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Transcriptional regulation of styrene degradation in *Pseudomonas putida* CA-3

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The styrene degradative pathway in *Pseudmonas putida* CA-3 has previously been shown to be divided into an upper pathway involving the conversion of styrene to phenylacetic acid and a lower pathway for the subsequent degradation of phenylacetic acid. It is reported here that expression of the regulatory genes *styS* and *styR* is essential for transcription of the upper pathway, but not for degradation of the lower pathway inducer, phenylacetic acid. The presence of phenylacetic acid in the growth medium completely repressed the upper pathway enzymes even in the presence of styrene, the upper pathway inducer. This repression is mediated at the transcription level by preventing expression of the *styS* and *styR* regulatory genes. Finally, an examination was made of the various stages of the diauxic growth curve obtained when *P. putida* CA-3 was grown on styrene together with an additional carbon source and it is reported that catabolite repression may involve a different mechanism to transcriptional repression by an additional carbon source.

Keywords: *Pseudomonas putida*, styrene, induction, catabolite repression

INTRODUCTION

Styrene is utilized by a variety of chemical industries, as a starting material in the production of synthetic polymers such as polystyrene and styrene-butadiene rubber and as a solvent in polymer processing. Gaseous and effluent emissions from these industries release large quantities of styrene into the environment, where, even at low concentrations, it can have toxic effects on living systems (Bond, 1989). Furthermore, mammalian metabolism of styrene produces a styrene oxide intermediate which is a known carcinogen (Foureman *et al*., 1989).

Styrene lacks true xenobiotic status however as it is naturally produced in the environment by the decarboxylation of cinnamic acid in decaying plant material (Shirai & Hisatsuka, 1979). Given the potential long-term exposure of various microbial communities present in the environment to styrene, it is not surprising perhaps that a variety of microbial species capable of degrading this aromatic compound have been isolated and identified (Baggi *et al*., 1983; Hartmans *et al*., 1989; O'Connor *et al*., 1995; Warhurst *et al*., 1994; Panke *et al*., 1998; Cox *et al*., 1993). Two major routes of aerobic styrene degradation are known to exist: (1) initial oxidation of the vinyl side-chain and (2) direct cleavage of the aromatic nucleus (Baggi *et al*., 1983; Hartmans *et al*., 1990; Warhurst *et al*., 1994; O'Connor *et al*., 1995). Several intermediates are sequentially produced during side-chain oxidation, which proceed through phenylacetic acid (PAA) (Baggi *et al*., 1983; Hartmans *et al*., 1990; O'Connor *et al*., 1995). Ring oxidation results in the formation of styrene *cis*-glycol and 3-vinyl catechol (Warhurst *et al*., 1994). It has been demonstrated previously that styrene degradation by *Pseudomonas putida* CA-3 proceeds via initial side-chain oxidation and can be divided into an upper pathway involving styrene, styrene oxide and PAA, and a lower pathway which begins with PAA (O'Connor *et al*., 1995).

Genetic studies have identified the genes involved in the upper pathway conversion of styrene to PAA in a number of styrene degraders of the genus *Pseudomonas* (Panke *et al*., 1998; Beltrametti *et al*., 1997; Marconi *et al*., 1996; Velasco *et al*., 1998). However, information regarding the lower pathway involved in PAA degradation is limited despite the identification of the first

Abbreviations: PAA, phenylacetic acid; PACoA, phenylacetate–CoA; SMO, styrene monooxygenase.

The GenBank accession number for the sequence determined in this work is AF257095.

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gene which encodes a phenylacetate–CoA (PACoA) ligase enzyme in a number of different *Pseudomonas* strains (Martinez-Blanco *et al.*, 1990; Vitovski, 1993; Minambres *et al.*, 1996; Velasco *et al.*, 1998; Ferrandez *et al.*, 1998). Potential regulatory genes *styS* and *styR* have also been isolated and sequence homology analyses have suggested strong links between these genes and those involved in other two-component regulatory systems (Velasco *et al.*, 1998).

