Physiological Analysis of the Expression of the Styrene Degradation Gene Cluster in *Pseudomonas fluorescens* ST

PEDRO MIGUEL SANTOS,¹ JANET MARTHA BLATNY,² ILARIA DI BARTOLO,¹ SVEIN VALLA,³ AND ELISABETTA ZENNARO¹*  

Department of Biology, Third University of Rome, 00146 Rome, Italy,¹ and Laboratory of Microbial Gene Technology, Department of Biotechnological Sciences, Agricultural University of Norway, 1432 Aas,² and UNIGEN Center for Molecular Biology and Laboratory of Biotechnology, Norwegian University of Science and Technology, 7489 Trondheim,³ Norway  

Received 23 September 1999/Accepted 11 January 2000

The effects of different carbon sources on expression of the styrene catabolism genes in *Pseudomonas fluorescens* ST were analyzed by using a promoter probe vector, pPR9TT, which contains transcription terminators upstream and downstream of the β-galactosidase reporter system. Expression of the promoter of the *stySR* operon, which codes for the styrene two-component regulatory system, was found to be constitutive and not subject to catabolite repression. This was confirmed by the results of an analysis of the *stySR* transcript in *P. fluorescens* ST cells grown on different carbon sources. The promoter of the operon of the upper pathway, designated *P*α, was induced by styrene and repressed to different extents by organic acids or carbohydrates. In particular, cells grown on succinate or lactate in the presence of styrene started to exhibit β-galactosidase activity during the mid-exponential growth phase, before the preferred carbon sources were depleted, indicating that there is a threshold succinate and lactate concentration which allows induction of styrene catabolic genes. In contrast, cells grown on glucose, acetate, or glutamate and styrene exhibited a diauxic growth curve, and β-galactosidase activity was detected only after the end of the exponential growth phase. In each experiment the reliability of the reporter system constructed was verified by comparing the β-galactosidase activity and the activity of the styrene monooxygenase encoded by the first gene of the styrene catabolic operon.

Styrene is a chemical that is used extensively in the manufacturing of plastics and synthetic rubbers. This toxic compound is released into the environment mainly through factory wastewater, evaporation, and pyrolysis of polystyrene. Different routes for styrene catabolism in different microorganisms have been described (8, 9, 17, 21, 29, 31). Recently, strains belonging to the genus *Pseudomonas* have been studied more extensively both at the physiological level (21–23) and the molecular level (2, 17, 24, 30). In these strains the catabolic genes are organized in a cluster whose expression requires the presence of two genes, *styS* and *styR*, which are organized in an operon and code for a sensor kinase and a regulatory DNA binding protein, respectively. Two-component regulatory systems for genes involved in aromatic hydrocarbon degradation have been described previously only for toluene degradation in *Pseudomonas putida* F1 and *Thauera* sp. strain T1 (6, 15) and for degradation of biphenyl in *Rhodococcus* sp. strain M5 (14).

In our laboratory, *Pseudomonas fluorescens* ST, which is able to grow on styrene as a sole carbon source, has been characterized, and both the regulatory genes (*styS* and *styR*) and the upper pathway genes (*styA*, *styB*, *styC*, and *styD*), which code for conversion of styrene into phenylacetic acid, have been sequenced (2, 17, 18). At the moment, our interest is focused on characterization of the regulatory system and, in particular, on the effects of different carbon sources on styrene-induced expression of the regulatory and structural genes. Several examples of carbon catabolite repression of expression of catabolic pathways for aromatic and nonaromatic compounds have been described in *Pseudomonas* spp. (12, 20, 32). However, none of these studies dealt with catabolic operons regulated by a two-component regulatory system.

In this paper we describe the effects of growth on different carbon sources on expression of the styrene regulatory and degradative operons.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, media, and chemicals.** The bacterial strains and plasmids used in this study are listed in Table 1. *P. fluorescens* ST and *Escherichia coli* cells were routinely grown at 30 and 37°C, respectively, in Luria-Bertani (LB) medium (19) or mineral salts medium (9) containing different carbon sources at the following concentrations: 0.2% succinate, 0.05% glucose, 0.1% lactate, and 0.1% acetate. In induction studies, styrene was added via the gas phase as previously described (17). When necessary, cultures were supplemented with ampicillin (100 μg/ml), tetracycline (15 μg/ml), or chloramphenicol (30 μg/ml). Isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM), 5′-bromo-4′-chloro-3′-indolyl-β-D-galactopyranoside (X-Gal) (1 mM), and 2-nitrophenyl-β-D-galactopyranoside (1 mM) were added to the media when appropriate.

