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Cloning and Characterization of Styrene Catabolism Genes from *Pseudomonas fluorescens* ST†

ANDREA M. MARCONI,[1] FABRIZIO BELTRAMETTI,[2] GIUSEPPINA BESTETTI,[2] FRANCESCA SOLINAS,[1] MAURIZIO RUZZI,[3] ENRICA GALLI,[2] AND ELISABETTA ZENNARO[1,4]*

Department of Cellular and Developmental Biology, "La Sapienza" University of Rome, 00185 Rome,[1] Department of Genetics and Biology of Microorganisms, University of Milan, 20133 Milan,[2] Department of Biology, Third University of Rome, 00154 Rome,[4] and Department of Agrobiology and Agrochemistry, "La Tuscia" University, 01100 Viterbo,[3] Italy

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A gene bank from *Pseudomonas fluorescens* ST was constructed in the broad-host-range cosmid pLAFR3 and mobilized into *Pseudomonas putida* PaW340. Identification of recombinant cosmids containing the styrene catabolism genes was performed by screening transconjugants for growth on styrene and epoxystyrene. Transposon mutagenesis and subcloning of one of the selected genome fragments have led to the identification of three enzymatic activities: a monooxygenase activity encoded by a 3-kb *Pst*I-*Eco*RI fragment and an epoxystyrene isomerase activity and an epoxystyrene reductase activity encoded by a 2.3-kb *Bam*HI fragment. *Escherichia coli* clones containing the 3-kb *Pst*I-*Eco*RI fragment were able to transform styrene into epoxystyrene, and those containing the 2.3-kb *Bam*HI fragment converted epoxystyrene into phenylacetaldehyde or, only in the presence of glucose, into 2-phenylethanol. The three genes appear to be clustered and are probably encoded by the same DNA strand. In *E. coli*, expression of the epoxystyrene reductase gene was under the control of its own promoter, whereas the expression of the other two genes was dependent on the presence of an external vector promoter.

Styrene is an important chemical used in large quantities in the manufacturing of plastics, synthetic rubber, and resins. In view of their potential applications as biocatalysts in the removal of styrene in industrial wastes, styrene-utilizing microorganisms have been isolated (32). However, the data concerning styrene catabolism in bacteria are not abundant, and only during recent years have the enzymatic activities of some styrene-degrading strains been identified and partially characterized (10, 11, 19, 28, 30, 31). Two main styrene catabolism routes have been proposed: the first involves the oxidation of the side chain to phenylacetate (2, 11, 19, 22–24, 26, 28), and the second involves the initial oxidation of the aromatic nucleus (1, 4, 10, 30, 31). As far as the lateral-chain oxidation is concerned, an initial oxidation to epoxystyrene, which is subsequentially isomerized to phenylacetaldehyde, has been proposed (11, 19, 28). However, among several styrene-degrading strains analyzed, only in the culture broth of one *Pseudomonas* strain was a small amount of epoxystyrene detected (22). Recently, Nöthe and Hartmans isolated a *Pseudomonas putida* mutant which accumulates epoxystyrene, thus demonstrating that this compound is a real intermediate of styrene oxidation in that strain (18). 2-Phenylethanol has been associated with the microbial degradation of styrene, as this compound is often present in culture broths of styrene-grown cells and a 2-phenylethanol dehydrogenase activity can be detected in their cell extracts (2, 22, 24, 28). However, the question as to whether this compound is an obligate intermediate of styrene metabolism is still unanswered (10).

Because of these uncertainties and difficulties in the identification of the intermediates, we decided to isolate the catabolic genes from a styrene-degrading strain. So far, no information regarding the organization of the styrene-catabolic genes is available. Cloning of these genes could enable us to determine the catabolic pathway and its regulation and to express the single enzymatic activities; this information will be interesting in view of their potential applications in the chemical and pharmaceutical industries, utilization in biotransformations and in the production of metabolic intermediates. The cloning of styrene-catabolic genes has a good chance of being successful, since genes for the utilization of other aromatic compounds which have been isolated so far are not scattered on the chromosome but are organized in catabolic operons (29). The *Pseudomonas fluorescens* strain ST is able to grow on styrene as its sole carbon source. This strain was previously partially characterized, and the catabolic pathway proposed consists of an initial oxidation of the lateral chain to phenylacetate (2). In this paper, we report the isolation of the *P. fluorescens* ST genes involved in the first steps of styrene degradation and the identification of the intermediates accumulated by single recombinant clones.

MATERIALS AND METHODS

The bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown at 37°C (*Escherichia coli*) or 30°C (*P. putida* PaW340 and *P. fluorescens* ST) in Luria-Bertani medium (17), nutrient agar (Difco), or mineral M9 (17) medium supplemented with glucose (0.2%), tryptophan (40 μg per ml), thiamine (1 μg per ml), and proline (40 μg per ml), if required. The antibiotics and concentrations used were as follows: ampicillin, 100 μg per ml; kanamycin, 50 μg per ml; tetracycline, 25 μg per ml; chloramphenicol, 50 μg per ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM) and indole (100 μg per ml) were added to the media when appropriate. Phenylacetaldehyde and 2-phenylethanol at a final concentration of 0.1% were added to M9 liquid or solid media. Growth on styrene or epoxystyrene of *P. fluorescens* ST and of the

* Corresponding author. Mailing address: Department of Cellular and Developmental Biology, "La Sapienza" University, P.le A. Moro 5, 00185 Rome, Italy. Phone: 39-6-49912215. Fax: 39-6-49912351. Electronic mail address: Zennaro@axcasp.caspur.it.

