universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

„ORF79 - a putative regulator of gene expression in ϕCH1 and analysis of $gp34_{52}$ as a tail fibre protein of *Nab. magadii*“

Verfasserin

Judith Beraha

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag. rer. nat.)

Wien, 2013

Studienkennzahl lt. Studienblatt:	A 441
Studienrichtung lt. Studienblatt:	Diplomstudium Genetik und Mikrobiologie
Betreuerin / Betreuer:	Ao. Univ.-Prof. Dipl.-Biol. Dr. Angela Witte

Abstract

For a considerable time, the group of the Archaea did not attract interest until Woese determined it as the third domain of life in 1987. Thereafter, work with Archaea became increasingly intensive as the Archaea harbor features not known for other organisms. In this study, the halophilic virus ϕCh1 which infects the haloalkalophilic Archaeum *Nab. magadii* is examined in more detail. Two open reading frames of ϕCh1, ORF49 and ORF79, are of particular interest as they seem to be repressive- as well as regulative elements in the lysis of *Nab. magadii*. ORF49 stands in the focus of this work because of the finding of a *Nab. magadii* strain carrying a mutant version of ϕCh1, ϕCh1-1, with amongst others an earlier onset of lysis and increased plaque formation. Further studies showed that ϕCh1-1 carries a mutation in the ORF49 leading to a more detailed research of the open reading frame. Repression of lysis as well as the ability for binding DNA by gp49 could be proven. Furthermore, the exact position of the DNA binding domain is determined as it is the case for the region responsible for the repressor activity. However, loss of the DNA binding domain of gp49 does not lead to the loss of the ability for repression. Amongst others this led to the idea that gp49 has the ability to dimerize with other viral proteins which is examined in this thesis. Otherwise, the assumption that gp79 has repressor activity was created by the deletion of ORF79 with unknown function at that time. The ORF79 deletion strain showed an earlier onset of lysis and an abnormal regulation of the expression of certain ϕCh1 proteins. In this thesis the complementation of L11-ΔORF79 is examined as well as studies about the localization of the promoter region of ORF79.

The discovery of a gene encoding for a site specific recombinase, Integrase1 of ϕCh1, which supports the exchange of 3´ parts of the surrounding open reading frames ORF34 and ORF36 led to the finding of different gp34 and gp36 variants. In contrast to gp36, one variant of gp34, $gp34_{52}$, has the ability to bind the cell surface of *Nab. magadii*, which could be the key to the mechanism by which ϕCh1 infects the host cell. In this thesis overexpression of int1 with the help of the inducible promoter *tnaN* of *Nab. magadii* is discussed. *tnaN* represents the promoter of the tryptophanase and thus is inducible with tryptophan. In addition, the binding of $gp34_{52}$ to a different halophilic Archaeon, *Nbt. gregoryi* is examined.

Zusammenfassung

Lange Zeit stand die Gruppe der Archaeen nicht im Fokus der Wissenschaft bis Woese sie 1987 als dritte Domäne des Lebens definierte. Danach gewann die Arbeit mit Archaeen mehr und mehr an Bedeutung, da sie Eigenschaften besitzen, die andere Organismen nicht aufweisen. In dieser Studie wird der halophile Virus ϕCh1, der das haloalkalophile Archaeum *Nab. magadii* infiziert, genauer untersucht. Zwei offene Leserahmen von ϕCh1, ORF49 and ORF79, sind von besonderem Interesse, da sie scheinbar repressive- sowie regulatorische Elemente in der Lyse von *Nab. magadii* darstellen. ORF49 steht im Mittelpunkt dieser Arbeit aufgrund der Entdeckung eines *Nab. magadii* Stammes, der eine mutierte Version von ϕCh1, ϕCh1-1, trägt. Der mutierte Stamm weist unter anderem eine frühere Lyse sowie eine vermehrte Plaque Rate auf. Weitere Studien zeigten, dass ϕCh1-1 eine Mutation in ORF49 trägt, was Veranlassung war, diesen offenen Leserahmen detailliert zu untersuchen. Die Unterdrückung der Lyse durch gp49 sowie die Fähigkeit von gp49, DNA zu binden, konnten nachgewiesen werden. Weiterhin konnte die exakte Position der DNA Bindungsdomäne sowie die Region, die zuständig für die repressive Aktivität ist, bestimmt werden. Der Verlust der DNA Bindungsdomäne führt jedoch nicht zum Verlust des repressiven Effekts von gp49. Das gibt Anlass zu der Vermutung, dass gp49 die Fähigkeit besitzt, Dimere mit anderen viralen Proteinen zu bilden. Auch dies wird in dieser Arbeit genauer untersucht. Die Annahme, dass gp79 eine repressive Aktivität besitzt, wurde anders als bei gp49 durch die Erstellung einer Deletion von ORF79 getroffen. Zu dieser Zeit war die Funktion von ORF79 noch unbekannt. Der Stamm der die Mutation trägt zeigt ebenfalls eine frühere Lyse sowie eine abnormale Regulation der Expression bestimmter Proteine von ϕCh1. Die Komplementierung von L11-ΔORF79 sowie eine Studie über die Position der Promoterregion von ORF79 werden in dieser Arbeit genauer untersucht.

Die Untersuchung eines Gens, das eine site specific Rekombinase, die Integrase1 von ϕCh1, codiert, führte zu der Entdeckung von verschiedenen gp34 und gp36 Varationen. Die integrase1 fördert den Austausch von Teilen der 3´ Sequenz von den offenen Leserahmen ORF34 und ORF36, die ORF35 (int1) umgeben. Im Gegensatz zu gp36 hat eine Variante von gp34, $gp34_{52}$, die Fähigkeit an die Zelloberfläche von *Nab. magadii* zu binden. Diese Strategie könnte der Schlüssel zum Verständnis darüber sein in welcher Weise die Infektion der Wirtszelle durch ϕCh1 erfolgt. In dieser Arbeit wird die Überexpression von int1 mit Hilfe des induzierbaren Promoters *tnaN* von *Nab. magadii* diskutiert. *tnaN* stellt den Promoter der Tryptophanase dar und ist deshalb mit

Tryptophan induzierbar. Zusätzlich wird die Bindung von $gp34_{52}$ an ein anderes halophiles Archaeum, *Nbt. gregoryi*, untersucht.

Index of Contents

1 Introduction

1.1 Classification and phylogeny of organisms

To introduce the world of Archaea, a look at the classification and phylogeny of organisms provides a good starting point.

The very early division of life into the two categories “animals” and “plants” was revolutionized at the latest in 1866 by Ernst Haeckel. He expanded the tree of life with the category of Protista (single celled forms) which could not be assigned either to the kingdom of Animals or the kingdom of Plants (Haeckel, 1866).

Between 1938 and 1959 two new kingdoms were appended. Copeland first introduced the category of Bacteria as the fourth kingdom (Copeland, 1938) and Whittaker determined the group of Fungi as the fifth kingdom (Whittaker, 1959).

This five kingdom system dividing all organisms into Animalia, Plantae, Fungi, Protista and Monera (Bacteria) is still accepted but phylogenetically not entirely correct. In this scheme the Monera as the group of the Prokaryotes has the same taxonomic range than the other four kingdoms which were summarized as Eukaryotes even though the bacterial members differ in nature because of their smaller complexity. Therefore the differentiation of life into the groups Eukaryotes and Prokaryotes was established (Woese *et al*., 1990). Chatton already proposed the assignment of the Bacteria to the Prokaryotesin in 1938 (Chatton, 1938), but to that time it was not possible to find a definition for Prokaryotes without assuming their non-eukaryotic characteristics.

This problem was first solved by molecular studies about the relationship between organisms which were based on the comparison of the 16S/18S rRNA sequences. rRNA´s as parts of ribosome constitute a representable chronometer because they appear in every living cell and the sequences change very slowly during evolution (Woese & Fox, 1977). In the course of the studies it was possible to compare organisms on a very different level and a new kingdom, the one of the Archaea was created. Erroneously, the group of the Archaea was assigned to the kingdom of Prokaryotes because of cytological accordance but with the finding of a unique structure in the archaebacterial 16S rRNA it became an own group (Woese, 1987).

The classification of all organisms into Eubacteria, Archaebacteria and Eukaroytes demanded a new higher level taxon which stands above the conventionally kingdoms. Woese proposed “domain” as the highest taxon and used the common ending *–a* as the formal suffix resulting in Bacteria, Archaea and Eubacteria. Because there never was a kingdom associated with Archaea before a new classification could be created. Archaea therefore was divided into the kingdoms Euryarchaeota as the group of the methanogens and the kingdom Crenarchaeota representing the group of thermophilic Archaea as shown in figure 1 (Woese *et al*., 1990).

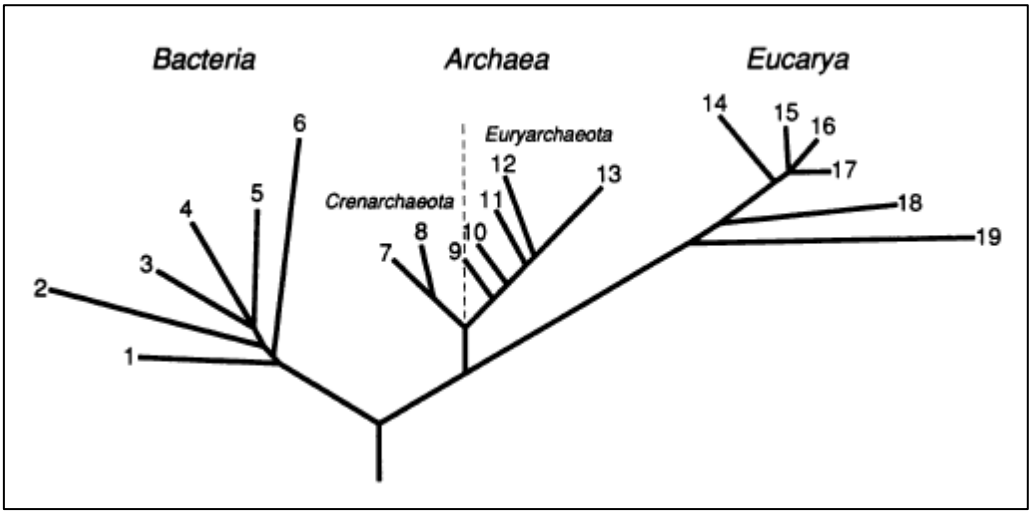

Fig. 1 The universal phylogenetic tree dividing live into the three domains Bacteria, Archaea and Eucarya based on the comparison of rRNA sequences. Groups of organisms underlying the domains are indicated by numbers: Bacteria: 1, the Thermotogales; 2, the flavobacteria and relatives; 3, the cyanobacteria; 4, the purple bacteria; 5, the Gram-positive bacteria; and 6, the green nonsulfur bacteria. Archae: the kingdom Crenarchaeota: 7, the genus Pyrodictium; and 8, the genus Thermoproteus; and the kingdom Euryarchaeota: 9, the Thermococcales; 10, the Methanococcales; 11, the Methanobacteriales; 12, the Methanomicrobiales; and 13, the extreme halophiles. Eucarya: 14, the animals; 15, the ciliates; 16, the green plants; 17, the fungi; 18, the flagellates; and 19, the microsporidia (Woese *et al*., 1990).

1.2 Archaea vs. Eubacteria

The dotted line within the domain of Archaea in figure 1 indicates a closer relationship between the Archaea and the Eucarya than between the Archaea and the Bacteria. This is reasonable by regarding their characteristics on a molecular level – in contrast to the original assumption that Archaea belong to the Bacteria because of their, in

comparison to Eukaryotes, simpler cytological structure (Woese, 1987). But there are other common characteristics shared by Archaea and Bacteria: mostly they carry a large circular chromosomal DNA, a nuclear membrane as well as organelles are lacking (Brown & Doolittle, 1997). Furthermore archaeal operons and gene cluster seem to resemble those of Bacteria as it is the case for the ribosomal operons and operons that encode the three largest subunits of the DNA-dependent RNA polymerase (Puhler *et al*., 1989). They look very similar with the exception of some archaeal variations. Also the type II restriction enzyme systems of some archaeal members show homologies to those of Bacteria (Brown *et al*., 1989), and restriction/modification enzymes, endodeoxyribonuclease and DNA methyltransferase found in the archaeal species *Methanobacterium thermoformicium* show high similarities to those of the Bacterium *Neisseria gonorrhoeae* (Nolling, 1992). By studying the cell division of Aarchaea, an archaeal protein was found related in sequence to the cell division protein FtsZ of Bacteria. As FtsZ is similar in sequence and function to the GTP-binding tubulin proteins of Eukaryotes it is unsure which of the systems evolved earlier and at which state Archaea have developed a FtsZ homolog (Baumann & Jackson, 1996).

However by studying the organisms more precisely, there are as many differences between Archaea and Bacteria as commonalities. First of all, Archaea and Bacteria usually prefer different environments. Most members of Archaea live in niches with high temperatures, especially the group of the thermophils, whereas Bacteria are adapted to low temperatures with rare exceptions (Achenbach-Richter *et al*., 1987; Nicolaus *et al*., 1991). Furthermore there are just a few members of the Bacteria that live anaerobically like chemotrophic anaerobic Bacteria (Thauer *et al*., 1977), the majority lives under aerobic conditions in contrast to the anaerobic live style of some Archaea. There are also differences in metabolism: Eubacteria have a variety in metabolisms whereas Archaea are metabolically monotonous with some exceptions (e.g. one group of methanogens use acetate and methyl amines). Also the existence of a unique coenzyme involved in the sulfur metabolism of extreme halophilic Archaea was never observed in Bacteria (Woese, 1987).

1.3 Archaea vs Eucarya

It seems that there are many commonalities in gene organisation, restriction/modification systems and cell division between Archaea and Bacteria, but most of the archaeal genes involved in transcription and translation are closer relative

to those of the Eukaroytes. The first step in transcription is the recognition of the initiation site of the gene to be transcribed. In Archaea as in Eukaryotes there is a TATA-binding box ~ 30 bp upstream of the site of transcription initiation (Hudepohl, 1990) which is bound by a highly conserved TATA-box binding protein (TBP) (Rowlands *et al.*, 1994). In Eukaryotes this binding protein is associated with a number of transcription initiation factors which stabilize the complex and finally build a bridge between the TBP and the RNA polymerase II (RNAP II). In Archaea just one of these transcription initiation proteins was found, the TFB, which is a homolog to TFIIB of Eukaryotes (Ouzounis & Sander, 1992). By comparing the RNA polymerase of Archaea and Eukaryotes it is noticeable that the archaeal polymerase has more similarities with the eukaryotic nuclear RNAP II than with bacterial enzymes by regarding the subunit complexity (Zillig *et al.*, 1979). The bacterial RNAP consists of only four major subunits whereas archaeal and eukaroytic RNAP´s have at least seven subunits with several homologies (Langer *et al.*, 1995). With the finding of an archaeal homolog to the eukaryotic transcription elongation factor TFIIS in *Sulfolobus* a further common characteristic of transcription was thought to have been found (Langer & Zillig, 1993). But with the identification of the genes encoding the small subunit of eukaryotic RNAP II, the RPB9, it was shown that the archaeal homolog of TFIIS is closer related to the RPB9 family (Kaine *et al.*, 1994). As there is one subunit of the archaeal RNAP which is still unidentified this subunit could represent the TFIIS homolog (Bell & Jackson, 1998). On the other hand homologues of the bacterial elongation factors NusA and NusG were found in Archaea (Klenk *et al.*, 1998) but it is unknown which function these proteins have in archaeal transcription.

Also in translation archaeal homologs to almost all eukaryotic initiation and elongation factors (Bell & Jackson, 1998) were found, the recruitment of the ribosomes to the mRNA however occurs differently. In Eucaryotes a 5´cap of the mRNA serves as recognition site for the ribosomes whereas in Archaea such a structure does not exist instead a bacterial-like shine dalgarno sequence is observable (Dennis, 1997).

1.4 Characteristics just Archaea

Many archaeal features appear to be a mosaic of eubacterial and eukaryotic characteristics, but there exist properties that are unique to Archaea. Especially the archaeal membrane structure differs from those of Bacteria and Eukarya with respect to the molecular organization of lipids. Bacterial and eukaryotic cytoplasmic membranes have

ester linkages between glycerol and hydrocarbon chains in contrast to the predominant ether linkages that are present in Archaea (Makula & Singer, 1978). Furthermore the prokaryotic and eukaryotic hydrocarbons consist of fatty acyl chains whereas archaeal hydrocarbons have highly methylbranched isopranyl chains (Gambacorta *et al*., 1994). Another difference lies within the existence of lipid tetraether in Archaea, a structure that is not known for ester lipids of the other two domains. The formation of tetraehters and the cyclization of the fatty acyl chains are an adaption to keep the cytoplasmic membrane stabile when exposed to extreme environments (Van de Vossenberg *et al*., 1998). Also, the glycerol linkages differ in nature as in Archaea glycerol ethers consist of 2,3-*sn*-glycerol in contrast to the 1,2-*sn*-glycerol present in prokaryotic and eukaryotic glycerol ester (Brown & Doolittle, 1997).

In addition to the differences concerning the cytoplasmatic membrane there are as well differences in the organization of the cell wall of Archaea, Bacteria and Eukaryotes. The archaeal cell wall lacks murein, a peptidoglycan that is present in all bacterial cell walls with the exception of some archaeal groups (e.g. *Methanobacteriales*) who possess pseudopeptidoglycan (pseudomurein). The bacterial peptidoglycan is composed of N-acetlymuramic acid with a ß-1,4 linkage to D-N-actelyglucosamine (GlcNAc) in contrast to the pseudomurein backbone wich is composed of L-N-acetylalosaminuronic acid with a ß-1,3 linkage to GlcNAc (Kandler & König, 1979). Another structure which is also found in Bacteria, the surface layer (S-layer), can be observed in the majority of Archaea, either as the only layer surrounding the cell or coupled with the pseudomurein layer. This archaeal S-layer consists of a single protein or glycoprotein species and serves for osmoprotection (Sleytr *et al*., 1999).

1.5 Kingdoms and Phyla

With the help of the rRNA sequence comparison Woese could not only distinguish the Archaea from the two other domains, he was also able to create archaeal kingdoms and phyla. First, the three major groups of methanogens and one group of extreme halophiles could be defined with the help of analysis of full sequences. They showed that the extreme halophiles are related to one of the groups of the methanogens, the *Methanomicrobiales*. Thus, the three groups of methanogens and the group of the extreme halophiles were subsumed and distinguished from the group of extremely thermophilic Archaea (Woese, 1987).

Further 16S rRNA analysis showed that there are three more groups that are related to the methanogens, the sulfate reducing species and two types of thermophiles (Woese, 1987; Achenbach-Richter *et al.*, 1988). For the three groups of methanogens, the group of the extreme halophiles, the sulfate reducing species and the two types of thermophiles Woese proposed the formal name *Euryarchaeota.* The methanogens are divided into the phyla *Methanobacteriales, Methanococcales* and *Methanomicrobiales*. For two of them, subdivisions exist and each group has a variety of species. The methanogens produce methane under strict anaerobe conditions with the help of unique enzymes and cofactors (DiMarco *et al*., 1990). The extreme halophiles are characterized by a very high internal salt concentration of about 5 M potassium ion (Kushner, 1985) and they are the only Archaea that are able to run photosynthesis with the help of a proton pump based on a unique pigment, the bacteriorhodopsin. The sulfate reducing species were assigned to the phylum *Euryarchaeota* because 16S rRNA sequence analysis showed that they arise from the methanogens which explains the presence of cofactors characteristic for methanogens in sulfate reducing species. Further it can be assumed that the sulfur metabolism existed first and then changed into methanogenesis. The two thermophiles, *Thermococcus* and *Thermoplasma* were added to the *Euryarchaeota* based on rRNA. However, phenotypically they seem to belong to the *Crenarchaeota*, the second kingdom of Archaea collecting the extreme thermophiles (Woese, 1987). The main characteristics of this group are the anaerobic life style (Stetter & Zillig, 1985) at extremely high temperatures (Stetter, 1982), at least one unique coenzyme, the insensitivity to most of the antibiotics (Cammarano *et al*., 1985) and their highly modified tRNAs and rRNAs (Woese *et al.*, 1984).

With the finding of a new hyperthermophilic archaeal organism, *Nanoarchaeum equitans*, a new archaeal kingdom was created. *N. equitans* was discovered by cultivating a new autotrophic sulfur-reducing species, *Ignococcus* (Huber *et al*., 2000), which was found to be covered by very tiny cocci, the *N. equitans*. These cells with a cell diameter of about 400 nm and a regular S-layer are loosely connected to *Ignococcus* creating a co-culture. It was not possible to characterize *N. equitans* based on commonly used ecological studies because of its unique DNA sequence which contains base exchanges even in high conserved regions. The very low sequence identities with Bacteria and some other features characteristic for Archaea gave rise to the assumption that this organisms are of archaeal nature but with a low accordance to the two archaeal kingdoms. Therefore they form a new kingdom, the *Nanoarchaeota* (Huber *et al.*, 2002).

With the help of rRNA analysis of environmental samples new lineages of mesophilic and even psychrophilic Archaea were identified. They were categorized in two groups, group I as a sister group of the *Crenarchaeota*, group II as part of the *Euryarchaeota* (DeLong, 1992; Fuhrman *et al*., 1992). Later on group I was fully added to the *Crenarchaeota* because of DNA polymerase sequence analysis, further rRNA analysis however showed that it forms a distinct lineage (Schleper *et al.*, 1997). This assumption was strengthened by the finding of eukaryotic like histone genes in *C. symbosium*, a member of group I, (L'ubomíra Cubonová *et al.*, 2005) as well as the absence of most of the hyperthermophilic specific proteins and the lack of the type I DNA topoisomerase (present in all known Archaea). Further ribosomal protein analysis confirmed the idea of *C. symbosium* and the other three group I members *Nitrosopumilus maritimus, Nitrososphaera viennensis and Nitrososphaera gargensis* as an own archaeal group, the kingdom *Thaumarchaeota* (Brochier-Armanet *et al*., 2008).

A fifth kingdom, the *Korarchaeota,* complete the archaeal domain. The fragmented chromosomal DNA of *Candidatus Korarchaeum cryptofilum* as a member of the *Korarchaeota* was characterized by shotgun sequencing and it was found to share features with *Euryarchaeota* and *Crenarchaeota*. *Korarchaeota* became an own kingdom because it seems to represent an ancestral archaeal form (Elkins *et al*., 2008).

1.6 Halophile- and alkalophile- Archaea

Hypersaline environments as the Dead Sea or as a consequence of evaporated seawaters exist all over the world and highly adapted microorganisms can be found in all of the three domains of life. The aerobic, heterotrophic archaeal family *Halobacteriaceae* of the kingdom *Euryarchaeota* is a great example of halophile organisms as they are highly present in such hypersaline environments. As there was no definition for the salt concentration that determines halophilic organisms, Donn Kushner distinguished following categories: organisms living in media with 2.5 to 5.2 M salt are extreme halophiles, moderate halophiles show an optimal growth in media with 0.5 to 2.5 M salt and halotolerant microorganisms don´t require salt for living but increase growth when exposed to high salt concentrations (Kushner, 1978). The biggest challenge for halophilic organisms is to withstand the osmotic pressure caused by the highly saline surrounding medium and maintain the cytoplasm at least isosmotic with the medium. The main mechanism preventing turgor is the exclusion of sodium ions from the cytoplasm and high concentrations of potassium ions within the cells, so called the "high-salt-in strate-

gy". In other words a high osmotic pressure in the cytoplasm has to be adhered to while the Na^+ concentration has to be kept low. In aerobic halophilic Archaea (of the order *Halobacteriales*) and anaerobic halophilic Bacteria (of the order *Haloanaerobiales*), this is accomplished by accumulation of KCl at concentrations at least as high as the NaCl concentration in the surrounding medium (Oren, 2002).