**Conjugative mating.** Plasmids were transferred from *E. coli* S17.1 to *P. fluorescens* ST by mating on membranes, and the mixtures were incubated on nutrient-yeast extract agar at 30°C for 14 h. The mating mixtures were then plated onto selective media.

**DNA manipulation.** Transformations of *E. coli*, restrictions, and ligations were carried out by using standard procedures (26). Plasmid DNA was prepared by the alkaline lysis protocol (26) or with a QIAGEN Midi isolation kit (Qiagen). DNA fragments were purified from agarose by using a Qiaquick gel extraction kit or with a QIAEXII kit (Qiagen). DNA 5′ protruding ends and 3′ protruding ends were made blunt by using Klenow polymerase and T4 DNA polymerase, respectively. PCR amplification of the regulatory gene promoter region (designated *P*α, from pTPE30 (17) was performed by using the following synthesized primers: 5′ GCTTCAAGATGCCAGATCTCTGCG 3′ and 5′ CGGCTATCTGGTCCTGCTG 3′ containing an *Xba*I site and a *Kpn*I site (underlined nucleotides), respectively. PCR amplification of the regulatory gene promoter region (designated *P*α, from pTPE30 (17) was performed by using the following synthesized primers: 5′ TAAGCTTGAATGCTTCAAGCTTGAAGATCTCCTGCTG 3′ and 5′ GGAATCCCAGATCTGCCAGATCTCG 3′ containing an *Hind*I site and an *EcoRI* site (underlined nucleotides), respectively. PCR amplifications were performed by using standard procedures and, unless otherwise specified, *Pfu* polymerase from Stratagene.

* Corresponding author. Mailing address: Department of Biology, Third University of Rome, Viale Marconi 446, 00146 Rome, Italy. Phone: 39 0655176318. Fax: 39 0655176321. E-mail: zennaro@bio.uniroma3.it.
and 600 nucleotides of
by monitoring the OD600.

The styrene-grown cells were transferred to styrene mineral medium, while the
pPR9TTPa, pPR9Ps, or pPR9TTPs were pregrown overnight at 30°C in mineral
purified with a Sephadex G-50 spin column. Filter hybridization and washing
sham Corp.) by using a random priming labeling kit (Boehringer) and was

Plasmids

P. fluorescens

pPR9
pBluescriptII KS+

lucZ promoter probe vector RK2 replication; Ap r; 8.9 kb

CoIE1 replication; Ap r; 2.9 kb

Our laboratory

Our laboratory

pPR9TT

pPR9 derivative in which a 410-bp BanHI-BglII fragment containing the mmbTT2 terminators
was cloned into a BglII site of pPR9; Ap r; 9.3 kb

Our laboratory

pTZ19R
pTE30

pTZ19R derivative containing a chromosomal fragment of P. fluorescens ST carrying two trun-
cations for paaK and styR; Ap r; 6.0 kb

Our laboratory

pBSPs
pBluescriptII KS+ derivative in which a 409-bp PCR blunted fragment containing PnySR
was cloned into the HinII site of the vector; Ap r; 3.3 kb

This study

pPR9Ps
pPR9 derivative in which the 411-bp fragment from pBSPs containing PnySR was cloned as a
HindIII-PstI fragment into the same sites of the vector; Ap r; 9.4 kb

This study

pPR9TTPs
pPR9 derivative in which a 411-bp fragment from pBSPs containing PnySR was cloned as a
HindIII-PstI fragment into the same sites of the vector; Ap r; 9.7 kb

This study

pTE30
pTZ19R derivative containing a chromosomal fragment of P. fluorescens ST carrying the genes
coding for styR, styA, and styB; Ap r; 6.0 kb

This study

pBSPa
pBluescriptII KS+ derivative in which a 492-bp PCR blunted fragment containing PaaA was
cloned into the HinII site of the vector; Ap r; 3.3 kb

This study

pPR9TTPa
pPR9 derivative in which a 560-bp BanHI-XhoI fragment from pBSPa containing PaaA was
cloned into BglII-XhoI sites of the vector; Ap r; 9.9 kb

This study

\* Ap r, ampicillin resistance; Tc r, tetracycline resistance; Cm r, chloramphenicol resistance; Sty, styrene metabolic phenotype; Sty r, streptomycin resistance.

