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SHORT CONTRIBUTION

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Heterologous expression of the acyl–acyl carrier protein thioesterase gene from the plant *Umbellularia californica* mediates polyhydroxyalkanoate biosynthesis in recombinant *Escherichia coli*

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Abstract The acyl–acyl carrier protein (ACP) thioesterase cDNA from the plant *Umbellularia californica* was functionally expressed in various recombinant *Escherichia coli* strains in order to establish a new metabolic route toward medium-chain-length polyhydroxyalkanoate (PHA_{MCL}) biosynthesis from non-related carbon sources. Coexpression of the PHA synthase genes from *Ralstonia eutropha* and *Pseudomonas aeruginosa*, or only the PHA synthase gene from *P. aeruginosa*, respectively, showed PHA_{MCL} accumulation when the type II PHA synthase from *P. aeruginosa* was produced. Both wild-type *E. coli* and various *fad* mutants were investigated; and only when the β-oxidation pathway was impaired PHA_{MCL} accumulation from gluconate was observed, contributing to about 6% of cellular dry weight. Thus coexpression of type II PHA synthase gene with cDNA encoding the medium-chain acyl-ACP thioesterase from *U. californica* established a new PHA_{MCL} biosynthesis pathway, connecting fatty acid de novo biosynthesis with fatty acid β-oxidation, using a non-related carbon source.

Introduction

The composition of the energy storage compound polyhydroxyalkanoate (PHA) depends on the PHA synthases, the carbon source and the metabolic routes involved (Madison and Huisman 1999; Rehm and Steinbüchel 1999). Various metabolic pathways have been described for the biosynthesis of PHA_{MCL} (composed of medium-chain-length 3-hydroxy fatty acids ranging over 6–14 carbon atoms). The fatty acid de novo biosynthesis is the main route during growth on carbon sources, like gluconate, acetate or ethanol, which are metabolized to acetyl-CoA (Huijberts et al. 1994; Rehm et al. 1998). The transacylase PhaG from *Pseudomonas putida*, which catalyses the transfer of the (*R*)-3-hydroxydecanoyl moiety from the ACP thioester to CoA (Rehm et al. 1998) has been identified and characterized. Thus, PhaG directly links fatty acid de novo biosynthesis with PHA biosynthesis. This metabolic pathway was recently established in the non-PHA-accumulating bacterium, *P. fragi*, which functionally expresses the *phaC1* gene from *P. aeruginosa* and the *phaG* gene from *P. putida* (Fiedler et al. 2000). *phaG* genes have also been identified in two other *Pseudomonas* species, which indicates that the pathway represents a general principle (Hoffmann et al. 2000a, b). Recombinant PHA_{MCL} synthesis was first obtained in recombinant *Escherichia coli*, when β-oxidation mutants LS1298 (*fadB*) or RS3097 (*fadR*) expressing PHA synthase genes from *P. aeruginosa* were applied. This indicated that the β-oxidation pathway in *E. coli* provides precursors for PHA_{MCL} synthesis (Langenbach et al. 1997; Qi et al. 1997, 1998). The substrate for PHA_{MCL} synthases (type II) is (*R*)-3-hydroxyacyl-CoA in pseudomonads; and this was confirmed when purified PHA_{MCL} synthases from *P. aeruginosa* exhibited in vitro enzyme activity with (*R*)-3-hydroxydecanoyl-CoA as substrate (Qi et al. 2000).

Coexpression of the cytosolic thioesterase I gene *tesA*, which encodes an acyl-CoA thioesterase, with a PHA synthase encoding gene in *E. coli* (*fadB*, *fadR*) causes synthesis of PHA mainly composed of 3-hydroxyoctanoate, using gluconate as carbon source (Klinke et al. 1999). These data suggest that both the fatty acid de novo synthesis and the β-oxidation pathway are involved. However, only a low accumulation (up to 2.3% of cellular dry weight; CDW) was obtained. Interestingly, *tesA* has a strong preference for acyl-CoA thioesters (>12 carbon atoms, preferring C16 and C18), which makes this enzyme less favourable for the release of free fatty acids from the acyl-ACP intermediates of fatty acid de novo biosynthesis (Spencer et al. 1978). In this study for the first time, we used an acyl-ACP thioesterase which strongly prefers the lauroyl-ACP thioester

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instead of the corresponding CoA thioester, in order to efficiently establish a new metabolic link between PHA_{MCL} synthesis and fatty acid de novo biosynthesis (Davies and Pollard 1994).

