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Role of Fatty Acid De Novo Biosynthesis in Polyhydroxyalkanoic Acid (PHA) and Rhamnolipid Synthesis by Pseudomonads: Establishment of the Transacylase (PhaG)-Mediated Pathway for PHA Biosynthesis in *Escherichia coli*

BERND H. A. REHM,[1]* TIMOTHY A. MITSKY,[2] AND ALEXANDER STEINBÜCHEL[1]

Institut für Mikrobiologie, Westfälische Wilhelms-Universität Münster, D-48149 Münster, Germany,[1] and Monsanto Company, St. Louis, Missouri 63167[2]

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Since *Pseudomonas aeruginosa* is capable of biosynthesis of polyhydroxyalkanoic acid (PHA) and rhamnolipids, which contain lipid moieties that are derived from fatty acid biosynthesis, we investigated various *fab* mutants from *P. aeruginosa* with respect to biosynthesis of PHAs and rhamnolipids. All isogenic *fabA, fabB, fabI, rhlG*, and *phaG* mutants from *P. aeruginosa* showed decreased PHA accumulation and rhamnolipid production. In the *phaG* (encoding transacylase) mutant rhamnolipid production was only slightly decreased. Expression of *phaG* from *Pseudomonas putida* and expression of the β-ketoacyl reductase gene *rhlG* from *P. aeruginosa* in these mutants indicated that PhaG catalyzes diversion of intermediates of fatty acid de novo biosynthesis towards PHA biosynthesis, whereas RhlG catalyzes diversion towards rhamnolipid biosynthesis. These data suggested that both biosynthesis pathways are competitive. In order to investigate whether PhaG is the only linking enzyme between fatty acid de novo biosynthesis and PHA biosynthesis, we generated five Tn*5* mutants of *P. putida* strongly impaired in PHA production from gluconate. All mutants were complemented by the *phaG* gene from *P. putida*, indicating that the transacylase-mediated PHA biosynthesis route represents the only metabolic link between fatty acid de novo biosynthesis and PHA biosynthesis in this bacterium. The transacylase-mediated PHA biosynthesis route from gluconate was established in recombinant *E. coli*, coexpressing the class II PHA synthase gene *phaC1* together with the *phaG* gene from *P. putida*, only when fatty acid de novo biosynthesis was partially inhibited by triclosan. The accumulated PHA contributed to 2 to 3% of cellular dry weight.

A wide variety of microorganisms accumulate polyhydroxyalkanoic acids (PHAs), mostly polyhydroxybutyrate, as metabolic storage materials, which are deposited as intracellular water-insoluble inclusions (1, 22). Meanwhile, more than 150 constituents of PHAs have been found (38). Recently, it was shown that provision of 3-mercaptopropionic acid as a carbon source resulted in biosynthesis of a novel sulfur-containing polyester with thioester linkages by *Ralstonia eutropha* (21). Most fluorescent pseudomonads belonging to rRNA homology group I, e.g., *Pseudomonas aeruginosa* and *Pseudomonas putida*, are able to synthesize and accumulate large amounts of PHAs consisting of various 3-hydroxy fatty acids with carbon chain lengths ranging from 6 to 14 carbon atoms (medium chain length [MCL] PHAs [PHA_{MCL}]) as carbon and energy storage compounds from cheap carbon sources, e.g., low-rank coal liquefaction products or waste oil from biotechnological rhamnose production (1, 9, 10, 22, 39, 40). The composition of PHA depends on the PHA synthases, the carbon source, and the metabolic routes involved (29, 31, 32). β-Oxidation is the main pathway when fatty acids are used as a carbon source, and fatty acid de novo biosynthesis is the main route during growth on carbon sources which are metabolized to acetyl coenzyme A (acetyl-CoA), like gluconate, acetate, or ethanol (16, 28, 32). Recently, recombinant PHA_{MCL} synthesis was also obtained in β-oxidation mutants of *Escherichia coli* LS1298 (*fadB*) or RS3097 (*fadR*) expressing PHA synthase genes from *P. aeruginosa* (20, 24, 25), indicating that the β-oxidation pathway in *E. coli* provides precursors for PHA synthesis. It has also been recently shown that coexpression of the thioesterase genes with a PHA synthase gene in *E. coli fad* mutants causes synthesis of PHA_{MCL} from the carbon source gluconate (18, 30). These data suggested that the fatty acid de novo synthesis as well as the β-oxidation pathways were involved. It was recently confirmed that the purified PHA_{MCL} synthases from *P. aeruginosa* exhibit in vitro enzyme activity with (*R*)-3-hydroxydecanoyl-CoA as the substrate (26). Thus, to serve as a substrate for the PHA synthase, (*R*)-3-hydroxyacyl-acyl carrier protein [(*R*)-3-hydroxyacyl-ACP], which is an intermediate of fatty acid de novo synthesis, must be converted to the corresponding CoA-derivative. Recently, the transacylase $PhaG_{Pp}$ from *P. putida*, which catalyzes the transfer of the (*R*)-3-hydroxydecanoyl moiety from the ACP thioester to CoA, has been identified and characterized (28). Thus, PhaG directly links fatty acid de novo biosynthesis with PHA biosynthesis (Fig. 1). Meanwhile, *phaG* genes were isolated and characterized from *Pseudomonas oleovorans* and *P. aeruginosa* and evidence was obtained that this transacylase-mediated pathway is widespread among pseudomonads (14, 15). Interestingly, in *P. aeruginosa* about 40% of the accumulated PHA is provided via alternative pathways from gluconate as the carbon source independent of the transacylase PhaG (14). In non-PHA-accumulating *Pseudomonas fragi* the coexpression of $phaG_{Pp}$ with

* Corresponding author. Mailing address: Institut für Mikrobiologie, Westfälische Wilhelms-Universität Münster, Corrensstrasse 3, D-48149 Münster, Germany. Phone: 49 251 8339848. Fax: 49 251 833 8388. E-mail. rehm@unimuenster.de.

