



# HÁSKÓLI ÍSLANDS

**B.Sc. thesis  
in Biochemistry**

## **Construction of a *Rhodothermus marinus* expression vector for *Sulfolobus* Tip49**

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**FACULTY OF LIFE AND ENVIRONMENTAL SCIENCES**



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Faculty of Life and Environmental sciences

School of Engineering and Natural sciences

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Tip49**

This thesis satisfies 15 credits towards an B.Sc. in Biochemistry in the Faculty of Life and Environmental Sciences,  
University of Iceland, School of Engineering and Natural Sciences

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# Útdráttur

ATPasarnir Pontin (RUVBL1) og Reptin (RUVBL2) eru hliðstæð prótein í AAA+ prótein fjölskyldunni. Próteinin eru talin hafa siðvörðsluprótein-líka virkni og aðstoða við samsetningu og stöðugleika lífsnauðsynlegra próteinflóka. Pontin og Reptin eru mjög vel varðveitt í heilkjörnungum og deila sameiginlegum forföður með fornbakteríugeninu *tip49*, en virkni þess er óþekkt. Á rannsóknarstofunni okkar hefur bakterían *E. coli* áður verið notuð til að tjá Tip49 próteinið úr *S. acidocaldarius* með það að markmiði að hreinsa virkt prótein til lífefnafræðilegra mælinga. Prótein einangrað á þennan hátt reyndist hins vegar að miklu leyti óleysanlegt og hafði líkleg ekki rétta þrívíddarbyggingu. Þar sem *S. acidocaldarius* er hitakær lífvera þá á það líklega betur við að tjá genið í annarri hitakærri lífveru til þess að auka líkurnar á réttri svipmótun og virkni próteinsins. Nýlega var þróað kerfi til þess að erfðabreyta hitakæru bakteríunni *R. marinus*. Í þessu verkefni var gerð tilraun til þess að klóna *tip49* genið úr *S. acidocaldarius* í *R. marinus-E. coli* skutluferju. Til þess að síðar væri hægt að rannsaka Tip49 beint í upprunalegu lífverunni, var reynt að rækta nokkra *Sulfolobus* stofna, en þær tilraunir báru ekki árangur.

## Abstract

The ATPases Pontin (RUVBL1) and Reptin (RUVBL2) are paralogous proteins of the AAA+ protein family. The proteins are suggested to partake in chaperone-like activities and aid in the assembly and stability of several essential protein complexes. Pontin and Reptin are highly conserved in eukaryotes and share a common ancestor with the archaeal *tip49*, whose functions are unknown. Our lab previously used *E. coli* to express the *tip49* gene from *S. acidocaldarius*, however, this yielded mostly insoluble protein which most likely was not correctly folded. As *S. acidocaldarius* is a thermophilic organism, a thermophilic host could be more appropriate for the expression to ensure the proper folding and function of the protein. Recently, a protein expression system was developed for the thermophilic bacterium *R. marinus*. In this study attempts were made to clone the *tip49* gene from *S. acidocaldarius* into a *R. marinus-E. coli* shuttle vector with. Furthermore, to be able to study Tip49 directly in the original organism in the future we tried to culture several *Sulfolobus* strains. However, none of those attempts was successful.











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# 1 Introduction

## 1.1 Pontin and Reptin

The proteins Pontin (or RUVBL1) and Reptin (RUVBL2) are part of the AAA+ (ATPases Associated with various cellular activities) protein family. Pontin and Reptin contribute to the assembly and stability of several complexes and aid in the recruitment of subunits, such as the chromatin remodeling complex Ino80 (Jónsson et al., 2004; Zhou et al., 2017), box C/D snoRNP during biogenesis (McKeegan et al., 2009), telomerase (Venteicher et al., 2008), mRNA surveillance complexes during nonsense-mediated mRNA decay (Izumi et al., 2010) and aggresomes (Zaarur et al., 2015). In addition, Pontin and Reptin stimulate protein aggregate disassembly (Zaarur et al., 2015) and are suggested to be molecular chaperones (Jónsson et al., 2004; Izumi et al., 2010; Nano & Houry, 2013; Zaarur et al., 2015; Zhou et al., 2017). The proteins also form the complex R2TP, comprising of a RNA polymerase-associated protein 3 (RPA-P3) and PIH1 domain-containing protein 1 (PIH1D1) heterodimer bound to a Pontin/Reptin hetero-hexameric ring (Muñoz-Hernández et al., 2019; Rivera-Calzada et al., 2017). This R2TP complex is a co-chaperone of Hsp90, which is highly conserved in eukaryotes from yeasts to humans (Kakihara & Houry, 2012; Rivera-Calzada et al., 2017; Zhao et al., 2008). However, Pontin and Reptin independently assemble together into a hetero-dodecameric complex, comprised of two hexameric rings with interchanging Pontin and Reptin, which has been shown using electron microscopy (Puri et al., 2007; Torreira et al., 2008) and X-ray crystallography (Gorynia et al., 2011; Lakomek et al., 2015).

Interestingly, Pontin and Reptin likely evolved from a common ancestor of the archaeal gene *tip49*, which seems to have split into two paralogous lineages in the eukaryotic group (Afanasyeva et al., 2014; Iyer et al., 2004; Kurokawa et al., 1999). However, the functions of TIP49 in archaea have not been studied extensively. The essential residues required for ATP hydrolysis appears to be evolutionally conserved between the archaeal and eukaryotic orthologs. (Afanasyeva et al., 2014).

Our lab recently examined the Tip49 protein from the thermoacidophilic archaea *Sulfolobus acidocaldarius*. Tip49 was not found to complement the gene loss of *rvb1* (Pontin) and *rvb2* (Reptin) in yeast (Ísabella Ögn Þorsteinsdóttir, 2021). Furthermore, recombinant Tip49 expressed in *E. coli* did not exhibit ATPase activity. Studying thermophilic proteins in a mesophilic host can be problematic as it can yield inactive misfolded products (Hidalgo et al., 2004). Hence, to achieve more reliable results, it would possibly be more appropriate to express Tip49 under conditions and in a host in which the internal environment resembles the proteins' native environment, such as the thermophilic bacterium *Rhodothermus marinus* (*R. marinus*) or study it in the original *Sulfolobus* host.

