Biochemistry, genetics and physiology of microbial styrene degradation

Niall D. O’Leary a,*, Kevin E. O’Connor b, Alan D.W. Dobson a

a Microbiology Department, National Food Biotechnology Centre, National University of Ireland, Cork, Ireland
b Department of Industrial Microbiology, National University of Ireland, Dublin, Ireland

Received 24 January 2002; received in revised form 27 June 2002; accepted 8 August 2002

First published online 11 September 2002

Abstract

The last few decades have seen a steady increase in the global production and utilisation of the alkenylbenzene, styrene. The compound is of major importance in the petrochemical and polymer-processing industries, which can contribute to the pollution of natural resources via the release of styrene-contaminated effluents and off-gases. This is a cause for some concern as human over-exposure to styrene, and/or its early catabolic intermediates, can have a range of destructive health effects. These features have prompted researchers to investigate routes of styrene degradation in microorganisms, given the potential application of these organisms in bioremediation/biodegradation strategies. This review aims to examine the recent advances which have been made in elucidating the underlying biochemistry, genetics and physiology of microbial styrene catabolism, identifying areas of interest for the future and highlighting the potential industrial importance of individual catabolic pathway enzymes.

© 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Phenylacetic acid; Two-component; Regulation; Biodegradation; Biotransformation

Contents

1. Introduction .......................................................... 404
2. Aerobic styrene degradation ............................................ 404
   2.1. Side-chain oxidation ............................................ 404
   2.2. Direct ring cleavage ............................................ 405
3. Anaerobic styrene metabolism ............................................ 405
4. Genetic organisation of the styrene upper pathway ....................... 406
5. The upper pathway regulatory apparatus .................................. 406
   5.1. StyS ........................................................... 407
   5.2. StyR ........................................................... 407
6. Pathway-specific regulation of the styrene catabolic operon ............... 408
7. Physiological factors affecting styrene degradation ........................ 410
   7.1. Catabolite repression ............................................ 410
   7.2. Nutrient limitation in continuous culture ........................ 411
8. Application of styrene-degrading microorganisms in biofilters ........... 411
9. Biotechnological applications of pathway enzymes ........................ 412
   9.1. Styrene oxide production ............................................ 412
   9.2. Indigo production ................................................. 412
   9.3. Other potential biotransformations .................................. 413
10. Conclusions ........................................................... 414
References ............................................................... 415

* Corresponding author. Tel.: +353 (21) 490 2952; Fax: +353 (21) 490 3101. E-mail address: ndolearyace@hotmail.com (N.D. O’Leary).
1. Introduction

The extensive use of aromatic hydrocarbons in industrial processes, coupled with inadequate waste management strategies, has led to the widespread introduction of these compounds into our environment [1]. Styrene, the simplest of the alkenylbenzenes, is one such compound which is employed both as a starting material for synthetic polymers and as a solvent in the polymer-processing industry, leading to its release in a variety of industrial effluents. Styrene is known to be genotoxic while styrene oxide, the major in vivo metabolite of styrene, is classified as a probable carcinogen in humans [2]. Recent reports indicate that styrene may also have an immuno-modulatory effect on workers exposed to gaseous emissions in an industrial setting [3]. As a result of concerns arising from these findings, the last decade has seen intense investigation into various aspects of the microbial degradation of this compound.

One feature of styrene which has undoubtedly been influential in the evolution of microbial styrene catabolic routes is the natural occurrence of this compound, e.g. via fungal decarboxylation of cinnamic acid [4,5]. This is perhaps reflected in the diverse range of styrene-degrading microbes that have been isolated from various soil locations around the globe since the late 1970s. While a dependence on mixed cultures has been reported, pure culture isolates capable of styrene degradation have included species of Pseudomonas, Rhodococcus, Nocardia, Xanthobacter and Enterobacter as well as the black yeast Exophiala jeannelii (for a review see Hartmans [6]). However, as the aim of this review is to present the current understanding of microbial styrene catabolism, the primary focus will repeatedly centre upon the genus Pseudomonas, from which significant insights have come in recent years.

2. Aerobic styrene degradation

Two main pathways for the aerobic degradation of styrene have been described, one involving an initial oxidation of the vinyl side-chain [7] and the other based on direct attack on the aromatic nucleus [8] (Fig. 1).

2.1. Side-chain oxidation

The side-chain oxidation pathway involves epoxidation of the vinyl side-chain by a flavin adenine dinucleotide-dependent, two-subunit monooxygenase followed by isomerisation of the epoxystyrene formed to phenylacetaldehyde (PAAL). This compound is subsequently oxidised to phenylactic acid (PAA) through the action of either an NAD\(^+\)- or phenazine methosulfate-dependent dehydrogenase [6]. This conversion of styrene to PAA is generally referred to as the upper pathway of styrene degradation and appears to operate in many of the bacterial strains studied to date; examples include Pseudomonas putida CA-3 [9], Xanthobacter strain 124X [10], Xanthobacter strain S5 [11], Pseudomonas fluorescens ST [12], Pseudomo-

![Fig. 1. A summary of the major pathways of bacterial styrene degradation. The number(s) associated with each pathway identify the organism(s) that have been shown to perform the particular transformation: 1, P. putida CA-3; 2, Xanthobacter strain 124X; 3, Xanthobacter strain S5; 4, P. fluorescens ST; 5, Pseudomonas sp. strain Y2; 6, Corynebacterium strain ST-10; 7, Rhodococcus rhodochrous NCIMB 13259. Dotted lines indicate proposed degradative routes yet to be formally demonstrated.](https://academic.oup.com/femsre/article/26/4/403/684625/fig1)
nas sp. strain VLB120 [13] and Pseudomonas sp. strain Y2 [14,15] (Fig. 1).

