cells' transformed with plasmid pfrank-mCherry-orb using the $CaCl_2$ heat-shock method (heat-shock 45ºC). Total DNA was used as the PCR template to detect the presence of the plasmid-borne *mCherry* gene with an expected amplicon size of 1326 bp. Both replicates for each culture were screened (e.g. 0 ng (a) and (b)). **Lane 1 and 10:** 1kb Generuler ladder (L). **Lane 2:** Positive control (P), **Lane 3 and 5:** Blank wells (B), **Lane 4:** no template control (N), **Lane 6 and 7:** Cells transformed with 0 ng of plasmid DNA. **Lane 8 and 9:** Cells transformed with 2000 ng plasmid DNA. Cultures for which an amplicon was visible are highlighted in red. Amplicons were resolved on a 0.8% (w/v) TBE agarose gel.

6.5 Electroporation of '*Ca*. N. franklandus C13'

Prior to the transformation of '*Ca*. N. franklandus C13' with plasmid DNA using electroporation, the response of this strain to different electric field strengths (FS) and varying numbers of pulses was investigated. The effect of FS on '*Ca*. N. franklandus C13' cells was investigated using exponential-decay pulses whereas number of pulses were manipulated using the square wave pulses.

The overall aim of these initial tests was to determine whether any of the electrical parameters used resulted in a significant decrease in cell viability following electroporation using nitrite production as an indicator. In addition, the use of chemical pre-treatments (i.e. CTAB) as a means to weaken the outer cell wall prior to electroporation and facilitate DNA entry into the cell was also investigated.

6.5.1 Effect of field strength on '*Ca*. N. franklandus C13'

Using the exponential-decay pulse, '*Ca*. N franklandus C13' cells were exposed to a FS of either 20, 25 or 28 kV/cm. The effect of the FS was tested in the absence of both plasmid DNA and antibiotics. Following electroporation, total nitrite production was monitored to determine whether any of the FS had any effect on the growth of this strain. No differences in nitrite production were evident between the pulsed and unpulsed control cultures suggesting that the FS used had no effect on cell viability (*Figure 6.5*).

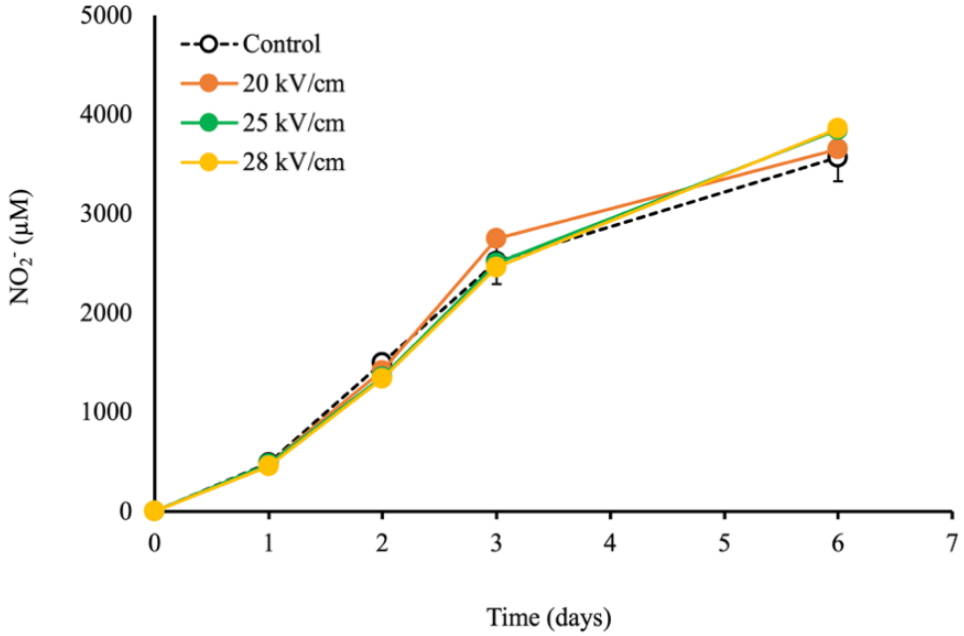
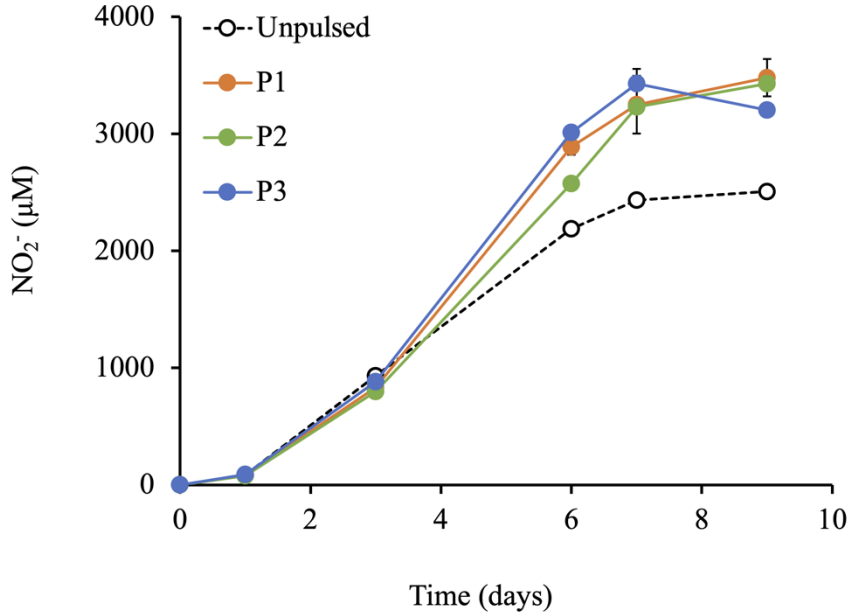


Figure 6.5: Effect of field strength on nitrite production. '*Ca*. N. franklandus C13' cells were electroporated using exponential-decay wave forms using a field strength of either 20, 25 or 28 kV/cm. No plasmid DNA or selective agent was used for these experiments. The control cultures were not electroporated. The nitrite (NO_2^-) concentrations plotted represent the average of three replicates. Error bars represent the standard error of the mean (SEM) and may be smaller than the symbols.

6.5.2 Response of '*Ca*. N. franklandus C13' cells to square-wave pulses

The use of a square wave protocol allows one to manipulate parameters such as the number and duration of the pulses used. In addition, when multiple pulses are to be discharged, one can also manipulate the time between each pulse (i.e. pulse interval). We proceeded to determine the effect of discharging two pulses at 2400 volts (equivalent to a FS of 24 kV/cm) for a duration of either 1, 2 or 5 ms on the growth of '*Ca*. N. franklandus C13'. Under these electrical parameters, doubling the number of pulses had no effect on nitrite production regardless of the duration of the pulse. (*Figure 6.6*). **Note:** The pulse interval was constant at 5 sec.



Parameters	P1	P2	P3
Voltage (V)	2400	2400	2400
Pulse length (ms)	1	2	5
No. of pulses	2	2	2
Pulse interval (sec)	5	5	5

Figure 6.6: Response of '*Ca*. N. franklandus C13' to square wave pulses. '*Ca*. N. franklandus C13' was pulsed (in the absence of plasmid DNA and antibiotics) using three different sets of parameters including P1, P2 and P3. **(P1**) Two pulses of 2400 volts for a duration of 1 ms. **(P2)** Two pulses of 2400 volts for a duration of 2 ms and **(P3)** Two pulses of 2400 volts for a duration of 5 ms. The pulse interval was maintained at 5 sec for all experiments. The unpulsed cells i.e. those not exposed to any electrical conditions served as a control. The total Nitrite (NO_2^-) concentrations plotted represent the average of three cultures. Error bars represent the standard error of the mean (SEM) and may be smaller than the symbol.

6.5.3 Electroporation of '*Ca*. N. franklandus C13' with plasmid DNA

Despite no response of '*Ca*. N. franklandus C13' to any of the field strengths tested (based on total nitrite production), transformation with the plasmids pfrank-CRISPR-amoB (*Figure 6.7*) and pfrank-mCherry-Cdc-orb (*Figure 6.8*) was attempted using electroporation with exponential-decay wave form.

Additionally, in an attempt to disrupt the outer cell membrane, '*Ca*. N. franklandus C13' was treated with the cationic detergent CTAB at a concentration of 20 µM. This was based on the protocol reported by Rajagopal and colleagues (2014) in which CTAB was used as a permeabilization agent for various bacterial species. Following CTAB treatment, a significant number of cells were lost in subsequent wash steps compared to the untreated cells. This was based on visualisation of the resulting cell pellet. **Note:** The untreated cells underwent similar wash steps so that the only difference was the CTAB treatment step. Therefore, it is possible that CTAB affected the ability of the cells to pellet at the bottom of the tubes and were subsequently lost in between wash steps.

Following transformation, it became evident that CTAB was toxic to '*Ca*. N. franklandus C13' at the concentration used. Additionally, the lack of exponential nitrite production and no difference in the growth pattern between the transformed and control cultures indicated that the transformation was unsuccessful. This was true for cells transformed with either pfrank-CRISPR-amoB and pfrank-mCherry-Cdc-orb (*Figure 6.7* and *Figure 6.8* respectively).

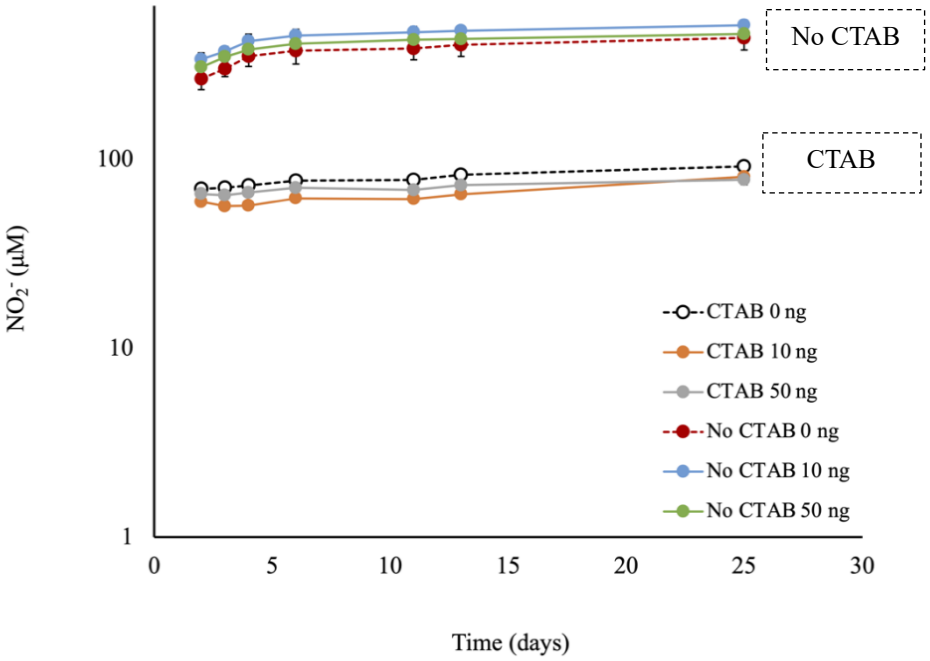


Figure 6.7: Nitrite production of '*Ca*. N. franklandus C13' cells transformed with the plasmid pfrank-CRISPR-amoB using electroporation (exponential-decay wave form). Electroporation of '*Ca*. N. franklandus C13' was performed on both CTAB treated and untreated cells. For the CTAB treated cells, a concentration of 20 µM was used. The total nitrite (NO_2^-) concentrations plotted are the averages of two replicates on a logarithmic scale. Error bars represent the standard error of the mean (SEM) and may be smaller than the symbol.

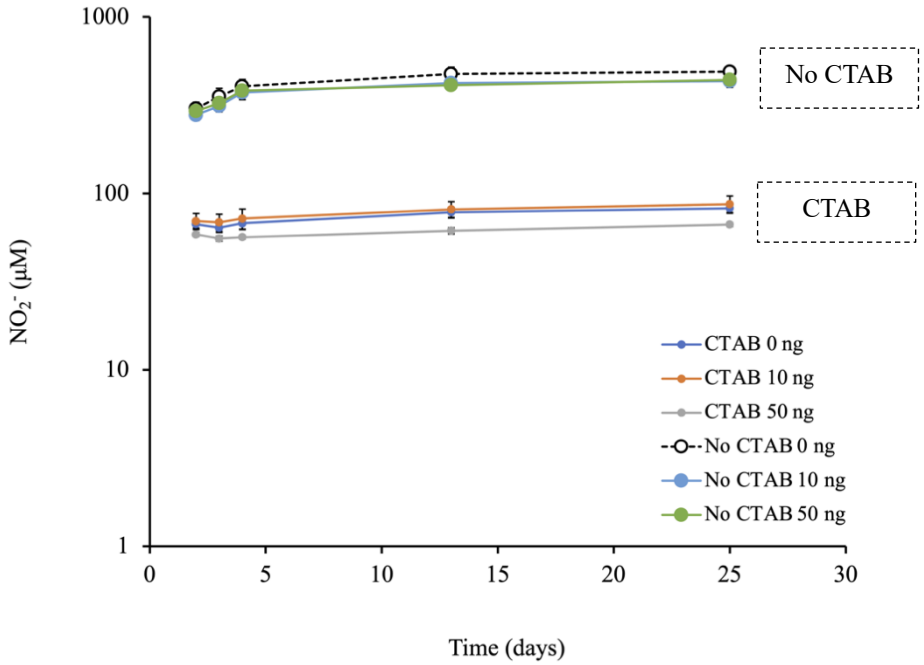


Figure 6.8: Nitrite production of '*Ca*. N. franklandus C13' cells transformed with plasmid pfrank-mCherry-Cdc-orb using electroporation (exponential-decay wave form). Electroporation of '*Ca*. N. franklandus C13' was performed on both CTAB treated and untreated cells. For the CTAB treated cells, a concentration of 20 µM was used. The total nitrite (NO_2^-) concentrations plotted represent the average of two replicates on a logarithmic scale. Error bars represent the standard error of the mean (SEM) and may be smaller than the symbol.

6.6 PEG-mediated transformation of '*Ca*. N. franklandus C13'

The transformation of '*Ca*. N. franklandus C13' with plasmid pfrank-mCherry-Cdc-orb using the PEG method involved the manipulation of two main factors including (i) the PEG concentration (35% or 60%) and (ii) the PEG molecular weight (PEG_{600} or PEG_{6000}). A total of 1000 ng of plasmid DNA was used for each transformation experiment. The control cultures were incubated in the presence of PEG but in the absence of plasmid DNA. No prior PEG toxicity tests were performed.

Exponential nitrite production was not evident for any of the PEG transformed cultures. In addition, no differences in nitrite production between the transformed and control cultures could be discerned indicating that the transformations were unsuccessful (*Figure 6.9* and *Figure 6.10*). As seen with previous transformation attempts, nitrite production did not cease following the addition of puromycin.

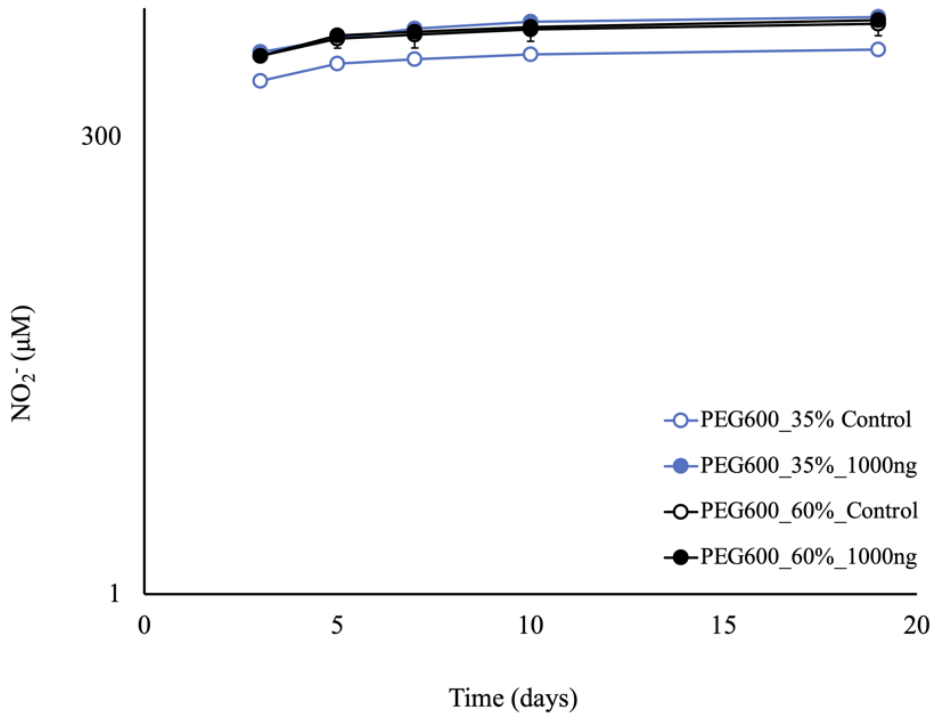


Figure 6.9: Nitrite production of '*Ca*. N. franklandus C13' cells transformed with the plasmid pfrank-mCherry-Cdc-orb using PEG_{600}. PEG was used at a concentration of either 35% or 60% and the total plasmid mass used for all experiments was 1000 ng. The control cultures were treated with PEG but in the absence of plasmid DNA. The total nitrite (NO_2^-) concentrations plotted are the mean of two replicates on a logarithmic scale. Error bars represent the standard error of mean (SEM) and may be smaller than the symbols.

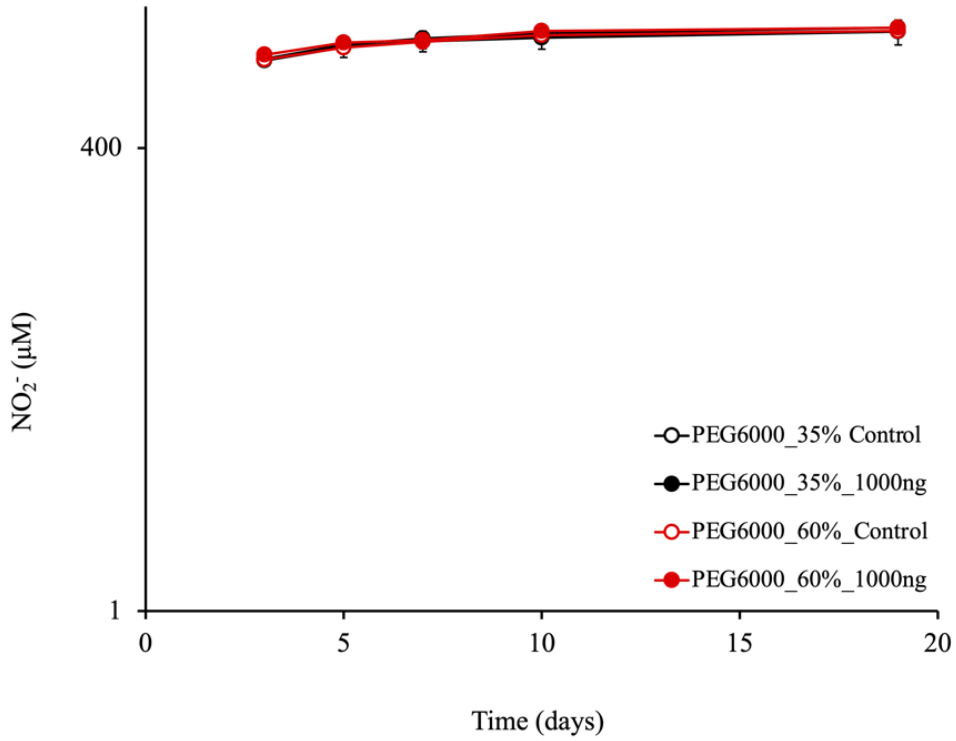


Figure 6.10: Nitrite production of *Ca*. N. franklandus C13' cells transformed with the plasmid pfrank-mCherry-Cdc-orb using PEG_{6000}. PEG was used at a concentration of either 35% or 60% and the total plasmid mass used for all experiments was 1000 ng. The control cultures were treated with PEG in the absence of plasmid DNA. The total nitrite (NO_2^-) concentrations plotted are the mean of two replicates on a logarithmic scale. Error bars represent the standard error of mean (SEM) and may be smaller than the symbols.

6.6.1 PCR screening of PEG transformed cells

All PEG-transformed '*Ca*. N. franklandus C13' cultures were screened by PCR targeting the plasmid-contained *mCherry* gene. As described previously, extracellular DNA was degraded using a DNase I treatment prior to total DNA extraction. No amplicons could be detected in any of the cultures except for those transformed with PEG_{600} (35%) 1000 ng (a) and PEG_{600} (60%) 1000 ng (b) (highlighted in red in *Figure 6.11A*).

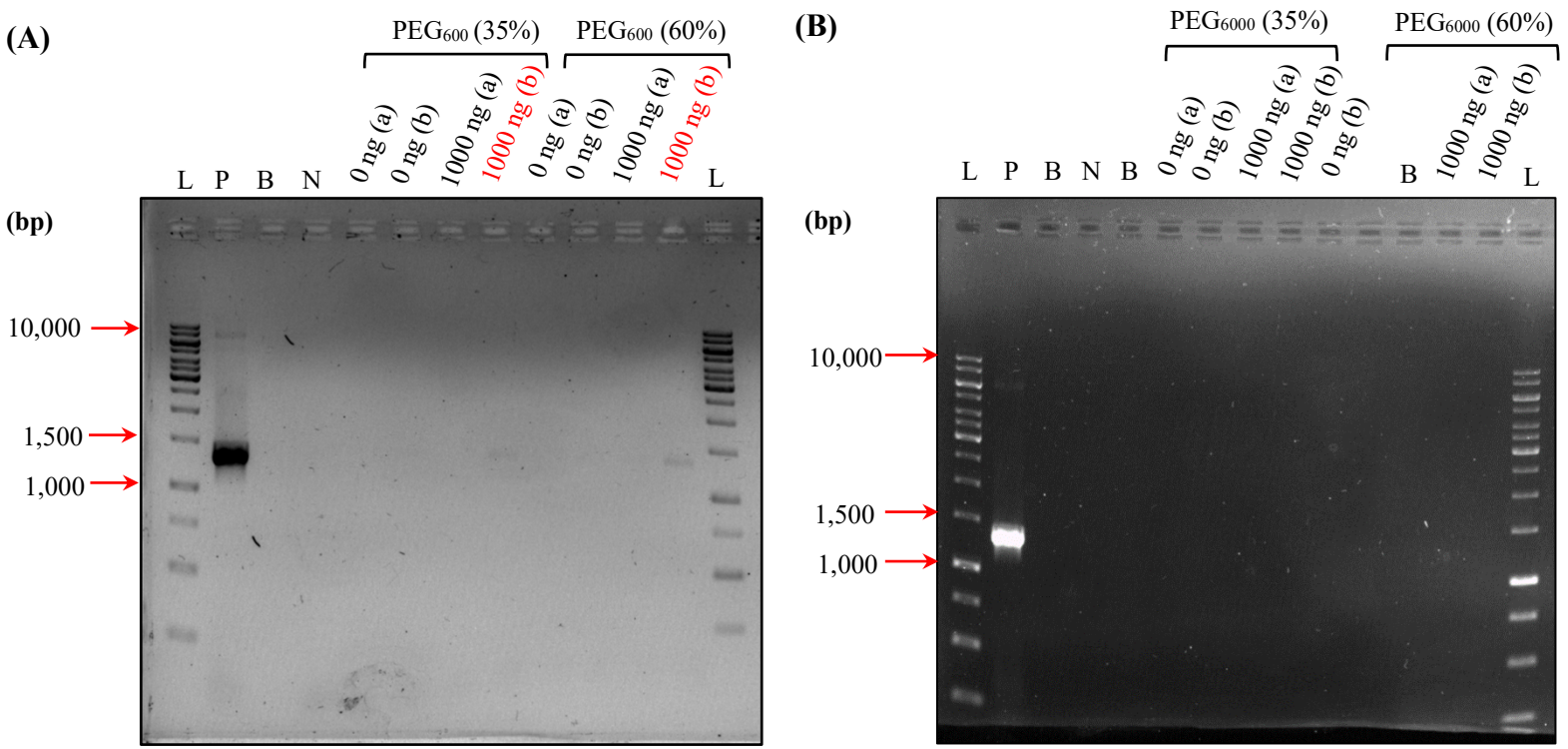


Figure 6.11: PCR screening of '*Ca*. N. franklandus C13' cells transformed using polyethylene glycol (PEG). Total genomic DNA was extracted from the liquid cultures and screened by PCR using primers targeting the *mCherry* gene with an expected product size of 1326 bp. **(A)** Cells transformed with 1000 ng of plasmid pfrank-mCherry-Cdc-orb using PEG_{600} at a concentration of either 35% or 60%. **Lanes 1 and 13:** 1kb generuler ladder (L). **Lane 2:** Positive control (P). **Lane 3:** Blank well (B). **Lane 4:** No template control (N). **Lanes 5/6 and 9/10:** No DNA transformants. **Lanes 7/8 and 11/12:** pfrank-mCherry-Cdc-orb transformants. **(B)** Cells transformed with 1000 ng of plasmid pfrank-mCherry-Cdc-orb using PEG_{6000} at a concentration of either 35% or 60% (v/v). **Lane 1/13:** 1kb generuler ladder (L). **Lane 1:** Positive control (P). **Lane 3/5:** Blank wells (B). **Lane 4:** No template control (N). **Lane 6/7** and **11/12:** No DNA transformants. **Lane 8/9** and **13/14:** pfrank-mCherry-Cdc-orb transformants. **Lane 10:** Blank well (B). PCR amplicons were resolved on a 0.8% (w/v) TBE agarose gel.

6.7 The '*Ca*. N. franklandus C13' restriction-modification system

R-M systems are important determinants of the transformation efficiency of an organism as they are responsible for the degradation of foreign DNA lacking the appropriate modification (i.e. methylation). The R-M systems of '*Ca*. N. franklandus C13' are yet to be characterised. Nevertheless, an *in silico* approach using the 'REBASE' database detected three methyltransferase genes in this strain (*Table 6.1*). **Note:** The cognate restriction endonucleases could not be detected in the database.

The methyltransferase encoded by the ORF NFRAN_1366 (MjaIII), was predicted to methylate the sequence motif 'GATC'. Interestingly, this recognition sequence is identical to that of the Dam methyltransferase. The remaining two methyltransferases encoded by ORF NFRAN_0126 and NFRAN_0696 are predicted to target 'AGCT' and 'GTAC' respectively and are not present in *E. coli* DH5α.

Table 6.1: Predicted methyltransferases in the genome of '*Ca*. N franklandus C13'.

ORF	R-M type	Closes relative and percent identity	Recognition sequence
Modification methylase (NFRAN_0126, yhdJ)	II	A putative Type II N4-cytosine or N6-adenine DNA methyltransferase from *Nitrosocosmicus hydrocola* G61 (93%)	AGCT
Modification methylase (NFRAN_0696, MjaV)	II	A putative Type II N4-cytosine or N6-adenine DNA methyltransferase from *Nitrosocosmicus hydrocola* G61 (72%)	GTAC
Modification methylase (NFRAN_1366, MjaIII)	II	A putative Type II N4-cytosine or N6-adenine DNA methyltransferase from *Nitrosocosmicus hydrocola* G61 (72%)	GATC (same as Dam)

6.8 Discussion

The main goal of this study was to identify a suitable transformation method that can be used to induce DNA uptake in the AOA strain '*Ca*. N. franklandus C13'. Three methods were tested including $CaCl_2$ heat-shock, electroporation, and PEG-mediated transformation. Following transformation, rather than plating the cells on solid growth medium, '*Ca*. N. franklandus C13' was maintained in liquid cultures. The purpose of this will be discussed in detail later on. In theory, exponential nitrite production was expected in successfully transformed cultures, since the cells would have gained resistance to puromycin and therefore continue to replicate and increase in biomass. Unfortunately, due to the absence of exponential nitrite production in all of the cultures and no discernible differences between the control and transformed cultures, it was concluded that the transformation efforts in '*Ca*. N. franklandus C13' were unsuccessful for all three methods.

Interestingly, despite unsuccessful transformation efforts, PCR screening for the plasmid-contained *mCherry* gene yielded the correct amplicons for a handful of the transformed cultures. Whether this suggests that the plasmids gained entry into the cells but were not expressed or replicated is unclear. The PCR products could also result from the incomplete digestion of extracellular DNA by DNase I prior to total DNA extraction. The inconsistent amplification of the *mCherry* gene between replicates could be due to poor quality DNA as no quality controls were performed on the total DNA that was extracted.

Nitrite production is an experimentally validated indicator of growth of AOA cells (Tourna *et al.*, 2011; Lehtovirta-Morley *et al.*, 2016). It was reported that for all '*Ca*. N. franklandus C13' cultures (i.e. transformed and controls) maintained under

selective conditions, did not cease to produce nitrite (albeit at a significantly lower rate). It is unclear whether this leaky nitrite production in the presence of a selection agent would make it difficult to detect successfully transformed cultures. For example, if the *pac* resistance gene is expressed at low levels, it may not confer high level resistance and may not be discernible from the control. The leaky nitrite accumulation also raises the question whether puromycin is indeed a suitable selection agent.

Perhaps the expectation that successful transformation would only be evident as exponential nitrite production is too idealistic or simplistic. It is also plausible that plasmid uptake slows the growth rate due to the metabolic burden of maintaining a plasmid. Furthermore, the expression of the selective marker (*pac*) may be suboptimal thus offering the cells limited resistance. Additionally, puromycin may have a so-called 'archaeal-static' effect (akin to bacteriostatic) whereby the cells are not killed but instead have significantly reduced growth rate. Based on bacterial studies, protein synthesis inhibitors can act as bacteriostatic antibiotics which are those that result in growth inhibition rather than cell death (Bernatová *et al.*, 2013). This would provide a logical explanation for the very slow growth rates of '*Ca*. N. franklandus C13' despite nitrite still being produced.

Alternatively, perhaps puromycin, an inhibitor of *de novo* protein synthesis causes decoupling of ammonia-oxidation and growth. In such a scenario, the AMO present in the cells prior to the addition of puromycin would be responsible for the observed nitrite production. Since no new protein synthesis would be expected to occur following the addition of puromycin, the rate of accumulation of nitrite would remain constant. However, if new protein were to be synthesised, then the nitrite would be expected to increase exponentially due to an increase in cellular and protein biomass.

