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Genetic and Functional Analysis of the Styrene Catabolic Cluster of *Pseudomonas* sp. Strain Y2

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The chromosomal region of *Pseudomonas* sp. strain Y2 involved in the conversion of styrene to phenylacetate (upper catabolic pathway) has been cloned and sequenced. Four catabolic genes, *styABCD*, and two regulatory genes, *stySR*, were identified. This gene cluster when transferred to *Escherichia coli* W confers to this phenylacetate-degrading host the ability to grow on styrene as the sole carbon and energy source. Genes *styABCD* are homologous to those encoding the styrene upper catabolic pathway in *Pseudomonas fluorescens* ST. Northern blot analyses have confirmed that genes *styABCD* constitute a transcription unit. The transcription start site of the *sty* operon was mapped 33 nucleotides upstream of the *styA* translational start codon. The *styS* and *styR* genes, which form an independent transcriptional unit, are located upstream of the *styABCD* operon, and their gene products show high similarity to members of the superfamily of two-component signal transduction systems. The *styS* gene product is homologous to histidine kinase proteins, whereas the *styR* gene product exhibits similarity at its N-terminal domain with cluster 1 of receiver modules and at its C terminus with the LuxR/FixJ family 3 of DNA-binding domains. Expression of the catabolic operon decreased significantly in the absence of the *stySR* genes and was restored when the *stySR* genes were provided in *trans* in the presence of styrene, suggesting that the *stySR* system behaves as a styrene-inducible positive regulator of the *styABCD* operon. Finally, a gene encoding a phenylacetyl-coenzyme A ligase that catalyzes the first step in the phenylacetate catabolism (styrene lower catabolic pathway) has been identified upstream of the *styS* gene. This activity was found to be induced in *Pseudomonas* sp. strain Y2 cells grown on styrene but not present in cells grown on glycerol. These results strongly suggest that the genes responsible for the complete mineralization of styrene are clustered in the chromosome of *Pseudomonas* sp. strain Y2.

Styrene is used in large quantities by the chemical industry, but it can also occur naturally, mostly by decarboxylation of cinnamic acid (42). Airborne emissions of styrene, even at low concentrations, often cause a problem because of their malodorous character and their toxic and carcinogenic effects (42). The removal of styrene from industrial waste gases could be accomplished by using styrene-degrading bacteria as biocatalysts; however, little is known concerning the microbial metabolism of styrene (6, 42). Two main routes of aerobic styrene breakdown have been described: (i) oxidation of the vinyl side chain with the formation of phenylacetate and (ii) initial oxidation of the aromatic nucleus (42).

The only information about the organization of the styrene catabolic genes has been recently obtained for *Pseudomonas fluorescens* ST (6, 25). This strain degrades styrene by oxidation of its lateral chain, and it has been shown that the upper pathway for the conversion of styrene to phenylacetate is encoded by four catabolic genes, *styABCD* (Fig. 1A). Although 2-phenylethanol accumulates in styrene-grown cells (25), genetic and biochemical analyses of the *styABCD* cluster have suggested that this compound is not an intermediate of the styrene catabolism in *P. fluorescens* ST (6). However, 2-phenylethanol appears to be an intermediate of styrene catabolism in *Corynebacterium* sp. strain ST-10 (19). In this sense, it has been also proposed that *Pseudomonas* sp. strain Y2 degrades styrene via 2-phenylethanol and phenylacetate (40). Therefore, it appeared interesting to investigate if the styrene upper catabolic pathway of this microorganism was different from that of *P. fluorescens* ST. Moreover, although there is now some biochemical and genetic information about the catabolism of styrene via lateral chain oxidation (6, 10, 16, 17, 25, 29, 40, 42), there are still many aspects that need to be studied. For instance, there are no genetic data about the regulation of the styrene upper pathway, and the genes responsible for the catabolism of phenylacetate (lower pathway) remain to be investigated.

This report describes the genetic characterization of the styrene upper catabolic cluster of *Pseudomonas* sp. strain Y2 and provides evidence of a mechanism of regulation that is unusual for the catabolism of aromatic compounds. In addition, the gene encoding the first step for phenylacetate degradation has been located and characterized.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used were *Escherichia coli* W ATCC 11105 (13), *E. coli* W14 (an *E. coli* W mutant deficient in the catabolism of phenylacetate) (13), *E. coli* DH5α (36), and *Pseudomonas* sp. strain Y2 (40). Plasmids pUC18 and pUC19 were used for cloning purposes (36). Bacteria were grown with shaking at 30 or 37°C in Luria-Bertani (LB) medium (36) or at 30°C in minimal medium M63 (27), using as carbon source 20 mM glycerol or a styrene-saturated atmosphere. Where appropriate, ampicillin (100 μg/ml), thiamine (1 μg/ml), vitamin B_{12} (5 ng/ml), and 1 mM indole were added.

DNA and RNA manipulations. DNA and RNA manipulations and other molecular biology techniques were carried out essentially as described elsewhere (36). Total RNA was extracted as previously described (1). Southern and Northern blotting as well as colony hybridization analyses were performed as previously reported (36), using as probes DNA fragments labeled with digoxygenin or [α-^{32}P]dCTP by using a Dig Luminescent Detection kit (Boehringer) or the random primer method (Pharmacia), respectively. Primer extension reactions were carried out with avian myeloblastosis virus reverse transcriptase (34). Pulsed-field gel electrophoresis was performed as previously described (37). Nucleotide sequences were determined by using a model 377 automated DNA

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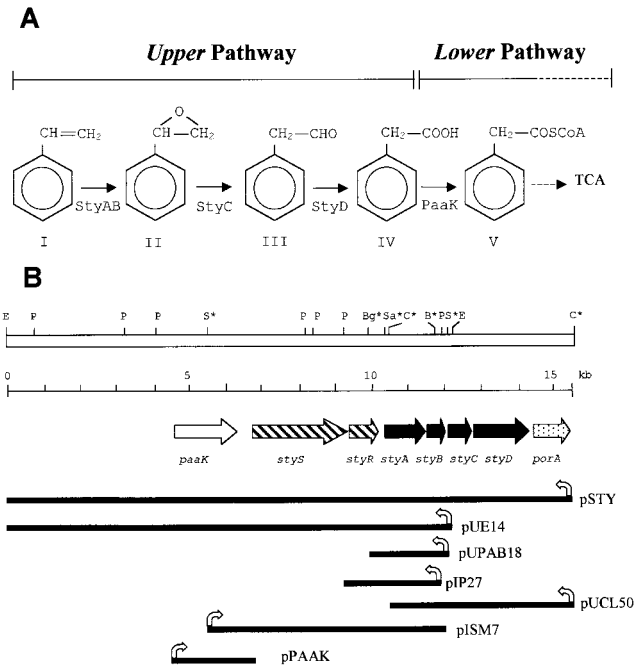

