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Towards a Genetic System for Ammonia Oxidizing Archaea

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Abstract

Ammonia oxidizing archaea (AOA) play an important role in the global geochemical nitrogen cycle. They are globally distributed in diverse environments and contribute to the loss of reactive nitrogen species in agricultural soils. However, little is known about the key metabolic enzymes responsible for their main energy metabolism, the conversion of ammonium to nitrite. Therefore, research on the establishment of a genetic system for these organisms is crucial to allow future studies on their gene and protein functions.

This project focused on *Candidatus* Nitrosocosmicus franklandianus C13, a soil species from the order of Nitrososphaerales. Several methods of DNA delivery into cells were tested, namely transformation via $CaCl_2$-heat shock, electroporation and a combination of both. Additionally, conjugation using *Escherichia coli* as donor species was attempted.

Neither transformation nor conjugation experiments yielded cultures growing due to acquired resistance to the inhibitor 6-MP by introduction of foreign plasmid DNA. However, spontaneous resistance occurred multiple times during this study. In contrast to prior research, a broader spectrum of mutations in *N. franklandianus* was revealed. They included single nucleotide polymorphisms (SNPs) and insertions leading to a frame shift and the distinct resistant mutations exhibited different phenotypes. This effect might be mitigated by the addition of AMP or GMP, as the lack could underlie their retarded growth.

Although *N. franklandianus* does not appear to be successfully transformed or conjugated, this study provides a basis on different strategies for future efforts towards the establishment of the first genetic system for AOA.

Zusammenfassung

Ammoniak oxidierende Archaeen (AOA) spielen eine wichtige Rolle im globalen geochemischen Stickstoffkreislauf. Sie sind weltweit in vielfältigen Lebensräumen verbreitet und tragen zum Verlust von reaktiven Stickstoffspezies in landwirtschaftlich genutzten Böden bei. Allerdings ist nur wenig über die Schlüsselenzyme bekannt, welche verantwortlich für ihren Hauptenergiestoffwechsel, die Umwandlung von Ammonium zu Nitrit, sind. Folglich ist Forschung zur Etablierung eines genetischen Systems für diese Organismen entscheidend, um künftige Studien zu ihren Gen- und Proteinfunktionen zu erlauben.

Diese Arbeit beschäftigte sich mit *Candidatus* Nitrosocosmicus franklandianus C13, einer Bodenspezies aus der Ordnung der Nitrososphaerales. Verschiedene Methoden zur Einführung von DNA in die Zellen wurden getestet, nämlich $CaCl_2$-Hitzeschock, Elektroporation und eine Kombination aus beidem. Zusätzlich wurde Konjugation unter Verwendung von *Escherichia coli* als Donorspezies durchgeführt.

Weder Transformations- noch Konjugationsexperimente führten zu Kulturen, die durch Aufnahme fremder Plasmid-DNA eine Resistenz gegen den Inhibitor 6-MP erworben haben. Allerdings trat im Verlauf dieser Studie mehrfach eine spontane Resistenz auf. Im Gegensatz zu vorangegangenen Untersuchungen zeigte sich ein breiteres Spektrum an Mutationen in *N. franklandianus*. Diese beinhalten Einzelnukleotid-Polymorphismen (SNPs) und Insertionen, welche zu einer Verschiebung des Leserahmens führen und die einzelnen Resistenzmutationen zeigten unterschiedliche Phänotypen. Dieser Effekt könnte durch die Zugabe von AMP oder GMP abgeschwächt werden, da dieser Mangel Grund für ihr vermindertes Wachstum sein könnte.

Obwohl *N. franklandianus* keine Anzeichen zeigte, erfolgreich transformiert oder konjugiert worden zu sein, lieferte diese Arbeit eine Basis für verschiedenen Strategien künftiger Bemühungen zur Herstellung des ersten genetischen Systems für AOA.

Contents

1 Introduction

1.1 Overview of archaeal discovery and diversity

1.1.1 Historical context and characteristics

Archaea were only distinguished from bacteria in 1977 by Carl Woese using ribosomal RNA analysis. The reason, why they were not determined to be distinct from bacteria in the past is because they share morphological similarities. As bacteria, they are single celled organisms and lack a nucleus, indicating that they together can be classified as prokaryotes. Originally, they were named Archaebacteria, but were subsequently renamed to Archaea. This name derives from the Greek word 'archaios' which means 'primitive' or 'ancient'. This revelation ultimately led to the model of three domains of life (Woese et al., 1990).

As archaea were only identified relatively recently in the history of biology, it is not surprising, that not many model organisms are available in this domain of life. There are several aspects that make archaea different from bacteria and eukaryotes. First, they contain different lipids in their membranes, which distinguishes them from eukaryotes and bacteria, because they contain ether-linked isoprenoids as side chains (Mayberry-Carson et al., 1974; Langworthy et al., 1974). Further, their cell walls do not contain murein, as it could be found in bacterial cells (Kandler, 1979). Astonishingly, there are strong similarities to eukaryotes when it comes to information transfer processes, such as replication, transcription and translation. These findings provide strong evidence, that archaea possess a mosaic of both bacterial and eukaryotic characteristics and therefore represent the third domain of life (Rivera et al., 1998; Bell & Jackson, 1998). Interestingly, the current knowledge supports the theory, that eukaryotes evolved from an archaeal ancestor (Spang et al., 2015, Zaremba-Niedzwiedzka et al., 2017).

Most of the earliest identified archaea were extremophiles, thriving in ecological niches that were inhospitable to most of the organisms living on earth. The first recognized archaea were either strictly anaerobic methanogens (Fox et al., 1977), thermoacidophiles (Darland et al., 1970; Brock et al., 1972; Fox et al., 1980) or halophiles (Magrum et al., 1978). These findings led to the realization that almost all of earths habitats are populated

by some kind of organism and that there is a huge 'microbial dark matter', of which we do not know much (Whitman et al., 1998; Rappé & Giovannoni, 2003; Marcy et al., 2007).

1.1.2 Environments with Archaea

Since then, archaea were found to thrive in many diverse environments, including moderate and extreme habitats. Examples for moderate environments can be agricultural soils, freshwater and marine waters and sediments (H. Wang et al., 2020). Extreme environments include hydrothermal vents (Zierenberg et al., 2000), hot springs (Kvist et al., 2007, Abby et al., 2018), salt lakes (Sorokin et al., 2015), and acidic mine drainages (Méndez-García et al., 2015). In addition, they are found in biogas reactors (Nettmann et al., 2010) and sewage treatment plants (Park et al., 2006). Further, they are part of the human microbiome (Eckburg et al., 2003). Most astonishingly, they are able to survive in global spacecraft clean rooms and could therefore be a source of contamination in search of extraterrestrial life (Moissl-Eichinger, 2011).

1.2 Ammonia oxidation in archaea and bacteria

Ammonia oxidation, the oxidation of ammonia to nitrite, is a crucial process in the global nitrogen cycle and often referred to as the first and rate limiting step (Lehtovirta-Morley, Sayavedra-Soto, et al., 2016). Nitrite is then further oxidized to nitrate in the global geochemical nitrogen cycle. The oxidation from ammonia to nitrite and further nitrate is called nitrification. Ammonia oxidation can be performed by bacteria and archaea, whereas the pathway from nitrite to nitrate is so far only known to be carried out by bacteria (Könneke et al., 2005). Some bacteria, known as complete ammonia oxidizers (Commamox) are able to carry out the full nitrification process from ammonia to nitrate (Daims et al., 2015, van Kessel et al., 2015). Although it remained unknown for a long time, archaea are now acknowledged to play a key role in diverse biogeochemical cycles (Offre et al., 2013).

Ammonia is first converted into hydroxylamine via the ammonia monooxygenase (AMO) using oxygen as reductant. Afterwards, ammonia oxidizing bacteria use the hydroxylamine oxidoreductase (HAO), that takes the hydroxylamine and converts it to nitric oxide which is further processed to nitrite via unknown enzymes (Lehtovirta-Morley, 2018). In AOA, this step is not unravelled yet and is carried out by an unknown enzyme. By

now, it is not even known, if it is a three-step pathway via nitric oxide as in bacteria or a two-step pathway (Figure 1). The two-step pathway was proposed in 2016 (Kozlowski et al., 2016), whereas the three-step model was proposed in 2018 (Lehtovirta-Morley, 2018).

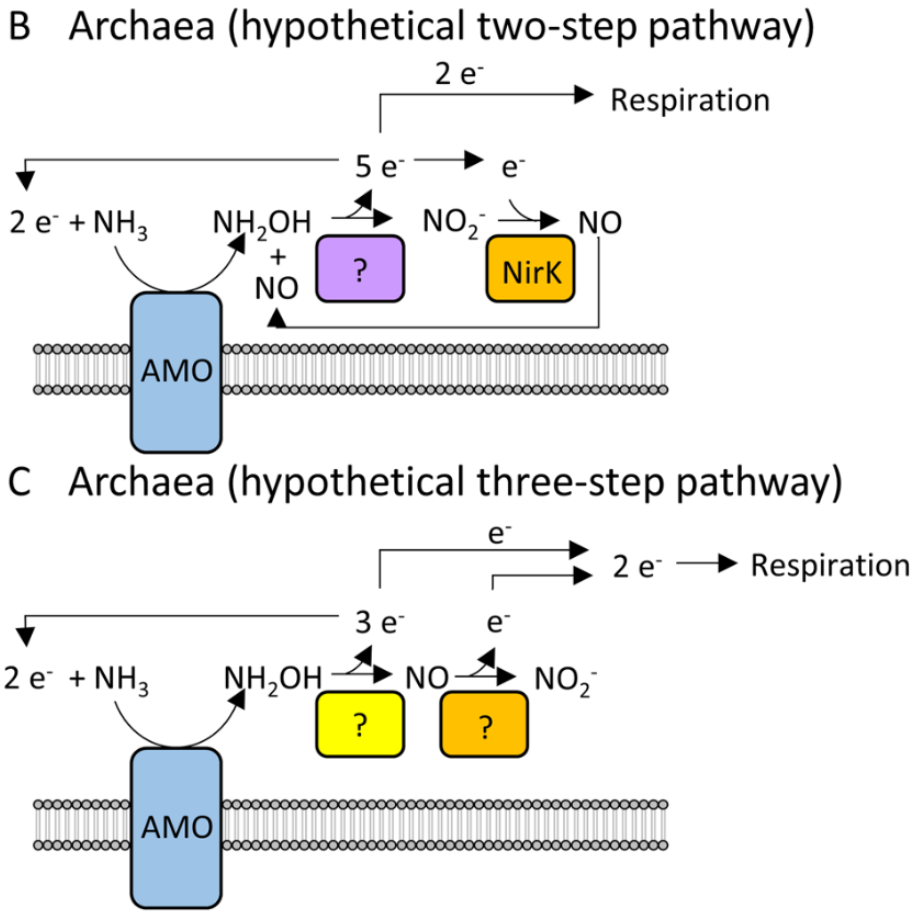


Figure 1 **Ammonia oxidation in archaea** Shown are the hypothetical two-step or three-step pathways of ammonia oxidation in AOA. Adapted from Lehtovirta-Morley, 2018.

1.3 Significance of AOA in nitrification

AOA belong to the order of Nitrososphaerales (Rinke et al., 2021) and are able to thrive in many different environments, ranging from moderate to extreme conditions. They are able to manage complex environmental parameters like high or low temperature (de la Torre et al., 2008; Yin et al., 2018), extreme pressure (J. Wang et al., 2017), low ammonia (Wang et al., 2017; Li et al., 2024) and low oxygen (J. Wang et al., 2017). The environments, in which AOA can be found in, include soil (Leininger et al., 2006; Tourna et al., 2011), oceans (Karner et al., 2001), waste water treatments plants (Park et al., 2006) and hot springs (Reigstad et al., 2008; Abby et al., 2018).

Until the discovery of AOA, it was believed, that ammonia oxidation is solely performed by bacteria, so called ammonia-oxidizing bacteria (AOB). It has become clear that AOA dominate over AOB in soil (Qiang et al., 2024). However, studies on both revealed that their distribution corresponds to distinct ecological niches (Lehtovirta-Morley, Ross, et al., 2016). The oxidation of ammonia to nitrite by AOA is of high importance for agriculture, because AOA can cause nitrogen loss from soils and therefore loss of soil fertility and degradation of agricultural lands (Zhalnina et al., 2012).

1.4 Physiological and genomic features of *Nitrosocosmicus franklandianus*, a representative of the Nitrosocosmicus clade

Candidatus Nitrosocosmicus franklandianus C13 was isolated from arable soil and belongs to the genus Nitrosocosmicus clade in the order of Nitrososphaerales. It has a generation time of 20-24 hours and a size of approximately 1 µm. In laboratory cultures it tends to form bigger aggregates of several cells (Lehtovirta-Morley, Ross, et al., 2016).

Its 2.84 Mb large genome encodes metabolic pathways for energy generation via ammonia oxidation and carbon dioxide fixation using the 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) pathway. It does not encode for surface layer (S-layer) proteins (Nicol et al., 2019), as it can be seen in other AOA like *Nitrososphaera viennensis* (Stieglmeier et al., 2014). *N. franklandianus* produces extracellular polymeric substances (EPS) which are hypothesized to enhance biofilm formation and give protection to external influences (Dreer et al., 2024).

1.5 Prerequisites for establishing a genetic system in AOA

To date, no genetic system for any representative of the group of AOA is available. Establishing a genetic system will be important to obtain information about essential genes and their functionality. One of the most important concerns will be the elucidation of the missing steps of the archaeal pathway of ammonia oxidation. Until now, only the first step, which is the oxidation of ammonia to hydroxylamine by the AMO is known (Lehtovirta-Morley, 2018).

