universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Construction of mutants of *Natrialba magadii* and ϕCh1

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer. nat.)

Verfasserin:	Regina Selb
Matrikel-Nummer:	0301844
Studienrichtung /Studienzweig (lt. Studienblatt):	A 441 Diplomstudium Genetik – Mikrobiologie (Stzw) UniStG
Betreuerin / Betreuer:	Ao. Univ.-Prof. Dipl.-Biol. Dr. Angela Witte

Wien, im April 2010

Table of contents

1. Introduction

1.1 Introduction to the world of viruses

Viruses are particles. Controversy rages on question whether they belong to the living or non-living world. Since viruses are not able to replicate without infecting a host cell, their status as organisms can be questioned. Nevertheless, viruses contribute to numerous amounts of the world's biomass, taken the fact that their estimated number on earth is 10^{31} (Breitbart and Rohwer, 2005). Transmission electron microscopy showed that one millilitre of sea water contains approximately 10 million virus like particles (Bergth *et al.*, 1989). Moreover, every cellular organism studied so far appeared to have its own viruses or, at least, virus-like selfish genetic elements (Van Regenmortel *et al.*, 2000).

Viruses can be seen as obligate intracellular parasites that do not replicate by cell division, but are assembled from pre-formed viral components (Skern, 2006). Since viruses basically consist of genetic information, their general aim can be summarised as follows: replication of the genetic material and the controlled expression of its inherent information (Baltimore, 1971). According to Baltimore, six classes of viral genomes exist (Baltimore, 1971) using the relationship of the viral genome to messenger RNA (figure 1).

Class I: dsDNA: double stranded DNA genome

Class II: ssDNA: single stranded DNA genome

Class III: dsRNA: double stranded RNA genome

Class IV: +ssRNA: positive sense single stranded RNA genome

Class V: -ssRNA: negative sense single stranded RNA genome

Class VI: dsRNA: positive sense single stranded RNA genome with a DNA intermediate (Retroviruses)

The evolution of viruses is driven by two characteristics, a large number of mutants and a huge amount of descendants (Skern, 2006). These two features allow a rapid adoption to new environments and hosts.

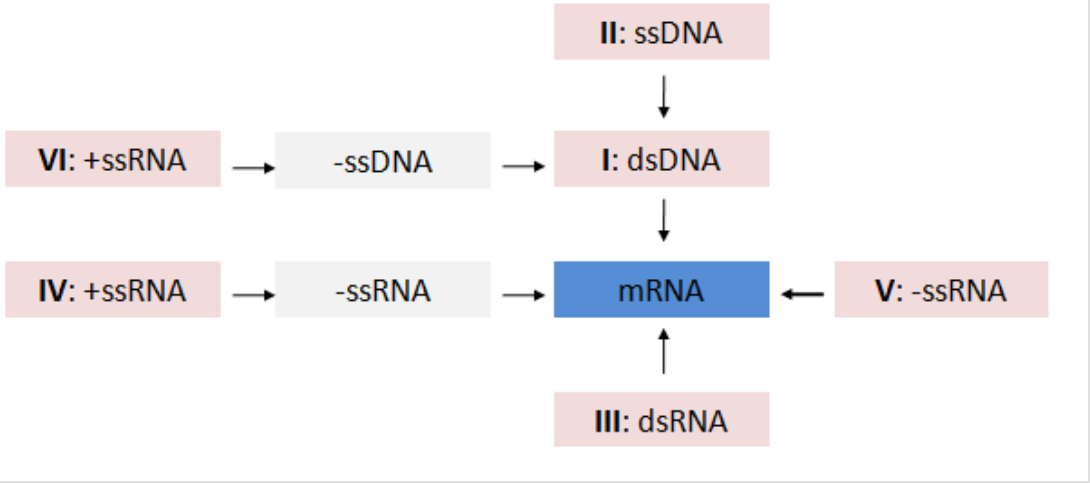


Figure 1: Baltimore's classification system
The six classes of (animal) viruses according to Baltimore: (I) double stranded DNA viruses, (II) single stranded DNA viruses, (III) double stranded RNA viruses, (IV) positive sense single stranded RNA viruses, (V) negative sense single stranded RNA viruses, (VI) positive sense single stranded RNA viruses that replicate via a DNA intermediate (retroviruses).
(Baltimore, 1971)

1.2 Archaeal Viruses

Contributing to the theory that viruses in general had multiple origins (Bamford, 2003) nearly all described archaeal viruses are unrelated to other known viruses. Indeed, genome sequencing revealed very few gene products having similarities with known proteins (Prangishvili *et al.*, 2006).

Interestingly, up to now all cultured archaeal viruses contribute to Baltimore's class I, consisting of a dsDNA genome (Prangishvili *et al.*, 2006), missing out a recently discovered virus infecting species of the genus *Halorubrum*. *Halorubrum* pleomorphic virus 1 (HPRV1) is the first virus of *Archaea* ever discovered that is harbouring a ssDNA chromosome (Pietilä *et al.*, 2009). In contrast to the mostly common characteristic of a dsDNA genome, numerous virion morphotypes can be monitored. Moreover, most of these often exceptional forms have not yet been observed for any other dsDNA virus (Prangishvili *et al.*, 2006).

1.2.1 Viruses of the *Crenarchaeota*

Most of the unique morphotypes described above are found in the group of crenarchaeal viruses (Häring *et al.*, 2005a). Examples that can be emphasised are the bottle-shaped virions of the *Ampullaviridae* (figure 2), the droplet-shaped virions of the *Guttaviridae* (figure 2) as well as the two-tailed virions of the *Bicaudaviridae* (Prangishvili *et al.*, 2006).

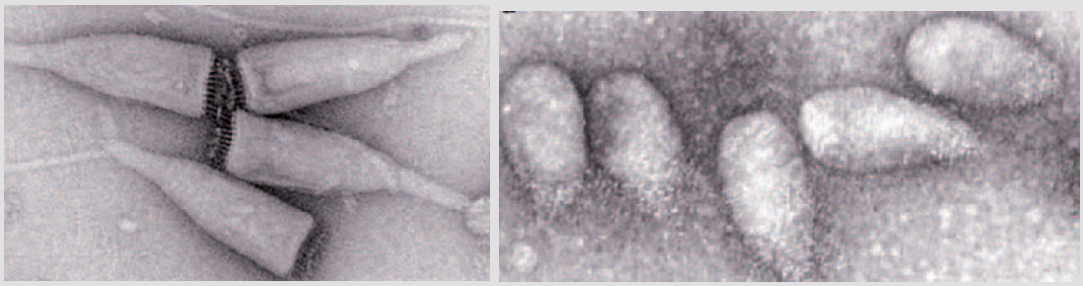


Figure 2: Bottle and droplet shaped viruses
Extraordinary forms of crenarchaeal viruses are found for example in the families *Ampullaviridae* (*Acidianus* bottle shaped virus; **left**) or *Guttaviridae* (*Sulfolobus neozealandicus* droplet-shaped virus; **right**).
(adapted from Prangishvili *et al.*, 2006)

As a typical property, the genome of most crenarchaeal viruses is linear and does not integrate into the host's chromosome while persisting at a low copy number in the cell. Another common characteristic is the stable relationship with the host, thereby avoiding a direct exposure of the virus particles to the often harsh environments. Only two crenarchaeal viruses, *T. tenax* virus 1 and *Acidianus* two tailed virus, are known to lyse their host cell (Häring *et al.*, 2005b).

1.2.2 Viruses of the *Euryarchaeota*

As well as in crenarchaeal viruses, the typical form of viral dsDNA in this group is linear. In contrast to crenarchaeal viruses, euryarchaeal viruses are often temperate, meaning they are able to undergo lysogeny, or their live cycle is generally lytic. The greatest amount of members of the euryarchaeal viruses belong to the families *Myoviridae* and *Siphoviridae*, hence possessing a classical head-tail morphology. While the genome size of these viruses can differ from 30 to 230 kbp, their G+C content is typically high. 60–70 % of the viral genome consists of guanine and cytosine as a result of their hosts over proportional G+C composition. All viruses of these two families seem to infect mesophilic or moderately thermophilic extreme halophiles or methanogens (Prangishvili *et al.*, 2006).

1.2.2.1 Haloarchaeal viruses in general

Haloarchaeal viruses or haloviruses are a group within the euryarchaeotal viruses infecting halophilic *Archaea*. The term "halophilic" refers to the fact that these organisms are able to survive in environments with high salt concentrations. While investigating flagella of *Halobacterium salinarum*, Torsvik and co-workers by coincidence detected phage particles in their work samples. This observation turned out to be the first haloarchaeal virus ever discovered (Torsvik and Dundas, 1974). Up to 1990, eight other haloviruses, all of them infecting *Hbt. salinarum* were found. The total number of haloarchaeal viruses increased to 15 in 2003, demonstrating little scientific progress in this field (Dyall-Smith *et al.*, 2003).

The discovery of the phages HF1 and HF2 in 1993 revolutionised the view on haloarchaeal viruses. Interestingly, these two examples were found to infect a wide range of haloarchaeal hosts, including the genera *Holoferax*, *Halobacterium*, *Haloarcula*, *Natrialba* and *Halorubrum*, while previously well-studied phages were found to be able to infect only different strains of one species. Moreover, these two viruses were the first haloviruses identified having a lytic live cycle (Nuttall and Dyall-Smith, 1993). Further investigation of HF1 and HF2 revealed complete sequence identity in 60 % of the whole genome, leading to the assumption of occurring recombination events between the viruses. Indeed, rapid evolution of haloviruses can be explained by this fact (Dyall-Smith *et al.*, 2003).

All haloarchaeal viruses described until 1998 consist of a classical head-tail morphology, described for the viral families *Myoviridae* and *Siphoviridae* mentioned earlier (Bath and Dyall-Smith, 1998). However, electron microscopy of salt water samples taken from the Dead Sea revealed a different picture. Indeed, most of the obtained virus particles found in the sample were lemon-shaped (fusiform), whereas only a minority of monitored viruses showed a head-tail morphology (Dyall-Smith *et al.*, 2003). A typical lemon-shaped virus, halophilic virus His1, can be seen in figure 3.

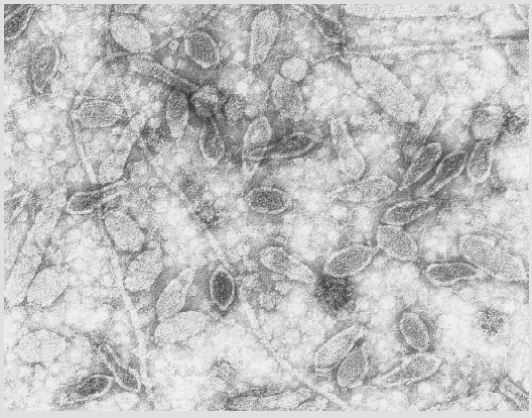


Figure 3: Halophilic virus His1
adapted from Bath and Dyall-Smith, 1998

1.2.2.2 Haloarchaeal viruses detail: ϕH

ϕH is a halophage infecting the halophilic archaeon *Hbt. salinarum* (formerly *Hbt. halobium*) and was first described by Heinke Schnabel and co-workers in 1982. It is a head and tail virus of the family *Myoviridae* and counts as one of the best studied halophages. The head form of the phage is icosahedral and 64 nm in diameter, while the contractile tail is 170 nm long and 18 nm wide. For structural integrity of ϕH a total NaCl or KCl concentration of 3 M is crucial. As it is peculiar for halophages and their hosts, the G+C content of the viral 59 kbp genome is over proportional (65 %) (Schnabel *et al.*, 1982). The phage DNA shows little sequence similarities to the genomes of *Bacteria*, bacteriophages or eukaryotic viruses. Nevertheless, it resembles P1 bacteriophages at the level of replication, morphology and control of lysogeny (Dyall-Smith *et al.*, 2003). The capsid of the phage contains a linear dsDNA genome (Schnabel *et al.*, 1982). The prophage genome, on the contrary, is a covalently closed circle of 57 kbp (Schnabel, 1984). This data was obtained, when restriction analysis suggested that the DNA of the phage consists of partially circularly permuted, terminally redundant DNA molecules (Schnabel *et al.*, 1982), reflecting those of phage P22 (Jackson *et al.*, 1978). The life cycle of ϕH is lysogenic; therefore, the phage is a temperate virus.

Interestingly, some resistant strains of *Hbt. salinarum* could be obtained from culture lysates. DNA hybridisation revealed that these resistant colonies contain much more ϕH homologous DNA than sensitive strains, indicating a stable lysogenic state of the virus in its host's DNA. Moreover, variations in the integrated ϕH DNA were observed in different sub-clones, suggesting that the physical structure is not static (Schnabel *et al.*, 1982).

Generally, substantial variation of ϕH DNA is possible. Six phage variants had been described by 1984 that can be distinguished by several insertions, a deletion and an inversion. Interestingly, the insertion element ISH1.8 (1.8 kb of size) also occurs at least twice in the genome of *Hbt. salinarum.*

Two variants of ϕH contain this insertion element twice in their DNA in inverted orientation, thus functioning as inverted repeats. This leads to the frequent inversion of the enclosed element, called "L segment". This segment is able to circularise, resulting in a 12 kb plasmid called pϕHL, furthermore resulting in the loss of all other viral DNA. Strains of *Hbt. salinarum* that have lost the phage DNA of ϕH except this plasmid are resistant to further infections with the virus. Since the plasmid is able to proliferate, the ϕH origin of replication has to be encoded in the L region (Schnabel, 1984).

1.2.2.3 Haloarchaeal viruses detail: Haloalkaliphilic virus ϕCh1

ϕCh1 was isolated in 1997 by Angela Witte and co – workers as the first virus of haloalkaliphilic *Archaea* ever detected. Furthermore, no other virus infecting this archaeal branch is known so far. After spontaneous lysis of the haloalkaliphilic archaeon *Natrialba magadii* (formerly *Natronobacterium magadii*) wild type strain, the existence of phage particles could be observed. Up to this point, *Nab. magadii* remains the only known host of the virus ϕCh1 (Witte *et al.*, 1997).

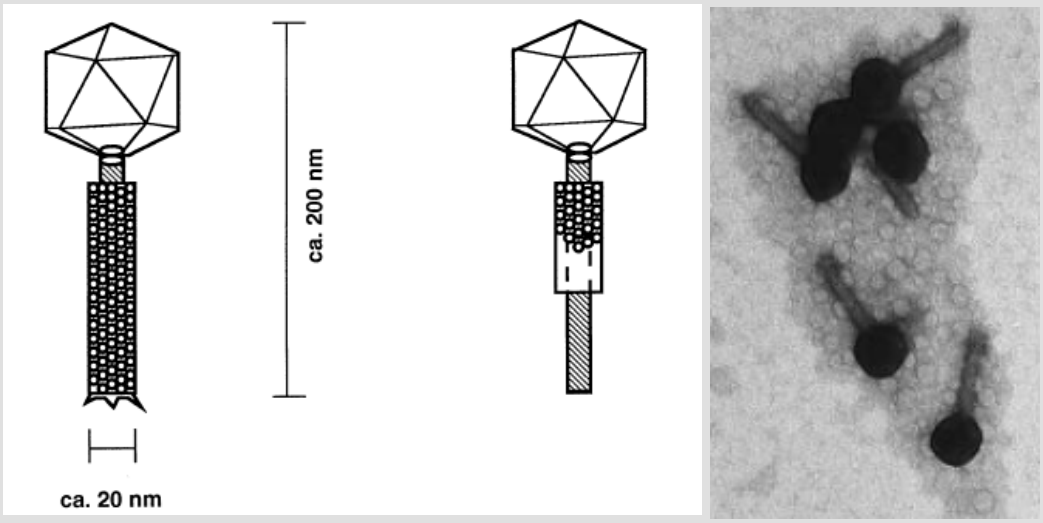


Figure 4: The virus ϕCh1
Schematic drawings of the virus ϕCh1 (**left**) and electron microscope picture of ϕCh1 phage particles (**right**). (adapted from Witte *et al.*, 1997)

1.2.2.3.1 General properties

Belonging to the family of the *Myoviridae*, ϕCh1 is a virus with an icosahedral head of 70 nm in diameter and a contractile tail with the length of 130 nm, summarising to a total length of 200 nm. The width of the tail is approx. 20 nm (figure 4). Investigation of the salt dependency of the virus was achieved by dialysing the phage particles against solutions with different NaCl concentrations, ranging from 1-4 M. It was observed that salt concentrations below 2 M result in complete loss of infectivity of the phage, indicating either conformational changes of the capsid proteins, or even dissociation of the phage particles at these concentrations. Lysis of the host *Nab. magadii* occurs after growth until stationary phase, signifying a growth phase depending lysis behaviour of ϕCh1 (Witte *et al.*, 1997).

1.2.2.3.2 Nucleic acids and genetic properties

ϕCh1 consists of a linear dsDNA genome, approximately 58 kbp of size. The ϕCh1 DNA is terminally redundant and circularly permuted, resulting in a so called "headful mechanism" packaging (Klein *et al.*, 2002). Further investigation by high-pressure liquid chromatography (HPLC) showed the ϕCh1 DNA to have a G+C content of approx. 62 %. Surprisingly, RNA particles packed in the viral capsid could also be obtained. Nevertheless, several hybridisation experiments revealed these nucleic acids not to descent from the virus, but instead being of host origin. The function of the RNA has to be determined further, a possible feature might be involvement in viral DNA packaging (Witte *et al.*, 1997).

Furthermore, the live cycle and genomic DNA of ϕCh1 was of interest. A close relative of ϕCh1, ϕH, is known to be a temperate phage, not integrating into the host's chromosome but, as a prophage, persisting in an episomal state in the archaeal cell (Schnabel, 1984). DNA hybridisation of phage DNA to total *Nab. magadii* L11 DNA showed ϕCh1 to be integrated into the host's chromosome (Witte *et al.*, 1997).

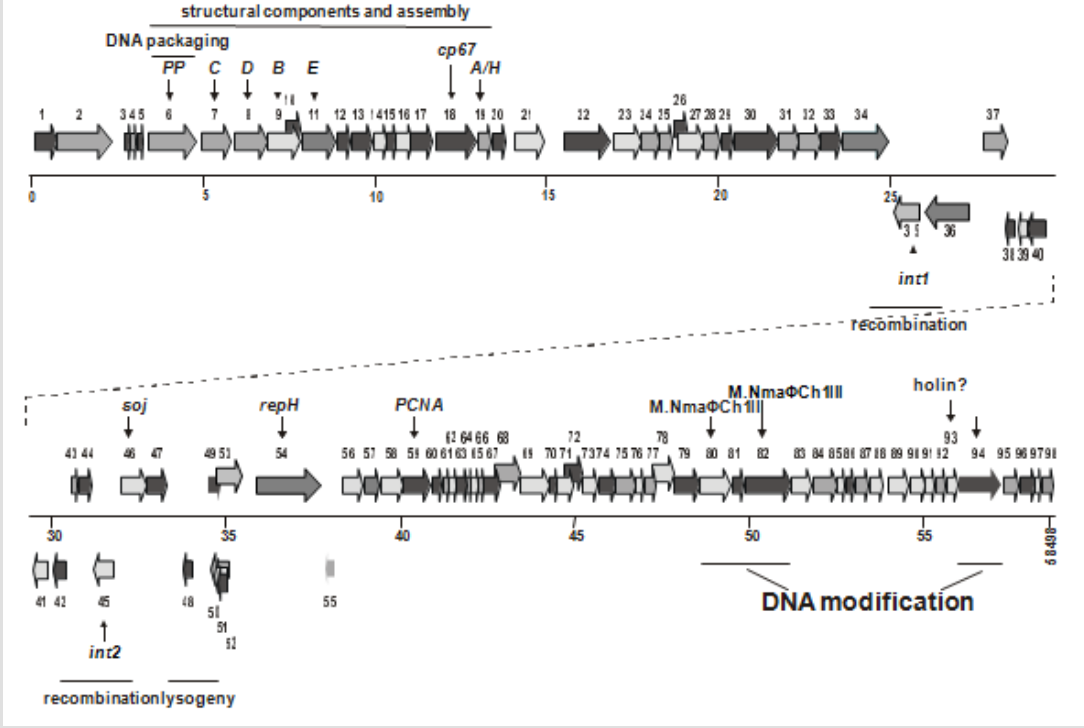


Figure 5: Genomic organisation of the virus ϕCh1
This figure shows a map of the 58 kbp genome of the virus ϕCh1. Arrows indicate the 98 ORFs proposed. The arrow's different shades of grey portend to the reading frame of the respective ORF. Genes of known or suggested function are inscribed.
adapted from Klein *et al.*, 2002

In 2002, Klein *et al.* achieved the first ever complete sequence of a halophage by determining the DNA composition of ϕCh1 (figure 5). They could obtain a total number of 98 open reading frames (ORFs) probably coding for proteins, most of which start with ATG, whereas four ORFs are predicted to have GTG as a start codon. The general codon usage was shown to reflect these of halophilic *Archaea*. Since a considerable amount of ORFs face the same reading direction, transcriptional units can be proposed. While the left part as well as the right part of the genome only consist of rightward-transcribed genes (ORF1-34 and ORF56-98 respectively), the middle part consists of a mixture of left- and rightward transcribed genes (ORF35-55). Further sequence analysis and comparison to data bases revealed 48 of the total 96 ORFs to have sequence similarities with known gene sequences. Moreover, 17 ORFs matched with genes or gene products of known function, whereas the other 31 sequences were compared to conserved proteins of unknown function. A majority of these conserved domains were similar to known sequences of *Hbt. salinarum* phage ϕH (Klein *et al.*, 2002). Further comparison of the ϕCh1 chromosome with ϕH followed. As mentioned above, the L – segment of the phage ϕH can circularise and replicate autonomously (Gropp *et al.*, 1992). A central part of the ϕCh1 sequence showed to be of high similarity with this ϕH region, even though the close genomic relation of the two phages stands in contrast to the different environments their hosts live in. For instance, ϕCh1 host *Nab. magadii* lives in salt lakes of high pH values whereas *Hbt. salinarum* does only tolerate neutral pH. Nevertheless, pH gradients in salt lakes do exist and can bring the two hosts in close proximity to each other. It is suggested that the two phages diverged at some point in evolution (Klein *et al.*, 2002).

Restriction analysis of the ϕCh1 genome visualised a typical modification pattern. In detail, 5'-GATC-3' and related sequences are methylated at adenine residues in a fraction of ϕCh1 genomes (Dam-like methylation). Since the *Nab. magadii* genome is not methylated, the virus is coding for its own methyltransferase (Witte *et al.*, 1997). A fifth base, N6-methyladenosine was found by liquid chromatography; furthermore, the corresponding N6-adenine methyltransferase gene was identified. The methyltransferase mRNA and protein showed to occur in the lysogenic phase of the ϕCh1 life cycle, designating it as "late gene". In the lytic state of the phage, only 5 % of the viral genomes carry the specific methylation. This number increases greatly to approx. 50 % in lysogenic phase (Baranyi *et al.*, 2000). The methyltransferase corresponds to ORF94 of the ϕCh1 genome (Klein *et al.*, 2002). Its function is not known so far, yet as a possibility, escaping the restriction system of an unknown host can come into account (Baranyi *et al.*, 2000). Up to this point, two more methyltransferases are known in ϕCh1, representing the ORFs 80 and 82 (Klein *et al.*, 2002).

1.2.2.3.3 Protein composition

Angela Witte and colleagues identified 4 major and 5 minor proteins of the phage ϕCh1 in early stages of their investigations. The gene products revealed to have quite low isoelectric points, ranging from pH 3.3 to pH 5.2. This is due to the fact that halophilic proteins consist of an over proportionally high number of acidic amino acids. Therefore, the virus reflects the protein build up of its host (Witte *et al.*, 1997).

1.2.2.3.4 Further characterisation of selected open reading frames

ORF11 – protein E:

ORF11 codes for the predominant structural protein of the phage ϕCh1 that is expressed in an extensive amount in the host cell. The corresponding gene (start to stop codon) has a size 966 bp (total ϕCh1 DNA: nucleotide 7756 – 8721), coding for the 35.8 kDa protein E. The G+C content of 63.36 % reflects the overall ϕCh1 G+C proportion. The calculated isoelectric point of 3.74 is due to the over proportional amount of acidic amino acids in the protein, typical for halophilic proteins. The gene and gene product respectively are highly conserved structures, strongly resembling the structural protein HP32 of *Hbt. salinarum* phage ϕH. Its function was determined experimentally (Klein *et al.*, 2000; Klein *et al.*, 2002). Apart from the HP32 sequence similarity of approx. 80 % at amino acid level, further relationships with structural proteins were observed. ORF13 of *Methanococcus thermoautotrophicum* phage cM2, coding for a 35 kDa structural protein (Pfister *et al.*, 1998) as well as the ORF xkdG gene product of *Bacillus subtilis* prophage PBSX (Krogh *et al.*, 1996) seem to resemble protein E (Klein *et al.*, 2000).

In the host's wild type strain L11, harbouring the prophage, messenger RNA (mRNA) of ORF11 can be detected in a late phase of the ϕCh1 live cycle, occurring 72 h after inoculation of the culture. Interestingly, when infecting the host strain L13 (formerly cured of the virus) transcripts of ORF11 can be detected earlier. A possible explanation might be that in L13 the virus enters the lytic stage directly instead of going into lysogeny first. Protein E was detected by western blotting about 24 hours before onset of lysis. Proteins that are associated with the host's membrane are often involved in DNA packaging and progeny assembly. Typically, these proteins are cleaved to relieve assembled phages (Siegel and Schaechter, 1973). As expected, experiments revealed that the majority of protein E is associated with the host's membrane after expression, and that cleavage of the protein occurs. Western blot analysis showed two bands, corresponding to the cleaved and uncleaved form of protein E, respectively (Klein *et al.*, 2000).

ORF93 – a putative holin?

From start to stop codon, open reading frame 93 has a size of 332 bp, corresponding to the bases 55431 to 55763 in the overall ϕCh1 sequence. The assumed ORF codes for a small protein of 11.8 kDa. Computer analyses revealed three *trans*-membrane helices (figure 6) as well as a dual-start motive, typical for holin proteins (Klein *et al.*, 2002). So far, no holin protein from an archaeal phage has been confirmed (Wang *et al.*, 2000), yet the observed features of ORF93 strongly indicate a relatedness to the family of holins.

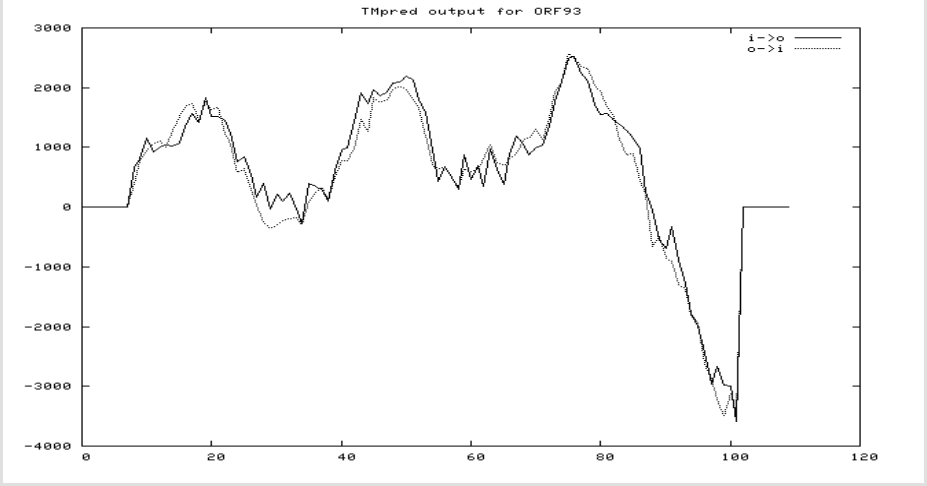


Figure 6: Proposed TM domains of ORF93
Three *trans*-membrane domains are proposed for ORF93. Here, the program TMpred was used. The strongly preferred model predicted by the program includes an N-terminus outside of the cell as well as the three *trans*-membrane helices.
(http://www.ch.embnet.org/software/TMPRED_form.html)

Concerning bacteriophages, holins are proteins that act in concert with endolysins (murein hydrolases) to achieve bursting of the viral host cell and therefore release of progeny phage particles. The endolysins are usually accumulating in an active and fully folded state in the host's cytosol, not gaining access to their substrate, the cell wall. At this point the holin protein comes into play. Holins are small membrane proteins that are associated with permeabilisation of the cell membrane and collapse of its potential. It is unknown whether holins actually form a defined hole or channel. However, the length of the vegetative cycle is determined by holins, since they control the access of endolysins to the cell wall murein (Wang *et al.*, 2000).

