A New Metabolic Link between Fatty Acid de Novo Synthesis and Polyhydroxyalkanoic Acid Synthesis

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To investigate the metabolic link between fatty acid de novo synthesis and polyhydroxyalkanoic acid (PHA) synthesis, we isolated mutants of Pseudomonas putida KT2440 deficient in this metabolic route. The gene phaG was cloned by phenotypic complementation of these mutants; it encoded a protein of 295 amino acids with a molecular weight of 33,876 Da, and the amino acid sequence exhibited 44% amino acid identity to the primary structure of the rhlA gene product, which is involved in the rhamnolipid biosynthesis in Pseudomonas aeruginosa FG201. S1 nuclease protection assay identified the transcriptional start site 239 base pairs upstream of the putative translational start codon. Transcriptional induction of phaG was observed when gluconate was provided, and PHA synthesis occurred from this carbon source. No complementation of the rhlA mutant of P. aeruginosa UO299-harboring plasmid pBHR81, expressing phaG gene under lac promoter control, was obtained. Heterologous expression of phaG in Pseudomonas oleovorans, which is not capable of PHA synthesis from gluconate, enabled PHA synthesis on gluconate as the carbon source. Native recombinant PhaG was purified by native polyacrylamide gel electrophoresis from P. oleovorans-harboring plasmid pBHR81. It catalyzes the transfer of the acyl moiety from in vitro synthesized 3-hydroxydecanoyl-CoA to acyl carrier protein, indicating that PhaG exhibits a 3-hydroxyacyl-CoA-acyl carrier protein transferase activity.

Fluorescent pseudomonads belonging to the rRNA homology group I are able to synthesize and accumulate large amounts of polyhydroxyalkanoic acids (PHA) consisting of various saturated 3-hydroxy fatty acids with carbon chain length ranging from 6 to 14 carbon atoms as carbon and energy storage compound (1). PHA isolated from these bacteria contained also constituents with double bonds or with functional groups such as branched, halogenated, aromatic, or nitrile side chains (2). The composition of PHA depends on the PHA synthases, the carbon source, and the involved metabolic routes (2–6). In Pseudomonas putida at least three different metabolic routes occur for the synthesis of 3-hydroxyacyl coenzyme A thioesters, which are the substrates of the PHA synthase (7). (i) β-Oxidation is the main pathway when fatty acids are used as carbon source. (ii) Fatty acid de novo biosynthesis is the main route during growth on carbon sources that are metabolized to acetyl-CoA, like gluconate, acetate, or ethanol. (iii) Chain elongation reactions in which acetyl-CoA moieties are condensed to 3-hydroxyacyl-CoA is involved in the PHA synthesis during growth on hexanoate. Recently, recombinant PHA_MCL (MCL = medium chain length) synthesis was also obtained in a β-oxidation mutant of Escherichia coli LS1298 (fadB) expressing PHA synthase genes from Pseudomonas aeruginosa (8, 9), indicating that the β-oxidation pathway in E. coli provides precursors for PHA synthesis (8). From extended homologies of the primary structures of PHA_MCL synthases to PHA_SCL (SCL = short chain length) synthases (1), which occur in bacteria accumulating poly(3-hydroxybutyric acid) such as Alcaligenes eutrophus, it seems also likely that the substrate of PHA_MCL synthases is (R)-3-hydroxyacyl-CoA in pseudomonads. The main constituent of PHA of P. putida KT2442 from unrelated substrates such as gluconate is (R)-3-hydroxydecanoate (7, 10, 11). Thus, to serve as substrate for the PHA synthase, (R)-3-hydroxyacyl-ACP must be converted to the corresponding CoA derivative. This can be mediated in a one step reaction by an (R)-3-hydroxyacyl (ACP to CoA) transferase. Another possibility is the release of (R)-3-hydroxydecanoic acid by a thioesterase, and subsequent activation to the CoA derivative. Only few enzymes have been described catalyzing a similar reaction. Examples are the malonyl-CoA-transferase, which catalyzes the transfer of the malonyl moiety from CoA to ACP (12), and (R)-3-hydroxydecanoyl-ACP-dependent UDP-GlcNAc acyltransferase, which catalyzes the transfer of hydroxycanoyl moiety from ACP to UDP-GlcNAc (13, 14). In this study, we describe the isolation and characterization of P. putida KT2440 mutants, which are defective in the PHA synthesis via fatty acid de novo biosynthesis, and we identified and characterized the gene locus, which phenotypically complements these mutants. The gene product of phaG was purified, and the catalyzed reaction was identified.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth of Bacteria—Pseudomonads and Escherichia coli strains as well as the plasmids used in this study are listed in Table I. E. coli was grown at 37 °C in Luria-Bertani (LB) medium. Pseudomonads were grown at 30 °C either in nutrient broth complex medium (0.8%, w/v) or in a mineral salts medium with 0.05% (w/v) ammonia (15). Nitrosoguanidine Mutagenesis—Mutagenesis was performed according to Miller (16). Cells were incubated for 15 min in the presence of 200 µg of N-methyl-N′-nitro-N′-nitrosoguanidine/ml.

