universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„The toxin gp44 of *N. magadii* virus ΦCh1: is it a RNase or protease?"

verfasst von / submitted by

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

Master of Science (MSc)

Wien, 2023 / Vienna, 2023

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

Acknowledgement

I would like to express my deepest gratitude to my supervisor, Ao. Univ.-Prof. Dipl.-Biol. Dr. Angela Witte, for her guidance and support throughout my master's thesis. She always has a sympathetic ear and helped me grow as a person and as a scientist. Her mentorship and expert advice were instrumental in enabling me to complete this work.

Furthermore, I am grateful to my lab colleagues, Gregor, Clara, Julian, and Richard, as well as the members of Bläsi and Moll groups for their helpful discussions and laughs inside and outside the lab.

A special thanks goes to my friend Lena, without whom this path would not have been with that much sunshine. And also to Arvid for being my constant source of motivation and comfort.

Finally, I would like to thank my mother for her endless support and encouragement throughout my academic journey.

Abstract

The halo- and alkaliphile *N. magadii* archaeon infecting temperate virus φCh1 has one co-translated and co-transcribed putative toxin-antitoxin system type II, encoded by ORF43/44. ORF44 contains a PiN-domain and ORF43 no sequence of known function. The corresponding gene products gp43/44 have been shown to enhance ORF48/49 involved in the regulation of the φCh1 cell cycle. An endoribonuclease activity is expected for gp44, due to the truncation of gp34 in the presence of gp44, and ORF43 is expected to be autoregulated.

To analyze the putative nuclease activity and the regulating effect on ORF48 and ORF49 the toxin ORF44 and antitoxin ORF43 were cloned under control of the inducible promoter ptnaH. *BgaH* (β-galactosidase like activity from *H. volcanii*) was used as reporter gene. To analyze ORF43/44 for nuclease activity RNA samples were taken to test via RT-PCR if cleavage of *bgaH* mRNA is happening. For investigation of the autoregulation of ORF43 the promoter p43 was fused with the reporter gene *bgaH* and on a second plasmid ORF43 was cloned under control of the constitutive promoter p34 from ORF34 which encodes for the tail fiber protein. Putative RNase activity of gp44 on gp34 was investigated with constructs containing ORF44 under the control of the inducible promoter ptnaN (tryptophanase promoter from *N. magadii*).

No autoregulation of ORF43 could be identified. The effects of ORF43/44 on ORF48 and ORF49 could not be measured, because of a lack of reporter gene (bgaH) activity. All plasmids with the inducible promoter seemed to have lost expression of the reporter gene (*bgaH*). The expression of ORF43/44 leads to a truncated version of gp34 (Hofbauer, 2015). RNA analyses were inconsistent and need further improvement.

KEYWORDS: φCh1, *Archaea*, *Natrialba magadii*, *Haloferax volcanii* L11, Toxin-antitoxin system, BgaH

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1. Introduction

1.1 Archaea

Archaea are the third domain of life along with the Bacteria and Eukarya (Cavicchioli, 2007). They were first discovered in the 1880s, but the formal proposal as the third domain of life only happened in 1990. Pioneering work from Carl Woese led to the reorganization of the tree of life based on small subunit ribosomal RNA sequences. This led to the discovery that archaea are distinct from bacteria (Woese et al., 1990). Recent discoveries even support eukaryotes emerged from within the archaea clade, indicating a two-domain tree of life (Williams et al., 2020), thus making archaea an even more fascinating field of research.

Archaea are prevalent in extreme environments and many archaeal species (so called ‘extremophiles’) hold records in distinctive extreme living conditions. They have commonly been grouped into extreme halophiles, methanogens and thermoacidophiles. Methanogens can perform methanogenesis and live in anaerobic aquatic environments and gastrointestinal tracts of animals. Thermoacidophiles grow fastest at temperatures above 80 °C like at hydrothermal vents and volcanic terrestrial environments. Halophiles, which will be discussed in more detail shortly, reside in hypersaline environments like salterns, saline lakes and the Dead Sea. So far no pathogenic archaea are known, but they have been associated with colorectal and periodontal disease (Cavicchioli et al., 2003).

Haloarchaea belong to the superphylum Euryarchaeota (Woese et al., 1990). They are capable of growing in salt concentrations from 3 to 5 M NaCl – common in salt lakes and marine salterns. Neutral lakes like the Dead Sea develop where Ca^{2+} and Mg^{2+} levels are high while the development of alkaline lakes like the soda Lake Magadi in Kenya is dependent on low Ca^{2+} and Mg^{2+} levels (Grant, 2004). The name ‘halophile’ indicates that they require salt to grow and are not able to grow in the absence of high salt concentrations. In contrast halotolerant organisms are able to survive at high salt concentration but do not need it to thrive (terms were discussed by Kushner (1978) and Vreeland (1987)). Survival at these

high salt concentrations is possible through adaptation to low water availability and high ionic strength. Their main strategy is the accumulation of inorganic ions (mostly KCl) to counterbalancing levels for osmotic stability, up to 5 M (Sehgal & Gibbons, 1960), (Lanyi et al., 1979). Proteins of haloarchaea are mostly adapted to contain high contents of acidic amino acids (glutamate and aspartate) and low amounts of basic amino acids (lysine and arginine) (see Lanyi (1974), Mevarech et al. (2000) and Oren (2002))

High internal salt concentrations of halophiles and the adaptions of the protein composition to this environment make working with halophile proteins rather difficult. It causes disadvantages for common protein/enzyme purification and protein separation which are ineffective at molar salt concentrations, especially if enzyme activity needs to be preserved (Holmes et al., 1997). As well as the rather long generation time of 9 h and 4 h in the logarithmic growth phase for *Natrialba magadii* and *Haloferax volcanii*, respectively. Storage of haloarchaea cultures is fairly easy by simply drying them down in salt and storing them desiccated at room temperature for considerable periods of time (Grant, 2004) or in liquid culture RT.

1.1.1 Haloalkaliphilic archaeon *Natrialba magadii*

Natrialba magadii was isolated form a Soda Lake in Magadi, Kenia, in 1980. Genera *Natrialba* and *Natronococcus* have a pH optimum at 9 to 10 and therefore represent the alkaliphilic group among the family of Halobacteriaceae. *N. magadii* has rod shaped cells, is strict aerobe, and as a dual extremophile has an optimal growth at alkaline (pH 9.5), hypersaline (3.5 M NaCl) conditions at a temperature of 42 °C (Tindall et al. (1980), Tindall et al. (1984)). Its generation time in the logarithmic growth phase is approximately 9 h.

For research two different *N. magadii* strains are in use, the L13 strain which was cured from the ΦCh1 virus by sub-culturing and strain L11 which carries the virus ΦCh1 and is immune to superinfection.

Two shuttle vectors are to date available for *N. magadii,* pRo-5 and pNB102. The vector pRo-5 (Mayrhofer-Iro et al., 2013) contains ΦCh1 operon ORF53/ORF54

(*repH*) as origin of replication (ori) and carries an ampicillin and novobiocin resistance. On the other hand, pNB102 (Zhou et al., 2004) is based on plasmid pNB101, which was isolated from the haloalkaliphilic archaeon *Natronobacterium sp.* strain AS7091 and contains an ampicillin and mevinolin resistance as well as the ColE1 *ori* in addition.

1.1.2 Halophile archaeon *Haloferax volcanii*

H. volcanii was isolated in 1975 from the Dead Sea. The disc-shaped cells grow best in 1.7-2.5 M NaCl (Mullakhanbhai & Larsen, 1975) and can grow on glycerol and organic acids (Kauri et al., 1990). Their genome is polyploid with up to 20 genome copies per cell and a high GC content with ~65%. To survive at high salt concentrations *H. volcanii* uses a 'salt-in' mechanism to equilibrate the internal salt concentration to that outside the cell. *H. volcanii* possesses a glycoprotein surface (S-) layer, like many archaea, instead of a cell wall (see review Perez-Arnaiz et al. (2020)).

H. volcanii is suited for molecular biological studies in archaea due to available tools for genetic manipulation, like novobiocin and mevinolin resistances as selective markers (Allers et al., 2004), *bgaH* as a β-galactosidase like reporter gene (Holmes & Dyall-Smith, 2000), controlled gene expression with the promoter of the tryptophanase gene (ptna) (Large et al., 2007), and shuttle vectors based on different *H. volcanii* replication origins (e.g. pMDS24) (Holmes et al., 1991) just to name a few (see review Perez-Arnaiz et al. (2020)).

The *H. volcanii* laboratory strain WD2 has a 2.8 Mbp circular chromosome and three circular mini-chromosomes (plasmids) with size from 85 kbp to 636 kbp and in contrast to the wildtype strain WD2 is cured from the pHV2 (6 kbp) plasmid. (Wendoloski et al., 2001).

Mechanisms of DNA replication and repair are well studied, but still leave a lot of open questions. Available tools make it possible to get a better understanding of *H. volcanii* as well as other haloarchaea (Perez-Arnaiz et al., 2020).

1.1.3 Genetic tools for manipulating halo(alkali)philic archaea

Tools for gene manipulation and expression are very important for the study of halo(alkali)philic archaea, given halobacterial genetic elements are not functioning in bacteria due to their difference in gene structure. Additionally, fusion genes are generally inactive (Hausner et al., 1991). One of the most important tools are vectors, of which only a few are available for halo(alkali)phile archaea but are necessary for studying halophile genes and enzymes.

The shuttle vector pMDS24 for *H. volcanii* contain resistances for ampicillin and mevinolin (Jolley et al., 1996). The shuttle vector pMI-1 was constructed by Iro et al (2007) by cloning the intergenic region of ORF48 and ORF49 of virus ΦCh1, whereas ORF49 was replaced with the promoter-less β-galactosidase gene (BgaH) (Holmes & Dyall-Smith, 2000) in pMDS24. Similar to that, pMI-2 was created with the presence of ORF48 and pMI-2Δ with the deleted start codon AUG of ORF48, while pMI-2-EΔ has a silent mutation in a repeat of ORF48. These four vectors will be summarized as pMI-* in this thesis for simplicity.

1.1.3.1 Tryptophane inducible promoter

Controlled induction of gene expression is an important tool for studying toxins. Regulatory elements like origins of replication (Ori), strong promoters, and transcriptional repressors can be used to establish inducible expression systems. Large et al. (2007) have identified a strong and fast inducible promoter via a shotgun DNA microarray, the promoter of the tryptophanase gene in *H. volcanii*. It is a tightly controlled promoter, which is induced by adding tryptophane. Expression increases 50-fold within 5 minutes, with maximum induction achieved with 4 mM tryptophane.

1.1.3.2 Reporter genes for halophile archaea

Available reporter genes for haloarchaea are the β-galactosidase-like *bgaH* gene (Holmes & Dyall-Smith, 2000; Holmes et al., 1997), the genes encoding the fluorescence proteins (Born & Pfeifer, 2019) and luciferase (Davis et al., 2020). In this study *bgaH* was used as a reporter gene. As already mentioned, *E. coli* reporter genes like β-galactosidase are not functioning in Haloarchaea because of the high internal salt concentration. Holmes et al. (1997) generated a *bgaH*

overproducing *Haloferax lucentense* strain (formerly called *Haloferax alicantei*) by UV-mutagenesis and screening on plates containing X-Gal as well as an ONPG assay. The isolated “super-blue” strain has a 140-fold BgaH activity compared to the wildtype *H. lucentense*. Substrates can be ONPG, X-Gal and lactulose. Activity optimum is at 4 M NaCl, and storage of the isolated BgaH is possible for several days in 3 M NaCl at RT. If stored at 4 °C or -20 °C overnight or with additional glycerol (20 % v/v) the specific activity decreases. Storage for several months is possible in 30 % sorbitol at -20 °C.

bgaH is a useful tool for investigation of gene expression and regulation and for investigation of promoters, all on a quantitative basis. It is used to study halophile promoters from related halophile archaea like *H. mediterranei* and *H. salinarum* in the model organism *H. volcanii*. The lack of an endogenous β-galactosidase like activity in *H. volcanii* provides a background-free measurement of gene expression. Patenge et al. (2000) reported that BgaH activity does not always reflect the mRNA data, but BgaH activity remains constantly high even though mRNA amounts were low during stationary phase, indicating that BgaH is relatively stable and remains active (Gregor & Pfeifer, 2001). Stability of *bgaH* mRNA and protein deviate from each other, but this observation diverges from results by Patenge et al. (2000).

1.2 Viruses of archaea

Haloarchaeal viruses include many different virus families like *Myoviridae, Siphjoviridae, Podoviridae, Pleioviridae, Pleilipoviridae, Sphaerolipoviridae and Fuselloviridae* with all their different morphologies. Some are like other viruses or bacteriophages and others carry unique lifecycle and morphological features.

There has been relatively little research into this compared to bacteriophages and viruses infecting eukaryotes, in part due to the difficulty in cultivating haloarchaea. But a great deal of novelty has been documented about certain haloarchaeal viruses from studies so far (see review Luk et al. (2014)).

Similar to their hosts, the haloarchaeal viruses have proteins adapted to high salt concentrations. Their proteins are mostly acidic below pH 6 and the low pI results in a negative charge which is thought to promote the formation of salt bridges, the attraction of water molecules, and the formation of hydration shells around the proteins (Luk et al., 2014).

With their high abundance, viruses have the ability to put a strong selective pressure on their hosts (Bamford, 2003). Haloarchaea are relatively slow-growing but they demonstrate high levels of genetic variation where haloarchaeal viruses may play an important role in their continuing evolution. Viruses are traditionally associated with having a predator-prey relationship with their hosts, but haloarchaeal viruses may have more of a mutualistic association. Being not only drivers of selection of host defense mechanisms but also assistants in genetic exchange, they are a positive influence to the population (Oren et al. (1997), Wais et al. (1975), Luk et al. (2014)).

Viruses can be classified by their modes of infection: Virulent, temperate, and persistent. Virulent viruses are unable to form stable lysogens with their hosts, performing a lytic cycle where they form progeny after infecting their host followed by lysis of the host and release of virions. Temperate viruses can form stable lysogens with their host and switch into a lytic cycle at a certain point. The control of lysogenic state and lytic life cycle is essential for their survival. In contrast, persistent infection does not lead to the formation of a provirus via integration of

the viral DNA into the host DNA, but unstable carrier states are formed where virus-progeny are produced and released continuously without lysis of the host.

1.2.1 Head-tailed viruses

Head-tailed viruses (*Caudovirales*) are one of the most well studied viral morphotypes and comprise 96% of known bacteriophages (Ackermann & Prangishvili, 2012). *Caudovirales* have no envelope. Their viral DNA is encapsulated by the icosahedral head to which a flexible hollow tail is attached by a connector. The tail adsorbs to the host cell and injects the viral DNA (Ackermann, 1998). Mechanisms of haloarchaeal head-tailed viruses have not yet been as well studied as the head-tailed bacteriophages. With only 1 % in some environments, head-tailed viruses are the least abundant morphotype, but the most studied. This shows a bias in favor of lytic viruses which can be identified by plaque formation in combination with the difficulty of host cultivation. There are three distinct head-tailed virus families, namely *Myoviridae, Siphoviridae* and *Podoviridae*, with long contractile tails, long non-contractile tails and short tails, respectively (Luk et al., 2014).

1.2.2 Haloarchaeal virus ΦH of *Halobacterium salinarum*

The first described phages in halophilic archaea were found in the genus Halobacteria, whereas ΦH of *Halobacterium salinarum* is the most extensively studied archaeal virus (Schnabel & Zillig, 1984). ΦH is a temperate virus and exists as a lysogen in a non-integrated circular form (Schnabel et al., 1982). It is either present as a 57 kbp circular extrachromosomal provirus or only the 12 kbp L circular form with only the L region of ΦH genome, where most of the phage's regulatory functions are encoded (P. Stolt & W. Zillig, 1993). ΦH requires high salt concentrations (3 M KCl or NaCl) for structural integrity (Pelle Stolt & Wolfram Zillig, 1993). ΦH DNA was used for the polyethylene glycol mediated spheroplast transformation of *H. salinarum* or *H. volcanii* which otherwise cannot be infected by the virus. This further led to the development of vectors suitable for haloarchaea using the replicon region of ΦH (Cline & Doolittle, 1987; Cline et al., 1989). ΦH regulates host abundance and protect itself during unfavourable

growth conditions e.g., changes in salinity (P. Stolt & W. Zillig, 1993). Being the most studied halophage, ΦH helps identifying potential gene and protein functions through sequence similarities.

