Interaction between poly(3-hydroxybutyrate) granule-associated proteins as revealed by two-hybrid analysis and identification of a new phasin in *Ralstonia eutropha* H16

Daniel Pfeiffer and Dieter Jendrossek

Correspondence
Dieter Jendrossek
dieter.jendrossek@imb.
uni-stuttgart.de

Institut für Mikrobiologie, Universität Stuttgart, Allmandring 31, Stuttgart, Germany

A large number of polypeptides are attached to poly(3-hydroxybutyrate) (PHB) granules of *Ralstonia eutropha*, such as PHB synthase (PhaC1), several PHB depolymerases (PhaZs) and phasins (PhaPs), the regulator protein PhaR_{Reu}, and possibly others. In this study we used the bacterial adenylate cyclase-based two-hybrid assay to investigate interactions between known PHB granule-associated proteins (PGAPs) and to screen for new PGAPs. The utility of the system was tested by the *in vivo* verification of previously postulated interactions of the PHB synthase subunits of *R. eutropha* (PhaC1 homo-oligomerization) and of *Bacillus megaterium* (PhaC_{Bmeg}–PhaR_{Bmeg} hetero-oligomerization). Nine proteins (PhaA, PhaB1, PhaC1, PhaP1–PhaP4, PhaZ1 and PhaR), with established functions in PHB metabolism of *R. eutropha*, were tested for interaction in all combinations. While no significant interaction was detected between the PHB synthase PhaC1 and any of the other eight tested Pha proteins, strong interactions were found between all phasin proteins, in particular between PhaP2 and PhaP4. When PhaP2 was used as bait in a two-hybrid screening experiment with a genomic library of *R. eutropha*, the B1934 gene product was identified in 24 out of 53 isolated clones. B1934 encodes a hypothetical protein (15.7 kDa) with similarity to phasins of PHB-accumulating bacteria. A fusion protein of eYfp and the B1934 gene product colocalized with PHB granules, confirming that B1934 represents a new phasin (PhaP5). PhaP5 was not essential for PHB granule formation, but overexpression of PhaP5 increased the number of cells with PHB granules at the cell poles.

Received 25 May 2011
Revised 4 July 2011
Accepted 6 July 2011

INTRODUCTION

Ralstonia eutropha is the model organism for accumulation of poly(3-hydroxyalkanoates) (PHA) (Pohlmann *et al.*, 2006; Pötter & Steinbüchel, 2006; Steinbüchel & Hein, 2001; Stubbe *et al.*, 2005; Sudesh *et al.*, 2000). At least 17 proteins have been described that are important for the biosynthesis, maintenance and intracellular utilization (mobilization) of poly(3-hydroxybutyrate) (PHB): four phasin proteins [PhaP1–PhaP4; phasins are PHB granule-associated proteins (PGAPs) with putative structural functions] (Pieper-Fürst *et al.*, 1994; Pötter *et al.*, 2002, 2004, 2005; York *et al.*, 2001a, b), seven intracellular PHB depolymerases (PhaZs) (Abe *et al.*, 2005; Handrick *et al.*, 2000; Saegusa *et al.*, 2001; York *et al.*, 2003), two 3-hydroxybutyrate oligomer hydrolases (PhaZb, PhaZc or PhaYs) (Kobayashi *et al.*, 2003, 2005), a regulator protein,

PhaR (Pötter *et al.*, 2002; York *et al.*, 2002), and the three main PHB biosynthetic enzymes [β -ketothiolase (PhaA), acetoacetyl-CoA reductase (PhaB1) and PHB synthase (PhaC1)] of the *phaCAB* operon. Some of the 17 proteins mentioned above have been shown experimentally to be PHB granule-bound or are likely to be PHB granule-bound. There may even be other as-yet-unidentified proteins involved in PHB metabolism and PHB granule formation. The *R. eutropha* genome has many isogenes encoding putative reductase proteins (e.g. *phaB3*) and thiolase proteins, as well as a second PHA synthase gene (*phaC2*) and proteins with phasin-like sequence motifs (Budde *et al.*, 2010; Pohlmann *et al.*, 2006). *In vitro* studies using isolated native PHB (nPHB) granules suggest that all activities that are necessary to synthesize PHB from acetyl-CoA and to mobilize PHB are attached to isolated nPHB granules (Uchino *et al.*, 2007). Reports for other PHA-accumulating organisms show that acyl-CoA synthetase (Acs1) is bound to the PHA granule surface *in vivo* in *Pseudomonas putida* (Ruth *et al.*, 2008). These findings suggest that PHA granules are much more than simple

Abbreviations: BACTH system, bacterial adenylate cyclase-based two-hybrid system; MU, Miller units; nPHB, native PHB; PGAP, PHB granule-associated protein; PHA, poly(3-hydroxyalkanoates); PHB, poly(3-hydroxybutyrate).

storage tanks for carbon end energy; rather, they are highly organized supramolecular complexes for which we have proposed the designation 'carbonosome' to indicate an organelle-like function (Jendrossek, 2009).

While considerable knowledge of the number of different PGAPs has accumulated, little is known about the organization of these PGAPs on the PHA granule surface. In particular, it is not known whether these proteins are randomly organized on the PHB granule surface or whether there are specific protein–protein interactions between some of the PGAPs that result in an ordered surface structure. To address this question experimentally, we used a two-hybrid approach for identification of potentially interacting proteins. Two-hybrid assays were first described for yeasts (Fields & Song, 1989), but bacterial counterparts of two-hybrid assays have been developed in recent years (e.g. Dove & Hochschild, 2001, 2004; Wang *et al.* 2011). In this study we used the bacterial two-hybrid system described by Karimova *et al.* (1998). This method takes advantage of the reconstitution of adenylate cyclase activity in an adenylate cyclase-deficient *Escherichia coli* reporter strain (Karimova *et al.*, 2000a, b). For this, two plasmids are used, each encoding one half of an adenylate cyclase (from *Bordetella pertussis*, named T18 and T25 fragments). Physical interactions between two test proteins that are fused to the T18 and T25 fragments result in the reconstitution of adenylate cyclase activity and in the formation of red colonies on MacConkey agar, or in growth and formation of blue colonies on X-Gal-containing mineral salts medium with lactose or maltose (Fig. 1).

Earlier investigations of the PHB synthase of *R. eutropha* *in vitro* (class I PHB synthase) have suggested that PHB synthase activity depends on homo-oligomerization of PhaC_{1_{Reu}} subunits (Stubbe & Tian, 2003; Stubbe *et al.*, 2005; Wodzinska *et al.*, 1996). In *Bacillus megaterium*, PHB synthase (class IV) consists of two different subunits (PhaC_{Bmeg} and PhaR_{Bmeg}) (McCool & Cannon, 1999, 2001; Pötter & Steinbüchel, 2006; Rehm, 2003, 2006), and only the heterodimer PhaC–PhaR has PHB synthase activity. We first tested the versatility of the adenylate cyclase two-hybrid system by confirmation of homo-oligomerization of *R. eutropha* PhaC1 subunits and by hetero-oligomerization of *B. megaterium* phaC_{Bmeg} and phaR_{Bmeg} PHB synthase subunits before the system was used to identify new interactions.

METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are shown in Table 1. *E. coli* strain JM109 was used in cloning experiments, and was routinely grown on Luria broth (LB) supplemented with the appropriate antibiotics at 37 °C. *R. eutropha* strains were routinely grown on nutrient broth (NB) medium at 30 °C. Sodium gluconate (0.2 %, w/v) was added to promote PHB accumulation.

Two-hybrid assays were performed with *E. coli* BTH101 harbouring the pUT18C and pKT25 plasmid combination of interest on LB agar

containing 100 µg ampicillin ml⁻¹, 50 µg kanamycin ml⁻¹, 0.5 mM IPTG and 40 µg X-Gal ml⁻¹ at 30 °C or room temperature. Alternatively, MacConkey agar containing 1 % (w/v) lactose, 100 µg ampicillin ml⁻¹, 50 µg kanamycin ml⁻¹ and 0.5 mM IPTG, or M63 mineral salts agar containing 50 µg ampicillin ml⁻¹, 25 µg kanamycin ml⁻¹, 0.5 mM IPTG, 40 µg X-Gal ml⁻¹ and 0.2 % (w/v) maltose as sole carbon source was also used. Depending on the degree of protein–protein interaction, the development of colonies and of a blue colour took 2–8 days at 30 °C.

Two-hybrid assay. For construction of pUT18C and pKT25 variant plasmids, the gene of interest was PCR-amplified from isolated chromosomal DNA of *R. eutropha* H16 and *B. megaterium*, respectively, using a proof-reading DNA polymerase. The primers used for PCR are summarized in Table 2. The primers additionally encoded two restriction endonuclease sites suitable for cloning in pUT18C and pKT25 vectors. The PCR products were cut with the respective endonucleases, purified, and cloned into pUT18C and pKT25. The DNA sequences of the inserts were determined. Only clones encoding correct amino acid sequences were used for two-hybrid assays. The two-hybrid assay was principally performed in both combinations, meaning that each gene was cloned into both pUT18C and pKT25, and that both combinations of each test pair were investigated. Only if both combinations gave signals that were significantly more intense than controls (pUT18C fusion with pKT25 and pKT25 fusion with pUT18C) was the outcome regarded as positive.

Two-hybrid screening of a genomic library of *R. eutropha*.

A genomic library of *R. eutropha* H16 fused to the 3' end of the gene for the *Bordetella pertussis* adenylate cyclase T18 domain was constructed. Isolated genomic DNA of *R. eutropha* H16 was partially digested with DNase I in the presence of 50 mM Tris/HCl, pH 7.5, supplemented with 1 mM MnCl₂. The resulting DNA fragments, ranging from 400 to 2000 bp, were isolated by agarose gel electrophoresis, end-repaired by Klenow DNA polymerase and subsequently ligated with BstXI adaptors (annealed oligonucleotides BstXC and BstXD). Remaining adaptor molecules and adaptor dimers were removed by multiple rounds of agarose gel electrophoresis and spin-column purification. Subsequently, the DNA fragments were ligated to a BstXI-digested pUT18BC-Kan1 vector and transformed into electrocompetent *E. coli* 10B cells (TFB Electro 10B, Transformation Biosciences). In total, approximately 2.6 × 10⁶ clones were obtained. Amplification of inserted DNA fragments from 76 randomly chosen clones by colony PCR confirmed the presence of inserts ranging from 300 to 1700 bp. Of 76 clones, 72 harboured chromosomal DNA inserts, and only four clones had an adaptor dimer as insert. All colonies of the genomic library were resuspended in buffer, and plasmid DNA was isolated from the resulting pool using a NucleoBond AX 500 cartridge (Qiagen). This plasmid library was used for two-hybrid screening experiments. Fig. 1 shows the procedure schematically.

The bait construct (pKT25-*phaP2*) was transformed into the adenylate cyclase-deficient reporter strain *E. coli* BTH101, and electrocompetent cells were prepared from a representative transformant. About 100 ng of the genomic library in pUT18BC-Kan1 was used for transformation of 40 µl of electrocompetent bait cells (Bio-Rad Gene Pulser II System, 0.2 cm cuvette, 25 µF, 2.5 kV, 200 Ω). A 960 µl volume of SOB medium (5 g yeast extract l⁻¹, 20 g tryptone l⁻¹, 0.6 g NaCl l⁻¹, 0.2 g KCl l⁻¹, 10 mM MgSO₄, 10 mM MgCl₂) was added and cells were incubated at 37 °C for 90 min. Cells were washed three times with M63 mineral salts medium, and 2 × 10⁸ to 6 × 10⁸ c.f.u. per experiment (approx. 10⁷ transformants per plate) was plated on M63 mineral salts maltose (0.2 %, w/v) agar supplemented with X-Gal (40 µg ml⁻¹), 0.5 mM IPTG, ampicillin (50 µg ml⁻¹) and kanamycin sulfate (25 µg ml⁻¹). Blue-coloured Cya⁺ colonies appeared after 3–8 days of incubation at 30 °C.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics or features*	Reference or source
Strains		
<i>E. coli</i> JM109	Cloning strain	
<i>E. coli</i> BTH101	Cya-BACTH expression strain; F ⁻ <i>cya-99 araD139 galE15 galK16 rpsL1</i> (Str ^r) <i>hsdR2 mcrA1 mcrB1</i>	Karimova <i>et al.</i> (2005)
<i>E. coli</i> S17-1	Conjugation strain	Simon <i>et al.</i> (1983)
<i>E. coli</i> 10B	Transformation strain	Transformation Biosciences
<i>B. megaterium</i>	PHB synthase type IV	DSMZ 168
<i>R. eutropha</i> H16	PHB synthase type I	DSMZ 428
<i>R. eutropha</i> HF39	Streptomycin-resistant mutant of <i>R. eutropha</i> H16 used for conjugation	Srivastava <i>et al.</i> (1982)
<i>R. eutropha</i> H16 Δ <i>phaP5</i>	Chromosomal deletion of <i>h16_b1934</i>	This study
<i>R. eutropha</i> HF39 Δ <i>phaP5</i>	Chromosomal deletion of <i>h16_b1934</i>	This study
Plasmids		
pJM9238	Runaway replication vector harbouring the <i>R. eutropha</i> <i>phaCAB</i> operon, Cm ^r	Kidwell <i>et al.</i> (1995)
pEYFP-C1	Source of <i>eyfp-c1</i>	Clontech
pUT18C	BACTH vector designed to express a given polypeptide fused in-frame at its N-terminal end with the T18 fragment; ColE1 ori; Amp ^r	Karimova <i>et al.</i> (2001)
pUT18BC-Kan1	pUT18C with two <i>Bst</i> XI sites	D. Ladant
pKT25	BACTH vector designed to express a given polypeptide fused in-frame at its N-terminal end with the T25 fragment; p15 ori; Km ^r	Karimova <i>et al.</i> (2001)
pUT18C- <i>zip</i> , pKT25- <i>zip</i>	Derivatives of pUT18C and pKT25 with a 114 bp DNA fragment encoding a leucine zipper (positive control for two-hybrid assays)	Karimova <i>et al.</i> (2001)
pUT18C- <i>phaR</i> _{Bmeg} pKT25- <i>phaC</i> _{Bmeg}	<i>B. megaterium</i> <i>phaR</i> and <i>phaC</i> cloned into pUT18C and pKT25 via <i>Pst</i> I and <i>Kpn</i> I restriction sites	This study
pUT18C- <i>phaC1</i> _{Reu} pKT25- <i>phaC1</i> _{Reu}	<i>R. eutropha</i> <i>phaC1</i> cloned into pUT18C and pKT25 via <i>Xba</i> I and <i>Sma</i> I restriction sites	This study
pUT18C/pKT25- <i>phaP1</i> , - <i>phaP2</i> , - <i>phaP3</i> , - <i>phaP4</i> , - <i>phaR</i> , - <i>phaA</i> , - <i>phaB1</i> , - <i>phaZ1</i> , - <i>phaP5</i>	<i>R. eutropha</i> <i>pha</i> ORFs cloned into pUT18C and pKT25 via <i>Xba</i> I and <i>Kpn</i> I restriction sites	This study
pBBR1MCS-2	Broad-host-range vector, Km ^r	Kovach <i>et al.</i> (1995)
pBBR1MCS-2-P _{<i>phaC</i>}	pBBR1MCS-2 containing a PCR fragment with the wild-type promoter and Shine-Dalgarno sequence of <i>phaCAB</i>	This study
pBBR1MCS-2-P _{<i>phaC</i>} - <i>eyfp-c1</i>	Universal vector for construction of fusions C-terminal to eYfp under the control of the <i>phaCAB</i> promoter	This study
pBBR1MCS-2-P _{<i>phaC</i>} - <i>eyfp-phaP2</i> , - <i>phaP4</i> , - <i>phaP5</i>	<i>R. eutropha</i> H16 <i>phaP2</i> , <i>phaP4</i> or <i>phaP5</i> (<i>h16_b1934</i>) cloned in-frame to <i>eyfp</i> via the <i>Xho</i> I and <i>Bam</i> HI restriction sites of pBBR1MCS-2-P _{<i>phaC</i>} - <i>eyfp-c1</i>	This study
pLO3	Deletion vector; Tc ^r	Lenz & Friedrich (1998)
pLO3- Δ <i>phaP5</i>	Deletion vector for <i>h16_b1934</i> ; 500 bp upstream and downstream of the gene were connected via overlap-PCR and cloned between the <i>Sac</i> I and <i>Xba</i> I restriction sites of pLO3	This study