Polyester Analysis—3–5 mg of lyophilized cell material was subjected
to methanolysis in the presence of 15% (v/v) sulfuric acid. The resulting methyl esters of the constituent 3-hydroxyalkanoic acids were assayed by gas chromatography according to Brandl et al. (17) and as described in detail recently (10).

Isolation, Analysis, and Manipulation of DNA—Plasmid DNA was prepared from crude lysates by the alkaline extraction procedure (18). Total genomic DNA was isolated according to Ausubel et al. (19). All genetic procedures and manipulations of DNA were conducted as described by Sambrook et al. (20). DNA sequencing was carried out by the dideoxy chain termination method (21) with single-stranded or with double-stranded alkali-denatured plasmid DNA but with 7-deaza-guanosine 5'-triphosphate instead of dGTP (22) and with α-35S-dATP using a T7 polymerase sequencing kit according to the manufacturer's protocol (Amersham Pharmacia Biotech). Synthetic oligonucleotides were used as primers, and the “primer-hopping strategy” (23) was employed. Analysis was done in 8% (w/v) acrylamide gels in buffer, pH 8.3, containing 100 mM hydrochloride, 83 mM boric acid, 1 mM EDTA, and 42% (w/v) urea in a S2-sequencing apparatus (Life Technologies, Inc.). Nucleic acid sequence data and deduced amino acid sequences were analyzed with the sequence analysis software package (version 6.2, June 1990) according to Devereux et al. (24). The nucleotide and amino acid sequence data reported here have been submitted to GenBank™ under accession number AF052507.

**Determination of the Transcriptional Start Site**—Total RNA was isolated as described by Oelmüller et al. (25). The determination of the transcriptional start site was done by a S1 nuclease protection assay. The hybridization conditions for the S1 nuclease protection assays were done as described by Berk and Sharp (26) and Sambrook et al. (20), and the S1 nuclease reactions were conducted as described by Aldea et al. (27). DNA probes and dideoxynucleotide sequencing reactions for sizing the signals were performed with pBluescript SK+BH13 DNA as a template. In the annealing reaction, the oligonucleotide (5'-GGGTATTCGCGTCACCT-3') complementary to positions 887 to 871 and the oligonucleotide 5'-CCGCATCCGCGCGATAG-3' complementary to positions 986 to 970, respectively, were used for 35S labeling. For all mapping experiments, 25 μg of RNA was mixed with the labeled DNA fragments (10^7 cpm/μg of DNA).

**Polymerase Chain Reaction**—Polymerase chain reaction amplifications were performed in 100-μl volumes according to Sambrook et al. (20) in an Omnigene thermocycler (Hybaid Ltd., Teddington, U. K.) with Vent polymerase (New England Biolabs GmbH, Schwalbach, Germany). The following oligonucleotides were used as primers to amplify the coding region of phaG to construct plasmids pBHR-QG (derivative of pQE60 (Qiagen), insertion into NcoI/BamHI sites) and pBHR81 (derivative of pBBR1MCS-2 (28), insertion into EcoRI/BamHI sites), respectively: 5'-CATGCGCATGGGAAGCCAGAAGATCCGGATGTA-3', 5'-
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CGGGATCCAGTGCGAATTCGATGACATG-3'