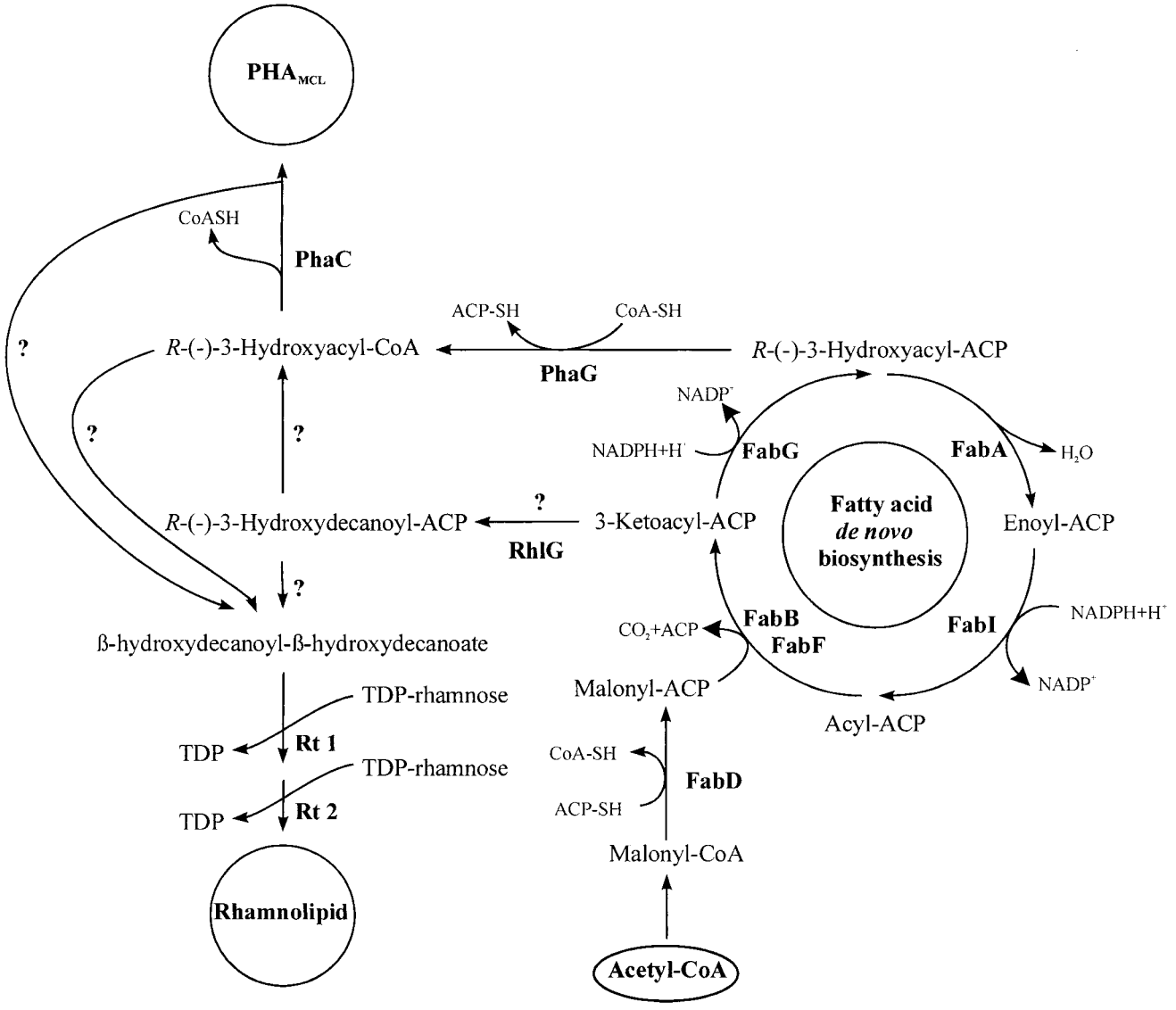

FIG. 1. Proposed pathways for PHA$_{MCL}$ and rhamnolipid biosynthesis. PhaC, PHA synthase; PhaG, 3-hydroxydecanoyl-ACP–CoA transacylase; RhlG, β-ketoacyl reductase; FabG, β-ketoacyl-ACP reductase; FabA, 3-hydroxydecanoyl-ACP dehydrase; FabB, β-ketoacyl-ACP synthase I; FabF, β-ketoacyl-ACP synthase II; FabI, enoyl-ACP reductase; FabD, malonyl-CoA–ACP transacylase. The question marks indicate hitherto-unconfirmed metabolic routes.

phaC1$_{Pa}$ established a new pathway for PHA$_{MCL}$ biosynthesis from fatty acid de novo biosynthesis using nonrelated carbon sources, e.g., gluconate or acetate (8).

