Heterologous Expression of Various PHA Synthase Genes in *Rhodospirillum rubrum*

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The phototrophic non-sulfur purple bacterium *Rhodospirillum rubrum* is known for its metabolic versatility. Particularly, *R. rubrum* is able to synthesize PHA under heterotrophic or even autotrophic growth with carbon monoxide as carbon and energy source. *R. rubrum* has therefore become a promising candidate for future cheap PHA production. However, *R. rubrum* synthesizes lower amounts of PHAs in comparison to well-known PHA producers like *Ralstonia eutropha* H16 or recombinant *Escherichia coli* strains. Since the PHA synthase is the key enzyme of PHA biosynthesis, genes encoding for twelve different PHA synthases were heterologously expressed in two generated *phaC* deletion mutants of *R. rubrum* in this study. To clearly see the effect of the foreign PHA synthases, PHA-negative mutants were required. The single mutant *R. rubrum* Δ*phaC2* showed a PHA-leaky phenotype (< 1 % PHA, wt/wt, of CDW), while the double mutant *R. rubrum* Δ*phaC1*Δ*phaC2* accumulated no measurable PHA. Eight different PHA synthase genes of class I, and four of class IV were chosen for heterologous expression. All recombinant *R. rubrum* strains showed significant PHA synthesis and accumulation, although PHA contents in the recombinant strains of the single mutant *R. rubrum* Δ*phaC2* were generally higher in comparison to those of the double mutant *R. rubrum* Δ*phaC1*Δ*phaC2*. Recombinant strains of the single mutant could be divided into two groups according to the accumulation of PHA in the cells. While recombinant strains dedicated to group one showed an increased PHA synthesis when compared to the wild type carrying an empty vector, strains of group two accumulated less PHA than the wild type. Finally, it was possible to increase the accumulation of PHA by up to 25 % due to heterologous expression of PHA synthase genes compared to the wild type.

Key words:

Rhodospirillum rubrum, Polyhydroxybutyrate (PHB), PHA-synthase genes, heterologous expression, PHB-negative deletion mutants

Introduction

The economic importance of recovery and regeneration of alternative energy and carbon sources has evolved into an ever-growing global challenge in the recent decades. Polyhydroxyalkanoates (PHAs) could be a forward-looking alternative to petrochemical-based plastics since they are biobased, biodegradable, biocompatible, and derived from renewable resources[1,2,3,4]. PHAs are synthesized by more than 300 different microorganisms as storage of carbon and energy, if an excess of carbon source is available but an essential nutrient is limited at the same time[5,6,7]. The most commonly accumulated and best studied PHA is polyhydroxybutyrate (PHB)[7]. Due to their elastomeric and thermoplastic character, PHAs have attracted much industrial attention for economic usage[8]. Various applications for PHAs as packaging material, medical implant material[9], textile fibers[10], feed supplements or even as precursor feedstock for biofuels have been reported[4].

Although there is intensive research on bacterial PHAs, their production costs are still not competitive to conventional plastics[6,11]. One of the main contributors to these costs arises from pure carbon sources used as feedstock for bacterial fermentations, which can comprise up to 50 % of the total production costs[12]. Therefore, bulk production of PHA requires feasible and economical fermentation processes from cheap carbon sources. One promising and widely investigated approach is the utilization of industrial waste products[13]. Besides complex industrial wastes, gasification processes enable the conversion of any carbonaceous material into synthesis gas (syngas)[13,14,15]. Syngas mainly consists of

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hydrogen (H_2), carbon monoxide (CO) and carbon dioxide (CO_2), and has attracted great biotechnical interest as a cheap but undefined feedstock for the fermentation of various microorganisms in the last years[14].

The phototrophic non-sulfur purple bacterium *Rhodospirillum rubrum* is able to synthesize PHA under heterotrophic and even autotrophic growth with carbon monoxide (CO) and carbon dioxide (CO_2) as carbon and energy source[14,15]. Growth of *Rhodospirillum rubrum* utilizing syngas has already been intensively investigated[14,15,16]. In 2008, a profitable and technically feasible concept for PHA accumulation of *R. rubrum* based on the utilization of synthesis gas was presented for the first time[16]. PHA biosynthesis through syngas fermentation was carried out, and the costs were $ 2 – 4 per kg less than in the case of using sugar fermentation and recombinant *E. coli* strains. Furthermore, H_2 was produced by *R. rubrum* under these conditions, which is also of great industrial interest[16]. These properties indicate the great biotechnological potential of *R. rubrum*. However, *R. rubrum* synthesizes lower amounts of PHAs than well-known PHA producers like *Ralstonia eutropha* or recombinant *E. coli* strains.

Since the PHA synthase is known to be the key enzyme of PHA biosynthesis, many studies aimed at heterologous expression of foreign PHA-synthase genes to increase PHA accumulation in the cells. Based on their substrate specificity and subunits, PHA synthases are divided into four classes[17]. Class I PHA synthases utilize short chain length hydroxyalkanoate (HA_{SCL}) monomers with three to five carbon atoms and are found in *R. eutropha* H16, *R. rubrum* S1 and many other α- and *β*-proteobacteria[18,19]. Medium chain length hydroxyalkanoates (HA_{MCL}) with a carbon backbone of five to fourteen carbon atoms are polymerized by PHA synthases of class II. Representative bacteria of class II PHA synthases are pseudomonads[20]. PHA synthases of class III and IV consist of two different subunits. Class III PHA synthases consist of two subunits which are encoded by the genes *phaC* and *phaE*. Enzymes of this class are known from *Allochromatium vinosum* and cyanobacteria. As precursors, substrate HA_{SCL} and HA_{MCL} are accepted[21]. Class IV PHA synthases are based on the subunits PhaC and PhaR. Until now, this class has been found only in *Bacillus* strains, such as *Bacillus megaterium* or *Bacillus cereus*[22]. PHA synthases of class IV are described to polymerize HA_{SCL}[19].

