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„Construction of virus ϕCh1 ORF43 and ORF43/44 deletion mutants "

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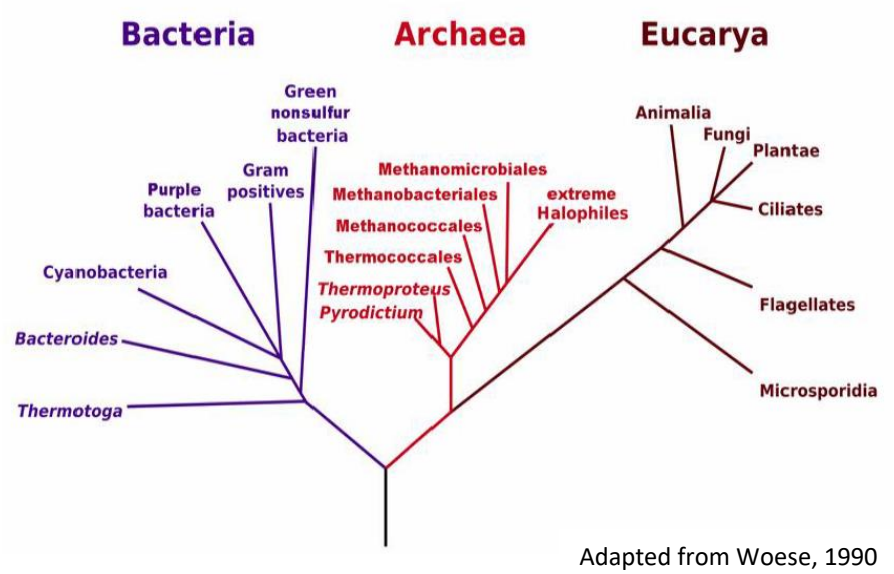
1. INTRODUCTION

1.1. Archaea

1.1.1. The timeline of archaeal discovery

The *Archaea* evolved as a third kingdom that has quintessential role in shaping the evolutionary path of life. Even though studies related to existence of *Archaea* in the scientific literature began 140 years ago, Carl Woese and George Fox proved the Archaea are unique organism which distinguishable from the *Bacteria* and *Eucarya* (Woese & Fox, 1977). Additionally, in 1990 the phylogenetic tree of life was created by Woese's method which classify species according to the number of shared oligonucleotide sequence that related to 16S rRNA (Woese, 1990).



Adapted from Woese, 1990

Figure 1: Carl Woese's model for a phylogenetic tree of life based on 16S (18S) ribosomal RNA sequences

As illustrated in *Figure 1*, Archaea share traits with both Bacteria and *Eucarya*: they have common ancestors with *Eucarya* which led them to have also common information-processing systems like DNA replication, transcription, translation; although archaeal metabolism and bacterial metabolism are alike. The classification system separates the prokaryotic phylum into *Bacteria* and *Archaea*, even though *Archaea* appear to be closer related to eukaryotes than to *Bacteria* (Woese, 1990). Related to archaeal phylogenetic tree,

first Woese subdivided Archaea into the kingdom *Euryarchaeota* which comprising methanogens, and the kingdom *Crenarchaeota* which comprising extremely thermophilic Archaea. Later studies described new kingdoms of Archaea: *Nanoarchaeota* are represented by the only member *Nanoarchaeum equitans* (Huber *et al*, 2002), and *Korarchaeota* are indicated only by DNA sequences of environmental samples of un-culterable archaea deriving from volcanic hot springs (Barns *et al*., 1996).

1.1.2. Halophilic and haloalkaliphilic *archaea*

Most of the archaeal species are able to survive in extreme environments (Rothschild *et al*., 2001). While the halophilic *Archaea* has extremophilic nature as a high salt concentration which is called hypersaline environments, the haloalkaliphilic *Archaea's* additionally needs high pH. The hypersaline environments have higher concentration of NaCl compared to the seawater that for example saline lakes, saltern ponds or hypersaline soils. Halophilic *Archaea* can be classified in two categories: moderate and extreme halophiles. Moderate halophiles reside with the salt concentration round about 0.5-2.5 M NaCl, but extreme halophilic *Archaea* need 2.5-5.2 M NaCl in their environment for proper growth (Andrei *et al.*, 2012). In such environment that extreme halophiles may grow, the evaporation rates are higher than precipitation rate which leading to ion concentrations are different the seawater therefore environments combining with alkaline pH. Haloalkaliphilic *Archaea* are obligate organisms that are found in so-called hypersaline alkaline habitats (Grant and Larsen, 1989). The most known example of hypersaline alkaline habitat is Lake Magadi in Kenya, Africa.

1.1.3. Haloalkaliphilic Archaeon - *Natriallba magadii*

The haloalkaliphilic archaeon *Natrialba magadii*, which belongs to family of Halobacteriaceae within the phylum Euryarchaeota, first been isolated from Lake Magadi, Kenya (Tindall *et al*., 1984). Although *N. magadii* was initially placed into new genus as a genus *Natronobacterium*, the phylogenetic relationships between the species by comparing the 16S rRNA led to the re-classification of *Natronobacterium magadii* into the genus *Natrialba* (Kamekura *et al*., 1997). The natural habitat of *N. magadii*, Lake Magadi, is called soda lake due to a salt concentration of up to 300 g/L with a pH exceeding 11. (Oren A., 2002).

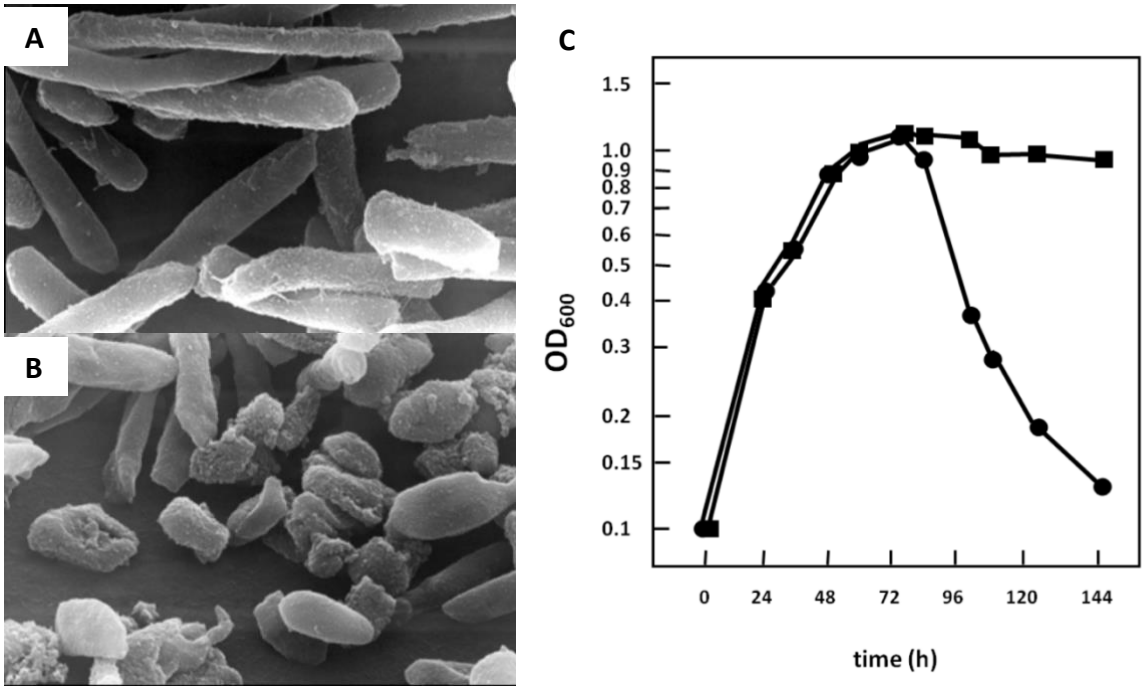


Figure 2: Electron micrographs of N. magadii strains & Growth Kinetics

A: *Natrialba magadii* L13, the cured strain
B: *Natrialba magadii* L11, the wildtype strain carrying Ch1 as a provirus
C: *N. magadii* L11 is represented by circles and *N. magadii* L13 is represented by squares. Exponential growth behaviour is almost identical for both strains but virus-induced lysis occurs after roughly three days in the case of *N. magadii* L11 whereas no lysis can be observed for the cured strain *N. magadii* L13
(M. Iro, 2006)

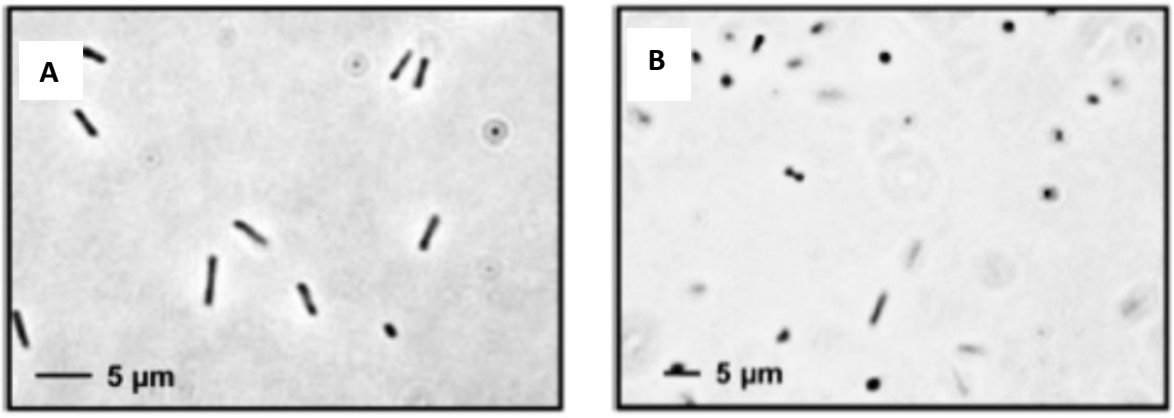
Therefore, in order to provide optimal growth conditions in laboratory, *N. magadii* requires a medium containing 3.5-4 M of NaCl that concentrations below 1.5 M result in cell lysis through osmotic pressure. Also, a pH between 9.5-11 and a temperature optimum of 37°C-42°C is essential in laboratory conditions. Additionally, a low Mg^{2+} ion concentration is required. *N. magadii* is strict aerobe and chemoorganotroph, that using peptides and amino acids as an energy source. (Tindall *et al*., 1984).

N. magadii cells are rod-shaped, 5-7 µm in length and motile through the presence of polar flagella. The cells are incorporated with carotenoid pigments in their membranes therefore they have reddish color not only in their growth cultures but also in colonies. In terms of genome characteristics of *Halobacteriacaea* family, they contain more than one copy of the genome per cell which is called polyploidy (Breuert *et al.,* 2006). *Natrialba magadii* up to 50 copies of chromosomal DNA per cell with roughly 9 hours generation time (Tindall *et al*., 1984).

In this study two *N. magadii* strains was used; *N. magadii* L11 was lysogenic wildtype strain that ϕCh1 integrated into its genome as a provirus, *N. magadii* L13 was called "cured" strain that lacking the provirus by continuous passaging. Also *N. magadii* L13 strain was used as an indicator strain that may still be re-infected by Ch1.

1.1.3.1. Genetic manipulations of N. magadii

Not only consisting of 50 copies of genome per cell but also 9 hours generation time makes a challenge in order to study with *N. magadii.* Additionally, one of the major challenge is the lack of genetic tools for use in haloalkaliphilc *Archaea.* Therefore, including this study some studies are performed in *E. coli* with its comparatively short generation time of 20-30 minutes before performing the actual genetic manipulations in *N. magadii*. Although researches were in slow progressing discipline, first successful transformation of haloalkaliphilc *Archaea* was done in 1987 by Cline and Doolittle where a polyethylene glycol-based method of transformation using spheroplasts was adopted in order to successfully transform ϕH DNA into *Halobacterium salinarum* (Cline and Doolittle, 1987). Also, by the help of same method transformation of *Haloferax volcanii* was accomplished at the same year (Charlebois *et al*., 1987). However, for the tranasformation of haloalkaliphilic species *N. magadii* its been discovered that for the removal of S-layer EDTA was not enough. Therefore, the polyethylene glycol-based transformation method was used with additional step in order to generate competent cells of *N. magadii. N. magadii* cells were incubated in bacitracin and proteinase K in order to inhibit glycosylation of S-layer which resulted in competent *N. magadii* spheroblasts (Mayrhofer-Iro *et al*., 2013).



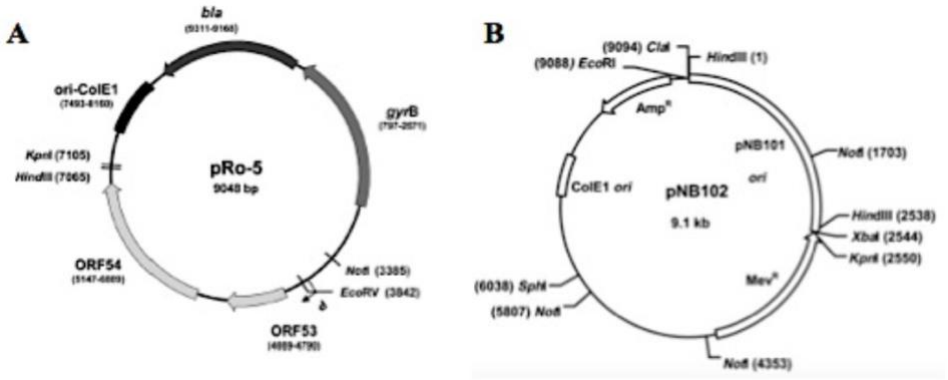
Adapted from Mayrhofer-Iro *et al.,* 2013.

Figure 3: Micrographs of spheroplasts of N. magadii L13

A: The rod-shaped *Natrialba magadii* cells in rich medium

B: The round-shaped *Natrialba magadii* cells with the treatment of bacitracin and proteinase K as they lose their S-layer

Also, there are specific shuttle vectors for the transformation of *N. magadii.* One of them is pRo-5 that the *E. coli* vector pKSII+ containing the point-mutated *gyrB* gene from the halophilic archaeon *Haloferax alicantei* with resistance to the novobiocin, which is specific marker for halophilic *Archaea*. Novobiocin is a DNA gyrase inhibitor inhibiting the B subunit of DNA gyrase by blocking the ATP-binding site (Holmes *et al*., 1991). Additionally, pRo-5 contains ampicillin resistance for selection in *E. coli* (Mayrhofer-Iro *et al*., 2013). Furthermore, it contains parts of ϕCh1 ORF53 and ORF54 that are necessary for origin of replication in order to achieve autonomous replication in *N. magadii* (Mayrhofer-Iro *et al*., 2013). The second shuttle vector is pNB102 from the haloarchaeal plasmid pNB101 in which the *E. coli* replicon ColE1 has been introduced with ampicillin as well as mevinolin (Zhou *et al*., 2004). Mevinolin is a HMG-CoA inhibitor, thereby blocking synthesis of archaeal isoprenoid lipids (Lam and Doolittle, 1992). Therefore, pNB102 is suitable to work with both *E. coli* and *N. magadii.*



Adapted from Mayrhofer-Iro *et al.,* 2013, Zhou *et al*., 2004

1.1.4. Haloalkaliphilic virus - ϕCh1

The discovery of viruses infecting haloarchaea was in 1974 that named as a ϕH which infects the archaeon *Halobacterium salinarum* (Torsvik, Dundas, 1974). By 2006, more than fifteen haloarchaeal viruses were described with their isolation and cultivation methods (Smith, Oren

et al., 2006). Even though ϕH was best studied virus among the all haloarchaeal viruses, the closest relative was ϕCh1 which infect *N. magadii* discovered by Witte in 1997 (Witte *et al*., 1997). ϕCh1 is a temperate virus and persists as prophage that integrated into chromosome of *N. magadii* (Klein *et al*., 2002; Schnabel *et al*., 1984; Witte *et al*., 1997).

1.1.4.1. Morphology of ϕCh1

As described above ϕCh1 was discovered in 1997 as a first phage that infects haloalkaliphilic archaeon. Upon spontaneous lysis of *N. magadii* culture ϕCh1 was isolated (Witte *et al*., 1997). The electron microscopy analysis descripted that ϕCh1 has morphologically resemblances to family of *Myoviridae*. ϕCh1 has a classic head-tail morphology with the size of roughly 200 nm in length by 20 nm in width. The viral head consists 58.498 bp viral genome which is roughly 70 nm in diameter and exhibits an icosahedral structure, whereas the virus' contractible tail measures approximately 130 nm (Witte *et al*., 1997; Klein *et al*., 2002).

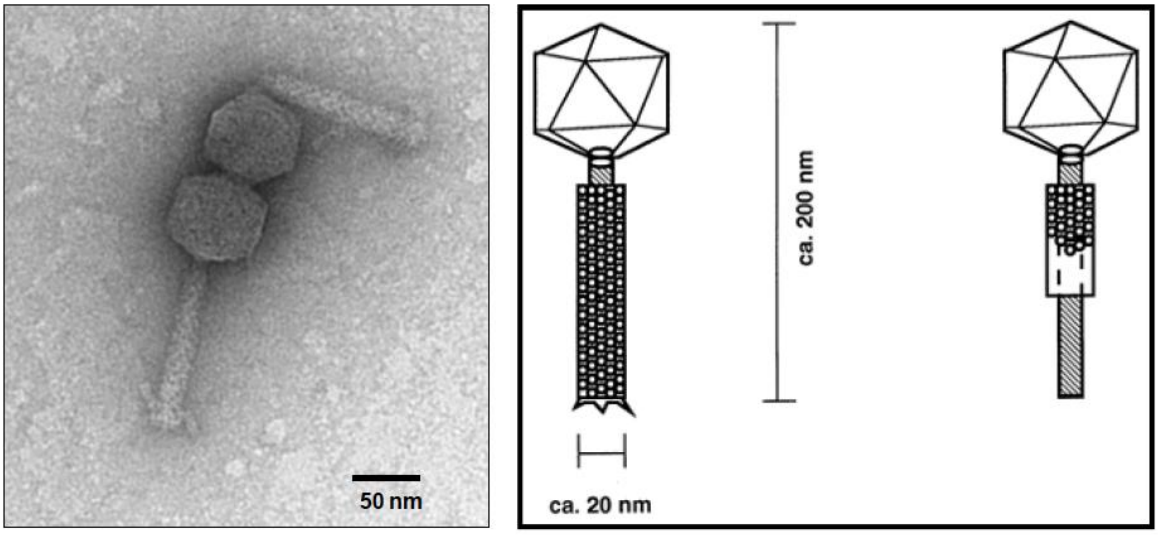


Figure 4: Morphology of ϕCh1

A: Electron micrograph of isolated ϕCh1 viral particles (Witte *et al*., 1997)
B: Schematic representation of a ϕCh1 virus particle alongside its dimensions (Witte *et al*., 1997)

1.1.4.2. General characteristics of ϕCh1

The host growth conditions, high salt concentration and pH, effects infectivity and stability of ϕCh1. It was tested that a low salt concentrations (lower than 2 M NaCl) lead to segregation of the virus particles or conformational changes of the capsid proteins, therefore resulting to loss of infectivity (Witte et al., 1997). The protein composition of ϕCh1 was determined by SDS-PAGE analysis. There are four major (A, E, H and I) and five minor proteins (B, C, D, F and G) with a size ranging from 15-80 kDa and having isoelectric points between pH 3.3 and pH 5.2 similar to various proteins of other halophilic Archaea (Lanyi, 1974).