## 1.2 Genetic engineering of *Rhodothermus marinus*

A genetic manipulation method was developed for *R. marinus* to study its biotechnological potential to express genes encoding thermostable proteins (Bjornsdottir et al., 2007). Due to a lack of antibiotic resistance genes known for *R. marinus*, prototrophic selection was chosen for the cloning. Consequently, tryptophan auxotrophs (*trpB* mutants) were generated (Bjornsdottir et al., 2005). The *R. marinus*–*E. coli* shuttle vector pRM3000 was constructed from the pRM21 plasmid, with the addition of the *trpB* gene, restriction sites, and the *E. coli* *ori* from pUC19 (Bjornsdottir et al., 2007). Recently, an ampicillin resistance marker was added to the plasmid for selection in *E. coli* instead of using the *trpB* gene, producing pRM3000.0 (Kristjansdottir et al., 2021). The plasmid map of pRM3000.0 can be seen in figure 1.1. When the gene of interest has been inserted into the plasmid and cloned in *E. coli*, *R. marinus* can be transformed via electroporation and the transformants selected for on a medium lacking tryptophan (Bjornsdottir et al., 2005).

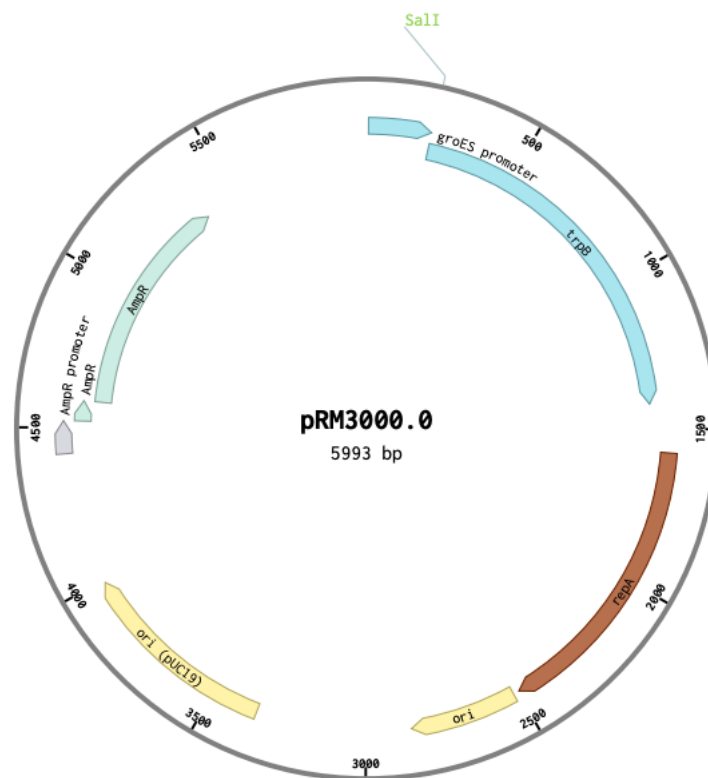


Figure 1.1: Plasmid map of the *R. marinus*–*E. coli* shuttle vector pRM3000.0.

## 1.3 Aims of the project

The paralogous AAA+ ATPases Pontin and Reptin are found to be a factor in various cellular activities and implied to have chaperone-like functions. Pontin and Reptin evolved from a shared progenitor of the archaeal protein Tip49. However, the functions of Tip49 in the archaea *S. acidocaldarius* are unknown. Furthermore, the usage of mesophilic hosts to study the protein has been inefficient. To address this, we aimed to generate an *R. marinus* expression plasmid with the *S. acidocaldarius tip49* gene (*sactip49*). Additionally, we aimed to set up *Sulfolobus* cultivation to be able to study the functions of Tip49 in future studies.



## 2 Materials and methods

### 2.1 Strains, plasmids, and primers

Listed in the following tables are the main components used in the study. Bacterial and archaeal strains are listed in table 2.1, plasmids in table 2.2, and PCR primers in Table 2.3

*Table 2.1: Bacterial and archaeal strains used in this study. The genotype of the strains is listed for non-commercial strains if known.*

Strain	Genotype	Source
<i>Escherichia coli</i>		
NEB 5 $\alpha$ (C2987I)		New England Biolabs
SN1187	MG1655 with $\Delta hsdR$ $\Delta endA$ $\Delta recA(\Delta 2,820,759-2,821,785)$	(Nozaki & Niki, 2019)
<i>Unidentified Sulfolobus isolates</i>		
ISCAR-1227 (B-12-82)		Matís ISCAR collection
ISCAR-1228 (B-12-83)		Matís ISCAR collection
<i>Sulfolobus acidocaldarius</i>		
SK-1	MR31 with $\Delta suaI$	(Suzuki & Kurosawa, 2016)
DP-1	SK-1 with $\Delta phr$	(Suzuki & Kurosawa, 2017)

Table 2.2: List of the plasmids used in this study.

Plasmids	Source
pET28a_sacTip49	(Ísabella Ögn Þorsteinsdóttir, 2021)
pRM3000.0	(Kristjansdottir et al., 2021)
pRM3000.0_sacTip49	This study

Table 2.3: Primers used in PCR amplification *sactip49*.

Primers	Sequence 5'-3' (overlap/spacer/ANNEAL)	Source
6His-SacTip49_fwd	ccatggaggtgcgcgatatgGGCAGCAGCCATCATC	This study
6His-SacTip49_rev	cagcagttcgggtctcggcgggtcgacatTCATTTCAATAAT AGATTCTCATATTCCTTTAC	This study

## 2.2 *Sulfolobus* media

### 2.2.1 Medium 88

To cultivate the unidentified *Sulfolobus* ISCAR strains, medium 88 was prepared according to DSMZ instructions (DSMZ, 2020). The composition of medium 88 is listed in table 2.4.

