JOURNAL OF BACTERIOLOGY, May 1995, p. 2513–2523 Vol. 177, No. 9
0021-9193/95/$04.00+0
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Identification of the Region of a 14-Kilodalton Protein of *Rhodococcus ruber* That Is Responsible for the Binding of This Phasin to Polyhydroxyalkanoic Acid Granules

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Received 31 January 1995/Accepted 3 March 1995

The function of the polyhydroxyalkanoic acid (PHA) granule-associated GA14 protein of *Rhodococcus ruber* was investigated in *Escherichia coli* XL1-Blue, which coexpressed this protein with the polyhydroxybutyric acid (PHB) biosynthesis operon of *Alcaligenes eutrophus*. The GA14 protein had no influence on the biosynthesis rate of PHB in *E. coli* XL1-Blue(pSKCO7), but this recombinant *E. coli* strain formed smaller PHB granules than were formed by an *E. coli* strain that expressed only the PHB operon. Immunoelectron microscopy with GA14-specific antibodies demonstrated the binding of GA14 protein to these mini granules. In a previous study, two hydrophobic domains close to the C terminus of the GA14 protein were analyzed, and a working hypothesis that suggested an anchoring of the GA14 protein in the phospholipid monolayer surrounding the PHA granule core by these hydrophobic domains was developed (U. Pieper-Fürst, M. H. Madkour, F. Mayer, and A. Steinbüchel, J. Bacteriol. 176:4328–4337, 1994). This hypothesis was confirmed by the construction of C-terminally truncated variants of the GA14 protein lacking the second or both hydrophobic domains and by the demonstration of their inability to bind to PHB granules. Further confirmation of the hypothesis was obtained by the construction of a fusion protein composed of the acetaldehyde dehydrogenase II of *A. eutrophus* and the C terminus of the GA14 protein containing both hydrophobic domains and by its affinity to native and artificial PHB granules.

Polyhydroxyalkanoic acids (PHAs) represent a widespread storage material of bacteria and occur as granules in the cell (for reviews, see references 1 and 30). In *Bacillus megaterium*, these granules contain, in addition to polyhydroxybutyric acid (PHB), proteins and lipids (7). Hitherto, the molecular architecture of the granules has not been well understood. PHAs are biodegradable thermoplastics and can be produced from renewable resources. The pathways of many bacteria for the biosynthesis of PHAs have been investigated, and the genes of several biosynthetic enzymes have been analyzed (for reviews, see references 30 and 32). The biosynthesis of PHB has been studied in most detail in *Alcaligenes eutrophus* (33). Two molecules of acetyl-coenzyme A (CoA) are converted to acetoacetyl-CoA by a 3-ketothiolase (EC 2.3.1.9). Subsequent to an NADPH-dependent reduction to D-(−)-3-hydroxybutyryl-CoA by an acetoacetyl-CoA reductase (EC 1.1.1.36), the PHB synthase catalyzes the polymerization reaction. The genes of these three enzymes are organized in a single operon; after transformation of the PHB operon into *Escherichia coli*, the cells of the recombinant strain produce PHB (19, 27, 29).

Recently, the coryneform bacterium *Rhodococcus ruber* NCIMB 40126 was found to accumulate a copolyester of 3-hydroxybutyric acid and 3-hydroxyvaleric acid from single unrelated carbon sources (10). Molecular analysis identified the PHA synthase gene locus of this bacterium (20). Sequence analysis revealed the PHA synthase gene, open reading frame 3 that codes for the GA14 protein, and open reading frame 4 that codes for a protein with unknown function (20). N-terminal amino acid analysis of proteins which were copurified with the granules identified the PHA synthase and the GA14 protein as PHA granule-associated proteins in *R. ruber* (20, 21). Immunoelectron microscopic studies with GA14-specific antibodies demonstrated that the GA14 protein is mostly bound to the surface of PHA granules in *R. ruber* (21). Analysis of the primary sequence of the GA14 protein revealed two domains consisting of 10 and 9 hydrophobic or amphiphilic amino acids, respectively, close to the C terminus. It was suggested that these two domains are responsible for the anchoring of the GA14 protein in a phospholipid monolayer surrounding the PHA granule core (21). In this study, we investigated the function of the GA14 protein during biosynthesis of PHA and the molecular basis for its binding to PHA granules.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strain and plasmids used in this study are listed in Table 1.

Growth conditions and preparation of crude extract. Recombinant strains of *E. coli* XL1-Blue were grown for 12 h at 37°C in 10 ml of Luria-Bertani medium (24) supplemented with 100 μg of ampicillin and 12.5 μg of tetracycline per ml. Two or 10 ml of these precultures was used to inoculate 50 or 300 ml of Luria-Bertani medium, respectively, containing 100 μg of ampicillin and 12.5 μg of tetracycline per ml and 1% (wt/vol) glucose or 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) plus 0.2% (wt/vol) glucose. These cultures were incubated for 24 h at 37°C. Growth was monitored with a Klett-Summerson photometer (filter no. 54 [520 to 580 nm]). Cells were harvested by centrifugation (2,800 × *g*, 10 min, 4°C), washed, and resuspended in 0.1 volume of 10 mM Tris-HCl (pH 7.0). Cells were broken by a twofold French press passage (110 × 10^6 Pa), and soluble protein fractions were prepared from the resulting crude extracts by ultracentrifugation (100,000 × *g*, 1 h, 4°C).

Preparation of native and artificial PHB granules and binding assays of proteins. Native PHB granules were isolated from the cells of recombinant *E. coli* strains by loading approximately 3 ml of crude extract onto discontinuous glycerol gradients (2 ml of glycerol at 88% [vol/vol], 5 ml of glycerol at 50% [vol/vol]), which were centrifuged for 1 h at 49,000 × *g* and 4°C. PHB granules were collected at the 88 to 50% interphase and removed from the gradient,

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TABLE 1. Bacterial strain and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi1 hsdr17* (r_K^- m_K^+) *supE44 relA1* λ^- *lac*$^-$ [F′ *proAB lacI*^{q}ZΔM15 Tn*10*(Tet)]	5
Plasmids		
pBluescript SK$^-$	Ap^r *lacPOZ′*	Stratagene
pBluescript KS$^-$	Ap^r *lacPOZ′*	Stratagene
pSK2665	Harbors the PHB operon of *A. eutrophus*	28
pSKXA10*	Harbors wild-type $phaP_{Rr}$ (414 bp)	21
pSKXA501	Harbors truncated $phaP_{Rr}$ (372 bp)	This study
pSKXA477	Harbors truncated $phaP_{Rr}$ (348 bp)	This study
pSKXA462	Harbors truncated $phaP_{Rr}$ (333 bp)	This study
pSKXA432	Harbors truncated $phaP_{Rr}$ (303 bp)	This study
pSKCO7	Corresponds to pSKXA10* but also contains the PHB operon of *A. eutrophus*	This study
pSKCO501	Corresponds to pSKXA501 but also contains the PHB operon of *A. eutrophus*	This study
pSKCO477	Corresponds to pSKXA477 but also contains the PHB operon of *A. eutrophus*	This study
pSKCO462	Corresponds to pSKXA462 but also contains the PHB operon of *A. eutrophus*	This study
pSKCO432	Corresponds to pSKXA432 but also contains the PHB operon of *A. eutrophus*	This study
pDel1074	Harboring *acoD* of *A. eutrophus*	23
pDel1074PHB	Corresponds to pDel1074 but also contains the PHB operon of *A. eutrophus*	This study
pDel1074B	Harbors modified *acoD*	This study
p74B11	Harbors a fusion gene of modified *acoD* and the C terminus of GA14 protein of *R. ruber*	This study
p74B11PHB	Corresponds to p74B11 but also contains the PHB operon of *A. eutrophus*	This study

pelleted (12,000 × *g*, 20 min, 4°C), washed, and resuspended in 300 μl of 10 mM Tris-HCl (pH 7.0).