Despite the fact that much of the styrene side-chain oxidation degradative pathway has been elucidated both at the biochemical and genetic level, little attention has focused on studying the physiological factors affecting the regulation of the pathway. Information such as this may help to facilitate the potential use of styrene-degrading strains in biological filters with the potential to convert styrene, present in a variety of industrial emissions, to less recalcitrant or innocuous pathway intermediates. In addition, it may help in the manipulation of the metabolic pathways for biotransformation applications, such as in the production of optically pure chemicals with broad chemical versatility (Panke *et al.*, 1998; Di Gennaro *et al.*, 1999).

In this study, we report on the inducive and repressive effects of various culture conditions on the styrene catabolic pathway(s) of *P. putida* CA-3 at both the physiological and genetic level by examining variations in catabolic enzyme activities, and in the transcription levels of genes encoding these enzymes, under different culture conditions.

METHODS

Media and growth conditions. The *P. putida* CA-3 strain utilized in this study was initially isolated from a bioreactor following enrichment on styrene and has been shown to utilize styrene as a sole source of carbon and energy (O'Connor *et al.*, 1995). Cultures were grown in 100 ml minimal salts (MS) medium in 1 litre Erlenmeyer flasks at 30 °C, with shaking at 120 r.p.m. MS medium contained 7·0 g K_2HPO_4, 3·0 g KH_2PO_4, 1·0 g $(NH_4)_2SO_4$ and 2 ml 10 % $MgSO_4.7H_2O$ per litre demineralized water; the latter was added post-autoclaving. Carbon sources were added to the medium (w/v) as follows: 0·1 % PAA, 0·05 % glucose and 0·1 % citrate. All were added pre-autoclaving. Growth on styrene required the addition of 70 µl liquid styrene to a test tube fixed centrally to the bottom of a baffled 1 litre Erlenmeyer flask. Cell growth was monitored by increasing OD_{540} with a Beckman DU640 spectrophotometer. For whole-cell enzyme assays and RNA isolations, utilized in RT-PCR studies, cells were harvested at mid-exponential phase (OD_{540} 0·5) unless otherwise stated.

Enzyme assays. Styrene monooxygenase (SMO) activity was monitored using the indole to indigo assay as previously described (O'Connor *et al.*, 1997). PACoA ligase activity was measured using the assay method of Martinez-Blanco *et al.* (1990). Activities are expressed as nmol product formed min^{-1} (mg protein)$^{-1}$ for both assays. Cells were harvested at mid-exponential phase unless otherwise stated.

Physiology and induction/repression studies. *P. putida* CA-3 was cultured solely on styrene, PAA, citrate and glucose, and the effects of these various carbon sources on the degradative pathway(s) examined. In the catabolite repression studies, cells were cultured in the presence of styrene + citrate, styrene + glucose, PAA + citrate, PAA + glucose and styrene + PAA. All carbon sources were added at the concentrations previously outlined. In experiments to assess the effect of adding PAA to a styrene-growing culture, PAA was added in early exponential phase (OD_{540}) to a final concentration of 10 mM. Cultures were then incubated for a further 30 min, after which time cells were harvested and subjected to enzyme assays and RNA isolation for RT-PCR. A second styrene-growing culture, to which no PAA was added, acted as a control.

HPLC analysis. In the catabolite repression studies, samples were taken for HPLC analysis to monitor concentrations of the repressing carbon sources. Sampling was performed by taking 1 ml of culture and filtering through a 0·45 µm sterile syringe filter (Schleicher & Schuell) into a HPLC vial stored on ice. Samples were then analysed on an LKB Bromma 2150 HPLC system with a Shodex R1-71 refractive index detector and a Highchrom heating block. A Rezex 8m % H organic acid column (300 × 7·8 mm; Phenomenex) was used with 0·005 M H_2SO_4 as the elution fluid, at a flow rate of 0·6 ml min^{-1}. The temperature of the column was maintained at 65 °C. Peaks and concentrations were determined by comparison of retention times with known standards.