† Dedicated to the memory of Francesca Solinas, who died suddenly on 16 September 1995. She contributed greatly to this paper. She remains in the thoughts of all of her colleagues as a scientist and, above all, as a friend.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description[a]	Reference or source
Strains		
E. coli		
JM109	*recA1 endA1 gyrA96 thi hsdR17* (r_k^+ m_k^+)*supE44 relA1* λ^- Δ(*lac-proAB*)(F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)	33
S17-1	*recA pro thi hsdR* with chromosomally integrated RP4-2-*tet*::Mu-*kan*::Tn*7* Tra^+ Tri Str	25
RU4420	*thi-1 endA1 hsdR17 supE44 trp*::Tn*1725* Cm^r	27
P. fluorescens ST	Sty^+ Hg^r	2
P. putida PaW340	Trp Str^r	8
Plasmids		
pTZ18/19R	Ap^r	U.S. Biochemicals
pLAFR3	Broad-host-range cosmid vector derived from IncP1 plasmid pRK290; RK2 replicon λcos^+ *lacZ*α; Mob^+ Tra^- Tc^r	3
p907	Tc^r; pLAFR3 with a 27-kb fragment from *P. fluorescens* ST chromosome	This study
p3B6	Tc^r; pLAFR3 with a 32-kb fragment from *P. fluorescens* ST chromosome	This study
pTD9	Ap^r; pTZ19 with the 9-kb *Dra*I fragment from p907, containing the styrene-catabolic genes	This study
pTE50	Ap^r; pTZ19 with the 5-kb *Eco*RI fragment from pTD9	This study
pTE50R	Ap^r; this plasmid has the same structure as pTE50 but the 5-kb *Eco*RI fragment is in an inverted orientation	This study
pTX40	Ap^r; pTZ19 with the 4-kb *Xho*I fragment from pTE50	This study
pTV46	Ap^r; pTZ19 with the 4.6-kb *Eco*RV fragment from pTE50	This study
pTPE30	Ap^r; pTZ19 with the 3.0-kb *Pst*I-*Eco*RI fragment from pTE50	This study
pTB23	Ap^r; pTZ19 with the 2.3-kb *Bam*HI fragment from pTD9	This study
pTB23R	Ap^r; this plasmid has the same structure as pTB23 but the 2.3-kb *Bam*HI fragment is in an inverted orientation	This study

[a] Sty, styrene-metabolic phenotype; Ap^r, ampicillin resistance; Str^r, streptomycin resistance.

recombinant clones was performed in M9 solid medium under a saturated atmosphere of these aromatic compounds.

Construction and screening of *P. fluorescens* ST cosmid genomic library. High-molecular-weight genomic DNA from *P. fluorescens* ST was prepared by the procedure of Goldberg and Ohman (9). After partial digestion with *Sau*3A (0.025 U/μg of genomic DNA for 1 h at 37°C), the DNA was size fractionated by ultracentrifugation (26,000 rpm for 24 h at 18°C in a Beckman SW28 rotor) on a linear sucrose gradient (10 to 40%) in TS buffer (1 M NaCl, 20 mM Tris-HCl, 5 mM EDTA [pH 8]). Fractions were collected from the bottom of the tube and analyzed for DNA size in a 0.7% agarose gel in Loening buffer (15) at 1 V/cm. Those fractions containing molecules predominantly of 15 to 30 kb were pooled, dialyzed against TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.2]), and precipitated with ethanol. The cosmid pLAFR3 was digested with *Bam*HI to completion and treated with calf intestinal alkaline phosphatase. After phenol-chloroform extraction, the cosmid was precipitated and ligated in a 1:3 molar ratio to *P. fluorescens* ST DNA partially digested with *Sau*3A, by using T4 DNA ligase for 24 h at 14°C and for a further 24 h at 4°C. The ligation solution was packaged in vitro in lambda heads by using commercial extracts (Amersham). The bacteriophage particles were stored in chloroform-saturated phage dilution buffer (10 mM Tris-HCl, 10 mM $MgSO_4$, 0.01% gelatin [pH 7.4]) and used to transduce *E. coli* S17-1 according to the supplier's protocol. Approximately 10^5 tetracycline-resistant (Tc^r) colonies per μg of inserted DNA was obtained. A total of 3,500 individual cosmid clones were stored at −80°C in 96-well microtiter plates containing 200 μl of growth medium supplemented with 25 μg of tetracycline per ml. The library was screened by mating each cosmid clone from *E. coli* S17-1 to *P. putida* PaW340 by biparental mating. Briefly, each storage plate containing 96 *E. coli* S17-1 clones harboring the gene bank was replicated in a microtiter plate containing (per well) 100 μl of Luria-Bertani medium supplemented with 25 μg of tetracycline per ml, by using a 96-prong replicator. After growth, each clone was conjugated to *P. putida* PaW340 on Luria-Bertani solid medium and transconjugants were replica plated on M9 medium supplemented with tryptophan (40 μg per ml), tetracycline (25 μg per ml), and styrene or epoxystyrene as the sole carbon sources. The positive clones were used for further analysis.

DNA manipulation. Plasmid preparations, restrictions, and ligations were carried out by standard procedures (16). *E. coli* was transformed with plasmid DNA by the standard $CaCl_2$ method. DNA was transferred onto nylon filters (Hybond N; Amersham) according to the supplier's instructions. Purified DNA fragments were labelled with [α-^{32}P]dCTP (3,000 Ci/nmol; Amersham) by using the Random Priming Labelling kit (Boehringer). DNA deletions were performed with DNase I by following the method described by Lin et al. (14).

