universität wien

MASTERARBEIT/MASTER´S THESIS

Titel der Masterarbeit/titel of master´s thesis:

"Evaluation of *rep* and ORF49 of φCh1: regulation of gene expression in dependence of growth conditions"

verfasst von/ submitted by

Julian Szalay, BSc

angestrebter akademischer Grad / in partial fulfillment of the requirements for the degree of

Master of Science (MSc)

Wien 2022 / Vienna 2022

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 066 830
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Molekulare Mikrobiologie, Mikrobielle Ökologie und Immunbiologie
Betreut von / Supervisor	Ao. Univ.- Prof. Dipl.-Biol. Dr. Angela Witte

Acknowledgments

First and foremost I would like to express my deepest thankfulness towards my supervisor Ao. Univ.-Prof. Dipl.-Biol. Dr. Angela Witte for her guidance, her patience and her assistance throughout my time at Max Perutz Laboratories and even beyond that.

Furthermore, I want to thank my colleagues Alexandros Athanasiou, Laxmikant Wali, Seyda Kigili, Richard Manning, Gregor Sommerkamp and Anna Koch, who have helped me over so many little hurdles and have filled hundreds of memories with jokes and laughter.

In addition I want to thank the members of Blaesi and Moll groups, especially, Armin Resch and Elisabeth Sonnleitner, for providing helpful hints and advice.

Finally, I would like to thank my mother, that she gave me the opportunity to follow my study by supporting me throughout all those years.

Table of content

Introduction

The whole diversity of life can be grouped by molecular comparison into three domains, Bacteria, Eukarya and Archaea (Woese *et al.*, 1990). However regardless the most common entity on planet earth are viruses, due to the fact that viruses infect all three domains of life and have, like their hosts, adapted to almost every environment (Suttle, 2007).

Viruses of Archaea are the least understood part of the global virosphere, whereas until now more than 6200 different bacteriophages are known, just around 100 viruses are described, which infect members of the domain Archaea (Ackermann, Prangishvili, 2012).

The first archaeal virus was specified 1974 in spontaneously lysing *Halobacterium halobium* cultures. *H. halobium* is a halophilic archaeaon, which requires 15% NaCl for growth (Torsvik, and Dundas, 1974). Archaeovirus research has increased in the last decades, and up to now 17 archaeal virus families have been discovered, which infect about 16 different genera of archaea (Krupovic *et al,* 2018).

Until now only archaeal viruses have been isolated, which possess a DNA genome, in either single stranded or double-stranded, linear or circular form (Prangishvili *et al*., 2017). Nevertheless meta- genomic analysis of hot springs in Yellowstone National Park let suggest that there might be uncultivated RNA archaeoviruses out there (Bolduc *et al.*, 2012).

Archaeoviruses can be grouped in cosmopolitan viruses, which are able to infect Bacteria as well as Archaea and into archaespecific viruses (Iranzo, *et al*., 2016).

The genome size of archaeal viruses can vary between 5.3 kbp in APBV1(C*lavavirus*, which is the smallest known double-stranded (=ds) DNA virus) to 143.8 kbp in HGTV-1 (*Myovirus*), whereas viruses specific for archaea tend to have smaller genomes than cosmopolitan viruses, like members of the *Caudavirales* (Krupovic *et al,* 2018).

Traditionally, phages can be classified upon their life cycle as well. There are virulent viruses, which build their progeny directly after infection and lyse the host cell immediately and there are temperate viruses, which possess in addition the opportunity to integrate stable into the host genome as provirus or remain outside the genome as plasmid. This lysogenic strategy allows them to persevere until the environmental circumstances are again favorable to exit their host cell. One advantage of such a lysogenic infection is, that the provirus is also transmitted to the daughter cells of the host, due to the fact, that the viral genome is replicated along with the host genome during replication (Bail, 1925, Bordet, 1925). Such persistent infections appear to be the most abundant viral strategy of halo-archaeal viruses (Luk *et al.*, 2014).

Nevertheless, since the discovery of temperate viruses around 100 years ago, the switch between lytic and lysogenic life-cycle was in the scope of many studies. One of the best understood temperate virus is the *Escherichia coli* phage λ. The bacteriophage λ was first described by Lederberg in 1951, and can infect its host either lytically or by integration into the host genome as provirus. The switch between these two viral strategies got studied excessively (Lwoff, 1953; Ptashne, 2004). Therefore the main players, which built this rather complex molecular switch are known and are namely the two proteins C1 and Cro as well as three promoters (*pR*, *pL*, *pRM*) and six operators (*oR1*, *oR2*, *oR3*, *oL1*, *oL2*, *oL3*). C1 is thought to be the main repressor, which binds the different operator sites to block transcription from the promoter sequences downstream and therefore represses the majority of all λ genes, which leads to the establishment or maintenance of lysogeny. In addition C1 is also up-regulating its own expression, this is achieved by different affinities of C1 to the quite similar promoter sequences. C1 has the lowest affinity for the *oR3/oL3* operator, upstream of the *pRM* promoter, which triggers the transcription of *c1* itself by the RNA-Polymerase (Sarai, Takeda, 1989). Therefore, if the amount of C1 is low, C1 prefers the other operator sites and more C1 protein will be produced. If the intracellular level of C1 increases, also the operator sites of its own promoter *pRM* will be blocked by C1 and the production of C1 will be stopped. Therefore C1 is auto-regulating its own expression (Lewis *et al.*, 2016).

In contrast, Cro is assumed to be the antagonist of C1. Cro acts against lysogeny by indirectly derepressing genes responsible for the establishment of the lytic life-cycle. Cro binds the same operators like C1, but with different affinities, whereas the promoter *pRM,* responsible for *c1* expression, is blocked first. The switch between lytic and lysogenic pathway can be induced by environmental stressors like e.g. UV-radiation. UV-radiation triggers in *E. coli* the so called SOS-response, where Rec A gets activated and cuts the Lex A repressor, which regulates effector genes of the SOS-response. The C1 protein of λ evolved in such a way, that C1 can be cut by Rec A as well, which allows the activation of lysis of λ upon UV-radiation, cause the promoters *pR* and *pL* get de-repressed by the removal of C1 through Rec A. This leads to the expression of Cro, and the blockage of *pRM,* by binding of Cro to its strongest operator site *oR3* and therefore the expression of C1 is inhibited and the lytic life cycle will be favored (Bailone *et al.*, 1979; Cohen *et al.*, 1981). In addition phage λ uses a terminator-antiterminator (Q, N) system to switch between early and late genes to be expressed. The RNA-Pol can just skip the terminator Q, if the early gene product N, which serves as antiterminator and is transcribed from the *pL* promoter, is expressed (Oppenheim, 2005).

Nevertheless there are also archaeal viruses, which use a budding mechanism to continuously release their viral progeny without lysing the host cell, such as the pleolipovirus His 1 (Porter *et al.*, 2007).

Archaeal viruses can be further roughly distinguished into those, who possess a unique morphology and genetic setup referring to archaea and those, who show significant homologies to bacteriophages and eukaryotic viruses (Krupovic *et al,* 2018). Viruses, which infect the archaeal phylum *Crenarchaeota* show often unique morphologies, like the bottle-shaped virons of *Ampullaviridae,* and have genomic features, not observed in viruses, which infect bacteria or eukaryotes (Häring *et al.,* 2005). In addition most archaeal viruses, which infect *Crenarchaeota,* possess viral proteins, which show less or none homologies to sequences of either other viral proteins or cellular proteins (Prangishvili *et al.*, 2006). Up to now there are no icosahedral tailed viruses described, which infect members of *Crenarchaeota* (Pangishvili, 2013).

In contrast viruses infecting members of the phylum *Euryarchaeota* share often great genomic and morphological homologies to bacteriophages. Most of them appear as tailed viruses, just the minority within this group refer to polyhedral, filamentous and pleomorphic viruses (Ackermann, Prangishvili, 2012). Viruses which possess an icosahedral symmetric head and a tail morphology can be classified in three families: *Myoviridae, Siphoviridae,* and *Podoviridae,* those families can be unified to the order *Caudovirales* (Ackermann, 2007). The vast majority of all known archaeal viruses infect hosts, which have adapted to extreme environments such as extreme halophiles, methanogens, or hyperthermophiles (Ackermann and Prangishvili, 2012).

Hypersaline environments can be found all over the world and include salt lakes, soda pods and artificial salt evaporation ponds. Haloarchaea can live in those environments, with salinity ranging between 15% up to salt saturation. In addition to salinity those environments can also differ in their limnology, geography and chemistry (Atanasova *et al.*, 2012).

Haloarchaea (= halophilic members of Archaea) are the dominant form in those hypersaline environments, whereas halobacteria contribute just a quarter of the total hypersaline biomass. Nevertheless viruses over count the archaeal cells in those hypersaline environments from ten to hundred fold (Rodriguez-Valera *et al.*, 2009) and, with 10^9 virus like particles (=VLP) per ml, hypersaline environments possess the highest viral density of all aquatic systems (Santos *et al.,* 2012).

Viruses, which infect Haloarchaea, belong mostly to the families *Myoviridae*, *Siphoviridae*, *Podoviridae*, *Pleolipoviridae*, *Sphaerolipoviridae* and *Fuselloviridae* (Luk *et al*., 2014). Haloviruses are the best described viruses of all extremophils, however compared to bacteriophages or viruses, which infect eukaryotes, our knowledge about them is lagging far behind (Ackermann, Prangishvili, 2012).

Two studies performed in 2012, one culture-dependent one culture-independent, revealed 33 haloarchaeoviruses (Atanasova *et al.*, 2012) and 34 putative haloarchaeoviruses (Mizuno *et al.*, 2012).

For those haloviruses spindle-shaped, spherical and filamentous viron morphotypes are described, which range in size between 40 to 100 nm (Oren *et al.,* 1997).

Nevertheless most cultivated viruses, which infect halophilic archaea are tailed viruses. However observations of viral morphology in hypersaline environments let suggest, that tailed viruses are just the minority and that irregular shaped viruses are dominant, which are not cultivable. This discrepancy might be caused by the fact that most isolation methods for haloviruses favor lytic, tailed viruses (Porter *et al.*, 2007).

All those icosahedral tailed viruses belong to the order of *Caudovirales.* If their tail is contractile they are attributable to the family *Myoviridae*. Tailed viruses, which possess a non-contractile tail are either ascribed to the family *Siphoviridae* (with a long tail) or *Podoviridae* (possess a short tail) (Pietilä *et al.*, 2013). Most isolated haloviruses belong to the *Myoviridae* family. All these tailed viruses possess a linear dsDNA genome and until now all genomes of tailed haloviruses, which got sequenced, were structured like mosaics- a typical feature of bacteriophages (Senčilo *et al*., 2014).

Its thought, that all phages show these mosaic like genomes, due to former recombinational events caused by super-infections (Dion *et al*., 2020).

In addition to those icosahedral tailed viruses, hypersaline environments are inhabited by pleomorphic archaeal viruses. Although pleomorphic viruses seem quite more abundant in those environments, until now it was just possible to isolate 11 such viruses, whereas only 8 are described in detail (Senčilo *et al*., 2012). Those 8 pleomorphic viruses can be unified to the family *Pleolipoviridae* (Pietilä *et al.*, 2016). Members of this family are characterized by enveloped pleomorphic membrane vesicles, which serve as virons. The genomes of pleolipoviruses are either composed of circular or linear dsDNA or circular single-stranded (=ss) DNA and their size is ranging between 7 to 16 kbp. Pleolipoviruses are not lytic, but release their virons constantly from the host cell by budding (Bamford *et al,* 2017).

The family of *Sphaerolipoviridae* comprises viruses, which infect Archaea as well as thermophilic bacteria. The viruses, which infect halophilic euryarchaea are grouped within the genera *Alphasphaerolipovirus* and the *Betasphaerolipovirus.* Characteristic for the *Sphaerolipoviridae* family is a tailless icosahedral capsid with an internal membrane (Demina *et al.,* 2017), those viruses posses either a linear or circular dsDNA genome or a circular ssDNA genome (Dellas *et al.*, 2014).

Until now there are many lytic members of *Sphaerolipoviridae* described like for example PH1 infecting *Haloarcula hispanica* (Porter *et al.*, 2013) as well as one temperate virus namely SNJ1, which infects the haloarchaea *Natrinema sp* (Zhang *et al., 2012).*

In addition, halophilic archaea can be infected with members of the *Fuselloviridae* family. Viruses within this family hold virus particles, which appear lemon-shaped with a short tail at one site and possess a circular dsDNA genome (Bath and Dyall-Smith, 1998). *Fuselloviruses* do not lyse their host cells, but release their virons consistently via a budding mechanism (Baquero *et al.,* 2020).

One of the best described temperate halo-viruses is φH, which infects the halophilic archaeon *H. salinarum* (Torsvik, Dundas, 1974). φH can favor either lysis or can remain as autonomous replicating plasmid within the cytosol of the host cell, and serves as model organism for temperate halo viruses. The regulation of lysogeny is mediated by the protein T6 (Rep) and T4, which are oriented head-to-head in the L-region of the φH genome. This arrangement is highly similar to the orientation of repressor/antirepressor C1 and Cro in the bacteriophage λ. T6 (*rep*) gets expressed in the lysogenic state, whereas T4 is acting upon the start of lysis (Gropp *et al.*, 1989).

Rep is assumed to bind upstream of early genes, which are involved in the onset of lysis, and represses their transcription by negatively regulating the promoter of T4. This mode of action allows Rep to confer immunity against super-infections with other φH viruses (Stolt, Zillig, 1992). Rep is also thought to auto-regulate itself, because the Rep repressor recognizes and binds to direct repeats (RRGAAG), which are also present upstream of its own promoter (Ken, Hackett, 1991). The study of Gropp in 1992 unveiled, that *rep* negatively regulates the expression from the T4 promoter, which promotes lysogeny. The switch to lysis is achieved by downregulation of *rep* through the transcription of the T4 promoter, but not due to the action of the gene product of T4 itself. The T4 promoter is namely a stronger promoter than the *rep* promoter, which is orientated in the opposite direction, whereas transcription of the two promoters is mutually exclusive (Stolt, Zillig, 1994). However the action of Rep can be enhanced by the φH gene *per* by tenfold (Stolt, Zillig, 1993).

φCh1, the virus, which is in focus of this study, infects the haloalkaliphilic Archaeon *Natrialba magadii.* The term haloalkaliphilic was first used by Soliman and Trüper in 1982 to describe the newly discovered archaeon *Halobacterium pharaonis*, which appeared to be halophilic as well as alkaliphilic. Until now this term is used to describe archaea, which depend on high salt concentrations and a quite alkaline pH of 8-11. *N. magadii* was first isolated by Tindall in 1984 from the alkaline Soda lake Magadi in Kenia and requires salt concentrations from 3.5 to 4 M and a high pH of 9.5 to 11. According to 16S RNA comparison *N. magadii* was grouped within the family *Halobacteriacaea,* which belongs to the phylum *Euryarchaeota* (Kamekura *et al.*, 1997).

N. magadii is an obligate aerobe, mesophil and rod-shaped microorganism, which requires temperatures between 37 to 42 °C to achieve optimal growth. This archaeon is chemoorganotroph, but is unable to use carbohydrates for its metabolism and therefore utilizes exclusively amino acids

and peptides as carbon source. The cells of *N. magadii* have a length of 5-7 µm and are flagellated, which allows motion. The generation time of this archaeal specimen is about 9 hours. (Tindall *et al.*, 1984).

φCh1 is a temperate halo-virus, which is able to integrate into the chromosome of its host. The only known host of φCh1 is *N. magadii*. *N. magadii* cells, infected lysogenic with φCh1 are called *N. magadii* L11 and are immune to superinfection by φCh1. Nevertheless the provirus in wild-type *N. magadii* L11 can be lost by passaging, in this way the cured *N. magadii* L13 strain arose, which was further used as indicator strain. φCh1 possesses a head-tail morphology and therefore belongs to the viral family *Myoviridae* in the Order *Caudovirales*. Its virus particles compromise a size of around 200 nm, whereas the tail, which is contractible, has a width of 20 nm and includes tail fiber proteins, which are necessary for the adsorption to the host. The temperate virus φCh1 has adapted to the salty environment of its host *N. magadii* and therefore requires 2 M NaCl, otherwise its infectivity is quickly lost, due to structural changes within its proteins (Witte *et al.*, 1997). The viral particle is composed of four major capsid proteins (A, E, H, I) and of about five minor capsid proteins (B, C, D, F, G), which range in size of 15 to 80 kDa and have isoelectric points typically for proteins isolated from halophils. The φCh1 genome consists of linear dsDNA of about 58.5 kbp size and has a G+C content of 62%, which is more or less the same G+C content, which is typically for *N. magadii* (Witte *et al.*, 1997; Tindall *et al.*, 1984). In addition φCh1 incorporates RNA species into its virus particle, which are thought to serve for packaging its dsDNA genome. The φCh1 genome is fully sequenced and contains 98 Open reading frames (ORFs), from which only 48 ORFs appear to have significant similarities to known proteins in public databases, therefore this let suggest, that φCh1 encodes for quite unique proteins. Nevertheless there is 80% sequence similarity with the genome of the halovirus φH. The genome itself is organized rather similar to other tailed dsDNA viruses, like those of the *E. coli* bacteriophage λ (Klein *et al.*, 2002).

φCh1 is a temperate virus, which is able to switch between lytic and lysogenic life-cycle. This molecular switch is achieved by the repressors ORF48, ORF49 and ORF79, which regulate gene expression of φCh1 throughout its lifecycle. This transcriptional regulators share sequence similarities as well as conserved gene organization with bacteriophage λ and the halo-virus φH (Iro *et al.*, 2007).

The previous study of Selb in 2017 unveiled ORF79 as crucial regulator within the lytic-lysogenic switch, because *N. magadii* L11 cells, which lack ORF79, appear to have a 24 hours earlier onset of lysis compared to wild-type. In addition, if ORF79 is over expressed, this leads to total inhibition of the lytic life-cycle and repression of viral genes. Therefore ORF79 is assumed to repress genes, responsible for the onset of lysis.

ORF48 (*rep*) was assumed to act as repressor, due to high sequence similarities with the φH repressor Rep, which role was discussed previously. In the sequence of ORF48 the amino acid pair Ala^{73}-Gly^{74} is present, which is known to serve as binding site of Rec A-mediated cleavage (Roberts and Roberts, 1975). ORF48 encodes also a helix-turn-helix motif within the Rep protein, which is known to be essential for sequence specific binding to DNA. Further RT-qPCR analysis have unveiled that *rep* is constitutively expressed during the whole life cycle of φCh1. This observation let assume, that ORF48 is a weak repressor, which is under the control of other regulators, because commonly such repressors are just expressed during lysogeny. Rep possesses binding sites for ORF43/44, these direct repeats (DR1 and DR2) are also involved in the auto-regulation of Rep itself. Therefore gp43/44 is thought to inhibit the action of ORF48. In addition, ORF43 and ORF44 are highly related to the *per* of φH and are thought to have also an enhancing effect on the transcription of the ORF49 promoter and therefore ORF48 seems to be indirectly repressed by gp43/44 (Iro *et al.*, 2007). To further elucidate the role of Rep and ORF49, BgaH assays (related to β-Galactosidase assay) with different transcriptional fusions of the putative promoter sequence within the intergenic region between ORF48 and ORF49 have been performed. The results of these experiments let suggest, that Rep blocks the transcription from the ORF49 promoter (Iro *et al.*, 2007).

In her Master thesis of 2019 Sabic proposed, that ORF48 and ORF49 act on the same set of genes, whereas ORF48 blocks the expression of lytic genes, and is ORF49 a transcription factor, which transiently activates the transcription of lytic genes. So therefore, in the lysogenic state ORF48 (Rep) is considered to block the expression of lytic genes as well as the transcription from the ORF49 promoter. To exit the lysogenic state ORF43/43 acts negatively on Rep, which stops repressing lytic genes and promotes the transcription from the ORF49 promoter, which thereafter results in the expression of ORF49, which is thought to be the promoting transcription factor for lytic gene expression. Nevertheless, it seems there are also other regulators involved like ORF79, which act on the switch between lytic and lysogenic life-cycle of φCh1. Sabic performed all experiments in NVM media, here NVM CAA^{+} media came into use, which provides *N. magadii* with a better accessible carbon source.

ORF49 was discovered upon analyzing the φCh1 variant φCh1-1, which has an earlier onset of lysis and a distinct lysis behavior compared to wild-type *N. magadii* L11. *N. magadii* cultures, which are infected with this natural arising mutant of φCh1 are called *N. magadii* L11-1 Sequence analysis unveiled a 223 bp duplication within ORF49, which led to the assumption, that ORF49 is a regulatory element as well. The duplicated and inserted 223 bp fragment consists of 109 bp from the 5'-UTR, and 114 bp of the coding region of ORF49 (nu. 34371-34593) (Iro *et al.*, 2007). This duplication is named ORF49′ and might get expressed as the putative protein gp49′. The question,

if this is indeed the case and if this protein plays a role within the switch between lytic and lysogenic life cycle will be in the scope of this study.

Results and Discussion

Evaluation of φCh1-1

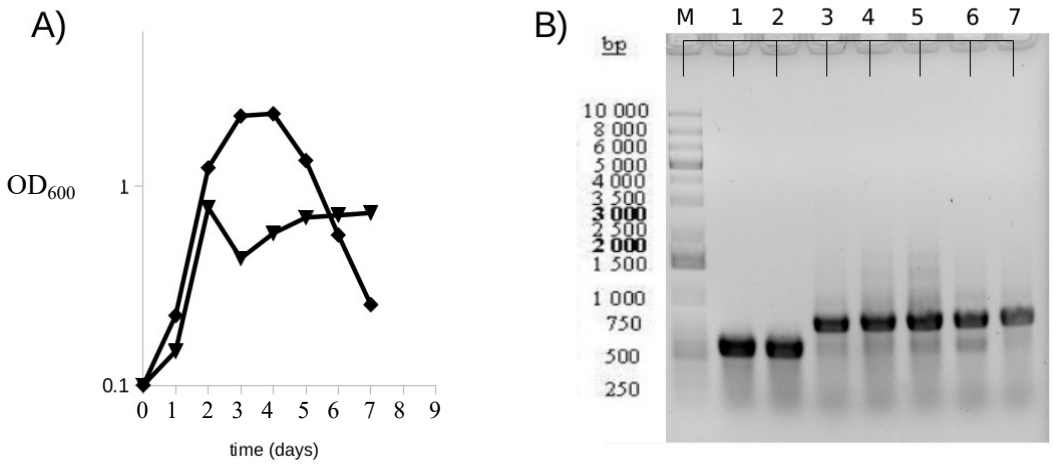


Figure 1: A) Growth properties of *N. magadii* L11-1 (marked by triangle) compared to wild-type *N. magadii* L11 (labeled with squares). Both cultures were inoculated to OD_{600}= 0.1 and grew at 37°C under agitation in NVM-CAA^+ Media. OD_{600} values were taken throughout the course of one week daily at a certain time point.
B) PCR reaction to verify ORF49´ in φCh1-1. Lane 1 and 2= Negative control (= *N. magadii* L11 template DNA), Lane 3: positive control (= pNB102-ORF49/49' template DNA); Lane 4-7: *N. magadii* L11-1 samples. As Marker served Gene Ruler 1kb DNA Ladder

In contrast to the previous performed experiments by Dina Sabic the here examined *N. magadii* cultures grew in NVM CAA^+ media instead of normal NVM media.
Previous studies of Iro *et al.* in 2007 unveiled a φCh1 variant, that exhibits an altered lysis behavior compared to wild-type, this variant is named φCh1-1. Those cultures show an earlier onset of lysis, which takes place on day 3, in contrast the wild-type culture starts lysing on day 5 (see Figure 1a). However, the lysis behavior of *N. magadii* L11-1 seems to be incomplete, because after this single decrease in optical density, the culture regenerated, perhaps because lysis moves aside to lysogeny. This observation let suggest that the regulation of lysogeny is altered in the φCh1-1 variant, due to changes in gene expression, caused by the mutation responsible for the phenotype of this mutant viral strain. Sequence analyses showed up that a part of ORF49, which encompasses 223 bp, is duplicated in the φCh1-1 variant (Iro *et al.*, 2007). This mutated form of ORF49 is described as ORF49'. ORF49 encodes for a transcription factor, which is thought to build together with ORF48

the molecular switch between lysogenic and lytic life cycle of φCh1. The role of ORF49 in this interplay seems to be the activation of the lytic life cycle by promoting the expression of lytic genes as a transcription factor.

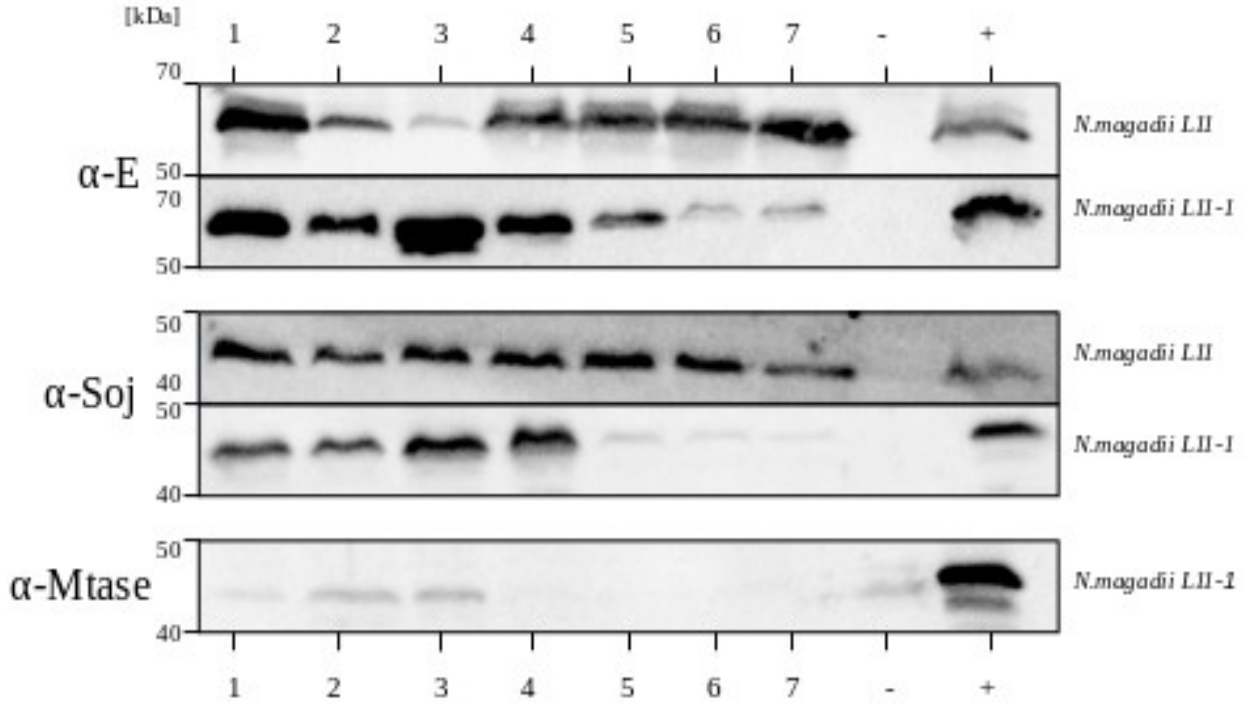


Figure 2: Western blot analysis to follow the lysis behavior of the *N. magadii* cultures L11-1 and L11. In addition to the optical density measurements, protein crude extracts were taken for Western blot. To obtain those western blots primary antibodies against Protein E (upper panel), Soj (middle panel) and Mtase (lower panel) were used. Protein samples of cured *N. magadii* L13 cultures served as negative control, whereas *N. magadii* L11 samples were used as positive control.

Furthermore, this mutation appeared as very unstable and is rapidly lost, only three passages are necessary to reestablish the wild-type phenotype of *N. magadii* L11 (Iro *et al.,* 2007). Therefore, the thawed L11-1 cultures were examined by PCR for the presence of the duplication in ORF49 (see Figure 1b), before they were used for further analysis. This PCR reaction made use of the primer 49-Kpn and 49-XB, which amplify a 539 bp fragment in the case that just ORF49 is present or amplify a 763 bp large fragment, if ORf49 is mutated to ORF49´. The clone, which template DNA was loaded in lane 7 was selected for further analyses, due to the fact that it has exclusively the larger PCR product of 763 bp in size and no smaller band of 539 bp, which would refer to the presence of wild-type ORF49. In contrast this smaller PCR product, which refers to ORF49 is present in the negative controls and also slightly in the samples taken from the other *N. magadii* L11-1 cultures. Therefore, this culture appeared as the most homogeneous *N. magadii* L11-1 culture and was used to determine the growth curve depicted in Figure 1A and was analyzed by Western

blotting (see Figure 2). To follow the lysis behavior of the cultures *N. magadii* L11 and *N. magadii* L11-1, antibodies against the viral proteins major capsid protein E, Soj, and Mtase were used.