P. aeruginosa is capable of producing various exoproducts, such as exoenzymes, pyocyanine, the exopolysaccharide alginate, and rhamnolipids. Rhamnolipids are glycolipids, which reduce water surface tension and emulsify oil. These rhamnolipids produced by *P. aeruginosa* in liquid cultures are mainly rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (monorhamnolipid) and rhamnosyl-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate (dirhamnolipids). Rhamnolipid biosynthesis proceeds through transfer of two rhamnose moieties from TDP-L-rhamnose (3). For the synthesis of monorhamnolipid, the enzyme rhamnosyltransferase 1 (Rt 1) catalyzes the rhamnose transfer to β-hydroxydecanoyl-β-hydroxydecanoate, while Rt 2 synthesizes dirhamnolipid from TDP-L-rhamnose and monorhamnolipid. Genes coding for biosynthesis, regulation, and induction of the Rt 1 enzyme are organized in tandem in the *rhlABRI* gene cluster (23). The gene *rhlC*, which encodes the Rt 2 enzyme, has been very recently described (27). This enzyme is homologous to rhamnosyltransferases involved in lipopolysaccharide biosynthesis. Recently, Campos-Garcia et al. (4) identified the *rhlG* gene encoding a β-ketoacyl reductase, which is presumably involved in the biosynthesis of rhamnolipids. RhlG is supposed to catalyze the NADPH-dependent reduction of β-ketodecanoyl-ACP, which is an intermediate of fatty acid de novo biosynthesis, resulting in β-hydroxydecanoyl-ACP, a putative precursor for rhamnolipid biosynthesis (Fig. 1).

Since both PHA and rhamnolipid contain lipid moieties which are derived from fatty acid biosynthesis, we investigated various *fab* mutants from *P. aeruginosa* with respect to the biosynthesis of PHA and rhamnolipid. Furthermore, the influ-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
P. putida		
KT2440	Wild type	43
GPp104	PHA synthase negative mutant of *P. putida* KT2442; mt-2 *hsdR1* ($r^- m^+$) without TOL plasmid	17
$PHAG_N$-21	PhaG-negative mutant of *P. putida* KT2440	28
B349/*Tn5*-1	Tn*5* mutant, PhaG negative	This study
B349/*Tn5*-2	Tn*5* mutant, PhaG negative	This study
B349/*Tn5*-3	Tn*5* mutant, PhaG negative	This study
B349/*Tn5*-4	Tn*5* mutant, PhaG negative	This study
B349/*Tn5*-5	Tn*5* mutant, PhaG negative	This study
P. aeruginosa		
PAO1	Wild type	ATCC 15692
KO2	PAO1 with *phaG*::Gm^r	14
ACP5	PAO1 with *rhlG*::Tc^r	4
PAO191	PAO1 with *fabA*::Gm^r	12
PAO192	PAO1 with *fabB*::Gm^r	12
PAO235	PAO1 with *fabI*::*FRT*	13
E. coli		
S17-1	*recA*; harbors the *tra* genes of plasmid RP4 in the chromosome; *proA thi-1*	3
JM109	*recA1 endA1 gyrA96 thi-1 hsdR17* ($r_K^- m_K^+$) *supE44 relA1* λ− *lac* [F′ *proAB lacI*^{q}ZΔM15]	33
RS3097	*el4- fadR*(Ts) *zcg-101::*Tn*10 fabA fadR tyrT mel-1*	36
DC170		6
IP1111	*Hfr galE45 fabI*(Ts) *relA1 spoT1*	42
Plasmids		
pBBR1MCS-2	Km^r; broad host range; *lacPOZ′*	19
pBHR81	pBBR1MCS-2 containing coding region of *phaG* gene from *P. putida* downstream of *lac* promoter	28
pUCP20	Cb^r; broad host range; *lacPOZ′*	35
pJC3	pUCP20 containing *rhlG* gene from *P. aeruginosa* PAO1	4
pBHR71	pBluescript SK(−) containing *phaC1* gene from *P. aeruginosa* PAO1	20
pBHR75	pUCP27 containing *phaG* gene from *P. putida* KT2440	28
pBHR86	pBBR1MCS-2 containing coding region of *phaC1* gene from *P. aeruginosa* downstream of *lac* promoter and the coding region of *phaG* from *P. putida* downstream of *phaC1* including the native promoter	8
pBHR87	pBBR1MCS-2 containing coding region of *phaC1* gene from *P. aeruginosa* downstream of *lac* promoter and the coding region of *phaG* from *P. putida* downstream of *phaC1*	This study

ence of the transacylase PhaG and the β-ketoacyl reductase RhlG on the synthesis of PHA and rhamnolipid, respectively, was studied. Moreover, we generated Tn*5* mutants of *P. putida* deficient in PHA_{MCL} biosynthesis from gluconate in order to investigate whether other genes are required for this specific pathway. Finally, the transacylase-mediated pathway for PHA_{MCL} synthesis from gluconate was established in recombinant *E. coli*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth of bacteria. Pseudomonads and *E. coli* strains as well as the plasmids used in this study are listed in Table 1. *E. coli* was grown at 37°C in complex Luria-Bertani (LB) medium. Pseudomonads were grown at 30°C in 300-ml baffled flasks containing 50 ml of either LB medium, PPGAS medium (NH_4Cl, 0.02 M; KCl, 0.02 M; Tris-HCl, 0.12 M; $MgSO_4$, 0.0016 M; glucose, 0.5% [wt/vol]; peptone, 1% [wt/vol] [44]), or mineral salts medium (MM) containing 0.05% (wt/vol) ammonium chloride and a carbon source as indicated (34), and if required antibiotics were added to appropriate concentrations. The amounts of antibiotics used for *P. aeruginosa* were as follows (per milliliter): 300 μg of carbenicillin, 250 μg of gentamicin, 150 μg of tetracycline, and 300 μg of kanamycin. The amounts of antibiotics used for *P. putida* were as follows (per milliliter): 10 μg of gentamicin and 50 μg of kanamycin. The amounts of antibiotics used for *E. coli* were as follows (per milliliter): 50 μg of kanamycin and 100 μg of ampicillin.

Isolation, analysis, and manipulation of DNA. DNA sequences of new plasmid constructs were confirmed by DNA sequencing according to the chain termination method using the model 4000L automatic sequencer LI-COR (MWG-Biotech, Ebersberg, Germany). All other genetic techniques were performed as described by Sambrook et al. (33).

