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„Complementation and characterisation of PCNA deletion mutant of ϕCh1"

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1. Introduction

1.1. Archaea

Archaea are unicellular prokaryotic organisms that form one of the three domains of life and can be found in many extreme environments (Woese, Kandler and Wheelis, 1990). The first organisms to be defined as *Archaea* were methanogenic and thought to have evolved before the oxygenation of the Earth's atmosphere, thus were given their name from the ancient Greek ἀρχαῖα meaning "ancient things", however it is now known that archaea can be found in many pedestrian biotopes (Woese, Kandler and Wheelis, 1990)(DeLong, 1998). The archaea domain can be divided into three main branches: Crenarchaeota, Euryarchaeota, and Thaumarchaeota (Woese, Kandler and Wheelis, 1990; Brochier-Armanet *et al.*, 2008). Archaea share characteristics with bacterial and eukaryotic organisms, which comprise the other two domains of life, such as similar metabolic pathways with bacteria and similar genes involved in gene expression with Eukarya (Koonin *et al.*, 1997).

1.2. Haloalkaliphilic Archaea

Haloalkaliphilic *Archaea* can be found hypersaline and hyperalkaline lakes such as Lake Magadi from which both *Natrialba magadii* and *Natronobacterium gregoryi* were isolated (Tindall, 1980)(Tindall, Ross and Grant, 1984). Both *N. magadii* and *N. gregoryi* have an optimal growth rate at a pH of 9.5 and a NaCl concentration of 3.5-4M (Tindall, 1980). Haloalkaliphilic archaeal membrane proteins are complex and highly glycosylated to maintain a neutral intracellular pH (Reed *et al.*, 2013) Halophilic *Archaea* are polyploid organisms and can contain upwards of 25 chromosome copies per cell (Breuert *et al.*, 2006).

N. magadii and *N. gregoryi* can be transformed using a polyethylene glycol based transformation protocol (Mayrhofer-Iro *et al.*, 2013). Two shuttle vectors for gene expression are available for the transformation of *N. magadii (*pNB102 and pRO-5) these shuttle vectors contain origins of replication for both halophilic archaea and for *E. coli* as well as antibiotic resistance markers for both halophilic *Archaea* and *E. coli.* (Zhou *et al.*, 2004; Mayrhofer-Iro *et al.*, 2013).

1.1. pNB102

In silico reconstruction of the sequence of pNB102 revealed an approximately 2000bp upstream region of the mevinolin resistance conferring *hmg* gene of *Haloferax volcanii* and an inactive 5' truncated *tet* gene. A single nucleotide polymorphism in the promoter region of the *hmg* gene conveys resistance to mevinolin (Mev^R) (Lam and Doolittle, 1989). The DNA upstream of the promoter of *hmg* and downstream of the *tet* gene fragment were thought non-essential for the function of the shuttle vector and may even decrease the efficiency of transformation. It was hypothesised that the identified

extraneous regions could be removed to increase the transformation efficiency and stability of pNB102. In addition, and as a proof of concept, this new smaller plasmid would be used to construct a new shuttle vector which had increased stability in both *E. coli* and halophiles by alternating the mesophilic and halophilic regions of in the shuttle vector to reduce internal recombination and including new cloning sites to increase the options for restriction ligation.

1.2. ϕCh1

The novel virus φCh1 was isolated from wild-type *N. magadii* L11 upon spontaneous lysis (Witte *et al.*, 1997). It was the first described virus of a haloalkaliphilic organism and is known only to infect *N. magadii* (Klein *et al.*, 2002). A strain of *N. magadii, N. magadii* L13, that was cured by repeated passaging can be reinfected by φCh1 (Witte *et al.*, 1997). φCh1 is a double stranded DNA virus with a genome size of 58,498bp and consists of 98 open reading frames and was the first virus found to contain both RNA and DNA in mature phage particles (Klein *et al.*, 2002) (Witte *et al.*, 1997). φCh1 shares a 63% nucleotide identity with the model halovirus φH, which infects the halophilic archaea *Halobacterium salinarum* (Dyall-Smith *et al.*, 2018). Genetic manipulation of φCh1 can be performed by transforming *N. magadii* L11 with a suicide plasmid containing an antibiotic resistance cassette with flanking homologies to the gene of interest to be mutated to induce targeted homologous recombination (Mayrhofer-Iro *et al.*, 2013). Due to the polyploid nature of *N. magadii* mutants obtained from this method require further steps to obtain a homogenous mutant, this is performed by isolating virus particles from the homogenous mutants and using them to infect the cured *N. magadii* L13 strain and plating the newly infected cells on selective media plates (Selb *et al.*, 2017).

1.3. Pilin

It is currently unknown to which surface protein of *N. magadii* φCh1 binds. Pilins are used by viruses to bind to their host cells such as *Neisseria gonorrhoeae* and bacteriophage φ6 (Kallstrom *et al.*, 2001)(Romantschuk and Bamford, 1985). ORF1379 of *Natrialba* magadii has a high degree of similarity to pilins in other organisms and was hypothesised to be a potential binding target of the virus ΦCh1. It was therefore chosen to be deleted by homologous recombination and determine if there was a change in the adsorption rate of ΦCh1 to *N. magadii* L13 during infection of the mutant cells.

1.4. Interaction of PCNAϕCh1 and ϕCh1 ori

Although most *Archaea* belonging to the euryarchaeota and thaumarchaeota branches encode a single PCNA gene that yields homotrimers, some members of the crenarchaeota domain contain three distinct genes that result in the formation of active heterotrimers (Ladner *et al.*, 2011). All known functions of PCNA require the duplex to encircle DNA, no function for PCNA off DNA has been reported (Pan, Kelman and Kelman, 2011).

In contrast with the PCNA proteins from thermophilic *Archaea* and eukaryotic PCNA, the PCNA from the halophilic archaeon *H. volcanii* has very few positively charged residues within the central cavity, and only one of the four found in eukaryotic PCNA (McNally *et al.*, 2010; Pan, Kelman and Kelman, 2011). This suggests a different mode of interaction halophilic PCNAs possibly mediated by bivalent cation interactions between the core of PCNA and the negatively charges backbone of DNA (Morgunova *et al.*, 2009; Winter *et al.*, 2009). During replication of DNA PCNA interacts with the origin of replication after formation of a replication fork and unwinding of the DNA by helicases (Moldovan, Pfander and Jentsch, 2007).

φCh1 and *N. magadii* each have a gene encoding for PCNA sharing 63% amino acid identity (Klein *et al.*, 2002; Siddaramappa *et al.*, 2012). Closer examination of the PCNA encoding ORF59 of φCh1 reveals an alternate GTG start codon encoded 63 bp upstream of the canonical ATG which is in consensus with the ATG start codon of *N. magadii.*

An experiment was designed to determine 1) if the removal of the GTG and 63 nucleotides upstream of the ATG and 2) changing the GTG to an ATG start codon affects the interaction of the PCNA of φCh1 and the origin or replication of φCh1. Using a BgaH reporter gene(Holmes et al. 1997) which can be used similarly to the beta-galactosidase assay of *E. coli* (Lim and Chae, 1989) located on a plasmid containing the origin of replication of ΦCh1 and plasmids expressing the PCNA encoding gene of φCh1 on separate co-transformed plasmids. The specific activity of the strains containing the different plasmids can be measured and used as a proxy for the copy number of the plasmid containing the reporter gene due to the gene dosage effect.

1.5. NgAgo

This protein was reported by Goa *et al.* to be a DNA guided endonuclease that could specifically cleave human genomic DNA *in vivo* (Gao *et al.*, 2016). This is a similar function to the revolutionary gene editor CRISPR Cas9, differing in the use of DNA as a guide as opposed to RNA in the Cas9 system(Hsu, Lander and Zhang, 2014). The reported result of the Gao paper was significant as it would have simplified the process of generating guides for the editing of DNA. Several groups attempted to replicated the results and were not able to find evidence of DNA-guided genome editing(Lee *et al.*, 2016) (Javidi-Parsijani *et al.*, 2017).The paper in which the original results were reported was retracted in August 2017. However, there was curiosity as to what the function of this NgAgo in its host *Natronobacterium gregoryi.* Argonaut proteins are known to be involved in the innate immune systems of cells, particularly in the RNA induced silencing system where they cleave double stranded RNA (Hutvagner and Simard, 2008) NgAgo is encoded by the gene ORF2597 of *N. gregoryi* was hypothesised as a protein that may function as a part of the immune system of *Natronobacterium gregoryi* and because of the reported results of Gao et al. may cleave exogenous dsDNA. It was therefore decided to delete the gene encoding NgAgo ORF2597 and investigate if there was any change in phenotype when exposed to double stranded

plasmid and halovirus φCh1 DNA via transformation and infection. It has since been reported that NgAgo plays a role in reducing pre-genomic RNA levels of the hepatitis B virus (Wu *et al.*, 2017).

1.6. F' and Lemo21(DE3)

Due to the high number of negative charges on the surface of haloalkaliphilic proteins they are prone to aggregation when expressed in mesophiles like *E. coli* (Karan, Capes and DasSarma, 2012). Lemo21(DE3) cells are designed for the production of proteins that are difficult to express in standard expression strains, such as those that are prone to aggregation (*Lemo21(DE3) Competent E. coli | NEB*). The standard protein expression plasmids express protein encoding genes under the control of the T7 promotor, which is repressed by the LacI repressor protein (Dubendorf and Studier, 1991). There is a mutant version of the *lacI* repressor, *lacI*^{Q} which is able to more tightly repress the expression of proteins under the control of the T7 promoter (Glascock and J. Weickert, 1998). While attempting to use Lemo(DE3) cells for halophile protein expression it was found that the protein of interest was being produced under non-inducing conditions. Therefore, it was desired to more tightly regulated the expression of proteins under the control of the T7 promoter in Lemo(DE3) by introducing a second copy of the *lacI*^{Q} into the strains. The F factor of the *E. coli* XL1-Blue strain encodes for *lacI*^{Q} and was transferred by mating conjugation to Lemo(DE3).

2. Materials

2.1. Strains

2.1.1. Bacterial strains

Strain	Features and characteristics	Source
E. coli XL1-Blue	*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17(r_K^-, m_K^+), supE44, *relA*1, *lac*, [F',*proAB*+, *lacI*^{q}ZΔM15, *Tn10*(Tet_r)]	Stratagene
E. coli Top 10	F- mcrA Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *nup*G *rec*A1 *ara*D139 Δ(ara-leu)7697 *gal*E15 *gal*K16 *rps*L(*Str*^{R}) *end*A1 λ^-	Invitrogen
E. coli Lemo21 (DE3)	f*huA*2 [lon] *omp*T gal (λDE3) [*dcm*] Δ*hsd*S/pLemo(Cam^R) λ DE3 = λ s*Bam*HIo Δ*Eco*RIB int::(*lac*I::P*lac*UV5::T7 gene1) *i21* Δ*nin*5 pLemo = pACYC184-p*rha*BAD-*lysY*	Novagen

E. coli Rosetta	F^- *omp*T *gal dcm lon hsd*S_B($r_B^-m_B^-$) λ(DE3 [*lac*I *lac*UV5-T7p07 *ind*1 *sam*7 nin5]) [*mal*B^+]$_{K-12}$(λ^S) pLysSRARE[T7p20 *ile*X *arg*U *thr*U *tyr*U *gly*T *thr*T *arg*W *met*T l*eu*W *pro*L *ori*$_{p15A}$](Cm^R)	Novagen
E. coli Lemo21 (DE3)	F^+ *fhuA*2 [lon] *omp*T *gal* (λDE3) [dcm] Δ*hsd*S/pLemo(Cam^R) λ DE3 = λ s*Bam*HIo Δ*Eco*RIB int::(*lac*I::P*lac*UV5::T7 gene1) *i21* Δ*nin*5 pLemo = pACYC184-p*rha*BAD-lysY	This study

2.1.2. Archaeal Strains

Strain	Features and characteristics	Source
N. magadii L13	cured strain	(Witte *et al.*, 1997)
N. magadii L11	wild-type strain, ϕCh1 provirus	(Witte *et al.*, 1997)
N. gregoryi	Wild type	DSM3393
N. gregoryi	Δ*NgAgo*::*gyr*B(NovR)	This study

2.2. Media

2.2.1. LB Medium (E. coli)

Peptone	10g
Yeast extract	5g
NaCl	5g
pH 7	
ddH2O ad 1L	
15g/L agar for plates	

2.2.2. Natrialba Vollmedium (NVM) for N. magadii and N. gregoryi

Casamino acids	8.8g	After Autoclaving the medium with or without agar	
Yeast Extract	11.7g	the medium was complemented with the following:	
Tri-Na Citrate	0.8g		
KCl	2.35g	0.57 M Na_2CO_3 (in sterile ddH_2O)	65ml
NaCL	235g	1 M $MgSO_4$ (Autoclaved)	1ml
pH 9.5		20 mM $FeSO_4$ (Dissolved in sterile ddH_2O)	1ml
ddH_2O ad 0.933L			
10g/L agar for normal plates			
5g/L agar for soft agar plates			

2.3.Additives to media

2.3.1. Antibiotics for E. coli

Antibiotic	**Stock [mg/ml]**	**End [µg/ml]**	**Dissolved in**	**Stored**
Ampicillin	20	100	Sterile ddH_2O	4°C
Tetracycline	10	10	70% EtOH	-20°C
Chloramphenicol	40	20	MetOH ad 96% EtOH	-20°C
Streptomycin	10	10	Sterile ddH_2O	4°C
Kanamycin	10	50	Sterile ddH_2O	4°C

2.3.2. Antibiotics for N. magadii and N. gregoryi

Antibiotic	Stock [mg/ml]	End [µg/ml]	Dissolved in	Stored
Novobiocin	3	3	Sterile ddH_2O	-20°C
Mevinolin	10	7.5	96% EtOH	-20°C
Bacitracin	70	7	ddH_2O, Filter sterile	4°C

2.3.3. Additional additives

Additive	[Stock]	[End]	Dissolved in	Stored
IPTG	1 M	0.1-1 M	ddH_2O	-20°C
X-Gal	20mg/ml	40µg/ml	DMSF	-20°C

2.4. Plasmids

Plasmid	Features and Characteristics	Source
pRSET-A	*bla*, pUC ori, T7 promoter, Nterminal 6x His-tag	Invitrogen
pNB102	*bla*, ColE1 ori, *hmg* (Mev^R), pNB101 ori	Zhou *et al*., 2004
pNBRM1	*bla*, ColE1 ori, *hmg* (Mev^R), pNB101 ori-deletion of	This study
pNBRM2	*bla, ColE1 ori, hmg (Mev^R), pNB101 ori, tet (Tet^R)*	This study
pNBRM2-34up	*bla*, ColE1 ori, *hmg* (Mev^R), pNB101 ori, *tet* (Tet^R), pORF34	This study

pBlueScript II KS(+) (pKSII+)	mcs, bla, ColE1, ori, LacZa	Stratagene^{+}
pKS_{II}^{+}34up	Promotor of ORF34 in pKS_{II}^{+}	
pKS_{II}^{+}34up-PCNA-1VE	ΦCh1-ORF59 with a point mutation of GTG>ATG under the control of the promoter of ΦCh1-ORF34 in pKS_{II}^{+}	This study
pKS_{II}^{+}34up-PCNA-1ME	5' truncated ΦCh1-ORF59 under the control of the promoter of ΦCh1-ORF34 in pKSII+	This study
pKS_{II}^{+}34up-PCNA-2VE	ΦCh1-ORF59 under the control of the promoter of ΦCh1-ORF34 in pKS_{II}^{+}	This study
pP-ATG	ΦCh1-ORF59 with a point mutation of GTG>ATG under the control of the promoter of ΦCh1-ORF34 in pNB102	This study
pP-Tr	ΦCh1-ORF59 under the control of the promoter of φ Ch1-ORF34 in pNB102	This study
pP-GTG	5' truncated φCh1-ORF59 under the control of the promoter of ORF34 in pNB102	This study
pNBRM1-34up-PCNA-1VE	φCh1-ORF59 with a point mutation of GTG>ATG under the control of the promoter of φCh1-ORF34 in pNBRM1	This study
pNBRM1-34up-PCNA-1ME	φCh1-ORF59 under the control of the promoter of φCh1-ORF34 in pNBRM1	This study
pNBRM1-34up-PCNA-2VE	5' truncated φCh1-ORF59 under the control of the promoter of ORF34 in pNBRM1	This study
pRo-5	*bla, gyrB (Nov^{R}), ColE1 ori, ϕCh1 ori*	(Mayrhofer-Iro *et al.*, 2013)

pRO-5-MI-I	*bla, gyrB (Nov^R), ColE1 ori, ϕCh1 ori, ϕCh1 IGR ORF48-49, bgaH*	C. Meissner, 2008