1.2.3 N. magadii infecting virus ΦCh1

The *N. magadii* infecting virus ΦCh1 is a temperate one which integrates as a provirus in the chromosomal DNA of its host. It is used as a model for haloalkaliphilic viruses (Witte et al., 1997). ΦCh1 is a head tail virus, assigned to the family *Myoviridae* because of its tail consisting of an internal shaft covered by a contractible tail with a width of ~20 nm and an approximate total size of 200 nm, whereas the head measures about 70 nm and the tail 130 nm. The head linear double-stranded DNA (58.5 kbp) is packed together with host encoded RNA (~80-700 nt). The function of the RNA is yet unknown, but it is possibly involved in DNA packaging. There are 98 assigned open reading frames (ORFs), whereas genes encoding for structural components and assembly, DNA replication and

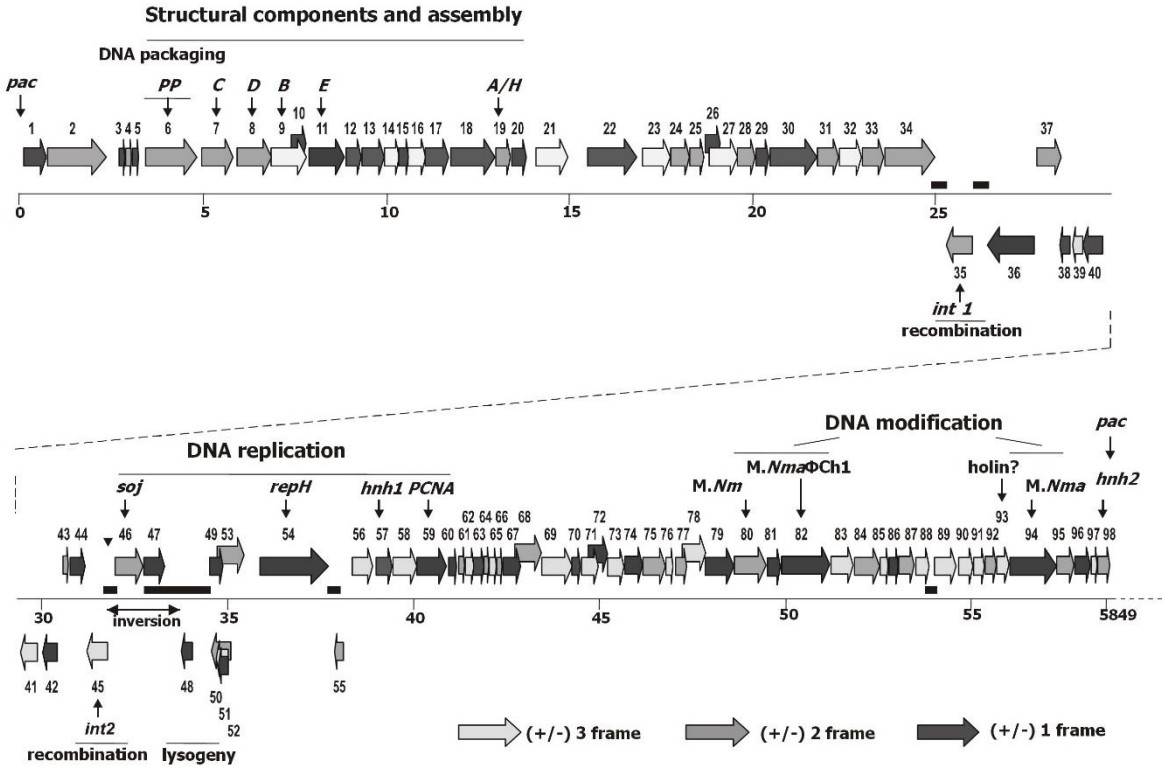

Figure 1 ***Schematic representation of the ΦCh1 genome****. ORFs are indicated by arrows and the three shades of grey indicate the different reading frames. Three functional modules responsible for encoding structural components, DNA replication and DNA modification are also indicated (Adapted from Klein et. al. 2002)*

DNA modification are clustered in three functional modules (see Fig.1) and the DNA has a GC content of 61.9% (Klein et al., 2002).

In contrast to its host, the virus DNA is partly methylated probably as protection of the genome (Baranyi et al., 2000; Bickle & Kruger, 1993; Kruger & Bickle, 1983). Until now, 27 ORFs have been experimentally identified (A. Witte, personal communication, March 9, 2023). ΦCh1 is like its host adapted to high salt concentrations, with virus stability and infectivity dependent on salt concentration which drops at below 2 M NaCl (Witte et al., 1997). Four major and five minor proteins with an isoelectric point between pH 3.3-5.2 indicate lots of acidic amino acids were identified and partly characterized (see Witte et al. (1997) and Klein et al. (2002)). Induction of the lytic cell cycle is spontaneously induced during the shift into the stationary phase and mature phage particles are released by cell lysis. ΦCh1 virus population densities are lowest during high salinity when host populations are high, and highest during low salinity when host populations are low (Witte et al., 1997).

1.3 Toxin-antitoxin (TA) Systems

Toxin-antitoxin (TA) Systems are genetic modules consisting of a stable toxin and a cognate unstable antitoxin. They are ubiquitous in genomes of bacteria and archaea (Pandey & Gerdes, 2005). Under proper growing conditions the antitoxin prevents the toxin from releasing its toxic effect. If stress conditions occur the unstable antitoxin degrades, and the toxin exerts its toxic effect. Currently TA-systems are classified into eight types based on their kind (protein or RNA) and mode of action (Jurenas et al., 2022). Their biological roles are very diverse, ranging from maintaining plasmids and genetic elements like genomic islands and prophages, to being involved in virulence of pathogenic bacteria and regulating biofilm and persister cell formation (Qiu et al., 2022).

1.3.1 Toxin-antitoxin system type II

The most abundant and extensively studied TA system is the one of type II which is arranged as bicistronic loci where the toxin is located downstream of the antitoxin insuring the efficient inhibition of the toxin through translational coupling. The genes usually overlap by a few bases or are separated by just a few (Yamaguchi & Inouye, 2009). Toxin and antitoxin form a stable complex where the toxins' function is inhibited. The antitoxin is less stable and therefore must be produced continuously to prevent the toxin from taking action. In stress conditions the antitoxin gets degraded, and the toxin is set free, resulting in cell growth inhibition or death (Aizenman et al., 1996). The TA operon is autoregulated by the TA complex binding to a palindromic sequence in the promoter sequence and repressing the TA expression. Therefore, TA systems type II are also named addiction modules (Yamaguchi et al., 2011).

Almost half of TA systems type II belong to the family of VapBC (Pandey & Gerdes, 2005), which stands for virulence associated proteins B and C and was first discovered in *Salmonella diblin* (Pullinger & Lax, 1992). The VapBC operon encodes two genes: the toxin VapC a PiN domain protein with putative ribonuclease activity and VapB the cognate antitoxin (Cooper et al., 2009).

1.3.2 PiN Domain

Originally the PiN (PilT N-terminus) domain was described as required for type IV pilus polymerization in motile, gram-negative bacteria (Wall & Kaiser, 1999). It is a 120-140 residue protein domain which is conserved across all domains of life and usually not associated with cell motility. Eukaryotic PiN domains exist as parts of larger proteins which are tightly regulated through protein-protein interactions and subcellular localization. Whereby archaeal and prokaryotic PiN domains are mostly standalone proteins commonly acting as the toxin part of a toxin-antitoxin system (Makarova et al., 1999). Members of the PiN domain-like super family [CATH 3.40.50.101] share three-layered α/β/α as a core motif with a central, parallel five-stranded β-sheet with two helices on either side, similar to the Rossman fold.

The superfamily can be divided in three structural clusters (SC) whereas SC:1 harbours the functional family T4 RNase H, SC:2 VapC toxins, other PiN domain proteins and rRNA endonuclease Nob1 and SC:3 Flap endonuclease 1 (FEN-1) and DNA polymerase I. Sequence conservation in SC:2 is minimal but nonetheless structural similarity is relatively high. The active site is well conserved with 4-5 canonical acidic residues (D, E, D, D /N) clustered together and the ability to bind divalent metal ions (Mg^{2+} or Mn^{2+}). Able to cleave RNA in sequence and/or structure-specific and divalent cation-dependent manner (Xu et al., 2016). Activity ranges from 5'-3' exonuclease activity; nonsense mediated RNA degradation in eukaryotes (Clissold & Ponting, 2000) to surveillance and bacterial stress response and pathogenesis in prokaryotes (Anantharaman & Aravind, 2003).

Examples for PiN-domain proteins in archaea are the 133-residue protein PAE2754 from the hyperthermophilic crenarchaeon *Pyrobaculum aerophilum* which has structural homology with the T4 RNase H, and conserved acidic residues in a similar position to the active site of T4 RNase H and a divalent metal dependent nuclease activity as well as conserved threonine close to the active site that is likely crucial for substrate recognition. Deactivation of the active site is achieved by binding of the antitoxin and insertion of a positively charged arginine residue which displaces the divalent metal ion and disables the PiN domain (Xu et al., 2016).

Or *Sulfulobus solfataricus* which encodes 26 PiN domain proteins of which most constitute the toxin part of (bona fide) toxin-antitoxin systems that are involved in cell growth and thermal stress adaption. (Cooper et al., 2009)
In *Acidanus hospitalis* toxin-antitoxin gene pairs of the vapBC were proposed as maintainer of CRISPR components in the genome by surrounding the CRISPR/Cas/Cmr immunity systems and therefore helping to adapt to environmental stress.

1.3.3 ΦCh1 ORF43 and ORF44 putative toxin-antitoxin system and its regulatory effect on ORF48/49

Sequence analysis of ORF43/44 showed high identity to the co-operative element of the repressor transcripts T9/T10 from ΦH and were identified as putative toxin-antitoxin system Type II. Both ORFs are under the control of one promoter and overlap one codon. Hence they are co-translated and co-transcribed (Iro et al., 2007). Sequence analysis of the corresponding proteins, gp43 and gp44, revealed that gp44 contains a PiN-domain belonging to the VapC family, spanning almost the entire protein. However, gp43 does not contain any domain of known function.

A study by Iro et al. (2007) showed gp43/44 have an enhancing effect on the intergenic region of ORF48 (*rep*) and ORF49. ORF48 (*rep*) and ORF49 were identified as possible regulators of ΦCh1 life cycle. ORF49 was identified by a ΦCh1 mutant with changed lysis behavior. ORF48 shows significant sequence similarities to a repressor molecule from the related virus ΦH and acts as a transcriptional repressor for ORF49. These two ORFs have a head-to-head orientation and the intergenic region comprises a promoter/operator sequence. That intergenic region was used for the construction of an expression vector (see 1.1.3.2 Reporter genes for halophile archaea) and is active in the model organism *H. volcanii*. It was identified that Rep itself binds within the coding region of *rep* to a repeat and acts as a repressor. Inducing a silent mutation in a repeat in the coding *rep* sequence revises the repression by Rep partially. Therefore, Rep and gp43/44 bind to the same direct repeat but gp43/44 with a lower specificity (Klein et al., 2002) (Iro et al., 2007). Nevertheless, Rep seems to have a higher

repression potency than gp43/44 has an enhancement effect which could be due to different binding affinities or competition between these two resulting in a reduced enhancement effect (Iro et al., 2007).

Expression of ORF49 is not constitutively, its weak at the beginning of the log phase and increases during phage development. Until now it is not known how ORF49 is triggered. ORF48 (*rep*) expression is constitutive throughout the entire ΦCh1 life cycle. Gp48 belongs to the family of winged helix repressor proteins, contains a predicted site for RecA-mediated self-cleavage and the self-cleavage of ORF48 (*rep*) would lead to the induction of ORF49 expression.

1.3.4 Tail fibre protein gp34 and gp36

ΦCh1 ORF34 and ORF36 encode tail fibre proteins, they are inverse oriented with their 3'end facing each other. In between is the putative site-specific recombinase encoding ORF35. Recombination in that region can lead to heterogenous variants of gp34 and gp36 with variable length (Rossler et al., 2004). Experiments from Hofbauer (2015) in *N. magadii* suggest endoribonuclease activity of gp44, because in its presence truncated gp34 was produced.

1.4 Thesis aim

The aim of this thesis was to get a better understanding of the function of ORF43 and ORF44. First the putative autoregulation of promoter ORF43 was tested in *N. magadii*, based on the master's thesis of Schmal (2018), but with constitutive expression of ORF43 under promoter p34 instead of its native promoter. Next, both genes were cloned under control of the inducible promoter ptna in the pMI-* vectors and transformed in *H. volcanii*. Especially important for the study of the putative toxin ORF44, and its putative nuclease activity. Attempting to test the putative nuclease activity with reverse transcriptase PCR (RT-PCR), to see if cleavage of BgaH mRNA happens. As well as trying to analyze the putative RNase activity of gp44 on gp34 via Western Blot and RT-PCR based on Athanasiou (2020) previous work and constructs for *H. volcanii.* Plus growing of *H. volcanii* in minimal media and induction of ptna promoter, as well as optimizing the BgaH assay for analysis in 96 well plates.

2. Materials and Methods

2.1 Materials

2.1.1 Bacterial and archaeal strains

Escherichia coli

Strain	Modifications	Source
XL1-Blue	*endA1, gyrA96, hsdR17* ($r_{k-}m_{K+}$), *lac, recA1, relA1, supE44, thi-1,* [F´, *lacI*^{q}*, lacZ*ΔM15*, proAB*$^{+}$, Tn10 (Tet^{R})]	Stratagene

Natrialba magadii

Strain	Modifications	Source
L13	φCh1 cured strain	(Witte et al., 1997)
L11	Contains wild type φCh1	(Witte et al., 1997)
L13 (pRo-5)	With plasmid pRo-5	
L13 (pRo-5-p34-ORF43)	With plasmid pRo-5 containing ORF43 under the control of constitutive promoter from ORF34	

Haloferax volcanii

Strain	Modifications	Source
WD2	ΔpHV2	(Charlebois et al., 1987)

2.1.2 Growth media

2.1.2.1 E. coli

2.1.2.1.1 Lysogeny broth medium (LB) for *E. coli*

NaCl ..... 5 g
Yeast extract ..... 5 g
Peptone ..... 10 g
pH 7.0
dH_2O ad 1 l
15 g/l agar for plates

2.1.2.2 H. volcanii

2.1.2.2.1 Concentrated Salt Water (SW) solution 30% (w/v)

NaCl ..... 240 g
$MgCl_2.6H_2O$ ..... 30 g
$MgSO_4.7H2O$ ..... 35 g
KCl ..... 7 g
1 M $CaCl_2.2H_2O$ (sterile)..... 5 ml
1 M Tris/HCl buffer (pH 7.5) ..... 10 ml
dH_2O ad 1 l

2.1.2.2.2 YPC Media

30% SW solution ..... 600 ml
Yeast Extract ..... 5 g
Peptone (Oxoid) ..... 1 g
Casamino Acids..... 1 g
pH 7.5 with 1 M KOH
dH_2O ad 995 ml

after autoclaving, complement with:
trace elements SL-6 ..... 1 ml
1M $CaCl_2$ (autoclaved)..... 3 ml

2.1.2.2.3 18% MGM Media for *H. volcanii*

30% SW solution ..... 600 ml
Yeast Extract ..... 1 g
Peptone (Oxoid) ..... 5 g
pH 7.5 with 1M Tris.Cl pH 7.5 (30 mM final)... 25 ml/L
dH_2O ad 1 L, autoclave

2.1.2.2.4 Minimal Media for *H. volcanii* (H.v.-Min)

Use a sterile bottle for 1 L

30% SW Solution .....600 ml
dH_2O (autoclaved).....310 ml
1M Tris.Cl pH 7.5 .....30 ml
H.v.-Min Carbon Source.....25 ml
H.v.-Min Salt Solution.....12 ml
0.5 M KPO_4 buffer pH 7.5.....2 ml
Thiamine & Biotin Solution .....900 µl
Alanine (2.23g/20ml, sterile filtered).....20 ml

2.1.2.2.5 H.v.-Min Media for plates

In 500ml bottles

Agar ..... 5 g
30% SW .....300 ml
dH2O.....160 ml
1M Tris/HCl pH 7.5.....15 ml
Add magnetic stirrer and autoclave
Put on 65°C after autoclave, if not immediately poured.

