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Purification and Characterization of a 14-Kilodalton Protein That Is Bound to the Surface of Polyhydroxyalkanoic Acid Granules in *Rhodococcus ruber*

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The N-terminal amino acid sequence of the polyhydroxyalkanoic acid (PHA) granule-associated M_r-15,500 protein of *Rhodococcus ruber* (the GA14 protein) was analyzed. The sequence revealed that the corresponding structural gene is represented by open reading frame 3, encoding a protein with a calculated M_r of 14,175 which was recently localized downstream of the PHA synthase gene (U. Pieper and A. Steinbüchel, FEMS Microbiol. Lett. 96:73–80, 1992). A recombinant strain of *Escherichia coli* XL1-Blue carrying the hybrid plasmid (pSKXA10*) with open reading frame 3 overexpressed the GA14 protein. The GA14 protein was subsequently purified in a three-step procedure including chromatography on DEAE-Sephacel, phenyl-Sepharose CL-4B, and Superose 12. Determination of the molecular weight by gel filtration as well as electron microscopic studies indicates that a tetrameric structure of the recombinant, native GA14 protein is most likely. Immunoelectron microscopy demonstrated a localization of the GA14 protein at the periphery of PHA granules as well as close to the cell membrane in *R. ruber*. Investigations of PHA-leaky and PHA-negative mutants of *R. ruber* indicated that expression of the GA14 protein depended strongly on PHA synthesis.

Polyhydroxyalkanoic acids (PHAs) are synthesized by many bacteria and function as intracellular carbon and energy storage compounds (1, 28). Inside the cell, the polyesters occur in granules which also contain proteins and lipids as described for *Bacillus megaterium* (8). PHA are considered substitutes for conventional plastic materials because of their physical properties and biodegradability. A copolyester of 3-hydroxybutyrate and 3-hydroxyvalerate (P[3HB-*co*-3HV]) is now produced by ZENECA BioProducts with *Alcaligenes eutrophus* (10). The PHA biosynthesis genes of many bacteria have been analyzed (29), and four basic biosynthetic pathways have been discussed (28). The biosynthesis of poly(3-hydroxybutyric acid) (PHB) in *A. eutrophus* has been studied in most detail (for reviews, see references 1, 28, and 30). Following the condensation of two molecules of acetyl coenzyme A (acetyl-CoA) to acetoacetyl-CoA by a 3-ketothiolase (EC 2.3.1.9), an NADPH-dependent acetoacetyl-CoA reductase (EC 1.1.1.36) catalyzes the reduction to D-(−)-3-hydroxybutyryl-CoA, which is the substrate of the polymerizing enzyme PHB synthase. The structural genes of these enzymes are organized in a single operon.

Recently, biosynthesis of the copolymer P(3HB-*co*-3HV) from single, unrelated carbon sources by several coryneform bacteria such as *Rhodococcus ruber* ATCC 40126 was described (9). Molecular analysis of the biosynthesis of PHA in *R. ruber* had identified the clone pRPS2o, which restored the ability for PHB biosynthesis in the PHB-negative mutant PHB$^-$4 of *A. eutrophus* H16 (23). The hybrid plasmid pRPS2o contained a 6.9-kbp genomic DNA fragment of *R. ruber*, and sequence analysis revealed the PHA synthase structural gene ($phaC_{Rr}$) and two additional open reading frames (ORF3 and ORF4) with unknown function (Fig. 1a). Furthermore, separation of granule-associated proteins of *R. ruber* in a sodium dodecyl sulfate (SDS)-polyacrylamide gel had exhibited four major bands representing proteins with M_rs of 61,000, 42,000, 35,000, and 15,500 (23). N-terminal amino acid sequence analysis identified the M_r-61,000 protein as the PHA synthase (23). In this study, we identified the granule-associated M_r-15,500 protein as the translation product of ORF3, which is located downstream of the PHA synthase gene. This protein was purified and characterized, and specific antibodies were raised against this protein in order to analyze its function in the biosynthesis of PHA and in the assembly of PHA granules in *R. ruber*.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *R. ruber* NCIMB 40126 (9), *Escherichia coli* XL1-Blue (3), and the pBluescript plasmids (Stratagene GmbH, Heidelberg, Germany) were used in this study. *R. ruber* was cultivated for 24 h at 30°C in thiamine-supplemented mineral salts medium (MSM) (26) which was inoculated with a 24-h Luria-Bertani (LB) preculture (25). To stimulate PHA accumulation, the concentration of ammonium chloride was reduced to 0.05% (wt/vol). *E. coli* was grown at 37°C in LB medium.

Quantitative and qualitative analysis of PHA. PHAs were converted to the methyl esters of constituent hydroxyalkanoic acids, which were analyzed by gas chromatography as described elsewhere (2, 31).

Chemical mutagenesis and isolation of mutants defective in the accumulation of PHA. Mutagenesis with sodium nitrite was done essentially as described by Kaudewitz (12). *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis was performed by the method of Schlegel et al. (27). After mutagenesis, cells were collected by centrifugation (2,800 × *g*, 10 min, 4°C), washed twice in 0.9% (wt/vol) NaCl, and resuspended in 10 ml of MSM with 0.05% (wt/vol) ammonium chloride and 0.2% (wt/vol) sodium valerate. Cells were cultivated at 30°C for 24 h, harvested, and washed, and the cell density was adjusted to an optical density at 436 nm of 10. Mutants defective in the

