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

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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*styA*, 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 biphenyls 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.

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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 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 styrene monooxygenase promoter region (designated P*styA*) from pTPE30 (17) was performed by using the following synthesized primers: 5′ GCTCTAGAATGTCAGATCTCTGGC 3′ and 5′ GGGGTACCTACGTAGTAGTAGTGG 3′ containing an *Xba*I site and a *Kpn*I site (underlined nucleotides), respectively. PCR amplification of the regulatory gene promoter region (designated P*stySR*) from pTE30 (our laboratory) was performed by using the following synthesized primers: 5′ CAAGCTTGAATGCTTCATGTCGGC 3′ and 5′ GGAATTCCGATCCAGAATGATCCG 3′ containing an *Hin*dIII site and an *Eco*RI site (underlined nucleotides), respectively. PCR amplifications were performed by using standard procedures and, unless otherwise specified, *Pfu* polymerase from Stratagene.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype and/or phenotype[a]	Reference or source
Strains		
P. fluorescens ST	Sty$^+$	1
E. coli DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA* (Nal^r) (*lacIZYA-argF*)*U169 deoR* [80*dlac* (*lacZ*)*M15*]	Bethesda Research Laboratories
E. coli S17.1	*recA pro thi hsdR* RP4-2-Tc::Mu-Km::Tn*7* Tra$^+$ Tp^r Str^r	27
Plasmids		
pPR9	*lacZ* promoter probe vector RK2 replicon; Ap^r Cm^r; 8.9 kb	Our laboratory
pBluescriptII KS+	ColE1 replicon; Ap^r; 2.9 kb	Strategene
pPR9TT	pPR9 derivative in which a 410-bp *Bam*HI-*Bgl*II fragment containing the *rrnBT1T2* terminators was cloned into a *Bgl*II site of pPR9; Ap^r Cm^r; 9.3 kb	Our laboratory
pTZ19R	ColE1 replicon; Ap^r; 2.9 kb	MBI Fermentas
pTE30	pTZ19R derivative containing a chromosomal fragment of *P. fluorescens* ST carrying two truncated genes coding for *paaK* and *styS*; Ap^r; 6.0 kb	Our laboratory
pBSPs	pBluescriptII KS+ derivative in which a 409-bp PCR blunt-ended fragment containing P*stySR* was cloned into the *Hin*cII site of the vector; Ap^r; 3.3 kb	This study
pPR9Ps	pPR9 derivative in which the 411-bp fragment from pBSPs containing P*stySR* was cloned as a *Hin*dIII-*Pst*I fragment into the same sites of the vector; Ap^r Cm^r; 9.4 kb	This study
pPR9TTPs	pPR9TT derivative in which a 411-bp fragment from pBSPs containing P*stySR* was cloned as a *Hin*dIII-*Pst*I fragment into the same sites of the vector; Ap^r Cm^r; 9.7 kb	This study
pTPE30	pTZ19R derivative containing a chromosomal fragment of *P. fluorescens* ST carrying the genes coding for *styR*, *styA*, and *styB*; Ap^r; 6.0 kb	17
pBSPa	pBluescriptII KS+ derivative in which a 492-bp PCR blunt-ended fragment containing P*styA* was cloned into the *Hin*cII site of the vector; Ap^r; 3.3 kb	This study
pPR9TTPa	pPR9TT derivative in which a 560-bp *Bam*HI-*Xho*I fragment from pBSPa containing P*styA* was cloned into *Bgl*II-*Xho*I sites of the vector; Ap^r Cm^r; 9.9 kb	This study

[a] Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; Cm^r, chloramphenicol resistance; Sty, styrene metabolic phenotype; Str^r, streptomycin resistance.