Another strategy, the "low-salt-in strategy" is to exclude salts from the cytoplasm while accumulating organic solutes as glycerol, amino acids or simple sugars like sucrose and trehalose to provide osmotic balance (Galinski, 1995). Common characterizations of the different organic solutes are: they exhibit a low molecular weight, are soluble at high concentrations in water and are either uncharged or zwitterionic at the physiological pH. The benefit of using such organic solutes is that it requires much less far-reaching adaptions of the intracellular enzymatic machinery than does the accumulation of KCl. On the other hand, the production of such solutes requires considerable of energy (Oren, 1999).

In cells that use the "high-salt-in strategy" an adaption of all enzymes and structural components of the cells to the presence of high salt concentrations can be observed. Such halophilic proteins contain a large excess of acidic amino acids and small amounts of hydrophobic amino acids (Lanyi, 1974) whereupon newer studies showed that there is an increase of small hydrophobic residues like glycine, alanine and valine, but a decrease of aliphatic residues (Madern *et al.*, 2000). It is known that the biological activity of these halophilic enzymes is dependent on the presence of salts. Under mesophilic conditions high salt concentrations destabilize the folded form of proteins and lead to the loss of activity, stability and solubility. It is not fully understood how halophilic proteins stay active, stabile and soluble in high salt concentrations as this behaviour is not dependent on one single feature but on an interaction of different ones. One is the shielding of negative charges of these proteins which leads to the protective effect of salt as in the absence of salt charge repulsing causes unfolding and loss of activity. Other protein stabilizing effects are the formation of hydrophobic bonds under high salt conditions and the interaction of cations with the highly negatively charged haloproteins (Lanyi, 1974). In cells using the compatible-solute strategy proteins show no adaptations to high salt concentrations as compatible solutes protect them but the underlying mechanism is not known yet (Brown, 1990).

Concerning the metabolism of halophilic organisms it may be mentioned that most microbial processes are the same as in organisms that live under low salt concentrations,

as it is the case for *e.g.* photosynthetic processes or dissimilatory processes which result in high energy production. However, there are dissimilatory processes that occur only in organisms living at low salt concentrations but not in organisms exposed to high salt concentrations, such as methanogenesis based on reduction of CO_2 with H_2 as electron donor, methanogenesis from acetate, oxidation of acetate by sulfate reducing bacteria and autotrophic nitrification. These are processes that result in low energy production which is not accordable with the production of organic solutes which is energetically expansive (Oren, 2002).

Alkaliphiles can be found in all three domains of life whereas a haloalkaliphilic lifestyle is reserved for Archaea. Alkaliphiles require a pH of at least 9 for their growth and have an optimal growth at a pH of 10. Haloalkaliphilic Archaea were isolated from extremely alkaline saline environments such as the western soda lakes of the United States or the Rift Valley lakes of East Africa and require an alkaline pH of at least 9 and additionally a high salinity. By studying the internal cytoplasmic pH of alkaliphilic organisms, it was discovered to be neutral which leads to the conclusion that there have to be features that maintain the internal neutral environment separate from the surrounding alkaline medium (Horikoshi, 1999). The key lies within the cell surface. The cell walls of alkaliphilic organisms contain peptidoglycan and additionally acidic polymers like galacturonic acid, gluconic acid, glutamic acid, aspartic acid and phosphoric acid (Aono & Horikoshi, 1983). Compared to the peptidoglycan of neutrophilic organisms the peptidoglycan composition of alkaliphilic organisms is very similar with the exception of an excess of hexosamines and amino acids in the cell walls (Horikoshi, 1999). In contrast to neutrophilic organisms alkaliphilic organisms require Na^+ for growth (Kurono *et al*., 1973). This is explained by regarding the energy production via the proton motive force. Usually it is generated by the electron transport chain or by excreted H^+ which is derived from ATP metabolism by the ATPase. The reincorporation of H^+ into the cells occurs with the help of co-transport of various substrates. In alkaliphilic organisms the proton motive force is displaced by the sodium motive force whereupon H^+ is exchanged with Na^+ by Na^+/H^+ antiporter systems. The pH value at the cell surface is reduced by the acidic polymers of the cell walls which function as a negatively charged matrix. Also the pH of the surface of the plasma membrane has to be kept below pH 9 because of its instability at pH´s above. The pH homeostasis is maintained by using the Na+/H+ antiporter system, the K^+/H^+ antiporter and ATPase–driven H^+ expulsion (Horikoshi, 1999). The first alkaline enzyme, an alkaline protease, was discovered 1971 and more alkaline enzymes were described later on. The extracellular alkaline serine protease was isolated from the alkaliphilic *Bacillus* sp. strain 221 and was found to have an

optimal pH of 11.5. More alkaline enzymes were analyzed like amylasen, cellulasen or lipasen.

1.7 *Natrialba magadii*

As mentioned before the family of the *Halobacteriaceae* belongs to the kingdom *Euryarchaeota* and is composed of 40 genera known to data: *Haladaptatus, Halalkalicoccus, Halarchaeum, Haloarchaeobius, Haloarcula, Halobacterium, Halobaculum, Halobellus, Halobiforma, Halococcus, Haloferax, Halogeometricum, Halogranum, Halolamina, Halomarina, Halomicrobium, Halonotius, Halopelagius, Halopenitus, Halopiger, Haloplanus, Haloquadratum, Halorhabdus, Halorientalis, Halorubrum, Halosarcina, Halosimplex, Halostagnicola, Haloterrigena, Halovenus, Halovivax, Natrialba, Natrinema, Natronoarchaeum, Natronobacterium, Natronococcus, Natronolimnobius, Natronomonas, Natronorubrum, Salarchaeum* (List of Prokaryotic names with Standing in Nomenclature).

The species *Natrialba magadii* was first classified as a member of the genera *Natronobacterium* from Tindall in 1984 (Tindall *et al*., 1984). Further 16S rRNA analysis however showed that it belongs to the genus *Natrialba* which was introduced by Kamekura and Dyall-Smith in 1996 (Kamekura & Dyall-Smith, 1996; Kamekura *et al*., 1997). The cells have a length of 0.5 to 0.7 mm and an optimal growth at 4 M sodium chloride where upon a salt concentration of 2 M is absolutely required as lysis occurs at a value below. To cultivate *Nab. magadii*, the medium has to show low concentrations of Mg^+, at most 10 mM. The optimal growth temperature is at 37°C to 42°C (Tindall *et al.*, 1984), one generation cycle takes about nine hours in the logarithmic phase. Lake magadi as other hypersaline lakes have a red coloration due to the carotenoid pigments (e.g. α-bacterioruberin) which are incorporated into the membranes of the members of *Halobacteriales*. As discussed above halophile organisms use the „high-salt-in strategy“ or the „low-salt-in strategy“ to deal with the surrounding high salt concentrations. *Nab. magadii* combines both strategies as an organic osmolyte, 2-sulfotrehalose, has been detected as well as high KCl concentrations (Oren, 2002).

Spontaneously lysis of *Nab. magadii* gave rise to the assumption that it is infected by a virus. This could be verified by the isolation and analysis of a prophage termed ϕCh1. A non-lysogenic strain, L13, could be found and isolated by repeated subculturing. This strain is first cured from the virus and can then be infected with ϕCh1 resulting in lyso-

genization and lysis. The result of lysis can be observed as plaque formation and a colony which produces phages can be isolated from a single plaque resulting in an infected strain, named L11. This strain contains the ϕCh1 DNA integrated into the chromosome of *Nab. magadii* till lysis occurs whereas L13 serves as an indicator strain (figure 2).

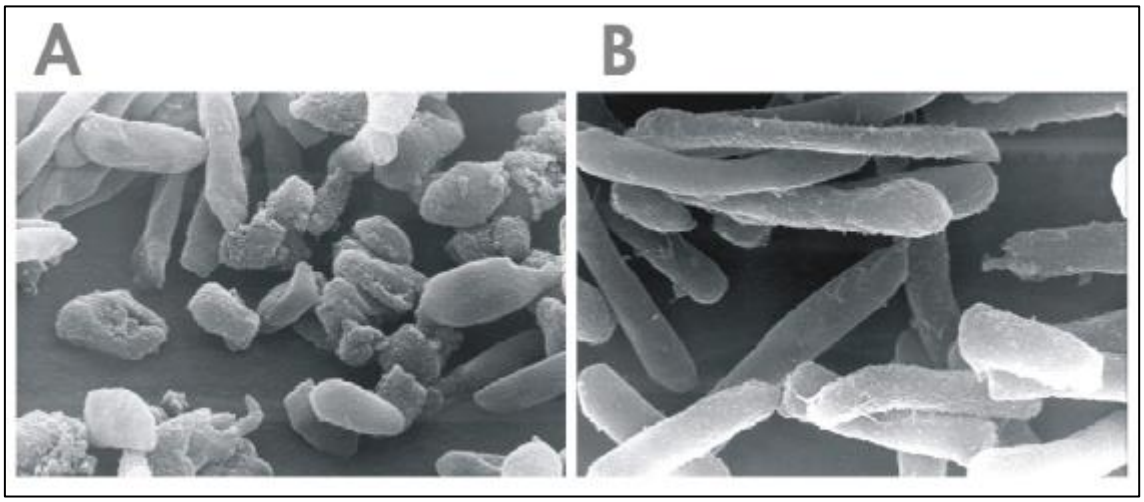

Fig. 2 Elektron micrographs of *Nab. magadii*. A): strain L11 carrying the prophage ϕCh1; B): indicator strain L13 cured from ϕCh1.

1.8 Transformation of *Nab. magadii*

The first transformation system for Archaea was introduced by Cline and Doolittle in *Halobacterium halobium*. This halophilic archaeon is known to be infected by virus ϕH. As there were no genetic markers available the ϕH DNA was used for transformation. Therefore the archaeal cells were treated with EDTA to remove the paracrystalline glycoprotein surface layer (S-layer) leading to a change of the morphology of the cells from rod-shaped to spherical, called spheroblasts. Transfection with the ϕH DNA then was performed by using polyethylene glycol 600 (PEG 600). With this method, not just *Hbt. halobium* but also other halophiles as *Halobacterium salinarum* and *Haloferax volcanii* could be transformed with the ϕH DNA (Cline & Doolittle, 1987). As it is known that the halovirus ϕCh1 infects *Nab. magadii* the polyethylene glycol-mediated transformation was used to introduce ϕCh1 into *Nab. magadii* strain L13 which is cured from the virus, thus a successful transformation should lead to plaque formation. However, neither spehroblasts nor plaque formation could be observed (Mayrhofer-Iro *et al*.,

2013). Another method to remove the S layer *of Hbt. salinarum* is the treatment of the cells with the antibiotic bacitracin as it interferes with the glycosylation of the S-layer, resulting in spheroblast formation (Wieland *et al*., 1981). Used for the removal of the S-layer of *Nab. magadii* no spheroblast formation could be achieved but cells with pleiotrophic morphology (Mayrhofer-Iro *et al*., 2013). This effect is due to the lower stability of bacitracin in alkaline media (Pavli & Kmetec, 2006). Another finding was the spheroblast formation of *Hbt. salinarum* by treatment of the cells with insoluble protease which removes the S-layer proteins (Strominger, 1976). This method was used for the transformation of *Nab. magadii* leading to spheroblast formation as well as a successful transfection with the ϕCh1 DNA, but with a low transformation rate of 10^3 PFU per microgram ϕCh1 DNA (compared to the transformation rate of *Hbt. salinarum*). However, the combination of treatment with bacitracin and proteinase K led to a high value of spheroblasts and a satisfying transformation rate of 10^4 PFU/µg DNA.

Additionally a shuttle vector system for *E. coli*/*Nab. magadii* was developed. First the *gyrB* gene of the halophilic Archaea *Haloferax alicantei*, which is responsible for a novobiocin resistance (Holmes *et al*., 1991) was cloned into the vector pKS_{II}^+ resulting in pNov-1 (Mayrhofer-Iro *et al*., 2013). pKS_{II}^+ is a vector with an origin of replication active in *E. coli* and additionally contains an ampicillin resistance as a selective marker. Sequence similarity analysis showed that there is an open reading frame within the ϕCh1 DNA, ORF54, which is related to *repH* (Klein *et al*., 2002), a gene that is required for the minimal replication origin of halophile plasmids (Wai-Leung & DasSarma, 1993). Also the ORF53, an open reading frame located upstream of ORF54 shows similarity to *repH* with a lower value as for ORF54. Subsequently genes ORF53 and ORF54 were added to pNov-1, resulting in vectors capable of replication and transformation with acceptable rates: pRo-3, pRo-5, pRo-7 and pRo-10. pRo-5 is now used exclusively, as it shows the highest transformation rate (Mayrhofer-Iro *et al*., 2013). In 2004 a novel shuttle vector was introduced, the pNB102. It is an expansion of pNB101, the first plasmid that was isolated from *Natronobacterium*, an haloalkaliphilic Archaeon. With the insertion of the ColE1 replicon the replication of the vector is ensured, the resistance against ampicillin and mevinolin serves as selective marker in both, *E.coli* and Haloarchaea.

1.9 *Natronomonas pharaonis*

Natronomonas pharaonis was isolated from saline soda lakes in Kenya and Egypt (Tindall *et al*., 1984; Soliman *et al.*, 1982). It is an extreme haloalkaliphilic Archaeon and thus requires a surrounding medium with high concentrations of salt, at least 2 M NaCl whereupon optimal growth occurs at 3.5 M NaCl. As it is also an alkaliphilic organism a pH of 8.5 to 9.5 is required but survival is warranted up to pH 11. Furthermore *N. pharaonis* has a very low tolerance against magnesium (Staley *et al*., 1989). Genome analysis including proteomic data showed that *N. pharaonis* has one of the densest coverage whereupon 60% of the cytosolic proteins are involved in metabolism and genetic information processing (Konstantinidis & Tebbe, 2007).

1.10 *Haloferax volcanii*

Haloferax volcanii was the first organism discovered in the Dead Sea. Till then it was believed that no life could be possible in such extremely salty environments. Cultivated in media, *H. volcanii* requires at least 2.6 M NaCl but it was found to grow in media made up with Dead Sea water which just contains 1.7 M NaCl but additionally high amounts of $MgCl_2$. The reason for growth at 1.7 M in Dead Sea water was thought to be the replacement of Na^+ with either Mg^{2+} or Cl^{2+} but studies showed that this is not entirely true. *H. vocanii* has the ability to replace NaCl with $MgCl_2$ but just in low amounts. A full replacement could not be observed but partially replacing supports normal growth. Later on it was discovered that *H. volcanii* grows also in media with 1.7 M NaCl when the temperature was increased to 30°C, which is equal to the Dead Seas temperature (Mullakhanbhai & Larsen, 1975).

1.11 *Natronobacterium gregoryi*

The alkaliphilic members of the *Halobacteriaceae* were separated by Tindall into two genera, *Natronobacterium* and *Natronococcus* (Tindall *et al*., 1984). The genera *Natronobacterium* contained amongst others *Nbt. magadii* (later it was classified to the genera *Natrialba*) as well as *Nbt. gregoryi* which represents the type species within the *Natronobacteria*. 16S rRNA analysis of *Nbt. magadii* and *Nbt. gregoryi* showed a 91.1% similarity within the rRNAs of the two organisms (Kamekura *et al*., 1997). However, this was no evidence for a close relationship between them as Devereux *et al.*

and Fry *et al.* proposed that only a 16S rRNA similarity above 93% indicates that two organisms belong to the same genera (Devereux *et al*., 1990; Fry *et al*., 1991). By reconstructing phylogenetic trees, Kamekura clearly separated *Nbt. gregoryi* and *Nbt. magadii* from each other (Kamekura *et al*., 1997). As *Nbt. gregoryi* is almost unexplored until now there is very few information about the live style and habituality of this organism.

1.12 Archaeal viruses

1974 Torsvik and Dundas discovered the first archaeal virus, infecting the Haloarchaeon *Hbt. salinarium.* As well as the few following viruses found in Archaea, it has an icohasedral head with a contractile helical tail and a double-stranded DNA genome. Because of the head-tail morphology of the until then discovered viruses, they were thought to be a group of the head-tail bacteriophages (Torsvik &Dundas, 1974). This erroneous classification was corrected by the finding that most of the archaeal viruses do not have a head-tail phenotype (Rohrmann, 1983; Dyall-Smith, 2003). Quite on the contrary, most of the members have a range of morphotypes which until then had not been observed for dsDNA viruses. Genome sequence analysis showed that the archaeal viruses are not related to other known viruses, thus must have evolved independently (Prangishvili, 2003). A phenotype that is exclusive for archaeal viruses is the fusiform including single or two-tailed viruses. This group of viruses is classified into the *Fuselloviridae* family, the *Bicaudaviridae* family and the genus *Salterprovirus* (Prangishvili *et al*., 2006) whereat most of the members carry a circular genome and genes encoding an integrase for the integration into the host chromosome (Muskhelishvili *et al.*, 1993).

Members of the *Fuselloviridae* family are known to infect the hyperthermophilic Crenarchaeon *Sulfolobus*. The *Sulfolobus* virus SSV1 *e.g*. has a positively supercoiled circular genome (Nadal *et al*., 1986) that integrates into a tRNA gene of the host chromosome. While expressing an integrase the tRNA gene of the host stays intact (Muskhelishvili *et al*., 1993), and the host cell does not undergo lysis while replication and release of the viruses takes place (Martin *et al*., 1984). The only known member of the *Bicaudaviridae* family and also the only known virus infecting acidophilic, hyperthermophilic Archaea is the Acidianus two-tailed virus (ATV). It is unique in the world of viruses because it is released from the host cell as a tail-less, fusiform particle. Tail formation then takes place at temperatures above 75°C, the temperature of the natural habit of the host cells. It is

still unclear how the tails can develop independently from the host or from any energy source (Haring *et al*., 2005; Prangishvili *et al.*, 2006). His1 and His2 are two distantly related viruses representing the *Salterprovirusgenus.* They infect strains of the extremely halophilic genus *Haloarcula* of the *Euryarchaeota*, are lytic and have linear genomes which show no similarities to the other fusiform viruses. A characteristic feature is the expression of a DNA polymerase (Bath & Dyall-Smith, 1998; Bath *et al*., 2006). Two more families were introduced, both consisting of just one virus because of their unique morphological features. The *Acidianus* bottle-shaped virus (ABV) has been assigned to the family *Ampullaviridae* and infects the hyperthermophilic genus *Acidianus*. In contrast from all other viruses, it has a supercoiled nucleoprotein filament that forms the cone-shaped core which is encased by the envelope. Additionally it forms short thick filaments attached to a disk at the broader end yet with unknown function (Haring *et al*., 2005). The only member of the family *Guttaviridae* is the *Sulfolobus neozealandicus* droplet-shaped virus (SNDV) which infects the hyperthermophilic genus *Sulfolobus*. It has multiple long, thin fibres that are attached at its apex with a helically ribbed surface (Arnold *et al*., 2000). Archaeal linear viruses are classified into two new families because of their dsDNA genome that was never observed in linear viruses. They have in common the lack of an integrase gene thus are thought to persist stably in host cells. The members of the *Rudiviridae* family have copies of a single, glycosylated, basic DNA-binding protein and a tube-like superhelix. The tube-like structure is associated with plugs carrying short tail fibres (Prangishvili *et al*., 1999). The rudivirus *Sulfolobus islandicus* rod-shaped virus 1 (SIRV1) *e.g.* encodes a Holiday junction resolvase probably needed for the dissolution of the head-to-head and tail-to-tail linked replicative intermediates (Birkenbihl *et al*., 2001). The *Lipothrixviridae* family consists of flexible, filamentous linear viruses that possess envelopes with lipids acquired from the host. As the members use different mechanisms for replication and differ in structure, the *Lipothrixviridae* family is subdivided into four genera. The last group of archaeal viruses comprises the head-tail viruses that are relatively rare in the world of archaeal viruses. They have icosahedral heads, helical tails and fail an envelope. They infect methanogens that are either mesophilic or moderately thermophilic as well as extreme thermophiles and thus are associated with the kingdom *Euryarchaeota*. Until now sixteen head-tail viruses were discovered and assigned to the dsDNA bacteriophage families *Myoviridae* and *Siphoviridae*. The most representable viruses of the head-tail viruses are the Haloarchaea-infecting phages ϕH and his closely relative ϕCh1 as well as HF1 and HF2 (Dyall-Smith, 2003; Prangishvili *et al*., 2006).

1.12.1 Haloviruses

In view of the number of genera of Halobacteria and their species, it is surprising that just a small number of haloviruses have been reported and analyzed. This is intensified by the finding of virus-like particles in high concentrations (about 10^7 per ml) in the Dead Sea (A Oren *et al*., 1997). An interesting couple of head-tail viruses are HF1 and HF2 as they are, in contrast to most of the other members of the haloviruses, lytic and not lysogenic and because of their extraordinary genome composition. They are able to infect different Haloarchaea like *Haloferax, Halobacterium, Haloarcula, Natrialba* and *Halorubrum* and were isolated from the same lake. Studies showed that there are sequence similarities between HF1 and HF2 of at least 80% which was surprisingly because they are infecting different hosts. By comparing the genome of HF1 and HF2 it is conspicuous that the first 60% of the genome are identically but the rest differs (87% similarity) because of base exchanges, deletions and insertions. This could be explained by a recent recombination event between one of the two HF viruses and a third HF like particle. The part of the sequence which is not identical could also be the reason for the infection of different hosts (Dyall-Smith *et al*., 2003). Furthermore, sequence similarity analysis showed that there are just a few features HF1 and HF2 have in common with other phages or organisms like affiliations from some Haloarchaea, Bacteria and bacteriophages. It seems that the genome is a mosaic of different genetic sections from different sources (Tang *et al*. 2002). Such mosaics are common for bacteriophages but were never observed for haloviruses.

The best studied, although only about 60% sequenced, is the genome of the virus ϕH. ϕH, which infects *Hbt. Salinarum* is a lysogenic virus with a icosahedral head, a contractile tail and a dsDNA of 59 kbp (Schnabel *et al*., 1982). Sequence similarity analysis showed that there are little similarities with Bacteria, bacteriophages (a few methylase genes) and eukaryotic viruses but the morphology, features of replication and lysogeny resemble those of the bacteriophage P1 (Dyall-Smith *et al*., 2003). ϕH requires a high concentration of salt for maintenance (3 M KCl or NaCl) and the lytic as well as the lysogenic cycle is controlled by an antisense-RNA-mechanism (Stolt & Zillig, 1992). In the host cell ϕH is present as a prophage. Its dsDNA is circular and contains an invertible DNA segment, the L fragment. It was found to have the ability to circulate and has lost most of the phage DNA. The R_1-L strain which carries just the L fragment does not produce phages and furthermore is immune against virus infection. The high frequency of inversion of the invertible L fragment leads to the variation of genome structures (Schnabel, 1984).

