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„Investigating the life cycle of a haloalkaliphilic virus: Further characterization of the regulatory ORF79 of halovirus ϕ Ch1 as well as the construction of *aglB* and *pilin* deletion mutants in its host *Natrialba magadii*.“

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Abstract

ϕ Ch1 of *Natrialba magadii* was the first virus to be described that infects an haloalkaliphilic archaeon. Research on halophilic and extremophilic organisms in general contributes significantly to a better understanding of the evolution of life on earth, as well as to the development of industrial applications. This thesis examines molecular regulators and elements of the ϕ Ch1 life cycle.

In preliminary studies, the ORF79 gene product of ϕ Ch1 was shown to act as a repressor on the lytic cycle of the virus. An ORF79-deficient strain ϕ Ch1- Δ ORF79 showed an earlier onset of lysis, as well as a deregulation of at least one essential structural virus capsid protein. Here this regulatory element was further characterized. A transcription analysis of the ORF79 mRNA using RT-PCR combined with Southern blotting showed a constitutive expression of the gene.

Analysis of the structural protein composition in the ORF79-deficient virus strain ϕ Ch1- Δ ORF79 revealed no evident differences between the mutant and the wild-type strains. These findings, combined with preliminarily conducted electron micrographs of the two strains, show that the ORF79 has no influence on the morphology of ϕ Ch1. In this thesis, growth parameters of ϕ Ch1 and ϕ Ch1- Δ ORF79 were also determined and compared. The ORF79-deficient strain shows a prolonged eclipse period as well as a three-fold reduced burst size. The studies on the ORF79 conducted during the course of this thesis will contribute to a more comprehensive understanding of this regulator of the ϕ Ch1 live cycle.

An important question related to research on ϕ Ch1 is what determines the virus receptor on the surface of its host, *Natrialba magadii*. Glycosylated surface proteins often serve as viral receptors involving recognition and adhesion to the host cell. Archaeal AglB and Pilin proteins are involved in the assembly and composition of glycosylated surface structures. The second part of this thesis details the construction of *aglB* and *pilin* deletion mutants in the archaeon *N. magadii*. Since the mutants'

construction was not completed in the course of this thesis, it will provide a meaningful task for future studies.

Zusammenfassung

Der *Natrialba magadii* Virus ϕ Ch1 war der erste beschriebene Virus, der haloalkaliphile *Archaeen* infiziert. Die Forschung an halophilen und generell extremophilen Organismen trägt signifikant zum Verständnis der Evolution des Lebens auf der Erde sowie zur Entwicklung von industriellen Anwendungen bei. Diese Arbeit befasst sich mit den molekularen Regulatoren und Elementen des Lebenszyklus von ϕ Ch1.

In vorangegangenen Studien wurde gezeigt, dass das ORF79 Genprodukt von ϕ Ch1 als ein Repressor des lytischen Zyklus dieses Virus wirkt. Der ORF79-Deletionsstamm ϕ Ch1- Δ ORF79 zeigte einen früheren Beginn der Lyse sowie eine Deregulation mindestens eines essentiellen strukturellen Viruscapsidproteins. Dieses regulatorische Element wurde hier näher untersucht. Eine transkriptionelle Analyse des ORF79 mRNA mithilfe von RT-PCR kombiniert mit Southern Blot zeigte eine konstitutive Expression des Gens.

Untersuchungen der Zusammensetzung von strukturellen Proteinen im ORF79-Deletionsstamm ϕ Ch1- Δ ORF79 offenbarten keine augenscheinlichen Unterschiede zwischen der Mutante und dem Wildtyp. Diese Beobachtungen in Kombination mit vorangegangenen Elektronenmikroskopaufnahmen der beiden Stämme zeigen, dass der ORF79 keine Auswirkungen auf die Morphologie des Virus ϕ Ch1 hat. Des Weiteren wurden Wachstumsparameter von ϕ Ch1 und ϕ Ch1- Δ ORF79 untersucht. Die ORF79-Mutante weist eine verlängerte Eclipseperiode sowie eine um den Faktor drei reduzierte Burst Size auf. Die Untersuchungen des ORF79 im Rahmen dieser Arbeit werden zu einem umfassenderen Verständnis dieses Regulators der Entwicklung von ϕ Ch1 beitragen.

Eine wichtige Fragestellung in der Forschung mit ϕ Ch1 ist die Identifizierung des Virusrezeptors auf der Oberfläche des Wirtes *Natrialba magadii*. Glykosylierte Oberflächenproteine dienen oft als Virusrezeptoren, die für die Erkennung und Anhaftung an der Wirtszelle verantwortlich sind. AglB und Pilin-Proteine in

Archaeen sind im Zusammenbau und dem strukturellen Aufbau von glykosylierten Oberflächenstrukturen beteiligt. Der zweite Teil dieser Arbeit befasste sich mit der Konstruktion von *aglB* und *pilin* Deletionsmutanten in dem Archaeon *N. magadii*. Da die Konstruktion der Mutanten im Rahmen dieser Arbeit nicht fertig gestellt werden konnte, wird dies eine wichtige Aufgabe für zukünftige Studien darstellen.

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1. Introduction

1.1 *Archaea*

1.1.1 *Archaea* and their standing in the living world

On our planet, manifold cellular life is divided into three domains: *Bacteria*, *Archaea* and *Eukarya*. The *Eukarya*, which can be unicellular or multicellular, include the following kingdoms: Protista, Plantae, Fungi and Animalia. The *Bacteria* and *Archaea* are unicellular prokaryotic organisms which, compared to the *Eukarya*, lack cell organelles such as the nucleus [1]. This wide variety of life needs adequate means of classification. The relationship between the organisms and the three domains of life is of fundamental importance for understanding the origins of life on earth. This chapter focuses on historic and modern models that propose where to place the *Archaea* on the tree of life.

Modern biological nomenclature was introduced by Carl Linnaeus in the eighteenth century. He divided the living world into two kingdoms: the *Regnum Animale* for animals and the *Regnum Vegetabile* for plants [2]. Single-celled organisms were first included in phylogenetic systems in 1866 when Ernst Haeckel proposed a third kingdom, the *Protista* [3]. In 1938, Édouard Chatton distinguished between eukaryotes and prokaryotes based on the presence or absence of cell nuclei [4]. After their discovery, the *Archaea* were classified as part of the kingdom *Bacteria*. They were first distinguished from the *Bacteria* as a different group of prokaryotes and termed *Archaeobacteria* by Woese and Fox in 1977 [5].

In the early twentieth century, bacteria were characterised based on their metabolic and physiological features, as well as their morphological traits [6].

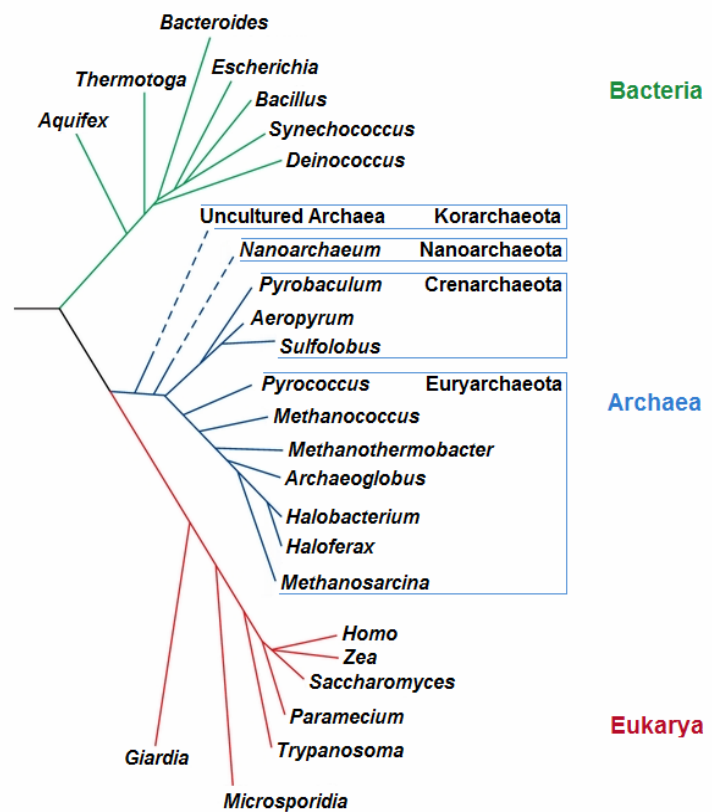
Later, DNA was discovered to be an important comparative feature, as it contains large amounts of information and exhibits variable as well as conserved elements. The use of the G+C composition ratios and DNA-DNA hybridization temperature differences marked a significant improvement in the characterization of organisms and microorganisms in particular [7]. An important breakthrough in the late twentieth century was the use of macromolecular sequences, mainly DNA and proteins, for phylogenetic analysis. In 1965, Zuckerkandl and Pauling proposed using gene sequences as a way to determine the relationship between organisms [8]. Woese's use of 16S/18S rRNA sequences marked a breakthrough in evolutionary research and phylogenetic studies. This molecule is present in all cellular organisms and is easy to isolate. Its large sequence conservation, along with adequately variable regions, provides an excellent tool for studying the relationships between organisms. By comparing 16S/18S rRNA sequences, Woese proposed the *Archaea* as a third domain, alongside the *Bacteria* and the *Eukarya*. He found that all three domains had specific signature features in their 16S/18S rRNA sequences. The rRNA sequence analyses also provided evidence that the *Archaea* were more closely related to the *Eukarya* than to the *Bacteria*. This modern three-domain system was introduced by Woese in 1990 and is largely accepted throughout the scientific community [9].

In contrast to the three-domain system, there are also other biological classifications present and still being developed. One of these alternative classifications is the Eocyte hypothesis. In 1984, it was proposed that the *Eukarya* originated from the *Crenarchaeota*, based on the discovery of new ribosomal structures. These structures showed greater similarities between the *Eukarya* and the *Crenarchaeota* than between the *Eukarya* and the *Crenarchaeota*; this was compared to the *Bacteria* and even other archaeal phyla [10].

The tree of life will continue to undergo changes in the future as new discoveries will reveal new relationships between organisms and challenge existing theories.

Figure 1: The three-domain system of life in a phylogenetic tree based on 16S/18S rRNA sequences.

Schematic representation of the living world on earth. The division of life into the three domains *Bacteria*, *Archaea* and *Eukarya* is based on the classification by Carl Woese. The classification within the tree of life relies on the comparison of 16S ribosomal RNA sequences. This system classifies the *Archaea* as more closely related to the *Eukarya* through a common ancestor. The *Archaea* consist of several phyla, with the *Euryarchaeota* being the most extensively studied. The *Crenarchaeota* comprise numerous extremophiles that are adapted to even the most extreme biological habitats. New sequencing and phylogenetic methods lead to the discovery of novel phyla, such as the *Korarchaeota*. Therefore, the tree of life will continue to be changed and complemented in the future [11].



1.1.2 Origin and diversity of the *Archaea*

On our approximately 4.54 billion year-old planet, life seems to be a rather ancient phenomenon. As microfossils pointed to the beginning of life around 3.5 billion years ago [12], more recently, biogenic chemofossils with an age of approximately 3.8 billion years were discovered [13]. The 'Late Heavy Bombardment' theory and new findings of organic carbon in 4.1 billion year-old zircons suggest that life on earth could have been possible earlier than 4 billion years ago, in the Hadean period [14]. Prokaryotic cell and sediment fossils were found to be as old as 3.5 billion years [15];

however, as *Archaea* cannot be distinguished from *Bacteria* by their cell shapes or sediment remains, chemical fossils in the form of unique lipids have to be used to date back the origin of the *Archaea* [16]. The oldest chemofossils that were proposed to be of Archaeal descent were found in Western Greenland and were dated at 3.7 billion years old [17]. Reconstructions of phylogenetic trees of proteomes suggest that the *Archaea* were the first of the three domains that segregated from their common ancestor, which would make the archaeal lineage the oldest on earth [18].

The contemporary diversity of the *Archaea* can be divided into two major phyla: *Euryarchaeota* and *Crenarchaeota*, as well as in different minor phyla, such as the *Korarchaeota* and the recently discovered *Thaumarchaeota* [19].

It should be mentioned that the majority of archaeal species has not been discovered yet, so the tree of archaeal lineage will most definitely continue to change in the future.

1.1.2.1 *Euryarchaeota*

The *Euryarchaeota* consist of eight classes: *Archaeoglobi*, *Halobacteria*, *Methanobacteria*, *Methanococci*, *Methanomicrobia*, *Methanopyri*, *Thermococci* and *Thermoplasmata*. It is the largest and best described phylum that comprises most of today's known species of *Archaea* [19].

The classes *Archaeoglobi* and *Thermococci* consist of species that are all anaerobic. They are all hyperthermophilic inhabitants of deep-sea hydrothermal vents. In these environments the *Thermococci*, unlike the *Archaeoglobi*, can use molecular sulphur as an electron acceptor [19].

The class *Thermoplasmata* consists exclusively of extreme acidophiles that have very low pH optima. While the genus *Ferroplasma* has a pH maximum of 2.2, the genus *Acidiplasma* has a pH minimum as low as 0. All members of the class *Thermoplasmata* are aerobic and capable of sulphur respiration; most are moderately thermophilic [19].

The methanogens within the phylum *Euryarchaeota* contain the classes *Methanobacteria*, *Methanococci*, *Methanomicrobia* and *Methanopyri*. All members of the four classes are obligate anaerobes that use CO₂ as an electron acceptor. About half of these methanogenic species are lithoautotrophes that can build essential molecules from inorganic sources of carbon only. The class *Methanomicrobia* contains some psychrophilic species that grow at a temperature range of 0-17°C. The class *Methanopyri* is represented by only one species, the hyperthermophile *Methynopyrus kandleri*, that has its optimal growing temperature at around 100°C, and was shown to survive and replicate at 122°C [19], [20].

Members of the class *Halobacteria* are extreme halophiles that require high salt concentrations of up to 30-36% NaCl for growth. All *Halobacteria* species are strictly aerobic and mesophilic organisms. Some genera are alkaliphilic, while others are at least alkalitolerant. Many strains appear in various shades of red, which is due to the presence of C₅₀ carotenoid pigments [19].

Nanoarchaeum equitans, one of the smallest known cellular organisms, was believed to be the first discovered member of a new phylum, the *Nanoarchaeota* [21]. However, recent analysis of its genome provided evidence for placing the species inside the *Euryarchaeota*, next to the *Thermococcales* [22]. *Nanoarchaeum equitans* is an obligate symbiont or parasite of the thermophilic Archaeon *Ignicoccus hospitalis* [23].

1.1.2.2 *Crenarchaeota*

Crenarchaeota are extremophile organisms that are adapted to life in some of the most inhospitable environments on our planet. The phylum consists of only one single class, the *Crenarchaeota* or *Thermoprotei*, and 26 genera. All *Crenarchaeota* are hyperthermophiles, and most species have an optimal temperature range of 80-100°C; most species are also acidophilic [19].

The most widely studied member of the *Crenarchaeota* is *Sulfolobus solfataricus*, a hyperthermophilic acidophile [24]. Studies on *Sulfolobus solfataricus* target, for example, the mechanisms of repairing DNA in high-temperature environments [25]. It is estimated that *Crenarchaeota* account for 20% of the oceanic pico plankton mass [26].

1.1.2.3 *Korarchaeota*

Analyses of samples obtained from hydrothermal vents contained species whose 16S rDNA sequences suggested placing them in a new archaeal phylum, the *Korarchaeota* [27].

So far, the *Korarchaeota* consist only of uncultivated species that are endemic to hydrothermal vents. The *Korarchaeota* are considered a deep-branching phylum inside the *Archaea*; however, their evolutionary relationship with the *Euryarchaeota* and *Crenarchaeota* will have to be studied further [28].

1.1.3.4 *Thaumarchaeota*

The recently discovered group of the *Thaumarchaeota* was first described as mesophilic *Crenarchaeota*, but meanwhile it has been proposed to constitute a new archaeal phylum [29]. *Korarchaeota* are widely distributed in terrestrial and marine habitats and are believed to play an important role in the global carbon and nitrogen cycles [30].

1.1.3 The *Archaea* compared to the other two domains

When compared to the *Bacteria* and *Eukarya*, the *Archaea* exhibit unique features as well as similarities to both of the other domains.

Cell structure

In comparison to the *Eukarya*, the inner cell structure of the *Archaea* does not comprise membrane-bound organelles or a nucleus [31]. The composition of the archaeal cell membrane differs from the other two domains with regard to their membrane lipids. The membrane lipids of *Bacteria* and *Eukarya* consist of fatty acid chains that are ester-linked to a glycerol backbone. In comparison, archaeal membrane lipids consist of branched isoprenyl chains that are ether-linked to the glycerol backbone [32]. Another unique feature of the *Archaea* is the presence of tetraether lipids in thermoacidophilic species. These tetraether lipids can form lipid monolayers, in contrast to the membrane bilayers of the *Bacteria* and *Eukarya* [33]. Ether bonds, as opposed to ester bonds, and monolayers formed by tetraether lipids are more resistant to hydrolysis and denaturation; therefore they are important for adaptation to high-temperature environments [32], [33].

Both the archaeal and bacterial cell envelope typically consist of an S-layer structure that is formed by a single species of proteins arranged in multimers [34]. In contrast to the *Bacteria*, the *Archaea* do not possess peptidoglycan, although a similar structure, pseudopeptidoglycan, is found in some species [35].

Transcription and Translation

Some mechanisms and factors involved in archaeal transcription and translations share similarities with their bacterial counterparts, while others more closely resemble the systems of the *Eukarya* [36]. While the general transcription factors of the *Archaea* resemble those of the *Eukarya*, archaeal transcription regulators are more closely related to their bacterial counterparts [37]. Like in *Bacteria*, AUG is the most abundant start codon in *Archaea*, but *Archaea* also use GUG and UUG as start codons. Leaderless mRNA appears to be more abundant in *Archaea* than in the other two domains [36].

Ribosomes

Both *Archaea* and *Bacteria* possess 70S ribosomes that consist of a large 50S and a small 30S subunit. Although archaeal and bacterial chromosomes are similar in size and composition, the primary structure of the archaeal ribosomal proteins and rRNA are closer related to the *Eukarya* [38].

Reproduction and genetic exchange

Archaea, like *Bacteria*, reproduce asexually by fission, budding, or fragmentation of the cells [39]. Genetic material can be exchanged between archaeal cells via transfection using archaeal DNA viruses as the genetic shuttles [40]. Also, direct genetic exchange between two archaeal cells (e.g. in the form of cytoplasmatic bridges) has been reported [41].

1.1.4 Extreme environments

Archaea are widespread in various biotopes and can survive in some of the harshest known environments on this planet [19]. The following list shall provide a quick overview of some extreme conditions which can still be endured by specialised extremophilic members of this domain.

Heat

Members of thermophilic and hyperthermophilic *Archaea* are found, for example, in deep-sea hydrothermal vents, hot springs or volcanoes [42]. By definition, thermophiles live at a temperature ranging from approximately 55°C to 80°C, while hyperthermophiles require temperatures above 80°C [43]. The current record holder is the *Euryarchaeon Methynopyrus kandleri* that has its optimal growing temperature at around 100°C and was shown to survive and replicate at 122°C [20].

Life at high temperatures requires cellular adaptations regarding membrane composition, DNA, and protein protection. Membranes of thermophilic *Archaea* show a reduction in fluidity caused by branched isoprenyl chains and tetraether monolayers [44]. DNA protection can be facilitated by DNA-binding proteins, DNA supercoiling and DNA repair mechanisms [45]. Proteins can be protected from heat damage by chaperone folding, an increase in the number of hydrophobic cores and salt bridges [46].

Cold

Psychrophilic or cryophilic organisms adapt to temperatures below 10°C. *Archaea* adapted to very low temperatures can be found in environments like permafrost soil or deep sea mountains [47].

In contrast to heat stress, the membranes of psychrophilic *Archaea* show an increase in membrane fluidity. Their enzymes exhibit more structural flexibility, lower thermostability and a higher specific activity at lower temperatures. In addition, the expression of cold-shock proteins and the presence of cryoprotectant molecules and antifreeze or ice-binding proteins contribute to cold-resistance [48].

Acidic habitats

Acidophilic *Archaea* have been isolated, for example, from acidic mine drainages or volcanoes [49]. Acidophilic organisms can pre-exist at pH levels below 2; the genus *Picrophilus* was shown to live at a pH as low as 0 [50].

Adaptations in the membrane of acidophiles to acidic stress can involve the presence of sterols and lipoglycans. Ether-linkages and impermeable tetraether membranes contribute further to acid resistance. Proteins can be protected from acid through chaperone folding and structural changes. Additionally, it was also proposed that acidophilic *Archaea* possess proton antiporter systems similar to the AR-system of *E. coli* [51]. The expression of DNA-repair enzymes appears upregulated. Many acidophiles also face the challenge of toxic metals which is countered by efflux ATPases, metal chelation or the reduction or oxidation of metals by enzymes [50].

Alkaline habitats

Alkaliphilic organisms live at a pH of approximately 8.5-11; archaeal representatives inhabit, for example, alkaline soda lakes [52]. Like acidophiles, alkaliphiles have to maintain their pH homeostasis. This involves the use of Na^+/H^+ antiporter systems to keep the intracellular pH constant [53].

Salt

Salt tolerance in the *Archaea* will be discussed in Chapter 1.1.5.

Pressure

Piezophile (sometimes called barophile) organisms can inhabit high-pressure environments such as deep sea hydrothermal vents or deep sea trenches [54]. *Thermococcus barophilus*, an inhabitant of deep sea vents, sustains hydrostatic pressure up to 40 Mpa [55].

Many mechanisms concerning adaptation to high pressure are not completely understood so far. From already isolated and characterised piezophiles, it seems that major cellular processes are altered, e.g. shifts in the respiratory systems and the electron transport chains [55].

