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Characterization of the DNA methyltransferase M.*Nma*φCh1I
and
further characterization of a transformation system
of haloalkaliphilic *Archaea*

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For Baba Loca

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Zusammenfassung

Abstract

Curriculum Vitae

1 Introduction

1.1 Haloalkaliphilic *Archaea*

1.1.1 Taxonomy and phylogeny of haloalkaliphilic *Archaea*

Through the molecular work of Woese et al. in the 1970's (Woese et al., 1977 and 1990) *Archaea* were brought into use as an individual phylogenetic group. Woese recommended the three-domain consideration of life. To modernize haloarchaeal taxonomy and terminology proposed to use the notion halobacteria just for halophiles, which are affiliated to the bacterial domain. The term haloarchaea should be applied for halophiles that are dependants of the archaeal domain (DasSarma and DasSarma, 2008).
On the basis of ribosomal RNA sequence analysis the archaeal domain could be distinguished into four phyla. The domain is subdivided into Euryarchaeota (e. g. *Haloferax*, *Methanopyrus, Halobacterium*), which include methanogens, their phenotypically various members and halophilic *Archaea*, Crenarchaeota (e. g. *Sulfolobus*), encompassing extremly thermophilic microorganisms and Korarchaeota (the only known examples, which not have been grown in pure culture, pJP27/pJP78) (Barns et al., 1996). In the early 21st century arguments arised to create a fourth sublineage called Nanoarchaeota. *Nanoarchaeum equitans* is till now the only known representative (Huber et al., 2003 and Di Giulio M., 2007).

The family *Halobacteriaceae*, which belongs to the phlyum Euryarchaeota, is made up of 27 genera. Those genera are cut into 119 species, including a group of *Archaea* living in extreme conditions (J.P. Euzéby, *Formerly List of Bacterial names with Standing in Nomenclature (LBSN*). Aerobic haloalkaliphilic *Archaea* are such organisms, which have their optimal growth in extreme environments. They grow best in surroundings containing at least 12% (2M) NaCl (Kamekura, 1998).
Some alkaliphilic members of the family require by the side of high salt concentrations also high pH values (between 8.5 and 11) and less than 10mM Mg^{2+} concentrations.

The isolates arise from a plethora of alkaline, hypersaline lakes and soils (Kamekura et al, 1997). These organisms are not resumed in one genus, although they show a lot of similarities in their physiology. In some genera in which they are findable there are alkaliphilc and neutrophilic species pooled, e.g. *Natrialba* implicates the alkaliphilic *Natraialba magadii* and the neutrophilic *Natrialba asiatica* (Horokoshi, 1999; Enache, 2007).

The most stable high-pH environments on Earth are the naturally occuring Soda lakes and Soda deserts. Because of the large amounts of carbonate minerals these lakes show pH values up to 11.5. The best studied ones are those of the East African Rift Valley. The most contributing coefficient is the absence of rocks of the sediemtary origins, which implies the deficiency of alkaline earth cations (Ca^{2+} and Mg^{2+}). These and other conditons lead to forming a closed drainage basin. Salts accumulate by evaporation and as a result of the evaporation the satiation of the alkaline earth cations is in a timely manner reached. By reason of the aggregation of CO_3^{2-} and the low concentrations of Ca^{2+} and Mg^{2+} the pH is able to increase.
An amazing attribute of these lakes is their color. By the blooms of different microorganisms in the water the lakes seem to be green or red. The nearly indefinite offer of CO_2 in connection with high ambient temperature and daily light intensities make the East African soda lake to the most productive aquatic surroundings.

Supposably provide the thick populations of cyanobacteria the rest of the microbial organization with photosynthetic primary productivity (Jones et al, 1998). In lake Magadi (Kenya) cyanobacterial blooms behave just after exhaustive rainfalls, whereby the salt concentration is reduced (Florenzano et al., 1985). Red anoxygenic, phototrophic and halophilic bacteria have also been isolated from the lake Magadi and other soda lakes (Grant and Tindall, 1986; Imhoff et al., 1979). The large number of the haloalkaliphilic *Archaea* are the reason for the red colored lakes and are those who are best adjusted to the extreme living conditions, the high concentration of salt and values of pH (Jones et al., 1998).

1.1.2 *Archaea* - In general

In 1977 *Archaea* were classified as an onw domain, next to the two already exisitng ones. Three classes of ribisomal RNA and ribosomes were discovered by new molecular techniques, the analyses of the 16S rRNA sequence. Up to that point procaryotes implicated two domains, which are equatable to the third one, the eukaryotes (Woese et al., 1977 and 1990). Further studies in this field revealed that *Archaea* share features with *Bacteria* and eukaryotes. With regard to the cellular structure and the genome organisation they resemble *Bacteria*, against what the DNA replikation, transcription and tranlsation procedure concerns *Archaea* conform eukaryotes (Brown and Doolittle, 1997).

1.1.2.1 Compared to bacteria and eukaryotes

Archaea and *Bacteria* are dependants of the kingdom procaryotes. They are similar to each other with regard to their cell size, morphology and membrane structure. Both miss a cytoskeleton and organells. They are either in possession of a single circular chromosome, which can be attended by one or more plasmids. Even the organisation of the genes is conformable (Brown and Doolittle, 1997). The archaeal genes and gene clusters are arranged in operons, similar to that of *Bacteria*. The order of these operons in bacteria is 16S-23S-5S. *Archaea* have a small difference in this arrangement. Methanogens and halophils have a insertion of a $tRNA^{Ala}$ gene among the 16S and the 23S gene. In some methanogens and thermoacidophiles the 5S rRNA gene is located distal (Brown et al., 1989). Additional *Archaea* use methionine as their initiator tRNA, whereas *Bacteria* us Formylmethionine. Their ribosomes are sensitive to diphtheria toxin, those in *Bacteria* are not (Saruyama and Sasaki, 1988).

The supposed Shine-Dalgarno ribosome binding site sequences in *Archaea* relative to the translational initiation codon are further diverging concering their location (Amils et al., 1993). On the basis of the interaction among SD motifs in the mRNA and anti-SD sequences in the 16S rRNA it appears that Archaea utlizises sometimes a bacterial procedure of mRNA respectively ribosome cognition (Condò et al., 1999; Tolstrup et al.,

2000). Sequencing various archaeal genomes has shown up that *Archaea* are invested with roughly 10 reputed translation initiation factors, which bear more analogies to eukaryotes (Dennis, 1997; Bell and Jackson, 1998). *Archaea* seem to subsist two various translational initiation mechanisms. One is competent to recognize leaderless mRNAs and the other deals with SD/anti-SD interaction (Tolstrup et al., 2000).

While *Archaea* and *Bacteria* share a lot of conformities in genome organization, some archaeal genes show a far bit of homologies to eukaryotes as in DNA replication, transcription and translation (Brown and Doolittle, 1997). E. g. the archaeal and bacterial DNA polymerase or RNAPs (Gropp et al., 1986), which have 12 subunits in *Archaea* and eukaryots. Or TATA-box like binding sites and some transcription factors are lacking in *Bacteria*. TBP and TFB, the two main transcription factors, needed for *in vitro* transcription in *Archaea* are equals of the TATA-box binding proteins and TFIIB in *Eukarya* (Bell et al., 2001).

A special feature of *Archaea* is the absence of peptidoglycan (murein) with various chemical divergences but the morphology, structure and function of the peptidoglycan is similar to the of *Bacteria*.
Another archaeal unique feature, which neither *Bacteria* nor *Eukarya* have, are ether linkages among hydrocarbons and glycerol. This linkages at the alpha and gamma carbon leads to the formation of long and branched fatty acid molecules. The ester linkages (at the alpha and beta carbon) in Bacteria lead to the formation of linear fatty acid molecules. Even tetraethers are formed in some archaeal membranes, which is not assimilable in bacterial structure (Gambacorta et al., 1993).

1.1.2.2 Archaeal promoter elements

In the late 80's the meaning was established that archaeal promoters show more homologies with the eukaryal RNA polymerase II promoters than with the bacterial model. Through linker scanning mutagenesis it was indicated that two regions are important for the transcription initiation *in vitro*, a sequence about 30 nucleotides upstream the transcriptional start sequence and the start site itself (Reiter et al., 1990).
In *Archaea* the TATA- box is the significant promoter component, which is essential for both transcription initiation at solid RNA and for protein encoding genes. Three princi-

pal units were found in all *Archaea*, INR (an initiator element) round the start site of the transcription, the TATA-box located at circa -26/-27 and a part upstream of the TATA-box (-34 AA -33) composed of two adenines, which is called BRE (transcription factor B recognition element). But the downstream promoter unit (DPE) is not found in archaeal groups like in eukaryotes (Soppa, 1999). There it is positioned +30 nucleotides (Hampsey, 1998) and is recognized by TBP (TATA box binding protein), wich is provided by the TAF. Statistical analysis point up that the consensus sequences of TATA-boxes, initiator elements and transcription factor B recognition elements vary for Euryachaeota (halophilic and methanogenic *Archaea*) and Crenarchaeaota. This indicates that the molecular details for the transcriptional processes are changeable, whereas the transcription factors are conserved in the archaeal domain (Soppa, 1999).

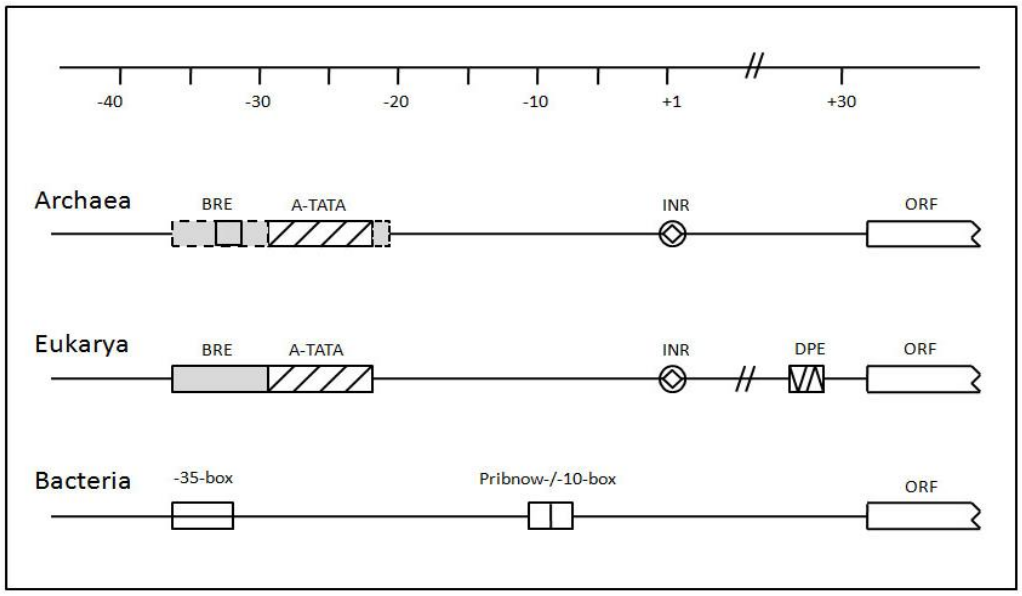

Fig. 1 Comparison of promoter elements.

Compared are promoter elements from *Archaea*, *Eukarya* and *Bacteria*. They are posed shematically aligned to the benchmark on the top. The numbering relates to the transcription start point (+1). BRE, TFB recognition element; TATA-box; INR, initiator compound; DPE, downstream promoter element; ORF, open reading frame.

(Soppa, 1999 with modifications)

1.1.3 Assimilation of haloalkaliphilic *Archaea*

1.1.3.1 Adaption to extreme living conditions

Halophilic and halotolerant microorganisms have to get over high salt concentrations in their surroundings. They overcome this problem by accumulating intracellular KCl close to saturation and extrusion of Na^+. Proteins, which are stable, soluble and active in high NaCl concentrations unfold after isolation from this Archaea and decreasing of the salt concentration (Oren, 1999 and Richard, 2000). Specific mechanisms were educed from the halophilic proteins to be both soluble and stable in the high cytoplasmic KCl concentration.

The second substantial factor in such extreme environments is the high pH value. Through the work of Horikoshi et al. (1999) it is conjecturable that the cell wall and the outer layers are responsible for the cytoplasmic pH regulation. Acidic polymers, which are located in the cell wall act as negatively charged matrix and seem to cut the pH value at the cell surface. Below the optimal growth pH (alkaline pH value) the plasma membrane is very unstable. That is the reason why the surface of the plasma membrane has to be kept below pH 9.0 (Horikoshi et al., 1999).

1.1.3.2 Halophilic proteins and their secretion to a hypersaline environment

Since the number of publications on halophilic proteins has increased the meaning has prevailed that salt is required for their solubility, activity and stability (Madern et al., 2000). For some halophilic enzymes even the nature of the salt is an important factor, because their activity is in KCl much higher than in NaCl e.g. 3-hydroxy-3-methylglutaryl-coenzyme A reductase from *Haloferax volcanii*. Increasing KCl concentrations increases the enzyme activity and is decreasing by increasing NaCl concentrations (Bischoff and Rodwell, 1996).
High salt concentration of some ions lead to protein stability, but also to precipitation. This event is called „salting-out" and utilizes by biochemists to get protein crystals.

Contrary to that are „saltin-in" events relevant for more solubility but also for unfolding the protein at high NaCl concentrations (von Hippel and Schleich, 1969). Even the activity is unavailable when the KCl or NaCl concentrations decrease to a value of less than 2 M. Heavy water (D_2O) or glycerol or members of the betaine family are able to stabilize halophilic enzymes when the salt concetration is to low (Madern et al., 2000).

Halophilic enzymes display a higher due of acidic amino acid remains (Lanyi, 1974). Statistical analysis reveals that these analyzed 26 soluble proteins from halophiles possess less lysine (Lys). Although they have a minor proportion of aliphatic residuals there is an increase in small hydrophobic residues like Gly, Ala and Val to notice (Madern et al., 1995). The acidic nature cannot be the only reason for the halophilism (Madern et al., 2000).
Experiments from Franzetti et al. (2001) imply that may be chaperones are liable for the correct folding of halophilic proteins.

Bacteria and *Eukarya* are using for most secreted proteins the Sec (secretory) pathway, which allows to keep the proteins in an unfolded status (Pohlschröder et al., 1997). To keep the proteins unfolded would be in halophile *Archaea* very adventurous. Folding of secreted proteins before secretion would give the haloarchaeal proteins the chance to be furthermore stable, prevent intra- and extracellulary aggregation (Berks et al., 2000).

The translocation apparatus in eukaryotic and bacterial plasma membranes are called Sec61αβγ SecYEG. Analysis of the archaeal genome revealed that the archaeal putative SecY/Sec61α proteins are more homologous to the eukaryotic than to the bacterial ones. Also found in *Archaea*, SecE/Sec61γ, the second important element of the apparatus is appropriate for the translocation function (Eichler, 2000 and references within).

Commonly it is not much known about the secreation pathway in such extreme living conditions like high salt concentrations. But present data lead to the suggestion that the archaeal secretion machinery differs from that of non-haloarchaeal. The most used pathway, which allows a correct cytoplasmic folding of the proteins is the sec-independent twin-arginine translocation (Tat) pathway. In Archaea this Tat-pathway is restricted to redox-proteins. Whole genome analysis of the Halobacterium sp. NRC-1 and a lot of other prokaryotes with TATFIND were done by Rose et al. (2002) to find

the putative Tat subrates. Their work suggest that most of the secreted proteins of halophile Archaea are putative subrates of the Tat-pathway.
The extreme usage of the Tat-pathway has grounds to believe that haloarchaea have adapted this machinery to the high salt environment by folding secreted proteins in the cytoplasm before their secretion (Rose et al., 2002).

1.1.4 Opportunity of genetic use of haloalkaliphilic *Archaea*

1.1.4.1 Marker and reporter genes

Like shortly described in 1.1.2.1 *Archaea* miss the peptidoglycan in their cell wall as one of their features. To work with *Archaea* there is the need of antibiotics for selection, but current antibiotics like ampicillin, which affects the bacterial cell wall or kanamycin, which inhibits the protein synthese after accumulation to the 30S subunit of membrane assosiated ribosomes can not be used for them. Indeed there are some available marker and reporter genes for *Archaea*. A few of them also work in halophilic *Archaea*, e.g. novobiocin and mevinolin. *GyrB* was isolated from *Haloferax alicantei* reulting in a antibiotic resistance to novobiocin, which exists in the nature retards the activity of the eukaryal DNA gyrase. The inhibition occurs through the binding to GyrB and hindering the entry of ATP to its binding site on this subunit, the type IIA topoisomerase (Mizuuchi et el., 1978 and Sugino et al., 1978).
Both strands of a DNA duplex are divided and a second duplex is relayed trough the double stranded split by the type II topoisomerases (Champoux, 2001). For DNA repilcation, transcription and recombination a introduction of negative supercoils into circular doublestranded DNA is important. DNA gyrase is responsible for this ATP-dependent supercoiling. In an ATP-independant way gyrase eases supercoiled DNA (Reece and Maxwell, 1991).

The second most commonly used antibiotic resistance for gentic analysis is mevinolin. The availability reaches from the insertion in plasmid vectors, gene knockouts, transposons, gene expression studies and much more (Wendoloski et al., 2001).

Mevinolin (1,2,6,7,8,8a-hexahydro-β, δ-dihydroxy-2, 6-dimethyl-8-(2-methyl-1-oxobutoxy)-1-naphtalene-heptanoic acid β-lactone) inhibits the 3-hydroxy-3-methylglutaryl coenzym A (HMG-CoA) reductase in *Eukarya* and in *Haloarchaea*. As a result of the obstruction of the production of isoprenoid lipids (Cabrera et al., 1986), which are the essential lipids in the cell membrane, the organisms is not able to growth in liquid media. The gene *hmg* was isolated from the *Haloferax volcanii* genome (Lam and Doolittle, 1989).
Lovastatin is a synonym for mevinolin and causes reduction of cholesterol synthesis in humans, by converting acetyl-CoA to mevalonic acid (Miller and Wolin, 2001). Because of the effects on the cholesterol in humans (Endo, 1992) including the formation of mevanolate it is a member of the chemical class „drugs" (Miller and Wolin, 2001).

The usage of novbiocin and mevinolin (Iro et al., in prep) could be established for haloalkaliphile *Archaea*.

1.1.4.2 Transformation

To find a successfull method to introduce foreign DNA into *Archaea*, Cline and Doolittle started with the transfection of *Halobacterium halobium* with phage ϕH DNA. Because of the abscence of selectable marker they trusted in the results of a plaque assay, to derivate the DNA uptake. This was the first transformation achievement in *Archaea* (Cline and Doolittle, 1987). Through the treatment with chelating agents like EDTA they could remove the S-layer (surface-layer), because the protein subunits are kept together by Mg^{2+}. By the use of the PEG-mediated (polyethylen glycol) method they were able to transform *Hbt. salinarum*. Until now this PEG-600 procedure is the best one to transform *Halobacteria*. But to transform haloalkaliphilic *Archaea* like *Nab. magadii* or *Nmn. pharaonis* there have to be done some changes in the protocol, which has been collected with other procedures, media compositions and things like that in the „The Halohandbook" by M. L. Dyall-Smith. In *Nab. magadii* and *Nmn. pharaonis* and some other haloalkaliphilic *Archaea* the treatment with EDTA is not enough to strip of the S-layer and to form spheroplasts. In cases like that the utilization of proteases is needed. After this attention the cells are prepared to be transformed (Iro et al., in prep).

1.1.4.3 Methylation in *Archaea*

By site-specific ribose methyltransferase proteins, which are able to detect both sequence and structure within the unmodified precursor tRNA substratum is the tRNA ribose methylation accelerated in *Bacteria* and eukaryotes. A few years ago a scientific group could identifiy the first tRNA ribose methyltransferase (2'-*O*-Mtase) in *Archaea*. *Archaea* as the only one of the three kingdoms seem to use also a RNA guide-dependant technique, which is called C/D box sRNP guide machinery. Obviously it finds and modifies the corresponding site within the fulllength tRNA substrates and procures the 2'-*O*-ribose methylation in distinct tRNAs (Clouet-d' Orval et al., 2005 and references within).

1.2 *Natraialba magadii*

1.2.1 In general

In 1984 *Nab. magadii* and eight other halophilic aerobic *Archaea* were described. These organisms were split into two genera *Natronobacterium* and *Natronococcus*, because as their names imply the original isolates contained rods and cocci (Tindall et al., 1984). Resulting the 16S rRNA analysis a rod shaped aerobic halophil archaeal isolate from the Lake Magadi in Kenya was transferred from the genus *Natronobacterium* to the genus *Natrialba* by Tindall et al. (1984).

Nab. magadii is a haloalkaliphilic member of the family of the *Halobacteriaceae* and requires temperatures among 37 and 42°C for its optimal growth. The pH values should not fall below 8.5 and not exceed 10.5. Another important growth condition is the salt concentration in the environment. To prevent cell lysis *Nab. magadii* requires a NaCl concentration between 2 and 4 M and a magnesium concentration less than 10 mM. Their cells are rod shaped, 0.5 till 0.7 µm long, motile and because of the carotinoides in their cell membrane orange-red (Tindall et al., 1984).

In the laboratory there are two strains available. In particular the strain L11, which

carries the prophage ϕCh1 and the other one called L13 (Fig.2 A, B). The original isolate (L11) lyses by gaining the stationary phase (Fig.2 C). Then the release of the descendant phages appears. By sit-specific recombination integration into the host chromosome occurs often and leads to a symbiosis of the phages and the host. The strain L13 was cured from ϕCh1 by Witte et al. through repeated subculturing. This results to be in possession of the indicator strain L13 receptive to infection with ϕCh1.

