Chapter 3
Microbial Styrene Degradation: From Basics to Biotechnology

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3.1 Introduction

Styrene, the simplest representative of alkenylbenzenes, is one of the most important monomers produced by the chemical industry today. The compound shows a distinct toxicological behavior and is classified as a possible carcinogen due to its metabolism in human via a highly mutagenic epoxide. Considerable amounts of styrene are released by emissions and effluents during production and usage in polymer manufacture and by deposition of industrial wastes. A high chemical reactivity as well as its natural occurrence are reasons for the ubiquitous presence of styrene-catabolic activities among microorganisms. Rapid breakdown of styrene occurs in soils and aquifers under aerobic as well as under anaerobic conditions. As a consequence, styrene can be classified as readily biodegradable. Furthermore, a high volatility and susceptibility to photooxidation prevent bioaccumulation of styrene.

Since first studies in the late 1970s revealed insights about the microbial styrene metabolism in the presence of oxygen, several pathways of aerobic and anaerobic degradation have been reported in the recent years. In addition to basic principles of bacterial carbon metabolism, certain enzymes of aerobic degradation like styrene monooxygenases and styrene oxide hydrolases were found to be of high biotechnological relevance.

This review intends to give a current overview on basics and applied aspects of microbial styrene degradation comprising: (i) physico-chemical and toxicological properties of styrene, (ii) distribution as well as biochemical, genetic, and regulatory

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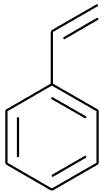
Table 3.1 Physico-chemical properties of styrene (CAS no. 100-42-5)

Property	Value	Chemical structure
Molecular formula	C_8H_8	
Molecular weight	104.15 g mol^{-1}	
Boiling point	145.15°C	
Freezing point	−30.6°C	
Flash point (closed cup)	31°C	
Heat of polymerization	–69.8 kJ mol^{-1}	
Density	0.906 g ml^{-1}	
Vapor pressure (25°C)	0.67 kPa	
Solubility in water	300 mg l^{-1}	
Henry partition coefficient (air/water)	0.21	
Octanol/water partition coefficient (K_{ow})	3.02 (log value)	

aspects of identified pathways, and (iii) usage of styrene-degradative consortia and styrene-catabolic enzymes in waste gas treatment and the preparation of chiral building blocks, respectively.

3.2 Physico-Chemical Properties, Toxicological Aspects, Industrial Usage, and Environmental Fate of Styrene

The monoaromatic hydrocarbon styrene was first isolated from natural balsam storax in the 1830s and is also known under the following common synonyms: cinnamene, ethenylbenzene, phenylethene, phenylethylene, and vinylbenzene. Important physico-chemical properties of the flammable colorless oily liquid styrene are summarized in Table 3.1. Styrene monomer has a penetrating sweetish odor and is miscible with most organic solvents in any ratio, but is only slightly soluble in water.

The high reactivity of the exocyclic double bond brings about a distinct tendency of spontaneous polymerization which requires stabilization even at room temperature (e.g. by addition of 4-*tert*-butylcatechol) for long-term storage. This property favors styrene as a monomer and solvent in the industrial production of the polymer polystyrene (PS) and co-polymers like styrene-butadiene rubber (SBR), styrene acrylonitrile copolymer (SAN), and acrylonitrile butadiene styrene resins (ABS). Styrene is also used in combination with unsaturated polyester resins for fiber reinforced synthetics. In 2004, the global styrene demand was reported to be over 24,000 Kt (data calculated from 2006 World Styrene Analysis) (CMAI 2005) making this monomer to one of the most important organic chemicals worldwide.

Due to its widespread usage, styrene has been a subject of extensive toxicological investigation (Gibbs and Mulligan 1997; Rueff et al. 2009). The acute neurotoxic effects of high levels of styrene to human and mammals are related to

prenarcotic central nervous depression which resembles with action of many other solvents which are probably mediated by the direct effect of the lipophilic hydrocarbon on nerve cell membranes (Bond 1989). In contrast, chronic toxicity of styrene may result from the action of reactive metabolites, i.e. its highly reactive epoxide styrene oxide to components of the central nervous system (Mutti 1988; Mutti et al. 1988). This initial metabolite is formed by the action of hepatic microsomal cytochrome P450 monooxygenases and its further metabolism is mediated to a major extent by microsomal epoxide hydrolases (mEH or EH) and different dehydrogenases (Hartmans 1995). The urinary products mandelic acid and phenylglyoxylic acid both represent important biomarkers for styrene exposure (Guillemin and Berode 1988). The International Agency for Research on Cancer (IARC) has classified styrene as carcinogenic to humans (group 2B) and the potential of carcinogenicity largely depends on the ability of the reactive epoxide to covalently modify DNA.

