Regulation of phasin expression and polyhydroxyalkanoate (PHA) granule formation in *Ralstonia eutropha* H16

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Regulation of expression of the phasin PhaP, which is the major protein at the surface of polyhydroxyalkanoate (PHA) granules in *Ralstonia eutropha* H16, was studied and analysed at the molecular level. The regulation of PhaP expression is achieved by an autoregulated repressor, which is encoded by phaR in *R. eutropha*. The occurrence of PhaR homologues and the organization of phaR genes was analysed in detail in 29 different bacteria. Three kinds of molecule to which PhaR binds were identified in cells of *R. eutropha*, as revealed by gel-mobility-shift assays, DNaseI footprinting, cell fractionation, immunoelectron microscopy studies employing anti-PhaR antibodies raised against purified N-terminal hexahistidine-tagged PhaR and in vitro binding studies employing artificial PHA granules. PhaR binds upstream of phaP at two sites comprising the transcriptional start site plus the −10 region and a region immediately upstream of the −35 region of the σ70 promoter of phaP, where two imperfect 12 bp repeat sequences (GCAMMAAWTMMD) were identified on the sense and anti-sense strands. PhaR also binds 86 bp upstream of the phaR translational start codon, where the σ54-dependent promoter was identified. PhaR also binds to the surface of PHA granules. In the cytoplasm of a phaRΩKm mutant of *R. eutropha* H16, increased quantities of PhaP were detected and the cells formed by this strain were much smaller and had many more PHA granules present than the wild-type. These data support the following model for the regulation of phaP expression. Under cultivation conditions not permissive for PHA biosynthesis or in mutants defective in PHA biosynthesis, PhaR binds to the phaP upstream region and represses transcription of this gene. After the onset of PHA biosynthesis, under conditions that are permissive for the formation of nascent granules, PhaR binds to PHA granules and phaP is transcribed. At the later stages of PHA accumulation, PhaR no longer binds to the granules and the transcription of phaP is again repressed. In addition to this, phaR expression is subject to autoregulation. Excess PhaR that has not bound to the phaP upstream region or to PHA granules binds to the phaR upstream region, thereby repressing its own transcription.

**Keywords:** phaR, phaP regulation, repressor, inclusion bodies, autoregulation of phaR

INTRODUCTION

Polyhydroxyalkanoates (PHAs) represent storage compounds for carbon and/or energy and occur as water-insoluble inclusions (PHA granules) in a large variety of prokaryotes. In most bacteria, PHAs are accumulated in the cytoplasm if a carbon source is provided in excess and if any other essential nutrient is limited (Anderson & Dawes, 1990). These biopolymers reveal thermoplastic and/or elastomeric properties similar to those of petrochemically produced polypropylene (Steinbüchel, 1991; Müller & Seebach, 1993). Due to their biodegrad-

Abbreviations: GARG, goat-anti-rabbit IgG-gold; His6, hexahistidine; MM, mineral salts medium; PHA, polyhydroxyalkanoate; PHB, polyhydroxybutyrate; poly(3HB), poly(3-hydroxybutyrate).
ability and origin from renewable resources, PHAs have attracted much interest, and many technical applications have been developed for them in the last few decades (Anderson & Dawes, 1990; Hocking & Marchessault, 1994). Since the discovery of poly(3-hydroxybutyrate) [poly(3HB)], in *Bacillus megaterium* over 70 years ago (Lemoigne, 1926), many different hydroxyalkanoic acids have been identified as constituents of PHAs, comprising different carbon chain lengths and also various substrates at different positions of the R-pendant groups attached to the backbone of the polymer (Steinbüchel & Valentin, 1995). One of the best studied microorganisms with respect to PHA accumulation is the Gram-negative bacterium *Ralstonia eutropha*. It synthesizes PHAs that consist of hydroxyalkanoic acids with a short carbon chain length, with 3-hydroxybutyric acid as the predominant constituent. Other hydroxyalkanoic acids have also been identified as constituents of PHAs (e.g. Holmes *et al.*, 1981; Kunioka *et al.*, 1988; Doi *et al.*, 1990). Just recently, the biosynthesis of 3-mercaptoalkanoic-acid-containing polymers by *R. eutropha* has also been demonstrated and, with reference to the different linkage type in the polymer backbone, these biopolymers were designated as polythioesters (Lütke-Eversloh *et al.*, 2001a, b).

*R. eutropha* is regarded as a model organism, along with *Allochromatium vinosum*, for studying short-carbon-chain-length PHA biosynthesis in bacteria (Steinbüchel & Schlegel, 1991). The genes responsible for PHA biosynthesis in *R. eutropha* have been cloned and characterized (Slater *et al.*, 1988; Schubert *et al.*, 1988; Peoples & Sinskey, 1989a, b) and comprise a β-ketothiolase (phaA), an acetoacetyl-CoA reductase (phaB) and the PHA synthase (phaC). β-Ketothiolase (EC 2.3.1.9) catalyses the first step of poly(3HB) biosynthesis, i.e. the condensation of two molecules of acetyl-CoA to acetoacetyl-CoA. Acetoacetyl-CoA is then reduced to R-(-)-3-hydroxybutyryl-CoA by an acetoacetyl-CoA reductase (EC 1.1.1.36). In the last step of the reaction, R-(-)-3-hydroxybutyryl-CoA is polymerized to poly(3HB) by PHA synthase, the key enzyme of PHA biosynthesis. The resulting PHA granules are coated with a layer of phospholipids and proteins, with phasins as the predominant compound. Phasins are a class of proteins of between 14 and 28 kDa in size that form a layer at the surface of the hydrophobic core of PHA granules; they also influence the number and size of PHA granules (Wieczorek *et al.*, 1995; Pieper-Fürst *et al.*, 1995; Steinbüchel *et al.*, 1995). The phasin of *R. eutropha* is encoded by *phaP*, and the formation of PhaP is dependent on PHA biosynthesis and accumulation (Wieczorek *et al.*, 1995). In *R. eutropha*, the amount of phasin present in cells has been shown to parallel the amount of PHA present in cells (York *et al.*, 2001a, b).