All PCR fragments were controlled by sequencing them with an Applied Biosystems automated sequencer (model 373 Stretch) and a DyeDeoxy terminator 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, succinate, and styrene to an optical density at 600 nm (OD_{600}) of approximately 0.3. RNA was prepared and electrophoresis was performed essentially as described by Leoni et al. (16). RNAs were transferred onto nitrocellulose filters (Optitran BA-S 83; Schleicher & Schuell) as described by Sambrook et al. (26) and heat fixed. A 1.7-kb *Xho*I-*Bgl*II DNA fragment containing 1,100 nucleotides of *styS* and 600 nucleotides of *styR* was labeled with [α-^{32}P]dATP (3.0 Ci/nmol; Amersham 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 pregrown overnight at 30°C in mineral salts medium supplemented with succinate, lactate, glucose, acetate, or styrene. The styrene-grown cells were transferred to styrene mineral 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 OD_{600}.

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 OD_{600} 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 indole in 1.5-ml polypropylene microcentrifuge tubes. The samples were incubated 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 OD_{600} of the supernatants were 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 was expressed in Miller units. The data presented below are to the results obtained from at least three independent experiments with a standard deviation of 10%.

RESULTS

Construction of P*stySR-lacZ* fusions. In order to study the activity of the promoter of the styrene regulatory operon, designated P*stySR*, 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-xylS* expression system has been replaced by the *lacZ* gene from PMC1871 (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^r marker was inserted into the unique *Hin*dIII site downstream of the *trfA* gene. pPR9TT is a derivative of pPR9 in which the strong ribosomal terminators *rrnBT1T2* from pBTac1 are inserted upstream of the polylinker region. No β-galactosidase activity was detected with pPR9TT in the *E. coli* or *Pseudomonas* sp. strains tested, while low levels of such activity (5 to 20 Miller units) were observed with pPR9 depending on the host strain and the growth phase, indicating a possible weak readthrough from the vector (data not shown). The putative promoter region, P*stySR*, was obtained by PCR as described above. This region is located upstream of the *stySR* genes, which encode a sensor histidine kinase and a response regulator (Fig. 1). The 409-bp PCR product included the stop codon of the upstream gene, *paaK* (18, 30), the intergenic region containing P*stySR*, and the first 46 codons of *styS*. We cloned P*stySR* in both pPR9 and pPR9TT, which generated pPR9Ps and pPR9TTPs, respectively (see below). To do this, P*stySR*

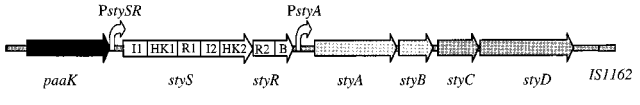

FIG. 1. Regulatory and catabolic operons of the styrene degradation system in *P. fluorescens* ST. *styS*, sensor; *styR*, regulator; *styAB*, SMO gene; *styC*, epoxystyrene isomerase gene; *styD*, phenylacetaldehyde dehydrogenase gene; *paaK*, phenylacetyl-coenzyme A ligase gene; *IS1162*, insertion sequence. P*stySR*, promoter of *stySR*; P*styA*, promoter of *styABCD*; I1, putative styrene-sensing domain; HK1 and HK2, histidine kinase domains; R1, sensor receiver domain; I2, putative oxygen- and/or redox potential-sensing domain; R2, regulator receiver domain; B, DNA binding domain. The bent arrows indicate the promoter regions and the orientation of gene transcription.

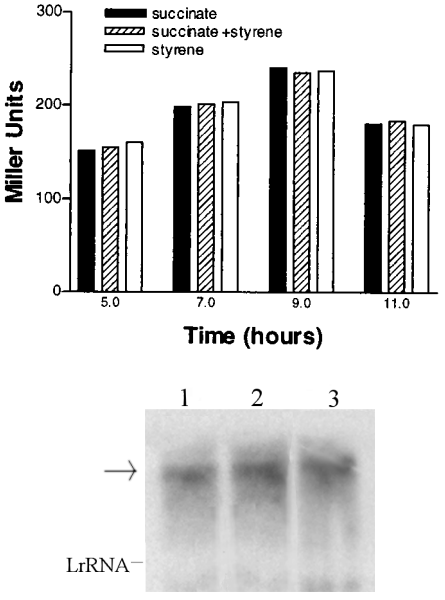

FIG. 2. Activity of the P*stySR* promoter under induced and uninduced conditions. The graph shows the β-galactosidase activities of *P. fluorescens* ST(pPR9Ps) at different times during growth on 0.2% succinate, 0.2% succinate and styrene, and styrene. The gel shows *stySR* expression as determined by Northern blot analysis. *P. fluorescens* ST cells were grown on glucose (lane 1), succinate (lane 2), and styrene (lane 3) to an OD_{600} of 0.3. The experimental procedures used for RNA preparation and detection are described in the text. Each lane contained 20 μg of RNA. The probe was a 1.7-kb DNA fragment containing both *styS* and *styR* sequences. LrRNA, large rRNA. The arrow indicates the position of the *StySR* transcript.