As a consequence of substantial industrial application, the polymer-processing industry represents a major source of anthropogenic styrene release. Furthermore, styrene is released to a lower extent during its production, the disposal of polymers, and combustion processes, of which most important sources are automobile exhausts. In addition to its presence in cigarette smoke, trace levels of styrene are naturally found in certain plants and food (fruits, vegetables, nuts, meat) and are generated from a number of natural processes like the decarboxylation of cinnamic acid (plant decomposition) and the metabolism of microorganisms (Sielicki et al. 1978; Smith 1994; Warhurst and Fewson 1994; Lafeuille et al. 2009). The annual total release of styrene from all above mentioned processes was estimated for the European Union to exceed 23,500 tons and approximately 96% of this load is released directly into the air (European Union Risk Assessment Report Styrene 2002). As a consequence of its high volatility, the atmosphere is the main environmental compartment for the fate of styrene. Photooxidation of styrene by hydroxyl radicals and ozone are the main abiotic routes of degradation and depending on the conditions, half-lives between 3.5 and 9 h were estimated (Alexander 1990). A similar fate can be expected for a substantial amount of styrene dissolved in aqueous systems due to its volatility and its low octanol/water coefficient of 3.02 (log value) which considerably limits the risk of geo-accumulation. Fu and Alexander (1992) have investigated the fate of styrene in different environmental compartments. They determined that 50% styrene is being lost within 3 h in the shallow layers of lake water whereas only 26% gets evaporated within 31 days from 1.5 cm soil depth. In addition, it was shown that styrene, which remained trapped in soil, was a subject of microbial mineralization. Other studies report 87–95% degradation from different types of soil within 16 weeks (US Inventory of Toxic Compounds 2001).

The distribution of styrene metabolism among microorganisms as well as the genetic and biochemical principles will be reviewed in detail in the following headings.

Table 3.2 Distribution of styrene-degrading capabilities among different classes of pro- and eukaryotic microorganisms

Class	Genus	References
Bacteria		
Actinobacteria	*Corynebacterium*	Itoh et al. (1996)
	Gordonia	Alexandrino et al. (2001)
	Mycobacterium	Burback and Perry (1993)
	Nocardia	Hartmans et al. (1990)
	Rhodococcus	Hartmans et al. (1990), Jung and Park (2005), Patrauchan et al. (2008), Tischler et al. (2009), Warhurst et al. (1994), Zilli et al. (2003)
	Streptomyces	Przybulewska et al. (2006)
	Tsukamurella	Arnold et al. (1997)
Bacilli	*Bacillus*	Przybulewska et al. (2006)
Clostridia	*Clostridium*	Grbić-Galić et al. (1990)
α-Proteobacteria	*Sphingomonas*	Arnold et al. (1997)
	Xanthobacter	Hartmans et al. (1989, 1990)
γ-Proteobacteria	*Enterobacter*	Grbić-Galić et al. (1990)
	Pseudomonas	Alexandrino et al. (2001), Beltrametti et al. (1997), Ikura et al. (1997), Kim et al. (2005), Lin et al. (2010), Marconi et al. (1996), O'Connor et al. (1997), Panke et al. (1998), Park et al. (2006a), Rustemov et al. (1992), Velasco et al. (1998)
	Xanthomonas	Arnold et al. (1997)
Sphingobacteria	*Sphinogobacterium*	Przybulewska et al. (2006)
Fungi		
Agaricomycetes	*Bjerkandera*	Braun-Lüllemann et al. (1997)
	Phanerochaete	Braun-Lüllemann et al. (1997)
	Pleurotus	Braun-Lüllemann et al. (1997)
	Trametes	Braun-Lüllemann et al. (1997)
Eurotiomycetes	*Aspergillus*	Paca et al. (2001)
	Exophiala	Cox et al. (1996)
	Penicillium	Cox (1995), de Jong et al. (1990), Paca et al. (2001)
Sordariomycetes	*Gliocladium*	Cox (1995)
	Sporothrix	Cox (1995), René et al. (2010)