Adjacent to the *pha*ABC gene cluster, an ORF (designated *phaR*) has been detected in *R. eutropha* (Slater *et al.*, 1998). In this bacterium, PhaR seems to be involved in the regulation of pha and PHA biosynthesis (York *et al.*, 2002). Other genes homologous to *phaR* have been found in several other PHA-accumulating bacteria (Rehm & Steinbüchel, 1999), such as *Sinorhizobium meliloti* (Tombolini *et al.*, 1995; Povolo & Casella, 2000). Recently, a *phaR* homologue (encoding PhaR) was identified in *Paracoccus denitrificans* which was capable of binding to the intergenic regions of *phaC–phaB and phaP–phaR*, and it was proposed that PhaR functions as a negative regulator of phasin synthesis (Maehara *et al.*, 1999, 2001). Whereas much knowledge of PhaR has been gained by studying *P. denitrificans*, less is known about PhaR in the model organism *R. eutropha*. York *et al.* (2002) suggested that PhaR promotes poly(3HB) synthesis in *R. eutropha* by regulating the expression of PhaP and by possibly regulating the expression of additional proteins. The aim of this study was to reveal the molecular mechanisms by which PhaR couples the synthesis of PhaP to the presence of poly(3HB) in *R. eutropha*. To achieve this, the *phaR* gene product of *R. eutropha* was analysed by electron microscopy investigations and DNA-binding experiments and by detailed analyses of a knock-out mutant. In combination with the results of a study that has been published recently (York *et al.*, 2002), a comprehensive model for the regulation of PHA granule formation in *R. eutropha* is suggested and discussed in this work.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Cells of *R. eutropha* were grown at 30 °C in mineral salts medium (MM) supplemented with filter-sterilized sodium gluconate (Schlegel *et al.*, 1961). Solid media contained 1.5% (w/v) purified agar. Tetracycline and kanamycin were used at final concentrations of 25 and 160 µg ml⁻¹, respectively. Cells of *Escherichia coli* were cultivated at 37 °C in Luria–Bertani (LB) medium (Sambrook *et al.*, 1989). Growth was monitored photometrically at 600 nm or with a Klett–Summersom photometer (Manostat, catalogue no. 76-550-220) using filter no. 54 (520–580 nm).

**Isolation and manipulation of DNA.** Chromosomal DNA of *R. eutropha* H16 was isolated by the method of Marmur (1961). Plasmid DNA was isolated by the method of Birnboim & Doly (1979). DNA restriction fragments were purified with Bio-Rad ultrapure agarose. DNA manipulation was performed using filter no. 54 (520–580 nm).

**Transfer of DNA.** Competent cells of *E. coli* were prepared and transformed by using the CaCl₂ procedure described by Hanahan (1983).

**DNA sequencing.** This procedure was done by using the SequiTherm EXCEL TM II Long Read Cycle Sequencing Kit (Epicentre Technologies), IRD800-labelled oligonucleotides (MWG-Biotech) and a Li-Cor 4000L (Li-Cor Biosciences) automated sequencer (MWG-Biotech).

**PCR amplifications.** All PCR amplifications of DNA were carried out as described by Sambrook *et al.* (1989), employing *Pfx* (DNA-polymerase (Gibco-BRL) and an Omigene HBT/R3CM DNA Thermal Cycler (Hybaid).

**Inactivation of phaR in R. eutropha H16 by insertion of ωKm.** To amplify *phaR* from the genomic DNA of *R. eutropha* H16,
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotypic/phenotypic characteristics</th>
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<tr>
<td><strong>Ralstonia eutropha</strong></td>
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<tr>
<td>H16</td>
<td>Wild-type</td>
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<tr>
<td>pphaRΩKm</td>
<td>PhaR-negative; Km'</td>
<td>This study</td>
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<td>H2275</td>
<td>pphaR: Tn5 mutant derived from <em>R. eutropha</em> HF39, Tn5 insertion 156 bp upstream of pphaR</td>
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<td>Stratagene</td>
</tr>
<tr>
<td>TOP10</td>
<td>recA1 endA1 gyrA96 tbl1 badR17 supE44 relA1 ΔlacI ΔM15</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>S17-1</td>
<td>thi proA bsdR17 hsdM' recA RP4-tra-function</td>
<td>Simon et al. (1983a)</td>
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<td><strong>Plasmids</strong></td>
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<td>Amp lacZp/o, T7 and T3 promoters</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pMa/c5-914</td>
<td>Amp cat cI857ts lacZp/o, TIR, P_L and P_R</td>
<td>SK 6772</td>
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<td>pSUP202</td>
<td>mobI, Tcφ Ap' Cm'</td>
<td>Overhage et al. (1999)</td>
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<td>ΔKm in pSKsym</td>
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<td>Ndel-XbaI fragment of <em>R. eutropha</em> harbouring His6-tagged pphaR in</td>
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* DSM, Deutsches Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1B, D-38124 Braunschweig, Germany; SK, Culture Collection of the Institute of Microbiology, Münster, Germany.