Nucleic acid isolation and manipulation. Genomic DNA, isolated from *P. putida* CA-3 by the method of Ausubel *et al.* (1987), was used together with oligonucleotide primer pairs in the PCR cloning steps. The following primer pairs were successful in identifying target gene homologues in our strain: S51/K51 (S51, 5′-GGTTGAGCATGTAGGACGGT-3′, and K51, 5′-GCCAATACCGCCTTGCTTGA-3′, produced a 540 bp fragment of the *paak* gene); styS R1/F1 (R1, 5′-TGCGGGCAGCTCTACTTGGAAAAT-3′, and F1, 5′-CTGGCGGAAGGGCGGAACATC-3′, generated a 750 bp *styS* gene fragment); styR R1/F1 (R1, 5′-CGCCCCTTTCAAACGATTCAT-3′, and F1, 5′-ATGACCACAAAGCCCACAGTA-3′, generated a 590 bp *styR* gene fragment); sma R1/F1 (R1, 5′-GGCCGCGATAGTCGGTGCGTA-3′, and F1, 5′-AGAAAAAGCGTATCGGTATT-3′, generated the complete 1247 bp *styA* gene); styD R1/F1 (R1, 5′-GTAGGCGATAACCAACGAGCG-3′, and F1, 5′-ATGACAAGGAGCCTAACCATGAAC-3′, amplified the complete 1508 bp *styD* gene); crc R1/F1 (R1, 5′-GCGGCGCATGCTGGGAGAA-3′, and F1, 5′-TGTGATCAGCGGCTTAGGTTT-3′, generated a 900 bp fragment of the *crc* gene). These PCR products were cloned into Topo TA vector (Invitrogen), according to the manufacturer's instructions. Sequencing reactions were performed via CEQ 2000 dye terminator cycle sequencing (Beckman Coulter) and analysed on a 373 DNA stretch sequencer (Perkin Elmer Biosystems).

The above primers (with the exception of crc R1/F1) were also utilized in the analysis of gene transcription levels by RT-PCR. RNA was isolated according to Ausubel *et al.* (1987) and 1 µg reverse-transcribed with 1 µl 10 mM Random Primer (Boerhinger Mannheim), 1 µl 10 mM dNTPs (Boerhinger Mannheim), 2 µl BSA (1 mg ml^{-1}), 4 µl 5× buffer (Promega), 40 U RNasin (Promega) and 200 U MMLV-RT (Promega). Reactions were made up to 20 µl with diethylpyrocarbonate (DEPC)-treated demineralized water and incubated for 1 h at 37 °C to generate cDNA. Two microlitres of the RT reaction was then used as a template for subsequent PCR with the appropriate primer pair(s). The number of amplification cycles used was optimized to avoid reaching a point at which band intensities, representing differing gene expression levels within cells, would be misleading due to a plateau of amplification having been reached.

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Selected oligonucleotide primers were recombined into pairs suitable for PCR analysis of genomic DNA and cDNAs generated by the reverse transcription process. The recombined pairs were: K51/stySR1, stySF1/styRR1, styRF1/smaR1 and smaF1/styDR1. PCR products amplified were partially sequenced to establish their identity.

RESULTS

PCR amplification of gene homologues from the *P. putida* CA-3 genome

A PCR-based approach was used to clone the complete *styA* gene from CA-3 together with fragments of the *styS*, *styR* and *paak* genes from the strain. PCR primers were designed by analysis of existing GenBank sequences from other styrene-degrading strains of the genus *Pseudomonas*. The primer pair smaR1/F1 generated the full-length 1247 bp SMO gene encoding a protein of 47 kDa which exhibits 92 % homology at the amino acid level with the StyA protein from *Pseudomonas* sp. strain Y2 (Velasco *et al*., 1998). Similarly, primer pairs were designed to amplify portions of the *paak*, *styS* and *styR* genes. Comparison of nucleotide sequences obtained from these homologues with available GenBank sequence data revealed identities of 96, 98 and 97 %, respectively, with corresponding genes in *Pseudomonas* species strain Y2.

