APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2006, p. 1302–1309 Vol. 72, No. 2
0099-2240/06/$08.00+0 doi:10.1128/AEM.72.2.1302–1309.2006

Cloning and Functional Characterization of the *styE* Gene, Involved in Styrene Transport in *Pseudomonas putida* CA-3

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Received 29 June 2005/Accepted 18 November 2005

A 1.5-kb region immediately downstream of the *styABCD* operon involved in styrene degradation in *Pseudomonas putida* CA-3 has been cloned. Sequence analysis revealed a 1,296-bp open reading frame, designated *styE*, and BLAST P database comparisons of the deduced StyE amino acid sequence revealed 33 to 98% identity with several membrane-associated ATPase-dependent kinase proteins involved in the active transport of aromatic hydrocarbons across bacterial membranes and also with FadL, an outer membrane protein necessary for the uptake of long-chain fatty acids in *Escherichia coli*. Transcription of *styE* is styrene dependent, and the gene is cotranscribed with the *styABCD* structural genes. StyE appears to be membrane associated, with a corresponding 45.9-kDa band being identified following sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of membrane preparations from styrene-grown cells. *P. putida* CA-3 cells in which the *styE* gene had been interrupted were no longer capable of growth on styrene. In contrast, overexpression of *styE* in *P. putida* CA-3 resulted in a 4.2-fold increase in styrene monooxygenase activity compared with wild-type cells grown on styrene, with a concomitant 8-fold increase in *styA* mRNA transcript levels. Experiments with the classic, ATPase inhibitor vanadate revealed that growth of wild-type cells on styrene was inhibited at a concentration of 1 mM, while 1.75 mM was required to achieve a similar effect in the StyE overexpression strain. Growth of either strain on citrate was not inhibited in the presence of up to 7 mM vanadate. These findings suggest a role for StyE in the active transport of styrene in *Pseudomonas putida* CA-3 and identify styrene transport as a potentially limiting factor with respect to mRNA transcript levels and associated enzymatic activity of the styrene degradative pathway.

Styrene, the simplest of the alkenylbenzenes, is used extensively in the petrochemical industry both as a solvent in polymer processing and as a starting material in the production of many synthetic polymers. Gaseous and effluent emissions from these industries release significant quantities of styrene into the environment, which is a cause for some concern due not only to the malodorous properties of the compound but also to the toxic and potentially carcinogenic nature of styrene (11, 18). Consequently, much interest has focused on the elucidation of different microbial catabolic pathways involved in styrene degradation, with a view to potentially developing whole-cell-based bioremediation strategies (2, 17). In this respect, the styrene-degradative pathways in a number of different bacterial strains have been studied at both the biochemical and genetic levels (22), including *Pseudomonas fluorescens* ST (17), *Pseudomonas* sp. strain Y2 (31, 32), *Pseudomonas* sp. strain VLB120 (27), *Xanthobacter* strain 124X (9), *Xanthobacter* strain S5 (10), and *Pseudomonas putida* CA-3 (20, 23).

Styrene degradation in *P. putida* CA-3 involves two distinct catabolic pathways. The *styABCD* operon, which is regulated at the transcriptional level by the StySR two-component sensory apparatus, encodes the enzymes necessary for the initial, stepwise conversion of styrene to phenylacetic acid (PAA) (24). This intermediate is subsequently activated to phenylacetyl (PA) coenzyme A (CoA), the sole substrate of the PA-CoA catabolic operon found in a wide variety of microorganisms capable of PAA catabolism (16, 26). Enzymatic manipulations within the pathway reduce the aromatic compound to acetyl-CoA, which is subsequently shuttled to the tricarboxylic acid cycle (25).

Despite recent advances in our understanding of microbial styrene catabolism, little is currently known about the mechanism by which styrene enters the cell, i.e., whether passive diffusion occurs or whether the compound is actively transported across the outer membrane. In an attempt to address this issue, we cloned a 1.5-kb region downstream of the *styD* gene in *P. putida* CA-3 and identified a 1,296-bp open reading frame (*styE*), the product of which displayed significant similarity at the deduced amino acid level with other membrane proteins that are known to be involved in the active uptake of aromatic hydrocarbons (8, 12, 13, 33) and also with the fatty acid transporter, FadL, a reported ABC transporter (4, 6). Here, we describe the functional characterization of this membrane-associated StyE protein and demonstrate its likely involvement in the facilitated uptake of styrene in *P. putida* CA-3.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture media, and growth. *Pseudomonas putida* CA-3 cultures were grown in 100 ml of minimal salts (MS) medium in 1-liter Erlenmeyer flasks, incubated at 30°C, and aerated via continuous agitation in an orbital shaker at 120 rpm. MS medium contained 7.0 g K_2HPO_4, 3.0 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, and 2 ml of 10% (wt/vol) $MgSO_4$ (added postautoclaving) per liter of demineralized water. Carbon sources were added as follows (wt/vol): 0.1% PAA, 0.1% succinate, and 0.1% citrate. Growth on styrene required the addition of 70 μl of liquid styrene to a test tube fixed centrally to the bottom of a baffled 1-liter Erlenmeyer flask. Cell growth was monitored by measuring

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TABLE 1. Primer sequences and annealing temperatures

