universität wien

MASTERARBEIT

Titel der Masterarbeit

„The function of gp34 and its regulation by ORF79 of ϕCh1 as well as the influence of other regulation elements“

verfasst von

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1 Introduction

1.1 Halophilic *Archaea*

Microorganism can thrive in environments with salt concentration ranging from fresh water up to habitats near the saturation point. Halophilic microorganisms are adapted to higher salt concentrations. The moderate halophiles grow with 0.5-2.5 M NaCl, whereas extreme halophiles need a concentration of 2.5-5.2 M NaCl (Andrei *et al.*, 2012). The main environments in which halophiles can be found are: evaporated seawater (thalassohaline environments), athalassohaline hypersaline environments (differ in the ion composition, e.g. the Dead Sea) and alkaline soda lakes with a pH value around 11 (Oren, 2002). Halophilic organisms exist in all three domains of life, but in the group of extreme halophiles, *Archaea* are the most numerous group. Most halophilic *Archaea* belong to the family *Halobacteriaceae* in the phylum *Euryarchaeota*. Although they are all adapted to high salt concentrations, the metabolism and nutrition is very diverse. *Archaea* of the *Halobacteriaceae* grow usually heterotrophic and aerobic and use amino acids as energy and carbon source. For this purpose they often export enzymes for degradation of substances, e.g. proteases or amylases (Andrei *et al.*, 2012).

To survive in a halophilic environment adaptations to the high salt concentration are necessary. Especially the cell surface has to withstand the surrounding osmotic pressure. Halophilic eubacteria and *Archaea* have different strategies for osmoregulation. Halophilic *Archaea* have a high salt concentration in the cytoplasm, whereas halophilic eubacteria use organic compounds to balance the high osmolality (Margesin and Schinner, 2001). A characteristic of archaeal membranes is the ether linkage and the isoprenoid chains. The high salinity in extremely halophilic habitats requires an altered composition of the polar lipids. The membrane destabilizing effect caused by NaCl is prevented by a special composition of the polar lipids. The major polar lipid in extreme halophiles is the archaetidylglycerol methylphosphate (PGP-Me) and is mainly responsible for the

membrane stability. However, there are also other lipids that play a role in the stabilizing mechanism (Tenchov *et al.*, 2006).

As mentioned above, halophilic *Archaea* accumulate high concentrations of Na^+ or K^+ in the cytoplasm for osmoregulation. For this reason, the enzymes have to be adapted to this environment to be active and stable. Normal proteins precipitate at 3-4 M NaCl or KCl, but enzymes of halophiles require this concentration (Fendrihan *et al.*, 2006). Firstly, these enzymes have a high amount of acidic amino acids and therefore an isoelectric point of approximately 4.2. Secondly, halophilic enzymes contain only few hydrophobic amino acids and hydrophobic interactions (Lanyi, 1974).

A group of halophilic *Archaea* does not only need a high salt concentration but also a high pH value around 10. This group is called haloalkaliphilic and is found in soda desserts and soda lakes. Although they live in an environment with a high pH value, the internal pH value is nearly neutral (Aono *et al.*, 1997). It has been shown, that alkaliphilic *Bacillus* protoplasts are not viable in an alkaline environment. Hence, the cell wall has to protect it from the alkaline outside. Acidic polymers in the cell wall cause a negative charge and reduce the pH value. Na^+/H^+ antiporter systems are responsible for regulation of the pH value at the cell surface. The import of substances into the cell is driven by the influx of Na^+. Therefore H^+ and Na^+ are exchanged by an H^+/Na^+ antiporter, which creates a sodium motive force (Horikoshi, 1999).

1.2 The haloalkaliphilic arachaeon *Natrialba magadii*

1.2.1 General features

Natrialba magadii belongs to the family of *Halobacteriaceae* of the phylum *Euryarchaeota*. Other members of this family are *Haloferax volcanii* or *Halobacterium salinarum*, which both are very well studied (Soppa *et al.*, 2008). *Nab. magadii* has been isolated from Lake Magadi in Kenya in 1984 (Tindall *et al.*, 1984). Originally, *Nab. magadii*

was classified into the genus *Natronobacterium*. Subsequent sequence analysis led to re-classification in the genus *Natrialba* because of 16s rRNA comparison (Kamekura *et al.*, 1997). *Nab. magadii* is haloalkaliphilic and requires therefore for growth 4 to 5 M sodium chloride and a pH value between 8.5 and 11. Further a low Mg^{2+} concentration is needed and the optimal growth temperature is between 37-42°C. *Nab. magadii* is chemoorganotrophic and obligate aerobe. It grows proteolytically and therefore does not utilize carbohydrates but instead amino acids and peptides as carbon and energy source (Tindall *et al.*, 1984).

1.2.2 Genetic manipulation

There are some aspects, which complicate the genetic manipulation of *Nab. magadii*. The copy number of the chromosome is up to 50 (Breuert *et al.*, 2006) and a generation time of approximately 9 hours in the logarithmic growth phase is quite slow (Tindall *et al.*, 1984). Furthermore, there are only two selection markers, novobiocin and mevinolin, available.

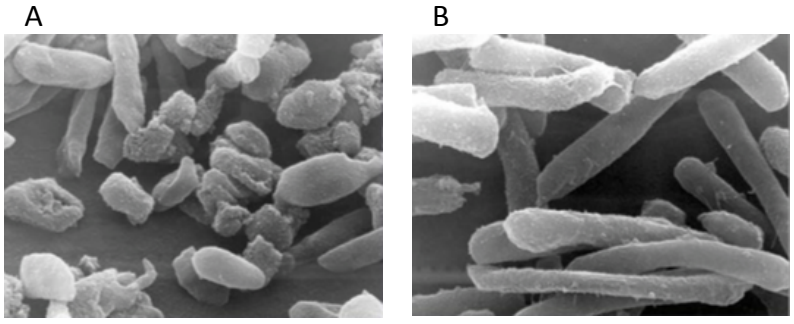

Figure 1: Morphology of *Nab. magadii* L11 and *Nab. magadii* L13.
Electron micrographs of A: wild type strain L11 after lysis and B: the cured strain L13 (Iro, 2006).

In this thesis, two strains were used *Nab. magadii* L11 and *Nab. magadii* L13. The strain L11 is the wild type strain, which carries the provirus ϕCh1, whereas L13 is the cured strain. The wild type strain starts to lyse after approximately four days (Witte *at al.*, 1997).

Transformation

The first method for transformation of a halophilic archaeon was performed by polyethylene glycol with *Halobacterium salinarum* (Cline and Doolittle, 1987). This method was adapted for *Haloferax volcanii* (Charlebois *et al.*, 1987) but did not work for *Nab. magadii*. However, incubation with bacitracin and treatment with proteinase K lead to formation of spheroblasts by removal of the glycoprotein surface layer. The spheroblasts are still viable and are able to take up DNA. This method demonstrated a successful method for transforming *Nab. magadii*, although the efficiency is lower than in other halophilic strains (Mayrhofer-Iro *et al.*, 2013).

Shuttle vectors

For *Nab. magadii*, two shuttle vectors are available. The plasmid pRo-5 has been constructed by fusion of a novobiocin resistance gene with the *Escherichia coli* vector pKS_{II}^{+}. For autonomous replication in *Nab. magadii* the minimal replicon of the virus ϕCh1 was used (Mayrhofer-Iro *et al.*, 2013). The replication in halophilic *Archaea* requires an AT-rich region and the gene *repH* (Ng and DasSarma, 1993). In ϕCh1 two ORFs were found, which have similarities to proteins involved in the plasmid replication. ORF54 and ORF53 have homologous parts to the RepH protein of *Haloarcula marismortui*. Up- and downstream of these ORFs are AT-rich regions located. Therefore, this region represents the minimal replicon of ϕCh1 and was used for the plasmid pRo-5. This plasmid is stable in *Nab. magadii* and is also replicated in other halophilic *Archaea* (Mayrhofer-Iro *et al.*, 2013). The second shuttle vector pNB102 has been constructed from the plasmid pNB101. The plasmid pNB101 has been isolated from the strain *Natronobacterium* sp. AS7091. To allow the replication in *E. coli* the ColE1 replicon was integrated. For selection in *Archaea* the mevinolin resistance gene was used (Zhou *et al.*, 2004).

1.3 Viruses of haloarchaea

Two of the best-studied viruses of *Halobacteriaceae* are ϕH of *Halobacterium salinarum* and ϕCh1 of *Natrialba magadii*. Both are temperate viruses and have head-tail morphology. Although ϕH has only few sequence similarities to bacteriophages, the mechanisms of replication and control of lysogeny is very similar to P1 (Schnabel *et al.*, 1982). In spite of the fact that their hosts are phylogenetical distant, the virus ϕH has a high sequence similarity to ϕCh1 (Klein *et al.*, 2002). One of the main differences of ϕCh1 and ϕH is that ϕH has a covalently closed DNA molecule (Schnabel *et al.*, 1984), whereas ϕCh1 integrates into the host chromosome (Witte *et al.*, 1997). The repressor *rep* of ϕH causes immunity to super-infection by inhibiting the transcription of early lytic transcripts by RNA polymerase (Ken and Hackett, 1991). The mode of action of both viruses is not very different from the well-studied temperate bacteriophages (Porter *et al.*, 2007).

Other viruses from hypersaline environments have been isolated, like the haloviruses HF1 and HF2. These viruses have a head-tail morphology and infect the cells persistently. HF2 is able to superinfect the cell and produce a permanent low virus titer. Since often only a thin surface layer protects the cell surface of *Archaea*, release of viral particles without lysis is possible (Porter *et al.*, 2007). HF1 and HF2 infect various host strains, for example *Haloferax*, *Halobacterium* or *Natrialba*. Although, they infect different hosts their genome is at minimal 80 % identical. The genome of HF2 contains many regions from different haloarchaea and this fact indicates an exchange of genes (Dyall-Smith *et al.*, 2003). Studies have shown, that there are also lytic viruses infecting halophilic Archaea, like virus Ja.1 or B10. These viruses infect *Hbt. salinarum*, but only little is known so far about their significance in the environment (Porter *et al.*, 2007).

1.4 The virus ϕCh1

1.4.1 General features

The virus ϕCh1 was first isolated from Witte *et al.* in 1997 and belongs to the family of *Myoviridae*. So far the only known host is the haloalkaliphilic archaeon *Nab. magadii*. The virus has a head-tail morphology and a length of approximately 200 nm. The head is icosahedral shaped and the tail is contractible. Since the virus is adapted to the environment of its host, the particle needs a salt concentration higher than 2 M. Otherwise the virus become non-infective and structural instable. Two strains of the host *Nab. magadii* were available, the wild type strain L11, which contains the provirus integrated into the host chromosome and the cured strain L13. ϕCh1 is a temperate virus with a lysogenic and lytic state. The virus starts to lyse in the stationary growth phase, which normally occurs from day 4 to 5. In the cell the first mature particles appear after an eclipse time of 5 hours (Witte *et al.*, 1997).

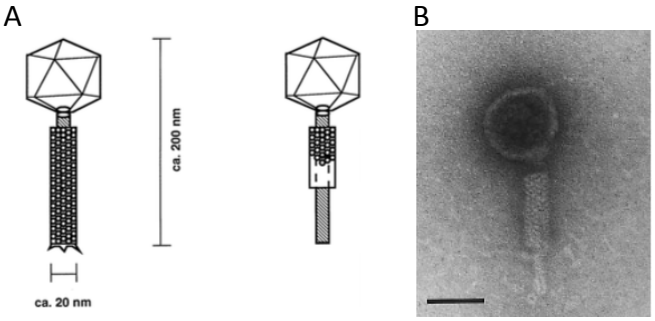

Figure 2: Morphology of ϕCh1.
A: Schematic drawing of the viral particle with the icosahedral head and the contractible tail.

B: Electron micrograph of the virus. The line represents 50 nm (Witte *et al.*, 1997).

The analysis of viral genomes leads often to no significant matches in sequence databases. However, ϕCh1 and other euryarchaeal viruses with head-tail morphology have ORFs with homologies to other genes, for example transcriptional regulators (Prangishvili *et al.*, 2006).

The viral particle contains a double-stranded DNA genome and also RNA. The genome contains 98 putative open reading frames (ORFs). The already known and annotated

functions of the proteins are indicated in Figure 3. The right and the left part contain only rightward-transcribed ORFs, whereas the part in the middle also harbors leftward-transcribed genes. The left part mainly codes for structural proteins and proteins for the virus assembly. The ORFs in the central region are responsible for replication, regulation and stabilization. ORFs of the right part have mainly no annotated function except for some DNA modifying enzymes (Klein *et al.*, 2002).

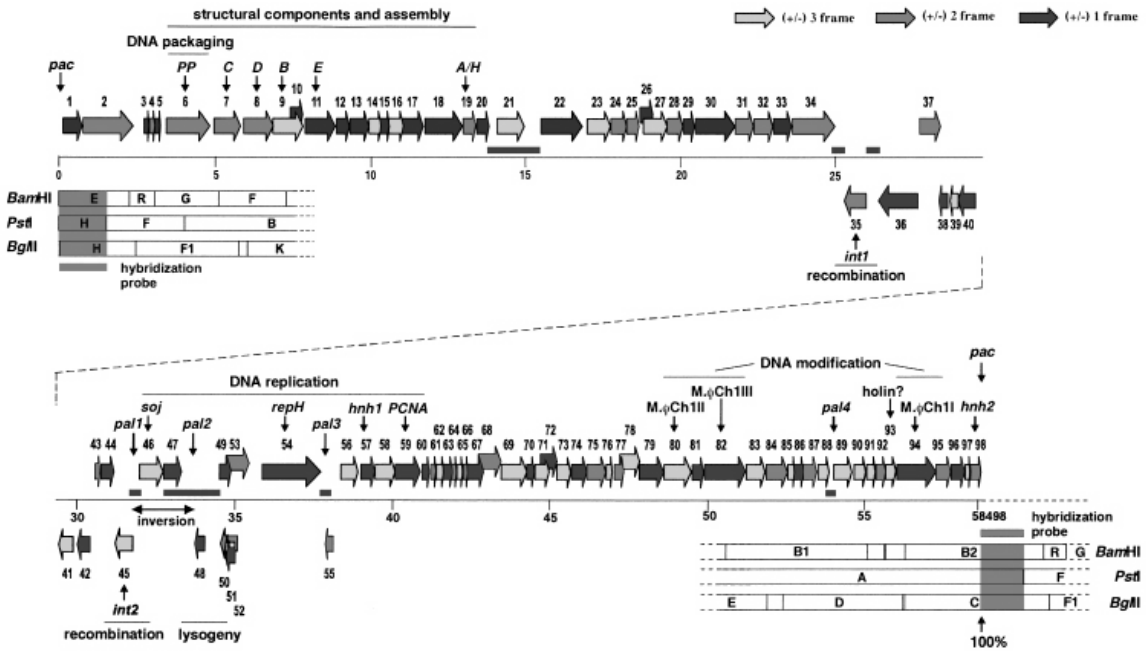

Figure 3: Genome of ϕCh1.
The scheme represents the linear 58,498 bp long genome. The arrows represent open reading frames (ORF). Putative or verified functions are indicated. The genome could be divided into three parts: the left part codes mainly for structural components. The ORFs of central region are mainly responsible for replication, gene regulation and stabilization. The right part contains ORFs with unknown or DNA modification functions. The origin of replication is localized in the region of ORF53 and ORF54 (Klein *et al.*, 2002).

1.4.2 The invertible region

The integrase Int1

The invertible region of ϕCh1 contains three open reading frames. ORF35 is located in the middle and codes for the integrase Int1. This enzyme is a site-specific recombinase of the λ integrase family. The *int1* gene is homologous to integrases of other *Archaea* or *Bacteria*, e.g. *Mesorhizobium loti*. According to the amino acid in the catalytic center and the amino acid sequence, the site-specific recombinases are divided into two groups. The tyrosine recombinases, also known as λ type integrases, use a tyrosine for DNA cleavage. On the contrary, serine invertases/resolvases have a catalytically serine. Both families differ in the cleavage mechanism and in their structure. Examples for well-studied tyrosine recombinases are the λ integrase or the Cre recombinase. Representatives of the serine family are the ϕC31 integrase or the γδ recombinase (Groth and Calos, 2004; Esposito and Scocca, 1997; Smith *et al.*, 2002). The λ recombinases have a conserved C-terminal end with three conserved boxes (A, B and C). Within these conserved boxes are four highly conserved amino acids.

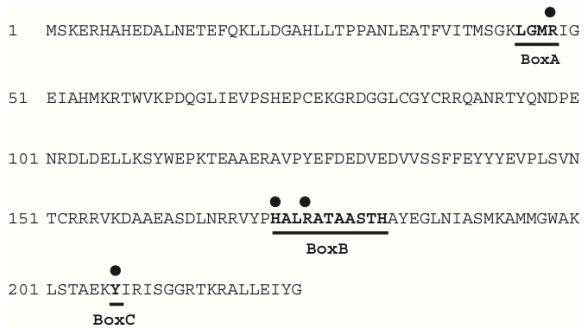

Figure 4: Amino acid sequence of Int1.
The conserved sequences are underlined and the highly conserved amino acids from the tetrad are marked (Rössler *et al.*, 2004).

The conserved tetrad consists of the amino acids R-H-R-Y, at which the tyrosine is catalytically active. The conserved boxes are also responsible for the conserved secondary structure (Argos *et al.*, 1986; Nunes-Düby *et al.*, 1998). Figure 4 shows the sequence of Int1 of ϕCh1, with the conserved boxes and the tetrad.

The tail fibre protein of ϕCh1

Downstream of ORF35 is ORF34 located and upstream ORF36. These ORF represents the viral tail fibre proteins. ORF34 contains the repeat cluster IR-L and ORF36 the cluster IR-R. The clusters consist of 30 bp direct repeats (Rössler *et al.*, 2004). The typical length of the recognition site of recombinases is 30 to 40 bp (Groth and Calos, 2004). The cluster IR-L is built up of 15 repeats and the cluster IR-R of 10 repeats and both are orientated in an inverse orientation. The first 8 nucleotides from the 5' end are highly conserved, whereas the following 22 nucleotides were less conserved and contain two to four mismatches. Int1 is able to use these repeat sequences to inverse the complete region. This reaction cannot only occur intramolecular but also intermolecular between two DNA molecules. Since the reaction is possible between different pairs of repeats, there are many different variants of the protein with different length. Hence, this reaction increases the genetic variability and produces many different proteins. This allows the virus to adapt to different conditions or to switch between patterns (Rössler *et al.*, 2004). In T-even phages like T4, the exchange of segments of the tail fibre protein allows to extent the host range of the phage. This leads to higher selective pressure on the tail fibre proteins than on the other genes of the genome (Tétart *et al.*, 1998).

An antibody against the viral particle recognizes both proteins from ORF34 and ORF36. Therefore, these proteins represent a part of the viral particle. However, only upstream of ORF34 is a promoter sequence located and only this protein is used for transcription. The inversion reaction exchanges the C-terminal part of the protein and causes the production of different tail fibre proteins. Gp34 could be first detected at the beginning of the stationary phase. Figure 5 shows the invertible region and the two possible orientations. The unchanged version is indicated by (+) and the inversed orientation by (-). Analysis showed that the (-) orientation could be detected by PCR over the whole life cycle. However, on day 3 and later the (+) orientation could also be detected after infection. This fact is explained either by the occurrence of different types in one cell or by different cells in the population, whereas one cell produces only one type (Klein *et al.*, 2012).