When CA-3 genomic DNA was subjected to PCR analysis using recombined oligonucleotide primers the following observations were made. K51/stySR1 generated a 2329 bp product containing N-terminal *paak* and C-terminal *styS* homologous regions while styRF1/smaR1 produced a 2052 bp fragment which, when subjected to nested PCR with the appropriate primers, was found to contain the *styR* and *styA* genes. Thus the genetic organization of the styrene catabolic operons in CA-3 appears identical to those of the highly homologous styrene-degrading *Pseudomonas* strains *Pseudomonas fluorescens* ST and *Pseudomonas* sp. strain Y2 (Fig. 1a).

RT-PCR analysis of total RNA from a styrene-grown culture of CA-3 with these recombined primer pairs was also performed. The ability to detect *paak*, *styS*, *styR* and *styA* mRNA transcripts indicates that all elements of the pathway are actively transcribed under this growth condition. However, the primer pairs K51/stySR1 and styRF1/smaR1 failed to generate any products; thus readthrough transcription does not occur between either *paak* and *styS*, or *styR* and *styA*. In contrast, stySF1/styRR1 amplified a 3571 bp product found to contain both the *styS* and *styR* genes, and smaF1/styDR1 generated a 3932 bp product which contained N-terminal *styA* gene and C-terminal *styD* gene homologous regions. This suggests that *stySR* are co-transcribed and that the upper pathway genes are transcribed in a single polycistronic mRNA (Fig. 1b).

Induction studies

Table 1 shows the results obtained when an overnight culture of *P. putida* CA-3 was inoculated into MS media containing one of the following carbon sources: styrene, PAA, glucose or citrate. Both SMO and PACoA ligase activities were detected in the MS media containing styrene with mRNA transcripts being detected for the *stySR* regulatory genes as well as the *styA* and *paak* upper and lower pathway genes, respectively, by RT-PCR. While culturing on PAA did result in PACoA ligase activity, no detectable SMO activity was present. These effects were mirrored at the transcription level with only *paak*, and not *styA* or *stySR*, mRNA transcripts being detected under these culture conditions. Growth of the organism on glucose or citrate did not induce any detectable enzymic activity or expression at the transcriptional level from the styrene catabolic operon.

