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Analysis of 4-Phosphopantetheinylation of Polyhydroxybutyrate Synthase from *Ralstonia eutropha*: Generation of β-Alanine Auxotrophic Tn*5* Mutants and Cloning of the *panD* Gene Region

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The postulated posttranslational modification of the polyhydroxybutyrate (PHA) synthase from *Ralstonia eutropha* by 4-phosphopantetheine was investigated. Four β-alanine auxotrophic Tn*5*-induced mutants of *R. eutropha* HF39 were isolated, and two insertions were mapped in an open reading frame with strong similarity to the *panD* gene from *Escherichia coli*, encoding L-aspartate-1-decarboxylase (EC 4.1.1.15), whereas two other insertions were mapped in an open reading frame (ORF) with strong similarity to the NAD(P)$^+$ transhydrogenase (EC 1.6.1.1) alpha 1 subunit, encoded by the *pntAA* gene from *Escherichia coli*. The *panD* gene was cloned by complementation of the *panD* mutant of *R. eutropha* Q20. DNA sequencing of the *panD* gene region (3,312 bp) revealed an ORF of 365 bp, encoding a protein with 63 and 67% amino acid sequence similarity to PanD from *E. coli* and *Bacillus subtilis*, respectively. Subcloning of only this ORF into vectors pBBR1MCS-3 and pBluescript KS$^-$ led to complementation of the *panD* mutants of *R. eutropha* and *E. coli* SJ16, respectively. *panD*-encoded L-aspartate-1-decarboxylase was further confirmed by an enzymatic assay. Upstream of *panD*, an ORF with strong similarity to *pntAA* from *E. coli*, encoding NAD(P)$^+$ transhydrogenase subunit alpha 1 was found; downstream of *panD*, two ORFs with strong similarity to *pntAB* and *pntB*, encoding subunits alpha 2 and beta of the NAD(P)$^+$ transhydrogenase, respectively, were identified. Thus, a hitherto undetermined organization of *pan* and *pnt* genes was found in *R. eutropha*. Labeling experiments using one of the *R. eutropha panD* mutants and [2-^{14}C]β-alanine provided no evidence that *R. eutropha* PHA synthase is covalently modified by posttranslational attachment of 4-phosphopantetheine, nor did the *E. coli panD* mutant exhibit detectable labeling of functional PHA synthase from *R. eutropha*.

Polyhydroxyalkanoic acids (PHA) represent a rather complex and diverse class of bacterial storage compounds; more than 100 different hydroxyalkanoic acids, which occur as insoluble cytoplasmic inclusions in the cells, have been identified as constituents of these polyesters (30). PHA synthases, the key enzymes of PHA synthesis, catalyze the polymerization of hydroxyalkanoic acids from corresponding coenzyme A (CoA) thioesters to PHA. The PHA synthase gene (*phaC*) of *Ralstonia eutropha* is part of the *phaCAB* operon, which also encodes the β-ketothiolase (*phaA*) and the acetoacetyl-CoA reductase (*phaB*) (19, 20, 24). There is some evidence that the *R. eutropha* PHA synthase is posttranslationally modified by 4-phosphopantetheine in *Escherichia coli* SJ16 (*panD*), thus presumably providing a second thiol group (7). One thiol group (Cys-319) has been identified by site-specific mutagenesis and covalent labeling of the corresponding PHA synthase peptide fragment to be directly involved in the catalytic mechanism and to be essential for enzymatic activity (7, 38). However, the serine residue, or another amino acid residue, to which 4-phosphopantetheine might be attached has not been identified, nor has it been shown whether the proposed posttranslational modification of the PHA synthase occurs also in *R. eutropha*. Therefore, the putative modification of the *R. eutropha* PHA synthase was studied in its natural host in order to gain a better understanding of the reaction mechanism of PHA synthases and to evaluate further requirements for effective expression of PHA synthase genes in other organisms. 4-Phosphopantetheine is used primarily for the synthesis of CoA and acyl carrier protein (ACP), which are the predominant acyl group carriers in the cell (5). The acyl moiety is attached to the terminal sulfhydryl of the 4-phosphopantetheine prosthetic group of these cofactors. 4-Phosphopantetheine is also a prosthetic group of other enzyme systems, such as the *entF* gene product involved in serine activation in the biosynthesis of *E. coli* siderophore enterobactin (21). Specific labeling of 4-phosphopantetheinylated proteins occurred in β-alanine auxotrophic *E. coli* (*panD*) fed with [2-^{14}C]β-alanine (18), since β-alanine is a precursor of 4-phosphopantetheine (2). The *panD* gene encodes the aspartate-1-decarboxylase, which catalyzes the conversion of L-aspartate to CO_2 and β-alanine (3, 37). Pantoate is synthesized from ketoisovalerate via two enzymatic steps, catalyzed by ketopantoate hydroxymethyltransferase (*panB*) and ketopantoate reductase (*panE*) (13, 36). Pantothenate is then synthesized by an ATP-dependent condensation of pantoate and β-alanine catalyzed by the pantothenate synthetase (*panC*) (11). In this study, we isolated β-alanine auxotrophic mutants of *R. eutropha* in order to (i) investigate whether a posttranslational modification of the PHA synthase occurs in *R. eutropha* and (ii) clone genes involved in 4-phosphopantetheine synthesis.