An active state of holin proteins is resulting in death of the host cell. Subsequently, the schedule of holin action has to be tightly controlled. If the host cell is lysing too early, progeny virions might be assembled only insufficiently, therefore resulting in non-functional phage particles. On the other hand, delaying the escape from the host's cell is also disadvantageous.

A prolonging of the viral live cycle results in decrease of the total number of progeny viruses, taking into account that every released phage particle immediately infects a new host. To maximise the viral progeny, holin proteins should not have toxic properties until the perfect time for host's lysis. It is of importance that the holin ensures prompt release of an excess of endolysins, therefore resulting in immediate and complete collapse of the host's cell wall (Wang *et al.*, 2000).

Two major classes of holins exist. Class I holins, like *Escherichia coli* phage λ holin protein S^{λ}, have three potential *trans*-membrane (TM) domains. On the contrary, class II holins, represented by the holin protein S^{21} of phage 21 infecting also *E. coli*, only have two TM domains. The TM domains consist of 20 or more residues. Even though the two proteins show no sequence similarity, they display correlation in a variety of features (Wang *et al.*, 2000). Typically, the C-terminal domain of holin proteins is extremely hydrophilic and charge-rich. Moreover, beta-turn motifs are strongly predicted between the first and second intramembrane domain. Early studies of the holin structures of phages infecting gram negative *Bacteria* suggested that the N terminus may be periplasmic while the C terminus is cytoplasmic (Young, 1992). The location of the C-terminus in the cytoplasm later confirmed to be the most conserved feature of holins. Instead, the N terminus location seems to be dependent on the number of TM domains. While proteins with 3 TM domains are predicted to have an N-terminal periplasmic location, a cytoplasmic location for the N-terminus of proteins with 2 TM domains is suggested. Further studies showed that the C-terminus might be bound to the inner surface of the cell membrane, involving Coulombic interactions. Wang and co-workers further investigated the structure of the holin. The "hole", the interruption of the cell membrane by the holin protein, seems to be a homo-oligomer of the protein. Experiments suggested 8-mer to 10-mer structures, possibly forming an aqueous pore walled by the TM domains. The certain structure of holin proteins yet has to be confirmed. Nevertheless, they are thought to be membrane-disrupting molecules, forming a hole that has to be big enough to release the quite large (>70 kDa) endolysin proteins (Wang *et al.*, 2000).

A feature that has been well studied in the holin protein of coliphage λ, S^{λ}, is the dual-start motif. Surprisingly, S^{λ} codes for two different proteins named $S^{\lambda}105$, the actual holin protein, and $S^{\lambda}107$ that showed to be a holin inhibitor. While the translation of the actual holin starts at codon three, the holin inhibitor $S^{\lambda}107$ is translated from the first start codon. This results in an additional strong positively charged residue in the inhibitor protein. Usually, the actual holin is present to a greater extent (2:1). Interestingly, the time point of cell lysis stays nearly the same when the translation of $S^{\lambda}107$ is inhibited. Indeed, the destruction of the host's cell happens slightly earlier, nevertheless, the lysis timing stays precisely defined. This

data indicate that the holin / holin-inhibitor system operates as a fine-tuning system rather than as a total control system of holin activity. It was also shown for phage 21 holin to have a dual-start motif, serving the same purpose as in phage λ (Wang *et al.*, 2000).

ORF79:

The putative ORF79 ranges from nucleic base pair 47669 until base pair 48460 in the total ϕCh1 genome, thus consisting of 791 bp from start to stop codon. The corresponding protein has a size of 28.7 kDa. The function of ORF79 is completely unknown. Sequence alignment studies did not reveal any related genes or sequences known so far (Klein *et al.*, 2002). Therefore, the putative ORF79 does not seem to code for a conserved protein. It is not clear, weather the open reading frame actually codes for a protein at all, or if it is so called "nonsense DNA" without function. Nevertheless, viruses commonly have the tendency to save genome space. As an example, numerous viruses are known to produce polyproteins that give rise to several smaller proteins. Other strategies in order to keep the viral genome small are alternative splicing, usage of several open reading frames on one DNA sequence or coding proteins on both DNA stands (Skern, 2006). Subsequently, the idea of nonsense DNA in viruses is questionable, and even though unknown so far, the ORF79 gene product most likely does have a function.

1.3 Hosts of Archaeal Viruses

1.3.2 *Archaea*: general overview

In 1990, Carl Woese and co – workers proposed a new system of classifying the living world. After studies on the small subunit ribosomal RNA, they established the scheme of the three domains of life, *Eukarya*, *Bacteria*, and *Archaea* (Woese *et al.*, 1990). Up to this point, the *Archaea*, traditionally known as archaebacteria, were pooled together with the *Bacteria* using the term prokaryotes. However, it was already stated in the early 20th century that *Archaea* and *Bacteria* might only be distant relatives. Stanier encapsulated this in 1971: "Indeed the major contemporary prokaryotic groups could well have diverged at an early stage in cellular evolution, and thus be almost as isolated from one another as they are from eucaryotes as a whole" (Stanier, 1971). *Archaea* are classified into three phyla, the *Euryarchaeota*, the *Crenarchaeota* and the *Nanoarchaeota*, as it was revealed by small subunit ribosomal RNA investigations (Gribaldo and Brochier-Armanet, 2006). The *Nanoarchaeota* so far contain one species exclusively, *Nanoarchaeum equitans*, that has a genome of only 500

kbp and is living attached to the surface of an archaeal host (Huber *et al.*, 2002). A fourth phylum is suggested, termed *Korarchaeota*. Up until now, the members of this candidate division were not cultivated and their existence was revealed by environmental gene sequences only. Nevertheless, a proposed korarchaeal member was characterised in 2008 (Elkins *et al.*, 2008). A phylogenetic tree of the three domains of life and the phyla of the *Archaea* is shown in figure 7.

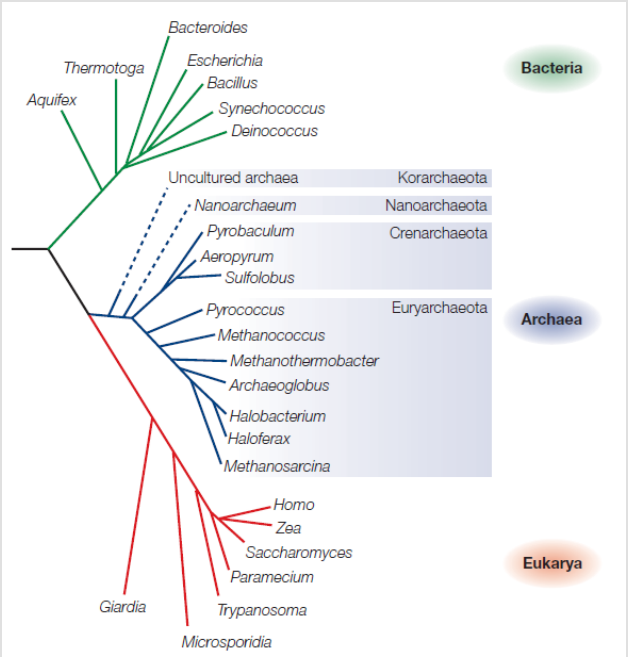


Figure 7: Phylogenetic tree of the domains of life

The three domains of life, *Bacteria*, *Archaea* and *Eukarya* are indicated in the scheme. Furthermore, the different phyla of the *Archaea* are shown. The two biggest groups of *Archaea* known, the *Euryarchaeota* and the *Crenarchaeota* can be recognised as well as the small group of the *Nanoarchaeota*. Furthermore, the suggested phylum of the *Korarchaeota* is shown in the scheme.

adapted from Allers and Mevarech, 2005

Archaea seem to resemble eukaryotes as well as *Bacteria* in many features. Gribaldo and co-workers summarised this fact in 2006: "[...] the *Archaea* look like organisms that use eukaryotic-like proteins in a bacterial-like context." (Gribaldo and Brochier-Armanet, 2006). Nevertheless, there are numbers of unique archaeal features one has to take into account. Therefore, the *Archaea* form a distinct domain, rather than only being a mixture of *Eukarya* and *Bacteria*.

1.3.2.1 Unique archaeal characteristics

Possibly one of the most striking characteristics, suggesting that *Archaea* are a distinctive group of organisms, is their unique cell membrane composition. Archaeal membrane phospholipids consist of glycerol-1-phosphate (G1P) and isoprenol ethers. Membranes of eucaryotes and *Bacteria* are, in contrast, esters of glycerol-3-phosphate (G3P) and fatty acids. Moreover, the enantiomeric configuration of the archaeal glycerophosphate backbone is reversed, compared to those of the other two domains. A prominent characteristic of *Bacteria*

is their murein. In contrast, *Archaea* display numerous of different cell envelopes, whereas murein has never been observed for any of their members. Nevertheless, the murein related molecule pseudomurein was shown in archaeal cell wall compositions in *Methanobacteriales*. Other possible cell wall components are heteropolysaccharides, for example in *Halococcus*. Nonetheless, not all *Archaea* actually do have a cell wall. The group of *Thermoplasmatales* have a cytoplasmic membrane including glycoproteins, whereas most of the *Archaea* cover their cell membrane with a surface-layer (S-layer) of glycoproteins (Forterre *et al.*, 2006).

1.3.2.2 Characteristics reflecting *Bacteria* or eucaryotes

Since the chromosome structure in *Archaea* is similar to the one of *Bacteria*, DNA replication was thought to be related as well. Surprisingly, the archaeal replication machinery is quite distinct to the one of *Bacteria* and seems to resemble the eukaryotic model. It came as a surprise that nearly every discovered archaeal DNA replication protein has a eukaryotic homologue whereas only one investigated protein reflected a bacterial structure (Forterre *et al.*, 2002).

In contrast to *Bacteria*, at least some of the *Archaea* seem to contain multiple origins of replication like eukaryotes. It is proposed that in replication initiation, a *trans*-acting factor binds to the origin of replication and furthermore recruits other replication agents. The DnaA protein was shown to act as this factor in *Bacteria*, whereas in eukaryotes the ORC, the origin recognition complex, binds to the replication origin. Nearly all so far observed archaeal species revealed to have ORC related proteins. Moreover, Cdc6, a protein that is recruited by the eukaryotic ORC, has close archaeal homologues. Further similarities of archaeal replication molecules with those of eukaryotes are found in DNA helicase proteins. Even though their function is not proven, MCM proteins (minichromosome maintenance complex) are the proposed DNA helicases functioning in eukaryotic replication. Up to this point, all sequenced archaeal species showed to contain at least one MCM like locus. This also indicates that, even though the exact function is not yet clear, MCM proteins are strongly conserved and therefore most likely have an important function in both domains, *Archaea* and *Eukarya*. Single strand binding proteins (SSBs), have an important role in protecting single stranded DNA from degradation and modification while for example DNA replication occurs. SSBs are present in *Archaea* as well as in the other two domains, yet one is able to notice quite some differences. While bacterial SSB is a homotetramer, replication protein A (RPA), the eukaryotic SSB complex, is a heterotrimer. SSB proteins are known to bind DNA by oligonucleotide / oligosaccharide-binding (OB). While OB folds are known in both domains, *Bacteria* and *Eukarya*, every bacterial

homotetramer additionally contains an acidic C-terminal domain for protein-protein interactions. Investigated archaeal SSB proteins show a stronger similarity to RPA proteins than to bacterial SSBs. The best studied SSB in *Archaea*, a protein of *Sulfolobus solfataricus*, has a single OB fold. Surprisingly, the domain structure seems to resemble bacterial SSB proteins, especially since a related acidic C-terminal domain was observed. Nevertheless, the OB fold of *S. solfataricus* is more related to human RPA than to bacterial SSBs, as it was determined by crystal structure. It was also shown that *Archaea* have proteins homologous to eukaryotic primases. Moreover, the size of archaeal Okazaki fragments reflects those of eukaryotes, being recognizably shorter than in *Bacteria*. Additionally, a family of replicative DNA polymerases unique to *Euryarchaeota* shows similarities with eukaryotic polymerases in one of the subunits. Further similarities to eukaryotes were revealed in sliding clamp proteins and, concerning *Euryarchaeota*, in the organisation of the chromosome. *Euryarchaeota* showed to have histone structures similar to the eukaryotic H3 / H4 tetrasome (Barry and Bell, 2006).

A feature *Archaea* share with *Bacteria* is the co-directional transcription of frequently transcribed genes with DNA replication, in order to avoid collision of the different machineries (Forterre *et al.*, 2002).

Unlike eukaryotes, *Archaea* have only one type of RNA Polymerase (RNAP). However, its overall structure as well as the subunit composition resembles the eukaryotic RNAPII to a great extent. It is suggested that the function of the archaeal RNAP lies close to the common ancestor polymerase of *Archaea* and eukaryotes respectively (Werner, 2007). Surprisingly, a sequence commemorative of the eukaryotic TATA box was detected. This sequence in the transcription promoter, called box-A, contains the nucleotides TTTAWA (W= A or T). It is localised 27 ±4 bp upstream of the start codon, resembling the distance of the eukaryotic TATA box to its start. Further homologies can be detected in archaeal and eukaryotic general transcription factors, respectively (Langer *et al.*, 1995).

In contrast to the archaeal transcription initiation machinery, resembling the eukaryotic apparatus as mentioned above, most of the candidate transcriptional regulators so far have bacterial homologues. The family of Lrp proteins (leucine-responsive regulator protein) seems to be widely distributed throughout *Archaea* and *Bacteria*. These DNA binding proteins have a size of about 15 kDa and function as important regulators of the amino acid metabolism as well as related processes. Even though most of these regulators show to influence gene transcription in a negative way, positive regulators were also observed. Lrs14, a distant relative of the Lrp family in *S. solfataricus*, binds to the attachment site of the TATA-box binding protein, therefore inhibiting transcription. In contrast, the LrpA protein of *Pyrococcus furiosus* prevents the binding of the RNA polymerase also resulting in transcription inhibition. Further

proteins inhibiting RNAP recruitment have been studied. Nevertheless, so far only one archaeal transcriptional activator is biochemically characterised. Ptr2 protein of *Methanococcus jannaschii*, seemingly facilitating the binding of basal transcription factors, also shows to be a member of the Lrp family (Brinkman *et al.*, 2003; Bell, 2005).

Key players in archaeal translation initiation seem to be closely related to eukaryotic proteins. So far, approx. ten proteins thought to be involved in translation initiation are known. Interestingly, all of them show to have a eukaryotic homologue. Investigations carried out with *S. solfataricus* revealed that the thermophilic archaeon possesses two quite different models of translation initiation. Concerning the first option, a Shine-Dalgarno (SD) sequence on mRNAs with a 5' untranslated region (5'UTR) is compulsory for its translation. SD sequences are known to be typical features of *Bacteria*; however, in *Bacteria* those sequences are not required by any means. Using polycistronic mRNAs, *S. solfataricus* employs the described way of translation initiation. In contrast, a more eukaryotic feature is exerted concerning the translation of monocistronic mRNAs and the first cistron of polycistronic mRNAs, respectively. These mRNAs lacking a 5' UTR are translated by a so called "leaderless" mechanism. Involving the initial transfer RNA (tRNAi), the 30S subunit of the archaeal ribosome is able to bind the mRNA by codon-anticodon interaction (Benelli *et al.*, 2003).

Archaea and *Bacteria* are traditionally united in the term "prokaryotes". Of course, many common characteristics are recognisable if molecular biological methods are not taken into account. Prokaryotes share a similar cell size, they do not have a nucleus and the chromosome is usually circular. As mentioned above, the archaeal chromosome structure is similar to *Bacteria*, both domains organise their genes in so called operons; moreover, the consisting genes are often disposed alike. The operon for ribosomal RNAs in *Archaea* and *Bacteria*, as an example, is arranged in the corresponding pattern 16S-23S-5S rDNA. Furthermore, high similarities in DNA gyrases and toposiomerases of the members of both domains provide additional proof for the obverse genome organisation. Interestingly, *Archaea* possess, unlike eukaryotes, 70S and 30S ribosomal subunits, therefore sharing the bacterial ribosome structure. More correlation between *Bacteria* and *Archaea* can be found in a certain cell division protein, called FtsZ. FtsZ is typical for *Bacteria*, a GTPase that is able to polymerize into filaments, yet it was identified in several archaeal species (Brown and Doolittle, 1997).

1.3.3 *Archaea* under extreme conditions – overview

Organisms living under extreme conditions, so called "extremophiles" are found in all three domains of life, *Archaea, Eukarya* and *Bacteria*. In contrast to mesophilic conditions,

extreme conditions can be defined as surroundings that are generally thought to be hostile to live. Extremophiles need special adaptations to require precise control over biomolecules and electric currents, taking into account that the term “special adaptation” is seen from a human, mesophilic point of view.

Temperature:

Whereas high temperatures can cause problems by for example denaturing proteins, low temperatures, on the other hand, may lead to ice crystal formation within cells, further demolishing biological structures. More problems occur concerning the temperature dependant solubility of gases in water, the degradation of chlorophyll over 75°C and many more. Nevertheless, a member of the *Archaea*, *Pyrolobus fumarii*, was observed living at temperatures up to 113°C. On the contrary, some so called psychrophilic organisms are able to live at temperatures below freezing point (Rothschild and Mancinelli, 2001).

Radiation:

Most importantly, radiation is able to introduce considerable damage to biomolecules, especially nucleic acids. The hyperthermophile *Thermococcus gammatolerans* is the most radiotolerant member of the *Archaea* known so far, enduring gamma ray doses up to 30 kGy. Since no unusual DNA repair enzymes could be found in the archaeon, the features responsible for the high radiotolerance yet remain to be characterized (Zivanovic *et al.*, 2009).

High pressure:

Living in considerable water depths in marine environments goes hand in hand with high amounts of pressure. While the atmospheric pressure on the earth surface amounts to about 100 kPa (= 0.1 MPa), microorganisms living in the deep sea are used to considerable higher pressures. The barophilic archaeon *Thermococcus barophilus* was isolated from a hydrothermal vent at 3550 m of depth and its enrichment was performed under 40 MPa of hydrostatic pressure (Marteinsson *et al.*, 1999).

Salinity:

So called “halophiles” are organisms that can tolerate or strictly require high salt concentrations. Most importantly, the problem of osmolarity has to be taken into account, as well as the problematic ionic requirements of these organisms (Rothschild and Mancinelli, 2001). Halophilic *Archaea* and salinity will be discussed in more detail in the next chapter of this work.

pH:

Acidophiles are organisms that are able to live at very low pH values. As an example, the extreme acidophilic archaeon *Ferroplasma acidarmanus* is able to live at pH values ranging from 0.2 to 2.5, while the optimal growth requires a pH of 1.2 (Dopson *et al.*, 2004). In such conditions, organisms usually face problems like denaturation of proteins and inhibition of biological processes (Rothschild and Mancinelli, 2001). On the other hand, alkaliphilic *Archaea*, *Bacteria* and eukaryotes are adapted to high pH values. Alkaliphily will be discussed in detail in the next chapter.

1.3.4 Halophilic and haloalkaliphilic *Archaea*

The extreme environment containing enormous amounts of salt is, from a mesophilic point of view, impossible to live in. Nevertheless, some *Archaea* adapted to concentrations of more than 20 % of salt, where their growth is at an optimum; therefore not only tolerating, but requiring high salinity (Lanyi, 1974).

The species of the extremely halophilic *Archaea*, belonging to the taxonomic family *Halobacteriaceae*, are divided into 19 genera known until now. 16S rDNA gene sequence studies revealed the haloarchaea to be a monophyletic group, showing sequence similarities of at least 83.2 %. Until 16S rDNA sequencing became an important tool for systematic analyses, the haloarchaeal genera were divided by their membrane polar lipid composition. Interestingly, the data observed by 16s rDNA sequencing mirrored the taxonomy data based on the membrane build-up in a remarkable way (Fendihan *et al.*, 2006).

As mentioned above, the key feature of halophilic *Archaea* is their osmotic adaption to the environment. It is crucial for the organisms to keep their cytoplasms at least isoosmotic with their environment, therefore prohibiting intracellular water to stream out of the cell. Interestingly, within the microbiological world, two different strategies seem to have evolved. The so called “compatible-solute strategy” refers to the tactic where organic compatible solutes are balancing the osmotic pressure of the outer medium, therefore not requiring a high internal salt concentration. To this end, no special adaptation of the biomolecules in the cell is necessary. The second strategy is the so called “salt-in strategy”, therefore referring to a high intracellular salt concentration that mirrors the one of the environment. All members of the *Halobacteriaceae* were shown to apply to this second scheme. While the main salty compound of the surrounding media is usually sodium chloride, the haloarchaea use potassium chloride as an osmotic balance internally. Indeed, all cellular components and biomolecules have to adapt to the high intracellular salt concentration (Oren, 1999). Interestingly, members of the

haloalkaliphilic *Archaea* (discussed in detail below) like *Natrialba*, *Natronomonas* and *Natronobacterium* were shown to additionally use a certain compatible organic solute next to inorganic salts. Grown under optimum conditions but in minimal medium, the organic compound sulfotrehalose was observed and, furthermore, its function as an osmolyte has been proven (Desmarais *et al.*, 1997).

1.3.4.1 Stability of membranes

To maintain stability in high salt environments, extremely halophilic *Archaea* have evolved to use unique characteristics concerning the composition of their cell membranes. Most importantly, the phospholipid archaetidylglycerol-3-methylphosphate (PGP-Me) was observed, that showed to be ubiquitous for this group of organisms. This archaeal analogue of phosphatidylglycerol methylphosphate cannot be found in moderate halophiles and non-halophiles, while it is common in the polar membrane lipids of extreme halophiles to an over proportional extent of 50-80 %. Since PGP-Me is found in the membranes of extreme halophiles exclusively, it is thought to be the key player of membrane stability in high salinity environments. Nevertheless, the mechanism has not yet been understood. In *in vitro* experiments using a water soluble marker molecule, Tenchov and colleagues could observe that the insolubility of the membranes increased strongly with the percentage of PGP-Me molecules. Moreover, membranes with high PGP-Me content showed not to aggregate at high salt concentrations up to at least 4 M and remained stable, suggesting involvement of steric repulsion. On the other hand, this effect could not be observed for membranes with polar lipids of moderately halophilic *Archaea* (Tenchov *et al.*, 2006).

1.3.4.2 Proteins of Halophiles

Most importantly, halophilic proteins face the challenge of being active, soluble and stable in high salt environments, therefore evolving special adaptations. It was shown that specific interactions of salt ions with water molecules as well as the protein itself are required to guarantee solubility and stability (Madern *et al.*, 2000).

Generally, the proteins were found to have an excess in acidic amino acids like aspartic and glutamic acid, contributing to more than 10 mole-% of the overall content. Furthermore, the typical isoelectric points lie around 4.2, while basic proteins are almost completely excluded. The composition concerning hydrophobic amino acids seems to be object of discussion. On the one hand, it was stated by Fendihan and co-workers in 2006 that the content of hydrophobic amino acids is generally low, followed by a poor quantity of

hydrophobic interactions (Fendihan *et al.*, 2006), while Madern and colleagues observed different results. They monitored an increase in small hydrophobic residues like glycine, alanine and valine, and a decrease in aliphatic residues in halophilic proteins (Madern *et al.*, 2000). Nevertheless, the importance of hydrophobic interactions was underlined in the work of Lanyi in 1974, suggesting that the role of these is much more important than examined in other cases. The author observed that in investigated halophilic proteins high concentrations of sodium chloride and potassium chloride are crucial for their stability, which reaches its maximum at salt concentrations over 2 M. By the direct binding of salt molecules to the polypeptide backbone of the proteins, they are thought to effect hydrophobic interactions close to the binding site by ordering water molecules. Therefore, high salt concentrations seem to exhibit a protective effect on halophilic proteins, since their unfolding ability decreases by the formation of new hydrophobic bonds (Lanyi, 1974).

1.3.4.3 (Halo-) Alkaliphily

Soda lakes like Lake Magadi in Kenya or Wadi Natrun in Egypt are considered to be one of the most extreme environments that can be found. These highly alkaline hypersaline environments exhibit pH ranges from 10.5 to 12 and are populated by haloalkaliphilic *Archaea* reaching numbers of 10^7 or 10^8 per ml of water.

While investigating the function of a protein originating in the alkaliphilic *Micrococcus* sp., the observations revealed an optimal pH value of 7.5. Thus, it can be concluded that the internal pH of the organism is around neutral, even though living in a highly alkaline environment. Moreover, *in vitro* studies of alkaliphilic ribosomes showed amino acids to be incorporated into protein at a pH only 0.5 values higher than in common neutrophiles. Therefore it can be concluded that the outer cell components play a crucial role in maintaining the internal pH at a neutral state, excluding the alkaline environment. The role of the cell wall was investigated by comparison of different bacilli, involving alkaliphilic and neutrophilic *Bacillus* species. It was observed that the cell wall of alkaliphilic organisms can contain acidic polymers, suggesting a role of their negative charges. These polymers like galacturonic, gluconic, glutamic or aspartic acid might act as adsorbents of sodium and hydronium ions, while repulsing hydroxide ions, therefore enabling the cell to grow in alkaline environments (Horikoshi, 1999).

Special adaptations of proteins seem to be expendable, since the internal pH shows to be neutral. Nevertheless, this is not the case in terms of proteins that are segregated into the surrounding media, like for example extracellular proteases. As an example, the extracellular alkaline serine protease of an investigated *Bacillus* strain has its optimal functionality at a pH of

11.5, while 75 % of the activity was sustained still at pH 13 (Horikoshi, 1999). One strategy to remain activity is, like in cell walls of alkaliphiles, the extent of amino acids with negative charges that are exposed to the environment. Hereby, as well as in the matter of cell walls, hydroxide ions can be rejected (Shirai *et al.*, 2008).

For energy transduction, the electrochemical gradient of Na^+ and H^+ is used by alkaliphilic organisms. Due to the pH difference outside and inside the cell, the H^+ gradient is able to drive a proton motif force. The high transmembrane pH gradient (ΔpH) is compensated by a strong electrical transmembrane protential (Δψ) leaving the inside of the cell considerably negative. Since many aerobic alkaliphiles are dependent on sodium ions in the surrounding media, they are able to use a Na^+/H^+ antiporter system to regulate the internal pH. This antiporter system is able to provide the necessary H^+ ions inside the cell as well as excluding sodium ions from the cytoplasm. Important for maintenance of this system is the impermeability of the cell membranes concerning the mentioned ions (van de Vossenberg *et al.*, 1999).

1.3.5 Host of ϕCh1: *Natrialba magadii*

Natrialba magadii, originally termed *Natronobacterium magadii*, is a member of the haloalkaliphilic *Archaea*. *Nab. magadii* was isolated from Lake Magadi in Kenya that was also responsible for the archaeon's designation. The isolation of the rod shaped archaeon together with archaeal cocci led to the establishment of the two genera *Natronobacterium* and *Natronococcus* within the haloarchaea by Tindall and colleagues (Tindall *et al.*, 1984). Only in 1997, analysis of the rDNA genes of several alkaliphilic haloarchaea led to reconsideration of their taxonomy. To this end, the former *Natronobacterium magadii* was transferred to the genus *Natrialba*, showing high similarities to its type strain *Nab. asiatica* (Kamekura *et al.*, 1997).

Optimal growth of *Nab. magadii* occurs at 4 M sodium chloride and high pH of 8.5 to 10.5, classifying the organism as a typical member of the haloalkaliphilic *Archaea*. Moreover, the optimum of Mg^{2+} ions lies at concentrations below 10 mM, while the ideal growth temperature ranges from 37 to 42° C. NaCl concentrations below 2 M as well as temperatures somewhat beneath 15° C result in lysis of the cells. The size of the rod shaped archaeal cells lies between 5 and 7 µm. The reddish colour of *Nab. magadii* indicates the carotenoids occurring in the cell membrane, moreover cells are motile due to flagella, which will be discussed in more detail below (Tindall, *et al.*, 1984 and personal observations).