The lower pathway is thought to involve the conversion of PAA to Krebs cycle intermediates but this has not been formally demonstrated in any styrene-degrading bacterium thus far. The current understanding of the microbial metabolism of PAA owes itself to studies in P. putida U [16] and Escherichia coli W [17]. In both strains, PAA is first activated to phenylacetyl-CoA (PACoA), before passing through a β-oxidation-like, enzyme-catalysed conversion process to yield acetyl-CoA moieties. Similarly, it has been reported for both strains that PACoA, not PAA, acts as the true inducer of the catabolic core. It alleviates the negative regulation imposed on the pathway by the DNA-binding transcriptional repressor PaaX in E. coli and PaaN in P. putida U [18]. This degradative route, referred to as the PACoA-catabolon, represents the hybridisation of a typically anaerobic step such as coenzyme A activation, with an aerobic β-oxidation-like process. Analysis of genome sequences has revealed that genetic elements of this catabolon are found in various bacterial genera, suggesting that it may represent a common, central catabolic route for bacterial PAA metabolism [18]. There are several, cautious lines of evidence to suggest that this catabolon may also operate in styrene-degrading strains. Genetic analysis of Pseudomonas sp. strain Y2 [15] and P. putida CA-3 [19] identified a PACoA ligase gene, paaK, directly upstream from the genes responsible for the conversion of styrene to PAA, which would facilitate activation of PAA to PACoA. In addition, Baggi and co-workers reported in P. fluorescens ST the accumulation of 2-hydroxyphenylacetic acid (2-OHPAA) in the culture media of cells grown on styrene and PAA [20]. The authors proposed that 2-OHPAA could be metabolised via homogentisate to acetoacetate based on the detection of homogentisate-1,2-dioxygenase activity. However, it has been demonstrated that disruption of the PACoA-catabolon paaZ gene, encoding an aldehyde dehydrogenase involved in ring cleavage of 2-OHPCoA in E. coli W, results in the hydrolysis of accumulated 2-OHPCoA to 2-OHPAA, which can be detected in the growth medium [17]. Thus the presence of 2-OHPAA in styrene- and PAA-grown cultures of P. fluorescens ST may reflect the role of a PACoA-catabolon in this strain also [20].

It should be noted that in the styrene-degrading Corynebacterium strains AC-5 and ST-10, there appears to be a modified form of the upper pathway with PAAL being reduced to 1-phenylethanol by a PAAL reductase enzyme [21,22]. Interestingly, an accumulation of 2-phenylethanol was reported in E. coli JM109 cells when transformed with the P. fluorescens ST genes encoding styrene monooxygenase and epoxysyrene isomerase, due to the presence of endogenous PAAL reductase activity, despite the inability of the natural host to utilise styrene [12,23]. A relatively minor pathway involving 1-phenylethanol, acetophenone, salicylate and 2-OHPAA has also been reported to occur in Pseudomonas sp. strain Y2 [14], suggesting that more than one degradative route may operate in a single strain.

2.2. Direct ring cleavage

Rhodococcus rhodochrous NCIMB 13259, a chemical dump isolate capable of growth on a diverse range of aromatic hydrocarbons, degrades styrene via direct oxidation of the aromatic nucleus. Thin layer chromatography and nuclear magnetic resonance studies revealed that styrene metabolism in this organism proceeded via 3-vinylcatechol [8] (Fig. 1). An NAD⁺-dependent cis-glycol dehydrogenase activity was detected in cells grown on nutrient broth and styrene. Cells grown under these conditions were also able to oxidise toluene cis-glycol. The styrene cis-glycol enzyme activity behaves very similarly to toluene cis-glycol dehydrogenase and is believed to be the same enzyme with a broad substrate specificity. 3-Vinylcatechol is further degraded via meta-cleavage to acetaldehyde and pyruvate. The role of catechol 2,3-dioxygenase in this process was established when 3-vinylcatechol accumulated in cells incubated with styrene and 3-fluorocatechol, an inhibitor of the dioxygenase. Evidence of direct ring cleavage has also been reported in P. putida MST where cells grown on styrene release 1,2-dihydroxy-3-ethenyl-3-cyclohexene into the culture medium [24]. Ring attack has also been proposed in Xanthobacter strain 124X [10]. The transient accumulation of a yellow colour in the media of cells grown on styrene and 1-phenylethanol, but not styrene oxide, corresponds with observations made in Nocardia species T5 grown on 1-phenylethanol. Cripps et al. [25] proposed that the 1-phenylethanol degradation in this Nocardia strain involved initial oxidation of the aromatic nucleus by a dioxygenase.