After autoclaving complement with:
H.v.-Min carbon source .....13 ml
H.v.-Min salt solution.....6 ml
0.5 M KPO_4 buffer pH 7.5.....1 ml
Thiamine & Biotin Solution .....450 µl
Alanine (2.23g/20ml, sterile filtered)10 ml

2.1.2.2.6 H.v-Min Carbon Source

(10% Lactic Acid, 9% Succinic acid, 1% Glycerol)

dH_2O .....~150 ml
~90 % S-Lactic acid .....27.5 ml
Succinic acid, Na_2-salt *6 H_2O .....37.5 g
89 % Glycerol.....2.93 ml
Adjust pH with NaOH to pH 7.5
dH_2O .....ad 250 ml
sterile filter with 0.2 µm

2.1.2.2.7 H.v.-Min Salt Solution

Use sterile bottle

Sterile dH_2O ..... 30 ml
Sterile 0.5 M $CaCl_2$ ..... 36 ml
Sterile filtered Trace Elements (H. v.)..... 6 ml
Store at 4°C

The original formula uses 1 M NH_4Cl as NH_3^+ source, but here it was replaced with dH_2O and substituted with Alanine because of its better NH_3^+ availability 2.23 g Alanine per 1L H. v.-Min-Media corresponds to 25 mM of nitrogen source (Large et al., 2007).

2.1.2.2.8 Alanine Solution

Alanine .....2.23 g
dH_2O..... ad 20 ml
sterilized by filtration

2.1.2.2.9 Trace Element Solution for *H. volcanii* (Duggin et al., 2015)

EDTA.....5 g
$FeCL_3$ * 6 H_2O.....1.325 g
$ZnCl_2$.....0.05 g
$CuCl_2$ * 2 H_2O .....0.0127 g
$CoCl_2$ .....0.01 g
H_3Bo_3.....0.01 g
$MnCl_2$.....1.6 g
$NiSo_4$ * 6 H_2O.....0.017 g
Na_2MoO_4 * 2 H_2O.....0.015 g
dH_2O..... ad 1000 ml
adjust pH with NaOH to pH 7
sterilized by filtration
Solution changes color from green to brown to red/orange.

2.1.2.2.10 0.5 M KPO_4 buffer (pH 7.5)

1 M K_2PO_4 ..... 83.4 ml
1 M KH_2PO_4..... 16.6 ml
dH_2O..... 100 ml
autoclave

1 M K_2PO_4 ..... 34.84g/200ml
1 M KH_2PO_4..... 13.6g/100ml
autoclave

2.1.2.2.11 Thiamine and Biotin Solution

Thiamine (1mg/ml)

dH_2O .....45 ml
thiamine Stock (10 mg/ml) .....5 ml
sterilized by filtration
final concentration 1 mg/ml

Biotin (1mg/ml)
0.1 g biotin in 10 ml dH_2O
add some 1 N NaOH for dissolving
sterilized by filtration

Thiamine & Biotin Solution
thiamine [1 mg/ml].....9.6 ml
biotin [1 mg/ml].....1.2 ml
stored at 4°C

2.1.2.3 N. magadii

2.1.2.3.1 NVM-CAA$^+$ / NVM-CH$^+$

NaCl .....235 g
KCl .....2.4 g
yeast extract.....11.7 g
casamino acids (CAA)/ casein hydrolysate (CH) 8.8 g
sodium citrate tribasic dehydrate.....0.8 g
pH 9-9.5 with NaOH
ddH_2O ad 935 ml
for plates: 10 g/l agar
autoclave

After autoclaving complemented with:
0.57 M Na_2CO_3 (dissolved in sterile ddH_2O) .....63 ml
1 M $MgSO_4$ (autoclaved) .....1 ml
20 mM $FeSO_4$ (dissolved in sterile ddH_2O) .....1 ml
trace elements SL-6 .....1 ml

2.1.2.3.2 1000x Trace elements SL-6

$CoCl2 * 6 H_2O$ .....0.2 g
$CuCl_2 * 6 H_2O$ .....10 mg
H_3BO_3 .....0.3 g
$MnCl_3 * 4 H_2O$ .....30 mg
$Na_2MoO * 4 H_2O$ .....30 mg
$NiCl2 * H_2O$ .....20 mg
$ZnSO_4 * 7 H_2O$ .....0.1 g
pH 3-4 with HCl
dH_2O ad 1000 ml
sterilized by filtration

2.1.3 Additives

Compound	**Stock concentration**	**Final Concentration**	**Preparation**
Ampicillin	20 mg/ml	100 µg/ml	dissolved in dH_2O, sterile filtered, stored at 4°C
Tetracycline	10 mg/ml	10 µg/ml	dissolved in half of EtOH and half dH_2O, stored at -20 °C
Mevinolin	5 mg/ml	4 µg/ml	Lovastatin Powder dissolved 96 % EtOH, stored at -20 °C
Novobiocin	3 mg/ml	0.3 µg/ml for *H. volcanii* 3 µg/ml for *N. magadii*	dissolved in dH_2O, sterile filtered, stored at -20 °C
Bacitracin	7 mg/ml	70 µg/ml	dissolved in dH_2O, sterile filtered, stored at 4°C
L-tryptophane (for plates)	40 mM	0.15-4 mM	dissolved in dH_2O sterile filtered, storage at 4°C
L-tryptophane	0.6 M	0.15-4 mM	dissolved in 1 M NaOH, storage at -20 °C

2.1.4 Plasmids

2.1.4.1 *H. volcanii* Plasmids

Construct	**Features**	**Source**
pMDS24	*bla*, ColE1, (mevR), DHFR, pHV2 ori	Jolley et al. (1996)
pMDS24-34_1	pMDS24 with ORF34 (+ orientation of *int1*) of φCh1 under the control of native promoter	Athanasiou (2020)

Construct	Features	Source
pMDS24-34$_1$-ORF43	pMDS24 with ORF34 (+ orientation of *int1*) and ORF43 of φCh1 under the control of native promoters	Athanasiou (2020)
pMDS24-34$_1$-tnaN-ORF44	pMDS24 with ORF34 (+ orientation of *int1*) of φCh1 under the control of native promoter and with ORF44 under the control of tryptophan inducible promoter	Athanasiou (2020)
pMDS24-34$_1$-ORF43/44	pMDS24 with ORF34 (+ orientation of *int1*) and ORF43/44 of φCh1 under the control of native promoters	Athanasiou (2020)
pMLH32	*NovR*, *bgaH*, *bla*, ColE1, pHV2 ori	Holmes and Dyall-Smith (2000)
pRV1-pTna	*bla*, *gyrB* (NovR), p*tna* promoted *bgaH*	Large et al. (2007)
pMI-1	*bla*, ColE1, *MevR*, pHV2 ori, intergenic region of ORF48 and ORF49, *bgaH*	Iro et al. (2007)
pMI-2	*bla*, ColE1, *MevR*, pHV2 ori, *rep* (ORF48), intergenic region of ORF48 and ORF49, *bgaH*	Iro et al. (2007)
pMI-2Δ	*bla*, ColE1, *MevR*, pHV2 ori, *rep* (ORF48), intergenic region of ORF48 and ORF49, AUG of *rep* deleted, *bgaH*	Iro et al. (2007)
pMI-2-EΔ	*bla*, ColE1, *MevR*, pHV2 ori, *rep* (ORF48), intergenic region of ORF48 and ORF49, *bgaH*, nu. 30 of rep changed from T to C	Iro et al. (2007)
pMI-2/43-44	pMI-2 with ORF43/44 under its native promoter	Iro et al. (2007)
pMI-2Δ/43-44	pMI-2Δ with ORF43/44 under its native promoter	Iro et al. (2007)
pMI-2/43	pMI-2 with ORF43 under its native promoter	Iro et al. (2007)
pMI-2Δ/43	pMI-2 Δ with ORF43 under its native promoter	Iro et al. (2007)

Construct	Features	Source
pMI-2/44	pMI-2 with ORF44 under promoter of ORF43	Iro et al. (2007)
pMI-2Δ/44	pMI-2 Δ with ORF44 under promoter of ORF43	Iro et al. (2007)
pRV1-ptna-ORF43	pRV1-pTna with *bgah* exchanged to ORF43	This study
pRV1-ptna-ORF43-ORF44	pRV1-pTna with *bgah* exchanged to ORF43/44	This study
pRV1-ptna-ORF44	pRV1-pTna with *bgah* exchanged to ORF44	This study
pMI-1-ptnaH-ORF43	pMI-1 with ORF43 under control of p*tna*	This study
pMI-2-ptnaH-ORF43	pMI-2 with ORF43 under control of p*tna*	This study
pMI-2Δ-ptnaH-ORF43	pMI-2Δ with ORF43 under control of p*tna*	This study
pMI-2-EΔ-ptnaH-ORF43	pMI-2-EΔ with ORF43 under control of p*tna*	This study
pMI-1-ptnaH-ORF43/44	pMI-1 with ORF43/44 under control of p*tna*	This study
pMI-2-ptnaH-ORF43/44	pMI-2 with ORF43/44 under control of p*tna*	This study
pMI-2Δ-ptnaH-ORF43/44	pMI-2Δ with ORF43/44 under control of p*tna*	This study
pMI-2-EΔ-ptnaH-ORF43/44	pMI-2-EΔ with ORF43/44 under control of p*tna*	This study
pMI-1-ptnaH-ORF44	pMI-1 with ORF44 under control of p*tna*	This study
pMI-2-ptnaH-ORF44	pMI-2 with ORF44 under control of p*tna*	This study
pMI-2Δ-ptnaH-ORF44	pMI-2Δ with ORF44 under control of p*tna*	This study

Construct	Features	Source
pMI-2-E Δ -ptnaH-ORF44	pMI-2Δ-E with ORF44 under control of p*tna*	This study
pMI-1-ORF43ΔATG	pMI-1 with ORF43 and ORF44 under control of p*tna*, ATG of ORF43 changed to CTG	This study
pMI-2Δ-ORF43ΔATG	pMI-2Δ with ORF43 and ORF44 under control of p*tna*, ATG of ORF43 changed to CTG	This study
pMI-2-EΔ-ORF43ΔATG	pMI-2Δ with ORF43 and ORF44 under control of p*tna*, ATG of ORF43 changed to CTG	This study
pMI-2-ORF44ΔATG	pMI-2Δ with ORF43 and ORF44 under control of p*tna*, ATG of ORF44 changed to CTG	This study
pMI-2Δ-ORF44ΔATG	pMI-2Δ with ORF43 and ORF44 under control of p*tna*, ATG of ORF44 changed to CTG	This study

2.1.4.2 *N. magadii* Plasmids

Construct	Features	Source
pRo-5	*bla*, ColE1 ori, *gyrB* (NovR), ΦCh1 derived ori	Mayrhofer-Iro et al. (2013)
pNB102	*bla*, ColE1 ori, *hmg* (mevR), φCh1 derived ori	Zhou et al. (2004)
pNB102-43up-bgaH	bgaH under promoter of ΦCh1 ORF43/44 operon in pNB102	Schmal (2018)
pRo-5-34up-ORF43	ORF43 under the control of the promoter region of ORF34 in pRo-5	Papek (2018)

2.1.5 Primer

Name	Sequence
HV2	GGTGTCGGTTGTAGTCGGGT
HV1	GACGACCACCGACCGAC
44-hind	CAGCAAGCTTGATTTAGGACTCGAGGACC
43-hind	CAGCAAGCTTTCATTCGCGCTCG
49-Kpn	CAGCGGTACCTTGCGTTCAGTTCCG
BgaH-3i	GAGTGAAAAACCACC CATG
44-3-Bam	GTTAGGATCCACAGAACGGACGTACGAC
44-Nde	GATCCATATGACGCTGTTCGTCGACA
43-3-B	GAATGGATCCACGAACAGCGTCATTCG
43-3-Bam	GCAGGGATCCGTGTCGACGAACAGC
Hfx-Tna	GACCGCGAGGAGACG
43-5-N	GTAGCATATGGCAATCGTCACCA
TR-1X	AATTTCTAGACCGCGTTGAAGGCAGCT
TR-2	AATTTCTAGATCCTGGGCCTCTTTGAA
34-Kpn	CAGCAGGGTACCCGGCGTTCGAGGTCA
43-hind	CAGCAAGCTTTCATTCGCGCTCG
43-5	CAGCAGTCTAGACGTTGTGCCAGCCGT
49-up-seq	AGGAGATCCGTCGAGTCCC
pMDS24-KpnI	TCCCATGCCACTCTTCACA

Sequences for restriction endonucleases are underlined

2.1.5.1 Primer for Gibson Assembly

Name	Sequence
pMDS_ORF43	AACAACCCCCCATGCCAAGCTTCTTTCATTCGCGCTCGCGCAGCTCGCGG
pMDS_ORF44	AACAACCCCCCATGCCAAGCTTCTTTTAGGACTCGAGGACCTCCTCCGGG
bgaH_3_pTnaH	GAGGTGTCGGACTCGCGTCCGAGTGTTTCCGTCGGGCCGAGTCGACTCGG
43-DATG-1	TACTACGATTGGTGACGATTGCCAGATGCGCAATAGGTCCGCGAATGTGA
43-DATG-2	TCACATTCGCGGACCTATTGCGCATCTGGCAATCGTCACCAATCGTAGTA
44-DATG-1	TGTTCGTGTCGACGAACAGCGTCAGTCGCGCTCGCGCAGCTCGCGGACCG
44-DATG-2	CGGTCCGCGAGCTGCGCGAGCGCGACTGACGCTGTTCGTCGACACGAACA

2.1.5.2 Primer for RT-PCR

Name	Sequence
RT-Bga-3C	GACGCGAGTCCGACACCT
RT-Bga-5C	GTACAACCGGCTTCACGAGTC
RT-Bga-3	GTGTCTGCTATTTCCCGGAG
RT-Bga-5	GCGTCGTTGAGGTCGG
Bga-M-N	GTGATGGAGCAACAGCCG
Bga-M-C	GACGTAGTCGGTCAGCGC
RT-44-3	GATTTAGGACTCGAGGACCTCC
RT-44-5	ATGACGCTGTTCGTCGAC
RT-34_1-5-5	ATGAGTAAAATCTGGGAACCG
RT-34_1-5-3	GTCCATTGCGGTCTGC
RT-34_1-3-5	GCACACCTACGACGACAG
RT-34_1-3-3	GCATTGGTTCAGATCAGGT
RT-43-5	GCAATCGTCACCAATCGTAGT
RT-44-5-2	CTGTTCGTCGACACGAACAT
16S-Hfx-1	GCATATAGTCCTCGCTTTTCTC
16S-Hfx-2	GCACAACACAGGCTCGTAG

2.1.6 Kits

Name	Usage	Manufacturer	Product number
Promega Wizard® SV Gel and PCR Clean-Up System	Purification of gel-eluted DNA fragments and PCR products	Promega	A9281
Wizard® Plus SV Minipreps DNA Purification System	Isolation of plasmid DNA	Promega	A1460
One Step Ahead RT-PCR Kit	RT-PCR	Qiagen	220213
Monarch Total RNA Extraction Kit	RNA Extraction	New England Biolabs	T2010S

