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Genetic Characterization of Accumulation of Polyhydroxyalkanoate from Styrene in *Pseudomonas putida* CA-3

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***Pseudomonas putida* CA-3 is capable of accumulating medium-chain-length polyhydroxyalkanoates (MCL-PHAs) when growing on the toxic pollutant styrene as the sole source of carbon and energy. In this study, we report on the molecular characterization of the metabolic pathways involved in this novel bioconversion. With a mini-Tn*5* random mutagenesis approach, acetyl-coenzyme A (CoA) was identified as the end product of styrene metabolism in *P. putida* CA-3. Amplified flanking-region PCR was used to clone functionally expressed phenylacetyl-CoA catabolon genes upstream from the *sty* operon in *P. putida* CA-3, previously reported to generate acetyl-CoA moieties from the styrene catabolic intermediate, phenylacetyl-CoA. However, the essential involvement of a (non-phenylacetyl-CoA) catabolon-encoded 3-hydroxyacyl-CoA dehydrogenase is also reported. The link between de novo fatty acid synthesis and PHA monomer accumulation was investigated, and a functionally expressed 3-hydroxyacyl-acyl carrier protein-CoA transacylase (*phaG*) gene in *P. putida* CA-3 was identified. The deduced PhaG amino acid sequence shared >99% identity with a transacylase from *P. putida* KT2440, involved in 3-hydroxyacyl-CoA MCL-PHA monomer sequestration from de novo fatty acid synthesis under inorganic nutrient-limited conditions. Similarly, with *P. putida* CA-3, maximal *phaG* expression was observed only under nitrogen limitation, with concomitant PHA accumulation. Thus, β-oxidation and fatty acid de novo synthesis appear to converge in the generation of MCL-PHA monomers from styrene in *P. putida* CA-3. Cloning and functional characterization of the *pha* locus, responsible for PHA polymerization/depolymerization is also reported and the significance and future prospects of this novel bioconversion are discussed.**

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters produced by numerous bacterial species, whose varied thermoplastic and elastomeric properties offer the potential for industrial, medical, and bulk consumer applications (34). PHA accumulation typically occurs when a suitable bacterium encounters a relative abundance of utilizable carbon, offset by an inorganic nutrient limitation, (e.g., nitrogen). The physicochemical properties of PHAs depend on their constituent (*R*)-3-hydroxyacyl-coenzyme A (CoA) monomer compositions; >100 monomers have been identified to date (27, 33). PHA synthase enzymes responsible for (*R*)-3-hydroxyacyl-CoA monomer polymerization are divided into class I, short-chain-length PHA synthases, polymerizing C_4-C_5 carbon-length monomers; and class II medium-chain-length (MCL) PHA synthases, whose substrate monomer lengths range between C_6 to C_{12} (23). The subsequent release of (*R*)-3-hydroxyacyl-CoA monomers from accumulated PHAs is facilitated by a PHA-associated depolymerase enzyme, PhaZ (13, 34).

Pseudomonas putida CA-3 exhibits the unique ability to accumulate MCL-PHAs when grown on the industrial waste pollutant styrene as the sole source of carbon and energy (32). Previously, we reported that styrene degradation in *P. putida* CA-3 involved an upper pathway converting styrene to phenylacetic acid (PA), and an independently regulated lower pathway initiated via activation of PA to phenylacetyl-CoA (21). A catabolic operon specifically responsible for the metabolism of phenylacetyl-CoA was first identified in *P. putida* U and *Escherichia coli* W (22, 5). This pathway, referred to as the PACoA catabolon, reportedly involves oxidation of the aromatic nucleus, followed by ring cleavage and β-oxidation of the alicyclic compound via a multienzyme complex.

In this study, we set out to identify and characterize the genetic apparatus necessary for metabolism of styrene post-phenylacetic acid, the subsequent generation of PHA monomers, and the accumulation of PHAs in *P. putida* CA-3. This novel conversion of the toxic environmental pollutant styrene to PHA is of biotechnological significance, as it (i) identifies the potential to exploit styrene waste as a low-cost starting material for value-added PHA accumulation and (ii) may also represent an economically attractive incentive for the bioremediation of stored styrene wastes. Ultimately, it will be the generation of recombinant strains capable of PHA overaccumulation from styrene that will dictate the potential application of this technology. In this regard, the identification and molecular characterization of some of the key structural and regulatory components involved, such as those described here, will allow the development of future recombinant strategies.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture media, and growth. *Pseudomonas putida* CA-3, a styrene-degrading, bioreactor isolate, has been described previously (18, 20). *E. coli* CC118λ*pir* hosted the mini-Tn*5* derivative pUT-Km1. This suicide plasmid has the R6K origin of replication and encodes resistance to kanamycin and ampicillin (3). Plasmid pRK600 (Cm^r) was used as a helper in triparental

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mating experiments and encodes the *tra* functions facilitating pUT-Km1 mobilization. PCR2.1-TOPO vector (Invitrogen, California) was used in the cloning of PCR amplification products.

Pseudomonas putida CA-3 was routinely grown on E2-minimal medium (30) containing either 10 mM citrate or 15 mM PA as the sole carbon source. Growth on styrene was facilitated by the addition of 70 μl of liquid styrene to a test tube fixed centrally to the bottom of a 1-liter Erlenmeyer flask. PHA accumulation was achieved by reducing the nitrogen content of the E2 medium in the form of NH_4SO_4 from 8 mM to 1.5 mM and allowing cultures to grow for 48 h. All *E. coli* vector hosts were maintained on standard LB agar plates containing the appropriate antibiotic(s) and were inoculated into 10 ml LB overnight broths prior to desired applications.