Radiation

Habitats that are exposed to high doses of ionizing radiation constitute another challenge for extremophilic microorganisms. The archaeon *Thermococcus radiotolerans*, which was isolated from hydrothermal chimneys, can survive acute doses of 30 kGy [56].

DNA, proteins and membranes must be repaired effectively and quickly in order to counteract the destructive effects of high doses of radiation on the cell. Radiotolerant microorganisms also exhibit many functional redundancies within vital pathways and cellular systems. *Thermococcus radiotolerans* is known to reassemble and repair shattered chromosomes without loss of viability [56].

Research concerning extremophilic microorganisms will become more and more important in the future with regard to applications in biotechnology [52]. In addition, extremophilic microorganisms serve as models in basic research related to the origins of life on earth and the search for extraterrestrial life-forms [57], [58].

1.1.5 Haloalkaliphilic lifestyle

Many extremophiles face more than one challenging condition at once. Haloalkaliphiles have to adapt to saline and alkaline conditions that are present, for example, in African alkaline soda lakes such as Lake Magadi in Kenya [59]. Most haloalkaliphilic *Archaea* that have been identified so far belong to the class *Halobacteria* inside the *Euryarchaeota* [19]. Extremely halophilic and alkaliphilic representatives are adapted to life in salt concentrations of 2.5 – 5.2 M NaCl (up to NaCl saturation) and pH values ranging from 8.5 to 11 [60], [52].

Halobacteria are typically aerobes and heterotrophs. Members of this class exhibit a large variety of metabolic pathways; many grow proteolytically and use amino acids as a source of energy and carbons [60].

Saline environments create a high degree of osmotic pressure which the cell has to counteract by adapting the cell membrane lipid composition and balancing the turgor pressure [59]. The cell membranes of halophilic *Archaea* display a special composition of phospholipids, with archaetidylglycerol methylphosphate being the dominant membrane lipid. The altered membrane lipid composition together with ether linkages and isoprenoid chains are important for cell membrane stability of *Archaea* living in high-salt environments [61].

Halophilic organisms have to exclude sodium ions from their cytoplasm and balance the osmotic pressure inside the cell by using either a 'salt-in' or 'salt-out' strategy. The 'salt-in' strategy involves accumulation of K^+ and Cl^- ions inside the cell to maintain the osmotic balance; some halophiles accumulate KCl concentrations inside the cell that are equal to or higher than the NaCl concentrations in the environment. Organisms that use the 'salt-out' strategy accumulate organic solutes such as glycerol or amino acids in the cytoplasm [59]. In both cases, the enzymes have to be prevented from denaturation caused by the high salt conditions. Enzymes of halophiles contain high amounts of acidic and only few hydrophobic amino acids [62].

Apart from maintaining stability in high salt concentrations, haloalkaliphiles also have to adapt to high pH levels and keep the pH homeostasis within the cell. Na^+/H^+ antiporter systems import protons into the cell to keep the intracellular pH constant, and acidic polymers inside the cell wall serve as a barrier protection against the high extracellular pH [52], [53].

An interesting feature of several halophilic *Archaea* is the presence of **polyploidity**. The species *Halobacterium salinarum* and *Haloferax volcanii*, for example, harbour between 15 and 25 copies of their genome inside the cell in all growth stages [63]. Several chromosomal copies per cell constitute an adaptive advantage in high salt environments. The higher genomic copy number contributes to resistance against DNA double-strand breakage caused by desiccation. Heterozygotes are also better suited to surviving mutations in crucial genes through the presence of intact wild-type copies. The high amount of genomic DNA may also serve as storage of phosphor polymers [64].

1.2 The haloalkaliphilic archaeon *Natrialba magadii*

1.2.1 General features of *Natrialba magadii*

Natrialba magadii is a haloalkaliphilic archaeon that was isolated from the alkaline soda lake Lake Magadi in Kenya. It belongs to the family of *Halobacteriaceae* inside the *Euryarchaeota* [65]. The species was originally termed *Natronobacterium magadii*, but phylogenetic analysis of its rRNA led to reclassification and introduction of the genus *Natrialba* [66]. *N. magadii* cells are rod-shaped in liquid media and motile through the use of flagella.

Carotinoid pigments in the cell membrane give them a slightly reddish/orange colour. The organism requires high salt concentrations and a high pH for growth and survival. The optimal NaCl concentration for growth lies around 3.5 M, whereas NaCl concentrations below 1.5 M cause lysis of the cells; the pH optimum is around 9.5. *N. magadii* is an obligate aerobe that grows optimally at temperatures between 37-42°C. It is a chemoorganotrophe that grows proteolytically by using amino acids and peptides as energy and carbon sources. Carbohydrates are not used by *N. magadii* [65]. Like several other representatives of halophilic *Archaea*, *N. magadii* is a polyploid organism that possesses up to 50 copies of its chromosome in one single cell [67].

Spontaneous lysis of *N. magadii* cultures led to the discovery of the halovirus ϕ Ch1. It was the first virus described that infects an haloalkaliphilic archaeon, with *N. magadii* being the only known host thus far [68].

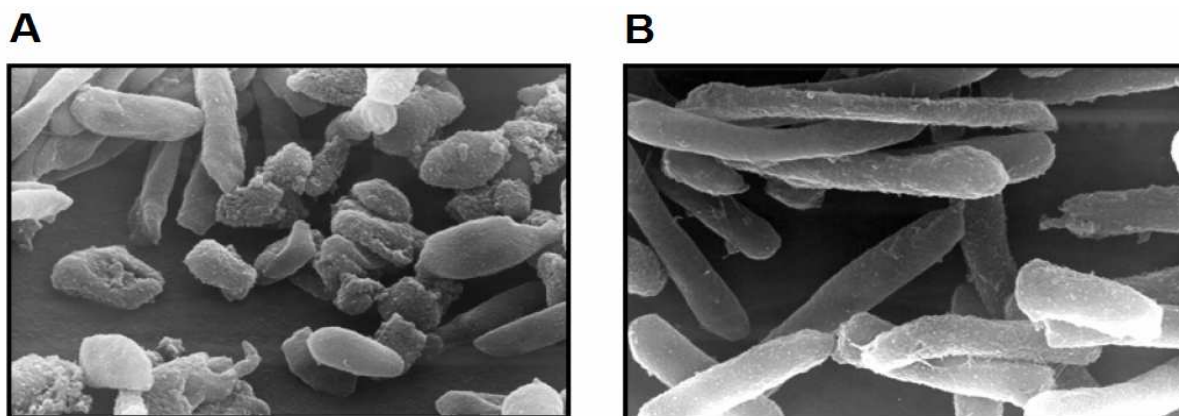


Figure 2: Electron micrographs of the *Natrialba magadii* laboratory strains L11 and L13

A: *N. magadii* strain L11 harbouring the virus Φ Ch1 **B:** *N. magadii* strain L13 'cured' of the virus ϕ Ch1 (adapted from Iro, 2006 [69]).

1.2.2 Working with *Natrialba magadii*

Two laboratory strains are available for studies of *N. magadii* and its virus ϕ Ch1. The wild-type strain *N. magadii* L11 harbours ϕ Ch1 as a chromosomally integrated provirus. The strain *N. magadii* L13 that was ‘cured’ of the virus through repeated sub-culturing of the wild-type strain can be re-infected with ϕ Ch1 and is used as an indicator strain [68]. The slow growth of *N. magadii* (generation time approximately 9 h in the logarithmic growth phase) demands careful planning of experiments that involve culturing of the archaeon [65]. The high number of chromosomal copies (up to 50) provides a challenge for obtaining deletion mutants, as successfully transformed cells need to be homozygotized through repeated passaging [70].

The first established transformation method for *Archaea* included the use of EDTA to remove the archeal S-layer and the transformation of these cells with DNA coupled to polyethylene glycol [71]. As this method failed to remove the S-layer of *N. magadii* efficiently, a new approach had to be developed. Treatment with bacitracin prevents glycosylation of the S-layer and the addition of proteinase K digests the S-layer. With this method, competent *N. magadii* cells are generated which can be transfected with DNA coupled with polyethylene glycol [70].

Two shuttle vectors, pRo-5 and pNB102, are available for use in *N. magadii*. The *Escherichia coli* plasmid pK_{Sn}⁺ was fused with the novobiocin resistance gene (mutated *gyrB* gene) to obtain a selectable marker in *N. magadii*. For replication in *N. magadii*, parts of ϕ Ch1 open reading frames ORF53 and ORF54 that exhibit sequence similarities to the *repH* gene of *N. magadii* were included in the vector, resulting in the plasmid pRo-5 [70]. The plasmid pNB102 was obtained by integrating the ColE1 replicon of *E. coli* into the plasmid pNB101, which was obtained from the strain *Natronobacterium* sp. AS7091. It features the mevinolin resistance gene for use in *Archaea* [72].

1.3 Viruses of the *Archaea*

1.3.1 Diversity of archaeal viruses

Archaeal viruses were discovered before the taxonomic introduction of the domain *Archaea*. In the early 1970s, the first head-tail shaped archaeal viruses infecting extremely halophilic species of *Halobacterium* were described. The first archaeal virus described was ϕ H of *Halobacterium salinarum* [73]. Viruses became important for studying archaeal mechanisms of transcription regulation and the development of viral genome-based vectors for the transformation of *Archaea*. As more and more archaeal viruses were discovered, their remarkable diversity became evident [74]. So far, all archaeal viruses studied are DNA viruses; most have been isolated from extreme geothermal and hypersaline environments [75], [76].

Viruses of *Euryarchaeota* contain the families of *Myoviridae* and *Siphoviridae*. Both show a typical head-tail morphology, with the main difference being that the *Myoviride* possess a contractible tail, whereas the tail of the *Siphoviridae* is non-contractible [77]. Euryarchaeal viruses infect halophilic or methanogenic euryarchaeal species; they can either be temperate or purely lytic. The most widely studied are ϕ H of *Halobacterium salinarum* and ϕ Ch1 of *Natrialba magadii*, both exhibiting the typical head-tail morphology of the *Myoviridae* and infecting extremely halophilic *Archaea*. Only very few other morphological types are found within euryarchaeal viruses. Examples would be the spindle-shaped viruses His-1 and His-2, and the spherical virus SH1, both infecting *Haloarcula* species. Apart from their often similar morphology, viruses of *Crenarchaeota* share homologous genes for structural capsid parts and virion assembly [75].

Crenarchaeal viruses display considerable morphological variety. Several of their morphotypes are not found in bacterial or eukaryal viruses. They consist of seven families: the spindle-shaped *Fuselloviridae* (e.g. SSV1 of *Sulfolobus solfataricus*), the lemon-shaped *Bicaudoviridae* that exhibit two tails (e.g. *Acidianus* two-tailed virus), the bottle-shaped *Ampullaviridae* (e.g. *Acidianus* bottle-shaped virus), the droplet-shaped *Guttaviridae* (e.g. SNDV of *Sulfolobus neozealandicus*), the staff-shaped *Rudiviridae* (e.g. SIRV1 of *Sulfolobus islandicus*), the filamentous *Lipothrixviridae* (e.g. TTV2 of *Thermoproteus tenax*), the spherical *Globuloviridae* (PSV of *Pyrobaculum* and *Thermoproteus*) [78], [77]. Crenarchaeal viruses often occur in a so-called ‘carrier-state’ in which the virions are actively produced within the host without lysis of the cell [77]. Upon lysis of the host cell, crenarchaeal viruses often produce outward-pointing, pyramid-shaped lysis structures that open up when the virions are released [79].

Since only few archaeal viruses in extreme environments were identified thus far, the future certainly holds many new and fascinating discoveries in this field. Advances in cultivation techniques, environmental DNA sequencing, and molecular biology in general with the subsequent new discovery of archaeal viruses will surely increase our knowledge and understanding of the viral world [78].

Viral Morphology	Family	Host Species	Notable References
Spindle	Fuselloviridae 	<i>Sulfolobus</i> , <i>Acidianus</i> , <i>Haloarcula</i> , <i>Pyrococcus</i> , <i>Aeropyrum</i> , <i>Stygiolobus</i> and <i>Thermococcus</i>	Krupovic et al. 2014
	Bicaudoviridae 	<i>Acidianus</i>	Haring et al. 2005b
	Spiraviridae 	<i>Aeropyrum</i>	Mochizuki et al. 2012
Spherical	"Halosphaerovirus" 	<i>Haloarcula</i> and <i>Halorubrum</i>	Luk et al. 2014 Porter et al. 2013
Pleomorphic	Pleolipoviruses 	<i>Haloarcula</i> , <i>Halorubrum</i> , and <i>Haloquadratum</i>	Pietila et al. 2012
Head and Tail	Myoviridae 	<i>Halorubrum</i> , <i>Natrialba</i> , <i>Halobacterium</i> , <i>Haloarcula</i> , and <i>Methanobacterium</i>	Pietila et al. 2012 Ackerman et al. 2012
	Podoviridae 	<i>Haloarcula</i>	Pietila et al. 2012 Ackerman et al. 2012
	Siphoviridae 	<i>Haloarcula</i>	Pietila et al. 2012 Ackerman et al. 2012
Bottle	Ampullaviridae 	<i>Acidianus</i>	Haring et al. 2005a
Bacilliform	Clavaviridae 	<i>Aeropyrum</i>	Mochizuki et al. 2010
Droplet	Guttaviridae 	<i>Sulfolobus</i> and <i>Aeropyrum</i>	Arnold et al. 2000a Mochizuki et al. 2011
Linear	Lipothirixviridae 	<i>Acidianus</i> and <i>Sulfolobus</i>	Arnold et al. 2000b
	Rudoviridae 	<i>Sulfolobus</i> , <i>Stygiolobus</i> , and <i>Acidianus</i>	Prangishvili et al. 1999 Vestergaard et al. 2005
Spherical	Globulaviridae 	<i>Pyrobaculum</i> and <i>Thermoproteus</i>	Ahn et al. 2006 Haring et al. 2004
Icosahedral	"Turriviridae" 	<i>Sulfolobus</i>	Rice et al. 2004 Happonen et al. 2010

Figure 3: Classified viruses of the Archaea

The list comprises the families of euryarchaeal and crenarchaeal viruses. Classification of the virus families is based on their morphology. Within the variety of archaeal viruses, remarkable and often unique morphotypes are found ranging from the typical head-tail structure of the *Myoviridae* to more and more 'exotic' shapes such as the lemon-shaped, two-tailed viruses of the family *Bicaudoviridae* (adapted from *Snyder et al.*, 2015 [78]).

1.3.2 Viruses of *haloarchaea*

Haloarchaeal viruses infect halophilic and extreme halophilic *Archaea* that live in environments close to or up to salt saturation, e.g. soda lakes or saline deposits. In these hypersaline environments, *Haloarchaea* seem to be the dominant cellular organisms. Haloviruses and their hosts rely on high salt concentrations; the virus particles will lose their structural integrity and infectivity below a specific salt concentration [80], [81].

The first halovirus to be described which now serves as a model organism is the virus ϕ H. It infects the archaeon *Halobacterium salinarum*. ϕ Ch1, which infect *Natrialba magadii*, is the first virus described that infects an haloalkaliphilic archaeon. Both belong to the family of *Myoviridae* and exhibit the typical head-tail structure comprising isometric heads, a contractible tail and tail fiber proteins for attachment to the host cell [82], [68]. The two viruses show a high sequence similarity, despite their hosts being phylogenetically distant [83]. Their life cycles consist of both a lysogenic and a lytic state. In the lysogenic state, ϕ H exists as a circular DNA molecule in a plasmid-like state in its host, whereas ϕ Ch1 is integrated into the host chromosome as a provirus [68]. The virus ϕ Ch1 infecting *N. magadii* will be described in more detail in the following chapters.

Although the head-tail morphotype seems to be dominant in haloarchaeal viruses, new discoveries suggest that spindle-shaped viruses, such as His1 infecting *Haloarcula*, appear to be predominant in hypersaline environments [81].

Head-tail shaped viruses like ϕ Ch1 often exist as proviruses within the host chromosome, whereas other members of the haloviruses, e.g. HF1, often only exhibit a lytic life cycle. Haloviruses can also differ significantly in their host range. While some like ϕ Ch1 have only one known host, others such as HF1 and HF2 have a large host range and can infect several genera of halophilic *Archaea* [84], [68].

1.4 The virus ϕ Ch1

1.4.1 ϕ Ch1: The first halolalkaliphilic virus identified

ϕ Ch1 is a halovirus that infects the haloalkaliphilic archaeon *Natrialba magadii*. So far, *N. magadii* is the only known host of ϕ Ch1. The virus was discovered by Witte *et al.* in 1997 upon spontaneous lysis of host cultures. ϕ Ch1 belongs to the *Myoviridae* family of viruses. The mature virus particles are approximately 200 nm in length and exhibit the typical head-tail morphology of the *Myoviridae*. The virions feature an icosahedral shaped head that contains the viral genome and a contractible tail including tail fiber proteins at its distal end. The virus relies on high salt concentrations; below 2 M NaCl the virus particles lose their morphological stability and infectivity. [68]

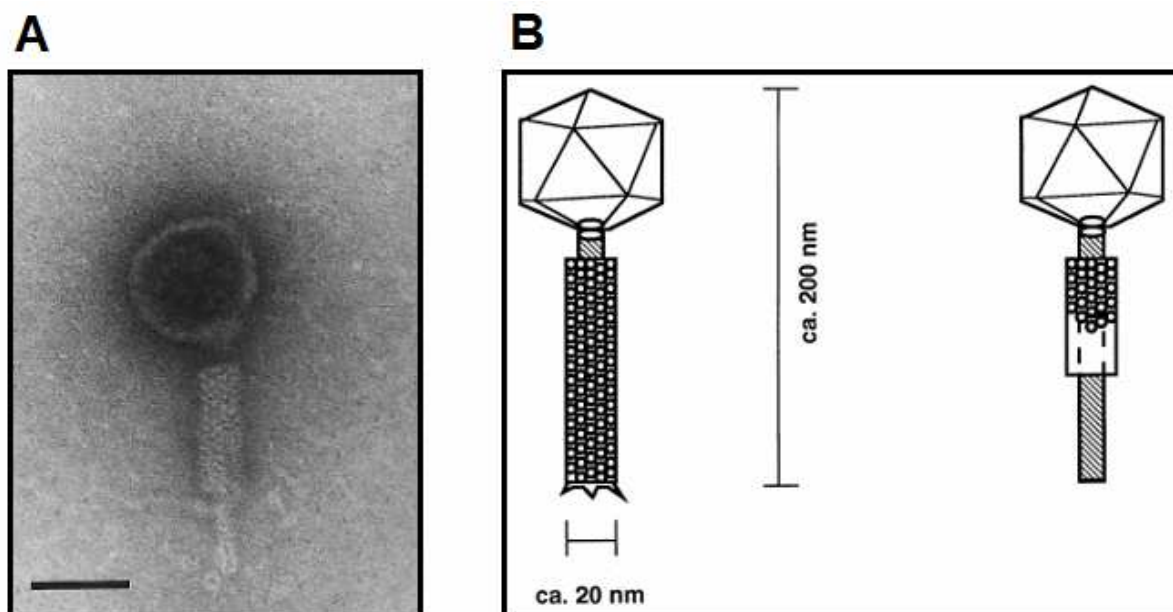


Figure 4: Morphology of the *Natrialba magadii* virus ϕ Ch1

A: Electron micrograph of ϕ Ch1. The head-tail morphology that is typical for members of the *Myoviridae* is clearly visible. The black bar represents 50nm. **B:** Schematic drawings of the mature virus particles. The mechanism of the contractible tail is illustrated (adapted from Witte *et al.*, 1997 [68]) .

Introduction

ϕ Ch1 is a temperate virus that exists as chromosomally integrated provirus; therefore, it has a lysogenic and lytic state. Lysis occurs in the stationary growth phase of *N. magadii*, typically from day 4 to day 5 after inoculation. After an eclipse period of 5 h, mature virus particles appear in the cell. The virus particles contain double-stranded DNA as well as several RNA species, making ϕ Ch1 the first described virus containing both DNA and RNA in mature virus particles. The RNA packed in the virus particle was shown to be host-encoded; its function is not fully understood yet. The DNA of ϕ Ch1 is partially methylated by the virus' own methyltransferase M.Nma ϕ Ch1-I [68], [85].

Two host strains are available for use in the laboratory. The wild-type strain L11 harbours ϕ Ch1 as a provirus integrated into the host chromosome. The non-lysogenic strain L13 that was 'cured' from ϕ Ch1 can be reinfected, making it an indicator strain for studying ϕ Ch1 [68].

1.4.2 Genome organization of Φ Ch1

ϕ Ch1 particles contain a linear dsDNA genome that is 58,498 bp in length as well as several RNA species. The RNA strands that are 80-700 nucleotides long were shown to be host-encoded, and it is assumed that they are involved in packaging the viral DNA. The G+C content of the genomic DNA is approximately 62%. As already mentioned, ϕ Ch1 DNA is partially methylated. As the chromosomal DNA of *N. magadii* is not methylated, it was assumed that ϕ Ch1 possesses its own methyltransferase. Analysis of the viral genome led to the discovery of the virus-encoded methyltransferase M.Nma ϕ Ch1-I, which facilitates dam-like methylation of adenine residues [68]. The chromosomal DNA is terminally redundant and circularly permuted, suggesting that the viral genome is brought into the capsid via the headful packaging mechanism [83]. 98 ORFs were identified in ϕ Ch1's genome.

Most, but not all genes start with the codon ATG; only four start with GTG. It is suggested that the genes form transcriptional units because of close arrangement in the same direction and slight overlapping of the ORFs [83].