Transposon Tn*1725* mutagenesis. *E. coli* RU4420 harboring a chromosomal insertion of Tn*1725* (27) was transformed with p907. Transformants were selected on nutrient agar (Difco) containing 50 μg of chloramphenicol per ml and 25 μg of tetracycline per ml. Individual transformants were grown at 30°C to the stationary phase in Luria-Bertani medium supplemented with 50 μg of chloramphenicol and 25 μg of tetracycline per ml. Aliquots of 100 μl from each culture were plated on nutrient agar-chloramphenicol gradient plates (0 to 1,000 μg of chloramphenicol per ml) in the presence of 25 μg of tetracycline per ml. Colonies showing the highest levels of chloramphenicol resistance (Cm^r) were isolated on nutrient agar containing 500 μg of chloramphenicol and 25 μg of tetracycline per ml and further analyzed by restriction mapping to determine the location of the Tn*1725* insertions. Plasmids containing Tn*1725* in the genomic insert of p907 were used to transform *P. putida* PaW340, and transformants were tested for growth on styrene or epoxystyrene.

Culture conditions for metabolite analysis. Cells of *E. coli* JM109 transformed with pTB23, pTB23R, and pTPE30 were grown at 37°C with shaking in a 500-ml flask containing 100 ml of M9 medium supplemented with glucose (0.2%), thiamine (1 μg per ml), and ampicillin (100 μg per ml). When the cultures reached an A_{600} of 0.6, L-malic acid and IPTG were added at final concentrations of 0.2% and 1 mM, respectively. The cultures were then incubated at 37°C with shaking for 16 h. These growth conditions (addition of malic acid and cell growth to stationary phase) gave the best yield in terms of biotransformation products and reproducibility of results. After growth, cells were harvested, washed twice with phosphate buffer (0.1 M, pH 7), and resuspended in the same buffer to an A_{600} of 2.0. The cell suspensions (20 ml in a 100-ml flask) were incubated at 30°C with shaking in the presence of the appropriate aromatic compound, at a concentration of 30 mM, added directly into the media. When necessary, 0.2% of glucose was added to the incubation mixture. The flasks were tightly sealed, and aliquots (0.5 ml each) were withdrawn at different incubation times through a flexible plastic tube connecting the culture to a disposable syringe. Incubation of *E. coli* JM109(pTPE30) in the presence of styrene was also performed in a BM3000 2.5-liter reactor (Bioindustrie Mantovane, Mantua, Italy). Cells, grown as described previously, were resuspended in phosphate buffer to an A_{600} of 2.0. The cell suspension (2 liters) was transferred into the reactor and incubated at 30°C with stirring. Styrene was supplied via the gas phase by passing the incoming air flow (1.5 liters per min) through a flask containing 100 ml of dibutyl phthalate and 20 ml of styrene. The use of dibutyl phthalate as a substrate reservoir allowed the maintenance of styrene, in the incoming gas phase, at a nontoxic concentration for *E. coli* cells. To overcome the high rate of evaporation of the aromatic substrate and products, the exhaust air was chilled on dry ice and the condensed vapors were collected and analyzed.

Metabolite analyses. Styrene, epoxystyrene, phenylacetaldehyde, and 2-phenylethanol biotransformations were monitored at different times of incubation up to 24 h. Condensed vapors from the reactor and supernatants from the culture samples were directly analyzed by reverse-phase high-pressure liquid chromatography (HPLC), performed with a Waters 600E delivery system equipped with a Waters 486 detector, a Waters 746 integrator, and a Waters reverse-phase μBondapak C_{18} column (3.9 by 300 mm) eluted with acetonitrile-water (50:50) at a flow rate of 1 ml/min. The eluted compounds were detected at A_{254}, and their

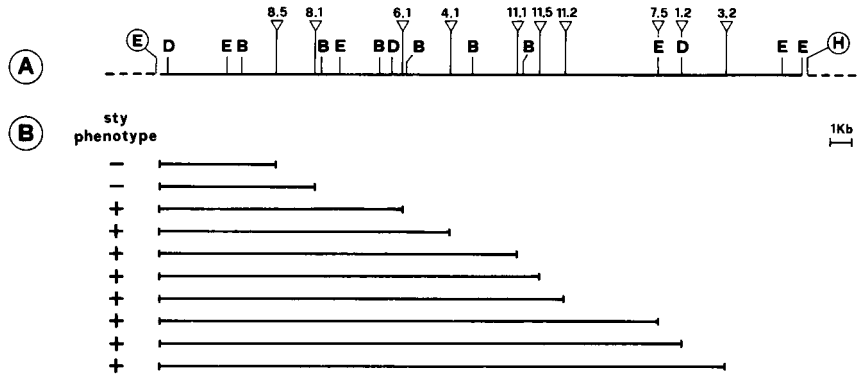

FIG. 1. (A) Restriction map of the 27-kb genomic insert of p907. Restriction sites: E, *Eco*RI; D, *Dra*I; B, *Bam*HI; H, *Hin*dIII. The circled restriction sites belong to the pLAFR3 vector. Insertions of Tn*1725* obtained by transposon mutagenesis are indicated by triangles, above which the names of the corresponding clones are shown. (B) Deletions of the 27-kb insert obtained as described in the text. The styrene degradation phenotype of the corresponding clones, positive (+) or negative (−), is also indicated.

retention time was compared with that of the authentic compounds. For proton nuclear magnetic resonance (^{1}H-NMR) analysis (carried out with Gemini XL200; Varian), cells were prepared as described above, exposed for 24 h to the substrate, and then removed by centrifugation. Supernatants from JM109 (pTB23) and JM109(pTB23R) cell suspensions were extracted with ethylacetate, and those from JM109(pTPE30) were extracted with hexane. Condensed vapors from the reactor were extracted when required. Aliquots of these samples were analyzed by thin-layer chromatography (Merck Silica Gel 60F254 plates), with hexane-acetate (8:2) as the solvent system. Compounds on thin-layer chromatography plates were detected by UV light and sulfuric acid and identified by comparison with standard chemicals run on the same plates. If more than one compound was present in a sample, the single components were purified by flash chromatography, performed with Merck Silica Gel 60 (40 to 63 μM, pH 7), eluted with the same solvent system as that used for thin-layer chromatography analysis. For ^{1}H-NMR analysis, 10 mg of each purified compound was dried under reduced pressure at 30°C and dissolved in deuterated chloroform. All chemicals used for the preparation of buffers and culture media were reagent grade or better; the solvents were from Merck, and the standards were from Sigma or Fluka.