first was cloned into the *Hin*cII site of pBluescriptII KS(+), which generated pBSPs, and then was transferred to pPR9 and pPR9TT as a 411-bp *Hin*dIII-*Pst*I fragment at the same sites of the vectors, in frame with the *lacZ* gene.

Activity of the P*stySR* promoter under different growth conditions. Previously, it was reported that a transcription termination-like sequence is present just downstream of the *paaK* stop codon (30). However, the effectiveness of this putative terminator has not been proven. In order to assess the functioning of such a putative terminator, we cloned P*stySR* in both pPR9 and pPR9TT. In order to study the activity of P*stySR* under different growth conditions in a homologous system, we transferred plasmids pPR9Ps and pPR9TTPs into strain ST, 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 *styR* gene, the intergenic region containing P*styA*, and the first 81 codons of the *styA* gene was cloned into the *Hin*cII site of pBluescriptII KS(+), generating pBSPa. pPR9TTPa was constructed by cloning the 560-bp *Bam*HI-*Xho*I fragment from pBSPa into the *Bgl*II-*Xho*I sites of pPR9TT in frame with the *lacZ* gene.

Effects of different carbon sources on P*styA* activity. pPR9TTPa 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

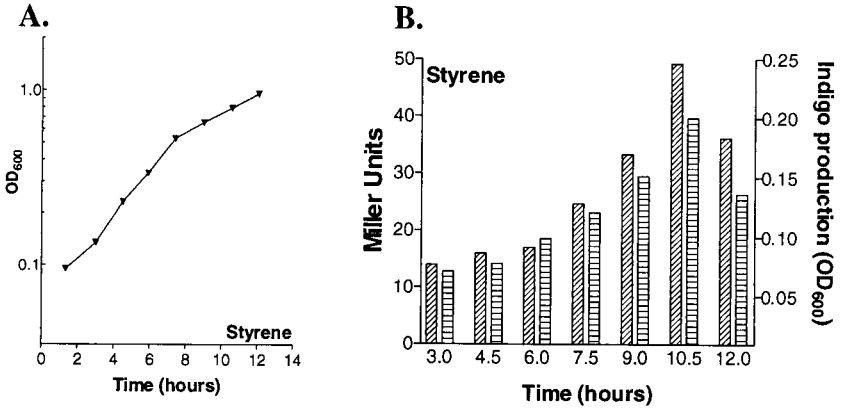

FIG. 3. Activity of the P*styA* promoter under induction conditions. (A) Growth curve for *P. fluorescens* ST(pPR9TTPa) when styrene was the sole carbon source. (B) β-Galactosidase activity (diagonally cross-hatched bars) and indigo production (horizontally cross-hatched bars) at different times.

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 P*styA* 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 P*styA* 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 not mutual exclusion 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 P*styA* induction, while glutamate and citrate strongly repressed P*styA* 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 P*styA* 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 pPR9Ps and in the transcript analysis showed that expression of the P*stySR* promoter is constitutive and does not depend on the type of carbon source. P*stySR* 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 P*styA* induction (24, 30). If there is no control at the translation level, StyS and StyR are constitutively present in a cell. This suggests that some steps in the signal transduction from styrene to P*styA* 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 IIA^{Ntr}, a protein of the PTS-like transport system (4), on carbon catabolite repression of the σ^{54}-dependent Pu promoter has been described (4). This protein seems to play a role in the relationship between some σ^{54}-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