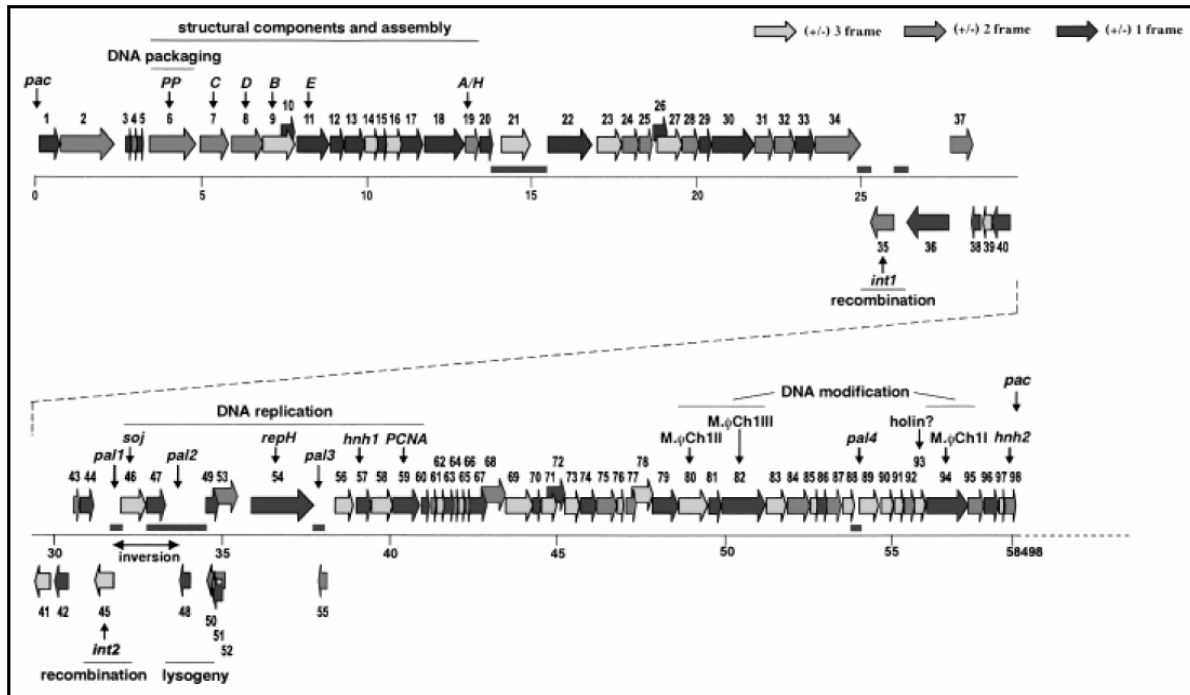


Figure 5: Genome organization of ϕ Ch1

Functional scheme of the 58,498 bp genome comprising 98 ORFs that were predicted to be protein-coding genes. The ORFs are numbered and depicted as arrows; the frames of the ORFs are indicated by different colours (adapted from *Klein et al.*, 2002 [83]).

The viral genome of ϕ Ch1 can be divided into three regions. The central region comprises rightward- and leftward-transcribed genes that are involved in gene regulation, gene replication and plasmid stabilization. The left (5') region contains only rightward-transcribed (5'→3') genes that encode mostly structural components of the viral capsid. The right (3') region also contains only rightward-transcribed genes. Genes for DNA modification and DNA restriction are found here, although many genes in the right region are still of unknown function. The clustering of genes in functional groups and the division of the genes in early, middle and late genes allows a precise and efficient timing of gene expression [83].

1.4.3 Life cycle and gene regulation of Φ Ch1

As previously mentioned, ϕ Ch1 is a temperate virus that is integrated into the *N. magadii* chromosome as a provirus until the lytic phase is induced. Lysis of the wild-type strain *N. magadii* L11 occurs from Day 4 to Day 5 after inoculation, whereas mature virus particles can be found inside the cells after an eclipse period of 5 h [68]. Viruses need control mechanisms to regulate their lysogenic and lytic state. Many viruses possess transcriptional repressors to regulate their life cycle. The protein structures of these repressors typically show a helix-turn-helix motive [86].

The most widely studied and characterised virus control system involving the two repressor proteins Cro and C1 belongs to the virus λ , which serves as a model organism for gene regulation in viruses [87]. The halovirus ϕ H of *Halobacterium salinarum*, a close relative of ϕ Ch1, possesses a similar control system. Its repressor molecules are organised head to head in the genome, which is also a typical feature of virus lysogeny/lysis regulators [88].

The ORF48 (*rep*) of ϕ Ch1 shows similarities to a repressor of ϕ H. It features the amino acid pair Ala⁷³-Gly⁷⁴, which is conserved among repressors of many viruses. However, it was observed that *rep* is expressed in both the lysogenic and lytic states. The gene product Rep also possesses no DNA-binding, helix-turn-helix motive that is typical for gene regulators in viruses. It is therefore assumed that Rep is not solely responsible for maintaining the lysogenic state [89].

A virus mutant that possesses a duplicated part of the ORF49, termed ϕ Ch1-1, was isolated. The corresponding *N. magadii* strain, L11-1, showed an earlier onset of lysis and larger plaques as compared to the wild-type strain L11. Compared to the expression of *rep*, the expression of the ORF49 is delayed in the lysogenic strain L11 but increases throughout the virus life cycle.

It is therefore concluded that the ORF49 is involved in the gene regulation of ϕ Ch1, possibly acting as a switch from the lysogenic to the lytic state [89].

Both the ORF48 and ORF49 are arranged head to head in the viral genome as it is typically observed in viral regulator protein pairs. The intergenetic region of the ORF48-ORF49 was revealed to have promoter activity in the halophilic archaeon *Haloferax volcanii*. It was shown that Rep acts as a transcriptional repressor by shutting down expression of the ORF49 [89].

The ORF48 and ORF49 do not feature the DNA-binding helix-turn-helix motive of other viral gene regulators. However, the DNA binding mechanism of these proteins could be facilitated through other secondary protein structures or motives which are active in high salt environments. In addition, other regulatory mechanisms such as antisense RNAs that were identified in the *H. salinarum* virus ϕ H could play a role in the regulation of the viral life cycle [89].

The ORF79 was shown to be another regulatory element of ϕ Ch1. The ORF79-disrupted virus strain ϕ Ch1 Δ -ORF79 showed an earlier onset of lysis compared to the wild-type strain. Negative stain electron microscopy revealed no morphological difference between the mutant and the wild-type strain. In previous studies, the regulatory influence of the ORF79 on the lytic cycle was studied. It was shown that disruption of the ORF79 results in de-regulated expression of viral genes. In ϕ Ch1- Δ ORF79, the expression of the major capsid protein E (a structural gene from the 5' region of the viral genome) was detected 24 hours earlier than in the wild-type strain, which corresponded with the earlier onset of lysis in the mutant strain. The expression of the viral methyltransferase M.Nma ϕ Ch1-I (a late gene of the 3' region) also occurred earlier in the mutant strain. In addition the gene product of the ORF79, gp79, directly regulates the expression of the ORF34 that encodes the tail-fiber protein gp34. Overexpression of the ORF79 also strongly reduces the infectivity of ϕ Ch1.

When infecting the cured strain *N. magadii* L13, the plating efficiency of the virus is reduced by seven orders of magnitude in the presence of gp79. Overexpression of the ORF79 in the lysogenic strain *N. magadii* L11 represses the induction of the lytic life cycle of ϕ Ch1.

In summary, it was shown that the ORF79 directly regulates the expression of the ORF34 coding for the viral tail fiber protein, but it is also assumed that gp79 represses the lytic cycle of ϕ Ch1 by influencing the expression of at least one activator [90], [91], [92], [93], [94].

In the course of the research for this thesis, the role of the ORF79 in the life cycle of ϕ Ch1 was investigated further. The results of this study, as well as a more detailed overview of the results of preliminary studies on the ORF79, will be given in Chapter 4.2.

1.4.4 Interaction with the host *Natrialba magadii* (Infection process)

The most widely studied member of the *Myoviridae* is the bacteriophage T4 that infects *Escherichia coli*. T4 features the typical morphology and structural elements of the *Myoviridae*. Its viral capsid consists of an isometric head, a long tail, a base plate as well as tail fiber proteins at the basal end of the tail. The tail consists of a contractible outer shaft and an internal tube that delivers the phage DNA. Tail fiber proteins are responsible for host recognition and facilitate contact with the host cell receptor in the initial stage of the infection process. Attachment of the tail fibers to the host receptor causes the phage tail to undergo a rapid conformational change. The tail shaft contracts and the internal tube is pushed through the opened basal plate, causing the tube to penetrate the outer membrane of the host cell. The viral DNA is then injected into the host cell through the open canal created by the phage's tail tube [95].

The morphology and infection mechanism of ϕ Ch1 are comparable to the bacteriophage T4. ϕ Ch1 features the typical morphology including the contractible tail [68]. The tail fiber proteins of ϕ Ch1 are composed during an exchange of the 3' ends of the ORFs 34 and 36. These ORFs, together with the integrase *int1*, constitute an invertible region in the viral genome. The inversion and subsequent recombination of the tail fiber genes creates two different versions of the tail fiber protein gp34. The availability of different versions of structural virus components increases genetic variability and helps to adapt to environmental changes or changes of the host cell receptor [96].

The function of gp34 as the tail fiber protein of ϕ Ch1 was confirmed by secretion of the gp34 variants in *N. magadii* L13 and subsequent reinfection. It was shown that the gp34 variant gp34₅₂ exported from the cells prevents reinfection with ϕ Ch1 [97]. Both variants of the tail fiber protein contain the predicted galactose-binding domains. It is therefore concluded that the receptor for ϕ Ch1 on the cell surface of *N. magadii* contains a galactose residue [98]. Therefore, possible receptors on the cell surface of *N. magadii* include the archaeal flagellum, Pilin protein as well as S-layer proteins. All three structures serve as virus receptors in several archaeal species [99]. The search for the ϕ Ch1 receptor on *N. magadii*'s cell surface is currently a major question in the research concerning the relationship between the virus and its host.

1.4.5 Possible receptors for Φ Ch1 on the host cell

As mentioned before, it appears that the receptor for the virus ϕ Ch1 on the cell surface of *N. magadii* contains a galactose residue and therefore constitutes a glycosylated protein [98]. Glycosylated structures on the archaeal cell surface include the archaeal flagellum, pili and the S-layer [99].

The properties of the *N. magadii* proteins AglB and Pilin were described in Chapters 1.2.3.1 and 1.2.3.2, respectively. In the course of this study, attempts were made to delete the glycosyltransferase AglB as well as a Pilin protein of *N. magadii*. Identification of the ϕ Ch1 receptor in *N. magadii* could contribute to the general understanding of biological mechanisms in halophilic and haloalkaliphilic microorganisms.

1.4.5.1 AglB

AglB is an oligosaccharyltransferase catalysing the process of protein glycosylation. In *Archaea*, it is involved in the assembly and transfer of archaeal S-layer and flagellum glycoproteins [100], [101].

N-glycosylation occurs in all three domains, but with regard to prokaryotes, it appears to be much more widespread in the *Archea* as compared to the *Bacteria*. So far little is known about the process in most archaeal species. N-glycosylation in *Archaea* is best studied in the model organisms *Haloferax volcanii*, *Methanococcus voltae* and *Methanococcus maripaludis*. AglB fulfils the role of an oligosaccharyltransferase in all three species. The enzyme transfers assembled oligosaccharides to selected asparagine residues of the target proteins [102]. In *Archaea*, AglB is therefore involved in the assembly and transfer of structural glycoproteins such as archaeal S-layer and flagellum glycoproteins [100], [101].

As already mentioned, AglB is involved in the post-translational modification of flagellar proteins [101]. In many cases, prokaryotic flagella serve as the receptor for prokaryotic viruses during the process of host recognition and adhesion of the virus to the host cell [103], [99].

Removal of the flagellum by deletion of the genes that encode the glycosylated flagellum proteins FlaB1 and FlaB2 in *N. magadii* was not successful in past studies, as no homozygous mutant was obtained [90], [91]. In this thesis, an indirect approach was chosen by preventing glycosylation of *N. magadii* cell surface proteins through deletion of *aglB*. The results are shown in Chapter 4.1.

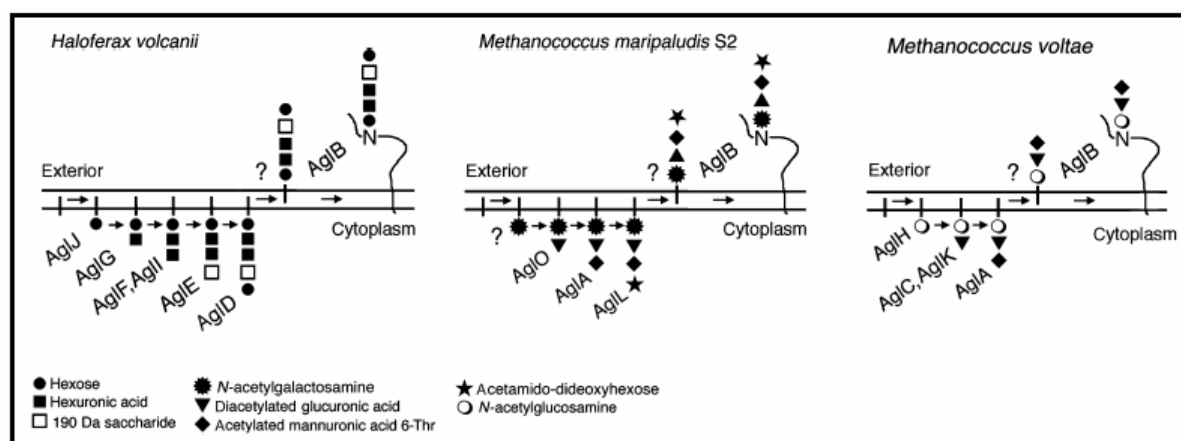


Figure 6: The role of AglB in the process of archaeal protein glycosylation

Schematic representation of the N-glycosylation pathways in the *Archaea* *Haloferax volcanii*, *Methanococcus maripaludis* and *Methanococcus voltae*. AglB catalyzes the last step in the protein glycosylation of archaeal surface proteins by transferring assembled oligosaccharides to asparagine residues of the target proteins (adapted from *Magidovich*, 2009 [102]).

1.4.5.2 Pilin

Archaeal pili, as well as other archaeal surface structures, are homologous to bacterial type IV pili systems. Pili are made up of several different protein species including minor pilins, major pilins and ATPases. The main functions of pili in *Archaea Bacteria* are to enable twitching cell motility, establishing cell-cell contact and the adhesion to surface structures [104]. Archaeal pili were also show to serve as receptors for archaeal viruses. Examples include the viruses SIRV1 and SIRV2 of *Sulfolobus* as well as AFV1 of *Acidianus*. The virus-host contact is established by attaching viral fiber or claw proteins to cellular pili [99].

Within the course of this thesis, deletion of the *N. magadii* pilin protein Nmag_0291 was attempted in order to investigate the role of the pilus in the infection process of *N. magadii* with ϕ Ch1.

2. Materials

2.1 Strains

2.1.1 Escherichia coli

Strain	Genotype	Source
XL1-Blue	<i>endA1, gyrA96(nal^R), thi-1, recA1, relA1, lac, glnV44, F'[::Tn10(tet^R), proAB⁺ lacI^q Δ(lacZ)M15] hsdR17(r_K⁻ m_K⁺)</i>	Stratagene
Tuner	<i>F-, ompT, hsdS_B(r_B⁻ m_B⁻), gal, dcm, lacY1(DE3)</i>	Novagene
Rosetta(DE3) pLysS	<i>F-, ompT, hsdS_B(r_B⁻ m_B⁻), gal dcm λ(DE3), pLysSRARE (Cam^R)</i>	Novagene
Rosetta pRep4	Rosetta strain containing plasmid pRep4(<i>lacI</i> , Neo ^R /Kan ^R)	Novagene
BL21	<i>F- dcm, ompT, hsdS(r_B⁻ m_B⁻), gal [malB⁺]_{K-12}(λ^S)</i>	Novagene
BL21(DE3) pLysS	<i>F-, ompT, gal, dcm, lon, hsdS_B(r_B⁻ m_B⁻), λ(DE3), pLysS(cm^R)</i>	Novagene
BL21 pRep4	BL21 strain containing plasmid pRep4(<i>lacI</i> , Neo ^R /Kan ^R)	Strain collection

2.1.2 *Natrialba magadii*

Strain	Genotype	Source
L11	wild type, provirus ϕ Ch1 integrated into the chromosome	Witte <i>et al.</i> , 1997
L13	derivate of L11, 'cured' of ϕ Ch1	Witte <i>et al.</i> , 1997
L13-pNB102-mtase, pRo-5-ptnaN-ORF79	<i>N. magadii</i> L13 with vector pNB102-mTase and vector pRo-5-ORF79 under the inducible promoter ptnaN	Hofbauer, 2014
L13-pNB102-ORF34, pRo-5-ptnaN-ORF79	<i>N. magadii</i> L13 with vector pNB102-ORF34 and vector pRo-5-ORF79 under the inducible promoter ptnaN	Schönfelder, 2013

2.2 Growth media

LB medium (*E. coli*)

Peptone	10 g
Yeast extract	5 g
<u>NaCl</u>	<u>5 g</u>
pH: 7.0	

for agar plates 15 g agar/L were added
ddH₂O added to a final volume of 1 L, autoclaved

NVM⁺ rich medium (*N. magadii*)

Yeast extract	11.7 g
Casamino acids	8.8 g
Tri-Na citrate	0.8 g
NaCl	235 g
<u>KCl</u>	<u>2.35 g</u>
pH: 9-9.5	

for agar plates 10 g agar/1 L were added

for top agar 5 g agar/1 L were added

ddH₂O added to a final volume of 935 ml, autoclaved

After autoclaving, the medium was complemented with:

0.57 M Na ₂ CO ₃ (dissolved in sterile dH ₂ O)	65 ml
1 M MgSO ₄ (autoclaved)	1 ml
20 mM FeSO ₄ (dissolved in sterile dH ₂ O)	250 µl

NMMII⁺ defined medium (*N. magadii*)

NaCl	205 g
KCl	2 g
Na ₂ HPO ₄	0.28 g
NaH ₂ PO ₄	0.28 g
Alanine	2.23 g
Leucine	0.66 g
Arginine	0.81 g
Histidine	0.778 g
Lysine	0.731 g
Na ₃ -citrate dihydrate	0.8 g
Na acetate	1.66 g
<u>Na pyruvate</u>	<u>1.1 g</u>
pH: 9.5	

for agar plates 8 g agar/1 L were added
 for top agar 4 g agar/1 L were added
 ddH₂O added to a final volume of 900 ml, autoclaved

After autoclaving, the medium was complemented with:

1.75 M Na ₂ CO ₃ (dissolved in sterile dH ₂ O)	100 ml
1 M MgSO ₄ (autoclaved)	1 ml
20 mM FeSO ₄ (dissolved in sterile dH ₂ O)	250 µl
1000x trace elements	1 ml

1000x trace elements: (100ml)

MnCl ₃	93 mg	4 mM
CaCl ₂	44 mg	3 mM
CuSO ₄	64 mg	4 mM
ZnSO ₄	86 mg	3 mM

2.3 Additives

E. coli

Additive	Stock concentration	Final concentration	Annotations
Ampicillin	20 mg/ml	100 µg/ml	dissolved in ddH ₂ O, sterile filtered, stored at 4°C
Tetracyclin	10 mg/ml	10 µg/ml	dissolved in ½ Vol. ddH ₂ O, followed by ½ Vol. 96 % EtOH, stored at -20°C (protected from light)
IPTG	1 M	0.5-1 mM	dissolved in ddH ₂ O, sterile filtered, stored at -20°C

N. magadii

Additive	Stock concentration	Final concentration	Annotations
Bacitracin	7 mg/ml	7 µg/ml	dissolved in ddH ₂ O, sterile filtered, stored at 4°C
Novobiocin	3 mg/ml	3 µg/ml	prepared from Lovastatin tablets *), stored at -20°C (protected from light)
Mevinolin	10 mg/ml	7.5 µg/ml	dissolved in ddH ₂ O, sterile filtered, stored at -20°C (protected from light)

*) 6 tablets (20 mg each) were powdered, dissolved in 12 ml 96% ethanol, stirred for 20 min at RT, centrifuged for 15 min at 4°C and the supernatant was collected.