The preparation of artificial PHB granules from *A. eutrophus* was performed as described by Jendrossek et al. (12). The binding assays with proteins were done according to the method of Liebergesell et al. (15).

Protein determination and enzyme assays. Protein concentrations were determined as described by Bradford (2). β-Ketothiolase activity in the soluble protein fraction was measured in the thiolysis direction as described by Nishimura and coworkers (18). The activity of the NADPH-dependent acetoacetyl-CoA reductase in the soluble protein fraction was monitored by measuring the oxidation of NADPH (9). PHB synthase activity was determined spectroscopically in the crude extract according to the method of Valentin and Steinbüchel (35). The activity of acetaldehyde dehydrogenase II (AcDH-II) was monitored in the soluble protein fraction by measuring the initial rate of NAD reduction (13).

Quantitative and qualitative analysis of PHAs. PHAs were converted to the methyl esters of constituent hydroxyalkanoic acids, which were analyzed by gas chromatography as described elsewhere (3, 34).

Electrophoresis of proteins. Samples were resuspended in gel loading buffer (0.6% [wt/vol] sodium dodecyl sulfate [SDS], 1.25% [vol/vol] β-mercaptoethanol, 0.25 mM EDTA, 10% [vol/vol] glycerol, 0.001% [wt/vol] bromophenol blue, 12.5 mM Tris-HCl [pH 6.8]), and the proteins were separated in SDS-polyacrylamide (10 to 15% [wt/vol]) gels as described by Laemmli (14). Proteins were stained with Coomassie brilliant blue R 250 (37).

Immunoblotting. Electrophoresis of proteins in SDS-polyacrylamide gels, blotting onto nitrocellulose membranes, and antibody reactions were done as described previously (21). Immunoglobulins directed against the GA14 protein of *R. ruber* were affinity purified (21). Antibodies directed against the AcDH-II of *A. eutrophus* were available from a previous study (13).

Electron microscopy. Postembedding immunogold labeling of the wild-type GA14 protein and a truncated variant of the GA14 protein on ultrathin sections of recombinant *E. coli* XL1-Blue cells was done as described previously (21).

For demonstration of the granule size, the cells were washed in 10 mM Tris (pH 7.0)–0.15 M NaCl and contrasted essentially as described previously (16). A 2% (wt/vol) aqueous solution of phosphotungstic acid neutralized with NaOH was used for negative staining.

Isolation, manipulation, and transformation of DNA. Isolation of plasmid DNA, agarose gel electrophoresis of DNA, and the use of restriction endonucleases, T4 DNA ligase, the Klenow fragment of DNA polymerase I, and mung bean nuclease were done by standard procedures (24). DNA was extracted from agarose as described by Vogelstein and Gillespie (36). Preparation of frozen competent cells of *E. coli* XL1-Blue by the RbCl-$CaCl_2$ method and their use for transformation were done according to protocol 3 described by Hanahan (8).

PCR. For PCR, 1 μM (each) oligonucleotide–200 μM (each) deoxynucleoside triphosphate (Stratagene)–250 ng of template DNA–2 U of Vent polymerase (New England Biolabs) in 100 μl of buffer supplied by the manufacturer were incubated 30 times under oil at 98°C for 1 min and 70°C for 5 min (6). The DNA was extracted with chloroform-isoamyl alcohol, precipitated with ethanol, and resuspended in Tris-EDTA buffer (24). Sequence analysis was done according to the dideoxy-chain termination method (25) to confirm the accuracy of the determination of the PCR products.

Synthesis of oligonucleotides. Oligonucleotides were synthesized in a Gene Assembler Plus apparatus according to the manufacturer's protocol (Pharmacia Biotech, Freiburg, Germany).

RESULTS

Construction of the hybrid plasmid pSKCO7 containing $phaP_{Rr}$ and $phaCAB_{Ae}$. In order to investigate the function of the PHA granule-associated GA14 protein of *R. ruber*, it was coexpressed with the PHB operon of *A. eutrophus*. First, plasmid pSKXA10*, which was previously constructed for the overexpression of the GA14 protein (21), was digested with *Kpn*I, the restriction site of which is localized downstream of $phaP_{Rr}$. $phaP_{Rr}$ represents the structural gene of GA14 protein and was previously referred to as open reading frame 3 (20, 21). Blunt ends were prepared by the exonuclease activity of the Klenow fragment of DNA polymerase I. Second, a 5.2-kbp *Sma*I-*Eco*RI restriction fragment of plasmid pSK2665 (27) that contained the PHB biosynthesis operon of *A. eutrophus* ($phaCAB_{Ae}$) was isolated, and blunt ends were created by mung bean nuclease. The ligation product of linearized pSKXA10* and of the 5.2-kbp restriction fragment was referred to as pSKCO7 and contained both $phaP_{Rr}$ and $phaCAB_{Ae}$ in an antilinear orientation (Fig. 1). The PHB operon includes the genes $phaC_{Ae}$, $phaA_{Ae}$, and $phaB_{Ae}$ encoding PHB synthase, 3-ketothiolase, and NADPH-dependent acetoacetyl-CoA reductase, respectively. These genes are expressed from their own promoter (26), whereas the expression of $phaP_{Rr}$ is controlled by the *lac* promoter (21).

Investigation of the effect of GA14 protein on PHB biosynthesis in *E. coli*. The hybrid plasmids pSKCO7 and pSK2665 were transformed into *E. coli* XL1-Blue, and cells of the recombinant strains were cultivated in Luria-Bertani complex medium. Growth of the cells was monitored by measuring the optical density of the cultures. Cells were collected at the end of the logarithmic phase and at the beginning and end of the stationary-growth phase, and the PHB contents and activities of the PHB biosynthesis enzymes were measured. The expression of GA14 protein in *E. coli* XL1-Blue(pSKCO7) over the period of growth was monitored by immunodot blotting (data not shown). The growth rate of *E. coli* XL1-Blue(pSK2665)

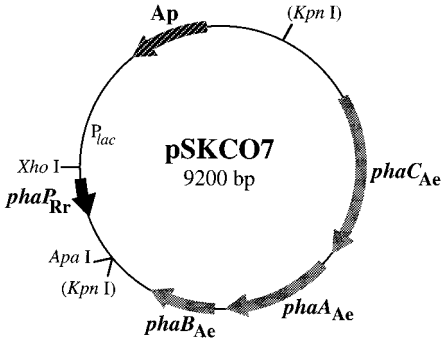

FIG. 1. Physical map of the hybrid plasmid pSKCO7. The plasmid contained the structural gene of the GA14 protein of *R. ruber* (*phaP*$_{Rr}$) and the PHB operon of *A. eutrophus* (*phaCAB*$_{Ae}$). The *Kpn*I restriction sites are shown in brackets because they were destroyed by manipulations during the construction of the plasmid. Ap, ampicillin resistance gene; P$_{lac}$, *lac* promoter.

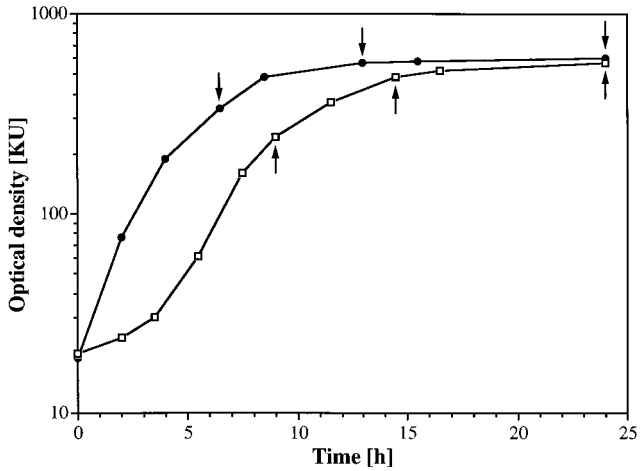

FIG. 2. Growth of *E. coli* XL1-Blue(pSKCO7) (□) and XL1-Blue(pSK2665) (●). Cells were cultivated at 37°C in 300 ml of Luria-Bertani medium with ampicillin, tetracycline, and 1% (wt/vol) glucose. Aliquots of the cultures (50 ml) were collected at the times marked by arrows. KU, Klett units.

seemed to be higher than the growth rate of *E. coli* XL1-Blue(pSKCO7) (Fig. 2). However, accumulation of PHB in *E. coli* XL1-Blue(pSK2665) had already started during the logarithmic-growth phase, whereas it started later in *E. coli* XL1-Blue(pSKCO7). Therefore, the stronger increase in optical density during the growth of *E. coli* XL1-Blue(pSK2665) could be due to the refraction of PHB granules. The maximal PHB content was more than 80% (wt/wt) of the cellular dry matter at the end of the stationary-growth phase (Table 2) and was similar in both strains. Similarly, the activities of the PHB biosynthesis enzymes were comparable in both strains and depended on the respective phase of PHB accumulation (Table 2). We therefore conclude that GA14 protein did not either positively or negatively affect the overall PHB accumulation in recombinant strains of *E. coli*, except for causing a brief delay in the onset of PHB accumulation.