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Characteristics	Reference or source
Strains		
E. coli		
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*)	39
S17-1	*thi1 proA hsdR17 recA1 tra* (RP4)	27
SJ16	F$^-$ *panD2 zad-220*::Tn*10* λ$^-$ *216 relA1 spoT1 metB1* λ^r Tc^r	10
R. eutropha		
HF39	Parent strain Sm^r	29
C3	*pntAA*::Tn*5* derived from *R. eutropha* HF39, β-alanine auxotroph	This study
M30	*pntAA*::Tn*5* derived from *R. eutropha* HF39, β-alanine auxotroph	This study
O22	*panD*::Tn*5* derived from *R. eutropha* HF39, β-alanine auxotroph	This study
Q20	*panD*::Tn*5* derived from *R. eutropha* HF39, β-alanine auxotroph	This study
Plasmids		
pVK100	Cosmid, Tc^r, Km^r	14
pBluescript SK$^-$	Ap^r, ColE1	Stratagene
pBluescript KS$^-$	Ap^r, ColE1	Stratagene
pBBR1MCS-3	Tc^r, RK2, Mob$^+$, Tra$^-$	15
pBHR68	Derivative of pBluescript SK$^-$ containing the 5.2-kb *Sma*I/*Eco*RI fragment comprising the PHA operon from *R. eutropha*	28
pBHR68(S260A)	Like pBHR68 but containing site-specific mutant of *phaC* gene (serine at position 260 changed to alanine)	This study
pBHR68(S546I)	Like pBHR68 but containing site-specific mutant of *phaC* gene (serine at position 546 was changed to isoleucine)	This study
pVK100-C20	Derivative of pVK100 containing a multiple 33.5-kb *Eco*RI fragment from *R. eutropha*	This study
pSKE15	Derivative of pBluescript SK$^-$ containing a 15-kb *Eco*RI fragment subcloned from pVK100-C20	This study
pKSKX0.76	Derivative of pBluescript KS$^-$ containing a 0.76-kb PCR-amplified *Kpn*I/*Xho*I fragment comprising the coding region of *panD* from *R. eutropha*	This study
pBBR1MCS-3KX0.76	Derivative of pBBR1MCS-3 containing a 0.76-kb PCR-amplified *Kpn*I/*Xho*I fragment comprising the coding region of *panD* from *R. eutropha*	This study

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Strains and plasmids used in this study are listed in Table 1. *E. coli* cells were grown at 37°C in Luria-Bertani (LB) broth or on LB agar supplemented with an antibiotic(s) (ampicillin [75 μg/ml], kanamycin [50 μg/ml], and/or tetracycline [12.5 μg/ml]) if required. *E. coli* SJ16 was cultivated in Dex-E-B1-met medium (3) supplemented with antibiotic(s) and β-alanine (20 μM) when relevant. *R. eutropha* was grown at 30°C in nutrient broth (NB) medium or mineral salt medium (MSM) supplemented with an antibiotic(s) (kanamycin [160 μg/ml], streptomycin [500 μg/ml], and/or tetracycline [25 μg/ml]) and/or β-alanine (10 mg/liter) when relevant.

DNA manipulations and cloning of *panD*. Standard recombinant DNA procedures were performed as specified by Sambrook et al. (22). Tn*5*-induced β-alanine auxotrophic mutants of *R. eutropha* HF39 were generated by using the suicide vector pSUP5011 (26), which was transferred to *R. eutropha* by conjugation. Tn*5* insertion sites were mapped after subcloning of *Sal*I restriction fragments of the Tn*5* fragment (specifying kanamycin resistance) plus adjacent chromosomal DNA from the corresponding mutant genomic DNA into pBluescript SK$^-$ by DNA sequencing using a Tn*5*-specific sequencing primer (5′-GTTCAGGACGCTACTTG-3′).

The *panD* gene was cloned by phenotypic complementation to β-alanine prototrophy of the β-alanine auxotrophic Tn*5* mutant *R. eutropha* Q20. The genomic library was constructed by using partially or completely *Eco*RI-hydrolyzed chromosomal DNA from *R. eutropha* HF39 or cosmid pVK100, respectively, and a packaging system from Promega (Madison, Wis.). Single cosmids from a genomic library were transferred to *R. eutropha* Q20 by conjugation and screened for the ability to mediate β-alanine prototrophy on mineral medium.

DNA was sequenced by the method of Sanger et al. (23). The DNA sequence of the *panD* gene region was determined either by subcloning into pBluescript SK$^-$ and use of the universal/reversal sequencing primers or by applying the sequencing primer hopping strategy with custom-made primers. The coding region of the *panD* gene was amplified by PCR using the oligonucleotides 5′-CGGGGTACCTATAAGGACGTATCACCC-3′ (N terminus) and 5′-TGCTCTAGAGAATTCTTATTGCTGCATT-3′ (C terminus). After digestion with *Kpn*I and *Xho*I, the PCR product was inserted into *Kpn*I/*Xho*I restriction sites of the vectors pBluescript KS$^-$ and pBBR1MCS-3 (15), respectively (Table 1).

Site-specific mutagenesis of the two conserved serine residues in the PHA synthase was done with a USE mutagenesis kit (Pharmacia, Uppsala, Sweden) and pBHR68 containing the 5.2-kb *Sma*I/*Eco*RI fragment comprising the PHA operon from *R. eutropha* as the template DNA (28). The mutagenic primers 5′-ATCCTGGACTTGCAGCCGGAGAGCGCGCTGGTGCG-3′ (S260A) and 5′-ATCGAGCATC-ACGGCATCTGGTGGCCG-3′ (S546I) were used.