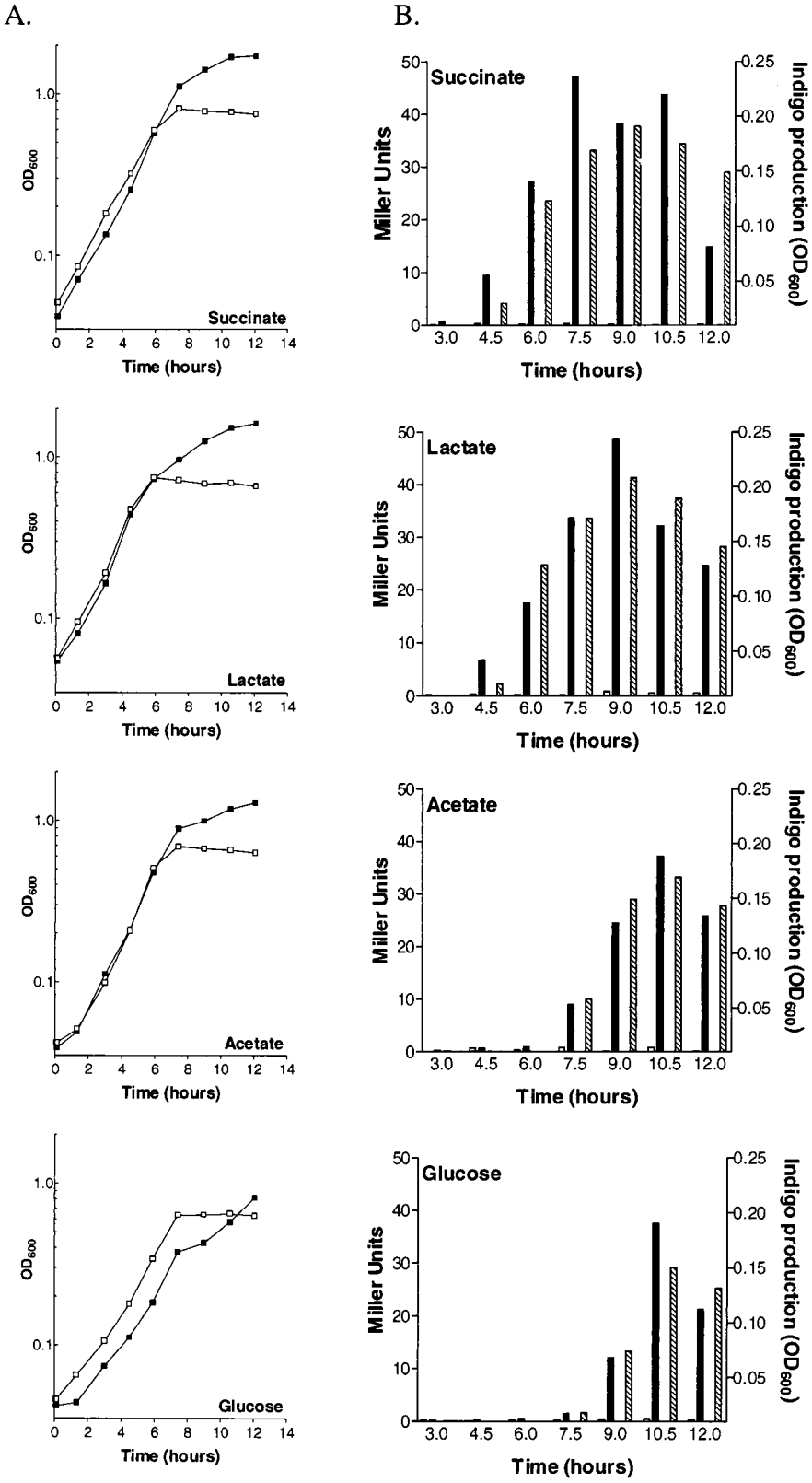

FIG. 4. Effects of different carbon sources on P*styA* promoter activity. (A) *P. fluorescens* ST cells harboring pPR9TTPa were grown on different carbon sources in the presence (■) or in the absence (□) of styrene. (B) β-Galactosidase activity in the presence (solid bars) or in the absence (open bars) 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.