RESULTS

Isolation of recombinant cosmids containing genes for styrene degradation. Total DNA from *P. fluorescens* ST was partially digested by *Sau*3A and ligated into the cosmid vector pLAFR3, cleaved by *Bam*HI. The ligated DNA was packaged into lambda phages and transfected into *E. coli* S17-1. The median size of the inserts was approximately 25 kb. About 3,500 recombinant cosmids were individually transferred by conjugation to *P. putida* PaW340, and transconjugants were screened for the ability to grow on styrene and epoxystyrene as their sole carbon source. *P. putida* PaW340 does not grow on styrene and epoxystyrene but can utilize phenylacetaldehyde, 2-phenylethanol, and phenylacetate. So, by this screening, only the first steps of the catabolic pathway could be selected, thus increasing the probability of detecting positive clones. We isolated six clones whose recombinant cosmids conferred a positive styrene and epoxystyrene phenotype to *P. putida* PaW340. For further analyses, we chose to utilize one of these recombinant cosmids, named p907. During the screening of *P. putida* PaW340 recombinant clones, we also isolated a clone able to grow on epoxystyrene, but not on styrene, named p3B6. Since strain ST contains a plasmid, named pEG (5), we performed Southern blot experiments with the selected fragments against chromosomal and purified plasmid DNAs. Results showed that the cloned fragments are contained in the chromosome (data not shown).

Localization of the styrene-catabolic genes in p907 by Tn*1725* mutagenesis and subcloning. A preliminary restriction map of the p907 recombinant cosmid showed that the inserted fragment was about 27 kb in length (Fig. 1A). To localize the catabolic genes within the cloned chromosomal fragment, we performed a transposon mutagenesis with Tn*1725*. The transposon source for mutagenesis was *E. coli* RU4420::Tn*1725* (27). This strain was transformed with p907, and after transposition occurred, cells were selected for growth on high concentrations of chloramphenicol, the selective marker of Tn*1725*. The resulting clones were screened, by restriction analysis, for the insertion of Tn*1725* into the chromosomal insert of p907. The positive clones were conjugated to PaW340, and transconjugants were analyzed for growth on styrene. Only two clones, 8-1 and 8-5, failed to grow on styrene. In these two clones, Tn*1725* was inserted in a 5-kb *Eco*RI fragment (Fig. 1A). The subcloning of this fragment showed that it was not sufficient to confer a styrene-positive phenotype (data not shown). To verify if the styrene-catabolic genes were clustered in a unique chromosomal region or scattered on the 27-kb fragment of p907, we performed a series of deletions of the chromosomal insert. To do this, we used the p907::Tn*1725* mutagenized cosmids, taking advantage of the presence of a *Hin*dIII site in the polylinker of pLAFR3 and of a *Hin*dIII site located inside Tn*1725* but outside its Cm^r gene. No other *Hin*dIII sites were present in p907::Tn*1725* recombinant cosmids. By digestion with this restriction enzyme and religation, we obtained several clones in which the chromosomal regions between the two *Hin*dIII sites had been deleted (Fig. 1B). The clones bearing deletions were screened for growth on styrene. Results (Fig. 1B) were consistent with a localization of the styrene-catabolic genes, probably organized in a cluster, in a limited region. In fact, the catabolic genes were found to be contained in a 9-kb region corresponding to a *Dra*I fragment, localized at one extremity of p907 (Fig. 1A). The deletion of the rest of the insert did not affect growth on styrene. The 9-kb *Dra*I fragment was cloned in pTZ19, and the recombinant plasmid (pTD9) was used to obtain a more detailed restriction map of this region (Fig. 2A). To localize the styrene-catabolic genes inside this fragment, we compared the other selected positive clones of the *P. fluorescens* ST genomic bank for the presence of sequences belonging to the 9-kb fragment. We also analyzed a clone, named 3B6, which grew on epoxystyrene but not on styrene. All the clones analyzed, except 3B6, retained two contiguous *Bam*HI fragments of 4.6 and 2.3 kb, indicated in Fig. 2A. The 4.6-kb fragment is included in the 5-kb *Eco*RI fragment whose inactivation by Tn*1725* insertion caused the loss of the styrene-positive phenotype. In the 3B6 clone, which grew on epoxystyrene but not on styrene, only the 2.3-kb *Bam*HI