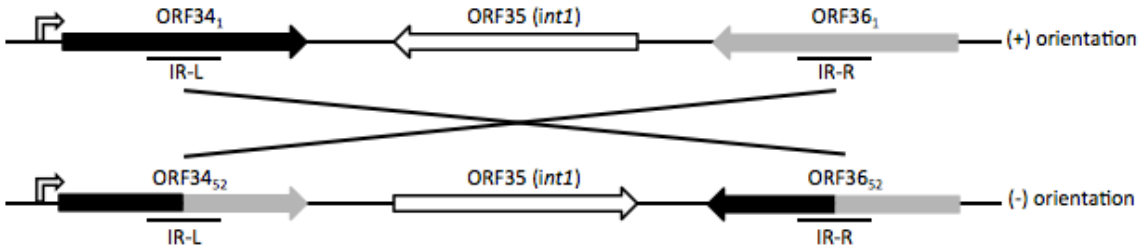

Figure 5: Invertible region of ϕCh1.
The invertase Int1 is able to exchange the 3'-ends of ORF34 and ORF36. The (+) orientation represents the unchanged situation, whereas the exchange version is represented by (-).

So far in all known tail fibre proteins the amino-terminal end connects the protein to the viral particle and the carboxy-terminal end contains the cell surface-binding domain (Casjens and Hendrix, 1988). Binding experiments with gp34 of both orientations showed, that only the (-) type gp34$_{52}$ with the ORF36 C-terminus is able to bind to the cell surface of *Nab. magadii*. Due to a galactose-binding domain, which is only present in C-terminus of ORF36, the virus is able to bind to the cell surface. Hence, the S-layer of *Nab. magadii* is very likely to be the target for the tail fibre protein. Further, the incubation of ϕCh1 with α-D-galactose reduces the infectivity according to the concentration. Other saccharides, e.g. α-D-lactose or α-D-glucose, do not have an influence on the infectivity. The galactose binds to the receptor and this prevents infection of the cells. Although, it is no other host for ϕCh1 known, usually phase variation systems are used to extend the host range (Klein *et al.*, 2012). The bacteriophages Mu or P1 use the switch in the production of their tail fibre proteins to infect other host cells. The genome of P1 related plasmid p15B codes for six possible tail fibre proteins and therefore might have six different hosts (Sandmeier *et al.*, 1992). In P1 the site-specific recombinase *cin* is responsible for the inversion of the C-segment. This enzyme is located directly upstream of the segment. On each end of the C-segment are the inverted repeats IR-L and IR-R and the crossover site *cix* present. Inversion of the C-segment changes the host range of P1. This system is similar to G-inversion in bacteriophage Mu (Hiestand-Nauer and Iida, 1983).

1.4.3 The lysogenic region

As mentioned above, ϕCh1 has a lysogenic and lytic life cycle. In the early exponential growth phase the virus exists as a provirus. At transition to the late exponential phase the lysis starts. The regulation of this transition is mediated by sequence specific DNA-binding proteins, which allow selective gene expression. These proteins typically have a helix-turn-helix structure (Pabo and Sauer, 1984). One of these repressors is ORF48 (*rep*), which is homologous to the ϕH repressor (Klein *et al.*, 2002). The repressor of ϕH is homologous to helix-turn-helix DNA-binding proteins and is able to block the transcription of major early lytic genes (Ken and Hackett, 1991; Stolt and Zillig, 1994). The protein of ϕCh1 ORF48 contains a winged helix-turn-helix-binding motif. This motif allows the sequence-specific contact to the DNA (Iro *et al.*, 2007).

Another possible regulator was discovered by the mutant strain *Nab. magadii* L11-1. Figure 6 shows the growth behavior of this strain. Strain L11-1 forms more plaques and the lysis starts earlier compared to the wild type strain. Analysis showed that the mutant strain differs from the wild type in the lysogenic region. In this strain ORF49 has a 223 nucleotides long duplication, which consists of a part of ORF49 and its upstream region. This results in an additional open reading frame ORF49' followed by ORF49. After some passages the mutation disappears and the lysis behavior is restored (Iro *et al.*, 2007). Infection of *Nab. magadii* L13, which produce gp49 constitutively from a plasmid, causes a reduction of the virus titer compared to the infection of the wild type strain. Experiments with shortened gp49 variants revealed that the N-terminus comprises the DNA binding domain and the C-terminus posses the repressor activity (Reiter, 2010).

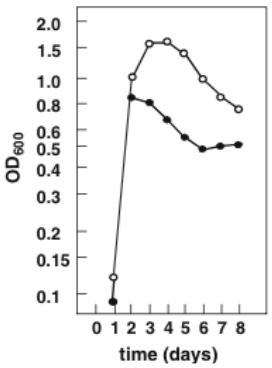

Figure 6: Growth curve of *Nab. magadii* L11 and L11-1.
The mutant strain L11-1 shows an earlier onset of lysis. The wild type cells start to lyse between day 4 and 5, but in the mutant strain it starts already at day 3 (Iro *et al.*, 2007).

The analysis of the expression pattern of ORF49 and ORF48 (*rep)* showed, that Rep is produced during the whole life cycle. However, ORF49 is expressed after 32 hours and then the level increases during virus development and leads to the production of particles and lysis (Figure 7). The two ORFs are orientated head-to-head with an intergenic region between. This region contains a promoter sequence and a ribosome-binding site (Iro *et al.*, 2007).

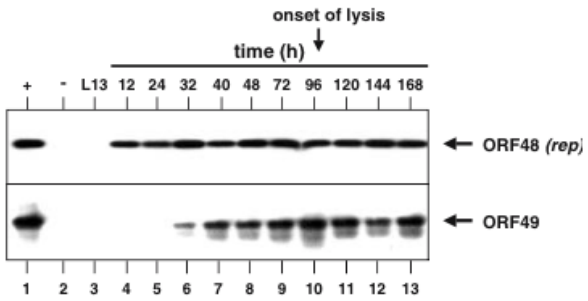

Figure 7: Expression pattern of ORF48 (*rep*) and ORF49.
ORF48 is constitutively expressed during the whole observed time. The expression of ORF49 starts after 32 hours and increases afterwards (Iro *et al.*, 2007).

Studies of the promoter activity showed, that Rep transcriptional suppresses the activity of the promoter (Iro *et al.*, 2007). In the virus ϕH the repressor binds to repeat sequences upstream of its own start codon and thereby may auto-regulate its own expression (Ken and Hackett, 1991). In the case of Rep of ϕCh1 the protein binds to a direct repeat within its own coding region. A mutation within the repeat sequence caused a loss of the repressing effect. So far it is not known what stops the repressing effect of Rep on ORF49. It is possible that a predicted site for self-cleavage of the repressor is responsible for the switch to the lytic life cycle (Iro *et al.*, 2007). This mechanism is known from the phage λ, where a stress response leads to recA-mediated cleavage of the repressor cI and causes the switch from lysogeny to lytic growth (Johnson *et al.*, 1981).

1.4.4 ORF43/44 – a putative toxin-antitoxin system

The ORF43 and ORF44 of ϕCh1 represent an operon. The stop and start codons of the ORFs overlap and therefore they will be co-transcribed and co-translated. Upstream of ORF43 is a promoter sequence located. Promoter studies in *Hfx. volcanii* showed, that

ORF43/44 has an enhancing effect on the ORF49 promoter. The plasmid contained the promoter from the intergenic region between ORF48 and ORF49, the reporter gene *bgaH*, ORF48 and ORF43/44. The enhancing effect was strongest with the construct containing the sequence of ORF48 with a mutated start codon. This could be explained by the absence of the repressor protein Rep and the binding of gp43/44 to a direct repeat within the coding sequence of ORF48. In this case the missing repressor and gp43/44 cause a higher expression from the ORF49 promoter. The enhancing effect was abolished with constructs, which did not contain ORF48 or the repeat sequence (Iro *et al.*, 2007). If ORF44 alone was present on the plasmid the reporter gene activity was completely eliminated in all cases. However, ORF43 alone causes an enhancement of the activity (Witte, in prep.).

Further experiments show, that ORF44 alone degrades ORF48 mRNA. In this experiment, a plasmid with ORF48, the promoter from the intergenic region and ORF44 was used. The amount of mRNA was monitored by RT-PCR. Although, the repressor *rep* was only expressed in a very low amount, there was no mRNA from the intergenic region detectable. Hence, gp44 also degrades this mRNA. The presence of gp44 also has an influence on the amount of plasmid DNA in *Hfx. volcanii*. (Witte, in prep.). Sequence analysis revealed homology of ORF44 to a VapC PIN domain nuclease.

VapBC toxin-antitoxin system

The VapBC (virulence associated proteins) operon represents a toxin-antitoxin (TA) system. There are two types of bacterial toxin-antitoxin systems. In type I TA systems the antitoxin is a small RNA, which prevents the translation of the toxin mRNA by binding to it. In type II the antitoxin is an unstable protein and inhibits the function of the toxin by binding to the protein. The VapBC TA system belongs to type II together with eight other systems, e.g. CcdB or MazF (Van Melderen and Saavedra De Bast, 2009). VapBC is one of the largest TA system families and nearly the halve of known loci are found in *Archaea* (Pandey and Gerdes, 2005). Type II systems are built up as an operon of two genes. The expression of this operon is transcriptionally regulated by autoregulation. The

downstream gene represents usually the toxin and the upstream gene the antitoxin (Van Melderen and Saavedra De Bast, 2009). In case of cellular stress, the antitoxin is proteolytically degraded and the toxin becomes active. Toxin-antitoxin systems have different functions. They are often involved in the regulation of important function in the cell (Daines *et al.*, 2007).

Another function is to prevent the loss of natural occurring plasmids. In this case the stable toxin, which is inherited to the daughter cell, will kill the cell if the plasmid is lost and no unstable inhibitor protein is newly produced. This is called post-segregational killing mechanism or addiction module (Gerdes *et al.*, 1986; Van Melderen *et al.*, 1994). The toxin VapC contains a PIN (PilT N terminus) domain. This domain has a nuclease activity and degrades RNA. Therefore, the protein can cause a retardation of the growth rate by cleavage of mRNA and inhibit the synthesis of proteins (Daines *et al.*, 2007). In stress situation, the slower growth and the degradation of existing transcripts will allow the cell to adapt to other conditions (Ramage *et al.*, 2009).

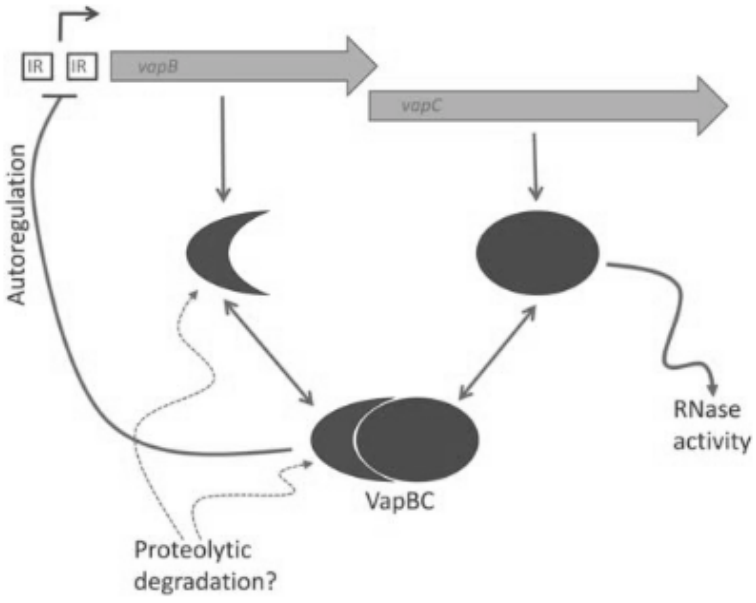

Figure 8: VapBC toxin-antitoxin system.
This TA system consists of two overlapping genes organized in an operon. The instable inhibitor VapB build a complex with the stable toxin VapC. The VapBC complex auto-regulates its own expression. The toxin becomes active if the inhibitor is degraded (Arcus *et al.*, 2010).

The PIN domain

The name PIN domain is derived from the sequence similarity to the N-terminal domain of a PiltT protein. PIN domains are small proteins with approximately 130 amino acids and are found frequently in different species in all three domains of life. Although the sequence similarity in the family is low, three acid amino acids are highly conserved. These three amino acids form the active site and coordinate an Mg^{2+} or Mn^{2+} ion. The PIN domain functions as a sequence specific ribonuclease and cleaves single-stranded RNA (Arcus *et al.*, 2010)

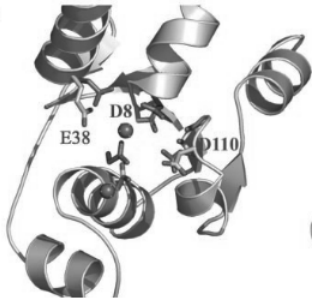

Figure 9: Active site of the PIN domain PAE2754.
Three-dimensional structure of the active site of ORF PAE2754 of *Pyrobaculum aerophilum*. The three acid amino acids, which coordinate the Mg^{2+} ion, are indicated (Arcus *et al.*, 2004).

1.4.5 ORF79 – a regulator of ϕCh1

The function of ORF79 was completely unknown because no homology to other proteins has been annotated (Klein *et al.*, 2002). Therefore, a deletion of ORF79 was created to study the function of this protein. In this mutant strain *Nab. magadii* L11-ΔORF79 the cells start to lyse earlier compared to the wild type cells (Selb, 2010). Accordingly, ORF79 may be involved in the regulation of the life cycle of ϕCh1. The production of the tail fibre protein gp34 and the major capsid protein E is altered in this strain. In the mutant strain protein E is produced after 43 hours, whereas in the wild type the production starts after 52 hours. So the regulation of this protein is only slightly influenced. However, the regulation of ORF34 is complete deregulated. The protein gp34 is produced through the whole life cycle. In the wild type strain this protein is produced approximately after 50 hours (Alte, 2011). ORF79 seems to have a critical role in the regulation of the tail fibre

protein. By complementing the deletion with a plasmid, constitutively producing gp79, the wild type situation was restored and the expression pattern of ORF34 and ORF11 was the same as could be seen within the lysogenic wild type strain *Nab. magadii* L11 (Beraha, 2013).

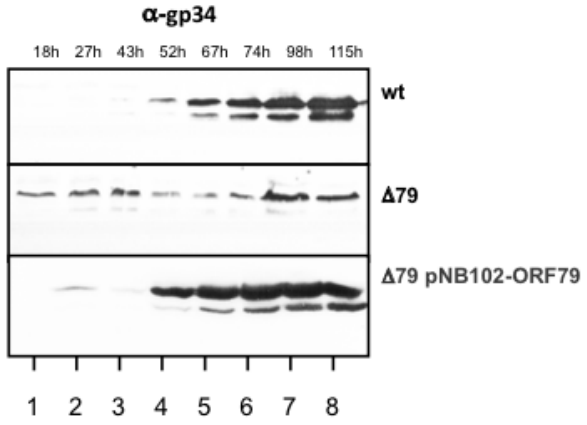

Figure 10: Expression pattern of ORF34 in the mutant strain *Nab. magadii* L11-ΔORF79.
In the wild type the protein is produced after 52 hours. The deletion of ORF79 caused a deregulation of the expression pattern. Complementing with the plasmid pNB102-ORF79 could restore the wild type situation (Schöner, 2013).

Another result, which emphasize the role of ORF79 as a regulator, showed that the infection of *Nab. magadii* L13 with ϕCh1-ΔORF79 lead to a higher virus titer than with the wild type ϕCh1. On the contrary, no plaques were obtained by infecting the strain *Nab. magadii* L13 containing the plasmid pNB102-p49-ORF79 neither with ϕCh1 nor ϕCh1-ΔORF79. In this strain ORF79 is constitutively expressed from the ORF49 promoter (Alte, 2011). The level of gp34 and E was also monitored in this strain. Protein E was not detectable and the amount gp34 was reduced. The effect of ORF79 on ORF34 was further characterized by a co-expression experiment in *Nab. magadii* L13. ORF79 was therefore cloned under an inducible promoter and ORF34 under its own promoter. In this situation the induction of the expression of ORF79 caused a repression of gp34, whereas in the not induced state gp34 was produced over the whole time. These results further confirm the role of ORF79 as a regulator of the life cycle of ϕCh1 (Schöner, 2013). In summary, the absence of ORF79 leads to a deregulated gene expression, earlier lysis and higher plaque formation, whereas constitutive expression to no plaques and repression of ORF34.

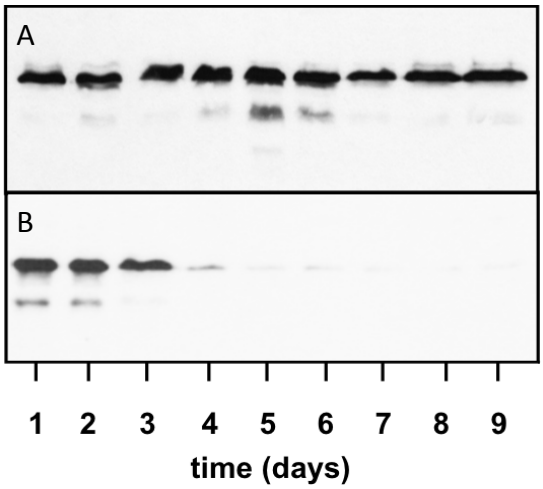

Figure 11: Co-expression of ORF79 and ORF34 in *Nab. magadii* L13.
Analysis of the expression pattern of ORF34 in *Nab. magadii* L13 (pRo5-ptnaN-ORF79/pNB102-$ORF34_{52}$). A: Expression of ORF79 is not induced. ORF34 is produced over the whole time. B: Expression of ORF79 is induced and the production of ORF34 decreases (Schöner, 2013 modified).

In this thesis I investigated the role of ORF79 as a regulator of ϕCh1 and its expression pattern in the wild type strain *Nab. magadii* L11. Further, I also showed the influence of different regulator proteins on the expression of ORF34. Another part of this work was the deletion of ORF49 of ϕCh1, which is involved in the regulation of lysis.

