JOURNAL OF BACTERIOLOGY, May 2000, p. 2978–2981 Vol. 182, No. 10
0021-9193/00/$04.00+0

FabG, an NADPH-Dependent 3-Ketoacyl Reductase of *Pseudomonas aeruginosa*, Provides Precursors for Medium-Chain-Length Poly-3-Hydroxyalkanoate Biosynthesis in *Escherichia coli*

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Received 22 October 1999/Accepted 3 February 2000

Escherichia coli **hosts expressing** ***fabG*** **of** ***Pseudomonas aeruginosa*** **showed 3-ketoacyl coenzyme A (CoA) reductase activity toward** ***R*****-3-hydroxyoctanoyl-CoA. Furthermore,** ***E. coli*** **recombinants carrying the poly-3-hydroxyalkanoate (PHA) polymerase-encoding gene** ***phaC*** **in addition to** ***fabG*** **accumulated medium-chain-length PHAs (mcl-PHAs) from alkanoates. When** ***E. coli fadB*** **or** ***fadA*** **mutants, which are deficient in steps downstream or upstream of the 3-ketoacyl-CoA formation step during β-oxidation, respectively, were transformed with** ***fabG*****, higher levels of PHA were synthesized in** ***E. coli fadA*****, whereas similar levels of PHA were found in** ***E. coli fadB*****, compared with those of the corresponding mutants carrying** ***phaC*** **alone. These results strongly suggest that FabG of** ***P. aeruginosa*** **is able to reduce mcl-3-ketoacyl-CoAs generated by the β-oxidation to 3-hydroxyacyl-CoAs to provide precursors for the PHA polymerase.**

Bacteria have developed several approaches to store carbon and energy in the form of polysaccharides, polyamino acids, and polyesters (17). One well-known polyester is poly-3-hydroxyalkanoate (PHA), a bioplastic material. It is known that PHAs are synthesized by polymerization of coenzyme A (CoA)-linked *R*-3-hydroxy fatty acids through a PHA polymerase (PhaC) (for a recent review, see reference 10). The synthesis of such CoA substrates can occur by a variety of pathways (see review in reference 10), the simplest of which uses a β-ketothiolase (PhbA) and an NADPH-dependent acetoacetyl-CoA reductase (PhbB) to synthesize precursors for short-chain-length PHAs such as poly-3-hydroxybutyrate (PHB) in *Ralstonia eutropha*. Precursors for so-called medium-chain-length PHAs (mcl-PHAs) can be generated through fatty acid β-oxidation, which produces acyl-CoA intermediates such as enoyl-CoA, 3-ketoacyl-CoA, and/or *S*-3-hydroxyacyl-CoA when fatty acids are used as the sole carbon sources (see review in reference 10). Recently, Campos-Garcia and coworkers have cloned and characterized an NADPH-dependent reductase (RhlG)-encoding gene from *Pseudomonas aeruginosa* (2). It was demonstrated that RhlG is involved in rhamnolipid and PHA synthesis, presumably converting 3-ketoacyl esters to 3-hydroxyacyl esters (2). However, their results also suggested that reductases other than RhlG might be present in *P. aeruginosa*. This prompted us to investigate the existence of other reductases that might be specifically involved in mcl-PHA synthesis. Since *Escherichia coli* with a complete and not inhibited β-oxidation cycle is unable to synthesize mcl-PHAs, even when equipped with a PHA polymerase-encoding gene of *Pseudomonas* (9, 12, 13), we chose *E. coli* as a model system to study the role of ketoacyl reductases from *P. aeruginosa* in mcl-PHA production. While the present paper was being reviewed, *E. coli* NADPH-dependent 3-ketoacyl reductase (FabG), which catalyzes ketoacyl-ACP to 3-hydroxyacyl-ACP during fatty acid synthesis and has been well studied (3), was overproduced in *E. coli* recombinants carrying *phaC*, and PHA accumulation was observed in these recombinants (18). In this study, we demonstrated that *P. aeruginosa* FabG, the sequence of which has been determined, but the enzymatic properties and functions of which have not been addressed experimentally, can be involved in mcl-PHA synthesis.