3.3 Degradation Pathways

The capability of styrene metabolism seems to be widespread among pro- and eukaryotic microorganisms. A large number of gram-negative and gram-positive bacteria as well as several fungi of the phyli basidiomycota and ascomycota were found to utilize styrene as (sole) source of carbon and energy (Table 3.2). Most frequently, degradation was shown to occur under oxic conditions and a lot of styrene-degrading isolates have initially been found to belong to the genus *Pseudomonas* (Warhurst and Fewson 1994; Mooney et al. 2006b;

O'Leary et al. 2002b). The predominance of styrene degradation among these proteobacterial r-strategists does not automatically reflect its true phylogenetic distribution and can probably be attributed to the rapid growth rate of pseudomonads during conventional enrichment techniques. In fact, more recently the identification of an increasing number of styrene-degrading Actinobacteria of the genera *Corynebacterium*, *Rhodococcus*, *Nocardia*, *Gordonia*, and related ones indicates that the capability of styrene catabolism is a common feature for that class of microorganisms, too.

In general, two major strategies of mineralization have been identified under oxic as well as anoxic conditions which differ with respect to type and location of initial attack. Epoxidation and hydratation of the vinyl side-chain seem to be favored mechanisms of initial attack during aerobic and anaerobic degradation, respectively, whereas dioxygenation and monohydroxylation of the aromatic nucleus are not frequently found under these conditions. There is certain evidence that the degradation pathway acting primarily on the aromatic nucleus has most likely its origin in the breakdown of other aromatic compounds like biphenyl or toluene (Warhurst et al. 1994; Cho et al. 2000; Patrauchan et al. 2008), whereas side-chain attack is more specific and restricted to styrene mineralization. Most microorganisms investigated for styrene degradation were shown to harbor only one single route for styrene breakdown.

Initial processes of aerobic and anaerobic styrene degradation lead to the formation of central intermediates, like 2-hydroxyphenylacetic acid, benzoic acid, 3-vinylcatechol, and 2-ethylhexanol. Since the subsequent conversion of these compounds into intermediates of the TCA cycle is mediated by the regular metabolism, lower degradation pathways are not discussed here.

It should be noted that in addition to the parent compound styrene, a few bacteria are also able to degrade substituted derivatives (Omori et al. 1974; Bestetti et al. 1989) or oligomers (Tuschii et al. 1977; Higashimura et al. 1983). The conversion of substituted derivatives is basically achieved by enzymes of the regular styrene catabolism, while most likely other enzymes are responsible for mineralization of the unsaturated styrene dimer by *Alcaligenes* sp. 559 (Tuschii et al. 1977).

3.3.1 Aerobic Styrene Degradation

Under aerobic conditions, side-chain oxygenation of styrene seems to be the favored mechanism, since most microorganisms investigated in that respect were found to follow this degradation pathway (Hartmans et al. 1990; Cox et al. 1996; Itoh et al. 1996; Beltrametti et al. 1997; Panke et al. 1998; Velasco et al. 1998; Park et al. 2006b). In the first reaction step, styrene is oxygenated into styrene oxide by the action of a monooxygenase (Fig. 3.1). Styrene monooxygenases (SMOs) of bacteria are flavin-dependent, whereas this reaction is typically catalyzed by heme-containing cytochrome P450 monooxygenases in fungi (Cox et al. 1996). Bacterial styrene monooxygenases were shown to be highly enantioselective leading in almost all