Two strains of *Nab. magadii* are known, called L11 and L13 respectively (figure 8). By repeated sub-culturing and single colony streaking of wild type cells, a non lysogenic strain was

obtained and called L13. *Nab. magadii* strain L13 can be re-infected with the phage ϕCh1, resulting in turbid plaque formation on the agar plate's cell lawn. Furthermore, a colony that is producing phages was isolated from a single plaque. These cells, termed L11, are harbouring the virus and therefore resemble wild type *Nab. magadii* (Witte *et al.*, 1997).

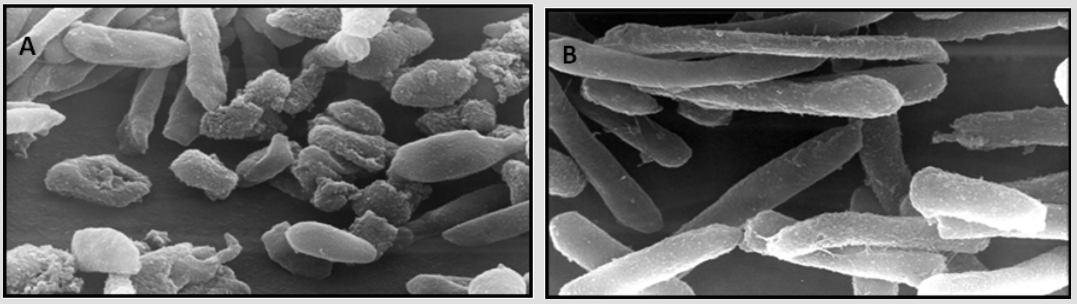


Figure 8: *Natrialba magadii*
Electron micrograph of *Nab. magadii* L11, carrying ϕCh1 as a prophage (A) and *Nab. magadii* L13, cured from the virus ϕCh1 (B).

1.3.5.1 A soda lake as natural habitat

Lake Magadi is a soda lake located in the Rift Valley of Kenya-Tanzania, a highly alkaline extreme environment. Due to the lack of alkaline earth cations like Ca^{2+} and Mg^{2+} in the surrounding topography, carbonate or carbonate complexes become the major anions in solution. Furthermore, a standing body of water deploys because of the shallow depression that forms a closed drainage basin with a high marginal relief. The high rate of evaporation in the arid region of Lake Magadi is exceeding the water inflow by rain; therefore salts can accumulate in the lake. The composition of the surrounding topography in the Rift Valley of Kenya-Tanzania is high in sodium ions, while nearly lacking calcium and magnesium ions. Since the ground water is saturated with CO_2, the molar concentration of Ca^{2+} and Mg^{2+} is greatly exceeded by HCO_3^-/CO_3^{2-} ions. These large amounts of carbonate minerals can generate pH values greater than 11.5 (Jones *et al.*, 1998).

1.3.5.2 Transformation of *Nab. magadii*

Transformation of halophilic *Archaea* was first achieved in the laboratory of Cline, where *Hbt. salinarum* was transfected with naked DNA from phage ϕH. Essential for this method is the removal of the archaeal S-layer, therefore generating spheroblastic cells, what was achieved by EDTA treatment. The established polyethylene glycol (PEG) mediated

transformation method was furthermore adapted for several archaeal species (Cline *et al.*, 1989).

The so called "Halohandbook", a manuscript by M. Dyall-Smith harbours a valuable collection of transfection methods for halophilic *Archaea* (Dyall-Smith, 2009). Nevertheless, an efficient transformation method for *Nab. magadii* yet had to be established, what was accomplished by M. Iro and colleagues in the laboratory of Angela Witte. Since EDTA treatment is not sufficient concerning the removal of the S-layer in *Nab. magadii*, a different approach was used. A combined treatment of cells using bacitracin and proteinase K proved to be the appropriate method to achieve the formation of spheroblasts. By adapting the method of PEG mediated transformation of DNA, successfully transformed cells were obtained (Iro *et al.*, in prep.).

1.3.5.3 Antibiotic resistances

Currently, two antibiotic resistances are known that can be used for molecular work in *Nab. magadii*, novobiocin and mevinolin resistance, respectively.

Novobiocin (nov) is an antibiotic that inhibits the B subunit of the bacterial DNA gyrase. It was also shown to be efficient inhibiting the growth of many *Archaea*, including halophilic *Archaea*. While investigating nov resistant strains of *Haloferax*, the responsible gene was isolated and revealed strong sequence homology to the bacterial gyrase B (*gyrB*). When this sequence was compared to the wild type *gyrB* gene of *Haloferax*, three base changes were displayed. These mutations were revealed to result in base substitutions in the highly conserved N-terminus sequence of the gyrase. Novobiocin is a competitor molecule, binding to the GyrB subunit and inhibiting its activity by blocking ATP access. This mechanism was observed for *Bacteria*, yet the same function can be assumed for *Archaea* (Holmes and Dyall-Smith, 1991).

Mevinolin (mev) is a drug inhibiting 3-methylglutaryl-coenzyme A (HMG-CoA) reductase in eukaryotes and halophilic *Archaea*. By the HMG-CoA reductase, mevalonate is synthesised that is necessary for the isoprenoid side chains of archaeal lipids. As it was described for the nov resistance, mev resistance was also observed by the spontaneous mutation in a member of *Haloferax*. Two possibilities of mev resistance can occur, excessive gene amplification on the one hand, and, on the other hand, up-promoter mutations. Both ways increase the expression of the HMG-CoA reductase and therefore present an excess of the enzyme, leaving the organism resistant to the drug (Lam and Doolittle, 1992).

1.3.5.4 Mutants of *Nab. magadii*

The first ever deletion mutant of a haloalkaliphilic archaeon was achieved by Christian Derntl in the laboratory of Angela Witte in 2009. This was accomplished by disrupting the wild type gene of the *Natrialba* extracellular protease (NEP) by a novobiocin resistance cassette (see above) and further transformation of the harbouring suicide plasmid into *Nab. magadii*. By homologous recombination, gene replacement was achieved. Since *Nab. magadii* is known to be polyploid and therefore contains up to 25 genome copies, passaging of the culture took place in order to receive a homozygote mutant strain. The NEP deficient strain, called P3, is not able to degrade proteins and peptides in the surrounding media anymore. Therefore the nutrient access, especially in the stationary phase, is limited compared to the wild type (Derntl, 2009).

1.3.5.5 Flagellin genes of *Nab. magadii*

Archaea are able to swim by their usage of flagella, as it is often seen in *Bacteria*. Yet in the domain *Archaea* the flagella assembly and mechanism of construction is unique (figure 9).

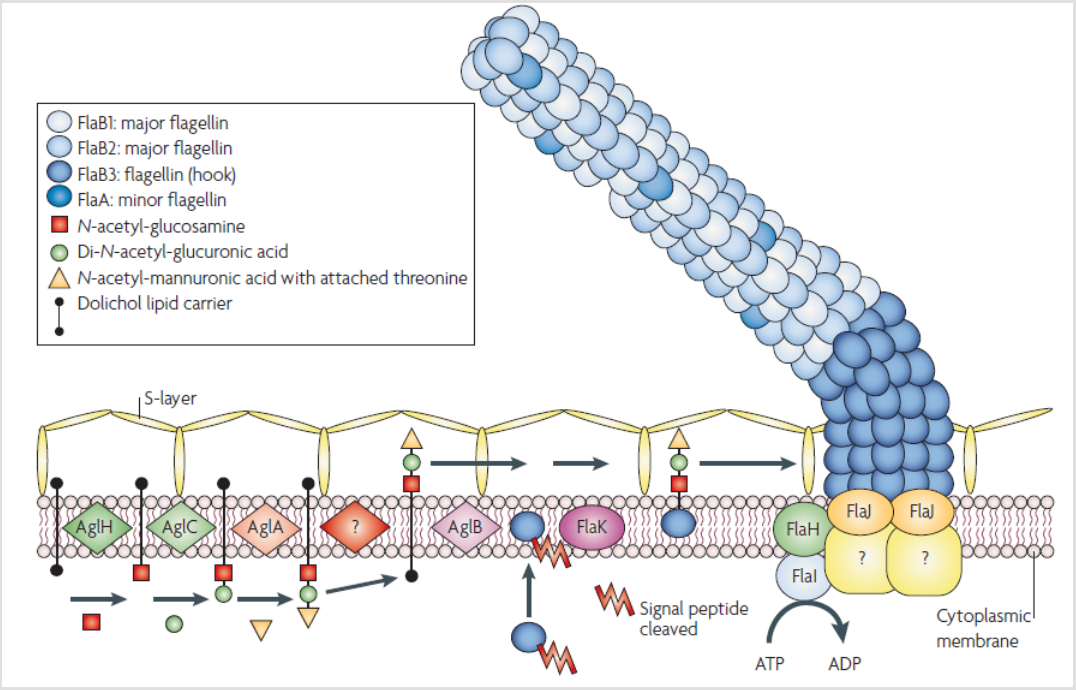


Figure 9: Archaeal flagellum
Model of the structure and assembly of archaeal flagella. Addition of archaeal flagellin subunits is thought to occur at the structural base. Post-translational modifications are known to occur to the archaeal flagellins. For example the indicated FlaK is an archaeal prepilin peptidase like enzyme, cleaving the type IV pilin-like signal peptide on the flagellin.
(Jarrell and McBride, 2008)

Considering their appearance, archaeal flagella seem to be quite similar to their bacterial counterpart. Nevertheless, various studies so far proved the two flagella types to be of different origin, what was shown by molecular analyses. Interestingly, the flagellins of *Archaea* exhibit signal peptides known for bacterial type 4 pili (T4P), and are, moreover, processed by a peptidase similar to the one of T4P. Other homologues to components of T4P have also been observed. While bacterial flagella are assembled by incorporating the respective subunits at the tip, archaeal flagella subunits are assorted at their base. This theory was encouraged by the fact that *Methanococcus maripaludis* deficient in the gene *flaB3* produces filaments without a hook structure. This is only possible if the hook components are the last structures that are added. Typical for archaeal flagella is an N-linked glycosylation, whereas glycosylation in general has rarely been reported for bacterial flagella (Jarrell and McBride, 2008).

The search for flagellin genes in *Nab. magadii* by Serganova and co-workers in 2002 revealed four ORFs in close proximity seemingly coding for flagella components. These ORFs were termed *flaB1- flaB4* and showed strong homology with known archaeal flagellin genes. Another ORF was also observed, named ORF5, displaying some sequence relations to putative flagella related genes of *Methanococcus* species. The organisation of the *Nab. magadii* flagellin operon can be seen in figure 10.

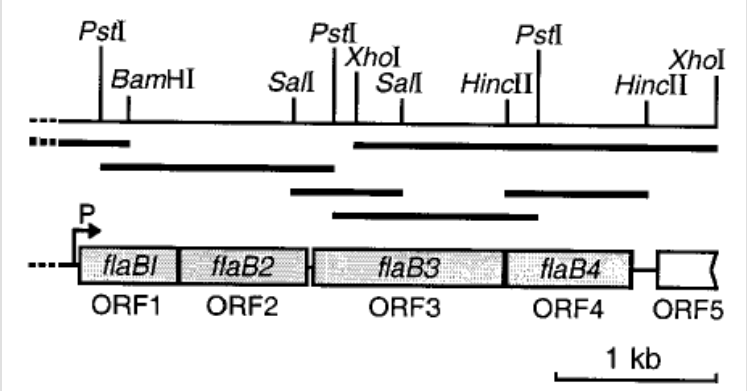


Figure 10: Flagellin genes of *Nab. magadii*
Organisation of the *Nab. magadii* flagellin operon.

(Serganova *et al.*, 2002)

Remarkably, the G+C content of the DNA locus *flaB1-flaB4* is only 54 %, which stands in contrast to the average *Nab. magadii* G+C content of 63 %. This fact was assigned to the low G+C frequency in the third codon position, resulting in further investigations of this unusual matter. Surprisingly, mathematical analysis revealed the codon usage of *Nab. magadii* flagellin genes to resemble that typical for *E. coli*, a mesophilic bacterium. These observations raise the question of the origin of the flagellin genes, suggesting a probable unique evolutionary history. Moreover, the gene *flaB3* contains a large insert, not showing any similarities to known

archaeal flagella genes. This sequence reveals to have an even lower G+C content than the rest of the *flaB* genes, therefore a unique origin can be assumed. Surprisingly, sequence alignment tools exhibited a homology to parts of known *E. coli* flagellin genes. The hypothesis of flagellin gene transfer between *Bacteria* and *Archaea* is supported by the close proximity of a certain *E. coli* strain and *Nab. magadii* that was observed in some geographical areas for example in Kenya (Serganova *et al.*, 2002).

2. Materials and Methods

2.1 Materials

2.1.1 Strains

2.1.1.1 Bacterial Strains

Strain	*Characteristics*	*Source*
E. coli XL-1 Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB $lacI^q$ ZΔM15 T*10* (Tet^r)]	Stratagene
E. coli Rosetta	F^- ompT $hsdS_B$ ($r_B^-m_B^-$) gal dcm, lacY1 (DE3) $pRARE^6$ (Cm^R)	Novagen
E. coli pop2135 F'	endA thi hsdR malt cI857 pR malPQ lac [F' proAB $lacI^q$ ZΔM15 T*10* (Tet^r)]	Institute Pasteur, Paris, France

2.1.1.2 Archaeal Strains

Strain	*Characteristics*	*Source*
Nab. magadii L11	virus ϕCh1 integrated; wild type strain	(Witte *et al.*, 1997)
Nab. magadii L13	cured of ϕCh1	(Witte *et al.*, 1997)
Nab. magadii L11Δ79	virus ϕCh1 deficient in ORF79	this thesis

2.1.2 Media

LB rich medium for *E. coli:*

Peptone	10 g
Yeast Extract	5 g
NaCl	5 g

pH 7.0
add $_{d}H_2O$ to a final volume of 1 litre, autoclave

for agar plates: addition of 15 g Agar per litre

NVM+ rich medium for *Nab. magadii*:

Casamino acids	8.8 g
Yeast extract	11.7 g
Tri-Na citrate x $2H_2O$	0.8 g
KCl	2.35 g
NaCl	235 g

pH 9.0
add $_{d}H_2O$ to a final volume of 934ml, autoclave

for agar plates: addition of 8 g Agar per litre

for soft agar plates: addition of 4 g Agar per litre

After autoclaving, 1 litre of medium/agar was complemented with:

0.57 M Na_2CO_3 (dissolved in sterile $_{dd}H_2O$)	65 ml
1 M $MgSO_4$ (autoclaved)	1 ml
20 mM $FeSO_4$ (dissolved in sterile $_{dd}H_2O$)	1 ml

2.1.3 Antibiotics

2.1.3.1 Antibiotics for *E. coli*

Antibiotic	*Stock conc.*	*Final conc.*	*Explanatory note*
Ampicillin	20 mg/ml	100 µg/ml	diluted in $_{dd}H_2O$, filter sterile, store at 4° C
Tetracycline	10 mg/ml	10 µg/ml	diluted in 70 % EtOH, store at -20° C

2.1.3.2 Antibiotics for *Nab. magadii*

Antibiotic	*Stock conc.*	*Final conc.*	*Explanatory note*
Novobiocin	3 mg/ml	3 µg/ml	diluted in $_{dd}H_2O$, filter sterile, store at -20° C, light sensitive
Mevinolin	10 mg/ml	7.5 µg/ml	diluted in 96 % EtOH, store at -20° C
Bacitracin	7 mg/ml	70 µg/ml	diluted in $_{dd}H_2O$, filter sterile, store at 4° C

2.1.4 Plasmid vectors

Plasmid	*Characteristics*	*Source*
pKSII+	mcs, *bla* colE1 ori, *lacZa*	Stratagene
pUC19	*bla*, pMB1ori *lacZa*, mcs	Yanisch-Perron, Vieira and Messing, 1985
pRo-5	*bla*, ColE1 ori, *gyrB*, ϕCh1 derived ori	Iro *et al.*, in prep.
pMDS11	*bla*, f1 ori, ColE1 ori, *gyrB*, pHK2 ori	Holmes, Nuttall and Dyall-Smith, 1991
pKS-pE	pKSII+ harbouring ORF11 of ϕCh1	this thesis
pΔE::Nov^R	pKSII+ with *gyrB* gene disrupting ORF11 of ϕCh1	this thesis
pKS-holin	pKSII+ harbouring ORF93 of ϕCh1	this thesis
pΔhol::Nov^R	pKSII+ with *gyrB* gene disrupting ORF93 of ϕCh1	this thesis
pKS-ORF79	pKSII+ harbouring ORF79 of ϕCh1	this thesis
pΔ79::Nov^R	pKSII+ with *gyrB* gene disrupting ORF79 of ϕCh1	this thesis
flaB 3/2	pRSET vector contains gene *flaB3*	Lab Angela Witte
pNB102	*bla*, ColE1 ori, *hmg* (mev^R), pNB101 ori	Zhou *et al.*, 2004

pKS-16S Prom	pKSII+ with 16S promoter of *Nab. magadii*	this thesis
pKS-16S Prom-MevR	pKS-16S Prom with mevR under control of the 16S promoter	this thesis
p16S-MevR	16S promoter and mevR moved from MCS	this thesis
p16S-MevR-pRo5Ori	p16S-MevR plasmid with additional origin of replication derived from plasmid pRo-5	this thesis
pRR007	derived from pKSII+ MevR under 16S promoter control, ϕCh1 derived ori, MCS is free for cloning purposes	this thesis
pRSET-C	mcs, *bla*, EK, PT7, rbs, His-tag, pUC ori, f1 ori	Invitrogen
pRSET-nuc	nuclease domain of ORF94 cloned into pRSET-C	this thesis
pPL-RSET-C	derived from pRSET-C, temperature sensitive promoter λ pL	Angela Witte
pPL-RSET-nuc	nuclease domain of ORF94 cloned into pPL-RSET-C	this thesis

2.1.5 Primer

Primer	***Sequence and restriction site (underlined)***	
E-HB-51	5’ – CAGCAGTCTAGACCAGGCGGTGGC – 3’	*Xba*I
E-HB-3	5’ - GACGACAAGCTTTCAGCCATCGTTCACC – 3’	*Hind*III
93-Nae	5’ - GATAGCCGGCCAGTATCGAGATCGCGC – 3’	*Nae*I (*Pdi*I)
93-Pst	5’ - GATACTGCAGGGTATCTGCGTTC GTGG – 3’	*Pst*I
79-Nae	5’ - GATAGCCGGCGACTCTCACAAGATCTC – 3’	*Nae*I (*Pdi*I)
79-Pst	5’ - GATACTGCAGCTCTTTGTACCGATGCGTC – 3’	*Pst*I
Nov-6	5’ - GGGATCGCAGAGGAG C – 3’	

Nov-9	5’ - GATGTCGGTCATCGCGG – 3’	
TR-2	5’ - AATTTCTAGATCCTGGGCCTCTTTGAA – 3’	*Xba*I
79-N1	5’ - GCGCTGTCAGAATCCC – 3’	
79-N2	5’ - GTGGGTACACCGCTTTCC – 3’	
pKS-5	5’ - GTAGCTCTTGATCCGGCA – 3’	
pKS-3	5’ - GCCCTCCCGTATCGTAGT – 3’	
FlaB1-1	5’ - CAGCAAGATCTATGTTCGAACAAAACGACG – 3’	*Xba*I
FlaB2-2	5’ - CAGGAAGCTTAGAGTCGGACCGCTTC – 3’	*Hind*III
FlaB3-1	5’ - GACCGGATCCATGTTCACATCCAATACAGATGA – 3’	*Bam*HI
FlaB4-3	5’ - GGTCGTCGGTAGTTTCGTT – 3’	
16S-5Hin	5’ - CAGGTAAAGCTTGTCCGCCGTACAGATGGGATC – 3’	*Hind*III
16S-3Bam	5’ – CCACGGATCCATAGGGTAGTTTGGCCAC – 3’	*Bam*HI
Mev-5Bam	5’ - GTACGGATCCGGCGCGGGAAACG– 3’	*Bam*HI
Mev-3Xba	5’ - CGTAGTCTAGAGGTGCGAAGTCGGAGGAAC– 3’	*Xba*I
N6-1b	5’-CAGGTGATCATGCAACTTGAAGAACTACCAACACCG-3’	*Bcl*I
N6-540Stop	5’-GACCGGTACCTCAGCCCTCGATGCAGTCCTCG-3’	*Kpn*I

2.1.6 DNA ladders

DNA ladder	***Fragments***	***Explanatory note***
λ *Eco*91I (*Bst*EII)	8454, 7242, 6369, 5687, 4822, 4.324, 3.675, 2.323, 1.929, 1.371, 1264, 0702 [bp]	λ DNA digested with restriction enzyme *Eco*91I (DNA and enzyme: Fermentas)
pUC19 *Hae*III	587, 458, 434, 298, 257, 174, 102, 80, 18, 11 [bp]	plasmid pUC19 digested with restriction enzyme *Hae*III
Biotinylated 2-log DNA ladder	10, 8, 6, 5, 4, **3**, 2, **1**, 1.5, 1.2, 1, 0.9, 0.8, 0.7, 0.6, **0.5**, 0.4, 0.3, 0.2, 0.1 [kbp]	New England Biolabs #N7554S
Gene Ruler^{TM} 1 kb DNA ladder	10, 8, **6**, 5, 4, 3.5, **3**, 2.5, 2, 1.5, 1, 0.75, 0.5, 0.25 [kbp]	Fermentas SM0311

2.1.7 Enzymes and buffers

2.1.7.1 PCR

Enzyme	*Company*	*Explanatory note*
Pwo	PeqLab	origin: *Pyrococcus woeseii* mainly used for cloning techniques
Go*Taq*	Promega	origin: *Thermus aquaticus* mainly used for analytic PCRs
Pfu	Promega	origin: *Pyrococcus furiosus* mainly used for analytic PCRs

Enzymes were used with supplied buffers.

2.1.7.2 Restriction

Enzyme	*Company*	*Explanatory note*
diverse	Fermentas	used with supplied buffers, double digests performed as suggested on the Fermentas webpage
diverse	New England Biolabs	used with supplied buffers

2.1.7.3 Others

Enzyme	*Company*	*Explanatory note*
T4 DNA ligase	Fermentas	used with supplied buffer
Calf intestine alkaline phosphatase	Fermentas	used with supplied buffer or adequate buffers suggested by the company
Klenow fragment	Fermentas	used with supplied buffer or adequate buffers suggested by the company 5’ overhang fill-in after restriction to create blunt ends

T4 DNA polymerase	Fermentas	used with supplied buffer or adequate buffers suggested by the company 3’ overhang removal after restriction to create blunt ends

2.1.8 Buffers and solutions

2.1.8.1 Gel electrophoresis

DNA Fragments < 600 bp	***DNA Fragments > 600 bp***
10x TBE buffer: tris base 108 g boric acid 55 g 0.5 M EDTA pH 8 add $_{dd}H_2O$ to a final volume of 1 L and adjust pH to 8.0 with solid boric acid	50x TAE buffer: 2 M tris- HCl 1 M acetic acid 0.1 M EDTA adjust pH to 8.2
6 % PAA gel: 30 % PAA 1.2 ml 1x TBE 4.8 ml 10 % APS 60 µl TEMED 6 µl 30 % PAA: 29 % acrylamide 1 % N,N'-methylenebisacrylamide	0.8 % agarose gel: solid agarose 1x TAE melted in the microwave

DNA loading dye:

50 mM	Tris-HCl
0.1 %	SDS
0.05 %	bromphenol blue
(0.05 %	xylene cyanol blue)
25 %	sucrose (sterile filtered, add after autoclaving)

adjust to pH 8.2

2.1.8.2 Transformation of *E. coli* – Competent cells

MOPSI:

100 mM	MOPS
10 mM	$CaCl_2$
10 mM	$RbCl_2$

adjust pH to 7.0 with KOH

MOPSIIa:

100 mM	MOPS
70 mM	$CaCl_2$
10 mM	$RbCl_2$
15 %	glycerol

adjust pH to 6.5 with KOH

MOPSII:

100 mM	MOPS
70 mM	$CaCl_2$
10 mM	$RbCl_2$

adjust pH to 6.5 with KOH

2.1.8.3 Transformation of *Nab. magadii*

Buffered high salt spheroblasting solution:

2 M	NaCl
27 mM	KCl
50 mM	Tris-HCl pH 8
15 %	Sucrose

Unbuffered high salt spheroblasting solution:

2 M	NaCl
27 mM	KCl
15 %	Sucrose

Buffered high salt spheroblasting solution with glycerol:

2 M	NaCl
27 mM	KCl
50 mM	Tris-HCl pH 8
15 %	Sucrose
15 %	Glycerol

2.1.8.4 Isolation of *Nab. magadii* chromosomal DNA

High salt alkaline solution:

4 M	NaCl
50 mM	Tris-HCl

adjust to pH 9.5

CsCl solution

1 g solid CsCl / ml $_{dd}H_2O$ (sterile)

14 mM deoxycholate

phenol: chloroform (1:1)

2.1.8.5 Southern Blot

50x Denhardt's solution:

Ficoll 400	1 g
Polyvinylpyrolidone	1 g
BSA	1 g
ad $_{dd}H_2O$	100 ml

store at -20° C

Hybridisation buffer:

$_{dd}H_2O$	60 ml
20x SSC	25 ml
50x Denhardt's sol.	10 ml
1 M NaH_2PO_4	5 ml
20 % SDS	500 µl
0.5 M EDTA	200 µl
solid BSA	0.5 g

store at -20°C

20x SSC:

3 M	NaCl
0.3 M	Na-citrate

pH 7.2

Blocking solution:

NaCl	7.3 g
Na_2HPO_4	2.41 g
NaH_2PO_4	0.96 g
SDS	50 g
pH 7.2	
ad $_{dd}H_2O$	1 L

Washing solution I:

1:10 dilution of blocking solution

10x washing Solution II:

Tris base	12.1 g
NaCl	5.85 g
$MgCl_2$	2.03 g
pH 9.5	
ad $_{dd}H_2O$	1 L

2.1.8.6 Isolation of ϕCh1 phage particles

High salt alkaline solution:

4 M	NaCl
50 mM	Tris-HCl

adjust to pH 9.5

Solution 1.1:

2 M	NaCl
50 mM	Tris HCl pH 8.5 – 9
0.6 M	CsCl

Solution 1.3:

2 M	NaCl
50 mM	Tris HCl pH 8.5 – 9
3.7 M	CsCl

Solution 1.5:

2M	NaCl
50 mM	Tris HCl pH 8.5 – 9
4 M	CsCl

2.1.8.7 Buffers for SDS Page and protein expression methods

4x separation gel buffer

1.5 M	Tris HCl pH 8.8
0.4 %	SDS

4x stacking gel buffer

0.5 M	Tris HCl pH 6.8
0.4 %	SDS

30 % PAA:

29 %	acrylamide
1 %	N,N'-methylenebisacrylamide

10 x SDS running buffer

0.25 M	Tris base
1.92 M	glycine
1 %	SDS

2x Laemmli (protein sample buffer)

0.12 mM	Tris HCl pH 6.8
4 %	SDS
17.4 %	glycerol
2 %	β-mercaptoethanol
0.02 %	bromphenol blue

5mM sodium phosphate buffer

A)	0.2 M	$NaH_2PO_4 \times 2H_2O$
B)	0.2 M	Na_2HPO_4

2.55 ml of A) and 2.45 ml of B) are mixed, $_{dd}H_2O$ is added to a final amount of 200 ml

Coomassie staining solution

25 %	methanol
10 %	acetic acid
0.15 %	Coomassie brilliant blue R-250

Coomassie destaining solution

25 %	methanol
10 %	acetic acid

2.2 Methods

2.2.1 Plasmid preparation from *E. coli*

Plasmid preparation from *E. coli* was performed according to the Fermentas GeneJET™ Plasmid Miniprep Kit. 3 ml of an *E. coli* overnight culture harbouring the desired plasmid were used as source material. All further steps were accomplished by the usage of the supplied buffers and according to the Fermentas protocol. The finally gained plasmid DNA was resolved in 50 µl of $_{dd}H_2O$.

2.2.2 PCR

Polymerase chain reaction is a method aspiring to amplify a certain DNA fragment. This can be used either in order to gain a considerable amount of the DNA for further work, e.g. cloning work, or, on the other hand, to verify the occurrence of a defined DNA sequence in a sample. The 5' and 3' ends, respectively, have to be known in order to perform PCR amplification, as this information is essential for the design of primers.