3. Anaerobic styrene metabolism

Degradation of styrene under anaerobic conditions was observed in an anaerobic consortium enriched on styrene and ferulic acid [26]. Although a wide range of aromatic intermediates was detected, the principal route of styrene degradation under these conditions was proposed to involve conversion to PAA via 2-phenylethanol and PAAL. This pathway bears similarity to the aerobic transformations of styrene. The initial step in aerobic degradation of styrene utilises molecular oxygen while it is thought that anaerobic bacteria derive the necessary oxygen from available water. Divergence of the aerobic and anaerobic pathways arises with the respective downstream processing of PAA. Anaerobic consortia are thought to convert PAA to benzoic acid via benzyl alcohol and benzoic dehydrogenase, much like the meta-cleavage pathway of toluene degradation encoded by the TOL plasmid, pWW0 [26].
4. Genetic organisation of the styrene upper pathway

Marconi and co-workers were the first to report the cloning and identification of genes involved in styrene catabolism [12]. They cloned two genes from the *P. fluorescens* ST chromosome, encoding enzymes with styrene monooxygenase and epoxystyrene isomerase activities. Further genetic characterisation of the locus by this group identified an upper pathway catabolic operon containing all enzymes necessary for the conversion of styrene to PAA [23]. The genes involved were *styA* and *styB*, encoding a two-subunit styrene monooxygenase responsible for the transformation of styrene to epoxystyrene, while *styC* was reported to encode an epoxystyrene isomerase that converted styrene oxide to PAAL. The concluding step of the upper pathway, the formation of PAA, was performed by the *styD*-encoded PAAL dehydrogenase (PAALDH). This operonic organisation was subsequently reported in three other styrene-degrading *Pseudomonas* strains, namely *Pseudomonas* sp. strain Y2 [15], *Pseudomonas* sp. strain VLB120, [13], and *P. putida* CA-3 [19]. The high degree of sequence similarity between the *styA* genes thus far cloned and sequenced (Table 1) suggests a common evolutionary origin for this catabolic route. This is further supported by the conserved operonic structure of the upper pathway between these *Pseudomonas* strains, which corresponds with the order of the catabolic steps (Fig. 2). This observation contrasts sharply with the diverse organisation and sequence variation observed in different *paa* genes from various species capable of PAA degradation [18], suggesting that independent evolution of the styrene upper and lower pathways may have occurred.

5. The upper pathway regulatory apparatus

The initial observation in *P. fluorescens* ST that the *‘sty’* genes were clustered on the chromosome raised the possibility that a pathway-specific regulatory apparatus could also be in close proximity. This was indeed established by Velasco et al. following complementation studies in *E. coli* W with elements of the *Pseudomonas* sp. strain Y2 *sty* operon [15]. It was reported that expression of the catabolic operon in *E. coli* W was significantly reduced in the absence of two genes, *styS* and *styR*, located upstream from the styrene catabolic operon. Expression was restored when these genes were provided in *trans*. Several studies have since reinforced the essential function of *stySR* in styrene catabolism and their role in pathway regulation will be dealt with in more detail below. *styS* and *styR* (Table 1; Fig. 2) encode two proteins which display a high level of amino acid similarity with members of the superfamily of two-component signal transduction systems found in both prokaryotes and eukaryotes [27,32]. Based on sequence analysis and RT-PCR investigations, it appears that both genes are expressed in a transcriptionally coupled fashion [19].

Table 1

Comparison of *P. putida* CA-3 styrene pathway gene products with other proteins of known function

<table>
<thead>
<tr>
<th>Gene</th>
<th>% G+C</th>
<th>Product(aa)</th>
<th>Similar polypeptide</th>
<th>% ID</th>
<th>Organism</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>paaK</td>
<td>62.7</td>
<td>437</td>
<td>PaaK (437)</td>
<td>96</td>
<td><em>Pseudomonas</em> sp. strain Y2</td>
<td>AJ000330</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PheA (439)</td>
<td>85</td>
<td><em>Pseudomonas</em> putida U</td>
<td>AF029714</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PaaK (447)</td>
<td>68</td>
<td><em>Escherichia coli</em> W</td>
<td>X97452</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PaaK (440)</td>
<td>67</td>
<td><em>Azotobacter vinelandii</em> KB740</td>
<td>AF176259</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PaaK (445)</td>
<td>49</td>
<td><em>Bacillus halodurans</em></td>
<td>AP001507</td>
</tr>
<tr>
<td>styS</td>
<td>55.5</td>
<td>226 (frag)</td>
<td>StyS (982)</td>
<td>99</td>
<td><em>Pseudomonas</em> sp. strain Y2</td>
<td>AJ000330</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>StyS (983)</td>
<td>88</td>
<td><em>Pseudomonas</em> flavescens ST</td>
<td>AF024619</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TutC (979)</td>
<td>49</td>
<td><em>Thauera aromatica</em></td>
<td>U57900</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TodS (978)</td>
<td>41</td>
<td><em>Pseudomonas</em> putida F1</td>
<td>AF180147</td>
</tr>
<tr>
<td>styR</td>
<td>52.2</td>
<td>207</td>
<td>StyR (207)</td>
<td>98</td>
<td><em>Pseudomonas</em> sp. strain Y2</td>
<td>AJ000330</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>StdR (207)</td>
<td>96</td>
<td><em>Pseudomonas</em> sp. strain VLB120</td>
<td>AF031161</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>StyR (207)</td>
<td>90</td>
<td><em>Pseudomonas</em> flavescens ST</td>
<td>AF024619</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TutB (218)</td>
<td>55</td>
<td><em>Thauera aromatica</em></td>
<td>U57900</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TodT (227)</td>
<td>49</td>
<td><em>Pseudomonas</em> putida F1</td>
<td>AF180147</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NodW (227)</td>
<td>51</td>
<td><em>Bradyrhizobium japonicum</em></td>
<td>AF322013</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FixJ (211)</td>
<td>40</td>
<td><em>Azorhizobium caulinodans</em></td>
<td>X56658</td>
</tr>
<tr>
<td>styA</td>
<td>57.9</td>
<td>416</td>
<td>StdA (415)</td>
<td>98</td>
<td><em>Pseudomonas</em> sp. strain VLB120</td>
<td>AF031161</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>StyA (415)</td>
<td>98</td>
<td><em>Pseudomonas</em> putida S12</td>
<td>Y13349</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>StyA (415)</td>
<td>95</td>
<td><em>Pseudomonas</em> sp. strain Y2</td>
<td>AJ000330</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>StyA (415)</td>
<td>96</td>
<td><em>Pseudomonas</em> flavescens ST</td>
<td>AF292524</td>
</tr>
</tbody>
</table>