Formation of mini granules by GA14 protein in *E. coli*. Interestingly, the PHB granules occurring in *E. coli* XL1-Blue(pSKCO7) were considerably smaller than those occurring in *E. coli* XL1-Blue(pSK2665) (Fig. 3a, b, and e and Fig. 4a and f). PHB granules were isolated from both strains, and the associated proteins were separated in SDS-polyacrylamide gels (Fig. 5a, lanes A3 and F3). Immunodetection with specific antibodies revealed that the GA14 protein is bound to PHB granules of strain *E. coli* XL1-Blue(pSKCO7) (Fig. 5b, lane A3). Postembedding immunogold labeling clearly demonstrated the localization of GA14 protein at the surface of the PHB granules (Fig. 3a), as previously shown for this protein and for PHA granules isolated from cells of *R. ruber* (21). In *E. coli* XL1-Blue(pSK2665), which expressed only *phaCAB*$_{Ae}$, no GA14 protein was detectable (Fig. 3e and Fig. 5, lanes F1 and F3).

Construction of truncated variants of the GA14 protein. We postulated that the two C-terminal hydrophobic domains of the GA14 protein (HD1 and HD2) mediate the association of this protein with a phospholipid monolayer that surrounds the core of the PHA granules in *R. ruber* (21). In order to prove this hypothesis, we constructed truncated variants of the GA14 protein, which had various parts of the C-terminal region deleted, and investigated the ability of these modified forms to bind to the surface of granules in recombinant *E. coli* cells coexpressing the PHB biosynthesis operon of *A. eutrophus*. DNA fragments encoding truncated variants of GA14 protein were synthesized by PCR (Fig. 6).

The PCR primer US3 (Fig. 6) was homologous to the 5′ end of the 1.0-kbp *Xho*I-*Apa*I fragment of plasmid pSKXA10* and therefore contained an *Xho*I restriction site. Four different oligonucleotides (DS3, DS3aI, DS3aII, and DS3a) (Fig. 6) that were homologous to different parts of the C-terminal nucleotide sequence of *phaP*$_{Rr}$ were used as secondary primers. These secondary primers contained, in addition to the matching bases, one stop codon as well as one *Apa*I restriction site to allow the same cloning strategy that was used for the construction of the clones pSKXA10* and pSKCO7. The PCR products were digested with *Xho*I and *Apa*I, and the *Xho*I-*Apa*I fragments generated were ligated to pBluescript SK$^-$ DNA that

TABLE 2. PHB contents and specific activities of PHB biosynthetic enzymes during growth of *E. coli* XL1-Blue(pSKCO7) and XL1-Blue(pSK2665)

Strain	Time (h)	PHB (% [wt/wt])	Sp act of:		
			PHB synthase (U/g of protein)	3-Ketothiolase (U/mg of protein)	NADPH-dependent acetoacetyl-CoA reductase (U/g of protein)
E. coli XL1-Blue(pSKCO7)	9	8	14	2.8	11
	14.5	57	66	10.9	44
	24	81	19	6.2	27
E. coli XL1-Blue(pSK2665)	6.5	34	60	6.0	16
	13	61	<10	11.4	28
	24	85	<10	3.2	13

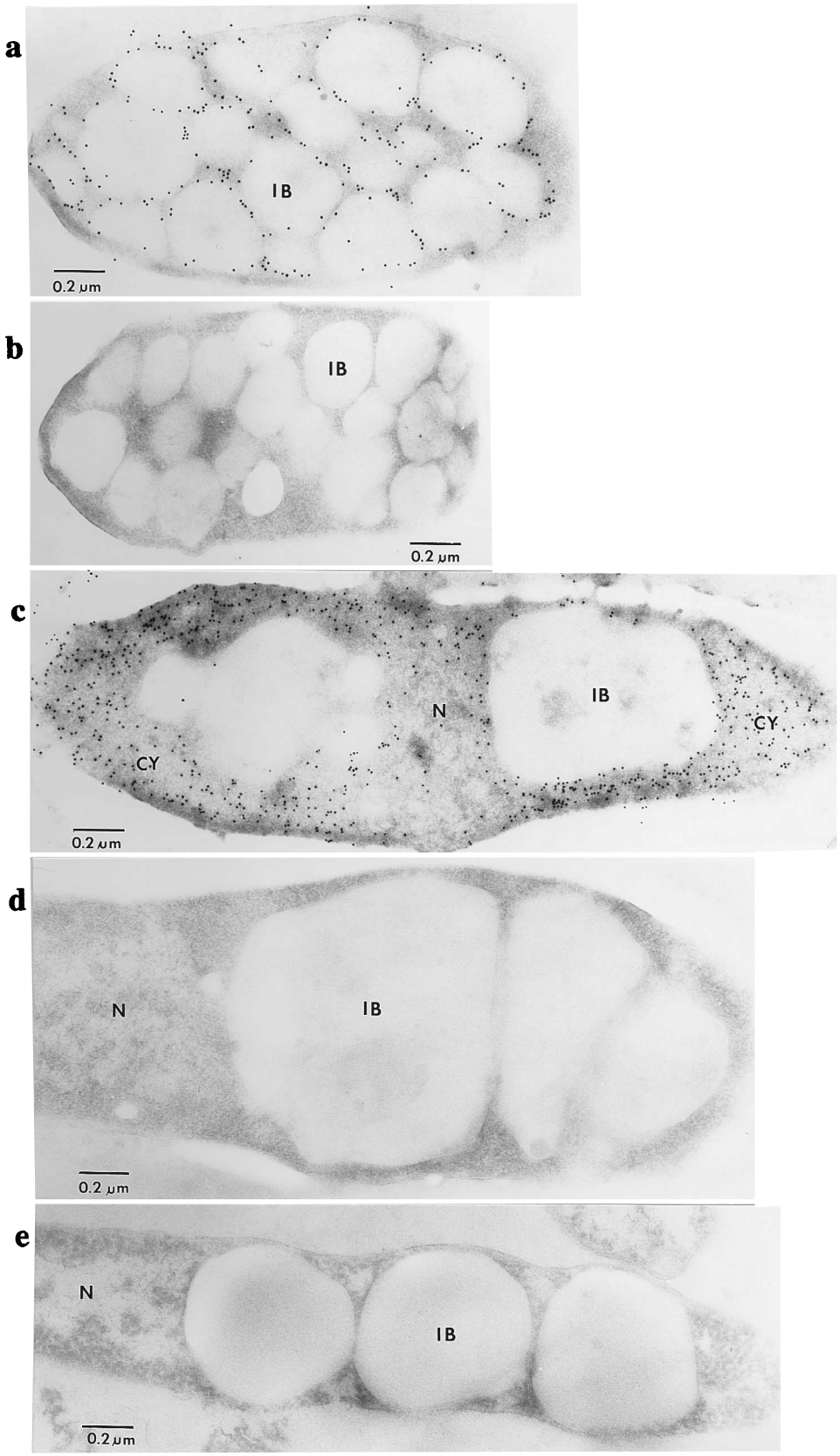

FIG. 3. Immunoelectron microscopic localization of the wild-type GA14 protein and a truncated variant of GA14 protein (M_r, 10,255) in recombinant strains of *E. coli* by postembedding immunogold labeling. Cells were cultivated for 24 h at 37°C in 50 ml of Luria-Bertani medium with ampicillin, tetracycline, and 1% (wt/vol) glucose. (a) Section of *E. coli* XL1-Blue(pSKCO7) cell labeled with antibodies against the GA14 protein and with goat anti-rabbit immunoglobulin G–gold (GARG) complexes. (b) Control section of *E. coli* XL1-Blue(pSKCO7) cell labeled with GARG complexes only. (c) Section of *E. coli* XL1-Blue(pSKCO432) cell labeled with antibodies against the GA14 protein and with GARG complexes. (d) Control section of *E. coli* XL1-Blue(pSKCO432) cell labeled with GARG complexes only. (e) Section of *E. coli* XL1-Blue(pSK2665) cell labeled with antibodies against the GA14 protein and GARG complexes. N, nucleolus; IB, inclusion body (PHB granule); CY, cytoplasm.