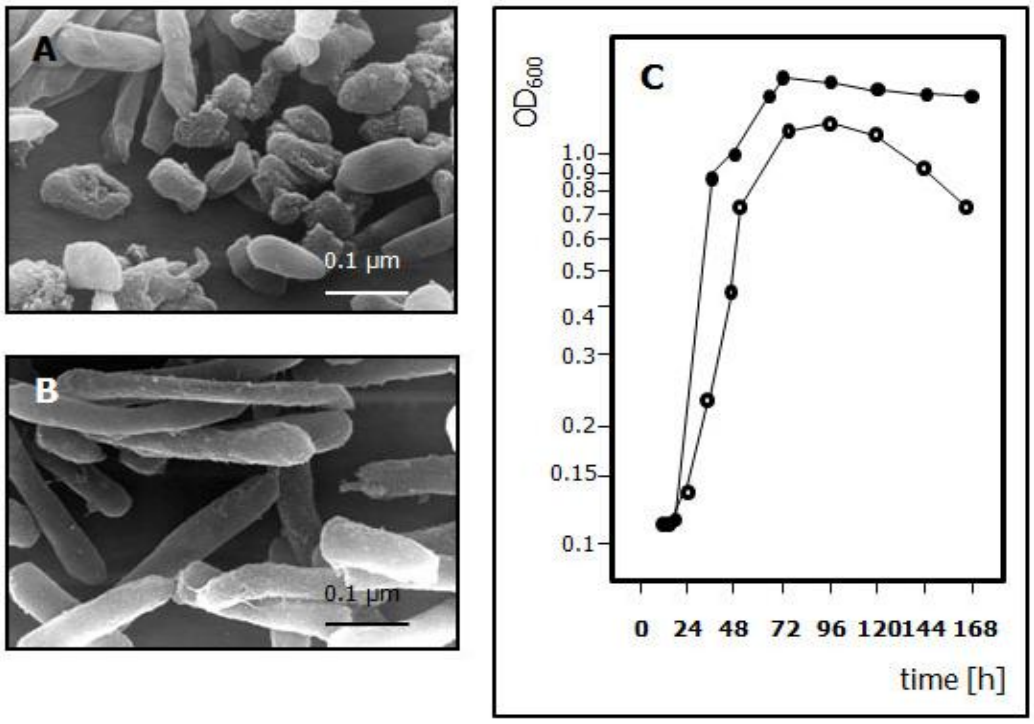

Fig. 2 Electron micrograph and growth curve

A Picture of the wild type Nab. magadii L11, which carries the prophage ϕCh1

B Image of the cured strain of Nab. magadii L13

C The diagramm shows the growth manner in optimal living conditions.
● indicator strain L13; ○ lysogenic strain L11

1.2.2 Genetical prospect

Until now the genome of *Nab. magadii* was not complete sequenced, because there are not so many scientists, who deal with this haloalkaliphilic archaeaon. The labora-

tory work would be easier when the sequence gets published.
Our laboratory tried to do the best with the current knowledge and were successfull in establishing a transformation system utilizing an exsiting method with modifications (chapter 1.1.4.2) and constructing an available phage derived plasmid with novobiocin as an antibiotic resistance (Iro et al., in prep).

Only two antibiotic resistances have enforced in the last few years, novobiocin and mevinolin. The resistance to novobiocin is the result of the *gyrB* gene from *Haloferax alicantei*, to mevinolin of the *hmg* from *Haloferax volcanii* (chapter 1.1.4.1).

1.3 *Natronomonas pharaonis*

1.3.1 In general

Nmn. pharaonis is an extremely haloalkailiphilic archaeaon and was isolated from saline soda lakes in Kenya and Egyt (Soliman et al., 1982 and Tindall et al., 1984). Just as *Nab. magadii Nmn. pharaonis* also needs extreme living conditions for its optimal growth. It requires salt concentrations between 2 and 3.5 M and alkaline pH values of 8.5 till 9.5 (able to survive up to pH 11.0). Its media should have low magnesium concentrations for a optimal process of growth (Staley et al., 1989).

Nmn. pharaonis is a member of the halophilic group of the euryarchaeota. A lot of proteins are highly homologous between the phylogenetic branch of the *Haloarchaea* (Konstantinidis et al., 2007). Trough genome analysis it seems that the metabolism is highly flexible and reflects an adaptation to extreme living conditions like high NaCl concentrations and high pH values (Falb et al., 2005).

1.3.2 Genetical prospect

Complete genome sequence analysis of *Nmn. pharaonis* concluded that their excist two

megaplasmids (PL131 and PL23) beside the 2.6 Mb GC-rich cromosome (63%). The plasmids conquer sizes of 131 kb and 23 kb and the theoretical proteome is composed of 2843 protein coding genes (Falb et al., 2005). In 2007 Konstantinidis et al. identified 43% of the theoretical proteome, that means 1226 proteins under high strictness.

There excist four rhodpsin-like (retinal) proteins in the membrane of *Halobacteria*. Bacteriorhodopsin (BR) and halorhodopsin (HR), which operate as light driven ion pump, sensory rhodpsin I (sRI) and phoborhodopsin (pR, synonym sensory rhodopsin II, sRII), which are photosensory receptors. All four proteins have a common tertiary structure. They have 7 transmembrane helices a retinal chromophore bound to a conserved lysine residue, located on the G-helix (the seventh one) through a protonated Schiff base (Sasaki et al., 2009 and references within). The first success in overexpressing rhodopsin in *E.coli* was achieved for phoborhodopsin from *Nmn. pharaonis* (ppR) (Shimono et al., 1997).

1.4 The halovirus ϕCh1

1.4.1 Haloviruses – general view

In the 1980's studies about haloviruses in *Archaea* started. The research concentrated on ϕH infecting *Hbt. salinarum* (Schnabel et al., 1982) and four other species. This halovirus is a temperate head tail virus with a genome of 59 kb dsDNA (Dyall-Smith et al., 2003). It belongs to the family of *Myoviridae*. The polyhedral head obtains a size of 64 nm in diameter and the tail a size of 170 nm in length and 18 nm in thickness (Schnabel et al., 1982). But till now the sequence has not been published, because it is only about 60% complete (Dyall-Smith et al., 2003).

In 1993 Dyall-Smith et al. isolated two other head-tail haloviruses from hypersaline lakes in Australia, which are called HF1 and HF2. They are able to infect more than one *Haloarchaea*, namely *Natrialba*, *Halobacterium*, *Halorubrum*, *Haloferax* and *Haloarcula* (Nuttal and Dyall-Smith, 1993). Although they show a few diffences like their host ranges were both isolated from the same lake and have 80% genome homologies.

Around 1997 the group isolated two more haloviruses in the australian outback, which bear the name His1 and His2. Infecting *Haloarcula hispanica* they reveal a small linear dsDNA genome of 15 kb and have felxible capsids. Whereas His1 has more morphological similarities with the lemon-shaped SSV1 virus, His2 more pleomorphic (Dyall-Smith et al., 2003). The virus particles are about 74 nm long and 44 nm wide, their tailsize is 7 nm (Bath and Dyall-Smith, 1998).

1.4.2 ϕCh1 in general

The laboratory of Witte et al. was successfull in detecting a new halovirus, named ϕCh1, which is as against the above mentioned haloviruses not lytic. ϕCh1 is the prophage, which infects the strain L11 of *Nab. magadii*. After creating a cured indicator strain L13, the halovirus could be characterized.
Till now *Nab. magadii* is the only known host of ϕCh1 (Witte et al., 1997).

1.4.2.1 Morphology

Morphological features of ϕCh1, the first described bachteriophage for *Nab. magadii* simulate other phages like ϕH of *Hbt. salinarum* or (Schnabel et al., 1982) T4 of *E. coli*. Trough electron mikroscopic analysis it is to tell that ϕCh1 has an icosahedral head and a contractible tail, which is used for the insertion of the genome in the host cell.
The phage head is about 70 nm in length and the tail about 130 nm, what results in a whole size of 200 nm. The tail (20 nm in wideness) is in possess of an internal shaft, which is capped by the contractible tail. At the end of the tail is a structure located which seems to be responsible for the adsorption, the first step of infection. In relation to the morphology the halovirus ϕCH1 belongs to the family of *Myoviridae* (Fig. 3).
The high salt concentrations (at least 2 M) made the morphology stable and infective. These characteristics classifiy ϕCh1 as a typical virus of *Haloarchaea* adapted to the extreme living conditions of its host (Witte et al., 1997).

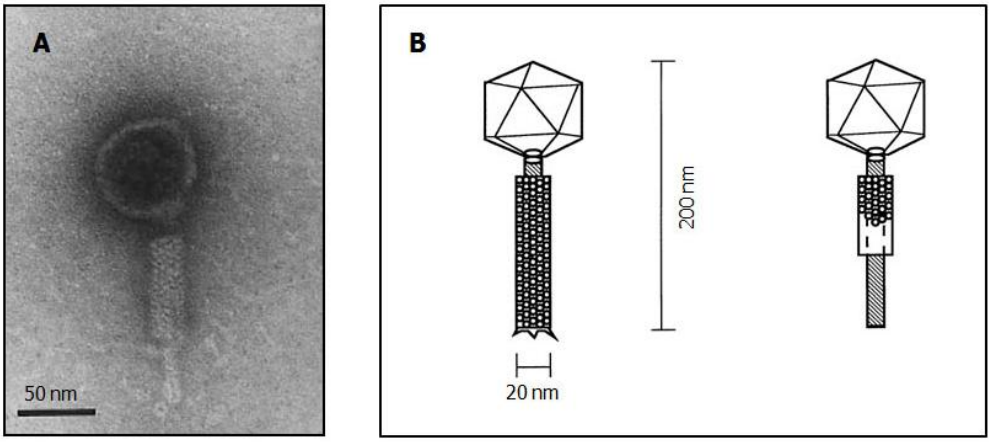

Fig. 3 Electron micrograph and shematic description of ϕCh1

A Phage particles negatively stained phospho-tungstate. The icosahedreal head, the contructile tail and the anchor pattern among head and tail are visible.

B All halovirus particles as contracted tail including dimensions are shematically shown.

(Witte et al., 1997 with modifications)

1.4.2.2 Genome organisation

The genome of ϕCH1 is composed of 58 498 bp linear double stranded DNA and has in total G+C content of 61.9%. Analysis have revealed that the RNA present in ϕCH1 could be host specific and encoded by the chromosomal DNA of *Nab. magadii* not by the phage itself (Witte et al., 1997).

In 2002 the complete sequence of ϕCh1 was determined by what 98 ORFs consiting of at least 30 codons were found by Klein et al. All of these open reading frames start with an ATG except of four of them (ORFs 3, 41, 79 and 83) which have to begin with a GTG. After closer consideration of the ORF they suggested that the genome is arranged in 3 transcriptional units, where the ORFs are either in the same reading direction after each other or overlapping by some nucleotides (left and rightwars-transcribed respectively) or in a mixture of right and leftward transcribed organized. Just 48 out of the 98 ORF have similarities to other already known sequences and only 17 of them are like proteins of noted function (Fig. 4) (Klein et al., 2002).

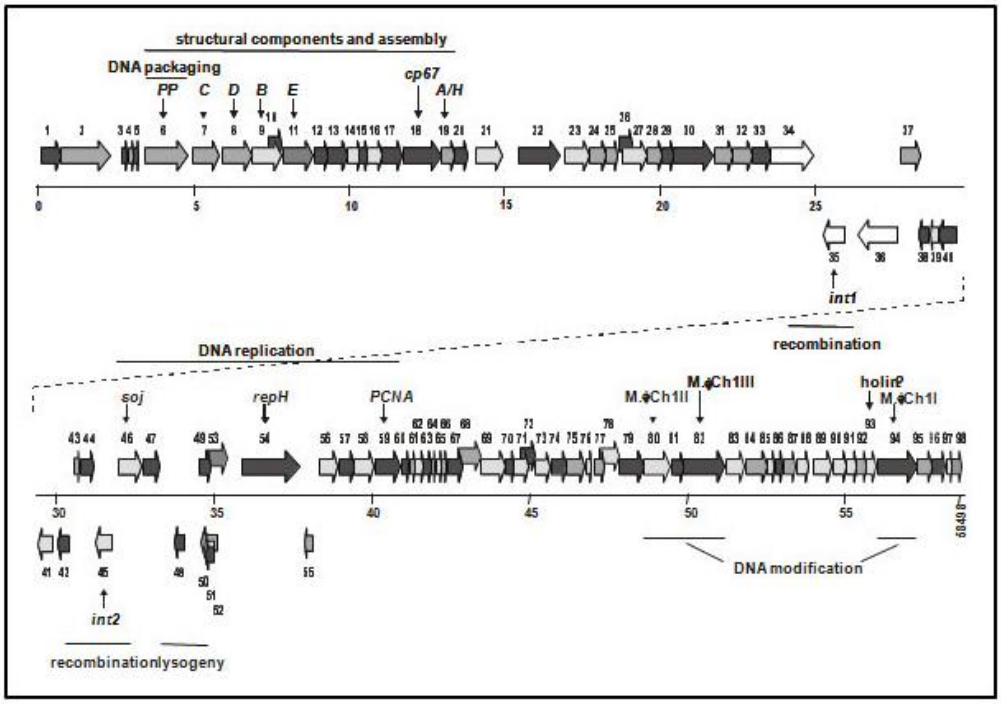

Fig. 4 Shematically representation of the ϕCh1 genome

This image show the 58 498 base pairs of the phage ϕCh1 and the ORFs, represented by arrows and numbered. Some putative and verified sites are indicated.The three parts are also shown: left part contains units for structural proteins and for virion morphogenesis, middle part contains genes for replication, plasmid stabilization and regulation of gene expression, right part contains genes for DNA methylation and restriction and several genes with unknown function. The grey shades represent the three open reading frames (dark grey = 3rd, middle grey = 2nd, light grey = 1st).

(Klein et al., 2002 with modifications)

Comparisons between the terminally redundant and circulary permuted available DNA sequences of ϕH of *Hbt. salinarum* and that of ϕCh1 show similarities in the mechanism of packaging of DNA into preheads. The central unit of ϕCh1 was assembled with the L-fragment of ϕH (Klein et al., 2002), which can circularize and replicate as an autonomous plasmid (pϕHL) (Gropp et al., 1992). These two sequences seemed to be highly analog over the entire of length varying between 50% and 97% (Klein et al., 2002). The hosts of these two haloviruses are geographilcally far apart from each other and are distinct concerning the pH values in their environments, but the homologies of their genome are more noticeable (Hendrix et al., 2000).

1.4.2.3 ϕCh1 – a temperate virus

After infection a phage can be differentiate between two different states, virulent and temperate. The virulent way results in destroying the host cell. A temperate virus is able to choose among destroying the cell by lysis and setting free its newly built particles and integrating its DNA into the host genome to be replicated without killing the cell. As a so called prophage the genome is replicated but the translation of virus protein like those which cause lysis are suppressed. Till the induction of spontanous lysis a repressor protein encoded on the virus is remaining the phage in this state and also preventing superinfection by other phage particles (Brock, 2006).

Particles of the ϕCh1 phage were obtained after spontanous lysis of *Nab. magadii*. Derived from this fact and other experimental results like the blurring of the plaques Witte et al. suggested that the phage is a temperate one. Through hybridization analysis they approved that the integration in the chromosome occurs. Southern blot analyses could reveal that the genome of ϕCh1 is detectable in the chromosome of *Nab. magadii* L13, but they get no results except one day previously inception of lysis with non-chromosomal DNA.
To date it is not clear what persuades in *Nab. magadii* infected with ϕCh1 the change from lysogenic to lytic cycle after reaching the stationary phase (Witte et al., 1997).

1.4.3 Gene expression of ϕCh1

As for all temperate viruses an important fact is the control of the lysogenic status verus the lytic one. Like reported by Klein et al. (2002) the genome of ϕCh1 consists not less than one putative repressor gene. The ORF48 show up homologies to the repressor of the phage ϕH infecting *Hbt. salinarum* and to other putative repressors of *Halobacterium sp*. strain NRC-1 or *Haloarcula marismortui*. It also shows similarities to repressors like LexA of *E. coli*. Because of that ORF48 was named *rep* (Iro et al., 2007).

After inspecting cultures deduced from sinlge plaques a virus variant named ϕCh1-1 was found. Compared to the wild type it formed larger plaques. Sequence analyses

lead to the conclusion that a duplication of 223 bp is present. This duplication implies a part of ORF49, a second putative repressor, and a small part of its upstream reagion. This results in creating a new ORF called ORF49‘ and is follwed by the original ORF49. Because of marginally overlapping these two ORFs are co-transcribed and co-translated.

Putative promoter sequencences seem to be located between ORF48 and ORF 49. Gene products of two other ORFs, ORF43 and 44 appear also to have an influence on this reagion. Both ORFs overlapp with their respective start and stop codons, what leads to the suggestion that they are also co-transcribed and co translated (Iro et al., 2007).

1.5 M.*Nma*ϕCh1I – a Dam-like methyltransferase

1.5.1 Methyltransferases in general

DNA methyltransferases detect specific in the DNA molecule and transfer methyl groups from S-adenosyl-L-methionine (AdoMet), which represents the methyl donor to adenine or cytosine remains. A lot of Mtases belong to the group of restriction/modification (R/M) systems to defend the DNA from their indogenous restriction endonucleases (Wilson, 1991; Krüger and Reuter, 1999).

There are two major groups of methyltransferases which have to be distinguishable, the C-Mtases and the N-Mtases. Whereas C-Mtases methylate a ring carbon and form C^5-methylcytosine, methylate the N-Mtases exocyclic nitrogens from N^4-methylcytosine or N^6-methyladenine. The ones are named N^4-Mtases and the other are called N^6-Mtases. Both Mtases use AdoMet as their cofactor and are active as monomeric enzymes (Baranyi et al., 2000).

1.5.2 Mtase of ϕCh1

By comparative digestion of genomic DNA of ϕCh1 Witte et al. (1997) assayed for attendance of adenine or cytosine methylation. It seemed that the phage DNA consists of adenine residues which are methylated (Dam-like methylation). These results were unforeseen, because it was confirmed that the sequence 5‘-GATC-3‘ from *Nab. magadii* is not methylated (Lodwick et al., 1986). Within the third part of the ϕCh1 genome three genes were identified as methyltransferases, the ORF 94, 80 and 82, which are named M.*Nma*ϕCh1I, II and III. The protein expressed of M.*Nma*ϕCh1I is the best studied one (Baranyi et al., 2000).
The protein of this identified *mtase* gene of ϕCh1 is a member of the β-subgroup of the N^6-mtases (Wilson, 1991; Malone et al., 1995) and experimental data show that it is expressed as a late virus gene (briefly before the release of the progeny particles). The late expression of the gene could be the explanation for the fact that not all virus genomes are methylated. Within about 50% of the resulting particles are methylated of the genome, but compared to the newly infected strain results in just 5% methylated genomes.
The experimental data above also proved that the Mtase is able to functionally complement the dam^- phenotype in the low-salt environment of *E. coli*, which is shared in DNA mismatch repair despite the low NaCl concentrations (Baranyi et al., 2000).

After new DNA strand syntheses at the replication fork the Mtases start to methylate its substrate. Through the hemimethylation the mismatch repair system is able to distinguish between the template and the daughter strand. Following this process dam methylation takes place. But in dam^- strains all 5‘-GATC-3‘ sequences are unmethylated what means that the uncorrect base pairs in the freshly synthesized strand are not replaced (Baranyi et al., 2000). *Nab. magadii's* own DNA is not modified and the methylation of plasmid DNA, which is used for transformation does not have a bearing on transformation efficiency (Iro et al., in prep).
Witte et al. (1997) did some restriction analysis which show that the methylation protect not only for the restriction endonuclease *MboI* but also to others like *PstI*, *ClaI*, *EcoRI* or *EcoRV*, which are delayed by adenine methylation. So the assumption arise that not only 5‘-GATC-3‘ sequences are methylated (Baranyi et al., 2000), what was tried to confirm by binding assays (Haider et al., in prep).

1.5.2.1 Structure of M.*Nma*ϕCh1I

The three-dimensional structure of M.*Nma*ϕCh1I is till now not determined and has not been crystallized. But in general it is known that Mtases are bilobal structures folded into two domains. The first larger catalytic one with both the active site for methyl transfer and the AdoMet binding site, the second one is a smaller DNA-recognition domain, which occupies loops included in sequence specific DNA recognition and the infiltration of the DNA to flip the target base. Several loops are flanking the two domains. In the gap of them the DNA is attached (Bheemanaik et al., 2006).

The Mtases are classified in nine conserved motifs named motif I-IX and the tenth motif X. Exocyclic methyltransferases are once more partitioned into six groups called *α*, *β*, *γ*, *ζ*, *δ* and *ε*. Depending on how the linear composition of the three conserved motifs, the AdoMet binding domain (FxGxG), the target recognition domain (TRD) and the catalytic domain (DPPY) is (Bheemanaik et al., 2006).

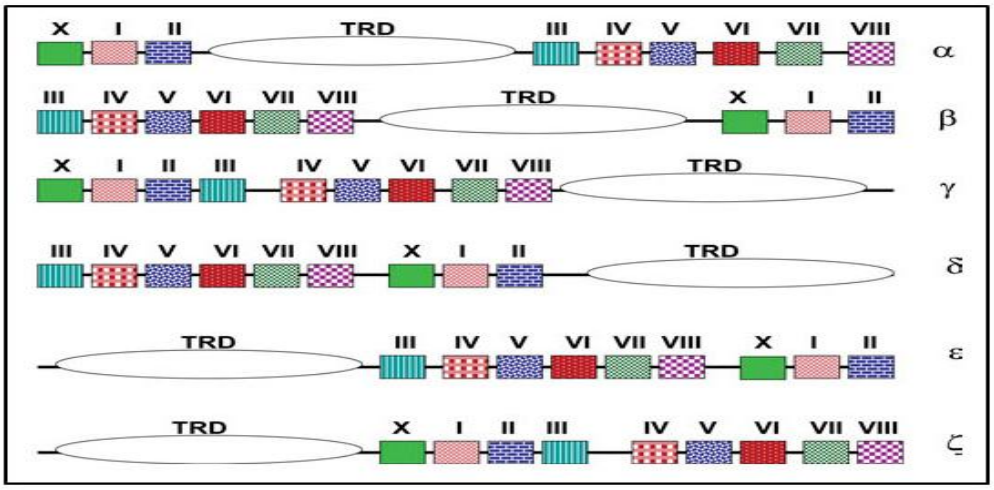

Fig. 5 Composition of conserved motifs in the primary structure of exocyclic DNA methyltransferases

M.*Nma*ϕCh1I belongs to the β subgroup with the motif IV N-terminally and the motif I at the C-terminus.