2.1.7 Enzymes

Enzyme Name	**Manufacturer**	**Product number**
2xGoTag Green Master Mix	Promega	M7122
Phusion Flash PCR Master Mix	Thermo Scientific	F548S
Restriction enzymes	Thermo Scientific	
T4 DNA Ligase	Thermo Scientific	EL0011
Fast AP Alkaline Phosphatase	Thermo Scientific	EF0652
Proteinase K	Qiagen	Lot. No. 160020092 Mat No. 1019499
RNasin	Promega	N2511
DNase I	Thermo Scientific	EN0525

2.1.8 Size Markers

2.1.8.1 Protein Marker

Marker	**Manufacturer**	**Product Number**	**Fragments [kDa]**
PageRuler™ Prestained Protein Ladder, 10 to 180 kDa	Thermo Scientific	26617	~**10**, ~15, ~25, ~35, ~40, ~55, ~**70**, ~100, ~130, ~180
PageRuler™ Plus Prestained Protein Ladder, 10 bis 250 kDa	Thermo Scientific	26619	~**10**, ~15, ~**25**, ~35, ~55, ~**70**, ~100, ~130, ~250

2.1.8.2 DNA Marker

Marker	Manufacturer	Product Number	Fragments [bp]
GeneRuler 50 bp DNA Ladder	Thermo Scientific	SM0373	50, 100, 150, 200, **250**, 300, 400, **500**, 600, 700, 800, 900, 1000
GeneRuler 1 kb DNA Ladder	Thermo Scientific	SM0311	250, 500, 750, **1000**, 1500, 2000, 2500, **3000**, 3500, 4000, 5000, **6000**, 8000, 10000
GeneRuler 1 kb Plus DNA Ladder	Thermo Scientific	SM1331	75, 200, 300, 400, **500**, 700, 1000, **1500**, 2000, 3000, 4000, **5000**, 7000, 10000, 20000

2.1.9 Antibodies

2.1.9.1 Primary Antibodies

Antibody	Target	Application	Source
α-gp34$_{52}$ (rabbit #20)	gp34	Diluted 1:2500 in 1x TBS, 0.3% BSA, 0.02% NaN_3	Till (2011)

2.1.9.2 Secondary Antibodies

Antibody	Target	Application	Source
ECL™ Anti-Rabbit IgG, Horseradish peroxidase linked whole antibody (from donkey) Lot 17434242 NA934V	Rabbit IgG	Diluted 1:5,000 in 1x TBS	GE Healthcare

2.1.10 SDS-Polyacrylamide gel

Compound	12% Separating Gel	4% Stacking Gel
Acrylamide solution 30% (37.5:1) *	2 ml	267 µl
Separating Gel Buffer	1.25 ml	-
Stacking Gel Buffer	-	0.5 ml
dH_2O	1.75 ml	1.233 ml
Total Volume	5 ml	2 ml
APS 10 %	60 µl	20 µl
TEMED	10 µl	5 µl

*37.5:1 for Archaea samples, 29:1 for *E. coli* samples

2.1.11 Membrane

Nitrocellulose Blotting Membrane Amersham Protean 0.2 µm GE Healthcare Life Science (Catalogue Number 10600001)

2.1.12 General Buffers and Solutions

2.1.12.1 Buffers for DNA methods

50x TAE

Tris-HCl pH 8.2 ..... 2 M
acetic acid ..... 1 M
EDTA ..... 0.1 M

50x Superbuffer

NaOH ..... 0.5 M
boric acid ..... 1.82 M

6x DNA loading dye

orange G .....0.144% m/v
xylene cyanol .....0.036% m/v
Tris-HCl pH 8.0 .....0.025 M
glycerol ..... 60 %

Ethidiumbromide bath

ethidiumbromide .....10 µg/ml

2.1.12.2 Buffers for Protein methods

10x SDS-running buffer

tris base ..... 0.25 M
glycine ..... 1.92 M
SDS ..... 1 %

4x Stacking gel buffer

Tris-HCl pH 6.8 ..... 0.5 M
SDS ..... 0.4 %
autoclaved

4x Separating gel buffer

Tris-HCl pH 8.8 ..... 1.5 M
SDS ..... 0.4 %
autoclaved

10 % APS

ammonium persulphate ..... 1 g
dH_2O ad 10 ml
stored at 4°C

2x Laemmli sample buffer

Tris-HCl pH 6.8 ..... 0.12 mM
SDS ..... 2 %
glycerol ..... 17.4 %
β-mercaptoethanol ..... 2 %
bromphenolblue ..... 0.02 %

100 ml Na-Phosphate buffer pH 6.8

1 M Na_2HPO_4 ..... 46.4 ml
1 M NaH_2PO_4 ..... 53.7 ml

Coomassie staining solution

methanol ..... 40 %
acetic acid ..... 10 %
coomassie Brilliant Blue R-250 .0.25 %

Coomassie destaining solution

acetic acid ..... 10 %

Blocking solution

Milk powder ..... 5 %
In 1x TBS

10x TBS

Tris-HCl pH 8.0 .....0.25 M
NaCl .....1.37 M
KCl .....27 mM

1x Towbin transfer buffer

methanol .....20% v/v
tris base .....25 mM
glycine .....192 mM

ECL buffer

1.5 M Tris pH 8.5 ..... 13,3 ml
1 aliquot Luminol ..... 500 µl
1 aliquot Coumaric acid ..... 250 µl
ad 200 ml dH_2O
stored at 4°C, protected from light

Coumaric acid

Coumaric acid..... 0.148 g
DMSO..... 10 ml
250 µl aliquots stored at -20 °C

Luminol

3-Aminophthalthydrazide .... 0.886 g
DMSO ..... 20 ml
500 µl aliquots stored
at -20 °C protected from light

2.1.12.3 Buffers for *E. coli* methods

MOPS I

MOPS..... 100 mM
$CaCl_2$ ..... 10 mM
RbCl ..... 10 mM
pH adjusted to 7.0 with KOH

MOPS II

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

MOPS IIa

MOPS ..... 100 mM
$CaCl_2$..... 70 mM
RbCl..... 10 mM
glycerol ..... 15 %
pH adjusted to 6.5 with KOH

2.1.12.4 Buffers for *H. volcanii* methods

Sucrose Stock Solution 70 %

sucrose..... 700 g
dH_2O ad 1 L
sterilized by filtration

Buffered low salt spheroplasting solution with glycerol

NaCl ..... 1 M
KCl..... 27 mM
Tris-HCl pH 8.0..... 50 mM
glycerol ..... 15 % v/v
sucrose*..... 15 % v/v
*added after autoclaving

Unbuffered low salt spheroplasting solution (UBSS-LS)

NaCl..... 1 M
KCl ..... 27 mM
sucrose* ..... 15 % v/v
*added after autoclaving

60 % PEG 600

PEG-600 ..... 60 % v/v
UBSS-LS..... 40 % v/v

2.1.12.5 Buffers for *N. magadii* methods

Buffered high salt spheroplast solution

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

Unbuffered high salt spheroplast solution UBSS-HS

NaCl.....2 M
KCl.....27 mM
after autoclaving, 15 % sucrose (sterile filtered) are added

60 % PEG 600

PEG-600* ..... 60 % v/v
UBSS-HS ..... 40 % v/v
*PEG-600 aliquots of 600µl are stored at -80°C for a maximum duration of six months

2.1.12.6 Solutions for BgaH

BgaH buffer

NaCl ..... 2.5 M
Tris-HCl pH 7.2 ..... 50 mM
$MnCl_2$ ..... 10 µM
added after autoclaving:
β-mercaptoethanol ..... 0.1 %
added on day of use, stored at 4 °C up to two weeks

2 % Triton X-100

in H_2O
prepare fresh

ONPG for BgaH Assay

ONPG ..... 4 mg/ ml
in BgaH-Buffer
store at -20°C

X-Gal 10 mg/ml

in Dimethylformamid
(Stock solution 40 mM)
stored at 20°C

2.1.12.7 Glycerol for Cryo-Strain collection

***E. coli* glycerol**

glycerol ..... 80 % v/v
in H2O; autoclaved

***H. volcanii* glycerol**

glycerol ..... 80 % v/v
salt water..... 6 % v/v

2.1.12.8 Buffers for RNA Methods*

*all solutions for RNA were prepared with DMSO- H_2O

DMSO-H_2O

0.1 % of DEPC were added to 1 L autoclaved dH_2O followed by incubation overnight at 37°C and autoclaving again.

Tris-HCl NaCl for resuspension of Pellet

NaCl .....4 M
Tris-HCl pH 9.5.....50 mM
in DMSO-H_2O

2x RNA Loading Dye

Formamide..... 95 % v/v
Na_2EDTA ..... 0.5 mM
SDS..... 0.02 % v/v
Xylene Cylene.....0.01 % w/v
Bromphenol Blue .....0.02 % w/v

10xTBE

Tris-Base..... 108 g
boric acid..... 55 g
EDTA pH 8, 0.5 M..... 40 ml
adjust pH with boric acid to 8.0
dH_2O ad 1000 ml

2.1.12.9 Gibson Assembly Master Mix

5x ISO reaction buffer

PEG-8000 (25%w/v)..... 2.5 g
1 M Tris-HCl, pH 7.5 (500 mM)..... 5 ml
1 M $MgCl_2$ (50 mM)..... 500 µl
DL-Dithiothreitol (50 mM)..... 77 mg
100 mM dATP (1mM) ..... 100 µl
100 mM dGTP (1mM)..... 100 µl
100 mM dCTP (1mM) ..... 100 µl
100 mM dTTP (1mM) ..... 100 µl
NAD (5 mM)..... 33.2 mg
Nuclease-free water ..... ad 10 ml

self-made Gibson Assembly Master mix

5x ISO reaction buffer..... 100 µl
T5 Exonuclease [10U/µl] (NEB M0363S) ..... 0.2 µl
Phusion polymerase [2 U/µl] (NEB M0530S)... 6.25 µl
Taq DNA ligase [40 U/µl] (NEB M0208S)..... 50 µl
Nuclease-free water ..... 218.55 µl

2.2 Methods

2.2.1 DNA methods

2.2.1.1 Agarose gel electrophoresis

DNA fragments were separated in 0.8 % agarose gel with 1xTAE (big fragments >1500) or 1x Super Buffer (fragments up to 1500 bp). For RNA samples 1xTBE 1% agarose gels were used.

2.2.1.2 Staining and visualization of DNA

Agarose gels were stained with ethidium bromide (10 µg/ml in dH_2O).

2.2.1.3 Preparative PCR

Thermo Scientific Phusion Flash High-Fidelity PCR Master Mix was used for preparative PCR.

Component	**Amount [µl]**
Phusion Flash Master Mix High-Fidelity	25
dH_2O	19
Primer 1 (0.1 µg/µl)	2.5
Primer 2 (0.1 µg/µl)	2.5
DNA template	1
Total	50

Phusion Flash High-Fidelity PCR Program:

Step	**Temperature [°C]**	**Time [mm:ss]**
1 Initial Denaturation	98	03:00 or 05:00
2 Denaturation	98	00:10
3 Annealing	T_m primer - 4°C	0:07
4 Elongation	72	15 sec/ 1 kbp
5 Final Elongation	72	05:00
6 Hold	16	∞

2.2.1.4 Analytical PCR

Promega GoTag Green Maser Mix was used for analytical PCR.

Component	Amount [µl]
GoTag Master Mix [2x]	12.5
ddH_2O	8.5
Primer 1 [0.1 µg/µl]	1.5
Primer 2 [0.1 µg/µl]	1.5
DNA template	1
Total	25

GoTag Master Mix PCR Program:

Step	Temperature [°C]	Time [mm:ss]
1 Initial Denaturation	95	05:00
2 Denaturation	95	00:10
3 Annealing	T_m primer - 4°C	0:07
4 Elongation	72	1min/ 1kbp
5 Final Elongation	72	05:00
6 Hold	16	∞

2.2.1.5 PCR templates

2.2.1.5.1 *E. coli* templates

For analytical PCR of E. coli 1 µl of culture was used directly as template.

2.2.1.5.2 *N. magadii* and *H. volcanii* templates

To use *N. magadii* or *H. volcanii* as template for analytical PCR 30 µl of culture were centrifuged at 14 000 rpm for 2 min, supernatant removed, and the pellet resuspended in 100 µl dH_2O.

2.2.1.6 DNA gel elution

If preparative PCR resulted in multiple bands, the desired band was isolated from an agarose gel using the Promega Wizard® SV Gel and PCR Clean-Up System according to manufacturer's protocol and eluted in 30 µl dH_2O.

2.2.1.7 DNA restriction

Restrictions were performed at 37 °C for at least 3 hours or overnight. Restriction enzymes from Thermo Fisher were used with their recommended buffer and double digests were done with the recommended buffer from Thermo Fischer DoubleDigest Calculator.

Dephosphorylation of pMI-vectors

Component	Amount [µl]
10x reaction buffer for AP	2
FastAP Thermosensitive Alkaline Phosphatase [1 U/µl]	1
Vector DNA	1 µg
Total	20

Incubation 10 min at 37°C followed by an inactivation step at 75°C for 10 min.

2.2.1.8 DNA Ligation

Ligation was done at 4°C overnight using T4 DNA Ligase. Ligation was done with 10-fold excess of insert, and approximately similar concentrations of vector and insert.

Component	Amount [µl]
Insert	11
Vector	1
Ligase Buffer	1.5
T4 DNA Ligase	1.5
Total	15

Transformation of the whole ligation mix in *E. coli*

2.2.1.9 Gibson Assembly

To ligate one or more fragments into a vector Gibson assembly was used. Therefore, the vector was linearized, dephosphorylated and mixed with a three- to five-fold molar excess of fragments (0.5 – 1 pmol per fragment) and 10 µl of Gibson assembly master mix followed by incubation at 50°C for 15 min. *E. coli* XL-1 Blue cells were than transformed with 10 µl of the Gibson assembly mix (see section 2.2.2.2 below).

2.2.1.10 DNA Purification

2.2.1.10.1 Purification of PCR fragments

After checking preparative PCRs on 0.8 % Agarose gels, fragments were isolated using the Promega Wizard® SV Gel and PCR Clean-Up System according to manufacturer's protocol and eluted in 30 µl dH2O.

2.2.1.10.2 Isolation of Plasmid DNA

Plasmids were isolated from *E. coli* overnight cultures using Wizard® Plus SV Minipreps DNA Purification System according to the manufactures protocol.

The solutions from the kit were also used for Plasmid isolation from *H. volcanii.* Therefore 3 ml of culture were pelleted 4 min at 14 krpm and resuspended in 50 µl 1 M NaCl, 200 µl lysis solution added, mixed by inversion, and left for 5 minutes at RT. 150 µl neutralization solution were added, mixed by inversion, and centrifuged 5 minutes at 14 krpm. Supernatant was transferred to a fresh reaction tube, precipitated with 1:1 Isopropanol, incubated for 2 minutes at RT and centrifuged 30 minutes at 4°C and 10 krmp. After removing the supernatant, the white pellet was resuspended in 20 µl dH_2O. Plasmids isolated from *H. volcanii* were then transformed in *E. coli* for plasmid isolation and sequencing.

2.2.2 Generation of competent cells and transformation procedures

2.2.2.1 **Generation of competent *E. coli***

Chemical competent *E. coli* were produced by inoculation of 200 ml LB medium with tetracycline from an overnight preculture to an OD_{600} (Optical density at 600 nm wavelength) of 0.1. Incubation of the culture at 37°C with agitation until an OD_{600} of 0.6-1.0 was reached. The cells were harvested by centrifugation at 10 krpm for 10 minutes at 4 °C and afterwards the pellet was resuspended in 80 ml MOPS I and 10 minutes incubated on ice. Second centrifugation step followed by resuspension of the pellet in 80 ml MOPS II and 30-minute incubation on ice. After carrying out the third centrifugation step the pellet was resuspended 4 ml MOPS IIa and aliquoted in 100 µl to store at -80 °C.

2.2.2.2 Transformation of *E. coli* competent cells and screening

After thawing the competent cell aliquot, DNA was added (15 µl ligation, 10 µl Gibson assembly, or up to 1 µg plasmid) and incubated on ice for 30 min followed by a heat shock at 42°C for two minutes and immediately cooled on ice. Then adding 300 µl LB-medium (without selection) and regenerating cells for 20-30 min at 37 °C. Cells were plated on selective medium and incubated at 37 °C overnight.

