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Analysis of a 24-Kilodalton Protein Associated with the Polyhydroxyalkanoic Acid Granules in *Alcaligenes eutrophus*

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A 5.0-kbp genomic *Eco*RI restriction fragment which complemented a third subclass of polyhydroxyalkanoic acid (PHA)-leaky mutants of *A. eutrophus* that accumulated PHA at a lower rate than the wild type was cloned from *Alcaligenes eutrophus* H16. A 687-bp $phaP_{Ae}$ gene on this fragment encoded a 24-kDa protein (M_r = 23,963), which was referred to as the GA24 protein. The GA24 protein was solubilized from the granules and purified to electrophoretic homogeneity, and antibodies against the GA24 protein were obtained. The GA24 protein bound to the surface of PHA granules, as revealed by immunoelectron microscopy of whole cells and of artificial PHA granules. The GA24 protein contributed approximately 5% (wt/wt) of the total cellular protein, and it was the predominant protein present in the granules. It was synthesized only in cells accumulating PHA and only in amounts that could be bound to the granules; no soluble GA24 protein was detected. Tn*5*::*mob*-induced $phaP_{Ae}$ mutants which were unable to synthesize intact GA24 protein formed only one large PHA granule per cell. The amino acid sequence of the GA24 protein revealed two closely related stretches consisting exclusively of nonhydrophilic amino acids at the C-terminal region, which are presumably involved in the binding of GA24 to the granules, as was recently proposed for a similar protein in *Rhodococcus ruber*. The GA24 protein seems to be a representative of phasins, which are a new class of protein that form a layer at the surface of PHA granules, like oleosins, which form a layer at the surface of triacylglycerol inclusions in oilseed plants.

A wide range of different polyhydroxyalkanoic acids (PHA) are synthesized by many bacteria; these polyesters function in most bacteria as energy and carbon storage compounds or as a sink for reducing equivalents (1, 46). Since PHA are biodegradable thermoplastics and/or elastomers, and since they are chiral components because of the incorporation of *R*(−)-3-hydroxyalkanoic acids, they are currently considered for many applications (47). A copolyester of 3-hydroxybutyric acid (3HB) and 3-hydroxyvaleric acid is already biotechnologically produced on the industrial scale by ZENECA Bioproducts, utilizing a derivative strain of *Alcaligenes eutrophus* H16 (6).

A. eutrophus synthesizes PHA consisting of hydroxyalkanoic acids with short chains, and besides 3HB and 3-hydroxyvaleric acid, 3-hydroxypropionic acid, 4-hydroxybutyric acid, 4-hydroxyvaleric acid, and 5-hydroxyvaleric acid are also incorporated into the polyester. Strain H16 has been investigated in most detail regarding physiological, biochemical, and molecular aspects of the metabolism of PHA (references 10 and 50 and references cited therein). Isolation of Tn*5*-induced mutants of *A. eutrophus* which were affected in the accumulation of poly(3HB) revealed not only poly(3HB)-negative mutants, which were completely impaired in the synthesis of poly(3HB), but also many poly(3HB)-leaky mutants (30, 41). Mutants exhibiting the poly(3HB)-leaky phenotype were still able to synthesize poly(3HB) but accumulated less polyester than the wild type. Molecular analysis and complementation studies of poly(3HB)-leaky mutants of *A. eutrophus* have so far revealed two different subclasses of genotypes. In mutants of the first subclass, Tn*5*::*mob* has inserted into two genes whose translational products are highly homologous to the protein HPr and the enzyme I component of bacterial sugar:phosphoenol pyruvate phosphotransferase systems. In analogy to *ptsH* and *ptsI*, which in enterobacteria encode these proteins, the *A. eutrophus* genes were referred to as *phaH* and *phaI*, respectively. It was proposed that these proteins are involved in the regulation of mobilization of poly(3HB) (35). In mutants of the second subclass, Tn*5*::*mob* has inserted into the structural gene of a dihydrolipoamide dehydrogenase (*phaL*) (34), which is located downstream of the structural genes encoding the other components of the pyruvate dehydrogenase multienzyme complex (14) and which represents only one of at least two different dihydrolipoamide dehydrogenase genes identified in *A. eutrophus* (14a).

Of 35 Tn*5*-induced poly(3HB)-leaky mutants, did not belong to either genotype. The aims of this study were the analysis of these mutants, the identification of the gene locus affected in these mutants, and elucidation of the function of the gene product.

MATERIALS AND METHODS

Bacterial strains and plasmids. *A. eutrophus* strains H16 (DSM 428), PHB$^-$4 (PHA-negative mutant of H16; DSM 541), H1489 (PHA-leaky mutant of Sm^r derivative strain HF39 of H16 defective in *phaI* [35]), and H1474 (PHA-leaky mutant of HF39 defective in *phaL* [34]) and the PHA-leaky mutants H2262, H2271, H2272, H2273, and H2275 (34), which were also obtained from HF39, were used in this study. For immunoscreening, we also used *A. eutrophus* strains SP1 (spontaneous PHA-negative mutant of H16 [41]) and PSI (Tn*5*-induced PHA-negative mutant of HF39 [41]).

In addition, we used *Escherichia coli* strains S17-1 (42), XL1-Blue (5), and DH1 (13) as well as plasmids pVK100 (21), pVDZ′2 (8), pSUP202 (43), pHC79 (17), pSUP5011 (43), pBluescript KS$^-$ and SK$^-$ (Stratagene, San Diego, Calif.), pVK101::PP1 (41), pHB4550 (35), and pVK6300 (34).

Determination of PHA synthase activity. The activity of PHA synthase (no EC number assigned) was measured in crude extracts, which were obtained as described by Schubert et al. (41) by a radiometric assay employing [^{14}C]-3-hydroxybutyryl-coenzyme A as a substrate, as described recently (52). Protein was determined as described by Lowry et al. (23).

Analysis of PHA. To determine the polyester content of bacteria, 3 to 5 mg of lyophilized cell material was subjected to methanolysis in the presence of 15% (vol/vol) sulfuric acid, and the hydroxyacyl methyl esters were analyzed by gas chromatography as described in detail by Brandl et al. (4) and Timm et al. (51).

Isolation and analysis of DNA. Total genomic DNA and plasmid DNA were

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isolated by standard procedures (37), and DNA-manipulating enzymes were used according to the manufacturers' protocols.

Hybridization experiments. DNA restriction fragments were separated electrophoretically in 0.8% (wt/vol) agarose gels in TBE buffer (50 mM Tris hydrochloride, 50 mM boric acid, 1.25 mM EDTA; pH 8.5). Denatured DNA was transferred from agarose gels or from plaques to positively charged nylon membranes (pore size, 0.45 μm; Pall Filtrationstechnik, Dreieich, Germany) and was hybridized with biotinylated probes as described by Oelmüller et al. (28).

Transfer of DNA. Transformation of *E. coli* was done by the $CaCl_2$ procedure as described by Hanahan (13). Matings of *A. eutrophus* (recipient) with *E. coli* S17-1 (donor) harboring hybrid donor plasmids were done on nutrient broth agar medium as described by Friedrich et al. (9).

Growth of bacteria. *A. eutrophus* was grown at 30°C either in a complex medium (0.8% [wt/vol] nutrient broth from GIBCO-BRL) or in a mineral salts medium (MM) (40) which was supplemented by a filter-sterilized carbon source as indicated below. To promote extensive accumulation of PHA, the concentration of ammonium chloride in the MM was reduced to 0.02% (wt/vol). *E. coli* was grown at 37°C in Luria-Bertani medium (37).

Cloning of genes. Genomic DNA was partially digested with *Eco*RI, ligated to *Eco*RI-digested pVK100 DNA, packaged with λ coat proteins by using an in vitro packaging kit, and transfected into *E. coli* S17-1 (17). From *E. coli* S17-1, the hybrid cosmids were mobilized into mutants H2275 and H2262, which were provided as a lawn on an MM agar plate supplemented with 0.5% (wt/vol) fructose and containing 12.5 μg of tetracycline per ml but a reduced amount of ammonium chloride. Phenotypic complementation of the mutants was indicated by colonies which exhibited increased opalescence (35).

Determination of metabolites. The concentrations of pyruvate and 3-hydroxybutyrate in cell-free fermentation broth were determined by spectrometric assays which were done at 365 nm employing lactate dehydrogenase (7) or 3-hydroxybutyrate dehydrogenase (55), respectively.