Tn*5* mutagenesis. In order to generate mutants of *P. putida*, which are defective in PHA_{MCL} biosynthesis from nonrelated carbon sources, such as gluconate, we performed Tn*5* mutagenesis. The suicide plasmid pMON5302 (Monsanto, St. Louis, Mo.) was constructed by insertion of the Tn*5* IS*50L* and IS*50R* regions comprising a gentamicin resistance cassette [AAC(3)-I gene] into plasmid pACYC (Monsanto). The plasmid was transferred into *P. putida* KT2440 by conjugation as previously described (27), and Tn*5* mutants were screened on MM agar plates containing 1.5% (wt/vol) gluconate as the sole carbon source. Colonies which appeared nonopaque were isolated, and after cultivation in the same medium the cells were analyzed with respect to PHA accumulation.

Plasmid construction. The coding region of the *P. putida* $phaG_{Pp}$ gene was amplified by tailored PCR using primers with noncomplementary 5′ ends, introducing *Bam*HI and *Xba*I restriction sites at either end of the PCR product by using plasmid pBHR75 as the template (28). The coding region of the PHA synthase gene $phaCl_{Pa}$ from *P. aeruginosa* was amplified by tailored PCR using plasmid pBHR71 as the template (20), introducing the *Eco*RI restriction site and the ribosome binding site at the 5′ end and the *Bam*HI restriction site at the 3′ end. The following oligonucleotides were applied for the PCRs: 5′-CCCGAATTCAATAAGGAGATATACATATGAGTCAG-3′ (5′ end) and 5′-TGCTCTAGAGGGCCCCCCTCGAGGTC-3′ (3′ end) ($phaCl_{Pa}$); and 5′-CGCGGATCCAAGGAGTCGATGACATG-3′ (5′ end) and 5′-GCGTCTAGACTACAAGGCGCCGAGCCG-3′ (3′ end) ($phaG_{Pp}$). Both PCR products were simultaneously subcloned into restriction sites *Eco*RI and *Xba*I of the vector pBBR1MCS-2, resulting in the insertion of the PHA synthase gene and the transacylase gene collinear to the *lac* promoter. The resulting plasmid, pBHR87, enabled functional coexpression of $phaCl_{Pa}$ and $phaG_{Pp}$.

Functional expression of PHA_{MCL} synthase gene. PHA synthase activity was confirmed by expression of the respective PHA synthase gene in various metabolic backgrounds favoring PHA_{MCL} synthesis, e.g., *E. coli* RS3097 and *P. putida* GPp104 (25, 26). Recombinant bacteria harboring the respective plasmid were cultivated in the presence of 0.25% (wt/vol) decanoate. PHA accumulation was

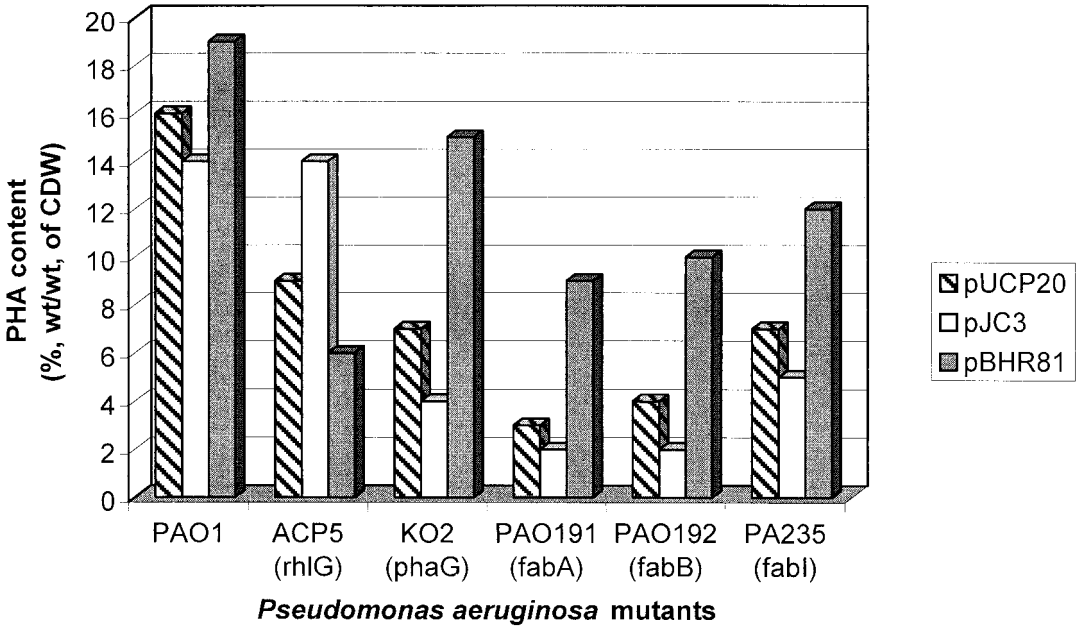

FIG. 2. PHA_{MCL} accumulation by mutants of *P. aeruginosa* from gluconate. Cultivations were performed under PHA-accumulating conditions on MM containing 1.5% (wt/vol) sodium gluconate, 0.01% (vol/vol) oleate, and 0.05% (wt/vol) ammonium chloride. Cells were grown for 48 h at 37°C. PHA content and composition of comonomers were analyzed by GC. The gene affected by each mutation is given in parentheses. CDW, cellular dry weight.

determined by gas chromatography (GC) analysis of lyophilized cells and indicated in vivo PHA synthase activity.

