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„Characterization of the PHA-Operon of *H. hispanica* and *H. marismortui* in *N. magadii*“

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1 Introduction

1.1 *Archaea*

There are many theories speculating about how the first forms of life emerged on earth 3.8 to 4.5 billion years ago. There is no definition where chemistry ends and life starts. In 1953 the famous experiment facilitated by Stanley Miller and Harold Urey showed, that in a possible, rudimentary early earth environment basic building blocks of life, amino acids, can be synthesized within the borders of this system (meaning temperature, pressure and availability of H_2O, CH_4, NH_3, CO and H_2 plus energy) (Miller, 1953). After this experiment others followed and theories were proposed as the iron-sulfur world by Günter Wächtershäuser (Wächtershäuser, 2006)or the PAH world (polycyclic aromatic hydrocarbons) by Pascale Ehrenfreund (Ehrenfreund *et al.*, 2006). Inbetween the term of the RNA world was coined by Walter Gilbert, which suggests an early stage of life based on the catalytical and self replicating properties of RNA, which is subsequently substituted by DNA (Gilbert, 1986).

After this chemical and early biological evolution the first primitive cell appeared on earths surface, which was given different names in the last decades: LUCA (last universal common ancestor) or progenote are just two abstract concepts which were chosen, I will stick with the latter one for further considerations. This point in time is the initial signal for the development of life on earth and furthermore has been the subject of discussion for generations of scientists in the past and future.

Todays perception of the tree of life comprised of the three domains *Archaea*, *Bacteria* and *Eukarya*, is based mostly on one molecule: 16S rRNA. Carl Woese was the first to become aware of the significance of this outstanding marker in an evolutionary context and the interrelationships of species. The properties of 16S rRNA predestine it as phylogenetic marker: universal distribution among species, high conservation with moderate variability and low rates of lateral gene transfer (DeLong and Pace, 2001); there are exceptions to the rule (Amann *et al.*, 2000). After a comparison of different 16S rRNA sequences, the datasets were rearranged and clustered. The results painted a very different picture from what was state of the art. The 5-kingdom theory, consisting of *Monera*, *Protista*, *Fungi*, *Animalia* and *Plantae*, was equally dismissed as a simple prokaryote-eukaryote dichotomy (Woese and Fox, 1977; Woese *et al.*, 1990).

Currently one of the most highly disputed aspects of the tree of life is its rooting. There are exponents who set the root within *Archaea* (Guilio, 2007), *Bacteria* (Brown and Doolittle, 1995) or *Eukarya* (Forterre and Philippe, 1999; Fournier *et al.*, 2011). Each theory has its pros and cons, additionally lateral gene transfer and lineage

fusions complicate an exact determination of the trees root.

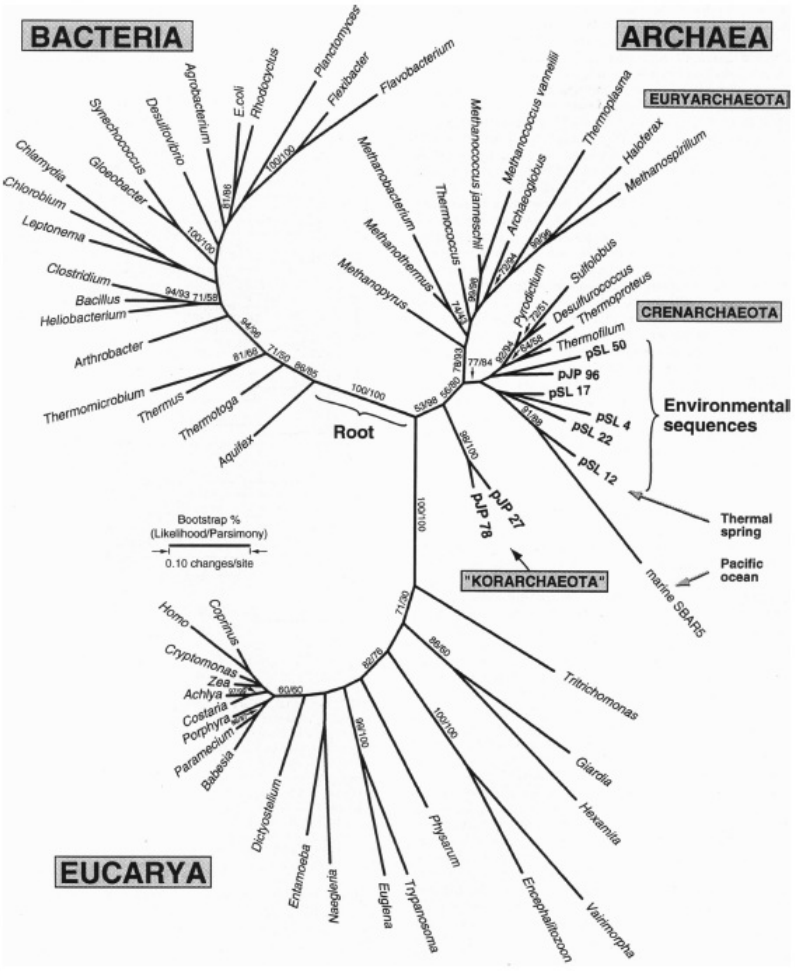


Figure 1: *Phylogenetic tree depicting the three kingdoms (Barns et al., 1996). The phylum of "Korarchaeota" is not established according to Bergey's Outline.*

In the final step of this short overview of the phylogentic tree of life is the focus on *Archaea*.

The next considerations are conducted according to Bergey's Outline (Garrity *et al.*, 2003). *Archaea* are devided into two phyla, the *Euryarchaeota*, which contain nine orders, and the *Crenarchaeota*, which contain four orders.

1. *Euryarchaeota*
   (a) *Methanobacteriales*
   (b) *Methanococcales*
   (c) *Methanomicrobiales*
   (d) *Methanosarcinales*
   (e) *Methanopyrales*

   (f) *Halobacteriales*

   (g) *Thermoplasmatales*

   (h) *Thermococcales*

   (i) *Archaeoglobales*

2. *Crenarchaeota*

   (a) *Thermoproteales*

   (b) *Desulfurococcales*

   (c) *Caldisphaerales*

   (d) *Sulfolobales*

Concerning *Euryarchaeota* there are five methanogenic and anaerobic phyla *(1a - e)* (whereas *Methanopyrales* is also a hyperthermophile and in contrast *Methanomicrobiales* is mesophile and closest related to *Halobacteriales*), one halophilic phylum *(1f)*, one thermoacidophilic phylum *(1g)* and two hyperthermophilic phyla *(1h, i)* (whereas *Archaeoglobales* posess artefacts of methanogenic metabolism). Focusing on the phylogenetic tree it suggests that the methanogenic metabolism might have developed secondary, because *Thermococcales* branch earliest and its next relative *Archaeoglobales* possesses rudimentary proteins involved in this way of life (Forterre *et al.*, 2002).

The second kingdom, *Crenarchaeota*, consists of two anaerobic, hyperthermophilic phyla *(2a, b)* and two thermophilic, acidophilic phyla *(2c, d)*

Investigating the properties of both kingdoms some evolutionary conclusions can be drawn:

1. The archael ancestor might have been minimally tolerant to oxygen: tolerance to oxygen was a secondary adaptation, as there are anaerobic and aerobic orders in both branches, therefore this was divergent evolution.

2. Another property of a possible archaeal progenote was chemiolithoautotrophy and it did not possess any methanogenic pathways.

3. Even when hyperthermophiles branch early the archaeal ancestor might have been mesophile, because it lacks, an otherwise very conserved protein among hypterthermophiles, reverse gyrase.

(Forterre *et al.*, 2002)

For the last ten years there have been considerations by different research groups to shape three more phyla for the domain *Archaea*. First there was the discovery of

Nanoarchaeum equitans which is the smallest prokaryote known so far (0.4 μm in diameter, genome 490 kb, 95% coding sequences). This species is only propagating in co-culture with an other archaeon *Ignicoccus hospitalis* and it is no yet clear whether its lifestyle is muatualistic, commensalistic or parasitic. The 16S rRNA is not detectable with FISH probes for other archaea (Huber *et al.*, 2003). In another paper the high number of split genes is analysed (11 protein coding, 6 tRNA coding sequences) and this fact is interpreted, in regard of the intron-exon structure of eukaryotic genes, as a hint for the eukaryal rooting of the tree of life (Guilio, 2007). Lately it was discovered that *N. equitans* might not be an own phylum but a fast-evolving euryarchaeal lineage (Brochier-Armanet *et al.*, 2008). Secondly after the investigation of a series of 16S rRNA sequences from environmental samples taken from thermal vents by Ken Takai and collegues, they created a new cluster called *Korarchaeota* (Takai *et al.*, 2001a,b). Further research on this cluster was done in 2006 by TA Auchtung and his team. They were still not able to cultivate the strains and their analysis showed a basal position of this cluster, but the bootstrap value was below 50% (Auchtung *et al.*, 2006). This fact led to another speculation that these *Korarchaeota* might just be a deep branch of *Crenarchaeota* (Fournier *et al.*, 2011). The third and most recently proposed phylum are the so called *Thaumarchaeota* which depict properties (presence or absence of certain ribosomal proteins) of the two established archaeal phyla Fournier *et al.* (2011).

1.1.1 Delimitation of *Archaea* with regard to *Bacteria* and *Eukarya*

The following description of aspects which delimitates archaea from bacteria and eukaryotes has to be incomplete by definition and in the course of this diploma thesis I am going to focus on - in my opinion - the most prominent facets of microbial live, as is DNA replication, transcription, translation, chemical composition of the cell envelope, environmental conditions and metabolism. Strikingly, properties which delimitate archaea from one other phylum are often shared with the third, and vice versa.

DNA replication

The archael DNA replication machinery is very similar to the eukaryotic one and subsequently quite different from bacterial DNA replication. But there are certain differences along the road ranging from initiation to archaeal histones.

Origin recognition. The archaeal origin recognition complex (ORC) consists of more than ten subunits and resembles the eukaryotic ORC containing homologs of

eukaryotic Orc1 and Cdc6. The number of origins itself can vary from one, bacterial-like, to very few. Investigations in *"Sulfolobus solfataricus"* showed that the three origins of replications fired synchronously (Barry and Bell, 2006).

DNA unwinding and single strand binding. It is not yet clear whether archaea possess a Mcm-homolog or not, on the other hand it is known that the unwinding of DNA occurs in 3'-5' direction as in eukaryotes and not in 5'-3' as in bacteria. The primary sequence of archaeal single strand binding proteins (SSBs) resembles bacterial ones, but the structure of the important oligonucleotide binding motif looks like the eukaryotic version of SSBs (Barry and Bell, 2006).

Replications machinery. The archael primase is a dimer consisting of PriS and PriL as in eukaryotes, bacteria have got a monomer called DnaG. PriL inhibits PriS in its synthetic ability leading to short RNA primers for DNA polymerase. Looking at the proliferating cell nuclear antigen (PCNA) and DNA polymerase of archaea it is possible to distinguish the two phyla euryarchaeota and crenarchaeota. Euryarchaeota exhibit DNA polymerases of family B and D, which is unique to archaea, and one PCNA homolog, which does just not apply in rare exceptions. On the other hand crenarchaeota possess 2 or more different DNA polymerases of family B and the same is true for PCNA homologs for the majority of species of this phylum (Barry and Bell, 2006).

Transcription

Comparing archaeal transcription with bacterial and eukaryotic transcription it makes sense to seperate the machinery itself from the regulatory processes, because the machinery itself resembles the eukaryotic RNA polymerase II complex, when the regulation mostly looks bacterial-like.

Transcriptional machinery. Archaeal TBP binds the TATA-box at the promotor site, TFB associates with the upstream, purine-rich TFB responsive element (BRE) and subsequently a RNA polymerase (RNAP) is recruited. These proteins have their counterparts in eukaryotes (Bell and Jackson, 2001). An important difference of archael and eukaryotic RNAP is the lack of the regulatory C-terminus of RNAP in archaea.

Regulation of transcription. This is a key step in gene expression and can be accomplished via negative, positive regulation or a combination of these two.

Some examples for negative regulation are metal-dependent MDR1 and TrmB, which does not interfere with the TBP/TFB complex but prevents RNAP from entering the complex (Bell *et al.*, 1999; Lee *et al.*, 2003). A similar function is featured by NipR, which plays a regulatory role in nitrogen fixation. When the nitrogen levels are sufficient copies of this protein cooperatively bind random operators to prevent transcription of the operon. At low cellular nitrogen condition 2-oxoglutarate will be bound by NipR decreasing its affinity to DNA (Lie *et al.*, 2005). Ligand-independent Lrs14 and LrpA, first mentioned will bind the TATA-box and BRE rendering it unaccessible for TBP and TFB (Bell and Jackson, 2000) and the last mentioned will interfere with the promotor in an unknown fashion (Brinkman *et al.*, 2000). A mixed form of negative and positive regulation is depicted by GrpD and GrpE in species of haloarchaea. These two proteins are a classic positive-negative regulator pair (Paget and Helmann, 2003). Positive regulators are for instance LysM which binds upstream of the TATA-box and the BRE and promote the transcription of the lysine operon. Counteractor is lysine if cellular concentration is high enough (Brinkman *et al.*, 2002). Ptr2 displays overall a known eukaryotic regulatory mechanism done by a bacterial-like protein: Ptr2 binds two elements upstream of a weak TATA-box turning it into a stronger motif. The term of upstream activating site (UAS) was used for this principle in archaea (Ouhammouch *et al.*, 2004; Geiduschek and Ouhammouch, 2005).

Translation

There are three mechanisms of translation initiation taking place in archaea: a bacteria-like mechanism, a eukaryote-like process and a novel one, which was observed and investigated in haloarchaea. In addition it can be noted that - again as in DNA replication and transcription - the number and homology of translation initiation factors resembles those of eukaryotes.

Leadered mRNA containing a Shine-Delgarno (SD) sequence. This mechanism is best known from bacteria. The leader or 5'-UTR (untranslated region) of the mRNA contains the SD sequence which is complementary to the anti-SD sequence within the 16S rRNA. Facilitating the translation off this template the 30S subuning of the ribosome is sufficient to from a stable complex. No cofactors or initiator tRNA (tRNAi) are needed. When the SD sequence is replaced by a random sequence the mRNA will no be translated. It has been proposed that this mechanism might be preferably used to translate distal genes on polycistronic mRNAs (Benelli *et al.*, 2003).

Leaderless mRNA. The 5'-UTR is not longer than 5 nucleotides upstream of the start codon AUG. This process is eukaryote-like because this short 5'-UTR does not abolish a very short scan as in eukaryotes until the first AUG is localized. To form a stable complex the 30S ribosomal subunit needs tRNAi, the association of these two factors might be aided by other cofactors. The translation of monocistronic or 5'-localized genes on polycistronic leaderless mRNAs might be favoured by this mechanism (Benelli *et al.*, 2003).

Novel mechanism in *Haloarchaea*. After an investigation of 63 mRNAs in haloarchaea a group surrounding Oliver Hering found that two thirds of the mRNAs were leaderless and almost all of the last third leadered mRNAs did not include a SD sequence. They proposed this new mechanism after they could exclude the two already mentioned processes and the two typical eukaryotic ones called scanning and IRES (internal ribosomal entry site) (Hering *et al.*, 2009).

Chemical composition of the cellular envelope

This aspect of archaeal phenotype is a very strong criteria for delimitation. Archaea do not synthesize murein as bacteria do which suggests that murein synthesis was acquired later in evolutionary terms. The next distinctive feature of archaea is that they synthesize isoprenoid L-glycerol diethers or di-L-glycerol tetraethers instead of diacyl D-glycerol diesters. In fact different genera of archaea developed different cell envelope polymers in their course of development, cf. figure 2 (Kandler and König, 1998).

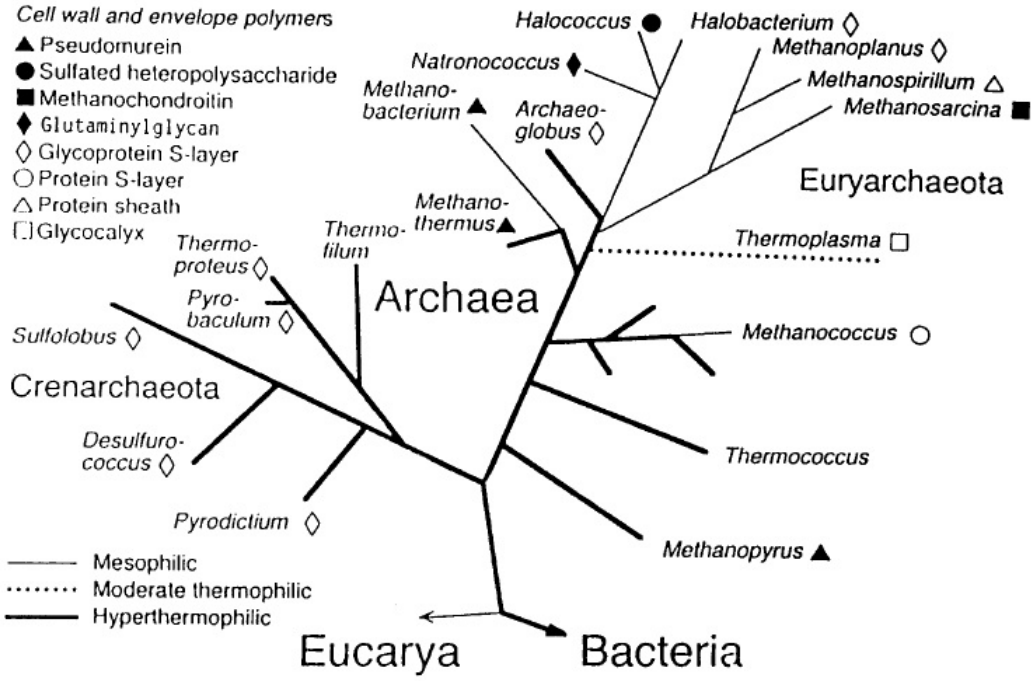


Figure 2: *Overview of cell wall polymers synthesized in different genera of archaea (Kandler and König, 1998)*

Environmental conditions and metabolism

The diversity of archaea is reflected by the variety of environments inhabited by exponents of this kingdom and their metabolic adaptation to these conditions. The following brief overview of different environments and metabolism I base on Chaban *et al.*, 2006, who chose to divide archaea into three groups for their considerations: methanogens; thermophiles, hyperthermophiles and acidophiles; and extreme halophiles.

Methanogens. The members of this division belong to the euryarchaeota and are defined as strict anaerobes. Different species live in temperatures ranging from 0°C to 100°C, NaCl-concentrations from freshwater to hypersaline and the pH varies from weakly acidic to about 9. As electron donor are used Fe, Al and Zn. Furthermore they are the only organisms which can produce methane from CO_2/H_2, formate and acetate. These microorganisms live in complex ecosystems with other bacteria, using their excretion products. They are also found in the gastrointestinal tract of animals and geological active regions as hydrothermal vents and hot springs metabolizing inorganic compounds (Chaban *et al.*, 2006).

Thermophiles, hyperthermophiles and acidophiles. The four orders of crenarchaeota and three orders of euryarchaeta (*Thermoplasmatales, Thermococcales* and *Archaeoglobales*) fall into this realm. It is the most heterogenous grouping with some obligate aerobes and about three fourth faciltative and obligate anaerobes. The metabolism ranges from heterotroph to facultative heterotroph, even chemoautotrophs metabolizing CO_2 are found. Hence there is a manifold of electron donors, for instance H_2, ferrous iron and reduced sulfur compounds and electron acceptors, e.g. molecular oxygen, ferric iron, CO_2, NH_3 and oxidized sulfur compounds. One feature these microorganisms have in common is the ability to withstand temperatures above 80°C, the highest temperature an organism was able to survive is 121°C. This amazing fact lets the scientific community hypothesize to which extent life is possible, an upper temperature limit is suggested around 150°C above this temperature the half-life of necessary biomolecules as ATP is decreased dramatically (Chaban *et al.*, 2006).

Extreme halophiles. This division is mostly comprised of euryarchaeota including some methanogens and haloalcaliphilic species. The exponents of this group are facultative anaerobe and grow heterotrophically. Under aerobe conditions the electron acceptor is molecular oxygen, under anaerobe conditions it is thiosulfate, fumarate or molecular sulfur. They are often able to metabolize sugars, glycerol, amino and organic acids originating from primary producers (Chaban *et al.*, 2006).

1.1.2 *Halobacteriaceae* and their adaptations to high concentrations of NaCl

Extremely halophilic archaea have to deal with NaCl concentrations ranging from 2 M to saturation, what constitutes a strong osmotic force. In order to survive or propagate a high amount of NaCl is needed, otherwise the integrity of these cells is severely diminished, which can lead to lysis. So the main challenges for archaea living

under those conditions is their structural integrity regarding the cellular envelope and osmotic pressure on one hand and on the other hand the maintenance of protein stability/functionality and metabolism.