Unfortunately, whether the AMO remains active for an extended period time in the absence of *de novo* protein synthesis has not been experimentally proven. It would have been informative to determine the cell counts to provide an indication of whether the nitrite produced post puromycin treatment, corresponded to an increase in biomass or was merely due to independent enzyme activity. Although puromycin proved to be a useful selection agent, further work is needed to understand this leaky nitrite production. It will also be necessary to test hygromycin B as an alternative selective agent to determine whether any differences exist between these two antibiotics.

In addition to qRT-PCR and cell counting, an interesting experiment that could provide some insight into whether puromycin decouples ammonia oxidation from growth, would be to grow '*Ca*. N. franklandus C13' on solid growth medium in the presence of puromycin. If colonies eventually develop, that means there was an increase in biomass and thus the nitrite produced post-selection is still coupled to growth. In contrast, if nitrite is accumulation occurs but no colonies develop then the decoupling hypothesis could hold true.

As mentioned previously, '*Ca*. N. franklandus C13' cells were maintained in liquid cultures and not plated on solid medium following transformation. The decision to maintain the transformants in liquid cultures as opposed to plating on gel plates, at first glance seems counterintuitive since we have previously developed a method to grow '*Ca*. N. franklandus C13' on solid growth medium (refer to Chapter 3). However, the enrichment of potential positive transformants in liquid growth medium as opposed to plating on gel plates offers several advantages. Firstly, although Phytagel™ was identified to be the only known gelling agent that is suitable for AOA, its use is dependent on the brand and batch. This makes the gellan gum batches that are known

to be suitable rather precious. Therefore, it was a safer option to initially maintain the transformants in liquid cultures to enrich for positive mutants. An added advantage of using liquid cultures is that the transformed cultures can be maintained in smaller volumes. The use of smaller volumes permits the testing of a wider range of physical parameters for each of the transformation methods. In addition, it is much faster to screen smaller volumes of liquid cultures to identify the suitable conditions before moving onto solid medium which is much more technically challenging to handle and more time-consuming. The use of liquid cultures also allows for the measurement of nitrite production in the cultures and visualisation of the growth patterns that could reveal subtle differences between the transformation parameters. Although nitrite can also be measured from cultures grown using the Liquid-Solid (LS) method, the larger volumes needed for this method could hinder the detection of subtle difference in nitrite due to the diluting effect of the large volumes. Secondly, it is less technically challenging to extract DNA from cells in liquid cultures as opposed to those growing within a gel matrix. Lastly, cells transformed with the pfrank-mCherry series of plasmids were to be screened by fluorescent microscopy. This would require harvesting of the cells and subsequent preparation for visualisation. If the cells had been plated in solid growth medium, it would be very difficult to minimise the carry-over of the gel debris which can interfere with the preparation of the microscopy samples. Despite these advantages of using liquid cultures for these initial transformation studies, the LS-method will be invaluable once successful transformation has been achieved.

Numerous factors could contribute to the failed transformation of '*Ca*. N. franklandus C13'. One such factor specific to this work is related to the plasmids used. As discussed in Chapter 5, the homology between the plasmid and the genome created

by the presence of the chromosomal replication origin and/or the *cdc* gene may result in unwanted plasmid integration. This potential integration would likely be detrimental to the cell because the sequence used as the putative replication origin in this work also spans a portion of the coding sequence of the *cdc* gene. The integration of the plasmids into the genome may provide an additional explanation (albeit speculative) as to why *mCherry* amplicons were obtained following PCR screening despite no evidence of a successful transformation (i.e. exponential nitrite production). A further drawback associated with the plasmids used is the potential competition for the initiator protein (Orc1/Cdc6) by the two identical origins (Zakrzewska-Czerwińska, *et al*., 1995) which could negatively impact the transformation efficiency. In hindsight, a suitable control experiment would have been to perhaps use an integrative plasmid that lacks the chromosomal replication origin.

As discussed previously, various mobile genetic elements (MGE) such as viruses and even integrative conjugative elements have been discovered in certain AOA strains (Abby *et al.*, 2018; Kim *et al*., 2019). MGE elements have been highly valuable in archaeal genetics particularly the Sulfolobales (Jonuscheit *et al*., 2003; Schleper *et al*., 1992). Such genetic elements could be a source of exogenous replication origins and would avoid dependence on the chromosomal replication origin. For example, the shuttle vector pEXSs used in *S. solfataricus* was constructed using the replication origin of the SSV1 virus which can also exist as an extrachromosomal element (Cannio *et al.*, 1998). The mesophilic growth conditions of '*Ca*. N. franklandus C13' are conducive to investigating conjugation as a potential transformation method with commonly used donor strains such as *E. coli*. Alternatively, it may also be possible to utilise broad range conjugative plasmids such as the IncPα plasmid RP4 that have already been characterised and extensively used (Grahn *et al*., 2000). Interestingly, the

broad range conjugative plasmid RP4 was successfully used to mediate successful conjugative DNA transfer from *E. coli* to the methanogen *Methanococcus maripaludis* (Dodsworth *et al*., 2010).

The constructs used in the current study were all circular DNA molecules. In certain archaeal organisms, it has been demonstrated that the topology of the constructs (linear vs circular) plays a major role in the determining the transformation efficiency. For example, in the methanogen *M. voltae*, transformation with linearized DNA resulted in a 19-fold improvement when compared to circular DNA (Patel *et al.*, 1994). A similar observation was made in *S. solfataricus*, where a drastic difference in transformation efficiency was obtained when using linear DNA of plasmid pKL (Deng *et al.*, 2009). In this work, the use of linearized plasmids was not tested but will need to be considered for any future attempts to transform this strain. More recently, a method to transform archaea using multimeric DNA generated by prolonged overlap enhanced PCR (POE-PCR) was developed and showed a drastic increase in the transformation of *T. kodakarensis* KOD1 and *P. yayanosii* A1 (Song *et al.*, 2021). Future work on the transformation of '*Ca*. N. franklandus C13' and perhaps other strains may need to consider the DNA topology of the constructs when designing transformation experiments.

'*Ca*. N. franklandus C13' is an unusual AOA strain with regards to its cell envelope architecture. Unlike most AOA strains available in culture, '*Ca*. N. franklandus C13' lacks genes encoding S-layer proteins (Nicol *et al.*, 2019) Based on high-resolution electron microscopy imaging (refer to Chapter 3), '*Ca*. N. franklandus C13' seems to be enveloped in two membranes i.e. an inner and an outer cell membrane with a large periplasmic space. This structure is reminiscent of the hyperthermophilic archaeon

Ignicoccus hospitalis (Heimerl *et al.*, 2017). In addition, '*Ca*. N. franklandus C13' also exhibits similar vesicular protrusions from its cytoplasm. This double-membrane cell structure may provide an explanation for the recalcitrant nature of this AOA strain to transformation. In fact, '*Ca*. N. franklandus C13' is a very difficult strain to lyse and requires both chemical and physical disruption methods to ensure sufficient lysis (Laura Lehtovirta-Morley, personal communication). In yeast and fungi, the cell wall is often removed or disrupted using enzymatic digestion or chemical treatments prior to transformation (Schiestl *et al*., 1993; Kawai *et al*., 2010; Li *et al.*, 2017). Similarly, the S-layer in some archaea is often removed using chemical treatment with EDTA prior to transformation (Dyall-Smith, 2009; Haque *et al*., 2020). Unfortunately, removal of the outer cell membrane in '*Ca*. N. franklandus C13' is currently not possible as no suitable method has been identified yet. This is further compounded by the unknown composition of the outer cell membrane.

Efforts to increase the cell permeability of '*Ca*. N. franklandus C13' using CTAB were evidently not successful. More importantly, this detergent exhibited toxic effects on the cells (at the concentrations used) and is unlikely to be a suitable approach for future experiments. However, the use of physical methods such as sonication could negate the need for enzymes or toxic chemicals (as seen with CTAB) to disrupt the outer cell membrane. Sonication method relies on the use of ultra-sound to increase cell permeability (Wyber *et al*., 1997). Interestingly, unlike electroporation whose primary target is the cell membrane, sonication is non-specific and targets both cell barriers (Azencott *et al*., 2007). Since electroporation does not affect the cell wall, the failed attempts to transform '*Ca*. N. franklandus C13' using this method could suggest that the cell wall or outer cell membrane does not readily permit transit of DNA. In archaeal strains such as *Sulfolobus*, the S-layer is not removed prior to transformation with

electroporation (Schleper *et al*., 1992; Kurosawa and Grogan, 2005). This could suggest that while S-layers have large enough pores that facilitate DNA entry in other archaea, the unknown outer cell membrane of '*Ca*. N. franklandus C13' does not.

An important factor that affects the transformation efficiency is the R-M system which targets foreign DNA. In fact, in some organisms, the R-M system is the sole barrier to transformation (Chung *et al*., 2013). To our knowledge no R-M system has yet been studied or characterised in any AOA strain. A bioinformatics-based search of the '*Ca*. N. franklandus C13' genome predicted the presence of three standalone methyltransferases belonging to the type II R-M system. The cognate restriction endonucleases to these methyltransferases (if any) were not detected in the genome. In type II R-M systems, the methylation and restriction endonucleases are encoded in separate genes and may even be found in different genomic loci (Vasu and Nagaraja, 2013; Phillips *et al.*, 2019). The absence of cognate restriction endonucleases could also suggest that these are orphan methyltransferases (e.g. Dam methylase) which are not associated with an R-M system (Broadbent *et al.*, 2007). Out of the three methyltransferases detected in '*Ca*. N. franklandus C13', only one shares a recognition sequence with a methyltransferase from *E. coli* DH5α (i.e. Dam methylase). This means that the plasmids used in this work lacked the necessary methylation modification provided by the remaining two methyltransferases. Therefore, assuming these are not orphan methyltransferases, the plasmids would still be sensitive to the cognate restriction endonucleases of these two methyltransferases which could have a significant impact on the transformation efficiency. This may provide an additional explanation for the lack of transformants. Future work will require the characterisation of the '*Ca*. N. franklandus C13' methylation which is typically done using long-read sequencing data such as that generated by Pacific Biosciences (PacBio) sequencing

(Rhoads and Au, 2015). However, an interesting experiment to conduct in the absence of such sequencing data would be to test whether cell-free extracts of '*Ca*. N. franklandus C13' are able to cleave or fragment the plasmid DNA. A similar approach was previously used in *Halobacterium salinarium* (formerly known as *Halobacterium halobium*) to detect the presence of a restriction endonuclease in cell-free extracts of this strain (Schinzel and Burger, 1986).

Lastly, although highlighted in Chapter 5, it is necessary to acknowledge that the functionality of the gene expression elements (e.g. promoters, terminators and RBS) needs to be considered. This is because even if DNA uptake was successfully introduced, the inadequate expression of the *pac* selection marker would result in cell death or reduced growth and thus low transformation efficiency.

6.9 Summary and outlook

It can be concluded from the data, that the efforts to transform '*Ca*. N. franklandus C13' using methods such as electroporation, PEG, and $CaCl_2$ heat-shock and their corresponding parameters were unsuccessful. Consequently, it is not possible to determine whether the chromosomal replication origin facilitated autonomous replication of the three plasmids or whether *pac* is a suitable selection marker for this strain.

While we have explored various factors that could contribute to the overall failure of the transformation in '*Ca*. N. franklandus C13', it is challenging to pinpoint specific factors at such an early stage. Nonetheless, this work has highlighted key aspects that need to be considered and prioritised for future transformation efforts of '*Ca*. N. franklandus C13' and perhaps alternative strains. Firstly, it is paramount that focus is

placed on characterising the outer cell membrane of '*Ca*. N. franklandus C13'. This will be important in developing methods to disrupt this barrier and enable DNA uptake as seen in other cell wall-containing models. Secondly, efforts to functionally characterise the R-M system will need to be prioritised to ensure the plasmids are not being degraded upon entry. It is also essential that characterisation of the various MGE discovered in other AOA strains are studied and characterised. Lastly, it may also be worthwhile to test these transformation methods on alternative AOA strains particularly those enveloped in an S-layer.

Chapter 7: General discussion and conclusion

7.1 General summary and project aims

Ammonia-oxidising archaea (AOA) catalyse the oxidation of ammonia to nitrite, the first and rate-limiting step of nitrification. These archaea are widely distributed in the environment and as such are considered to be key microorganisms in the nitrogen cycle. Although nitrification is integral to the nitrogen cycle, it can also be detrimental to the environment as well as agricultural output (Wrage *et al.*, 2001; Lehtovirta-Morley, 2018). For example, nitrification is associated with nitrous oxide emissions which is a potent greenhouse gas. With regards to agriculture, the oxidation of ammonia-based nitrogen fertilisers has a significant impact on crop yields due to reduced fertiliser use efficiency (Norton and Ouyang, 2019). Additionally, the oxidation of the ammonia in nitrogen-based fertiliser increases the presence of highly mobile nitrates that leach into aquatic systems and contribute towards eutrophication (Lehtovirta-Morley, 2018). Therefore, a better understanding of AOA biology could contribute towards the development of innovative mitigation strategies that improve nitrogen fertiliser use and minimise greenhouse gas emissions (Norton and Ouyang, 2019).

Among the various gaps in our knowledge regarding AOA biology are those pertaining to the archaeal ammonia oxidation pathway and the physiological adaptations to different ecological niches. Notable examples include the identity of the missing enzymes of the archaeal ammonia oxidation pathway and the specific mechanisms by which these archaea successfully adapt to different ecological niches such as acidic pH. Unfortunately, due to the lack of a genetic system, it is challenging to investigate these knowledge gaps and paint a complete picture of these processes. To our

knowledge, nobody has reported a genetic system for the Thaumarchaeota, and this highlights a major research bottleneck.

The overarching aim of this study was to lay the foundation for the establishment of a genetic toolbox for AOA. The specific objectives addressed in this work are: **(i)** the development of a novel cultivation method that allows AOA to be grown on a solid growth medium as single colonies **(ii)** to identify antibiotics that can be used as selective agents **(iii)** construct an artificial plasmid(s) and **(iv)** develop a transformation protocol to induce DNA uptake in AOA. Each of the empirical chapters (i.e. Chapters 3, 4, 5 and 6) address these specific objectives. These objectives represent the four main components of a genetic toolbox that together form a functional genetic system.

This chapter aims to provide a summary of the key findings of the empirical chapters and emphasize the contributions this work has made to field of AOA research. Furthermore, this chapter aims to acknowledge some of the limitations and caveats associated with the methodological approach and discuss various recommendations for the future of this project.

7.2 Cultivation of AOA on solid growth medium as single colonies

The main objective addressed in Chapter 3 was to develop a method to grow AOA on solid growth medium as distinct single colonies. The ability of archaea for whom genetic tools have been developed to readily form single colonies on solid growth medium has been instrumental in the establishment of a genetic system. This is because growth on solid medium allows efficient and low-cost mutant screening and isolation (see *section 1.5*). The approach used in this study was to first identify factors that preclude the growth of these archaea on solid growth medium. It was subsequently

determined that the growth of AOA on solid medium is dependent on the gelling agent used. To our knowledge, this phenomenon has only been previously reported in bacteria (Janssen *et al.*, 2002; Tamaki *et al.*, 2009). Among the archaeal strains that readily form colonies on solid growth medium, the choice of gelling agent is usually dictated by the growth conditions (e.g. temperature) of the particular strain. For example, gellan gum is almost exclusively used for hyperthermophilic strains as it is suited to elevated temperatures (> 60ºC). In comparison, mesophilic methanogens and haloarchaea are typically grown on agar. Thus, the findings that the growth of archaea is affected by gelling agents could better inform future efforts to grow not only AOA but also archaea from newly discovered or under studied phyla.

It still remains unclear whether the gel inhibition observed in this study is due to the gelling agent itself, or perhaps interactions of the gel with the media components during media preparation as has been reported by Tanaka and colleagues (2014). An interesting experiment would be to supplement the growth medium with the individual sugar components of the gels to determine how they impact growth. This approach was used by Tuovinen and Kelly (1973) who determined that galactose, one of the sugar components of agar, was inhibitory to *Thiobacillus ferrooxidans*. The individual sugar components of agar were released due to acid hydrolysis at low pH. This would be particularly interesting to test for the acidophilic AOA strain '*Ca*. N. sinensis Nd2' as it was inhibited by both agar and gellan gum.

Chapter 3 also describes a novel cultivation method, the Liquid-Solid (LS) method that enables the growth of AOA on solid growth medium as single colonies. The general principle of the LS-method involves the immobilisation of the cells in Phytagel™ (akin to the pour plate method) and overlaying this with liquid growth medium. The liquid

layer can be decanted off and replaced with fresh medium thus minimising the decrease of pH and the accumulation of nitrite to toxic levels. However, the LS-method is currently only amenable to two AOA strains including '*Ca*. N. franklandus C13' and *N. viennensis* EN76. It will be interesting to test whether this method is applicable to other AOA isolates.

A further limitation of the LS-method is the lack of a consistent and reliable brand (or batch) of gellan gum. Therefore, further tests are needed to identify suitable and reliable suppliers of gellan gum. It will also be interesting to expand the gel screening efforts to other strains which could perhaps reveal additional precluding factors to the growth of these archaea of solid medium.

Overall, Chapter 3 has successfully addressed one of the main objectives of this work, which is to identify the factors affecting the growth of AOA on solid growth medium and develop a novel cultivation method that allows AOA to be grown on solid growth medium as single colonies.

7.3 Identification of suitable selection agents

A selection system for mutants is an essential component of a genetic toolbox. Selection among archaea is primarily done using either antibiotics or auxotrophy (refer to *Table 1.1*). In the absence of a pre-existing genetic system and a lack of spontaneous auxotrophic mutants for the model AOA strain used in this study, it was decided that an antibiotic-based selection system was the most appropriate and more importantly the simplest option. However, reports on the sensitivity of the Thaumarchaeota to antibiotics are scarce and the few available reports are limited to a handful of strains and a narrow range of antibiotic families (Vajrala *et al.*, 2014; Zhao *et al.*, 2020).

Therefore, if an antibiotic-based selection system was to be developed, it was necessary to gain some insight into the sensitivity of the AOA to antibiotics. The primary objective of Chapter 4 was to identify suitable antibiotic that can be used as a selective agent for '*Ca*. N. franklandus C13'.

Two protein synthesis inhibitors, puromycin and hygromycin B were identified as potent inhibitors of '*Ca*. N. franklandus C13'. However, key questions still need to be answered regarding these antibiotics. For example, it remains to be determined whether spontaneously resistant mutants of '*Ca*. N franklandus C13' eventually develop. Extended monitoring of puromycin and hygromycin B treated cultures did not exhibit any evidence of resistance. This information is particularly important for the AOA as they are relatively slow growing microorganisms, and the selection agent needs to be highly effective to prevent a high background of untransformed cells. It will also be important to explore how cell density affects the observed sensitivity to puromycin and hygromycin B. This is because higher cell densities are usually favourable for transformation experiments.

Archaea represent a unique model for testing novel compounds because in addition to uniquely archaeal features, they are also known to harbour bacterial and eukaryotic elements. As a result, archaea could harbour novel targets, and even represent simplified models for complex eukaryotic processes (Barry and Bell, 2006). Through a collaborative effort with Matt Hutchings' research group (John Innes Centre, Norwich), two novel compound classes i.e. the fasamycins and formicamycins, were tested against '*Ca*. N. franklandus C13' and *N. viennensis* EN76. These compounds are rapidly emerging as important drug candidates and testing them in an archaeal model not only expands our knowledge of the scope of their biological activity but

may also reveal novel molecular targets. Both the fasamycins and formicamycins are known to target the type II topoisomerase enzyme DNA gyrase in bacteria. However, DNA gyrase is absent in the phylum Thaumarchaeota (Villain *et al*., 2022). Therefore, the potent activity of these compounds against the two AOA strains strongly suggests the presence of a novel target. The sole type II topoisomerase present in the AOA, topoisomerase VI, was proposed as a likely target, but since no *in vitro* assays have been performed this is still too speculative. It will therefore be necessary to conduct *in vitro* assays with purified topoisomerase VI to determine if it is the true target of these compounds. This work highlights the importance of including archaeal models in the efforts to characterise novel compounds.

In addition to being abundant in archaea, topoisomerase VI is also present in plants (McKie *et al.*, 2022). Interestingly, this topoisomerase is currently being studied as a possible target for herbicides (Allen, 2023). This raises the question whether the topoisomerase VI in AOA could present as a target for developing inhibitors of the nitrification reaction to mitigate the loss of nitrogen-based fertilisers?

Overall, puromycin and hygromycin B have been identified as potential selective agents for '*Ca*. N. franklandus C13' which successfully addresses the second objective of this work.

7.4 Artificial plasmids for '*Ca*. N. franklandus C13'

The availability of native plasmids in various archaea from the major phyla has been instrumental in the establishment of genetic tools (see *section 1.5*). This is because these native plasmids have been repurposed into molecular vehicles to transfer heterologous DNA into desired host strains. However, the availability of plasmids also

plays an important role during the development of transformation methods as they can reveal successful DNA uptake. It was therefore necessary to construct artificial plasmids for use in '*Ca*. N. franklandus C13' as no naturally occurring plasmids are currently available for the Thaumarchaeota.

In chapter 5, the rationale behind the design of three artificial vectors for '*Ca*. N franklandus C13' is described. To our knowledge, this is the first report on the assembly of plasmids for use in an AOA strain. All three plasmids were designed to self-replicate under the control of the '*Ca*. N franklandus C13' chromosomal replication origin. Unfortunately the use of a chromosomal replication origin for plasmid replication is associated with various limitations which have been extensively discussed. Future work will need to explore the use of integrative vectors. The use of integrative vectors has the added advantage of possibly being used across different AOA strains as it is unclear whether the plasmids designed in this work, under the control of '*Ca*. N. franklandus C13' chromosomal replication origin, could replicate in other AOA strains.

As discussed previously, native plasmids (specifically extrachromosomal plasmids) have not been identified in any of the AOA strains currently available in culture. This is in contrast to archaeal groups such as the methanogens, Sulfolobales, halophiles and even certain hypethermophilic Euryarchaeota, all for whom several naturally occurring plasmids have been discovered. It may be that the Thaumarchaeota primarily rely on other mobile genetic elements such as viruses or transposons to mediate genetic exchange. This may be supported by two recent studies that have reported the discovery of Thaumarchaeota viruses in metagenomic sequence data as well as in culture (Ahlgren *et al*., 2019; Kim *et al*., 2019). In addition, integrative mobile genetic

elements such as proviruses and integrated-conjugative elements have been discovered in the genomes of certain AOA (Krupovic *et al.*, 2011, 2019). Conjugative plasmids and viruses have played an instrumental role in establishing genetic tools in the Sulfolobales (see *section 1.5.2.1*). It will therefore be worthwhile to focus on efforts characterising these elements as they could be developed into robust DNA transfer and transformation tools. The use of conjugation also has an added advantage as it has been used to evade R-M systems. This is because conjugative transfer initially introduces single stranded DNA into the recipient host which can evade degradation by the endonuclease allowing its methylation at a later stage (Riley and Guss, 2021). This has successfully been reported for *Clostridium difficile* (Purdy *et al*., 2002). This is particularly relevant for the AOA as their R-M systems are yet to be characterised.

CRISPR-Cas systems have rapidly become the preferred tool for introducing genetic manipulations in eukaryotic and bacterial systems. Unlike its predecessors such as the zinc-finger nucleases (ZFN) and the transcription-activator like effector nucleases (TALEN), CRISPR-Cas is relatively easy to use and highly specific. While homologous recombination is still widely used for archaeal genetic studies, CRISPR-Cas is rapidly becoming a tool of choice. This is because unlike homologous recombination, CRISPR-Cas systems are not only efficient but also versatile. For example, in addition to genome-editing, CRISPR-Cas can be used to regulate the transcription of genes within a cell (Zebec *et al*., 2014; Larson *et al*., 2013). However, the majority of the CRISPR-Cas platforms currently in use are endogenous to the strains being studied (Zebec *et al.*, 2014; Peng *et al.*, 2015). A major disadvantage of relying on such endogenous systems is that due to the variation of CRISPR-Cas systems between strains, they may have limited use in alternative strains (Nayak and

Metcalf, 2017). In addition, CRISPR-Cas system endogenous to an extremophilic strain may require extensive adaptation to function in mesophilic strains.

For this reason, the development of class 2 CRISPR-Cas systems for the archaea has been a major breakthrough for archaeal genetic studies (Bao *et al*., 2022; Nayak and Metcalf, 2017). Class 2 CRISPR-Cas systems are easy to use as they rely on a single effector protein and may be used across different strains as they are not present in archaea (Makarova *et al*., 2020; Nayak and Metcalf, 2017). These factors played a role in the decision to construct a CRISPR-Cas9-based plasmid in this study.

Overall, this work has contributed towards the efforts aiming to establish a genetic toolbox by making available three distinct plasmids including a CRISPR-Cas9-based plasmid and two reporter gene-based plasmids.

7.5 Transformation of '*Ca.* N. franklandus C13

In Chapter 6, the transformation of '*Ca*. N. franklandus C13' using three artificial plasmids designed in this work was attempted. This formed part of the final objective of this study which was to explore different transformation methods to induce DNA uptake. Due to the lack of any reports on the transformation of the AOA, it was necessary to cast a 'wide net' and test different methods and conditions simultaneously. Based on the available literature on archaea of the major phyla, the three most commonly used methods are electroporation, $CaCl_2$ heat-shock and PEG-mediated transformation (refer to *Table 1.1*). These methods were consequently tested against '*Ca*. N. franklandus C13' in this study.

It was concluded from the data presented in chapter 6, that none of the three transformation methods yielded any successful transformants. The main basis for this conclusion was **(i)** the absence of exponential nitrite production in the cultures transformed with plasmid DNA and **(ii)** no difference in growth patterns between the control and transformed cultures.

An interesting observation was that puromycin, used as the selective agent, did not fully inhibit nitrite production. Although nitrite was expected to accumulate during the recovery period prior to the addition of puromycin, no significant nitrite production was expected following the addition of the antibiotic. This seems to be contrary to the results in Chapter 4, where the puromycin concentration that was highly inhibitory was lower (100 μM) than that used in the transformation experiments (200 μM) (Chapter 6). However, although cell counts were not performed in these experiments, it is likely that the cell density was higher in the transformation experiments and could explain this residual nitrite production. Furthermore, if the effect of puromycin is bacteriostatic, rather than bacteriocidal, a higher cell density and residual enzymatic activity may also explain why nitrite accumulation was only detectable in the transformation experiments. It is yet to be determined whether this 'post-selection' nitrite production is associated with cellular growth or merely the enzyme activity of the ammonia monooxygenase. Two main explanations have been proposed for nitrite production in the presence of the selective agent (i) puromycin only inhibits growth but does not kill the cells (akin to bacterial-static antibiotics) and/or (ii) puromycin decouples ammonia oxidation from growth. These can easily be investigated using methods such as quantitative real-time PCR and cell counts to determine whether the puromycin also inhibits an increase in cellular biomass.

Unfortunately, it was challenging if not impossible to pinpoint the exact cause of the inability to induce DNA uptake in '*Ca*. N. franklandus C13' with these methods at this stage. While nitrite production is a useful indicator of growth, it does not provide any information on for example the effects that the different treatments have on the permeability of the cell. A more informative approach to determining the ability of the different treatments to induce DNA uptake would be to use fluorescent molecules. For example, while comparing the effects of electroporation and sonication on cell permeability, Azencott and colleagues (2007) utilised the fluorescent molecules calcein and FITC-bovine serum albumin to determine their uptake following electroporation or sonication. Other commercial kits such as the LIVE/DEAD® BacLight™ (Invitrogen™, USA) work on a similar principle whereby the dye SYTO® 9 indiscriminately stains all cells regardless of viability or state of the cell membrane whereas the second dye (propidium iodide) selectively stains cells with a compromised cell membrane. This kit could be useful in determining the ability of the transformation conditions to increase cell permeability. Another DNA-binding dye, YOYO-1 has been successfully used to visualise interaction of plasmid DNA with the yeast cell wall as well as its uptake into the cell following treatments with PEG, heat-shock and lithium (Zheng *et al.*, 2005). Such methods may be much more informative with regards to the effects or response of '*Ca*. N. franklandus C13' (or an alternative strain) to the different transformation methods and their parameters than nitrite production alone. Furthermore, the use of such fluorescent methods has an added advantage over the use of reporter genes as they do not depend on gene expression elements such as promoters and terminators that are yet to be characterised in the AOA.

The cell wall in organisms such as unicellular algae has been shown to be a major barrier to the uptake of various molecules (Azencott *et al*., 2007). Similarly, in yeast

and fungi, protocols exist that rely on the disruption of the cell wall (e.g. via enzymatic digestion) (Schiestl *et al*., 1993) while in archaea, disruption of the S-layer forms the basis of various transformation protocols (e.g. methanogens and haloarchaea) (Dyall-Smith, 2009). High-resolution images of '*Ca*. N. franklandus C13' have revealed the presence of an unusual double-membrane (refer to Chapter 3). This means that for exogenous DNA to enter the cell, it must cross both these barriers. It may be worthwhile exploring the use of physical disruption methods such as sonication which have been shown to non-specifically disrupt cell barriers unlike electroporation which is specific to the cell membrane (Azencott *et al*., 2007). The use of non-selective permeabilization methods such as sonication is particularly relevant for this strain because it lacks an S-layer and its composition is currently unknown.

To our knowledge, this is the first publicly available literature on the transformation of an AOA. It is therefore premature to disregard the transformation methods used for future attempts. Furthermore, the different transformation parameters investigated in this work were far from exhaustive and consequently, there is still room for further investigation in future experiments. Lastly, regardless of the unsuccessful transformation efforts of an AOA in this study, this work will serve as a much needed point of reference for future transformation attempts of this strain and perhaps even other AOA.

7.6 '*Ca.* N. franklandus C13' as a model strain for an AOA genetic system

'*Ca*. N. franklandus C13' was selected as the model strain for the establishment of an AOA genetic system in this work. The decision to use this strain was attributed to its physiological properties which include a relatively fast growth rate and its mesophilic

nature. For example, the neutral pH and mesophilic temperature range (37ºC - 42ºC) are conducive to the use of antibiotics as selective agents. More importantly however, strains in the genus '*Nitrosocosmicus*' are known to tolerate higher ammonia concentrations which is typical of agricultural soils receiving high input of nitrogen-based fertilisers making this genus a relevant model to study in the context of agricultural management (Lehtovirta-Morley *et al.*, 2016; Liu *et al.*, 2021).