Cloning of a ketoacyl reductase-encoding gene of *P. aeruginosa* PAO1. To investigate whether a specific mcl-ketoacyl-CoA reductase which is exclusively involved in mcl-PHA synthesis is present in *P. aeruginosa*, the genomic DNA sequence of *P. aeruginosa* PAO1 (www.pseudomonas.com [release of March 1999]) was searched for homologous regions to the acetoacetyl-CoA reductase-encoding genes from *Alcaligenes* sp. strain SH-69 (accession no. AF002014), *Acinetobacter* sp. strain RA3849 (accession no. L37761), *Paracoccus denitrificans* (accession no. D49362), *Chromatium vinosum* D (accession no. A27012), *Ralstonia eutropha* H16 (accession no. J04987) and *Rhizobium meliloti* 41 (accession no. U17226). One fragment located in contig 50 of the Pseudomonas Genome Project was found to have the highest homology to the known acetoacetyl-CoA reductase-encoding genes. Sequence analysis revealed that this fragment contains the *fabG* gene (GenBank database accession no. U91631), which was recently identified as part of a *fabD-fabG-acpP-fabF* gene cluster and encodes the 3-ketoacyl-acyl carrier protein (ACP) reductase (7). The deduced amino acid sequences of FabG showed an overall 30 to 35% identity to the known acetoacetyl-CoA reductases and 32 to 55% identity to FabG enzymes from other origins. The entire *fabG* gene was subsequently cloned from PAO1 by using the PCR primers 5′-TGGCTCGAGAGAGAGAAAGGAGA-3′ and 3′-CAGATCTTAAGCAACGCCTT-5′. PCR amplifications using total *P. aeruginosa* PAO1 DNA, *Taq* DNA polymerase (Promega), and a Perkin-Elmer GeneAmp PCR System 9600 were carried out. After restriction with *Xho*I and *Eco*RI, the PCR product was cloned into *Sal*I- and *Eco*RI-digested pUC19 to generate pET201. Since pET201 was found unstable in *E. coli* recombinants (data not shown), plasmid

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TABLE 1. Accumulation of PHA in recombinant *E. coli* strains carrying pET200 and pBTC2[a]

Strain	Plasmid(s)	Gene (relevant markers)	PHA content (% cell dry wt)	Composition (mol%)[b]			M_w
				3HHx	3HO	3HD	
MF4100	pBTC2	*phaC*$^+$	—[c]	—			—
	pBTC2, pET200	*phaC*$^+$*fabG*$^+$	2.8	10	90	0	48,200
RS3338	pBTC2	*phaC*$^+$	—	—			—
	pBTC2, pET200	*phaC*$^+$*fabG*$^+$	3.1	9	91	0	51,100
JMU193	pBTC2	*phaC*$^+$	4.6	11	76	13	56,400
	pBTC2, pET200	*phaC*$^+$*fabG*$^+$	5.2	10	77	13	52,700
JMU194	pBTC2	*phaC*$^+$	14.2	20	67	13	68,300
	pBTC2, pET200	*phaC*$^+$*fabG*$^+$	20.6	23	65	12	67,000

[a] *E. coli* recombinants were cultivated as described in the text. Determination of the PHA content and the PHA molecular weight was carried out as described previously (8, 11). Average data of three independent experiments are given.
[b] 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate.
[c] —, <0.1%.

pET200 was constructed as follows. pET201 was digested with *Eco*RI and *Hin*dIII. The 750-bp *fabG*-containing fragment obtained was then inserted into pBCKS (Promega) to get pET202, which was further cut with *Bam*HI and *Kpn*I, and the *fabG*-containing fragment was then ligated into pCK01 (4), resulting in pET200.