Single colonies were inoculated in 5 ml selective medium, incubated at 37 °C with agitation and directly used as template for screening by analytical PCR. Plasmid DNA from positive clones was isolated and sent for sequencing (Microsynth).

2.2.2.3 *E. coli* glycerol stock

For longtime storage at -80 °C 1 ml culture with an OD_{600} of 0.6-0.8 were mixed with 0.8 ml 50 % glycerol in a cryogenic vial (1.8 ml skirted cryovial with internal thread, silicone seal cap, star lab) and put in the -80 °C freezer.

2.2.2.4 Transformation of *H. volcanii*

Generation of H. volcanii *competent cells*

H. volcanii overnight culture was inoculated in 50 ml MGM^+ until OD_{700} of 0.8 to 1 was reached. Cells were harvested by centrifugation at 6 krpm for 15 min at RT, followed by resuspension in 10 ml buffered low salt spheroplasting solution. Cell suspension was subsequently centrifuged at 6 krpm for 10 min at RT and the resulting pellet was resuspended in 5 ml buffered low salt spheroplasting solution with 15% glycerol. 150 µl aliquots were stored at -80 °C.

Transformation of H. volcanii *competent cells and screening*

All steps for the PEG-600 mediated transformation of *H. volcanii* are performed at room temperature. To 150 µl competent *H. volcanii* 15 µl of 0.5 M EDTA solution were added and incubated 10 minutes. Then 1-3 µg of DNA in a maximum volume of 10 µl were added and incubated 5 minutes.

150 µl of 60 % PEG-600 (with unbuffered low-salt spheroplasting solution) were added to the transformation and incubated for 30 minutes. Subsequently the cells were washed twice with 1 ml of Media and centrifugation at 10 krpm 5 minutes to remove PEG-600.

The pellet was resuspended in 1 ml of media and plated on selective media; regeneration of the cells is not mandatory. Plates were dried over night at RT, followed by incubation at 42 °C until colonies appear after 4-5 for rich media (YPC and MGM) or 7+ days for minimal media (H.v.-Min) plates.

H. volcanii *storage and glycerol stock*

H. volcanii clones were stored in falcon tubes on the bench and four aliquots at -80 °C by mixing 1.3 ml culture with 0.5 ml 80 % Glycerol 6 % SW in a cryotube.

2.2.2.5 Transformation of *N. magadii*.

Generation of competent N. magadii *cells*

To generate competent *N. magadii* cells first a preculture in 25 ml NVM CAA^+ was grown in a baffled flask at 42 °C with agitation to an OD >1, of which 4 and 6 ml were used to inoculate 50 ml of NVM-CAA^+ containing bacitracin (70 µg/ml) in a baffled flask at 37 °C with agitation until OD_{600} of 0.6 to 1 was reached.

Cells were harvested by centrifugation at 6 krpm for 15 min at RT, followed by resuspension in 25 ml buffered high-salt spheroplasting solution (half volume of the bacitracin culture) and addition of proteinase K to a final concentration of 0.1 % v/v. Cell suspension was incubated for 2 days at 42 °C with agitation approximately 48 hours until the cells lose the S-layer reflected in the formation of spheroplasts which was observed under the microscope. The competent cells were used and stored on the bench for up to one week.

PEG-600 based Transformation of N. magadii *cells*

1.5 ml of competent cells were centrifuged 3 minutes at 10 krpm RT and the pellet resuspended in 150 µl high salt buffered spheroplast solution. DNA of interest was added (1-3 µg; maximum 10 µl) and incubated 5 minutes at RT. 150 µl of 60 % PEG-600 (in HS unbuffered spheroplasting solution) were added to the transformation mix and incubated 20 to 30 minutes. Afterwards the cell mix was washed twice with 1 ml NVM CAA^+ (10 krpm, 5 minutes, RT) to remove PEG-600 residues, then the final pellet was resuspended in 1 ml NVM CAA^+ and incubated at 37 °C with agitation until cells regenerated their original rod shaped morphology, which takes about two to three days. Finally, 100 µl of the cell suspension (undiluted, 1:10 and 1:100 dilution) was plated on NVM CAA^+ agar plates with antibiotics and incubated at 42 °C in sealed plastic bags to prevent desiccation of the plates.

Single colonies were inoculated in 500 µl NVM CAA^+ with antibiotics and incubated at 37 °C with agitation followed by screening with analytical PCR (see 2.2.1.4).

2.2.3 Protein Methods

2.2.3.1 Crude extract protein samples for SDS-PAGE

Protein crude extract samples of *H. volcanii* were taken daily for the duration of one week. 1.5 ml of culture were collected by centrifugation at 13 0000 rpm for 3 min, supernatant removed and quick spin to remove the excess supernatant. To the pellet OD_{700} x 0.75 = µl 5 mM Na-PO_4 buffer (pH 6.8) was added, and the pellet scraped from the reaction tube. Same amount of 2x Laemmli buffer was added. To dissolve the pellet, it was incubated at 37 °C overnight. Protein crude extract samples were stored at -20 °C. Denaturation at 95 °C for 10 min prior to loading the SDS-Gel. Same procedure for *N. magadii* protein crude extract samples, except OD_{600} is used.

2.2.3.2 SDS-PAGE

2.2.3.2.1 SDS-Gel

Discontinuous SDS-Gels with 12 % separating gel and 4 % stacking gel were prepared in a BioRad Mini Protean apparatus with 0.75 mm spacers.
Gels were run covered in 1xSDS-Buffer either in Mini-PROTEAN Tetra Vertical Electrophoresis Cell or if more than four gels at once Mini-PROTEAN® 3 Dodeca™ Cell. *H. volcanii* samples were separated at 40 V and voltage increased to 50 V after entering the separating gel until the bromphenol blue left the gel.

2.2.3.2.2 Coomassie Gel

Coomassie gel was done to determine the sample concentration and equalize for the Western Blot later, therefore 5 µl of Protein Ladder and 10 µl of samples were loaded.
Staining the gel for 5 to 10 min with Coomassie stain, then decolorizing the gel with destain solution until proper destaining.

2.2.3.2.3 Western Blot

SDS-Page

For the Western Blot 8 µl of protein ladder and 7-14 µl of sample as determined with the Coomassie gel were loaded according to the coomassie stain gel. As positive control *N. magadii* L11 (8 µl) and as negative control *H. volcanii* pMDS24 (10 µl) were loaded on each gel.

Blotting, Blocking and Antibodies

Semi-dry blotting with Towbin transfer buffer 83 V for 20 min (2 gels) or 35 min (4 gels). Therefore, membrane and Whatman filter paper were soaked in transfer buffer and stacked the following way, from the anode at the bottom to the cathode on top: three Whatman paper, membrane, gel, three Whatman paper. Slightly rolled over the sandwich with a tube to remove air and excess and dab off any excess liquid. After the transfer, the marker lines on the membrane were marked with an indelible pen, for easier visualisation later.

The membrane was blocked overnight with 100 ml of blocking solution at 4 °C, or for longer with additional 0.02 % NaN_3, with agitation.

Then the membrane was washed three times for ten minutes with 1xTBS, followed by incubation with 10 ml of primary antibody for one hour at room temperature and subsequently washing with 1xTBS. For the secondary antibody 10 ml 1xTBS and 2 µl of α-Anti-Rabbit coupled with Horseradish peroxidase were added to the membrane and incubated at room temperature for 1 h, after that again washing with 1x TBS.

Detection

Right before detection 3 ml ECL and 2 µl H_2O_2 were added directly to the membrane, followed by tilting to cover the whole membrane evenly. Excess liquid was removed, the membrane placed it in the BioRad Chemidoc and detected with setting High Sensitivity Chemiluminescence, 5-180 sec exposure, 30 pictures. Also a colorimetric picture was taken to merge with and visualise the marker.

Membrane Stripping

For detection with multiple primary antibodies membrane was stripped by adding 10 ml of Roti® Free stripping buffer, incubation for 30 min at 37°C with agitation followed by thoroughly washing with 1xTBS at least 5 times for 10 min and subsequently re-blocking.

2.2.4 RNA Methods

All solutions were prepared with DEPC-H_2O, tips and microtubes were autoclaved twice, or nuclease-free filter tips were used.

2.2.4.1 RNA sampling and preparation for RT-PCR

1 ml of culture was pelleted at 13 krpm for 5 minutes and resuspend in 500 µl of 4 M NaCl 50mM Tris-Cl pH 9.5. To the resuspended cells 500 µl TRIzol reagent were added and vortexed thoroughly 1 minute and continued with RNA-Isolation or storage at -80 °C.

2.2.4.2 RNA-Isolation (Phenol/Chloroform extraction)

All centrifugation steps were performed at 4 °C and 12000 g.

TRIzol samples from -80 °C were thawed on ice and vortexed again. Added 100 µl chloroform and shaked well for 15 sec. Incubated 3-5 min on RT to let the phases settle. After centrifugation for 15 min the upper aqueous phase was transferred to a new tube without interrupting the DNA containing white interphase. Then repeated the chloroform step to remove phenol residues. Equal amounts of Isopropanol were added to the aqueous phase for precipitation of RNA, followed by incubated for 10 min at room temperature or storage at -20 °C or -80 °C temporary. Then centrifugation for 15 min and removal of the supernatant. Because of the high salt solution used for resuspending the pellet at the beginning at this step the pellet was white and porous instead of translucent and gel-like. The pellet was washed with 500 µl of 70 % ethanol, by pipetting up and down thoroughly followed by centrifugation for 15 min and removing the supernatant carefully. Subsequently drying the pellet at RT or 5 min at 65°C followed by resuspending it in 50 µl of DEPC-H_2O.

2.2.4.3 RNA Isolation (Monarch Total RNA extraction Kit)

Extraction was performed as the manufacturer protocol states except the following changes:

Sample Preparation:

To pre-extracted samples with phenol/chloroform extraction method double amount of Lysis Buffer was added.

To TRIzol extracted samples after centrifugation and transfer of the aqueous phase double amount of lysis buffer was added.

Continued with part 2 RNA binding and elution.

Washing step before on-column DNase I treatment was performed twice to remove excess salt from the column.

DNase I treatment:

On-column DNase I treatment for enzymatic removal or residual gDNA was performed.

Before adding DNaseI and after the last washing step before elution an extra dry spin for 30 seconds was included.

Elution from column was performed by adding 60 µl nuclease free water, incubating 5 minutes at room temperature, and eluate was reapplied to the column.

2.2.4.4 RNA Quality control

For loading on Agarose gels 5 µl RNA isolate were mixed with 10 µl 2x RNA loading dye denatured 3 min at 90 °C, cooled on ice and then loaded. Isolated RNA was loaded on a 0.8 % agarose gel (1xTBE).

2.2.4.5 DNase I treatment

RNA samples were treated with DNase I as seen in Table 1 and incubated over night at 37 °C followed by phenol/chloroform extraction (see 2.2.4.2)

Table 1 DNase I treatment of RNA samples

Component	**Amount [µl]**	**Final concentration**
RNA sample	4 µg	
DNase I [1 U/µl]	10	0.1 U/µl
10x DNase Buffer	10	1x
RNasin [40 U/µl]	2.5	1 U/µl
DEPC-H_2O	add 100	

2.2.4.6 RT-PCR

To analyze gene expression and cleavage of mRNA an RT-PCR was performed.

For one RT-PCR reaction

Component	**Amount [µl]**	**Final concentration**
One Step Ahead RT-PCR MM 2.5x	10	1x
One Step Ahead RT-MM 25x	1	1x
primer A	1.5	0.5 µM
primer B	1.5	0.5 µM
RNase-free H_2O	10	Ad 25µl
template	1	0.1pg-1µg per reaction
total volume	25	

RT-PCR program

Step	Temperature [°C]	Time [mm:ss]
1 Reverse Transcription	50	10:00
2 Initial PCR activation	95	05:00
3 Denaturation	95	00:10
4 Annealing	T_m - 4	00:10
5 Extension	72	00:10
6 Final Extension	72	02:00
7 Stop	4	infinite

2.2.5 BgaH Assay

2.2.5.1 BgaH samples

N. magadii BgaH samples

50 ml NVM CAA^+ were inoculated from preculture to an OD_{600} of 0.1 and inoculated at 37 °C with agitation. Samples were taken every 24 h for 7 days directly from the culture (0.5 ml) and stored at room temperature.

H. volcanii BgaH samples

100 ml 18% MGM were inoculated with *H. volcanii* to an OD_{700} of 0.1. Every 24 h 1 ml of culture was centrifuged at 8 krpm for 5 minutes, the pellet resuspended in 500 µl BgaH buffer and at least two aliquots stored at -80 °C.

2.2.5.2 BgaH-assay using a 96 well plate

Measurements were performed in at least duplicates. To a 96 well plate (flat bottom) solutions were put in the following order: 20 µl BgaH-sample, 20 µl 2xTriton X-100, 120 µl BgaH buffer and 40 µl 4 mg/ml ONPG to start the assay.

Measurement was performed in a Synergy H1 plate reader starting with 10 minutes shake double orbital (Frequency: 237 cpm (4 mm)) followed by a kinetic read at 405 nm wavelength for 60 minutes in 1-minute intervals.

Analysis was done in excel, calculating SLOPE and RSQ, BgaH activity was calculated only from samples with RSQ above 0.8 with the formula depicted in Figure 2.

$$BgaH\ activity = \frac{1000 * \mathrm{SLOPE}}{0{,}01 * x * OD}$$

x... *factor of sample concentration*

OD... Optical Density

Figure 2 ***Formula for the calculation of BgaH activity*** *based on the Optical Density*

2.2.5.3 Plate reader OD measurement

OD measurement in the Synergy H1 plate reader 50 µl culture were mixed with 150µl medium and three times OD measurement with double orbital shake for 10 seconds in between.

2.2.6 Spot assay for qualitative BgaH activity analysis using X-Gal

5 µl *H. volcanii* containing the different constructs were spotted on different medium plates (YPC, H.v.-Min) containing various amounts of tryptophane (Trp), incubated at 42°C until spots are visible followed by spraying with 10mg/ml X-Gal in dimethylformamid (DMF).

2.3 Cloning Strategy

2.3.1 Cloning of ORF43, ORF43/44 and ORF44 under the control of inducible promoter p*tna* in pMI-*

First, *H. volcanii* was transformed with the following plasmids constructed by Iro et al. (2007), pMI-2/44, pMI-2Δ/44, pMI-2/43-44, pMI-2Δ/43-44, pMI-2/43, pMI-2Δ/43 and as a control plasmid pMLH32. Plasmid pMI-2 which contains *rep* and the intergenic region, and pMI-2Δ which contains *rep* with AUG deleted and the intergenic region. Both contain the different variants of ORF43 and ORF44 together under its native promoter as well as both ORFs by themselves.
The resulting clones were screened with primer BgaH-3i and 49-Kpn and clones with plasmid pMLH32 were screened with primer HV2 and HV1.
Even though >500 clones were tested it was not possible to obtain clones containing plasmid pMI-2/44 pMI-2Δ/44, which contain the putative toxin ORF44

under its native promoter, presumably because of its toxic effect and there for making the cells non-viable.

This led to the change of strategy and cloning the same constructs with the inducible promoter p*tna*.

To link the genes of interest (ORF43 and ORF44) with the inducible promoter p*tna* from *Haloferax*, they were subcloned into the plasmid pRV1-p*tna* which was restricted with *Bam*HI and *Nde*I to remove the *bgaH* sequence and linearize the plasmid. The insert was amplified with Phusion High fidelity polymerase using φCh1 as a template, adding restriction sites on both sites, an *Nde*I on the 5'-end and *Bam*HI at the 3'-end, using the primers listed in Table 2.