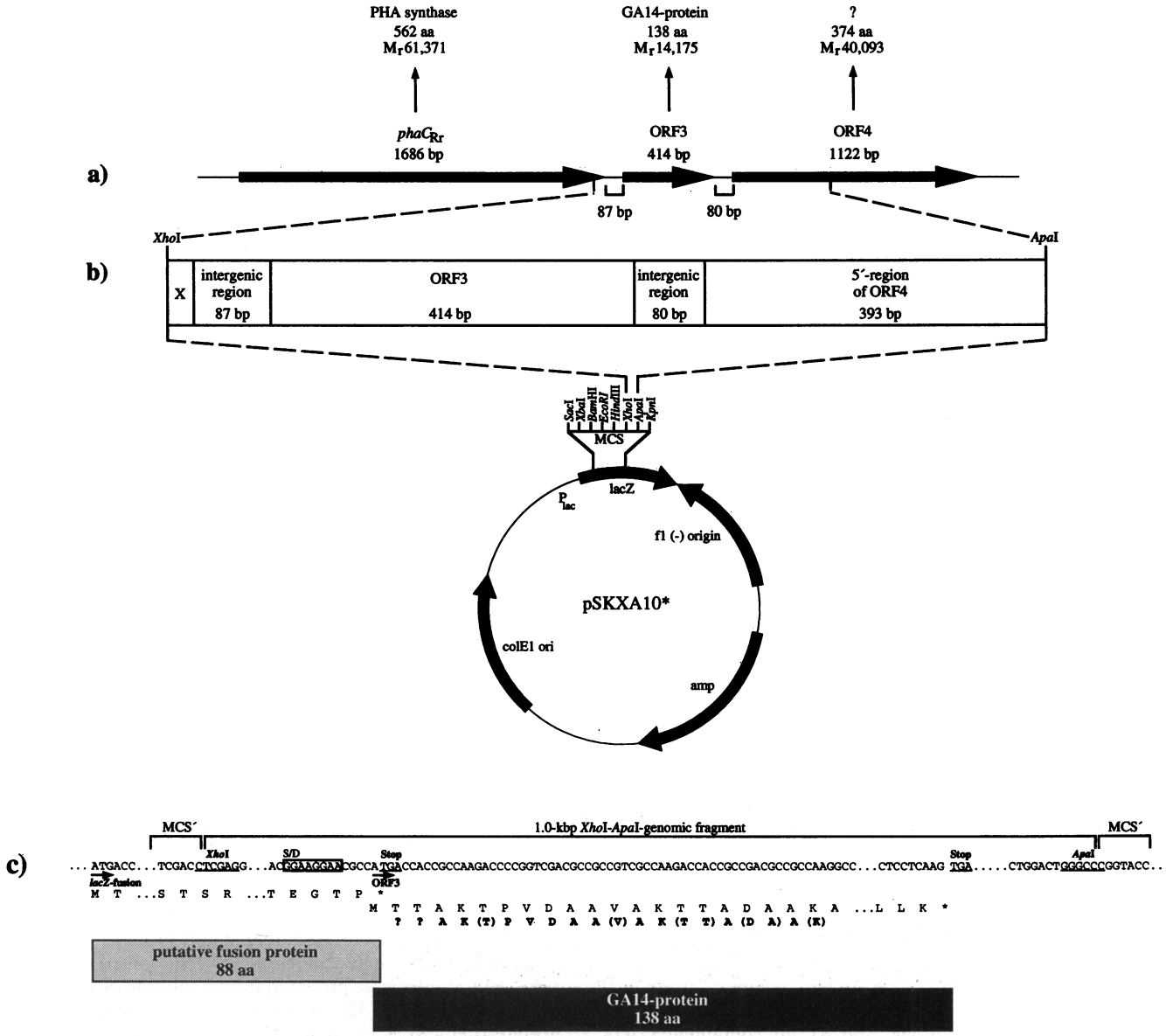


FIG. 1. Construction of the hybrid plasmid pSKXA10* and sequence data related to expression of the GA14 protein. (a) PHA synthase gene locus of *R. ruber*. aa, amino acids. (b) Detailed structure of the 1.0-kbp *XhoI-ApaI* fragment and its location in the multiple cloning site of pBluescript SK$^-$, yielding pSKXA10*. X, 32 bp of the 3' region of $phaC_{Rr}$. (c) Depiction of the putative *lacZ* fusion gene and of ORF3. The deduced amino acid sequences are partially shown in the one-letter code below the nucleotide sequence; the N-terminal amino acid sequence of the GA14 protein from granule preparations as determined by Edman degradation is marked in boldface. MCS, multiple cloning site.

accumulation of PHA were enriched in Percoll density gradients (22). This method utilizes the observation that PHA-free mutants of *A. eutrophus* have a lower density than PHA-containing cells (22). To obtain clearly visible differences between *R. ruber* wild-type cells and the mutants, as described for the wild-type and PHA-negative mutants of *A. eutrophus* (27), the conditions to achieve a maximum level of polymer accumulation were optimized. Since previous studies had demonstrated that *R. ruber* accumulated PHA up to 90% of cell dry matter from sodium valerate (9), cells of each fraction above the wild-type band were plated on MSM containing 0.02% (wt/vol) sodium valerate and incubated at 30°C for 2 days. PHA-leaky and PHA-negative mutants could be distinguished from colonies of the wild type on the basis of their transparent reddish colony phenotype.

Electrophoresis of proteins. Samples were resuspended in gel loading buffer (0.6% [wt/vol] 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 were separated in 11.5% (wt/vol) SDS-polyacrylamide gels as described by Laemmli (16). Proteins were stained with Coomassie brilliant blue R 250 (34).

Preparation of PHA granules and analysis of the N-terminal amino acid sequence of the GA14 protein. PHA granules of *R. ruber* were isolated by density gradient centrifugation as described previously (23). The associated proteins were released

from the granule core by resuspension in gel loading buffer and were separated in an SDS-polyacrylamide gel. For determination of the N-terminal amino acids of the GA14 protein, the protein was extracted from the gel as described by Weber and Osborn (34). For removal of SDS, the freeze-dried proteins were precipitated with acid acetone (13). The N-terminal amino acid sequence was determined by automated Edman degradation.

Isolation, manipulation, and transformation of DNA. Isolation of plasmid DNA, agarose gel electrophoresis of DNA, and use of restriction endonucleases and of ligase were done by standard procedures (25). DNA was extracted from agarose as described by Vogelstein and Gillespie (33). *E. coli* XL1-Blue was transformed by the $CaCl_2$ method (25).

Overexpression and purification of the GA14 protein. A 1.0-kbp *Xho*I-*Apa*I fragment containing the gene for the GA14 protein of *R. ruber* (23) was ligated to pBluescript SK$^-$ DNA which had been treated with *Xho*I and *Apa*I. Ligation products were transformed into *E. coli* XL1-Blue, and transformants harboring the construct (pSKXA10*) were used to inoculate 10 ml of LB medium containing 12.5 μg of tetracycline and 75 μg of ampicillin per ml. After incubation at 37°C for 10 h these precultures were transferred into 100 ml of LB medium containing 12.5 μg of tetracycline per ml, 75 μg of ampicillin per ml, and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 20 h of cultivation at 37°C, a total of 4 g of cells was collected from 10 100-ml cultures by centrifugation.

Cells were washed and resuspended in 25 ml of 20 mM sodium phosphate buffer (pH 6.5). All steps were carried out at 4°C in this buffer, and proteins were eluted from the columns at a flow rate of 0.5 ml/min. Cells were broken by twofold French press passage (110×10^6 Pa), and cell debris and membranes were removed from the crude extract by centrifugation (100,000 × *g*, 1 h, 4°C). The supernatant (25 ml) was dialyzed for 2 h against 2 liters of buffer and applied to a column (2.6 by 12.2 cm, 65-ml bed volume) of DEAE-Sephacel (Pharmacia Biosystems GmbH, Freiburg, Germany). The GA14 protein eluted in the wash fractions. Fractions containing GA14 protein were identified by SDS-polyacrylamide gel electrophoresis (PAGE), pooled, and concentrated 15-fold in a Diaflo chamber, using a PM10 membrane (Amicon, Witten, Germany). Six milligrams (1.25 ml) of protein was loaded onto a column (1.6 by 9.0 cm, 18-ml bed volume) of phenyl-Sepharose CL-4B (Pharmacia Biosystems). Again, the protein eluted in the wash fractions, and 41 ml containing GA14 protein was collected and concentrated 80-fold in a Diaflo chamber (see above) as well as a Centricon 10 concentrator (Amicon). From this concentrate, 250 μl (0.6 mg of protein) was applied onto a Superose 12 fast pressure liquid chromatography (FPLC) column (Pharmacia Biosystems) equilibrated with buffer; 1.5 ml containing GA14 protein was collected and concentrated in a Centricon 10 concentrator.