Enzymatic assay of L-aspartate-1-decarboxylase. The activity of L-aspartate-1-decarboxylase was determined as described by Williamson and Brown (37). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 5 mM EDTA (dipotassium salt), 3 mM L-[U-^{14}C]aspartate (220 mCi/mmol), and 50 μg of protein (crude extract) in a total volume of 100 μl. After 2 h of incubation at 42°C, the reaction was stopped by the addition of 10 μl of 50% trichloroacetic acid. Precipitated protein was sedimented by centrifugation, and the reaction products in the supernatant were analyzed by thin-layer chromatography (TLC), using cellulose TLC plates and 1-propanol–water–28% ammonium (80:19:1) as the solvent; the spots were identified by autoradiography. L-[U-^{14}C]aspartate and [1-^{14}C]β-alanine (Sigma, Deisenhofen, Germany) served as reference compounds.

In vitro PHA synthase activity. PHA synthase activity, at substrate concentrations of up to 130 μM, was measured spectrophotometrically at 412 nm in 25 mM Tris-HCl (pH 7.5) containing 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) as described by Valentin and Steinbüchel (35).

Polyester analysis. Three to 5 mg of lyophilized cell material 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 gas chromatography by the method of Brandl et al. (1) as described in detail recently (33).

SDS-PAGE and Western immunoblotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as described by Sambrook et al. (22). Proteins were separated in 12.5 or 15% (wt/vol) SDS-polyacrylamide gels and stained with Coomassie brilliant blue R-250. On Western blots using polyvinylidene difluoride membranes (34), PhaC1 from *Pseudomonas aeruginosa* and PhaC from *R. eutropha* were detected with anti-PhaC1 and anti-PhaC antisera, respectively, and an alkaline phosphatase-conjugated secondary antibody. Bound antibodies were detected with nitroblue tetrazolium chloride and the toluidine salt of 5-bromo-4-chloro-3-indolylphosphate.

^{14}C-labeling of 4-phosphopantetheinylated proteins. The procedure of Rusnak et al. (21) was followed, with the modifications indicated below. *R. eutropha* was cultivated in MSM containing 0.05% (wt/vol) NH_4Cl and 0.5% (wt/vol) sodium gluconate, whereas *E. coli* was cultivated in Dex-E-B1-met medium containing 0.5% (wt/vol) glucose, 1 mM thiamine, and 0.002% (wt/vol) methionine. Media contained the appropriate antibiotic, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and 20 μM [U-^{14}C]β-alanine (220 mCi/mmol). Cells were cultivated for 24 h. Crude extracts were prepared, and proteins were separated by SDS-PAGE. Autoradiography was performed to visualize 4-phosphopantetheinylated proteins. Immunoblotting was conducted to identify the PHA synthases. Cells were also analyzed with respect to PHA accumulation to obtain evidence for in vivo activity of PHA synthases.