2.5. Primers

Primer	Sequence(5'-3')
2597-5en	GAACCCAGCGTCCATCTAG
2597-3en	GCCTGCTGGCCAGCT
2597-Up-Gi-F	CTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGATCAACCGAGCGCGAAGCTGGC
2597-Up-Gi-R	TCAGGAAATGACCTCGTTCCAGTCGACACACCCGGGGATCGGGTGGTATCGTAGAACAAT
2597-Do-Gi-F	TAACCAGACCGTTCAGCTGGATATTACGGCCTGCAGTAACCCGGAGCGTTCGAGTCTAAT
2597-Do-Gi-R	CGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATCTCGCCTGCCGCTGACCCAG
2597-GI-NR_U	TAACCAGACCGTTCAGCTGGATATTACGGCCTGCAGTAACGGGTGGTATCGTAGAACAAT
2597-GI-NR_D	TCAGGAAATGACCTCGTTCCAGTCGACACACCCGGGGATCCCGGAGCGTTCGAGTCTAAT
1379-11	GAATTCTAGATGAACTGGCCGCGTC
1379-2	GAGAGGATCCGAGTCGGTTGCGGAGTGA
1379-3	GAATAAGCTTGAACCAGAGCCTGAGTCAGAAT
1379-4	GAGAGGTACCGGTCGATAGTCGGTAACAGTCC
Nov-12	GCCGGTGAGTACTTAACGC
2597-3en	GCCTGCTGGCCAGCT

Nov-13	GACGCCGAATGGGTAGAC
2597-5en	GAACCCAGCGTCCATCTAG
2597-int5	GCTCCGGGTGAAGACGAC
2597-int3	GAACATATCTGCGTCCCCAG
1379-11	GAATTCTAGATGAACTGGCCGCGTC
1379-2	GAGAGGATCCGAGTCGGTTGCGGAGTGA
1379-3	GAATAAGCTTGAACCAGAGCCTGAGTCAGAAT
1379-4	GAGAGGTACCGGTCGATAGTCGGTAACAGTCC
1379-11	GAATTCTAGATGAACTGGCCGCGTC
1379-2	GAGAGGATCCGAGTCGGTTGCGGAGTGA
1379-3	GAATAAGCTTGAACCAGAGCCTGAGTCAGAAT
1379-4	GAGAGGTACCGGTCGATAGTCGGTAACAGTCC
NovR-1p	GATCCTGCAGTGTCGACTGGAACGAGG
NovR-1s	GATCCCCGGGTGTCGACTGGAACGAGG
NovR-2p	GTTACTGCAGGCCGTAATATCCAGCTGA
NovR-2s	GTTACCCGGGCCGTAATATCCAGCTGA
pNB102_2NdeIBamHI2	GATCGGATCCCATATGGGCAAAACGGTTCGGGAGG
pNB102_2NdeIBamHI	GATCGGATCCCATATGCCGGCCGGGTCGGTGGCGGCC
pBR322_tet_F	CACCCGCTGTAGACGCCATGTTTGACAGCTTATCATCG

pBR322_tet_R	CATGCCACTCTTCACACGCGGTACGCTCCAATTCTTGGAGTGG
pKS_MCS_ori_F	CCGGTACAGGCTCTGCAACTTGGTCTGACAGTTACCAAT
pKS_MCS_ori_R	GGATCTTCACCTAGATCCTTTTCCCGACTGGAAAGCG

2.6.Solutions and reagents for DNA methods

2.6.1. Solutions and reagents for gel electrophoresis

50x TAE	
Tris HCl	2 M
Acetic Acid	1M
EDTA	0.1 M
pH 8.2 (NaOH)	

5x loading dye	
Tris HCl	50mM
SDS	0.1%
Bromophenol blue	0.05%
Xylene cyanol	0.05%
pH 8.2 (NaOH)	
Add sucrose to 25% after autoclaving	

λ /*BstE*II marker		
	5µg digested λ DNA (*BstE*II)	
	20µl of 5x DNA loading dye	
	ddH_2O ad 100 µl	
	denature at 60°C for 10 minutes	
	Store at -20°C	
	Store at -20°C	

2.6.2. Kits

Name	Company	Product #	Usage
GeneJET PCR Purification Kit	Thermo Scientific	K0701	Purification of PCR products
GeneJET Plasmid MiniPrep Kit	Thermo Scientific	K0503	Isolation of plasmid DNA in *E. coli*
Promega Wizard® SV Gel and PCR Clean-Up System	Promega	A9280/A9281/ A9282/A9285	Purification of PCR products and gel-eluted DNA fragments
QIAquick® Gel Extraction Kit	QIAGEN	28706	Purification of geleluted DNA fragments
Wizard® Plus SV Minipreps DNA Purification System	Promega	A1460	Isolation of plasmid DNA in *E. coli*

2.6.3. DNA ladders

DNA ladder	Company	Size range in bp
Lambda DNA *Bst*EII Digest	Thermo Scientific	702, 1264, 1371, 1929, 2323, 3675, 4324, 4822, 5686, 6369, 7242, 8454
GeneRuler 1kb DNA Ladder	Thermo Scientific	250 to 10000
GeneRuler 100 bp DNA Ladder	Thermo Scientific	100 to 3000
GeneRuler 50 bp DNA Ladder	Thermo Scientific	50 to 1000

2.7.Solutions and reagents for protein methods

2.7.1. Solutions for SDS-PAGE and Western blots

2x Protein sample buffer (Laemmli)	
Tris HCl pH 6.8	0.12 mM
SDS	4%
glycerol	17%
β-mercaptoethanol	2%
bromophenol blue	0.02%

4x separating gel buffer	
Tris HCl pH 8.8	1.5 M
SDS	0.4%
ad 250ml ddH_2O	

4x Stacking gel buffer	
Tris HCl pH 6.8	0.5 M

10x SDS-PAGE running buffer	
Tris base	0.25 M
Glycine	1.92 M
SDS	1%

30% Acrylamide solution	
Acrylamide	73g
N,N´-methylene bisacrylamide	2g
ad 250ml ddH_2O	

Ponceau S Solution		
	Ponceau S	0.5%
	TCA	3%

SDS	0.4%
ad 250ml ddH_2O	

Transblot buffer	
Tris base	48 mM
Glycine	39 mM
SDS	0.037%
Methanol	20%

Coomassie Staining solution	
Methanol	25%
Acetic acid	10%
Coomassie Brilliant Blue R-250	0.15%

Coomassie destaining solution	
Methanol	25%
Acetic acid	10%

2.7.2. Protein Ladders

Protein ladder name	**Source**	**Size range in kDa**
PageRuler™ Prestained Protein Ladder	Thermo Scientific	10, 15, 25, 35, 40, 55, 70, 100, 130, 170

2.7.3. Antibodies used in this study

Primary Antibody	**Target protein**	**Dilution**	**Source**
α-Pilin (from rabbit)	Pilin protein encoded by ORF1379 of *N. magadii*	1:750	This Study

2.7.4. Solutions for purification of proteins under denaturing conditions

Buffer B (lysis buffer) 1L	
NaH_2PO_4	100 mM
Tris HCl	10 mM
Urea	8M
pH 8.0	

Buffer C (washing buffer) 1L	
NaH_2PO_4	100 mM
Tris HCl	10 mM
Urea	8M
pH 6.3	

Buffer D (elution buffer 1) 1L	
NaH_2PO_4	100 mM
Tris HCl	10 mM
Urea	8M
pH 5.9	

Buffer E (elution buffer 2) 1L	
NaH_2PO_4	100 mM
Tris HCl	10 mM
Urea	8M
pH 4.5	

2.8. BgaH assay solutions

BgaH Buffer	2.5 M NaCl	
	50mM Tris pH7,2	
	10µM $MnCl_2$	
	0.1% 2-mercaptopethanol	Add before use.

ONPG	8mg/ml in BgaH Buffer	Stored at -20°C
Triton X-100	2% in ddH_2O	Prepare fresh

2.9. Transformation solutions

2.9.1. Solutions for the transformation of E. coli

MOPS solution I	
MOPS	100 mM
$CaCl_2$	10 mM
$RbCl_2$	10 mM
pH 7.0 (KOH)	

MOPS solution II	
MOPS	100 mM
$CaCl_2$	70 mM
$RbCl_2$	10 mM
pH 6.5 (KOH)	

MOPS solution IIa	
MOPS	100 mM
$CaCl_2$	70 mM
$RbCl_2$	10 mM
glycerol	15%
pH 7.0 (KOH)	

2.9.2. N. magadii and N. gregoryi transformation solutions

High salt buffered spheroblasting solution	
NaCl	2 M

High salt unbuffered spheroblasting solution	
NaCl	2 M

KCl	27 mM
Tris HCl pH 8.2	50 mM
Sucrose (sterile filtered)	15%

KCl	27 mM
Sucrose (sterile filtered)	15%

2.10. Solutions for isolation and storage of ϕCh1

CsCl solution 1.5	
NaCl	2 M
Tris HCl pH 8.5-9.5	50 mM
CsCl	4 M

CsCl solution 1.3	
NaCl	2 M
Tris HCl pH 8.5-9.5	50 mM
CsCl	2.7 M

CsCl solution 1.5	
NaCl	2 M
Tris HCl pH 8.5-9.5	50 mM
CsCl	0.6 M

High salt alkaline solution	
NaCl	4M
Tris HCl pH 9.5	50mM

3. Methods

3.1. DNA methods

3.1.1. Agarose gel electrophoresis

DNA fragments were separated using agarose gel electrophoresis. A standard agarose concentration of 0.8% was used for fragments over 700bp in size. For fragments smaller than 700bp an agarose concentration of 2% was used. The agarose gel was prepared by completely dissolving the required

amount of agarose in 1x TAE buffer in a microwave, the dissolved agarose and TAE solution cooled to ~60°C with constant gentle agitation before being poured into a horizontal tray. For larger gels (300ml) a running voltage of ~150-170V was used, for smaller gels (100ml) a voltage of ~100V was used.

3.1.2. Staining and visualisation of DNA

DNA fragments separated on a gel were visualised by staining the whole gel in 5mg/L of Ethidium bromide for 5-15 minutes. The gel was rinsed with ddH_2O and loaded into a GelDoc for visualisation under a UV light.

3.1.3. Polymerase Chain Reaction (PCR)

For the preparation of DNA fragments, for cloning experiments Phusion polymerase (Thermo Scientific) was used according to the following protocol

Component	**Vol(µl)**
Phusion HiFi buffer	20
2mM dNTPs	5
Forward Primer	5
Reverse Primer	5
Template	1
ddH_2O	63
Phusion Polymerase	1

Step	**t(s)**	**T(°C)**	**#cycles**
Initial Denaturation	180	98	1
Denaturation	7	98	35
Annealing	7	T_m*	
Extension	15/kb	72	
Final Extension	120	72	1
Soaking	∞	15	

For testing of plasmids and mutants, an analytical PCR protocol was used as follows:

Component	Vol(µl)
GoTaq 2x MM	12.5
Forward Primer	1.5
Reverse Primer	1.5
Template	1
ddH_2O	8.5

Step	t(s)	T(°C)	#cycles
Initial Denaturation	180	98	1
Denaturation	7	98	35
Annealing	7	T_m*	
Extension	60s/kb	72	
Final Extension	2x Extension	72	1
Soaking	∞	15	

*T_m the annealing temperature was set as 2°C below the melting temperature of the primer with the lowest melting temperature. Melting temperatures were calculated using GeneRunner from Hastings Software Inc.

3.1.4. PCR template preparation

E. coli: 1µl of culture was used directly as a template.

N. magadii and *N. gregoryi*: 50µL of culture was centrifuged at 16000g for 3 minutes, the supernatant discarded, and the pellet resuspended in 50µL of ddH_2O and incubated while shaking at 300rpm in a thermoblock at 98°C for 10 minutes.

ϕCh1 DNA was used at a concentration of between 10-30ng/µL

Plasmid DNA was used at a concentration of between 10-30ng/µL

3.1.5. PCR product quality control

For analytical PCR products 12µL of the PCR mixture was loaded directly onto an agarose gel and run next to an appropriate DNA marker.

For a preparative PCR 5µL of the PCR mix was mixed with 5µL of 5x loading dye and loaded onto an agarose gel and run next to an appropriate DNA marker. If the visualisation showed that there were no off-target bands then the desired fragment could be directly isolated from the PCR mix via column purification. If there were off-target bands then the band must be gel eluted.

3.1.6. DNA elution from an agarose gel

The gel was stained with ethidium bromide and the desired fragment cut out from the gel using a UV table for visualisation of the fragment. The gel piece containing the fragment was placed in an Eppendorf tube and the weight of the gel piece calculated. To the gel piece an equal volume to the weight of the gel of membrane binding solution was added, the Eppendorf tube was then incubated at 63°C shaking until the gel had completely dissolved. The resultant solution was then loaded onto a silica column and centrifuged at 13k rpm for 1 minute. The flowthrough was discarded at 600µl of wash solution loaded onto the column and centrifuged again at 13k rpm for 1 minute. The flowthrough was again discarded and 350µl of Wash solution loaded onto the column and centrifuged again at 13k rpm for 1 minute. The flowthrough was discarded and centrifuged for two minutes and max rpm to remove any excess ethanol from the column. The column was then transferred into a clean Eppendorf tube and 30µl of ddH_2O was added directly to the glass membrane and allowed to incubate for one minute. The column and Eppendorf tube were then centrifuged for 1 minute at max rpm to elute the DNA off the column. The column was then discarded, and the DNA could be measured for concentration and stored at -20°C.

3.1.7. Measuring DNA concentration

DNA concentrations were estimated using a NanoDropTM ND-2000c spectrophotometer from Thermo Scientific.

3.1.8. DNA restriction

Restriction of DNA was most commonly performed using restriction enzymes sourced from Thermo Scientific. The restrictions were most commonly performed in volumes of 50µl for DNA fragments with a maximum DNA content of 2 µg. For plasmid restrictions, a total volume of 20µl was used. Most restrictions were performed at 37°C O/N, unless the restriction enzyme had a different alternate temperature for optimal activity.

3.1.9. DNA ligation

For ligation of DNA fragments T4 ligase and 10x T4 ligase buffer from Promega was used. Ligations were incubated either at room temperature for 3 hours or overnight at 4°C. For ligation of a DNA fragment into a plasmid the following protocol was used. DNA was used in concentrations between 30-50ng/µl. For self-ligations 13.5µl of backbone DNA was used.

DNA fragment	11.5 µl
Plasmid	1 µl
T4 ligase buffer	1.5 µl
T4 ligase	1 µl

3.1.10. Gibson Assembly

Gibson assemblies were designed so that two fragments were isolated using restriction digests and two fragments were isolated after PCR amplification with primers designed to give the fragments 20bp homologies with the restricted fragments. Following isolation and purification of the fragments the concentrations were measured. Using the measured concentrations, the volumes of the fragment containing solutions required to have equimolar concentrations of the larger fragments (>2kb) and three to four times high molar concentration of the smaller fragments were calculated. The concentration of the largest fragment was in the range of 0.01-0.1pmol. The calculations were made so that the total volume of the fragments combined was no greater than 5µl. The total 5µl of fragments were then added to 15µl of completely thawed Gibson Master Mix. The mix was incubated at 50°C for one hour without shaking and was then either used immediately for transformation or stored at -20°C.

3.2. *Transformation of* E. coli

3.2.1. Generation of competent E. coli *cells*

100ml of LB medium was inoculated to an OD_{600} of 0.1 with an overnight culture of the *E. coli* strain from which competent cells were to be prepared. The culture was incubated at 37°C shaking at 160rpm until on OD_{600} of 0.6-0.8 was reached. The cells were harvest by centrifugation at 6krpm and 4°C for 10 minutes. The pellet was resuspended in 40ml of ice cold MOPS I solution and incubated on ice for 10

minutes. The cells were again pelleted by centrifugation at 6k rpm and 4°C for 10 minutes before the pellet was resuspended in 40ml of MOPS II solution and incubated on ice for 30 minutes. The cells were then pelleted for a final time by centrifugation at 6k rpm and 4°C for 10 minutes, the pellet was then resuspended in 2ml of MOPS IIa solution. 100µl of the now competent cells were the aliquoted out, these were either used directly for transformation or stored at -80°C until needed.

3.2.2. Transformation of competent E. coli *cells*

If frozen aliquots of competent cells were to be used the cells were thawed on ice, freshly transformed cells were used directly for transformation. The amount of DNA to be added to the competent cells varied depending on the DNA to transformed. For ligations the 15µl total reaction volume was added to the competent cells, for the Gibson assembly prepared plasmids the total 20µl reaction volumes was added, for transformation of intact plasmids 5-10µl containing ~100ng of DNA was added to the competent cells. Following addition of the DNA the cells plus DNA were gently mixed and then left to incubate on ice for 30 minutes. The cells were then heat shocked at 42°C for 2 minutes, and then cooled on ice for 30 seconds. Once the cells had cooled, 300µl of LB medium was added to the cells and gently mixed, the cells were then left to regenerate at 37°C without shaking. Following regeneration, the freshly transformed cells were plated out onto three selective plates, which were then incubated at 37°C overnight.

3.3. Transformation of N. magadii *and* N. Gregoryi

3.3.1. Generation of competent cells

Fresh preculture of the strain to be transformed was inoculated NVM^+ with bacitracin to an OD_{600} of 0.1 and incubated for a maximum of 24 hours or until the culture reached on OD_{600} of 0.5-0.6. The culture was centrifuged at 5500g for 15 minutes at room temperature. The supernatant was discarded, and the pellet resuspended in 30ml of buffered high salt spheroblasting solution with glycerol and proteinase K (0.1%) and incubated at 42°C shaking until the majority of the cells were spherical when viewed under a microscope.