Name	Primer sequence (5′–3′)	Annealing temp (°C)
StyD-BIO	CTGGAACTGGGTGGCAAGAGC	68
StyDF	TCCAAGAAACACCACGAGAATG	64
StyDR	ACGCCCGAGTCCTTGAAC	58
StyDF(1074)	TGCTTCGGCACATCCGTAATG	62
Primer A	CAGTTCAAGCTTGTCCAGGAATTCNNNNNNNCGCGGT	78
Primer B	CAGTTCAAGCTTGTCCAGGAATTC	72
StyEF	CAACAGATGTTCTGCGCCTCG[a]	70
StyER	TCAGAAATTATGGGTATACGA[b]	56
StyABF	CAACAGCTTAGTCAGCAGCAACCCACAACA[a]	84
StyABR	TCAGAAAGATTTTGTTCGTTGCACACTTAA[b]	80
CitF	AACTTCCTGCACATGATGTT	56
CitR	GCGAAGATCACGGTGAACAT	60
StyAF	CATCTTGGCCTCTTCCTC	60
StyAR	GATTTCCAGGTCGCTACC	60
GentF	CTGTCCAACCCGGCT	40
GentR	CTGCGGGGCG	36

[a] The sequences underlined indicate an XbaI restriction site.
[b] The sequences underlined indicate an HindIII restriction site.

optical density at 540 nm with a Beckman DU640 spectrophotometer. Cells were harvested at mid-log phase (optical density at 540 nm, 0.6) for both whole-cell enzyme assays and RNA isolations. When necessary, the following antibiotic concentrations were added to the different media: ampicillin (100 μg/ml), kanamycin (50 μg/ml), and gentamicin (20 μg/ml). *Escherichia coli* strains were grown on Luria-Burtani (LB) medium at 37°C. *E. coli* DH5α (Invitrogen) and XL-1 Blue (Stratagene) were used for routine transformation and maintenance of plasmids. TOPO pCR 2.1 (Invitrogen) was used for cloning of PCR products (according to the manufacturer's instructions).

Cloning and sequencing of the *styE* gene. A 1.5-kb region downstream from the *styD* gene was cloned using amplified flanking-region PCR. The method used was a modification of that previously described (29) and included the following steps. (i) An initial round of PCR using a 5′ biotinylated gene specific primer, StyD-BIO, and a degenerate flanking primer, primer A (Table 1), was carried out. (ii) Purification of biotinylated (i.e., gene-specific) PCR products involved physicochemical separation with streptavidin-coated magnetic beads (DYNAL). (iii) The purified, biotinylated amplicons served as the template for a second PCR with an internal *styD* gene-specific primer (StyDF) and a nondegenerate oligonucleotide, primer B, identical to the 5′ region of primer A (Table 1). PCR products from this PCR were then separated by electrophoresis on a 1% agarose gel, purified (QIAGEN Gel Extraction), and cloned into a pCR 2.1TOPO vector (Invitrogen). Transformants in *E. coli* were analyzed by PCR using the internal primers StyDF and StyDR (Table 1). Sequencing reactions were performed on positive transformants using Big Dye Terminator cycle sequencing and analyzed on 5.75% and 4.75% Long Ranger gels for ABI 377 (Lark Technologies, Inc.). Sequence data were assembled (accession number AY450871) and processed using DNASTAR (Madison, WI) software.

RT-PCR Analysis. Total RNA was isolated (QIAGEN RNeasy Mini kit) from cells grown on styrene, PAA, and succinate, respectively; and 1 μg was reverse transcribed (RT) in a reaction containing 100 ng random hexamer primers (Boehringer), 1 mM deoxynucleoside triphosphates, 10.5 mM dithiothreitol, 1× RT buffer, 50 U of Expand reverse transcriptase (Roche), and 40 U of RNase inhibitor (Roche) as previously described (24). The final reaction volume was adjusted to 20 μl using diethyl pyrocarbonate-treated water. Reverse transcription was performed at 30°C for 10 min, followed by 45 min at 45°C, prior to incubation on ice. Two microliters of the cDNA generated was then employed in subsequent PCR amplifications, carried out with a PTC200 DNA thermal cycler (MJ Research) using the primer pairs StyDF-StyDR and StyDF(1074)-StyER (Table 1). Primers specific for the constitutively expressed citrate synthase gene in *P. putida* (CitF and CitR) (Table 1) were used as a positive control. In each case, the number of cycles used in the PCR was varied to avoid reaching a point at which bands representing different conditions would have equal intensities, due to a plateau effect. Amplified RT-PCR products were partially sequenced confirm their identity.

Inactivation of *styE* in *P. putida* CA-3 by homologous recombination. (i) Construction of the *styE*-targeted pKnockout-G derivative pKGmE1. The use of pKnockout vectors for rapid and efficient inactivation of genes in *Pseudomonas aeruginosa* has been previously reported (34). In this study, the full-length *styE* gene was PCR amplified from CA-3 genomic DNA as described above and subjected to a double restriction digest with BamHI and SalII (New England Biolabs) at 37°C for 1 h in the presence of 1× NEB buffer 4. The suicide vector pKnockout-G (ColE1*ori mob*$^+$; lacking *tra*), containing the complementary restriction enzyme sites in the polylinker region was similarly digested, and both samples were subjected to electrophoresis and visualization, following ethidium bromide staining on a 1% agarose gel. The resulting 813-bp, 5′- and 3′-truncated internal *styE* fragment and the ~6-kb linearized pKnockout-G vector were purified using Qiaquick gel purification columns (QIAGEN) and an overnight ligation (insert:vector ratio, 3:1), performed at 16°C using T4 DNA ligase (Promega) in accordance with the manufacturer's instructions. Subsequently, Top10F′ chemically competent *Escherichia coli* DH5α (Invitrogen, Calif..) was transformed with 2 μl of the ligation mixture and plated out on LB agar with isopropyl-β-D-thiogalactopyranoside, X-galactosidase, and 20-μg/ml gentamicin. Blue-white screening was employed to identify ligation of the 813-bp *styE* fragment into the polylinker region of pKnockout-G. Several white colonies (10 in total) were selected for overnight growth at 37°C in 10 ml liquid LB medium (Gm20); 2-ml aliquots of each were subjected to plasmid purification (QIAGEN Miniprep kit), BamHI/SalII digestion, and visualization in an ethidium bromide-stained 1% agarose gel to confirm the presence of the insert. This process generated the *styE*-targeted pKnockout derivative pKGmE1.