Table 2 Primers used for Phusion PCR to amplify fragments for ligation in pRV1-ptna

Insert	**Primer A**	**Primer B**	**Size [bp]**
ORF43	43-5-N	43-3-Bam	176
ORF43-44	43-5-N	44-3-Bam	592
ORF44	44-Nde	44-3-Bam	430

Subsequently the amplicon was isolated from agarose gel and restricted with *Nde*I and *Bam*HI followed by a PCR clean up. Then the linearized pRV1-p*tna* vector was ligated with the insert, respectively, resulting in the constructs pRV1-p*tna*-ORF43, pRV1-p*tna*-ORF43-44 and pRV1-p*tna*-ORF44. Competent *E. coli* XL-1 Blue were transformed with the ligation mix and resulting clones were screened with primer listed in Table 3 followed by sequencing of positive clones with primer Hfx-Tna.

Table 3 Primers used for screening of the pRV1-ptna-ORF43/44 constructs and their corresponding fragment size

Construct	**Primer A**	**Primer B**	**Size [bp]**
pRV1-p*tna*-ORF43	Hfx-Tna	43-3-Bam	~550
pRV1-p*tna*-ORF43-44	Hfx-Tna	44-3-Bam	~600
pRV1-p*tna*-ORF44	Hfx-Tna	44-3-Bam	823

In the next step the genes of interest linked with the p*tna* were inserted into four different pMI vectors (pMI-1, pMI-2, pMI-2Δ and pMI-2EΔ, abbreviated as pMI-*) via Gibson Assembly. The vectors were restricted with *Xba*I and dephosphorylated. The inserts for the Gibson assembly were amplified using the template and primers listed in Table 4, for the ΔATG constructs (ATG mutation to CTG) two fragments were amplified and assembled. The Gibson assembly was performed using a fivefold excess of insert, and afterwards transformed in *E. coli* XL-1 Blue and resulting clones were screened using the primer pairs listed in Table 5, lastly the plasmid was isolated from positive clones and transformed in *H. volcanii* and screened with the same primers.

Table 4 Primers used for the amplification of the ptna-ORF43/44 fragments for Gibson Assembly and their corresponding fragment size [bp]

Fragment	**Template**	**Primer A**	**Primer B**	**Size [bp]**
p*tna*-ORF43	pRV1-p*tna*-ORF43	bgaH_3_pTnaH	pMDS_ORF43	550
p*tna*-ORF43/44	pRV1-p*tna*-ORF43/44	bgaH_3_pTnaH	pMDS_ORF44	942
p*tna*-ORF44	pRV1-p*tna*-ORF44	bgaH_3_pTnaH	pMDS_ORF44	831
ORF43 dATG-pMDS	pRV1-p*tna*-ORF43/44	43-DATG-2	pMDS_ORF44	582
*tna*H-ORF43-dATG	pRV1-p*tna*-ORF43/44	bgaH_3_pTnaH	43-DATG-1	410
ORF44 dATG-pMDS	pRV1-ptna-ORF43/44	44-DATG-2	pMDS_ORF44	421
*tna*H-ORF44-dATG	pRV1-ptna-ORF43/44	bgaH_3_pTnaH	44-DATG-1	571

Table 5 Primers used for screening of the pMI--ptna-ORF43/44 clones*

Template	**Primer A**	**Primer B**	**Size [bp]**
pMI-*-p*tna*-ORF43	Hfx-Tna	43-hind	~550
pMI-*-p*tna*-ORF43/44	Hfx-Tna	44-hind	~600
pMI-*-p*tna*-ORF44	Hfx-Tna	44-hind	831

2.4 Influence of ORF43 and ORF44 on gp34

H. volcanii was transformed with the plasmids, pMDS24, pMDS24-34$_1$, pMDS24-34$_1$-p43-ORF43, pMDS24-34$_1$-p43-ORF43/44 and pMDS24-34$_1$-p43-ORF44 constructed by Athanasiou 2020.

Clones were screened using primer 34-Xba and 34-NcoI which targets ORF34 which is present in all plasmids (size 442 bp, annealing temperature 58°C), except pMDS24 which was tested with primer HV1 and HV2 (fragment size 628 bp) which bind to the ori of pMDS24.

2.5 Autoregulation of promoter ORF43

N. magadii (pRo-5) and *N. magadii* (pRo-5-p34-ORF43) were inoculated from the bench culture to prepare competent cells (see 2.2.2.5) and transform them with plasmid pNB102-p43-BgaH. Clones were selected using NVM CAA$^+$ medium with Novobiocin (3 µg/ml) and Mevinolin (4 µg/ml).

The resulting clones *N. magadii* (pRo-5/pNB102-p43-BgaH) and *N. magadii* (pRo-5-p34-ORF43/pNB102-p43-BgaH) were screened using the primer pairs listed in Table 6.

Table 6 Screening of the clones N. magadii (pRo-5/pNB102-p43-BgaH) and N. magadii (pRo-5-p34-ORF43/pNB102-p43-BgaH)

Primer A	**Primer B**	**Binding Site**	**Fragment Size**
BgaH-M-N	BgaH-M-C	BgaH internal	492 bp
34-Kpn	43-Hind	34up-ORF43	~600bp
TR-2	TR-1X	Ori of pRo-5	535 bp

Positive clones were used for growth curve in NVM CAA$^+$ Nov$^+$ Mev$^+$ and taking BgaH samples (see 2.2.5.1)

3. Results and Discussion

3.1 Autoregulation of promoter ORF43

The Hypothesis of this experiment was based on the master's thesis of Schmal (2018), where the autoregulation of ORF43 was analyzed by cloning the reporter gene bgaH under the control of the promoter from ORF43 (pNB102-p43-bgaH) and subcloning a vector with ORF43 under its own promoter (pRo-5-p43-ORF43) in *N. magadii*. Due to equivocal results the experiment was repeated with subcloning of ORF43 under control of the constitutive promoter 34up of ORF34 as seen in Figure 3. As control the empty pRo-5 vector was used with the proper pNB102 subcloned.

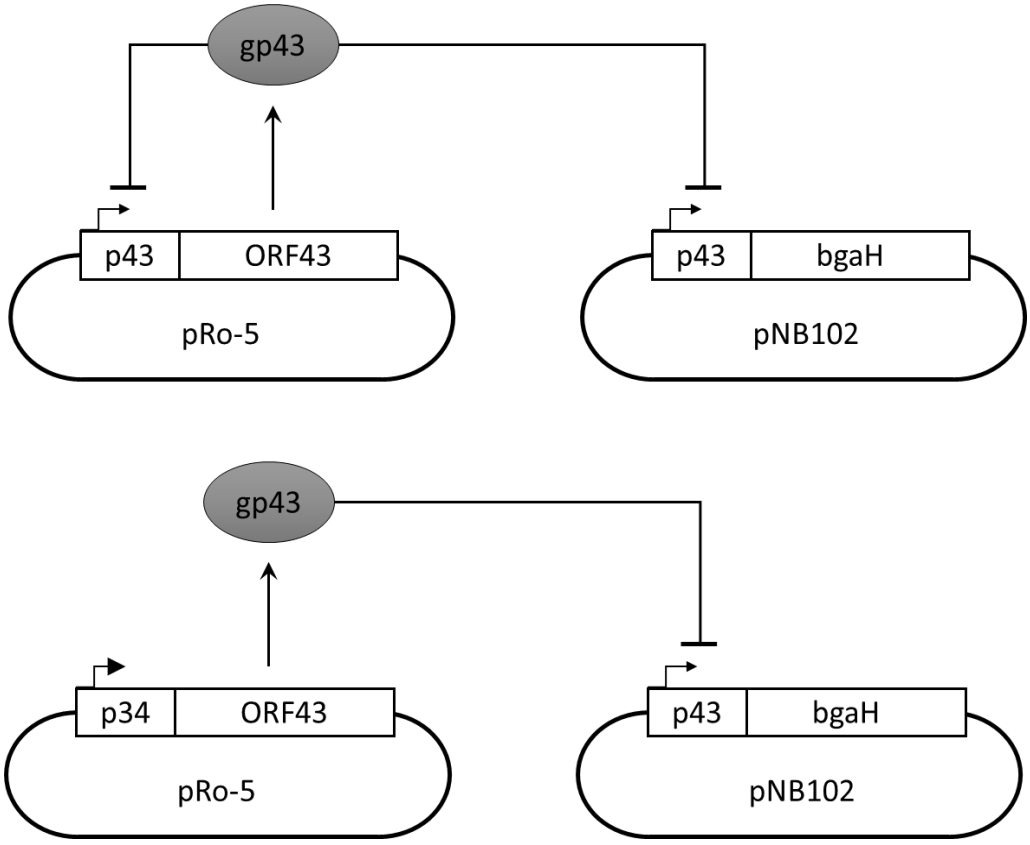

Figure 3 ***Schematic hypothesis of plasmids used for the identification of autoregulation of ORF43*** *and the potential regulatory effect of ORF43.Top: ORF43 and reporter gene bgaH under the control of p43 as performed by Schmal (2018). Bottom: ORF43 under control of the constitutive promoter of ORF34 (34up) and reporter gene bgaH under control of p43.*

For *N. magadii* (pRo-5, pNB102-p43-bgaH) and *N. magadii* (pRo-5-34up-ORF43, pNB102-p43-bgaH) three clones each were analyzed over the time of four days (96h), Every 24 h OD_{600} was measured and samples for BgaH activity measurements were taken. No difference in growth behavior was observed (see Figure 4A). Stationary phase is starting from day 3 (72 h).

BgaH activity of the different days and clones can be seen in Figure 4B, where the BgaH activity generally seems lowest after 48h, during the exponential phase, and increases over the next days. On day 1 (24 h) the activity is similar to day 3 (72 h), which could be because of carryover from the preculture.

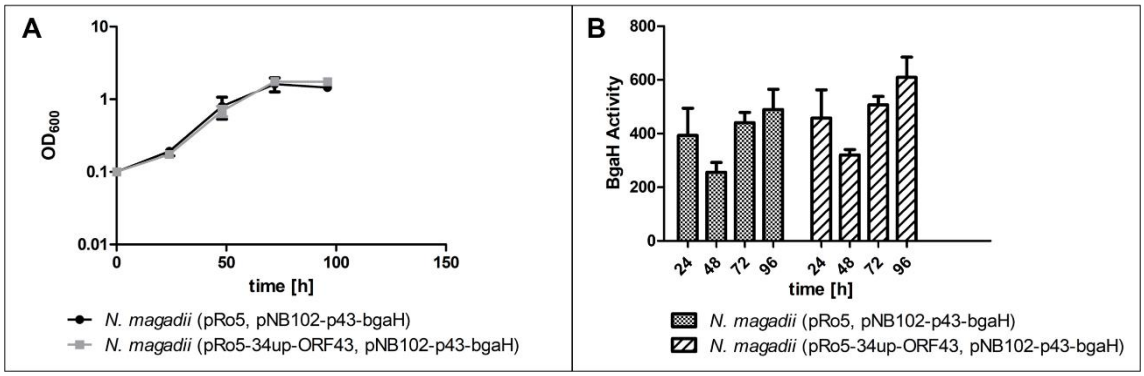

Figure 4 A: ***Growth curve*** *(OD_{600}) of* N. magadii *(pRo-5, pNB102-p43-bgaH) and* N. magadii *(pRo-5-34up-ORF43, pNB102-p43-bgaH) B:* ***BgaH activity*** *of* N. magadii *(pRo-5, pNB102-p43-bgaH) and* N. magadii *(pRo-5-34up-ORF43, pNB102-p43-bgaH). Median ± SD*

Comparing the two different strains, the activity is quite similar, indicating that there is no autoregulation of ORF43 via p43. Otherwise, *N. magadii* (pRo-5-34up-ORF43; pNB102-p43-bgaH) should have lower BgaH activity.

Usually type II TA systems are transcriptionally autoregulated. Molar excess of antitoxin (in this case ORF43) should lead to a transcriptional repression, and molar excess of the toxin (ORF44) should lead to transcriptional activation (Jurenas et al. 2022). But there are, as always, exceptions, for example there are noncanonical type II TA systems where the antitoxin is located downstream of the toxin gene, there are three-component type II TA systems, where a third upstream encoded gene is responsible for the regulation. As well as “incomplete” TA systems which lack an antitoxin DNA-binding domain or regulator (Loris & Garcia-Pino, 2014). For the antitoxin ORF43 no known domain has been

identified, so we do not know if a DNA binding site is present. And it could be possible that a third gene (host or phage encoded) is involved in the regulation of ORF43/44. Furter investigation into how the ORF34/44 TA operon is regulated is needed.

3.2 ORF43/44 under control of ptnaH

Construction of the pMI-*constructs with ORF43/44 under control of the ptnaH promoter to investigate the enhancing effect on the intergenic region of ORF48 and ORF49 was successful as far as the PCR and sequencing results indicate. Testing BgaH activity and inducing ptnaH turned out to be rather challenging. Adopting the medium to a higher buffer capacity with increased Tris (30 mM) for the induction with tryptophane in 1 N NaOH only helped partially. It prevented precipitation of the culture, which happened after inducing multiple times. Testing different storage buffers and temperatures for the BgaH samples was also tried for improving the BgaH activity measurement. Because of the inability to measure the qualitative BgaH activity for most clones a screening with X-gal was performed.

3.2.1 Screening of the ptnaH constructs (X-gal spot assay)

H. volcanii clones containing the constructed pMI-* plasmids were spotted on minimal media and complex media containing different amounts of tryptophane to qualitatively analyze the BgaH activity (Figure 5). Two clones of each construct were tested and one of the plasmids without insert as control. As seen in Table 7 the plasmids without inserts (pMI-1, pMI-2, pMI-2Δ and pMI-2EΔ) all turned dark blue, which indicates BgaH activity. All other constructs did not turn blue and therefore have no BgaH activity.

As example spot assay plates with vectors pMI-1 and pMI-2EΔ can be seen in Figure 5. Constructs pMI-1 and pMI-2EΔ containing ORF44 did not grow at all (except one pMI-2EΔ clone showed very limited growth) on H.v.-Min 4 mM Trp plates. It indicates that the amount of tryptophane was sufficient to induced ORF44 to an amount that inhibits/prevents growth. And minimal medium with

0.25 mM tryptophane and YPC was not sufficient to properly induce ORF44. The putative toxin ORF44 was cloned under control of the inducible promoter ptnaH because it was not possible to obtain clones with constructs containing ORF44 under its own promoter even after testing over 500 clones.

Growth behavior generally varies between the different media. Colonies on YPC are rather thin and pale pink compared to H.v.-Min where they are colorful bright grapefruit colored and raised. On H.v.-Min 4 mM Trp they are smaller and almost of brownish red color.

The question is, why was growth not inhibited in the pMI-2-ptnaH-ORF44 where *rep* is present and pMI-2Δ-ptnaH-ORF44 where *rep* has a mutated start codon under the same induction conditions (4 mM trp)? In pMI-1-ptnaH-ORF44 *rep* is not present at all and in pMI-2-E∆-ptnaH-ORF44 *rep* is present but with a silent mutation of a repeat. So why is ORF44 not able to act putatively toxic in the first two instances while the contrary is true in the second two? Could it be that the presence of *rep* leads to a repression of ORF44 and in its absence ORF44 is able to interfere with cell growth? But there is even growth in the strain containing the constructs with the start codon mutation *rep,* which would mean that the presence of the *rep* sequence alone interferes with the expression of ORF44. Or the repeat from *rep* is the interfering entity, because in the construct with a mutation in the repeat sequence of *rep* (pMI-2EΔ-ptnaH-ORF44) the lack of growth indicates that ORF44 is expressed.

Besides that, it is also unclear why the constructs with the ptnaH promoter have no BgaH activity. One approach to that problem could be to use a different reporter gene (e.g., ORF34).