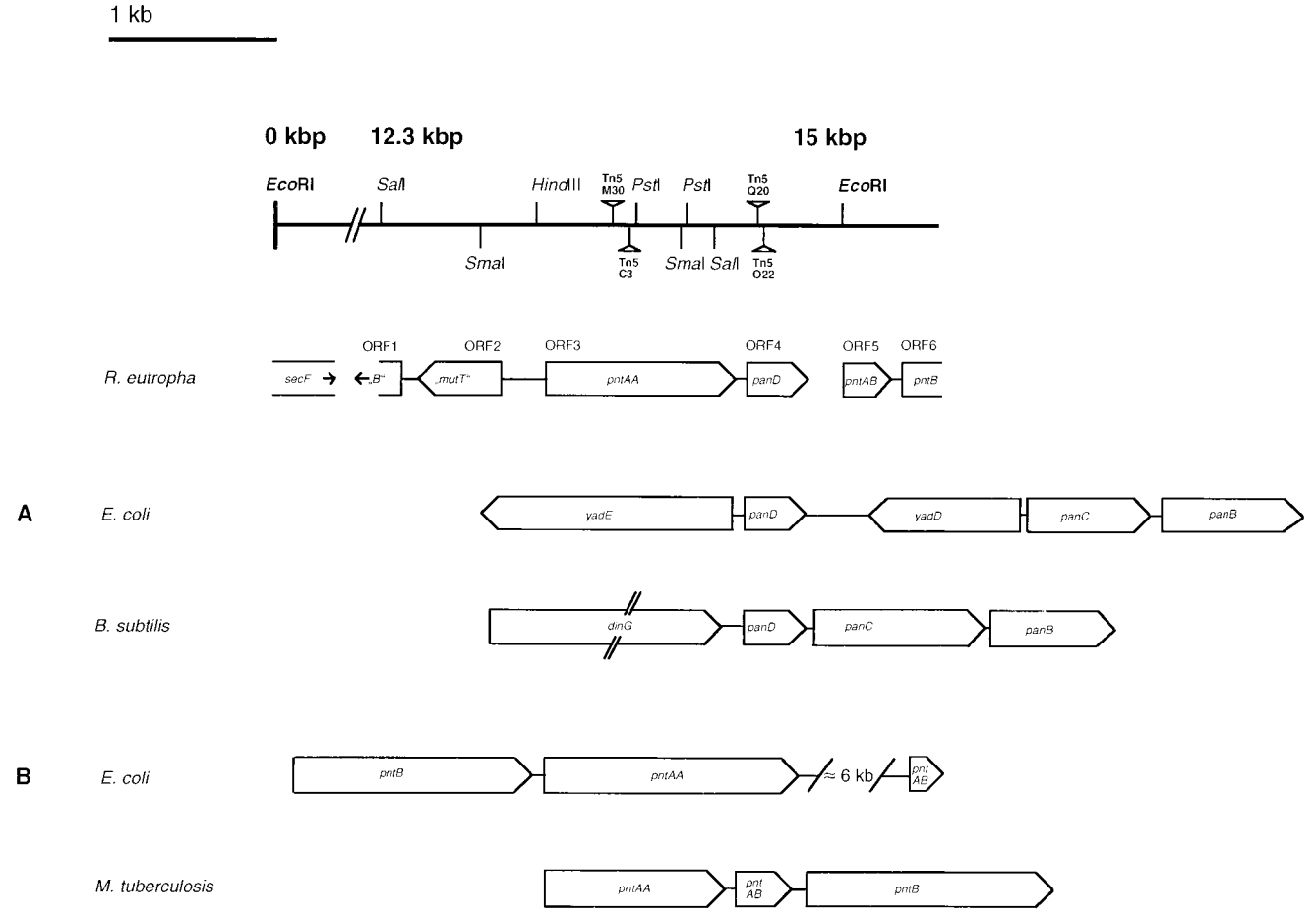

FIG. 1. Partial physical map of the genomic 15-kb *Eco*RI fragment and comparison of the localization of *panD* (A) and *pntAA*(B) with the localization of *panD* and *pntAA* in *E. coli* (accession no. AE000122 and AE000255) and *panD* in *B. subtilis* (accession no. Z99115) as well as *pntAA* in *Mycobacterium tubercolosis* (accession no. Z92770). ORFs from *R. eutropha* were designated according to the strongest similarity of the derived amino acid sequences to databases. Tn*5* insertions sites are labeled with Tn*5* and the corresponding mutant designation (M30, C3, Q20, or O22).

Nucleotide sequence accession number. The *panD* gene nucleotide sequence data were deposited in the GenBank database under the accession no. AF061246.

RESULTS

Isolation and characterization of β-alanine auxotrophic Tn*5* mutants of *R. eutropha*. To investigate the posttranslational covalent modification of the PHA synthase in *R. eutropha*, we isolated four β-alanine auxotrophic Tn*5*-induced mutants (C3, M30, O22, and Q20), using suicide vector pSUP5011 (26). In all four mutants, wild-type growth could be recovered when β-alanine was added to the medium (data not shown). The DNA regions of all four mutants harboring the Tn*5* insertions were subcloned via *Sal*I digestion and selection for Tn*5*-mediated kanamycin resistance. DNA sequence analysis mapped two Tn*5* insertions (mutants Q20 and O22) in an open reading frame (ORF) at positions 2235 and 2279, respectively, with strong similarity to the *panD* gene from *E. coli*, encoding the aspartate-1-decarboxylase (4); two Tn*5* insertions (mutants C3 and M30) occurred in an ORF at positions 1470 and 1399, respectively, with strong similarity to the *pntAA* gene from *E. coli*, encoding the NAD(P)$^+$ transhydrogenase subunit alpha 1 (8) (Fig. 1).

Cloning and DNA sequencing of the *panD* gene region. The *panD* gene region was cloned by phenotypic complementation to β-alanine prototrophy of the *panD* mutant *R. eutropha* Q20 and by transferring recombinant cosmids (pVK100) of a genomic library of strain HF39 to this mutant by conjugation. The four recombinant cosmids (C5, C9, C10, and C20) isolated harbored a common 15-kb *Eco*RI fragment beside the cosmid and other genomic fragments and complemented the *R. eutropha* mutant Q20. Subcloning of the 15-kb *Eco*RI fragment into pBluescript SK$^-$ resulted in plasmid pSKE15; DNA sequencing revealed strong similarity to *panD* from *E. coli* at one end and strong homology of about 44% identity to SecF (integral inner membrane protein involved in protein translocation) from *E. coli* at the other end (Fig. 1). Analysis of subfragments of the 15-kb *Eco*RI fragment revealed the nucleotide sequence of an approximately 3.3-kb region comprising five ORFs including the entire *panD* gene (Fig. 1).

Physical organization of the *panD* gene in *R. eutropha* and DNA sequence analysis. The *panD* derived amino acid sequence revealed the strongest similarity, about 63 and 67%, to *panD*-encoded L-aspartate-1-decarboxylases from *E. coli* and *Bacillus subtilis*, respectively (Fig. 2). In contrast to *E. coli* and *B. subtilis*, the 4-phosphopantetheine biosynthesis genes in *R. eutropha* are not colocalized (4). *panD* is localized 55 bp downstream of ORF3, the derived amino acid sequence of which exhibited strong similarity (42%) to the *pntAA*-encoded transhydrogenase subunit alpha 1 from *E. coli* (Fig. 1). Interestingly, ORF5 and incomplete ORF6 were identified directly 273 bp

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                 1                                                  50
R. eutropha      .MQRIMLRAK LHRVTVTQAD LNYEGSCGID QDLLDAADMK EFEKIELYNV
B. subtilis      .MYRTMMSGK LHRATVTEAN LNYVGSITID EDLIDAVGML PNEKVQIVNN
E. coli          .MIRTMLQGK LHRVKVTHAD LHYEGSCAID QDFLDAAGIL ENEAIDIWNV
H. pylori        .....MLYSK IHRATITDAN LNYIGSITID EDLAKLAKLR EGMKVEIVDV
M. tuberculosis  .MLRTMLKSK IHRATVTCAD LHYVGSVTID ADLMDAADLL EGEQVTIVDI
Synechocystis sp. .MGSIRLMHAK LHRVCVTEAN VNYVGSITID PVMLERVGLL PLEEVDIINL
consensus         m r ml  K lHR tvT A  lnY GS tID  dl daa  l e e v i n