2.2.2.1 Primers

Primers are short DNA sequences able to hybridise with the 5' and 3' ends of the desired DNA fragment, respectively, therefore acting as the starting points for DNA polymerase. All primers used in this work were obtained from the company VBC genomics and diluted to a stock concentration of 0.1 µg / µl. The annealing temperature of the performed PCR reaction is calculated by the G+C content of the primers used.

2.2.2.2 Templates

Phage DNA, chromosomal DNA or plasmid DNA was diluted according to the quantity of the purification; generally, a 1:30 dilution was performed. For quick examination of cultures, raw extracts were used as PCR templates. For *E. coli* raw extracts, 5 µl of culture were diluted in $_{dd}H_2O$ and heated at 95° C for 5 min. PCR templates of *Nab. magadii* were acquired as follows: 100 µl of a dense culture were centrifuged for 3 min at 13.3 krpm. The pellet was resolved in 100 µl $_{dd}H_2O$ (less if the culture was not dense) and briefly vortexed.

2.2.2.3 Preparative PCR

Preparative PCR was performed using peqGold *Pwo* DNA polymerase from PeqLab. *Pwo* polymerase, originating in *Pyrococcus woeseii,* possesses 3'-5' exonuclease activity. This so called proofreading activity is important for cloning purposes, since sequence replication mistakes are kept at a minimum.

The elongation time depends on the length of the DNA fragment that is amplified. *Pwo* DNA polymerase is able to elongate 1000 bp per minute.

PCR mix solution: 100 µl		***PCR programme***		
68 µl	$_{dd}H_2O$	1.	95° C	5 '
10 µl	10x *Pwo* buffer	2.	95° C	1 '
10 µl	2mM dNTPs (each)	3.	x ° C	1 ' (primer annealing)
5 µl	Primer 1	4.	68° C	x ' (elongation)
5 µl	Primer 2	5.	68° C	10 '
1 µl	Template	6.	4° C	infinite
1 µl	*Pwo* polymerase (1 u / µl)			
		Steps 2 – 4 were repeated 33x		

2.2.2.4 Analytical PCR

Analytical PCR was performed using Go*Taq* DNA polymerase from Promega. In contrast to *Pwo* polymerase, *Taq* polymerase from *Thermus aquaticus* does not have a proofreading function. Since this feature is irrelevant concerning analytical PCR assays and, moreover, Go*Taq* polymerase seems to be more efficient than *Pwo* polymerase, *Taq* is the enzyme of choice in this matter. *Taq* polymerase elongates 1000 bp per minute.

Alternatively, *Pfu* polymerase, originating in the archaeon *Pyrococcus furiosis*, was used. This polymerase proved to be a good option especially for long DNA fragments. For elongation with *Pfu* polymerase, 2 minutes per 1000 bp should be allowed.

PCR mix solution: 50 µl		***PCR programme***		
28.75 µl	$_{dd}H_2O$	1.	95° C	5 '
10 µl	5x Go*Taq* buffer	2.	95° C	1 '
5 µl	2mM dNTPs (each)	3.	x ° C	1 ' (primer annealing)
2.5 µl	Primer 1	4.	72° C	x ' (elongation)
2.5 µl	Primer 2	5.	72° C	10 '
1 µl	Template	6.	4° C	infinite
0.25 µl	Go*Taq* polymerase (5 u / µl)			
		Steps 2 – 4 were repeated 33x		

PCR mix solution: 50 µl		*PCR programme*		
33 µl	$_{dd}H_2O$	1.	95° C	5 ‘
5 µl	10 x *Pfu* Buffer with $MgSO_4$	2.	95° C	1 ‘
5 µl	2mM dNTPs (each)	3.	x ° C	1 ‘ (primer annealing)
2.5 µl	Primer 1	4.	72° C	x ‘ (elongation)
2.5 µl	Primer 2	5.	72° C	10 ‘
1 µl	Template	6.	4° C	infinite
1 µl	*Pfu* polymerase (2 u / µl)			
		Steps 2 – 4 were repeated 33x		

2.2.3 DNA electrophoresis

2.2.3.1 Agarose gel electrophoresis

Gel electrophoresis using an agarose gel was performed concerning DNA fragments > 600 bp. Usually, an agarose concentration of 0.8 % was chosen. The agarose as well as 1 x TAE buffer were melted in the microwave until the solution was clear; subsequently the solution was poured into an appropriate gel tub after cooling down to about 40 - 50 ° C. To obtain the slots for application of the DNA, a comb was applied to the gel tub before pouring the gel. After complete quenching, a solid, approx. 1 cm thick gel was acquired. The gel was completely covered with 1 x TAE buffer and after removing of the comb, the gel is ready for loading DNA samples. DNA samples were mixed with 5 x DNA loading buffer in the appropriate ratio. An electric potential of about 6 V per cm of gel was applied; and, after the run, the DNA was visualised by ethidium bromide.

2.2.3.2 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was used for DNA fragments <600 bp. For this matter, gel casting equipment of the company Bio-Rad (Mini-PROTEAN®) was used and applied as suggested in the protocol. A 6 % PAA gel (for recipe see “materials” section) was poured between two standing glass plates and a fitting comb was applied immediately afterwards. After polymerisation, the gel was covered with 1 x TBE buffer, and the comb was removed. DNA samples were mixed with 5 x DNA loading buffer in the appropriate ratio and loaded onto the gel. A current strength of 15 mA was applied for 26 minutes. Afterwards, the gel was stained in an ethidium bromide solution and visualised under UV light.

2.2.4 Cloning

2.2.4.1 DNA purification

Purification of DNA was performed in order to remove unwanted components after chemical reactions like restrictions, as well as to eliminate small DNA fragments like dNTPs or primers after PCR reactions. Moreover, samples were cleared of bigger, unwanted DNA fragments by the use of a gel extraction kit.

Simple DNA purification was performed with the help of QIAquick PCR purification kit, gel extraction with QIAquick gel extraction kit, both from Qiagen. All steps were accomplished as suggested by the company. The purified DNA sample was eluted in an appropriate amount of $_{dd}H_2O$, depending on the initial sample and the final concentration desired.

2.2.4.2 DNA restriction

DNA restriction was implemented with the help of enzymes and the supplied buffers of the company Fermentas. Additionally, occasional restrictions were performed by supplies of the company New England Biolabs. All enzymes were used at the suggested temperatures and with the supplied buffers; the incubation time was usually 3 hours or overnight. Double restrictions with Fermentas enzymes were, if possible, accomplished with the help of buffer *Bam*HI.

2.2.4.3 Further DNA modifications

For blunt end ligations, the vector DNA was pre-treated with calf intestine alkaline phosphatase (CIAP), acquired from Fermentas. The hydrolysis of the free 5' phosphate groups on the linear vector DNA prevents a re-closure of the plasmid, therefore increasing the possibility of positive clones harbouring a DNA insert. Vector DNA was incubated with CIAP and the appropriate buffer for 30 minutes at 37° C, subsequently the enzyme was inactivated at 85° C for 10 minutes.

Filling-in reactions of 5' overhangs for blunt-end ligations were performed by the Klenow Fragment obtained from the company Fermentas. The reaction was incubated with the supplied buffer and dNTPs (0.05 mM final concentration) for 30 minutes at 37 °C. Inactivation of the enzyme was accomplished by incubation at 75° C for 10 minutes.

Digestion of 3' overhangs for blunt-end ligations was implemented by T4 DNA polymerase acquired from Fermentas. Hereby, the enzyme deploys its 3'-5' exonuclease activity, therefore

removing 3’ overhangs on DNA. The reaction was performed with the supplied buffer at RT for 10 minutes, inactivation of the enzyme followed (70° C, 10 minutes).

2.2.4.4 Ligation

The ligation reaction is performed to link linear vector DNA with a DNA fragment, therefore resulting in a plasmid with DNA insert. For the reaction, T4 DNA ligase was used with the supplied buffer, obtained from Fermentas.

Sticky end ligation:

11.5 µl	DNA fragment (insert)
1.5 µl	10x Ligase buffer
1 µl	Vector DNA
1 µl	T4 DNA ligase (1 u / µl)

Blunt end ligation:

18.5 µl	DNA fragment (insert)
2.5 µl	10x Ligase buffer
1 µl	Vector DNA (CIAP treated)
3 µl	T4 DNA ligase (1 u / µl)

The ligation mix was incubated at 16° C overnight or at RT for 3 hours. The incubation at RT was not performed concerning blunt end ligations. The ligation batch was transformed immediately after incubation, or deep-frozen at -20° C.

2.2.4.5 Transformation of *E. coli*

2.2.4.5.1 $CaCl_2$ competent cells

100 ml of LB medium were inoculated with *E. coli* at a start absorbance (A_{600nm}) of 0.1 at 37°C (shaking), and grown until an A_{600nm} of approx. 0.6 was reached. For pop2135 F’ cells, inoculation was performed at 28° C instead of 37° C. The cells were centrifuged at 10 krpm for 10 minutes at 4° C; the resulting cell pellet was resuspended in 40 ml of chilled MOPSI solution. Subsequently, the cells were incubated on ice for 10 minutes. Further centrifugation, using the same parameters as before, followed. The cell pellet was resuspended in 40 ml of chilled buffer MOPSII and incubated on ice for 30 minutes. Final centrifugation succeeded, again at 10 krpm, 4° C and for 10 minutes. Afterwards, the resulting cell pellet was resuspended in 2 ml MOPSIIa solution that comprises glycerol for storage purposes. The cell solution was divided into aliquots of 100 µl and, if not needed for immediate transformation, stored at -80° C.

2.2.4.5.2 Transformation

For transformation of plasmids gained after plasmid preparation, 1 µl of plasmid DNA (approx. 300 ng / µl) was used. In case of transformation after ligation, the whole ligation batch was applied.

100 µl of competent *E. coli* cells were defrosted on ice for 5 minutes. The DNA for transformation was added and the batch was incubated on ice for another 30 minutes. Afterwards, a heat shock at 42° C was applied for 2 minutes, followed by short incubation on ice (2-5 minutes). 300 µl of LB medium were added, and the cells were incubated at 37 °C for 30 minutes. After regeneration, the cells were plated on LB agar plates with the appropriate antibiotics for selection and incubated overnight at 37° C. For pop2135 F' cells, all 37° C steps were done at 28° C instead.

2.2.4.5.3 Determination of positive clones

Pre-selection of possibly positive clones was achieved by a quick preparation method. Usually, twenty single colony clones were tested for each transformation experiment in the first place, more followed if no positive clone was obtained. The single colonies were inoculated in glass tubes with 5 ml of rich medium with selective compounds and grown overnight at 37° C (28° C for pop2135 F').

300 µl of liquid *E. coli* culture (originating from a single colony) were centrifuged for 3 minutes at 13.2 krpm. The pellet was resuspended in 30 µl of 5x DNA loading buffer by pipetting or vortex. 14 µl of Phenol / Chloroform (1:1) was added, followed by heavy vortexing for at least 20 seconds. After centrifugation for 7 – 10 minutes at 13.2 krpm, the upper phase (aqueous phase) was loaded onto an agarose gel. After visualisation with ethidium bromide, possible positive clones could be recognised by different running behaviour of the plasmid DNA compared to the (mostly) majority of the clones. Depending on the size of the insert, these clones run slightly or considerably higher than empty vectors. Of course, verification of the clones was obtained by PCR analysis of the plasmids, and / or by restriction analysis. Cultures of positively verified clones harbouring the right plasmid were mixed with 50 % sterile glycerol (1 ml of culture, 800 µl of glycerol) and stored at -80°C.

2.2.5 Cloning strategies

2.2.5.1 Clonings for deletions of ϕCh1

The initial vector for all clonings, pKSII+, does not harbour an origin of replication for *Nab. magadii*, moreover, no replication origin is cloned into the plasmid. Therefore, all constructions described here are suicide plasmids in *Nab. magadii*, aiming at homologous recombination of the described ϕCh1 loci into the chromosomal DNA. The orientation of the

Nov^R resistance cassette, since cloning occurs by blunt-end ligation, had to be determined. This was achieved by PCR analysis.

2.2.5.1.1 Deletion of ORF11

pKS-pE:

The ORF11 (protein E) of ϕCh1 was obtained by PCR amplification of purified ϕCh1 DNA, using the primers E-HB-51 and E-HB-3. The resulting DNA fragment of about 1180 bp was cut with the restriction enzymes *Xba*I and *Hind*III placed on the primer sequences. As vector DNA, pKSII+ was used and restricted according to the fragment. The ORF11 DNA fragment was cloned into the vector, resulting in the plasmid pKS-pE.

pΔE::Nov:

The Nov^R (*gyrB*) resistance cassette, harbouring the Nov^R itself as well as the promoter sequence, was obtained from plasmid pMDS11. By cutting the plasmid with the restriction enzyme *Hind*III and following fill-in of the 5' overhangs, the vector was linearised. Further restriction with *Sma*I resulted in excision of the blunt end Nov^R fragment from the rest vector. The fragment was purified by gel extraction.

The plasmid pKS-pE was cut with *Bam*HI within the ORF11 sequence, and the 5' overhangs were filled-in by the Klenow fragment. The NovR cassette was cloned into the plasmid by blunt-end ligation; therefore pΔE::Nov was generated, a plasmid with ORF11 disrupted by the Nov^R.

Determination of Nov^R orientation: primers Nov-6 / E-HB-51; Nov-9 / E-HB-51

2.2.5.1.2 Deletion of ORF93

pKS-holin:

The proposed holin gene, ORF93 of ϕCh1, was acquired by PCR amplification of purified ϕCh1 DNA with the primers 93-Nae and 93-Pst. The fragment of about 410 bp as well as the vector DNA pKSII+ were restricted with *Nae*I and *Pst*I and ligated. The resulting plasmid pkS-holin was the first step in cloning the final pΔhol::Nov vector desired.

pΔhol::Nov:

The Nov^R resistance cassette was obtained as described for plasmid pΔE::Nov. The plasmid pkS-holin was restricted within the ORF93 by the enzyme *Bam*HI. *Bam*HI cuts twice in the ORF93 sequence; the small fragment of approx. 60 bp excised from the plasmid was

removed by DNA purification. The 5' overhangs generated by the restriction enzymes were filled-in to allow blunt end ligation with the Nov^R resistance cassette, resulting in the plasmid pΔhol::Nov.

Determination of Nov^R orientation: primers Nov-6 / 93-Nae; Nov-9 / 93-Nae

2.2.5.1.3 Deletion of ORF79

pKS-ORF79:

The approx. 830 bp DNA sequence of ORF79 of ϕCh1 was obtained by DNA amplification of purified ϕCh1 DNA with the primers 79-Nae and 79-Pst. The DNA was furthermore restricted with the enzymes *Nae*I and *Pst*I; the corresponding cut sites were placed on the primer sequences. As a cloning vector, the plasmid pKSII+ was also restricted with *Nae*I and *Pst*I. Ligation of vector and insert DNA resulted in the plasmid pKS-ORF79.

pΔ79::Nov:

The Nov^R resistance cassette was obtained as described for plasmid pΔE::Nov. Interruption of ORF79 in the plasmid pKS-ORF79 was achieved by restriction with the enzyme *Sac*I. *Sac*I generates 3' overhangs, therefore the DNA had to be treated with T4 polymerase to digest these overhangs in order to achieve blunt end DNA. In the next step, the Nov^R resistance cassette was inserted into the vector, therefore disrupting the ORF79 gene in the plasmid pΔ79::Nov.

Determination of Nov^R orientation: primers Nov-6 / 79-Nae; Nov-9 / 79-Nae

2.2.5.2 Cloning of pRR007

pKS-16SProm:

The 16S promoter fragment or 280bp was amplified from *Nab. magadii* L13 DNA with use of the primers 16S-5Hin and 16S-3Bam. The fragment was cut with *Hind*III and *Bam*HI, as well as the vector DNA pkSII+. The small fragment excised from the vector by this double restriction was removed by DNA purification. Subsequently, the 16S promoter DNA fragment was cloned into pKSII+.

pKS-16S Prom-MevR:

As a vector, pkS-16SProm was used and restricted with *Bam*HI and *Xba*I and purified. The mevinolin resistance gene was amplified from plasmid pNB102 by using the primers Mev-5Bam and Mev3-Xba and subsequently cut with the same enzymes as the vector DNA. The

fragment was cloned into the plasmid, resulting in pKS-16S Prom-MevR, were the mevinolin resistance is under control of the 16S promoter.

p16S-MevR:

The construct of 16S promoter and mevinolin resistance gene was excised from the plasmid pKS-16S Prom-MevR with the enzymes *Hind*III and *Xba*I and the resulting sticky ends of the plasmid were filled up with the use of the Klenow fragment. The plasmid pKSII+ was used as vector DNA and cut with *Nae*I, giving blunt ends, and the fragment of 16S promoter and mevinolin resistance gene was cloned into the site. This work resulted in the plasmid p16S-MevR, harbouring the two genes but leaving the MCS free for further cloning purposes.

p16S-MevR-pRo5Ori

The origin of the plasmid pRo-5, having a size of approx. 3.1 kbp, was excised with the enzymes *Not*I and *Hind*III. The plasmid p16S-MevR, functioning as vector DNA, was cut with the same enzymes. Subsequently, the origin of replication was cloned into the vector, resulting in the plasmid p16S-MevR-pRo5Ori.

pRR007:

The plasmid p16S-MevR was cut with the enzyme *Ecl*136II, leaving it with blunt ends. The pRo-5 origin was gained as described for p16S-MevR-pRo5Ori, but, in a further step, the sticky ends were filled-up with Klenow polymerase and the fragment was cloned into p16S-MevR.

2.2.5.3 Cloning of the nuclease-domain of the M.*Nma*ϕCh1I

pRSET-nuc:

The approx. 450 bp nuclease domain of the methyltransferase gene was amplified by using the primers N6-1b and N6-540Stop. The fragment was first restricted with *Bcl*I and furthermore with the enzyme *Kpn*I. The vector DNA, pRSET-C, was restricted with *Bam*HI and *Kpn*I and the fragment was cloned into the plasmid. For expression analysis, the plasmid was transformed into *E. coli* Rosetta.

pPL-RSET-nuc:

All cloning steps were done like in plasmid pRSET-nuc, but instead of pRSET-C, the vector pPL-RSET-C was used. For expression analysis, the plasmid was transformed into *E. coli* pop2135 F'.

2.2.6 Transformation of *Nab. magadii* L11

2.2.6.1 Generation of competent cells

Three baffled 500 ml Erlenmeyer flasks were filled with 60 ml of NVM+ medium each and bacitracin was added at a final concentration of 70 µg /ml. Each flask was completed with a different amount of a fresh *Nab. magadii* culture (A_{600} = 0.9 – 1.2); 8 ml, 6 ml, and 5 ml respectively, and furthermore incubated at 37° C, shaking at a speed of 160 rpm. Approximately 18 – 24 hours later, the culture that reached the optimum A_{600} of 0.55 – 0.6 was used for all further steps; the other two flasks were discarded.

The *Nab. magadii* culture was centrifuged at 6 krpm for 15 minutes; the resulting cell pellet was resuspended in 35 ml of high salt buffered spheroblasting solution with glycerol. 35 µl of the enzyme proteinase K (final concentration: 20 µg /ml) were added, and the culture was incubated in a 250 ml Erlenmeyer flask at 42° C, shaking at a speed of 140 rpm. After 18 – 24 hours, the cells were usually spheroblastic. Due to the destabilisation and digest of the archaeal S-layer by bacitracin and proteinase K, formerly rod shaped cells change into spheroblastic form, as can be seen by microscopic analysis.

At this stage, the *Nab. magadii* cells are ready for immediate transformation. For storage of the cells, 1.5 ml of spheroblasts are centrifuged at 10 krpm for 3 minutes (RT); the pellet is resuspended in 150 µl of high salt buffered spheroblasting solution with glycerol. Cells can be stored at -80° C in this form. It has to be remarked that the transformation quality decreases with every day of storage; the maximum storage time should not exceed one week. For optimal results, competent cells were always freshly prepared for the work in this thesis.

2.2.6.2 Transformation

Preparation:

The DNA to be transformed was used at a concentration of 0.5 µg /µl; therefore the application of 10 µl DNA resulted in transformation of a total amount of 5 µg DNA. The DNA batch was pre-warmed to 37° C before transformation.

An aliquot of PEG600 (stored at -80° C) was defrosted at 65° C and mixed with high salt unbuffered spheroblasting solution (60 % PEG600; 40 % HS solution). The buffer was kept available at 37° C until use.

Implementation:

- centrifuge 1.5 ml of competent cells at RT and 10 krpm for 3 minutes
- resuspend the cell pellet in 150 µl high salt unbuffered spheroblasting solution (w/o glycerol)
- carefully add 15 µl of 0.5 M EDTA
- incubate at RT for 10 min.
- add 10 µl (5 µg) of DNA to be transformed
- incubate at RT for 5 min.
- add 150 µl of PEG600 / high salt unbuffered spheroblasting solution mix, blend carefully
- incubate at RT for 25 min.
- add 1 ml of NVM+ and mix carefully
- centrifuge for 3 min. at 10 krpm and RT
- carefully resolve the pellet in 1 ml NVM+
- add the culture to a baffled 25 ml Erlenmeyer flask with 19 ml NVM+
- incubate the culture for 24 hours on a shaker at 37° C for regeneration
- add a final concentration of 3 µg / ml novobiocin for selection of positive clones

2.2.6.3 Test transformation of *Nab. magadii* L11

All transformations of *Nab. magadii* had, until this point, involved the strain L13 only. Therefore, test transformation of *Nab. magadii* L11 had to take place in order to expand the approved method of transformation to this strain. As a transformation vector, plasmid pRo-5 was used. Occurrence of the plasmid replicating in the culture was tested with the primers TR-2 and Nov-6, binding to the plasmid's origin of replication for *Nab. magadii* and the novobiocin resistance respectively.

2.2.7 Quick growth and screening of *Nab. magadii* clones

Single colonies of *Nab. magadii* were inoculated in Eppendorf tubes with 750 µl of NVM+ medium and the adequate selective components. The Eppendorf tubes were placed in an Erlenmeyer flask which was inoculated at 42° C shaking heavily. The Eppendorf tubes were opened once per day to allow oxygen ventilation of the strictly aerobe cultures. Using this method, single colonies usually grew to an A_{600} that allows PCR analysis in 2-3 days. For quick examination by PCR, 100 µl each of ten liquid cultures were pooled, mixed well and used for

further preparation of template DNA. This method allowed quick screening of a considerable amount of *Nab. magadii* single colonies. If a positive signal was obtained, the PCR was repeated, whereas each of the ten cultures was tested in a distinct PCR batch.

2.2.8 Determination of L11 harbouring mutant ϕCh1 genes

Since the transformed vectors are suicide plasmids, the occurrence of recombination events was tested. This was achieved by PCR analysis of culture samples, where the primer sequences bind to the desired gene disrupted and the novobiocin resistance respectively.

Primers for disrupted ORF11: Nov-9 / E-HB-51
Primers for disrupted ORF93: Nov-9 / 93-Pst
Primers for disrupted ORF79: Nov-6 / 79-Nae

Since negative controls, batches that were not transformed with DNA, were also growing in the NVM + medium with novobiocin, the occurrence of spontaneous novobiocin resistant cells had to be taken into account. Therefore, it could not be assumed that a positively tested transformation culture only consisted of cells harbouring a positive recombination event. The solution was growing of single colonies (originating in a single cell) on agar plates and further testing by PCR, using the same primers as mentioned above.

2.2.9 Infection of *Nab. magadii* L13 with (mutant) ϕCh1 virus particles

2.2.9.1 Harvesting of ϕCh1 phage particles

The *Nab. magadii* L11 culture harbouring disrupted gene copies was inoculated in NVM+ medium at 37° C and grown until a dense culture was obtained. The culture was inoculated further, until complete lysis of *Nab. magadii* L11 occurred by ϕCh1 and an A_{600} of about 0.4 – 0.5 was reached.

The lysed culture was centrifuged at 10 krpm for 15 min and RT. The culture supernatant was sterile filtered (0.45 µm or 0.20 µm) to dispose of possible occurring L11 cells and obtain a solution with phage particles only.

2.2.9.2 Infection of *Nab. magadii* L13

A freshly grown culture A_{600} = 0.5 – 0.6 was used for the infection. 500 – 700 µl of L13 culture were gently mixed with 100 – 500 µl of phage solution obtained as described above. The batches were incubated at 37° C without shaking for one hour. Afterwards, 200 µl were plated on NVM+ agar plates with 3 µg /ml novobiocin as selective compound, and inoculated at 37° C.

2.2.9.3 Selection of homozygous mutants

Single colonies were obtained as soon as 1.5 - 2 weeks after inoculation of the agar plates. The colonies were picked and grown in Eppendorf tubes with 750 µl NVM+ (3 µg / ml novobiocin) at 42° C while shaking heavily. The grown cultures were tested by PCR analyses as follows:

- Occurrence of the disrupted gene
- no occurrence of the wild type gene (primers used for cloning of the respective ORF)
- no occurrence of rests of the suicide plasmid (primers pKS-5 / pKS-3)

Single colony - derived cultures that showed the desired PCR results were further tested via southern blot analysis (see section southern blot).

2.2.10 Deletion of flagellin genes in *Nab. magadii* L13

2.2.10.1 Homozygation of deletion mutants

Since *Nab. magadii* is known to have up to 25 genome copies per cell, cells harbouring disrupted (flagellin) genes are not necessarily homozygote. In order to enhance the number of mutant genes, so called passaging took place. Cultures containing the novobiocin resistance cassette (*gyrB* gene) disrupting their flagellin genes were observed by PCR analysis. These cultures were grown in 25 ml NVM+ medium with 3 µg / ml novobiocin at 37° C. As soon as the cells achieved an A_{600} of approx. 1, what usually occurred 2 – 3 days after inoculation, 200 µl of the culture were transferred to a fresh Erlenmeyer flask with selective medium. This was repeated 25 times; whereas testing of the passages by PCR was performed approx. every 5 passages (see section below). Finally, single colony streaking was accomplished and testing of liquid cultures originating in single colonies followed, again by PCR analysis.

2.2.10.2 Determination of occurring wild type flagellin genes

As mentioned above, the single colony derived cultures as well as the passaged cultures were tested by PCR analysis. Since two ways of DNA integration by single homologous recombination are feasible, also two different types of mutant cells are possible, assuming that only one recombination event happened per cell. In order to obtain a PCR strategy, the variation of recombination has to be known, what was tested by southern blot analysis (see southern blot section).

Using the mentioned primers, no PCR product is obtained in a cell only containing mutant gene copies. For each possibility of recombination, a different PCR is performed:

- recombination possibility a) : Primer FlaB3-1 / FlaB4-3
- recombination possibility b): Primer FlaB1-1 / FlaB2-2

Both primer pairs result in a PCR band when wild type *Nab. magadii* L13 is used as a template.

2.2.11 Isolation of *Nab. magadii* chromosomal DNA

- 500 ml of a *Nab. magadii* culture were grown until optimally an A_{600} of more than 1 was reached. The cells were centrifuged at 8 krpm and RT for 20 minutes and the cell pellet was resuspended in high salt alkaline solution and completely resolved.
- The solution was shared between two SS34 centrifuge tubes and 5 ml 14 mM deoxycholate were added to each tube. The tubes were carefully rolled / twisted on the table /in the hand until the cells were utterly mixed with the solution, and a slimy, transparent liquid was obtained.
- 7.5 ml of sterile $_{dd}H_2O$ were added to each tube. The tubes were again gently rolled / turned like above, until the content was mixed well. Perfectly mixed batches could be recognised by the appearance of slimy filaments at the tube wall.
- 12.5 ml phenol / chloroform (1:1) were added to each tube. The solution was mixed carefully but thoroughly by repeated inverting of the tubes. Centrifugation followed at 4°C and 10 krpm for 30 – 40 minutes.
- The aqueous, uppermost phase was carefully transferred to a sterile 50 ml Erlenmeyer flask and overlaid with isopropanol (approx. 0.6 x the amount of the transferred phase).

- The DNA was coiled onto a sterile, bended Pasteur pipette, and resuspended in approx. 3ml of sterile $_{dd}H_2O$.
- Sterile EDTA was added at an end concentration of 10 mM.
- Per ml of sample, 1 g of solid CsCl was added and resolved. Moreover, per ml of sample approx. 5 µl of ethidium bromide (stock: 10 mg / ml) were added. The solution should have a gentle pinkish colour after addition.
- The solution was transferred to an ultra centrifuge tube and sealed. Ultra centrifugation took place at 60 krpm and RT for 20 hours.
- The tube was carefully cut open, and the DNA band (= upper pink band, lower pink band = RNA) was cautiously and precisely removed with a pipette and transferred to an Eppendorf tube.
- Removal of ethidium bromide was achieved by extraction with water saturated butanol. Approx. half a ml of butanol was added to the DNA solution and the content was carefully mixed by inverting the tube. The butanol was removed and the procedure was repeated until the DNA lost its pink colour.
- Removal of CsCl and remaining butanol was accomplished by dialysis against sterile $_{dd}H_2O$ for 24h. For optimum results, the water was exchanged once or twice.