Nucleic acid manipulations. Nucleic acid isolation (DNA/RNA), was performed according to Ausubel et al. (2). All oligonucleotide primers used in this study were synthesized by Sigma-Genosys, Ltd. (United Kingdom). Nucleic acid sequence determination involved BigDye Terminator cycle sequencing (Lark Technologies, Inc., England), followed by analysis on 5.75% and 4.75% LongRanger gels for ABI 377.

Amplification of genomic DNA located upstream of the phenylacetyl-CoA ligase *paaK* gene. A modified version of the two-step PCR method reported by Sorensen was used to amplify flanking DNA upstream of the phenylacetyl CoA ligase *paaK* gene adjoining the styrene degradative operon in *P. putida* CA-3 (19, 26). A degenerate flanking primer, AFR-Rdm1 (5′-CAGTTCAAGCTTGTCC AGGAATTCNNNNNNNCGCGGT-3′), was combined with a *paaK*-specific biotinylated primer, *S51*-Bio (5′-CTGGAACTGGGTGGCAAGAGC-3′) in the first round of PCR. Thermal cycling conditions and reagents were standard; a 52°C annealing temperature was used. Biotinylated fragments were purified from the random amplification pool via overnight binding at 30°C to superparamagnetic Dynabeads M-280 Streptavidin (Dynal), followed by extraction using a magnetic particle concentrator (Dynal). Bound fragments were released by washing with 0.1 M NaOH, followed by neutralization with 0.1 M HCl; 2 μl of this suspension was used as a template for the second round of PCR. The primers in this instance were a nondegenerate flanking primer, AFR-Ndg1 (5′-CAGTTCA AGCTTGTCCAGGAATTC-3′), designed to bind within the 5′ region of amplicons generated with AFR-Rdm1; and *AltS*-nb1 (5′-ATGTCGTTCTGGGTG TAG-3′), a *paaK*-specific primer located upstream from the *S51*-Bio priming site. The annealing temperature of the reaction mixture was 50°C; all other conditions and reagents were standard. PCR products were visualized on 1% agarose gels, and those of >1.5 Kb in size were purified (Qiaex II kit; QIAGEN) and cloned into pCR2.1-TOPO prior to M13 primed sequencing.

Random mini-Tn*5* mutagenesis. A triparental mating approach was used to introduce pUT-Km1 into *P. putida* CA-3. The mating mixture (1 ml), containing mid-log-phase LB-grown recipient, donor, and helper strains at a ratio of 7:2:1, was centrifuged for 2 min at 16,400 relative centrifugal force, resuspended in 50 μl fresh LB medium, spotted onto an LB agar plate, and incubated at 30°C for 24 h. *P. putida* CA-3 transconjugants expressing kanamycin resistance were subsequently isolated by plating serial dilutions of the mating mixture onto E2-citrate medium containing kanamycin (50 μg/ml). Approximately 10^5 isolated colonies were individually transferred to 96-well microtiter plates containing 200 μl of E2-citrate broth in each well. These master plates were subsequently used to identify transposition events, resulting in a loss of the wild-type *P. putida* CA-3 strain's ability to (i) utilize PA as a sole carbon source and (ii) accumulate PHAs from unrelated carbon sources such as styrene, PA, and citrate. PA-negative phenotype strains were identified by direct transfer to E2-PA agar plates and subsequent screening for the presence or absence of colony growth after 24 h at 30°C. These were subsequently grown overnight in E2-citrate broth, and cells were harvested by centrifugation, washed with E2 medium lacking any carbon source, and used to inoculate E2-styrene broth to assess their ability to utilize styrene. PHA-negative phenotypes were identified by first growing colonies in E2-citrate broth containing 1.5 mM nitrogen (30°C; 24 h), followed by transfer to E2-citrate agar plates lacking any nitrogen, incubation for 48 h at 30°C, and monitoring thereafter for an absence of colony opacity associated with PHA-accumulating microorganisms.

Mapping of transposon insertion sites. Arbitrarily primed PCR was employed to identify gene disruption sites by using two consecutive rounds of amplification. The primer sequences and appropriate thermal cycling parameters have been published previously (4). The resulting amplicons were visualized on 1% agarose gels prior to purification with a Qiaex II gel extraction kit (QIAGEN, California). Sequencing was performed using a mini-Tn*5* internal primer.

Gas chromatography-tandem mass spectrometry (GC-MS) analysis of Tn*5* mutant PHA percent cell (dry weight) (% CDW) content. PHA monomers were extracted from freeze-dried cells by a method previously described (15). Samples were analyzed on a Fisons GC-8000 series gas chromatograph equipped with a 30-m by 0.32-mm HP-1 0.25 μm column (Hewlett Packard) operating in split mode (split ratio, 5:1) with temperature programming (60°C for 2 min, increments of 5°C/min up to 200°C, increments of 40°C/min up to 280°C, and 5 min at 280°C). For peak identification, PHA standards from *Pseudomonas oleovorans* were used.

