Genome-wide transcriptome analyses of the 'Knallgas' bacterium *Ralstonia eutropha* H16 with regard to polyhydroxyalkanoate metabolism

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Ralstonia eutropha H16 is probably the best-studied 'Knallgas' bacterium and producer of poly(3-hydroxybutyrate) (PHB). Genome-wide transcriptome analyses were employed to detect genes that are differentially transcribed during PHB biosynthesis. For this purpose, four transcriptomes from different growth phases of the wild-type H16 and of the two PHB-negative mutants PHB⁻4 and Δ *phaC1* were compared: (i) cells from the exponential growth phase with cells that were in transition to stationary growth phase, and (ii) cells from the transition phase with cells from the stationary growth phase of *R. eutropha* H16, as well as (iii) cells from the transition phase of *R. eutropha* H16 with those from the transition phase of *R. eutropha* PHB⁻4 and (iv) cells from the transition phase of *R. eutropha* Δ *phaC1* with those from the transition phase of *R. eutropha* PHB⁻4. Among a large number of genes exhibiting significant changes in transcription level, several genes within the functional class of lipid metabolism were detected. In strain H16, *phaP3*, *accC2*, *fabZ*, *fabG* and H16_A3307 exhibited a decreased transcription level in the stationary growth phase compared with the transition phase, whereas *phaP1*, H16_A3311, *phaZ2* and *phaZ6* were found to be induced in the stationary growth phase. Compared with PHB⁻4, we found that *phaA*, *phaB1*, *paaH1*, H16_A3307, *phaP3*, *accC2* and *fabG* were induced in the wild-type, and *phaP1*, *phaP4*, *phaZ2* and *phaZ6* exhibited an elevated transcription level in PHB⁻4. In strain Δ *phaC1*, *phaA* and *phaB1* were highly induced compared with PHB⁻4. Additionally, the results of this study suggest that mutant strain PHB⁻4 is defective in PHB biosynthesis and fatty acid metabolism. A significant downregulation of the two *cbb* operons in mutant strain PHB⁻4 was observed. The putative polyhydroxyalkanoate (PHA) synthase *phaC2* identified in strain H16 was further investigated by several functional analyses. Mutant PHB⁻4 could be phenotypically complemented by expression of *phaC2* from a plasmid; on the other hand, in the mutant H16 Δ *phaC1*, no PHA production was observed. PhaC2 activity could not be detected in any experiment.

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Abbreviations: ACP, acyl-carrier proteins; CBB cycle, Calvin–Benson–Bassham cycle; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); GPC, gel permeation chromatography; PHA, polyhydroxyalkanoate; PHB, poly(3-hydroxybutyrate).

Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MEXP-2440.

A supplementary figure, showing multiple DNA sequence alignments of *phaC1* and *phaC2* in *R. eutropha* H16, and four supplementary tables, showing changes in gene expression profiles, are available with the online version of this paper.

INTRODUCTION

Ralstonia eutropha H16 is a Gram-negative, facultative chemolithoautotrophic and hydrogen-oxidizing bacterium belonging to the order *Betaproteobacteria*. This strain was isolated from a spring near Göttingen about 50 years ago (Schlegel *et al.*, 1961; Walde, 1962) and is probably the best-examined microorganism with regard to polyhydroxyalkanoate (PHA) metabolism. *R. eutropha* H16 possesses a remarkable metabolic versatility; the strain can use organic

compounds, preferably organic acids, as well as molecular H₂ as electron donors, and it can shift between heterotrophic and autotrophic growth modes. CO₂ is fixed via the Calvin cycle (reviewed by Bowien & Kusian, 2002). Under anoxic conditions, *R. eutropha* H16 can switch to anaerobic respiration, exploiting alternative electron acceptors such as NO₃⁻ and NO₂⁻ by a complete denitrification pathway (Pfitzner & Schlegel, 1973).

Under unbalanced growth conditions, whenever carbon is available in excess but when another macroelement, including oxygen, is growth-limiting, *R. eutropha* H16 stores large amounts of organic carbon in the cytoplasm as poly(3-hydroxybutyrate) (PHB). PHB biosynthesis from the central intermediate acetyl-CoA occurs in three steps and is mediated by a β -ketothiolase (PhaA), which catalyses the conversion of two molecules of acetyl-CoA to acetoacetyl-CoA, an acetoacetyl-CoA reductase (PhaB), which reduces acetoacetyl-CoA to R-3-hydroxybutyryl-CoA, and finally the key enzyme PHB synthase (PhaC), which catalyses the polymerization of the hydroxyacyl moiety of R-3-hydroxybutyryl-CoA. The genes for the three enzymes are encoded by the *phaCAB* operon which was cloned during the 1980s (Schubert *et al.*, 1988; Slater *et al.*, 1988; Peoples & Sinskey, 1989). Since then, several additional genes involved in PHB metabolism have been identified. Homologues of *phaA*, encoding alternative β -ketothiolases (for example *bktB* and *bktC*) with differing substrate specificities, have been reported (Haywood *et al.*, 1988; Slater *et al.*, 1998). More recently, a genome analysis of strain H16 revealed 14 additional homologues of *phaA*, 39 *phaB* homologues and a second gene for a putative PHA synthase, *phaC2* (Pohlmann *et al.*, 2006; Reinecke & Steinbüchel, 2009). However, the physiological function of the latter remained obscure, as no PHA production occurred in cells of *R. eutropha* H16 Δ *phaC1* after transformation with *phaC2* (Reinecke & Steinbüchel, 2009).

The PHB granules in *R. eutropha* H16 are coated with a layer consisting of phospholipids and four different types of proteins. The PHA synthase PhaC, the intracellular PHB depolymerase PhaZ as well as phasins are the predominant components of the layer (Pötter & Steinbüchel, 2005). Phasins are a class of structural proteins, consisting of a hydrophobic domain, which binds to the granule surface, and hydrophilic or amphiphilic domains, which are exposed to the cytoplasm (Wieczorek *et al.*, 1995). In *R. eutropha* H16, four phasin genes have been identified: *phaP1*, *phaP2*, *phaP3* and *phaP4*; PhaP1 is the major and best-studied phasin and it contributes to approximately 3–5 % of the total cell protein (Pötter *et al.*, 2004; Wieczorek *et al.*, 1995). Genetic studies with Tn5-induced *phaP1* mutants demonstrated a significantly lower rate of PHB biosynthesis and the appearance of only one single large granule in the cells (Wieczorek *et al.*, 1995). The three PhaP1 homologues PhaP2, PhaP3 and PhaP4 exhibit 42, 49 and 45 % identity or 61, 62 and 63 % similarity to PhaP1, respectively, (Schwartz *et al.*, 2003) and occur in minor amounts in the cells (Pötter *et al.*, 2004; Zeller *et al.*, 2005). Due to their similarities, PhaP1 and PhaP3 are homologues,

but may represent paralogues of PhaP2 and PhaP4. In a *phaP1* disruption mutant, the amount of PhaP3 in the cells increased significantly (Pötter & Steinbüchel, 2005). Transcription of *phaP1* and *phaP3* is controlled by an autoregulative transcription repressor PhaR, which is the fourth protein that is at least partially bound to the granules (Pötter *et al.*, 2002; Pötter & Steinbüchel, 2005).

Mobilization of PHB is induced when the limiting macroelement that caused PHB biosynthesis is supplied again. Nine isoenzymes are putatively suspected to be involved in PHB mobilization. Five PHB depolymerases containing a DepA domain (PhaZ1 to PhaZ5) (Saegusa *et al.*, 2001; York *et al.*, 2003; Schwartz *et al.*, 2003; Pötter *et al.*, 2004) and two additional depolymerases (PhaZ6 and PhaZ7) sharing a LpqC domain (Abe *et al.*, 2005) have been identified. In addition, two hydroxybutyrate oligomer hydrolases (*phaY1* and *phaY2*) have been detected, further degrading the cleavage products formed by the PHB depolymerases (Bork, 1993; Kobayashi *et al.*, 2005; Saegusa *et al.*, 2002). In contrast with extracellular degradation of PHAs (Jendrossek *et al.*, 1996; Jendrossek, 2002; Jendrossek & Handrick, 2002), relatively little is known about the intracellular mobilization of the storage polymer.

Surprisingly, a relationship has recently been found between PHB metabolism and flagellation of *R. eutropha* H16, depending on nutrient supply. Strong flagellation appears under nutrient limitation that is permissive for PHB accumulation, but flagella are lost in the stationary growth phase under conditions permissive for PHB mobilization, and no flagella assembly was observed during cultivation in rich complex media. In the PHB-negative mutant *R. eutropha* PHB⁻4 flagella assembly did not occur at all (Raberg *et al.*, 2008).

The genome of *R. eutropha* H16 is comprised of three replicons: chromosome 1 (4.1 Mbp), chromosome 2 (2.9 Mbp) and the megaplasmid pHG1 (0.45 Mbp) (Schwartz & Friedrich, 2001) adding up to a total genome size of about 7.5 Mbp with 6626 coding sequences. The sequence of the megaplasmid was published first (Schwartz *et al.*, 2003), whereas the whole genome of strain H16 was sequenced and annotated later (Pohlmann *et al.*, 2006). Sequence analysis revealed that all essential genes for metabolism and essential cell functions are located on chromosome 1, whereas genes for an extended substrate range and for the use of different terminal electron acceptors are located on chromosome 2 and the megaplasmid (Pohlmann *et al.*, 2006; Reinecke & Steinbüchel, 2009).

The availability of the entire genome sequence and its annotation enabled the application of DNA microarrays for genome-wide transcriptome analyses. It affirms the role of *R. eutropha* as a model organism for PHB metabolism and for hydrogen-based chemolithoautotrophy. The aim of the present study was to further extend the knowledge of PHB metabolism in *R. eutropha* H16 and the behaviour of this strain during PHB biosynthesis by using microarrays. For this purpose, transcription analyses were performed,

comparing different growth phases of the PHB-producing wild-type *R. eutropha* H16, the non-directional chemically generated PHB-negative mutant *R. eutropha* PHB⁻4 and a PHB-negative *phaC1* deletion mutant. In addition, the transcriptome data were used to verify recent proteome data (Raberg *et al.*, 2008). In line with the genome analysis of strain H16, a second gene for a putative PHB synthase was identified. The gene *phaC2* is obviously not the result of a gene duplication of *phaC1* (Pohlmann *et al.*, 2006). In a closely related strain, *R. eutropha* JMP222, a second PHA synthase gene was also identified (sequence available online <http://www.jgi.doe.gov>) that is neither a homologue of the *phaC1* family of synthase genes nor similar to *phaC2* from strain H16. To unravel whether or not PhaC2 is active in the cell and to unravel the function of this putative PHA synthase, several functional analyses were performed.

METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Cells of *R. eutropha* were cultivated at 30 °C in 2 l Erlenmeyer flasks with baffles containing 400 ml mineral salts medium (Schlegel *et al.*, 1961) supplemented with 1% (w/v) sodium gluconate as sole carbon source. The concentration of NH₄Cl was reduced to 0.05% (w/v) to provide conditions permissive for PHB accumulation. Cell growth was measured photometrically in a Klett–Summerson photometer (Manostat) using filter no. 54 (520–580 nm).

RNA isolation and cDNA synthesis. Cells were harvested after 4 (exponential growth phase), 7 (transition phase) and 12 (stationary growth phase) h by centrifugation (15 min, 6000 g, 4 °C). The harvested cells were directly shock-frozen in liquid nitrogen and stored at –70 °C. For isolation of total RNA, the cell pellet was resuspended in 350 µl RLT buffer from an RNeasy mini kit (Qiagen) containing 1% (v/v) β-mercaptoethanol and 50 mg 0.1 mm-diameter zirconia–silica beads (Roth). The cells were mechanically disrupted by 4 × 15 s agitation in a Silamat S5 bead mill (Vivadent). RNA isolation was performed by using the RNeasy mini kit (Qiagen) according to the manufacturer's protocol. RNA molecules were eluted from RNeasy mini spin columns with 60 µl RNase-free water, and were then incubated with DNase I (Roche) according to the manufacturer's protocol. The purity of isolated RNA and the absence of DNA was demonstrated by the lack of product formation in a standard PCR using the primers *ptsH_fw* and *ptsH_rv* (Table 1). Subsequently, phenol extraction and ethanol precipitation were performed. The quantity of the isolated RNA was determined using a NanoDrop spectrophotometer (NanoDrop Technologies), and its quality was investigated by denaturing formaldehyde agarose gel electrophoresis.

Total RNA (25 µg) was used for random-hexamer-primed synthesis of fluorescence-labelled cDNA by reverse transcription using the CyScribe first-strand cDNA labelling kit and the fluorescent nucleotide analogues FluoroLink Cy3-dUTP (green) and Cy5-dUTP (red) (GE Healthcare). Subsequently, the labelled cDNA was purified with the CyScribe GFX purification kit (GE Healthcare), and the amount of incorporated fluorescent nucleotides was determined by measuring the absorption of the labelled cDNA at 550 nm for Cy3 and at 650 nm for Cy5 using the NanoDrop spectrophotometer (NanoDrop Technologies).

Generation of *R. eutropha* oligonucleotide microarrays. Full genomic *R. eutropha* H16 oligonucleotide microarrays were constructed by spotting a 15 µM solution of 5' amino-C6-modified

oligonucleotides in 50 mM sodium phosphate (pH 8.5) with a length of 60–70 bases on CodeLink microarray slides (SurModics) using a MicroGrid II microarray spotter (Zinsser Analytic). Each spot contains around 1.5 fmol oligonucleotide. Oligonucleotides were covalently coupled to the slide surface and blocked using ethanol-amine according to the supplier's instructions. The array consisted of 6676 different oligonucleotides and all annotated ORFs of *R. eutropha* were spotted in duplicate. All oligonucleotides were designed to have a predicted melting point of 81 ± 5 °C and are devoid of internal hairpin structures in order to ensure uniform hybridization. The specificity of each oligonucleotide for its target gene was checked by BLAST analysis against all other ORFs of the organism.

Microarray hybridization and scanning. Before hybridization, samples were denatured by incubation at 98 °C for 5 min. The hybridization was done in Tom Freeman hybridization buffer (Fitzpatrick *et al.*, 2005) for 15 h at 58 °C with cDNA containing approximately 80 pmol Cy-3 and Cy-5 in an automatic Lucidea slide processor (GE Healthcare). Slides were washed using a program of consecutive washes twice with 1 × SSC buffer containing 0.2% SDS and then with 0.1 × SSC. At the end, hybridization chambers were flushed with 2-propanol and this was evaporated with air to dry the slides. Scanning was done using a GenePix 4000B microarray scanner (Molecular Devices) using the GenePix Pro 6.0 software. To achieve reliable data, each hybridization was repeated on a second slide using the reverse combination of dye labels (Ehrenreich, 2006).

Data normalization and filtering. Data were normalized by multiplication of a constant factor so that the mean of the ratio of medians of all features became equal to 1. Arrays that required a normalization factor larger than 2 were excluded from further analysis to eliminate experiments with major deviations in the applied dye incorporated in the labelled cDNA. After determining the fluorescence intensities of both channels, a background correction was made by subtracting the local background value from the foreground intensity (Benes & Muckenthaler, 2003). Data with an intensity value smaller than the background in both channels were excluded from further analyses. Features with an intensity value smaller than the background in only one channel and an intensity value ≥ 300 in the other channel were not excluded but 'floor' values were assigned which were equivalent to the local background (Ehrenreich, 2006). To exclude irregular spots, dye precipitates or misplaced feature indicators, the ratio of medians, the ratio of means and the regression ratios were calculated for each feature. When these ratios deviated more than 30% from each other, the corresponding feature was excluded from further analyses. A threefold cut-off value was set to identify genes with significant regulation. Finally, the normalized ratio of the median was log-transformed to base two and was taken to reflect the relative RNA abundance. The original raw data files have been deposited in the ArrayExpress database under accession number E-MEXP-2440. Data were generated from two independent biological experiments.

In silico characterization of novel PHA synthases. The BLAST algorithm (Altschul *et al.*, 1997) was applied to identify novel PHA synthases in publicly available genome sequences using various protein sequences of known PHA synthases as a query. Similarities of protein sequences of well-characterized PHA synthases and recently discovered additional synthases were calculated from a multiple alignment performed applying the CLUSTAL W software package (Thompson *et al.*, 1994) with a Blosom45 matrix (Henikoff & Henikoff, 1993). The existence of conserved residues known to contribute to catalysis (reviewed by Rehm, 2003) was checked manually.

Construction of the mutant *R. eutropha* H16Δ*phaC1*. Upstream and downstream regions of *phaC1* encoding the PHA synthase were

Table 1. Bacterial strains, plasmids and oligonucleotides used in this study

Strain, plasmid or primer	Description	Reference, source or location
<i>R. eutropha</i> H16	Wild-type, PHB accumulating	DSM 428; Walde (1962)
<i>R. eutropha</i> PHB ⁻ 4	Chemically induced mutant of H16; PHB-negative	DSM 541; Schlegel <i>et al.</i> (1970a)
<i>R. eutropha</i> Δ <i>phaC1</i>	Precise deletion mutant of H16; PHB-negative	Reinecke & Steinbüchel (2009)
<i>R. eutropha</i> JMP222	Wild-type, pJp4-free derivative of strain JMP134 (DSM 4058); PHB-positive	Don & Pemberton (1981)
<i>Burkholderia xenovorans</i> LB400	Wild-type	NRRL-B 18064
<i>Escherichia coli</i> TOP10	F ⁻ , <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> , <i>araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU galK rpsL</i> (Str ^R) <i>endA1 nupG</i>	Invitrogen
<i>E. coli</i> S17-1	<i>recA</i> ; harbouring the <i>tra</i> -genes of plasmid RP4 on the chromosome, <i>proA thi-1</i>	Simon <i>et al.</i> (1983)
<i>Pseudomonas putida</i> GPp104	PHA-negative derivative of <i>P. putida</i> KT2440 (DSM 6125)	Huisman <i>et al.</i> (1991)
Plasmids		
pCR2.1-TOPO	Ap ^R Km ^R	Invitrogen
pJO2	Km ^R , <i>mob</i>	Overhage <i>et al.</i> (2002)
pBHR68	Ap ^R , <i>phaCAB</i>	Spiekermann <i>et al.</i> (1999)
pBHR69	Ap ^R , <i>phaAB</i>	Spiekermann <i>et al.</i> (1999)
pJQ200mp18Tc	Tc ^R , <i>sacB mob</i>	Pötter <i>et al.</i> (2005)
pBHR69:: <i>phaC2</i> _{H16}	Ap ^R , <i>phaAB phaC2</i> _{H16}	This study
pBHR69:: <i>phaC2</i> _{JMP134}	Ap ^R , <i>phaAB phaC2</i> _{JMP134}	This study
pJO2:: <i>phaC2</i> _{H16}	Km ^R , <i>phaC2</i> _{H16}	This study
pJO2:: <i>phaC2</i> _{JMP134}	Km ^R , <i>phaC2</i> _{JMP134}	This study
pJO2:: <i>phaC3</i> _{LB400}	Km ^R , <i>phaC3</i> _{LB400}	This study
pJQ Δ <i>phaC1</i>	Tc ^R , <i>sacB mob</i> , regions flanking <i>phaC1</i> _{H16}	This study
Oligonucleotides		
<i>ptsH</i> _{fw}	ATGCTGCAGAGGGACACCACC	Upstream <i>ptsH</i>
<i>ptsH</i> _{rv}	TCACTCTCCCTCACCAAAGCGG	Downstream <i>ptsH</i>
C1US-5'	AAAAGAATTCTGCGCGCGCTCGGCTTCAGCCTTGC	<i>EcoRI</i> , 5' upstream <i>phaC1</i> _{H16}
C1US-3'	AAAAGGATCCGATTGTGATTGTCTCTCTGCCGTCAC	<i>BamHI</i> , 3' upstream <i>phaC1</i> _{H16}
C1DS-5'	AAAAGGATCCCGCTTGCATGAGTGCCGGCGTGCG	<i>EcoRI</i> , 5' downstream <i>phaC1</i> _{H16}
C1DS-3'	AAAAGAATTCTTATTTGCGCTCGACTGCCAGCGC	<i>BamHI</i> , 3' downstream <i>phaC1</i> _{H16}
C1Gen-5'	CGGTCGCTTCTACTCCTATCGG	Binds upstream of C1US-5'
C1Gen-3'	ACCACACGAAAGCCATCCTTGG	Binds downstream of C1DS-3'
C2H16-5'	CGCGAATTCTGCCATCAACGGAGGGCAAC	<i>EcoRI</i> , 5' <i>phaC2</i> _{H16}
C2H16-3'	TTGCGAATTTCGAAGGTTGCCAGGCG	<i>EcoRI</i> , 3' <i>phaC2</i> _{H16}
C2JMP-5'	GGATCGAATTTCGGTACGAACAAGAAGACGAGGGTAGTAGC	<i>EcoRI</i> , 5' <i>phaC2</i> _{JMP134}
C2JMP-3'	TTACTAAGCTTACAGATTACAGTTGGCGTTACAGAAATCG	<i>HindIII</i> , 3' <i>phaC2</i> _{JMP134}
C3LB-5'	AAAAGAATTCTTGGTCCAGAGCCACGAAGACTTTC	<i>EcoRI</i> , 5' <i>phaC3</i> _{LB400}
C3LB-3'	GGGAAGCTTATACTGAAGCACATAGGTGCCG	<i>HindIII</i> , 3' <i>phaC3</i> _{LB400}
RT- <i>phaC2</i> _{fw}	ATGACGGGACCGAACCCGTTCTGTGG	Upstream <i>phaC2</i> _{JMP222}
RT- <i>phaC2</i> _{rv}	ACTCGAACGTCTTCCGCAATGCAGC	Downstream <i>phaC2</i> _{JMP222}
RT- <i>phaC1</i> _{fw}	ATGGCGACCGGCAAAGGTGCGGCAG	Upstream <i>phaC1</i> _{JMP222}
RT- <i>phaC1</i> _{rv}	GCCAGGAAATTGGCCGCGCACATCG	Downstream <i>phaC1</i> _{JMP222}