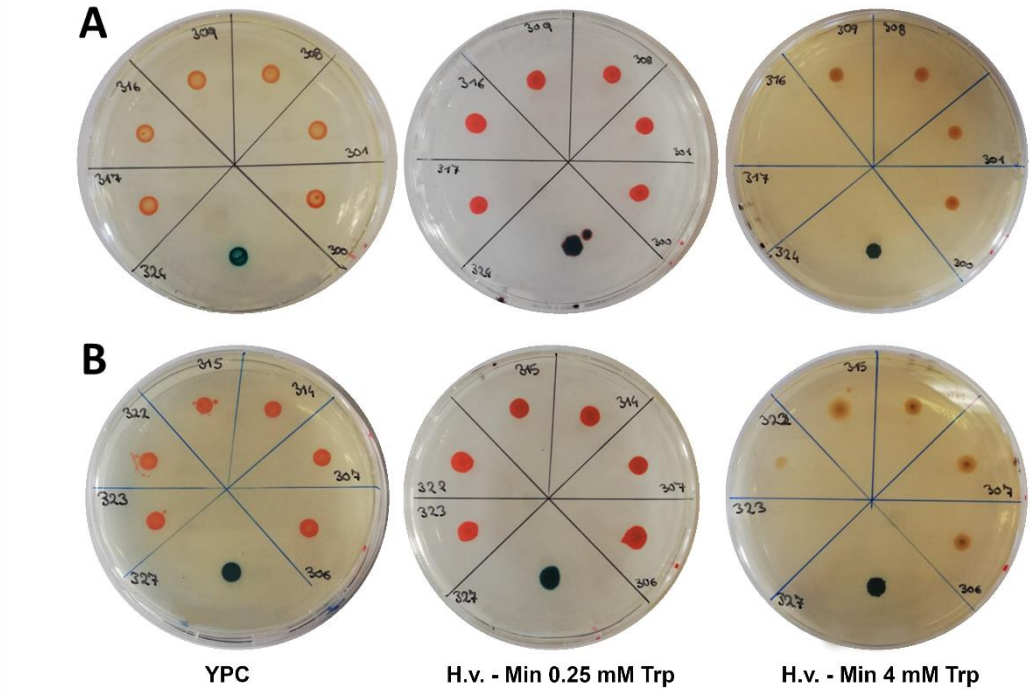

Figure 5 ***Qualitative BgaH activity screening of*** **H. volcanii** ***transformants*** *sprayed with X-Gal. Transformants containing pMI constructs with ORF43 and/or ORF44 under control of ptnaH promoter spotted on different media plates (left to right: YPC, H.v.-Min 0.25 mM Trp, H.v.-Min 4 mM Trp). Row A: clockwise starting from the bottom pMI1 "324" and two clones each pMI-1-ptnaH-ORF43 "317" "316", pMI-1-ptnaH-ORF43/44 "309" "308" and pMI-1-ptnaH-ORF44 "301" "300" Row B: clockwise starting from the bottom pMI-2ΔE "327" and two clones each pMI-2ΔE-ptnaH-ORF43 "323" "322", pMI-2ΔE-ptnaH-ORF43/44 "315" "314" pMI-2ΔE-ptnaH-ORF44 "307" "306"*

Table 7 Qualitative BgaH activity of H. volcanii clones containing vector pMI with ORF43 and/or ORF44 under control of the ptnaH promoter on minimal medium and complex medium plates with tryptophane (Trp) concentrations of 0, 0.25 and 4 mM. (–) *no BgaH activity,* (+) *BgaH activity (blue colony).*

***H. volcanii* with plasmid:**	**H.v.-Min 0.25 mM Trp**	**H.v.-Min 4 mM Trp**	**YPC**	**YPC 0.25 mM Trp**
pMI-1-ptnaH-ORF43	-	-	-	-
pMI-1-ptnaH-ORF43/44	-	-	-	-
pMI-1-ptnaH-ORF44	-	no growth	-	-
pMI-1	+	+	+	+
pMI-2-ptnaH-ORF43	-	-	-	-
pMI-2-ptnaH-ORF43/44	-	-	-	-
pMI-2-ptnaH-ORF44	-	-	-	-
pMI-2	+	+	+	+
pMI-2Δ-ptnaH-ORF43	-	-	-	-
pMI-2Δ-ptnaH-ORF43/44	-	-	-	-
pMI-2Δ-ptnaH-ORF44	-	-	-	-
pMI-2Δ	+	+	+	+
pMI-2EΔ-ptnaH-ORF43	-	-	-	-
pMI-2EΔ-ptnaH-ORF43/44	-	-	-	-
pMI-2EΔ-ptnaH-ORF44	-	little/no growth	-	-
pMI-2EΔ	+	+	+	+

3.2.2 Sequencing of the plasmid

Due to the absence of activity from all transformants with pMI-* and insert, two were isolated from *E. coli* sequenced (pMI-2Δ-ptnaH-ORF43 and pMI-2-ptnaH-ORF43/44) with Primer pMDS24-KpnI, RT-Bga-5c and RT-Bga-3 49-up-seq, to screen for the presence of the *bgaH* gene and ORF48 (see Figure 6). All sequencing results from both plasmids were alright and the orientation of ptna-ORF43 was checked as well with primer RT-Bga-3 and 43-Hind (binding 3'-end of ORF43), showing no alterations or mutations.

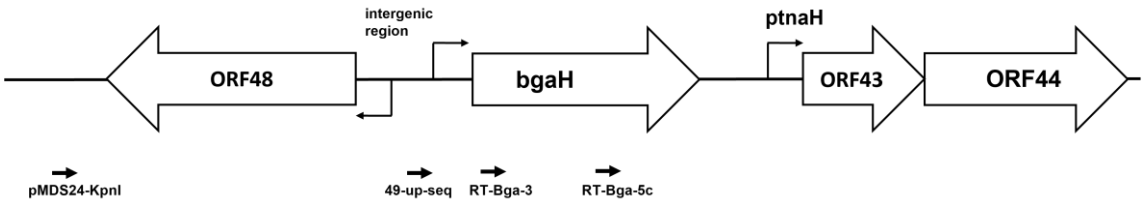

Figure 6 ***Schematic diagram of the pMI-construct*** *and positions of the used sequencing primers.*

3.2.3 BgaH activity of the *H. volcanii* with pMI-* vectors

For the pMI-* clones, growth curves over the course of 7 days were performed and samples were taken every day. Due to the absent BgaH activity different storage options for samples of *H. volcanii* were tested, and the best results with least background noise were achieved with samples stored in BgaH buffer at -80 °C. Storing samples in medium taken direct from the culture is also possible at 4 °C but the H.v.-Min medium can lead to a lot of background noise if there is not much activity, which can lead to false positive results. Still, as long as activity was there, there was no problem with background noise for the measurement.

In the growth curve of *H. volcanii*, all strains can be seen in Figure 7 for the ones with BgaH activity and Figure 8 for the start codon deletion (ΔATG) constructs which all had no detectible BgaH activity. All strains had similar growth, reaching the stationary phase after ~48 h.

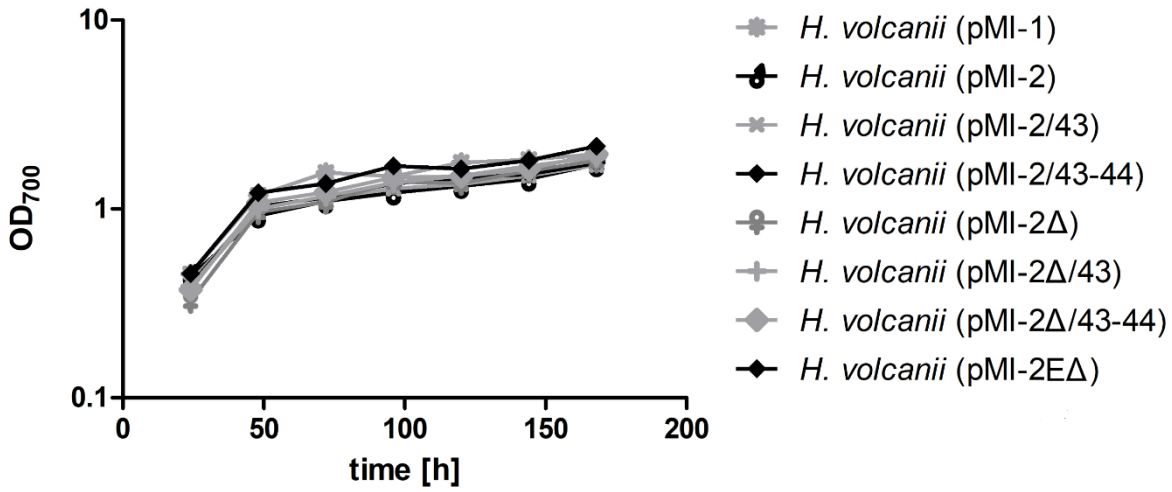

Figure 7 ***Growth curve*** *of* H. volcanii *transformants over the course of seven days.* Strains were inoculated in 18 % MGM medium with an optical density of 0. 1 and grown at 37°C with agitation. The optical densities were measured at 700 nm.

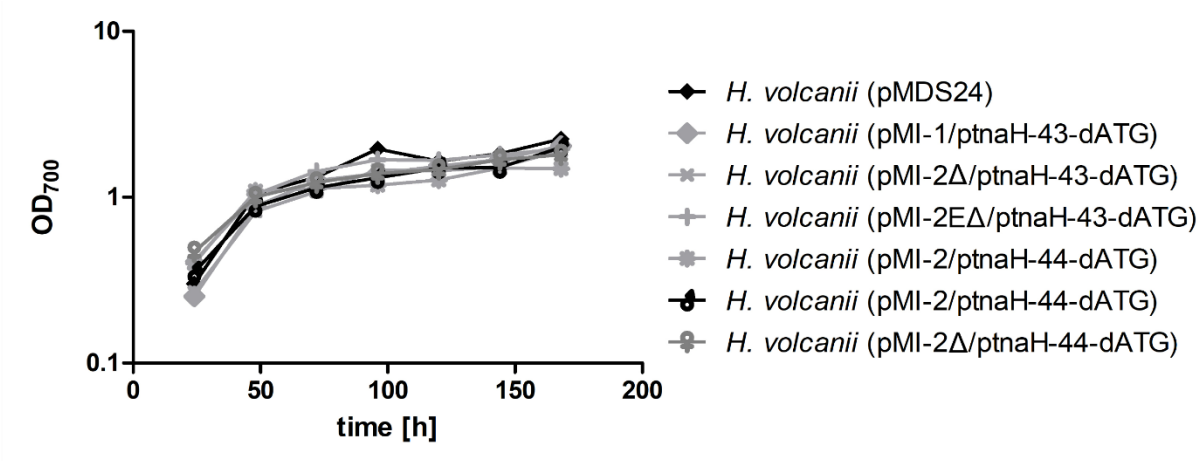

Figure 8 ***Growth curve*** H. volcanii *transformants with start codon deletion over the course of seven days. All without BgaH activity.* Strains were inoculated in 18 % MGM medium with an optical density of 0. 1 and grown at 37°C with agitation. The optical densities were measured at 700 nm.

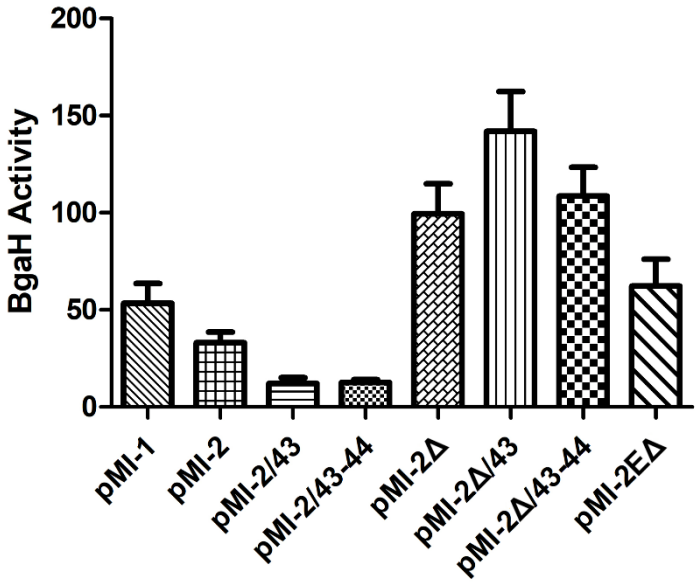

Figure 9 ***BgaH activity*** *of* H. volcanii *strains grown in 18 % MGM containing different vectors mean of day 5 +SD.*

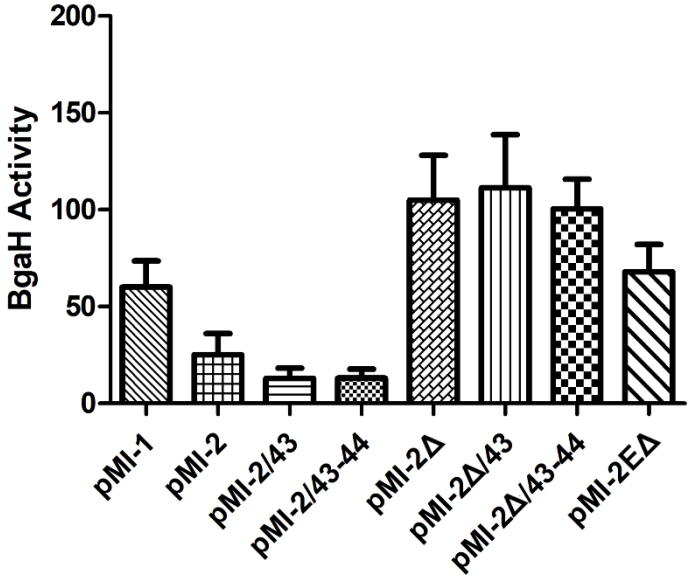

Figure 10 ***BgaH activity*** *of* H. volcanii *strains grown in 18% MGM containing different vectors mean of day 1 to 7 +SD.*

The BgaH activities for the *H. volcanii* transformants can be seen in Figure 9 for day 5 and in Figure 10 the mean for day one to seven. The results for the vectors without insert are similar to Iro et al. (2007) with activity lowest in pMI-2, higher activity in pMI-1 and pMI-2EΔ and highest in pMI-2Δ. A direct comparison to the data from Iro is not reasonable because they calculated the BgaH activity with

the protein amount measured with Bradford assay and here it was calculated only with the OD_{700}. An enhancing effect of ORF43 and ORF43/44 is not visible for pMI-2, but rather the opposite. For pMI-2Δ there is a slight increase with ORF43 but not really for ORF43/44. It would have been more reasonable to measure the protein amount for calculating the BgaH activity, because the OD_{700} is probably not the most accurate information in that regard.

3.3 Effect of ORF43/44 on ORF34

3.3.1 Growth and induction

The growth of the *H. volcanii* strains containing pMDS24, pMDS24-34_1, pMDS24-34_1-p43-ORF43, pMDS24-34_1-p43-ORF43/44 and pMDS24-34_1-ptnaN-ORF44 were observed over the course of seven days by measuring the OD_{700} as seen in Figure 11. Split of the culture for different induction is indicated in Figure 11B. There is no difference in growth visible for the strains containing different constructs.

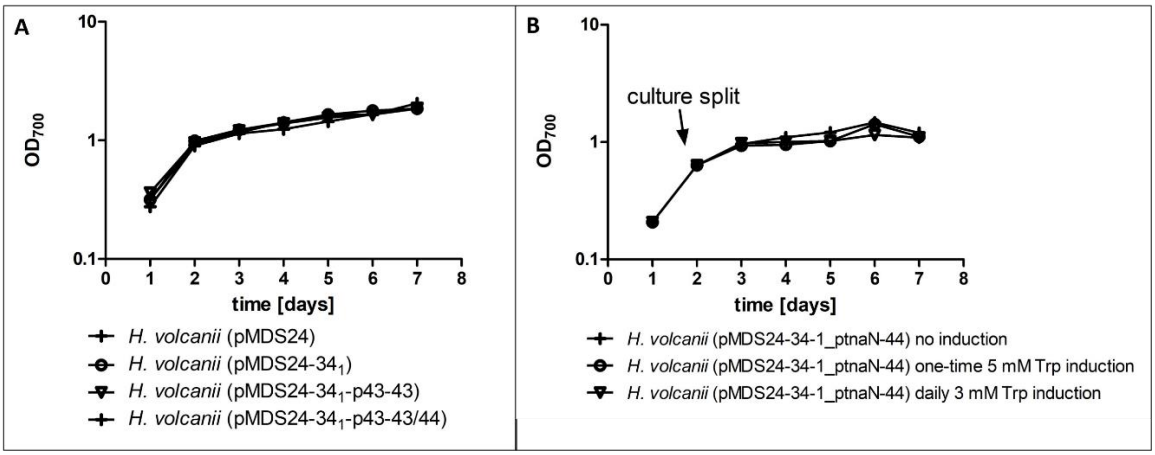

Figure 11 ***Growth curve*** *of* H. volcanii *clones.* Strains were inoculated in 18 % MGM medium with an optical density of 0. 1 and grown at 37°C with agitation. The optical densities were measured at 700 nm.