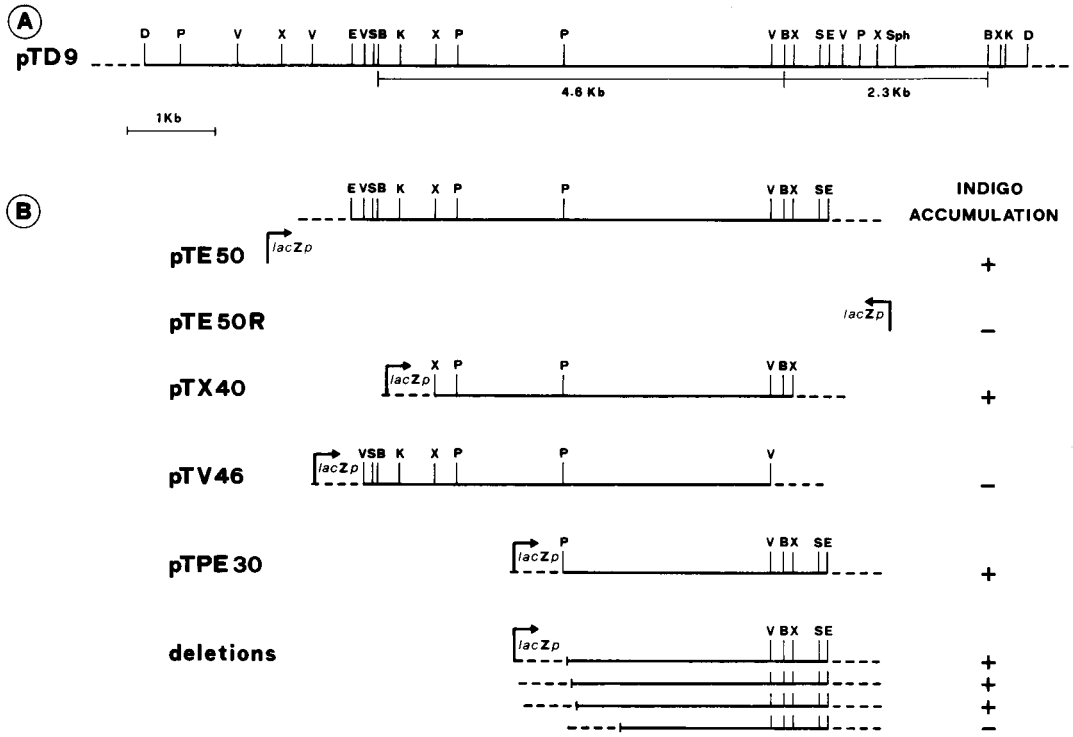

FIG. 2. (A) Restriction map of the 9-kb *Dra*I fragment cloned in pTZ19. The 4.6- and 2.3-kb *Bam*HI fragments common to all the styrene-positive clones of the *P. fluorescens* ST genomic bank are also shown. (B) Subcloning of the 9-kb *Dra*I fragment in pTZ19. The orientations of fragments in respect to the *lacZp* of pTZ19 are indicated by arrows. Deletions of the pTPE30 insert were obtained with DNase I. The ability of each subclone to accumulate indigo is indicated as positive (+) or negative (−). Restriction sites: E, *Eco*RI; B, *Bam*HI; D, *Dra*I; K, *Kpn*I; P, *Pst*I; S, *Sal*I; V, *Eco*RV; X, *Xho*I.

fragment was present. These data indicate that the genes essential for the first steps of styrene and epoxystyrene utilization, probably coding for a styrene monooxygenase and an epoxystyrene isomerase, are located in these two *Bam*HI fragments.

Identification of the p907 region coding for a styrene monooxygenase activity. To preliminarily identify the presence of the proposed styrene oxygenase gene, we cloned, in both orientations, the 5-kb *Eco*RI fragment (in which the 4.6-kb *Bam*HI fragment is included) in pTZ19, and we tested the ability of the *E. coli* JM109 transformants to oxidize indole to indigo, whose formation gives rise to blue colonies. The same test has been successfully used to detect other mono- and dioxygenase activities (7, 12, 13). As shown in Fig. 2B, we obtained indigo-positive clones only with the 5-kb *Eco*RI fragment in one orientation, indicating that the expression of the oxygenase gene(s) was dependent on the pTZ19 *lac* promoter (*lacZp*) activity. In fact, we could not observe blue colonies when pTE50R was used, even in the presence of the possible inducers, styrene and epoxystyrene, suggesting that promoter and/or regulatory elements are not present in this cloned fragment or are not effective in *E. coli* cells. To further restrict this region, we performed subcloning and deletion experiments on the 5-kb *Eco*RI fragment, and by the indole test, we could localize the putative oxygenase gene(s) inside the 2.5-kb region extending about 100 bp downstream from the *Pst*I site to the *Xho*I site (Fig. 2B). To obtain further evidence for the presence of the styrene monooxygenase activity being encoded by this region, we used JM109(pTPE30) to monitor the conversion of styrene into epoxystyrene by HPLC analysis. Styrene biotransformation assays were performed either in flasks or in a reactor, as described in Materials and Methods. Supernatants and condensed vapors from the reactor were analyzed for the presence of the biotransformation products, at different times of incubation up to 24 h. Styrene biotransformation was observed only when a high oxygen concentration was maintained in the culture by continuous air flow into the reactor. Under these conditions, small amounts of epoxystyrene, detected by HPLC analysis, were found in the cultural media (data not shown), probably because of the low solubility and the high volatility of this compound, while larger amounts of epoxystyrene were present in the condensed vapors emitted from the reactor (approximately 85% of the total compounds eluted from the column, as derived from absorbance and extinction coefficients of the two compounds) (Fig. 3). The styrene biotransformation product was also identified as epoxystyrene by ^{1}H-NMR analysis (data not shown). No epoxystyrene formation was observed in JM109(pTZ19) cultures used as a negative control. Results obtained from the identification of epoxystyrene as a styrene biotransformation product demonstrate that the 3-kb *Pst*I-*Eco*RI genomic fragment of *P. fluorescens* ST present in pTPE30 contains the styrene monooxygenase structural gene. It is probable that the entire fragment is not required for the expression of the enzymatic activity, as suggested by the indigo accumulation tests performed on the clones bearing deletions obtained from pTPE30 (Fig. 2).