oligonucleotides pphaR_up (5'-AAAAAGCATGCGACGCACCAT-ATGTCGTCGACGCA-3') and pphaR_down (5'-AAAAGCATGCGACGCACCAT-ATTGCGTCGTCGACGCA-3') were designed (*Pvu* restriction sites are shown in bold). The 987 bp PCR product was cloned into pSK Bluescript, to create pSKphaR. This plasmid was then digested with *Stu*I and the linearized plasmid (pSKphaR) was ligated with ΔKm that had been recovered from *Sma*I-digested pSKsymΩKm DNA, whose construction has been described by Overhage et al. (1999). *E. coli* XL-1 Blue was transformed with the ligation mixture and transformants harbouring the resulting hybrid pSKphaRΩKm, conferring kanamycin resistance, were obtained. To exchange the functional *phaR* gene for the inactivated one, pphaRΩKm was cloned into the suicide vector pSUP202 (Simon et al., 1983a). To achieve this, pphaRΩKm was isolated from pSKphaRΩKm after its digestion with *Pvu*I and the fragment was ligated with *Pvu*I-digested pSUP202. *E. coli* S17-1 was transformed with the ligation mixture and transformants harbouring the hybrid pSUP202phaRΩKm, conferring resistance to tetracycline and kanamycin, were obtained. Subsequently, pSUP202phaRΩKm was transferred to *R. eutropha* H16 by conjugation. Homogenates resulting from a double crossover exhibiting a kanamycin-resistant phenotype were selected and distinguished from heterologotes resulting from only a single crossover and exhibiting a tetracycline- plus kanamycin-resistant phenotype on MM agar plates containing the respective antibiotics. The genotype of homogenotes was controlled by PCR and DNA sequencing.

**Cloning of pphaR and purification of recombinant *phaR***. For the cloning of pphaR into *E. coli*, PCR was done using pphaR_His6_5 (5'-AAAAAATCATGATGACACCAACACCAACACATGGCCACCGACACAAAAAGGCGC-3') as the sense primer and pphaR_His6_3 (5'-AAAAAATCTAGACAGCGTGCGGATATGCC-3') as the anti-sense primer. These primers were deduced from the upstream and downstream regions, respectively, of the *phaR* gene of *R. eutropha* H16 (Slater et al., 1998). The pphaRHis6 PCR product obtained was purified and ligated into pMa/c5-915 (Table 1), which harbours the cI857ts gene encoding the temperature-sensitive λ repressor. The recombinant His6-PhaR (N-terminal fusion) was purified from *E. coli* TOP10 harbouring pMa/c5-914phaRHis6. Protein purification under native and denaturing conditions was conducted with Ni-NTA Spin Columns (Qiagen), as described by the manufacturer.

**PAGE and Western immunoblotting**. Protein samples were resuspended in gel loading buffer (0.6% (w/v) SDS, 1.25% (w/v) β-mercaptoethanol, 0.25 mM EDTA, 10% (v/v) glycerol, 0.08% (w/v) bromophenol blue, 12.5 mM Tris/HCl, pH 6.8) and separated in SDS 12.5% (w/v) polyacrylamide gels, as described by Laemmli (1970). Proteins were stained with Coomasie brilliant blue R-250 (Weber & Osborn, 1969) or with silver (Heukeshoven & Dernick, 1985). Immunological detection of the PhaR protein blotted from the SDS-polyacrylamide gel onto PVDF membranes was done exactly as described by Towbin et al. (1979).

**Preparation and purification of antibodies**. Approximately 850 µg of the PhaR-His6 protein was dissolved in 500 µl Tris/HCl (pH 7.0). This was then separated by SDS-PAGE and blotted onto a PVDF membrane. The membrane was sent to Eurogentec (Herstal, Belgium) for the production of polyclonal anti-PhaR antiserum in rabbits. The IgG fraction of the serum was purified on a protein A-Sepharose CL-4B affinity column (Hjelm et al., 1972). To obtain highly monospecific antibodies against PhaR, the antiserum was subjected to an affinity purification according to the method of Olmsted (1981) with the modifications of Pieper-Fürst et al.
(1994). Antibodies against PhaP were available from previous studies (Wieczorek et al., 1995).

**Primer extension.** The oligonucleotide primer PE.phaR (5'-ATCGCTTTGCGGCCTGCTGCACC-3') was 5'-labelled with IRD800. Total RNA (1 µg) extracted from R. eutropha H16 cells, which were grown under the storage conditions described above, was purified by using the RNeasy Mini Kit (Qiagen). The extension reaction was carried out at 50 °C for 50 min in a total volume of 50 µl of the presence of 10 U of Superscript II reverse transcriptase, as described by the manufacturer (Invitrogen). After its digestion with RNase A for 30 min at 37 °C, the sample was precipitated with ethanol, dissolved in H₂O and then analysed on a 6% (w/v) polyacrylamide sequencing gel containing 8 M urea, employing the Li-Cor 4000L automated DNA sequencer. Performing the reaction using the same labelled primer was run alongside the sample to determine the size of the primer-extension product. The phaR PCR product described in the ‘gel-mobility-shift assay’ was used as template for the sequencing reaction.