2.4 Plasmids

Construct	Features	Source
pBlueScriptII KS(+) (pKS _{II} ⁺)	mcs, bla, ColE1 ori, lacZa	Stratagene
pRSET-A	bla, pUC ori, T7 promoter, N-terminal 6x His-tag	Invitrogen
pUC-19	bla, pMB1ori, lacZa, mcs	Yanish-Perron et. al., 1985
pQE30	bla, ColE1 ori, N-terminal 6x His-tag	Quiagen
pNB102	bla, ColE1 ori, hmg (MevR), pNB101 ori	Zhou et. al., 2004
pRo-5	bla, ColE1 ori, gyrB (Nov ^R), ϕCh1 derived ori	Mayrhofer-Iro et. al., 2013
pNB102-ORF34	pNB102 with ORF34 of ϕCh1	Hofbauer, 2014
pNB102-Mtase	pNB102 with <i>mTase</i> gene of ϕCh1	Hofbauer, 2014

pRo-5-ptnaN-ORF79	pRo-5 with ORF79 of ϕ Ch1 under the inducible promoter ptnaN	Stan Schafellner
pKS _{II} ⁺ -agIB 1-2	pKS _{II} ⁺ with <i>agIB</i> upstream region	this thesis
pKS _{II} ⁺ -agIB 1-2 Nov ^R forward	pKS _{II} ⁺ with <i>agIB</i> upstream region followed by the novobiocin cassette in forward orientation	this thesis
pKS _{II} ⁺ -agIB 1-2 Nov ^R reverse	pKS _{II} ⁺ with <i>agIB</i> upstream region followed by the novobiocin cassette in reverse orientation	this thesis
pKS _{II} ⁺ -agIB 1-4 Nov ^R forward	pKS _{II} ⁺ with <i>agIB</i> up- and downstream regions, novobiocin cassette inserted in between in forward orientation	this thesis
pKS _{II} ⁺ -agIB 1-4 Nov ^R reverse	pKS _{II} ⁺ with <i>agIB</i> up- and downstream regions, novobiocin cassette inserted in between in reverse orientation	this thesis
pKS _{II} ⁺ -Pil 1-2	pKS _{II} ⁺ with <i>pilin</i> upstream region	this thesis
pKS _{II} ⁺ -Pil 1-4	pKS _{II} ⁺ with <i>pilin</i> up- and downstream regions	this thesis
pKS _{II} ⁺ -Pil 1-4 Nov ^R forward	pKS _{II} ⁺ with <i>pilin</i> up- and downstream regions, novobiocin cassette inserted in between in forward orientation	this thesis
pKS _{II} ⁺ -Pil 1-4 Nov ^R reverse	pKS _{II} ⁺ with <i>pilin</i> up- and downstream regions, novobiocin cassette inserted in between in reverse orientation	this thesis

pRSET-A-AglB-C	pRSET-A with AglB C-terminus	this thesis
pRSET-A-Pil-C	pRSET-A with Pilin C-terminus	this thesis
pNB102-aglB	pNB102 with <i>aglB</i>	this thesis

2.5 Primer

Name	Sequence ^{*)}	Restriction site
AglB-1	GATT <u>TCTAGA</u> AAGCGACCGAGAACGAATC	<i>Xba</i> I
AglB-2	GAAT <u>GGATCC</u> GGATATGGTACTGCCGGC	<i>Bam</i> HI
AglB-Cla	GATC <u>ATCGAT</u> GAGACTGCCTCGGTCAAG	<i>Cla</i> I
AglB-Kpn	GAAT <u>GGTACC</u> GACCGCTACAGAACAGATTCA	<i>Kpn</i> I
AlgB-5E	GATTGAATTCATCGCTGGTCCCAACCAG	<i>Eco</i> RI
AlgB-5B	GAAT <u>AGATCT</u> ATGGCTGCGGGCAAGT	<i>Bgl</i> II
AlgB-3H	GATTAAGCTTTCAGGTGTCCGTGTCCGT	<i>Hind</i> III
AlgB-3C	GATTATCGATTCAGGTGTCCGTGTCCGT	<i>Cla</i> I
Pil-1	GCCATCTAGAGAGGAACACCGTGAACCAG	<i>Xba</i> I
Pil-2	GTTAGGATCCGTTCCGCCAAGGTGTCA	<i>Bam</i> HI
Pil-3	GCCAAGCTTCTTCCTTACTGCGATTCCGGTG	<i>Hind</i> III
Pil-4	GTTAGGTACCGGCTTCCGTTGCGCTC	<i>Kpn</i> I
Pil-5en	GTGGGCTGTCTGTACTCCG	-
Pil-3en	GTTTCTGACGAGGACCCG	-
0291_3H	GTTCAAGCTTCAGTCTTTCTCTTCAACGAAAT	<i>Hind</i> III
0291_5B	GATT <u>GGATCC</u> GGTGGTTCAGTTGGAGAAGA	<i>Bam</i> HI
Nov-6	GGGATCGCAGAGGAGC	-
Nov-8	TCATCGAGGCCGGCTACG	-
Nov-9	GATGTCGGTCATCGCGG	-
Nov-11	GCATGTCGTGGCTGTTCG	-
Nov-12	GCCGGTGAGTACTTAACGC	-
Nov-13	GACGCCGAATGGGTAGAC	-

NovR-1p	GATC <u>CTGCAGT</u> GTCGACTGGAACGAGG	<i>Pst</i> I
NovR-1s	GATC <u>CCCCGGG</u> TGTCGACTGGAACGAGG	<i>Sma</i> I
NovR-2p	GTTA <u>CTGCAGG</u> CCCGTAATATCCAGCTGA	<i>Pst</i> I
NovR-2s	GTTA <u>CCCCGGG</u> CCCGTAATATCCAGCTGA	<i>Sma</i> I
79-RT-1	GACGAACCTCACCGACTT	-
79-RT-2	GCATCGGTCGAGTCAC	-
527f	ACCGCGGCCKGCTGG	-
1406r	ACGGGCGGTGTGTRC	-

*) Underlined sections represent the recognition sites for the restriction enzymes

2.6 Enzymes

PCR:

Enzyme	Remarks	Reference
Pfu DNA Polymerase	Used for preparative PCR, buffer supplied by Promega	Promega
GoTaq® DNA Polymerase	Used for analytical PCR, prepared as 2X Master Mix, containing polymerase, nuclease-free water, dNTPs, buffer and loading dye	Promega
Reverse Transcriptase	Used for reverse transcription during RT-PCR's	Thermo Scientific

Restriction:

Enzyme	Remarks	Reference
FastDigest™ enzymes	Used for analytical restrictions, used with FastDigest™ buffer	Thermo Scientific
Conventional restriction enzymes	Used for preparative restrictions, buffers were chosen according to the recommendations in the manufacturers' protocols	Thermo Scientific, Promega

Other:

Enzyme	Remarks	Reference
T4 DNA Ligase	Used for ligation of linear DNA in cloning	Promega
Proteinase K	Used for the digestion of archaeal S-layers	QUIAGEN
DNase I	Used for digestion of DNA in RNA samples	Thermo Scientific
RNasin Ribonuclease Inhibitor	Used for inhibition of ribonucleases in RNA samples	Promega

2.7 Size marker

DNA marker

Marker	Fragments	Annotations/Reference
λ - <i>Bst</i> EII (<i>Eco</i> 91I)	8453, 7242, 6369, 5687, 4822, 4324, 3675, 2323, 1929, 1371, 1264, 702 [bp]	λ DNA (Fermentas/ Thermo Scientific) digested with <i>Bst</i> EII (Thermo Scientific), DNA concentration of the marker: 50ng/ μ l
pUC19 <i>Hae</i> III (<i>Bsu</i> R1)	587, 458, 434, 298, 257, 174, 102, 80, 18, 11 [bp]	pUC19 DNA (prepared from E.coli XL1-Blue) digested with <i>Hae</i> III (<i>Bsu</i> R1)
pUC19 <i>Sau</i> 3AI	955, 585, 341, 258, 141, 105, 78/75, 46, 36, 18/17, 12/11.8 [bp]	pUC19 DNA (prepared from E.coli XL1-Blue) digested with <i>Hae</i> III (<i>Sau</i> 3AI)
GeneRuler™ 1 kB DNA Ladder	10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250 [bp]	Thermo Scientific

Protein marker

Marker	Fragments	Annotations/Reference
Page Ruler Prestained Protein Ladder	180, 130, 100, 70, 55, 40, 35, 25, 15, 10 [kDa]	Thermo Scientific

2.8 Antibodies

Primary antibodies

Antibody	Target	Application	Reference
Anti-HIS Epitop-Tag (mouse)	C-terminal, N-terminal and internal His-tags	Diluted 1:5000 in 1xTBS, 0.02% NaN ₃	Dianova
α -AglB (rabbit)	AglB of <i>N. magadii</i>	Diluted 1:5000 in 1xTBS, 0.02% NaN ₃	Protein purification: This thesis Immunisation: Moravian-Biotechnology
α -Pil (rabbit)	Pilin of <i>N. magadii</i>	Diluted 1:5000 in 1xTBS, 0.02% NaN ₃	Protein purification: This thesis Immunisation: Moravian-Biotechnology
α -gp34	gp34 of ϕ Ch1	Diluted 1:2500 in 1xTBS, 0.02% NaN ₃	Till P., 2011 Immunisation: Moravian-Biotechnology
α -gp79	gp79 of ϕ Ch1	Diluted 1:200 in 1xTBS, 0.02% NaN ₃	Schönfelder K., 2015 Immunisation: Moravian-Biotechnology
α -E	protein E of ϕ Ch1	Diluted 1:1000 in 1xTBS, 0.02% NaN ₃	Klein <i>et al.</i> , 2015 Immunisation: Moravian-Biotechnology

Secondary antibodies

Antibody	Target	Application	Reference
α -mouse IgG, horseradish peroxidase linked (sheep)	mouse IgG	Diluted 1:5000 in 1xTBS	GE Healthcare
α -rabbit IgG, horseradish peroxidase linked (donkey)	rabbit IgG	Diluted 1:5000 in 1xTBS	GE Healthcare

2.9 Kits

Kit	Company	Product number
GeneJET Plasmid Miniprep Kit	Thermo Scientific	K0502
GeneJET PCR Purification Kit	Thermo Scientific	K0701
QIAquick Gel Extraction Kit	QIAGEN	28706
NEBlot® Phototope ® Kit	New England Biolabs	7550S
Phototope®-Star Detection Kit for Nucleic Acids	New England Biolabs	7020S
Super Signal® West Pico Chemiluminescent Substrate	Thermo Scientific	34080
OneStep RT-PCR Kit	QIAGEN	210210

2.10 Buffers and solutions

2.10.1 Solutions and reagents for DNA methods

Gel electrophoresis

50X TAE

Tris/HCl pH 8.2	2 M
Acetic acid	1 M
EDTA	0.1 M

5X DNA loading dye

Tris/HCl pH 8.2	50 mM
Sucrose	25 %
SDS	0.1 %
Bromphenol blue	0.05 %
Xylene cyanol	0.05 %

0.8 % Agarose gel

Agarose melted in 1X TAE

Southern blot

50X Denhardt's solution

Ficoll 400	1 g
Polyvinylpyrrolidone	1 g
<u>BSA</u>	<u>1 g</u>
filled up with ddH ₂ O to 100 ml	

Blocking solution

NaCl	125 mM
Na ₂ HPO ₄	17 mM
NaH ₂ PO ₄	8 mM
<u>SDS</u>	<u>0.5 %</u>
pH 7.2	

20X SSC solution

NaCl	3 M
<u>Sodium citrate</u>	<u>0.3 M</u>
pH 7.2, autoclaved	

1X Wash solution I

1:10 dilution of blocking solution

Hybridization buffer

20X SSC	25 ml
50X Denhardt's solution	10 ml
BSA 10 %	5 ml
Na ₂ HPO ₄ 1M	5 ml
SDS 20 %	500 µl
EDTA 0.5M	200 µl
<u>ddH₂O</u>	<u>55 ml</u>

stored at -20°C

10X Wash solution II

Tris	100 mM
NaCl	100 mM
<u>MgCl₂ hexahydrate</u>	<u>10 mM</u>
pH 9.5	

2.10.2 Solutions and reagents for Protein methods**Protein purification under denaturing conditions (*E. coli*)****Buffer B (Lysis Buffer)**

NaH ₂ PO ₄	100 mM
Tris	10 mM
<u>Urea</u>	<u>8 M</u>
pH adjusted to 8.0 with NaOH prior to use	

Buffer D (Elution Buffer)

NaH ₂ PO ₄	100 mM
Tris	10 mM
<u>Urea</u>	<u>8 M</u>
pH adjusted to 5.9 with NaOH prior to use	

Buffer C (Wash Buffer)

NaH ₂ PO ₄	100 mM
Tris	10 mM
<u>Urea</u>	<u>8 M</u>
pH adjusted to 6.3 with NaOH prior to use	

Buffer E (Elution Buffer)

NaH ₂ PO ₄	100 mM
Tris	10 mM
<u>Urea</u>	<u>8 M</u>
pH adjusted to 4.5 with NaOH prior to use	

10X PBS

NaCl	1.37 M
KCl	27 mM
Na ₂ HPO ₄	81 mM
<u>NaH₂PO₄</u>	<u>14.7 mM</u>
pH 7.4	

SDS PAGE

2X Laemmli sample buffer

Tris/HCl pH 6.8	60 mM
SDS	2 %
Glycerol	10 %
β -mercaptoethanol	5 %
Bromphenol blue	0.01 %

1M Sodium phosphate buffer

Na ₂ HPO ₄ 1M	46.3 ml
<u>NaH₂PO₄ 1M</u>	<u>53.7 ml</u>
= 100 ml	

30% Acrylamide

Acrylamide	29 %
N,N'-methylenebisacrylamide	1 %

10X SDS PAGE running buffer

Glycine	1.92 M
Tris	0.25 M
SDS	1 %

Separating gel buffer

Tris/HCl pH 8.8	1.5 M
<u>SDS</u>	<u>0.4 %</u>
autoclaved	

Coomassie staining solution

Methanol	25 %
Acetic acid	10 %
Coomassie Brilliant Blue R250	0.15 %

Stacking gel buffer

Tris/HCl pH 6.8	0.5 M
<u>SDS</u>	<u>0.4 %</u>
autoclaved	

De-staining solution

Acetic acid	10 %
-------------	------

Western Blot

Transblot buffer

Tris	48 mM
Glycine	39 mM
SDS	0.037 %
Methanol	20 %

10X TBS

Tris/HCl pH 8.8	0.25 M
NaCl	1.37 M
<u>KCl</u>	<u>27 mM</u>
autoclaved	

Blocking solution

5 % skim milk powder
dissolved in 1X TBS

2.10.3 Solutions and reagents for *E. coli* methods

Generation of competent *E. coli* cells

MOPS I

MOPS 100 mM
 CaCl₂ 10 mM
RbCl 10 mM
 pH adjusted to 7.0 with KOH

MOPS IIa

MOPS 100 mM
 CaCl₂ 70 mM
 RbCl 10 mM
Glycerol 15 %
 pH adjusted to 6.5 with KOH

MOPS II

MOPS 100 mM
 CaCl₂ 70 mM
RbCl 10 mM
 pH adjusted to 6.5 with KOH

2.10.4 Solutions and reagents for *N. magadii* methods

Generation of competent *N. magadii* cells

Buffered high salt spheroplast solution Proteinase K (QIAGEN®)

+ glycerol

Stored at room temperature

Tris/HCl pH 9.5 50 mM
 NaCl 2 M
 KCl 27 mM
 Glycerol 15 %
 after autoclaving, 15 % sucrose
 (sterile filtered) was added

Transformation of *N. magadii*

Buffered high salt spheroplast solution

Tris/HCl pH 9.5	50 mM
NaCl	2 M
KCl	<u>27 mM</u>

after autoclaving, 15 % sucrose
(sterile filtered) was added

60% PEG in high salt spheroplast solution

Aliquotes were stored at -80°C

EDTA 0.5 M

Unbuffered high salt spheroplast solution

NaCl	2 M
KCl	<u>27 mM</u>

after autoclaving, 15 % sucrose
(sterile filtered) was added

Isolation of chromosomal DNA of *N. magadii*

High salt alkaline solution

NaCl	4 M
Tris	50 mM

EDTA 10 mM

CsCl (solid)

Desoxycholat 14 mM

Ethidium bromide (10mg/ml)

Phenol/Chloroform 1:1

n-Butanol

Isopropanol

2.10.5 Solutions and reagents for Φ Ch1 Methods

Isolation of virus particles

High salt alkaline solution

NaCl	4 M
Tris	50 mM

CsCl solution 1.1

CsCl	20 g
NaCl	23.4 g
<u>Tris/HCl pH 9.5</u>	<u>50 mM</u>

Filled up with ddH₂O to 200 ml

CsCl solution 1.3

CsCl	90 g
NaCl	23.4 g
<u>Tris/HCl pH 9.5</u>	<u>50 mM</u>

Filled up with ddH₂O to 200 ml

CsCl solution 1.5

CsCl	135 g
NaCl	23.4 g
<u>Tris/HCl pH 9.5</u>	<u>50 mM</u>

Filled up with ddH₂O to 200 ml

3. Methods

3.1 DNA and RNA Methods

3.1.1 PCR

3.1.1.1 Preparative PCR

For cloning, the preparative PCR using the *Pfu* polymerase was used. The *Pfu* Polymerase is an enzyme derived from the thermophilic archaebacterium *Pyrococcus furiosus*. It features a proofreading activity; therefore, its high fidelity makes it suitable for cloning. Its optimal working temperature is 72°C, and it is capable of adding 500bp per minute.

The preparative PCR batch contained:

Component	Final concentration	Final volume
10X <i>Pfu</i> Buffer	1X	10 µl
<i>Pfu</i> Polymerase	2-3 U/µl	2 µl
Primer 1	0.05 µg/µl	10 µl
Primer 2	0.05 µg/µl	10 µl
dNTP's	0.2 mM	10 µl
Template DNA	variable	1 µl
ddH ₂ O	-	67 µl
		= 100 µl

The PCR program consisted of the following steps:

- 1) Preheating at 95°C for 5 minutes
- 2) Melting the strands at 95°C for 1 minute
- 3) Annealing the primers at a primer-specific temperature for 1 minute
- 4) Elongation at 72°C for a time that was chosen according to the length of the desired PCR product
- 5) 72°C for 2 - 8 minutes according to the length of the desired PCR product

The reaction was run in the thermocycler for 35 cycles. After the PCR, the products and the quality of the PCR were analysed on an agarose gel.

3.1.1.2 Analytical PCR

For analytical purposes, the preparative PCR using the GoTaq® polymerase obtained from Promega was used. The GoTaq® polymerase has its optimal working temperature at 72°C and is capable of adding 1000 bp per minute.

The reaction was set up as follows:

Component	Final concentration	Final volume
GoTaq® master mix (containing the GoTaq® polymerase, reaction buffer and dNTPs)	1X	12.5 µl
Primer 1	0.03 µg/µl	1.5 µl
Primer 2	0.03 µg/µl	1.5 µl
Template DNA	variable	1 µl
ddH ₂ O	-	8.5 µl
		= 25 µl

The PCR program consisted of the following steps:

- 1) Preheating at 95°C for 5 min (10min for colony PCR)
- 2) Melting the strands at 95°C for 1 minute
- 3) Annealing the primers at a primer-specific temperature for 1 minute
- 4) Elongation at 72°C for a time that was chosen according to the length of the desired PCR product
- 5) 72°C for 2 - 8 minutes according to the length of the desired PCR product

The reaction was run in the thermocycler for 20 cycles. After the PCR, the products were analysed on an agarose gel.

3.1.1.3 Colony PCR

The colony PCR was used for rapid screening of *E. coli* cultures. Here, a scrap of a single colony on an agar plate or one microliter of a liquid culture was used directly as a template for PCR. The PCR programs were not changed as compared to normal Test PCR programs, but the initial heating step was extended to 10 min instead of 5 min in order to break open the cells.

In the case of agar plates, single colonies were chosen, marked with numbers on the bottom side of the petri dish and a small amount of each colony was picked with a sterile 200 µl pipette tip and transferred to the reaction tube containing the PCR reaction mix. Clones that were positively tested could be identified by their number on the bottom of the petri dish and grown in LB medium for further use.

In the case of liquid cultures, 1 µl of the culture was used as a template.

3.1.1.4 Reverse transcription PCR (RT-PCR)

RT-PCRs were performed using the OneStep RT-PCR Kit from Quiagen according to the manufacturer's protocol. Template RNA used for the reaction was isolated and treated as described in 3.1.10 and 3.1.11.

3.1.2 DNA purification

In order to remove unwanted components that might interfere with subsequent reactions, PCR products were purified using the QIAquick® PCR Purification Kit according to the manufacturer's protocol. If there were unwanted DNA bands visible after the preparative PCR, the desired DNA bands were eluted from an agarose gel prior to the purification step.

3.1.3 DNA restriction

For restrictions used for cloning, conventional restriction enzymes from Thermo Scientific were used. For vector restrictions, the concentration of the vector DNA was estimated on an agarose gel and the DNA diluted if the concentration was found to be too high. The restriction reactions were incubated at 37°C for three hours. Vector restrictions were tested on an agarose gel with the unrestricted vector serving as a negative control.

Methods

The conventional restriction batch consisted of:

Component	Final concentration	Final volume
DNA in H ₂ O	variable	30 µl
10X Restriction buffer	1X	5 µl
Restriction enzyme 1	variable	2 µl
Restriction enzyme 2	variable	2 µl
ddH ₂ O	-	11 µl
		= 50 µl

For test restrictions, FastDigest enzymes from Thermo Scientific were used. The reactions were incubated at 37°C for about one hour and the restricted DNA was tested on an agarose gel.

The test restriction batch consisted of:

Component	Final concentration	Final volume
DNA in H ₂ O	variable	10 µl
10X FastDigest buffer	1X	2 µl
Restriction enzyme 1	variable	1 µl
Restriction enzyme 2	variable	1 µl
ddH ₂ O	-	11µl
		= 20 µl

3.1.4 DNA ligation

For ligation of vector and insert DNA the T4 ligase (Promega) was used. This ligase is capable of ligating blunt or sticky end DNA. Prior to ligation, the digestion of the vector DNA was controlled on an agarose gel and the vector was diluted (e.g. 1:20, 1:50), depending on the strength of the vector bands on the gel.

The ligation reaction was set up as follows:

Component	Final concentration	Final volume
Insert DNA	variable	11.5 µl
Vector DNA	variable	1 µl
10X T4 ligase buffer	1X	1.5 µl
T4 ligase	About 0.1 U/2-3 U/µl	1 µl
		= 15 µl

The reaction was incubated at room temperature for three hours at 4°C overnight.

3.1.5 Plasmid preparation

Plasmid preparations were performed using the GeneJET Plasmid Miniprep Kit from Thermo Scientific according to the manufacturer's protocol. The DNA was eluted in 30-60 µl ddH₂O and stored at -20°C.

3.1.6 Nucleic acids concentration measurements

The concentration of DNA and RNA was measured using a NanoDrop® photospectrometer. In either case, one microliter per sample was used, and the A260/280 ratio was measured. The water in which the particular nucleic acids were dissolved was used as blanks.