Establishing a genetic system calls for these four tools: 1) A method to deliver DNA into cells, 2) a selective agent 3) a shuttle vector and optionally 4) the ability to grow and obtain monoclonal colonies on plates.

There are several options to deliver DNA into cells. Plasmids can enter cells via natural or artificial transformation. Natural transformation is possible and actively occurs (Wagner et al., 2017). The latter can be achieved by several methods, for example via $CaCl_2$-heat shock in *Methanococcus voltae, Pyrococcus furiosus* and *Sulfolobus acidocaldarius* or electroporation in *Methanosarcina acetivorans*, *M. voltae* and *Sulfolobus solfataricus* (Allers & Mevarech, 2005). DNA can also be incorporated using conjugation, via direct cell-cell contacts from another organism. Conjugation was already successfully used twice between archaea and bacteria, using *E. coli* as donor and *Methanococcus maripaludis* (Dodsworth et al., 2010) or *Methanothermobacter thermautotrophicus ΔH* (Fink et al., 2021) as recipient. Moreover, DNA can be brought into cells via transduction, which would require viruses that are able to infect AOA cells. There are indeed viruses, that are known to infect archaea (Wirth & Young, 2020). A successful transfection for the establishment of a genetic system in archaea was already done (Schleper et al., 1992).

Secondly, a selective agent is needed. For bacterial applications, a wide array of antibiotics is available. These antibiotics are mostly not applicable to archaea, because they target bacteria specific features, which are fundamentally different in transcription and translation processes, cellular structures and the often extreme growth conditions archaea are thriving in. Consequently, specific targets and thermostability of possible inhibiting compounds have to be considered. For example, archaea are resistant to antibiotics targeting peptidoglycan synthesis, because of their cell wall composition. In addition, antibiotics, targeting the 30S and 50S ribosomal subunits are often not useful in archaea (Khelaifia & Drancourt, 2012).

Therefore, other compounds targeting archaeal structures had to be identified. One of those compounds is simvastatin, which targets the HMG-CoA reductase. This is a key enzyme in the membrane lipid biosynthesis of Archaea, which is essential for survival of the cells. The inhibition of archaeal growth by simvastatin could be shown in *Sulfolobus islandicus* (Zheng et al., 2012) and *Methanobrevibacter smithii*. Unfortunately, simvastatin targets a complex mechanism in archaeal cells. The inhibition not only

depends on several aspects like the ability to cross the cell wall and enzymatic activation. A significant disadvantage of simvastatin is, that it targets the membrane lipid biosynthesis which severely interferes with membrane formation and can lead to growth defects or asymmetric cell division (Gottlieb et al., 2016).

In contrast, compounds like 6-Methylpurine (6-MP) seem to be a simpler choice for this cause. It works more straightforward than statins because it is a toxic adenine analogue that gets incorporated into DNA. Active phosphoribosyltransferases (*apt*) catalyse a purine salvage reaction, where toxic purine analogues get incorporated into molecules like adenosine monophosphate (AMP). This salvage reaction happens, because the synthesis of molecules via this pathway is less costly than de novo synthesis. Normal AMP, for instance, is essential for the synthesis of ADP or ATP (Zhang et al., 2016).

One drawback, that 6-MP inherits, is, that resistance mutations occur. As mentioned before, the *apt* gene is essential for toxic analogues to get incorporated. Mutations in the *apt* gene, rendering it dysfunctional, therefore lead to resistance against 6-MP. On the other hand, the resistance does not occur instantaneously, rather, it takes some time to arise, depending on the cell count and the concentration of the selective agent.

6-MP as a selective agent was already used for other archaea. First, it was employed in the hyperthermophilic euryarchaeote *Thermococcus kodakarensis* to create a protocol, where nonessential genes can be deleted from the genome (Santangelo et al., 2010). Further, it was applied to *Thermococcus barophilus*. *T. barophilus* is less sensitive to 6-MP, which is most likely due to a site difference in the gene encoding a hypoxanthine/guanine phosphoribosyltransferase, that is targeted by 6-MP. However, it was shown, that the deletion of the gene encoding for the xanthine-guanine phosphoribosyltransferase in *T. barophilus* is responsible for the resistance to 6-MP (Birien et al., 2018). A counterselection system based on the inactivation of the *apt* gene was created in *S. islandicus*. Due to the fact, that *apt* is conserved in crenarchaea, it was assumed, that this system might be applicable to other archaeal species too (Zhang et al., 2016).

What can be concluded from previous studies, is that 6-MP was mainly used as selective agent in the studies of thermophilic archaea. On the other hand, it greatly aided in the understanding and development of genetic tools for these species based on either the

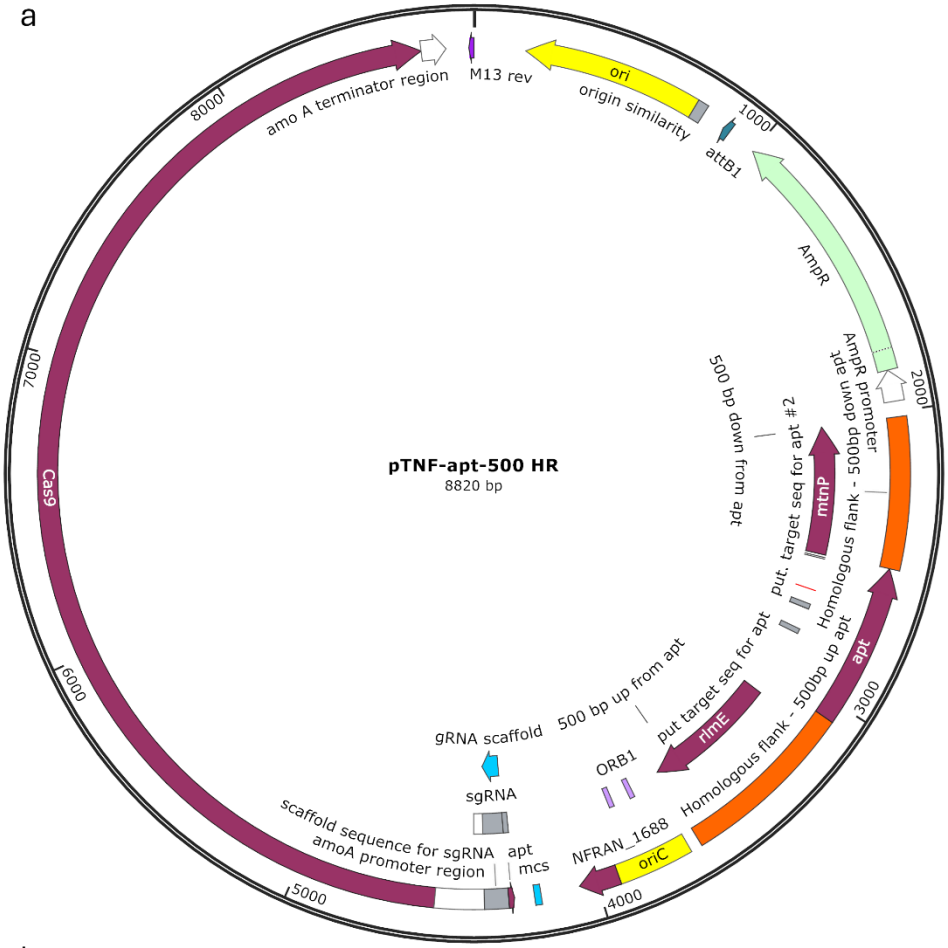
hypoxanthine phosphoribosyltransferase (*hpt*) or *apt* as selection marker. Consequently, 6-MP appeared to be a promising selective agent for investigating *N. franklandianus* and for the establishment of a genetic system for AOA in the context of this thesis, as *N. franklandianus* also encodes an *apt* gene and was shown to be inhibited by 6-MP for a period of up to three weeks (Schleper lab, unpublished data).

The third requirement for a genetic system is a shuttle vector. For the experiments during this thesis, four different transformation plasmids (Figure 2) and one conjugation plasmid were used (Figure 5). They were engineered to confer resistance to 6-MP upon incorporation of a mutated *apt* gene via homologous recombination. In addition, the *apt* gene included synonymous changes to distinguish transformants from spontaneous resistance mutations. The transformation and conjugation plasmids consisted of three and four main parts, respectively. They are composed of an *E. coli* backbone including an ori and an ampicillin resistance, homologous recombination templates including the mutated *apt* gene with either a SNP or a deletion and its flanking regions and a Cas9 with its sgRNA. The Cas9/sgRNA system was already used in methanogens and showed a vast increase in efficiency of transformation (Nayak & Metcalf, 2017). In the conjugation plasmid, the *traJ* gene, essential for the process of conjugation, is present additionally.

For most organisms, the last requirement for the generation of a genetic system is the ability to grow and obtain monoclonal colonies on plates. On the other hand, for some species, the growth on plates to gain single colonies is simply not possible or comes with difficulties. *N. franklandianus* can be grown to single colonies in a combination of liquid and solid medium, containing Phytagel™, for a short time now (Klein et al., 2022). Unfortunately, this approach is tedious and time-consuming, which is why it was impractical and not applicable to routine use for this thesis. Therefore, all except for one experiment were done in liquid culture.

To date, few genetic systems have been developed for archaea. Moreover, all of them were created only in extremophiles like *Sulfolobales*, *Thermococcales*, halophiles and methanogens. Currently, there is no genetic system available for archaea derived from aerobic moderate environments, which makes it even more difficult to find suitable methods for this task (Leigh et al., 2011).

a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Homologous flank - 500bp up apt
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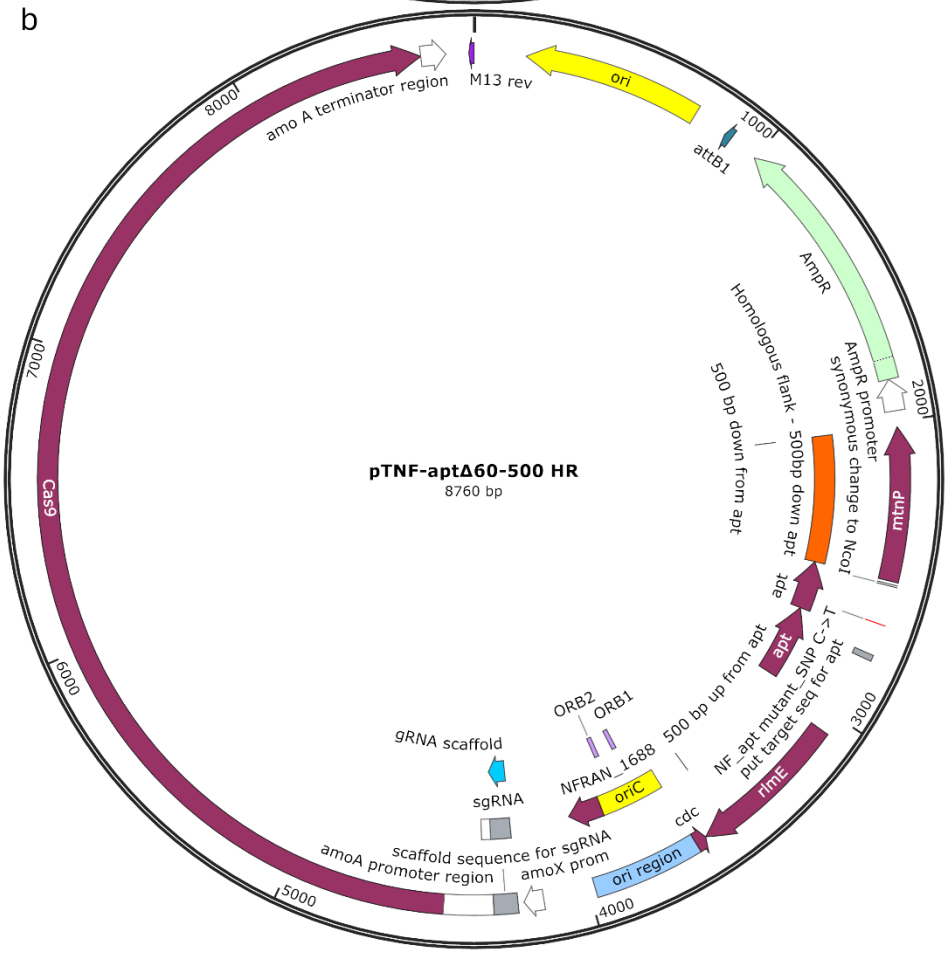
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apt
NF_apt mutant_SNP C->T
put target seq for apt
rlmE
500 bp up from apt
ORB2
ORB1
gRNA scaffold
sgRNA
NFRAN_1688
oriC
cdc
ori region
scaffold sequence for sgRNA
amoA promoter region
amoX prom
Cas9
1000
2000
3000
4000
5000
6000
7000
8000

Figure 2 **Map of transformation plasmids pTNF-apt-500HR and pTNF-aptΔ60-500 HR (Provided by Maximilian Dreer)** Both plasmids consist of the following three main parts: *E. coli* backbone, the *apt* gene with recombination flanks and the Cas9 with its sgRNA. (a) pTNF-apt-500HR occurs to only hold a SNP from C to T in its *apt* gene. (b) pTNF-aptΔ60-500 HR comprises the same SNP and a 60 bp deletion in the *apt* gene. The plasmid maps were created with SnapGene.