Materials and methods

Bacterial strains, plasmids and growth of bacteria

The *E. coli* strains and plasmids used in this study are described below. *E. coli* was grown at 37 °C in complex Luria-Bertani (LB) medium containing 1.5% sodium gluconate and, if required, the appropriate antibiotic as indicated.

Isolation, analysis and manipulation of DNA

DNA sequences of new plasmid constructs were confirmed by DNA sequencing, employing the chain termination method and using the automatic sequencer LI-COR model 4000L (MWG-Biotech, Ebersberg, Germany). All other genetic techniques were performed as described by Sambrook et al. (1989).

Plasmid construction

PCR was employed to amplify a DNA fragment (about 1.3 kb) which encoded a N terminal *Lac*Z′ fusion protein of the acyl-ACP thioesterase from *Umbellularia californica*. Plasmid pCGN3823 served as template (Voelker and Davies 1994) and the following primers were used: 5′-CGCGGATCCTCTTGCTACTGCTACTACTAC-3′ and 5′-CGCGGATCCAAATGACTACTTCGATAACCTTGAC-3′. These tailored primers introduced a *Bam*HI site at either end of the PCR product, which was subsequently subcloned into the restriction enzyme site *Bam*HI of vector pBBR1MCS-2. This has been modified by the insertion of a tetracycline cassette into the *Xba*I site (Kovach et al. 1995; Hoffmann et al. 2000a), resulting in plasmid pBHR85. The insert orientation was analysed by *Eco*RI digestion and DNA sequencing.

Functional expression of the acyl-ACP thioesterase gene

In vivo thioesterase activity was confirmed by expression of the thioesterase gene in various metabolic backgrounds favouring the accumulation of fatty acids (such as *E. coli* RS3097) in the presence of either the β-oxidation inhibitor acrylic acid (0.24 mg ml^{-1}) or the *fadB* mutant LS1298. Recombinant bacteria harbouring plasmid pBHR85 were cultivated in the presence of 1.5% (w/v) sodium gluconate. Fatty acid accumulation was determined by GC analysis of lyophilized cells; and this indicated in vivo thioesterase activity.

Enzymatic thioesterase assay

Recombinant *E. coli* cells harbouring either vector pBBR1MCS-2-tet or pBHR85 were disrupted by French press in 7 mM KH_2PO_4–KOH, pH 8, containing 20% glycerol, 1 mM dithiothreitol and 0.1% Triton X100. The same buffer was applied for the assay, but it additionally contained 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid; DTNB), crude extract with 0.2 mg ml^{-1} total protein and 0.5 mM lauroyl-CoA as substrate, which was synthesized as described previously (Rehm et al. 1998). The total volume of the reaction mixture was 500 µl and all experiments were performed at 30 °C. Enzyme activity was spectrophotometrically monitored by an increase of absorption of reduced DTNB at 412 nm, exhibiting an extinction coefficient of 13.6 cm^2 μmol^{-1} (Ellman 1959). Three independent measurements were performed in order to provide a mean value of enzymatic activity. The enzyme activitiy of one unit corresponded to the amount of enzyme required to hydrolyse 1 µmol substrate min^{-1}.

Gas chromatographic analysis of polyester and fatty acids in cells

PHA and fatty acids in cells were qualitatively and quantitatively analysed by GC as described previously (Brandl et al 1988).