(ii) Conjugation and screening for *styE* homologous recombination with pKGmE1. Conjugal transfer of the mobilizable pKGmE1 suicide vector into *P. putida* CA-3 involved triparental mating in a 50-μl volume spotted onto the surface of an LB agar plate overnight at 30°C. The pRK2013 helper strain provided the transfer function (*tra* genes) for pKGmE1. Following incubation, the mixture was lightly scraped from the plate surface with a sterile pipette tip and washed twice, each time in 1 ml minimal salts medium lacking any carbon source. Aliquots (each, 50 μl) were subsequently spread plated onto minimal salts agar containing 15 mM citrate and Gm20 to remove the non-citrate-utilizing *E. coli* donor and helper strains. Colonies capable of growth on citrate in the presence of Gm20 were subsequently screened for altered indole-to-indigo conversion phenotypes, as previously reported in the assessment of styrene monooxygenase (SMO) activity (21), following replica plating onto minimal salts agar containing 10 mM glucose (which does not impose catabolite repression on the *sty* operon in CA-3) and the gratuitous inducer 3 mM indole. Two colonies completely incapable of indole-to-indigo conversion were identified (A6 and D9). Ten-milliliter minimal salts-citrate broth overnight cultures of A6, D9, and the parent strain were washed twice in MS broth lacking any carbon source prior to inoculation into minimal salts broth with styrene as the sole carbon source. Growth at 30°C was monitored over a 24-h period. Genomic DNA was isolated from 10 ml LB broth cultures of A6, D9, and the wild-type CA-3 strain grown overnight at 30°C (1). Analysis of the respective genomic DNA samples for an intact *styE* gene was performed via PCR screening using the styEF-styER primers and amplification conditions described above. Screening for the 5′- and 3′-truncated gene duplications arising from homologous recombination was performed by Southern blot analysis with the [α-^{32}P]dATP-labeled (30 μCi) 813-bp *styE* internal fragment.

TABLE 2. Other membrane-associated proteins sharing homology with StyE

Protein	Organism	% Identification	Accession no.	Proposed function
StyE	*Pseudomonas* sp. strain Y2	98.1	CAA04004	Putative active transporter
CumH	*P. fluorescens* IP01	51.4	BAA12149	Transport of cumene across the cell membrane
XylN	*P. putida* PAW1	46.5	BAA09665	Transport of xylene across the cell membrane
TbuX	*Ralstonia pickettii* PK01	45.1	AAF03168	Transport of toluene across the cell membrane
TodX	*P. putida* F1	38.4	AAC43318	Transport of toluene across the cell membrane
FadL	*E. coli*	33.3	CDD11775	Transport of fatty acids across the cell membrane

Homologous overexpression of StyE and StyAB in *P. putida* CA-3. Full-length *styE* gene primers StyEF and StyER (Table 1) were designed, which allowed the mispriming incorporation of XbaI and HindIII restriction enzyme sites at the 5′ and 3′ ends of the gene, respectively, permitting *styE* to be cloned as an XbaI/HindIII fragment into the *lacZ* expression vector pBBR1MCS (15) to form the pStyEF expression vector. *styE* was also cloned into pBBR1MCS in the reverse orientation to generate pStyER, which acted as a negative control. StyABF and StyABR primers (Table 1) were employed to generate the full-length *styAB* genes. Again, these primers allowed the mispriming incorporation of XbaI and HindIII restriction enzyme sites at the 5′ and 3′ ends of the gene, respectively, which allowed *styAB* to be cloned as an XbaI/HindIII fragment into pBBR1MCS to form the pStyAB expression vector. pRK2013 was employed as a helper for the mobilization of plasmids pStyEF, pStyER, and pStyAB into *Pseudomonas putida* CA-3 via triparental mating (15). *P. putida* CA-3 transconjugants resulting from triparental mating were selected on MS citrate solid medium supplemented with gentamicin. The presence of the plasmid was subsequently verified by PCR using the primers GentF and GentR (Table 1).

Extraction of outer membrane fraction proteins. To isolate the membrane fraction, the cultures used were grown as described above, but samples were harvested by centrifugation, washed with a 100 mM potassium phosphate buffer (pH 7.0), and resuspended in the same buffer. Lysozyme (5 mg/ml) was added to the cell suspension, and the resulting suspension was incubated at 30°C for 30 min. Cells were ruptured using a Mini-BeadBeater-8 (BIOSPEC Products) (30 s at force 6). $MgSO_4$ and DNase were added to a final volume of 10 mM and 0.1 mg/ml, respectively, and the cell suspension was incubated at 30°C for a further 15 min. K-EDTA was added to a final concentration of 15 mM and large-cell debris was removed by centrifugation at 14,600 × *g* for 5 min at 4°C. After this step, the supernatant was removed and centrifuged at 4°C for 1 h at 60,000 × *g* in a Beckman L-60 ultracentrifuge. The membrane preparations were then washed with 50 mM potassium phosphate buffer and collected by centrifugation for 1 h at 60,000 × *g*. The resulting pellet was resuspended in 50 mM potassium phosphate buffer, and the protein concentration was determined by the Bio-Rad assay (5). Five micrograms of each protein sample was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini-Protean II system (Bio-Rad, California) according to the manufacturer's instructions. Gels were run for 2 h at 30 mA through a 5% acrylamide stacking gel and a 10% separating gel. Prestained protein marker weight standards (New England Biolabs) were used for molecular mass estimation. Gels were stained with Coomassie brilliant blue and destained in methanol/acetic acid/water (7:3:7).