In the samples taken from *N. magadii* L11 the signal for protein E is quite weak within the first three days, whereas the strong signal in lane 1 is just a remnant of the pre-culture, and therefore a left over from a former lysis cycle. On day 2 and 3 only a weak signal for protein E was detectable in the blots obtained from the *N. magadii* L11 culture. The signal then increased in intensity on day 4 to day 7, whereas the signal was most intense on day 7. In the samples taken from the *N. magadii* L11-1 culture, protein E, and therefore virus particles are detectable on day 1 to 5, whereas the signal is significantly strongest on day 3, where also a harsh decrease in optical density was observed. This is in line with the observation, that on this day the culture started to lyse. On day 6 and 7 protein E is only barely detectable in the *N. magadii* L11-1 samples. In each *N. magadii* L11 sample it was possible to detect the protein Soj (see Figure 2), which is typically constitutively expressed in φCh1, nevertheless this is not the case for the samples taken from the culture infected with φCh1-1, here no signal in Western blot is detectable for Soj upon day four, despite there is a signal for protein E in samples taken at these days. To not detect a signal for Soj is very unlikely dealing with *N. magadii* L11 cultures during Western blot, therefore an additional western blot with antibodies against the main Methyltransferase Mtase of φCh1-1 has been performed. Also this blot unveiled just a weak signal for the *N. magadii* L11-1 protein samples taken on the first 3 days of the measurement. The missing signal on the right side of the Soj and Mtase blots could also be traced back to degraded protein samples.

To ensure that this absence of crucial viral proteins is in fact reasoned by the nature of φCh1-1 and to exclude the possibility of invalid protein crude extracts, Figure 3 depicts the SDS-PAGE, obtained with the same *N. magadii* L11-1 samples, like used for Western blot. Apparently, the samples used for the SDS-PAGE as well as for the Western blots discussed above, are not degraded and therefore this observation is indeed caused by φCh1-1 itself. It seems, that the duplication within ORF49 is responsible for this altered lysis behavior, and that gp49´ somehow negatively effects the lytic life cycle of φCh1. The duplication is located enclosed to ORF48 (*rep*), which encodes for the repressor Rep, that inhibits the expression of lytic genes as well as the expression from the ORF49 promoter. The putative protein gp49', for which ORF49' might code for, has a molecular weight of 13.3 kDa and consists of the first 38 amino acids of gp49 and 41 unrelated amino acids (Iro *et al.*, 2007).

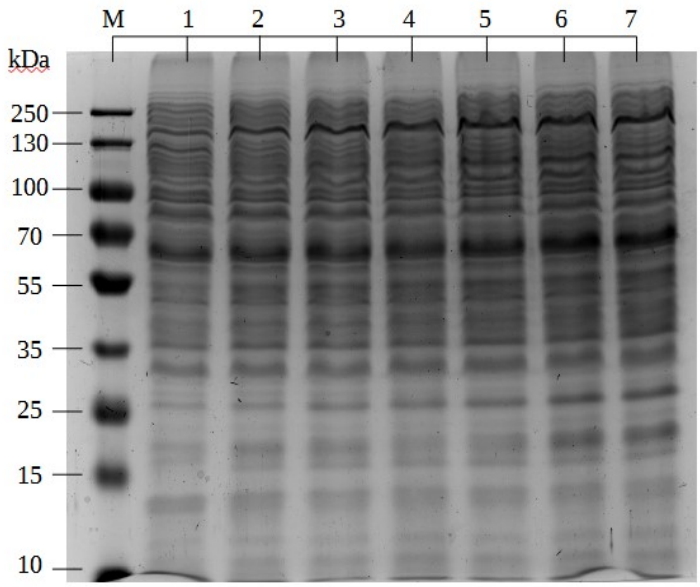


Figure 3: SDS-PAGE of *N. magadii* L11-1 stained with Coomassie brilliant blue. The protein crude extracts were separated at 40-50 V for 5-6 h after denaturing the samples for 10 minutes at 95°C. As Marker the Page Ruler pre-stained plus protein ladder was used. The *N. magadii* L11-1 samples taken from day 1 to 7 were loaded into the Lanes 1 to 7.

An explanation for the altered lysis behavior of this mutant strain could be, that this gene product of ORF49´ could indirectly promotes lysogeny of φCh1-1, by hindering the action of the transcription factor gp49, which activates the expression of lytic genes. This mutation could display a mechanism for enforcing enhanced lysogeny of φCh1 during unfavorable conditions for the virus. Nevertheless until now it is not clear, if ORF49´ is indeed an Open reading frame, that is actually translated into protein, which can influence the molecular switch between lytic and lysogenic life cycle of φCh1. The aim of this thesis is to proof if ORF49‘ indeed encodes for a truncated variant of the transcription factor ORF49, which is able to alter the lysis behavior of φCh1-1 in a suppressive manner.

Therefore, the start codon of ORF49´ was deleted by conventional cloning. The two constructs pNB102-ORF49/49'ΔAUG and pRO5/mev-ORF49/49'ΔAUG were transformed into *N. magadii* L13 cells, infected with different deletion mutants of φCh1 (wt, ΔORF48, ΔORF49, ΔORF49/48). Eventually the lysis behaviors and growth kinetics of the resulting cultures were examined by growth curves obtained by taking OD_{600} values for 7-10 days. In addition, the viral proteins Capsid protein E (ORF11), Soj and the main Methyltransferase were detected via Western blot. Furthermore, virus titers of lysing cultures were determined via plaque assay. The results obtained from the two constructs, each infected with the four different deletion mutants, were compared with control strains (constructed in previous studies), in which either just ORF49´ or ORF49/ 49´ are over expressed.

Evaluation of ΔORF48 and ΔORF49 Deletion Mutants of φCh1

Previous studies identified ORF48 as repressor encoding gene, ORF48 was identified as repressor by sequence comparison, which unveiled high similarities to the *rep* repressor of φH, because ORF48 possesses 13 bp direct repeats interrupted by 43 bp spacer in the 5´- end of the coding region, which are characteristic for rep-like repressors (Iro *et al.*, 2007). The role of ORF49 was investigated, because the 223 bp duplication, which seems to be responsible for the dysregulated gene expression in φCh1-1, and which impact should get elucidated more further within the curse of this thesis, gave a hint that ORF49 might have a regulatory function as well. ORF49 seems to be a transcription factor, that promotes lysis.

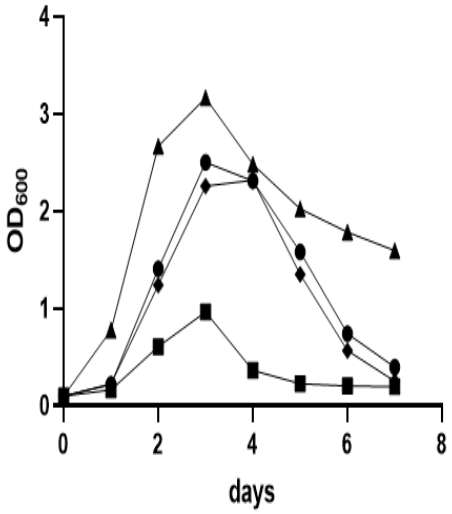


Figure 4: Growth properties of ORF48 and ORF49 Deletion Mutants. The growth curve obtained from wild-type *N. magadii* L11 is labeled with diamonds, the *N. magadii* L11-ΔORF48 culture is marked with circles, the *N. magadii* L11-ΔORF49 culture is marked with triangles and the double mutant *N. magadii* L11-ΔORF48/49 highlighted by squares. *A*ll cultures were grown at 37° with agitation for 7 days in NVM CAA^{+} media, each day at the same time protein samples were taken and optical density was measured at 600 nm.

In this section the results are displayed, which were obtained from *N. magadii* L13 cultures, which were infected with ORF48 and ORF49 φCh1 deletion strains, namely φCh1-ΔORF48, φCh1-ΔORF49 and the double mutant φCh1-ΔORF48/49 and are placed in comparison to the results from the wild-type culture *N. magadii* L11.

These deletion strains of φCh1 were created during the Master thesis of Dina Sabic in 2019 and were also used to infect cultures containing the two constructs pRo5/mev-ORF49/ORF49´ΔAUG and pNB102-ORF49/ORF49´ΔAUG, which were cloned in this thesis. And served in addition also for the infection of the control strains L13 (pRo5-ORF49/ORF49´), L13 (pRo5-ORF49´) and L13 (pNB102-ORF49/ORF49´). These control strains were also created by Dina Sabic.

The growth properties of the *N. magadii* cultures infected with the different φCh1 mutant strains are displayed in Figure 4. The *N. magadii* culture infected with wild-type φCh1 (see Figure 4) grows slightly over an OD_{600} of 2.3, before it starts lysing on day five, where the optical density decreased to OD_{600}= 1.3. During the next two days of lysis OD_{600} value of the wild-type culture decreased further to 0.2 and was therefore completely lysed.

In comparison the culture, which was infected with the φCh1-ΔORF48 deletion strain of φCh1 (marked by circles), started lysing on day 4 after it reached an OD_{600} of 2.5. The slight decrease in optical density on day four down to OD_{600}= 2.3 was followed by a much huger loss in optical density on day 5, where the value decreased to OD_{600}= 1.5. The next day the value dropped again to OD_{600}=0.7 and stabilized at OD_{600} = 0.4 on the last day of this experiment. Despite this culture lysed one day earlier, it seems to possess a slightly decelerated lysing rate compared to wild-type φCh1.

The *N. magadii* L11-φCh1-ΔORF49 culture lysed as well one day earlier compared to wild-type *N. magadii* L11 (see Figure 4). Of all three deletion mutants this culture reached the highest optical density of OD_{600} = 3.2. On day 4 the optical density dropped to OD_{600}= 2.5 and again the day after to OD_{600}= 2.0 but lysed after this harsh decrease only weakly the following day down to OD_{600}= 1.8. The remaining days of this experiment the OD_{600} value stayed more or less stable at this value. This observation of incomplete lysis refers to a significant reduced lysis rate in comparison to the wild-type *N. magadii* L11 culture.

The *N. magadii* culture, which was infected with the double deletion mutant φCh1-ΔORF48/49 just reached an OD_{600} of 1 after three days of growth, before the optical density dropped at day 4 down to OD_{600}= 0.4, and the optical density subsequently decreased the following days to OD_{600}= 0.2. This culture appeared as growth deficient, because this culture never reached a high optical density and it was challenging to even get this double mutant in culture. All three examined mutant strains started to lyse on day 4, so one day earlier compared to wild-type φCh1.

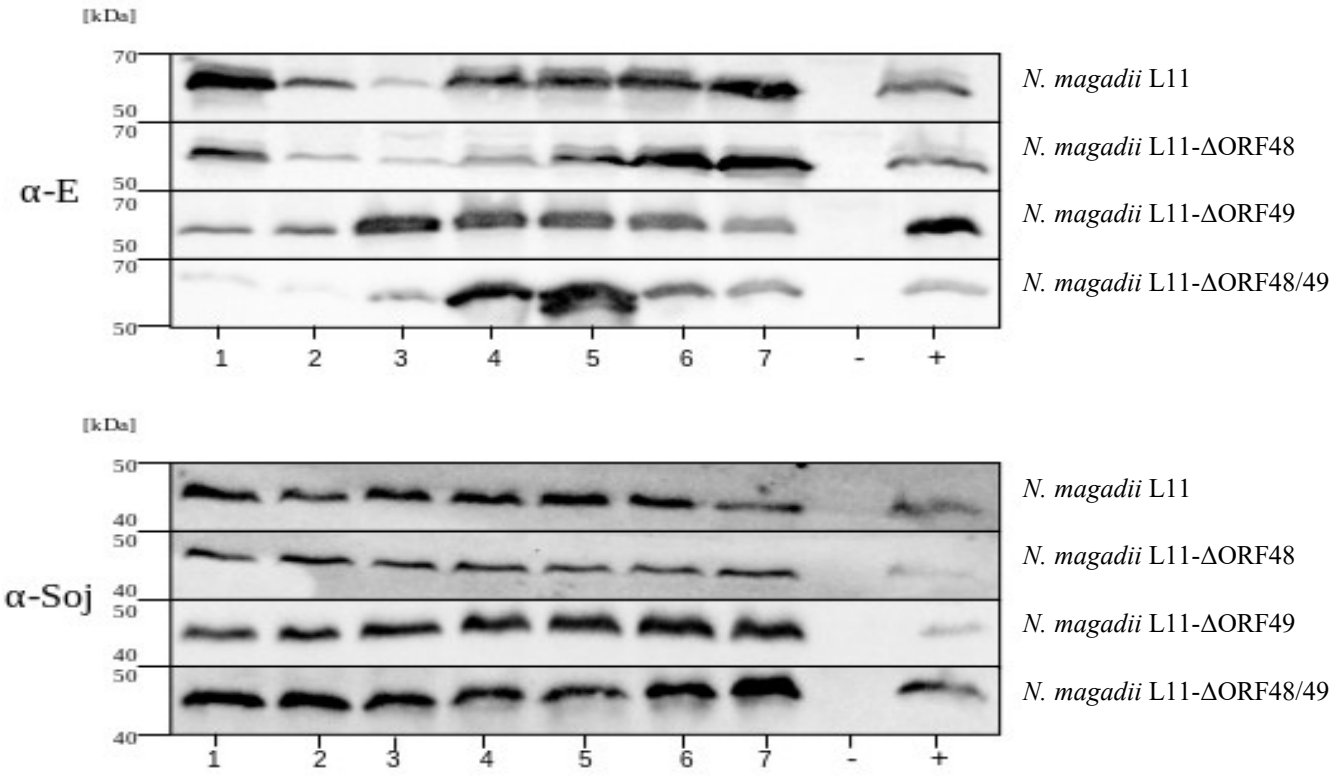


Figure 5: Western Blot analysis of ORF48 and ORF49 Deletion Mutants. Protein extracts were taken from the *N. magadii* cultures infected with the φCh1-ΔORF48, φCh1-ΔORF49 and φCh1-ΔORF48/49 deletion strains of φCh1, to display the presence of the viral protein Soj (depicted in lower panel) and protein E (depicted in upper panel) via western blot. As negative control an *N. magadii* L13 sample was used, as positive control served an *N. magadii* L11 sample, on Lane 1 to 7 the samples taken from day 1 to 7 were applied.

The Western blot analysis of the *N. magadii* L11-ΔORF48 and *N. magadii* L11-ΔORF49 deletion mutants, as well as for the double mutant *N. magadii* L11-ΔORF48/ΔORF49 are displayed in Figure 5. To compare the lysis behavior of these deletion mutants, the Western blots obtained from an *N. magadii* L11 wild-type culture are displayed as well. All examined cultures show a constant signal in western blot, when the primary antibodies against Soj was used. The Soj protein, which is thought to be involved in plasmid partitioning, is constitutively expressed in φCh1. Therefore it can serve as a loading control, to ensure that the same amount of sample was applied to the blots. As positive control, a sample taken from a lysing *N. magadii* L11 culture was used, as negative control a sample of an uninfected *N. magadii* L13 culture was applied to the blots.

In the blots obtained from the *N. magadii* L11 samples the signal for protein E is quite weak the first three days, whereas the strong signal on day one is misleading, because it is just a remnant

from the former lysis cycle carried over from the pre- culture, which was used to inoculate the examined *N. magadii* L11 culture. On day four the signal gets more intense, which is in line with the observation that the culture started lysing the next day. The signal stayed strong within the remaining days of this measurement, which is in line with the observation that the culture lysed here nearly completely.

The *N. magadii* L11-ΔORF48 culture shows a weak signal for protein E the first 4 days, also here a weak left over signal of the pre-culture is visible. Between day 5 and 7 the signal is strongest and therefore the most viral particles have been produced, this is the same time period within it was observed that the culture lysed. The Western blot analysis makes also apparent, that in comparison to wild-type lysis is one day delayed.

In contrast to the samples taken from wild-type or the *N. magadii* L11-ΔORF48 culture, the *N. magadii* L11-ΔORF49 samples appear to have a signal for protein E on each day of this measurement, whereas the signal increases on day 3, so one day before this culture started to lyse. The signal in Western blot against protein E intensifies and seems to be strongest on day 5, when this culture started lysing and decreases on day 6 and 7, where the lysis rate decreased as well, which leads to an incomplete lysis of this culture just until an OD_{600} of 1.7. In contrast the wild-type culture lysed completely down to OD_{600} of about 0.2.

The signal for protein E is only barely detectable within the samples taken from the *N. magadii* L11-ΔORF48/49 culture on day 1 and 2. On day three just a weak signal is apparent, the next day a loss in optical density was observed in the growth curve analysis and also the signal for protein E increased on this day significantly, the signal then intensified further on day five, where the signal got so strong, that the blot appears to be overloaded. Nevertheless there was no huge loss in optical density detected after this day and the OD_{600} value of this culture stabilizes at around 0.2 on the last two days of this experiment. Also the signal for protein E decreased drastic on day 6 and 7 where just a faint band was detected.

Discussion

ORF48 and ORF49 are thought to build together the molecular switch between lytic and lysogenic life cycle of φCh1 (Iro *et al*., 2007). The deletion of ORF48 as well as those of ORF49 seem to deregulate gene expression, in such a way that all three deletion strains of φCh1 started one day earlier to lyse, than the culture, infected with wild-type φCh1. This earlier onset of lysis is apparent in the growth curves as well as in the Western blot analysis of the three evaluated cultures.

In case of the culture infected with the ORF48 deletion strain of φCh1, this might be caused due to the proposed feature of ORF48 to repress lytic genes. The lack of this crucial player seems to accelerate lysis and hinder lysogeny. In Western blot analysis it is apparent to see, that protein E appeared one day earlier, than on the Western blots of wild-type *N. magadii* L11.

Whereas the deletion of ORF49 results in an incomplete and less rapid lysis behavior of the *N. magadii* L11-ΔORF49 culture, nevertheless the major capsid protein E of φCh1 was detectable via Western blot on each day of this experiment, this is in line with the assumption, that the lack of the transcription factor gp49 does not allow proper expression of lytic genes, thereafter lysis appear to be insufficient.

The culture infected with the φCh1 strain harboring the double knock-out in ORF48 and ORF49 was growth deficient, but lysed sharply, which let assume, that in this mutant strain gene expression is completely disrupted due to the lack of ORF48 and ORF49 and therefore this *N. magadii* L11-ΔORF48/49 culture was not able to reach a higher optical density like the other cultures within this comparison did. Due to the fact that in this culture unregulated lysis seems to have taken place.

In the upcoming sections the results will be displayed and discussed, which were obtained from the *N. magadii* pNB102 and pRo5/mev cultures L13-pRo5/mev-ORF49/49´ΔAUG and L13-pNB102-ORF49/49´ΔAUG as well as from the control strains, which led to the over-expression of ORF49´, without the mutated AUG. These cultures were infected with different deletion strains of φCh1, which either lack ORF48 or ORF49 or both, to visualize the effect of the introduced mutation without complementation of ORF48 or ORF49.

Encodes ORF49´ the putative protein gp49´, which can alter the lysis behavior of φCh1?

Verification of the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG), L11 (pRO5/mev-ORF49/49´) and L11 (pRo5/mev-ORF49´)

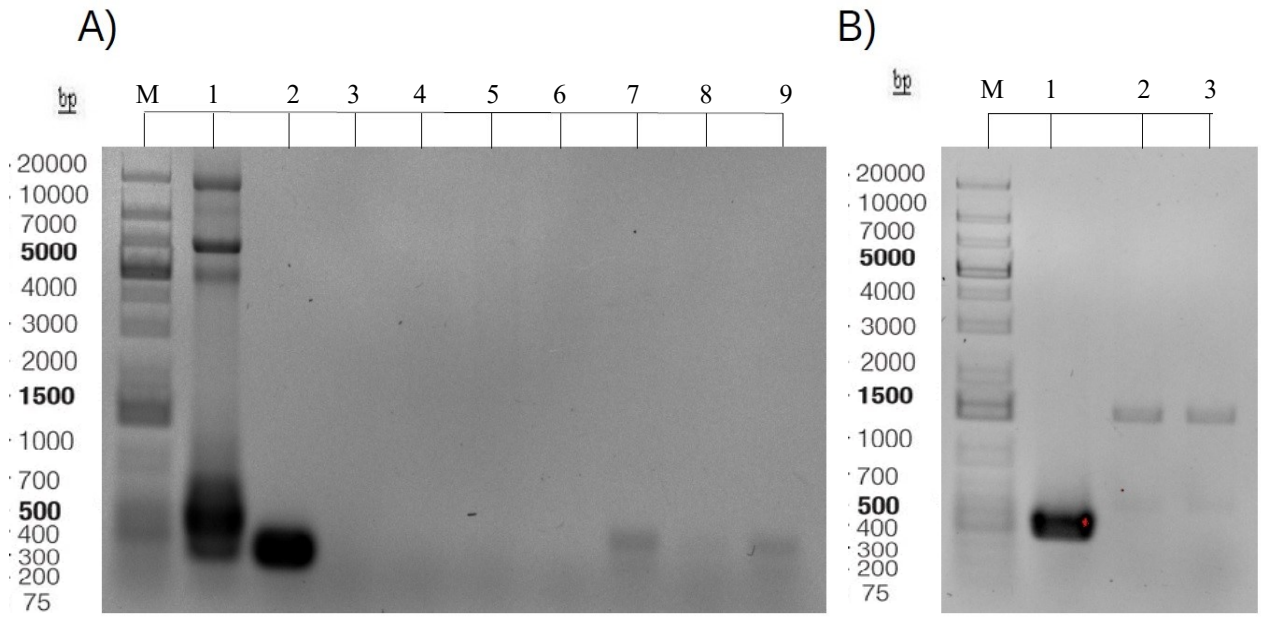


Figure 6: A) Colony PCR after Transformation of the construct pRo5/mev-ORF49/49´ΔAUG into competent *N. magadii* L13 cells: M= Marker Gene Ruler 1kB plus, Lane 1= negative control (= vector pRO5/mev), Lane 2= positive control (=shuttle vector pRo5/mev-ORF49/49´ΔAUG), Lane 3-9: DNA templates of *N. magadii* transformants, whereas Lane 7 and 9 appear as positive transformants
B) ORF56 PCR reaction in advance of plaque assay to verify absence of φCh1: M= Marker GeneRuler 1kB plus DNA ladder, Lane 1= positive control (= φCh1 template DNA), Lane 2 and 3= template DNA of L13 (pRo5/mev-ORF49/49´ΔAUG) positive clones, in which ORF56 was not detected.

After successfully cloning, the construct pRo5/mev-ORF49/49´ΔAUG got transformed into uninfected *N. magadii* L13 cells. To verify the success of this transformation, a colony PCR with the primers 49-*Kpn* and 49-*Kpn*-3 has been performed. The agarose Gel obtained after this PCR reaction, is displayed in Figure 6A. This primer pair amplifies a DNA sequence, which has a size of

547 bp. In two of the examined transformants a PCR product of the same size like the positive control was obtained, which proofs, that those cultures have taken up the plasmid pRo5/mev-ORF49/49´ΔAUG successfully and therefore those cultures were used to be further infected via plaque assay.

To ensure that both cultures have not been accidentally infected by φCh1 in advance of this plaque assay, a PCR reaction to detect ORF56 has been performed. The detection of ORF56, is a significant proof for an infection with φCh1. This PCR reaction makes use of the primer 56-RT5 and 56-RT3, which amplify a PCR product of 478 bp. The agarose gel of this PCR reaction is displayed in Figure 6B and shows a strong signal for the positive control, but no signal within the examined cultures, which would refer to the presence of ORF56. Therefore, this result proofs the absence of φCh1. The presence of an uncertain φCh1 strain within these cultures, due to contamination, would have hindered a super-infection with the different ORF48 and ORF49 deletion strains of φCh1 in the following step, which was crucial to further elucidate the impact of the duplication in ORF49.

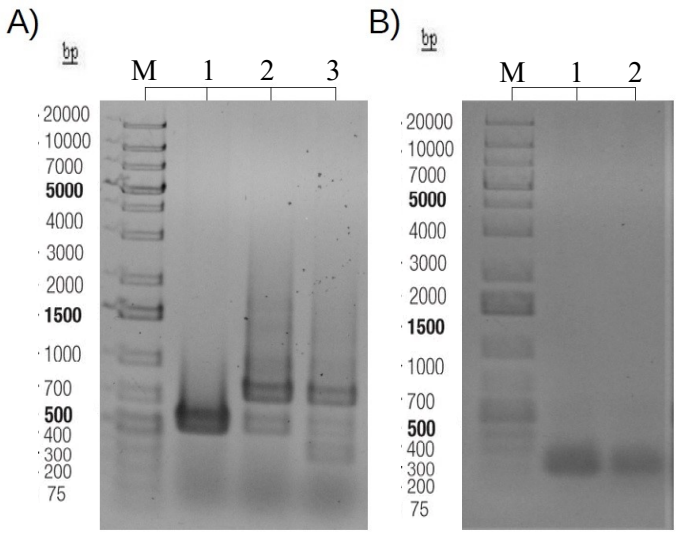


Figure 7:A) Verification of the genetic identity of the culture *N. magadii* L13 (pRo5/mev-ORF49/49´): Lane M= Marker gene ruler 1kB plus, Lane 1= negative control (= *N. magadii* L11 template DNA), Lane 2= positive control (= *N. magadii* L11-1 template DNA), Lane 3= *N. magadii* L13 *(*pRo5/mev-ORF49/49´) template DNA

B) Proof for the presence of pRo5/mev-ORF49´ via PCR reaction: Lane M= Marker Gene Ruler 1kB plus DNA ladder, Lane 1= positive control (= *N. magadii* L11-1 DNA template), Lane 2= *N. magadii* L13 (pRo5/mev-ORF49´) DNA template

The constructs pRo5/mev-ORF49/49´ and pRo5/mev-ORF49´, which serve as controls for this study, have been created by Dina Sabic during her Master thesis back in 2019. The cultures were stored for more than one year at Room temperature, due to the risk that those cultures might have lost their plasmids during this time, it was necessary to verify that those cultures have not genetically changed. Therefore, a PCR reaction with template DNA, obtained from those cultures. The agarose gel of this PCR reaction, which detects the open reading frame ORF49/49´ on the plasmid pRo5/mev is displayed in Figure 7A. In this PCR reaction the primers 49-*Kpn* and 49-*Kpn*-3 got used to generate a PCR product, which has a length of 757 bp. A PCR product of that size is clearly apparent in the positive control (see Figure 7A: Lane 2) and also got obtained from the DNA template of the examined culture (applied to Lane 3), but it got not detected in the template DNA of the negative control (see Lane 1). Here exclusively a smaller band is visible, which refers to the presence of wild-type ORF49. It was possible to use this culture for further experiments, due to the fact, that the examined culture exclusively possesses the fragment of 757 bp size, which proofs the presence of ORF49´.

In Figure 7B the agarose gel picture of the PCR reaction to determine the genetic identity of *N. magadii* L13 (pRo5/mev-ORF49´) is depicted. This PCR reaction makes use of the primer pair 49-*Kpn* and 49-K-*Kpn,* together those primers amplify a DNA sequence of 420 bp, which refers to the presence of ORF49´. An amplicon of such a size is visible in the positive control and is also present in the PCR product of the examined culture *N. magadii* L13 *(*pRo5/mev-ORF49´). This result proofs the presence of ORF49´ within the examined culture and therefore this culture got selected to be further infected with the different ORF48 and ORF49 deletion strains of φCh1, to get deeper insights in the lysis behavior of the resulting cultures.

In this following step the *N. magadii* L13 cultures transformed with the plasmids pRo5/mev-ORF49/49´ΔAUG, pRo5/mev-ORF49/49´ and pRo5/mev-ORF49´, were infected with different φCh1 deletion strains, namely the ΔORF48, ΔORF49 and the double mutant ΔORF48/49. To verify that this infection took place successfully an ORF56 PCR has been performed, with the DNA templates obtained from the cultures, which resulted from these plaque assays.

The agarose gel of this PCR reaction to proof the presence of φCh1 is displayed in Figure 8A. Here the same primers were used as discussed previously. In all examined cultures a clear and strong signal for ORF56 is apparent, except for the DNA template of the *N. magadii* culture L11 (pRo5/mev-ORF49/49´ΔAUG), which was supposed to get infected with the ΔORF48/49 deletion strain of φCh1. In this case no PCR product was obtained and therefore it was not possible to detect the virus within this culture. Due to that with this culture an additional plaque assay has been performed to infect with the ΔORF48/49 deletion strain of φCh1.