Cloning of the PHA operon in *P. putida* CA-3. Genomic DNA was isolated from *P. putida* CA-3 as previously described (2) and PCR screened with oligonucleotide primers designed from existing GenBank PHA gene sequences of various *Pseudomonas* species to PCR clone *phaC1*, *phaZ*, *phaC2*, and *phaG* gene homologues from our strain. The primers employed were C1-F (5′-ATGAGTA ACAAGAACAACGAT-3′), C1-R (5′-TCAGCGCTCGTGAACGTAGGT-3′), Z-189 (5′-CGGGGTCGGCGGCTCGTCTAC-3′), Z-631 (5′-GCCGGATCTT GTGCAGCCAGTG-3′), C2-F (5′-ATGACAGAAAAACCGGGCAAA-3′), C2-R (5′-TCATCGGGTCAGCACGTAGGT-3′), G-F (5′-ATGAGGCCAGA AATCGCTGT-3′), and G-R (5′-TCAGATGGCCAATGCATGCT-3′). PCR products were cloned into pCR2.1-TOPO (Invitrogen, California), according to the manufacturer's instructions. Sequencing was performed as outlined above using M13 universal primers. The operonic nature of the *pha* locus was assessed by recombining primers C1-F/C2-R, C1-F/Z-631, and Z-189/C2-R. All PCR products were subsequently cloned and sequenced as described above.

RT-PCR analysis. Total RNA was isolated from *P. putida* CA-3 cells grown under differing carbon and nitrogen (C/N) ratios. For each growth condition, 1 μg of RNA was reverse transcribed as previously reported (21), and 2 μl of the resulting cDNA pool was used as a template for PCR amplifications of *phaC1*, *Z*, *C2*, and *G* with the relevant primer pairs. To assess the transcriptional status of *paaX*, total RNA was isolated from *P. putida* CA-3 cells growing on citrate and PA, and reverse transcription-PCR (RT-PCR) was performed with the following *paaX* internal primers: *pX*-F1 (5′-CGCCATAACGCCAAACCCCT-3′) and *pX*-R1 (5′-TGCCCCACTGAACAACCTGAT-3′). In the case of all cDNA generated, PCR with oligonucleotides specific to the citrate synthase housekeeping gene of *P. putida* CA-3 acted as a positive control for each reverse transcription reaction. Total RNA was used as template in negative-control PCRs to demonstrate the absence of contaminating genomic DNA.

Nucleotide sequence accession numbers. Nucleotide sequence data generated in this study were deposited in GenBank under accession numbers AY714618, AY714619, and AY726000.

RESULTS

Identification of PACoA catabolon genes in *P. putida* CA-3. Amplified flanking-region PCR was used to clone a 2.8-kb region immediately upstream of the phenylacetyl-CoA ligase gene *paaK*, located 413 bp upstream from the styrene catabolic operon in *P. putida* CA-3. Sequence analysis identified full-length *paaX* and *paaY* gene homologues, which appeared to encode the archetypal PACoA catabolon transcriptional repressor and an associated regulatory enzyme (currently of unknown function), respectively (Table 1). RT-PCR analysis of total RNA from *P. putida* CA-3 revealed constitutive expression of *paaX* during growth on E2 minimal salts medium with styrene, phenylacetic acid, or citrate as a sole carbon source (data not shown). A 795-bp *paaN* gene homologue fragment was also identified, which appeared to encode a putative aldehyde dehydrogenase involved in ring cleavage of 2-hydroxyphenylacetyl-CoA (22). Table 1 shows the results of BLAST P screening of protein databases using the predicted amino acid sequences of the three genes. All three amino acid sequences displayed >99% similarity to reported PACoA catabolon enzymes, located upstream of the styrene degradative operon in *Pseudomonas* sp. Y2 (1, 29). The *paaXYN* genes also displayed similar structural organization in both *Pseudomonas* strains. These findings strongly support the involvement of an archetypal PACoA catabolon in degradation of the styrene catabolic intermediate, phenylacetic acid, in *P. putida* CA-3.