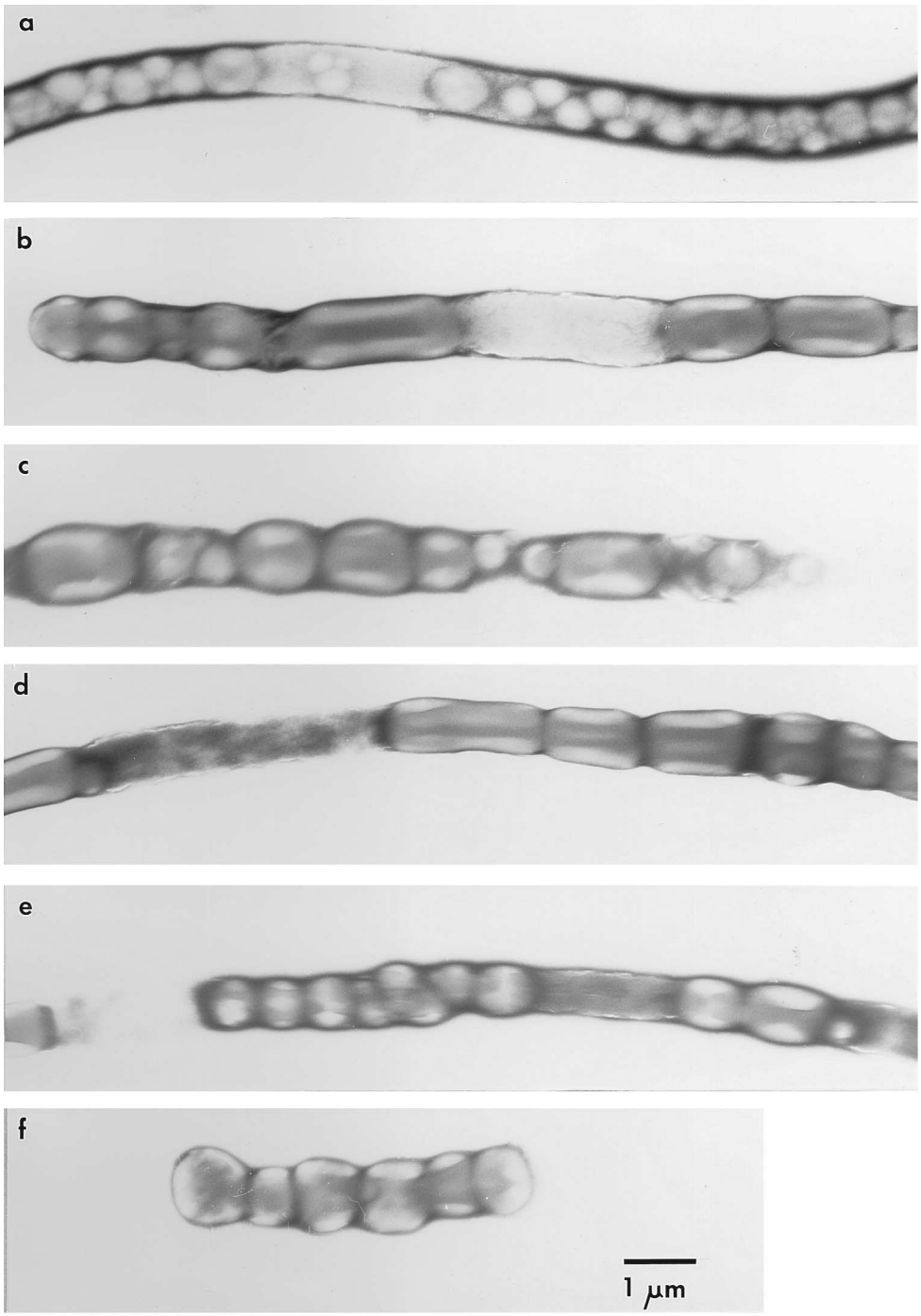

FIG. 4. Electron microscopic demonstration of granule size in recombinant *E. coli* strains. Cells were cultivated as described in the legend to Fig. 3. (a) *E. coli* XL1-Blue(pSKCO7); (b) *E. coli* XL1-Blue(pSKCO501); (c) *E. coli* XL1-Blue(pSKCO477); (d) *E. coli* XL1-Blue(pSKCO462); (e) *E. coli* XL1-Blue(pSKCO432); (f) *E. coli* XL1-Blue(pSK2665).

was treated with *Xho*I and *Apa*I; this yielded the hybrid plasmids pSKXA432, pSKXA462, pSKXA477, and pSKXA501, respectively.

The combination of the primers US3 and DS3 (Fig. 6) yielded the clone pSKXA432, which harbored a 432-bp *Xho*I-*Apa*I fragment encoding a truncated GA14 protein without HD1 plus HD2 (M_r, 10,255 [101 amino acids]). The 462-bp *Xho*I-*Apa*I fragment of clone pSKXA462 encoded a truncated GA14 protein (M_r, 11,222 [111 amino acids]) that stopped immediately downstream of HD1. The 477-bp *Xho*I-*Apa*I fragment of pSKXA477 encoded a truncated GA14 protein (M_r, 11,808 [116 amino acids]) containing HD1 plus 5 additional amino acids. This variant was constructed because we found a high degree of similarity (underlined) between the five amino acids downstream of HD1 (E<u>DLTK</u>) and HD2 (R<u>DLLK</u>) of wild-type GA14 protein. Therefore, this sequence motif might