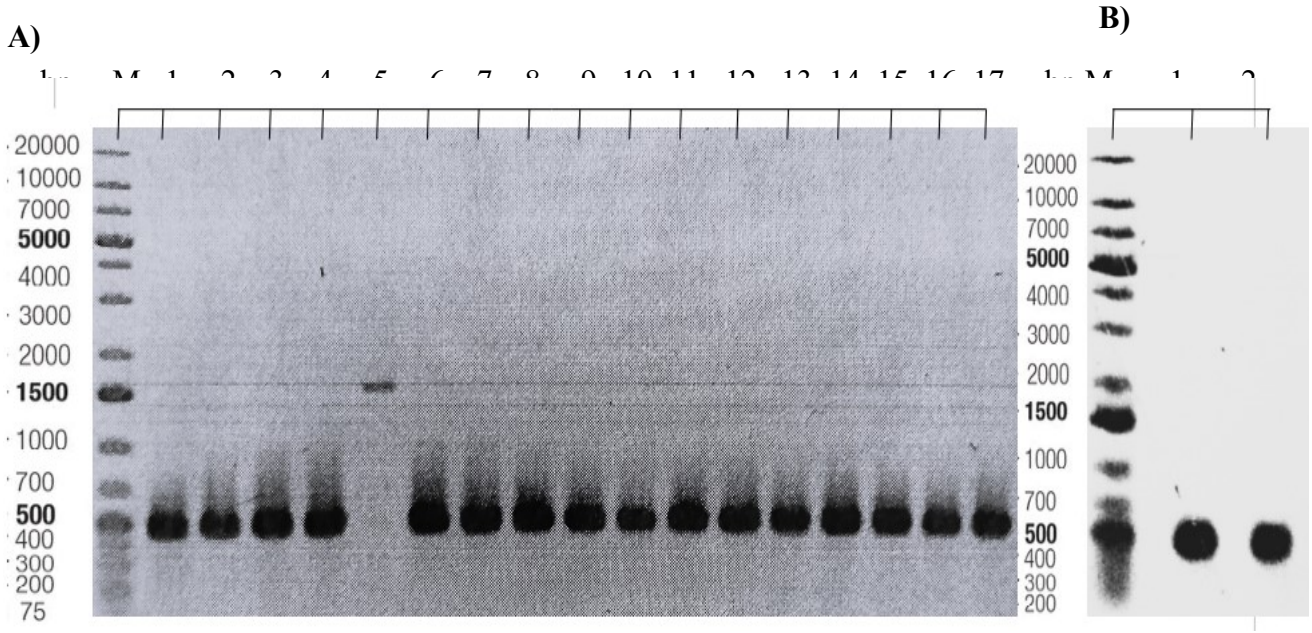


Figure 8:A) Post-plaque assay ORF56 PCR to verify the presence of φCh1 in all pRo5/mev-cultures: Lane M= Marker Gene Ruler 1kb plus, Lane 1= positive control (= φCh1 template DNA), Lane 2=*N. magadii* L11 *(*pRo5/mev-ORF49/49´ΔAUG), Lane 3= *N. magadii* L11-ΔORF49 *(*pRo5/mev-ORF49/49´ΔAUG), Lane 4= *N. magadii* L11-ΔORF48 *(*pRo5/mev-ORF49/49´ΔAUG), Lane 5= *N. magadii* L11-ΔORF48/49 *(*pRo5/mev-ORF49/49´ΔAUG), Lane 6= *N. magadii* L11 (pRo5/mev-ORF49/49´) Lane 7= *N. magadii* L11-ΔORF49 (pRo5/mev-ORF49/49´), Lane 8= *N. magadii* L11-ΔORF48 (pRo5/mev-ORF49/49´), Lane 9= *N. magadii* L11-ΔORF48/49 (pRo5/mev-ORF49/49´), Lane 10 = *N. magadii* L11 *(*pRo5/mev-ORF49´), Lane 11=*N. magadii* L11-ΔORF49 *(*pRo5/mev-ORF49´), Lane 12=*N. magadii* L11-ΔORF48 *(*pRo5/mev-ORF49´), Lane 13= *N. magadii* L11-ΔORF48/49 *(*pRo5/mev-ORF49´), Lane 14= *N. magadii* L11 *(*pRo5/mev-ORF49Δ4), Lane 15= *N. magadii* L11-ΔORF48 *(*pRo5/mev-ORF49Δ4), Lane 16= *N. magadii* L11-ΔORF49 *(*pRo5/mev-ORF49Δ4), Lane 17= *N. magadii* L11-ΔORF48/49 *(*pRo5/mev-ORF49Δ4)

B) Post-plaque assay ORF56 PCR of *N. magadii* L11-ΔORF48/49 *(*pRo5/mev-ORF49/49´ΔAUG) after additional plaque assay. M= Marker Gene Ruler 1kb plus, Lane 1= positive control (= φCh1 template DNA), Lane 2= *N. magadii* L11-ΔORF48/49 *(*pRo5/mev-ORF49/49´ΔAUG) positive for infection with φCh1.

In Figure 8B the results of the ORF56 PCR reaction are displayed, which was performed after the second plaque Assay. After this plaque assay it was possible to detect the viral ORF56 free of doubt in the examined cultures as well as in the positive control (see Figure 8B).

Therefore, it was possible to use the culture *N. magadii* L11-ΔORF48/49 (pRo5/mev-ORF49/49´ ΔAUG), among all other examined *N. magadii* cultures, which were successfully infected with a distinct deletion strain of φCh1, for further examinations. In which their growth kinetics and lysis behaviors were followed by growth curve, Western blot and virus titer analysis. The results of this experiments are displayed in the upcoming chapters of this thesis.

The impact of the deleted Start codon in ORF 49´ examined on the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG), L11 (pRo5/mev-ORF49/49´) and L11 (pRo5/mev-ORF49), infected with wild-type φCh1

To verify, if the 223 bp duplication in ORF49´indeed encodes for an open reading frame, which is expressed as the putative protein gp 49, two constructs were cloned. Both constructs pRo5/mev-ORF49/49´ΔAUG as well as pNB102-ORF49/49´ ΔAUG carry the open reading frames ORF49 and ORF49´, but the start codon (AUG) is deleted in ORF49´. To evaluate how the lysis behavior of φCh1 is altered by this introduced mutation, both constructs were transformed into *N. magadii* L13 cultures, which ultimately got infected with the different ORF48 and ORF49 deletion mutants of φCh1, along with wild-type φCh1.

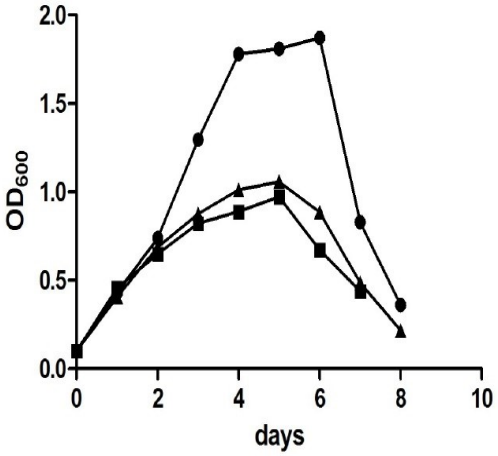


Figure 9: Growth kinetics and lysis behavior of *N. magadii* L11 *(*pRo5/mev-ORF49/49´ΔAUG) (marked with triangle), *N. magadii* L11 (pRo5/mev-ORF49/49´) (labeled with circle) and *N. magadii* L11 (pRo5/mev-ORF49´) (marked with square). The cultures were inoculated to a start OD_{600} of 0.1. The OD_{600} values were measured each day at the same time for the course of one week. The cultures were grown at 37°C under agitation in NVM CAA^{+} media.

In this section the results are displayed, which got obtained from the culture *N. magadii* L11 *(*pRo5/mev-ORF49/49´ΔAUG), and the control cultures *N. magadii* L11 (pRo5/mev-ORF49/49´) and *N. magadii* L11 *(*pRo5/mev-ORF49´). These three cultures were infected with φCh1 via plaque assay, to then examine their lysis behavior. The growth properties of these cultures are depicted in Figure 9. All cultures were inoculated to a Start OD_{600} of 0.1 and were grown at 37°C under agitation. To follow growth, the optical density was measured each day at a certain time point

at 600 nm. In this experiment the introduced mutation is complemented by ORF48 as well as by ORF49, due to the presence of both open reading frames are in wild-type φCh1.

The *N. magadii* L11 (pRo5/mev-ORF49/49´ΔAUG) culture just reached an OD_{600} value of about 1 after 4 days of growth, before this culture started to lyse on day 6, where the optical density decreased to OD_{600} = 0.88. This slight decrease in optical density was followed by a bigger loss the following day, on which an OD_{600} value of 0.5 was determined. On the last day of this measurement the culture was lysed barely completely to OD_{600}= 0.21.

The culture *N. magadii* L11 (pRo5/mev-ORF49´) shows a quite similar growth and lysis behavior than the culture, which harbors the deleted start codon in ORF49´. Indeed the OD_{600} values of the control strain *N. magadii* L11 *(*pRo5/mev-ORF49´) were slightly beneath those of the construct, but the shape of both growth curves is obviously similar, and therefore the lysis behavior is quite comparable. In addition, the control strain *N. magadii* L11 (pRo5/mev-ORF49´) started lysing on day 6, the same day like the culture *N. magadii* L11 (pRo5/mev-ORF49/49´ΔAUG) did.

Nevertheless the lysis rate of *N. magadii* L11 (pRo5/mev-ORF49´) is slightly higher compared to the evaluated construct, but of course the differences here are much smaller than, in comparison to the lysis behavior of *N. magadii* L11 (pRo5/mev-ORF49/49´).

In contrast, the culture, harboring the plasmid pRo5/mev-ORF49/49´, reached after 6 days of growth a much higher optical density of around OD_{600}= 1.88. Before the OD_{600} value of this culture dropped to 0.83 on day 7. The optical density was decreased even further the following day, where eventually an OD_{600} value of 0.36 was determined. The culture *N. magadii* L11 (pRo5/mev-ORF49/49´) shows a higher growth rate and lysed one day later than the construct *N. magadii* L11 *(*pRo5/mev-ORF49/49´ΔAUG), therefore the lysis behavior of this culture can be declared as indeed delayed, but on the other hand also as rapid and more or less complete.

Next to the determination of the optical density, each day a protein sample for Western blot analysis was prepared from the cultures *N. magadii* L11 *(*pRo5/mev-ORF49/49´ΔAUG), *N. magadii*-L11 (pRo5/mev-ORF49/49´) and *N. magadii* L11 (pRo5/mev-ORF49´), which were infected with wild-type φCh1 (except for *N. magadii* L11 (pRo5/mev-ORF49/49´) on day 8 no protein sample was prepared). To achieve the western blots, which are displayed in Figure 10, antibodies against the major capsid protein E, sized 55 kDa, and the constitutively expressed viral protein Soj, which has a size of 42 kDa, came into use. For a proper positive control, a sample obtained from a lysing *N. magadii* L11 culture was applied to the blots, also a protein sample from an uninfected *N. magadii* L13 culture was applied to serve as negative control.

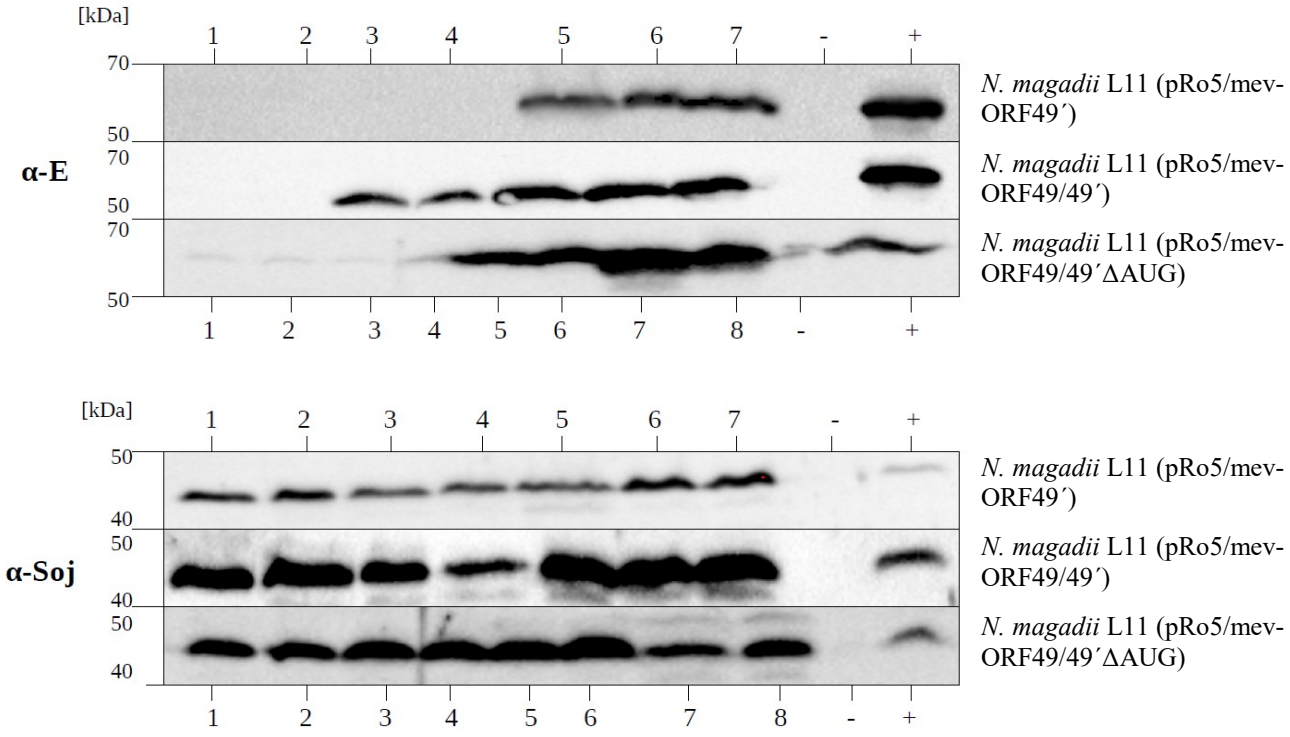


Figure 10: Western blot analysis of the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG), L11 (pRo5/mev-ORF49/49´) and L11 (pRo5/mev-ORF49´). Protein crude extracts for SDS-PAGE were taken in addition to the daily optical density measurement. The upper figure displays the Western blots, in which a primary antibodies against the major capsid protein E were used. The lower panel depicts the blots, in which primary antibodies against Soj came into use.

Samples, obtained from the culture *N. magadii* L11 (pRo5/mev-ORF49/49´ΔAUG) appear to have a faint signal for protein E on day 1, 2, 3 and 4, whereas a clear signal against protein E on day 5, 6, 7 of this measurement got detected, which refers to the production of virus particles within this time period. The signal has the same intensity on day 5 like on day 6, and is most intense on day 7. On day 8 the signal is less intense than on day 7, but still stronger than on day 6. This is in line with the observations made during growth curve analysis, where the first decrease in optical density was measured on day 6 and therefore virus particles apparently start forming one day in advance. Also the much greater loss in optical density, which was detected on day 7, is in line with the observation that on this day the protein E signal in western blot has intensified.

In contrast to the growth curve analysis the *N. magadii* culture L11 (pRo5/mev-ORF49´) possesses a distinct lysis behavior according to western blot comparison, which differs slightly from the protein E expression pattern observed in *N. magadii* L11 (pRo5/mev-ORF49/49´ΔAUG).

Here, exclusively on day 5, 6 and 7 a signal for protein E was detected, This signal appeared to be constant in intensity within these three days, in contrast to the up-swinging signal observed in the samples of the culture harboring the deleted AUG in ORF49´.

Contrary to the two cultures discussed first, in which capsid protein E was detected just one day before a decrease in optical density was apparent, the culture *N. magadii* L11 (pRo5/mev-ORF49/49´), infected with wild-type φCh1, displays a clear signal against protein E much earlier, namely upon day 3 till the end of this measurement. The signal on day 3 and 4 is slender but intense, increased on day 5, and stayed at this intensity until day 7. Despite this culture started lysing on day 7, with a significant decrease in the OD_{600} values, virus particles are detectable upon day 3. This observation let suggest, that lysis and growth within this culture hold balance until the drop in optical density on day 7, this would also explain the quite stable OD values of the *N. magadii* L11 (pRo5/mev-ORF49/49´) culture on day 4, 5, and 6 in advance of lysis.

The Western blots, which were obtained with primary antibodies against protein Soj, show an almost homogeneous signal for all three cultures at each day of this experiment.

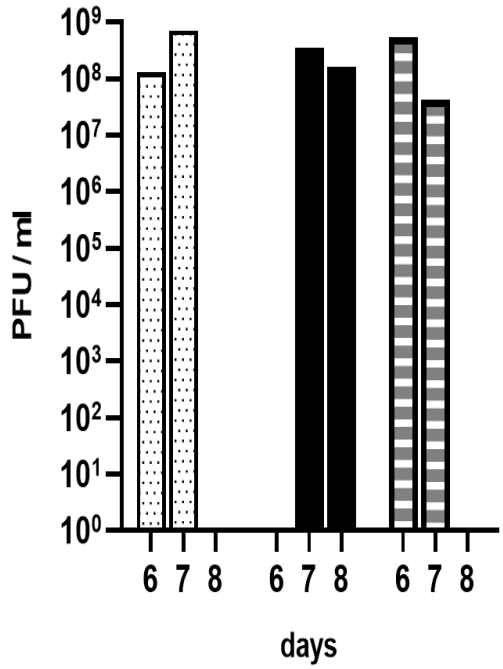


Figure 11: Virus titer analaysis of the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG) (labeled with points), L11 (pRo5/mev-ORF49/49´) (marked in black) and L11 (pRo5/mev-ORF49´) (marked with lines). The plaques, which arose in the lawn of *N. magadii* L13 cells, where counted 7 days after infection with the supernatants taken from the lysing cultures

In addition to the Western blot comparison and the determination of growth curves, virus titers of the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG), L11 (pRo5/mev-ORF49/49´)

and L11 (pRo5/mev-ORF49´), infected with wild-type φCh1, have been determined. To determine these virus titers plaque assays were performed to infect *N. magadii* L13 cells with supernatants, taken from the lysing pRo5/mev cultures. After incubating the resulting plaque assays for 7 days at 37°C, the arising plaques were counted to eventually calculate the plaque forming unit.

Figure 11 displays the plaque forming unit (= PFU) of all three examined cultures. The samples for the virus titer, where taken from the L11 (pRo5/mev-ORF49´) and the L11 (pRo5/mev-ORF49/49´ΔAUG) were obtained on day 6 and 7, those from the *N. magadii* L11 (pRo5/mev-ORF49/49´) culture on day 7 and 8.

On day 6 of lysis for the culture L11 (pRo5/mev-ORF49/49´ΔAUG) a plaque forming unit of $1.3*10^8$ PFU/ml was determined, this value increased on the following day up to $7.2*10^8$ PFU. The supernatant of the *N. magadii* culture L11 (pRo5/mev-ORF49/49´) formed $3.5*10^8$ plaques on day 7. This value is higher compared to the PFU obtained from the culture, which harbors the deleted start codon in ORF49´. On day 8 the culture *N. magadii* L11 (pRo5/mev-ORF49/49´) has an PFU of $1.6*10^8$, which is significant lower than the value of the L11 (pRo5/mev-ORF49/49´ΔAUG) culture, which got determined on the same day. The PFU of the *N. magadii* culture L11 (pRo5/mev-ORF49´) is highest on day 6 with a plaque forming unit of $5.5*10^8$. This value is in a quite comparable range like the value obtained from the L11 (pRo5/mev-ORF49/49´ΔAUG) culture, but the virus titer drops much harsher to $4.3*10^7$ on day 7.

Discussion

The growth kinetic comparison of the pRo5/mev containing *N. magadii* cultures, which were infected with wild-type φCh1 made apparent, that the culture, which carried the plasmid harboring the introduced mutation, has quite similar growth properties like the *N. magadii* L11 (pRo5/mev-ORF49´) culture. The shape of both growth curves, and therefore the lysis behaviors of both cultures is highly related, both cultures started to lyse on the same day.
Whereas the lysis behavior of the third examined *N. magadii* culture, which carries the plasmid pRo5/mev-ORF49/49´, appears to be delayed. Nevertheless, this culture lysed more sharply than the other two cultures did.

Interestingly, the Western blot analysis unveiled, that the *N. magadii* L11 (pRo5/mev-ORF49/49´) culture has a constant signal for protein E upon day three of this measurement. Also a clear difference between the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG) and L11 (pRo5/mev-ORF49´) could have been disclosed by Western blotting. Samples from the control possess a constant protein E signal from day 5 to 7, whereas the signal for protein E in the samples obtained from the culture carrying the construct increased definitely on day 7. This increase is also visible in the number of plaques, which arose from the supernatant of this culture. In contrast the virus titer of the other two examined cultures dropped on the second day of lysis.

These results let assume, that lytic genes are not properly expressed in the case of the *N. magadii* L11 (pRo5/mev-ORF49/49´) culture, and therefore this culture appeared to have a delayed onset of lysis. Whereas the other two examined cultures behaved the same at first sight, nevertheless the lysis behavior of the *N.magadii* L11 (pRo5/mev-ORF49/49´ΔAUG) culture seems to be more aggressive, due to the fact, that the protein E signal increased on day 7, where also more virus particles were produced, than within the *N. magadii* L11 (pRo5/mev-ORF49´) culture. However, in this experimental setup the introduced mutation is complemented by ORF48 as well as from ORF49, due to the infection with wild-type φCh1. This circumstances might abates the effect of gp49´ on the lysis behavior of φCh1.

Impact of the deleted AUG in ORF49´ on the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG), L11 (pRo5/mev-ORF49/49´) and L11 (pRo5/mev-ORF49´) infected with the ΔORF48 strain of φCh1

Here the results will be displayed, which got accomplished from the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG), L11 (pRo5/mev-ORF49/49´) and L11 (pRo5/mev-ORF49´), which have been infected with the ORF48 deletion strain of φCh1 via plaque assay. In this φCh1 strain ORF48 is deleted, but ORF49 is still present.

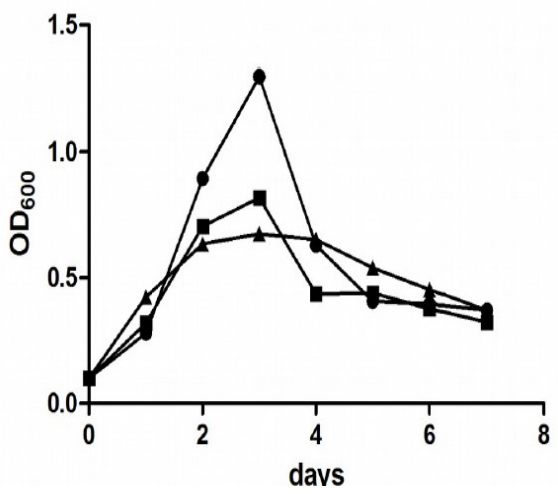


Figure 12: Growth curve analysis of the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG) (marked with circle), L11 (pRo5/mev-ORF49/49´) (labeled with square) and L11 (pRo5/mev-ORF49´) (highlighted with triangle) infected with the ΔORF48 deletion strain of φCh1. All three cultures were inoculated with a starting OD_{600} of 0.1 and were grown on 37°C under agitation in NVM CAA^+ media. The optical density values got obtained each day at a distinct time point for the course of one week.

The growth curves of these three cultures are displayed in Figure 12. All three growth curves relay on OD_{600} values, which were measured through the curse of one week at a certain time point at 600nm.

The *N. magadii* culture L11 (pRo5/mev-ORF49/49´ΔAUG) reached the highest optical density of all three cultures of this comparison of about OD_{600}= 1.3, before it lysed sharply to an OD_{600} value of 0.63 on day 4. The day after the optical density decreased again to OD_{600}= 0.4, but after this decrease on day 5, the optical density values stayed quite stable at an OD_{600} of around 0.4 for the remaining days of this experiment.

In contrast to the sharp shape of the *N. magadii* L11 (pRo5/mev-ORF49/49´ΔAUG) growth curve, the growth curve of L11 (pRo5/mev-ORF49´) is much more flat. This is reasoned by the fact that this culture reached an optical density of OD_{600}= 0.67 on day 4, and lysed just slightly on day 5 to OD_{600}= 0.65 before the optical density decreased further each day of round about 0.1 of OD_{600} values for the rest of this experiment. In addition, this culture reached the lowest optical density and also lysed one day later compared to the other two cultures.

The *N. magadii* culture L11 (pRo5/mev-ORF49/49´) grew to an OD_{600} of 0.82, before lysis took place on day 4, which is the same day like the evaluated construct did. In this case lysis took place exclusively on day 4, after this onetime decrease in optical density of about 0.4 OD_{600} values the optical density stabilized at around 0.35 until the end of this measurement.

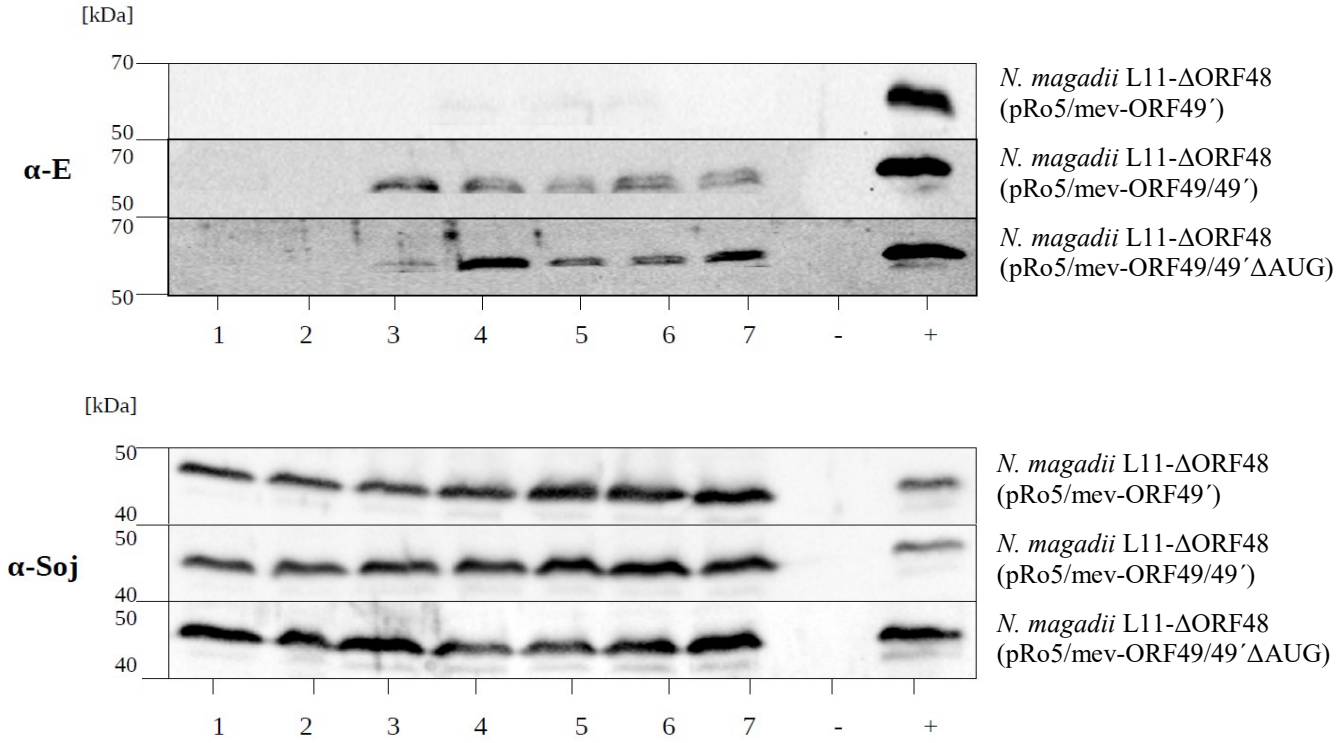


Figure 13: Western Blots analysis of the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG), L11 (pRo5/mev-ORF49/49´) and L11 (pRo5/mev-ORF49´),infected with the ΔORF48 strain of φCh1. Western blots using antibodies against the major capsid protein E (upper panel) and protein Soj (lower panel) Lane 1-7= protein samples obtained from the cultures on day1 to 7; positive control= *N. magadii* L11 sample; negative control= *N. magadii* L13 sample

Figure 13 displays the results of the Western blot analysis obtained from the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG), L11 (pRo5/mev-ORF49/49´) and L11 (pRo5/mev-ORF49´), infected with the ΔORF48 strain of φCh1. During Western blotting primary antibodies

came into use, which targeted protein E, one of the major capsid proteins of φCh1, as well as protein Soj, which is constitutively expressed and is thought to be involved in plasmid partitioning. The protein crude extracts, which were applied to the blots, were taken each day next to the OD_{600} measurement.