Table 2.4: Chemical composition of the DSMZ medium 88 for *Sulfolobus* cultivation

Medium 88	
Components	Amount per liter
$(\text{NH}_4)_2\text{SO}_4$	1.3 g
$\text{KH}_2\text{PO}_4$	0.28 g
$\text{MgSO}_4 \times 7\text{H}_2\text{O}$	0.25 g
$\text{CaCl}_2 \times 2\text{H}_2\text{O}$	0.07 g
$\text{FeCl}_3 \times 6\text{H}_2\text{O}$	0.02 g
$\text{MnCl}_2 \times 4\text{H}_2\text{O}$	1.8 mg
$\text{Na}_2\text{B}_4\text{O}_7 \times 10\text{H}_2\text{O}$	4.5 mg
$\text{ZnSO}_4 \times 7\text{H}_2\text{O}$	0.22 mg
$\text{CuCl}_2 \times 2\text{H}_2\text{O}$	0.05 mg
$\text{Na}_2\text{MoO}_4 \times 2\text{H}_2\text{O}$	0.03 mg
$\text{VOSO}_4 \times 2\text{H}_2\text{O}$	0.03 mg
$\text{CoSO}_4 \times 7\text{H}_2\text{O}$	0.01 mg

### 2.2.2XTU medium

The cultivation of *S. acidocaldarius* strains SK-1 and DP-1 required the preparation of a xylose and tryptone (XT) medium with pH 3 and supplemented with 0.02 g/L of uracil for an XTU medium (Grogan D. W., 1996). The components of the XT medium are listed in table 2.5.

Table 2.5: Chemical composition of XTU medium for the cultivation of *S. acidocaldarius*

XTU Medium	
Components	Amount per liter
K <sub>2</sub> SO <sub>4</sub>	3.0 g
NaH <sub>2</sub> PO <sub>4</sub>	0.5 g
MgSO <sub>4</sub> × 7H <sub>2</sub> O	0.3 g
CaCl <sub>2</sub> × 2H <sub>2</sub> O	0.1 g
D-Xylose	2.0 g
Tryptone	1.0 g
FeCl <sub>3</sub> × 6H <sub>2</sub> O	0.1 mg
CuCl <sub>2</sub> × 2H <sub>2</sub> O	0.01 mg
CoCl <sub>2</sub> × 6H <sub>2</sub> O	0.01 mg
MnCl <sub>2</sub> × 4H <sub>2</sub> O	0.01 mg
ZnCl <sub>2</sub>	0.01 mg
Uracil	20 mg

### 2.2.3 Solid medium

Solid medium *Sulfolobus* plates were prepared by mixing equal parts of boiling 1.4% (w/v) Phytigel with a heated 2X *Sulfolobus* medium. This solution must be supplemented with a 100X Ca/Mg stock solution containing, 0.3 M CaCl<sub>2</sub> and 1 M MgCl<sub>2</sub>, to promote gelation of the gellan gum polymers (Alfastsen et al., 2021). About 30 mL of the hot mixture was poured onto each plate, resulting in a 0.7% Phytigel with either medium 88 or XTU medium in 1X concentrations.

## 2.3 Cultivation of *Sulfolobus*

Two unidentified *Sulfolobus* strains were obtained from the Matís ISCAR collection, ISCAR-1227 (B-12-82) and ISCAR-1228 (B-12-83). The strains were originally isolated from volcanic hot springs in Iceland and are known to be of the *Sulfolobus* genus. However,

the species has not been identified. Before attempting the cultivation of other *Sulfolobus* strains, we wanted to test out the medium and our equipment by cultivating the ISCAR strains.

ISCAR-1227 and ISCAR-1228 were inoculated from -80°C stocks into glass tubes with 5 mL of fresh liquid medium 88 and streaked onto 0.7% Phytigel/medium 88 solid medium plates. Both liquid and solid cultures were incubated at 65°C without shaking.

Additionally, two genetically modified *S. acidocaldarius* strains were provided from Dr. Norio Kurosawa of Soka University. The strains SK-1 (MR31 with  $\Delta suaI$ ) and DP-1 (SK-1 with  $\Delta phr$ ) were inoculated from room temperature liquid cultures into polypropylene culture tubes containing 1.5 ml XTU medium and streaked onto 0.7% Phytigel/XTU medium solid media plates. Liquid and solid cultures were incubated at 75°C without shaking.

## 2.4 Cultivation of *E. coli*

*E. coli* strains were cultured in Luria-Bertani (LB) liquid medium and LB agar plates for solid medium. Liquid and solid cultures were supplemented with 50 µg/mL of carbenicillin for selection when needed.

## 2.5 PCR of *sac-tip49*

The primer pair 6His-SacTip49\_fwd and 6His-SacTip49\_rev (see table 2.3) was designed using the NEBuilder Assembly Tool v2.5.6. The primers were designed with overlapping regions to ligate between the *groESL* promoter and *trpB* gene on the pRM3000.0 plasmid, see figure 2.1.

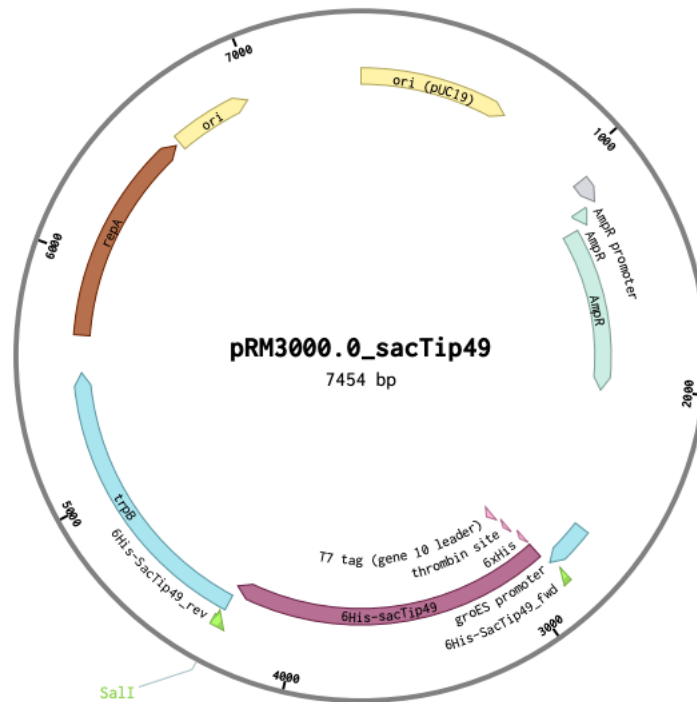


Figure 2.2: Plasmid map of pRM3000.0\_sacTip49, *R. marinus*–*E. coli* shuttle vector with the *sactip49* gene (purple).