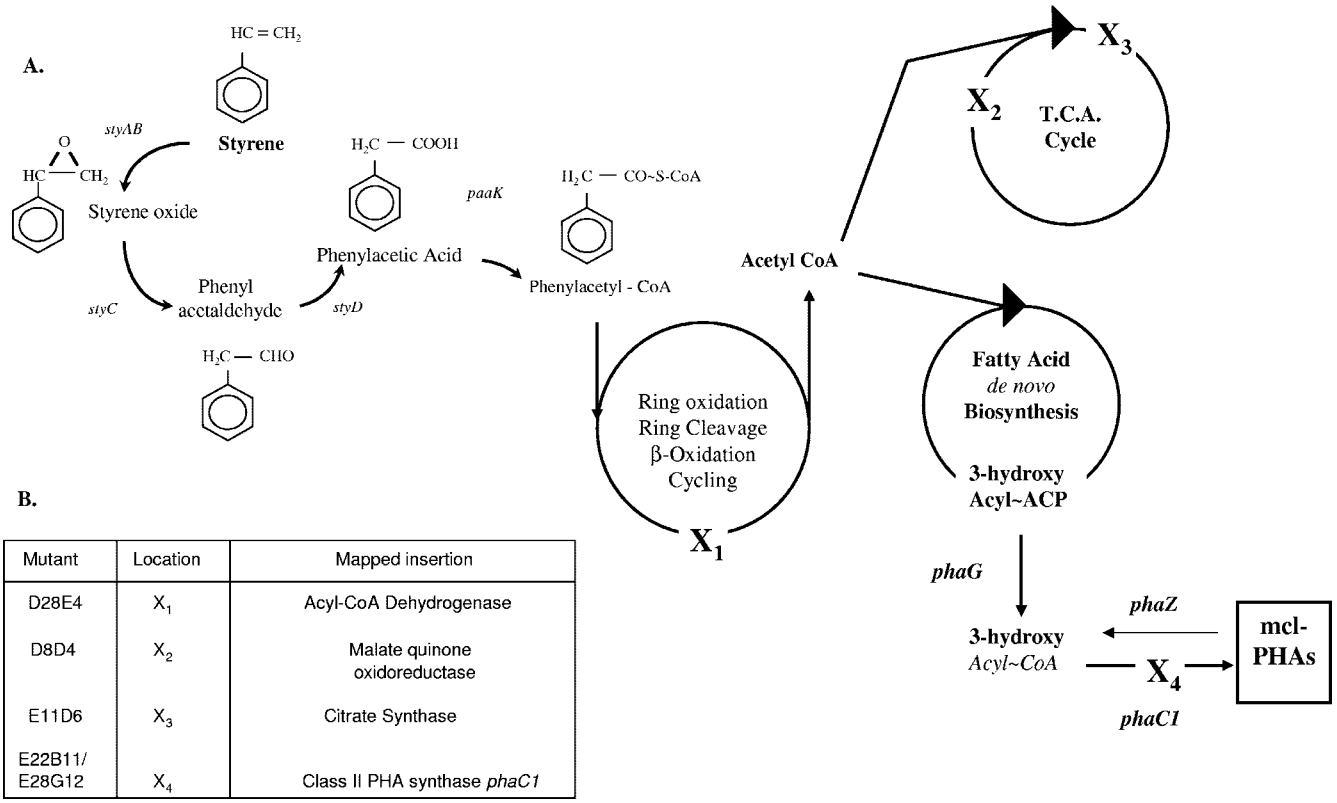
Styrene/phenylacetic acid mini-Tn*5* mutants. In an attempt to identify functional genes essential to metabolism of the PA

TABLE 1. BLAST P GenBank comparison of amino acid sequences of genes identified upstream of *paaK* in *P. putida* CA-3

Gene	Product (no. of amino acids)	Similar polypeptide	% Identification	Organism	Accession no.
paaX	307	PaaX	99	*Pseudomonas* sp. strain Y2	CAD76909
		PaaX2	86	*Pseudomonas* sp. strain Y2	CAE45100
		PaaX	79	*P. putida* U	AAC24342
		paaX	41	*E. coli* W	CAA66101
paaY	198	PaaY	99	*Pseudomonas* sp. strain Y2	CAD76911
		PaaY	76	*P. putida* U	AAC24341
		PaaY2	73	*Pseudomonas* sp. strain Y2	CAE45101
		PaaY	60	*E. coli* W	CAA66102
paaN	265[a]	PaaN	99	*Pseudomonas* sp. strain Y2	CAD76913
		PaaZ	52	*Azoarcus evansii*-	AAG28964
		PaaN3	19	*Pseudomonas* sp. strain Y2	CAE45118
		PaaN	15	*P. putida* U	AAC24340
		PaaZ	13	*E. coli* W	CAA66089

[a] Fragment.

intermediate produced during styrene degradation, random mini-Tn*5* mutagenesis of *P. putida* CA-3 was performed. This generated three *P. putida* CA-3 mutants that were no longer capable of utilizing styrene or phenylacetic acid as a source of carbon and energy but retained the ability to grow on citrate. In two of these mutants (D8D4 and E11D6), mapping of the Tn*5* insertion sites revealed the disruption of two tricarboxylic acid (TCA) cycle genes essential for the incorporation of acetyl-CoA into the TCA cycle, namely, the NAD-dependent malate dehydrogenase, responsible for oxaloacetate formation, and the citrate synthase facilitating condensation of acetyl-CoA and oxaloacetate to citrate (Fig. 1A and B). These mutants provide the first evidence that the end product of styrene metabolism in *P. putida* CA-3 is acetyl-CoA. GC-MS analysis



B.

Mutant	Location	Mapped insertion
D28E4	X_1	Acyl-CoA Dehydrogenase
D8D4	X_2	Malate quinone oxidoreductase
E11D6	X_3	Citrate Synthase
E22B11/ E28G12	X_4	Class II PHA synthase *phaC1*

FIG. 1. (A) Proposed route of PHA accumulation from styrene in *P. putida* CA-3 under nitrogen-limiting conditions. Genes responsible for the stepwise conversions are indicated by italics, where known. *styAB*, two-component styrene mono-oxygenase gene; *styC*, styrene oxide isomerase gene; *styD*, phenylacetaldehyde dehydrogenase gene; *paaK*, phenylacetyl-CoA ligase gene; *phaG*, 3-hydroxyacyl-ACP-CoA transacylase gene; *phaC1*, class II MCL-PHA synthase gene; *phaZ*, PHA depolymerase gene. X's indicate pathway interruptions by mini-Tn*5* insertions. (B) Table identifying enzymes disrupted by mini-Tn*5* insertion sites and respective locations within proposed pathway. D28E4, acyl-CoA dehydrogenase; D8D4, malate-quinone oxidoreductase; E11D6, citrate synthase; E22B11/E28G12, both mapped to a class II MCL-PHA synthase.