Isolation of PHA granules. PHA granules were isolated by a modified method of Preusting et al. (33). The poly(3HB) granules were obtained from cells of *A. eutrophus* H16 which were grown in MM containing 1% (wt/vol) fructose. After 48 h of incubation, cells of six 500-ml cultures were harvested by centrifugation (20 min, 6,000 × *g*, 4°C). The cells were washed and resuspended in 30 ml of Tris-HCl buffer (100 mM, pH 7.5), and after a twofold French press passage (100×10^6 Pa), 5 ml of the lysate was loaded on top of a linear glycerol gradient. This gradient was obtained from a discontinuous gradient prepared from 1 ml of 88% and 4 ml of 44% glycerol in Tris-HCl (pH 7.5), which was frozen at −70°C and then thawed at 4°C. After centrifugation (0.5 h, 210,000 × *g*, 4°C), a granule layer was obtained at about 88% glycerol. The granules were isolated from this region and were washed with 100 mM Tris-HCl (pH 7.5) by centrifugation (10 min, 100,000 × *g*, 4°C). The granules were resuspended in 100 mM Tris-HCl (pH 7.5) and were loaded on top of a linear sucrose gradient, prepared from 2 ml each of 2.0, 1.66, 1.33, and 1.0 M sucrose in 100 mM Tris-HCl (pH 7.5). After centrifugation (2 h, 210,000 × *g*), the granules sedimented at a density of approximately 1.2 g/ml. The granules of this band were washed twice with 100 mM Tris-HCl (pH 7.5) and were then stored at −20°C.

Solubilization and purification of the GA24 protein. For isolation of the GA24 protein, a 100 mM Tris-HCl buffer (pH 7.5 or 8.0) containing EDTA and phenylmethylsulfonyl fluoride (each 0.5 mM) was used throughout all purification steps, and all steps were performed at 4°C. The GA24 protein was solubilized from the native granules of *A. eutrophus* H16 by treatment with Triton X-114 (1.5% [wt/vol] in Tris-HCl buffer). The suspension was stirred for 90 min at 4°C, and the solubilized proteins were separated from the granules by centrifugation (68,000 × *g*, 20 min). After 5 min of incubation at 37°C, the solution became turbid, and the Triton X-114 micelles were collected by centrifugation (2,800 × *g*, 10 min). The upper phase, which was referred to as the soluble fraction, was isolated and stored at −20°C.

The soluble fraction was applied to a column (65-ml bed volume; 5-cm diameter) of Procion Blue H-ERD (ICI, Frankfurt, Germany) which was coupled to Sepharose CL-6B and equilibrated with buffer. After the column was washed with 500 ml of buffer and a linear gradient of NaCl (1,000 ml; 0 to 1 M; pH 7.5) was applied, the GA24 protein eluted from the column between 250 and 400 mM NaCl. Fractions (10 ml each) were collected, those which contained the GA24 protein as revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were pooled and concentrated by ultrafiltration with a YM5000 membrane (Amicon, Witten, Germany), and ammonium sulfate was added to 35% saturation. This solution was applied to a column of butyl Sepharose CL-4B (40-ml bed volume; 26-mm diameter; Pharmacia LKB Biotechnology, Uppsala, Sweden). The column was washed with 100 ml of buffer, and the GA24 protein was eluted with an ammonium sulfate gradient (35 to 0% ammonium sulfate saturation). Fractions (7.5 ml each) containing the GA24 protein, which was eluted from 24 to 20% saturation of ammonium sulfate, were pooled, concentrated by ultrafiltration with a YM5000 membrane, and dialyzed overnight against 100 mM Tris-HCl, pH 8.0. The dialyzed sample was then applied to a Q-Sepharose column (40-ml bed volume; 26-mm diameter; Pharmacia LKB Biotechnology). The column was washed with 120 ml of buffer, the proteins were eluted from the column with a linear gradient of NaCl (500 ml; 0 to 500 mM; pH 7.5), and fractions (6 ml each) were collected. The GA24 protein was eluted from 15 to 50 mM NaCl. Fractions containing only the GA24 protein were pooled, concentrated with a YM5000 membrane, and stored at −20°C.

Preparation of poly(3HB) and poly(3HO) granules. Granules consisting of the homopolyester poly(3HB) were isolated from sodium gluconate-grown cells of *A. eutrophus* H16 with hypochlorite as described previously (19). The preparation of poly(3-hydroxyoctanoic acid) [poly(3HO)] granules was done as described by Schirmer et al. (39).

Determination of nucleotide sequence. DNA sequencing was performed by the dideoxy-chain termination method of Sanger et al. (38) (see Fig. 7) with alkali-denatured double-stranded plasmid DNA, using 7-desazaguanosine 5′-triphosphate instead of dGTP, and with α-^{35}S-dATP by using a T7 polymerase kit as specified by the manufacturer (Pharmacia LKB Biotechnology). Universal and synthetic oligonucleotides were used as primers. Nucleic acid sequence data and deduced amino acid sequences were analyzed with DNA-Inspector and DNA-Strider (24).

Nucleotide sequence accession number. The nucleotide and amino acid sequence data reported here have been submitted to the EMBL, GenBank, and DDBJ nucleotide sequence databases and are available under accession no. X85729.

SDS-PAGE. Samples were resuspended in gel loading buffer (0.6% [wt/vol] SDS, 1.25% [wt/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 were separated in SDS–12.5% (wt/vol) polyacrylamide gels as described by Laemmli (22). Proteins were stained with Coomasie brilliant blue R-250 (54) or with silver (15).

Preparation and purification of antibodies. Antigen (600 μg of GA24 protein in 500 μl of 100 mM Tris-HCl, pH 7.5) was mixed with incomplete Freund's adjuvant (500 μl) and was injected subcutaneously into the back of a rabbit (New Zealand White, female, 2.5 kg). A booster injection with the same amount of antigen mixed with incomplete Freund's adjuvant was given 3 weeks later. After 15 days, a second booster injection with the same amount of antigen was administered in one of the front paws. Two weeks later, the rabbit was bled, and the immunoglobulin G fraction of the serum was purified on a protein A-Sepharose CL-4B affinity column (16). To obtain highly monospecific antibodies against the GA24 protein, the antiserum was subjected to an affinity purification done by a modification of the method of Olmsted (29) as described by Pieper-Fürst et al. (31).

Western blotting (immunoblotting). Immunological detection of the GA24 protein blotted from SDS-polyacrylamide gels onto nitrocellulose membranes was done exactly as described recently (31).

Electron microscopic studies. Cells which had been washed and suspended in 50 mM potassium phosphate buffer (pH 6.8) were fixed in the presence of a mixture of 0.2% (vol/vol) glutaraldehyde plus 0.3% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS) and embedded in Spurr's low-viscosity resin (45) as described by Walther-Mauruschat et al. (53). For postembedding and immunogold labelling of the GA24 protein, the cells were embedded in Lowicryl K4M (Lowi, Waldkraiburg, Germany) as described previously (36) except that methanol instead of ethanol was used for dehydration. Immunological detection of the GA24 protein in ultrathin sections employing the primary antibody and goat anti-rabbit immunoglobulin G-gold (GARG) complex (Dakopatts, Hamburg, Germany) and metal shadowing of the sections was done exactly as described recently (31). The specificity of the labelling was demonstrated by a control experiment using only the GARG complexes and monoclonal antibodies against the cap proteins of Sendai virus. Micrographs were taken with a Philips EM 301 electron microscope at an acceleration voltage of 80 kV. Magnifications were calibrated with a cross-lined grating replica (Balzers Corp., Liechtenstein).

For on-grid labelling, the poly(3HB) granules were resuspended in PBS buffer and adsorbed onto glow-discharged Formvar-coated carbon grids for 15 s at room temperature. The grids were incubated for 15 min on a PBS drop with 1% (wt/vol) casein and then incubated for 1 h in a solution of antibody diluted in PBS. The grids were then washed once with PBS-Tween buffer and were floated for 2 h at room temperature over a series of dilutions of GARG complex. The grids were washed again with PBS, floated for 30 s over double-distilled water, and stained with 4% (wt/vol) aqueous uranyl acetate (pH 4.5) for 2 min. The specificity of the labelling was demonstrated by a control experiment using only the GARG complexes.