1.12.2 ϕCh1

The halophage ϕCh1 of the familiy *Myoviridae* was discovered by Witte in 1997. It infects the above described haloalkalophilic Archaeon *Nab. magadii* for which no phage infection was reported yet. It is a head-tail virus with a total length of about 200 nm including a head of about 70 nm and a tail of about 130 nm. The contractible tail covers an internal shift and has a width of about 20 nm. Additionally structures for adsorption of the host cell are located at the end of the tail. ϕCh1 requires at least 2 M NaCl for infection and was not found to infect other halophiles (e.g. *Natronobacterium pharaonis*, *Natronobacterium gregoryi* and *Natronococcus occultus*). Four major and five minor proteins of ϕCh1 were reported with isoelectric points from 3.3 to 5.2, which indicates that there is an excess of negative charges as it is the case for proteins of halophiles (Reistad, 1970; Lanyi, 1974). When the culture enters the stationary-growth phase viruses are released, which is a sign for a growth-phase dependent lysis. Virus particle formation can be observed after 5 h and viruses can be detected in the supernatant after 11 h. About 150 phage particles can be found per cell after lysis of cells, which happens after approximate 96 h. Surprisingly, analysis of the nucleic acid shows that ϕCh1 does not just contain a linear double-stranded DNA of approx. 55 kbp but presumably also a RNA fraction of about 700 to 800 nucleotides. Cleavage with RNase A of purified virus particles does not lead to degradation of the RNA indicating that it is located within the virus (Witte *et al*., 1997). As the finding of DNA and RNA packaged within a virus was never observed for any bacteriophage before (Reanny & Ackermann, 1982), further analyses were performed. In order to exclude a contamination of the phage suspension with archaeal ribosomal RNA, 16S rDNA from *Nab. magadii* was hybridized with the RNA found in the phage particles. No matches could be found leading to the assumption that the RNA fragment is not part of the ribosomes of *Nab. magadii*. Additionally antibodies against 50S and 30S ribosomal proteins gave no signal when exposed virus samples. Further RNA hybridization analyses were performed and signals occurred when hybridized with chromosomal DNA fragments of both *Nab*. *magadii* strains, L11 and L13, but there were just rare signals when hybridized with the DNA of ϕCh1. That indicates that the ϕCh1 RNA is host specific and is encoded by the chromosomal DNA of *Nab. magadii*. In contrast to the provirus ϕH whose DNA is present in the host cell as a plasmid (Schnabel *et al*., 1982), the ϕCh1 DNA is integrated into the host chromosome until onset of lysis. This could be demonstrated by hybridization experiments with the phage DNA and the chromosomal DNA of both *Nab. magadii* strains, L11 and L13. As expected hybridization of virus DNA with DNA of L11 was positive in contrast to the hybridization with DNA of L13, the ϕCh1 cured strain, which

did not lead to any signal. Thus the assumption of the provirus ϕCh1 as a temperate virus with his DNA integrated into the host chromosome was verified. Further restriction analysis showed that ϕCh1 contains completely Dam-methylated molecules and Dam-related sequences with additional methylated adenines but no modifications of cytosine residues. As the DNA of *Nab. magadii* is not Dam-methylated (Lodwick *et al*., 1986) it seems possible that ϕCh1 activates a silent, host-encoded methyltransferase or encodes its own DNA methylase (Witte *et al*., 1997).

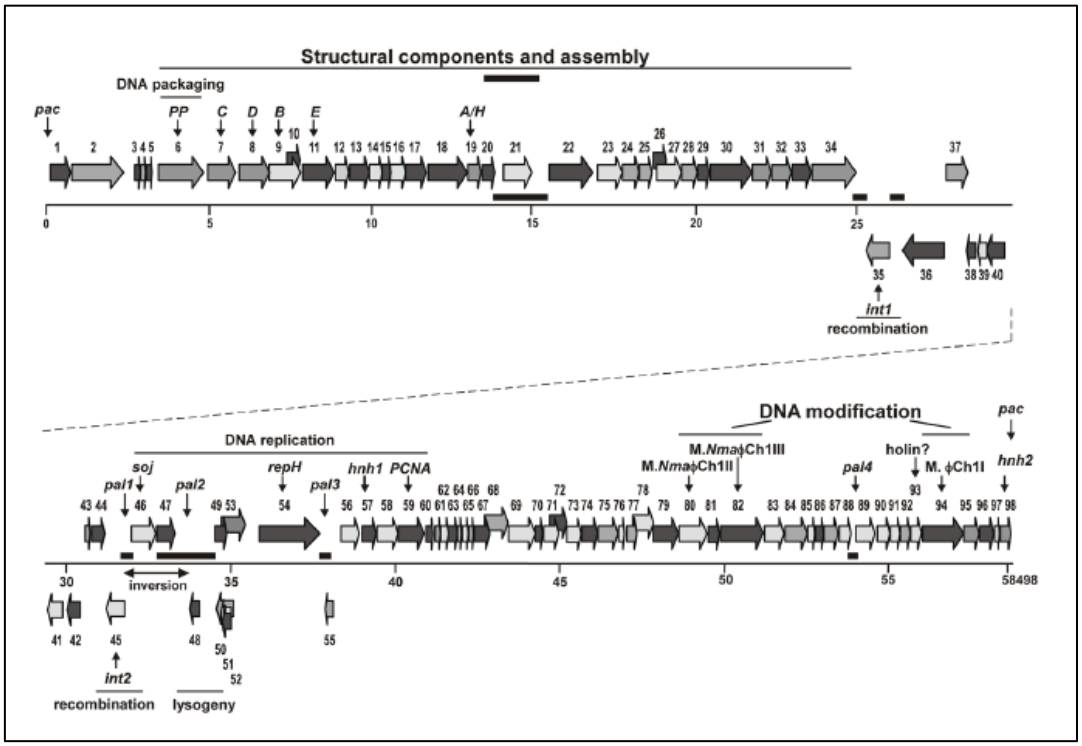

Fig. 3 Overall organization of the ϕCh1 genome. The arrows represent open reading frames, dark-grey arrows indicate the first reading frame, mid-grey the second reading frame and light-grey the third reading frame. The 58 498 bp genome with 98 ORFs can be divided into three regions: the left part harbors genes coding for structural proteins and genes putative involved in virion morphogenesis, the middle part consists of genes involved in replication, plasmid stabilization and regulation for gene expression and the right part comprises genes coding for DNA methylation and restriction and genes of unknown function (Klein *et al*., 2002).

The complete genome of ϕCh1 was sequenced in 2002. The 58 498 bp sequence has an overall G + C content of 61.9% whereupon two major low G + C (< 55%) regions were found. One of these two regions contains open reading frames whereas the other harbors a putative binding site for a site specific recombinase and putative cis-acting regulatory elements. By analyzing the open reading frames of the ϕCh1 genome sequence 570 potential ORFs were found, 98 seem to be genes. When compared to proteins in the databases, 48 of the 98 ORFs have been found to be similar to other se-

quences including 17 proteins similar to proteins of known function and 31 proteins similar to conserved proteins of unknown function. All together the ϕCh1 genome can be divided into three regions: the left part consists of rightward transcribed genes coding for structural proteins and genes putative involved in virion morphogenesis, the middle part contains a region of a mixture of leftward and rightward transcribed genes and a region involved in replication, plasmid stabilization and regulation for gene expression and the right part of the genome harbors rightward transcribed genes coding for DNA methylation, restriction and genes of unknown function (Figure 3). By comparing the genome of ϕCh1 to that of ϕH it is noticeable that the L-fragment of ϕH and the central part of ϕCh1 are highly similar with an extent of similarity between 50% and 97% (Klein *et al*., 2002). This seems remarkable as ϕH and ϕCh1 are infecting different organisms and inhabit different environments.

2 Material and Methods

2.1 Material

2.1.1 Strains

2.1.1.1 Bacterial strains

Strains	*Characteristics*	*Source*
E. coli XL1-Blue	*endA1, gyrA96, hsdR17 (r_k-m_{K+}), lac, recA1, relA1, supE44, thi, (F´, lacI^q, lacZΔM15, proAB$^+$, tet)*	*Stratagene*
E. coli Rosetta	*F-, ompT, hsdS$_B$ ($r_B^-m_B^-$), gal, dcm, lacY1, (DE3), pRARE$_6$, (Cm^R)*	*Novagen*
E. coli JM110	*rpsL (Str^r), thr, leu, thi-1, lacY, galK, galT, ara, tonA, tsx, dam, dcm, supE44, Δ (lac-proAB), [F, traD36 proAB, lacI^{q}Z, ΔM15]*	*Stratagene*

2.1.1.2 Archaeal strains

Strains	*Characteristics*	*Source*
Nab. magadii L11	*wild type, prophage of ΦCh1*	*Witte et al., 1997*
Nab. magadii L13	*cured of ΦCh1*	*Witte et al., 1997*
Nab. magadii P3	*NEP deficient derivate of L13*	*Derntl, 2009*
Nbt. gregoryi	*wild type*	*DSM 3393*

2.1.2 Media

2.1.2.1 LB (rich medium for *E. coli*)

Peptone	10.0 g
NaCl	5.0 g
Yeast Extract	5.0 g

pH 7.0

add ddH_20 to a final volume of 1 litre then autoclave

for plates add 15 g/l agar

2.1.2.2 **NVM** (rich medium for *Nab. magdii* and *Nbt. gregoryi*)

Casaminoacids	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 ddH_2O to a final volume of 935 ml then autoclave

for plates add 8 g/l agar

for soft agar plates add 4 g/l agar

After autoclaving, the medium has to be complemented by addition of:

0.57 M Na_2CO_3	65 ml
1 M $MgSO_4$	1 ml
20 mM $FeSO_4$	1 ml

2.1.2.3 **DSM medium 372** (rich medium for *Nab. asiatica*)

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 983 ml then autoclave

for plates add 10 g/l agar

After autoclaving, the medium has to be complemented by addition of:

1 M $MgSO_4$	8 ml
0.02 M $FeCl_2$	9 ml
0.02 M $MnCO_2$	90 µl

2.1.3 Antibiotics

2.1.3.1 Antibiotics for *E. coli*

Antibiotics	*Stock conc.*	*Final conc.*	*Comments*
Ampicillin	*20 mg/ml*	*100 µg/ml*	*in ddH_2O, filter sterile,*
Tetracyclin	*10 mg/ml*	*10 µg/ml*	*in 70% EtOH, store at -20°C*
Chloramphenicol	*40 mg/ml*	*20 µg/ml*	*in 96% EtOH, store at -20°C*

2.1.3.2 Antibiotics for *Nab. magadii*

Antibiotics	*Stock conc.*	*Final conc.*	*Comments*
Novobiocin	*3 mg/ml*	*3 µg/ml*	*in ddH_2O, filter sterile, store at -20°C*

Mevinolin	*10 mg/ml*	*7,5 µg/ml*	*in 96% EtOH, store at -20°C*
Bacitracin	*7 mg/ml*	*7 µg/ml*	*in ddH_2O, filter sterile, store at -4°C*

2.1.4 Plasmids

Plasmid	*Feature*	*Source*
pUC19	*bla, pMB1ori, lacZa, mcs*	*Yanisch-Perron et al.,1985*
pQE30	*bla, ColE1, N-terminal Poly(His)6-tag*	*Quiagen*
pKSII+	*mcs, bla, ColE1 ori, lacZa*	*Stratagene*
pRSET-A	*mcs, bla, EK, PT7, RBS, His-tag, pUC ori, f1 ori*	*Invitrogen*
pRo-5	*bla, ColE1 ori, gyrB (NovR), ΦCh1 derived ori*	*Mayrhofer-Iro et al., 2013*
pNB102	*bla, ColE1 ori, hmg (MevR), pNB101 ori*	*Zhou et al., 2004*
pBAD-24-tnaN	*bla, araC, rrnB, mcs, PBAD promoter, pBR322 ori, tryptophane promotor of Nab. magadii*	*Alte, 2012*
pRo-5-tnaN-int1-6xHis	*bla, ColE1 ori, gyrB (NovR),ΦCh1 derived ori, Nab. magadii promotor tnaN, ORF35, 6xHis*	*Lab Angela Witte, 2012*
pRo-5-ORF49	*bla, ColE1 ori, gyrB (NovR), ΦCh1 derived ori, ORF49*	*Meissner, 2008*
pRo-5-ORF49Δ1	*bla, ColE1 ori, gyrB (NovR), ΦCh1 derived ori, ORF49 Δ1 (nu. 34321-34795)*	*Reiter, 2010*
pRo-5-ORF49Δ2	*bla, ColE1 ori, gyrB (NovR), ΦCh1 derived ori, ORF49 Δ2 (nu. 34321-34760)*	*Reiter, 2010*

pRo-5-ORF49Δ3	*bla, ColE1 ori, gyrB (NovR), ΦCh1 derived ori, ORF49 Δ3 (nu. 34321-34723)*	*Reiter, 2010*
pRo-5-ORF49Δ4	*bla, ColE1 ori, gyrB (NovR), ΦCh1 derived ori, ORF49 Δ4 (nu. 34321-34686)*	*Reiter, 2010*
pRo-5-ORF49Δ5	*bla, ColE1 ori, gyrB (NovR), ΦCh1 derived ori, ORF49 Δ5 (nu. 34321-34650)*	*Reiter, 2010*
pRo5-ORF49Δ1 -ORF54	*bla, ColE1 ori, gyrB (NovR), ΦCh1 derived ori, ORF49 Δ1 (nu. 34321-34795), ORF54 (nu.36300-36481)*	*Lab Angela Witte, 2012*
pRo5-ORF49Δ2 -ORF54	*bla, ColE1 ori, gyrB (NovR), ΦCh1 derived ori, ORF49 Δ1 (nu. 34321-34760), ORF54 (nu. 36300-36481)*	*Lab Angela Witte, 2012*
pRo5-ORF49Δ3 -ORF54	*bla, ColE1 ori, gyrB (NovR), ΦCh1 derived ori, ORF49 Δ1 (nu. 34321-34723), ORF54 (nu. 36300-36481)*	*Lab Angela Witte, 2012*
pRo5-ORF49Δ4 -ORF54	*bla, ColE1 ori, gyrB (NovR), ΦCh1 derived ori, ORF49 Δ1 (nu. 34321-34686), ORF54 (nu. 36300-36481)*	*Lab Angela Witte, 2012*
pRo5-ORF49Δ5 -ORF54	*bla, ColE1 ori, gyrB (NovR), ΦCh1 derived ori, ORF49 Δ1 (nu. 34321-34650), ORF54 (nu. 36300-36481)*	*Lab Angela Witte, 2012*
pQE30-ORF34$_{52}$	*bla, ColE1, N-terminal Poly(His)6-tag, ORF34$_{52}$*	*Rössler et al., 2004*
pQE30-p_79	*bla, ColE1, N-terminal Poly(His)6-tag, ORF79 with extended promotor region (nu. 46106-48460)*	*this thesis*
pRo-5-p_79	*bla, ColE1 ori, gyrB (NovR),ΦCh1 derived ori, ORF79 with extended promotor region (nu. 46106-48460)*	*this thesis*
pNB102-p_79	*bla, ColE1 ori, hmg (MevR),pNB101 ori, ORF79 with extended promotor region (nu. 46106-48460)*	*this thesis*
pQE30-p_79-1	*bla, ColE1, N-terminal Poly(His)6-tag, ORF79 with extended promotor region (nu. 46106-48460), deletion of*	*this thesis*

	nu. 46791-47081	
pRo-5-p_79-1	*bla, ColE1 ori, gyrB (NovR), ΦCh1 derived ori, ORF79 with extended promotor region (nu. 46106-48460), deletion of nu. 46791-47081*	*this thesis*
pNB102-p_79-1	*bla, ColE1 ori, hmg (MevR), pNB101 ori, ORF79 with extended promotor region (nu. 46106-48460), deletion of nu. 46791-47081*	*Lab Angela Witte, 2012*
pQE30-p_79-2	*bla, ColE1, N-terminal Poly(His)6-tag, ORF79 with extended promotor region (nu. 46106-48460), deletion of nu. 46752-47316*	*this thesis*
pRo-5-p_79-2	*bla, ColE1 ori, gyrB (NovR),ΦCh1 derived ori, ORF79 with extended promotor region (nu. 46106-48460), deletion of nu. 46752-47316*	*this thesis*
pNB102-p_79-2	*bla, ColE1 ori, hmg (MevR), pNB101 ori, ORF79 with extended promotor region (nu. 46106-48460), deletion of nu. 46752-47316*	*Lab Angela Witte, 2012*
pNB102-p_79-3	*bla, ColE1 ori, hmg (MevR), pNB101 ori, ORF79 (nu. 47106-48460)*	*Lab Angela Witte, 2012*
pRo-5-p_79-3	*bla, ColE1 ori, gyrB (NovR), ΦCh1 derived ori, ORF79 (nu. 47106-48460)*	*Lab Angela Witte, 2012*
pNB102-p_79-4	*bla, ColE1 ori, hmg (MevR), pNB101 ori, ORF79 (nu. 47158-48460)*	*Lab Angela Witte, 2012*
pRo-5-p_79-4	*bla, ColE1 ori, gyrB (NovR), ΦCh1 derived ori, ORF79 (nu. 47158-48460)*	*Lab Angela Witte, 2012*
pNB102-p49-ORF79	*bla, ColE1 ori, hmg (MevR), pNB101 ori, ORF49 promotor, ORF79*	*Alte, 2012*

2.1.5 Primer

Primer	*Sequence*
79-XH	GAC GTC TAG AAG CTT GAC GTC ATC AAC CTG CTA AAC C
79-Kpn	GAC GGG TAC CTC AAG CAT CGG TCG AGT CA
49-Kpn	CAG CGG TAC CTT GCG TTC AGT TCC G
54-CC-3	GAC CAA GCT TCA CAG GTC TGC GGC GAT
79-XH-2	GACGTCTAGAAGCTTACTGCGGTTCTGACTGCC
79-XH-3	GACGTCTAGAAGCTTCGGTCGGCACCTGCAT
79-Pst	GATACTGCAGCTCTTTGTACCGATGCGTC
36-3	CAG CAG AAG CTT ATT CAG GTT TCA TGT CGC TG
34-XbaI	CAG CAG TCT AGA CGG CGT TCG AGG TCA
NB-1	TCTACCGGGTGCTGAACG
NB-2	CGCTGATGTACGAACCGAG
79-Nae	GATAGCCGGCGACTCTCACAAGATCTC
34-Kpn	CAG CAG GGT ACC CGG CGT TCG AGG TCA

2.1.6 Ladder

2.1.6.1 DNA ladder

DNA- ladder	*Fragment*	*Source*
λ BstEII	*8453, 7242, 6369, 5687, 4822, 4324, 3675, 2323, 1929, 1371, 1264, 702 [bp]*	*λ DNA from Ferm digested with Eco91I (Fermentas)*
λ PstI	*11501, 5077, 4749, 2838, 2556, 2443, 2140, 1986, 1700, 1159, 1093, 805, 514, 468, 448, 339, 264, 247, 216, 211, 200, 164, 150, 94, 87, 72, 15 [bp]*	*λ DNA from Fermentas, digested with PstI (Fermentas)*
pUC19/HaeIII	*587, 458, 434, 298, 257, 174, 102, 80, 18, 11 [bp]*	*puc19 plasmid DNA, digested with BsuRI (Fermentas)*
pUC19/Sau3AI	*955, 585, 341, 258, 141, 105, 78, 46, 36, 18, 12 [bp]*	*puc19 plasmid DNA, digested with Sau3AI (New England BioLabs)*

2.1.6.2 Protein ladder

Protein ladder	*Fragment*	*Source*
Unstained Protein Molekular Weigth Marker	*166, 66, 45, 35, 25, 18.4, 14.4 [kDa]*	*Fermentas #SM0431*

2.1.7 Enzymes

2.1.7.1 PCR

Enzymes	*Company*	*Comments*
Pwo	*PeqLab*	*polymerase reactions were performed as recommended by PeqLab*
Pfu	*Promega*	*polymerase reactions were performed as recommended by Promega*
GoTaq	*Promega*	*polymerase reactions were Performed as recommended by Promega*

2.1.7.2 Restriction

Enzymes	*Company*	*Comments*
Diverse	*Fermentas*	*digests were performed as recommended by Fermentas*
Diverse	*New England BioLabs*	*digests were performed as recommended by New England BioLabs*

2.1.7.3 Other

Enzymes	*Company*	*Comments*
T4 DNA Ligase	*Fermentas*	*ligation was performed as recommended by Fermentas*
T4 DNA Polymerase	*Fermentas*	*reaction was performed as recommended by Fermentas*
Klenow Fragment	*Fermentas*	*reaction was performed as recommended by Fermentas*
Proteinase K	*Roche*	*reaction was performed as recommended by Rocher*

2.1.8 KITs

Product	*Company*	*Comment*
QIA® Gel Extraction Kit	*Qiagen*	*DNA purification, PB and PE buffer*
QIA® Gel Extraction Kit	*Qiagen*	*DNA purification – elution from gel, PE and Elution buffer*
Gene JETTM Plasmid Miniprep KIT	*Fermentas*	*Miniprep*
Super Signal® West Pico Chemiluminescent Substrate	*Thermo Scientific*	*Western Blot – development of the blot*

2.1.9 Solutions and Buffer

2.1.9.1 Electrophoresis

50x TAE

Tris-HCL	2 M
Acetic acid	1 M
EDTA, pH8.2	0.1 M

10x TBE

Tris- base	108 g
Boric acid	55 g
EDTA	0.5 M

Adjust pH 8.0 with Boric acid

30% PAA solution

Acrylamide	29%
N, N´- Bisacrylamide	1%

6% PAA gel

30% AA solution	1.2 ml
1x TBE	4.8 ml
10% APS	60 µl
TEMED	6 µl

5x DNA loading dye

Tris-HCL pH 8.0	50 mM
SDS	0.1%
Bromophenol blue	0.05%
Xylene blue	0.05%

After autoclaving add 25% sucrose

2.1.9.2 SDS-PAGE and Western blot analysis

10x TBS (-T)

Tris-HCL	250 mM
NaCl	1.37 M
KCl	27 mM

Add 0.05% Tween20 for TBS-T

SDS running buffer

Tris- base	0.25 M
Glycine	1.92 M
SDS	1%

4x Separation gel buffer

Tris-HCL, pH 8.8	1.5 M
SDS	0.4%

4x Stacking gel buffer

Tris-HCL, pH 6.8	0.5 M
SDS	0.4%

2x Protein sample buffer (Lämmli)

Tris-HCL, pH 6.8	0.12 mM
SDS	4%
Glycerol	17.4%
ß- mercaptoethanol	2%
Bromphenol bule	0.2%

5 mM Sodium phosphate buffer (pH 6.8)

A) NaH_2PO_4	0.2 M
B) Na_2HPO_4	0.2 M

2.55 ml of solution A) and 2.45 ml of solution B) are mixed and filled up to 200 ml with H_2O

Coomassie staining solution

Methanol	25%
Acetic acid	10%
Coomassie Brillant (Blue R-250)	0.15%

Coomassie destaining solution

Methanol	25%
Acetic acid	10%

Transblot buffer		*Ponceau S solution*	
Tris- base	48 mM	Ponceau S	0.5%
Glycine	39 mM	TCA	3%
SDS	0.037%		
Methanol	20%		

2.1.9.3 Protein purification (denaturing)

Buffer B (Lysis buffer)		*Buffer C (Washing buffer)*	
NaH_2PO_4	100 mM	NaH_2PO_4	100 mM
Tris- HCL	10 mM	Tris- HCL	10 mM
Urea	8 M	Urea	8 M
pH 8.0		pH 6.3	

Buffer E (Elution buffer)		*Dialysis buffer 1*	
NaH_2PO_4	100 mM	Urea	4 M
Tris- HCL	10 Mm	NaCl	2 M
Urea	8 M	Tris-base	50 mM
pH 4.5		pH 9.5	

Dialysis buffer 2	
NaCl	4 M
Tris- base	50 mM
pH 9.5	

2.1.9.4 Transformation of *E. coli* – competent cells

MOPS I		*MOPS II*	
MOPS	100 mM	MOPS	100 mM
$CaCl_2$	10 mM	$CaCl_2$	70 mM
$RbCl_2$	10 Mm	$RbCl_2$	10 mM
pH 7.0 (KOH)		pH 6.5 (KOH)	

MOPS IIa

MOPS	100 mM
$CaCl_2$	70 mM
$RbCl_2$	10 mM
Glycerol	15%
pH 6.5 (KOH)	

2.1.9.5 Transformation of *Nab. magadii* – competent cells

Buffered high salt spheroblasting sol.		*Unbuffered high salt spheroblasting sol.*	
NaCl	2 M	NaCl	2 M
KCL	27 mM	KCl	27 mM
Tris-HCL, pH 8.0	50 mM	Sucrose	15%
Sucrose	15%		
(Glycerol)	15%		

2.1.9.6 Isolation of chromosomal DNA of *Nab. magadii*

2.1.9.7 Plasmid preparation from *Nab. magadii*

Resuspension solution (solution I)		*Lysis solution (solution II)*	
NaCl	2 M	SDS	1%
		NaOH	0.2 M

Precipitation solution (solution III)	
K-Acetate	5 M

2.1.9.8 Phage isolation

CsCl solution 1.1		*CsCl solution 1.3*	
NaCl	2 M	NaCl	2 M
Tris-HCL, pH 8.5	50 mM	Tris-HCL, pH 8.5	50 mM
CsCl	0.6 M	CsCl	2.7 M

CsCl solution 1.5		*High salt alkaline solution*	
NaCl	2 M	NaCl	4 M
Tris-HCL, pH 8.5	50 mM	Tris-HCL, pH 9.5	50 mM
CsCl	4 M		

2.2 Methods

2.2.1 DNA electrophoresis

DNA electrophorese was performed to separate, define and visualize DNA fragments accordingly to their size. Fragments with more than 700 bp were observed on a 0.8% agarose gel. Small fragments with less than 700 bp were separated on a 6% polyacrylamide gel.