Also, unusually for viruses, ϕCh1 particle contains RNA with the length range of 80-700 nucleotides. Even though mainly host-derived RNAs were detected, there were virus specific RNAs obtained from purified virus particles which still functionally unknown (Witte et al., 1997). Furthermore, although the sequence identity between ϕCh1 and ϕH has 97% relativity, the main difference is; ϕH acts as an episomal prophage, whereas ϕCh1 acts as an integrated provirus (Schnabel & Zillig, 1984; Witte *et al.,* 1997). Thus, ϕCh1 is temperate virus.

Moreover, the restriction analysis via *Dpn*I/*Mob*I/*Sau*3A indicated that while the DNA of ϕCh1 is partially dam-like methylated within 5'-GATC-3', the chromosomal DNA of *N. magadii* is not Dam methylated. Therefore, this result lead to the idea; ϕCh1 might code for its own methylation. Further genetic analysis led to discovery of *mtase* gene, with the gene product M.*Nma*ΦCh1I Baranyi *et al*., 2000).

1.1.4.3. Genome organization & life cycle of ϕCh1

The first complete sequence of ϕCh1 genome was published in 2002 (Klein *et al*., 2002). ϕCh1 DNA has a size of 58.498 bp with with an overall G+C content of 61.9% and distinct 98 open reading frames (ORFs) which concluded to be protein-coding genes starting with ATG, except four ORFs (3, 41, 79 and 83) starting with GTG. As indicated in *Figure 5*, the linear genome is organized into three parts; the 5' region (ORFs 1-34) are for structural components and assembly, the middle part with the invertible region involved in DNA replication, and the 3' region (ORFs 56-98) generally involved in DNA modifications which still unknown function.

There are three identified region of ϕCh1 methyltransferases; the main ORF94-encoded methyltransferase M.*Nma*ϕCh1I, ORF80-encoded M.*Nma*ϕCh1II, and ORF82-encoded M.*Nma*ϕCh1III (Klein *et al*., 2002). Most of the ORFs encoding proteins in ϕCh1 genome are similar to the ones with known function share sequence similarities with the virus ϕH which infects *H. salinarum* (Klein *et al*., 2002). These similarities between ϕH and ϕCh1 might rely on their host habitat. However, the identification of all these unknown function gene still is an ongoing study.

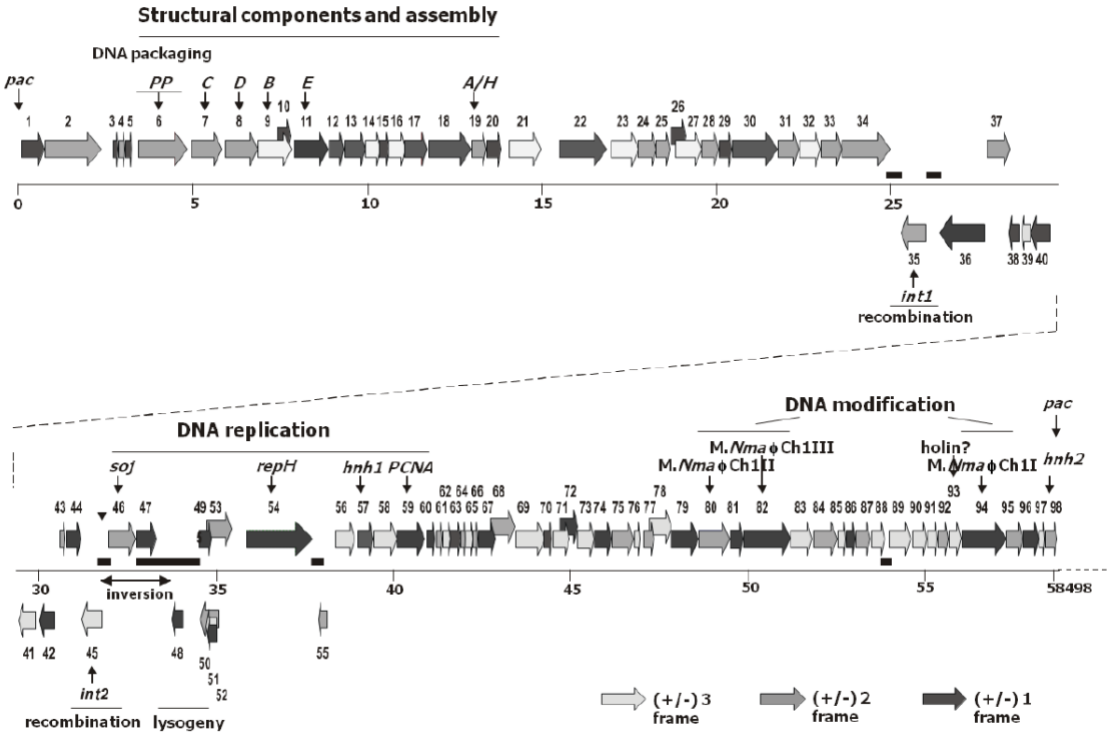


Figure 5: The illustration of linear genome of ϕCh1

The ORFs are represented by arrows, and the putative, along with the established functions of the gene products, are shown. The greyscale of the arrows indicates the frame of the ORFs: light grey – third frame, mid grey – second frame, dark grey – first frame. The genome consists of three functional modules: On the left side, the genes coding for structural proteins, which are presumably involved in virion morphogenesis, are present. The central part is involved in replication, regulation of gene expression and plasmid stabilization. The genes on the right side are responsible for DNA modification. (Klein *et al*., 2002).

As a temperate virus ϕCh1 need a control system to enter and keep the lysogenic state and to switch to the lytic life cycle. ϕCh1 exists as a provirus until occurrence of stationary growth phase, then newly synthesized viral particles are released through lysis of the host cells (Witte

et al., 1997). The regulation of switching lysogenic to lytic cycle is controlled generally by transcriptional repressors like in bacteriophage λ (Ptashne M., 1967). The regulation in bacteriophage λ is controlled by two repressor proteins; Cro and CI. The two corresponding genes are organized head to head and are transcribed leftwards and rightwards. The operator region includes three repressor binding sites also promoter regions for two repressor genes. The repressor genes bind to those binding sites within the operator with different affinity order. Lysogeny of virion is controlled by transcription of CI. Moreover, CI not only regulates and controls its own expression but also blocks the promoter region which is responsible for transcription of Cro and early lytic genes. The Cro is essential for the lytic life cycle (Ptashne M., 2004).

In the case of ϕCh1 mechanism of regulation between lysogenic and lytic cycle is resemble to bacteriophage λ. The proteins Rep (ORF48) and T4 are organized head to head similar to Cro and CI of bacteriophage λ. The repressor, Rep, is transcribed during lysogeny and blocks transcription of the early lytic genes from the promoter for T4. T4 is produced during the lytic life cycle and block transcription of Rep (Stolt and Zillig, 1994). Further studies done by Iro *et al*. investigate that gp48 actually contains a helix-turn-helix DNA-binding domain which led to description of repressor-operator system (ORF48/49) making up the lysogenic region of ϕCh1 (Iro *et al*., 2007).

1.1.4.4. ϕCh1 ORF43/44 – Putative Toxin-Antitoxin System

Toxin-antitoxin system in general is effective for plasmid maintenance or stability modules (Holcik & Iyer, 1997), protection of DNA loss (Van Melderen, 2010), and stress regulation via apoptosis (Lewis, 2010). The toxin part can prevent cell growth by targeting substances that essential in fundamental cellular processes, like DNA replication, mRNA stability and protein synthesis (Pandey & Gerdes, 2005; Yamaguchi et al., 2011). If toxin and antitoxin part are co-transcribed and/or co-translated, they can be called as TA operon. Generally, there are three types of TA systems: The type I and III TA systems have antitoxin part as a noncoding RNAs, which bind to the toxin mRNA and prevents the toxin production, whereas type II TA system has an unstable antitoxin protein, which binds to the toxin and inhibits its production (Van Melderen & Saavedra De Bast, 2009).

Upon hypothesis that related to ϕCh1, ORF43/44 region has similarity to virulence associated protein, VapBC, toxin-antitoxin system which belongs to type II systems. This VapBC system demonstrates represent the largest family of bacterial toxin-antitoxin systems (Arcus *et al*., 2011). The VapBC TA system has operon that VapC part contains a PIN domain which has ribonuclease function whereas VapB represents the stable antitoxin (inhibitor) containing a transcription factor domain (Van Melderen and Saavedra De Bast, 2009).

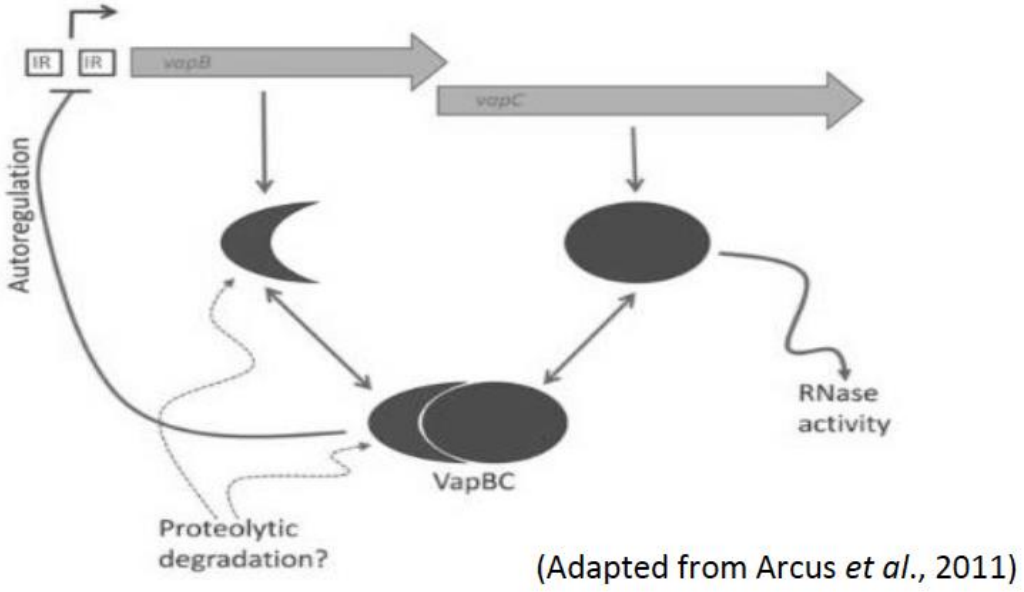


Figure 6: The illustration of VapBC Toxin-Antitoxin System
The start-and-stop codons of the two genes encoding for the stable antitoxin (*vapB*) and for the RNase (*vapC*) which forms together operon system. The *vapBC* operon encodes proteins forming a tight complex together which binds to inverted repeats (IR) in the promoter region of the *vapBC* operon. This binding results in the autoregulation of *vapBC* operon transcription (Arcus *et al*., 2011).

As illustrated in *Figure 6*, two overlapping genes forming an operon that *vapB* represents the stable antitoxin whereas *vapC* for the RNase. The transcription of *vapBC* is autoregulated by promoter binding. However, activation of toxin VapC is unknown although it is believed to result from proteolytic degradation of the stable VapB part (Arcus *et al*., 2011).

In the matter of ϕCh1, ORF43 and ORF44 have overlapping start and stop codons which means they are co-transcribed and co-translated (Marcotte et al., 1999). In order to conduct more information about TA system on the region ORF43/44, Experiments first were done by Michaela Iro in *H. volcanii* which is a halophilic model organism that including gene products of ORF43 (gp43) and ORF43/44 (gp43/44). The results showed that both gp43 and gp43/44 have a repressor effect on ORF49 transcription via binding to the 5' repeats in the ORF48 (*rep*) sequence (Iro M., 2006). Also, the gp44 alone has ability to degrade mRNAs belongs to ORF48 and the intergenic region between ORF48 and ORF49 (Iro M., 2006). These results are demonstrated that gp44 has a predicted PIN-like domain, therefore making gp44 capable of exhibiting a nuclease function. Additionally, according to the Pfam analysis, it's been conducted that there is a PIN domain in the gene product of ORF44. Therefore, hypothesis arose that while gp44 is a putative toxin of type II VapBC TA system, gp43 is a potential antitoxin.

2. MATERIALS & METHODS

2.1. MATERIALS

2.1.1. Strains & Related Medias

2.1.1.1. Bacterial Strain - Escherichia coli

Strain	Genotype	Source
XL1-Blue	*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*(r_K^-, m_K^+), *supE44*, *relA1*, *lac*, [F′, *proAB*+, *lacI*^{q}ZΔM15, Tn10(Tet^r)]	Stratagene

Lysogeny broth (LB)

Components	Amount
Yeast extract	5 g
NaCl	5 g
Peptone	10 g

pH 7.0, ddH_2O add 1 l, 15 g agar for plates

2.1.1.2. Archaeal Strains - *Natrialba magadii*

Strain	Genotype	Source
N. magadii L11	Wild type carrying provirus ϕCh1	Witte *et al*., 1997
N. magadii L13	Cured strain lacking provirus ϕCh1	Witte *et al*., 1997

Natrialba rich medium (NVM)

Components	Amount
Casein hydrolysate	8.8 g
Yeast extract	11.7 g
Tri-sodium citrate dehydrate	0.8 g
KCl	2.35 g
NaCl	235 g

pH 9.5, ddH_2O add 935 ml, 10 g/l agar for plates, 5 g/l agar for top agar.
Upon autoclaving, medium complemented with:

Components	Volume
0.57 M Na_2CO_3	63 ml
1 M $MgSO_4$	1 ml
20 M $FeSO_4$	1 ml

2.1.2. Antibiotics & Additives

Domain	Compound	Stock Conc.	Final Conc.	Preparation
Bacteria	Ampicillin	20 mg/ml	100 μg/ml	Dissolved in ddH_20, sterile filtered, stored at 4°C
	Tetracycline	10 mg/ml	10 μg/ml	Dissolved in half of ethanol and half ddH_20, stored at -20°C
	IPTG	1 M	0.5 – 1 mM	Dissolved in ddH_20, sterile filtered, stored at -20°C
	X-Gal	10 mg/mL	20 μg/ml	Dissolved in N-N-dimethylformamide, Stored at -20°C
Archaea	Novobiocin	7 mg/ml	3 μg/ml	Dissolved in ddH_20, sterile filtered, stored at -20°C
	Mevinolin	10 mg/ml	6 μg/ml	isolated from pulverized Lovastatin* tablets, dissolved in 96 % ethanol, stored at -20°C
	Bacitracin	3 mg/ml	70 μg/ml	Dissolved in ddH_20, sterile filtered, stored at 4°C

*120 mg of Lovastatin tablets (6 tablets*20 mg) are dissolved in 12 mL of 96% EtOH via stirring for 15-20 minutes at room temperature, then centrifugation at 12 krpm for 15 minutes at 4 ° C.
The supernatant was collected as a final concentration 10mg/ml and stored -20°C.