2 Materials

2.1 Strains

Escherichia coli

Strain	Genotype	Source
XL1-Blue	endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB$^+$ lacI^q Δ(lacZ)M15] hsdR17(r_K^- m_K^+)	Stratagene
Rosetta(DE3)pLysS	F^- ompT $hsdS_B$(R_B^- m_B^-) gal dcm λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) pLysSRARE (Cam^R)	Novagen
BL21(DE3)pLysS	F^- ompT gal dcm lon $hsdS_B$(r_B^- m_B^-) λ(DE3) pLysS(cm^R)	Stratagene

Natrialba magadii

Strain	Genotype	Source
L11	wild type, carry prophage ϕCh1	Witte *et al.*, 1997
L13	cured strain, not infected by ϕCh1	Witte *et al.*, 1997

2.2 Media

2.2.1 LB

Peptone	10 g/l
Yeast extract	5 g/l
NaCl	5 g/l
pH 7.2	
Agar for plates	15 g/l

2.2.2 Rich medium for *Nab. magadii* (NVM)

Casaminoacid	8.8 g/l
Yeast extract	11.7 g/l
Trisodium citrate	0.8 g/l
KCl	2.4 g/l
NaCl	235.0 g/l
pH 9.5	
Agar for plates	8 g/l
Agar for soft agar plates	4 g/l

After autoclaving the medium is complemented with:

Na_2CO_3	37 mM
$MgSO_4$	1 mM
$FeSO_4$	20 µM

2.2.3 Minimal medium for *Nab. magadii* (NMMb)

NaCl	3.5 M
KCl	27.0 mM
Na_2HPO_4	2.0 mM
NaH_2PO_4	2.0 mM
Alanine	25.0 mM
Leucine	5.0 mM
Arginine	5.0 mM
Histidine	5.0 mM
Lysine	5.0 mM
Trisodium citrate	2.7 mM
Sodium acetate	20.0 mM
Sodium pyruvate	10.0 mM

pH 9.0-9.5

After autoclaving the medium is complemented with:

Na_2CO_3	175 mM
$MgSO_4$	1 mM
$FeSO_4$	5 µM
1000 x Trace elements	1 ml

1000 x Trace elements

$MnCl_3$	4 mM
$CaCl_2$	3 mM
$CuSO_4$	4 mM
$ZnSO_4$	3 mM

2.3 Antibiotics and additives

Substance	Used concentration	Note
Ampicillin	100 µg/ml	dissolved in ddH_2O, sterile filtered, stored at 4 °C
Tetracycline	10 µg/ml	dissolved in ½ vol. ddH_2O, ½ vol. 96 % Ethanol added, stored at 4 °C
ITPG	0.5-1 mM	dissolved in ddH_2O, stored at -20 °C
X-Gal	40 µg/ml	dissolved in DMF, stored at -20 °C
Novobiocin	3 µg/ml	dissolved in ddH_2O, sterile filtered, stored at -20 °C
Mevinolin	6 µg/ml	isolated from pulverized tablets, dissolved in 96 % Ethanol, stored at -20 °C
Bacitracin	70 µg/ml	dissolved in ddH_2O, sterile filtered, stored at 4 °C
Tryptophan	2 mM	dissolved in 1 M NaOH, stored at -20 °C

2.4 Primer

Primer	Sequence	Restriction site	Tm [°C]
Δrep-13	5'-AATTGGATCCATGTACTCCGGGCCGAG-3'	*Bam*HI	62.5
Δrep-23	5'-AATTAAGCTTTCGTCGACGAGTTGATCG-3'	*Hind*III	62.5
12-7-3	5'-CAGCAGAAGCTTTCATCCTGCGGTTTCG-3'	*Hind*III	60.7
12-7-5	5'-CAGCAGGGATCCATGAACACCCCCAATAGACAC-3'	*Bam*HI	61.3

Primer	Sequence	Restriction site	Tm [°C]
34-3	5’-CAGCAGAAGCTTCAGATCAGGTTTATATTGCTGAAGT-3’	*Hind*III	60.2
34-Kpn	5’-CAGCAGGGTACCCGGCGTTCGAGGTCA-3’	*Kpn*I	62.2
36-3X	5’-GCAGTCTAGACCATCGGTTATTCGAGTTTC-3’	*Xba*I	59.7
43-5	5’-CAGCAGTCTAGACGTTGTGCCAGCCGT-3’	*Xba*I	62.1
43-Hind	5’-CAGCAAGCTTTCATTCGCGCTCG-3’	*Hind*III	55.5
44-Hind	5’-CAGCAAGCTTGATTTAGGACTCGAGGACC-3’	*Hind*III	56.4
49-Kpn	5’-CAGCGGTACCTTGCGTTCAGTTCCG-3’	*Kpn*I	56.2
79-N2	5’-GTGGGTACACCGCTTTCC-3’	-	61.8
BAD24-3-C	5’-GTTCATCGATCTTCTCTCATCCGCCA-3’	*Cla*I	56.9
BAD24-5-C	5’-GATCATCGATGCACGGCGTCACAC-3’	*Cla*I	57.7
D49-1	5’-GACCGGATCCCGATGATCAATCGAAGCG-3’	*Bam*HI	65.8
D53-1	5’-GACCGAATTCGGATGCAAGCTGCTCGTGG-3’	*Eco*RI	62.7
DR-R-Xba	5’-GCTATCTAGAGTAGGCCGTCTCGGACGT ACTTACCGCGTCCATGCCATCGCAACGAAC-3’	*Xba*I	58.4
Int-3-C	5’-GTTCATCGATTCAGCCGTAGATCTCGAG-3’	*Cla*I	57.9
Int-E-3	5’- GATTGGACTTGGTACCTCAGCCGTAGATCTCGAGTA-3’	*Kpn*I	58.9
Int-E-5	5’- GATCCATATGCATCATCATCATCA TCACATGTCCAAAGAGAGACATGC-3’	*Nde*I	58.0
IR-L-Kpn	5’-GACTGGTACCCATATGGACGCGGTGAGC GACTCCCAGACGGCACGCCGAGGAGGTTCT-3’	*Kpn*I	58.6
IR-M1	5’-GCTATCTAGAGTAATAGACGCGGTAAGT ACGTCCGAGACGGCCGCCATCGCAACGAAC-3’	*Xba*I	58.4
IR-M11	5’-GCTATCTAGAGTAATGGAAGCGGTAAGT ACGTCCGAGACGGCCGCCATCGCAACGAAC-3’	*Xba*I	58.4
IR-M4	5’-GCTATCTAGAGTAATGAACGCGGTAAGT ACGTCCGAGACGGCCGCCATCGCAACGAAC-3’	*Xba*I	58.4
IR-M7	5’-GCTATCTAGAGTAATGGTCGCGGTAAGT ACGTCCGAGACGGCCGCCATCGCAACGAAC-3’	*Xba*I	58.4
IR-R-Xba	5’-GCTATCTAGAGTAATGGACGCGGTAAGT ACGTCCGAGACGGCCGCCATCGCAACGAAC-3’	*Xba*I	58.4
IR-R-XbaΔ	5’-GCTATCTAGAGTAATGGACGCGCCATCG GCCATCGCAACGAAC-3’	*Xba*I	58.4
MevR-5	5’-GTCGTGATGGCGTTCGC-3’	-	65.6
MevR-6	5’-GAGACGTAGAGGTCGCCG-3’	-	62.4
MevR-7	5’-GAACCGACGGCGATGC-3’	-	65.5
MT-Kpn-5	5’-GAATGGTACCGCGAGTCGGACAACGTTC-3’	*Kpn*I	63.4
MT-Xba-3	5’-GATCTCTAGATCACTCATTATCACCGGCGT-3’	*Xba*I	64.2
NB-1	5’-TCTACCGGGTGCTGAACG-3’	-	63.7
NB-2	5’-CGCTGATGTACGAACCGAG-3’	-	63.2
NB-3	5’-GCCACAAGTTCCTCGC-3’	-	59.2
Nov-11	5’-GCATGTCGTGGCTGTTCG-3’	-	65.6
Nov-12	5’-GCCGGTGAGTACTTAACGC-3’	-	65.1

Primer	Sequence	Restriction site	Tm [°C]
Nov-13	5'-GACGCCGAATGGGTAGAC-3'	-	61.9
P28-	5'-GAGCCGTGTTCGTTCTG-3'	-	59.2
P28+	5'-TCTGGCCTGAATGACGA-3'	-	60.3
pQE-T	5'-GCTTTGCATCGCATACAGGAT-3'	-	64.9
PR-2K	5'-GACGACGGTACCTCCTGGGCCTCTTTG-3'	*Kpn*I	56.7
PR-3H	5'-GACGACAAGCTTGATGCGATCTCCTCTGG-3'	*Hind*III	58.0
repc-2	5'-CAGCAGCTGCAGAGTGTCTGTGCTGTCGATGCC-3'	*Pst*I	66.6
SC-2	5'-AGATCTTCACCGTGCTCACG-3'	*Bgl*II	55.0
Soj-3	5'-CAGCAGCTGCAGCAGCAGTCAGCCATGGAATCCCT-3'	*Pst*I	60.0
Soj-5	5'-GCAGCAAGATCTATCGGAGTTACCAACCAGAAA-3'	*Bgl*II	61.1
Soj-Hind	5'-GATCAAGCTTGATCTTCCCACGGGTTGC-3'	*Hind*III	64.3
Soj-Kpn	5'-GATCGGTACCCGCGTTGCTCATCTGTTTG-3'	*Kpn*I	64.4
Tna-N-1	5'-GATTAAGCTTCTGAGGAATCGACCGGTT-3'	*Hind*III	61.5

2.5 Plasmids

Plasmid/Construct	Features	Source
pBAD24	*bla*, *araC*, *rrnB*, mcs, PBAD promoter, pBR322 ori	Guzman *et al*., 1995
pBAD24-ptnaN	pBAD24 with tryptophanase promoter	Alte, 2011
pBAD24-ptnaN-int1-his	pBAD24 with ϕCh1 ORF35 (*int1*) under tryptophanase promoter and 6 x His-tag	This thesis
pBluescript II KS(+)	mcs, *bla*, ColE1 ori, *lacZa*	Stratagene
pET-E-his	pET with ORF11 (E) and 6 x His-tag	Klein *et al.*, 2000
pKSIIΔ49-Nov^{R}T Orientation 1	pKSIIΔ49$_{1-4}$ with novobiocin resistance cassette and terminator sequence in ϕCh1 ORF49 orientation	This thesis
pKSIIΔ49-Nov^{R}T Orientation 2	pKSIIΔ49$_{1-4}$ with novobiocin resistance cassette and terminator sequence in inverse ϕCh1 ORF49 orientation	This thesis
pKSIIΔ49$_{1-4}$	pBluescript II KS(+) with up- and downstream flanks of ϕCh1 ORF49	Svoboda, 2011
pMDS11	*bla*, ColE1 ori, *gyrB* (nov^R), pHK2 ori	Holmes *et al.*, 1991
pNB102	*bla*, ColE1 ori, *hmg* (Mev^R), pNB101 ori	Zhou *et al.*, 2004
pNB102-Bgb34$_1$	pNB102 with ϕCh1 ORF34$_1$	Till, 2011
pNB102-Bgb34$_{52}$	pNB102 with ϕCh1 ORF34$_{52}$	Till, 2011
pNB102-int1-DR-R	pNB102-ptnaN-int1-his with repeat IR-L and DR-R	This thesis
pNB102-int1-IR-M1	pNB102-ptnaN-int1-his with repeat IR-L and IR-M1	This thesis
pNB102-int1-IR-M11	pNB102-ptnaN-int1-his with repeat IR-L and IR-M11	This thesis
pNB102-int1-IR-M4	pNB102-ptnaN-int1-his with repeat IR-L and IR-M4	This thesis

Plasmid/Construct	Features	Source
pNB102-int1-IR-M7	pNB102-ptnaN-int1-his with repeat IR-L and IR-M7	This thesis
pNB102-int1-IR-R	pNB102-ptnaN-int1-his with repeat IR-L and IR-R	This thesis
pNB102-int1-IR-RΔ	pNB102-ptnaN-int1-his with repeat IR-L and IR-RΔ	This thesis
pNB102-mtase	pNB102 with ϕCh1 ORF94 (*mtase*)	This thesis
pNB102-ptnaN-int1-his	pNB102 with ϕCh1 ORF35 (*int1*) under tryptophanase promoter and 6 x His-tag	This thesis
pNB102-ptnaN-ORF79	pNB102 with ϕCh1 ORF79 under tryptophanase promoter	Schafellner, 2012
pQE-30	*bla*, ColE1 ori, N-terminal Poly(His)6-tag	QIAGEN
pQE30-Nov^{R}T	pQE30 with novobiocin resistance cassette and terminator sequence	This thesis
pQE30-ORF34	pQE30 with ORF34$_{52}$ of ϕCh1	Rössler *et al.*, 2004
pRep4	Neo^R/Kan^R, *lacI*, p15A ori	QIAGEN
pRo-5	*bla*, ColE1 ori, *gyrB* (Nov^R), ϕCh1 derived ori	Mayrhofer-Iro *et al.*, 2013
pRo5-48	pRo5 with ϕCh1 ORF48 (*rep*)	Meissner, 2008
pRo5-49	pRo5 with ϕCh1 ORF49	Meissner, 2008
pRo5-49’	pRo5 with mutated ϕCh1 ORF49 version	Meissner, 2008
pRo5-P$_{43}$-43	pRo5 with ϕCh1 ORF43 under ϕCh1 ORF43 promoter	Meissner, 2008
pRo5-P$_{43}$-43/44	pRo5 with ϕCh1 ORF43/44 under ϕCh1 ORF43 promoter	Meissner, 2008
pRo5-P$_{43}$-44	pRo5 with ϕCh1 ORF44 under ϕCh1 ORF43 promoter	Meissner, 2008
pRo5-soj	pRo-5 with ϕCh1 ORF46 (*soj*)	This thesis
pRo5-ptnaN-ORF79	pRo5 with ϕCh1 ORF79 under tryptophanase promoter	Schafellner, 2012
pRSET-A	mcs, *bla*, EK, PT7, RBS, His-tag, pUC ori, f1 ori	Invitrogen
pRSETA-soj	pRSET-A with ϕCh1 ORF46 (*soj*)	This thesis

2.6 Enzymes

Name	Company	Product number	Used buffer
FastDigest *Bam*HI	Thermo Scientific	FD0054	1x FastDigest buffer
FastDigest *Bgl*II	Thermo Scientific	FD0083	1x FastDigest buffer
FastDigest *Eco*32I (*Eco*RV)	Thermo Scientific	FD0303	1x FastDigest buffer
FastDigest *Eco*RI	Thermo Scientific	FD0274	1x FastDigest buffer
FastDigest *Hind*III	Thermo Scientific	FD0504	1x FastDigest buffer
FastDigest *Kpn*I	Thermo Scientific	FD0524	1x FastDigest buffer
FastDigest *Pst*I	Thermo Scientific	FD0614	1x FastDigest buffer

Name	Company	Product number	Used buffer
FastDigest *Sma*I	Thermo Scientific	FD0663	1x FastDigest buffer
FastDigest *Xba*I	Thermo Scientific	FD0684	1x FastDigest buffer
*Bsu*15I (*Cla*I)	Thermo Scientific	ER0141	1x Tango buffer
*Eco*91I (*Bst*EII)	Thermo Scientific	ER0391	1x O buffer
*Nhe*I	Thermo Scientific	ER0971	1x Tango buffer
Pfu DNA Polymerase	Promega	M7741	1x Reaction buffer
Go*Taq*®	Promega	M3001	1x Go*Taq*® Reaction buffer
Go*Taq*® Green Master Mix	Promega	M7123	1x Go*Taq*® Green Reaction buffer
FastAP Thermosensitive Alkaline Phosphatase	Thermo Scientific	EF0654	1x FastDigest buffer
Klenow Fragment	Thermo Scientific	EP0051	1x Reaction buffer
T4 DNA Ligase	Promega	M1801	1x T4 DNA Ligase buffer
Proteinase K	QIAGEN	19133	-

2.7 Size Markers

Name	Company	Product number	Size of bands
Lambda DNA BstEII Digest			Size of fragments in bp: 8454, 7242, 6369, 5686, 4822, 4324, 3675, 2323, 1929, 1371, 1264, 702
GeneRuler™ 1kb DNA Ladder	Thermo Scientific	SM0311	10000 to 250 bp
Unstained Protein Molecular Weight Marker	Thermo Scientific	26610	Size in kDa: 116.0, 66.2, 45.0, 35.0, 25.0, 18.4, 14.4
PageRuler Prestained Protein Ladder	Thermo Scientific	26616	Size in kDa: 170, 130, 100, 70, 55, 40, 35, 25, 15, 10

2.8 Kits

Name	Company	Product number
GeneJET Plasmid Miniprep Kit	Thermo Scientific	K0503
GeneJET PCR Purification Kit	Thermo Scientific	K0701
QIAquick Gel Extraction Kit	QIAGEN	28706
SuperSignal West Pico Chemiluminescent Substrate	Thermo Scientific	34080
Phototope®-Star Detection Kit	New England BioLabs	N7020S

2.9 Antibodies

Name	Target	Dilution	Source
α-Soj (from rabbit)	Soj of ϕCh1	1:250	This thesis
α-gp79 (from rabbit)	gp79 of ϕCh1	1:100	This thesis
α-gp34 (from rabbit)	gp34 of ϕCh1	1:2500	Till, 2011
α-Mtase (from rabbit)	M.*Nma*ϕCh1I	1:1000	Baranyi *et al.*, 2000
α-E (from rabbit)	E of ϕCh1	1:1000	Klein *et al.*, 2000
Penta-His Antibody (from mouse)	His-tag	1:500	QIAGEN (product number: 34660)
ECL Anti-mouse IgG, horseradish peroxidase linked (from sheep)	mouse IgG	1:5000	GE Healthcare (product number: NA931)
ECL Anti-rabbit IgG, horseradish peroxidase linked (from donkey)	rabbit IgG	1:5000	GE Healthcare (product number: NA934)

3 Methods

3.1 DNA methods

3.1.1 Agarose gel electrophoresis

Separation of DNA of different size was achieved by electrophoresis in 0.8 % agarose gel in 1x TAE buffer. The sample was mixed with 5x DNA loading buffer. Afterwards, the DNA was visualized by ethidium bromide staining and exposed to UV light.

50x TAE	
Tris/HCl pH 8.2	2.0 M
Acetic acid	1.0 M
EDTA	0.1 M

5x DNA loading buffer	
Tris/HCl pH 8.2	50.0 mM
Sucrose	25.0 %
SDS	0.1 %
Bromphenol blue	0.05 %
Xylene cyanol	0.05 %

3.1.2 Polymerase Chain Reaction

For preparative PCR the *Pfu* polymerase was used due to the 3'-5' exonuclease or proofreading activity. Analytical PCRs were performed with GoTaq polymerase since no high accuracy is necessary. The annealing temperature was calculated with the program GeneRunner (Version 3.05, Hastings Software, Inc.). The extension time depends on the polymerase and is for the *Pfu* polymerase 2 min/1 kb and for the GoTaq 1 min/1 kb.

Batch for *Pfu* polymerase (100 µl)	
Forward primer (0.05 µg/µl)	5 µl
Reverse primer (0.05 µg/µl)	5 µl
10x Reaction buffer	10 µl
2 mM dNTP mixture (0.2 mM)	10 µl
Template DNA	1 µl
Pfu Polymerase (2-3 U/µl)	2 µl
ddH_2O	67 µl

Batch for GoTaq (25 µl)	
2x GoTaq Green Master Mix	12.5 µl
Forward Primer (0.1 µg/µl)	2.5 µl
Reverse Primer (0.1 µg/µl)	2.5 µl
Template DNA	1.0 µl
ddH_2O	6.5 µl

PCR program

Step	Temperature [°C]	Duration [min]	Number of cycles
Initial denaturation	95	4	1
Denaturation	95	1	35 (preprative) 20 (analytical)
Annealing	Tm	1	
Extension	72	t	
Final extension	72	2 x t	1

Tm … calculated annealing temperature, t … calculated extension time

3.1.3 Cell extract for PCR template

To allow a fast screening of cultures a crude cell extract of *E. coli* or *Nab. magadii* was used. The cell extract was directly taken as PCR template.

E. coli:

To 100 µl ddH_2O were 5 µl culture added and heated for 10 min at 95 °C.

Nab. magadii:

From the culture 50 µl were centrifuged (3 min, 13 krpm) and the pellet was resuspended in 100 µl ddH_2O

3.1.4 DNA purification, restriction and modification

Purification

DNA was purified after PCR or restriction with GeneJET PCR Purification Kit. DNA fragments of a particular size were purified by agarose-gel electrophoresis and gel elution. The gel elution was performed with the QIAquick Gel Extraction Kit.

Restriction

Restriction of DNA was performed with enzymes from Thermo Scientific according the manufactures protocol with some modifications in certain cases. The incubation time was 45 min for FastDigest enzymes and 4 hours to overnight for conventional enzymes. For most batches FastDigest enzymes was used because of the easier handling of double digests.

Dephosphorylation

Dephosphorylation of 5' ends of the linearized vector was performed in blunt end cloning to avoid re-ligation of the plasmid. For this purpose, Fast-AP enzyme was added to the restriction digest for the last 10 min incubation time, since this enzyme has 100 % activity in 1x FastDigest buffer.

Filling of 5'-overhang

To fill the 5'-overhang produced by restriction was necessary in blunt end ligation. For this purpose the Klenow fragment was used. The reaction was carried out for 10 min at 37 °C according the suggestions from Thermo Scientific.

Ligation

The ligation reaction was performed with T4 DNA ligase from Promega according the protocol from the manufacturer with an insert to vector ration of 3:1. Blunt end ligation reactions were incubated over night at 16 °C and reactions with sticky ends 3 hours at room temperature or over night at 4 °C.