Cellular envelope. The lipid core of halophilic archaea consists mostly of $C_{20}C_{25}$ (*sn*-2-sesterpanyl-3-phytanylglycerol), $C_{25}C_{25}$ (*sn*-2,3-diseseterterpanylglycerol) and $C_{20}C_{20}$ (*dn*-2,3-diphytanylglycerol) depending on the genus. Phosphatidylglycerol phopsphate-methyl ester, phosphatidylglycerol and phosphatidic acid are the most common polar lipids found in all genera of *halobacteriaceae*. Glycolipids are even more diverser than the aforementioned polar lipids (Kamekura and Kates, 1999). This extraordinary membrane composition leads to a negatively charged outer membrane which is highly impermeable for H^+ and Na^+. This fact is very important for the following considerations concerning the osmotic pressure. Furthermore increasing pH has only minimal influence on the integrity of the cellular envelope (van de Vossenberg *et al.*, 1999).

Osmotic pressure. There are two strategies to guarantee a balanced or slightly increased turgor pressure. The first is called high-salt-in: meaning that the intracellular concentration of anorganic salts equals the outer one. But instead of NaCl KCl is the most common intracellular salt. The second strategy is called organic-solutes-in. This approach is facilitated by synthesizing organic solutes increasing the turgor pressure, e.g. ectoine, glycerols, amino acid derivatives. The synthesis of 2-sulfotrehalose in molar amounts is very specific for haloalkalophilic species and therefore *N. magadii* (Oren, 2002). *Halobacteriaceae* use a mixture of these two strategies to ensure structural integrity (Oren, 2008).

Protein stability/functionality. The proteosome of halophiles generally trends to be more acidic than the one of mesophiles. This is accompanied by a higher incidence of acidic and small hydrophobic amino acids and a decrease of lysine and aliphatic amino acids which leads to a better solubilization and folding of proteins. After a biochemical survey of certain halophilic proteins it was stated that their activity did not change within a salt gradient but was more dependent on the temperature of the environment (Madern *et al.*, 2000).

Metabolism. All metabolic pathways are possible under high concentrations of NaCl except for autotrophic nitrification, methanogenesis based on the reduction of CO_2/H_2 or acetate and the oxidation of acetate have never been observed. The limit for these processes might be 100 - 150 g NaCl per litre (Oren, 2002).

1.1.3 *Haloarcula hispanica*, *Haloarcula marismortui* and *Natrialba magadii* - An Overview

Next I want to present an overview of phenotypic characteristics of the different species of *halobacteriaceae*, phylum *euryarchaeota*, used in this study.

	Haloarcula hispanica	***Haloarcula marismortui***	***Natrialba magadii***
Basonym	*Halobacterium marismortui*		*Natronobacterium magadii*
Morphology	Flat pleomorphic	Pleomorphic rod	Rod
Cell size	1-2 x 2-3μm	0.3 x 0.5-1μm	0.7-0.9 x 2-4μm
Motility	- or $\pm$	+	+
Gas vesicles	-	-	-
NaCl optimum (M)	3.4-3.9	2.6	3.5
NaCl range (M)	1.7-5.1	2.0-5.2	2.0-5.2
Mg optimum (M)	>0.05-0.1	>0.005	<0.01
pH optimum	Neutral	Neutral	9.5
Temp. optimum (°C)	40-50	35-40	37-40
Nitrite from nitrate	+	+	-
Gas from nitrate	+	+	-
Anaerobic growth on nitrate	+	NR	-
Anaerobic growth on L-arginin	-	NR	NR
Acids from carbohydrates	+	+	-
Growth on single carbon sources	+	+	-
Indole from tryptophan	-	a	NR
Starch hydrolysis	+[a]	+	-
Tween 80 hydrolysis	NR	+	NR
Gelatin hydrolysis	-	+	+[a]
Casein hydrolysis	NR	a	NR
Pigmentation	Red	Red	Red
Major glycolipids	TGD-2	TGD-2	-
Presence of PGS	+	+	-
G+C of DNA	62.7 (major) 54.7 (minor)	62.7	63.0 (major), 49.5 (minor)
16S rRNA sequence	X61688	U68541, X61689	X72495, D14124
Type strain	ATCC43049	ATCC33960, DSM 4426 NCIMB 2187, VKM B-1755	ATCC43099, DSM 3739 NCIMB 2190, VKM B-1751 JSM 8861, CCM 3739
Source of isolation	Dead Sea, Israel	Saltern, Spain	Lake Magadi, Kenya

+: all strains tested positive; -: all strains tested negative; $\pm$ weak reaction; NR: not reported
[a] conflicting reports exist
TGD-2 Glcp-β-(1$\rightarrow$6)-Manp-α(1$\rightarrow$2)-Glcp-α(1$\rightarrow$1)-sn-glyceroldiether
PGS phosphatidylglcerol sulfate

Table 1: *Phenotypic characteristics of H. hispanica, H. marismortui and N. magadii adapted from Oren (2006)*

1.2 Polyhydroxyalkanoates (PHAs)

PHAs are polyesters of carbon compounds containing a hydroxy and a terminal carboxy group. An example for the simplest compound to meet these criteria would be polyglycolic acid (PGA), the monomer is glycolic acid COH−COOH.
The monomers of PHAs synthesized in microorganisms are commonly constituted of 3 - 16 carbon atoms and the position of the hydroxy group can vary between 3 (3-HA) and 5 (5-HA). Additionally R-pendant groups containing saturated or unsaturated fatty acids, aliphatic or aromatic functional groups, straight or branched sidechains can be found among the incorporated monomers. For further considerations PHAs have been categorized depending on the chain length of their constituting monomers: PHAs consisting of monomers which are comprised of 3 - 5 carbon atoms are called short-chain-length (SCL-) PHAs, medium-chain-length (MCL-) PHAs are built from monomers containing 6 - 14 carbon atoms and monomers with more than 14 carbon atoms build long-chain-length (LCL-) PHAs (Singh and Mallick, 2009).
Some exemplary monomers are given in figure 3.

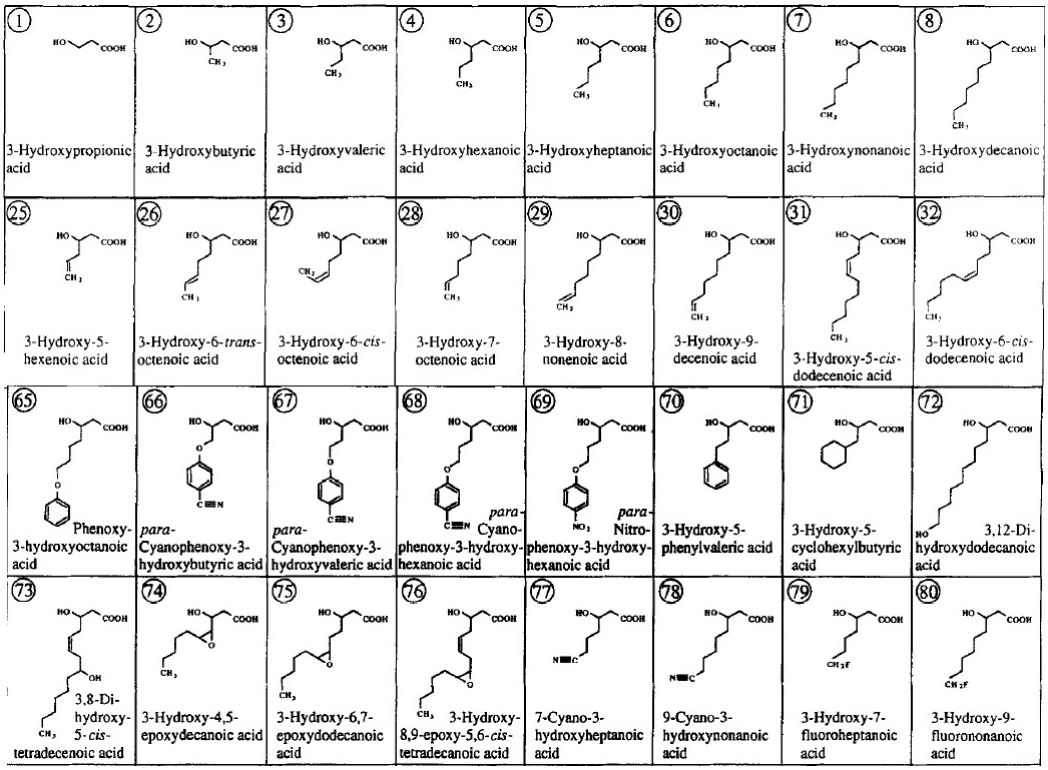


Figure 3: *Examples for bacterial hydroxyalkanoates incorporated into PHAs adapted from Steinbüchel and Valentin (1995). Today more than 150 different compounds are known to be polymerized into PHAs (Reddy et al., 2003; Rehm, 2003)*

PHAs or to be more precise poly-3-hydroxy butyrate was first discoverd in 1926 by M. Lemoigne in *Bacillus megaterium* (Lemoigne, 1926). Over time more and more

species were identified which would utilize different monomers for polymerisation and up to now exponents of 115 different genera were reported to be able to synthesize PHAs (Koller *et al.*, 2010). Generally PHAs are synthesized under nutrient stress as carbon and energy storage. They are deposited as water-insolulable granules intracellularly and they can make up to 90% of cell dry weight. Their molecular mass ranges from 50 to 5,000 kDa. An increased degree of polymerization is accompanied by thermoplastic and elastomeric features, always keeping in mind that also the composition of the polymer has strong influence on its properties (Rehm, 2003). These properties of course vary from species to species. There are also non-storage PHAs in the membrane of *Escherichia coli*, yeasts, plants and animals (Reddy *et al.*, 2003), their concentrations is 3 to 4 orders lower than the PHA content of PHA accumulating species. So far no genes have been identified which would encode for proteins producing these non-storage PHAs (Rehm, 2003).

Archaea	***Bacteria***	***Eukarya***
Halobacterium	*Actinomycetes*	*Aureobasidium*
Haloarcula	*Caulobacter*	*Penicillium*
Haloferax	*Clostridium*	*Physarum*
Haloquadratum	*Legionella*	
Haloterrigena	*Nocardia*	
	...	

All archael and eukaryal genera which are able to synthesize PHAs are inidcated. In case of the archael genera, they are all members of the Halobacteriaceae, the eukaryal genera are able to synthesize poly-β-malic acid. The list of bacterial genera is only fragmenterary.

Table 2: *Fragmentary and exemplary list of PHA synthesizing organisms adapted from Koller et al. (2010), for the complete list look ibid.*

PHA synthesis can be divided into three steps:

1. Uptake of a suitable carbon source by active transport or diffusion across the membrane (SCL-PHA is source-dependent)
2. Metabolic processing of the carbon source leads to a hydroxyacyl coenzyme A thioester (MCL- and LCL-PHA subunits are catabolized)
3. This hydroxyacyl coenzyme A thioester can be polymerased by a PHA synthase which leads to an elongated PHA and the release of coenzyme A

(Steinbüchel and Valentin, 1995; Reddy *et al.*, 2003)

Most PHAs are composed of different monomers which are differently distributed. Most species capable of PHA synthesis only produce SCL- or MCL-PHA but no

mixture of them. Exceptions from the rule are for instance *Rhodococcus ruber* which synthesizes a copolymer consistent of three different monomers, and *Pseudomonas* sp. A33 which is able to build a very heterogenic composition of 3-HA SCL- and 3-HA MCL-PHA (Steinbüchel and Valentin, 1995).

The next two tables are given to illustrate the influence of carbon source on PHA composition and yield.

Microorganism	**Carbon source**	**PHA**	**Yield (% CDW)**
Alcaligenes latus	Sucrose	P(3HB)	88%
Azetobacter vinelandii	Glucose	P(3HB)	79.8%
Bacillus megaterium	Glucose	P(3HB-co-3HV)[a]	58.6%[1]
	Glycerol	P(3HB)	62.4%[1]
Halomonas boliviensis	Glucose	P(3HB)	81%
Ralstonia eutropha[d]	Glucose	P(3HB)	>80%[2]
	Fructose	P(3HB)	81%[3]
Pseudomonas oleovorans	Glucose	P(3HD-3HDD)	31.3%[3]
Haloferax mediterranei	Glucose	P(3HB-co-3HV)[b]	48.6%
	Hydrolyzed whey	P(3HB-co-3HV)[c]	72%

3HB *3-hydroxy butyrate;* ***3HV*** *3-hydroxy valerate;* ***3HD*** *3-hydroxdecanoate;* ***3HDD*** *3-hydroxydodecanoate;* ***CDW*** *cell dry weight*

[a] *97.5% 3HB, 2.5% 3-HV;* [b] *89.3% 3HB, 10.7% 3HV;* [c] *94% 3HB, 6% 3HV incorporated into PHA;* [d] *formerly known as Alcaligenes eutrophus*

[1] *Reddy et al. (2009);* [2] *Chen (2009);* [3] *Tian et al. (2009)*

Table 3: *Exemplary overview of PHA producing microorganisms adapted from Quillaguamán et al. (2010) and expanded*

Table 3 shows how the carbon source can influence on one hand the PHA yield of certain species and on the other hand the composition of the PHA. *H. mediterranei* is so far the best archael PHA producer. The polymerization mostly takes place in the logarithmic growing phase of the species (Quillaguamán *et al.*, 2010).

Pseudomonas aeruginosa	Polymercomposition (mol %)				
Carbon source	**3HB**	**3HV**	**3HHD**	**3HOD**	**Yield (% CDW)**
Glucose	92	2.4	2.4	3.2	43.1
Palm oil	86.6	5.8	3.2	4.4	62.2
Palm oil, Palm oil cake	87.3	5.1	3.6	4	74.4

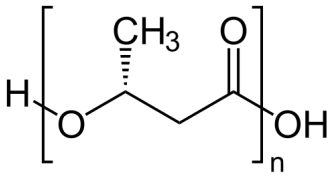
3HB *3-hydroxy butyrate;* ***3HV*** *3-hydroxy valerate;* ***3HHD*** *3-hydroxyhexadecanoic acid;* ***3HOD*** *3-hydroxyoctadecanoic acid;* ***CDW*** *cell dry weight*

Table 4: *PHA composition in Pseudomonas aeruginosa depending on carbon source, adapted from Singh and Mallick (2008) and (2009)*

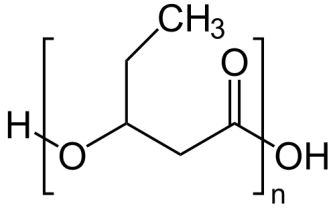
Table 4 shows on one hand again that the PHA yield depends on the carbon source and on the other hand that also the composition of the PHA is substrate specific.

1.2.1 Poly-3-Hydroxy Butyrate (PHB) and Poly-3-hydroxybutyrate-co-3-hydroxyvalerate (P(3HB-co-3HV))

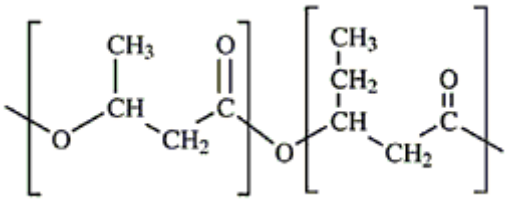
The focus of this study lies on the PHA synthases of *H. hispanica* and *H. marismortui*. These two species are able to synthesize PHB and P(3HB-co-3HV) under nutrient limiting conditions from glucose, hence I will focus on the synthesis and properties of these two biopolymers (Han *et al.*, 2009).



(a) ***PHB****; reproduced from http://en.wikipedia.org/wiki/Polyhydroxybutyrate, September 8th 2011, public domain*



(b) ***PHV****; reproduced from http://de.wikipedia.org/wiki/Polyhydroxyalkanoate, September 8th 2011, public domain*



(c) ***P(3HB-co-3HV)****; adapted from Tan (2003)*

Figure 4: *Chemical structure of PHB, PHV and P(3HB-co-3HV)*

Figure 4 depicts the chemical structure of the investigated PHAs.

The fact that PHA synthases usually polymerize either SLC- or MLC-PHA does not imply that they are substrate specific within the SLC- or MLC-borders. Quite the contrary, they can and will use other available precursors and incorporate them into the polymer.

Synthesis of PHB - Common Pathway

When I mentioned PHA synthesis before, I did that in very loose fashion, now I would like to focus on the second and third step of the pathway without the basic metabolic processes which take place before hand.

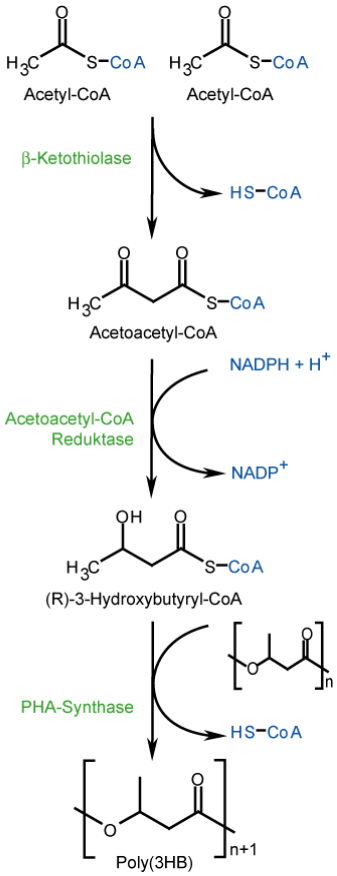


Figure 5: *Common pathway of PHB synthesis starting from acetyl-CoA thioester, carbon source metabolism up to this point is not depicted. Adapted from http://de.wikipedia.org/wiki/Polyhydroxyalkanoate, September 8th 2011, public domain*

Acetyl-CoA descends from metabolic processes, e.g. glycolysis or β-oxidation of fatty acids. After a Claisen condensation facilitated by β-ketothiolase, the resulting acetoacetyl-CoA is reduced by acetoacetyl-CoA reductase which needs NADPH as energy. Finally the PHA synthase uses the 3-hydroxybutyryl-CoA to elongate the PHB by one monomer. In case of PHV the two reactants are acetyl-CoA and Propionyl-CoA which are condensated to 3-ketovaleryl-CoA. Subsequently this compound is reduced to 3-hydroxyvaleryl-CoA which is incorporated into PHV or P(3HB-co-3HV) (Aldor *et al.*, 2002; Steinbüchel and Lütke-Eversloh, 2003; Han *et al.*, 2009).

Properties of PHB and P(3HB-co-3HV)

As PHAs polymerize to longer chains, their properties become more and more distinguished and resemble those of common plastics.

Property	**PHB**	**P(3HB-co-3HV)[1]**	**Polypropylene**
M/(10^5 g/mol)	1-8	3	2.2-7
ρ/(kg/dm^3)	1.25	1.2	0.905
melting point/°C	171-182	75-172	176
crystallinity/%	80	55-70	70
glass transition temperature/°C	5-10	-13-8	-10
O_2-permeability/(cm^3/(m^2 kPa day))	0.4	n.d.	17
UV-resistance	good	good	bad
resistance to solvents	bad	bad	good
tensile strength/MPa	40	25-30	38
elongation to break/%	6	8-1200	400
Young's modulus	3.5	2.9 (3% 3HV) 0.7 (25% 3HV)	1.7
biodegradability	yes	yes	no

n.d. not determined; [1] calculations for 3HV percentage ranging from 4-95%

Table 5: *Properties of PHB and P(3HB-co-3HV) compared to polypropylene, reproduced from Koller et al. (2010)*

Table 5 shows how similar PHAs and conventional plastics can be. But this list also highlights the advantages of PHB and P(3HB-co-3HV): same resilience and processability as polypropylene but at the same time biodegradability. Biodegradability is an attribute which cannot be stressed enough thinking about the increasing pollution of oceans and overflowing landfills by traditional plastics.

1.2.2 Classes of PHA Synthases

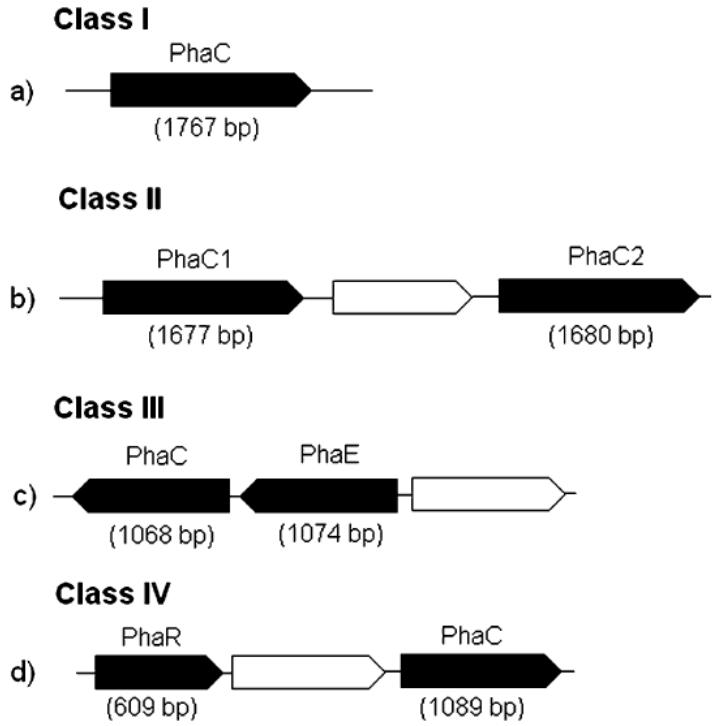
PHA synthases can be categorized based on the number of involved proteins, the nature of monomers they are able to polymerize within their hosts and the molecular mass of the resulting PHA. Table 6 gives an overview over synthase composition, exemplary species which possess this synthase, possible substrates and molecular weight of the polymer.