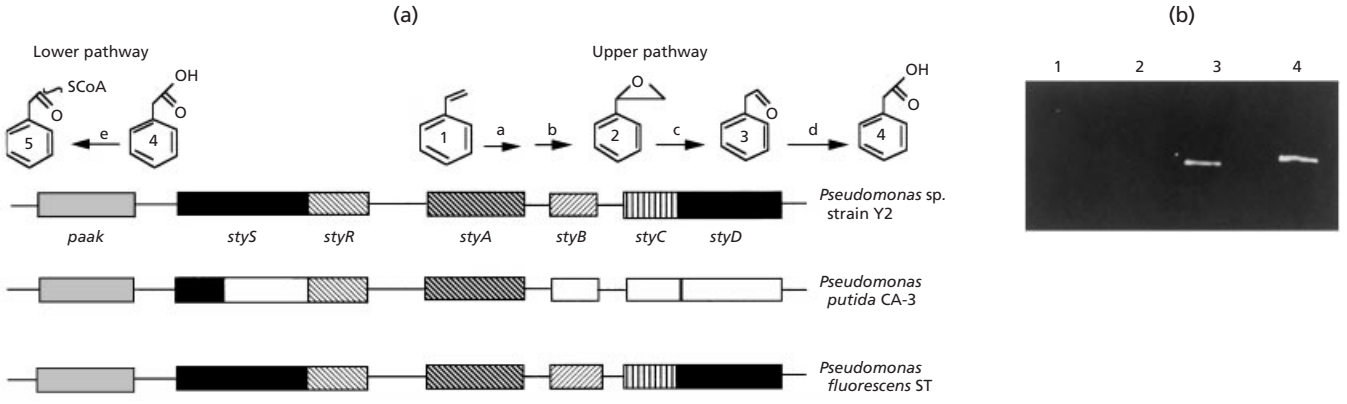

Fig. 1. (a) Comparison of genetic organization of the styrene-degradative genes (*paak*, *stySR* and *styABCD*), from a variety of *Pseudomonas* strains. Individual steps in the upper and lower pathway are shown above the genes responsible for the respective steps. a, SMO A subunit; b, SMO B subunit; c, styrene oxide isomerase; d, phenylacetaldehyde dehydrogenase; e, PACoA ligase. 1, styrene; 2, styrene oxide; 3, phenylacetaldehyde; 4, PAA; 5, phenylacetyl–CoA. Unshaded regions represent sequence data yet to be determined. (b). RT-PCR analysis of total RNA from a styrene-grown culture of *P. putida* CA-3 assessing operonic expression of pathway elements. Lanes: 1, K51/stySR1; 2, styRF1/smaR1; 3, 3571 bp product obtained with stySF1/styRR1; 4, 3932 bp product generated by smaF1/styDR1.

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Table 1. Induction and repression of the styrene catabolic operon in *P. putida* CA-3 under different growth conditions

Substrate	Enzyme activity*		RT-PCR†			
	SMO	PACoA ligase	*styA*	*paak*	*styS*	*styR*
Styrene	3·7	2·5	+	+	+	+
PAA	ND	2·8	−	+	−	−
Glucose	ND	ND	−	−	−	−
Citrate	ND	ND	−	−	−	−

* Specific enzyme activity expressed as nmol product formed min^{-1} (mg protein)$^{-1}$. Data are means of at least three independent determinations. ND, Not detected.

† RT-PCR analysis of mRNA transcripts for *styA* (encoding SMO), *paak* (encoding PACoA ligase), *styS* and *styR*. +, Transcripts detected; −, transcripts not detected.

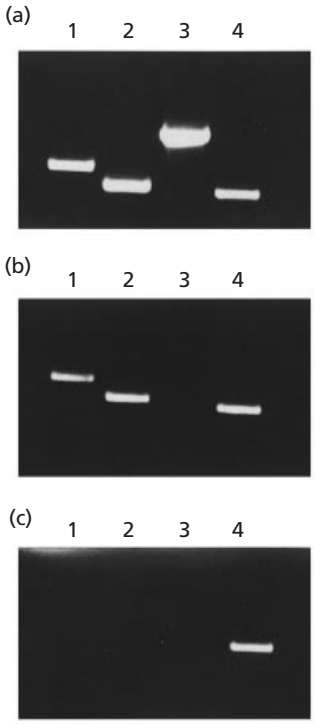

Fig. 2. RT-PCR analysis of RNA from cells grown on (a) styrene alone, (b) styrene with PAA added in mid-exponential phase, and (c) styrene and PAA. Lanes 1, 2, 3 and 4 correspond to the products for *styS*, *styR*, *styA* and *paak* under the respective growth conditions.

Repression of the upper pathway by PAA

When *P. putida* CA-3 was cultured in MS media containing both styrene and PAA, it was found that only the lower pathway *paak* gene was transcriptionally active (Fig. 2c). Despite the presence of styrene, the upper pathway inducer, in the media neither SMO activity nor *styA* mRNA transcripts could be detected. In addition, transcription of the *stySR* regulatory genes was not detected under these growth conditions (Fig. 2c). Furthermore, the addition of 10 mM final concentration PAA to a culture already growing on styrene as the sole carbon source caused a complete loss of expression of SMO, the key enzyme in the upper pathway involved in the conversion of styrene to PAA, within 30 min (Fig. 2b). While there still appear to be detectable levels of *stySR* mRNA transcripts, the RT-PCR products generated were consistently of much lower intensity than those obtained when CA-3 was cultured on styrene alone (Fig. 2a) (as determined by densitometric comparison of samples run on a single agarose gel; data not shown). These residual levels of *stySR* gene transcripts may be attributable to residual mRNAs generated during growth on styrene alone rather than continued expression of the genes following the introduction of PAA to the media. This reduction in transcription of both *styA* and *stySR* was not observed in CA-3 cells grown on styrene alone without the addition of PAA at mid-exponential phase (Fig. 2a).