PHB biosynthesis in *R. rubrum* has already been investigated intensively, and it shares some general similarities with PHB biosynthesis in *R. eutropha* H16, which represents the best studied model organism[23]. Basically, the PHB synthesis is divided into three steps[24,25,26]. First, two molecules of acetyl-CoA are converted by a *β*-ketothiolase (PhaA) in a claisen condensation to yield acetoacetyl-CoA. The NADPH-dependent acetoacetyl-CoA reductase (PhaB) subsequently reduces acetoacetyl-CoA stereospecifically to *R*-(–)-3-hydroxybutyryl-CoA. In a last step, the PHA synthase (PhaC) polymerizes *R*-(–)-3-hydroxybutyryl-CoA to PHB, and CoA is released again[17,27]. However, PhaB of *R. rubrum* has been described in literature as an NADH-dependent isoenzyme, which converts acetoacetyl-CoA to *S*-(–)-3-hydroxybutyryl-CoA[28]. As PHA synthases are known to be stereospecific for *R*-(–)-3-hydroxybutyryl-CoA, two enoyl-CoA hydratases obviously convert *S*-(–)-3-hydroxybutyryl-CoA via crotonyl-CoA to *R*-(–)-3-hydroxybutyrate in *R. rubrum* S1[28,29]. Since the entire genome sequence of *R. rubrum* S1 was published in 2011, a first basis for specific manipulations of the genome of *R. rubrum* S1 was achieved[30].

Since 2000, it has been known that *R. rubrum* S1 carries at least two genes encoding for PHA synthases[31,32]. However, another homologous PHA synthase gene (*phaC3*) was identified recently[32]. All three PHA synthase genes were characterized, and it turned out that PhaC2 had a significantly lower enzyme activity than PhaC1 and PhaC3 *in vivo*. PhaC2 however, was detected in ten-fold higher concentration in the cytoplasm of the cell. Furthermore, single and multiple deletion mutants of the genes *phaC1*, *phaC2* and *phaC3* were generated and investigated by Jin and Nikolau (2012)[32]. It was shown that PhaC1 and PhaC3 were only marginally involved in the PHB synthesis, although only gene *phaC1* (Rru_0275) is located in a putative PHB operon.

In this study, two *phaC* deletion mutants of *R. rubrum* S1 were generated. Twelve different PHA synthase genes were expressed heterologously in both mutants to investigate PHB biosynthesis. Appropriate synthases were identified by an intensive literature research with special emphasis on high enzyme activities, successful previous heterologous expression, and strong PHB accumulation in host strains or in the wild type, respectively. This is the first detailed study dealing with the effect of foreign PHA synthase genes on PHB biosynthesis in *R. rubrum* S1.

Materials and methods

Chemicals and enzymes. All chemicals used were supplied by Carl Roth (Karsruhe, G), Merck (Darmstadt, G) or VWR (Langenfeld, G). Enzymes for DNA-manipulation were obtained by Thermo Fisher Scientific (Dreieich, G).

Bacterial strains. *Rhodospirillum rubrum* S1 (ATCC 11170) was used for generating deletion mutants. Cells of *Escherichia coli* TOP10 were applied for cloning and transformation methods. *E. coli* S17–1 was used for spot agar mating technique. The strains *Alcaligenes latus* H1, *Burkholderia cepacia* H111, *Chromobacterium sp.*USM2, *Bacillus cereus* E33L, *Bacillus megaterium* QMB1551, *Delftia acidovorans* SPH-1, *Paracoccus denitrificans* PD1222, *Ralstonia eutropha* H16 and *Rhodospirillum rubrum* S1 were used as donor strains for PHA synthase genes. All selected strains, corresponding PHA synthase genes, and references are shown in Table 1.

Cultivation of microorganisms. *E. coli* cells were grown in LB-Medium at 37 °C. Cultivation of *R. rubrum* strains was carried out in supplemented succinate fructose nitrogen (SSFN) medium (modified from Bose *et al*.[33]) with the omission of malate and a reduced concentration of ammonium sulfate (0.5 g L^{-1}). An amount of 10 g L^{-1} of fructose, 2 g L^{-1} of di-sodium succinate hexahydrate, and 0.3 g L^{-1} of yeast extract and casamino acids were added to the media. For cultivation, baffled flasks were used with a medium volume/flask volume ratio of 1:5 – 1:10. Precultures of recombinant *R. rubrum* strains were incubated for 48 h in 20 mL SSFN medium at 125 rpm on a gyratory shaker. For main cultures, 100 mL SSFN medium with reduced concentration of yeast extract, casamino acids, and ammonium sulfate was inoculated with 10 % (vol/vol) of precultures in 1000 mL baffled KLETT flasks. Cultivation was carried out for 50 h at 30 °C and 125 rpm. Growth was measured via optical density (OD) measurement using a KLETT-Summerson photometer (Manostat) at 520 to 580 nm. Cultures with a volume of 5 mL were centrifuged in 1.5 mL reaction tubes at 10.000 × *g* for 5 minutes. Cultures with a volume of 50 mL and more were centrifuged in 50 mL reaction tubes for 15 minutes at 4.000 x *g* and 4 °C. The amount of 25 μg mL^{-1} kanamycin was applied for recombinant *R. rubrum* strains, and 50 μg mL^{-1} kanamycin for recombinant *E. coli* strains.

Cloning of PHA synthase genes

The chosen PHA synthase genes were amplified from genomic DNA of the respective host strain (Table 1) by PCR applying proofreading Phusion polymerase (Thermo Fischer Scientific). Primers used for amplification are shown in Table 2. After subcloning PCR fragments into vector pJET1.2/blunt, fragments were excised and ligated into the broad host range expression vector pBBR1MCS-2[34]. Recombinant vectors (Table 3) were controlled to harbor correct PHA synthase gene inserts applying sequence analysis. Obtained sequences were analyzed using Chromas software (version 1.45, Technelysium Pty. Ltd.), Genamics Expression software (version 1.100 [http://genamics.com/expression/index.htm]), BLAST online service available on NCBI (National Center for Biotechnology Information [http://blast.ncbi.nlm.nih.gov/Blast.cgi]), and BioEdit[35]. Genomic DNA was isolated using the "DNeasy Blood&Tissue Kit" (QIAGEN; Hilden, G.). Plasmid DNA was isolated by the method of Birnboim and Doly[36] or highly purified using the „peqGOLD Plasmid Miniprep Kit" (Peqlab Biotechnologie GmbH, Erlangen, G). Isolation of DNA-fragments from agarose gels was carried out with the "High Pure PCR Cleanup Micro Kit" (Hoffmann-La Roche Ltd., S). For agarose gel electrophoreses, 0.8 % (wt/vt) agarose gels were prepared. Tris-Borate-EDTA-Buffer (TBE-Buffer) was used as buffer. Electrophoresis was carried out at 100 to 170 V, and 80 mA for 50 to 90 min. Competent cells of *E. coli* were prepared and transformed by the $CaCl_2$ procedure as described by Hanahan[37].