*Abbreviations: Amp^r, ampicillin-resistant; Cm^r, chloramphenicol-resistant; Km^r, kanamycin-resistant; Str^r, streptomycin-resistant; Tc^r, tetracycline-resistant.

Selected Cya⁺ colonies were purified on M63-maltose medium and characterized by DNA sequencing of the inserts. Transformation efficiency was determined by plating aliquots on LB-ampicillin-kanamycin agar.

Construction of fusion proteins with eYfp and of chromosomal knockouts. A universal vector based on the broad-host-range

plasmid pBBR1MCS-2 (Kovach *et al.*, 1995), which allows expression of proteins fused to the C terminus of eYfp under the control of the constitutive *phaCAB* promoter, was constructed. First, a 466 bp PCR-generated DNA fragment that included the promoter and Shine-Dalgarno (SD) sequence of the *phaCAB* operon was cut with *Kpn*I and cloned between the *Kpn*I and *Sma*I restriction sites of pBBR1MCS-2. The resulting plasmid, pBBR1MCS-2-P_{*phaC*}, was cut

Table 2. Oligonucleotides used in this study

Restriction sites are underlined. Start and stop codons are shown in bold type.

Oligonucleotide	Sequence (5'–3')
Two-hybrid analysis	
B.m._phaR_C_forward_PstI	AACTGCAGTTTGAACAGCAAAAAGTATTTGATCC
B.m._phaR_C_reverse_KpnI	GGGGTACCTACTTGCGAGCCGGCTG
B.m._phaC_C_forward_PstI	AACTGCAGTCGTGGCAATTCCTTACGTG
B.m._phaC_C_reverse_KpnI	GGGGTACCTATTTAGAGCGTTTTTCTAGCCAATCG
R.e._phaC1_C_forward_XbaI	GCTCTAGACATGGCGACCGGCAAAGG
R.e._phaC1_C_reverse_SmaI	TCCCCCGGGCGTCATGCCTTGGCTTTGACG
R.e._phaP1_C_forward_XbaI	GCTCTAGAAATGATCCTCACCCCGGAAC
R.e._phaP1_C_reverse_KpnI	GGGGTACCTCAGGCAGCCGTCGTC
R.e._phaP2_C_forward_XbaI	GCTCTAGAGATGACTCAGTGGACAGCAG
R.e._phaP2_C_reverse_KpnI	GGGGTACCCTACTTTGCAGCTGCCGG
R.e._phaP3_C_forward_XbaI	GCTCTAGACATGTCCCCTTTTATGCCCG
R.e._phaP3_C_reverse_KpnI	GGGGTACCTTATTTGCTTGAAGCGCGG
R.e._phaP4_C_forward_XbaI	GCTCTAGAGATGACTCAGTGGTCCCCC
R.e._phaP4_C_reverse_KpnI	GGGGTACCTTAATTTGCAGCTGCCTTTGAGG
R.e._phaA_C_forward_XbaI	GCTCTAGAAATGACTGACGTTGTCATCGTATC
R.e._phaA_C_reverse_KpnI	GGGGTACCTTATTTGCGCTCGACTGCC
R.e._phaB1_C_forward_XbaI	GCTCTAGACATGACTCAGCGCATTGCG
R.e._phaB1_C_reverse_KpnI	GGGGTACCTCAGCCCATATGCAGGCC
R.e._phaR_C_forward_XbaI	GCTCTAGAGATGGCCACGACCAAAAAAGG
R.e._phaR_C_reverse_KpnI	GGGGTACCTTACTTCTTGTCGGCTGGTTG
R.e._phaZ1_C_forward_XbaI	GCTCTAGACATGCTCTACCAATTGCATGAGTTC
R.e._phaZ1_C_reverse_KpnI	GGGGTACCTTACCTGGTGGCCGAGG
R.e._H16_B1934_C_forward_XbaI	GCTCTAGACATGGCCACGCCTCCC
R.e._H16_B1934_C_reverse_KpnI	GGGGTACCCTAGCCCTTGGATTTGCGCTTG
BstXC	p-CTGGTTCGCGGGG
BstXD	p-GCCCGCGGAACAGCTCC
eyfp fusions	
phaC-promoter_forward_KpnI	GGGGTACCCAAAAATTCATCCTTCTCGCCTATGC
phaC-promoter_reverse_NdeI	GGGAATTCCATATGTTGATTGTCTCTCTGCCGTAC
EYFP-C1_forward_NdeI	GGGAATTCCATATGGTGAGCAAGGGCGAGG
EYFP-C1_reverse_SpeI	GGACTAGTTGATCAGTTATCTAGATCCGGTGGATCC
R.e._phaP2_C_forward_XhoI	CCGCTCGAGGCATGACTCAGTGGACAGCAG
R.e._phaP2_C_reverse_BamHI	CGGGATCCCTACTTTGCAGCTGCCGG
R.e._phaP4_C_forward_XhoI	CCGCTCGAGGCATGACTCAGTGGTCCCCC
R.e._phaP4_C_reverse_BamHI	CGGGATCCCTTAATTTGCAGCTGCCTTTGAGG
R.e._H16_B1934_C_forward_XhoI	CCGCTCGAGGCATGGCCACGCCTCCC
R.e._H16_B1934_C_reverse_BamHI	CGGGATCCCTAGCCCTTGGATTTGCGCTTG
phaP5 deletion	
B1934upstreamF	CGATCAGAGCTCCACTGCGCGCCACCGCCG
B1934upstreamR	GCCGTTAATTAAGCCGGCTGGGTCTCCTTCTGGAAGGACTGGC
B1934downstreamF	CGGCTTAATTAACGGCCCCAGGCCACCGCTAGCGCTGC
B1934downstreamR	GCTCTAGACGCGGCCACCGACCCGTCC