PaaK, phenylacetyl-coenzyme A ligase (PACoAL) of the styrene degradative pathway [13]; PaaK and PheA, aerobic PACoAL from PAA catabolic pathways [18]; StyS and StyR, sensor histidine kinase and response regulator from styrene degradative pathway [15,34,41]; StdR and StdA, regulator and styrene monooxygenase (large component) [41]; TodS and TodT, sensor kinase and response regulator, respectively, involved in aerobic toluene degradation [28]; TutC and TutB, sensor kinase and response regulator of toluene degradation [29]; NodW and FixJ, response regulators involved in nodulation and nitrogen fixation, respectively [38,39]; StyA, oxygenase component of styrene monooxygenases [12,15,41].
5.1. StyS

The StyS proteins exhibit amino acid sequence similarity to a number of sensor kinase proteins (Table 1), particularly the TodS and TutC sensor kinases that regulate toluene catabolism in *P. putida* F1 and DOT-T1E [28,29]. Interestingly, recent work on toluene degradation by *P. putida* F1 and DOT-T1E indicates that styrene can act as an inducer of the *tod* operon [30,31]. StyS consists of five distinct domains (Fig. 3). Two of these are histidine kinase domains, HK1 and HK2, both containing the characteristic kinase amino acid blocks H, N, G1, F and G2 (Fig. 3). The occurrence of two perfectly duplicated kinase cores was previously reported as being unique to the TodS sensor kinase [31]. It should be noted that the HK1 and HK2 domains in StyS differ slightly and have been further classified into the kinase subfamilies 1a and 4, respectively [27]. The receiver domain of StyS contains the D, D, S, K amino acid residues typical of bacterial response regulators, and belongs to the RA2 receiver subfamily [27]. Adjacent to the receiver is an input region (Input 2), with significant similarity to the putative oxygen-sensing domain of TodS [28]. Located within this domain are the S1 (PAS) and S2 (PAC) sensory boxes, typical of PAS domains found in a variety of prokaryotic and eukaryotic sensory mechanisms [33]. Zennaro and co-workers suggest that the presence of the PAS domain may play a role in the detection of styrene as a stress factor, perhaps as a consequence of the redox potential generated in the cell due to the toxicity of the intermediates styrene oxide or PAAL [34]. However, the observation that input domains are usually located at the N-terminus of a sensor kinase has led to the suggestion that another input domain (Input 1) may also be present in StyS [15]. It is not yet known precisely how the input domain responds to the presence of styrene.

5.2. StyR

The StyR proteins display amino acid identity with many response regulators of other two-component systems. Given the similarity between StyS and the sensor kinases of the toluene degradation regulatory apparatus, TodS and TutB, it is not surprising that StyR also exhibits high level amino acid identity with the cognate response regulators, TodT and TutC (Table 1). StyR contains a receiver domain and a DNA-binding domain joined by a Q-linker region (Fig. 4). The receiver domain belongs to the RA4 receiver subfamily and contains the essential, conserved D, D, T and K amino acid residues found in a variety of bacterial response regulators [27,35]. A consensus sequence of putative DNA-binding domains in the family 3 response regulators is also present in the carboxyl-terminal region of the proposed protein [36]. The StyS and StyR proteins are one of only four two-component regulatory systems known to be involved in aromatic hydrocarbon catabolism [37]. Interestingly, the StyR proteins also display amino acid similarity with the response regulators NodW and FixJ, involved in nodulation and nitrogen fixation, respectively [38,39] and may, as has been proposed by Diaz and co-workers, represent another example of domain shuffling which could have occurred during the parallel evolution of family 3 DNA-binding domains with cluster 1 receiver modules [15,36,40].
6. Pathway-specific regulation of the styrene catabolic operon

Styrene-dependent induction of the sty operon has been demonstrated by several groups. Northern blotting and transcription start site analysis by primer extension in Pseudomonas sp. strain Y2 revealed that the presence of styrene was essential for upper pathway gene transcription [15]. Zennaro and co-workers have reported that β-galactosidase activity was only detectable in P. fluorescens ST cells harbouring a plasmid-borne styA promoter::lacZ fusion when styrene was present in the medium [34]. Furthermore, reverse-transcription analysis of total RNA from P. putida CA-3 grown on various carbon sources also indicated that there were no detectable mRNA transcripts from the sty operon in the absence of styrene [19]. Complementation studies performed in E. coli with elements of the sty operon from both Pseudomonas sp. strain...
Y2 and Pseudomonas sp. strain VLB120, demonstrated that the StyS and StyR proteins were required for the styrene-dependent induction of the sty upper pathway genes [15,41]. It has also been reported in P. putida CA-3 that transcription of the styrene upper pathway genes is entirely dependent upon expression of stySR [19].