Styrene monooxygenase assay. To quantify styrene monooxygenase activity, indole-to-indigo conversion was assayed as previously described (21). Assays were performed in triplicate to facilitate standard deviation calculations.

Quantification of gene transcript levels. Real-time PCR analysis of styrene monooxygenase *styA* gene transcript levels was performed with the LightCycler (Roche Diagnostics, Germany) using cDNA templates generated from styrene-grown wild-type *P. putida* CA-3, the pStyE overexpression strain, and *P. putida* CA-3 harboring the negative control vector pStyER. Primers StyAF and StyAR (Table 1) were used with the LightCycler FastStart DNA Master SYBER Green I kit (Roche) in precooled centrifuge adapters per the manufacturer's instructions. The final concentrations of Mg^{2+} and primers were 2.5 mM and 50 pmol, respectively. Citrate synthase gene transcript levels were also monitored as a control with the primer pair CitF-CitR (Table 1). Thermal cycling was as follows: initial denaturation of 95°C for 10 min; 40 cycles, each consisting of denaturation (10 s at 95°C), annealing (10 s at 55°C), and extension (12 s at 72°C); and melting curve analysis (65 to 95°C at 0.2°C/s). Products were visualized on a 1% agarose gel (660 bp for *styA* and 540 bp for citrate synthase, respectively). *P. putida* CA-3 genomic DNA acted as a template in the generation of *styA* and citrate synthase amplification standards. The appropriate band was excised and purified (QIAGEN; gel extraction). Serial dilutions of each gene were then prepared with LightCycler-grade water, where the *styA* dilution series ranged from 1×10^{18} to 1×10^{7} copies and the citrate synthase ranged from 2.5×10^{16} to 2.5×10^{6} copies.

The LightCycler software generated a standard curve by plotting crossing cycle number versus logarithms of the given concentration for each standard. A regression curve was plotted between these points and used to calculate the copy number of the *styA* and citrate synthase genes in each sample.

Addition of the ATPase inhibitor vanadate. Vanadate (Sigma) a classic p-type ATPase inhibitor (14, 30), was added to the culture medium of *P. putida* CA-3 wild-type cells and those containing the pStyE expression vector, cultured on MS medium with styrene as the sole carbon source, at vanadate concentrations of 0.25 mM to 2.00 mM. As a control, *P. putida* CA-3 cells were also grown on MS citrate medium in the presence of vanadate (0.25 to 1.75 mM). In addition to the growth inhibition studies, vanadate was also added to washed-cell suspensions of styrene-grown wild-type, StyE, and StyAB overexpression strains prior to carrying out the styrene monooxygenase assay. The inclusion of the StyAB overexpression strain was aimed at highlighting differences between increased monooxygenase activity and any potential increase in substrate availability as a result of StyE overexpression.

RESULTS AND DISCUSSION

Cloning and sequence analysis of the *styE* gene in *P. putida* CA-3. Amplified flanking-region PCR was used to clone a 1.5-kb region immediately downstream of the *styABCD* operon involved in styrene degradation in *P. putida* CA-3. The region was found to contain a 1,296-bp open reading frame designated *styE*, encoding a 434-amino-acid peptide with an estimated molecular mass of 45.9 kDa. BLAST P database comparisons of the deduced StyE amino acid sequence revealed significant identity (ranging from 33 to 98%) with proposed transport proteins previously reported in a number of hydrocarbon-degrading bacteria, namely, StyE from *Pseudomonas* sp. strain Y2 (32), CumH from *Pseudomonas fluorescens* IP01 (8), XylN from *Pseudomonas putida* PAW1 (13), TbuX from *Ralstonia picketti* PK01 (12), and TodX from *Pseudomonas putida* F1 (33), together with FadL from *E. coli*, which is involved in the transport of long-chain fatty acids across the outer membrane (Table 2) (4, 6). These proteins contained a high number of hydrophobic amino acids, XylN and StyE in particular, which are composed of >30% hydrophobic amino acids and few cysteinyl residues, properties common among membrane proteins. XylN is an outer membrane protein involved in the transport of *m*-xylene and its analogues across the outer membrane of *Pseudomonas putida* F1 (13). Hydropathy analysis of XylN and StyE using DNASTAR software indicates the comparable location of hydrophobic regions within both proteins (19). Further analysis of StyE using both PSORTb (version 2.0; http://psort.nibb.ac.jp) (7) and SignalP (version 3; Center for Biological Sequence Analysis [http://www.cbs.dtu.dk]) (3) predicted a signal sequence at the N-terminal region with a proposed cleavage site between position 9 and 10. Based on these initial observations, it was decided to further investi-