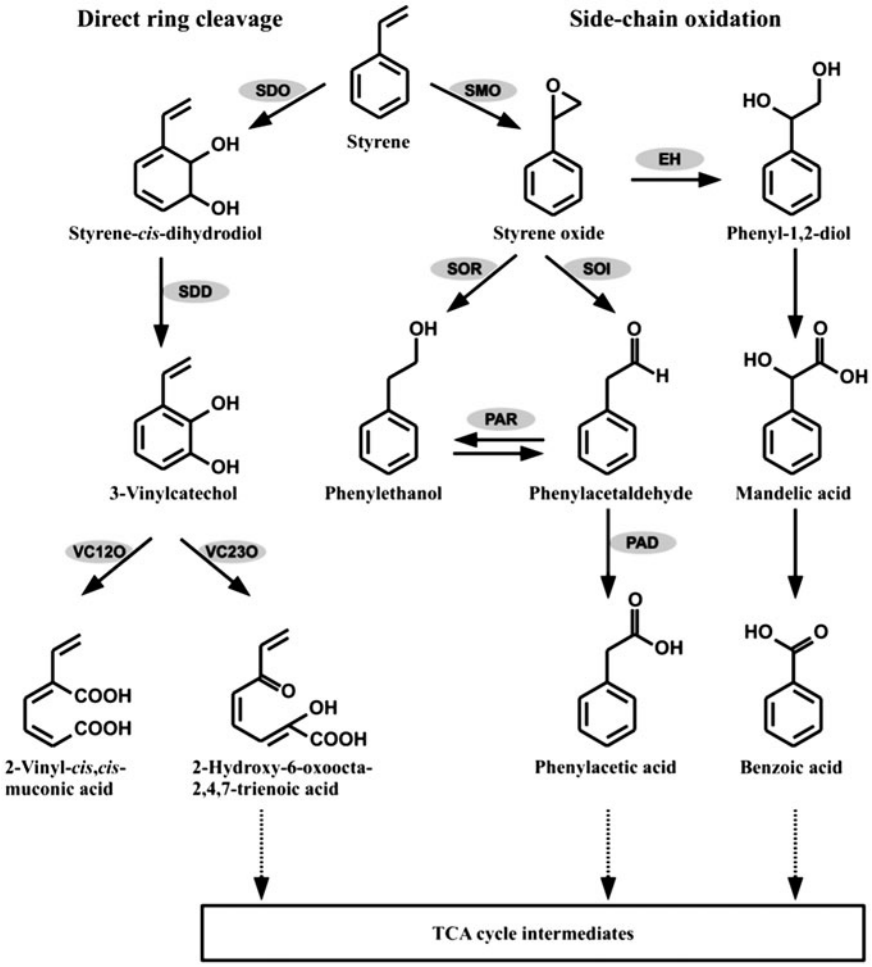


Fig. 3.1 Microbial pathways of aerobic styrene degradation. Characterized enzymes are indicated by their *abbreviation*

cases to the formation of the (*S*)-enantiomer of styrene oxide (Otto et al. 2004; van Hellemond et al. 2007; Tischler et al. 2009). Most commonly, the reactive oxirane is then isomerized to phenylacetaldehyde by a styrene oxide isomerase (SOI). In contrast to cytosolic SMOs, several studies indicated a membrane-bound localization of SOIs in *Pseudomonas*, *Corynebacterium* (Itoh et al. 1997a), and *Rhodococcus* (unpublished). The formed phenylacetaldehyde is then converted to phenylacetic acid by a phenylacetaldehyde dehydrogenase (PAD) (Fig. 3.1). It must be mentioned that *E. coli* strains are able to convert phenylacetaldehyde into 2-phenylethanol, too, which might interfere with studies using whole-cell assays (Beltrametti et al. 1997).

A slight modification of the above pathway comprises the additional action of both a styrene oxide reductase (SOR) and a phenylacetaldehyde reductase (PAR). Styrene oxide is reduced to 2-phenylethanol by SOR and oxidized to phenylacetaldehyde by PAR or another dehydrogenase. In *Pseudomonas fluorescens* ST, this variant was shown to be a side reaction (Marconi et al. 1996), whereas in *Pseudomonas* sp. 305-STR-1-4, *Pseudomonas* sp. Y2, and *Xanthobacter* sp. strain 124X, 2-phenylethanol was identified as one major metabolite (Shirai and Hisatsuka 1979; Hartmans et al. 1989; Utkin et al. 1991). According a current hypothesis, this route might belong to ethylbenzene degradation via 2-phenylethanol and thus reflects an unspecific conversion of styrene by enzymes of this pathway.