Analysis of the promoter region upstream from the styA gene has identified a number of key features that support the proposed role of StyR as the transcriptional activator of the styrene upper pathway genes. A potential DNA-binding site with the palindromic sequence ATAAAC-CATGGTTTAT, centered at position 341, was located in the styA promoter region of Pseudomonas sp. strain Y2 [15]. This sequence is almost identical to the inverted repeat ATAAACCATGGTTTAT of the TodT-binding site in the tod operon [28]. Sequence analysis of the potential styA promoter region in P. fluorescens ST [23] and P. putida CA-3 [19] revealed that this is also likely to be the case in these strains. In Pseudomonas sp. strain VLB120, a 'sty' box was located 75 bp upstream from the styA start codon and it was shown that 5' deletion of this region prevented styA expression [41]. As these strains do not possess a putative −35 σ70 binding site in their styA promoter region, it is likely that StyR exerts control over the upper pathway by binding at this −10 region sty box and attracting RNA polymerase to the extended −10 box (TGTTAGCTT) present in the promoter. A proposed model of the styrene-induced phosphorylation cascade, leading to transcriptional activation of the upper pathway genes by the two-component StyS and StyR proteins, is presented in Fig. 5.
in P. putida CA-3, reverse transcription analysis of total RNA revealed that stySR mRNA transcripts were not detectable in the absence of styrene, suggesting basal level transcription and/or poor transcript stability in the absence of inducer [19]. The authors also reported that in CA-3 the addition of the lower pathway substrate, PAA, into styrene-growing cultures caused a complete loss of detectable upper pathway activity and that this was linked to transcriptional repression of stySR. This would also lead us to the conclusion that stySR have no role in the regulation of the lower pathway. This hypothesis is further supported by reports of the transcriptional repressors PaaN and PaaX controlling expression of the PAcO A catabolic core in P. putida U and E. coli W, respectively [16,17]. It is unclear as to the mechanism by which PAA imposes this negative regulation on the upper pathway, but the phenomenon is not universal in styrene degradation. The presence of PAA does not prevent detection of styrene oxide isomerase activity in the styrene-degrading Xanthobacter strain 124X [10]. The authors of the latter work also report detectable PAA dehydrogenase activity. However, it may be worth noting that early work in CA-3 reported similar activity during growth on PAA [9], until it was later demonstrated that there was clearly no transcription of the CA-3 upper pathway structural genes when PAA was present [19]. Thus, PAA or its metabolic intermediates may induce a non-styD-encoded acetaldehyde dehydrogenase, resulting in false-positive upper pathway activity under potentially repressive growth conditions.

7. Physiological factors affecting styrene degradation

7.1. Catabolite repression

Catabolite repression has been reported in a number of Pseudomonas strains for a variety of aromatic carbon assimilation routes including toluene [42], phenol [43], benzylamine [44], chloroaromatics [45] and ethylbenzene [46]. Similarly, it has been reported that a number of non-aromatic carbon sources such as organic acids and/or carbohydrates repress styrene degradation in both P. putida CA-3 and P. fluorescens ST [9,19,34]. Interestingly, the catabolite repression substrate profiles of the latter two organisms, while sharing some common carbon sources, are affected quite differently by others. In CA-3, glucose, succinate and acetate have no repressing effects [9], while the same carbon sources strongly repress induction of the styA promoter in P. fluorescens ST [34]. It is unclear as yet how catabolite repression is specifically imposed on sty gene expression in these strains. Santos and co-workers postulate that since styS and styR appear to be constitutively expressed in strain ST, catabolite repression may involve some factor(s) affecting signal transduction of the two-component system, either through repression of the sensor kinase activity or by direct repression of the styA promoter [34]. However, in CA-3, the repressive effect of citrate was shown to involve reduced transcription of stySR and styA for as long as the alternative carbon source persisted in the medium. It was also reported in