TABLE 2. GC-MS analysis of *P. putida* CA-3 mini-Tn*5* mutants[a]

P. putida CA-3	Growth in NH_4^+ concentration of:		
	0	0.2 mM	0.4 mM
Wild type	2.3 ± 0.9	9.35 ± 1.75	5.5 ± 0.5
D8D4	0	0	0
E11D6	3.45 ± 0.75	5.75 ± 0.25	1.8 ± 0.8

[a] E11D6, citrate synthase-negative mutant; D8D4, malate dehydrogenase mutant. All cells were grown on 10 mM citrate (8 mM NH_4^+) for 18 h and resuspended in 10 mM PA for 36 h with 0, 0.2, and 0.4 mM NH_4^+, respectively, before PHA (% CDW) analysis.

of D8D4 and E11D6 revealed a marked reduction in the PHA accumulation capacity of mutant E11D6 and a complete loss of PHA production in mutant D8D4 (Table 2). These observations are interesting, given the recent report on PHA accumulation from styrene in *P. putida* CA-3, implicating de novo fatty acid synthesis in the generation of PHA monomers (32). Thus, the potential availability of additional acetyl-CoA moieties to fatty acid synthesis in these TCA cycle mutants offered the potential for increased PHA output from these strains. It is not clear at this point why disruption of the malate dehydrogenase should result in a complete loss of PHA accumulation.

In a third mutant displaying the styrene negative phenotype (D28E4), the Tn*5* insertion site was located 375 bp downstream from the ATG translational start codon of a 3-hydroxyacyl-CoA dehydrogenase gene typically involved in β-oxidation (Fig. 1). Together, these mutants demonstrated that acetyl-CoA is the end product of styrene metabolism in *P. putida* CA-3, which involves β-oxidation enzymes. It should be noted that BLAST P comparative protein database analysis of the deduced 125-amino-acid, N-terminal acyl-CoA dehydrogenase sequence did not produce any significant percentage identity (<16%) with previously reported PACoA catabolon-associated acyl-CoA dehydrogenases (Table 3). Thus, it would appear that β-oxidation enzymes other than those encoded by the PACoA catabolon may be involved in styrene/PA catabolism in *P. putida* CA-3.

Cloning of a *phaG* homologue; the link between de novo fatty acid synthesis and 3-hydroxyacyl-CoA PHA monomers. In conjunction with our investigation of styrene metabolism to assess its contribution to PHA accumulation in *P. putida* CA-3, we also sought to identify the genetic apparatus responsible for PHA monomer sequestration, polymerization, and depolymerization. As mentioned previously, PHA accumulation from styrene in *P. putida* CA-3 was recently reported to involve de novo fatty acid synthesis, based on biochemical inhibition studies (32). Rehm et al. were the first to report the role of a 3-hydroxyacyl-acyl carrier protein (ACP)-CoA transacylase, PhaG, responsible for PHA monomer sequestration from de novo fatty acid biosynthesis during growth of *P. putida* KT2440 on the unrelated carbon source, gluconate (24). With this in mind, an 888-bp *phaG* gene homologue was cloned from the *P. putida* CA-3 genome, which displayed >98% similarity at the amino acid level to PhaG from *P. putida* KT2440 (Table 4). In *P. putida* CA-3, PHA accumulation to 23.2% CDW only occurs under nitrogen-limiting conditions (Table 5, "Wild type"). Analysis of the transcriptional profile of *phaG* during growth of *P. putida* CA-3 under limiting and nonlimiting conditions revealed that maximal expression of the gene also occurred only under nitrogen limitation (Fig. 2B, lane 5). This provides a molecular basis for the inhibition of PHA accumulation in *P. putida* CA-3 by 2-bromo-octanoate, a transacylase inhibitor (32), and suggests that this transcriptionally regulated *phaG* homologue is likely to play a key role in PHA monomer sequestration during nitrogen-limited growth of *P. putida* CA-3 on styrene.

Cloning and characterization of the *pha* operon. With degenerate PCR primer pairs, the PHA gene cluster was cloned from the wild-type CA-3 strain; sequencing revealed the commonly reported operon structure of two class II MCL-PHA synthases (*phaC1* and *phaC2*) flanking the PHA depolymerase-encoding *phaZ* gene (Table 4) (23). To further characterize the functionality of these PHA synthetic genes, *P. putida* CA-3 was subjected to mini-Tn*5* random mutagenesis, and colonies were screened for a loss of opacity during growth on N-limiting E2-citrate medium. This approach has been used previously to examine the role of de novo fatty acid biosynthesis in PHA and rhamnolipid synthesis by pseudomonads (25). Two PHA-negative *P. putida* CA-3 mutants, E22B11 and E28G1, were isolated by this method (Fig. 3), and mapping of the transposon insertion sites revealed disruption of the *phaC1* gene in both (Fig. 1A and B). GC-MS analysis of these cells grown under PHA-accumulating conditions (N limitation) revealed a complete loss of detectable PHA accumulation (Table 5), confirming the solitary role of PhaC1 in MCL-PHA monomer polymerization in our strain.