1.6 Goal of thesis

The goal of this thesis was to try to create a genetic system for the archaeal species *Nitrosocosmicus franklandianus*. For this purpose, cloning vectors were provided that contained the mutated *apt* gene obtained from earlier screenings with 6-MP. The introduction of genetic material into the cells should be attempted by several methods, namely transformation via $CaCl_2$-heat shock, electroporation or conjugation using an *E. coli* strain as donor. After transformation and conjugation, respectively, the cultures should be investigated by DNA extraction and subsequent sequencing.

2. Results

2.1 Heat shock conditions

Before transformation experiments were attempted, different heat shock temperatures were tested. From *E. coli*, it is known, that heat shock is ideally done at 42°C (Arsène et al., 2000), which is 5°C above its optimal growth temperature of 37°C. The first chosen heat shock temperature for *N. franklandianus* was 52°C, which is already 10°C above its optimal growth temperature. However, it could be shown that temperatures up to 62°C did not show an effect on growth of *N. franklandianus* (Appendix figure A). Only heat shock temperatures starting from 64°C showed an effect (Figure 3). Growth of AOA is followed by nitrite measurements in µM, as the cells do not grow dense enough to be measured by OD_{600}, as it is usually done for other microorganisms.

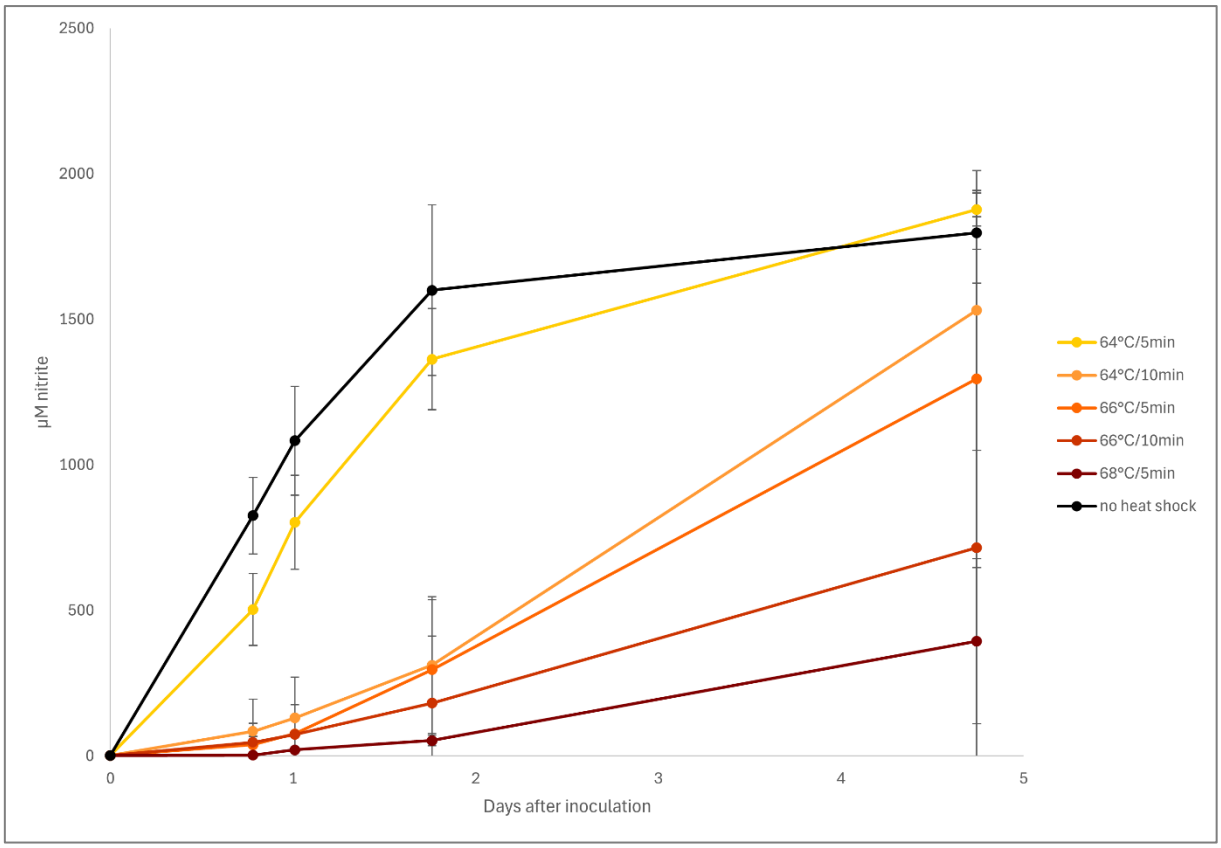


Figure 3 **Effect of different heat shock temperatures on growth of *N. franklandianus*** Nitrite production of cultures with heat shock temperatures ranging from 64°C-68°C are shown. Before inoculation, the cells were concentrated and divided into ~ $2x10^6$ cells per sample. There was no plasmid added to the cultures. All cultures were inoculated in growth medium after the heat shock.

2.2 Transformation experiments with heat shock or electroporation and a combination of both

As a starting point for transformation experiments, different transformation parameters and settings were examined. All experiments shown were done with four different transformation plasmids with either 500 bp or 1000 bp long homologous recombination flanks and the *apt* gene with either only a SNP (*apt*130 Glycine → Aspartic Acid) or the same SNP together with a 60 bp deletion (*apt*192-252). Additionally, plasmid free negative and positive controls were done. The selective agent 6-MP was added to all samples except the positive controls. The experiments were always performed in triplicates. A detailed summary of all conditions is shown in Appendix table A.

The transformation efficiency is among other parameters positively correlated with the number of cells present (Dower et al., 1988). Therefore, high cell numbers of at least $1x10^9$

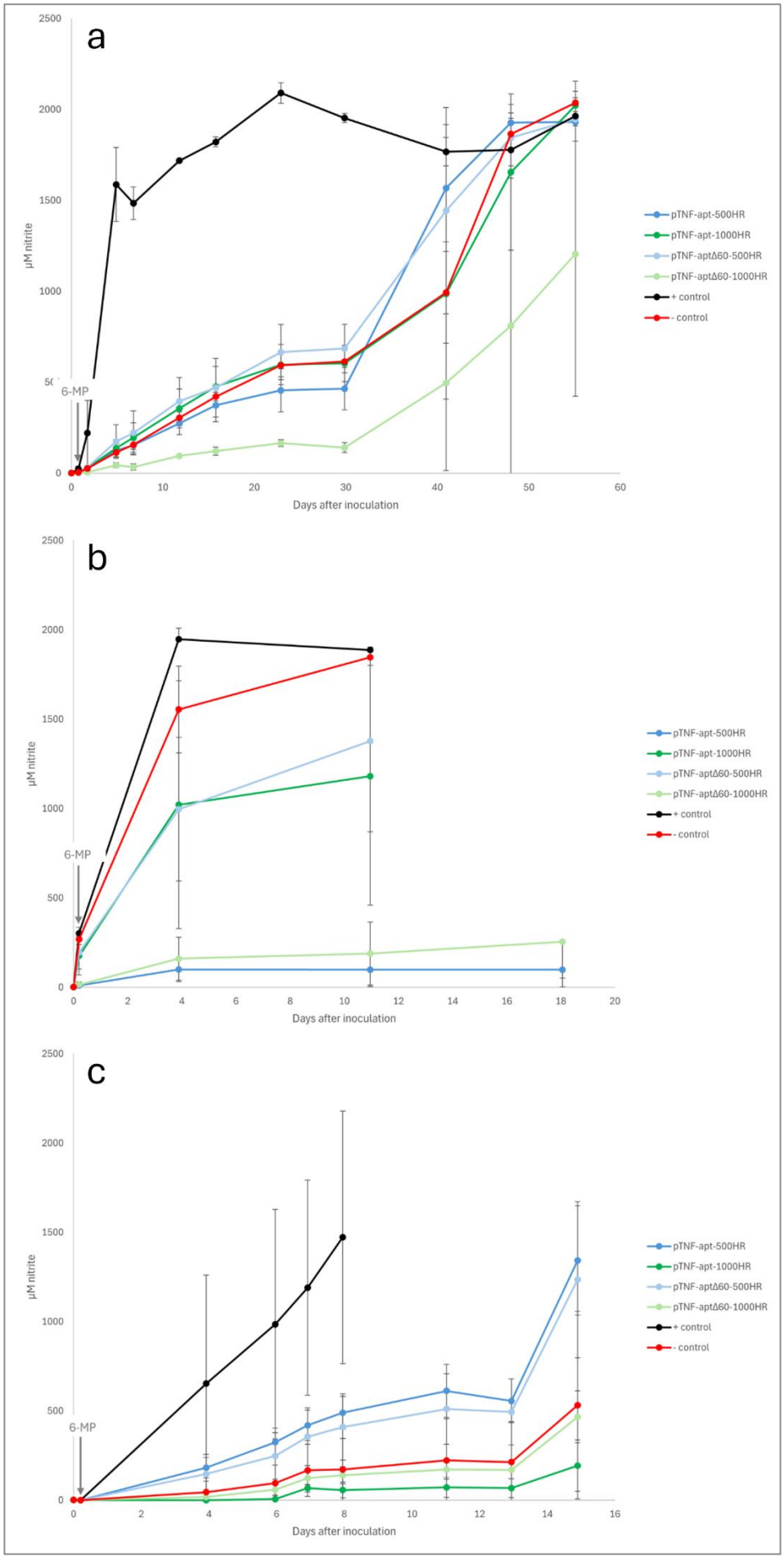
cells per replicate are typically used (Metcalf et al., 1997). *N. franklandianus*, like other AOA strains, only grows to cell concentrations of ~1x10^7 cells/ml (Tourna et al., 2011) and thus had to be upscaled and subsequently concentrated ~100x times prior to transformation experiments. The effects of these high cell numbers on the effectiveness of 6-MP as selective agent and transformation parameters like heat-shock and electroporation were tested prior to experiments (Appendix figure B).

The first thoroughly tested method was transformation via $CaCl_2$-heat shock. The heat shock was performed at 66°C for five minutes and 6-MP was added after 18 hours of regeneration. The positive controls grew very rapidly after inoculation, while cells inhibited by 6-MP displayed retarded growth until approximately 30 hours, followed by faster growth. There was no difference between negative controls and cultures to which plasmid DNA was added (Figure 4a).

Secondly, transformation via electroporation was carried out. The electroporator was set to 2000V, 25mF and 1000Ω. For the samples of pTNF-apt-500HR and one sample of pTNF-aptΔ60-500HR, 2000V was too high, because the electroporator always detected an arc, so it was changed to 1500V. As output, the time values were between 6.0 – 15.6 ms for the transformation samples and 20.5 – 24.1 ms for the positive and negative controls. In this case, regeneration lasted five hours. The positive and negative controls grew very fast. Both positive and negative controls grew faster than samples containing transformation plasmids (Figure 4b).

Lastly, the combination of electroporation and heat shock was tested as delivery method for the transformation experiments, as this was shown to increase transformation efficiency by four orders of magnitude (van der Rest et al., 1999). Electroporation was done at 1500V, 25mF and 1000Ω. The time values for the transformation samples were 7.8 ms – 15.6 ms and for the positive and negative controls 19.6 ms – 22.8 ms. Heat shock was performed afterwards at 64°C for ten minutes. Regeneration was done for five hours. Contrasting to the transformation settings before, two sample settings, containing the pTNF-apt-500HR and pTNF-aptΔ60-500HR plasmids, started to grow earlier than the negative controls. The other two sample settings, containing the remaining plasmids, grew later than the negative controls (Figure 4c).

The samples, that started growing earlier than the negative controls, were sent for sequencing. The sequencing data showed none of the changes derived from the designed plasmids, but several other changes (Figure 9, Appendix table C and Appendix figure C).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µM nitrite
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Days after inoculation
pTNF-apt-500HR
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pTNF-aptΔ60-500HR
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Days after inoculation
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pTNF-aptΔ60-500HR
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Days after inoculation
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pTNF-apt-1000HR
pTNF-aptΔ60-500HR
pTNF-aptΔ60-1000HR
+ control
- control

Figure 4 **Transformation via heat shock, electroporation, and a combination of both** Nitrite production of *N. franklandianus* transformation cultures. (a) Transformation via heat shock at 66°C for 5 minutes and 18 hours regeneration. (b) Transformation via electroporation at 1500/2000V, 25mF and 1000Ω and 5 hours regeneration. (c) Transformation via a combination of electroporation at 1500V, 25mF and 1000Ω and heat shock at 64°C for ten minutes and five hours regeneration. The grey arrow indicates the addition of 6-MP and therefore the end of regeneration. The transformation cultures contained four different plasmids and were grown in selective medium. The negative and positive controls did not contain a plasmid. The negative controls were grown in selective medium, too.

2.3 Conjugation proof-of-concept with *E. coli*

The conjugation plasmid was constructed using the provided pTNF-aptΔ60-500 HR (Figure 2b). Therefore, the new pCNF-aptΔ60-500 HR contains all the changes to the wildtype, that confer resistance to 6-MP too (Figure 5). To assess the theoretical feasibility of successful conjugation with the newly constructed plasmid, a test run between two *E. coli* strains was conducted. To reach this goal, a workflow for the conjugation was established (Figure 6a). The recipient strain (“NEB stable”) exhibits resistance to streptomycin, and upon successful conjugation, the plasmid is expected to confer additional resistance to ampicillin. Successful conjugation could be proven by growth on selective medium plates (Figure 6b) and subsequent colony PCR (Figure 6c).