3.3.2. Transformation of competent cells

For each transformation 1.5ml of the spheroblasts was used. The cells were pelleted by centrifugation at 10 000g for 15 minutes at room temperature and resuspended in 150µl of buffered high salt Spheroblast solution. 15µl of 0.5M EDTA was added to the cells and incubated for 10 minutes at room temperature. DNA for transformation was added to the cells in a maximum volume of 10µl of H_2O. For shuttle vector transformations 3-5µg of plasmid DNA was used. For suicide plasmids for the purposes of homologous

recombination and minimum of 30µg of DNA was used. The cells plus DNA were incubated at room temperature for 5 minutes. 150µl of 60% PEG-600 in unbuffered high salt spheroblasting solution was added and incubated at room temperature for 30 minutes. The cells were then washed by the addition on 1ml of NVM^+ followed by gentle mixing and then centrifugation at 10000g for 5 minutes at room temperature. The supernatant was discarded, and the pellet resuspended in 1ml of NVM^+ and incubated at 37°C shaking at 160rpm. Every 24 hours the cells were examined, if the majority of cells were still spheroblasts the cells were washed again in 1ml of NVM^+ and re-incubated. Once the majority of cells had regenerated their rod-shaped morphology the cells were plated out. NVM^+ selective media plates were used for plating, 9 plates containing 100µl of undiluted regenerated cells and 10 plates containing 100µl of a 1:10 dilution of the regenerated cells in NVM^+. The plates were incubated at 42°C until colonies were visible.

3.4. Virus particle isolation

N. magadii L11 was inoculated at $_{OD600}$ 0.1 and grown to until the culture had maximally lysed as measured by the OD_{600}.The culture was centrifuged at 10,000g for 15 minutes at room temperature and the supernatant decanted into a sterile flask. The supernatant was incubated with 10%PEG 6000 and stirred overnight. Centrifuge at 6000rpm for 30 min. Discard the supernatant and dissolve the pellet with a minimal volume of 4M NaCl, 50mM tris pH9.4. Ultracentrifuge (30krpm, 20hrs, 20°C) with a CsCl gradient made by layering 2ml of 1.5 CsCl, 4ml of 1.3 CsCl, 6-7ml of L11 slurry, topped off with CsCl 1.1 solution.

Solutions (200 ml):

1.5: 135g CsCl, 23.4 g NaCl, 50 mM Tris/HCl, pH 9.5

1.3: 90g CsCl, 23.4 g NaCl, 50 mM Tris/HCl, pH 9.5

1.1: 20g CsCl, 23.4 g NaCl, 50 mM Tris/HCl, pH 9.5

3.5. Construction of a heterogenous NgAgo mutant

50µl of *N. gregoryi* was inoculated from a storage culture at room temperature into 5ml of NVM^+ and grown to an optical density (OD_{600}) of 1.0. The culture was used as a stock for wild type controls strains and for the generation of competent cells for transformation.

Two plasmids were constructed for the deletion of NgAgo(ORF2597 of *N. gregoryi)*; pKS-Ng2597KO-NovR-F and pKS-Ng2597KO-NovR-R were created for the deletion of NgAgo substituting the gene for a novobiocin resistance gene in either the forward or reverse direction.

Plasmid pKS-Ng2597KO-NovR-F was constructed via Gibson assembly from 4 fragments: pKS_{II}^{+} digested with *Eco*RV, a 2688bp fragment released from pUC19-NovR-R by restriction digestion (*Dra*I, *Sma*I and *Pst*I), and 5' and 3' flanking fragments amplified from *N. gregoryi* template DNA using the primer pairs 2597-Up-Gi-F/2597-Up-Gi-R (991bp), and 2597-Do-Gi-F/2597-Do-Gi-R (559bp).

pKS-Ng2597KO-NovR-R was constructed as with the forward deletion plasmid, differing in the use of primer pairs 2597-Up-Gi-F/2597-GI-NR_U (982bp), and 2597-GI-NR_D/2597-Do-Gi-R (559bp) for the flanking region amplifications.

Competent cells of *N. gregoryi* were prepared for transformation and transformed with 30µg of either deletion plasmid as described for *N. magadii.* Once the cells had regenerated at 37°C they were transferred to NVM^{+}nov plates and incubated at 42°C until colonies were visible (~9 days). Colonies were incubated in 500µl of NVM^{+} medium in Eppendorf tubes and incubated shaking at 42°C for 3 days.

Clones were tested for 3' and 5' crossover via PCR, primer pairs as follows: those transformed with pKS-Ng2597KO-NovR-F were tested with Nov-13/2597-3en (1104bp) for 3' crossover, and 2597-5en/Nov-12 (1201bp) for 5' crossover; those transformed with pKS-Ng2597KO-NovR-R were tested with 2597-3en/Nov-12 (669bp) for 3', and 2597-5en/Nov-13 (1636bp) for 5' crossovers.

Positive clones were passaged by inoculating 100µl of OD_{600} 0.5-1 culture into 10ml of NVM^{+}Nov liquid medium and incubating at 42°C shaking, this was repeated until no ORF2597 could be detected using PCR (2597-3int/2597-5int (994bp)).

3.6. Infection analysis of N gregoryi

To test for infectivity by ΦCh1 of 25ml of OD600 0.1 cultures of L13, *N. gregoryi*, and NgΔ*NgAgo*::gyrB(NovR) were prepared, to which 10µl of purified ΦCh1 particles were introduced, together at 37°C shaking, and the OD600 measured daily for six days.

3.7. Transformation efficiency assay

Competent cells were prepared for each of the strains to be analysed. The cells were transformed with 20µg of DNA in triplicate. Once regenerated the cells were plated out in triplicate and incubated at 37°C until colonies were visible on all plates at which point colony counts were performed.

3.8. His-Tag purification of proteins from E. coli

E. coli strains containing expression vectors were inoculated from overnight cultures to an optical density of 0.1 at 600nm and incubated at 37°C until OC_{600} 0.1 at which point 1mM IPTG was added to

induce protein production. A series of samples were taken at time points up to 360 minutes and run on an SDS gel to determine the optimum timepoint for protein isolation. The inoculation procedure was repeated, and the culture centrifuged at the optimum timepoint for 15 minutes 4°C at 6000g. The pellet was frozen at -20°C and then resuspended in Buffer B and incubated overnight at room temperature. The lysate was centrifuged for 20 minutes at 10,000g at room temperature. The supernatant was then incubated with 1.5ml of Ni-NTA and stirred overnight at 4°C. The resultant solution was run through a silica column and the flowthrough collected. The column was washed twice with 4ml of buffer C, and the proteins eluted 4 times with 0.5ml of buffer D, and four times with 0.5ml of Buffer E, collecting the flowthrough for each other the wash and elution steps. All flowthrough samples, and samples of the culture taken before induction and at the end of the induction period, were analysed via SDS-PAGE. The fractions containing the protein of interest were pooled and dialysed with 1xPBS for one hour, and then against fresh 1XPBS overnight. The concentration of the purified protein was measured by running samples alongside known concentrations of bovine serum albumin standards.

3.9. α-Pilin production

A previously constructed plasmid with the 3´-end of ORF1379 cloned into the plasmid pRSET-A, was used to produce antibodies against the putative pilin. The plasmid was transformed into *E. coli* strain Rosetta.

Protein production of the transformed strain was induced with 1mM IPTG at an optical density of 0.3 and incubated for 105 minutes at 37°C. The protein was isolated under denaturing conditions using Ni-NTA affinity purification columns as described. The protein was isolated from 7L of culture and dialysed in 1x PBS. The concentration of the protein in the eluents were measured using SDS-PAGE and BSA standards from 0.05-1µg/µl (New England Biolabs) and sent to an antibody production facility (Moravian-Biotechnology, Ltd., Brno, Czech Republic).

3.10. Construction of Pilin deletion plasmids

Two plasmids for the deletion of ORF1379 of *N. magadii*, plasmids pKS-1379-1-4-NovR-F and pKS-1379-1-4-NovR-R were constructed. The 5’ flanking region of ORF1379 was amplified with primers 1379-11/1379-2 (1035bp) from L13 template DNA. The fragment was restricted in parallel with pKS_{II}^{+} by *Xba*I and *Bam*HI. The PCR fragment and the restricted plasmid were purified by gel extraction, ligated together with T4 polymerase, and transformed into XL1-Blue. Clones were screened and positive transformants identified via restriction digest analysis with *Xba*I and *Bam*HI. The resultant plasmid (pKS-

1379-1-2) was ligated with the 3' flanking region amplified from L13 DNA with primers 1379-3/1379-4 (1450bp) after both had been digested with *Hind*III and *Kpn*I. Positive clones were controlled with a *Hind*III and *Kpn*I restriction and labelled pKS-1379-1-4. The resistance cassette containing the *gyrB* gene was amplified for the reverse variant with primers NovR-1p/NovR-2s (2800bp) from pUC19-NovR and the forward variant with primers NovR-1s/NovR-2p (2800bp) from pUC19-NovR "forward". These fragments were restricted with *Sma*I and *Pst*I and ligated into pKS-1379-1-4 digested with the same enzymes. Positive clones were identified by restriction analysis with *Sma*I and *Pst*I and named pKS-1349-1-4-NovR-F and pKS-1349-1-4-NovR-R.

3.11. Construction of N. magadii *L13::1379*

The suicide plasmids pKS-1379-1-4-NovR-F and pKS-1379-1-4-NovR-R were transformed into competent *N. magadii* L13 cells using the standard protocol. Clones were tested for homologous recombination using the PCR primers Nov-12/1379-3en (669bp) for the reverse mutant and Nov-9/1379-3en(972bp)

3.12. Infection of N magadii *L13 with ϕCh1 virus particles*

500µ aliquots of OD_{600} *N. magadii* L13 add 100, 200,300, 400, and 500µl of isolated virus particles were mixed and incubated for 2hrs at 37°c no shaking. Then the cells were washed by centrifuging for 5 min, RT 16krcf, resuspended for 30 minutes at 37°C with shaking. The washing step was repeated 3 times and 200µl of the final resuspension were plated on NVM^+ plates with antibiotics if required. the plates were dried over night at RT and transferred to 42°C for incubation until colonies were visible. Colonies were incubated in 500µl of NVM^+ shaking at 37°C with required antibiotics for 3 days and then tested with PCR for the presence of the virus.

3.13. Adsorption assay

Strains were inoculated to an optical density of 0.1 from cultures growing in the exponential phase and incubated at 37°C until an OD_{600} of 0.5. 20ml of the cultures was transferred to new Erlenmeyer flasks and 200µl of purified virus particles were introduced. Samples were taken at 0 65 and 120 minutes. The samples were centrifuged (16,000rpm, RT 3') and the supernatant removed into a clean Eppendorf tube, to which 20µl of chloroform was added. These samples were used to perform a virus titre assay to determine the amount of free virus particles in the supernatant.

3.14. Construction of Lemo f' (DE3) cells

XL1-Blue and Lemo21(DE3) Cells were inoculated in LB tetracycline and LB chloramphenicol respectively and incubated at 37°C overnight. These cultures were streaked into plates containing either tetracycline

or chloramphenicol, one strain on each half of the plate to check from resistances. The strains were mated by mixing 1ml total volumes of 1.0 OD_{600} Lemo21(DE3) and 0.5 OD_{600} XL1-Blue cultures in ratios of 1:1, 9:1, and 2:1, and incubating them at 37°C for 1hr. 10µl of the mated strains were plated out on LB cam/tet plates and incubated at 37°C overnight. Overgrown plates were streaked out on new plates and incubated at 37°C overnight. Individual colonies were picked into 5ml of LB cam/tet.

Colonies were inoculated in liquid media, LB amp/tet, and grown overnight. The culture was then streaked onto IPTG plates X-gal plates along with XL1-blue and Lemo(DE3) cultures as controls to verify the presence of the gene encoding the alpha fragment on the F factor.

3.15. pNBRM1 Construction

The amplification of the fragment was performed by PCR using Phusion Hi-Fi polymerase (Thermo Scientific) and the primers pNB102_2NdeIBamHI2/pNB102_1PvuIIBamHI (6258bp). It was essential for this PCR that the denaturing and annealing times were sub 10 seconds. The PCR product was column purified. The purified PCR product was digested with *BamH*I and purified by gel extraction. The restricted PCR product was ligated with T4 ligase and transformed into XL1-Blue. Colonies were incubated in 5ml LB amp/tet 37°C shaking overnight and plasmid isolated from each culture the following day. Positive clones were identified using restriction digest analysis using the enzymes: *Pst*I (3663bp, 2019bp, 563bp, 30bp), *Hind*III (3737bp, 2538bp) and *Bam*HI (6275bp). The resultant plasmid was termed pNBRM1.

3.16. pNBRM2 Construction

Constructed of four fragments ligated together in a Gibson assembly: 1) 2232bp fragment resulting from digest of pNB102 (*Hind*III-*Sgs*I-*Blp*I)(1X Tango buffer, with 2 fold *Hind*III) 2) Tetracycline resistance gene amplified from pBR322 with primers pBR322_tet_F/pBR322_tet_R (1324bp) 3) 2561bp fragment released from pNBRM1 with *Dra*I and *Kpn*I (Tango 1X) containing a mevinolin resistance conferring gene and an *E. coli* origin of replication 4) Multiple cloning site and an ampicillin resistance gene amplified from pKS_{II}^{+} with primers pKS_MCS_ori_F/pKS_MCS_ori_R (2003bp).

The fragments were isolated and purified by gel extraction then assembled using Gibson assembly. The assembled plasmid was transformed into *E. coli* Top10 cells and plated on LB amp/tet plates.

Plasmids isolated from the resultant colonies were by tested by restriction digest analysis with *Pst*I (4552bp, 2939bp, 563bp, 30bp) and *Bam*HI (4298bp, 2356bp, 1250bp). The positive plasmid was termed pNBRM2.

3.17. Construction of PCNA expression plasmids in pNB102 and pNBRM1

The variants of ORF59 were amplified with PCNA-2VE/PCNA-22 (827bp), PCNA-1VE/PCNA-22 (827bp), and PCNA-1ME/PCNA-22 (754bp), restricted with *EcoR*I and *Kpn*I. Fragments were ligated into pKS-34up restricted with *EcoR*I and *Kpn*I resulting in plasmids pKS-34up-PCNA-2VE, pKS-34up-PCNA-1VE, pKS-34up-PCNA-1ME. The promotor region of ORF34 and ORF59 were released from the plasmids with *Xba*I and *Kpn*I, purified by gel extraction and T4 ligated into pNB102 or pNBRM1 likewise restricted with *Xba*I and *Kpn*I. The resultant plasmids were named pP-GTG(pNB102-34up-PCNA-2VE), pP-ATG(pNB102-34up-PCNA-1VE), pP-Tr(pNB102-34up-PCNA-1ME), pNBRM1-34up-PCNA-2VE, pNBRM1-34up-PCNA-1VE, and pNBRM1-34up-PCNA-1ME.

3.18. Bgah Assay

700µl of BgaH buffer, 100µl of cell culture, and 100µl of 2% Triton X-100 were mixed together in an Eppendorf tube. This mixture was then transferred into a cuvette and 100µl of ONPG solution added and mixed by pipetting. Following addition of the ONPG the OD_{405} of the solution mixture was then measured continuously until the OD_{405} was between 0.7 and 1.0.

The specific activity (SA) of BgaH in the culture was then calculated with the following formula:

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

Variable	**Meaning**
ΔA_{405}	change in absorption at 405 nm
Δt	time-span of change of the absorption (in minutes)
V	volume of culture added to the assay (in ml)

N. magadii and N. gregoryi PCR template preparation*:*

Spin down 50µl of culture, 16kG, RT 5', remove supernatant and resuspend in 100µl of autoclaved ddH_2O, incubate 5' at 98°C, use directly as template, can be stored long term at -20°C.

4. Results and Discussion

4.1. The deletion of NgAgo

In order to determine the function of NgAgo, the suicide plasmid pKS-Ng2597KO-NovR-R was transformed into competent *N. gregoryi cells.* The 48th and 50th tested colonies returned positive PCR signals for homologous recombination of the deletion cassette in the ORF2597 locus encoding for NgAgo. The two positive clones were passaged 10 times resulting in to homozygous strains of *N. gregoryi* *Δ*NgAgo*::gyrB* were produced: Ng4810 and Ng5010, the 48th and 50th colonies tested which had each been passaged for 10 generations to homozygosity

Wild type and *ΔNgAgo* strains were inoculated with ϕCh1 particles and the growth behaviour analysed (Figure 1). There was no significant change in the growth behaviour of the mutant strains compared to the wildtype strain.