RESULTS

Characterization of *phaP* mutants. Recent studies had revealed two different genomic fragments of *A. eutrophus* H16 which phenotypically complemented most of the Tn*5*-induced PHA-leaky mutants of H16 (34, 35). Of a total of 35 PHA-leaky mutants, only 5 (H2262, H2271, H2272, H2273, and H2275) were not complemented by either fragment. This group of mutants is referred to hereafter as *phaP* mutants. No pleiotropic phenotypic effects were detected with PHA-leaky mutants with the *phaP* genotype. The opacity of colonies on MM-fructose (1% [wt/vol]) agar plates starved for nitrogen was much less than the opacity of wild-type colonies and was comparable to that of colonies of *phaHI* or *phaL* mutants.

TABLE 1. Activity of poly(3HB) synthase in PHA-negative and PHA-leaky mutants of *A. eutrophus*

Strain	Mutant class	Gene affected	Specific activity of poly(3HB) synthase (U/g of protein)
H16	Wild type		29.1
PHB$^-$4	PHB-negative	*phaC*	<0.1
H1489	PHB-leaky	*phaI*	11.3
H1474	PHB-leaky	*phaL*	25.6
H2275	PHB-leaky	*phaP*	7.3

When these mutants were cultivated under nitrogen starvation in liquid MM containing fructose, the cells accumulated poly(3HB) up to a maximum of only 50% (wt/wt) of the cellular dry matter. The rate of poly(3HB) accumulation was less than with the wild type, and D-(−)-3HB was transiently excreted into the medium up to a concentration of approximately 1 mM; in contrast, pyruvic acid, which was excreted by *phaL* mutants (34), was not detected. Cells of mutant H2275 expressed only approximately 25% of the poly(3HB) synthase activity that was detected in wild-type cells. Similar values were also obtained for independent *phaP* mutants. In contrast, *phaHI* and *phaL* mutants expressed approximately (40 ± 5)% or (90 ± 5)% of the poly(3HB) synthase activity of the wild type. Therefore, expression of poly(3HB) synthase activity was more affected in *phaP* mutants than in *phaHI* or *phaL* mutants (Table 1).

Cells of *phaP* mutants which had been cultivated under the conditions described above were clearly distinguished from the wild type and from both other subclasses of genotypes of PHA-leaky mutants by the size and number of the poly(3HB) granules occurring in the cells. As shown in Fig. 1 for mutant H2275, in most cells only a single granule which filled almost the entire cytoplasm was detected, whereas cells of the wild type contained usually 6 to 15 smaller granules.

Identification of the GA24 protein as the major PHA granule-associated protein. When poly(3HB) granules were isolated from *A. eutrophus* H16 cells cultivated for 48 h in MM containing 1.0% (wt/vol) fructose plus 0.02% (wt/vol) ammonium chloride, by employing centrifugation of crude cells extracts containing the granules in glycerol or sucrose density gradients as described in Materials and Methods, the granule preparation contained a major 24-kDa protein along with several other, minor proteins (Fig. 2). This protein was referred to as the GA24 protein. Interestingly, the electropherogram of crude extracts of the wild-type H16 revealed a 24-kDa protein which was one of the predominant proteins and which contributed approximately 5% (±2%) of the total protein as estimated from the intensity of the stained protein band. Immunological studies clearly showed that this protein band is lacking in PHA-negative mutants as well as in *phaP* mutants (see below and Fig. 3). The proteins of wild-type granules were separated in SDS-polyacrylamide gels and were blotted onto a polyvinylidene difluoride membrane; the region comprising the GA24 protein of the wild-type granules was cut off and was subjected to microsequencing. A sequence of 25 amino acids was obtained [MILTPEQVAAAQKANLETLFG(L)A(T)K].

Purification of the GA24 protein. Several detergents were investigated for their capability to solubilize the GA24 protein from poly(3HB) granules isolated from *A. eutrophus*. Whereas Triton X-100 (0.5%, wt/vol), Tween 20 (0.5%, wt/vol), and Brij 58 (0.5%, wt/vol) were less effective and solubilized this protein only partially, SDS (0.15%, wt/vol) and Triton X-114 (0.5%, wt/vol) solubilized the GA24 protein almost completely after 90 min of incubation at 30 or 4°C, respectively. For routine purification, the GA24 protein was solubilized from *A. eutrophus* H16 granules by treatment with Triton X-114, and the solubilized protein was subsequently purified to electrophoretic homogeneity in a three-step procedure (Fig. 2).

Chromatography on triazine dye affinity media was chosen as the first purification step. Of nine affinity media which were tested for the capability to bind the GA24 protein, only Procion Blue H-ERD-Sepharose and Matrex gel Blue A bound the protein; Procion Blue Mx-2G-Sepharose CL-4B, Blue A, Matrex gel Orange A, Procion Yellow Mx8G-Sepharose CL-4B, Matrex gel Green A, Procion Brown Sepharose CL-4B, and Procion Green HE4BD-Sepharose CL-4B did not bind the GA24 protein. Because of the strength of the binding, Procion Blue H-ERD-Sepharose was chosen for further purification. The GA24 protein was eluted from the column at approximately 350 mM NaCl. SDS-PAGE of the purified protein revealed four bands, which represented proteins with M_rs of 43,000, 24,000, 15,000, and 10,000. Subsequent hydrophobic interaction chromatography on butyl Sepharose CL-4B starting with 35% ammonium sulfate saturation eluted the protein at approximately 20% ammonium sulfate saturation. After chromatography on a Q-Sepharose column, from which the GA24 protein was eluted with an NaCl linear gradient at 20 mM, an electrophoretically homogeneous preparation (Fig. 2) was obtained. A purification procedure starting with 9 liters of cell suspension or approximately 40 g of wet cells, of which corresponded to approximately 12 g of dry cell mass, yielded approximately 10 mg of purified GA24 protein.

The purified GA24 protein was again subjected to microsequencing, and an amino acid sequence identical to that described above was obtained. The preparation of the purified GA24 protein was also used to raise polyclonal antibodies and to investigate binding to artificial PHA granules.

Regulation of *phaP* expression. Crude extracts of wild-type *A. eutrophus* cells that were cultivated in MM under nitrogen deficiency as well as PHA granules isolated from these cells exhibited the presence of a strong 24-kDa protein band on any carbon source that was provided in excess (fructose, gluconate, 4-hydroxyvalerate, laevulinate, octanoate, and succinate) as revealed by the electropherograms of SDS-polyacrylamide gels. In contrast, *A. eutrophus* cells which were grown in nutrient broth medium did not accumulate PHA and did not express the GA24 protein. Spontaneously occurring PHA-negative mutants such as SP1 or Tn*5*-induced negative mutants such as PSI, both of which resulted from a single mutation within the PHA synthase structural gene, also did not express the GA24 protein. The same was true for *A. eutrophus* H16 PHB$^-$4, which was also a PHA synthase mutant and which was complemented by the expression of PHA synthase genes from many other bacteria, such as, e.g., *Rhodococcus ruber* or *Rhodobacter sphaeroides* (reference 49 and references cited therein). Immunoblotting analysis of proteins which were separated in an SDS-polyacrylamide gel did not reveal the presence of any GA24 protein (Fig. 3).