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REFERENCES

1. **Baggi, G., M. M. Boga, D. Catelani, E. Galli, and V. Treccani.** 1983. Styrene catabolism by a strain of *Pseudomonas fluorescens*. Syst. Appl. Microbiol. **4:** 141–147.
2. **Beltrametti, F., A. M. Marconi, G. Bestetti, C. Colombo, E. Galli, M. Ruzzi, and E. Zennaro.** 1997. Sequencing and functional analysis of styrene catabolism genes from *Pseudomonas fluorescens* ST. Appl. Environ. Microbiol. **63:** 2232–2239.
3. **Blatny, J. M., T. Brautaset, H. C. Winther-Larsen, K. Haugan, and S. Valla.** 1997. Construction and use of a versatile set of broad-host-range cloning and expression vectors based on the RK2 replicon. Appl. Environ. Microbiol. **63:** 370–379.
4. **Cases, I., J. Pérez-Martín, and V. de Lorenzo.** 1999. The IIA^{Ntr} (PtsN) protein of *Pseudomonas putida* mediates the C source inhibition of the σ^{54}-dependent Pu promoter of the TOL plasmid. J. Biol. Chem. **274:**15562–15568.
5. **Collier, D. N., P. W. Hager, and P. V. Phibbs, Jr.** 1996. Catabolite repression control in the pseudomonads. Res. Microbiol. **147:**551–561.
6. **Coschigano, P. W., and L. Y. Young.** 1997. Identification and sequence analysis of two regulatory genes involved in anaerobic toluene metabolism by strain T1. Appl. Environ. Microbiol. **63:**652–660.
7. **Eaton, R. W., and P. J. Chapman.** 1995. Formation of indigo and related compounds from indolecarboxylic acids by aromatic acid-degrading bacteria: chromogenic reactions for cloning genes encoding dioxygenases that act on aromatic acids. J. Bacteriol. **177:**6983–6988.
8. **Hartmans, S., J. P. Smits, M. J. Van der Werf, F. Volkering, and J. A. M. de Bont.** 1989. Metabolism of styrene oxide and 2-phenylethanol in the styrene-degrading *Xanthobacter* strain 124X. Appl. Environ. Microbiol. **55:**2850–2855.
9. **Hartmans, S., M. J. Van der Werf, and J. A. M. de Bont.** 1990. Bacterial degradation of styrene involving a novel flavin adenine dinucleotide-dependent styrene monooxygenase. Appl. Environ. Microbiol. **56:**1347–1351.
10. **Heipieper, H. J., and J. A. M. de Bont.** 1994. Adaptation of *Pseudomonas putida* S12 to ethanol and toluene at the level of the fatty acid composition of membranes. Appl. Environ. Microbiol. **60:**4440–4444.
11. **Heipieper, H. J., G. Meulenbeld, Q. van Oirschot, and J. A. M. de Bont.** 1996. Effect of environmental factors on the *trans*/*cis* ratio of unsaturated fatty acids in *Pseudomonas putida* S12. Appl. Environ. Microbiol. **62:**2772–2777.
12. **Holtel, A., S. Marqués, I. Mohler, U. Jakubzik, and K. Timmis.** 1994. Carbon source-dependent inhibition of *xyl* operon expression of the *Pseudomonas putida* TOL plasmid. J. Bacteriol. **176:**1173–1176.
13. **Isken, S., and J. A. M. de Bont.** 1998. Bacteria tolerant to organic solvents. Extremophiles **2:**229–238.
14. **Labbé, D., J. Garmon, and P. C. K. Lau.** 1997. Characterization of the genes encoding a receptor-like histidine kinase and a cognate response regulator from a biphenyl/polychlorobiphenyl-degrading bacterium, *Rhodococcus* sp. strain M5. J. Bacteriol. **179:**2772–2776.
15. **Lau, P. C. K., Y. Wang, A. Patel, D. Labbé, H. Bergeron, R. Brousseau, Y. Konisshi, and M. Rawlings.** 1997. A bacterial basic region leucine zipper histidine kinase regulating toluene degradation. Proc. Natl. Acad. Sci. USA **94:**1453–1458.