All PCR fragments were controlled by sequencing them with an Applied Biosystems automated sequencer (model 373 Stetch) and a DyeDeoxy termi-
nator cycle sequencing kit (Perkin-Elmer). Both commercially available and synthetic primers were used for sequencing reactions.

Northern blot analysis. P. fluorescens ST cells were grown on glucose, succi-
nate, and styrene to an optical density at 600 nm (OD600) of approximately 0.3. RNA was prepared and electrophoresed was performed essentially as described by Leoni et al. (16). RNAs were transferred onto nitrocellulose filters (Schleicher & Schuell) as described by Sambrook et al. (26) and heat
fixed. A 1.7-kb XhoI-BglII DNA fragment containing 1,100 nucleotides of styS and 600 nucleotides of styR was labeled with [γ-32P]ATP (30 Ci/mmol; Amer-
sham Corp.) by using a random priming labeling kit (Boehringer) and was purified with a Sephadex G-50 spin column. Filter hybridization and washing were performed by using standard procedures (26).

Induction conditions. In induction assays, P. fluorescens ST cells harboring pPR9TTPa, pPR9Ps, or pPR9TTPs were grown overnight at 30°C in mineral salts medium supplemented with succinate, lactate, glucose, or styrene. The styrene-grown cells were transferred to styrene minimal medium, while the succinate-, lactate-, acetate-, and glucose-grown cells were inoculated into the corresponding mineral media with or without styrene. Cell growth was measured by monitoring the OD600.

SMO assays. To quantify styrene monooxygenase (SMO) activity, production of indigo was assayed essentially as described by O'Connor et al. (23). Cells were harvested in the exponential and stationary phases by centrifugation, washed with 50 mM potassium phosphate buffer (pH 7.0), and resuspended in the same buffer to an OD600 of 3.0. One hundred to 600 µl of concentrated cells was added to 400 µl of 50 mM potassium phosphate buffer (pH 7) containing 0.25 mM
methylene blue in 1.5-ml polypropylene microcentrifuge tubes. The samples were incu-
bated horizontally at 30°C with vigorous shaking for 30 min. The samples were then centrifuged at 14,000 rpm for 2 min, and the supernatants were carefully
discarded. The cell pellets were resuspended in 1 ml of dimethylformamide and extracted by shaking for 15 min. The tubes were then centrifuged to remove the cell debris, and the OD600 of the supernatants was determined. The data presented below are the results obtained from at least three independent experiments with standard deviations ranging from 5 to 10%.

β-Galactosidase assay. β-Galactosidase activity was measured as described by Miller (19) and expressed in Miller units. The data presented below are the results obtained from at least three independent experiments with a standard deviation of 10%.

RESULTS

Construction of PstySR-lacZ fusions. In order to study the activity of the promoter of the styrene regulatory operon, designated PstySR, we used two new promoter probe vectors, pPR9 and pPR9TT, which were based on the RK2 replicon
and contained lacZ as a reporter gene (Santos et al., unpublished data). pPR9 is a derivative of pJB653 (3) in which the Pm-xybF expression system has been replaced by the lacZ gene from P. putida ATCC 802 (Pharmacia), which lacks transcription and translation signals. This plasmid contains the polylinker of pBluescriptII KS+(+) and the transcriptional terminators of Ω-Km (3) located downstream of lacZ. Moreover, the Cm m
marker was inserted into the unique HindIII site downstream of the trfA gene. pPR9TT is a derivative of pPR9 in which the strong ribosomal terminators mBT2 from pBTac1 are inser-
ted upstream of the polylinker region. No β-galactosidase activity was detected with pPR9TT in the E. coli or Pseudomo-

FIG. 1. Regulatory and catabolic operons of the styrene degradation system in P. fluorescens ST. styA, sensor; styR, regulator; styAB, SMO gene; styC, cypoy-