amplified using *Taq* polymerase and primer pairs C1US-5'/C1US-3' and C1DS-5'/C1DS-3' (Table 1). Products were digested with *Bam*HI, ligated and directly cloned into pCR2.1-TOPO (Invitrogen). The fragment containing both flanks was prepared by digestion with *Sac*I and *Xho*I and cloned into the vector pJQ200mp18Tc (Pötter *et al.*, 2005) which was digested with the same enzymes. The resulting plasmid pJQ Δ *phaC1* was transferred to *R. eutropha* H16 by conjugation using *Escherichia coli* S17-1 as donor. Transconjugants were first selected on mineral medium plates containing 25 μ g tetracycline ml⁻¹ and 1.5 % (w/v) sodium gluconate as a carbon source. Single colonies were spread several times on the same plates to ensure that no *E. coli* contaminants remained. Heterogenotes were propagated in low salt Luria-Bertani (10 g tryptone l⁻¹, 5 g yeast extract l⁻¹, 2.5 g NaCl l⁻¹) medium

supplemented with 0.5 % (w/v) sodium gluconate without antibiotics to enable the second crossover event. Dilutions of overnight cultures were spread on 0.8 % (w/v) nutrient broth plates containing 10 % (w/v) sucrose to select for strains lacking the *sacB* gene. Colonies showing a reduced opacity were selected and strains that lost the ability to grow on tetracycline-containing plates were checked by PCR using primers C1Gen-5' and C1Gen-3', which hybridize outside the flanks used for recombination and subsequent sequencing of the amplified region.

Cloning *phaC* genes and transfer of DNA. Genes encoding putative PHB synthases were amplified by PCR using primer pairs as listed in Table 1. Locus tags of the amplified genes and accession numbers for the translated products are as follows: *phaC2*_{H16}

(H16_A2003), PhaC2 (CAJ93103.1); *phaC2*_{JMP134} (Reut_A2138), PhaC2_{JMP134} (AAZ61502.1); *phaC3*_{LB400} (Bxe_C0053), PhaC3 (YP_555330.1). Fragments and the target vector were digested using the restriction enzymes given in Table 1. Transformation of *E. coli* cells was performed by using chemically competent cells (Hanahan, 1983). Clones were verified by restriction mapping and sequencing of inserted DNA fragments. Plasmid transfer to *Pseudomonas putida* and *R. eutropha* was achieved by conjugation with *E. coli* S17-1 as plasmid donor. The recipient strain was plated on mineral salts medium supplemented with 1.5% (w/v) filter-sterilized sodium gluconate (Schlegel *et al.*, 1961) and kanamycin (50 µg ml⁻¹ for *P. putida*, 250 µg ml⁻¹ for *R. eutropha*) before the donor strain was streaked across with sterile toothpicks. Transconjugants were propagated several times on mineral salts medium plates to ensure purity of the strain.

Expression of genes homologous to *phaC*. For expression of proteins homologous to PhaC in strains of *E. coli*, derivatives of vector pBHR69 were used. The medium used for *E. coli* strains was Luria–Bertani (10 g tryptone l⁻¹, 5 g yeast extract l⁻¹, 10 g NaCl l⁻¹) supplemented with 1 mM IPTG and 1% (w/v) glucose. Cells were cultivated aerobically in flasks with baffles at 37 °C. For expression in *Ralstonia* and *Pseudomonas* strains, derivatives of the vector pJ02 were used. *R. eutropha* and *P. putida* were grown at 30 °C in mineral salts medium supplemented with 1.5% (w/v) filter-sterilized sodium gluconate in baffled flasks. To promote accumulation of polymer by nitrogen limitation, the concentration of ammonium chloride in the medium was reduced to 0.02% (w/v).

RT-PCR. Total RNA was isolated as described above. One-step RT-PCR was applied, using the oligonucleotides listed in Table 1 and the OneStep RT-PCR kit (Qiagen) according to the manufacturer's protocol. To exclude any contaminating DNA that might have served as a template for PCR, template RNA was added in a control experiment, after inactivating the reverse transcriptase and activating the *Taq* polymerase with an initial heating step at 95 °C for 15 min. Absence of PCR products in the control indicated that the RT-PCR products were not derived from contaminating DNA.

Analysis of the PHA contents of whole cells. Polymer contents of cells were analysed by gas chromatography. Cells were harvested by 15 min centrifugation at 10 000 g and were then washed twice in saline solution (0.9%, w/v, NaCl). Pellets were lyophilized overnight. About 10 mg dry cells was subjected to methanolysis in presence of 15% (w/v) sulfuric acid. The resulting methyl esters of 3-hydroxyalkanoates were quantified by a Hewlett Packard gas chromatography system (Brandl *et al.*, 1988; modified by Schubert *et al.*, 1991). Polymer content is given as a percentage of the total cellular dry mass of the cells analysed.

Isolation of PHA from lyophilized cells. PHA was extracted from lyophilized cells using chloroform extraction in a Soxhlet apparatus. Excess chloroform was removed using a rotary evaporator. Polymer was precipitated by dripping the remaining highly viscous chloroform solution onto ice-cold ethanol. The precipitate was harvested by centrifugation, dissolved in chloroform and precipitated with ethanol to obtain highly purified polymer.

Gel permeation chromatography (GPC). Molecular mass analysis of extracted polymer was conducted with purified PHA dissolved in chloroform at a concentration of about 10 mg polymer ml⁻¹. Separation was performed in a Waters GPC system equipped with Styragel Guard and a Styragel HR3-6 separation column. Eluted polymer was detected by differential refraction (model 410 detector, Waters). A polystyrene molecular mass standard (Sigma) was used for calibration.

PHB synthase assay. Cells were disrupted by a threefold passage through a French pressure cell at 1000 MPa. Protein concentrations were determined applying a bicinchoninic acid assay (Sigma). The *in vitro* activity of PHA synthase in crude extracts was assayed by the photometric method applying 5,5'-dithiobios-(2-nitrobenzoic acid) (DTNB, Acros, ϵ_{412} 13 600 M⁻¹·cm⁻¹) (Valentin & Steinbüchel, 1993) and using a racemic mixture of 3-hydroxybutyryl-CoA (Sigma) as substrate.

RESULTS AND DISCUSSION

Genome-wide transcription analyses with a focus on PHB biosynthesis

To investigate global regulatory mechanisms of PHB metabolism in *R. eutropha* H16, a genome-wide transcription analysis was performed. A full-genome microarray with immobilized synthetic oligonucleotides representing the whole genome of *R. eutropha* H16 was used to identify genes involved in or affected by PHB metabolism. For this purpose, the transcripts from different growth phases of strain H16 and of two PHB-negative mutants were compared. The latter included the chemically induced mutant *R. eutropha* PHB⁻4 (Schlegel *et al.*, 1970a), which has not been characterized at a molecular level, and a precise *phaC1* deletion mutant, which was generated in this study. Mutant *R. eutropha* H16Δ*phaC1* did not accumulate any PHB. Growth of the three strains in mineral salts medium permissive for PHB accumulation is shown in Fig. 1. Four hours after inoculation, cells were still in the exponential growth phase where no limitation of nutrients occurred. After 7 h cultivation, the cells were in transition to the stationary growth phase; PHB synthesis started as a consequence of conditions becoming permissive for PHB accumulation (a surplus of the carbon source and limitation of nitrogen). Cultures of both PHB-negative mutants had yielded optical densities of about 350 Klett units and remained at this value, whereas the optical density of strain H16 increased further. This is due to the accumulation of PHB, which is stored as refractile grana, causing a further increase of the light scattering of the cells and thereby of the optical density of the solution (Schlegel *et al.*, 1970b; Sreenc *et al.*, 1984). After 12 h cultivation, the wild-type had reached an optical density of about 570 Klett units.

Samples for transcriptome analyses were withdrawn during the different growth phases as indicated in Fig. 1; the experimental design of the entire transcription analyses is summarized in Fig. 2. Four transcriptome comparisons were carried out in this study as follows: (i) cells from the exponential growth phase (4 h after inoculation) with cells from the transition phase (7 h), and (ii) cells from the transition phase (7 h) with cells from the stationary growth phase (12 h) of *R. eutropha* H16. By comparing these three stages, the changes in the transcriptome of the wild-type during transition from growth towards PHB accumulation should be unravelled. In addition, we compared (iii) cells from the transition phase of *R. eutropha* H16 with those

Fig. 1. Growth curves of the wild-type *R. eutropha* H16 (▲) and the PHB-negative mutants *R. eutropha* PHB⁻4 (■) and *R. eutropha* H16Δ*phaC1* (●) in mineral salts medium containing 1 % (w/v) sodium gluconate and 0.05 % (w/v) ammonium chloride. Arrows indicate the time points at which samples were withdrawn: (#1) exponential growth phase, 4 h after inoculation; (#2) transition phase, 7 h after inoculation; (#3) stationary phase, 12 h after inoculation.

from the transition phase of *R. eutropha* PHB⁻4 and (iv) cells from the transition phase of *R. eutropha* Δ*phaC1* with those from the transition phase of *R. eutropha* PHB⁻4. These comparisons aimed to unravel differences between the transcriptomes of the wild-type and of the two mutants under conditions permissive for the onset of PHB accumulation. Two independent biological experiments were performed, resulting in mean RNA ratios of eight replicates. Genes with an increase or decrease in expression greater than threefold are considered here. The RNA levels were log-transformed to base 2 and were taken to reflect the relative RNA abundance.

Pathways linking PHA synthesis with central metabolism have been intensively studied, particularly in *R. eutropha*

and *P. putida*, revealing connections with fatty acid β-oxidation, fatty acid *de novo* synthesis and the citric acid cycle (Madison & Huisman, 1999; Steinbüchel, 2001; Taguchi *et al.*, 2002). For example, an acyltransferase encoded by *phaG* serves as a metabolic link between fatty acid *de novo* synthesis and PHA biosynthesis in pseudomonads (Rehm *et al.*, 1998). In *Aeromonas caviae* PhaJ supplies 3-hydroxyacyl-CoA monomer units from fatty acid β-oxidation to poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) biosynthesis (Fukui *et al.*, 1998). However, it is obvious that lipid metabolism is providing metabolites for PHB metabolism, and genes involved in lipid metabolism might play a role in PHB metabolism as well. For this reason, genes within the functional class of lipid metabolism were also of great interest for this study.