Class	**Subunits**	**Molecular weight**	**Example**	**Substrate**	**Properties of PHA**
I	PhaC	61-73 kDa	*R. eutropha*	3/4/5HA 3-5C	500-5,000 kDa
II	PhaC	60-65 kDa	*P. aeruginosa*	4/5HA + 3HA 6-14C +3HB	50-500 kDa
III	PhaC PhaE	40 kDa 40 kDa	*Allochromatum violosum*	3/4/5HA 3-5C + 3HA 6-8C	molecular mass lies between I and II
IV	PhaC PhaR	40 kDa 22 kDa	*B. megaterium*	3HA 3-5C	no data available

3/4/5HA 3-, 4- or 5-hydroxyalkanoate respectively; #C describes the chain length of the monomers (number of C atoms)

Table 6: *Classification of bacterial PHA synthases (Rehm, 2003; Quillaguamán et al., 2010)*

Figure 6 shows the organization of the PHA operon on the chromosome.



(a) R. eutropha, (b) P. aeruginosa, A. violosum, B. megaterium

Figure 6: *Chromosomal organisation of PHA synthase classes only showing the synthase subunits, reproduced from Quillaguamán et al. (2010)*

What figure 6 misses are other genes usually also organized within the PHA operon, like the β-ketothiolase, the β-ketoacyl-CoA reductase and the PHA depolymerase. Class I and II synthases show high identity and share a hydrophobic C-terminus, which might facilitate the attachment to the polyester core. Concerning class III and IV PHA synthases, PhaC show high identity, but PhaE and PhaR do not except for their hydrophobic C-termini. *In vitro* studies with class I PHA synthase taken from *R. eutropha* show a very broad substrate range, which indicates that the polymer synthesized in certain species might only be dependent on the metabolic activity of the species. Using acetyl-CoA as precurser the *in vitro* production of PHB resulted in granules which had a ten times greater diameter than *in vivo* synthesized (3μm instead of 0.3 μm). Adding $MgCl_2$ to the mixture even lead to macroscopic PHB granules. Using more synthase resulted in more low molecular mass PHA. CoA which is realeased from the synthase during the process of polymerization acts a competitive inhibitor of the synthase (Rehm, 2003). Experiments using other PHA synthases also showed that they accept a broad range of substrates (Quillaguamán *et al.*, 2010).

Archael PHA Synthases

Looking at different species of *Halabacteriaceae* which are able to synthesize PHAs, there is one remarkable point: the archael PHA synthase is split into two subunits called PhaC and PhaE. Phylogenetic analysis showed that this sort of synthases best fit with bacterial class III synthases. Looking back at table 6 on page 20, the sizes of the PhaC and PhaE subunites are 40 kDa, when the archael subunits PhaC and PhaE are between 50 and 58 kDa and around 20.5 kDa respectively. After a closer phylogenetic investigation by Han *et al.* (2010) they would decide to introduce a new type of PHA synthase called IIIA for archael synthases aside of IIIB for bacterial synthases because they would not cluster well.
In vitro experiments with a haloarchael PHA synthase brought up interesting findings:

1. the enzyme was active up to a temperature of 60°C
2. the PHA synthase needed high concentrations of NaCl, which would not be interesting except for the fact that
3. PHA bound PHA synthase activity was independent of NaCl

(Rehm, 2003)

1.2.3 PHA synthesis in *Haloarcula hispanica* and *Haloarcula marismortui*

Cultures grown in rich medium do not accumulate PHA, but transferring them into defined mineral medium supplemented with 2% glucose PHA is accumulated over time according to Han *et al.* (2007). The key enzymes in *H. hispanica* which facilitate the granule formation have been identified by Han *et al.* (2009) (cf. figure 5, page 18):

1. In *H. hispanica* there are 6 paralogs of the FabG gene, called fabG1 to 6, which are entitled to be β-ketoacyl-CoA reductases. These enzymes are meant to reduce acetoacetyl-CoA to 3-hydroxybutyrate-CoA using NADPH as energy source. After knock-out studies in *H. hispanica* and reconstruction of PHA synthesis in *Haloferax volcanii*, it was stated that only fabG1 is the only significantly active reductase, even when there were amounts of fabG3 and fabG4 similar to those of fabG1.
2. PhaC (53 kDa, pI 3.8) and PhaE (20.6 kDA, pI 3.8) which are organized whithin the PHA operon and reconstitute the haloarchael PHA synthase. Polymerization results in P(3HB-co-3HV) with a low molar fraction of PHV.

The process of PHA synthesis in *H. marismortui* is possibly very similar, the PHA synthases share more than 95% homology according to BLAST and are able to complement each other (Han *et al.*, 2007). In addition there is also a fabG1 homolog present.

1.3 Objective Targets of this Study

Characterization of the PHA operon of *H. hispanica* and *H. marismortui* in *N. magadii*

The goal of this study was to reconstruct the PHA synthesizing pathway of *H. hispanica* and *H. marismortui* in *N. magadii*.

What does this statement account for? The task was to clone the two PHA operons in different configurations on to a cloning vector and transform these into the host, resulting in different new strains of *N. magadii*.
On the one hand each of the two PHA operons were cloned with their native promoters, on the other this native promoter was replaced by the *N. magadii* 16S rDNA promoter. The resulting four constructs were cloned onto a vector for transformation and onto a suicide plasmid for chromosomal integration. Transformation of *N. magadii* leads to a copy number of 3, whereas recombination into the chromosome would enhance this ten times after reaching a state of homozygousity (*N. magadii* might have up to 50 copies of its genome). Table 7 displays a summary of my different cloning approaches.

Promoter	Genes	Vector
Native Promotor	$PhaEC_{Hh}$[1]	Plasmid and suicide plasmid for chromosomal integration
	$PhaEC_{Hm}$[2]	
16S rDNA $Promotor_{Nm}$[3]	$PhaEC_{Hh}$	
	$PhaEC_{Hm}$	

[1] *Hh - Haloarcula hispanica;* [2] *Hm - Haloarcula marismortui;* [3] *Nm - Natrialba magadii*

Table 7: *Overview of PHA synthase constructs and cloning strategy*

How to detect PHA synthesis?

First, PHA can be stained using Sudan Black V or Nile Red. Sudan Black V is visible using a phase contrast microscope, Nile Red is a fluorescence marker.

Second, if no signal can be obtained, the recombinant subunits of *H. hispanica* PHA synthase are purified and sent off to antibody production for western blot analysis to prove translation of the two subunits.

Third, should this detection approach also fail we would have to take another step down on the ladder and perform northern blot analysis to examine whether a mRNA is produced or not.

These tests will just show preliminary whether there is mRNA, protein or visibly PHA or not.
Final evidence of PHA accumulation will be provided by gas chromatography of methanolysed biomass.

Why use *N. magadii* as desired host of PHA synthesis? On one hand there is the scientific thirst for knowledge: whether halophilic proteins can function in a haloalkaliphilic environment in a quite different organism. On the other hand there are two very economic reasons, which concern the possible production costs and applications of PHA in halophiles:

First, contrary to the two *Haloarcula* species *N. magadii* is able to grow and proliferate proteolytically instead of saccharalotyically. Glucose is an expensive substrate whereas proteins and amino acids can be waste products of other biologic processes.

Second, the extraction of PHA is possibly easier from the host of choice due to no known expression of exopolysaccharide (EPS) on the cell surface. EPS strongly interferes with the extraction of PHA from *Haloarcula* species making the process very difficult and therefore elaborate, which leads to higher costs.

Third, *N. magadii* does not posess any lipopolysaccharide which would make it necessary to purify the PHA in case of medical applications. Nor is any antigenic component known.

Fourth, the use of extreme halophiles makes strict sterility obsolete and therefore autoclaving of all components is not necessary anymore.

Strictly speaking the last two arguments do meet also the properties of other haloarchaea and the last argument demands corrosion resistant culture equipment for endured use.

2 Material and Methods

2.1 Material

2.1.1 Strains, Growth Media and Antibiotics

E. coli Strains, their Growth Media and Antibiotics

***E. coli* Strains**

Strain	Genotype	Supplied by/Reference
XL-1-Blue	endA1, GyrA96, hsdR17 ($r_{k\text{-}}m_{K+}$, lac, recA1, relA1, supE44, thi, (F', $lacI^q$, lacZDM15, $proAB^+$, tet)	Stratagene
BL21(DE3) pLysE	F^-, ompT, $hsdS_B$ ($r_{B\text{-}}m_{B\text{-}}$), gal, decm, (DE3) pLysE (Cm^R)	Novagen
Rossetta	F^-, ompT, $hsdS_B$ ($r_{B\text{-}}m_{B\text{-}}$), gal, dcm, lacY1, (DE3), $pRARE^6$, (Cm^R)	Novagen
$Tuner^{tm}$	F^-, ompT, $hsdS_B$ ($r_{B\text{-}}m_{B\text{-}}$), gal, dcm, lacY1	Novagen
C41 pLysS	F^-, ompT, $hsdS_B$ ($r_{B\text{-}}m_{B\text{-}}$), gal, dcm, pLysS ($Cm^R$)	Miroux and Walker (1996)
C43 pLysS	F^-, ompT, $hsdS_B$ ($r_{B\text{-}}m_{B\text{-}}$), gal, dcm, pLysS ($Cm^R$)	Miroux and Walker (1996)
GM48F'	F^-, thr, leu, thi-1, lacY, galKL, galT, ara, tonA, tsx, dam, dcm supE44	(Palmer and Marinus, 1994)
PC1363	δ(lac pro), thi, cap, cya, rpsL, recA, F', $laci^{q1}$, lac^+, pro^+	Phabagen Collection
PC1363 p4a-PHB	δ(lac pro), thi, cap, cya, rpsL, recA, F', $laci^{q1}$, lac^+, pro^+, PHB-Operon of *R. eutropha*	Schroll (2005)

Table 8: *Overview of used E. coli strains*

Growth Media LB, rich medium

Peptone	10 g
Yeast extract	5 g
NaCl	5 g
pH	7.0
ad 1l dH_2O	

For plates 15 g/l agar were added. For PHA synthesis in PC1363 p4a-PHB 1% glucose were added after autoclaving.

Antibiotics

Antibiotic	**Final concentration**	**Assembly**
Ampicillin	100 μg/ml	aq., sterile filtrated; storage at 4°C
Tetracycline	10 μg/ml	dissolved in 70% ethanol; dark storage at -20°C
Chloramphenicol	20 μg/ml	dissolved in 96% ethanol; storage at -20°C

Archael Strains, their Growth Media and Antibiotics

Archael Strains

Species	**Genotype**	**Reference**
H. hispanica	wildtype	(Juez *et al.*, 1986)
H. marismortui	wildtype	(Oren *et al.*, 1990)
N. magadii L13	prophage cured wildtype	(Witte *et al.*, 1997)

Table 9: *Overview of used archael species*

Growth Media

NVM, rich medium for *N. magadii*

Casamino acids	8.8g
Yeast extract	11.7 g
Tri-Na citrate	0.8 g
KCl	2.35 g
NaCl	235 g
pH	9.0
ad 933 ml dH_2O	

After autoclaving 1l of medium or agar respectively were complemented with the following solutions:

0.57 M	Na_2CO_3	65 ml
1 M	$MgSO_4$ (autoclaved)	1 ml
20 mM	$FeSO_4$ (sterile filtrated)	1 ml

NMM, defined mineral medium for *N. magadii*

205 g	NaCl
2 g	KCl
0.28 g	Na_2HPO_4
0.28 g	NaH_2PO_4
0.64 g	NH_4CL
0.66 g	Leucine
1.66 g	Sodium acetate
1.1 g	Sodium pyruvate
	pH 9.0
	ad 900 ml dH_2O

After autoclaving 1 l of medium or agar respectively were complemented with the following solutions:

1.75 M	Na_2CO_3	100 ml
1 M	$MgSO_4$ (autoclaved)	1 ml
20 mM	$FeSO_4$ (sterile filtrated)	250 μl
1000x	Trace elements (sterile filtrated)	1 ml

1000x Trace elements

4 mM	$MnCl_2$
3 mM	$CaCl_2$
4 mM	$CuSO_4$
3 mM	$ZnSO_4$

Dissolved in ddH_2O and sterile filtrated.

NMM media which were supplemented with x g casamino acids were called NMMx (NMM including 4 g casamino acids was called NMM4).
For NVM and NMM plates 8 g/l agar were added.

23% MGM, rich medium for *H. hispanica* and *H. marismortui*

184.08 g	NaCl
23.01 g	$MgCl_2 \cdot 6\,H_2O$
26.85 g	$MgSO_4 \cdot 7\,H_2O$
5.36 g	KCl
5 g	Peptone
1 g	Yeast extract
0.15 g	$NaHCO_3$
3.83 ml	1 M Tris-HCl pH 7.5
3.83 ml	1 M $CaCl_2$
	pH 7.5 (1 M Tris base)
	ad 1 l dH_2O

Precipitate as a result of autoclaving was dissolved using a few drops of 37% HCl. The pH was readjusted using 1 - 2 ml of 1 M Tris base (>45 mM of Tris might act inhibitory on archael growth) .

MG, defined minimal medium for PHA accumulation of *H. hispanica* and *H. marismortui*

200 g	NaCl
2 g	KCl
1 g	Sodium glutamate
1 g	Yeast extract
6.05 g	Tris-base
20 g	$MgSO_4 \cdot 7\,H_2O$
	pH 7.2 (NaOH)
	ad 960 ml dH_2O

1 M	$FeSO_4$ (sterile filtrated)	179.8 μl
2 mM	$MnCl_2$ (sterile filtrated)	909.5 μl
	50% Glucose (sterile filtrated)	40 ml

After autoclaving 1 l of medium or agar respectively is complemented with the following solutions: First add the $FeSO_4$ and the $MnCl_2$ to the glucose, mix it and pour the mixture into the rest of the media to avoid precipitates.
For plates add 8 g/l agar to 23% MGM or MG.

Antibiotics

Antibiotic	**Final concentration**	**Assembly**
Novobiocin	3 μg/ml	aq., sterile filtrated; storage at -20°C
Bacitracin	70 μg/ml	aq., sterile filtrated; storage at 4°C

2.1.2 Vectors, Primer Sequences and DNA/Protein Markers

Vectors

Plasmid	Properties	Reference
pKSII+	mcs, bla, ColE1 ori, LacZa	Stratagene
pKS-rrnb	mcs, bla, ColE1 ori, LacZa, 16S rDNA promoter$_{Nm}$[1]	Selb (2010)
pMDS11	bla, f1 ori, ColE1 ori, gyrBm pHK2 ori	Holmes *et al.* (1991)
pKS-rrnb PhaEC$_{Hh}$[2]	mcs, bla, ColE1 ori, LacZa, 16S rDNA promoter$_{Nm}$ upstream of PhaEC$_{Hh}$	this thesis
pKS-rrnb PhaEC$_{Hm}$[3]	mcs, bla, ColE1 ori, LacZa, 16S rDNA promoter$_{Nm}$ upstream of PhaEC$_{Hm}$	this thesis
pKSII+HR	mcs, bla, ColE1 ori, LacZa, 2 $_{Nm}$-homolog flanks downstream of protease	this thesis
pKSII+HR PHB$_{Hh}$	mcs, bla, ColE1 ori, LacZa, ($_{Nm}$-homolog flank, PHA-Operon$_{Hh}$, *gyrB*, $_{Nm}$-homolog flank)	this thesis
pKSII+HR PHB$_{Hm}$	mcs, bla, ColE1 ori, LacZa, ($_{Nm}$-homolog flank, PHA-Operon$_{Hm}$, *gyrB*, $_{Nm}$-homolog flank)	this thesis
pKSII+HR 16S prom$_{Nm}$ PhaEC$_{Hh}$	mcs, bla, ColE1 ori, LacZa, ($_{Nm}$-homolog flank, 16S rDNA prom$_{Nm}$ PhaEC$_{Hh}$, *gyrB*, $_{Nm}$-homolog flank)	this thesis
pKSII+HR 16S prom$_{Nm}$ PhaEC$_{Hm}$	mcs, bla, ColE1 ori, LacZa, ($_{Nm}$-homolog flank, 16S rDNA prom$_{Nm}$ PhaEC$_{Hh}$, *gyrB*, $_{Nm}$-homolog flank)	this thesis
pRSET-A	mcs, bla, EK, PT7, RBS, His-tag, pUC ori, f1 ori	Invitrogen
pRSET-A PhaC$_{Hh}$	mcs, bla, EK, PT7, RBS, His-tag, pUC ori, f1 ori, PhaC$_{Hh}$	this thesis
pRSET-A PhaE$_{Hh}$	mcs, bla, EK, PT7, RBS, His-tag, pUC ori, f1 ori, PhaE$_{Hh}$	this thesis
pRo-5	*bla*, ColE1 ori, *gyrB*, ϕCh1 derived ori	(Iro *et al.* in prep.)
pRo-5 16S prom PhaEC$_{Hh}$	*bla*, ColE1 ori, *gyrB*, ϕCh1 derived ori, 16S rDNA promoter$_{Nm}$ upstream of PhaEC$_{Hh}$	this thesis
pRo-5 16S prom PhaEC$_{Hm}$	*bla*, ColE1 ori, *gyrB*, ϕCh1 derived ori, 16S rDNA promoter$_{Nm}$ upstream of PhaEC$_{Hm}$	this thesis

[1] *Nm - N. magadii;* [2] *Hh - H. hispanica;* [3] *Hm - H. marismortui*

Plasmid	Properties	Reference
pRo-5 PHB_{Hh}	*bla*, ColE1 ori, *gyrB*, ϕCh1 derived ori, PHA-$Operon_{Hh}$	this thesis
pRo-5 PHB_{Hm}	*bla*, ColE1 ori, *gyrB*, ϕCh1 derived ori, PHA-$Operon_{Hm}$	this thesis

[1] Nm - N. magadii; [2] Hh - H. hispanica; [3] Hm - H. marismortui

Table 10: *Overview of used and assembled plasmids*

Primer Sequences

Name	Sequence	Restriction site
PHB-1	5'-GACCATCGATGCAAAACGTCAACAGCG-3'	*Cla*I
PHB-1-Kpn	5'-GACCGGTACGGCAAAACTTCAACATCG-3'	*Kpn*I
PHB-2	5'-GACCGGTACCGAATTCTTACAGTTGGTCGAGCCAG-3'	*Eco*RI, *Kpn*I
PHB-3	5'-GACCATCGATGAGCAGATCCGCGAGAC-3'	*Cla*I
PHB-3-KpnI	5'-GACCGGTACCGAGCAGATCCGCGAGAC-3'	*Kpn*I
PHB-3-KpnI-K	5'-GAAAGGTACCGAGCAGATCCGCGAGAC-3'	*Kpn*I
PHB-4	5'-GACCGGTACCGAATTCAGACGCGCATGGCTG-3'	*Eco*RI, *Kpn*I
PHB-5	5'-GACCTCTAGAAACCGATAGCCATTGGCA-3'	*Xba*I
PHB-6	5'-GAATGGATCCGGGAGTGGAACGTACGAACT-3'	*Bam*HI
PHB-7	5'-GACCATCGATCGTCGCAGTGATGAGAATC-3'	*Cla*I
PHB-8	5'-GACCGGTACCGACAGGGTGATGCCTTCAG-3'	*Kpn*I
PHB-9	5'-CAGGTAGAATTCGTCCGCCGTACAGATGGGATCC-3'	*Eco*RI
PHB-10	5'-GACCATCGATTTACAGTTGGTCGAGCCAG-3'	*Cla*I
PHB-11	5'-GACCATCGATTTACAGTTGATCGAGCCAGTC-3'	*Cla*I
PHB-12	5'-GAAAATCGATGAGCAGATCCGCGAGAC-3'	*Cla*I
PhaC-Xba	5'-GACCTCTAGATTACAGTTGGTCGAGCCAG-3'	*Xba*I
PhaC-5	5'-GACCGGATCCATGTCCAGCAACCCGTT-3'	*Bam*HI
PhaE5	5'-GACCGGATCCATGAGTAATACAAACAACATTCAGG-3'	*Bam*HI
PhaE3	5'-GACCGGTACCTTACTCTTCCAGGTGTTCGA-3'	*Kpn*I
Pha-Hm-5	5'-GACCGGATCCATGAGTAATACAAACAACATTCAGGAG-3'	*Bam*HI
Pha-Hm-3	5'-GACCTCTAGATTACAGTTGATCGAGCCAGTC-3'	*Xba*I

Name	Sequence	Restriction site
Hm-PHB1	5'-ATGAGTAATACAAACAACATTCAGGAGGAA-3'	not used
Hm-PHB2	5'-TTATTCTTCTAAGTGTTCGAGAACGCG-3'	not used
16S-3Pro	5'-GTCGTTGCTGGCAATTAGGAG-3'	not used
16S-5Pro	5'-GTAAACCGGCAGCACGAGAT-3'	not used

DNA Markers

DNA ladder	Fragments (bp)
λ:*BstE*II	8454, 7242, 6369, 5687, 4822, 4324, 3675, 2323, 1929, 1371, 1264, 702
λ:*Pst*I	11501, 5077, 4749, 4507, 2838, 2556, 2459, 2443, 2140, 1986, 1700, 1159, 1093, 805, 514, 468, 448, 339, 264, 247, 216, 211, 200, 164, 150, 94, 87, 72, 15
pUC19:*Hae*III	587, 458, 434, 298, 257, 174, 102, 80, 18, 11

Protein Markers

Protein ladder	Fragments (kDA)	Supplied by/Reference
Unstained Protein Molecular Weight Marker	116, 66.2, 45, 35, 25, 18.4, 14.4	Fermentas
PageRuler^{TM} Unstained Protein Ladder	200, 150, 120, 100, 85, 70, 60, 50, 40, 30, 25, 20, 15, 10	Fermentas
PageRuler^{TM} Plus Prestained Protein Ladder	250, 130, 100, 70, 55, 35, 25, 15, 10	Fermentas

2.1.3 Enzymes

All enzymes were used in appropriate, always included buffers and according to supplier specifications.