FIG. 1. Pathway for the catabolism of styrene in *Pseudomonas* sp. strain Y2 and genetic organization of the corresponding structural and regulatory genes. (A) Biochemistry of the pathway. Metabolites: styrene (compound I), epoxystyrene (compound II), phenylacetaldehyde (compound III), phenylacetate (compound IV), and phenylacetyl-coenzyme A (compound V). Enzymes: StyAB, styrene monooxygenase; StyC, epoxystyrene isomerase; StyD, phenylacetaldehyde dehydrogenase; PaaK, phenylacetyl-coenzyme A ligase. (B) Physical and genetic map of the chromosomal region encoding styrene catabolism. Locations of the genes are shown relative to those of some relevant restriction sites. Arrows indicate the direction of gene transcription. The cloned fragments (thick line) and their orientations with respect to the *lacZ* promoter (bent arrow) of plasmid pUC18 or pUC19 are indicated. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; P, *Pst*I; S, *Sma*I; Sa, *Sal*I. Asterisks mean that other identical restriction sites are present.

sequencer (Applied Biosystem Inc.). Nucleotide and protein sequence similarity searches were done with the BLASP, BLASTN, and BLASTX programs (2) via the National Institute for Biotechnology Information server. Pairwise and multiple protein sequence alignments were done with the ALIGN (44) and CLUSTAL W (38) programs, respectively, at the Baylor College of Medicine-Human Genome Center server.

Phenylacetate production assay. *E. coli* W14(pSTY) cells were incubated overnight at 30°C in minimal medium M63 containing 20 mM glycerol, ampicillin, and vitamin B_{12}, in a styrene-saturated atmosphere. Products accumulated in the culture medium were analyzed spectrophotometrically at 220 nm with Gilson high-pressure liquid chromatography (HPLC) equipment, using a Lichrosphere SRP-8 column (150 by 4.6 mm) (mobile phase, 40% methanol; flow rate, 1 ml/min).

Production of PaaK protein in *E. coli*. The *paaK* gene was PCR amplified from plasmid pSTY by using oligonucleotides K5 (5′-GGGAATTCCACCAGCTAT CGGCGCTCTTC-3′) (the *Eco*RI site introduced is underlined) and S5 (5′-CC CAACACTTCGAACGGAG-3′) as primers. The 1.6-kb amplified fragment was digested with *Eco*RI and ligated to *Eco*RI-*Hin*cII double-digested pUC18, such that in the resulting plasmid pPAAK (Fig. 1), expression of the *paaK* gene was under the control of the *lac* promoter. To determine the production of PaaK, *E. coli* W14(pPAAK) cells were grown overnight at 30°C in LB medium containing 0.5 mM isopropylthiogalactopyranoside. Culture was harvested by centrifugation, washed, and resuspended in 0.05 volume of 0.5 M potassium phosphate buffer (pH 8.0) prior to disruption by passage through a French press. The cell debris was removed by centrifugation, and the clear supernatant fluid was used as a crude extract.

Phenylacetyl-coenzyme A ligase assay. Phenylacetyl-coenzyme A ligase was assayed as previously described (28). One unit of enzyme activity is defined as the catalytic activity leading to the formation of 1 nmol of phenylacetylhydroxamate in 1 min. Protein concentration was determined by the method of Bradford (8), using bovine serum albumin as standard.

Indigo production assay. To quantify styrene monooxygenase activity, indigo production was assayed in resting cells essentially as previously described (6) but using chloroform instead of ethyl acetate to extract the culture samples.

Nucleotide sequence accession number. The nucleotide sequence reported in this study (nucleotides [nt] 1 to 10743) has been submitted to the GenBank/EMBL data bank (accession no. AJ000330).

RESULTS AND DISCUSSION

Cloning and expression of a gene cluster for the catabolism of styrene. We had observed that styrene induces in *Pseudomonas* sp. strain Y2 an activity that oxidizes indole to the blue dye indigo. To isolate the genes involved in the first steps of the styrene catabolism in *Pseudomonas* sp. strain Y2, a screening strategy based on the appearance of a blue phenotype in the presence of indole was followed (20, 25, 30). Thus, an *Eco*RI DNA library of *Pseudomonas* sp. strain Y2 was constructed in *E. coli* DH5α by using *Eco*RI-digested pUC19 vector; and after 2 days of growth on indole-containing LB medium at 30°C, three indigo-positive clones were isolated. All of them showed a plasmid, pUE14, that contained a 12.2-kb DNA insert (Fig. 1). The subcloning of this fragment revealed that the gene(s) encoding the oxygenase activity were localized within the 2.6-kb *Pst*I fragment of pUE14 (plasmid pIP27 [Fig. 1]).

When total DNA from *Pseudomonas* sp. strain Y2 was analyzed by pulsed-field electrophoresis, no plasmid was observed. The chromosomal location of the *Eco*RI fragment was confirmed by Southern blot experiments. Therefore, the styrene catabolic pathway of *Pseudomonas* sp. strain Y2 seems to be, as in the case of *P. fluorescens* ST (25), chromosome encoded.

To determine whether the cloned 12.2-kb *Eco*RI fragment encoded the complete styrene upper catabolic pathway, we checked the ability of plasmid pUE14 to confer to *E. coli* the capacity to grow on styrene as the sole carbon and energy source. Since *E. coli* DH5α cannot use phenylacetate as a carbon source, we transformed plasmid pUE14 into *E. coli* W, a strain able to mineralize this intermediate in the catabolism of styrene. However, *E. coli* W(pUE14) cells were unable to grow on styrene as the sole carbon source, suggesting that the cloned *Eco*RI fragment probably did not contain all of the genes needed for the conversion of styrene to phenylacetate. Since the gene(s) encoding the putative styrene monooxygenase was located at the right end of the *Eco*RI fragment (Fig. 1; see above), we decided to clone the contiguous chromosomal region of *Pseudomonas* sp. strain Y2. A *Cla*I DNA library of *Pseudomonas* sp. strain Y2 was constructed in pUC19 and its screening by colony hybridization using the terminal 0.6-kb *Bam*HI-*Eco*RI fragment of pUE14 (Fig. 1) as a probe allowed us to isolate plasmid pUCL50, which harbored a 4.6-kb *Cla*I insert (Fig. 1). To reconstruct the 15.5-kb chromosomal region shown in Fig. 1, plasmid pSTY was engineered by inserting the purified 12.2-kb *Eco*RI fragment of pUE14 into *Eco*RI-digested pUCL50. Remarkably, *E. coli* W harboring plasmid pSTY was now able to grow on styrene as the sole carbon and energy source. Moreover, when plasmid pSTY was transformed into *E. coli* W14 (an *E. coli* W mutant having a complete deletion of the phenylacetate catabolic pathway) (13) and the recombinant cells were grown on glycerol in the presence of styrene, we observed the accumulation in the culture medium of large amounts of a compound that cochromatographed on HPLC with standard phenylacetate. Gas chromatography-mass spectrometry analyses of the accumulated product confirmed its identity with phenylacetate. Taken together, all of these results suggested that plasmid pSTY contained the complete gene cluster of the styrene upper catabolic