This indicated that the expression of the GA24 protein depended strongly on the presence of on intact PHA biosynthesis apparatus and that the expression of *phaP* was regulated. Evidence for strong regulation of the expression of the GA24 protein was also obtained from other experiments. When the PHA granules in crude cell extracts of PHA-accumulating cells of *A. eutrophus* were centrifuged at a low speed, which was sufficient to sediment the granules, and when the soluble proteins of the supernatant were separated in an SDS-polyacrylamide gel and subjected to immunoblot analysis, no GA24 protein was detected by Western blotting (Fig. 4). This indicated that the cells synthesized only as much GA24 protein as

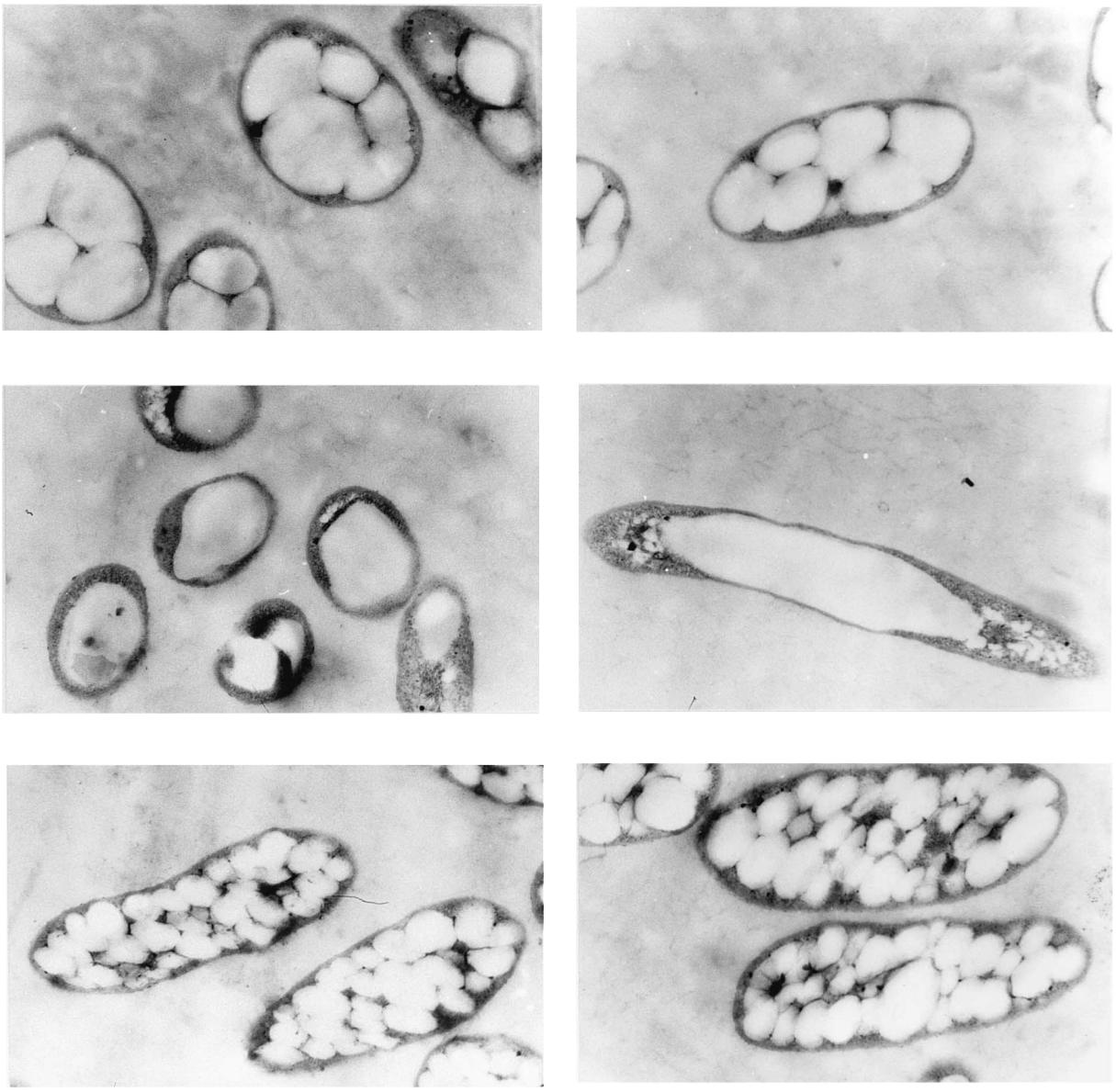

FIG. 1. Morphology of cells of *A. eutrophus phaP* mutants. Cells were cultivated in MM containing 0.05% (wt/vol) ammonium chloride plus 1.0% (wt/vol) fructose and were harvested in the stationary growth phase. The poly(3HB) content of the cells was determined gas chromatographically, thin sections were prepared, and electron micrographs were taken as described in Materials and Methods. Representative cells of the following strains with the indicated approximate percentages of poly(3HB) are shown: wild-type *A. eutrophus* H16 (top), 75%; PHA-leaky mutant H2275 (center), 42%; and H2275 harboring plasmid pVK5000 (bottom), 77%. Bar, 1 μm.

could be bound to the granules. The cells obviously did not synthesize an excess of GA24 protein which remained unbound.

Immunoelectron microscopic localization of the GA24 protein. The purified polyclonal antibodies against GA24 protein were highly specific for the GA24 protein. Only one band was obtained in Western blots prepared from crude extracts or from the proteins solubilized from the granule fractions of *A. eutrophus* (Fig. 4). These antibodies were used to localize the protein in the cells at the ultrastructural level by immunoelectron microscopy. Cells of *A. eutrophus* H16, PHA-negative mutant PHB$^-$4, and PHA-leaky mutant H2275 were embedded, and ultrathin sections were subjected to immunogold labelling. The labelling was confined only to the periphery of the PHA granules in cells of the wild type, and there was no evidence that the GA24 protein occurred within the core of the granules. Metal shadowing of the ultrathin sections confirmed that the surface of the granules was coated with GA24 protein (Fig. 5). With cells of PHB$^-$4 or H2275, no specific labelling was observed.

Cloning of genomic fragments phenotypically complementing PHA-leaky mutants. In order to clone the gene(s) which were insertionally inactivated in five PHA-leaky mutants, two independent cosmid libraries of strain H16 genomic DNA were prepared from *Eco*RI-digested genomic DNA in *E. coli* S17-1. The hybrid cosmids were mobilized into mutant H2262 or H2275, and clones were screened for their capability to complement these mutants phenotypically, i.e., to form colo-

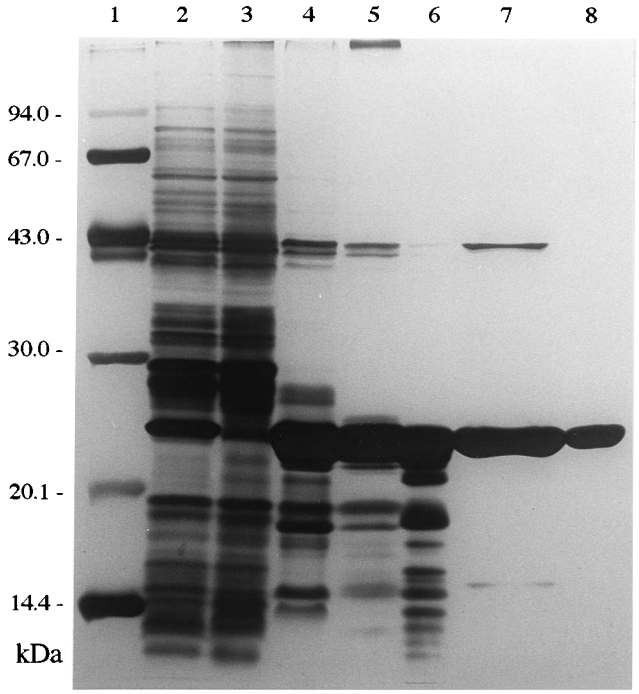

FIG. 2. Purification of the GA24 protein. Proteins were separated by SDS-PAGE. Lanes: 1, molecular mass standard proteins with the masses indicated on the left (from top to bottom, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin, respectively); 2, crude extract proteins of *A. eutrophus* H16; 3, crude extract proteins of *A. eutrophus* H16 PHB$^-$4; 4, isolated poly(3HB) granules; 5, proteins solubilized from the granules; 6, Procion Blue H-ERD eluate; 7, butyl Sepharose eluate; 8, purified GA24 protein after chromatography on Q-Sepharose.