                 51                                                 100
R. eutropha      NNGERFSTYI IKGERGSGEI SLNGAAARRA HLGDQLIICT YAPMS..DEE
B. subtilis      NNGARLETYI IPGKRGSGVI CLNGAAARLV QEGDKVIIIS YKMMS..DQE
E. coli          TNGKRFSTYA IAAERGSRII SVNGAAAHCA SVGDIVIIAS FVIMP..DEE
H. pylori        NNGERFSTYV ILGK..KGEI CVNGAAAARV AIGDVVIILA YASMN..EDE
M. tuberculosis  DNGARLVTYA ITGERGSGVI GINGAAAHLV HPGDLVILIA YATMD..DAR
Synechocystis sp. SNGNRFSTYV LPGQAHSKEI CPNGGAALLC QPGDRLIIFA YELCDRREVL
consensus         NG RfsTY  i g rgsg I   NGaAA      GD vIi   y  m   d e

                 101                                                145
R. eutropha      IAAYKPKVIL VNEKNGIKEI KKF..... ..... ..... .....
B. subtilis      AASHEPKVAV LNDQNKIEQM LGNEPARTIL ..... ..... .....
E. coli          ARTWRPNVAY FEGDNEMKRT AKAIPVQVA. ..... ..... .....
H. pylori        INAHKPSIVL VDEKNEILE. ..... ..... ..... .....
M. tuberculosis  ARTYQPRIVF VDAYN..... ..... ..... ..... .....
Synechocystis sp. QKGHQAKVLV TNESNETVDF YLQELIPKED GVKFINNICS EAIPH
consensus              p v       N
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FIG. 2. Comparison of the PanD sequence from *R. eutropha* with various PanD sequences from other bacteria. Accession numbers for the PanD amino acid sequences used were AE000122 (*E. coli*), Z99115 (*B. subtilis*), P56065u (*Helicobacter pylori*), O06281 (*Mycobacterium tuberculosis*), and Q55382 (*Synechocystis* sp.).

downstream of *panD*, which on the amino acid sequence level share strong similarity (about 60 and 57%) with PntAB (transhydrogenase subunit alpha 2) and PntB (transhydrogenase subunit beta) from *E. coli*, respectively (Fig. 1). Upstream of ORF3 and putatively transcribed in the opposite direction, we identified ORF2. The ORF2-encoded amino acid sequence revealed about 44% similarity to 7,8-dehydro-8-oxoguanine-triphosphatase (MutT) from *E. coli* (32). Downstream of ORF2 an incomplete ORF1 was identified, possessing 63 and 48% amino acid sequence similarity to hypothetical proteins in the *purB* 5′ regions of *Haemophilus influenzae* and *E. coli*, respectively. Therefore, in *R. eutropha*, *panD* is separated from other genes required for the synthesis of 4-phosphopantetheine and localized within a cluster of genes encoding the transhydrogenase. Such an organization of *pan* or *pnt* genes has not previously been described.

Identification of the complementing unit. The putative coding region of *panD* was amplified by PCR and subcloned into vectors pBluescript SK$^-$ and pBBR1MCS-3 downstream of and colinear to the *lac* promoter, leading to plasmids pKSKX0.76 and pBBR1MCS-3KX0.76, respectively (Table 1). A ribosome-binding site was inserted by PCR at a position optimally relative to the putative start codon of *panD* (25). Plasmid pBBR1MCS-3KX0.76 complemented all four β-alanine auxotrophic Tn*5* mutants of *R. eutropha* (Table 1), and plasmid pKSKX0.76 mediated β-alanine prototrophy to *E. coli* SJ16 (*panD*), whereas plasmid pSKE15 did not enable complementation of *E. coli* SJ16 (*panD*). Complementation of *panD* mutants was demonstrated in growth experiments and by determination of L-aspartate-1-decarboxylase activity (Fig. 3).

Determination of L-aspartate-1-decarboxylase activity. L-Aspartate-1-decarboxylase activity was qualitatively analyzed in crude extracts from various *E. coli* recombinants by using L-[U-^{14}C]aspartate as the substrate. The reaction product (β-alanine) was analyzed by TLC and autoradiography. L-Aspartate was converted to β-alanine when *E. coli* S17-1 was cultivated on NB medium but not when it was cultivated on Dex-E-B1-met medium containing 20 μM β-alanine. *E. coli* SJ16 exhibited no L-aspartate-1-decarboxylase activity when grown in Dex-E-B1-met medium containing 20 μM β-alanine, but when harboring plasmid pKSKX0.76 and in the presence of 1 mM IPTG, it showed enzyme activity (Fig. 3). Omission of IPTG significantly decreased L-aspartate-1-decarboxylase activity. No activity was obtained with plasmid pSKE15 in *E. coli* SJ16 in the presence of β-alanine (Fig. 3).