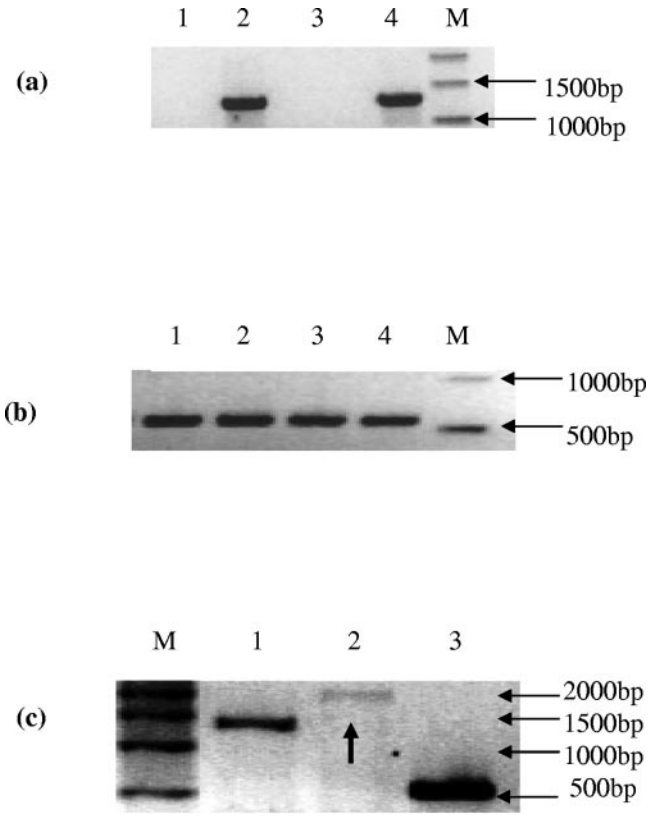

FIG. 1. (a) RT-PCR analysis of *styE* mRNA expression in *P. putida* CA-3 grown on various carbon sources. Lanes: 1, succinate; 2, styrene; 3, phenylacetic acid; 4, *P. putida* CA-3 genomic DNA as positive control; M, molecular weight markers. (b) RT-PCR analysis of citrate synthase mRNA expression in *P. putida* CA-3. Lanes: 1, succinate; 2, styrene; 3, phenylacetic acid as a sole carbon source; 4, *P. putida* CA-3 genomic DNA as a positive control; M, molecular weight markers. (c) RT-PCR analysis of total RNA from a styrene-grown culture of *P. putida* CA-3, assessing operonic expression of *styE*. Lanes: 1, 1,508-bp product obtained with styDF/styDR; 2, black arrow indicating 1,730-bp product obtained with styDF(1074)/styER; 3, 540-bp product obtained with CitF/CitR; M, molecular weight markers.

gate the potential role of StyE as a membrane-associated transport protein involved in styrene metabolism in *P. putida* CA-3.

Transcriptional analysis of the *styE* gene. RT-PCR analysis of total RNA from *P. putida* CA-3 cells cultured on styrene, phenylacetic acid, and succinate, respectively, revealed that transcription of *styE* only occurs in the presence of styrene, (Fig. 1a, lane 2). We have previously shown styrene-dependent, polycistronic expression of the *styABCD* operon genes (24) and therefore investigated cotranscription of *styE* as part of the *sty* catabolic operon. RT-PCR analysis of total RNA from styrene-grown *P. putida* CA-3 cells with the StyDF-StyER primer pair generated a 1,730-bp product, indicating read-through transcription between *styD* and *styE* (Fig. 1c, lane 2). This finding was in marked contrast to the *sty* catabolon in *Pseudomonas* species Y2, where a putative transcriptional termination sequence was reported to exist between *styD* and *styE* (32). A similar termination sequence also appears to exist between *styD* and *styE* in *P. putida* CA-3, but our ability to detect *styE* mRNA transcripts in styrene-grown cells indicated that it may not be functional. Indeed, a similar putative transcriptional termination sequence also appears to exist between *styB* and *styC*, despite cotranscription of these genes being previously reported in both *Pseudomonas* species Y2 and *P. putida* CA-3 (24, 32).

Inactivation of *styE* by homologous recombination with pKGmE1. The pKnockout-G suicide vector derivative, pKGmE1, carried an 813-bp, 5′- and 3′-truncated internal fragment of the wild-type *styE* gene. Conjugal transfer into *P. putida* CA-3 generated 43 recombinant colonies capable of growth on MS citrate agar in the presence of 20-μg/ml gentamicin. The recombinant colonies were replica plated onto MS glucose agar plates containing 3 mM indole to monitor for altered indole-to-indigo conversion phenotypes. Two well-defined colonies (A6 and D9) (Fig. 2a) incapable of indole-to-indigo conversion were selected for further analysis. Subsequent PCR screening of genomic DNA from A6 and D9, using the *styE* full-length gene primers, failed to amplify the anticipated 1,296-bp gene product (Fig. 2b). This result was consistent with disruption of *styE* via insertion of the 6-kb pKGmE1 vector by homologous recombination in these strains, which was confirmed by Southern blot analysis of EcoRI genomic digests from cultures of the *styE* mutants A6 and D9 and the *P. putida* CA-3 wild type (data not shown). The effects of *styE* disruption on styrene utilization were also investigated, and it was observed that the *styE* mutant strains A6 and D9 were no longer capable of utilizing styrene as a sole carbon source, compared with the wild-type *P. putida* CA-3 (data not shown). These findings indicate that the *styE* gene product is essential for styrene degradation in *P. putida* CA-3.