Over the course of this study, further technical advantages of '*Ca*. N. franklandus C13' as a model strain became evident. Firstly, we demonstrate that this strain is one of two AOA strains that were amenable to growth on solid growth medium using the LS-method. In addition, the cells of '*Ca*. N. franklandus C13' are relatively easy to harvest on membrane filters which is often desirable when using large volumes and it also yields visible cell pellets. This is in comparison to other strains such as *N. viennensis* EN76 that are preferably harvested by centrifugation which can be rather time-consuming particularly when working with larger volumes. It was also determined in this work that '*Ca*. N. franklandus C13' is sensitive to multiple selective agents in comparison to *N. viennensis* EN76 that was only significantly sensitive to hygromycin B. Consequently, a larger pool of selection markers is currently available for '*Ca*. N. franklandus C13'.

In conclusion, '*Ca*. N. franklandus C13' and related strains may be considered to be suitable models for a genetic system in the phylum Thaumarchaeota.

7.7 Conclusion

Overall, while not successful in establishing a functional genetic system for an AOA, this work has made a significant contribution towards this goal by developing a novel

cultivation method for two AOA strains, identified promising antibiotic-based selective agents and the construction of a CRISPR-Cas9 and reporter gene plasmids. Lastly, although unsuccessful in the transformation of '*Ca*. N. franklandus C13', this work serves a reference point for future efforts into the developing transformation protocols.

Appendices

Appendix A: Modified non-chelated trace element solution

Component	Mass/volume (per litre)	Concentration
dH_2O	987 ml	-
HCl (~12.5 M)	8 ml	100 mM
H_3BO_3	30 mg	0.5 mM
$MnCl_2.4H_2O$	100 mg	0.5 mM
$CoCl_2.6H_2O$	190 mg	0.8 mM
$NiCl_2.6H_2O$	24 mg	0.1 mM
$CuCl_2.2H_2O$	2 mg	0.01 mM
$ZnSO_4.7H_2O$	144m mg	0.5 mM
$Na_2MoO_4.2H_2O$	36 mg	0.15 mM

Note: The trace element solution is autoclaved as described previously and stored in the dark at 4ºC.

Appendix B: AOA vitamin solution

Component	Mass (g/L)
Biotin	0.02
Folic acid	0.02
Pyridoxine HCl	0.10
Thiamine HCl	0.05
Riboflavin	0.05
Nicotinic acid	0.05
DL Pantothenic acid	0.05
P Aminobenzoic acid	0.05
Choline Chloride	2
Vitamin B12	0.01

Note: Using KOH, adjust the pH to 7 and filter sterilise using a 0.22 μM pore size filter. Store in the dark at 4ºC.

Appendix C: Effects of gelling agents on the growth of '*Ca.* N. sinensis Nd2' and *N. viennensis* EN76

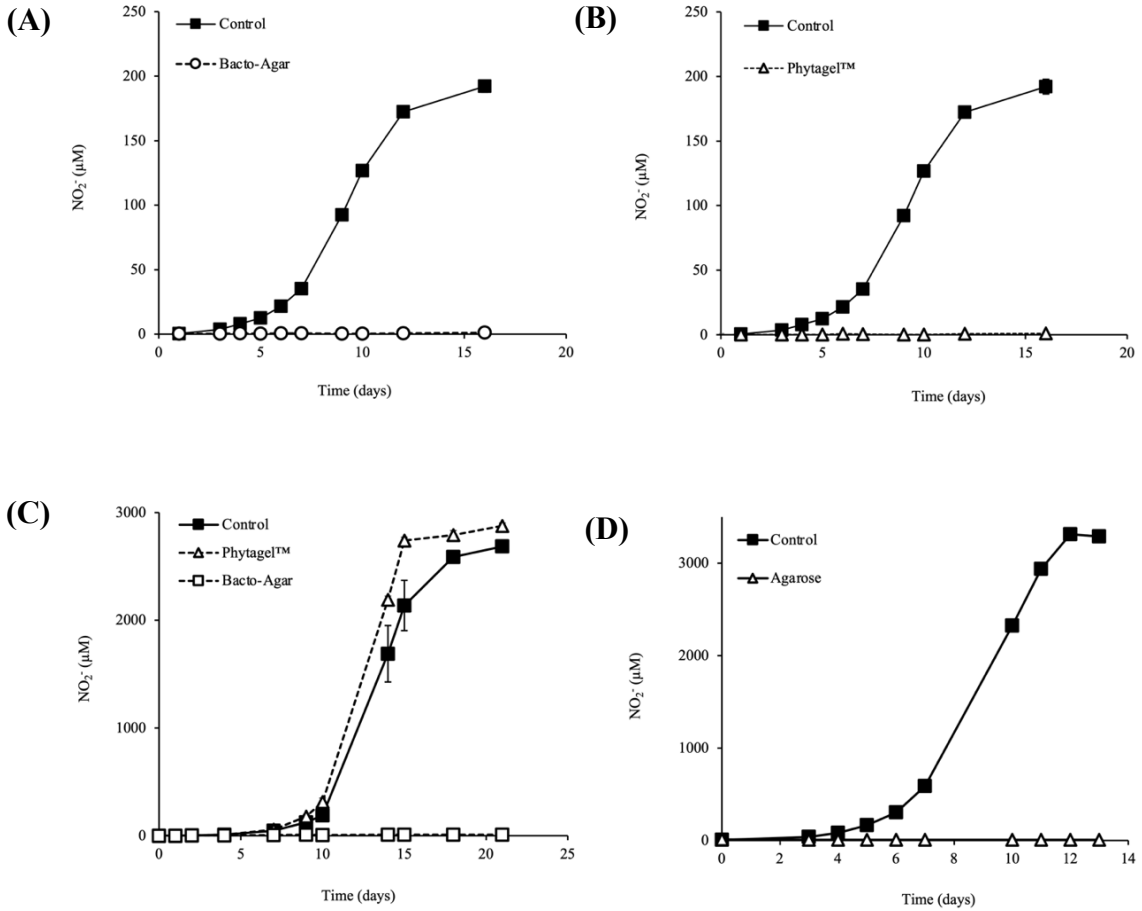


Figure C-1: Effects of Phytagel™ and Bacto-agar on the growth of '*Ca.* N. sinensis Nd2' (**A and B**) and *N. viennensis* EN76 (**C and D**). The control cultures were grown in the absence of a solidifying agent. Nitrite concentrations represent the average of three replicate cultures. Error bars are standard errors of the means and where they are not visible are smaller than the size of the symbol.

Appendix D: Nitrite accumulation of *N. viennensis* EN76 cells growing in Phytagel™

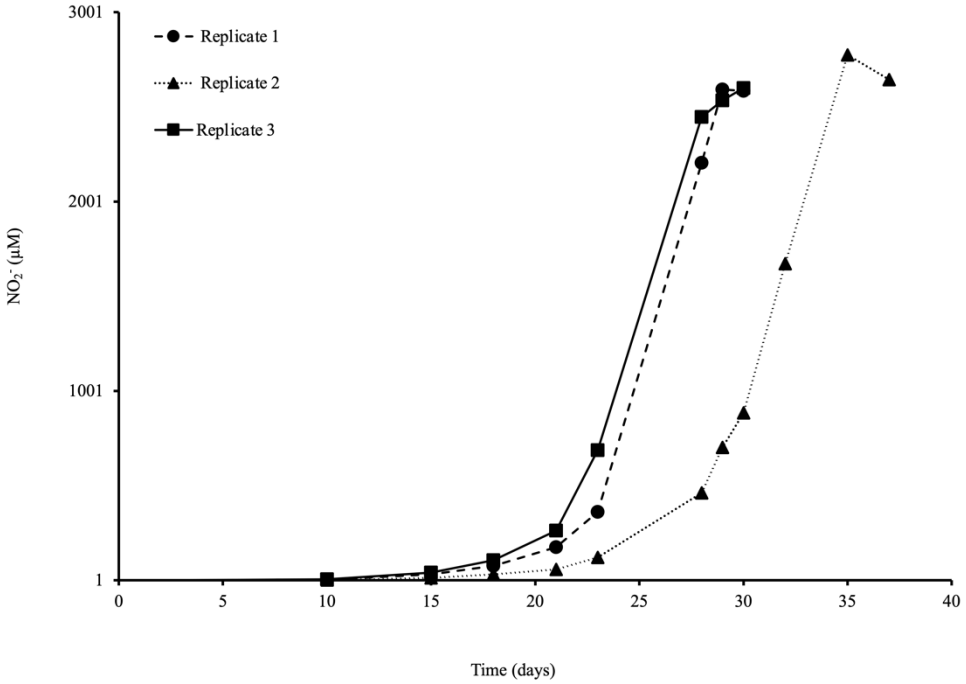


Figure D-1: Nitrite accumulation by *N. viennensis* EN76 cells embedded in Phytagel™. The detection of nitrite accumulation in all three cultures of Phytagel™ embedded *N. viennensis* cells suggested viable cells. Nitrite concentrations for each of the three replicate cultures were plotted separately.

Appendix E: Images of *N. viennensis* EN76 colonies and fluorescent micrographs gel embedded cells

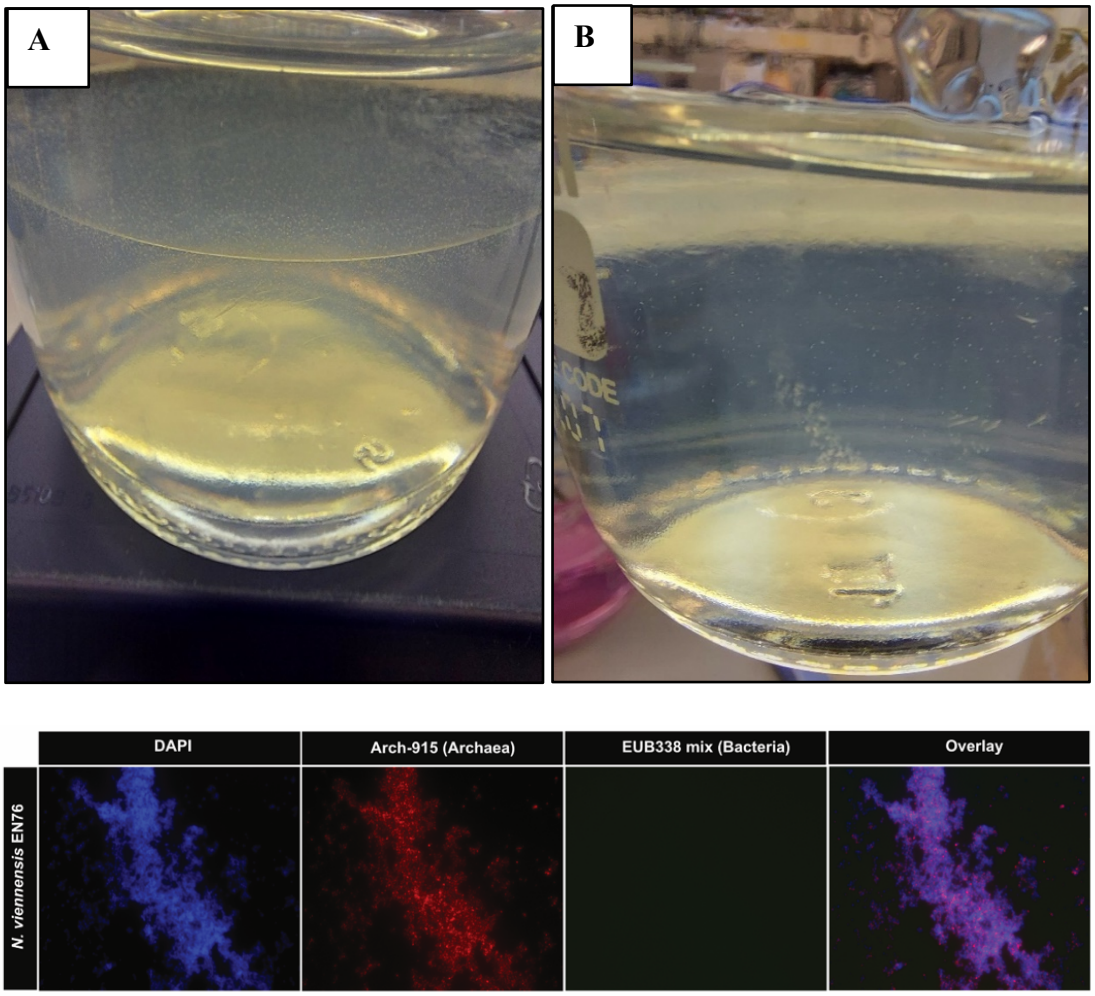


Figure E-1: Photographs of *N. viennensis* EN76 colonies growing within Phytagel™. **(A)** Colonies arising from a high-inoculum and **(B)** Colonies arising from a low-density inoculum. Fluorescent micrographs of *N. viennensis* EN76 cells growing within the Phytagel™. Cells are stained with FISH probes for archaea (Arch-915, red), bacteria (EUB338 mix, green) and DAPI (blue). Images were viewed at 1000 X magnification.

Appendix F: Long-term incubation of '*Ca*. N. franklandus C13' cells in the presence of antibiotics

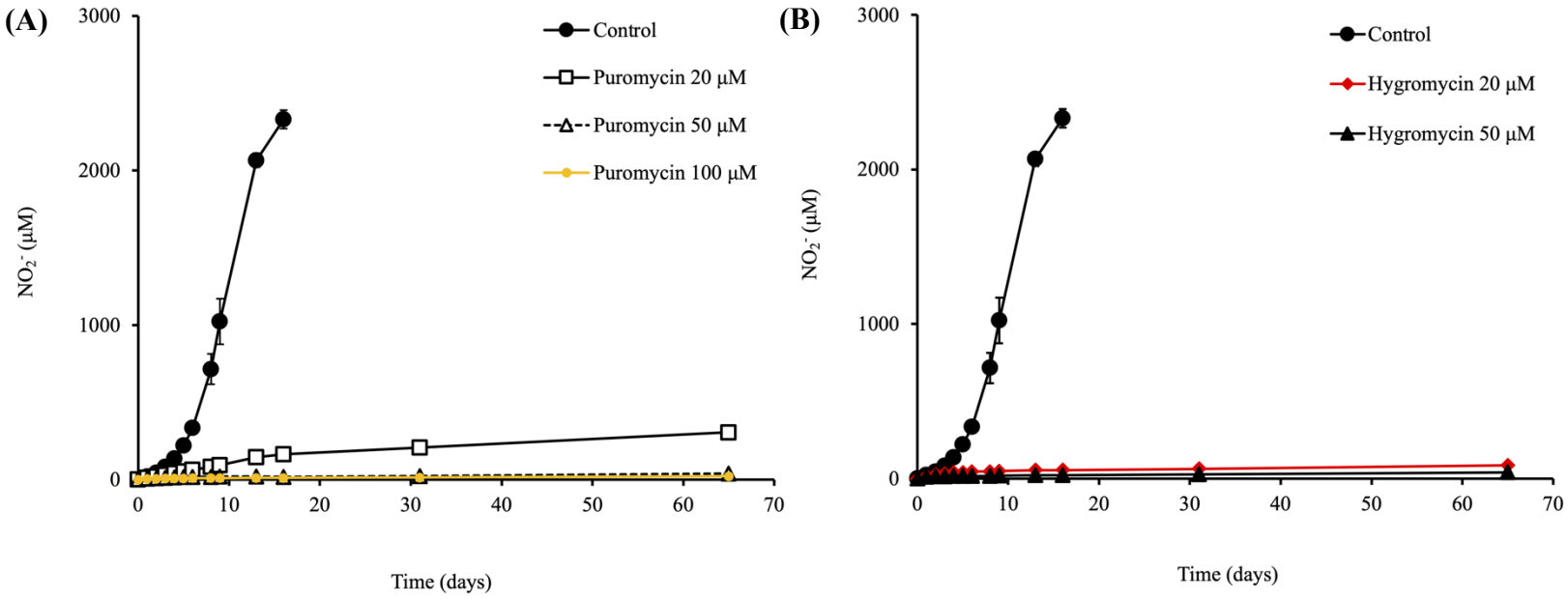
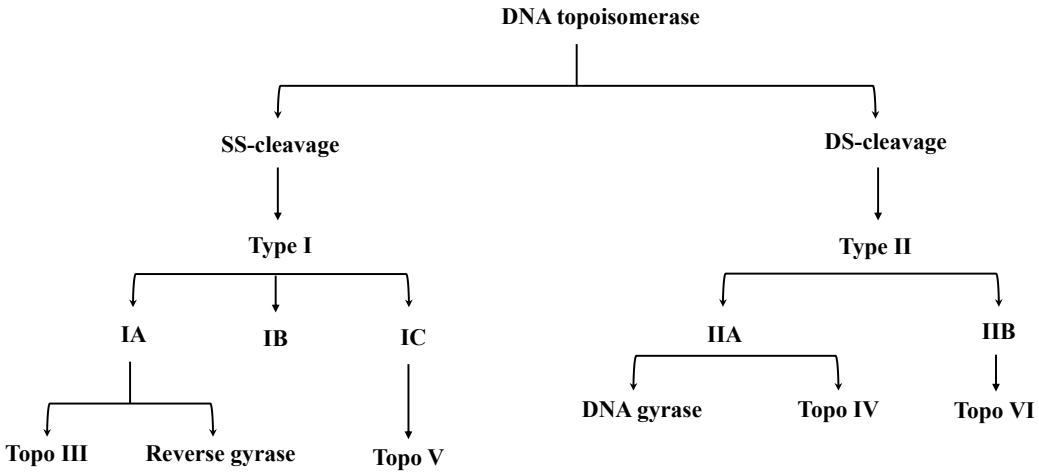


Figure F-1: Extended incubation of '*Ca*. N franklandus C13' in the presence of antibiotics. Both puromycin and hygromycin B strongly inhibit liquid cultures of '*Ca*. N franklandus C13'. No evidence of spontaneous mutant development could be detected following the extended incubation (~65 days) with **(A)** Puromycin (20 µM, 50 µM and 100 µM **(B)** Hygromycin B (50 µM and 100 µM). Nitrite (NO_2^-) concentrations plotted are the averages of three replicate cultures. Error bars are standard errors of the means and where they are not visible are smaller than the size of the symbol.

Appendix G: Classification of topoisomerases



References

Aagaard, C. Leviev, I., Aravalli, R.N., Forterre, P., Prieur, D and Garrett, R.A. (1996) 'General vectors for archaeal hyperthermophiles: Strategies based on a mobile intron and a plasmid', *FEMS Microbiology Reviews*, 18(2-3), pp. 93-104. Available at: https://doi.org/10.1111/j.1574-6976.1996.tb00229.x.

Abby, S.S., Melcher, M., Kerou, M., Krupovic, M., Stieglmeier, M., Rossel, C., Pfeifer, K. and Schleper, C. (2018) 'Candidatus Nitrosocaldus cavascurensis, an ammonia oxidizing, extremely thermophilic archaeon with a highly mobile genome', *Frontiers in Microbiology*, 9. Available at: https://doi.org/10.3389/fmicb.2018.00028.

Abuaita, B.H. and Withey, J.H. (2011) 'Genetic screening for bacterial mutants in liquid growth media by fluorescence-activated cell sorting', *Journal of Microbiological Methods*, 84(1), pp. 109-113. Available at: https://doi.org/10.1016/j.mimet.2010.11.003.

Adam, P.S., Borrel, G., Brochier-Armanet, C. and Gribaldo, S. (2017) 'The growing tree of Archaea: New perspectives on their diversity, evolution and ecology', *ISME Journal*, 11, pp. 2407-2425. Available at: https://doi.org/10.1038/ismej.2017.122.

Adli, M. (2018) 'The CRISPR tool kit for genome editing and beyond', *Nature Communications*, 9(1), p. 1911. Available at: https://doi.org/10.1038/s41467-018-04252-2.

Ahlgren, N.A., Fuchsman, C.A., Rocap, G. and Fuhrman, J.A. (2019) 'Discovery of several novel, widespread, and ecologically distinct marine Thaumarchaeota viruses that encode amoC nitrification genes', *The ISME Journal*, 13(3), pp. 618-631. Available at: https://doi.org/10.1038/s41396-018-0289-4.

Alangaden, G.J., Kreiswirth, B.N., Aouad, A., Khetarpal, M., Igno, F.R., Moghazeh, S.L., Manavathu, E.K., Lerner, S.A. (1998) 'Mechanism of Resistance to Amikacin and

Kanamycin in Mycobacterium tuberculosis', *Antimicrobial Agents and Chemotherapy,* 42(5), pp. 1295-1297. Available at: https://doi.org/10.1128/AAC.42.5.1295.

Algire, M.A., Lartigue, C., Thomas, D.W., Assad-Garcia, N., Glass, J.I. and Merryman, C. (2009) 'New selectable marker for manipulating the simple genomes of Mycoplasma species', *Antimicrobial Agents and Chemotherapy*, 53(10), pp. 4429-4432. Available at: https://doi.org/10.1128/AAC.00388-09.

Allen, A. (2023) *DNA topoisomerase VI: characterisation and investigation as a target for inhibitors*. PhD thesis. University of East Anglia. Available at: https://ueaeprints.uea.ac.uk/id/eprint/92305.

Allers, T., Ngo, H.P., Mevarech, M. and Lloyd, R.G. (2004) 'Development of Additional Selectable Markers for the Halophilic Archaeon *Haloferax volcanii* Based on the *leu*B and *trp*A Genes', *Applied and Environmental Microbiology*, 70(2), pp. 943-953. Available at: https://doi.org/10.1128/AEM.70.2.943-953.2004.

Allison, S.M and Prosser, J.I. (1993) 'Ammonia oxidation at low pH by attached populations of nitrifying bacteria', *Soil Biology and Biochemistry*, 25(7), pp. 935-941. Available at: https://doi.org/10.1016/0038-0717(93)90096-T.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) 'Basic Local Alignment Search Tool', *The Journal of Molecular Biology*, 215, pp. 403-410.

Alves, R.J.E., Minh, B.Q., Urich, T., Von Haeseler, A. and Schleper, C. (2018) 'Unifying the global phylogeny and environmental distribution of ammonia-oxidising archaea based on *amo*A genes', *Nature Communications*, 9(1), p. 1517. Available at: https://doi.org/10.1038/s41467-018-03861-1.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R. and Stahl, D.A. (1990) 'Combination of 16S rRNA-Targeted Oligonucleotide Probes with Flow Cytometry for Analyzing Mixed Microbial Populations', *Applied and Environmental Microbiology*, 56(6), pp. 1919-1925.

Amitai, G. and Sorek, R. (2016) 'CRISPR-Cas adaptation: Insights into the mechanism of action', *Nature Reviews Microbiology*, 14(2), pp. 67-76. Available at: https://doi.org/10.1038/nrmicro.2015.14.

Andersson, D.I. and Hughes, D. (2014) 'Microbiological effects of sublethal levels of antibiotics', *Nature Reviews Microbiology*, 12(7), pp. 465-478. Available at: https://doi.org/10.1038/nrmicro3270.

Argyle, J.L., Tumbula, D.L. and Leigh, J.A. (1996) 'Neomycin Resistance as a Selectable Marker in *Methanococcus maripaludis*', *Applied and Environmental Microbiology*, 62(11), pp. 4233-4237.

Arp, D.J. and Stein, L.Y. (2003) 'Metabolism of Inorganic N Compounds by Ammonia-Oxidizing Bacteria', *Critical Reviews in Biochemistry and Molecular Biology*, 38(6), pp. 471-495. Available at: https://doi.org/10.1080/10409230390267446.

Arp, D.J., Chain, P.S.G. and Klotz, M.G. (2007) 'The Impact of Genome Analyses on Our Understanding of Ammonia-Oxidizing Bacteria', *Annual Review of Microbiology*, 61(1), pp. 503-528. Available at: https://doi.org/10.1146/annurev.micro.61.080706.093449.

Arp, D.J., Sayavedra-Soto, L.A. and Hommes, N.G. (2002) 'Molecular biology and biochemistry of ammonia oxidation by *Nitrosomonas europaea*', *Archives of Microbiology*, 178(4), pp. 250-255. Available at: https://doi.org/10.1007/s00203-002-0452-0.

Asif, A., Mohsin, H., Tanvir, R. and Rehman, Y. (2017) 'Revisiting the mechanisms involved in calcium chloride induced bacterial transformation', *Frontiers in Microbiology*. 8, p. 2169, Available at: https://doi.org/10.3389/fmicb.2017.02169.

Atomi, H., Imanaka, T. and Fukui, T. (2012) 'Overview of the genetic tools in the Archaea', *Frontiers in Microbiology*, 3, p. 337. Available at: https://doi.org/10.3389/fmicb.2012.00337.

Ausiannikava, D. and Allers, T. (2017) 'Diversity of DNA replication in the archaea', *Genes*, 8(2), p. 56. Available at: https://doi.org/10.3390/genes8020056.

Aviner, R. (2020) 'The science of puromycin: From studies of ribosome function to applications in biotechnology', *Computational and Structural Biotechnology Journal*. 18, pp. 1074-1083. Available at: https://doi.org/10.1016/j.csbj.2020.04.014.

Azencott, H.R., Peter, G.F. and Prausnitz, M.R. (2007) 'Influence of the Cell Wall on Intracellular Delivery to Algal Cells by Electroporation and Sonication', *Ultrasound in Medicine and Biology*, 33(11), pp. 1805-1817. Available at: https://doi.org/10.1016/j.ultrasmedbio.2007.05.008.

Bai H, Deng A, Liu S, Cui D, Qiu Q, Wang L, Yang Z, Wu J, Shang X, Zhang Y. and Wen T. (2018) 'A Novel Tool for Microbial Genome Editing Using the Restriction-Modification System', *ACS Synthetic Biology*, 7(1), pp. 98-106. Available at https://doi: 10.1021/acssynbio.7b00254.

Baker, N.M., Rajan, R. and Mondragńn, A. (2009) 'Structural studies of type I topoisomerases', *Nucleic Acids Research*, 37(3), pp. 693-701. Available at: https://doi.org/10.1093/nar/gkn1009.

Ban, C. and Yang, W. (1998) 'Crystal Structure and ATPase Activity of MutL: Implications for DNA Repair and Mutagenesis', *Cell*, 95(4), pp. 541-552. Available at: https://doi.org/10.1016/s0092-8674(00)81621-9.

Bao, J., De Dios Mateos, E. and Scheller, S. (2022) 'Efficient CRISPR/Cas12a-Based Genome-Editing Toolbox for Metabolic Engineering in *Methanococcus maripaludis*', *ACS Synthetic Biology*, 11(7), pp. 2496-2503. Available at: https://doi.org/10.1021/acssynbio.2c00137.

Barns, S.M., Delwichet, C.F., Palmert, J.D. and Pace, N.R. (1996) 'Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences', *Proceedings of the National Academy of Sciences of the United States of America*, 93(17), pp. 9188-9193. Available at: https://doi.org/10.1073/pnas.93.17.9188.

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D.A. and Horvath, P. (2007) 'CRISPR provides acquired resistance against viruses in prokaryotes', *Science*, 315(5819), pp. 1709-1712. Available at: https://doi.org/10.1126/science.1138140.

Barry, E.R. and Bell, S.D. (2006) 'DNA Replication in the Archaea', *Microbiology and Molecular Biology Reviews*, 70(4), pp. 876-887. Available at: https://doi.org/10.1128/mmbr.00029-06.

Bartossek, R., Spang, A., Weidler, G., Lanzen, A. and Schleper, C. (2012) 'Metagenomic analysis of ammonia-oxidizing archaea affiliated with the soil group', *Frontiers in Microbiology*, 3, p. 208. Available at: https://doi.org/10.3389/fmicb.2012.00208.

Bayer, B., Vojvoda, J., Reinthaler, T., Reyes, C., Pinto, M. and Herndl, G.J. (2019) '*Nitrosopumilus adriaticus* sp. nov. and *Nitrosopumilus piranensis* sp. nov., two ammonia-

oxidizing archaea from the Adriatic Sea and members of the class *Nitrososphaeria*', *International Journal of Systematic and Evolutionary Microbiology*, 69(7), pp. 1892-1902. Available at: https://doi.org/10.1099/ijsem.0.003360.

Becker, B. and Cooper, M.A. (2013) 'Aminoglycoside antibiotics in the 21st century', *ACS Chemical Biology*. American Chemical Society, pp. 105-115. Available at: https://doi.org/10.1021/cb3005116.

Beeckman, F., Motte, H. and Beeckman, T. (2018) 'Nitrification in agricultural soils: impact, actors and mitigation', *Current Opinion in Biotechnology*, 50, pp. 166-173. Available at: https://doi.org/10.1016/j.copbio.2018.01.014.

Bell, S.D. and Jackson, S.P. (1998) 'Transcription and translation in Archaea: a mosaic of eukaryal and bacterial features', *Trends in Microbiology*, 6(6), pp. 222-228. Available at: https://doi.org/10.1016/s0966-842x(98)01281-5.

Bell, S.D., Kosa, P.L., Sigler, P.B. and Jackson, S.P. (1999) 'Orientation of the transcription preinitiation complex in Archaea', *Proceedings of the National Academy of Sciences of the United States of America*, 96(24), pp. 13662-13667. Available at: https://doi.org/10.1073/pnas.96.24.13662.

Bergerats, A., Gadelle, D. and Forterre, P. (1994) 'Purification of a DNA Topoisomerase I1 from the Hyperthermophilic Archaeon *Sulfolobus shibatae*', *The Journal of Biological Chemistry,* 269(44), pp. 27663-27669. Available at: https://doi.org/10.1080/15476286.2020.1723328.

Berkemer, S.J., Maier, L.K., Amman, F., Bernhart, S.H., Wörtz, J., Märkle, P., Pfeiffer, F., Stadler, P. F. and Marchfelder, A. (2020) 'Identification of RNA 3′ ends and termination sites

in *Haloferax volcanii*', *RNA biology*, 17(5), pp. 663-676. https://doi.org/10.1080/15476286.2020.1723328.

Berkner, S. and Lipps, G. (2008) 'Genetic tools for *Sulfolobus* spp.: Vectors and first applications', *Archives of Microbiology*, 190(3), pp. 217-230. Available at: https://doi.org/10.1007/s00203-008-0392-4.