Functional expression of PhaG (transacylase) gene. Functional expression of *phaG* [encoding the (*R*)-3-hydroxydecanoyl-CoA–ACP transacylase] based on pBHR86 or pBHR87 was confirmed by complementation of *phaG* mutants *P. aeruginosa* KO2 and *P. putida* $PhaG_N$-21 and establishment of the PhaG-mediated pathway in *P. oleovorans* (8, 14, 28). Recombinant cells were cultivated in MM plus 1.5% (wt/vol) sodium gluconate, and after 48 h of incubation at 30°C the PHA content of lyophilized cells was determined by GC analysis. PHA accumulation from gluconate indicated in vivo activity of PhaG.

Functional expression of Rh1G (β-ketoacyl reductase) gene. Functional expression of *rhlG* (encoding the β-ketoacyl reductase) based on pJC3 was confirmed by complementation of the *rhlG* mutant *P. aeruginosa* ACP5 (4). Recombinant cells were cultivated on PPGAS medium, and after 24 h of incubation at 37°C the rhamnolipid concentration in the cell supernatant was determined and indicated in vivo activity of β-ketoacyl reductase.

GC analysis of polyester and fatty acids in cells. PHAs and fatty acids were qualitatively and quantitatively analyzed by GC. Liquid cultures were centrifuged at 10,000 × *g* for 15 min, and then the cells were washed twice in saline and lyophilized overnight. Lyophilized cell material (8 to 10 mg) was subjected to methanolysis in the presence of 15% (vol/vol) sulfuric acid. The resulting methyl esters of the constituent 3-hydroxyalkanoic acids were assayed by GC according to the method of Brandl et al. (2) and as described in detail recently (40). GC analysis was performed by injecting 3 μl of sample into a Perkin-Elmer (Überlingen, Germany) 8420 gas chromatograph using a 0.5-μm-diameter Permphase PEG 25 Mx capillary column 60 m in length.

Analysis of rhamnolipids. The orcinol assay (5) was used to directly assess the amount of rhamnolipids in the sample: 333 μl of the culture supernatant was extracted twice with 1 ml of diethyl ether. The ether fractions were pooled and evaporated to dryness, and 0.5 ml of H_2O was added. To 100 μl of each sample 900 μl of a solution containing 0.19% orcinol (in 53% [vol/vol] H_2SO_4) was added; after being heated for 30 min at 80°C, the samples were cooled for 15 min at room temperature, and the A_{421} was measured. The concentration of rhamnolipids was calculated by comparing the data with those obtained with rhamnose standards between 0 and 50 μg/ml.

RESULTS

Analysis of various isogenic *P. aeruginosa* mutants with respect to PHA and rhamnolipid synthesis. Since precursors for PHA and rhamnolipid biosynthesis are derived from fatty acid de novo biosynthesis, various *fab* mutants of *P. aeruginosa* were analyzed. For PHA biosynthesis analysis cells were cultivated under PHA-accumulating conditions on MM containing 1.5% (wt/vol) sodium gluconate, 0.01% (vol/vol) oleate, and 0.05% (wt/vol) ammonium chloride. For rhamnolipid biosynthesis analyses cells were cultivated in PPGAS medium containing 0.5% (wt/vol) glucose as the carbon source. The *fabA* mutant PAO191 carries a Gm^r cassette in the *fabA* gene, which encodes β-hydroxyacyl-ACP dehydratase. The *fabB* mutant PAO192 was constructed by insertion of the Gm^r cassette into the *fabB* gene, which encodes β-ketoacyl-ACP synthase I (12). Moreover, the isogenic *fabI* mutant PAO235 was employed, which carries an insertionally inactivated *fabI* gene that encodes enoyl-ACP reductase (13). In addition to the various *fab* mutants, we used the isogenic *phaG* mutant KO2, which is impaired in PHA_{MCL} accumulation from nonrelated carbon sources (14), and the isogenic *rhlG* mutant ACP5, which is almost deficient of rhamnolipid production, i.e., it produced less than 1.3% of the rhamnolipid produced by the wild type (4). These mutants were studied with respect to their capability to produce PHA_{MCL} and rhamnolipids. The *fab* mutants were strongly impaired in PHA_{MCL} accumulation and rhamnolipid production, whereas the the *phaG* mutant showed only 40% of wild-type PHA_{MCL} accumulation and only a slightly decreased rhamnolipid production (Fig. 2 and 3). The *rhlG* mutant showed a decreased PHA_{MCL} accumulation and rhamnolipid production. Transfer of the *phaG* gene into the *fab* mutants showed a strong increase in PHA_{MCL} accumulation, whereas rhamnolipid biosynthesis was almost abolished (Fig. 2 and 3). In the *rhlG* mutant PHA_{MCL} accumulation was slightly decreased and no effect on rhamnolipid synthesis was observed when *phaG* was expressed (Fig. 2 and 3). Transfer of the *rhlG*