Crude extracts from all seven days were used for Western Blot analysis with antibody against gp34 using *H. volcanii* (pMDS24) and *N. magadii* L11 as controls (see Figure 12).

Bands of gp34 at ~60 kDa for *H. volcanii* containing plasmid pMDS24-34_1, pMDS24-34_1-p43-ORF43 and pMDS24-34_1-p43-ORF43/44 are clearly visible,

with a decline of intensity starting from day 4, which corresponds to the beginning of the stationary phase.

A second (little bit smaller) band is clearly visible for *H. volcanii* strains containing plasmids pMDS24-34$_1$, or pMDS24-34$_1$-p43-ORF43. This could not be detected for *H. volcanii* strains containing plasmids pMDS24-34$_1$-p43-ORF43/44, but there the upper band seems to be slightly lower than the others.

Within the different induction states of *H. volcanii* (pMDS24-34$_1$-ptnaN-ORF44) there is not much difference. All have the same two bands which are also visible in *H. volcanii* (pMDS24), indicating unspecific binding of the antibody and no expression of gp34.

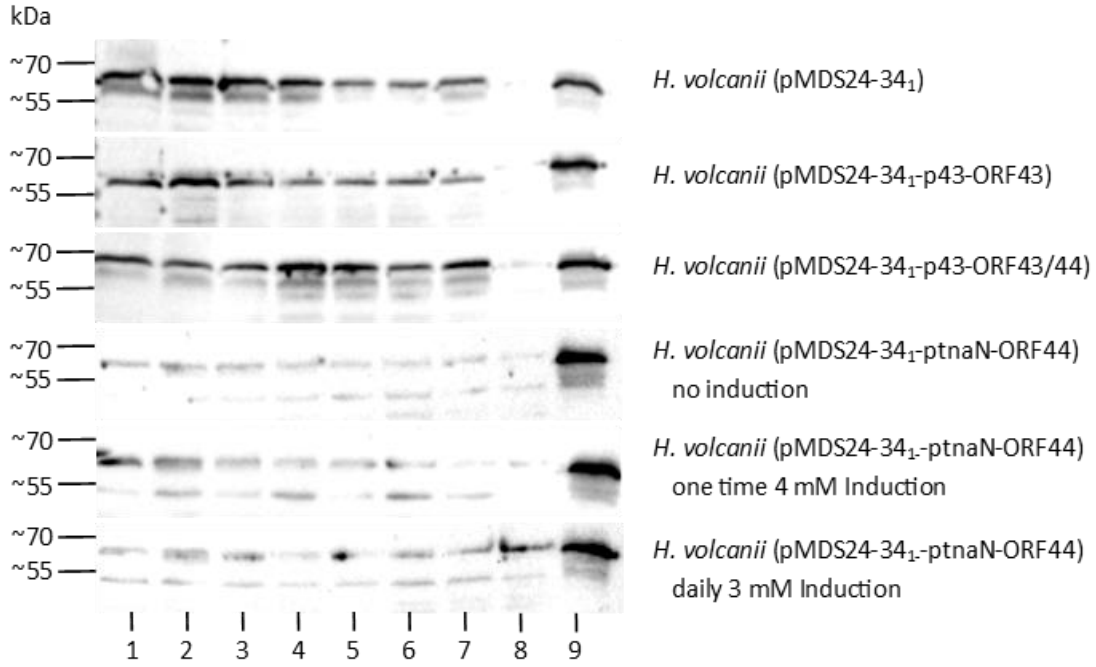

Figure 12 ***Western Blot*** *of* H. volcanii *protein samples with antibody against gp34$_{52}$ 1-7* H. volcanii *protein samples day 1-7; 8:* H. volcanii *(pMDS24) 9:* N. magadii *L11*

For better visibility of the different sizes of gp34 the samples from day 2 (after 48 h) were loaded next to each other (see Figure 13) which shows a thick band at ~65 kDa for *H. volcanii* (pMDS24-34$_1$) and *H. volcanii* (pMDS24-34$_1$-p43/44) as well as *N. magadii* L11.

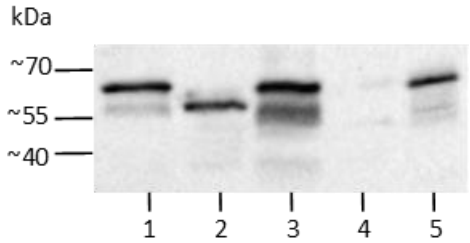

Figure 13 ***Western Blot with antibody against gp34$_{52}$*** *to compare gp34 sizes of* H. volcanii *with different constructs 1: pMDS24-34$_1$ 2: pMDS24-34$_1$-p43-ORF43 3: pMDS24-34$_1$-p43-ORF43/44 4: pMDS24 5:* N. magadii *L11*

H. volcanii pMDS24-34$_1$-p43-ORF43 does not have a band at all at that height, but there is an intense band a little bit lower which is also faintly visible for the other two strains in pMDS24-34$_1$ as well as the positive control *N. magadii* L11 and intense but blurry in pMDS24-34$_1$-p43-ORF43/44. Very faint bands are present in the negative control *H. volcanii* (pMDS24) similar to the Western Blots in Fig. 11. This indicates that the presence of ORF43 leads to a smaller gp34 as well as the presence of ORF43/44, but not to the same extent.

3.3.2 RNA isolation and RT-PCR

RNA from the strains containing the constructs for investigation of the effect of ORF43/44 on ORF34 (e.g., pMDS24-34$_1$) were isolated, treated with DNase I and screened with PCR to see if they are DNA free. Then an RT-PCR was performed with a control without the reverse transcriptase as a double check for DNA free samples. As well as multiple primer pairs (see Table 8) were used. 16S rRNA for quantification, ORF43 and ORf43/44 as control for its presence and 34$_1$ 5'-end as well as 34$_1$ 3'-end to screen for presence and cleavage of the mRNA. It was not possible to obtain RNA in adequate quality and quantity with the Phenol/Chloroform extraction method.

Then the Total RNA extraction kit was used for RNA isolation of the *H. volcanii* (pMDS24-34$_1$-ptnaN-ORF44) sample, including on column DNase I treatment. This RNA isolate was tested for DNA residues via a 40-cycle PCR using the primer pair targeting the 34$_1$ 5'-end (data not shown), there no DNA could be detected.

Table 8 Amplified targets of RT-PCR with their corresponding primer pairs.

Target	**Primer A**	**Primer B**
16S rRNA	16S-Hfx-1	16S-Hfx-2
34_1 5'-end	RT-34_1-5-5	RT-34_1-5-3
34_1 3'-end	RT-34_1-3-5	RT-34_1-3-3
ORF44	RT-44-5-2	RT-44-3
ORF43/44	RT-43-5	RT-44-3

But with the obtained RNA inconsistent RT-PCR results were obtained (see Figure 14) RT-PCR without RT-Mix was negative, as desired for the absence of DNA, for primer pair 16S rRNA, 34_1 5'-end and ORF44. But on the contrary primer pair 34_1 3'-end indicates the presence of DNA. Apart from that, it can be assumed that 34_1 mRNA is present even if gp34 could not be detected in the Western Blot because there it seemed only like unspecific binding (see Fig. 11).

Intense bands can be seen for the RT-PCR targeting 16S rRNA and 34_1 3'-end and a faint band for 34_1 5'-end. As expected, there is no band in the lane with the primer pair targeting ORF43/44, because ORF43 is not present in this construct. These results indicate that RNA isolation works better with the kit, but using more culture would be beneficial to extracting higher amounts of RNA for proper RT-PCR screening which is still in need of improvement. As well as results for the other constructs are still pending.

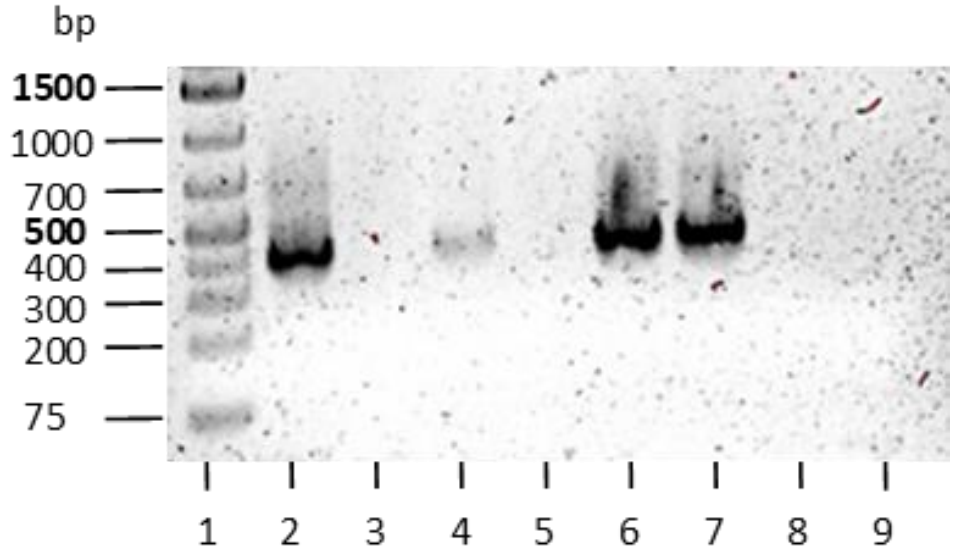

Figure 14 ***Agarose Gel of RT-PCR from RNA sample*** *pMDS24-34$_1$-ptnaN-ORF44*
1: 1kb plus marker, Lane 2 ,4 ,6 ,8 with RT-Mix, Lane 3,5,7,9 without RT-Mix
Primer pairs: 2+3 16S rRNA, 4+5 ORF34$_1$ 5'-end, 6+7 ORF34$_1$ 3'-end, 8+9 ORF44;

4. Conclusion

The aim of this thesis was to get a better understanding of the function of ORF43 and ORF44 from the *N. magadii* infecting virus ΦCh1. A putative toxin-antitoxin system type II with overlapping start and stop codons leading to co-transcription and co-translation. Based on the sequence similarity of ORF44, the entire ORF43/ORF44 operon was classified as a type II toxin-antitoxin. These are characterised by self-regulation. Here, the antitoxin binds to its own promoter in order to regulate gene expression (Yamaguchi et al., 2011). Using a reporter gene, however, no self-regulation could be detected for this system. This suggests that this system either does not belong to type II or is an exception to already known systems in this aspect.

Western blot analyses of crude extracts obtained from *H. volcanii* also showed little or no expression of ORF34 once ORF44 was also present under the control of the ptnaH promoter. This may possibly be due to a lack of regulation, as medium with a proportion of peptone was used here. A renewed investigation of crude extracts obtained from strains growing in mineral medium may provide information as to whether this system is applicable at all. mRNA of ORF34 could be partially detected, so that it can be assumed that gp44 is the cause for the absence of a signal of gp34 in the western blot. Which could

mean that maybe the gp34 was degraded and therefore it’s is not visible on the western blot. No final statement can be made regarding the nuclease or protease activity of ORF44 because of the insufficient RNA data and the need of further investigation.

Because of the incapability of transforming ORF44 under control of its native promoter, it was cloned under control of a tryptophane inducible promoter (ptnaH). But although these constructs were PCR screened and sequenced indicating the correctness of the construct no reporter activity could be detected, neither qualitatively nor quantitatively. It is unknown why the exchange of the native promoter p34 with ptnaH alone led to an absolute absence of reporter activity (BgaH) in the pMI-* plasmids compared to those constructs from Iro et al. (2007). Therefore, the effect of ORF43/44 and especially of ORF44 on the intergenic region of ORF48 and ORF49 could not be investigated. Despite all efforts it is unknown why that happened and maybe using a different reporter gene (e.g., gp34) or another inducible promoter that first must be discovered would solve that problem.

Zusammenfassung

Das halo- und alkaliphile Archaeon *N. magadii*, wird von dem gemäßigten Virus φCh1 infiziert. Der Virus besitzt ein co-translatiertes und co-transkribiertes mutmaßliches Toxin-Antitoxin-System vom Typ II, das von ORF43/44 kodiert wird. ORF44 enthält eine PiN-Domäne und ORF43 keine Sequenz mit bekannter Funktion. Die entsprechenden Genprodukte gp43/44 verstärken nachweislich ORF48/49, welche an der Regulierung des φCh1-Zellzyklus beteiligt sind. Außerdem wird für gp44 eine Endoribonuklease-Aktivität erwartet, da gp34 in Anwesenheit von gp44 abgeschnitten wird. Es wird vermutet, dass ORF43 autoreguliert wird.

Um die mutmaßliche Nukleaseaktivität und den regulierenden Effekt auf ORF48 und ORF49 zu analysieren, wurden das Toxin ORF44 und das Antitoxin ORF43 unter Kontrolle des induzierbaren Promotors ptnaH kloniert. *bgaH* (β-Galaktosidase aus *H. volcanii*) wurde als Reportergen verwendet. Zur Überprüfung der Nukleaseaktivität von ORF43/44 wurden RNA-Proben entnommen, um mittels RT-PCR zu testen, ob sie *bgaH* mRNA schneiden.

Zur Untersuchung der Autoregulation von ORF43 wurde dieser unter Kontrolle des konstitutiven Promotors p34, dem Promotor von ORF34 der für das Schwanzfaserproteins kodiert, kloniert und auf einem zweiten Plasmid der Promotor p43 mit dem Reportergen *bgaH* fusioniert. Die mutmaßliche RNase-Aktivität von gp44 auf gp34 wurde mit Konstrukten untersucht, die ORF44 unter der Kontrolle des induzierbaren Promotors ptnaN (Tryptophanase-Promotor von *N. magadii*) enthalten.

Es konnte keine Autoregulation von ORF43 festgestellt werden. Die Auswirkungen von ORF43/44 auf ORF48 und ORF49 konnten nicht gemessen werden, da die Aktivität des Reportergens (*bgaH*) fehlte. Alle Plasmide mit dem induzierbaren Promotor schienen die Expression des Reportergens (*bgaH*) verloren zu haben. Die Expression von ORF43/44 führt zu einer verkürzten Version von gp34. Die RNA-Analysen waren wiedersprüchlich und müssen weiter verbessert werden.

KEYWORDS: φCh1, *Archaea*, *Natrialba magadii*, *Haloferax volcanii* L11, Toxin-antitoxin System, BgaH

Table of figures

Figure 1 **Schematic representation of the ΦCh1 genome**. ORFs are indicated by arrows and the three shades of grey indicate the different reading frames. Three functional modules responsible for encoding structural components, DNA replication and DNA modification are also indicated (Adapted from Klein et. al. 2002)..... 8

Figure 2 **Formula for the calculation of BgaH activity** based on the Optical Density..... 46

Figure 3 **Schematic hypothesis of plasmids used for the identification of autoregulation of ORF43** and the potential regulatory effect of ORF43.Top: ORF43 and reporter gene bgaH under the control of p43 as performed by Schmal (2018). Bottom: ORF43 under control of the constitutive promoter of ORF34 (34up) and reporter gene bgaH under control of p43. 51

Figure 4 A: **Growth curve** (OD_{600}) of N. magadii (pRo-5, pNB102-p43-bgaH) and N. magadii (pRo-5-34up-ORF43, pNB102-p43-bgaH) B: **BgaH activity** of N. magadii (pRo-5, pNB102-p43-bgaH) and N. magadii (pRo-5-34up-ORF43, pNB102-p43-bgaH). Median ± SD 52

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