Ketoacyl-CoA reductase activity of FabG in recombinant *E. coli*. In order to determine whether the cloned *fabG* gene product of *P. aeruginosa* is active toward CoA esters, thus providing a substrate for the PHA polymerase, pET200 was transformed into *E. coli* hosts MF4100 (*fadR*) (derived from MC4100 [Biolabs]), RS3338 (*fadR fadL*; B. J. Bachman), JMU193 (*fadR fadB*) (15), and JMU194 (*fadR fadA*) (15). The *fadR* gene encodes a protein that exerts negative control over genes necessary for fatty acid oxidation (see review in reference 1). A mutation in *fadR* derepresses transcription of these genes, as a result of which the *fad* genes are constitutively expressed, rendering *E. coli* capable of growth on mcl fatty acids (1). Mutations in *fadA* or *fadB* block fatty acid oxidation and result in accumulation of specific acyl-CoA intermediates (15). *E. coli* hosts carrying pET200 were then analyzed for ketoacyl-CoA reductase activity. In analogy to the previously reported acetoacetyl-CoA reductase assay using different-chain-length 3-hydroxyacyl-CoA substrates (5), the FabG reductase was assayed with glycine-NaOH buffer (50 μmol [pH 9.0]), $NADP^+$ (100 nmol), and 3-hydroxyoctanoyl-CoA (100 nmol), which was prepared according to the method described previously (6). The reaction was initiated with 3-hydroxyoctanoyl-CoA at 30°C. 3-Ketoacyl-CoA reductase activity was monitored by examining A_{340} due to the reduction of $NADP^+$ during oxidation of 3-hydroxyoctanoyl-CoA (5).

E. coli MF4100, RS3338, JMU193, and JMU194 carrying pET200 exhibited a relatively high ketoacyl-CoA reductase activity (from 50 to 90 mU/mg of total protein), whereas the control strains harboring vector pCK01 showed only a background reductase activity (10 to 20 mU/mg of protein), which is probably caused by the FabG of *E. coli*. These results strongly indicate that FabG of *P. aeruginosa* is able to use CoA esters as substrates.

mcl-PHA synthesis in *E. coli* recombinants expressing *phaC* and *fabG* of *Pseudomonas*. To synthesize mcl-PHA in recombinant *E. coli*, plasmid pBTC2 (14), which contains *phaC2* of *Pseudomonas oleovorans* Gpo1, was used. MF4100 and RS3338 carrying pBTC2 and/or pET200 were cultivated in M9 minimal medium (16) with 10 mM sodium octanoate as a carbon source. For growth of the *fadB*- or *fadA*-negative *E. coli* strains JMU193 and JMU194 carrying pBTC2 and/or pET200, 0.2% (wt/vol) yeast extract was used as a carbon source and 2 mM hexadecanoate was added as a cosubstrate for PHA formation and growth. Cell cultures were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in the early exponential growth phase and harvested in the stationary phase. PHA accumulation and PHA composition were analyzed as previously described (8). No detectable PHA (less than 0.1% PHA of cell dry weight) was found in either *E. coli* MF4100 or RS3338 harboring *phaC* only (Table 1). Addition of *fabG* in both *E. coli* recombinants resulted in about 3% PHA with a composition similar to that found in wild-type *Pseudomonas* strains (8) (Table 1). Therefore, we concluded that *fabG* of *P. aeruginosa* facilitated PHA production in these strains, supporting the notion that FabG channels β-oxidation intermediates, probably 3-ketoacyl-CoAs, to the PHA polymerase in *E. coli fadR* recombinants (Fig. 1B versus A). To further verify this hypothesis, *E. coli* mutants JMU193 and JMU194, which are deficient in β-oxidation steps downstream and upstream of the formation of 3-ketoacyl-CoA, respectively, were further investigated for PHA production when carrying the *phaC* and/or *fabG* gene (Fig. 1C to F). Without the *fabG* gene, JMU193 and JMU194 carrying *phaC* (Fig. 1C and E) accumulated PHA to levels of about 5 and 14%, respectively (Table 1). Addition of *fabG* in JMU193 carrying *phaC* did not cause significant changes in PHA content or composition (Table 1). Introduction of *fabG* to recombinant JMU194 carrying *phaC* resulted in increased PHA production to 20%, however, confirming that FabG might be capable of converting 3-ketoacyl-CoAs to 3-hydroxyacyl-CoAs (Fig. 1F). To prove that the 3-hydroxyalkanoate compounds obtained are not due to accumulated monomers, we isolated and determined the M_w of the polymers described above as detailed in reference 11. The M_w of the polymers isolated from the recombinants was significantly lower (Table 1) than the M_w of the polymer produced by the wild-type organisms (11). In all *E. coli* recombinants tested, PHA was never found in cells harboring the ketoacyl reductase-encoding *fabG* gene without *phaC*.