3.2 Transformation of *E. coli*

3.2.1 Competent cells

For producing competent *E. coli* cells 400 ml overnight culture were inoculated at an OD_{600} of 0.1. At an OD_{600} of 0.6 the cells were harvested by centrifugation. The pellet was resuspended in 160 ml MOPS I and incubated for 10 min on ice. After centrifugation the pellet was resuspended in 160 ml MOPS II and incubated for 30 min on ice. After the last centrifugation step the pellet was resuspended in 8 ml MOPS IIa and 100 µl aliquots were stored at -80 °C. Centrifugation was done for 15 min at 6 krpm at 4 °C.

MOPS I

MOPS	100 mM
$CaCl_2$	10 mM
RbCl	10 mM
pH 7 adjusted with KOH	

MOPS II

MOPS	100 mM
$CaCl_2$	70 mM
RbCl	10 mM
pH 6.5 adjusted with KOH	

MOPS IIa

MOPS	100 mM
$CaCl_2$	70 mM
RbCl	10 mM
Glycerol	15 %
pH 6.5 adjusted with KOH	

3.2.2 Transformation

The complete ligation reaction batch was added to the competent cells, which were thawed 10 min on ice before. The cells were incubated for 30 min on ice followed by a heat shock for 2 min on 42 °C. After short incubation on ice 300 µl LB were added and the cells were regenerated 30 min at 37 °C. Then the cells were plated on LB-agar plates.

3.2.3 Quick prep for screening

This method allows a quick screening of a larger amount of transformed *E. coli* cells to reduce the number of candidates. Therefore, 300 µl over night culture was centrifuged at 13 krpm for 5 min. The pellet was resuspended in 30 µl 5x DNA sample buffer and 14 µl 1:1 phenol/chloroform was added. The cells were vortexed and after centrifugation for 5 min at 13 krpm 12 µl of the supernatant was applied to an agarose gel.

3.2.4 Plasmid extraction

The extraction of plasmids from *E. coli* was performed from fresh over night cultures with the GeneJET Plasmid Miniprep Kit according to the manufacturer protocol.

3.3 Transformation of *Nab. magadii*

3.3.1 Competent cells

Cells were grown with 70 µg/ml bacitracin overnight to an OD600 of 0.5 to 0.6. The cells were harvested by centrifugation for 15 min at 6 krpm and room temperature. The pellet was resuspended in ½ volume buffered spheroblasting solution high salt with glycerol and 0.1 % Proteinase K was added. The culture was incubated on 42 °C until cells become spheroblasts.

Buffered spheroblasting solution high salt with glycerol

Tris-HCl pH 9.5	50 mM
NaCl	2 M
KCl	27 mM
Glycerol	15 %

After autoclaving 15 % sucrose (sterile filtered) was added

3.3.2 Transformation

1.5 ml of competent spheroblasts were centrifuged for 3 min at 10 krpm and room temperature. The pellet was resuspended in 150 µl spheroblasting solution high salt without glycerol and 15 µl 0.5 M EDTA was added. After incubation for 10 min at room temperature 10 µl DNA (5-10 µg) at maximum was added and the cells were incubated for 5 min at room temperature. 150 µl of 60 % PEG 600 and 40 % unbuffered spheroblasting solution high salt was added followed by 30 min incubation at room temperature.

Then 1 ml medium were added to the cells and centrifuged 5 min at 10 krpm at room temperature to wash the PEG 600 away. The washing step was repeated with 1 ml medium and then the pellet was resuspended in 1 ml medium. The spheroblasts were regenerated at 37 °C with agitation up to two days until the cells had their normal rod shape. Every day during the regeneration phase, the medium was changed. Afterwards the cells were plated on rich medium agar plates.

Buffered spheroblasting solution high salt

Tris-HCl pH 8.0	50 mM
NaCl	2 M
KCl	27 mM

After autoclaving 15 % sucrose (sterile filtered) was added

Unbuffered spheroblasting solution high salt

NaCl	2 M
KCl	27 mM

After autoclaving 15 % sucrose (sterile filtered) was added

3.3.3 Screening of positive transformants

To allow a fast screening of colonies after the transformation, 700 µl rich medium was inoculated with colonies from the plates in Eppendorf tubes. Since *Nab. magadii* is obligatory aerobe the cultures has to be supplied with fresh air every 1-2 days. Once the cells start to grow a cell extract was prepared and the cultures were tested by PCR.

3.3.4 Plasmid isolation

Plasmids were extracted from 3 ml of a growing culture. The sample was centrifuged 3 min at 13 krpm and the pellet was resuspended in 50 µl solution 1. Cells were lysed by addition of 200 µl solution 2 and gently mixed by inversion. After incubation for 5 min at room temperature, 150 µl of solution 3 were added and again gently mixed by inversion. The sample was centrifuged 10 min at 13 krpm at 4 °C and the supernatant was transferred into a new Eppendorf tube. The DNA was precipitated by 1x volume 2-propanol and incubation of 2 min. After centrifugation for 30 min at 13krpm and 4 °C the pellet was washed twice with 1 ml 70 % ethanol. The pellet was air-dried and dissolved in 30 µl ddH_2O.

Solution 1

NaCl	2.0 M

Solution 2

NaOH	0.2 M
SDS	1.0 %

Solution 3

Potassium acetate	5.0 M

3.4 Southern Blot

Synthesis of the probe

The probes were synthesized by PCR and marked with biotinylated dUTP (Bio-11-dUTP, GeneON). After amplification, the probes were purified by gel extraction. The purity and the concentration were controlled by agarose-gel electrophoresis.

PCR batch:

2x GoTaq Green Master Mix	50.0 µl
Forward primer (0.1 µg/µl)	10.0 µl
Reverse primer (0.1 µg/µl)	10.0 µl
Bio-11-dUTP (25 µmol)	2.5 µl
ddH_2O	26.5 µl
Template DNA	1.0 µl
	100.0 µl

Blotting the DNA to the membrane

The target DNA was applied on an agarose gel and then visualized by ethidium bromide. Before the blotting process the gel was incubated for 30 min in 0.25 M HCl and for 30 min in 0.4 M NaOH/0.6 M NaCl. The first step facilitates transferring larger DNA fragments to the membrane and the second step denatures the DNA. The neutralization was done for 30 min in 1.5 M NaCl/0.5 M Tris-HCl pH7.5. The DNA was transferred to Amersham Hybond nylon membrane (GE Healthcare) by capillary blotting over night with 10x SSC. After the blotting step the membrane was incubated in 0.4 M NaOH for 1 min and 0.2 M Tris-HCl pH 7.5 also for 1 min. The DNA was fixed by UV-cross-linking.

Pre-hybridization and Hybridization

To prevent unspecific binding the membrane was incubated in hybridization buffer with salmon sperm (0.1 mg/ml) for 3 hours at 65 °C. Before the hybridization the probe was denatured at 95 °C for 5 min. Afterwards the probe was added to the membrane and hybridized at 65 °C overnight.

Development of the blot

The membrane was washed twice with 2x SSC/0.1 % SDS for 5 min at room temperature and twice with 0.1x SSC/0.1 % SDS (pre-warmed) at 65 °C for 15 min. The blot was then developed with Phototope®-Star Detection Kit and exposed to an x-ray film (Amersham Biosciences)

20x SSC	
NaCl	3.0 M
Sodium citrate	0.3 M
pH 7.2	

50x Denhardt's solution	
Ficoll 400	0.1 g/l
Polyvinylpyrolidone	0.1 g/l
BSA	0.1 g/l

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

Blocking solution	
NaCl	125.0 mM
Na_2HPO_4	17.0 mM
NaH_2PO_4	8.0 mM
SDS	0.5 %
ph 7.2	

1x Wash solution I
1:10 dilution of blocking solution

10x Wash solution II	
Tris-base	100 mM
NaCl	100 mM
Mg_2Cl*6H_2O	10 mM

3.5 Construction of a *Nab. magadii* homozygote deletion mutant by passaging

This method was used to obtain a *Nab. magadii* homozygote deletion mutant by applying a constant selection pressure. This is necessary because *Nab. magadii* has up to 80 copies of its genome. Therefore, the heterozygote transformants were grown in rich medium with novobiocin to exponential growth phase (OD_{600} at approx. 1.0) and then 200 µl of the culture were transferred to 20 ml fresh medium with novobiocin. Since, the deletion cassette contains a novobiocin resistance gene, this selects for cells, which have more

copies of the deleted gene. Every fifth passage the culture was tested for homozygote mutants.

3.6 ϕCh1 methods

3.6.1 Isolation of virus particles

To isolate viral particles *Nab. magadii* L11 culture was grown at 37 °C until lysis was completed. Afterwards, the culture was centrifuged 20 min at 8 krpm and room temperature. The particles were precipitated with PEG-6000. Therefore, 10 % w/v PEG-6000 was added to the supernatant and dissolved overnight by stirring at room temperature. The ϕCh1 particles coated with PEG-6000 was centrifuged at 7 krpm for 30 min at room temperature. The pellet was resuspended in high salt alkaline solution (4 M NaCl, 50 mM Tris/HCl, pH 9.5).

The purification of the particles was achieved by a discontinuous CsCl density gradient centrifugation. The gradient was obtained by 2 ml CsCl solution 1.5 on the bottom overlaid by 5 ml CsCl solution 1.3 and 6 ml phage suspension. The tubes were filled up with CsCl solution 1.1 and centrifuged for 20 hours at 30 krpm at room temperature. After isolation the band containing the viral particles was isolated. A continuous CsCl was used to concentrate and purify the ϕCh1 particles further. The particles were mixed with CsCl solution 1.3 and again centrifuged at 30 krpm for 20 hours at room temperature. The isolated particles were dialyzed twice against high salt alkaline solution first for one hour and then overnight.

3.6.2 Plaque assay

Dilutions of the supernatant of a lysed *Nab. magadii* L11 culture or of purified ϕCh1 particles were prepared, usually 10^{-4}, 10^{-6}, 10^{-8}, 10^{-10}. 100 µl of the dilution were mixed with 300 – 500 µl well growing *Nab. magadii* L13 cells in 5 ml rich medium soft agar at 55 °C. Then the soft agar was poured onto a rich medium agar plate. After the agar

become solid the plates were incubated at 37 °C in sealed plastic bags. After one week, the number of plaques was counted and the pfu/ml was calculated.

3.6.3 Infection of *Nab. magadii*

After complete lysis of the culture, the sample was centrifuged for 20 min at 10 krpm and room temperature. The supernatant was sterile filtered and one drop chloroform was added to avoid growth of remaining cells. 1 ml of well growing *Nab. magadii* L13 (OD_{600} of 0.4-0.8) were infected with 100 respectively 250 µl supernatant. To allow adsorption of the virus particle to the cell surface the culture was incubated for 45 min at 37 °C without agitation. Then the cells were plated on rich medium agar plates.

3.7 Protein methods

3.7.1 Preparation of crude protein extract

Crude protein extracts from *E. coli* or *Nab. magadii* were used for detection of production of certain proteins. Therefore, 1.5 ml culture was centrifuged (3 min, 13 krpm) and the pellet was resuspended x µl sodium-phosphate-buffer and x µl Laemmli buffer (OD of culture x 75 = x µl). Whereas the *E. coli* samples could be directly heated for 10 min to 95 °C the samples from *Nab. magadii* has to be incubated at 37 °C until the pellet has dissolved. Since *Nab. magadii* contains up to 50 copies of its chromosome the large amount of DNA has to be degraded to allow pipetting the sample. Then the probes were loaded onto a SDS-PAGE gel.

2x Laemmli sample buffer	
Tris-HCl pH 6.8	60 mM
SDS	2 %
Glycerol	10 %
β-mercaptoethanol	5 %
Bromphenol blue	0.01 %

Sodium-phosphate buffer	
1 M Na_2HPO_4	46.3 ml
1 M NaH_2PO_4	53.7 ml
pH 6.8	

3.7.2 SDS-PAGE

The separation of proteins according to their size was achieved by a discontinuous polyacrylamid gel electrophoresis. The stacking gel contains 4 % polyacrylamid whereas the separating contains 12 %. Separation of the sample was performed at 40 V at the beginning and after the sample entered the separating gel the voltage was shifted to 100 V for proteins from *E. coli* and 80 V for *Nab. magadii* proteins. To visualize the proteins the gel was stained with coomassie staining solution. The gel was destained with destaining solution so that only the proteins remained stained.

10x SDS Running buffer	
Tris-base	0.25 M
Glycin	1.92 M
SDS	1.0 %

Separating gel buffer	
Tris-HCl pH 8.8	1.5 M
SDS	0.4 %

Stacking gel buffer	
Tris-HCl pH 6.8	0.5 M
SDS	0.4 %

30 % Acrylamide	
Acrylamide	29 %
N,N'-methylenebisacrylamide	1 %

Destaining solution	
Methanol	25 %
Acetic acid	10 %

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

3.7.3 Western blot

After the separation of the proteins by SDS-PAGE they were transferred to a nitrocellulose membrane by semi-dry blotting with 1x transfer buffer. In case of an unstained protein marker the membrane was stained by Ponceau S to mark the bands. To prevent unspecific binding of the antibody the membrane was blocked with 1x TBS with 5 % milk overnight at 4 °C.

The next day, the membrane was washed with 1x TBS for 20 min and then incubated with the primary antibody for 1 hour at room temperature. Then the membrane was washed

three times with 1x TBS for 10 min and incubated with the secondary antibody for 1 hour at room temperature. This was followed by three washing steps with 1x TBS for 10 min. The blot was developed with SuperSignal West Pico Chemiluminescent Substrate and exposed to an x-ray film.

Transfer buffer	
Tris-base	48.0 mM
Glycine	39.0 mM
SDS	0.037 %
Methanol	20.0 %

10x TBS	
Tris-HCl pH 8	0.25 M
NaCl	1.37 M
KCl	27.0 mM

Ponceau S staining solution	
Ponceau S	0.5 %
TCA	3.0 %

3.7.4 Over-expression of His-tagged proteins

For over-expressing of His-tagged proteins in *E. coli* a fresh culture was inoculated at an OD_{600} of 0.1 with an overnight culture. The production of the protein was induced at an OD_{600} of 0.3 with IPTG (0.5-1 mM). After 3-4 hours the cells were harvested by centrifugation (6 krpm, 15 min, 4 °C) and the pellet was frozen over night at -20 °C.

The pellet was thawed on ice for 10 min and then resuspended in buffer B. The cells were stirred for 2-4 hours at room temperature. In case of insufficient lysis the cells were sonicated. The lysate was centrifuged at 10,000 x g for 30 min at room temperature and the supernatant was incubated with Ni-NTA over night at room temperature by stirring. Binding of Ni-NTA agarose to the polyhistidine tag allows purification by affinity chromatography.

The lysate-resin mixture was loaded onto an empty column. This was followed by two washing steps with 4 ml buffer C. The proteins were eluted by four times 0.5 ml buffer D and four times 0.5 ml buffer E. Each fraction was collected in a separate tube. To find the fractions that contain the desired protein, samples of each fraction were analyzed by SDS-

PAGE. The fractions with the protein where pooled and dialyzed in 1x PBS at room temperature for 1 hour followed by overnight.

Buffer B	
NaH_2PO_4	100 mM
Tris base	10 mM
Urea	8 M
pH 8.0 adjusted with NaOH	

Buffer C	
NaH_2PO_4	100 mM
Tris base	10 mM
Urea	8 M
pH 6.3 adjusted with HCl	

Buffer D	
NaH_2PO_4	100 mM
Tris base	10 mM
Urea	8 M
pH 5.9 adjusted with HCl	

Buffer E	
NaH_2PO_4	100 mM
Tris base	10 mM
Urea	8 M
pH 4.5 adjusted with HCl	

10x PBS	
NaCl	1.37 M
KCl	27.0 mM
Na_2HPO_4	81.0 mM
NaH_2PO_4	14.7 mM
pH 7.4	

3.8 Cloning strategies

3.8.1 pQE30-Nov^{R}T

The plasmid pMDS11, which contains the novobiocin resistance cassette with terminator sequence, was cut with *Hind*III and *Sma*I and the gene was isolated by gel electrophoresis. The vector pQE-30 was cut with the same enzymes and then ligated with the novobiocin fragment.

3.8.2 pKSIIΔORF49

The already existing plasmid pKSIIΔ$49_{1\text{-}4}$ was cut with *Sma*I and dephosphorylated to prevent re-ligation. The novobiocin resistance cassette with the terminator sequence was cut out from pQE30-Nov^{R}T with *Sma*I and *Nhe*I. After purification of the insert by agarose

gel electrophoresis the 5'-overhang produced by *Nhe*I was filled by Klenow fragment. The blunt ends of vector and insert allowed insertion of the $Nov^{R}T$ fragment in both directions. To avoid altered gene expression of up- and downstream located genes due to the orientation, for both orientations of the insert were screened.

3.8.3 pRo5-soj

The *soj* gene was amplified by PCR from ϕCh1 DNA with primer Soj-Kpn and Soj-Hind and cut with *Kpn*I and *Hind*III. The primer Soj-Kpn binds approximately 350 bp upstream of the gene to ensure that the promoter sequence is included. The vector pRo-5 was also cut with *Kpn*I and *Hind*III. Afterwards, the insert and the vector were ligated.

3.8.4 pRSETA-soj

For protein production of Soj in *E. coli* the gene was amplified by PCR from ϕCh1 DNA with primers Soj-5 and Soj-3. The insert was restricted with *Bgl*II and *Pst*I whereas the vector pRSET-A was linearized with *Bam*HI and *Pst*I. The gene was ligated into the vector under the T7 promoter. The N-terminal polyhistidine tag allows purification by affinity chromatography.

3.8.5 pNB102-mtase

The ϕCh1 ORF 94 (*mtase*) with its promoter region was amplified from ϕCh1 DNA by PCR with primer MT-Kpn-5 and MT-Xba-3. The PCR product and the vector pNB102 were both cut with *Kpn*I and *Xba*I and ligated.

3.8.6 pBAD24-ptnaN-int1-his

The gene *int1* was amplified from ϕCh1 DNA with primer Int-E-3 and Int-E-5. The latter contains a sequence coding for a polyhistidine tag. The plasmid pBAD24-tnaN and the PCR product were cut with *Nde*I and *Bam*HI and ligated.

3.8.7 pNB102-int-repeat

The first step was to insert the *int1* gene under tryptophanase promoter into the plasmid pNB102. Therefore the fragment was amplified from the plasmid pBAD24-tnaN-int-his with primer Bad24-3-C and Int-3-C. Then the fragment and the vector pNB102 was cut with *Cla*I and ligated. The next step was to insert the spacer sequence with the different repeats. The repeats were added to a part of ϕCh1 ORF82 by PCR. The reverse primer IR-R-Xba, DR-R-Xba, IR-R-XbaΔ, IR-M1, IR-M4, IR-M7 and IR-M11 contain different repeats whereas the forward primer IR-L-Kpn stayed the same in all PCRs. The seven PCR products and the vector pNB102-tnaN-int-his was cut with *Kpn*I and *Xba*I and ligated.

4 Results and Discussion

4.1 Influence of gp34 variants on the infectivity of ϕCh1

4.1.1 Aim

The aim of this project was to investigate the influence of different gp34 types on the infectivity of virus ϕCh1 on *Nab. magadii* L13 cells. ORF34, encoding the tail fibre protein, is located in the invertible region of ϕCh1. Within this region there are three ORFs, ORF34 and ORF36 and the integrase ORF35 (*int1*), flanked by ORF34 and ORF36. The integrase Int1 is able to exchange the 3'-ends of ORF34 and ORF36 by an inversion reaction taken place within the clusters of 30 bp repeats, which are located in both ORFs. This reaction results in different types of both proteins, depending on the used repeats. It has been shown, that ϕCh1 expresses various types of ORF34. However, only the version with the ORF36 C-terminus is able to bind to the cell surface of *Nab. magadii* (Rössler *et al.*, 2004; Klein *et al.*, 2012). Here, the overexpression of proteins $gp34_1$ and $gp34_{52}$ was investigated towards the effects on ϕCh1 infection. Therefore, *Nab. magadii* L13 (pNB102) as well as *Nab. magadii* (pNB102-$ORF34_1$) and *Nab. magadii* L13 (pNB102-$ORF34_{52}$) were grown in rich medium until an optical density of 1.0 was achieved. The cultures were used for a virus titer assay.