Table 1 – *Evaluated phaC genes for heterologous expression in R. rubrum strains. Donor strain, PHA-synthase class, annotation, gene size and references dealing with the respective phaC gene are mentioned.*

Organism	Strain	PHA-Synthase class	Gene	Size (bp)	Locus tag	Reference
Burkholderia cepacia	H111	I	*phaC*	1866	I35_3401	[51]
Chromobacterium sp.	USM2	I	*phaC*	1704	HM989943	[45]
Bacillus cereus	E33L	IV	*phaC/phaR*	1086/483	BCE33L1210/BCE33L1208	[48]
Bacillus megaterium	QMB1551	IV	*phaC/phaR*	1089/600	BMQ_1231/ BMQ_1229	[48]
Alcaligenes latus	DSM 1123/ H1	I	*phaC*	1611	AF004933	[52]
Delftia acidovorans	SPH-1	I	*phaC*	1826	Daci_3600	[49]
Paracoccus denitrificans	PD1222	I	*phaC*	2322	Pden_0958	[49]
Ralstonia eutropha	H16	I	*phaC1*	1770	H16_A1437	[49]
Rhodospirillum rubrum	S1	I	*phaC2*	1776	Rru_A2413	[32]
Rhodospirillum rubrum	S1	I	*phaC1*	1245	Rru_A0275	[32]

Table 2 – *Generated oligonucleotide primers used for PHA synthase gene amplification and sequencing*

Primer	Discription
A. latus phaC forward-Primer	ATCGATAGGAGGCGCGCGATGTCGGGCCTGAACC
A. latus phaC reverse-Primer	ACTAGTTCAGGCCTTCTGCTTCACGTAACGCCC
B. cepacia phaC forward-Primer	AAGCTTAGGAGGCAGGTAATGACAGCATCGAAAAATTCGTCG
B. cepacia phaC reverse-Primer	ACTAGTTCAATCGCGCTGCAACACGTAGCG
B. cereus phaC forward-Primer	ATCGATAGGAGGAGAAAAATGACTACATTCGCAACAGAATGGG
B. cereus phaC reverse-Primer	ACTAGTTTACTTCGAACGCTCGTCAAGCCAATC
B. cereus phaR forward-Primer	ACTAGTAGGAGGGGGAGTGTAGGTAAAGTGATTGATC
B. cereus phaR reverse-Primer	GAGCTCTCACTTTTTATTTTCTGGTTTATTCGTAGG
B. megaterium phaC forward-Primer	ATCGATAGGAGGATATTCGTGGCAATTCCTTACGTGCAAGAG
B. megaterium phaC reverse-Primer	ACTAGTTTATTTAGAGCGTTTTTCTAGCCAATCGCC
B. megaterium phaR forward-Primer	ACTAGTAGGAGGGGAGTTTTGGAACAGCAAAAAGTATTTGATCCG
B. megaterium phaR reverse-Primer	GAGCTCTTACTTGCGAGCCGGCTGCTCAG
C. sp. phaC forward-Primer	ATCGATAGGAGGAACCAGATGCAGCAGTTTGTCAATTCCC
C. sp. phaC reverse-Primer	ACTAGTTCAGTTCAAGGCGGCGGCGAC
D. acidovorans phaC forward-Primer	ATCGATAGGAGGTGCAGCATGAATTTTGACCCACTCGC
D. acidovorans phaC reverse-Primer	ACTAGTTCAGGCCTTGACCAGTACGTAGCGGC
D. denitrificans phaC reverse-Primer	ACTAGTTTATGCCCGCTCGTGAACGTAGAGCCC
P. denitrificans phaC forward-Primer	ATCGATAGGAGGTCAATTATGGCGGGCAAGGACAAAAAACC
R. eutropha phaC1 forward-Primer	ATCGATAGGAGGCAAATCATGGCGACCGGCAAAGG
R. eutropha phaC1 reverse-Primer	ACTAGTTCATGCCTTGGCTTTGACGTATCGC
R. rubrum phaC1 forward-Primer	ACTAGTAGGAGGAGGCCATGAGCAACCCAACCGACCC
R. rubrum phaC1 reverse-Primer	GAGCTCCCGTTGGTTGCTGAAGCGGGGG
R. rubrum phaC2 forward-Primer	AAGCTTAGGAGGATCAACATGGCCAATCAGGGCAGCG
R. rubrum phaC2 reverse-Primer	ACTAGTCTAGCCGGCGCGCACCTTCACG

Table 3 – *Recombinant vectors carrying PHA synthase genes generated in this study*

Recombinant vector
pBBR1MCS-2::$phaC2_{Rhodospirillum\ rubrum}$
pBBR1MCS-2::$phaC1_{Rhodospirillum\ rubrum}$
pBBR1MCS-2::$phaC2_{Rhodospirillum\ rubrum}$::$phaC1_{Rhodospirillum\ rubrum}$
pBBR1MCS-2::$phaC1_{Ralstonia\ eutropha}$
pBBR1MCS-2::$phaC_{Burkholderia\ cepacia}$
pBBR1MCS-2::$phaC_{Chromobacterium\ sp.}$
pBBR1MCS-2::$phaC_{Delftia\ acidovorans}$
pBBR1MCS-2::$phaC_{Paracoccus\ denitrificans}$
pBBR1MCS-2::$phaC_{Alcaligenes\ latus}$
pBBR1MCS-2::$phaC_{Bacillus\ cereus}$:: $phaR_{Bacillus\ cereus}$
pBBR1MCS-2::$phaC_{Bacillus\ cereus}$:: $phaR_{Bacillus\ megaterium}$
pBBR1MCS-2::$phaC_{Bacillus\ megaterium}$:: $phaR_{Bacillus\ megaterium}$
pBBR1MCS-2::$phaC_{Bacillus\ megaterium}$:: $phaR_{Bacillus\ cereus}$