\hline
<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype and/or phenotype*</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. fluorescens</em> ST</td>
<td>Sty'</td>
<td>1</td>
</tr>
<tr>
<td>E. coli DH5a</td>
<td>endA1 hsdR17 supE44 thi-1 recA1 gyrA (Nal') (lacIZYA-argF) U169 deoR [80lacI (lacZ)M15]</td>
<td>Bethesda Research Laboratories</td>
</tr>
<tr>
<td>E. coli S17.1</td>
<td>recA pro thi hsdR RP4-2-Tc:Mm-Km:Trn7 Tra' Ty' Str'</td>
<td>27</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pPR9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBluescriptII KS+</td>
<td>lucZ promoter probe vector RK2 replication; Ap r</td>
<td>8.9 kb</td>
</tr>
<tr>
<td>pPR9TT</td>
<td>pPR9 derivative in which a 410-bp BanHI-BglII fragment containing the mmbTT2 terminators was cloned into a BglII site of pPR9; Ap r</td>
<td>9.3 kb</td>
</tr>
<tr>
<td>pTZ19R</td>
<td>CoIE1 replication; Ap r</td>
<td>2.9 kb</td>
</tr>
<tr>
<td>pTE30</td>
<td>pTZ19R derivative containing a chromosomal fragment of P. fluorescens ST carrying two truncations for paaK and styR; Ap r</td>
<td>6.0 kb</td>
</tr>
<tr>
<td>pBSPs</td>
<td>pBluescriptII KS+ derivative in which a 409-bp PCR blunted fragment containing PnySR was cloned into the HinII site of the vector; Ap r</td>
<td>3.3 kb</td>
</tr>
<tr>
<td>pPR9Ps</td>
<td>pPR9 derivative in which the 411-bp fragment from pBSPs containing PnySR was cloned as a HindIII-PstI fragment into the same sites of the vector; Ap r</td>
<td>9.4 kb</td>
</tr>
<tr>
<td>pPR9TTPs</td>
<td>pPR9 derivative in which a 411-bp fragment from pBSPs containing PnySR was cloned as a HindIII-PstI fragment into the same sites of the vector; Ap r</td>
<td>9.7 kb</td>
</tr>
<tr>
<td>pTE30</td>
<td>pTZ19R derivative containing a chromosomal fragment of P. fluorescens ST carrying the genes coding for styR, styA, and styB; Ap r</td>
<td>6.0 kb</td>
</tr>
<tr>
<td>pBSPa</td>
<td>pBluescriptII KS+ derivative in which a 492-bp PCR blunted fragment containing PaaA was cloned into the HinII site of the vector; Ap r</td>
<td>3.3 kb</td>
</tr>
<tr>
<td>pPR9TTPa</td>
<td>pPR9TT derivative in which a 560-bp BanHI-XhoI fragment from pBSPa containing PaaA was cloned into BglII-XhoI sites of the vector; Ap r</td>
<td>9.9 kb</td>
</tr>
</tbody>
</table>

* Ap r, ampicillin resistance; Tc r, tetracycline resistance; Cm r, chloramphenicol resistance; Sty, styrene metabolic phenotype; Sty r, streptomycin resistance.

Downloaded from http://aem.asm.org/ on January 13, 2016 by guest
which contained the stySR and styABCD operons (Fig. 1) in its chromosome. The β-galactosidase activity of ST cells harboring pPR9Ps did not depend on the presence of styrene and was not influenced by additional carbon sources in any of the growth phases analyzed. The β-galactosidase activities of *P. fluorescens* ST(pPR9Ps) cells grown on succinate, succinate plus styrene, and styrene are shown in Fig. 2. The same results were obtained when cells were grown on glucose, on glutamate, or in LB medium (data not shown). To confirm that these results were not due to read-through from the vector because of the inefficiency of the putative terminator located upstream of *P*stySR, the same experiments were performed with pPR9TTPs. The results obtained with this vector were identical to the results obtained with pPR9Ps, indicating that the natural terminator is effective. However, since RK2-based vectors, such as pPR9 and pPR9TT, occur at levels of five to seven copies per chromosome (3), we examined the possibility that the presence of multiple copies of *P*stySR could result in apparent constitutive expression of *P*stySR. Therefore, we analyzed the transcripts of *stySR* genes in *P. fluorescens* ST grown on glucose, succinate, and styrene. The results obtained (Fig. 2) showed that a comparable amount of the *stySR* transcript (length, approximately 3.6 kb) was present under the growth conditions examined, which confirmed the data obtained with the β-galactosidase assay.