with *NdeI* and *SpeI*, and ligated with a DNA fragment encoding the polylinker and *eyfp*-coding sequence of pEYFP-C1, yielding pBBR1MCS-2-P_{phaC}-*eyfp-cl*. The B1934 gene and the *phaP2* and *phaP4* genes were each cloned in-frame to the *eyfp* gene between the *XhoI* and *BamHI* restriction sites of pBBR1MCS-2-P_{phaC}-*eyfp-cl*. The resulting plasmids were conjugatively transferred from *E. coli* S17-1 to *R. eutropha* HF39. A precise chromosomal deletion of B1934 (Δ phaP5) was constructed in *R. eutropha* H16 and HF39 using the *sacB*-sucrose selection method and pLO3 as deletion vector, as described elsewhere (Lenz & Friedrich, 1998). Fluorescence microscopy analysis was done as described previously (Jendrossek, 2005; Jendrossek *et al.*, 2007).

Determination of β -galactosidase activity. All assays were performed in triplicate and the means were calculated. The strain of interest was grown in 2.5 ml LB medium supplemented with 100 μ g ampicillin ml⁻¹, 50 μ g kanamycin ml⁻¹ and 0.5 mM IPTG at 30 °C and 150 r.p.m. for 16 h. The β -galactosidase assay was performed according to the protocol described by the manufacturer (Euromedex). Alternatively, cells were grown in 96-deep-well plates (1 ml volume per well). A 100 μ l volume of 1:5 diluted cells was mixed with 900 μ l Z buffer (10.7 g Na₂HPO₄·2H₂O l⁻¹, 5.5 g NaH₂PO₄ l⁻¹, 0.75 g KCl l⁻¹, 0.246 g MgSO₄·7H₂O l⁻¹, 2.7 ml β -mercaptoethanol l⁻¹, pH 7) before 20 μ l of 0.1% (w/v) SDS and 20 μ l toluene were added for permeabilization. Toluene was

Fig. 1. Principles of the adenylate cyclase-based two-hybrid approach (a), and screening of a genomic library for interactions with a bait gene of interest (b).

evaporated by shaking at 150 r.p.m. and 37 °C for 40 min. A 30 μ l volume of ONPG solution (4 mg ml⁻¹ in Z buffer without β -mercaptoethanol) was added to 150 μ l of permeabilized cells, and activity measurement was carried out by recording A_{420} for 40 min using a microplate reader [all steps with prewarmed (28 °C) solutions]. β -Galactosidase activity in Miller units (MU) was then calculated (Thibodeau *et al.*, 2004). For conversion of microplate reader A_{600} and A_{420} values into standard photometer values, correction factors were determined using serial dilutions of cells and an *o*-nitrophenyl (ONP) solution. One unit of β -galactosidase activity per milligram corresponds to 1.5 MU.

RESULTS

The ability of the PHB synthase of *R. eutropha* (PhaC1_{Reu}) and those of *B. megaterium* (PhaC_{Bmeg}, PhaR_{Bmeg}) to form PhaC1_{Reu} homo-oligomers and PhaC_{Bmeg}-PhaR_{Bmeg} hetero-oligomers *in vivo* was tested by two-hybrid assay. When the empty plasmids (pUT18C+pKT25), or one of the empty plasmids in combination with the respective pUT18C or pKT25 hybrid plasmid carrying one of the PHB synthase gene fusions, were tested, the streaks generally were white on IPTG-X-Gal LB medium, indicating that no

significant adenylate cyclase activity was restored (negative controls, Fig. 2a). In contrast, the strain with the positive control (leucine zipper) became blue within 30 h of incubation at 30 °C. When the plasmid combinations [(pUT18C-*phaC1*_{Reu}+pKT25-*phaC1*_{Reu}) or (pUT18C-*phaR*_{Bmeg}+pKT25-*phaC*_{Bmeg})] were tested the colonies of the three strains turned blue within 2–3 days of incubation (Fig. 2a). Apparently, the *R. eutropha* PHB synthase PhaC1_{Reu} forms homo-oligomers *in vivo*, resulting in reconstitution of adenylate cyclase activity. Similarly, the fusions with PhaC_{Bmeg} and PhaR_{Bmeg} associated to hetero-dimers, as indicated by formation of blue colonies. The same principal results were obtained when the experiments were repeated in a PHB-producing *E. coli* background (pJM9238) (data not shown). Apparently, the presence of PHB did not significantly influence the two-hybrid assay. However, colour development was less intense, probably because of the accumulation of (white) PHB. Determination of specific β -galactosidase activities after growth of two-hybrid strains in liquid cultures (Fig. 2b) showed that the activities for the combinations of the *R. eutropha* *phaC1* gene were twofold [100 U (mg cell dry

Fig. 2. (a) BACTH analysis of PHB synthase subunits. Co-transformants of *E. coli* BTH101 expressing T18 and T25 fusions of (A) *B. megaterium* *phaR*_{Bmeg} and *phaC*_{Bmeg}, and (B) *R. eutropha* *phaC1*_{Reu} and control strains, were grown on LB-X-Gal medium at 30 °C for 2 days. Zip, leucine zipper (positive control). (b) β -Galactosidase activity assay. The efficiency of functional complementation was quantified in suspensions of SDS/toluene-treated *E. coli* BTH101 cells harbouring the indicated constructs. Values shown are the mean results from three independent cultures; error bars, SD. cdw, cell dry weight.

weight)⁻¹] and those for the *B. megaterium* PHB synthase subunits were five- to sixfold [280 U (mg cell dry weight)⁻¹] higher in comparison with the negative controls [~50 U (mg cell dry weight)⁻¹]. Several repetitions of the experiments using different growth temperatures or different growth times always led to similar results, i.e. the specific activities were between 40 and 70 U (mg cell dry weight)⁻¹ for all negative controls; the values for the PhaC1_{Reu} homologomerization experiment were between 80 and 140 U (mg cell dry weight)⁻¹ and those for the *B. megaterium* PHB synthase PhaC and PhaR fusions were between 140 and 280 U (mg cell dry weight)⁻¹. In conclusion, the adenylate cyclase-based two-hybrid system was able to detect protein–protein interactions *in vivo*.