Berkner, S., Grogan, D., Albers, S.V., Lipps, G. (2007) 'Small multicopy, non-integrative shuttle vectors based on the plasmid pRN1 for *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus*, model organisms of the (cren-)archaea', *Nucleic Acids Research*, 35(12). e88. Available at: https://doi.org/10.1093/nar/gkm449.

Bernatová, S., Samek, O., Pilát, Z., Šerý, M., Ježek, J., Jákl, P., Šiler, M., Krzyžánek, V., Zemánek, P. and Holá, V. (2013) 'Following the mechanisms of bacteriostatic versus bactericidal action using raman spectroscopy', *Molecules*, 18(11), pp. 13188-13199. Available at: https://doi.org/10.3390/molecules181113188.

Berquist, B.R. and DasSarma, S. (2003) 'An archaeal chromosomal autonomously replicating sequence element from an extreme halophile, *Halobacterium* sp. strain NRC-1', *Journal of Bacteriology*, 185(20), pp. 5959-5966. Available at: https://doi.org/10.1128/JB.185.20.5959-5966.2003.

Bertani, G. and Baresi, L. (1987) 'Genetic Transformation in the Methanogen *Methanococcus voltae* PS', *Journal of Bacteriology,* 169(6), pp. 2730-2738. Available at: https://doi.org/10.1128/jb.169.6.2730-2738.1987.

Bhaya, D., Davison, M. and Barrangou, R. (2011) 'CRISPR-Cas Systems in Bacteria and Archaea: Versatile Small RNAs for Adaptive Defense and Regulation', *Annual*

Review of Genetics, 45(1), pp. 273-297. Available at: https://doi.org/10.1146/annurev-genet-110410-132430.

Bitan-Banin, G., Ortenberg, R. and Mevarech, M. (2003) 'Development of a gene knockout system for the halophilic archaeon *Haloferax volcanii* by use of the *pyr*E gene', *Journal of Bacteriology*, 185(3), pp. 772-778. Available at: https://doi.org/10.1128/JB.185.3.772-778.2003.

Bitinaite, J., Wah, D.A., Aggarwal, A.K., and Schildkraut, I. (1998) '*Fok*I dimerization is required for DNA cleavage'. *Proceedings of the National Academy of Sciences of the United States of America*, 95(18), pp. 10570-10575. Available at: https://doi.org/10.1073/pnas.95.18.1057.

Boch, J. and Bonas, U. (2010) 'Xanthomonas AvrBs3 family-type III effectors: Discovery and function', *Annual Review of Phytopathology*, 48, pp. 419-436. Available at: https://doi.org/10.1146/annurev-phyto-080508-081936.

Bogdanove, A.J., Schornack, S. and Lahaye, T. (2010) 'TAL effectors: finding plant genes for disease and defense', *Current Opinion in Plant Biology*, 13(4), pp. 394-401. Available at: https://doi.org/10.1016/j.pbi.2010.04.010.

Bogdanove, A.J. and Voytas, D.F. (2011) 'TAL Effectors: Customizable Proteins for DNA Targeting', *Science*, 333(6051), pp. 1843-1846. Available at: https://doi.org/10.1126/science.1204094.

Bokranz, M., Klein, A. and Meile, L. (1990) 'Complete nucleotide sequence of plasmid pME2001 of *Methanobacterium thermoautotrophicum* (Marburg)', *Nucleic Acids Research*, 18(2), p. 363. Available at: https://doi.org/doi.org/10.1093/nar/18.2.363.

Bollmann, A., Bullerjahn, G.S. and McKay, R.M. (2014) 'Abundance and diversity of ammonia-oxidizing archaea and bacteria in sediments of trophic end members of the Laurentian Great Lakes, Erie and Superior', *PLOS ONE*, 9(5), e97068. Available at: https://doi.org/10.1371/journal.pone.0097068.

Bolotin, A., Quinquis, B., Sorokin, A. and Dusko Ehrlich. (2005) 'Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin', *Microbiology*, 151(8), pp. 2551-2561. Available at: https://doi.org/10.1099/mic.0.28048-0.

Borovinskaya, M.A., Shoji, S., Fredrick, K. and Cate, J.H.D. (2008) 'Structural basis for hygromycin B inhibition of protein biosynthesis', *RNA*, 14(8), pp. 1590-1599. Available at: https://doi.org/10.1261/rna.1076908.

Bosher, J.M. and Labouesse, M. (2000) 'RNA interference: genetic wand and genetic watchdog', *Nature Cell Biology,* 2, e31-36. Available at: https://doi.org/10.1038/35000102.

Broadbent, S.E., Balbontin, R., Casadesus, J., Marinus, M.G. and Van Der Woude, M. (2007) 'YhdJ, a nonessential CcrM-like DNA methyltransferase of *Escherichia coli* and *Salmonella enterica*', *Journal of Bacteriology*, 189(11), pp. 4325-4327. Available at: https://doi.org/10.1128/JB.01854-06.

Brochier-Armanet, C., Boussau, B., Gribaldo, S. and Forterre, P. (2008) 'Mesophilic crenarchaeota: Proposal for a third archaeal phylum, the Thaumarchaeota', *Nature Reviews Microbiology*, 6(3), pp. 245-252. Available at: https://doi.org/10.1038/nrmicro1852.

Brock, T.D., Brock, K.M., Belly, R.T. and Weiss, R.L. (1972) '*Sulfolobus:* A New Genus of Sulfur-Oxidizing Bacteria Living at Low pH and High Temperature', *Archives of Microbiology*, 84, pp. 54-68. Available at: https://doi.org/10.1007/BF00408082.

Brown, J.W., Daniels C.J., Reeve, J.N. and Konisky, J. (1989) 'Gene structure, organization, and expression in archaebacteria', *Critical Reviews in Microbiology*, 16(4), pp. 287-337. Available at: https://doi.org/10.3109/10408418909105479.

Brzobohatý, B. and Kovác, L. (1986) 'Factors Enhancing Genetic Transformation of Intact Yeast Cells Modify Cell Wall Porosity', *Journal of General Microbiology*, 132(11), pp. 3089-3093. Available at: https://doi.org/10.1099/00221287-132-11-3089.

Buan, N.R. (2018) 'Methanogens: Pushing the boundaries of biology', *Emerging Topics in Life Sciences*. 2(4), pp. 629-646. Available at: https://doi.org/10.1042/ETLS20180031.

Burton, S.A.Q. and Prosser, J.I. (2001) 'Autotrophic Ammonia Oxidation at Low pH through Urea Hydrolysis', *Applied and Environmental Microbiology*, 67(7), pp. 2952-2957. Available at: https://doi.org/10.1128/AEM.67.7.2952-2957.2001.

Cammarano, P., Teichner, A., Londei, P., Acca, M., Nicolaus, B., Sanz, J.L. and Amils, R. (1985) 'Insensitivity of archaebacterial ribosomes to protein synthesis inhibitors*', Evolutionary implications*', *EMBO Journal*, 4(3), pp. 811-816.

Campbell, M.A., Chain, P.S.G., Dang, H., El S.A.F., Norton, J.M., Ward, N.L., Ward, B.B. and Klotz, M.G. (2011) '*Nitrosococcuswatsoniisp*. nov., a new species of marine obligate ammonia oxidizing bacteria that is not omnipresent in the world's oceans: calls to validate the names '*Nitrosococcus halophilus'* and '*Nitrosomonas mobilis'*, *FEMS Microbiology Ecology*, 76(1), pp. 39-48. Available at: https://doi.org/10.1111/j.1574-6941.2010.01027.x.

Campbell, M.A., Chain, P.S.G., Dang, H., El Sheikh, A.F., Norton, J.M., Ward, N.L., Ward, B.B. and Klotz, M.G. (2011) '*Nitrosococcus watsonii* sp. nov., a new species of marine obligate ammonia-oxidizing bacteria that is not omnipresent in the world's oceans: Calls to validate the names "*Nitrosococcus halophilus*" and "*Nitrosomonas mobilis*"', *FEMS Microbiology Ecology*, 76(1), pp. 39-48. Available at: https://doi.org/10.1111/j.1574-6941.2010.01027.x.

Cannio, R., Contursi, P., Rossi, M. and Bartolucci, S. (1998) 'An autonomously replicating transforming vector for *Sulfolobus solfataricus*', *Journal of Bacteriology*, 180(12), pp. 3237-3240.

Cannio, R., Contursi, P., Rossi, M. and Bartolucci, S. (2001) 'Thermoadaptation of a mesophilic hygromycin B phosphotransferase by directed evolution in hyperthermophilic Archaea: Selection of a stable genetic marker for DNA transfer into *Sulfolobus solfataricus*', *Extremophiles*, 5(3), pp. 153-159. Available at: https://doi.org/10.1007/s007920100189.

Capecchi, M.R. (1989) 'Altering the Genome by Homologous Recombination', *Science*, 244(4910), pp. 1288-1292. Available at: https://doi.org/10.1126/science.2660260.

Caputo, A.T., Eder, O.M., Bereznakova, H., Pothuis, H., Ardevol, A., Newman, J., Nuttall, S., Peat, T.S. and Adams, T.E. (2021) 'Structure-guided selection of puromycin N-acetyltransferase mutants with enhanced selection stringency for deriving mammalian cell lines expressing recombinant proteins', *Scientific Reports*, 11(1), p. 5247. Available at: https://doi.org/10.1038/s41598-021-84551-9.

Caranto, J.D. and Lancaster, K.M. (2017) 'Nitric oxide is an obligate bacterial nitrification intermediate produced by hydroxylamine oxidoreductase', *Proceedings of*

the National Academy of Sciences of the United States of America, 114(31), pp. 8217-8222. Available at: https://doi.org/10.1073/pnas.1704504114.

Carte, J., Wang, R., Li, H., Terns, R.M. and Terns, M.P. (2008) 'Cas6 is an endoribonuclease that generates guide RNAs for invader defense in prokaryotes', *Genes and Development*, 22(24), pp. 3489-3496. Available at: https://doi.org/10.1101/gad.1742908.

Catchpole, R., Gorlas, A., Oberto, J. and Forterre, P. (2018) 'A series of new E. coli–Thermococcus shuttle vectors compatible with previously existing vectors', *Extremophiles*, 22(4), pp. 591-598. Available at: https://doi.org/10.1007/s00792-018-1019-6.

Catchpole, R.J., Barbe, V., Magdelenat, G., Marguet, E., Terns, M., Oberto, J., Forterre, P. and Da Cunha, V. (2023) 'A self-transmissible plasmid from a hyperthermophile that facilitates genetic modification of diverse Archaea', *Nature Microbiology*, 8(7), pp. 1339-1347. Available at: https://doi.org/10.1038/s41564-023-01387-x.

Chaban, B., Logan, S.M., Kelly, J.F. and Jarrell, K.F. (2009) 'AglC and AglK are involved in biosynthesis and attachment of diacetylated glucuronic acid to the N-glycan in *Methanococcus voltae*', *Journal of Bacteriology*, 91(1), pp. 187-195. Available at: https://doi.org/10.1128/JB.00885-08.

Chain, P., Lamerdin, J., Larimer, F., Regala, W., Lao, V., Land, M., Hauser, L., Hooper, A., Klotz, M. and Norton, J. (2003) 'Complete genome sequence of the ammonia-oxidizing bacterium and obligate chemolithoautotroph *Nitrosomonas europaea*', *Journal of Bacteriology*, 185(9), pp. 2759-2773. Available at: https://doi.org/10.1128/JB.185.9.2759-2773.2003.

Champoux, J.J. (2001) 'DNA topoisomerases: Structure, function, and mechanism', *Annual Review of Biochemistry*, 70, pp. 369-413. Available at: https://doi.org/10.1146/annurev.biochem.70.1.369.

Charlebois, R.L., Lam, W.L., Cline, S.W. and Doolittle, W.F. (1987) 'Characterization of pHV2 from *Halobacterium volcanii* and its use in demonstrating transformation of an archaebacterium', *Proceedings of the National Academy of Sciences of the United States of America*, 84(23), pp. 8530-8534. Available at: https://doi.org/10.1073/pnas.84.23.8530.

Charpentier, E. (2015) 'CRISPR-Cas9: how research on a bacterial RNA -guided mechanism opened new perspectives in biotechnology and biomedicine', *EMBO Molecular Medicine*, 7(4), pp. 363-365. Available at: https://doi.org/10.15252/emmm.201504847.

Charpentier, E., Richter, H., van der Oost, J. and White, M.F. (2015) 'Biogenesis pathways of RNA guides in archaeal and bacterial CRISPR-Cas adaptive immunity', *FEMS Microbiology Reviews*, 39(3), pp. 428-441. Available at: https://doi.org/10.1093/femsre/fuv023.

Chen, L., Brügger, K., Skovgaard, M., Redder, P., She, Q., Torarinsson, E., Greve, B., Awayez, M., Zibat, A., Klenk, H.P. and Garrett, R.A. (2005) 'The genome of *Sulfolobus acidocaldarius*, a model organism of the Crenarchaeota,' *Journal of Bacteriology*, 187(14), pp. 4992–4999. Available at: https://doi.org/10.1128/JB.187.14.4992-4999.2005.

Chicaybam, L., Barcelos, C., Peixoto, B., Carneiro, M., Limia, C.G., Redondo, P., Lira, C., Paraguassú-Braga, F., Vasconcelos, ZFMD. and Barros, L. (2017) 'An Efficient Electroporation Protocol for the Genetic Modification of Mammalian Cells', *Frontiers in*

Bioengineering and Biotechnology, 4. p. 99. Available at: https://doi.org/10.3389/fbioe.2016.00099.

Christman, G.D., Cottrell, M.T., Popp, B.N., Gier, E. and Kirchman, D.L. (2011) 'Abundance, diversity, and activity of ammonia-oxidizing prokaryotes in the coastal arctic ocean in summer and winter', *Applied and Environmental Microbiology*, 77(6), pp. 2026-2034. Available at: https://doi.org/10.1128/AEM.01907-10.

Chu, Y.J., Lee, J.Y., Shin, S.R. and Kim, G.J. (2015) 'A Method for Cell Culture and Maintenance of Ammonia-Oxidizing Archaea in Agar Stab', *Indian Journal of Microbiology*, 55(4), pp. 460-463. Available at: https://doi.org/10.1007/s12088-015-0536-6.

Chung, D., Farkas, J. and Westpheling, J. (2013) 'Overcoming restriction as a barrier to DNA transformation in Caldicellulosiruptor species results in efficient marker replacement', *Biotechnology for Biofuels*, 6(1), p. 82. Available at: https://doi.org/10.1186/1754-6834-6-82.

Chutivisut, P., Isobe, K., Powtongsook, S., Pungrasmi, W. and Kurisu, F. (2018) 'Distinct microbial community performing dissimilatory nitrate reduction to ammonium (DNRA) in a high C/NO_3^- reactor', *Microbes and Environments*, 33(3), pp. 264-271. Available at: https://doi.org/10.1264/jsme2.ME17193.

Cline, S.W., Lam, W.L., Charlebois, R.L., Schalkwyk, L.C. and Ford Doolittle, W. (1989a) 'Transformation methods for halophilic archaebacteria', *Canadian Journal of Microbiology,* 35(1), pp. 148-152. Available at: https://doi.org/10.1139/m89-022.

Cline, S.W., Schalkwyk, L.C. and Doolittle, W.F. (1989b) 'Transformation of the archaebacterium *Halobacterium volcanii* with genomic DNA', *Journal of Bacteriology,* 171(9), pp. 4987-4991. Available at: https://doi.org/10.1128/jb.171.9.4987-4991.1989.

Cobb, R.E., Wang, Y. and Zhao, H. (2015) 'High-Efficiency Multiplex Genome Editing of *Streptomyces* Species Using an Engineered CRISPR/Cas System', *ACS Synthetic Biology*, 4(6), pp. 723-728. Available at: https://doi.org/10.1021/sb500351f.

Coker, J.A., DasSarma, P., Capes, M., Wallace, T., McGarrity, K., Gessler, R., Liu, J., Xiang, H., Tatusov, R., Berquist, B.R. and DasSarma, S. (2009) 'Multiple replication origins of *Halobacterium* sp. strain NRC-1: Properties of the conserved orc7-dependent oriC1', *Journal of Bacteriology*, 191(16), pp. 5253-5261. Available at: https://doi.org/10.1128/JB.00210-09.

Confreres, A. and Maxwell, A. (1992) 'gyrB mutations which confer coumarin resistance also affect DNA supercoiling and ATP hydrolysis by *Escherichia coli* DNA gyrase', *Molecular Microbiology*, 6(12), pp. 1617-1624. Available at: https://doi.org/10.1111/j.1365-2958.1992.tb00886.x.

Conway de Macario, E., Guerrini, M., Dugan, C.B. and Macario, A.J. (1996) 'Integration of foreign DNA in an intergenic region of the archaeon *Methanosarcina mazei* without effect on transcription of adjacent genes', *Journal of Molecular Biology,* 262(1), pp. 12-20. Available at: https://doi.org/10.1006/jmbi.1996.0494.

Corbett, K.D. and Berger, J.M. (2003) 'Structure of the topoisomerase VI-B subunit: implications for type II topoisomerase mechanism and evolution', *The EMBO journal*, 22(1), pp. 151-163.

Coronado, M.J., Vargas, C., Kunte, H.J., Galinski, E.A., Ventosa, A. and Nieto, J.J. (1995) 'Influence of salt concentration on the susceptibility of moderately halophilic bacteria to antimicrobials and its potential use for genetic transfer studies', *Current Microbiology*, 31(6), pp. 365-371. Available at: https://doi.org/10.1007/BF00294701.

Daebeler, A., Herbold, C.W., Vierheilig, J., Sedlacek, C.J., Pjevac, P., Albertsen, M., Kirkegaard, R.H., de la Torre, J.R., Daims, H. and Wagner, M. (2018) 'Cultivation and Genomic Analysis of "*Candidatus* Nitrosocaldus islandicus," an Obligately Thermophilic, Ammonia-Oxidizing Thaumarchaeon from a Hot Spring Biofilm in Graendalur Valley, Iceland', *Frontiers in Microbiology*, 9. p. 193. Available at: https://doi.org/10.3389/fmicb.2018.00193.

Daims, H., Brühl, A., Amann, R., Schleifer, K.H. and Wagner, M. (1999) 'The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set', *Systematic and Applied Microbiology*, 22(3), pp. 434-444. Available at: https://doi.org/10.1016/S0723-2020(99)80053-8.

Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinszky, M., Vierheilig, J. and Bulaev, A. (2015) 'Complete nitrification by *Nitrospira* bacteria', *Nature*, 528(7583), pp. 504-509. Available at: https://doi.org/10.1038/nature16461.

Dar, D., Prasse, D., Schmitz, R.A. and Sorek, R. (2016) 'Widespread formation of alternative 3' UTR isoforms via transcription termination in archaea', *Nature Microbiology*, 1(10), p. 1643. Available at: https://doi.org/10.1038/nmicrobiol.2016.143.

Das, N., Triparthi, N., Basu, S., Bose, C., Maitra, S. and Khurana, S. (2015) 'Progress in the development of gelling agents for improved culturability of microorganisms',

Frontiers in Microbiology, 6. p.698. Available at: https://doi.org/10.3389/fmicb.2015.00698.

Datta, S., Costantino, N. and Court, D.L. (2006) 'A set of recombineering plasmids for gram-negative bacteria', *Gene*, 379(1-2), pp. 109-115. Available at: https://doi.org/10.1016/j.gene.2006.04.018.

De la Luna, S., Soria, I., Pulido, D., Ortín, J. and Jiménez, A. (1988) 'Efficient transformation of mammalian cells with constructs containing a puromycin-resistance marker', *Gene,* 62(1), pp. 121-126. Available at: https://doi.org/10.1016/0378-1119(88)90585-9.

de la Torre, J.R., Walker, C.B., Ingalls, A.E., Könneke, M. and Stahl, D.A. (2008) 'Cultivation of a thermophilic ammonia oxidizing archaeon synthesizing crenarchaeol', *Environmental Microbiology*, 10(3), pp. 810-818. Available at: https://doi.org/10.1111/j.1462-2920.2007.01506.x.

Deltcheva, E., Chylinski, K., Sharma, C.M., Gonzales, K., Chao, Y., Pirzada, Z.A., Eckert, M.R., Vogel, J. and Charpentier, E. (2011) 'CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III', *Nature*, 471(7340), pp. 602-607. Available at: https://doi.org/10.1038/nature09886.

Deng, L., Zhu, H., Chen, Z., Liang, Y.X. and She, Q. (2009) 'Unmarked gene deletion and host-vector system for the hyperthermophilic crenarchaeon *Sulfolobus islandicus*', *Extremophiles*, 13(4), pp. 735-746. Available at: https://doi.org/10.1007/s00792-009-0254-2.

Deweese, J.E., Osheroff, M.A. and Osheroff, N. (2009) 'DNA topology and topoisomerases: Teaching A "knotty" subject', *Biochemistry and Molecular Biology Education*, 37(1), pp. 2-10. Available at: https://doi.org/10.1002/bmb.20244.

Dhamad, A.E. and Lessner, D.J. (2020) 'A CRISPRi-dCas9 System for Archaea and Its Use To Examine Gene Function during Nitrogen Fixation by *Methanosarcina acetivorans*', *Applied and Environmental Microbiology*, 86(21). e01402-20. Available at: https://doi.org/10.1128/AEM.01402-20.

Di, H.J., Cameron, K.C., Shen, J.P., Winefield, C.S., O'Callaghan, M., Bowatte, S. and He, J.Z. (2010) 'Ammonia-oxidizing bacteria and archaea grow under contrasting soil nitrogen conditions', *FEMS Microbiology Ecology*, 72(3), pp. 386-394. Available at: https://doi.org/10.1111/j.1574-6941.2010.00861.x.

Di, H.J., Cameron, K.C., Shen, J.P., Winefield, C.S., Ocallaghan, M., Bowatte, S. and He, J.Z. (2009) 'Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils', *Nature Geoscience*, 2(9), pp. 621-624. Available at: https://doi.org/10.1038/ngeo613.

Dickson, G. (1995) Molecular and cell biology of human gene therapeutics. Royal Holloway, University of London, Surrey, Springer Dordrecht, Molecular and Cell Biology of Human Diseases.

Dimitriu, T., Szczelkun, M.D. and Westra, E.R. (2020) 'Evolutionary Ecology and Interplay of Prokaryotic Innate and Adaptive Immune Systems', *Current Biology*, 30(19), R1189–R1202. Available at: https://doi.org/10.1016/j.cub.2020.08.028.

Dodsworth, J.A., Li, L., Wei, S., Hedlund, B.P., Leigh, J.A. and de Figueiredo, P. (2010) 'Interdomain conjugal transfer of DNA from bacteria to archaea', *Applied and*

Environmental Microbiology, 76(16), pp. 5644–5647. Available at: https://doi.org/10.1128/AEM.00967-10.

Dong M.J, Luo H. and Gao F. (2022) 'Ori-Finder 2022: A Comprehensive Web Server for Prediction and Analysis of Bacterial Replication Origins,' *Genomics Proteomics Bioinformatics*. 20(6), pp. 1207-1213. Available at: https://doi: 10.1016/j.gpb.2022.10.002.

Doudna, J.A. and Charpentier, E. (2014) 'The new frontier of genome engineering with CRISPR-Cas9', *Science*, 346(6213). Available at: http://science.sciencemag.org/.

Dower, W.J., Miller, J.F. and Ragsdale, C.W. (1988) 'High efficiency transformation of *E. coli* by high voltage electroporation', *Nucleic Acids Research*. 16(3), pp. 6127-6145. Available at: https://doi.org/10.1093/nar/16.13.6127.

Drlica, K., Hiasa, H., Kerns, R., Malik, M., Mustaev, A. and Zhao, X. (2009) 'Quinolones: Action and Resistance Updated', *Current Topics in Medicinal Chemistry*, 9(11), pp. 991-998. Available at: https://doi.org/10.2174/156802609789630947.

Dutta, R. and Inouye, M. (2000) 'GHKL, an emergent ATPase/kinase superfamily', *Trends in biochemical Sciences*, 25(1), pp. 24-28. Available at: https://doi.org/10.1016/s0968-0004(99)01503-0.

Dupuis MÈ, Villion M, Magadán A.H. and Moineau S. (2013) 'CRISPR-Cas and restriction-modification systems are compatible and increase phage resistance', *Nature Communications*, 4, p.2087. Available at: https://doi: 10.1038/ncomms3087.

Dyall-Smith, M. (2009) *Protocols for Halobacterial genetics*, [Online]. Martinsried: Dr. Mike Dyall-Smith. Available at: https://haloarchaea.com/wp-content/uploads/2018/10/Halohandbook_2009_v7.3mds.pdf.

Elevi Bardavid, R. and Oren, A. (2008) 'Sensitivity of *Haloquadratum* and *Salinibacter* to antibiotics and other inhibitors: Implications for the assessment of the contribution of Archaea and Bacteria to heterotrophic activities in hypersaline environments', *FEMS Microbiology Ecology*, 63(3), pp. 309-315. Available at: https://doi.org/10.1111/j.1574-6941.2007.00433.x.

Elferink, M.G.l., Schleper, C. and Zillig, W. (1996) 'Transformation of the extremely thermoacidophilic archaeon *Sulfolobus solfataricus* via a self-spreading vector', *FEMS Microbiology Letters*, 137(1), pp. 31-35. Available at: https://doi.org/10.1111/j.1574-6968.1996.tb08078.x.

Elmore, J., Deighan, T., Westpheling, J., Terns, R.M. and Terns, M.P. (2015) 'DNA targeting by the type I-G and type I-A CRISPR-Cas systems of *Pyrococcus furiosus*', *Nucleic Acids Research*, 43(21), pp. 10353-10363. Available at: https://doi.org/10.1093/nar/gkv1140.

Elmore, J.R., Yokooji, Y., Sato, T., Olson, S., Glover, C.V., 3rd, Graveley, B.R., Atomi, H., Terns, R.M. and Terns, M.P. (2013) 'Programmable plasmid interference by the CRISPR-Cas system in *Thermococcus kodakarensis*', *RNA Biology*, 10(5), pp. 828-840. Available at: https://doi.org/10.4161/rna.24084.

Erguder, T.H., Boon, N., Wittebolle, L., Marzorati, M. and Verstraete, W. (2009) 'Environmental factors shaping the ecological niches of ammonia-oxidizing archaea',

FEMS Microbiology Reviews, 33(5), pp. 855-869. Available at: https://doi.org/10.1111/j.1574-6976.2009.00179.x.

Evans-Roberts, K. and Maxwell, A. (2009) 'DNA Topoisomerases', *E. coli, Salmonella and the Enterobacterales*, 3(2). Available at: https://doi.org/10.1128/ecosalplus.4.4.9.

Farias, W.R.L., Valente, A.P., Pereira, M.S. and Mourão, P.A.S. (2000) 'Structure and anticoagulant activity of sulfated galactans. Isolation of a unique sulfated galactan from the red algae *Botryocladia occidentalis* and comparison of its anticoagulant action with that of sulfated galactans from invertebrates', *Journal of Biological Chemistry*, 275(38), pp. 29299-29307. Available at: https://doi.org/10.1074/jbc.M002422200.

Farkas, J., Chung, D., DeBarry, M., Adams, M.W. and Westpheling, J. (2011) 'Defining components of the chromosomal origin of replication of the hyperthermophilic archaeon *Pyrococcus furiosus* needed for construction of a stable replicating shuttle vector', *Applied and Environmental Microbiology*, 77(18), pp. 6343-6349. Available at: https://doi.org/10.1128/AEM.05057-11.

Fatma, Z., Schultz, J.C. and Zhao, H. (2020) 'Recent advances in domesticating non-model microorganisms', *Biotechnology Progress*, 36(5), e3008. Available at: https://doi.org/10.1002/btpr.3008.

Fauser, F., Schiml, S. and Puchta, H. (2014) 'Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in *Arabidopsis thaliana*', *Plant Journal*, 79(2), pp. 348-359. Available at: https://doi.org/10.1111/tpj.12554.

Fendrihan, S., Legat, A., Pfaffenhuemer, M., Gruber, C., Weidler, G., Gerbl, F. and Stan-Lotter, H. (2006) 'Extremely halophilic archaea and the issue of long-term

microbial survival', *Reviews in Environmental Science and Biotechnology*, 5(2-3), pp. 203-218. Available at: https://doi.org/10.1007/s11157-006-0007-y.

Feng, Z., Chakraborty, D., Dewell, S.B., Reddy, B.V.B. and Brady, S.F. (2012) 'Environmental DNA-encoded antibiotics fasamycins A and B inhibit FabF in type II fatty acid biosynthesis', *Journal of the American Chemical Society*, 134(6), pp. 2981-2987. Available at: https://doi.org/10.1021/ja207662w.

Fiala, G. and Stetter, K.O. (1986) '*Pyrococcus furiosus* sp. nov. represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100°C', *Archives of Microbiology*. 145, pp. 56-61. Available at: https://doi.org/10.1007/BF00413027.

Fields, S. (2004) 'Global Nitrogen: Cycling out of control', *Environmental health perspectives*, 112(10), pp. A556-A563. Available at: https://doi.org/10.1289/ehp.112-a556.

Fink, C., Beblawy, S., Enkerlin, A.M., Mühling, L., Angenent, L.T. and Molitor, B. (2021) 'A Shuttle-Vector System Allows Heterologous Gene Expression in the Thermophilic Methanogen *Methanothermobacter thermautotrophicus* ΔH. *mBio*, 12(6), e0276621. Available at: https://doi.org/10.1128/mBio.02766-21.

Fonfara, I., Richter, H., BratoviÄ, M., Le Rhun, A. and Charpentier, E. (2016) 'The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA', *Nature*, 532(7600), pp. 517-521. Available at: https://doi.org/10.1038/nature17945.

Fonseca, D.R., Halim, M.F.A., Holten, M.P. and Costa, K.C. (2020) 'Type IV-Like Pili Facilitate Transformation in Naturally Competent Archaea', J*ournal of Bacteriology,* 202(21), e00355-20. Available at: https://doi.org/10.1128/JB.00355-20.

Forterre, P., Krupovic, M., Raymann, K. and Soler, N. (2014) 'Plasmids from Euryarchaeota', *Microbiology Spectrum*, 2(6). Available at: https://doi.org/10.1128/microbiolspec.plas-0027-2014.