As mentioned above, styrene metabolism by bacteria and fungi shares the initial step of monooxygenation. However, all following metabolic reactions in fungi differ (Braun-Lüllemann et al. 1997) and are quite similar to styrene detoxification route in human (Warhurst and Fewson 1994; Rueff et al. 2009). Styrene oxide is hydrolyzed to phenylethan-1,2-diol by the action of an epoxide hydrolase (EH) and oxidized to mandelic acid by a dehydrogenase. Enzymatic decarboxylation then yields benzoic acid. Further, metabolites, like 2-phenylethanol, were detected and might be side products as observed for other organisms.

Initial dioxygenation of the aromatic nucleus and ring cleavage is another type of mechanism through which various bacteria degrade styrene (Bestetti et al. 1989; Hartmans et al. 1989; Warhurst et al. 1994; Patrauchan et al. 2008). In this case, a styrene 2,3-dioxygenase (SDO) introduces two oxygen atoms adjacent to the vinyl group and a styrene *cis*-glycol is formed. Subsequently, a styrene-2,3-dihydrodiol dehydrogenase (SDD) catalyzes re-aromatization to 3-vinylcatechol. These steps are consistent with the peripheral pathways of benzene-, toluene- and ethylbenzene degradation (Smith 1990; Warhurst et al. 1994; Mars et al. 1997) which yield catechol, 3-methylcatechol, and 3-ethylcatechol, respectively, as the central intermediates. Since the involved 2,3-dioxygenases usually show a relatively high substrate tolerance, conversion of styrene may be the result of fortuitous metabolism. The central intermediate 3-vinylcatechol then may undergo *ortho*- or *meta*-cleavage by the action of a vinylcatechol 1,2-dioxygenase (VC12O) or a vinylcatechol 2,3-dioxygenase (VC23O) yielding 2-vinyl-*cis,cis*-muconic acid and 2-hydroxy-6-oxoocta-2,4,7-trienoic acid, respectively. Further degradation of 2-vinyl-*cis,cis*-muconate by *ortho*-pathway fails and as a result this compound accumulates as a dead-end metabolite (Warhurst et al. 1994). A similar observation was made for methylaromatics which are mineralized by most bacteria through the *meta*-cleavage pathway (Marín et al. 2010). For example, if 4-methylcatechol undergoes *ortho*-cleavage, 4-methylmuconolactone accumulates in the growth medium (Knackmuss et al. 1976). Therefore, the *ortho*-cleavage pathway is usually unsuited for the degradation of alkylcatechols.

In contrast, the intensively yellow-colored semialdehyde from *meta*-cleavage of 3-vinylcatechol seems to be subject of further turnover. First indication for the presence of a styrene-catabolic route by *meta*-cleavage may be drawn from the preliminary occurrence of a yellow-colored intermediate from a styrene-growing