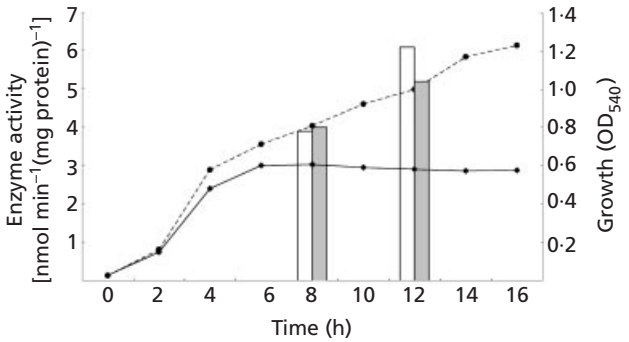

Fig. 3. Catabolite repression by citrate of styrene degradation in strain CA-3. ---, Growth on citrate+styrene; ——, growth on citrate alone. Growth is expressed as changes in OD_{540}. Enzyme activities are expressed as nmol product formed (mg protein)$^{-1}$. ▩, SMO activity; □, PACoA ligase activity.

Catabolite repression of the catabolic operon by a non-aromatic C source

Growth of *P. putida* CA-3 in the presence of both styrene and an additional carbon source such as citrate produced a diauxic growth pattern, as opposed to the typical growth curve obtained when the strain was cultured on styrene alone (Fig. 3). During the first stage of exponential growth (up to 5 h), it was not possible to detect either SMO (Fig. 3) or PACoA ligase enzyme activity. RT-PCR analysis of total RNA, isolated at the same time points as the enzyme assays were carried out, did identify the presence of mRNA transcripts from *stySR*, *styA* and *paak*, but at very low levels. Analysis of cells during the second stage of exponential growth (Fig. 3; 10–16 h) resulted in a marked increase in both SMO

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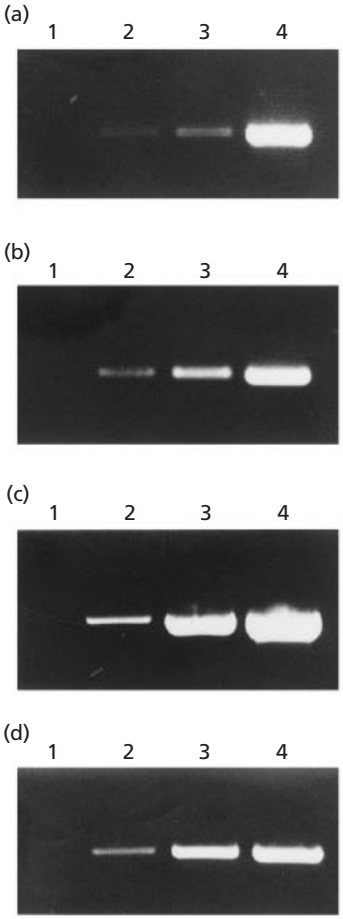

Fig. 4. RT-PCR analysis of total RNA isolated at different time points during diauxic growth on citrate+styrene (Fig. 3). (a) *styA*, (b) *paak*, (c) *styS*, (d) *styR*. Lanes: 1, cells grown on citrate; 2, cells grown on citrate+styrene (3 h); 3, cells grown on citrate+styrene (10 h); 4, cells grown on styrene.

and PACoA ligase enzyme activities. There was a concomitant increase in both upper and lower pathway gene transcription, which also corresponded with an increase in *stySR* expression (Fig. 4a–d). HPLC analysis of the growth media indicated that the increased activity from the catabolic operons coincided with exhaustion of the citrate in the MS media (data not shown).

DISCUSSION

Research to date on microbial styrene degradation has mainly concentrated on the genetic characterization of the catabolic operons together with functional analysis of key enzymes from the pathways in a number of strains (Beltrametti *et al.*, 1997; Velasco *et al.*, 1998; Martinez-Blanco *et al.*, 1990; Ferrandez *et al.*, 1998). While this has provided valuable information on the organization of the genes in the operons, little information is available on the physiological factors or specific environmental conditions that influence expression of these genes. The aim of this study therefore was to elucidate the genetic components of styrene degradation in *P. putida* CA-3, and to study the regulatory effects of different physiological batch culture growth conditions on the styrene catabolic operons in the strain.