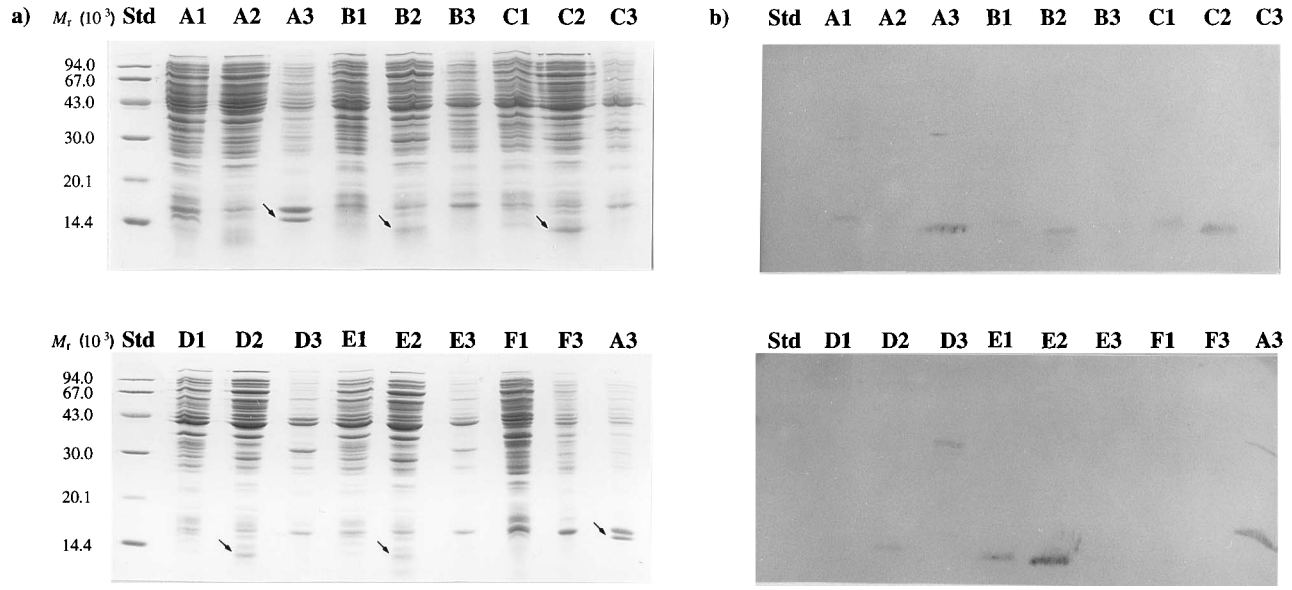

FIG. 5. Analysis of the ability of wild-type GA14-protein and of truncated variants of GA14 protein to bind to PHB granules. Cells were cultivated as described in the legend to Fig. 3. Crude extracts (A1 to F1), soluble protein fractions (A2 to F2), and granule protein fractions (A3 to F3) were prepared as described in Materials and Methods. (a) SDS-polyacrylamide (15% [wt/vol]) gel stained with Coomassie brilliant blue. M_rs of standard proteins (phosphorylase *b*, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100; α-lactalbumin, 14,400) are provided in the left margin. Wild-type and putative truncated GA14 proteins are marked by arrows. (b) Immunoblot with GA14-specific antibodies. Std, standard proteins; A, *E. coli* XL1-Blue(pSKCO7); B, *E. coli* XL1-Blue(pSKCO501); C, *E. coli* XL1-Blue(pSKCO477); D, *E. coli* XL1-Blue(pSKCO462); E, *E. coli* XL1-Blue(pSKCO432); F, *E. coli* XL1-Blue(pSK2665).

be involved in the binding of the GA14 protein to the granules, or it might be required for the stability of the protein. Finally, the 501-bp *Xho*I-*Apa*I fragment of pSKXA501 encoded a truncated GA14 protein (M_r, 12,697 [124 amino acids]) that stopped immediately upstream of HD2.

Analysis of the PHB granule surface binding site of GA14 protein. The hybrid plasmids pSKXA432, pSKXA462, pSKXA 477, and pSKXA501 were cut at their single *Kpn*I restriction sites, and blunt ends were created with the Klenow fragment of DNA polymerase I. Subsequently, the 5.2-kbp fragment containing the PHB biosynthesis operon of *A. eutrophus* was ligated to these linearized plasmids, yielding pSKCO432, pSKCO462, pSKCO477, and pSKCO501, respectively. These plasmids contained the truncated variants of $phaP_{Rr}$ and $phaCAB_{Ae}$ in an antilinear orientation. After transformation into *E. coli* XL1-Blue, the ability of the different truncated variants of the GA14 protein to bind to native PHB granules was investigated (Fig. 5). Proteins of crude extracts, the soluble protein fraction after ultracentrifugation, and the granule-associated proteins of the different strains were separated in SDS-polyacrylamide gels and blotted onto nitrocellulose. The wild-type GA14 protein and the different truncated variants of the GA14 protein were detected with polyclonal antibodies raised against the wild-type GA14 protein. Wild-type GA14 protein could be detected in crude extracts and in granule preparations of *E. coli* XL1-Blue(pSKCO7) (Fig. 5a and b, lanes A1 and A3), but it was absent from the soluble protein fraction (Fig. 5a and b, lane A2). This indicated that in this strain, the GA14 protein was completely bound to the PHB granules. The amount of other proteins, which were unspecifically bound to the granules, was not reduced. All truncated variants of the GA14 protein lacking HD1 or both HD1 and HD2 were only detected in crude extracts and in the soluble protein fractions, as revealed by the electropherograms and immunoblots obtained from cells of the strains *E. coli* XL1-Blue(pSKCO501), XL1-Blue(pSKCO477), XL1-Blue(pSKCO462), and XL1-Blue(pSKCO432) (Fig. 5a and b, lanes B1 and B2, C1 and C2, D1 and D2, and E1 and E2, respectively), but they were never detected in the granule protein fraction (Fig. 5a and b, lanes B3, C3, D3, and E3). Therefore, these proteins have obviously lost the ability to bind to PHB granules.

Interestingly, the expression of the M_r 12,697, 11,808, and 11,222 variants of the GA14 protein, which lacked only HD2, was lower than the expression of the M_r 10,255 variant, which lacked both HD1 and HD2. Therefore, the antibody reaction with these proteins in the crude extract was very weak (Fig. 5b, lanes B1, C1, and D1), and the bands corresponding to the truncated variants of the GA14 protein in the soluble protein fraction were rather faint (Fig. 5b, lanes B2, C2, and D2). No signals were obtained with crude extracts or granule protein fractions prepared from cells of *E. coli* XL1-Blue(pSK2665) expressing the PHB operon only (Fig. 5 b, lanes F1 and F3). An unspecific antibody reaction was observed with a higher-M_r protein of the crude extract and/or granule fraction of strains *E. coli* XL1-Blue(pSKCO7) and XL1-Blue(pSKCO462) (Fig. 5b, lanes A1, A3, and D3). The nature of this protein, however, is unknown.

Postembedding immunogold labeling of the strains *E. coli* XL1-Blue(pSKCO7) and XL1-Blue(pSKCO432) with GA14-specific polyclonal antibodies confirmed the results of the immunoblot analysis. Whereas in *E. coli* XL1-Blue(pSKCO7), the wild-type GA14 protein was found only at the surface of PHB granules (Fig. 3a), in *E. coli* XL1-Blue(pSKCO432), the truncated GA14 protein lacking both HD1 and HD2 was randomly distributed in the cytoplasm but was not attached to the PHB granule surface (Fig. 3c). The inability to bind to the granules significantly affected the size of the granules. In the recombinant strains *E. coli* XL1-Blue(pSKCO501), XL1-Blue(pSKCO477), XL1-Blue(pSKCO462), and XL1-Blue(pSKCO432) (Fig. 4b, c, d, and e, respectively), the granules were considerably larger than those in *E. coli* XL1-Blue