The effects of StyE overexpression on upper pathway transcriptional activation in *Pseudomonas putida* CA-3. The effect of StyE overexpression on the rate of styrene degradation in *P. putida* CA-3 was assessed via SMO-dependent indigo formation, which has previously been shown to correlate directly with styrene utilization in this strain (21, 23). *P. putida* CA-3 cells harboring pStyEF demonstrated a 4.26-fold maximal increase in SMO activity following growth on styrene, compared to wild-type *P. putida* CA-3 cells (see Table 4, 0 mM vanadate) or cells harboring the negative control vector pStyER (data not shown) grown under similar conditions. This increased SMO activity was mirrored at the transcriptional level by real-time quantitative PCR revealing an eightfold maximal increase in transcriptional activity of the *sty* operon, as represented by *styA* transcript levels, with little variation observed for mRNA transcript levels from the constitutively expressed citrate synthase gene (Table 3). In each case, transcript copy numbers were calculated from standard curves where regression coefficients of 0.98 and 0.96 were obtained for *styA* and citrate synthase, respectively. Thus, it appears that overexpression of StyE stimulates enhanced transcriptional activation of the upper pathway *styABCD* genes by increasing styrene dependent activation of these genes in *P. putida* CA-3 cells. One potential way in which this may be achieved is by increasing the levels of styrene entering the cell. To test this hypothesis, we further investigated the potential of StyE to act as a membrane-associated transport protein.

Localization of the *styE* gene product in the outer membrane of *P. putida* CA-3. In an attempt to identify the localization of StyE, we performed SDS-PAGE analysis of outer membrane protein preparations from citrate-grown *P. putida* CA-3 wild-

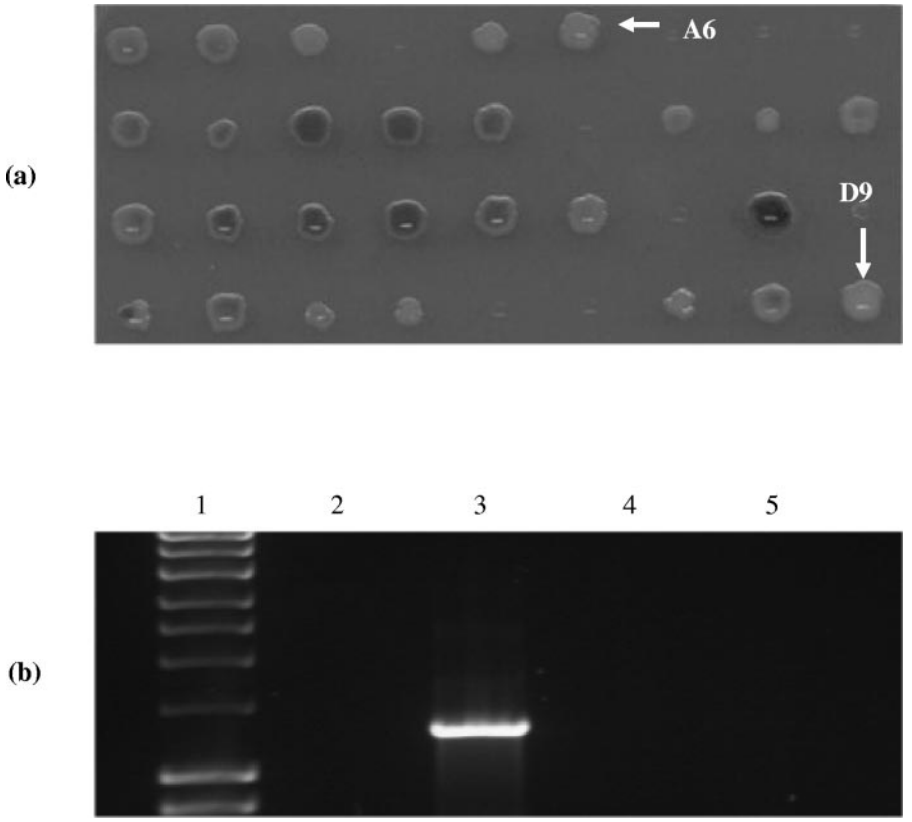

FIG. 2. (a). Screening of pKGmE1 recombinant colonies for altered indole-to-indigo conversion phenotypes on minimal salts glucose with 3 mM indole. White arrows indicate colonies A6 and D9. (b) PCR screening of genomic DNA for the intact 1,296-bp *styE* gene. Lanes: 1, Hyperladder I (Promega); 2, negative control; 3, *P. putida* CA-3 genomic DNA; 4, A6; 5, D9.

type and StyE-overexpressing strains. A 45.9-kDa band, corresponding to StyE, was identified in the membrane protein fraction of the overexpression strain only (Fig. 3, lane 1). The membrane protein fraction from the wild-type strain did not contain a corresponding 45.9-kDa band (Fig. 3, lane 2), which was consistent with earlier RT-PCR data suggesting that *styE* transcription in the wild-type strain is induced only in the presence of styrene.