and the reaction mixture was analyzed by HPLC. Five mutants (PhAG<sub>N</sub>) were identified, which accumulated PHA only up to 3% of the cellular dry weight (CDW) from gluconate but up to 85% PHA of CDW when cultivated on octanoate as the sole carbon source. The composition of the polymer was not affected. We constructed a library of EcoRI-digested <i>P. putida</i> KT2440 genomic DNA with the cosmid vector pVK100 (32) and the Gigapack II Gold Packaging Extract (Stratagene Cloning Systems, La Jolla, CA) in <i>E. coli</i> S17-1. Approximately 5,000 transductants were applied to minicomplementation experiments, with mutant PHAG<sub>N</sub>-21 as recipient. One of the hybrid cosmids (pVK100-K18) harbored three EcoRI-frgments (3, 6, and 9 kbp) and enabled PHAG<sub>N</sub>-21 to accumulate PHA from gluconate. Subcloning revealed that the 3-kbp EcoRI fragment (E3, pME3) complemented PHAG<sub>N</sub>-21 and any other PHAG<sub>N</sub> mutant exhibiting this phenotype. Complementation was not achieved by the hybrid cosmid pH1016::PP2000 comprising the entire 7.3-kbp PHA synthase locus of <i>P. aeruginosa</i> PAO1 plus approximately 13 kbp of the upstream region or by the hybrid cosmid pH1016::PP180 comprising thephaC2 gene of <i>P. aeruginosa</i> PAO1 plus approximately 16 kbp of the adjacent downstream region (10).

FIG. 2. Homology of the phaG gene product to RhA (40) and the putative qin gene product (GenEMBL data library, accession number L021605) of <i>P. aeruginosa</i>. That part of the amino acid sequence that was deduced from the improved open reading frame analysis of the qin nucleotide sequence is given in lowercase letters. Matching amino acids are boxed. Dashes indicate gaps, which were introduced to improve the alignment. Numbers indicate the positions of the amino acids in the respective proteins.

RESULTS

Complementation of Mutants Affected in the PHA Synthesis via de Novo Fatty Acid Biosynthesis—Mutants of <i>P. putida</i> KT2440, which are only deficient in the metabolic route-linking fatty acid de novo synthesis, were generated with nitrosoguanidine according to Miller et al. (16). Five mutants (PhAG<sub>N</sub>) were identified, which accumulated PHA only up to 3% of the cellular dry weight (CDW) from gluconate but up to 85% PHA of CDW when cultivated on octanoate as the sole carbon source. The composition of the polymer was not affected. We constructed a library of EcoRI-digested <i>P. putida</i> KT2440 genomic DNA with the cosmid vector pVK100 (32) and the Gigapack II Gold Packaging Extract (Stratagene Cloning Systems, La Jolla, CA) in <i>E. coli</i> S17-1. Approximately 5,000 transductants were applied to minicomplementation experiments, with mutant PHAG<sub>N</sub>-21 as recipient. One of the hybrid cosmids (pVK100-K18) harbored three EcoRI-frgments (3, 6, and 9 kbp) and enabled PHAG<sub>N</sub>-21 to accumulate PHA from gluconate. Subcloning revealed that the 3-kbp EcoRI fragment (E3, pME3) complemented PHAG<sub>N</sub>-21 and any other PHAG<sub>N</sub> mutant exhibiting this phenotype. Complementation was not achieved by the hybrid cosmid pH1016::PP2000 comprising the entire 7.3-kbp PHA synthase locus of <i>P. aeruginosa</i> PAO1 plus approximately 13 kbp of the upstream region or by the hybrid cosmid pH1016::PP180 comprising thephaC2 gene of <i>P. aeruginosa</i> PAO1 plus approximately 16 kbp of the adjacent downstream region (10).