4.1.2 Growth kinetics and expression of ORF34

As shown in Figure 12, all strains mentioned above had nearly the same growth rates. The strains expressing ORF34 indicate a slight slower growth compared to the control strain *Nab. magadii* L13 (pNB102). However, the production of gp34 affected the growth rate only minimal. The western blot with α-gp34 antibody confirms the production of the protein in the investigated strains and the absence of the protein in the control strain (Figure 12B).

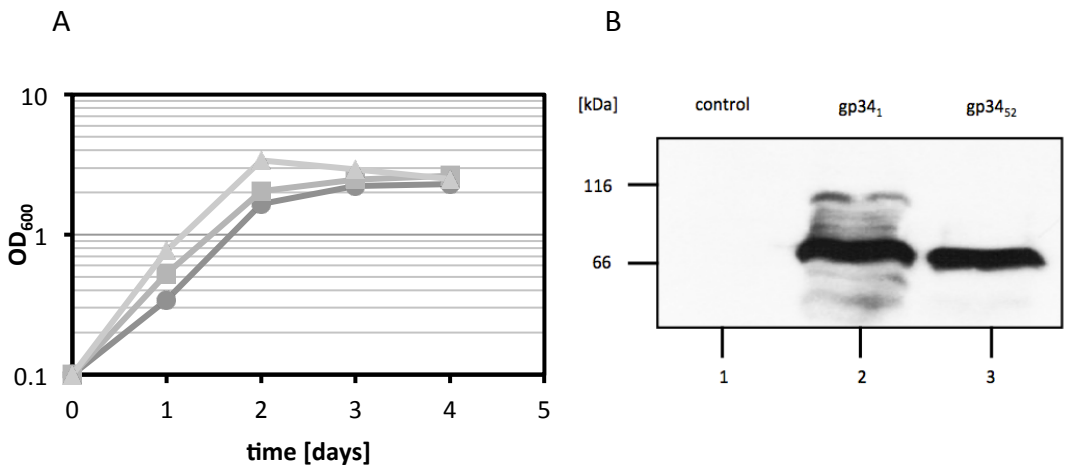

Figure 12: Control of ORF34 expression and growth curves.
A: Growth and lysis of strains *Nab. magadii* L13 (pNB102) [▲], *Nab. magadii* L13 (pNB102-34_{52}) [■] and *Nab. magadii* L13 (pNB102-34_1) [●]. All strains were inoculated in rich medium and incubated at 37 °C with agitation, the optical density was measured at 600 nm.

B: Western blot analysis of proteins $gp34_1$ and $gp34_{52}$ using α-ORF34 antibodies. Samples were taken at the beginning of the stationary growth phase, prepared as described (3.7.1) and analyzed by western blotting. Lane 1: *Nab. magadii* L13 (pNB102), lane 2: *Nab. magadii* L13 (pNB102-34_1), and lane 3: *Nab. magadii* L13 (pNB102-34_{52}). The sizes of the marker proteins are indicated on the left.

4.1.3 Virus titer analysis

At the same time point, were the samples were taken for western blotting, cells were used to determine the virus titer of ϕCh1 (Table 1).

Strain	**Virus titer [pfu/ml]**
Nab. magadii L13 (pNB102)	3.4×10^{11}
Nab. magadii L13 (pNB102-ORF34_1)	$> 1 \times 10^{1}$
Nab. magadii L13 (pNB102-ORF34_{52})	1.7×10^{11}

Table 1: Virus titer after infection of strains expressing different ORFs34 with ϕCh1.
The plaque assay was performed with different dilutions of virus ϕCh1 and plates were incubated for one week at 37 °C in sealed plastic bags.

As shown in Table 1, the infectivity of ϕCh1 is drastically reduced when plated on strain *Nab. magadii* L13 (pNB102-ORF34_1) compared to the control strain *Nab. magadii* L13

(pNB102) or to strain *Nab. magadii* L13 (pNB102-ORF34$_{52}$). For the first strain no plaques were visible, whereas a titer of 2 – 3 x 10^{11} was determined for the other two strains. Strain *Nab. magadii* L13 (pNB102-ORF34$_1$) expressed the ORF34$_1$ variant which was shown not to bind to the cell surface of *Nab. magadii* (Rössler *et al.*, 2004).

4.1.4 Discussion

This experiment showed that the expression of ORF34$_1$ during the life cycle of ϕCh1 reduces the infectivity of the virus. As shown earlier a virus variant with ORF34$_{52}$ can infect *Nab. magadii* (Rössler *et al.*, 2004). Within the logarithmic phase an inversion reaction takes place to ensure that both forms, ORF34$_1$ and ORF34$_{52}$, are present in a population of ϕCh1 genomes. At the end of the life cycle the ratio between these genes is approximately 1:1. It is not clear whether this is caused by a mixed population or by different inversion reactions in one cell. The type gp34$_1$ has no binding capacity to the *Nab. magadii* cell surface (Klein *et al.*, 2012). If this protein is already present from the beginning at a large amount, this protein is mainly attached to the viral particle. The inverse orientated protein, produced by the virus itself, is replaced and not or in very rare cases built into the particle. Therefore, the produced viral particles were not infective and the viral spread in a *Nab. magadii* culture is inhibited.

The inverse orientated type, ORF34$_{52}$, represents one version with an ORF36 C-terminus. The C-terminus of ORF36 harbors a galactose-binding domain. This domain is necessary for the infection of *Nab. magadii* cells (Klein *et al.*, 2012). In the described experiment gp34$_1$, containing the C-terminus of ORF34, was provided in trans from the plasmid pNB102. This plasmid has a copy number of 20, thereby causing an over-expression of ORF34$_1$. The over-expression of ORF34$_1$ led to an out titrating of the expression of ORF34$_{52}$ from the genome of ϕCh1. As a consequence, progeny virus particles exhibit only gp34$_1$ or at least a majority of the tail fibres containing gp34$_1$. This leads to non-infectious virus particles unable to from plaques on an agar lawn. This experiment clearly shows that only gp34$_{52}$, as a tail fibre protein, is able to adsorb to *Nab. magadii* L13 cells. Other phages like bacteriophage P1 use site-specific recombination by invertases to produce

different tail fibre proteins. This allows the phage to adapt to different hosts und extend the host range (Sandmeier, 1994). So far, there is no other known host for ϕCh1. However, the phase variation system would suggest that other hosts could be infected by the various types of tail fibre proteins.

4.2 Regulation of ORF34

4.2.1 Aim

In this project I investigated the regulation of ORF34, the tail fibre protein of ϕCh1. The invertible region of ϕCh1 harbors two putative tail fibre proteins, ORF34 and ORF36. However, only ORF34 has an upstream promoter sequence, and only this ORF is expressed (Klein *et al.*, 2012). In a previous thesis, it has been shown that ORF79 has an influence on the expression of ORF34. After induction of ORF79 expression in a co-expression experiment with ORF34 in *Nab. magadii* L13, the production of gp34 is repressed (Schöner, 2013).

The next step was to determine if other known regulators of ϕCh1 also have an effect on ORF34. Therefore, putative regulators under their own promoter sequence were transformed in *Nab. magadii* L13 cells together with $ORF34_{52}$. In this co-expression experiment induction of the putative regulator is not necessary, because the promoters were constitutive active. The investigated regulators were: ORF49, ORF48, ORF43 and ORF44 During a time course experiment samples were taken and then analyzed by western blot with a α-gp34 antibody. Due to an internal standard on every blot, it was possible to calculate the relative signal intensity by densitometry analysis with the program ImageJ (version 1.48v, NIH).

4.2.2 Regulator ORF48 and ORF49

ORF48 (*rep*) and ORF49 of ϕCh1 are known regulators of the lysogenic and the lytic life cycle and are orientated head-to-head with an intergenic region between. This intergenic

region contains a promoter sequence. The protein Rep represses the expression of ORF49 by binding to a direct repeat sequence within its own sequence. ORF49 may be involved in the switch between lysogenic and lytic life cycle. The mutant strain *Nab. magadii* L11-1, which has a mutation in ORF49, shows an earlier onset of lysis (Iro *et al.*, 2007). In a co-expression experiment, it was investigated if these two regulators also have an influence on the expression of ORF34.

The western blot analysis of ORF49 and ORF48 showed that the expression of ORF34 did not change over the time in comparison to the control strain with the empty plasmid. Thus, both regulators do not have an influence on the regulation of the tail fibre protein. It seems that these proteins are specific for the intergenic region of ϕCh1 so far.

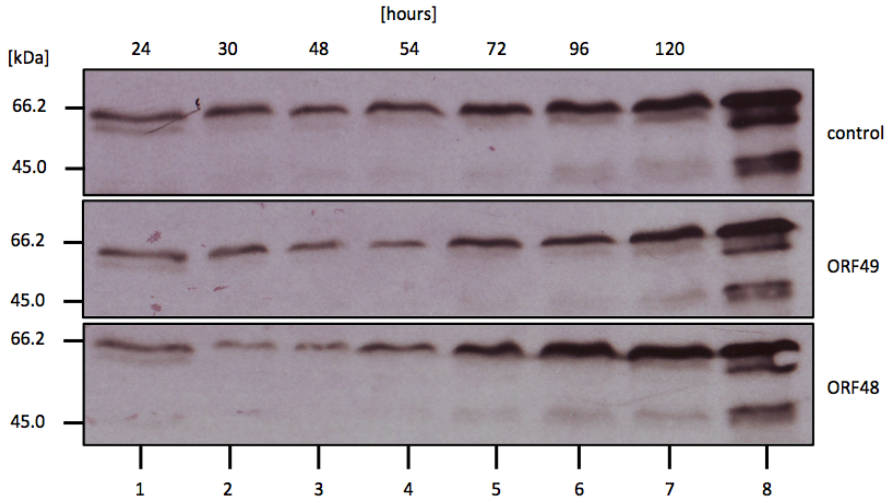

Figure 13: Influence of ORF49 and ORF48 on the expression of ORF34.
Western blot with α-gp34 antibody of the strains *Nab. magadii* L13 (pNB102-ORF34/pRo-5) (control), *Nab. magadii* L13 (pNB102-ORF34/pRo-5-ORF49) and *Nab. magadii* L13 (pNB102-ORF34/pRo-5-ORF48). Lane 1-7: samples from the time course experiment; lane 8: standard for quantification (*E. coli* XL1-Blue (pQE30-ORF34)). The applied amount of protein samples was controlled by coomassie staining. The cells were grown at 37 °C with agitation in rich medium. The expression of ORF34 does not change with ORF49 or ORF48 in comparison to the control.

4.2.3 ORF43 and ORF44

ORF43 and ORF44 represent an operon with a promoter sequence upstream of ORF43. Both ORFs overlap in one nucleotide and are located upstream of ORF49 and ORF48. It has been shown, that protein gp43 has an enhancing effect on the expression of genes under the control of the ORF49 promoter (Iro *et al.*, 2007). Analysis of the DNA sequence revealed a homology to VapBC toxin-antitoxin system. This system contains the inhibtor VapB and the PIN-domain nuclease VapC. Those two proteins build an operon and overlap in their sequence. Both proteins form an inactive complex, which autoregulates its own expression. When the inhibtor is proteolytically degraded, the nuclease gets active and cleaves mRNAs (Arcus *et al.*, 2011). Here, the effect of ORF43/44 was investigated.

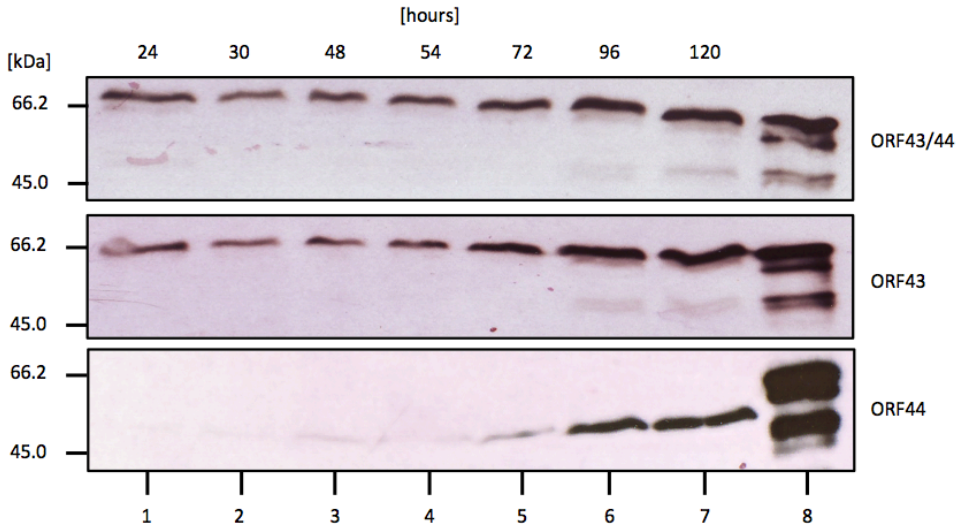

Figure 14: Western blot analysis of ORF43/44, ORF43 and ORF44
The blot shows the expression of ORF34 in *Nab. magadii* L13 (pNB102-ORF34$_{52}$/pRo5-p$_{43}$-ORF43/44), *Nab. magadii* L13 (pNB102-ORF34$_{52}$/pRo5-p$_{43}$-ORF43) and *Nab. magadii* L13 (pNB102-ORF34$_{52}$/pRo5-p$_{43}$-ORF44). Lane 1-7 represents the different time points where samples were taken. Lane 8: crude protein extract from E. coli XL1-Blue (pQE30-ORF34$_{52}$) as a control for quantification. The amount of applied sample has been controlled by coomassie staining before western blotting. The cells were grown at 37 °C in rich medium with agitation. The signal of gp34$_{52}$ with gp43/44 or gp43 does not differ from the control. The expression of gp44 prevents the signal until 48 hours. Cells of *Nab. magadii* L13 (pNB102-ORF34$_{52}$/pRo5-p$_{43}$-ORF44) express ORF34$_{52}$ 98 hours after inoculation of the culture. Here the same intensities were detected as for the other strains.

Figure 14 shows the production of gp34 in presence of gp43/44, gp43 and gp44. In contrast to the other observed regulators ORF49 and ORF48 only gp44 has an effect on ORF34. At 24 hours the expression of ORF34 is not visible and after 30 hours the signal steadly increases until 96 hours where it is as strong as in the control strain. Gp43/44 and gp43 does not show this regulation effect.

4.2.4 Densitometry analysis

The signal of the western blot was quantified by densitometry analysis. Therefore, the applied amount of the protein probes were controlled by coomassie staining and on every gel the same amount of a standard probe was applied. This allowed the quantification by measuring the signal intensity and calculation of relative values.

This method allowed quantifying the expression levels more accurate than only by visual evaluation. The value of the signal was calculated by the intensity of the pixel of each band on every lane. The program gives a graphical depiction of this pixel intensity. The area under the graph represents the density of the band. The background level of the signal was not included. The area under the graph has been integrated and this value was divided by the result of the control band. These relative values were used for the comparison of the different strains.

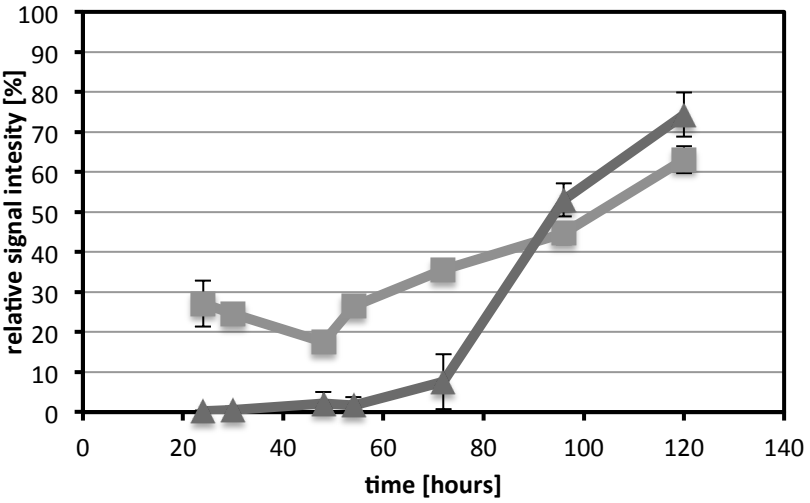

Figure 15: Densitometry analysis of the expression of ORF34.
The graph shows the relative signal intensity of the western blot with a α-gp34 antibody. The intensity was normalized to a standard, which was the same on every gel. The control (■) and all other investigated regulators (data not shown) show similar increase of the intensity after 48 hours. Whereas, ORF44 (▲) prevents the expression of ORF34 at the beginning completely and after 72 hours the expression increased. Error bars represent +/- 1 SD.

The investigated proteins, except gp44, caused no change in the signal intensity compared to the control and therefore have no influence on the expression of this protein. However, in presence of gp44 the expression of ORF34 is not detectable at the beginning and increased slowly for 72 hours. After 96 hours, the signal is as high as the control and no repression does occur.

4.2.5 Discussion

It has been shown, that the known regulators ORF49 and ORF48 of ϕCh1 does not have an influence on the expression of ORF34. Nevertheless, ORF79 is not the only protein, which affects the expression of ORF34 because ORF44 represses also the expression. ORF44 builds an operon with ORF43 and has no own promoter sequence (Iro *et al.*, 2007). Therefore, it was necessary for this experiment to use the constitutive active promoter of ORF43. Sequence analysis of ORF44 revealed a PIN domain, which has a nuclease activity. The expression of ORF44 in *Hfx. volcanii* has an effect on the amount of plasmid in the cell. The presence of ORF44 also inhibits the expression of ORF48 and from the intergenic

region of ϕCh1. Here, specific RNA could not be detected in the presence of gp44. This suggests a degradation of RNA and/or plasmid DNA (Witte, in prep.).

It took several attempts to find a strain, which has taken up the plasmid encoding ORF44. The inhibitory effect of gp44 could only be observed when ORF43 is not present. If ORF43 does not repress the expression of ORF44, the protein with the nuclease activity is produced and this could have severe consequences for the cell. This could explain why it was so difficult to find a pRo5-P_{43}-ORF44 positive transformed strain. The PIN-VapC like region of ORF44 could be a part of archaeal toxin-antitoxin (TA) like operon. Gp43 in this case is the tight regulated repressor of the toxin gp44 with the nuclease activity.

Gp43/44 has an enhancing effect on the expression from the ORF49 promoter. This effect is depended on a repeat sequence within the coding sequence of ORF48, which could be a binding site for the protein. The enhancing effect on the ORF49 promoter was only seen with gp43/44, otherwise gp44 also have a reducing effect (Iro *et al.*, 2007). This could also be explained by the missing repressor and the nuclease activity of gp44, which degrades mRNA.

The signal of gp34 increased after 72 hours to the level of the control. Thus, the repression of gp44 must be somehow overcome. In this experiment, ORF44 was cloned under the ORF43 promoter, which is constitutive active, and not under an inducible promoter. Since strong expression of ORF44 will cause severe problems in the cells, only cells, which produce the protein in a sub-lethal dosage, could survive. Therefore, expression of ORF34 is only possible in the late growth phase where it overrides the expression of ORF44. However, only the activity of the ORF34 promoter is known (Iro *et al.*, 2007).