Labeling of 4-phosphopantetheinylated proteins. To investigate the postulated posttranslational modification of the PHA synthase from *R. eutropha* in its natural host, we used the *R. eutropha panD* mutant Q20. This *panD* mutant was cultivated under conditions permissive for PHA accumulation in the presence of [2-^{14}C]β-alanine, and crude extracts were subjected to SDS-PAGE (autoradiography) and immunoblot analysis. Furthermore, recombinant *E. coli* SJ16(pBHR68), functionally expressing the wild-type PHA synthase from *R. eutropha*, was analyzed with respect to 4-phosphopantetheinylation of PHA synthase (Fig. 4) (28). We also analyzed two site-specific mutants (pBHR68S260A and pBHR68S546I) of the PHA synthase from *R. eutropha*, carrying mutations at the only two highly conserved serine residues which might function as targets for covalent modification by 4-phosphopantetheine. Immunoblot analysis with anti-PHA synthase antibodies demonstrated expression of either PHA synthase gene (Fig. 4C). The corresponding PHA-expressing cells revealed in vivo activity of only the wild-type PHA synthases, whereas neither site-specific mutation caused accumulation of PHA at a detectable level. In addition, the in vitro PHB synthase activity of the two site-specific mutants was almost completely abrogated (Table 2). Autoradiography of SDS-PAGE-separated proteins derived from *R. eutropha* Q20 (*panD*) revealed no specific labeling of proteins corresponding in size to PHA synthase proteins (apparent molecular weight of 65,000) (Fig. 4). Use of β-alanine by the cells is indicated by very weak labeling of any protein, which became visible only after prolonged exposure of the gels to X-ray films (Fig. 4). In contrast, *E. coli* SJ16 enabled specific labeling of 4-phosphopantetheinylated proteins, as indicated by the presence of a strongly labeled protein with an apparent molecular weight of 8,000, which presumably corresponds to holo-ACP (Fig. 4). However, no specific labeling of either PHA synthase protein was observed (Fig. 4).

Effect of β-alanine auxotrophy on PHA accumulation in *R. eutropha*. The four β-alanine auxotrophic Tn*5* mutants of *R. eutropha*, recombinant strains of these mutants harboring plasmid pBBR1MCS-3KX0.76 and the wild type were cultivated under conditions permissive for PHA accumulation on MSM containing 1% (wt/vol) gluconate and 0.05% NH_4Cl as well as in the presence of 10 mg of β-alanine per liter. In the absence or presence of β-alanine, the wild type accumulated PHA to a level of about 55 or 65% of cell dry weight (CDW), respectively (Fig. 5). The *panD* mutants exhibited a strong decrease in PHA accumulation, to approximately 20% of the wild-type level, when β-alanine was omitted. However, wild-type-level PHA accumulation was recovered when the cells

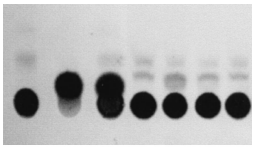

FIG. 3. In vitro activity of the *panD*-encoded L-aspartate-1-decarboxylase from *R. eutropha* in *E. coli* SJ16 harboring various plasmids. L-[U-^{14}C]aspartate was used as the substrate for L-aspartate-1-decarboxylase (crude extracts), and the reaction products were separated by TLC. Spots were identified by autoradiography. Reference substances were L-[U-^{14}C]aspartate (lane 1), [2-^{14}C]β-alanine (lane 2), and a mixture of L-[U-^{14}C]aspartate and [2-^{14}C]β-alanine (1:1) (lane 3). Crude extracts from various *E. coli* cells were subjected to this assay for L-aspartate-1-decarboxylase activity: lane 4, *E. coli* SJ16 (plus β-alanine); lane 5, *E. coli* SJ16 harboring plasmid pKSKX0.76 (plus β-alanine and IPTG); lane 6, *E. coli* SJ16 harboring plasmid pKSKX0.76 (plus β-alanine, minus IPTG); lane 7, *E. coli* SJ16 harboring plasmid pSKE15 (plus β-alanine).