Results

Construction of plasmid pBHR85 for stabilized expression of the acyl-ACP thioesterase gene from *U. californica* in recombinant *E. coli*

Because the multicopy plasmid pCGN3823, which was previously constructed by Voelker and Davies (1994), showed a toxic effect on the investigated *E. coli* strains, i.e. growth and plasmid stability were strongly impaired, the coding region of the thioesterase gene from the Californian bay plant, *U. californica*, was subcloned in the medium copy number vector pBBR1MCS-2-tet. This vector was compatible with plasmids containing the ColE1 or p15A origins of replication. Subcloning was performed as described in detail in Materials and methods, resulting in plasmid pBHR85. In order to monitor the functional expression of the thioesterase gene, plasmid pBHR85 was transferred into *E. coli* LS1298 (*fadB*) and *E. coli* RS3097 (*fadR*; DiRusso 1990; Simon et al. 1980). Cultivation in LB medium containing 1.5% (w/v) sodium gluconate resulted in the accumulation of laurate (12:0), contributing to about 4% of the CDW (Fig.1). Furthermore, strain RS3097 harbouring plasmid pBHR85 only showed an accumulation of laurate (C12:0) when the β-oxidation pathway was inhibited by

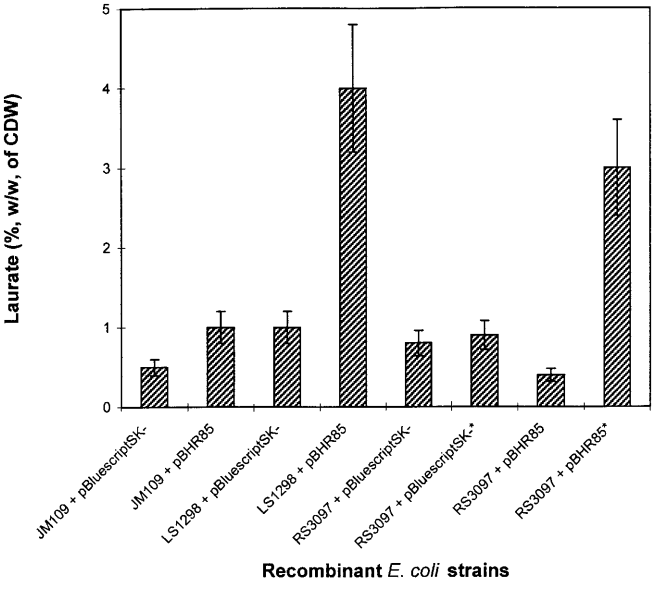

Fig. 1 Acyl-ACP thioesterase-mediated accumulation of laurate. Recombinant *Escherichia coli* was cultivated in LB medium containing 1.5% (w/v) gluconate, 1 mM isopropyl thiogalactose and either (for plasmids pBHR71/pBHR77) pBluescript SK^- 75 µg ampicillin ml^{-1} or (for plasmid pBHR85) either 50 µg kanamycine ml^{-1} or 12.5 µg tetracycline ml^{-1}. Cultivations were performed at 37 °C for 48 h. * Medium also contained 0.24 mg acrylic acid ml^{-1}