Summary. Since *P. aeruginosa* may have several reductases which are involved in PHA synthesis (2), attempts to knock out *fabG* in *P. aeruginosa* have failed (Q. Ren et al., unpublished data), and deletion of *fabG* in *E. coli* was lethal to the cells (19), we chose a heterologous system in *E. coli* to study the function of *P. aeruginosa* FabG and its involvement in PHA production.

Our data confirmed the results obtained in parallel by Tagu-

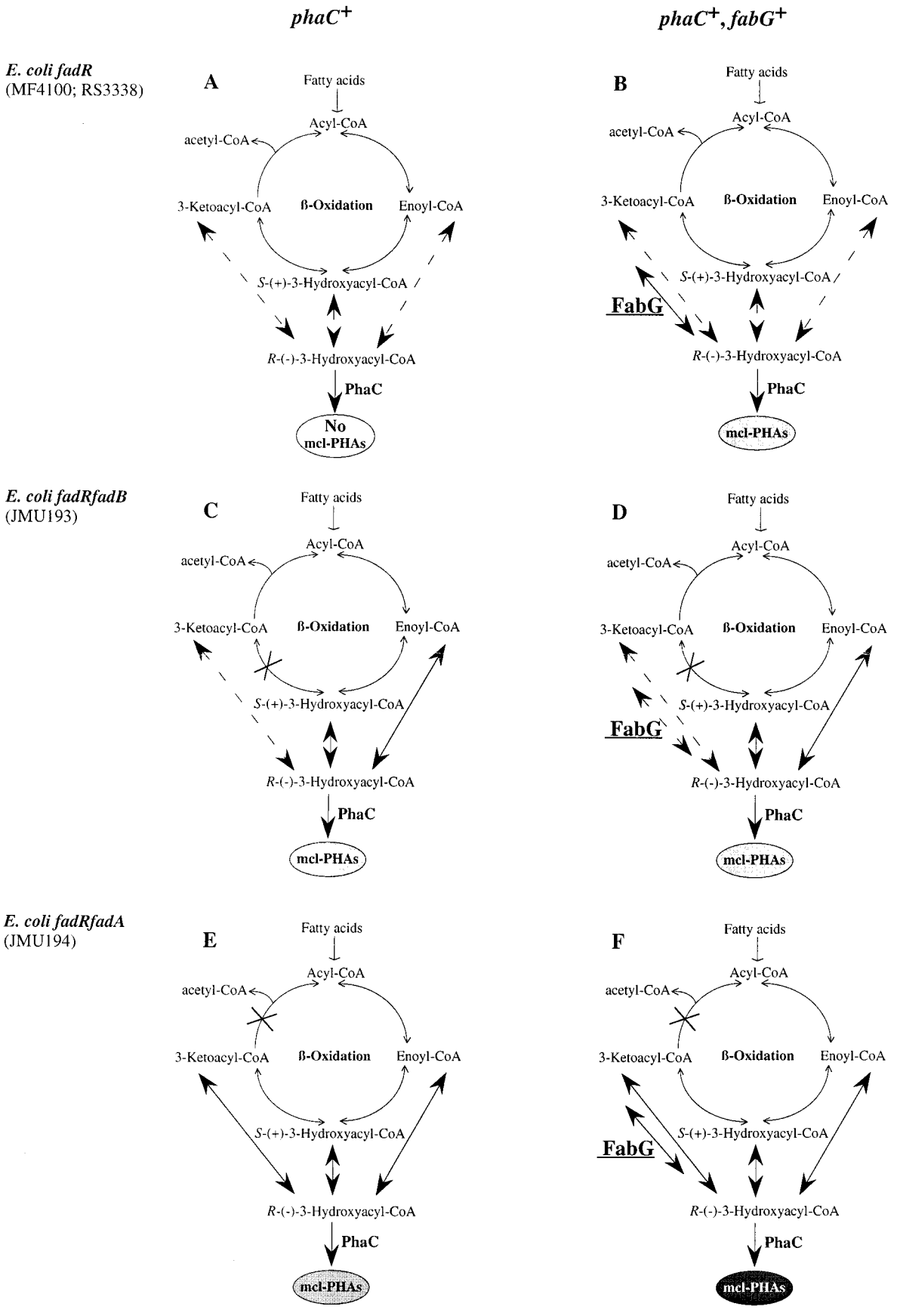

FIG. 1. Possible pathways for PHA synthesis in recombinant *E. coli* from alkanoates via β-oxidation cycle. Dashed lines indicate pathways or reactions that are theoretically possible, but the precursor concentrations might be too low to permit the reaction to take place. Crosses indicate a blocking of the enzymatic reaction. Amounts of PHA are indicated by the density of shading: higher intensity corresponds to larger amounts of PHA.

chi et al. (18), i.e., reduction of 3-ketoacyl-CoA is a channeling pathway for supplying 3-hydroxyacyl-CoA monomer units for PHA synthesis through fatty acid degradation and established a role for FabG in PHA synthesis. In addition to the in vivo studies and the in vitro NADPH-dependent reductase activity toward acetoacetyl-CoA determined for *E. coli* FabG (18), we could demonstrate in vivo and in vitro that FabG of *P. aeruginosa* has activity toward mcl-ketoacyl-CoA substrates. Furthermore, we proved that a polymer was produced, albeit, with low molecular weight. According to our data presented in this paper combined with data from previous research (9, 13), we propose the following model for mcl-PHA synthesis in *E. coli* (Fig. 1). In *E. coli fadR* hosts, acyl-CoAs of different chain lengths that are derived from alkanoates are degraded via the β-oxidation cycle, resulting in the formation of ketoacyl-CoA