Determination of the Gene Locus and Nucleotide Sequence of phaG—Fragment E3 was cloned into pBluescript SK, and the entire nucleotide sequence was determined (Fig. 1). It comprised 3,061 nucleotides with three ORFs (Fig. 1). The only ORF that was completely localized on this fragment was ORF2 (Fig. 1) and comprised 3,061 nucleotides with three ORFs (Fig. 1). The only ORF that was completely localized on this fragment was ORF2 (Fig. 1) and comprised 3,061 nucleotides with three ORFs (Fig. 1). The only ORF that was completely localized on this fragment was ORF2 (Fig. 1) and comprised 3,061 nucleotides with three ORFs (Fig. 1). The only ORF that was completely localized on this fragment was ORF2 (Fig. 1) and comprised 3,061 nucleotides with three ORFs (Fig. 1). The only ORF that was completely localized on this fragment was ORF2 (Fig. 1) and comprised 3,061 nucleotides with three ORFs (Fig. 1). The only ORF that was completely localized on this fragment was ORF2 (Fig. 1) and comprised 3,061 nucleotides with three ORFs (Fig. 1).
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![Table I: Bacterial strains and plasmids](http://example.com/table1)

| Strains and plasmids | Relevant characteristics | Source or reference |
|----------------------|--------------------------|---------------------|
| *P. putida*           |                          |                     |
| KT2440               |                           |                     |
| PHAG<sub>r</sub>-21   |                           |                     |
| P. oleovorans        |                           |                     |
| E. aeruginosa        |                           |                     |
| S17–1                | recA<sup>+</sup> harbors the *tra* genes of plasmid RF4 in the chromosome; *proA*, *thi-1* | (46) |
| JM109                | recA<sup>+</sup> endA1 gyrA96 thi hsdR17 (rk<sup>+</sup>,mk<sup>+</sup>) supE44 relA1, λ<sup>-</sup> lac<sup>[F proAB lacIqZΔM15]</sup> | (21) |
| Plasmids             |                           |                     |
| pH1016::PP180        |                           | (47) |
| pH1014::PP2000       |                           | (47) |
| pVK100               |                           | (32) |
| pVK100::K18          |                           |                     |
| pMP92                |                           | (48) |
| pMP3                |                           |                     |
| pUCP27               |                           | (49) |
| pBLUESKRIPT SK       |                           |                     |
| pBluescript SK<sup>BH13</sup> |                 |                     |
| pQ60                 |                           |                     |
| pBBR1MCS-2           |                           | (28) |
| pBBR51               |                           |                     |
| pBluescript SK<sup>BH13</sup> |                 |                     |
| pQ60                 |                           |                     |
| pQ60                 |                           |                     |
| pQ60                 |                           |                     |
| pQ60                 |                           |                     |

| Strains and plasmids | Relevant characteristics | Source or reference |
|----------------------|--------------------------|---------------------|
| *P. putida*           |                          |                     |
| KT2440               | m<sup>−2</sup>, hsdR1 (r<sup>−</sup> m<sup>−</sup>), ohne | (45) |
| PHAG<sub>r</sub>-21   |                           |                     |
| P. oleovorans        |                           |                     |
| E. aeruginosa        |                           |                     |
| S17–1                |                           |                     |

Characterization of the *phaG* Translational Product—The codon usages in *phaG*, ORF1 and ORF3 agreed well with typical *P. putida* codon preferences. The G + C content of 59.2 mol % for *phaG* was similar to the value of 60.7 to 62.5 mol % determined for total genomic DNA of *P. putida* (35). The *phaG* gene encodes a protein of 295 amino acids with a molecular mass of 33,876 Da. Sequence alignments of the amino acid sequence deduced from *phaG* revealed 44% overall identity to the *rhlA* gene product of *P. aeruginosa* PG201 (Fig. 2). RhlA also consists of 295 amino acids and has a molecular mass of 32.5 kDa. This gene represents the 5′-terminal gene of a gene cluster consisting of the genes *rhlA*, *rhlB*, and *rhlR*. The first two genes encode proteins involved in rhamnolipid biosynthesis. The *rhlB* gene product exhibited rhamnosyltransferase activity, whereas the function of RhlA is not yet characterized but is necessary for effective rhamnolipid biosynthesis. RhlR represents a transcriptional activator acting upon *pha*-dependent promoters (36). The C-terminal region of RhlA and PhaG revealed high homology to a gene region (**gin**) of *P. aeruginosa* encoding the so-called “quinolone-sensitivity protein” (GenBank data library, accession number L02105) amounting to 50.6 and 40.1% to PhaG or to RhlA, respectively, in 249 overlapping residues (Fig. 2). This region consists of 1503 nucleotides. The N terminus of the *gin* gene was not exactly determined, and the homology as depicted in the data base extents only from nucleotide 207 to 566 of this sequence (Fig. 2). However, translation of this sequence in all six reading frames and a subsequent tBLASTn search resulted in the identification of homologies also in the upstream region of the suggested **gin** translational start codons but in different reading frames with the N-terminal region of PhaG and RhlA.