3.1.7 Southern blot

3.1.7.1 Synthesis of the probe

The probes for the Southern blot were synthesised by PCR using the GoTaq® polymerase. The probes were labelled by using biotinylated dUTP. The dNTP mixture consisted of:

Component	Final concentration	Final volume
dATP	0.25 mM	10 µl
dCTP	0.25 mM	2 µl
dGTP	0.25 mM	1 µl
dTTP	0.125 mM	1 µl
dUTP	0.125 mM	11 µl
		= 20 µl

The probe PCR was set up as follows:

Component	Final concentration	Final volume
5X GoTaq® Reaction buffer	1X	20 µl
GoTaq® polymerase	0.05 U/µl	0.5 µl
dNTP mixture	0.2 mM	20 µl
Primer 1	0.025 µg/µl	5 µl
Primer 2	0.025 µg/µl	5 µl
Template DNA	variable	1 µl
ddH ₂ O	-	48.5 µl
		= 100 µl

Following the PCR, the quality of the product was controlled on an agarose gel. The probes were then purified by gel extraction and eluted in 100 µl ddH₂O.

3.1.7.2 Target DNA electrophoresis and blotting

The target DNA was separated on an agarose gel and controlled by staining with ethidium bromide. The gel was then incubated in 0.25 M HCl for 30 min to help transfer larger fragments to the membrane. In order to denature the DNA, the gel was incubated in 0.4 M NaOH/0.6 M NaCl for 30 min. It was then neutralised by incubating in 1.5 M NaCl/0.5 M Tris-HCl, pH 7.5 for 30 min.

The DNA was blotted to an Amersham nylon membrane (GE healthcare) by O/N capillary blotting using Whatman paper, paper towel and 10X SSC buffer. After blotting, the membrane was incubated in 0.2 M NaOH for one minute then in 0.2 M Tris-HCl pH 7.5 for one minute, and the DNA was fixed by UV-crosslinking.

3.1.7.3 Blocking and hybridisation

For blocking and hybridisation, the membrane was placed in a hybridisation tube. It was blocked by adding 12 ml hybridisation buffer and 120 µl herring sperm DNA (10 µg/µl). Prior to the hybridisation, the probes were denatured at 95°C for 5 min. Hybridisation of the membrane and the probe took place in an hybridisation oven at 65°C overnight.

3.1.7.4 Blot development

After hybridisation, the membrane was washed two times with 2X SSC/0.1% SDS for 5 min at room temperature and two times with 0.1X SSC/0.1% SDS (pre-warmed) for 15 min at 65°C. The blot was developed using the Phototope®-Star Detection Kit for Nucleic Acids (New England Biolabs) according to the manufacturer's protocol. The signals were detected by exposing the membrane to an x-ray film.

3.1.8 Agarose gel electrophoresis

0.8% agarose gels were prepared by melting the appropriate amount of agarose in 1X TAE buffer. The molten gel was poured into an electrophoresis trough and after solidification was covered with 1X TAE buffer. After loading the DNA mixed with loading buffer containing sucrose, bromphenol blue and xylene cyanol, a voltage of 6 V/cm of gel was applied. The duration of the electrophoresis was estimated by comparing the migration of the bromphenol blue in the buffer with the size of the DNA fragment. After electrophoresis, the gel was stained in an ethidium bromide bath containing 10 µg/ml ethidium bromide and visualised using an UV-transilluminator apparatus.

3.1.9 Agarose gel extraction

The agarose gel was loaded with DNA and loading buffer and run at 6 V/cm. The separated gel was stained with ethidium bromide solution and the desired fluorescent bands were cut out under 70% UV light. The pieces were filled in reaction tubes and purified using the QIAquick® gel extraction kit. Purified DNA was eluted with 30–60 µl ddH₂O according to the desired concentration and stored at -20°C.

3.1.10 RNA isolation

For all RNA steps, pipette tips were autoclaved twice and only used for RNA. Additionally, Eppendorf tubes and solutions and chemicals for RNA manipulation were marked and not used for other procedures. DEPC-treated water was used for each step.

Cell lysis:

Pellets of cultures that had a corresponding OD₆₀₀ of 0.6 - 0.8 were resuspended in 500 µl 4M NaCl, 50mM Tris/HCl, pH 9.5. 500µl Trizol® reagent was added and the reaction tube was vortexed for one minute.

Phase separation:

Chloroform was added 1/5 volume of TRIzol® volume and shaken (no vortexing) for 15 sec. The tubes were incubated for 5 min at room temperature and centrifuged for 15 min at 12.000 g, 4°C. The upper aqueous phase was transferred to a new tube; care was taken not to aspire any DNA-containing white interphase.

Precipitation:

The RNA was precipitated with isopropanol. Therefore, 70% of aqueous phase isopropanol was added and the samples incubated for 10 min at room temperature. The precipitated RNA was pelleted by a centrifugation for 15 min, 12.000 g, 4°C and the supernatant was removed.

Washing the RNA:

The pellets were washed by adding 500 µl 70% ethanol, quickly vortexing the tubes, and centrifuging them for 15 min, 12.000 g, 4°C. The supernatant was removed and the pellets air-dried at room temperature.

Re-dissolving the RNA:

The RNA pellet was re-dissolved in 50 µl DEPC-treated water and stored at -80°C.

3.1.11 DNase digestion

In order to remove DNA that was still present after the RNA extraction, the RNA samples that were used for RT-PCR had to be treated with DNase beforehand. RQ1 RNase-Free DNase from Promega was used for the digest.

The RNA concentration in the samples and, by extension, the volume of RNA that was used in the reaction was estimated by running the samples on an agarose gel.

The reaction was set up as follows:

Component	Final concentration	Final volume
RNA in DEPC ddH_2O	variable	1-8 μl (depending on the RNA concentration)
RQ1 RNase-Free DNase 10X Reaction Buffer	1X	1 μl
RQ1 RNase-Free DNase	about 1 U/ μg RNA	1 μl
Nuclease-free water	variable	0-7 μl (depending on the RNA concentration)
Recombinant RNasin® Ribonuclease Inhibitor (Promega)	about 1 U/ μl	1 μl
		= 11 μl

The reaction was incubated at 37°C for 2 h.

Adding 1 μl of RQ1 DNase Stop Solution and incubation at 65°C for 15 min inactivated the DNase and stopped the reaction.

To test if the DNA digestion of the samples was complete, and the RNA did not degrade significantly, the samples were applied on an agarose gel. Since very small amounts of nucleic acids would not be detectable on a stained agarose gel, the samples were tested with PCR. Therefore, the same primes that were used for the RT-PCR were used in this test PCR. ϕCh1 DNA or *N. magadii* L11 chromosomal DNA was used in the positive control.

3.2 Protein methods

3.2.1 Over-expression and purification of His-tagged proteins in *E. coli*

The particular *E. coli* expression strain harbouring the expression vector was inoculated from an overnight culture at an OD600 of 0.1. When the culture reached the exponential growth phase (around OD600 of 0.3), the cells were induced by the addition of 0.5 mM IPTG. After 2-4 hours, the cells were harvested by centrifugation (6,400 × g, 15 min, 4°C) and the pellet was frozen and thawed rapidly in order to aid destruction of the cells. The thawed pellets were resuspended in buffer B and stirred overnight. Completeness was controlled with phase contrast microscopy. If the cells were not lysed sufficiently they were sonificated until lysis was complete. The lysate was centrifuged (10,000 × g, 20 min, RT). In order to purify the proteins via affinity chromatography, Ni-NTA was added to lysate and stirred gently overnight at room temperature. The lysate-Ni-NTA mixture was loaded on an empty 20 ml column and the flow-through was collected. The column was then washed twice with 4 ml buffer C. The proteins were eluted four times with 0.5 ml buffer D and four times with 0.5 ml buffer E. The flow-through, the wash fraction and all elution fractions were analysed by SDS-PAGE and Western blot. Fractions that contained the protein of interest were pooled and dialysed against PBS buffer for one hour at room temperature and overnight after changing the buffer. Purified proteins were stored at -20°C.

3.2.2 Preparation of crude protein extracts from *E. coli* and *N. magadii*

1.5 ml of the culture were centrifuged (20,000 × g, 5 min, RT) and the pellet was resuspended in x µl sodium-phosphate-buffer and x µl 2X Laemmli buffer. (x = OD of culture * 75). *E. coli* samples were directly resuspended in sodium-phosphate-buffer and stored at -20°C for SDS-PAGE and Western blot analysis. *N. magadii* possesses up to 50 chromosome copies, and the samples would be too viscous for direct resuspension due to the large amount of DNA. In order to break down the chromosomal DNA, the sodium-phosphate-buffer and Laemmli buffer were added to the pellets without resuspension, and the tubes were incubated with shaking at 37°C overnight. The next day, the *N. magadii* samples were resuspended and stored at -20°C.

3.2.3 TCA precipitation of virus proteins

Virus particles were isolated as described in 3.4.4. 1/10 volume of 100% TCA was added to the virus, mixed well and incubated in ice for one hour. The precipitate was centrifuged (20,000 × g, 5 min, 4°C) and the supernatant removed. The pellet was rinsed with ice-cold acetone (pre-cooled at -20°C), centrifuged again, and the supernatant removed. The resulting pellet was air-dried, resuspended in 1X PBS buffer and stored at -20°C.

3.2.4 SDS-PAGE

Proteins were separated according to their mass by discontinuous polyacrylamide electrophoresis. The stacking gel contained 4 % acrylamide, whereas the separating gel contained 12 %. At first, 40 V were applied to the gel until the samples reached the stacking gel. The voltage was then increased to 60 V, until the samples reached the separating gel, and then increased to 100 V. In the case of samples and proteins derived from *N. Magadii*, 40 V were applied on the gel during the entire electrophoresis process.

The separated gels were either used for Western blotting or stained with Coomassie Blue. To visualise proteins directly, the gels were stained with Coomassie staining solution for about 15 min. The gels were de-stained with 10 % acetic acid for 3-5 h on a shaker or overnight without shaking. De-staining of the gels was complete when the blue protein bands were clearly visible and the background was clear.

3.2.5 Western Blot

After separating the proteins by SDS-PAGE, the proteins were blotted onto a nitrocellulose membrane. The blot was build up by overlaying 3 gel-sized whatman papers with the polyacrylamide gel, followed by the gel-sized nitrocellulose membrane and 3 more whatman papers. The whatman paper and the nitrocellulose membrane were soaked in transblot buffer prior to building the blot. The blot was placed in a semi-dry blotting apparatus and 20 V were applied for 20 min (one gel) or 30 min (two gels).

To prevent unspecific binding on the membrane, it was blocked by incubation in 5 % milk powder/1X TBS at 4°C overnight.

After blocking, the membrane was washed 3 times with 1 X TBS for 15 min and incubated together with the primary antibody for 1 h at room temperature. The membrane was then washed again 3 times with 1 X TBS for 15 min and incubated together with the secondary antibody for 1 h at room temperature. After three additional washing steps, the blot was developed with the Super Signal® West Pico Chemiluminescent Substrate kit and the membrane was exposed to an x-ray film.

3.3 *E. coli* methods

3.3.1 Competent cells

The *E. coli* strain of choice was incubated in 100ml LB medium containing the appropriate antibiotics at 37°C to an OD600 of approximately 0.6. The cells were centrifuged (6,000 × g, 10 min, 4°C), the pellet was resuspended in 40ml MOPS I and incubated on ice for 10 min. The culture was centrifuged (6,000 × g, 10 min, 4°C), pellet resuspended in 40 ml MOPS II and incubated on ice for 30 min. The culture was centrifuged once again, finally resuspended in 2 ml MOPS IIa and aliquoted (100 µl).

3.3.2 Transformation of *E. coli*

100 µl of competent cells (frozen at -80°C) were thawed for about 10 min on ice. 15 µl ligation preparation (for test transformations, one µl of plasmid DNA with known concentration) was added to the competent cells, and they were incubated on ice for 30 min. The cells were then heat-shocked for 2 min at 42°C and then quickly put on ice for 30 sec. 300 µl LB medium without antibiotics were added to the cells, which were then regenerated for 30 min at 37°C without agitation. Finally, 100 µl of the transformed *E. coli* cells were plated thrice on LB plated with the proper antibiotics. The plates were incubated at 37°C overnight.

3.3.3 Screening of transformants

After transformations, single colonies were tested with colony PCR (see Chapter 3.1.1.3). Potential positive candidates were inoculated and grown overnight. They were then further tested by test restriction.

3.3.4 Reference stocks

1 ml of fresh overnight culture was mixed with 800µl glycerol and stored in cryovials at -80°C.

3.3.5 Acetone powder from *E.coli*

1 l of the desired *E.coli* expression strain was incubated at 37°C until the culture reached the stationary phase and then centrifuged (6,000 × g, 20 min, RT). The resulting pellet was resuspended in 0.9 % saline water (1 ml saline water per 1 g pellet) and incubated on ice for 5 min. 2 ml acetone (pre-cooled at -20°C) per 1 ml cell suspension were added, mixed and incubated on ice for 30 min. The acetone/cell suspension mix was then centrifuged (10,000 × g, 10 min, RT), resuspended in fresh, pre-cooled acetone, incubated on ice for 10 min and centrifuged again (10,000 × g, 10 min, RT). The pellet was dried on filter paper, powdered with a mortar and stored at room temperature.

3.4 Halophiles methods

3.4.1 Competent cells

Three baffled Erlenmeyer flasks containing 60 ml NVM⁺ medium were inoculated with a fresh and dense overnight culture of *N. magadii* (2/4/6 ml) and incubated at 37°C. When one of the cultures reached an OD₆₀₀ of 0.5-0.6, the cells were harvested by centrifugation (5,500 × g, 15 min, RT) and the pellet was resuspended in 30 ml buffered, high-salt spheroplast solution + glycerol (Proteinase K was added to a final concentration of 0.1%). The resuspended cells were incubated at 42°C until the cells became spheroplasts. The competent cells were used directly for transformation. Alternatively, the competent cells could be stored in 1.5 ml aliquotes at -80°C up to a week.

3.4.2 Transformation of *N. magadii*

1.5 ml of competent cells were centrifuged (10,000 × g, 3 min, RT) and the pellet was resuspended in 150 µl buffered high salt spheroplast solution. 15 µl 0.5 M EDTA were added and the cells were incubated at room temperature for 10 min. Next, the DNA (3-10 µg in max. 10 µl ddH₂O) was added and after a 5 min incubation at RT 150 µl, 60% PEG600 (in unbuffered high salt spheroplast solution) were added. After an additional incubation step for 30 min, 1 ml of NVM⁺ medium was added to the cells and they were centrifuged (10,000 × g, 3 min, RT). The pellet was resuspended in 1 ml NVM⁺ and after an additional centrifugation step, the cells were resuspended in 1 ml NVM⁺ medium. The transformed *N. magadii* cells were regenerated at 37°C with agitation until the cells had a rod-like shape again. 10 undiluted and 10 1:10 plates were plated per transformation. (100 µl per plate) The plates were incubated at 42°C for 2-3 weeks until colonies were visible.

3.4.3 Screening of transformants

Single colonies were inoculated in 700 µl NVM⁺ medium and were incubated at 37°C with agitation for about a week until the growth was visible. Every second day the tubes were opened to supply the cells with fresh air. Once the cells were growing, they were tested with PCR. For PCR templates, 100 µl of the culture was centrifuged (20,000 × g, 3 min, RT) and the pellet was resuspended in 100 µl ddH₂O.

3.4.4 Isolation of virus particles

The strain *N. magadii* L11 was inoculated in 1.5L NVM⁺ rich medium and incubated at 37°C. The OD₆₀₀ was measured every day and listed on a growth curve. When the growth curve indicated the lysis of the strains, the medium containing the ϕ Ch1 particles was centrifuged at 10,000 g for 20 min at 4°C and 150 g PEG-6000 was added to the pooled supernatants of the lysed cultures. The PEG-6000 was dissolved by slowly stirring it overnight at room temperature. The medium containing the virus particles attached to the PEG-6000 was centrifuged at 10,000 g for 30 min at room temperature, and the pellet was resuspended in high salt alkaline solution (4M NaCl, 50mM Tris/HCl, pH 9.5), resulting in a final volume of approximately 30 ml. The pellets were collected in a centrifugation tube. In order to purify the ϕ Ch1 particles from the suspension, a discontinuous CsCl density gradient was established by overlaying 2 ml CsCl solution 1.5 with 4 ml CsCl solution 1.3, followed by 5 ml of the suspension containing the virus particles and at least approximately 1ml of the CsCl solution 1.1. The gradient was centrifuged at 160,000 g for 20 h at room temperature. The accumulated ϕ Ch1 particles could be seen as a light blue band between the two CsCl solutions and were removed using a Gilson pipette. Additional purification and concentration using a continuous CsCl density gradient (virus particles mixed with solution 1.3 and centrifuged at 160,000 g for 20 h), as well as dialysis against high salt alkaline solution in order to remove the Cs⁺, was carried out. The purified virus particles were stored at room temperature.

3.4.5 Virus titer

To determine the virus titer, *N. magadii* L13 were infected by soft agar technique: a dilution series (10^{-2} , 10^{-4} , 10^{-6} , 10^{-8} , 10^{-10}) was prepared and 100 μ l of each dilution was mixed with 500 μ l of well growing *N. magadii* L13 cells and plated together with 5 ml soft agar on NVM⁺ plates. The plates were stored at room temperature for one day and then incubated at 37°C for one to two weeks. The virus plaques were then counted and the virus titer determined (plaque-forming units or pfu per ml).

3.4.6 One-step growth curve

N. magadii L13 was grown in non-baffled flasks until the culture reached the exponential growth phase. The culture was then infected with ϕ Ch1 and ϕ Ch1 Δ 79 respectively at a multiplicity of three virus particles per cell. After an adsorption period of 30 min at 37°C without agitation, non-adsorbed virus particles were removed by centrifugation (5000 $\times$ g, 15 min, RT). The pellet was resuspended in prewarmed NVM⁺ medium and the culture was incubated at 37°C with agitation. Samples were taken at different points in time. At each point, one sample of 1 ml was vortexed for 1 min with 200 μ l chloroform and centrifuged (20,000 $\times$ g, 15 min, RT). 900 μ l of the supernatant were transferred in a new tube and stored at room temperature with 20 μ l chloroform. At each point in time, a second sample was taken without adding 200 μ l. The samples were then used for a virus titer.

3.5 Cloning strategies

3.5.1 *aglB*

3.5.1.1 *pKS_{II}⁺-aglB 1-4 Nov^R forward*

The upstream fragment (amplified with primers *AgIB*-1/*AgIB*-2) was restricted with *Xba*I/*Bam*HI and ligated with the vector *pKS_{II}⁺*, digested with *Xba*I/*Bam*HI, resulting in the plasmid *pKS_{II}⁺-aglB 1-2*.

pKS_{II}⁺-aglB 1-2 was restricted with *Sma*I/*Pst*I and ligated with the *Nov^R*-forward fragment (amplified with primers *Nov^R*-1p/*Nov^R*-1s) restricted with *Sma*I/*Pst*I, resulting in the plasmid *pKS_{II}⁺-aglB 1-2 Nov^R forward*. *pKS_{II}⁺-aglB 1-2 Nov^R forward* and the *aglB* downstream fragment (amplified with primers *AgIB*-Cla/*AgIB*-Kpn) were digested with *Cla*I/*Kpn*I and ligated, resulting in the plasmid *pKS_{II}⁺-aglB 1-4 Nov^R forward*.

3.5.1.2 *pKS_{II}⁺-aglB 1-4 Nov^R reverse*

The upstream fragment (amplified with primers *AgIB*-1/*AgIB*-2) was restricted with *Xba*I/*Bam*HI and ligated with the vector *pKS_{II}⁺*, digested with *Xba*I/*Bam*HI, resulting in the plasmid *pKS_{II}⁺-aglB 1-2*.

pKS_{II}⁺-aglB 1-2 was restricted with *Sma*I/*Pst*I and ligated with the *Nov^R*-reverse fragment (amplified with primers *Nov^R*-2p/*Nov^R*-2s) restricted with *Sma*I/*Pst*I, resulting in the plasmid *pKS_{II}⁺-aglB 1-2 Nov^R reverse*. *pKS_{II}⁺-aglB 1-2 Nov^R reverse* and the *aglB* downstream fragment (amplified with primers *AgIB*-Cla/*AgIB*-Kpn) were digested with *Cla*I/*Kpn*I and ligated, resulting in the plasmid *pKS_{II}⁺-aglB 1-4 Nov^R reverse*.

3.5.1.3 pRSET-A-AglB-C

The C-terminal fragment for expression (amplified with primers AglB-5B/AglB-3H) was restricted with *BglII/HindIII* and ligated with the vector pRSET-A digested with *BamHI/HindIII*, resulting in the vector pRSET-A-AglB-C.

3.5.1.4 pNB102-aglB

The fragment for complementation (amplified with primers AglB-5E/AglB-3C) was restricted with *Clal/EcoRI* and ligated with the vector pNB102 digested with *Clal/EcoRI*, resulting in the vector pNB102-aglB.

3.5.2 pilin

3.5.2.1 pKS_{II}⁺-pil 1-4 Nov^R forward

The upstream fragment (amplified with primers Pil-1/Pil2) was digested with *XbaI/BamHI*, and ligated with the vector pKS_{II}⁺ cut with *XbaI/BamHI*, resulting in the plasmid pKS_{II}⁺-pil 1-2. pKS_{II}⁺-pil 1-2 was restricted with *HindIII/KpnI* and ligated with the pil downstream fragment (amplified with primers Pil-3/Pil-4) restricted with *HindIII/KpnI*, resulting in the plasmid pKS_{II}⁺-pil 1-4.

pKS_{II}⁺-pil 1-4 and the Nov^R-forward fragment (amplified with primers Nov^R-1p/Nov^R-1s) were digested with *SmaI/PstI* and ligated, resulting in the plasmid pKS_{II}⁺-pil 1-4 Nov^R forward.