2.1.3. Plasmids

Construct Name	Feature	Source
pBlueScript II KS(+)	mcs, bla, ColE1 ori, lacZa	Stratagene
pNB102	*bla*, ColE1 ori, *hmg* (Mev^R) pNB101 ori	Holmes *et al.*, 1991
pUC19-NovR "reverse"	pUC19 containing the novobiocin resistance cassette in reverse orientation	Wurzer, 2015
pUC19-NovR "forward"	pUC19 containing the novobiocin resistance cassette in forward orientation	Wurzer, 2015
pKS^+II-43up-ORF44	Shuttle vector containing promoter region of ϕCh1 ORF43 and the region ΦCh1 ORF44	Michi, 2005

Construct Name	Feature	Source
pKS$^+$II-ΔORF43/44-F	Suicide plasmid for ϕCh1 ΔORF43/44 with novobiocin resistance cassette in forward orientation	Manning, 2017
pKS$^+$II-ΔORF43/44-R	Suicide plasmid for ϕCh1ΔORF43 with novobiocin resistance cassette in reverse orientation	Manning, 2017
pNB102-MI-1/43/44	pNB102 containing only intergenic region and ϕCh1 ORF43/44	This Study
pNB102-MI-1/43	pNB102 containing only intergenic region and ϕCh1 ORF43	This Study
pNB102-MI-2/43/44	pNB102 containing intergenic region, repressor and ϕCh1 ORF43/44	This Study

2.1.4. Primers

Primer Name	Sequence (5'-3')
40-3	GCACGGTACCTCATATCTTCGTCACGACCC
40-5	CAGCCGGATCCATGTGCTGGATGAGCATCTAC
43-3up-NB	GAACGGATCCCATATGAGGTCTTACTCGGCCTCCTCG
43-Hind	CAGCAAGCTTTCATTCGCGCTCG
43-Kpn-3	CAGCAGGGTACCCGTGTCGACGAACAGCG
43-Kpn-5	CAG CAG GTA CCG TTG TGC CAG CCG T
43-probe	GCGGTGATCTCGTCGT
44-3en	GACGCCTGCAGAACTACCTT
44-Hind	CAGCAAGCTTGATTTAGGACTCGAGGACC
49-Kpn	CAGCGGTACCTTGCGTTCAGTTCCG
BgaH-3i	GAG TGA AAA ACC ACC CATG
D43-2	GCTGGATATTACGGCCTGCACGACCACGCCGATTCGTCCG
D43-4	TCGTTCCAGTCGACACCGACCACGCCGATTCGTCCG
D44-2	GAATGGATCCCTCCATCACGTTCAGCA
D44-3	GCAGAAGCTTAGGTCCTCGAGTCCTAAATC
D44-4	GAATGGTACCCAGTGACAGTGAAGGTTGC
IR-BgaH-2	GTGTATACGGCCGGTTTAAAGCTTCTAAGTCCGACAACACAATTCCTGTTTT
IR-BgaH-3	CCCTCCCATGCCACTCTTCACACGCTCATTCGCGCTCGCGCAGCTCGCGGA
IR-BgaH-4	CCCTCCCATGCCACTCTTCACACGCTTAGGACTCGAGGACCTCCTCCGGGG
Nov-12	GCCGGTGAGTACTTAACGC
Nov-9	GATGTCGGTCATCGCGG
ORF-45-3-F	GCTGGATATTACGGCCTGCAAGGTCCTCGAGTCCTAAATC
ORF-45-5	ACGGTATCGATAAGCTTGATACAGTGACAGTGAAGGTTGC
ORF41-42-3-R	GCTGGATATTACGGCCTGCAcctgcaggaggcctaaaacg
ORF41-42-5	CCGGGCTGCAGGAATTCGATtaggcctcctcctcggcggt

p43-ORF44-3-R	TCGTTCCAGTCGACACACCCcgttttaggcctcctgcagg
p43-ORF44-5	ACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGG
pBAD-X-1	GATCTCTAGAGACCGCTTCTGCGTTCTGAT
pro5-mev	CCTTATCAAACACGCACAC

2.1.5. Kits

For the efficient usage of Kits, buffers and solutions amounts were arranged according to company instructions.

Purpose of Usage	Name of Product	Company
Isolation/Purification of plasmid DNA in *E. coli*	Wizard.®Plus SV Minipreps DNA Purification System	Promega
Purification of PCR products and gel-eluted DNA fragments	Wizard® SV Gel and PCR Clean-Up System	Promega
Purification of gel eluted DNA fragments	QIAquick® Gel Extraction Kit	QIAGEN
Detection of biotin-labeled nucleic acids (Southern Blot)	Chemiluminescent Nucleic Acid Detection Module	BioRad
Chemiluminescent substrate for HRP visualization on immunoblots (Western Blot)	ClarityTM Western ECL Substrate	BioRad

2.1.6. Enzymes & Nucleotides

Enzymes	Company	Product Number
Restriction enzymes	Thermo Scientific	
DNA polymerases		
GoTagâ Green Master Mix	Promega	M0531S/M0532L
Phusion. Flash PCR Master Mix	Thermo Scientific	F548S/F548L
DNA modifying enzymes		
T4 DNA ligase	Promega	M1801
Alkaline Phosphatase	Thermo Scientific	EF0652
Other enzymes		
Proteinase K	QIAGEN	19133
Nucleotides		
Biotin-11-dNTP	GeneON	110

2.1.7. Antibodies

Dilutions of antibodies were prepared in in 1XTBS, 0.3% BSA, 0.02% NaN_3

Primary Antibodies

Name	Target Protein	Dilution	Source
α-E	ϕCh1 major capsid protein E	1:2500	Klein *et al.*, 2000
α- M.*Nma*ϕCh1-I	ϕCh1 main DNA methyltransferase	1:500	Till, 2011
α -Soj	Soj of ϕCh1	1:250	Hofbauer, 2015

Secondary Antibody

Name	Target Protein	Dilution	Source
ECL™ Anti-Rabbit IgG, HRP linked whole antibody from donkey	Rabbit Immunoglobulin G	1:5000	GE Healthcare UK Limited

2.1.8. DNA & Protein Markers

DNA Markers	Company	Size range in bp
λ/BstEII	λ DNA (Thermo Scientific) digested with BstEII	Fragment size range: 702–8454 bp
GeneRuler 1kb DNA Ladder	Thermo Scientific	Fragment size range: 250 to 10000
Protein ladder	**Company**	**Size range in kDa**
PageRuler™ Prestained Protein Ladder	Thermo Scientific	Protein size range: 10 proteins spanning 10 - 180 kDa

2.1.9. Solutions & Buffers

2.1.9.1. Competent cells

- ✓ ***Escherichia coli***

MOPSI		MOPSII		MOPSIIa	
MOPS	100 mM	MOPS	100 mM	MOPS	100 mM
KCl	10 mM	KCl	10 mM	KCl	10 mM
RbCl	10 mM	RbCl	10 mM	RbCl	10 mM
pH 7		pH 6.2		pH 6.2	
				Glycerol 15 %	

- ✓ **Natrialba magadii**

Buffered High Salt Spheroblasting Solution		**Buffered High Salt Spheroblasting Solution with Glycerol**		**Unbuffered High Salt Spheroblasting Solution**	
NaCl	2 M	NaCl	2 M	NaCl	2 M
KCl	27 mM	KCl	27 mM	KCl	27 mM
Tris/HCl	pH 8	Tris/HCl 15 % glycerol	pH 9.5		

After autoclaving: add 15% sterile filtered sucrose
0.5 M EDTA Proteinase K Commercially available from QIAGEN

2.1.9.2. Isolation of virus ϕCh1 particles

High-salt alkaline Solution	
Tris/HCl pH 9.5	50 mM
NaCl	4 M

1.1 CsCl Solution		**1.3 CsCl Solution**		**1.5 CsCl Solution**	
Tris/HCl pH 8.5-9	50 mM	Tris/HCl pH 8.5-9	50 mM	Tris/HCl pH 8.5-9	50 mM
NaCl	2 M	NaCl	2 M	NaCl	2 M
CsCl	0.6 M	CsCl	3.7 M	CsCl	4M

2.1.9.3. DNA gel electrophoresis

50x TAE		**5x DNA Loading Dye**	
Tris/HCl pH 8.2	2 M	Tris/HCl pH 8.2	50 mM
Acetic acid	1 M	SDS	0.1 %
EDTA	0.1 M	Bromphenol Blue	0.05%
		Xylene cyanol	0.05%

2.1.9.4. Polyacrylamide gel

- ✓ **For protein crude extracts**

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

5 mM sodium phosphate buffer pH 6.8	
NaH_2PO_4	1 M
Na_2HPO_4	1 M

- ✓ **SDS-PAGE**

30 % Acrylamide Solution	
Acrylamide	29 %
N, N'-methylenebisacrylamide	1 %
Add 250 ml ddH_2O	

10x SDS Running Buffer	
Tris	0.25 M
Glycin	1.92 M
SDS	1 %

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 %

- ✓ **Coomassie Stainning Solutions**

Coomassie Staining Solution	
Methanol	25 %
Acetic acid	10 %
Coomassie Briliant Blue R-250	0.15 %

Coomassie Destaining Solution	
Acetic acid	10 %

2.1.9.5. Western Blot

Transfer buffer	
Tris	48 mM
Glycine	39 mM
Methanol	20 %
SDS	0,037 %

10x TBS	
Tris/HCl pH 8.8	0.25 M
NaCl	1.37 M
KCl	27 M

Luminol

Luminol	0.886 g
DMSO	Add 20 ml

ECL

Tris/HCl pH 8.8	1.5 M
Luminol	500 µl
Coumaric acid	250 µl
Add 200 ml ddH_2O	

Blocking Solution

Milk powder in 1x TBS	5 %

Coumaric acid

Coumaric acid	0.184 g
DMSO	Add 10 ml

2.1.9.6. Southern Blot

50x Denhardt's solution

BSA	1 g
Ficoll 400	1 g
Polyvinylpyrolidone	1 g
Add 100 ml ddH_2O	

20x SSC

Na-citrate	0.3M
NaCl	3 M
pH 7.2, autoclave	

Hybridization Buffer

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

Blocking solution

NaH_2PO_4	0.96 g
Na_2HPO_4	2.41 g
NaCl	7.3 g
SDS	49.89 g
pH 7.2	

10x Wash Solution

Tris	12.1 g
NaCl	5.85 g
$MgCl_2$	2.03 g
pH 9.5	

2.1.9.7. BgaH Assay Solutions

BgaH Buffer		**ONPG Solution**
Tris/HCl pH 7.2	50 mM	8 mg/ml dissolved in BgaH-buffer
NaCl	2.5 M	
$MnCl_2$	10 µM	
β-mercaptoethanol	0.1 %	

2.2. METHODS

2.2.1. DNA METHODS

2.2.1.1. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a widely used technique that allows scientist to amplify specific DNA fragments. In this study, different polymerases were used according to reactions purposes; either 'preparative' PCR or 'analytical' PCR.

2.2.1.1.1. Preparation of PCR templates

Two different protocols were followed to prepare PCR templates. For the generation of *N. magadii* template, first the cells were collected from growth culture with centrifugation at 13 krpm for 3 minutes at room temperature, then followed by resuspension of the pellet in 100 µl ddH_2O. To amplify *N.magadii* DNA, 1 µl of the cell suspension was used directly as a PCR template. Additionally, in order to perform a colony PCR, a single colony was boiled at 95 °C for 5 minutes in 50 µl ddH_2O then 1 µl was used for PCR. For the preparation of PCR template from *E. coli* began either taking 1 µl of the overnight cultures and directly used as a DNA template, or in the case of the colony PCR, single colony was used directly from agar plate as a template.

2.2.1.1.2. Preparative PCR

For preparative PCRs, *Phusion* polymerase was used. During amplification reaction, *Phusion* is capable of incorporating 4 kb per minute which is significantly faster than *Taq* polymerase. Additionally, it also has ability of proofreading due to its 3'-5' exonuclease activity.

The reaction mixture was prepared in 50 µl batches:

Component	Volume
Sterile ddH_2O (PCR-quality H_2O)	19 µl
Forward primer*	2.5 µl
Reverse primer*	2.5 µl
Phusion. Flash PCR Master Mix	25 µl
DNA template	1 µl

Standard preparative PCR (*Phusion* PCR) program:

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	98°C	5’	1
Denaturation	98°C	15’’	
Annealing	T_A**	15’’	35
Extension	72°C	t***	
Final extension	72°C	5’	1

*: Primers were obtained from Eurofins Genomics, they were used 100 ng/ml.
**: Annealing temperatures of the primers were calculated using Tm Calculator from Thermo Scientific.
***: Extension time depends on the expected size of the fragment.

2.2.1.1.3. Analytical PCR

In order to observe the success rates of transformations or confirmation of the correct constructions analytical PCRs were done. PCRs were performed with thermostable *GoTaq*® DNA polymerase which contains *Taq* DNA polymerase that lacks the 3’-5’ exonuclease activity, and all the necessary PCR reagents without the template and the primers.

The reaction mixture was prepared in 25 µl batches:

Component	Volume
Sterile ddH_2O (PCR-quality H_2O)	8.5 µl
Forward primer*	1.5 µl
Reverse primer*	1.5 µl
GoTaq® Master Mix	12.5µl
DNA template	1 µl

Standard analytical PCR program:

Step	Temperature (°C)	Time	Number of cycles
Initial denaturation	95°C	2’	1
Denaturation	95°C	25’’	
Annealing	T_A**	25’’	25
Extension	72°C	t***	
Final extension	72°C	5’	1

*: Primers were obtained from Eurofins Genomics, they were used 100 ng/ml.
**: Annealing temperatures of the primers were calculated using Tm Calculator from Thermo Scientific.
***: Extension time depends on the expected size of the fragment.

2.2.1.1.4. Quality Control of PCR Product

The quality of PCR product was observed by loading samples on a 0.8% agarose gel. For preparative PCR products, 5 µl of product was mixed 5x DNA loading dye with 5 µl then applied onto the gel. However, for analytical PCR product did not need to be mixed with 5x DNA loading dye, since the *GoTaq*® Green Master Mix already contained reagents for loading and visualizing, therefore out of the PCR mix 12 µl were directly applied onto the 0.8% agarose gel. After gel separation was done, products were visualized under UV light upon staining with ethidium bromide.

2.2.1.1. Agarose Gel Electrophoresis

Agarose gel electrophoresis is a widely method that separating DNA fragments based on their molecular weight. The amount of agarose relies on size of the fragment therefore in this thesis, 0.8% agarose (since fragments generally were above 700 bp) was dissolved in 1x TAE buffer, melted in microwave approximately 5 minutes and then poured into the tray after cooling at the room temperature. As indicated above to observe of DNA probes from preparative PCR, aliquots were mixed with 5 µl 5x DNA loading dye, loaded onto DNA gels and separated in electric fields round about 100-150 V. Additionally, from analytical PCR probes 12 µl directly applied, afterwards the DNA was stained by incubation of the gel in an ethidium bromide bath (10 µg/ml) and, before visualizing under a UV-trans illuminator gel was rinsed in the water for a minute.

2.2.1.2. DNA Modification

2.2.1.2.1. DNA Restriction

Every restriction enzymes to digest DNA fragments was from Thermo Scientific company. Reaction mix included desired DNA, buffer, ddH_2O, and the restriction enzyme. The volume of ingredients was determined generally according to the manufacturer's recommendation. In case of double restrictions protocol was followed according to *the Double Digest Calculator* from Thermo Scientific. Additionally,

recommendation of reaction time is 3 hours at 37°C, depending on restriction quality reactions were performed overnight at 37°C until entire DNA was restricted.

2.2.1.2.2. Dephosphorylation

In order to avoid re-circulization of plasmid DNA the removal of the 5'- and/or 3'-phosphate group from the fragment FastAP Thermosensitive alkaline phosphatase was used from Thermo Scientific company. 1U of FastAP alkaline phosphatase was added to linearize 1 µg of DNA and incubated for 10 minutes at 37 °C, then incubation at 65 °C for 15 minutes for the deactivation of phosphatase and restriction enzymes. At the last step, plasmid DNA was purified via using Wizard. SV Gel and PCR Clean-up System.

2.2.1.2.3. Ligation

The T4-DNA Ligase was used to clone digested DNA fragment of interest into the desired plasmid which was digested with the same restriction enzymes. The ligation reaction was incubated for 3 hours at room temperature or overnight at 4°C

Component	Volume
Restricted DNA Fragment	11.5 µl
Restricted Plasmid	1 µl
T4-DNA ligase	1 µl
10x T4 Ligase buffer	1.5 µl

2.2.1.3. DNA Purification

2.2.1.3.1. PCR Product Purification

In order to get rid of unspecific residues of PCR reagents, PCR products were eluted from gel loading prior to using "Promega Wizard® SV Gel and PCR Clean-Up System" kit. However, if there were no unspecific bands present in the PCR reaction, desired DNA was isolated using directly kit protocol.

2.2.1.3.2. Plasmid Isolation

Plasmid preparations were performed using the GeneJET Plasmid Miniprep Kit from Thermo Scientific according to the manufacturer's protocol. The DNA was eluted in 50-100 µl ddH_2O and stored at -20°C.

2.2.1.3.3. The concentration of purified products

The concentration of purified DNA was measured by the help of a NanoDrop® photospectrometer. The ratio A260/280 was measured.

2.2.1.4. Gibson Assembly

Nowadays one of the most rapid method on molecular cloning is Gibson assembly which discovered and named by Daniel G. Gibson (Gibson *et al*., 2009). This alternative cloning technique have ability to assemble multiple DNA fragments in a single isothermal reaction by including three enzymatic activities: T5 exonuclease generates 3'-overhangs in fragment DNA, Phusion polymerase fills in the gaps of annealed regions, and finally T4 DNA ligase assembles annealing fragments. In this thesis, home-made Gibson assembly master mix was used to join up to three DNA fragments. Firstly, the fragments were amplified and purified. For efficient reaction, same molar amounts of fragments were mixed with 100 ng of the linearized vector and incubated at 50 °C for 1 hour. After incubation, sample was transformed into XL1-Blue cells.

2.2.1.5. Southern Blot

Southern Blot technique was used to identify specific DNA sequence in a DNA sample. Firstly, the particular DNA was separated on an agarose gel and denaturation the ssDNA was blotted on a nylon membrane. Afterwards, the membrane was incubated with a biotinylated probe binding the sequence of interest. Finally, blot was developed using Chemiluminescent Nucleic Acid Detection Module kit from BioRad related to the manufacturer's recommendations.

2.2.1.5.1. Synthesis of Probes

For the probe synthesis GoTag® Green Master Mix protocol was followed, additionally biotinylated dUTP was used with 1:5 dilution in 1X TE buffer. The probe was purified by gel extraction in order to remove unbound biotinylated-dNTPs. The total volume of reaction was arranged as a 100 µl.

Component	Volume/Amount
Sterile ddH_2O	26.5 µl
Forward primer*	10 µl
Reverse primer*	10 µl
GoTaq® Master Mix	50 µl
Biotin-11-dNTP 1:5	2.5 µl
DNA template	100 ng

2.2.1.5.2. DNA Restriction and Separation

The DNA sample was digested with New England Biolabs High Fidelity restriction enzymes was incubated overnight and separated on a 0.8% agarose gel. Prior to transforming DNA from gel to nylon membrane, gel picture was taken with a ruler in order to identify the fragment sizes where the probes would anneal.