**Identification and Regulation of the Promoter**—244 bp upstream of *phaG*, a putative ω<sup>−</sup>-dependent promoter structure TTGCGCN<sub>T</sub>TGGAAAT (where N is a nucleoside) was identified. The promoter was verified by complementation studies of mutant PHAG<sub>r</sub>-21 with subfragments of E3. The 2.2-kbp SalI-*EcoRI* fragment harboring *phaG* comprising *phaG* including the native promoter *phaG* without promoter *phaG* downstream of lac promoter *phaG* containing coding region of *phaG* promoter control in *E. coli* This study This study This study This study This study PhaG expression in E. coli—A plasmid expressing a C-terminal His<sup>6</sup> tag fusion protein of PhaG was constructed. The resulting plasmid pBBR-QG enabled overexpression of *phaG* under lac promoter control in *E. coli* JM109 (Fig. 4). The fusion protein could only be purified under denaturing conditions by immobilized metal ion affinity (Fig. 5) and was used as antigen to raise antibodies.
Functional Homologous and Heterologous Expression of phaG—Functional expression, as revealed by complementation of mutant PHAG$_{N21}$, was obtained from plasmid pBHR81, a derivative of vector pBBR1MCS-2 (28) containing the coding region of phaG in sites EcoRI/BamHI (Fig. 4, Table II). Additionally, transfer of pBHR81 into P. oleovorans ATCC 29347, which is not capable of PHA synthesis from simple carbon sources, resulted in PHA accumulation from gluconate contributing to about 55% of CDW (Table II). Thus only functional expression of phaG in P. oleovorans established a metabolic link between fatty acid de novo biosynthesis and PHA synthesis.

Expression of phaG in P. aeruginosa PAO1 based on plasmid pBHR81 revealed an ~40% increase in PHA accumulation (Table II). We also investigated functional expression of phaG in E. coli JM109-harboring plasmids pBHR81 and pBHR71 allowing functional expression of PHA synthase gene phaC1 (8), but no PHA accumulation was observed when cells were grown on glucose. Furthermore, transfer of pBHR81 into P. aeruginosa UO299 (rhlA) did not result in complementation of this mutant with respect to rhamnolipid synthesis (data not shown). Thus PhaG does not functionally replace RhlA. To evaluate whether PhaG exhibits PHA synthase activity, we cultivated the P. putida PHAG$_N$ mutants harboring pBHR81 under nonlimited nitrogen conditions, which resulted in decreased PHA synthase levels and decreased PHA accumulation (37). No increase in PHA accumulation was observed when cells were grown on gluconate in the presence of PhaG (data not shown).

Enzymatic Assay of PhaG—Native PhaG was purified from crude extracts of P. oleovorans (pBHR81) by native PAGE as described under “Experimental Procedures.” Recombinant PhaG showed high mobility in native PAGE, which could be utilized for one-step purification (Fig. 5). PhaG was also identified by N-terminal amino acid sequencing.