The potential role of StyE in ATPase-dependent transport of styrene. Attempts were made to inhibit any potentially active styrene transport by including a classic p-type ATPase inhibitor, vanadate, in the culture medium (28). We observed that the growth of the wild-type *P. putida* CA-3 on styrene as the sole carbon source was inhibited in the presence of 1 mM vanadate, while higher levels of vanadate (1.75 mM) were required to markedly affect cell growth in *P. putida* cells overexpressing *styE* (Fig. 4a). To assess whether these observations related specifically to styrene utilization or occurred simply as a result of general physiological stress imposed by the compound, citrate-grown cultures were also exposed to vanadate. Under these conditions, the MIC for both the wild-type cells

TABLE 3. Transcript copy numbers quantified by real-time PCR

Strain	Transcript level		
	styA	*citS*	*styA*/*citS*
Pseudomonas putida CA-3 (wild type)	4.076×10^6	13.700×10^{15}	$1{:}3.36 \times 10^9$
Pseudomonas putida CA-3 (StyEF)	3.233×10^7	11.130×10^{15}	$1{:}3.44 \times 10^8$
Pseudomonas putida CA-3 (StyER)	6.492×10^6	11.090×10^{15}	$1{:}1.71 \times 10^9$

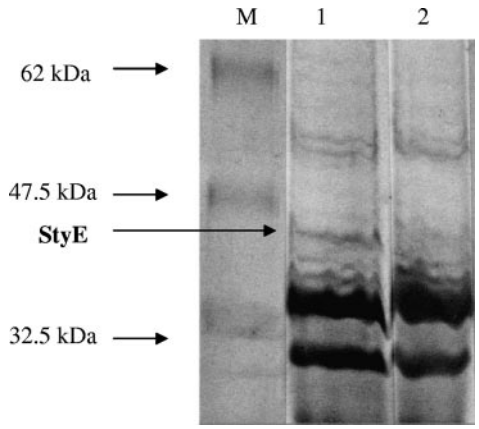

FIG. 3. SDS-PAGE analysis of outer membrane protein fraction of *Pseudomonas putida* CA-3 cells harboring pStyEF (lane 1) and *Pseudomonas putida* CA-3 wild-type cells (lane 2). M, protein markers (New England Biolabs); 62 kDa, glutamic dehydrogenase (bovine liver); 47.5 kDa, aldolase (rabbit muscle); 32.5 kDa, triosephosphate isomerase (*E. coli*). StyE (~46 kDa) is indicated by a black arrow.

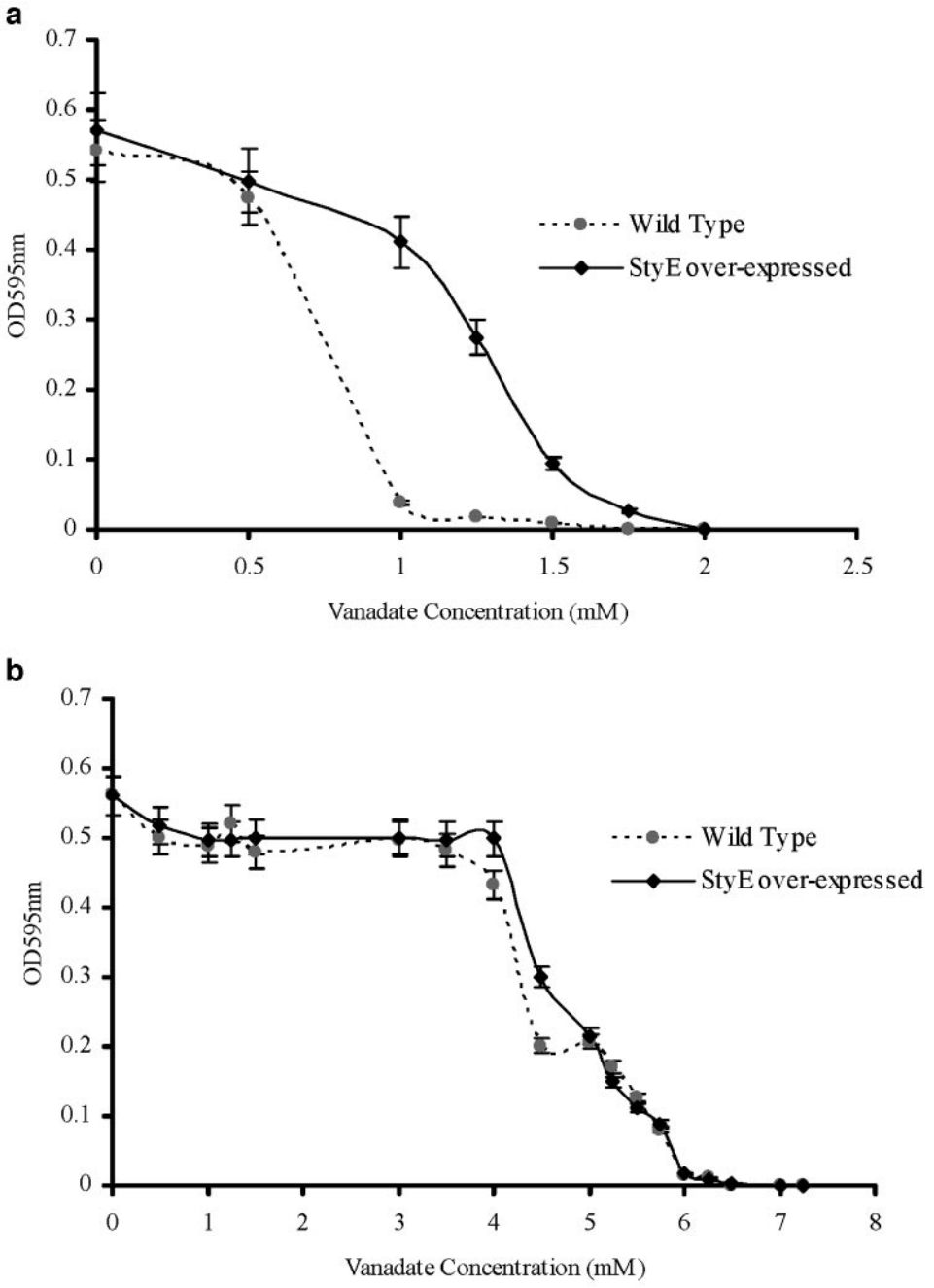

FIG. 4. Determination of the MIC of vanadate on *P. putida* CA-3 wild-type and StyE-overexpressing strains. (a) Effects of vanadate on cells growing on minimal salts medium with styrene alone. (b) Effects of vanadate when the cultures were fed citrate as the sole carbon source. ◆, *styE*-overexpressing strain; ■, CA-3 wild-type strain.