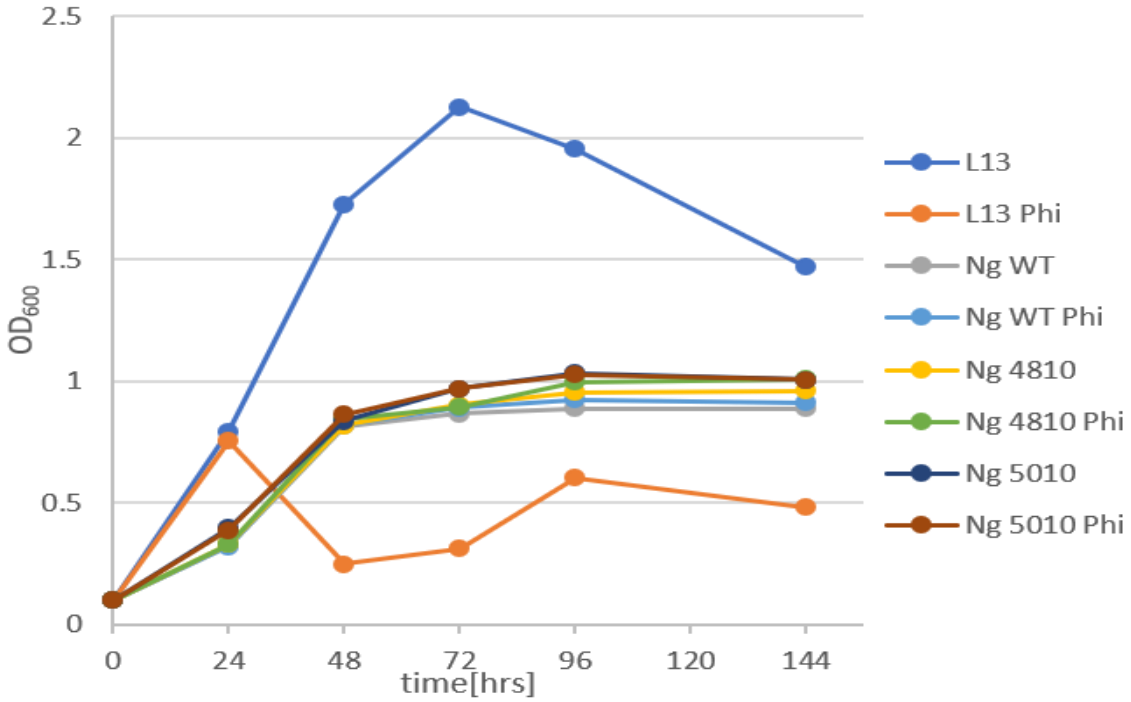

Figure 1: The growth curves of *N. gregoryi* and *N. magadii* L13 cells incubated with and without ΦCh1 virus particles. Ng WT, wild type strain of *N. gregoryi*. Ng 4810 and Ng 5010 homogenous deletion mutant strains (Δ*NgAgo*::*gyr*B(NovR)). Strains inoculated with ϕCh1 particles have the designation Phi.

Wild type and *ΔNgAgo* strains were transformed with purified ϕCh1 DNA and plated on plates without antibiotics to determine if any plaques were formed on the resultant lawns. No plaques were detected that contained ΦCh1 DNA as tested by PCR.

A transformation efficiency assay was also undertaken. There was no significant difference between the number of colonies on the wild type and mutant plates. Therefore, NgAgo seems to have not function in the defence of uptake of foreign DNA. However, since no virus strain is available for *N. gregoryi*, a possible function in the defence of a virus infection cannot be ruled out completely.

4.2. Pilin

4.2.1. Homogenisation of pilin mutant

Suicide plasmids for the deletion of ORF1379 a putative pilin encoding gene of *N. magadii* were created with the *gyr*B gene in the forward and reverse orientations. These strains should be used for the analysis of a putative receptor for ϕCh1 on the surface of *N. magadii*. These plasmids were termed pKS-1349-1-4-NovR-F and pKS-1349-1-4-NovR-R. The plasmids were transformed into *N. magadii* L13. Positive signals for homologous recombination were only detected in one colony with the insertion of the *gyr*B gene in the reverse orientation in the locus of ORF1379. Passaging was performed to obtain a homogenous mutant. After 38 passages the wild type ORF1379 was still detectable with PCR (data not shown).

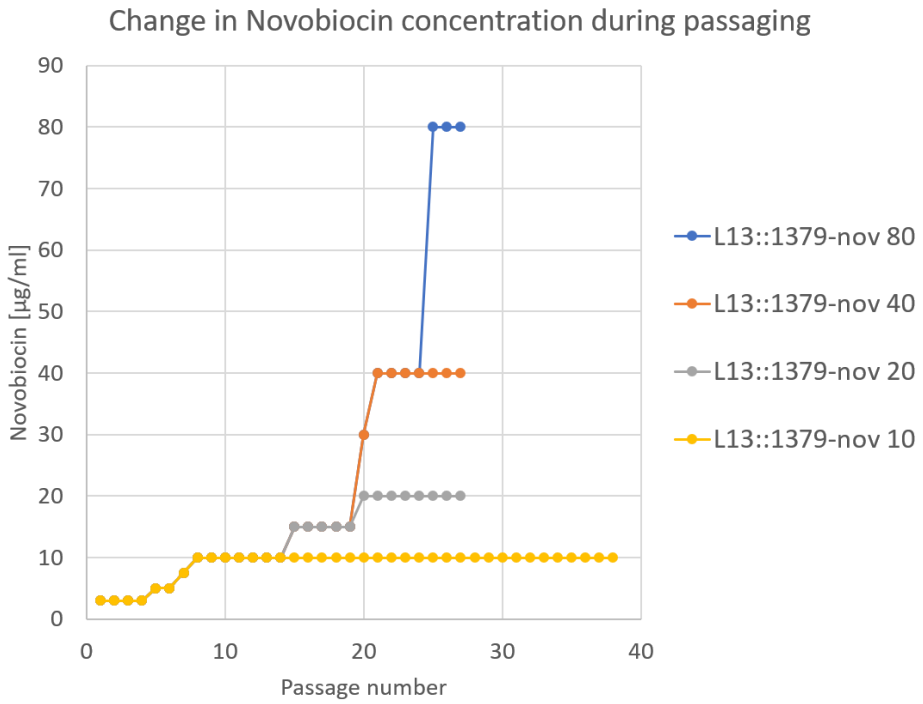

Figure 2: The number of passages of the four resultant strains and the concentration of novobiocin in which they were incubated

As can be seen in Figure 2 in addition to the multiple passages the concentration of novobiocin in the cultures was raised to increase the selective pressure for chromosomes containing the *gyr*B gene in the ORF1379 locus. This resulted in four heterogenous mutant strains *N. magadii* L13::1379-10, *N. magadii*

L13::1379-20, *N. magadii* L13::1379-40, and *N. magadii* L13::1379-80. 10, 20, 40 and 80 refers to the concentration of novobiocin in the cultures.

Using the antibody against the pilin protein that was produced from the expression of ORF1379 western blots against pilin were performed to analyse the amount of pilin present in each of the mutant strains.

4.2.2. Pilin Western blot

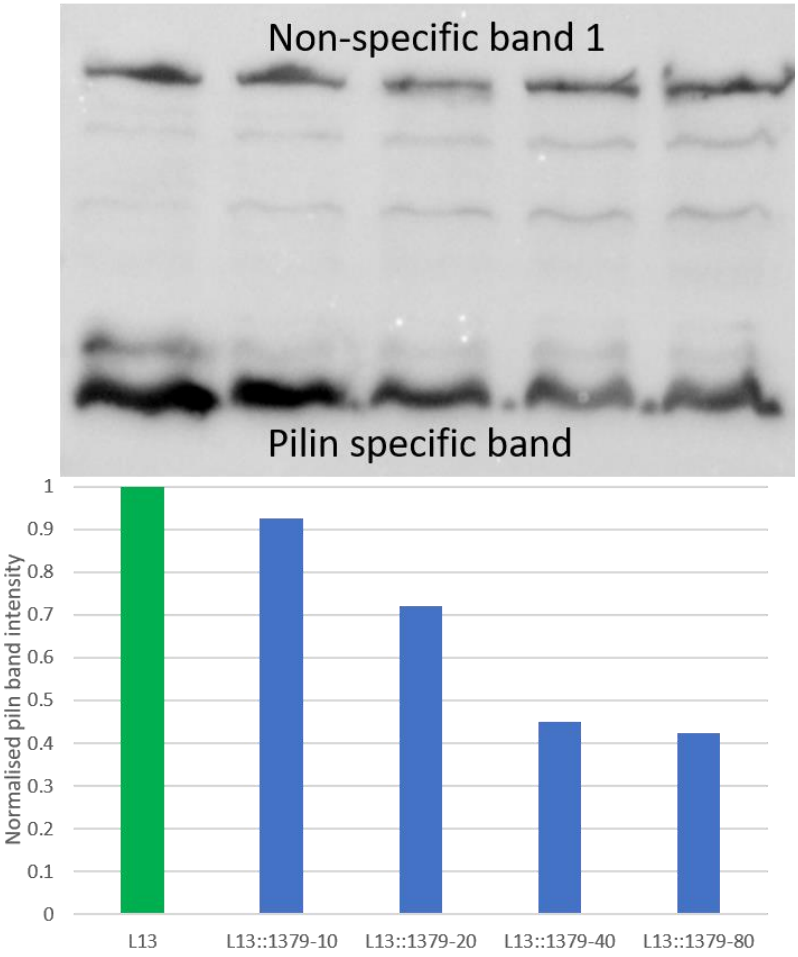

Figure 3: Western blot image of crude protein extract from WT L13 and each of the heterologous knockout mutants and a graphical representation of the band intensities of the mutant strains. The titles of the column in the graph represent the lane in the WB image directly above.

The results of the western blot can be seen in Figure 3. The results indicate that increasing the concentration of novobiocin in the cultures was successful in decreasing the amount of pilin detectable in the strains.

4.2.3. Adsorption assay

This experiment was designed to determine if ORF1379 encodes a membrane protein to which ϕCh1 can bind. ϕCh1 has previously been shown not to adsorb to *N. magadii* L11 strain (Iro et al., 2007). Inoculate L13, L11, and the different *N. magadii* L13::1379 cultures. Incubate strains at 0D600 0.5 with equal quantities of virus particles. Take samples of cell culture supernatants at 0, 60, and 120 minutes Perform a virus titre assay to determine virus particle concentration within the supernatant

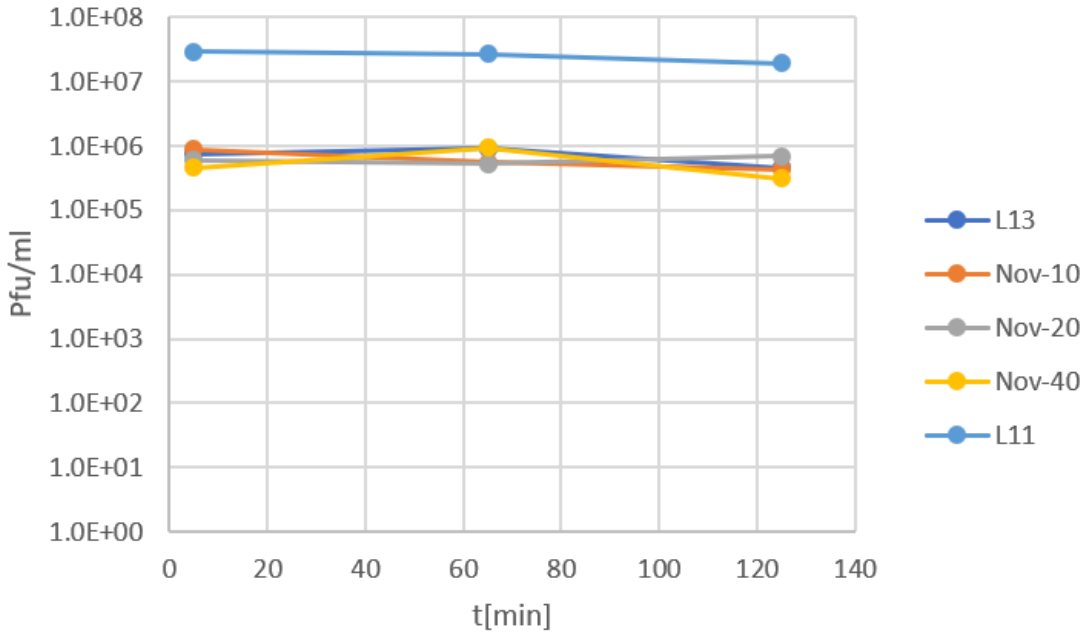

Figure 4: The pfu over time following the incubation of L13 and the pilin knockdown strains. pfu is used as a proxy for the amount of virus particles in the supernatant that had not bound to the surface of *N. magadii.*

The results of the adsorption assay can be seen in Figure 4. They do not demonstrate any discernible difference between the amount of virus particles adsorbed onto the surface of the wild type *N. magadii* L13 and the strains inoculated under progressively higher concentrations of novobiocin. This is not definitive evidence and further analyses should be carried out, additionally a control using only NVM^+ medium without cells should be included if the experiment in repeated. It is unlikely that Pilin acts a binding receptor for ΦCh1. Although is it still possible that pilin acts a secondary receptor which the virus uses for insertion into L13.

4.3. Construction of F' containing Lemo cells

LemoBL12(DE3) containing the F' phage were constructed via mating with XL1-Blue cells. Lemo21(DE3) cells containing the F factor of XL1-Blue were identified and introduced into the strain collection. These cells can be used for blue white selection of positive transformants and growth using tetracycline as a selection marker which was not possible in the LemoBL21(DE3) cells obtained from New England Biolabs.

4.4. Redesign of pNB102

The plasmids were created as designed and were able to be detected propagating after transformation in strains of *N. magadii* and pNBRM1 was able to be detected propagating in *Nbt. Gregoryi.* The transformation efficiency of pNB102, pNBRM1, and pNBRM2 still requires testing in both *E. coli* and *N. magadii*. It would also be suggested to analyses differences in copy number in both *E. coli*, and haloalkaliphiles to see if it is advantageous.

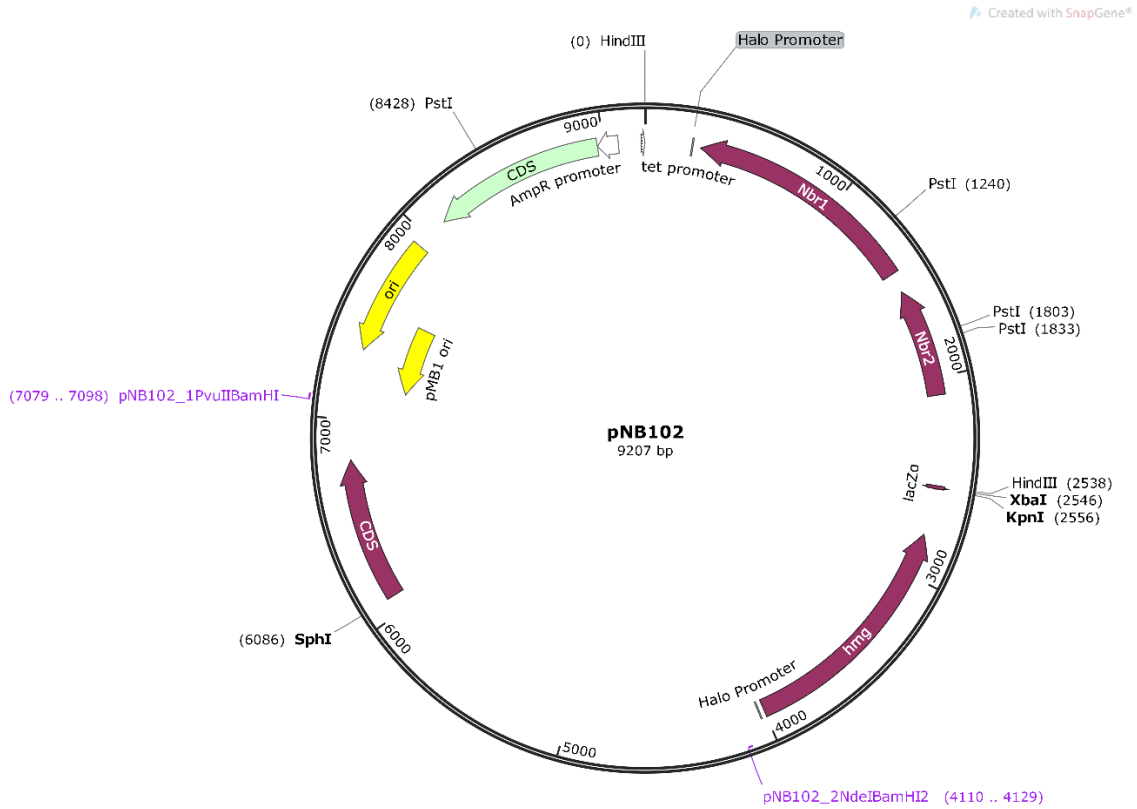

Figure 5: Plasmid map of pNB102 including the location of the primers used for the generation of pNBRM1