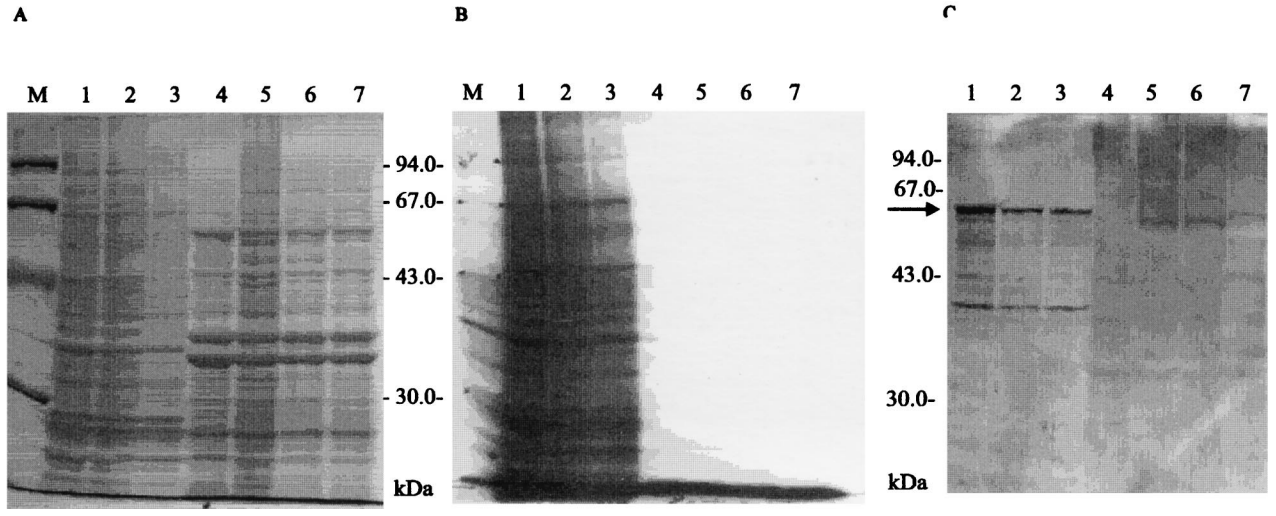

FIG. 4. ^{14}C-labeling of 4-phosphopantetheinylated proteins from *R. eutropha* (β-alanine auxotrophic mutants Q20 and M30) and recombinant *E. coli* SJ16. (A) SDS-PAGE of crude extracts from *R. eutropha* Q20 and M30 mutants as well as *E. coli* SJ16 harboring various plasmids, which were cultivated in the presence of [2-^{14}C]β-alanine. Lane M, molecular weight standard; lane 1, *R. eutropha* HF39 (negative control); lane 2, *R. eutropha* Q20; lane 3, *R. eutropha* M30; lane 4, *E. coli* SJ16(pBluescript KS$^-$); lane 5, *E. coli* SJ16(pBHR68, expressing PHA synthase from *R. eutropha*); lane 6, *E. coli* SJ16(pBHR68S260A), expressing site-specific mutant of PHA synthase from *R. eutropha*); lane 7, *E. coli* SJ16(pBHR68S546I, expressing site-specific mutant of PHA synthase from *R. eutropha*). (B) Autoradiography of the gel in panel A. (C) Immunoblot of the gel in panel A with polyclonal anti-PhaC (*R. eutropha*) antibodies. The arrow indicates the position of PHA synthase.

harbored plasmid pBBR1MCS-3KX0.76 and when the cells were cultivated in the presence of β-alanine (Fig. 5).

DISCUSSION

To investigate posttranslational covalent modification of the PHA synthase from *R. eutropha* by 4-phosphopantetheine in its natural host, we isolated four independent β-alanine auxotrophic Tn*5*-induced mutants of *R. eutropha*. These mutants, analogous to the *E. coli* SJ16 *panD* mutant, should enable specific labeling of 4-phosphopantetheinylated proteins in *R. eutropha* when fed with [2-^{14}C]β-alanine, a precursor of CoA, which serves as a donor of 4-phosphopantetheine to apo-ACP (5). Subcloning of the DNA regions containing the Tn*5* insertions and DNA sequence analysis indicated that two insertions occurred, one in an ORF with strong similarity to the transhydrogenase subunit: alpha 1 and the other in ORFs with strong similarities to L-aspartate-1-decarboxylases from *E. coli* and *B. subtilis*. The L-aspartate-1-decarboxylase (encoded by *panD*) converts L-aspartate to CO_2 and β-alanine, which is an intermediate of 4-phosphopantetheine synthesis. A 15-kb *Eco*RI fragment complementing all four β-alanine auxotrophic Tn*5* mutants was cloned from genomic DNA of *R. eutropha*. DNA sequence analysis of a 3.3-kb DNA region of the 15-kb *Eco*RI fragment revealed that the *panD* gene was located directly downstream of an ORF with strong homology to *pntAA*-encoded transhydrogenase subunit alpha 1. No consensus promoter sequence was detected upstream of the *panD* gene coding region, but upstream of *pntAA* a weakly conserved σ^{70}-specific promoter was identified (9). These data indicate that *pntAA* and *panD* are cotranscribed, which explains the β-alanine auxotrophy of mutants with Tn*5* insertions in the *pntAA* gene. Thus, Tn*5* insertions in the putative *pntAA* gene have negative polar effects on *panD* expression in mutants M30 and C3. Downstream of *panD* we identified two further ORFs, pre-

TABLE 2. PHB synthase, β-ketothiolase, and acetoacetyl-CoA reductase activities in *E. coli* harboring various plasmids

Plasmid	PHB synthase (mU/g)	β-Ketothiolase (U/g)	Acetoacetyl-CoA reductase (U/mg)
pBluescript SK$^-$	ND[a]	12.5	0.01
pBHR68	63	241	0.32
pBHR68(S260A)	0.5	205	0.3
pBHR68(S546I)	2.3	123	0.2

[a] ND, not detectable.

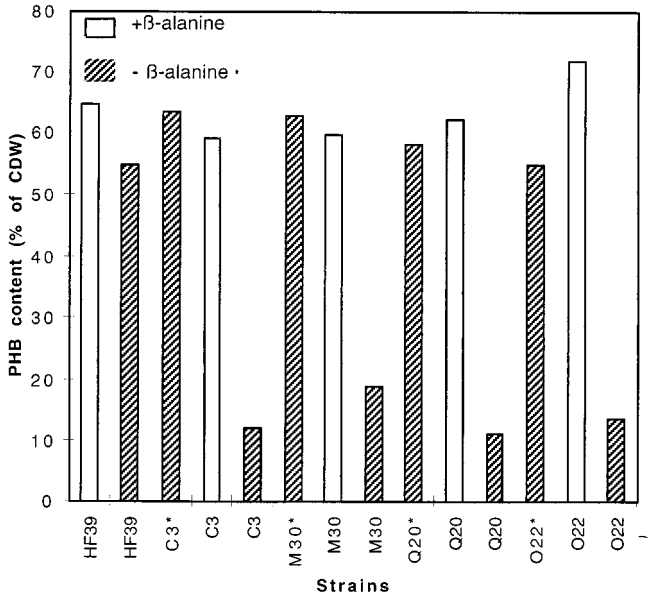

FIG. 5. PHB accumulation of β-alanine auxotrophic Tn*5* induced mutants of *R. eutropha* (M30, C3, Q20, and O22). The parent strain *R. eutropha* HF39 served as a control. Cells were cultivated in MSM containing 0.05% (wt/vol) NH_4Cl plus 1% (wt/vol) sodium gluconate and in the presence or absence of β-alanine (10 mg/liter). Each mutant harboring *panD*-expressing plasmid pBBR1MCS-3KX0.76 (indicated as *) was also analyzed.

sumably encoding transhydrogenase subunits alpha 2 (*pntAB*) and beta (*pntB*). No evidence for colocalization of *panD* with *panB* and *panC*, encoding ketopantoate-hydroxymethyltransferase and pantothenate synthetase, respectively, as shown for *E. coli* and *B. subtilis*, was found (4). Hybrid plasmids comprising the *panD* region from *R. eutropha* complemented the β-alanine auxotrophic Tn*5* mutants of *R. eutropha* and *E. coli* SJ16, and activity of the L-aspartate-1-decarboxylase was demonstrated in *E. coli* SJ16 harboring plasmid pKSKX0.76 (Fig. 3), strongly suggesting that the *panD* gene from *R. eutropha* encodes a L-aspartate-1-decarboxylase (37).