16. **Leoni, L., A. Ciervo, N. Orsi, and P. Visca.** 1996. Iron-regulated transcription of the *pvdA* gene in *Pseudomonas aeroginosa*: effect of Fur and PvdS on promoter activity. J. Bacteriol. **178:**2299–2313.
17. **Marconi, A. M., F. Beltrametti, G. Bestetti, F. Solinas, M. Ruzzi, E. Galli, and E. Zennaro.** 1996. Cloning and characterization of styrene catabolism genes from *Pseudomonas fluorescens* ST. Appl. Environ. Microbiol. **62:**121–127.
18. **Marconi, A. M., M. Ruzzi, S. Iurescia, and E. Zennaro.** 1998. Genetics of styrene in bacteria. Rec. Res. Dev. Microbiol. **2:**95–105.
19. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Müller, C., L. Petruschka, H. Cuypers, G. Burchhardt, and H. Herrmann.** 1996. Carbon catabolite repression of phenol degradation in *Pseudomonas putida* is mediated by the inhibition of the activator protein PhlR. J. Bacteriol. **178:**2030–2036.
21. **O'Connor, K., C. M. Buckley, S. Hartmans, and A. D. W. Dobson.** 1995. Possible regulatory role for nonaromatic carbon source in styrene degradation by *Pseudomonas putida* CA-3. Appl. Environ. Microbiol. **61:**544–548.
22. **O'Connor, K., W. Duetz, B. Wind, and A. D. W. Dobson.** 1996. The effect of nutrient limitation on styrene metabolism in *Pseudomonas putida* CA-3. Appl. Environ. Microbiol. **62:**3594–3599.
23. **O'Connor, K. E., A. D. W. Dobson, and S. Hartmans.** 1997. Indigo formation by microorganisms expressing styrene monooxygenase activity. Appl. Environ. Microbiol. **63:**4287–4291.
24. **Panke, S., B. Witholt, A. Schmid, and M. Wubbolts.** 1998. Towards a biocatalyst for (s)-styrene oxide production: characterization of styrene degradation pathway of *Pseudomonas* sp. strain VLB120. Appl. Environ. Microbiol. **64:**2032–2043.
25. **Parkinson, J. S., and E. C. Kofoid.** 1992. Communication modules in bacterial signaling proteins. Annu. Rev. Genet. **26:**71–112.
26. **Sambrook, J., E. F. Fritsch, and T. Manniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria. Bio/Technology **1:**784–791.
28. **Terpe, K., K. Kerkhoff, E. Pluta, and D. Jendrossek.** 1999. Relationship between succinate transport and production of extracellular poly(3-hydroxybutyrate) depolymerase in *Pseudomonas lemoignei*. Appl. Environ. Microbiol. **65:**1703–1709.
29. **Utkin, I. B., M. M. Yakimov, L. N. Matveeva, E. I. Kozlyak, I. S. Rogozhin, Z. G. Solomon, and A. M. Bezborodov.** 1991. Degradation of styrene and ethylbenzene by *Pseudomonas* species Y2. FEMS Microbiol. Lett. **77:**237–242.
30. **Velasco, A., S. Alonso, J. L. García, J. Perera, and E. Díaz.** 1998. Genetic and functional analysis of the styrene catabolic cluster of *Pseudomonas* sp. strain Y2. J. Bacteriol. **180:**1063–1071.
31. **Warhurst, A. M., K. F. Clarke, R. A. Hill, R. A. Holt, and C. A. Fewson.** 1994. Metabolism of styrene by *Rhodococcus rhodochrous* NCIMB 13259. Appl. Environ. Microbiol. **60:**1137–1145.
32. **Yuste, L., I. Canosa, and F. Rojo.** 1998. Carbon-source-dependent expression of the P*alk*B promoter from the *Pseudomonas oleovorans* alkane degradation pathway. J. Bacteriol. **180:**5218–5226.
33. **Zhulin, I. B., B. L. Taylor, and R. Dixon.** 1997. PAS domain S-boxes in *Archaea*, bacteria and sensors for oxygen and redox. Trends Biochem Sci. **22:** 331–333.