Francis, C.A., Roberts, K.J., Beman, J.M., Santoro, A.E. and Oakley, B.B. (2005) 'Ubiquity and diversity of ammonia-oxidizing archaea in water columns and sediments of the ocean', *Proceedings of the National Academy of Sciences of the United States of America*, 102(41), pp. 14683–14688. Available at: https://doi.org/10.1073/pnas.0506625102.

Friman, V.P., Guzman, L.M., Reuman, D.C. and Bell, T. (2015) 'Bacterial adaptation to sublethal antibiotic gradients can change the ecological properties of multitrophic microbial communities', *Proceedings Biological Sciences*, 282(1806), p. 20142920. Available at: https://doi.org/10.1098/rspb.2014.2920.

Frost, L.S., Leplae, R., Summers, A.O. and Toussaint, A. (2005) 'Mobile genetic elements: The agents of open source evolution', *Nature Reviews Microbiology*, 3(9), pp. 722-732. Available at: https://doi.org/10.1038/nrmicro1235.

Furuhata, Y., Sakai, A., Murakami, T., Morikawa, M., Nakamura, C., Yoshizumi, T., Fujikura, U., Nishida, K. and Kato, Y. (2019) 'A method using electroporation for the protein delivery of Cre recombinase into cultured Arabidopsis cells with an intact cell wall', *Scientific Reports*, 9(1), p. 2163. Available at: https://doi.org/10.1038/s41598-018-38119-9.

Gadelle, D., Bocs, C., Graille, M. and Forterre, P. (2005) 'Inhibition of archaeal growth and DNA topoisomerase VI activities by the Hsp90 inhibitor radicicol', *Nucleic Acids Research*, 33(7), pp. 2310-2317. Available at: https://doi.org/10.1093/nar/gki526.

Gao, F. (2014) 'Recent Advances in the Identification of Replication Origins Based on the Z-curve Method', *Current Genomics*, 15(2), pp. 104-112. Available at: https://doi.org/10.2174/1389202915999140328162938.

Gao F. and Zhang C.T. (2008) 'Ori-Finder: a web-based system for finding oriCs in unannotated bacterial genomes,' *BMC Bioinformatics*, 9(79). Available at: https://doi: 10.1186/1471-2105-9-79.

Garg, A., Lohmueller, J.J., Silver, P.A. and Armel, T.Z. (2012) 'Engineering synthetic TAL effectors with orthogonal target sites', *Nucleic Acids Research*, 40(15), pp. 7584-7595. Available at: https://doi.org/10.1093/nar/gks404.

Garnier, F., Couturier, M., Débat, H. and Nadal, M. (2021) 'Archaea: A Gold Mine for Topoisomerase Diversity', *Frontiers in Microbiology*, 12, 661411. Available at: https://doi.org/10.3389/fmicb.2021.661411.

Gernhardt, P., Possot, O., Foglino, M., Sibold, L. and Klein, A. (1990) 'Construction of an integration vector for use in the archaebacterium *Methanococcus voltae* and expression of a eubacterial resistance gene', *Molecular and General Genetics*, 221(2), pp. 273-279. Available at: https://doi.org/10.1007/BF00261731.

Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison, C.A. and Smith, H.O. (2009) 'Enzymatic assembly of DNA molecules up to several hundred kilobases', *Nature Methods*, 6(5), pp. 343-345. Available at: https://doi.org/10.1038/nmeth.1318.

Gichner, T., Ptáček, O., Stavreva, D.A. and Plewa, M.J. (1999) 'Comparison of DNA Damage in Plants as Measured by Single Cell Gel Electrophoresis and Somatic Leaf Mutations Induced by Monofunctional Alkylating Agents', *Environmental and*

Molecular Mutagenesis, 33(4), pp. 279-286. Available at: https://doi.org/10.1002/(sici)1098-2280(1999)33:4<279::aid-em4>3.0.co;2-k.

Gichner, T., Ptácek, O., Stavreva, D.A., Wagner, E.D. and Plewa, M.J. (2000) 'A comparison of DNA repair using the comet assay in tobacco seedlings after exposure to alkylating agents or ionizing radiation', *Mutation Research*, 470(1), pp. 1-9. Available at: https://doi.org/10.1016/s1383-5718(00)00081-4.

Gietz, R.D., Schiestl, R.H., Willems, A.R. and Woods, R.A. (1995) 'Studies on the Transformation of Intact Yeast Cells by the LiAc/SS-DNA/PEG Procedure', *Yeast*, 11(4), pp. 355-360. Available at: https://doi.org/10.1002/yea.320110408.

Goldstein, J.L. and Brown, M.S. (1990) 'Regulation of the mevalonate pathway', *Nature*, 343(6257), pp. 425-430. Available at: https://doi.org/10.1038/343425a0.

Gophna, U., Allers, T. and Marchfelder, A. (2017) 'Finally, Archaea Get Their CRISPR-Cas Toolbox', *Trends in Microbiology*, 25(6), pp. 430-432. Available at: https://doi.org/10.1016/J.TIM.2017.03.009.

Grahn, A.M., Haase, J., Bamford, D.H. and Lanka, E. (2000) 'Components of the RP4 conjugative transfer apparatus form an envelope structure bridging inner and outer membranes of donor cells: implications for related macromolecule transport systems', *Journal of Bacteriology*, 182(6), pp. 1564–1574. Available at: https://doi.org/10.1128/JB.182.6.1564-1574.2000.

Griffiths A.J. (1995) 'Natural Plasmids of Filamentous Fungi', *Microbiology Reviews*, 59(4), pp. 673-685. Available at: https://doi.org/10.1128/mr.59.4.673-685.1995.

Gritz, L. and Davies, J. (1983) 'Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*', *Gene*, 25(2-3), pp. 179-183. Available at: https://doi.org/10.1016/0378-1119(83)90223-8.

Großkopf R., Stubner, S. and Liesack, W. (1998) 'Novel euryarchaeotal lineages detected on rice roots and in the anoxic bulk soil of flooded rice microcosms', *Applied and Environmental Microbiology*, 64(12), pp. 4983-4989. Available at: https://doi.org/10.1128/AEM.64.12.4983-4989.1998.

Gubry-Rangin, C., Hai, B., Quince, C., Engel, M., Thomson, B.C., James, P., Schloter, M., Griffiths, R.I., Prosser, J.I. and Nicol, G.W. (2011) 'Niche specialization of terrestrial archaeal ammonia oxidizers', *Proceedings of the National Academy of Sciences of the United States of America*, 108(52), pp. 21206-21211. Available at: https://doi.org/10.1073/pnas.1109000108.

Hall, T.A. (1999) 'BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT', *Nucleic Acids Symposium Series*, 41(41), pp. 95-98.

Hanahan, D. (1983) 'Studies on transformation of *Escherichia coli* with plasmids', J*ournal of Molecular Biology*, 166(4), pp. 557-580. Available at: https://doi.org/10.1016/s0022-2836(83)80284-8.

Haque, R.U., Paradisi, F. and Allers, T. (2020) '*Haloferax volcanii* for biotechnology applications: challenges, current state and perspectives', *Applied Microbiology and Biotechnology*, 104(4), pp. 1371-1382. Available at: https://doi.org/10.1007/s00253-019-10314-2.

Hasty, P., Rivera-Perez, J. and Bradley, A. (1992) 'The role and fate of DNA ends for homologous recombination in embryonic stem cells', *Molecular and Cellular Biology*, 12(6), pp. 2464-2474. Available at: https://doi.org/10.1128/mcb.12.6.2464-2474.1992.

Hatzenpichler R. (2012) 'Diversity, physiology, and niche differentiation of ammonia-oxidizing archaea', *Applied and environmental microbiology*, 78(21), 7501-7510. Available at: https://doi.org/10.1128/AEM.01960-12.

Hatzenpichler, R., Lebedeva, E.V., Spieck, E., Stoecker, K., Richter, A., Daims, H. and Wagner, M. (2008) 'A moderately thermophilic ammonia-oxidizing crenarchaeote from a hot spring', *Proceedings of the National Academy of Sciences of the United States of America*, 105(6), pp. 2134-2139. Available at: https://doi.org/10.1073/pnas.0708857105.

Hayatsu, M., Tago, K., Uchiyama, I., Toyoda, A., Wang, Y., Shimomura, Y., Okubo, T., Kurisu, F., Hirono, Y., Nonaka, K., Akiyama, H., Itoh, T. and Takami, H. (2017) 'An acid-tolerant ammonia-oxidizing γ-proteobacterium from soil', *ISME Journal*, 11(5), pp. 1130-1141. Available at: https://doi.org/10.1038/ismej.2016.191.

Head, I.M., Hiorns, W.D., Embley, T.M., McCarthy, A.J. and Saunders, J.R. (1993) 'The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences', *Journal of General Microbiology*, 139(6), pp. 1147-1153. Available at: https://doi.org/https://doi.org/10.1099/00221287-139-6-1147.

Heimerl, T., Flechsler, J., Pickl, C., Heinz, V., Salecker, B., Zweck, J., Wanner, G., Geimer, S., Samson, R.Y., Bell, S.D., Huber, H., Wirth, R., Wurch, L., Podar, M., and Rachel, R. (2017) 'A complex endomembrane system in the archaeon *Ignicoccus*

hospitalis tapped by *Nanoarchaeum equitans'*, *Frontiers in Microbiology*, 8. p. 1072. Available at: https://doi.org/10.3389/fmicb.2017.01072.

Herbert, R.A. (1999) 'Nitrogen cycling in coastal marine ecosystems', *FEMS Microbiology Reviews*, 23(5), pp. 563-590. Available at: https://doi.org/10.1111/j.1574-6976.1999.tb00414.x.

Herbold, C.W., Lehtovirta-Morley, L.E., Jung, M.Y., Jehmlich, N., Hausmann, B., Han, P., Loy, A., Pester, M., Sayavedra-Soto, L.A., Rhee, S.K., Prosser, J.I., Nicol, G.W., Wagner, M. and Gubry-Rangin, C. (2017) 'Ammonia-oxidising archaea living at low pH: Insights from comparative genomics', *Environmental Microbiology*, 19(12), pp. 4939-4952. Available at: https://doi.org/10.1111/1462-2920.13971.

Hileman, T.H. and Santangelo, T.J. (2012) 'Genetics techniques for *Thermococcus kodakarensis'*, *Frontiers in Microbiology*, 3. Available at: https://doi.org/10.3389/fmicb.2012.00195.

Hinnen, A., Hicks, J.B. and Fink, G.R. (1978) 'Transformation of yeast, Genetics', *Proceedings of the National Academy of Sciences of the United States of America*, 75(4), pp. 1929-1933. Available at: https://doi.org/10.1073/pnas.75.4.1929.

Hodgskiss, L.H., Melcher, M., Kerou, M., Chen, W., Ponce-Toledo, R.I., Savvides, S.N., Wienkoop, S., Hartl, M. and Schleper, C. (2023) 'Unexpected complexity of the ammonia monooxygenase in archaea', *The ISME Journal*, 17(4), pp. 588-599. Available at: https://doi.org/10.1038/s41396-023-01367-3.

Holden, J.F., Takai, K., Summit, M., Bolton, S., Zyskowski, J. and Baross, J. A. (2001) 'Diversity among three novel groups of hyperthermophilic deep-sea *Thermococcus*

species from three sites in the northeastern Pacific Ocean', *FEMS Microbiology Ecology*, 36(1), pp. 51-60. Available at: https://doi.org/10.1111/j.1574-6941.2001.tb00825.x.

Holmes, M., Pfeifer, F. and Dyall-Smith, M. (1994) 'Improved shuttle vectors for *Haloferax volcanii* including a dual-resistance plasmid', *Gene*, 146(1), pp. 117-121. Available at: https://doi.org/10.1016/0378-1119(94)90844-3.

Holmes, M.L. and Dyall-Smith, M.L. (1990) 'A plasmid vector with a selectable marker for halophilic archaebacteria', *Journal of Bacteriology*, 172(2), pp. 756-761. Available at: https://doi.org/10.1128/jb.172.2.756-761.1990.

Holmes, N.A., Devine, R., Qin, Z., Seipke, R.F., Wilkinson, B. and Hutchings, M.I. (2018) 'Complete genome sequence of *Streptomyces formicae* KY5, the formicamycin producer', *Journal of Biotechnology*, 265, pp. 116-118. Available at: https://doi.org/10.1016/j.jbiotec.2017.11.011.

Hommes, N.G., Sayavedra-Soto, L.A. and Arp, D.J. (2003) 'Chemolithoorganotrophic Growth of *Nitrosomonas europaea* on Fructose', *Journal of Bacteriology*, 185(23), pp. 6809-6814. Available at: https://doi.org/10.1128/JB.185.23.6809-6814.2003.

Horz, H.P., Barbrook, A., Field, C.B. and Bohannan, B.J. (2004) 'Ammonia-oxidizing bacteria respond to multifactorial global change', *Proceedings of the National Academy of Sciences of the United States of America*, 101(42), pp. 15136-15141. Available at: https://doi.org/10.1073/pnas.0406616101.

Hu, B.L., Shen, L.D., Xu, X.Y. and Zheng, P. (2011) 'Anaerobic ammonium oxidation (anammox) in different natural ecosystems', in *Biochemical Society Transactions*, 39(6), pp. 1811-1816. Available at: https://doi.org/10.1042/BST20110711.

Huber, H., Hohn, M.J., Rachel, R., Fuchs, T., Wimmer, V. C. and Stetter, K.O. (2002) 'A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont', *Nature*, 417(6884), pp. 63-67. Available at: https://doi.org/10.1038/417063a.

Isalan M. (2011) 'Zinc-finger nucleases: how to play two good hands', *Nature Methods*, 9(1), 32-34. Available at: https://doi.org/10.1038/nmeth.1805.

Ishino, S., Skouloubris, S., Kudo, H., l'Hermitte-Stead, C., Es-Sadik, A., Lambry, J.C., Ishino, Y. and Myllykallio, H. (2018) 'Activation of the mismatch-specific endonuclease EndoMS/NucS by the replication clamp is required for high fidelity DNA replication', *Nucleic Acids Research*, 46(12), pp. 6206-6217. Available at: https://doi.org/10.1093/nar/gky460.

Ishino, Y., Shinagawa, H., Makino, K., Amemura, M. and Nakata, A. (1987) 'Nucleotide Sequence of the iap Gene, Responsible for Alkaline Phosphatase Isozyme Conversion in *Escherichia coli*, and Identification of the Gene Product', *Journal of Bacteriology*, 169(12), pp. 5429-5433. Available at: https://doi.org/10.1128/jb.169.12.5429-5433.1987.

Iwaki, T. and Umemura, K. (2011) 'A single plasmid transfection that offers a significant advantage associated with puromycin selection, fluorescence-assisted cell sorting, and doxycycline-inducible protein expression in mammalian cells', *Cytotechnology*, 63(4), pp. 337-343. Available at: https://doi.org/10.1007/s10616-011-9357-6.

Jacques, C.N., Hulbert, A.K., Westenskow, S. and Neff, M.M. (2020) 'Production location of the gelling agent Phytagel has a significant impact on Arabidopsis thaliana

seedling phenotypic analysis', *PLoS ONE*, 15(5), e0228515. Available at: https://doi.org/10.1371/journal.pone.0228515.

Jaeger, P.A., McElfresh, C., Wong, L.R. and Ideker, T. (2015) 'Beyond agar: Gel substrates with improved optical clarity and drug efficiency and reduced autofluorescence for microbial growth experiments', *Applied and Environmental Microbiology*, 81(16), pp. 5639-5649. Available at: https://doi.org/10.1128/AEM.01327-15.

Jana, S. and Deb, J.K. (2006) 'Molecular understanding of aminoglycoside action and resistance', *Applied Microbiology and Biotechnology*, 70(2), pp. 140-150. Available at: https://doi.org/10.1007/s00253-005-0279-0.

Jansen, R., Embden, J.D., Gaastra, W. and Schouls, L.M. (2002) 'Identification of genes that are associated with DNA repeats in prokaryotes', *Molecular Microbiology*, 43(6), pp. 1565-1575. Available at: https://doi.org/10.1046/j.1365-2958.2002.02839.x.

Janssen, P.H., Yates, P.S., Grinton, B.E., Taylor, P.M. and Sait, M. (2002) 'Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions Acidobacteria, Actinobacteria, Proteobacteria, and Verrucomicrobia', *Applied and Environmental Microbiology*, 68(5), pp. 2391-2396. Available at: https://doi.org/10.1128/AEM.68.5.2391-2396.2002.

Jasin, M. and Berg, P. (1988) 'Homologous integration in mammalian cells without target gene selection', *Genes and Development*, 2(11), pp. 1353-1363. Available at: https://doi.org/10.1101/gad.2.11.1353.

Javaid, D., Ganie, S.Y., Hajam, Y.A. and Reshi, M.S. (2022) 'CRISPR/Cas9 system: a reliable and facile genome editing tool in modern biology', *Molecular Biology*

Reports. 49, pp. 12133-12150. Available at: https://doi.org/10.1007/s11033-022-07880-6.

Jia, Z. and Conrad, R. (2009) 'Bacteria rather than Archaea dominate microbial ammonia oxidation in an agricultural soil', *Environmental Microbiology*, 11(7), pp. 1658-1671. Available at: https://doi.org/10.1111/j.1462-2920.2009.01891.x.

Jiang, F. and Doudna, J.A. (2015) 'The structural biology of CRISPR-Cas systems. *Current Opinion in Structural Biology*', 30, pp. 100–111. Available at: https://doi.org/10.1016/j.sbi.2015.02.002.

Johnson, D.B. (1998) 'Biodiversity and ecology of acidophilic microorganisms', *FEMS Microbiology Ecology*, 27(4), pp. 307-317. Available at: https://doi.org/10.1111/j.1574-6941.1998.tb00547.x.

Johnston, C.D., Cotton, S.L., Rittling, S.R., Starr, J.R., Borisy, G.G., Dewhirst, F.E. and Lemon, K.P. (2019) 'Systematic evasion of the restriction-modification barrier in bacteria', *Proceedings of the National Academy of Sciences of the United States of America*, 166(23), pp. 11454-11459. Available at: https://doi.org/10.1073/pnas.1820256116.

Jones, W.J., Whitman, W.B., Fields, R.D. and Wolfe, R.S. (1983) 'Growth and Plating Efficiency of *Methanococci* on Agar Media', *Applied and Environmental Microbiology*, 46(1), pp. 220-226. Available at: https://doi.org/10.1128/aem.46.1.220-226.1983.

Jonuscheit, M., Martusewitsch, E., Stedman, K.M. and Schleper, C. (2003) 'A reporter gene system for the hyperthermophilic archaeon *Sulfolobus solfataricus* based on a selectable and integrative shuttle vector', *Molecular Microbiology*, 48(5), pp. 1241-1252. Available at: https://doi.org/10.1046/j.1365-2958.2003.03509.x.

Jordan, E.T., Collins, M., Terefe, J., Ugozzoli, L. and Rubio, T. (2008) 'Optimizing Electroporation Conditions in Primary and Other Difficult-to-Transfect Cells', *Journal of Biomolecular Techniques*, 19(5), pp. 328-334.

Joung, J.K. and Sander, J.D. (2013) 'TALENs: A widely applicable technology for targeted genome editing', *Nature Reviews Molecular Cell Biology*, pp. 49-55. Available at: https://doi.org/10.1038/nrm3486.

Jung, M.Y., Sedlacek, C.J., Kits, K.D., Mueller, A.J., Rhee, S.K., Hink, L., Nicol, G.W., Bayer, B., Lehtovirta-Morley, L. Wright, C., de la Torre, J.R., Herbold, C.W., Pjevac, P., Daims, H. and Wagner, M. (2022) 'Ammonia-oxidizing archaea possess a wide range of cellular ammonia affinities', *ISME Journal*, 16(1), pp. 272-283. Available at: https://doi.org/10.1038/s41396-021-01064-z.

Junier, P., Kim, O.S., Junier, T., Ahn, T.S., Imhoff, J.F. and Witzel, K.P. (2009) 'Community analysis of betaproteobacterial ammonia-oxidizing bacteria using the amoCAB operon', *Applied Microbiology and Biotechnology*, 83(1), pp. 175-188. Available at: https://doi.org/10.1007/s00253-009-1923-x.

Juven-Gershon, T., Hsu, J.Y., Theisen, J.W. and Kadonaga, J.T. (2008) 'The RNA polymerase II core promoter - the gateway to transcription', *Current Opinion in Cell Biology*, 20(3), pp. 253-259. Available at: https://doi.org/10.1016/j.ceb.2008.03.003.

Kalivoda, E.J., Horzempa, J., Stella, N.A., Sadaf, A., Kowalski, R.P., Nau, G.J. and Shanks, R.M. (2011) 'New vector tools with a hygromycin resistance marker for use with opportunistic pathogens', *Molecular Biotechnology*, 48(1), pp. 7-14. Available at: https://doi.org/10.1007/s12033-010-9342-x.

Kang, K.S., Veeder, G.T., Mirrasoul, P.J., Kaneko, T. and Cottrell, I.W. (1982) 'Agar-Like Polysaccharide Produced by a Pseudomonas Species: Production and Basic Properties', *Applied and Environmental Microbiology*, 43(5), pp. 1086-1091. Available at: https://doi.org/10.1128/aem.43.5.1086-1091.1982.

Kartal, B., Kuypers, M.M., Lavik, G., Schalk, J., Op den Camp, H.J., Jetten, M.S. and Strous, M. (2007) 'Anammox bacteria disguised as denitrifiers: Nitrate reduction to dinitrogen gas via nitrite and ammonium', *Environmental Microbiology*, 9(3), pp. 635-642. Available at: https://doi.org/10.1111/j.1462-2920.2006.01183.x.

Kawagishi, I., Okunishi, I., Homma, M. and Imaet, Y. (1994) 'Removal of the periplasmic DNase before electroporation enhances efficiency of transformation in the marine bacterium *Vibrio alginolyticus*', *Microbiology*, 140(9), pp. 2355-2361. Available at: https://doi.org/10.1099/13500872-140-9-2355.

Kawai, S., Hashimoto, W. and Murata, K. (2010) 'Transformation of *Saccharomyces cerevisiae* and other fungi: methods and possible underlying mechanism.', *Bioengineered Bugs*, 1(6), pp. 395-403. Available at: https://doi.org/10.4161/bbug.1.6.13257.

Kerou, M., Offre, P., Valledor, L., Abby, S.S., Melcher, M., Nagler, M., Weckwerth, W. and Schleper, C. (2016) 'Proteomics and comparative genomics of *Nitrososphaera viennensis* reveal the core genome and adaptations of archaeal ammonia oxidizers', *Proceedings of the National Academy of Sciences of the United States of America*, 113(49), pp. E7937-E7946. Available at: https://doi.org/10.1073/pnas.1601212113.

Khan, T., Sankhe, K., Suvarna, V., Sherje, A., Patel, K. and Dravyakar, B. (2018) 'DNA gyrase inhibitors: Progress and synthesis of potent compounds as antibacterial agents',

Biomedicine and Pharmacotherapy, 103, pp. 923-938. Available at: https://doi.org/10.1016/j.biopha.2018.04.021.

Khelaifia, S. and Drancourt, M. (2012) 'Susceptibility of archaea to antimicrobial agents: Applications to clinical microbiology', *Clinical Microbiology and Infection*, 18(9), pp. 841-848. Available at: https://doi.org/10.1111/j.1469-0691.2012.03913.x.

Kieper, S.N., Almendros, C. and Brouns, S.J.J. (2019) 'Conserved motifs in the CRISPR leader sequence control spacer acquisition levels in Type I-D CRISPR-Cas systems', FEMS *Microbiology Letters*, 366(11), fnz129. Available at: https://doi.org/10.1093/femsle/fnz129.

Kita K, Kotani H, Sugisaki H. and Takanami M. (1989) 'The fokI restriction-modification system. I. Organization and nucleotide sequences of the restriction and modification genes', *The Journal of Biological Chemistry*, 264(10), pp. 5751-6. Available at: https://doi.org/10.1016/S0021-9258(18)83613-4.

Kim, J.G., Park, S.J., Sinninghe Damsté, J.S., Schouten, S., Rijpstra, W.I., Jung, M.Y., Kim, S.J., Gwak, J.H., Hong, H., Si, O.J., Lee, S., Madsen, E.L. and Rhee, S.K. (2016) 'Hydrogen peroxide detoxification is a key mechanism for growth of ammonia-oxidizing archaea', *Proceedings of the National Academy of Sciences of the United States of America*, 113(28), pp. 7888-7893. Available at: https://doi.org/10.1073/pnas.1605501113.

Kim, J.G., Kim, S.J., Cvirkaite-Krupovic, V., Yu, W.J., Gwak, J.H., López-Pérez, M., Rodriguez-Valera, F., Krupovic, M., Cho, J.C. and Rhee, S.K. (2019) 'Spindle-shaped viruses infect marine ammonia-oxidizing thaumarchaea', *Proceedings of the National Academy of Sciences of the United States of America*, 116(31), pp. 15645–15650. Available at: https://doi.org/10.1073/pnas.1905682116.

Kim, M.S. and Kini, A.G. (2017) 'Engineering and application of zinc finger proteins and TALEs for biomedical research', *Molecules and Cells*. *Molecules and Cells*, 40(8), pp. 533-541. Available at: https://doi.org/10.14348/molcells.2017.0139.

Kirchman, D.L. (2012) 'Marine archaea take a short cut in the nitrogen cycle', *Proceedings of the National Academy of Sciences of the United States of America*, pp. 17732-17733. Available at: https://doi.org/10.1073/pnas.1215654109.

Klebe, R.J., Harriss, J.V., Sharp, Z.D. and Douglas, M.G. (1983) 'A general method for polyethylene-glycol-induced genetic transformation of bacteria and yeast', *Gene*, 25(2-3), pp. 333-341. Available at: https://doi.org/10.1016/0378-1119(83)90238-x.

Kohler, P.R.A. and Metcalf, W.W. (2012) 'Genetic manipulation of *Methanosarcina* spp.', *Frontiers in Microbiology*. 3, p. 259. Available at: https://doi.org/10.3389/fmicb.2012.00259.

Könneke, M., Bernhard, A.E., De La Torre, J.R., Walker, C.B., Waterbury, J.B. and Stahl, D.A. (2005) 'Isolation of an autotrophic ammonia-oxidizing marine archaeon', *Nature*, 437(7058), pp. 543-546. Available at: https://doi.org/10.1038/nature03911.

Könneke, M., Schubert, D.M., Brown, P.C., Hügler, M., Standfest, S., Schwander, T., Schada von Borzyskowski, L., Erb, T.J., Stahl, D.A. and Berg, I.A. (2014) 'Ammonia-oxidizing archaea use the most energy-efficient aerobic pathway for CO_2 fixation', *Proceedings of the National Academy of Sciences of the United States of America*, 111(22), pp. 8239-8244. Available at: https://doi.org/10.1073/pnas.1402028111.

Kotnik, T., Rems, L., Tarek, M. and Miklavčič, D. (2019) 'Annual Review of Biophysics Membrane Electroporation and Electropermeabilization: Mechanisms and Models', *Annual Review of Biophysics*, 48, pp. 63-91. Available at: https://doi.org/10.1146/annurev-biophys-052118.

Kozlowski, J.A., Stieglmeier, M., Schleper, C., Klotz, M.G. and Stein, L.Y. (2016) 'Pathways and key intermediates required for obligate aerobic ammonia-dependent chemolithotrophy in bacteria and Thaumarchaeota', *ISME Journal*, 10(8), pp. 1836-1845. Available at: https://doi.org/10.1038/ismej.2016.2.

Krause, K.M., Serio, A.W., Kane, T.R. and Connolly, L.E. (2016) 'Aminoglycosides: An overview', *Cold Spring Harbor Perspectives in Medicine*, 6(6), a027029. Available at: https://doi.org/10.1101/cshperspect.a027029.

Krishna, S.S., Majumdar, I. and Grishin, N.V. (2003) 'Structural classification of zinc fingers: survey and summary', *Nucleic Acids Research*, 31(2), pp. 532-550. Available at: https://doi.org/10.1093/nar/gkg161.

Krupovic, M., Spang, A., Gribaldo, S., Forterre, P. and Schleper, C. (2011) 'A thaumarchaeal provirus testifies for an ancient association of tailed viruses with archaea', *Biochemical Society Transactions*, 39(1), pp. 82-88. Available at: https://doi.org/10.1042/BST0390082.

Krupovic, M., Makarova, K.S., Wolf, Y.I., Medvedeva, S., Prangishvili, D., Forterre, P. and Koonin, E.V. (2019) 'Integrated mobile genetic elements in Thaumarchaeota', *Environmental Microbiology*, 21(6), pp. 2056-2078. Available at: https://doi.org/10.1111/1462-2920.14564.

Kulik, M., Goral, A.M., Jasiński, M., Dominiak, P.M. and Trylska, J. (2015) 'Electrostatic interactions in aminoglycoside-RNA complexes', *Biophysical Journal*, 108(3), pp. 655-665. Available at: https://doi.org/10.1016/j.bpj.2014.12.020.

Kurosawa, N. and Grogan, D.W. (2005) 'Homologous recombination of exogenous DNA with the *Sulfolobus acidocaldarius* genome: Properties and uses', *FEMS Microbiology Letters*, 253(1), pp. 141-149. Available at: https://doi.org/10.1016/j.femsle.2005.09.031.

Lam, W.L. and Doolittle, W.F. (1989) 'Shuttle vectors for the archaebacterium *Halobacterium volcanii', Proceedings of the National Academy of Sciences of the United States of America,* 86(14), pp. 5478-5482. Available at: https://doi.org/10.1073/pnas.86.14.5478.

Lam, W.L. and Doolittle, W.F. (1992) 'Mevinolin-resistant mutations identify a promoter and the gene for a eukaryote-like 3-hydroxy-3-methylglutaryl-coenzyme A reductase in the archaebacterium *Haloferax volcanii*', *Journal of Biological Chemistry*, 267(9), pp. 5829-5834. Available at: https://doi.org/10.1016/S0021-9258(18)42628-2.

Lane D.J (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, (eds). Nucleic Acid Techniques in bacterial systematics. New York: Wiley. pp. 115-175.

Lange, M. and Ahring, B.K. (2001) 'A comprehensive study into the molecular methodology and molecular biology of methanogenic Archaea', *FEMS Microbiology Reviews*, 25(5), pp. 553-571. Available at: https://doi.org/10.1111/j.1574-6976.2001.tb00591.x.