**Gel-mobility-shift assay.** To generate fragments comprising the upstream and downstream regions of phaP and phaR that could be employed in this assay, PCR was done using the genomic DNA of R. eutropha H16 and the appropriate primers. Amplification using primers USP.phaR-shift (5'-GGATGATCTGCAGCAAGA-3') and DSP.phaR-shift (5'-CCCAATGGCCCGATCCTGAG-3') with subsequent digestion of the PCR products with PstI and Smal produced fragments of 126, 232, 518 and 775 bp. PCR products generated using primers USP.phaR-shift (5'-GCAATGGCCGATCCTGAG-3') and DSP.phaR-shift (5'-CCAATGGCCCGACAGA-3') were digested with PstI and give fragments of 330 and 685 bp. These DNA fragments (1-5 µg) were mixed with purified PhaR–His₉ (10–2000 ng) in binding buffer [1 mM EDTA, 10 mM Tris/HCl (pH 7.0), 80 mM NaCl, 10 mM β-mercaptoethanol, 5% (w/v) glycerol] in a total volume of 20 µl. Incubation was carried out for 40 min at room temperature. After this incubation, 10 × loading dye (250 mM Tris/HCl (pH 7.5), 0.2% (w/v) bromophenol blue, 0.2% (w/v) xylene cyanol, 40% (w/v) glycerol) was added to the samples. DNA–protein complexes were separated from unbound DNA fragments in 8% (w/v) native polyacrylamide gels, using 2 × Tris/borate/EDTA buffer (Sambrook et al., 1989). After electrophoresis, the gels were stained with ethidium bromide and the bands were visualized with a UV light.

**DNasel footprinting.** DNasel footprinting was performed by using non-radioactive probes containing the IRD800 label in combination with a Li-Cor sequencer. For this purpose, DNA probes were prepared as follows. The PCR products described in the ‘gel-mobility-shift assay’ were used as template DNA in PCR reactions with an IRD800-labelled primer (footprint III, 5'-CCCGGATTTGGCTGCACCCGCCGATGC-3') in combination with DSP.phaR-shift (5'-GCCCAGATTTGCTGCGATCCCGAG-3') and USP.phaR-shift (5'-CTGGCAGGCCGCCAG-3') in combination with PE.phaR (5'-ATCGCTTTGCGGCCTGCTGCACC-3'). Ten nanograms of the IRD800-labelled fragment was used per reaction. The binding reaction conditions for DNasel footprinting were identical with the conditions used in the ‘gel-mobility-shift assay’. DNasel cleavage was done by adding 20 µl of a solution containing 5 mM CaCl₂, 10 mM MgCl₂ and 2.5 µl of DNasel (Gibco-BRL) to the PCR products. After 1 min, the DNasel reaction was stopped by the addition of 20 µl of 4 M ammonium acetate and 30 mM EDTA. The DNA was extracted with 60 µl of phenol, precipitated with 96% ethanol in the presence of 40 µl of 50% glycyogen and washed with 70% (v/v) ethanol. The pellet was dissolved in 1 µl of formamide loading buffer, heated at 95 °C for 5 min and then chilled on ice. Subsequently, 0.8 µl of the sample was analysed on a Li-Cor 4000L sequencer using a 6% denaturing sequence gel with 0.2 mm spacers and settings at 2000 V, 25 mA, 50 W and 45 °C.

**Electron microscopy studies.** Cells were washed and suspended in 50 mM potassium phosphate buffer (pH 6.8), fixed in the presence of a mixture of 0.2% (v/v) glutaraldehyde plus 0.3% (w/v) paraformaldehyde and embedded in Spurr’s low-viscosity resin (Spurr, 1969), as described by Walther-Mauruschat et al. (1977). For the post-embedding and immunogold labelling of PhaR and PhaP, cells were embedded in Lowicryl K4M (Lowri) as described by Roth et al. (1981), except that methanol was used instead of ethanol for dehydration. Immunological detection of PhaR and PhaP in ultra-thin sections, employing the primary antibodies and goat-anti-rabbit IgG–gold (GARG) complex (Dakopatts), was done exactly as described by Pieper-Fürst et al. (1994). The specificity of the labelling was demonstrated by a control experiment using only the GARG complexes and mAbs against the Cap proteins of Sendai virus. Micrographs were taken with a Philips EM301 electron microscope at an acceleration voltage of 80 kV. Magnifications were calibrated with a cross-grated lining replica (Balzers).

**Analysis of the PHA.** The samples (i.e. their poly(3HB)) were subjected to methanolysis in the presence of 15% (v/v) sulfuric acid. The resulting methyl esters of 3-hydroxybutyric acid were analysed by gas chromatography, as described by Brandl et al. (1988) and Timm & Steinbüchel (1990).

**Preparation of PHA granules.** Granules consisting of poly(3HB) were isolated from R. eutropha H16 cells that had been grown in sodium gluconate, by employing the hypochlorite treatment described previously (Jendrossek et al., 1993). PHA granules were also isolated by centrifugation in sucrose gradients according to the method of Preusting et al. (1993) with the modifications of Wieczorek et al. (1995).