Generation of deletion mutants of *R. rubrum* S1

Flanking regions of 700 to 1000 bp upstream and downstream of the target genes *phaC1* (Rru_A0275) and *phaC2* (Rru_A2413) were amplified by PCR, adding *Xba*I and *Eco*RI restriction sites to the resulting fragments. The fragments were then restricted with *Xba*I and *Eco*RI and ligated, forming fragments of approximately 1800 (for Δ*phaC1*) and 2000 (for Δ*phaC2*) bp. The fragments were again amplified by PCR, digested with *Xba*I and ligated into an *Xba*I digested pJQ200mp18 vector, yielding gene replacement vectors pJQ200mp18::Δ*phaC1* and pJQ200mp18::Δ*phaC2*. The plasmids pJQ200mp18::Δ*phaC1* and pJQ200mp18::Δ*phaC2* were mobilized from *E. coli* S17–1 as the donor strain to the corresponding recipient *R. rubrum* strain by the spot agar mating technique[38]. Mutants of *R. rubrum* were identified on SSN solid media (SSFN media without fructose) with 10 % (wt/vol) sucrose and SSN solid media containing 20 μg mL^{-1} gentamycine[39]. Successful gene replacement was confirmed by

PCR analyses and DNA sequencing, with the primers used for generating the flanking region fragments of *phaC1* and *phaC2*. *R. rubrum* Δ*phaC2* was used as the initial strain for generating the double deletion mutant *R. rubrum* Δ*phaC2*Δ*phaC1*.

Electroporation of *R. rubrum* cells. The transfer of generated vectors to cells of *R. rubrum* was carried out by electroporation[40]. However, a modified Super Optimal Catabolite Repression-Medium (SOC medium) containing fructose instead of glucose was used. Regeneration of the cells was carried out for 5 – 6 h at 125 rpm and 30 °C. Cells were harvested, resuspended in 100 µL sterile saline, and transferred to SSFN solid medium containing kanamycin. Single colonies of electroporated *R. rubrum* cells were obtained after 3 – 5 days at 30 °C.

PHB Analysis. Samples of 25 and 50 mL were collected from liquid cultures by centrifugation at 4.000 × *g* and 4 °C for 15 minutes. An amount of 5 – 10 mg of dried cells were subjected to acid methanolysis in the presence of 85 % (vol/vol) methanol and 15 % (vol/vol) sulfuric acid for 3 h at 100 °C. The resulting methyl esters of 3-hydroxybutyrate were characterized by gas chromatography according to[20,41]. For gas-chromatographic analysis, an Agilent 6850 gas chromatograph (Agilent Technologies) equipped with a BP21 capillary column (50 m by 0.22 mm; film thickness, 250 nm; stationary phase: polyethylene glycol [PEG]), and a flame ionization detector (Agilent Technologies) were used. The evaluation of the data analysis was based on the Agilent Cerity QA-QC software. As reference, retention times of commercial 3-hydroxy fatty acid standards were used for the identification and quantification of the fatty acids present in the samples. The PHB content in % (wt/wt) of the CDW was determined from a standard curve generated, based on using purified PHB samples.

Growth experiments. For each recombinant strain, two independent biological experiments were performed. Growth experiments started with pre-cultures (20 mL SSFN supplemented with 1.5 g L^{-1} casamino acids/ yeast extract/ 0.5 g L^{-1} ammonium sulfate) in baffled flasks with a total volume of 100 mL, which were grown for 48 h at 30 °C and 125 rpm. Main cultures (two replicate flasks for each strain in each biological experiment) were cultivated in baffled KLETT flasks with a total volume of 1000 mL filled with 100 ml of SSFN medium (0.3 g L^{-1} casamino acids/yeast extract) for 50 h at 30 °C and 125 rpm. To inoculate main cultures, pre-cultures were harvested by centrifugation (15 min at 4000 rpm, 4 °C), and resuspended in 4 mL medium. Each replicate main culture was inoculated with 2 mL of this suspension. Samples for PHB content determination were taken after 27 h and 50 h, representing cells of the early and late stationary growth phase, respectively. Cells were harvested by centrifugation (15 min at 4000 rpm, 4 °C), frozen, and used for GC analysis as described previously. Therefore, all recombinant strains of *R. rubrum* were tested four times for PHB accumulation in the early, and four times in the late stationary growth phase in this study.

Results

Heterologous expression of foreign PHA synthases in the single mutant *R. rubrum* Δ*phaC2*.

To characterize possible differences in growth and other properties of the recombinant *R. rubrum* strains, cultivations were performed in SSFN liquid media (Bose *et al*., 1961)[33], which was modified according to the requirements of this study. The purpose was to apply media that allow good growth as well as high PHB synthesis rates. To reach this goal, 2 g L^{-1} di-sodium succinate hexahydrate was added to the medium as additional and rapidly convertible carbon source beside fructose (10 g L^{-1}) to support cell growth. In order to promote PHB synthesis by limiting the N-sources and providing the mentioned C-sources in excess, limiting amounts of $(NH_4)_2SO_4$ and only low amounts of the supplements yeast extract and casamino acids were provided. This adjusted media composition showed best results in initial growth experiments (data not shown).

Growth curves were generated based on average values of measured optical cell densities (OD in Klett-units, KU). Growth curves for recombinant strains of *R. rubrum* Δ*phaC2* are shown in Fig. 1.