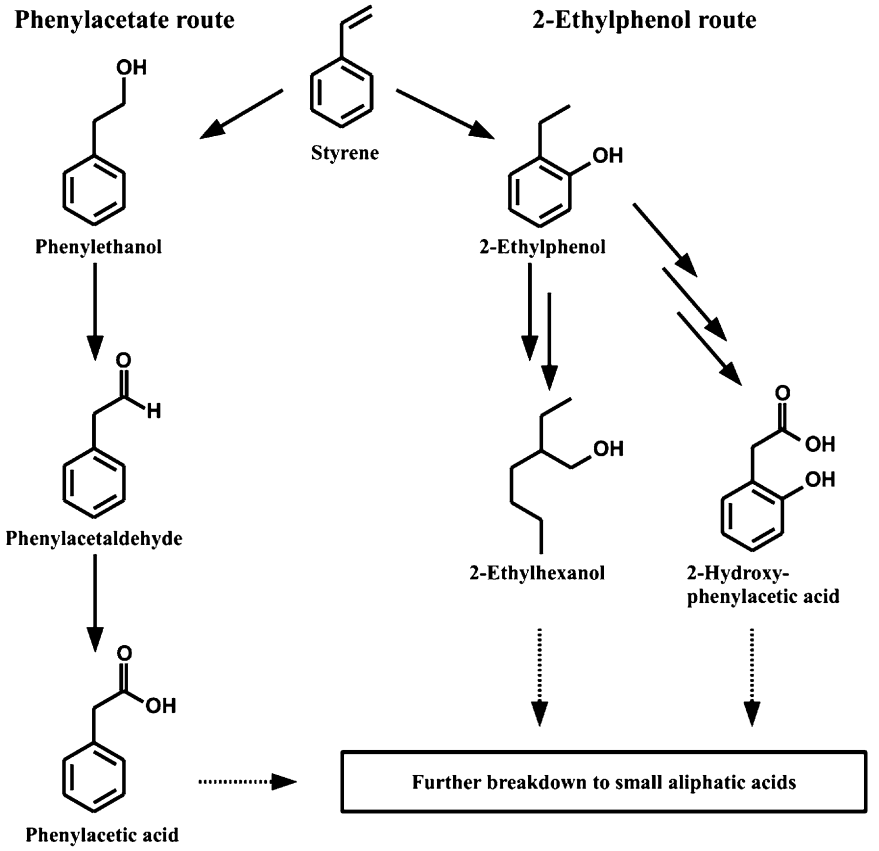


Fig. 3.2 Proposed steps of anaerobic styrene degradation pathways (adapted from Grbić-Galić et al. 1990). The herein shown routes for anaerobic styrene breakdown are based on identified metabolites from pure or mixed cultures. No data about involved enzymes are available so far

culture. However, co-existence of styrene catabolic pathways of side-chain oxygenation and direct ring attack might be possible (Hartmans et al. 1989).

3.3.2 Anaerobic Styrene Degradation

Compared to aerobic metabolism, less information is available on mechanisms of anaerobic styrene breakdown and especially on involved enzymes. Enriched consortia from anaerobic sludge as well as pure bacterial cultures were characterized for their ability to utilize styrene as sole source of carbon and energy (Grbić-Galić et al. 1990; Araya et al. 2000). Till now, three hypothetical pathways were assigned on the basis of identified intermediates rather than on enzymatic activities (Fig. 3.2).

A pathway similar to the aerobic breakdown by side-chain oxygenation was supposed to occur in *Enterobacter* as well as in anaerobic consortia (Grbić-Galić et al. 1990). According to current hypothesis, oxygenation of the vinyl group by a hydratase-catalysed addition of water leads to 2-phenylethanol which is subsequently oxidized by different dehydrogenases via phenylacetaldehyde to phenylacetic acid (Grbić-Galić et al. 1990).

An alternative anaerobic degradation sequence starts with a water-mediated hydroxylation of the aromatic ring, followed by reduction of vinyl side-chain to yield 2-ethylphenol. This intermediate may be subjected to (i) ring cleavage yielding 2-ethylhexanol or (ii) side-chain oxidation yielding 2-hydroxyphenylacetic acid.

Both anaerobic styrene degradation pathways are supposed to be important and seem to occur contemporary in microbial consortia. Pure cultures and microbial communities are able to degrade styrene almost completely under anaerobic conditions into carbon dioxide. Only small amounts of aromatic and alicyclic compounds remain. Most of the anaerobic styrene degradation is still unclear, especially due to the lack of detailed information about involved enzymes and corresponding genes or gene cluster.

3.4 Genetics and Regulatory Mechanisms

Detailed knowledge about the organization of genes and regulatory elements involved in upper styrene degradation by side-chain oxygenation is currently limited to pseudomonads like *P. fluorescens* ST (Marconi et al. 1996; Beltrametti et al. 1997), *P. putida* strain CA-3 (O'Connor et al. 1995, 1997; O'Leary et al. 2001, 2002a), strain S12 (O'Connor et al. 1997; Kantz et al. 2005), and strain SN1 (Park et al. 2006b), and *Pseudomonas* sp. strain LQ26 (Lin et al. 2010), strain VLB120 (Panke et al. 1998; Otto et al. 2004), and strain Y2 (Utkin et al. 1991; Velasco et al. 1998). The styrene-catabolic *sty* genes were found to be both, clustered on the chromosome (Velasco et al. 1998) or on plasmids (Bestetti et al. 1984; Ruzzi and Zennaro 1989) (Fig. 3.3a, b). Regulatory elements (*styS*/*styR*) as well as a styrene transporter (*styE*) were found in direct neighborhood to the catabolic genes *styABCD*. Thus, at least for few *Pseudomonas* strains, a complete *sty(rene)*-operon could be described.