DNA Polymerases

Polymerase	Velocity (bp/min)	Supplied by/Reference
Pwo	1000	Peqlab (cloned from *Pyrococcus woesii*)
GoTaq	1000	Promega
Pfu	500	Promega (cloned from *Pyrococcus furiosus*)

Restriction Enzymes

All restriction enzymes were supplied by Fermentas, except for *Hae*III, it was supplied by New England Biolabs. Double digestion using Fermentas restriction enzymes was carried out in buffers suiting both enzymes. To obtain the correct buffer the DoubleDigest^{TM} tool from the Fermentas homepage was used.

Other Enzymes

Enzyme	Supplied by/Reference
Calf Intestine Phosphatase	Fermentas
DNaseI	Fermentas
Klenow Fragment	Fermentas
Proteinase K	Roche
T4 Ligase	Fermentas

2.1.4 Antibodies

Detection of His-tagged proteins a goat-α-His antibody was used. The antibody was conjugated with horseradish peroxidase (HRP) for developing. Another antibody for detection of *H. hispanica*-PhaE was ordered: rabbit-α-PhaE$_{Hh}$. After three immunizations the whole serum was sent to the lab, for developing of the blot a HRP conjugated sheep-α-rabbit antibody was used. All antibodies were diluted in TBS-T containing 0.6% BSA. Grade of dilution depended on the antibody: goat-α-His was diluted 1:5000, rabbit-α-PhaE$_{Hh}$ 1:10000 and sheep-α-rabbit 1:5000.

2.1.5 Buffers and Solutions

DNA Gel Electrophoresis

50x TAE

2 M	Tris-HCl
1 M	Acetic acid
0.1 M	EDTA
	pH 8.2

5x DNA loading buffer

50 mM	Tris-HCl
0.1%	SDS
0.05% (w/v)	Bromphenol blue
0.05% (w/v)	Xyclene cyanol
	pH 8.2

After autoclaving sucrose is added to a final concentration of 25% (v/v).

10x TBE

108 g	Tris base
55 g	Boric acid
0.5 M	EDTA pH 8
	pH 8.0 (Boric acid)
	ad 1l ddH_2O

30% AA-solution

29% (w/v)	Acryl amide
1% (w/v)	N,N'-methylene bisacrylamide

6% Polyacrylamide gel

1.2 ml	30% AA-solution
4.8 ml	1x TBE
60 μl	10% APS
6 μl	TEMED

Northern Blot

Diethylpyrocarbonate (DEPC) ddH_2O

0.1% DEPC

DEPC ddH2O is incubated overnight at 37°C and then autoclaved.

10x MOPS running buffer

0.4 M	MOPS
0.1 M	Sodium acetate
0.01 M	EDTA
	pH 7.0

5x RNA loading buffer (denat.)

80 μl	0.5 M EDTA pH 8.0
720 μl	37% Formaldehyde
2 ml	100% Glycerol2 ml
3084 μl	Formamide
4 ml	10x MOPS running buffer
5 mg	Bromphenol blue
100 mg	Ethidium bromide
ad 10 ml	DEPC ddH_2O

20x SSC

3 M	NaCl
0.3 M	Sodium citrate
	pH 7.2
ad 1 l	DEPC ddH_2O

50x Denhardts Solution

1 g	Ficoll 400
1 g	Polyvinylpyrolidone
1 g	BSA
ad 100 ml	DEPC ddH_2O

Hybridization buffer

55 ml	DEPC ddH_2O
25 ml	20x SSC
10 ml	50x Denhardts solution
0.5 g	BSA
5 ml	1 M Na_2HPO_4
500 μl	20% SDS
200 μl	0.5 M EDTA

10x Wash buffer

12.1 g	Tris-base
5.85 g	NaCl
2.03 g	$MgCl_2$
	pH 9.5 (HCl)
ad 1 l	DEPC ddH_2O

Blocking solution

7.3 g	NaCl
2.41 g	Na_2HPO_4
0.96 g	NaH_2PO_4
49.89 g	SDS
	pH 7.2
ad 1 l	DEPC ddH_2O

SDS-Polyacrylamide Gel Electrophoresis (PAGE)

30% AA-solution has been described on page 36.

4x Seperation gel buffer

1.5 M	Tris-HCl pH 8.8
0.4%	SDS

4x Stacking gel buffer

0.5 M	Tris-HCl pH 6.8
0.4%	SDS

2x Laemmli buffer

0.12 mM	Tris-HCl pH 6.8
4%	SDS
17.4% (v/v)	Glycerol
2% (v/v)	β-mercaptoethanol
0.02% (w/v)	Bromphenol blue

10x SDS-PAGE running buffer

0.25 M	Tris base
1.92 M	Glycine
1%	SDS

Coomassie staining solution

25% (v/v)	Methanol
10% (v/v)	Acetic acid
0.15% (w/v)	Coomassie Brilliant Blue R-250

5 mM Sodium phosphate buffer
Solution A: 0.2 M $NaH_2PO_4 \cdot 2\,H_2O$
Solution B: 0.2 M Na_2HPO_4
Mix 2.55 ml of A and 2.45 ml of B, add ddH_2O up to 200 ml.

Native PAGE

30% AA-solution has been described on page 36.

	Native stacking gel buffer	Native seperation gel buffer
Tris-HCl	0.5 M	1.5 M
pH	6.8	8.8

Native running buffer

3 g	Tris-base
14.4 g	Glycine
	pH 8.3
ad 1 l	ddH_2O

Destain solution

100 ml	Methanol
100 ml	Glacial acetic acid
ad 1 l	ddH_2O

5x Sample buffer

15.5 ml	1 M Tris-HCl pH 6.8
2.5 ml	1% Bromphenol blue aqueous solution
7 ml	ddH_2O
25 ml	100% Glycerol

Western Blot

10x TBS

250 mM	Tris-HCl
1.37 M	NaCl
27 mM	KCl
	pH 8.0

To obtain 1 l 1x TBS-T, add 500 μl Tween20 to 100 ml of 10x TBS and ad 1 l ddH_2O (final concentration of Tween20 in 1x TBS-T is 0.05%).

Transblotbuffer

48 mM	Tris base
39 mM	Glycine
0.037%	SDS
20% (v/v)	Methanol

Ponceau S solution

0.5% (w/v)	Ponceau S
3% (v/v)	TCA

Mild stripping buffer

15 g	Glycine
1 g	SDS
10 ml	Tween20
	pH 2.2 (37% HCl)
ad 1 l	ddH_2O

Generation of Competent *E. coli*

	MOPS I	MOPS II	MOPS IIa
MOPS	100 mM	100 mM	100 mM
$CaCl_2$	10 mM	70 mM	70 mM
$RbCl_2$	10 mM	10 mM	10 mM
Glycerol (v/v)			15%
pH (KOH)	7.0	6.5	6.5

Generation and Transformation of Competent *N. magadii*

	Buffered High Salt Spheroblasting Solution	Buffered High Salt Spheroblasting Solution with glycerol	Unbuffered High Salt Spheroblasting Solution
NaCl	2 M	2 M	2 M
KCl	27 mM	27 mM	27 mM
Sucrose (v/v)	15%	15%	15%
Tris-HCl pH 8.5	50 mM	50 mM	
Glycerol (v/v)		15%	

Overexpression and Purification of His-Tagged Proteins under Denaturing Conditions

1 M IPTG, aq., sterile filtrated (storage at -20°C), for induction of protein overexpression from pRSET-A plasmids. Final concentration in culture 0.5 mM.

	Buffer B (lysis buffer)	Buffer C (washing buffer)	Buffer E (elution buffer)
NaH_2PO_4	100 mM	100 mM	100 mM
Tris-HCl	10 mM	10 mM	10 mM
Urea	8 M	8 M	8 M
pH	8.0 (NaOH)	6.3 (HCl)	4.5 (HCl)

Before each use, the pH of each solution has to be checked and in case of deviation from target value, the pH has to be readjusted using HCl.

10x PBS (for dialysis)

1.36 M	NaCl
26 mM	KCl
101 mM	Na_2HPO_4
17 mM	KH_2PO_4
	pH 7.4

Fixed Phase Liquid Chromatography (FPLC)

All solutions for FPLC had to be filtrated through a 0.22 μm filter and precooled to 4°C.

Elution buffers

1x PBS
1x PBS containing 200 mM NaCl
1x PBS containing 300 mM NaCl
1x PBS + 2% glycerol
1x PBS + 5% glycerol

Washing buffer

0.5 M NaOH

Storage buffer

20% Ethanol

Acidic Depolymerization of PHA

Acidified methanol

3% (v/v) Sulfuric acid

Chloroform including benzoic acid as internal standard for gas chromatography (GC)

50 mg/ml Benzoic acid

Microscopy

Sudan Black V

0.3% Sudan Black V in 60% ethanol

Nile Red

20 mg/ml Nile Blue in 96% ethanol

Nile Blue

0.5% Nile Blue in aqueous solution

Safranin O

0.5% Safranin O in aqueous solution

30% SW

240 g	NaCl
30 g	$MgCl_2 \cdot 6\,H_2O$
35 g	$MgSO_4 \cdot 7\,H_2O$
7 g	KCl
0.8 g	NaBr
0.2 g	$NaHCO_3$
5 ml	1 M Tris-Cl pH 7.5
	pH 7.5 (Tris-base)
ad 1 l	ddH_2O

2.2 Methods

2.2.1 DNA Gel Electrophoresis

Agarose gel electrophoresis. Usually 0.8% agarose gels are cast. Therefore an appropriate volume of agarose in 1x TAE is prepared and heated in the microwave. After downcooling of the solution it is cast into an adequate tray prepared with combs. When the gel is solid it is put into a chamber containing 1x TAE as running buffer and the comb is removed. The gel is now ready for loading and seperation of DNA. 3 - 5 μl of DNA are mixed with 5 μl of DNA loading buffer and pipetted into slots. The gel is going to run for 45 - 70 minutes at 10 V/cm and then stained in an ethidium bromide bath (5 mg/l) for several minutes. After a wash step with water the gel is put onto the gel documentation device or an UV table for preparative processing.

PAGE. This is done for DNA fragments smaller than 600 bp, facilitated in the Biorad Mini - Protean®3 system. The gel is 6% PAA in 1x TBE buffer which lead to the use of 1x TBE also as running buffer. The gels are going to run at 15 mA per gel for approximately 40 minutes. Then the gel is stained in an ethidium bromide bath (5 mg/l) for several minutes and subsequently washed in a tray filled with water. Analysis of the gel is done via gel documentation device.

DNA Extraction from Agarose Gels. When the desired DNA fragment amplified by PCR or after a restriction reaction is not the sole prominent band on an agarose gel it has to be purified. Therefore the whole DNA sample is applied to a gel, adequate current is applied and afterwards it is stained using ethidium bromide as shortly as possible. After washing using water the gel is put onto an UV table and the desired bands are as precisely and quickly as possible cut out using a scalpel. The gel slices are put into Eppendorf tubes and the extraction using the QIAquick Gel Extraction kit supplied by Qiagen can commence. Purity again was analyzed using an agarose gel.

2.2.2 Chromosomal DNA Preparation from Haloarchaea

200 μl of densely grown culture are centrifuged for 5 minutes at 10 krpm. The pellet is resuspended in 200 μl ddH_2O, this leads to cell lysis. Then 200 μl of water-saturated phenol are added and the sample is cautiously vortexed. Then it is heated up to 65°C for 1 hour. After another centrifugation the aqueous top phase is transfered into a new tube and 400 μl of ice cold 96% ethanol are added and mixed. Precipitation of

the DNA takes place at -20°C for 20 minutes. After a centrifugation at 10 krpm for 20 minutes the DNA pellet is washed with cold 70% ethanol and centrifuged again. The pellet is dried at room tempereture and taken up in 50 μl ddH_2O. This protocol is adapted from Dyall-Smith (2009).

2.2.3 Polymerase Chain Reaction (PCR)

PCR is employed on one hand for cloning using *Pwo* and *Pfu* and on the other for analytical purposes, the confirmation of clones, using GoTaq.

Primers. Primers are supplied in a lyophylized state by VBC genomics. They are diluted with ddH_2O to achieve a concentration of 1 $\mu g/\mu l$, this represents the primer stock which is kept at -20°C for storage purposes. Upon use the stock is diluted 1:10. Melting temperature (T_M) is calculated using Gene Runner Version 3.01 by Hastings Software, Inc. Annealing temperature (T_A) is calculated as T_M - 4°C, but in certain cases it had to be determined experimentally either by PCR, gradient PCR or in case of low product yield touch-down PCR.

Template DNA. As template served either plasmid DNA or crude extracts from *E. coli*, *H. hispanica*, *H. marismortui* or *N. magadii*. In case of *H. marismortui* it is recommended to do a phenol-chloroform extraction of chromosomal DNA because in crude extracts components are present which would inhibit or interfere strongly with PCR.
Crude extract of *E. coli* is prepared using 5 μl of culture plus 100 μl of ddH_2O. The mix is heated to 100°C for 10 minutes and then applicable for PCR. Crude extracts of haloarchaea are prepared using 100 μl of culture. The cells are centrifuged at 10 krpm for 5 minutes and the pellet resuspended in 100 μl ddH_2O. This is usually sufficient but not for a stock of *H. marismortui*. Therefore I had to use a protocol from the halohandbook described on page 44 (Dyall-Smith, 2009).

PCR batch and programs

Pwo:

Batch	
10 μl	10x *Pwo* buffer
10 μl	2 mM dNTPs
5 μl	Forward primer
5 μl	Reverse primer
1 μl	Template DNA
2 μl	*Pwo* polymerase
ad 100 μl	ddH_2O

Program		
1:	5 min	94°C
2:	45 sec	94°C
3:	45 sec	T_A
4:	1 min/kbp	68°C
5:	5 min	68°C
6:	infinite	4°C

Steps 2-4 are repeated 33 times, duration of step 5 always depends on the size of the desired polymerized fragment.

Pfu:

Batch	
10 μl	10x *Pfu* buffer
10 μl	2 mM dNTPs
5 μl	Forward primer
5 μl	Reverse primer
1 μl	Template DNA
2 μl	*Pfu* polymerase
ad 100 μl	ddH_2O

Program		
1:	5 min	94°C
2:	45 sec	94°C
3:	45 sec	T_A
4:	2 min/kbp	72°C
5:	10 min	72°C
6:	infinite	4°C

Steps 2-4 are repeated 33 times, duration of step 5 always depends on the size of the desired polymerized fragment.

GoTaq:

	Batch
4 μl	5x GoTaq buffer
2 μl	2 mM dNTPs
1 μl	Forward primer
1 μl	Reverse primer
0.5 μl	Template DNA
0.2 μl	GoTaq polymerase
ad 20 μl	ddH_2O

	Program	
1:	5 min	94°C
2:	45 sec	94°C
3:	45 sec	T_A
4:	1 min/kbp	72°C
5:	5 min	72°C
6:	infinite	4°C

Steps 2-4 are repeated 33 times, duration of step 5 always depends on the size of the desired polymerized fragment.

Fragment analysis and further processing. Analysis of PCR is mostly done on 0.8% agarose gels, in few cases, when the amplified fragment is smaller than 600 bp, it is analyzed on a 6% polyacrylamide gel. For cloning purposes the PCR fragment is purified using the Qiagen PCR Purification kit. In case of a small yield the fragment is concentrated using less than 50 μl ddH_2O for elution. When more than one band is visible on the gel, the whole PCR batch was applied to an agarose gel, the bands are cut out and processed via the Qiagen QIAquick Gel Extraction kit.

2.2.4 Northern Blot

Northern blotting allows detection of specific RNAs using any user-defined DNA probe. DNA probes for untranslated and translated parts of mRNAs are possbile as are probes for other sorts of ncRNAs.

Isolation of RNA. 1.5 ml of a logarithmic growing culture are taken and centrifuged at 11 krpm for 3 minutes. The pellet is resuspended in 250 μl of 4 M NaCl and 750 μl of Trizol reagent are added. Incubation lasts 5 minutes at room temperature. After further addition of 20 μl chloroform the sample is mixed and again incubated at room temperature for 15 minutes. When centrifugation at 10 krpm for 15 minutes at 4°C is performed the aqueous phase is transferred to a new tube an 500 μl 2-propanol are added. After another centrifugation at 10 krpm for 10 minutes at 4°C the pellet is washed using 1 ml 75% ethanol. When the final centrifugation at 6 krpm for 5 minutes at 4°C is performed the pellet is carefully dried and dissolved in 30 μl DEPC ddH_2O. To get rid of any residual DNA which might tamper with the results a DNase I digest is carried out.

26 μl	RNA
3 μl	10x DNAse I reaction buffer
1 μl	DNAse I

Incubation at 37°C for 30 minutes, inactivation by addition of 1 μl of 25 mM EDTA and heating to 65°C for 10 minutes. A sample of RNA is mixed 5x RNA loading buffer and denature for 5 minutes at 70°C is performed. The sample is applied to a 1% agarose (in 1x MOPS buffer/6.5% formaldehyde) gel. The separation of RNA is performed at 7 V/cm until the bromphenol blue reaches the lower end of the gel. Additionally the RNA concentration is measured using nanodrop because the final amount of RNA for the blot should be 3 mg.

Blotting procedure. For blotting the RNA sample containing 5x RNA loading buffer is denatured for 15 minutes. The seperation of RNA is performed at the same conditions as described before and the RNA is controlled using a gel documentation device. Protran nitrocellulose, Whatman papers and paper towels are cut the same size of the gel. The blot is assembled as follows: a glass tray is filled with transferbuffer. An agarose gel tray is put inside in a upside down fashion (comparable to a bridge or table inside the glass tray). Two long stripes of Whatman paper are put on the upside of the agarose gel tray in a way that the two ends of the stripes reach the transferbuffer in the glass tray. Afterwards the agarose gel is positioned. On top the nitrocellulose membrane is placed. Using a glass pipette any occurring air bubbles are removed. Upon the membrane 5 layers of Whatman paper are put, on top of those a big stack of normal paper towels is adjusted. The nitrocellulose, Whatman papers and paper towels are cut that way that their size resembles the size of the gel. A prepared cardboard box is put over the blot. The next day the membrane is dried and the RNA is cross linked using an UV oven.

Probes and labelling. Probes are isolated using PCR. For detection of mRNAs originating from the PHA operon of *H. hispanica* in *N. magadii* transformants primer set PhaE5/PhaE3 and corresponding chromosomal DNA are used. For detection of mRNAs originating from the PHA operon of *H. marismortui* in *N. magadii* transformants primer set Hm-PHB1/Hm-PHB2 and corresponding chromosomal DNA are used. As control primers 16S-3Pro/16S-5Pro and chromosomal DNA from *N. magadii* are used which would clone parts of the 16S rDNA. This housekeeping gene is used as positive control. The probes are labelled according to New England Biolabs Phenotype Star Detection kit.

Prehybridization, hybridization and detection. Prehybridization of the membrane was done at 65°C for 3 hours using 12 ml of hybridization buffer and 120 μl salmon sperm DNA in a flask. This is done to block any free parts of the membrane where no RNA is bound, otherwise unspecific detection would be imminent. Before the probes are added to the flask they are denatured at 95°C for 5 minutes and put back on ice for another minute. Hybridization takes place at 65°C over night. The next day the membrane is washed two times using 2x SSC/0.1% SDS at room temperature for 5 minutes. Then it is washed another two times using 0.1x SSC/0.1% SDS at 65°C for 15 minutes.

Development. The membrane is incubated using blocking solution for 5 minutes. The development is carried out according to the kits protocol. The development is carried out in the dark room using a cassette and a X-ray hyper film. The film is positioned on the membrane and the exposure time is determined by trial and error.

2.2.5 SDS-Polyacrylamide Gel Electrophoresis (PAGE)

This method separates a mixture of proteins depending on their molecular weight. Protein samples are denatured by heating and β-mercaptoethanol. SDS (sodiumdodecylsulfate) masks the proteins native charges with its own strong negative one. After applying an electric current to the gel the proteins migrate depending on their molecular weight instead of their net charge. This applies for mesophilic proteins, but not necessarily for halophilic proteins which are strongly negatively charged in general. This circumstance is due to a higher incidence of acidic amino acids which leads to lower isoelectric points.

Preparation of a discontinuous PAA gel. BioRad's Mini Protean 3 system is used for gel casting. A discontinuous gel consists of a low PAA percentage stacking gel on top of a high PAA percentage separation gel.