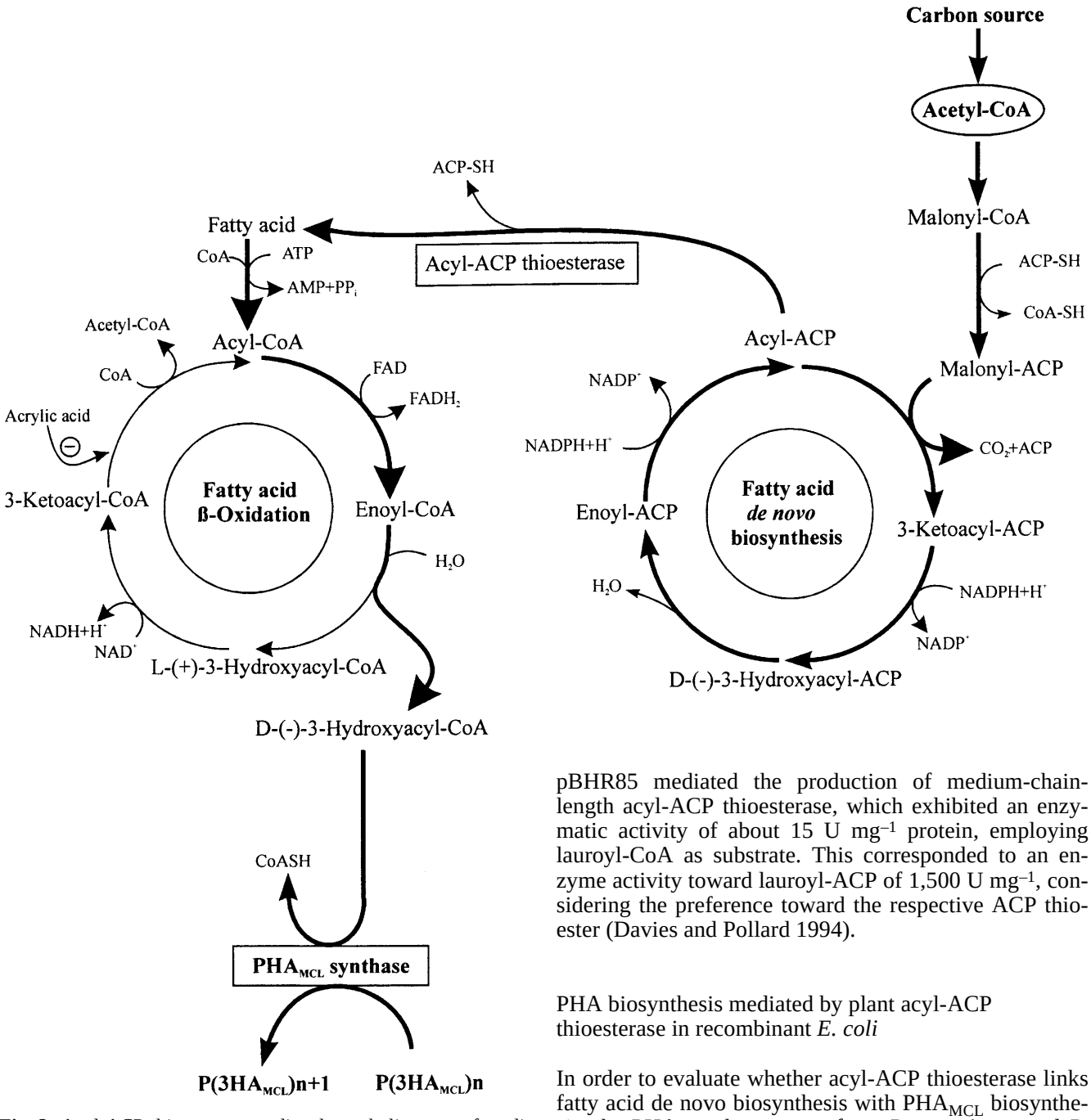

Fig. 2 Acyl-ACP thioesterase-mediated metabolic route of medium-chain-length polyhydroxyalkanoate (PHA_{MCL}) synthesis from acetyl-CoA

acrylic acid (Thijsse 1964; Qi et al. 1998; Figs. 1, 2). Acrylic acid did not affect the growth of recombinant *E. coli* RS3097 when cultivated in LB medium plus gluconate. When wild-type *E. coli* JM109 harbouring plasmid pBHR85 was employed, laurate accumulation was much lower (Fig. 1). Functional expression of the thioesterase gene was also confirmed by determining the enzymatic activity in crude extracts of *E. coli* harbouring either pBBR1MCS-2-tet or pBHR85 only. Plasmid pBHR85 mediated the production of medium-chain-length acyl-ACP thioesterase, which exhibited an enzymatic activity of about 15 U mg^{-1} protein, employing lauroyl-CoA as substrate. This corresponded to an enzyme activity toward lauroyl-ACP of 1,500 U mg^{-1}, considering the preference toward the respective ACP thioester (Davies and Pollard 1994).