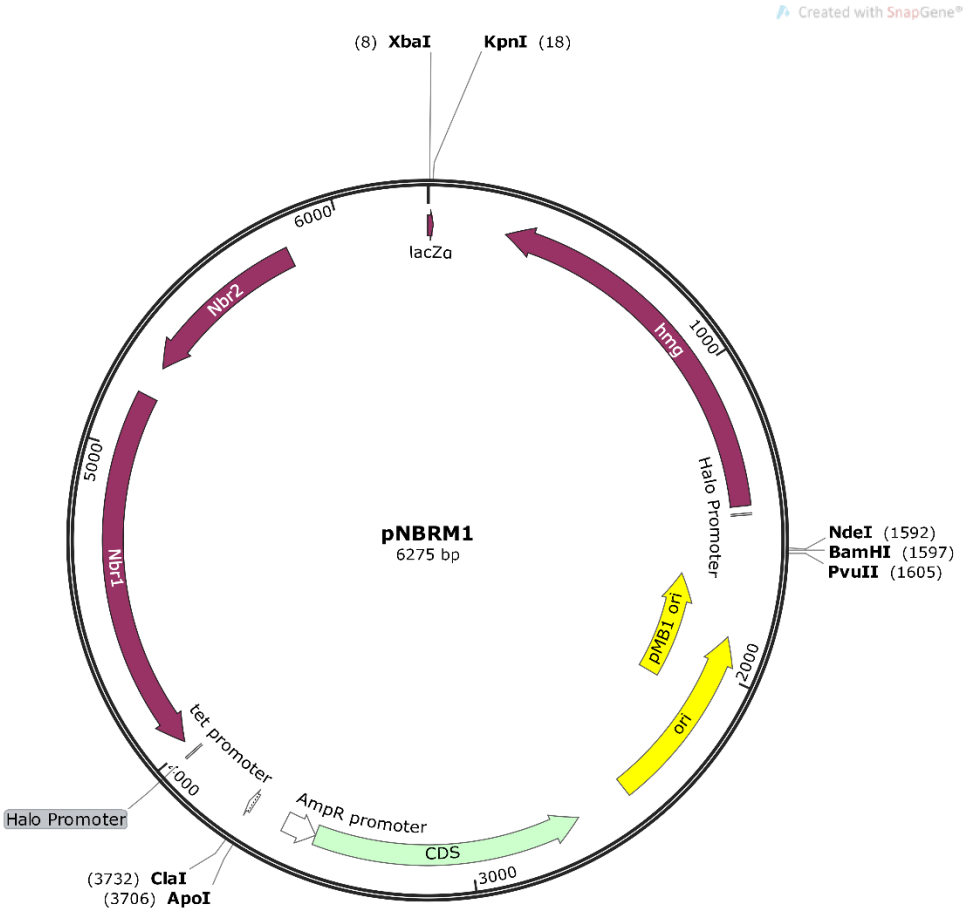

Figure 6: Plasmid map of pNBRM1 including potential restriction sites

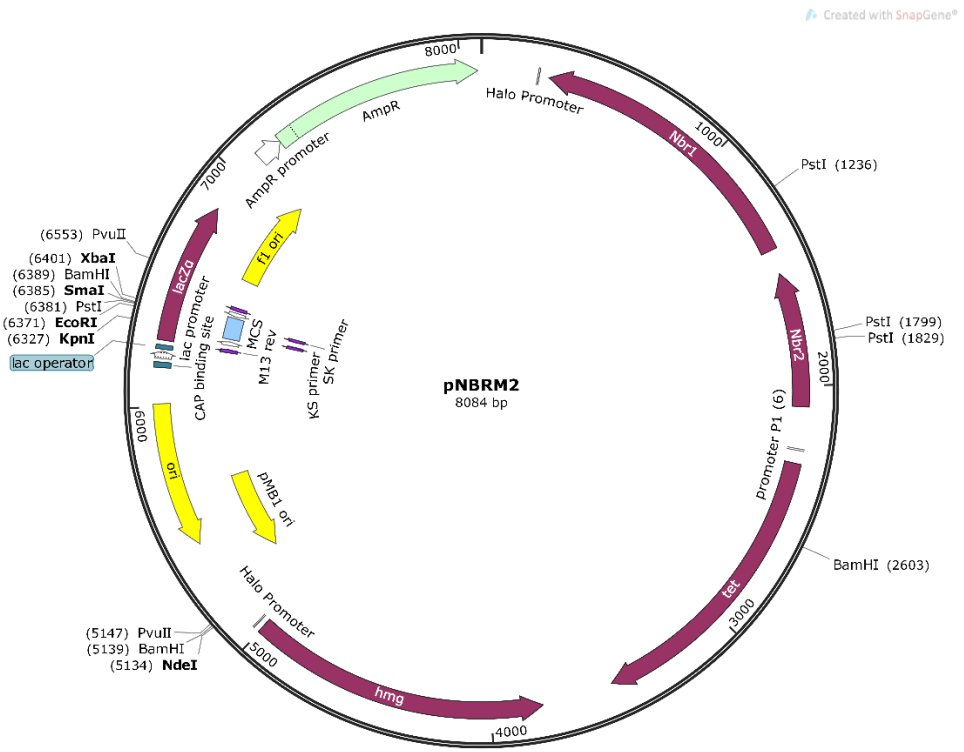

Figure 7: Plasmid map of pNBRM2 including restriction sites for cloning and for identification

The strains containing the new plasmids should also be tested to see if there is any change in the minimum inhibitory concentration(MIC) of *N. magadii* transformed with the redesigned pNB102 plasmids. A change in MIC would indicate that the concentration of mevinolin in liquid and solid growth media should be increased or decreased to compensate for any changes in MIC and maintain maximum efficiency and specificity of transformation.

4.5. PCNA of ΦCh1

pRO-5-MI-1 plasmids containing the BgaH gene under the control of the promotor region of ORF34 were co transformed with pNB102 containing one of three variants of $PCNA_{\Phi Ch1}$ under the control of the promotor region of ORF34 (pP-GTG, pP-ATG, pP-Tr). The plasmids were transformed into the cells and positive colonies isolated and grown for testing. empty plasmid controls were also transformed. A strain containing the BgaH gene under the control of the fdx promotor was used. This strain was used to determine that the Assay conditions were correct to obtain the previously reported activity levels

The following experimental strains were created by double transformation of *N. magadii* L13

- *N. magadii* L13 (pP-ATG, pRO5-MI-I)
- *N. magadii* L13 (pP-GTG, pRO5-MI-I)
- *N. magadii* L13 (pP-Tr, pRO5-MI-I)

These strains were transformed to determine 1) if the PCNA of ΦCh1 interacts with the origin of replication of ΦCh1 which is used in the shuttle vector plasmid pRO5.

The following control strains were created by double transformation of *N. magadii* L13

- *N. magadii* L13 (pP-ATG, pRO5)
- *N. magadii* L13 (pP-GTG, pRO5)
- *N. magadii* L13 (pP-Tr, pRO5)
- *N. magadii* L13 (pNB102, pRO5-MI-I)
- *N. magadii* L13 (pNB102, pRO5)

The following control strains were grown to be used as control strains that had previously been created by single transformations of *N. magadii* L13

- *N. magadii* L13 (pRO5-MI-I)
- *N. magadii* L13 (pRO5)
- *N. magadii* L13 (pNB102)
- *N. magadii* L13 (pRO5-fdx-BgaH)

All strains were positively identified by test PCRs as listed in Table 1 and precultures grown for inoculation of test cultures.

Test PCRs performed as follows:

Plasmid name	Primer 1	Fragment size
pNB102	NB-32/MevR-4	785bp
pRO5	D49-3/D49-4	1154bp
pP-ATG, pP-GTG, pP-Tr	34-p5-XbaI/PCNA-22	1105bp (pP-Tr) 1155bp (pP-GTG, pP-ATG)
pRO5-MI-I	BgaH-P/BgaH-3i	1006bp
pRO5-fdx-BgaH		

Table 1: The plasmids contained in strains used to assay the interaction of $PCNE_{\Phi Ch1}$ and the origin of replication of ϕCh1.

As can be seen in Fig. XY, an increase in BgaH activity could be detected when strains contain an additional copy of the virus-encoded PCNA. However, there were no differences between these variations of PCNA, which could not be seen in the lysogenic strain *N. magadii* L11 (see manuscript of this thesis). In addition, the increase in activity of BgaH was very low. Therefore, these experiments have to be repeated.

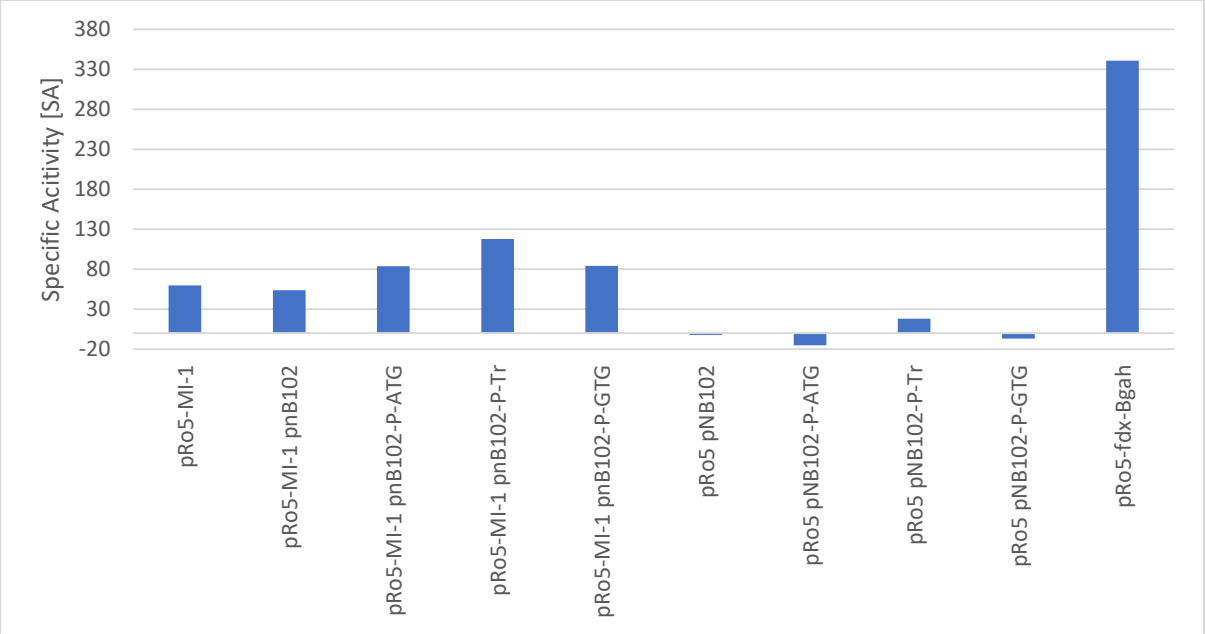

Figure 8: BgaH assay results for strains that grew to a maximal OD_{600} of 0.2.

When attempting to obtain a measurement on the BgaH activity for pRo-5-fdx-BgaH that matched previously reported values is was found that the addition of trace elements was required to recapitulate those results.

5. Conclusion

5.1. NgAgo

A homologous strain of *Natronobacterium gregoryi* ΔNg*Ago*::*gyrB*(Nov^R) as verified by analytical PCR was obtained. It displayed no overt changes in behaviour of the wildtype strain when compared for induction of lysis when incubated with ΦCh1 particles. viral DNA transformation, and plasmid transformation efficiency. Its interaction with pregenomic RNA indicates that it could possibly be a DNA guided RNAse, or targets DNA/RNA duplexes. In which case neither the dsDNA halovirus ΦCh1 or plasmid DNA would be the type of foreign nucleic matter NgAgo defends against.

A southern blot is required to verify the genotype of the strain.

Western blots are still required to determine if there is a knockout of NgAgo production

5.2. Pilin

The protein encoded by *N. magadii* ORF1379 is a likely essential gene and a homologous knockout strain was not able to be isolated. As such only heterologous deletion mutant strains could be obtained. Using

increasingly high concentration of the antibiotic Novobiocin during passaging, strains produced increasingly less Pilin protein as verified by Western blot. It is possible that further passages at increasingly high concentrations of novobiocin may lead to a homogenous knockout strain. The cultures obtained by passaging were analysed using an Adsorption assay to determine if there was a variation in the rate at which virus particle adsorbed to the surface of the WT and the heterologous deletion mutants. The results of the adsorption assay strongly suggest that pilin is not involved in the initial binding of ΦCh1 to the cell surface of *N. magadii.*

5.3. Redesign of pNB102

pNB102 was redesigned into a 3000bp smaller shuttle vector termed pNBRM1 without any significant loss off function.

From pNBRM1 a vector was created with alternating genes for mesophilic bacteria and haloalkaliphilic archaea to potentially decrease recombination of the plasmids in *E. coli.* This plasmid requires further testing

5.4. PCNA

The strains required for the designed experiment have all been created. Preliminary BgaH results indicated that there may be an increase in the specific BgaH activity of strains containing plasmids expressing ΦCh1-ORF59 from pNB102 and the BgaH gene on pRO5.This may indicate that $PCNA_{\Phi Ch1}$ interacts, directly or indirectly, with the ΦCh1 origin of replication. BgaH assay need to be repeated with cultures of OD_{600}. 0.5-0.6 to obtained meaningful results.

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7. The PCNA of halovirus ϕCh1 precisely regulates viral activity with a 21-amino acid N-terminal extension relative to the highly homologous host-specific PCNA

Contribution to the paper “The PCNA of halovirus φCh1 precisely regulates viral activity with a 21-amino acid N-terminal extension relative to the highly homologous host specific PCNA”

The expression plasmid construction, complementation and overexpression strain construction, complementation and overexpression strain analysis, growth curves, virus titre, Western blots of Mtase and gp-34, and manuscript preparation of the following paper was performed by Richard Manning. Sonja Sabitzer performed the initial Western blots of PCNA. Michael Tschurtschenthaler constructed the homogenous mutant *N. magadii* L11-ΔP. Dina Ada Sabitzer performed the Western blots of PCNA and Protein E. Angela Witte designed the experiments, performed the Southern blot, virus titre, and edited the manuscript.

Article

The PCNA of halovirus ϕCh1 precisely regulates viral activity with a 21-amino acid N-terminal extension relative to the highly homologous host-specific PCNA

Richard Manning , Michael Tschurtschenthaler, Sonja Sabitzer, Dina Ada Sabic, and Angela Witte.

Abstract: Proliferating Cell Nuclear Antigen (PCNA) is a scaffold protein principally found at the center of replication, coordinating with a wide array of interaction partners. ϕCh1, unusually for a virus, encodes a putative PCNA. *Natrialba magadii,* the only known host of ϕCh1, also encodes a putative PCNA of high sequence similarity, differing majorly in the presence of an alternate GTG start codon in the ϕCh1 variant 5′ of the ATG start codon resulting in a larger protein. Homologous recombination was used to delete the viral PCNA gene. Complementation and overexpression strains with plasmid expressed viral PCNA variants were created and analyzed for growth and lytic behavior, viral protein levels, and virus titer. Deletion of ϕCh1-ORF59 encoding PCNA$_{\Phi Ch1}$ resulted in a significant reduction in the virus titer, reduced viral protein load, and reduced lysis. Complementation and overexpression revealed that the 5′ GTG to ATG sequence enables ϕCh1 to finely control its equilibrium with the host. The different variants have a broad range of variable effects on each of the phenotypes observed. The virally encoded PCNA is not essential, but is crucial, to the behavior of the virus ϕCh1. The 5′ region upstream of the primary ATG codon and its regulation with an alternate start codon is essential to the homeostasis of the virus-host relationship.

Keywords: PCNA; *Natrialba magadii*; ΦCh1

1. Introduction

Proliferating Cell Nuclear Antigen (PCNA) is a crucial processivity factor and binding scaffold in the replication fork from which point in regulates the activity of a wide array of proteins and plays a key role in cell function and activity including cancer genesis [1][2][3]. PCNA forms a trimeric (Eukarya and Archaea), or dimeric (Bacteria) sliding clamp, primarily known as an essential component of the replication fork, but also has roles in DNA repair, DNA methylation, chromatin remodeling, and cell cycle regulation[2][4]. All activities described for the PCNA proteins require them to encircle the duplex; no biochemical function for PCNA off DNA has been reported.[4]

Natrialba magadii is a member of the Halobacteriaceae family of the euryarchaeota branch of the archaeal domain which also includes the crenarchaeota and thaumarchaeota branches [4]. Most members of the euryarchaeota and thaumarchaeota encode for a single PCNA gene that forms homotrimers, however some members of the crenarchaeota branch contain three distinct PCNA genes that yields an active heterotrimeric form[5][6]. The crystal structure of the PCNA of *Haloferax volcanii* (HvPCNA), another member of the *Halobacteriaceae* family, was solved revealing that in comparison to PCNA found in other organisms the positive surface charge at the interior surface of the PCNA trimer was significantly reduced. It is hypothesized that bound cations within the shell of HvPCNA may reduce the effect of electrostatic repulsion and allow sliding along the negatively charged DNA [7].

ϕCh1 has only one known host and is the only virus known to infect the haloalkaliphile *Natrialba magadii* [1]. It has a head and tail morphology, the capsid contains both RNA and a 96kbp dsDNA genome

packed with a headful mechanism[8]. Unusually for a virus, ϕCh1 encodes a putative PCNA, with a high sequence similarity to a putative PCNA produced by the host indicating recent horizontal gene transfer[9]. Intriguingly, ϕCh1 also encodes an alternate GTG start codon 63bp upstream of the primary ATG start codon. The canonical start codon AUG is the LacI repressor has GUG start codon[10]

Homologous recombination was used to disrupt ϕCh1-ORF59. Complementation and overexpression strains containing ϕCh1-ORF59 plasmid expressed variants, were analysed for lytic behaviour, viral protein levels with Western blots, and virus titres.