2.2.1.1 Agarose gel electrophorese

The agarose was melted in 1 x TAE running buffer by heating up till the solution was clear. When cooled down but still liquid the gel was casted in sealed trays equipped with combs which served as loading pockets for the DNA samples. After solidification the gel was removed to a corresponding gel electrophoresis apparatus and covered with 1 x TAE. The DNA samples were mixed with 5 x loading dye and loaded onto the gel. The loading dye was additionally provided with bromphenol blue and xylene cyanol to estimate the location of the DNA. The fragments were separated in electric fields (10 V/cm) and then stained in an ethidium bromid bath with a concentration of 10 µg/ml approximately for 15 minutes. Visualization of the DNA occurred under UV light and a picture of the stained gel was taken using a GelDoc.

2.2.1.2 Polyacrylamide gel electrophorese

To cast 6% PAA gels the Mini – Protean® 3 system from Biorad was used and applied as suggested in the protocol. The components of the gel solution were mixed as described in 2.1.9.1 and poured between two vertical glass plates fixed in the casting system. The polymerized gels were transferred to a corresponding apparatus and covered with 1 x TBE running buffer. After removing the comb the DNA samples were mixed with 5 x loading dye, loaded on the gel and separated by a voltage of 20 mA/gel for 20 minutes.

2.2.2 Plasmid preparation from *E. coli*

Preparation (isolation) of Plasmid-DNA from *E .coli* was performed by using the GeneJET^{TM} Plasmid Miniprep KIT from Fermentas. The procedure was done accordantly to manufacturer`s instructions. Plasmids used for further cloning steps were isolated from 3 ml of an overnight culture and eluted with 50 µl of ddH_2O.

2.2.3 PCR

Polymerase chain reaction (PCR) is a method to amplify DNA fragments with known 3´ and 5´ ends. With the help of the program Genrunner from Hestings Software small DNA fragments homologue to the 3´ and 5´ end of the sequence of interest were designed. These 3´ and 5´ primer bind to the template DNA and serve as docking sites for the polymerase which elongates new DNA strands.

The lyophilized primer ordered and delivered from VBC genomics were diluted in ddH_2O to a final concentration of 100 µg/ml. The annealing temperature which depends on the G – C content of the primer was calculated by “Gene Runner” whereupon the melting temperature of the primer was estimated by subtraction 4°C from the annealing temperature.

Either purified plasmid DNA, chromosomal DNA and phage DNA were used as templates or haloarchaeal raw extracts for quick examinations of DNA samples. Therefore 20 µl of a culture was centrifuged and the pellet was dissolved in 100 µl ddH_2O.

The used polymerases varied in nature depending on the performance of a preparative or analytic PCR.

2.2.3.1 Preparative PCR

In the preparative PCR the amount of specific DNA sequences is amplified for further steps, e.g. cloning strategies. Therefore the recombinant polymerases *Pfu* or *Pwo* were used. Both have proofreading activity and thus low error rates.

Batch for preparative PCR (Pfu, Pwo), 100 µl		*preparative PCR program*	
68 µl	ddH_2O	95°	C5´
10 µl	2 mM dNTP	95°	C1´
10 µl	10 x *Pfu/Pwo* buffer	x°C*	1´
5 µl	forward primer	y°C*	z´ *
5 µl	reverse primer	y°C*	5´
1 µl	template DNA	4°C	infinite
1 µl	polymerase (*Pfu/Pwo*)		

* x: the annealing temperature was calculated based on the G – C content of the primer

y: temperature specific for highest activity of polymerase:

Pfu: 72°C, *Pwo*: 68°C

z: elongation time depends on the efficiency of the polymerase:

Pfu: 500 bp/min., *Pwo*: 100 bp/min.

2.2.3.2 Analytical PCR

To proof cloning constructs or verify the existence of specific DNA sequences in an organism/sample, the analytic PCR was performed. The quality of the PCR product is not as important as in the preparative PCR therefore a polymerase without proofreading activity was chosen, the *GoTaq*® polymerase.

Batch for analytic PCR (GoTaq®), 50 µl		*PCR program*	
28 µl	ddH_2O	95°	C5´
10 µl	5 x G*oTaq*® buffer	95°	C1´
5 µl	2 mM dNTP	x°C*	1´
2.5 µl	forward primer	y°C	z´ *
2.5 µl	reverse primer	y°C	5´
1 µl	template DNA	4°C	infinite
1 µl	*GoTaq*® polymerase		

* x: the annealing temperature was calculated based on the G – C content of the primer

y: temperature specific for highest activity of polymerase:

GoTaq®: 72°C

z: elongation time depends on the efficiency of the polymerase:

GoTaq®: 1000 bp/min.

2.2.4 Cloning

2.2.4.1 DNA purification

DNA purification was performed to purify DNA from a sample for e.g. further cloning steps or sequencing. Therefore certain buffers, enzymes and other components of previous reactions had to be removed. For the purification of DNA from a sample the QIAquick PCR purification kit (Qiagen) was used according to manufacturer´s instructions. To purify DNA e.g. from a PCR batch or for removal of a plasmid backbone after restriction a gel elution was performed by using the QIAquick Gel Extraction kit (Qiagen). In both cases the DNA was eluted in an appropriate amount of ddH_2O, depending on the final concentration desired.

2.2.4.2 DNA restriction

DNA restriction reactions were performed with restriction endonucleases and corresponding buffers from Fermentas and New England Biolabs. Digests were done at recommending temperatures for 3 hours or 1 hour for analytic analysis, respectively.

2.2.4.3 Further DNA modifications

Some cloning strategies require the restriction of DNA with different endonucleases resulting in blunt ends that can´t be ligated. Therefore the Klenow fragment (Fermentas) was used. By adding dNTPs (final concentration of 0.05 mM) the enzyme fills up the 3´ overhangs and creates sticky ends. Contrary the generation of sticky ends out of blunt ends was performed by using the T4 DNA polymerase from Fermentas which digests 3´ overhangs with the help of its exonuclease activity. Both reactions were done as recommended from Fermentas.

2.2.4.4 Ligation

Restricted DNA fragments were ligated by the T4 DNA ligase from Fermentas in order to generate novel plasmids. The reaction was performed with the recommended buffers and incubated either 3 hours at room temperature or overnight at 16°C.

Batch composition

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

2.2.4.5 Transformation of *E.coli*

2.2.4.5.1 Generation of $CaCl_2$ competent cells

For the generation of competent *E. coli* cells, 400 ml of LB medium with appropriate antibiotics was inoculated with an overnight culture resulting in an optic density (600 nm) of 0.1. The culture was incubated at 37°C till an $OD_{600\ nm}$ of 0.6, centrifuged at 4°C for 15 minutes at 6 krpm and the pellet was resuspended in 160 ml of the solution MOPS I (components described in 2.1.9.4). After incubation on ice for 10 minutes the cell solution was centrifuged at 4°C for 15 minutes at 6 krpm and the pellet was adjusted in 160 ml MOPS II. After another incubation on ice for 30 minutes followed by a centrifugation step at 4°C for 15 minutes at 6 krpm the pellet was dissolved in 8 ml of the solution MOPS IIa and samples of 100 µl were aliquoted and stored at –80°C.

2.2.4.5.2 Transformation

Either 1 – 2 µl of a purified DNA sample or the whole ligation batch was mixed with 100 µl of competent *E. coli* cells and incubated on ice for 30 minutes. A heat shock at 42°C for 2 minutes and a step on ice for a few seconds followed.

300 µl of LB medium without antibiotics was added and the cells were regenerated for 30 minutes on 37°C. 100 µl of the cells were plated in triplets on LB plates containing the corresponding antibiotics and inoculated at 37°C overnight. The next day 5 ml LB medium (with corresponding antibiotics) were inoculated with single colonies and incubated at 37°C overnight.

2.2.4.5.3 Screening and verifying positive clones

To screen for positively transformed clones 300 µl of the incubated cultures as well as a control culture carrying the empty vector DNA was centrifuged at 13 krpm and room temperature for 5 minutes and the pellets were resuspended in 30 µl 5 x loading dye. 14 µl of a phenol/chloroform solution (1:1) was added and after vortexing the final centrifugation step at 13 krpm and room temperature for 5 minutes occurred. 12 µl of the upper phase was loaded on a gel and the DNA was visualized under UV light. With the help of the control consisting of the empty vector DNA a comparison between the samples is possible. Plasmid DNA with insert is larger as without and thus appears higher

on the gel as the control. To finally verify the positive clones an analytic restriction or PCR was performed. Cultures of positive clones were mixed with 50% glycerol and stored at –80°C.

2.2.5 Cloning strategies

2.2.5.1 pQE30-p_79/pRo-5-p_79/pNB102-p_79

The ORF79 with additional 1033 nucleotides upstream of the putative promoter region was amplified by PCR using ΦCh1 DNA as template. 79-XH served as 3´ primer while 79- Kpn was used as 5´ primer. The out coming product with a length of 2380 bp was isolated, digested with *Xba*I and *Kpn*I and cloned into the vector pQE30. The resulting plasmid then was digested with *Xba*I and *Kpn*I to ligate it with the vector pNB102 as well as restriction was performed with *Hind*III and *Kpn*I for the cloning in pRo-5.

2.2.5.2 pQE30-p_79-1/pRo-5-p_79-1 and pQE30-p_79-2/pRo-5-p_79-2

To find out more about the exact position of the promoter region of ORF79 parts of the putative promoter sequence were excluded. This was done by digesting the plasmid pQE30-p_79 with *Nae*I and a following relegation (resulting in pQE30-p_79-1). The ORF79 with the deletion within the promoter region was then cloned into the vector pRo-5 by performing digests with *Hind*III and *Kpn*I (resulting in pRo-5-p_79-1). The same process emanate from pQE30-p_79 was repeated with the restriction enzyme ClaI instead of *Nae*I resulting in the plasmids pQE30-p_79-2 and pRo-5-p_79-2 to exclude another part of the putative promoter region.

2.2.6 Methods for Archaea

2.2.6.1 Transformation of *Nab. magadii*

2.2.6.1.1 Generation of competent cells

60 ml of NVM+ medium was inoculated with an overnight culture of *Nab. magadii* and bacitracin (final concentration: 70 µg/ml) was added. The cultures were incubated

overnight at 37°C till an $OD_{600\ nm}$ of 0.5 – 0.6 was measured. After centrifugation at 6 krpm for 15 minutes at room temperature the pellet was resuspended to half volume (30 ml) of buffered high salt spheroblasting solution (with glycerol). 30 µl of proteinase K (final concentration: 20 µg/ml) was added and the cultures were incubated at 42°C until the cells have lost their S-layer resulting in the formation of spheroblasts which can be inspected by microscopy.

2.2.6.1.2 Transformation

For the transformation of *Nab. magadii,* 1.5 ml of the spheroblast cells were centrifuged at 10 krpm for 3 minutes at room temperature and the pellet was resuspended in 150 µl buffered high salt spheroblasting solution (without glycerol). After addition of 15 µl 0.5 M EDTA and incubation for 10 minutes at room temperature at most 10 µl of DNA (with a concentration of about 500 ng/µl) was added followed by incubation for 5 minutes. The batch was inoculated with 150 µl of a 60% PEG600 solution (diluted in unbuffered spheroblasting solution) for 30 minutes and centrifuged (10 krpm, 5 minutes, room temperature) after addition of 1 ml NVM+ medium. Finally the pellet was resuspended in 1 ml NVM+ and incubated at 37°C till the cells were regenerated.

The whole sample, a 1:10 dilution and a batch without DNA as control was plated on NVM+ plates (100 µl/plate) with corresponding antibiotics. Single colonies could be observed after incubation at 42°C for 1 – 2 weeks.

2.2.6.1.3 Screening for positive clones

Colonies of the transformed cells were picked from the plates, transferred in 1 ml NVM+ and incubated at 42°C. At high density a template for an analytic PCR was prepared as described in 2.2.3. For a quicker screening the method of colony PCR was performed. Therefore the colonies were not transferred in liquid medium but streaked on a fresh plate and a small volume of the adnated culture was used directly for PCR analysis.

2.2.6.2 Plasmid preparation from *Nab. magadii*

3 ml of a culture with an $OD_{600\ nm}$ of about 1 was centrifuged at 13 krpm for 1 minute at room temperature and the pellet was resuspended in 200 µl solution I. Subsequently

200 µl of the solution II was added followed by incubation at room temperature for 5 minutes. For the precipitation of proteins, membranes and chromosomal DNA the sample was mixed with 350 µl of solution III and centrifuged at 13 krpm for 10 minutes. The clear supernatant was transferred into a new 1.5 ml reaction tube and the same volume of phenol/chloroform (1:1) was added. After gently inverting, a centrifugation step at 16.4 krpm and 4°C occurred resulting in a formation of an aqueous and an organic phase. The aqueous phase was transferred into a new 1.5 ml reaction tube and 0.8% volumes of ice-cold isopropanol was added. The precipitation of the DNA occurred by freezing the sample at -20°C for 15 minutes and a following centrifugation step at 16.4 krpm and 4°C for 15 minutes. The pellet was washed once with 70% EtOH and dissolved in 30 µl ddH_2O.

2.2.6.3 Isolation of chromosomal DNA of *Nab. magadii*

2.2.7 Virus methods

2.2.7.1 Isolation of virus particles

For the isolation of ΦCh1 particles 4.5 L NVM+ was inoculated with 30 – 40 ml of a dense culture of *Nab. magadii* and incubated at 37°C. Every day the $OD_{600\ nm}$ was measured and with the help of a growth curse the time point for the complete lysis of the cells could be observed. At this point the culture was centrifuged at 8 krpm for 20 minutes at room temperature and the supernatant was inoculated with 10% PEG 6000 by stirring slightly overnight. The PEG-bound phages were harvested by centrifugation at 8 krpm for 20 minutes at room temperature and the pellet was resuspended in about 30 ml of 4 M NaCl/50 mM Tris- HCl (pH 9.5).

For the separation of the virus particles a discontinuous CsCl gradient was performed. The gradient was built up in ultracentrifugation tubes by covering 3 ml of a 1.5 CsCl solution with 4 ml of a 1.3 CsCl solution. After addition of 6 ml phage solution the tubes were balanced with a 1.1 CsCl solution and ultracentrifuged at room temperautre for 20 h at 30 krpm using the swinging bucket rotor SW40Ti.

Centrifugation results in the formation of two bands within the gradient. The upper band mainly contains archaeal flagella and the virus particles are located in the lower band

which was harvested carefully for purification with the help of a continuous CsCl gradient. Therefore the virus particles were mixed with the 1.3 CsCl solution and again unltracentrifuged at room temperature for 20 h at 30 krpm resulting in a single band. The purified phages were finally dialyzed against 4 M NaCl/50 mM Tris- HCl pH 9.5 overnight and stored at room temperature.

2.2.7.2 Isolation of virus DNA

100 µl of purified virus was mixed with 300 µl ddH_2O. For the extraction of the DNA 200 µl Phenol/Chloroform (1:1) was added and the batch was centrifuged at 13.2 krpm for 3 minutes. This step was repeated 3 times whereupon the aqueous phase was transferred to a new reaction tube each time. After precipitation of the DNA with 2 x volume 96% EtOH a centrifugation step at 18 krpm for 20 minutes at 4°C occurred. The pellet was washed twice with 1 ml 70% EtOH, dried carefully and resolved in an appropriate volume of ddH_2O.

2.2.7.3 Virus titer analysis

Virus titer analysis was performed to determine the plaque forming units and thus the concentration of ΦCH1 viruses in a solution. Therefore 400 µl of a *Nab. magadii* L13 culture residing in a late exponential or early stationary phase was mixed with 5 ml NVM+ softagar and 100 µl of a phage dilution (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}). For every dilution the batch was plated on NVM+ plates in triplets and incubated at 37°C for about 10 days until plaque formation was visible.

2.2.8 Protein methods

2.2.8.1 Protein overexpression in *E. coli*

Overexpression of recombinant proteins in *E. coli* was performed by using the pQE30 vector carrying the lac promoter which controls the gene of interest and is recognized by *E. coli* polymerase.

1 L LB full medium was inoculated with an overnight culture of *E. coli* to an $OD_{600\ nm}$ of 0.1. The culture was incubated at 37°C until an $OD_{600\ nm}$ of 0.3 was reached and sub-

sequently protein expression was induced by adding 1 M IPTG (final concentration 0.5 mM). After 3 hours (time point of highest expression, already determined for used culture/ plasmid) the cells were harvested by centrifugation at 6 krpm at 4°C for 15 minutes and the pellet was frozen at -80°C overnight. The next day the pellet was resuspended in 50 ml lysis buffer and stirred for at least 4 hours.

2.2.8.2 Protein purification under denaturing conditions

The cells were additionally lysed by sonification (3x 4 minutes) and centrifuged at 9 krpm and 4°C for 20 minutes. The supernatant was incubated with 1 ml Ni-NTA, stirred overnight and applied to a column from the QIAexpressionist^{TM} Kit (Qiagen). In the first step the flow through was collected followed by two wash steps using 4 ml washing buffer. Finally the proteins were eluted six times with 500 µl elution buffer. To determine the fractions with the highest protein concentration samples of each fraction were mixed with 2x Laemmli buffer, boiled at 95°C for 5 minutes and analysed via SDS-PAGE. The fractions chosen for further analysis were then dialysed to exchange the buffer and remove urea. The concentration of the proteins was determined by SDS-PAGE analysis with the help of BSA-standards.

2.2.8.3 Protein binding assay

To test the binding of a protein to the surface of different archaeal strains 100 to 300 ng purified proteins were mixed with 1,5 ml of a culture ($OD_{600\ nm\ =}$ 0,5) and incubated at 37°C for 1 hour. The batch was centrifuged at 13,2 krpm for 5 minutes at room temperature and the supernatant was transferred into a new reaction tube. The pellet was resuspended with 5 mM Na-phosphate buffer whereupon the amount in µl was calculated by multiplying the $OD_{600\ nm}$ with the factor 75 and the same amount of Laemmli buffer was added. The supernatant was precipitated with 5% of a 100% trichloroacetic acid solution (TCA) at 4°C overnight and was centrifuged at 16,4 krpm at 4°C for 30 minutes the next day. The pellet was resolved in the calculated amount of Na- phosphate buffer and Laemmli buffer. The samples were incubated at 95°C for 5 minute and analyzed by SDS-PAGE and Western blot. Signals of surface bound proteins could be detected in the pellet samples in contrast to no bound proteins which could be detected in the precipitated supernatants.

2.2.8.4 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze proteins. Therefore the Mini Protean 3 system provided by BioRad was used as described in manufacturer´s instruction. The components of the separating gel (described in 2.1.9.2) were mixed and poured between the two vertical glass plates fixed in the casting system and covered immediately with isopropanol. After solidification of the gel the isopropanol was removed, the stacking solution (components described in 2.1.9.2) was casted on top of the separating gel and a comb was added. The polymerized gel was transferred to a corresponding apparatus and covered with 1 x SDS running buffer. After removing the comb the protein samples were loaded on the gel and a voltage of 40 V was adjusted till the samples congregated at the stripline between the stacking and the separating gel. Separation of the gels then occurred at 80 V and 100 V respectively.

2.2.8.5 Coomassie staining

To visualize all proteins separated by SDS-PAGE the gel was stained with a coomassie staining solution for 5 to 15 minutes. To increase the contrast between proteins and background the gel was destained by water or a 20% acetic acid solution.

2.2.8.6 Western blot analysis

To visualize certain proteins from a sample the western blot analysis was performed. The first step was the transfer of proteins to a nitrocellulose membrane using the semi dry method. Therefore a nitrocellulose membrane was covered with the polyacrylamide gel resulting from a SDS-PAGE and both were flanked with 3 layers of filter papers on each side.

After incubation of the composition in transblot buffer it was transferred into a semi- dry transblotting apparatus and blotting occurred at 20 V for 20 minutes. The membrane then was stained with Ponceau S to visualize proteins and verify blotting. To block non protein bound spots on the membrane it was incubated with 5% milk powder overnight and washed with 1x TBS for 10 minutes the next day. After incubation with the first antibody (diluted in 1x TBS containing 0,3% BSA) for 1 hour at room temperature the membrane was washed 3 times for 10 minutes with 1x TBS. The membrane was then

incubated with the second antibody and again washed 3 times for 10 minutes. For visualization of the proteins the SuperSignal® West Pico Chemiluminescent Substrate Kit from Thermo Scientific was used according to manufacturer´s instructions. An X- ray hiper film provided by Amersham Biosciences was exposed to the membrane and developed in the dark room.

3 Results and Discussion

3.1 ORF49

The finding of a *Nab. magadii* strain with an earlier onset of lysis, an altered lysis rate with increased plaque formation including larger plaques compared to the wild type, led to the isolation of a mutant of ϕCh1, ϕCh1-1. Sequence analysis of ϕCh1-1 showed a duplication of 223 bp containing a region of ORF49 and a small part of its upstream region creating a new ORF. This ORF49´ lies next to ORF49 with overlaps, resulting in co-transcription and co-translation (figure 4). Expression of ORF49 is not constitutive as expression starts 32 h after inoculation with an increase in the logarithmic growth phase of the infected strain. The increase of the gene product of ORF49, gp49, during the virus life cycle and the fact that the duplication of the 223 bp is not stable but has the tendency to revert into the wild type form indicates a role of ORF49 in the switch between the lysogenic and the lytic life cycle of ϕCh1, associated with a putative repressor activity (Iro *et al*., 2006).