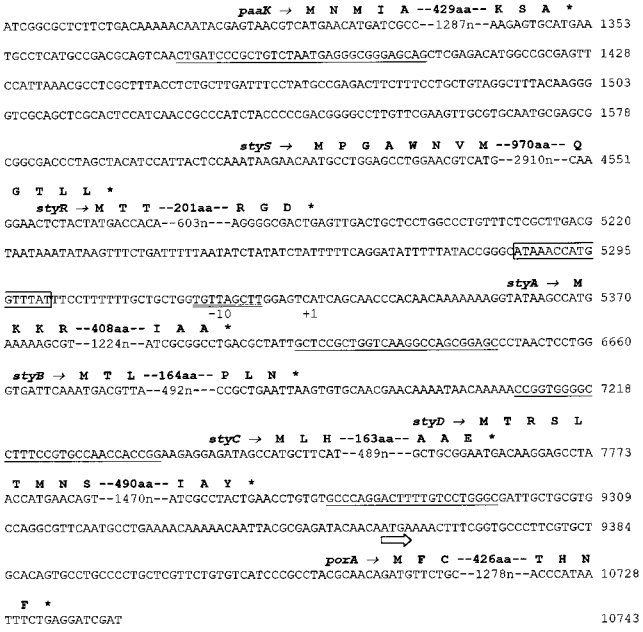

FIG. 2. Nucleotide and derived amino acid sequences of the styrene catabolic pathway. Only the sequences of the 5′- and 3′-end coding regions of the genes are shown. Small arrows show the direction of gene transcription. Asterisks indicate the stop codons. Sequence comparison between the region upstream of *porA* and the equivalent region in the *sty* operon from *P. fluorescens* ST (6) revealed that in *Pseudomonas* sp. strain Y2, a duplication of seven nucleotides (GCGAGCCgcgagcc) at position 9469 could be responsible for the truncation of a longer reading frame that probably started at nt 9357 (large open arrow). The putative transcription termination sequences are underlined. The proposed extended −10 promoter box is double underlined. +1 indicates the transcription start site of the *sty* operon. The postulated StyR-binding site is boxed.

pathway involved in the transformation of styrene into phenylacetate.

Sequence analysis. To genetically characterize the styrene upper catabolic cluster of *Pseudomonas* sp. strain Y2, the 15.5-kb insert of plasmid pSTY was sequenced. The nucleotide sequence of the 10,743-bp right region of the insert is shown in Fig. 2. Computer analysis of this sequence revealed the presence of eight open reading frames (ORFs) which may encode the putative PaaK, StyS, StyR, StyA, StyB, StyC, StyD, and PorA proteins (Fig. 2). Databases were searched for similar proteins, and those showing the highest similarity values were then retrieved and compared with the sequences obtained in this study (Table 1). An overall analysis revealed that genes *styABCD* were nearly identical to the *styABCD* genes of the styrene catabolic cluster from *P. fluorescens* ST (Table 1), and the order of these genes corresponded to that of the catabolic steps (Fig. 1). Two regulatory genes (*stySR*), an ORF (*porA*) encoding a potential truncated outer membrane protein, and the *paaK* gene, which codes for a phenylacetyl-coenzyme A ligase, were also identified (Fig. 1 and 2).

(i) Catabolic genes. The *styA* gene encodes a protein of 46,639 Da (415 amino acids [aa] long) (Fig. 2) that shows similarity to several bacterial flavin-type aromatic hydroxylases (Table 1) and contains the three flavin adenine dinucleotide-binding regions also observed in other flavin monooxygenases (11, 43).

The *styB* gene encodes a protein of 18,364 Da (170 aa long) (Fig. 2) that shows similarity to the SnaC, ActRV, NtaB, and NmoB flavin mononucleotide (FMN) oxidoreductases (Table 1) (7, 21, 39, 46). On the other hand, a significant similarity was also observed between StyB and the coupling proteins of the two-component 4-hydroxyphenylacetate-3 monooxygenases from *E. coli* (33) and *Klebsiella pneumoniae* (14) (Table 1). Although no enzymatic activity has been ascribed to these coupling proteins, they appear to play an important role to discriminate substrate analogs that can be hydroxylated by the flavin-containing component (33). Remarkably, it has been shown that StyB is essential for the complete activity of styrene monooxygenase (6). Therefore, all of these data strongly suggest that StyB may be the small subunit of a two-component monooxygenase.

The *styC* gene encodes a protein of 18,028 Da (169 aa long) (Fig. 2) which shows no significant similarity to other known proteins with the exception of the epoxystyrene isomerase of *P. fluorescens* ST (6). The lack of similarity between StyC and the isomerases of other aromatic catabolic pathways could be ascribed to the unusual substrate of this enzyme.

The *styD* gene shows two putative ATG start codons at nt 7759 and 7777. However, while the second start codon was preceded by a putative ribosome-binding site (RBS) (AAGG AG), the first one overlaps the stop codon of the preceding *styC* gene (Fig. 2). The *styD* stop codon was located at nt 9265 (Fig. 2). The putative largest version of StyD is a protein of 53,502 Da (502 aa long) that shows similarity to many prokaryotic and eukaryotic aldehyde dehydrogenases (Table 1). Surprisingly, the similarity between StyD and the PadA and FeaB phenylacetaldehyde dehydrogenases recently characterized in *E. coli* W (13) and *E. coli* K-12 (15), respectively, is not higher than that observed between StyD and other aldehyde dehydrogenases with different substrate specificity. The StyD sequences 249-FTGSTEVG-256 and 298-AIFFNHGQVCTA-309, respectively, match the consensus NAD^+-or $NADP^+$-binding site motif and the active-site motif spanning the catalytic cysteine (underlined) of aldehyde dehydrogenases (18, 24). Moreover, the conserved glutamic acid residue that has been shown to bind to the adenine ribose of NAD^+ (24) is also found (Glu-201) in StyD.