Next, we investigated the interaction between different known ‘PHB players’, and the following genes were

PCR-amplified and each was fused to the pUT18C and pKT25 vectors: the phasin genes *phaP1*, *phaP2*, *phaP3* and *phaP4*; the intracellular PHB depolymerase gene *phaZ1*; the thiolase and reductase genes *phaA* and *phaB1* of the PHB biosynthetic operon; and the regulatory gene *phaR* (Table 1). Please note that PhaR of *R. eutropha* (regulator) is not related to PhaR of *B. megaterium* (PHA synthase subunit). Each of the constructs was transformed in combination with the corresponding empty pendant vector (control) and in all possible permutations into the adenylate cyclase-deficient reporter strain *E. coli* BTH101. An agar-based two-hybrid assay was performed with all 101 individual *E. coli* clones using MacConkey agar and M63-maltose medium at 30 °C. Overall, the two indicator media gave similar results (Fig. 3a, b). The M63-maltose medium (Fig. 3b) was more sensitive than MacConkey agar (Fig. 3a), as growth of the strains on M63-maltose agar depends on

Fig. 3. BACTH analysis of proteins involved in PHB metabolism of *R. eutropha*. Co-transformants of *E. coli* BTH101 expressing the indicated T18 and T25 fusions were spotted on MacConkey agar (a) or M63-maltose-mineral salts agar (b), and plates were incubated for 1.5 days at 30 °C. -, Empty vector.

functional interactions of T18 and T25 fragments, while on MacConkey agar all strains can grow and the degree of protein interaction is indicated by (red) colour development only.

All negative controls in which the pKT25 bait constructs were tested in combination with the empty pUT18C vector and most pUT18C bait constructs with empty pKT25 resulted in no significant growth, similar to the strain with empty pKT25 or pUT18C plasmids (left lane

of Fig. 3b). The control strains with the pUT18C-*phaR*, pUT18C-*phaA* and pUT18C-*phaB1* constructs together with empty pKT25 showed weak growth and weak blue colour development, indicating minor background activity. The reverse experiments, i.e. the pKT25-*phaR*, pKT25-*phaA* and pKT25-*phaB1* constructs plus empty pUT18C, did not show a positive reaction. Therefore, we considered only those assays as positive which showed growth and colour development in both plasmid combinations.

Homo-oligomerization

All clones in which the same gene was fused to the T18 and T25 fragments were able to develop blue colonies (diagonals in Fig. 3). The strongest growth was obtained for clones harbouring PhaP2 or PhaR constructs. Apparently, the respective gene products are able to form oligomers *in vivo*.

Interactions between different PGAPs

(i) Phasins. All clones in which combinations of different phasin genes were tested for interaction gave more or less intense positive results. In particular, two-hybrid assays in which *phaP2* was combined with any of the other three phasin genes resulted in strong growth and colour development. The PhaP2–PhaP4 interaction was notably strong, comparable with the positive control (leucine zipper). Apparently, phasins not only are able to form homo-oligomers but also can associate with each other and can form hetero-oligomers. Phasins also showed weak interactions with the intracellular PHB depolymerase PhaZ1.

(ii) PHB depolymerase PhaZ1. When *E. coli* clones harbouring the *phaZ1* gene were assayed for their interaction potential with other Pha proteins, a weak signal was found with phasins, in particular with PhaP2 and PhaP3. A weak two-hybrid reaction near the detection limit was also found for PhaZ1 with the PHB synthase PhaC1 and with the regulator PhaR.

(iii) PHB synthase PhaC1. PhaC1 showed no interaction with any of the other tested gene products, except for a poor degree of homo-oligomerization and a weak reaction with PhaZ1.

(iv) Regulator PhaR. PhaR gave a very strong result in the homo-oligomerization experiment that was comparable with the positive control (leucine zipper), and a weak reaction near the detection limit with PhaZ1.

(v) PhaA and PhaB. Clones harbouring *phaA* or *phaB1* fusions gave no growth or colour development on maltose agar in any of the tested combinations, except for homo-oligomerization.

Screening for proteins capable of interaction with PhaP2

The results described above indicated that some PHB granule-associated proteins are able to interact *in vivo* with each other. In particular, phasin PhaP2 revealed the highest degree of interaction with other PHB granule-associated proteins. This finding prompted us to investigate whether PhaP2 can interact with other proteins that have not yet been identified as PGAPs. The pKT25-*phaP2* construct was used as bait to screen a *R. eutropha* H16 genomic library fused to pUT18C (see Methods and Fig. 1) to address this question. Several dozen blue-coloured clones developed within 4–6 days at 30 °C, out of which 53 were isolated and sequenced. Interestingly, 24 clones that revealed the

strongest growth and colour development on M63-maltose agar all contained parts of the coding sequence of the B1934 gene fused in-frame to the adenylate cyclase T18 fragment. The B1934 gene product is annotated as a hypothetical protein in the database and has a ‘phasin protein’ motif. The other sequenced clones that showed less intense growth on M63-maltose agar harboured parts of different genes, such as putative chemotaxis proteins (B0232, B2044), hypothetical transcriptional regulators (B0535, B1446, B1787), putative acyl-CoA dehydrogenases (A1530, B0703), and several other hypothetical proteins. The frequent identification of the B1934 gene and the phasin protein motif in the deduced amino acid sequences suggested that B1934 could encode a novel PGAP. The complete coding sequence (429 bp, corresponding to a polypeptide of 15.7 kDa) was fused to the T25 and T18 fragments, and the two-hybrid assay was repeated with all nine other *pha* genes. The interaction of the B1934 gene product with PhaP2 was confirmed in both plasmid combinations (Fig. 4a). Interestingly, the B1934 gene product also interacted with the other phasins, with PhaR and with PhaZ1, and showed high homo-oligomerization potential. No interaction was detected with the PHB synthase PhaC1 and with PhaA or PhaB1. These results were confirmed by quantitative determination of β -galactosidase activities after growth of the respective *E. coli* clones (Fig. 4b): the highest values were obtained for the homo-oligomerization (2100 MU), for interaction with PhaP2 (1150 MU), PhaR (530 MU), PHB depolymerase PhaZ1 (360 MU) and the other phasins (100–250 MU). The values for the controls (T18-PhaP5 with T25 and T25-PhaP5 with T18) amounted to 55 and 40 MU, respectively.

Subcellular localization of the B1934 gene product

Our results indicate that the B1934 gene product is probably a new ‘PHB player’, and presumably is attached to the PHB granule surface. To find evidence for this assumption, an *eyfp-b1934* fusion was constructed in pBBR1MCS-2 (under the control of the constitutive *phaC* promoter). The resulting construct, pBBR1MCS-2::P_{phaC}-*eyfp-b1934*, was conjugatively transferred to *R. eutropha* and investigated for PHB granule formation and localization of fusion proteins. PHB-free cells of *R. eutropha* transconjugants harbouring the constructs with and without the fusion were transferred to medium permissive for PHB accumulation (30 °C), and samples were analysed by fluorescence microscopy. As shown in Fig. 5(a), free eYfp (control) was distributed in the cytoplasm independently of the presence or absence of PHB granules. PHB-free (stationary) cells of *R. eutropha* harbouring pBBR1MCS-2::P_{phaC}-*eyfp-b1934* could not be distinguished from eYfp-expressing cells (Fig. 5b). However, most cells of samples that had been taken after incubation under conditions permissive for PHB accumulation harboured PHB granules (stained with Nile red) that were almost exclusively located at the cell poles or in the

Fig. 4. BACTH analysis of B1934 (PhaP5) and known Pha proteins of *R. eutropha*. (a) Co-transformants of *E. coli* BTH101 expressing T18 and T25 fusions were spotted on M63-maltose-mineral salts agar, and plates were incubated at 30 °C for about 2 days at 30 °C. (b) The efficiency of functional complementation was quantified by measuring β -galactosidase activities in suspensions of SDS/toluene-treated *E. coli* BTH101 cells harbouring the corresponding plasmids after growth at 30 °C for 16 h. Values shown are the mean results from three independent cultures; error bars, SD. The Zip/Zip control yielded 2600 MU in this experiment (results not shown). -, Empty vector.

area of future septum formation (Fig. 5c, d). The eYfp fluorescence colocalized with PHB granules and confirmed our assumption that the B1934 gene product is a PHB granule-associated phasin *in vivo*. At later stages of growth, PHB granules always colocalized with eYfp fluorescence (not shown). A chromosomal deletion mutant of B1934 was constructed for strains HF39 and H16 and investigated for PHB granule formation. However, the number, size and localization of PHB granules did not differ significantly from those of the respective wild-type (not shown). When an eYfp-PhaP2 or an eYfp-PhaP4 fusion was expressed in *R. eutropha*, a colocalization of eYfp fluorescence and PHB granules was observed, confirming that PhaP2 and PhaP4 also represent true PGAPs (phasins) (Fig. 5e-h). However, formation of only cell pole-localized granules as for the eYfp-B1934 fusion was not observed for the PhaP2 and PhaP4 fusions.