Preparation and purification of antibodies. The antigen (500 μg in 500 μl 20 mM sodium phosphate [pH 6.5]) was mixed with complete Freund's adjuvant (500 μl) and injected subcutaneously along the back of a rabbit (New Zealand, female, 2.5 kg). A booster injection with the same amount of antigen mixed with incomplete Freund's adjuvant was given after 4 weeks. After an additional 11 days, the rabbit was bled. To obtain monospecific antibodies against the GA14 protein, the antiserum was subjected to an affinity purification done by a modification of the method described by Olmsted (21). Approximately 3.5 mg of the antigen was separated in an SDS-polyacrylamide gel and blotted onto a nitrocellulose BA83 membrane (pore size, 0.2 μm; Schleicher & Schuell, Dassel, Germany), using a Semidry Fast Blot B33 apparatus (Biometra, Göttingen, Germany). The membrane was stained with 0.2% (wt/vol) Ponceau S (Sigma, Deisenhofen, Germany) in 3% (wt/vol) trichloroacetic acid, and the region harboring the GA14 protein was cut out. Free protein-binding sites on this membrane were blocked with 2.5% (wt/vol) skim milk in phosphate-buffered saline (PBS); (10 mM potassium phosphate [pH 7.2], 0.5% [wt/vol] NaCl, 0.05% [wt/vol] Tween 20) at 37°C for 1 h. The membrane was incubated with 3 ml of the antiserum at room temperature for 3 h. After three washing steps for 10 min with PBS, GA14-specific antibodies were eluted twice for 2 min with 2 ml of elution buffer (5 mM glycine [pH 2.3], 0.5 M NaCl, 0.05% [wt/vol] Tween 20). The antibody solution (approximately 30 μg/ml) was neutralized with 1 M potassium phosphate (pH 8.0) and after addition of 1% (wt/vol) bovine serum albumin stored at −20°C.

Western blotting (immunoblotting). Proteins were separated in an SDS-polyacrylamide gel (11 cm by 10 cm by 1 mm) and blotted onto a nitrocellulose membrane (see above). Free binding sites were blocked with 2.5% (wt/vol) skim milk in PBS at 37°C for 1 h. Antibodies were diluted 100-fold in PBS containing 0.5% (wt/vol) Tween 20 and incubated with the immobilized antigen at room temperature for 3 h. Following three washing steps (each 10 min in PBS), the membrane was transferred into a solution of anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Sigma) in PBS containing 0.5% (wt/vol) Tween 20. After 1 h of incubation at room temperature, the membrane was washed three times in PBS and once in reaction buffer (0.1 M Tris-HCl [pH 8.8], 0.1 M NaCl, 5 mM $MgCl_2$). Bound antibodies were detected with 22 μl of a solution of nitroblue tetrazolium (75 mg/ml in 70% [vol/vol] dimethylformamide) and 17 μl of a solution of 5-bromo-4-chloro-3-indolylphosphate (50 mg/ml in 100% [vol/vol] dimethylformamide) in 20 ml of reaction buffer.

Molecular weight determination. The M_r of the GA14 protein was determined by gel filtration on a Superose 12 FPLC column (Pharmacia Biosystems) that was equilibrated with 20 mM sodium phosphate buffer (pH 6.5). RNase A from bovine pancreas (M_r, 13,700), chymotrypsinogen A from bovine pancreas (M_r, 25,000), ovalbumin from hen egg (M_r, 43,000), and albumin from bovine serum (M_r, 67,000) (Pharmacia Biosystems) were used as standard proteins.

Electron microscopic studies. Negatively stained samples of the GA14 protein were prepared from a solution of 10 to 25 μg of protein per ml (32), using 4% (wt/vol) aqueous uranyl acetate (pH 4.8) as the stain (20). For postembedding immunogold labeling of the GA14 protein, cells and PHA granules were washed twice with 50 mM potassium phosphate (pH 7.0) and were subsequently fixed with a mixture of 0.3% (vol/vol) glutaraldehyde and 0.2% (wt/vol) paraformaldehyde in the buffer described above (24). This fixation method preserved both the ultrastructure of the cells and the antigenicity of the GA14 protein, as controlled by Western blotting. The samples were embedded in Lowicryl K4M (Lowi, Waldkraiburg, Germany) as described previously (24) except that methanol instead of ethanol was used for dehydration. Ultrathin sections were mounted onto Formvar-covered nickel grids, and free protein-binding sites were blocked with skim milk (5). The samples were incubated overnight at 4°C with a series of different dilutions of the primary antibody. Sections were washed by a mild spray of PBS-Tween (50 mM potassium phosphate, 0.9% [wt/vol] NaCl, 0.05% [vol/vol] Tween 20 [pH 6.9]) and subsequent incubations on drops of PBS-Tween (three times for 3 min each time). The grids were then incubated with a series of dilutions of goat anti-rabbit immunoglobulin G–gold complex (GARG; Dakopatts, Hamburg, Germany) at room temperature for 2 h. The sections were then

rinsed as described above and washed in H_2O. Poststaining was performed in 4% (wt/vol) aqueous uranyl acetate (pH 4.5) for 3 to 5 min. The specificity of the labeling was demonstrated by a control experiment using only the GARG complexes. For metal shadowing, the sections were coated with platinum-carbon evaporated at an angle of 30° (metal shadowing apparatus, type EPA100; Leybold-Heraeus, Hanau, Germany). Micrographs were taken on a Philips EM 301 electron microscope at 80-kV acceleration voltage. Magnifications were calibrated with a cross-lined grating replica (Balzers Corp., Liechtenstein).

RESULTS

Identification of the GA14 protein. Analysis of the N terminus of the granule-associated M_r-15,500 protein, the predominant representative of four proteins bound to isolated granules of *R. ruber* (23), yielded the following amino acid sequence: ??AK(T)PVDAA(V)AK(T)(T)A(D)(A)AK (question marks and parentheses indicate unidentified and uncertain amino acids, respectively). It was in agreement with the amino acid sequence deduced from the nucleotide sequence of the 5′ region of ORF3, which maps downstream of the PHA synthase structural gene (*phaC*$_{Rr}$) (Fig. 1a and c). The N-terminal methionine residue is probably removed in vivo. ORF3 comprised 414 bp and encoded a protein of 138 amino acids with an M_r of 14,175 (Fig. 1a) (23). The ORF3 product will hereafter be referred to as GA14 protein.