It seems likely that the effect of gp44 is not specific for ORF34 and the intergenic region. If it represents a toxin-antitoxin system it would rather degrade different mRNAs. The function of a toxin-antitoxin system is very diverse, for example to hinder the loss of

mobile genetic elements, response to environmental changes or DNA damage (Arcus *et al.*, 2011). TA systems were discovered with proteins, which protect plasmids from loss during cell division (Gerdes *et al.*, 1986). This mechanism is called “death upon curing” (Van Melderen and De Bast, 2009). It is possible that in ϕCh1 this system protects the provirus in the genome of *Nab. magadii*. If the viral DNA with ORF43/44 is not inherited to the daughter cell after division, the unstable inhibitor is degraded after a short time and the stable PIN-domain nuclease becomes active and cause cell death.

Another possible function would be a role in thermal or nutritional stress. Host cells sometimes use TA systems, originally introduced form mobile elements, for their own purpose (Van Melderen and De Bast, 2009). Nevertheless, further investigation of this operon is necessary. Especially, more insight into the expression of ORF43/44 and the interaction between those two proteins are needed. This will show how gp43 inhibits the activity of gp44 and if these two proteins rally represents a toxin-antitoxin system.

4.3 Purification of ϕCh1 Soj for antibody production

4.3.1 Overexpression in *E. coli* and purification of the protein Soj

To detect the protein Soj of ϕCh1 in a western blot, an antibody, which recognizes the protein, is needed. Soj is homologous to plasmid partitioning proteins and is of interest in one of my other projects. Therefore, the gene was overexpressed in *E. coli* and the protein was purified under denatured conditions. Due to the relative slow growth rate of *Nab. magadii* and the lack of suitable strong inducible promoter, a purification within the native environment is not possible. Therefore, the *soj* gene was cloned into the vector pRSET-A, resulting in plasmid pRSETA-soj, and transformed into *E. coli*.

The first step within the purification process was to find a strain with strong expression of the gene. The advantage of the plasmid pRSET-A is the 6xHis tag and the T7 promoter. Here the *E. coli* strains Rosetta and BL21 were used because these strains posses a T7

polymerase under control of the *lac* promoter. Both strains expressed the *soj* gene, but BL21 showed a stronger expression (data not shown). One major problem was that the cells already expressed the gene before induction of the *lac* promoter with IPTG.

Expression of a foreign gene at a very high level is a stress moment for the cell. For this reason it is necessary to induce the gene expression in the exponential growth phase, when the cells are in good condition. After the induction, the cells produce the protein and the cell division slows down. As induction starts in this case at the beginning, the cells do not become the chance to get in exponential growth phase. The result is a very slow growing culture with few cells or in contrast the selection of faster growing cells, which do express the gene in low amount. Both *E. coli* strains contain the plasmid pLysS, which codes for the T7 lysozyme. The lysozyme should repress the T7 polymerase completely in the not induced state. However, expression was detected before induction. To overcome this problem I used a strain with the additional plasmid pRep4, which contains an additional copy of the *lac* repressor. This plasmid should repress the *lac* promoter completely before induction. However, the situation remained unchanged and the gene was still constitutive expressed (data not shown).

Another attempt was to express the gene in *E. coli* XL1-Blue from the plasmid pQE-30 or pQE-60. In these two plasmids the gene was cloned directly under the *lac* promoter and therefore the strain XL1-Blue was used, which contains no T7 polymerase. The plasmid pQE-30 codes for an N-terminal 6xHis tag, whereas on pQE-60 the tag is on the C-terminal side. Both plasmids were used to exclude an influence of the His tag on the expression. However, the expression of the gene within this strain failed completely (data not shown). Therefore, the only possibility was to purify several liters of BL21 culture to obtain the needed concentration of the protein for the antibody production.

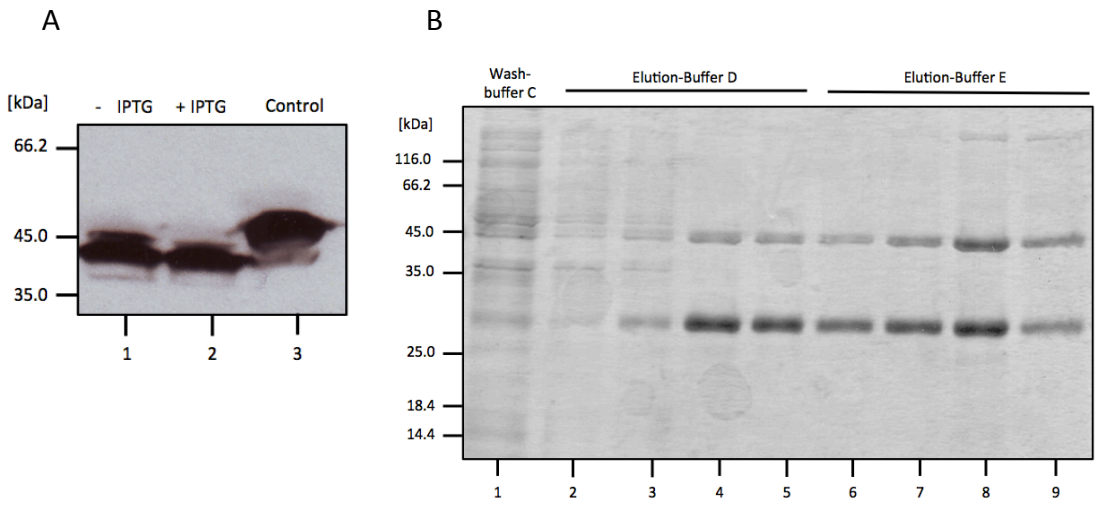

Figure 16: Overexpression and purification of Soj
A: Western blot with Penta-His antibody for controlling the protein expression. Lane 1: *E. coli* BL21 (pRSETA-soj) without IPTG; lane 2: *E. coli* BL21 (pRSETA-soj) with 1 mM IPTG; lane 3: positive control for Penta-His antibody *E. coli* XL1-Blue (pET-E). The protein is already expressed without IPTG.

B: SDS-PAGE after protein purification under denatured condition. Lane 1: wash buffer C; lane 2-5: eluate of buffer D; lane 6-9: eluate of buffer E. The protein Soj has a size of approximately of 45 kDa. The highest amount of protein is in buffer E. The other band at 30 kDa is typically for *E. coli*, especially if the heterologous expressed gene is very weak.

4.3.2 Examination of the specificity of the antibody

After the needed amount of protein has been purified, the lyophilized protein was sent for antibody production. Therefore, a rabbit was immunized three times with the protein to induce an immune reaction to Soj. The serum of the rabbit was tested for recognizing the protein. Figure 17 shows the test of the antibody in a western blot with *Nab. magadii* L11 and *Nab. magadii* L13. The antibody was saturated with acetone powder from *Nab. magadii* L13 to reduce the unspecific binding.

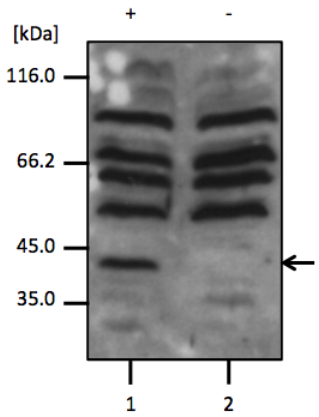

Figure 17: Test of α-Soj antibody.
The antibody was diluted 1:250 and unspecific binding was saturated with acetone powder of *Nab. magadii* L13. Lane 1: *Nab. magadii* L11; lane 2: *Nab. magadii* L13. There is still unspecific binding of the antibody, but the band for Soj at 45 kDa (indicated by an arrow) is only visible in lane 1.

4.3.3 Discussion

The purification of the protein Soj in *E. coli* was complicated by the constitutive expression of the gene, caused by an insufficient regulation of the repression. This was also not prevented by the additional plasmid pRep4, which harbors the repressor *lacI*. The massive production of Soj caused the cells to grow very slow. However, this slow growth rate leads to a low cell density and to a low amount of protein. The cells also tend to lose the plasmid because of the stress caused by the over-expression.

Accordingly, I made a compromise between cultures with and acceptable growth rate and expression of the gene in an acceptable amount. Otherwise, very slow growing cells, which express the gene in high amount, stayed at a very low optical density and the culture died after a time. This made it necessary to purify several liters of culture to obtain the needed protein concentration. To prevent the problem of constitutive expression, it would be necessary to over-express the gene in the natural host. Thus, this will prevent that a foreign sequences alter the regulation of gene expression. Therefore, a strong and inducible promoter and a fast and efficient purification method for *Nab. magadii* are necessary.

The specificity of the antibody was improved by saturation with acetone powder of *Nab. magadii* L13, but there is still binding of unspecific proteins. The powder contains all cell components from *Nab. magadii* and binds all unspecific antibodies. Since the remaining unspecific bands occur in both strains, the acetone powder cannot eliminate these. However, the band of Soj at 45 kDa is only recognized in *Nab. magadii* L11. Therefore, the antibody can be used for detecting the Soj protein in western blot analysis.

4.4 The regulator ORF79

4.4.1 Aim

ORF79 is a gene of unknown function (Klein *et al.*, 2002). For this reason a deletion mutant of this ORF has been created to study the function of the encoded protein. This mutant strain showed an earlier onset of lysis compared to the wild type *Nab. magadii* L11 (Selb, 2010). The deletion of ORF79 leads to an altered expression of ORF34 and ORF11, encoding the major capsid protein E (Alte, 2011). Further, it is known from a co-expression experiment in *Nab. magadii* L13 that ORF79 has a direct influence on the expression of ORF34 (Schöner, 2013).

Within this thesis I showed, that ORF48, ORF49 and ORF43 are not involved in the regulation of ORF34. The next question is whether ORF79 is a universal regulator, which regulates the expression of other genes or it is a specific regulator for ORF34. Therefore, the same experimental setup was used as described for ORF34 with the modification that ORF46 (*soj*) was used instead of ORF34. This ORF has been chosen because it is known to have an upstream promoter sequence. This protein is annotated as a putative plasmid partitioning protein (Klein *et al.*, 2002). If ORF79 is a specific regulator, the expression of *soj* should not be effected.

4.4.2 Co-expression of ORF79 and ORF46 (*soj*)

After the transformation of plasmid pRo5-soj and pNB102-ptnaN-ORF79 into *Nab. magadii* L13, a time course experiment was performed. ORF79 was controlled by the inducible tryptophanase promoter ptnaN of *Nab. magadii* whereas ORF46 was transcribed from its own promoter. The induction of the tnaN promoter with tryptophan also induces the tryptophanase of *Nab. magadii*, thus the inducer is degraded and this makes it necessary to add tryptophan every day.

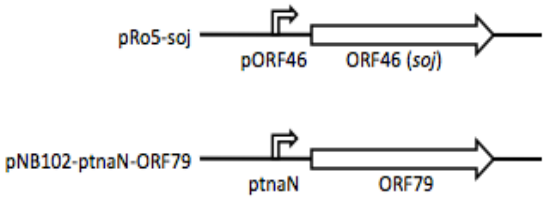

Figure 18: Scheme of plasmids pRo5-soj and pNB102-ptanN-ORF79
ORF46 (*soj*) was cloned in the plasmid pRo-5 with its 500 bp upstream sequence to include its promoter sequence. ORF79 was cloned under the inducible tryptophanase promoter ptnaN.

Both constructs were transformed into *Nab. magadii* L13 at the same time. Transformed cells were grown in minimal medium to avoid induction of the ptnaN promoter. The culture was inoculated with an OD_{700} of 0.1. The cell density was measured at 700 nm because in minimal medium the cells produce β-carotene, which has its absorption maximum at around 580 nm. By measuring the cell density at 600 nm, mainly the β-carotene and not the cells would be measured. One culture was induced every day with 2 mM tryptophan and the control culture was not induced.

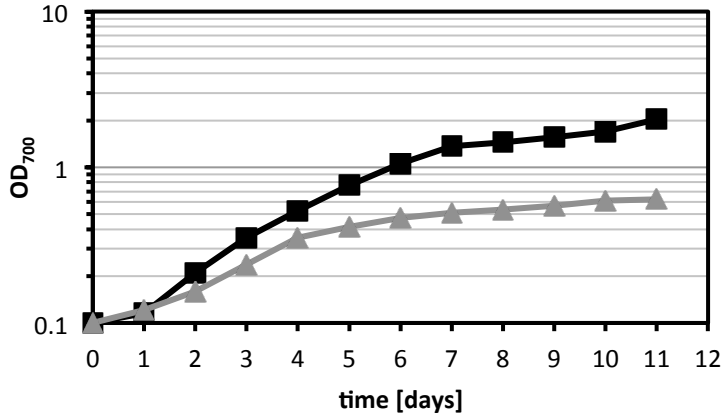

Figure 19: Growth curve of *Nab. magadii* L13 (pRo5-soj/pNB102-ptnaN-ORF79).
The control culture (■) was not induced. The other (▲) was induced with 2 mM tryptophan every day. Due to the protein production the induced culture grew slower. Cells were grown in minimal medium at 37 °C with agitation.

Samples from the cultures were taken from day 1 to 9 (Figure 19) and analyzed by western blot using a α-Soj antibody to determine the expression of *soj*.

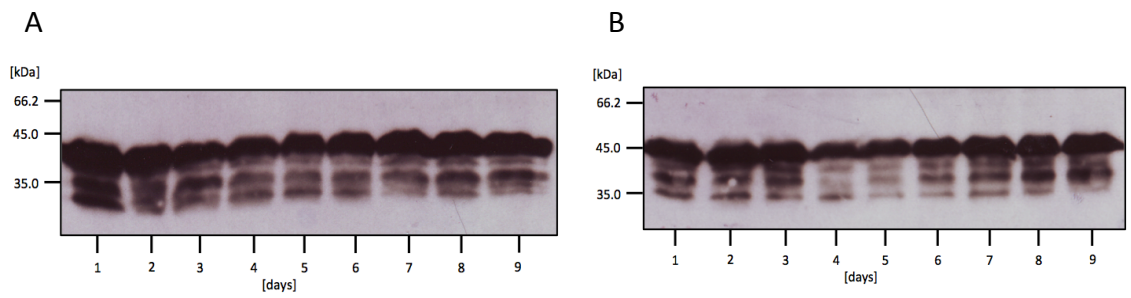

Figure 20: Influence of ORF79 on the expression of *soj*.
Western blot with α-Soj antibody with samples taken from cultures of *Nab. magadii* L13 (pRo5-soj/pNB102-ptnaN-ORF79). The amount of the applied sample was controlled by coomassie staining. A: None induced control culture. B: Induced with 2 mM tryptophan every day. The expression of *soj* does not change over the time and there is no difference of the not induced to the induced culture.

The analysis of the induced and not induced culture showed that the expression of *soj* does not change over the time and therefore, the expression of ORF79 does not have an influence on Soj. At the end of the experiment, the cultures were tested for both plasmids by PCR. Although the loss of the plasmid should be avoided by the addition of antibiotics, spontaneous resistance followed by the loss of the plasmid is possible. The PCR revealed that the signal for pNB102-ptnaN-ORF79 was very weak (data not shown). Therefore, it could be possible that the missing regulation effect of ORF79 is caused by a low copy number of the plasmid.

4.4.3 Influence of Soj on plasmid stability

A reason for the loss of the plasmid could be that Soj somehow affects the stability of pNB102. The protein Soj of ϕCh1 has sequence similarities to plasmid partitioning proteins (Klein *et al.*, 2002). It has been shown, that Soj in *E. coli* is able to bind to DNA in an ATP dependent manor. It is involved in plasmid segregation and co-localize to the nucleoid. The binding of Soj to plasmid DNA may influence the plasmid stability (Hester and Lutkenhaus, 2007).

To determine whether the plasmid itself is unstable under these conditions or Soj is responsible, two strains were passaged several times. Here, *Nab. magadii* L13 (pRo5-ptnaN-ORF79/pNB102-ORF34) and *Nab. magadii* L13 (pRo5-soj/pNB102-ptnaN-ORF34) were used. Both strains were grown to stationary phase in minimal medium with antibiotics. Afterwards they were incubated into fresh medium again with antibiotics. After each passaging step, the culture was tested by PCR. The used amount of cells for template preparation was adjusted by the optical density of the culture. This compensated the different cell densities and ensured that a weaker signal is not caused by a smaller amount of template DNA.

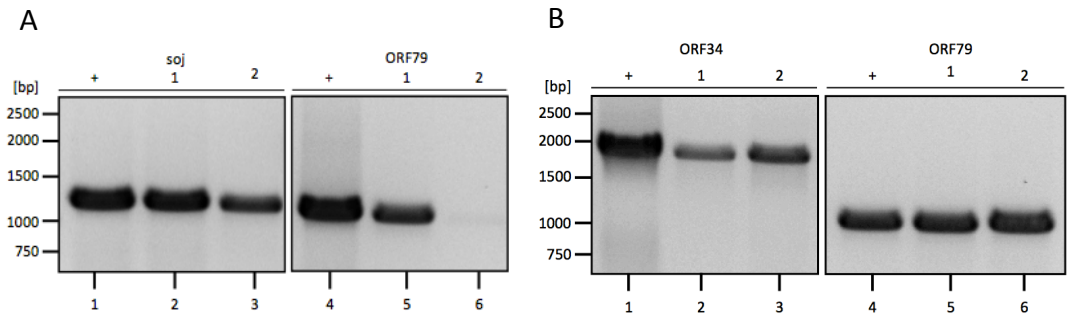

Figure 21: Loss of plasmid pNB102-ptnaN-ORF79
Instability of pNB102-ptnaN-ORF79 in dependence on the protein Soj. Strains were grown in minimal medium at 37 °C with antibiotics. Both strains were passaged three times. After each passage nucleic acids were isolated and used as templates for PCR analysis. Primer used for PCR: soj: Soj-Hind and Soj-Kpn; ORF79: Tna-N-1 and 79-N2 and ORF34: 34-Kpn and 36-3X.

A: *Nab. magadii* L13 (pRo5-soj/pNB102-ptnaN-ORF79). Lane 1: positive control *soj*; lane 4: positive control ORF79; lane 2 and 5: first passage; lane 3 and 6: second passage.

B: *Nab. magadii* L13 (pRo5-ptnaN-ORF79/pNB102-ORF34). Lane 1: positive control ORF34; lane 4: positive control ORF79; lane 2 and 5: passage 1; lane 3 and 6: passage 2.

Figure 21 shows that the plasmids of strain *Nab. magadii* L13 (pNB102-ORF34/pRo5-ptnaN-ORF79) were stable and the signal intensity did not change over the time. However, strain *Nab. magadii* L13 (pNB102-ptnaN-ORF79/pRo5-soj) lost the plasmid carrying ORF79 nearly completely within passage 2. The third passage led to a culture that did not grow anymore (data not shown). This may be caused by the added antibiotics and the missing

mevinolin resistance gene due to the loss of plasmid pNB102. In contrast, plasmid pRo5-soj seems to be stable and the signal intensity is nearly equal.

4.4.4 Discussion

The deletion mutant *Nab. magadii* L11-ΔORF79 has an altered production of the tail fibre protein gp34 and the major capsid protein E (Beraha, 2013). Although it is possible that other ORFs of ϕCh1 can be also regulated by this protein. Therefore, the influence of gp79 on the expression of the *soj* gene of ϕCh1 was analyzed. The western blot indicated that *soj* is not regulated by gp79, indicating a specific regulatory function of gp79 towards ORF34. However, a problem was the instability of the plasmid pNB102-ptnaN-ORF79 in the presence of Soj. This made it difficult to ensure that the missing regulation is not caused by a low amount of gp79. It is possible that Soj, in the function as a plasmid partitioning protein, causes the loss of the plasmid, because without Soj the plasmid is stable. All experiments were performed with the addition of antibiotics. Consequently, there must be a reason why the cells lost the plasmid despite selection with antibiotics and died afterwards. The protein Soj is homologous to partitioning proteins, which are involved in the segregation of plasmids during cell division (Hester and Lutkenhaus, 2007). Thus, Soj could affect the stability of the plasmid pNB102. It is possible that Soj interacts with the origin of replication of pNB102 and causes the loss of the plasmid.