**RESULTS**

**Sequence analysis of the R. eutropha H16 phaR gene**

Genes encoding putative proteins homologous to the R. eutropha phaR translational product are found in many PHA-accumulating bacteria (Fig. 1, and see Fig. I, supplementary data at http://mic.sgmjournals.org). However, the function of PhaR has not yet been revealed. The organization of phaR-like genes and of adjacent regions reported in the literature or as found by homology searches of microbial genomes are shown in Fig. II (supplementary data at http://mic.sgmjournals.org). Genes encoding PhaR homologues have, so far, only been found in the proteobacterial branch of the eubacteria (Fig. 1). An amino-acid-sequence alignment of PhaR homologues (Fig. I, supplementary data at http://mic.sgmjournals.org) revealed an overall high level of sequence identity in the N-terminal regions (amino acids 24–125), whereas the C-terminal regions (amino acids 126–225) lacked this striking similarity. Prediction of the secondary structure of the R. eutropha H16 PhaR sequence at the EMBL server (http://www.embl.de) revealed a putative helix–turn–helix motif in the region comprising amino acids 95–114 (Fig. I, supplementary data at http://mic.sgmjournals.org).
With the exception of the PhaR homologue StdC, present in Comamonas testosteroni, all of the phaR homologues occurred within clusters of PHA biosynthesis genes (Fig. II, supplementary data at http://mic.sgmjournals.org). The location of these phaR homologues within the PHA biosynthesis gene clusters is variable; however, phaR is located close to phaA only in the genomes of the α-Proteobacteria Rhizobium etli, S. meliloti, Mesorhizobium loti, Caulobacter crescentus and Methylobacterium extorquens.

Phenotypic characterization of the R. eutropha phaRΩKm strain

Although a large number of transposon-induced mutants affected in PHA biosynthesis and accumulation have been screened and characterized at the molecular level (e.g. Schubert et al., 1988), so far no mutants of R. eutropha have been found that are defective in phaR. Therefore, no phenotype can be assigned to this gene. In this study, we generated a phaR knock-out mutant of R. eutropha by inserting a kanamycin-resistance cassette into phaR, employing a respective derivative of the suicide vector pSUP202 for homogenization. When cells of the phaRΩKm mutant were grown on MM containing 0-02 % (w/v) NH₄Cl (storage conditions) and sodium gluconate as sole carbon source, the cells accumulated poly(3HB) to 48 % (w/w) of the cell dry weight in the stationary phase of growth, whereas the wild-type strain and the phaP polyhydroxybutyrate(PHB)-leaky mutant H2275 contained poly(3HB) to 76 or 31 % (w/w) of the cell dry weight, respectively, if cultivated under the same conditions. In addition, cells of the phaRΩKm mutants were clearly distinguishable from the wild-type and from the PHB-leaky mutant H2275 by the size and number of the poly(3HB) granules occurring in their cells. As shown in Fig. 2, cells of the phaR knock-out mutant contained a large number of small granules that filled almost the entire cytoplasm, whereas cells of the wild-type and of the PHB-leaky mutant H2275 contained fewer but larger granules or only one single, large granule, respectively.

Expression of the N-terminal His₆-tagged fusion protein of PhaR and its purification by metal-ion-chelating chromatography

Electropherograms of SDS-polyacrylamide gels in which crude extracts of the wild-type and a phaRΩKm mutant had been separated did not reveal the presence or...
absence of a protein exhibiting an apparent molecular mass expected for the putative phaR translational product. Because PhaR probably has a regulatory function, only low concentrations of the protein were expected to be detected. Therefore, to generate a PCR product comprising the DNA regions directly upstream and downstream of the start and stop codons of phaR, respectively, tailed PCR was employed to amplify the coding region of phaR and to insert restriction sites for NdeI and XbaI and an 18 nt region encoding six His residues. This PCR product was inserted into the NdeI–XbaI sites of pMa/c-5-914. The resulting plasmid, pMa/cphaRHis', contained phaR under the control of the temperature-sensitive λ-repressor cI857ts, with an artificial ribosome-binding site conserved for E. coli.

The His<sup>6</sup>–PhaR fusion protein could be detected in E. coli TOP10 crude extracts upon raising the temperature of the culture from 32 to 42 °C (Ausubel et al., 1987) and was purified from the recombinant cells of E. coli by applying the soluble fraction of crude extracts to a Ni-NTA/agarose column. The His<sub>6</sub>–PhaR fusion protein was eluted from the matrix with 250 mM imidazole; the imidazole was then removed by dialysis against 10 mM Tris/HCl (pH 7–0). The purity of phaR was analysed by SDS-PAGE (Fig. 3) and by immunoblotting (Fig. 4a, lane 5).

**Fig. 3.** Purification of PhaR. Following its expression in E. coli TOP10, the His<sub>6</sub>-tagged PhaR fusion protein was purified using a Ni-NTA Spin Column under native conditions [lysis and wash in a solution containing 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl and 20 mM imidazole (pH 8.0); elution with a solution containing 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl and 250 mM imidazole (pH 8.0)].

Proteins were separated in 12.5% SDS-polyacrylamide gels and stained with Coomasie brilliant blue. Lanes: 1, crude protein extract from E. coli TOP10 pMa/c5-914; 2, crude protein extract from E. coli TOP10 pMa/cphaRHis<sub>6</sub>; 3, flow-through; 4, first wash step; 5, second wash step; 6–8, eluates; M, molecular mass marker.

**Specific binding of PhaR to the phaP and phaR upstream regions**

To investigate the interaction of PhaR with DNA, gel-mobility-shift assays were performed (Fig. 5). Purified His<sub>6</sub>–PhaR was incubated with a mixture of fragments derived from PCR amplification and comprising phaP, phaR and their respective adjacent regions, as described in Methods. A 518 bp SmaI–PstI DNA fragment containing the phaP upstream and 5' phaP region clearly shifted, depending on the concentration of PhaR added. In contrast to this result, no retardations of the 128, 232 and 775 bp fragments were observed (Fig. 5a). A 330 bp DNA fragment containing the phaR upstream and 5' phaR region clearly shifted at increasing concentrations of PhaR (Fig. 5b). None of the Sau3AI digestion products (i.e. the 119, 168 and 243 bp fragments) of the shifted 518 bp fragment shifted in presence of PhaR (data not shown). The gel-mobility-shift assays clearly revealed the binding of PhaR to phaP upstream regions, as well as to phaR upstream regions.
Regulation of phasin and granule formation

DNAseI footprinting experiments

To identify the PhaR-binding sites at the phaP and phaR upstream regions exactly, we performed DNAseI footprinting with purified His<sub>6</sub>–PhaR and PCR fragments containing the phaP or phaR promoter region (Fig. 6). The addition of PhaR to these samples resulted in DNAseI protection at a region of −49 to −36 and at a region of −12 to +21 relative to the phaP transcriptional start site (+1; Fig. 6a). Corresponding experiments were done to identify the exact binding site upstream of phaR. PhaR also mediated DNAseI protection at a region of +11 to +18 upstream of the phaR transcriptional start site (Fig. 6b).