Construction of the hybrid plasmid pSKXA10* and overexpression of GA14 protein in *E. coli*. A 1.0-kbp *Xho*I-*Apa*I subfragment of clone pRPS2o, which contained 32 bp of the 3′ region of *pha*C$_{Rr}$, an intergenic region of 87 bp, the entire ORF3, an intergenic region of 80 bp, and 393 bp of the 5′ region of ORF4, was ligated into pBluescript SK$^-$ to generate the hybrid plasmid pSKXA10* (Fig. 1b). By this construction, a new ORF comprising the 5′ region of the β-galactosidase gene (*lacZ*) up to the *Xho*I site of the multiple cloning site (144 bp), 32 bp of the 3′ region of *pha*C$_{Rr}$, the 87-bp intergenic region upstream of ORF3, and the first nucleotide of ORF3 was obtained. The stop codon (TGA) of this *lac*Z fusion gene overlapped with the start codon (ATG) of ORF3 (Fig. 1c). Cells of *E. coli* XL1-Blue(pSKXA10*) containing ORF3 downstream and colinear to the *lac* promoter (P$_{lac}$) synthesized a protein with the same electrophoretic mobility as the GA14 protein. In the presence of IPTG, this protein was overexpressed (Fig. 2, lane B). Cells of *E. coli* XL1-Blue containing pKSAX10 (a construct of pBluescript KS$^-$ and the 1.0-kbp *Xho*I-*Apa*I fragment) with ORF3 downstream but in antilinear orientation to P$_{lac}$ produced no GA14 protein (data not shown). Therefore, the transcription of ORF3 was under the control of P$_{lac}$. The proposed fusion protein composed of 88 amino acids and exhibiting an M_r of 9,217 was not detected.

Purification of the GA14 protein. Purification of the GA14 protein started from 4 g of wet cells of *E. coli* XL1-Blue(pSKXA10*) which had been cultivated in the presence of IPTG. The GA14 protein constituted approximately up to 10% of the total soluble protein in crude extracts, as estimated from the electropherogram (Fig. 2, lane B). Following dialysis, the proteins were loaded onto a DEAE-Sephacel column. Because the GA14 protein has an isoelectric point of 4.66 (see below), a buffer of pH 6.5 was chosen. It was expected that the native GA14 protein would have a negative net charge and would therefore bind to the anion-exchange matrix. However, it was eluted in the wash fractions with high purity (Fig. 2, lane E). The concentrated protein was then applied to a column of phenyl-Sepharose CL-4B. Again, the GA14 protein did not bind to the column and was eluted in the wash fractions, whereas contaminating proteins were efficiently retained on the hydrophobic matrix (Fig. 2, lane F). Subsequent gel filtration on a Superose 12 FPLC column (Fig. 2, lane G) yielded 830 μg of GA14 protein. When this protein was subjected to Edman degradation, an N-terminal amino acid sequence (??AK?PVDAAVAK) which corresponded to the amino acid sequence deduced from the nucleotide sequence of ORF3 was obtained (Fig. 1c). The protein that appeared below the GA14 protein in SDS-polyacrylamide gels was a proteolytic fragment emerging during the purification, as demonstrated by Western blotting (data not shown).

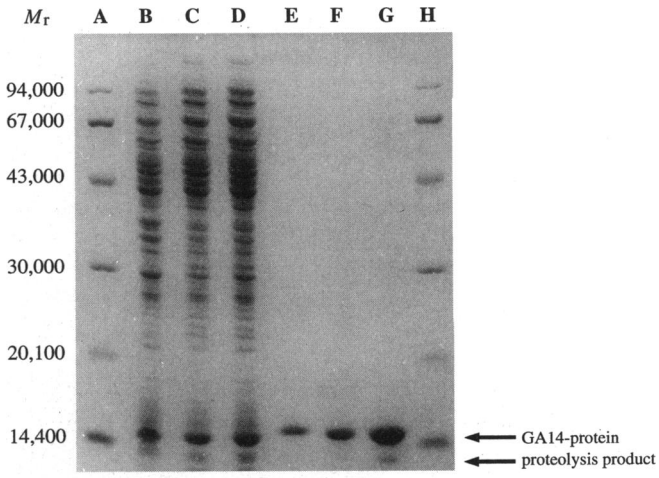


FIG. 2. Purification of the GA14 protein. Proteins were separated by SDS-PAGE, and the positions of the GA14 protein and its proteolysis product are marked by arrows. Relative M_rs of standard proteins (phosphorylase *b*, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100; and α-lactalbumin, 14,400) are given at the left. Lanes: A and H, molecular mass standard proteins; B, crude extract proteins of *E. coli* XL1-Blue(pSKXA10*); C, proteins of the supernatant after ultracentrifugation; D, proteins after dialysis of the supernatant; E, protein concentrate after DEAE-Sephacel anion-exchange chromatography; F, protein concentrate after hydrophobic chromatography on phenyl-Sepharose CL-4B; G, purified GA14 protein following gel filtration on Superose 12.

Properties of the GA14 protein. The M_r of the native GA14 protein, isolated from the recombinant *E. coli* strain, as determined by gel filtration on a Superose 12 FPLC column was 53,500 ± 3,700 irrespective of the presence or absence of NaCl (0 to 1 M) during chromatography. Following negative staining of the native GA14 protein with uranyl acetate, electron micrographs showed particle projections of a size ranging from 7 to 12 nm in diameter and with three or four intensity maxima. These images can be interpreted as triangular and square projections of one type of tetrameric protein resulting from different orientations of the protein complex (Fig. 3a, c, d, and f) (20). We therefore concluded that the quaternary structure of the native GA14 protein in *E. coli* is a tetramer. Structures 3 nm in diameter were also frequently revealed; they may represent monomers of the GA14 protein (Fig. 3a and b) which could result as products of partial decomposition taking place during the negative staining procedure. In addition, noticeable irregular structures with diameters of more than 12 nm occurred; these were interpreted as aggregates of a large number of GA14 protein molecules (Fig. 3a and e). The tendency of the GA14 protein to form