DISCUSSION

In this study we used the bacterial adenylate cyclase-based two-hybrid (BACTH) system (Karimova *et al.*, 2000b) to

investigate interactions between proteins involved in PHB metabolism. The utility of the system was first tested for interactions between the class I model PHB synthase subunits (PhaC1_{Reu} of *R. eutropha*) and the class IV model PHB synthase of *B. megaterium* (PhaC_{Bmeg} and PhaR_{Bmeg}) (McCool & Cannon, 2001). Two-hybrid assays with phaC_{Bmeg} and phaR_{Bmeg} resulted in the formation of blue colonies on indicator media (Fig. 1) and in levels of β -galactosidase activity that were three to five times higher than in the negative control. β -Galactosidase activity was significantly lower than the activity for the positive control (leucine zipper). However, one should consider that PhaC_{Bmeg} (40 kDa) and PhaR_{Bmeg} (22 kDa) are relatively large molecules that probably reduce the physical association of the two adenylate cyclase fragments by spatial hindrance. The positive control, the leucine zipper, is a domain of only ~6 kDa, and is therefore unlikely to influence the association of adenylate cyclase fragments.

The two-hybrid assay with the *R. eutropha* class I PHB synthase (64 kDa, PhaC1_{Reu}) also resulted in the formation of blue colonies and in specific β -galactosidase activities that

Fig. 5. Microscopic analysis of cells of *R. eutropha* HF39 harbouring pBBR1MCS-2- P_{phaC} -*eyfp-c1*. Note the uniform fluorescence of the cells, indicating soluble localization of free eYfp (a). *R. eutropha* HF39 harbouring pBBR1MCS-2- P_{phaC} -*eyfp-phaP5* (b–d): PHB-free cells (24 h NB seed culture) (b) were transferred to conditions permissive for PHB accumulation (0.1 vol. seed culture to fresh NB + 0.2% sodium gluconate) (c, d) and incubated at 30 °C. Note the formation of eYfp foci predominantly at the cell periphery (cell poles) (c). The green foci colocalize with PHB granules, as indicated by staining with Nile red (d). *R. eutropha* H16 harbouring pBBR1MCS-2- P_{phaC} -*eyfp-phaP2* (e, f) or pBBR1MCS-2- P_{phaC} -*eyfp-phaP4* (g, h) after transfer to conditions permissive for PHB accumulation. (e, g) eYfp fluorescence, (f, h) Nile red fluorescence. Bars, 3 μ m.

were about two to three times higher than background. Earlier studies by others have suggested that homo-dimerization of PhaC1_{Reu} is required for PHB synthesis *in vitro* (Stubbe *et al.*, 2005; Wodzinska *et al.*, 1996; Zhang *et al.*, 2000, 2003). It is assumed that dimerization and binding of 3-hydroxybutyrate are the rate-limiting steps in the initiation of PHB synthesis by *R. eutropha* PHB synthase, and are responsible for the occurrence of a lag phase before maximal *in vitro* activity is obtained. The lower β -galactosidase activity in comparison with the two-hybrid assay of *B. megaterium* PHB synthase can be explained by homo-dimerization of the T18-PhaC_{Reu} and T25-PhaC_{Reu} constructs, which reduces the probability of forming mixed dimers of T18-PhaC_{Reu} with T25-PhaC_{Reu}. Only the mixed dimer will lead to reconstitution of adenylate cyclase activity and to formation of β -galactosidase activity. To our knowledge, the data confirm the postulated dimerization of PHB synthase *in vivo* for the first time.

A remarkable finding of the two-hybrid assays between nine known 'PHB players' (PhaA, PhaB1, PhaC1, PhaP1–4, PhaR and PhaZ1) was the strong interaction between PhaP2 and other phasins, in particular with PhaP4 (Pötter *et al.*, 2004). Previous work by Pötter and co-workers has shown that *in vivo* PhaP2 is apparently not detectably bound to isolated nPHB granules, although it is expressed in PHB-accumulating cells and has the capacity to bind to (artificial) PHB (Kuchta *et al.*, 2007; Pötter *et al.*, 2004, 2005; Pötter & Steinbüchel, 2006). Srinivasan *et al.* (2002)

detected PhaP2 by 2D PAGE of whole-cell extracts of fructose-induced cells, but at that time the *R. eutropha* genome sequence had not been published, and the protein was not identified as a phasin. In this study we confirmed that PhaP2 and PhaP4 clearly are attached to PHB granules *in vivo* and represent true phasins (Fig. 5). One could speculate that PhaP1 and/or other phasins control the access of PHB depolymerases such as PhaZ1 and/or PGAPs to the PHB granule surface. This assumption is supported by the finding that nPHB granules isolated from *R. eutropha* Δ *phaP1* mutants have a threefold higher PHB depolymerase activity than nPHB granules of the wild-type (unpublished results). Dennis and co-workers recently found a net-like structure on the surface of isolated PHB granules by atomic force microscopy that was reminiscent of a cytoskeleton (Dennis *et al.*, 2003, 2008). It could be that PhaP2 and/or other PGAPs are part of this structure. This question was addressed by screening a genomic library for proteins interacting with PhaP2. The B1934 gene was identified in 24 clones with the strongest growth in the two-hybrid screening experiment. Analysis of published microarray data (Budde *et al.*, 2010) indicates that B1934 is expressed under conditions permissive for PHB accumulation. The genes up- and downstream of B1934 on the chromosome are both transcribed in the opposite direction from B1934. Therefore, B1934 is not part of an operon. The eYfp–B1934 fusion protein was clearly identified as a PHB granule-bound protein (Fig. 5). We conclude that

Table 3. Similarity of *R. eutropha* PhaP5 to other phasins

The identity values (above the diagonal of 100 % values) and similarity values (below the diagonal) for the global alignment of PhaP5 (B1934 gene product) with other phasins are given.