Transcriptional analysis of *pha* genes. A transcriptional profile for each of the PHA operon genes was generated by RT-PCR analysis of total RNA from *P. putida* CA-3 cultures grown under nonlimiting, nitrogen-limiting, and carbon-limiting growth conditions with styrene as the sole carbon source. Figure 2A to C, lanes 2, revealed constitutive expression of *phaC1*; however, PHA accumulation was only observed under nitrogen limitation (Fig. 2B, lane 2; Table 5), in conjunction with maximal expression of the transacylase homologue PhaG (Fig. 2B, lane 5). Transcription of the *phaZ* depolymerase was detectable only under carbon limitation (Fig. 2C, lane 4) and coin-

TABLE 3. Amino acid comparison of the *P. putida* CA-3 mini-Tn*5*-disrupted 3-hydroxyacyl-CoA dehydrogenase with PACoA catabolon associated 3-hydroxyacyl-CoA dehydrogenases from a variety of *Pseudomonas* species

Mini-Tn*5*-disrupted 3-hydroxyacyl-CoA dehydrogenase	% Identification	PACoA catabolon-3-hydroxyacyl-CoA dehydrogenase (no. of amino acids)	Organism	Accession no.
125-amino-acid fragment	16.3	PaaC (507)	*Pseudomonas* sp. strain Y2	CAD76920
	14.7	PaaC2 (505)	*Pseudomonas* sp. strain Y2	CAE45104
	14.3	PaaC (505)	*P. putida* U	AAC24331
	14.3	PaaH (475)	*E. coli* W	CAA66097

TABLE 4. BLAST P comparison of the deduced amino acid sequences of *pha* genes cloned from *P. putida* CA-3[a]

Gene	Product (no. of amino acids)	Similar polypeptide	% Identification	Organism	Accession no.
phaC1	559	PhaC1	98	*P. putida* KT2440	AAM63407
		PhaA	95	*P. oleovorans*	M58445
		PhaC1	94	*P. putida* U	AF150670
		PhaC1	77	*Pseudomonas* sp. strain 61-3	AB014758
phaZ	256	PhaZ	97	*P. putida* KT2440	AAM63408
		PhaB	96	*P. oleovorans*	M58445
		PhaZ	96	*P. putida* U	AF150670
		PhaZ	89	*Pseudomonas* sp. strain 61-3	AB014758
phaC2	560	PhaC2	97	*P. putida* KT2440	AAM63409
		PhaC	92	*P. oleovorans*	M58445
		PhaC2	87	*P. putida* U	AF150670
		PhaC2	73	*Pseudomonas* sp. strain 61-3	AB014758
phaG	296	PhaG	98	*P. putida* KT2440	AF052507
		PhaG	95	*P. oleovorans*	AF169252
		PhaG	72	*Pseudomonas* sp. strain 61-3	AB047080

[a] PhaC1, PHA synthase 1; PhaZ, PHA depolymerase; PhaC2, PHA synthase 2; PhaG, (*R*)-3-hydroxyacyl-ACP coenzyme A transferase. Accession numbers for the sequences used in these comparisons are AY714618 and AY714619.

cided with the disappearance of detectable PHAs, previously accumulated under nitrogen limitation (21). Transcription of *phaC2* was not detected under any of the growth conditions tested (Fig. 2A to C, lanes 3).

DISCUSSION

Metabolism of phenylacetic acid. The ability of *P. putida* CA-3 to metabolise styrene to PA with subsequent activation to PACoA has been reported previously (18, 21). Currently, we are interested in investigating metabolism beyond this point to understand the influence of this pathway on PHA accumulation from styrene in this strain. The discovery in *E. coli* W and *P. putida* U of a novel catabolic route solely for PACoA metabolism (5, 22) is of particular interest for styrene metabolism in *P. putida* CA-3, as PACoA ligase activities have been reported previously in our strain during growth on styrene or its metabolic intermediate, phenylacetic acid (21). The route, referred to as the PACoA catabolon, reportedly involves oxidation of the PACoA aromatic nucleus, ring cleavage, and β-oxidation cycling of the alicyclic compound. The catabolon genes appear widespread among bacterial genera, although their structural organization varies considerably (16). Investigation of the genomic region directly upstream of the PACoA ligase *paaK* gene in *P. putida* CA-3 identified three PACoA catabolon gene homologues, *paaXYN* (Table 1). The genes encode a PACoA catabolon, negative transcriptional regulator, an associated regulatory protein of unknown function, and an aldehyde dehydrogenase, reportedly involved in ring cleavage, respectively. With *E. coli* W, it has previously been shown that constitutively expressed PaaX inhibits transcription of the PACoA catabolon in the absence of its inducer, PACoA (5, 6). In this study, RT-PCR analysis revealed that the *paaX* homologue identified in *P. putida* CA-3 is constitutively expressed when grown on E2 minimal salts medium with citrate, styrene, or phenylacetic acid as the sole carbon source (results not shown). Furthermore, PACoA ligase activity and *paaK* gene transcription are only detectable in CA-3 when cultures are grown on substrates generating a PACoA intermediate, i.e., styrene and phenylacetic acid (21). Thus, PaaX may have a similar regulatory role in transcription of a PACoA catabolic operon in *P. putida* CA-3. As Table 1 indicates, the PACoA catabolon genes identified in *P. putida* CA-3 are almost identical (>99%) with those reported in the styrene degrading *Pseudomonas* sp. Y2. The similarity between the CA-3 and Y2 PACoA catabolon genes also extends to their unique genetic organization upstream of the styrene degradative operon. On the basis of these similarities, the recent cloning of a second, functional phenylacetic acid catabolic gene cluster in *Pseudomonas* sp. Y2 indicated the potential existence of additional PACoA degradation routes in *P. putida* CA-3 (1). Therefore, in an attempt to functionally define the metabolic fate of PA in *P. putida* CA-3, mini-Tn*5* mutagenesis was used to generate mutants no longer capable of styrene or phenylacetic acid utilization.