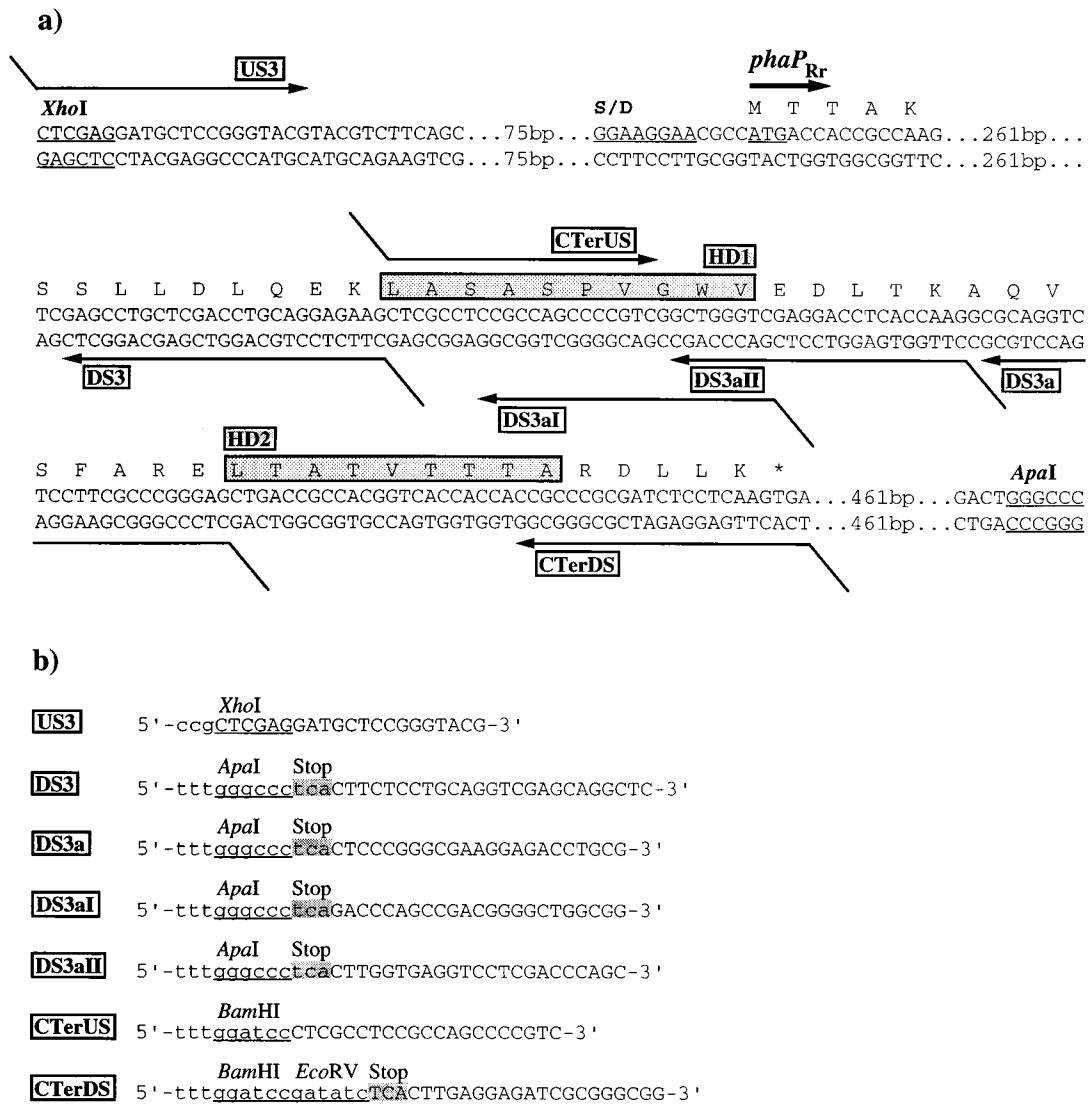

FIG. 6. Construction of truncated forms of $phaP_{Rr}$ and of a DNA fragment containing the 3′-terminal region of $phaP_{Rr}$ by PCR. (a) Relevant part of the nucleotide sequence of the 1.0-kbp *Xho*I-*Apa*I fragment of pSKXA10* containing $phaP_{Rr}$. The deduced amino acid sequence of the GA14 protein is partially shown above the nucleotide sequence in the one-letter code. The C-terminal hydrophobic domains HD1 and HD2 are highlighted. PCR primers used to construct truncated forms of $phaP_{Rr}$ and the product containing the 3′-terminal region of $phaP_{Rr}$, which encodes the C terminus of the GA14 protein, are depicted as arrows. S/D, putative ribosome binding site. (b) Nucleotide sequences of the PCR primers. Parts homologous to pSKXA10* are shown in capital letters, and nonhomologous parts are shown in lowercase letters. Original or additional stop codons are highlighted. Restriction sites are underlined.

(pSKCO7) (Fig. 4a), which expressed the granule-associated wild-type GA14 protein.

Construction of a fusion protein composed of AcDH-II and the C terminus of the GA14 protein. A second approach was applied to confirm that the two C-terminal hydrophobic domains of the GA14 protein are responsible for the anchoring of the protein to the PHB granules. For this, we constructed a fusion gene that was composed of the gene *acoD*, which encoded the AcDH-II of *A. eutrophus* (22), and the C-terminal region of $phaP_{Rr}$. This construct was based on a pBluescript KS^- derivative, pDel1074, that overproduced AcDH-II in *E. coli* XL1-Blue (23).

First, we eliminated the stop codon of *acoD*. For this, the 1.3-kbp *Bgl*II-*Bam*HI restriction fragment was removed from pDel1074 (Fig. 7a). This fragment contained 314 bp of the 3′ region of *acoD* and approximately 1 kbp of genomic DNA of *A. eutrophus* located downstream of *acoD*. With the primers AcDHUS and AcDHDS (Fig. 7a and e), a PCR that produced a 320-bp fragment containing the 3′ region of *acoD* modified by the insertion of a *Bam*HI restriction site instead of the stop codon of *acoD* was performed. This PCR product was ligated to the remaining *Bgl*II-*Bam*HI restriction fragment of pDel1074 that contained the 5′ region of *acoD*, yielding the plasmid pDel1074B (Fig. 7b). In this construct, the translation of *acoD* mRNA stopped 54 nucleotides downstream of *acoD* at the TAG stop codon in the T7 promoter region of pBluescript KS^-. Second, a PCR product was synthesized by employing the oligonucleotides CTerUS and CTerDS as primers and plasmid pSKXA10* as template DNA (Fig. 6). The resulting 132-bp *Bam*HI fragment contained the 3′-terminal region of $phaP_{Rr}$ encoding the C-terminal region of the GA14 protein, including HD1 and HD2, the stop codon downstream of $phaP_{Rr}$, and an additional *Eco*RV restriction site. This *Bam*HI fragment was ligated into *Bam*HI-digested pDel1074B, yielding the plasmid