The ORF named *porA* shows a potential ATG start codon, which it is not preceded by a typical RBS, and a stop codon at nt 9434 and 10733, respectively (Fig. 2). The deduced PorA is a protein of 45,906 Da (433 aa long) that shows a significant similarity to some outer membrane proteins (Table 1), although it lacks the typical N-terminal signal sequence involved in secretion and integration of the protein into the membrane. This fact, together with the observation that a similar ORF is completely truncated in *P. fluorescens* ST, strongly suggests that PorA may not play a relevant role in the catabolism of styrene in these bacteria.

It had been proposed that styrene was catabolized via 2-phenylethanol in *Pseudomonas* sp. strain Y2 (40). However, the similarity of the *sty* catabolic pathway reported here and that of *P. fluorescens* ST (6) suggests that styrene in strain Y2 can be degraded by a pathway that does not involve 2-phenylethanol as an intermediate.

(ii) Regulatory genes. The *styS* and *styR* genes encode two proteins that show a high similarity with members of the superfamily of two-component signal transduction systems found both in eukaryotes and prokaryotes (3, 32, 35). The *styS* gene shows two putative ATG start codons at nt 1618 and 1639, respectively (Fig. 2). The stop codon of *styS* was located at nt 4564 and overlaps the putative ATG start codon of *styR* (Fig. 2), suggesting that the two genes are likely expressed in a translationally coupled fashion. The large version of StyS is a protein of 108,758 Da (982 aa long) that shows similarity to many sensor histidine kinase proteins (Table 1). Specially relevant was the high similarity between StyS and the TodS and

TABLE 1. Styrene pathway genes, their products, and identities with other proteins[a]

Gene	% G+C content	Gene product (aa/kDa)	Similar polypeptide (aa)	% Identity	Organism	Accession no.
paaK	63.6	437/49.1	Pcl (439)	86.3	*Pseudomonas putida* U	Z71175
			o437 (437)	67.3	*Escherichia coli* K-12	AE000236
			PaaK (437)	67.3	*E. coli* W	X97452
styS	56.8	982/108.8	TutC (979)	46.5	*Thauera* sp. strain T1	U57900
			TodS (978)	41.2	*P. putida* F1	U72354
			NodV (889)	24.5	*Bradyrhizobium japonicum*	P15939
			BpdS (1576)	18.3	*Rhodococcus* sp. strain M5	U85412
			FixL (505)	18.3	*Rhizobium meliloti*	P10955
styR	52.9	207/23.3	TutB (218)	51.4	*Thauera* sp. strain T1	U57900
			NodW (227)	45.8	*B. japonicum*	P15940
			TodT (227)	42.4	*P. putida* F1	U72354
			FixJ (204)	41.1	*R. meliloti*	P23221
			BpdT (209)	26.7	*Rhodococcus* sp. strain M5	U85412
styA	58.8	415/46.6	StyA (415)	94.5	*P. fluorescens* ST	Z92524
			PobA (394)	22.3	*P. aeruginosa*	X68438
			NahG (434)	20.6	*P. putida* PpG7	P23262
			MhpA (554)	18.1	*E. coli* K-12	Y09555
styB	57.3	170/18.4	StyB (170)	94.1	*P. fluorescens* ST	Z92524
			SnaC (176)	29.2	*Streptomyces pristinaespiralis*	P54994
			ActRV (177)	24.1	*S. coelicolor* A3(2)	X63449
			HpaH (170)	22.3	*Klebsiella pneumoniae* M5a1	L41068
			HpaC (170)	21.2	*E. coli* W	Z29081
			NmoB (322)	18.6	*Chelatobacter heintzii*	L49438
			NtaB (335)	17.9	*C. heintzii*	U39411
styC	55.9	169/18.0	StyC (169)	92.3	*P. fluorescens* ST	Z92524
styD	59.4	502/53.5	StyD (502)	97.8	*P. fluorescens* ST	Z92524
			CymC (494)	45.9	*P. putida* F1	U24215
			ALDH Mt (498)	45.5	*Leishmania tarentolae*	S43184
			PadA (499)	45.3	*E. coli* W	X97453
			FeaB (500)	45.1	*E. coli* K-12	X99402
			ALDH Ct (501)	44.6	*Ovis aries*	P51977
porA	58.8	433/45.9	CumH (457)	51.0	*P. fluorescens* IPO1	D83955
			XylN (464)	47.6	*P. putida* mt-2	D63341
			TodX (453)	41.8	*P. putida* F1	P10384
			CymD (460)	40.3	*P. putida* F1	U24215

[a] Sequences included in this analysis: Pcl, phenylacetyl-coenzyme A ligase of the phenylacetate catabolic pathway; PaaK, phenylacetyl-coenzyme A ligase of the phenylacetate catabolic pathway (12); o437, protein of unknown function; TutC and TutB, sensor histidine kinase and response regulator of the toluene anaerobic catabolic pathway, respectively; TodS and TodT, sensor histidine kinase and response regulator of the toluene aerobic catabolic pathway, respectively; BpdS and BpdT, sensor histidine kinase and response regulator of the biphenyl/polychlorobiphenyl catabolic pathway, respectively; NodV and NodW, sensor histidine kinase and response regulator of the nodulation genes, respectively; FixL and FixJ, sensor histidine kinase and response regulator of the nitrogen fixation genes, respectively; StyA and StyB, styrene monooxygenase components; PobA, 4-hydroxybenzoate 3-hydroxylase; NahG, salicylate hydroxylase; MhpA, 3-(3-hydroxyphenyl) propionate hydroxylase; SnaC, NADH:FMN oxidoreductase of the pristinamycin II_A synthase; ActRV, NADH:FMN oxidoreductase of actinorhodin synthesis, dimerase; HpaH and HpaC, coupling proteins of 4-hydroxyphenylacetate 3-hydroxylase of the 4-hydroxyphenylacetate catabolic pathway; NmoB and NtaB, NADH:FMN oxidoreductase of the nitrilotriacetate catabolic pathway; StyC, epoxystyrene isomerase; StyD, phenylacetaldehyde dehydrogenase of the styrene catabolic pathway; CymC, *p*-cumic aldehyde dehydrogenase; ALDH Mt, mitochondrial aldehyde dehydrogenase (NAD^+) precursor; PadA and FeaB, phenylacetaldehyde dehydrogenases of the 2-phenylethylamine catabolic pathway; ALDH Ct, cytosolic aldehyde dehydrogenase; CumH, XylN, TodX, and CymD, proposed outer membrane proteins and putative transporters of cumene, toluene/xylene, and *p*-cymene, respectively.