A similar effect has been observed in *Acidianus hospitalis* W1 from the phylum *Crenarchaeota*. This strain harbors the conjugative plasmid pAH1 and is a host for the *Acidianus* filamentous virus 1 (AFV1). The infection with the virus causes a loss of the free plasmid. An explanation for this occurrence is a protein, which cure the cell from the plasmid (Basta *et al.*, 2009). This example shows another possibility for interacting of genetic elements in *Archaea*. The interaction of the plasmid pNB102 and the protein Soj need to be further investigated. Therefore, further experiments are planned, which will show if the plasmid is also lost in other conditions. Nevertheless, the specific regulatory effect of ORF79 on ORF34 is not clearly demonstrated. For this reason, I also investigated the expression of another gene, the gene of the methyltransferase M.*Nma*ϕCh1-I.

4.4.5 Co-expression of ORF79 and the methyltransferase M.*Nma*ϕCh1-I

The aim of this experiment was to test if another protein of ϕCh1 is regulated by ORF79. Since Soj had an influence on the plasmid stability, it was necessary to choose another ORF with a known promoter. Therefore, the plasmids pRo5-ptnaN-ORF79 and pNB102-mtase were transformed into *Nab. magadii* L13. During a time course experiment the expression of the methyltransferase M.*Nma*ϕCh1-I, encoded by ORF94, was analyzed by western blot using a α-Mtase antibody.

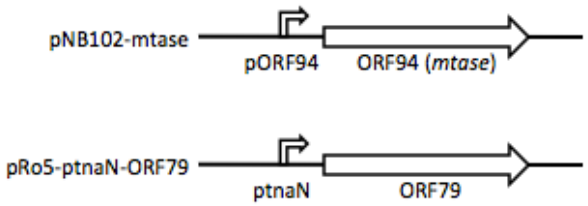

Figure 22: Scheme of plasmids pNB102-mtase and pRo5-ptnaN-ORF79
The cloned sequence of ORF94 (*mtase*) contains its own upstream promoter sequence. ORF79 was cloned under the inducible tryptophanase promoter ptnaN.

The time course experiment was done in minimal medium at 37 °C with two different cultures. One was not induced and served as a control and the other culture was induced. Therefore, every day 2 mM tryptophan was added to the induced culture to counteract the degradation of the inducer by the tryptophanase of *Nab. magadii*.

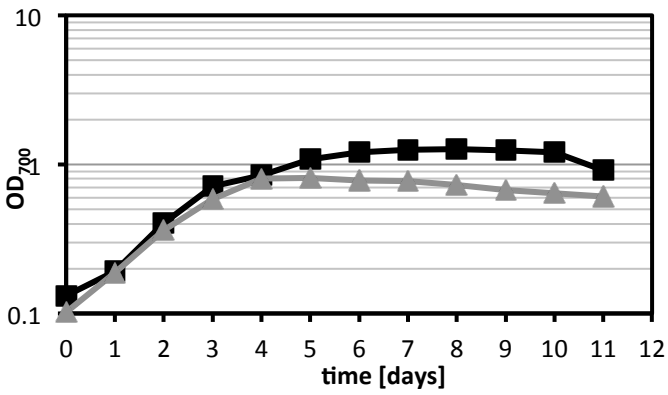

Figure 23: Growth curve of *Nab. magadii* L13 (pRo5-ptnaN-ORF79/pNB102-mtase).
The control strain was not induced (■) and the other strain (▲) was induced with 2 mM tryptophan every day. Cells were grown in minimal medium at 37 °C and agitation.

Samples taken at day 1 to 9 were analyzed by western blot with a α-Mtase antibody to show a possible regulating effect of ORF79 on the expression of the methyltransferase.

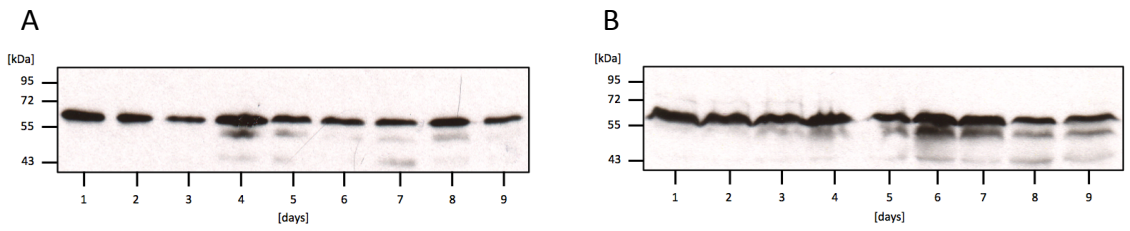

Figure 24: Co-expression experiment of ORF79 and Mtase.
Western blot with α-Mtase antibody for samples from the time course experiment of *Nab. magadii* L13 (pRo5-ptnaN-ORF79/pNB102-mtase). The amount of applied sample was controlled by coomassie staining. A: None induced control culture. B: Culture induced with 2mM tryptophan every day. The signal of the control and induced culture do not change during the time.

The signal of the expression of the Mtase in the not induced control culture does not change during the observed period. The co-expression of ORF79 was induced with tryptophan. In this culture the methyltransferase is also expressed without changes in the observed time.

4.4.6 Discussion

In this experiment, both plasmids were stable over the whole time. So the missing repressing effect could not be caused by a lost plasmid. The expression of the methyltransferase gene did not change over the observed time neither with nor without ORF79. Hence, the regulator ORF79 does not regulate the expression of this gene. This shows that ORF79 is not a universal regulator of ϕCh1 and there are proteins, which were produced independently of this regulator. This supports the hypothesis that ORF79 is a specific regulator of the tail fibre protein gp34.

4.5 Expression of ORF79 in *Nab. magadii* L11

4.5.1 Aim

In previous thesis it has been shown, that ORF79 is an important regulator of ϕCh1. One target of gp79 is ORF34, which encodes the tail fibre protein (Beraha, 2013; Schöner, 2013). Since an antibody against gp79 is now available, it is possible to study the expression pattern of this regulator and compare it to its target protein gp34. Within the co-expression experiment of ORF79 and ORF34, the expression of ORF34 is repressed after induction of ORF79 (Schöner, 2013). This result suggests that both ORFs are not expressed at the same time. Since the mutant strain *Nab. magadii* L11-ΔORF79 also have an slight altered expression of ORF11 (Beraha, 2013), also the major capsid protein E was compared to gp79 production.

4.5.2 Analysis of the expression pattern

The strain *Nab. magadii* L11 was grown in rich medium at 37 °C with agitation. Every day the cell density was measured. The lysis of the strain occurs after day 3. Samples were taken at different time points, especially at the transition to another growth phase. The expression of ORF79, ORF34 and ORF11 were analyzed by western blot.

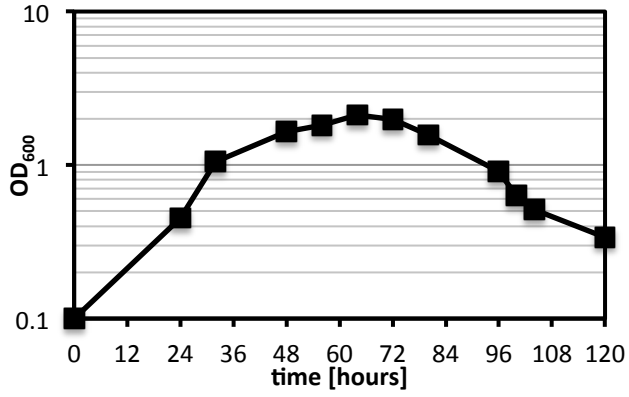

Figure 25: Growth curve of *Nab. magadii* L11.
The cells were grown in rich medium at 37 °C with agitation.

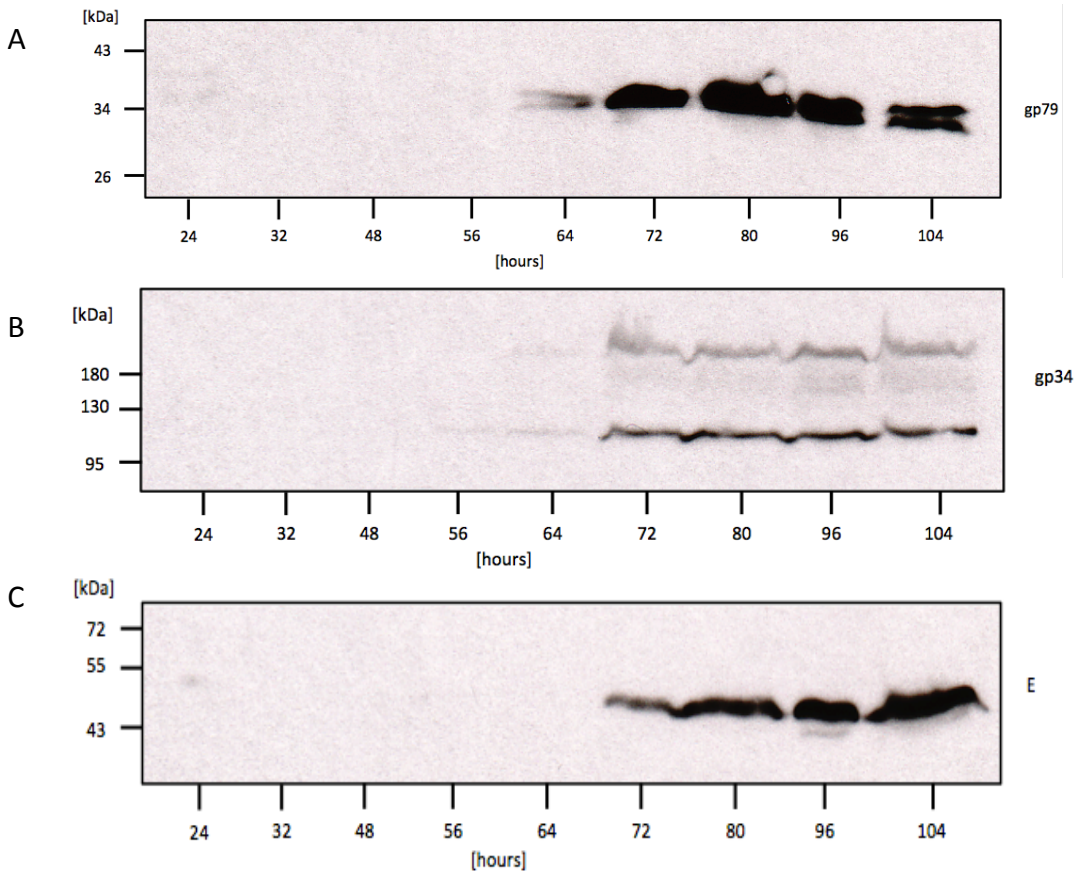

Figure 26: Expression of ORF79, ORF34 and ORF11.
The figure shows the western blot analysis of the expression of A: ORF79, B: ORF34 and C: ORF 11 in *Nab. magadii* L11. The expression of ORF79 starts after 56 hours and increases until 72 hours. ORF34 is also expressed after 56 hours, but the production of protein E starts at 72 hours. Applied amounts of samples for western blotting were controlled by coomassie staining.

In contrast to the expected results, ORF79 is expressed not before 64 hours after inoculation. The production of gp34 starts at the same time, after 64 hours. The capsid protein E is produced after 72 hours. The amount of produced gp79 stays the same from 72 to 96 hours and decreases a little at 104 hours. The expression of ORF34 and ORF11 remains constant after the induction phase over the observed period.

4.5.3 Discussion

The result of the expression pattern of ORF79 was unexpected. The protein is produced after 64 hours and then remains constant. The same pattern is also observed at ORF34. Previous experiments showed clearly a repression of gp34 after induction of ORF79 (Schöner, 2013). In the mutant strain *Nab. magadii* L11-ΔORF79 the missing regulator caused a constitute expression of ORF34 (Beraha, 2013).

In this time course experiment with the wild type *Nab. magadii* L11 it has been shown that the repressor is expressed at the same time than the target. Unfortunately, it was not possible to detect gp79 in the samples from the co-expression experiment in *Nab. magadii* L13. This would have shown the amount and the time point of the expression of ORF79 under this condition. However, this could be caused from the antibody, which has a very poor affinity for its target. In the mutant strain *Nab. magadii* L11-ΔORF79 the production of protein E is also slightly altered. The protein E in the mutant strain is produced few hours earlier compared to the wild type. But also this protein is produced at the same time as ORF79.

The experiment was repeated three times with different strains and always showed the same results. A reason for the inconsistent results could be that the unnatural expression of both ORFs from plasmids under an inducible promoter leads to an altered proportion, which does normally not occur. Another possibility is that in the model system an additional factor is missing. In contrast to the lysogenic wild type strain, in *Nab. magadii* L13, all other ORFs of ϕCh1 are missing. These factors may have an influence on the activity of the repressor. Therefore, one must be careful at the direct comparison of the wild type and the artificial system. There could be lot of unknown factors and conditions, which cause an altered regulation in the artificial system. To determine the interaction between these proteins it is necessary to gain more insight into the mode of action of the wild type strain.

4.6 Deletion mutant strain of ϕCh1 ORF49

4.6.1 Aim

The mutant strain *Nab. magadii* L11-1 shows an earlier onset of lysis. This mutant strain contains an additional short ORF called ORF49'. This ORF consists of a duplication of a part of ORF49 and the upstream region. After a few passages the duplication disappears. This suggests a role of ORF49 in regulation of the life cycle of ϕCh1 (Iro *et al.*, 2007). In a previous diploma thesis the attempt to create a homozygote deletion mutant of ORF49 failed. The strain still contains the wild type ORF49 and additional altered sequences (Svoboda, 2011)

For this reasons I tried once again to construct a homozygote deletion mutant strain of ORF49, to further determine the function of this regulator. Therefore, I constructed a plasmid with a deletion cassette containing a novobiocin resistance marker to delete ORF49 by homologous recombination. Since *Nab. magadii* contains up to 50 copies of its chromosome it is necessary to make sure that every copy of ORF49 has been deleted.

4.6.2 Construction of the deletion plasmid and heterozygote mutant strains

The plasmid pKSIIΔORF49

In a previous thesis it was possible to delete gens of ϕCh1 by homologous recombination (Selb, 2010). For this purpose a plasmid was constructed, which contains the up- and downstream flanks of ORF49 for the homologous recombination. The plasmid pKS_{II}^{+} was used, because it has no suitable origin of replication for *Nab. magadii*. This suicide plasmid will cause the death of the cell if it is not integrated into the host chromosome. Into this plasmid the novobiocin selection marker (*gyrB*) with a terminator sequence has been inserted. This terminator sequence was included to ensure that transcription ends after the resistance gene. This was necessary because it is not known, if the gene already contains a terminator. Otherwise, the expression of the surrounding ORFs could be

altered. To further exclude that the orientation of the deletion cassette has an influence on other ORFs, the constructs were made with both orientations of the selection marker.

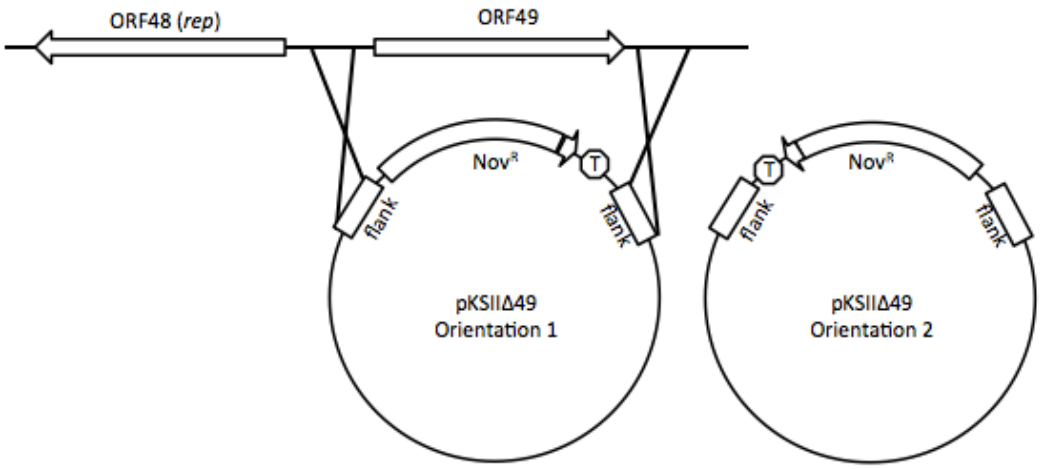

Figure 27: Scheme of the suicide plasmid for the deletion of ORF49.
The plasmid pKS_{II}^{+} contains the up- and downstream flanking sequence of ORF49 and the novobiocin resistance gene (*gyrB*) with a terminator sequence between. The resistance marker has been inserted in both orientations.

Finding a strain with a double crossover event

After the transformation of both constructs into *Nab. magadii* L11, there are three possible outcomes. One is that there is no recombination between the flanking region of ORF49 and the plasmid is lost because of the lack of a suitable origin of replication. Due to selection by novobiocin these cells will die. The other possibilities are a single crossover and a double crossover. In this case the cells will survive but for a homozygous deletion mutant a double crossover is needed. Figure 28 shows that only a double crossover causes an exchange of the resistance marker and ORF49. Candidates where tested by PCR to distinguish between single and double crossover event. This was achieved with primer pairs, which cover the 3' and 5' end of the deletion cassette. Only if both primer pairs lead to a PCR product a recombination on both ends has occurred.

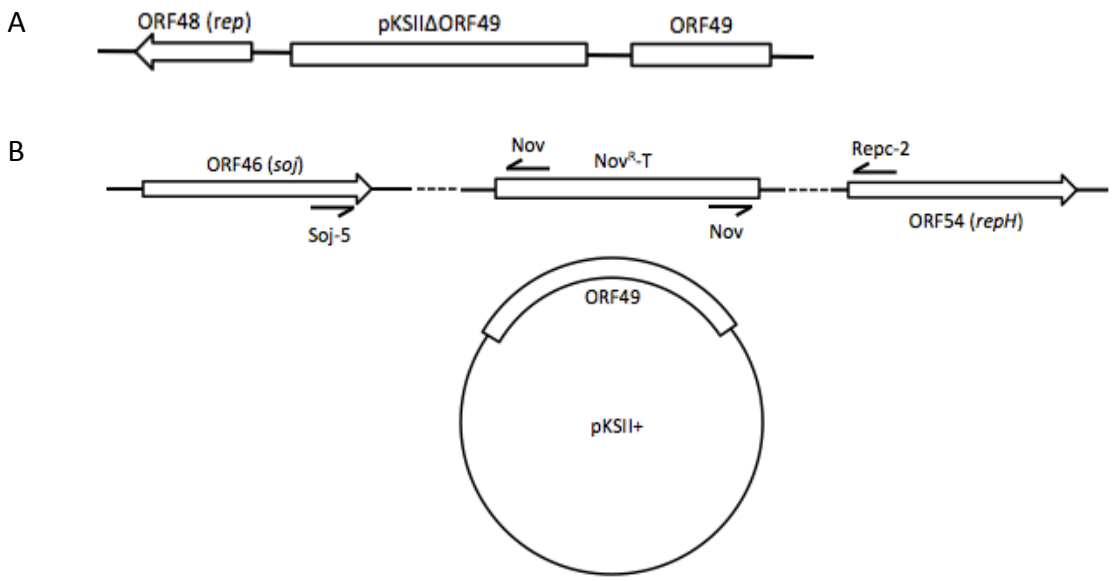

Figure 28: Scheme of possible crossover events.
A: A single crossover on one site will lead to an integration of the complete plasmid but ORF49 is not deleted. B: A recombination event on the 3' and the 5' end will cause an exchange of the novobiocin resistance gene and ORF49. Arrows indicate the primers used for PCR.