(Bheemanaik et al., 2006 with modifications)

Baranyi et al. (2000) did some sequence comparison studies using the program BLASTP and obtained results like there are similarities of 30.2% (over 242 amino acids

of the 419 codons) between the site specific DNA Mtase M.*Bst*F5I-1 (Degtyrev et al., 1997), which recognizes the specific sequence 5‘-GGATG-3‘ of *Bacillus stearothermophilus* and the M.*Nma*ϕCh1I.

The mtase gene of ϕCh1 has a derived molecular mass of 46 604 Da and an isoelectric point of 4.17. It also shows motifs specific for N^6-adeninemethyltransferases like the conserved motif IV (DPPY) N-terminally located (between the amino acid 196-199 of the 419 amino acid sequence) and the motif I (FxGxG) which has its location among the amino acid 360-364. The complete sequence of the gene is available in GenBank with the accession no. AF172444 (Baranyi et al., 2000).

2 Materials and Methods

2.1 Materials

2.1.1 Strains

2.1.1.1 Bacterial strains

Strain	Features and characteristics	Source
E. coli XL-1-Blue	*endA1, gyrA96, hsdR17* ($r_{k-}m_{K+}$), *lac, recA1, relA1, supE44, thi,* (F´, *lacI*^{q}*, lacZ* M15*, proAB*$^+$*, tet*)	Stratagene
E. coli Rossetta	F$^-$, *ompT, hsdS*$_B$ *(*$r_B^-m_B^-$*), gal, dcm, lacY1, (DE3),* pRARE^6, (Cm^R)	Novagen
E.coli GM48F‘	*F‘, thr, leu, thi-1, lacY, galK, galT, ara, tonA, tsx, dam, dcm, supE44*	Palmer and Marinus, 1994
E.coli JM110	*F‘, traD36, lacI*^{q} *(lacZ)M15, proA*$^+$*B*$^+$*/rpsL, thr, leu, thi, lacY, galK, galT, ara, fhuA, dam, dcm, glnV44, (lac-proAB)*	Yanisch-Perron *et al.*, 1985
E.coli SCS110	*rpsL* (Strr) *thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*) [F´ *traD36 proAB lacI*qZΔ*M15*	Stratagene

2.1.1.2 Archaeal strains

Strain	Features and characteristics	Source
Nab. magadii L11	wt, ϕCh1 as prophage	Witte *et al.*, 1997
Nab. magadii L13	ϕCh1 cured derivate of L11	Witte *et al.*, 1997
Nmn. pharaonis	wt	DSM 2160

Nmn. pharaonis 205	wt	DSM 2160
Hrr. sodomense	wt	DSM 3755
Hbt. salinarium R1	wt	Schnabel *et al.*, 1982
Nab. asiatica	wt	DSM 12278
Nbt. gregoryi	wt	DSM 3393
Hrr. coriense	wt	DSM 10284
Hrr. lacusprofundi	wt	DSM 5037
Hrr. saccharovorum	wt	Tomlinson and Hochstein, 1976

2.1.2 Media

2.1.2.1 Medium for *E. coli*

LB medium (rich medium)

Peptone	10 g
Yeast extract	5 g
NaCl	5 g

pH 7.0

add dH_2O to a final volume of 1 l
for plates 15 g/l agar were added

2.1.2.2 Medium for *Nab. magadii*, *Nmn. pharaonis*, *Nbt. gregoryi*

NVM+ (rich medium)

Casamino acids	8.8 g
Yeast extract	11.7 g
Tri-Na citrate	0.8 g
KCl	2.35 g

NaCl	235 g

pH 9.0

add dH_2O to a final volume of 934ml

for plates 8 g/l agar were added
for soft agar 4 g/l agar were added

After autoclaving the medium or the agar were completed with:

0.57 M Na_2CO_3 (dissolved in steril dH_2O)	65 ml
1 M $MgSO_4$ (autoclaved)	1 ml
20 mM $FeSO_4$ (dissolved in steril dH_2O)	1 ml

2.1.2.3 Medium for *Nmn. pharaonis* 205

DSM Medium 205 (rich medium)

Casamino acids	15.0 g
Tri-Na citrate	3.0 g
Glutamic acid	2.5 g
$MgSO_4 \times 7H_2O$	2.5 g
KCl	2.0 g
NaCl	250.0 g

add dH_2O to a final volume of 961.5ml

for plates 8 g/l agar were added

After autoclaving the medium or the agar were completed with:

20% Na_2CO_3 to change the pH to 8.7-8.8	
(dissolved in steril dH_2O)	65 ml

2.1.2.4 Medium for *Hbt. salinarum*

HSVM+ (rich medium)

Casamino acids	5.0 g
Yeast extract	5.0 g

Tri-Na citrate	3.0 g
KCl	2.0 g
NaCl	250.0 g
1M Tris-HCl pH 7.4	50 ml

add dH_2O to a final volume of 920ml

for plates 15 g/l agar were added

After autoclaving the medium or the agar were completed with:

1 M $MgSO_4$ x $7H_2O$ (dissolved in steril dH_2O)	80 ml

2.1.2.5 Medium for *Nab. asiatica*

DSM medium 372

Casamino acids	5.0 g
Yeast extract	5.0 g
Na-glutamate	1.0 g
Tri-Na citrate	3.0 g
KCl	2.0 g
NaCl	200 g

pH 7.0-7.2

add dH_2O to a final volume of 983ml

for plates 10 g/l agar were added

After autoclaving the medium or the agar were completed with:

1 M $MgSO_4$ (dissolved in steril dH_2O)	8 ml
0.02 M $FeCl_2$ (dissolved in steril dH_2O)	9 ml
0.02 M $MnCO_2$ (dissolved in steril dH_2O)	90 µl

2.1.2.6 Medium for *Hrr. coriense*, *Hrr. lacusprofundi*, *Hrr. saccharovorum*

18% MGM

30% salt water	600 ml
dH_2O	367 ml
Peptone	5.0 g
Yeast extract	1.0 g

adjust pH to 7.5 with 1 M Tris-HCl pH 9.5 and autoclave

for plates 10 g/l agar were added

2.1.3 Antibiotics

2.1.3.1 Antibiotics for *E. coli*

Antibiotic	Stock concentration	Final concentration	Comment
Ampicilin	20 mg/ml	100 µg/ml	in ddH_2O, filter sterile, store at 4°C
Tetracyclin	10 mg/ml	10 µg/ml	in 70% EtOH, store at -20°C, light sensitive
Chloramphenicol	40 mg/ml	20 µg/ml	in MeOH or 96% EtOH, store at -20°C
Streptomycin	50 mg/ml	30 µg/ml	in ddH_2O, filter steril, store at 4°C

2.1.3.2 Antiobiotics for *Archaea*

Antibiotic	Stock concentration	Final concentration	Comment
Novobiocin	3 mg/ml	3, 6 or 9 µg/ml	in ddH_2O, filter sterile, store at -20°C
Mevinolin	10 mg/ml	5 - 10 µg/ml	in 70% EtOH, store at -20°C
Bacitracin	7 mg/ml	70 µg/ml	in ddH_2O, filter sterile, store at 4°C

2.1.4 Plasmids

Plasmid	Feature	Reference
pUC19	*bla*, pMB1ori, *lacZα*, mcs	Yanisch-Perron et al., 1984
pKS_{II}^{+}	mcs, *bla*, ColE1, *lacZα*	Stratagene
pRSETC	mcs, *bla*, EK, P_{T7}, RBS, His-tag, pUC ori, f1 ori	Invitrogen
pQE32	P_{T5}, His-tag, mcs, lambda t_0, *rrn*B T1, ColE1 ori, β-lac	Qiagen
pNov1	*gyrB* (nov^R) introduced into pKS_{II}^{+}	Iro *et al.*, in prep
pRo-4	nu. 33951-37978 of ϕCh1 introduced into pNov-1	Iro *et al.*,in prep
pRo-4/mev	nu. 33951-37978 of ϕCh1 introduced into pNov-1, *hmg* (mev^R)	Iro *et al.*, in prep
pRo-5	*bla*, ColE1 ori, *gyrB*, ϕCh1 derived ori	Iro *et al.*, in prep
pNB102	*bla*, ColE1 ori, *hmg* (mev^R), pNB101 ori	Zhou *et al.*, 2004
pNov1/101	*gyrB* (nov^R), pNB101 ori	this thesis
pMDS11	*bla*, ColE1, *gyrB* (nov^R), pHK2 ori	Holmes *et al.*, 1991
pMDS24	*bla,* ColE1, (mev^R), DHFR, pHV2 ori	Jolley *et al.*, 1996
pRo-5/Bop	*bla*, ColE1 ori, *gyrB*, ϕCh1 derived ori, Bop gene pMG100 derived	this thesis
pMG100	pUS-Mev and pNB102 derived, pNB101 ori, amp^R, mev^R,*bop*	Kolog-Gulko *et al.*, in prep

pMG200	pMKK-100 and pNB102 derived, pNB101 ori, amp^R, mev^R, *bga*H	Kolog-Gulko *et al*., in prep
pMG300	pMKK-100 derived, amp^R, mev^R, *bga*H	Kolog-Gulko *et al*., in prep
pUCpN6M	*bla*, pMB1ori, *lacZα*, mcs, P (ORF34 of ϕCh1 derived)	this thesis
pUCpN6M/1b	*bla*, pMB1ori, *lacZα*, mcs, P (ORF34 of ϕCh1 derived), mtase gene ϕCh1 derived	this thesis
pUCpN6M/124b	*bla*, pMB1ori, *lacZα*, mcs, P (ORF34 of ϕCh1 derived), middle mtase fragment ϕCh1 derived	this thesis
pUCpN6M/541b	*bla*, pMB1ori, *lacZα*, mcs, P (ORF34 of ϕCh1 derived), small mtase fragment ϕCh1 derived	this thesis
pRo-5pN6M/1b	*bla*, ColE1 ori, *gyrB*, ϕCh1 derived ori, P (ORF34 of ϕCh1 derived), mtase gene ϕCh1 derived	this thesis
pRo-5pN6M/124b	*bla*, ColE1 ori, *gyrB*, ϕCh1 derived ori, P (ORF34 of ϕCh1 derived), middle mtase fragment ϕCh1 derived	this thesis
pRo-5pN6M/541b	*bla*, ColE1 ori, *gyrB*, ϕCh1 derived ori, P (ORF34 of ϕCh1 derived), small mtase fragment ϕCh1 derived	this thesis
pRSETC/1b	mcs, *bla*, EK, P_{T7}, RBS, His-tag, pUC ori, ϕCh1 ori, mtase gene ϕCh1 derived	this thesis
pRSETC/124b	mcs, *bla*, EK, P_{T7}, RBS, His-tag, pUC ori, ϕCh1 ori, middle mtase fragment ϕCh1 derived	this thesis
pRSETC/541b	mcs, *bla*, EK, P_{T7}, RBS, His-tag, pUC ori, ϕCh1 ori, small mtase fragment ϕCh1 derived	this thesis
pQE32/1b	P_{T5}, His-tag, mcs, lambda t_0, *rrn*B, mtase gene ϕCh1 derived	this thesis
pQE32/124b	P_{T5}, His-tag, mcs, lambda t_0, *rrn*B, middle mtase fragment ϕCh1 derived	this thesis
pQE32/541b	P_{T5}, His-tag, mcs, lambda t_0, *rrn*B, small mtase fragment ϕCh1 derived	this thesis

2.1.5 Primers

Primer	Sequence
MT-Kpn	5'-GACCGGTACCTATTCCCCCGGCGCTC-3´
34-3-XBcl	5‘-CAGGTCTAGATGATCACTTGTGTTACCTCGTAGCTCTGG-3‘
3mt-1b	5‘-GACGACAAGCTTTCACTCATTATCACCGGC-3‘
N6-1b	5‘-CAGGTGATCATGCAACTTGAAGAACTACCAACACCG-3‘
N6-124b	5‘-CAGGTGATCATGCAGACGAAGGGCTGGATC-3‘
N6-541b	5‘-CAGGTGATCATGTCCCAGCGCCTCGAG-3‘
TR1	5‘-AATTGCGGCCGCCGCGTTGAAGGCA-3‘
TR2	5‘-AATTTCTAGATCCTGGGCCTCTTTGAA-3‘
NB-1	5‘-TCTACCGGGTGCTGAACG-3‘
NB-2	5‘-CGCTGATGTACGAACCGAG-3‘
NB-3	5‘-CAGCCCACCGCGAGATT-3‘
MevR-1	5‘-CAGCAGGGATCCCCCAGCTTCTTCTAGA-3‘
MevR-2	5‘-TTGCATGCCACGCGTCT-3‘
MevR-3	5‘-CCGCGACGACCGACC-3‘
MevR-4	5‘-GCGAACGCCATCACGAC-3‘
Bop-1H	5‘-GACCAAGCTTGGATCCGACGTGAAGATGG-3‘
Bop-2H	5‘-GACCAAGCTTCGCGATCGTGCGAGTAC-3‘
MT-f	5‘-ATGGCGAGCATCGCT-3‘
Mt-rev	5‘-CGATCGCGCCGTACTCG-3‘
MT-RT1	5‘-GAACCGGAGCGTGATCG-3‘
MT-RT2	5‘-TACCGCACGACGACTACTTCG-3‘
MT-RT3	5‘-GCTACAAGATCCGGCCCTC-3‘
MT-RT4	5‘-CAGAATGCTTACCAGACGGC-3‘
MT-RT5	5‘-ATCTGTGGTCACTGCGGTAAC-3‘
MT-RT6	5‘-ACGGACCTGGGCGC-3‘
MT-RT7	5‘-TGGCTCGCATCTACCCCT-3‘
MT-RT8	5‘-GATTCCTCTCGTTCCGGTGT-3‘
MT-RT9	5‘-CTACTTCCAGATGATCCGCC-3‘
MT-RT-I1	5‘-GGACGAGGTCAACCGAGTCACC-3‘
Nov-6	5‘-GGGATCGCAGAGGAGC-3‘
MT-PE2	5‘-TCTCTTCGTTCTGCTCGTTCGGGTT-3‘

The underlined sequences represent restriction endonuclease sites used for cloning.

2.1.6 DNA ladders

Lambda/*Bst*EII marker (DNA from New England Biolabs or Fermentas)

add 20 µl 5x DNA loading dye to 5 µg digested Lambda DNA
add ddH_2O to final volume of 100 µl
Denaturation at 65°C for 10 min

8454, 7242, 6369, 5687, 4822, 4324, 3675, 2323, 1929, 1371, 1264, 702, bp

100 bp Ladder (New England Biolabs)

1517, 1200, 1000, 900, 800, 700, 600, 500/517, 400, 300, 200, 100 bp

1 kb DNA Ladder (New England Biolabs)

10.0, 8.0, 6.0, 5.0, 4.0, 3.0, 2.0, 1.5, 1.0, 0.5 kbp

pUC19/*Hae*III

add 20 µl 5x DNA loading dye to 5 µg digested pUC19 DNA
add ddH_2O to final volume of 100 µl
Denaturation at 65°C for 10 min

587, 458, 434, 298, 267/257, 174, 102, 80, 18,11 bp

Quick-Load® 2-Log DNA Ladder - biotinylated (New England BioLabs)

10, 8, 6, 5, 4, 3, 2, 1.5, 1.2, 1 kbp 900, 800, 700, 600, 500, 400, 300, 200, 100 bp

2.1.7 Protein ladders

Unstained Protein Molecular Weight Marker from Fermentas

116, 66.2, 45, 35, 25, 18.4, 14.4 kDa

2.1.8 Restriction enzymes and buffers

Restriction endonucleases and their appropriate buffers were used from Fermentas and New England Biolabs. Concentrations were used as recommended.

2.1.9 DNA, RNA and protein modifing enzymes

Calf intestine alkaline phosphatase, T4 DNA Ligase, Klenow Fragment, RNAse I, DNAse I from Fermentas, Proteinase K from Roche and Pronase E from Merck as well as γ-^{32}P ATP, ATP, NAD were used in suggested concentrations.

2.1.10 PCR and RT-PCR required enzymes and buffers

Pwo Polymerase from Peqlab, GoTaq Polymerase from Promega, RiboLock™ RNAse Inhibitor and Revert Aid™ M-MuLV Reverse Transcriptase purchased from Fermentas were applied.

2.1.11 Induction needed substances

To overexpress proteins in *E. coli* IPTG (1 M, in sterile water, stored at -20%) was used.

2.1.12 Antibodies

Primary antibodies

Antibody	Annotation	Dilution prior use
α-His-tag	mouse, against His tag epitope	1:2500 till 1:500
α-His-tag conjugated	mouse, against His tag epitope, no 2nd antibody needed	1:2500 till 1:500
α-Mtase	rabbit, against ORF94 of ϕCh1	1:2500 till 1:5000

All primary antibodies were diluted in 1 x TBS except α-His-tag conjugated, which was diluted in 1 x TBS T and incubated for 1 h.

Secondary antibodies

Antibody	Annotation	Dilution prior use
α-mouse IgG	conjugated to horseradish peroxidase	1:5000
α-rabbit IgG	conjugated to horseradish peroxidase	1:5000

All secondary antibodies were diluted in 1 x TBS and incubated for 1 h.

2.1.13 Solutions and buffers

2.1.13.1 Gelelectrophoresis

5x loading dye

0.1 %	SDS
0.05 %	Bromphenol blue
50 mM	Tris-HCl pH 8.2
after autoclaving add 25% steril sucrose	

50x TAE

2 M	Tris-HCl
1 M	Acetic acid
0.1 M	EDTA
adjust pH 8.2	

10x TBE

108 g	Tris base
55 g	boracic acid
0.5 M	EDTA pH
adjust pH 8.0 with boracic acid	

6 % PAA gel

1.2 ml	30 % PAA
4.8 ml	ddH_2O
60 µl	10 % APS
6 µl	TEMED

10 % PAA gel

2 ml	30 % PAA
4 ml	ddH_2O
60 µl	10 % APS
6 µl	TEMED

2.1.13.2 Southern blot analysis

20x SSC

3 M	NaCl
0.3 M	Sodium citrate
adjust pH 7.2 with HCl and add ddH_2O to a final volume of 1 l	

Blocking solution

7.3 g	NaCl
2.41 g	Na_2HPO_4
0.96 g	NaH_2PO_4
adjust pH 7.2 and add ddH_2O to a final volume of 1 l	

10x Washing solution II

12.1 g	Tris base
5.85 g	NaCl
2.03 g	$MgCl_2$

adjust pH 9.5 and add ddH_2O to a final volume of 1 l

1x Washing solution I

1:10 dilution of Blocking solution

50x Denhardt's solution

1 g	Ficoll 400
1 g	Polyvinylpyrolidone
1 g	BSA

add ddH_2O to a final volume of 100 ml

Hybridisation buffer (store at -20°C)

55 ml	ddH_2O
25 ml	20x SSC
10 ml	50x Denhardt's sol.
5 ml	10 % BSA
5 ml	1 M Na_2HPO_4
500 µl	20 % SDS
200 µl	0.5 M EDTA

2.1.13.3 SDS Page

2x Laemmli buffer

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

10x SDS running buffer

0.25 M	Tris base
1.92 M	Glycine
1 %	SDS

30 % PAA (29:1)

29 %	Acryl amide
1 %	N,N`-methylene bisacrylamide

10 % APS

0.5 g	Ammonium persulfate

add ddH_2O to a final volume of 5 ml

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

Coomassie staining solution

25 %	Methanol

Coomassie destaining solution

25 %	Methanol

10 %	Acetic acid
0.15 %	Coomassie Brillant Blue R-250

10 %	Acetic acid

2.1.13.4 Western blot analysis

Ponceau S staining solution

1 %	Acetic acid
0.5 %	Ponceau S

10x TBS

8 %	NaCl
3 %	Tris base
0.2 %	KCl
adjust pH 8.0	

Transblotbuffer

48 mM	Tris base
39 mM	Glycine
0.037 %	SDS
20 %	MeOH

2.1.13.5 Native protein purification

Lysis buffer

50 mM	NaH_2PO_4
300 mM	NaCl
10 mM	Imidazole
adjust pH 8.0 using NaOH	

Washing buffer

50 mM	NaH_2PO_4
300 mM	NaCl
20 mM	Imidazole
adjust pH 8.0 using NaOH	

Elution buffer

50 mM	NaH_2PO_4
300 mM	NaCl
250 mM	Imidazole
adjust pH 8.0 using NaOH	

Mtase dialyse buffer

50 mM	Tris-HCl pH 7.5
5 mM	β-mercaptoethanol
10 mM	EDTA pH 8.0
store at RT	

2.1.13.6 Plasmid isolation from *E. coli* and *Archaea*

Solution 1 (*E. coli*)

50 mM	Glucose
25 mM	Tris-HCl pH 8.0
10 mM	EDTA pH 8.0
10 µg/ml	RNase A

Solution 2

0.2 N	NaOH
1 %	SDS

Solution 3

3 M	KAc
adjust pH 4.8	

Solution 1 (*Archaea*)

1 M	Tris-HCl pH 9.0
2 M	NaCl
10 µg/ml	RNase A
store at 4°C	

2.1.13.7 RNA isolation

4 M	NaCl

2.1.13.8 Primer extension

10x RT- buffer

0.5 M	Tris-HCl pH 8.3
0.6 M	NaCl
0.1 M	DTT
solve in DEPC-H_2O	
store at -20°C	

10x RT+ buffer

0.5 M	Tris-HCl pH 8.3
0.6 M	NaCl
0.1 M	DTT
for primer extension	
0.02-0.03 M	$MgCl_2$
for toeprint	
0.06 M	$MgCl_2$

5x VD-buffer +/- Mg^{2+}

0.05 M	Tris-HCl pH 7.4
0.3 M	NH_4Cl
30 mM	β-mercaptoethanol
0.05 M	MgOAc

MMLV-loading dye

188 µl	10 M Urea
8 µl	10x TBE
4 µl	2 % BB + XX
6 µl	0.5 M EDTA

8 % PAA-Urea gel

24 g	Urea
10 ml	40 % PAA
5 ml	10x TBE

fill up with DEPC water to 50 ml
after dissolving urea, add 300 µl 10 % APS and 30 µl TEMED

2.1.13.9 Transformation of *E. coli*

MOPS I solution

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

adjust pH 7.0 using KOH

MOPS II solution

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

adjust pH 6.5 using KOH

MOPS IIa solution

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

adjust pH 6.5 using KOH

2.1.13.10 Transformation of *Archaea*

2.1.13.10.1 Transformation of *Nab. magadii*, *Nmn. pharaonis*, *Hbt. salinarum*, *Nab. asiatica*

Buffered spheroplasting soltion high salt with glycerol

2 M	NaCl
27 mM	KCl
50 mM	Tris-HCl pH 8.2
15 %	Sucrose
15 %	Glycerol

Buffered spheroplasting soltion high salt without glycerol

2 M	NaCl
27 mM	KCl
50 mM	Tris-HCl pH 8.2
15 %	Sucrose

Unbuffered spheroplasting soltion high salt (pH neutral)

2 M	NaCl
27 mM	KCl
15 %	Sucrose

2.1.13.10.2 Transformation of *Hrr. sodomense*, *Hrr.saccharouvorum*, *Hrr. coriense*, *Hrr. lacusprofundi*

Buffered spheroplasting soltion low salt with glycerol

1 M	NaCl
27 mM	KCl
50 mM	Tris-HCl pH 8.2
15 %	Sucrose
15 %	Glycerol

Buffered spheroplasting soltion low salt without glycerol

1 M	NaCl
27 mM	KCl
50 mM	Tris-HCl pH 8.2
15 %	Sucrose

Unbuffered spheroplasting soltion low salt with glycerol

1 M	NaCl
27 mM	KCl

Unbuffered spheroplasting soltion low salt with glycerol

1 M	NaCl
27 mM	KCl

15 %	Sucrose	15 %	Sucrose

2.1.13.11 Phage isolation

CsCl solution 1.1

2 M	NaCl
50 mM	Tris-HCl pH 8.5-9.0
0.6 M	CsCl

CsCl solution 1.3

2 M	NaCl
50 mM	Tris-HCl pH 8.5-9.0
2.7 M	CsCl

CsCl solution 1.5

2 M	NaCl
50 mM	Tris-HCl pH 8.5-9.0
4 M	CsCl

High salt alkaline solution

4 M	NaCl
50 mM	Tris-HCl pH 9.5

2.2 Methods

2.2.1 DNA Methods

2.2.1.1 Plasmid isolation

2.2.1.1.1 from *E. coli*

Plasmid DNA isolation for cloning or restriction analysis were done by the use of GeneJet™ Plasmid Miniprep Kit from Fermentas. The procedure was performed as recommended in the protocol. The isolate derived from 1.5 or 3 ml overnight culture, depending on the densityof the culture. The DNA was eluted in 50 µl ddH_2O. The silica-columns were brought into use again for isolation of the same plasmid.