TutC sensor hybrid histidine kinases that regulate the aerobic and anaerobic catabolism of toluene in *P. putida* F1 (23) and *Thauera* sp. strain T1 (9), respectively (Table 1 and Fig. 3). These three proteins, together with the BpdS sensor kinase from *Rhodococcus* sp. strain M5, which responds to biphenyl and polychlorobiphenyl (22), are the unique sensors of the two-component regulatory systems involved in the metabolism of aromatic compounds that have been described so far. The N terminus of StyS (aa 1 to 75) resembles the basic region leucine zipper motif that mediates protein dimerization and DNA binding of TodS (23) (Fig. 3). Another interesting feature of StyS, shared only by TodS and TutC, is the presence of a receiver domain flanked by two canonical kinase domains (Fig. 3). The sequence (≈aa 570 to 754; Fig. 3, INPUT2) that resembles the putative oxygen-sensing domain of TodS (23) contains the highly conserved S1 and S2 sensory boxes present in PAS domains (47) (Fig. 3), suggesting that this region may act as an input domain in StyS. On the other hand, the N-terminal location of the input domains in most sensor kinases (32, 35) points to the existence of another input domain in StyS (Fig. 3, INPUT1; ≈aa 76 to 187).

The *styR* gene encodes a protein of 23,343 Da (207 aa long) (Fig. 2) that shows a significant similarity to many response regulators of two-component systems (Table 1). Amino acid sequence alignments revealed that StyR was highly similar to the response regulators TodT (23) and TutB (9) (Table 1 and Fig. 4), a result that is in agreement with the high similarity found among the cognate sensory proteins StyS, TodS, and TutC (see above). The residues Asp-11, Asp-12, Asp-55, Thr-83, and Lys-105 of StyR are highly conserved in the receiver domains of other response regulators (4, 32, 41) (Fig. 4). By analogy with TodT (23) and other receiver modules, Asp-11, Asp-12, and Asp-55 are predicted to form an acid pocket where Asp-55 may be the acceptor of the phosphoryl group

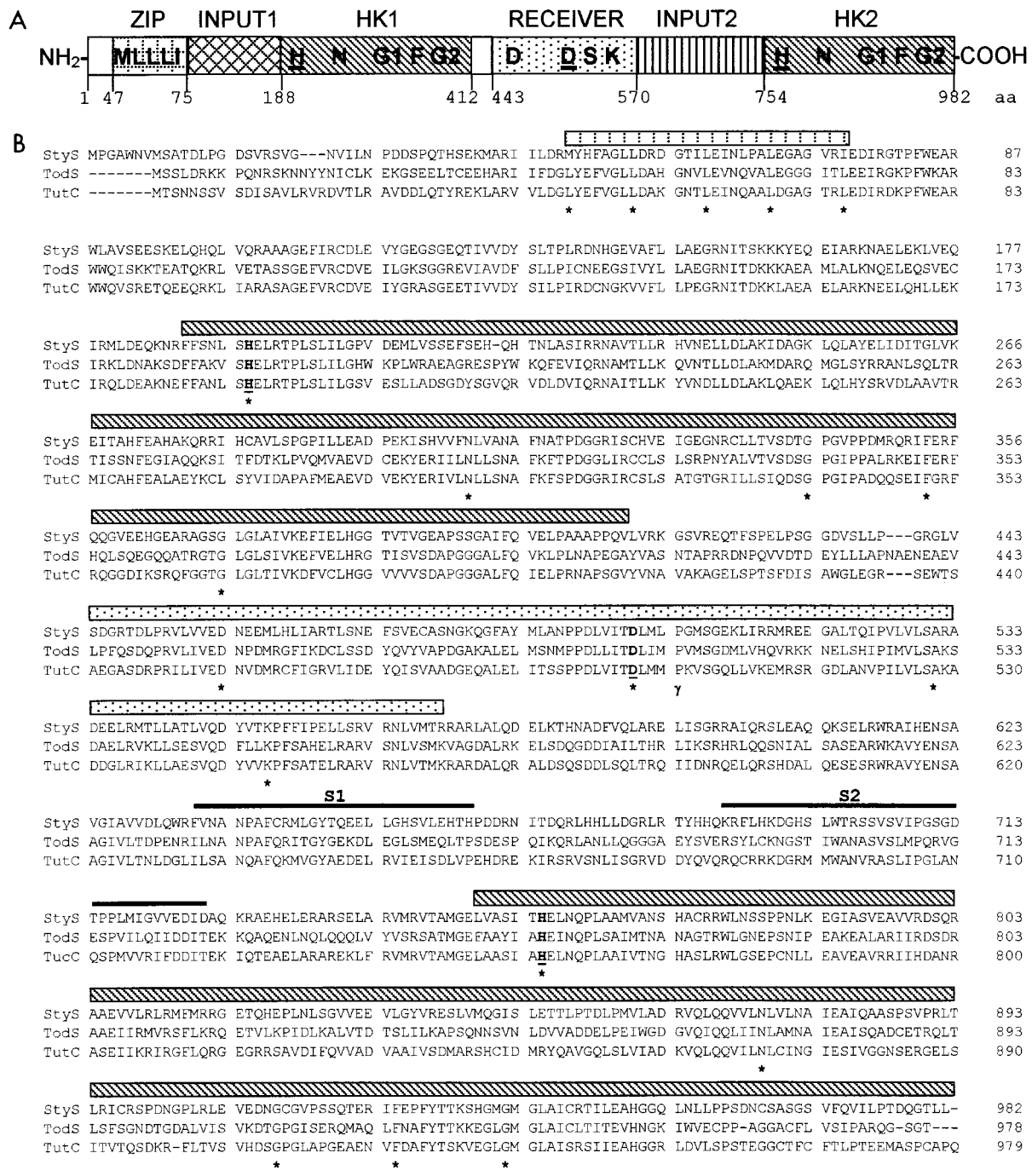

FIG. 3. Schematic domain structure of StyS (A) and alignment of its amino acid sequence with those of TodS and TutC sensor kinases (B). Amino acid residues, indicated by the standard one-letter code, are numbered on the right. Separate domains are indicated by differently shaded rectangles. A putative leucine zipper (ZIP) characterized by the repeating heptads (MLLLI) is shown. HK1 and HK2 are two histidine kinase domains characterized by the conserved amino acid blocks known as **H**, N, G1, F, and G2 (32). Histidine residues that may be phosphorylated in HK1 and HK2 are underlined and in boldface. The receiver domain (RECEIVER) contains the conserved D**D**SK residues characteristic of bacterial response regulators (4, 32, 41); the conserved serine (S) is often replaced by threonine (T) (Fig. 4). The aspartic acid residue that may be phosphorylated is underlined and indicated in boldface. The rigid group of the γ-turn loop is shown (γ). The putative input domains are represented as INPUT1 and INPUT2. Asterisks show the position of the most relevant conserved residues detailed in the StyS domain structure. S1 and S2 indicate the locations of the sensory boxes (47).