Purified PhaG and crude extracts from P. oleovorans (pBHR81) were employed to demonstrate enzymatic activity of PhaG. As substrate we provided in vitro synthesized (R,S)-3-hydroxydecanoyl-CoA and analyzed the reaction products by HPLC (Fig. 6). P. oleovorans harboring only vector pBBR1MCS-2 and heat-inactivated purified PhaG served as negative control. The HPLC data clearly demonstrate that, applying either crude extract or purified PhaG, a transfer of the 3-hydroxydecanoyl moiety from CoA to ACP occurs (Fig. 6). The omission of MgCl$_2$ resulted in a loss of enzymatic activity, indicating that MgCl$_2$ is an important cofactor. Furthermore, we applied the straight chain octanoyl-CoA and decanoyl-CoA thioesters as substrate. None of these CoA thioesters yielded the corresponding ACP thioester, and they were therefore not accepted as substrate by PhaG.
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Phenotypic complementation of P. putida KT2440 PHAG₈ mutants, which are affected in PHA biosynthesis based on fatty acid de novo biosynthesis, led to the identification and characterization of phaG as a new gene locus relevant for PHA biosynthesis in P. putida. The PHA synthesis pathway via β-oxidation was not impaired in the PHAG₈ mutants. PHAG₈ mutants were not complemented with the PHA synthase locus of P. oleovorans P AO1 and adjacent genomic region. Therefore, PHAG₈ mutants are not defective in the PHA synthase locus, and most probably phaG is not closely linked to the PHA synthase locus. Furthermore, phaG is not in general essential for the synthesis of PHA in P. putida KT2440 but is only required for PHA synthesis and accumulation from gluconate or other simple carbon sources, which are catabolized to acetyl-CoA in this organism before PHA synthesis starts.

From results of labeling studies, nuclear magnetic resonance spectroscopy and gas chromatography-mass spectroscopy Eggink et al. (4) and Huijberts et al. (7, 38) concluded that the precursors of PHAₘₛₐₚₖₐₘ₃ biosynthesis from simple carbon sources are predominantly derived from (R)-3-hydroxyacyl-ACP intermediates occurring during the fatty acid de novo biosynthetic route. Since the constituents of PHB and PHA represent the R configuration, and since PHAₘₛₐₚₐₘ₃ and PHAₘₛₐₚₐₘ₃ synthases are highly homologous, the intermediates in fatty acid metabolism are presumably converted to (R)-3-hydroxyacyl-CoA before polymerization. Nevertheless, some other routes of PHA synthesis are also possible. Other conceivable alternatives are the release of free fatty acids by the activity of a thioesterase with a thiolase, subsequently activating these fatty acids to the corresponding hydroxyacyl-CoA thioesters or chain elongation with β-ketothiolase, or β-oxidation of synthesized fatty acids. Evidence for the latter pathways in P. putida (7) was obtained and explains why phaG mutants are not completely defective in PHAMₘₐₚₐₘ₃ biosynthesis from gluconate. Functional expression of either PHA synthase and accumulation of PHAMₘₐₚₐₘ₃ from fatty acids indicate that PHA synthases are not utilizing (R)-3-hydroxyacyl-ACP derivatives as substrate (8, 9).

All mutants analyzed and complemented by phaG synthesized PHA to some extent (0.5–3% CDW) with a typical monomer composition of polyester derived from simple carbon sources, as far as detectable. However, analysis of mutant complementation studies and the genomic organization of phaG revealed no indication for the existence of another protein essential for the PHA synthesis from simple carbon sources in P. putida KT 2440. Therefore, most probably only one additional specific enzymatic step is required for PHA synthesis from gluconate that is not required for PHA synthesis from octanoate. This hypothesis was supported by the observation that only PhaG conferred the ability to synthesize PHA from gluconate to P. oleovorans, which lacks this capability (Table II). Furthermore, the analysis of enzymatic activity of PhaG strongly suggests that one enzyme is sufficient to link fatty acid de novo synthesis with PHA synthesis (Fig. 6). Evidence that PhaG is not directly involved in synthesis of PHAMₘₐₚₐₘ₃ was provided by cultivations of the P. putida PHAG₈ mutants (pBHR81) under nitrogen limited and nonlimited conditions. Under nonlimited conditions the level of PHA synthases and PHAMₘₐₚₐₘ₃ accumulation is significantly decreased (37), and even in the presence of PhaG, no increase in PHAMₘₐₚₐₘ₃ synthesis was observed.