The Western blots resulting from the usage of antibodies against protein Soj, displayed in the lower panel of Figure 13, show a homogeneous signal in the samples from all three cultures on each day of this measurement. This verifies, that the same amount of sample has been applied to the blots, and therefore ensures that the protein E Western blots reflect the actual amount of protein E, which results from the interplay of φCh1 with its host.

The *N. magadii* culture L11 (pRo5/mev-ORF49/49´ΔAUG), infected with the ΔORF48 deletion mutant of φCh1, shows a strong signal for protein E on day 4. This is in line with the observation made during growth curve analysis, where a decrease in optical density was observed on this day too. On day 3, one day before lysis started, just a faint signal was detectable. On day 5, 6 and 7 the signal appears weaker than on day 4. This is in line with the observed OD_{600} values, which decreased between day 4 and 7 due to lysis.

In the samples taken from the *N. magadii* L11 (pRo5/mev-ORF49/49´) culture a signal for protein E got detected on days 3 to 7. Nevertheless, these signals are much fainter compared to those on the Western blot obtained from the *N. magadii*- L11-ΔORF48 (pRo5/mev-ORF49/49ΔAUG) culture. Only one day in advance of the singular lysis event on day 3 the protein E signal was slightly more intense than on the other days. The detection of these weak signals for protein E in Western blots obtained from the protein crude extracts of this culture are in line with the observations made during the growth curve analysis of this culture, where a decelerated lysis rate was apparent.

In the Western blot obtained from the *N. magadii* culture L11 (pRo5/mev-ORF49´) infected with the ΔORF48 deletion mutant of φCh1, the signal for protein E is just hardly detectable. Only on day 4 and 5 a faint shadow is visible, which would refer to the presence of protein E. Nevertheless, this Western blot is except the positive control more or less empty, which let assume that no real lysis event has taken place within this culture. The Western blot against protein Soj proofs that this weak signal against protein E is not artificial, but is caused by suppressed gene expression. This observation is compliant with the growth curve analysis, where also only a small decrease in optical density has been measured.

To further evaluate the growth properties of these three cultures the plaque forming units were determined via plaque assay. The results of this virus titer analysis are displayed in Figure 14. The samples for the virus titer determination were prepared on day 4, 5 and 6 of this experiment, where all three cultures lysed.

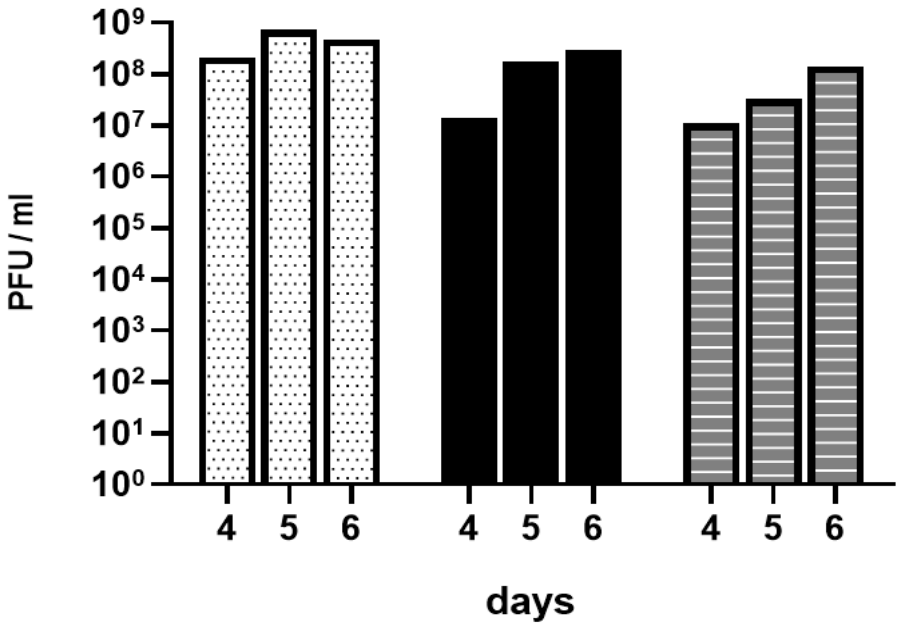


Figure 14: Virus titer analysis of the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG) (highlighted with points), L11 (pRo5/mev-ORF49/49´) (marked in black) and L11 (pRo5/mev-ORF49´)(labeled with lines) infected with the φCh1-ΔORF48 strain. The Plaque assays were incubated for one week at 37°C, before plaques were counted to determine the plaque forming unit per milliliter.

The L11 (*N. magadii*-pRo5/mev-ORF49/49´ΔAUG) culture infected with the ΔORF48 viral strain, produces the most viral particles, which meets expectations with the previous evaluations of the lysis behavior of this culture by growth curve and Western blot analysis. Plaques of this culture appear as frizzled and middle big in size. On day 4 the PFU of this culture reached about $2.1*10^{8,}$ plaques per ml, where the *N. magadii* culture L11 (pRo5/mev-ORF49/49´) only reached a PFU of about $1.4*10^{7}$.

Plaques, which arose from the supernatant of this culture, are also frizzled, but smaller than those of the *N. magadii* culture L11 (pRo5/mev-ORF49/49´ΔAUG), infected with the ΔORF48 deletion mutant (data not shown). The plaque forming unit for this day, derived from the L11 (pRo5/mev-ORF49´) culture, ranges with $1.1*10^{7}$ in the same order of magnitude as the *N. magadii* L11 (pRo5/mev-ORF49/49´) culture, but both cultures stay behind one order of magnitude compared to the value obtained from the L11-ΔORF48 (pRo5/mev-ORF49/49´ΔAUG) culture. In contrast to the other two *N. magadii* cultures the plaques of L11 (pRo5/mev-ORF49´) appear as small and round. Also on day 5, where lysis took place, the L11 (pRo5/mev-ORF49/49´ΔAUG) culture appears to have the highest PFU/ml of $7.3*10^{8}$, the value of the L11 (*N. magadii*-pRo5/mev-ORF49/49´) is lower but has the same order of magnitude with $1.8*10^{8}$.

Again, the PFU value of the *N. magadii* L11-ΔORF48 (pRo5/mev-ORF49´) culture is clearly lower with $3.24*10^7$ compared to the other two examined cultures. On day 6 of this experiment the PFU value of the *N. magadii* L11 (pRo5/mev-ORF49´) culture increased to $1.4*10^8$, but the value stayed below the values obtained from the other two cultures. Again, the culture *N. magadii* L11 (pRo5/mev-ORF49/49´ΔAUG) has the highest plaque forming unit of $4.7*10^8$ PFU/ml. The value for the *N. magadii* L11 (pRo5/mev-ORF49/49´) has the same order of magnitude but is slightly lower with $3*10^8$ PFU/ml.The obtained virus titers are in line with the observations taken from the western blot analysis and the growth curves, cause the *N. magadii* L11 (pRo5/mev-ORF49/49´ΔAUG), which lysed most sharply, produced the most virus particles, whereas the culture *N. magadii* L11-ΔORF48 (pRo5/mev-ORF49´), which lysed only slightly, produced much less plaques.

Discussion

In the previously discussed experiment ORF48 was absent within the *N. magadii* L11-ΔORF48 (pRo5/mev) cultures, nevertheless ORF49, which is thought to be a transcription factor involved in the expression of lytic genes is still present, and is acting on the lysis behaviors of the three examined cultures.

The *N. magadii* culture L11 (pRo5/mev-ORF49/49´ΔAUG), infected with the ΔORF48 deletion strain of φCh1, lysed in a sharp and proper manner. Despite this culture has not lysed completely, the observation taken from growth curve, Western blot and virus titer analysis let assume, that in the φCh1 variant, which has infected this culture, the lytic genes are not repressed by ORF48 and also not indirectly repressed by the gene product of ORF49´, which results in a normal lysis behavior.

In the control strain *N. magadii* L11 (pRo5-ORF49/49´), even though ORF48 is not repressing lytic genes, the expression of lytic genes appear to be affected in a suppressive manner due to the fact that this culture lysed just on one single day and the protein E signal was detected only weakly in Western blot, compared to the culture carrying the construct. However, this culture lysed as well on day 4, like the *N. magadii* L11 (pRo5/mev-ORF49/49´ΔAUG) and the *N. magadii* L11-ΔORF48 (see Fig. 4) cultures did.

Interestingly the second control strain *N. magadii* L11 (pRo5/mev-ORF49´), where more or less no lysis took place, when ORF49´ was over expressed, produced just around one order of magnitude less plaques as the other two strains. The lysis behavior of this culture seems to be completely decelerated, there is only a pour decrease in optical density visible in growth curve analysis and there is no detectable signal against the major capsid protein E in Western blot. This observations would be in line with the assumption that gp49´ might affect lysis in a suppressive manner by hindering the activation of lytic genes by ORF49.

How the deletion of AUG in ORF49´ influences the lysis behaviors of the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG), L11 (pRo5/mev-ORF49/49´) and L11 (pRo5/mev-ORF49´), infected with the ORF49 deletion strain of φCh1

In this section the results obtained from the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG), L11 (pRo5/mev-ORF49/49´) and L11 (pRo5/mev-ORF49´), which were infected with the ΔORF49 deletion strain of φCh1. Therefore within this viral strain the introduced deletion of the start codon in ORF49´ is not complemented by ORF49, nevertheless ORF48 is still present, and had influenced the lysis behavior of the examined cultures.

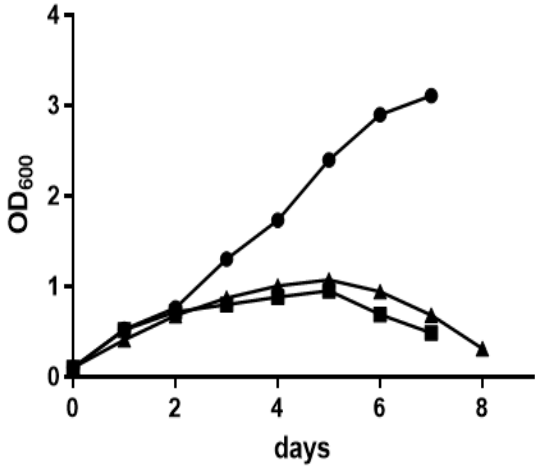


Figure 15: Growth curve analysis of the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG) (marked by triangles), L11 (pRo5/mev-ORF49/49´) (labeled with circles) and L11 (pRo5/mev-ORF49´) (indicated with squares), infected with φCh1-ΔORF49. The optical density was measured at 600 nm each day at a distinct time point. All cultures were inoculated to a start OD_{600} of 0.1 and grew at 37°C under agitation.

In Figure 15 the growth curves are displayed, which relay on the daily determination of the optical density. Next to this measurement also samples for SDS- PAGE (data not shown) and Western Blot got prepared.

The *N. magadii* culture L11 (pRo5/mev-ORF49/49´ΔAUG), which got infected with φCh1-ΔORF49, reached an OD_{600} value of about 1.1, before lysis started on day 6, with a slight decrease in optical density to OD_{600} = 0.94. The optical density decreased further the day after to OD_{600}=0.68, before the value eventually dropped to OD_{600}= 0.31 on the last day of this measurement.

The *N. magadii* culture L11 (pRo5/mev-ORF49´) behaved in a quite similar way like the culture, which harbors the virus with the deleted start codon in ORF49´. Also this culture took 5

days of growth until it reached an OD_{600} of 0.95, before the value decreased the day after to OD_{600}= 0.70, which is a higher decrease than observed in the *N. magadii* L11 (pRo5/mev-ORF49/49´ΔAUG) culture on the first day of lysis. Eventually, on day 7 the optical density has decreased to OD_{600}=0.48.

The *N. magadii* culture L11 (pRo5/mev-ORF49/49´) infected with the ΔORF49 strain of φCh1, grew most dense of all three examined cultures and reached an optical density of OD_{600}= 3.1 on day 7 of this measurement. This culture showed no sign of lysis, because no decrease in optical density got detected, and seems to grow unaffected by the infection of the φCh1 variant, which carries the ORF49/49´ duplication.

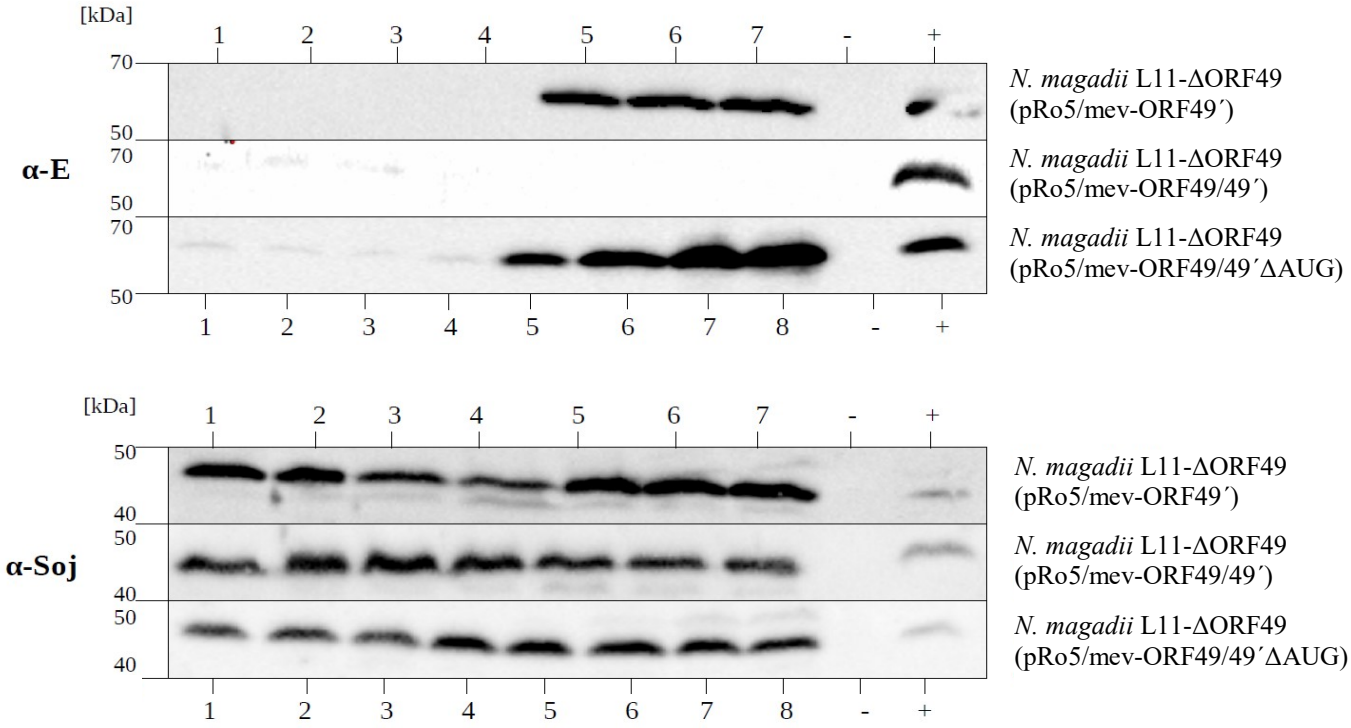


Figure 16: Western blot comparison of the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG), L11 (pRo5/mev-ORF49/49´) and L11 (pRo5/mev-ORF49´), infected with the ORF49 deletion strain of φCh1. The lower panel displays the Western blots, in which primary antibodies against protein Soj came into use, the upper panel shows the Western blots were primary antibodies against protein E were used. Samples taken from day 1-7, got applied to lanes 1-7; negative control= *N. magadii* L13 sample; positive control= *N. magadii* L11 sample

Next to the growth curve analysis, Western blots have been performed, to follow the lysis behavior of the three pRo5/mev cultures, infected with ΔORF49 φCh1, along with the protein levels

of the viral proteins E and Soj. Those Western blots are displayed in Figure 16. To see the complete lysis cycle of the *N. magadii* culture L11 (pRo5/mev-ORF49/49´ΔAUG), from this culture an additional protein sample was prepared on day 8. The Western blots, which got obtained with primary antibodies against protein Soj, show a signal, with constant intensity for all samples taken on each day from the three cultures during this measurement. This ensures that all samples have been applied in an equal amount to the Western blots, and therefore the protein E signal is not artificially influenced.

In samples from he *N. magadii* culture L11 (pRo5/mev-ORF49/49´ΔAUG), which was infected with the ΔORF49 strain of φCh1, on the first 4 days of this experiment only a very weak signal for protein E was detectable. On day 5 a clear signal is visible on the blot, this is one day before a decrease in optical density could have been determined. The signal for protein E increased in intensity on day 6, further on day 7 and appeared strongest on the last day of this experiment. So it is apparent, that in samples taken from this culture the signal for protein E and the lysis rate increased during the course of the last three days of this experiment.

Also the *N. magadii* culture L11 (pRo5/mev-ORF49´), which appeared to have a quite similar lysis behavior like the culture carrying the construct, according to growth curve analysis, shows the first signal for protein E on day 5 of this experiment, also one day in advance of the first detected decrease in optical density. However in the samples obtained from this culture the protein E signal stayed stable on day 6 and 7, and therefore no increase in protein E levels got observed, like in the *N. magadii* L11-ΔORF49 (pRo5/mev-ORF49/49´ΔAUG). Also contrary, here no faint signal for protein E was detectable on day 1 to 4.

In contrast to these two cultures, which clearly lysed according to growth curve and Western blot analysis, it was not possible to detect a signal for protein E in samples obtained from the *N. magadii* L11-ΔORF49 (pRo5/mev-ORF49/49´) and therefore it can be assumed that this culture has not produced virus particles. However the fact that the protein E blot for this culture appears, except the positive control, as empty, is in line with the previous growth curve analysis, where no decrease in optical density got observed. That the samples have not been degraded and that the culture is infected with φCh1 is ensured by the Western blot against protein Soj, which has been performed with the same samples, and where a clear signals for protein Soj was detectable.

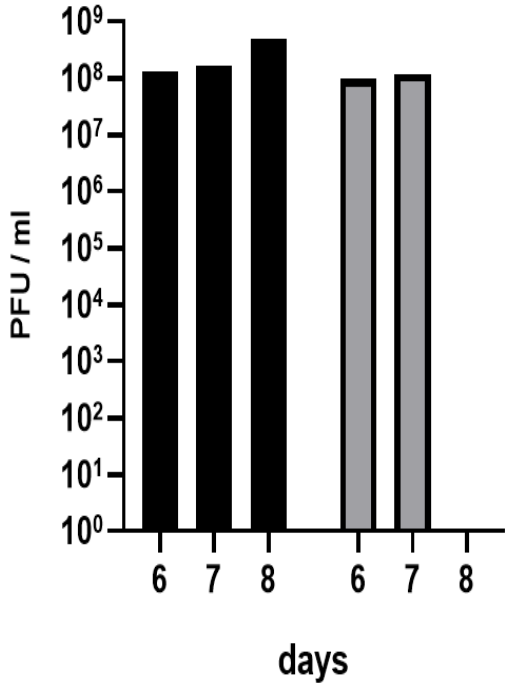


Figure 17: Virus titer comparison of the *N. magadii* culture L11 (pRo5/mev-ORF49/49´ΔAUG) (black bars) and *N. magadii* L11 (pRo5/mev-ORF49´) (gray bars), infected with the ORF49 deletion strain of φCh1. The plaque forming unit was determined via plaque assay using the supernatants of the lysing cultures to infect *N. magadii* L13 cells. The plaque assays were incubated for 7 days on 37°C, before the plaques were counted and the titers were determined.

In addition to the growth curve determination and Western blot analysis, virus titers were determined via plaque assay. The obtained virus titers of the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG) and L11 (pRo5/mev-ORF49´), infected with φCh1-ΔORF49 are displayed in Figure 17. Due to the fact that the culture *N. magadii* L11 (pRo5/mev-ORF49/49´) has not lysed, no virus titer was determined for this culture. The samples from which the virus titers got obtained, were taken from the *N. magadii* L11 (pRo5/mev-ORF49´) culture on day 6 and 7 of this experiment, because on this two days this culture lysed. In case of the *N. magadii* L11-ΔORF49 (pRo5/mev-ORF49/49´ΔAUG) culture an additional sample on day 8 got prepared.

On the first day, on which the culture with the deleted Start codon in ORF49´ lysed, the supernatant forms $1.3*10^8$ plaques per milliliter, the PFU value than increased slightly on the second day of lysis to a plaque forming unit of $1.7*10^8$, eventually on the last day of this experiment the PFU value for the *N. magadii*-L11-ΔORF49 (pRo5/mev-ORF49/49´ΔAUG) culture increased significantly to $5*10^8$ plaques per milliliter. Therefore also in the virus titer comparison an increase in virus particle formation is observed for this culture.

In contrast the control strain *N. magadii* L11-ΔORF49 (pRo5/mev-ORF49´) showed a PFU of $9.88*10^7$ on day 6. The plaque forming unit has not increased in a significant amount for this

culture on the following day, therefore no clear increase in the number of formed plaques could have been determined here like for the *N. magadii*-L11-ΔORF49 (pRo5/mev-ORF49/49´ΔAUG) strain. Also this observation is in line with the previous results obtained from the Western blot analysis, where also an increase in lysis rate was observed.

Discussion

Again, like in the comparison of the pRo5/mev containing *N. magadii* cultures, which were infected with wild-type φCh1, the growth curves of *N. magadii* L11 (pRo5/mev-ORF49/49´ΔAUG) and L11 (pRo5/mev-ORF49´), infected with ΔORF49 φCh1, appear to be quite similar in shape, nevertheless the further examination via Western blot and virus titer analysis, showed that the production of viral particles clearly increased throughout the lysis cycle of *N. magadii* L11-ΔORF49 (pRo5/mev-ORF49/49´ΔAUG), whereas the signal for protein E and the obtained PFU values for the *N. magadii* L11-ΔORF49 (pRo5/mev-ORF49´) culture stayed constant. Both cultures started to lyse on day 6 and appear to have a suppressed lysis behavior.

In contrast, the culture *N. magadii* L11-ΔORF49 (pRo5/mev-ORF49/49´) showed no sign of lysis neither in growth curve nor in Western Blot analysis. This observation, that, if ORF49/49´ is expressed in the absence of ORF49, lysis does not take place in this *N. magadii* culture, leads to the assumption, that the gene product of ORF49´ hinders ORF49 to activate lytic genes, while ORF48, which is present in this setup, represses lytic genes, this combination than results in complete lysogeny. The fact that in the second control strain as well as in the *N. magadii* L11-ΔORF49 (pRo5/mev-ORF49/49´ΔAUG) culture lysis took place, although apparently in a suppressed manner, let suggest that the mechanism of action of gp49´ might relies on the presence of ORF49, even though the increase in virus particle formation in the *N. magadii* L11-ΔORF49 *(*pRo5/mev-ORF49/49´ΔAUG) culture would give a hint, that lytic genes get more proper expressed than in the control strain *N. magadii* L11-ΔORF49 *(*pRo5/mev-ORF49´).

Nevertheless this seemingly minor difference and the fact that *N. magadii* L11 (pRo5/mev-ORF49/49´ΔAUG) behaves exact contrary to the culture *N. magadii* L11 (pRo5/mev-ORF49/49´), gives an additional evidence, that ORF49´ might be translated into protein and have an impact on gene expression of φCh1.

Examining the deletion of AUG in ORF49´ on the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG), L11 (pRo5/mev-ORF49/49´) and L11 (pRo5/mev-ORF49´) infected with the ΔORF48/ORF49 strain of φCh1

This chapter displays the results, which were obtained from the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG), L11 (pRo5/mev-ORF49/49´) and L11 (pRo5/mev-ORF49´), which were infected with a φCh1 strain, that harbors a double deletion of ORF48 and ORF49. Therefore, in this strain the main players in the regulation of lysis and lysogeny in φCh1 are missing, and the effect of the deleted start codon in ORF49´ is neither complemented by ORF48 nor by ORF49. This means that the following section will give the most meaningful statement about the effect of the introduced mutation on the lysis behavior of φCh1.

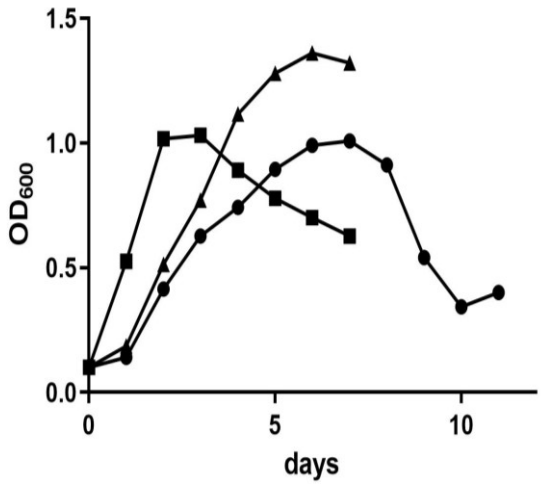


Figure 18: Growth curve analysis of the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG) (marked with circles), L11 (pRo5/mev-ORF49/49´)(labeled with triangles) and L11 (pRo5/mev-ORF49´) (highlighted by squares) infected with ΔORF48/49 deletion strain of φCh1. The growth curves rely on the optical density values, which were measured at 600nm each day at a certain time point. All three cultures were grown at 37°C with agitation, after they were inoculated to start OD_{600} of 0.1.

In Figure 18 the growth properties of the pRo5/mev containing cultures, which got infected with the ΔORF48/49 double mutant of φCh1, are displayed. The *N. magadii* culture L11-ΔORF48/49 (pRo5/mev-ORF49/49´ΔAUG) grew in seven days just up to an OD_{600} of about 1, before it started to lyse on the eight day to an OD_{600} of 0.91. The OD_{600} value decreased further on the following day to OD_{600}= 0.54 and dropped eventually down due to lysis to an OD_{600} of 0.34 on

day 10 of this experiment. The optical density than stabilized again with a slight increase on the eleventh day of this experiment.

The *N. magadii* culture L11-ΔORF48/49 (pRo5/mev-ORF49/49´) reached the highest optical density of the cultures evaluated in this section of OD_{600} =1.36, which is still a rather low OD_{600} value compared to wild-type *N. magadii* L11, however all cultures, which are infected with the ΔORF48/49 deletion strain of φCh1 typically appear to possess deficits in growth. Nevertheless, this culture showed no sign of lysis according to growth curve analysis.

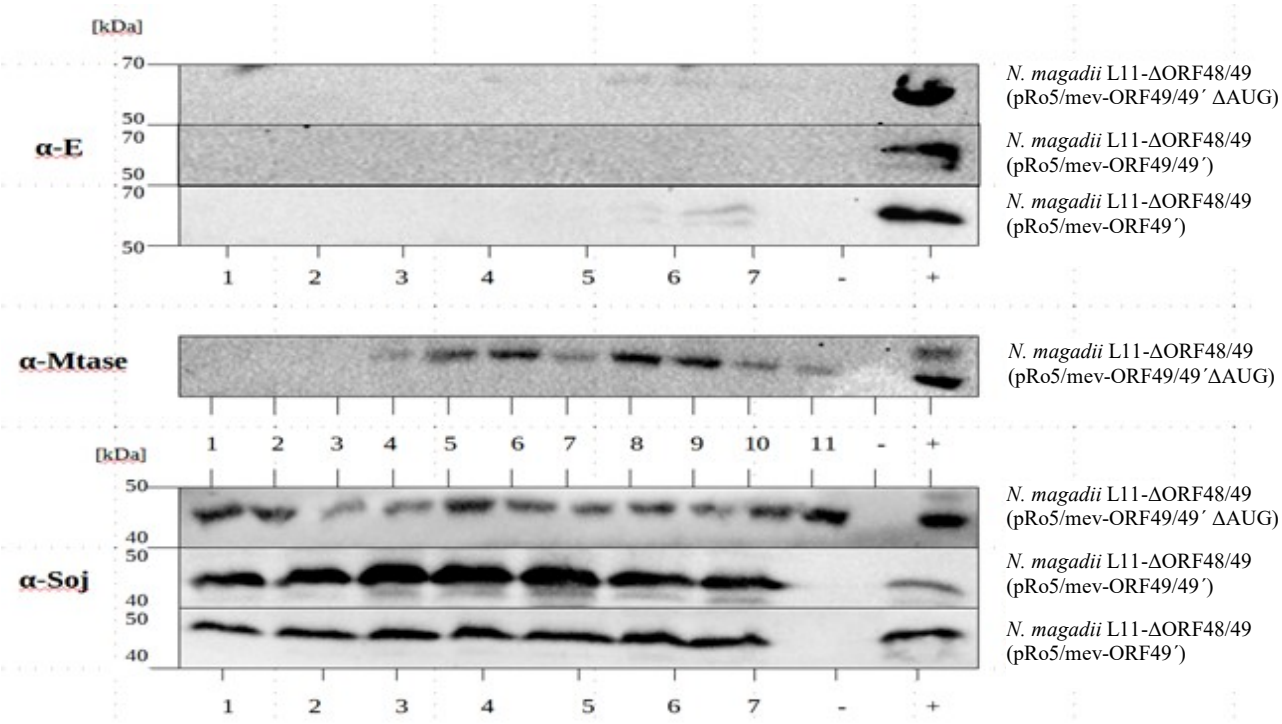


Figure 19: Western blot analysis of the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG), L11 (pRo5/mev-ORF49/49´) and L11 (pRo5/mev-ORF49´), infected with the ΔORF48/49 deletion strain of φCh1. To obtain these blots primary antibodies against the φCh1 proteins E (see upper panel), Mtase (middle panel) and protein Soj (lower panel) got used. As positive control an *N. magadii* L11 protein sample was used, as negative control served an *N. magadii* L13 sample. Protein extracts collected from day 1-7 or 1-11 were applied to the corresponding lanes.