2.2.1.1.2 from *Archaea*

As a substrate 3 ml of a ~ 4 days old culture, which had an OD_{600} of 1.2 was centrifuged for 2 min and 11.8 krpm. The pellet was resuspended in 50 µl Solution 1 (*Archaea*), which is locateable in the fridge at 4°C. 200 µl of Solution 2 are added and several times inverted. After an incubation of 5 min 150 µl of Solution 3 were subjoined and again inverted. Afterwards a centrifugation of 3 min and 11.8 krpm followed. The supernatant (~400 µl) was transferred into a new eppendorf tube and blend with 2x volume (800 µl) of 96 % EtOH. The incubation of 2 min at room temperature was followed by a centrifugation of 5 min and 11.8 krpm. The pellet was washed with 1 ml 70 % EtOH and towards drying it admitted in 30 µl RNAse free ddH_2O.
The original alkaline lysis procedure from this modified version coined by "The Halo-Handbook" (2006) by Dyall-Smith.

2.2.1.2 PCR – Polymerase chain reaction

The polymerase chain reaction is a possiblitiy to amplifiy DNA or to identify specific sequences contained in the sample of interest. A forward (5') and reverse (3') primer were designed with the help of the program Generunner. The primers attach to the corresponding reagion. As a result, the polymerase binds to the duet of primer and template and starts to elongate a new DNA strand beginning from the primer. The primer were ordered from VBC genomics or Sigma Aldrich were delivered lyophilized and deluted in ddH_2O to a concentration of 100 µg/ml.
The elongation time rose as a result of the sequence length of interest. Per 1000 bp were 1 min estimated. The annealing temperature was calculated also with the help of computer program. From the annealing temperature of the primer with the lower one 4 °C were subtracted and used for the PCR program. If there were to much unspecific bands, the PCR was repeated and 1 °C increased.

Analytical and preparative PCR batches were done utilizing *Pwo* Polymerase of Peqlab and GoTaq Polymerase of Promega in the submitted buffers. Whereas the analytical PCRs were done with *Pwo*, except getting no sufficient intense signal, the preparative were performed using the GoTaq.

Tentative batch with *Pwo*

10 µl	10x Pwo complete buffer
10 µl	2 mM dNTPs
5 µl	3' primer (=500 ng)
5 µl	5' primer (=500 ng)
1 µl	template
2 µl	Pwo
67 µl	ddH_2O

PCR program

99°C	Lid temperature
Preheating	ON
94°C	5 min
94°C	1 min
Annealing	1 min
72°C	1 min per 1000 bp product
72°C	5 min
33 cycles	
4°C	limitless

Tentative batch with GoTaq

2.5 µl	5x GoTaq™ reaction buffer
5 µl	2 mM dNTPs
2.5 µl	3' primer (=500 ng)
2.5 µl	5' primer (=500 ng)
1 µl	template
1 µl	GoTaq
36 µl	ddH_2O

PCR program

99°C	Lid temperature
Preheating	ON
94°C	5 min
94°C	1 min
Annealing	1 min
72°C	1 min per 1000 bp product
72°C	5 min
33 cycles	
4°C	limitless

2.2.1.2.1 Purification of PCR and DNA fragments

After a successful PCR the removal of dNTPs, buffers, salts and enzymes is needed. E. g. after PCR the polymerase has to be removed and the buffer has to be replaced. Towards restriction cuts the buffers have to be substituted and short DNA sequences have also be removed from the batch.
Sometimes there have to be purified DNA sequences from similar length. These procedures have been done using the QIAquick Purification kit and the QIAquick gel extraction kit from QIAGEN, respectively. In both cases the fragments were eluted in half of the introduced volume ddH_2O. The silica-columns have been resused for the same sequence template.

2.2.1.2.2 Template preparation of *E. coli* and *Archaea*

For PCR templates were isolated plasmid DNA, chromosomal DNA or raw extracts used. The template concetration had to have around 50 µg/ml. For raw extracts in *Archaea* 100 µl were centrifuged for 1 min and 11.8 krpm and the pellet resuspended in 100 µl ddH_2O. The other possibility to get raw extracts from *Archaea* was to mix up 5 µl culture with 100 µl ddH_2O and to vortex it. *E. coli* cells had to be boiled for 10 min at 95°C subsequently.

2.2.1.3 Restriction of DNA

Restriction endonucleases and their corresponding buffers were utilized from Fermentas and New England Biolabs. The batches were done using the recommended concentrations and incubated either over night or for 3 h. Analytical analysis were incubated for just 1 h.

2.2.1.4 Ligation of DNA

To construct novel plasmids, which carry the insert of interest the plasmids and inserts were pretreated with endonucleases or other modifing enzymes and ligated using the T4 DNA ligase from Fermentas and the recommended buffer at 16°C over night.

Batch composition

11.5 µl	Insert DNA
1 µl	Vector DNA
1.5 µl	T4 DNA ligase buffer
1 µl	T4 DNA ligase (1 u/µl)

This constellation was altered when doing blund end ligations; therefore the vector DNA was pre-treated with CIAP (calf intestine alkaline phosphatase) and 2 µl each of vector DNA, ligase and buffer were applied.

2.2.1.5 Additional DNA modifications

Like above mentioned CIAP is needed for blunt end ligations. Calf intestine alkaline phosphatase catalyzes the release of 5‘-phosphates from the DNA and prevents therefor recircularization of the vector without insert. The procedure takes 30 min at 37°C and is follwed by an inactivation of 20 min at 80°C.

In presence of sticky ends some cloning strategies could not work. For that reason they Klenow fragment was appointed, which fills up the 3‘ overhangs. For this padding were 100 to 200 µM dNTPs (each) required. The incubation took 30 min at 37°C and

afterwards the reaction was inactivated at 65°C for 10 min.

2.2.1.6 Agarose gel electrophoresis

The agarose (purchased from QIAgen) concentration was dependent on the length of the DNA fragments, which should be separated. For fragments till 700 bp a 1.5 % or 2 % gel was made, lengths over 700 bp were resolved on 0.8 % or 1.1 % gels.

The agarose was melted in 1x TAE using a microwave. The gel trays and the casting gates were set up in the electrophoresis chamber for casting. When casting gates were not available the ends of the gel trays were taped with strips of autoclavings tape. The comb was placed and the cooled down agarose was poured into the trays. After the gel was solidified the casting gates were removed and the tray was placed into a corresponding gel electrophoresis apparatus, which was filled with 1x TAE. Applying voltage separated the DNA fragments. To estimate the migration of the fragments the loading dye was mixed with bromphenol blue. Thereupon the gel was stained in a ethidium bromide bath (4 µg/ml) for a few minutes, visualized under UV light and a picture was taken of it.

2.2.1.7 PAA- polyacrylamid gel electrophoresis

These kind of gels were performed using Mini-Protean 3 cells from Bio-Rad. With the help of casting frames and casting stand the gelsolution (components see 2.1.13.1) was poured between the glass plates and the comb was adjusted. After polymerization the gel cassette sandwich was placed into the electrode assembly and the inner chamber was lowered into the „Mini Tank". The seperation of the DNA fragments took also place trough-applying voltage (15 mA per gel).

The gel concentration was adjusted to the expected length of fragments. For DNA sequences below 150 bp a 10 % gel was cast. DNA fragments of a size over 150 bp to 550 bp were applied on a 6 % gel.

2.2.1.8 Southern blot analysis

A Southern blot is a method to check the existence of a DNA sequence in a DNA sample. Before blotting an agarose gel electrophoresis is done to separate the DNA. Afterwards the size separated fragments are transferred to a nylon membrane by capillar blotting. The sequence of interest is visualized after hybridization of a labelled DNA probe. The technique is called after his originator Edwin Southern (1975).

2.2.1.8.1 Sample preparation and blotting process

For this analyis DNA fragments descending from restriction batches or PCR products and a biotinylated marker were separated on a 1.1 % agarose gel of about 15 cm length. After the size separation the gel was stained with ethidium bromide (see 2.2.1.6) and a picture was taken. The gel was treated with 0.25 M HCl for 10 min to depurinate the DNA fragments. Following a flush with ddH_2O the agarose gel was incubated in 0.4 M NaOH/0.6 M NaCl for 30 min to denature the double stranded DNA. The denaturation enhances the binding of the negatively DNA to the positively membrane. Residuals RNA that may still excist in the DNA are destroyed by this step.
Subsequently a bath in 1.5 M NaCl/0.5 M Tris-HCl pH 7.5 for 30 min pursued. Over night the DNA was transferred by the ion exchange interactions among the membrane and the DNA and blotted on a nylon membrane trough capillary forces using 10x SSC.

2.2.1.8.2 Labelling of the probe

The labeling reaction was performed as recommended in the NEBlot® Phototope® Kit protocol from New England Biolabs. About 500 ng purified DNA descending from a preparative PCR were applied and biotinylated. The reaction was stopped by the addition of 2 µl of 0.5 M EDTA. Precipitation of the biotinylated DNA was obtained by adding 10 µl 4 M LiCl and 200 µl 96 % EtOH. After an incubation time of 20 min at -20°C a centrifugation of 30 min, at 4°C and 16.4 krpm followed. Subsequently the pellet was washed with 1 ml 70 % EtOH and resuspend in 20 µl ddH_2O.

2.2.1.8.3 Prehybridization and hybridization procedure

Before cross-lining the membrane cross-linked under UV, it was incubated for 1 min each in 0.4 M NaOH and 0.2 M Tris-HCl pH 7.5. After that the membrane was prehybridized in 20 ml hybridisation buffer contaning 100 µg/ml salmon sperm DNA at 65°C for 3 h. The biotinylated probe was added and over night at 65°C the hybridization took place.
On the next day the incubated membrane was twice washed with 2x SSC/0.1 % SDS for 5 min at room temperature and twice with 0.1x SSC/0.1 % SDS for 15 min at 65°C.

2.2.1.8.4 Blot development and vizualization

For 5 min the membrane was incubated in blocking solution and afterwards kept for 5 min in a mixture of 7 ml blocking solution and 7 µl streptavidin. Streptavidin was taken from the used Phototope® Star Detection kit from New England Biolabs. There upon the membrane with the biotinylated probe DNA was washed three times with Washing solution I for 5 min each. Then it was incubated in 7 ml blocking solution combined with 7 µl biotinylated alkaline phosphatase from the kit for 5 min and again washed, but this time first with blocking solution for 5 min and afterwards three times with 1x washing solution II for 5 min each. The last step of the detection was the treatment with 6 µl CDP Star™ reagent in 3 ml diluted buffer from the Phototope® Star Detection kit, namly an incubation of 5 min. The visualization happened by exposing an X-ray film to the membrane and subsequently developing it in the darkroom.

This method replaces radioactive detection and is based on chemiluminescence. Streptavidin is first binding to the biotinylated target, the biotinylated alkaline phosphatase binds in turn to streptavidin. The phosphatase catalyzes the removal of the phosphate from the CDP Star™ reagent, so the emitted light is detectable.

2.2.2 RNA methods

2.2.2.1 RNA purification from *Archaea*

After pelleting 1.5 ml of a circa 4 day old culture for 3 min and 10 krpm it was resuspended in 250 µl 4 M NaCl. The cells were lysed with 750 µl TRIZOL reagent, which was incubated on room temperature for half an hour. After shaking the batch an incubation of 5 min at RT followed. A volume of 200 µl chlorophorm was added and mixed for 15 seconds shaking by hand. Past another incubation of 15 min at room temperature the aqueous and the organic phase were separated by centrifugation at 4°C for 15 min and 10 krpm. The equous phase was transferred into a new eppendorf tube and the RNA presipitated with isopropanol. A centrifugation of 10 min at 4°C and 10 krpm followed an incubation of 10 min at RT. The supernatant was discarded and the pellet washed with 1 ml 96 % EtOH. The washed pellet was dried and incorporated in 30 µl RNase free DEPC water.

2.2.2.1.1 DNAse I treatment

To use the isolated mRNA for e.g. RT-PCR it has to be purified of excess of chromosomal DNA, because halophilic *Archaea* store more than one chromosome inside their cell. To check the the success of the treatment a PCR and an analyse by gel electrophorese was done.

Batch formula (enzyme and buffer purchased from Fermentas)

20.5 µl	mRNA
2.5 µl	10x reaction buffer with $MgCl_2$
2 µl	DNAse I, RNAse free

The reaction was incubated for 30 min at 37°C and stopped by adding 1 µl 25 mM EDTA. Ending with an heat up to 65°C for 10 min. In the absence of chelating agents the RNA hydrolyzes during heating.

2.2.2.2 RT-PCR – Reverse Transkription Polymerase chain reaction

2.2.2.2.1 Two step method

The RT-PCR was performed as a two-step process. The RNA strand is first reverse transcribed into its DNA complement (cDNA) using the enzyme reverse transcriptase and primers, because the DNA polymerase of the following traditional PCR can act only on DNA templates. Between the two steps a denaturation occurs to separate the two strand and allow the primers to bind again. Afterwards the DNA extension takes place.

1st step

5 µl	DNAse I treated RNA (correspond 5 µg)
2 µl	random primer
5.5 µl	DEPC water

The reaction was stoked up to 65°C for 5 min and following components were added.

4 µl	5x reaction buffer MulV
0.5 µl	Ribolock Inhibitor
2 µl	10 mM dNTPs
1 µl	M-MuLV

The reaction mix was incubated at 25°C for 10 min, afterwards for one hour at 45°C, because it was a GC-rich sequence, in other cases this step would be done at 42°C and thereupon heated up to 70°C for 10 min.
The 2nd step of the RT-PCR was done like described in 2.2.1.2.

2.2.2.3 Primer extension

Primer extension can be used to determine the start site of RNA transcription of a known gene. This method requires a radioactive labelled primer, which is usually among 20 and 50 base pairs in length and complementary to a reagion near the 5‘ end of the gene.

2.2.2.3.1 Primer labelling

2 µl primer (5 pmol/µl)
2 µl 10x PNK-buffer
13 µl DEPC H_2O
2 µl γ-^{32}P ATp (3.3 pmol/µl)
1 µl PNK (10 u/µl)

The batch was incubated at 37°C for 1 h. Then the reaction was inactivated by incubation at 65°C for 10 min, followed by precipitation adding 1 µl of glycogen, 10 µl 3 M NaAc and 80 µl 96 % EtOH. The batch was kept at -20°C for 20 min and centrifuged for 5 min at 12 krpm. The supernatant was discarded and the pellet dried for about 15 min at RT and resuspended in 20 µl DEPC water (final concentration of 0.5 pmol/µl).

2.2.2.3.2 Primer extension inhibition reaction

To anneal the primer 5x VD-buffer without Mg^{2+}, 0.25 pmol/µl mRNA, 0.5 pmol/µl primer and DEPC water was heated for 3 min on 80°C, quickly frozen in liquid N_2. Finally 10 mM MgOAc in 1x VD were added. The extension reaction was composed of 1x VD with Mg^{2+}, 2 pmol/µl 30S, 8 pmol/µl tRNA-fMet and 0.04 pmol of the annealing mix. This reaction was incubated at 37°C for 10 min. After that 2 µl MMLV mix (5x VD with Mg^{2+}, MMLV (200 u/µl), BSA (1 mg/ml) and dNTPs (100 mM) in 1x VD+) was added and another 10 min at 37°C incubated. An aliquote was mixed with MMLV-loading dye and 8 µl were loaded on a 8 % PAA-Urea gel.

2.2.2.3.3 Sequencing reaction and sequencing gel

1 µl 10x RT-Mg^{2+}
2.4 µl mRNA (0.25 pmol/µl)
2.4 µl primer (0.5 pmol/µl)
4.2 µl DEPC water

The annealing reaction was incubated for 3 min on 80°C and frozen in liquid N_2. After defrosting 2 µl of 36 mM $MgCl_2$ in 1x RT were added.

The followed sequencing reaction had an incubation time of 10 min on 42°C and was mixed up with 10 µl of AMV-loading dye. 8 µl were loaded on a 8 % PAA-Urea gel.

1 µl	1x RT+
2 µl	Annealing mix
4 µl	5x dNTPs
4 µl	5x ddNTPs
1 µl	AMV-RT-mix

2.2.2.3.4 Vizualization

The reaction was vizualized using the Phosphoimager.

2.2.3 Protein methods

2.2.3.1 Overexpression of proteins in *E.coli* and protein sample preparation

The over expression was done in E.coli using the strains Rosetta and GM48F', which express the T7-polymerase and the sequence of interest within the expression vectors pRSET-C and pQE32. The protein expression is in both strains incuible with IPTG.

An over night culture, incubated at 28°C was used to inoculate 1 l LB with an OD_{600} of 0.1. The culture was incubated at 28°C shaking till an OD_{600} of 0.3 was reached. Then the induction by adding 0.5 ml of 1 M IPTG (final concentration 0.5 mM) followed. Cells were grown at 28°C for over nigth in case of Rosetta and 3 h in that of GM48F'. The culture was centrifuged for 15 min at 6 krpm and 4 °C. Afterwards the pellet was used for protein purification.

2.2.3.1.1 Protein purification under native conditions

The resulting pellet was resuspended in 50 ml lysis buffer (native) and put on -20°C over night or for 1 h on -80°C. After that the lysate was defrost on room temperature and mixed up with a spatulatip of lysozyme. This mixture was kept on 4°C panning for two hours. This step was followed by sonification three times 5 min each.
Remaining cells and cell debris was removed by centrifugation at 4°C, 6 krpm for 15 min. The supernatant was collected in a 100 ml flask and combined with 500 µl Ni-NTA solition from QIAgen. This approach was stirred over night at room temperature.
The mixture was filled into a Poly-Prep-column from BioRad. The flow through was collected and also the 5 ml washing solution flown trough. Afterwards the protein was eluted using the following shema.

1 ml	900 µl Washing buffer/100 µl Elution buffer
1 ml	800 µl Washing buffer/200 µl Elution buffer
1 ml	700 µl Washing buffer/300 µl Elution buffer
1 ml	600 µl Washing buffer/400 µl Elution buffer
1 ml	500 µl Washing buffer/500 µl Elution buffer
1 ml	400 µl Washing buffer/600 µl Elution buffer
1 ml	300 µl Washing buffer/700 µl Elution buffer

All fuids, which were flown trough were stored at RT and aliquotes were mixed up with 2x Laemmli, boiled for 10 min at 95°C and applied on a SDS-page and visualized by staining with Coomassie. For binding assays or enzyme activity assays the buffers had to be exchanged by dialysis.

2.2.3.2 Protein sample preparation from *Nab. magadii*

For protein samples 1.5 ml to 4.5 ml culture were used. The cells were centrifuged at room temperature for 3 min at 11.8 krpm. The pellet was dissolved in 5 mM Na-phosphate buffer, which consists of 0.2 M NaH_2PO_4 and 0.2 M Na_2HPO_4 at pH 6.8. It was not really resuspended because the pellet was extremely pasty. The volume of the buffer was calculated concidering the OD_{600}. The OD value was multiplied by 75 and the result revealed the µl per introduced 1.5 ml culture. The same amount of 2x

Laemmli buffer was mixed up. There upon the samples were boiled at 95°C for 15 min and then incubated for 24 h at 37°C shaking. Prior use the samples were kept on 65°C for about 2 h.

2.2.3.3 SDS-Page – Polyacrylamid gel electrophoresis

Polyacrylamid gel electrophoresis is a technique used to separate proteins accordging to their electrophoretic mobility (function of molecular weight). SDS, an anionic detergent denatures secundary and non-disulfide-linked tertiary structures of the proteins and applies in proportion to its mass a negative charge to each protein. Voltage is applied and the proteins can be sparated on the SDS-PAGE, which is utilized as matrix. This method is mostly followed by a Coomassie staining or Western blot analysis.

2.2.3.3.1 Laemmli method – casting and running a gel

The SDS-PAGE was performed using a vertical system from BioRad named Mini Protean 3 cell. The acompanying materials were availed for casting and running the gel.