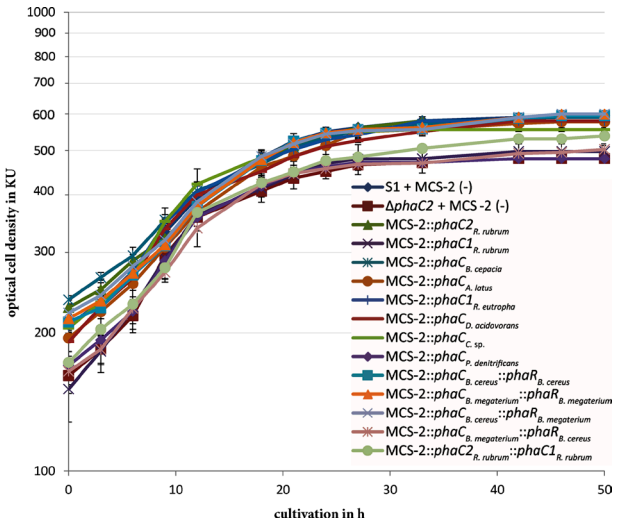

Fig. 1 – *Growth curves of all tested recombinant strains of R. rubrum ΔphaC2. Cells were cultivated for 50 h at 30 °C and 125 rpm in 100 mL SSFN-Medium at conditions promoting PHA accumulation. Pre-cultures were grown in SSFN-Medium for 48 h. Optical cell density was measured in Klett-units [KU]. All strains were tested in two independent biological experiments in duplicate. All data shown here are based on mean values. The strains R. rubrum S1 + MCS-2 (–) and R. rubrum ΔphaC2 + MCS-2 (–) were cultivated as reference strains. MCS-2 = pBBR1MCS-2; MCS-2 (–) = pBBR1MCS-2 without insert; h = hour; KU = Klett-units.*

An exponential growth phase (0 – 27 h) and a stationary growth phase starting after 27 h of cultivation were observed in all cultures, whereas *lag*-phases were not detected due to the inoculation of the cultures with actively growing cells. No significant differences with regard to growth were identified during the exponential growth phase. All strains reached the stationary growth phase after 27 h but showed different maximum optical cell densities (OD). According to this, the recombinant strains could be subdivided into two groups based on the shapes of their growth curves and the maximum OD. The highest maximum OD was reached by the reference strain *R. rubrum* S1 pBBR1MCS-2 (–) and amounted to 600 KU. In contrast, with reference strain *R. rubrum* Δ*phaC2* pBBR1MCS-2 (–), the lowest maximum OD (480 KU) was obtained. All other generated recombinant strains behaved quite similar concerning growth when compared to *R. rubrum* S1 pBBR1MCS-2 (–) (group 1) or *R. rubrum* Δ*phaC2* pBBR1MCS-2 (–) (group 2), respectively. Therefore, the recombinant strains carrying the PHA synthase genes $phaC1_{R.\ rubrum}$, $phaC_{P.\ denitrificans}$ and $phaC_{B.\ megaterium}$::$phaR_{B.\ cereus}$ were referred to group 1. With the exception of the strain *R. rubrum* Δ*phaC2* pBBR1MCS-2:: $phaC2_{R.\ rubrum}$:: $phaC1_{R.\ rubrum}$, all other strains exhibited similar growth curves like *R. rubrum* S1 pBBR1MCS-2 (–). Representative for group 1, *R. rubrum* S1 pBBR1MCS-2(–) showed a growth rate of μ = 0.1935 h^{-1} and a doubling time of t_d = 3.58 h. For *R. rubrum* Δ*phaC2* pBBR1MCS-2(–) (group 2), a growth rate of μ = 0.1898 h^{-1} and a doubling time of t_d = 3.65 h was determined. Maximum OD values and growth rates of recombinant *R. rubrum ΔphaC2* strains are shown in Table 4. Deviations in OD for the repeated cultivations of recombinant strains were low (see error bars Fig. 1).

Nearly all strains showed higher PHB content in the late stationary growth phase than in the early stationary phase (Fig. 2). While the wild type strain accumulated a maximum of 26 % PHB (CDW), the complemented strain *R. rubrum* Δ*phaC2* pBBR1MCS-2::$phaC2_{R.\ rubrum}$ showed the highest PHB synthesis of all strains and accumulated PHB to 32 % of CDW. Expression of several other foreign *phaC* genes resulted in comparable cellular PHB content. Expression of gene $phaC1_{R.\ rubrum}$ was not able to restore the phenotype of the wild type. Expression of genes $phaC1_{R.\ rubrum}$ and $phaC2_{R.\ rubrum}$ likewise did not result in a positive effect on PHB accumulation. While three recombinant strains harboring vectors with shuffled *phaC* and *phaR* genes encoding, the different subunits of type IV PHA synthases of *B. megaterium* and *B. cereus* stored more PHB than the wild type, strain *R. rubrum* Δ*phaC2* pBBR1MCS-2:

Table 4 – *Maximum optical cell densities (OD) and growth rates of recombinant R. rubrum ΔphaC2 strains after 50 h cultivation in SSFN-Medium under PHB accumulation conditions*

Heterologously expressed gene(s) in *Rhodospirillum rubrum* Δ*phaC2*	Maximum OD of recombinant strains after 50 h cultivation	Growth rate
$phaC2_{Rhodospirillum\ rubrum}$	600.0 KU	0.1902 h^{-1}
$phaC1_{Rhodospirillum\ rubrum}$	497.5 KU	0.1894 h^{-1}
$phaC2_{Rhodospirillum\ rubrum}$::$phaC1_{Rhodospirillum\ rubrum}$	537.5 KU	0.1906 h^{-1}
$phaC1_{Ralstonia\ eutropha}$	580.0 KU	0.1925 h^{-1}
$phaC_{Burkholderia\ cepacia}$	590.0 KU	0.1861 h^{-1}
$phaC_{Chromobacterium\ sp.}$	555.0 KU	0.1948 h^{-1}
$phaC_{Delftia\ acidovorans}$	580.0 KU	0.1891 h^{-1}
$phaC_{Paracoccus\ denitrificans}$	480.0 KU	0.1907 h^{-1}
$phaC_{Alcaligenes\ latus}$	577.5 KU	0.1924 h^{-1}
$phaC_{Bacillus\ cereus}$:: $phaR_{Bacillus\ cereus}$	595.0 KU	0.1931 h^{-1}
$phaC_{Bacillus\ cereus}$:: $phaR_{Bacillus\ megaterium}$	600.0 KU	0.1927 h^{-1}
$phaC_{Bacillus\ megaterium}$:: $phaR_{Bacillus\ megaterium}$	600.0 KU	0.1940 h^{-1}
$phaC_{Bacillus\ megaterium}$:: $phaR_{Bacillus\ cereus}$	502.5 KU	0.1910 h^{-1}
Wild type*	600.0 KU	0.1935 h^{-1}
Deletion mutant Δ*phaC2**	480.0 KU	0.1898 h^{-1}