Larson, M.H., Gilbert, L.A., Wang, X., Lim, W.A., Weissman, J.S. and Qi, L.S. (2013) 'CRISPR interference (CRISPRi) for sequence-specific control of gene expression', *Nature Protocols*, 8(11), pp. 2180-2196. Available at: https://doi.org/10.1038/nprot.2013.132.

Le Guellec, S., Leroy, E., Courtine, D., Godfroy, A. and Roussel, E.G. (2021) 'H_2-dependent formate production by hyperthermophilic Thermococcales: an alternative

to sulfur reduction for reducing-equivalents disposal', *ISME Journal*, 15(12), pp. 3423-3436. Available at: https://doi.org/10.1038/s41396-021-01020-x.

Lee, S.W., Browning, G.F. and Markham, P.F. (2008) 'Development of a replicable oriC plasmid for *Mycoplasma gallisepticum* and *Mycoplasma imitans*, and gene disruption through homologous recombination in *M. gallisepticum*', *Microbiology*, 154(9), pp. 2571-2580. Available at: https://doi.org/10.1099/mic.0.2008/019208-0.

Lehtovirta-Morley, L.E., Stoecker, K., Vilcinskas, A., Prosser, J.I. and Nicol, G.W. (2011) 'Cultivation of an obligate acidophilic ammonia oxidizer from a nitrifying acid soil', *Proceedings of the National Academy of Sciences of the United States of America*, 108(38), pp. 15892-15897. Available at: https://doi.org/10.1073/pnas.1107196108.

Lehtovirta-Morley, L.E., Ge, C., Ross, J., Yao, H., Nicol, G.W. and Prosser, J.I. (2014) 'Characterisation of terrestrial acidophilic archaeal ammonia oxidisers and their inhibition and stimulation by organic compounds', *FEMS Microbiology Ecology*, 89(3), pp. 542-552. Available at: https://doi.org/10.1111/1574-6941.12353.

Lehtovirta-Morley, L.E., Sayavedra-Soto, L.A., Gallois, N., Schouten, S., Stein, L.Y., Prosser, J.I. and Nicol, G.W. (2016) 'Identifying potential mechanisms enabling acidophily in the ammonia-oxidizing archaeon "Candidatus Nitrosotalea devanaterra"', *Applied and Environmental Microbiology*, 82(9), pp. 2608-2619. Available at: https://doi.org/10.1128/AEM.04031-15.

Lehtovirta-Morley, L.E., Ross, J., Hink, L., Weber, E.B., Gubry-Rangin, C., Thion, C., Prosser, J.I. and Nicol, G.W. (2016) 'Isolation of "Candidatus Nitrosocosmicus franklandus", a novel ureolytic soil archaeal ammonia oxidiser with tolerance to high

ammonia concentration', *FEMS Microbiology Ecology*, 92(5). fiw057. Available at: https://doi.org/10.1093/femsec/fiw057.

Lehtovirta-Morley L.E. (2018) 'Ammonia oxidation: Ecology, physiology, biochemistry and why they must all come together', *FEMS Microbiology Letters*, 365(9), fny058. Available at: https://doi.org/10.1093/femsle/fny058

Leigh, J.A. (2000) 'Nitrogen Fixation in Methanogens', *Current Issues in Molecular Biology,* 2(4), pp. 125–131.

Leigh, J.A., Albers, S.V., Atomi, H. and Allers, T. (2011) 'Model organisms for genetics in the domain Archaea: Methanogens, halophiles, Thermococcales and Sulfolobales', *FEMS Microbiology Reviews*, 35(4), pp. 577-608. Available at: https://doi.org/10.1111/j.1574-6976.2011.00265.x.

Lepage, E., Marguet, E., Geslin, C., Matte-Tailliez, O., Zillig, W., Forterre, P. and Tailliez, P. (2004) 'Molecular Diversity of New Thermococcales Isolates from a Single Area of Hydrothermal Deep-Sea Vents as Revealed by Randomly Amplified Polymorphic DNA Fingerprinting and 16S rRNA Gene Sequence Analysis', *Applied and Environmental Microbiology*, 70(3), pp. 1277-1286. Available at: https://doi.org/10.1128/AEM.70.3.1277-1286.2004.

Lestini, R., Delpech, F. and Myllykallio, H. (2015) 'DNA Replication Restart in Archaea', in C.C Clark (ed.) *Advances in DNA repair*. San Diego: InTech. pp. 381-404. Available at: DOI 10.5772/58716.

Li, D., Tang, Y., Lin, J. and Cai, W. (2017) 'Methods for genetic transformation of filamentous fungi', *Microbial Cell Factories*, 16(1), p. 168. Available at: https://doi.org/10.1186/s12934-017-0785-7.

Li, H., Yang, Y., Hong, W., Huang, M., Wu, M. and Zhao, X. (2020) 'Applications of genome editing technology in the targeted therapy of human diseases: mechanisms, advances and prospects', *Signal Transduction and Targeted Therapy*, 5. p. 1 Available at: https://doi.org/10.1038/s41392-019-0089-y.

Li, X., Fu, L., Li, Z., Ma, X., Xiao, X. and Xu, J (2015) 'Genetic tools for the piezophilic hyperthermophilic archaeon *Pyrococcus yayanosii*', *Extremophiles*, 19(1), pp. 59-67. Available at: https://doi.org/10.1007/s00792-014-0705-2.

Li, Y., Chapman, S.J., Nicol, G.W., Yao, H. (2018) 'Nitrification and nitrifiers in acidic soils', *Soil Biology and Biochemistry*. 116, pp. 290-301. Available at: https://doi.org/10.1016/j.soilbio.2017.10.023.

Li, Y., Lin, Z., Huang, C., Zhang, Y., Wang, Z., Tang, Y. J., Chen, T. and Zhao, X. (2015) 'Metabolic engineering of *Escherichia coli* using CRISPR-Cas9 meditated genome editing', *Metabolic Engineering*, 31, pp. 13-21. Available at: https://doi.org/10.1016/j.ymben.2015.06.006.

Li, Y., Pan, S., Zhang, Y., Ren, M., Feng, M., Peng, N., Chen, L., Liang, Y.X. and She, Q. (2016) 'Harnessing Type I and Type III CRISPR-Cas systems for genome editing', *Nucleic Acids Research*, 44(4), p. e34. Available at: https://doi.org/10.1093/nar/gkv1044.

Lin, J., Zhou, D., Steitz, T.A., Polikanov, Y.S. and Gagnon, M.G. (2018) 'Ribosome-Targeting Antibiotics: Modes of Action, Mechanisms of Resistance, and Implications for Drug Design', *Annual Review of Biochemistry*. 87, pp. 451-478. Available at: https://doi.org/10.1146/annurev-biochem-062917-011942.

Lipscomb, G.L., Stirrett, K., Schut, G.J., Yang, F., Jenney, F.E., Jr, Scott, R.A., Adams, M.W. and Westpheling, J. (2011) 'Natural competence in the hyperthermophilic archaeon *Pyrococcus furiosus* facilitates genetic manipulation: construction of markerless deletions of genes encoding the two cytoplasmic hydrogenases', *Applied and Environmental Microbiology*, 77(7), pp. 2232-2238. Available at: https://doi.org/10.1128/AEM.02624-10.

Liu, H., Han, J., Liu, X., Zhou, J. and Xiang, H. (2011) 'Development of pyrF-based gene knockout systems for genome-wide manipulation of the archaea *Haloferax mediterranei* and *Haloarcula hispanica*', *Journal of Genetics and Genomics*, 38(6), pp. 261-269. Available at: https://doi.org/10.1016/j.jgg.2011.05.003.

Liu, L., Li, S., Han, J., Lin, W. and Luo, J. (2019) 'A two-step strategy for the rapid enrichment of Nitrosocosmicus-like ammonia-oxidizing Thaumarchaea', *Frontiers in Microbiology*, 10, p. 875. Available at: https://doi.org/10.3389/fmicb.2019.00875.

Liu, L., Liu, M., Jiang, Y., Lin, W. and Luo, J. (2021) 'Production and Excretion of Polyamines To Tolerate High Ammonia, a Case Study on Soil Ammonia-Oxidizing Archaeon "*Candidatus* Nitrosocosmicus agrestis", *mSystems*, 6(1), e01003-20. Available at: https://doi.org/10.1128/msystems.01003-20.

Loenen W.A, Dryden D.T, Raleigh E.A, Wilson G.G. and Murray N.E. (2014) 'Highlights of the DNA cutters: a short history of the restriction enzymes', *Nucleic Acids Research*. 42(1), pp. 3-19. Available at https://doi: 10.1093/nar/gkt990.

Londei, P., Altamura, S., Sanz, J.L. and Amils, R. (1988) 'Aminoglycoside-induced mistranslation in thermophilic archaebacteria', *Molecular and General Genetics*, 214(1), pp. 48-54. Available at: https://doi.org/10.1007/BF00340178.

Lucas, S., Toffin, L., Zivanovic, Y., Charlier, D., Moussard, H., Forterre, P., Prieur, D. and Erauso, G. (2002) 'Construction of a shuttle vector for, and spheroplast transformation of, the hyperthermophilic archaeon *Pyrococcus abyssi*', *Applied and Environmental Microbiology*, 68(11), pp. 5528-5536. Available at: https://doi.org/10.1128/AEM.68.11.5528-5536.2002.

Lundgren, M., Andersson, A., Chen, L., Nilsson, P. and Bernander, R. (2004) 'Three replication origins in *Sulfolobus* species: synchronous initiation of chromosome replication and asynchronous termination', *Proceedings of the National Academy of Sciences of the United States of America*, 101(18), pp. 7046-7051. Available at: https://doi.org/10.1073/pnas.0400656101.

Luo, H., Quan, C. L., Peng, C. and Gao, F. (2019) 'Recent development of Ori-Finder system and DoriC database for microbial replication origins', *Briefings in Bioinformatics*, 20(4), pp. 1114-1124. Available at: https://doi.org/10.1093/bib/bbx174.

Luo, H., Zhang, C.T. and Gao, F. (2014) 'Ori-Finder 2, an integrated tool to predict replication origins in the archaeal genomes', *Frontiers in Microbiology*, 5. Available at: https://doi.org/10.3389/fmicb.2014.00482.

Luo, M.L., Leenay, R.T. and Beisel, C.L. (2016) 'Current and future prospects for CRISPR-based tools in bacteria', *Biotechnology and Bioengineering*, 113(5), pp. 930-943. Available at: https://doi.org/10.1002/bit.25851.

MacLeod, K.C. and Holland, P.L. (2013) 'Recent developments in the homogeneous reduction of dinitrogen by molybdenum and iron', *Nature Chemistry*, pp. 559-565. Available at: https://doi.org/10.1038/nchem.1620.

Magnet, S. and Blanchard, J.S. (2005) 'Molecular insights into aminoglycoside action and resistance', *Chemical Reviews*, pp. 477-497. Available at: https://doi.org/10.1021/cr0301088.

Maguin P, Varble A, Modell J.W. and Marraffini L.A. (2022) 'Cleavage of viral DNA by restriction endonucleases stimulates the type II CRISPR-Cas immune response', *Molecular Cell*, 82(5), pp. 907-919, Available at: https://doi: 10.1016/j.molcel.2022.01.012.

Makarova, K.S., Haft, D.H., Barrangou, R., Brouns, S.J., Charpentier, E., Horvath, P., Moineau, S., Mojica, F.J., Wolf, Y.I., Yakunin, A.F., van der Oost, J. and Koonin, E.V. (2011) 'Evolution and classification of the CRISPR-Cas systems', *Nature Reviews Microbiology*, 9(6), pp. 467-477. Available at: https://doi.org/10.1038/nrmicro2577.

Makarova, K.S., Wolf, Y.I., Alkhnbashi, O.S., Costa, F., Shah, S.A., Saunders, S.J., Barrangou, R., Brouns, S.J., Charpentier, E., Haft, D.H., Horvath, P., Moineau, S., Mojica, F.J., Terns, R.M., Terns, M.P., White, M.F., Yakunin, A.F., Garrett, R.A., van der Oost, J., Backofen, R. and Koonin, E.V. (2015) 'An updated evolutionary classification of CRISPR-Cas systems', *Nature Reviews Microbiology*, 13(11), pp. 722-736. Available at: https://doi.org/10.1038/nrmicro3569.

Makarova, K.S., Zhang, F. and Koonin, E.V. (2017) 'SnapShot: Class 2 CRISPR-Cas Systems', *Cell*, 68(1-2), pp. 328-328.e1. Available at: https://doi.org/10.1016/j.cell.2016.12.038.

Makarova, K.S., Wolf, Y.I. and Koonin, E.V. (2018) 'Classification and Nomenclature of CRISPR-Cas Systems: Where from Here?', *The CRISPR Journal*, 1(5), pp. 325-336. Available at: https://doi.org/10.1089/crispr.2018.0033.

Makarova, K.S., Wolf, Y.I. and Koonin, E.V. (2019) 'Towards functional characterization of archaeal genomic dark matter', *Biochemical Society Transactions*, 47(1), pp. 389-398. Available at: https://doi.org/10.1042/BST20180560.

Makarova, K.S., Wolf, Y.I., Iranzo, J., Shmakov, S.A., Alkhnbashi, O.S., Brouns, S.J.J., Charpentier, E., Cheng, D., Haft, D.H., Horvath, P., Moineau, S., Mojica, F. J.M., Scott, D., Shah, S.A., Siksnys, V., Terns, M.P., Venclovas, Č., White, M.F., Yakunin, A.F., Yan, W., Zhang, F., Garrett, R.A., Backofen R., van der Oost, J., Barrangou, R. and Koonin, E.V. (2020) 'Evolutionary classification of CRISPR–Cas systems: a burst of class 2 and derived variants', *Nature Reviews Microbiology*. Nature Research, pp. 67-83. Available at: https://doi.org/10.1038/s41579-019-0299-x.

Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E. and Church, G.M. (2013) 'RNA-guided human genome engineering via Cas9', *Science*, 339(6121), pp. 823-826. Available at: https://doi.org/10.1126/science.1232033.

Martens-Habbena, W., Berube, P.M., Urakawa, H., de la Torre, J.R. and Stahl, D.A. (2009) 'Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria', *Nature*, 461(7266), pp. 976-979. Available at: https://doi.org/10.1038/nature08465.

Maisnier-Patin S, Malandrin L, Birkeland N.K. and Bernander R. (2002)'Chromosome replication patterns in the hyperthermophilic euryarchaea *Archaeoglobus fulgidus* and *Methanocaldococcus* (*Methanococcus*) *jannaschii*', *Molecular Microbiology*, 45(5), pp. 1443-50. Available at: https://doi: 10.1046/j.1365-2958.2002.03111.x.

Matsunaga, F., Forterre, P., Ishino, Y. and Myllykallio, H. (2001) 'In vivo interactions of archaeal Cdc6Orc1 and minichromosome maintenance proteins with the replication origin', *Proceedings*

of the National Academy of Sciences of the United States of America, 98(20), pp. 11152-11157. Available at: www.pnas.orgcgidoi10.1073pnas.191387498.

Matt, T., Ng, C.L., Lang, K., Sha, S.H., Akbergenov, R., Shcherbakov, D., Meyer, M., Duscha, S., Xie, J., Dubbaka, S.R., Perez-Fernandez, D., Vasella, A., Ramakrishnan, V., Schacht, J. and Böttger, E.C. (2012) 'Dissociation of antibacterial activity and aminoglycoside ototoxicity in the 4-monosubstituted 2-deoxystreptamine apramycin', *Proceedings of the National Academy of Sciences of the United States of America*, 109(27), pp. 10984-10989. Available at: https://doi.org/10.1073/pnas.1204073109.

Maxwell, A. (1997) 'DNA gyrase as a drug target', *Trends in Microbiology*, 5(3), pp. 102-109. Available at: https://doi.org/10.1016/S0966-842X(96)10085-8.

Maxwell, A. and Lawson, D.M. (2003) 'The ATP-Binding Site of Type II Topoisomerases as a Target for Antibacterial Drugs', *Current Topics in Medicinal Chemistry*, 3(3), pp. 283-303. Available at: https://doi.org/10.2174/1568026033452500.

Mcclintock, B. (1950) 'The origin and behavior of mutable loci in maize', *Proceedings of the National Academy of Sciences of the United States of America*, 36(6), pp. 344-355. Available at: https://doi.org/10.1073/pnas.36.6.344.

Meaking, W.S., Edgerton, J., Wharton, C.W. and Meldrum, R.A. (1995) 'Electroporation-induced damage in mammalian cell DNA', *Biochimica et Biophysica Acta*, 1264(3), pp. 357-362. Available at: https://doi.org/10.1016/0167-4781(95)00177-8.

Metcalf, W.W., Zhang, J.K., Apolinario, E., Sowers, K.R. and Wolfe, R.S. (1997) 'A genetic system for Archaea of the genus *Methanosarcina*: Liposome-mediated

transformation and construction', *Proceedings of the National Academy of Sciences of the United States of America*, 94(6), pp. 2626-2631. Available at: https://doi.org/10.1073/pnas.94.6.2626.

Miao, L., Liu, W., Qiao, Q., Li, X. and Xu, Z. (2020) 'Fluorescent antibiotics for real-time tracking of pathogenic bacteria', *Journal of Pharmaceutical Analysis*, 10(5), pp. 444-451. Available at: https://doi.org/10.1016/j.jpha.2020.09.003.

Micheletti, P.A., Sment, K.A. and Konisky, J. (1991) 'Isolation of a Coenzyme M-Auxotrophic Mutant and Transformation by Electroporation in *Methanococcus voltae*', *Journal of Bacteriology*, 173(11), pp. 3414-3418. Available at: https://doi.org/10.1128/jb.173.11.3414-3418.1991.

Mingeot-Leclercq, M.P., Glupczynski, Y. and Tulkens, P.M. (1999) 'Aminoglycosides: Activity and Resistance', *Antimicrobial Agents and Chemotherapy*, 43(4), pp. 727-737. Available at: https://doi.org/10.1128/AAC.43.4.727.

Mir, A., Edraki, A., Lee, J. and Sontheimer, E.J. (2018) 'Type II-C CRISPR-Cas9 Biology, Mechanism, and Application', *ACS Chemical Biology*. 13(2), pp. 357-365. Available at: https://doi.org/10.1021/acschembio.7b00855.

Mojica, F.J., Díez-Villaseñor, C., García-Martínez, J. and Soria, E. (2005). Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *Journal of Molecular Evolution*, 60(2), pp. 174-182. Available at: https://doi.org/10.1007/s00239-004-0046-3.

Mondorf, S., Deppenmeier, U. and Welte, C. (2012) 'A novel inducible protein production system and neomycin resistance as selection marker for *Methanosarcina mazei*', *Archaea*, 2012, 973743. Available at: https://doi.org/10.1155/2012/973743.

Moriya, S., Atlung, T., Hansen, F.G., Yoshikawa, H. and Ogasawara, N. (1992) 'Cloning of an autonomously replicating sequence (ars) from the *Bacillus subtilis* chromosome', *Molecular Microbiology*, 6(3), pp. 309-315. Available at: https://doi.org/10.1111/j.1365-2958.1992.tb01473.x.

Mullakhanbhai, M.F. and Larsen, H. (1975) '*Halobacterium volcanii* spec. nov., a Dead Sea halobacterium with a moderate salt requirement', *Archives of Microbiology*, 104(1), pp. 207-214. Available at: https://doi.org/10.1007/BF00447326/METRICS.

Muller, H.J. (1927) 'Artificial Transmutation of the Gene', *Science*, 66(1699), pp. 84-87. Available at: https://doi.org/10.1126/science.66.1699.84.

Muschiol, S., Balaban, M., Normark, S. and Henriques-Normark, B. (2015) 'Uptake of extracellular DNA: Competence induced pili in natural transformation of *Streptococcus pneumoniae*', *BioEssays*, 37(4), pp. 426-435. Available at: https://doi.org/10.1002/bies.201400125.

Naitou, A., Kato, Y., Nakanishi, T., Matsuura, T. and Watanabe, H. (2015). 'Heterodimeric TALENs induce targeted heritable mutations in the crustacean Daphnia magna', *Biology Open*, *4*(3), 364-369. Available at: https://doi.org/10.1242/bio.20149738.

Nakagawa, T. and Stahl, D.A. (2013) 'Transcriptional response of the archaeal ammonia oxidizer *Nitrosopumilus maritimus* to low and environmentally relevant ammonia concentrations', *Applied and Environmental Microbiology*, 79(22), pp. 6911-6916. Available at: https://doi.org/10.1128/AEM.02028-13.

Nam, K.H., Haitjema, C., Liu, X., Ding, F., Wang, H., DeLisa, M. P. and Ke, A. (2012) 'Cas5d protein processes Pre-crRNA and assembles into a cascade-like interference

complex in subtype I-C/Dvulg CRISPR-Cas system', *Structure*, 20(9), pp. 1574-1584. Available at: https://doi.org/10.1016/j.str.2012.06.016.

Nayak, D.D. and Metcalf, W.W. (2017) 'Cas9-mediated genome editing in the methanogenic archaeon *Methanosarcina acetivorans*', *Proceedings of the National Academy of Sciences of the United States of America*, 114(11), pp. 2976-2981. Available at: https://doi.org/10.1073/pnas.1618596114.

Nemudryi, A.A., Valetdinova, K.R., Medvedev, S.P. and Zakian, S.M. (2014) 'TALEN and CRISPR/Cas Genome Editing Systems: Tools of Discovery', *Acta Naturae,* 6(3), pp. 19-40.

Nercessian, O., Reysenbach, A.L., Prieur, D. and Jeanthon, C. (2003) 'Archaeal diversity associated with in situ samplers deployed on hydrothermal vents on the East Pacific Rise (13 degrees N)', *Environmental Microbiology*, 5(6), pp. 492-502. Available at: https://doi.org/10.1046/j.1462-2920.2003.00437.x.

Ngo, D.H. and Kim, S.K. (2013) 'Sulfated polysaccharides as bioactive agents from marine algae', *International Journal of Biological Macromolecules,* 62, pp. 70-75. Available at: https://doi.org/10.1016/j.ijbiomac.2013.08.036.

Nicol, G.W., Hink, L., Gubry-Rangin, C., Prosser, J.I. and Lehtovirta-Morley, L.E. (2019) 'Genome Sequence of "*Candidatus* Nitrosocosmicus franklandus" C13, a Terrestrial Ammonia-Oxidizing Archaeon', *Microbiology Resource Announcements*, 8(40), e00435-19. Available at: https://doi.org/10.1128/MRA.

Norais, C., Hawkins, M., Hartman, A.L., Eisen, J.A., Myllykallio, H. and Allers, T. (2007) 'Genetic and physical mapping of DNA replication origins in *Haloferax*

volcanii', *PLoS Genetics*, 3(5), pp. 729-743. Available at: https://doi.org/10.1371/journal.pgen.0030077.

Norton, J. and Ouyang, Y. (2019) 'Controls and adaptive management of nitrification in agricultural soils', *Frontiers in Microbiology*, 10(1931). Available at: https://doi.org/10.3389/fmicb.2019.01931.

Notman, R., Noro, M., O'Malley, B. and Anwar, J. (2006) 'Molecular basis for dimethylsulfoxide (DMSO) action on lipid membranes', *Journal of the American Chemical Society*, 128(43), pp. 13982-13983. Available at: https://doi.org/10.1021/ja063363t.

Nuñez, J.K., Kranzusch, P.J., Noeske, J., Wright, A.V., Davies, C.W. and Doudna, J.A. (2014) 'Cas1-Cas2 complex formation mediates spacer acquisition during CRISPR-Cas adaptive immunity', *Nature Structural Molecular Biology*, 21(6), pp. 528-534. Available at: https://doi.org/10.1038/nsmb.2820.

Nuñez, J.K., Lee, A.S., Engelman, A. and Doudna, J.A (2015) 'Integrase-mediated spacer acquisition during CRISPR-Cas adaptive immunity', *Nature*, 519(7542), pp. 193-198. Available at: https://doi.org/10.1038/nature14237.

Ochsenreiter, T., Selezi, D., Quaiser, A., Bonch-Osmolovskaya, L. and Schleper, C. (2003) 'Diversity and abundance of Crenarchaeota in terrestrial habitats studied by 16S RNA surveys and real time PCR', *Environmental Microbiology*, 5(9), pp. 787-797. Available at: https://doi.org/10.1046/j.1462-2920.2003.00476.x.

Oelgeschläger, E. and Rother, M. (2009) 'In vivo role of three fused corrinoid/methyl transfer proteins in *Methanosarcina acetivorans*', *Molecular Microbiology*, 72(5), pp. 1260-1272. Available at: https://doi.org/10.1111/j.1365-2958.2009.06723.x.

Offre, P., Kerou, M., Spang, A. and Schleper, C. (2014) 'Variability of the transporter gene complement in ammonia-oxidizing archaea', *Trends in Microbiology*. 22(12), pp. 665-675. Available at: https://doi.org/10.1016/j.tim.2014.07.007.

Offre, P., Prosser, J.I. and Nicol, G.W. (2009) 'Growth of ammonia-oxidizing archaea in soil microcosms is inhibited by acetylene', *FEMS Microbiology Ecology*, 70(1), pp. 99-108. Available at: https://doi.org/10.1111/j.1574-6941.2009.00725.x.

Offre, P., Spang, A. and Schleper, C. (2013) 'Archaea in Biogeochemical Cycles', *Annual Review of Microbiology*, 67(1), pp. 437-457. Available at: https://doi.org/10.1146/annurev-micro-092412-155614.

Oelgeschläger, E. and Rother, M. (2009) 'In vivo role of three fused corrinoid/methyl transfer proteins in *Methanosarcina acetivorans*', *Molecular Microbiology*, 72(5), pp. 1260-1272. Available at: https://doi.org/10.1111/j.1365-2958.2009.06723.x.

Oren, A. and Ventosa, A. (1999) 'In Memoriam-Benjamin Elazari Volcani', *International Journal of Salt Lake Research*, 8, pp. 3-6. Available at: https://doi.org/10.1007/BF02442132.

Özcan, A., Pausch, P., Linden, A., Wulf, A., Schühle, K., Heider, J., Urlaub, H., Heimerl, T., Bange, G. and Randau, L. (2019) 'Type IV CRISPR RNA processing and effector complex formation in *Aromatoleum aromaticum*', *Nature Microbiology*, 4(1), pp. 89-96. Available at: https://doi.org/10.1038/s41564-018-0274-8.

Palatinszky, M., Herbold, C., Jehmlich, N., Pogoda, M., Han, P., von Bergen, M., Lagkouvardos, I., Karst, S.M., Galushko, A., Koch, H., Berry, D., Daims, H. and Wagner, M. (2015) 'Cyanate as an energy source for nitrifiers', *Nature*, 524(7563), pp. 105-108. Available at: https://doi.org/10.1038/nature14856.

Panja, S., Aich, P., Jana, B. and Basu, T. (2008) 'How does plasmid DNA penetrate cell membranes in artificial transformation process of *Escherichia coli*?', *Molecular Membrane Biology*, 25(5), pp. 411-422. Available at: https://doi.org/10.1080/09687680802187765.

Panja, S., Saha, S., Jana, B. and Basu, T. (2006) 'Role of membrane potential on artificial transformation of *E. coli* with plasmid DNA', *Journal of Biotechnology*, 127(1), pp. 14-20. Available at: https://doi.org/10.1016/j.jbiotec.2006.06.008.

Park, H.D., Wells, G.F., Bae, H., Criddle, C.S. and Francis, C.A. (2006) 'Occurrence of ammonia-oxidizing archaea in wastewater treatment plant bioreactors', *Applied and Environmental Microbiology*, 72(8), pp. 5643-5647. Available at: https://doi.org/10.1128/AEM.00402-06.

Patel, G.B., Nash, J.H., Agnew, B.J. and Sprott, G.D. (1994) 'Natural and Electroporation-Mediated Transformation of *Methanococcus voltae* Protoplasts', *Applied and Environmental Microbiology*, 60(3), pp. 903-907. Available at: https://doi.org/10.1128/aem.60.3.903-907.1994.

Peabody, C.R., Chung, Y.J., Yen, M.R., Vidal-Ingigliardi, D., Pugsley, A.P. and Saier, M.H. (2003) 'Type II protein secretion and its relationship to bacterial type IV pili and archaeal flagella', *Microbiology*, 149(11), pp. 3051-3072. Available at: https://doi.org/10.1099/mic.0.26364-0.

Pelve, E.A., Martens-Habbena, W., Stahl, D.A. and Bernander, R. (2013) 'Mapping of active replication origins in vivo in thaum- and euryarchaeal replicons', *Molecular Microbiology*, 90(3), pp. 538-550. Available at: https://doi.org/10.1111/mmi.12382.

Peng, W., Feng, M., Feng, X., Liang, Y.X. and She, Q. (2015) 'An archaeal CRISPR type III-B system exhibiting distinctive RNA targeting features and mediating dual

RNA and DNA interference', *Nucleic Acids Research*, 43(1), pp. 406-417. Available at: https://doi.org/10.1093/nar/gku1302.

Perevalova, A.A., Kolganova, T.V., Birkeland, N.K., Schleper, C., Bonch-Osmolovskaya, E.A. and Lebedinsky, A.V. (2008) 'Distribution of Crenarchaeota representatives in terrestrial hot springs of Russia and Iceland', *Applied and Environmental Microbiology*, 74(24), pp. 7620-7628. Available at: https://doi.org/10.1128/AEM.00972-08.

Pérez-Arnaiz, P., Dattani, A., Smith, V. and Allers, T. (2020) '*Haloferax volcanii*- A model archaeon for studying DNA replication and repair: *Haloferax volcanii*, a model archaeon', *Open biology*, 10(12), p. 200293. Available at: https://doi.org/10.1098/rsob.200293.

Pérez-González, J.A., Vara, J. and Jiménez, A. (1985) 'The mechanism of resistance to puromycin and to the puromycin-precursor O-demethyl-puromycin in *Streptomyces alboniger*', *Journal of General Microbiology*, 131(11), pp. 2877-2883. Available at: https://doi.org/10.1099/00221287-131-11-2877.

Pester, M., Rattei, T., Flechl, S., Gröngröft, A., Richter, A., Overmann, J., Reinhold-Hurek, B., Loy, A. and Wagner, M. (2012) 'AmoA-based consensus phylogeny of ammonia-oxidizing archaea and deep sequencing of amoA genes from soils of four different geographic regions', *Environmental Microbiology*, 14(2), pp. 525-539. Available at: https://doi.org/10.1111/j.1462-2920.2011.02666.x.