2.2.1.5.3. Blotting of the DNA to a Nylon Membrane

After separation of DNA fragments on a agarose gel, the DNA had to be denatured and neutralized. Therefore, the gel was incubated in 0.4 M NaOH/ 0.6 M NaCl to be denaturized for 30 minutes, then for the neutralization step the gel was incubated for 30 minutes in 1.5 M NaCl/ 0.5 M Tris/HCl pH 7.5. Afterwards, the capillary blot was arranged with the following steps; 20 cm x 20 cm Whatman paper was place in the agarose gel electrophoresis chamber, and the chamber was filled halfway in 10X SSC buffer. Three pieces of Whatman papers were cut exactly same size of the gel and placed in the middle of the chamber. Above the Whatman papers, the agarose gel was placed followed by insertion of nylon membrane (GE Healthcare Amersham Hybond™-N) which was same dimensions with the gel also previously equilibrated in 10X SSC. The additional three additional Whatman papers were placed on top of the

membrane and by the help of glass pipet rolling over the surface of the Whatman papers air bubbles between the gel and the membrane were removed. Paper towels were cut to the same size as membrane and placed on top of the chamber and compressed by putting maximum 1 kg weight. Transfer was done overnight.

The next day, nylon membrane was incubated. 4 M NaOH and 0.2 M Tris/HCl pH 7.5 for 1 minute. Afterwards, the membrane was crosslinked UV-irradiation in UV transilluminator.

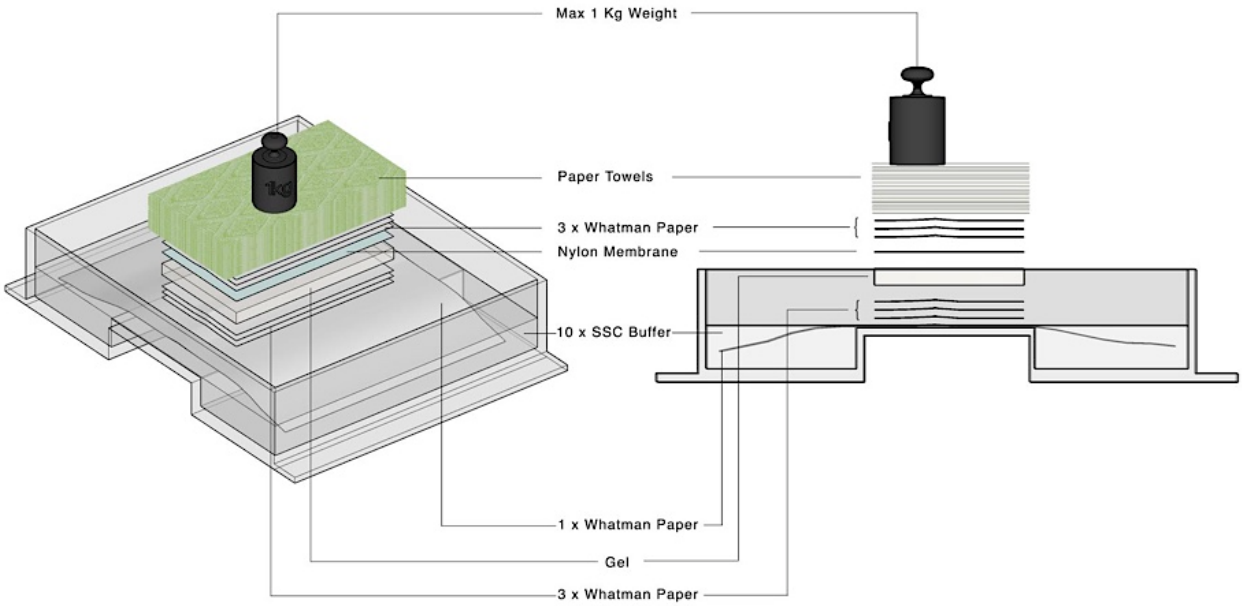


Figure 7: The Schematic Illustration of Blotting DNA into nylon membrane

2.2.1.5.4. Membrane Blocking

In order to avoid unspecific binding of the probe, 2 ml of hybridization buffer and 120 µl of salmon sperm DNA (10 mg/ml) were added to block the membrane for 3 hours at 65 °C. Before the usage of probes, they were denatured by incubation at 95 °C for 10 minutes, afterwards they were added in hybridization buffer then Hybridization was performed overnight at 65 °C.

2.2.1.5.5. Blot Development

After overnight hybridization, the first wash step was two times for five minutes in 2x SSC/ 0.1% SDS at room temperature, then the second wash was two times for 15 minutes in 0.1x SSC/ 0.1% SDS at 65 °C in hybridization oven. As a final step blot was developed by the help of Chemiluminescent Nucleic Acid Detection Module kit from BioRad.

2.2.1.6. Bacterial Strain Methods

2.2.1.6.1. Generation of competent *Escherichia coli* cells

The preparation of competent cells from *Escherichia coli*, the overnight culture of XL1-Blue strains was inoculated with and OD_{600} of 0.1 in 200 ml LB medium which supplemented with tetracycline. The culture was incubated at 37°C, shaking at 165 rpm, until it has reached an OD_{600} of 0.6. The cells were then harvested by centrifugation at 10 krpm for 10 minutes at 4°C and the pellet was resuspended in 80 ml MOPS I afterwards incubated on ice for 10 minutes. The cells were collected by centrifugation at 10 krpm for 10 minutes at 4 °C then the pellet was resuspended in 80 ml MOPS II and incubated on ice for 30 minutes. The final centrifugation step was carried out at 10 krpm for 10 minutes at 4°C and the pellet resuspended in 4 ml MOPS IIa. The generated *E.coli* competent cells were transferred into sterile Eppendorf tubes with a volume of 100 µl aliquots and stored at -80 °C for final usage.

2.2.1.6.2. Transformation of competent *Escherichia coli* cells

The frozen 100 µl aliquot of *Escherichia coli* cells was thawed on ice for 10 minutes, then 15 µl of ligation mixture was added and incubated on ice for 30 minutes. For a successful transformation heat shock was necessary therefore, the cells were incubated at 42°C for 2 minutes followed by incubation on ice shortly. After that transformation batch was enriched with 300 µl LB medium for 30 minutes at 37°C.

Finally, 130 µl of the transformation batch was plated on on LB plates with selective agent (amp+/tet+) and incubated overnight at 37°C.

2.2.1.6.3. Screening of *Escherichia coli* transformants

- **Quick-Prep**

In order to observe whether *E. coli* transformants contain plasmid of interest, Quick plasmid preparation (Quick-prep) method was used. First of all, each colony was inoculated in 5 ml LB (amp+/tet+) and incubated overnight at 37 °C. For the identification of positive colonies, 300 µl of overnight culture was centrifuged for 3 minutes at maximum speed at room temperature. The pellet was resuspended in 30 µl 5X DNA loading dye then vortexed well. After that 14 µl of the phenol/chloroform (1:1) was added and mixed by vortexing for 30 seconds. Prepared sample was then centrifuged at maximum speed for five minutes at room temperature. Soon after, 12 µl of the supernatant containing the chromosomal DNA, plasmid DNA and RNA were loaded on 0.8% agarose gel together with the bare plasmid of interest as a negative control.

- **Analytical PCR**

Potential positive clones which obtained as a result of Quick-prep confirmed by colony PCR.

2.2.1.7. Archaeal Strain Methods

2.2.1.7.1. Generation of competent *Natriallba magadii* cells

In order to generate competent *N. magadii* cells, first of all the pre-culture has to be prepared. For that aim, *N. magadii* was inoculated in 15 ml NVM+ and grown until late log phase. After that freshly grown *N. magadii* cells were incolauted in 60 ml NVM+ which supplemented with 70 µg/mL Bacitracin into the baffled 500 mL Erlenmeyer flask. They were incubated at 37°C until OD_{600} situated in between 0.5 and 0.6. Then cells were harvested by centrifugation at 6 krpm for 15 minutes at room temperature

and the supernatant was removed. Resulting pellets were resuspended in half the volume of the initial culture (30 ml) of the buffered high-salt spheroblasting solution, and proteinase K was added to the final concentration of 0.1% (v/v). The total suspension was incubated at 42°C with agitation up to 48 hours. The cells were checked by light microscope to be ensured whether spheroblasts were formed or not. Finally, if cells became spheroblasts, 1.5 mL aliquots of competent cells were prepared either could be used immediately or stored up to ten days at -80°C.

2.2.1.7.2. Transformation of competent *Natriallba magadii* cells

1.5 ml of the competent *N. magadii* cells were suspended at 10 krpm for 5 minutes at room temperature. The supernatant was discarded, and the resulting pellet was resuspended in 150 µl high-salt spheroblast solution. 15 µl of 0.5 M EDTA pH 8.0 were added and incubated at room temperature for 10 minutes. The protocol was followed by adding DNA of interests; 3-5 µg DNA, or 10-20 µg DNA for deletion mutants was transformed into the competent cells then incubated at room temperature for 10-15 minutes. The maximum volume should be 10 µl to be ensured constant NaCl concentration. Thereafter, 150 µl of 60% PEG 600 were added to transformation assay and incubated at room temperature for 30 minutes. 1 ml NVM+ was then added and the cells was centrifuged at 10 krpm for 5 minutes at room temperature. In order to wash the residual PEG-600, the resulting pellet was resuspended in 1 ml NVM+ and centrifuged again at 10 krpm for 5 minutes at room temperature. The cells were subsequently incubated at 37°C with agitation until the cells had regenerated and had regained their rod-shaped structure. Upon cell regeneration they were plated on NVM+ rich medium agar plates with the required antibiotics (100 µl per plate) and then incubated at 42°C in sealed plastic bags until colonies were visible (up to 15 days).

2.2.1.7.3. Screening of *Natriallba magadii* transformants

N. magadii colonies were inoculated in Eppendorf tubes in 500 µl NVM+ with 0.1% trace elements and incubated at 37 °C with agitation until increased turbidity. PCR template was then prepared (see Section 2.2.1.1.) and analytical PCR done to check for

the presence of the plasmid of interest. After identification of a positive clone, it was inoculated in rich medium containing the appropriate antibiotic for selection.

2.2.2. ϕCh1 METHODS

2.2.2.1. Isolation of ϕCh1 virus particle

Virus particles were isolated from lysogenic *N. magadii* strains, precipitated with polyethylene glycol-PEG and purified and further concentrated by CsCl-density gradient centrifugation. As a first step, the *Nab. magadii* strain which carrying ϕCh1 as a prophage was inoculated at OD_{600} of 0.1 with volume of 2 x 2.5 L at 37 °C with agitation until reaching lower limit of lysis. Growth and lysis of the cultures shaking at 37° C was reported by daily determination of the optical density. When the optical density decreasing was stopped, the phage particles were separated from the lysed culture by centrifugation 6000 g for 20 minutes at room temperature. The supernatant was transferred in fresh flasks, supplemented with 10 % (w/v) PEG 6000 and the virus particles were precipitated by stirring overnight. The phage particles coated by PEG particles were collected by centrifugation 6000 g for 20 minutes at room temperature and solved in 10-15 ml high alkaline salt solution (4 M NaCl, 50 mM Tris/HCl, pH 9.5). The second step was the discontinuous CsCl-density gradient centrifugation for the purification of virus particles. A discontinuous gradient approach was prepared in the following order; 4 ml of 1.5 CsCl, 5 ml 1.3 CsCl, and 5 ml of the viral suspension in the ultracentrifuge tubes. Before centrifugation, tubes were balanced using 1.1 CsCl solution and centrifuged at 30 000 rpm for 20 hours at room temperature in Beckman model ultracentrifuge system using the swinging bucket rotor SW40Ti. The virus particles were observed as a distinctive blue band at approximately 1.3 density interface. For further steps continuous CsCl gradient was used to purify virus particles. To this end, virus particles was mixed with 1.3 CsCl solution and centrifuged at 30 000 rpm for 16 hours at room temperature. The purified virus particles were incubated for 1 hour at room temperature in high-salt alkaline solution followed by overnight dialysis in fresh buffer. Finally, dialyzed virus particles were collected and stored at room temperature.

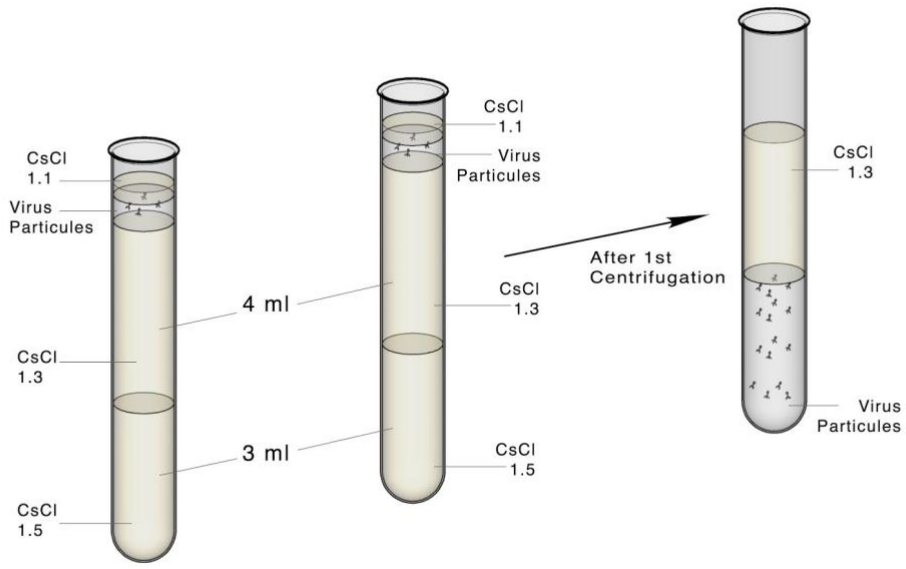


Figure 8: The illustration of CsCl gradient centrifugation method

2.2.2.2. Extraction of ϕCh1 DNA

100 µl of the viral suspension was mixed with 400 µl of ddH_2O and 400 µl of phenol/chloroform (1:1), then vortexed for 30 seconds, and centrifuged at 13 krpm for 2 minutes at room temperature. Upper phase containing DNA was collected, and the phenol/chloroform extraction was repeated until no white interphase was visible. For a final step viral DNA was precipitated from the upper phase with 1 volume of isopropanol by centrifugation at 13 krpm for 30 minutes at 4 °C. The supernatant was discarded, and the resulting pellet was washed with ice-cold ethanol, dried at room temperature and resuspended in 50-100 µl nuclease-free ddH_2O, depending on the expected yield and needed concentration.

2.2.2.3. Analyzation of ϕCh1 Deletion Mutants

2.2.2.3.1. Cell culture Passaging

The cell culture passaging technique was used to analyze stability of *N.magadii* L11-ΔORF43/44 deletion mutant. To do so, *N.magadii* L11 as a control, *N.magadii* L11-ΔORF44, and *N.magadii* L11-ΔORF43/44 plaques was inoculated as a pre-ulture of pre-culture in 10 ml NVM+ rich medium at 42°C with agitation . The deletion mutant strains were additionally inoculated with selective antibiotic which in this case was novobiocin. When preculture's optical density was round about 0.8, they were inoculated into 20 ml NVM^+ rich medium with beginning OD_{600} of 0.1 and incubated at 37°C, shaking at 165 rpm for 72 hours. Afterwards, the new inoculations were done into 40 ml NVM^+ to an OD600 of 0.1 called day 0, and incubated at 37°C, shaking at 165 rpm, until day 7. During those seven days' time interval, the optical density of the cultures was measured every day and the samples were taken as described *Figure 9.* After onset of lysis, the cultures were re-inoculated into 40 ml NVM^+ to an OD600 of 0.1 as a day 0 again, and the protocol was repeated until 7^{th} passage.

2.2.2.3.2. Virus Titer Analysis

As described above, in order to determine the concentration of ϕCh1 virus particles both wild type and deletion mutants, the virus titer analysis was done with the samples which were complemented with 20 µl $CHCl_3$. The samples were diluted with the ratio of 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-10}. Additionally, the *N. magadii* L13 strain which grown late log or stationary phase, the NVM^+ soft agar which complemented with half amount of agar, and the non-selective NVM^+ rich medium plates were needed. Then protocol was followed; 5 ml of NVM^+ soft agar was filled with 100 µl of diluted sample and 300 µl of *N. magadii* L13, then the mixture was vortexed and poured onto the NVM^+ plate. After 10 days of incubation at 37°C in sealed plastic bags, plaques were counted and calculated as "plaque-forming units per ml" (pfu/ml).

2.2.3. PROTEIN METHODS

2.2.3.1. Whole cell extracts

For the preparation of protein crude extracts, 1.5 ml of culture were centrifuged at 13 krpm for 3 minutes at room temperature. The pellet was resuspended in (OD600 x 75) µl of 5 mM sodium phosphate buffer and same volume of 2X Laemlli buffer. The cell extracts were incubated overnight at 37 °C. Additionally, samples were incubated at 95 °C for 10 minutes for denaturation of the proteins.

2.2.3.2. SDS-PAGE

In order to analyze protein of interest, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method was used. By the help of denaturation by SDS, proteins were separated according to their size. For this purpose, first polyacrylamide gel was prepared according to its compositions; separating and stacking gels via using BioRad-Mini-Protean apparatus. After assembly of glass plates, separating gel (12% PAA) was initially poured and was coated with isopropanol to avoid air bubbles.After polymerization of the separating gel, the isopropanol was removed and the stacking gel (4%) was poured and the 10-well comb was carefully inserted between glass plates. Upon complete polymerization, gel was placed in electrophoretic chamber and covered with 1X SDS-PAGE running buffer. 10 µl of cell extracts was loaded on the gel together with the protein marker. Due to high salt concentration in *N. magadii* crude extracts, the samples needed to be separated at a continuous 40 V.

2.2.3.3. Comassie Stainning

Comassie staining was used to verify total protein concentration for the quantitative analysis of the western blot signals. The intensity of Coomassie Brilliant blue dye and protein complex rely on the number of positive charges on the protein. In this study, the volume of the samples that added for western blot was determined by Comassie staining. After gel running at 40 V for 5 hours, the glass plates were separated, and the stacking gel was removed. The separating gel which containing separated proteins

was incubated in Comassie staining with gentle agitation at room temperature. The incubation time for comassie staining depending on freshness of comassie solution, if newly prepared and concentrated even one-minute staining will be enough. Upon staining, the gel was destained by overnight with Coomassie destain solution. After complete destaining, analyzation of the protein concentrations was determined.