PCR of pET28a\_sacTip49 was carried out using primers 6His-SacTip49\_fwd and 6His-SacTip49\_rev. Contamination of template and primers was tested using controls containing either no template or no primer. The reaction preparation and PCR program for sample J1 is listed in tables 2.6 and 2.7, and samples J2-J7 in tables 2.8 and 2.9. For the optimization of the PCR reaction, a temperature gradient was utilized for samples J2-J7.

Table 2.6: Volume of PCR reagents and components and initial and final concentrations in sample J1

Materials	Initial Concentration	Final concentration	Volume (μL) in 1x reaction
pET28a_sacTip49	26.6 ng/μL	5.32 ng	0.2
Q5® High-Fidelity 2X master mix	2X	1X	5.0
6His-SacTip49_fwd	10 μM	0.5 μM	0.5
6His-SacTip49_rev	10 μM	0.5 μM	0.5
MQ H <sub>2</sub> O			3.8
<b>TOTAL VOLUME</b>			<b>10</b>

*Table 2.7 PCR thermocycler program for sample J1*

<b>STEP</b>	<b>TEMP</b>	<b>TIME</b>
Initial Denaturation	98°C	30 sec
30 Cycles	98°C	10 sec
	72°C	20 sec
	72°C	1:20 min
Final Extension	72°C	5 min
Hold	15°C	

*Table 2.8: Volume of PCR reagents and components and initial and final concentrations in samples J2-J7*

<b>Materials</b>	<b>Initial Concentration</b>	<b>Final concentration</b>	<b>Volume in 1x reaction (μL)</b>	<b>Volume in 6.5x reaction mix (μL)</b>
Template DNA	26.6 ng/μL	0.81 ng	0.03	0.20
Q5 master mix	2X	1X	5.0	32.5
6His-SacTip49_fwd	10 μM	0.5 μM	0.50	3.25
6His-SacTip49_rev	10 μM	0.5 μM	0.50	3.25
<b>MQ H<sub>2</sub>O</b>			3.96	25.8
<b>TOTAL VOLUME</b>			10	65

Table 2.9: PCR thermocycler program for samples J2-J7

STEP	TEMP	TIME
Initial Denaturation	98°C	30 sec
30 Cycles	98°C	10 sec
	<i>see table 2.10</i>	30 sec
	72°C	2 min
	72°C	5 min
Final Extension	72°C	5 min
Hold	15°C	

Table 2.10: Annealing temperature gradient for the PCR of samples J2-J7

Sample	Annealing Temp (°C)
J2	72
J3	70
J4	68
J5	66
J6	64
J7	62

## 2.6 DNA gel electrophoresis

DNA samples were analyzed on 1% agarose gels containing: 50 mL 1X TAE buffer, 2.5  $\mu$ L ethidium bromide, and 0.5 g agarose. Before loading DNA samples onto the gels, 2  $\mu$ L DNA sample was mixed with 2  $\mu$ L 6X loading dye (NEB) and 8  $\mu$ L MQ H<sub>2</sub>O. To determine the size of the DNA fragments, a 1 kB DNA ladder (NEB) was used. After loading the DNA ladder and samples, the gel is subjected to 80 V for 40, 60, or 80 minutes. Gels were imaged under UV light for DNA size evaluation and analysis.

## 2.7 *Sal*I digestion of pRM3000.0 backbone

Plasmid pRM3000.0 was made linear by performing a *Sal*I digest. First, the plasmid was quantified using NanoDrop. Then, the components in Table 2.11 were mixed and incubated at 37°C for 2 hours. The digested sample was heat-inactivated at 65°C for 20 min to hinder any further enzymatic activity. DNA gel electrophoresis was used to assess the digestion and compare it to an uncut pRM3000.0 plasmid.

Table 2.11: Volume of components in the *Sal*I restriction enzyme digestion of pRM3000.0

Materials	Volume in 1x reaction (μL)
pRM3000.0 (109.57 ng/μL)	9.10
10X NEBuffer Cutsmart (NEB)	5.00
<i>Sal</i> I-HF (NEB)	1.00
MQ H <sub>2</sub> O	34.9
<b>TOTAL VOLUME</b>	<b>50</b>

## 2.8 Gibson Assembly cloning

The Gibson Assembly cloning method was utilized to clone the *S. acidocaldarius tip49* gene (*sactip49*) into the *R. marinus* expression vector (pRM3000.0), as described in table 2.12. The sample was incubated at 50°C for 15 minutes and stored at -20°C overnight.

Table 2.12: Volume of Gibson Assembly reagents and DNA components mixed

Materials	Volume (μL)	Amount (ng)
pRM3000.0 (23.24 ng/μL)	2.15	50.0
sacTip49 (53.18 ng/μL)	0.44	23.4
Gibson Assembly Master Mix (2X)	10.0	
<b>MQ H<sub>2</sub>O</b>	7.41	
<b>TOTAL VOLUME</b>	<b>20.0</b>	

Next, NEB-5 $\alpha$  Competent *E. coli* C2987I (NEB) was transformed with the Gibson Assembly product according to steps 1-8 in the High-Efficiency Transformation protocol for C2987H/C2987I supplied by the manufacturer. After step 8 in the protocol, 100  $\mu$ L of the culture was spread onto LB agar plates with carbenicillin using glass beads. The remaining cells in SOC were centrifuged at 11 kG for 30 seconds until a pellet had formed at the bottom of the sample. Next, the liquid was discarded except ca. 100  $\mu$ L, which was mixed with the pellet and 100  $\mu$ L of the supernatant was spread onto LB carbenicillin plates. Plates were incubated overnight at 37°C after which they were stored at 4°C. The colonies were streaked onto new plates to avoid false positives during colony PCR.

## 2.9 Colony PCR

Screening clones for pRM3000.0\_sacTip49 was carried out by colony PCR. Colonies transformed with the Gibson Assembly product were picked with a sterile pipette tip and submerged briefly in 15  $\mu$ L of colony PCR lysis buffer (TE + 0.1% Triton-X100). Then the pipette tip was dropped into LB medium with carbenicillin. These liquid cultures were incubated overnight at 37°C with shaking and refrigerated briefly before proceeding with plasmid purification the same day.