Identification of the phaR promoter

Sequence analysis of phaR revealed a region 95 bp upstream of the translational start codon of phaR that exhibited extended homology with the eubacterial σ<sub>E</sub>-promoter consensus sequence (Reitzer & Magasanik, 1985). The transcriptional start site of phaR was identified as being downstream of this region, by primer-extension assays with total RNA isolated from R. eutropha H16 that had been grown under storage conditions and harvested in the stationary phase of growth (Fig. 7). At 228 bp upstream of the translational start codon of phaR the nucleotide sequence TGT-N<sup>1</sup>ACA was identified, which may represent a putative upstream activator sequence.

Studies on the binding of PhaR to artificial poly(3HB) granules

Another putative location of PhaR is the PHA granule. Therefore, emulsions of artificial poly(3HB) granules and water (1.5%, w/v) were incubated with the purified His<sub>6</sub>–PhaR fusion protein for 90 min on ice (Wieczorek et al., 1995). After this incubation, the granules were collected by centrifugation, washed twice with 1 ml of 10 mM Tris/HCl (pH 7.0) and resuspended in denaturing buffer. The granule suspension and supernatants of each washing step were analysed by SDS-PAGE (Fig. 4b). The His<sub>6</sub>–PhaR fusion protein exhibited a high level of affinity for poly(3HB) (Fig. 4b, lane 5).

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**Fig. 4.** Localization of PhaR and binding of the PhaR His<sub>6</sub>-tagged protein of R. eutropha H16, overexpressed in E. coli TOP10 harbouring pMa/c5-914phaRH<sub>His</sub><sub>6</sub> to artificial poly(3HB) granules. (a) R. eutropha H16 was cultivated in MM containing 0.02% (w/v) NH<sub>4</sub>Cl plus 1.5% (w/v) sodium gluconate; cells were harvested in the stationary phase of growth. Crude protein extracts or proteins from solubilized granules were separated by SDS-PAGE and subjected to Western-blot analysis employing antibodies raised against PhaR. Lanes: 1, crude extract (300 µg total protein); 2, soluble fraction (55 µg); 3, membrane fraction (60 µg); 4, granule-associated proteins (32 µg); 5, purified PhaR His<sub>6</sub>-tagged protein overexpressed in E. coli TOP10 harbouring pMa/cphaRHis<sub>6</sub> (15 µg). (b) Proteins were separated in 12.5% SDS-polyacrylamide gels and stained with silver. The purified PhaR His<sub>6</sub>-tagged protein (100 µg in 130 µl of 100 mM Tris/HCl, pH 7.0) was incubated for 90 min on ice with poly(3HB) granules. The mixtures were subsequently separated into pellets and supernatants by centrifugation and washed twice with 10 mM Tris/HCl, pH 7.5. Lanes: M, molecular mass marker; 1, artificial poly(3HB) granules incubated with the purified PhaR His<sub>6</sub>-tagged protein of E. coli TOP10 harbouring pMa/cphaRHis<sub>6</sub>; 2, supernatant after centrifugation; 3, supernatant after first wash step; 4, supernatant after second wash step; 5, pellet with PhaR His<sub>6</sub>-tagged protein after second wash step.
Fig. 5. Gel-mobility-shift assays of PhaR binding to DNA fragments containing the upstream regions of phaP (a) or phaR (b). (a) Different amounts of purified PhaR protein were incubated with 1–5 ng of DNA containing phaP and/or its upstream region. Lanes: 1, 0 ng; 2, 10 ng; 3, 100 ng; 4, 500 ng; 5, 1000 ng; 6, 2000 ng; M, 100 bp ladder. (b) Different amounts of purified PhaR protein were incubated with 1–5 ng of DNA containing phaR and/or its upstream region. Lanes: 1, 0 ng; 2, 100 ng; 3, 500 ng; 4, 2000 ng; M, 100 bp ladder. The DNA–protein complex was separated from unbound DNA on an 8% native polyacrylamide gel. DNA fragments were stained with ethidium bromide and visualized with UV light.

Washing with 1 ml of 10 mM Tris/HCl (pH 7.0) did not remove the fusion protein from the poly(3HB) granules (Fig. 4b, lanes 2 and 3). To demonstrate that PhaR did not precipitate during the assay, PhaR was incubated without granules under the same conditions as described above. During the time course of this control experiment PhaR did not precipitate.