3.5.2.2 pKS_{II}⁺-pil 1-4 Nov^R reverse

The upstream fragment (amplified with primers Pil-1/Pil2) was restricted with *XbaI/BamHI*, and ligated with the vector pKS_{II}⁺ cut with *XbaI/BamHI*, resulting in the plasmid pKS_{II}⁺-pil 1-2. pKS_{II}⁺-pil 1-2 was restricted with *HindIII/KpnI* and ligated with the *pilin* downstream fragment (amplified with primers Pil-3/Pil-4) restricted with *HindIII/KpnI*, resulting in the plasmid pKS_{II}⁺-pil 1-4.

pKS_{II}⁺-pil 1-4 and the Nov^R-reverse fragment (amplified with primers Nov^R-2p/Nov^R-2s) were digested with *SmaI/PstI* and ligated, resulting in the plasmid pKS_{II}⁺-pil 1-4 Nov^R reverse.

3.5.2.3 pRSET-A-Pil-C

The C-terminal fragment for expression (amplified with primers 0291_3H/0291_5B) was restricted with *BglII/HindIII* and ligated with the vectors pRSET-A digested with *BamHI/HindIII*, resulted in the plasmid pRSET-A-Pil-C .

4. Results

4.1 *aglB* and *pilin* deletion mutants

4.1.1 Generation of *aglB* and *pilin* deletion mutants in *N. magadii*

4.1.1.1 Aim

One of the main tasks regarding the relationship between the halovirus ϕ Ch1 and *Natrialba magadii* is to determine the virus binding site on the host surface. It was shown that one of the virus' tail fiber proteins, gp34₅₂, possesses a C-terminal galactose binding domain and binds to the surface of *N. magadii*. The observation that the presence of α -D-galactose in the medium prevents the viral gp34₅₂ protein from binding to the cell surface further supports the theory that the ϕ Ch1 binds to its host at a surface structure that exhibits galactose residues [98].

Candidate surface structures with galactose residues include the archaeal S-layer, flagella and pili. The genes in *N. magadii* that make up the S-layer have not yet been identified, which precludes the construction of S-layer deletion mutants. Furthermore, the S-layer gives the cells their morphological structure and stability. Therefore, depletion of S-layer proteins in the medium through the use of enzymes might strongly interfere with cell stability and viability.

Deleting the oligosaccharyltransferase AglB gene would constitute an indirect approach to deplete glycosylated surface structures, since AglB is involved in the assembly and transfer of structural glycoproteins such as archaeal S-layer and flagellum glycoproteins [100], [101].

Another candidate for the binding of ϕ Ch1 is the glycosylated pilin protein Nmag_0291. The genes are located on the *N. magadii* chromosome at 947948-950833 (*aglB*) and 280439-280969 (*pilin*). This project aims to construct *N. magadii* *aglB*- and *pilin*-deficient strains.

4.1.1.2 Experimental setup

A method of obtaining deletion mutants is the introduction of suicide plasmids that replace the gene of interest with a resistance marker. For this project, suicide plasmids containing a novobiocin resistance cassette flanked by the *aglB/pilin* up- and downstream sequences were used. For both genes, the novobiocin resistance cassette was cloned in forward as well as in reverse direction, since it has no terminator sequence which could lead to unpredictable effects on neighbouring genes or gene elements. Transformation of *N. magadii* with these plasmids and growth on a selective medium that contains novobiocin should lead to a homologous recombination between the up- and downstream sequences on the plasmid through their homologous sequences on the chromosome. This double crossover event is schematically illustrated in Figure 7. Successful recombination should lead to clones that have the *aglB/pilin* genes replaced by the novobiocin resistance cassette. Since *N. magadii* is a polyploid organism that contains up to 50 chromosomal copies, the transformants would be heterozygous. To obtain homozygous mutants, heterozygous mutants would need to be passaged to select for chromosomal copies that possess the novobiocin cassette. This can be achieved by transferring exponentially growing cultures to fresh selective medium. The cultures would be passaged until no wild-type gene signal would be detected by PCR and Southern blot. Homozygous mutants would then be tested for their growth behaviour and infected with ϕ Ch1 to determine virus titers. A decrease in the virus titer of the

aglB or *pilin* deficient strains could suggest the gene as a receptor of the viral tail fiber protein.

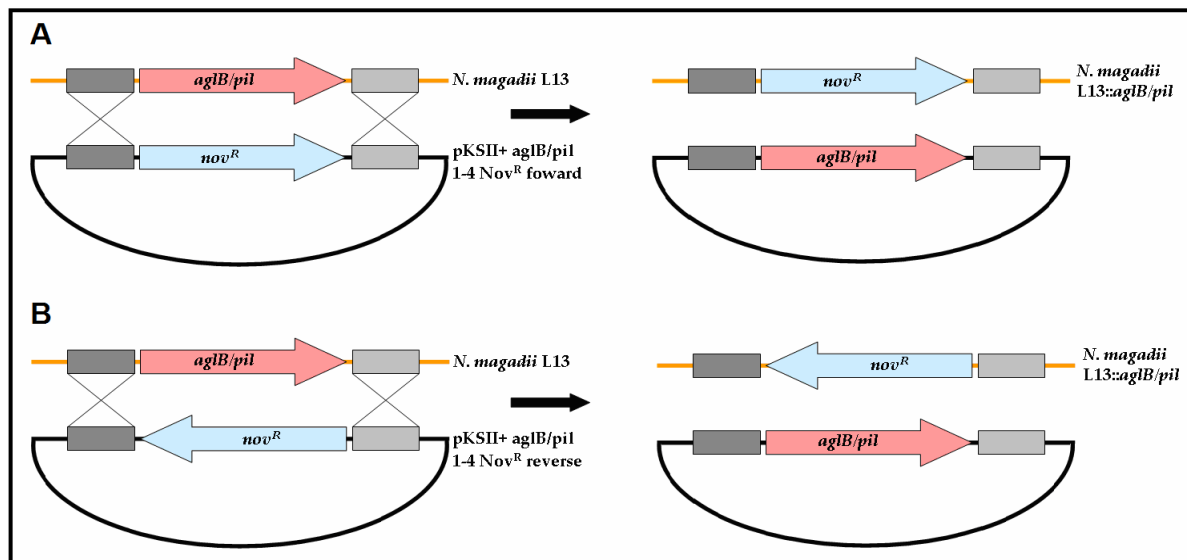


Figure 7: Schematic illustration of the crossover event

The hypothetical, desired outcome of the crossover event between the *aglB/pilin* up-and downstream sequences on the suicide plasmid and their homologous sequences in the *N. magadii* chromosome. Successful double crossovers would lead to the *aglB/pilin* genes being replaced by the novobiocin cassette on the chromosome. **A** represents the hypothetical outcome when using the novobiocin cassette in forward direction, whereas **B** represents the cassette in reverse direction.

4.1.1.3 Results

The vectors pKSII+-*aglB* 1-4 Nov^R forward, pKSII+-*aglB* 1-4 Nov^R reverse, pKSII+-Pil 1-4 Nov^R forward and pKSII+-Pil 1-4 Nov^R reverse were constructed and cloned in *E. coli* XL1-Blue as described in the Material and Methods sections. The vectors pKSII+-*aglB* 1-4 Nov^R forward and pKSII+-*aglB* 1-4 Nov^R reverse include the Nov^R resistance cassette flanked by the 1027 bp upstream region and the 999 bp downstream region of the *aglB* gene. The vectors pKSII+-Pil 1-4 Nov^R forward and pKSII+-Pil 1-4 Nov^R reverse include the novobiocin resistance cassette flanked by the 473 bp upstream region and the 1415 bp downstream region of the *pil* gene.

The successful cloning and the presence of the upstream regions, downstream regions and the novobiocin resistance cassette were verified by analytical PCR and test restrictions.

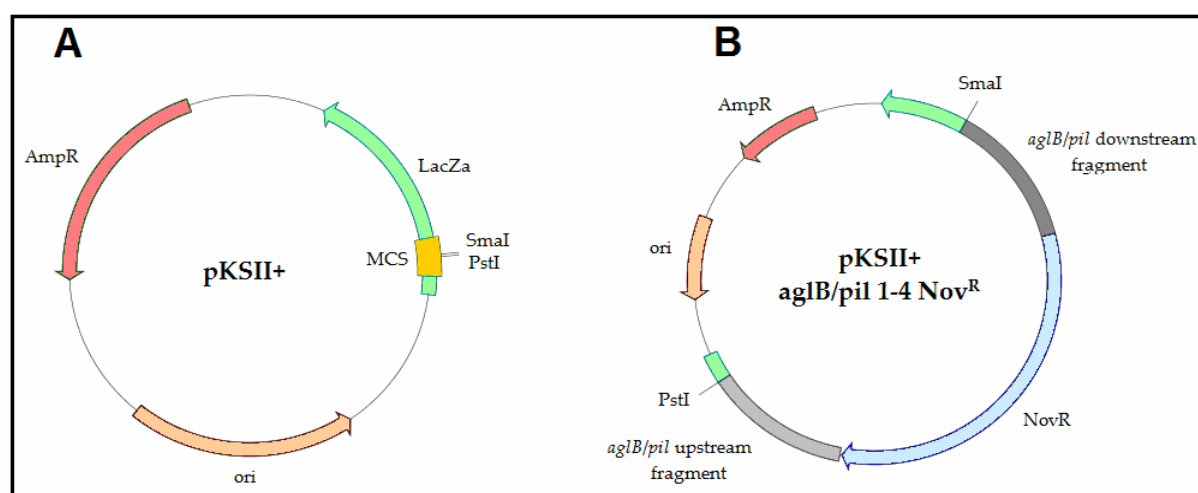


Figure 8: The constructed suicide plasmids

A: pKSII+ plasmid featuring ampicillin resistance for selection in *E. coli*, origin of replication (*E. coli*) and the multiple cloning site containing the restriction sites for *SmaI* and *PstI* enzymes

B: Plasmids for deletion of *aglB* and *pilin* in *N. magadii*. Nov^R here in forward direction, flanked by the upstream and downstream sequences of the respective gene

Competent *N. magadii* cells were generated and transformed as described in 3.4. They were plated on NVM⁺ agar plates containing 3 µg/ml novobiocin and incubated at 37°C. After two to three weeks, colonies were screened as described in 3.4.3. To test the occurrence of a double crossover event, clones were tested with a primer that includes a region upstream of the gene, along with a primer that binds inside of the novobiocin cassette, and also with a primer that includes a region downstream of the gene, along with a primer that binds inside of the novobiocin cassette. For the resistance cassette in forward orientation the primer pairs AglB-5E/Nov-12, AglB-Cla/Nov-10, Pil-5en/Nov-12 and Pil-3en/Nov-10 were used. For the resistance cassette in reverse orientation the primer pairs AglB-Cla/Nov-12, AglB-5E/Nov-13, Pil-3en/Nov-6, Pil-3en/Nov-11, Pil-3en/Nov-12, Pil-5en/Nov-8, Pil-5en/Nov-9 and Pil-5en/Nov-13 were used. When colonies were found, around 30 to 90 colonies were

tested for every gene in every transformation attempt. In some cases, a single crossover was detected although no double crossover was found in any of the transformants. All plasmids were tested again for the presence of the correct up-and downstream sequences, as well as the correct insertion of the novobiocin resistance cassette. Analytical PCRs, test restrictions and sequencing of the plasmids verified the correct construction of the plasmids (data not shown). For typical results of the PCR screenings, see Figure 9.

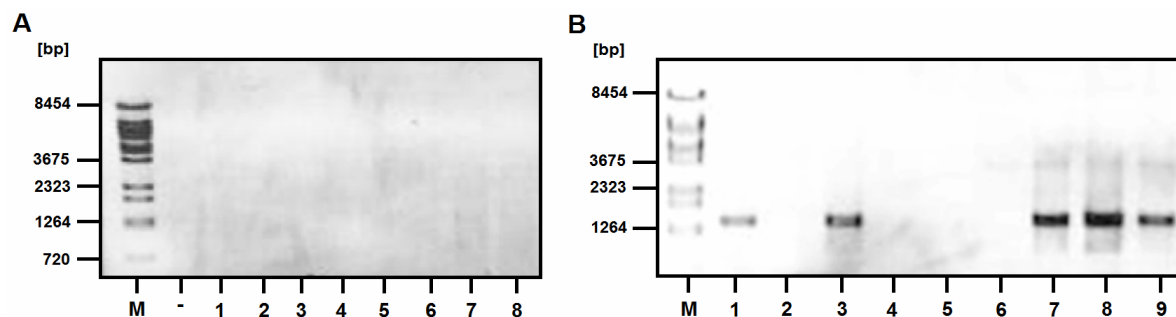


Figure 9: Screening of *aglB* and *pilin* transformation attempts

The pictures show typical agarose gels of the PCR screenings. **A:** *N. magadii* L13 transformed with pKSII+*aglB* 1-4 Nov^R reverse. Primer: *AgIB*-*Cla*/*Nov*-12. No signals were detected in the expected 1126 bp fragment size. **B:** *N. magadii* L13 transformed with pKSII+*Pil* 1-4 Nov^R reverse, primer: *Pil*-3en/*Nov*-12. In some transformants, signals were detected in the expected fragment size of 1542 bp. Subsequent tests of these clones with upstream-primers yielded no signals, indicating single crossover events. M: Marker (λ -*Bst*EII), -: Negative control (L13 DNA template), numbered lanes: PCR screening of single colonies.

The transformations were repeated six times, with different amounts of DNA ranging from 0.5 to 5 μ g of total transformed DNA. In three out of seven transformations, no colonies were visible on the plates even after eight weeks of incubation at 42°C. In these cases, the regeneration of the transformed cells also took three times as long, up to five days. Several reasons for the absence of colonies were considered. In order to get rid of remaining washing detergent traces, the flasks for the transformations were washed with ddH₂O, autoclaved, sterilized and then washed with sterile medium prior to use. When all the solutions for the transformation in *N.*

magadii were replaced by freshly made solutions, the transformed cells were able to regenerate in one to two days again. When the new solutions were used, also more viable cells were found when examining the regenerating cells with identical starting optical density under a light microscope. As for the NVM⁺ agar, the suppliers for the casamino acids, yeast extract and agar were changed and a new batch of NaCl was ordered and only used for *N. magadii* media in the halophiles laboratory. The combination of newly prepared transformation solutions and new chemicals for the NVM⁺ medium restored growth on the agar plates. In one case where no growth occurred on the agar plates, it was found that the temperature of the 42°C incubator was increased to 55°C. This change took place without informing the author beforehand. The length of time that the temperature was set to 55°C could not be verified, nor was it possible to confirm that this was the reason that no colonies were obtained in said transformation attempt. In another transformation attempt, it was discovered that the initial culture that was used to generate the competent cells was infected with ϕ Ch1. The infection was detected by PCR using primers that are specific for ϕ Ch1 genes. The experiment was ultimately stopped because no positive transformants were found, even after seven transformation attempts.

4.1.1.4 Discussion

The first step of the experiment, which involved the construction of the suicide plasmids for the gene deletion in *N. magadii*, yielded the correct plasmids for replacement of either the *aglB* or the *pilin* gene with the novobiocin resistance cassette in both forward and reverse direction.

However, none of the seven transformation attempts led to a positive *N. magadii* clone that had either the *aglB* or *pilin* gene replaced with the novobiocin cassette. Several reasons were considered for the lack of positive clones. One reason could be

that the number of tested colonies was simply too low. In this case, repeating the transformations and testing for a higher number of colonies could eventually lead to a positive clone. As all four plasmids were tested positively for the up- and downstream sequences as well as for the presence of the novobiocin cassette with analytical PCR, test restrictions and sequencing of the plasmids, the plasmids constructed in this thesis could be used directly for future experiments.

Deletion attempts of *aglB* in the thermophiles *S. acidocaldarius* and *S. islandicus* were unsuccessful and lead to a lethal phenotype indicating that the gene is essential for cell viability at least in these organisms [105]. When taking these results into account, the possibility that the *aglB* gene is essential in *N. magadii* could also be considered. In order to test this theory, a second copy of *aglB* could be introduced into the cells which would allow deletion of the first *aglB* copy on the original gene site.

It is also conceivable that important regulatory sequences of unknown function are located in the immediate proximity of the *aglB* and *pilin* genes. If this is the case, the introduction of the novobiocin cassette through homologous recombination could impair the function of these regulatory sequences and thus prevent the generation of viable deletion mutants.

The experiment clearly exposed the challenges when working with *N. magadii* as compared to easy-to-culture mesophilic organisms like *E. coli* laboratory strains. Small changes in the chemicals that are used for growth media and even using chemicals from different suppliers appear to have a significant impact on the growth and/or viability of the cells. Replacing the solutions for the transformation in *N. magadii* that were two to five years old with freshly prepared solutions improved the time that the cells needed for regeneration, from up to five days to one or two days, as well as the cell's viability in the regeneration step. This observation makes it evident that the solutions for transformations in *N. magadii* should be changed and freshly prepared on a regular basis to ensure proper viability and regeneration of the cells.

4.1.2 Antibody production for AglB and Pilin proteins

4.1.2.1 Aim and experimental setup

When studying a gene of interest, specific antibodies against the gene product are needed in order to monitor the expression of the gene of interest. This would, for example, involve comparing expression patterns of different strains or verifying a complementation strain. In any case, specific antibodies against a given gene product are needed when using the protein as a control in Western blots. The aim of this part of the thesis was to create antibodies against the gene products of *aglB* and *pilin* of *N. magadii* for further use in experiments that involve revealing the function of these genes in the process of infecting *N. magadii* with ϕ Ch1. The first step in this task involved the construction of expression vectors harbouring the C-terminal regions of the *aglB* or *pilin* gene fused to an N-terminal His-tag. Subsequently, an *E. coli* expression strain that expresses the correct gene products should have been found and the proteins purified for immunisation of rabbits. Immunised rabbits were screened for the presence of specific and sensitive antibodies.

4.1.2.2 Results

AglB and Pilin are transmembrane proteins that exhibit a transmembrane spanning topology consisting of at least one (Pilin) or several (AglB) hydrophobic transmembrane portions [104], [102]. Heterologous expression of transmembrane proteins in *E. coli* would likely result in protein misfolding and/or the formation of inclusion bodies. Therefore, only the C-termini of the AglB and Pilin proteins were expressed in *E. coli* and used for antibody production. Predictions of transmembrane regions for the proteins AglB and Nmag_0291 (Pilin) can be seen in Figure 10,

indicating the C-terminal regions used for protein expression and the production of antibodies.

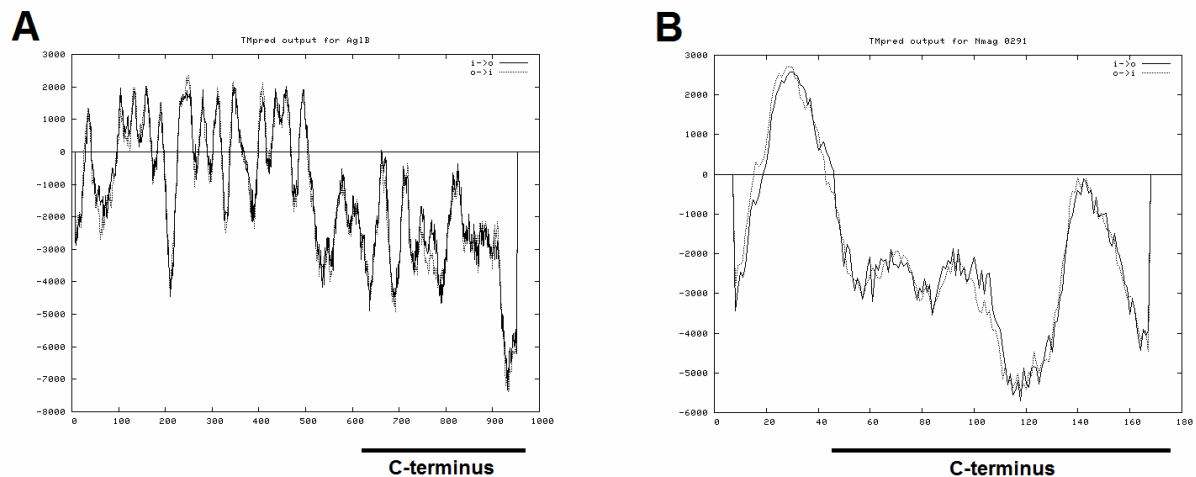


Figure 10: Prediction of transmembrane regions for the proteins AglB and Nmag_0291 (Piln)

Analysis of the AglB (A) and Nmag_0291 (Piln) (B) proteins of *N. magadii* using the bioinformatics program TMpred. The program uses algorithms based on the statistical database analysis of naturally occurring transmembrane proteins. Areas above 0 on the x-axis indicate hydrophobic regions. The C-terminal regions used for protein isolation and antibody production are indicated by black bars.

The expression vectors pRSET-A-AglB-C and pRSET-A-Pil-C were constructed as described in 3.5.1.3 and 3.5.2.3. The plasmid pRSET-A features ampicillin resistance, the T7 promoter for induction of the expression with IPTG, and a 6x-His-tag for detection and purification of the gene products of interest. The plasmids were tested for the correct inserts with analytical PCR and test restriction. The vectors were also sequenced to ensure that the open reading frames were placed in the plasmids in-frame. As for expression strains, *E. coli* BL21, BL21(DE3) pLysS, BL21 pRep4, Rosetta(DE3) pLysS and Tuner were tested for the expression of the C-terminal fragments. These expression strains possess the T7 polymerase under the control of the *lac* promoter. For both the *aglB* and *pilin* gene, several transformants of each strain had to be tested with Western blotting using secondary antibodies against the N-terminal 6xHis-tag. This resulted in the strains *E. coli* Tuner pRSET-A-AglB-C and

E.coli Rosetta pRep4 pRSET-A-Pil-C. With these strains, it was possible to express the C-terminal gene products of both *aglB* and *pilin*. The expressed proteins were purified under denaturing conditions via affinity chromatography, as described in 3.2.1, and lyophilised. The results were monitored by visualising the resulting fractions on SDS gels that were stained with Coomassie Brilliant Blue. BSA standards with different concentrations ranging from 0.5 to 0.05 mg/ml were used to estimate the AglB and Pilin protein concentrations. In the case of Pilin, ten litres of culture were needed, whereas in the case of AglB, twenty-four litres were needed in order to obtain at least one milligram of lyophilised protein that was needed for the immunisation of rabbits. The lyophilised proteins were sent to Moravian Biotechnology Ltd. for the production of polyclonal antibodies. For both proteins, two rabbits were immunised. After the second and third immunisation, rabbit antisera were obtained from the company and tested for specificity and sensitivity with Western blots using purified proteins and protein raw extracts from *N. magadii*. One rabbit was chosen for each protein and the final antisera obtained. The final antisera were diluted with 1X TBS and saturated with acetone powder obtained from the *E. coli* expression strains. The appropriate final dilutions were adjusted with Western blots. The diluted antisera were stored at 4°C for further use.