Each colony sample in the lysis buffer was incubated at 95°C for 5 minutes and centrifuged at 11 kG for 10 minutes. For the PCR, 2  $\mu$ L of supernatant was used as template DNA in each reaction as shown in table 2.13. The PCR thermocycler program used is shown in table 2.14.

*Table 2.13: Volume of the PCR reagents and components for the colony PCR screening of pRM3000.0\_sacTip49*

Materials	Volume in 1x reaction ( $\mu$ L)
Colony in lysis buffer	2.00
Eppendorf Taq Master Mix (2.5X)	10.0
6His-SacTip49_fwd (10 $\mu$ M)	1.25
6His-SacTip49_rev (10 $\mu$ M)	1.25
MQ H <sub>2</sub> O	10.5
<b>TOTAL VOLUME</b>	<b>25 <math>\mu</math>L</b>

Table 2.14: PCR thermocycler program for the colony PCR

STEP	TEMP	TIME
Initial Denaturation	95°C	30 sec
30 Cycles	95°C	30 sec
	64°C	60 sec
	68°C	4 mins
	68°C	5 mins
Final Extension	68°C	5 mins
Hold	15°C	

In the first colony PCR, the negative control was produced by touching the solid medium instead of colonies before going into the lysis buffer and LB to test the recombinant DNA contamination on the plate itself, which can result in false positives in the samples. During the second colony PCR, the negative control did not touch the medium or colonies; instead, clean pipette tips went straight into the lysis buffer and LB.

## 2.10 Plasmid DNA purification

Purification of potential positive clones harboring pRM3000.0\_sacTip49 was performed using NucleoSpin Plasmid (NoLid) (Macherey-Nagel) according to manufacturer protocol Plasmid DNA purification: User manual (Nov 2012/Rev. 08), in section 5.1 (Isolation of high-copy plasmid DNA from *E. coli*). During washing in step 5, the optional wash with buffer AW was carried out. In the DNA elution step, a higher efficiency elution procedure was performed, described in section 2.4 as “Higher yield in general, especially for larger constructs”. After completion, samples were quantified using a NanoDrop spectrophotometer.

## 2.11 Carbenicillin test

To test the carbenicillin stock, LB medium with and without selection was inoculated with the *E. coli* strain SN1187. For comparison, a freshly made stock of carbenicillin was prepared and tested as described above. The strain does not possess an ampicillin resistance gene and should not survive if the carbenicillin is not degraded. The liquid cultures were incubated overnight at 37°C while shaking, after which they were inspected for signs of growth.



## 3 Results

### 3.1 Attempts to cultivate several *Sulfolobus* strains

#### 3.1.1 Matís ISCAR strains

One of our goals was to cultivate *Sulfolobus* strains at the lab for further studies. To start testing *Sulfolobus* cultivation, we received two *Sulfolobus* strains from the ISCAR collection. The strains, ISCAR-1227 and ISCAR-1228, are unidentified strains of *Sulfolobus* isolated at Matís from volcanic hot springs with a temperature of 65°C. ISCAR-1227 and ISCAR-1228 were streaked onto 0.7% Phytigel/medium 88 solid medium plates and inoculated into liquid medium 88. During incubation at 65°C, the cultures were checked for growth and dehydration/moisture on days 5 and 10. Then on the 15<sup>th</sup> day, the cultures were discarded. No growth was visible in the cultures.

#### 3.1.2 *Sulfolobus acidocaldarius* strains SK-1 and DP-1

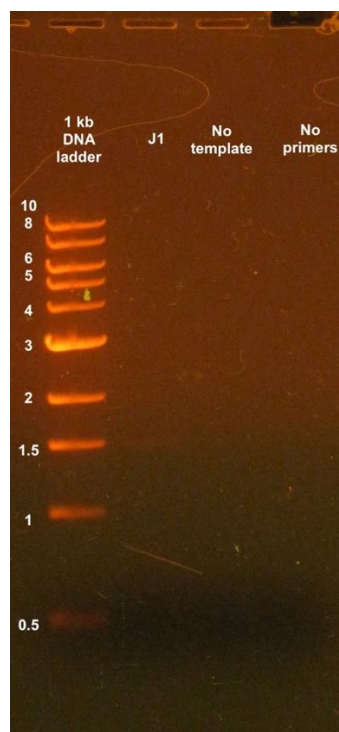
Two *Sulfolobus acidocaldarius* strains, SK-1 and DP-1, were received from Dr. Norio Kurosawa's lab at Soka University. The strains were shipped in 1 mL liquid cultures in Eppendorf tubes for 19 days and cultivated on arrival at 75°C. After 3 days in the incubator, the liquid cultures of SK-1 and DP-1 in the XTU medium had evaporated completely. However, the solid medium cultures did not dehydrate but showed no signs of growth after two weeks of incubation.

### 3.2 *Rhodothermus marinus* expression vector with *sactip49*

#### 3.2.1 PCR of *sactip49* insert

The first step in producing a *R. marinus*–*E.coli* shuttle vector with the *sactip49* gene was to PCR the *tip49* gene using the Gibson Assembly primers 6His-SacTip49\_fwd and 6His-SacTip49\_rev (see sequence in table 2.3).

The size of the desired PCR product is 1505 bp. A recommended annealing temperature of 72°C was used with insufficient success for the first PCR. In figure 3.3 a very faint ghost band can be seen around 1500 bp in sample J1. Contamination was checked with both no template and no primer controls. These controls did not have any DNA bands visible on the gel.



*Figure 3.3: DNA gel electrophoresis of PCR sample J1. The annealing temperature was 72°C, as was recommended. The first well contains the 1 kb DNA ladder, the size of each band is shown on the left in kb. Sample J1 contains a very weak band of the correct size, around 1500 bp, but it is hardly noticeable. Neither negative control had visible bands.*

To achieve a successful PCR, we decided to optimize the annealing temperature of the primers by using a temperature gradient (Table 2.10). In addition, annealing and extension times were increased (Table 2.9). As shown in figure 3.1, samples J2-J7 all had bands of correct sizes. The sample well for J4 was shifted lower due to a bent comb tooth; thus, the band looks smaller but should be around 1500 bp. However, sample J4 was not be used further due to this error. Sample J6 had an annealing temperature of 64°C, which produced the most efficient reaction. For this reason, products from sample J6 were used as the insert for the Gibson Assembly.