Identification of the p907 region coding for epoxystyrene isomerase and epoxystyrene reductase activities. The p3B6 cosmid of the *P. fluorescens* ST genomic bank allowed growth on epoxystyrene but not on styrene. Since *P. putida* PaW340 is able to grow on phenylacetaldehyde and 2-phenylethanol, this clone should at least carry the information required to catalyze the first step of epoxystyrene degradation through the side chain oxidation pathway. The p3B6 cosmid retains only the 2.3-kb *Bam*HI fragment (indicated in Fig. 2A) of the 9-kb

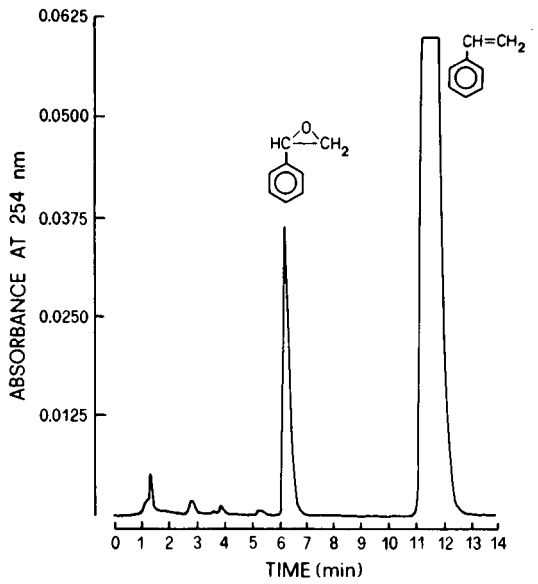

FIG. 3. Reverse-phase HPLC analysis of the styrene biotransformation products by JM109(pTPE30). Cells were grown in the presence of IPTG, resuspended in phosphate buffer, and incubated in a reactor, into which styrene was supplied via the gas phase by the incoming air flow, as described in Materials and Methods. After 24 h of incubation, the vapors emitted by the reactor were condensed and analyzed by reverse-phase HPLC. The eluted compounds were detected at A_{254}, and their retention time was compared with that of authentic compounds. The resulting peaks were further identified by ^{1}H-NMR analysis. Retention times: epoxystyrene, 6.2 min; styrene, 11.6 min. Extinction coefficients (per millimole per centimeter): styrene, 10.53; epoxystyrene, 0.158. Elution conditions: flow rate, 1 ml/min with acetonitrile-water (50:50).

region identified as sufficient to support growth on styrene. This *Bam*HI band was subcloned in pTZ19 in both orientations, and the resulting recombinant plasmids, pTB23 and pTB23R (Table 1), were used to transform *E. coli* JM109. In pTB23, the 2.3-kb *Bam*HI fragment has the same orientation as the partially overlapping 3-kb *Pst*I-*Eco*RI insert of pTPE30 (Fig. 2).

JM109(pTB23) and JM109(pTB23R) were grown with IPTG as a *lacZp* inducer, resuspended in phosphate buffer, and divided between two flasks. Glucose was added to one flask to increase the biotransformation yields, as suggested by Brand et al. (6). Epoxystyrene was then added to each cell suspension. The supernatants of samples collected at different times of incubation were analyzed by HPLC, and the biotransformation products were further identified by ^{1}H-NMR (data not shown) as described in Materials and Methods. Both the JM109 (pTB23) cell suspensions used (cells incubated with or without glucose) were able to isomerize epoxystyrene to phenylacetaldehyde. Moreover, in cell suspensions where glucose was present, a small amount of 2-phenylethanol could also be detected, as shown in Fig. 4. Epoxystyrene isomerization occurred at a very high rate: in fact, this compound was completely transformed into phenylacetaldehyde in approximately 2 h of incubation. At this time, the HPLC elution profile was the same as the one detected at 5 h of incubation (shown in Fig. 4) and did not exhibit appreciable variations up to 24 h. The rate of 2-phenylethanol formation appeared to be slower, even if the small final amount of this compound was likely to be due to the rapid depletion of epoxystyrene, by the isomerase activity, in the incubation mixture. JM109(pTB23R) cells never isomerized epoxystyrene to phenylacetaldehyde. However, when the cells were incubated in the presence of glucose, a reduction to 2-phenylethanol was observed (Fig. 5).

In order to verify the origin of 2-phenylethanol and phenylacetaldehyde, JM109(pTB23) and JM109(pTB23R) were exposed to phenylacetaldehyde and to 2-phenylethanol, under the different conditions described above. In each case, no biotransformation was detected (data not shown), indicating that the two metabolites analyzed originated from a direct transformation of epoxystyrene. As a whole, the data showed that the formation of phenylacetaldehyde occurred only in JM109(pTB23), while cells of both JM109(pTB23) and JM109(pTB23R) were able to reduce epoxystyrene to 2-phenylethanol only in the presence of glucose, which probably supports this reaction by increasing the reducing power of the cells. The amount of 2-phenylethanol detected at the different times of incubation of JM109(pTB23R) confirmed that the rate of this biotransformation was lower than that of isomerization, indicating that no interference, other than substrate availability, occurred between the two routes, isomerization and reduction, of epoxystyrene transformation.