Fig. 2. Overview of the experimental design of transcriptomic analyses. Each ellipse represents one sample, and each arrow points from the Cy3-labelled sample to the Cy5-labelled sample. The Roman numerals indicate the four transcriptome comparisons that were carried out in this study: (i) *R. eutropha* H16 cells from the exponential growth phase (#1, 4 h after inoculation) compared with cells from the transition phase (#2, 7 h); (ii) *R. eutropha* H16 cells from the transition phase (#2, 7 h) compared with cells from the stationary growth phase (#3, 12 h); (iii) *R. eutropha* H16 cells from the transition phase compared with those from the transition phase of *R. eutropha* PHB⁻4; (iv) cells of *R. eutropha* PHB⁻4 from the transition phase compared with those from the transition phase of *R. eutropha* Δ*phaC1*.

Global gene expression in *R. eutropha* H16 with a focus on PHB biosynthesis

(i) Exponential phase versus transition phase. In this experiment, only one and three genes were detected with an increased and decreased transcription level, respectively. None of these genes is involved in or related to PHB metabolism (for more details, see Supplementary Table S1 available with the online version of this paper). In the exponential growth phase, a highly increased metabolic activity was expected in comparison with that at the transition phase. The detection of only three genes with high expression in the exponential phase suggests that after 7 h of cultivation, the growth of strain H16 did not come to rest completely and that the metabolism was still active despite the beginning of PHB biosynthesis.

(ii) Transition phase versus stationary growth phase. In total, 184 genes with a decreased transcription level were detected in the stationary growth phase compared with the transition phase. These genes fall into 20 functional classes used by the *R. eutropha* genome project (Pohlmann *et al.*, 2006). The largest group of genes (47) are involved in (i)

translation, ribosomal structure and biogenesis; another large group of genes (16 genes) is currently categorized as (ii) hypothetical, uncharacterized or unknown. Other repressed genes can be classified as (iii) energy conversion (32 genes), (iv) amino acid transport and metabolism (20 genes), (v) cell envelope biogenesis, outer membrane (nine genes), (vi) intracellular trafficking and secretion (seven genes), (vii) nucleotide transport and metabolism, (viii) post-translational modification, protein turnover, chaperones (six genes in each case), (ix) coenzyme metabolism, (x) inorganic ion transport and metabolism, (xi) lipid metabolism, (xii) transcription (five genes in each case), (xiii) cell motility and secretion, (xiv) NAD-reducing [NiFe] hydrogenase (four genes in each case), (xv) carbohydrate transport and metabolism, (xvi) DNA replication, recombination and repair, (xvii) [NiFe] hydrogenase maturation (three genes in each case), (xviii) signal transduction mechanism (two genes), (xix) cell division and chromosome partitioning, and (xx) secondary metabolites biosynthesis, transport and catabolism (one gene in each case) (for more details, see Supplementary Table S2 available with the online version of this paper).

In the stationary growth phase, five genes with a decreased transcription level were detected within the functional class of lipid metabolism. *accC2* (H16_A3172, twofold) encodes a biotin carboxylase which represents a component of the acetyl-CoA carboxylase multi-component enzyme, catalysing the first step in fatty acid synthesis from acetyl-CoA to malonyl-CoA, as shown in Fig. 3. Fatty acid synthesis is then continued by acyl-carrier protein (ACP)-dependent enzymes. The 3-oxoacyl-[ACP] reductase *FabG* (H16_A2567) and the 3-hydroxymyristoyl/3-hydroxydecanoyl-[ACP] dehydratase *FabZ* (H16_A2044) were found to be significantly repressed in the stationary growth phase (each approximately twofold). These results are of course partially due to the increased metabolic activity in the transition phase, but additionally, it is a very interesting finding as *fabG* represents one of the 39 *phaB* homologues (Reinecke & Steinbüchel, 2009) but is known to provide no precursors for PHB biosynthesis. This is due to a lack of a *PhaG* homologue in *R. eutropha*, which would act as a link between fatty acid *de novo* synthesis and PHA synthesis, as, for example, in *P. putida* (Rehm *et al.*, 1998). Additionally, *accC2* and *fabG* were also detected in the third substudy, comparing the transition phases of strain H16 and the mutant strain PHB⁻4, as discussed later. So there are reasons for the assumption that *AccC2* and *FabG* are somehow involved in PHB biosynthesis. Further studies are required to clarify the functions of these enzymes in *R. eutropha*.

In addition, a putative enoyl-CoA hydratase (H16_A3307) exhibited an approximately twofold decreased transcription level in the stationary growth phase of strain H16. This form of enzyme catalyses the second step of β -oxidation in fatty acid metabolism, hydrating the double bond between the second and the third carbon atoms of acyl-CoA. In PHB metabolism, H16_A3307 is putatively involved in the mobilization of PHB as shown in Fig. 3.

In this study, *phaP1* (H16_A1381) was detected in the stationary phase of strain H16 with an approximately threefold increased transcription level. Furthermore, *phaP3* (H16_A2172) was found to be repressed threefold. The expression of *PhaP3* under conditions permissive for PHB accumulation and the binding to the surface of PHB granules has been reported previously (Pötter *et al.*, 2004). *PhaP3* represents a homologue to the major and best-studied phasin *PhaP1*, but occurs normally only in minor amounts in the cells under conditions permissive for PHB accumulation. Hence, it was assumed that *PhaP3* probably serves a different function in the cell to *PhaP1* (Reinecke & Steinbüchel, 2009). Hitherto, a significantly increased amount of *PhaP3* has only been shown in *phaP1* deletion mutants. Such mutants accumulate less PHB than the wild-type and possess only one single large granule per cell (Pötter *et al.*, 2004). Until now, one can only speculate on the functions of the multiplephasins in *R. eutropha* H16. For example, *phaP3* may be transcribed in the very early stages of PHB biosynthesis in the transition phase when only low amounts of polymer are accumulated, whereas *PhaP1* may become predominant in the stationary growth phase, mediating correct granule formation in the cell.

An influence of PHB metabolism on flagellation of *R. eutropha* H16 has been reported previously (Raberg *et al.*, 2008). Flagellation appeared in the exponential growth phase, stagnated under conditions permissive for PHB biosynthesis and disappeared under conditions permissive for PHB mobilization. These findings were confirmed by the transcriptome analyses in this study. *fliC* (H16_B2360) encoding flagellin, the main structural component of bacterial flagella (Macnab, 2003) and *flgE* (H16_B0264), which encodes the flagellar hook protein, were found to be significantly repressed in the stationary growth phase (approximately four- and twofold, respectively). These results are in agreement with the observation of stopped flagellin synthesis in stationary growth phase, when cells are fully packed with PHB granules and when nitrogen is limiting (Raberg *et al.*, 2008).

Beside genes involved in or related to PHB metabolism, genes of the citric acid cycle exhibited a repressed transcription level as well, including *sucC* (H16_A0547, threefold), *acnA*, *mdh1* and *fumA* (H16_A2638, H16_A2634, H16_A2528, each twofold) as shown in Fig. 3. Furthermore, genes encoding proteins that are involved in primary metabolism, such as amino acid biosynthesis, nucleotide or coenzyme metabolism were repressed in the stationary growth phase (see Supplementary Table S2). Here, the strongest repression was found for the heat-shock protein GroEL (H16_A0706, fivefold) and the cochaperone GroES (H16_A0705, fourfold). These findings reflect the still increased metabolic activity in the transition phase and the corresponding higher demand for chaperones for correct folding of nascent proteins (Lund, 2001), which is due to the accumulation of PHB.

In contrast, 113 genes were found to be induced in the stationary growth phase. They fall into 13 functional classes

Fig. 3. Overview of the regulation of genes encoding enzymes of PHB biosynthesis, Entner–Doudoroff pathway, citric acid cycle, pentose phosphate pathway, Calvin–Benson–Bassham (CBB) cycle and fatty acid metabolism in *R. eutropha*. Results of the transcriptome analyses are given in log₂ transformed ratios next to each gene. White rectangles, comparison of the transition phase and stationary growth phase of *R. eutropha* H16; light grey rectangles, comparison of *R. eutropha* H16 and strain PHB⁻4 in transition phase; dark grey rectangles, comparison of *R. eutropha* Δ*phaC1* and strain PHB⁻4 in transition phase. The complete lists of all studied genes are given in Supplementary Tables S2, S3 and S4.

with the majority (68) of the genes categorized as (i) hypothetical, uncharacterized or unknown, followed by (ii) inorganic ion transport and metabolism (10 genes), (iii) transcription (nine genes), (iv) signal transduction mechanism (eight genes), (v) amino acid transport and metabolism (four genes), (vi) energy conversion (four genes), (vii) lipid metabolism (three genes), (viii) DNA replication, recombination and repair (two genes), (ix) coenzyme metabolism, (x) intracellular trafficking and secretion, (xi) nucleotide transport and metabolism, (xii) post-translational modification, protein turnover, chaperones, and (xiii) secondary metabolites biosynthesis, transport and catabolism (one gene in each) (see Supplementary Table S2).

In cells from the stationary growth phase, striking changes were detected in three genes known to be involved in PHB metabolism: *phaP1* (H16_A1381), encoding the major and best-studied phasin, exhibited a threefold increased transcription level, as already discussed above. Moreover, the PHB depolymerases PhaZ2 and PhaZ6 (H16_A2862, H16_B2073) were found to be induced by a factor of about three, as shown in Fig. 3. Recent genome analysis of strain H16 revealed nine PHB depolymerase isoenzymes (Pohlmann *et al.*, 2006), but up to now, little was known about the intracellular degradation of PHB. Studies of *phaZ* deletion mutants revealed that PhaZ2 contributes to intracellular degradation of PHB during carbon starvation (York *et al.*, 2003), and Uchino *et al.* (2008) detected PHB depolymerase activity only for PhaZ1. However, the results lead to the assumption that in the stationary growth phase, the mobilization of stored PHB granules occurred, although no nitrogen source was added to promote growth. It is possible that the degradation of small amounts of PHB contributes to maintaining the basal metabolism of the cell. This creates a picture of the PHB granules as a complex and dynamic organelle with biosynthesis and degradation in the stationary growth phase. PhaZ6 contribution to PHB degradation has not been described yet.

Furthermore, an enoyl-CoA hydratase encoded by H16_A3311 exhibited a threefold increased transcription level. This enzyme is involved in PHB mobilization, as discussed above (Fig. 3).

In addition, the detection of seven transcriptional regulators that exhibited increased transcription levels [H16_A2259, H16_B2080 (each threefold increased), H16_A1157, H16_A3607, H16_B0114, H16_B0858 and H16_B1594 (each twofold increased)] was a striking finding. An interpretation of the changes of the transcriptional regulators is not possible currently and requires further investigations.