2.2.12 Southern Blot: method

The southern blot is a method of DNA detection named after its inventor Edwin Southern. By immobilizing single stranded DNA on a nitrocellulose membrane and further hybridizing with a known DNA probe, certain desired DNA sequences can be detected within an often heterogeneous DNA sample. Therefore, the probe is visualised for example by radioactivity or chemiluminescence methods.

2.2.12.1 Labelling of the probe

Labelling of the probe was accomplished with the help of the NEBlot® Phototope® Kit obtained from New England Biolabs.

A DNA fragment able to hybridise to the DNA of question was first obtained by PCR or restriction, purified and resolved in $_{dd}H_2O$. 34 µl of the DNA solution were heated to 95° C for 5 minutes to obtain single stranded DNA and further incubated on ice for 5 minutes. The following kit components were added to the tube: 10 µl of 5 x labelling mix; 5 µl of dNTPs (harbouring biotinylated ATP); 1 µl of Klenow fragment (3' – 5' exo$^-$). The batch was incubated at 37° C for 3 hours. Afterwards, the reaction was stopped by addition of 2 µl 0.5 M EDTA (pH =

8.2). The DNA was precipitated with 10 µl of 4 M LiCl and 200 µl 96 % EtOH and by incubation at -20° C for 30 minutes. Next, the batch was centrifuged at 16.4 krpm and 4 °C for 30 minutes. Subsequently, the DNA pellet was washed with 1 ml of 70 % EtOH and dried at 65° C for 15 minutes; afterwards it was resolved in 20 µl of $_{dd}H_2O$.

2.2.12.2 Day 1: Separation of DNA sample and blotting

The DNA sample to be tested for the occurrence of a certain sequence was applied to an agarose gel and therefore the fragments were separated by size. A biotinylated DNA ladder was also applied. The gel was set and run according to the above described protocol for gel electrophoresis and the DNA was visualised by ethidium bromide staining.

After the run, the gel was incubated in 0.25 M HCl for 10 minutes, enabling an easier transfer of the nucleic acids to the nitrocellulose membrane by breaking them into smaller fragments. Furthermore, the gel was bathed in 0.4 M NaOH / 0.6 M NaCl for 30 minutes and additionally for another 30 minutes in 1.5 M NaCl / 0.5 M TrisHCl (pH 7.5) solution, in order to achieve and retain separation of the DNA strands.

Transfer of the DNA to a nitrocellulose membrane was achieved by a capillary blot in 10 x SSC buffer overnight. The agarose gel was stained by ethidium bromide, where no longer occurring DNA fragments were proof for a positive transfer.

The membrane was incubated in 0.4 M NaOH and 0.2 M Tris / HCl for 1 minute each. The DNA was fixed on the membrane by applying UV light using the Stratalink cross - linker obtained from Stratagene, thereby achieving cross - linking of the nucleic acids to the membrane.

2.2.12.3 Day 2: Hybridisation with the probe

The membrane was applied to a southern blot tube and 20 ml of hybridisation buffer were added, as well as salmon or herring sperm DNA at a concentration of 100 µg / ml. Therefore, blocking of the membrane by the sperm DNA is achieved in order to inhibit unspecific binding of the DNA probe. The tube was placed into a hybridisation oven for 3 hours at 65° C. After pre - hybridisation, the total 20 µl of the DNA probe prepared were applied to the tube, and incubated in the hybridisation oven over night or at least 15 hours.

2.2.12.4 Day 3: Detection

Detection of the blot is accomplished with the help of the Phototope®-Star detection kit from New England Biolabs. The kit aims at chemiluminescent detection of the biotinylated

DNA probe. Biotinylations on the probe are bound by streptavidin that is itself bound by the biotin linked with an alkaline phosphatase. The alkaline phosphatase used in this kit removes phosphate residues from a specific reagent that is subsequently emitting light.

In a first step, the membrane was washed twice with 2x SSC / 0.1 % SDS buffer at RT for five minutes. Concerning the southern blot verification of a PCR fragment, 4x SSC / 0.1 % SDS buffer was used instead. Subsequently, the membrane was again washed twice with 0.1x SSC / 0.1 % SDS buffer at 65 °C for 15 minutes each.

The membrane was moved to a small basin; wash buffers and detection reagents were used according to the kit. To this end, the membrane was first incubated with blocking solution for 5 minutes; subsequently, 7 ml of blocking solution together with 7 µl of streptavidin were pipetted over the membrane. In the next step, the membrane was washed thrice with washing solution I for 5 minutes each. Afterwards, 7 ml of blocking solution and 7 µl of biotinylated alkaline phosphatase were together pipetted over the membrane for 5 minutes. Incubation with pure blocking solution for five minutes was then followed by three washing steps using 1x washing solution II for five minutes each. Finally, 3 ml of 1x CDP-Star® dilution buffer as well as 6 µl of CDP-Star® reagent were added and pipetted over the membrane for another 5 minutes. Visualisation of the blot was achieved by application and development of a photo film. The exposure time had to be determined experimentally; usually, one to five minutes were sufficient for detection of a PCR fragment, whereas visualisation of fragments in chromosomal DNA samples can take one to 3 hours.

2.2.13 Southern Blot: strategies

2.2.13.1 Strategy for ORF79 deficient ϕCh1

As a southern blot probe, the wild type gene of ORF79 was amplified by PCR, using the primers 79-Pst and 79-Nae and ϕCh1 DNA as a template. Chromosomal DNA of the *Nab. magadii* strains Δ79 and L11 (control) was prepared. The DNA was restricted with the enzyme *Age*I, that cuts upstream and downstream of ORF79 respectively, resulting in the following fragments:

- *Nab. magadii* L11 (wild type control): 2880 bp
- homozygous Δ79 mutant (incorporated Nov^R): 5338 bp
- non homozygous Δ79 mutant: 2880 bp (preferred binding), 5338 bp

2.2.13.2 Strategy for flagellin deficient *Nab. magadii* L13

As probe, the gene *flaB3* of *Nab. magadii* L13 was obtained by restriction of the plasmid flaB3/2, comprising the gene. The vector was cut with the enzymes *Bam*HI and *Hind*III, and the excised DNA fragment was purified. Chromosomal DNA of the strains containing a mutant gene copy, as well as *Nab. magadii* L13 (control), was isolated and restricted with *Pst*I. *Pst*I is cutting the *flaB3* sequence in the chromosomal DNA in two dissimilar parts. Therefore, the southern blot detection of bands is uneven because hybridisation of the probe with the larger part of the *flaB3* gene is preferential. The following southern blot bands can be detected:

- *Nab magadii* L13 (wild type control): 1387 bp (weak signal); 1258 bp
- homozygous recombination variant a): 10841 bp (weak signal); 1258 bp
- recombination variant a) plus wt genes: 10841 bp (weak signal); 1258 bp; 1387 bp (weak signal)
- homozygous recombination variant b): 10712 bp; 1387 bp (weak signal)
- recombination variant b) plus wt genes: 10712 bp; 1387 bp (weak signal); 1258 bp

2.2.14 ϕCh1 phage methods

2.2.14.1 Phage isolation

A pre-culture of *Nab. magadii* was inoculated in NVM+ medium. This L11 culture was either grown from a fresh plaque on a L11 lawn or, alternatively, an L11 culture stock, as well originating from a single plaque, was used. Referring to the second strategy, L11 cultures can be kept well sealed at room temperature for several months.

The pre-culture was used to inoculate 4.5 L of fresh medium and *Nab. magadii* L11 was grown until complete lysis of the cells occurred. The lysis behaviour of the culture was determined by regular measurements of the culture's A_{600}; as a thoroughly lysed culture exhibits an A_{600} of 0.4 – 0.6. In order to remove remaining cells and cell debris, centrifugation of the culture at 8 krpm for 20 minutes at room temperature followed. The supernatant harbouring the ϕCh1 phage particles was moved to a large Erlenmeyer flask; PEG6000 was added at a concentration of 10 % and stirred gently overnight. PEG6000 binds to the phage particles and enables their precipitation by centrifugation, what was accomplished at 8 krpm and RT for 20 minutes. Furthermore, the resulting phage pellet was resuspended in 50 ml of high salt alkaline solution.

A discontinuous CsCl gradient was applied in order to purify the phage particles. The gradient was performed in the following order: 2 ml of solution 1.5, 5 ml of solution 1.3, 6 ml

of phage suspension and approx. 1 ml of solution 1.1 (rest of the tube) were filled into ultracentrifugation tubes. Centrifugation itself took place at 30 krpm, RT and for 20 hours. Two bands could clearly be distinguished in the tubes after centrifugation, one referring to flagella particles of *Nab. magadii* and the other one representing the phage particles. The phage band was carefully removed by pipetting, while trying to keep the quantity as low as possible. In the next step, a continuous CsCl gradient was applied to the phage particles in order to achieve further purification. To this end, the phage solution was mixed with an equal amount of CsCl solution 1.3 and further centrifuged, again at 30 krpm for 20 minutes at room temperature. The phage band was carefully removed like above and finally dialysed against high salt alkaline solution overnight twice. The quantity of phages in the purified solution was figured by phage titre analysis described below.

2.2.14.2 Phage titre analysis

Different dilutions of the phages purified were made in NVM+ rich medium; usually 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-8} and 10^{-9}. 100 µl of each phage dilution were mixed with 400 µl of a *Nab. magadii* culture that was grown until late exponential or early stationary phase. The mixture was added to 5 ml of NVM+ soft agar and poured onto NVM+ agar plates. After growth at 37° C for approx. 2 – 3 weeks, plaques were visible. The number of plaques per plate allows calculation of the plaque forming units per ml (pfu / ml) of the phage suspension; therefore, the quantity of the phage particles in the purification at hand can be concluded.

2.2.14.3 DNA isolation

In order to lyse the phages, 300 µl of phage solution were mixed with 900 µl of sterile $_{dd}H_2O$. DNA isolation was achieved by two phenol / chloroform extractions, whereas the two components are present in equal amounts. The aqueous phase was separated and the containing DNA was precipitated by addition of 96 % EtOH (2.5 x the volume) and incubation at -80° C for 30 minutes. Centrifugation followed at 16.4 krpm at 4 ° C, again for 30 minutes. The resulting DNA pellet was washed in 70 % EtOH twice and dried at 65° C, followed by resuspension in 25 µl of sterile $_{dd}H_2O$. The quantity of the DNA isolation was controlled on an agarose gel.

2.2.15 Protein methods

2.2.15.1 Protein expression studies

An *E. coli* expression strain was transformed with a plasmid harbouring the specific gene to be expressed. A freshly grown single colony containing the expression vector was inoculated in LB medium and the respective selective compounds and grown overnight. This culture was used to inoculate a fresh culture at an A_{600} = 0.1 and 37° C (for Rosetta) or 28° C (for pop2135). At an A_{600} of 0.3 – 0.4, induction of the protein expression was achieved by addition of 0.5 mM IPTG (Rosetta, lac promoter) or heat shift to 42° C (pop 2135 F', heat inducible promoter). After induction, the A_{600} was measured and samples of 1.5 ml were taken every 30 minutes for 2 – 3 hours. The samples were centrifuged at 13.2 krpm for 3 minutes, and the resulting pellet was resuspended in sodium phosphate buffer and 2x Laemmli sample buffer. The volume of sodium phosphate buffer and sample buffer respectively was calculated with the formula [A_{600} x 75 = the amount of buffer [µl]]. By comparing the protein pattern of the culture of choice with a negative control, not harbouring the expression plasmid, the occurrence of the protein could be measured. Therefore, the proteins of the samples were separated by SDS-PAGE.

2.2.15.2 SDS-PAGE

Protein samples were separated by the use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In this method, the proteins in a sample are separated by their molecular weight, since all protein charges are identical due to the use of SDS. The proteins are denatured with the help of SDS and β-mercaptoethanol being part of the protein sample buffer; further denaturation is achieved by heating the samples to 95° C.

For casting of the gel, equipment of the manufacturer Bio-Rad (Mini-PROTEAN®) was used as instructed by the company. First, the reagents for the separation gel (see table below) were mixed on ice and casted between two glass plates about 5 cm high, leaving space for the stacking gel to follow. The separation gel was immediately overlaid with a thin layer of isopropanol, therefore achieving a perfectly horizontal surface. After polymerisation of the gel, the isopropanol was removed. The stacking gel (see table below) was again mixed on ice and poured on top of the separation gel. A comb was fitted between the glass plates immediately, in order to achieve loading slots for the protein samples. After polymerisation, the gel was covered with 1 x SDS running buffer and after removing the comb once again, was ready for loading of the samples.

12 % Separation gel		4 % Stacking gel	
$_{dd}H_2O$	1750 µl	$_{dd}H_2O$	1233 µl
Separation gel buffer	1250 µl	Stacking gel buffer	500 µl
30 % PAA solution	2000 µl	30 % PAA solution	267 µl
10 % APS	60 µl	10 % APS	20 µl
TEMED	10 µl	TEMED	5 µl

Usually, 10 µl of a protein sample were mixed with another 10 µl of 2x Laemmli buffer (protein loading buffer) and heated at 95° C for 10 minutes. Afterwards, 10 µl of the mix were loaded onto the gel. By applying amperage of 15 mA per gel, the sample proteins were separated. Visualisation of the proteins was achieved by bathing the gel in Coomassie staining solution for approx. 20 minutes, followed by destaining of by the respective destain solution. Coomassie brilliant blue is a dye binding to aromatic amino acids, as well as histidine and arginine, therefore making the proteins visible on the gel.

3. Results and Discussion

3.1 Deletion mutants of virus ϕCh1

In the thesis at hand, we aspired to achieve the first ever deletion mutants of a halo(alkali)philic virus by deleting different ORFs of ϕCh1. So far, no deletion mutant of a halo(alkali)philic virus has ever been published.

In 2009, the first ever deletion mutant of a haloalkaliphilic archaeon was achieved by disrupting the gene of an extracellular protease of *Nab. magadii* (Derntl, 2009). This successful deletion attempt followed the establishment of an efficient transformation method for the archaeon, what was also achieved in the laboratory of Angela Witte (Iro *et al.*, in prep.). Indeed, the step to come next was expanding the method by deleting viral genes of ϕCh1, a temperate phage integrated into the *Nab. magadii* L11 chromosomal DNA. The method of deletion deployed by Derntl (Derntl, 2009) was adopted and adjusted for the phage mutations. Hereby, the target gene for deletion was disrupted with a novobiocin resistance cassette, by replacing the respective ORF with the mutant version involving homologous recombination.

Three different ORFs were chosen for the deletion attempt. The ORF11 is a gene of known function, coding for the structural protein E (Klein *et al.*, 2000). This ORF is advantageous because the proposed phenotype of an ORF11 deletion mutant, deficient in protein E, is clear. A ϕCh1 mutant without operating protein E would most likely result in a packing deficiency, therefore the viral progeny would not be able to re-infect cells. The ORF93 is proposed to code for a holin (Klein *et al.*, 2002). By constructing an ORF93 deletion mutant, this theory could be verified. Moreover, a phenotype would possibly be obtained, since a holin is responsible for the host's lysis. ORF79 is a gene of unknown function (Klein *et al.*, 2002). A deletion mutant of this ORF could advance the studies on the gene, and probably even result in elucidation of its function.

3.1.1 Transformability of *Nab. magadii* L11

The established transformation method for *Nab. magadii* (Iro *et al.*, in prep.) so far had only been demonstrated for the strain L13 that is cured from the virus ϕCh1. In order to delete virus genes while the phage is integrated into its host's DNA, transformation of strain L11 had

to be done. Here, we confirm that transformation of *Nab. magadii* L11 is possible, and the same method proposed for the strain L13 can be applied. Transformation of the plasmid pRo-5 resulted in novobiocin resistant cultures, since the respective resistance cassette is included in the plasmid. Moreover, PCR analysis proved the resistant colonies to possess the vector DNA, what is shown in figure 11.

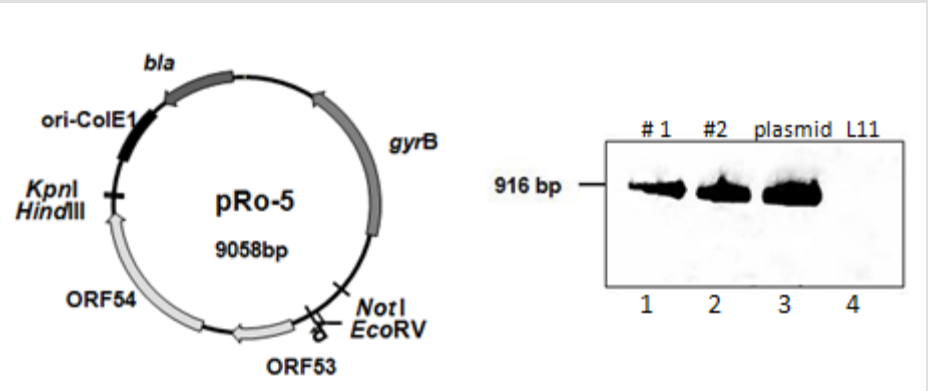


Figure 11: Test transformation of *Nab. magadii* L11
The plasmid pRo-5 on the left hand of this picture was transformed into *Nab. magadii* L11, leaving the strain novobiocin resistant due to the *gyrB* gene. As a control, a PCR analysis was done, screening the *Archaea* for the plasmid (right hand side of the picture). The primers Nov-6 and TR-2 were used, binding within the sequence of the *gyrB* gene and the origin of replication of the plasmid (ORF53/54), respectively. The PCR results in a 916 bp fragment in the tested colonies #1 and #2 (seen in lanes 1 and 2, respectively) as well as the in plasmid (lane 3). When *Nab. magadii* L11 DNA is used as a template, no PCR product is obtained (negative control, lane 4).

3.1.2 ϕCh1 Δ79 – the first ever deletion mutant of a haloalkaliphilic virus

3.1.2.1 Transformation of the suicide plasmid pΔ79::Nov and recombination

The plasmid pΔ79::Nov was constructed and transformed into *Nab. magadii* L11 as described in section “Materials and methods”, including a gene copy of ORF79 that is disrupted with a novobiocin resistance cassette. A map of construction of the plasmid is shown in figure 12. Since the vector is not able to replicate in the archaeon, recombination of the disrupted ORF79 gene with a wild type gene integrated in the chromosomal DNA is necessary in order to achieve novobiocin resistance. Several options of recombination are possible, whereas the most desired way is the mechanism of double recombination. Recombination involving both halves of the ORF79 results in a deleted gene, while the plasmid on the other hand harbours the wild type ORF79 version and is lost due to its lack of an origin of replication.

Therefore, linear plasmid DNA of pΔ79::Nov was transformed in order to force double recombination. This method showed to be of no success, since no recombination event was recognised. Another possibility is a one sided recombination event that results in integration of the whole plasmid in the *Nab. magadii* chromosomal DNA. This one sided recombination can be followed by a second recombination event, excluding the plasmid from the chromosomal DNA once more. As it was determined in the work of C. Derntl, concievably because of the considerable size of the plasmid, this second recombination event immediately follows the first (Derntl, 2009 and personal communication). This leads to either wild type genotypes that are negatively selected in the next step, or to the desired gene disruption event. One possible mechanism of one sided recombination followed by the desired second recombination is shown in figure 13. Transformation of circular plasmid DNA resulted in recombination events in at least three of seven attempts (compare to figure 14). This data was obtained by PCR analysis of the transformed cultures, using the primers 79-Nae and Nov-6. The primers bind sequences in the ORF79 and the novobiocin resistance cassette respectively, resulting in a DNA fragment of about 450 bp, while with an intact ORF79 no PCR product is received (compare to figure 12). The obtained recombination events were also verified by southern blot analysis (data not shown).

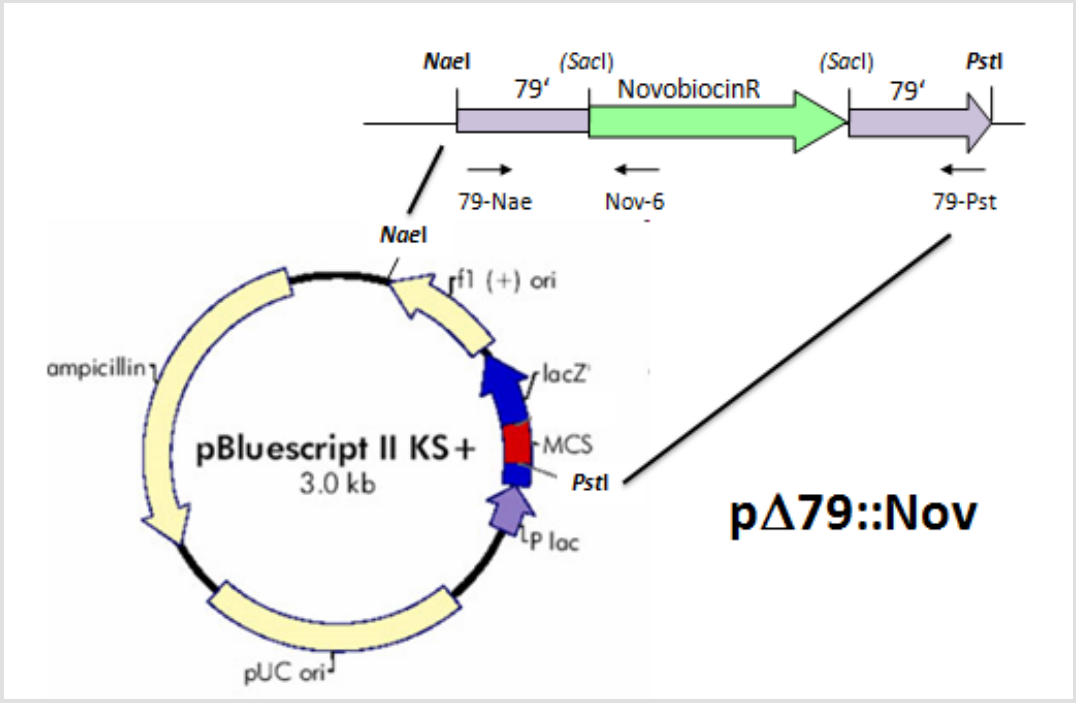


Figure 12: The plasmid pΔ79::Nov
In this picture, the construction of the plasmid pΔ79::Nov is shown. A Bluescript vector (pKSII+) was used as basic vector and the ORF79, amplified by the primers 79-Nae and 79-Pst, was cloned into the plasmid with the respective cut sites. The ORF79 was disrupted with the restriction enzyme *Sac*I, and the novobiocin resistance cassette was cloned into the gap by blunt end cloning. For control PCRs, the primers 79-Nae and Nov-6 were used, binding in the 5' region of the ORF79 gene and the novobiocin resistance cassette, respectively.

An unexpected problem arose, when the negative controls of the transformation experiment were well growing in novobiocin selective medium. Therefore, it could not been assumed that all cells in a culture where a recombination event was positively verified actually are as well positive themselves. In order to solve this issue, streak dilution technique of the transformed cultures was applied and single colonies, most likely deriving from a single cell, were re-tested. After PCR screening of about 60 colonies, a positive colony carrying the deletion was confirmed.

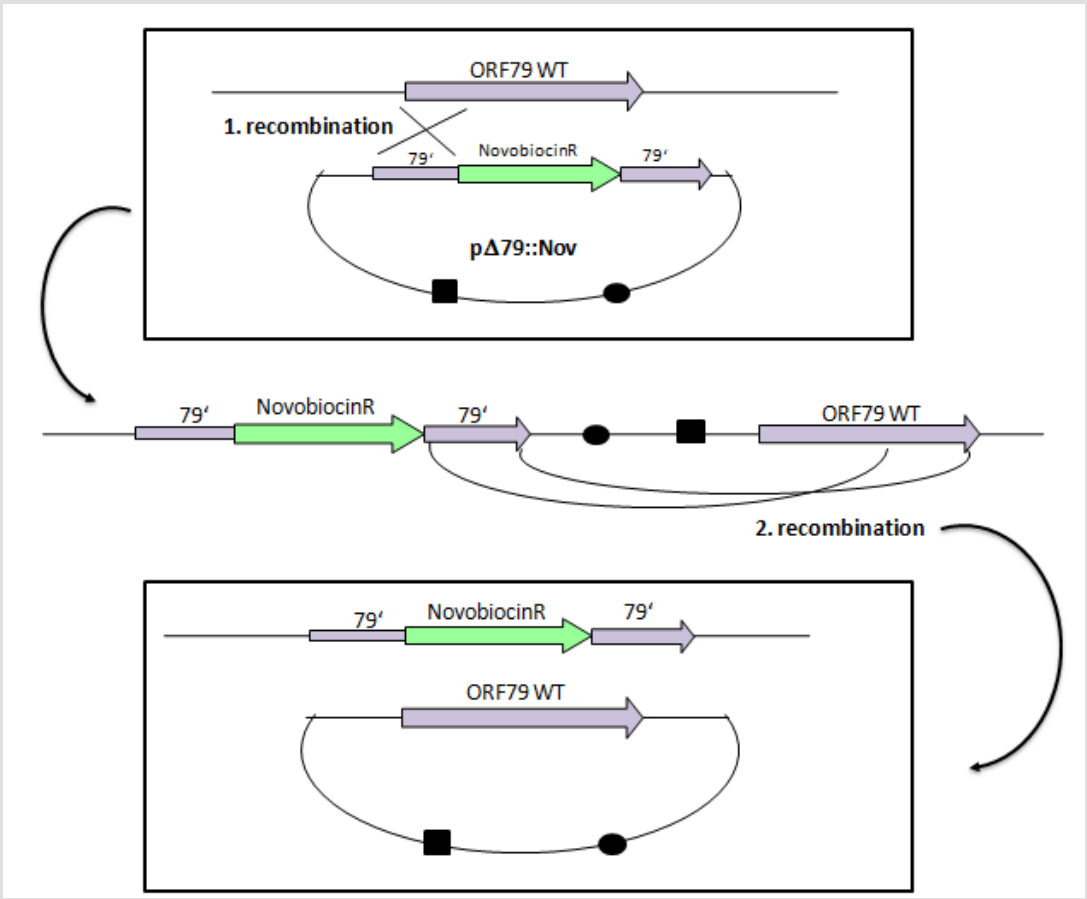


Figure 13: Possible recombination of pΔ79::Nov

In the initial step, the first part of the ORF79 being part of the plasmid pΔ79::Nov recombines with its homologue sequence of the wild type ORF79 gene integrated into the chromosome. The plasmid is therefore incorporated into the chromosome, whereas the wild type ORF79 is still present in the genome. The second recombination event proposed happens between the 3' part of the deleted ORF79 gene and its counterpart in the wild type genome copy. This leads to excision of the plasmid vector, harbouring the wild type ORF79 gene, while the chromosome is left with the disrupted gene version.