The *N. magadii* L11 culture carrying the plasmid pRo5/mev-ORF49´ reached within for days of growth an optical density of OD_{600}= 1.03. The measured OD_{600} values decreased than during the following day slightly to an OD_{600} of 0.89, and decreased continuously further the day after to OD_{600}= 0.78. On the last day of this experiment the OD_{600} value dropped eventually to OD_{600}= 0.63.

To sum up, the *N. magadii* L11-ΔORF48/49 (pRo5/mev-ORF49/49´ΔAUG) culture lysed late but with a significant decrease in optical density, whereas the *N. magadii* L11-ΔORF48/49 *(*pRo5/mev-ORF49/49´) did not lyse at all, and the *N. magadii* L11-ΔORF48/49 (pRo5/mev-ORF49´) culture lysed early. but apparently in a quite suppressed manner.

The results of the Western blot analysis of the *N.magadii* L11 (pRo5/mev) cultures, infected with the ΔORF48/49 strain of φCh1, are displayed in Figure 19. Those blots got obtained to detect the φCh1 proteins major capsid protein E, the main methyltransferase, and protein Soj, which is involved in plasmid partitioning. All blots, for which primary antibodies against Soj came into use, show a constant signal on each day of this experiment, for all three examined cultures, from which samples were taken. The detection of this viral protein Soj ensures, that indeed a viral infection is present in the culture as well as that the protein crude extracts, which got used to achieve the Western blots have not been degraded.

The *N. magadii* culture L11 (pRo5/mev-ORF49/49´ΔAUG), infected with the ΔORF48/49 variant of φCh1, appear to have no clear signal for protein E in western blot, only a very weak shade is apparent on day 8 and 9 of this measurement. Nevertheless, for this culture an additional Western blot has been performed, which aimed to detect the main methyltransferase Mtase. This blot showed a clear signal for Mtase, which started on day 4, and increased until day 6, before it decreased in intensity on day 7. Eventually the signal again intensified on day 8 and 9 of this measurement, before the signal then decreased on the last two days.

The blots against protein E obtained from the samples taken from the *N. magadii* culture L11 (pRo5/mev-ORF49/49´), which got infected with the double mutant, show no signal for protein E at all, despite a signal on the Soj Western blots is clearly detectable. This observation from Western blot is in line with the fact that this culture showed no sign of lysis during the previous discussed OD_{600} measurement.

Also the protein E western blots of the *N. magadii* L11 (pRo5/mev-ORF49´) culture infected with the ΔORF48/49 stain of φCh1, showed no clear signal on the first 4 days of this evaluation. Nevertheless it was possible to detect a faint signal for protein E on day 6 and 7. This result is not in line with the observed decrease in optical density on day 4 visible in growth curve analysis.

In addition to the previous discussed growth curve and Western blot analysis, the lysis behaviors of *N. magadii* L11 (pRo5/mev-ORF49/49´ΔAUG) and L11 (pRo5/mev-ORF49´), which were infected with the ΔORF48/49 deletion strain of φCh1, were also followed by the determination of virus titers via plaque assay.

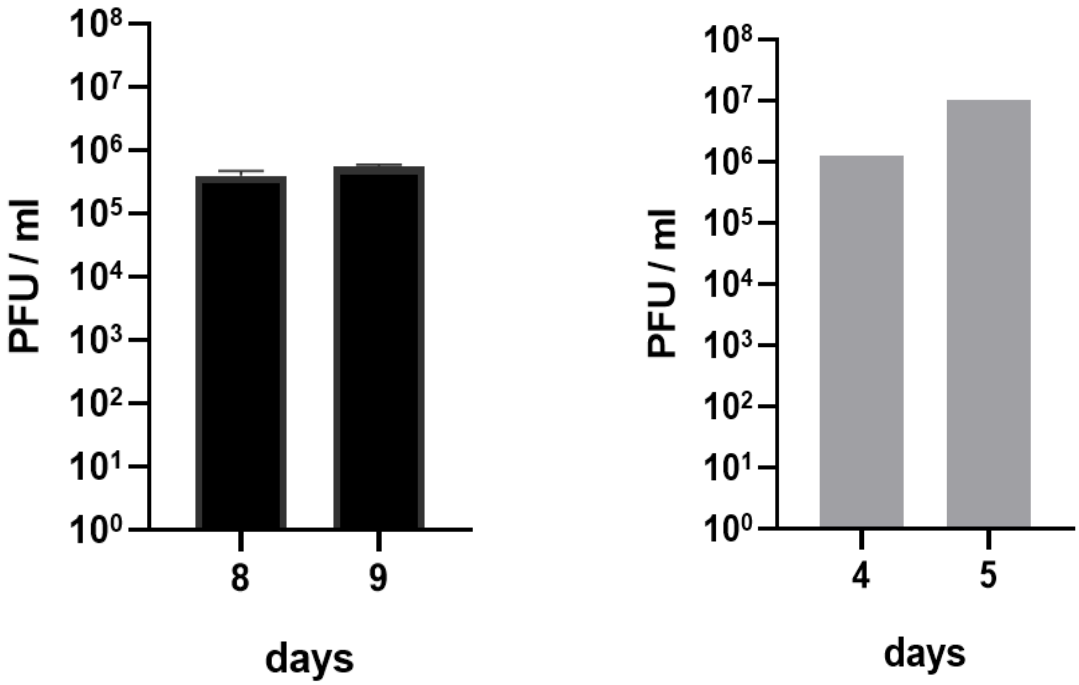


Figure 20: Virus titer comparison of the *N. magadii* cultures L11 (pRo5/mev-ORF49/49´ΔAUG) (labeled in black) and L11 (pRo5/mev-ORF49´) (marked in gray) infected with the ΔORF48/49 φCh1 deletion strain. The plaque assays were incubated at 37°C for one week, before the plaques got counted to determine the plaque forming unit. Error bars indicate SEM.

Samples, to achieve these titers, were taken from *N. magadii* L11-ΔORF48/49 (pRo5/mev-ORF49´) on day 4 and 5 (see Figure 18) and from the *N. magadii* culture L11-ΔORF48/49 (pRo5/mev-ORF49/49´ΔAUG) on the day 8 and 9 of this experiment. The resulting virus titers are displayed in Figure 20. On day 8, the first day, on which the culture *N. magadii* L11-ΔORF48/49 (pRo5/mev-ORF49/49´ΔAUG) lysed, the plaque forming unit was determined as $4*10^5$ PFU/ml. This value increased slightly on the following day to $5.5*10^5$ PFU/ml. This values are lower than the virus titers obtained from the *N. magadii* L11 (pRo5/mev-ORF49´) culture, which was infected with ΔORF48/49 φCh1. Namely, here the PFU value on the first day of lysis, namely day 4, was determined as $1.3*10^6$ PFU/ml. The virus titer for the second day, on which this culture lysed is even higher with a PFU/ml of $1.1*10^7$.

The plaque forming unit of the *N. magadii* culture L11 (pRo5/mev-ORF49/49´), infected with the ΔORF48/49 deletion mutant, was not determined, due to the fact that this culture showed no sign of lysis neither in growth curve nor in Western blot analyses.

Discussion

Due to the lack of ORF48 and ORF49 in the mutant φCh1-ΔORF48/49 strain, which was used to infect the here examined *N. magadii* cultures, the introduced mutation in ORF49´ is not complemented and therefore its effect on the lysis behavior of the infected culture should be here most apparent.

The *N. magadii* culture L11 (pRo5/mev-ORF49/49´ΔAUG) infected with φCh1-ΔORF48/49, lysed on day 8, which is much later than *N. magadii* L11 (pRo5/mev-ORF49´) culture, where lysis started on day 4. In contrast optical density values of the *N. magadii* culture L11-ΔORF48/49 (pRo5/mev-ORF49/49´) have not decreased within seven days of measurement.

On the Western blot against protein E only on day 8 and 9 a weak signal was detectable in the protein samples of *N. magadii* L11-ΔORF48/49 (pRo5/mev-ORF49/49´ΔAUG), nevertheless, when antibodies against Mtase was used a clear signal was obvious, which accompanied the measured decrease in optical density. The Western blot of the culture *N. magadii*-L11 (pRo5/mev-ORF49´), infected with φCh1-ΔORF48/49 also shows just a weak signal for protein E on day 7, whereas the Western blot against protein E obtained from the *N. magadii* culture L11-ΔORF48/49 (pRo5/mev-ORF49/49´) appears to be completely empty.

The virus titers obtained from the *N. magadii* culture L11-ΔORF48/49 (pRo5/mev-ORF49/49´ΔAUG) is rather low compared to the values of the control strain *N. magadii* L11-ΔORF48/49 (pRo5/mev-ORF49´, which lay at least one order of magnitude higher.

These results let assume, that lytic genes are not proper expressed in *N. magadii* L11-ΔORF48/49 *(*pRo5/mev-ORF49/49´), and therefore no lysis takes place due to the proposed action of gp49´ on the lysis promoting transcription factor ORF49. In the culture, which carries the introduced mutation in the start codon of ORF49´, lytic genes seem to get expressed even though delayed, due to the presence of ORF49, which results in complete lysis. gp49´ might act itself as transcription factor if expressed alone but seems to be able to hinder also the action of gp49. The *N. magadii* culture L11-ΔORF48/49 (pRo5/mev-ORF49´), therefore favored early lysis, which is also visible in detected the increase in the plaque forming units.

Verification of the two *N. magadii* cultures L11 (pNB102-ORF49/49´ΔAUG) and L11 (pNB102-ORF49/49´)

In this section the PCR gels are displayed, which verify the genetic identity of the *N. magadii* cultures L11 (pNB102-ORF49/49´ΔAUG) and L11 (pNB102-ORF49/49´). In case of the construct pNB102-ORF49/49´ΔAUG, which was cloned during this study, the agarose gel after transformation into *N. magadii* L13 cells is depicted in Figure 21A. For the control strain *N. magadii* L11 *(*pNB102-ORF49/49´), which was created in the course of the former Master thesis of Dina Sabic (Dina A. Sabic; „The regulation of gene expression in haloalkaliphilic virus

φCh1: further characterization of the role of *rep* and ORF49."; Vienna, 2019; page 75), the agarose gel of the PCR reaction, which proofs the presence of ORF49/49´, is displayed in Figure 21B.

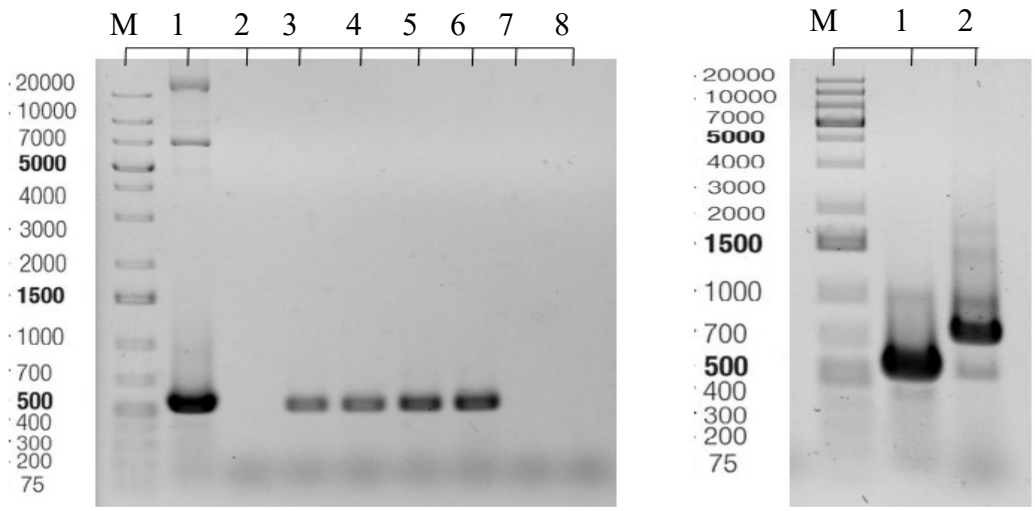


Figure 21: A) PCR gel to verify the transformation of pNB102-ORF49/49´ΔAUG into *N. magadii* L13 cells*:* M= Marker gene ruler 1kb plus, Lane 1= positive control (=*N. magadii* L11-1 template DNA), Lane 2= negative control (=empty vector pNB102), Lane 3 – Lane 8 template DNA of *N. magadii* L13 *(*pNB102–ORF49/49´ΔAUG) transformants.

B) Analytical PCR gel to verify the genetic identity of *N. magadii*-L13 (pNB102-ORF49/49´): M= Marker gene ruler 1kb plus, Lane 1= negative control (= *N. magadii* L11 template DNA), Lane 2= DNA template of *N. magadii* L13 (pNB102-ORF49/49´)

In addition the results of the ORF56 PCR reaction, which proofs the absence of φCh1 within the *N. magadii* L13 (pNB102-ORF49/49´ΔAUG) are displayed in Figure 22 as well as the same PCR reaction for both cultures after plaque assay, to verify the infection with the different ORF48 and ORF49 deletion strains of φCh1.

The PCR reaction, which came into use to screen for positive L13 (pNB102-ORF49/49´ΔAUG) transformants, used the same primers, like for cloning the mutated ORF49/49´, into the plasmid pNB102, namely the forward primer 49-*Kpn* and the reverse primer 49-XB. With this primer set it was possible to obtain the same PCR product like from the template DNA of the positive control, with a size of 561 bp. This DNA fragment got detected within the template DNAs of the checked transformants 3, 4, 5 and 6. This result proofs the success of the transformation of pNB102-ORF49/49´ΔAUG into *N. magadii* L13 cells, therefore this cells were used to perform the further experiments.

But before the transformed *N. magadii* L13 cells got infected with the different ORF48 and ORF49 deletion strains via plaque assay, an additional PCR reaction to detect ORF56 was performed to verify the absence of φCh1.

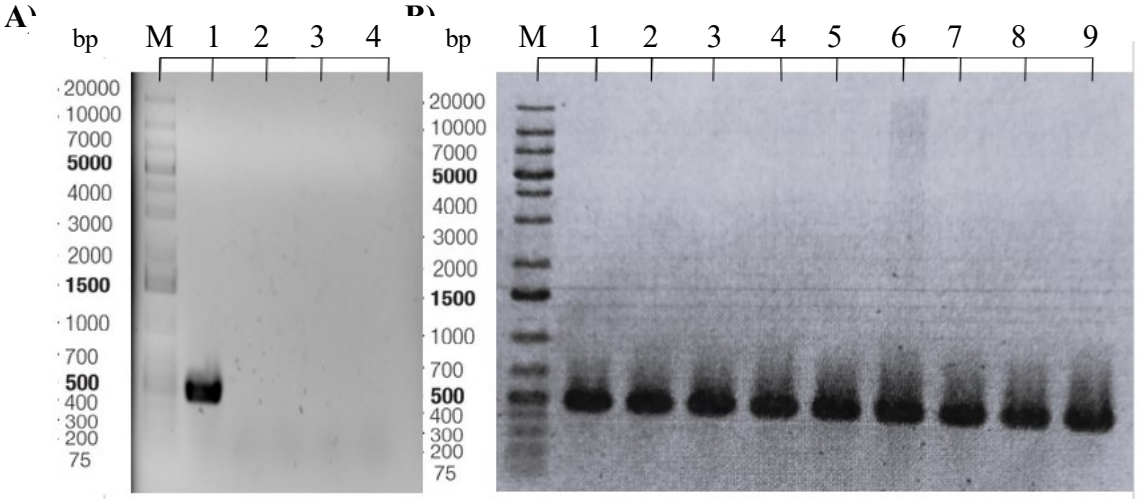


Figure 22: A) ORF56 PCR to detect absence of φCh1: M= Marker gene ruler 1kB plus, Lane 1= positive control (=*N. magadii* L11 template DNA), Lane 2 – 4= *N. magadii* L13 (pNB102-ORF49/49´ΔAUG) templates

B) ORF56 PCR to detect presence of φCh1: M= Marker gene ruler 1kB plus, Lane 1= positive controll (= *N. magadii* L11 template DNA), Lane 2=*N. magadii* L11 (pNB102-ORF49/49´ΔAUG) template, Lane 3= *N. magadii* L11-ΔORF49 (pNB102-ORF49/49´ΔAUG) template, Lane 4= *N. magadii* L11-ΔORF48 (pNB102-ORF49/49´ΔAUG) template, Lane 5= *N. magadii* L11-ΔORF48/49 (pNB102-ORF49/49´ΔAUG) template DNA, Lane 6= *N. magadii* L11 (pNB102-ORF49/49´) template, Lane 7= *N. magadii* L11-ΔORF49 (pNB102-ORF49/49´) template, Lane 8= *N. magadii* L11-ΔORF48 (pNB102-ORF49/49´) template, Lane 9= *N. magadii* L11-ΔORF48/49 (pNB102-ORF49/49´) template,

The agarose gel to visualize this PCR reaction is displayed in Figure 22A and shows no signal for ORF56, when the primers 56-RT5 and 56-RT3 were used, therefore this proofs, that the examined cultures were uninfected, and could be used further for infecting with a distinct φCh1 strain.

In Figure 21B a quite similar PCR reaction, like for the verification of the transformation of pNB102-ORF49/49´ΔAUG into *N. magadii* L13 cells, is displayed. This PCR reaction makes also use of the primer pair 49-*Kpn* and 49-XB, but amplifies a PCR product of 763 bp length, if pNB102-ORF49/49´ is present in the provided template DNA. As negative control template DNA, obtained from an *N. magadii* L11 culture, came into use. With this template an PCR fragment sized about 500 bp is amplified. The culture, which was examined for the presence of pNB102-ORF49/49´, has except a weak signal referring to wild-type ORF49, exclusively a strong PCR product of 763 bp, which proofs the genetic identity of this culture and therefore this culture was used to be further infected with different deletion strains of φCh1 via plaque assay.

Figure 22B displays the agarose gel of the ORF56 PCR reaction, which should ensure, that the infection of the *N. magadii* cultures L13 (pNB102-ORF49/49´ΔAUG) and L13 (pNB102-ORF49/49´) with the φCh1 strains wild-type, ΔORF48, ΔORF49 and ΔORF48/49, took place successfully. To detect the viral ORF56, and therefore φCh1, this PCR reaction uses the primer 56-RT5 and 56-RT3, which amplifies a PCR fragment of 478 bp size. *N. magadii* L11 template DNA served as positive control, each examined *N. magadii* L11 (pNB102) culture, appears to have a clear signal of 478 bp, in same intensity like the positive control. Therefore the lysis behavior of those cultures was further examined via growth curve, Western blot and virus titer analysis. The results, which got obtained from these experiments will be discussed in the following sections.

Investigation of the *N. magadii* culture L11 (pNB102-ORF49/49´ΔAUG) and the *N. magadii* culture L11 (pNB102-ORF49/49´) infected with φCh1

In this chapter the results will be illustrated, which got obtained from the *N. magadii* cultures L11 (pNB102-ORF49/49´ΔAUG) and *N. magadii* L11 (pNB102-ORF49/49´), which were infected with the wild-type strain of φCh1. The wild-type strain of φCh1, possesses ORF48 as well as ORF49, therefore the introduced mutation is in this case complemented by both. In contrast to pRo5/mev, which is a low copy plasmid, the examined open reading frames got expressed on the plasmid pNB102, which is a high-copy plasmid.

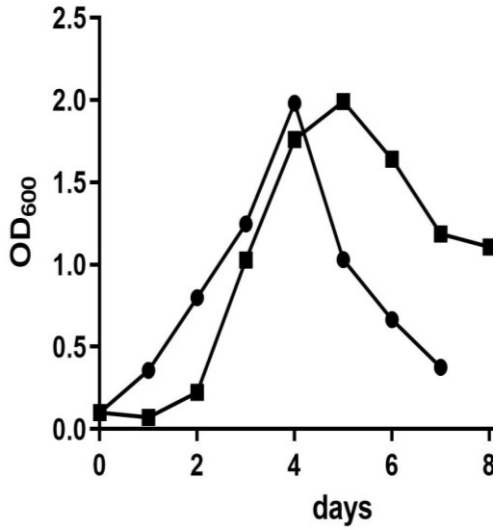


Figure 23: Growth curve analysis of the *N. magadii* cultures L11 (pNB102-ORF49/49´ΔAUG) (marked with circles) and *N. magadii* L11 (pNB102-ORF49/49´) (highlighted by squares). Both cultures were inoculated to OD_{600}= 0.1 and grew at 37°C under agitation in NVM CAA^{+} media, the optical density was determined each day at a certain time point at 600nm.

The growth properties of both *N. magadii* cultures are depicted in Figure 23. The OD_{600} values of the *N. magadii* culture L11 (pNB102-ORF49/49´ΔAUG) were determined through the course of one week, whereas growth of the *N. magadii* L11 (pNB102-ORF49/49´) culture was followed for 9 days. Each day the optical density was measured at 600 nm. To follow the growth and lysis behavior of these cultures further, in addition a protein crude extract for Western blot and supernatant of lysing cultures for virus titer determination got prepared.

The *N. magadii* culture L11 (pNB102-ORF49/49´ΔAUG), infected with wild-type φCh1, reached an optical density of OD_{600}= 1.98 after four days of growth, before lysis decreased this value the day after to an OD_{600} of 1.0. Eventually, on day 7 the optical density decreased further to

OD_{600}= 0.38. The lysis behavior of this culture seems aggressive and not suppressed, also lysis was completed within one week.

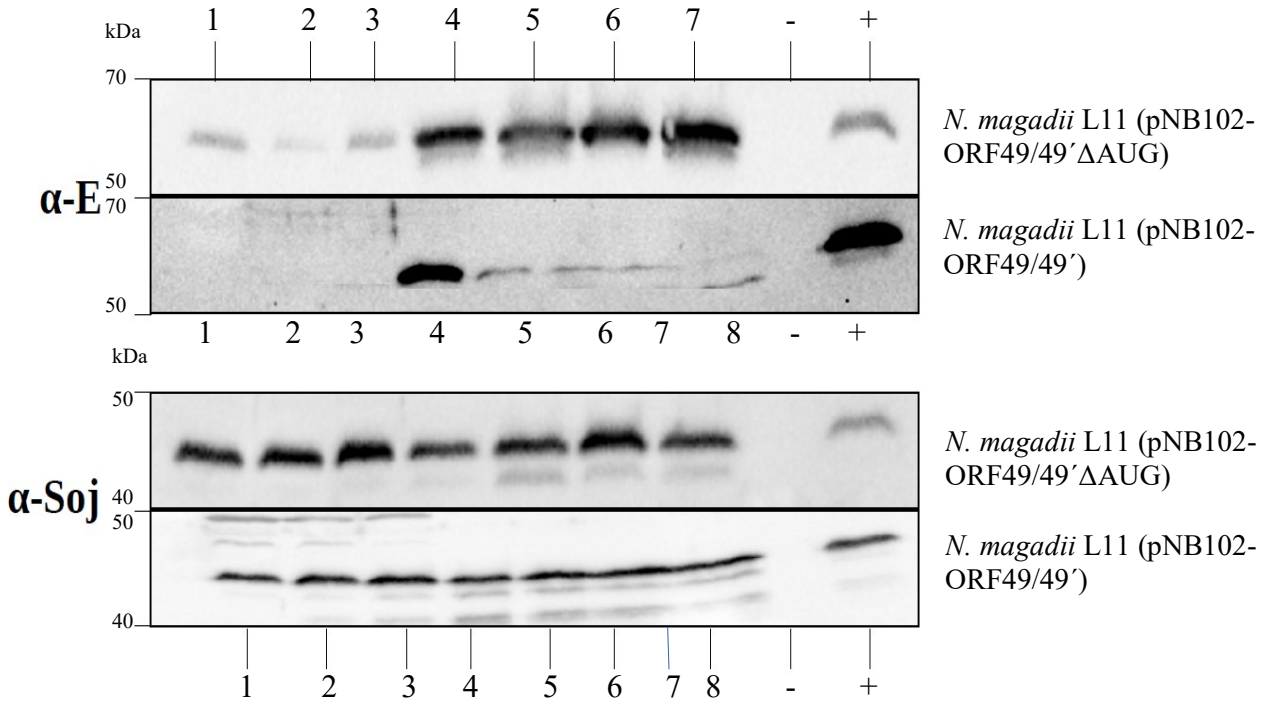


Figure 24: Western Blot analysis of the *N. magadii* cultures L11 (pNB102-ORF49/49´ΔAUG) and L11 (pNB102-ORF49/49´). The upper panel shows the Western blots obtained with the primary antibody against protein E, the lower panel depicts those made with the primary antibody against protein Soj. Into Lanes 1 to 7 the samples, taken from the cultures on day 1 to 7, were applied. As positive control an *N. magadii* L11 protein sample was used, as negative control served an *N. magadii* L13 sample.

In contrast to *N. magadii* L11 (pNB102-ORF49/49´) culture, which was infected with wild-type φCh1. This culture started to lyse on day 6, therefore one day later than the *N. magadii* L11 (pNB102-ORF49/49´ΔAUG) culture. In addition, the optical density decreased on the first day of lysis just from OD_{600}= 2.0 to OD_{600}= 1.65, in comparison the other culture lost within the same time period more or less twice as much in optical density due to lysis. Also, lysis took place less sharply, because the OD_{600} value decreased further to OD_{600}=1.2 on day 7 but decreased not further and stabilized at around OD_{600}= 1.1 on the last day of this experiment, which means lysis took place incomplete.

The fact, that the control strain lysed one day later compared to the *N. magadii* L11 (pNB102-ORF49/49´ΔAUG) culture, as well as the observation, that lysis is incomplete and less

efficient, let assume that the lysis behavior of *N. magadii* L11 (pNB102-ORF49/49´), is suppressed in a manner, which reminds to the growth properties of *N. magadii* L11-ΔORF49 cultures.

Next to the determination of OD_{600} values, protein samples for Western blot have been prepared, the resulting Western blots are depicted in Figure 24. The lower panel of Figure 24 displays the blots, in which primary antibodies against the viral protein Soj got used. With both Western blots it was possible to detect a consistent signal for Soj on each day of this experiment, which ensures that the same amount of sample has been applied to the blots, and therefore the protein E levels, which was detected on the blots of the upper panel, refer to the natural abundance of protein E within the samples taken from the *N. magadii* cultures L11 (pNB102-ORF49/49´ΔAUG) and L11 (pNB102-ORF49/49´).

Whereas the signal for protein E builds up in a successive manner within the *N. magadii* L11 (pNB102-ORF49/49´ΔAUG) culture, a clear signal for protein E is only detectable on day 4 in samples of the *N. magadii* L11 *(*pNB102-ORF49/49´) culture. On the first 3 days of the measurement the samples of *N. magadii* L11 *(*pNB102-ORF49/49´ΔAUG) show just a weak signal for protein E, whereas there is no signal detectable in the *N. magadii* L11 (pNB102-ORF49/49´) culture on these days. On day 4 the protein E signal increased in intensity. Upon this day the signal for protein E stays intense within the *N. magadii* L11 (pNB102-ORF49/49´ΔAUG) samples for the last days of this experiment, while in contrast the signal for protein E decreased on day 5 and stabilized on a weak level of intensity for the remaining days 6 and 7.