(iii) Comparison of global gene expression in *R. eutropha* H16 and PHB-negative mutant PHB⁻4 in the transition phase. A large number of genes (194) were induced in *R. eutropha* H16 compared with mutant

PHB⁻4 when the cells from the exponential phase entered the transition phase. The major group of genes (52) is involved in translation, ribosomal structure and biogenesis. A further 36 genes encode proteins for energy conversion. The remaining genes are spread among 17 further functional classes: (i) amino acid transport and metabolism (17 genes), (ii) hypothetical, uncharacterized or unknown (17 genes), (iii) post-translational modification, protein turnover and chaperones (11 genes), (iv) cell envelope biogenesis and outer membrane (10 genes), (v) lipid metabolism (eight genes), (vi) transcription (seven genes), (vii) intracellular trafficking and secretion (six genes), (viii) carbohydrate transport and metabolism (five genes), (ix) cell motility and secretion (four genes), (x) coenzyme metabolism (four genes), (xi) inorganic ion transport and metabolism (four genes), (xii) DNA replication, recombination and repair (three genes), (xiii) [NiFe] hydrogenase maturation (three genes), (xiv) subunit of NAD-reducing [NiFe] hydrogenase (three genes), (xv) signal transduction mechanism (two genes), (xvi) nucleotide transport and metabolism (one gene) and (xvii) secondary metabolites biosynthesis, transport and catabolism (one gene) (see Supplementary Table S3 available with the online version of this paper).

In comparison with mutant PHB⁻4, nine genes involved in lipid metabolism were found to be induced in the wild-type. Two of these, *phaA* (H16_A1438; induced twofold) and *phaB1* (H16_A1439; induced twofold), are known to encode key enzymes of PHB metabolism, mediating the first two steps of PHB biosynthesis from acetyl-CoA to *R*-3-hydroxybutyryl-CoA (Schubert *et al.*, 1988; Oeding & Schlegel, 1973) as shown in Fig. 3. These data indicate that either the mutation in strain PHB⁻4 comprises the *phaCAB* operon completely or the mutation in *phaC1* exerts a polar effect on the downstream genes *phaA* and *phaB*. Furthermore, *paaH1* (H16_A0282), catalysing the step from acetoacetyl-CoA to the *S*-stereoisomer of 3-hydroxybutyryl-CoA, and the putative enoyl-CoA hydratase (H16_A3307) which putatively further dehydrates (*S*)-3-hydroxybutyryl-CoA to crotonyl-CoA, were both induced approximately twofold.

The biotin carboxylase *accC2* (H16_A3172), catalysing the first step in fatty acid synthesis and the 3-oxoacyl-[ACP] reductase *fabG* (H16_A2567), also involved in fatty acid biosynthesis, were induced approximately threefold and twofold, respectively, in the wild-type as shown in Fig. 3. As discussed above, in substudy (ii), *accC2* and *fabG* were found to be repressed in the stationary growth phase of strain H16 compared with the transition phase; moreover, *fabG* is one of the *phaB* homologues. These findings led to the assumption that the mutation in the genome of the PHB-negative strain PHB⁻4 either comprises, besides the PHB biosynthesis genes, parts of the fatty acid metabolism genes or that the translational products of *accC2* and/or *fabG* serve as a metabolic link between fatty acid *de novo* synthesis and PHB biosynthesis.

In addition, *phaP3* (H16_A2172) exhibited a threefold elevated transcription level in strain H16. As already discussed above, *phaP3* was found to be repressed in the stationary growth phase compared with the transition phase. These results support the assumption that *phaP3* may be transcribed in early stages of PHB biosynthesis.

In comparison with strain PHB⁻4, four transcripts for flagella assembly were detected in the wild-type (see Supplementary Table S3). A threefold increased transcription level was found for *fliC* (H16_B2360), followed by *flgC* and *flgE* (H16_B0262; H16_B0264; each twofold), which encode the flagellar basal body rod protein and the hook protein, respectively. Moreover, *flhC1* (H16_B0236) encoding the flagellar transcriptional activator was twofold upregulated. These results confirm recent proteomic analyses in our laboratory, demonstrating not only an influence of PHB metabolism on flagellation of *R. eutropha* H16 (see above) but also an absence of flagellation in the mutant PHB⁻4 (Raberg *et al.*, 2008).

Beside the described alterations in the expression levels of genes of PHB metabolism, detecting changes in 11 genes involved in CO₂ fixation via the Calvin–Benson–Bassham (CBB) cycle was a surprising finding. The genes for this pathway are located in duplicate in two *cbb* operons on the megaplasmid pHG1 and on chromosome 2 (Bowien & Kusian, 2002). *cbbG2* (H16_B1386), *cbbP2*, *cbbF2* (H16_B1389–1390) and *cbbS2* (H16_B1394) and the corresponding duplicates on the megaplasmid (PHG418, PHG421–422, PHG426), as well as *cbbY2*, *cbbX2* (H16_B1392–1393), *cbbAp*, *cbbKp* (PHG416–417) and *cbbEp* (PHG423), showed changes ranging from twofold to fourfold, as shown in Fig. 3. Enzymes of autotrophic metabolism were also found in *R. eutropha* H16 cells grown heterotrophically on gluconate. Here, activities were up to 20 % higher than in autotrophic cells (Friedrich *et al.*, 1981; Jeffke *et al.*, 1999). In the present study, a significant downregulation of the *cbb* operons in the mutant strain PHB⁻4 was detected.

In strain PHB⁻4, 117 genes exhibited increased transcription levels. Genes belonging to the following functional classes were identified: (i) hypothetical, uncharacterized or unknown (56 genes), (ii) amino acid transport and metabolism (12 genes), (iii) inorganic ion transport and metabolism (10 genes), (iv) lipid metabolism (seven genes), (v) energy conversion (five genes), (vi) signal transduction mechanism (five genes), (vii) nucleotide transport and metabolism (four genes), (viii) post-translational modification, protein turnover and chaperones (four genes), (ix) transcription (three genes), (x) carbohydrate transport and metabolism (three genes), (xi) cell envelope biogenesis, outer membrane (two genes), (xii) coenzyme metabolism (two genes), (xiii) intracellular trafficking and secretion (two genes), (xiv) DNA replication, recombination and repair (one gene) and (xv) secondary metabolites biosynthesis, transport and catabolism (one gene) (see Supplementary Table S3).

A set of genes that contributes to PHB metabolism exhibited an increased transcription level in strain PHB⁻4 compared with the wild-type. This was true for the phasin-encoding *phaP1* and *phaP4* (H16_A1381, H16_B2021, each increased approximately twofold) and two PHB depolymerases encoded by *phaZ2* and *phaZ6* (H16_A2862, increased fourfold; H16_B2073, increased twofold) as shown in Fig. 3. As mentioned above, *phaP3* exhibited an elevated transcription level in strain H16 when compared with PHB⁻4 and was found to be repressed in the stationary growth phase of the wild-type compared with the transition phase. These data are a striking finding. The expression of PhaP1, PhaP3 and PhaP4 under conditions permissive for PHB accumulation and their binding to PHB granules has been shown for the wild-type strain H16 (Pötter *et al.*, 2004). Due to their similarity, PhaP1 and PhaP3 are homologues, but may represent paralogues to PhaP2 and PhaP4. Rather small amounts of PhaP3 and PhaP4 occurred in cells of the wild-type, hence, it was assumed that they probably serve a different function from PhaP1 in the cells (Reinecke & Steinbüchel, 2009). The increased transcription level for *phaP4* (although no PHB biosynthesis occurred in strain PHB⁻4) may support this assumption. The upregulation of *phaP1*, which is the predominant phasin under storage conditions in strain H16, is possibly also caused by a mutation in the genome of strain PHB⁻4. However, *R. eutropha* PHB⁻4 is a chemically induced mutant which has not been characterized at a molecular level. Hence, one can only speculate on the cause that mediates the transcriptome data obtained. Anyway, it has become evident that the situation regarding the phasins is much more complex and that an interpretation of the functions of the multiple phasins is not possible so far.

The increased transcription level for the two PHB depolymerases encoded by *phaZ2* and *phaZ6* was surprising, as no PHB biosynthesis occurred in strain PHB⁻4. Furthermore, both depolymerases were detected in the wild-type with increased transcription levels in the stationary growth phase, as discussed in substudy (ii). These results led to the assumption either that the mutation in strain PHB⁻4 affects the depolymerases *phaZ2* and *phaZ6* despite their location on different chromosomes or that the corresponding enzymes serve a different function in the cells than the depolymerization of stored PHB.

(iv) Comparison of global gene expression in *R. eutropha* Δ *phaC1* and *R. eutropha* PHB⁻4 in the transition phase. A precise deletion mutant of *R. eutropha* H16 lacking *phaC1* only was constructed as described in Methods, and the deletion was confirmed by PCR and subsequent sequencing of the amplified region (data not shown). Neither mutant *R. eutropha* H16 Δ *phaC1* nor PHB⁻4 accumulated any PHB. Compared with the chemically induced mutant PHB⁻4, 14 genes with significantly increased transcription levels were detected

in the *phaC1* deletion mutant in the transition phase (see Supplementary Table S4, available with the online version of this paper). The genes *phaA* and *phaB1*, known to encode key enzymes in PHB biosynthesis, were highly induced, as shown in Fig. 3 (H16_A1438, H16_A1439, each threefold). These results again support the assumption either that a mutation in the key components of PHB biosynthesis *phaA* and *phaB1* had occurred or that the mutation in *phaC1* exerts a polar effect on the downstream genes in strain PHB⁻4, as discussed above. Furthermore, the expression of 10 genes involved in the CBB cycle was upregulated. Transcription of *cbbK2* (H16_B1385), *cbbF2*, *cbbE2*, *cbbY2* and *cbbX2* (H16_B1390–1393), as well as *cbbS2* and *cbbL2* (H16_B1394–1395) and the corresponding duplicates on the megaplasmid (PHG426–427) and *cbbPp* (PHG421), was found to be upregulated at levels between twofold and fourfold (Fig. 3). The data emphasize the assumption of a downregulation of the *cbb* operons in the mutant strain PHB⁻4, as discussed above (Fig. 3).

In strain *R. eutropha* PHB⁻4, only one gene exhibited an increased expression level (H16_A2818, *gcdH*, about threefold). The much lower number of differentially expressed genes in the precise deletion mutant Δ *phaC1* than in the chemically induced mutant PHB⁻4 compared with the wild-type, clearly indicates that mutant PHB⁻4 has a pleiotropic character. On the other hand, after 7 h of cultivation, the culture of mutant Δ *phaC1* is in an advanced stage of the transition phase, rather than in an early stationary growth phase compared with the culture of mutant PHB⁻4.

Correlation between transcriptomic and proteomic data

Recently, the proteome of *R. eutropha* H16 was analysed with regard to proteins of PHB metabolism using 2D-PAGE and MALDI-TOF analysis. Cells were cultivated in a Biostat D650 fermenter under conditions permissive for PHB biosynthesis and samples from (i) the exponential growth phase (8 h after inoculating), (ii) the stationary phase permissive for PHB accumulation (18 and 21 h), and (iii) the phase permissive for PHB mobilization (33 h) were analysed after a nitrogen source was added to the medium to induce PHB mobilization (Raberg *et al.*, 2008). A total of 15 proteins with significantly changed expression levels under two different conditions were detected, as listed in Table 2. To reveal whether or not there is a correlation between the transcriptomic and the proteomic data, the obtained data were compared with each other. This comprises the proteome data ranging from 18 to 21 h and 33 h taken from the paper by Raberg *et al.* (2008), and the transcriptome data obtained in substudy (ii) ranging from 7 to 12 h. Due to the differing cultivation conditions and time points of sampling in both studies, the comparison of the datasets can only provide some hints.