Fig. 5. Schematic depiction of a possible mode of signal transduction in the regulation of the styrene catabolic genes. (1) Styrene enters the cell membrane and (2) interacts with Input 1, a sensory input domain in StyS. (3) Oxygen changes indicating a reduction in cell energy levels may also be sensed by another input domain, Input 2. (4) Depending on the input domain activated, one of the kinases phosphorylates its associated His residue (bold face). (5) The phosphoryl group is ultimately transferred to an Asp residue in the receiver domain of StyR, activating the carboxy DNA-binding domain of the response regulator StyR. (6) The phosphorylated response regulator binds to the 8-bp inverted repeat shown centred at position −41 upstream from the styA start codon. (7) This attracts RNA polymerase (RNA pol) to the promoter region and transcription of the upper pathway genes (ABCD) takes place.
CA-3 that the lower pathway paaK, which appears to be regulated independently of stySR, is also subject to catabolite repression, with effects being mediated at the transcriptional level [19]. It has been reported that cAMP is not involved in catabolite repression in P. putida CA-3 [47] and other Pseudomonas species where cAMP pools do not fluctuate with carbon source, nor does the addition of cAMP relieve repression of catabolite responsive pathways [48]. Recent reports have, however, documented the involvement of a catabolite repression control protein (Crc) in both P. putida and Pseudomonas aeruginosa [49]. Catabolite repression of the P. putida branched-chain keto acid pathway by Crc was proposed to involve post-transcriptional regulation of the pathways positive regulator, BkdR [50]. Hester et al. have postulated that Crc might act as a structure-specific ribonuclease degrading bkdR mRNA or, alternatively, hindering the efficiency of bkdR mRNA translation [50]. The former hypothesis supported observations made in P. putida CA-3, where the addition of citrate caused a dramatic reduction in detectable levels of stySR mRNA transcripts [19]. However, a recent study by Yuste and Rojo into catabolite repression of the P. putida GPO1 alkane degradation pathway provided a further insight [51]. Using wild-type and Crc-deficient Pseudomonas strains harbouring key genetic elements of the alkane degradation pathway, the authors demonstrated that mRNA transcript stability during catabolite repression was not significantly different in the presence or absence of crc. These findings suggest that Crc does not function as a ribonuclease and is unlikely to be responsible for the low levels of stySR mRNA transcripts detected during catabolite repression of styrene degradation in P. putida CA-3. In addition, and perhaps more interestingly, Yuste and Rojo demonstrated that Crc-dependent repression was only observed in Luria–Bertani rich media and was not involved in the catabolite repression observed in GPO1 when mineral salts medium containing a single, repressing organic acid was used. Thus, catabolite repression of the hydrocarbon degradative pathway in this P. putida GPO1 strain, and potentially in others, involves at least two independent mechanisms. In fact, what is currently emerging from various studies on catabolite repression in other microbial systems is that an interplay of cellular factors may be involved including integration host factor (IHF), PtsN (IIA)f protein, the alarmone guanine 5-diphosphate 3-diphosphate (ppGpp), σ factor stability and interaction with RNA polymerase and alterations in σ factor dependence [37,52,53]. This will be a complex issue to resolve but its importance is obvious if one intends to apply the styrene catabolic potential of an organism to environments where the carbon composition is varied.

7.2. Nutrient limitation in continuous culture

Investigations of styrene catabolism under conditions of organic and inorganic nutrient limitation have thus far only been performed with P. putida CA-3. However, the findings reported strongly resemble observations made with the TOL pathway during continuous culture of P. putida mt-2 harbouring pWW0 and may therefore be representative of a common feature in the regulation of catabolic pathways in Pseudomonas species which, through evolution, have become integrated with overall cell physiology. O’Connor and co-workers reported that growth of P. putida CA-3 on limiting concentrations of styrene resulted in far higher upper pathway enzyme activities than those recorded in batch systems [54]. Subsequent investigation revealed that this effect coincided with increased stySRABCD and paaK gene transcript levels in response to limiting concentrations of pathway substrates. It was not possible to detect transcription of the upper or lower pathway genes when succinate was the limiting carbon source, thereby ruling out the possibility that increased transcription of the two-component stySR apparatus was due to non-specific signal induction during carbon starvation [55]. This finding was almost identical to that reported in P. putida mt-2, where limiting concentrations of the TOL pathway inducer, m-xylene, resulted in increased transcription of the necessary degradative genes [56]. According to Harder and Dijkhuizen, microorganisms may overcome low concentrations of carbon by either (a) increased uptake and intracellular accumulation of the substrate, or (b) enhanced initial metabolism of intracellular substrate [57]. Although little is currently known with respect to active intracellular styrene accumulation, the observed increase in the levels of sty mRNA transcripts supports the use of the latter mechanism.

Conversely, it has been reported that inorganic nutrient limitations such as phosphate, sulfur and nitrogen, which are common environmental conditions, have strikingly similar repressive effects on the catabolic activities of the P. putida CA-3 sty operon and the P. putida mt-2 TOL pathway, as well as making them highly sensitive to catabolite repression [54–56]. It is thought that a variety of global regulatory factors are involved in these co-ordinated responses but the concentration trends and potential roles of these factors are poorly understood at present [37,58]. Nevertheless, the transcriptional repression of the sty operon in CA-3 and potentially in other styrene degraders during inorganic nutrient-limiting growth has serious implications with respect to the development of environmentally-based bioremediation/biodegradation strategies.

8. Application of styrene-degrading microorganisms in biofilters

Despite these challenges, several groups have already begun to investigate the potential of biofilters for the removal of styrene from contaminated waste gases. Cox and co-workers, using perlite-packed filters to enrich styrene-
degrading fungi, reported styrene elimination capacities of 70 g m⁻³ filter bed h⁻¹ when exposed to influent gas containing styrene amounts ranging from 290 to 675 mg m⁻³ [59]. In a subsequent study, Weigner et al. achieved 85% elimination of styrene from an organic load of 170 g m⁻³ h⁻¹, 18 days after the start-up of four serially aligned, perlite-packed filters that had been inoculated with a long-term adapted mixed microbial culture [60]. The potential of low cost natural filters has also been assessed. Reittu and co-workers, using peat as filter material, reported maximal styrene elimination capacities of 30 g m⁻³ filter material h⁻¹. The group identified several bacterial isolates capable of styrene degradation from the genera Tsukamurella, Pseudomonas, Sphingomonas, Xanthomonas and an unidentified member of the Proteobacteria [61]. However, an elimination capacity of 63 g m⁻³ h⁻¹ was subsequently reported using a filter packed with a mixture (4:1) of peat and glass beads inoculated with the styrene degrader R. rhodochrous NCIMB 13259 [62]. All of the studies thus far have been based either upon the functional utilisation of natural microflora in the filter-packing material or upon inoculation with individual and/or mixed cultures capable of styrene degradation (followed by careful control of the nutrient, pH, and temperature conditions). However, the continued elucidation of mechanisms regulating the bacterial degradation of styrene may permit the construction of enhanced styrene-degrading strains capable of maximising the degradative potential of these filter systems.