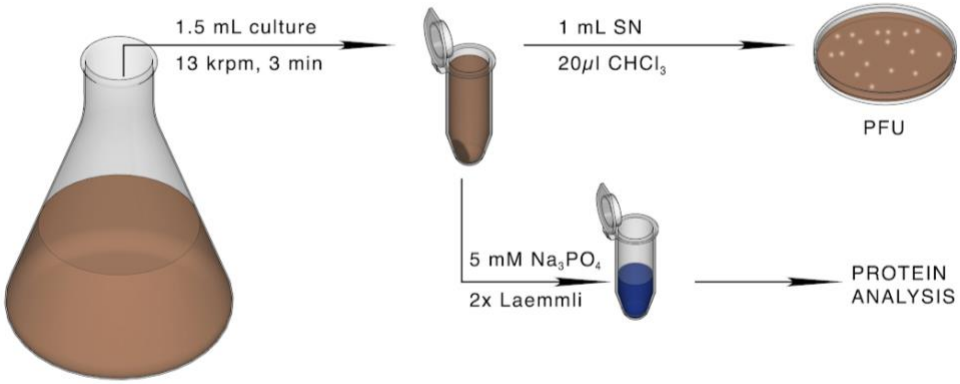


Figure 9 : 1.5 ml of sample was taken after onset of lysis and centrifuged at 13 krpm for 3 minutes at room temperature. While 1 ml of supernatant was separated and complemented with 20 µl $CHCl_3$ for virus titer analysis, the pellet was centrifuged with quick-spin to get rid of the rest supernatants completely. First the resuspension of pellet was done with 5 mM sodium phosphate buffer, then 2X Laemlli buffer was added to perform protein analysis.

2.2.3.4. Western Blot

For detection and quantification of specific proteins, western blotting technique was used. This technique consists of three main steps; fractionation of proteins according to their size on an SDS-PAGE gel, protein transfer to a membrane, detection of signals according to specific antibodies.

- **Transfer of the proteins via semi-dry blotting system**

Proteins were transferred to the nitrocellulose membrane via semi-dry blotting using Transblot Turbo Transfer apparatus from BioRad. For the transfer procedure nitrocellulose membrane and six pieces of Whatman paper were cut same size of the gel. Each of them was rinsed in transblot buffer and the blot was built up in a semi-dry blotting apparatus as following order: 3 layers Whatman paper -nitrocellulose

membrane – polyacrylamide gel – further 3 layers Wathman paper. Transfer was performed at 20V for 30 min. To ensure successful blotting, the membrane was shortly incubated in Ponceau S solution, causing reversible staining. After marking visible protein ladder bands, the blot was completely destained again with water.

- **Blocking of the membrane**

  After blotting, nitrocellulose membranes were blocked by incubation in 1x TBS containing 5 % milk powder at 4°C overnight with gently shaking in order to prevent unspecific binding of the antibodies.

- **Incubation with antibodies**

  After overnight blocking of the membrane, the membrane was washed with 1x TBS three times for 10 minutes until all remains of the blocking solution were eliminated. Antibody solutions were prepared according to their desired dilutions and complemented to a final concentration of 0.3% BSA and 0.02% NaN_3 as indicated material section. 10 ml of primary antibody solution were pipetted onto the membrane and incubated for 1 hour gently shaking at the room temperature. After incubation, the primary antibody solution was collected as it may be re-used and the membrane was washed three times for 10 minutes in 1x TBS with agitation. 10 ml of freshly prepared secondary antibody solution were poured onto the membrane and again incubated for 1 hour gently shaking at room temperature. The incubation was followed 10-minute washing steps with 1x TBS (on shaker).

- **Detection**

  In this study, for the detection of proteins horseradish peroxidase (HRP) was used as a reporter enzyme and luminol was the substrate. Detection of the signal was done using Bio-Rad Clarity™ Western ECL Substrate Kit from BioRad.

2.2.4. BgaH Activity Measurement

For the measurement of β- galactosidase activities in *N.magadii* culture, first of all the sterile plastic-cuvette was filled with 700 µl of BgaH buffer then, 100 µl of growth culture of *N.magadii* (optical density was not higher than 1) was added and the cuvette was vortexed for 3 seconds. The next step was addition of 100 µl of a 2 % Triton X-100 solution to lyse the cells and vortexed for 10 seconds. The reaction will be started by adding 100 µl of ONPG solution. Therefore, prior to addition of ONPG solution, the spectrophotometer was settled as following; absorption at 405 nm and time scan was every 5 seconds up to 3 minutes. When the ONPG was added, the cuvette was vortexed for 3 second then immediately transferred into the spectrophotometer.

The specific activity (SA) of β- galactosidase was calculated with the following formula:

$$SA = \frac{\Delta A_{405}}{\Delta t * V * OD_{600}} * 1000$$

Variable	
ΔA_{405}	change in absorption at 405 nm
Δt	time-span of change of the absorption (in minutes)
V	volume of culture (in ml)
OD_{600}	optical density at 600 nm

2.2.5. PLASMID CONSTRUCTIONS

2.2.5.1. Suicide plasmids for deletion mutant of ϕCh1 ORF43

In order to create a suicide plasmid for ΔORF43, PCR was performed using ϕCh1 DNA as a template and primers ORF41-42-5 and Δ 43-2 that yielded a length of approximately 1089 bp for an upstream homology. Also, pKS$^+$II was resctricted by *Eco*RV with a length of roughly 2700 bp for backbone plasmid and the resistance of the construct novobiocin cassette restricted with SmaI, *Dra*I, and *Pst*I yielded by gel elution approximately 2690 bp. Additionally for downstream fragment pKS$^+$II-43ap-ORF44 plasmid was aligned via PCR with

primers p43-ORF44-5 and D43-4. All fragments were assembled by Gibson, resulting in pKS$^+$II-ΔORF43-R.

2.2.5.2. Suicide plasmids for deletion mutant of ϕCh1 ORF43/44

In order to identify the operon system of ϕCh1 ORF43/44, the suicide plasmid pKS$^+$II-ΔORF43/44-F was created by R.Manning, 2017. In this study, the construct pKS$^+$II-ΔORF43/44-F was transformed to *Natrialba magadii* and relative experiments were done.

2.2.5.3. Plasmids for BgaH measurements

In order to verify the influence of ORF43/44 on BgaH activity, the fragments ORF43and ORF43/44 amplified via PCR and they were introduced into plasmid pNB102 with *bgaH* gene under the control of the ORF49 promoter, and intergenic region between ORF48 and ORF49, resulted in respectively pNB102-MI-1/43 and pNB102-MI-1/43/44. Additionally, the construct pNB102-MI-2/43/44 including a mutated ORF48 start codon.

3. RESULTS & DISCUSSION

In this thesis, the main idea was the analysis the of putative toxin antitoxin system of ϕCh1 in more detail. As mentioned in the introduction, ORF43 and ORF44 of ϕCh1 were co-transcribed and co-translated with a promoter sequence upstream of ORF43. Even though there were different studies about TA system of ϕCh1, the focus of this thesis relied on deletion of putative antitoxin part, ORF43, and the deletion of complete operon region, ORF43/44. Additionally, the illustration of enhancing effect of gp43/44 on expression of the regulatory sequence, ORF48 (rep) and ORF49 has been identified by cloning *Natrialba magadii.*

3.1. Construction of an ORF43 deletion mutant – N. magadii L11-ΔORF43

3.1.1. Aim and cloning strategy

In order to identify the putative antitoxin function of ORF43, first aim was to create the deletion mutant of *N. magadii* L11-ΔORF43. Therefore, the constructs were combined by using Gibson assembly which contained both the upstream and the downstream region of ϕCh1 ORF43 which flanked by a novobiocin resistance cassette (*gyrB*) in 'forward and 'reverse' orientation. The 'suicide' plasmid pBlueScript II KS (+), (pKS$^+$II), does not contain an applicable origin of replication for *N. magadii*, which means the cells that have no integration into host chromosome will be death when they are transformed into *N. magadii.* About creation of upstream and downstream regions detail information can be found in Materials & Methods part in 2.2.5.1.

Gibson assembly cloning method was worked only in 'reverse' orientation of novobiocin resistant cassette. Therefore, in order to transform into *N. magadii* the generation of construct pKS$^+$II- ΔORF43 was successfully created.

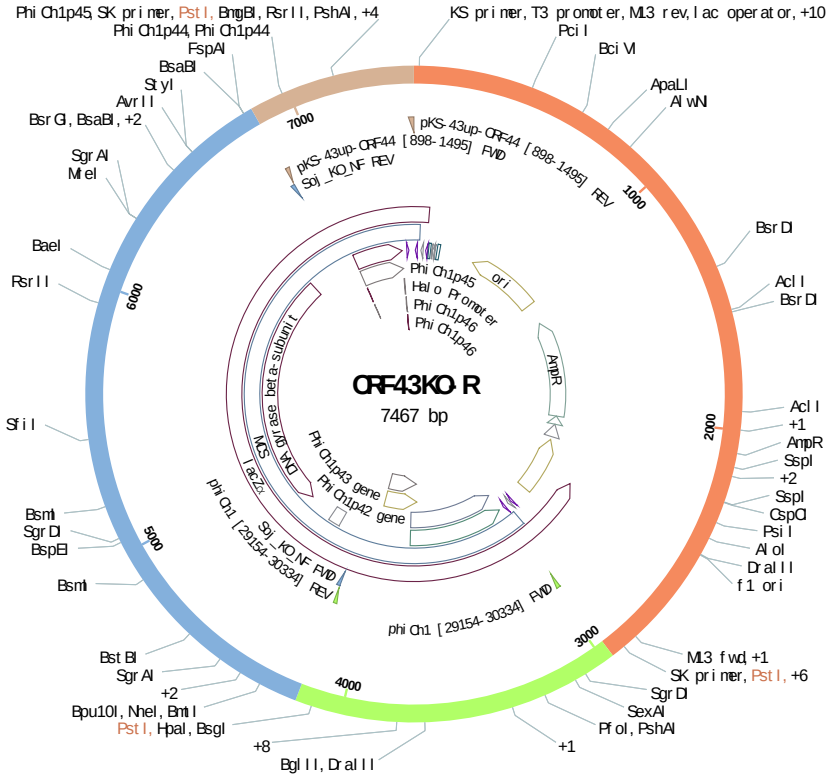


Figure 210: Schematic presentation of the suicide plasmid pKS-ORF43KO-R

3.1.2. Screening of putative clones of *N. magadii* L11-ΔORF43

After transformation of construct pKS⁺II-ΔORF43 into *N. magadii* L11, the analytical PCR was used to teste successful result of transformation. In order to identify positive clones a set of primer was chosen; for the detection of novobiocin resistance cassette Nov-9 and Nov-12, for upstream homology 40-5 and downstream homology 44-3en were used as shown in *Figure 11.*

An agarose gel picture of analytical PCR results was illustrated in *Figure 12*. Analysis related to the identification of *N. magadii* L11-ΔORF43-R revealed that there was no homologous recombination both upstream and downstream region. As shown on the agarose gel picture, no signal was detected in specific region that assembled by primers; for upstream homology 40-5 and Nov-12, and for downstream homology Nov-9 and 44-3en. There was some unspecific signal detection in 3'-end.

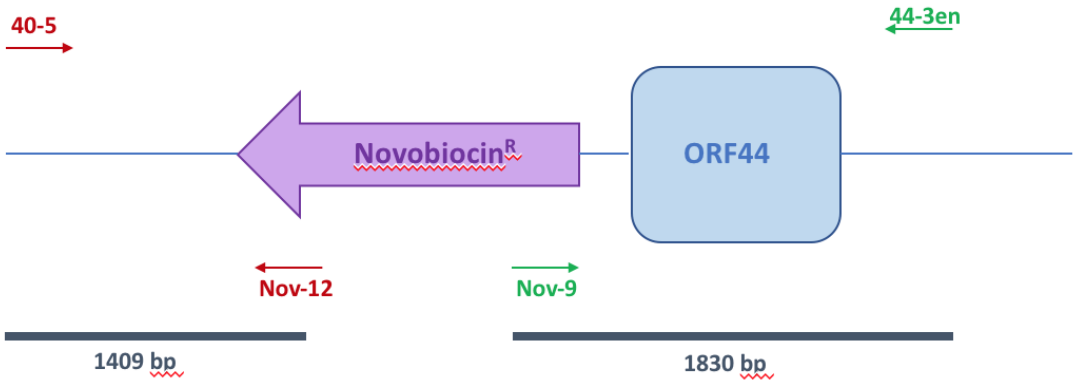


Figure111: The schematic representation of primer binding sites to identify N. magadii* L11-*ΔORF43
In order to identify transformation result, in the analytical PCR the chosen primers were used: for the upstream homology primers 40-5 and Nov-12 were used and the expected length was 1409 bp, and for the downstream homology primers Nov-9 and 44-3en were used and the expected length was 1830 bp.

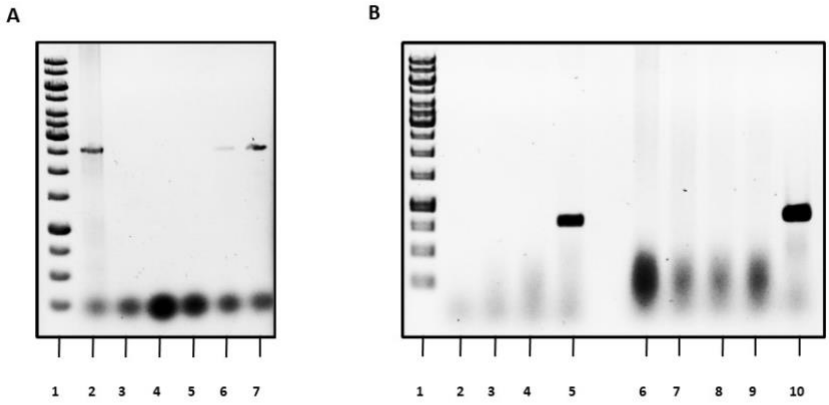


Figure 12: Analysis of putative positive clones of N. magadii* L11-*ΔORF43
In order to identify transformation result, in the analytical PCR the chosen primers were used: for the upstream homology primers 40-5 and Nov-12 were used (A) and the expected length was 1409 bp, and for the downstream homology primers Nov-9 and 44-3en (B) were used and the expected length was 1830 bp. A: lane 1: 1 kbp ladder, lanes 2 to 7: different clones after transformation of pKS-ΔORF43 in *N. magadii* L11. B: lane 1: 1 kbp ladder, lanes 2 to 10: different clones after transformation of pKS-ΔORF43 in *N. magadii* L11.

The observation of these results remained same for analysis of 500 transformants. Therefore it's been concluded that with this technique no deletion mutants of ORF43 could be created in *N. magadii* L11.

3.1.3. Discussion

In order to describe putative antitoxin function of ORF43 from ϕCh1, deletion mutant of this open reading frame was created first in suicide plasmid then tried to be transferred into host organism *Natrialba magadii*. The technique that used for the transformation was the homologous recombination that helped to create successfully for different ϕCh1 deletion mutants so far: *N. magadii* L11-ΔORF79*,* an ORF79 deletion mutant (Selb 2010), *N. magadii* L11-ΔORF34*,* an ORF34 deletion mutant (Till, 2011 & Dimmel 2013) and *N. magadii* L11-ΔORF44 (Gillien, 2017). In this thesis the same technique was used to create *N. magadii* L11-ΔORF43. However, the results demonstrated that homologous recombination only and mostly occurred in downstream region of ORF43 (pKS^{II}-Δ43-NovR-R), data is not shown, which lead to the integration of the whole construct into the genome of *N. magadii* L11 rather than a deletion of ORF43. For the future outlook of the results of this part of thesis, one-way transformations might be repeated, or the construct might be created in different manner such as taking upstream region further from promoter region like beginning of ORF40. Also, it might be that operon system could completely coordinate each other meaning that the deletion of ORF43 could lead to non-viable cells.

3.2. Characterization of an ORF43/44 deletion mutant – *N. magadii* L11-ΔORF43/44

3.2.1. Aim & Homogenization of *N. magadii* L11-ΔORF43/44

One of the ways to identify putative toxin- antitoxin system of ϕCh1 was to delete hall operon system therefore, to create deletion mutant as *N. magadii* L11-ΔORF43/44. In 2017, R. Manning was able to create constructs as a pKS^{+}II-ΔORF43/44-F and pKS^{+}II-ΔORF43/44-R. He also transformed them into *N. magadii* to achieve deletion mutant. In this thesis, the lysate of *N. magadii* L11-ΔORF43/44-F was used to demonstrate characterization of deletion of

operon system. To do so, first step was generation of homozygous *N. magadii* L11-ΔORF43/44-F mutants.

3.2.2. Screening of putative clones of *N. magadii* L11-ΔORF43/44

Single colonies were inoculated and analyzed for the integration of the fragment replacing ORF43/44 with the novobiocin cassette with primers 43-5 and 44-3en. The sequence of primer 43-5 is homologue to the upstream of ORF43, whereas primer 44-en to the downstream sequence of ORF44, thereby covering the complete sequence of the region to be deleted.

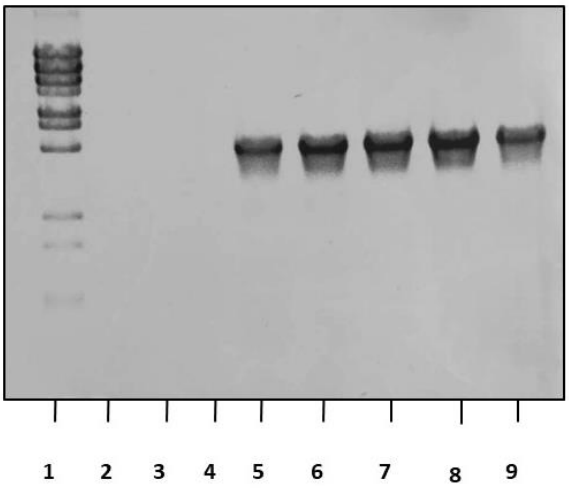


Figure 13: Screening of N. magadii-ΔORF43/44 clones

Clones were incubated in complex medium after transformation and crude extracts were used for PCR analysis using primers 43-5 and 44-3en. Aliquots were separated on a 0.8% agarose gel and strained with ethidium bromide. Lane 1: λ *Bst*EII, lanes 2 to 9: different clones after transformation with the suicide plasmid pKS-ΔORF43/44.