Ingredients	12 % Seperation gel	4 % Stacking gel
30 % AA Solution	2000 µl	267 µl
Seperation gel buffer	1250 µl	-
Stacking gel buffer	-	500 µl
ddH_2O	1750 µl	1233 µl
10 % APS	60 µl	20 µl
TEMED	10 µl	5 µl

The seperation gel mixture was prepared on ice in a beaker glass and prior casting APS and TEMED was added. These two substances are responsible for gel formation and polymerisation. Next to that the gel was overlayed with isopropanol to get a flat surface and prevent bubbles. Afterwards polymerisation the isopropanol was discarded and the fresh prepared stacking gel was casted. Again polymerization followed. After removing the comb 10 µl samples and an unstained molecular weight marker were

applied to the slots.
The apparatus chamber was filled with 1x SDS running buffer. By applying voltage (15 mA per gel) the proteins could be seperated by size till the bromphenol blue from the 2x laemmli buffer reached the end of the matrix.

2.2.3.4 Detection by Coomassie R-250

The apparatus was relieved and the gel put in a small staining tub and was overlyed with the staining solution for 30 min. Coomassie Brilant Blue R-250 is an anionic dye which staines the proteins on the gel while the acetic acid from the solution fixes them. Subsequently the Coomassie solution was poured back into the bottle and the gel was rinsed with water and kept shaking over night in it. On the next day only the protein-bands were visible and the gel was dried using a geldrier. Sometimes destaining solution for 30 min was used instead of the water method.

2.2.3.5 Western blot analysis

The western blot technique is a method to detect specific proteins in a given sample. By gel electrophoresis proteins are separated by size and then transferred to nitrocellulose membrane which is treated with antibodies specific to the target protein afterwards. To visualize the protein of interest the second antibody which binds to the first one is labeled mostly.

2.2.3.5.1 Semi-dry-blotting procedure

After a SDS-page the proteins were transferred to nitrocellulose membrane Protran purchased from Whatman through a semi dry blotting system. For that six whatman papers and the membrane were cut in the size of the gel. For the given situation 3 sheets of whatman flanked the gel and the membrane. This construction was incubated for a few minutes in transblot buffer and then put in the blotting apparatus. The

transfer occurs by applying 20 V for 20 min (for two gels 20 V for 35 min, 3 gels 20 V for 40 min). The membrane was shortly rinsed with water and put in a staining tub.

2.2.3.5.2 Ponceau S staining and blocking

Ponceau S is a hydrophilic stain for reversible staining of proteins fixed on a membrane. The membrane was incubated in the Ponceau S solution until the proteinbands get visible. The bands of the protein marker were scribed and the blot was destained with water subsequently.

Over night the membrane was incubated in 5 % skim milk powder which was solved in 1x TBS by shaking. This blocking step was done to prevent the interactions between the membrane and the antibody used for detection of the target protein. The „blocking protein" attaches in all places where the target protein has not attached. Thus the antibody has no other possibility as to bind to the protein of interest.

2.2.3.5.3 Antibody titers

The membrane was rinsed with 1x TBS on the next day and incubated for 1 h at room temperature with the first or the conjugated antibody which was diluted in 1x TBS, 1x TBS-T respectively under shaking. Thereupon the membrane was washed three times with 1x TBS for 10 min each and treated with the second antibody, if necessary for one hour under the same conditions. At the end the membrane was also washed three times with 1x TBS for 10 min each.

2.2.3.5.4 Blot development and vizualization

The bound antibodies on the membrane were detected using the ECL kit from PIERCE observing the suggested instructions. The membrane was placed in a exposure cassette and a X-ray hyper film purchased from Amersham Biosciences was exposed on

the membrane and developed in the dark room.

2.2.4 Determination of DNA, RNA and protein concentration

The amounts of DNA, RNA and proteins was determined by spectroscopic mesurements at 260 nm utilizing Nanodrop from Peqlab.

2.2.5 Transformation

2.2.5.1 Transformation in *E. coli*

2.2.5.1.1 Preparation of MOPS competent cells

50 ml LB were inoculated with an over night culture of E. coli strains (XL1-Blue, Rosetta, GM48F', SCS110, JM110) so that the culture has an OD_{600} of 0.1 mixed up with the corresponding antibiotics. The cells were incubated at 37°C rocking till the OD_{600} reached 0.6. At that moment they were centrifuged at 4°C and 10 krpm for 10 min. The pellet was resuspended in 20 ml ice cold MOPS I solution and incubated on ice for 10 min. Another centrifugation of 10 min at 4°C and 10 krpm followed. The supernatant was discarded and the resulting pellet in 20 ml MOPS II solution dissolved. After an incubation of 30 min on ice the cells were centrifuged 10 min at 4°C and 10 krpm. The small cell pellet was carefully resuspended in MOPS IIa solution and aliquoted 100 µl each. The resulted competent cells were stored at -80°C for further useableness.

2.2.5.1.2 Transformation of MOPS competent cells

Shortly after preparation or after thawing on ice the competent cells were used for transformation. Constructing new plasmids the whole ligation batch was added to an aliquote of 100 µl competent cells. For intact plasmids it was enough to transform 1 µl. To retransform plasmids like pRo-5 isolated from halophilic *Archaea* 7 µl have to be taken. After an incubation of 30 min on ice a heat shock for 2 min on 42°C followed. The cells were put back on ice and then combined with 300 µl sterile LB. Thereupon the cells regenerated on 37°C for 30 min. Finally 134 µl were plated on three LB plates each, also containing the corresponding antibiotics and incubated over night on 37°C.

2.2.5.1.3 Screening by Quick-Prep and control of positive clones

After transformation of E. coli XL1-Blue cells with novel plasmids 20 colonies were picked and grown in 5 ml LB, of course containing the corresponding antibiotics at 37°C. 300 µl of the resulting over night cultures were cetrifuged for 3 min at RT and 13.2 krpm. The pellets were resuspended in 30 µl loading dye by vortexing and combined with 14 µl of a 1:1 phenol/chlorophorm solution. Each sample was vortexed for half a minute and centrifuged for 7 min at 13.2 krpm and RT. The aqueous phase was applied to a 0.8 % agarose gel. Plasmids with the insert of interest are larger then those without, so that putative positive clones could be screened very quickly. These were analysed by either restriction batches or PCR reactions or both.

Proved clones were stored at -80°C. Therefor 1 ml culture was added to 800 µl 50 % glycerol and labeled with a number.

2.2.5.2 Transformation of halo- and haloalkaliphilic *Archaea*

2.2.5.2.1 Preparation of competent cells

Archaeal cells were grown in their correlated rich medium in the presence of 70 µg/ml bacitracin till an OD_{600} of 0.5-0.6 was reached. This took normally about 3 days. The cells were then collected by centrifugation at room temperature and 6 krpm for 15 min and carefully resuspended in half of the introduced volume buffered shperoplasting solution with glycerol. At this point the addition of Proteinase K (e.g. *Nab. magadii*) in a concentration of 20 µg/ml or Pronase E (e.g. *Nmn. pharaonis*) resulting in a final concetration of 0.3 mg/ml was needed. To form sheroplasts the cells were incubated at 42°C for about 48 h. Sheroplast formation was inspected by microscopy.

2.2.5.2.2 Transformation using PEG-600 - Polyethylene Glykol

As soon as the cells had lost their S-layer, reflected in the formation of spheroplasts they were transformed. Each batch was executed in triplicates including one batch without plasmid as a control. For each sample 1.5 ml were centrifuged for 3 min at RT and 10 krpm. The pellet was solved in 150 µl buffered spheroplasting solution without glycerol and mixed with 15 µl EDTA pH 8.0. After an incubation of 10 min at room temperature the DNA was added and again kept on RT for 5 min. Thereafter 150 µl 60 % PEG, which was prepared before starting the transformation (60 % in unbuffered spheroplasting solution and stored at 65°C) were added to the batch. Subsequently a centrifugation of 5 min at RT and 10 krpm took place and the pellet was resuspended in another 1 ml rich medium after discarding the supernatant. To regenerate the affected cells the samples were put on a shaker a incubated for 2 days at 37°C with agitation.
At the end 100 µl of the cells were plated. Three plates were performed for each batch and incubated at 37°C till colonies were visibles.

2.2.5.2.3 Screening for positive clones

For *Archaea* a quick apply was not performed. The colonies were grown in 15 ml rich medium till the culture was dense enough and following PCRs from raw extracts, which proved the existence of novel plasmids.

2.2.6 Phage methods

2.2.6.1 Isolation of phage (ϕCh1) particles

NVM+ was inoculated with a plaque of the lysogenic strain of *Nab. magadii* L11. It was grown until an OD_{600} of 0.5 was reached and used for inoculation of a larger volume of rich medium. The new culture was grown till an OD_{600} of 1.0 and used for inoculation of three times 1.5 l NVM+. When no further lysis could be observed the culture was pelleted twice at RT for 15 min at 6 krpm. 10 % PEG 6000 was added to the supernatant and stirred at RT over night. By cetrifugation the virus particles were collected and resuspended in high salt alkaline solution.

After that a discontinuous CsCl gradient was used to purify the virus particles. The gradient was performed by overlaying 2 ml CsCl solution 1.5 with 5 ml solution 1.3, 6 ml of the solution containing the virus particles and at the end by 1 ml CsCl solution 1.1. The gradient centrifugation was carried out for 20 h at 30 krpm and RT. The upper of the two resulting bands was collected and again purified by a continuous gradient. Therefore the same volume of ϕCh1 particle solution and CsCl solution 1.3 was cetrifuged for further 20 h at 30 krpm and RT.

By dialysis CsCl was removed from the suspension against a high salt alkaline solution and the concetration of the virus particles was determined by a following phage titer infecting the strain L13 of *Nab. magadii*.

2.2.6.2 Phage titers – appropriation by soft plating technique

5 ml NVM+ soft agar were combined with 300 µl culture of *Nab. magadii* L13 and 100 µl of a ϕCh1 dilution (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} in NVM+), which was prepared before starting the titer. This mixture was poured on NVM+ plates and incubated at 37°C till plaques were visible. Each batch was performed in triplicates.

2.2.7 Cloning strategies

pRSET-C/1b, pRSET-C/124b, pRSET-C/541b

The mtase fragments (1b, 124b and 541b) were amplified by a preparative PCR using the 3mt-1b, and N6-1b, N6-124b, N6-541b primers, respectively, and isolated ϕCh1 DNA was used as template. They were cloned into the expression vector pRSET-C. Therefore the restriction enzymes *Bcl*I, *Hind*III were used to cut the fragments and *Bam*HI and *Hind*III were applied for the vector.

pQE32-1b, pQE32-124b, pQE32-541b

The same cloning strategie as above were performed for this constructs.

pUCpN6M

In a first step the promoter sequence of ORF34 was amplified with the primers Mt-Kpn and 34-3-Bcl. It was cloned into pUC19 restricted with *Xba*I and *Kpn*I and *Kpn*I and *Xba*I to restrict the insert.

pUCpN6M/1b, pUCpN6M/124b, pUCpN6M/541b

The mtase gene fragments already amplified were cut with *Bcl*I and *Hind*III like the vector including the promoter sequence (pUCpN6M) and ligated.

pRo-5pN6M/1b, pRo-5pN6M/124b, pRo-6pN6M/541b (also called pRo-5/1b, pRo-

5/124b and pRo-5/541b)

The promoter sequence plus the mtase fragments were isolated from the pUC19 vector and cloned into pRo-5 by a restriction using the enzymes *Kpn*I and *Hind*III.

pNov1/101

Two vectors namely pNov-1 and pNB102 were both digested using the restriction enzyme *Hind*III. The resulting fragment of 2.5 kbp of the pNB102 cut, which represents the origin of replication of pNB101 was ligated with pNov-1.

pRo-5/Bop

The *bop* gene which encodes the protein bacteriorhodopsin (Shand and Betlach, 1994) was amplified using a preparative PCR with the primers Bop-1H and Bop-2H. The plasmid pMG100 served as a template. pRo-5, as the vector and the insert were restricted with *Hind*III and ligated. The restriction enzyme *Bam*HI was finally used to identify the orientation of the gene in the plasmid.

2.2.8 Testing the methyltransferase activity

To investigate the activity of the M.*Nma*φCh1I different points were determined. Baranyi et al. (2000) already started with such experiments, which was the basis of the following assays.

2.2.8.1 *In vivo*

The constructs with the different sequences of the mtase gene were transformed into GM48F‘ to investigate if the Mtase fragments are functionally active in *E. coli*.
30 ml LB (amp/strep) was inoculated with an over night culture of GM48F‘ containing the plasmid pQE32 and GM48F‘ containing the plasmid pQE32-mtase fragment. Both were grown until an OD_{600} of 0.3 was reached. Then they were induced with IPTG resulting in a final concetration of 0.5 mM and let them grow for 180 min. At this time plasmid DNA was isolated using the GeneJet plasmid isolation kit from Fermentas. The eluates were utilized for restriction analysis.

2.2.8.2 *In vitro* / binding assays

Under native conditions protein was isolated from *E. coli* strain Rosetta containing the plasmids pRSET-C-mtase fragments (see: 2.2.3.1.1). The isolate was used to perfom binding and activity assays *in vitro* to determine which and how much of the *mtase* gene is nessecary to bind to DNA and to be active.

3 Results and Discussion

3.1 Characterization of the M.*Nma*ϕCh1

The methyltransferase M.*Nma*ϕCh1I, encoded by the halophage ϕCh1 infecting the haloalkaliphilic archaeon *Nab. magadii*, is composed of 2 domains: beside the methyltransferase domain, a ParB like nuclease domain was identified. In this study, the different domains were analysed in more detail.

3.1.1 Determination of methyltransferase activity

As shown before, M.*Nma*ϕCh1I was able to methlyate DNA in the low environment of *E. coli*. Here, only 5´-GATC-3´ sequences could be methylated and not additional once, not strictely conserved to the sequence recognized by the DAM methyltransferase of *E. coli* (Baranyi et al., 2000). With respect to the different domains of M.*Nma*ϕCh1I two truncated versions of this methyltransferse were produced and expressed in *E. coli* in order to investigate the activities of these proteins. An schematically representation of the domain structure and the truncated versions of the protein is shown in Fig. 6.

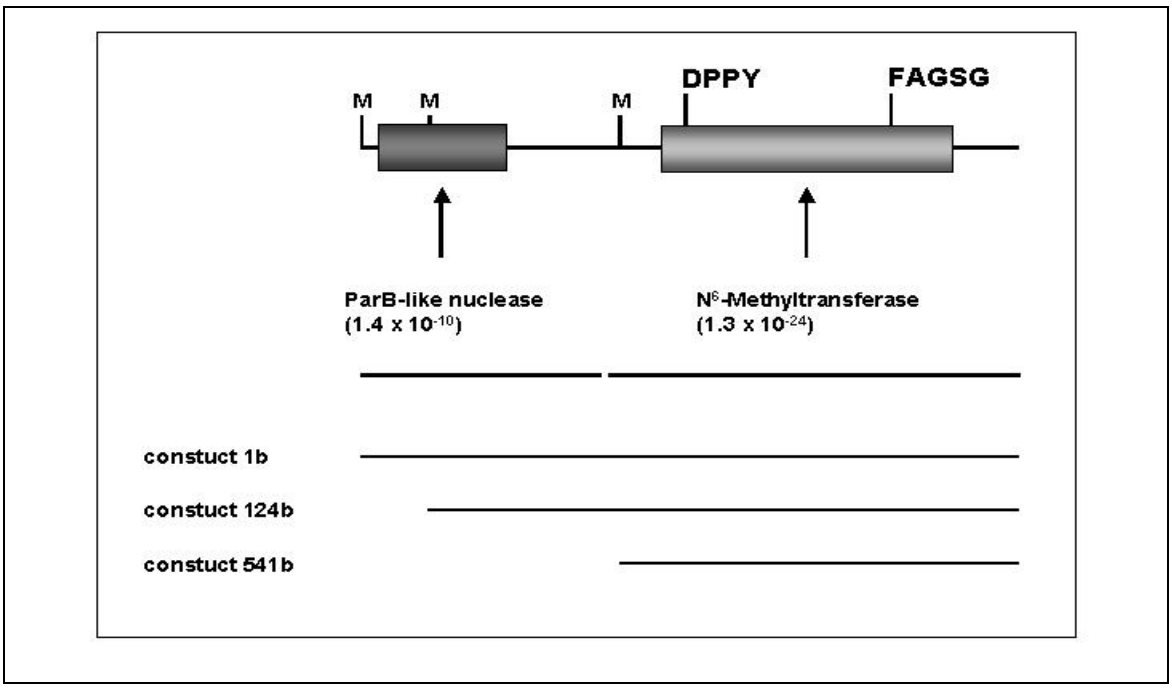

Fig. 6 Schematically representation of M.*Nma*φCh1I.

Boxes indicate the domains of M.*Nma*φCh1I (ParB-like-domain and N^6-Methyltransferase, similarities to known proteins are mentioned above). The methionine codons as well as the active domains of the Mtase are indicated above. Parts of the gene were cloned into different vectors and are indicated by lanes. The abbreviations of the constructs are given on the left.

3.1.1.1 Activity of a truncated version of M.*Nma*φCh1I in *E. coli*

The protein M.*Nma*φCh1I methylates 5´-GATC-3´sites in plasmids, isolated from *E. coli*. In order to investigate the role of the Mtase domain, truncated versions of the gene of M.*Nma*φCh1I were constructed in the expression vectors pQE-32 and pRSET-A to express the genes in *E. coli*. After expression of the gene encoding the construct 124b, plasmid DNA was isolated from strain SCS110, a strain of *E. coli* with deficiencies in the genes *dam* and *dcm*. These mutations do not allow the methylation of DNA in this strain. After restriction of plasmid DNA isolated after expression of the truncated M.*Nma*φCh1I, only a partial modification of the plasmid DNA was found (data not shown). This is in contrast to the full-length protein. Here a complete methylation of the DNA was observed (Baranyi et al., 2000). For this reason, the shortest version of the protein, construct 541b was not investigated.

In order to understand the different Mtase activities of the truncated M.*Nma*φCh1I proteins, where only the N-terminal part was reduced not interfering with the Mtase domain, the theoretical 3D structure of the different versions of the M.*Nma*φCh1I were predicted using the LOOPP program (Fig. 7). As shown in Fig. 7, the flexibility of the protein with the different domains is reduced in the shorter versions of the protein. Thereby this increasing compactness could be a reason for the reduced activity of the Mtase domain.

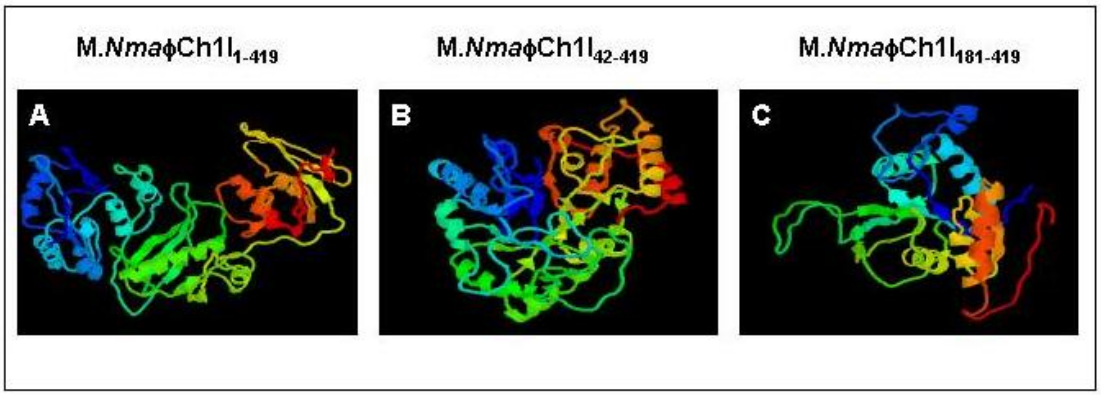

Fig. 7 The 3-D structure of M.*Nma*φCh1I and derivatives.

The structures were predicted using the LOOPP program available at http://cbsuapps.tc.cornell.edu/index.aspx. **A**: the complete protein, **B**: construct 124b, **C**: construct 541b.

3.1.1.2 Expression of M.*Nma*φCh1I in *Nab. magadii* L13

In order to investigate M.*Nma*φCh1I and its derivatives in their natural environment, the corresponding DNA fragments were cloned into the shuttle vector pRo-5 and transformed into *Nab. magadii* L13 (details for cloning can be found in chapter 2.2.7). The transformation rates are nearly in the same range as for the cloning vector pRo-5, regardingless if the ParB nuclease domain was expressed or not (Fig. 8A). After incubation in rich medium samples were taken from cultures *Nab. magadii* L13 (pRo-5pN6M/1b) and *Nab. magadii* L13 (pRo-5pN6M/124b). Crude extracts were prepared and analysed by Western blotting using a α-M.*Nma*φCh1I antiserum (Baranyi et al., 2000). As shown in Fig. 8B, expression of M.*Nma*φCh1I could only observed in the stationary phase. This is in concordance with the expression of the protein in the lysogenic strain *Nab. magadii* L11 (Baranyi et al., 2000).

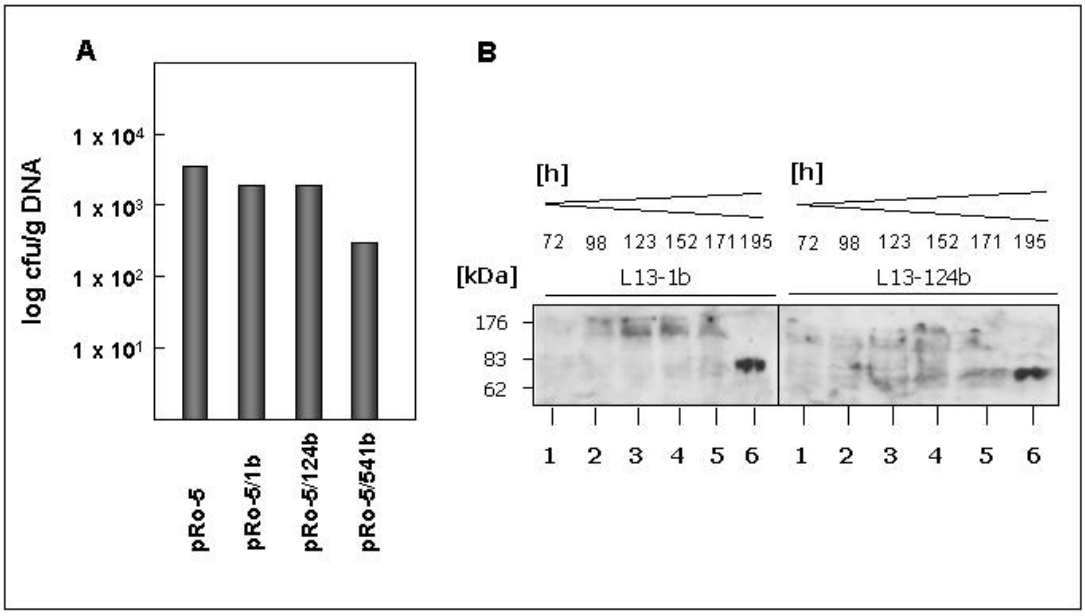

Fig. 8 Transformation of M.*Nma*ϕCh1I and its derivatives in *Nab. magadii* L13.