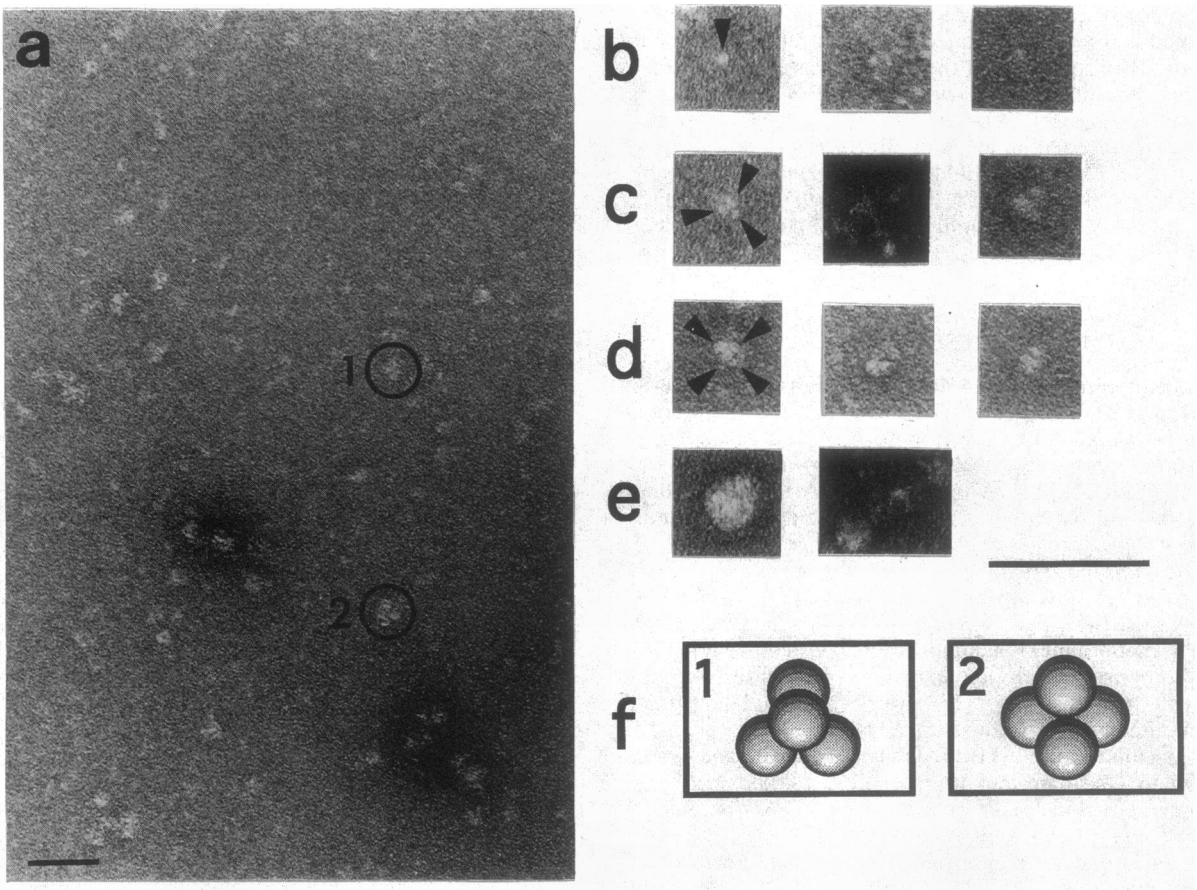


FIG. 3. Electron micrographs of native GA14 protein from *E. coli* negatively stained with uranyl acetate. Micrographs were taken from samples with protein concentrations of 10 to 25 μg/ml. (a) Overview. Two projection types with three intensity maxima (1) or four intensity maxima (2) are indicated; the scale bar represents 40 nm. (b) to (e) Detailed depictions of the different projections that have been observed. The scale bar represents 60 nm. (b) Monomers of the GA14 protein; (c) tetrameric structure of the GA14 protein with triangular projections; (d) tetrameric structure of the GA14 protein with square projections; (e) aggregates of a large number of GA14 proteins. (f) Schematic illustration of the tetrameric structure of the GA14 protein to demonstrate the triangular projection with three intensity maxima (1) and the square projection with four intensity maxima (2).

aggregates may be the reason for irreversible precipitation of a part of the protein during the purification.

An absorption spectrum of the purified GA14 protein revealed only protein-specific maxima at 214 and 280 nm and did not indicate the presence of cofactors. The isoelectric point of the GA14 protein was calculated to be 4.66 on the basis of the primary structure deduced from the nucleotide sequence of ORF3, using the program ISOELECTRIC of the Genetics Computer Group package (4). This value was confirmed by isoelectric focusing (data not shown). Hitherto, no homologies of the GA14 protein to other proteins had been detected by comparison of primary structures of proteins in the EMBL gene bank. Analysis of the amino acid sequence of the GA14 protein deduced from ORF3 (23) revealed that the GA14 protein is composed of 16 mol% of extremely hydrophobic (I, V, and L), 29 mol% of hydrophobic (F, M, and A), 24 mol% of amphiphilic (G, T, W, S, Y, and P), and 31 mol% of hydrophilic (R, N, D, Q, E, H, and K) amino acids. No protein segment comprising 19 amino acids with amphiphilic and/or hydrophobic properties that could represent a typical bilayer membrane-spanning part of a protein as described by Kyte and Doolittle (15) was identified. However, two segments of 10 and 9 hydrophobic and/or amphiphilic amino acids were detected between amino acid positions 102 to 111 and 125 to 133 of the polypeptide, respectively (23). The length of each of these sections would be sufficient to span a phospholipid monolayer.

Rabbit antiserum raised against the GA14 protein showed cross-reactivity with several other proteins from *R. ruber* (data not shown), presumably as a result of the use of complete Freund's adjuvant with *Mycobacterium* cell material. After the antiserum was purified as described in Materials and Methods, it was highly specific for the GA14 protein, as demonstrated by Western blotting (Fig. 4). Cross-reactivity of the GA14-specific antibodies with PHA granule-associated proteins of *A. eutrophus* or *Chromatium vinosum* D was not observed (results not shown).