3.2.3. Homogenization of N. magadii L11-ΔORF43/44

N. magadii is a polyploid organism that carries up to 50 copies of chromosomal DNA. Furthermore, *N. magadii* L11 carries up to 50 copies of ϕCh1 DNA. Therefore, upon transformation many copies of the *N. magadii* genome will still have wild-type alleles of the target gene. In order to get homogenous population of *N. magadii* L11-ΔORF43/44, every existing copy of ORF43/44 should be deleted. First of all, heterogenous deletion mutant of *N.*

magadii L11-ΔORF43/44-F was propagated in NVM$^+$ until complete lysis, then virus particles were harvested by centrifugation. Then, virus particles were incubated in liquid culture of *N. magadii* L13 strain and plated NVM$^+$ plates complemented with novobiocin, which resulted in homogenous population since ϕCh1 virus particles have a single copy of the genome and only a single virus particle can infect one non-infected cell. The single novobiocin-resistant colonies were incubated in NVM$^+$ at 37^{o}C and tested for conformation of presence of provirus with defective ORF43/44 loci. First analytical PCR was done to check for provirus presence. Then confirmed pools tested with second PCR with 43-5 and 44-3en primers which anneal at the 5'-end respectively the 3'-end of ORF43/44 region. The analytical PCR results helped to confirm identification of putative homozygous ORF43/44 deletion mutants.

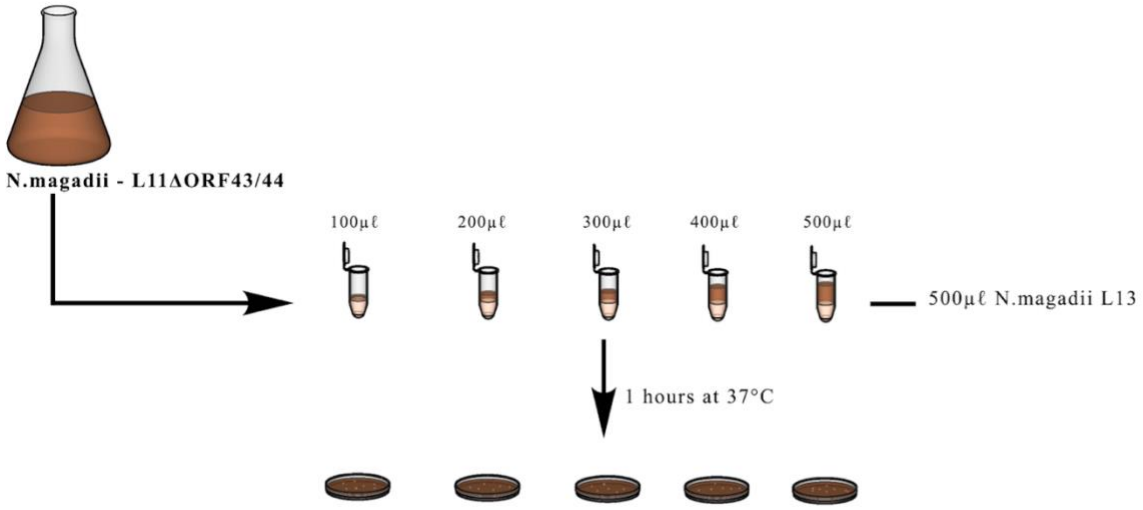


Figure 14: Homogenization of N. magadii L11-ΔORF43/44

N. magadii L11-ΔORF43/44 strain was incubated in NVM$^+$ without novobiocin at 37 ^{o}C until late stationary phase. After complete lysis, strain was added the amount as shown 100, 200, 300, 400, 500 µl into the liquid culture of *N. magadii* -L13 in order to infect cured strain. The dilution series was then incubated for 1 hour at 37 ^{o}C. Afterwards, 100 µl per dilution were plated on rich medium plates containing novobiocin. This technique was resulted in homozygous *N. magadii* L11-ΔORF43/44.

After passaging single colonies were again incubated and used for a PCR analysis. Here, the presence of ORF43/44 were analyzed with primers 43-Bam and 44-Hind spanning the complete operon. To be sure, that no copies were left, 50 cycles of PCR were performed.

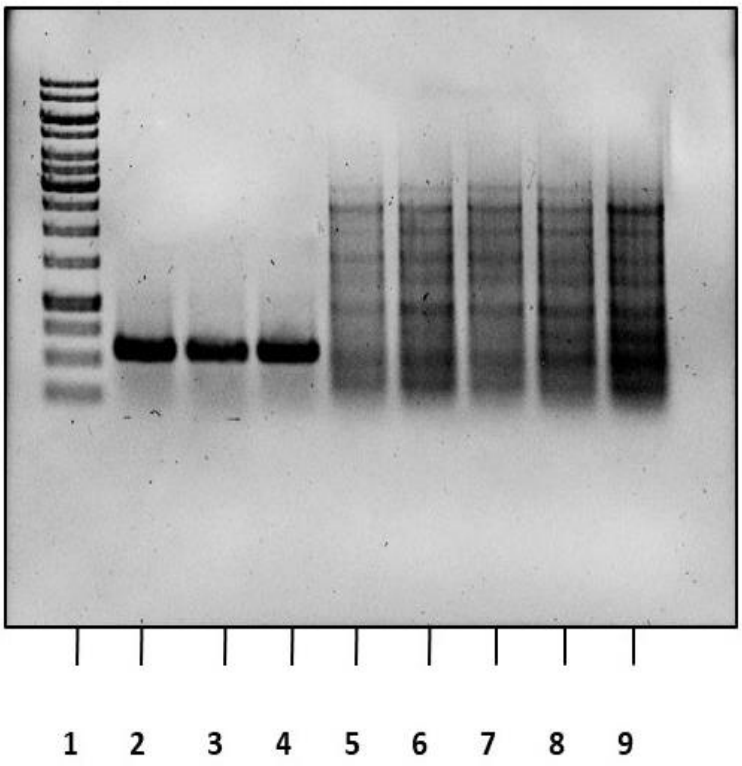


Figure 15: Screening of N. magadii-ΔORF43/44 clones

Clones were incubated in complex medium after transformation and crude extracts were used for PCR analysis using primers 43-Bam and 44-Hind. Aliquots were separated on a 0.8% agarose gel and strained with ethidium bromide. Lane 1: 1 kbp ladder, lanes 2 to 9: different clones after homogenization.

As shown in Figure 15, only clones applied in lanes 6 to 9 do not contain the wild-type fragment as in lanes 2 to 4. Therefore, one of these clones was chosen for further work.

3.2.4. Confirmation of the homozygous *N. magadii* L11-ΔORF43/44 – Southern Blot

The Southern Blot method was used to confirm that total deletion of ORF43/44, putative toxin-antitoxin system locus of ϕCh1, was achieved properly. To do so, both virus DNA and *N. magadii* L11-ΔORF43/44 DNA were isolated and digested with *PstI* restriction enzyme. The restricted fragments then were separated on agarose gel to adapt concentration of DNA for blotting. After that arranged volume of restricted DNAs were separated on 0.8% agarose gel to be transferred to nylon membrane. The designing of biotinylated probes was done by PCR by using primer pairs; while 43-probe and ΔORF44-2 primers were used for the 5´ upstream region, ΔORF44-3 and ΔORF44-4 primers were used for the 3´downstream region which

resulting in 798 bp and 659 bp respectively. The representation of probe analysis and hybridization patterns is depicted in following figures.

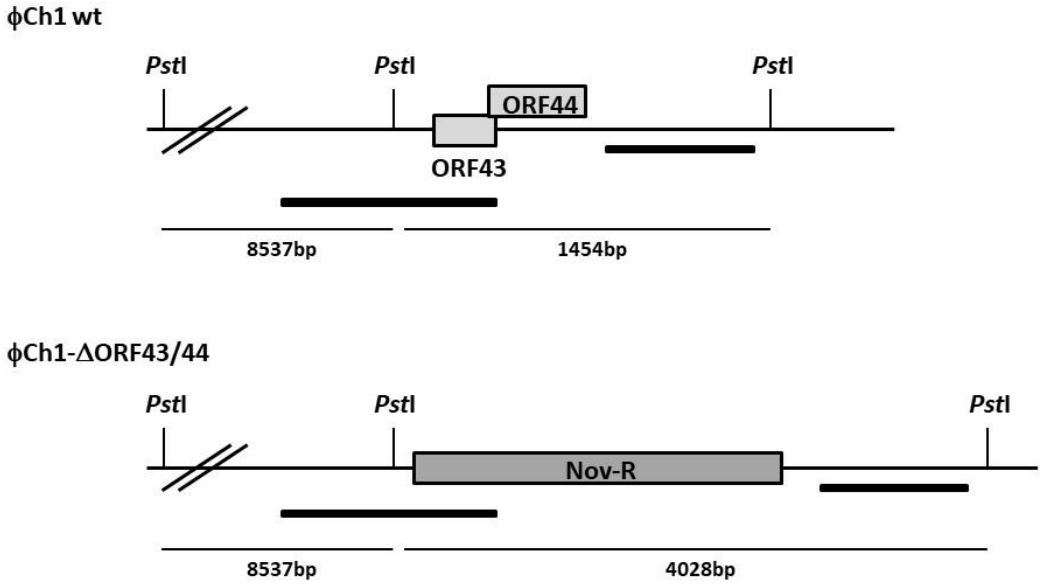


Figure 16: Schematic representation of the ORF43/44 locus for Southern Blot

Parts of the genome of ϕCh1 wt and ϕCh1-ΔORF43/44 is shown. The open reading frames 43 and 44 as well as the novobiocin resistance cassette are indicated as gray boxes. *Pst*I restriction sites used for the digestion of both DNAs are indicated. The lengths of the expected fragments are indicated below the presentations of both genomes. The position of the 5´- as well as the 3´-probes are given as bold lines.

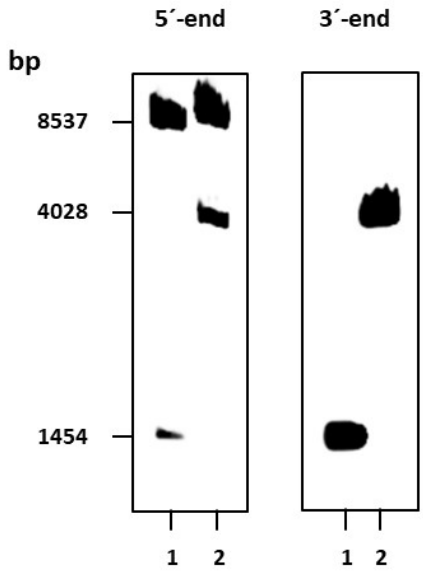


Figure 17: Southern Blot of ϕCh1 wt and ϕCh1-ΔORF43/44

After separation of restricted DNA of ϕCh1 wt and ϕCh1-ΔORF43/44 with *Pst*I, the fragments were transferred to a nylon membrane and hybridized with probes for the 5´-end (left) and the 3´-end (right). The sizes of the hybridized fragments are given on the left in bp. Lane 1: ϕCh1 wt, lane 2: ϕCh1-ΔORF43/44

As can be seen in Figure 17, all signals obtained with the DNA of ϕCh1 wt and ΔCh1-ORF43/44 were obtained by the southern hybridization. More important, there were no signals for the wt within the DNA of Ch1-ORF43/44 or vice versa. This indicated again, that the resulting virus Ch1-ORF43/44 is a homogenous strain and was used in further studies to elucidate the deletion of the operon ORF43/44.

3.2.5. Characterization of *N. magadii* L11-ΔORF43/44

To determine the effect of the deletion of ORF43/44 onto growth of *N. magadii*, growth kinetic analysis was performed. Growth and lysis behavior of wild-type and mutant strain was compared by measuring everyday optical density and taking sample for protein extract.

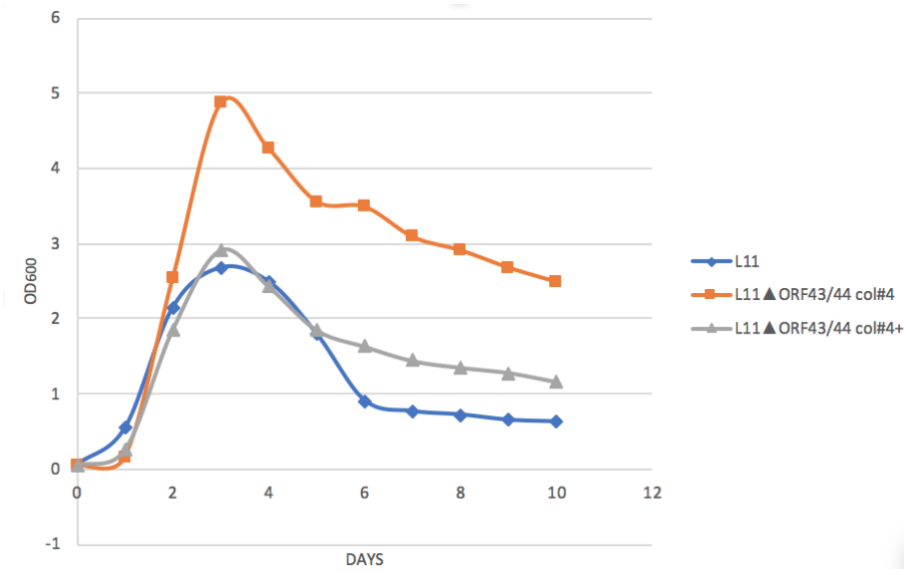


Figure 18: Growth Kinetics analysis of N. magadii L11-ΔORF43/44

The blue line represents *N. magadii* L11 while the orange and grey line represent mutant strains respectively *N. magadii* L11-ΔORF43/44 and *N. magadii* L11-ΔORF43/44(+) which inoculated with additional novobiocin.

3.2.5.1. Growth kinetics analysis

For characterization of *N. magadii* L11-ΔORF43/44-F, *N. magadii* L11 and *N. magadii* L11-ΔORF43/44-F strains were incubated NVM^+, additional *N. magadii* L11-ΔORF43/44-F (+) strain was incubated with novobiocin. After incubations were arranged with beginning optical density 0,05, the OD_{600} of samples were measured every day at the same time. As data shown in *Figure 16*, the *N. magadii* L11 strain was demonstrated at blue color whereas *N. magadii* L11-ΔORF43/44 was shown in orange color, *N. magadii* L11-ΔORF43/44(+) was stated in grey color.

It's been outlined at the growth kinetic analysis that *N. magadii* L11-ΔORF43/44 (+) strain behaved almost identical to *N. magadii* L11 wild-type strain. Also, compared to the *N. magadii* L11, the deletion mutant *N. magadii* L11-ΔORF43/44 showed more dense culture although lysis nehaviour of them were alike. The initial growth and lysis behavior of the strains were similar, whereas the differences occur with respect to the lytic behavior of the deletion mutant strain without additional antibiotic. In the case of *N. magadii* L11-ΔORF43/44 F after

lysis occurs, culture density continuously decreased. However, it can be concluded that the deletion of operon system of ϕCh1 has no strict difference from wild-type strain, so there is no regulatory effect of lysis of virus neither reduced nor accelerated.

During growth kinetic analysis, while measuring OD_{600} there were sample taken from *N. magadii* L11-ΔORF43/44 to demonstrate the number of viruses released at given time points. Phage titer analysis from mutant strain is illustrated in Figure 1. Obtained results showed that virus release particles had relative increase with growth curve analysis: when culture lysed at day 4, also plaque number had increased, then continued in following days.

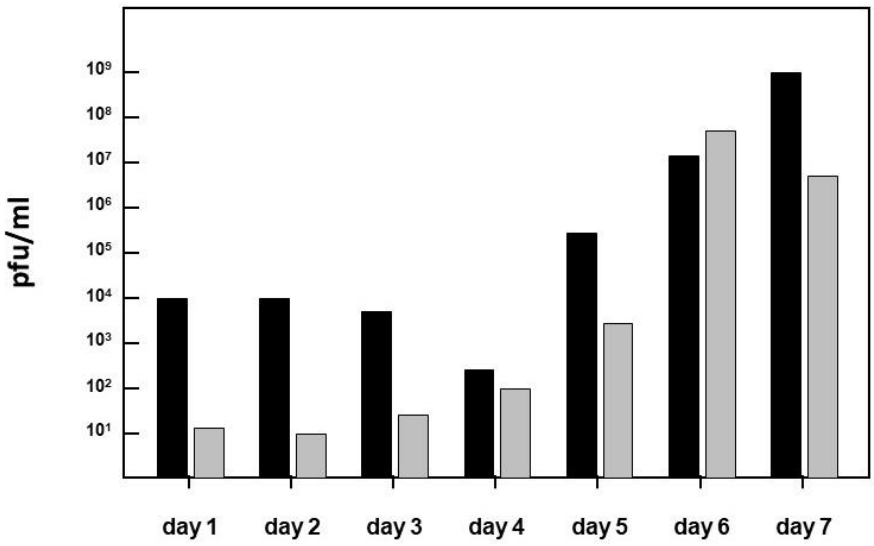


Figure 19: Production of virus particles from N. magadii L11-ΔORF43/44

Samples of the cultures were taken every day as indicated. After centrifugation, dilutions of the supernatants were prepared and plated on *N. magadii* L13 with top agar. After incubation of 7 to 9 days at 37°C, the plaque forming units were determined. Black bars represent virus release of strain *N. magadii* L11 wt, gray bars *N. magadii* L11-ΔORF43/44.