A: Transformation rates per µg DNA. Plasmid DNA of the different constructs were transformed into *Nab. magadii* L13 and the efficiency was determined as transformants per µg plasmid DNA.

B: Expression of M.*Nma*ϕCh1I: crude extracts were separated on a SDS-PAGE and used for western blotting. Lanes 1 – 6: different samples of *Nab. magadii* L13 (pRo-5pN6M/1b) in the left part and *Nab. magadii* L13 (pRo-5pN6M/124b) in the right part of the picture. The time points were samples were taken are indicated on top.

However, up to now the expression of the shortest construct 541b could not be demonstated as well as a methylation of DNA in these strains. These investigations will be performed in future.

3.1.2 Identification of the promoter sequence of M.*Nma*ϕCh1I

In order to identify the promoter sequence of the M.*Nma*ϕCh1I two different appoaches were performed. First of all, reverse transcription coupled with an PCR assay (RT-PCR) was performed. Here, primer pairs covering the 5´-region of ORFs 86 to an entire sequence of M.*Nma*ϕCh1I were used (for details see Fig. 9). Allthough a signal was detected for an internal M.*Nma*ϕCh1I fragment (Fig. 9A), the used primer pairs were not successful (Fig. 9B, C). A reason for this could be the organisation of transcripts in halophilic Archaea: here transcripts normally start without a 5´-sequence, in some

cases with a very short one (Benelli et al. 2003).

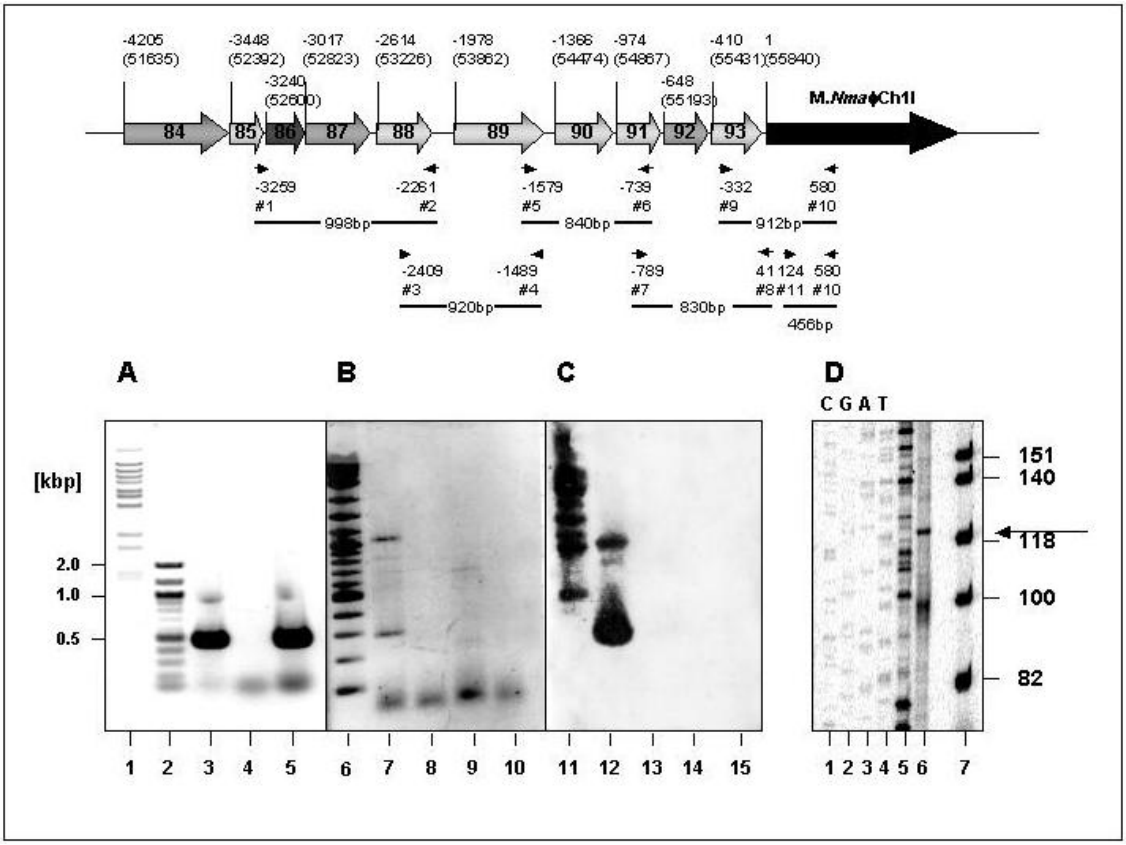

Fig. 9 Identification of the promoter sequences of the M.*Nma*ϕCh1I gene

In the upper part of the figure, the genetic organisation of the M.*Nma*ϕCh1I is shown including 10 open reading frames upstream of the M.*Nma*ϕCh1I gene. The coordinates with respect to the ϕCh1 sequence as well as to the AUG start codon of M.*Nma*ϕCh1I are given on top of the schematically representation of the investigated region of ϕCh1. Beneath the schematically drawing of the 5´sequence the primers as well as the expected fragment length and the coordinates of the promers are given.

RT-PCR: reactions for the RT-PCR were performed as described in Materials and Methods.
A: positive control internal fragment of M.*Nma*ϕCh1I: lane 1: λ *Bst*EII, lane 2 1 kb ladder, lane 3: PCR reaction with primers 10 and 11, lane 4: same primers using RNA as a template, lane 5: same primers using cDNA as a template
B: RT-PCR with primers 9 and 10: lane 6: 2 log DNA ladder, lane 7: PCR reaction with primers 9 and 10, lane 8: same primers using RNA as a template, lane 9: same primers using cDNA as a template, lane 10: same primers using cDNA as a template, different RNA sample.
C: lanes 11 – 15 Southern hybridisation of B using the positive control from lane 7.
D: primer extension using RNA from the lysogenic strain *Nab. magadii* L11 and primer MT-PE2. lanes 1 – 4 Sequencing reactions with ϕCh1 and primer MT-PE2 (CGAT), lane 5: all sequencing reactions in one lane, lane 6 extension reaction with primer MT-PE2 and RNA, lane 7: size marker, The length of the marker bands are indicated on the right as well as the position of the extension signal with an arrow on the right.

Therefore, a second approach was performed: a primer extension reaction to map the

5´-end of the M.*Nma*ϕCh1I gene assuming a very short untranslated region. As shown in Fig. 9D, a positive signal could be detected 24 nucleotides (G) upstream from the AUG start codon of M.*Nma*ϕCh1I. In a range of 25 nucleotides upstream of the start of the transcript, a typical halophilic promoter sequence (Soppa, 1999) was observed with the sequence 5´-CAGATA-3´. This arrangement, the distance of the promoter sequence to the translational start site as well as the spacing of the untranslational region of the M.*Nma*ϕCh1I gene is in complete agreement with most of the organisation of halophilic genes described so far (Soppa, 1999).

3.1.3 *In vitro* activity of M.*Nma*ϕCh1I

The methyltransferase of ϕCh1, M.*Nma*ϕCh1I, showed a modification activity in vivo: the genomic DNA of ϕCh1 is methylated in the sequence 5´-GATC-3´ and in related sequences (Witte et al., 1997). Even in the low salt environment of *E. coli* M.*Nma*ϕCh1I methylates the sequence 5´-GATC-3´ (Baranyi et al., 2000). In order to investige the methyltransferase M.*Nma*ϕCh1I in more detail two different proteins were purified after expression in *E. coli*: the full length protein as well as the truncated 124b protein. Both proteins were incubated with different DNA substrates and the samples were analysed by agarose gel electrophoresis.

In a first approach both proteins were incubated with closed circular plasmid DNA (pKS_{II}^{+}) isolated from strain XL1-Blue and strain SCS110. While the DNA from the first strain is methylated the DNA from the second one showed no modifications, nor from the DAM like neither from the DCM like modification, typically for *E. coli* strains. As shown in Fig. 10A, both proteins seem to introduce nicks into the DNA when compared to samples without protein. Both proteins reacted in the same way, independently of the complete ParB domain, which is not complete in protein 124b (see Fig. 6 for details). Even though the addition of EDTA, chelating Mg^{2+} ions, did not change the activity of both proteins. Moreover, the methylation rate of the substrate did not change the protein activity: both proteins seem to react with the DNA and seem to introduce nicks in the circular plasmid pKS_{II}^{+}. However, M.*Nma*ϕCh1I restricted the plasmid DNA completely in the case the plasmid was linearized and free 5´-end and free 3´-OH groups were present (Fig. 10B). Again there were no differences in activity when methylated

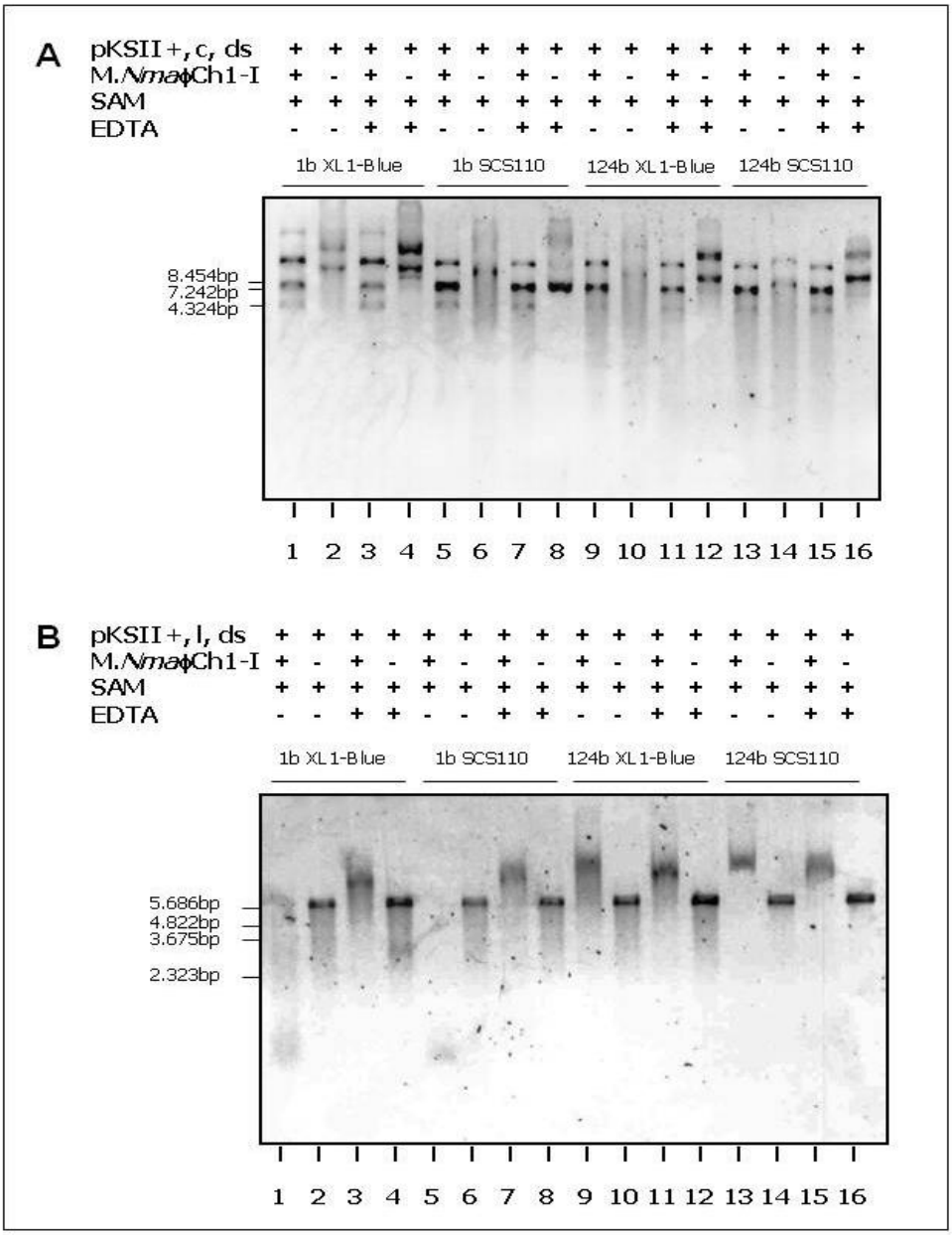

Fig. 10 *In vitro* activity of M.*Nma*φCh1I using plasmid pKS_{II}^{+} as a substrate.

Protein samples were incubated with plasmid DNA of pKS_{II}^{+} for 1 h at 37°C and the samples were analysed on a 0.8% agarose gel. The composition of the samples is indicated on top of the figures. XL1-Blue: plasmid DNA isoloated from strain XL1-Blue was used, SCS110: plasmid DNA isoloated from strain SCS110 was used. 1b: full length protein, 124b: truncated form. The composition of the buffer is indicated in Materials and methods. On the left, the molecular weight marker is indicated.

A: DNA substrate: closed circular pKS_{II}^{+},
B: linear pKS_{II}^{+} (restricted with *Bam*HI). SAM: 80µM, EDTA: 10mM.

or non-methylated DNA was used. On the other hand, EDTA completely blocked the

nuclease activity of M.*Nma*ϕCh1I. Here, a mobility shift of the linearized fragment of pKS_{II}^{+} could be detected, indicating that the protein is reacting with the DNA substrate. Using protein 124b, in all cases no nicking or nuclease activity could be seen with linear pKS_{II}^{+} plasmid DNA. However, in all cases protein 124b was able to bind to the plasmid DNA (Fig. 10B).

In the two experiments mentioned above, double stranded DNA was used as a substrate. In order to test single stranded DNA, the genomic DNA of the phage ϕX174 was used: this DNA is a closed circular, single stranded DNA. Incubation of the two proteins described above did not give a uniform activity (data not shown).

As shown before, the activity of the ParB like nuclease domain seems to depend on the concentration of Mg^{2+} ions in the reaction sample. In order to prove this, different concentrations of EDTA were used (Fig. 11A). Here, only the same concentration of EDTA as the one of Mg^{2+} ions, the nuclease activity was blocked and protein binding resulted in a mobility shift of the DNA substrate. Concentrations of EDTA below 10 mM resulted in a complete digestion of the substrate.

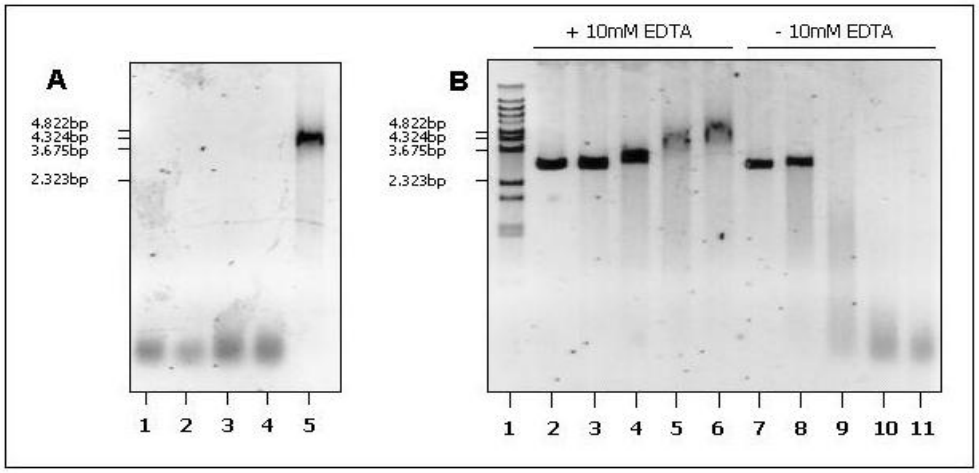

Fig. 11 M.*Nma*ϕCh1I activity depending on Mg^{2+} and protein concentration.

Protein samples were incubated with linear plasmid DNA of pKS_{II}^{+} for 1 h at 37°C and the samples were analysed on a 0.8% agarose gel.
A: different concentrations of EDTA: lane 1: 0mM EDTA, lane 2: 1mM EDTA, lane 3: 2.5mM EDTA, lane 4: 5mM EDTA, lane 5: 10mM EDTA.
B: Different protein concentrations with and without EDTA: lane 1: λ *Bst*EII, lane 2: 0 µg M.*Nma*ϕCh1I, lane 3: 1 µg M.*Nma*ϕCh1I, lane 4: 2.5 µg M.*Nma*ϕCh1I, lane 5: 5 µg M.*Nma*ϕCh1I, lane 6: 8 µg M.*Nma*ϕCh1I, lanes 2 – 6: with 10mM EDTA, lanes 7 – 11: same as lanes 2 – 6, without EDTA.
The molecular weight markers are indicated on the left.

This dependence on free Mg^{2+} ions could also be seen when different concentrations of M.*Nma*φCh1I were used (Fig. 11B): here a complete binding of the protein to the DNA was seen when concentrations of 5 and 8 µg/assay were used. The same concentrations of protein were necessary for the digestion of DNA without EDTA.

In addition the influence of NAD and ATP as a energy source was investigated (Fig. 12 A and B). The addition of more ATP, here increased from 0.5 mM to 1 mM, did not show any differences to the protein activity as shown before (Fig. 12A). However, the addition of 26 µM NAD as an energy source resulted in an endonuclease activity although the addition of EDTA in this samples inhibited this nuclease activity under the described conditions without NAD.

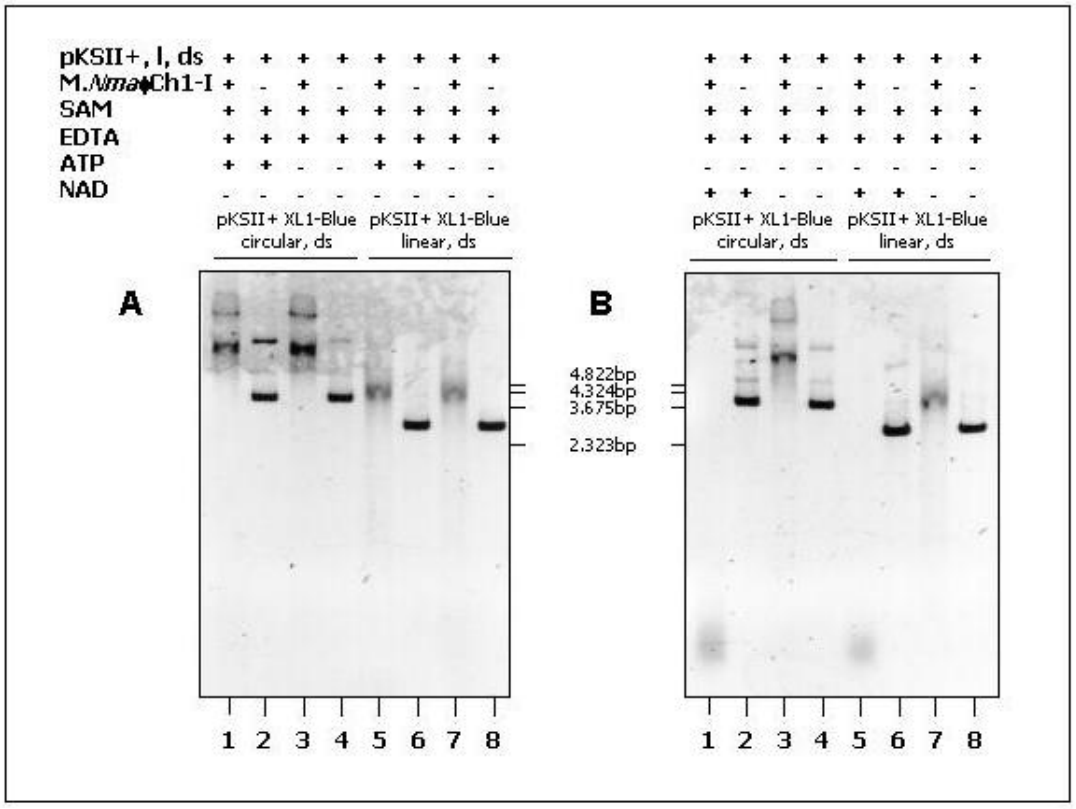

Fig. 12 M.*Nma*φCh1I activity depending on ATP and NAD.

Protein samples were incubated with plasmid DNA of pKS_{II}^{+} for 1 h at 37°C and the samples were analysed on a 0.8% agarose gel. The composition of the samples are indicated on top of the figures. XL1-Blue: plasmid DNA isoloated from strain XL1-Blue was used. The composition of the buffer is indicated in Materials and methods. On the left, the molecular weight marker is indicated.
A: ATP: 500 µM,
B: NAD: 26 µM.
The molecular weight markers are indicated in the middle.

3.1.4 Discussion

Here, the activity of the M.*Nma*φCh1I was investigated in more detail. Allthough the studies for the *in vivo* activities are at the beginning, the expression of the protein in *Nab. magadii* L13 could be shown and will be the basis for detailed studies in the future.

The *in vitro* activity of M.*Nma*φCh1I showed the following results up to now: first the nuclease domain could be described in more detail: the acitivity could be shown for the first time. This activity is depending on free Mg^{2+} ions. Allthough the function of the ions could be replaced by the addition of NAD, but not by ATP. Again this phenomenon has to be studied in more detail in the future. In addition, the form of the substrate – circular versus linear double stranded DNA – has an influence on the activity of M.*Nma*φCh1I. Beside the nuclease domain, the methyltransferase activity has to be studied in more detail. An appropriate candidate will be the second truncated version of M.*Nma*φCh1I, 541b.