Isolation and characterization of mutants defective in the biosynthesis of PHA. Chemical mutagenesis and Percoll density gradient centrifugation were applied to obtain mutants of *R. ruber* which lack the ability to accumulate PHA (referred to as PHA-negative mutants) or which accumulate less PHA than the wild type (referred to as PHA-leaky mutants). The frequency of mutations varied between 0.5×10^{-7} and 3.5×10^{-7} in different experiments. When glucose was used as the sole carbon source during cultivation for characterization of the mutants, cells of the wild type accumulated PHA up to 45%

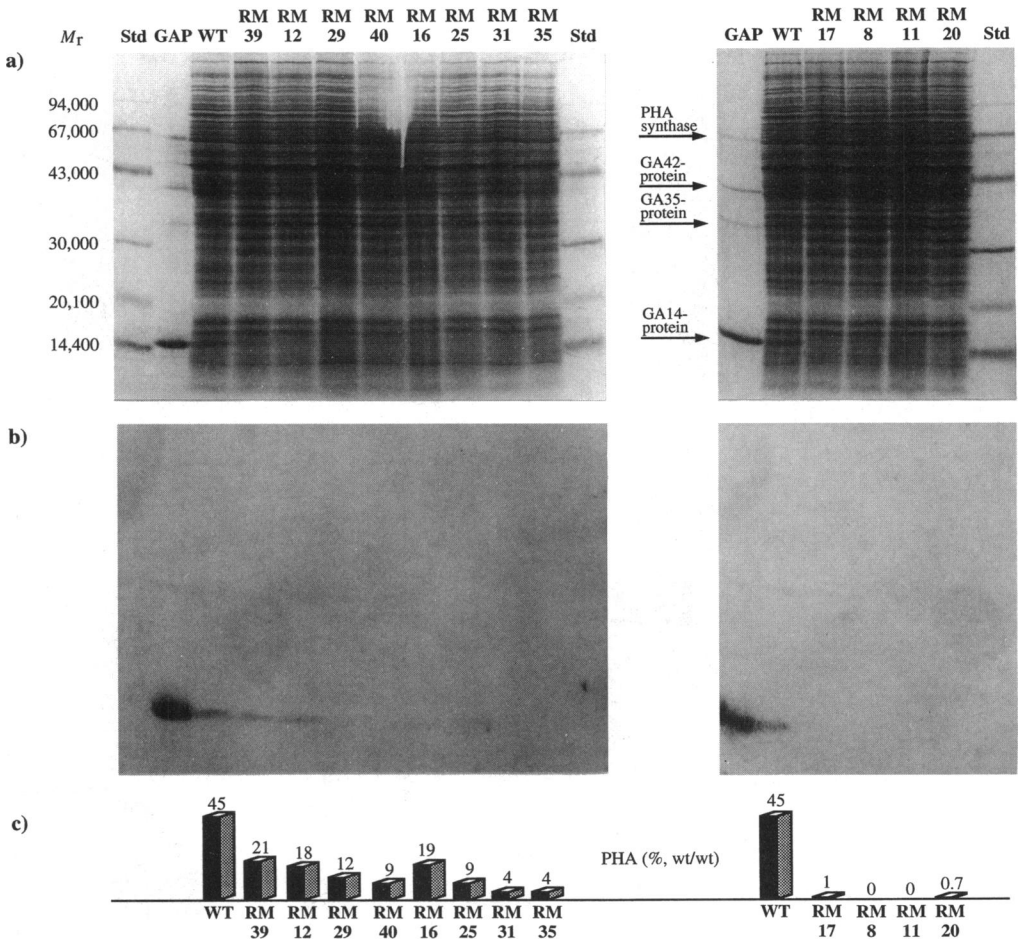
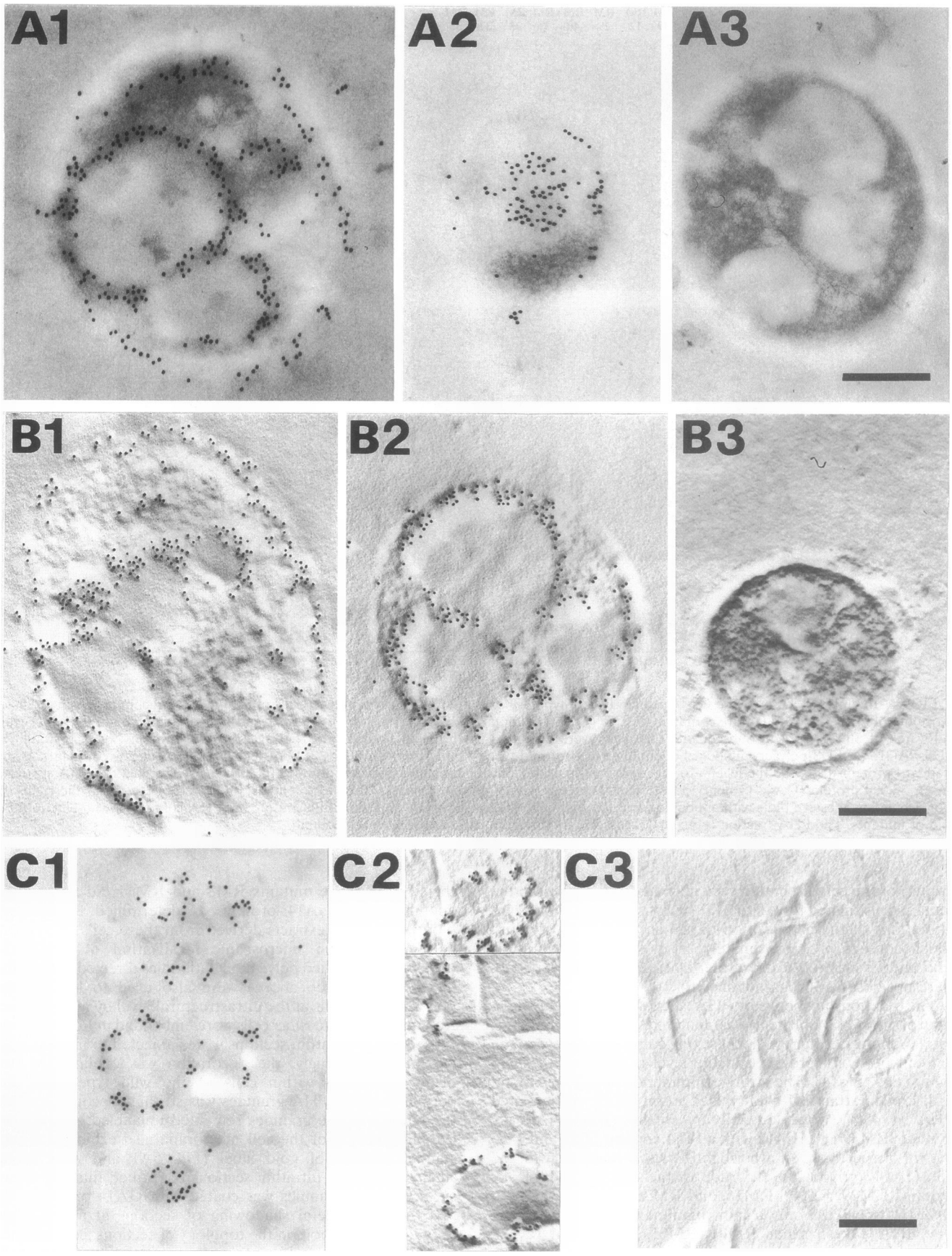


FIG. 4. Immunodetection of the GA14 protein in wild-type *R. ruber* and PHA-leaky mutants. Cells of a 10-ml LB preculture were used as an inoculum of a 100-ml MSM culture containing 0.05% (wt/vol) ammonium chloride, 1% (wt/vol) glucose, and 1 μg of thiamine per ml, which were cultivated at 30°C for 24 h. Subsequently, the cells were disrupted by threefold French press passage, and the proteins were separated by SDS-PAGE. (a) SDS-polyacrylamide gel of crude extracts of wild-type (WT) *R. ruber* as well as of PHA-leaky and PHA-negative mutants. M_rs of standard proteins (Std; see the legend to Fig. 2) are given at the left; the granule-associated proteins (GAP) of *R. ruber*, i.e., the PHA synthase, an M_r 42,000 protein (GA42 protein), an M_r 35,000 protein (GA35 protein), and the GA14 protein, are indicated in the gel at the right. (b) Corresponding Western blots with antibodies directed against the GA14 protein. (c) Diagram illustrating the PHA content of wild-type *R. ruber* and of mutants. The PHA fraction (percent) of the cell dry matter is indicated above each column.