The observed increasing protein E signal in the samples, taken from *N. magadii* L11 (pNB102-ORF49/49´ΔAUG) on day 4, and the strong signal on day 5, 6 and 7 are inline with the previous observations from growth curve analysis, where this culture lost optical density rapidly on these days. In contrast the *N. magadii* L11 (pNB102-ORF49/49´) culture, appears to have a clear signal against protein E only on day 4, on the following days of this experiment the signal for protein E is only barely detectable as faint bands. Also this fits to the assumption that lysis was suppressed within this culture, which also got apparent as incomplete lysis during growth curve analysis.

In addition to Growth curves and Western blots, the plaque forming unit got determined for the *N. magadii* cultures L11 (pNB102-ORF49/49´ΔAUG) and L11 (pNB102-ORF49/49´). In Figure 25 the virus titers are displayed, which got obtained on the two initial days of lysis. In case of *N. magadii* L11 (pNB102-ORF49/49´ΔAUG) the PFU/ml is displayed from samples taken on day 5 and 6, for the culture *N. magadii* L11 (pNB102-ORF49/49´) the samples were taken on day 6 and 7.

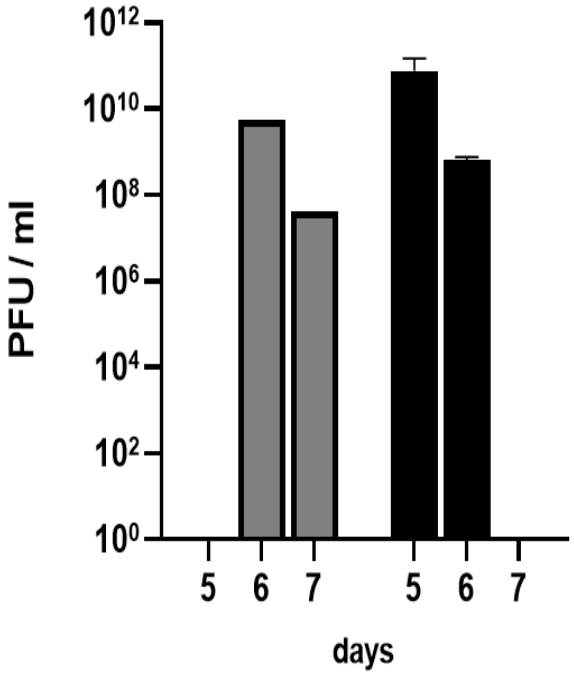


Figure 25: Virus Titer comparison of the *N. magadii* cultures L11 (pNB102-ORF49/49´ΔAUG) (marked black) and L11 (pNB102-ORF49/49´) (labeled in gray), infected with wild-type φCh1. *N. magadii* L13 cells were infected with different dilutions, of supernatants taken on two days where both cultures lysed. Plaque assays got incubated at 37° C for one week, before plaques were counted and PFU vales got determined. Error bars indicate SEM.

On the first day of lysis the *N. magadii* culture L11 (pNB102-ORF49/49´ΔAUG) formed $7.54*10^{10}$ plaques per milliliter, whereas in contrast for the control strain a PFU/ml of just $5.73*10^{9}$ plaques/ml was determined. This virus titer, therefore lays one order of magnitude lower compared to the culture carrying the introduced mutation. Despite the PFU value decreased for the L11 (pNB102-ORF49/49´ΔAUG) *N. magadii* culture on the second day of lysis to $6.5*10^{8}$ PFU/ml, it is still one orders of magnitudes higher than the plaque forming unit of the control strain *N. magadii* L11 (pNB102-ORF49/49´), which is about $4.34*10^{7}$ PFU/ml.

Discussion

The previously displayed results of the *N. magadii* cultures L11 (pNB102-ORF49/49´ΔAUG) and L11 (pNB102-ORF49/49´) made a suppressed and incomplete lysis behavior of the control strain apparent, whereas *N. magadii* L11 (pNB102-ORF49/49´ΔAUG) lysed in unaffected manner on day 5, referring to the lysis behavior, which is typically for an infection with wild-type φCh1. However in this experiment the introduced mutation is complemented by ORF49. The *N. magadii* L11 (pNB102-ORF49/49´) culture lysed on day 6, so one day later than the culture carrying the pNB102-ORF49/49´ΔAUG plasmid. Also in Western blot analysis this culture shows an intense signal against protein E upon day 4, whereas the *N. magadii* culture L11 (pNB102-ORF49/49´) has exclusively on day 4 a strong signal for protein E in Western blot.

Again, like readable from the growth curves and from the Western blot analysis, the virus titer of the *N. magadii* L11 (pNB102-ORF49/49´ΔAUG) culture lies significantly higher, than the value obtained from the control strain, even though both titers decreased on the second day of lysis. This observations refer to a normal lysis behavior, of *N. magadii*-L11 (pNB102-ORF49/49´ΔAUG), which is comparable to wild-type φCh1, whereas lysis of the control strain *N. magadii* L11 (pNB102-ORF49/49´), seems to be suppressed and delayed for one day. This would be in line with the assumption that gp49´, influences the lysis behavior of φCh1 in a suppressive manner by hindering ORF49 to activate lytic genes, which than results in delayed and incomplete lysis.

In addition this effect seems to be vanished within the *N. magadii* culture L11 (pNB102-ORF49/49´ΔAUG), where the start codon of ORF49´ was deleted via cloning, due to the fact that this culture lysed in a proper manner, which reminds to wild-type φCh1. In contrast the shape of the growth curve of the *N. magadii* L11 (pNB102-ORF49/49´) culture shows similarities to the growth curve of *N. magadii* L11-ΔORF49 (compare with Figure 4). This observation gives an additional hint to the assumed inactivation of ORF49 by gp49´.

Evaluation of the *N. magadii* cultures L11 (pNB102-ORF49/49´ΔAUG) and L11 (pNB102-ORF49/49´) infected with the ΔORF48 strain of φCh1

In this chapter the results will be presented, which got obtained from the *N. magadii* cultures L11 (pNB102-ORF49/49´ΔAUG) and L11 (pNB102-ORF49/49´), infected with φCh1-ΔORF48. Again ORF49/49´ΔAUG and ORF49/49´ are expressed from the high copy plasmid pNB102, but other than in the previous experiment, where the cultures got infected with wild-type φCh1, here ΔORF48 φCh1 was used for infection and therefore the introduced mutation into ORF49´ is not complemented by ORF48.

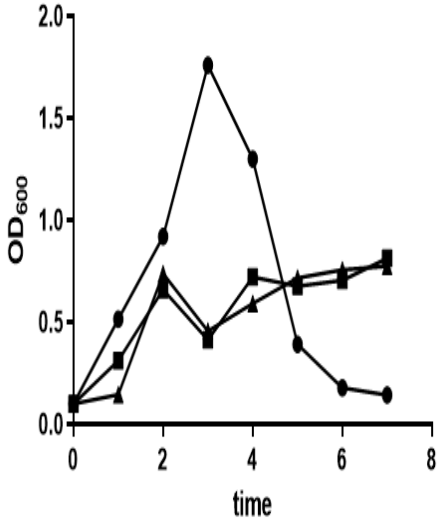


Figure 26: Growth Properties of the *N. magadii* cultures L11-ΔORF48 (pNB102-ORF49/49´ΔAUG) (labeled with circle), L11-ΔORF48 (pNB102-ORF49/49´) (highlighted by squares) and-*N. magadii* L11-1 (marked with triangle). All cultures got inoculated to OD_{600}=0.1 and grew at 37°C under agitation, the optical density was determined at 600nm each day at a certain time point.

Figure 26 displays the growth properties of the examined cultures *N. magadii* L11 (pNB102-ORF49/49´ΔAUG) and *N. magadii* L11 (pNB102-ORF49/49´), infected with the ORF48 deletion strain of φCh. In addition to these growth curves, also the growth curve of *N. magadii* L11-1 is plotted, because of the high similarity of this growth curve to the growth curve of *N. magadii* L11-ΔORF48 (pNB102-ORF49/49´).

The *N. magadii* L11-ΔORF48 (pNB102-ORF49/49´ΔAUG) culture reached after three days of growth an optical density of OD_{600}= 1.74 and lysed on the following day down to OD_{600}=1.3. On

the fifth day of this experiment the optical density was just about OD_{600}= 0.4 and decreased the following days further to OD_{600}= 0.18. Therefore this culture appears to have a lysis behavior, comparable to wild-type, which seems not suppressed by the introduced mutation.

In contrast, the growth curve obtained from the strain *N. magadii* L11-ΔORF48 (pNB102-ORF49/49´), appears to have high similarities to the typical growth curve of *N. magadii* L11-1(see Figure 26). The control culture reached an optical density of OD_{600}= 0.67 after two days of growth, before it lysed in a singular lysis event, on the third day to OD_{600}= 0.4, but then recovered and started growing again until the culture eventually reached OD_{600}= 0.8 at the last day of the experiment. This kind of lysis, dominated by a singular decrease in optical density followed by lysogeny, is typically for φCh1-1 (see Iro *et al.*, 2006). This similarity between the control culture and *N. magadii* L11-1 is not surprising, due to the fact, that this strain harbors ORF49/49´, which is thought to be decisive for the phenotype of φCh1-1.

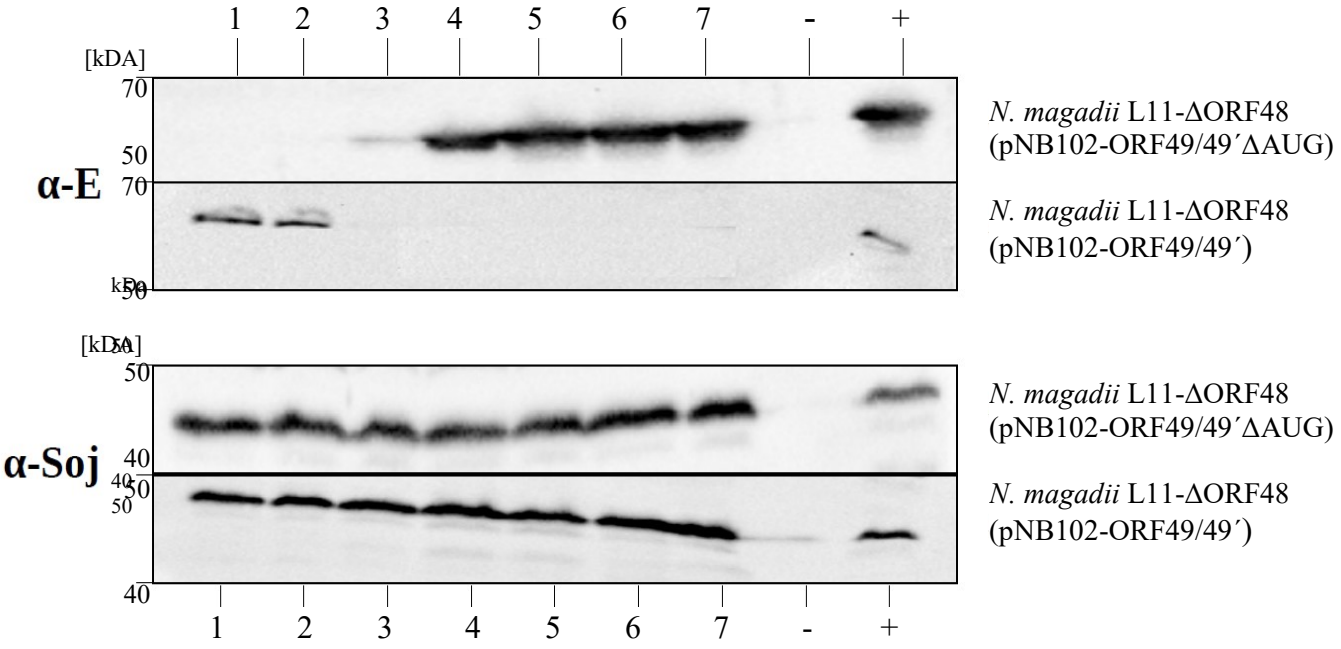
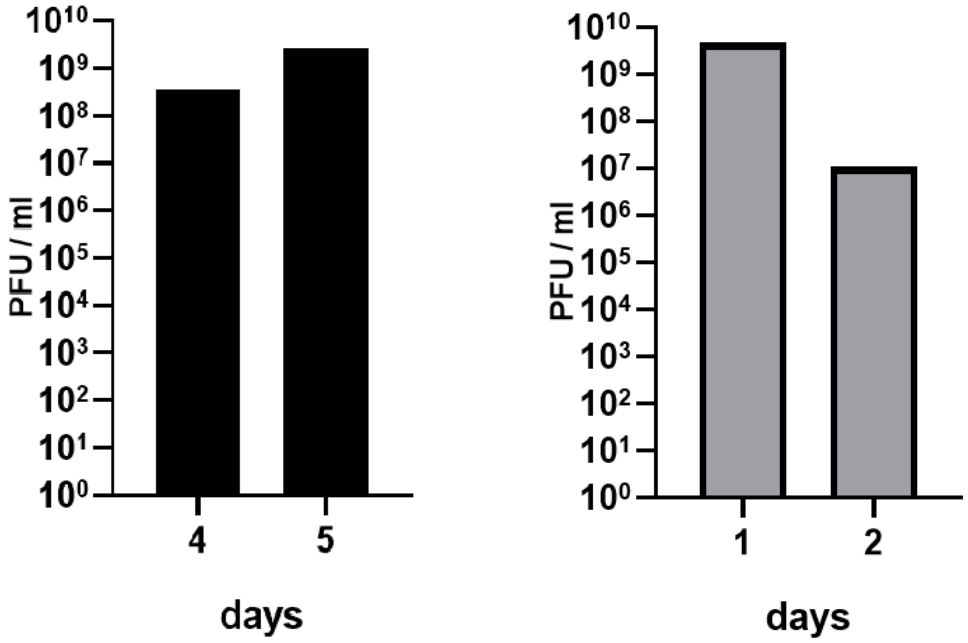


Figure 27: Western Blot analysis of the *N. magadii* cultures L11 (pNB102-ORF49/49´ΔAUG) and L11 (pNB102-ORF49/49´) infected with the ΔORF48 deletion strain of φCh1. The upper panel depicts the blots against protein E, the lower panel displays those, where a primary antibody against protein Soj got used. As positive control an *N. magadii* L11 sample was used, as negative control served an *N. magadii* L13 sample. In Lane 1 to 7, the samples, which were taken on day 1 to 7, were applied to the Western blots.

Next to the determination of optical density also Western blots got performed to follow the lysis behaviors of the two *N. magadii* cultures, infected with φCh1-ΔORF48. These Western blots are depicted in Figure 27 and confirm the picture we already got from the growth curve analysis.

The Western blots, obtained with primary antibodies against Soj, show a homogeneous Signal for this viral protein on all days of this measurement, for samples of the *N. magadii* cultures L11-ΔORF48 (pNB102-ORF49/49´ΔAUG) as well as L11-ΔORF48 (pNB102-ORF49/49´. Therefore the protein amount was applied to the blots in an equal manner, and the fluctuations in the protein E signal are therefore not artificial, but reflect the natural circumstances within the examined cultures.

On the first two days no signal for protein E got detected in Western blot, in samples taken from the *N. magadii* culture L11-ΔORF48 (pNB102-ORF49/49´ΔAUG). On day 3 just a weak signal became apparent, this day was the last day, on which the optical density increased. The protein E signal intensified on day 4, which is in line with the observation, that on this day a decrease in optical density was detected for this culture. The protein E level increased further on day 5, but appears with constant intensity the remaining days of this experiment.



2 **Figure 28: Virus titer comparison** of *N. magadii* L11-ΔORF48 (pNB102-ORF49/49´ΔAUG) (labeled black) and *N. magadii* L11-ΔORF48 (pNB102-ORF49/49´) (marked in gray). The plaque assays were incubated for 7 days at 37°C, after *N. magadii* L13 cells were infected with the supernatant taken from the lysing cultures.

Therefore, protein E was detected each day on which the *N. magadii* culture L11-ΔORF48 (pNB102-ORF49/49´ΔAUG) lysed, and the lyis behavior of this culture seems not to be affected in a suppressive manner.

In contrast, the L11-ΔORF48 (pNB102-ORF49/49´) culture appears to have a distinct lysis behavior, which is highly similar to the lysis behavior of *N. magadii* L11-1. Here, only on day 1 and 2 a signal for protein E was detectable, so one day in advance of the single lysing event on day 3. This is exactly the opposite, what was observed in the culture, where the start codon of ORF49´ is deleted. The *N. magadii* L11-ΔORF48 (pNB102-ORF49/49´) culture, seems to build virus progeny just in the beginning, before gp49´ perhaps suppresses the lytic life cycle and lysogeny is favored.

In addition, the plaque forming unit was determined for both cultures on two days, where those cultures lysed. The resulting virus titers are depicted in Figure 28. This figure displays the virus titers of *N. magadii* L11-ΔORF48 (pNB102-ORF49/49´ΔAUG), obtained on day 4 and 5, and puts them in comparison with the titers of *N. magadii*-L11-ΔORF48 (pNB102-ORF49/49´) culture, from which samples were taken on day 1 and 2. The plaque forming unit obtained from the *N. magadii* culture L11-ΔORF48 (pNB102-ORF49/49´ΔAUG) increased from day 4 to day 5 from $3.6*10^8$ to $2.8*10^9$ PFU/ml. In contrast the virus titer of the control strain L11-ΔORF48 (pNB102-ORF49/49´) decreased from day 1 to 2 from $4.8*10^9$ to $1.1*10^7$ PFU/ml.

Discussion

This result is again in line with the previous observations, where it was apparent to see that lysis and the production of viral particles is down regulated in the control strain *N. magadii* L11-ΔORF48 (pNB102-ORF49/49´) culture except for a singular lysis event, whereas the culture, carrying the construct with the deleted start codon in ORF49´, seems to lyse in a not suppressed manner, because here lysis was complete accompanied by strong signals against protein E in Western Blot, and a significant increase in the production of viral particles in the virus titer experiment.

Despite the lack of ORF48, interestingly the control strain, which expresses ORF49 and ORF49´ behaved in growth curve comparison exactly the same like*N. magadii* L11-1 cultures do (see Figure 26), whereas in contrast the culture with the introduced mutation lysed in a proper manner. This might be reasoned by the suppressive action of gp49´ on ORF49, which results in early just temporary activation of lytic genes, before lysogeny is eventually favored. The *N. magadii* L11-ΔORF48 (pNB102-ORF49/49´ΔAUG) culture lysed like expected thereafter the lack of ORF48 to repress lytic genes and ORF49, which promotes their expression.

Examination of the *N. magadii* cultures L11 (pNB102-ORF49/49´ΔAUG) and L11 (pNB102-ORF49/49´) infected with the ORF49 deletion strain of φCh1

In this section the results are displayed, which were obtained from the *N. magadii* cultures L11 (pNB102-ORF49/49´ΔAUG) and L11 (pNB102-ORF49/49´), both cultures were infected with the ORF49 deletion strain of φCh1, here the introduced mutation is not complemented by ORF49. In Figure 29 the growth curves, which got obtained from these two cultures are displayed. The optical density was measured daily at the same time point, to follow the lysis behavior of the examined culture also on a molecular level, protein crude extracts for Western blot have been prepared in addition.

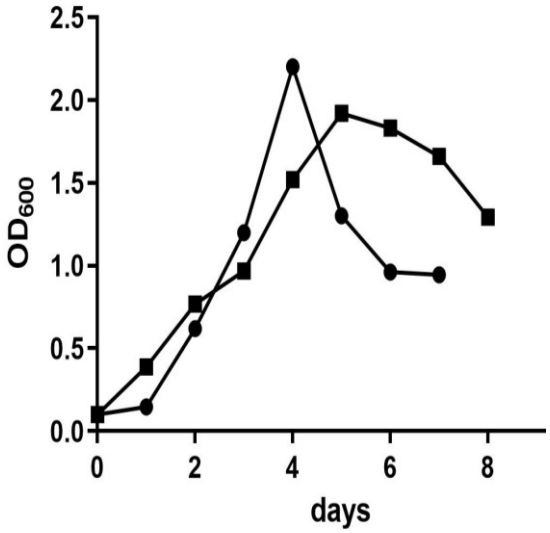


Figure 29: Growth curve analysis of the *N. magadii* cultures L11 (pNB102-ORF49/49´ΔAUG) (marked with squares) and L11 (pNB102-ORF49/49´) (labeled with circles), both infected with φCh1-ΔORF49. Both cultures were inoculated to OD_{600}= 0.1 agitation in NVM CAA^+ media and were incubated at 37° under. Optical density was determined each day at 600 nm, to follow the lysis behavior of the evaluated cultures.

The *N. magadii* culture L11-ΔORF49 (pNB102-ORF49/49´ΔAUG) reached an optical density of OD_{600}= 1.9 on the 5th day of growth, before it started to lyse slightly on day 6, where the optical density decreased to OD_{600}= 1.8. On the following days lysis decreased the optical density of this culture continuously to OD_{600}= 1.3 on day 8 of this experiment. The growth curve of the *N.*

magadii culture L11-ΔORF49 (pNB102-ORF49/49´ΔAUG) is rather flat, therefore the lysis behavior of this culture seems to be affected in a suppressive manner.

In contrast to this growth curve the control strain *N. magadii* L11-ΔORF49 (pNB102-ORF49/49´) lysed much sharper, this culture reached an optical density of OD_{600}= 2.2 on day 4, before it lysed down to OD_{600}=1.3 the following day, the optical density decreased than again to OD_{600}= 0.95, but stayed stable at this value for the rest of this experiment. This growth properties refer to a more aggressive, though incomplete lysis behavior, due to the fact, that the OD_{600} decreased first sharply but the value has not dropped below OD_{600}= 0.9, which refers to incomplete lysis.

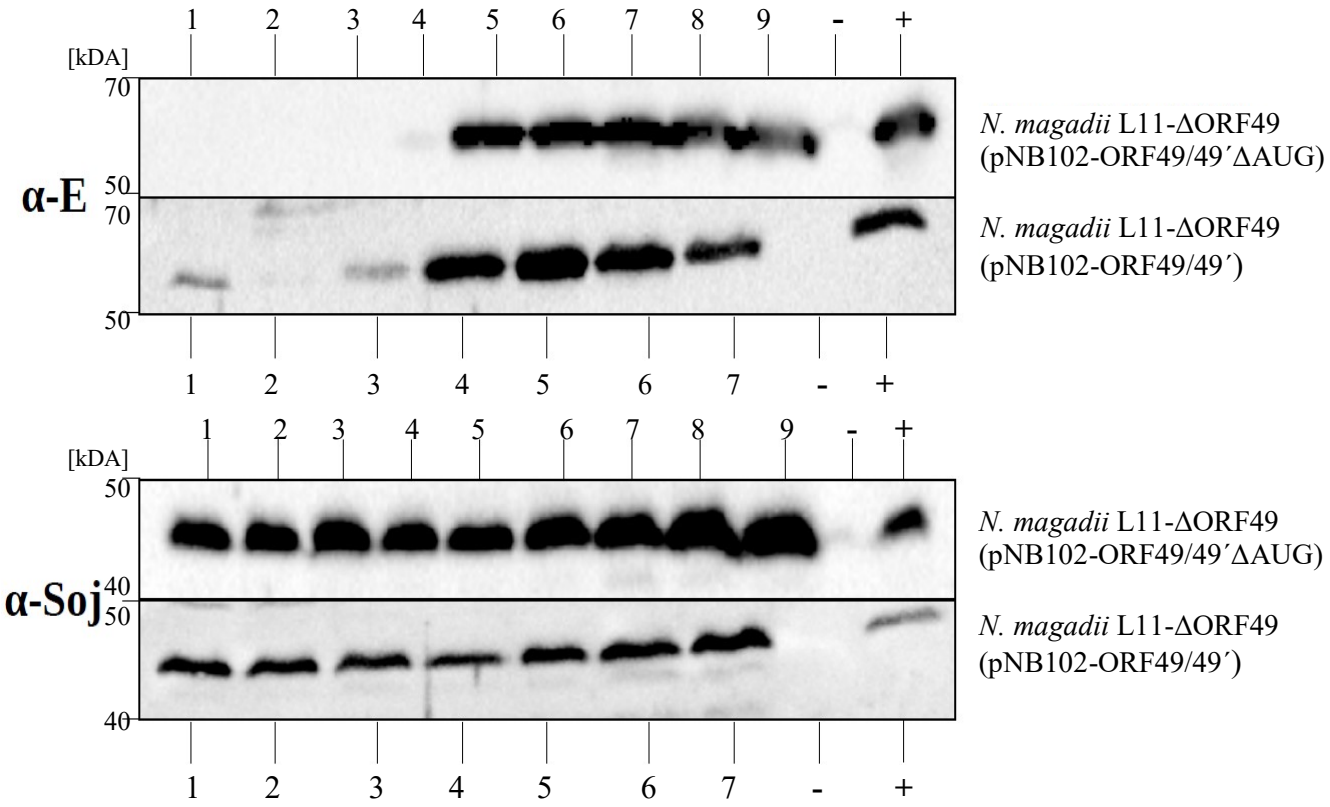


Figure 30: Western blot comparison of the *N. magadii* cultures L11 (pNB102-ORF49/49´ΔAUG) and L11 (pNB102-ORF49/49´), infected with the deletion strain φCh1-ΔORF49. The upper panel depicts the Western blots, obtained with primary antibodies against protein E, in the lower panel the Western blots, which used α-Soj as primary antibody are depicted. The depicted slot number refers to the day, where the protein crude extracts got prepared and the optical density was measured.

Next to the determination of optical density for both cultures, also each day a protein sample for Western blot got prepared, the resulting blots, which used antibodies against the viral proteins E and Soj are depicted in Figure 30.

The Western blots, which got obtained with the primary antibody against the viral protein Soj, a constitutively expressed protein of φCh1, are depicted in the lower panel of Figure 30. Those blots show a more or less homogeneous signal for both examined cultures on all days, on which samples were taken from the examined cultures. This proofs, that the protein amount was applied in an equal manner to the blots, and therefore the protein E signals refer to the upcoming conditions within the examined variants of φCh1.

In the samples taken from the *N. magadii* L11-ΔORF49 (pNB102-ORF49/49´ΔAUG) culture, infected with the ORF49 deletion strain of φCh1, protein E is first detectable on day 5, one day before lysis was visible by a decrease in the optical density values. The signal neither lost or gained intensity over the next 4 days of this experiment. Nevertheless, the signal appears to be slightly weaker on day 9.

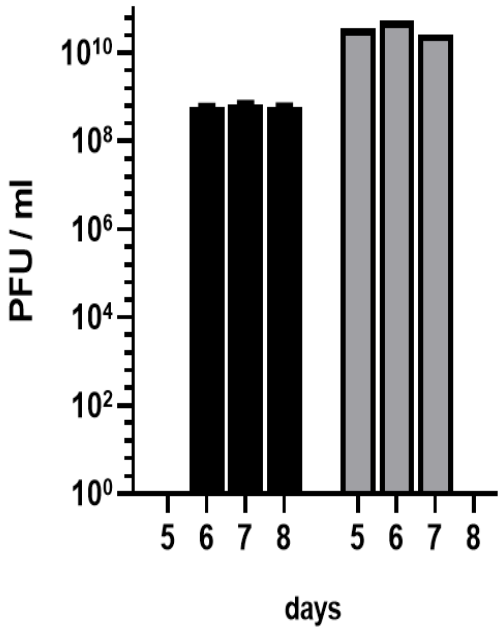


Figure 31: Virus titer comparison of the *N. magadii* cultures L11-ΔORF49 (pNB102-ORF49/49´ΔAUG) (labeled by black bars) and L11-ΔORF49 (pNB102-ORF49/49´) (highlighted in gray). *N. magadii* L13 cells were infected with the supernatant taken from the lysing cultures. After the plaque assays were incubated at 37°C for seven days, the plaques were counted and the PFU/ml values were calculated, the error-bars reflect SEM.

In contrast to this blot, the blot obtained from the *N. magadii* L11-ΔORF49 (pNB102-ORF49/49´), shows weak signals for protein E on day one, which might be a remnant from the pre-culture and on day 3, so also one day in advance of the start of lysis. Here, the signal got more intense on day four, before it intensified on day 5 of this measurement, where the most drastic loss

in optical density was observed during growth curve analysis. On day 6 and 7 the protein E signal decreased but stayed more intense than the signal on day 3.

Next to the previously presented results of Western blot and growth curve analysis, also the plaque forming units for both examined cultures have been determined via plaque assay. This virus titers are displayed in Figure 31. From the *N. magadii* L11-ΔORF49 (pNB102-ORF49/49´ΔAUG) culture the titer was determined from samples taken on day 6, 7 and 8 and set into comparison with the titers obtained from the control strain *N. magadii* L11-ΔORF49 (pNB102-ORF49/49´). From this culture virus titer samples were taken on day 5, 6 and 7 next to the OD_{600} Measurements (see Fig 29).