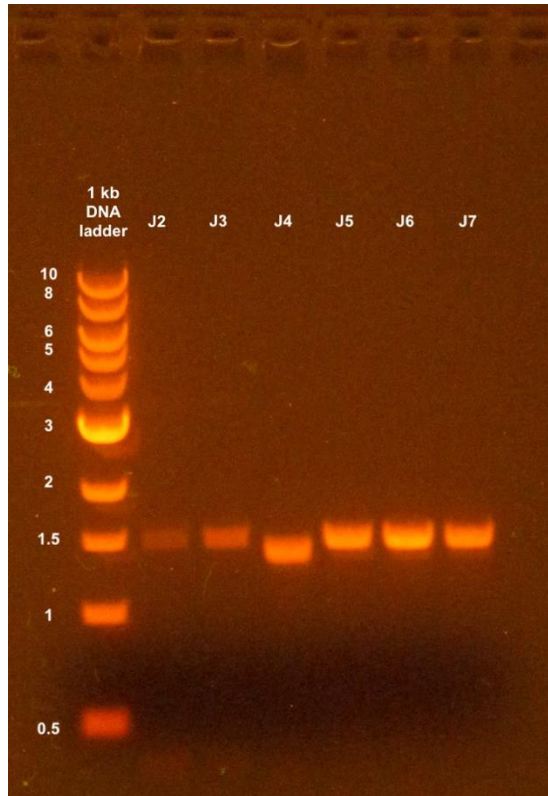


Figure 3.4: DNA gel electrophoresis of PCR samples J2-J7. The annealing temperature of the samples are 72°C for J2, 70°C for J3, 68°C for J4, 66°C for J5, 64°C for J6, and 62°C for J7. The first well contains the 1 kb DNA ladder, the size of each band is shown on the left in kb. Samples J2-J7 contain each a band around 1500 bp, which is the expected size of the *sactip49* PCR product. Sample J2 has the weakest band and J6 the brightest. The comb tooth for the J4 well was bent, resulting in the band being lower on the gel than it should be.

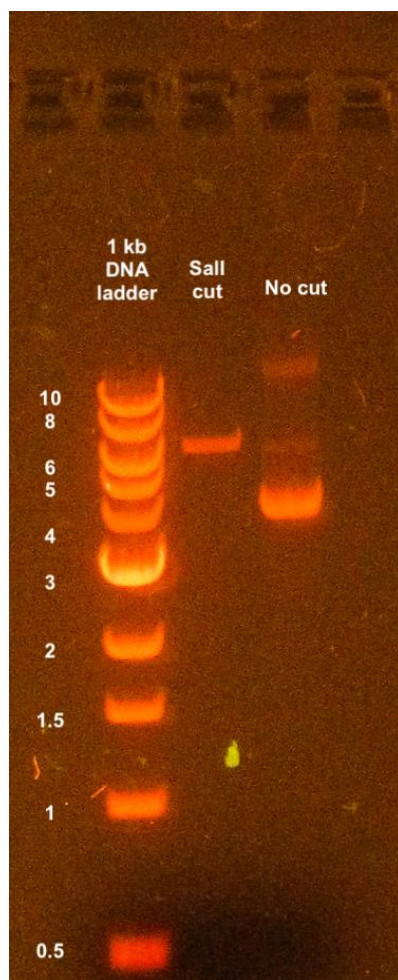
### 3.2.2 Digestion of pRM3000.0 backbone

To produce a linear DNA strand for the Gibson Assembly, pRM3000.0 was digested with SalI which cuts the plasmid once, opening it up between *groESL* promoter and *trpB*. Before starting the digestion, the plasmid sample received from Matís was quantified with NanoDrop (table 3.15).

Table 3.15: Nucleic acid quantification of plasmid pRM3000.0 using a NanoDrop spectrophotometer

Sample ID	DNA concentration (ng/μl)	A <sub>260</sub>	A <sub>280</sub>	A <sub>260</sub> /A <sub>280</sub>	A <sub>260</sub> /A <sub>230</sub>
pRM3000.0	109.57	2.191	1.192	1.84	1.92

The digested pRM3000.0 was analyzed and compared to an uncut plasmid on a gel (figure 3.5). In the cut sample, only a single band appears around 6000 bp, which is the size of pRM3000.0 (5993 bp). On the other hand, three bands are visible in the uncut sample and are most likely supercoiled, linear, and nicked DNA. As only one band appeared in the cut sample, there should be no need to purify the digested sample.



*Figure 3.5: DNA gel electrophoresis of a SalI digested and an uncut sample of pRM3000.0. The first well contains the 1 kb DNA ladder, the size of each band is shown on the left in kb. In the digested sample, there appears only one band around 6000 bp. The size of the plasmid is 5993 bp. The uncut sample has three bands; the brightest band is likely the supercoiled form of the plasmid, and the band above would be the linear form.*

### 3.2.3 Gibson assembly and transformation of *E.coli*

DNA quantification was performed on the digested pRM3000.0 and *sactip49* PCR product, see table 3.16. As the samples have not been purified, they contain components from the PCR and digest and are not pure DNA.