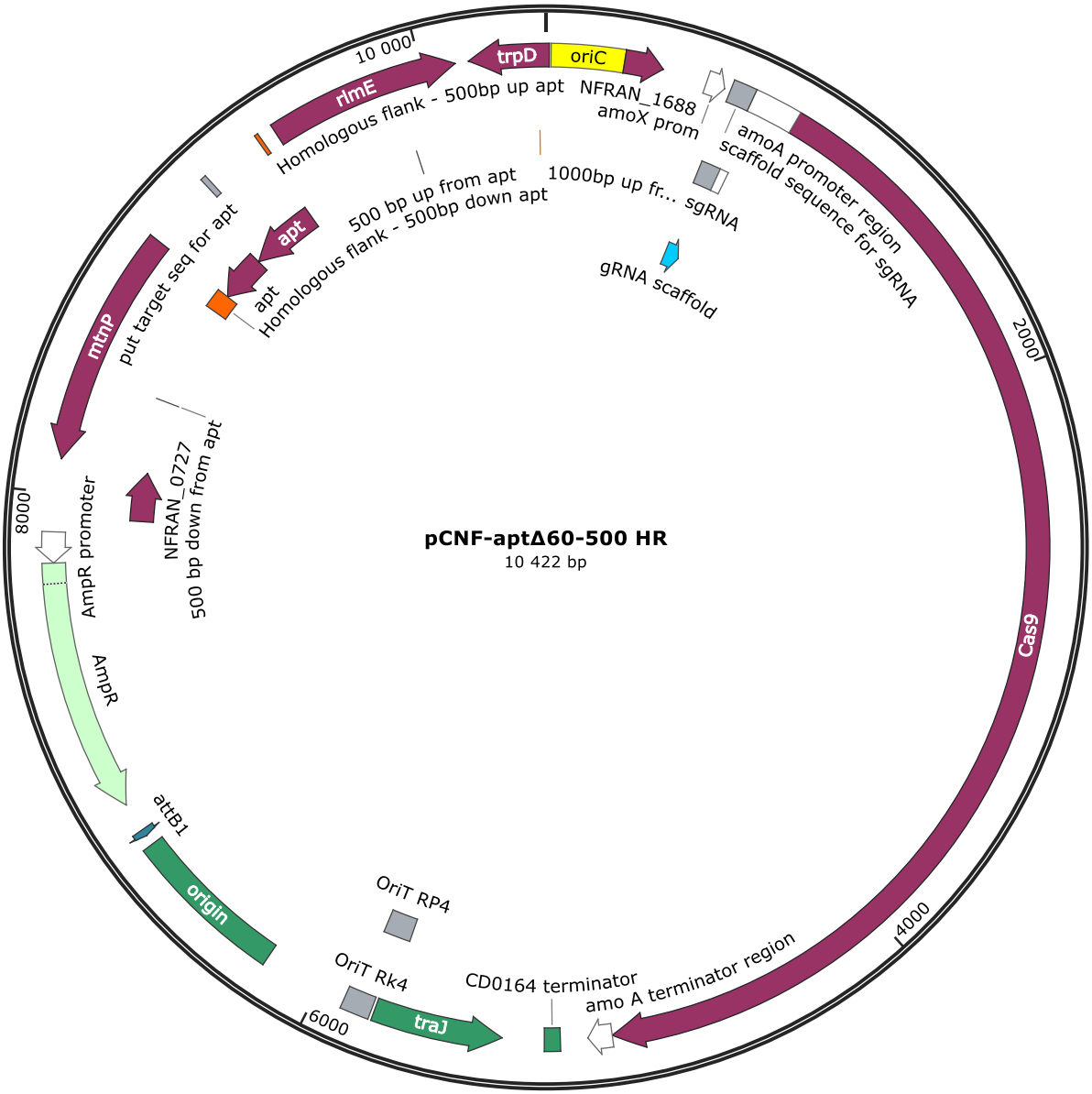


Figure 5 **Map of conjugation plasmid pCNF-aptΔ60-500 HR** The plasmid pCNF-aptΔ60-500 HR was constructed from plasmid pTNF-aptΔ60-500 HR and an *E. coli* backbone containing an ori and the *traJ* gene needed for conjugation. The plasmid map was created with SnapGene.

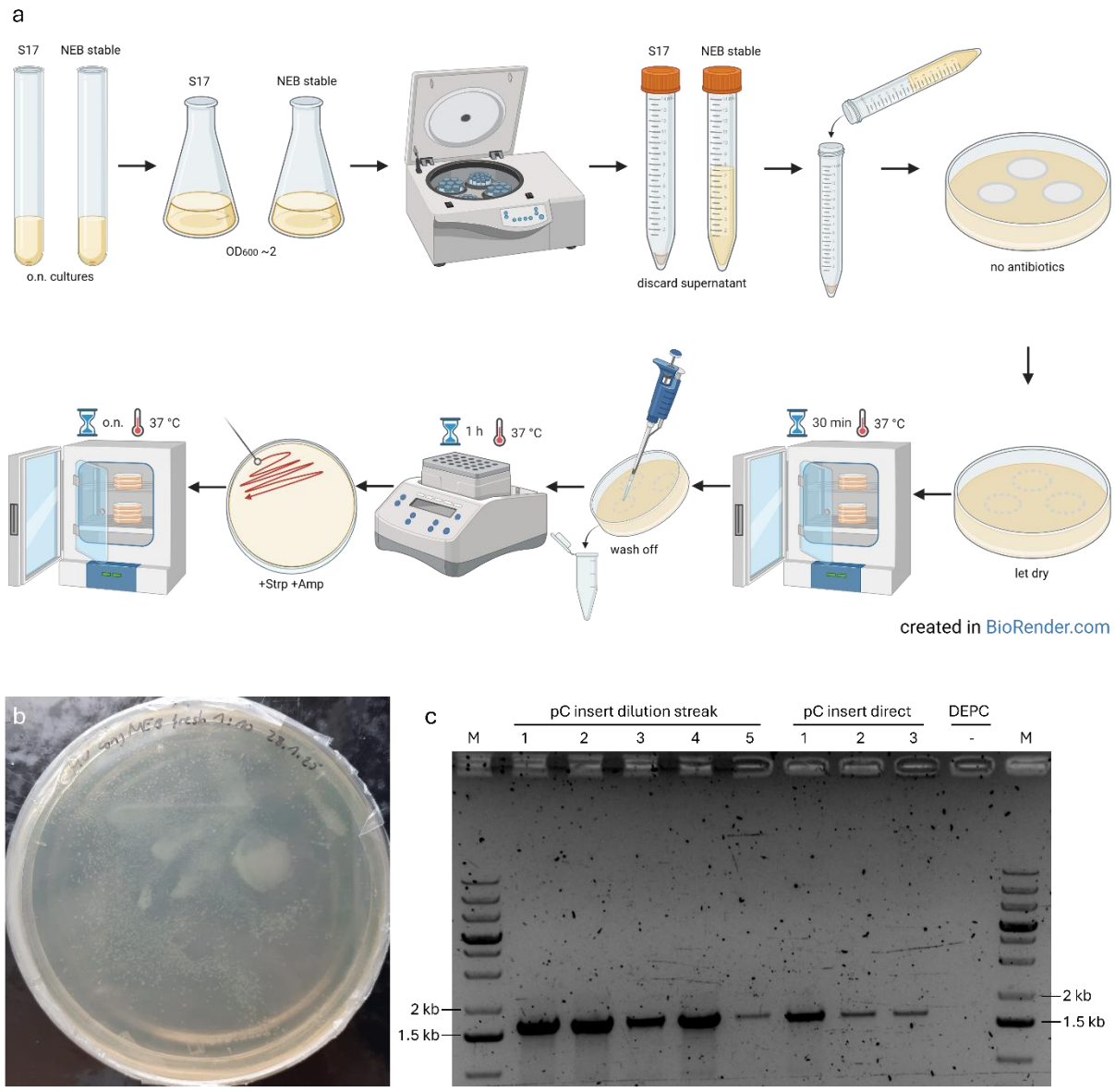


Figure 6 **Conjugation proof-of-concept from *E. coli* S17 to *E. coli* NEB stable** (a) Workflow of proof-of-concept *E. coli* conjugation. Workflow was created using BioRender.com (b) Colonies on selective medium plate after conjugation. (c) 1% agarose gel of colony PCR of several colonies either from a dilution streak or from the original growth plate after conjugation directly. The PCR was conducted using primers specific for the insert containing the *traJ* gene, needed for conjugation (Appendix table B). The expected PCR product was 1644 bp long. The negative control was done with DEPC instead of a colony, therefore, no band was expected here. As indicated above the lane numbers, the colonies functioning as templates for the PCR were either taken from the dilution streak plate or directly from the overnight plate after conjugation. Abbreviations: -, negative control; M, Marker. The used marker was GeneRuler™ 1 kb Plus DNA Ladder (Thermo Fisher).

2.4 Conjugation into *N. franklandianus*

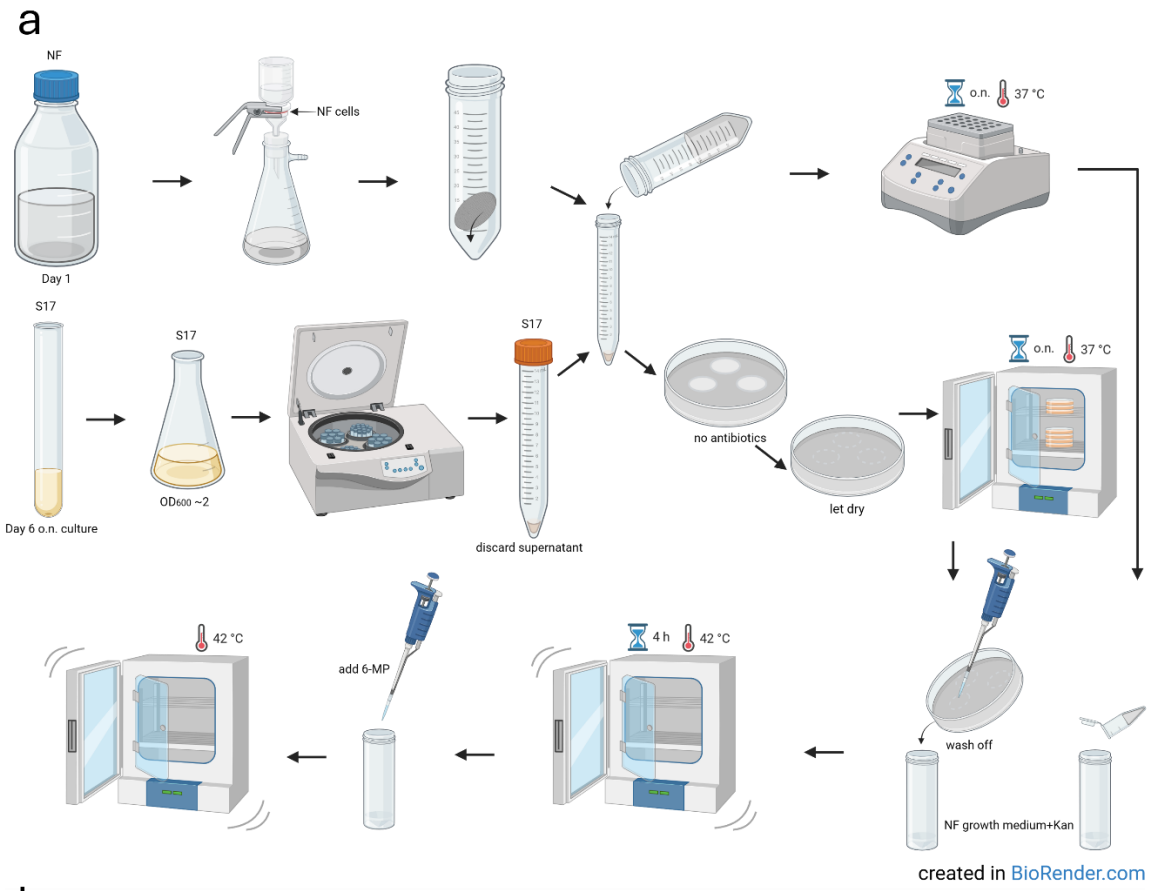
The functional conjugation plasmid was used for conjugation experiments between *E. coli* and *N. franklandianus*. All experiments were done with conjugation plasmid pCNF-aptΔ60-500 HR. Conjugation samples, positive and negative controls were carried out at recipient:donor ratios of 1:5 and 1:10. Over the course of the project, conjugation in liquid and on plates and different regeneration durations were attempted. Furthermore, plasmid free positive controls were done and 6-MP was added to all the samples except for the positive controls. Negative controls were only included in the experiment that followed a cultivation on solid medium using the overlay technique. Conjugation experiments were always performed in triplicates. A summary of all used conditions can be found in Appendix table A.

At first, conjugation in liquid medium with a regeneration of four hours was attempted. The positive controls grew rapidly after inoculation, while samples inhibited by 6-MP showed growth until a nitrite value of approximately 800 µM. Afterwards, there seemed to be growth retardation. Growth reoccurred in some samples only after one month.

The second conjugation attempt was carried out via conjugation on solid gelrite/conjugation medium plates. The duration of regeneration remained unchanged to the first experiment. Again, positive controls exhibited fast growth after inoculation and 6-MP inhibited cells resumed growth after one month.

Lastly, conjugation was carried out on solid gelrite/conjugation medium plates without regeneration and subsequent cultivation on solid medium using the overlay technique (Klein et al., 2022), further described in the Materials and Methods section of this work. Contrasting to the previous conjugation experiments, negative controls, which instead of a conjugation plasmid, harboured the transformation plasmid pTNF-apt-500HR, were included. The positive controls grew rapidly. The conjugation samples and negative controls stayed inhibited by 6-MP (Figure 7b).