The proteome analyses (Raberg *et al.*, 2008) revealed three heat-shock proteins with decreased expression levels

during the course from the stationary growth phase (18 h) to the mobilization phase (33 h), including DnaK (H16_A3089), GroEL (H16_A0706) and the cochaperone GroES (H16_A0705). These findings were confirmed by the transcriptome analyses: in substudy (ii), comparing cells in the transition phase (7 h) to cells in stationary growth phase (12 h) of *R. eutropha* H16, *dnaK* exhibited a threefold decreased transcription level, whereas *groEL* and *groES* exhibited five and fourfold decreased transcription levels (Table 2; Supplementary Table S2). Furthermore, the significant decrease in *fliC* (H16_B2360) observed in proteome analyses could be confirmed by transcriptome analyses: *fliC* showed a fourfold decrease in the stationary growth phase. Finally, similar results for protein and transcript expression were obtained for the translation elongation factor TS (*tsf*; H16_A2054, threefold decrease in transcriptome data) and the nitrogen-regulatory protein PII GlnK (H16_A0320, fivefold increase in transcriptome data). In contrast, contradictory results were obtained in the two types of analyses for *phaP1* (H16_A1381) and *hoxU* (PHG089). Seven proteins exhibited changing expression levels in proteomic but not in transcriptomic analyses: GalU (H16_A2752), a β -ketothiolase (H16_B1369), an ankyrin repeat protein (H16_A1574), a nitrogen-regulatory protein PII (H16_A0750), a sigma 54 modulation protein S30EA (H16_A0386) and two conserved hypothetical proteins (H16_A1618 and H16_B0194). Finally, the comparison of 15 proteins with significantly different gene expression ratios revealed six ORFs showing the same expression trends in proteomic and transcriptomic analyses (*dnaK*, *groEL*, *groES*, *fliC*, *tsf* and *glnK*) whereas for two genes, contradictory results were obtained (*phaP1* and *hoxU*). Seven ORFs were detected in proteomic analyses, but these did not exhibit significantly changed transcription levels (*galU*, H16_B1369, H16_A1574, H16_A1618, H16_A0750, H16_B0194 and H16_A0386).

Functional analysis of an additional putative PHB synthase *phaC2* identified in *R. eutropha* H16

Many bacteria accumulate PHAs in the form of intracellular granules. Analysis of several bacterial genome sequences revealed the presence of more than one PHA synthase in many species. In strains of the genus *Burkholderia*, the presence of multiple PHA synthase genes is common, all sharing similar neighbouring genes (Chain *et al.*, 2006; Holden *et al.*, 2004; Nierman *et al.*, 2004). In *R. eutropha* strain JMP222, a *phaC2* gene has been identified (sequence available online <http://www.jgi.doe.gov>) that is neither a homologue of the *phaC1* family of PHA synthase genes nor similar to *phaC2* from strain H16. In *R. eutropha* H16, the key component of PHB biosynthesis is the PHB synthase encoded by *phaC1*. The gene is constitutively expressed, and the enzyme occurs as a soluble protein only at the beginning of PHB biosynthesis; upon onset of PHB biosynthesis, the enzyme becomes granule-bound (Haywood *et al.*, 1989; Gerngross *et al.*, 1993). The constitutive expression of *phaC1* is affirmed by the first

Table 2. Verification of *R. eutropha* H16 proteomic data by transcriptomic expression ratios

ND, Not detected.

ID	Name	Annotation	Change in the amount of protein during cultivation of <i>R. eutropha</i> H16*	Fold change†
A3089	DnaK	HSP70; chaperone	Decreasing	3.21
A0706	GroEL	HSP60; chaperone	Decreasing	5.39
B2360	FliC	Flagellin	Decreasing	4.03
A2752	GalU	UTP-glucose-1-phosphate uridylyltransferase	Decreasing	ND
A2054	Tsf	Translation elongation factor TS	Decreasing	3.10
B1369		β -Ketothiolase	Increasing	ND
A1381	PhaP1	Phasin	Decreasing	−3.32
A1574		Ankyrin repeat protein	Increasing	ND
PHG089	HoxU	NAD-reducing diaphorase moiety small subunit	Constant	2.96
A1618		Conserved hypothetical protein	Constant	ND
A0750		Nitrogen-regulatory protein PII	Decreasing	ND
A0320	GlnK	Nitrogen-regulatory protein PII	Increasing	−5.06
A0705	GroES	HSP10; cochaperone	Decreasing	4.92
B0194		Conserved hypothetical protein	Increasing	ND
A0386		Sigma 54 modulation protein S30EA	Increasing	ND

*Data were taken from the paper by Raberg *et al.* (2008).†Gene expression changes in *R. eutropha* H16 during shift from the transition phase to the stationary growth phase. Results are given in log₂ transformed ratios. Only ORFs with relative RNA levels equal to or greater than 1.58 or less than −1.60 are shown, according to a threefold cut-off value. The relative RNA levels are the mean from eight replicates resulting from two independent biological experiments.

two transcription analyses presented in this study, comparing (i) transcriptomes from exponential growth phase with transcriptomes from the transition phase and (ii) transcriptomes from transition phase with transcriptomes from the stationary growth phase of *R. eutropha* H16. Both analyses exhibited high intensity values up to 2800 for both the Cy3 and the Cy5 channel in fact, but the ratios of approximately 1 reflect no significant changes in transcription for *phaC1* during PHB biosynthesis (data not shown).

The recently identified second putative PHB synthase encoded by *phaC2* is obviously not the result of a gene duplication of *phaC1* (Pohlmann *et al.*, 2006). For an overview, a multiple DNA sequence alignment of *phaC1* and *phaC2* is shown in Supplementary Fig. S1, available with the online version of this paper. Transcription analyses exhibited intensity values equal to or lower than the background value for both channels (data not shown). These results led to the assumption that *phaC2* is, in contrast with *phaC1*, not transcribed at all under the conditions investigated. To explore the transcription of the additional PHA-synthase-encoding gene *phaC2* in the related bacterium *R. eutropha* JMP222, one-step RT-PCR was employed for qualitative analysis. For this, cells were cultivated in mineral salts medium containing 1 % (w/v) sodium gluconate as a sole carbon source, i.e. in conditions permissive for PHB biosynthesis and accumulation. Cells were harvested after 24 h, total RNA was isolated and RT-PCR was carried out as described in Methods. RT-PCR analysis clearly demonstrated that *phaC1* and *phaC2* were

transcribed under storage conditions in *R. eutropha* JMP222 (data not shown). In contrast, constitutive expression of *phaC1* in *R. eutropha* H16 has been demonstrated (Pötter & Steinbüchel, 2006).

***In silico* characterization of novel PHA synthases**

Based on sequence similarities to well-defined enzymes, PHA synthases can be assigned a substrate specificity and classified into one of the established classes of type I or type II enzymes, preferring either hydroxyacyl-CoA thioesters of short or medium carbon chain length. Further functional analyses were done, to unravel whether or not PhaC2 is active in the cell and to determine the function of this putative PHB synthase. To classify and further investigate the putative PHA synthase gene *phaC2*, a global alignment of various protein sequences of known PHA synthases and those identified in *R. eutropha* H16, *R. eutropha* JMP134, *Bukrholderia xenovorans*, *Pseudomonas aeruginosa*, *P. putida* KT2440, *Allochrochromatium vinosum*, *Thiocystis violacea* and *Bacillus megaterium* was done, as shown in Table 3. The presence of catalytic residues was checked (based on data reviewed by Rehm, 2003). It became evident that all amino acids of the catalytic triad (C319, D480 and H508) and an additional residue (W425), which is thought to be essential for dimerization of active PHA synthases, are present in all sequences. A similarity tree based on the alignment of protein sequences of well-characterized PHA synthases and recently discovered additional synthases was constructed using the neighbour-joining method (Saitou & Nei, 1987)

Table 3. Alignments of catalytic amino acids and their surrounding regions of different types of PHA synthases

Protein*	Class†	Cysteine 319 (C)‡	Aspartic acid 480 (D)	Histidine 508 (H)	Tryptophan 425 (W)
PhaC1 _{H16}	I	313-INVLF C VGGTIVST-327	473-YIYGSRE D HIVPWT-487	501-FVLGASG H IAGVINP-515	418-PFDLLF W NGDATNLP-433
PhaC1 _{JMP134}	I	310-VNLHGACSGAMTMSA-324	464-YVLAGMT D HITPWKG-478	492-FVLSSSG H VQSLINP-506	410-AFDILY W NNDTTRL-425
PhaC1 _{LB400}	I	321-INTLG F CVGGTMLAT-335	481-FIYGSRE D HIVPWQT-495	509-FVLGASG H IAGVINP-523	426-PFDLLY W NSDSTSLP-441
PhaC1 _{PAO1}	II	290-LNLLGACSGGITTAT-304	444-YCVAGLN D HITPWES-458	472-FILSN S G H IQSILNP-486	390-AFDILY W NNDTTRL-405
PhaC2 _{PAO1}	II	290-VNLAGACAGGLTVAA-304	445-FHVAGIT D HITPWDA-459	473-FILSN S G H IQSILNP-487	391-AFDILY W NNDNTRL-406
PhaC1 _{KT2440}	II	290-LNMLGACSGGITCTA-304	444-YSLAGTN D HITPWPS-458	472-FVL S NSG H IQSILNP-486	390-VFDILF W NNDTTRL-405
PhaC2 _{KT2440}	II	290-PNLMGACAGGLTMAA-304	445-FTVAGSN D HITPWDA-459	473-FVLANS G H I QSIINP-487	391-AFDILY W NADSTR-406
PhaC _{Av}	III	143-VNLLG I CQGGAFSLM-157	295-LNIFALQ D HLVPPDA-309	324-ELAFPGG H IGIYVSG-338	241-FLRMEK W IFDSPDQA-256
PhaC _{Tv}	III	142-VNILG I CQGGAFSLM-156	294-LNIYALQ D HLVPPDA-308	323-ELAFPGG H IGIYVSG-337	240-FLRMEK W IFDSPDQA-255
PhaC _{Bm}	IV	146-LSVLGY C MGGTMTSI-160	300-LNIAASR D HIAMPHQ-314	329-YKLLQT G HVSVVFGP-343	246-WKLMQK W VADGIPFA-261
PhaC2 _{H16}	U	322-VHAAGY C LGGTLLAT-336	477-FVVGTE H DHVSPWRS-491	505-FLLTSGG H NAGIVSE-519	423-ANDLV S WNRDTRL-438
PhaC2 _{JMP134}	U	318-INTLG F CVGGTIIST-332	475-YIYGSRE D HIVPWT-489	503-FVLGASG H IAGVINP-517	420-PFDLLF W NGDATNLP-435
PhaC2 _{LB400}	U	326-IHATGY C LGGTLLSI-340	480-FVVGTER D HIAPWRS-494	508-FVLTS G G H NAGIVSE-522	426-MIDLMA W NADATRMP-441
PhaC3 _{LB400}	U	323-VHATGY C LGGTLLSI-337	477-FVVGTD R DHIAPWRS-491	505-FVLTS G G H NAGIVSE-519	423-MFDLMA W NADSTRMP-438

*Index designates the strain, species key: H16, *R. eutropha*; JMP134, *R. eutropha*; LB400, *Burkholderia xenovorans*; PAO1, *Pseudomonas aeruginosa*; KT2440, *Pseudomonas putida*; Av, *Allochrochromatium vinosum*; Tv, *Thiocystis violaceae*; Bm, *Bacillus megaterium*. Accession numbers are included in Fig. 4.