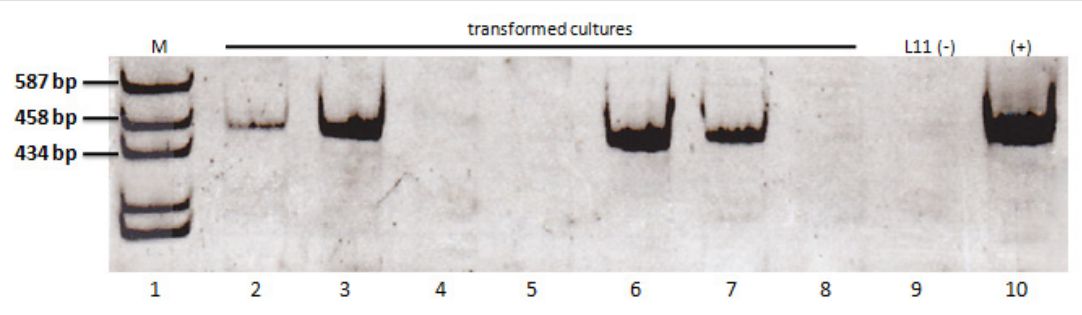


Figure 14: PCR analysis of *Nab. magadii* L11 transformed with pΔ79::Nov
Different cultures of *Nab. magadii* L11 were tested by PCR analysis using the primers 79-Nae and Nov-6. After transformation of the plasmid pΔ79::Nov, recombination occurred in three or four of the seven cultures (see lanes 2, 3, 6 and 7), resulting in a PCR product of about 450 bp. The plasmid was used as a positive control (lane 10), as negative control, an untransformed culture *of Nab. magadii* L11 was analysed (lane 9). Not transformed or positively transformed cultures where no recombination event happened would not give a PCR product (lanes 4, 5 and 8), since pΔ79::Nov is a suicide plasmid and cannot replicate in the *Archaea*. For further steps in this thesis, the culture seen in lane 6 was used, since the strongest PCR signal was obtained here.
6% PAA gel with the marker (M) pUC19/*Hae*III (lane 1)

3.1.2.2 Infection of *Nab. magadii L13* with (mutant) phage particles

Since *Nab. magadii* is known to have up to 25 genome copies, it can be concluded that the phage ϕCh1 is also present in several copies integrated into the chromosomes. Therefore, *Nab. magadii* L11 carrying a deleted gene copy of the viral gene ORF79 might still have a considerable amount of wild type ORF79 genes, what was confirmed by PCR analysis. Indeed, a homozygote deletion of the gene is necessary in order to monitor possible phenotypes and impacts on the viral life cycle. In the work at hand, we were focussing on the so called helper phage effect. The established theory says that the possible function of the deficient gene is taken over by the wild type ORF79 genes, still present in the archaeon's DNA, and the viral life cycle is not altered. Therefore, the mutant phage DNA is packed into virions as regularly as non deleted phage DNA copies, resulting in progeny phages carrying the deletion.

To this end, the L11 culture carrying a deleted gene copy of ORF79 was grown until lysis and the cell supernatant, containing the progeny phage particles, was collected and freed of archaeal cells and cell debris. The supernatant was used to infect fresh *Nab. magadii* L13 cells that are not carrying the virus until this point. It is known that every cell is infected by only one viral particle; therefore, L11 cells are obtained not carrying wild type ORF79 gene copies but only the deleted version. By growth on rich medium agar plates with novobiocin as a selective compound, non infected L13 cells or cells that were infected with wild type virus particles were excluded, since only deleted ORF79 genes carry the novobiocin resistance cassette.

3.1.2.3 Verification and characterisation of the *Nab. magadii* L11Δ79 genotype

Notably, single colonies were obtained as soon as 1.5 weeks after inoculation of the agar plates. Two colonies were picked and grown in rich medium with novobiocin until a dense culture was obtained, suitable for PCR analysis of raw extracts. Both single colony derived cultures showed to contain the mutated ORF79 gene (figure 15), while a PCR product of the wild type gene could not be obtained. Surprisingly, using the primers 79-Nae and 79-Pst, usually amplifying the wild type ORF79 gene (compare to figure 12), a large PCR product of 3200 bp was obtained, even though the elongation time of the PCR cycle was appointed to only one minute. Therefore, it could be assumed that PCR products considerably larger than 1000 bp are not amplified. Nevertheless, this large DNA band was obtained and the size actually fitted perfectly to the whole length of the deleted ORF79, including the novobiocin resistance cassette (compare to figure 16). Since the much shorter wild type gene of ORF79 would preferably be amplified, the existence of wild type genes is very unlikely. The non-existing wild type ORF79 also confirms the realisation of the second recombination event discussed above, where the transformed plasmid including the wild type gene is excluded from the chromosomal DNA once more (compare to figure 13). Nonetheless, the next step to take was the analysis of a properly occurred recombination event, therefore inquiring the occurrence of plasmid DNA in the cell. To this end, the primes pks-5 and pks-3 were used, amplifying a fragment of the originally used vector DNA pKSII+. Surprisingly, rests of the vector could be attested for one of the clones. The second clone was free of vector DNA, and was therefore used for all further purposes (figure 15).

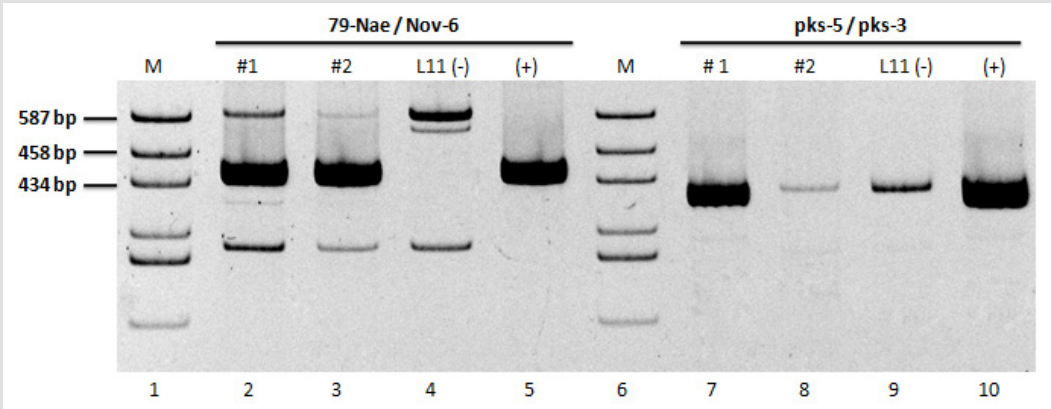


Figure 15: PCR analyses of *Nab. magadii* L13 clones after infection with viral particles ϕCh1Δ79 (1)
On the left hand side of the figure, a PCR analysis with the primers 79-Nae and Nov-6 can be seen. The obtained PCR products in both tested clones, #1 and #2 (seen in lane 2 and 3), confirmed the existence of phages with an ORF79 deletion that infected the cells. The right hand side of the picture shows the PCR analysis with the primers pks-5 and pks-3. Herby, rests of the plasmid pΔ79::Nov were detected in clone #1 (lane 7). Clone #2 was free of rest vector (lane 8), confirming a proper recombination event of the plasmid, excising it from the chromosome. As positive control, the plasmid pΔ79::Nov was used (lanes 5 and 10, respectively), *Nab. magadii* L11 served as negative control (lanes 4 and 9, respectively).
6% PAA gel; marker (M) pUC19/*Hae*III (lanes 1 and 6)

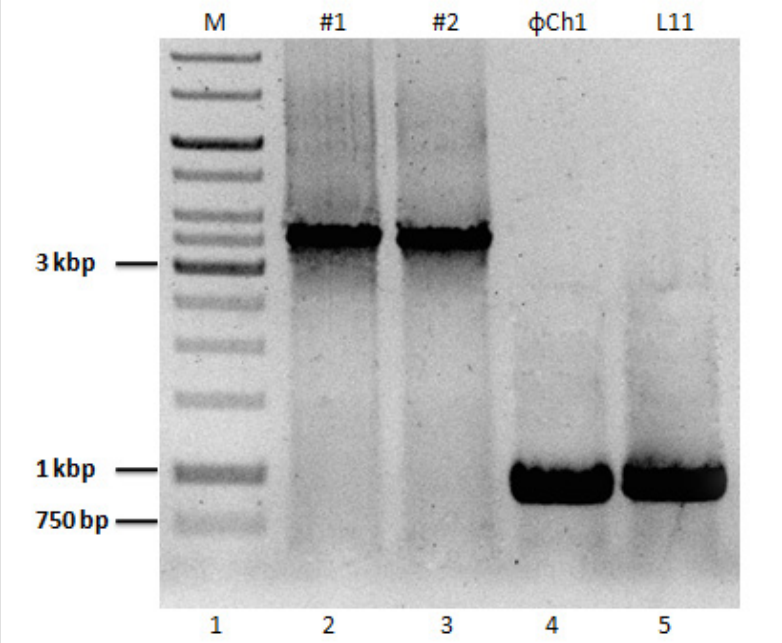


Figure 16: PCR analysis of *Nab. magadii* L13 clones after infection with viral particles ϕCh1Δ79 (2)
PCR analysis of ϕCh1 (lane 4) or *Nab. magadii* L11 (lane 5), using the primers 79-Nae and 79-Pst, resulted in amplification of the wild type ORF79 gene; a PCR product of about 830 bp. When the same primers were used analysing the *Nab magadii* clones #1 (lane 2) and #2 (lane 3) that were infected with ϕCh1Δ79, a PCR product of approx. 3.2 kbp was received. This indicates that the wild type ORF79 gene is not present in these clones. In contrast, the large PCR product represents the deleted ORF79 that is interrupted with the novobiocin resistance cassette.
0.8 % agarose gel, marker (M) Gene Ruler 1 kb DNA Ladder (lane 1)

Further verification of the homozygote *Nab. magadii* L11Δ79 clone was achieved by southern blot analysis. Therefore, chromosomal DNA of the strain, as well as chromosomal DNA of L11 as a control, was isolated and restricted with the enzyme *Age*I. *Age*I cuts sequences in regions up- and downstream of ORF79 in the ϕCh1 DNA, while within the ORF79 or the novobiocin resistance cassette, no restriction site occurs. This leads to a fragment, including ORF79, of 2880 bp in the wild type *Nab. magadii* L11 DNA sequence. On the contrary, the fragment is considerably larger in the mutant strain L11Δ79, since the novobiocin resistance cassette is incorporated into ORF79. Hereby, the length of the DNA fragment amounts to 5330 bp. As a southern blot probe, the wild type gene of ORF79 was used and therefore amplified by PCR. If non disrupted ORF79 genes would still be present in L11Δ79, this probe would preferably bind these wild type genes, since the whole length of the probe is complementary. Deleted gene copies are only bound by half of the probe on each site of the novobiocin resistance cassette. The results revealed that the strain *Nab. magadii* L11Δ79 is definitely free of wild type ORF79 genes, showing a distinct southern blot signal at 5330 bp, referring to the disrupted ORF79, while no signal was obtained at 2880 bp. Concerning the negative control, *Nab. magadii* L11, a signal at 2880 bp could clearly be observed (figure 17).

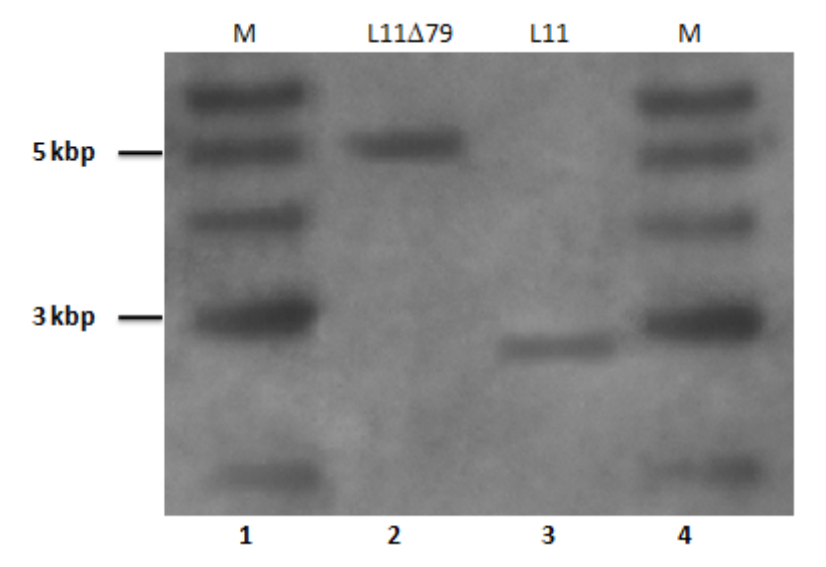


Figure 17: Southern blot analysis of *Nab. magadii* L11Δ79
This figure shows the results of a southern blot analysis. In *Nab. magadii* L11, the wild type control, the wild type ORF79 is detected by a signal at 2880 bp (lane 3). This signal was not obtained in L11Δ79. Instead, a southern blot signal at 5330 bp was received (lane 2), proving the integrated novobiocin resistance cassette deleting the ORF79 gene.
The marker (M) used was the NEB biotinylated 2log DNA ladder (lanes 1 and 4).

3.1.2.4 Phenotype of *Nab. magadii* L11Δ79

First experiments did not reveal a distinct phenotype of the mutant *Nab. magadii* L11Δ79 until this point. The obtained growth curves showed that the archaeal culture is still lysing, therefore a major influence on the lysis behaviour can be excluded, as it would, in contrast, for example be expected for a holin mutant. In the growth curve shown in figure 18, the onset of lysis occurs earlier than in the control culture, *Nab. magadii* L11. This effect is most likely due to the freshly infected Δ79 culture, resulting in fast lysis of the cells, and does presumably not mean a distinct phenotype. Nevertheless, the Δ79 deletion mutant offers a great opportunity for several experiments to be done in the future. As mentioned in a previous section, the probability that ORF79 has a function for ϕCh1 is considerably high and the strain L11Δ79 could provide further insight regarding the viral features and the live cycle of the phage.

3.1.2.5 Discussion

Our achievement to delete the ORF79 in ϕCh1 and to subsequently accomplish the strain *Nab. magadii* L11Δ79 resulted in the first ever deletion mutant of a haloalkaliphilic phage. Since the ORF79 is disrupted by the novobiocin resistance cassette in a region about in the middle of the DNA sequence, the probability that a functioning protein can be translated is very unlikely. The ORF79 gene amounts to approx. 790 bp, resulting in a hypothetical protein

of about 29 kDa. The correctly translated part of the gene in the L11Δ79 deletion mutant would therefore only be about 15 kDa in size. It can be seen as unlikely that this much smaller protein part can fulfil the function of the 29 kDa wild type protein. As mentioned above, the function of the ORF79 derived wild type protein has yet to be determined. The strain *Nab. magadii* L11Δ79 will expectedly enable and encourage a range of future experiments to achieve this designation.

Apart from the possibility to gain insight into the role of ORF79 for the virus ϕCh1, another major point of discussion is the future generation of haloalkaliphilic phage mutants. In the work at hand, we established a method to achieve specific deletions of viral genes incorporated into haloalkaliphilic *Archaea*. This method can be used for further phage mutants; therefore considerable insight into the viruses of haloalkaliphilic *Archaea* in general can be gained. The progress in the research field of haloalkaliphilic *Archaea* and their viruses can generally be regarded to as rather slow; consequently, this established approach could contribute to a future plethora of new knowledge.

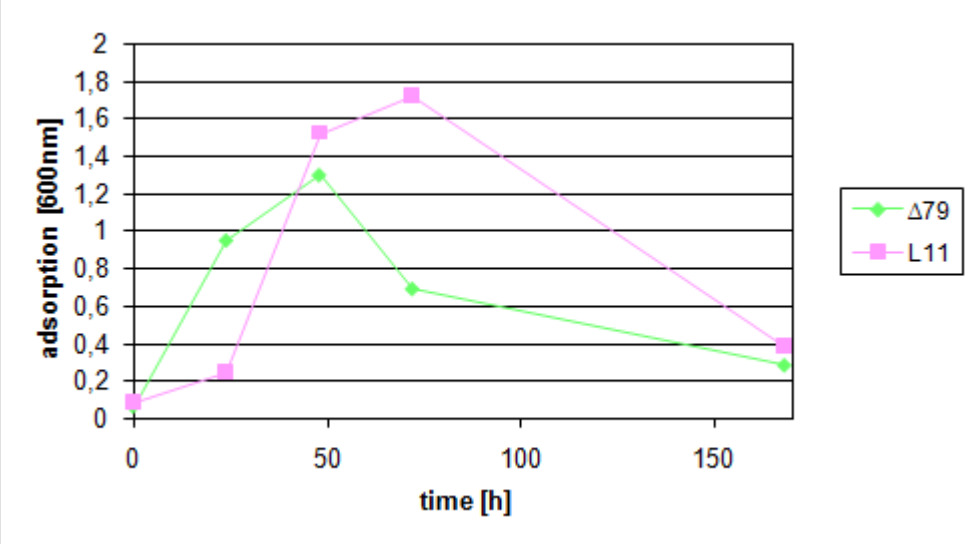


Figure 18: Growth curve of L11Δ79 compared to wild type *Nab. magadii* L11
The figure shows a 7 day (168 h) growth curve of *Nab. magadii* L11 and L11Δ79. In L11Δ79, the cells grew faster and lysis occurred earlier, since the absorption was decreasing strongly 72h after inoculation already. The fresh infection of the *Nab. magadii* strain with the Δ79 phages might be responsible for this fast lysis, and the obtained data does not necessarily mean a general phenotype for L11Δ79.

3.1.3 Further ϕCh1 deletion attempts: ΔE

3.1.3.1 Transformation of the suicide plasmid pΔE::Nov and recombination

Because of the negative experiences while trying to transform *Nab. magadii* L11 with restricted, linear pΔ79::Nov DNA, described above, this approach was not applied. Nevertheless, after several attempts to achieve transformation of the plasmid pΔE::Nov and subsequent recombination of ΔE with the wild type ORF11, still no success could be registered. The following troubleshooting attempts resulted in a test series of transforming different amounts of circular plasmid, using quantities between 3 µg and 10 µg of DNA. Surprisingly, these approaches led to recombination events in nearly every batch, whereas no used amount of DNA could be seen as advantageous for an occurring recombination event, what can be seen in figure 19. Indeed, this successful try of transformation and recombination is notably not due to a specifically used DNA quantity, but contributes to the fact that baffled Erlenmeyer flasks were used to grow transformation competent *Nab. magadii* L11 cells. Because of the advantageous ventilation for the strictly aerobe cells, they are growing faster and their fitness seems to be beneficial concerning their transformability. Subsequently, the probability for recombination is also increasing. Verification of the positive clones was done by analytic PCR analysis, using the primers E-HB-51 and Nov-9 that bind in the 5' region of ORF11 and the novobiocin resistance cassette, respectively.

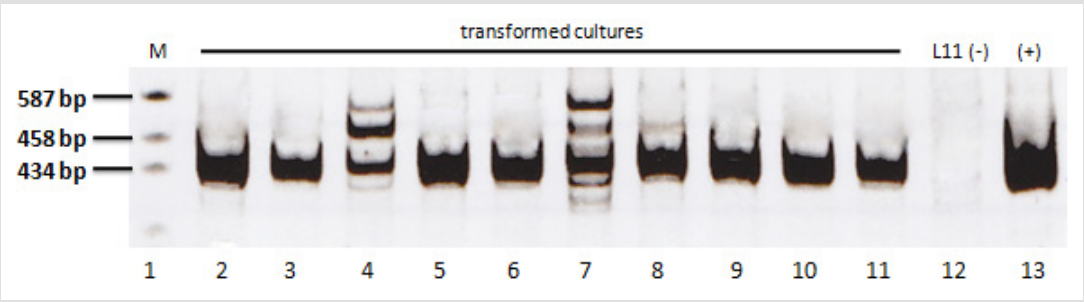


Figure 19: PCR analysis of *Nab. magadii* L11 transformed with the plasmid pΔE::Nov
The PCR analysis with the primers E-HB-51 and E-HB-3 resulted in a product of approx. 450 bp in cultures where transformation of the plasmid and recombination with the wild type ORF11 had occurred. Cultures tested in lanes 2 and 3 were transformed with 10 µg of DNA, in lanes 4 and 5 with 8 µg, cultures in lanes , 6, 7, 8 and 9 with 5 µg and lanes 10 and 11 with 3 µg of plasmid DNA. Except the cultures in the lanes 4 and 7, all cultures are clearly positive, negating an influence of the transformed amount of DNA to the recombination probability. Cultures that were successfully transformed with the plasmid but where no recombination happened, would not give a positive result, since pΔE::Nov is a suicide plasmid. As a positive control, the plasmid pΔE::Nov was used (lane 13). PCR analysis using wild type L11 as a template (negative control) did not result in a PCR product (lane 12).
6% PAA gel, marker (M) pUC19/*Hae*III digest (lane 1)

Again, like in the attempt to transform pΔ79::Nov, the negative control was also growing in novobiocin selective medium, making the application of streak dilution technique necessary. In order to find a culture where all of the cells are harbouring the deleted gene version of ORF11, single cells were quick grown and the resulting cultures were tested by PCR, again using the primers E-HB-51 and Nov-9. In contrast to the attempt of the Δ79 deletion, where approx. 60 single colonies had to be grown and tested for the deletion, several hundred clones had to be screened over nearly four months in order to gain a colony deriving from a single cell harbouring the deleted ORF11 gene.

3.1.3.2 Infection of *Nab. magadii* L13 with (ΔE) phage particles

The L11 culture of cells all harbouring a ΔE deletion in the ϕCh1 chromosome was grown until complete lysis and the supernatant was freed of L11 cells by the use of a filter with the pore size of 0.45 nm. Infection of a fresh *Nab. magadii* L13 culture with the cell supernatant containing (mutant) phages resulted in single colonies that were containing deleted copies of ORF11 as well as, unfortunately, wild type copies of the gene. This observation implied that the pore size of the 0.45 µm filter is probably not tight enough to utterly hold back the archaeal rod shaped cells that have a width of about 0.5 µm. Instead, by using a filter size of 0.20 µm, ϕCh1 phage particles might have a problem fitting trough the filter. Nevertheless, viruses were detected to pass the filter, and the mutant gene copy of ORF11 was detected in the filtered culture supernatant (see figure 20), and infection of *Nab. magadii* L13 was again attempted.

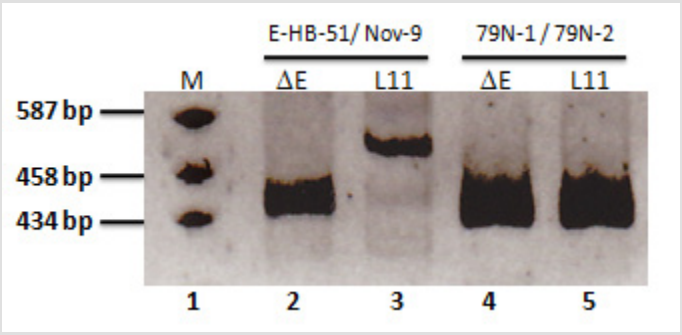


Figure 20: PCR analysis of filtered culture supernatants
The primers 79N-1 and 79N-2 were applied in a PCR in order to prove whether the ϕCh1 phages do fit through the 0.20 µm filter. PCR products were obtained for the L11 supernatant (lane 5) as well as for the supernatant of the culture harbouring the ΔE deletion (lane 4).
The primers E-HB-51 and Nov-9 detected the deleted gene in the supernatant of the ΔE harbouring culture (lane 2), therefore the deleted phage DNA is probably packed into virions. The negative control, wild type L11, did not give a result with these primers (lane 3).
6% PAA gel, marker (M) pUC19/*Hae*III digest (lane 1)

3.1.3.3 Discussion

The infection of *Nab. magadii* L13 with phage particles present in the 0.20 µm filtered culture supernatant was attempted, and the results of this experiment are still outstanding. Since the culture supernatant was obtained to harbour mutant phage DNA, the probability that ϕCh1 phages with a ΔE deletion are packed into virions is high. Nevertheless, the ability of the mutant phage particles to infect *Nab. magadii* L13 has still to be determined. As mentioned above, an obtained strain *Nab. magadii* L11ΔE would probably not be able to produce functioning progeny viruses. Since the virus would be completely deficient in producing the major capsid protein E, packing of the virus particles would be prohibited.

3.1.4 Further ϕCh1 deletion attempts: Δ93

3.1.4.1 Transformation of the suicide plasmid pΔhol::Nov and recombination

Again, after several attempts of achieving transformation and recombination, a striking success could be gained as soon as baffled flasks were used to grow the *Nab. magadii* L11 cells during the process of making them competent for DNA transformation. This supports the theory mentioned above that the good ventilation and the resulting faster growth of the cultures in these flasks achieves fitter cells that can furthermore be transformed easier. Successfully transformed cells were recombination had taken place were confirmed by PCR analysis, using the primers 93-Pst and Nov-9 (figure 21). The primers 93-Nae and Nov-6, formerly employed to verify the positive cloning of the plasmid, were not used due to a PCR product occurring in the negative control.

As well as in the other two phage deletion attempts, streak dilution was done in order to get hold of a single colony, only consisting of cells carrying the mutant phage genes. Unfortunately, until now the screening of nearly 500 clones has not been successful, and the search to find a positive clone is still ongoing.

3.1.4.2 Infection of *Nab. magadii* L13 with (Δ93) phage particles

Nevertheless, an infection of freshly grown L13 cells with culture supernatant has been attempted twice. One of the successfully transformed cultures where recombination was positively confirmed, giving the strongest PCR signal of all, was grown until lysis. The cell supernatant was cleared of occurring cells and cell debris by filtering and infection of the L13 culture followed. The cell supernatant most likely consists of at least a few mutant phage particles, if the helper phage effect can be taken into account. Selection for the cells that were

infected with a mutant virus was done by streaking the culture on rich medium plates with novobiocin. Unfortunately, this attempt did not achieve any positive results.

3.1.4.3 Discussion

A possible explanation for the unsuccessful infection described above might be, that the number of phages in the supernatant carrying the ORF93 deletion was simply too low in order to have an impact on the result. Given the fact, that in nearly 500 tested single colonies of an overall positive culture no *Nab. magadii* L11 cell with the ORF93 deletion was found, and that moreover only one in up to 25 genome copies might carry the mutation per cell, the number of wild type phage particles in the supernatant is enormous compared to deleted viruses. Therefore, the probability that a mutant virus is infecting one of the L13 cells is obviously too low.

The logical consequence is further screening of single colonies in order to gain a culture where every cell has at least one deleted ORF93 copy, and again try infection of *Nab. magadii* L13. *Nab. magadii* L11Δ93, a strain where the virus ϕCh1 is integrated into the archaeon only carrying mutated versions of ORF93, would be expected to have an interesting phenotype. As it was already described in the introduction to this work, ORF93 is very likely to code for a holin protein, responsible for host's cell lysis at the end of the viral live cycle. An ORF93 deletion mutant would therefore probably involve readily packed progeny phage particles that are not able to leave the host cell due to the holin deficiency. If this phenotype would not occur, the proposed function of ORF93 as a holin protein could be questioned.

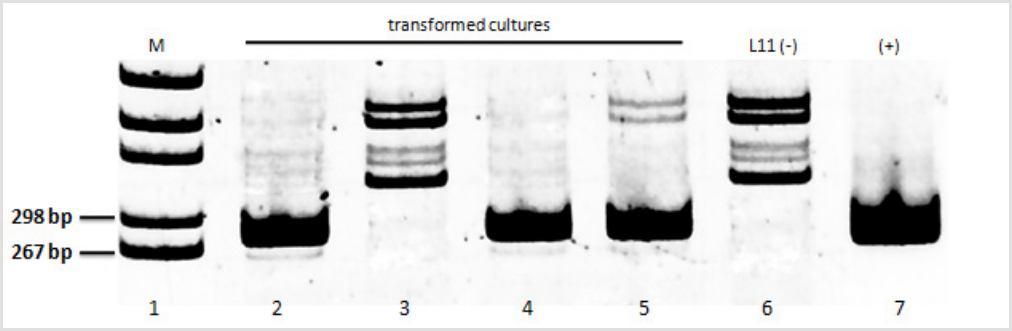


Figure 21: PCR analysis of *Nab. magadii* L11 after transformation with the plasmid pΔhol::Nov
This picture shows a PCR analysis of the *Nab magadii* L11 cultures transformed with the plasmid pΔhol::Nov, using the primers 93-Pst and Nov-9. The PCR resulted in a product of approx. 290 bp in cultures were transformation of the plasmid was successful and recombination of the disrupted ORF93 with the wild type gene had taken place (lanes 2, 4 and 5). Like in the deletion attempt of ORF11, transformation and recombination was accomplished in nearly all batches (not all transformed cultures are shown here), independent of the transformed amount of DNA. Again, cultures where transformation had taken place but recombination did not occur do not result in a PCR product since the suicide plasmid cannot replicate in the *Archaea*. The plasmid pΔhol::Nov was used as a positive control (lane 7), wild type L11 (negative control) did not result in a PCR product (lane 6).
6% PAA gel; marker (M) pUC19/*Hae*III (lane 1)

3.2 Deletion of *Nab. magadii* flagellin genes

This project was already started by Christian Derntl, after achieving the first deletion mutant of *Nab. magadii* by disrupting the gene of its extracellular protease. The search for another gene that's deletion would be followed by an easily detectable phenotype resulted in mutation attempts of the archaeon's flagellin genes.