Styrene- or phenylacetic acid-negative mini Tn*5* mutants. Two mutants, D8D4 and E11D6, carried insertions in two tricarboxylic acid cycle enzymes involved in acetyl-CoA metabolism (Fig. 1A and B), providing the first definitive proof that styrene is degraded to acetyl-CoA in *P. putida* CA-3. This finding, together with the identification of functionally expressed PACoA catabolon genes, strongly suggested that a PACoA β-oxidation mechanism may be involved in the generation of acetyl-CoA moieties in *P. putida* CA-3. Indeed, a

TABLE 5. GC-MS analysis of *P.* putida CA-3 mini-Tn*5* mutants

P. putida CA-3	Growth substrate (mM)	PHA (% CDW)
Wild type	PA (10)	23.2
E28G12[a]	PA (10)	0
E22B11[a]	PA (10)	0

[a] E28G12 and E22B11 are both PhaC1 synthase-negative mutants. Cells were grown on PA for 48 h on E2 medium–8 mM NH_4^+ before assessment for PHA accumulation.

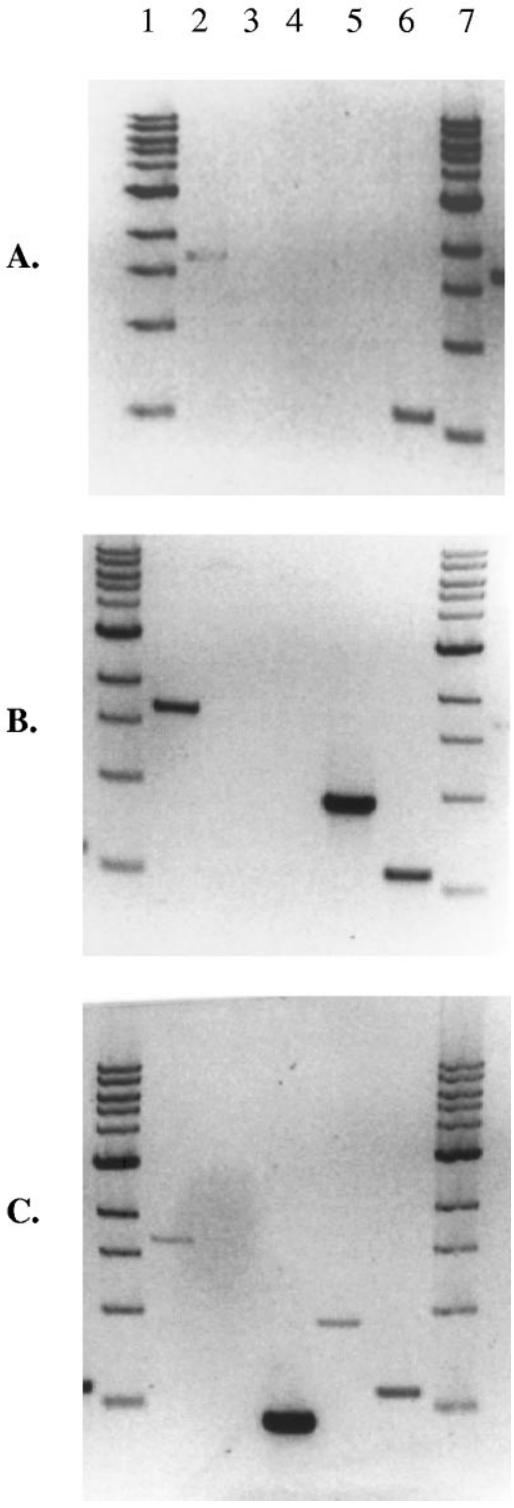


FIG. 2. Influence of varied C/N ratios on transcriptional activity of the *pha* operon genes. Medium conditions are as follows: +C/+N (nonlimited) (A), +C/−N (nitrogen limited) (B), −C/+N (carbon limited) (C). In the case of all growth conditions, lanes 1 and 7 show 10-kb molecular weight markers; lanes 2 to 5 represent the results of *phaC1*/*C2*/*Z* (lanes 2 to 4) and *phaG* (lane 5) RT-PCRs; lane 6 shows the citrate synthase positive-control RT-PCR.

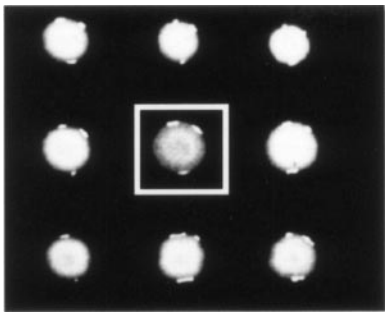


FIG. 3. *P. putida* CA-3 mini-Tn*5* mutants grown on E2-N-limited media for 72 h. The framed colony demonstrates the loss of opacity used as a phenotypic screen for PHA nonaccumulation.

third styrene/phenylacetic acid-negative mutant, D28E4, carried a Tn*5* insertion in a (*S*)-3-hydroxyacl-CoA dehydrogenase, typical of β-oxidation and also found in the multienzyme complex of the PACoA catabolon (22). However, the BLAST P results shown in Table 3 demonstrate that this Tn*5*-disrupted 3-hydroxyacyl-CoA dehydrogenase does not appear to be encoded by the PACoA catabolon, as the deduced, N-terminal amino acid sequence does not share significant (<16%) similarity with the N termini of reported PACoA catabolon-associated 3-hydroxyacyl-CoA dehydrogenases. To our knowledge, this is the first study to demonstrate the involvement of non-PACoA catabolon β-oxidation enzyme activity in aerobic styrene-phenylacetic acid degradation in a *Pseudomonas* species harboring functionally expressed PACoA catabolon genes. It also demonstrates that noncatabolon β-oxidation is involved in PHA accumulation from these unrelated carbon sources in *P. putida* CA-3.