The samples, that started growing after a month, were analysed by sequencing. Sequencing data showed none of the changes from the designed plasmids, but several other changes (Figure 9, Appendix table C and Appendix figure C).



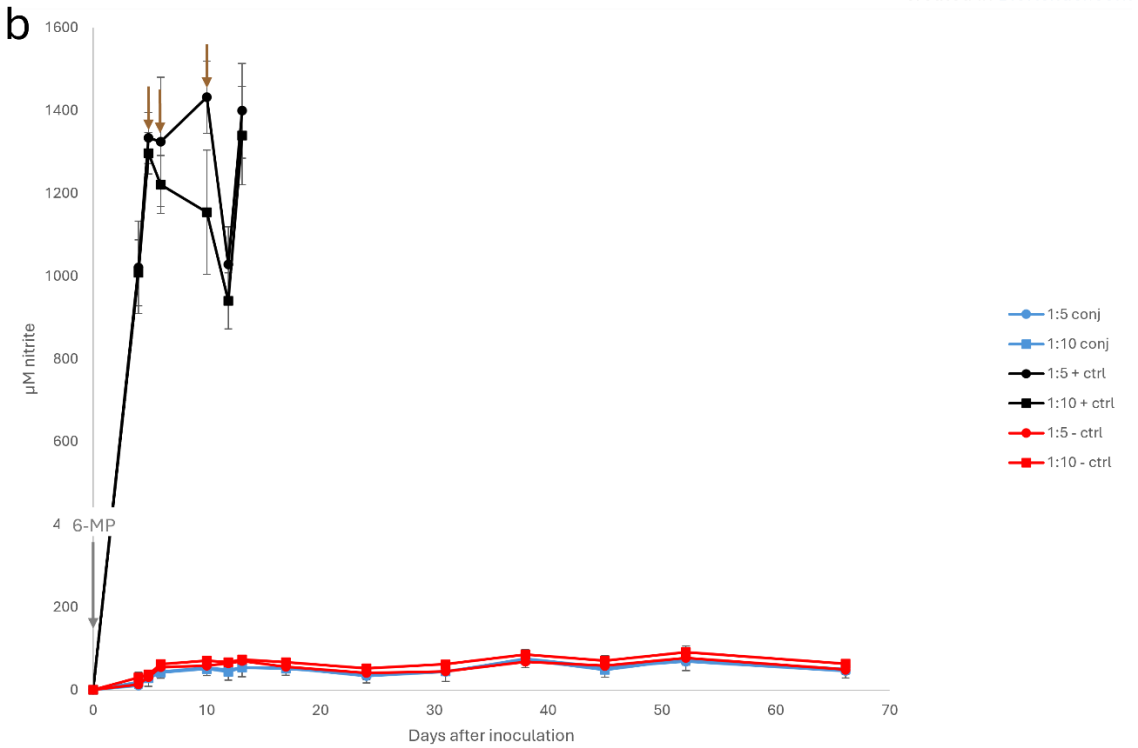


Figure 7 **Conjugation on solid plates and subsequent cultivation on solid medium** (a) Workflow of conjugation between *E. coli* and *N. franklandianus*. Workflow was created using BioRender.com (b) Nitrite production of *N. franklandianus* conjugation samples. Conjugation was performed on solid

gelrite/conjugation medium plates without regeneration. The grey arrow indicates the addition of 6-MP, the brown arrows indicate the medium exchange of the positive controls. The conjugation samples contained the pCNF-apt-500HR plasmid and were grown in selective medium. The positive controls did not contain a plasmid. The negative controls contained a transformation plasmid, not capable of performing conjugation and were grown in selective medium.

2.5 6-MP phenotypes

Over the course of this master project, several mutated strains of *N. franklandianus* with an acquired 6-MP resistance were obtained. They were not successfully transformed or conjugated using the constructed plasmids, but were able to grow in selective medium, even after several passages into fresh selective medium. To remove traces of wildtype DNA remnants in the sample, mutated strains were transferred multiple times using 1% (v/v) inoculation volumes before being sequenced. Sample A was transferred into new selective medium four times, sample G was transferred five times, samples F and H were transferred six times, samples B, C, I, J and K were transferred seven times and samples D and E were transferred eight times.

Acquired resistance cultures B, D, E, H, I, J and K grew rapidly after each new inoculation. The cultures A, F and G displayed retarded growth (Figure 8a). All samples were sent for sequencing with the HR check 11.9 FW and RV primers (Appendix table B). The sequencing data showed two major different mutations in the samples. B, D, E H, I, J and K seemed to have acquired a SNP in the *apt* gene (*apt*107 Glutamic acid → Glycine). The other three samples, A, F and G seem to have obtained a frame shift mutation in an adenine rich region in the *apt* gene (Figure 8b). The sequencing data display a correlation of the mutation to the according growth curve, as the seven samples with a SNP show rapid growth and the three samples with the frame shift mutation show slower growth after inoculation (Figure 8a).

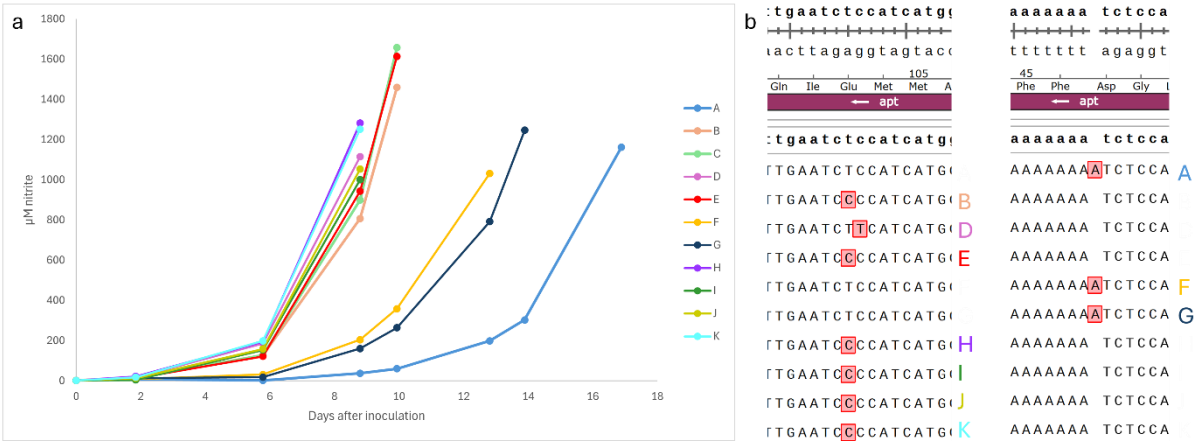


Figure 8 **6-MP resistant *N. franklandianus* samples** (a) Nitrite production of 11 *N. franklandianus* acquired resistance cultures. Cultures were grown in selective medium until growth occurred. These cultures were then transferred several times into new selective medium and grown under usual conditions. Most cultures grew rapidly and some of them took more time to reach the same nitrite levels. (b) The sequencing data from these samples shows two major different mutations. The cultures B, D, E, H, I, J and K seem to have achieved a SNP in the *apt* gene, leading to resistance. Samples A, F and G seem to have acquired a frame shift mutation in an adenine stretch of the *apt* gene.

Additionally, some other *N. franklandianus* cultures from transformation and conjugation experiments, that grew earlier than the negative controls were isolated and transferred once or twice. DNA extraction and sequencing was performed upon them to analyse if resistance was acquired by transformation or conjugation. Several different mutations and two distinct types of mutations, namely SNPs and single base pair insertions could be recognized (Figure 9 and Appendix table C and Appendix figure C).

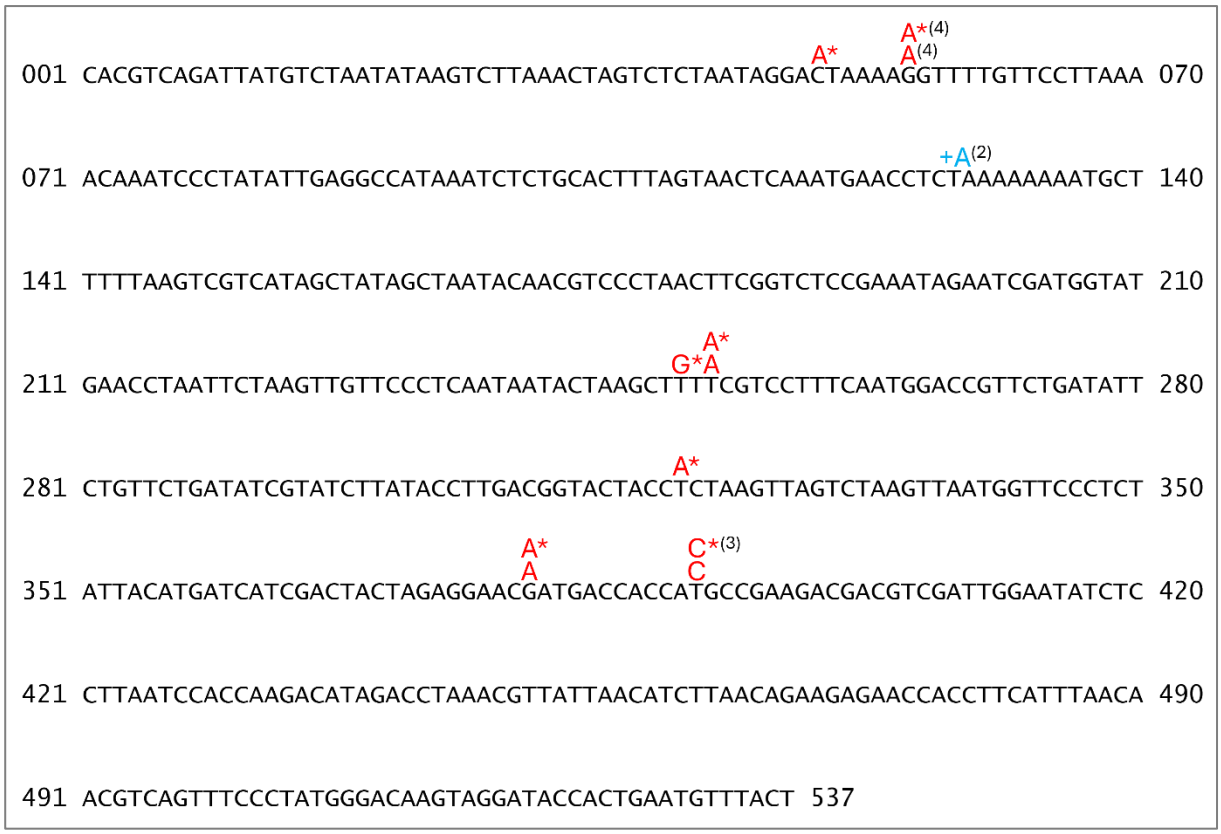


Figure 9 **Mutations of spontaneous 6-MP resistant samples in the *apt* gene** Depicted is the whole *apt* gene, starting from base 1 to base 537. SNPs are indicated in red above the sequence. Single base pair insertions are indicated in blue with a plus sign and the inserted base. The asterisks depict suboptimal clarity of the chromatogram of the sequencing at this position. The numbers in parentheses show how often a mutation occurred.

3 Discussion

3.1 Natural resistance to incorporation of foreign DNA

In this study, different heat shock conditions were shown to influence the growth of *N. franklandianus*. Methods for the exploration of a genetic system for the AOA species *N. franklandianus* were investigated. Based on pre-tests, an optimal heat shock temperature was aspired. Transformation experiments, either with $CaCl_2$-heat shock, electroporation or a combination of both did not seem to lead to a successful introduction of DNA into the genome. By the proof-of-concept conjugation between two *E. coli* strains with the designed conjugation plasmid cells, growth of cells with the right antibiotic resistance were received, however, applying this concept to *N. franklandianus* as recipient was not shown to achieve the creation of a genetic system. As for the obtained *N. franklandianus*

cultures with spontaneous acquired resistance, two different mutation types could be isolated.

For standard transformation procedures, as done with *E. coli*, heat shock is done at 5°C above its optimal growth temperature of approximately 37°C. *E. coli* is already effectively inactivated at temperatures beginning from 55°C, meaning this is only 18°C above its optimal growth temperature (Singh et al., 2010). For *N. franklandianus* it could be seen that with a heat shock up to 64°C, which is already 22°C above its optimal growth temperature of 42°C, there is no effect on growth at all (Figure 3). They are effectively inhibited at temperatures starting from 80°C, which is 38°C above the preferred growth temperature (Data not shown). This shows, that *N. franklandianus* is far more heat resistant than *E. coli*. Interestingly, this fits the fact, that the optimal growth temperature of all isolated AOA is much higher than the environment they were isolated in. Soil has a temperature of 10-20°C, but in the lab the strains are cultivated at 42°C, marine environments harbour temperatures of under 10°C and the strains are grown at around 30°C. Therefore, it was assumed, that for the experiments in this thesis, the heat shock temperature had to be higher than 5°C above the optimal growth temperature of *N. franklandianus*. Otherwise, the pores in the cell membrane formed through heat shock might not be large enough or not assembled at all.