3.2.1 Method

The *Nab. magadii* flagellin genes consist, as mentioned in the introduction to this work, of the genes *flaB1 – flaB4* and a fifth ORF. *FlaB1-flab4* are organised in an operon and therefore a mutation of two or more genes at once can be done in one step. To this end, a plasmid was constructed, where a part of the genes *flaB2* and *flaB3* was excised, respectively. A novobiocin resistance cassette also being included on the plasmid resulted in resistance to the antibiotic, if recombination had taken place after transformation (figure 22). The gene *ura3* was cloned into the plasmid in order to select for a second recombination to follow the first. Nevertheless, this attempt was shown not to work in *Nab. magadii* and will therefore not be explained further.

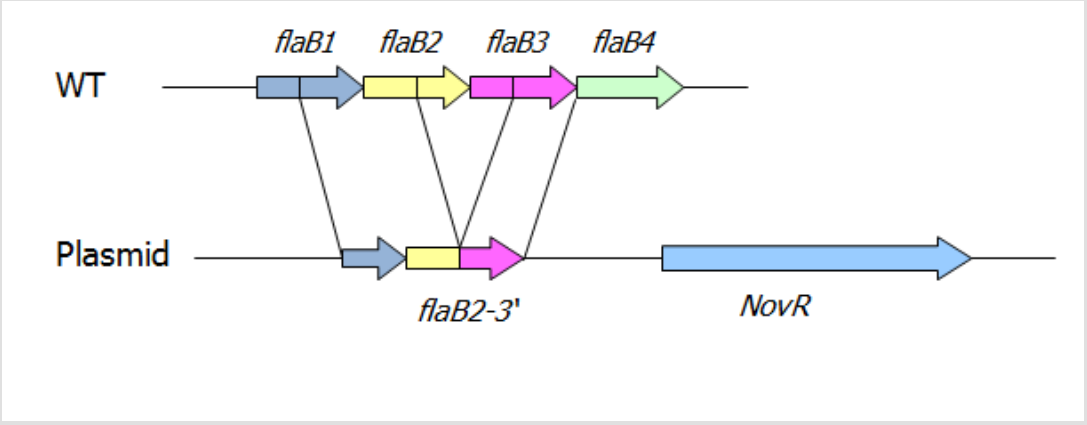


Figure 22: Wild type flagellin operon of *Nab. magadii* and the plasmid FlaB2'Ura3
The flagellin genes *flaB1 – flaB4* of *Nab. magadii* are organised in an operon. A plasmid was constructed, harbouring the 3' region of *flaB1*, the 5' region of *flaB2* and the 3' region of *flaB3*. The plasmid also contains a novobiocin resistance cassette. In order to gain the novobiocin resistance, one sided recombination of the *fla* genes on the plasmid has to take place with the wild type sequence, leading to integration of the plasmid into the chromosome. The plasmid is not able to replicate in the *Archaea*, since it is lacking an origin of replication for *Nab. magadii*.

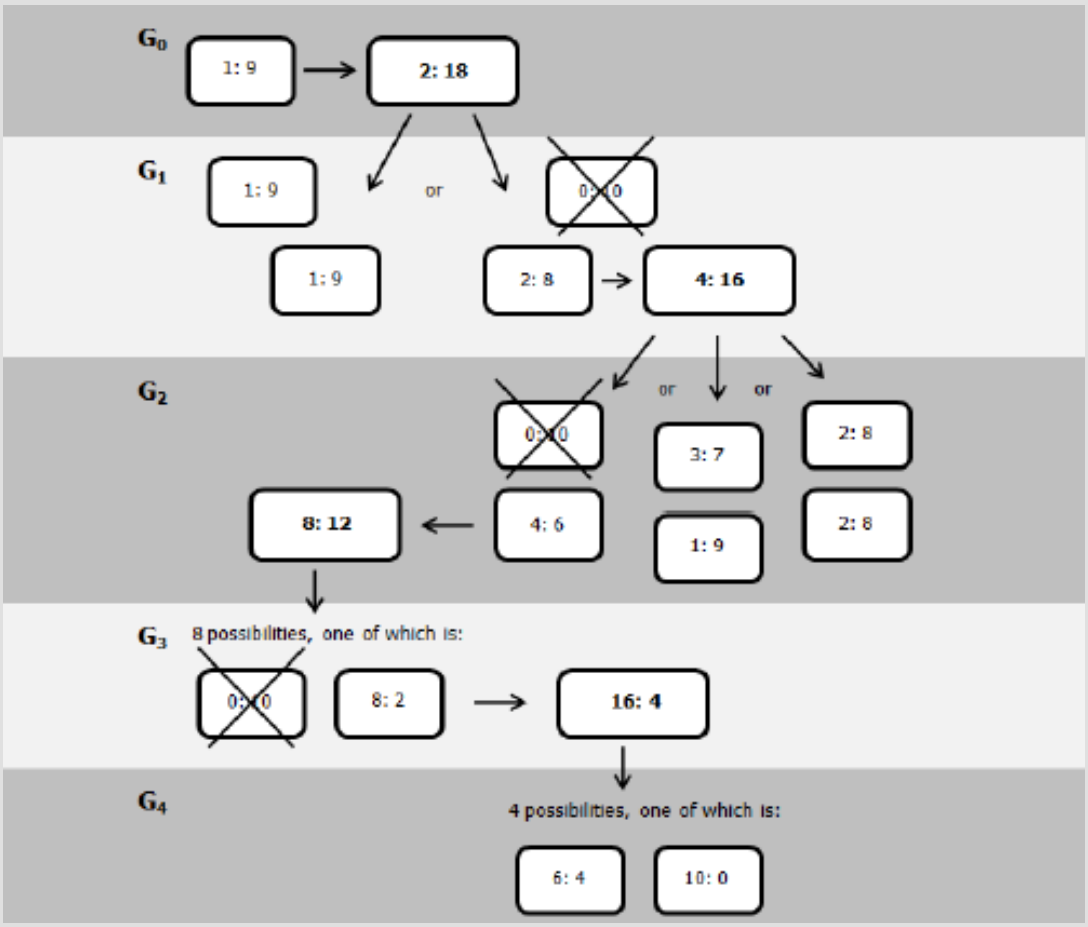


Figure 23: Model for homozygation in polyploid microorganisms

This graphical representation of the homozygation model is based on a theoretical microorganism with a multiplicity of its chromosome of ten copies after cell division. During growth each copy is duplicated once resulting in exactly 20 copies prior to cell division. Further, the chromosomes segregate completely independently and randomly. The model starts with a cell in the starting generation (G_0) having acquired a mutation on one chromosome facing nine wild-type chromosomes. Prior to cell division the cell contains two mutant chromosomes and 18 wild-type chromosomes. Cell division can have two different outcomes. The first option for the next generation (G_1) is an equal distribution of the two mutant chromosomes resulting in two alike daughter cells having the same genotype like the mother cell. Alternatively, the two mutant chromosomes allocate in the same daughter cell. The other daughter cell is then completely wild-type. In the case of gene displacement this genotype can be counter selected. Cell division of the 2:8 cell can end in four alternatives, two of which are equal and one of which is the generation of 4:6 cell. Expanding on this idea, the first completely mutant cell can emerge already in the fourth emerging generation (G_4). The degree of probability for this is very low. Due to the selection against wild-type cells, the ratio of mutant to wild-type chromosomes in sum of all cells in one generation raises with each generation starting with 1:9 in G_0. Respecting all possibilities and the likelihood of each, in G_1 one mutant chromosome opposes only 6.5 wild type chromosomes and in G_2 even mere 4.375. Therefore, the more generations emerge the higher the probability to achieve pure mutant cells. This model does not comment on or consider putative recombination events between chromosomes throughout growth.

Thankfully adopted from Derntl, 2009

3.2.2 Transformation of the plasmid flaB2'ura3 and recombination

Cloning of the plasmid, transformation and the following recombination was achieved by Christian Derntl. The transformation of *Nab. magadii* L13 was done as described for phage mutants in this thesis and an occurring recombination event was controlled by PCR analysis. In 14 transformation attempts, recombination could be observed in six samples.

As it was mentioned in previous sections *Nab. magadii* has up to 25 genome copies, leading to the fact that a single recombination event leaves a considerable number of wild type genes. In order to achieve a gene deletion in each of the genome copies, passaging of the cultures was performed, as described in the methods section of this thesis. By the addition of novobiocin to the growth medium, theoretically some cells have lost all their wild type chromosome copies after a certain number of passages. Since the novobiocin resistance cassette is harboured by the plasmid, wild type copies of the chromosomes are negatively selected (figure 23). As it was shown by Christian Derntl, 15 passages were sufficient in order to achieve cells that have gene copies harbouring the mutant gene only. Since in every passage five generations can be assumed, the total number of generations within 15 passages amounts to approx. 75 (Derntl, 2009).

3.2.3 Genotype of flagellin mutants

Compared to the other methods, deletion of ϕCh1 phage genes and the extracellular protease, there is a striking difference in the attempt to mutate the *Nab. magadii* flagellin genes. While in former ventures the novobiocin resistance cassette was disrupting the gene to be deleted, here, in contrast, the resistance gene was cloned into the plasmid separately. This leads to novobiocin resistant cells only if a one sided recombination event takes place and the plasmid is therefore incorporated into the chromosomal DNA as a whole. Double recombination of the homologue sites in the genes *flaB2* and *flaB3* is not followed by novobiocin resistance, since the cassette is lost with the suicide plasmid. Two possible genotypes can be obtained after one sided recombination, what can be seen in figure 24. Of course, both possible genotypes still contain all the wild type flagellin genes, since only one sided recombination took place. Nevertheless, in possibility a) a cell without wild type chromosomes might already be deficient in *flaB2* and *flaB3*, since the wild type genes are disconnected with their promoter due to the incorporated plasmid. The same might be true for possibility b), where the cells might be deficient in *flaB4*. Nonetheless, in order to verify a possible phenotype, all the wild type chromosomes have to be lost by passaging.

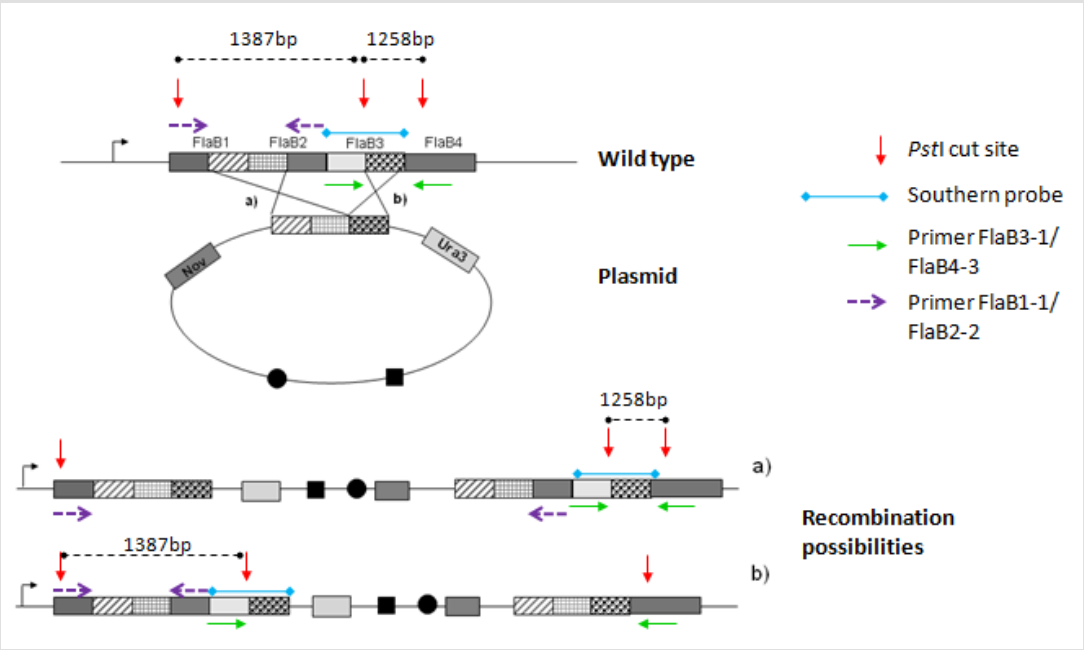
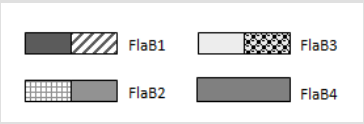


Figure 24: Possible genotypes of flagellin mutants after one sided recombination

In this figure, the possible genotypes of flagellin mutants after one sided recombination with the plasmid flaB2'ura3 are shown. Double recombination is excluded, since it does not lead to novobiocin resistance of the strains and is negatively selected. Concerning recombination possibility **a)**, the truncated gene sequences of *flaB1* and *flaB2* recombine with their counterparts on the chromosome. This leads to a genotype, where a copy of the *fla* operon with partially deleted *flaB2* and *flaB3* genes and complete deficient in the *flaB4* sequence is followed by the rest sequence of the plasmid flaB2'Ura3 and a near wild type copy of the *fla* operon, only missing the 5' sequence part of *flaB1*. The other recombination possibility, **b)**, involves the 3' region of *flaB3* only, whereas the sequence on the plasmid again recombines with its counterpart on the chromosome. This leads to a nearly wild type *fla* operon under the control of the promoter, but deficient in *flaB4*. This is followed by the rest of the plasmid and a *fla* sequence consisting of truncated *flaB1, flaB2* and *flaB3* versions, but a complete sequence of *flaB4*.

*Pst*I cut sites in the sequences are shown with an upright arrow. Chromosomal DNA restricted with *Pst*I was used for a southern blot analysis. Using the gene *flaB3* as a southern blot probe (shown by a horizontal line with squared ends), two different signals can be obtained concerning the wild type chromosome, a band of 1258 bp and one of 1387 bp. A *Nab. magadii* culture that only harbours chromosomes of the recombination type **a)** and is free of wild type chromosome copies, only shows the 1258 bp signal. On the other hand, cells only harbouring chromosomes of recombination type **b)** exclusively show the signal at 1387 bp.

The primers FlaB3-1 and FlaB4-3 are shown as horizontal arrows. In the wild type chromosome as well as in recombination possibility **a)**, a PCR product of 1226 bp is obtained. No PCR product can be obtained in recombination possibility **b)**. Using the primers FlaB1-1 and FlaB2-2 (shown as horizontal arrows with disrupted lines), a PCR product of 1390 bp is received in the wild type as well as in chromosomes that recombined with the plasmid according to possibility **b)**. In chromosomes with possibility **a)**, no PCR product is obtained.



The genes *flaB1, flaB2, flaB3* and *flaB4* as shown in the figure above. For better understanding of the plasmid construction (flaB2'ura3) and the recombination events occurring, the first 3 genes of the operon are divided in two parts each.

3.2.4 Analysis of passaged flagellin mutants

3.2.4.1 Southern Blot

After 18 passages of the clones where recombination had taken place, a southern blot analysis of the cell's chromosomal DNA was done. By restricting the isolated DNA with the enzyme *Pst*I, information concerning the type of one sided recombination could be gained, as well as insight into the number of still occurring wild type chromosomes. Using the wild type gene *flaB3* as a probe for the southern blot, in the wild type chromosome two bands were detected: a strong signal of 1258 bp and a weak signal of 1387 bp. The enzyme *Pst*I cuts the 1190 bp gene *flaB3* in two dissimilar parts. A small DNA fragment of approx. 130 bp, responsible for the weak binding and the faint signal at 1387 bp, and a much bigger fragment of about 1060 bp, resulting in a strong southern blot signal at 1258 bp, are obtained.

In *Nab. magadii* clones were recombination already took place, a different picture can be seen. If recombination possibility a) has happened and no wild type chromosomes are present any more, only the 1258 bp signal is received. Moreover, a large band of approx. 10.8 kbp can be monitored. If the same is true for possibility b), only the 1387 bp signal as well as a large signal of 10.7 bp is obtained (figure 24).

As it can be seen in figure 25, the tested clone #1 only revealed a signal at 1258 bp and the large signal of presumably 10.8 kbp, suggesting that recombination possibility a) had taken place in these cells. The missing signal of 1387 bp could hint to the fact, that hardly any wild type chromosomes are still present in the clone. Nevertheless, it has to be taken into account that this 1387 bp signal is not preferably bound by the probe and might therefore not necessarily mean non-existing wild type chromosomes.

On the other hand, in the tested clone #2 only the band of 1387 bp and a large signal were obtained, being indicative of recombination possibility b). The missing band of 1258 bp led to the conclusion that only a few or even no wild type chromosomes are present any more in this clone. The absent wild type signal in this case was especially remarkable, since the *flaB3* probe would bind the 1258 bp band preferably, because the homologue sequence is considerably longer.

3.2.4.2 PCR analysis

After several passages, streak dilution was applied and single colonies were tested for the occurrence of wild type chromosomes still present in the cell. The binding sites of the primers can be reviewed in figure 24.

Clone #1 was analysed by using the primers FlaB1-1 and FlaB2-2, resulting in a 1390 bp fragment in the wild type *fla1-fla2* genes. The same band would also be obtained in culture, were no wild type chromosomes are present but recombination possibility b) had taken place. In clone #1, as was determined by southern blot analysis, most likely recombination possibility a) had taken place. Therefore, if no wild type chromosomes are present any more, no PCR product could be obtained. The monitoring of several single colonies revealed the presence of wild type genes in all of the clones. Nevertheless, this was not unexpected since the results of the former southern blot analysis were inconclusive.

Concerning clone #2 that was shown to appeal to recombination possibility b), the primers FlaB3-1 and FlaB4-3 were used. In wild type cells, as well as in clones correlating with the recombination possibility a), a PCR signal of 1226 bp was obtained. In contrast, analysis of the clone #2 without any present wild type chromosomes would not result in a PCR signal. Nevertheless, even though the southern blot analysis was indicating the opposite, wild type chromosomes were obtained in every tested single colony of clone #2. This came as a surprise, since in the former southern blot experiment no wild type chromosomes could be detected. Therefore, a very small number can still be present in the cell, or, in theory, there even have to be some single cells without any wild type chromosomes at all, respectively.

3.2.5 Discussion of flagellin mutants

It has to be considered that out-recombination of the plasmid is possible at any time. Therefore the cells loose the novobiocin resistance cassette in the respective chromosome, but also regain wild type function of the flagellin genes. The cells can still survive the selective pressure of novobiocin, since the plasmid might be incorporated in another chromosome copy. To this end, it might be quite challenging to achieve cells that have lost all wild type chromosomes. Nevertheless, concerning the experiments performed, it can be considered that the flagellin genes of *Nab. magadii* might be essential for the archaeon, and complete loss of function of the genes might be lethal. This hypothesis is supported by the results obtained from the tested clone #2. As mentioned above, southern blot analysis clearly showed that at the uttermost a very small number of wild type chromosomes were present in the cells, or even none at all, while the majority of the chromosomes had the plasmid incorporated according to recombination type b). Since PCR analysis still revealed wild type chromosome copies in all tested colonies after several passages, it could be assumed that as soon as a "homozygote" is achieved, the respective cell dies.

The theory of homozygote flagellin genes could be verified in further experiments, by using the method applied for the extracellular protease. If the novobiocin resistance interrupts the target genes instead of only being present on the plasmid, double recombination is possible and still results in novobiocin resistant chromosome copies. Passaging of these clones theoretically enriches the cells in deleted gene copies and back recombination to wild type is not possible. Therefore, if wild type chromosomes are still present after several passages the essentiality of the *Nab. magadii* flagellin genes could be assumed.

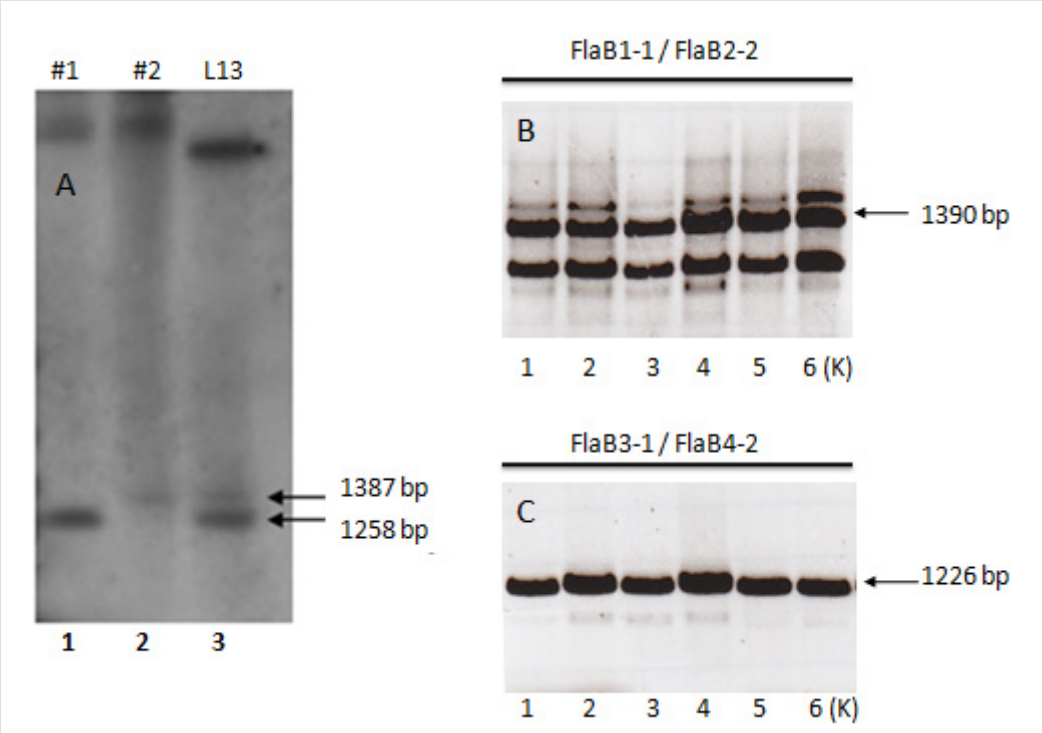


Figure 25: Results of the attempted flagellin deletion
Figure **A** shows the southern blot analysis of two passaged colonies, having the plasmid flaB2'ura3 integrated into the chromosome and a wild type control. In *Nab. magadii* L13, the wild type control, two southern blot bands are detectable, a strong signal of 1258 bp and a weaker signal of 1387 bp (lane 3). Concerning clone #1, only the band of 1258 bp is visible (lane 1). This could be due to the fact that the plasmid is integrated as proposed in recombination possibility a) (figure 24), and wild type chromosomes are not available in large copy numbers. Looking at clone #2, only the band of 1387 bp can be detected (lane 2). This is interesting, since the 1258 bp signal would be detected preferably in this southern blot analysis. Therefore it can be assumed, that wild type copies of the chromosome are rare in this clone, and that the plasmid is integrated according to recombination type b) (compare to figure 24). Figure **B** shows the screening of single colonies of the clone #1. Since recombination type a) is proposed for this clone by the southern blot, the primers FlaB1-1 and FlaB2-2 do only give a product in remaining wild type chromosome copies in this clone. Screening of several colonies showed the wild type band of 1390 bp to occur in every clone tested (lanes 1-5). The wild type control is *Nab. magadii* L13 (lane 6). Figure **C** shows the screening of single colonies of the clone #2. Hereby, recombination type b) was suggested by the southern blot analysis. Therefore, PCR analysis with the primers FlaB3-1 and FlaB4-3 only results in a PCR product if wild type chromosomes are still present. All tested single colonies showed to still contain wild type chromosomes (lanes 1-5). As control the wild type strain *Nab magadii* L13 was used (lane 6).

3.3 pRR007- a new shuttle vector for *Nab. magadii* and *E. coli*

As mentioned in the introduction to this work, two possible antibiotic resistances can be used for work in the haloalkaliphilic archaeon *Nab. magadii* until now. The method of using the novobiocin resistance coded on the plasmid pRo-5 was established in our lab previously (Iro *et al.*, in prep.) and has been practiced successfully since. In contrast, the use of the plasmid pNB102 in *Nab. magadii*, harbouring a mevinolin resistance gene, was not that straightforward from time to time. Problems occurred especially concerning false positive clones growing in selective media, whereas increasing of the antibiotic's concentration resulted in death of all cells.

3.3.1 Construction

Therefore, it was the idea to construct a new plasmid, where the mevinolin resistance gene was cloned under the control of a strong promoter. To this end, the 16S promoter of *Nab. magadii* was used, and the construct of 16S promoter and mevinolin resistance gene was cloned into pKSII+, already harbouring an origin of replication for *E. coli*. In order to gain an origin of replication for *Nab. magadii*, the one of the plasmid pRo-5 was used. This origin is composed of the ϕCh1 operon ORF53/ORF54 that proved to be the minimal replication origin of the virus (Iro *et al.*, in prep.). The different DNA fragments were cloned into the vector while leaving the original multiple cloning site (MCS) of pKSII+ free for further purposes. In the achieved new shuttle vector, pRR007, several restriction sites in the MCS are still available for cloning.

3.3.2 Mevinolin concentration

Transformation experiments of pRR007 in *Nab. magadii* L13 were performed, using different amounts of mevinolin as selective compound. The concentrations of 5 µg / ml, 7.5 µg / ml, 10 µg / ml and 15µg / ml were used. Contrary to our expectations, the optimal mevinolin concentration for pRR007 was only 7.5 µg / ml, as it was also determined for pNB102. No colonies could be observed growing on 10 µg / ml or even 15 µg / ml. This was rather unexpected, since the used 16S promoter is thought to be a strong promoter. Nevertheless, it could be the case that this promoter is preferably active during the exponential growth phase of the *Archaea*, and the mevinolin resistance gene is expressed to a small amount only in later stages.

3.3.3 Discussion

With pRR007 (figure 26), we achieved a new shuttle vector for the work in *Nab. magadii* and *E. coli*. It harbours an ampicillin resistance gene functioning in *E. coli* and a mevinolin resistance for *Natrialba*. Even though an increase of the applicable mevinolin concentration compared to the plasmid pNB102 is not possible in transformation assays, another advantage is obtained. The plasmid pRR007 contains a MCS with several restriction sites that make future cloning work much easier due to the greater possibilities of restriction enzymes that can be used.

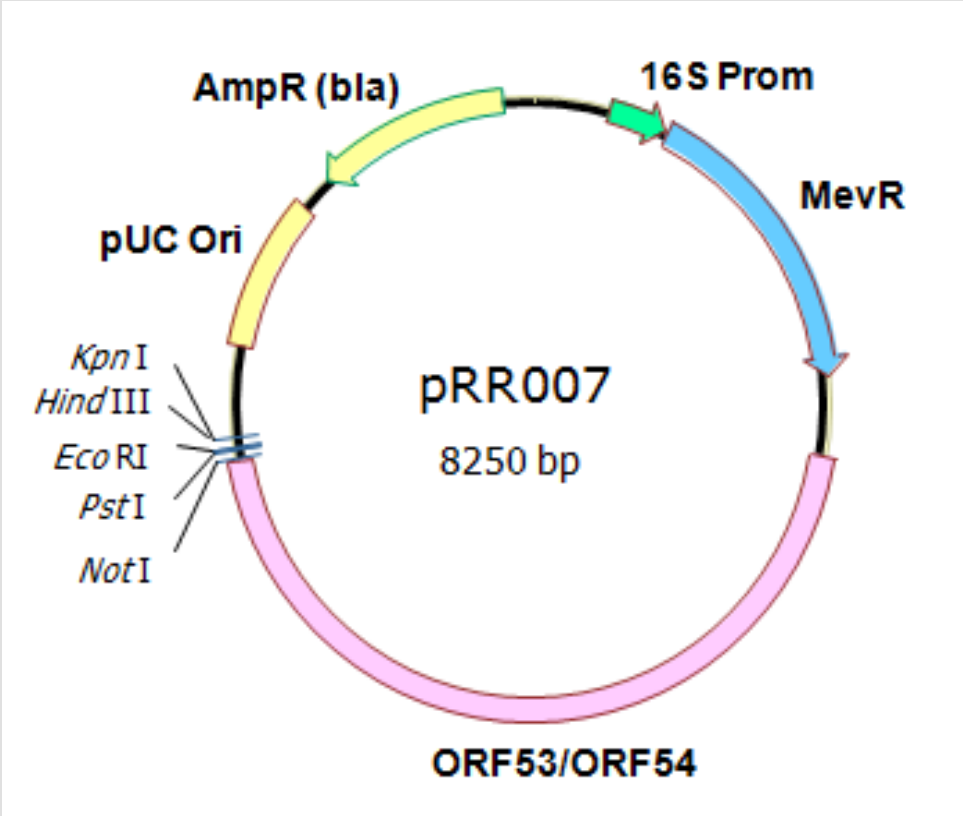


Figure 26: Plasmid pRR007
This picture shows a schematic drawing of the plasmid pRR007, our new shuttle vector for *Nab. magadii* and *E. coli*. The ORF53 / ORF54 gene sequence is derived from the phage ϕCh1 and allows replication of the plasmid in *Nab. magadii*. The MevR designated sequence is a mevinolin resistance gene functioning *in Nab. magadii* that is under the control of the archaeon's own 16S promoter. The pUC origin of replication functions in *E. coli*, whereas the ampicillin resistance gene (AmpR) allows selection of the plasmid in the bacterium. The plasmid also harbours a multiple cloning site, consisting of the restriction sites *Kpn*I, *Hind*III, *Eco*RI, *Pst*I and *Not*I.