In general, here it could be demonstrated for the first time, that a methyltransferase with a nuclease domain from a haloalkaliphilic virus is active in low salt environments and that both activities can be investigated under different conditions. This will lead to more informations about DNA methyltransferases containing a nuclease domain. This kind of protein could be found in a variety of different microorganisms (e.g. *Dehalococcoides ethenogenes* 195, Seshadri et al., 2005), however, none of the proteins were studied in detail so far.

3.2 Further characterization of a transformation system

Since Iro et al. (in prep) could establish an effiecient transformation system for haloalkaliphilic *Archaea,* it was easier to perform experiments in their natural living conditions and was able to manipulate these organisms gentically. The virus ϕCH1, whose only known host is the haloalkaliphilic archaeon *Nab. magadii* was taken as a model system.

3.2.1 Forming spheroplasts

To form spheroplasts cells were incubated in rich medium with bacitracin until an optical density of app. 0.6 was reached, followed by an incubation with proteinase K for about 48 h at 42°C. In halophilic *Archaea* like in Nmn. pharaonis proteinase K is not efficient (data not shown). For those organisms an alternative enzyme was tested. The so called pronase E from *Streptomyces griseus* led to the formation of spheroplasts in *Nmn. pharaonis*. The necessity came up if the enzyme pronase E is able to remove the S-layer with the same efficiency or may be better than proteinase K in *Nab. magadii*. For that two transformation batches were perfomed in parallel, one treating with proteinase K and one with pronase E. This experiment was also done for *Hrr. sodomense*.

It seemed that in the case of *Nab.magadii* the use of proteinase K is more efficient than pronase E because with the use of proteinase K more spheroplasts were formed. In the case of *Hrr. sodomense* the difference in time in which spheroplasts were developed was observed. Using proteinase K the S-layer was removed in half of the time (24 h) than by the use of pronase E.

3.2.2 Transformation of pRo5 – a shuttle vector system

Sequence analysis of halophilic plasmids like pNC100 from *Hbt. halobium* revealed that a gene called *repH* and an AT-rich sequence 5‘ of the open reading frame is needed for autonomous replication in their host organism (Ng and DasSarma, 1993).

ORF53 and ORF54 showed after similarity searches with ϕCh1 revealed homologies with *repH* encoded by *Hal. marismortui*, *Hbt. salinarum* and some others. This lead to the assumption that the replication protein of ϕCh1 is constructed of two ORFs. This region was taken to create a shuttle vector system for *E. coli/Nab. magadii*. pNov-1 is the plasmid, which was created by cloning a novobiocin resitance into pKS_{II}^{+}. pNov-1 was used to introduce different fragments of the central region of ϕCh1. Plasmids with different deletions were created like pRo-4 and pRo-5. Both are carrrying the promoter sequence, but while pRo-4 is containing the region from ORF53 till ORF55 pRo-5 has two deletions in this sequence. One in the ATG of ORF49 and on the other hand one in the start-codon of ORF55 (Ladurner, 2008). Both ORF49 and ORF55 represent a putative repressor.

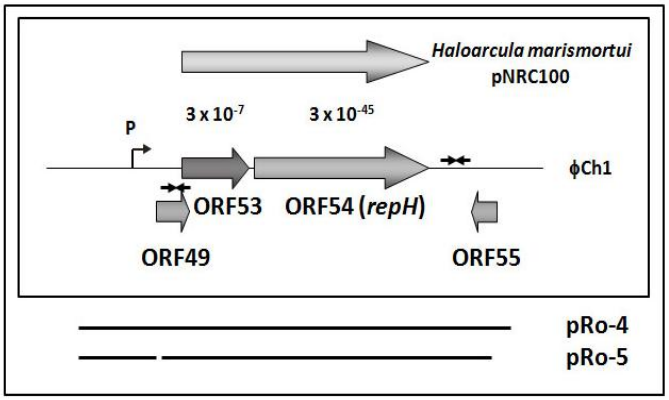

Fig. 13 Shematic drawing of puptaive origin of replication of ϕCh1 compared to *repH*

Large arrows indicate the open reading frames of the virus as well as that of *repH* of pNRC100. Black small arrows display palindromic sequences.

(Ladurner, 2008 with modifications

3.2.2.1 Transformation of *Nab. magadii* L13, *Nmn. pharaonis* and *Hrr. sodomense* with pRo-5

The strains *Nab. magadii* L13, *Nmn. pharaonis* and *Hrr. sodomense* were transformed with the shuttle vector pRo-5 to specifiy the host range. The transformation protocol is as described in 2.2.5.2. The procedure was successful for all three strains. This was established using analytical PCRs with the primers TR-1 and TR-2 from isolated plasmid DNA and raw extracts. The developed system utilizing the putative replication of origin can be used to introduce DNA into different halophilic and haloalkaliphilic *Archaea*. The results led to the assumption that the host range is not limited to *Nab. magadii*.

3.2.2.1.1 Stability of pRo-5

After efective transformation of *Nab. magadii* L13, *Nmn. pharaonis*, *Hrr. sodomense* (this thesis), *Nab. asiatica*, *Nbt. gregoryi*, *Hbt. salinarium*, *Hrr. lacusprofuni*, *Hrr. saccharovorum* and *Hrr. coriense* (Ladurner, 2008) the aim of studying the plasmid stability arised. Therefor plasmid DNA was isolated from all archaea mentioned above and 7 µl each were retransformed into the *E. coli* strain XL1-Blue. Afterwards a plasmid isolation of the retransformants was performed and used for restriction analysis with the enzyme *Sac*I. Five transformants of the archaeal strains were tested and all of them showed a positive result (Fig. 7, not all data shown). The indicated bands on the left in Fig.7 were obtained of all tested retransformants. These results show a 100 % stability of pRo-5 in this host organisms.

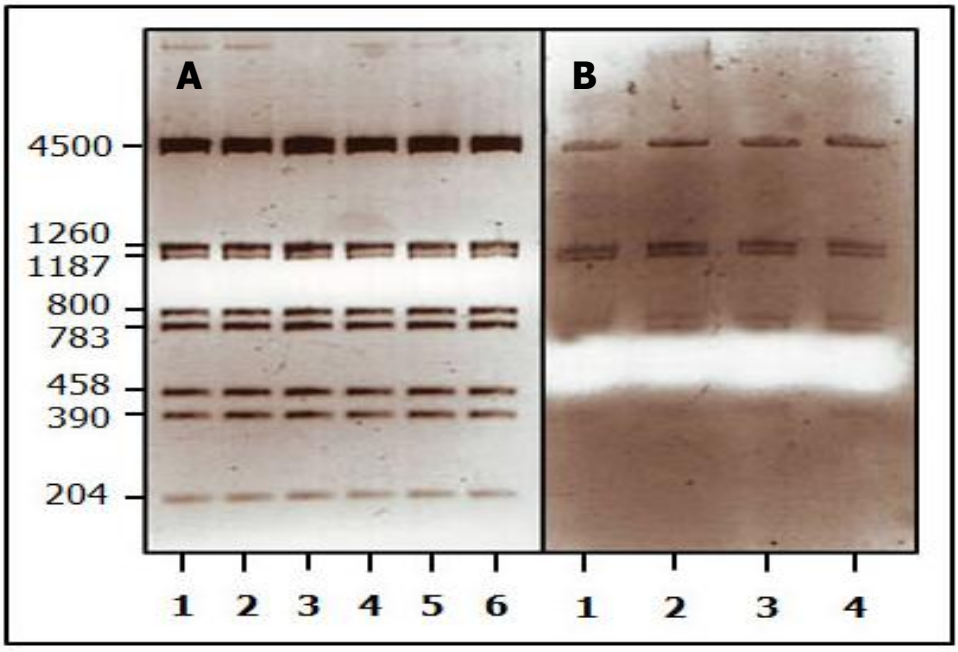

Fig. 14 Picture of restransformants after digestion

The pictures show the specific band pattern after restriction analyse with *Sac*I.

A Retransformants from *Nbt. asiatica*, *Nbt. gregoryi*, *Hrr. lacusprofundi*, *Hrr. saccharovorum*, *Hrr. coriense* and *Hrr. sodomense* (in numerical order)

B Four samples of *Hbt. salinarum*
The molecular weight markers are indicated on the left.

3.2.3 Transformation of plasmids with different antibiotic resistance – novobiocin and mevinolin

As described in 1.1.4.1 novobiocin and mevinolin are the prefered antibiotic resistances in the genetical work with halo(alkali)philic *Archaea*. In our laboratory novobiocin was mostly used but the urge for another available marker came up.

3.2.3.1 Determination of the adequate concentration of mevinolin

Growth experiments were done to establish the adequate concentration for *Nab. magadii* and other *Archaea* like *Nmn. pharaonis*. The indicator strain *Nab. magadii* L13 was grown in rich medium in 5 different batches. They had mevinolin concetrations of 0.5 µg/ml, 2 µg/ml, 5 µg/ml and 15 µg/ml. The 5th batch was the control without antibiotic. For one week the OD_{600} was mesuared every 12 hours.

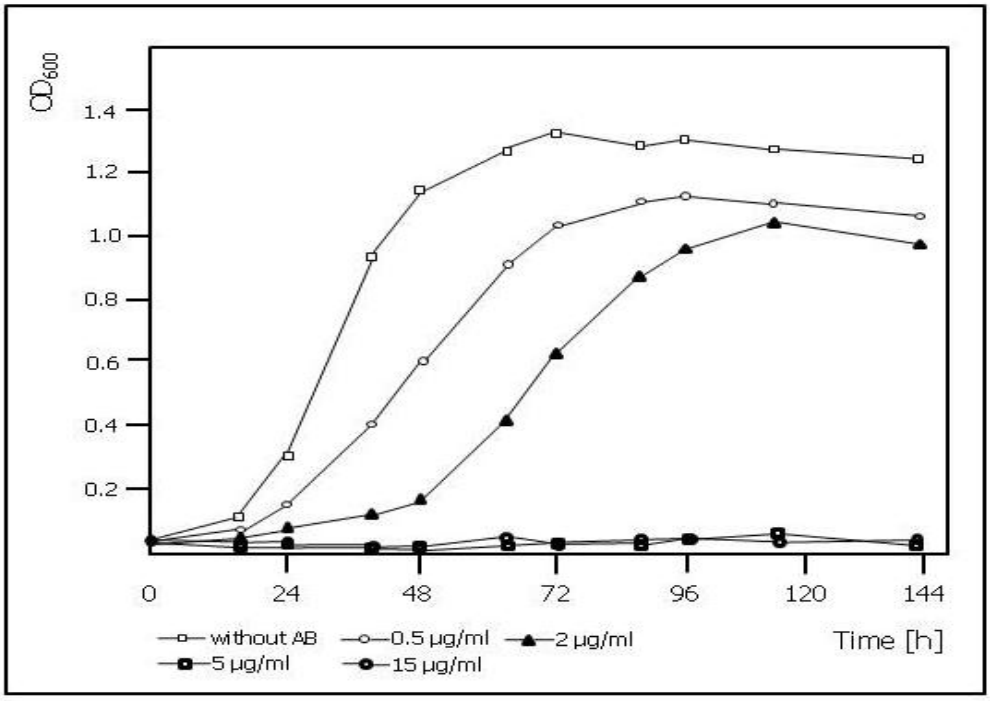

Fig. 15 *Nab. magadii* L13 growth experiment – mevinolin

Rich medium was inoculated with *Nab. magadii* L13 and different mevinolin concentrations. Every 12 h the OD_{600} was measured.

Up to 2 µg/ml mevinolin growth of *Nab. magadii* L13 could be observed, however with a decrease rate compared to the control without mevinolin. Concentrations 2 µg/ml led to a complete loss of growth (Fig. 8).

Because of these results the following experiments were performed using 5 µg/ml, 7.5 µg/ml and 10 µg/ml mevinolin. Whereby it became apparent that utilizing 7.5 µg/ml most transformants were obtained (data not shown).

3.2.3.2 Transformation of various plasmids in *Nab. magadii* L13

To determine if other plasmids than pRo-5 are transformable, transformation experiments in *Nab. magadii* L13 were perfomed. Some of the chosen plasmids had novobiocin as their antibiotic resistance and others mevinolin. The transformation were done in doublicates. All positive transformants were inspected by a PCR with the following primers

pRo-5 and pRo-4:	TR-1 and TR-2
pNB102 and pRo-4/Mev:	NB-3 and MevR-4
pNov-1/101:	Nov-6 and NB-1
pRo-5/Bop:	Bop-1H and Bop-2H

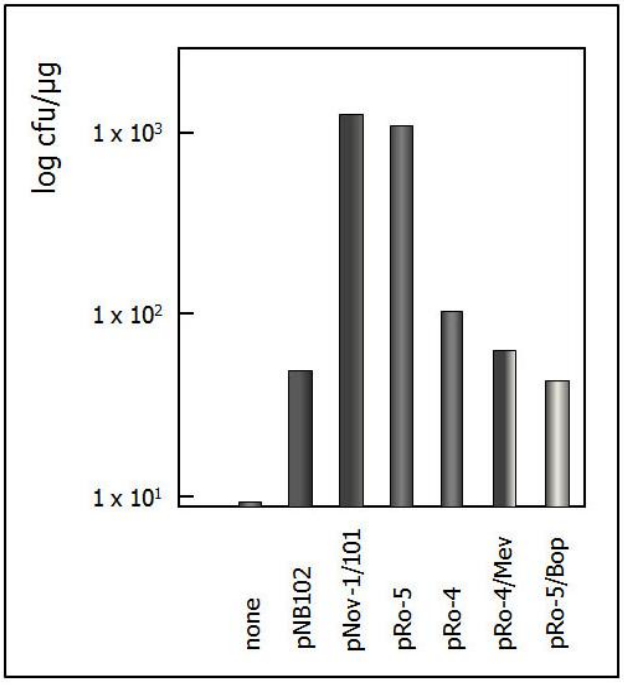

Fig. 16 Transformation efficiency of different constructs

The efficiency was calculated in colony forming units per µg plasmid DNA (cfu/µg)

For this assay the following plasmids were used, pNov-1/101 was the only plasmid which had approximately the same transformation efficiency like pRo-5. pNov-1/101 has also novobiocin as antibiotic resistance but has an other origin of replication, that from pNB101. pNB102 (Zhou et al., 2004) is as well in possession of this origin. The important differnece between pNB102 and pNov-1/101 is their marker concerning the antibiotic resistance.

The construct pRo-5/Bop was also successfully transformed, but showed no red colonies on the plate like anticipated. The aim was to have a marker which could be selectionized just by inspecting on the plates. *Nab. magadii* colonies are normally coulorless but the *bop* gene encodes the protein bacteriorhodopsin, which results in the red colour of *Hbt. salinarum*.

Additionally the plasmids pMDS11 (Holmes et al., 1991) and pMDS24 (Jolley et al., 1996) were transformed into *Nab. magadii* L13, but neither the one nor the other was taken up.

3.2.3.3 Double-transformation

As shown before, plasmids with different origins of replication and different resistance marker could be transformed successfully in *Nab. magadii* L13. The next step was to transform two plasmids into *Nab. magadii*. pRo-5 including the *gyr*B gene for the novobiocin resistance and pNB102 containing the *hmg* gene for the mevinolin resistance. First pRo-5 was transformed. The resulting new strain was used for the following second transformation with pNB102. Antibiotic concetrations which were taken for this experiment were 3 µg/ml novobiocin and 7.5 µg/ml mevinolin.
To manifest the existence of both plasmids in the archaeal indicator strain PCRs with the primers TR-1 and TR-2 for pRo-5 and the primers NB-3 and MevR-4. To be sure that the resulting bands are really the expected ones a Southern blot analysis followed. The probes were performed using a PCR with the primers TR-1 and TR-2 in case of pRo-5 and the probe for the pNB102 sample was obtained through a restriction of pNB102 with the enzymes *Bam*HI and *Hind*III.

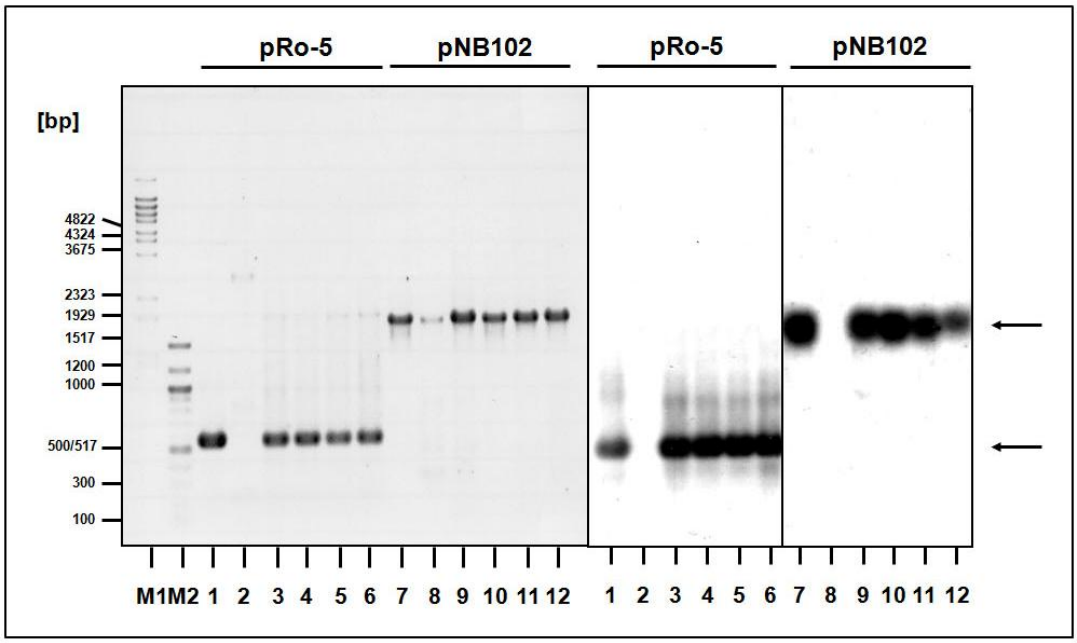

Fig. 17 PCR results and Southern blot analysis after double transformation

Four transformants (lane 3 till 6 and 9 till 12) were taken to verify the double-transformation. On the left the picture after perfoming the PCRs is shown. The right picture was obtained after performing a Southern blot (marked with a black arrow).
(M1, M2) used ladders were Lambda/*Bst*EII and the 2-Log DNA ladder from NEB. Lane number 1 and 7 represent the corresponding positive controls (pRo-5 and pNB102 plasmids as a template) whereas 2 and 8 the negative (*Nab. magadii* L13 as a template).

The doubletransformation succeed and the selction based on two markers concerning the antibiotic resistance. It seems that *Nab. magadii* L13 is able to take up more than one plasmid without integrating it into its genome.

3.2.3.4 Transformation of *Nmn. pharaonis* with different plasmids

Nmn. pharaonis was the second organism which was observed a bit more in this thesis. After successful transformation of pRo-5 the question came up if other plasmids are also transforable like in *Nab. magadii*.
The transformation procedure was done like for *Nab. magadii* L13 with a small difference. *Nmn. pharaonis* needed a higher optimal antibiotic concentration namely 6 µg/ml. Used plasmids were pRo-5 as a control, pRo-4, pRo-4/Mev, pNov-1/101, pNB102 and pRo-5/Bop.

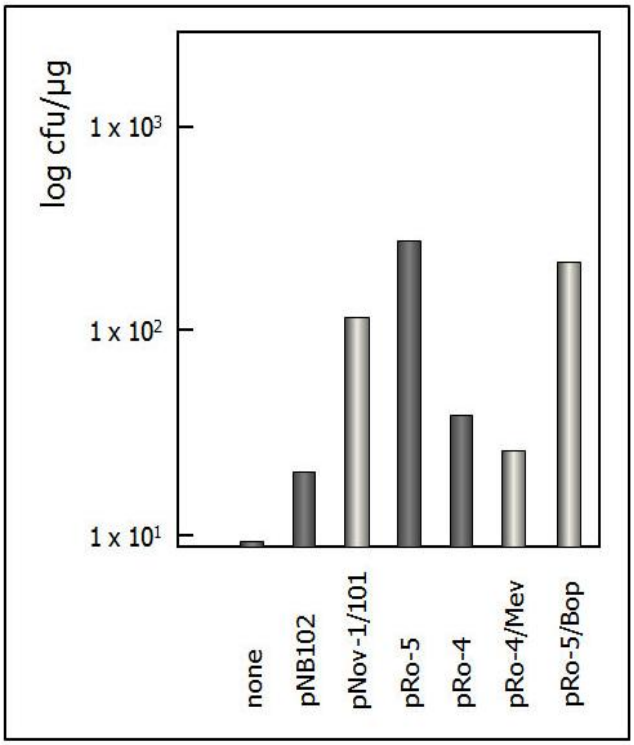

Fig. 18 Transformation efficiency of different constructs in *Nmn. pharaonis*

The efficiency was calculated in colony forming units per µg plasmid DNA (cfu/µg)

All transformants which were taken for further experiments were proved using PCRs with the primers named in 3.2.3.2.

Compared to *Nab. magadii* L13 transformation the effieciency is generally lower. But the important point is that the transformation worked.

Additionally plasmids which our laboratory got from the Max Planck Institute named pMG100, pMG200 and pMG300 were also transformed. The efficiency was very low. Only about five transformants were obtained, but all of them which were tested by PCR using the primers NB-3 and MevR-4 appeared as positive. The results were established by performing a Southern blot analysis utilizing a PCR product with pNB102 as template (primers NB-3 and MevR-4) as probe. After exposing a x-ray film the expected bands got visible (data not shown) and confirmed the results obtained through the PCRs before.