from the phosphorylated StyS sensor. The C terminus of StyR contains a sequence (aa 143 to 185) that has 17 of the 19 consensus residues of the DNA-binding domain of the LuxR/FixJ family 3 DNA-binding domains (31, 35) (Fig. 4). A putative Q-linker (aa 121 to 136) (45) between the receiver and DNA-binding modules was also observed in StyR (Fig. 4). Interestingly, StyR shows a significant similarity with NodW and FixJ (Table 1 and Fig. 4), two response regulators that

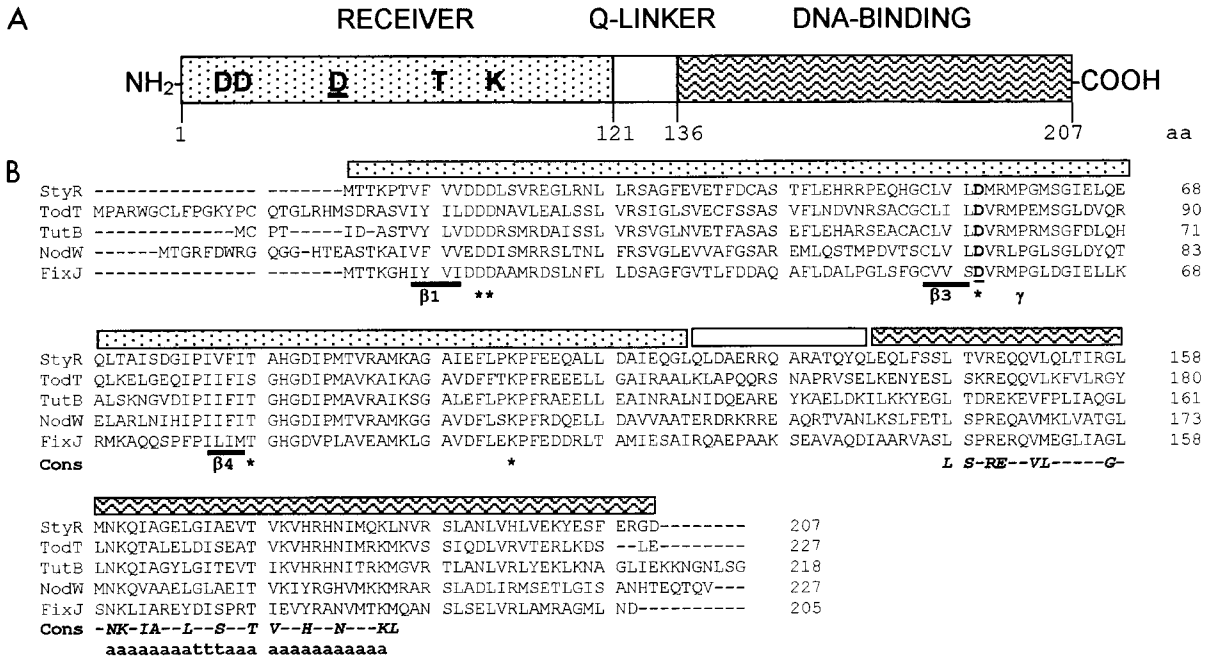

FIG. 4. Schematic domain structure of StyR (A) and alignment of its amino acid sequence with that of other bacterial response regulators (B). The sources of TodT, TutB, NodW, and FixJ sequences are indicated in Table 1. Amino acid residues, indicated by the standard one-letter code, are numbered on the right. Separate domains are shown by differently shaded rectangles. Shading code corresponds to that of the bars at the top of the aligned sequences. The receiver domain (RECEIVER) contains the conserved D**D**TK residues characteristic of bacterial response regulators and are located in the alignment by asterisks (4, 32, 41). The aspartic acid residue that may be phosphorylated is underlined and indicated in boldface. The rigid group of the γ-turn loop is also shown (γ). A predicted Q-Linker region (45) between the receiver and DNA-binding domains is indicated. Amino acids underlined by solid bars correspond to the three internal β-strands (β1, β3, and β4) of the prototype CheY response regulator structure (4). The consensus sequence (Cons) of putative DNA-binding domains in the family 3 response regulators as tabulated by Pao and Saier (31) is in boldface italics. The α-helix (a)/turn (t)/α-helix (a) secondary structure typical of DNA-binding domains is also indicated.

have been classified within the cluster 1 receiver modules that contain family 3 DNA-binding domains (35). Thus, StyR could be considered another example of domain shuffling that occurred during the parallel evolution of family 3 DNA-binding domains with cluster 1 receiver modules (35).

(iii) The first gene of the styrene lower pathway. The *paaK* gene shows two putative ATG start codons at nt 39 and 45, but only the first one is preceded by a putative RBS (Fig. 2). The *paaK* stop codon was located at nt 1350, and 23 bp downstream of this codon a 32-bp palindromic sequence ($\Delta G = -17.2$ kcal/mol) that may act as a transcription terminator can be found (Fig. 2). The large version of PaaK is a protein of 49,074 Da (437 aa long) that shows high similarity to the phenylacetyl-coenzyme A ligase (Pcl) of *P. putida* U, an enzyme that catalyzes the first step, i.e., the activation of phenylacetate to phenylacetyl-coenzyme A, in the aerobic phenylacetate degradation pathway of this strain (28). The AMP-binding site typical of acyl-coenzymeA-activating enzymes (28) can be also found in the primary structure of PaaK (96-SSGTTGKPTV-105).