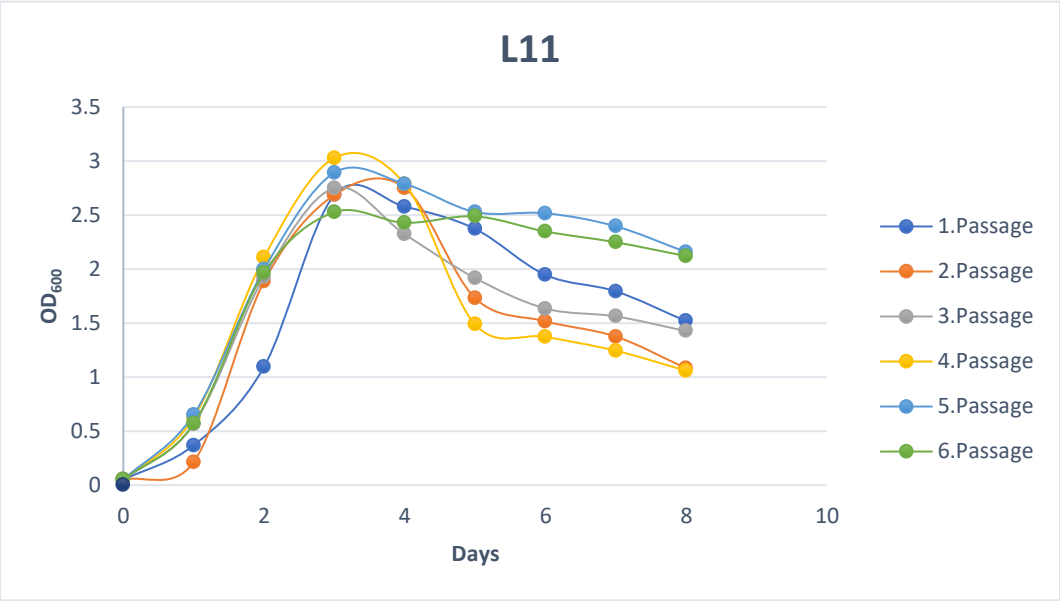
3.2.5.2. Stability of *N. magadii* L11-ΔORF43/44

Previous studies done by Michaela Edwards in 2018 have shown that the deletion of the ORF44 leads to the increased instability of the lysogenic strain (Edwards, 2018). In order to confirm the same for hall putative toxin-antitoxin genome of ϕCh1, the same passaging experiment was conducted in this thesis. To do so, fresh lysogenic strains of *N. magadii* L11-

ΔORF43/44 were retrieved by inoculation of the plaques obtained by the infection of the N. magadii L13. Afterwards, obtained plaques were inoculated in 15 ml NVM^+ and subsequent propagation at 42 °C with aeration until cultures were dense. Then cultures were transferred into 40 ml NVM^+ with the beginning OD_{600} of 0,05 and supplemented with novobiocin and propagated with aeration at 37 °C. Growth kinetics analysis of both wildtype *N. magadii* L11 and deletion mutant *N. magadii* L11-ΔORF43/44 strains were analyzed by measuring optical density at 600 nm every 24 hours until lysis onset. Additionally, during everyday OD_{600} measurements, the samples were taken from both cultures for protein analysis and virus titers. Further passages done by re-inoculation in 40 ml selective NVM^+ with the beginning OD_{600} of 0,05 and growth kinetic analysis were done in the same manner. Passaging assay was done until 6^{th} re-inoculation.

3.2.5.2.1. Growth kinetics analysis

As shown in growth kinetics analysis data (Figure 20), the continues passaging did not significantly affect growth and lysis behavior of wild-type strain of ϕCh1, *N. magadii* L11. There were no significant changes in growth until passage 4. The last 2 passages showed that, lysis behavior of *N. magadii* L11 was decreased. Therefore, the strain seems to be cured by every passage resulting in strain *N. magadii* L13 stain. Passaging of the deletion mutant N. magadii L11-ΔORF43/44 strain resulted in changes in growth and lysis behavior including 4^{th} passage and continued in further passages as depicted in Figure 20.



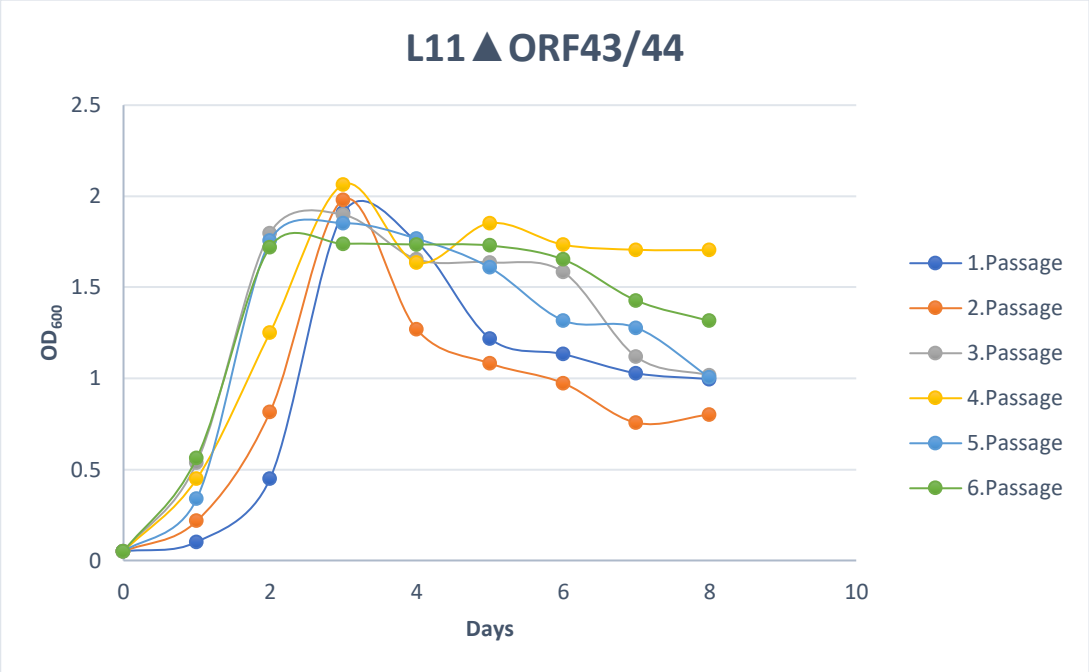


***Figure 20: Growth kinetics analysis of the mutant strain N. magadii* L11 wt and *N. magadii* L11 ΔORF43/44 during the first six cell culture passages**

The passaging of cultures mutant strain *N. magadii* L11 wt and *N. magadii* L11 ΔORF43/44 was performed as described above by measuring everyday optical density at 600 nm. The first three passage were similar lysis behavior with wild-type strain however further three passages had different lysis behavior.

3.2.5.2.2. Virus titer analysis

During passaging, the samples were taken for quantitative assessment of the virus release. Virus titer of six passages for wild-type strain and deletion mutant strains was analyzed and shown in Figure 21. The viral titer of wild-type strain *N. magadii* L11 increased until the fourth passage then decreased almost with the same order until the last passage. The deficient putative toxin-antitoxin strain, *N. magadii* L11-ΔORF43/44, had non-sustainable results that almost no virus particles at the last passage. However, it can be concluded that for the *N. magadii* L11-ΔORF43/44 strain which supplemented with novobiocin had similar plaque forming with wild-type strain even though with less numbers except first two passages.

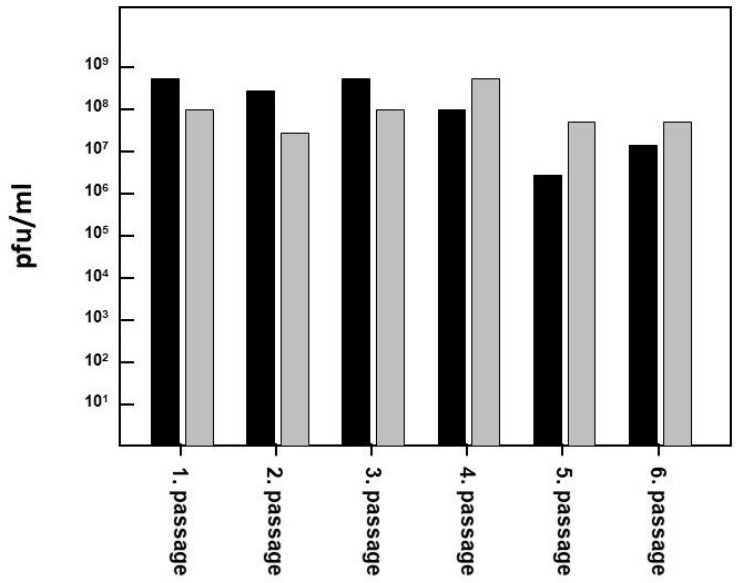


Figure 21: Virus titer analysis of passaging assay of deletion mutant

Cell-free supernatants obtained from the *N. magadii* L11, *N. magadii* L11-ΔORF43/44 and *N. magadii* L11-ΔORF43/44 strain complemented with novobiocin were used to infect cured strain of *N. magadii* L13. During measurements of optical density, samples were taken from cultures when they began to lyse. Then viral titer data was collected via counting plaques related to passaging assay. Black bars represent virus release of strain *N. magadii* L11 wt, gray bars *N. magadii* L11-ΔORF43/44. The *N. magadii* L11-ΔORF43/44 strain complemented with novobiocin is not represented.

3.2.5.2.3. Morphology of virus titers

Moreover, it was proceeded with the characterization of the morphology and the turbidity of the plaques formed by *N. magadii* L11-ΔORF43/44. Plaques collected during passaging assay showed unusual morphology. As depicted in *Figure 21*, in the first passage of deletion mutant there were turbid plaques on the lawn of the susceptible *N. magadii* L13 cells. In the fourth passage plaques were bigger but not clear than first passage. It's been considered that the formation of either turbid or clear plaques rely on lysogeny of the virus particles; if viruses lysogenize host cell turbid plaques were formed whereas when virus lost its ability to lysogenize clear plaques were formed. On the last passage there were turbid and clear plaques meaning that virus already began to lose its lysogeny. Additionally, plaque size was different between passages, it became bigger significantly that were clearly visible to the naked eye. The plaques formed by the mutant strain at the first passage had approximately diameter of 1 mm whereas at the last passage plaques were in the shape that with the diameter of > 1,5 mm.

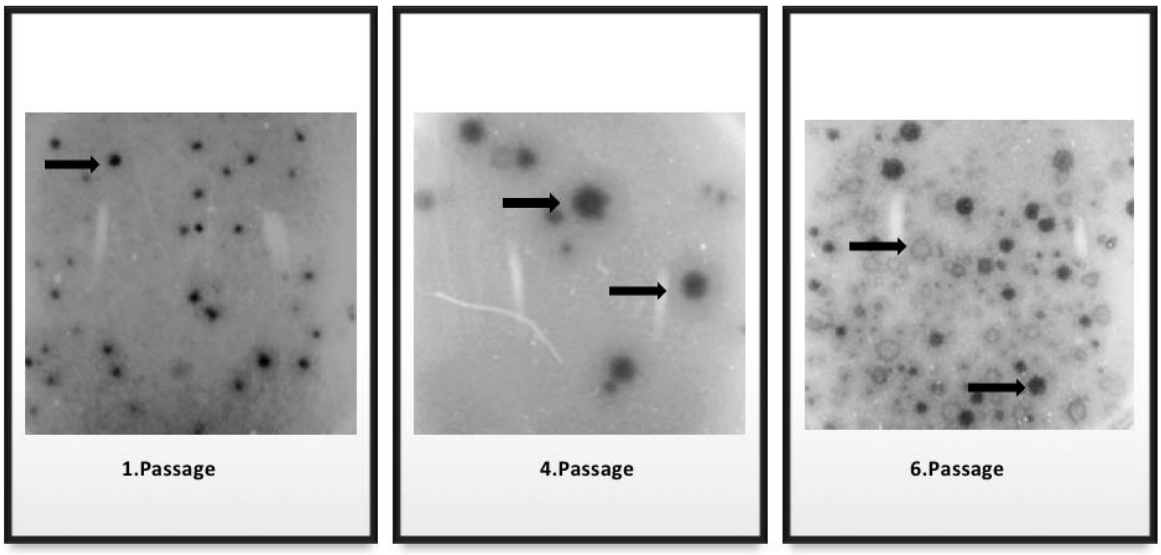


***Figure 21: Plaque morphology of passaging assay of N. magadii* L11 ΔORF43/44**

Plaque assay was done via infection of the cured strain of *N .magadii*, N. magadii L13, with the ϕCh1 ΔORF43/44 virus particles. After incubation at 37°C in ten days plaques were counted and imaged using ChemiDoc Imaging System from Biorad

3.2.6. Discussion

One of the main outlines of this thesis was the investigation of putative toxin-antitoxin system of ϕCh1. The first study about ORF43/44 was performed by Iro *et al.* in 2007 that resulted in this operon system and ORF43 alone have an enhancing effect on the intergenic region between ORF48 and ORF49 by binding directly or indirectly to the 5' repeats of ORF48 sequence which encodes a repressor protein rep, and thereby enhances the transcription of ORF49, which has a role in the activation of the lytic life cycle of ϕCh1 (Iro et al., 2007). Then in 2015 Hofbauer studies indicated that ORF44 has an impact of the expression of the genes; $ORF34_{52}$ encoding a tail-fibre protein and ORF94 encoding the main methyltransferase. (Hofbauer, *et al.,* 2015). Moreover, Pfam analysis clarified that there was a PIN domain in the gene product of ORF44, therefore the idea was revealed that gp44 may cleave single-stranded RNA in a sequence specific manner like in in the toxins of type II TA system (Arcus *et al.,* 2011). Further studies done by Gillien related to investigations on the characterization of ϕCh1 ORF44 in 2017 and by Edwards about the stability of ORF44 in 2018.

For investigation of regulatory role in operon system of ϕCh1, the deletion mutant of *N. magadii* L11-ΔORF43/44 was created and submitted by Southern Blotting. The growth kinetic analysis resulted in similar lysis behavior with wild-type strain *N. magadii* L11. However, the comparison of passaging assay result of both strains indicated that after 4^{th} passage the deletion mutant *N. magadii* L11-ΔORF43/44 had closer to the cured strain *N. magadii* L13. Not only virus titer analysis but also morphology of plaques suggested that passaging assay might be repeated and should be continued till at least 12^{th} passage. Additionally, for more reliable results in case of effect of novobiocin resistance cassette, it might be tried with different amounts of novobiocin on the deletion mutant cultures. However, it can be concluded that the prolongation of passaging led to *N. magadii* L11-ΔORF43/44 become non-lysogenic.

3.3. The demonstration of ORF43/44 enhancing effect in Natrialba magadii

One of the studies about gene expression regulation of archaeal virus was done in virus ϕH infecting *H. salinarum* (Gropp *et al*., 1992). It was concluded that different fragments of an

immunity-conferring construct pϕHL conferred immunity against ϕH infection to *H. salinarum* when cloned individually. However, these constructs had different immunity-conferring efficiencies from the ones observed for a strain having been transformed with the entire pϕHL construct (Gropp *et al*., 1992). Especially regions that responsible for encoding two ORFs of 54 AA and 131 AA acted co-operatively with the ϕH repressor (Stolt and Zillig *et al*., 1993). It's been discovered that those two ORFs has high sequence similarity with ϕCh1 ORF43/44, 90% and 94%, respectively. Therefore, the ORF43/44 of ϕCh1 thought to be good candidates of the regulation of gene expression in ϕCh1 alongside ORF48/49. (Iro *et al*., 2007). Further discoveries showed that promoter sequences could only be found upstream of ORF43 (p_{43}) and stop codons of ORF43 and ORF44 overlap which become an idea that ORF43 and ORF44 are co-transcribed as well as co-translated, thus forming an operon together (Klein *et al*., 2002).

Additional analysis demonstrated that both ORFs revealed a similarity to the so-called VapBC (Virulence-associated proteins) toxin-antitoxin system, where ORF43 would code for the antitoxin VapB as an inhibitor and ORF44 would code for the toxin VapC as a nuclease activity which also contains a PIN-like domain in their sequence (Hofbauer *et al*., 2015).VapBC (Virulence-associated proteins) toxin-antitoxin systems are described in an operon system that related genes overlap in start- and stop codons. The unstable VapB antitoxin, putatively ORF43, builds a complex with the stable toxin VapC, putatively ORF44, and the complex auto-regulates its own expression. Upon degradation of the inhibitor, the toxin becomes active (Arcus *et al*., 2011).

According to the hypothesis of the ORF43/44 toxin-antitoxin system of ϕCh1, first a preliminary binding site for gp43/44 has been found in the sequence of ORF48 encoding for the repressor protein Rep. In 2007, experiments were done to conclude hypothesis via testing *bgaH* expression on the constructs : pMI-2Δ/43-44 was containing the intergenic region between ORF48 and ORF49, a mutated ORF48 start codon, the *bgaH* gene under the control of the ORF49 promoter as well as ORF43/44 cloned downstream of the *bgaH* gene, pMI-2Δ was the same construct not containing the ORF43/44 operon, pMI-2Δ/43 was the same construct only containing ORF43. All constructs were transformed into *H. volcanii*, then tested for BgaH activity (Iro *et al*. 2007). The measurements were shown in Figure 22. While the

construct pMI-2Δ/43-44 and pMI-2Δ/43 had higher BgaH activity than the construct pMI-2Δ, there was no detectable BgaH activity on the pMI-2Δ/44 construct which only containing ORF44. Additional observation was BgaH activity dropped in the manner of non-mutated start codon of ORF48. When all results combined, the hypothesis was resulted in that ORF43/44 exhibits an enhancing effect on *bgaH* expression with an enhancing activity of ORF43 on the intergenic region and a putative repressing activity of ORF44, all in the presence of the ORF48 coding sequence. The putative repressing- or degrading activity of ORF44 would then be further evidence for the ORF43/44 toxin-antitoxin hypothesis. (Iro *et al*. 2007).