The plaques, which arose from the supernatant of the culture *N. magadii* L11-ΔORF49 (pNB102-ORF49/49´) were larger than usually observed in plaques formed by wild-type φCh1 and had a round shape (data not shown). On the first day of lysis the PFU/ml was $3.56*10^{10}$, also the value from the sample obtained the next day laid in order of magnitude by ten to the power ten and had an even higher PFU/ml of $5.42*10^{10}$, also on the third day, on which this culture lysed, the value was above 10^{10}, whereas the value decreased to PFU/ml= $2.85*10^{10}$ compared to the day before.

In contrast the supernatant of the *N. magadii* L11-ΔORF49 (pNB102-ORF49/49´ΔAUG) contains two orders of magnitude less virus particles, which appear to be regular in size and shape. On the first day on which this culture lysed, the determined PFU/ml was $5.73*10^{8}$, the following day more virus particles were produced, here the PFU/ml lies by $6.73*10^{8}$ on the following day this value increased again to about $5.83*10^{8}$ PFU/ml.

Discussion

These results show that the control strain, infected with the ΔORF49 strain of φCh1, lysed sharper compared to the lysis behavior of the *N. magadii* L11-ΔORF49 (pNB102-ORF49/49´ΔAUG) culture. The growth curve of the culture carrying the construct is much flatter and lysis took place two days later, than in the control strain. However, the growth curve of *N. magadii* L11-ΔORF49 (pNB102-ORF49/49´), appears to be quite similar to the growth curve of *N. magadii* L11-ΔORF49, which let suggest that gp49´ needs ORF49 to show up its influence on the life-cycle of φCh1.

In addition, in Western blot the signal for protein E got detected two days earlier in samples taken from the control strain, compared to the *N. magadii* L11-ΔORF49 (pNB102-ORF49/49´ΔAUG) culture. The lysate of this culture also generated two orders of magnitudes less plaques, than the control did.

However, this observation is the opposite of what was observed in the results obtained from the other pNB102 cultures, which were infected with wild-type and the ORF48 deletion strain. There the control showed a clear suppressed lysis behavior compared to the cultures, carrying the construct with the deleted start codon in ORF49´. This contrary result might be reasoned by the feature of ORF49 to act in a dose depended manner.

Conclusion

The evaluation to show up, if the deletion of the start codon AUG in ORF49´ has an impact on the lysis behaviors of the examined pRo5/mev and pNB102 *N. magadii* L11 cultures made apparent, that cultures, which carried the introduced mutation lysed more often, compared to the control cultures, which expressed functional ORF49´. Previous studies unveiled that ORF49 encodes for a transcription factor, which promotes the expression of genes responsible for the onset and maintenance of lysis and is shown to act in a dose dependent manner (Sabic, 2019). In most pRo5/mev cultures the construct behaved rather similar to the control strain, which only expresses ORF49´, whereas further molecular examination showed up differences, where the lysis behavior of the cultures carrying the introduced mutation seems to be better controlled. The control, which expressed ORF49/49´ however behaved always contrary to the culture carrying the deletion in the Start codon, and appeared to have a more suppressed lysis behavior. In all pNB102 cultures the control *N. magadii* cultures L11 (pNB102-ORF49/49´) and the cultures carrying the introduced mutation appear to have a contrary lysis behavior, whereas ORF49/49´ΔAUG *N. magadii* L11 cultures, lysed more likely in a non-suppressed manner. This suppressed lysis behavior characteristic for φCh1-1 might represent a lysogenic strategy to overcome periods of unfavorable circumstances for the halo-virus.

The fact that *N. magadii* L11 cultures, in which ORF49´ gets expressed, show a suppressed lysis behavior, which is characterized by just one early singular lysis event or even absence of lysis, makes an effect of gp49´ obvious. The fact, that this effect on the lysis behavior of φCh1 is vanished in the majority of the strains carrying the deletion in AUG within ORF49´, leads to the assumption that ORF49´ is indeed translated into the protein gp49´, which represents a truncated form of the transcription factor gp49. The duplication of ORF49, which results in ORF49´, encompasses a part of the 5´ end of gp49, which previous studies showed up to be crucial for the action of gp49 (Reiter 2007, Sabis, 2019). Therefore it can be speculated further, that gp49´ might possess a DNA binding domain, which allows specific binding to ORF49 binding sites, but lacks a trans-acting domain, which would be required for the communication with the RNA polymerase. A trans-acting domain is also a crucial feature for a functional transcription factor and its lack could result in the block of binding sites, which gp49 needs to promote the transcription of lytic genes. This might result in the suppressed lysis behavior, that is typically observed in φCh1-1. Nevertheless, to proof this assumption further experiments have to be performed, to evaluate the question, if gp49´ indeed

possesses a DNA binding domain, which allows sterically inhibition of gp49 binding sites and are the reason for the suppressed lysis behavior.

Materials

Strains:

Organism	Genotype	Source
Escherichia coli		
XL1-Blue	*endA1, gyrA96, hsdR17* (r_k-m_{K+}), *lac, recA1, relA1, supE44, thi,* (F′, *lacI*^{q}, *lacZ*ΔM15, *proAB*$^+$,, Tn10 (Tet^R)]	Stratagene
Natrialba magadii		
L11	Wild type strain - φCh1	Witte *et al.*, 1997
L13	φCh1 cured strain	Witte *et al.*, 1997
Unknown		
K2-4	unknown archaeal species	Isolated from Crete, 2001

Growth Media:

LB (=Luria broth) Medium (1l):

5g	NaCl
10g	Peptone
5g	Yeast extract
pH= 7 15 g/l agar for plates	

M2 Medium (general growth media for K2-4)

5g	Casamino acids
5g	Yeast extract
200g	NaCl
2g	KCl
0.2g	$CaCl_2x2H_2O$
$_dH_2O$ ad 901.6 ml pH=7.4	
After sterilization add:	
98.4ml	1M $MgCl_2x6H_2O$

Natrialba rich medium (NVM CAA+) (1l):

235 g	NaCl
2.4 g	KCl
11.7 g	Yeast extract
8.8 g	Casamino acids
0.8 g	Sodium citrate tribasic dihydrate

After sterilization add:		
62ml	0.57 M Na_2CO_3	(4)
1 ml	1 M $MgSO_4$	(1)
1 ml	20 mM $FeSO_4$	(2)
1 ml	1x Trace elements SL-6	(3)

pH= 9.5

$_dH_2O$ ad 935 ml

8 g/l agar for plates or 4 g/l agar for top agar

1000x Trace elements	
0.2g	$CoCl_2.6H_2O$
10 mg	$CuCl_2.6H_2O$
0.3 g	H_3BO_3
30 mg	$MnCl_3.4H_2O$
30 mg	$Na_2MoO_4.H_2O$
20 mg	$NiCl_2.6H_2O$

Antibiotics and other additives:

Compound	Stock concentration	Final concentration	Preparation
Ampicillin	20 mg/ml	100 µg/ml	dissolved in $_dH_2O$, sterile filtered, stored at 4°C
Chloramphenicol	40 mg/ml	20 µg/ml	dissolved in 96 % EtOH, stored at -20 °C
Tetracycline	10 mg/ml	10 µg/ml	dissolved in half of EtOH and half $_dH_2O$, stored at -20 °C
Mevinolin	10 mg/ml	4,5 µg/ml	120 mg dissolved in 12 ml 96 % EtOH, storage at -20 °C
Novobiocin	3 mg/ml	3 µg/ml	dissolved in $_dH_2O$, sterile filtered, storage at -20 °C
Bacitracin	7 mg/ml	70 µg/ml	dissolved in $_dH_2O$, sterile filtered, storage at 4°C
IPTG	1 M	1 mM	dissolved in $_dH_2O$, sterile filtered, storage at -20 °C
X-Gal	40 mg/ml	20 mg/ml	Thermo scientific ready to use storage at -20 °C

Kits:

name	Purpose	company
Wizard® SV Gel and PCR Clean-Up System	Purification of gel-eluted DNA fragments and PCR products	Promega
Wizard® Plus SV Minipreps DNA Purification System	Isolation of plasmid DNA	Promega

Plasmids:

Name	Features	Source
pKS_{II}^{+}	mcs, *bla*, ColE1 ori, lacZα	Stratagene
pKS_{II}^{+}-176bp	pKS_{II}^{+} with 176bp fragment of ORF49	this study
pKS_{II}^{+}-ORF49/ORF49`ΔAUG	pKS_{II}^{+} with 176bp fragment of ORF49 and 371bp fragment of ORF49`ΔAUG	this study
pRo5	*bla*, ColE1 ori, *gyrB* (novR), φCh1 derived ori	Mayrhofer-Iro *et al.*, 2013
pRo5/mev	*bla*, ColE1 ori, *hmg* (mevR), φCh1-derived ori	Dina, 2019
pNB102	*bla*, ColE1 ori, *hmg* (mevR), φCh1 derived ori	Zhout *et al.,* 2004
pNB102-ORF49/ORF49`ΔAUG	pNB102 with 176bp fragment of ORF49 and 371bp fragment	this study

	of ORF49`ΔAUG	
pNB102-ORF 49/ORF 49´	pNB102 with ORF 49 and ORF 49´ derived from φCH1-1	Dina, 2019
pRo5-ORF49/ORF49`ΔAUG	pRo5 with 176bp fragment of ORF49 and 371bp fragment of ORF49`ΔAUG	this study
pRo5/mev-ORF49/ORF49`ΔAUG	pRo5/mev with 176bp fragment of ORF49 and 371bp fragment of ORF49`ΔAUG	this study
pRo5/mev-ORF 49/ORF 49´	pRo5/mev with ORF 49 and ORF 49´ derived from φCH1-1	Dina, 2019
pRo5/mev-ORF 49´	pRo5/mev with ORF 49´ derived from φCH1-1	Dina, 2019
pRo5/mev-ORF 49 Δ4	pRo5/mev with truncated ORF 49	Dina, 2019

Antibodies:

Primary Antibody (rabbit);			
antibody	**target**	**Dilution**	**source**
α- Protein E	φCh1 gp11	1:2500	Klein *et al.*, 2000
α- Soj	Soj of φCh1	1:250	Hofbauer, 2015
α-Mtase	Main Methyltransferase of φCh1	1:500	Till, 2011
Secondary Antibody (donkey):			
ECL™ Anti-Rabbit IgG, HRP linked	Rabbit IgG	1:5000	GE Healthcare

Primer:

Primer name	Sequence (5′>3′)
Δ49′-1	GAT CGA ATT CTG GGC CTC TTT GAA AGG
Δ49′-2	GTT CGA ATT CAA CAC CCC CAA TAG ACA CTG T
49-Kpn	CAGCGGTACCTTGCGTTCAGTTCCG
49-Kpn-Xb	CAGCTCTAGAGGATCCTCATCCTGCGGTTTCG
49-HindIII-3	CAG CAA GCT TTC GAG GCG TCA TCC T
49-Kpn-3	CAGCGGTACCTCATCCTGCGGTTTCG
527f	ACCGCGGCCKGCTGG
1406r	ACGGGCGGTGTGTRC
pNB-R	CTTTTCTTCGCCGTTGTTCC
pRo5-mev	CCTTATCAAACACGCACAC
pKS-5-H	GTACAAGCTTCTATAGGGCGAATTGGAGCTC
pKS-MCS-R	GCCAAGCGCGCAATTAA
56-RT5	GAACAGCGCCAGTCCA
56-RT3	GACCACCGGCTTCAGC
49K-Xba1	CAGCTCTAGACTATTGGGGGTGTTCATTC
49-K-Kpn	CAGCGGTACCCTATTGGGGGTGTTCATTC
49-4XB	CAGCTCTAGAGGATCCTCACAAGAACAGGAGAGTGTCCA
49Δ4-Kpn1	CAGCGGTACCTCACAAGAACAGGAGAGTGTCCA

DNA and Protein ladders:

DNA Ladders:	size (bp)	company
GeneRuler 1kb DNA Ladder	250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000, 6000, 8000, 10000	Thermo Scientific
GeneRuler 1kb plus DNA Ladder	100, 250,500, 750, 1000, 1500, 2000, 3000, 4000, 5000, 6000, 8000, 100000	Thermo Scientific

GeneRuler 50bp DNA Ladder	50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 100	Thermo Scientific
Protein Ladders:	**size(kDa)**	**company**
PageRuler™ Prestained Protein Ladder	10, 15, 25, 35, 40, 55, 70, 100, 130, 180	Thermo Scientific
PageRuler™ Plus Prestained Protein Ladder	10, 15, 25, 35, 55, 70, 100, 130, 250	Thermo Scientific

Enzymes:

Restiction enzymes:	**company**
*Eco*RI	Thermo Scientific
*Eco*RV	Thermo Scientific
*Kpn*I	Thermo Scientific
*Xba*I	Thermo Scientific
HindIII	Thermo Scientific
Polymerases:	
2x GoTaq green Master Mix	Promega
Phusion® Flash PCR Master Mix	Thermo Scientific
DNA modifying enzymes:	
T4 DNA ligase	Promega
Fast AP Alkaline Phosphatase	Thermo Scientific
other enzymes:	
Proteinase K	Quiagen

*All enzymes were used in combination with buffers, which are recommended by the manufacture

Buffers and solutions:

DNA Gels:

5x DNA loading dye

50 mM	Tris.Cl (pH 8.2)
0.1 % v/v	SDS
0.05 % w/v	Xylene cyanol
0.1 M	EDTA

10 x TBE

108 g	Tris
55g	Boric acid
9.3g	Na_2EDTA

Add $_dH_20$ to 1l (pH= 8)

PAA Gel

10 ml	6% PAA
150 µl	APS
20 µl	TEMED

Agarose Gel

0.8 g agarose in 300ml 1xTAE

50x TAE

2 M	Tris.Cl pH 8.2
1 M	acetic acid
0.05 % w/v	bromophenol blue

Ethidium Bromid bath

1000 ml H_20 + 1ml EtBr (stock= 10 mg/ml (final concentration 10 µg/ml)

50x Super Buffer (400ml)

8 g	NaOH
45 g	boric acid

6% Acrylamide solution

14.8 g	acrylamide
2 g	N, N´-methylene-bisacrylamide

Protein Gels:

2x Lämmli Buffer

125 mM	Tris.Cl pH 6.8
4 % v/v	SDS
10 % v/v	β-mercaptoethanol
20 % v/v	glycerol
0.04 % w/v	bromphenol blue

5mM Na-Phosphate buffer

1 M	Na_2HPO_4
1 M	NaH_2PO_4

pH= 6.8

30% Acrylamide solution

73 g	acrylamide
2 g	N, N´-methylene-bisacrylamide

add $_dH_2O$ to 250 ml and filter to sterilize

10x SDS Running buffer

250 mM	Tris
1.92 M	Glycine
1% w/v	SDS

Stacking gel buffer

0.5 M	Tris HCl (pH 6.8)
0.4% v/v	SDS

Separating gel buffer

1.5 M	Tris HCl (pH= 8.8)
0.4 % v/v	SDS

Coomassie solution

25 % v/v	Methanol
10% v/v	Acetic acid
0.25 % w/v	Coomassie brilliant blue R250

Destaining solution

10 % v/v	Acetic acid

Western Blot:

10x TBS

250 mM	Tris HCl (pH=8.0)
1.37 M	NaCl

1x Transfer Buffer (Towbin)

25 mM	Tris
192 mM	Gycine
20% v/v	Methanol

Ponceau-S solution

0.5 % w/v	Ponceau-S
3 % v/v	Trichloracetic acid

Blocking solution

5 % w/v	Milk powder in 1 x TBS

Coumaric acid solution

0.148 g	Coumaric acid
10 ml	DMSO

Luminol solution

0.886 g	Luminol
20ml	DMSO

ECL buffer

13.3 ml	1.5M Tris.HCl (pH=8.5)
500µl	Luminol
250µl	Coumaric acid
add 200ml	$_dH_2O$

Membrane stripping buffer

30 ml	1 M Tris.HCl (pH=6.8)
3.95 ml	β-mercaptoethanol
2 % v/v	SDS
$_dH_2O$	ad to 500ml

Generation of competent cells: *E. coli*

MOPS I

100 mM	MOPS
10 mM	KCl
10 mM	RbCl
	pH= 7

MOPS II

100 mM	MOPS
10 mM	KCl
10 mM	RbCl
	pH= 6.5

MOPSIIa

100 mM	MOPS
10 mM	KCl
10 mM	RbCl
15 % v/v	glycerol
	pH= 6.5

Generation of competent cells: *Natrialba magadii*

Buffered high salt spheroblasting solution

2 M	NaCl
27 mM	KCl
50 mM	Tris.HCl (pH= 9.5)

After autoclaving: 15 % v/v sucrose (filtered)

Unbuffered high salt spheroblasting solution

2M	NaCl
27 mM	KCl

After autoclaving: 15 % v/v sucrose (filtered)

60 % PEG 600

60 % v/v	PEG 600
40 % v/v	unbuffered high salt spheroblasting solution

0.5 M EDTA

0.5 M	EDTA

pH= 8 (NaOH pellets)

1.1 Solution (200 ml)

20 g	CsCl
23.4 g	NaCl
0.4 g	KCl
4 g	$MgCl_2$
0.04 g	CaCl2 x $2H_2O$

pH= 7.4

1.3 Solution (200 ml)

90 g	CsCl
23.4 g	NaCl
0.4 g	KCl
4 g	$MgCl_2$
0.04 g	CaCl2 x $2H_2O$

pH= 7.4

1.5 Solution (200 ml)

135 g	CsCl
23.4 g	NaCl
0.4 g	KCl
4 g	$MgCl_2$
0.04 g	CaCl2 x $2H_2O$

pH= 7.4

Virus Buffer (1l)

200 g	NaCl
2g	KCl
20 g	$MgCl_2.6xH_2O$
0.2 g	$CaCl_2.2xH_2O$

pH= 7.4

(also used for dialysis)

Methods:

DNA Methods

PCR (=Polymerase chain reactíon)

Template preparation

Template DNA from *N. magadii* was obtained by centrifuging 50 µl liquid culture for 3 minutes at 13 krpm. After discarding the supernatant the pellet was resuspended in 100 µl $_{dd}H_2O$. In general 1µl template was used for PCR. To generate *E. Coli* template DNA, it was sufficient to put 1µl liquid culture directly into a colony PCR reaction. For preparative PCR reactions, purified φCh1 DNA or plasmid DNA, with a concentration in the range of 60-100 ng/µl, has been used.

Primer

All primers used in this study were obtained from the company microsynth and delivered in a lyophilized state. All stock primers were resuspended in $_{dd}H_2O$ to a concentration of 1µg/µl. To achieve the target concentration for PCR, the stock primers have been diluted 1:10.
The annealing temperatures of the certain forward and reverse primer pairs were estimated by using the program T_M Calculator from Thermo Scientific (alternatively NEB T_M Calculator).

PCR-Reaction

Preparative PCR

For all preparative PCR reactions the Phusion Hifi© polymerase was used, this polymerase has a 3'→5' Exonuclease activity and therefore allows a reliable amplification of the desired PCR product.

Volume	Component
2.5 µl	Forward primer
2.5 µl	Reverse primer
variable	template
25 µl	Phusion Hifi© Master Mix
x	$_{dd}H_20$

ad 50 µl	

Time	Temperature	Step
5 min	98°C	Initial Denaturation
35 x { 10 sec	98°C	Denaturation
10 sec	variable	Annealing
30 sec	72°C	Extension
2 min	72°C	Final Extension

Analytical PCR

In contrast to preparative PCR reactions, where the incorporation of point mutations into the amplicon should be avoided, mutations are not a big issue in analytical PCR reactions. Therefore the error-prone *Taq* polymerase is used for analytical purposes. The *Taq* polymerase is part of the GoTaq© Master Mix, which has in addition also a loading dye incorporated.

Volume	Component
1,5 µl	Forward primer
1,5 µl	Reverse primer
1,0 µl	template
12,5 µl	GoTaq© Master Mix
8,5 µl	$_{dd}H_20$
25 µl	

Time	Temperature	Step
3 min	95°C	Initial Denaturation
24 x { 30 sec	95°C	Denaturation
30 sec	variable	Annealing
1 min	72°C	Extension
5 min	72°C	Final Extension

Quality Control and PCR product purification

To analyze the quality of the obtained amplicon, an aliquot of 5 µl was mixed with the same amount of loading dye and applied on a 0. 8 % Agarose gel. The gel was stained with EtBr (target concentration 10 µg/ml) for at least 10 minutes and visualized with UV light in the ChemiDoc XRS+ apparatus.

To make the obtained PCR product suitable for further applications, it got purified using the Wizard© SV Gel and PCR Clean up Kit. The concentration of the purified DNA was determined spectrophotometrically by Nano Drop (ND-2000c, Thermo Scientific).

DNA-Restriction

Restriction enzymes were used according to the recommendations set by the manufacturer Thermo Scientific. The buffer system for double digests got selected by using the restriction enzyme chart provided by the manufacturer. The restriction digest was incubated overnight at 37°C, alternatively 2-3h at 37°C. For vector restrictions it was possible to control the digestion on a 0. 8% agarose gel, due to the fact that a linearized vector migrates slower, than a non-restricted, circular one. To get rid of the restriction enzymes and their buffer system the digested insert got purified using the Wizard© SV Gel and PCR Clean up Kit. In case of digested vectors it was sufficient to inactivate the restrictions enzymes by heat (75°C, 10 min).

Vector-Dephosphorylation

To avoid self-ligation of a vector, which was cut by only one restriction enzyme, it was necessary to remove the phosphate groups of the 5´-end. Therefore the vector was incubated for 15 minutes at 37°C with Fast AP alkaline phosphatase, to further inactivate this enzyme the reaction mix was incubated at 75°C for 10 minutes.

Ligation

To ligate the restricted insert into the digested vector, the enzyme T4-DNA-ligase from Thermo Scientific has been used. This enzyme is derived from the phage T4 and is an ATP depended ligase. The ATP for this ligation reaction was provided by using the 10x T4-DNA-ligase buffer.

Instead of calculating the molar ratios of insert and vector a thermodynamic approach has been chosen. Therefore 11.5 µl of insert, with a concentration around 150 ng/µl, were mixed with 1µl vector (concentration between 35-75 ng/µl), 1.5µl 10x T4-DNA-ligase buffer and 1µl T4-DNA-ligase.

This ligation mix was either incubated for 3 h at room temperature or overnight at 4°C.

Gel Electrophoresis

Agarose Gels

Agarose Gel electrophoresis was used to separate DNA fragments larger than 300 bp. All agarose gels in this thesis had a percentage of 0. 8 % agarose. When 1x TAE was used as running buffer, a voltage between 100-140 V was used to separate the DNA in a large gel. In case 1x Super Buffer was used as gel electrophoresis buffer, a small gel was ran with 200 V for 20 min. To follow the migration of the DNA in the Gel, the probes got mixed with 5x loading dye. (This was not necessary for analytical PCR, because the GoTaq© Master Mix already contains a loading dye.) In addition to the samples, the DNA ladder GeneRuler 1kb was applied onto the gel, to allow the length determination of the separated DNA fragments. The agarose gels were stained with EtBr (c=10µg/ml) for at least 10 minutes and got afterwards visualized under UV in the ChemiDoc XRS+ Imager.

PAA Gels

To separate DNA fragments smaller than 300 bp Polyacrylamide (PAA) gel electrophoresis was used. The PAA gels consisted of 6% polyacrylamide. PAA gels were run with 35-50 V in 1x TBE. In contrast to SDS-PAGEs, PAA gels are not composed of Separating gel and Stacking gel, but of a continuously poured gel. As well like for agarose gels a loading dye was used to visualize the dye front. But instead of GeneRuler 1kb the GeneRuler 50 bp was used, to determine the length of the small DNA fragments. PAA gels were stained and analyzed like common agarose gels.

Escherichia coli Methods

Plasmid isolation

To obtain plasmid DNA, a *E. coli* strain, which contained the desired plasmid, was inoculated in 20 ml selective LB medium and grown overnight at 37°C under agitation. From this overnight culture 3-10 ml were applied to the Wizard® Plus SV Minipreps DNA Purification System. To increase the output of this purification, the plasmid DNA was eluted from the column with 30µl pre-warmed (42°C) nuclease-free $_{dd}H_2O$. The concentration of the purified plasmid DNA was determined by Nano Drop (ND-2000c, Thermo Scientific).

Preparation of competent *E.coli* cells

To generate competent XL1-blue *E. coli* cells, 200 ml of LB, supplemented with tetracycline, was inoculated to OD_{600}= 0,1. The inoculated culture was grown under agitation at 37°C until an OD_{600} of 0,6-0,8 was reached. The cells were harvested via centrifugation (10 krpm, 15 minutes at 4°C), the pellet got resuspended in 80 ml MOPS I, and incubated for 10 minutes on ice. After an additional centrifugation step the pellet got resuspended in 80 ml MOPS II and incubated for 30 minutes on ice. Eventually the cells got centrifuged again and the pellet was resuspended in 4 ml MOPS IIA, which contains glycerol and allows to store the competent cells in 100 µł aliquotes at -80°C for several months. Nevertheless the achieved competence should be controlled via a test transformation (= transforming a plasmid of known concentration and calculating the transformation efficiency).

Transformation of competent *E. coli* cells

To transform DNA into *E. coli*, a 100 µl aliquot of frozen competent cells was thawed on ice. After addition of 15 µl ligation mix to the competent cells, the transformation was incubated for 30 minutes on ice. The cells were than further stressed through a heat shock of 42°C, which lasted for 2 minutes. Upon that the cells were incubated on ice again to end the heat shock, after providing 300 µl LB to the cells, the transformation got incubated for 30 minutes at 37°C. Eventually the complete transformation was plated on 3 selective LB plates (Amp/Tet), which than got incubated at 37°C.

Single colonies were inoculated in 5 ml LB -Amp/Tet and analyzed the next day by an analytical PCR reaction. To discover the positive clones via colony PCR the same primers were used, like for creating the insert. Clones, which displayed a right-sized PCR product, were sent to sequencing (microsynth). If the obtained sequence was correct, the positive clone got stored with glycerol at -80°C in the strain collection.

Natrialba magadii Methods

Generation of competent *N. magadii* cells

To obtain competent *N. magadii* cells 50 ml NVM-CAA+ supplemented with Bacitracin (target concentration of 70 µg/ml) were inoculated with a L13 preculture and incubated at 37°C. After the culture reached an OD_{600} between 0.6-1.0, the cells were harvested by centrifugation (6 krpm, 20 minutes at RT). The resulting pelett was subsequently resuspended in 25 ml buffered high-salt spheroblasting solution and supplemented with 0.1% v/v Proteinase K. To allow the formation of spheroblasts, the cells were incubated at 42°C for 2-4 days. The *N. magadii* cells were checked each

second day under the microscope. The cells were ready for transformation, when the formation of spheroblasts was accomplished in 60-70 % of the population.

Transformation of competent *N. magadii* cells

In the first step of transforming plasmid DNA into *N. magdii,* 1.5 ml of competent cells got centrifuged for 10 minutes, 10 krpm at RT. The pellet was resuspended in 150 µl buffered high-salt spheroblasting solution. After the addition of 15 µl 0,5 M EDTA, the transformation was incubated for 10 minutes at room temperature. Upon that not more than 10 µl DNA ,with a concentration ranging between 0.7-3 µg ,was added and carefully mixed by inverting the test tube.
After leaving the reaction for 15 minutes at room temperature, 150 µl 60% PEG 600 (40% unbuffered high-salt spheroblasting solution) was added and the transformation again was incubated for 30 minutes at room temperature. In addition the transformation assay was washed twice with 1 ml NVM-CAA^+ (5min, 10krpm, RT), and the pellet got subsequently resuspended in 1 ml NVM-CAA^+. Eventually the transformed cells were regenerated at 37°C with agitation for 3-7 days. The regeneration process was observed via light microscopy, when more than 60 % of the cells, within the *N. magadii* population, regained their typical rod-shaped appearance, the transformed cells were plated on selective NVM-CAA^+ plates and incubated at 42°C for 1-3 weeks.

To check the transformation, single colonies were inoculated in 1 ml selective NVM-CAA^+, the test tubes were than incubated at 37°C under agitation for 5-12 days. After obtaining the template DNA of each clone like described before, a colony PCR as like for *E. coli* was performed. The positive L13 transformants were inoculated in 15 ml selective NVM-CAA^+ to further be infected with φCh1 via plaque assay.