Gerngross et al. (7) obtained evidence that the PHA synthase from *R. eutropha* is posttranslationally modified by 4-phosphopantetheine in *E. coli* SJ16, identifying radioactively labeled 4-phosphopantetheinylated proteins in the β-alanine auxotrophic *E. coli* SJ16. Since, only one essential cysteine residue (Cys-319) was identified by site-specific mutagenesis in the PHA synthase of *R. eutropha* and since no other cysteine residue is highly conserved in PHA synthases (7, 16), the second thiol group postulated for the catalytic mechanism might be provided by a 4-phosphopantetheine linked to a conserved serine residue. In this study, we investigated the putative 4-phosphopantetheinylation of PHA synthase in the β-alanine auxotrophic *R. eutropha* Q20 and therefore in the natural host for this PHA synthase. In addition, two site-specific mutants of the PHA synthase from *R. eutropha*, carrying mutations at the only two conserved serine residues (S260A and S546I), and wild-type PHA synthase were analyzed with respect to 4-phosphopantetheinylation in *E. coli* SJ16. All of the investigated PHA synthase genes were expressed to similar levels, as demonstrated by immunoblotting (Fig. 4C), but the two site-specific serine mutants of the PHA synthase from *R. eutropha* exhibited neither in vitro nor in vivo activity (Table 2). However, no specific labeling of the PHA synthase by 4-phosphopantetheine was obtained, whereas only 4-phosphopantetheinylated ACP was detected in the autoradiograms. Gerngross et al. (7) observed in *E. coli* SJ16, in addition to ACP and the PHA synthase, two 4-phosphopantetheinylated proteins: one unknown 35-kDa protein, which is presumably identical with the recently characterized EntB (isochorismate lyase), and the 140-kDa EntF protein (enterobactin synthase) (6, 21). Both enzymes are involved in enterobactin biosynthesis, and expression of the corresponding genes is strictly dependent on iron-limited growth conditions (21). Under iron starvation, EntB and EntF were identified as 4-phosphopantetheinylated proteins in *E. coli* SJ16 when [3-^{3}H]β-alanine was added to the growth medium (21). In the presence of 2 μM $FeSO_4$, only ACP was detected as a 4-phosphopantetheinylated protein (21). In addition labeling experiments with [^{14}C]pantothenic acid clearly indicated that ACP is the predominantly labeled protein in *E. coli* (18). Since we did not use iron-limited conditions, the observation of only ACP as a 4-phosphopantetheinylated protein is in good agreement with results of previous labeling experiments. Analysis of 4-phosphopantetheinylated proteins in *R. eutropha* Q20 did not reveal specific labeling of 4-phosphopantetheinylated proteins except ACP but indicated radiolabeling of all proteins detected. This suggests either that external [2-^{14}C]β-alanine, in contrast to the case for *E. coli* SJ16 (10, 11, 21), is not exclusively used for CoA synthesis in *R. eutropha* Q20 or intermediates of CoA biosynthesis are degraded and channeled to central metabolism. Since no evidence for 4-phosphopantetheinylation of PHA synthases was obtained and since the PHA synthase of *R. eutropha* was functionally expressed in various organisms from different kingdoms, 4-phosphopantetheinylation seems not to be required for enzymatic activity of PHA synthases. In addition, so far no pantetheinylated peptide of the PHA synthase from *R. eutropha* has been isolated (16). Calculations of specific activity and the extent of labeling (about 1%), as previously obtained, makes specific posttranslational modification by 4-phosphopantetheine of PHA synthase very unlikely (7, 16). Instead, during heterologous expression of the PHA synthase gene from *R. eutropha* in *E. coli*, most probably a minor fraction of the PHA synthase protein was covalently modified by the action a 4-phosphopantetheine transferase present in *E. coli*, which is obviously not relevant for PHA metabolism in *R. eutropha*. This finding is relevant to strategies for expressing PHA synthase genes in organisms, such as plants, (17, 31), which can be used for biotechnological production of PHA.

Based on these observation and on kinetic studies, Sinskey and coworkers are now postulating a new model of the PHA synthase reaction mechanism in which two subunits of PHA synthase from a homodimer, with each subunit providing one thiol group by Cys-319. Thus, the protein dimer is the active form of the enzyme (16). To investigate a metabolic link between CoA biosynthesis and PHA biosynthesis, we cultivated the β-alanine auxotrophic *R. eutropha* Tn*5* mutants under conditions permissive for PHA accumulation and in the presence or absence of β-alanine. Although growth in the absence of β-alanine was weak, all mutants accumulated PHA to a level of about 10% of the CDW (Fig. 5). The weak accumulation of PHA might be due to low concentrations of acetyl-CoA and other essential thioesters of the central metabolism as well as to the physiological state of the cells (12). Wild-type PHA accumulation in the absence of β-alanine was restored when *panD* (pBBR1MCS-3KX0.76) was expressed in the mutants, supporting the review that PHA accumulation relies on an intact CoA biosynthesis.

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