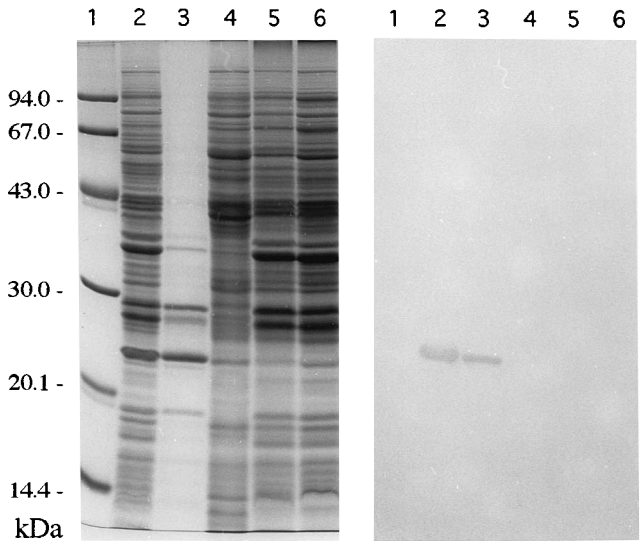

FIG. 3. Regulation of *phaP* expression. Cells of a 50-ml nutrient broth preculture were used as an inoculum for a 500-ml MM culture containing 0.05% (wt/vol) ammonium chloride plus 1.0% (wt/vol) fructose; the cells were cultivated at 30°C for 36 h. Crude extract proteins of *A. eutrophus* were separated by SDS-PAGE and stained with Coomassie brilliant blue (left panel) and subjected to Western blot analysis employing antibodies raised against the GA24 protein (right panel). Lanes 1, molecular mass standards (same as for Fig. 2); 2, crude extract of *A. eutrophus* H16; 3, proteins associated with granules isolated from strain H16; 4, cytoplasmic proteins of H16 obtained in the supernatant after sedimentation of poly(3HB) granules by centrifugation at 210,000 × *g* for 40 min; 5, crude extract of *A. eutrophus* H16 PHB$^-$4; 6, crude extract of the spontaneous PHA-negative mutant SP1.

nies which exhibited the same opalescence as colonies of the wild type. These studies revealed 1 positive clone (of 486 tested) complementing mutant H2275 and 2 positive clones (of 918 tested) complementing mutant H2262. Later, it was shown that either clone was also capable of complementing the other mutant.

The hybrid cosmids of all positive clones harbored a 5.0-kbp genomic *Eco*RI restriction fragment, which was referred to as E5000. This fragment was subcloned as a single fragment in various vectors, such as pVK100 or pSUP202. In addition, various subfragments of E5000 were also cloned in high-copy-number vectors, such as pBluescript SK$^-$ or KS$^-$ or pUC18. Interestingly, attempts to subclone E5000 or the subfragments P880, *E*/*Sma*I, or *E*/*Sal*I (Fig. 6) in high-copy-number plasmids failed; in addition, from the 0.78-kbp *Eco*RI-*Pst*I subfragment, which was referred to as EP780 (Fig. 6), only hybrid plasmids in which *phaP* (see below) was oriented antilinear to the *lacZ* promoter of the vector were obtained. This indicated that the expression of the gene of interest exerts a deleterious effect on recombinant *E. coli*.

The entire nucleotide sequence of E5000 was obtained from both strands by employing universal sequencing primers, custom-made primers, and Tn*5* primers and applying the primer-hopping strategy to E5000, subfragments of E5000, or genomic fragments which were cloned from the PHA-leaky mutants H2275 and H2271 and which harbored Tn*5*::*mob* insertions (Fig. 7). The nucleotide sequence revealed only one open reading frame which, according to the criteria of Bibb et al. (3), could represent a coding region. It was referred to as *phaP*, and it comprised 687 nucleotides (Fig. 7). The molar G+C contents of the first, second, and third codons were 65, 44, and 74%, respectively. A putative ribosomal binding site (GGAGA) was identified 6 nucleotides upstream from *phaP*. A putative promoter sequence almost identical to the *E. coli* σ^{70} promoter consensus sequence was detected approximately 160 nucleotides upstream of *phaP*. The sequence of the first 25 amino acids of the GA24 protein (see above) was identical to the amino acid sequence deduced from the nucleotide sequence at the 5′ region of the $phaP_{Ae}$ locus (Fig. 7). This confirmed that $phaP_{Ae}$ encoded the GA24 protein, which is the predominant granule-associated protein in *A. eutrophus*.

Cells of a recombinant strain of mutant H2275, which harbored plasmid pVK5000 (vector pVK100 ligated to E5000) and which was thus phenotypically complemented, contained many (approximately 30 to 50) small granules (Fig. 1). Crude extracts of this strain revealed a higher concentration of the GA24 protein in the cells than in the wild-type H16 (Fig. 4), and granules prepared from these crude extracts contained a greater proportion of the GA24 protein; in some preparations, the GA24 protein occurred as almost the only granule-associated protein. This and the occurrence of only one granule in *phaP* mutants (see above) demonstrated that the copy number of *phaP* affects the size and the number of the granules.

Mapping of Tn*5* insertions. In order to analyze the Tn*5* insertions in PHA-leaky mutants which were phenotypically complemented with plasmid pVK5000, we ligated *Eco*RI-digested genomic DNAs of mutants H2262, H2271, H2273, and H2275 to the *Eco*RI sites of pHC79 or pBluescript SK$^-$. From clones which grew in Luria-Bertani agar plates containing kanamycin, hybrid plasmids were isolated and the *Sal*I restriction fragments which conferred kanamycin resistance to the respective host because of the presence of the kanamycin phosphotransferase gene of Tn*5* (20) were subcloned. By employing an 18-mer oligonucleotide which hybridized at a distance of 130

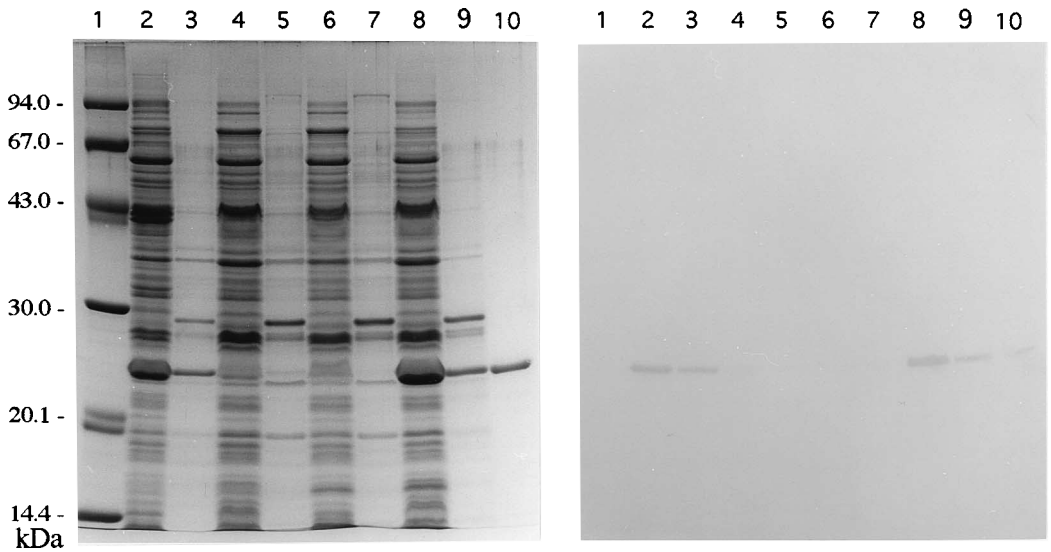

FIG. 4. Immunodetection of the GA24 protein. Cells of a 50-ml nutrient broth preculture were used as an inoculum for a 500-ml MM culture containing 0.05% (wt/vol) ammonium chloride plus 1.0% (wt/vol) fructose and were cultivated at 30°C for 36 h. Crude protein extracts or proteins from the solubilized granules were separated in SDS-polyacrylamide gels and stained with Coomassie brilliant Blue (left panel) or subjected to Western blot analysis employing antibodies raised against the GA24 protein (right panel). Lanes: 1, molecular mass standard (same as for Fig. 2); 2, crude extract of *A. eutrophus* H16; 3, granule-associated proteins of H16; 4, crude extract of *A. eutrophus* H2275; 5, granule-associated proteins of H2275; 6, crude extract of H2275(pVK1900); 7, granule-associated proteins of H2275(pVK1900); 8, crude extract of H2275(pVK5000); 9, granule-associated proteins of H2275(pVK5000); 10, purified GA24 protein.

nucleotides from the terminus of Tn*5*, the insertions of Tn*5*::*mob* in mutants H2271 and H2275 were mapped to 26 and 156 bp, respectively, upstream of the putative translational start site of *phaP*. When *Eco*RI-restricted total genomic DNAs of mutants H2271 and H2275 were hybridized with a 4.5-kbp *Bam*HI fragment which was derived from the Tn*5*-harboring *Eco*RI fragment of mutant H2275 and which comprises DNA of Tn*5* plus *A. eutrophus* genomic DNA (7,500 bp), a 12.5-kbp genomic fragment gave a signal. Since a 5.0-kbp fragment of *Eco*RI-digested DNA of the wild type hybridized with this fragment, Tn*5*::*mob* has inserted into fragment E5000 in these mutants.