Phillips, Z.N., Husna, A.U., Jennings, M.P., Seib, K.L. and Atack, J.M. (2019) 'Phasevarions of bacterial pathogens-phase-variable epigenetic regulators evolving

from restriction-modification systems', *Microbiology*, 165(9), pp. 917-928. Available at: https://doi.org/10.1099/mic.0.000805.

Picone, N., Pol, A., Mesman, R., van Kessel, M.A.H.J., Cremers, G., van Gelder, A.H., van Alen, T.A., Jetten, M.S.M., Lücker, S. and Op den Camp, H.J.M. (2021) 'Ammonia oxidation at pH 2.5 by a new gammaproteobacterial ammonia-oxidizing bacterium', *The ISME Journal*, 15(4), pp. 1150-1164. Available at: https://doi.org/10.1038/s41396-020-00840-7.

Plesniak, L.A., Botsch, K., Leibrand, M., Kelly, M., Sem, D., Adams, J.A. and Jennings, P. (2008) 'Transferred NOE and saturation transfer difference NMR studies of novobiocin binding to EnvZ suggest binding mode similar to DNA gyrase', *Chemical Biology and Drug Design*, 71(1), pp. 28-35. Available at: https://doi.org/10.1111/j.1747-0285.2007.00607.x.

Possot, O., Gernhardt, P., Klein, A. and Sibold, L. (1988) 'Analysis of drug resistance in the archaebacterium *Methanococcus voltae* with respect to potential use in genetic engineering', *Applied and Environmental Microbiology*, 54(3), pp. 734-740. Available at: https://journals.asm.org/journal/aem.

Potter H. and Heller R. (2003) 'Transfection by electroporation', *Current Protocols in Molecular Biology*, Chapter 9, Unit–9.3. Available at: https://doi.org/10.1002/0471142727.mb0903s62.

Pourcel, C., Touchon, M., Villeriot, N., Vernadet, J.P., Couvin, D., Toffano-Nioche, C. and Vergnaud, G. (2020) 'CRISPRCasdb a successor of CRISPRdb containing CRISPR arrays and cas genes from complete genome sequences, and tools to

download and query lists of repeats and spacers', *Nucleic Acids Research*, 48(D1), pp. D535-D544. Available at: https://doi.org/10.1093/nar/gkz915.

Prangishvili D., Albers S.V., Holz I., Arnold H.P., Stedman K., Klein T., Singh H., Hiort J., Schweier A., Kristjansson J.K. and Zillig W. (1998) 'Conjugation in archaea: frequent occurrence of conjugative plasmids in *Sulfolobus*', *Plasmid*, 40(3), pp. 190-202. Available at: https://doi.org/10.1006/plas.1998.1363.

Prosser, J.I. (1990) 'Advances in microbial physiology', *Advances in Microbial Physiology*, 30, pp. 125-181. Available at: https://doi.org/10.1016/s0065-2911(08)60112-5.

Prosser, J.I. and Nicol, G.W. (2012) 'Archaeal and bacterial ammonia-oxidisers in soil: The quest for niche specialisation and differentiation', *Trends in Microbiology*, pp. 523-531. Available at: https://doi.org/10.1016/j.tim.2012.08.001.

Prosser, J.I., Hink, L., Gubry-Rangin, C. and Nicol, G.W. (2020) 'Nitrous oxide production by ammonia oxidizers: Physiological diversity, niche differentiation and potential mitigation strategies', *Global Change Biology*, 26(1), pp. 103-118. Available at: https://doi.org/10.1111/gcb.14877.

Purdy, D., O'Keeffe, T.A., Elmore, M., Herbert, M., McLeod, A., Bokori-Brown, M., Ostrowski, A. and Minton, N.P. (2002) 'Conjugative transfer of clostridial shuttle vectors from *Escherichia coli* to *Clostridium difficile* through circumvention of the restriction barrier', *Molecular Microbiology*, 46(2), pp. 439-452. Available at: https://doi.org/10.1046/j.1365-2958.2002.03134.x.

Purdy, K.J., Cresswell-Maynard, T.D., Nedwell, D.B., McGenity, T.J., Grant, W.D., Timmis, K.N. and Embley, T.M. (2004) 'Isolation of haloarchaea that grow at low

salinities', *Environmental Microbiology*, 6(6), pp. 591-595. Available at: https://doi.org/10.1111/j.1462-2920.2004.00592.x.

Purkhold, U., Pommerening-Röser, A., Juretschko, S., Schmid, M.C., Koops, H.P. and Wagner, M. (2000) 'Phylogeny of All Recognized Species of Ammonia Oxidizers Based on Comparative 16S rRNA and *amo*A Sequence Analysis: Implications for Molecular Diversity Surveys', *Applied and Environmental Microbiology*, 66(12), pp. 5368-5382. Available at: https://doi.org/10.1128/AEM.66.12.5368-5382.2000.

Qin, W., Amin, S.A., Martens-Habbena, W., Walker, C.B., Urakawa, H., Devol, A.H., Ingalls, A.E., Moffett, J.W., Armbrust, E.V. and Stahl, D.A. (2014) 'Marine ammonia-oxidizing archaeal isolates display obligate mixotrophy and wide ecotypic variation', *Proceedings of the National Academy of Sciences of the United States of America*, 111(34), pp. 12504-12509. Available at: https://doi.org/10.1073/pnas.1324115111.

Qin, W., Heal, K.R., Ramdasi, R., Kobelt, J.N., Martens-Habbena, W., Bertagnolli, A.D., Amin, S.A., Walker, C.B., Urakawa, H., Könneke, M., Devol, A.H., Moffett, J.W., Armbrust, E.V., Jensen, G.J., Ingalls, A.E. and Stahl, D.A. (2017) '*Nitrosopumilus maritimus* gen. nov., sp. nov., *Nitrosopumilus cobalaminigenes* sp. nov., *Nitrosopumilus oxyclinae* sp. nov., and *Nitrosopumilus ureiphilus* sp. nov., four marine ammonia-oxidizing archaea of the phylum Thaumarchaeota', *International Journal of Systematic and Evolutionary Microbiology*, 67(12), pp. 5067-5079. Available at: https://doi.org/10.1099/ijsem.0.002416.

Qin, Z., Munnoch, J.T., Devine, R., Holmes, N.A., Seipke, R.F., Wilkinson, K.A., Wilkinson, B. and Hutchings, M.I (2017) 'Formicamycins, antibacterial polyketides produced by *Streptomyces formicae* isolated from African Tetraponera plant-ants',

Chemical Science, 8(4), pp. 3218-3227. Available at: https://doi.org/10.1039/c6sc04265a.

Qin, Z., Munnoch, J.T., Devine, R., Holmes, N.A., Seipke, R.F., Wilkinson, K.A., Wilkinson, B. and Hutchings, M.I. (2017) 'Formicamycins, antibacterial polyketides produced by *Streptomyces formicae* isolated from African *Tetraponera* plant-ants', *Chemical Science*, 8(4), pp. 3218-3227. https://doi.org/10.1039/c6sc04265a.

Quirke, J.C.K., Sati, G.C., Sonousi, A., Gysin, M., Haldimann, K., Böttger, E.C., Vasella, A., Hobbie, S.N. and Crich, D. (2022) 'Structure-Activity Relationships for 5″ Modifications of 4,5-Aminoglycoside Antibiotics', *ChemMedChem*, 17(13), e202200120. Available at: https://doi.org/10.1002/cmdc.202200120.

Rajagopal, K., Singh, P.K., Kumar, R. and Siddiqui, K.F. (2014) 'CTAB-mediated, single-step preparation of competent *Escherichia coli*, *Bifidobacterium* sp. and *Kluyveromyces lactis* cells', *Meta Gene*, 2, pp. 807-818. Available at: https://doi.org/10.1016/j.mgene.2014.10.002.

Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A. and Zhang, F (2013) 'Genome engineering using the CRISPR-Cas9 system', *Nature Protocols*, 8(11), pp. 2281-2308. Available at: https://doi.org/10.1038/nprot.2013.143.

Rath, D., Amlinger, L., Rath, A. and Lundgren, M. (2015) 'The CRISPR-Cas immune system: Biology, mechanisms and applications', *Biochimie*, 117, pp. 119-128. https://doi.org/10.1016/j.biochi.2015.03.025.

Raun, W.R. and Johnson, G.V. (1999) 'Improving nitrogen use efficiency for cereal production', *Agronomy Journal*, 91(3), pp. 357-363. Available at: https://doi.org/10.2134/agronj1999.00021962009100030001x.

Ravindran, S. (2012) 'Barbara McClintock and the discovery of jumping genes.', *Proceedings of the National Academy of Sciences of the United States of America*, 109(50), pp. 20198-20199. Available at: https://doi.org/10.1073/pnas.1219372109.

Recht, M.I. and Puglisi, J.D. (2001) 'Aminoglycoside resistance with homogeneous and heterogeneous populations of antibiotic-resistant ribosomes', *Antimicrobial Agents and Chemotherapy*, 45(9), pp. 2414-2419. Available at: https://doi.org/10.1128/AAC.45.9.2414-2419.2001.

Rehman, L., Su, X., Guo, H., Qi, X. and Cheng, H. (2016) 'Protoplast transformation as a potential platform for exploring gene function in *Verticillium dahliae*', *BMC Biotechnology*, 16(1). Available at: https://doi.org/10.1186/s12896-016-0287-4.

Reilly, M.S. and Grogan, D.W. (2001) 'Characterization of intragenic recombination in a hyperthermophilic archaeon via conjugational DNA exchange', *Journal of Bacteriology*, 183(9), pp. 2943-2946. Available at: https://doi.org/10.1128/JB.183.9.2943-2946.2001.

Reiter, W.D., Hudepohl, U. and Zilligt, W. (1990) 'Mutational analysis of an archaebacterial promoter: essential role of a TATA box for transcription efficiency and start-site selection *in vitro*', *Proceedings of the National Academy of Sciences of the United States of America*, 87(24), pp. 9509-9513. Available at: https://doi.org/10.1073/pnas.87.24.9509.

Rhoads, A. and Au, K.F. (2015) 'PacBio Sequencing and Its Applications', *Genomics, Proteomics and Bioinformatics,* 13(5), pp. 278-289. Available at: https://doi.org/10.1016/j.gpb.2015.08.002.

Riley, L.A. and Guss, A.M. (2021) 'Approaches to genetic tool development for rapid domestication of non-model microorganisms', *Biotechnology for Biofuels*, 14(1), p. 30. Available at: https://doi.org/10.1186/s13068-020-01872-z.

Rinke, C., Chuvochina, M., Mussig, A.J., Chaumeil, P.A., Davín, A.A., Waite, D.W., Whitman, W.B., Parks, D.H. and Hugenholtz, P. (2021) 'A standardized archaeal taxonomy for the Genome Taxonomy Database', *Nature Microbiology*, 6(7), pp. 946-959. Available at: https://doi.org/10.1038/s41564-021-00918-8.

Roberts, R.J., Vincze, T., Posfai, J. and Macelis, D. (2003) 'REBASE: Restriction enzymes and methyltransferases', *Nucleic Acids Research*, 31(1), pp. 418–420. Available at: https://doi.org/10.1093/nar/gkg069.

Roberts, R.J., Vincze, T., Posfai, J. and Macelis, D. (2010) 'REBASE-A database for DNA restriction and modification: Enzymes, genes and genomes', *Nucleic Acids Research*, 38(Database Issue), D234-D236. Available at: https://doi.org/10.1093/nar/gkp874.

Robinson, J.L., Pyzyna, B., Atrasz, R.G., Henderson, C.A., Morrill, K.L., Burd, A.M., Desoucy, E., Fogleman, R.E., 3rd, Naylor, J.B., Steele, S.M., Elliott, D.R., Leyva, K.J. and Shand, R.F. (2005) 'Growth kinetics of extremely halophilic Archaea (family Halobacteriaceae) as revealed by arrhenius plots', *Journal of Bacteriology*, 187(3), pp. 923-929. Available at: https://doi.org/10.1128/JB.187.3.923-929.2005.

Robinson, N.P., Dionne, I., Lundgren, M., Marsh, V.L., Bernander, R. and Bell, S.D. (2004) 'Identification of Two Origins of Replication in the Single Chromosome of the Archaeon *Sulfolobus solfataricus*', *Cell*, 116(1), pp. 25-38. Available at: https://doi.org/10.1016/s0092-8674(03)01034-1.

Rodgers K. and McVey M. (2016) ‘Error-Prone Repair of DNA Double-Strand Breaks’, *Journal of Cellular Physiology*, 231(1), pp. 15-24. Available at: https://doi: 10.1002/jcp.25053.

Rodionov, D.A., Dubchak, I.L., Arkin, A.P., Alm, E. J. and Gelfand, M.S. (2005) ‘Dissimilatory metabolism of nitrogen oxides in bacteria: comparative reconstruction of transcriptional networks.’, *PLoS Computational Biology*, 1(5), e55. Available at: https://doi.org/10.1371/journal.pcbi.0010055.

Rodrigues-Oliveira, T., Belmok, A., Vasconcellos, D., Schuster, B. and Kyaw, C.M. (2017) ‘Archaeal S-layers: Overview and current state of the art’, *Frontiers in Microbiology*, 8, p. 2597. Available at: https://doi.org/10.3389/fmicb.2017.02597.

Rose, A., Padovan, A., Christian, K., van de Kamp, J., Kaestli, M., Tsoukalis, S., Bodrossy, L. and Gibb, K. (2021) ‘The Diversity of Nitrogen-Cycling Microbial Genes in a Waste Stabilization Pond Reveals Changes over Space and Time that Is Uncoupled to Changing Nitrogen Chemistry. Microbial ecology’, 81(4), pp. 1029-1041. Available at: https://doi.org/10.1007/s00248-020-01639-x.

Rothstein, R.J. (1983) ‘One-Step Gene Disruption in Yeast’, *Methods in Enzymology*, 101, pp. 202-211. Available at: https://doi.org/10.1016/0076-6879(83)01015-0.

Rouet, P., Smih, F. and Jasin, M. (1994) ‘Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease.’, *Molecular and Cellular Biology*, 14(12), pp. 8096-8106. Available at: https://doi.org/10.1128/mcb.14.12.8096.

Rusinov I.S, Ershova A.S, Karyagina A.S, Spirin S.A. and Alexeevski A.V. (2018) ‘Avoidance of recognition sites of restriction-modification systems is a widespread but

not universal anti-restriction strategy of prokaryotic viruses', *BMC Genomics*, 19(1):885. Available at: https://10.1186/s12864-018-5324-3.

Sakai, H.D. and Kurosawa, N. (2017) '*Sulfodiicoccus acidiphilus* gen. nov., sp. nov., a sulfur-inhibited thermoacidophilic archaeon belonging to the order Sulfolobales isolated from a terrestrial acidic hot spring', *International Journal of Systematic and Evolutionary Microbiology*, 67(6), pp. 1880-1886. Available at: https://doi.org/10.1099/ijsem.0.001881.

Sakai, H.D. and Kurosawa, N. (2018) '*Saccharolobus caldissimus* gen. nov., sp. nov., a facultatively anaerobic iron-reducing hyperthermophilic archaeon isolated from an acidic terrestrial hot spring, and reclassification of *Sulfolobus solfataricus* as *Saccharolobus solfataricus* comb. nov. and *Sulfolobus shibatae* as *Saccharolobus shibatae* comb. nov', *International Journal of Systematic and Evolutionary Microbiology*, 68(4), pp. 1271-1278. Available at: https://doi.org/10.1099/ijsem.0.002665.

Sakoula, D., Koch, H., Frank, J., Jetten, M.S.M., van Kessel, M.A.H.J. and Lücker, S. (2021) 'Enrichment and physiological characterization of a novel comammox *Nitrospira* indicates ammonium inhibition of complete nitrification', *ISME Journal*, 15(4), pp. 1010-1024. Available at: https://doi.org/10.1038/s41396-020-00827-4.

Sanchez-Nieves R.L, Zhang C. and Whitaker R.J. (2023) 'Integrated conjugative plasmid drives high frequency chromosomal gene transfer in *Sulfolobus islandicus*', *Frontiers in Microbiology*, 14, p. 1114574. Available at: https://doi.org/10.3389/fmicb.2023.1114574.

Sander, J.D., Dahlborg, E.J., Goodwin, M.J., Cade, L., Zhang, F., Cifuentes, D., Curtin, S.J., Blackburn, J.S., Thibodeau-Beganny, S., Qi, Y., Pierick, C.J., Hoffman, E., Maeder, M.L., Khayter, C., Reyon, D., Dobbs, D., Langenau, D.M., Stupar, R.M., Giraldez, A.J., Voytas, D.F., Peterson, R.T., Yeh, J.J. and Joung, J.K. (2011) 'Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA)', *Nature Methods*, 8(1), pp. 67-69. Available at: https://doi.org/10.1038/nmeth.1542.

Sands, J.G. and Bennett, E.O. (1966) 'Effect of Different Commercial Agar Preparations on the Inhibitory Activities of Phenols', *Applied Microbiology*, 14(2), pp. 196-202. Available at: https://doi.org/10.1128/am.14.2.196-202.1966.

Santangelo, T.J. and Reeve, J.N. (2006) 'Archaeal RNA polymerase is sensitive to intrinsic termination directed by transcribed and remote sequences', *Journal of Molecular Biology*, 355(2), pp. 196-210. Available at: https://doi.org/10.1016/j.jmb.2005.10.062.

Santangelo, T.J., Cubonová, L., Matsumi, R., Atomi, H., Imanaka, T. and Reeve, J.N. (2008a) 'Polarity in archaeal operon transcription in *Thermococcus kodakaraensis*', *Journal of Bacteriology*, 190(6), pp. 2244-2248. Available at: https://doi.org/10.1128/JB.01811-07.

Santangelo, T.J., Čuboňová, L. and Reeve, J.N. (2008b) 'Shuttle vector expression in Thermococcus kodakaraensis: Contributions of cis elements to protein synthesis in a hyperthermophilic archaeon', *Applied and Environmental Microbiology*, 74(10), pp. 3099-3104. Available at: https://doi.org/10.1128/AEM.00305-08.

Santangelo, T.J., Cubonová, L., Skinner, K.M. and Reeve, J.N. (2009) 'Archaeal intrinsic transcription termination in vivo', *Journal of Bacteriology*, 191(22), pp. 7102-7108. Available at: https://doi.org/10.1128/JB.00982-09.

Sato, T., Fukui, T., Atomi, H. and Imanaka, T. (2003) 'Targeted gene disruption by homologous recombination in the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1', *Journal of Bacteriology*, 185(1), pp. 210-220. Available at: https://doi.org/10.1128/JB.185.1.210-220.2003.

Sato, T., Fukui, T., Atomi, H. and Imanaka, T. (2005) 'Improved and versatile transformation system allowing multiple genetic manipulations of the hyperthermophilic archaeon *Thermococcus kodakaraensis*', *Applied and Environmental Microbiology*, 71(7), pp. 3889-3899. Available at: https://doi.org/10.1128/AEM.71.7.3889-3899.2005.

Schauss, K., Focks, A., Leininger, S., Kotzerke, A., Heuer, H., Thiele-Bruhn, S., Sharma, S., Wilke, B.M., Matthies, M., Smalla, K., Munch, J.C., Amelung, W., Kaupenjohann, M., Schloter, M. and Schleper, C. (2009) 'Dynamics and functional relevance of ammonia-oxidizing archaea in two agricultural soils', *Environmental Microbiology*, 11(2), pp. 446-456. Available at: https://doi.org/10.1111/j.1462-2920.2008.01783.x.

Schelert, J., Dixit, V., Hoang, V., Simbahan, J., Drozda, M. and Blum, P. (2004) 'Occurrence and Characterization of Mercury Resistance in the Hyperthermophilic Archaeon *Sulfolobus solfataricus* by Use of Gene Disruption', *Journal of Bacteriology*, 186(2), pp. 427-437. Available at: https://doi.org/10.1128/JB.186.2.427-437.2004.

Schiestl, R., Manivasakam, P. and Woods, R. (1993) 'Introducing DNA into yeast by transformation', *Methods*, 5(2), pp. 79-85. Available at: https://doi.org/10.1006/meth.1993.1011.

Schinzel, R. and Burger, K.J. (1986) 'A site-specific endonuclease activity in *Halobacterium halobium*', *FEMS Microbiology Letters*, 37(3), pp. 325-329. Available at: https://doi.org/10.1111/j.1574-6968.1986.tb01818.x.

Schleper, C., Kubo, K. and Zillig, W. (1992) 'The particle SSV1 from the extremely thermophilic archaeon *Sulfolobus* is a virus: demonstration of infectivity and of transfection with viral DNA.', *Proceedings of the National Academy of Sciences*, 89(16), pp. 7645-7649. Available at: https://doi.org/10.1073/pnas.89.16.7645.

Schleper, C., Holz, I., Janekovic, D., Murphy, J. and Zillig, W. (1995) 'A Multicopy Plasmid of the Extremely Thermophilic Archaeon *Sulfolobus* Effects Its Transfer to Recipients by Mating', *Journal of Bacteriology*, 177(15), pp. 4417-4426. Available at: https://doi.org/10.1128/jb.177.15.4417-4426.1995.

Schmidt, C.M., Shis, D.L., Nguyen-Huu, T.D. and Bennett, M.R. (2012) 'Stable maintenance of multiple plasmids in *E. coli* using a single selective marker.', *ACS Synthetic Biology*, 1(10), pp. 445-450. Available at: https://doi.org/10.1021/sb3000589.

Schoeffler, A.J. and Berger, J.M. (2008) 'DNA topoisomerases: Harnessing and constraining energy to govern chromosome topology', *Quarterly Reviews of Biophysics*, 41(1), pp. 41-101. Available at: https://doi.org/10.1017/S003358350800468X.

Schumacher, M.A. (2012) 'Bacterial plasmid partition machinery: A minimalist approach to survival', *Current Opinion in Structural Biology*, 22(1), pp. 72-79. Available at: https://doi.org/10.1016/j.sbi.2011.11.001.

Scott, K.A., Williams, S.A. and Santangelo, T.J. (2021) '*Thermococcus kodakarensis* provides a versatile hyperthermophilic archaeal platform for protein expression', *Methods in Enzymology*, 659, pp. 243-273. Available at: https://doi.org/10.1016/bs.mie.2021.06.014

Serizawa, M., Sekizuka, T., Okutani, A., Banno, S., Sata, T., Inoue, S. and Kuroda, M. (2010) 'Genomewide screening for novel genetic variations associated with ciprofloxacin resistance in *Bacillus anthracis*', *Antimicrobial Agents and Chemotherapy*, 54(7), pp. 2787-2792. Available at: https://doi.org/10.1128/AAC.01405-09.

Sganzerla Martinez, G., Perez-Rueda, E., Kumar, A., Sarkar, S. and de Avila E Silva, S. (2023) 'Explainable artificial intelligence as a reliable annotator of archaeal promoter regions', *Scientific Reports*, 13(1). p. 1763. Available at: https://doi.org/10.1038/s41598-023-28571-7.

Shaner, N.C., Campbell, R.E., Steinbach, P.A., Giepmans, B.N., Palmer, A.E. and Tsien, R.Y (2004) 'Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein', *Nature Biotechnology*, 22(12), pp. 1567-1572. Available at: https://doi.org/10.1038/nbt1037.

Shariati, A., Arshadi, M., Khosrojerdi, M.A., Abedinzadeh, M., Ganjalishahi, M., Maleki, A., Heidary, M. and Khoshnood, S. (2022) 'The resistance mechanisms of bacteria against ciprofloxacin and new approaches for enhancing the efficacy of this antibiotic', *Frontiers in Public Health*, 10, p. 1025633. Available at: https://doi.org/10.3389/fpubh.2022.1025633.

Sharma, S.V, Agatsuma, T. and Nakano, H. (1998) 'Targeting of the protein chaperone, HSP90, by the transformation suppressing agent, radicicol', *Oncogene*, 16(20), pp. 2639-2645. https://doi.org/10.1038/sj.onc.1201790.

Shen, J.P., Zhang, L.M., Zhu, Y.G., Zhang, J.B. and He, J.Z. (2008) 'Abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea communities of an alkaline sandy loam', *Environmental Microbiology*, 10(6), pp. 1601-1611. Available at: https://doi.org/10.1111/j.1462-2920.2008.01578.x.

Sherba, J.J., Hogquist, S., Lin, H., Shan, J.W., Shreiber, D.I. and Zahn, J.D. (2020). 'The effects of electroporation buffer composition on cell viability and electro-transfection efficiency', *Scientific Reports*, 10(1), p. 3053. Available at: https://doi.org/10.1038/s41598-020-59790-x.

Shintani, M., Sanchez, Z.K. and Kimbara, K. (2015) 'Genomics of microbial plasmids: Classification and identification based on replication and transfer systems and host taxonomy', *Frontiers in Microbiology*. 6, p. 242. Available at: https://doi.org/10.3389/fmicb.2015.00242.

Silas, S., Mohr, G., Sidote, D.J., Markham, L. M., Sanchez-Amat, A., Bhaya, D., Lambowitz, A.M. and Fire, A.Z. (2016) 'Direct CRISPR spacer acquisition from RNA by a natural reverse transcriptase-Cas1 fusion protein', *Science*, 351(6276). Available at: https://doi.org/10.1126/science.aad4234.

Silva-Rocha, R., Pontelli, M.C., Furtado, G.P., Zaramela, L.S. and Koide, T. (2015) 'Development of new modular genetic tools for engineering the halophilic archaeon *Halobacterium salinarum*', *PLoS ONE*, 10(6), e0129215. Available at: https://doi.org/10.1371/journal.pone.0129215.

Silva, G., Poirot, L., Galetto, R., Smith, J., Montoya, G., Duchateau, P. and Pâques, F. (2011) 'Meganucleases and Other Tools for Targeted Genome Engineering: Perspectives and Challenges for Gene Therapy', *Current gene therapy*, 11(1), pp. 11-27. Available at: https://doi.org/10.2174/156652311794520111.

Singh, A. and Singh, A.K. (2017) 'Haloarchaea: worth exploring for their biotechnological potential', *Biotechnology Letters*, 39(12), pp. 1793-1800. Available at: https://doi.org/10.1007/s10529-017-2434-y.

Smith, J., Bibikova, M., Whitby, F.G., Reddy, A.R., Chandrasegaran, S. and Carroll, D. (2000) 'Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains', *Nucleic Acids Research*, 28(17), pp. 3361-3369. https://doi.org/10.1093/nar/28.17.3361.

Smithies, O., Gregg, R.G., Boggs, S.S., Koralewski, M.A. and Kucherlapati, R.S. (1985) 'Insertion of DNA sequences into the human chromosomal β-globin locus by homologous recombination', 317(6034), pp. 230-234. Available at: https://doi.org/10.1038/317230a0.

Solanki, M.K., Wang, Z., Wang, F.Y., Li, C.N., Gupta, C.L., Singh, R.K., Malviya, M.K., Singh, P., Yang, L.T., and Li, Y.R. (2020) 'Assessment of Diazotrophic Proteobacteria in Sugarcane Rhizosphere When Intercropped With Legumes (Peanut and Soybean) in the Field', *Frontiers in Microbiology*, 11, p. 1814. Available at: https://doi.org/10.3389/fmicb.2020.01814.

Song, Y., Zhu, Z., Zhou, W. and Zhang, Y.P.J. (2021) 'High-efficiency transformation of archaea by direct PCR products with its application to directed evolution of a

thermostable enzyme', *Microbial Biotechnology*, 14(2), pp. 453–464. Available at: https://doi.org/10.1111/1751-7915.13613.

Sorek, R., Kunin, V. and Hugenholtz, P. (2008) 'CRISPR--a widespread system that provides acquired resistance against phages in bacteria and archaea', *Nature Reviews Microbiology*, 6(3), pp. 181–186. https://doi.org/10.1038/nrmicro1793.

Sowers, K.R., Boone, J.E. and Gunsalus, R.P. (1993) 'Disaggregation of *Methanosarcina* spp. and Growth as Single Cells at Elevated Osmolarity', *Applied and Environmental Microbiology*, 59(11), pp. 3832-3839. Available at: https://doi.org/10.1128/aem.59.11.3832-3839.1993.

Spang, A., Hatzenpichler, R., Brochier-Armanet, C., Rattei, T., Tischler, P., Spieck, E., Streit, W., Stahl, D.A., Wagner, M. and Schleper, C. (2010) 'Distinct gene set in two different lineages of ammonia-oxidizing archaea supports the phylum Thaumarchaeota', *Trends in Microbiology*, 18(8), pp. 331-340. Available at: https://doi.org/10.1016/j.tim.2010.06.003.

Stachler, A.E. and Marchfelder, A. (2016) 'Gene repression in haloarchaea using the CRISPR (Clustered regularly interspaced short palindromic repeats)-Cas I-B system', *Journal of Biological Chemistry*, 291(29), pp. 15226-15242. Available at: https://doi.org/10.1074/jbc.M116.724062.

Stachler, A.E., Turgeman-Grott, I., Shtifman-Segal, E., Allers, T., Marchfelder, A. and Gophna, U. (2017) 'High tolerance to self-targeting of the genome by the endogenous CRISPR-Cas system in an archaeon', *Nucleic Acids Research*, 45(9), pp. 5208-5216. Available at: https://doi.org/10.1093/nar/gkx150.

Stedman, K.M., She, Q., Phan, H., Holz, I., Singh, H., Prangishvili, D., Garrett, R. and Zillig, W. (2000) 'pING Family of Conjugative Plasmids from the Extremely Thermophilic Archaeon *Sulfolobus islandicus*: Insights into Recombination and Conjugation in Crenarchaeota', *Journal of Bacteriology*, 182(24), pp. 7014-7020. Available at: https://doi.org/10.1128/JB.182.24.7014-7020.2000.

Stein, L.Y. and Klotz, M.G. (2016) 'The nitrogen cycle', *Current Biology*, 26(3), R94-R98. https://doi.org/10.1016/j.cub.2015.12.021.

Stein, L.Y., Arp, D.J., Berube, P.M., Chain, P.S., Hauser, L., Jetten, M.S., Klotz, M.G., Larimer, F.W., Norton, J.M., Op den Camp, H.J., Shin, M. and Wei, X. (2007) 'Whole-genome analysis of the ammonia-oxidizing bacterium, *Nitrosomonas eutropha* C91: Implications for niche adaptation', *Environmental Microbiology*, 9(12), pp. 2993-3007. Available at: https://doi.org/10.1111/j.1462-2920.2007.01409.x.