*carrying an empty vector

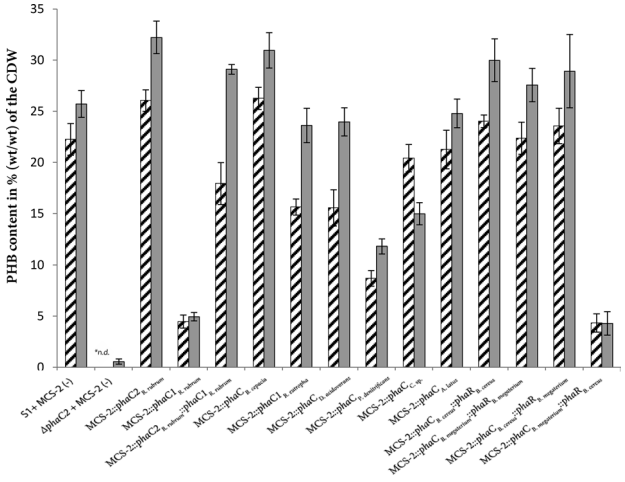

Fig. 2 – *PHB determination of investigated recombinant strains of the single mutant R. rubrum ΔphaC2. The maximum PHB content in % (wt/wt) of the cell dry weight (CDW) is shown as mean value of all tested samples. Striped bars indicate the PHB content after 27 h of cultivation and grey bars indicate the PHB content after 50 h of cultivation. The strains R. rubrum S1 + MCS-2 (–) and R. rubrum ΔphaC2 + MCS-2 (–) were cultivated as reference strains. MCS-2 = pBBR1MCS-2; MCS-2 (–) = pBBR1MCS-2 without insert. All recombinant strains were examined at identical conditions in two independent biological experiments. In addition, each strain was cultivated in duplicate in each experiment. Therefore, each strain was tested for PHB-accumulation four times after 27 h and 50 h cultivation. MCS-2 = pBBR1MCS-2;*n.d.= not detectable.*

:pBBR1MCS-2::$phaC_{B.\ megaterium}$::$phaR_{B.\ cereus}$ interestingly stored significantly lower amounts of PHB. Another surprising finding was that the heterologous expression of $phaC_{C.sp.}$ resulted in higher PHB content in the early stationary growth phase than in the late stationary growth phase. The deletion mutant *R. rubrum* Δ*phaC2* pBBR1MCS-2 (–) accumulated low content of PHB (< 1 %, wt/wt, of the CDW) after 50 h cultivation, however, no measureable PHB was found in the early stationary growth phase.

Heterologous expression of foreign PHA synthases in the double mutant *R. rubrum* Δ*phaC1*Δ*phaC2*. In contrast to the growth of the recombinant *R. rubrum* Δ*phaC2* strains (Fig. 1), lower maximum ODs were measured in cells of the recombinant strains of the double mutant *R. rubrum* Δ*phaC1*Δ*phaC2* (Fig. 3). The reference strain *R. rubrum* S1 pBBR1MCS-2 (–) again showed the highest maximum OD amounting to 600 KU. Lowest maximum ODs were observed in cultures of *R. rubrum* Δ*phaC1*Δ*phaC2* pBBR1MCS-2 (–) and *R. rubrum* Δ*phaC1*Δ*phaC2* pBBR1MCS-2::$phaC1_{R.\ rubrum}$, where only 480 KU and 450 KU were measured, respectively. All other strains showed maximum ODs between 480 and 600 KU. The deviations in OD in the repeated different cultivations were likewise low (see error bars Fig. 3).

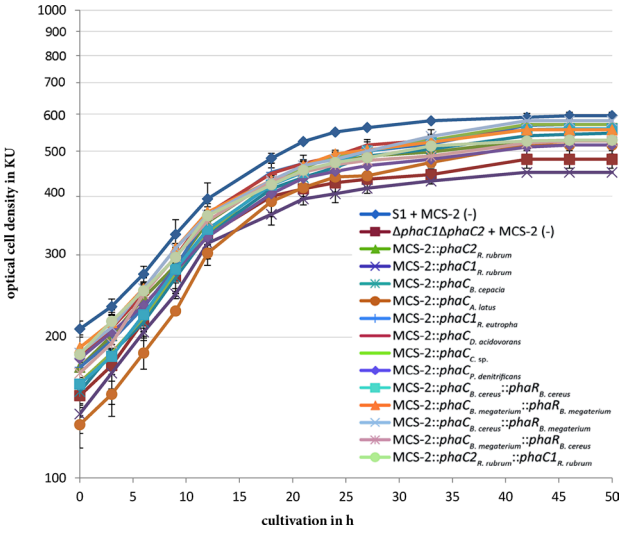

Fig. 3 – *Growth curves of all tested recombinant strains of R. rubrum ΔphaC1ΔphaC2. Cells were cultivated for 50 h at 30 °C and 125 rpm in 100 mL SSFN-Medium at conditions promoting PHA accumulation. Pre-cultures were grown in SSFN-Medium for 48 h. Optical cell density was measured in Klett-units [KU]. All strains were tested in two independent biological experiments in duplicate. All data shown here are based on mean values. The strains R. rubrum S1 + MCS-2 (–) and R. rubrum ΔphaC2 + MCS-2 (–) were cultivated as reference strains. MCS-2 = pBBR1MCS-2; MCS-2 (–) = pBBR1MCS-2 without insert; h = hour; KU = Klett-units.*