(wt/wt) of the cell dry matter, whereas the PHA content in the mutant cells ranged from 0 to 21% (wt/wt) (Fig. 4c). Significant alterations of the composition of the accumulated polyester, which consists of approximately 25 mol% 3-hydroxybutyric acid and 75 mol% 3-hydroxyvaleric acid in the wild type (9), were not observed (data not shown).

The relationship between PHA accumulation and expression of the GA14 protein was analyzed on several mutants. Immunoblots clearly revealed a strong correlation between the amount of GA14 protein and the level of PHA synthesis in these cells (Fig. 4). The strongest immunoreaction was found with crude extracts of wild-type *R. ruber*, which accumulated PHA at 45% (wt/wt) of cell dry weight. The PHA-leaky mutants RM39 and RM12 with a PHA content of 21 and 18% (wt/wt), respectively, showed slightly weaker reactions with the anti-GA14 antibody. With crude extracts of the PHA-leaky mutants RM29, RM40, RM16, and RM25, accumulating 9 to 19% (wt/wt) PHA, only a faint immunoreaction was visible. The PHA-leaky mutants RM31, RM35, RM17, and RM20, with a PHA content of between 0.7 and 4% (wt/wt), as well as the PHA-negative mutants RM8 and RM11 did not express any detectable GA14 protein, as determined by Western blotting of crude extracts derived from these cells.

Immunoelectron microscopic localization of the GA14 protein. The purified polyclonal antibodies directed against the GA14 protein (see above) were also used to localize the polypeptide in cells at the ultrastructural level by immunoelectron microscopy. *R. ruber* cells were embedded in Lowicryl, and subsequently ultrathin sections were subjected to immunogold labeling. Surprisingly, the decoration was confined to two distinct locations within cells of the wild type: first, the periphery of the PHA granules was strongly decorated, while the interior of the granules remained unlabeled; second, the cytoplasmic side of the cell membrane also exhibited a substantial amount of gold label (Fig. 5A1 and A2). Metal shadowing of the ultrathin sections confirmed that the entire surface of the granules was coated with GA14 protein (Fig. 5B1 and B2). Metal shadowing of sections at a low angle provides information on the topology of sections and indicates that the surface of a section is not completely flat; rather, in



those areas of the section where a cell is sectioned, this cell is somewhat elevated above the surrounding resin, and surfaces of PHA granules within these cells which are exposed at the surface of the section can be identified. With cells of the PHA-negative mutant *R. ruber* RM11, no specific label was observed (data not shown). This was in excellent agreement with the results obtained by Western blotting, which indicated a lack of expression of the GA14 protein in this mutant (see above). To correlate these results with data of other biochemical and immunological experiments, which indicated that the GA14 protein was enriched during the purification of the PHA granules, we also subjected isolated granules to immunoelectron microscopy. Figure 5C clearly demonstrates that antibodies decorated the surface of isolated granules exactly like granules in whole cells. Occasionally some irregularly shaped objects were labeled (Fig. 5C1 and C2). These most likely represent granules that were disrupted during the preparation. The high specificity of the immunogold labeling method used here is demonstrated in the control sections that were incubated only with the GARG complexes (Fig. 5A3, B3, and C3).

It was also of interest to investigate the subcellular localization of the GA14 protein in *E. coli* XL1-Blue(pSKXA10*), which was constructed for overexpression of this protein. Most of the cells exhibited strong decoration of their periphery, indicating that the GA14 protein seems to have a strong tendency to bind to membranous structures (data not shown). A substantial fraction of the cells was labeled rather uniformly throughout the cytoplasm (data not shown).

DISCUSSION

Analysis of PHA biosynthesis in *R. ruber* at the molecular level yielded a genomic fragment containing the genes for the PHA synthase and a second granule-associated protein, which is referred to as the GA14 protein. Since the structural gene of the GA14 protein maps downstream of the PHA synthase gene, and since the GA14 protein represents the major of four granule-associated proteins, an important function of this protein for the biosynthesis and/or the accumulation of PHA is most likely. An unspecific association of the GA14 protein to the PHA granules as described recently for lysozyme (17) can be excluded. Immunological experiments with the wild type as well as with PHA-leaky and PHA-negative mutants of *R. ruber* indicated a positive correlation between the expression of the GA14 protein and the PHA content. This could result from a mutation in a putative promoter upstream of the PHA synthase gene that prevents the transcription of the PHA synthase gene and ORF3. Promoter structures, however, have not yet been identified in the genus *Rhodococcus*, and therefore a clear statement about promoter structures in *R. ruber* is not possible at present. Putative transcriptional termination structures were identified upstream of the PHA synthase gene and downstream of ORF3 but not in the short intergenic region. Therefore, a transcription unit consisting of both genes is plausible. The analysis of a large number of mutants makes it very unlikely that all of these mutants have the same kind of genotype that is characterized by a mutation in the putative promoter region. Therefore, the question is rather whether the GA14 protein is expressed only if PHA is synthesized, or whether PHA synthesis depends on the presence of the GA14 protein. Since no PHA synthase activity was detected in the PHA-negative mutants RM8 and RM11 or in the PHA-leaky mutants RM17 and RM20 (data not shown), which are characterized by an extremely low PHA content, the mutations had most probably occurred in the PHA synthase gene. Therefore, it is most likely that the capability for PHA synthesis was inhibited first in the mutants and that subsequently expression of the GA14 protein was prevented. To confirm this hypothesis, extended and detailed studies on the regulation of PHA biosynthesis in *R. ruber*, including molecular analysis of the defects in PHA-negative and PHA-leaky mutants, are necessary.