2. Materials and Methods

All PCRs for the amplification of DNA for the purposes of cloning were performed using Phusion High-Fidelity DNA Polymerase according to the manufacturer's instructions (Thermo Fisher Scientific Inc., Waltham, MA, USA). PCRs for screening of *N magadii* and *E. coli* transformants were performed using GoTaq® DNA Polymerase according the to manufacturer's instructions (Promega Corporation, Madison, WI, USA).

ORF59 of ϕCh1 was amplified using primers p30-5/p30-3 (bp) and ϕCh1 DNA as a template, the amplified fragment was restricted with *Bgl*II and *Hind*III and ligated into pRSET-A also restricted with the *Bgl*II and *Hind*III, resulting in plasmid pPol5 which was transformed into *E. coli* Bl21(DE3) competent cells. Protein was expressed and purified using the same method as previously reported for Rosetta strains[11]. Protein was isolated twice and used for the generation of two separate rabbit polyclonal antibody sera (Moravian-Biotechnology, Ltd., Brno, Czech Republic).

Open Reading Frame 59 (ORF9) of ϕCh1 was deleted using the homologous recombination plasmid pKSII-PCNA1-5-NovR-R created as follows. The region upstream of ORF59 was amplified using ϕCh1 DNA as template with the primers ΔPCNA-1/ΔPCNA-2 (1240bp), the fragment and pKSII+ were separately restricted with *Xba*I and *Sma*I, purified, and ligated together. Positive clones were named pKSII-PCNA1-2. The flanking region downstream of ORF59 was PCR amplified with PCNA-3 /PCNA-5 (629bp), restricted with *Hind*III and *Kpn*I and ligated into pKSII-PCNA1-2 also digested with *Hind*III and *Kpn*I. Positive clones were named pKSII-PCNA-1-5. The *gyr*B gene was released from pMDS11[12] with *Hind*III and *Sma*I (2448bp) and ligated into plasmid pQE30 restricted with *Hind*III and *Sma*I resulting in plasmid pQE30-NovR. The *gyr*B was amplified from pQe-30-NovR with primers NovR-1p/NovR-1s(bp), restricted with *Pst*I and *Sma*I and ligated into pUC19 also restricted with *Pst*I and *Sma*I resulting in pUC19-NovR"reverse". The *gyr*B gene (2453 bp) was released from the vector pUC19-NovR "reverse" with *Dra*I, *Sma*I, and *Pst*I, and ligated into pKSII-PCNA-1-5 digested with *Sma*I and *Pst*I, creating pKSII-PCNA-1-5-novR "reverse"(pKSII-PCNA1-5-NovR-R).

pKSII-PCNA1-5-NovR-R was transformed into competent *N. magadii* L11 as previously reported[11]. Resultant colonies were PCR tested for the presence of 5′ homologous crossover with primers 56-5/Nov-13(1992bp) and 3′ crossover with Nov-12/PCNA-3en(1065bp). A homozygous or genetically pure mutant strain was obtained using the same method as previously reported for ORF79 of ϕCh1 [11]. Following homozygation of the mutant, individual colonies were again tested for the presence of both 3′ and 5′ crossover as well as for the absence or presence of the ORF59 with p30-5/p30-3(764bp). The results of the PCR data were confirmed by Southern blot genotyping was carried as previously reported [13] and the positive strain designated as L11-*ΔP*.

The promotor region of ORF34 of ϕCh1 was amplified from ϕCh1 template DNA with 34-p5-Xba1/34-p3(326bp), restricted with *BamH*1 and *Xba*I and ligated into pKSII+ also restricted with *BamH*1 and *Xba*I resulting in plasmid pKS-34up.

The variants of ORF59 were amplified with PCNA-GTG/PCNA-22 (827bp), PCNA-ATG/PCNA-22 (827bp), and PCNA-Tr/PCNA-22 (754bp), restricted with *Eco*RI and *Kpn*I. Fragments were ligated into pKS-

34up restricted with *EcoR*I and *Kpn*I resulting in plasmids pKS-34up-P-GTG, pKS-34up-P-ATG, pKS-34up-P-Tr. promotor region of ORF34 and ORF59 were released from the plasmids with *Xba*I and *Kpn*I and ligated in pNB102 likewise restricted with *Xba*I and *Kpn*I. Resulting in the construction of three complementation plasmids pP-GTG, pP-ATG, and pP-Tr respectively.

Each of the complementation plasmids and pNB102 were transformed into L13. Each of the four resultant strains was then infected with virus particles from either L11 or L11-ΔP using a reinfection strategy as previously reported[11] resulting in 8 strains for analysis (see Table 1).

All *N. magadii* strains used in this study were grown in rich medium containing 8.8 g l^{-1} of casein hydrolysate and 11.7 g l^{-1} yeast extract in 4M NaCl, 1mM $MgSO_4$, 0.02mM FeSO4, 36mM Na2CO3, 31.5mM KCl, and 3 mM Na3-citrate, at 37°C shaking at 160rpm. Optical density was measured at 600nm. Novobiocin and mevinolin were added to a final concentration of 3 and 7.5 μg/ml, respectively.

Spheroblast generation, transformation, and transformant screening in *N. magadii* strains was performed as previously reported [11].

DNA isolation from halophilic *Archaea*. Chromosomal DNA of *N. magadii* was isolated by phenol-chloroform extraction, followed by precipitation with isopropanol as previously described [14]. Southern blot genotyping was performed as previously described[15].

Virus titres were determined as described previously [14].

Cell extracts for Western blot analysis were prepared as described previously [14]. Western blot analyses were performed as previously described [16]. Detection was performed with 3ml of 0.113mM p-coumaric acid, 1.25mM 3-Aminophthalhydrazide in ddH_2O, and 1μl of H_2O_2 and imaged with Biolabs XRS ChemiDoc.

3. Results

Initial analysis

A polyclonal antibody with specificity to the ϕCh1 encoded PCNA (α-PCNA) was developed. Western blots using this antibody revealed that it's affinity to the PCNAs of *H. volcanii* and *H. salinarum,* and possibly the PCNA produced by the immunity conferring plasmid pϕHL derived from the central L-region of ϕH [17](Figure 1B).

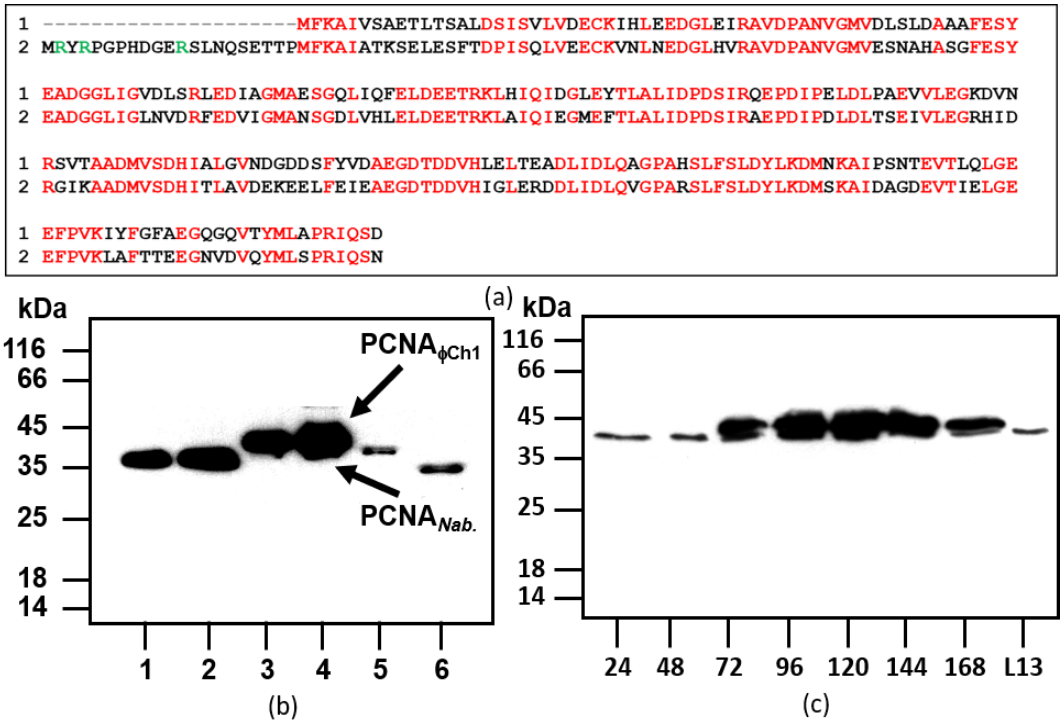

Figure 1: *N. magadii* L11 produces a second larger PCNA protein compared to L13. (a) Amino acid alignment of 1) PCNA$_{Nab}$ and 2) PCNA$_{\phi Ch1}$ including the N-terminal extension of PCNA$_{\phi Ch1}$. Identical AA are indicated in red. (b) Western blot with α-PCNA of closely related halophilic strains-Lanes: 1) *H. salinarum* R$_1$, 2) *H. salinarum* R$_1$-L, 3) *N. magadii* L11 (72hrs), 4) *N. magadii* L11 (120hrs) 5) *N. magadii* L13, 6) *H. volcanii*. (c)Western blot analysis of *N. magadii* L11 protein samples taken every 24 hours after inoculation with α-PCNA, the final lane contains L13 crude extract as a control.

Western blots of *N. magadii* L11 crude extracts using this antibody revealed a second higher signal, compared to the cured L13 strain, appearing 72 hours after inoculation with band intensity peaking at 120 hours (Figure 1C). This provides evidence that PCNA$_{\phi Ch1}$ is, at least in part, translated using the alternate GUG start codon encoded upstream of the ATG consensus sequence in ORF59. This raised the question as to whether PCNA$_{\Phi Ch1}$ is essential for the replication cycle of ϕCh1.

Construction and analysis of N. magadii *L11-ΔP*

To determine the relevance of PCNA$_{\Phi Ch1}$ a deletion mutant was created using homologous recombination via the suicide plasmid pKSII-PCNA1-5-NovR-R resulting in the knock out deletion of ORF59 and the insertion of the novobiocin resistance conferring gene *gyr*B in the ORF59 locus. PCNA$_{\Phi Ch1}$ is not necessary for the virus to replicate but does enhance the production of virus particle and viral proteins.

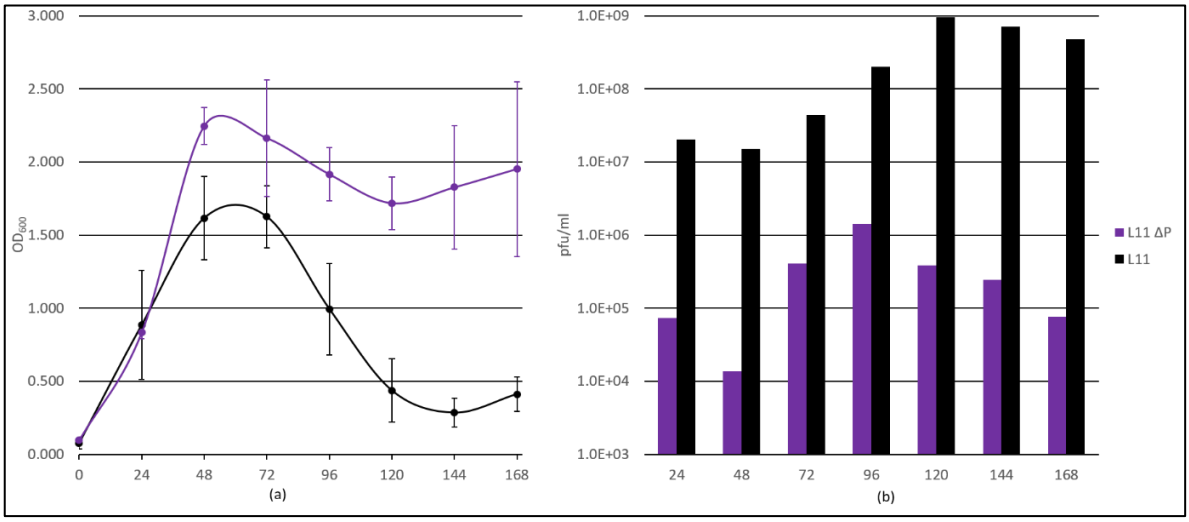

Figure 2: Growth and virus progeny production of the wild type strain *N. magadii* L11 and the mutant strain *N. magadii* L11-ΔP (a) Growth curves of strains *N. magadii* L11 and *N. magadii* L11-ΔP. (b) Virus titres of *N. magadii* L11 and *N. magadii* L11-ΔP.

The disruption of PCNA$_{\Phi CH1}$ does not eliminate the lysis phenotype, nor does it affect the timing of lysis induction, however it results in a significant increase in peak OD_{600}, and a highly significant increase in post lysis optical density (Figure 2A). Both *N. magadii* L11 and *N. magadii* L11-ΔP grow similarly for the first 24hrs of incubation indicating that the growth of the host during the exponential phase is not affected by PCNA$_{\Phi Ch1}$.

The disruption of ORF59 results in a minimum two magnitude decrease in virus titer over all sampled timepoints and an earlier peak titer compared to wildtype *N. magadii* L11(Figure 2B). The virus titre data in concert with the growth curve data reveals that PCNA$_{\Phi Ch1}$ is not essential for the replication cycle of ΦCh1, but it does play an important role in the ability of the strain to produce viral progeny.

To further investigate the function of PCNA$_{\Phi Ch1}$ *N. magadii* L11-ΔP and *N. magadii* L11 were transformed with pNB102 shuttle vectors expressing one of three variants of ORF59 under the control of the promoter region of ORF34 of ΦCh1[11]. Plasmid pP-GTG expresses the "wild-type" form of PCNA$_{\Phi Ch1}$, pP-ATG has a point mutation of the GTG to ATG compared to pP-GTG, and pP-Tr expresses a 5′ truncated variant starting from the ATG consensus sequence. In addition, both *N. magadii* L11 and *N. magadii* L11-ΔP were transformed with empty pNB102 plasmids as the presence of the mevinolin selection marker on pNB102 may results in a change in growth behavior, possibly due to growth rate limiting effects of the metabolically important mevalonate pathway. A comparison of the growth rates of the strains *N. magadii* L11, *N. magadii* L13, and *N. magadii* L11-ΔP with and without pNB102 can been see in figure S2.

strains	
Overexpression	***Complementation***
N. magadii L11 (pNB102)	*N. magadii L11-ΔP (pNB102)*
N. magadii L11 (pP-ATG)	*N. magadii L11-ΔP (pP-ATG)*
N. magadii L11 (pP-Tr)	*N. magadii L11-ΔP (pP-Tr)*
N. magadii L11 (pP-GTG)	*N. magadii L11-ΔP (pP-GTG)*

Table 1: Strains created for the analysis of PCNA$_{\Phi Ch1}$.

Analysis of complementation strains

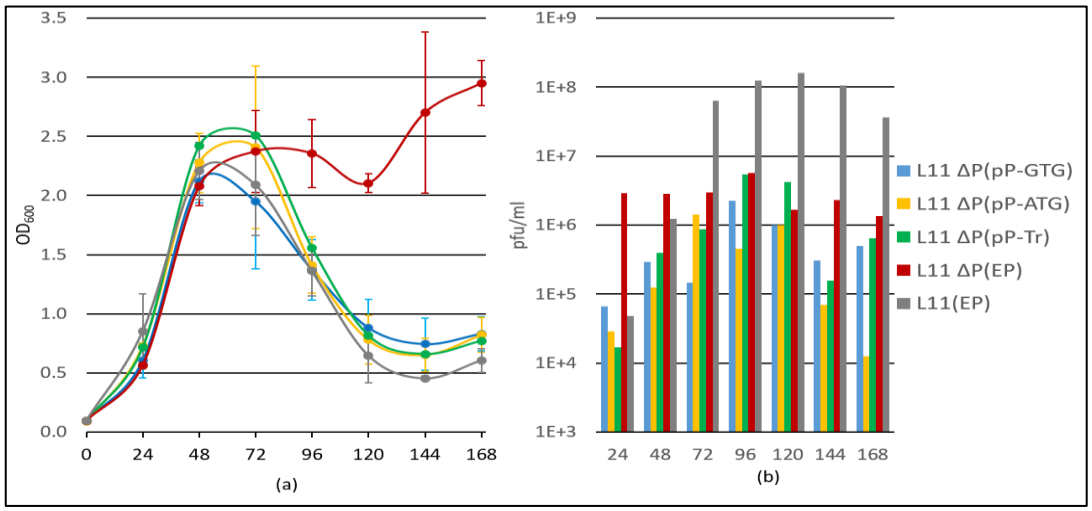

Figure 3: Growth and virus progeny production of the complemented strains (a) Growth curves of the complemented strains with *N. magadii* L11 (pNB102) and *N. magadii* L11-ΔP (pNB102) included as controls. (b) Virus titres of the complemented strains with *N. magadii* L11 (pNB102) and *N. magadii* L11-ΔP (pNB102) included as controls.