Under all culture conditions tested and with both clones exposed to epoxystyrene, we observed the presence of a metabolite identified as phenylethanediol (retention time, 2.8 min). This compound was not a microbial oxidation product but was derived from a spontaneous oxidation of epoxystyrene, since it could be detected in culture performed with JM109 carrying the vector and also in phosphate buffer without cells. No metabolite peaks corresponding to phenylacetaldehyde or 2-phenylethanol were detected with cell suspensions of JM109 carrying the vector, used as negative controls (data not shown). From these data, we can assume that two enzymatic activities are encoded by the 2.3-kb *Bam*HI fragment: an epoxystyrene isomerase activity, responsible for the accumulation of phenylacetaldehyde, and an epoxystyrene reductase activity which allows the formation of 2-phenylethanol only under specific incubation conditions. As far as the expression of the corresponding genes is concerned, we could detect epoxystyrene isomerase gene expression only in JM109(pTB23), in which the inserted fragment has the same orientation as the 3-kb *Pst*I-*Eco*RI styrene monooxygenase coding fragment with respect to the *lacZp* of pTZ19.

These results indicate that the styrene monooxygenase and the epoxystyrene isomerase genes are located in the same DNA strand and that their expression in the recombinant plasmids used is strictly dependent on the pTZ19 *lacZp* activity. Expression of the epoxystyrene reductase gene seems to be dependent on an internal promoter, since it is independent of the orientation of the 2.3-kb *Bam*HI fragment within the pTZ19 vector.

DISCUSSION

The present study provides the first example of the cloning and identification of styrene catabolism genes. The genes have been found to be located on the chromosome. This finding disproves our previous conclusions, based on preliminary evidence, that the styrene-positive phenotype of *P. fluorescens* ST was dependent on the presence of the pEG plasmid harbored by this strain (21). The enzymatic activities encoded by the cloned catabolic genes have confirmed that *P. fluorescens* ST degrades styrene through oxidation of the lateral chain. The first step consists of the oxidation of the vinyl group by a monooxygenase, with the formation of epoxystyrene.

We have identified an oxygenase activity indirectly by the accumulation of indigo in indole-containing media and directly by the identification of epoxystyrene as a styrene bioconversion product. The dependence of this reaction on the presence of the 3-kb *Pst*I-*Eco*RI *P. fluorescens* ST genomic fragment has

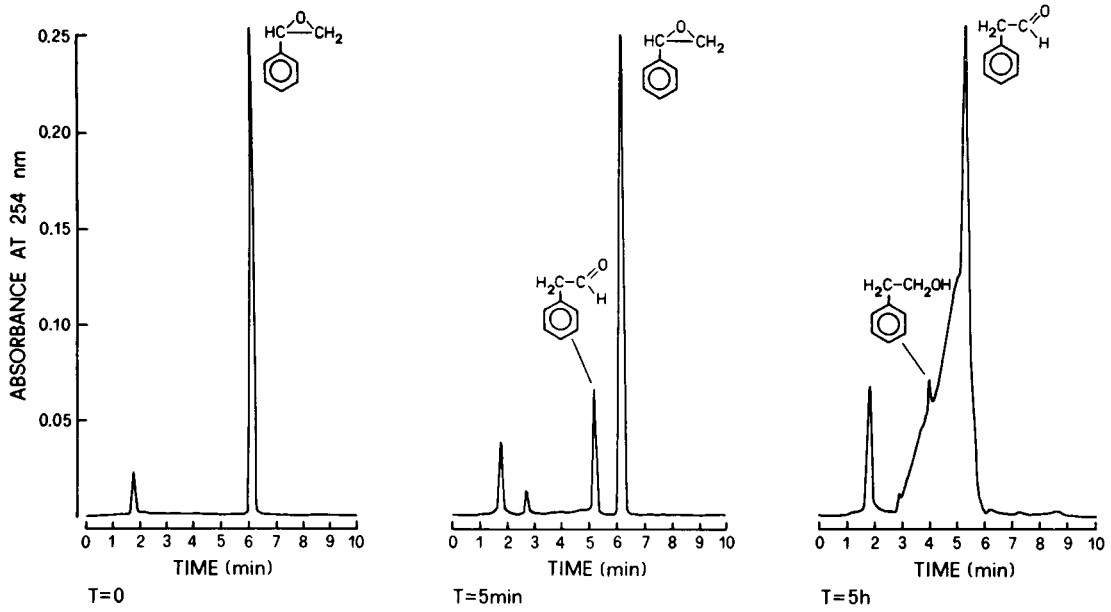

FIG. 4. Reverse-phase HPLC of the epoxystyrene biotransformation products by JM109(pTB23). Cells were grown in the presence of IPTG, resuspended in phosphate buffer in the presence of epoxystyrene (30 mM) and glucose (0.2%), and incubated in a flask at 37°C with shaking. At different times of incubation (T), aliquots were withdrawn, and after centrifugation supernatants were analyzed by reverse-phase HPLC. The resulting peaks were also analyzed by ^{1}H-NMR. Chromatography conditions were as described in the legend to Fig. 3. Retention times: epoxystyrene, 6.2 min; phenylacetaldehyde, 5.4 min; 2-phenylethanol, 4.0 min. The peak with the retention time of 1.8 min represents compounds deriving from cellular lysis. Extinction coefficients (per millimole per centimeter): epoxystyrene, 0.158; phenylacetaldehyde, 0.182; 2-phenylethanol, 0.163.

also been demonstrated. The 2.3-kb *Bam*HI genomic fragment confers to *E. coli* transformants the capability to convert epoxystyrene into phenylacetaldehyde and/or 2-phenylethanol. Direct transformation of epoxystyrene into 2-phenylethanol was suggested by Shirai and Hisatsuka (22), whereas Hartmans et al. (11) proposed that this transformation proceeds in two steps involving phenylacetaldehyde as an intermediate.