Similarity	Identity							
	PhaP1	PhaP3	PhaP2	PhaP4	H16_B1934	RALTA_B1632*	ApdA*	Mms16*
PhaP1	100.0 %	47.4 %	41.2 %	43.3 %	13.9 %	12.4 %	13.4 %	12.4 %
PhaP3	62.4 %	100.0 %	37.6 %	38.7 %	17.0 %	14.9 %	16.0 %	14.4 %
PhaP2	57.7 %	53.1 %	100.0 %	69.6 %	12.9 %	12.4 %	16.5 %	12.9 %
PhaP4	60.8 %	55.2 %	78.9 %	100.0 %	13.4 %	11.9 %	16.0 %	12.9 %
H16_B1934	22.2 %	25.2 %	20.6 %	19.6 %	100.0 %	84.5 %	41.8 %	54.1 %
RALTA_B1632	21.1 %	23.7 %	20.1 %	18.6 %	87.6 %	100.0 %	37.6 %	47.9 %
ApdA	24.2 %	26.3 %	27.8 %	27.3 %	56.2 %	52.6 %	100.0 %	58.2 %
Mms16	22.7 %	25.3 %	25.3 %	22.2 %	64.9 %	59.8 %	73.2 %	100.0 %

*See text.

B1934 is a novel *R. eutropha* phasin protein, for which we propose the designation PhaP5. PhaP5 was able to interact with other phasins, in particular with PhaP2, but also with PhaR and PhaZ1. Our results show that some but not all proteins necessary for PHB synthesis have the ability to bind to each other, and indicate that the spatial organization of at least some of the proteins on the PHB granule surface is controlled. Our data also indicate that several phasins and other PGAPs constitute a complex ordered surface layer on nPHB granules that might result in the network-like structure on the PHB granule surface observed by Dennis *et al.* (2008). The deduced amino acid sequence of B1934 (15.7 kDa) revealed significant similarities to phasin proteins of species related to *R. eutropha* (65–90 % identity): *Cupriavidus taiwanensis* (RALTA_B1632, 85 %), *Rhodospirillum rubrum* (ApdA, 42 %) and *Magnetospirillum magneticum* (Mms16, 54 %) (Handrick *et al.*, 2004). However, purified His-tagged B1934 protein did not increase the hydrolysis of nPHB granules by *R. rubrum* PhaZ1-like ApdA or Mms16 (unpublished data). The similarities of PhaP5 to *R. eutropha* phasins PhaP1 to PhaP4 were rather low (≤ 17 % identity, Table 3). In conclusion, PhaP5, together with RALTA_B1632, ApdA and Mms16, is a member of a new subgroup of phasin-like PGAPs in PHB-accumulating bacteria. The function of PhaP5 remains unclear: constitutive (over)expression of PhaP5 resulted in the formation of exclusively polar-localized PHB granules (Fig. 5). The same phenotype was observed for *R. eutropha* Δ phaP1–4: only polar-localized PHB granules were formed by this mutant (unpublished results). However, deletion of phaP5 alone showed no phenotype under the microscope. Apparently, a high concentration of PhaP5 (in relation to other phasins), obtained either by deletion of the other phasin genes or by overexpression of PhaP5, results in the formation of cell pole-localized PHB granules. The function of phasins PhaP2–PhaP4 remains unclear.

The detection of interactions between several classes of PGAPs is in agreement with the idea of a sophisticated 3D

order of proteins with functions in PHB metabolism, and supports the idea of PHB granules being organelles (carbonosomes) (Jendrossek, 2009). At present, we do not know whether the identification of chemotaxis proteins and of other hypothetical proteins as potential interaction proteins for PhaP2 is significant or whether these clones are false-positives. Interestingly, a connection between PHB metabolism and motility and flagellin synthesis has been described (Raberg *et al.*, 2008), and indicates that the two-hybrid clones with chemotaxis genes may encode proteins that interact with PGAPs. It will be important to extend the two-hybrid screening assays to the remaining PHB granule-associated proteins and to find out whether other PGAPs are still missing, as well as to confirm the associations identified between individual proteins in the PHB-accumulating strain itself, e.g. by Förster (fluorescence) resonance energy transfer (FRET) analysis.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft. We thank D. Ladant, Institut Pasteur, Paris, France, for providing plasmid pUT18BC, and Pia Kaufmann for technical assistance in certain experiments.

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.
- Budde, C. F., Mahan, A. E., Lu, J., Rha, C. & Sinskey, A. J. (2010). Roles of multiple acetoacetyl coenzyme A reductases in polyhydroxybutyrate biosynthesis in *Ralstonia eutropha* H16. *J Bacteriol* **192**, 5319–5328.
- Dennis, D., Liebig, C., Holley, T., Thomas, K. S., Khosla, A., Wilson, D. & Augustine, B. (2003). Preliminary analysis of polyhydroxyalkanoate inclusions using atomic force microscopy. *FEMS Microbiol Lett* **226**, 113–119.
- Dennis, D., Sein, V., Martinez, E. & Augustine, B. (2008). PhaP is involved in the formation of a network on the surface of