Analysis of the sequence obtained for the derivative fragment of mutant H2273 revealed the nucleotide sequence of pSUP5011 DNA, thus indicating that the vector of Tn*5*::*mob* has cointegrated into the genome of *A. eutrophus* in this mutant. This was confirmed by hybridization experiments (results not given in detail).

Hybridization experiments (results not given in detail) employing various DNA probes and *Eco*RI-digested total genomic DNA of mutant H2262 localized Tn*5* not in fragment E5000 but in a 17-kbp genomic *Eco*RI fragment. The nucleotide sequence which was obtained with the Tn*5* primer and subsequently with synthetic oligonucleotides for the region of the genomic DNA adjacent to the insertion of the transposon within a 4.5-kbp *Bam*HI fragment from mutant H2262, which contained DNA of Tn*5*, revealed a sequence of 540 bp which was not found in fragment E5000 or in any other sequence obtained in this study. Interestingly, the amino acid sequence deduced from this sequence exhibited 45.5% amino acid identity with the RecO protein of *E. coli* (26). Since H2265 was phenotypically not significantly different from all other subclass III PHA-leaky mutants, and since this mutant was complemented with pVK5000, like the other mutants, the function of this gene locus in *A. eutrophus* remained unclear. Most likely, a primary insertion of Tn*5*::*mob* has occurred in fragment E5000, and a subsequent nonprecise excision of Tn*5* was followed by a secondary insertion of Tn*5* into the *recO* homologous gene. Therefore, this gene locus is most likely not related to PHA metabolism in *A. eutrophus*.

Studies of binding of the GA24 protein to artificial poly(3HB) and poly(3HO) granules. Emulsions of artificial poly(3HB) or poly(3HO) granules in water (1.5 mg wt/vol) were incubated with the purified GA24 protein (150 μg in 120 μl of 100 mM Tris-HCl, pH 7.5) or with the soluble protein fraction of *A. eutrophus* H16 (1,500 μg in 94 μl of 10 mM Tris-HCl, pH 7.5). In addition, the artificial granules were incubated with both the GA24 protein (38 μg in 30 μl) and the soluble protein fraction (1,500 μg) for 90 min at 4°C. After the incubation, the granules were collected by centrifugation, washed twice with 1 ml of 10 mM Tris-HCl (pH 7.5), and resuspended in denaturing buffer. The granule suspension and the supernatants of each washing step were analyzed in an SDS–12.5% polyacrylamide gel (Fig. 8). The GA24 protein exhibited a high level of affinity for poly(3HB) as well as for poly(3HO) granules. Soluble proteins of *A. eutrophus* H16 bound only weakly to poly(3HB) granules; in contrast, many proteins bound unspecifically to the poly(3HO) granules. In both cases, the GA24 protein prevented other proteins from binding to the artificial granules [shown only for poly(3HB) granules in Fig. 8].

The same experiments were also carried out in the presence of phosphatidylcholine (600 μg in 50 μl) in order to simulate a phospholipid monolayer at the surface of the granules. Phosphatidylcholine did not weaken the binding of the GA24 protein to poly(3HB) granules (Fig. 8), but it prevented the binding of the GA24 protein to poly(3HO) granules (Fig. 8, lanes 6a to d). This indicated a higher affinity of GA24 protein for poly(3HB) than for poly(3HO). Interestingly, phosphatidylcholine also prevented unspecific binding of other cytoplasmic proteins, particularly to poly(3HB) granules (Fig. 8, lane 10b).

Analysis of a second genomic fragment which partially restored opalescence in *phaP* mutants. In addition to the two clones mentioned above, a clone which complemented the

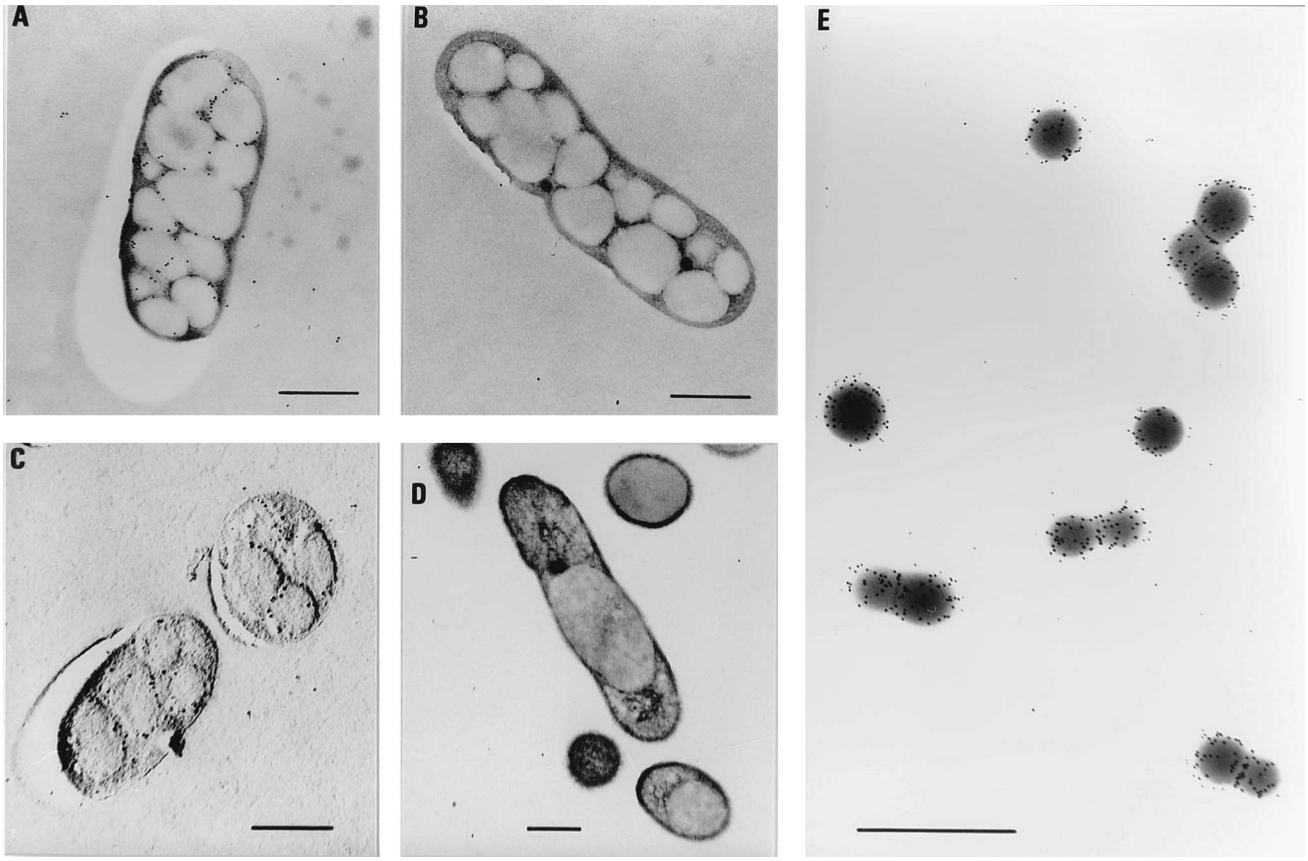

FIG. 5. Immunoelectron microscopic localization of the GA24 protein in *A. eutrophus*. Cells were cultivated in MM containing 0.05% (wt/vol) ammonium chloride plus 1.0% (wt/vol) fructose and were harvested in the stationary growth phase. Thin sections of cells (A, C and D) were labelled with antibodies raised against the GA24 protein and with the GARG complexes as described in Materials and Methods. (A) Wild-type *A. eutrophus* H16 cell labelled with antibodies against the GA24 protein plus GARG complexes. (B) H16 cell labelled with GARG complexes only. (C) Same specimen as in panel A but shadowed with metal to give a three-dimensional impression. (D) Sections of cells of the PHA-leaky mutant H2275 labelled with antibodies against the GA24 protein plus GARG complexes. (E) Isolated PHB granules from H16 cells labelled on grid with antibodies against the GA24 protein plus GARG complexes. Bars, 1 μm.