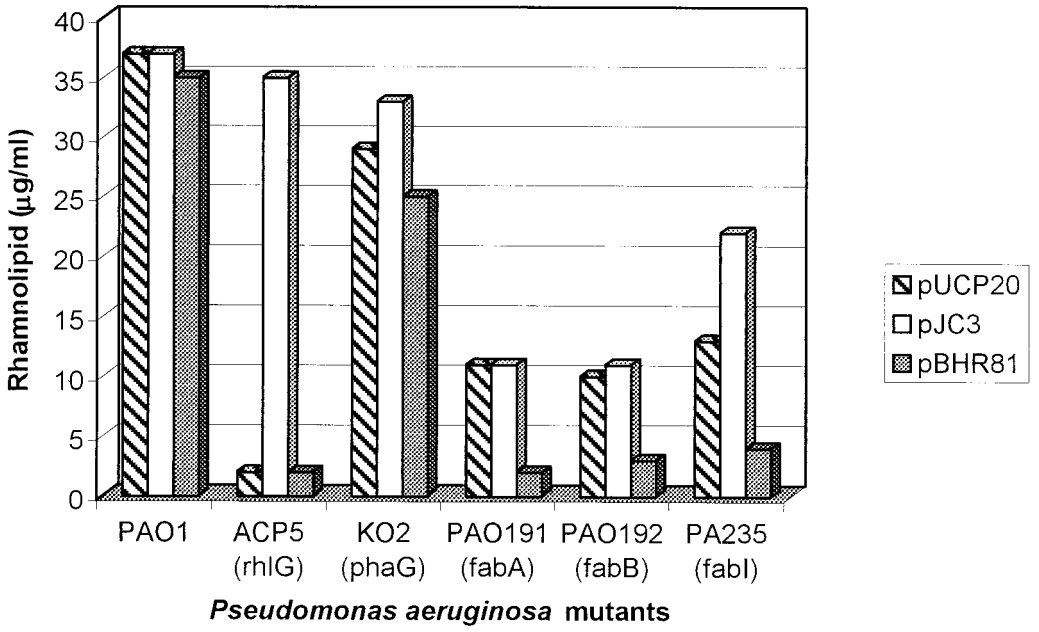

FIG. 3. Rhamnolipid production by mutants of *P. aeruginosa*. Rhamnolipid concentration is expressed as micrograms of rhamnose in rhamnolipids per milliliter of culture supernatant. The PPGAS medium contained 0.5% (wt/vol) glucose as a carbon source. The gene affected by each mutation is given in parentheses.

gene into these mutants mediated an increase of rhamnolipid production in mutants ACP5 (complementation of mutation), KO2, and PA235, whereas PHA_{MCL} accumulation was decreased in all mutants except in ACP5 (Fig. 2 and 3). In order to obtain evidence for the potential precursor of PHA_{MCL} synthesis, the PHA_{MCL} composition of all mutants was analyzed. GC analysis of the accumulated PHA showed that in the *phaG* knockout mutant KO2 the molar fraction of the 3-hydroxydecanoate (3HD) in PHA_{MCL} was decreased by approximately 20%, whereas in the *rhlG* mutant ACP5 this molar fraction was slightly increased (Table 2).

Generation of independent Tn*5* mutants of *P. putida* deficient in PHA_{MCL} accumulation. Since the transacylase PHA biosynthesis pathway was described in *Pseudomonas*, the only gene involved that has been identified so far is *phaG*, capable of restoring PHA_{MCL} biosynthesis in the *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine mutant $PhaG_N$-21 of *P. putida*, which was strongly impaired in PHA_{MCL} synthesis from nonrelated carbon sources. The establishment of this pathway in non-*Pseudomonas* species has not been achieved yet. In order to further analyze this possibility, we generated independent Tn*5* mutants of *P. putida*. For this, we constructed the suicide plasmid pMON5302, which enabled Tn*5*-mediated random insertion of a Gm^r cassette into the *P. putida* chromosome. After transfer of plasmid pMON5302 into *P. putida*, cells were screened on MM containing gluconate as the sole carbon source. Five nonopaque mutants (B349/Tn*5*-1 to -5) were isolated that were strongly impaired in PHA_{MCL} biosynthesis from nonrelated carbon sources but accumulated significantly higher levels of PHA_{MCL} from decanoate as the carbon source (data not shown). Transfer of plasmid pBHR81, which carries the $phaG_{Pp}$ gene under the *lac* promoter's control, into these Tn*5* mutants showed restoration of PHA_{MCL} accumulation from nonrelated carbon sources comparable to the level of PHA_{MCL} accumulation from decanoate as the carbon source (Fig. 4). Analysis of PHA_{MCL} composition by GC-mass spectrometry showed that the molar fraction of 3HD was decreased, whereas the molar fraction of 3-hydroxydodecanoate (3HDD) was increased in the Tn*5* mutants when compared with that in wild-type *P. putida* (Fig. 5). Functional expression of $phaG_{Pp}$ in these mutants again enhanced the molar fraction of 3HD and decreased the molar fraction of 3HDD of the accumulated PHA_{MCL}.

Establishment of transacylase-mediated PHA_{MCL} biosynthesis in recombinant *E. coli*. We recently reported the establishment of the transacylase-mediated PHA_{MCL} biosynthesis pathway in the non-PHA accumulating *P. fragi* by functional expression of $phaG_{Pp}$ plus $phaC1_{Pa}$ using plasmid pBHR86 (8). These data clearly demonstrated that diversion of intermedi-

TABLE 2. Accumulation of PHAs in various *P. aeruginosa* mutants from gluconate[a]

Strain	PHA content (% [wt/wt][b])	Composition of PHA (mol%)[c]				
		3HHx	3HO	3HD	3HDD	3HDD:1
PAO1	18.5	5	20	63	12	ND[d]
KO2 (*phaG*)[e]	7	4	32	50	14	ND
ACP5 (*rhlG*)[e]	9	6	20	70	4	ND
PAO191 (*fabA*)[e]	3	5	13	65	17	ND
PAO192 (*fabB*)[e]	3.5	2	18	66	16	ND
PA235 (*fabI*)[e]	7	5	24	63	8	ND

[a] Cultivations were performed under PHA-accumulating conditions on MM containing 1.5% (wt/vol) sodium gluconate and 0.05% (wt/vol) ammonium chloride. Cells were grown for 48 h at 30°C. PHA content and composition of comonomers were analyzed by GC.
[b] Dry weight.
[c] Abbreviations: 3HHx, 3-hydroxy-hexanoate; 3HO, 3-hydroxyoctanoate; 3HDD:1, 3-hydroxydodecenoate.
[d] ND, not detectable.
[e] Gene affected by insertional inactivation.