Here, we demonstrate that 2-phenylethanol can be produced directly from the reduction of epoxystyrene. We have found that this reaction was strictly dependent on incubation conditions, since we could detect 2-phenylethanol accumulation only in the presence of glucose. This incubation condition increases the reducing power of the cell, probably supporting the reduction of epoxystyrene to a less toxic compound. However, 2-phenylethanol is not an obligatory intermediate of styrene degradation, since we have selected *P. fluorescens* ST mutants which had lost the ability to grow on this compound but fully retained the ability to grow on styrene (data not shown). The formation

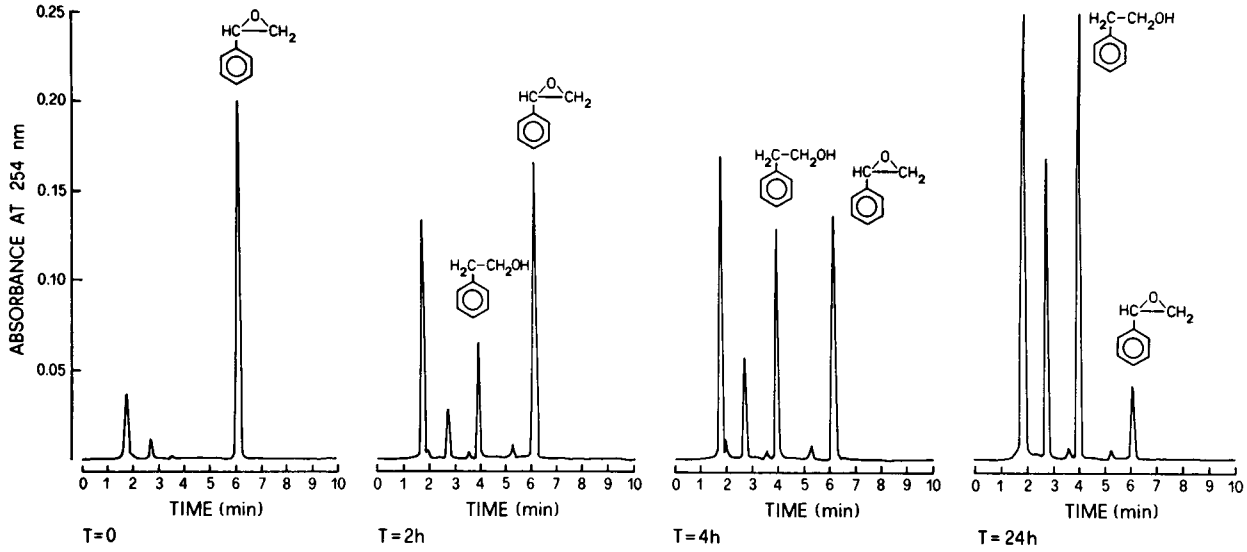

FIG. 5. Reverse-phase HPLC of the epoxystyrene biotransformation products by JM109(pTB23R). Cell growth, incubation, and chromatography conditions were as described in the legend to Fig. 4. Retention times: epoxystyrene, 6.2 min; 2-phenylethanol, 4.0 min; phenylethanediol, 2.8 min. The peak with a retention time of 1.8 min represents compounds deriving from cellular lysis. Extinction coefficients are as described in the legend to Fig. 4. T, time of incubation.

of phenylacetaldehyde was independent of the incubation conditions and occurred at a very high rate. This behavior is likely to be due to the fact that, in the recombinant plasmid used, the expression of the epoxystyrene isomerase gene was not regulated by an endogenous system but was dependent on the activity of the external *lacZp* promoter. Owing to the low solubility of the compounds tested, it is difficult to make any quantitative conclusions. Under our experimental conditions, epoxystyrene was supplied at an oversaturating concentration (30 mM), so that a continuous solubilization of this compound occurred during its biotransformation. Furthermore, the high instability of epoxystyrene in watery solutions always led to the spontaneous formation of large amounts of phenylethanediol, especially after a long incubation time. This compound is not degraded by *P. fluorescens* ST, and up to now, only *P. putida* R1 seems to degrade styrene through the formation of phenylethanediol (20).

As far as the organization of the styrene-catabolic genes is concerned, some indications may be obtained by considering the relationship between their expression, as measured by enzymatic activities, and the orientation of the cloned fragments. The genes encoding styrene monooxygenase and epoxystyrene isomerase are located in the partially overlapping 3-kb *Pst*I-*Eco*RI and 2.3-kb *Bam*HI fragments, respectively. These genes were expressed only when they were cloned in the same orientation with respect to the *lacZp* of the vector. This result indicates that the same DNA strand contains the two coding sequences and that not all the elements necessary for an autonomous expression of these genes are present in the cloned fragments or are effective in *E. coli* host cells. The 2.3-kb *Bam*HI fragment also encodes an epoxystyrene reductase activity. The expression of the corresponding gene was independent of the orientation of the cloned fragment, as shown by the same level of 2-phenylethanol accumulation obtained with JM109(pTB23) and JM109(pTB23R) in the presence of epoxystyrene and glucose.

These data suggest that expression of the epoxystyrene reductase gene is under the control of its own promoter. Whether this promoter contains regulatory elements cannot be established in our experimental system, because the high copy number of the recombinant plasmid used could mask a low level of promoter activity. An indication in favor of the hypothesis that the epoxystyrene reductase gene has the same orientation as the other two genes is its nonincreased expression by *lacZp* in the pTB23R plasmid. Together, these data indicate that the genes involved in the first steps of styrene degradation are organized in a cluster and are probably encoded by the same DNA strand.

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