3.4 M.*Nma*ϕCh1I ParB like nuclease domain

The methyltransferase M.*Nma*ϕCh1I of the virus ϕCh1 is composed of two parts. Apart from the methyltransferase domain, a ParB like nuclease domain contributes to the overall protein composition. Investigations of the methyltransferase activity as well as promoter identification studies were already implemented by Flora Haider in 2009 (Haider, 2009). In order to further characterise the ParB like nuclease domain, cloning of the respective gene and expression of the nuclease domain protein in *E. coli* had to be accomplished.

3.4.1 Expression in *E. coli* Rosetta

In a first approach, the nuclease domain gene was cloned into the expression vector pRSET-C. The plasmid is holding a T7 promoter sequence, controlling the expression of the gene of interest. Since the *E. coli* strain Rosetta was used for the experiment, the T7 polymerase is itself controlled by the lac promoter. The expression of the nuclease domain protein is therefore indirectly induced by the addition of IPTG.

Surprisingly, transformation of the completed vector pRSET-nuc resulted in a very low number of transformed colonies. Moreover, expression studies of the protein using the few positively transformed cells did not result in a gain of protein. None of the cultures seemed to express the nuclease domain protein, what lead us to the conclusion that an expressed protein might act as nuclease even in *E. coli*, and therefore destroys the cells. The surviving cells seem to not express the protein. The lac promoter is known to be not utterly tight and background expression of the T7 polymerase is still possible to a small extent without the addition of IPTG. Therefore, even leaky expression of the nuclease protein might be responsible for the death of the cells.

3.4.2 Expression in *E. coli* pop2135 F'

In order to be able to control the expression to a greater extent, the nuclease domain gene was cloned into the plasmid pPL-RSET-C. Here, the gene of interest is under the control of the temperature sensitive λ pL promoter. The promoter is bound by a repressor molecule of the strain pop2135 F' at 28° C and is destroyed at 37-42° C, leading to expression of the protein.

The transformation of the plasmid into pop2135 F' was not associated with a surprisingly small number of transformed clones this time. Nevertheless, in several expression experiments, the protein of the nuclease domain could not be observed.

3.4.3 Discussion

The experiments performed did not lead to a gain of the nuclease domain protein. Considering the attempts of expression in two different strains, it can be assumed that the nuclease protein is active in *E. coli* and is able to destroy the cells if expression takes place. In order to further study the nuclease domain of the M.*Nma*ϕCh1I, other experiments have to be considered.

4. List of figures

5. References

Allers, T. and Mevarech, M. (2005) 'Archaeal genetics - the third way', *Nature Review Genetics*, no. 6 (1), Jan, pp. 58-73.

Baltimore, D. (1971) 'Expression of animal virus genomes', *Bacteriological Reviews*, vol. 35 (3), Sep, pp. 235-41.

Bamford, D.H. (2003) 'Do viruses form lineages across different domains of life?', *Research in Microbiology*, no. 154 (4), May, pp. 231-6.

Baranyi, U., Klein, R., Lubitz, W., Krüger, D.H. and Witte, A. (2002) 'The archaeal halophilic virus-encoded dam-like methyltransferase M.ϕCh1-I methylates adenine residues and complements dam mutants in the low salt environment of *Escherichia coli*', *Molecular Microbiology*, no. 35 (5), Mar, pp. 1168-79.

Barry, E.R. and Bell, S.D. (2006) 'DNA replication in the *Archaea*', *Microbiology and molecular biology reviews*, no. 70 (4), Dec, pp. 876-87.

Bath, C. and Dyall-Smith, M.L. (1998) 'His1, an archaeal virus of the *Fuselloviridae* family that infects *Haloarcula hispanica*', *Journal of Virology*, no. 72 (11), Nov, pp. 9392-5.

Bell, S.D. (2005) 'Archaeal transcriptional regulation - variation on a bacterial theme?', *Trends in Microbiology*, no. 13 (6), Jun, pp. 262-5.

Benelli, D., Maone, E. and Londei, P. (2003) 'Two different mechanisms for ribosome/mRNA interaction in archaeal translation initiation', *Molecular Microbiology*, no. 50 (2), Oct, pp. 635-43.

Bergth, O., Børsheim, K., Bratbak, G. and Heldal, M. (1989) 'High abundance of viruses found in aquatic environments', *Nature*, no. 340 (6233), Aug, pp. 467-8.

Breitbart, M. and Rohwer, F. (2005) 'Here a virus, there a virus, everywhere the same virus?', *Trends in Microbiology*, no. 13 (6), Jun, pp. 278-84.

Brinkman, A.B., Ettema, T.J.G., de Vos, W.M. and van der Oost, J. (2003) 'The Lrp family of transcriptional regulators', *Molecular Microbiology*, no. 48 (2), Apr, pp. 287-94.

Brown, J.R. and Doolittle, W.F. (1997) '*Archaea* and the prokaryote-to-eukaryote transition', *Microbiology and Molecular Biology Reviews*, no. 61 (4), Dec, pp. 456-502.

Cline, S., Lam, W., Charlebois, R., Schalkwyk, L. and Doolittle, W. (1989) 'Transformation methods for halophilic archaebacteria', *Canadian Journal of Microbiology*, no. 35 (1), Jan, pp. 148-52.

Derntl, C. (2009) 'Construction of the first deletion mutant of a haloalkaliphilic archaeon and analysis of gene expression of the methyltransferase M.*Nma*ϕCh1I of the halophage ϕCh1', *Diploma thesis, University of Vienna*.

Desmarais, D., Jablonski, P.E., Fedarko, N.S. and Roberts, M.F. (1997) '2-Sulfotrehalose, a novel osmolyte in haloalkaliphilic *Archaea*', *Journal of Bacteriology*, no. 179 (10), May, pp. 3146-53.

Dopson, M., Baker-Austin, C., Hind, A., Bowman, J.P. and Bond, P.L. (2004) 'Characterization of *Ferroplasma* isolates and *Ferroplasma acidarmanus* sp. nov., extreme acidophiles from acid mine drainage and industrial bioleaching environments', *Applied and Environmental Microbiology*, no. 70 (4), Apr, pp. 2079-88.

Dyall-Smith, M. (2009) *The Halohandbook - protocols for haloarchaeal genetics (version 7.1)*.

Dyall-Smith, M., Tang, S.-L. and Bath, C. (2003) 'Haloarchaeal viruses: how diverse are they?', *Research in Microbiology*, no. 154 (4), May, pp. 309-13.

Elkins, J.G., Podar, M., Graham, D.E., Makarova, K.S., Wolf, Y., Randau, L., Hedlund, B.P., Brochier-Armanet, C., Kunin, V., Anderson, I., Lapidus, A., Goltsman, E., Barry, K., Koonin, E.V., Hugenholtz, P., Kyrpides, N., Wanner, G., Richardson, P., Keller, M. and Stetter, K.O. (2008) 'A korarchaeal genome reveals insights into the evolution of the *Archaea*', *Proceedings of the National Academy of Sciences of the United States of America*, no. 105 (23), Jun, pp. 8102-7.

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*, no. 5 (2-3), Aug, pp. 203-218.

Forterre, P., Brochier, C. and Philippe, H. (2002) 'Evolution of the *Archaea*', *Theoretical Population Biology Review*, no. 61 (4), Jun, pp. 409-22.

Gribaldo, S. and Brochier-Armanet, C. (2006) 'The origin and evolution of *Archaea*: a state of the art', *Philosophical Transactions of the Royal Society*, no. 361 (1470), Jun, pp. 1007-22.

Gropp, F., Grampp, B., Stolt, P., Palm, P. and Zillig, W. (1992) 'The immunity-conferring plasmid p phi HL from the *Halobacterium salinarium* phage phi H: nucleotide sequence and transcription.', *Virology*, no. 190 (1), Sep, pp. 45-54.

Haider, F. (2009) 'Characterization of the DNA methyltransferase M.*Nma*ϕCh1I', *Diploma thesis, University of Vienna*.

Häring, M., Rachel, R., Peng, X., Garrett, R. and Pragishvili, D. (2005a) 'Viral diversity in hot springs of Pozzuoli, Italy, and characterization of a unique archaeal virus, *Acidianus* bottle-shaped virus, from a new family,the *Ampullaviridae*', *Journal of Virology*, no. 79 (15), Aug, pp. 9904-11.

Häring, M., Vestergaard, G., Rachel, R., Lanming, C. and Prangishvili, D. (2005b) 'Independent virus development outside a host', *Nature*, no. 436 (7054), Aug, pp. 1101-2.

Holmes, M.L. and Dyall-Smith, M.L. (1991) 'Mutations in DNA gyrase result in novobiocin resistance in halophilic archaebacteria', *Journal of Bacteriology*, no. 173 (2), Jan, pp. 642-8.

Holmes, M., Nuttall, S. and Dyall-Smith, M. (1991) 'Construction and use of halobacterial shuttle vectors and further studies on Haloferax DNA gyrase.', *Journal of Bacteriology*, no. 173 (12), Jun, pp. 3807-13.

Horikoshi, K. (1999) 'Alkaliphiles: Some applications of their products for biotechnology', *Microbiology and Molecular Biology Reviews*, no. 63 (4), Dec, pp. 735-50.

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*, no. 417 (6884), May, pp. 63-7.

Iro, M., Ladurner, A., Meissner, C., Derntl, C., Reiter, M., Haider, F., Rössler, N., Klein, R., Baranyi, U., H, S. and Witte, A. (in prep.) 'Transformation of *Natrialba magadii*: application of ϕCh1 elements for a shuttle vector system for haloalkaliphilic *Archaea*'.

Jackson, E., Jackson, D. and Deans, R. (1978) '*Eco*RI analysis of bacteriophage P22 DNA packaging', *Journal Molecular Biology*, no. 118 (3), Jan, pp. 365-88.

Jarrell, K.F. and McBride, M.J. (2008) 'The surprisingly diverse ways that prokaryotes move', *Nature Review Microbiology*, no. 6 (6), Jun, pp. 466-76.

Jones, B.E., Grant, W.D., Duckworth, A.W. and Owenson, G.G. (1998) 'Microbial diversity of soda lakes', *Extremophiles*, no. 2 (3), Aug, pp. 191-200.

Kamekura, M., Dyall-Smith, M.L., Upasani, V., Ventosa, A. and Morris, K. (1997) 'Diversity of alkaliphilic halobacteria: proposals for transfer of *Natronobacterium vacuolatum* , N*atrono bacterium magadii*, and *Natronobacterium pharaonis* to *Halorubrum*, *Natrialba*, and *Natronomonas* gen. nov., respectively [...]', *International Journal of Systematic Bacteriology*, no. 47 (3), Jul, pp. 853-7.

Klein, R., Baranyi, U., Rössler, N., Greinder, B., Scholz, H. and Witte, A. (2002) '*Natrialba magadii* virus ϕCh1: first complete nucleotide sequence and functional organization of a virus infecting a haloalkaliphilic archaeon', *Molecular Microbiology*, no. 45 (3), Aug, pp. 851-63.

Klein, R., Greineder, B., Baranyi, U. and Witte, A. (2000) 'The Structural Protein E of the archaeal Virus phiCh1: Evidence for Processing in *Natrialba magadii* during Virus Maturation', *Virology*, no. 276 (2), Oct, pp. 376-87.

Krogh, S., O'Reilly, M., Nolan, N. and Devine, K.M. (1996) 'The phagelike element PBSX and part of the skin element, which are resident at different locations on the *Bacillus subtilis* chromosome, are highly homologous', *Microbiology*, no. 142 (Pt 8), Aug, pp. 2031-40.

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-coenzymeA reductase in the archaebacterium *Haloferax volcanii*', *The Journal of Biological Chemistry*, no. 267 (9), Mar, pp. 5829-34.

Langer, D., Hain, J., Thuriaux, P. and Zillig, W. (1995) 'Transcription in *Archaea*: Similarity to that in *Eucarya*', *Proceedings of the National Academy of Sciences of the United States of America*, no. 92 (13), Jun, pp. 5768-72.

Lanyi, J.K. (1974) 'Salt-dependent properties of proteins from extremely halophilic *Bacteria*', *Bacteriological Reviews*, no. 38 (3), Sep, pp. 272-90.

Madern, D., Ebel, C. and Zaccai, G. (2000) 'Halophilic adaptation of enzymes', *Extremophiles*, no. 4 (2), Apr, pp. 91-8.

Marteinsson, V.T., Birrien, J.-L., Reysenbach, A.-L., Vernet, M., Marie, D., Gambacorta, A., Messner, P., Sleytr, U.B. and Prieur, D. (1999) '*Thermococcus barophilus* sp. nov., a new barophilic and hyperthermophilic archaeon isolated under high hydrostatic pressure from a deep-sea hydrothermal vent', *lnternational Journal of Systematic Bacteriology*, no. 49 (Pt 2), Apr, pp. 351-9.

Nuttall, S.D. and Dyall-Smith, M.L. (1993) 'HF1 and HF2: novel bacteriophages of halophilic *Archaea*', *Virology*, no. 197 (2), Dec, pp. 678-84.

Oren, A. (1999) 'Bioenergetic aspects of halophilism', *Microbiology and Molecular Biology Reviews*, no. 63 (2), Jun, pp. 334-48.

Pfister, P., Wasserfallen, A., Stettler, R. and Leisinger, T. (1998) 'Molecular analysis of *Methanobacterium* phage psiM2', *Molecular Microbiology*, no. 30 (2), Oct, pp. 233-44.

Pietilä, M.K., Roine, E., Paulin, L., Kalkkinen, N. and Bamford, D.H. (2009) 'An ssDNA virus infecting *Archaea*: a new lineage of viruses', *Molecular Microbiology*, no. 72 (2), Apr, pp. 307-19.

Prangishvili, D., Forterre, P. and Garrett, R.A. (2006) 'Viruses of the *Archaea*: a unifying view', *Nature*, no. 4 (11), Nov, pp. 837-48.

Prangishvili, D., Garrett, R.A. and Koonin, E.V. (2006) 'Evolutionary genomics of archaeal viruses: Unique viral genomes in the third domain of life', *Virus Research*, no. 117 (1), Apr, pp. 52-67.

Rothschild, L.J. and Mancinelli, R.L. (2001) 'Life in extreme environments', *Nature*, no. 409 (6823), Feb, pp. 1092-101.

Schnabel, H. (1984) 'An immune strain of *Halobacterium halobium* carries the invertible L segment of phage ϕH as a plasmid', *Proceedings of the National Academy of Sciences of the United States of America*, no. 81 (4), Feb, pp. 1017-1020.

Schnabel, H., Zillig, W., Pfaiffle, M., Schnabel, R., Michel, H. and Delius, H. (1982) '*Halobacterium halobium* phage ϕH', *EMBO Journal*, no. 1 (1), pp. 87-92.

Serganova, I., Ksenzenko, V., Serganov, A., Meshcheryakova, I., Pyatibratov, M., Vakhrusheva, O., Metlina, A. and Fedorov, O. (2002) 'Sequencing of flagellin genes from *Natrialba magadii* provides new insight into evolutionary aspects of archaeal flagellins', *Journal of Bacteriology*, no. 184 (1), Jan, pp. 318-22.

Shirai, T., Kobayashi, T., Ito, S. and Horikoshi, K. (2008) 'Alkaline Adaptation of Proteins', in *Protein Adaptation in Extremophiles (Molecular Anatomy and Physiology of Proteins)*, Nova Science Publishers Inc.

Siegel, P.J. and Schaechter, M. (1973) 'The role of the host cell membrane in the replication and morphogenesis of bacteriophages', *Annual Review Microbiology*, no. 27, pp. 261-82.

Skern, T. (2006) *Coffee House Notes on Virology*, Facultas, Vienna, Austria

Stanier, R.Y. (1971) 'Toward an evolutionary taxonomy of the *Bacteria*', *Recent Advances in Microbiology; eds. Perez-Miravete, A. & Pelaez, D. (Assoc. Mex. Microbiol., Mexico City)*, pp. 595-604.

Tenchov, B., Vesico, E.M., Sprott, G.D., Zeidel, M.L. and Mathai, J.C. (2006) 'Salt tolerance of archaeal extremely halophilic lipid membranes', *The Journal of Biological Chemistry*, no. 281 (15), Apr, pp. 10016-23.

Tindall, B., Ross, H. and Grant, W. (1984) '*Natronobacterium* gen. nov. and *Natronococcus* gen. nov., two new genera of haloalkaliphilic archaebacteria', *Systematic and Applied Microbiology*, no. 5, pp. 41-57.

Torsvik, T. and Dundas, I.D. (1974) 'Bacteriophage of *Halobacterium salinarium*', *Nature*, no. 248 (450), Apr, pp. 680-1.

Van de Vossenberg, J.L.C.M., Driessen, A.J.M., Grant, W.D. and Konings, W.N. (1999) 'Lipid membranes from halophilic and alkali- halophilic *Archaea* have a low H+ and Na+ permeability at high salt concentration', *Extremophiles*, no. 3 (4), Nov, pp. 253-7.

Van Regenmortel, M., Fauquet, C., Bishop, D., Carstens, E., Estes, M., Lemon, S., Maniloff, J., Mayo, M., McGeoch, D., Pringle, C. and Wickner, R. (2000) '7th Report of the International Committee on Taxonomy of Viruses', *Academic Press*.

Wang, I.-N., Smith, D.L. and Young, R. (2000) 'Holins: the protein clocks of bacteriophage infections', *Annual Review Microbiology*, no. 54, pp. 799-825.

Werner, F. (2007) 'Structure and function of archaeal RNA polymerases', *Molecular Microbiology*, no. 65 (6), Sep, pp. 1395-404.

Witte, A., Baranyi, U., Klein, R., Sulzner, M., Luo, C., Wanner, G., Krüger, D. and Lubitz, W. (1997) 'Characterization of *Natronobacterium magadii* phage ϕCh1, a unique archaeal phage containing DNA and RNA', *Molecular Microbiology*, no. 23 (3), Feb, pp. 603-16.

Woese, C.R., Kandler, O. and Wheelis, M.L. (1990) 'Towards a natural system of organisms: Proposal for the domains *Archaea, Bacteria*, and *Eucarya*', *Proceedings of the National Academy of Sciences of the United States of America*, no. 87 (12), Jun, pp. 4576-9.

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) 'Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors.', *Gene*, no. 33 (1), pp. 103-19.

Young, R. (1992) 'Bacteriophage lysis: mechanism and regulation', *Microbiological Reviews*, no. 56 (3), Sep, pp. 430-81.

Zhou, M., Xiang, H., Sun, C. and Tan, H. (2004) 'Construction of a novel shuttle vector based on an RCR-plasmid from a haloalkaliphilic archaeon and transformation into other haloarchaea.', *Biotechnol Lett.*, no. 26 (14), Jul, pp. 1107-13.

Zivanovic, Y., Armengaud, J., Lagorce, A., Leplat, C., Guérin, P., Dutertre, M., Anthouard, V., Forterre, P., Wincker, P. and Confalonieri, F. (2009) 'Genome analysis and genome-wide proteomics of *Thermococcus gammatolerans*, the most radioresistant organism known amongst the *Archaea*', *Genome Biology*, no. 10 (6) :R70.

Acknowledgements

First of all I want to thank my supervisor and mentor Angela Witte for giving me the opportunity to work in her laboratory and to write this thesis. Angela's advice and support was immeasurable and very important to me, and her enthusiasm for my topic was a great motivation, especially in critical stages of my work. Angela is also a good friend and the heart of our "halolab", where scientific work and having fun are not two different things. I am very grateful for the time.

The department of Microbiology, Immunobiology and Genetics has been a great place to work. I am not only thankful for the scientific support of my co-workers, but also for several new friendships. My very special thanks go to my dear colleague Michael Reiter for his great support for my work and for the fun time we had together. Moreover, I want to thank my colleagues Christian Derntl, who was a crucial help for my thesis, and Flora Haider. Daniel Kiesenhofer and Tatjana Swoboda also were great co-workers in our "halolab". Many other people in the department were important to me. I want to thank Eliza Deltcheva, Sanda Pasc, Konstantin Byrgazov, Hermann Hämmerle, David Hasenöhrl and Elisabeth Sonnleiter. Moreover, I want to thank Tim Skern for his help concerning my skills for writing scientific English.

My family has always supported me in my choice of studying Microbiology. I am much obliged to my parents, Rosmarie and Michael Selb, for their mental and financial support. I also want to thank my sister, Karoline, and her two girls Florentina and Stephanie. You are my sunshine! My special thanks go to my boyfriend Joachim "Spezzi" Hofer and my dear friend Claudia "Honig" Haider for believing in me and keeping me distracted from my work from time to time.

Zusammenfassung

Diese Arbeit beschreibt die Entwicklung der ersten Deletionsmutante eines halo(alkalo)philen Phagen und des daraus hervorgehenden Stammes *Nab. magadii* L11Δ79. Der offene Leserahmen (ORF) 79 des ins Genom von *Nab. magadii* integrierten Phagen ϕCh1 wurde mit Hilfe einer Novobiocin Resistenzkassette unterbrochen. Ein „suicide" Plasmid, welches den disruptierten ORF79 trägt, wurde in *Nab. magadii* L11 transformiert, und Rekombination des mutanten Genes mit dem ORF79 Wildtyp Gen konnte nachgewiesen werden. Anschließend wurde die Kultur lysiert um mutante Phagenpartikel zu erhalten. Diese Viren wurden im darauffolgenden Schritt zur Infektion des Stammes *Nab. magadii* L13 verwendet. *Nab. magadii* L13 enthält keine virale ϕCh1 DNA und kann daher infiziert werden. Durch die Infektion des Stammes mit Virenpartikeln, welche die disruptierte Version des ORF79 besitzen, entstand der Stamm *Nab. magadii* L11Δ79. Die Funktion von ORF79 des Phagen ϕCh1 ist noch unbekannt, jedoch könnte die in dieser Arbeit entwickelte Mutante bei deren Entschlüsselung eine wichtige Rolle spielen. Deletionsmutanten von weiteren ϕCh1 ORFs, ORF11 und ORF93, wurden ebenfalls erreicht. Die Entwicklung der entsprechenden *Nab. magadii* Stämme, welche diese mutanten Viruskopien beinhalten, ist jedoch noch in Arbeit.

Der zweite Teil dieser Diplomarbeit beschäftigt sich mit dem Versuch zur Entwicklung eines *Nab. magadii* Stammes, welcher deletierte Versionen der Flagellin Gene enthält. Die Flagellin Gene *flaB1 – flaB4* von *Nab. magadii* sind in einem Operon angeordnet. Durch die Transformation eines Plasmids, welches verkürzte Versionen der Gene *flaB2* und *flaB3* enthält, und eine darauffolgende Rekombination mit den Wildtyp Flagellin Genen, konnten die mutanten Kopien in den Stamm eingebracht werden. Da *Nab. magadii* bis zu 25 Chromosomenkopien enthält, ist die Entwicklung einer homozygoten Flagellin-Mutante noch in Arbeit.

Der dritte Teil dieser Arbeit beschreibt die Klonierung eins neuen Shuttle- Vektors für *Nab. magadii* und *E. coli*. Das Plasmid beinhaltet Replikationsloci für beide Organismen sowie zwei Resistenzgene. Während Mevinolin für die Selektion in *Nab. magadii* verwendet werden kann, beinhaltet der Vektor weiters ein Ampicillin Resistenzgen für *E. coli*. Durch eine vorhandene „multipe cloning site" können auf einfache Weise mehrere Gensequenzen in das Plasmid kloniert werden.

Der vierte Teil der Arbeit beschäftigt sich mit dem Versuch der Charakterisierung der Nuklease Domäne der ϕCh1 Methyltransferase M.*Nma*ϕCh1I. Da das halophile Protein in *E. coli* aktiv zu sein scheint, waren Expressionsversuche jedoch nicht erfolgreich.

Abstract

This work describes the first ever deletion mutant of a halo(alkali)philic phage and the resulting archaeal strain, *Nab. magadii* L11Δ79, carrying the mutant virus. The ORF79 of the haloalkaliphilic phage ϕCh1 was disrupted with a novobiocin resistance cassette. *Nab. magadii* L11, the wild type strain of the archaeon carrying the phage ϕCh1 integrated its genome, was transformed with a suicide plasmid harbouring the deleted gene version of ORF79. Recombination of the gene with its wild type counterpart in the viral DNA was achieved. Phage particles carrying the deleted version of ORF79 were harvested and used for infection of *Nab. magadii* L13, a strain formerly cured of ϕCh1. This resulted in *Nab. magadii* L11Δ79, a strain that is infected with phage copies deficient in ORF79 exclusively. The function of ORF79 has yet to be determined, the generated archaeal strain might be a great support to decipher this in the future. Furthermore, deletion mutants of two other ORFs of the phage ϕCh1, ORF11 and ORF93, were aspired. Deletion mutants of the phage genes were achieved and the work to generate archaeal strains carrying these deletions will continue.

The second part of the work at hand describes the attempt to achieve a strain of *Nab. magadii* L13 deficient in two flagellum genes. The genes *flaB1 – flaB4* are arranged in an operon. A suicide plasmid carrying deleted gene versions of the genes *flaB2* and *flaB3* was constructed and transformed into *Nab. magadii*. Recombination of the deleted gene versions with their wild type counterparts was achieved. Since *Nab. magadii* is harbouring up to 25 genome copies, the still ongoing work is concerned with the generation of a homozygote mutant strain.

In the third part of this thesis, the cloning of a new shuttle vector for *Nab. magadii* and *E. coli* is described. The plasmid is carrying origins of replication for both organisms and a mevinolin and ampicillin resistance gene for selection in *Nab. magadii* and *E. coli*, respectively. A multiple cloning site allows easy cloning work and the integration of more than one gene into the plasmid.

The fourth part of this work describes the attempt to characterise the nuclease domain of the ϕCh1 methyltransferase M.*Nma*ϕCh1I. Expression of the gene was not successful in *E. coli*, since the halophilic nuclease protein seems to be active in the *Bacteria*, being a lethal factor for the cells.

Curriculum Vitae

Personal details:

Name:	Regina Maria Selb
Date and place of birth:	Jan., 17th, 1983 in Feldkirch, Austria
Nationality:	Austria

Education:

"Volksschule Feldkirch - Tisis", Austria
1989 – 1993

"Übungshauptschule Feldkirch", Austria
1993 – 1997

"BAKIP Feldkirch", Austria (with distinction)
1997 – 2002

Participation EVS program, "European voluntary service", of the European Commission
YMCA Belfast, United Kingdom
2002 – 2003

Studies of "Microbiology and Genetics"; University of Vienna
October 2003 – May 2010

Scholarship for excellent grades (2008)

Diploma thesis: Laboratory of Prof. Dr. Angela Witte; Department of Microbiology, Immunobiology and Genetics; University of Vienna
November 2008 – May 2010

Working experience:

University of Vienna:
Tutor of student practical trainings: "Übungen in Mikrobiologie", "Mikrobiologisches Laborarbeiten", "Übungen im Pflichtfach Mikrobiologie"
Winter term 2008/2009; summer term 2009, winter term 2009/2010, summer term 2010

FH Campus Wien (University of Applied Sciences, Vienna):
Tutor of student practical trainings "Molekularbiologische Übungen I und II"
Winter term 2008/2009; summer term 2009, winter term 2009/2010, summer term 2010

Ivoclar Vivadent; Schaan, Principality of Liechtenstein
Practical training, department of professional care and biotechnology: Bacteria causing dental caries
Summer 2007

Hilti AG, Schaan, Principality of Liechtenstein
Practical training, department of metallography
Summers of 2004, 2005, and 2006