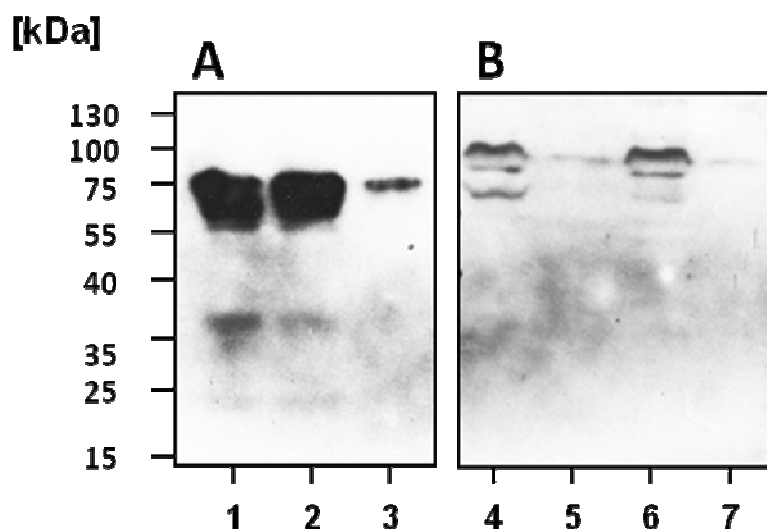


Figure 11: Evaluation of the polyclonal antibodies against AglB-C with Western blotting

A: Lane 1: Purified AglB-C; Lane 2: Purified AglB-C 1:10; Lane 3: Purified AglB-C 1:1000

B: Lane 4: L13 protein extract; Lane 5: L13 protein extract 1:10; Lane 6: L11 protein extract
Lane 7: L11 protein extract 1:10

4.1.2.3 Discussion

Constructing the expression plasmids resulted in the plasmids pRSET-A-AglB-C and pRSET-A-Pil-C, which allow heterologous expression of the C-terminal parts of the AglB and Pilin proteins. When transferred into *E. coli* expression strains, the strains *E. coli* Tuner pRSET-A-AglB-C and *E. coli* Rosetta pRep4 pRSET-A-Pil-C were obtained; they express these proteins. The proteins were successfully expressed, purified and sent to Moravian Biotechnology Ltd. for the production of polyclonal antibodies. The resulting antibodies showed sufficient specificity and sensitivity for the AglB and Pilin proteins in *N. magadii*. The antibodies that were created in the course of this study can now be used for further experiments involving the *aglB* or *pilin* genes in *N. magadii*.

4.2 The regulator ORF79

4.2.1 Preliminary results

The ORF79 of ϕ Ch1 is 791 bp in length, and the corresponding protein has a size of 28.7 kDA. Sequence alignment studies did not reveal conserved features and thus no related sequences of genes. Therefore, no certain function could have been predicted for the ORF79 [83]. This made the ORF79 an interesting candidate for the construction of a deletion mutant. The ORF79 mutant strain termed *N. magadii* L11- Δ ORF79 is the first-ever created deletion mutant of a haloalkaliphilic virus [90].

Phenotypic experiments with the mutant strain showed that the onset of lysis occurred 24 hours earlier in the L11- Δ ORF79 mutant as compared to the wild-type strain. [90] Complementation of the mutant strain with a plasmid that harboured an intact copy of ORF79 restored the original lysis behaviour in the complemented strain *N. magadii* L11- Δ ORF79 (pNB102-ORF79). This suggests that the ORF79 encodes a repressor that regulates the induction of lysis in ϕ Ch1 [91].

Constitutive expression of ORF79 in the cured strain *N. magadii* L13 completely inhibited infection of the host with the wild-type virus ϕ Ch1, as well as with the mutant strain ϕ Ch1- Δ ORF79 [92].

Comparisons of the protein expression patterns revealed differences between the *N. magadii* L11- Δ ORF79 and the *N. magadii* wild-type strain L11. The expression patterns of the major capsid protein E and the viral encoded methyltransferase M.Nma ϕ Ch1-I showed a slightly altered behaviour in a way that the expression of these proteins occurred about 10 hours earlier in the mutant as compared to the wild type. The expression of the viral tail fiber protein gp43 appears to be completely deregulated in the mutant. It was therefore suggested that ORF79 could be a regulator of the viral tail fiber protein gp34 [91], [92], [93].

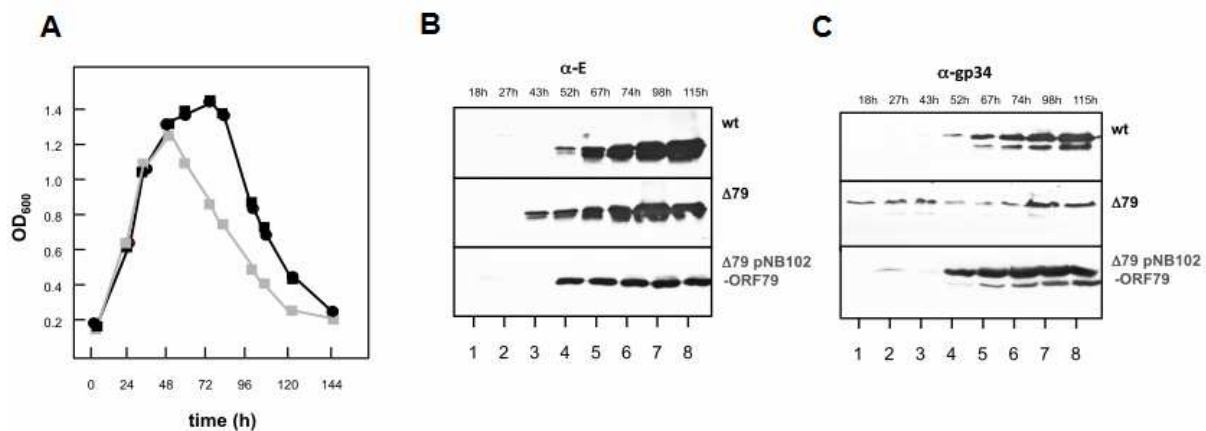


Figure 12: Influence of the ORF79 on the growth and lysis behaviour of *N. magadii* L11 and Western blot analysis of protein expression patterns

A: The onset of lysis of the mutant strain *N. magadii* L11-ΔORF79 (grey line) occurred 24 hours earlier as compared to the wild-type strain *N. magadii* L11 (black line, black squares). The complemented strain *N. magadii* L11-ΔORF79 (pNB102-ORF79) (black line, black circles) exhibited the original lysis behaviour of the wild-type strain. **B:** The expression of the major capsid protein E could be detected earlier in the mutant strain as compared to the wild-type strain. **C:** The expression of the tail fiber protein gp34 appears to be completely deregulated in the ORF79 deletion mutant (adapted from Schöner, 2013 and Beraha, 2013 [92], [93]).

Over-expression of the ORF79 in the strain *N. magadii* L13 (pRo5-ptnaN-ORF79, pNB102-ORF34₅₂) prevented the expression of gp34 [92].

Co-expression of the ORF79 with the viral methyltransferase M.*Nma*φCh1-I showed no apparent influence of the ORF79 on the expression of these proteins. This supports the theory that the ORF79 is not a universal regulator of φCh1 but is specific for either one or more proteins [94].

The preliminary studies on the ORF79 of φCh1 support the theory of the ORF79 as a regulator of the viral infection process and a repressor of the viral tail fiber protein gp34. In the course of this thesis, the ORF79 should be characterized further in order to supplement the existing results and to support the theory of the gene being a regulator of the infection process of *N. magadii* with φCh1.

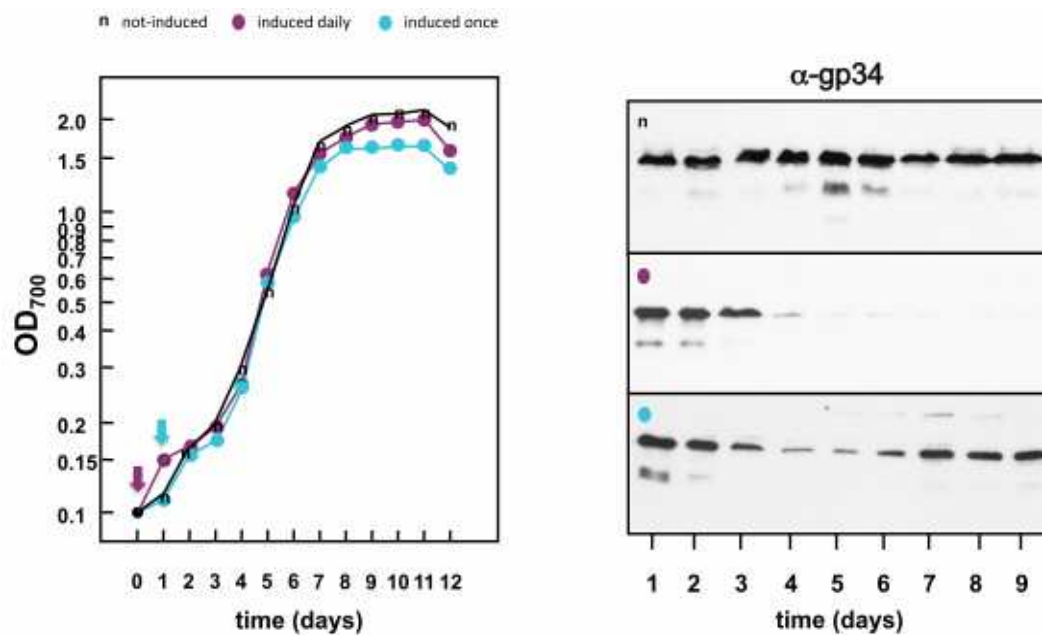


Figure 13: Growth kinetics and Western blot analysis of *N. magadii* L13 (pRo5-ptnaN-ORF79, pNB102-ORF34₅₂)

The ORF79 was over-expressed in three cultures by induction with tryptophane. One culture was induced daily, one only once and one culture was not induced. All three cultures show similar growth kinetics. In the un-induced culture, Western blot analysis shows a constant expression of the tail fiber protein gp34. In the culture that was induced daily, the signal for gp34 disappeared after Day 3, whereas the culture that was induced only on Day 1, the gp34 signal decreased on Day 3 and then later recovered (adapted from Schöner, 2013 [92]).

4.2.2 Transcriptional analysis of ORF79

4.2.2.1 Aim and summary

Although strong evidence supports the theory of the ORF79 being involved in the regulation of the tail fiber protein gp79, its mode of operation is still unclear. [90], [91], [92], [93], [94]. Analysis of regulatory elements may also be conducted at the level of transcription in addition to expression analysis, so further characterization of the regulatory ORF79 should also include transcriptional analyses.

In this study, the ORF79 transcription profile was monitored in the *N. magadii* wild-type strain L11 and compared to the expression profiles of gp79 and gp34. In a time course experiment, samples were prepared and the transcription and expression profiles monitored. Transcriptional activity was shown by reverse transcription PCR of the RNA samples. In addition, a Southern blot was conducted with the RT-PCR gel because of the higher specificity of the Southern blot probes as compared to the RT-PCR. Expression of the gp79 and gp34 proteins was monitored via Western blotting.

4.2.2.2 Results

For the time course, *N. magadii* L11 was inoculated in NVM+ rich medium at a starting OD₆₀₀ of 0.1 and incubated at 37°C with agitation for 8 days. Pre-cultures were in the logarithmic growth phase. Samples were collected daily for RNA and protein preparation. The RNA was isolated from these samples as described in 3.1.10, the protein samples were treated as described in 3.2.2. For use in reverse transcription PCR, the RNA samples had to be separated from enzymes; this was achieved by separating the organic and anorganic phases with TRIzol®/Chloroform. The DNA in the RNA samples was digested as described in 3.1.11, and the completeness of the digestion was controlled on an agarose gel and with test PCR using the primer pair 79-RT-1/79-RT-2. After three digestion steps, no traces of DNA were detectable with PCR. The concentration of the RNA was determined to ensure that enough RNA was remaining and to ensure the RNA concentrations were equal. 10 ng RNA dissolved in RNase-free ddH₂O were used as templates for the RT-PCR. The RT-PCR was conducted as described in 3.1.1.4, and the results were analyzed on an agarose gel. (See Figure 14) In addition to the RT-PCR for the ORF79 transcripts with the primer pair 79-RT-1/79-RT-2, a second RT-PCR with the primer pair

527f/1406r and the same samples was performed. This second RT-PCR that amplified 16S rDNA in the L11 samples was used to verify if the concentrations of the templates was equaled correctly. This is possible, because it is known that the levels of 16S rDNA transcriptions are equal in all growing stages of the cells. The 16S RT-PCR showed that an equal ratio of L11 RNA templates of the Day 1 to Day 8 samples was used. In the ORF79 RT-PCR, it appeared that the transcription of the ORF79 RNA began on Day 1 and had its highest levels at Days 3 and 4; the RNA level dropped again from Day 5 onwards. For both PCRs three controls were used: *N. magadii* L11 DNA was used as a template in the positive control. For the negative control, isolated RNA from Day 8 was used in a PCR reaction without the precedent reverse transcription step to ensure that all samples were DNA-free. The aim of a third control in which L13 DNA was used as a template was to yield a product only for the 16S DNA PCR but not for the ORF79 PCR. The positive controls resulted in products of the expected size; the negative controls showed no product, and the L13 control only led to a product in the 16s rDNA control PCR.

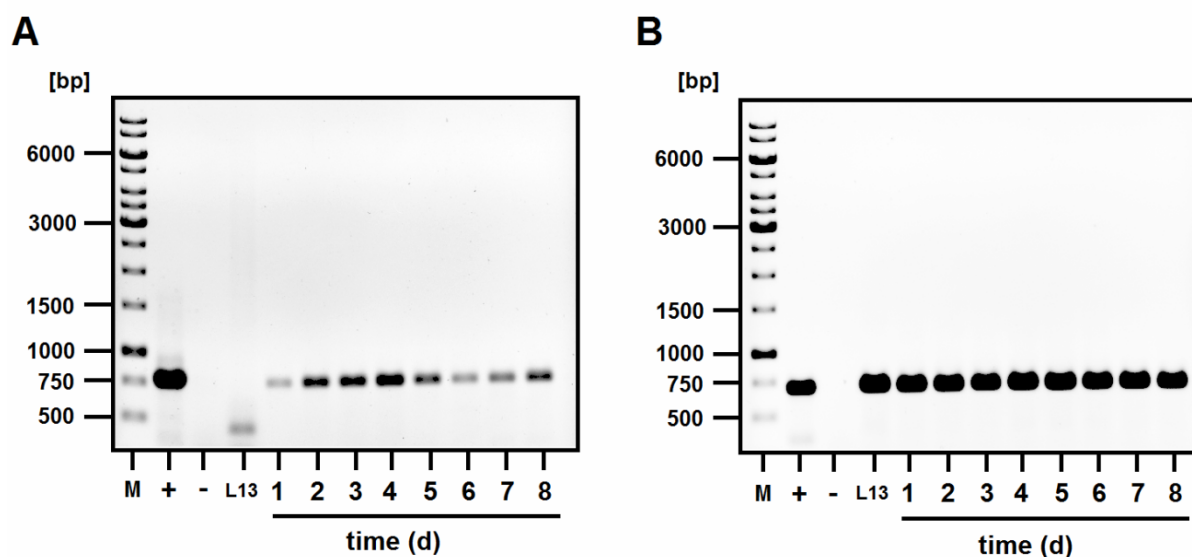


Figure 14: Reverse transcription PCRs with *N. magadii* L11 RNA samples

M: 1kB DNA ladder; +: Positive control with ϕ Ch1 template; -: Negative control: Day 8 without reverse transcription reaction; L13: control with L13 template; 1-8: L11 RNA samples Days 1-8

A: RT-PCR for the ORF79 transcripts with primers 79-RT-1/79-RT-2

B: 16S rDNA RT-PCR with primers 527f/1406r

The ORF79 transcripts were detected on all eight days with a peak transcription on Day 4 and drop in the amount from Day 5 onwards. In the 16S rDNA RT-PCR, equal signal strengths are visible for the samples of Days 1-8. This shows that equal amounts of RNA were used as templates for the daily samples in both RT-PCRs.

Both agarose gels with the products of the ORF79 and 16S rDNA RT-PCRs were used for Southern blotting as described in 3.1.7. For the ORF79 gel, probes generated with primers 79-RT-1/79-RT-2 and for the 16S gel probes generated with primers 527f/1406r were used. The Southern blots showed signals corresponding to the results of the RT-PCRs. All controls were to conform to the RT-PCR gels. The 16S Southern blot also showed an equal ratio of 16S rDNA in the L11 samples. When using the ORF79 probes in the Southern blot, the strongest transcription was monitored from Day 3 to Day 5 (see Figure 15).

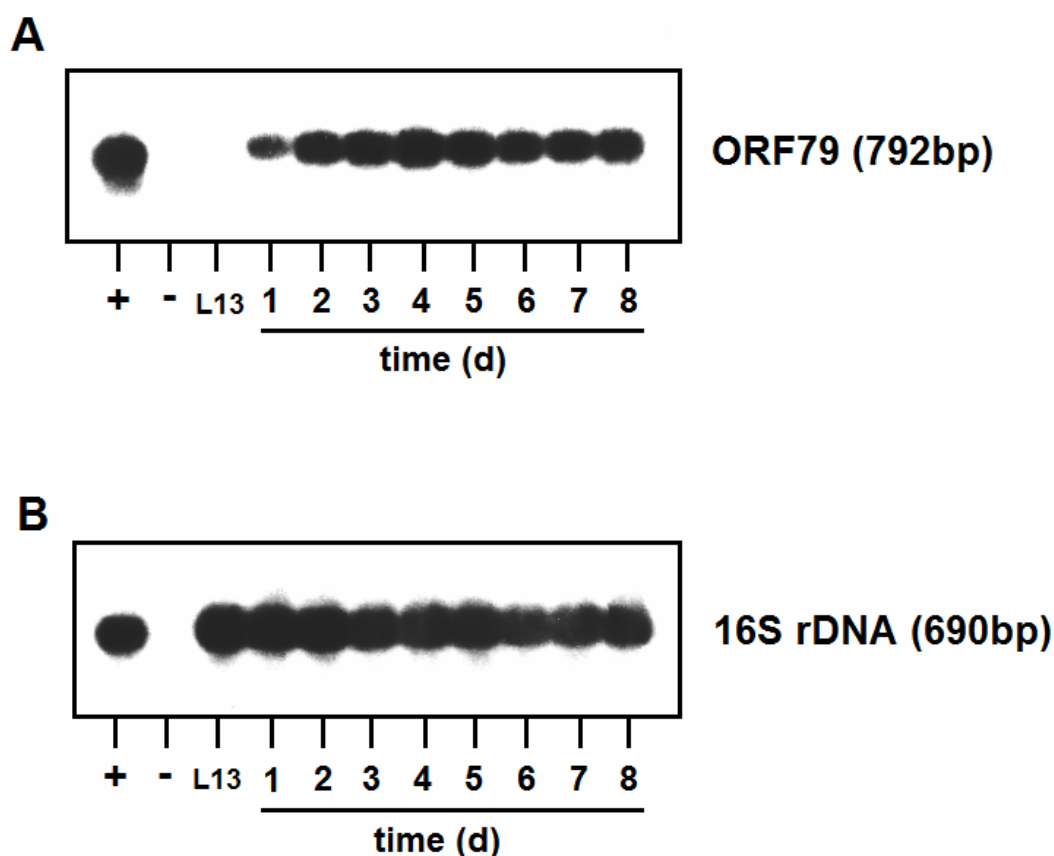


Figure 15: Southern blots of reverse transcription PCRs with *N. magadii* L11 RNA samples

M: 1kB DNA ladder; +: Positive control with *N. magadii* L11 template; -: Negative control: Day 8 without reverse transcription reaction; L13: control with L13 template; 1-8: L11 RNA samples Days 1-8

A: Southern blot of the ORF79 RT-PCR with probes generated with primers 79-RT-1/79-RT-2

B: Southern blot of the 16S rDNA RT-PCR with probes generated with primers 527f/1406r

The ORF79 transcripts were detected with specific probes on every day of the daily samples. As in the RT-PCR, the highest transcription appeared on Day 4. The results of the 16S Southern blot also confirmed the 16S RT-PCR with equal signal strengths on every day of the daily samples.

N. magadii L11 pellets from Days 1 to 8 were also used to perform Western blots with specific antibodies against the ORF79 gene product gp79 and the ORF34 gene product gp34. Expression of gp79 was detected from Day 2 onwards, with its peak on day 3 and declining expression from Day 4 onwards. When compared with the RT-PCR results (Fig.16), it is shown that the levels of gp79 drop significantly after

Day 3 and are not detectable any more after Day 6, even though the transcription of ORF79 is constant after Day 2 without decline.

The tail fiber protein gp34 was detected from the third day onwards with a continuous expression for the rest of the days. The protein showed the expected expression pattern of the wild-type strain L11 that begins approximately 3 days after the beginning of the logarithmic growth phase (see Figure 16).