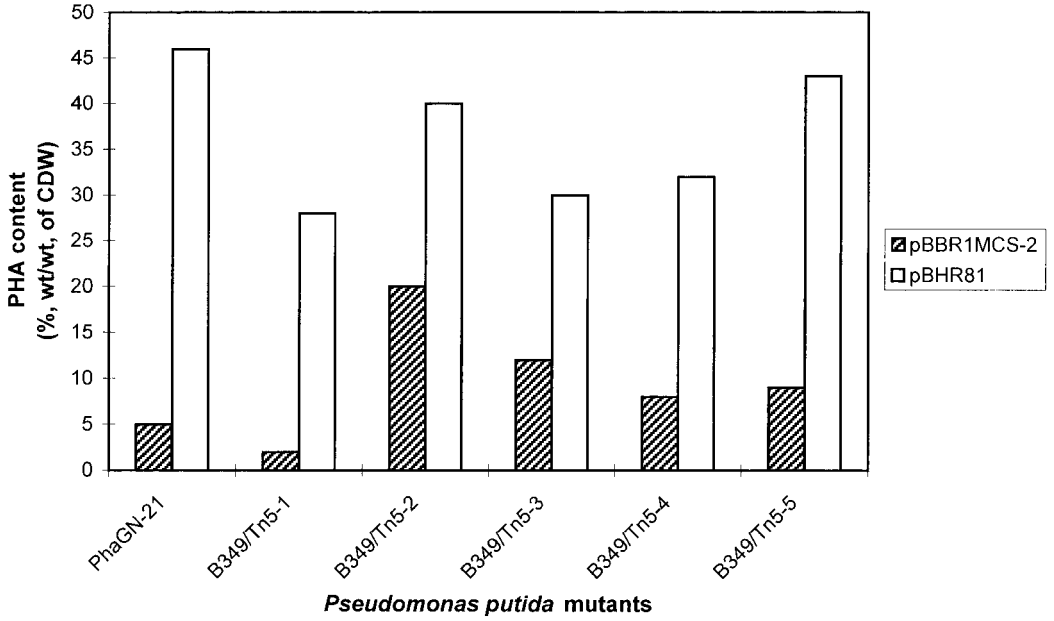

FIG. 4. PHA_{MCL} accumulation by Tn*5* mutants of *P. putida*. Cultivations were performed under PHA-accumulating conditions on MM containing 1.5% (wt/vol) sodium gluconate and 0.05% (wt/vol) ammonium chloride. Cells were grown for 48 h at 30°C. PHA content and composition of comonomers were analyzed by GC. Plasmid pBHR81 enables functional expression of the $phaG_{Pp}$ gene. CDW, cellular dry weight.

ates from fatty acid β-oxidation is not required to establish the transacylase-mediated pathway. Therefore, we introduced plasmid pBHR86, which contains $phaCl_{Pa}$ and $phaG_{Pp}$ collinear to the *lac* promoter with $phaG_{Pp}$ still preceded by its native promoter, into *E. coli* JM109. Cells were cultivated either in LB medium or M9 medium containing gluconate as the sole carbon source. No PHA_{MCL} accumulation was observed. To avoid transcriptional deficiency of $phaG_{Pp}$ in *E. coli* due to its native promoter, we constructed plasmid pBHR87, containing the $phaG_{Pp}$ gene without its native promoter. However, plasmid pBHR87 did not mediate PHA_{MCL} biosynthesis in *E. coli* JM109. Since (*R*)-3-hydroxyacyl-ACP, an intermediate of fatty acid de novo biosynthesis and substrate for the transacylase PhaG, has to be available for PHA_{MCL} biosynthesis from nonrelated carbon sources, we employed two *E. coli fab* mutants, which might contain higher levels of (*R*)-3-hydroxyacyl-ACP. The *fabA* mutant *E. coli* DC170 and the *fabI* mutant *E. coli* IP1111 were used to establish the transacylase-mediated pathway. However, transfer of plasmids pBHR86 and pBHR87 into each of the mutants, respectively, alone did not mediate PHA_{MCL} accumulation. Therefore, we used inhibitors of fatty acid de novo biosynthesis in order to generate an intermediate pool, which might favor provision of the substrate for the transacylase. We employed cerulenin, which specifically inhibits FabB (β-ketoacyl-ACP synthase I) and FabF (β-ketoacyl-ACP synthase II), which catalyze the condensation of malonyl-ACP with acyl-ACP (7). The application of the inhibitor cerulenin did not show any effect on PHA_{MCL} synthesis from nonrelated carbon sources in recombinant *E. coli* S17-1. However, application of triclosan, which specifically inhibits the enoyl-ACP reductase (11), led to PHA_{MCL} accumulation contributing to about 2 to 3% of cellular dry weight in recombinant *E. coli* harboring either pBHR86 or pBHR87, when grown on LB medium plus gluconate as the carbon source (Table 3).