Reagent	**4% Stacking gel**	**12% Separation gel**
ddH_2O	1233 μl	1750 μl
Stacking gel buffer	500 μl	
Seperation gel buffer		1250 μl
30% AA solution	267 μl	2000 μl
10% APS	20 μl	60μl
TEMED	5 μl	10 μl

All reagents are prepared on ice without APS and TEMED. After addition of these two reagents to the separation gel it is cast into the prepared apparatus. Afterwards the polymerizing gel is covered with a layer of 2-propanol. When the separation gel is solid the 2-propanol layer is removed and APS and TEMED are added to the stacking gel. After casting this upper part the comb is placed in the still liquid stacking gel. The gel is fixed in the apparatus and 1x SDS running buffer is added into the two chambers.

Sample preparation. 2x Laemmli buffer is added to a protein sample and it is heated to 95°C for 10 minutes. The samples are either frozen at -20°C for later use or just cooled down for immediate application on the gel along with a protein weight marker. After applying an electric current of 15 mA/gel to the apparatus the progress of the separation can be observed via the front of the bromphenol blue. Depending on the application the gel is run for 50 - 60 minutes.

Concentration of protein samples using trichloroacetic acid (TCA) precipitation. In case of low concentrated protein fractions after analytical FPLC it is necessary to concentrate them in order to detect any bands on SDS-PAGE gels. TCA is added to a protein sample until the final concentration of TCA in the sample is 5 - 10%. After incubation on ice for 30 minutes proteins are collected by centrifugation at 13.2 krpm for 10 minutes. The supernatant is discarded and the precipitate is dissolved in 1x Laemmli buffer. In case of changeover from blue to yellow, meaning that too much TCA is still present in the sample, it is vaporated using 25% ammonia solution, or 1 μl of the solution is added to the sample.

Staining of proteins. After disassembling the running chamber the stacking gel is removed and the separation gel is put into a dish containing coomassie staining solution for 5 - 20 minutes. Destaining of the gel is done using rinse water overnight on a shaking table. The next day the gel is scanned and dried.

2.2.6 Native PAGE

The only difference to SDS-PAGE is that the protein samples are not denatured and therefore the proteins are not only separated depending on their molecular weight but also depending on their native charge.

Preparation of a discontinuous PAA gel. The preparation procedure is the same as for SDS-PAGE gels.

Reagent	**3.75% Stacking gel**	**7.5% Separation gel**	**10% Separation gel**
ddH_2O	3.7 ml	7.4 ml	6.2 ml
Native stacking gel buffer	1.5 ml		
Native seperation gel buffer		3.7 ml	3.7 ml
30% AA solution	750 μl	3.7 ml	5 ml
10% APS	50 μl	150 μl	150 μl
TEMED	7.5 μl	15 μl	15 μl

The only difference to a SDS-PAGE gel is after the separation gel is cast it is covered with ddH_2O instead of 2-propanol. After the gel is solid the running chamber is assembled and native running buffer is filled into the two chambers.

Sample preparation. The proper amount of the 5x loading buffer is added to the samples. The separation takes place at 20 - 25 mA/gel for about 3 hours.

Staining of proteins. Staining of the gel is done using Coomassie staining solution for 2 hours or overnight. Afterwards its destained using destain solution.

2.2.7 Western Blot

Western blot is a technique which allows detection of a certain protein within a heterogeneous mixture by specific antibodies. Antibodies can be mono- or polyclonal, conjugated or non-conjugated. The used α-His tag antibody is conjugated with HRP, the other antibody which was ordered against $PhaE_{Hh}$ was not. Therefore a second conjugated antibody directed against the conserved region of the first is needed.

Blotting procedure. Preceding the blotting, protein samples of interest are applied to a SDS-PAGE gel and separated. 6 pieces of Whatman paper and 1 piece of Protran nitrocellulose are cut in the size of a gel. The blot is assembled in a dish

containing transblot buffer: from bottom up there are 3 pieces of Whatman paper, nitrocellulose, SDS-PAGE gel and again 3 pieces of Whatman paper. This stack is transferred onto the semi-dry blot apparatus and using a test tube as rolling pin air bubbles are removed. Depending on the number of blots the duration of the blot has to be adjusted. At 20 V 1 blot takes 20 minutes, 2 take 35 minutes and 3 blots are transferred in 50 minutes. Subsequently the semi-dry blot is disassembled and the nitrocellulose membranes are put into dishes containing Ponceau S. This reversible staining of protein has two purposes, on one hand to check whether the transfer worked out correctly and on the other to chart the protein marker using a pencil. The nitrocellulose membrane is destained using rinse water.

Detection and development. All steps are facilitated on a shaking table. The nitrocellulose membrane is blocked overnight using 5% milk powder in 1x TBS-T at 4°C. The next day the membrane is washed thoroughly using 1x TBS-T. Afterwards the first antibody is applied. The antibody is diluted 1:5000 in 0.6% BSA 1x TBS-T. The dilution depends on the quality of the antibody. The blot is incubated for 1 hour at room temperature. The blot is washed 3 times for 10 minutes using 1x TBS-T. In case of a one-step protocol, as is the used α-His tag antibody, the blot can be developed using Pierces ECL kit. Otherwise the second antibody has to be applied to the blot. The dilution is 1:5000 in 0.6% BSA 1x TBS-T. Incubation lasts for 1 hour at room temperature. The blot is washed again three times and then developed according to the protocol. Pierces ECL kit is used in combination with HRP which leads to the emission of light. For detection of the bands an X-ray hyper film is put upon the blot in a dark room. Exposure time is determined by trial-and-error. The last step is to copy the marker bands from the blot onto the X-ray hyper film for referencing.

Mild stripping. All steps are facilitated on a shaking table. To reuse a blot getting rid of the used antibodies is absolutely necessary. Therefore the blot was covered with mild stripping buffer for 5 - 10 minutes at room temperature. The buffer is replaced once. Washing is done using 1x PBS for 10 minutes. This step is repeated one time. Afterwards the blot is washed two times for 10 minutes using 1x TBS-T. Afterwards the blot is ready for blocking.

2.2.8 Generation and Transformation of Competent *E. coli*

Generation of competent *E. coli*. 100 ml LB including strain-adequate antibiotics are inoculated with an overnight culture to an OD_{600} of 0.1. The culture is incubated on a shaker at 37°C until it reaches an OD_{600} between 0.6 and 0.8. The

cells are harvested by centrifugation for 10 minutes at 10 krpm at 4°C. The supernatant (medium) is discarded and the pellet is resuspended in 40 ml of ice cold MOPS I solution for 10 minutes on ice. The cells are again centrifuged, the supernatant is discarded and the pellet is resuspended in 40 ml of ice cold MOPS II solution. After 30 minutes on ice the cells are again centrifuged. The supernatant is discarded and the pellet is resuspended in 2 ml ice cold MOPS IIa solution. After this final step aliquots á 100 μl are portioned. These aliquots can be used either for transformation right away or stored at -80°C for several months.

Transformation of competent *E. coli*. This is facilitated by thawing an aliquot on ice and adding an appropriate amount of plasmid DNA. In case of transformation of an existing plasmid, 1 μl of plasmid DNA is sufficient to obtain clones. Otherwise when a ligation reaction precedes the transformation the whole ligation mix of 15 μl is added to the competent cells. After 30 minutes of incubation on ice the cells are heat shocked at 42°C for 2 minutes. Then they are put back on ice and 300 μl LB is added per aliquot followed by 30 minutes of regeneration at 37°C. The final step of transformation is plating of the batch on 3 selective LB plates containing antibiotics.

2.2.9 Generation and Transformation of Competent *N. magadii*

Generation of Competent *N. magadii*. 100 ml NVM containing 70 μg/ml bacitracin are inoculated with several ml of a densely grown culture and incubated at 37°C until the culture reaches and OD_{600} of 0.5 - 0.6. For better growth of *N. magadii* baffled flasks are preferentially chosen. When the desired OD_{600} is reached the cells are harvested by centrifugation: 15 minutes at 6 krpm and room temperature. The pellet is resuspended in 50 ml of buffered high salt spheroblasting solution containing glycerol (half the volume of the original culture). At last proteinase K is added at a concentration of 20 μg/ml. The culture is shaken at 42°C until primarily spheroblasts can be observed under the light microscope. This process takes 24 to 48 hours.

Transformation of competent *N. magadii*. 1.5 ml of spheroblasts are taken and centrifuged for 3 minutes at 10 krpm. The pellet is resuspended in buffered high salt spheroblasting solution lacking glycerol. 15 μl of 0.5 M EDTA are added and the cells incubated for 10 minutes at room temperature. 3 μg of DNA in a maximum of 10 μl aqueous solution are added and again incubated for another 5 minutes at room temperature. 150 μl of a 60% PEG-600 in unbuffered high salt spheroblasting solution is added to the cells (PEG-600 is stored at -80°C up to a year and heated at 65°C before the unbuffered high salt spheroblasting solution is added). Again the cells are incubated at room temperature for 30 minutes. For washing purposes 1 ml of

NVM is added and the cells are pelleted by centrifugation at 10 krpm for 5 minutes. After discarding the supernatant 1 ml of NVM is added without resuspending the pellet actively. The tube is put on a shaking thermomixer for 24 to 48 hours at 37°C. Thereafter about 100 μl of cells are plated on selective NVM plates until colonies are visible. After 10 days to 3 weeks single colonies are visible. The whole transformation process is derived from the PEG-600 method described by Charlebois *et al.* (1987) and Cline and Doolittle (1987).
After colonies are visible, clones are picked and incubated in 1 or 15 ml selective NVM until the medium turns turbid. At this point 100 μl of culture are taken, pelleted at 10 krpm for 3 minutes and lysed with ddH_2O. Subsequently PCR is used to confirm positive clones.

Generation of homozygotes. As mentioned before *N. magadii* is supposed to possess up to 30 copies of its genome. When a suicide plasmid is used for transformation heterozygotes can survive in the selective medium. To achieve survival only one copy of the gyrB gene is necessary. Therefore a clone is passaged 15 to 20 times before a dilution is plated on selective NVM agar plates. Then again single colonies are picked. PCR using primers flanking the region of the insert is carried out to find homozygotes. If there is no PCR product or only one corresponding to the size of the insert the clone is homozygous.

2.2.10 Plasmid Preperation from transformed *E. coli* (MiniPrep)

Plasmid isolation from transformed *E. coli* is facilitated using the GeneJET^{TM} Plasmid Miniprep kit from Fermentas. Alterations compared to the original protocol are: use of 3 ml of an overnight culture and elution using water instead of the supplied buffer. Analysis of the plasmid is done via agarose gel electrophoresis: on one hand the plasmid is directly applied and on the other hand the restricted plasmid using adequate restriction enzymes to recover the insert.

2.2.11 Quick Plasmid Preperation from transformed *E. coli* for Screening Purpose

After transformation several colonies growing on selective agar are picked and incubated overnight at 37°C in 5 ml LB containing adequate antibiotics. The next day 300 μl culture per clone are taken and centrifuged at 13.2 krpm for 3 minutes. The pellet is resuspended in 5x DNA loading buffer and then 14 μl of Phenol/Chloroform (1:1) are added. After 10 seconds of vigorously vortexing, the samples are again centrifuged at 13.2 krpm for 7 minutes. 12 μl of the aqueous phase are applied to a agarose gel and analyzed. A qualification of the different samples is only conclusive within the context of this preparation method and in comparison to the applied samples. In general plasmids migrating slower and therefore higher in the gel are supposed to be positive clones. Afterwards presumably positive and negative candidates are chosen, a MiniPrep and subsequent a restriction analysis are carried out (cf. section 2.2.10, page 55 and section 2.2.12, page 55).

Storage of positive Clones. Positive clones are freshly inoculated in selective LB overnight at 37°C. On the following day 1 ml of culture is mixed with 800 μl of sterile 50% glycerol and stored at -80°C.

2.2.12 Cloning in *E. coli* Strains

1. Restriction of target plasmids and potentially purified PCR fragments is done using enzymes supplied by Fermentas. The restriction is done following the supplied protocols and in case of double digests following the buffer suggestions obtained from Double Digest^{TM} tool from the Fermentas homepage. For strictly analytical purposes the duration of the restriction is reduced to 1 hour.

2. Purification of plasmids and DNA fragments is done using either QIAgen Purification or QIAquick Gel Extraction kit (cf. section 2.2.3, page 47).

[opt.] In case of blunt end fragments the plasmid has to be processed using *Klenow* fragment, if the plasmid was not restricted using a blunt end cutter. *Klenow* fragment is supplied by Fermentas and application is carried out according to the protocol.

3. DNA ligation is facilitated using Fermentas T4 ligase predominantly overnight at 16°C, rarely for 3 hours at room temperature.

1 μl	Restricted vector
1.5 μl	10x T4 ligase buffer
1 μl	T4 ligase
1 μl	50% PEG-4000 solution, optional only for blunt end ligations
ad 15 μl	Restricted insert

The whole batch was used for transformation of one aliquot of competent *E. coli* (cf. section 2.2.9, page 53).

Cloning strategies

Following abbreviations apply: Nm for *N. magadii*, Hh for *H. hispanica*, Hm for *H. marismortui* and Nov^R for novobiocin resistance.

pRSET-A $PhaC_{Hh}$, pRSET-A $PhaE_{Hh}$

Step	Method			
1.	PCR	Primer set	Template DNA	Fragment
		PhaC-5/PHB-2 PhaE-5/PhaE-3	*H. hispanica*	$PhaC_{Hh}$ 1451 bp $PhaE_{Hh}$ 566 bp
2.	Restriction	Precursors	Enzymes	
		pRSET-A $PhaC_{Hh}$ $PhaE_{Hh}$	*Bam*HI/*Kpn*I	
3.	Ligation:	pRSET-A/$PhaC_{Hh}$ pRSET-A/$PhaE_{Hh}$	Host:	XL-1-Blue

pRo-5 PHB_{Hh}, pRo-5 PHB_{Hm}

Step	Method			
1.	PCR	Primer set	Template DNA	Fragment
		PHB-1/PHB-2	*H. hispanica*	PHB_{Hh} 2504 bp
		PHB-3/PHB-4	*H. marismortui*	PHB_{Hm} 2414 bp
2.	Restriction	Precursors	Enzyme	
		PHB_{Hh} PHB_{Hm}	*Kpn*I	
3a.	Restriction	Precursor pRo-5	Enzyme *Hind*III	
3b.	DNA modification	Precursor pRo-5:*Hind*III	Enzyme *Klenow* fragment	
3c.	Restriction	Precursor blunted pRo-5:*Hind*III	Enzyme *Kpn*I	
4.	Ligation:	pRo-5/PHB_{Hh} pRo-5/PHB_{Hm}	Host:	XL-1-Blue

pKS-rrnb $PhaEC_{Hh}$, pKS-rrnb $PhaEC_{Hm}$

Step	Method				
1.	PCR	Primer set	Template DNA	Fragment	
		PhaE-5/PhaC-Xba	*H. hispanica*	$PhaEC_{Hh}$	1994 bp
		Pha-Hm-5/Pha-Hm-3	*H. marismortui*	$PhaEC_{Hm}$	1968 bp
2.	Restriction	Precursors	Enzymes		
		pKS-rrnb $PhaEC_{Hh}$ $PhaEC_{Hm}$	*Bam*HI/*Xba*I		
3.	Ligation:	pKS-rrnb/$PhaEC_{Hh}$ pKS-rrnb/$PhaEC_{Hm}$	Host:	XL-1-Blue	

pRo-5 16S prom $PhaEC_{Hh}$, pRo-5 16S prom $PhaEC_{Hm}$

Step	Method		
1a.	Restriction	Precursors	Enzyme
		pKS-rrnb $PhaEC_{Hh}$	*Xba*I
		pKS-rrnb $PhaEC_{Hm}$	
1b.	DNA modification	Precursor	Enzyme
		pKS-rrnb $PhaEC_{Hh}$:*Xba*I	*Klenow* fragment
		pKS-rrnb $PhaEC_{Hm}$:*Xba*I	
1c.	Restriction	Precursors	Enzyme
		blunted pKS-rrnb $PhaEC_{Hh}$:*Xba*I	*Kpn*I
		blunted pKS-rrnb $PhaEC_{Hm}$:*Xba*I	
1d.	Gel extraction	Fragment	Size
		16S $prom_{Nm}$ $PhaEC_{Hh}$	2274 bp
		16S $prom_{Nm}$ $PhaEC_{Hm}$	2248 bp
2a.	Restriction	Precursor	Enzyme
		pRo-5	*Hind*III
2b.	DNA modification	Precursor	Enzyme
		pRo-5:*Hind*III	*Klenow* fragment
2c.	Restriction	Precursor	Enzyme
		blunted pRo-5:*Hind*III	*Kpn*I
3.	Ligation:	pRo-5/16S $prom_{Nm}$ $PhaEC_{Hh}$	Host: XL-1-Blue
		pRo-5/16S $prom_{Nm}$ $PhaEC_{Hm}$	

pKSII+HR

Step	Method			
1.	PCR	Primer set	Template DNA	Fragment
		PHB-5/PHB-6	*N. magadii*	PHB-56 357 bp
2.	Restriction	Precursors	Enzymes	
		pKSII+, PHB-56	*Xba*I/*Bam*HI	
3.	Ligation:	pKSII+/PHB-56	Host:	GM48F'
4.	PCR	Primerset	Template DNA	Fragment
		PHB-7/PHB-8	*N. magadii*	PHB-78 488 bp
5.	Restriction	Precursors	Enzymes	
		pKSII+PHB-56, PHB-78	*Bsu*15I(*Cla*I)/*Kpn*I	
6.	Ligation:	pKSII+PHB-56/PHB-78	Host:	GM48F'

pKSII+PHB-56 is isolated after step 3 from positive clones.

pKSII+HR PHB_{Hh}, pKSII+HR PHB_{Hm}

Step	Method			
1.	PCR	Primer set	Template DNA	Fragment
		PHB-1/PHB-2	*H. hispanica*	PHB_{Hh} 2504 bp
		PHB-4/PHB-12	*H. marismortui*	PHB_{Hm} 2414 bp
2.	Restriction	Precursor	Enzymes	
		pKSII+HR PHB_{Hh} PHB_{Hm}	*Bsu*15I(*Cla*I)/*Eco*RI	
3.	Ligation:	pKSII+HR/ PHB_{Hh} pKSII+HR/ PHB_{Hm}	Host:	XL-1-Blue

Step	Method			
4a.	Restriction	Precursor	Enzyme	
		pKSII+HR/ PHB_{Hh} pKSII+HR/ PHB_{Hm}	*SmaI*	
4b.	DNA modification	Precursor	Enzyme	
		pKSII+HR/ PHB_{Hh}:*SmaI* pKSII+HR/ PHB_{Hm}:*SmaI*	CIP	
5a.	Restriction	Precursor	Enzyme	
		pMDS11	*Hind*III/*Sma*I	
5b.	DNA modification	Precursor	Enzyme	
		pMDS11: *Hind*III/*Sma*I	*Klenow* fragment	
5c.	Gel extraction	Fragment		
		Nov^R 2.3 kbp		
6.	Ligation:	pKSII+HR PHB_{Hh}/Nov^R pKSII+HR PHB_{Hm}/Nov^R	Host:	XL-1-Blue

pKSII+HR/PHB_{Hh} and pKSII+HR/PHB_{Hm} are isolated after step 3 from positive clones.

pKSII+HR 16S $prom_{Nm}$ $PhaEC_{Hh}$, pKSII+HR 16S $prom_{Nm}$ $PhaEC_{Hm}$

Step	Method			
1.	PCR	Primer set	Template DNA	Fragment
		PHB-9/PHB-10	pKS-rrnb $PhaEC_{Hh}$	16S $prom_{Nm}$ $PhaEC_{Hh}$ 2274 bp
		PHB-9/PHB-11	pKS-rrnb $PhaEC_{Hm}$	16S $prom_{Nm}$ $PhaEC_{Hm}$ 2248 bp
2.	Restriction	Precursor	Enzymes	
		pKSII+HR 16S $prom_{Nm}$ $PhaEC_{Hh}$ 16S $prom_{Nm}$ $PhaEC_{Hm}$	*Bsu*15I(*Cla*I)/*Eco*RI	
3.	Ligation:	pKSII+HR/16S $prom_{Nm}$ $PhaEC_{Hh}$ pKSII+HR/16S $prom_{Nm}$ $PhaEC_{Hm}$	Host:	XL-1-Blue
4a.	Restriction	Precursor	Enzyme	
		pKSII+HR/16S $prom_{Nm}$ $PhaEC_{Hh}$ pKSII+HR/16S $prom_{Nm}$ $PhaEC_{Hm}$	*SmaI*	
4b.	DNA modification	Precursor	Enzyme	
		pKSII+HR/16S $prom_{Nm}$ $PhaEC_{Hh}$:*SmaI* pKSII+HR/16S $prom_{Nm}$ $PhaEC_{Hm}$:*SmaI*	CIP	

Step	Method			
5a.	Restriction	Precursor	Enzyme	
		pMDS11	*Hind*III/*Sma*I	
5b.	DNA modification	Precursor	Enzyme	
		pMDS11: *Hind*III/*Sma*I	*Klenow* fragment	
5c.	Gel extraction	Fragment		
		Nov^R 2.3 kbp		
6.	Ligation:	pKSII+HR 16S $prom_{Nm}$ $PhaEC_{Hh}/Nov^R$ pKSII+HR 16S $prom_{Nm}$ $PhaEC_{Hm}/Nov^R$	Host:	XL-1-Blue

pKSII+HR/16S $prom_{Nm}$ $PhaEC_{Hh}$ and pKSII+HR/16S $prom_{Nm}$ $PhaEC_{Hm}$ were isolated after step 3 from positive clones.