†The class of some putative PHA synthases are unknown (U).

‡Catalytic sites are summarized by Rehm (2003): C319, D480 and H508 form a catalytic triad, W425 is thought to be essential for dimerization. These residues are underlined and in bold type.

Fig. 4. Phylogenetic tree based on a multiple alignment of several PHA synthases. Accession numbers of the proteins are given in parentheses. In addition to the enzyme class (Roman numerals) the affiliation to a subclass of the source organism (in the case of proteobacteria) is indicated by the corresponding Greek letter. Enzymes marked with a black dot (●) were investigated in this study.

and plotted using TreeView (Page, 1996). The constructed tree shows that PhaC2 belongs to the large group of PHA synthases but can not be grouped into one of the established classes based on the sequences analysed thus far (Fig. 4).

Polymer accumulation of recombinant strains

To confirm that *phaC2* from *R. eutropha* H16 and *phaC2* from *R. eutropha* JMP134 encode active PHA synthases, these genes were cloned into plasmid pBHR69 downstream from the *lac* promoter. Plasmid pBHR69 has been previously used to study polymer accumulation in strains of *E. coli* and harbours the genes *phaA* and *phaB* from *R. eutropha* H16, which catalyse the two initial steps of PHB biosynthesis. The resulting hybrid plasmids pBHR69::*phaC2*_{JMP} and pBHR69::*phaC2*_{H16}, respectively, were then transferred into *E. coli* TOP10. In addition, the two *phaC2* genes mentioned above and *phaC3* from *B. xenovorans* LB400 were cloned into the vector pJO2. The resulting hybrid plasmids were also transferred into *E. coli* TOP10, *P. putida* GPp104, *R. eutropha* PHB⁻4 and *R. eutropha* H16Δ*phaC1*. Cells were cultivated as described in Methods, and the PHA contents were measured by gas chromatography. None of the recombinant strains was able to produce any PHB with the exception of *R. eutropha* PHB⁻4 pJO2::*phaC2*_{H16}. The polymer was isolated from the cells of this recombinant strain and purified to 99.8% as determined by gas chromatography. The only constituent of this polymer was 3-hydroxybutyrate. The molecular mass, as determined by GCP was about 1 MDa, which is a typical value compared with PHAs produced by wild-type strains of *R. eutropha*. Because *phaC2*_{H16} enables polymer synthesis only in *R. eutropha* PHB⁻4 and not in other bacteria, the encoded protein alone may be inactive, and some other factor present in *R. eutropha* but not in the other bacteria is required to form an active enzyme. Inactivity of PhaC2 in the *phaC1* deletion mutant may indicate that parts of PhaC1 contribute to this factor.

PHB synthase assays

To detect enzymes that catalyse the polymerization of 3-hydroxybutyrate-CoA in crude extracts of recombinant strains, enzyme assays were performed as described in Methods. All strains showed non-specific background activities of PHB synthases that did not correlate with the ability to produce PHA. Only enzyme activities measured in extracts of *R. eutropha* H16 were significantly higher than the background activity. A possible complementation on the protein level of the PHB-producing recombinant strain PHB⁻4 pJO2::*phaC2*_{H16} was checked using a special assay combination: crude extracts of *R. eutropha* H16Δ*phaC1* harbouring pJO2::*phaC2*_{H16} were mixed with cell-free extracts of PHB⁻4, but no enzyme activity higher than the background was detected. Fig. 5 shows a multi-step assay: (i) extracts of PHB⁻4 were added to the substrate, after this (ii) PhaC2_{H16}-containing extracts were added and finally, (iii) crude extracts from the wild-type strain H16 containing lower amounts of PhaC1_{H16} were added to the assay. A background activity of about 27.5 (± 6.7) mU (mg total protein)⁻¹ was determined, which only increased to a specific activity of 482 mU (mg total protein)⁻¹ by adding extracts of the wild-type containing PhaC1_{H16}.

The functional role of the additional PHA synthase PhaC2 with high homologies to classical PHA synthase genes remains unknown. Expression data obtained by transcription analyses led to the assumption that *phaC2* is not transcribed at all under the conditions investigated. An activity of the enzyme could not be demonstrated. Only expression in the chemically induced mutant PHB⁻4 led to the accumulation of PHB.

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Fig. 5. Example of an enzyme assay using crude extracts from 1, *R. eutropha* PHB⁻4 (final total protein concentration of 0.1 $\mu\text{g } \mu\text{l}^{-1}$ total assay volume); 2, *R. eutropha* Δ*phaC1* harbouring the plasmid pJO2::*phaC2*_{H16} (0.1 $\mu\text{g } \mu\text{l}^{-1}$); and 3, *R. eutropha* H16 (0.02 $\mu\text{g } \mu\text{l}^{-1}$). Release of CoA by enzymic cleavage of the substrate 3-hydroxybutyrate-CoA was followed by measurement of the increase of the reaction product of CoA with DTNB at 412 nm.

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REFERENCES

- Abe, T., Kobayashi, T. & Saito, T. (2005). Properties of a novel intracellular poly(3-hydroxybutyrate) depolymerase with high specific activity (PhaZd) in *Wautersia eutropha* H16. *J Bacteriol* **187**, 6982–6990.
- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Benes, V. & Muckenthaer, M. (2003). Standardization of protocols in cDNA microarray analysis. *Trends Biochem Sci* **28**, 244–249.
- Bork, P. (1993). Identification of a ring protein that can interact *in vivo* with the *BRCA1* gene product. *Proteins* **17**, 363–374.
- Bowien, B. & Kusian, B. (2002). Genetics and control of CO₂ assimilation in the chemoautotroph *Ralstonia eutropha*. *Arch Microbiol* **178**, 85–93.
- Brandl, H., Gross, R. A., Lenz, R. W. & Fuller, R. C. (1988). *Pseudomonas oleovorans* as a source of poly(β -hydroxyalkanoates) for potential applications as biodegradable polyesters. *Appl Environ Microbiol* **54**, 1977–1982.
- Chain, P. S. G., Deneff, V. J., Konstantinidis, K. T., Vergez, L. M., Agullo, L., Reyes, V. L., Hauser, L., Cordova, M., Gomez, L. & other authors (2006). *Burkholderia xenovorans* LB400 harbors a multi-replicon, 9.73-Mbp genome shaped for versatility. *Proc Natl Acad Sci U S A* **103**, 15280–15287.
- Don, R. H. & Pemberton, J. M. (1981). Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. *J Bacteriol* **145**, 681–686.
- Ehrenreich, A. (2006). DNA microarray technology for the microbiologist: an overview. *Appl Microbiol Biotechnol* **73**, 255–273.
- Fitzpatrick, J. M., Johnston, D. A., Williams, G. W., Williams, D. J., Freeman, T. C., Dunnea, D. W. & Hoffmann, K. F. (2005). An oligonucleotide microarray for transcriptome analysis of *Schistosoma mansoni* and its application/use to investigate gender-associated gene expression. *Mol Biochem Parasitol* **141**, 1–13.
- Friedrich, C. G., Friedrich, B. & Bowien, B. (1981). Formation of enzymes of autotrophic metabolism during heterotrophic growth of *Alcaligenes eutrophus*. *J Gen Microbiol* **122**, 69–78.
- Fukui, T., Shiomi, N. & Doi, Y. (1998). Expression and characterization of (R)-specific enoyl coenzyme A hydratase involved in polyhydroxyalkanoate biosynthesis by *Aeromonas caviae*. *J Bacteriol* **180**, 667–673.
- Gerngross, T. U., Reilly, P., Stubbe, J., Sinskey, A. J. & Peoples, O. P. (1993). Immunocytochemical analysis of poly- β -hydroxybutyrate (PHB) synthase in *Alcaligenes eutrophus* H16: localization of the synthase enzyme at the surface of PHB granules. *J Bacteriol* **175**, 5289–5293.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.
- Haywood, G. W., Anderson, A. J., Chu, L. & Dawes, E. A. (1988). Characterization of two 3-ketothiolases possessing differing substrate specificities in the polyhydroxyalkanoate synthesizing organism *Alcaligenes eutrophus*. *FEMS Microbiol Lett* **52**, 91–96.
- Haywood, G., Anderson, A. & Dawes, E. (1989). The importance of PHB-synthase substrate-specificity in polyhydroxyalkanoate synthesis by *Alcaligenes eutrophus*. *FEMS Microbiol Lett* **57**, 1–6.
- Henikoff, S. & Henikoff, J. G. (1993). Performance evaluation of amino acid substitution matrices. *Proteins* **17**, 49–61.
- Holden, M. T. G., Titball, R. W., Peacock, S. J., Cerdeno-Tarraga, A. M., Atkins, T., Crossman, L. C., Pitt, T., Churcher, C., Mungall, K. & other authors (2004). Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. *Proc Natl Acad Sci U S A* **101**, 14240–14245.
- Huisman, G. W., Wonink, E., Meima, R., Kazemier, B., Terpstra, P. & Witholt, B. (1991). Metabolism of poly(3-hydroxyalkanoates) (PHAs) by *Pseudomonas oleovorans*. Identification and sequences of genes and function of the encoded proteins in the synthesis and degradation of PHA. *J Biol Chem* **266**, 2191–2198.
- Jeffke, T., Gropp, N. H., Kaiser, C., Grzeszik, C., Kusian, B. & Bowien, B. (1999). Mutational analysis of the *ccb* operon (CO₂ assimilation) promoter of *Ralstonia eutropha*. *J Bacteriol* **181**, 4374–4380.
- Jendrossek, D. (2002). Extracellular polyhydroxyalkanoate depolymerases: the key enzymes of PHA degradation. In *Biopolymers*, vol. 3b: *Polyesters II – Properties and Chemical Synthesis*, pp. 41–84. Edited by Y. Doi & A. Steinbüchel. Weinheim: Wiley.
- Jendrossek, D. & Handrick, R. (2002). Microbial degradation of polyhydroxyalkanoates. *Annu Rev Microbiol* **56**, 403–432.
- Jendrossek, D., Schirmer, A. & Schlegel, H. G. (1996). Biodegradation of polyhydroxyalkanoic acids. *Appl Microbiol Biotechnol* **46**, 451–463.
- Kobayashi, T., Uchino, K., Abe, T., Yamazaki, Y. & Saito, T. (2005). Novel intracellular 3-hydroxybutyrate-oligomer hydrolase in *Wautersia eutropha* H16. *J Bacteriol* **187**, 5129–5135.
- Lund, P. A. (2001). Microbial molecular chaperones. *Adv Microb Physiol* **44**, 93–140.
- Macnab, R. M. (2003). How bacteria assemble flagella. *Annu Rev Microbiol* **57**, 77–110.
- Madison, L. L. & Huisman, G. W. (1999). Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. *Microbiol Mol Biol Rev* **63**, 21–53.
- Nierman, W. C., DeShazer, D., Kim, H. S., Tettelin, H., Nelson, K. E., Feldblyum, T., Ulrich, R. L., Ronning, C. M., Brinkac, L. M. & other authors (2004). Structural flexibility in the *Burkholderia mallei* genome. *Proc Natl Acad Sci U S A* **101**, 14246–14251.
- Oeding, V. & Schlegel, H. G. (1973). β -Ketothiolase from *Hydrogenomonas eutropha* H16 and its significance in the regulation of poly- β -hydroxybutyrate metabolism. *Biochem J* **134**, 239–248.
- Overhage, J., Steinbüchel, A. & Priefert, H. (2002). Biotransformation of eugenol to ferulic acid by a recombinant strain of *Ralstonia eutropha* H16. *Appl Environ Microbiol* **68**, 4315–4321.
- Page, R. D. (1996). TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.
- Peoples, O. P. & Sinskey, A. J. (1989). Poly- β -hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (*phbC*). *J Biol Chem* **264**, 15298–15303.
- Pfützner, J. & Schlegel, H. G. (1973). Denitrification in *Hydrogenomonas eutropha* strain H16. *Arch Mikrobiol* **90**, 199–211.
- Pohlmann, A., Fricke, W. F., Reinecke, F., Kusian, B., Liesegang, H., Cramm, R., Eitinger, T., Ewering, C., Pötter, M. & other authors (2006). Hydrogen-based biotechnology: genome sequence of the bioplastic-producing “Knallgas” bacterium *Ralstonia eutropha* H16. *Nat Biotechnol* **24**, 1257–1262.
- Pötter, M. & Steinbüchel, A. (2005). Poly(3-hydroxybutyrate) granule-associated proteins: impacts on poly(3-hydroxybutyrate) synthesis and degradation. *Biomacromolecules* **6**, 552–560.
- Pötter, M. & Steinbüchel, A. (2006). Biogenesis and structure of polyhydroxyalkanoate granules. In *Microbiology Monographs*, vol. 1:

- Inclusions in Prokaryotes*, pp 109–136. Edited by A. Steinbüchel & J. M. Shively. Berlin, Heidelberg: Springer Verlag.
- Pötter, M., Madkour, M. H., Mayer, F. & Steinbüchel, A. (2002). Regulation of phasin expression and polyhydroxyalkanoate (PHA) granule formation in *Ralstonia eutropha* H16. *Microbiology* **148**, 2413–2426.
- Pötter, M., Müller, H., Reinecke, F., Wieczorek, R., Fricke, F., Bowien, B., Friedrich, B. & Steinbüchel, A. (2004). The complex structure of polyhydroxybutyrate (PHB) granules: four orthologous and paralogous phasins occur in *Ralstonia eutropha*. *Microbiology* **150**, 2301–2311.
- Pötter, M., Müller, H. & Steinbüchel, A. (2005). Influence of homologous phasins (PhaP) on PHA accumulation and regulation of their expression by the transcriptional repressor PhaR in *Ralstonia eutropha* H16. *Microbiology* **151**, 825–833.
- Raberg, M., Reinecke, F., Reichelt, R., Malkus, U., König, S., Pötter, M., Fricke, W. F., Pohlmann, A., Voigt, B. & other authors (2008). *Ralstonia eutropha* H16 flagellation changes according to nutrient supply and state of poly(3-hydroxybutyrate) accumulation. *Appl Environ Microbiol* **74**, 4477–4490.
- Rehm, B. H. A. (2003). Polyester synthases: natural catalysts for plastics. *Biochem J* **376**, 15–33.
- Rehm, B. H., Krüger, N. & Steinbüchel, A. (1998). A new metabolic link between fatty acid *de novo* synthesis and polyhydroxyalkanoic acid synthesis. The *phaG* gene from *Pseudomonas putida* KT2440 encodes a 3-hydroxyacyl-acyl carrier protein-coenzyme A transferase. *J Biol Chem* **273**, 24044–24051.
- Reinecke, F. & Steinbüchel, A. (2009). *Ralstonia eutropha* strain H16 as model organism for PHA metabolism and for biotechnological production of technically interesting biopolymers. *J Mol Microbiol Biotechnol* **16**, 91–108.
- Saegusa, H., Shiraki, M., Kanai, C. & Saito, T. (2001). Cloning of an intracellular poly [D(–)-3-hydroxybutyrate] depolymerase gene from *Ralstonia eutropha* H16 and characterization of the gene product. *J Bacteriol* **183**, 94–100.
- Saegusa, H., Shiraki, M. & Saito, T. (2002). Cloning of an intracellular D(–)-3-hydroxybutyrate oligomer hydrolase gene from *Ralstonia eutropha* H16 and identification of the active site serine residue by site-directed mutagenesis. *J Biosci Bioeng* **94**, 106–112.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Schlegel, H. G., Gottschalk, G. & von Bartha, R. (1961). Formation and utilization of poly-β-hydroxybutyric acid by Knallgas bacteria (*Hydrogenomonas*). *Nature* **191**, 463–465.
- Schlegel, H. G., Lafferty, R. & Krauss, I. (1970a). The isolation of mutants not accumulating poly-β-hydroxybutyric acid. *Arch Mikrobiol* **71**, 283–294.
- Schlegel, H. G., Lafferty, R. & Krauss, I. (1970b). Bacterial mutants of *Hydrogenomonas* lacking poly-β-hydroxybutyric acid. *Experientia* **26**, 554–555.
- Schubert, P., Steinbüchel, A. & Schlegel, H. G. (1988). Cloning of the *Alcaligenes eutrophus* gene for synthesis of poly-β-hydroxybutyric acid and synthesis of PHB in *Escherichia coli*. *J Bacteriol* **170**, 5837–5847.
- Schubert, P., Krüger, N. & Steinbüchel, A. (1991). Molecular analysis of the *Alcaligenes eutrophus* poly(3-hydroxybutyrate) biosynthetic operon – identification of the N-terminus of poly(3-hydroxybutyrate) synthase and identification of the promoter. *J Bacteriol* **173**, 168–175.
- Schwartz, E. & Friedrich, B. (2001). A physical map of the megaplasmid pHG1, one of three genomic replicons in *Ralstonia eutropha* H16. *FEMS Microbiol Lett* **201**, 213–219.
- Schwartz, E., Henne, A., Cramm, R., Eitinger, T., Friedrich, B. & Gottschalk, G. (2003). Complete nucleotide sequence of pHG1: a *Ralstonia eutropha* H16 megaplasmid encoding key enzymes of H₂-based lithoautotrophy and anaerobiosis. *J Mol Biol* **332**, 369–383.
- Simon, R., Priefer, U. & Pühler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Biotechnology* **1**, 784–791.
- Slater, S. C., Voige, W. H. & Dennis, D. E. (1988). Cloning and expression in *Escherichia coli* of the *Alcaligenes eutrophus* H16 poly-β-hydroxybutyrate biosynthetic pathway. *J Bacteriol* **170**, 4431–4436.
- Slater, S., Houmiel, K. L., Tran, M., Mitsky, T. A., Taylor, N. B., Padgett, S. R. & Gruys, K. J. (1998). Multiple β-ketothiolases mediate poly(β-hydroxyalkanoate) copolymer synthesis in *Ralstonia eutropha*. *J Bacteriol* **180**, 1979–1987.
- Spiekermann, P., Rehm, B. H., Kalscheuer, R., Baumeister, D. & Steinbüchel, A. (1999). A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. *Arch Microbiol* **171**, 73–80.
- Srienc, F., Arnold, B. & Bailey, J. E. (1984). Characterization of intracellular accumulation of poly-β-hydroxybutyrate (PHB) in individual cells of *Alcaligenes eutrophus* H16 by flow cytometry. *Biotechnol Bioeng* **26**, 982–987.
- Steinbüchel, A. (2001). Perspectives for biotechnological production and utilization of biopolymers: metabolic engineering of polyhydroxyalkanoate biosynthesis pathways as a successful example. *Macromol Biosci* **1**, 1–24.
- Taguchi, K., Taguchi, S., Sudesh, K., Maehara, A., Tsuge, T. & Doi, Y. (2002). Metabolic pathways and engineering of PHA biosynthesis. In *Biopolymers – vol. 3A, Polyesters I – Biological Systems and Biotechnological Production*, pp. 217–248. Edited by Y. Doi & A. Steinbüchel. Weinheim: Wiley VCH.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Uchino, K., Saito, T. & Jendrossek, D. (2008). Poly(3-hydroxybutyrate) (PHB) depolymerase PhaZa1 is involved in mobilization of accumulated PHB in *Ralstonia eutropha* H16. *Appl Environ Microbiol* **74**, 1058–1063.
- Valentin, H. E. & Steinbüchel, A. (1993). Cloning and characterization of the *Methylobacterium extorquens* polyhydroxyalkanoic-acid-synthase structural gene. *Appl Microbiol Biotechnol* **39**, 309–317.
- Walde, E. (1962). Untersuchungen über Wachstum und Speicherstoffsynthese von *Hydrogenomonas eutropha*. *Arch Mikrobiol* **43**, 109–137.
- Wieczorek, R., Pries, A., Steinbüchel, A. & Mayer, F. (1995). Analysis of a 24-kilodalton protein associated with the polyhydroxyalkanoic acid granules in *Alcaligenes eutrophus*. *J Bacteriol* **177**, 2425–2435.
- York, G. M., Lupberger, J., Tian, J. M., Lawrence, A. G., Stubbe, J. & Sinskey, A. J. (2003). *Ralstonia eutropha* H16 encodes two and possibly three intracellular poly[D(–)-3-hydroxybutyrate] depolymerase genes. *J Bacteriol* **185**, 3788–3794.
- Zeller, M., Pötter, M., Reinecke, F., Steinbüchel, A., Burkitt, W. I., Derrick, P. J. & König, S. (2005). Mass spectrometry identifies homologues of phasin PhaP1 protein of *Ralstonia eutropha* on polyhydroxybutyrate granules. *J Biomacromol Mass Spectrom* **1**, 75–84.

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