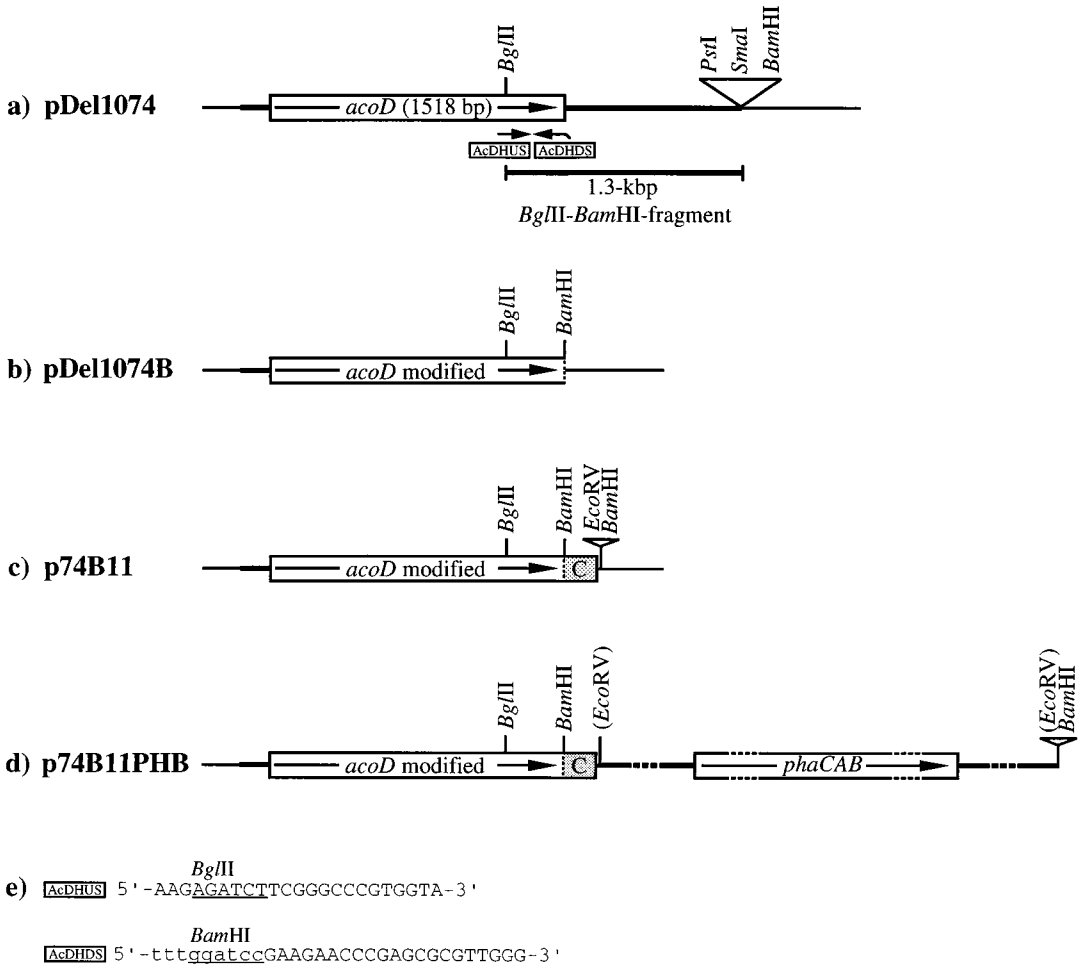

FIG. 7. Construction of the fusion gene composed of *acoD* and of the C-terminal nucleotide sequence of $phaP_{Rr}$ and coexpression with the PHB operon of *A. eutrophus*. Plasmid pBluescript KS$^-$ DNA is shown by a thin line, and the genomic DNA of *A. eutrophus* is shown by a thick line. C, C-terminal nucleotide sequence of $phaP_{Rr}$. (a to d) Different hybrid plasmids relevant for construction. (e) Nucleotide sequences of PCR primers. Parts homologous to *acoD* are shown in capital letters, and parts not homologous to *acoD* are shown in lowercase letters. Restriction sites are underlined.

p74B11 (Fig. 7c). The blunt-ended restriction site for *Eco*RV in p74B11 was ligated to the 5.2-kbp fragment encoding the PHB operon of *A. eutrophus*, yielding the plasmid p74B11PHB (Fig. 7d). In order to compare the ability of the modified AcDH-II protein to bind to PHB granules with that of the wild-type AcDH-II protein, the 5.2-kbp fragment with the PHB operon of *A. eutrophus* was also ligated to the blunt-ended *Sma*I restriction site of pDel1074 (Fig. 7a), yielding the plasmid pDel1074PHB. Both plasmids p74B11PHB and pDel1074PHB contained the modified or wild-type *acoD*, respectively, and $phaCAB_{Ae}$ in a colinear orientation.

Analysis of the ability of the modified AcDH-II to bind to the surface of PHB granules in vivo. The hybrid plasmids mentioned above were transformed into *E. coli* XL1-Blue, and the recombinant strains were cultivated in Luria-Bertani complex medium containing antibiotics, glucose, and IPTG. The specific activity of AcDH-II was determined both in the soluble protein fraction and in the granule fraction (Table 3). The specific activity of the wild-type AcDH-II was slightly higher (354 U/g) in *E. coli* XL1-Blue(pDel1074) that contained no PHB operon than in *E. coli* XL1-Blue(pDel1074PHB) that also expressed the PHB operon of *A. eutrophus* (216 U/g). Modifications at the C terminus of AcDH-II resulted in a sharp decrease in specific activity. *E. coli* XL1-Blue(pDel1074B) and *E. coli* XL1-Blue(p74B11) expressed only approximately 3% of AcDH II-activity (11 or 9 U/g, respectively). In *E. coli* XL1-Blue(p74B11PHB), which also expressed the PHB operon, the

TABLE 3. Specific activities of wild-type AcDH-II and modified AcDH-II containing the C terminus of GA14 protein

Sample[a]	Sp act of AcDH-II (U/g)
Soluble protein fraction of *E. coli*	
XL1-Blue(pDel1074)	354
XL1-Blue(pDel1074PHB)	216
XL1-Blue(pDel1074B)	11
XL1-Blue(p74B11)	9
XL1-Blue(p74B11PHB)	22
XL1-Blue(pSK2665)	0
PHB granule protein fraction of *E. coli*	
XL1-Blue(pDel1074PHB)	3
XL1-Blue(p74B11PHB)	5

[a] Protein fractions were prepared after a 24-h incubation at 37°C in 50 ml of Luria-Bertani medium with ampicillin, tetracycline, 0.1 mM IPTG, and 0.2% (wt/vol) glucose.

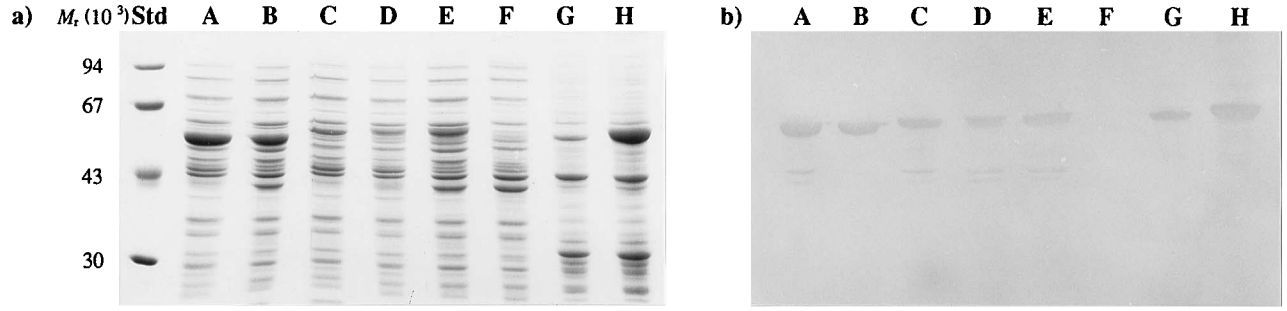

FIG. 8. Expression of the AcDH-II fusion protein and binding to native PHB granules. Cells were cultivated for 24 h at 37°C in 50 ml of Luria-Bertani medium with ampicillin, tetracycline, 0.1 mM IPTG, and 0.2% (wt/vol) glucose. Soluble protein fractions (A, B, C, D, E, and F) and granule proteins (G and H) were prepared as described in Materials and Methods. The M_rs of standard proteins (Std [see the legend to Fig. 5]) are given at the left. (a) SDS-polyacrylamide (10% [wt/vol]) gel Coomassie stained. (b) Immunoblot with AcDH-II-specific antibodies. Lanes: A, *E. coli* XL1-Blue(pDel1074); B and G, *E. coli* XL1-Blue(pDel1074PHB); C, *E. coli* XL1-Blue(pDel1074B); D, *E. coli* XL1-Blue(p74B11); E and H, *E. coli* XL1-Blue(p74B11PHB); F, *E. coli* XL1-Blue(pSK2665).

level of activity was slightly higher (22 U/g). The activities of AcDH-II in the granule protein fractions of the strains *E. coli* XL1-Blue(pDel1074PHB) and *E. coli* XL1-Blue(p74B11PHB) were 3 and 5 U/g, respectively.

In further experiments, the soluble proteins and the proteins of the granule preparations were separated in SDS-polyacrylamide gels and transferred onto nitrocellulose. Wild-type AcDH-II and modified AcDH-II were detected with polyclonal antibodies raised against the wild-type protein. In the soluble fraction of *E. coli* XL1-Blue(pDel1074) and *E. coli* XL1-Blue(pDel1074PHB), a strong band was visible in the electropherogram that corresponded to the wild-type AcDH-II exhibiting an M_r of 54,819 (Fig. 8a, lanes A and B [22]); the occurrence of AcDH-II was confirmed by immunoblot analysis (Fig. 8b, lanes A and B). No overproduction of modified AcDH-II containing the C terminus of GA14 protein (M_r, 58,908) in *E. coli* XL1-Blue(p74B11) and *E. coli* XL1-Blue(p74B11PHB) was observed (Fig. 8a, lanes D and E), but the antibody reaction detected a protein with an electrophoretic mobility slightly higher than that of wild-type AcDH-II (Fig. 8b, lanes D and E). In PHB granule preparations of *E. coli* XL1-Blue(pDel1074PHB), only a relatively small amount of wild-type AcDH-II protein (Fig. 8a and b, lane G) was observed, whereas in the electropherograms as well as in the immunoblots of the granule proteins of *E. coli* XL1-Blue(p74B11PHB), large amounts of the modified AcDH-II protein were detected (Fig. 8a and b, lane H). Therefore, we conclude that the C terminus of GA14 protein of *R. ruber* mediated a much higher affinity of the modified AcDH-II to PHB granules in recombinant *E. coli* cells. The detection of small amounts of wild-type AcDH-II at the PHB granules (Fig. 8a and b, lane G) is probably due to a tendency of the protein to bind weakly but unspecifically to PHB granules. This was not astonishing in view of the tendency of PHB granules to adsorb various proteins of the cytoplasm of recombinant *E. coli* cells (Fig. 5a, lanes A3, B3, C3, D3, E3, and F3 and Fig. 8a, lanes G and H) and in view of the strong overproduction of AcDH-II in *E. coli* XL1-Blue(pDel1074PHB).