The primers for the test PCR binds within an upstream or downstream ORF. The appropriate primers, which bind in the novobiocin resistance gene, where used according to the orientation of the deletion cassette.

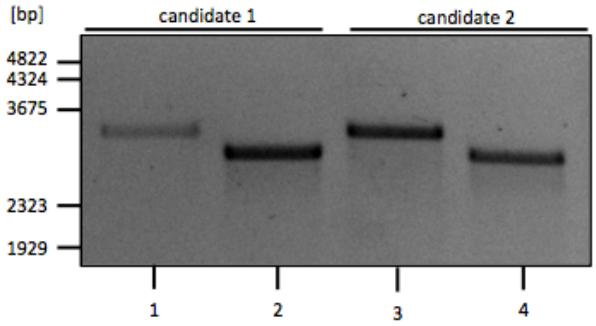

Figure 29: Test for double crossover for orientation 2.
The two candidate strains of *Nab. magadii* L11-ΔORF49 where tested by PCR for a double crossover event in at least one copy of its chromosome. For the 5' end primers Nov-13 and Soj-5 and for the 3' end primers Nov-12 and Repc-2 were used. Lane 1 and 3: 5' crossover of candidate 1 and 2; lane 2 and 4: 3' crossover of candidate 1 and 2.

In candidate 2 (orientation 2) occurred a double crossover event because for both ends the expected PCR products can be seen (Figure 29). Nevertheless, this strain is not homozygote for the deletion and there are still copies of ORF49 present (data not shown). For orientation 1 the candidates show a clear PCR product for the 5' crossover but the signal for the 3' end is very weak (Figure 30A). This weak band could be an unspecific band, so a southern blot was performed to control if the PCR product is caused from the 3' crossover (Figure 30B). The probes used in the southern blot were designed to hybridize to the 5' respectively to the 3' end of the DNA fragment, if it is a specific PCR product.

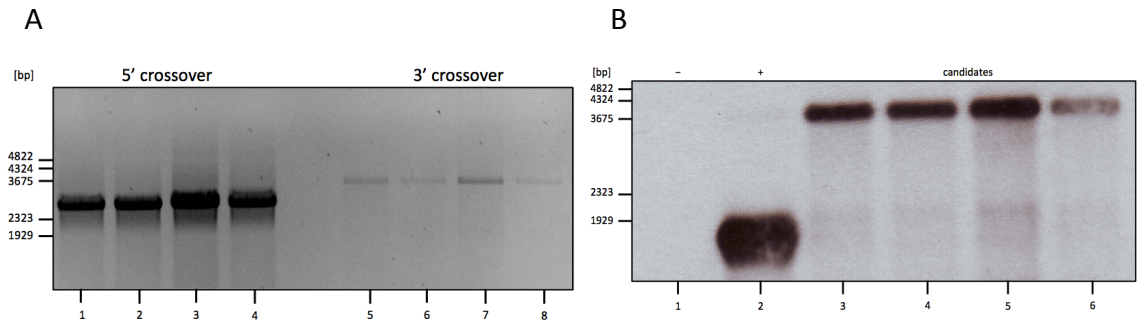

Figure 30: Control of double crossover event of orientation 1 by PCR and southern blot.
A: Test PCR for double crossover with primers Nov-12 and Soj-5 for the 5' end and Nov-13 and Repc-2 for the 3' end. Lane 1-4: 5' crossover of candidates 1-4; lane 5-6: 3' crossover of candidates 1-4.

B: Southern blot to control the specificity of the 3' crossover PCR product of candidates 1-4. Lane 1: negative control (PCR product from 5' crossover); lane 2: positive control (unlabeled probe); lane 3-6: PCR product of 3' crossover of candidates 1-4. The probe was synthesized with primer Δrep-13 and Δrep-23. The resulting probe is 1.8 kb large and hybridize to the 3' end of the PCR product.

The southern blot confirms that the PCR product from the 3' crossover is specific and the probes hybridize to both ends (data for 5' end not shown). The weak signal of the test PCR results from a crossover event at the 3' end. Candidate 1 was used for the further process, which is also not homozygous like the strain for orientation 2. Next, both strains have to become homozygous and deletion of every copy of ORF49 has to be achieved.

4.6.3 Homozygation of *Nab. magadii* L11-ΔORF49

Selection by size of the viral plaque

The first attempt to obtain a homozygous deletion mutant was to select for larger plaques. The mutant strain *Nab. magadii* L11-1 shows an earlier onset of lysis and therefore cells with deleted ORF49 may have a deregulated lysis behavior. One consequence of this deregulation is the production of larger plaques compared to plaques of the wild type strain (Iro *et al.*, 2007). First, a time course experiment was performed with both strains. Samples were taken at different time points. The viral particles can only contain either the deleted version or the wild type. Consequently, the cells, which are infected by ϕCh1-ΔORF49, will produce larger plaques compared to the wild type, caused by an earlier lysis. Therefore, a well growing *Nab. magadii* L13 culture has been infected with the supernatant. The size of the plaques was measured and the larger ones were picked an inoculated in fresh medium.

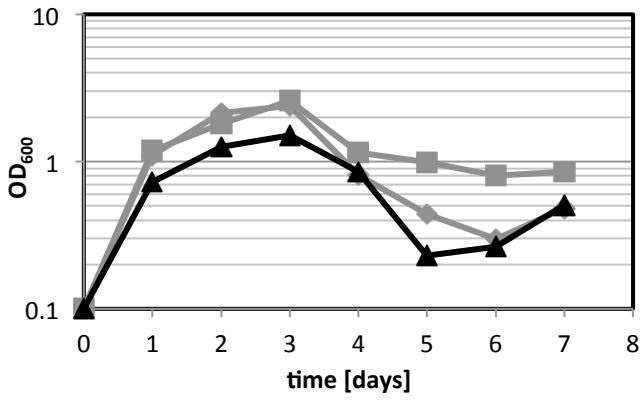

Figure 31: Growth curve of *Nab. magadii* L11-ΔORF49.
Growth curve of *Nab. magadii* L11 (▲), *Nab. magadii* L11-Δ49 (orientation 1) (♦) and *Nab. magadii* L11-ΔORF49 (orientation 2) (■). The mutant strains show the same lysis behavior than the wild type. Cells were grown in rich medium at 37 °C with agitation.

Figure 31 shows that the heterozygote mutant strains start lysis on day 4, the same day as the wild type. This could be explained by the wild type ORF49, which is still present in the cells. Analysis of the stains from the plaques showed, that no homozygous deletion mutant was obtained by this method. One reason for the failure of this method could be

that size of the plaque is not so much influenced by the deletion of ORF49. So it is very hard to differentiate between large and small plaques.

Infection of *Nab. magadii* L13 with ϕCh1-ΔORF49

As mentioned above, every ϕCh1 particle contains one copy of its genome. Therefore, each particle from a heterozygous *Nab. magadii* L11-ΔORF49 strain must carry the deleted or the wild type version. Upon infection of *Nab. magadii* L13 cells with supernatant of a lysed culture the cells will be infected with the wild type or the deletion mutant. However, this fact alone was not sufficient to separate the mutant from the wild type virus, as seen in the first attempt with the plaque size. Now I tried again to infect *Nab. magadii* L13 cells with the supernatant of a heterozygous strain. Here the different samples were plated after adsorption on rich medium agar plates with novobiocin. The incubation phase in liquid rich medium at 37 °C allows the virus to adsorb to the cell surface. Due to selection by novobiocin only cells, which are infected by mutant viral particle, will survive.

The colonies from the plates were transferred into fresh medium and tested for a homozygous deletion mutant. Therefore, a PCR was performed with primers, which binds in ORF49 respectively in the deletion cassette. After several tries and hundreds of tested colonies no homozygous deletion mutant was found. All tested cultures contained either both or only the wild type version. The reason for the heterozygous cells is not clear because super-infection of one cell by two viral particles does normally not occur. However, it could happen that the colonies are not produced by a single cell. The transfer of the colony to liquid medium then causes a heterozygous culture.

Passaging of *Nab. magadii* L11-ΔORF49

As a third method I tried to select for cells, which have more copies of the novobiocin resistance gene, by passaging. This was already successfully done in the deletion of genes in *Nab. magadii* (Derntl, 2009). The cells were grown in rich medium supplemented with novobiocin until they reach an OD_{600} around 1.0. This was important to passage the cells

before lysis occurs. Then a small amount was transferred into fresh medium and again grown to OD_{600} around 1.0. This gives a growth advantage to cells, which have more copies of the resistance gene. After some passages, the culture will become homozygous.

For each orientation a culture was used for the process. After some passages a dilution plate was made to separate the cells in the culture. The colonies from the plates were screened by PCR. Then the culture with the weakest signal for ORF49 and the strongest for the deletion cassette was used for further passaging. Additionally, the original culture was further passaged. Every fifth passage the culture was tested by PCR. Figure 32 and Figure 33 show the scheme of passaging and also the test PCR of the last culture of every passage.

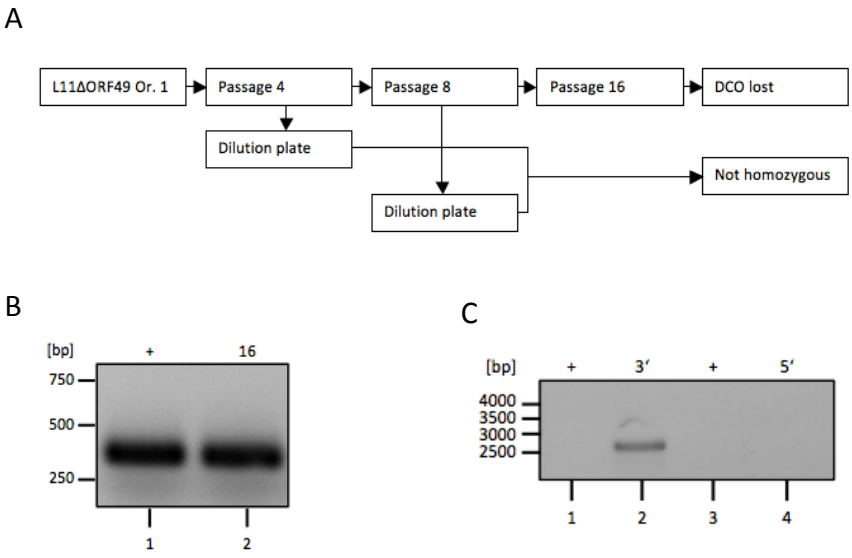

Figure 32: Passaging process for *Nab. magadii* L11-ΔORF49 (orientation 1).
A: Scheme of passaging process. A dilution plate was made from passage 4 and 8 and colonies were screened. B: Test PCR of passage 16 for ORF49. Lane 1: *Nab. magadii* L11 (positive control); lane 2: culture of passage 16. C: Control if cells, which contain a double crossover of the deletion cassette, are still present. Lane 1 and 3: *Nab. magadii* L11 (negative control); lane 2: test for 3' crossover of passage 16; lane 4: test for 5' crossover of passage 16. The wild type ORF49 is still present, whereas there was no signal for a double crossover anymore.

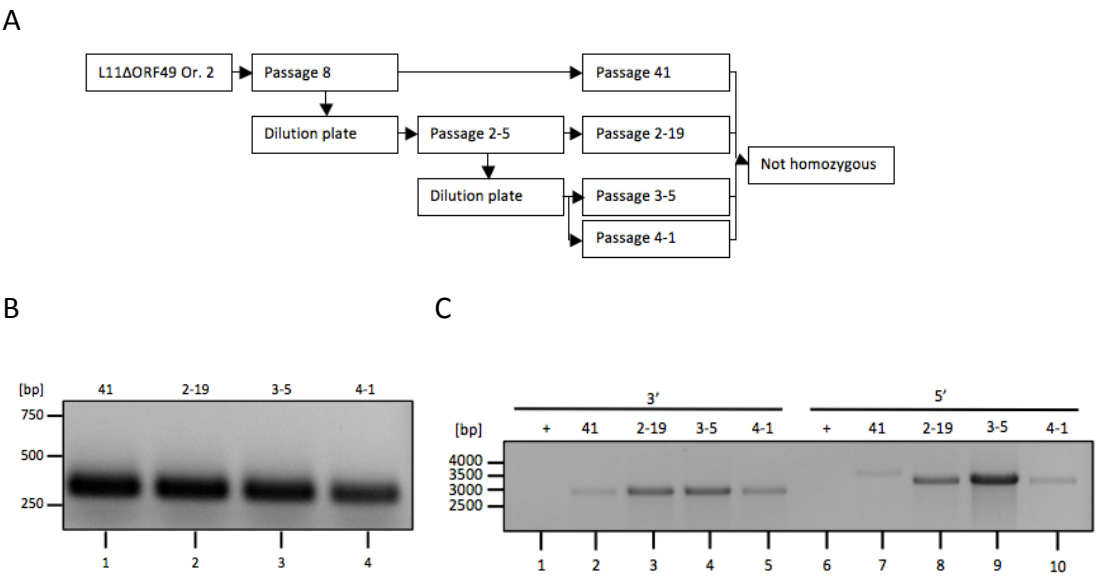

Figure 33: Passaging process of *Nab. magadii* L11-ΔORF49 (orientation 2).
A: Scheme of passaging process. A dilution plate was made from passage 8 and 2-5 and colonies were screened. With colonies from the dilution plates a new passaging was started. B: Test PCR for ORF49 after ending the passaging. All cultures still contain the wild type gene. C: Test PCR for a double crossover. Lane 1 and 6: *Nab. magadii* L11 (negative control); lane 2-5: control for 3' crossover; lane 7-10: test for 5' crossover. The signal for the deletion cassette is weak, whereas the signal for ORF49 is strong.

Unfortunately, it was not possible to delete ORF49 completely and it is still present after several passages. The process was stopped after the signal for the deletion cassette gets weaker or lost. The signal for ORF49 stayed as strong as on the beginning. The weak signal for the 3' crossover of orientation 1 disappears already after 12 passages. The passaging did not cause an increase in the amount of copies of the deletion, instead the cells become resistant to novobiocin. Therefore, also this method failed and the complete experiment was aborted.

4.6.4 Discussion

It was not possible to obtain a homozygous deletion mutant of ORF49 with different methods. None of the methods did lead to success and the heterozygous mutants did not lose the wild type gene. Since I was the second person who failed with this project, it

seems that deletion of ORF49 is quite impossible. A problem with the selection marker novobiocin is that the cells can become resistant. The resistance gene *gyrB* form *Haloferax* differs in only three mutations from the wild type gene (Holmes and Dyall-Smith, 1991). Spontaneous mutation followed by resistance can therefore happen, especially when the cells are exposed a longer time to the antibiotic. Perhaps a higher concentration of novobiocin could solve this problem, but this could cause cell death at the beginning of growth.

A reason for the failure could be that ORF49 is such an important regulator in the life cycle of ϕCh1 that a complete deletion is not possible. This is supported by the fact that the mutant *Nab. magadii* L11-1 lose the mutation after short time (Iro *et al.*, 2007). A complete loss of the regulator can also lead to a very early lysis of the cells and so a homozygote deletion mutant is not viable. This fact supports the theory that ORF49 is a regulator of ϕCh1 and is involved in lysis regulation. For further investigation of ORF49, another method then deletion has to be found.

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7 Appendix

7.1 Acknowledgment

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7.2 Abstract

Understanding of the regulation of the viral life cycle is important to develop tools for genetic manipulation or protein synthesis in the host organism. This is especially important for less studied organisms. This thesis therefore concerns the transcriptional regulation of the virus ϕCh1, which infects the haloalkaliphilic archaeon *Natrialba magadii*.

The first part of this thesis deals with the regulation of the tail fibre protein gp34. This protein is a part of a phase variation system, in which the C-terminal end can be exchanged to produce two different orientation of this protein. Production of each variant in the cured strain *Nab. magadii* L13, showed that the (+) orientation reduces the infectivity of ϕCh1. This result is another confirmation that only one orientation of gp34 is able to adsorb to *Nab. magadii*. It has been further shown, that the lysogeny regulators ORF48 and ORF49 have no influence on the production of the tail fibre protein. However, ORF44 has a repressing effect on ORF34. ORF44 is a part of a putative archeal toxin-antitoxin system and has homologies to a PIN-domain nuclease. The antitoxin of ORF43 prevents this repressing effect.

The focus of the second part of this thesis lies on the ORF34 regulator ORF79. Previous experiments revealed a repressing activity of ORF79 on the production of gp34. Here, the specificity of the regulator was tested by co-expression with other ϕCh1 genes. The first investigated gene was *soj*. In the course of this experiment an antibody against the protein Soj was created. It has been shown that the production of this protein is not regulated by ORF79, but this protein had an influence on plasmid stability. This could be explained by the homologies of Soj to partitioning proteins. For this reason the influence of ORF79 on a second protein was observed. Therefore, the methyltransferase was used, which is also not affected by ORF79. Additionally, the expression pattern of ORF79 was monitored and compared with the expression pattern of ORF34.

The last part of this thesis deals with the regulator ORF49, which is involved in the switch between lysogenic and lytic life style. To further characterize this ORF, I tried to construct a homozygous deletion mutant. This attempt failed, because the complete deletion of the wild type ORF49 was not possible.

7.3 Zusammenfassung

Das Verständnis der Regulation von Viren ermöglicht die Entwicklung von Methoden für genetische Manipulation sowie die Synthese von Proteinen in der Wirtszelle. Vor allem in noch weniger erforschten Organsimen ist die Entwicklung neuer Methoden essentiell. Diese Masterarbeit beschäftigt sich mit der Regulation des Virus ϕCh1, der das haloalkaliphile Archaeon *Natrialba magadii* infiziert.

Der erste Teil dieser Arbeit untersucht die Regulation des tail fibre Proteins gp34. Dieses Protein ist Teil eines Phasenvariationssystem und kann in zwei Orientierungen produziert werden. Die Produktion beider Varianten in dem vom Virus geheilten Stamm *Nab. magadii* L13 zeigte, dass bei einer Variante der Virustiter stark verringert wird. Das Ergebnis bestätigt somit, dass nur eine Version die Bindung an die Wirtszelle ermöglicht. Eine weitere Analyse der Regulation zeigte, dass die Regulatoren ORF48 und ORF49 keinen Einfluss auf ORF34 haben, jedoch ORF44 die Expression inhibiert. Eine mögliche Erklärung ist die Homologie von ORF44 zu einer PIN-Domäne Nuclease und somit zu einem Toxin-Antitoxin System.

Der zweite Teil beschäftigt sich mit dem ORF34 Regulator ORF79. In einer früheren Arbeit wurde die inhibierende Wirkung dieses Regulators auf ORF34 gezeigt. Um zu zeigen, ob ORF79 spezifisch auf ORF34 wirkt, wurde der Einfluss auf andere Proteine von ϕCh1 untersucht. Dazu wurde das Protein Soj ausgewählt und ein Antikörper dagegen hergestellt. Das Protein zeigte keinen Regulationseffekt durch ORF79, aber einen Einfluss auf die Stabilität des Plasmides. Dies kann durch die Homologie von Soj zu Partitionierungsproteinen erklärt werden. Deshalb wurde zusätzlich die Produktion der Methyltransferase unter Einfluss von ORF79 untersucht. Hier konnte erneut kein Regulationseffekt von ORF79 auf die Expression festgestellt werden. Weiters wurde das Expressionsmuster von ORF79 im Vergleich zu ORF34 untersucht.

Im letzten Teil dieser Arbeit wurde versucht eine Deletionsmutante des ORF49 zu konstruieren. Dieser ORF49 ist involviert in den Wechsel vom lysogenen zum lytischen Lebensstil des Virus. Eine homozygote Mutante sollte dieses Protein weiter charakterisieren. Doch der Versuch blieb ohne Erflog, da es nicht möglich war alle Kopien von ORF49 aus der Zelle zu löschen.

7.4 Curriculum vitae

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