DISCUSSION

In this study, we evaluated the role of fatty acid de novo biosynthesis for PHA_{MCL} synthesis and rhamnolipid production, particularly considering the role of the linking enzymes PhaG, transacylase, and Rh1G, β-ketoacyl reductase. Analysis of PHA_{MCL} synthesis and rhamnolipid production in various isogenic *fab* mutants of *P. aeruginosa*, impaired in fatty acid de novo biosynthesis, strongly suggested that precursors for

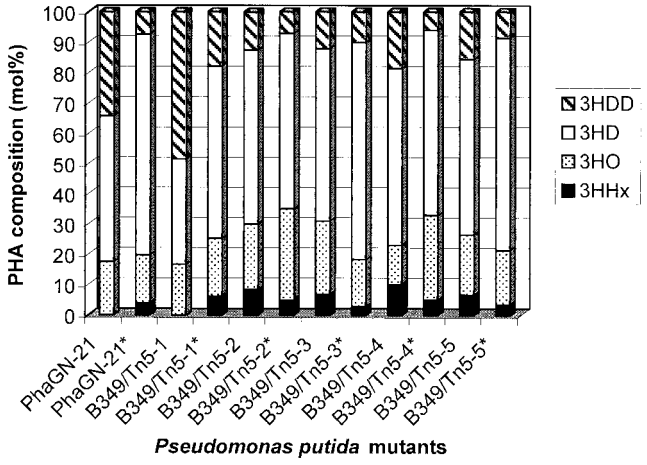

FIG. 5. Composition of PHA_{MCL} accumulated by Tn*5* mutants of *P. putida*. Cultivations were performed under PHA-accumulating conditions on MM containing 1.5% (wt/vol) sodium gluconate and 0.05% (wt/vol) ammonium chloride. Cells were grown for 48 h at 30°C. PHA content and composition of comonomers were analyzed by GC. 3HHx, 3-hydroxy-hexanoate; 3HO, 3-hydroxyoctanoate. An asterisk indicates that cells harbor plasmid pBHR81. Plasmid pBHR81 enables functional expression of the $phaG_{Pp}$ gene.

TABLE 3. Analysis of recombinant *E. coli* S17-1 harboring plasmids with respect to PHA accumulation[a]

E. coli-borne plasmid	Composition of PHA (% dry wt)[b]			
	3HB	3HO	3HD	3HDD
pBBR1MCS-1	ND	ND	ND	ND
pBHR86	ND	ND	3.3	ND
pBHR87	ND	ND	1.5	ND

[a] Recombinant *E. coli* was cultivated in LB medium containing 1.5% (wt/vol) gluconate, 1 mM isopropyl-β-D-thiogalactopyranoside and kanamycin (50 μg/ml). After 24 h of cultivation at 37°C, triclosan was added to the medium at a final concentration of 0.1 μg/ml, and cells were harvested after a further incubation of 24 h.

[b] Abbreviations: 3HB, 3-hydroxybutyrate; 3HDD, 3-hydroxydodecanoate.

PHA_{MCL} biosynthesis and rhamnolipid biosynthesis are provided via fatty acid de novo biosynthesis. This is consistent with the observation that the PHA_{MCL} biosynthesis transacylase PhaG and the rhamnolipid biosynthesis β-ketoacyl reductase Rh1G use intermediates of fatty acid de novo biosynthesis as the substrate, which are converted by the respective enzyme to a direct precursor of PHA_{MCL} or rhamnolipid biosynthesis, respectively (4, 28). It is still not clear why the *rhlG* mutant ACP5 is strongly impaired in PHA_{MCL} biosynthesis and why the *phaG* mutant KO2 showed a slightly decreased rhamnolipid production. Functional expression of $phaG_{Pp}$ in the *fab* mutants enhanced carbon flux towards PHA_{MCL} biosynthesis, whereas rhamnolipid production was almost abolished, which indicated that PhaG catalyzes conversion of a molecule that plays a role in rhamnolipid biosynthesis. Interestingly, expression of $phaG_{Pp}$ in *P. aeruginosa* KO2 (*phaG* mutant) did not abolish rhamnolipid biosynthesis, suggesting that the original genomic *phaG* gene is required in addition to $phaG_{Pp}$ gene copies, provided by plasmid pBHR81, for efficient diversion of intermediates towards PHA biosynthesis. The finding that expression of $rhlG_{Pa}$ decreased PHA_{MCL} accumulation in all mutants, except the ACP5 mutants, indicated that PHA_{MCL} biosynthesis and rhamnolipid biosynthesis interfere with each other, presumably by competing for intermediates. Mutant ACP5 (*rhlG*) harboring plasmid pJC3 ($rhlG_{Pa}$) might not exhibit the same phenotype, because of the missing genomic $rhlG_{Pa}$ gene.

Five independent Tn*5* mutants of *P. putida* which are deficient in PHA_{MCL} accumulation from nonrelated carbon sources were all at least to some extent complemented by constitutive expression of *phaG*, which indicated that PhaG is the only key enzyme linking fatty acid de novo biosynthesis with PHA_{MCL} biosynthesis. The compositional analysis of the PHA_{MCL} accumulated by the Tn*5* mutants suggested that PhaG contributed to PHA_{MCL} biosynthesis by strong provision of 3-hydroxydecanoyl–CoA, presumably reflecting the substrate specificity of PhaG. Since the transacylase PhaG seems to be the only required enzyme for PHA_{MCL} biosynthesis from nonrelated carbon sources, and since fatty acid de novo biosynthesis plays a crucial role in the provision of precursors for PHA_{MCL} biosynthesis from nonrelated carbon sources, we employed *fab* mutants of *E. coli* as well as specific inhibitors of fatty acid de novo biosynthesis in order to establish the transacylase-mediated route in recombinant *E. coli*. However, only the application of triclosan, a specific inhibitor of the enoyl-ACP reductase FabI, enabled weak PHA_{MCL} accumulation from nonrelated carbon sources in *E. coli* when $phaG_{Pp}$ and $phaCl_{Pa}$ were functionally expressed. Overall, this study indicates that carbon flux through the fatty acid de novo biosynthesis, i.e., the pool of intermediates, is crucial for PHA_{MCL} biosynthesis as well as rhamnolipid production and that fatty acid de novo biosynthesis in pseudomonads is different from that in *E. coli*.

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