In vitro binding of the modified AcDH-II to artificial PHB granules. Furthermore, the affinity of wild-type and modified AcDH-II to artificial PHB granules that contained no phospholipid monolayer was investigated. For this, crude extract proteins of the strains *E. coli* XL1-Blue(pDel1074) and *E. coli* XL1-Blue(p74B11) were incubated with artificial PHB granules. Subsequently, the granules were washed, and associated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with AcDH-II-specific antibodies. It was clearly demonstrated that the modified AcDH-II protein efficiently bound to the artificial granules (Fig. 9a and b, lane B3), whereas the wild-type AcDH-II protein was only visible as a faint band among several other proteins that were adsorbed unspecifically to the granules (Fig. 9a and b, lane A3).

The native GA14 protein, which was purified from *E. coli* XL1-Blue(pSKXA10*), was also able to associate with artificial PHB granules (data not shown).

DISCUSSION

The function of the PHA granule-associated GA14 protein from *R. ruber* was investigated in *E. coli* cells after coexpression with the PHB biosynthesis operon of *A. eutrophus*. This strat-

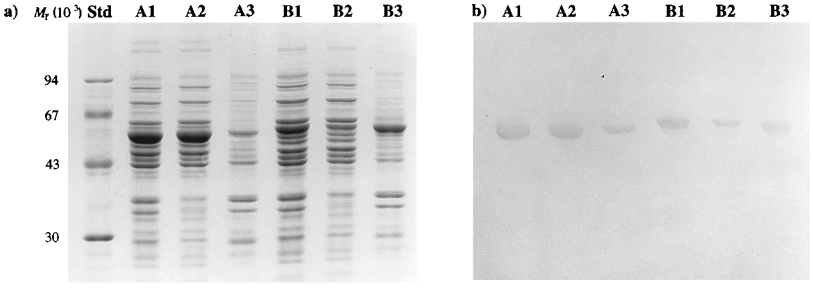

FIG. 9. Binding of wild-type AcDH-II and modified AcDH-II to artificial PHB granules. (a) SDS-polyacrylamide (10% [wt/vol]) gel with proteins of the incubation mixture of crude extract and artificial PHB granules (A1 and B1), proteins of the supernatant after centrifugation (A2 and B2), and proteins bound to artificial granules (A3 and B3). M_rs of standard proteins (Std [see legend to Fig. 5]) are given at the left. (b) Immunoblot with AcDH-II-specific antibodies. A, *E. coli* XL1-Blue(pDel1074); B, *E. coli* XL1-Blue(p74B11).

egy was necessary because of the genetic inaccessibility of the *R. ruber* wild type and mutants defective in PHA biosynthesis (21) and because of the inability of recombinant *E. coli* cells to accumulate PHA after transformation with the PHA synthase gene locus of *R. ruber* (20). Coexpression of the GA14 protein ($phaP_{Rr}$) plus the PHB biosynthesis pathway ($phaCAB_{Ae}$) in *E. coli* XL1-Blue(pSKCO7) did not increase the amount of accumulated PHB or the activities of the biosynthesis enzymes compared with *E. coli* XL1-Blue(pSK2665), which expressed only the PHB biosynthesis pathway. The only (but interesting) effect of the coexpression of $phaP_{Rr}$ and $phaCAB_{Ae}$ was the formation of minigranules in *E. coli* XL1-Blue(pSKCO7) due to the association of the GA14 protein with the PHB granule surface as demonstrated by immunoelectron microscopy. A decoration of the GA14 protein with gold particles at the cell membrane as described for the *R. ruber* wild type or *E. coli* XL1-Blue(pSKXA10*) (21) was not observed in *E. coli* XL1-Blue(pSKCO7). One explanation could be that the amount of GA14 protein in the cells of *E. coli* XL1-Blue(pSKCO7) was lower than that in the strains mentioned above and that the GA14 protein has a higher affinity for the granules than for the cell membrane. The formation of minigranules has already been observed in *A. eutrophus*. Mutants defective in the gene $phaP_{Ae}$ encoding the granule-associated GA24 protein contained only one large PHB granule. However, subsequent to the complementation of these mutants with a hybrid plasmid harboring $phaP_{Ae}$, a large number of rather small granules were detected (38). Interestingly, there are also similarities to the oil bodies of plants. Small oil bodies of pollen or seeds are surrounded by proteins that are referred to as oleosins and that are attached to the phospholipid monolayer on the surface of these oil bodies. Large oil bodies of fruits, on the other hand, lack these oleosins (17). These similarities of the GA14 protein of *R. ruber* and the GA24 protein of *A. eutrophus* to the oleosins of plants prompted us to refer to these PHA granule-associated proteins as phasins (31). The analysis of PHB-accumulating cells of *E. coli* expressing truncated forms of the GA14 protein clearly demonstrated that already the loss of only HD2 had impaired the binding of the modified GA14 protein to the surface of PHB granules.

The analysis of PHB-accumulating cells of *E. coli* expressing the fusion protein consisting of AcDH-II plus the C terminus of the GA14 protein and studies of this fusion protein regarding its binding to artificial PHB granules demonstrated that the fusion protein was bound very efficiently to native or artificial granules. AcDH-II was not the most suitable protein, because the experiments clearly revealed that modifications at the C terminus (i.e., the removal of the stop codon and extension of the enzyme by 18 amino acids in clone pDel1074B) reduced the specific activity drastically. Therefore, the PHB granule-bound enzyme was mostly inactive. In principle, however, these experiments revealed the possibility of immobilizing any suitable protein at a PHB matrix. This might be of biotechnological interest because PHB or other PHAs are a rather cheap material for this purpose and because these polyesters are accessible to biodegradation under mild conditions.

Previous results had suggested that the hydrophobic domains HD1 and HD2 at the C terminus of the GA14 protein might be responsible for anchoring this protein in a phospholipid monolayer surrounding the PHA granule core in *R. ruber* (21). The results obtained in this study confirmed this hypothesis on the basis of (i) the inability of the different truncated variants of the GA14 protein lacking HD2 or both HD1 plus HD2 to bind to PHB granules and (ii) the binding of the modified AcDH-II containing the C terminus of the GA14 protein to native PHB granules. The association of modified AcDH-II and wild-type GA14 protein with artificial PHB granules implies that HD1 and/or HD2 could also directly interact with the PHA granule surface even without the involvement of a phospholipid monolayer. In this context, HD2, which is a stretch of nine amphiphilic or hydrophobic amino acids, is of special interest because five of the nine amino acids are threonines. Similar threonine-rich sequences were also observed at the C termini of extracellular PHB depolymerases of *Pseudomonas lemoignei*, and they were considered to be the substrate-binding domain of these depolymerases (4, 11).

ACKNOWLEDGMENTS

This work was supported by ZENECA BioProducts and by the DFG-Graduiertenkolleg Chemische Aktivitäten von Mikroorganismen at the Georg-August-Universität Göttingen.

We thank D. Jendrossek for providing AcDH-II-specific antibodies and artificial PHB granules and H. Priefert for supplying plasmid pDel1074.

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