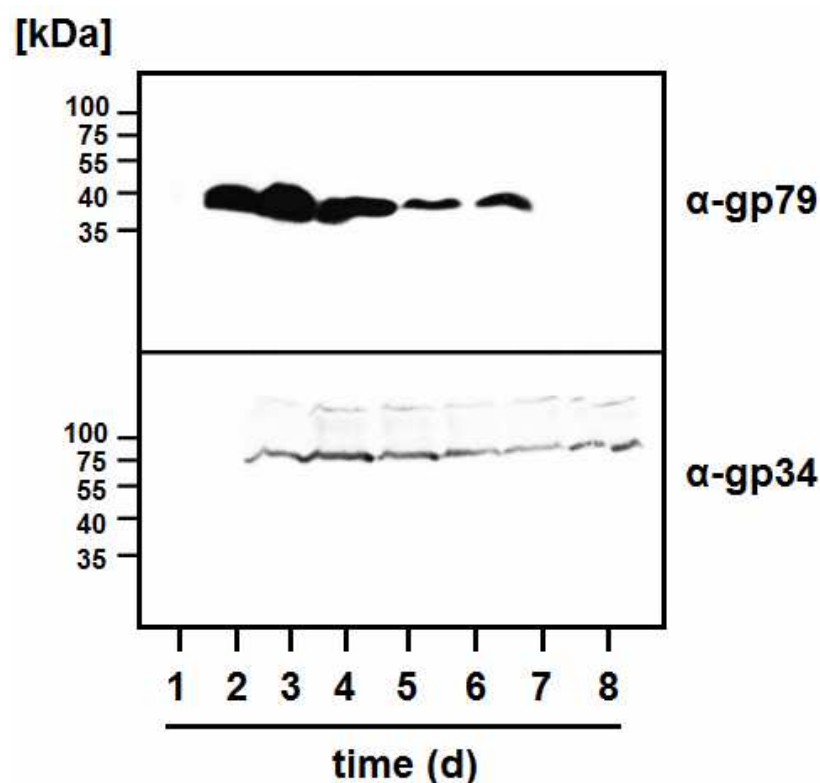


Figure 16: Expression of ORF79 and ORF34 in *N. magadii* L11

The results of the Western blots compare the expression of the ORF79 gene product gp79 with the results of the ORF79 RT-PCR and Southern blot. In addition, the results show the expression pattern of the ORF34 gene product gp34.

Lanes 1 to 8 represent Days 1 to 8 of the *N. magadii* L11 time course. gp79 was detected from Day 2 to Day 6, with a peak expression on Day 3 and a drop in the amount from Day 3 onwards. The expression of gp34 started a day later, on Day 3, and remained tolerably constant for the remaining days. Equal amounts of proteins were applied to all lanes and in both blots. This was monitored via Coomassie staining.

4.2.2.3 Discussion

This first transcription profile of the ORF79 in the wild-type strain *N. magadii* L11 contributes to the characterization of the regulator ORF79. Transcription of the regulator started the first day of growth and remains at a constant level from Day 2 until Day 8. In contrast, the translation analysis of the strain L11 shows an expression that starts on Day 2, has its maximum on Day 3 and declines from Day 4 onwards. The protein gp79 was no longer detected on Days 7 and 8. These results reveal that the transcription levels of the ORF79 in the wild-type host strain remain constant, whereas the levels of the corresponding protein gp79 drop significantly over time.

When looking at ORF79 as a repressor of ORF34, it can therefore be concluded that it is necessary for the constant expression of gp34 that the levels of gp79 drop, not the levels of the ORF79 transcripts.

As the levels of the ORF79 mRNA remain constant, the drop in the gp79 levels might be a consequence of mRNA degradation or the degradation of the protein itself. To gain more insight into the mode of operation of this important regulator of the ϕ Ch1 life cycle, the regulating factors of the ORF79 should be identified. It was proposed that the release of ϕ Ch1 virus particles might be a consequence of *N. magadii* cells entering the stationary growth phase. [68] This would suggest an interaction of the viral ORF79 with factors in the host cell. Identifying the 'trigger' for the depletion of gp79 over time could be an important step in understanding the ϕ Ch1 life cycle.

In addition, viral encoded binding partners of the ORF79 should be revealed e.g. through Far-Western blotting. The tail fiber protein gp34 would be a suitable first candidate for such studies, but also other viral proteins should be considered, as a regulatory effect of the ORF79 on other viral proteins cannot be excluded.

Future experiments could also include examining transcription profiles of the proteins gp34, protein E and M.Nma ϕ Ch1-I in the wild-type virus strain and the mutant virus strain ϕ Ch1- Δ ORF79.

4.2.3 Virus and host strain protein comparison

4.2.3.1 Aim and summary

Characterization of ϕ Ch1- Δ ORF79 also involves analyzing the morphology of the mutant virus strain. Therefore, the protein composition in mutant and wild-type strains should be compared. Here, the abundance of the structural proteins E (major capsid protein) and gp34 (tail fiber protein) were monitored in the mutant and wild-type virus strains via Western blotting. In addition, the expression of these proteins in the host strains *N. magadii* L11 and *N. magadii* L11- Δ ORF79, harbouring ϕ Ch1 or ϕ Ch1- Δ ORF79 as proviruses was compared. The results should provide evidence whether the morphology of the mutant virus strain differs significantly from the wild-type virus strain.

4.2.3.2 Results

To obtain the ϕ Ch1 and *N. magadii* proteins for Western blot analysis, protein samples of the virus and the host strains had to be prepared. Virus particles of ϕ Ch1 and ϕ Ch1- Δ ORF79 were isolated as described in 3.4.4, and the virus proteins were prepared as described in 3.2.3.

During the isolation of the virus particles, after the PEG-6000 precipitation (see Chapter 3.4.4), the Δ ORF79 virus strain was much more viscous as compared to the wild-type strain. It was speculated that the high viscosity resulted either from a high concentration of host DNA or a high concentration of free viral DNA in the virus suspension. The reason for this is still unknown. The host DNA was digested with 50 μ l DNase I per 5 ml virus suspension to ensure pipettability for the next centrifugation steps. The successful preparation of virus proteins was analysed with SDS-PAGE and Coomassie staining. Coomassie-stained gels were also used to

estimate the concentration of the virus proteins and to align the concentration of the ϕ Ch1 and ϕ Ch1- Δ ORF79 proteins for Western blot analysis. The Coomassie-stained gel after adjusting the virus protein concentrations can be seen in Fig.17, A. In this gel, no obvious differences in the composition of the most prominent viral proteins of ϕ Ch1 and ϕ Ch1- Δ ORF79 are evident. In addition, isolated ϕ Ch1- Δ ORF79 virus particles showed a slight decrease in plating efficiency when conducting a virus titer (Fig. 17, A).

N. magadii L13, *N. magadii* L11 and *N. magadii* L11- Δ ORF79 protein samples were prepared as described in 3.2.2. The crude proteins extracts were checked with SDS-PAGE and Coomassie staining, which was also used to align the concentrations of the three strains (Fig. 17, E). As the amount of *N. magadii*-encoded proteins in the host cells were many times higher than the amount of viral proteins, the *N. magadii* Coomassie-stained gels cannot be used to compare the amount of viral proteins in the different strains.

Western blots were performed to compare the amounts of the major capsid protein E, the tail fiber protein gp34 and the ORF79 gene product gp79. On each blot, the signal strength of one protein between the wild-type and the mutant virus or archaeal strain was compared. Equal concentrations of the proteins were used for each blot. The results of the Western blots are summarized in Figure 17.

In the virus protein blots, no significant differences in the abundance of the proteins E and gp34 were detected. Even though it did not appear significant, the intensity of the gp34 signal seemed to be slightly higher in the mutant virus strain ϕ Ch1- Δ ORF79 compared to the wild type (Fig. 17, B+C). However, this observation needs to be tested further by repeating the blot or measuring the protein concentrations via protein assays. For the protein gp79, no signal was detected in either the wild-type or the mutant strain (Fig. 17, D). The lack of a gp79 signal in the wild-type strain was expected for a protein that is a regulator that gets expressed within the host cell and

is not a component of the viral capsid. The strain *N. magadii* L11, expressing the protein gp79, served as a positive control in this blot.

In the *N. magadii* blots, signals for the proteins E and gp34 did not appear to differ significantly in strength (Fig. 17, F+G). The protein gp79 was detected in the wild-type strain L11 but not in the mutant strain L11- Δ 79 (Fig1 17, H). The faint band on the L11- Δ 79 lane is most likely due to a contamination with the L11 sample while loading the SDS-PAGE gel, since the mutant strain had been shown to be free of any copies of the ORF79 gene [90]. In these blots, the 'cured' strain *N. magadii* L13 was used as a negative control.

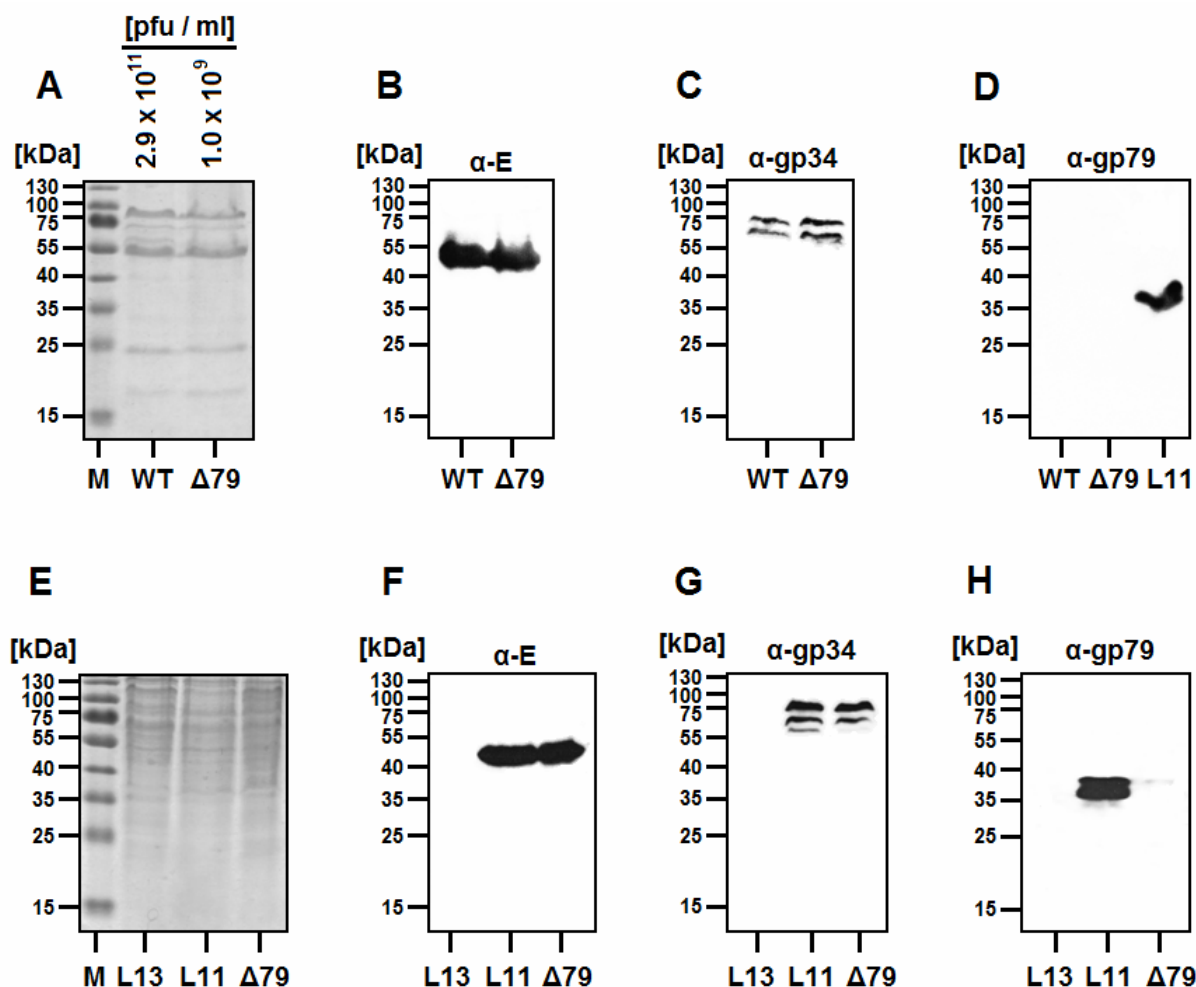


Figure 17: Western blots of viral and host protein abundance in Δ ORF79 mutants and wild-type strains

WT: Wild-type virus strain ϕ Ch1. **Δ 79:** Mutant virus strain ϕ Ch1- Δ ORF79 or mutant host strain *N. magadii* L11- Δ ORF79. **L11:** Wild-type host strain L11. **L13:** 'cured' host strain *N. magadii* L13.

A: SDS-PAGE of virus protein extracts stained with Coomassie Blue. This gel was used to ensure that equal amounts of total virus proteins are applied on the gels for virus Western blots. Virus titer results are included and shown above the lanes.

B: Abundance of the major capsid protein E in the wild-type and mutant virus strains. No significant differences in the intensity of the bands were detected.

C: Abundance of the tail fiber protein gp34 in the wild-type and mutant virus strains. The intensity of the gp34 band seemed to be slightly higher in the mutant strain, although this observation needs to be tested in the future.

D: Abundance of the ORF79 gene product gp79 in the wild-type and mutant virus strains. No gp79 signals were detected in the mutant strain and the wild-type strain which was expected for a protein that is not part of the virus capsid. The strain L11 served as a positive control.

E: SDS-PAGE of *N. magadii* protein extracts stained with Coomassie Blue. This gel was used to ensure that equal amounts of total proteins are applied on the gels for *N. magadii* Western blots.

F: Abundance of the major capsid protein E in the wild-type and mutant *N. magadii* strains.

No significant differences in the intensity of the bands were detected.

G: Abundance of the tail fiber protein gp34 in the wild-type and mutant *N. magadii* strains. No significant differences in the intensity of the bands were detected.

H: Abundance of the ORF79 gene product gp79 in the wild-type and mutant *N. magadii* strains. The cured strain *N. magadii* L13 served as a negative control.

4.2.3.3 Discussion

The results of this study show no significant changes in the abundance of the major capsid protein E and the tail fiber protein gp34 in the mutant strain $\phi\text{Ch1-}\Delta\text{ORF79}$. Therefore, it can be assumed that the deletion of the ORF79 does not lead to a stoichiometric change in these structural proteins.

Recent electron micrographs of $\phi\text{Ch1-}\Delta\text{ORF79}$ allow a visual examination of the mutant's morphology. The mutant virus strain shows no change in size and composition. The morphology of the $\phi\text{Ch1-}\Delta\text{ORF79}$ virus capsid's head, tail and tail fibers appears to be equal to those the wild type [Witte *et al.*, hitherto unpublished].

The results of this study, combined with the electron micrographs, present evidence for the assumption that the decreased fitness of the ΔORF79 mutant virus is not related to a change in the virus capsid's morphology. To completely confirm this assumption, the remaining ϕCh1 capsid proteins should be identified and their stoichiometric proportions analyzed in the wild-type and the mutant virus.

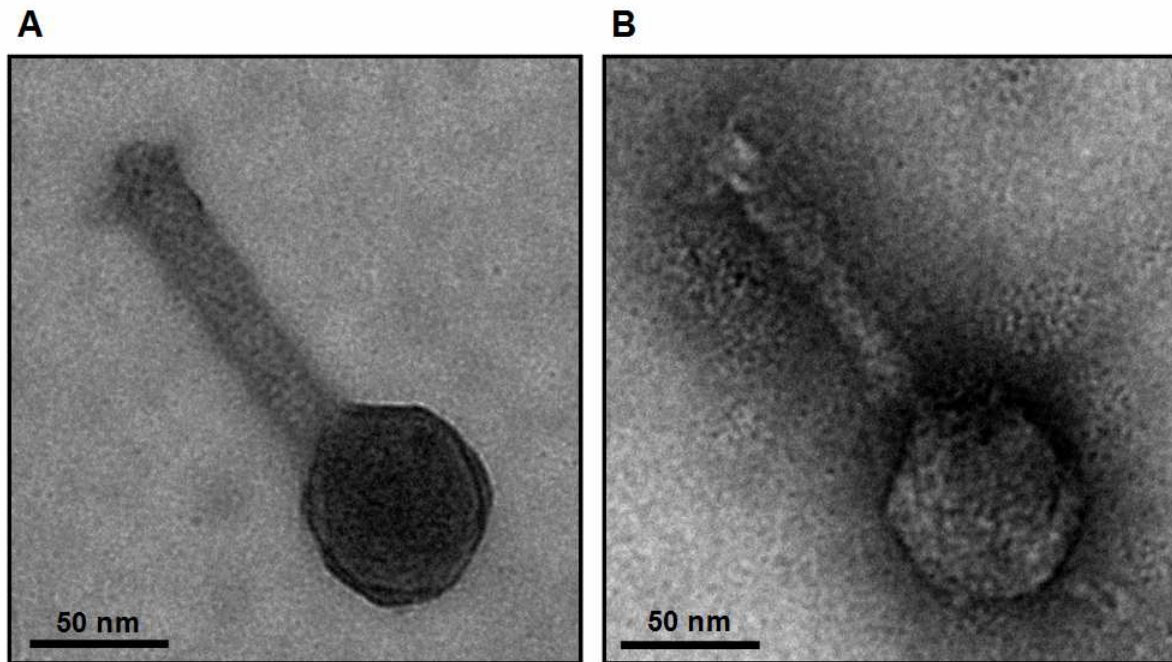


Figure 18: Electron micrographs of ϕ Ch1 and ϕ Ch1- Δ ORF79

A: ϕ Ch1, B: ϕ Ch1- Δ ORF79 No apparent change is visible in the size and morphology regarding the virus head, tail and tail fibers.

(Witte *et al.*, hitherto unpublished)

4.2.4 Virus burst size comparison

4.2.4.1 Aim and summary

Characterization of ϕ Ch1- Δ ORF79 should also include examination of its growth parameters. Therefore, the burst size and the length of the eclipse period of the mutant virus strain were determined in this study and compared to the wild-type strain ϕ Ch1.

Here, mutant virus particles were isolated, the virus titer determined, and a one-step growth curve experiment was conducted to determine burst size and eclipse.

4.2.4.2 Results

ϕ Ch1 and ϕ Ch1- Δ ORF79 virus particles were isolated as described in 3.4.4 (see also Chapter 4.2.3). During virus isolation, the lysis of the mutant strain *N. magadii* L11- Δ ORF79 occurred approximately 24 h earlier than the lysis of the wild-type strain *N. magadii* L11. This observation corresponds with previous experiments investigating the ORF79 mutant growth and lysis behaviour [92], [93].

Virus titers (as described in 3.4.5) were performed with the isolated mutant and wild-type viruses. The titers added up to 2.89×10^{11} pfu ml⁻¹ for the wild-type virus and 7.10×10^8 pfu ml⁻¹ for the Δ ORF79 mutant virus. Here, already a lower titer for the mutant strain was evident. In addition, the plaques of the Δ ORF79 mutant virus were more turbid in appearance as compared to the wild-type virus (data not shown).

The concentrations of the virus strains were adjusted for the one-step growth curve as a synchronous infection had to be ensured. The infection step in this experiment was done with a multiplicity of infection of 1; this means that the ratio of virus particles to cells was 1:1. The one step growth curve was performed as described in 3.4.6. The resulting wild-type and mutant virus samples from the different time points were used to perform virus titers. Pfu ml⁻¹ were counted for the ϕ Ch1 chloroform treated/non-treated samples and for the ϕ Ch1- Δ ORF79 chloroform treated/non-treated samples (data not shown). From the virus titer data, the eclipse and burst size values of both strains were determined.

The eclipse time for the mutant appears to be delayed by 2 h, whereas the burst size of the mutant was decreased significantly to only about a third as compared to the wild type. (see Table 1)

Virus	Burst Size	Eclipse
ϕ Ch1	150	8 h
ϕ Ch1- Δ ORF 79	50	10 h

Table 1: Comparison of burst size and eclipse periods between ϕ Ch1 and ϕ Ch1- Δ ORF79

4.2.4.3 Discussion

The results of this study took a closer look at the growth parameters of the ϕ Ch1- Δ ORF79 mutant.

The isolated mutant virus particles led to a reduced virus titer as compared to the wild type. This matches preliminary results.

The one-step growth curve experiment revealed altered growth characteristics of the Δ ORF79 mutant. The virus burst size of ϕ Ch1- Δ ORF79 was reduced by a factor of 3 when compared to ϕ Ch1. When examining the viral plaques, the Δ ORF79 mutant plaques also showed a clearly visible increase in turbidity. The increased turbidity of the ϕ Ch1- Δ ORF79 might also be a result of the reduced burst size. The eclipse period of ϕ Ch1- Δ ORF79 was found to be prolonged by 2 hours.

The reduced burst size and extended eclipse are likely the result of a deregulation in the viral life cycle. An optimal latent period is vital for viruses to ensure high viral fitness that is also expressed in a preferably high burst size [106]. Considering previous studies in which no change in the ϕ Ch1- Δ ORF79 capsid morphology was found (see 4.2.3.3), it can therefore be assumed that the decreased virus fitness of the Δ ORF79 mutant is a result of its altered latent period.

Different explanations are considered for the increased turbidity of the mutant virus plaques. As already mentioned above, the smaller burst size is thought to be one contributing factor. It can also not be excluded that slower adsorption of the mutant virus particles by the host cells led to the turbid plaques [107]. Additionally, a combination of these two and/or other factors would be possible.

This study demonstrates the effect of the deletion of an important regulator in the viral life cycle. Determining the ϕ Ch1 developmental parameters in the course of this thesis will contribute to an increasingly comprehensive characterisation of the virus strain ϕ Ch1- Δ ORF79.

5. References

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