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5 References

Amils R, Cammarano P, Londei P: **Translation in *Archaea***. In Kates M, Kushner DJ, Matheson AT (ed.), The biochemistry of *Archaea* (Archaebacteria). Elsevier Biomedical Press, Amsterdam, The Nederlands. 1993, p. 393-438

Baranyi U, Klein R, Lubitz W, Krüger DH, Witte A: **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***. Mol Microbiol. 2000, 35:1168-1179

Barns SM, Delwiche CF, Palmer JD, Pace NR: **Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences**. Proc Natl Acad Sci U S A. 1996, 93:9188-9193

Bath C, Dyall-Smith ML: **His1, an archaeal virus of the Fuselloviridae family that infects *Haloarcula hispanica***. J Virol. 1998, 72:9392-9395

Bell SD, Jackson SP: **Transcription and translation in archaea: a mosaic of eukaryal and bacterial features**. Trends Microbiol. 1998, 6:222-227

Bell SD, Magill CP, Jackson SP: **Basal and regulated transcription in *Archaea***. Biochem Soc Trans. 2001, 29:392-395

Benelli D, Maone E, Londei P: **Two different mechanisms for ribosome/mRNA interaction in archaeal translation initiation**. Mol Microbiol. 2003, 50:635-643

Bheemanaik S, Reddy YVR, Rao DN: **Structure, function and mechanism of exocyclic DNA methyltransferases**. Biochem J. 2006, 399:177-190

Bischoff K, Rodwell VW: **3-hydroxy-3-methylglutaryl coenzyme A reductase from *Haloferax volcanii*: purification, chracterisation and expression in *Escherichia coli*.** J Bacteriol. 1996, 178:19-23

Berks BC, Sargent F, Palmer T: **The Tat protein export pathway**. Mol Microbiol. 2000, 35:260-274

Brown JR, Doolittle WF: ***Archaea* and the prokaryote-to-eukaryote transition.** Microbiol Mol Biol Rev. 1997, 61:456-502

Brown JW, Daniels CJ, Reeve JN: **Gene structure, organisation and expression in archaebacteria**. Crit Rev Microbiol. 1989, 16:287-338

Cabrera JA, Bolds J, Shields PE, Havel CM, Watson JA: **Isoprenoid synthesis in *Halobacterium halobium*. Modulation of 3-hydroxy-3-methylglutaryl coenzyme A concentration in response to mevalonate availability**. J Biol Chem. 1986, 261, 3578-3583

Champoux JJ: **DNA topoisomerases: structure, function and mechanism**. Annu Rev Biochem. 2001, 70:369-413

Cline SW, Doolittle WF: **Efficient transfection of the archaebacterium *Halobacterium halobium***. J Bacteriol. 1987, 169, 1341-1344

Clouet d' Orval B, Gaspin C, Mougin A: **Two different mechanisms for tRNA ribose methylation in Archaea: a short survey**. Biochimie. 2005, 87:889-895

Condò I, Ciammaruconi A, Benelli D, Ruggero D, Londei P: **Cis-acting signals controlling translational initiation in the thermophilic archaeon *Sulfolobus solfataricus***. Mol Microbiol. 1999, 34:377-384

DasSarma P, DasSarma S: **On the origin of prokaryotic "species": the taxonomy of halophilic *Archaea.*** Saline Systems. 2008, 4:5. doi: 10.1186/1746-1448-4-5

Degtyrev SK, Netesova NA, Abdurashitov MA, Shevchenko AV: **Primary structure and strand specificity of *Bst*F5I-1 DNA methyltransferase which recognizes 5'GGATG-3`**. Gene. 1997, 187:217-219

Dennis PP: **Ancient ciphers: translation in archaea**. Cell. 1997, 89:1007-1010

Di Giulio M: **The tree of life might be rooted in the branch leading to Nanoarchaeota.** Gene 401. 2007, 108–113

Eichler J: **Archaeal protein translocation. Crossing membranes in the third domain of life**. Eur J Biochem. 2001, 267, 3402-3412

Enache M, Itoh T, Fukushima T, Usami R, Dumitru L, Kamekura M: **Phylogenetic relationships within the family Halobacteriaceae inferred from rpoB` gene and protein sequences.** Int J Syst Evol Microbiol. 2007, 57 (Pt10): 2289-95.

Endo A: **The discovery and development of HMG-CoA inhibitors**. J Lipid Res. 1992, 33:1569-1582

Euzéby JP, *Formerly List of Bacterial names with Standing in Nomenclature (LBSN)*

Falb M, Pfeiffer F, Palm P, Rodewald K, Hickmann V, Tittor J, Oesterhelt D: **Living with two extremes: conclusions from the genome sequence of *Natronomonas pharaonis***. Genome Res. 2005, 15 (10), 1336-1343

Florenzano G, Sili C, Pelosi E, Vinzenzini M: ***Cyanoapira rippkae* and *Cyanospira capsulata* (gen. nov. and spp. nov.): a new filamentous heterocystous cynobacteria from Magadi Lake (Kenya).** Arch Microbiol. 1985, 140:301-306

Franzetti B, Schoehn G, Ebel C, Gagnon J, Ruigrok RW, Zaccai G: **Characterization of a novel complex from halophilic archaebacteria, which displays chaperone-like activities in vitro**. J Biol Chem 2001, 276:29906-29914

Gambacorta A, Trincone A, Nicolaus B, Lama L, de Rosa M: **Unique features of lipds of *Archaea***. Syst Appl Microbiol. 1993, 16:18-27

Grant WD, Tindall BJ: **The alkaline, saline environment.** In: Herbert RA, Codd GA (eds) Microbes in extreme environment. Academic Press, London 1986, pp 22-54

Gropp F, Grampp B, Stolt P, Palm P, Zillig W: **The immunity-conferring plasmid pϕHL from the *Halobacterium salinarium* phage ϕH: nucleotide sequence and transcription**. Virology. 1992, 190:45-54

Gropp F, Reiter WD, Sentenac A, Zillig W, Schnabel R, Thomm M, Stetter KO: **Homologies of components DNA-dependent RNA polymerases of archaebacteria, eukaryotes and eubacteria.** Syst Appl Microbiol. 1986, 7:95-101

Hampsey M: **Molecular genetics of the RNA polymerase II general transcriptional machinery**. Microbiol Mol Biol Rev. 1998, 62:465-503

Hendrix RW, Lawrence JG, Hatfull GF, Casjens S: **The origins and ongoing evolution of viruses**. Trends Microbiol. 2000, 8:504-508

Holmes ML, Nuttall SD, Dyall-Smith ML: **Construction and use of halobacterial shuttle vectors and further studies on *Haloferax* DNA gyrase**. J Bacteriol. 1991, 173:3807-3813

Horikoshi K: **Alkaliphiles: some Applications of their products for Biotechnology**. Microbiol Mol Biol Rev. 1999, 63:735-750

Huber H, Hohn MJ, Stetter KO, Rachel R.: **The phylum Nanoarchaeota: present knowledge and future perspectives of a unique form of life**. Res Microbiol. 2003, 154:165-171

Imhoff JF, Sahl HG, Soliman GSH, Trüper HG**: The Wadi Natrun: Chemical composition and microbial mass developments in alkaline brines of Eutrophic desert lakes**. Geomicrobiology 1979, 1:219-234

Iro M, Klein R, Gálos B, Baranyi U, Rössler N, Witte A: **The lysogenic region of virus ϕCh1: identification of a repressor-operator system and determination of its activity in halophilic *Archaea***. Extremophiles 2007, 11:383-396

Jolley KA, Rapaport E, Hough DW, Danson NJ, Woods WG, Dyall-Smith ML: **Dihydrolipoamide dehydrogenase from the halophilic archaeon *Haloferax volcanii*: homologous overexpression of the cloned gene**. J Bacteriol. 1996, 178:3044-3048

Jones BE, Grant WD, Duckworth AW, Owenson GG: **Microbial diversity of soda lakes.** Extremophiles. 1998, 2:191-200

Kamekura M, Dyall-Smith M, Upasani V, Ventosa A, Kates M: **Diversity of Alkaliphilic Halobacteria: Proposals for Transfer of *Natronobacterium vacuolatum*, *Natronobacterium magadii*, and *Natronobacterium pharaonis* to *Halorubrum*, *Natrialba*, and *Natronomonas* gen. nov., Respectively, as *Halorubrum vacuolatum* comb. nov., *Natrialba magadii* comb. nov., and *Natronomonas pharaonis* comb. nov., Respectively.** International Journal of Systematic Bacteriology. 1997, p.853-857

Kamekura M: **Diversity of extremely halophilic bacteria.** Extremeophiles. 1998, 2:289-295

Klein R, Baranyi U, Rössler N, Greineder B, and Witte A: ***Natrialba magadii* virus ϕCh1: first complete nucleotide sequence and functional organization of a virus infecting a haloalkaliphilic archaeon**. Mol Microbiol. 2002, 45:851-863

Konstantinidis K, Tebbe A, Klein C, Scheffer B, Aivaliotis M, Bisle B, Falb M, Pfeiffer F, Siedler F, Oesterhelt D: **Genome-wide proteomics *Natronomonas pharaonis***. J Proteome Res. 2007, 6 (1), 185-193

Krüger DH, Reuter M: **Host-controlled modification and restriction**. In *Encyclopedia of Virology*. Webster R., Granoff A (eds.). London: Academic Press. 1999, p. 758-763

Lam WL, Doolittle WF: **Shuttle vectors for the archaebacterium *Halobacterium volcanii***. Proc Natl Acad Sci. 1989, 86:5478–5482

Ladurner A: **Characterization of the inversion reaction of ϕCh1 and the establishment of a transformation system for *Natrialba magadii***. Diploma thesis, University of Vienna. 2008

Lanyi JK: **Salt dependent properties of proteins from extremely halophilic bacteria**. Bacteriol Rev. 1974, 38:272-290

Lodwick D, Ross HNM, Harris JE, Almond JW, Grant WD: ***dam* methylation in the *archaebacteria***. J Gen Microbiol. 1986, 132:3055-3059

Madern D, Ebel C, Zaccai G: **Halophilic adaptation of enzymes**. Extremophiles 2000, 4:91-98

Madern D, Pfister C, Zaccai G: **A single acidic amino acid mutation enhances the halophilic behaviour of malate dehydrogenase from *Haloarcula marismortui***. Eur J Biochem. 1995, 230:1088-1095

Malone T, Blumenthal MR, Cheng X: **Structure-guided analysis reveals nine sequence motifs conserved among DNA amino-methyltransferases, and suggests a catalytic mechanism for these enzymes**. J Mol Biol. 1995, 253:618-632

Miller TL, Wolin MJ: **Inhibition of growth of methane-producing bacteria of the ruminant forestomach by hydroxymethylglutaryl~SCoA reductase inhibitors**. J Dairy Sci. 2001, 84:1445-1448

Mizuuchi K, O'Dea MH, Gellert M: **DNA gyrase: subunit structure and ATPase activity of the purified enzyme**. Proc Natl Acad Sci USA. 1978, 75:5690-5963

Nuttal SD, Dyall-Smith ML: **HF1 and HF2: novel bacteriophages of halophilic *Archaea***. Virology. 1993, 197:678-684

Oren A: **Bioenergetic aspects of halophilism**. Microbiol Mol Biol Rev. 1999, 63:334-348

Palmer BR, Marinus MG: **The *dam* and *dcm* strains of *Escherichia coli* – a review**. Gene. 1994, 143:1-12

Pohlschröder M, Prinz WA, Hartmann E, Beckwith J: **Protein translocation in the three domains of life: variations on a theme**. Cell 1997, 91:563-566

Reece RJ, Maxwell A: **DNA gyrase: structure and function**. Crit Rev Biochem Mol Biol. 1991, 26:335-375

Reiter WD, Hüdepohl U, Zillig W: **Mutational analysis of an archaebacterial promoter: Essential role of TATA box for transcription efficiency and start site selection *in vitro*.** Proc Natl Acad Sci USA. 1990, 87:9509-9513

Richard SB, Madern D, Garcin E, Zaccai G: **Halophilic adaptation: novel solvent protein interactions observed in the 2.9 and 2.6 Å resolution structures of the wild type and mutant of malate dehydrogenase from *Haloarcula marismortui*.** Biochemistry. 2000, 39, 992-1000

Rose RW, Brüser T, Kissinger JC, Pohlschröder M: **Adaptation of protein secretion to extremely high-salt conditions by extensive use of the twin-arginine translocation pathway**. Mol Microbiol. 2002, 45:943-950

Saruyama H, Sasaki S: **Purification and characterization of peptide-elongation factor 2 (aEF-2) from an extremely halophilic archaebacterium *Halobacterium halobium***. Eur J Biochem. 1988, 170:499-505

Sasaki T, Kubo M, Kikukawa T, Kamiya M, Aizawa T, Kawano K, Kamo N, Demura M: **Halorhodopsin from *Natronomonas pharaonis* forms a trimer even in the presence of a detergent, dodecyl-β-D-maltoside**. Photochemistry and Photobiology. 2009, 85:130-136

Seshadri R, Adrian L, Fouts DE, Eisen JA, Phillippy AM, Methe BA, Ward NL, Nelson WC, Deboy RT, Khouri HM, Kolonay JF, Dodson RJ, Daugherty SC, Brinkac, LM, Sullivan SA, Madupu R, Nelson KE, Kang KH, Impraim M, Tran K, Robinson JM, Forberger HA, Fraser CM, Zinder SH,

Heidelberg JF: **Genome sequence of the PCE-dechlorinating bacterium *Dehalococcoides ethenogenes*.** Science. 2005, 307, 105-108

Schnabel H, Zillig W, Pfäffle M, Schnabel R, Michel H, Delius H: ***Halobacterium halobium* phage ϕH**. EMBO J. 1982, 1:87-92

Shand R, Betlach MC: ***bop* gene cluster expression in bacteriorhodopsin-overproducing mutants of *Halobacterium halobium***. J Bacteriol. 1994, p. 1655-1660

Shimono K, Iwamoto M, Sumi M, Kamo N: **Functional expression of *pharaonis* phoborhodopsin in *Escherichia coli***. FEBS Lett. 1997, 420, 54-56

Soliman GSH, Trueper HG: ***Halobacterium-pharaonis* new-species a new extremely halo alkaliphilic archaebacterium with low magnesium requirement**. Zentralbl Mikrobiol. 1982, 3, 318-329

Soppa J: **Transcription initiation in Archaea: facts, factors and future aspects.** Mol Microbiol. 1999, 31, 1295-1305

Southern EM: **Detection of specific sequences among DNA fragments separated by gel electrophoresis**. J Mol Biol. 1975, 98:503-17

Staley JT, Bryant MP, Pfennig N, Holt JG: **Bergey's Manual of Systematic Bacteriology**. In *Archaeobacteria*; Koenig H, Stetter KO, Eds. Williams and Wilkins: Baltimore, MD. 1989, 3:2230-2232

Sugino A, Higgins NP, Brown PO, Peebles CL, Cozzarelli NR: **Energy coupling in DNA gyrase and the mechanism of action of novobiocin**. Proc Natl Acad Sci USA. 1978, 75:4838-4842

Tindall BJ, Ross HNM, Grant WD: ***Natronobacterium* gen. nov. and *Natronococcus* gen. nov., two new genera of haloalkaliphilic archaebacteria**. Syst Appl Microbiol. 1984, 5:41-57

Tolstrup N, Sensen CW, Garrett RA: **Two different and highly organized mechanisms translation initiation in the archaeon *Sulfulobus solfataricus*.** Extremophiles. 2000, 4:175-179

Tomlinson GA, Hochstein LI: ***Halobacterium saccharovorum* sp. nov., a carbohydrate-metabolizing, extremely halophilic bacterium**. Can J Mikrobiol. 1976, 22:587-591

von Hippel P, Schleich T: **The effects of neutral salts on the structure and conformational stability of macromolecules in solution.** In: Timasheff SN, Fasman GD (eds) Structure of biological macromolecules. Dekker, I New York. 1969, p. 417-575

Wendoloski D, Ferrer C, Dyall-Smith ML: **A new simvastatin (mevinolin)-resistance marker from *Haloarcula hispanica* and a new *Haloferax volcanii* strain cured of plasmid pHV2**. Microbiology. 2001, 147, 959-964

Wilson G: **Organization of restriction-modification systems**. Nucleic Acids Res. 1991, 19:2539-2566

Witte A, Baranyi U, Klein R, Sulzner M, Luo C, Wanner G, Krüger DH, Lubitz W: **Characterization of *Natronobacterium magadii* phage ϕCh1, a unique archaeal phage containing DNA and RNA**. Mol Microbiol. 1997, 23:603-616

Woese CR, Fox GE: **Phylogenetic structure of the prokaryotic domain: the primary kingdoms**. Proc Natl Acad Sci U S A. 1977, 74:5088-5090

Woese CR, Kandler O, Wheelis ML: **Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya**. Proc Natl Acad Sci U S A. 1990, 87:4576-4579

Yanisch-Perron C, Viera J, Messing J: **Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors**. Gene. 1985, 33:103-119

Zhou M, Xiang H, Sun C, Tan H: **Construction of a novel shuttle vector based on an RCR-plasmid from a haloalkaliphilic archaeon and transformation into other haloarchaea**. Biotechnol Lett. 2004, 26:1107-1113

Textbooks

Dyall-Smith ML: **The Halohandbook – protocols for haloarchaeal genetics**. Version 6.01. 2006

Madigan MT, Martinko JM (ed): **Brock Biology of Microorganisms**. 11th edition. Pearson Edication Inc. 2006

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Zusammenfassung

Diese Arbeit beschreibt den Versuch der Charakterisierung der M.*Nma*ϕCh1 und der weiteren Charakterisierung eines entwickelten Transformationssystems in haloalkaliphilien *Archaea*.

Der Virus aus dem das Methyltransferasegen isoliert wurde ist ein temperenter Phage, dessen einzig bekannter Wirt *Nab. magadii* ist. Die optimalen Lebensbedingungen dieses Archaeons sind hohe Salzkonzentrationen und extrem hohe pH Werte. Es wurden drei Konstrukte die entweder das ganze Methyltransferasegen oder Teile davon enthalten durch PCR angefertigt und in verschiedene Vektoren kloniert, nämlich pro-5, pQE32 und pRSET-C. Die drei Vektoren, die entweder das gesamte Mtase-Gen oder Teile davon enthielten, wurden benutzt, um die Transformationseffiezienz in *Nab. magadii* L13 zu prüfen. Allerdings mußte dafür zuerst die Promotorsequenz vom ORF34 davor kloniert weden, da bis dato der vermeintliche Initiationsstartpunkt der Mtase nicht bekannt war. Die pQE32 Konstrukte wurden verwendet um *in vivo* die Aktivität auf ihre Existenz zu prüfen. Zuletzt wurde anhand der pRSET-C Vektoren, die ebenfalls die Mtasefragmente enthielten die Bindung des Proteins an verschiedene unmethylierte DNA Sequenzen getestet. Dabei hat sich z.B. herausgestellt, dass für die erfolgreiche Bindung Chelat bindende Substanzen wie EDTA notwendig sind.

Der zweite Teil dieser Arbeit hat sich mit dem Transformationssystem und dessen weitere Charakterisierung beschäftigt. Zu Beginn wurde versucht heraus zu finden welches Enzym eine bessere Spheroplastenbildung hervorruft, Proteinase K oder Pronase E. Dabei hat sich heraus gestellt, dass durch den Gebrauch von Proteinase K mehr Spheroplasten gebildet werden, nur im Falle von *Nmn. pharaonis* kann auf den Gebrauch von Pronase E nicht verzichtet werden. Verschiedene Plasmide sogar mit unterschiedlichen Antibiotikaresistenzen wurden versucht zu transformieren. Diese Versuche zeigten dass es möglich ist Mevinolin als Selektionsmarker zu verwenden. Die optimalen Wachstumsbedingungen waren in diesem Fall 7.5 µg/ml Mevinolin.
Anhand dieser Ergebnisse wurden folgende Vektoren erfolgreich in *Nab. magadii* L13 transformiert: pRo-5, pRo-4, pro-4/Mev, pNB102 und zwei neu konstruierte, pNov-1/101 und pRo-5/Bop. Obwohl pNov-1/101 den gleichen Replikationsursprung wie pNB102 enthält wurde es effiezienter von den Zellen aufgenommen als pNB102.

Plasmide wie pMDS11 und pMDS24 konnten leider nicht transformiert werden.
Auch andere Organismen, nämlich *Nmn. pharaonis* wurden auch bezüglich ihrer Transformationsfähigkeit untersucht. Die oben genannten Plasmide wurden auch hier erfolgreich transformiert, allerdings mit einer geringeren Ausbeute. Zusätzlich wurden auch die Vektoren pMG100, pMG200 und pMG300 in die Testreihe miteinbezogen, deren Transformanten mit Southern blot Analysen bestätigt wurden.

Abstract

This thesis describes the analysis for the characterization of the M.*Nma*φCh1 and further characterization of a transformation system in haloalkaliphilic *Archaea*.

The virus from which the methyltransferase gene was isolated is a temerate one and its only known host is the archaeon *Nab. magadii*, whose optimal living conditions are in a high alkaine environment with high pH values. Three constructs including the whole or parts of the *mtase* gene were performed by PCR and cloning into different plasmids like pRo-5, pQE32 and pRSET-C. pRo-5 plasmids including the different fragments of the Mtase gene were used for transformation experiments in *Nab. magadii* L13 to test the efficiencv. Therefore, it was needed to clone the promotorsequence of ORF34 infront of the fragments because till this thesis it putative initiation sequence was not known.
The pQE32 constructs were used for *in vivo* assays to determine the activity of the gene. Binding assays with different unmethylated DNA substrates were performed with the pRSET-C constructs, which result in the assumption that a chelat binding agent like EDTA is required for binding of the protein.

The second part of this study is the further characterization of the established transformation system in *Archaea*. Therefore it was tested what kind of enzyme is better for forming spheroplasts, proteinase K or pronase E. It became apparent that proteinase K is the more efficient enzyme. Only in case of *Nmn. pharaonis* the pronase E was needed to successed in transformation. Different plasmids even with an other antibiotic resistance were tried to transform, which lead to the possibility to use mevinolin as a marker gene. Growth experiments and a few transformations showed that the optimal concentration of mevinolin is 7.5 µl/ml.
With these data plasmids like pRo-5, pRo-4, pro-4/Mev, pNB102 and two constructed ones pNov-1/101 and pRo-5/Bop were successfully transformed into *Nab. magadii* L13. pNov-1/101 which containes the same origin of replication as pNB102 was anyhow more efficient. Plasmids like pMDS11 and pMDS24 were not transformable.
Other organisms were also tested namely *Nmn. pharaonis*. The same plasmids were indeed with a lower efficiency but successfully transformed and additionally the constructs pMG100, pMG200 and pMG300 which were proven by Southern blot analysis.

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