- polyhydroxyalkanoate inclusions in *Cupriavidus necator* H16. *J Bacteriol* **190**, 555–563.
- Dove, S. L. & Hochschild, A. (2001). Bacterial two-hybrid analysis of interactions between region 4 of the σ^{70} subunit of RNA polymerase and the transcriptional regulators Rsd from *Escherichia coli* and AlgQ from *Pseudomonas aeruginosa*. *J Bacteriol* **183**, 6413–6421.
- Dove, S. L. & Hochschild, A. (2004). A bacterial two-hybrid system based on transcription activation. *Methods Mol Biol* **261**, 231–246.
- Fields, S. & Song, O. (1989). A novel genetic system to detect protein–protein interactions. *Nature* **340**, 245–246.
- Handrick, R., Reinhardt, S. & Jendrossek, D. (2000). Mobilization of poly(3-hydroxybutyrate) in *Ralstonia eutropha*. *J Bacteriol* **182**, 5916–5918.
- Handrick, R., Reinhardt, S., Schultheiss, D., Reichart, T., Schüler, D., Jendrossek, V. & Jendrossek, D. (2004). Unraveling the function of the *Rhodospirillum rubrum* activator of polyhydroxybutyrate (PHB) degradation: the activator is a PHB-granule-bound protein (phasin). *J Bacteriol* **186**, 2466–2475.
- Jendrossek, D. (2005). Fluorescence microscopical investigation of poly(3-hydroxybutyrate) granule formation in bacteria. *Biomacromolecules* **6**, 598–603.
- Jendrossek, D. (2009). Polyhydroxyalkanoate granules are complex subcellular organelles (carbonosomes). *J Bacteriol* **191**, 3195–3202.
- Jendrossek, D., Selchow, O. & Hoppert, M. (2007). Poly-(3-hydroxybutyrate) granules at the early stages of formation are localized close to the cytoplasmic membrane in *Caryophanon latum*. *Appl Environ Microbiol* **73**, 586–593.
- Karimova, G., Pidoux, J., Ullmann, A. & Ladant, D. (1998). A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc Natl Acad Sci U S A* **95**, 5752–5756.
- Karimova, G., Ullmann, A. & Ladant, D. (2000a). *Bordetella pertussis* adenylate cyclase toxin as a tool to analyze molecular interactions in a bacterial two-hybrid system. *Int J Med Microbiol* **290**, 441–445.
- Karimova, G., Ullmann, A. & Ladant, D. (2000b). A bacterial two-hybrid system that exploits a cAMP signaling cascade in *Escherichia coli*. *Methods Enzymol* **328**, 59–73.
- Karimova, G., Ullmann, A. & Ladant, D. (2001). Protein-protein interaction between *Bacillus stearothermophilus* tyrosyl-tRNA synthetase subdomains revealed by a bacterial two-hybrid system. *J Mol Microbiol Biotechnol* **3**, 73–82.
- Karimova, G., Dautin, N. & Ladant, D. (2005). Interaction network among *Escherichia coli* membrane proteins involved in cell division as revealed by bacterial two-hybrid analysis. *J Bacteriol* **187**, 2233–2243.
- Kidwell, J., Valentin, H. E. & Dennis, D. (1995). Regulated expression of the *Alcaligenes eutrophus pha* biosynthesis genes in *Escherichia coli*. *Appl Environ Microbiol* **61**, 1391–1398.
- Kobayashi, T., Shiraki, M., Abe, T., Sugiyama, A. & Saito, T. (2003). Purification and properties of an intracellular 3-hydroxybutyrate-oligomer hydrolase (PhaZ2) in *Ralstonia eutropha* H16 and its identification as a novel intracellular poly(3-hydroxybutyrate) depolymerase. *J Bacteriol* **185**, 3485–3490.
- 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.
- Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., II & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**, 175–176.
- Kuchta, K., Chi, L., Fuchs, H., Pötter, M. & Steinbüchel, A. (2007). Studies on the influence of phasins on accumulation and degradation of PHB and nanostructure of PHB granules in *Ralstonia eutropha* H16. *Biomacromolecules* **8**, 657–662.
- Lenz, O. & Friedrich, B. (1998). A novel multicomponent regulatory system mediates H₂ sensing in *Alcaligenes eutrophus*. *Proc Natl Acad Sci U S A* **95**, 12474–12479.
- McCool, G. J. & Cannon, M. C. (1999). Polyhydroxyalkanoate inclusion body-associated proteins and coding region in *Bacillus megaterium*. *J Bacteriol* **181**, 585–592.
- McCool, G. J. & Cannon, M. C. (2001). PhaC and PhaR are required for polyhydroxyalkanoic acid synthase activity in *Bacillus megaterium*. *J Bacteriol* **183**, 4235–4243.
- Pieper-Fürst, U., Madkour, M. H., Mayer, F. & Steinbüchel, A. (1994). Purification and characterization of a 14-kilodalton protein that is bound to the surface of polyhydroxyalkanoic acid granules in *Rhodococcus ruber*. *J Bacteriol* **176**, 4328–4337.
- Pohlmann, A., Fricke, W. F., Reinecke, F., Kusian, B., Liesegang, H., Cramm, R., Eitinger, T., Ewering, C., Pötter, M. & other authors (2006). Genome sequence of the bioplastic-producing “Knallgas” bacterium *Ralstonia eutropha* H16. *Nat Biotechnol* **24**, 1257–1262.
- Pötter, M. & Steinbüchel, A. (2006). Biogenesis and structure of polyhydroxyalkanoate granules. In *Inclusions in Prokaryotes*, pp. 109–136. Edited by 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. (2003). Polyester synthases: natural catalysts for plastics. *Biochem J* **376**, 15–33.
- Rehm, B. H. (2006). Genetics and biochemistry of polyhydroxyalkanoate granule self-assembly: the key role of polyester synthases. *Biotechnol Lett* **28**, 207–213.
- Ruth, K., de Roo, G., Egli, T. & Ren, Q. (2008). Identification of two acyl-CoA synthetases from *Pseudomonas putida* GPo1: one is located at the surface of polyhydroxyalkanoates granules. *Biomacromolecules* **9**, 1652–1659.
- 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.
- 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**, 794–791.
- Srinivasan, S., Barnard, G. C. & Gerngross, T. U. (2002). A novel high-cell-density protein expression system based on *Ralstonia eutropha*. *Appl Environ Microbiol* **68**, 5925–5932.

- Srivastava, S., Urban, M. & Friedrich, B. (1982). Mutagenesis of *Alcaligenes eutrophus* by insertion of the drug-resistance transposon Tn5. *Arch Microbiol* **131**, 203–207.
- Steinbüchel, A. & Hein, S. (2001). Biochemical and molecular basis of microbial synthesis of polyhydroxyalkanoates in microorganisms. *Adv Biochem Eng Biotechnol* **71**, 81–123.
- Stubbe, J. & Tian, J. (2003). Polyhydroxyalkanoate (PHA) homeostasis: the role of PHA synthase. *Nat Prod Rep* **20**, 445–457.
- Stubbe, J., Tian, J., He, A., Sinskey, A. J., Lawrence, A. G. & Liu, P. (2005). Nontemplate-dependent polymerization processes: polyhydroxyalkanoate synthases as a paradigm. *Annu Rev Biochem* **74**, 433–480.
- Sudesh, K., Abe, H. & Doi, Y. (2000). Synthesis, structure and properties of polyhydroxyalkanoates: biological polymers. *Prog Polym Sci* **25**, 1503–1555.
- Thibodeau, S. A., Fang, R. & Joung, J. K. (2004). High-throughput beta-galactosidase assay for bacterial cell-based reporter systems. *Biotechniques* **36**, 410–415.
- Uchino, K., Saito, T., Gebauer, B. & Jendrossek, D. (2007). Isolated poly(3-hydroxybutyrate) (PHB) granules are complex bacterial organelles catalyzing formation of PHB from acetyl coenzyme A (CoA) and degradation of PHB to acetyl-CoA. *J Bacteriol* **189**, 8250–8256.
- Wang, Z. H., Ma, P., Chen, J., Zhang, J., Chen, C. B. & Chen, G. Q. (2011). A transferable heterogeneous two-hybrid system in *Escherichia coli* based on polyhydroxyalkanoates synthesis regulatory protein PhaR. *Microb Cell Fact* **10**, 21.
- Wodzinska, J., Snell, K. D., Rhomberg, A., Sinskey, A. J., Biemann, K. & Stubbe, J. (1996). Polyhydroxybutyrate synthase: evidence for covalent catalysis. *J Am Chem Soc* **118**, 6319–6320.
- York, G. M., Junker, B. H., Stubbe, J. A. & Sinskey, A. J. (2001a). Accumulation of the PhaP phasin of *Ralstonia eutropha* is dependent on production of polyhydroxybutyrate in cells. *J Bacteriol* **183**, 4217–4226.
- York, G. M., Stubbe, J. & Sinskey, A. J. (2001b). New insight into the role of the PhaP phasin of *Ralstonia eutropha* in promoting synthesis of polyhydroxybutyrate. *J Bacteriol* **183**, 2394–2397.
- York, G. M., Stubbe, J. & Sinskey, A. J. (2002). The *Ralstonia eutropha* PhaR protein couples synthesis of the PhaP phasin to the presence of polyhydroxybutyrate in cells and promotes polyhydroxybutyrate production. *J Bacteriol* **184**, 59–66.
- York, G. M., Lupberger, J., Tian, J., 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.
- Zhang, S., Yasuo, T., Lenz, R. W. & Goodwin, S. (2000). Kinetic and mechanistic characterization of the polyhydroxybutyrate synthase from *Ralstonia eutropha*. *Biomacromolecules* **1**, 244–251.
- Zhang, S., Kolvek, S., Lenz, R. W. & Goodwin, S. (2003). Mechanism of the polymerization reaction initiated and catalyzed by the polyhydroxybutyrate synthase of *Ralstonia eutropha*. *Biomacromolecules* **4**, 504–509.

Edited by: V. Eijssink