Stephenson M. and Stickland L.H. (1933) 'Hydrogenase: The bacterial formation of methane by the reduction of one-carbon compounds by molecular hydrogen', *Biochemical Journal*, 27(5), pp. 1517-1527. Available at: https://doi.org/10.1042/bj0271517.

Stettler, R., Pfister, P. and Leisinger, T. (1995) 'Characterization of a Plasmid Carried by *Methanobacterium thermoautotrophicum* ZH3, a Methanogen Closely Related to *Methanobacterium thermoautotrophicum* Marburg', *Systematic and Applied Microbiology,* 17(4), pp. 484-491. Available at: ttps://doi.org/10.1016/S0723-2020(11)80066-4.

Stieglmeier, M., Klingl, A., Alves, R.J.E., Rittmann, S.K.R., Melcher, M., Leisch, N. and Schleper, C. (2014) '*Nitrososphaera viennensis* gen. nov., sp. nov., an aerobic and mesophilic, ammonia-oxidizing archaeon from soil and a member of the archaeal

phylum Thaumarchaeota', *International Journal of Systematic and Evolutionary Microbiology*, 64(8), pp. 2738-2752. Available at: https://doi.org/10.1099/ijs.0.063172-0.

Stillman, B. (2005) 'Origin recognition and the chromosome cycle', *FEBS letters*, 579(4), pp. 877-884. https://doi.org/10.1016/j.febslet.2004.12.011.

Stinchcomb, D.T., Thomas, M., Kelly, J., Selker, E. and Davis, R.W. (1980) 'Eukaryotic DNA segments capable of autonomous replication in yeast', *Proceedings of the National Academy of Sciences of the United States of America*, 77(8), 4559-4563. https://doi.org/10.1073/pnas.77.8.4559.

Suzuki, I., Dular, U. and Kwok, S.C. (1974) 'Ammonia or ammonium ion as substrate for oxidation by *Nitrosomonas europaea* cells and extracts', *Journal of Bacteriology*, 120(1), 556-558. Available at: https://doi.org/10.1128/jb.120.1.556-558.1974.

Takahashi, R., Kondo, N., Usui, K., Kanehira, T., Shinohara, M. and Tokuyama, T. (1992) 'Pure Isolation of a New Chemoautotrophic Ammonia-Oxidizing Bacterium on Gellan Gum Plate', *Journal of Fermentation and Bioengineering*, 74(1), pp. 52-54. Available at: https://doi.org/10.1016/0922-338X(92)90268-Y.

Takai, K., Sugai, A., Itoh, T. and Horikoshi, K. (2000) '*Palaeococcus ferrophilus* gen. nov., sp. nov., a barophilic, hyperthermophilic archaeon from a deep-sea hydrothermal vent chimney', *International Journal of Systematic and Evolutionary Microbiology*, 50, pp. 489-500. Available at: https://doi.org/10.1099/00207713-50-2-489.

Tamaki, H., Sekiguchi, Y., Hanada, S., Nakamura, K., Nomura, N., Matsumura, M. and Kamagata, Y. (2005) 'Comparative analysis of bacterial diversity in freshwater sediment of a shallow eutrophic lake by molecular and improved cultivation-based

techniques', *Applied and Environmental Microbiology*, 71(4), pp. 2162-2169. Available at: https://doi.org/10.1128/AEM.71.4.2162-2169.2005.

Tamaki, H., Hanada, S., Sekiguchi, Y., Tanaka, Y. and Kamagata, Y. (2009) 'Effect of gelling agent on colony formation in solid cultivation of microbial community in lake sediment', *Environmental Microbiology*, 11(7), pp. 1827-1834. Available at: https://doi.org/10.1111/j.1462-2920.2009.01907.x.

Tanaka, T., Kawasaki, K., Daimon, S., Kitagawa, W., Yamamoto, K., Tamaki, H., Tanaka, M., Nakatsu, C.H. and Kamagata, Y. (2014) 'A hidden pitfall in the preparation of agar media undermines microorganism cultivability', *Applied and Environmental Microbiology*, 80(24), pp. 7659-7666. Available at: https://doi.org/10.1128/AEM.02741-14.

Tavormina, P.L., Orphan, V.J., Kalyuzhnaya, M.G., Jetten, M.S. and Klotz, M.G. (2011) 'A novel family of functional operons encoding methane/ammonia monooxygenase-related proteins in gammaproteobacterial methanotrophs', *Environmental Microbiology Reports*, 3(1), pp. 91-100. Available at: https://doi.org/10.1111/j.1758-2229.2010.00192.x.

Taylor, A.E., Zeglin, L.H., Dooley, S., Myrold, D.D. and Bottomley, P.J. (2010) 'Evidence for different contributions of archaea and bacteria to the ammonia-oxidizing potential of diverse Oregon soils', *Applied and Environmental Microbiology*, 76(23), pp. 7691-7698. Available at: https://doi.org/10.1128/AEM.01324-10.

Teske, A., Alm, E., Regan, J.M., Toze, S., Rittmann, B.E. and Stahl, D.A. (1994) 'Evolutionary Relationships among Ammonia-and Nitrite-Oxidizing Bacteria',

Journal of Bacteriology, 176(21), pp. 6623-6630. Available at: https://doi.org/10.1128/jb.176.21.6623-6630.1994.

Tesson, F., Hervé, A., Mordret, E., Touchon, M., d'Humières, C., Cury, J. and Bernheim, A. (2022) 'Systematic and quantitative view of the antiviral arsenal of prokaryotes', *Nature communications*, 13(1), 2561. Available at: https://doi.org/10.1038/s41467-022-30269-9.

Thomm, M. and Wich, G. (1988) 'An archaebacterial promoter element for stable RNA genes with homology to the TATA box of higher eukaryotes', *Nucleic Acids Research*, 16(1), pp. 151-163. Available at: https://doi.org/10.1093/nar/16.1.151.

Thomm, M., Hausner, W. and Hethke, C. (1993) 'Transcription Factors and Termination of Transcription in *Methanococcus*', *Systematic and Applied Microbiology*, 16(4), pp. 648-655. Available at: https://doi.org/10.1016/S0723-2020(11)80336-X.

Tock M.R. and Dryden D.T. (2005) 'The biology of restriction and anti-restriction', *Current Opinion in Microbiology*, 8(4), pp. 466-72. Available at: https://doi: 10.1016/j.mib.2005.06.003.

Tolar, B.B., Wallsgrove, N.J., Popp, B.N. and Hollibaugh, J.T. (2017) 'Oxidation of urea-derived nitrogen by thaumarchaeota-dominated marine nitrifying communities', *Environmental Microbiology*, 19(12), pp. 4838-4850. Available at: https://doi.org/10.1111/1462-2920.13457.

Tourna, M., Stieglmeier, M., Spang, A., Könneke, M., Schintlmeister, A., Urich, T., Engel, M., Schloter, M., Wagner, M., Richter, A. and Schleper, C. (2011) '*Nitrososphaera viennensis*, an ammonia oxidizing archaeon from soil', *Proceedings*

of the National Academy of Sciences of the United States of America, 108(20), pp. 8420-8425. Available at: https://doi.org/10.1073/pnas.1013488108.

Treusch, A.H., Leininger, S., Kletzin, A., Schuster, S.C., Klenk, H.P. and Schleper, C. (2005) 'Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling', *Environmental Microbiology*, 7(12), pp. 1985-1995. Available at: https://doi.org/10.1111/j.1462-2920.2005.00906.x.

Tsai, A., Uemura, S., Johansson, M., Puglisi, E.V., Marshall, R.A., Aitken, C.E., Korlach, J., Ehrenberg, M. and Puglisi, J.D. (2013) 'The Impact of Aminoglycosides on the Dynamics of Translation Elongation', *Cell Reports*, 3(2), pp. 497-508. Available at: https://doi.org/10.1016/j.celrep.2013.01.027.

Tumbula, D.L., Bowen, T.L. and Whitman, W.B. (1997) 'Characterization of pURB500 from the Archaeon *Methanococcus maripaludis* and Construction of a Shuttle Vector', *Journal of Bacteriology*, 179(9), pp. 2976-2986. Available at: https://doi.org/10.1128/jb.179.9.2976-2986.1997.

Tumbula, D.L., Makula, R.A. and Whitman, W.B. (1994) 'Transformation of *Methanococcus maripaludis* and identification of a *Pst* I-like restriction system', *FEMS Microbiology Letters*, 121(3), pp. 309-314. Available at: https://doi.org/10.1111/j.1574-6968.1994.tb07118.x.

Tuovinen, O.H. and Kelly, D.P. (1973) 'Studies on the Growth of *Thiobacillus ferrooxidans*', *Archives of Microbiology*, 88(4), pp. 285-298. Available at: https://doi.org/10.1007/BF00409941.

Vajrala, N., Bottomley, P.J., Stahl, D.A., Arp, D.J. and Sayavedra-Soto, L.A. (2014) 'Cycloheximide prevents the de novo polypeptide synthesis required to recover from acetylene inhibition in *Nitrosopumilus maritimus*', *FEMS Microbiology Ecology*, 88(3), pp. 495-502. Available at: https://doi.org/10.1111/1574-6941.12316.

Vajrala, N., Martens-Habbena, W., Sayavedra-Soto, L.A., Schauer, A., Bottomley, P.J., Stahl, D.A. and Arp, D.J. (2013) 'Hydroxylamine as an intermediate in ammonia oxidation by globally abundant marine archaea', *Proceedings of the National Academy of Sciences of the United States of America*, 110(3), pp. 1006-1011. Available at: https://doi.org/10.1073/pnas.1214272110.

Van De Graaf, A.A., De Bruijn, P., Robertson, L.A., Jetten, M.S.M. and Gijs K.J. (1996) 'Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor', *Microbiology*, 142, pp. 2187-2189. Available at: https://doi.org/DOI:10.1099/13500872-142-8-2187.

van Wolferen, M., Wagner, A., van der Does, C. and Albers, S.V. (2016) 'The archaeal Ced system imports DNA', *Proceedings of the National Academy of Sciences of the United States of America*, 113(9), pp. 2496-2501. Available at: https://doi.org/10.1073/pnas.1513740113.

Vanden Broeck, A., Lotz, C., Ortiz, J. and Lamour, V. (2019) 'Cryo-EM structure of the complete *E. coli* DNA gyrase nucleoprotein complex', *Nature Communications*, 10(4935). Available at: https://doi.org/10.1038/s41467-019-12914-y.

Vara, J., Malpartida, F., Hopwood, D.A. and Jiménez, A. (1985) 'Cloning and expression of a puromycin N-acetyl transferase gene from *Streptomyces alboniger* in

Streptomyces lividans and *Escherichia coli*', *Gene*, 33(2), pp. 197-206. Available at: https://doi.org/10.1016/0378-1119(85)90094-0.

Vartak, N.B., Lin, C.C., Cleary, J.M., Fagan, M.J. and Saier, M.H., Jr. (1995) 'Glucose metabolism in '*Sphingomonas elodea'*: pathway engineering via construction of a glucose-6-phosphate dehydrogenase insertion mutant', *Microbiology*, 141(9), pp. 2339-2350. Available at: https://doi.org/10.1099/13500872-141-9-2339.

Vasu, K. and Nagaraja, V. (2013) 'Diverse Functions of Restriction-Modification Systems in Addition to Cellular Defense', *Microbiology and Molecular Biology Reviews*, 77(1), pp. 53-72. Available at: https://doi.org/10.1128/mmbr.00044-12.

Venter, J.C., Remington, K., Heidelberg, J.F., Halpern, A.L., Rusch, D., Eisen, J.A., Wu, D., Paulsen, I., Nelson, K.E., Nelson, W., Fouts, D.E., Levy, S., Knap, A.H., Lomas, M.W., Nealson, K., White, O., Peterson, J., Hoffman, J., Parsons, R., Baden-Tillson, H., Pfannkoch, C., Rogers, YH. and Smith, H.O. (2004) 'Environmental Genome Shotgun Sequencing of the Sargasso Sea', *Science*, 304(5667), pp. 66-74. Available at: https://doi.org/DOI: 10.1126/science.1093857.

Verhamme, D.T., Prosser, J.I. and Nicol, G.W. (2011) 'Ammonia concentration determines differential growth of ammonia-oxidising archaea and bacteria in soil microcosms', *ISME Journal*, 5(6), pp. 1067-1071. Available at: https://doi.org/10.1038/ismej.2010.191.

Vestergaard, G., Garrett, R.A. and Shah, S.A. (2014) 'CRISPR adaptive immune systems of Archaea', *RNA Biology*, 11(2), pp. 156-167. Available at: https://doi.org/10.4161/rna.27990.

Vicente, E.J. and Dean, D.R. (2017) 'Keeping the nitrogen-fixation dream alive', *Proceedings of the National Academy of Sciences of the United States of America*, 114(12), pp. 3009-3011. Available at: https://doi.org/10.1073/pnas.1701560114.

Vieille, C. and Zeikus, G.J. (2001) 'Hyperthermophilic Enzymes: Sources, Uses, and Molecular Mechanisms for Thermostability', *Microbiology and Molecular Biology Reviews*, 65(1), pp. 1-43. Available at: https://doi.org/10.1128/MMBR.65.1.1-43.2001.

Villain, P., Catchpole, R., Forterre, P., Oberto, J., da Cunha, V. and Basta, T. (2022) 'Expanded Dataset Reveals the Emergence and Evolution of DNA Gyrase in Archaea', *Molecular Biology and Evolution*, 39(8), msac155. Available at: https://doi.org/10.1093/molbev/msac155.

Vinokur, J.M., Korman, T.P., Cao, Z. and Bowie, J.U. (2014) 'Evidence of a novel mevalonate pathway in archaea', *Biochemistry*, 53(25), pp. 4161-4168. Available at: https://doi.org/10.1021/bi500566q.

Vižintin, A. and Miklavčič, D. (2022) 'The electropermeome: cellular response to electroporation', *Zdravniski Vestnik*. Slovene Medical Society, 91(11-12), pp. 483-495. Available at: https://doi.org/10.6016/ZdravVestn.3267.

Vos, S.M., Tretter, E.M., Schmidt, B.H. and Berger, J.M. (2011) 'All tangled up: How cells direct, manage and exploit topoisomerase function', *Nature Reviews Molecular Cell Biology*, 12(12), pp. 827-841.Available at: https://doi.org/10.1038/nrm3228.

Waege, I., Schmid, G., Thumann, S., Thomm, M. and Hausner, W. (2010) 'Shuttle vector-based transformation system for *Pyrococcus furiosus*', *Applied and Environmental Microbiology*, 76(10), pp. 3308-3313. Available at: https://doi.org/10.1128/AEM.01951-09.

Wagner, M., Berkner, S., Ajon, M., Driessen, A.J., Lipps, G. and Albers, S.V. (2009) 'Expanding and understanding the genetic toolbox of the hyperthermophilic genus *Sulfolobus*', *Biochemical Society Transactions*, 37(1), pp. 97-101. Available at: https://doi.org/10.1042/BST0370097.

Wagner, M., Hornt, M. and Daims, H. (2003) 'Fluorescence in situ hybridisation for the identification and characterisation of prokaryotes', *Current Opinion in Microbiology*, 6(3), pp. 302–309. Available at: https://doi.org/10.1016/s1369-5274(03)00054-7.

Walker, J.E., Luyties, O. and Santangelo, T.J. (2017) 'Factor-dependent archaeal transcription termination', *Proceedings of the National Academy of Sciences of the United States of America*, 114(33), pp. E6767-E6773. Available at: https://doi.org/10.1073/pnas.1704028114.

Wang, H. and Tor, Y. (1997) 'Electrostatic Interactions in RNA Aminoglycosides Binding', *Journal of the American Chemical Society*, 119(37), pp. 8734-8735. Available at: https://doi.org/10.1021/ja9715105.

Wang, H., Peng, N., Shah, S.A., Huang, L. and She, Q. (2015) 'Archaeal Extrachromosomal Genetic Elements', *Microbiology and Molecular Biology Reviews*, 79(1), pp. 117-152. Available at: https://doi.org/10.1128/mmbr.00042-14.

Wang, J., Kan, J., Zhang, X., Xia, Z., Zhang, X., Qian, G., Miao, Y., Leng, X. and Sun, J. (2017) 'Archaea dominate the ammonia-oxidizing community in deep-sea sediments of the Eastern Indian Ocean-From the Equator to the Bay of Bengal', *Frontiers in Microbiology*, 8. p. 415. Available at: https://doi.org/10.3389/fmicb.2017.00415.

Wang, J.Y., Pausch, P. and Doudna, J.A. (2022) 'Structural biology of CRISPR–Cas immunity and genome editing enzymes', *Current Opinion in Structural Biology,* 30, pp. 100-111. Available at: https://doi.org/10.1016/j.sbi.2015.02.002.

Wang, L., Yang, L., Wen, X., Chen, Z., Liang, Q., Li, J. and Wang, W. (2019) 'Rapid and high efficiency transformation of *Chlamydomonas reinhardtii* by square-wave electroporation', *Bioscience Reports*, 39(1), BSR20181210. Available at: https://doi.org/10.1042/BSR20181210.

Wang, X., Bai, J., Xie, T., Wang, W., Zhang, G., Yin, S., and Wang, D. (2021) 'Effects of biological nitrification inhibitors on nitrogen use efficiency and greenhouse gas emissions in agricultural soils: A review', *Ecotoxicology and Environmental Safety*. 220, p. 112338. Available at: https://doi.org/10.1016/j.ecoenv.2021.112338.

Ward, B.B. and O'Mullan, G.D. (2002) 'Worldwide distribution of *Nitrosococcus oceani*, a marine ammonia-oxidizing γ-proteobacterium, detected by PCR and sequencing of 16S rRNA and *amo*A genes', *Applied and Environmental Microbiology*, 68(8), pp. 4153-4157. Available at: https://doi.org/10.1128/AEM.68.8.4153-4157.2002.

Watson, S.W. (1965) 'Characteristics of a Marine Nitrifying Bacterium, *Nitrosocystis oceanus* sp. N', *Limnology and Oceanography*, 10, pp. 274-289. Available at: https://doi: 10.4319/lo.1965.10.suppl2.r274.

Wenderoth, M., Pinecker, C., Voß, B. and Fischer, R. (2017) 'Establishment of CRISPR/Cas9 in *Alternaria alternata*', *Fungal Genetics and Biology*, 101, pp. 55-60. Available at: https://doi.org/10.1016/j.fgb.2017.03.001.

Wich, G., Hummel, H., Jarsch, M., Bär, U. and Böck, A. (1986) 'Transcription signals for stable RNA genes in *Methanococcus*', *Nucleic Acids Research*, 14(6), pp. 2459-2479. Available at: https://doi.org/10.1093/nar/14.6.2459 https://doi.org/10.1093/nar/14.6.2459.

Wilkansky, B. (1936) 'Life in the dead sea', *Nature*, 138, p. 467. Available at: https://doi.org/https://doi.org/10.1038/138467a0.

Williams, T.A., Foster, P.G., Cox, C.J. and Embley, T.M. (2013) 'An archaeal origin of eukaryotes supports only two primary domains of life', *Nature*, 504(7479), pp. 231-236. https://doi.org/10.1038/nature12779.

Wimmer, F. and Beisel, C.L. (2020) 'CRISPR-Cas Systems and the Paradox of Self-Targeting Spacers', *Frontiers in Microbiology*, 10, p. 3078. Available at: https://doi.org/10.3389/fmicb.2019.03078.

Winkler, F.K. (2006) 'Amt/MEP/Rh proteins conduct ammonia', *Pflugers Archiv: European Journal of Physiology*, 451(6), pp. 701-707. Available at: https://doi.org/10.1007/s00424-005-1511-6

Woese, C.R. and Fox, G.E. (1977) 'Phylogenetic structure of the prokaryotic domain: the primary kingdoms', *Proceedings of the National Academy of Sciences of the United States of America*, 74(11), pp. 5088-5090. Available at: https://doi.org/10.1073/pnas.74.11.5088.

Woo, Y., Cruz, M.C. and Wuertz, S. (2022) 'Selective Enrichment of *Nitrososphaera viennensis*-Like Ammonia-Oxidizing Archaea over Ammonia-Oxidizing Bacteria from Drinking Water Biofilms', *Microbiology Spectrum*, 10(6). e0184522. Available at: https://doi.org/10.1128/spectrum.01845-22.

Wrage, N., Velthof, G.L., van Beusichem, M.L. and Oenema, O. (2001) 'Role of nitrifier denitrification in the production of nitrous oxide', *Soil Biology and Biochemistry*, 33(12-13), pp. 1723-1732. Available at: https://doi.org/10.1016/S0038-0717(01)00096-7.

Wright, D.A., Thibodeau-Beganny, S., Sander, J.D., Winfrey, R.J., Hirsh, A.S., Eichtinger, M., Fu, F., Porteus, M.H., Dobbs, D., Voytas, D.F. and Joung, J. K. (2006) 'Standardized reagents and protocols for engineering zinc finger nucleases by modular assembly', *Nature Protocols*, 1(3), pp. 1637-1652. Available at: https://doi.org/10.1038/nprot.2006.259.

Wu, Z., Liu, J., Yang, H., Liu, H. and Xiang, H. (2014) 'Multiple replication origins with diverse control mechanisms in *Haloarcula hispanica*', *Nucleic Acids Research*, 42(4), pp. 2282-2294. Available at: https://doi.org/10.1093/nar/gkt1214.

Wuchter, C., Abbas, B., Coolen, M.J., Herfort, L., van Bleijswijk, J., Timmers, P., Strous, M., Teira, E., Herndl, G.J., Middelburg, J.J., Schouten, S. and Sinninghe Damsté, J.S. (2006) 'Archaeal nitrification in the ocean', *Proceedings of the National Academy of Sciences of the United States of America*, 103(33), pp. 12317-12322. Available at: https://doi.org/10.1073/pnas.0600756103.

Würgler, F.E. and Maier, P. (1972) 'Genetic control of mutation induction in *Drosophila melanogaster*. I. Sex-chromosome loss in x-rayed mature sperm', *Mutation Research*, 15(1), pp. 41-53. Available at: https://doi.org/10.1016/0027-5107(72)90090-5.

Wyber, J.A., Andrews, J. and D'Emanuele, A. (1997) 'The Use of Sonication for the Efficient Delivery of plasmid DNA into Cells', *Pharmaceutical Research*, 14(6), pp. 750-756. Available at: https://doi.org/10.1023/a:1012198321879.

Xiaoshuai, L., Qiushi, W. and Rui, W. (2022) 'Advantages of CRISPR-Cas9 combined organoid model in the study of congenital nervous system malformations', *Frontiers in Bioengineering and Biotechnology*, 10, p. 932936. Available at: https://doi.org/10.3389/fbioe.2022.932936.

Xue C, and Greene E.C. (2021) 'DNA Repair Pathway Choices in CRISPR-Cas9-Mediated Genome Editing', *Trends in Genetics*, 37(7), pp. 639-656. Available at: https://doi: 10.1016/j.tig.2021.02.008.

Yan, Q. and Fong, S.S. (2017) 'Challenges and advances for genetic engineering of non-model bacteria and uses in consolidated bioprocessing', *Frontiers in Microbiology*, 8, p. 2060. Available at: https://doi.org/10.3389/fmicb.2017.02060.

Yang, L., Li, X., Wu, P., Xue, J., Xu, L., Li, H. and Wei, X. (2020) 'Streptovertimycins A–H, new fasamycin-type antibiotics produced by a soil-derived *Streptomyces morookaense* strain', *Journal of Antibiotics*, 73(5), pp. 283-289. Available at: https://doi.org/10.1038/s41429-020-0277-6.

Yang, Y., Xiao, T., Li, J., Cheng, P., Li, F., Yu, H., Liu, R., Muhammad, I. and Zhang, X. (2020) 'Wild-type cutoff for Apramycin against *Escherichia coli*', *BMC Veterinary Research*, 16(1), 309. Available at: https://doi.org/10.1186/s12917-020-02522-0.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S. and Madden, T.L. (2012) 'Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction', *BMC Bioinformatics*, 13, p. 134. Available at: https://doi.org/10.1186/1471-2105-13-134.

Yi, Y. and Kuipers, O.P. (2017). 'Development of an efficient electroporation method for rhizobacterial *Bacillus mycoides* strains', *Journal of Microbiological Methods*, 133, pp. 82-86. Available at: https://doi.org/10.1016/j.mimet.2016.12.022.

You, J., Das, A., Dolan, E.M. and Hu, Z. (2009) 'Ammonia-oxidizing archaea involved in nitrogen removal', *Water Research*, 43(7), pp. 1801-1809. Available at: https://doi.org/10.1016/j.watres.2009.01.016.

Young, J.L. and Dean, D.A. (2015) 'Electroporation-Mediated Gene Delivery', *Advances in Genetics*, 89, pp. 49-88. Available at: https://doi.org/10.1016/bs.adgen.2014.10.003.

Zakrzewska-Czerwińska, J., Majka, J. and Schrempf, H. (1995) 'Minimal Requirements of the *Streptomyces lividans* 66 oriC Region and Its Transcriptional and Translational Activities', *Journal of Bacteriology*, 177(16), pp. 4765-4771. Available at: https://doi.org/10.1128/jb.177.16.4765-4771.1995.

Zaremba-Niedzwiedzka, K., Caceres, E.F., Saw, J.H., Bäckström, D., Juzokaite, L., Vancaester, E., Seitz, K.W., Anantharaman, K., Starnawski, P., Kjeldsen, K.U., Stott, M.B., Nunoura, T., Banfield, J.F., Schramm, A., Baker, B.J., Spang, A. and Ettema, T.J.G. (2017) 'Asgard archaea illuminate the origin of eukaryotic cellular complexity', *Nature*, 541(7637), pp. 353-358. Available at: https://doi.org/10.1038/nature21031.

Zebec, Z., Manica, A., Zhang, J., White, M.F. and Schleper, C. (2014) 'CRISPR-mediated targeted mRNA degradation in the archaeon *Sulfolobus solfataricus*', *Nucleic Acids Research*, 42(8), pp. 5280-5288. Available at: https://doi.org/10.1093/nar/gku161.

Zhang R. and Zhang C.T. (1994) 'Z curves, an intuitive tool for visualizing and analyzing the DNA sequences,' *Journal of Biomolecular Structure and Dynamics*, 11(4), pp. 767-82. Available at https://doi: 10.1080/07391102.1994.10508031.

Zhang R. and Zhang C.T. (2002) 'Single replication origin of the archaeon *Methanosarcina mazei* revealed by the Z curve method,' *Biochemical and Biophysical*

Research Communications, 297(2), pp. 396-400. Available at: https://doi: 10.1016/s0006-291x(02)02214-3.

Zhang, R. and Zhang, C.T. (2003) 'Multiple replication origins of the archaeon *Halobacterium* species NRC-1', *Biochemical and Biophysical Research Communications*, 302(4), pp. 728-734. Available at: https://doi.org/10.1016/S0006-291X(03)00252-3.

Zhang R. and Zhang C.T. (2004) 'Identification of replication origins in the genome of the methanogenic archaeon, *Methanocaldococcus jannaschii*,' *Extremophiles*. 8(3), pp. 253-8. Available at https://doi: 10.1007/s00792-004-0385-4.

Zhang, R. and Zhang, C.T. (2005) 'Identification of replication origins in archaeal genomes based on the Z-curve method', *Archaea*, 1, pp. 335-346. Available at: https://doi.org/10.1155/2005/509646.

Zhang, R. and Zhang, C.T. (2014) 'The Z-curve Theory and its Application in Genome Analysis', *Current Genomics*, 15(2), pp. 78-94. Available at: https://doi.org/10.2174/1389202915999140328162433.

Zhang, Y., Heidrich, N., Ampattu, B.J., Gunderson, C.W., Seifert, H.S., Schoen, C., Vogel, J. and Sontheimer, E.J (2013) 'Processing-Independent CRISPR RNAs Limit Natural Transformation in *Neisseria meningitidis*', *Molecular Cell*, 50(4), pp. 488-503. Available at: https://doi.org/10.1016/j.molcel.2013.05.001.

Zhang, Y., Zhao, Z., Chen, C.T., Tang, K., Su, J. and Jiao, N. (2012) 'Sulfur Metabolizing Microbes Dominate Microbial Communities in Andesite-Hosted Shallow-Sea Hydrothermal Systems', *PLoS ONE*, 7(9), p. 44593. Available at: https://doi.org/10.1371/journal.pone.0044593.

Zhao, J., Bello, M.O., Meng, Y., Prosser, J.I. and Gubry-Rangin, C. (2020) 'Selective inhibition of ammonia oxidising archaea by simvastatin stimulates growth of ammonia oxidising bacteria', *Soil Biology and Biochemistry*, 141. 107673. Available at: https://doi.org/10.1016/j.soilbio.2019.107673.

Zhao, Y., Bian, S-M., Zhou, H-N., Huang, J-F. (2006) 'Diversity of nitrogenase systems in diazotrophs', *Journal of Integrative Plant Biology*, 48(7), pp. 745-755. Available at: https://doi.org/10.1111/j.1744-7909.2006.00271.x.

Zheng, Y., Li, J., Wang, B., Han, J., Hao, Y., Wang, S., Ma, X., Yang, S., Ma, L., Yi, L. and Peng, W. (2020) 'Endogenous Type I CRISPR-Cas: From Foreign DNA Defense to Prokaryotic Engineering', *Frontiers in Bioengineering and Biotechnology*, *8*, 62. Available at: https://doi.org/10.3389/fbioe.2020.00062.

Zheng, H.Z., Liu, H.H., Chen, S.X., Lu, Z.X., Zhang, Z.L., Pang, D.W., Xie, Z.X. and Shen, P. (2005) 'Yeast transformation process studied by fluorescence labeling technique', *Bioconjugate Chemistry*, 16(2), pp. 250–254. Available at: https://doi.org/10.1021/bc049833v.

Zillig, W., Holz, I., Janekovic, D., Schäfer, W. and Reiter, W.D. (1983) 'The Archaebacterium *Thermococcus celer* Represents, a Novel Genus within the Thermophilic Branch of the Archaebacteria', *Systematic and Applied Microbiology*, 4(1), pp. 88-94. Available at: https://doi.org/10.1016/S0723-2020(83)80036-8.