Growth curves of the complemented strains

Complementation of the deletion mutant *N. magadii* L11-ΔP with all three of the PCNA$_{\Phi Ch1}$ variants leads to rescue of the lysis phenotype. *N. magadii* L11 (pNB102) has a slightly lower final OD$_{600}$ than any of the complemented strains, indicating a greater overall degree of lysis. *N. magadii* L11-ΔP (pP-GTG) starts to lyse at a similar timepoint to *N. magadii* L11 (pNB102) while *N. magadii* L11-ΔP (pP-ATG) and *N. magadii* L11-ΔP (pP-Tr), the strains complemented with the truncated and altered start codon variants of ORF59, start to lyse at a slightly later time point, although these differences are not significant as there is a high degree of OD$_{600}$ variation within induvial strains at the onset on lysis.

Virus titre of the complemented strains

Unlike the growth and lysis, complementation of the knockout strains does not result in a rescue of the virus titre. Rather, the complemented strains have a lower virus titre than the uncomplemented deletion strain. The virus titre kinetics are altered in the complemented strains in comparison to the uncomplemented knockout strain. *N. magadii* L11-ΔP (pNB102) has a relatively static virus titre over the analysed time-period, in comparison to the WT strain with has a low virus titre over the first two days, increasing significantly on day three and peaking on day five. The complemented strains, like the WT strain, have a low virus titre on days one and two, with *N. magadii* L11-ΔP (pP-ATG) peaking on day three and the strains complemented with the WT and truncated variants of ϕCh1-PCNA having a peak virus titre on day four.

Protein production of complementation strains

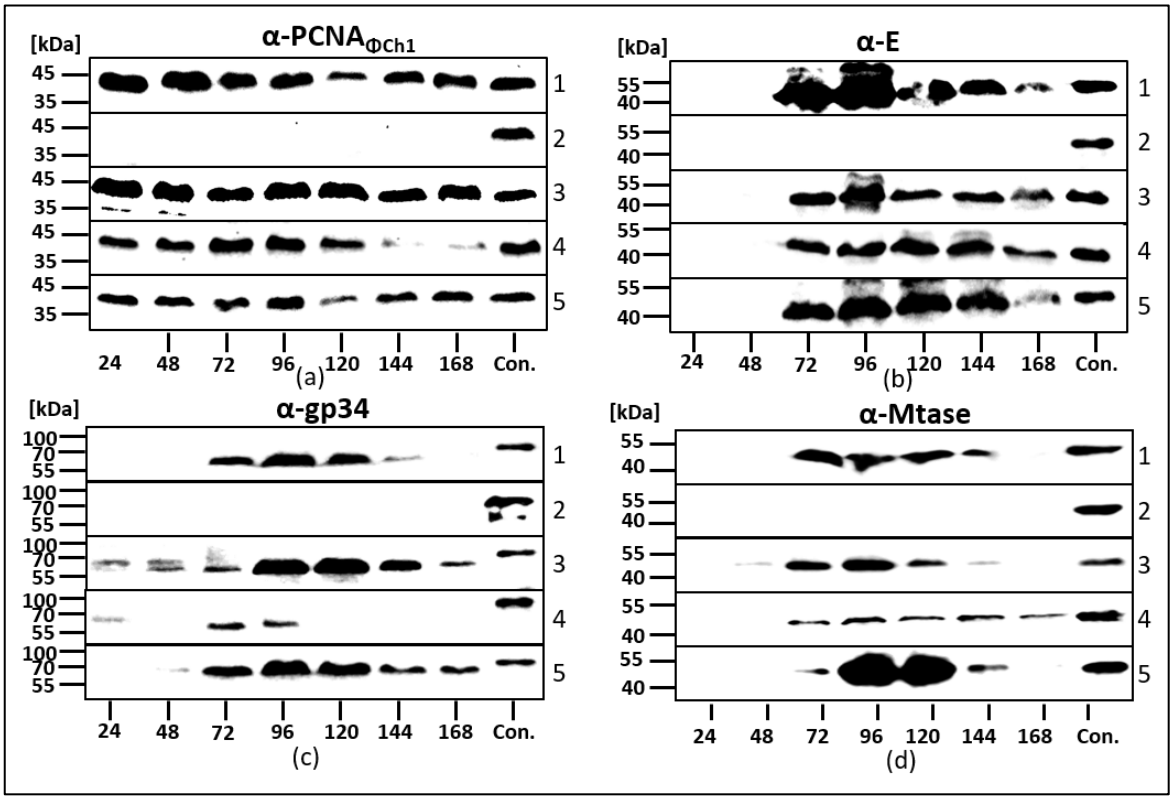

Figure 4: Protein levels of the complementation strains as measured by Western blot. (a) α-PCNA$_{ΦCh1}$ (b) α-E (c) α-gp34 (d) α-Mtase. 1) *N. magadii* L11 (EP), 2) *N. magadii* L11-ΔP(EP), 3) *N. magadii* L11-ΔP (pP-ATG), 4) *N. magadii* L11 (pP-Tr), 5) *N. magadii* L11 (pP-GTG). Lanes 1 to 7: samples were taken 24 to 168 h after infection. Con: proteins isolated from *E. coli* were taken as a control (lane 8 in each panel). Size markers are indicated at the left.

Deletion of ORF59 leads to undetectable levels of PCNA, Mtase, gp34, and protein E in *N. magadii* L11-ΔP (pNB102) when analysed via Western blot (Figure 4, panel 2). Complementation with each of the PCNA$_{ΦCh1}$ variants results in a return to detectable levels of all proteins (Figure 4, panels 3, 4, and 5). However, the protein production kinetics differ between strains and these differences also vary between the proteins analysed.

The blots with α-PCNA$_{ΦCh1}$ (Figure 4A) indicate that the wild type ORF59 expressing strain has a similar protein production kinetic to *N. magadii* L11 (pNB102) and the ATG exchange of strain *N. magadii* L11 (pP-ATG) has a higher production of protein than either the truncated or wild type of ORF59 expressing strains and higher even than the control strain at most time analysed time points. The truncated ORF59 expressing strain has similar levels of PCNA protein to the control, GTG and ATG exchanged expressing ORF 59 strains for the first five-time points but decreases considerably compared to the others 144 and 168 hours, possibly a sign that the GTG start codon is necessary for the production of PNCA$_{ΦCh1}$ under lytic stress conditions.

Western blots using α-E do not reveal significant differences in major capsid protein E production between the complemented strains. The wild type ORF59 expression strains produces slightly more protein than either the ATG exchanged or truncated strains. The blots also reveal that none of the strains produces as much capsid protein as *N. magadii* L11 (pNB102), possibly an indication as to why the virus titre levels in the complemented deletion strains are not as high as the wild type.

α-gp34 blots show a higher amount of protein in the GTG and ATG exchanged ORF59 expression strains than *N. magadii* L11 (pNB102) (Figure 4 c). The ATG exchanged ORF59 expressing strains has detectable levels of gp34 in each of the time points, in contrast to the wild type control which has no detectable levels in the first two and final time points. The GTG expressing strain has detectable gp34 levels 24, 48 and 168 hours after inoculation when it is not detectable in the wild-type strain. The truncated ORF59 expressing strain has the lowest levels of detectable protein of the complemented strains with a small amount detectable at 24 hours, likely remaining from the previous passage and otherwise detectable protein in only the 72 and 96-hour time point samples. This indicates that the 21-amino acid N-terminal extension of PCNA$_{\Phi Ch1}$ helps to regulate the expression of gp34.

Western blots with α-Mtase show an earlier production of Mtase in the ATG exchanged ORF59 complemented strains, lower levels and extended production in the truncated ORF59 expressing strain, and very high protein levels at 96 and 120 hours in the GTG variant expressing strain. Again, this demonstrates the differential regulation of proteins by the variants of PCNA$_{\Phi ch1}$.

Analysis of overexpressing strains

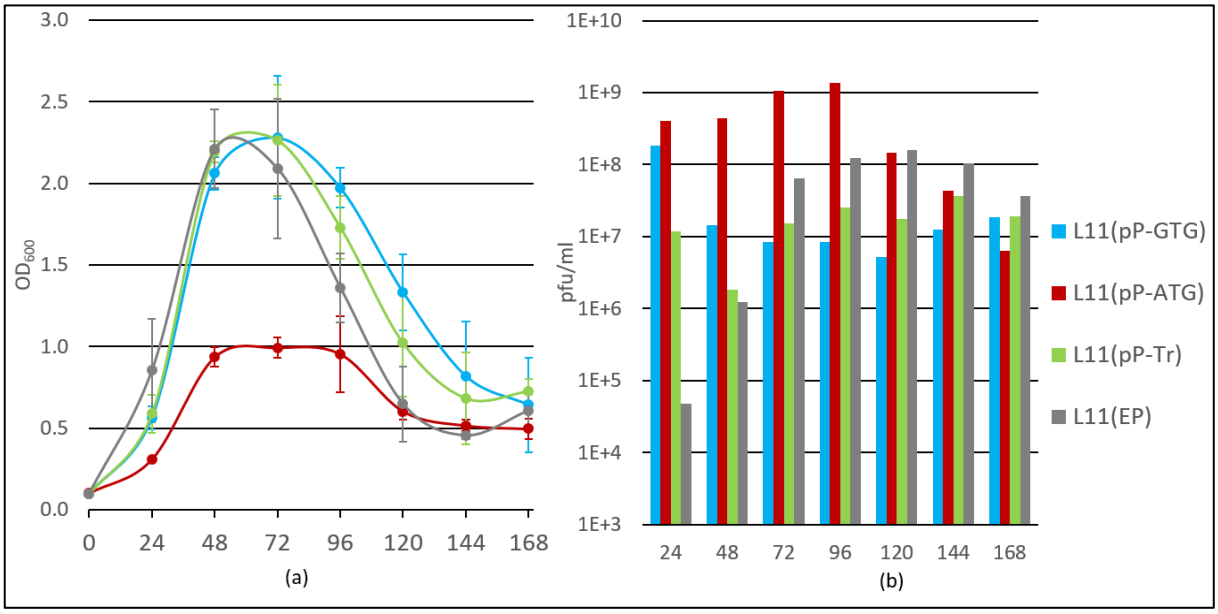

Figure 5: Growth and virus progeny production of the complemented strains (a) Growth curves of the complemented strains with *N. magadii* L11 (pNB102) and *N. magadii* L11-ΔP (pNB102) included as controls. (b) Virus titres of the complemented strains with *N. magadii* L11 (pNB102) and *N. magadii* L11-ΔP (pNB102) included as controls.

Growth curves of overexpressing strains

The presence of pP-ATG leads to a significant reduction of the growth of *N. magadii* L11 over the analysed time-period (Figure 5 a). The strains harbouring plasmids coding for the WT and truncated variants of ϕCh1-PCNA have a similar growth rate in the exponential phase and a similar rate of lysis after a slightly delayed onset in comparison to the WT strain. This is effect is slightly more noticeable in *N. magadii* L11 (pP-GTG). The strains transformed with the WT and truncated PCNA$_{\Phi Ch1}$ encoding plasmids showed a slightly reduction in total virus particle production compared to both *N. magadii* L11 (pNB102) and *N. magadii* L11.

Virus titre of the overexpressing strains

The virus titre of *N. magadii* L11 (pP-ATG) as with its growth curve, has an altered kinetic of virus particle production. Compared to the wild type it produces at least one magnitude more virus particles for the first three days of the time series and an equal amount on day four. Strains harbouring plasmids containing the WT and Truncated versions of ϕCh1-PCNA have pfu counts higher than the control strain on days one and two, while the control strains has higher virus titres on the final four time points. The strains transformed with the WT and truncated PCNAΦCh1 encoding plasmids showed a reduction in total virus particle production compared to both L11 pNB102 and L11.

The *N. magadii* L11 and *N. magadii* L11 (pNB102) strains produced the least virus particle in the first 48 hours after inoculation and the greatest number of virus particles 120 hours after inoculation. While the strains contained WT and truncated $PCNA_{\Phi Ch1}$ did not display a steadily increasing number of virus particles being produced following inoculation

The strain transformed with the plasmid containing the mutated start codon results has a significantly altered growth and lysis phenotype. This strain has a retarded growth profile and has a significantly lower optical density than all other strains including *N. magadii* L11. The virus titer of *N. magadii* L11 (pP-ATG), as with its growth curve, has an altered kinetic of virus particle production. Compared to the wild type it produces at least one magnitude more virus particles for the first three days of the time series and an equal amount on day four. Strains harboring plasmids containing the WT and Truncated versions of ϕCh1-PCNA have pfu counts higher than the control strain on days one and two, while the control strains has higher virus titers on the final four timepoints.

The strain containing the empty plasmid showed a decrease in the total amount of virus particles produced during the samples time-period as well as an order of magnitude decrease in the maximum measured virus titer. Overexpression with both the truncated and WT variants of ϕCh1-PCNA has comparable effects on the protein expression Mtase or gp34. The presence of the plasmid containing the mutated ORF59 with theGTG start codon in detectable levels of Mtase one day earlier than for the Expression of ORF59 with a point mutation of the GTG start codon to an ATG start codon when expressed from a plasmid in the wild type background results in a significant increase in the early virus titer and a reduction in peak culture density.

Protein production of overexpressing strains

Western blots using α-PCNA (Figure 6A). The images obtained for *N. magadii* L11 (pP-ATG) reveal an exceptionally high amount of detectable protein in comparison to any of the other strains. Indicating that a mutation of ATG>GTG at the 5′ end of the extension of ⊚Ch1-ORF59 results in a strong over production of $PCNA_{\Phi Ch1}$. The levels of protein in *N. magadii* L11(pP-GTG) and *N. magadii* L11(pP-Tr) are lower than in the wild type control LL(EP), in agreement with the virus titre levels.

Western blots with α-E (Figure 6 b) show as in the complemented deletion strains vary considerable between the strains. *N. magadii* L11 (pP-ATG) only has detectable protein E levels from 72-144 hours, which is unexpected given the high virus titre of the strain at the 24 and 72-hour time points. *N. magadii* L11 (pP-Tr) has detectable levels of protein E at all seven time points.

gp34 levels (Figure 6 c) do not alter significantly from *N. magadii* L11 (pNB102) in either *N. magadii* L11 (pP-GTG) or *N. magadii* L11 (pP-Tr). However, the levels in *N. magadii* L11 (pP-ATG) are reduced in comparison to *N. magadii* L11() and the other overexpression strains. This may not be due to a reduced production of gp34, but rather the increased rate of early lysis which reduces the build-up of the protein in the host cells and instead increased the amount of protein in the supernatant.

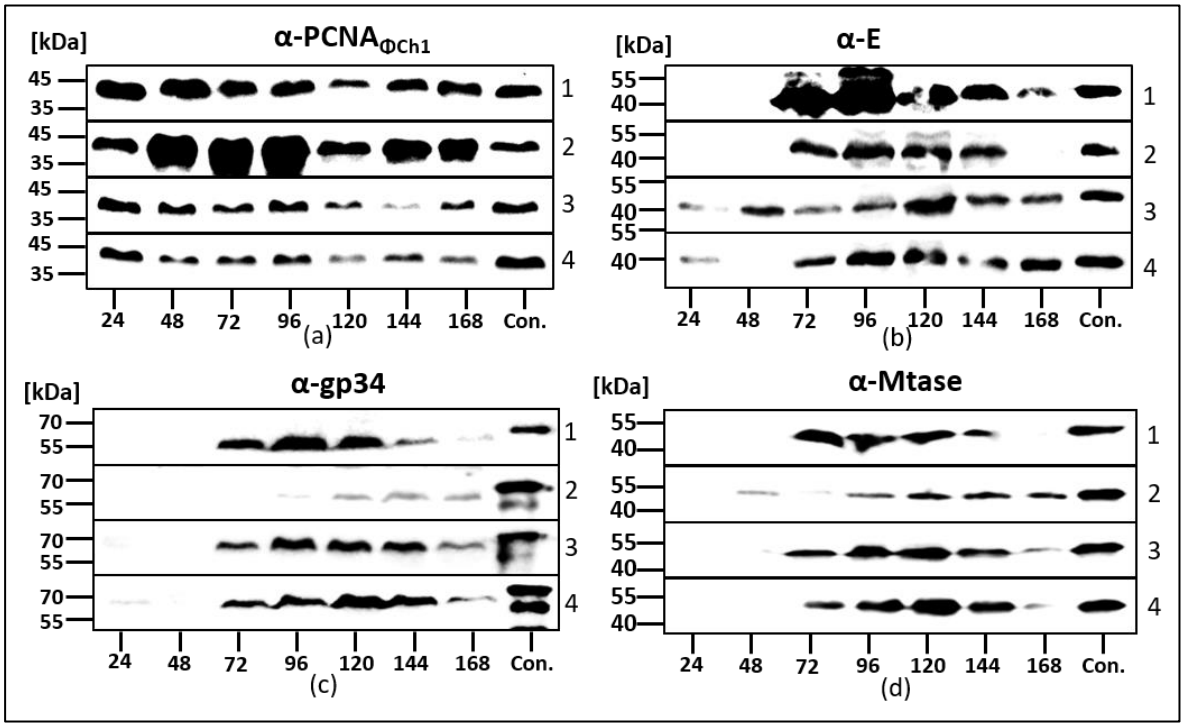

Figure 6: Protein levels of the over expressing strains as measured by Western blot. (a) α-PCNA$_{ΦCh1}$ (b) α-E (c) α-gp34 (d) α-Mtase. 1) *N. magadii* L11 (EP), 2) *N. magadii* L11-ΔP(EP), 3) *N. magadii* L11-ΔP (pP-ATG), 4) *N. magadii* L11 (pP-Tr), 5) *N. magadii* L11 (pP-GTG). Lanes 1 to 7: samples were taken 24 to 168 h after infection. Con: proteins isolated from *E. coli* were taken as a control (lane 8 in each panel). Size markers are indicated at the left.