**Construction of *P*styA- *lacZ* fusion.** The *P*styA promoter (Fig. 1) is induced in the presence of styrene and is responsible for expression of the styrene catabolic operon (2, 17, 24, 30). Sequence analysis of the DNA region upstream of *styA* has shown that there is an inverted repeat that is located 75 bp upstream of the start codon and contains a sequence identical to the *tod* box sequence involved in toluene utilization in *Pseudomonas putida* F1 (15). It has been shown that this box is the DNA binding site of TodT, which belongs to a two-component regulatory system that is highly homologous to the StyS-StyR system. To study the activity of *P*styA, a 492-bp PCR fragment that included 23 codons of the upstream gene was cloned into the *Hin*II site of *P*bla gene. The intergenic region containing *P*styA, and the first 81 codons of the *styA* gene was cloned into the *Hin*II fragment from pBSPa into the *Xho* I site of pPR9TT, generating pBSFa. *P*PR9TTa was constructed by cloning the 560-bp *Bam*HI-*Xho*I fragment from pBSFa into the *Bgl*II-*Xho*I sites of pPR9TT in frame with the *lacZ* gene.

**Effects of different carbon sources on *P*styA activity.** pPR9TTa was transferred by conjugation into *P. fluorescens* ST, and cells were grown on styrene mineral medium (Fig. 3A) and on mineral medium supplemented with different carbon sources, such as gluconate, glutamate, or in LB medium (data not shown). To confirm that these results were not due to read-through from the vector because of the inefficiency of the putative terminator located upstream of *P*stySR, the same experiments were performed with pPR9TTPs. The results obtained with this vector were identical to the results obtained with pPR9Ps, indicating that the natural terminator is effective. However, since RK2-based vectors, such as pPR9 and pPR9TT, occur at levels of five to seven copies per chromosome (3), we examined the possibility that the presence of multiple copies of *P*stySR could result in apparent constitutive expression of *P*stySR. Therefore, we analyzed the transcripts of *stySR* genes in *P. fluorescens* ST grown on glucose, succinate, and styrene. The results obtained (Fig. 2) showed that a comparable amount of the *stySR* transcript (length, approximately 3.6 kb) was present under the growth conditions examined, which confirmed the data obtained with the β-galactosidase assay.

**Construction of *P*styA-*lacZ* fusion.** The *P*styA promoter (Fig. 1) is induced in the presence of styrene and is responsible for expression of the styrene catabolic operon (2, 17, 24, 30). Sequence analysis of the DNA region upstream of *styA* has shown that there is an inverted repeat that is located 75 bp upstream of the start codon and contains a sequence identical to the *tod* box sequence involved in toluene utilization in *Pseudomonas putida* F1 (15). It has been shown that this box is the DNA binding site of TodT, which belongs to a two-component regulatory system that is highly homologous to the StyS-StyR system. To study the activity of *P*styA, a 492-bp PCR fragment that included 23 codons of the upstream gene was cloned into the *Hin*II site of *P*bla gene. The intergenic region containing *P*styA, and the first 81 codons of the *styA* gene was cloned into the *Hin*II fragment from pBSPa into the *Xho* I site of pPR9TT, generating pBSFa. **P**PR9TTa was constructed by cloning the 560-bp *Bam*HI-*Xho*I fragment from pBSFa into the *Bgl*II-*Xho*I sites of pPR9TT in frame with the *lacZ* gene.

**Effects of different carbon sources on *P*styA activity.** pPR9TTa was transferred by conjugation into *P. fluorescens* ST, and cells were grown on styrene mineral medium (Fig. 3A) and on mineral medium supplemented with different carbon sources, such as gluconate, glutamate, or in LB medium (data not shown). To confirm that these results were not due to read-through from the vector because of the inefficiency of the putative terminator located upstream of *P*stySR, the same experiments were performed with pPR9TTPs. The results obtained with this vector were identical to the results obtained with pPR9Ps, indicating that the natural terminator is effective. However, since RK2-based vectors, such as pPR9 and pPR9TT, occur at levels of five to seven copies per chromosome (3), we examined the possibility that the presence of multiple copies of *P*stySR could result in apparent constitutive expression of *P*stySR. Therefore, we analyzed the transcripts of *stySR* genes in *P. fluorescens* ST grown on glucose, succinate, and styrene. The results obtained (Fig. 2) showed that a comparable amount of the *stySR* transcript (length, approximately 3.6 kb) was present under the growth conditions examined, which confirmed the data obtained with the β-galactosidase assay.