Although no complementation of rhamnolipid synthesis in P. aeruginosa rhlA mutant UO299 was obtained with phaG expressed from plasmid pBHR81, the high degree of homology of phaG to rhlA and the qin region of P. aeruginosa, respectively, indicates a related function of these proteins. The exact function of the “quinolone sensitivity protein” has not yet been described. Quinolones such as nalidixic acid are synthetic antibiotics exhibiting strong antimicrobial effects on Gram-negative bacteria including P. aeruginosa. The rhlA gene product is involved in the rhamnolipid biosynthesis of P. aeruginosa PG201, which are synthesized as biosurfactants during the late exponential and stationary growth phases. Rhamnolipid biosynthesis proceeds by sequential glycosyl transfer reactions, each catalyzed by specific rhamnosyltransferases with TDP-rhamnose acting as a rhamnosyl donor, and 3-hydroxydecanoyl-3-hydroxydecanoate or l-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate acting as acceptors as proposed by Burger et al. (39, 40). 3-Hydroxydecanoate can be formed via β-oxidation or via fatty acid de novo biosynthesis (41). A dimer consisting of two 3-hydroxydecanoic acid molecules is formed by a hitherto unknown mechanism. RhlA significantly enhanced the level of rhamnolipids in rhamnolipid-negative mutants of P. aeruginosa PG201 when it was coexpressed with the rhamnosyltransferase (RhIB) as compared with the expression of the isolated rhlB gene.

3-Hydroxyacyl-ACP intermediates provided by fatty acid biosynthesis are presumably the common intermediates of PHA and rhamnolipid biosynthesis from gluconate. If the ACP derivatives themselves do not serve as substrates for PHA synthases or enzymes involved in rhamnolipid synthesis for the condensation of two 3-hydroxydecanoyl moieties, they must be either directly transesterified to the corresponding CoA derivatives or transferred to CoA thioesters by the combined action of a thioesterase and a thiokinase. Various transacylases and acyltransferases have been described and well characterized.

**Fig. 5.** a, heterologous expression of phaG-His tag in E. coli and purification. Cytoplasmic fractions obtained from cells of recombinant strains of E. coli grown in LB medium and fractions from batch purification with Ni²⁺-nitrilotriacetic-acid-agarose were separated in 11.5% (w/v) polyacrylamide gels and stained to visualize protein with Serva blue R. M, molecular weight standards. Lane 1, crude extract of E. coli JM109 (pQE60); lane 2, crude extract of E. coli JM109 (pBHR-QG); lane 3, eluate after washing with 20 mM imidazole; lane 4, purified PhaG-His tag after elution with 250 mM imidazole. b, heterologous expression of phaG in P. oleovorans and purification of native PhaG. P. oleovorans harboring pBHR81 was cultivated 16 h at 30 °C on mineral salts medium containing 1% (w/v) gluconate. Crude extracts were applied to native PAGE (PrepCell 491, Bio-Rad), and the first fraction with high absorption at 280 nm yielded purified PhaG was analyzed. M, molecular weight standards. Lane 1, crude extract of P. oleovorans (pBHR1MCS-2); lane 2, crude extract of P. oleovorans (pBHR81); lane 3, first protein eluate from native PAGE containing pure PhaG.
catalyzing the direct transfer of an acyl moiety, e.g. (i) the malonyl-CoA-ACP transferase, which catalyzes the transfer of the malonyl moiety from CoA to ACP (12) and (ii) the hydroxydecanoyl-ACP-dependent UDP-GlcNAc acyltransferase, which catalyzes the transfer of hydroxydecanoyl moiety from ACP to UDP-GlcNAc (13, 14). The bacterial acyltransferase LpxA is one representative of a large family that possesses conserved repeating hexapeptides (42). Sequence analysis of membrane-bound glycerolipid acyltransferases revealed that these proteins share a highly conserved domain containing invariant histidine and aspartic acid residues separated by four less conserved residues from an invariant histidine and aspartic acid residues separated by four less conserved residues from a repeating hexapeptide (43). Site-directed mutagenesis of the invariant histidine resulted in lack of activity, indicating an essential role of this residue (43). Although no significant homology of PhaG to transacylases and acyltransferases was found, this highly conserved H4X4D mini-motif is also present in PhaG at positions 176–181 of the amino acid sequence (Fig. 1), suggesting a similar function of PhaG.

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