2.2.13 Overexpression and Purification of His-Tagged Proteins

100 ml of fresh selective LB is inoculated with an overnight culture of the desired *E. coli* strain carrying the pRSET-A plasmid of choice with an OD_{600} of 0.1. The culture is then incubated at 37°C until it reaches an OD_{600} between 0.3 and 0.5. Then the gene expression is triggered by adding IPTG to a final concentration of 0.5 mM and the culture is now incubated at 28°C.

Investigation of best suiting Conditions for Overexpression Before induction, then every hour 5 hours in total and overnight samples are taken and the OD_{600} is measured. The samples are centrifuged at 13.2 krpm for 3 minutes and the pellet is resuspended in 75 x OD_{600} μl 5 mM sodium phosphate buffer and the same volume 2x Laemmli buffer. After boiling of the raw extracts at 95°C for 10 minutes the samples can be applied to a SDS-PAGE or frozen at -20°C for later usage.
To determine the best conditions for protein overexpression certain parameters can be examined:

***E. coli* strain.** Different strains of *E. coli* can display different behavior when expressing heterologous recombinant proteins. Other strains might also demand other culture conditions or additional plasmids.

IPTG concentration. IPTG concentration can be varied to achieve a higher yield.

Growth temperature. Changing the temperature from 37°C before induction to 28°C after leads to a heat shock. To avoid this the culture could also be grown at 28°C before induction.

Duration of Overexpression. This factor is associated with the growth temperature. Is it e.g. 16°C the overexpression could also take place overnight, when an overnight expression at 28°C does not yield any target protein.

OD_{600} at Induction. Especially applicable for toxic proteins, it can be beneficial to start overexpression later in log phase e.g. at OD_{600} 0.8.

Purification of His-Tagged Proteins under denaturing Conditions. 1 l of fresh selective LB is inoculated with an overnight culture. OD_{600} is 0.1. The culture is grown at 28°C or 37°C until the OD_{600} reaches a value between 0.3 and 0.5. The protein overexpression is induced by addition of IPTG to a final concentration of 0.5 mM. When the culture has grown long enough at 28°C the cells are harvested by centrifugation at 6 krpm for 15 minutes at 4°C. The pellet is frozen at -20°C overnight. The next day it is resuspended in buffer B (lysis buffer) and stirred for 3 hours. Then the solution is sonicated in an ice bath for 5 - 10 minutes (the solution should become clearer due to cell lysis). After centrifugation at 8 krpm for 20 min at 4°C the supernatant is collected and 500 μl of Ni-NTA are added. This mixture stirs slowly overnight. During this procedure samples are taken before and after sonication in order to retrace the overexpressed protein. This is necessary in case of loss of protein after purification. The next day the solution is applied to a column supplied by Qiagen QIAexpressionist^{TM} kit. The flow-through is collected in a separate tube. Then the column material is washed using 2 times 4 ml of buffer C (wash buffer). These fractions are also collected seperately in tubes. Last the protein is eluted using 6 times 0.5 ml of buffer E (elution buffer). Subsequently samples of collected fractions can be analyzed using a SDS-PAGE gel.

Dialysis. When fractions are pure enough the fractions are pooled and dialysed against 1x PBS. This is carried out as follows, first for 1 hour and then overnight using fresh 1x PBS. After dialysis the sample are again analyzed on a SDS-PAGE gel.

2.2.14 Fixed Phase Liquid Chromatography (FPLC)

FPLC is facilitated on ÄKTA^{TM} systems from GE Healthcare using UNICORN^{TM} 5.01 control software. Analytical FPLC is done using a Superdex 200 10/300 GL column, preparative FPLC is done using a HiLoad 26/60 Superdex 200 or a HiPrep Sephacryl S-200 26/60 HR column, all three also provided by GE Healthcare.

Property	**Superdex 200 10/300 GL**	**HiLoad 26/60 Superdex 200**
Column volume (CV)	24 ml	320 mml
Dead volume	8 ml	110 ml
Max. sample volume	500 μl	13 ml
Max. flow rate	1 ml/min	2.6 ml
Pressure limit	1.5 MPa	0.5 MPa
Separation range	10 - 600 kDa	10 - 600 kDa

Concerning their instruction manuals HiLoad 26/60 Superdex 200 and HiPrep Sephacryl S-200 26/60 HR columns are quite similar. For further information please visit GE Healthcares internet presence.

Protocol. The protocol is the same for the small analytical column as for the larger preparative columns because specifications are stated in column volumes. Equilibration of the column is done using 2 CVs of elution buffer. At the end of equilibration the baseline of the UV detector has to be checked whether it is stable or not. The sample is applied either per injection into the loop or using the sample pump and the elution is done for 1.2 CVs. Fractionation starts after the dead volume of the column when proteins are detected. After finishing one run the column can be washed using 0.5 CVs of elution buffer when another run in the same buffer is scheduled. In case of change of the elution buffer the column has again to be equilibrated using 2 CVs of elution buffer. After the last run the column has to be cleaned using 0.5 CVs 0.5 M NaOH. Afterwards 2 CVs of ddH_2O are applied and then for storage reason 1 CVs of 20% ethanol.

2.2.15 PHA Extraction from Recombinant *E. coli*

2 l of recombinant *E. coli* are grown overnight in LB supplemented with 2% glucose. After harvesting the cells by centrifugation at 6 krpm for 15 minutes at 4°C the wet pellet was put into approximately 150 ml chloroform. This flask is shaken for 48 hours. Then the chloroform and cells are filtrated using a folded filter and a vacuum flask. The PHA which is dissolved in chloroform leads to an increase in

viscosity. Afterwards up to 3 to 5 volumes of ice cold acetone are added, until the PHA precipitates in big clouds on the bottom of the jar. Again the whole conglomerate is filtrated through a folded filter. The extracted PHA is retained in the filter and can be put into a glass petri dish and dried by evaporation in the extractor hood. For better purity it is possible to dissolve the PHA again in chloroform and precipitate it using acetone again, this can be repeated several time. A good overview of extraction methods for PHA and yield improvement can be found at Koller *et al.* (2010) and Ramsay *et al.* (1990). I chose this method because it has already been proven successful and it is easy to handle.

Acidic Depolymerization of PHA

This method was chosen for subsequent gas chromatographic (GC) analysis of the PHA content of PHA synthesizing archaea. PHA extraction from archaea can be tricky due to exopolysaccharides or other transfectants on the surface of haloarchaea. Therefore I chose this method because biomass is taken as is and applied to the acidic digest of PHA. The method is adapted from Braunegg *et al.* (1978). Cells are harvested using centrifugation at 6 krpm for 15 minutes at room temperature. Then 2 ml of acidified methanol and 2 ml chloroform are added to the sample. The sample is cooked at 100°C for 3.5 hours. This leads to depolymerization of PHB to 3-hydroxybutyric acid methylester. Then the sample is cooled down to room temperature, 1 ml ddH_2O is added and the sample vigorously vortexed for 10 minutes. After settling of the aqueous and organic phase the organic phase is taken and injected into a GC. In this case the organic phase was taken and transferred into a teflon sealed test tube and stored at 4°C for later analysis at the TU Wien. GC was done by Andreas Posch from the group of Christoph Herwig.

2.2.16 Microscopy

For Sudan Black V staining a standard phase contrast microscope is used. For Nile Red, Nile Blue and Safranin O stainings a fluorscence microscope employing a mercury lamp is used. Later in the process a confocal microscope is used for examining cells stained with Nile Red or Nile Blue. The confocal microscope is a inverse Zeiss LSM510 exciting at 488 nm using an argon laser and emission was monitored at 530 - 615 nm due to possible autofluorescence of haloarchael cells.
Before any culture was submitted to microscopy the OD_{600} was measured and the amount of cells was normalized to 1.5. Then the staining or fixation could commence.

Dry fixation. 7 μl of the prepared sample are slightly spread on a microscopy slide and air dried. Afterwards the slide is desalted for 5 minutes in 2% acetic acid. The slide is air dried again and then stains can be applied for 3 minutes. After removing the stain the slide is again dried and observed under light or fluorescence microscope.

Agarose coated slides. 1% agarose in 18% SW is prepared the following way: 0.1 g of agarose is molten in 4 ml ddH_2O and then 6 ml of warm 30% SW is added. 500 μl of this agarose solution is spread on a slide. Afterwards stained cells could be put onto the agarose pad and observed under light or fluorescence microscope.

Live staining. Originally produced for cell culture observation I obtained 8-well chambers from Ibidi. These are used in combination with an inverted confocal microscope. 400 μl of prepared cells are put into a well and 2 μl of Nile Blue are added. Then the sample is ready for examination using the inverse, confocal microscope.

3 Results and Discussion

3.1 Cloning and Transformation

For the purpose of characterization of the two different PHB operons of *H. hispanica* and *H. marismortui* in *N. magadii* two different strategies of introduction were chosen: a stable plasmid and a suicide vector. The construct on the suicide vector would only be successfully introduced into *N. magadii* when homologous recombination took place. As the functionality of the native promoter of the two operons was not assured in the host the 16S rDNA promoter of *N. magadii* was used additionally, resulting in eight constructs overall.
For the purification of the two subunits of the PHA synthase of *H. hispanica* two pRSET-A constructs were cloned. For cloning strategies cf. section 2.2.12 page 56.

Description of cloned constructs:

pRSET-A $PhaC_{Hh}$, pRSET-A $PhaE_{Hh}$
: These two constructs were cloned for the overexpression of the two subunits of the PHA synthase of *H. hispanica*. Gene expression is induced by IPTG and the result is either a his-tagged large subunit PhaC or small subunit PhaE.

pRo-5 PHB_{Hh}, pRo-5 PHB_{Hm}
: These two constructs are designed for transformation of *N. magadii*. pRo-5 is a stable vector carrying a novobiocin resistence and either the PHB operon of *H. hispanica* or *H. marismortui*.

pRo-5 16S prom $PhaEC_{Hh}$, pRo-5 16S prom $PhaEC_{Hm}$
: These two constructs are also designed for transformation of *N. magadii*. The only difference to the already described two pRo-5 constructs is, instead of the native promoter of the two PHB operons the 16S rDNA promoter of *N. magadii* is used.

pKS+HR PHB_{Hh}, pRo-5 PHB_{Hm}
: These two constructs are designed for homologous recombination into the genome of *N. magadii*. The recombination takes place downstream of a protease and the insert contains a novobiocin resistance. The second part of the insert is either the PHB operon of *H. hispanica* or *H. marismortui*.

pKS+HR 16S prom $PhaEC_{Hh}$, pRo-5 16S prom $PhaEC_{Hm}$
: These two constructs are also designed for homologous recombination into the genome of *N. magadii*. The only difference to the already described pKS+HR constructs is, instead of the native promotor of the two PHB operons the 16S rDNA promoter of *N. magadii* is used.

Cloning in *E. coli* strains was successful, the Quick Plasmid Preparation for Screening Purpose was useful. It was possible to screen up to 500 clones per day, this was necessary for a few clonings. After retrieving all cloned constructs from *E. coli* strains the transformation of *N. magadii* L13 was successful using either pRo-5 vectors as the suicide plasmids based on pKSII+. Employing protocols and experience from former co-workers allowed for a transformation procedure which would take one week and therefore save time.

Also some problems arose which were not predictable from the start:

1. For the cloning of pKSII+HR it was obligatory to use strain GM48F' due to the fact that this strain lacks dam methylase. Without this genotype subsequent restriction of the plasmid using *Bsu*15I (*Cla*I) would be impossible, thes enzyme cannot cut dam methylated DNA. An interesting observation was made concerning this plasmid: containing two inserts the size of 845 bp the isolated plasmid runs further than the original pKSII+ in an 0.8% agarose gel, rendering it smaller.
2. Homozygotization of suicide plasmid transformants was not successful. After 20 passages I was not able to obtain single clones carrying any construct homozygously.

3.2 Purification of PHA synthase and Western blot

3.2.1 Protein Overexpression

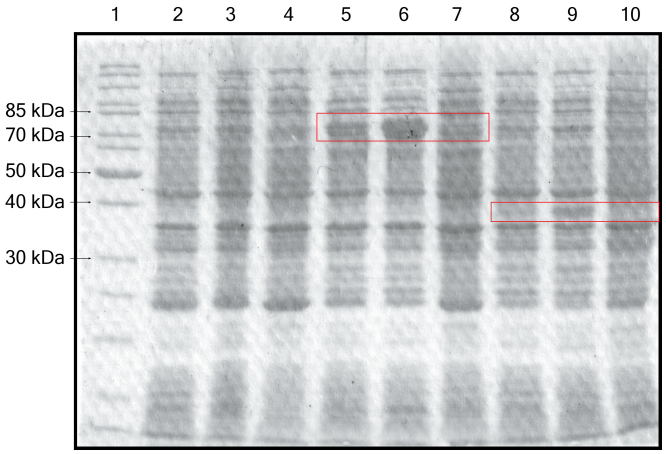
The first batch of overexpression of $PhaC_{Hh}$ and $PhaE_{Hh}$ was completed under standard conditions in *E. coli* strain Rosetta (cf. section 2.2.13 page 62). However the expression condition had to be improved.
Therefore the plasmids were transformed into BL21(DE3) pLysE, Tuner^{tm}, C41 pLysS and C43 pLysS. Tuner^{tm} needed an additional plasmid to be able to carry pRSET-A. pREP4, containing a stronger lac repressor, was required. Otherwise no transformants could be obtained. First, the concentration of the inducer IPTG was varied from 0.3 mM to 1 mM. There were no significant changes in gene expression, so the established IPTG concentration of 0.5 mM was maintained. Secondly, the temperature was adapted: 37°C before and after induction, 37°C before/28°C after induction, 28°C before and after induction. This resulted in a growth temperature of cultures at 28°C before and after induction up to 5 hours.
Experimentally discovered expression conditions:

- Strain: Rosetta
- Inducer (IPTG) concentration: 0.5 mM
- Growth temperature: 28°C before and after induction
- Induction duration: up to 5 hours
- Inducton OD_{600}: 0.8
- Quantitiy: 12 l

3.2.2 From Overexpression to Lyophilization of FPLC purified Protein

Starting with the protein overexpression and ending with the purified proteins $PhaC_{Hh}$ and $PhaE_{Hh}$, the experimental process can be divided into six steps:

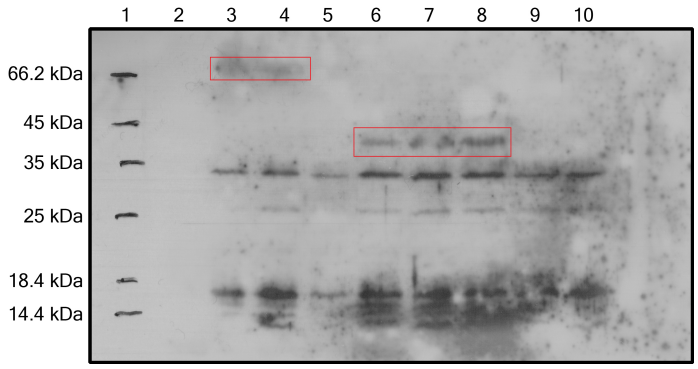
1. SDS-PAGE of both expression series, figure 7 page 72
2. Western blot using α-His antibodies, figure 8 page 73
3. His-tag purification using Qiagen QIAexpressionist^{TM} kit
4. Collection of useful fractions, dialysis and filtration for FPLC, figures 9 and 10 on pages 74 and 74
5. FPLC of His-tag purified protein
6. Collection of useful fractions and lyophilization, figures 11 and 12 on pages 75 and 76



Lane 1: PageRuler^{TM} Unstained Protein Ladder
Lane 2-4: Rosetta (neg. ctrl.) before induction, two hours after induction and over night
Lane 5-7: Rosetta pRSET-A/$PhaC_{Hh}$ before induction, two hours after induction and over night
Lane 8-10: Rosetta pRSET-A/$PhaE_{Hh}$ before induction, two hours after induction and over night

Figure 7: *SDS-PAGE of $PhaC_{Hh}$ and $PhaE_{Hh}$ expression series*

As shown in figure 7 the overexpression of $PhaC_{Hh}$ (53 kDa) and $PhaE_{Hh}$ (20.6 kDa) is successful (time course and proteins are indicated by red rectangles). However the protein bands are shifted upwards rendering the two proteins 15 - 20 kDa larger than they really are. This can be credited on one hand to the his-tag and on the other hand to the low pI of the two proteins which is 3.8. To confirm a western blot using α-His antibodies was performed. This is shown in figure 8.

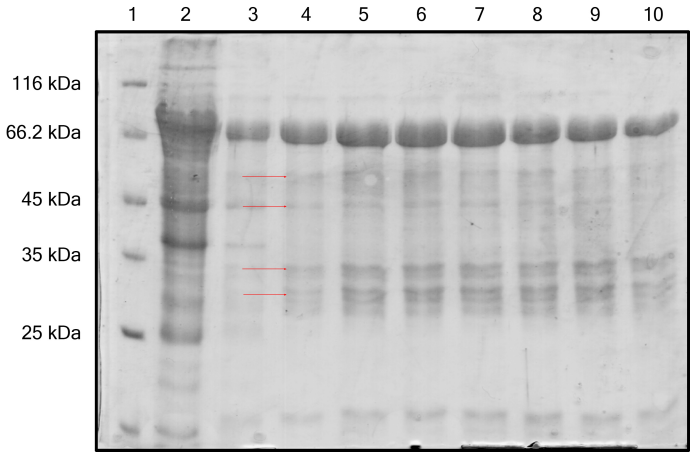


Lane 1: Unstained Protein Molecular Weight Marker
Lane 2-5: Rosetta pRSET-A/PhaC$_{Hh}$ before induction, two and four hours after induction and over night
Lane 6-9: Rosetta pRSET-A/PhaE$_{Hh}$ before induction, two and four hours after induction and over night
Lane 10: Rosetta (neg. ctrl.) two hours after induction

Figure 8: *Western blot of PhaC$_{Hh}$ and PhaE$_{Hh}$ samples using α-His-tag antibodies*

The western blot shown in figure 8 confirms that the in figure 7 observed protein bands are the two his-tagged proteins PhaC$_{\mathrm{Hh}}$ and PhaE$_{\mathrm{Hh}}$ (indicated by red rectangles). Lane 2 did not work out, there is no protein. Lane 6 shows that the promotor is leaky: there is a weak signal without induction. The other bands are unspecific, compare to negative control in lane 10.

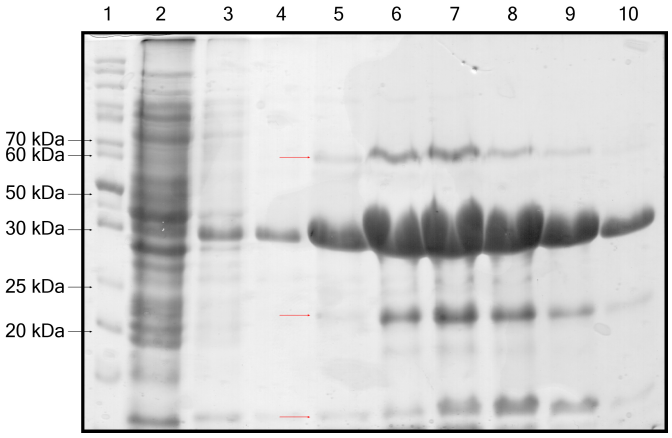
These two figures lead to the conclusion that PhaC$_{\mathrm{Hh}}$ behaves like a protein 65 - 70 kDa in size and PhaE$_{\mathrm{Hh}}$ behaves like a protein 35 - 40 kDa in size in a SDS-PAGE.



Lane 1: Unstained Protein Molecular Weight Marker
Lane 2: Flow-through
Lane 3: Wash fraction
Lane 4-10: Fractions 1-7

Figure 9: *SDS-PAGE of $PhaC_{Hh}$ samples after His-tag purification*

Figure 9 shows the process of his-tag purification of $PhaC_{Hh}$. The protein is the strongest band around 66.2 kDa in lanes 2 - 10. The fractions are not pure enough for antibody production (impurities are indicated by red arrows). Fractions containing enough protein of interest were pooled and dialyzed against 1x PBS. Afterwards they were applied to FPLC gel filtration.