PHA biosynthesis mediated by plant acyl-ACP thioesterase in recombinant *E. coli*

In order to evaluate whether acyl-ACP thioesterase links fatty acid de novo biosynthesis with PHA_{MCL} biosynthesis, the PHA synthase genes from *P. aeruginosa* and *R. eutropha* were introduced into the respective recombinant *E. coli fad* mutants. Only the type II PHA synthase gene *phaC1* from *P. aeruginosa* in plasmid pBHR71 in either *E. coli* LS1298 or *E. coli* RS3097 did not show PHA accumulation when the cells were grown in LB medium with sodium gluconate as non-related carbon source (Langenbach et al. 1997; Qi et al. 1998). However, when fatty acids were provided as carbon source, these recombinant *E. coli fad* mutants accumulated PHA_{MCL} as shown previously (Langenbach et al. 1997; Qi et al. 1998). Furthermore, we introduced plasmid pBHR77, which contained the *phaC1* gene from *P. aeruginosa* and collinearly the entire *PHB* operon from *R.*

Table 1 Accumulation of polyhydroxyalkanoate (*PHA*) in recombinant *Escherichia coli* strains. Cells of recombinant strains of *E. coli* were cultivated in LB medium containing 1.5% (w/v) gluconate, 1 mM isopropyl thiogalactose and either (for plasmids pBHR71/pBHR77) 75 µg ampicillin ml^{-1} or (for plasmid pBHR85) either 50 µg kanamycine ml^{-1} or 12.5 µg tetracycline ml^{-1}. Strain RS3097 (*fadR*) was cultivated in the presence of 0.24 mg acrylic acid ml^{-1}. This *fadR* mutation resulted in the constitutive expression of the *fadBA* operon. Strain LS1298 carried a *fadB* mutation, which resulted in the loss of detectable activities of β-oxidation enzymes. Cultivations were performed at 37 °C for 48 h. *3HB* 3-Hydroxybutyrate, *3HD* 3-hydroxydecanoate, *CDW* cellular dry weight, *nd* not detectable

Strain/plasmid	PHA content of the cells, as % (w/w) of CDW	PHA composition, as % (w/w)	
		3HB	3HD
JM109/pBHR77 + pBHR85	75.0	100	nd
JM109/pBHR71 + pBHR85	nd	nd	nd
JM109/pBHR77	76.0	100	nd
JM109/pBHR71	nd	nd	nd
JM109/pBluescriptSK–	nd	nd	nd
LS1298/pBHR77 + pBHR85	72.5	92.4	7.6
LS1298/pBHR71 + pBHR85	3.2	nd	100
LS1298/pBHR77	53.0	100	nd
LS1298/pBHR71	nd	nd	nd
LS1298/pBluescriptSK–	nd	nd	nd
RS3097/pBHR77 + pBHR85	63.4	94.6	5.4
RS3097/pBHR71 + pBHR85	3.4	nd	100
RS3097/pBHR77	50.0	100	nd
RS3097/pBHR71	nd	nd	nd
RS3097/pBluescriptSK–	nd	nd	nd

eutropha into recombinant *E. coli* (Antonio et al. 2000). Plasmid pBHR77 mediated strong polyhydroxybutyrate (PHB) accumulation (contributing to > 50% of CDW in all tested recombinant *E. coli* strains) when gluconate was provided as carbon source. However, no PHA_{MCL} accumulation was observed (Antonio et al. 2000). When fatty acids were provided as carbon source, a blend of PHB and PHA_{MCL} was accumulated (Table 1).