If the pores are large enough, the designed plasmids should in theory be able to cross the cell membrane of *N. franklandianus*. Contrasting to other AOA, *N. franklandianus* does not have an S-layer, which could display another protective mantle around the cells, that could prevent plasmids from entering cells (Han et al., 2024). On the other hand, additionally to the cell membrane as barrier to the exterior and protection, *N. franklandianus* embeds itself in a layer of extracellular polymeric substances (EPS). All isolated Nitrosocosmicus form aggregates in liquid culture. Moreover, via scanning electron microscopy (SEM), it was already shown that amongst other AOA, *N. franklandianus* cells often build up a compact, multilayered biofilm (Dreer et al., 2024). This implies that during transformation it could be more difficult for plasmids to cross the cell membranes of a high enough cell number due to cells, that stick together and therefore mask other cells, that are in the inner layers of a biofilm.

Besides the assumption, that the plasmids were not able to enter the cells across the EPS, possible biofilm and the cell membrane, there are more reasons, why transformation might did not work. The sole existence of plasmids in the cytosol of a cell does not mean it is always used as a template and further that their sequences are transcribed. Otherwise, conjugation, where the plasmid is directly delivered into the cytosol of the recipient, which was tested for this thesis too, could be a solution for this complication.

Additionally, *N. franklandianus* might encode of a restriction modification system (R-M system), to protect itself from uptake of foreign DNA. These systems are not present in eukaryotes, but it is estimated that approximately 95% of bacteria and archaea contain at least one R-M system (Roberts et al., 2015). R-M systems consist of methyl transferases or restriction endonucleases or a combination of both. The genomic DNA (gDNA) of the host gets epigenetically modified, typically through specific methylation patterns. This labels the DNA as its own DNA and prevents cleavage by the restriction enzymes. The restriction endonucleases therefore recognize unmethylated or foreign methylated DNA and cleave it, thus differentiating between self- and non-self DNA (Freed et al., 2018). The plasmids used for this thesis were amplified or constructed in either *E. coli* Top10 or *E. coli* DH5α. Therefore, they are all methylated by *E. coli*. *E. coli* uses Dam methylation, which adds a methyl group to the adenine in 5'-GATC-3' (Marinus & Morris, 1973) and DCM methylation which methylates the cytosine in 5'-CCAGG-3' or 5'-CCTGG-3' (Marinus, 1973). Hence, the plasmids might get delivered into the cytosol, but are digested promptly after entering due to their foreign character.

Overcoming these systems might be crucial for a successful introduction of DNA via transformation and conjugation into an organism. The presence of such R-M systems was not investigated during this study, but it might be an important indication where to focus on further insights for the development of genetic systems for AOA.

3.2 No successful transformation by a combination of heat shock and electroporation

Over the course of this study, many different settings for transformation and conjugation were tested (Appendix table A). There are already genetic systems for different archaea. In *M. maripaludis*, transformation can simply be done by natural transformation (Tumbula

et al., 1994). As a starting point for this thesis, transformation was attempted via heat shock alone (Figure 4a), as it is normally done for *E. coli* (Straus et al., 1987). Moreover, it is known, that *T. kodakarensis* was already successfully transformed via heat shock (Sato et al., 2005). Further, transformation via electroporation alone was attempted (Figure 4b), based on the achievement in *Saccharolobus solfataricus* (previously *Sulfolobus solfataricus*) (Schleper et al., 1992).

There is a possibility, that both of these methods are simply not suitable as a transformation method for AOA species due to the physical barriers discussed above, which is why a combination of both was attempted. One experiment with this combination resulted in plasmid cultures, that grew earlier than the negative controls. Growth could be recognized from day four, with a difference in nitrite levels of 100-140 µM nitrite compared to the negative control (Figure 4c). Further, for *Corynebacterium glutamicum* it is already known that electroporation followed by a heat shock increases the transformation efficiency (van der Rest et al., 1999). These findings implied, that there might have been a successful transformation in *N. franklandianus*.

This experiment setting was repeated with one deviation from the first one, namely the regeneration time. It was changed to either no regeneration time or to only two hours instead of five. Contrasting to the previous results, the negative controls again were the fastest to grow, which did not hint to a successful transformation in these experiments.

In addition to the repetition, the cultures growing in selection medium were newly inoculated one time to perform DNA extraction and sequencing. This was done to minimize the presence of wildtype DNA in the samples and obtain clearer data from sequencing of the mutated sequences. Even after multiple attempts using various cell harvesting techniques, the direct performance of culture PCRs with *N. franklandianus* samples without DNA extraction was never successful, probably due to the physical barriers discussed above (Data not shown).

Sequencing data showed no successful transformation of the cultures. The sequenced samples resembled the wildtype and did not harbour the changes of the designed plasmids. On the other hand, several different mutations, that likely lead to resistance against 6-MP could be obtained (Figure 9). The chromatogram of the sequencing of several samples exhibited suboptimal clarity. This could be a consequence of excessive

wildtype DNA still present in the samples, potentially resulting from an insufficient number of transfers prior to DNA extraction and sequencing.

3.3 Unsuccessful conjugation experiments

Based on a successfully created genetic system for the methanogen *Methanothermobacter thermautotrophicus* ΔH, conjugation was attempted for *N. franklandianus* too. Considering, that for *M. thermautotrophicus* ΔH there were similar hurdles to overcome, as natural transformation, several transformation methods including electroporation and interdomain conjugation using *E. coli* as a donor was attempted and only the latter resulted in a successful introduction of DNA (Fink et al., 2021).

The first conjugation cultures of *N. franklandianus* started to grow after approximately one month, which does not strongly suggest a successful introduction of the conjugation plasmid into the genome. Due to the fact, that in the first two conjugation experiments no negative controls were included, there is no data on how fast they would have grown and if they would have been faster than the plasmid cultures. However, given the possibility, that there were only very few transformants, which obviously would have taken more time to proliferate due to the low inoculation volume and the higher generation times, DNA was extracted after newly inoculating the samples two times and send for sequencing.

The sequencing data was comparable to the results from the transformation experiments and no successful transformation could be seen. The sequenced samples again resembled the wildtype. Several different resistance mutations could be obtained (Figure 9). The sequencing data from the conjugation experiments demonstrated slightly greater clarity compared to the transformation experiments, probably due to the implementation of two transfers instead of one prior to sequencing.

3.4 Different resistance mutations with different phenotypes

6-MP as a selective agent was already used for the exploration of the hyperthermophilic archaeon *S. islandicus*. Growing *S. islandicus* in medium with 6-MP resulted in a whole spectrum of mutations in the *apt* gene. There were many different types of mutations found, namely SNPs, single or multiple base pair insertions and deletions, tandem

duplications and some more. The mutations with the highest occurrence were SNPs, followed by frame shift mutations (Zhang et al., 2016). Further, the mutation profile of *Sulfolobus* species seems to correlate with that of *T. kodakarensis*, which was also treated with 6-MP using *hpt* as selection marker (Čuboňová et al., 2013). Based on these findings, it was assumed, that there will be a similar mutation pattern in AOA and that the *apt* gene might be a suitable target for a selective marker in the construction of a genetic system for AOA.

The approach was for the wildtype *apt* of *N. franklandianus* to be replaced by introducing a mutated version by transformation or conjugation and inoculation of the cultures into selective medium. Keeping in mind, that in *S. islandicus*, a whole mutation library was found in the *apt* gene, it should not matter, what type of mutation interrupts the functionality of the *apt* gene.

It was assumed, that the *apt* gene will gain similar mutations in *N. franklandianus* grown in selective medium as in *S. islandicus*. Nevertheless, *N. franklandianus* mostly only shows one specific mutation in the *apt* after introduction to selective medium, which is in stark contrast to what was discovered before. The transformation and conjugation plasmids were designed based on this mutation. Surprisingly, over the course of this project, several other mutations were found in the *apt* gene, rendering *N. franklandianus* resistant to 6-MP (Figure 8 and 9). It was astonishing, that one of these mutations, a frame shift mutation due to an insertion of an adenine, showed a different phenotype than the SNP that was found more often (Figure 8). This is not the case for *S. islandicus*, where every mutation in the *apt* led to a loss of function of the gene, theoretically making it an appropriate selection marker for the creation of a genetic system (Zhang et al., 2016).

Therefore, the findings of different phenotypes based on different mutations in the *apt* were not observed before and led to concerns, that the *apt* gene might not be a suitable target in *N. franklandianus* due to a possible residual activity. The strength of the residual activity would be dependent on the type of mutation in the gene.

3.5 Phenotype rescue

The *apt*/6-MP system is applicable for the investigation of the pattern of spontaneous mutations. From further *Sulfolobus* species besides *S. islandicus*, namely *S.*

acidocaldarius and *S. tokodaii* it is known that the *apt* is conserved in this genus and probably also in all crenarchaea that code for a respective homolog. That is why the *apt*/6-MP system should be tested for a broader application to more species. Interestingly, it was also found, that when using an *apt* deficient strain, independent of the inhibition with 6-MP, the growth was impaired. Recovery from this only happened after approximately three weeks after inoculation.

Intriguingly, when adding AMP or GMP to the growth arrested samples, they were rescued. AMP or GMP therefore seems to be required to maintain normal growth (Zhang et al., 2016). The reason why GMP can be used instead of AMP for this purpose seems to be due to the interconversion between GMP and AMP in numerous organisms (Zimmerman & Magasanik, 1964). Although the molecular mechanism for the dependence of *apt* deficient *S. islandicus* strains on AMP or GMP for growth is still unknown, a similar case was reported in the hyperthermophilic euryarchaeon *Pyrococcus furiosus*, where the growth of a mutant with a similar deficiency, namely *hpt*, was supported by the addition of GMP (Kreuzer et al., 2013).

There is a possibility, that something similar has happened to the experiments with *N. franklandianus* and the growth of transformed or conjugated cells was stalled due to the lack of AMP or GMP. Therefore, adding AMP or GMP to the cultures after transformation or conjugation might have led to a different growth behaviour of *apt* deficient strains.

4 Conclusions

This master thesis' overall goal was to contribute to the development of the first genetic system for an AOA. For this purpose, *N. franklandianus,* an organism belonging to the order of Nitrososphaerales was used and transformation and conjugation plasmids conferring resistance to the inhibitor 6-MP were employed. Transformation was attempted via $CaCl_2$-heat shock, electroporation and a combination of both; conjugation was done using *E. coli* as donor species. Optimal heat shock conditions were inspected and shown to be considerably higher than standardly used for *E. coli* (Figure 3).

Though several different conditions were examined, nothing hinted to the successful transformation of *N. franklandianus* during the conducted experiments. Furthermore, the

proof-of-concept conjugation between two *E. coli* species using the newly designed plasmid seemed to be successful, as the introduction of the plasmid led to acquired resistance and therefore growth on selective medium plates (Figure 6b). Further, the plasmid was then used for interdomain conjugation using *E. coli* as donor and *N. franklandianus* as recipient. However, a successful conjugation could not be demonstrated, as the cultures stayed inhibited by 6-MP (Figure 7b).

Over the course of this project, multiple spontaneous 6-MP resistant strains arose from different experiments. It could be shown that they harbour various distinct mutations that led to the acquisition of resistance. Amongst them, two different types of mutations could be identified, namely SNPs and insertions, respectively (Figure 8b). Moreover, it could be shown, that the two different kinds of mutations exhibited different growth patterns (Figure 8a).

5 Outlook

To this date, no genetic system for a representative of the group of AOA is available. Previous studies on this topic, using transformation as the mode of delivery were never successful, which is why in this project several adjustments to earlier attempts were examined. Transformation was done not only via electroporation, but also by $CaCl_2$-heat shock and a combination of both. As none of the transformation experiments yielded successful results, conjugation using *E. coli* as donor was pursued as the next step.

The conjugation experiments did not show signs of success, however the last experiment with subsequent plating instead of cultivation in liquid medium could not be evaluated yet. A possibly conjugated cell would take even longer than 65 days, which the experiment was followed, to proliferate enough, for growth to be recognized by nitrite values and colony formation similar to *E. coli* on plates. Therefore, this experiment could still be evaluated after growth can finally be perceived. In the future, this or similar simplified plating techniques could be a promising approach to receive single colonies of successfully transformed or conjugated cells.

The amplification of the transformation and conjugation plasmids was performed in E. *coli*. A possible hurdle that could have appeared in the experiments are the possibly

different methylation patterns between the two species. Therefore, *N. franklandianus* would recognize the foreign DNA and destroy it before the plasmids can be used as template. For further studies on a genetic system, it will be important to obtain more information about the differences in the methylation patterns of *E. coli* and *N. franklandianus*, or any other AOA species that one wishes to use for this purpose.

Finally, as it was seen in similar studies on other archaeal species, the addition of AMP or GMP to *apt* deficient strains could be important to maintain normal growth during transformation or conjugation experiments.

6 Materials and Methods

6.1 Culture conditions in liquid medium

The isolation from arable soil in Aberdeen and the growth conditions of *Candidatus* Nitrosocosmicus franklandianus C13 were described previously (Lehtovirta-Morley, Ross, et al., 2016). Briefly, stock cultures were routinely grown in 30ml aseptic polystyrene multipurpose containers (Greiner Bio-One) in 20ml growth medium. Biomass scale up cultures were grown in 1l glass schott bottles (DURAN) containing 600ml growth medium.

The standard growth medium consists of fresh water medium (FWM, 17.1116 mM NaCl, 1.9674 mM $MgCl_2$ $6H_2O$, 680.2258 µM $CaCl_2$ $2H_2O$, 1.4697 mM KH_2PO_4, 2.6827 mM KCl, diluted in MilliQ and autoclaved)(Lehtovirta-Morley, Ross, et al., 2016). The FWM was supplemented with 2 mM NH_4Cl, 2 mM $NaHCO_3$, 7.5 µM Fe-Na-EDTA, 0.1% dilution of Thiamine-HCl, 0.1% dilution of modified non-chelated trace element solution and 10 mM HEPES buffer, buffering to a pH of ~7.6 at 42°C (Tourna et al., 2011; Könneke et al., 2005). For selective medium, 40 µM 6-Methylpurine was added. For conjugation medium, 1 mM pyruvate was added as an energy source for *E. coli*. Counterselection medium was amended with 40 µM 6-MP and 50 µg/ml kanamycin, for the inhibition of *E. coli*.