PCR analysis of the CA-3 genome with various primer combinations has allowed us to map the structural organization of the styrene degradative pathway in this strain. Fig. 1(a) illustrates the high degree of structural conservation between the styrene catabolic operons of *P. putida* CA-3, *P. fluorescens* ST and *Pseudomonas* sp. strain Y2. One common structural feature of these pathways is that all genes thus far identified are transcribed in the same direction. We therefore attempted to determine if discrete operons existed within the pathway. Total RNA from a styrene-grown culture of CA-3 was chosen for analysis as this growth condition allows for detection of *paak*, *stySR* and *styA* gene mRNA transcripts (Table 1). Despite the success of the primer combinations K51/stySR1 and styRF1/smaR1 in amplifying appropriate regions of the CA-3 genome, their application in PCR analysis of cDNA produced from styrene-grown cells failed to generate products. Therefore, it appears that a transcriptional termination signal(s) exists between the *paak* and the *styR* genes which prevents readthrough transcription into the regulatory elements *styS* and *styR*. This conclusion is supported by recent work involving the promoter region of *stySR* from the styrene-degrading *P. fluorescens* ST (Santos *et al.*, 2000). Similarly, it would appear that a transcriptional termination signal(s) exists between *styR* and *styA*, which prevents the progress of RNA polymerase into the upper pathway genes beginning with *styA*. The primer pairs stySF1/styRR1 and smaF1/styDR1 generated RT-PCR products of 3571 and 3932 bp, respectively (Fig. 1b). This indicates that *styS* and *styR* are co-transcribed and expression of the upper pathway genes involves a single polycistronic mRNA. The pathway therefore appears to be composed of at least three discrete operons from which transcription occurs.

Complementation studies in *Escherichia coli* with elements of the *Pseudomonas* sp. strain Y2 styrene degradative pathway have identified a key role for *stySR* in the positive regulation of the upper pathway genes (Velasco *et al.*, 1998). The *styS* gene encodes a sensor kinase which becomes phosphorylated due to the intracellular presence of styrene. This results in a phosphorylation cascade event causing the response regulator, StyR, to become active. Amino acid comparisons of the gene products from the 3·5 kb *stySR* region of strain CA-3 with those of *Pseudomonas* sp. strain Y2 and *P. fluorescens* ST identified highly conserved regions consistent with functional domains identified in other two-component systems (Lau *et al.*, 1997; Coschigano & Young, 1997). The probability that *styR* functions as a response regulator in CA-3 is further supported by the observation that, as in strain Y2, a potential DNA-binding site with the pallindromic se-

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quence ATAAACCATGGTTTAT, centred at position −41 of the upper pathway promoter region, is also present in strain CA-3. As both strains lack a putative −35 σ-factor-binding site in the promoter region, it is likely that StyR exerts control over the upper pathway by binding at the −41 region and attracting RNA polymerase to the −10 TGTTAGCTT sequence upstream from *styA* (Barne *et al*., 1997; Velasco *et al*., 1998). This mechanism is very similar to the effect mediated by TodT, the response regulator of the *tod* operon (Lau *et al*., 1997). Given the degree of sequence homology between CA-3 and strain Y2 and the highly conserved structural features of the respective catabolic operons (Fig. 1a), a similar regulatory system is likely to function in our strain.

Induction experiments with strain CA-3 reveal the significance of styrene in expression of the upper and lower pathway enzymes. Table 1 clearly illustrates that detection of SMO and PACoA ligase activities occurs when cells are cultured on styrene, and not when glucose or citrate acts as the sole carbon source. Pathway induction in our strain is controlled at the transcriptional level since RT-PCR analysis of the respective genes indicated that transcription of *paak*, *stySR* and *styA* does not occur in the absence of the inducer styrene (Table 1). These results also indicate a key role for *stySR* as the two-component mechanism positively regulating the upper pathway given that expression of the upper pathway genes is not observed in the absence of *stySR* transcription. It should also be noted that cells grown on PAA, while showing increased levels of PACoA ligase enzyme expression, failed to induce transcription of either the upper pathway enzymes or the *stySR* regulatory molecules. Therefore, while *stySR* appear essential for induction of the upper pathway genes, they do not play a role in lower pathway induction by PAA.