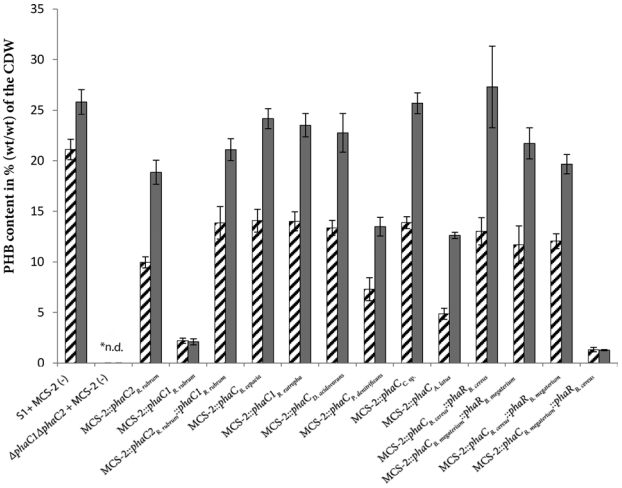

Fig. 4 – *PHB determinations of all recombinant strains of the double mutant R. rubrum ΔphaC1ΔphaC2. The maximum PHB content in % (wt/wt) of the cell dry weight (CDW) is shown as mean value of all tested samples. Striped bars indicate the PHB content after 27 h of cultivation and grey bars indicate the PHB content after 50 h of cultivation. The strains R. rubrum S1 + MCS-2 (–) and R. rubrum ΔphaC2 + MCS-2 (–) were cultivated as reference strains. MCS-2 = pBBR1MCS-2; MCS-2 (–) = pBBR1MCS-2 without insert. All recombinant strains were examined at identical conditions in two independent biological experiments. In addition, each strain was cultivated in duplicate in each experiment. Therefore, each strain was tested for PHB-accumulation four times after 27 h and 50 h of cultivation. MCS-2 = pBBR1MCS-2; *n.d.= not detectable.*

In contrast to the heterologous expression of *phaC* genes in the single mutant (Fig. 1 and Fig. 2), plasmid based *phaC* expression in the double mutant *R. rubrum* Δ*phaC1*Δ*phaC2* did not result in significantly increased PHB content in cells of the recombinant strains when compared to the wild type S1 harboring the empty pBBR1MCS-2 vector (Fig. 4). As expected, the recombinant strains *R. rubrum* Δ*phaC1*Δ*phaC2* carrying an empty vector, showed no measurable PHB content, while the plasmid-based expression of $phaC1_{R.\ rubrum}$ resulted in a PHB accumulation of ~ 2 % (wt/wt) in the early and late stationary growth phase. Heterologous expression of pBBR1MCS-2::$phaC2_{R.\ rubrum}$:: $phaC1_{R.\ rubrum}$ did not fully complement the wild type phenotype. Expression of the *B. cereus* PHA synthase genes *phaC* and *phaR* showed slightly higher PHB content of + 1 % (wt/wt) of CDW. Interestingly, the PHB content of nearly all recombinant strains of *R. rubrum* Δ*phaC1*Δ*phaC2* showed 90 to 100 % higher PHB content in the late stationary growth phase compared to the early stationary growth phase. This is not the case for the heterologous expressions in the single mutant, where PHB content was only 20 – 25 % higher in the late stationary phase.

Discussion

Several previous studies dealt with the growth of the wild type *R. rubrum* S1 under various cultivation conditions and the used carbon sources. Cultivations were carried out under heterotrophic and autotrophic conditions as well as in the absence or presence of light[14,15,16,18,42,43]. In most preceding studies, *R. rubrum* showed slow growth rates leading to long and inefficient cultivations. In order to acquire fast growth of the cultures and high content of accumulated PHB in the cells, cultivation and medium components were successfully optimized in our study.

PHB accumulation of recombinant *R. rubrum* strains

The heterologous expression of PHA synthase genes in the single and double deletion mutants *R. rubrum* Δ*phaC2* and *R. rubrum* Δ*phaC1*Δ*phaC2*, respectively, resulted in a significant accumulation of PHB in all generated strains (Fig. 2 and Fig. 4). Six of the thirteen generated *phaC* hybrid vectors led to an increased PHB accumulation in cells of *R. rubrum* Δ*phaC2* in comparison to the wild type strain carrying an empty vector (Table 5). In contrast, all recombinant strains of *R. rubrum* Δ*phaC1*Δ*phaC2* harboring heterologous *phaC* genes accumulated significantly less PHB than the recombinant single mutants and the wild type strain. Therefore, deleting the chromosomal $phaC1_{R.\ rubrum}$ in *R. rubrum* might have caused polar effects on other PHB biosynthesis genes. The $phaC1_{R.\ rubrum}$ [Rru_A0275] is located adjacent to *phaA* [Rru_A0274] and to *phaB* [Rru_A0273] in a putative PHB operon[32]. In addition, a putative *phaR* gene [Rru_A0276] was observed by *in silico* studies in close proximity (data not shown).

Table 5 – *Effect of heterologously expressed PHA synthase genes on PHB accumulation in R. rubrum* Δ*phaC2. All recombinant strains were examined under the same medium and growth conditions in two independent biological experiments. In addition, each strain was cultivated in duplicate. +: increase of PHB in %; –: decrease of PHB in %.*

Increase/decrease of the PHB accumulation compared to *R. rubrum* S1 pBBR1MCS-2 (–)	Heterologous expressed PHA synthase gene(s) in *R. rubrum* Δ*phaC2*
+ 25.3 %	$phaC2_{R.\ rubrum}$
+ 20.3 %	$phaC_{B.\ cepacia}$
+ 16.6 %	$phaC_{B.\ cereus}$:: $phaR_{B.\ cereus}$
+ 13.1 %	$phaC2_{R.\ rubrum}$::$phaC1_{R.\ rubrum}$
+ 12.4 %	$phaC_{B.\ megaterium}$:: $phaR_{B.\ cereus}$
+ 7.1 %	$phaC_{B.\ megaterium}$:: $phaR_{B.\ megaterium}$
– 3.6 %	$phaC_{A.\ latus}$
– 6.8 %	$phaC_{D.\ acidovorans}$
– 8.2 %	$phaC1_{R.\ eutropha}$
– 41.7 %	$phaC_{C.\ sp.}$
– 54.1 %	$phaC_{P.\ denitrificans}$
– 80.7 %	$phaC1_{R.\ rubrum}$
– 83.3 %	$phaC_{B.\ cereus}$:: $phaR_{B.\ megaterium}$

Growth experiments in this study (Fig. 1 and Fig. 3) showed that recombinant strains with higher content of accumulated PHB were linked to higher growth rates and higher maximum ODs, which is due to the light scattering effect of PHB granules. All recombinant strains of *R. rubrum* Δ*phaC1*Δ*phaC2* showed significantly decreased growth rates and maximum ODs when compared to the wild type carrying an empty vector. As the reductive step in PHB-synthesis is also known to act as an electron sink, the reduced ability to synthesize PHB might also lead to an imbalanced level of reducing agents, resulting in slower growth of the cells[44]. Our results are in line with alternative *phaC* deletion mutants of *R. rubrum* generated by Jin and Nikolau (2012)[33], which likewise showed that the deletion of *phaC2* resulted in decreased growth and strongly reduced PHB accumulation.