3.4.1 The Gene Cluster styABCD(E) of the Upper Styrene Degradation Pathway

The number and arrangement of genes within the *styABCD(E)* clusters of pseudomonads reflect the necessity and sequence of encoded enzymes, respectively,

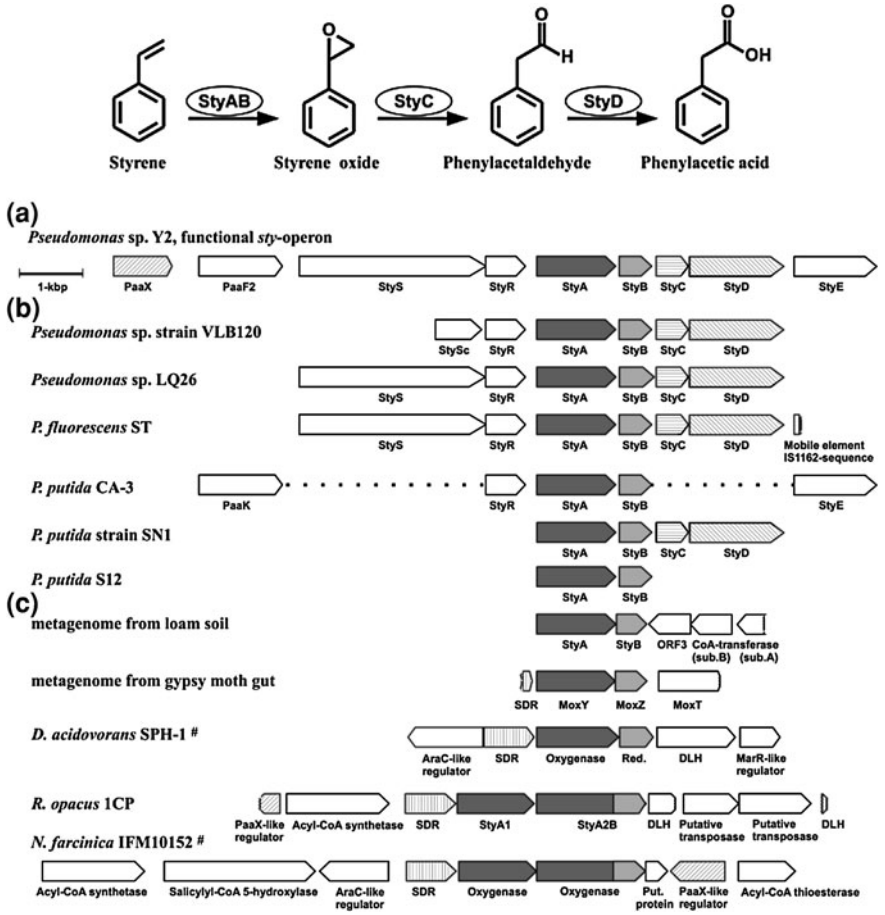


Fig. 3.3 Comparison of the organization of **a** the *styABCDE*-operon from *Pseudomonas* sp. Y2, **b** other (incomplete) styrene-catabolic gene clusters from pseudomonads, and **c** the genetic location of flavin monooxygenases with (# hypothetical) function as styrene monooxygenases. The upper pathway of styrene degradation by side-chain oxygenation is shown at the top and involved gene products are given. Identical types of filling of ORFs indicate similar function of encoded proteins

to catalyze the conversion of styrene into phenylacetic acid (Fig. 3.3a, b). The genes *styA* and *styB* encode the two-component flavin-dependent styrene monooxygenase (SMO, or StyA/StyB) which initially converts styrene to styrene oxide at the expense of NADH. A single gene s*tyC* encodes a styrene oxide isomerase (SOI, or StyC) which catalyzes the intramolecular rearrangement of styrene oxide to phenylacetaldehyde. Finally, *styD* which is located directly downstream to *styC* encodes an NAD- or phenanzine methosulfate-dependent phenylacetaldehyde dehydrogenase (PAD or StyD) oxidizing phenylacetaldehyde into phenylacetic acid, the final product of the upper styrene pathway. An additional gene *styE*