To experimentally demonstrate that *paaK* encodes a phenylacetyl-coenzyme A ligase, we have cloned the gene under the control of the *lac* promoter in plasmid pUC18. Expression of *paaK* from the resulting plasmid pPAAK (Fig. 1) was assayed in *E. coli* W14, a mutant of *E. coli* W devoid of the endogenous phenylacetyl-coenzyme A ligase activity (12, 13). Interestingly, while *E. coli* W14(pPAAK) cell extracts showed a high phenylacetyl-coenzyme A ligase activity (130 U/mg of protein), control extracts of *E. coli* W14(pUC18) cells exhibited no detectable activity. This result confirmed that PaaK is involved in the activation of phenylacetate to phenylacetyl-coenzyme A. On the other hand, while *Pseudomonas* sp. strain Y2 cultured in 20 mM glycerol-containing medium had no detectable phenylacetyl-coenzyme A ligase activity, cells grown in a styrene-saturated atmosphere or with 5 mM phenylacetate as the sole carbon source showed a significant activity (25 to 50 U/mg of protein). Thus, these results strongly suggest that a phenylacetyl-coenzyme A ligase is involved in the catabolism of styrene in *Pseudomonas* sp. strain Y2.

It is worth noting that PaaK shows also a high similarity to the putative products of the open reading frames o437 from *E. coli* K-12 and *paaK* from *E. coli* W (Table 1). The *paaK* gene of *E. coli* W has been located within the gene cluster encoding a phenylacetate catabolic pathway (12). This cluster has been cloned in a plasmid (pFA2) (13) which confers to the phenylacetate-minus mutant *E. coli* W14 strain the capacity to use this compound as the sole carbon and energy source (13). Analysis of the sequence upstream of *paaK* in *Pseudomonas* sp. strain Y2 revealed that this region encodes several proteins of unknown function that are also coded by plasmid pFA2 and that have been found to be essential for phenylacetate catabolism in *E. coli* W (12). All of these results strongly suggest that the styrene lower and upper catabolic pathways are contiguous in the chromosome of *Pseudomonas* sp. strain Y2, PaaK being the phenylacetyl-coenzyme A ligase that catalyzes the first step in the styrene lower pathway.

Although the overall G+C content (57.7%) of the 10.7-kb DNA fragment (Fig. 2) is close to that of the genus *Pseudomonas* (60%) (codon usage tabulated from GenBank), there is a difference between the G+C content of the *stySRABCDporA* genes (Table 1) and that of *paaK* (Table 1) and the upstream genes present in plasmid pSTY. The G+C content (87.6%) at the third position of the *paaK* codons is also higher than that of the *sty* codons (66.4%). Therefore, these values may suggest

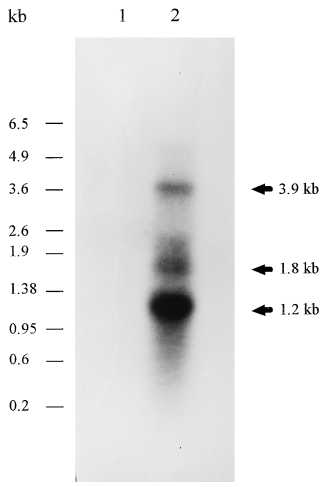

FIG. 5. Northern analysis of the *Pseudomonas* sp. strain Y2 *styABCD* genes. Total RNA (10 μg) was isolated from cells grown at 30°C in minimal medium M63 containing 20 mM glycerol (lane 1) or styrene (lane 2) as the sole carbon and energy source and then probed with a radioactively labeled *Sal*I-*Pst*I 1.5-kb fragment of plasmid pISM7 containing the *styAB* genes (Fig. 1). The sizes of RNA molecular weight markers (Promega) are indicated on the left; arrows on the right show the sizes and positions of the major transcripts.

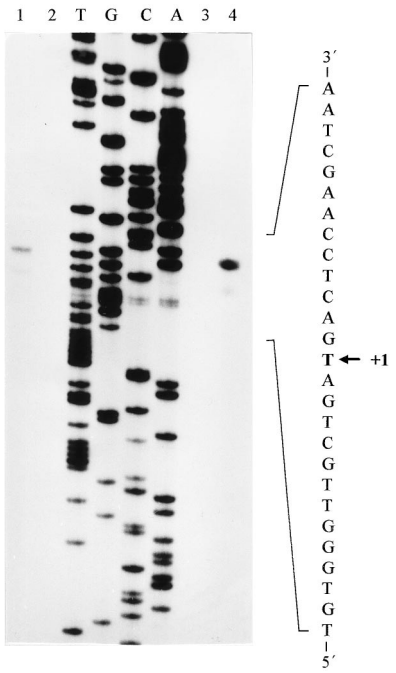

FIG. 6. Identification of the 5′ transcription start site of the *sty* operon. Total RNA from *Pseudomonas* sp. strain Y2 was purified from cells grown at 30°C in minimal medium M63 containing styrene (lane 1) or 20 mM glycerol (lane 2) as the sole carbon source. Total RNA from *E. coli* DH5α(pSTY) was purified from cells grown at 30°C in LB medium in the absence (lane 3) or presence (lane 4) of styrene. The size of the extended products was determined by comparison with a DNA-sequencing ladder of the *Psty* promoter region (T, G, C, and A), using plasmid pSTY as the template. The primer extension and sequencing reactions were performed with the same primer (5′-CGATCAGTGTACACAGTGACG TCG-3′), which hybridized at 80 nt downstream of the *styA* start codon. To the right, an expanded view of the nucleotide sequence surrounding the transcription initiation site (+1) is shown. Note that the sequence corresponds to the coding strand.

that the styrene upper and lower clusters have not evolved together.

Transcription analyses of the styrene catabolic genes. The genetic arrangement of the *styABCD* coding sequences suggests the possible cotranscription of these genes. To determine whether the genes *styABCD* form part of the same transcription unit, a Northern blot analysis was performed by using a *styAB*-specific probe and total RNA isolated from *Pseudomonas* sp. strain Y2 cells grown minimal medium containing glycerol or styrene as the sole carbon source. While three RNA bands of about 3.9, 1.8, and 1.2 kb were observed in styrene-grown cells, no hybridizing band was detected in the RNA sample isolated from cells cultivated in the absence of styrene (Fig. 5). The hybridizing band of 3.9 kb perfectly matches a styrene-induced transcription unit encompassing the *styABCD* genes. This 3.9-kb transcript is consistent with the presence of a palindromic sequence ($\Delta G = -21.2$ kcal/mol) at the 3′ end of the *styD* gene (Fig. 2) which could act as a transcriptional terminator. Interestingly, two putative stem-loop structures showing ΔG values of −25.3 and −27.0 kcal/mol were found downstream of the *styA* and *styB* genes, respectively (Fig. 2). Thus, the existence of potential *styA* and *styAB* transcripts could explain the 1.2- and 1.8-kb hybridizing bands (Fig. 5), respectively. Nevertheless, the possibility that these two bands were the result of incomplete transcription or of RNA degradation or processing cannot be ruled out.