**Construction of *P*styA-*lacZ* fusion.** The *P*styA promoter (Fig. 1) is induced in the presence of styrene and is responsible for expression of the styrene catabolic operon (2, 17, 24, 30). Sequence analysis of the DNA region upstream of *styA* has shown that there is an inverted repeat that is located 75 bp upstream of the start codon and contains a sequence identical to the *tod* box sequence involved in toluene utilization in *Pseudomonas putida* F1 (15). It has been shown that this box is the DNA binding site of TodT, which belongs to a two-component regulatory system that is highly homologous to the StyS-StyR system. To study the activity of *P*styA, a 492-bp PCR fragment that included 23 codons of the upstream gene was cloned into the *Hin*II site of *P*bla gene. The intergenic region containing *P*styA, and the first 81 codons of the *styA* gene was cloned into the *Hin*II fragment from pBSPa into the *Xho* I site of pPR9TT, generating pBSFa. **P**PR9TTa was constructed by cloning the 560-bp *Bam*HI-*Xho*I fragment from pBSFa into the *Bgl*II-*Xho*I sites of pPR9TT in frame with the *lacZ* gene.
sources in the presence or absence of styrene (Fig. 4A), as described above. Samples were harvested at different times during the exponential and stationary phases, and SMO and β-galactosidase activities were determined. SMO activity was determined by monitoring the conversion of indole to indoxyl, which spontaneously dimerizes to the blue dye indigo. Formation of indigo has been used extensively to select microorganisms that express dioxygenase or monooxygenase activities (2, 7). Previously, we demonstrated that E. coli expressing a DNA fragment containing styAB formed indigo from indole and styrene oxide from styrene, indicating that the two reactions are catalyzed by the same enzyme (2). In this way we could directly measure the activity of the styAB gene product together with cloned PsyA expression.

The results which we obtained showed that formation of indigo was induced only in the presence of styrene (Fig. 3B and 4B). When cells were grown on organic acids or carbohydrates, indigo was not formed and β-galactosidase activity was not detected (Fig. 4B). When styrene was used as the sole carbon source, formation of indigo and β-galactosidase activity were detected in the early exponential growth phase (Fig. 3B). However, cells grown on succinate or lactate and styrene started to accumulate indigo and to exhibit β-galactosidase activity during the mid-exponential growth phase (Fig. 4B). This suggests that cells started to grow by utilizing succinate or lactate and that the shift in substrate utilization from these organic acids to styrene occurred before the preferred carbon sources were depleted. The conclusion that during the early exponential growth phase these organic acids repressed PsyA induction was confirmed by the finding that the two enzymatic activities considered were easily detected in the early exponential phase of the growth when glycerol was the carbon source added (data not shown). The effects of succinate and lactate at concentrations ranging from 0.05 to 0.4% were also examined. A diauxic growth curve was not observed, indicating that there was no off period by the two substrates and that there probably is a threshold succinate and lactate concentration which allows induction of the styrene catabolic genes.

It has been reported that in Pseudomonas lemoignei uptake of succinate depends on the pH (28), and the optimum pH range is 5.6 to 7.0. We performed experiments with P. fluorescens ST cells grown in pH 6.0 buffered mineral medium supplemented with succinate and styrene, and we found that the styrene catabolic operon was expressed only at the end of the exponential phase (data not shown). This higher level of repression could have been a result of a higher concentration of succinate inside the cells due to greater efficiency of its transport system. However, we were not able to obtain diauxic growth even at higher concentrations.