Table 3.16: Nucleic acid quantification of the *SalI* digested pRM3000.0 and *sactip49* PCR sample J6 using a NanoDrop spectrophotometer

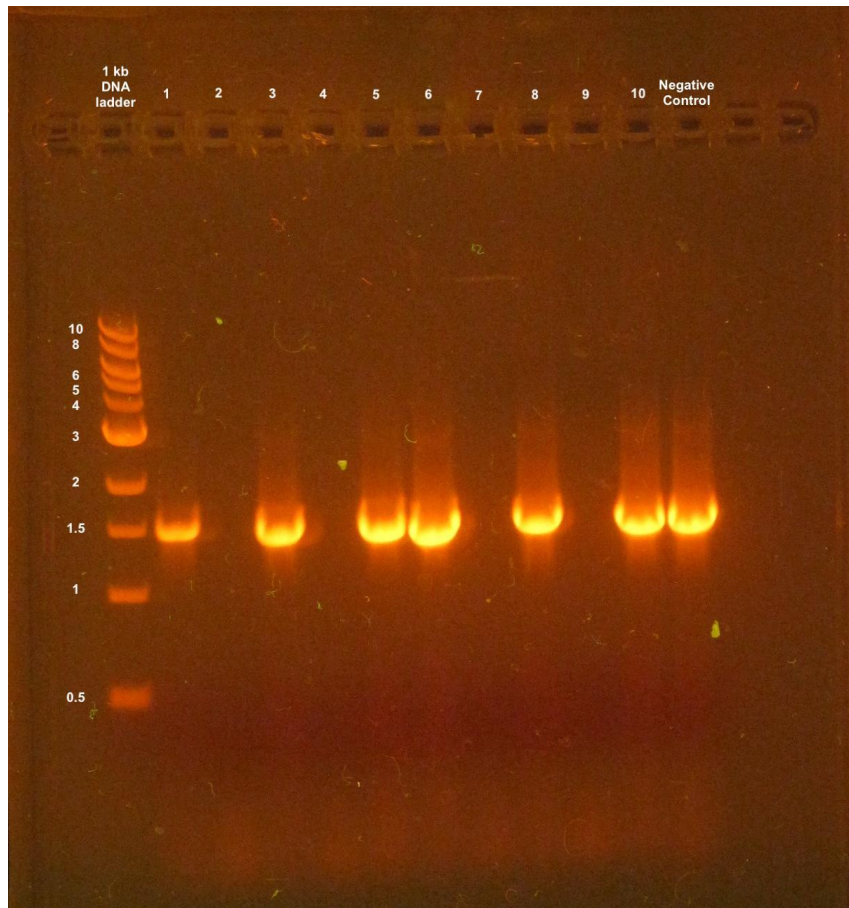
Sample ID	DNA concentration (ng/μl)	A <sub>260</sub>	A <sub>280</sub>	A <sub>260</sub> /A <sub>280</sub>	A <sub>260</sub> /A <sub>230</sub>
pRM3000.0 backbone ( <i>SalI</i> digested)	23.24	0.465	0.305	1.52	0.6
J6 <i>sactip49</i> insert (PCR)	531.82	10.636	6.201	1.72	1.01

The incubated selection plates displayed a dense cluster of transformed colonies from the Gibson Assembly. Non-transformed cells were tested on the same batch of selection plates with no growth exhibited. Demonstrating that the carbenicillin selection on the plates is functional and that the Gibson Assembly appears to have worked.

### 3.2.4 Recombinant plasmid screening

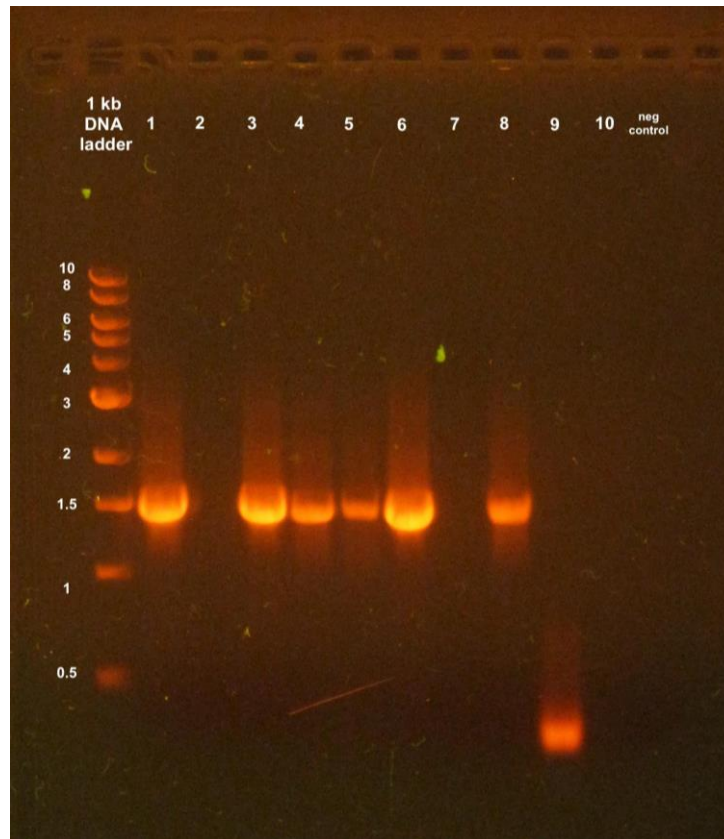
The next step was to test which colonies have the pRM3000.0\_ *sacTip49* plasmid from the Gibson Assembly cloning. Unfortunately, as the pRM3000.0 is not a commercially made plasmid, we could not use standard sequencing primers to screen the colonies for the recombinant plasmid. Consequently, it was decided to attempt to screen using the Gibson primers previously used to PCR amplify the *sactip49* insert.

In the first colony PCR, see figure 3.6, half of the colonies were positive with the correct band size. However, the negative control was also positive for PCR product, indicating the need to restreak the colonies to avoid potential false positives.



*Figure 3.6 DNA gel electrophoresis of colony PCR, screening clones for pRM3000.0\_sacTip49. The first well contains the 1 kb DNA ladder, the size of each band is shown on the left in kb. The expected band is 1505 bp. Sample clones no. 1, 3, 5, 6, 8, and 10 had one band around 1500 bp, thus, potentially positive for pRM3000.0\_sacTip49. However, the negative control also has a band the same size. The positive clones are therefore potentially false positives.*

The colony PCR was repeated after streaking colonies onto new plates (see figure 3.5). Samples 1, 3, 4, 5, 6, and 8 displayed a band around 1500 bp, making them potentially positive clones for pRM3000.0\_sacTip49. In sample 9, there appears to be a non-specific band around 300 bp. The negative control and samples 2, 7, and 10 have no visible bands. Therefore, the liquid cultures from samples 1, 3, 4, 5, 6, and 8 will be used to purify recombinant plasmid.