To enable PHA_{MCL} synthesis from gluconate in recombinant *E. coli* strains, we introduced the plasmid pBHR85, which functionally expressed the acyl-ACP thioesterase gene (cDNA) from the plant *U. californica* (Fig. 1), in addition to pBHR71 and pBHR77, respectively, into the respective recombinant *E. coli* strain. The transfer of plasmid pBHR85 into *E. coli* JM109 (wild type) harbouring either plasmid pBHR71 or pBHR77 did not result in PHA_{MCL} accumulation (Table 1). However, when we applied the recombinant *E. coli fadB* mutant LS1298 or the *fadR* mutant RS3097 (in the presence of acrylic acid) harbouring either plasmid pBHR71 or pBHR77 in addition to plasmid pBHR85, PHA_{MCL} accumulation contributing to a significant fraction of the CDW was obtained (Table 1). Recombinant strains of *E. coli* LS1298 and RS3097, which harboured pBHR77 plus pBHR85, also accumulated poly(3-hydroxybutyrate) (Table 1). Laurate accumulation contributing to about 4% of the CDW in *E. coli* harbouring pBHR85 as shown above (Fig. 1) was abolished when the PHA synthase of *P. aeruginosa* encoded by pBHR71 or pBHR77 was co-expressed with the acyl-ACP thioesterase gene, resulting in PHA_{MCL} synthesis. Since growth of recombinant *E. coli* harbouring plasmid pBHR85 in M9 medium was strongly impaired, all cultivations were performed in LB medium plus gluconate as carbon source.

Discussion

In the present study, we could clearly demonstrate that the acyl-ACP thioesterase from *U. californica* produced in *E. coli* mutants, impaired in fatty acid β-oxidation, mediated accumulation of laurate (Fig. 1). Furthermore, in the presence of the type II PHA synthase from *P. aeruginosa*, these fatty acids were channeled via truncated fatty acid β-oxidation into PHA_{MCL} (Fig. 2). Thus a PHA_{MCL} biosynthetic pathway from non-related carbon sources was established, releasing fatty acids from ACP intermediates of the fatty acid de novo biosynthesis by the activity of acyl-ACP thioesterase. These were then activated to CoA thioesters, employing a truncated fatty acid β-oxidation pathway (Langenbach et al. 1997; Qi et al. 1998). The (*R*)-3-hydroxyacyl-CoA thioesters, which were derived from fatty acid β-oxidation, served then as substrate for the PHA synthase. However, only a rather weak accumulation of PHA_{MCL} (contributing to about 6% of CDW) was obtained (Table 1). This is about three-fold higher than the accumulation produced by the application of the cytosolic acyl-CoA thioesterase TesA from *E. coli* (Klinke et al. 1999).

Acyl-ACP thioesterase releases free fatty acids from the intermediates of fatty acid de novo biosynthesis and thus strongly interferes with a central pathway of the bacterial metabolism. This is confirmed by the impaired growth of *E. coli* expressing the acyl-ACP thioesterase gene in M9 medium plus gluconate as carbon source. However, this study shows that acyl-ACP thioesterase serves as a tool to provide a precursor for PHA_{MCL} synthesis from non-related carbon sources, although the PHA_{MCL} content of the respective recombinant *E. coli* cells was rather low.

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References

Antonio RV, Steinbüchel A, Rehm BHA (2000) Analysis of in vivo substrate specificity of the PHA synthase from *Ralstonia eutropha*: formation of novel copolyester in recombinant *Escherichia coli*. FEMS Microbiol Lett 182:111–117

Brandl H, Gross RA, Lenz RW, Fuller RC (1988) *Pseudomonas oleovorans* as a source of poly(3-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl Environ Microbiol 54:1977–1982

Davies HM, Pollard M (1994) Plant thioesterase having preferential hydrolase activity toward C12 acyl-ACP substrate. U.S. Patent US5304481

DiRusso CC (1990) Primary sequence of the *Escherichia coli fadBA* operon, encoding the fatty acid-oxidizing multienzyme complex, indicates a high degree of homology to eukaryotic enzymes. J Bacteriol 172:6459–6468

Ellman GL (1959) Tissue sulhydryl groups. Arch Biochem Biophys 82:70–77

Fiedler S, Steinbüchel A, Rehm BHA (2000) PhaG-mediated synthesis of poly(3-hydroxyalkanoates) consisting of medium-chain-length constituents from non-related carbon sources in recombinant *Pseudomonas fragii*. Appl Environ Microbiol 66:2117–2124