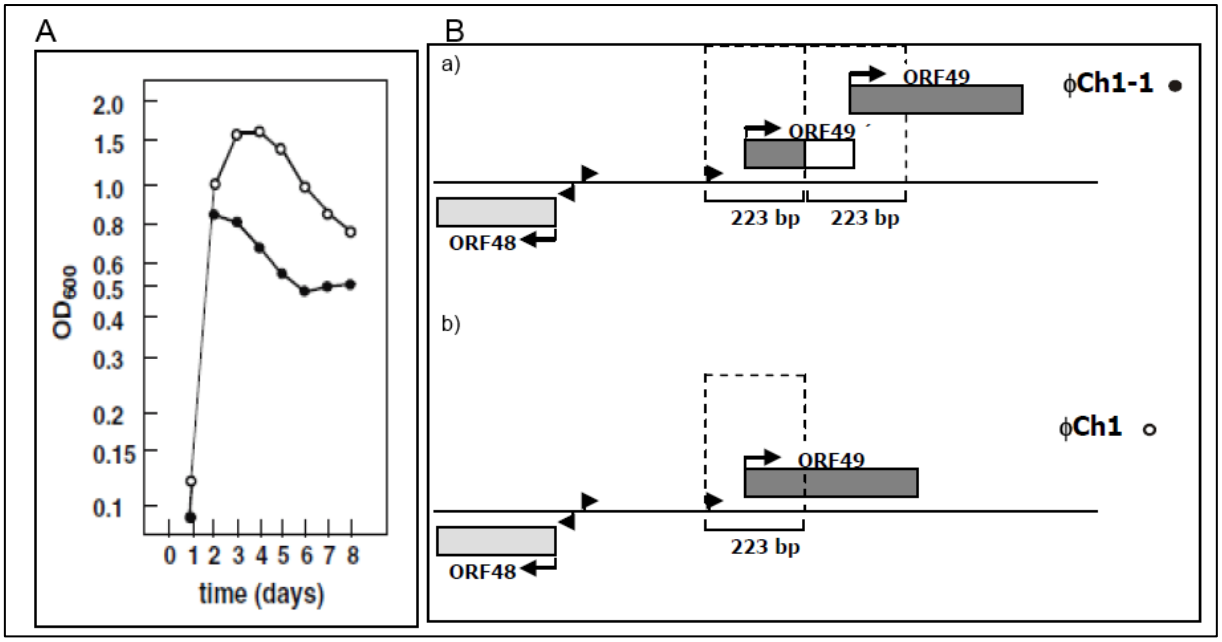

Fig. 4 A: growth curve of two different *Nab. magadii* strains L11. The white line represents the growth curve of L11 carrying the ϕCh1 wild type with a logarithmic growth phase until onset of lysis at about 72 h. The black line indicates the growth curve of L11 carrying the mutant version of ϕCh1, ϕCh1-1. It is remarkable that the logarithmic phase is shorter than in wt and an earlier onset of lysis at about 36 h could be observed. B: gene organization of ORF49 in ϕCh1 and ϕCh1-1. a) Rearranged form of ORF49 in ϕCh1-1. The duplication of the 223 bp is indicated by the grey and white box leading to a new open reading frame, ORF49´. Parts are overlapping with the ORF49 wt form. b) wild type form of ORF49 in ϕCh1. The dotted box indicates a sequence of 223 bp including a part of ORF49 and a part of its upstream region (Iro *et al*., 2006).

To observe the effect on lysis and plaque formation when ORF49 is expressed constitutively, virus titer analyses were performed. As shown in figure 5, plaque formation units decrease dramatically when a *Nab. magadii* strain containing pRo-5 with ORF49 is infected with ϕCh1 in contrast to the plaque formation unit generated by infection of a *Nab. magadii* strain carrying pRo-5 alone. This indicates that pg49 has repressor activity resulting in an aborting effect of ORF49 on the infectivity of ϕCh1.

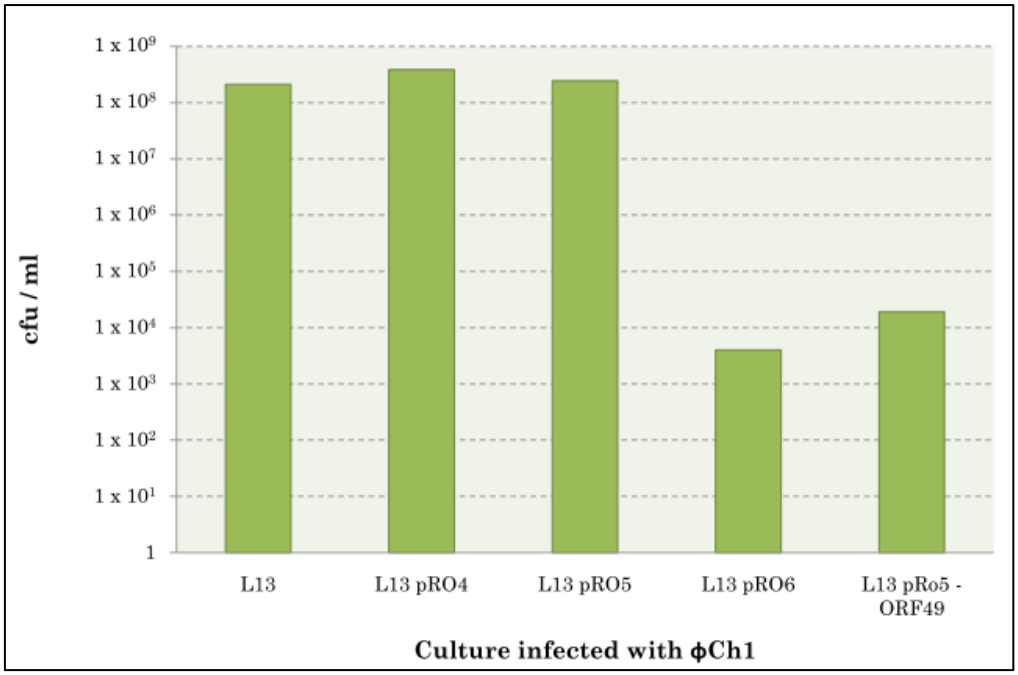

Fig. 5 Phage titer analysis of infected *Nab. magadii* strains expressing ORF49 constitutively from a plasmid. Certain interest applies to the plaque formation unit rate of the infected *Nab. magadii* strains carrying pRo-5 and pRo-5–ORF49. Additionally expression of ORF49 leads to a dramatically decrease of plaque formation compared to plaque formation by infecting *Nab. magadii* carrying pRo-5 alone (Reiter, 2010).

To confine the region responsible for the repressor activity of ORF49, five ORF49 variants with truncated 3´ C-terminals were constructed and virus titer analyses were repeated. The 3´ ends of the mutants differ in approximate 30 bp to 40 bp length, respectively (figure 6).

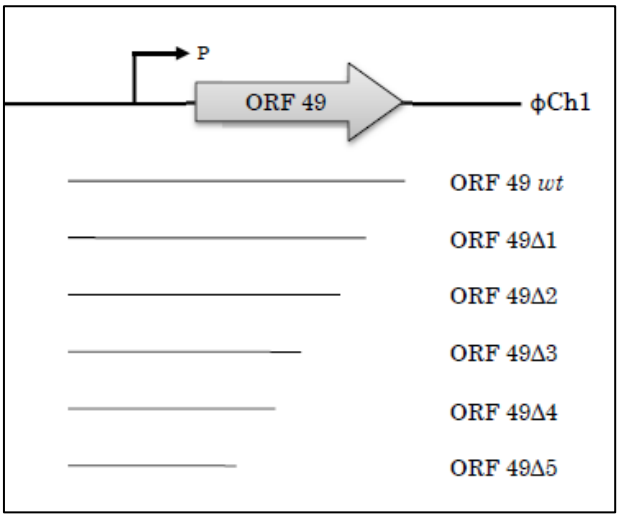

Fig. 6 Truncated versions of the ORF49 mutants. Parts of the C-terminal region of ORF49 were deleted. The variants differ in approximate 30 bp to 40 bp length.

The deletion of 3´ end parts in the first three mutants of ORF49, ORF49Δ1, ORF49Δ2 and ORF49Δ3, shows the same decreased plaque formation rate when expressed from the plasmid pRo-5 in *Nab. magadii* infected with ϕCh1 as the infected *Nab. magdii* strain carrying pRo-5 with the whole ORF49. In contrast, the deletion mutants of ϕCh1 containing ORF49Δ4 and ORF49Δ5 seem to have lost their repressor activity (Figure 7). The overall number of codons of ORF49 is 117 while the mutations ORF49Δ4 and ORF49Δ5 together covering the codons 1 to 81 thus this region is responsible for the repressive effect on lysis (Reiter, 2010).

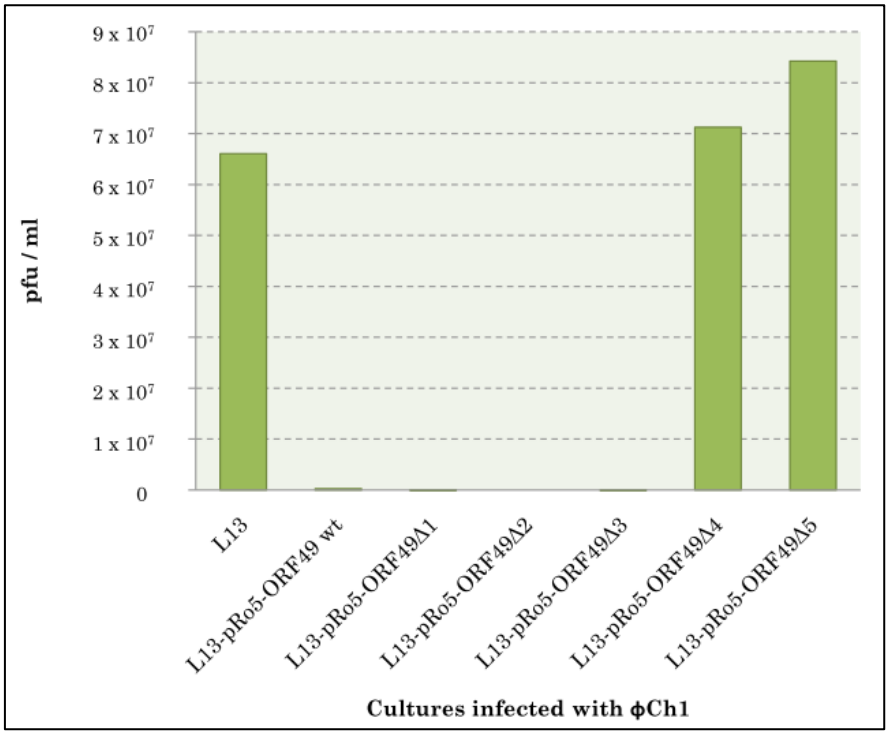

Fig. 7 Five deletion mutants of ϕCh1 with truncated 3´ ends lead to different plaque formation rates. L13-pRo-5-ORF49 serves as control which shows a decreased rate of infection due to the constitutive expression of ORF49. The infection of *Nab. magadii* strains carrying the deletion mutants ORF49Δ1, ORF49Δ2 and ORF49Δ3 leads to no effect on plaque formation compared to the control. On the contrary, the infection of the strains carrying the ORF49Δ4 and ORF49Δ5 mutations lead to increased plaque formation indicating that the deleted regions (codon 1 to 81) are responsible for the repressor activity of ORF49 (Reiter, 2010).

As proteins with repressor activity generally contain a DNA binding domain a band shift assay was performed. Therefore the binding of gp49 to the ϕCh1 DNA was tested and a shift could be observed. The band shift assay was repeated with the C-terminal truncated versions of gp49 leading to the same result. Thus, gp49 has a DNA binding site which is not located at the C-terminal region. To find out more about the location of the DNA binding site, a mutant of gp49 with the deletion of the N-terminus (first 52 codons)

was constructed and the band shift was repeated. In contrast to the gp49 wt which binds to the ϕCh1 DNA the deletion mutant gp49ΔN has lost the ability for DNA binding, indicating that the DNA binding site lies within the N-terminus (Reiter, 2010).

To exclude the possibility that ORF49 acts as regulatory RNA and not as a protein an AUG deletion mutant of ORF49 was constructed. The deletion of the codon AUG prevents translation of the mRNA to the protein level thus no protein expression occurs. A virus titer was performed with L13 wt, L13-pRo-5 as the control, L13-pRo-5-ORF49 and L13-pRo-5-ORF49ΔAUG (Svoboda, 2011). The strains were infected with ϕCh1 wt and plaque formation units were determined. As shown in figure 8 the plaque formation of the AUG deletion strain correlates with the one of L13-pRo-5, indicating that the RNA of ORF49 has no repressive effect on lysis and plaque formation, thus ORF49 acts on protein level.

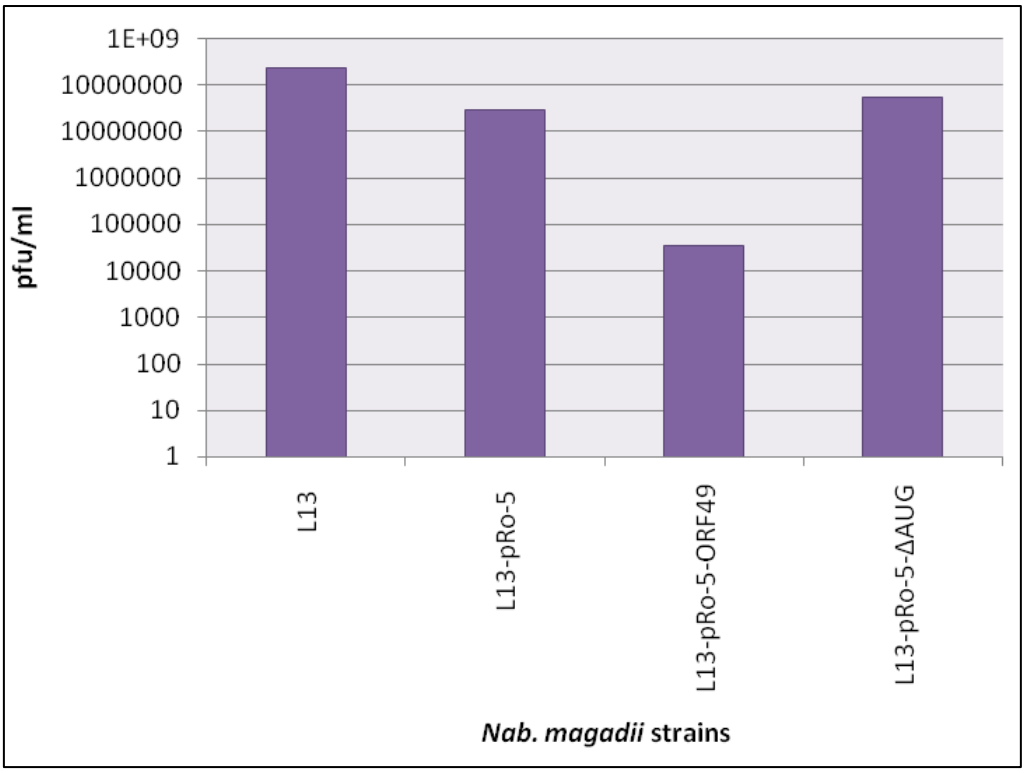

Fig. 8 Virus titer analysis of different *Nab. magadii* strains. Plaque formation units of L13 represent the wt while L13-pRo-5 serves as control. L13-pRo-5-ORF49 shows a reduced plaque formation rate as gp49 acts as a repressor. L13-pRo-5-ORF49ΔAUG shows a value of plaque formation as the control, indicating that no repression occurred (Svoboda, 2011).

The detection of gp49 via western blot analysis surprisingly led to two signals at different heights. The first showed gp49 with a size of 13.3 kDa as expected whereas the other signal represented a protein with a size of approximately 30 kDa. As the western

blot was performed with an antibody specific against gp49, the signal at a higher size could arise from dimerization of gp49. This seems possible as the size of the unknown protein is almost the double of gp49. This led to the idea that the loss of repressor activity of the mutants containing ORF49Δ4 and ORF49Δ5 is connected to the loss of a dimerization region. Therefore a protein of ϕCh1 with the ability for dimerization was determined with the help of the program “ch.EMBnet.org- COILS- Prediction of Coiled Coil Regions in Proteins”. Dimerization takes place due to the presence of a coiled coil structure which consists of several helices that form a superhelix within the protein structure. The program searches for coiled coil structures, and a protein encoded by ORF54 was found to have such a motif (figure 9). The ORF54 then was fused to ORF49Δ1 – ORF49Δ5, transformed into *Nab. magadii* L13 and the infectivity was again tested with the help of virus titer analysis (Svoboda, 2011).

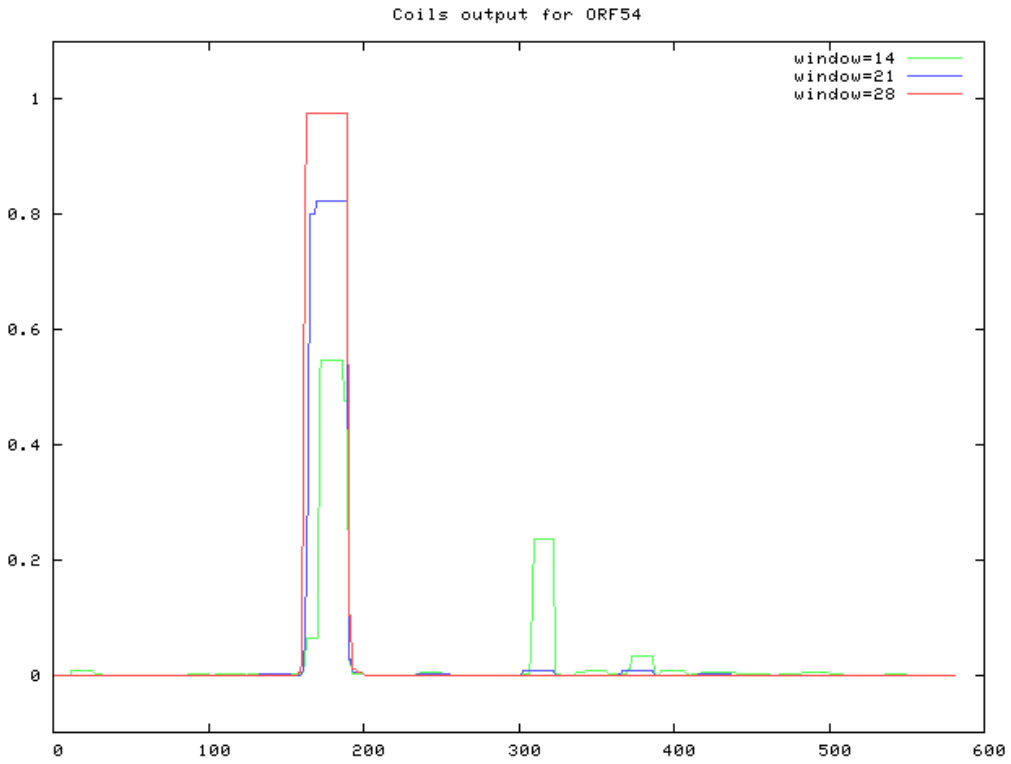

Fig. 9 Schematically illustration of ORF54 with predicted coiled coil structure. The horizontal line shows the position of the 581 amino acids of the entire ORF54. The vertical line indicates the coiled coil probability. A coiled coil structure can be observed at the amino acid position 161 to 192 (ch.EMBnet.org- COILS- Prediction of Coiled Coil Regions in Proteins).

Figure 10 shows the resulting phage formation units when different *Nab. magdii* strains containing the ORF49 deletion mutants fused to ORF54 were infected with ϕCh1. L13-pRo-5-ORF49 serves as control with a repressor activity of gp49 compared to the

higher plaque formation rate of L13-pRo-5 infected with ϕCh1. The fusion of the deletion mutants with ORF54 however leads to the total loss of repressor activity of gp49 in all mutants. This could be explained by the enormous size of the fusion protein, gp54, which contains 581 amino acids (Svoboda, 2011).

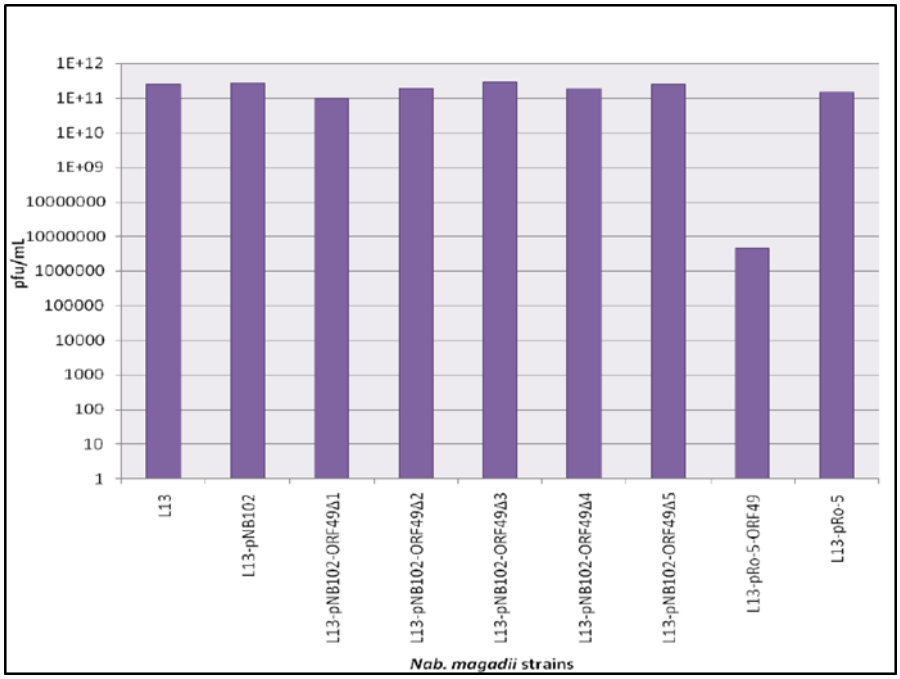

Fig. 10 Plaque formation units of different *Nab. magadii* strains containing ORF49 deletion mutants fused to ORF54 and infected with ϕCh1. The control strains L13 and L13-pRo-5 show a higher infection rate compared to the strain L13-pRo-5-ORF49 in which gp49 is expressed constitutively leading to a repressed infection. The fusion of ORF54 does not lead to the regeneration of the repressor activity of the mutants ORF49-Δ4 and ORF49-Δ5, in contrast, all fusion products have lost the ability for repression (Svoboda, 2011).

As the enormous size of ORF54 with 581 amino acids is presumably the reason for the loss of the repressor activity of all ORF49 mutants, a new strategy was chosen. The coiled coil structure of gp54 lies within the amino acids 161 to 192 therefore the region between AA 151 and 212 was isolated and fused to the ORF49 mutants. To keep the fusion product variable and to ensure a certain flexibility 5 glycine residues were integrated between the ORF49 variants and ORF54. Again the ORF49 mutants fused to the coiled coil region of ORF54 were infected with ϕCh1 and virus titer analyses were performed. However, it was not possible to isolate a positive *Nab. magadii* L13 strain carrying pRo-5-ORF49Δ5-ORF54 (coiled coil) thus the titer includes just the mutants ORF49Δ1-ORF54 (coiled coil) to ORF49Δ4-ORF54 (coiled coil).