Localization of PhaR by Western immunoblotting

Because the in vitro binding studies shown above demonstrated the high affinity of His₆–PhaR for artificial poly(3HB) granules, it was very likely that the native protein is one of the granule-associated proteins occurring in *R. eutropha*. Various methods were employed to study the in vivo localization of PhaR. The cellular localization of native PhaR was analysed by Western immunoblotting using monospecific polyclonal anti-PhaR antibodies to probe the native PHB granules fraction, the membrane fraction and the soluble protein fraction (see Methods for details of the fractionation procedure). In addition, the crude cellular extract was subjected to Western immunoblotting. Fig. 4(a) clearly shows that PhaR was detected in the crude extract (lane 1), in the insoluble fraction (lane 3) and in the poly(3HB) granule-associated protein fraction (lane 4), whereas no PhaR could be detected in the soluble protein fraction (lane 2). The experiments showed that the purified
Fig. 6. Footprinting analysis of PhaR binding. (a) DNasel footprinting analysis using purified PhaR and phaP promoter DNA. Purified PhaR (0, 50, 2000 and 0 ng) was incubated with IRD800-labelled DNA. (b) Purified PhaR and phaR promoter DNA. Purified PhaR (0, 50 and 2000 ng) was incubated with IRD800-labelled DNA. After DNasel treatment, the DNA was analysed in parallel with a sequencing reaction. The grey boxes indicate DNasel protection, the open boxes refer to the sequence repeats and the arrows along the boxes indicate the direction of the repeats. Sequence positions are relative to the transcriptional start site (+1) of phaP or phaR.
polyclonal antibodies against PhaR were highly specific for this protein. Only one band was obtained in Western blots prepared from crude extracts of *E. coli* TOP10 harbouring pMa/cphaRHis6 or of the poly(3HB) granule-associated protein fraction (Fig. 4a, lanes 5 and 4, respectively). To determine the quantities of the monospecific polyclonal anti-PhaR antibodies present, different concentrations (14, 28, 56, 112 and 224 µg) of the soluble protein fraction were subjected to Western immunoblotting and probing with the monospecific polyclonal anti-PhaR antibodies. The results clearly showed that PhaR was detected in the soluble protein fraction as a faint band only when 224 µg soluble protein was applied (data not shown).

**Immunoelectron microscopic localization of PhaR**

The monospecific polyclonal anti-PhaR antibodies were also used to localize PhaR in the cells at the ultrastructural level, by using immunoelectron microscopy. Cells of *R. eutropha* H16 and of the phaR knock-out mutant were embedded and ultra-thin sections were subjected to immunogold labelling. The labelling was mainly confined to only the periphery of the PHA granules in cells of the wild-type and there was no evidence that PhaR occurred within the core of the granules (Fig. 8). PhaR was not detected in the mutant.

**Fig. 7.** Primer-extension assay of the transcript of phaR. Lanes A, C, G and T represent standard sequencing reactions to size the primer-extension product. A dot and an arrow indicate the origin (+1) and direction of transcription; the −12 and −24 regions are boxed.

**Fig. 8.** Immunoelectron microscopic detection and localization of PhaR in wild-type *R. eutropha* H16 (a) and *R. eutropha* phaRΩKm (b). Cells were cultivated in MM containing 0.02% (w/v) NH₄Cl plus 1.5% (w/v) sodium gluconate and were harvested in the stationary phase of growth. Thin-sections of cells were labelled with antibodies against the PhaR plus GARG complex. (a) *R. eutropha* H16 cell labelled with antibodies against the PhaR plus GARG complex. (b) Sections of cells of the phaR knock-out mutant *R. eutropha* phaRΩKm labelled with antibodies against the PhaR plus GARG complex (control section). Bars, 0.2 µm.

**Fig. 9.** Immunoelectron microscopic localization of PhaP in wild-type *R. eutropha* H16 (a) and the phaR knock-out mutant *R. eutropha* phaRΩKm (b). Cells were cultivated as described in Fig. 8 and were labelled with antibodies against the PhaP plus GARG complex. Bars, 0.2 µm.
Immunoelectron microscopic comparison of the \textit{R. eutropha} H16 wild-type and \textit{R. eutropha phaR\Omega Km} cells with respect to the amount of PhaP present

Cells of the wild-type and of the \textit{phaR} knock-out mutant were cultivated on MM containing sodium gluconate as carbon source under storage conditions and harvested in the stationary phase of growth. The cells were labelled with antibodies raised against PhaP. In the knock-out mutant, the amount of PhaP present (Fig. 9b), as represented by the GARG complexes, was clearly higher than the amount of PhaP present in the wild-type (Fig. 9a).

DISCUSSION

Previous studies have revealed that cells of \textit{R. eutropha} are capable of synthesizing large amounts of PhaP, which contribute 3–5\% of the total cellular protein (Wieczorek \textit{et al.}, 1995; Steinbüchel \textit{et al.}, 1996). However, the occurrence of PhaP in the cells is strictly dependent on PHA biosynthesis, and cells cultivated under conditions not permissive for PHA synthesis or mutants defective in the PHA synthase structural gene do not synthesize detectable levels of PhaP (Steinbüchel \textit{et al.}, 1995; Wieczorek \textit{et al.}, 1995; York \textit{et al.}, 2001a). Moreover, even if PhaP is present in the wild-type in large quantities after employing permissive conditions, almost all of the PhaP is bound to the granules, and PhaP is hardly detected as a soluble protein in the cytoplasm (Wieczorek \textit{et al.}, 1995). To achieve all of the above, an effective mechanism for the regulation of PhaP formation is required, and this regulation most likely relies on at least one regulatory gene. Sequence analyses of the \textit{phaCAB} operon of \textit{R. eutropha} and the \textit{pha} regions of many other bacteria have revealed the presence of a gene that exhibits substantial homology across bacterial species, which in \textit{R. eutropha} H16 is referred to as \textit{phaR} (Fig. I, supplementary data at http://mic.sgmjournals.org). PhaR and its homologous proteins from other bacteria have been predicted to be DNA-binding proteins (Cabrera \textit{et al.}, 1997; Povolo & Casella, 2000; Maehara \textit{et al.}, 2001).