phaP mutants only partially, as revealed by enhanced opalescence of the recombinant mutants H2262 and H2275, was detected in the library. The hybrid cosmid of the clone, which was referred to as pVK6.26, contained a 1.9-kbp *Eco*RI genomic fragment, E1900, which was subcloned in pVK100, and the resulting hybrid plasmid was designated pVK1900. Fragment E1900 was subcloned in pBluescript vectors, and its entire nucleotide sequence was obtained by employing the primer-hopping strategy. The sequence revealed several open reading frames comprising more than 150 nucleotides; two of them were of interest. Only ORF1901, which comprised 516 nucleotides, conformed to the rules of Bibb et al. (3) for coding regions. The amino acid sequence deduced from the nucleotide sequence of ORF1901 did not exhibit any significant homology to the primary structure of protein found in data banks. ORF1907, which comprised 201 nucleotides, was preceded by a reliable Shine-Dalgarno sequence. The amino acid sequence deduced from it exhibited a remarkably homology to the primary structure of the cold shock proteins CS7.4 and CS7.0 (52% identity to each) of *E. coli* (11) and *Streptomyces clavuligerus* (2, 12, 44), respectively. However, the molar G+C content of ORF1907 was only 59.7%, which was significantly less than the G+C content of the *A. eutrophus* genome. Since biotinylated E1900 DNA hybridized with a 1.9-kbp fragment of *Eco*RI-digested total genomic DNA of the wild type as well as to that of any of the *phaP* mutants, Tn*5* had not inserted in the *phaP* locus. It was concluded that the presence of fragment E1900 slightly increased the opalescence of the colonies of the *phaP* mutant by a different mechanism, which is most probably not related to the metabolism of PHA in *A. eutrophus*. Therefore, this fragment was not investigated further, and the data

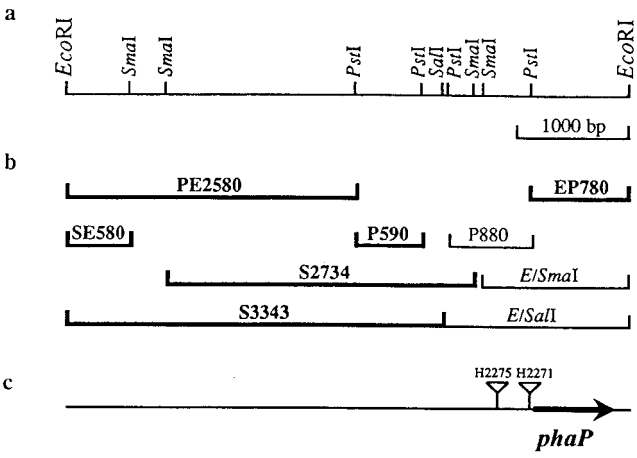

FIG. 6. Physical map of *phaP* and adjacent regions in the genome of *A. eutrophus*. (a) Restriction map. (b) Subfragments that were used for DNA sequence analysis; the only subfragments which could be subcloned in high-copy-number plasmids are indicated (boldface). (c) Position and orientation of *phaP* and Tn*5*::*mob* insertions.

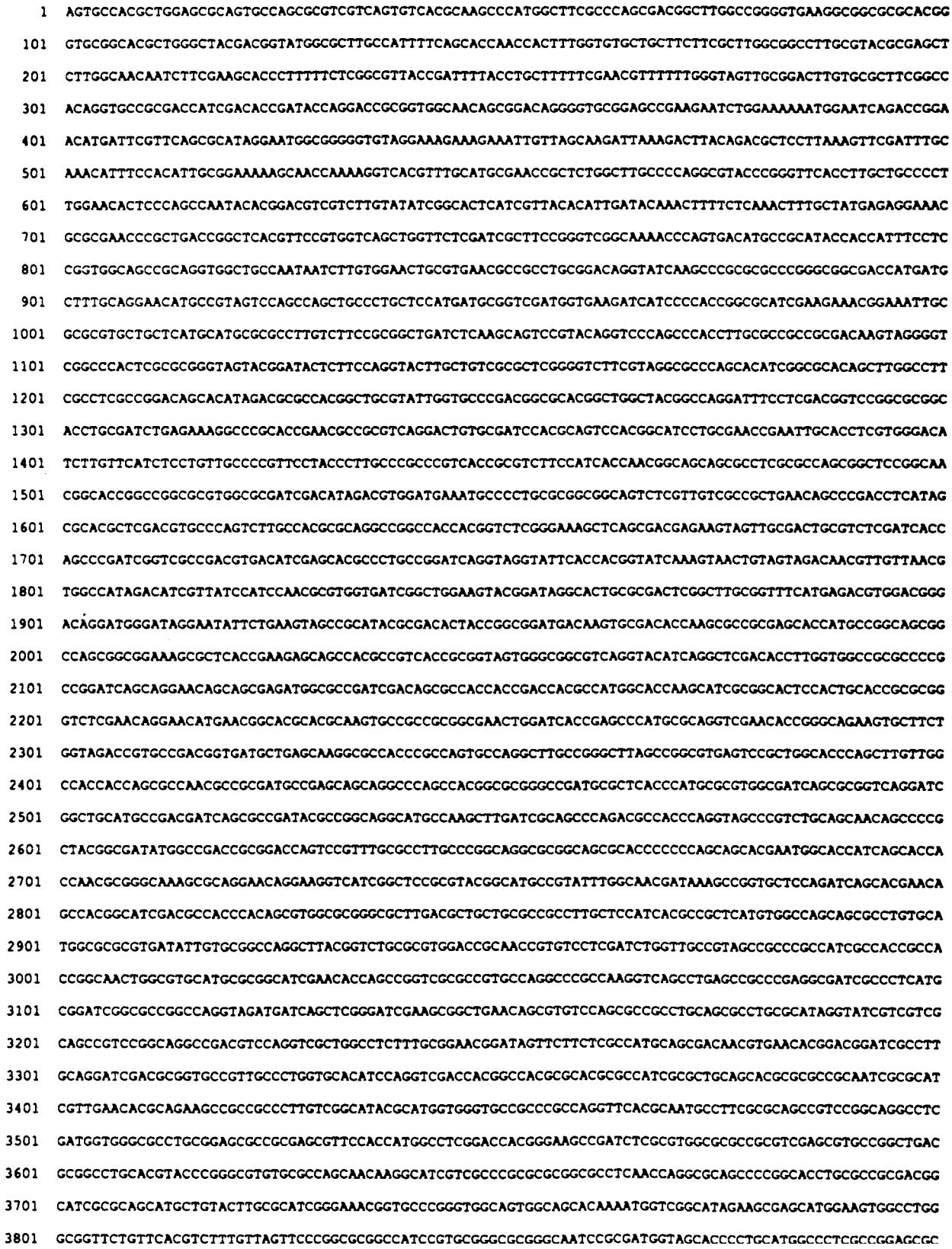

FIG. 7. Nucleotide sequence of the *A. eutrophus phaP* region. The primary structure of the putative *phaP* translational product as deduced from the nucleotide sequence is shown. The positions of transposon insertions in *phaP* in the PHA-leaky mutants H2275 and H2271 (triangles), the putative ribosomal binding site (boxed), and a hairpin-like structure (inverted arrows) are indicated.

FIG. 7—*Continued.*

were presented only because the homology of the putative translational product of ORF1907 to cold shock proteins was striking.

DISCUSSION

In the present study, we isolated the GA24 protein of *A. eutrophus* H16 in a three-step procedure by chromatography on Procion Blue H-ERD CL-6B, butyl Sepharose, and Q-Sepharose. Electrophoretic studies and immunological studies revealed the GA24 protein as one of the major proteins overall and as the major protein of the poly(3HB) granules of *A. eutrophus* that is located at the surface of the inclusions. The experiments done in this study clearly demonstrated that this protein affects the size and the number of PHA granules in the cells of *A. eutrophus*.