The FWM was autoclaved at 121°C and 103 kPa pressure. The additives were filter sterilized with a 0.22 µm MCE filter (Merck MF-Millipore™) and added afterwards. All the solutions, except for $NaHCO_3$, which was stored at room temperature in the dark, were stored at 4°C in the dark.

Stock cultures were grown aerobically at 42°C in the dark, shaking at 80 rpm and transferred to new growth medium at ~1200-1500 µM nitrite once a week with an inoculation volume of 1%.

6.2 Culture conditions in solid medium

The last conjugation experiment was performed using the cultivation on solid medium with a liquid overlay established by Klein et al., 2022. Instead of 30 ml aseptic polystyrene multipurpose containers (Greiner Bio-One), 250 ml glass schott bottles (DURAN) were used. As a base layer, 1% autoclaved Gelrite was mixed the same volume double concentrated counterselection medium, resulting in a 0.6% overlay. Afterwards, a Gelrite and counterselection medium cell overlay was applied. To prevent cells from escaping into the liquid phase, a small layer of Gelrite with counterselection medium was added on top. Finally, the solid medium was overlayed with a layer of liquid counterselection medium (Figure 10). The liquid overlay was exchanged to fresh medium, when the cultures reached nitrite values of approximately 1500 µM. Cultures were grown under normal growth conditions, though in a non-shaking incubator.

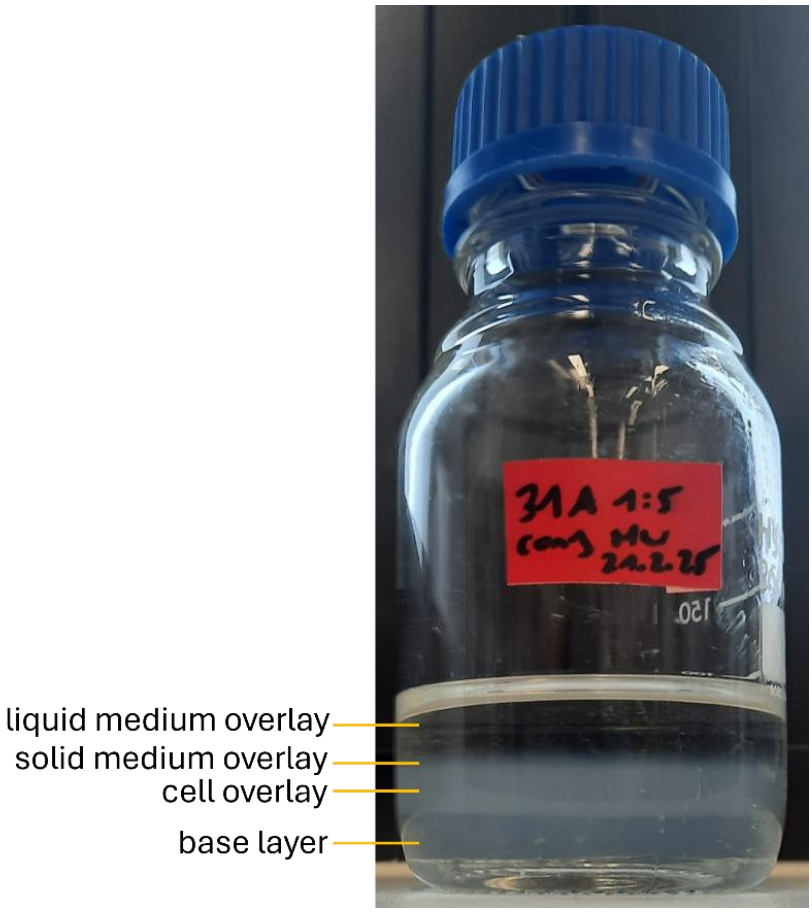


Figure 10 **Overlay technique for growth on solid plates** The base layer is a mixture of Gelrite and counterselection medium. The cell overlay is embedded in a mixture of Gelrite and counterselection medium. This layer is sealed with a small layer of a mixture identical to the base layer. Finally, liquid counterselection medium is added on top.

6.3 DNA manipulation

Several used standard techniques of molecular biology were carried out according to Green et al., 2012. For colony and culture PCRs, three different polymerases were used, namely Taq (New England BioLabs), Q5® (NEB) and Phire (ThermoFisher). The PCR reactions were performed in 20 µl for Phire polymerase and 25 µl for Taq and Q5 polymerases. For colony PCRs, single colonies were used as templates. For culture PCRs, different amounts varying from 1 µl to 8 µl of liquid culture were used as templates. The PCR machine settings for the Taq polymerase are the following: 95°C for 2 minutes; 30 cycles of 95°C for 10 seconds, 56°C-62°C for 30 seconds, 72°C for 1 minute per kb; and 72°C for 5 minutes. The PCR machine settings for the Q5 polymerase are as follows: 98°C for 30 seconds; 30 cycles of 98°C for 10 seconds, 65°C for 30 seconds with touchdown of 0.2°C per cycle, 72°C for 30 seconds per kb; and 72°C for 5 minutes. The PCR machine seetings for the Phire polymerase are the following: 98°C for 30 seconds; 30 cycles of 98°C for 5 seconds, 56°C for 5 seconds, 72°C for 15 seconds per kb; and 72°C for 1.5 minutes. The PCR products were investigated on 1% agarose gels. As ladder, GeneRuler 1 kb Plus (ThermoFisher) was used. PCR cleanup was done with Monarch® PCR & DNA Cleanup Kit (New England BioLabs). The used primers for this thesis can be examined in Appendix table B.

DNA sequencing was done by Eurofins (Ebersberg, Germany). Over the course of this project, two chemically competent *E. coli* strains were used for plasmid reproduction, namely, Top10 or DH5α. They were grown either in liquid or solidified Luria-Bertani (LB) medium at 37°C, shaking at 180 rpm and non-shaking, respectively. As selective agents, Streptomycin and Ampicillin were used. *E. coli* was transformed using heat shock at 42°C for 45 seconds. The small scale plasmid extraction was either done with the Monarch® Plasmid Miniprep Kit (New England BioLabs) or the PureYield™ Plasmid Miniprep System (Promega) and for larger scale with the PureYield™ Plasmid Midiprep System (Promega).

6.4 DNA extraction

DNA extraction was performed from late exponential cultures. The cells were centrifuged for 20 minutes. All centrifugation steps are carried out at 4°C and 21130 x g. The pellets were resuspended in SDS Extraction Buffer (0.7 M NaCl, 0.1 M Na_2SO_3, 0.1 M Tris/HCl, 50 mM EDTA and 1% SDS), transferred to Lysing Matrix B tubes and mixed with Phenol/Chloroform/Isoamylalcohol (25:24:1). The samples were treated in a FastPrep-24™ Classic bead beating grinder and lysis system (MP Biomedicals) for 30 seconds at speed 4 and centrifuged for 10 minutes. The supernatant was combined with Chloroform/Isoamylalcohol (24:1) and centrifuged again. The supernatant was mixed with glycogen and 2x volume Polyethylenglycol solution and incubated overnight at 4°C.

The samples were centrifuged for 30 minutes, and the pellet was washed with 70% ice cold ethanol, followed by another centrifugation step of 10 minutes. The supernatant was removed, and the pellet was dried and resuspended in 60°C pre-heated TE buffer. The extracted DNA was incubated at room temperature for 30 minutes and stored at -20°C.

6.5 Transformation plasmids

The transformation plasmids used in this study were provided by Maximilian Dreer. The plasmids consisted of three main parts: An *E. coli* backbone, comprising an ori and an ampicillin resistance; the *apt* gene with recombination flanks for homologous recombination; and a *cas9* gene with a sgRNA targeting the *apt* gene. They were designed to give resistance to 6-MP upon incorporation, and additionally contained synonymous changes to be able to distinguish them from spontaneous mutations leading to resistance.

All four plasmids were based on the same backbone but contained the following differences: (I) the lengths of the recombination flanks, which were either 500 bp or 1000 bp, (II) the *apt* gene: There was either only a SNP or a 60 bp deletion, that included the SNP, within the gene to render the protein product non-functional and therefore introduce resistance to 6-MP. Only the two plasmids with 500 bp recombination flanks, pTNF-apt-500HR and pTNF-aptΔ60-500 HR, are depicted (Figure 2).

6.6 Construction of conjugation plasmids

Construction of the conjugational plasmid was based on the pre-existing transformation plasmids. The assembly was performed using the NEBuilder® HiFi DNA Assembly Master Mix (New England BioLabs). The subsequent transformation into chemically competent *E. coli* DH5α was accomplished as mentioned above. The inserted fragment was derived from an *E. coli* backbone and contained the crucial *traJ* gene for conjugal DNA transfer. The conjugation plasmid was derived from the pTNF-aptΔ60-500 HR plasmid and was therefore called pCNF-aptΔ60-500 HR (Figure 5).

6.7 Transformation procedures

If not mentioned otherwise, all transformation experiments were performed in triplicates. Besides the transformation samples with plasmids, there were always positive and negative controls without plasmids. The positive controls were not treated with selective marker. The regeneration for all heat shock and electroporation transformation experiments was carried out at 42°C, shaking at 80rpm, in the dark.

6.7.1 Heat shock

N. franklandianus wildtype was grown in glass laboratory bottles (DURAN) and concentrated by filtration through an autoclaved MF-Millipore 0.22 µm MCE Membrane, 47 mm filter (Merck). The cells were taken up in 1 ml heat shock transformation buffer, which is comprised of modified FWM containing 17.11 mM NaCl, 1.9674 mM $MgCl_2$ $6H_2O$, 80 mM $CaCl_2$ $2H_2O$ and 6.7 mM KCl. It was stored at 4°C in the dark. The cells were divided into aliquots of ~ $2x10^6$ cells per sample. From each of the four different transformation plasmids, which were already provided, ~1 µg was added to the cells and heat shocked with different temperatures and durations over the course of this project. These settings can be seen in Appendix table A. After heat shock, the cells were regenerated in growth medium. The duration of regeneration varied for each experiment. After regeneration, the growth medium was altered into selective medium.

6.7.2 Electroporation

The cells were concentrated the same way as in the heat shock experiments and washed twice by pipetting with 1 ml of autoclaved ultrapure water. Between each washing step,

centrifugation for 15 minutes at 4°C and 21130 x g was performed. About 1 µg of plasmid DNA was added to the cells before electroporation.

Electroporation was done in cuvettes with a 1 mm gap utilizing a Gene Pulser XCell including a PC and CE module (BioRad). The chosen electroporation settings varied between the experiments and can be seen in Appendix table A. The resulting time values were between 7.8-21.6 ms for the transformation samples and 19.6-25.1 ms for the positive and negative controls. Subsequently, each sample was immediately transferred to growth medium for regeneration. The duration of regeneration was individual for each experiment. Regeneration was stopped by altering the current medium into selective medium.

6.8 Conjugation procedures

6.8.1 *E. coli* S17 to *E. coli* NEB stable

To investigate the functionality of the constructed conjugation plasmid, a conjugation test run from *E. coli* S17 to *E. coli* NEB stable was performed. S17 and NEB stable were grown to an OD_{600} of ~2. They both were harvested and combined afterwards in the following recipient:donor ratios: 1:1, 1:10, 1:100 and 1:1000. Spots were placed on LB plates without antibiotics. For the conjugation process, the cells were incubated at 37°C without shaking. After the conjugation step was accomplished, the cells were washed off into LB medium for recovery and afterwards transferred onto selective medium plates, containing the respective antibiotics for the receiving *E. coli* strain and the plasmid. For this overnight growth at 37°C, a serial dilution was executed. A detailed workflow can be seen in figure 6a.

6.8.2 *E. coli* S17 to *N. franklandianus*

N. franklandianus cultures were grown to a cell count of 1.8^{10} and *E. coli* S17 were grown to an OD_{600} of ~2. The archaeal cells were harvested by filtration and the bacterial cells were harvested via centrifugation, as both previously described. They were combined in conjugation medium in either 1:5 or 1:10 recipient:donor ratio. The conjugation step for conjugation in liquid culture was performed overnight, at 37°C, shaking at 300 rpm. For conjugation on solid medium, plates, made of one part Gelrite and one part growth

medium, were used. Spots of the mixed donors and recipients were placed on the plates and incubated overnight, at 37°C, non-shaking. The cells were recovered in counterselection medium. After recovery, selection via 6-MP is started. A detailed workflow can be seen in figure 7a.

6.9 Nitrite assay

Since OD_{600} measurements are not applicable to *N. franklandianus*, the measurement of nitrite production is used as a tool to determine cell growth. This technique was exerted as described previously by Tourna et al., 2011. Briefly, 10 µl culture samples were obtained and combined with 790 µl 1x FWM (as described above) and 200 µl Sulf-NED (2.19 M 85% ortho-phosphoric acid, 58.1 mM sulfanilamide, 1.91 mM α-naphthylethylenediamine dihydrochloride in H_2O). The absorption is measured at 545 nm using a Sunrise Microplate Absorbance Reader (Tecan).