9. Biotechnological applications of pathway enzymes

While most of the early work on elucidation of the pathways for microbial styrene degradation in a variety of microorganisms was primarily undertaken with a view to assessing the potential of these organisms for use in the bioremediation of contaminated environments, it has become clear that a number of the pathway enzymes are of potential use in organic synthesis.

9.1. Styrene oxide production

Of the enzymes involved in styrene degradation, styrene monoxygenase has perhaps received the most attention, in part due to the product of the reaction it catalyses, styrene oxide, but also due to its potential as a broad range catalyst for the production of epoxides. Styrene monoxygenase is found in a number of Pseudomonas species (Table 1), and the ability of the enzyme to produce styrene oxide from a cheap starting material is reflected in the number of studies undertaken to develop styrene-degrading strains, or bacterial hosts heterologously expressing styrene monoxygenase genes, as biocatalysts [63,64]. Optically active compounds such as epoxides are desired for their use as chiral building blocks (synthons) for the chemical synthesis of pharmaceutical drugs. The production of optically pure styrene oxide has been the focus of attention for a number of groups [65]. Bestetti and co-workers have recently developed a procedure for the production of enantiomerically pure epoxides from a variety of substituted styrenes with a recombinant E. coli strain expressing the P. fluorescens ST styrene monoxygenase gene [63,66].

Nöthe and Hartmans were the first to report in detail the biocatalytic production of styrene oxide from styrene with a nitrosoguanidine (NTG) mutant of P. putida S12 and a Mycobacterium strain E3 [67]. P. putida S12 mutant M2 produced S-styrene oxide while E3 produced R-styrene oxide. The rate of consumption of styrene was 40 times higher for the Pseudomonas strain (200 nmoles min⁻¹ (mg dry wt.)⁻¹) than for strain E3. Both strains produced styrene oxide to an enantiomeric excess (e.e.) of >98%. Wubbolts and co-workers had earlier reported that xylene oxygenase from P. putida nt-2 was capable of producing S-(+)-styrene oxide from styrene to an e.e. of 93 ± 5% [68]. The styrene degraders P. putida S12 and Mycobacterium E3 were also capable of transforming 4-chlorostyrene to 4-chloro styrene oxide to an e.e. of >98%, while P. putida nt-2 achieved an e.e. of 37% [67].

The most extensive studies on the production of optically pure styrene oxide have since been carried out by Panke and co-workers, who investigated the biocatalytic potential of cloned styrene monoxygenase from Pseudomonas sp. strain VLB120. E. coli JM101 (pT7ST-C) expressing styrene monoxygenase (StyAB) from strain VLB120 produced styrene oxide to an e.e. >99% at a rate of 79 nmoles min⁻¹ (mg dry wt.)⁻¹. While the rate of styrene oxide production was less than half that reported for P. putida S12 mutant strain M2, the e.e. value for the styrene oxide produced was greater [13]. Further work attempted to produce styrene oxide in a continuous two-liquid phase system by expressing the styrene monoxygenase on the chromosome of a recombinant P. putida KT2440 strain [64]. The rate of styrene oxide formation was 6 nmoles min⁻¹ (mg dry wt.)⁻¹. This compared to shake flask biotransformation rates of 30 nmoles min⁻¹ (mg dry wt.)⁻¹. While the generation of new biocatalysts through genetic engineering has produced valuable data on the genetic stability of heterologously expressed genes, it is the improved stereoselectivity and production rate that have primarily driven research in this area. Ironically, despite the progress that has been made in the area of engineered biocatalysts, the highest rate of styrene oxide formation achieved to date still remains that observed in the NTG-derived mutants of P. putida S12.

9.2. Indigo production

It has long been established that many microorganisms capable of growth on toluene, naphthalene and other aromatic hydrocarbons are capable of producing indigo
from indole [69]. However, the rate of indigo formation by these microorganisms was reported only in arbitrary units, due in part to the low transformation rates achieved. The biotransformation of indole to indigo by aromatic dioxygenases was hindered by an even greater problem – the appearance of undesired side products. Indirubin, a heterodimer of 2- and 3-hydroxyindole, is produced as a minor red by-product of indole biotransformation by organisms expressing naphthalene dioxygenase [70]. The appearance of indirubin during indigo biotransformation by naphthalene dioxygenase can be explained by the formation of cis-indole 2,3-dihydrodiol. Chemical dehydration of the dihydrodiol can result in the formation of the two compounds 3-oxindole (indoxyl) and 2-oxindole, dimerisation of which results in the formation of indirubin. Indigo is a dimer of 3-oxindole. The problems of transformation rate and end-product purity thus hindered the success of this biotransformation. However, it was possible that an enzyme expressing a monooxygenase rather than a dioxygenase activity could produce the desired 3-oxindole product and alleviate the problem of impurities. The first report of indigo formation by an organism expressing styrene-degrading genes was in an E. coli strain expressing the styrene monooxygenase gene from P. fluorescens ST [23]. In further work where the formation of indigo by a range of aromatic hydrocarbon-degrading organisms was performed, both P. putida CA-3 and P. putida S12 were shown to be capable of producing stoichiometric amounts of indigo from indole (1:2) in whole-cell biotransformation assays [71,72]. Thin layer chromatography analysis showed that the indigo produced by both Pseudomonas strains contained no detectable levels of the contaminating pigment indirubin. The maximum rate of indigo formation achieved by P. putida S12 and P. putida CA-3 was 10.9 nmoles indigo min⁻¹ (mg dry wt.)⁻¹ [71]. When the indigo formation rate of both P. putida strains was compared with that of a number of aromatic hydrocarbon-degrading strains, expressing both mono- and dioxygenases for either toluene or naphthalene degradation, it was shown to be as much as 83-fold higher [72]. Interestingly, P. putida PpG7 expressing the well characterised naphthalene dioxygenase gene was capable of producing indigo at a rate between 25% and 50% lower than that observed for P. putida CA-3 and S12 [72]. In addition, microorganisms degrading styrene through initial attack of the side-chain produce indigo from indole at rates higher than styrene-degrading microorganisms expressing a ring-hydroxylating dioxygenase [71].