The characteristic of recombinant PHA accumulation by each synthase is reflected in both mutants. In most cases, the heterologously expressed *phaC* genes, which led to the highest amount of accumulated PHB (wt/wt of the CDW) in the single deletion mutant *R. rubrum* Δ*phaC2*, also resulted in the highest PHB synthesis in the double deletion mutant *R. rubrum* Δ*phaC1*Δ*phaC2*. However, PHB content in recombinant strains of the double mutant was generally lower than in the wild type (see Fig. 2 and Fig. 4). The heterologous expression of the PHA synthase gene of *Chromobacterium* sp., $phaC_{C.\ sp.}$, showed unexpected low PHB content in both mutants. This PHA synthase was studied intensively in previous studies[45,46]. In these studies, the heterologous expression of pBBR1MCS-2::$phaC_{C.\ sp}$ in the PHB negative strain *R. eutropha* PHB^{-}4 resulted in a maximum PHB accumulation, and *in vivo* enzyme activity tests showed a high activity of 2462 ± 80 U g^{-1}. This was about 8-fold higher than the PHA synthase activity from *R. eutropha* H16[46]. Additionally, the heterologous expression of $phaC_{C.\ sp.}$ in *R. rubrum* Δ*phaC2* surprisingly showed a significantly higher PHB content in the early stationary growth phase than in the late stationary growth phase in our study (see Fig. 2). This is unusual, since PHB accumulation usually reaches its maximum in the late stationary growth phase[5]. The reasons for these results with $phaC_{C.\ sp.}$ in *R. rubrum* remain unclear.

Heterologous expression of class IV PHA synthases has been studied intensively in the past[22,47,48]. In our study, class IV PHA synthases of the PHB accumulating strains *Bacillus megaterium* and *Bacillus cereus* were investigated. In addition to the heterologous expression of $phaC_{B.\ megaterium}$::$phaR_{B.\ megaterium}$ and $phaC_{B.\ cereus}$::$phaR_{B.\ cereus}$, newly combined hybrid PHA synthases were generated. For this, the *phaR* genes were replaced and two further active PHA synthases encoded by $phaC_{B.\ megaterium}$::$phaR_{B.\ cereus}$ and $phaC_{B.\ cereus}$::$phaR_{B.\ megaterium}$ were generated. Similar hybrid vectors with shuffled *phaC* and *phaR* genes were heterologously expressed in *E. coli*[48]. Up to 80 % (wt/wt) PHB of the CDW were synthesized by the *B. cereus* PHA synthase, while the PHA synthase of *B. megaterium* showed less PHB accumulation of 26 % (wt/wt) of the CDW. Coexpression of $phaC_{B.\ megaterium}$ and $phaR_{B.\ cereus}$ resulted in a PHB accumulation of 39 % (wt/wt). Generated hybrid synthases with rearranged *phaR* and *phaC* genes resulted in significant differences in PHB content and molecular weight[48]. In our work, the heterologous expression showed a significant PHB content in each generated recombinant strain, however, the expression of $phaC_{B.\ megaterium}$::$phaR_{B.\ cereus}$ resulted in a reduced PHB content of 4.8 and 1.3 % (wt/wt) of the CDW in the single mutant and double mutant compared to the co-expression in *E. coli*[48]. Possibly, an impaired interaction of the subunits $PhaC_{B.\ megaterium}$ and $PhaR_{B.\ cereus}$ might be responsible for the observed reduced PHB content and cell growth. However, an experimentally proven explanation for the phenotype of *R. rubrum* pBBR1MCS-2::$phaC_{B.\ megaterium}$::$phaR_{B.\ cereus}$ is not yet available.

In summary, we engineered different strains of the metabolically versatile PHB-producing bacterium *R. rubrum* to heterologously express a number of twelve different *phaC*-Genes, thereby increasing the amount of accumulated PHB by up to 25 % compared to the wild type strain. Our study proves the PHA synthase to be a significant bottleneck in the biosynthesis of PHB, as well as evaluates the potential of several promising PHA synthases for application in PHB production in recombinant microorganisms for the first time in a single host strain.

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List of Abbreviations and Symbols

*n.d. – not detectable
Δ – delta, deletion
°C – Degrees Celsius
μ – micro growth rate, h^{-1}
µL – microliter
A. – *Alcaligenes*
ATCC – American type- and culture collection
B. – *Bacillus*
B. – *Burkholderia*
bp – base pair C – carbon
C. – *Chromobacterium*
CDW – cell dry weight
CO – carbon monoxide
CO_2 – carbon dioxide
CoA – Coenzyme A
C_x – carbon atom number x
D. – *Delftia*
DNA – deoxyribonucleic acid
E. – *Escherichia*
Fig. – figure
g – gravity 9.81 m s^{-2}
G – Germany
g – gram
g L^{-1} – gram per liter
h – hour
H_2 – hydrogen
KU – KLETT-Units
L – liter
mA – milliampere
MCS-2 – vector pBBR1MCS-2
Min – minute
mL – milliliter
N – nitrogen
O – oxygen
OD – optical density
P. – *Paracoccus*
PCR – Polymerase Chain Reaction
PEG – Polyethylene glycol
PHA – Polyhydroxyalkanoate
PHB – Polyhydroxybutyrate
R. – *Rhodospirillum, Ralstonia*
rpm – revolutions per minute
SSFN – Supplemented Succinate Fructose Nitrogen
Syngas – Synthesis gas
TBE – Tris-Bora-EDTA
td – doubling time
V – volt
vol/vol – volume per volume
wt/vol – weight per volume
wt/wt – weight per weight

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