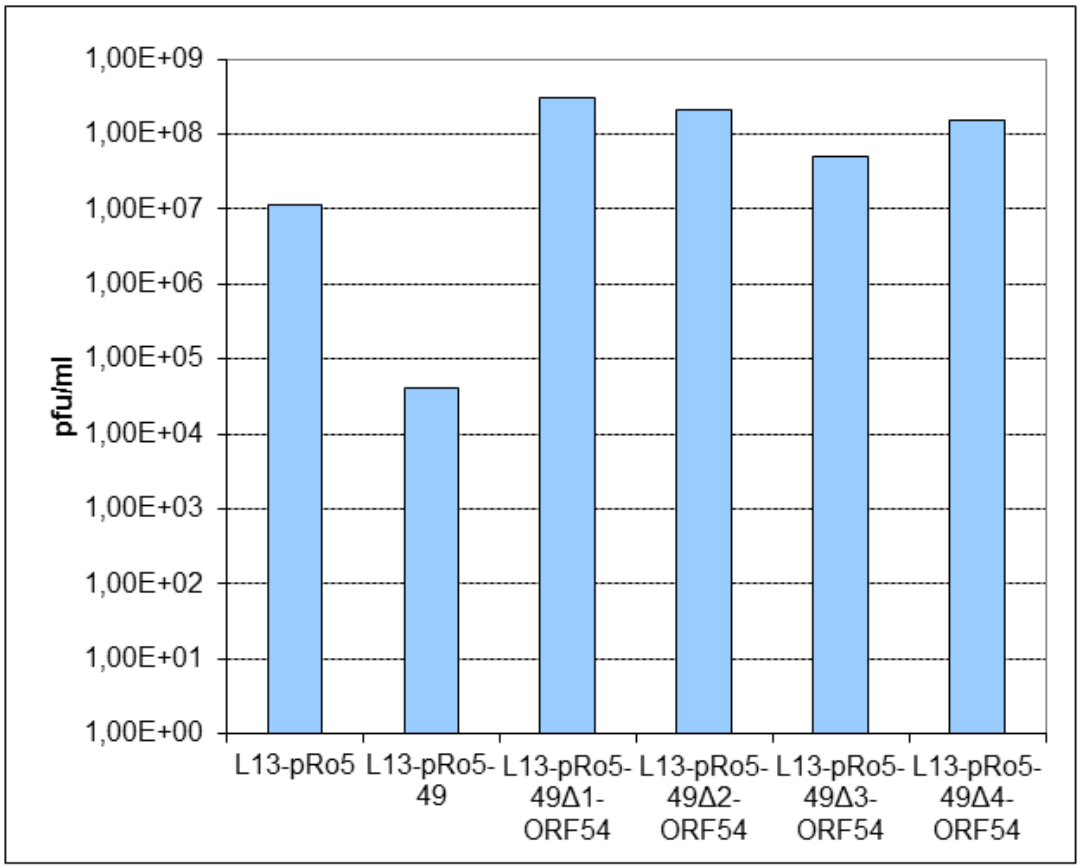

Fig. 11 Rate of plaque unit formation of ϕCh1 infected L13 strains carrying ORF49 mutants fused to the coiled coil structure (AA 151 to 212) of ORF54. L13-pRo-5 as the wt serves as negative control whereas L13-pRo-5-ORF49 represents the positive control as the plaque formation rate decreases dramatically when gp49 is expressed constitutively indicating a repressor activity of gp49 on infection. Fusion of the coiled coil region of ORF54 to ORF49Δ1 to ORF49Δ4 does not regenerate the repressor activity of gp49, in contrast, all fusion products have lost the ability for repression.

Infection of *Nab. magadii* strain L13 carrying pRo-5 with ϕCh1 shows a plaque formation rate typical for the wt whereas L13-pRo-5-ORF49 leads to decreased plaque formation and thus decreased infection of *Nab. magadii*. As shown in figure 7 the truncated versions ORF49Δ4 and ORF49Δ5 of ORF49 show no more repressor activity as the plaque formation rate increased dramatically when infected with ϕCh1. Figure 11 shows the plaque formation rate of the ϕCh1 infected ORF49Δ1-4 mutants fused to the coiled coil structure of ORF54. In this study it was not possible to regenerate the repressor activity of ORF49Δ4 by fusion to the coiled coil structure of ORF54 as the plaque formation rate is even higher than in wt. Furthermore the mutants ORF49Δ1 to ORF49Δ3 which had repressor activity when not fused to another protein lost this feature.

3.1.1 Discussion

It is evident that the gene product of ORF49, gp49, has a repressive effect on lysis of *Nab. magadii* as the virus titer analyses in figure 5 show that there is a decrease in plaque formation when ORF49 is expressed constitutively. Also, the region which is responsible for the repressor activity of gp49 could be determined with the help of truncated forms of ORF49 and following virus titer analysis. Furthermore the ability of gp49 to bind DNA was verified by band shift assays. The fact that the C-terminal deletion mutants of gp49 still bind DNA even without the ability for repression led to the idea that either ORF49 acts on RNA level or that the binding of DNA is compensated by fusion with other proteins containing dimerization domains. The hypothesis of ORF49 as a regulatory RNA could be excluded by virus titer analysis. Therefore it is reasonable to assume that the repressor activity is connected to the ability of gp49 for dimerization but this could not be proven. Neither the fusion of the truncated versions of ORF49 with the entire ORF54 nor the fusion with only the coiled coil region of ORF54 could regenerate the repressor activity of the ORF49-Δ4 and Δ5 versions. It seems that the dimerization of ORF49 with ORF54 leads to a secondary structure of the fusion product which prevents gp49 of any activity. The fact that the repressor activity of gp49 could not be restored but that in contrast the plaque formation rate is even higher could indicate a loss of every activity gp49 has. Furthermore the coiled coil region of ORF54 determined by the program “COILS Server” is not proven to be one. Coiled coil regions are determined by comparing the sequence to be analyzed to sequences of known proteins containing coiled coil regions like the mesophilic proteins myosin, tropomyosin or keratin. Generally such regions contain hydrophobic amino acid residues at certain positions leading to hydrophobic interactions promoting the coiled coil structure. Therefore the program “COILS Server” searches amongst others for such hydrophobic residues. As mentioned in the introduction some halophilic proteins form hydrophobic bonds under high salt conditions. Thus the search for a coiled coil region in a halophilic Archaea as *Nab. magadii* can lead to wrong results.

3.2 ORF79

3.2.1 Complementation of the deletion mutant strain L11-Δ79

A deletion mutant of the *Nab. magadii* strain L11 was constructed to determine the function of the protein gp79 of ϕCh1 which until then was unknown. The deletion strain L11-Δ79 showed an earlier onset of lysis compared to the wild type strain L11 (figure 12), indicating a regulatory function of gp79. That gave rise to the performance of western blot analysis with whole protein samples of L11 and L11-Δ79 taken at different time points and exposed to different antibodies.

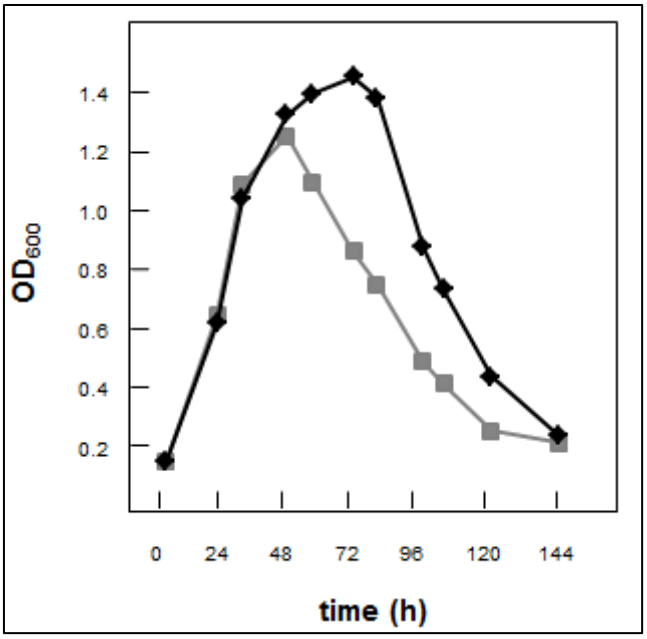

Fig. 12 Growth curve of *Nab. magadii* L11 and *Nab. magadii* L11-Δ79. The black line indicates the growth curve of the wt strain L11 with a logarithmic growth phase rising in the onset of lysis after about 72 hours. The grey line shows the growth curve of the mutated strain L11-Δ79. The logarithmic phase is shorter than in wt with an earlier onset of lysis after about 48 hours (Alte, 2011).

The western blot analyses (Figure 13) with antibodies against the capsid protein E show that there is a somewhat earlier expression of the protein in the deletion strain as expression starts after 13 h in contrast to the expression in wt which starts after 52 h. An even more dramatical effect can be seen on the expression of gp34 when using antibodies against the tail fibre protein. The expression of gp43 in the Δ79 strain seems to be almost unregulated as gp34 is expressed constantly from the beginning to the end of the time course compared to the expression in the wt strain which starts at about 52

h and increases at the onset till the end of lysis. This indicates a regulatory activity of ORF79 on the expression of gp34 and protein E (Alte, 2011).

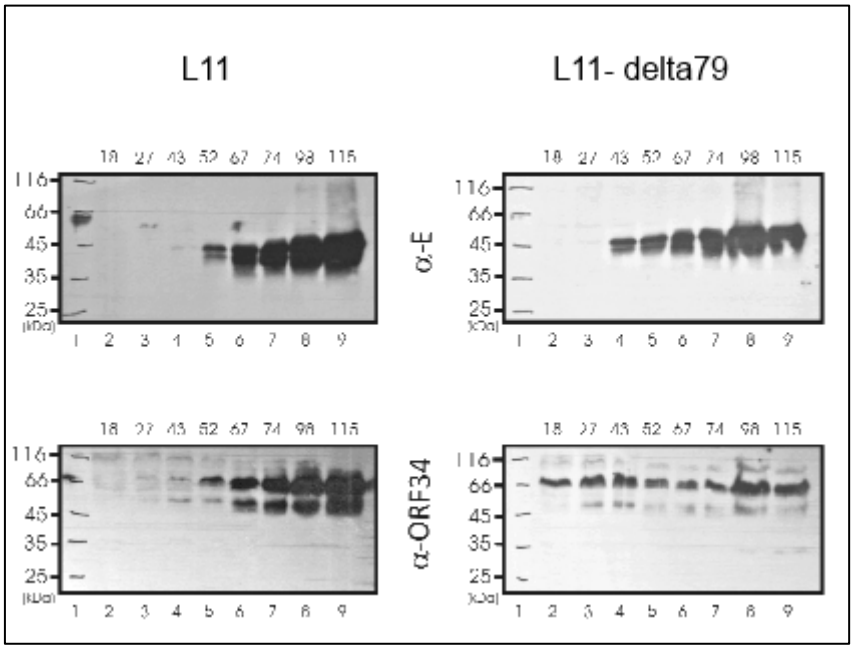

Fig. 13 Western blot analysis of the expression of different proteins in the *Nab. magadii* wt strain L11 and the mutant strain L11-Δ79 carrying a deletion of ORF79. The signals resulting from the exposure of whole protein samples with antibodies against the capsid protein E show that the expression of the protein starts earlier in the mutant strain than in the wt strain. A more dramatical effect of the deletion of ORF79 is the almost unregulated expression of gp34 compared to the expression in the wt strain (Alte, 2011).

To prove that the modified regulation of the gp34 and capsid E expression is caused by the deletion of ORF79, a complementation of the L11-Δ79 strain was performed. To compensate the deletion of ORF79 the vector pNB102- p_ORF49-ORF79 (with the promoter of ORF49 to ensure a constitutive expression of ORF79) was transformed into the mutant strain L11-Δ79 leading to the cultivation of the complemented strain L11-Δ79-pNB102-p_ORF49-ORF79 (Alte, 2011). To assert that the expression of the capsid E protein and gp34 in the complemented strain behaves like in wt again, western blot analyses were repeated.

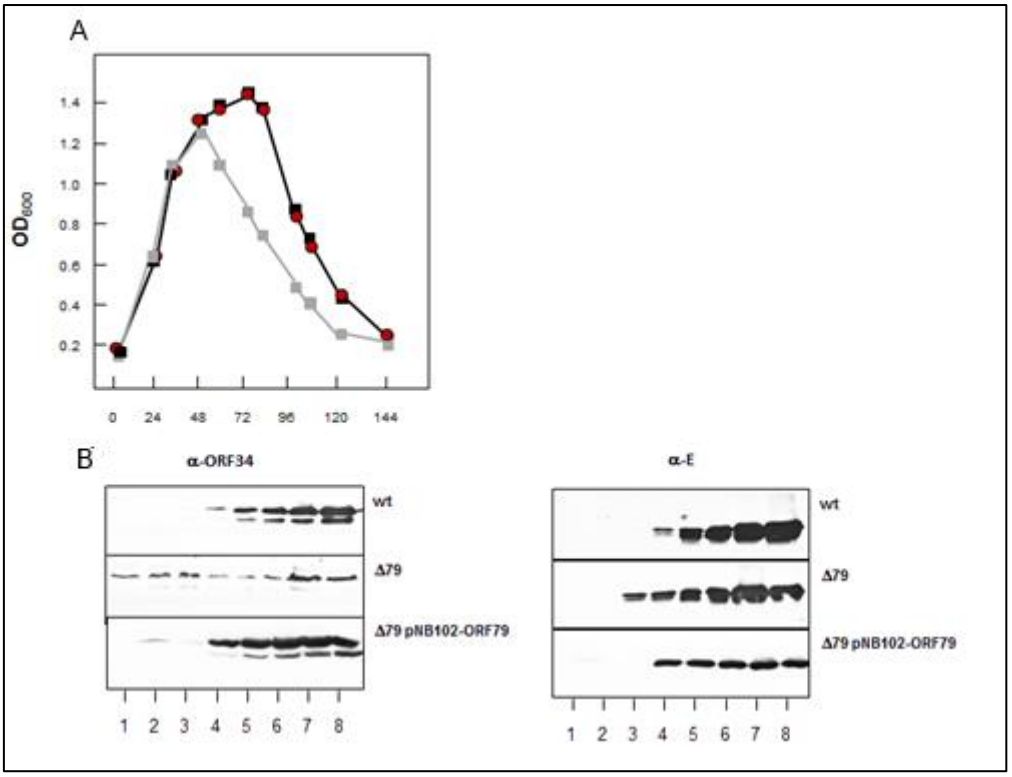

Fig. 14 A: growth curve of the *Nab. magadii* wt strain L11 and the mutant strain L11-Δ79-pNB102-p_ORF49-ORF79. The black line indicates the growth curve of the wt strain L11, the grey line shows the growth curve of the complemented strain L11-Δ79-pNB102-ORF79. The complemented strain behaves like wt again. B: Western blot analysis of the expression of different proteins in the *Nab. magadii* wt strain L11, the mutant strain L11-Δ79 carrying a deletion of ORF79 and the complemented strain L11-Δ79-pNB102-p_ORF49-ORF79. The expression of the tail fibre protein gp34 in L11-Δ79 seems almost unregulated compared to the expression in wt. the complementation of L11Δ79 with pNB102-p_ORF49-ORF79 leads to an expression of gp34 similar to that in wt, indicating a regenerated regulation of expression by gp79. The regulated expression of capsid protein E could also be restored by complementation.

As shown in figure 14 the regulatory effect of gp79 on the expression of the capsid E protein as well as on the expression of gp34 could be regenerated by complementing the mutant strain L11-Δ79 with pNB102-p_ORF49-ORF79. The constitutive expressed gp79 compensates the deletion of ORF79 within the ϕCh1 genome leading to a wt-like behavior of growth and protein expression. Therefore other factors leading to the abnormally expression of the inspected proteins can be excluded.

3.2.2 Promoter analysis of ORF79

As described in 3.2.1 the complementation of L11-Δ79 was performed by transforming the vector pNB102-p_ORF49-ORF79 in the ORF79 deletion strain. Because of the

tendency of L11-Δ79 to an earlier onset of lysis and the comparatively long time needed for the transformation of *Nab. magadii* another strategy for the complementation was chosen first. Thereby, the vector pNB102-p_ORF49-ORF79 was first transformed into L13 and then infected with the isolated ϕCh1-Δ79 virus particles, and a virus titer was performed. If the infection is successful, plaque formation should take place. The vicinity of the plaques includes colonies not lysed but infected with ϕCh1-Δ79 which could be exceled and transferred in NVM+ resulting in the cultivation of the strain L11-Δ79-pNB102-p_ORF49-ORF79. However, the infection of L13-ORF79 with ϕCh1-Δ79 was not successful as almost no plaque formation could be observed. To exclude a methodical failure, the phage titer was repeated with L13-pNB102-p_ORF49-ORF79 and L13-pNB102 as the control and infection occurred not just with ϕCh1-Δ79 but also with ϕCh1 wt particles. As shown in figure 15 the plaque formation rate of infected L13-pNB102 is between 10^7 and 10^8 which accords with the rate of an infected L13 wt strain. However, just a small amount of plaques could be achieved by infecting L13-pNB102-p_ORF49-ORF79 with ϕCh1-ΔORF79 and ϕCh1 wt, respectively.

strain	ϕCh1 wt (pfu/ml)	ϕCh1-ΔORF79 (pfu/ml)
Nab. magadii L13 (pNB102)	1×10^8	3×10^7
Nab. magadii L13 (pNB102-ORF79)	$<1 \times 10^1$	$<1 \times 10^1$

Fig. 15 Plaque formation units by infection of L13-pNB102 as control and L13-pNB102-p_ORF49-ORF79 with ϕCh1 wt and ϕCh1-ΔORF79. Infection of L13-pNB102 leads to a normal plaque formation rate of 10^7 to 10^8 whereas infection of L13-pNB102-p_ORF49-ORF79 leads to almost no plaque formation with a rate of $1x10^1$ (Alte, 2010).

The very low plaque formation rate of the virus titer of L13-pNB102-p_ORF49-ORF79 strengthened the idea of gp79 as a regulatory element in the lysogenic life cycle of ϕCh1.

As the promoter region of ORF79 is not defined until now the results of the virus titer can be used to find out more about the exact position of the ORF79 promoter sequence. Therefore ORF79 with its putative promoter region was cloned into the vector pNB102 as well as into pRo-5, and different parts of the promoter sequence were deleted resulting in four different clone variants, p_ORF79- 1 to p_ORF79-4. The first variant, p_ORF79-1 was constructed by restriction of the promoter region with the digest

enzyme *Cla*I and a following relegation. This leads to a deletion of 290 bp between the putative start codons. The digest with the restriction enzyme *Nae*I and following relegation lead to the loss of a 564 bp sequence including one of the assumed start codons, represented by p_ORF79-2. Two more deletions were introduced by PCR, one, p_ORF79-3 was constructed with the help of the primers 79-Kpn and 79-XH-2, leading to a promoter sequence of ORF79 with 563 bp including one of the putative start codons. p_ORF79-4 was constructed by PCR with the primers 79-Kpn and 79-XH-3 leading to a total loss of all start codons with a promoter length of 511 bp (see figure 16).

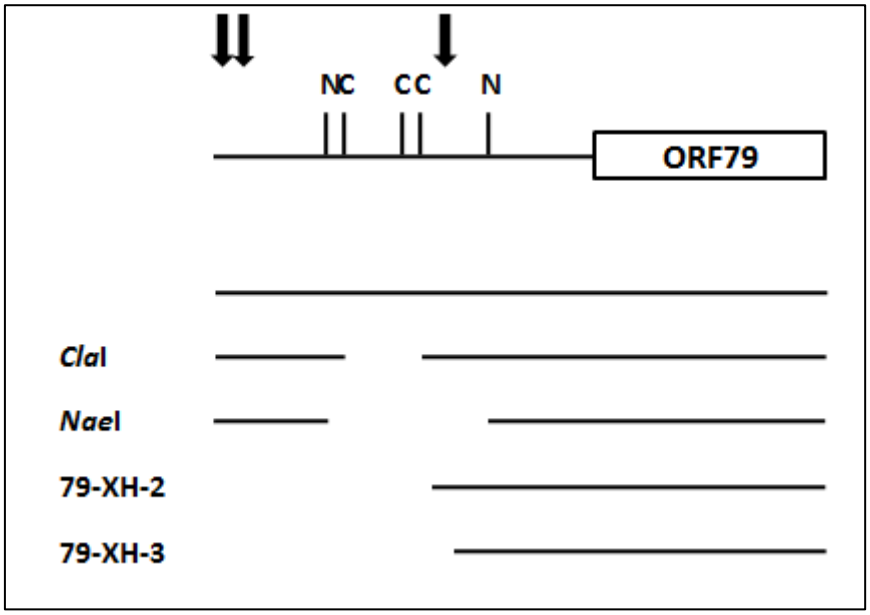

Fig. 16 Schematical illustration of ORF79 with its putative promoter region. The framed part represents the open reading frame 79 with its preceded promoter region indicated as a line. The black arrows show putative start codons with the amino nucleotide sequence WWWTTT, typical for Archaea. The capital letters C indicate the restriction sites for the digest enzyme *Cla*I, the capital letters N indicate the restriction sites for the digest enzyme *Nae*I. Restriction with *Cla*I and following relegation leads to a loss of a region between the putative start codons and consists of 290 bp. Restriction with *Nae*I and following relegation leads to the loss of a region consisting of 564 bp including one of the putative start codons. The region cloned with the help of the primer 79-XH-2 carries one of the three putative start codons within the shortened 563 bp promoter region while the region cloned with the help of the primer 79-XH-3 carries no start codon at all and consists of 511 bp of the promoter sequence.

All p_ORF79 variants were cloned into the vectors pNB102 and pRo-5 because of the different copy numbers of the vectors. The copy number of pRo-5 lies between 1 and 5 whereas pNB102 has 20 copies per cell and thus transformation with pNB102 should

lead to a higher expression of the cloned gene. The vectors then were transformed into *Nab. magdii* L13, infected with ϕCh1 and virus titers were performed.

As the virus titer of figure 15 led to no plaque formation when ORF79 is expressed constitutively, the phage titer with the p_ORF79 variants should lead to no plaque formation when the promoter is intact while plaque formation should be observed performing the phage titer with p_ORF79 variants carrying disrupted promoter regions.

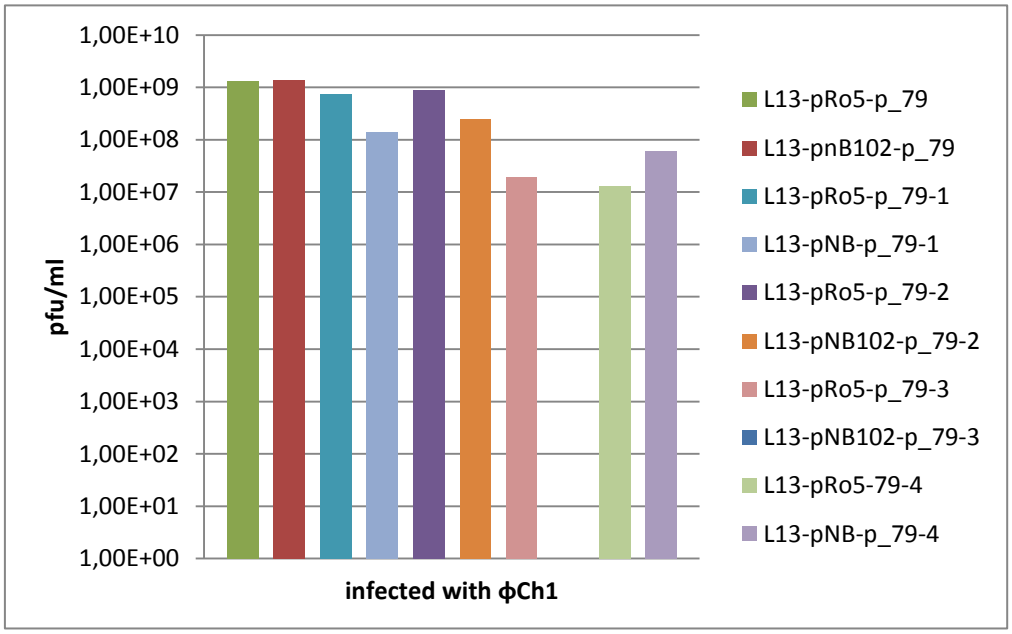

Fig. 17 Virus titer analysis of different *Nab. magadii* strains infected with ϕCh1. L13 serves as control with a plaque formation unit of about 10^9. The plaque formation unit of the infected strains carrying pRo-5 and pNB102 with p_ORF79-1 to p_ORF79-4 shows different values from 10^7 to 10^9 with the exception of L13-pNB102-p_ORF79-3 which shows no plaque formation at all.