Hoffmann N, Steinbüchel A, Rehm BHA (2000a) The *Pseudomonas aeruginosa phaG* gene product is involved in the synthesis of polyhydroxyalkanoic acid consisting of medium-chain-length constituents from non-related carbon sources. FEMS Microbiol Lett 184:253–259

Hoffmann N, Steinbüchel A, Rehm BHA (2000b) Homologous functional expression of cryptic *phaG* from *Pseudomonas oleovorans* establishes the transacylase-mediated polyhydroxyalkanoate biosynthetic pathway. Appl Microbiol Biotechnol 54:665–670

Huijberts GNM, Rijk TC de, Waard P de, Eggink G (1994) ^{13}C Nuclear magnetic resonance studies of *Pseudomonas putida* fatty acid metabolic routes involved in poly(3-hydroxyalkanoate) synthesis. J Bacteriol 176:1661–1666

Klinke S, Ren Q, Witholt B, Kessler B (1999) Production of medium-chain length poly(3-hydroxyalkanoates) from gluconate by recombinant *Escherichia coli*. Appl Environ Microbiol 65:540–548

Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM II, Peterson KM (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166:175–176

Langenbach S, Rehm BHA, Steinbüchel A (1997) Functional expression of the PHA synthase gene phaC1 from *Pseudomonas aeruginosa* in *Escherichia coli* results in poly(3-hydroxyalkanoate) synthesis. FEMS Microbiol Lett 150:303–309

Madison LL, Huisman GW (1999) Metabolic engineering of poly(3-hydroxy-alkanoates): from DNA to plastic. Microbiol Mol Biol Rev 63:21–53

Qi Q, Rehm BHA, Steinbüchel A (1997) Synthesis of poly(3-hydroxyalkanoates) in *Escherichia coli* expressing the PHA synthase gene *phaC2* from *Pseudomonas aeruginosa*: comparison of PhaC1 and PhaC2. FEMS Microbiol Lett 157:155–162

Qi Q, Steinbüchel A, Rehm BHA (1998) Metabolic routing towards polyhydroxyalkanoic acid synthesis in recombinant *Escherichia coli* (*fadR*): inhibition of fatty acid β-oxidation by acrylic acid. FEMS Microbiol Lett 167:89–94

Qi Q, Steinbüchel A, Rehm BHA (2000) In vitro synthesis of poly(3-hydroxydecanoate): Purification and enzymatic characterization of type II polyhydroxyalkanoate synthases PhaC1 and PhaC2 from *Pseudomonas aeruginosa*. Appl Microbiol Biotechnol 54:37–43

Rehm BHA, Steinbüchel A (1999) Biochemical and genetic analysis of PHA synthases and other proteins required for PHA synthesis. Int J Biol Macromol 13:83–88

Rehm BHA, Krüger N, Steinbüchel A (1998) A new metabolic link between fatty acid de novo synthesis and polyhydroxyalkanoic acid synthesis. J Biol Chem 273:24044–24051

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Simon RW, Egan PA, Chute HT, Nunn WD (1980) Regulation of fatty acid degradation in *Escherichia coli*: isolation and characterization of strains bearing insertion and temperature-sensitive mutations in gene *fadR*. J Bacteriol 142:621–632

Spencer AK, Greenspan AD, Cronan JE Jr (1978) Thioesterases I and II of *Escherichia coli*, hydrolysis of native acyl-acyl carrier protein thioesters. J Biol Chem 253:5922–5926

Thijsse GJE (1964) Fatty-acid accumulation by acrylate inhibition of β-oxidation in an alkane-oxidizing pseudomonads. Biochim Biophys Acta 84:195–197

Voelker T, Davies HM (1994) Alteration of the specificity and regulation of fatty acid synthesis of *Escherichia coli* by expression of a plant medium-chain acyl-acyl carrier protein thioesterase. J Bacteriol 176:7320–7327