To determine the transcription initiation site of the *styABCD* catabolic operon, primer extension analyses were performed with total RNA isolated from styrene-induced and uninduced *E. coli* DH5α(pUE14) and *Pseudomonas* sp. strain Y2 cells. Under induced conditions, a transcription initiation site located 33 nt upstream from the ATG translation initiation codon of the *styA* gene was identified both in the homologous and heterologous hosts (Fig. 6). In contrast no transcription initiation site of the *sty* operon was observed in the absence of styrene (Fig. 6). Therefore, these results revealed not only that expression of the styrene catabolic genes was very low in the absence of inducer but also that it was inducible in an heterologous host. On the other hand, all attempts to determine the transcription start site of the *stySR* operon were unfruitful, probably due to the low expression level of these genes, and further research needs to be done.

The low G+C content (36.4%) of the intergenic region upstream of *styA* is in agreement with the existence of a functional promoter. Thus, upstream of the identified transcriptional start site, a putative extended −10 box (TGTTAGCTT) (5) was observed (Fig. 2). The absence of a consensus −35 box typical of σ^{70}-dependent promoters would agree with StyR being a transcriptional regulator that binds to the *sty* promoter. In this sense, StyR contains a DNA-binding domain similar to that of the class 3 response regulators which appear to interact with RNA polymerases containing various sigma factors (35). Furthermore, a palindromic sequence (ATAAACCATGGTT TAT) centered at position −41 from the *styA* transcriptional start site (Fig. 2) was found to be nearly identical to the inverted repeat (ATAAACCATcGTTTAT) (lowercase letter indicates a mismatch) that binds to the analogous TodT response regulator in the *tod* operon (*tod* box) (23). This observation strongly suggests that the 8-bp inverted repeat in the *sty* promoter could be the StyR-binding site (*sty* box). However, a striking difference between the *sty* and *tod* boxes is that whereas the latter is centered at −105 bp from the transcriptional start site (23), the former overlaps the putative −35 promoter region. Hence, it can be predicted that in the *tod* and *sty* operons there are different mechanisms of interaction be-

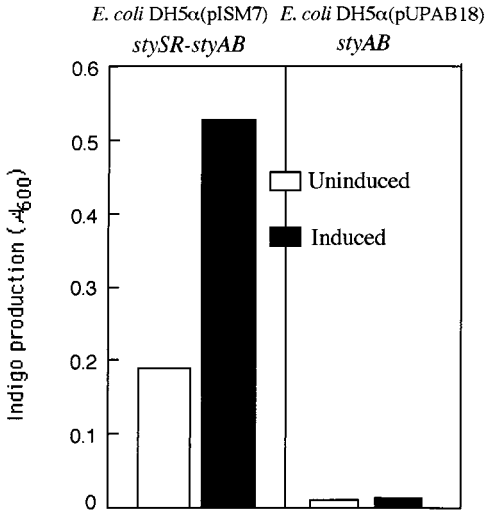

FIG. 7. Assessment of styrene monooxygenase expression in *E. coli*. Expression of the *styAB* genes in the presence (plasmid pISM7) or absence (plasmid pUPAB18) of *stySR* genes was monitored by measuring indigo production. *E. coli* DH5α(pUPAB) and *E. coli* DH5α(pISM7) cells were grown in LB medium at 30°C until the cultures reached an A_{600} of 0.4. Styrene was then added to half of each culture, and growth was resumed until cultures reached an A_{600} of 0.8. Cells were collected by centrifugation, washed with 0.1 M sodium phosphate buffer (pH 7.0), and resuspended in the same buffer (0.05 ml of buffer/ml of culture). The cell suspension was incubated with stirring at 30°C in the presence of 1 mM indole. After 60 min of incubation, indigo produced was assayed.

tween the RNA polymerase and the TodT and StyR regulators, respectively.

Regulation of the styrene catabolic cluster. Experimental evidence that the genes *styS* and *styR* were required for expression of the *styABCD* catabolic operon emerged from the genetic studies involving *E. coli* cells harboring plasmids pUPAB18 (pUC18 derivative that harbors a 2.2-kb *Bgl*II-*Sma*I fragment containing the *styAB* genes) (Fig. 1) and pISM7 (pUC18 derivative that harbors a 6.6-kb *Sma*I fragment containing the *stySR* and *styAB* genes) (Fig. 1). Despite the presence of the styrene monooxygenase-encoding genes (*styAB*) necessary for the indole-indigo conversion, the production of indigo was negligible in *E. coli* DH5α(pUPAB18) cells grown in the presence or absence of styrene (Fig. 7). However, when genes *styS* and *styR* were provided in *cis*, *E. coli* DH5α(pISM7) cells showed a high production of indigo that was inducible by styrene (Fig. 7). These results indicate that the StySR two-component system is functional in *E. coli* and behaves as a positive regulator of the *sty* promoter. A similar finding was observed with the homologous TodST system that also activates the expression of the *tod* structural genes in *P. putida* F1 (23).

It is worth noting that even in the absence of styrene, *E. coli* DH5α(pISM7) cells produced higher amounts of indigo than *E. coli* DH5α(pUPAB18) cells (Fig. 7). This observation can be explained by a low induction effect of indole on styrene monooxygenase expression, as it has been shown in *P. putida* CA-3 (30). Nevertheless, we cannot rule out the possibility that StyR can be phosphorylated by metabolic cross talk carried out either by a noncognate host sensor or by a chemical phosphorylating agent such as phosphoramidate or acetyl phosphate (32). The same argument has been used to explain the *trans*-acting effect of TodT in *E. coli* as a positive regulator of the *tod* operon in the absence of an intact *todS* gene (23). Finally, it is also known that positive regulators can activate their controlled genes in the absence of inducer when they are overproduced from high-copy-number vectors (26).

In summary, the results presented here represent the first report on the mechanism of regulation of a styrene catabolic pathway. A complex two-component regulatory system (StySR) has been identified and shown to be functional in a heterologous host. Engineering chimeric regulatory proteins by interchanging equivalent domains of the StySR, TodST, and TutCB systems will provide new insights into the structure-function relationships of these domains. On the other hand, we have shown here that the styrene upper catabolic cluster of *Pseudomonas* sp. strain Y2 confers to *E. coli* W the ability to grow on styrene as the sole carbon source. Thus, it will be of biotechnological interest engineering the styrene upper and lower catabolic pathways as a transposable DNA cassette to expand the catabolic abilities of environmentally relevant microorganisms endowed with a high solvent tolerance for styrene removal.

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