As with the protein levels of PCNA$_{Φch1}$, protein E and gp34, the levels of Mtase (Figure 6 d) are broadly similar in *N. magadii* L11 (pNB102), *N. magadii* L11 *N. magadii* (pP-GTG) and *N. magadii* L11 (pP-Tr), they do however peak in concentration 24-48 hours later in the two overexpression strains and Mtase is detectable in both at 168 hours which it is not in the control strain. As with the three other analysed proteins, the *N. magadii* L11 (pP-ATG) western blot with αMtase reveals significant differences to the wild type control and the other overexpression strains. Mtase is detectable 24 hours earlier than in the other strains and is otherwise present at much lower levels in the other time point sample. The lower levels may again be due to the high rate of lysis reducing the build-up of viral proteins in the host cells.

4. Discussion

Deletion of ORF59 resulted in a significant reduction in the virus titre, viral protein levels, and aberrant lytic behaviour. Three complementation variants resulted in reversal of the lytic phenotype, a variety of effects on the viral protein levels, and a failure to return virus titre levels comparable to the wild type. Overexpression with a full-length wild-type variant and a ‘5 truncation variant of ORF59 resulted in a reduction in the virus titre. Contrastingly, overexpression with the pP-ATG point mutation variant of ORF59 induced a significantly increased virus titre and a significantly reduced growth rate.

The analysis of growth reveals that ORF59 plays a critical role in the lytic behaviour of ΦCh1, and, that this behaviour is largely regulated by the region downstream of the ATG consensus sequence when compared to the host encoded PCNA. The results of the virus titre and western blot experiments demonstrate that the production of viral progeny is highly sensitive to the amount and variant of PCNA$_{ΦCh1}$ produced by the cells.

The western blot data provides evidence that the PCNA of ΦCH1 may have additional roles in gene expression and protein production. It can also be observed that the expression of different variants of the

ORF59 have differential effects on viral protein production. This possibly indicates that the 21-amino acid N-terminal extension has a significant direct or indirect effect on the proteome of ΦCh1

The GTG to ATG exchange of ORF59 does not alter the function of $PCNA_{\Phi Ch1}$, it does however, result in an increase of $PCNA_{\Phi Ch1}$ post lysis.

The alternate start codon GTG regulates production of $PCNA_{\Phi Ch1}$. A single point mutation of GTG to ATG results in a significant increase in early virus titre, proteins detectable with α-$PCNA_{\Phi Ch1}$ Western blots, and a significant reduction in the growth kinetics, possibly by dysregulation of lytic repressors and activators. Intriguingly, when the ATG variant is expressed in the mutant background it does not significantly differ in behaviours from the strains expressing the GTG and Truncated variants of ORF59 in the deletion mutant background. It may be that the full activity of the extended version of $PCNA_{\Phi Ch1}$ requires the formation of a PCNA heterotrimer with the shorter version of the host encoded PCNA.

5. Conclusion

The viral encoded PCNA is not essential, but is crucial, to the behaviour of the virus ϕCh1. The 5′ region upstream of the primary ATG codon is essential to the function of the viral homolog. Overexpression in a wild-type background with a GTG to ATG point mutation demonstrates that gross control that PCNA maintains over the replicative balance of the virus and host.

In general, the virus encoded PCNA leads to a dramatic increase of progeny viruses. Therefore, the function of the protein seems to direct the replication machinery of N. magadii towards a replication of the viral DNA instead of the replication of chromosomal DNA. However, this aspect has to determine in future experiments. The GTG start codon regulates the gene expression. Overexpression of a mutated ORF59 with an ATG start codon leads to an earlier production of virus particles and a reduced growth rate of the lysogenic strain.

In general, up to now it is not clear, if the described phenotypes of the deletion mutant as well as the overproduction strains show a direct or indirect of PCNA on the replication machinery of *N. magadii.*

Supplementary Materials: Supplementary Material can be found at Figure S1 and Figure S2.

Author Contributions:

R.M. expression plasmid construction, complementation and overexpression strain creation and analysis, growth curves, virus titre, Western blots of Mtase and gp-34, and manuscript preparation. S.S. performed the initial Western blots of PCNA. M.T. Creation L11-ΔP. D.S. Western blots of PCNA and Protein E. A.W.; Experimental design, Southern blot, virus titre, manuscript editing.

Appendix A

Plasmids used in this study

Name	***Description***	***Reference***
pRSET-A	*mcs, bla, ColE1, His-tag*	*Invitrogen*
pPol5	*ORF59 in pRSET-A*	*This study*
pKSII+	*Cloning vector for E. coli*	*Stratagene*
pUC19	*Cloning vector for E. coli*	*Yanisch-Perron et al [18]*

pUC19-NovR "reverse"	*gyrB gene in pUC19 in the reverse orientation*	*This study*
pKSII-PCNA1-5-NovR-R	*Suicide plasmid for the disruption of ORF59 with gyrB (bla)*	*This study*
pKS-34up	*Promotor of ORF34 in PKSII+*	*This study*
pKS-34up-P-ATG	*ΦCh1-ORF59 with a point mutation of GTG>ATG under the control of the promoter of ΦCh1-ORF34 in pKSII+*	*This study*
pKS-34up-P-Tr	*5' truncated ΦCh1-ORF59 under the control of the promoter of ΦCh1-ORF34 in pKSII+*	*This study*
pKS-34up-P-GTG	*ΦCh1-ORF59 under the control of the promoter of ΦCh1-ORF34 in pKSII+*	*This study*
pNB102	*Shuttle vector (bla, Mev^R)*	*Zhou et al[19]*
pP-ATG	*ΦCh1-ORF59 with a point mutation of GTG>ATG under the control of the promoter of ΦCh1-ORF34 in pNB102*	*This study*
pP-GTG	*ΦCh1-ORF59 under the control of the promoter of ΦCh1-ORF34 in pNB102*	*This study*
pP-Tr	*5' truncated ΦCh1-ORF59 under the control of the promoter of ORF34 in pNB102*	*This study*

Strains used in this study

Name	***Description***	***Reference***
N. magadii L13	*cured strain*	*Witte et al [14]*
N. magadii L11	*wild-type strain, ΦCh1 provirus*	*Witte et al [14]*
N. magadii L11-ΔP	*deletion of ΦCh1-ORF59 by gyrB*	*This study*
E. coli BL21(DE3)	*E. coli str. B F$^-$ ompT gal dcm lon hsdS$_B$($r_B^- m_B^-$) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB$^+$]$_{K-12}$(λ^S)*	*Novagen*
E. coli XL1-Blue	*endA1 gyrA96(nal^R) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB$^+$ lacI^q Δ(lacZ)M15 Amy Cm^R] hsdR17(r_K^- m_K^+)*	*Stratagene*

Primers used in this study

Primer name	***Primer Sequence (5'-3')***
p30-5	*CAGCAGAGATCTATGTTCAAAGCAATCGCTAC*
p30-3	*CAGCAGAAGCTTTCAGTTGCTCTGGATCCG*

ΔPCNA-1	*GATCTCTAGAGAAGTGCTACCGCTGCA*
ΔPCNA-2	*GAATCCCGGGAGCTCAGCGTGCTCGA*
ΔPCNA-3	*GTATAAGCTTCCAGAGCAACTGAGTGATCA*
ΔPCNA-5	*GATCGGTACCGTCCCGCATGCACTCAG*
NovR-1p	*GATCCTGCAGTGTCGACTGGAACGAGG*
NovR-1s	*GATCCCCGGGTGTCGACTGGAACGAGG*
56-5	*CAGCAGGATCCATGAGAGAGAACAATCC*
Nov-13	*GACGCCGAATGGGTAGAC*
Nov-12	*GCCGGTGAGTACTTAACGC*
PCNA-3en	*GTCGACGATCTCCGCTAGCT*
34-p5-XbaI	*CAG CAG TCT AGA ATC GGG TGG GAT CCC*
34-p3	*GACGACGGATCCTGTGTTACCTCGTAGCTCTGG*
PCNA-ATG	*GTTCGAATTCATGCGGTATCGACCAGGACC*
PCNA-22	*GACAGGTACCTCAGTTGCTCTGGATCCGC*
PCNA-Tr	*GTTCGAATTCATGTTCAAAGCAATCGCTACGA*
PCNA-GTG	*GTTCGAATTCGTGCGGTATCGACCAGGACC*

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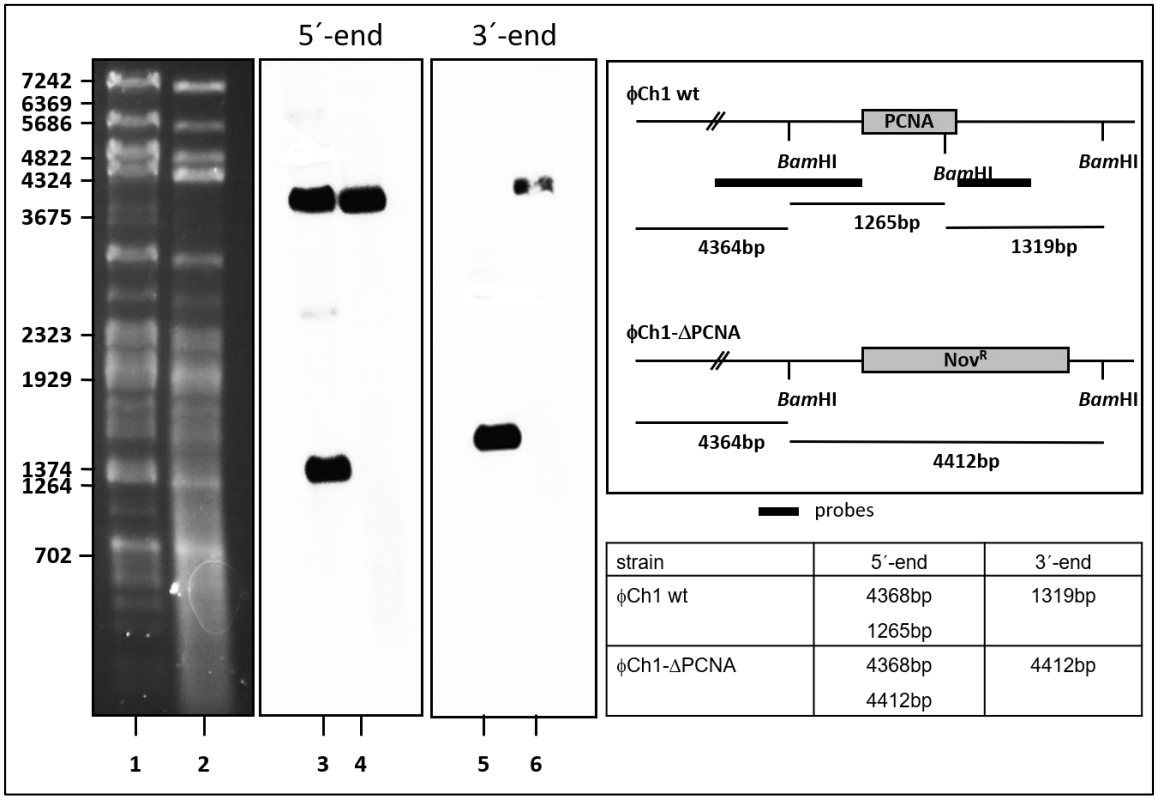
Supplementary materials



strain	5´-end	3´-end
ϕCh1 wt	4368bp 1265bp	1319bp
ϕCh1-ΔPCNA	4368bp 4412bp	4412bp

Figure S1: Southern blot of L11 and L11-ΔP. Lanes: 1), 3),5) *N. magadii* L11. Lanes: 2), 4), 6) *N. magadii* L11-ΔP

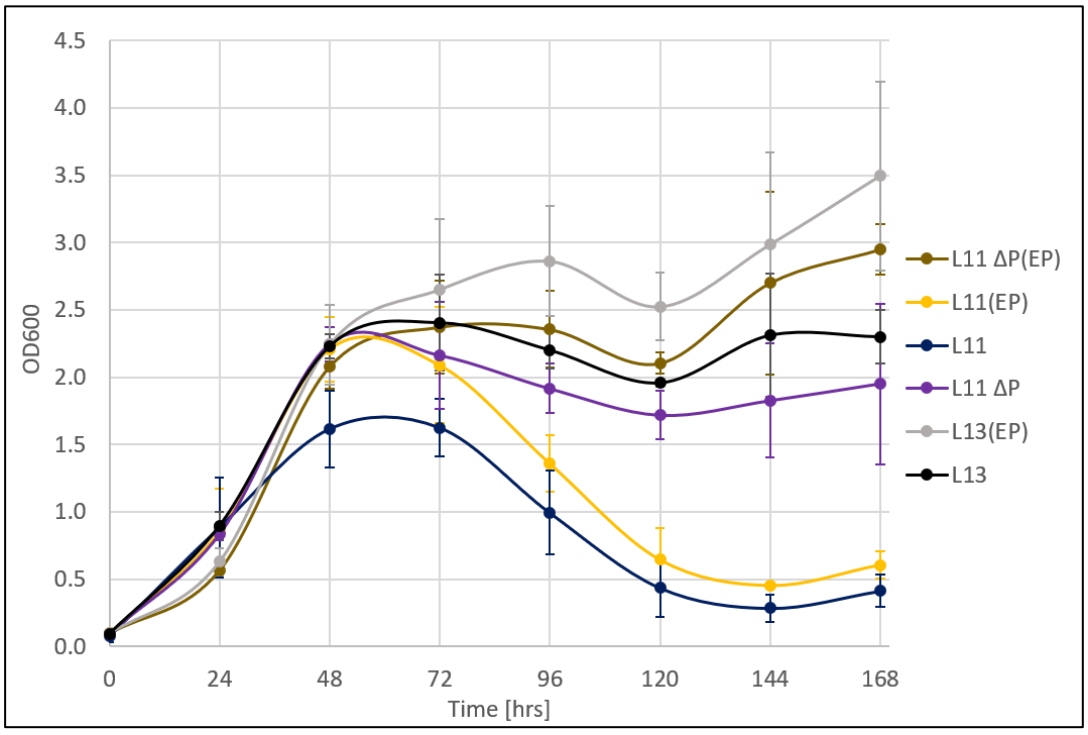


Figure S2: A comparison of the growth curves of *N. magadii* with and without pNB102

8. Abstract

The primary focus of this thesis was to characterise the function of the PCNA protein encoded by the halovirus ϕCh1 which infects the haloalkaliphilic archaeon *Natrialba magadii.* The PCNA of φCh1 has a high amino acid sequence homology to the PCNA encoded by its host *N. magadii* indicating it has been acquired by horizontal gene transfer. Results indicate that the addition of a 21 nucleotide N-terminal tag, relative to the PCNA of *N. magadii*, under the control of an alternate GTG start codon significantly increases the production of viral proteins, the extent of culture lysis, and the production of viral particles. The results of this research have been used for the construction of a manuscript which is included in this thesis.

This thesis also includes several secondary investigations. These include the creation of a heterozygous deletion mutant of a potential binding target for φCh1 on the surface of *N. magadii*, the creation and analysis of a homozygous deletion mutant of the gene encoding NgAgo in *Natronobacterium gregoryi*, the redesign and construction of halophilic shuttle vectors, and the creation of a new strain of *E. coli* for the expression of haloalkaliphilic proteins and the construction of strains for the investigation of the interaction of the PCNA of ϕCh1 and the origin of replication of ϕCh1.

9. Zusammenfassung

Das vorrangige Ziel dieser Arbeit war die funktionelle Charakterisierung des PCNA-Proteins, ein durch das Halovirus φCh1 kodiertes Protein. Die PCNA-Aminosäuresequenzen des φCh1 und seines Wirtes Natrialba magadii, ein halo- und alkaliphiles Archaeum, weisen einen hohen Ähnlichkeitsgrad auf, was auf einen horizontalen Gentransfer schließen lässt. Diese Untersuchungen haben gezeigt, dass das Hinzufügen von 21 Nukleotiden am N-Terminus unter der Kontrolle eines alternativen GTG Startcodons zu einer erhöhten Produktion an viralen Partikel und Proteinen sowie vermehrt zur Lyse der Kultur führt.(Diese in der Masterarbeit festgehaltenen Forschungsergebnisse wurden für die Erstellung eines Manuskriptes, welches auch inkludiert ist, verwendet.)
Darüber hinaus wurde eine heterozygote *N. magadii* L13 Mutante hergestellt, in welcher das Gen für einen Oberflächenrezeptor, der höchstwahrscheinlich eine Bindung des φCh1 ermöglicht, deletiert wurde. Des Weiteren wurde eine homozygotische Natronobacterium gregoryi Mutante erzeugt, bei der das Gen für NgAgo deletiert wurde. Außerdem wurde ein neuer *E.coli* Stamm, der haloalkaliphile Proteine exprimiert, kreiert. Zu guter Letzt wurden noch Stämme hergestellt, die für zukünftige Interaktionsstudien herangezogen werden können.