In *P. putida* CA-3 an additional level of control exists, where the presence of PAA in the growth medium results in complete repression of the upper pathway, even in the presence of the upper pathway inducer, styrene. RT-PCR analysis of cells grown under these conditions reveals that this effect is mediated by repressing transcription of the two-component *stySR* genes (Fig. 2b, c). Thus, PAA acts as a negative regulator of the upper pathway genes. This is in contrast to the styrene-degrading strain *Xanthobacter* 124X, as growth of the bacterium on PAA results in detectable levels of activity from upper-pathway-associated enzymes styrene oxide isomerase and phenylacetaldehyde dehydrogenase (Hartmans *et al*., 1989). Furthermore, a recent study with *P. fluorescens* ST suggested that *stySR* transcription is constitutive regardless of the carbon source (Santos *et al*., 2000). Therefore, while a common route for styrene catabolism is observed in many of the bacterial species studied to date, it is clear that there are significant differences in how these degradative pathways are regulated. The mechanism by which this repressive effect is elicited in strain CA-3 is as yet unknown. To our knowledge this is the first report of transcriptional repression of a two-component regulatory system controlling an aromatic hydrocarbon degradative pathway by an intermediate of the pathway.

We have previously reported on the repressive effect of citrate and other nonaromatic carbon sources such as glutamate on styrene degradation in *P. putida* CA-3, by assessing oxygen uptake rates by cell-free extracts (O'Connor *et al*., 1995). Here, we demonstrate that the effect of catabolite repression is reduced transcription of both upper and lower pathway genes together with a reduction in *stySR* transcript levels. HPLC analysis of cultures grown in the presence of both styrene and citrate reveals that this repressive effect is sustained only while citrate is present in the media (data not shown). Depletion of the alternative carbon source coincides with increased upper and lower pathway gene expression, together with detectable levels of SMO and PACoA ligase enzyme activity, indicating growth on styrene (Fig. 4a, b). However, catabolite repression by citrate does not result in complete inhibition of gene transcription as *paak*, *styS*, *styR* and *styA* mRNA transcripts were detected during the early growth phase (Fig. 4a–d). Despite the low levels of gene transcripts, no enzyme activities were detectable. These observations contrast with those made when CA-3 is cultured on styrene and PAA, where complete inhibition of the *stySR* regulatory genes, and subsequently the upper pathway genes, occurs (Fig. 2c). Therefore the repression of the upper pathway by PAA appears to be exerted by a different mechanism to catabolite repression mediated by citrate. PAA specifically inhibits expression of the StySR regulatory molecules while citrate affects transcription of both upper and lower pathway genes. Citrate also exerts a similar repressive effect on PAA metabolism in strain CA-3. Reduced gene transcription is observed in cultures grown on PAA and citrate as long as citrate is present in the media (data not shown). This suggests that catabolite repression involves a more general cellular regulation mechanism rather than inhibition of specific targets as exhibited during growth of CA-3 on styrene and PAA. Using PCR primers based on the *P. putida crc* gene, a 900 bp *crc* homologue has been isolated from the *P. putida* CA-3 genome. The catabolite repression control (Crc) protein has previously been shown in *P. putida* and *Pseudomonas aeruginosa* (Hester *et al*., 2000) to act as a structure-specific ribonuclease which down-regulates the expression of a branched-chain keto acid pathway, by degrading mRNA of branched-chain keto acid dehydrogenase, encoded by *bkdR*, the positive regulator of the pathway. Therefore, given that reduced mRNA transcript levels for both *styS* and *styR* regulatory genes are observed during catabolite repression, and that a *crc* homologue is present, the possibility exists that a similar mechanism of control is being exerted in our strain. Further work is currently under way to explore this possibility.

In conclusion, the results presented here on the transcriptional repression of the styrene catabolic operon by metabolic intermediates of the pathway, as well as by nonaromatic carbon sources, may have implications regarding the suitability of this, and other, styrene-

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degrading strains for use in a variety of biotechnological applications.

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