intermediates of different chain lengths. These intermediates are converted to *R*-3-hydroxyacyl-CoAs by an *R*-specific ketoacyl-CoA reductase encoded by *fabG*, and the resultant *R*-3-hydroxyacyl-CoAs with 6 to 10 carbon atoms are incorporated into a growing polyester chain by PHA polymerase. In the *fadB* mutant JMU193, blocking of the 3-hydroxyacyl-CoA dehydrogenase (Fig. 1C) reduces the available ketoacyl-CoA level (13). As a consequence, introduction of *fabG* (Fig. 1D) did not significantly increase the amount of PHA formed. In contrast, blocking of the ketoacyl-CoA thiolase in the *fadA* mutant JMU194 increased the available ketoacyl-CoA level (Fig. 1E) (13), resulting in an increased amount of PHA after introduction of *fabG* (Fig. 1F and Table 1). The substrate range of FabG of *P. aeruginosa* is not known yet. Since this enzyme is likely involved in fatty acid synthesis, it might process substrates with carbon chain length up to C_{18} as well as ACP-coupled substrates. If so, this explains why no significant changes of monomer composition could be detected in any of the *E. coli* recombinants tested (Table 1).

Although we could demonstrate that FabG of *P. aeruginosa* is capable of providing precursors for mcl-PHA in recombinant *E. coli*, we cannot rule out the possibility that another 3-ketoacyl-CoA reductase is present in *P. aeruginosa* that is exclusively responsible for mcl-PHA production in the native strain. This deserves further investigation.

We thank M. Röthlisberger for DNA sequencing, G. Frank for N-terminal sequencing of the protein, D. Dennis for providing strains JMU193 and JMU194, and M. A. Prieto for providing strain MF4100.

This work was supported by grants from the Swiss Federal Office for Education and Science (BBW no. 96.0348) to Q.R.

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