*Figure 3.7: DNA gel electrophoresis of colony PCR, screening clones for pRM3000.0\_sacTip49. The first well contains the 1 kb DNA ladder, the size of each band is shown to the left in kb. The expected band is 1505 bp. Sample clones no. 1, 3, 4, 5, 6, and 8 had one band around 1500 bp, thus, potentially positive for pRM3000.0\_sacTip49. An unspecific band is evident in sample 9. No band is visible for the negative control.*

### 3.2.5 Plasmid purification

Three attempts were made to extract and purify plasmid DNA from the cultures. However, all efforts yielded insignificant concentrations of DNA. After the first failed miniprep, see table 3.17, liquid cultures were prepared from the positive sample colony isolates that had previously been restreaked on fresh plates with selection. With the new liquid cultures, the plasmid purification was repeated, again without success, see table 3.18. Before attempting the purification once again, the carbenicillin stock used for selection in the liquid cultures was tested. Carbenicillin on the selection plates had already been confirmed to work correctly but the liquid culture selection had not been tested. The carbenicillin test showed that the old stock was no longer functional, as it allowed *E. coli* strain SN1187 to proliferate, a strain without ampicillin resistance. Conversely, the freshly prepared carbenicillin stock showed no growth after overnight incubation, signifying the results above.

The new functional stock of carbenicillin was then used for selection in liquid cultures inoculated with the positive sample clones. After an overnight incubation the cultures exhibited growth as routine and the third plasmid purification was performed, see table 3.19. Although the selection was functional the results were the same. Hence, the inability to isolate plasmid DNA is likely caused by something else. This DNA plasmid purification kit used is known to be working as other lab members have successfully used it after our attempts (data not shown).

*Table 3.17: Results from the first plasmid purification attempt. Nucleic acid quantification of samples using a NanoDrop spectrophotometer*

Sample clone	DNA concentration (ng/μl)	A <sub>260</sub>	A <sub>280</sub>	A <sub>260</sub> /A <sub>280</sub>	A <sub>260</sub> /A <sub>230</sub>
1	14.47	0.289	0.174	1.66	0.57
3	11.09	0.222	0.135	1.65	0.55
4	11.35	0.227	0.152	1.49	0.55
5	9.64	0.193	0.116	1.66	0.53
6	8.44	0.169	0.084	2.00	0.53
8	12.58	0.252	0.147	1.72	0.55

*Table 3.18: Results from the second plasmid purification attempt. Nucleic acid quantification of samples using a NanoDrop spectrophotometer*

Sample clone	DNA concentration (ng/μl)	A <sub>260</sub>	A <sub>280</sub>	A <sub>260</sub> /A <sub>280</sub>	A <sub>260</sub> /A <sub>230</sub>
1	13.66	0.273	0.156	1.75	0.59
3	10.31	0.206	0.112	1.84	0.54
4	10.84	0.217	0.103	2.11	0.57
5	21.87	0.433	0.271	1.60	0.61
6	11.12	0.222	0.121	1.83	0.54
8	11.43	0.229	0.132	1.74	0.52

*Table 3.19: Results from the third plasmid purification attempt. Nucleic acid quantification of samples using a NanoDrop spectrophotometer*

<b>Sample clone</b>	<b>DNA concentration (ng/μl)</b>	<b>A<sub>260</sub></b>	<b>A<sub>280</sub></b>	<b>A<sub>260</sub>/A<sub>280</sub></b>	<b>A<sub>260</sub>/A<sub>230</sub></b>
1	11.87	0.237	0.148	1.60	0.53
3	11.81	0.236	0.174	1.36	0.57
4	18.08	0.362	0.214	1.69	0.57
5	10.96	0.219	0.136	1.61	0.56
6	12.48	0.250	0.155	1.60	0.59
8	12.35	0.247	0.155	1.60	0.59

# Discussion

The preliminary efforts to cultivate *Sulfolobus* yielded unsatisfactory results. First, the uncharacterized *Sulfolobus* strains ISCAR-1227 and ISCAR-1228 from Matís were inoculated and incubated without any signs of growth on both liquid and solid medium. Likewise, attempts to cultivate *S. acidocaldarius* strains SK-1 and DP-1 were without success. However, at the time of inoculation the condition of the cultures may have been suboptimal. Strains from Matís were acquired directly from -80°C freezer aliquots, and SK-1 and DP-1 spent 19 days in unknown conditions during postage from Japan to Iceland. Thus, the integrity of the *Sulfolobus* cultures prior to cultivation might have been lacking. In addition, the incubation methods might need to be reconsidered, such as to optimize the airflow and humidity conditions within the incubator.

Efforts were made to construct an *R. marinus* expression vector with the *tip49* gene from *S. acidocaldarius*. First, *sactip49* was PCR amplified with Gibson primers designed with overlaps complementing the pRM3000.0 insert region between the *groESL* promoter and *trpB*. After confirming the correct size of the PCR product, the pRM3000.0 plasmid was linearized by a *SalI* digest, which was verified by DNA gel electrophoresis. Next, *sactip49* was ligated together with pRM3000.0 in a Gibson Assembly reaction and *E. coli* transformed with the product to clone the candidate pRM3000.0\_*sacTip49*. The transformed cells were cultured on selection plates, producing an abundance of single colony clones. Clones were restreaked onto fresh selection plates to avoid false positives in the colony PCR. The colony PCR was repeated on the freshly plated colonies and sample clones 1, 3, 4, 5, 6, and 8 produced PCR products of the correct size when screening for *sactip49*. Hence, these clones were possibly harboring the pRM3000.0\_*sacTip49* plasmid. To further validate the candidate plasmid via restriction enzyme digest and sequencing, plasmid purification was required. Three attempts were made to purify the plasmid from the positive sample clones, all without any success. As the DNA concentrations measured is very low in the samples, around 10 ng/μl, it might have resulted from the absorbance of salt in the elution buffer and not actual DNA in the sample. These samples are therefore not regarded as DNA samples. When investigating the possible causes of these results, the carbenicillin selection was functioning properly, colonies restreaked onto new plates and the purification kit confirmed to be working as well. However, the cause was not uncovered.

For future applications, I believe it would be beneficial to screen the colonies again with a new set of primers designed to screen this plasmid. The colony PCR performed in the study was using the same set of primers as was used in the initial PCR of the *sactip49* insert. In the past, this region has been PCR amplified repeatedly at the lab, which might cause a contamination in the PCR and produce false positives.

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