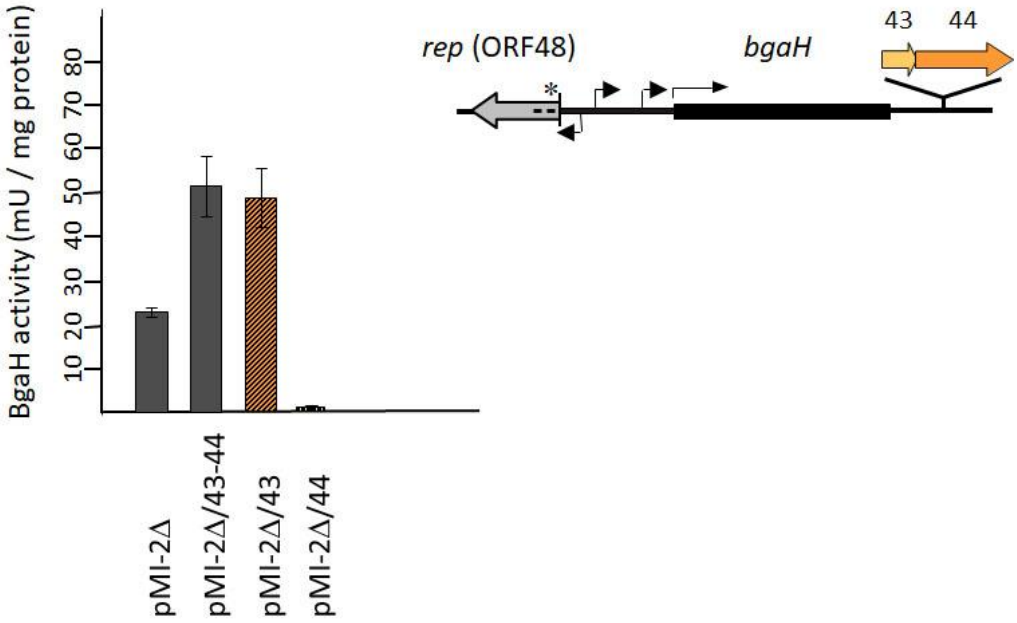


Figure 22: The influence of ORF43/44 on BgaH activity in presence of the ORF48 (rep) sequence with a mutated start codon (adapted from Iro et al., 2007)

Here, a plasmid constructs labelled pMI-2Δ containing ORF48 with a mutated start codon, the intergenic region of ORF48 and ORF49 as well as the *bgaH* gene (replacing ORF49) under control of the ORF49 promoter was constructed and transformed in *H. volcanii*. pMI-2Δ gives rise to BgaH activity because of promoter activity in the intergenic region. The enhancing effect of ORF43 as well as ORF43/44 on the BgaH activity in presence of the coding sequence of ORF48 is clearly visible. ORF44 alone exhibits a repressing activity in the presence of the ORF48 coding sequence. (Iro *et al*., 2007)

3.3.1. Aim

In this thesis, the constructs were arranged to make suitable transformants for *Natrialba magadii.* As illustrated below, the pNB102 used as a vector and the inserted regions were respectively; the intergenic region between ORF48 (rep) and ORF49, the *bgaH* gene under the control of the ORF49 promoter and containing hall operon region called pMI-1/43/44, the same construct that containing only ORF43 instead of hall operon region called pMI-1/43. The construct named as pMI-2/43/44 had ORF48 region differently than pMI-1/43/44.

3.3.2. BgaH activity & Discussion

After the constructs were created to test BgaH activity in *N.magadii*, first the analytical PCR was done for identification of correct plasmid with primers 49-Kpn and BgaH-3i. Then constructs were transferred into the cured strain *N. magadii* L13. Afterwards BgaH activities were measured as described in the section 2.2.4. BgaH Activity Measurement.

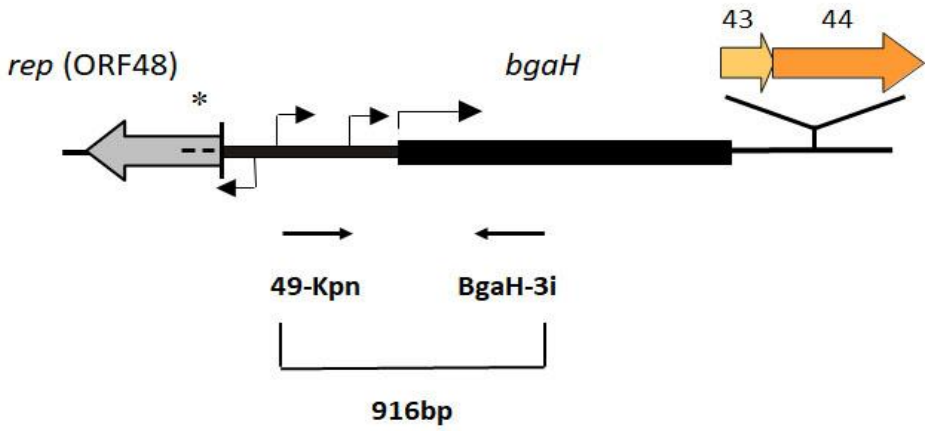


Figure 23: Schematically representation of region to be cloned and primers used for cloning

The illustration of primers that used for the analysis of the insertion for *bgaH* genes of the constructs pMI-1/43/44, pMI-1/43 and pMI-2/43/44.

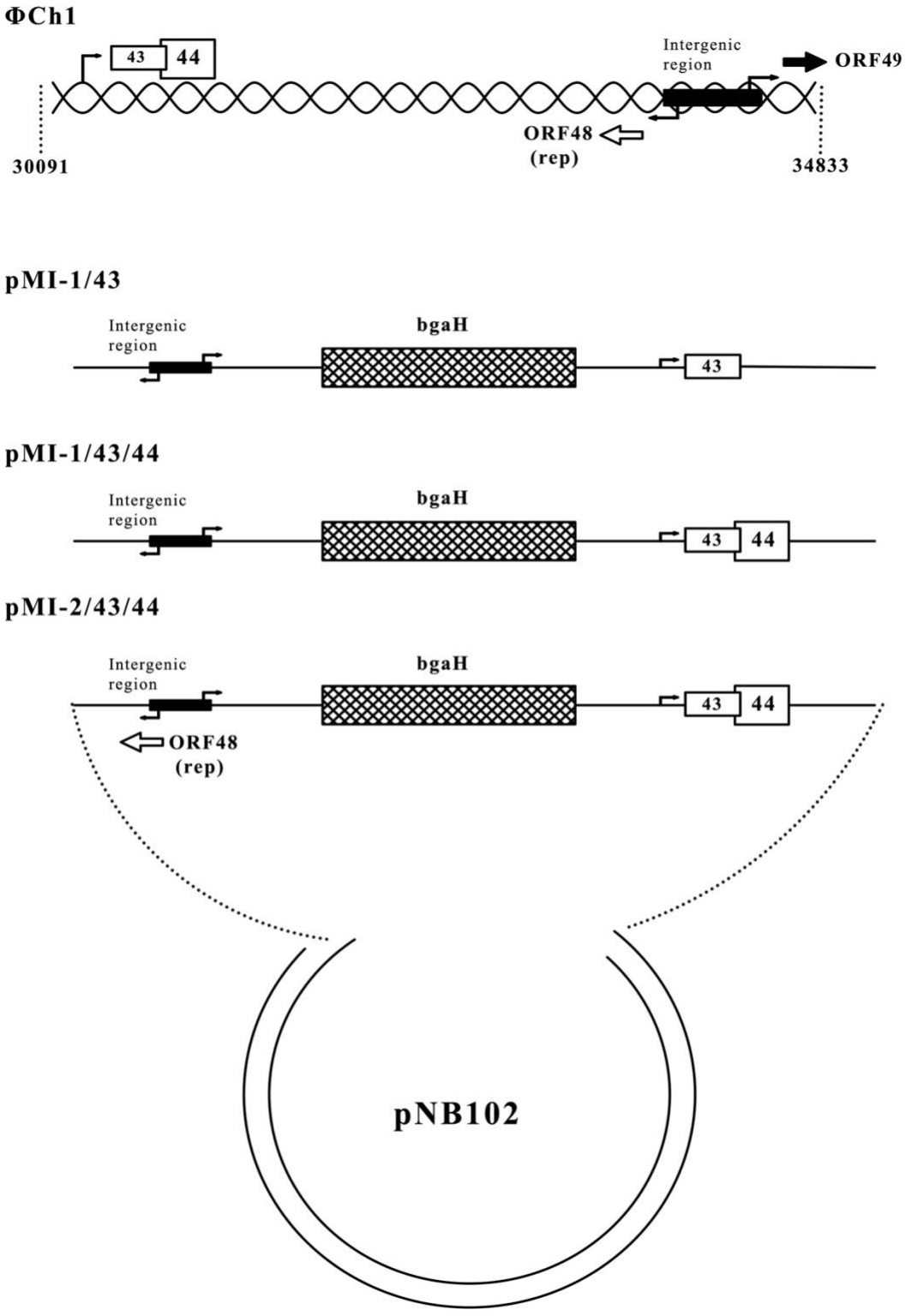


Figure 24: Schematically representation of creation of the constructs for BgaH activity

The pNB102 used as a vector and the inserted regions were respectively; the intergenic region between ORF48 (rep) and ORF49, the *bgaH* gene under the control of the ORF49 promoter and containing hall operon region called pMI-1/43/44, the same construct that containing only ORF43 instead of hall operon region called pMI-1/43. The construct named as pMI-2/43/44 had ORF48 region differently than pMI-1/43/44.

The plasmids indicated in Figure 24 were transformed into *N. magadii* L13 and PCR analysis using primers 49-Kpn and BgaH-3i as stated in *Figure 23*, indicate the successful transformation of *N. magadii*.

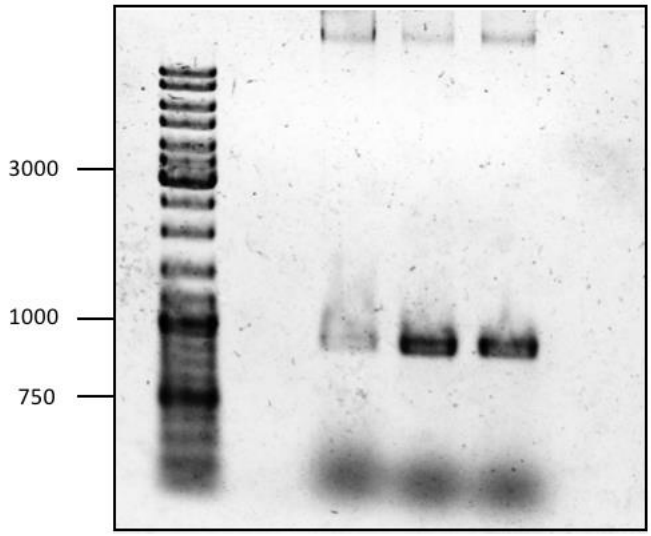


Figure212: The illustration of results of analytical PCR for the constructs

The identification of correct constructs was done via using primers 49-Kpn and BgaH-3i and the expected length was 916 bp as depicted above. After the marker line, the construct order was respectively pMI-1/43, pMI-1/43/44 and pMI-2/43/44.

As described above previous studies done in *H. volcanii* showed that ORF43 has an enhancing effect on the intergenic region between ORF48 and ORF49 by potentially binding directly or indirectly to 5' repeats within the ORF48 (*rep*) sequence and thereby enhancing transcription of ORF49 (Iro *et al*., 2007). Since on that time there were no suitable suitable transformation system for *N. magadii,* for the characterization of putative toxin-antitoxin system of ϕCh1 studies were done in a model Halophilic Archaea. When the constructs transformed into the *N. magadii*, as depicted in *Figure 26*, the similar results were collected.

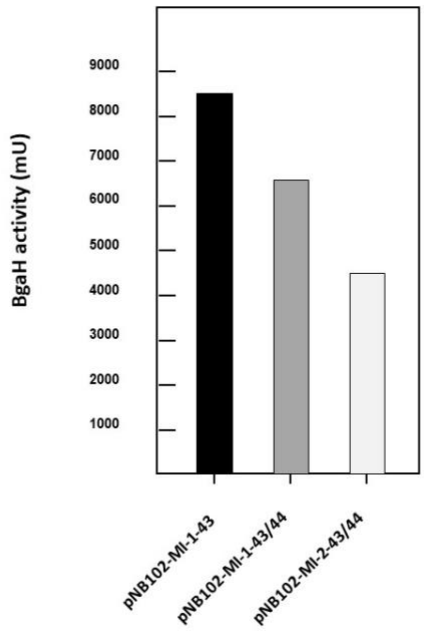


Figure 25: The illustration of results of BgaH activity of the constructs

The construct pMI-1/43/44 which containing hall operon region showed less BgaH activity than the same construct that containing only ORF43 instead of hall operon region called pMI-1/43. The construct named as pMI-2/43/44 which has additionally ORF48 region demonstrated significant difference within the other constructs.

The construct containing only antitoxin region called pMI-1/43 showed highest activity of BgaH. However, the construct containing additionally repressor gene, pMI-2/43/44, had significant dropped on the BgaH activity. Therefore, it can be concluded that ORF43/44 exhibits an enhancing effect on *bgaH* expression with an enhancing activity of ORF43 on the intergenic region. Additionally, in the presence of the ORF48 coding sequence the putative repressing activity of ORF44 was effective, thereby taken all together: The putative repressing- or degrading activity of ORF44 would then be further evidence for the ORF43/44 toxin-antitoxin hypothesis.

For further studies and more reliable results, the new constructs should be created and studied; such as the pNB102 plasmid containing the intergenic region between ORF48 and ORF49, a mutated ORF48 start codon, the *bgaH* gene under the control of the ORF49 promoter as well as ORF43/44 cloned downstream of the *bgaH* gene described as pNB102-MI-2Δ/43/44, and a construct only containing ORF43, pNB102-MI-2 Δ /43, or a construct only

containing ORF44, pNB102-MI-2Δ/44, and a construct not containing the ORF43/44 operon pNB102-MI-2Δ.

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5. Abstract

In order to gain more understanding about viral transcriptional regulation, the temperate virus ϕCh1 infecting the haloalkaliphilic archaeon *Natrialba magadii* is an important field of research. It was confirmed by previous studies that ORF43 and ORF44 have overlapping start and stop codons, indicating that both genes are co-transcribed and co-translated, thereby forming operon. Preliminary experiments performed with ORF44 revealed that it confers a repressing and an endoribonucleolytic function. Pfam analysis demonstrated a PIN domain in gp44 cleaves single-stranded RNA in a sequence specific manner, therefore making gp44 the VapC of a VapBC TA system which belongs to type II toxin-antitoxin system.

For investigation of regulatory role in operon system of ϕCh1, the deletion mutant of *N. magadii* L11-ΔORF43/44 was was constructed via homologous recombination by inserting the novobiocin resistance cassette. According to the growth kinetic analysis, lysis behavior of deletion mutant and wild-type strain are alike. However, the stability of deletion mutant showed different lysis behavior after 4th passage which closer to the cured strain *N. magadii* L13. Additionally, both virus titer analysis and morphology of plaques from deletion mutant suggested that passaging assay might be repeated and should be continued till at least 12th passage in order to get reliable results.

For further demonstration of regulatory function of ORF43/44, the BgaH measurements were done with the constructs; the construct containing only antitoxin region called pMI-1/43 showed highest activity of BgaH, however, the construct containing additionally repressor gene, pMI-2/43/44, had significant dropped on the BgaH activity. According to that ORF43/44 exhibits an enhancing effect on *bgaH* expression with an enhancing activity of ORF43 on the intergenic region. Furthermore, in the presence of the ORF48 coding sequence the putative repressing activity of ORF44 was effective. Thereby it can be concluded that the putative repressing- or degrading activity of ORF44 would then be further evidence for the ORF43/44 toxin-antitoxin hypothesis.

Keywords: ϕCh1, Archaea, Natrialba magadii, cured strain L13, lysogenic strain L11, Toxin-antitoxin system

6. Zusammenfassung

Um mehr Verständnis über virale Transkriptionsregulation zu erlangen, ist das temperente Virus ϕCh1, das das haloalkaliphile Archäon *Natrialba magadii* infiziert, ein wichter Modelorganusmus. In früheren Studien wurde bestätigt, dass ORF43 und ORF44 einen überlappenden Start- und Stoppcodon aufweisen, sodaß beide Gene co-transkribiert und co-translatiert werden. Eine Pfam-Analyse zeigte eine PIN-Domäne in der Sequenz von gp44, die einzelsträngige RNA auf sequenzspezifische Weise spalten könnte. Aufgrund dieser Homolgie könnte gp44 ein Teil eines Toxin/Antitoxin (TA) Systems der Klasse Typ-II sein. Diese Homologlie deutet auf ein VapBC-TA-System hin.

Zur Untersuchung der regulatorischen Rolle des Operons ORF43/44 von ϕCh1 wurde die Deletionsmutante *N. magadii* L11-ΔORF43/44 durch homologe Rekombination und Insertion einer Novobiocin-Resistenzkassette konstruiert. Nach der wachstumskinetischen Analyse des Wachstums des Stammes *N. magadii* L11-ΔORF43/44 ist das Lyseverhalten von Deletionsmutante und Wildtyp-Stamm gleich. Allerdings ist die Stabilität der Deletionsmutante nach der 4. Passage reduziert: ein unterschiedliches Lyseverhalten, das näher an dem Virus-losen Stamm *N. magadii* L13 lag, konnte hier beobachtet werden. Die Freisetzung von Virus-Partikeln ging dagegen nur leicht zurück. Eine Weiterführung des Experimentes mit einer signifikanten Erhhöhung der Passagen auf bis zu 12 ist deshalb erforderlich.

Zur weiteren Analyse der regulatorischen Funktion von ORF43/44 wurden die Reporterassays mit Bga durchgeführt. Hierfür wurden bereits fertiggestelle Konstrukte aus *Haloferax volcanii* in ein Plasmid für *N. magadii* transferriert. Here konnte gezeigt werden, das gpr43 eine Aktivierung der *bgaH* expression ergab. Hierbei stand die Kontrolle des *bgaH* Genes unter der intergenischen Region von ORF48 und ORF49 aus ϕCh1. Waren auf diesem Plasmid noch ebenfalls das Repressor Gen von ϕCh1 (ORF48) und ORF44 anwesent, so reduzierte sich die gemessene BgaH Aktivität signifikant.

Schlüsselwörter: ϕCh1, Archaea, Natrialba magadii, ausgehärter Stamm L13, lysogenischer Stamm L11, Toxin-Antitoxin System