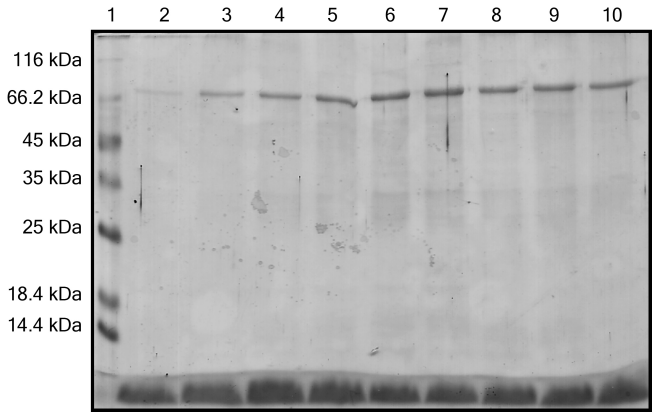
Lane 1: PageRuler™ Unstained Protein Ladder
Lane 2: Flow-through
Lane 3: Wash fraction
Lane 4-10: Fractions 1-7

Figure 10: *SDS-PAGE of $PhaE_{Hh}$ samples after His-tag purification*

Figure 10 shows the process of his-tag purification of $PhaE_{Hh}$. The protein is the strongest band around 27 kDa in lanes 2 - 10. The fractions are not pure enough

for antibody production (impurities are indicated by red arrows). The best known impurity is SlyD with a size of 25 kDa. This protein has also high affinity to Ni-NTA due to a poly-his stretch. Fractions containing enough protein of interest were pooled and dialyzed against 1x PBS. Afterwards they were applied to FPLC gel filtration.

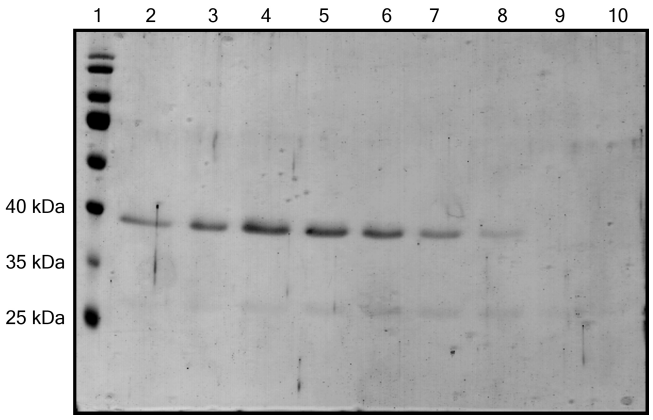
Before application of the two proteins to FPLC the protein concentrations were estimated using NanoDrop. The protein concentrations always were between 0.7 and 1.3 mg/ml. In order to get antibodies at least 5 mg of each protein had to be collected.



Lane 1: Unstained Protein Molecular Weight Marker
Lane 2-10: Exemplary fractions

Figure 11: *SDS-PAGE of $PhaC_{Hh}$-fractions after FPLC*

Figure 11 shows some fractions of $PhaC_{Hh}$ after preparative FPLC gel filtration. The protein is the band around 66.2 kDa. At the bottom of the figure very large and strong bands can be seen. These might be degradation products. These fractions are pure enough to be used for antibody production. All fractions containing $PhaC_{Hh}$ and no other impurites were pooled and the protein concentration was estimated using NanoDrop. The concentration was around 0.3 - 0.4 mg/ml, the volume of the sample was around 35 ml. 2% glycerol in the elution buffer leads to problems during lyophilization: the solution tends to blister and this might lead to the loss of sample volume. The lyophilized sample of $PhaC_{Hh}$ was stored at -20°C.



Lane 1: PageRuler™ Plus Prestained Protein Ladder
Lane 2-10: Exemplary fractions

Figure 12: *SDS-PAGE of $PhaE_{Hh}$-fractions after FPLC)*

Figure 12 shows some fractions of $PhaE_{Hh}$ after preparative FPLC gel filtration. The protein is the band around 37 kDa. At the bottom of the figure strong bands can be seen. These might be degradation products. These fractions are pure enough to be used for antibody production. All fractions containing $PhaE_{Hh}$ and no other impurites were pooled and the protein concentration was estimated using NanoDrop. The concentration was around 0.5 - 0.6 mg/ml, the volume of the sample was around 20 ml. The $PhaE_{Hh}$ sample is lyophilized and sent for antibody production.

3.2.3 Analytical and Preparative FPLC - Evaluation of appropriate Elution Buffer

Before this degree of protein purity after preparative FPLC was accomplished, analytical FPLC was performed to detect the adequate elution buffer for each protein. As mentioned in section 2.1.5 on page 42, the following elution buffers were used for analytical FPLC:

- 1xPBS
- 1xPBS 200 mM NaCl
- 1xPBS 300 mM NaCl
- 1xPBS + 2% Glycerol
- 1xPBS + 5% Glycerol

Each elution buffer was used for both proteins of interest on an analytical column (Superdex 200 10/300 GL). Afterwards the chromatograms were analyzed and compared to the SDS-PAGE of the fractions. Fraction size was 200 μl, in order to detect anything on a SDS-PAGE the samples were concentrated (cf. section 2.2.5 page 50).

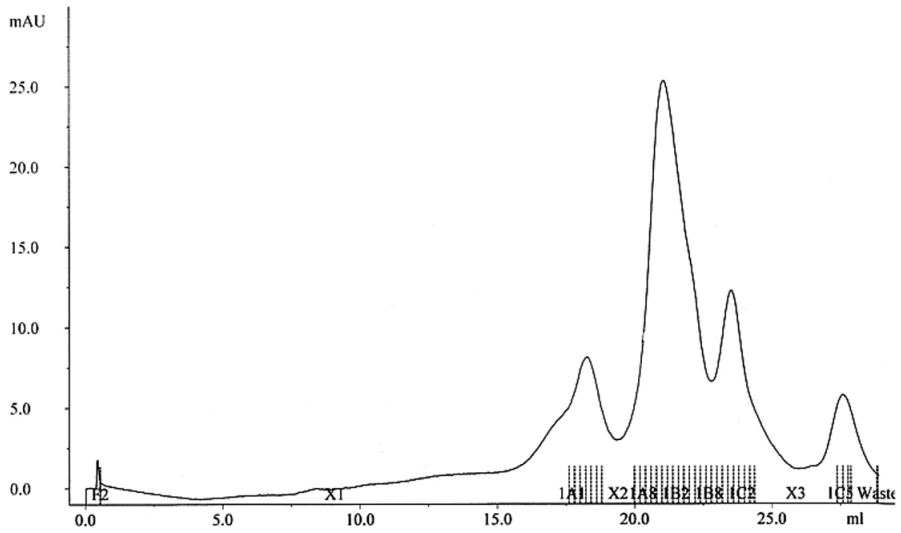


Figure 13: *Chromatogram of an analytical FPLC of $PhaC_{Hh}$ using 1xPBS + 2% glycerol as elution buffer*

Figure 13 shows the chromatogram of an analytical FPLC of $PhaC_{Hh}$ using 1xPBS + 2% glycerol as elution buffer. The segregation of peaks is very good, but the SDS-PAGE of the fractions after TCA precipitation was negative. Non the less this elution buffer was chosen for preparative FPLC.

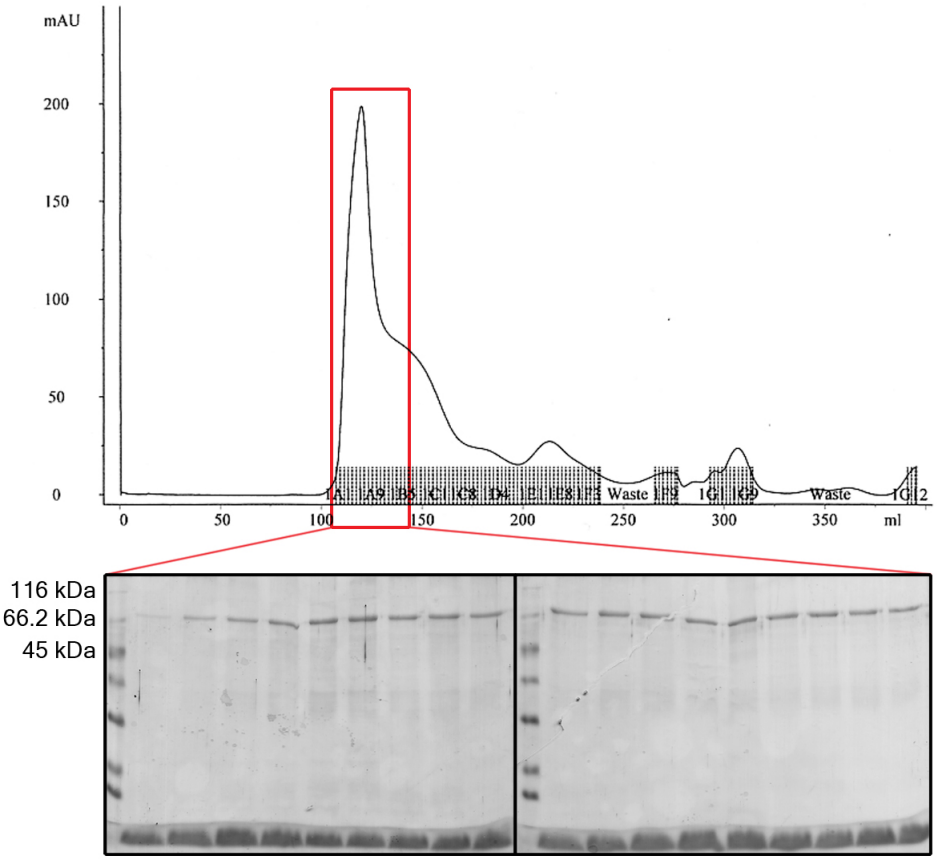


Figure 14: *Chromatogram and SDS-PAGE of PhaC$_{Hh}$ after preparative FPLC using 1xPBS + 2% glycerol as elution buffer. The SDS-PAGE shows fractions A1-B6 of the chromatogram.*

Figure 14 shows on one hand the chromatogram of preparative FPLC of PhaC$_{Hh}$ using 1xPBS + 2% glycerol as elution buffer and on the other hand a SDS-PAGE of exemplary fractions. Compared to the analytical FPLC in figure 13 the chromatograms differ completely. But the SDS-PAGE shows very pure fractions. This came unexpected because these first fractions should contain all the undelayed components of the sample. At the bottom of the gel strong bands can be seen which might be degradation products.

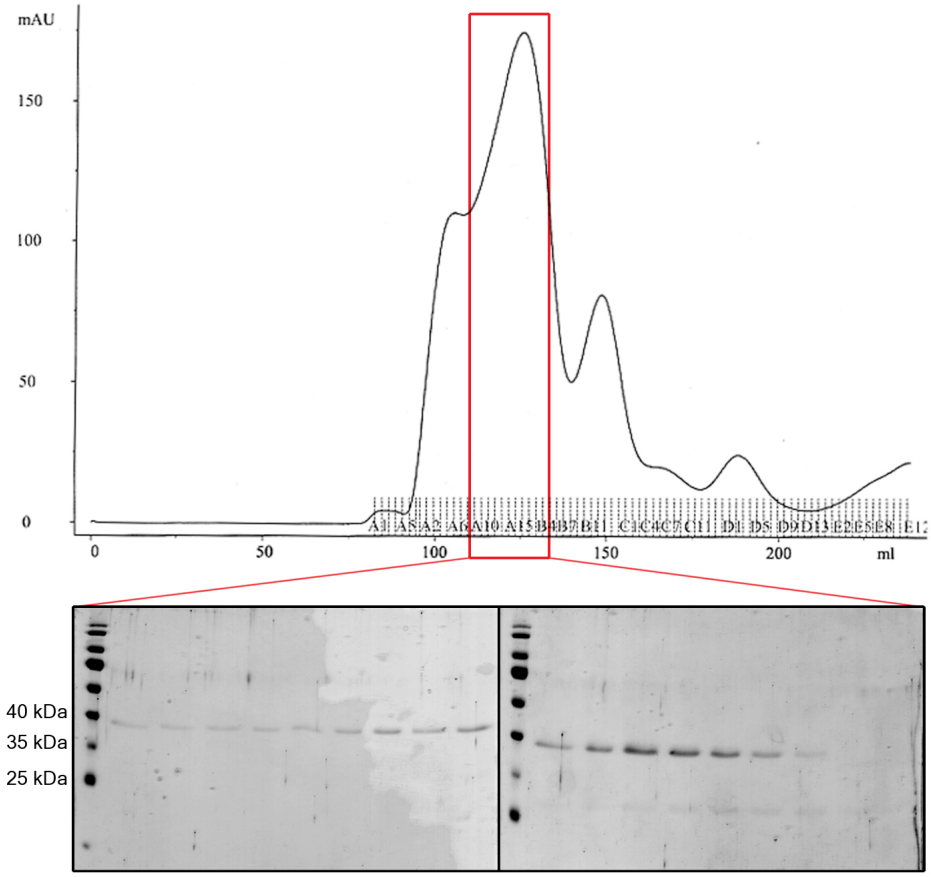


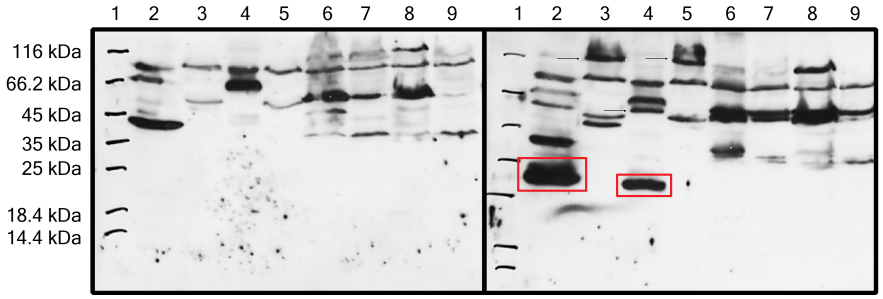
Figure 15: *Chromatogram and SDS-PAGE of PhaE$_{Hh}$ after preparative FPLC. The SDS-PAGE shows fractions A10-B12.*

Figure 15 shows on one hand the chromatpgram of preparative FPLC of PhaE$_{\mathrm{Hh}}$ using 1xPBS as elution buffer and on the other hand a SDS-PAGE of exemplary fractions. The segregation of peaks is good and the fractions are pure with few impurities.

To sum up the results of analytical FPLC: 1x PBS + 2% gylcerol is the adequate elution buffer for preparative FPLC of PhaC$_{\mathrm{Hh}}$ and 1x PBS is the adequate elution buffer for FPLC of PhaE$_{\mathrm{Hh}}$.

3.2.4 Western blot

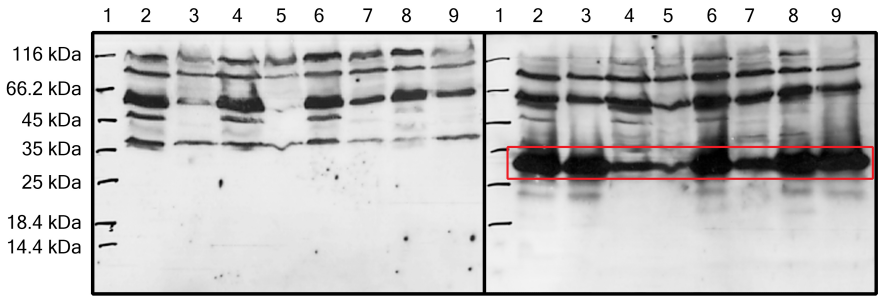
After the successful purification of $PhaE_{Hh}$ the lyophilized protein was sent off for antibody production. Two whole sera from two rabbits were obtained. After testing of both sera using protein samples from recombinant expressed $PhaE_{Hh}$ it became clear that only one serum contained α-PhaE antibodies. In the following consideration the serum of the rabbit before it was immunized with $PhaE_{Hh}$ is referred to as pre-serum and the serum after the immunization is referred to as α-PhaE.
Cultures of *H. hispanica* and *H. marismortui* grown in rich (23%MGM) and defined mineral medium (MG) are used as positive controls for the western blot. Cultures of *N. magadii* L13 and L13/pRo-5 (carrying only the empty plasmid) grown in rich (NVM) and defined mineral medium (NMM4) are used as negative controls for the western blot. All transformants and homolog recombinants grown in rich (NVM) and defined mineral medium (NMM4) are investigated using western blot. All samples were taken in a late logarithmic growth phase for comparability.



Both blots depict the same loading scheme, only difference is the left blot was incubated using pre-serum and the right blot was incubated using α-PhaE.
Lane 1: Unstained Protein Molecular Weight Marker
Lane 2/3: H. hispanica grown in rich (23%MGM) and defined mineral medium (MG)
Lane 4/5: H. marismortui grown in rich (23%MGM) and defined mineral medium (MG)
Lane 6/7: N. magadii L13 grown in rich (NVM) and defined mineral medium (NMM4)
Lane 8/9: N. magadii L13/pRo-5 grown in rich (NVM) and defined mineral medium (NMM4)

Figure 16: *Western blot of controls using pre-serum on the left and α-PhaE on the right blot*

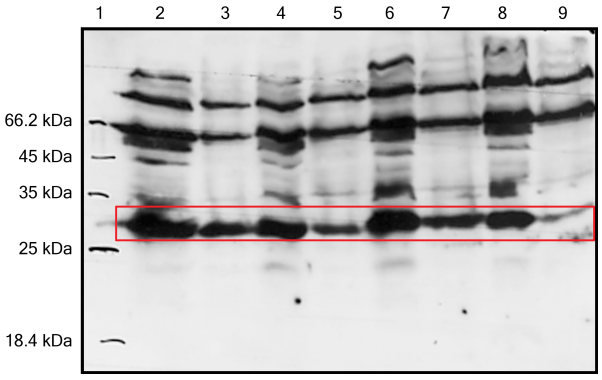
Figure 16 shows on the left side unspecific binding of the pre-serum in every sample. On the right blot the α-PhaE shows unspecific binding as the pre-serum. Additionally α-PhaE detects $PhaE_{Hh}$ in the positive controls in lanes 2 and 4 (indicated by red rectangles). The molecular mass of $PhaE_{Hh}$ can be estimated with 30 kDa. Other proteins which are detected by α-PhaE and not by the pre-serum are indicated by arrows. This figure also shows that α-PhaE detects PhaE from *H. marismortui*.
Contrary to expectations based on Han *et al.* (2007) the small subunit PhaE is not expressed in defined mineral medium (MG) but in rich medium (23%MGM).



Both blots depict the same loading scheme, only difference is the left blot was incubated using pre-serum and the right blot was incubated using α-PhaE.
Lane 1: Unstained Protein Molecular Weight Marker
Lane 2/3: L13 pRo-5 PHB_{Hh} grown in rich (NVM) and defined mineral medium (NMM4)
Lane 4/5: L13 pRo-5 16S prom $PhaEC_{Hh}$ grown in rich (NVM) and defined mineral medium (NMM4)
Lane 6/7: L13 pRo-5 PHB_{Hm} grown in rich (NVM) and defined mineral medium (NMM4)
Lane 8/9: L13 pRo-5 16S prom $PhaEC_{Hm}$ grown in rich (NVM) and defined mineral medium (NMM4)

Figure 17: *Western blot of transformants using pre-serum and α-PhaE*

Figure 17 shows on the left blot unspecific binding of the pre-serum in every transformant. The right blot shows that every transformant independent of medium and promoter expresses PhaE (indicated by red rectangle). There is no unspecific binding of α-PhaE on the right blot which pre-serum would not account for.
An important fact can be seen on the right blot: lanes 2/3 and 6/7 show that the native promoter of the operon is functional in *N. magadii* which is not the case generally.



Lane 1: Unstained Protein Molecular Weight Marker
Lane 2/3: L13 HR PHB_{Hh} grown in rich (NVM) and defined mineral medium (NMM4)
Lane 4/5: L13 HR 16S $prom_{Nm}$ $PhaEC_{Hh}$ grown in rich (NVM) and defined mineral medium (NMM4)
Lane 6/7: L13 HR PHB_{Hm} grown in rich (NVM) and defined mineral medium (NMM4)
Lane 8/9: L13 HR 16S $prom_{Nm}$ $PhaEC_{Hm}$ grown in rich (NVM) and defined mineral medium (NMM4)

Figure 18: *Western blot of homolog recombinants using pre-serum and α-PhaE*

Figure 18 shows a western blot of all homolog recombinants using α-PhaE. The blot shows that every homolog recombinant independent of medium and promoter ex-

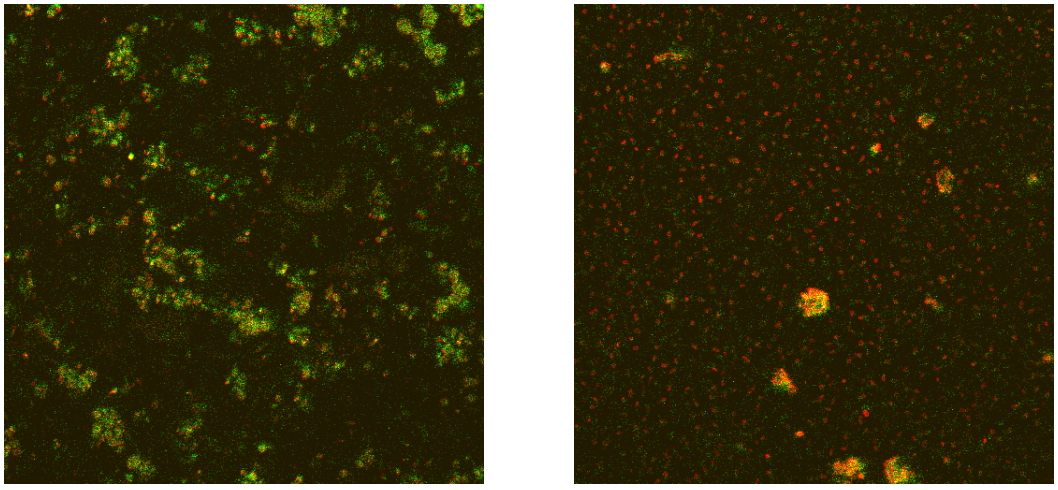
presses PhaE (indicated by red rectangle).
Lane 9 shows that this homolog recombinant does not express as much PhaE in defined mineral medium (NMM4) as in rich medium (23%MGM) and as other homolog recombinants.