With respect to PHA accumulation from unrelated carbon sources, the role of de novo fatty acid biosynthesis in generating (*R*)-3-hydroxyacyl-CoA monomers for PHA accumulation has been widely reported (34). The metabolism of styrene to acetyl-CoA is supportive of such a route, in conjunction with the inability of *P. putida* CA-3 cells to accumulate PHAs from styrene in the presence of the fatty acid synthesis inhibitor cerulenin (31). Furthermore, the monomer composition of the PHA accumulated from styrene in *P. putida* CA-3 clearly indicates the involvement of an anabolic process such as fatty acid synthesis, with 3-hydroxyhexanoic acid, 3-hydroxyoctanoic acid, and 3-hydroxydecanoic acid present at a ratio of 3:27:70 (32). Rehm et al. were the first to report on the activity of a 3-hydroxyacyl ACP-CoA transacylase encoded by *phaG*, responsible for the transfer of 3-hydroxydecanoate moieties from the fatty acid biosynthesis acyl carrier protein to coenzyme A, which were subsequently packaged by a class II PhaC1 synthase into MCL-PHAs (24). Several groups have subsequently reported an essential role for the enzyme in the accumulation of MCL-PHAs from unrelated carbon sources in wild-type and recombinant *Pseudomonas* species (7, 9, 10, 11, 17). In this study, a *phaG* homologue was cloned in *P. putida* CA-3, which was almost identical to its *P. putida* KT2440 equivalent (Table 4). Transcriptional analysis revealed that this putative transacylase was maximally expressed under nitrogen-limiting conditions (Fig. 2), with concomitant PHA accumulation from styrene in *P. putida* CA-3. These findings, together with the

earlier report of 2-bromo-octanoate inhibition of PHA synthesis from styrene in CA-3 (32), strongly suggest that this enzyme facilitates MCL-PHA substrate sequestering from fatty acid de novo synthesis when styrene-grown *P. putida* CA-3 cells are subjected to nitrogen-limiting conditions.

Identification and transcriptional regulation of the polyhydroxyalkanoate *pha* operon. Random mini-Tn*5* mutagenesis was used to generate PHA-negative mutants (Fig. 3), and two of these (E22B11 and E28G12) were found to involve disruption of a class II MCL-PHA synthase encoded by *phaC1* (Fig. 1A and B). GC-MS analysis revealed a complete loss of PHA accumulation in these mutants, identifying the essential role of PhaC1 in polyester production in *P. putida* CA-3. The *pha* operon was subsequently cloned and found to contain the typical organization of class II *pha* genes, a PhaC1 synthase, a *phaZ*-encoded depolymerase, and a second synthase, PhaC2 (Table 4) (24). Previous characterizations of *Pseudomonas pha* gene loci have reported the initiation of transcription of the operon genes from promoter elements upstream of *phaC1* (12, 28). It has also been demonstrated with *P. putida* U that PHA accumulation is facilitated by both synthases (8). These observations raised concerns that polar effects of the *phaC1*::Tn*5* disruption might inhibit transcription of the intact *phaC2*, thus masking its ability to accumulate PHAs in *P. putida* CA-3 grown on styrene. However, RT-PCR analyses revealed that in the wild-type strain, *phaC2* gene transcripts could not be detected under any of the growth conditions examined (Fig. 2A to C, lanes 3). Thus, PhaC1, which appears to be constitutively expressed, is the sole synthase involved in PHA packaging under nitrogen-limiting conditions in *P. putida* CA-3, (Table 5; Fig. 2A to C, lane 2). Only transcription of the *phaZ* depolymerase gene was observed under carbon-limiting conditions, concurrent with the absence of detectable PHAs (Fig. 2C, lane 4). The cotranscription of *phaC1* was also observed under this condition and similar findings have been reported in *P. oleovorans* and *P. aeruginosa* with the detection of dual transcripts by Northern blotting corresponding to *phaC1* mRNA alone and cotranscripts of *phaC1* and *phaZ* (12, 28). This has been attributed to altered transcription initiation from σ^{54} and σ^{70} consensus sequences localized upstream of *phaC1* in these strains; our observations suggest a similar control mechanism may operate in *P. putida* CA-3 (28).

Conclusions. The ability of *P. putida* CA-3 to synthesize biodegradable polyesters with potential medical and industrial applicability from the toxic industrial pollutant styrene represents a significant, undiscovered potential within toxic waste bioremediation. The recent identification of the phenylacetyl-CoA pathway as one of the four main pathways for the catabolism of core aromatic intermediates, based on sequence analysis of the metabolically versatile *P. putida* KT2440 genome, is also very significant in this regard (14). It highlights the potential for a wide variety of aromatic compounds producing a phenylacetic acid metabolic intermediate to be converted to MCL-PHAs. However, our discovery of the involvement of a non-PACoA catabolon β-oxidation enzyme indicates that the complete mechanism of PACoA metabolism has yet to be fully understood. We expect that the identification of essential structural and regulatory genes involved in accumulation of PHAs from styrene will allow us to further exploit this novel bioconversion through the generation of *P. putida* CA-3 PHA-overproducing strains, via targeted recombination strategies.

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