Cells grown on glucose or acetate and styrene started to accumulate indigo and to exhibit β-galactosidase activity only after the end of exponential growth phase (Fig. 4), indicating that these carbon sources do impose a high level of catabolite repression on expression of the styrene degradative operon. Furthermore, we examined the influence of the concentration of these carbon sources by using concentrations ranging from 0.05 to 0.4%, and we observed that an increase in concentration resulted in an increase in the time necessary for the shift to styrene utilization. This resulted in a prolonged second lag in diauxic growth (data not shown). Several other substrates were tested, and we found that arginine and glycerol did not affect PsyA induction, while glutamate and citrate strongly repressed PsyA induction (data not shown), as described above for glucose and acetate. Finally, the results of the assays performed in LB medium in the presence or in the absence of styrene showed that neither β-galactosidase nor SMO is expressed in this medium. A similar repressive effect of LB medium has been described previously for the majority of the aromatic or aliphatic catabolic operons that have been studied so far (12, 20, 32).

**DISCUSSION**

Our results show that PsyA expression is induced by styrene. In the presence of an additional carbon source, such as an organic acid or a carbohydrate, induction by styrene was affected, and the extent to which induction was affected depended on the carbon source and on its concentration. It is known that organic acids are usually the preferred carbon sources in Pseudomonas spp. cultures (5), but the mechanism of catabolite repression in these microorganisms is not understood yet. Our data confirm the results obtained for SMO activity in Pseudomonas putida CA-3 and support the hypothesis that also in this strain catabolite repression can occur at the transcriptional level (21).

Results obtained with pPRP9P's and in the transcript analysis showed that expression of the PsyA promoter is constitutive and does not depend on the type of carbon source. PsyA is the promoter of the operon coding for the two-component regulatory system, which includes a sensor (styS) and a regulator (styR), which are necessary for PsyA induction (24, 30). If there is no control at the translation level, StyS and StyR are constitutively present in a cell. This suggests that the signal transduction from styrene to PsyA activation are controlled by catabolite repression. The factor that is responsible for catabolite repression can affect the kinase activity of the sensor, can inhibit phosphorylation of the regulator or binding of the regulator to the promoter, or can directly bind to a specific sequence on the repressible promoter. However, analysis of different promoters of aromatic and aliphatic degradative operons did not reveal common sequences which could be the binding site for a common repressor. In Pseudomonas cultures, the presence of a solvent in the medium triggers a stress response which induces an overall readjustment of the cells through activation of defense mechanisms, including adaptation to the solvent (10, 11; for a review see reference 13). Many of these defense mechanisms are energy dependent so that growing cells in the presence of styrene leads to a requirement for more energy. This demand for extra energy is preferably met by using a readily utilizable carbon source rather than the solvent, whose utilization requires many steps to obtain an energy-yielding intermediate.

Finding a two-component regulatory system for degradation of aromatic compounds is not common. Such a system is usually associated with complex metabolic responses to environmental changes, such as nitrogen fixation, alginate production, nodulation, or virulence, or with a stress response (25, 33). It is possible that cells sense styrene as a stress factor or that styrene catabolism requires fine regulation linked to the redox status of the cell due to the toxicity of the catabolic intermediates styrene oxide and phenylacetaldehyde. We do not know if this kind of regulation is also associated with catabolite repression but it is possible to look at this process as a response to a specific energetic state of the cells. Recently, the effect of IAA₉⁶, a protein of the PTS-like transport system (4), on carbon catabolite repression of the σ₉⁶-dependent Pu promoter has been described (4). This protein seems to play a role in the relationship between some σ₉⁶-dependent promoters and nitrogen and carbon metabolism (4). This finding is consistent with the picture emerging from studies of catabolite repression of aromatic and aliphatic degradative operons in Pseudomonas spp. (12, 20, 32), which seems to indicate that the
FIG. 4. Effects of different carbon sources on PyrA promoter activity. (A) *P. fluorescens* ST cells harboring pPR9TTPa were grown on different carbon sources in the presence (■) or in the absence (□) of styrene. (B) β-Galactosidase activity in the presence (solid bars) or in the absence (open bars) of styrene and indigo production in the presence (cross-hatched bars) or in the absence (stippled bars) of styrene at different times.
mechanism involved is a general mechanism related to cell metabolism, since a single carbon source has different repressive effects depending on the strain and the growth conditions.

ACKNOWLEDGMENTS

We thank Hermann Heipieper for useful discussions. This work was supported by grant 9701252.40 from the Target Project on Biotechnology, Consiglio Nazionale delle Ricerche, Rome, Italy. P. M. Santos received a Ph.D. fellowship from the FCT, Portugal (grant PRAXIS XXI/BD/15899/98).

REFERENCES