Figure 17 shows the plaque formation units of the ϕCh1 infected strains carrying disruptions of the promoter region of ORF79. L13 serves as the control with a plaque formation unit of about 10^9, similar to that of the strains L13-pRo-5-p_ORF79 and L13-pNB102-p_ORF79. Infection of the strain pNB102-p_ORF79-3 leads to no formation of plaques whereas the plaque formation units of the other strains vary between 10^7 and 10^9.

3.2.3 Discussion

The expression of the tail fibre protein gp34 and of the capsid protein E is amongst others regulated by gp79. This is evidenced by the fact that the expression of gp34 is unregulated when detected in L13-ΔORF79 as well as by the earlier start of the expression of the protein E compared to wt. The complementation of L13-ΔORF79 with pNB102-p_ORF49-ORF79 again leads to an expression of the inspected proteins like in wt. This proves that the deletion of ORF79 is responsible for the divergent expression of gp34 and of the capsid protein E. As expression of the two proteins starts earlier when ORF79 is deleted, gp79 has not just a regulatory effect but also a repressor activity. To understand more about the way of repression further studies should be done. As most enzymes with repressor activity dispose of a DNA binding site, the binding of gp79 to certain DNA sequences like the promoter region of ORF34 and ORF11 (capsid protein E) could be examined by band shift assays. Furthermore the research with constitutively expressed gp79 could lead to a better understanding of the connection between ORF79 and ORF34. Therefore transformation of both, ORF34 and ORF79 into L13 and observation of expression of gp34 via western blot analysis could lead to more details about the functionality of gp79. In contrast to the expression of gp34 alone, gp79 should have a repressive effect on gp34 leading to a reduced expression of the tail fibre protein. Also the transformation of ORF79 into L11 should lead to an unregulated, late expression of gp34 even though it is not expressed vectorially but from the ϕCh1 genome. Furthermore, the expression of gp34 and gp79 on the transcriptional level is of interest. Such an analysis on the transcriptional level can be done by taking RNA samples at different time points within the growth curve of L11 and by a following reverse transcription via PCR. Hence, it is possible to get information about the beginning of transcription of ORF79 and ORF34. All these experiments should lead to more details about the features of ORF79 and thus gp79 which could also be used to detect other regulative elements of ϕCh1.

In the course of the complementation of the ORF79 deletion strain, ϕCh1 infection of L13 with constitutively expressed gp79 did not lead to plaque formation (figure 15). This gives rise to the idea that gp79 has a regulatory effect on lysis. On the other side the result was used to find out more about the promoter region of ORF79. Therefore ORF79 with its own putative promoter was cloned into L13 as well as ORF79 variants carrying deletions within the promoter sequence. The hypothesis was that gp79 prevents lysis when carrying an intact promoter while lysis should take place when the promoter is disrupted. However infection of L13-pRo-5-p_ORF79 as well as L13-

pNB102-p_ORF79 did not lead to a reduced plaque formation rate. Also the strains carrying the putative start codons show no decrease in plaque formation just as the strains that lost the start codons. This could be explained by the different protein concentration of gp79 when expressed constitutively from a vector or when expressed from its own promoter. A regulation of ORF79 from other factors can not be excluded as well.

A light decrease in plaque formation can be observed for the strains L13-pNB102-p_ORF79-1, L13-pRo-5-p_ORF79-3 and L13-pRo-5-p_ORF79-4 but there is no reasonable explanation as p_ORF79-1 carries all putative start codons, p_ORF79-3 contains one of the three possible start codons and p_ORF79-4 none of them. The only exception is L13-pNB102-p_ORF79-3 with a total absence of plaques. Within this strain the promoter region of ORF79 contains one of the three start codons and gp79 is expressed from the vector pNB102. As the expression from pRo-5 does not lead to a conspicuous decrease of plaque formation the virus titer should be repeated even though the copy number of pNB102 is almost 4 times higher than in pRo-5.

3.3 Integrase1 – gp34

3.3.1 ORF35 - Integrase1

The middle part of the ϕCh1 genome contains a region of leftward and rightward transcribed genes. Especially ORF34, ORF35 and ORF 36 are interesting parts of this region as ORF35 encodes for a putative site specific recombinase (integrase1) of the bacteriophage λ Int family. As shown in figure 3, ORF35 lies between ORF34 and ORF36, sequences that are orientated in an inverse direction while their 3´ ends are facing each other (Klein *et al*., 2002). By comparing the sequences of ORF34 and ORF35 high similarities at the DNA as well as at the amino acid (AA) level could be observed. In addition both sequences carry clusters of 30 bp direct repeats as it is known for genes of bacterial phages like λ and T4 encoding for tail fibre proteins (Sandmeier, 1994). Therefore the gene products of ORF34 and ORF36, gp34 and gp36 are thought to be putative tail fibre proteins of ϕCh1. The assumption is intensified by the finding that recombination events take place with the help of the highly similar repeat clusters of ORF34 and ORF36. This recombination event leads to the inversion of the int1 gene but also to the exchange of 3´ parts of the ORF34 and ORF36 sequences. The ex-

change of the 3´ sequences which are parts of the N-termini of the proteins lead to variants of gp34 and gp36 with N-termini that differ in length and in their composition (Rössler *et al*., 2004). The appearance of such variations of gp34 and gp36 with the assumption that they represent tail fibre proteins could be the key to the mechanism by which ϕCh1 infects the host cell. It is still unknown how archaeal tailed dsDNA viruses attach to their host but a possibility is the recognition of receptors of the host cell surface by the viral tail fibre proteins. Out of all protein variants two forms with the possible orientation of ORF34 and ORF36 are designated in figure 18. The (+) segment indicates the non-rearranged form of ORF34 and ORF36 resulting in the putative expression of the proteins $gp34_1$ and $gp36_1$ of the clone BgB1 while in the (-) segment 3´ parts of the sequences are exchanged resulting in the putative proteins $gp34_{52}$ and $gp36_{52}$ of the clone BgB52. However, analysis on the protein level showed that there is no expression of gp36 neither in BgB1 nor in BgB52 but an expression of both, $gp34_1$ (in BgB1) and $gp34_{52}$ (in BgB52) in the late-logarithmic phase and at the onset of lysis. The presence of int1 could be observed during the entire life cycle of ϕCh1 with low expression rates during the first 60 h after inoculation and an increased expression 36 h before the onset of lysis (Klein *et al*., 2012).

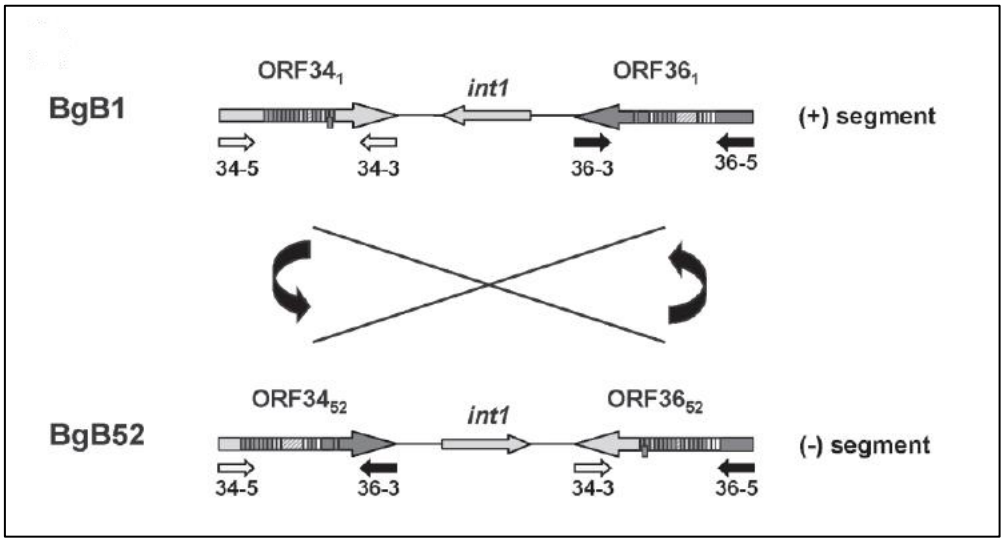

Fig. 18 Clone BgB1 contains the (+) segment with ORF34 and ORF36 orientated in opposite directions separated from each other by ORF35 (int1) with a leftward orientation (orientation is indicated by arrows). The (-) segment of the clone BgB52 is generated by a recombination event through the repeat clusters of ORF34 and ORF36 (boxes within ORF34 and ORF36 show repeat regions) resulting in an inversion of int1 and the exchange of 3´ parts of ORF34 and ORF 36 (indicated by the dark grey and light grey parts of the ORFs) (Klein *et al*., 2012).

To examine int1 in more details, an overexpression and isolation of the protein is sought. Overexpression in *E. coli* and following isolation as it is described in 2.2.8 was

not successfully when int1 was fused to a 6x His tag and detected by western blot with the help of anti-His antibodies as well as with anti-int1 antibodies. In the course of another study, Bea Alte introduced a tryptophan-inducible promoter for *Nab. magadii*, *tnaN*. Such an inducible promoter derived from a tryptophanase gene was first described in 2009 for the halophilic Archaeum *Hfx. volcanii* (Large *et al*., 2007). There, a tryptophanase gene was found within the sequence of *Nab. magadii* containing 450 amino acids with a weight of 50 kDa (Halolex, 2011). The fusion of the promoter *tnaN* to the halophilic ß-galactosidase which served as reporter gene and the following measure of the activity of this protein led to the finding that the promoter is inducible with tryptophan and that there is almost no background activity of the promoter itself. As addition of high concentrations of the inducer tryptophan has negative effects on the growth of *Nab. magadii*, the optimal concentration was determined to 2 mM tryptophan, dissolved in 1M NaOH (Alte, 2011).

To use the promoter *tnaN* for the overexpression of the Integrase, the int1 gene was cloned onto the vector pRo-5 carrying *tnaN* and a 6x His tag. The tag serves for the isolation of the protein via affinity chromatography with the help of the chelate Ni-NTA after overexpression. The vector then was transformed into L13 and a preparatory culture was cultivated in NVM+. For the inoculation with tryptophan a culture with an OD of 0.1 was estimated in 100 ml of the selective medium $NMMb^+$ which does not contain tryptophan. Induction occurred the first time after 12 hours with a final concentration of 2 mM tryptophan and then every 72 hours. 1 ml of the culture was taken as a sample before the first induction and then every time before tryptophan was added. Cultivation and induction was done in duplicates. The OD of one culture was measured with 600 nm while the other was determined with 700 nm. This was necessary because of the red discoloring of the culture in $NMMb^+$ deriving from the carotenoid pigments that are integrated in the membranes of *Nab. magadii*, as it can not be excluded that the red-color adulterates the value of the OD at 600 nm.

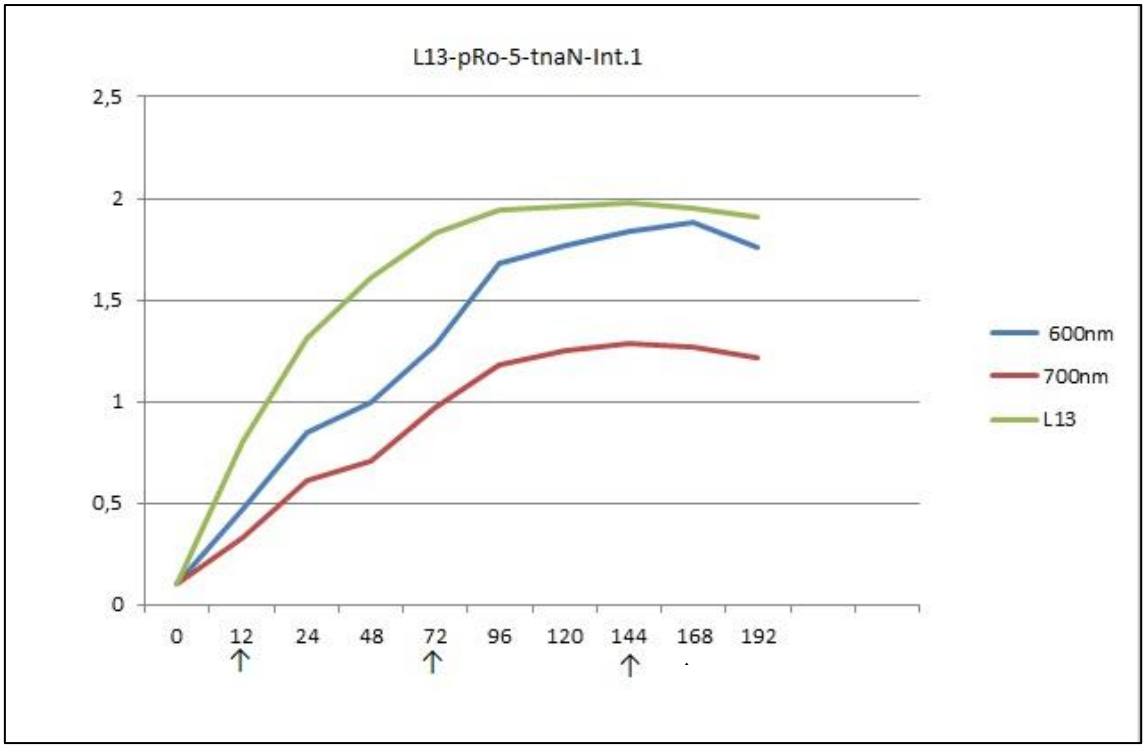

Fig. 19 Growth curve of L13-pRo-5-*tnaN*-int1 and L13 wt. The measurement of the optical density of L13-pRo-5-*tnaN*-int1 occurred at 600 nm (blue line) and 700 nm (red line). The arrows represent the time point at which the inducer tryptophan was added.

Figure 19 shows that the growth of L13-pRo-5-*tanN*-int1 is in accordance with the growth of L13 wt. The induction with tryptophan has no influence on growth.

The taken samples were centrifuged at 13 krpm for 5 minutes, the pellet was resuspended in 5 mM Na phosphate buffer (volume calculated by OD x 75, in µl) and the same volume of Laemmli buffer was added. The proteins then were separated via SDS page and int1 was detected by western blot analysis with anti-Integrase1 antibodies. However, it was not possible to detect any signal indicating the presence of int1 and thus no overexpression could be observed.

3.3.2 gp34

The gene products of ORF34 and ORF36, gp34 and gp36, represent putative tail fibre proteins with gp34 involved in the attachment of ϕCh1 to the cell surface of *Nab. magadii*. This assumption is strengthened by the finding of a C-terminal part of gp34 which shows similarities to a galactose-binding domain. Such galactose-binding domains are known to form the binding pocket of α-D-galactose and are parts of certain enzymes like the *E. coli* galactose oxidase or sialidase which are responsible for the

binding to specific cell surface-attached carbohydrate ligands (Ito *et al*., 1991). As shown in figure 20, the galactose-binding domain of the C-terminal part of $gp34_{52}$ contains conserved amino acid sequences which could not be found in $gp34_1$. Therefore, different binding assays were performed. Purified $gp34_1$ and $gp34_{52}$ samples were incubated with L13 *Nab. magadii* cells and after centrifugation $gp34_{52}$ could be detected in the pellet fraction in contrast to $gp34_1$ which was exclusively found in the supernatant. That indicates that just $gp34_{52}$ but not $gp34_1$ is responsible for the binding to the host cell. To prove that the galactose binding domain is the central part of binding the assay was repeated with $gp34_{52}$-C, a mutant protein lacking the N-terminal part. Again the protein could be detected in the pellet fraction and not in the supernatant, confirming that the galactose-binding domain is needed for attachment to the cell surface. To prove that, one of the conserved AA residues, a tryptophan residue involved in binding to the cell surface, was changed to an arginine leading to the loss of binding of $gp34_{52}$. As control residues of two amino acids located outside but close to the galactose-binding domain were exchanged, this caused no influence on the binding of $gp34_{52}$. By increasing the concentration of α-D-galactose in the presence of $gp34_{52}$-C a decrease in binding of not just gp34 but also of whole virions could be observed. The reason therefore is that the binding pockets of gp34 are occupied by the galactose and thus gp34 is not able to bind to the cell surface of the host. As the addition of α-D-glucose, α-D-mannose and α-D-sucrose have no influence on the binding of gp34 to *Nab. magadii*, α-D-galactose is the predominant binding ligand (Klein *et al*., 2012).

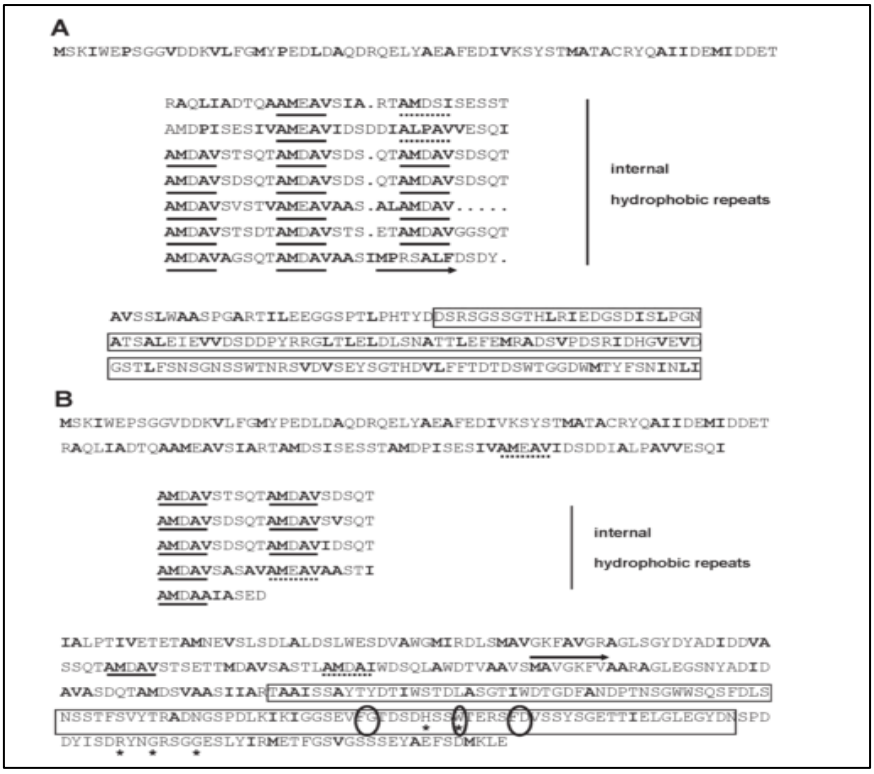

Fig. 20 Illustration of the C-terminal parts of $gp34_1$ (A) and $gp34_{52}$ (B) with the galactose-binding domains represented in boxes. Repeats are underlined and clustered and hydrophobic amino acid residues are indicated in bolt. B: Asterisks below the sequence show conserved amino acid residues within the galactose-binding domain. The circles indicate the exchanged amino residues resulting in point mutations (Klein *et al*., 2012).

In this study the binding of $gp34_{52}$ of *Nab. magadii* to another haloalkalophilic strain, *Nbt. gregoryi*, was analyzed. Therefore certain amounts of $gp34_{52}$ were added to 1.5 ml of the culture and incubated at 37°C for 1 hour. Thereupon the culture was centrifuged and protein samples from the pellet as well as from the supernatant were prepared. Detection of gp34 was performed via SDS-Page and following western blot analysis with anti-$gp34_{52}$ antibodies.

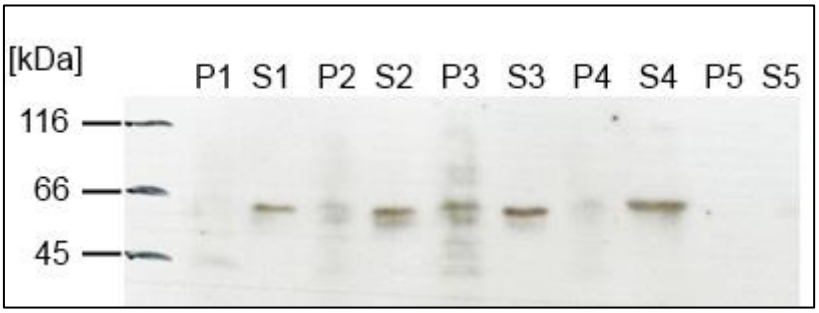

Fig. 21 Results of the western blot analysis of protein samples of *Nbt. gregoryi*, incubated with $gp34_{52}$. P: Pellet. S: Supernatant. Different amounts of $gp34_{52}$ were added, starting with 100 ng which complies with 3 µl (P1 and S1) going up to 300 ng which complies with 9 µl (P4 and S4). As control protein samples of *Nbt. gregoryi* were analyzed without the addition of $gp34_{52}$ (P5 and S5). With the exception of the control a signal can be observed in every fraction whereas a higher amount of $gp34_{52}$ can be detected in the supernatant compared to the pellet fractions.

Figure 21 shows the result of the western blot analysis. $gp34_{52}$ (~ 66 kDa) could be detected mainly in the supernatant fractions although a light signal can be observed in the pellet fractions. A protein sample without the addition of $gp34_{52}$ served as control which shows no signal at all.

3.3.3 Discussion

Archaea are heterotrophic organisms thus they are able to exploit organic compounds like the essential amino acid tryptophan with the help of the enzyme tryptophanase which catalyzes the cleavage of tryptophan to indole, pyruvic acid and ammonia. As the production of tryptophanase is energetically expensive for the cell, its promoter *tnaN* is only activated in the presence of tryptophan. Therefore the *tnaN* promoter was chosen to be the first inducible promoter for the overexpression of certain proteins of interest in *Nab. magadii*. With the help of the reporter gene encoding for the halophilic ß-galactosidase it was proven that *tnaN* shows only activity when induced with tryptophan and that there is no appreciable background activity. This turns *tnaN* into an ideal inducible promoter with which it should be possible to overexpress proteins of interest. In this study the overexpression of the integrase1 was aspired, but it was not possible to achieve a high expression. This could be explained by the expression of the tryptophanase of *Nab. magadii* which is still intact and activated by tryptophan resulting in the degradation of the amino acid when added to the medium. Consequently, a deletion of the archaeal tryptophanase should be aspired and overexpression should be performed in the deletion strain. With such arrangements it should be possible to overexpress a number of genes of interest in *Nab. magadii*.