Protein Methods

Protein crude extract preparation

N. magadii cultures, infected with different variants of φCh1, were inoculated to OD_{600} = 0. 1 and have been grown on 37°C for 7-12 days. The optical density was measured each day at the same time to obtain a growth curve. In addition protein samples for SDS-PAGE have been prepared. Therefore 1. 5 ml of culture were centrifuged at 13 krpm for 3 minutes. (1 ml of the supernatant was kept to determine the virus titer. The addition of 40µl Chloroform inhibited archael growth in these samples.) The remaining supernatant was removed completely. The pellet was then resuspended in 5mM Sodium-Phosphate buffer and 2x Laemmli Buffer. To calculate the values of these buffers, the OD_{600} value was multiplied by 75. (If the resulting value was lower than 50 µl, additional 1, 5 ml of culture were centrifuged like depicted above and the value got multiplied by two.) The proper

vortexed protein crude extract was then incubated for 24h in the 37°C room without agitation, and stored at
-20°C afterwards.

Preparation of *N. magadii* Acetone Powder

N. magadii Aceton powder was used to decrease unspecific binding of antibodies onto *N. magadii* proteins during Western blotting. All antibodies used in this study were polyclonal and therefore recognize not just a single epitope but a larger set of epitopes (Nakazawa *et al.*,2016). Due to structural similarities of the target viral protein, and proteins of *N. magadii* the primary antibody can bind unspecific to those archaeal proteins. To decrease this non-desirable effect in Western Blot, acetone powder can be added to the antibody. Acetone powder consists of *Natrialba magadii* proteins, which provide a target for antibodies, which would bind in an unspecific manner to the blot.

To obtain *N. magadii* acetone powder, 1l NVM-CAA^+ was inoculated with *N. magadii* L13. The culture was grown until late stationary phase. The culture was harvested by centrifugation (6krpm, 20 minutes, RT), the pellet got resuspended in 0,9% NaCl solution (1ml for 1g pellet) and incubated for 5-10 minutes on ice. For 2 ml cell suspension 8 ml acetone were added, the mixture was than incubated for 30 minutes on ice and centrifuged for 10 minutes at 6krpm at room temperature. Upon that the pellet was resuspended in ice-cold acetone and incubated on ice for 10 minutes. The suspension got eventually centrifuged (6krpm, 20 minutes, RT) and the resulting pellet was dried on filter paper overnight at room temperature. The dry pellet was pulverized and stored at room temperature.

SDS-PAGE with Coomassie Staining

SDS-Polyacrylamid Gel-electrophoresis was used to separate all proteins within the obtained *Natrialba magadii* crude extracts, according to their molecular weight. It was necessary to denature the protein samples before loading, therefore all samples were cooked at 95°C for 10 minutes. 10 µl of each sample got applied to a discontinuous SDS-PAGE, which consisted of a 12 % separating gel and a 4 % stacking gel. Due to the high salt concentration of the examined proteins, it was necessary to run the gel for 5-6 h on 35-45 V. The operation was done in a BioRad Mini Protean apparatus filled with 1x SDS-Running Buffer. The SDS-PAGE got stained with Coomassie staining solution for 3-10 minutes afterwards and got incubated with Destaining solution overnight under agitation.

Western Blot

The amount of protein sample loaded on the SDS-PAGE, used for blotting, was adjusted according to the SDS-PAGE seperated in advance. To transfer the fractionated proteins from the SDS-PAGE onto the nitrocellulose membrane (9, 5 × 6, 5 cm; GE Healthcare AmershamTM ProtranTM 0.2 µm NC), a semi-dry Western blot has been performed. Within the blotting device Trans-Blot® TurboTM Transfer System from BioRad, a Sandwich of 3 Whatman papers, the nitrocellulose membrane, the SDS-Gel, and three additional Whatman papers was assembled. The Whatman papers as well as the nitrocellulose membrane were soaked in 1x Transfer buffer, whereas the gel was shortly put in tap water to decrease the salt concentration before blotting. Blotting took place at 25 mA for 30 minutes. After blotting the transfer was ensured by staining the blot with Ponceau S solution. The blotting membrane got than blocked in milk (7,5 % milk powder in 100 ml 1x TBS) overnight at 4°C (alternatively: 2-3 h at 37°C).

To remove the remaining milk afterwards, the blot was washed three times with 1x TBS for 10 minutes under agitation at RT. Further the blot was incubated with a primary antibody against a certain protein of φCh1 for 1 hour under agitation at RT (alternatively: overnight at 4°C). After removing the primary antibody, the blot was again washed with 1x TBS and got incubated with the secondary antibody for 1 hour at RT. All primary antibodies in this study are derived from rabbit sera, therefore the secondary Antibody targets the FC-part of the primary rabbit antibodies. The secondary IgG antibody is coupled to Horseradish peroxidase (HRP). After washing the unbound secondary antibodies off the blot with 1x TBS, 3 ml ECL and 3 µl H_2O_2 have been applied to eventually develop the blot.

The HRP enzyme attached to the epitope of interest via primary and secondary antibody, catalyzes the oxidation of luminol (Thorpe *et al.*, 1985). This triggers a light reaction, which can be followed via the Chemidoc XRS+ imager apparatus. All blots where analyzed with the program Data Image lab and the setting “High sensitivity”. The maximal exposure time for all blots in these thesis was 180 seconds, within this time period every 5 seconds a picture was taken. Alternatively the exposure time was chosen automatically by the Chemidoc XRS+ imager to achieve the best resolution for intense bands.

If it was necessary to develop the same blot with a different primary antibody, the membrane was stripped. Therefore the blot was washed three times with 1x TBS for 10 minutes at RT under agitation after ECL treatment, and incubated for 30 minutes on 37°C with 10 ml ROTI® Free Stripping Buffer (Roth). To get rid of the stripping solution the blot again was washed three

times with 1x TBS and eventually got blocked again with milk (= 7,5 % milk powder in 100 ml 1x TBS) overnight.

Haloalkalophilic Virus Methods

Plaque assay

Plaque assays were performed to infect *N. magadii* L13 cultures with different variants of φCh1. This was either done to generate a new *N. magadii* L11 culture or to determine the virus titer of a certain φCh1 variant.

Therefore a *N. magadii* culture was grown until exponential growth phase at 37°C under agitation, Soft agar was prepared (unautoclaved NVM-CAA$^-$ supplemented with the half amount of agar normally used for plates) and purified φCh1 virus particles or alternatively supernatant of a lysed culture was serially diluted (10^{-2},10^{-4}, 10^{-6},10^{-8}).
After autoclaving, the soft agar was complemented and aliquoted into 65°C pre-warmed test tubes. To 5 ml of NVM-CAA$^+$-soft agar, 300-500 µl uninfected culture and 100 µl of a certain virus dilution were added. After vortexing the soft agar was poured onto a non-selective NVM-CAA$^+$ plate in a way that a consistent top layer of soft agar was formed. The plaque assay plates dried on room temperature over night and were eventually incubated at 37 °C for 1 week. Plaques, which arised in the archeael lawn, were either inoculated in 500 µl selective NVM-CAA$^+$ and grown at 37°C to obtain a culture for further analysis or were counted to determine the virus titer.

Plaque assays for virus titer determination were performed in duplicates. The virus titer was calculated according to the formula:

$$\text{PFU/ml} = \frac{\text{counted plaques}}{\text{volume of lysate} * \text{dilution}}$$

Virus isolation

To isolate virus particles a CsCl gradient centrifugation was performed, therefore 1 l of infected archaeal culture was inoculated to OD_{600} = 0,1 and grown at 37°C with agitation until the culture lysed nearly completely. The culture was harvested by centrifugation (8 krpm, 20 min, RT) and the supernatant was supplemented with 10 % PEG 6000 (w/v) and incubated stirring over night at RT, to allow binding of the virus particles to PEG. The virus precipitate was than again centrifuged for 20 minutes at RT and 8 krpm. The resulting pellet was resuspended in 7-15 ml phage precipitation solution. To obtain a discontinuous CsCl gradient, 4 ml of 1.5 solution was gently layered with 4 ml 1.3 solution and 5 ml of the phage precipitate within the Beckman test tube. The solution 1.1 was used to tare the test tubes against each other, before centrifuging them for 20 h at RT with 30 krpm

with a Beckman L-70 ultracentrifuge (rotor= SW40-Ti). Purified viruses appear as a light blue band within the CsCl gradient, and got further purified by a continuous CsCl gradient, therefore the virus particles were mixed with 1.3 solution and centrifuged again (30 krpm, 20 h, room temperature). After this centrifugarion step the viral particles got dialysed over night against high-salt dialysis solution.

Cloning strategies

Cloning the deletion of AUG of ORF49´

pKS$_{II}^{+}$-ORF49/ORF49´ΔAUG

To obtain the 5´-end of the construct pKS$_{II}^{+}$-ORF49/ORF49´ΔAUG, a 176 bp fragment was amplified with the primers Δ49´-1 and 49-Kpn (annealing temperature=53°C) from φCh1-1 template. After restricting the vector pKS$_{II}^{+}$ as well as the insert with *Kpn*1 and *Eco*RI, the 176 bp fragment was incorporated into pKS$_{II}^{+}$ via a ligation reaction. After transformation into XL1-blue, it was possible to make use of blue/white selection to choose candidates for a analytical PCR, to determine a positive clone.

From this positive clone plasmid DNA was isolated and restricted with *Kpn*1 1 and *EcoR*I. The insert for the 3´-end had a size of 371 bp and was amplified by PCR using the primers Δ49´-2 and

49-*Kpn-XB* and the φCh1-1 template. The 371 bp fragment got as well restricted with *Xba*1 and *Eco*RI and ligated into the pKS$_{II}^{+}$ vector, which already had the insert of the 5´-end incorporated. After Transformation the positive clone was than determined via colony PCR and sequencing.

Cloning of the mutated fragment into shuttle vectors

pRo5/mev-ORF49/ORF49´ΔAUG

The plasmid pKS$_{II}^{+}$-ORF49/ORF49´ΔAUG was purified and served as template for PCR amplification with the primers 49-Kpn and 49-Kpn-3. The obtained insert had a size of 555 bp and got restricted with *Kpn*1 as like the shuttle vector pRo5/mev. The vector was ligated with the insert and was than transformed into competent XL1-blue cells. pRo5/mev-ORF49/ORF49´ΔAUG plasmid DNA was purified from the positive clone, which was ensured by PCR and sequencing, and got eventually transformed into *N. magadii.*

pNB102-ORF49/ORF49´ΔAUG

To obtain the shuttle vector pNB102-ORF49/ORF49´ΔAUG the insert was amplified in a PCR reaction with the template pKS_{II}^{+}-ORF49/ORF49´ΔAUG and the primers 49-Kpn and 49-XB. The obtained amplicon had a size of 561 bp. The insert as well as the vector pNB102 were restricted with Kpn1 and *Xba*1. After ligation and transformation into XL1-blue, a colony PCR was performed to screen for positive clones. Plasmid DNA from this positive clone was purified, sequenced and transformed in *N. magadii.*

pRo5-ORF49/ORF49´ΔAUG

As well as for the other shuttle vectors the purified plasmid pKS_{II}^{+}-ORF49/ORF49´ΔAUG served as template to obtain the insert via PCR reaction using the primers 49-Kpn and 49-HindIII-3. The resulting PCR product of 555 bp got digested with the restriction enzymes Kpn1 and HindIII, again the same digest was performed for the vector pRo5. The insert and vector were ligated, transformed in *E. coli* and the clones were checked via colony PCR. Unfortunately it was not possible to transform the shuttle vector pRo5 with the incorporated construct obtained from this positive clone in *N. magadii.*

References

Ackermann HW. 5500 Phages examined in the electron microscope.*Arch Virol*. **2007**;152(2):227-243. doi:10.1007/s00705-006-0849-1

Ackermann HW, Prangishvili D. Prokaryote viruses studied by electron microscopy. *Arch Virol*. **2012**;157(10):1843-1849. doi:10.1007/s00705-012-1383-y

Arnold HP, Ziese U, Zillig W. SNDV, a novel virus of the extremely thermophilic and acidophilic archaeon Sulfolobus.*Virology*. **2000**;272(2):409-416. doi:10.1006/viro.2000.0375

Atanasova, N.S.; Roine, E.; Oren, A.; Bamford, D.H.; Oksanen, H.M. Global network of specific virus-host interactions in hypersaline environments. Environ. Microbiol. **2012**, 14, 426–440.

Bail O, Der kolistamm 88 von Gildmeister und Herzberg. *Med Klin*. 21:1271–3. **1925**.

Bailone A, Levine A, Devoret R. Inactivation of prophage lambda *in vivo. J Mol Biol.* **1979**; 131(3):553-572. doi:10.1016/0022-2836(79)90007-x

Bamford DH, Pietilä MK, Roine E, et al. ICTV Virus Taxonomy Profile: *Pleolipoviridae*.*J Gen Virol*. **2017**;98(12):2916-2917. doi:10.1099/jgv.0.000972

Bath C, Dyall-Smith ML. His1, an archaeal virus of the *Fuselloviridae* family that infects *Haloarcula hispanica*. *J Virol.* **1998**; 72(11);9392-9395. doi:10.1128/JVI. 72.11.9392-9395.1998

Baquero DP, Liu Y, Wang F, Egelman EH, Prangishvili D, Krupovic M. Structure and assembly of archaeal viruses.*Adv Virus Res*. **2020**;108:127-164. doi:10.1016/bs.aivir.2020.09.004

Bolduc B, Shaughnessy DP, Wolf YI, Koonin EV, Roberto FF, Young M. Identification of novel positive-strand RNA viruses by metagenomic analysis of archaea-dominated Yellowstone hot springs. *J Virol*. **2012**;86(10):5562-5573. doi:10.1128/JVI.07196-11

Bordet J. Le problème de l'autolyse microbienne transmissible ou du bactériophage. Ann Inst Pasteur 39:711-63. **1925**

Cohen S, Knoll BJ, Little JW, Mount DW, Preferential cleavage of phage lamda repressor moonomers by RecA protease. *Nature*. **1981**;294(5837):182-184. doi:10.1038/294182a0

Dellas N, Snyder JC, Bolduc B, Young MJ. Archaeal Viruses: Diversity, Replication, and Structure *Annu Rev Virol* **2014**; 1(1):399-426. doi: 10.1146/annurev-virology-031413-085357

Demina TA, Pietilä MK, Svirskaitė J, *et al*. HCIV-1 and Other Tailless Icosahedral InternalMembrane-Containing Viruses of the Family *Sphaerolipoviridae*. *Viruses*. **2017**;9(2):32. Published 2017 Feb 18. doi:10.3390/v9020032

Dion MB, Oechslin F, Moineau S. Phage diversity, genomics and phylogeny.*Nat Rev Microbiol*. **2020**;18(3):125-138. doi:10.1038/s41579-019-0311-5

Gropp F, Palm P, Zillig W. Expression and regulation of *Halobacterium halobium* phage phi H genes. *Can J Microbiol*. **1989**;35(1):182-188. doi:10.1139/m89-028

Gropp F, Grampp B, Stolt P, Palm P, Zillig W. The immunity-conferring plasmid p phi HL from the *Halobacterium salinarium* phage phi H: nucleotide sequence and transcription. *Virology*. **1992**;190(1):45-54. doi:10.1016/0042-6822(92)91191-v

Häring M, Rachel R, Peng X, Garrett RA, Prangishvili D. Viral diversity in hot springs of Pozzuoli, Italy, and characterization of a unique archaeal virus, *Acidianus* bottle-shaped virus, from a new family, the *Ampullaviridae*. *J Virol*. **2005**;79(15):9904-9911. doi:10.1128/JVI.79.15.9904-9911.

Hofbauer C, The function of gp34 and its regulation by ORF79 of φCh1 as well as the influence of other regulation elements. Master thesis. **2015**

Iranzo J, Krupovic M, Koonin EV. The Double-Stranded DNA Virosphere as a Modular Hierarchical Network of Gene Sharing. *mBio*. 2016;7(4):e00978-16. Published **2016** Aug doi:10.1128/mBio.00978-16

Iro M, Klein R, Gálos B, Baranyi U, Rössler N, Witte A. The lysogenic region of virus φCh1: identification of a repressor-operator system and determination of its activity in halophilic Archaea. *Extremophiles*. **2007**;11(2):383-396. doi:10.1007/s00792-006-0040-3

Kamekura M, Dyall-Smith ML, Upasani V, Ventosa A, Kates M. Diversity of alkaliphilic halobacteria: proposals for transfer of *Natronobacterium vacuolatum*, *Natronobacterium magadii*, and *Natronobacterium pharaonis* to *Halorubrum, Natrialba*, and *Natronomonas gen. nov*., respectively, as *Halorubrum vacuolatum* comb. *nov*., *Natrialba magadii* comb. *nov*., and *Natronomonas pharaonis* comb. *nov*., respectively. *Int J Syst Bacteriol*. **1997**;47(3):853-857. doi:10.1099/00207713-47-3-853

Ken R, Hackett NR. *Halobacterium halobium* strains lysogenis for phage phi H contain a protein resembling coliphage repressors. *J Bacteriol*. **1991**;173(3):955-960. doi:10.1128/jb. 173.3.955-960.

Klein R, Baranyi U, Rössler N, Greineder B, Scholz H, Witte A. *Natrialba magadii* virus phiCh1: first complete nucleotide sequence and functional organization of a virus infecting a haloalkaliphilic archaeon. *Mol Microbiol*. **2002**;45(3):851-863. doi:10.1046/j.1365-2958.2002.03064.x

Klein R, Greineder B, Baranyi U, Witte A. The structural protein E of the archaeal virus φCh1: evidence for processing in *Natrialba magadii* during virus maturation.*Virology*. **2000**;276(2):376-387. doi:10.1006/viro.2000.0565

Krupovic M, Cvirkaite-Krupovic V, Iranzo J, Prangishvili D, Koonin EV. Viruses of archaea: Structural, functional, environmental and evolutionary genomics. *Virus Res*. **2018**;244:181-193. doi:10.1016/j.virusres.2017.11.025

Lwoff A. Lysogeny. Bacteriol Rev. 17(4):269–337. **1953**

Lederberg EM. Lysogenicity in *E. coli* K-12. Genetics. 36:560. **1951**

Lewis DE, Gussin GN, Adhya S. New Insights into the Phage Genetic Switch: Effects of Bacteriophage Lambda Operator Mutations on DNA Looping and Regulation of *PR, PL,* and *PRM*. *J Mol Biol*. **2016**;428(22):4438-4456. doi:10.1016/j.jmb.2016.08.027

Luk AW, Williams TJ, Erdmann S, Papke RT, Cavicchioli R. Viruses of haloarchaea. *Life (Basel)*. 2014;4(4):681-715. Published **2014** Nov 13. doi:10.3390/life4040681

Maniatis T, Ptashne M. Multiple repressor binding at the operators in bacteriophage λ. **1973**; PNAS. 70(5):1531-5.

Mizuno CM, Rodriguez-Valera F, Garcia-Heredia I, Martin-Cuadrado AB, Ghai R. Reconstruction of novel cyanobacterial siphovirus genomes from Mediterranean metagenomic fosmids. *Appl Environ Microbiol*. **2013**;79(2):688-695. doi:10.1128/AEM.02742-12

Nakazawa M, Mukumoto M, Miyatake K, Production and Purification of Polyclonal Antibodies *Methods Mol Biol*. **2016**;1474:49-59. doi:10.1007/978-1-4939-6352-2_3

Oppenheim AB, Kobiler O, Stavans J, Court DL, Adhya S. Switches in bacteriophage lambda development. *Annu Rev Genet*. **2005**;39:409-429. doi:10.1146/annurev.genet.39.073003.113656

Oren A, Bratbak G,. Heldal M, Occurrence of virus-like particles in the Dead Sea. *Extremophiles* **1997**, 1, 143–149.

Pietilä MK, Laurinmäki P, Russell DA, et al. Insights into head-tailed viruses infecting extremely halophilic archaea. *J Virol*. **2013**;87(6):3248-3260. doi:10.1128/JVI.03397-12

Pietilä MK, Demina TA, Atanasova NS, Oksanen HM, Bamford DH. Archaeal viruses and bacteriophages: comparisons and contrasts.*Trends Microbiol*. **2014**;22(6):334-344. doi:10.1016/j.tim.2014.02.007

Pietilä, MK., Roine, E., Sencilo, A. *et al.Pleolipoviridae*, a newly proposed family comprising archaeal pleomorphic viruses with single-stranded or double-stranded DNA genomes.*Arch Virol* 161**,**249–256 **2016**. https://doi.org/10.1007/s00705-015-2613-x

Porter K, Russ BE, Dyall-Smith ML Virus-host interactions in salt lakes. Curr Opin Microbiol **2007**.10:418–424

Porter K, Tang SL, Chen CP, Chiang PW, Hong MJ, Dyall-Smith M. PH1: an archaeovirus of Haloarcula hispanica related to SH1 and HHIV-2.*Archaea*. **2013**;2013:456318. doi:10.1155/2013/456318

Prangishvili D, Forterre P, Garrett RA. Viruses of the Archaea: a unifying view. *Nat Rev Microbiol*. **2006**;4(11):837-848. doi:10.1038/nrmicro1527

Prangishvili D. The wonderful world of archaeal viruses. *Annu Rev Microbiol*. **2013**;67:565-585. doi:10.1146/annurev-micro-092412-155633

Prangishvili D, Bamford DH, Forterre P, Iranzo J, Koonin EV, Krupovic M. The enigmatic archaeal virosphere.*Nat Rev Microbiol*. 2017;15(12):724-739. doi:10.1038/nrmicro.**2017**.125

Ptashne M. A genetic switch: phage lambda revisited. Cold Spring Harbor Laboratory Press. 3rd edition. **2004**

Reiter M. Gene regulation of φCh1: characterization of ORF49 and further characterization of the origin of replication of the halophage φCh1. Master thesis. **2010**

Rodriguez-Valera F, Martin-Cuadrado AB, Rodriguez-Brito B, *et al.* Explaining microbial population genomics through phage predation. *Nat Rev Microbiol*. **2009**;7(11):828-836. doi:10.1038/nrmicro2235

Sabic D, The regulation of gene expression in haloalkaliphilic virus φCh1: further characterization of the role of rep and ORF49, Masters thesis.**2019**

Santos F, Yarza P, Parro V, Meseguer I, Rosselló-Móra R, Antón J. Culture-independent approaches for studying viruses from hypersaline environments. *Appl Environ Microbiol*. **2012**; 78(6):1635-1643. doi:10.1128/AEM.07175-11

Sarai A, Takeda Y. Lambda repressor recognizes the approximately 2-fold symmetric half-operator sequences asymmetrically. **1989**; PNAS. 86(17):6513-7.
Selb R, Derntl C, Klein R, *et al.* The Viral Gene ORF79 Encodes a Repressor Regulating Induction of the Lytic Life Cycle in the Haloalkaliphilic Virus ϕCh1. *J Virol*. **2017**;91(9):e00206-17. Published 2017 Apr 13. doi:10.1128/JVI.00206-17

Senčilo A, Paulin L, Kellner S, Helm M, Roine E. Related haloarchaeal pleomorphic viruses contain different genome types.*Nucleic Acids Res*. **2012**;40(12):5523-5534. doi:10.1093/nar/gks215

Senčilo A, Roine E. A Glimpse of the genomic diversity of haloarchaeal tailed viruses.*Front Microbiol*. **2014**;5:84. Published 2014 Mar 12. doi:10.3389/fmicb.2014.00084

Soliman GS, Trüper H. *Halobacterium pharaonis* sp. nov., a new, extremely haloalkaliphilic archaebacterium with low magnesium requirement. *Z Bakteriol Parasitenkd Infektionskr Hyg* Abt 1Orig Reihe C. **1982**; 3(2):318-29.

Stolt P, Zillig W. *In vivo* studies on the effects of immunity genes on early lytic transcription in the *Halobacterium salinarium* phage phi H. Mol Gen Genet. **1992**;235(2-3):197-204. doi:10.1007/BF00279361

Shao Y, Wang IN. Bacteriophage adsorption rate and optimal lysis time. *Genetics*. **2008**;180(1):471-482. doi:10.1534/genetics.108.090100

Stolt P, Zillig W. *In vivo* and *in vitro* analysis of transcription of the L region from the Halobacterium salinarium phage phi H: definition of a repressor-enhancing gene. *Virology*. **1993**;195(2):649-658. doi:10.1006/viro.1993.1416

Stolt P, Zillig W. Transcription of the halophage phi H repressor gene is abolished by transcription from an inversely oriented lytic promoter. *FEBS Let*t. **1994**;344(2-3):125-128. doi:10.1016/0014-5793(94)00347-5

Tindall B, Ross HN, Grant W. *Natronobacterium* gen. nov. and *Natronococcus* gen. nov., two new genera of haloalkaliphilic archaebacteria. Syst Appl Microbiol. **1984;** 5(1):41-57.

Torsvik T, Dundas ID, Bacteriophage of *Halobacterium salinarium*. Nature 248:680–681,**1974**

Witte A, Baranyi U, Klein R, *et al*. Characterization of *Natronobacterium magadii* phage phi Ch1, a unique archaeal phage containing DNA and RNA. *Mol Microbiol*. **1997**;23(3):603-616. doi:10.1046/j.1365-2958.1997.d01-1879.x

Zhang Z, Liu Y, Wang S, et al. Temperate membrane-containing halophilic archaeal virus SNJ1 has a circular dsDNA genome identical to that of plasmid pHH205. *Virology*. **2012**;434(2):233-241. doi:10.1016/j.virol.2012.05.036

Abstract

φCh1-1 is a naturally occurring mutant strain of the temperate halo-virus φCh1, which harbors a duplication within ORF49. The open reading frame ORF49 is thought to encode a transcription factor, which promotes the expression of genes responsible for the onset and maintenance of lysis. Studying ORF49´, so the partially duplicated ORF49, allows for deeper insights into the molecular switch between lysogenic and lytic life-cycle of φCh1.

To elucidate the question if ORF49´ is indeed translated into the protein gp49´ which might act on the life-cycle of φCh1, the start codon of ORF49´ was deleted via cloning and ORF49/ORF49´ΔAUG was transformed and expressed on low and high copy plasmids in different *N. magadii* L11 cultures. The lysis behavior of those *c*ultures was eventually followed via Growth curve analysis, Western blot and the determination of virus titers.

This evaluation made apparent that *N. magadii* L11 cultures in which ORF49´ was expressed appear to have a suppressed lysis behavior, whereas most cultures containing ORF49´ without AUG lysed in a moderate manner.

These observations let suggest that ORF49´ is expressed as the truncated protein gp49´, that can alter lysis in a suppressive manner. If the mode of action relies on the presence of a DNA binding domain, but the absence of a trans-acting domain in gp49´, which would result in the blockage of binding sites for gp49, no communication to the RNA polymerase and thereafter inefficient expression of lytic genes, must be in focus of further studies.

Zusammenfassung

φCh1-1 ist eine natürlich auftretende Mutante des temperaten Halovirus φCh1, die eine Duplikation im ORF49 besitzt. Es wird angenommen, dass der offene Leserahmen ORF49 für einen Transkriptionsfaktor kodiert, der für die Verstärkung der Expression von Genen verantwortlich ist, die für das Einsetzen und Beibehalten der Lyse zuständig sind. Studien über ORF49´, also den partiell duplizierten ORF49, erlauben tiefere Einblicke in den molekularen Schalter zwischen lysogenen und lytischen Lebenszyklus von φCh1.

Um die Frage zu beantworten ob ORF49´ tatsächlich in das Protein gp49´ translatiert wird, welches möglicherweise einen Einfluss auf das Lyse Verhalten von φCh1 hat, wurde das Start Codon in ORF49´ mittels Clonierung entfernt und der so veränderte ORF49/ORF49´ΔAUG auf low und high copy Plasmiden in verschiedene *N. magadii* L11 Kulturen transformiert und exprimiert. Schließlich wurde das Lyse Verhalten der resultierenden Kulturen mit Hilfe von Wachstumskurven, Western blots und Virus Titer Analysen verfolgt,

Diese Evaluierungen zeigten klar, dass Kulturen in denen ORF49´ exprimiert wurde ein supprimiertes Lyse Verhalten aufgewiesen haben, wohingegen die meisten Kulturen, die das gelöschte Start Codon in ORF49 beinhaltet haben, ein normales Lyse Verhalten zeigten.

Diese Beobachtungen lassen darauf schließen, dass ORF49´ tatsächlich als das verkürzte Protein gp49´ exprimiert wird, das einen die Lyse abschwächenden Effekt aufweist.
Ob der Wirkungsmechanismus dieses Effekts wirklich darauf beruht, dass zwar eine DNA binding Domäne, aber keine trans-acting Domäne in gp49´ vorhanden ist, und dieser Umstand dazu führt, dass die Bindungsstellen für gp49 durch gp49´ blockiert sind jedoch gp49´ nicht mit der RNA Polymerase kommunizieren kann, was zu ineffizienter Expression von lytischen Genen führt, müssen weitere Studien klären.