Immunoelectron microscopic studies localized the GA14 protein at the surface of PHA granules and at the cytoplasmic membrane of *R. ruber* as well as at the cytoplasmic membrane and in the cytoplasm of *E. coli* XL1-Blue(pSKXA10*) that overexpressed the GA14 protein. These results clearly demonstrated the tendency of the GA14 protein to bind to membranous structures. However, the GA14 protein does not have a completely hydrophobic character, as revealed by its solubility in the cytoplasm of *E. coli*, by its lack of affinity to the hydrophobic chromatography matrix phenyl-Sepharose during purification, and by the amino acid composition. On the assumption that PHA granules are surrounded by a phospholipid monolayer rather than by a bilayer (19), an anchoring and positioning of the GA14 protein to the granule surface may be achieved by the two segments of hydrophobic and amphiphilic amino acids of the GA14 protein mentioned above (Fig. 6B). This hypothesis requires the expression of the GA14 protein as a monomer in *R. ruber*, in contrast to the tetrameric structure determined for the recombinant protein in *E. coli*. Previous studies have revealed that PHB granules of *Bacillus megaterium* are composed of 97.7% PHB, 1.87% protein, and 0.46% lipid (8). For PHB granules which have diameters ranging from 0.2 to 0.5 μm and which are surrounded by a phospholipid monolayer of 2- to 3-nm thickness (18), however, a phospholipid content of 2.5 to 6.0% (wt/wt) is required. This discrepancy between the calculated and the experimentally determined phospholipid content makes it likely that other amphiphilic molecules contribute considerably to the granule surface that separates the hydrophobic core of the PHA granules and the hydrophilic cytoplasm. Amphiphilic proteins may represent putative candidates which, together with phospholipids and the PHA synthase, occur at the surface of the PHA granules. Amphiphilic proteins have been recently found at the surface of oil bodies of seeds from plants (11). Therefore, we propose that the models of PHA granule assembly as described previously by several laboratories (6, 7, 14) have to be modified by considering proteins which occur in addition to the PHA synthase at the surface of the granules. The latter enzyme constitutes only a relatively small amount of the granule-associated proteins (6a, 17, 23). In *R. ruber*, the GA14 protein investigated in this study is probably the main component of the granule surface, functioning as an amphiphilic

FIG. 5. Immunoelectron microscopic localization of the GA14 protein in wild-type *R. ruber* and at isolated PHA granules by postembedding immunogold labeling. Cells were cultivated as described in the legend to Fig. 4. The scale bar represents 0.3 μm. (A1 and A2) Sections of wild-type *R. ruber* labeled with antibodies against the GA14 protein and GARG complexes; (A3) control section of wild-type *R. ruber* labeled with GARG complexes only; (B1 and B2) specimens as in panels A1 and A2 but shadowed with metal to give a three-dimensional impression; (B3) control section as in panel A3 but metal shadowed; (C1) section of PHA granules from wild-type *R. ruber* labeled with antibodies against the GA14 protein and GARG complexes; (C2) specimen as in panel C1 but metal shadowed; (C3) control section of granules labeled with GARG complexes only and metal shadowed.

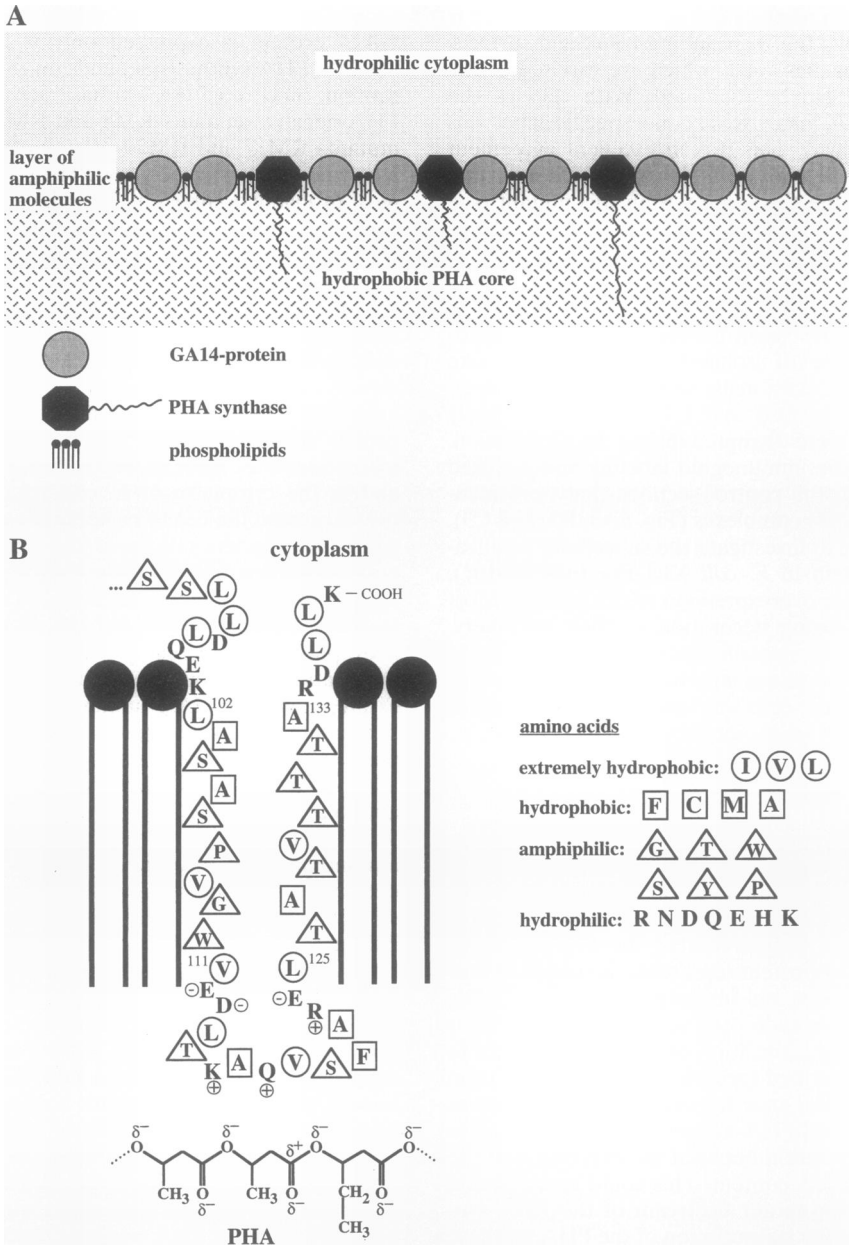


FIG. 6. Model for the structure and composition of the PHA granule surface in *R. ruber* (A) and for the anchoring of the GA14 protein monomer in a phospholipid monolayer (B). The grouping of amino acids is according to Huang (11). The circle for the GA14 protein in panel A does not reflect the quaternary structure of the GA14 protein since this is not known for the native protein as it occurs in *R. ruber*.

protein in the interphase between the hydrophilic cytoplasm and the hydrophobic polyester molecules (Fig. 6A). In addition, it may also function as an anchor for the binding of additional proteins such as the PHA synthase.

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