The ability of styrene-grown cells to produce indigo from indole stoichiometrically was due only in part to the presence of styrene monooxygenase, since NTG-derived mutants of P. putida S12 that lacked styrene oxide isomerase enzyme activity produced indigo following a lag phase and at a much lower rate [67]. This indicates that the product of styrene monooxygenase catalysis can be chemically converted to indigo by styrene oxide isomerase, and that this conversion is essential for the rapid production of indigo from indole. Since there is no contaminating indirubin produced, the product of styrene oxide isomerase catalysis must be 3-oxindole. To support this hypothesis, experiments with 2-oxindole as a substrate failed to yield any indigo for either P. putida CA-3 or S12 [71]. Thus, the conversion of indole to indigo is the result of a two-step biotransformation with indole oxide and 3-oxindole (indoxyl) as intermediates in the reaction (Fig. 6), and microorganisms expressing styrene monooxygenase and styrene oxide isomerase show a distinct advantage over organisms with other mono- or dioxygenases in that they produce a pure product at a higher rate of catalysis [71]. The use of whole cells for the regiospecific production of indigo from indole may be a better option than cell-free systems. In the latter, the indole epoxide formed from styrene monooxygenase is unstable. Accumulation of such a compound would result in chemical hydrolysis to produce a dihydrodiol. Chemical dehydration could result in mixed product formation (indoxyl and 2-oxindole). Dimerisation of the products would result in the formation of mixed products, creating the same by-product problem observed for whole-cell biocatalysts with wild-type dioxygenase activity [70]. In any case, the styrene-degrading P. putida CA-3 and S12 strains possess monooxygenase and styrene oxide isomerase activities that may prove useful in the future biological production of indigo.

9.3. Other potential biotransformations

Styrene monooxygenase has already been reported to allow conversion of indole to indole oxide [65], but the ability of styrene monooxygenase to produce epoxides from substrates structurally related to styrene has not been well studied. One report cites the ability of styrene monooxygenase to produce indene oxide from indene in whole cells of P. putida S12 [71]. Indene oxide is a precursor of cis-1S,2R-aminodindanol, an intermediate in the synthesis of the anti-HIV-1 drug Crixavin. The chemical synthesis of indene oxide is difficult, as reflected in the report by Zhang and co-workers [73]. Thus the bioconversion of indene to indene oxide may overcome this chemical bottleneck. However, the indene oxide formed by whole cells of P. putida S12 was through-converted to indanone by styrene oxide isomerase (Fig. 6) and subsequent manipulation of this strain either through mutation of the isomerase or the use of cell-free systems would be required in order to accumulate indene oxide from indene.

While the majority of attention has focused on the biotechnological potential of styrene monooxygenase and styrene oxide isomerase, Itoh and co-workers have reported the presence of a PAAL reductase from Corynebacterium strain ST-10 that, in addition to producing 2-phenylethanol from PAAL, has an extremely broad substrate specificity [22]. The enzyme shows potential as an interesting
biocatalyst given that it is capable of the production of a number of chiral alcohols, ketones and 2-alkanones, with yields varying from 29% to 100% [74]. The stereoisomers of these alcohols are important starting materials for the synthesis of agrochemicals, liquid crystals and pharmaceuticals [75]. Therefore, this enzyme, like styrene monooxygenase and styrene oxide isomerase, may also have significant future biotechnological applications.

10. Conclusions

While there have been significant advances over the last decade in understanding bacterial styrene degradation at the molecular level, and in the application of pathway enzymes to achieve various chemical conversions, there are numerous important aspects which remain unresolved. Despite the current understanding of styrene-induced transcriptional regulation of the catabolic pathways, it is at present unclear whether a specific transport system exists for this compound which could be manipulated to enhance an organism’s ability to accumulate the compound intracellularly. Similarly, while the requirement for StyS and StyR in the activation of the upper pathway genes has been clearly demonstrated, further work is required to shed light on the specific phosphorylation cascade governing signal transduction between these regulatory proteins. This information may be of use in the potential development of stySR-based reporter systems for the detection of styrene. Such reporter systems have already been constructed for the detection of BTEX compounds, phenols and polychlorinated biphenyls [76–78]. Finally, there remains the complex task of identifying how global regulatory molecules exert control over the styrene degradation...
pathway in response to the physiological and metabolic status of the cell. In this endeavour particular emphasis should be placed on those that exert negative regulatory influences as these currently represent the greatest obstacles to establishing feasible, environmentally based, styrene biodegradation strategies, as well as influencing potential methods of whole-cell biocatalysis.

References