and those overexpressing *styE* was determined to be 7.00 mM vanadate (Fig. 4b), with the growth rate and effect of vanadate addition on each culture being comparable. These findings suggest that vanadate-induced repression in *P. putida* cells relates specifically to styrene utilization and also appears to directly affect StyE activity, as overexpressing strains require greater vanadate concentrations than wild-type cells to cause growth inhibition on styrene.

To further characterize the inhibitory effects of vanadate on styrene metabolism in *P. putida* CA-3, we performed experiments using cultures grown on styrene, thereby ensuring transcription of the *styABCD* operon. Vanadate concentrations ranging from 0 to 1.75 mM were added to washed-cell suspensions from these cultures immediately prior to performance of the SMO assay. In addition to the wild-type *P. putida* CA-3- and StyE-overexpressing strains, we also analyzed a *P. putida* CA-3 strain overexpressing the *styAB* (styrene monooxygenase) genes. In particular, we wished to determine how vana-

TABLE 4. Effects of increasing vanadate concentrations on SMO activity from washed-cell suspensions of mid-log phase styrene-grown cultures

Vanadate [mM]	Styrene monooxygenase activity (nmol/min/mg cell [dry wt])		
	Wild-type CA-3	pStyAB	pStyEF
0	2.67 ± 0.27	3.86 ± 0.66	11.66 ± 0.98
0.5	2.54 ± 0.13	2.24 ± 0.52	4.48 ± 0.35
0.75	2.6 ± 0.12	1.94 ± 0.91	3.84 ± 0.61
1.25	0.51 ± 0.05	0.38 ± 0.14	1.73 ± 0.13
1.75	0.32 ± 0.03	0.28 ± 0.01	0.27 ± 0.03

date affected the strains overexpressing *styE* and *styAB*, where it was observed that while both exhibited increased *styAB* levels relative to the wild-type strain; higher levels of *styE* occurred only in the pStyEF culture. In contrast to our earlier observation of inhibition of growth on styrene, analysis of the data in Table 4 revealed that SMO activity in wild-type cells grown on styrene was largely unaffected at vanadate concentrations of up to 0.75 mM, (despite 1 mM inhibiting growth cultures) and still persisted at a basal level at 1.75 mM (Table 4, column "Wild-type CA-3"). At 0 mM vanadate, cells overexpressing *styAB* exhibited approximately 1.5-fold-higher levels of SMO activity than the wild type, consistent with higher levels of available enzyme. However, upon the addition of 0.5 mM vanadate, SMO activity in the culture fell back to levels comparable to those of the wild type; this trend continued throughout the subsequent additions of vanadate. Therefore, despite the presence of increased intracellular levels of functional styrene monooxygenase enzyme, the pStyAB culture was unable to transform indole to indigo above wild-type conversion rates in the presence of vanadate, suggesting a lack of substrate availability. As we have shown, earlier overexpression of *styE* resulted in an increase in *styABCD* mRNA transcript levels (Table 3). However, Table 4, column "pStyEF," reveals a very different profile from that presented by the pStyAB overexpression strain. The addition of 0.5 mM vanadate to washed-cell suspensions of styrene-grown pStyE cultures caused a significant reduction in SMO activity, although conversion rates were still almost twofold higher than both the wild-type and StyAB overexpression strains. Indeed, this relative trend continued for all subsequent vanadate additions until 1.75 mM was added preassay. The significantly different SMO activity profiles of the pStyAB and pStyEF cultures were interesting, revealing that while both cultures overexpress *styAB*, it is the availability of intracellular substrate which is the limiting factor; this limitation is overcome by overexpression of *styE*. However, it should be noted that at the final 1.75 mM vanadate concentration, all cultures demonstrated a comparative basal level of SMO activity, which would suggest that very low levels of passive diffusion of the styrene substrate may occur.

In conclusion, the data presented here indicate the likely involvement of StyE, a membrane-associated protein, in the facilitated uptake of styrene in *P. putida* CA-3. The styE gene is cotranscribed with the *styABCD* genes and is thus under the control of the two-component StySR regulatory system. Thus, it appears that batch culture growth of wild-type *P. putida* CA-3 cells with styrene as the sole carbon source is suboptimal with respect to extracellular substrate concentration. We hypothesize that this condition reflects a somewhat limited capacity for the facilitated uptake of substrate due to stringent, coordinated regulation of the StyE transport protein, together with the *styABCD* degradative genes. While further kinetic and regulatory characterization of this system is currently under way, it is clear to us at this stage that StyE may represent a valuable target for recombinant technologies aimed at improving whole-cell-based styrene bioremediation-bioconversion strategies.

ACKNOWLEDGMENT

This project was partially funded under the PRTLI program for Irish third-level institutions, administered by the Higher Education Authority.

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