The purification of both subunits of the PHA synthase of *H. hispanica* was successful. An α-$PhaE_{Hh}$ was obtained which also detected PhaE from *H. marismortui*. The native promoter of both PHA synthases is functional in *N. magadii*. Contrary to the initial expectation based on Han *et al.* (2007), that the PHA synthase is only expressed under starvation conditions in defined mineral medium (MG) it was only expressed in rich medium (23%MGM) by *H. hispanica* and *H. marismortui*. The proof of PhaE expression implies the expression of PhaC due to the operon organisation of the synthase.

3.3 Microscopy

An important step towards evidence of PHA synthase activity, PHA accumulation, is microscopic observation. It is possible to stain PHA granules specifically using Sudan Black V and Nile Blue. But these two stains are generally able to stain hydrophobic compounds as lipids. Therefore positive results are just a preliminary hint that PHA might be accumulated in cells of interest.

Cultures of *H. hispanica* and *H. marismortui* grown in rich (23%MGM) and defined mineral medium (MG) are used as positive controls for microscopy. Cultures of *N. magadii* L13 and L13/pRo-5 (carrying only the empty plasmid) grown in rich (NVM) and defined mineral medium (NMM4) are used as negative controls for microscopy. All transformants and homolog recombinants grown in rich (NVM) and defined mineral medium (NMM4) are investigated using microscopy. For reasons of comparability cultures were investigated at late logarithmic growth phase. Staining and microscopy of samples was done according to 2.2.16 on page 65. Live staining was used.



(a) Culture grown in rich medium (23%MGM)

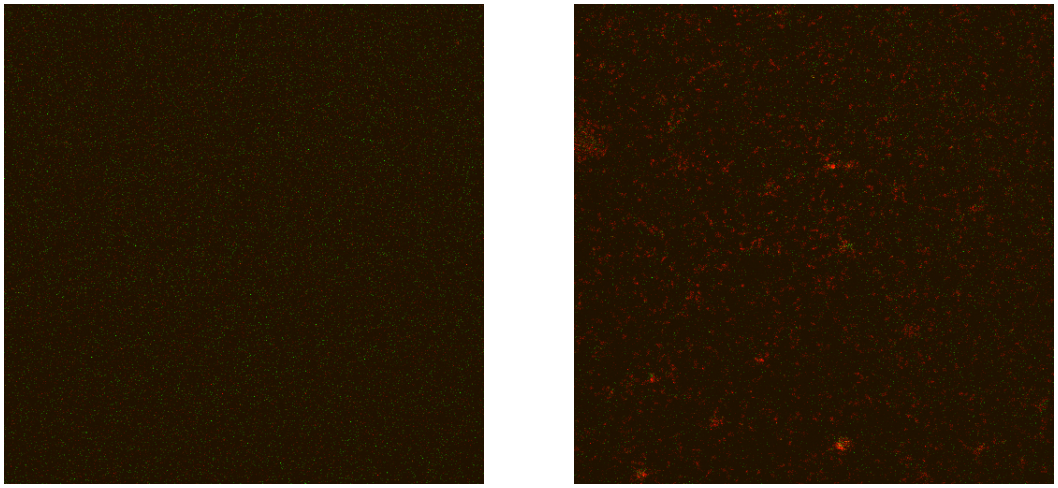
(b) Culture grown in defined mineral medium (MG)

Stained PHB (green); Autofluorescence (red)

Figure 19: *Confocal microscopy of stained H. hispanica grown in different media as positive controls*

Figure 19 shows *H. hispanica* grown in rich (23%MGM) and defined mineral medium (MG) as positive control. In picture 19(a) strong signals can be seen which originate from stained PHA granules. Additionally it can be seen that several cells aggregate. This might be an indication of surface transfectants which are expressed under these conditions. The right picture 19(b) also shows stained PHA granules but these are

only sporadically aggregated.
Contrary to expectations based on Han *et al.* (2007) much more PHA is accumulated in rich medium (23%MGM) than in defined mineral medium (MG).



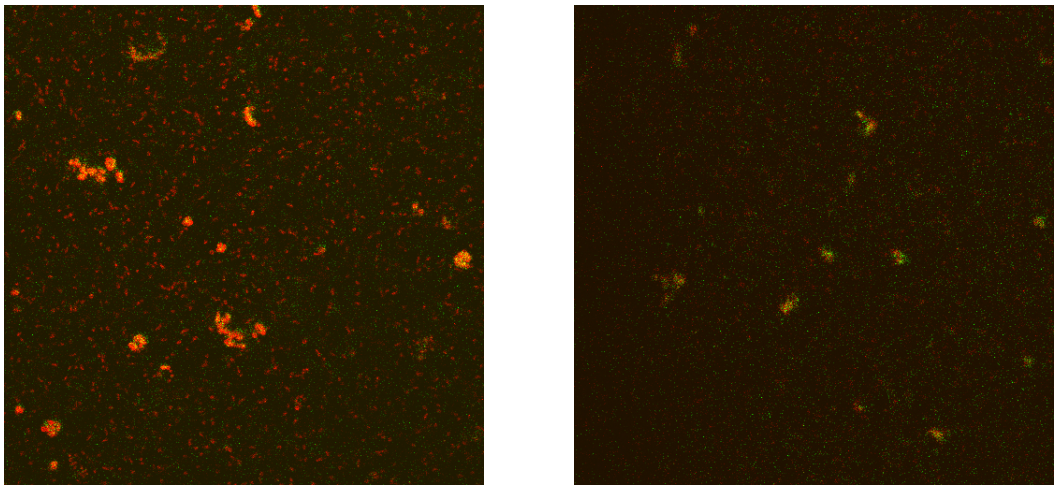
(a) Culture grown in rich medium (NVM)

(b) Culture grown in defined mineral medium (NMM4)

Stained PHB (green); Autofluorescence (red)

Figure 20: *Confocal microscopy of stained N. magadii L13/pRo-5 grown in different media as as negative controls*

Figure 20 shows stained *N. magadii* L13/pRo-5 as negative control. The negative control does not show any specific staining neither in rich (NVM) nor in defined mineral medium (NMM4).



(a) L13 pRo-5 16S prom $PhaEC_{Hh}$ *(b) L13 pRo-5 PHB_{Hm}*

Stained PHB (green); Autofluorescence (red)

Figure 21: *Confocal microscopy of stained transformants grown in defined mineral media (NMM4)*

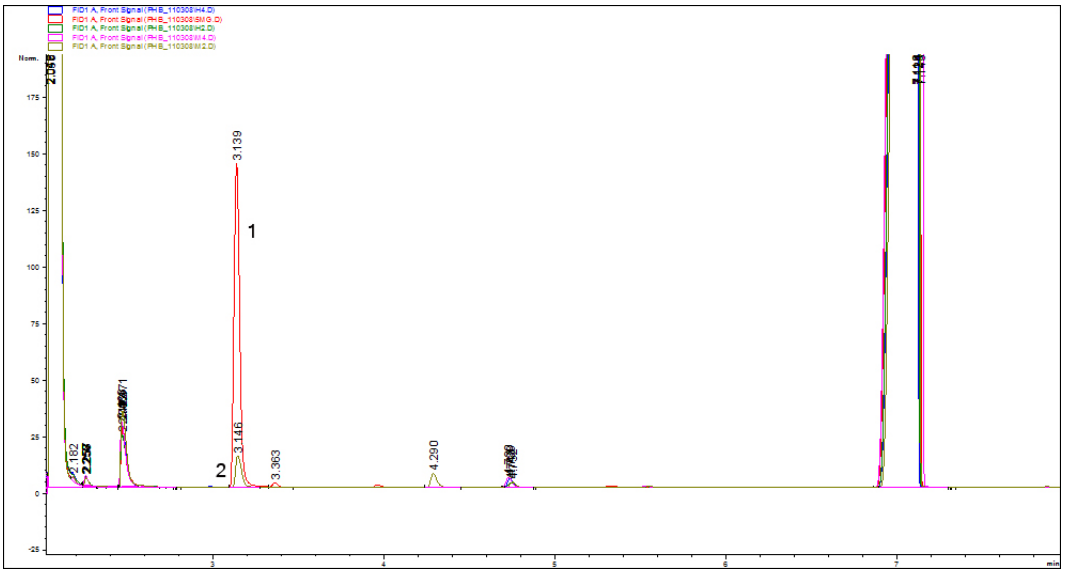
Figure 21 shows two exemplary transformants. There are L13 pRo-5 16S prom $PhaEC_{Hh}$, picture 21(a), and L13 pRo-5 PHB_{Hm}, picture 21(b), both showing possible PHA accumulation.
The homolog recombinants did not show good unspecific staining.

After microscopy it was possible to determine the most promising constructs for PHA accumulation. In general it was challenging to determine an adequate microscopy setup for halophilic archaea.

3.4 Gas Chromatography (GC)

GC is a very sensitive method to detect compounds of interest in a sample. Using an appropriate column the products of acidic depolymerization of PHB, 3-hydroxybutyric acid methylester, can be detected. In this thesis the qualitative analysis is sufficient, a quantification of PHB would also be possible using this technique. GC should have final authority whether PHB is synthesized by transformant and recombinant *N. magadii* or not.

Cultures of *H. hispanica*, *H. marismortui*, *N. magadii* L13 and L13/pRo-5 were grown in rich and defined mineral medium. When they reached the late logarithmic growth phase, they were harvested and prepared for gas chromatography (cf. section 2.2.15 page 65). The same was done for all transformants and homolog recombinants. Late logarithmic growth phase was chosen because there should be detectable amounts of PHA.



1: peak of 5 mg/ml PHB
2: peak of H. marismortui grown in rich medium (23%MGM)

Figure 22: *Chromatogram of GC: controls are 5 mg/ml PHB, H. hispanica and H. marismortui grown in rich (23%MGM) and defined mineral medium (MG)*

Figure 22 shows that only *H. marismortui* grown in rich medium (23%MGM) synthesizes detectable amounts of PHA. *H. hispanica* grown in rich medium (23%MGM) shows a mini peak, which is smaller than the threshold for peak detection. *H. hispanica* and *H. marismortui* grown in defined mineral medium (MG) did not show any PHA accumulation. These findings are contrary to expectations based on Han *et al.* (2007). GC of samples from transformants did also not detecte any PHA.

The peak for *H. marismortui* shows only little amounts of PHA. One could hypothesize that the used biomass is just too small to detect PHA. After this experiment it is hard to draw final conclusions because even the controls did not properly work out. Therefore it would be too early to dismiss the chance of PHA accumulation in transformants and homolog recombinants.

3.5 Summary and Criticism

3.5.1 Summary

Northern blot and Native Page. These two experiments were not mentioned above because they did not produce useful results.

Cloning and Transformation. Cloning and transformation of constructs was successful. pRo-5 plasmids were stable and suicide plasmids facilitated homologous recombination into the genome. Only homozygotization failed.

Purification of PHA synthase and Western blot. Conditions for protein over-expression were evaluated and applied. His-tag purification was only the first step in the purification process of the two subunits of the PHA synthase. Subsequent FPLC allowed pure protein to be sent off for antibody production. Western blotting proved that $PhaE_{Hh}$ was present in all transformants regardless of the promoter and growth medium.

Microscopy. Starting with phase contrast followed by fluorescence and confocal microscopy, this method gave a first hint of PHA accumulation in transformed and recombinant *N. magadii* L13.

Gas Chromatography (GC). GC should have been the final authority whether the preceding observations using microscopy and western blot were correct or not. But the results were inconclusive due to nearly undetectable amounts of PHA in the positive controls.

Results Overview.

Species	Medium	Microscopy	Western blot	GC
***Haloarcula* species**	23%MGM	+	+	+
Positive controls	MG	-	-	-
***N. magadii* L13, L13**	NVM	-	-	-
pRo-5 Negative controls	NMM4	-	-	-
Transformants and	NVM	-	+	-
recombinants	NMM4	+	+	-

Table 11: *Overview of results*

Table 11 sums up the results of all facilitated experiments.

Haloarcula species (positive controls) were consistent:

- PHA synthase was detected by western blot only in rich medium (23%MGM),
- microscopy revealed stained PHA granules and
- final GC detected small amounts of PHA.

N. magadii L13, L13 pRo-5 (negative controls) were also consistent:

- no PHA synthase,
- no signals detected by microscopes and
- no detection by GC.

Transformants and recombinants showed promising results:

- PHA synthase could be detected by western blot independent of medium and promoter,
- microscopy gave hints about which constructs might accumulate PHA and should be chose for GC
- but GC could not detect PHA in any of the analyzed samples

3.5.2 Criticism

As this thesis was based on the paper by Han *et al.* (2007), several inconsistencies were encountered:

- The described defined mineral medium MG for PHA accumulation suffered from the wrong buffer substance leading to a high amount of precipitates. Therefore instead of KH_2PO_4 as buffer substance 50 mM tris-base was used.
- The accumulation of PHA in *H. hispanica* and *H. marismortui* in MG could not be observed. Instead an accumulation of PHA could be proven in rich medium 23%MGM using microscopy, western blot and gas chromatography.

These discrepancies may be credited to the environmental conditions in this thesis which were different from those described in the paper: Han *et al.* (2007) used a continuous culture instead of batch growth.

3.6 Outlook

- The results of GC are not completely conclusive due to the weak signals of the positive controls. Also Han *et al.* (2010) showed in his paper that a reconstitution of the PHA synthesis pathway in *Haloferax volcanii* is only then successful when, additionally to the PHB operon, the fabG1 gene is also transformed. FabG1 represents the upstream β-ketoacyl-ACP reductase which supplies the substrate for the PHA synthase (cf. figure 5 page 18). BLAST showed that *N. magadii* does not possess a homolog of this gene.

- The preparation of cultures for microscopy has to be reviewed in a more methodical manner. Starting with *N. magadii* L13 (grown in NVM and NMM) at three different points the OD_{600} is normalized to 1. Then different amounts of Nile Red and Nile Blue are added and the slides are investigated using a standard fluorescence microscope (employing a mercury lamp). Then the Nile Red and Nile Blue concentrations are chosen which come closest to the point where non-specific signal can be observed. The same is done for *H. hispanica* and *H. marismortui* (grown in 23%MGM and MG).

- Another problem concerning gene expression encountered in *N. magadii* is, that different transformants can show different gene expression. According to Spiekermann *et al.* (1999) it is possible to screen PHA producing bacteria after addition of Nile Red to the agar before pouring plates. The plates and colonies can be investigated using a basic UV table. In case of halophiles Nile Red and Nile Blue could be added after complementation of the medium and before pouring plates. As mentioned in the preceding paragraph this should be done in a methodical manner. Starting with a not too high concentrated *N. magadii* L13 it can be plated on NVM and NMM agar plates containing different concentrations of Nile Red and Nile Blue. After single colonies appear the plates can be investigated and adequate concentrations of the two stains can be chosen. The same criterion applies as before: stain concentration should be as high as possible without unspecific signals. The same should be done for *H. hispanica* and *H. marismortui* grown on 23%MGM and MG agar plates.

  This might be a very elegant solution to kill two birds with one stone: it is possible to identify clones which carry the plasmid and additionally it is possible to identify clones which actually express the right genes and accumulate PHA. Therefore much time is saved without the necessity to take them into liquid culture to show possible PHA accumulation. Nonetheless this is just a preliminary screen, but one that might proof itself very useful.

- Further studies employing GC for the detection of PHA more biomass should

be used. Looking back at the results (cf. section 3.4 page 86) even the positive controls did show only very weak signals. It might be useful to use 200 ml or even more, harvest the cells by centrifugation and lyophylize them for acidic depolymerization. This way it would be also easier to harvest candidates and controls in different growth phases without the drawback of little biomass.

Concerning the pure PHB, isolated from recombinant *E. coli*, the maximum amount of PHB which is well dissolved in chloroform was 5 mg/ml (10 mg/ml did not dissolve properly and was therefore not useful). Although the viscosity of 5 mg/ml is quite high and the handling suffers. Additionally the concentration of banzoic acid as internal standard was chosen too high (50 mg/ml), a tenth would be adequate.

List of Figures

List of Tables

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Abstract

The aim of this diploma thesis was to transform the PHA synthase of *H. hispanica* and *H. marismortui* into *N. magadii*. Subsequently also PHA synthesis should be proven. The PHA operon consists of its native promoter and the two subunits of the PHA synthase PhaC (53 kDa) and PhaE (20.6 kDa). *Haloarcula* species and *N. magadii* live at the same high NaCl concentrations around 4 M.*Haloarcula* species thrive at a neutral pH and grow saccharolytically. They synthesize PHA under starvation from glucose. *N. magadii* lives at an alkaline pH between 9 and 11 and can grow proteolytically.

For the transformation of *N. magadii* different strategies and constructs were employed. On one hand a stable plasmid was used for transformation and on the other hand a suicide plasmid which would only become stable after homolog recombination took place. The constructs involved the two PHA synthases either with its native promoters or 16S rDNA promoter from *N. magadii*.

The two subunits of the PHA synthase were overexpressed and purified for antibody production. In case of PhaE it was successful and therefore western blots were facilitated. The results showed that PhaE was expressed in all transformants, due to the operon organisation probably PhaC also. The two *Haloarcula* species, positive controls, expressed PhaE contrary to the expectations only in rich medium and not in defined mineral medium.

At the same time PHA detection was promoted. First cells were stained using Nile Blue and then they were investigated using a fluorescence and confocal microscope. The positive controls displayed stronger PHA accumulation in rich medium compared to defined mineral medium and contrary to the expectations. The transformants also showed specific staining. Gas chromatography was employed to verify or falsify the observations made by microscopy. Concerning the positive controls GC verified the results of microscopy. PHA was detected only in rich medium but one species only showed a minipeak, therefore probably not enough biomass was applied. Concerning the transformants no PHA could be detected.

Possibly *N. magadii* misses β-ketoacyl-ACP reductase. This enzyme is set upstream of the PHA synthase supplying it with substrate. This might be a starting point for future projects.

Zusammenfassung

Ziel dieser Diplomarbeit war das PHA-Operon von *H. hispanica* bzw. *H. marismortui* in *N. magadii* zu transformieren und PHA-Synthese nachzuweisen. Das PHA-Operon besteht aus dem nativen Promotor und den beiden Untereinheiten der PHA-Synthase PhaC (53 kDa) und PhaE (20,6 kDa). Was die beiden *Haloarcula* Spezies mit *N. magadii* verbindet ist die hohe Salzkonzentration, die sie zum Leben benötigen, 4 M NaCl. Was sie trennt ist ihr bevorzugter pH-Wert, *Haloarcula* Spezies wachsen bei neutralem, *N. magadii* bei basischem pH (zwischen 9 und 11). Die Fähigkeit protelytisch zu wachsen ist *N. magadii* vorbehalten, während *Haloarcula* Spezies saccharolytisch wachsen und auch nur unter Mangelbedingungen aus Glukose PHA synthetiseren.

Die Transformation von *N. magadii* erfolgte mit unterschiedlichen Konstrukten, einerseits ein stabiles Plasmid, andererseits ein Vektor, der nur durch homologe Rekombination stabil in der Population verankert wird. Die Konstrukte umfassten die beiden PHA-Operone jeweils mit dem nativen und dem 16S rDNA Promotor aus *N. magadii*.

Die beiden Untereinheiten der PHA-Synthase wurden überexprimiert und aufgereinigt, um Antikörper herstellen zu lassen. Im Fall von PhaE war dies erfolgreich und so konnten Western Blots durchgeführt werden. Diese zeigten, dass die kleine Untereinheit, und somit auch die große Untereinheit, in allen Transformanten exprimiert wurde. Die Positivkontrollen, die beiden *Haloarcula* Spezies, exprimierten PhaE entgegen der Annahme jedoch nur im Vollmedium, nicht im Mangelmedium.

Parallel dazu wurde der Nachweis von PHA betrieben. Dies wurde mittels Färbung mit Nile Blue und anschließender Untersuchung unter dem Fluoreszenz- und Konfokalmikroskop versucht. Die Positivkontrollen synthetisierten entgegen den Erwartungen im Mangelmedium kaum, aber im Vollmedium viel PHA. Auch die Transformanten zeigten spezifische Färbung, daher wurde in weiterer Folge eine Gaschromatographie durchgeführt, um die gemachten Beobachtungen zu verifizieren. Die GC bestätigte die Beobachtungen der Mikroskopie betreffend der Positivkontrollen. Jedoch wurde wahrscheinlich zu wenig Biomasse verwendet, sodass bei einer Kontrolle nur ein minimaler Ausschlag detektiert werden konnte. Was die Transformanten betraf, so konnte in keiner PHA nachgewisesen werden.

Möglicherweise fehlt in *N. magadii* die β-ketoacyl-ACP Reduktase, ein Enzym, das, der PHA-Synthase vorgeschaltet, das Substrat für diese katalysiert. Dies wäre ein Ansatzpunkt für zukünftige Projekte rund um dieses Thema.

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