The binding of the tail fibre protein $gp34_{52}$ of the virus ϕCh1 to another Archaeum than *Nab. magadii* could not be observed yet. Even in the natural habitat of *Nab. magadii* and thus ϕCh1, lake magadii, no information about infection of other strains is documented. This led to the idea to examine the binding of $gp34_{52}$ to the haloalkalophilic Archaeum *Nbt. gregoryi* in the lab. The method of adding $gp34_{52}$ to a culture to prove the binding ability of the protein to the surface was already applied when the localization of

the binding pocket of gp34 was determined. However, binding analysis of $gp34_{52}$ to the surface of *Nbt. gregoryi* did not lead to an explicit result. A signal of $gp34_{52}$ can be observed in the supernatant fraction but also a light signal arises from the pellet fractions. This could be explained by taking of the supernatant not properly enough after incubation of *Nbt. gregoryi* with $gp34_{52}$, leading to a small amount of supernatant in the pellet fraction before preparing the protein samples. The absence of a signal in the control fractions indicates that the anti-34_{52} antibodies do not bind to other, *Nbt gregoryi* own proteins. Thus nonspecifically binding of the antibody can be excluded. The experiment should be repeated more precisely to obtain a definite result, which can then be used to examine also the binding of $gp34_{52}$ to other archaeal strains than *Nbt. gregoryi*.

4 References

Achenbach-Richter, L., Gupta, R., Stetter, K.O., Woese, C.R. (1987) Were the original eubacteria thermophiles? *Systematic and applied microbiology* **9**: 34–39

Achenbach-Richter, L., Gupta, R., Zillig, W., Woese, C.R. (1988) Rooting the archaebacterial tree: the pivotal role of Thermococcus celer in archaebacterial evolution. *Systematic and applied microbiology* **10**: 231–240

Alte, B. (2011) Konstruktion einer Flagellum-Deletionsmutante in *Natrialba magadii* und Charakterisierung bestehender *Natrialba magadii* Mutanten. *Diploma thesis, Universität Wien*

Aono, R., Horikoshi, K. (1983) Chemical Composition of Cell Walls of Alkalophilic Strains of Bacillus. *Microbiology* **129**: 1083–1087

Arnold, H.P., Ziese, U., Zillig, W. (2000) SNDV, a novel virus of the extremely thermophilic and acidophilic archaeon Sulfolobus. *Virology* **272**: 409–416

Bath, C., Cukalac, T., Porter, K., Dyall-Smith, M.L. (2006) His1 and His2 are distantly related, spindle-shaped haloviruses belonging to the novel virus group, Salterprovirus. *Virology* **350**: 228–239

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

Baumann, P., Jackson, S.P. (1996) An archaebacterial homologue of the essential eubacterial cell division protein FtsZ. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 6726–6730

Bell, S.D., Jackson, S.P. (1998) Transcription and translation in Archaea: a mosaic of eukaryal and bacterial features. *Trends in Microbiology* **6**: 222–228

Birkenbihl, R.P., Neef, K., Prangishvili, D., Kemper, B. (2001) Holliday junction resolving enzymes of archaeal viruses SIRV1 and SIRV2. *J. Mol. Biol.* **309**: 1067–1076

Brochier-Armanet, C., Boussau, B., Gribaldo, S., Forterre, P. (2008) Mesophilic Crenarchaeota: proposal for a third archaeal phylum, the Thaumarchaeota. *Nature reviews. Microbiology* **6**: 245–252

Brown, A.D. (1990) Microbial water stress physiology. Principles and perspectives. *John Wiley & Sons, Ltd., Chichester, United Kingdom*

Brown, J.R., Doolittle, W.F. (1997) Archaea and the prokaryote-to-eukaryote transition. *Microbiology and Molecular Biology Reviews* **61**: 456–502

Brown, J.W., Daniels, C.J., Reeve, J.N. (1989) Gene structure, organization, and expression in archaebacteria. *Critical Reviews in Microbiology* **16**: 287–338

Cammarano, P., Teichner, A., Londei, P., Acca, M., Nicolaus, B., Sanz, J.L., Amils, R. (1985) Insensitivity of archaebacterial ribosomes to protein synthesis inhibitors. Evolutionary implications. *The EMBO journal* **4**: 811–816

Chatton, E. (1938) Titre et travaux scientifique (1906-1937) de Edouard Chatton. *E. Sottano, Sète, France*

Cline, S.W., Doolittle, W.F. (1987) Efficient transfection of the archaebacterium Halobacterium halobium. *Journal of bacteriology* **169**: 1341–1344

Cubonová, L., Sandman, K., Hallam, S.J., DeLong, E.F., Reeve, J.N. (2005) Histones in crenarchaea. *Journal of Bacteriology* **187**: 5482–5485

DeLong, E.F. (1992) Archaea in coastal marine environments. *Proceedings of the National Academy of Sciences of the United States of America* **89**: 5685–5689

Dennis, P.P. (1997) Ancient ciphers: translation in Archaea. *Cell* **89**: 1007–1010

Devereux, R., He, S.H., Doyle, C.L., Orkland, S., Stahl, D.A., LeGall, J., Whitman, W.B. (1990) Diversity and origin of Desulfovibrio species: phylogenetic definition of a family. *J. Bacteriol*. **172**: 3609-3619

DiMarco, A.A., Bobik, T.A., Wolfe, R.S. (1990) Unusual coenzymes of methanogenesis. *Annual Review of Biochemistry* **59**: 355–394

Dyall-Smith, M., Tang, S.L., Bath, C. (2003) Haloarchaeal viruses: how diverse are they? *Res. Microbiol*. **154**: 309–313

Elkins, J.G., Podar, M., Graham, D.E., Makarova, K.S., Wolf, Y., Randau, L., Hedlund, B.P., et al. (2008) A korarchaeal genome reveals insights into the evolution of the Archaea. *Proceedings of the National Academy of Sciences of the United States of America* **105**: 8102–8107

Fry, N. K., Wanvick, S., Saunders, N.A., Embley, T.M. (1991) The use of 16s ribosomal RNA analyses to investigate the phylogeny of the family Legionrllaceae. *J. Gen. Microbiol*. **137**: 1215-1222

Fuhrman, J.A., McCallum, K., Davis, A.A. (1992) Novel major archaebacterial group from marine plankton. **Nature 356**: 148–149

Galinski E.A. (1995) Osmoadaptation in bacteria. *Adv Microb Physiol* **37**: 273–328

Gambacorta, A., Trincone, A., Nicolaus, B., Lama, L., Derosa, M. (1994) Unique features of lipids of Archaea. *Systematic and Applied Microbiology* **16**: 518–527

Haeckel, E. (1866) Generelle Morphologie der Organismen. *Reimer, Berlin*

Haring, M. et al. (2005) Virology: independent virus development outside a host. *Nature* **436**: 1101–1102

Haring, M., Rachel, R., Peng, X., Garrett, R.A., Prangishvili, D. (2005) Viral diversity in hot springs of Pozzuoli, Italy, and characterization of a unique archaeal virus, Acidianus bottle-shaped virus, from a new family, the Ampullaviridae. *J. Virol*. **79**: 9904–9911

Holmes, M.L., Nuttall, S.D., Dyall-smith, M.L. (1991) Construction and Use of Halobacterial Shuttle Vectors and Further Studies on Haloferax DNA Gyrase. *Journal of Bacteriology* **173**: 3807-3813

Horikoshi, K. (1999) Alkaliphiles: some applications of their products for biotechnology. *Microbiology and Molecular Biology Reviews* **63**: 735-750

Huber, H., Burggraf, S., Mayer, T., Wyschkony, I., Rachel, R., Stetter, K.O. (2000) Ignicoccus gen. nov., a novel genus of hyperthermophilic, chemolithoautotrophic Ar-

chaea, represented by two new species, Ignicoccus islandicus sp nov and Ignicoccus pacificus sp nov. and Ignicoccus pacificus sp. nov. *International journal of systematic and evolutionary microbiology* **50**: 2093–2100

Huber, H., Hohn, M.J., Rachel, R., Fuchs, T., Wimmer, V.C., Stetter, K.O. (2002) A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. *Nature* **417**: 63–67

Hudepohl, U.W.E. (1990) Mutational analysis of an archaebacterial promoter: Essential role of a TATA box for transcription efficiency and start-site selection. *Proc. Natl. Acad. Sci*. **87**: 9509–9513

Iro, M., Klein, R., Gálos, B., Baranyi, U., Rössler, N., Witte, A. (2006) The lysogenic region of virus phiCh1: identification of a repressor-operator system and determination of its activity in halophilic Archaea. *Extremophiles* **11**: 383–396

Ito, N., Phillips, S.E., Stevens, C., Ogel, Z.B., McPherson, M.J., Keen, J.N., et al. (1991) Novel thioether bond revealed by a 1.7 A crystal structure of galactose oxidase. *Nature* **350**: 87–90

Kaine, B.P., Mehr, I.J., Woese, C.R. (1994) The sequence, and its evolutionary implications, of a Thermococcus celer protein associated with transcription. *Proceedings of the National Academy of Sciences of the United States of America* **91**: 3854–3856

Kamekura, M. Dyall-Smith, M.L., Upasani, V., Ventosa, A., Kates, M. (1997) 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 com. nov., and Natronomonas Pharaonis cob. nov., respectively. *International journal of Systematic Bacteriology* **47**: 853–857

Kamekura, M., Dyall-Smith, M.L. (1996) Taxonomy of the family halobacteriaceae and the description of two new genera Halorubrobacterium and Natrialba. *Journal of General and Applied Microbiology* **41**: 333–350

Klein, R., Baranyi, U., Rössler, N., Greineder, B., Scholz, H., Witte, A. (2002) Natrialba magadii virus phiCh1: first complete nucleotide sequence and functional organization of a virus infecting a haloalkaliphilic archaeon. *Molecular microbiology* **45**: 851–863

Klein, R., Rössler, N., Iro, M., Scholz, H., Witte, A. (2012) Haloarchaeal myovirus φCh1 harbours a phase variation system for the production of protein variants with distinct cell surface adhesion specificities. *Molecular microbiology* **83**: 137–150

Klenk, H., Clayton, R.A., Tomb, J., Dodson, R.J., Gwinn, M., Hickey, E.K., Fleischmann, R.D., et al. (1997) The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon. *Nature* **390**: 364-370

König H, Kandler, O. (1979) N-Acetyltalosaminuronic acid aconstituent of the pseudomurein of the genus Methanobacterium. *Arch Microbiol* **123**: 295–299

Konstantinidis, K., Tebbe, A. (2007) Genome-wide proteomics of Natronomonas pharaonis. *Journal of Proteome Research* **6**: 185–193

Kurono, Y., Horikoshi, K. (1973) Alkaline catalase produced by Bacillus no. Ku-1. *Agric. Biol. Chem.* **37**: 2565–2570

Kushner, D.J. (1978) Life in high salt and solute concentrations. In Microbial Life in Extreme Environments. *London: Academic Press* **1978**: 317-368

Kushner, D.J. (1985) The Halobacteriaceae. *The bacteria* **8**: 171-214

Langer, D., Hain, J., Thuriaux, P., Zillig, W. (1995) Transcription in archaea: similarity to that in eucarya. *Proceedings of the National Academy of Sciences of the United States of America* **92**: 5768–5772

Langer, D., Zillig, W. (1993) Putative tfIIs gene of Sulfolobus acidocaldarius encoding an archaeal transcription elongation factor is situated directly downstream of the gene for a small subunit of DNA-dependent RNA polymerase. *Nucleic acids research* **21**: 2251

Lanyi, J.K. (1974) Salt-dependent properties of proteins from extremely halophilic bacteria. *Bacteriological reviews* **38**: 272–290

List of Prokaryotic names with Standing in Nomenclature. Internet: http://www.bacterio.cict.fr/index.html

Lodwick, D., Ross, H.N., Harris, J.E., Almond, J.W., Grant, W.D. (1986) Dam Methylation in the Archaebacteria. *Journal of general microbiology* **132**: 3055–3059

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

Makula, R.A., Singer, M.E. (1978) Ether-containing lipids of methanogenic bacteria. *Biochemical and Biophysical Research Communications* **82**: 716–722

Martin, A. et al. (1984) SAV1, a temperate u.v.-inducible DNA virus-like particle from the archaebacterium Sulfolobus acidocaldarius isolate B12. *The EMBO Journal* **3**: 2165–2168

Mayrhofer-Iro, M., Ladurner, A., Meissner, C., Derntl, C., Reiter, M., Haider, F., Dimmel, K., et al. (2013) Utilization of Virus Ch1 Elements To Establish a Shuttle Vector System for Halo(alkali)philic Archaea via Transformation of Natrialba magadii. *Applied and environmental microbiology* **79**: 2741–2748

Mullakhanbhai, M.F., Larsen, H. (1975) Halobacterium volcanii spec. nov., a Dead Sea halobacterium with a moderate salt requirement. *Archives of microbiology* **104**: 207–214

Muskhelishvili, G., Palm, P., Zillig, W. (1993) SSV1-encoded site-specific recombination system in Sulfolobus shibatae. *Mol. Gen. Genet*. **237**: 334–342

Nadal, M., Mirambeau, G., Forterre, P., Reiter, W.-D., Duguet, M. (1986) Positively supercoiled DNA in a virus-like-particle of an archaebacterium. *Nature* **321**: 256–258

Nölling, J., Willem, M. (1992) Characterization of the Archaeal, Plasmid-Encoded Type II Restriction-Modification System MthTI from Methanobacterium thennoformicicum THF: Homology to the Bacterial NgoPII System from Neisseria gonorrhoeae. *Journal of Bacteriology* **174**: 5719-5726

Oren, A. (1999) Bioenergetic Aspects of Halophilism. *Microbiol. Mol. Biol. Rev.* **63**: 334

Oren, A. (2002) Diversity of halophilic microorganisms: environments, phylogeny, physiology, and applications. *Journal of industrial microbiology & biotechnology* **28**: 56–63

Oren, A., Bratbak, G., Heldal, M. (1997) Occurrence of virus-like particles in the Dead Sea. *Extremophiles life under extreme conditions* **1**: 143–149

Ouzounis, C. and Sander, C. (1992) TFIIB, an Evolutionary Link between the Transcription Machineries of Archaebacteria and Eukaryotes. *Cell* **71**: 189–190

Pavli, V., Kmetec, V. (2006) Pathways of chemical degradation of polypep- tide antibiotic bacitracin. *Biol. Pharm. Bull*. **29**: 2160–2167

Prangishvili, D. (2003) Evolutionary insights from studies on viruses of hyperthermophilic archaea. *Res. Microbiol*. **154**: 289–294

Prangishvili, D., et al. (1006) Structural and genomic properties of the hyperthermophilic archaeal virus ATV with an extracellular stage of the reproductive cycle.J. *Mol. Biol*. **359**: 1203–1216

Prangishvili, D., et al. (1999) A novel virus family, the Rudiviridae: Structure, virus-host interactions and genome variability of the Sulfolobus viruses SIRV1 and SIRV2. *Genetics* **152**: 1387–1396

Prangishvili, D., Forterre, P., Garrett, R.A. (2006) Viruses of the Archaea: a unifying view. *Nature reviews. Microbiology* **4**: 837–848

Puhler, G., Lefferst, H., Gropp, F., Palm, P., Klenk, H., Lottspeich, F., Garrett, R.A., et al. (1989) Archaebacterial DNA-dependent RNA polymerases testify to the evolution of the eukaryotic nuclear genome. *Proc. Natl. Acad. Sci. USA* **86**: 4569–4573

Reanny, D.C., Ackermann, H.-W. (1982) Comparative biology and evolution of bacteriophages. *Adv Virus Res* **27**: 205–280

Reistad, R. (1970) On the composition and nature of the bulk protein of extremely halophilic bacteria. *Arch Microbiol* **71**: 353–360

Reiter, M. (2010) Gene regulation of φCh1: characterization of ORF49 and further characterization of the origin of replication of the halophage φCh1. *Diploma thesis, Universität Wien*

Rohrmann, G.F., Cheney, R., Pauling, C. (1983) Bacteriophages of Halobacterium halobium: virion DNAs and proteins. *Can. J. Microbiol*. **29**: 627–629

Rössler, N., Klein, R., Scholz, H., Witte, A. (2004) Inversion within the haloalkaliphilic virus phi Ch1 DNA results in differential expression of structural proteins. *Molecular Microbiology* **52**: 413-426

Rowlands, T., Baumann, P., Jackson, S.P. (1994) The TATA-binding protein: a general transcription factor in eukaryotes and archaebacteria. *Science* **264**: 1326–1329

Sandmeier, H. (1994) Acquisition and rearrangement of sequence motifs in the evolution of bacteriophage tail fibres. *Molecular Microbiology* **12**: 343–350

Schleper, C., Swanson, R.V., Mathur, E.J., DeLong, E. F. (1997) Characterization of a DNA polymerase from the uncultivated psychrophilic archaeon Cenarchaeum symbiosum. *Journal of Bacteriology* **179**: 7803–7811

Schnabel, H. (1984) An immune strain of Halobacterium halobium carries the invertible L segment of phage PhiH as a plasmid. *Proceedings of the National Academy of Sciences of the United States of America* **81**: 1017–1020

Schnabel, H., Zillig, W., Pfäffle, M., Schnabel, R., Michel, H., Delius, H. (1982) Halobacterium halobium phage øH. *The EMBO journal* **1**: 87–92

Sleytr, U., Messner, P., Pum, D., Sara, M. (1999) Crystalline bacterial cell surface layers (S layers): from supramolecular cell structure to biomimetics and nanotechnology. *Angew. Chem. Int. Ed*. **38**: 1034-1054

Soliman, G.S.H., Trueper, H.G. (1982) Halobacterium-pharaonis new- species a new extremely halo alkaliphilic archaebacterium with low magnesium requirement. *Zentralbl. Mikrobiol*. **3**: 318-329

Sprott, G.D., Sciences, B. (2011) Archaeal Membrane Lipids and Applications. *Encyclopedia of Life Sciences*

Staley, J.T., Bryant, M.P., Pfennig, N., Holt, J.G. (1989) Bergey's Manual of Systematic Bacteriology. **3**: 2230-2232

Stetter, K.0. (1982) Ultrathin mycelia-forming organisms from submarine volcanic areas having an optimum growth temper- ature of 105°C. *Nature (London)* **300**: 258-260

Stetter, K.O., Zillig, W. (1985) Thermoplasma and the thermophilic sulfur-dependent archaebacteria. *In C. R. Woese and R. S. Wolfe (ed.), The bacteria* **8**: 85-170

Stolt, P., Zillig, W. (1992) In vivo studies on the effects of immunity genes on early lytic transcription in the Halobacterium salinarium phage phi H. *Molecular general genetics* **235**: 197–204

Strominger, L. (1976) Structural (shape-maintaining) role of the cell surface glycoprotein of Halobacterium salinarium. *Proc. Natl. Acad. Sci. USA* **73**: 2687–2691

Svoboda T. (2011) Characterization of putative repressors of the temperate phage φCh1 and analysis of the flagellum operon as a putative receptor of φCh1. *Diploma thesis, Universität Wien*

Tang, S.L., Nuttall, S., Ngui, K., Fisher, C., Lopez, P., Dyall-Smith, M. (2002) HF2: A double-stranded DNA tailed haloarchaeal virus with a mosaic genome. *Mol. Microbiol*. **44**: 283–296

Thauer, R.K., Jungermann, K., Decker, K. (1977) Energy Conservation in Chemotrophic Anaerobic Bacteria. *Bacteriological Reviews* **41**: 100–180

Tindall, B., Ross, H. and Grant, W. (1984) Natronobacterium gen. nov. and Natronococcus gen. nov., two new genera of haloalkaliphilic archaebacteria. *Systematic and Applied Microbiology* **5**: 41-57

Torsvik, T., Dundas, I.D. (1974) Bacteriophage of Halobacterium salinarium. *Nature* **248**: 680–681

Van de Vossenberg, J.L., Driessen, A. J., Konings, W.N. (1998) The essence of being extremophilic: the role of the unique archaeal membrane lipids. *Extremophiles: life under extreme conditions* **2**: 163–170

Wai-Leung N.G., DasSarma S. (1993) Minimal replication origin of the 200- kilobase Halobacterium plasmid pNRC100. *J. Bacteriol.* **175**: 4584–4596

Whittaker, R.H. (1959) On the broad classification of organisms. *The Quarterly review of biology* 34: 210–226

Wieland, F., Lechner, J., Bernhardt, G., Sumper, M. (1981) Halobacterial glycoprotein saccharides contain covalently-linked sulphate. *FEBS Lett*. **132**: 319–323

Witte, A., Baranyi, U., Klein, R., Sulzner, M., Luo, C., Wanner, G., Krüger, D.H., et al. (1997) Characterization of Natronobacterium magadii phage phi Ch1, a unique archaeal phage containing DNA and RNA. *Molecular microbiology* 23: 603–616

Woese, C.R. (1987) Bacterial Evolution. *Microbiological Reviews* **51**: 221–271

Woese, C.R., Fox, G. E. (1977) Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proceedings of the National Academy of Sciences of the United States of America* 74: 5088–5090

Woese, C.R., Gupta, R., Hahn, C.M., Zillig, W., Tu, J. (1984) The phylogenetic relationships of three sulfur dependent archaebacteria. *Systematic and applied microbiology* **5**: 97–105

Woese, C.R., Kandler, O., Wheelis, M. L. (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proceedings of the National Academy of Sciences of the United States of America* **87**: 4576–4579

Zillig, W., Stetter, K.O., Janeković, D. (1979) DNA-dependent RNA polymerase from the archaebacterium Sulfolobus acidocaldarius. *The Federation of European Biochemical Societies Journal* 96: 597–604

5 List of Figures

Acknowlegement

First of all I want to thank Prof. Dr. Angela Witte for the continuous support of my diploma thesis, for her patience, motivation, enthusiasm and comprehensive knowledge.

In addition, I would like to thank my colleagues Katharina Dimmel and Lea Schöner for our enriching discussions about our work. They were not just valuable colleagues but became also friends of mine.

My thanks also go to Petra Till, Tatjana Svoboda and Beatrix Alte who accompanied and supported me through the first weeks of my work. Their theoretical and practical knowledge was a great enrichment and support for my further research.

Especial thanks go to my family who always was open minded and interested in my work. I am very grateful for the support of my parents Anna and David during my whole studies and for the emotional assistance of my sister Esther.

Last but not least I want to thank my friends Johanna Grubner and Agnes Raschauer as well as my friend Pascal Pal who encouraged me during the whole time of my diploma work.

Curriculum Vitae

Personal details:

Name:	Judith Beraha
Nationality:	German

Education:

1991 - 1995	Grundschule Grüner Markt, Erding, Germany
1995 - 2002	Gymnasium Erding, Germany
2002 - 2005	Franz-Marc-Gymnasium, Markt Schwaben, Germany
2006 - 2013	Studies of “Biology/Genetics and Microbiology” (Diploma course), University of Vienna, Austria
2011 - 2012	Diploma thesis in the laboratory of Dr. Angela Witte at the “Department for Microbiology, Immunobiology and Genetics” of the University of Vienna, Austria

Working Experience:

2005	Homework assistance at the Herzog Tassilo Realschule Erding, Germany
WS 2011/2012	Tutor of students for practical trainings in the course of the studies in “BioEngineering”, FH Campus Vienna (University of applied Sciences), Austria

SS 2012	Tutor of students for practical trainings in the course of the studies in “Molekulare Biotechnologie”, FH Campus Vienna (University of applied Sciences), Austria
SS 2012	Tutor of students for practical trainings in “Übung in Molekularer Mikrobiologie” and “Übungen III A – Molekularbiologische Laborarbeiten”, University of Vienna, Austria
WS 2012/2013	Tutor of students for practical trainings in the course of the studies in “Biotechnologie”, FH Campus Vienna (University of applied Sciences), Austria
WS 2012/2013	Tutor of students for practical trainings in “Übungen III A – Molekularbiologische Laborarbeiten”, University of Vienna, Austria
SS 2013	Tutor of students for practical trainings in “Übungen III A – Molekularbiologische Laborarbeiten”, University of Vienna, Austria