After cloning and analysis of the *phaC* locus, which encodes the PHA synthase structural gene (reference 50 and references cited therein) and which is affected in PHA-negative mutants, now all gene loci which are affected in PHA-leaky mutants have also been analyzed at a molecular level in *A. eutrophus*. Three different subclasses of genotypes of PHA-leaky mutants of *A. eutrophus* can be distinguished. (i) Subclass I genotypes are defective in genes encoding proteins exhibiting homology to the substrate-unspecific components HPr and EI of the bacterial sugar phosphoenol pyruvate phosphotransferase system (*phaH* and *phaI*). These mutants more readily exhibit mobilization of the accumulated polyester (35). (ii) Subclass II genotypes are defective in the structural gene coding for the dihydrolipoamide dehydrogenase component (*phaL*) of the pyruvate dehydrogenase multienzyme complex. These mutants obviously suffer from a less efficient supply of acetyl coenzyme A for the poly(3HB) biosynthesis route (14, 34). (iii) All other PHA-leaky mutants of *A. eutrophus* which are available in our laboratory belong to the subclass III genotype and are unable to synthesize the GA24 protein that is encoded by the $phaP_{Ae}$ locus.

Although the size of the GA24 protein of *A. eutrophus* is clearly different from the size of the GA14 protein of *R. ruber* (31), the two proteins share the presence of two closely related stretches consisting exclusively of hydrophobic plus amphiphilic amino acids at the C terminus. In the GA24 protein, these stretches are represented by the amino acid sequences Cys-192-Leu-Ile-Thr-Ala-Cys-Val-188 and Ala-203-Ala-Ala-Gly-Pro-Leu-Ala-209. Interestingly, the first stretch of nonhydrophobic amino acids in the GA24 protein was followed by the amino acids Glu-199 and Asp-200, which occurred also in the GA14 protein of *R. ruber* at these positions (31). According to our working hypothesis, this region in *R. ruber* is responsible for the binding of the GA14 protein to the surface of the PHA granules (31). Detailed studies of modified GA14 proteins which were recently done in our laboratory confirmed this hypothesis (32). From this structural homology of the GA24 protein to the GA14 protein, the localization at the surface of PHA inclusions, the high affinity for PHA granules as revealed in this study, and the large amounts of the GA24 protein occurring in PHA-accumulating cells, we conclude that the GA24 protein is the major component of the "membrane" of PHA granules (25) in *A. eutrophus*. In analogy to the oleosins at the surface of triacylglycerol inclusions in plants (18, 27), representatives of this type of granule-associated protein were recently referred to as phasins (48). The phenotype of the *phaP* mutants is in accordance with our phasin model, which states that phasins stabilize the PHA in water emulsion and prevent the granules from coalescing (48).

How the phasin molecules interact with the other granule-associated proteins, such as the PHA synthase or the PHA depolymerase, in *A. eutrophus* is presently unknown. $phaP_{Ae}$ mutants expressed PHA synthase at a lower specific activity than the wild type. This may be because of a lack of a coop-

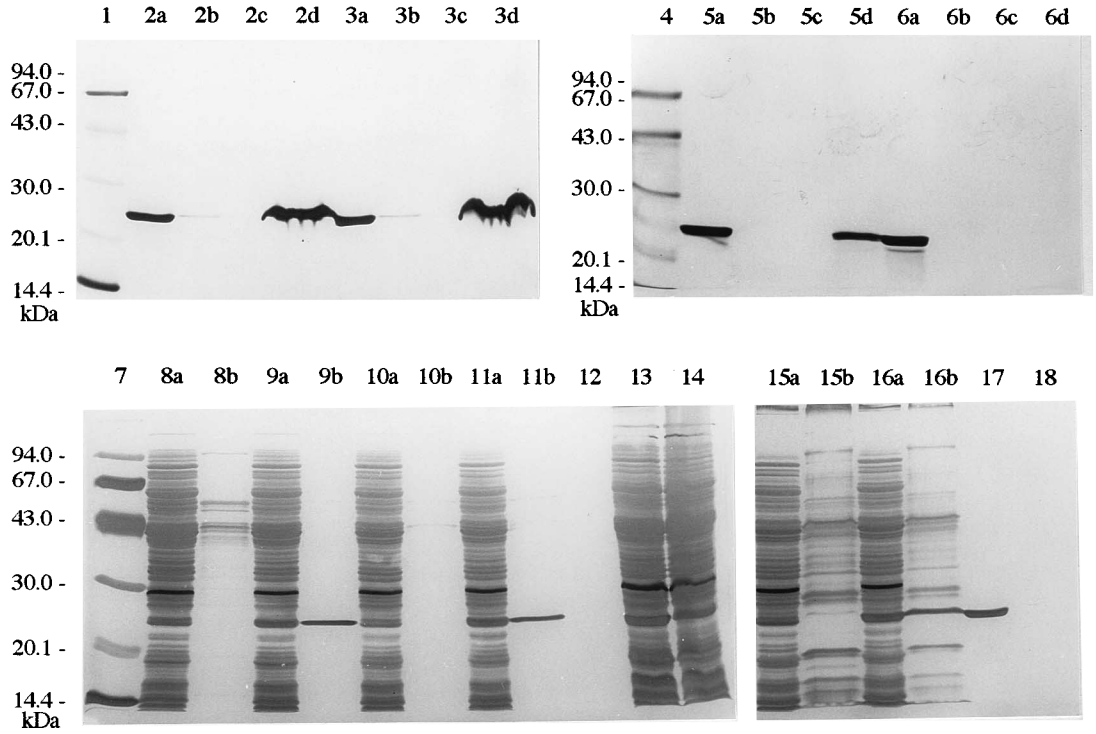

FIG. 8. Binding of the GA24 protein and cytoplasmic proteins of *A. eutrophus* H16 for artificial poly(3HB) and poly(3HO) granules. Proteins were separated in SDS–12.5% polyacrylamide gels and stained with silver. Lanes 1, 4, and 7 contain molecular mass standards (same as for Fig. 2). The isolated GA24 protein was incubated for 90 min with poly(3HB) (lanes 2 and 3) or poly(3HO) (lanes 5 and 6) granules, and subsequently the mixtures were separated into pellets and supernatants by centrifugation. The pellets were washed with buffer. Supernatants after 90 min of incubation (lanes 2a and 5a), after the first wash (lanes 2b and 5b), and after the second wash (lanes 2c and 5c) and pellets (lanes 2d and 5d) are shown. The isolated GA24 protein was incubated with poly(3HB) or poly(3HO) granules also in the presence of phosphatidylcholine and separated into a pellet and supernatant as described above; supernatants after 90 min of incubation (lanes 3a and 6a), after the first wash (lanes 3b and 6b), and after the second wash (lanes 3c and 6c) and pellets (lanes 3d and 6d) are shown. Crude protein extracts of H16 from which the poly(3HB) granules had been removed by centrifugation were incubated with isolated poly(3HB) granules alone (lanes 8) or in the presence of the GA24 protein (lanes 9), phosphatidylcholine (lanes 10), or the GA24 protein plus phosphatidylcholine (lanes 11); supernatants after 90 min of incubation (lanes a) and pellets (lanes b) are shown. Poly(3HB) granules alone (lane 12), crude protein extract from *A. eutrophus* H16 (lane 13), and crude protein extract from H16 plus GA24 protein (lane 14) are also shown. Crude protein extracts of H16 were isolated with poly(3HO) granules alone (lanes 15) or in the presence of phosphatidylcholine (lanes 16), and subsequently the mixtures were separated into pellets and supernatants by centrifugation; supernatants after 90 min of incubation (lanes a) and pellets (lanes b) are shown. GA24 protein alone (lane 17) and poly(3HO) granules alone (lane 18) are also shown.

erative effect between the GA24 protein and the PHA synthase protein or because *phaP* mutants have much less granule surface, since they have only one large granule instead of several small granules. Since the size of PHA granules occurring in a PHA-producing cell might be important for studies undertaken to separate the polyester from other cell constituents and to obtain PHA of high purity, knowledge about phasins will be relevant for establishing efficient downstream processes for the biotechnological production of PHA in bacteria as well as in transgenic plants. Knowledge of the regulation of phasin expression might also be relevant for establishing efficient biosynthesis routes in these organisms. First, the presence of phasins obviously prevents other proteins which are not related to PHA metabolism and which have a different function in the PHA-accumulating cell from binding unspecifically to the surface of PHA granules. Unspecific binding of other proteins could cause deleterious effects to the cells (48). Second, unbalanced overexpression of phasins may also cause deleterious effects. In this study and a previous study, evidence that recombinant strains of *E. coli* overexpressing phasins are not stable was obtained, and it was shown that the *R. ruber* GA14 protein bound to some extent also to the cytoplasmic membrane (31).

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