6.10 Screening for transformants

To check for possible transformants, DNA extraction was executed as described above. Afterwards, PCR was performed and the samples were checked on a 1% agarose gel. The PCR was cleaned up as mentioned above and a restriction digest using NcoI was carried out. The restriction digest was done in a 25 µl reaction, using ~ 500 ng of DNA. The restriction digest was checked on a 1% agarose gel too and sent for sequencing.

7 Appendix

	temperature	time	voltage	capacity	resistance	time constant	time constant	regeneration time
	°C	min	V	mF	Ohm	ms samples	ms ctrls	h
Transformation only heat shock								
	52	2	/	/	/	/	/	16
	70	10	/	/	/	/	/	16
	66	5	/	/	/	/	/	5.7
	66	5	/	/	/	/	/	18
	64	5	/	/	/	/	/	18
	64	5	/	/	/	/	/	18
	64	5	/	/	/	/	/	17.5
	52	2	/	/	/	/	/	16.25
	64	10	/	/	/	/	/	no/5
Transformation only electroporation								
	/	/	1500	25	1000	10.8-21.6	20.5-24.1	5
Transformation both								
	64	10	1500	25	1000	13.5-15.9	23.3-24.2	5
	64	10	1500	25	1000	13.9-18.1	23.1-25.1	no
	64	10	1500	25	1000	7.8-15.6	19.6-22.8	2
Conjugation liquid								
	/	/	/	/	/	/	/	4
Conjugation solid								
	/	/	/	/	/	/	/	4
	/	/	/	/	/	/	/	no

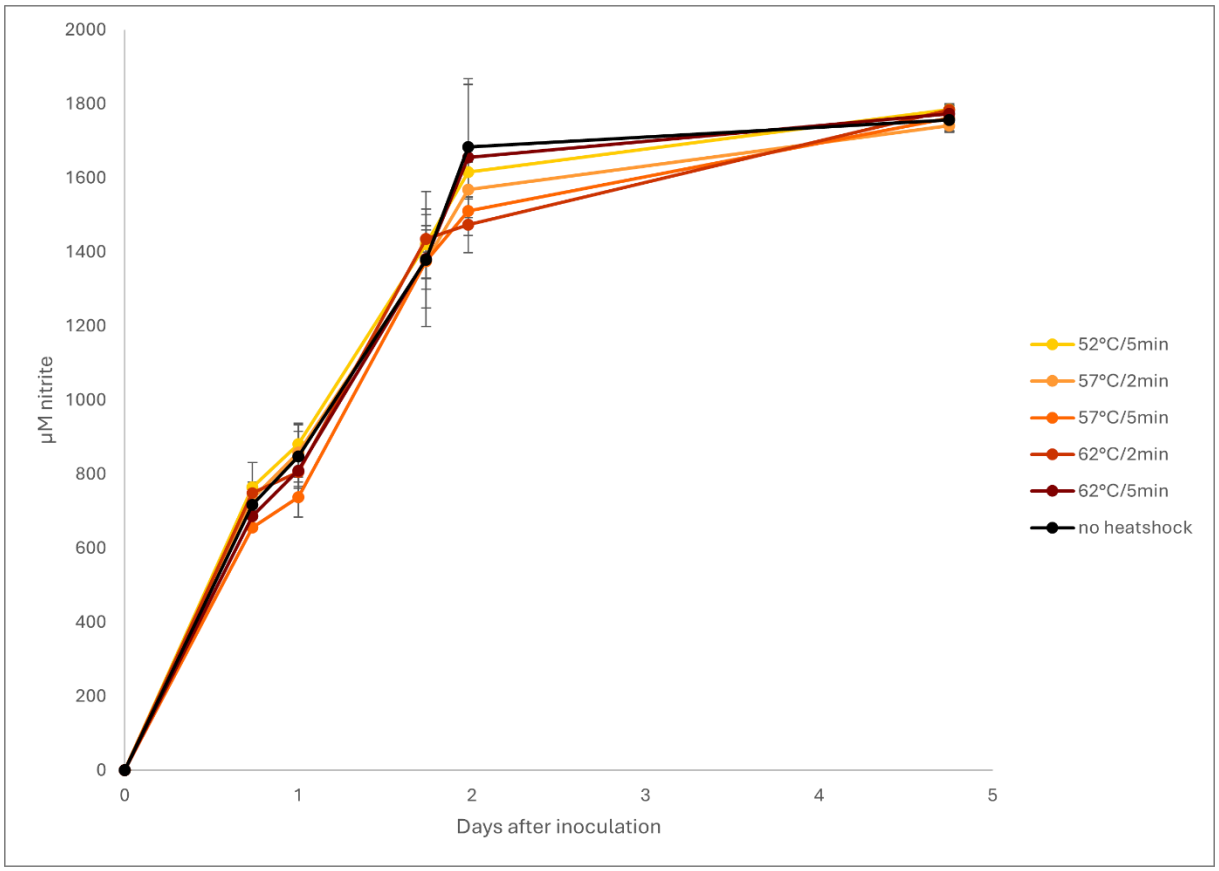
Appendix table A **Transformation and conjugation conditions used during this study**

Primer	Sequence
NF_apt.F	GGCCTAATTGTTCGTGTCTCCT
NF_apt.R	GCAGCTCCTATGTCTACAATTTTCATCC
aptΔ30.F	TTGTCTTATAGTCTTGCCAGGT
aptΔ30.R	GGAGATAATGTACTAGTAGCTGATGA
pTNF.inv.del2.F	AGATAATACCATGTTAATTTACTCTTTGTTAT
pTNF.inv.del1.R	AAGCTTGCATGCGGC
HR check 11.09 FW	CGGTGCAGGTAGAAAAGTATCAAG
HR check 11.09 RV	CTGCTATCATTGCCTCGTCTTG
pC_vec_MD3_FW	CGCGCAGTGATACCAATTCGGAGCC
pC_vec_MD3_RV	GACCACTAGTACATGCATCCACCATCG
pC_ins_MD3_FW	TGGTATCACTGCGCGTAATCTGC
pC_ins_MD3_RV	GATGCATGTACTAGTGGTCATAGCTGTTTC
pC_ins_MD1_FW	ATGATAAGTGCTAGCAGTGGGCAAG
pC_ins_MD1_RV	GGAAATGTGACTAGTGGTCATAGCTGTTTC
pC_ins_MD2_FW	ATGATAAGTGCTAGCAGTGGGCAAG
pC_ins_MD2_RV	GCCATTATCAACTAGTGGTCATAGCTGTTTC
pT#1.pT.HiFi.F	TGTTAAGTTAAAAGGAAGTCTCCAATAACTGTG
InsCheck1.FW	CCATCACTGGTCTTTATGAAACACGG
InsCheck1.RV	CAGGATTTTGCCAAAGGGTTCGTG

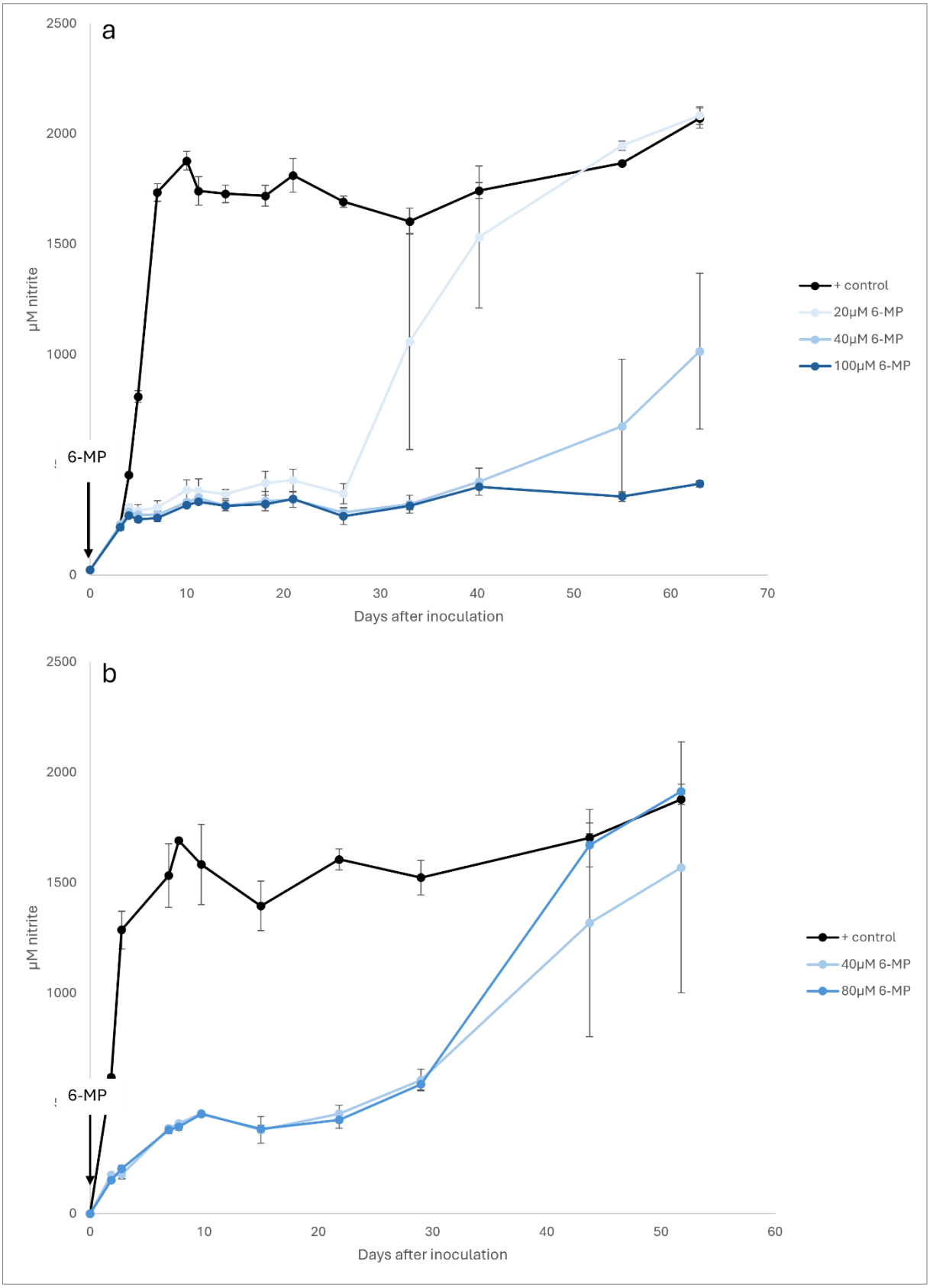
Appendix table B **Primers used for this thesis**

Appendix table C **Mutations of spontaneous 6-MP resistant samples**

	FW primer			RV primer			
Sample number	Mutation in *apt* (base)	Quality chromatogram	Amino acid change	Mutation in *apt* (base)	Quality chromatogram	Amino acid change	FW and RV same?
26_2B	49C>A	not good	y, Asp->Tyr	apt49C>A	not good	y, Asp->Tyr	y
26_2G	128_129insA	good	frame shift	128_129insA	good	frame shift	y
26_2H	380G>A	good	y, Ala->Val	380G>A	good	y, Ala->Val	y
26_2.2A	320T>A	not good	y, Glu->Val	320T>A	not good	y, Glu->Val	y
26_2.2B	380G>A	not good	y, Ala->Val	/	/	/	n
26_2.2C	/	/	/	/	/	/	y
26_2.2G	250T>G	not good	y, Lys->Gln	/	/	/	n
26_2.2H	/	/	/	/	/	/	y
26_2.2I	252T>A	good	y, Lys->Asn	252T>A	good	y, Lys->Asn	y
26_2.3A	55G>A	not good	y, Pro->Ser	55G>A	not good	y, Pro->Ser	y
26_2.3B	/	/	/	252T>A	not good	y, Lys->Asn	n
26_2.3C	/	/	/	/	/	/	y
26_2.3G	/	/	/	/	/	/	y
26_2.3H	/	/	/	/	/	/	y
26_2.3I	128_129insA	good	frame shift	128_129insA	good	frame shift	y
29_3A	55G>A	good	y, Pro->Ser	55G>A	good	y, Pro->Ser	y
29_3B	55G>A	good	y, Pro->Ser	55G>A	good	y, Pro->Ser	y
29_3C	55G>A	not good	y, Pro->Ser	/	/	/	n
30_3A	391T>C	good	y, Thr->Ala	391T>C	good	y, Thr->Ala	y
30_3B	391T>C; 55G>A	not good	y, Thr->Ala; Pro->Ser	391T>C; 55G>A	not good	y, Thr->Ala; Pro->Ser	y
30_3C	55G>A	good	y, Pro->Ser	55G>A	good	y, Pro->Ser	y
30_3D	55G>A	good	y, Pro->Ser	55G>A	good	y, Pro->Ser	y
30_3E	391T>C; 55G>A	not good	y, Thr->Ala; Pro->Ser	391T>C	not good	y, Thr->Ala	n
30_3F	391T>C	not good	y, Thr->Ala	391T>C	not good	y, Thr->Ala	y



Appendix figure A **Too low temperatures do not have an effect on growth of *N. franklandianus***



Appendix figure B **Effect of 6-MP on unconcentrated and concentrated *N. franklandianus* cells** (a) Unconcentrated cells. (b) ~100x concentrated cells

Appendix figure C **Sequencing of spontaneous 6-MP resistant mutant samples** (a) *apt*49C>A (b) *apt*55G>A, (c) *apt*128_129insA, (d) *apt*250T>G (e) *apt*252T>A (f) *apt*320T>A (g) *apt*380G>A (h) *apt*391T>C

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