As the conclusion of their physiological study, York \textit{et al.} (2002) proposed a rather simple and straightforward mechanism for the regulatory action of PhaR in \textit{R. eutropha}. According to these authors, PhaR is a repressor that binds to a regulatory sequence upstream of \textit{phaP}; however, DNA-binding studies were not done by this group. Upon the onset of PHB synthesis, PhaR was postulated to be removed from the binding region, thus allowing the formation of PhaP; however, the ‘titrating factor’ was not identified. In the current study, we also found that PhaR binds upstream of \textit{phaP}, as revealed by gel-mobility-shift assays (Fig. 5). Moreover, the exact location of the binding site was identified by DNaseI footprinting experiments. The latter experiments showed that PhaR protects two DNA regions: one region is located directly upstream of the −35 region of the promoter that was identified previously (Steinbüchel \textit{et al.}, 1996) and the second region maps from position −12 to +21. The protected regions comprise 14 and 33 bp and exhibit G+C ratios of 35 and 40 mol\%, respectively. When the 14 and 33 bp binding sequences were analysed for sequence motifs that could serve as targets for DNA-binding proteins (e.g. palindromes, inverted or direct repeats), a 12 bp repeat sequence – GCAMMAAWTMMD – was identified on the sense and anti-sense strands, with the −10 and −35 regions of \textit{phaP} located in between. These data clearly demonstrate (by the application of various independent methods) that PhaR binds to the promoter region of \textit{phaP}. Maehara \textit{et al.} (2001) have also shown in gel-mobility-shift assays that the PhaR protein of \textit{P. denitrificans} specifically binds to the intergenic \textit{phaC–phaP} and \textit{phaP–phaR} regions.
This study also revealed that the PHA granules are a very plausible candidate for the ‘titrating factor’ of PhaR. The capability of PhaR to bind to PHA granules was demonstrated by in vitro experiments employing artificial granules as well as by in vivo cell fractionation and an immunoelectron microscopic approach employing specific anti-PhaR antibodies. Therefore, it seems plausible that upon the onset of PHA biosynthesis (i.e. when the nascent granules lack PhaP) PhaR is bound to the granules. Because of this, repression is removed from the phaP upstream binding region and phaP can be transcribed. The surface of the PHA granules is sufficiently large to bind the relatively small amounts of PhaR protein. However, the phasin PhaP most probably possesses a higher affinity for PHA granules than PhaR does. This could explain why in the later stages of PHA accumulation excess PhaR protein can again bind to the phaP upstream region, thus preventing the formation of more PhaP than can be bound to the granules.

By use of gel-mobility-shift assays, this study also clearly showed that PhaR binds not only to the phaP upstream region and to the PHA granules, but it also binds to the phaR upstream region. DNaseI footprinting experiments mapped the PhaR binding site to being approximately 86 bp upstream of the translational start site of phaR, which was also identified in this study. The transcriptional start site was identified as being 8 bp upstream of the putative −12 region. Normally, the distance from the transcriptional start to the −12 region is 4−8 bp (Hawley & McClure, 1983; Rosenberg & Court, 1979). Since the number of copies of the phaR upstream region is limited by the number of copies of chromosomes in the cell, this region has a lower capacity to titrate significant quantities of PhaR than the surface of the PHA granules. However, this binding may allow the efficient autoregulation of PhaR expression and, thus, may prevent the synthesis of more PhaR than is required for the repression of PhaP expression. Steinbûchel et al. (1996) have already suggested an autoregulation mechanism for the expression of PhaP; however, we have found that phaR (the repressor gene) is itself autoregulated and that phaP is not. Fig. 10 shows a model for the regulatory action of PhaR and its interactions with the phaP and phaR upstream regions and the PHA granules. This model is based on the results of this study and is also consistent with the results of previous studies (Wieczorek et al., 1995; Steinbûchel et al., 1996; Maehara et al., 2001; York et al., 2002).

The regulatory model presented in Fig. 10 is consistent with the phenotype of phaP and phaR mutants. phaP mutants do not synthesize phasin and, since the naked PHA granules exhibit a hydrophobic surface and are therefore not protected from coalescence, at the end of the accumulation phase the cells contain only one single, large granule. It has been shown previously that the introduction of multiple copies of phaP into R. eutropha results in the formation of many more, much smaller PHA granules than seen in the wild-type (Wieczorek et al., 1995). The phaR mutant generated in this study exhibited exactly this phenotype. This phenotype is explained by the lack of the repressor (PhaR) in this knock-out mutant, allowing the formation of increased amounts of PhaP; this explanation was confirmed by the immunoelectron microscopic studies performed here.

Due to the higher concentration of PhaP in the cells, a larger surface area of PHA granules can be covered by PhaP, thus allowing stabilization of the PHA in water dispersion with a smaller size of granule than seen in the phaR mutant.

In conclusion, the interactions of the autoregulated repressor PhaR with the PHA granules and the phaP and phaR upstream promoter regions allow R. eutropha to regulate the expression of the phasin PhaP very efficiently. On the one hand, this regulation guarantees that the required large amounts (3−5% of total cellular protein) of PhaP can be synthesized if PHAs are synthesized. On the other hand, it ensures that PhaP is not synthesized in excess, thereby decreasing the burden imposed on the cells.

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