(or *porA*) was found in few pseudomonads to be located directly downstream to the catabolic cluster *styABCD* and high similarities to several membrane-associated ATPase-dependent kinase proteins (Velasco et al. 1998; Mooney et al. 2006a) suggested a function as styrene transporter. This hypothesis was strengthened by the detection of (i) a co-expression of *styE* (*P. putida* CA-3) with *styABCD*, (ii) a styrene-dependent transcription, (iii) a membrane association, and (iv) an increased styrene degradation rate in the presence of additionally overexpressed *styE* copies (Mooney et al. 2006a). Furthermore, basic necessity of the transporter was shown by a *styE*-negative mutant of strain CA-3 which lost its ability to grow on styrene. However, Nikodinovic-Runic and coworkers detected only minor levels of StyE from a styrene-grown pseudomonad under both non-limiting and nitrogen-limiting conditions (Nikodinovic-Runic et al. 2009). These findings implicate that both, membrane diffusion as well as active styrene transport, are important for the uptake of styrene. In addition, it cannot be excluded that other transporters support active styrene uptake.

It is supposed that the upper pathway of styrene degradation may have evolved in a different way as the lower route necessary for phenylacetic acid conversion to TCA cycle intermediates (Ferrández et al. 1998; Olivera et al. 1998; Alonso et al. 2003a; Di Gennaro et al. 2007).

Considering the low number of identified styrene-catabolic gene clusters and their apparently restricted occurrence in pseudomonads, styrene degradation by side-chain oxygenation seems to be not widely distributed in nature. This assumption is currently strengthened by available genome data which did not allow the identification of *styABCD*-like gene clusters from other bacterial phyla. However, taking into account on one hand the widespread ability of styrene utilization (Table 3.2), and on the other hand the preferred isolation of pseudomonads during classical enrichment procedures, current knowledge probably does not reflect the true distribution. In fact, single enzymatic activities or genes with hypothetical function in upper styrene degradation could be found in several other organisms than pseudomonads including gram-positive bacteria.

Starting with the initial flavin-dependent styrene monooxygenase StyA/StyB, the distribution frequency among bacteria is supposed to be generally low (based on representatives per genome, van Berkel et al. 2006). The only gene products showing significant homology to StyA/StyB from pseudomonads and activity on styrene or analogous compounds are found in two metagenoms (Guan et al. 2007; van Hellemond et al. 2007), as well as in the actinobacterium *Rhodococcus opacus* 1CP (Tischler et al. 2009, 2010) (Fig. 3.3c). *In silico*-screening for further representatives yielded several homologous proteins with similarities either to SMOs from pseudomonads or to that one from *Rhodococcus opacus* strain 1CP which was exemplarily shown by van Hellemond et al. (2007) and Tischler et al. (2009) (Fig. 3.3c). Interestingly, only in few Actinobacteria, a novel type of styrene monooxygenase was found so far which will be discussed later.

Biochemical evidence for styrene oxide isomerases was provided for several bacteria like *Xanthobacter* sp. 124X (Hartmans et al. 1989), *Corynebacterium* spp. (Itoh et al. 1996, 1997a), *Rhodococcus opacus* 1CP (Tischler et al. 2009),

Rhodococcus sp. S5, and others (Hartmans et al. 1990). However, none of the corresponding genes has been described so far and homology search of StyC towards available databases did not indicate significant similarities to gene products others than StyC-homologs from pseudomonads.

It should be mentioned that in *Rhodococcus opacus* 1CP, no *styC*-homologous gene could be found in direct neighborhood to the styrene monooxygenase genes *styA1*/*styA2B* (Fig. 3.3c), indicating that the genetic organization of styrene catabolism is different to that in pseudomonads (Tischler et al. 2009).

Phenylacetaldehyde dehydrogenases should be much more common in bacteria, since their substrate phenylacetaldehyde originates from different catabolic pathways apart from styrene degradation. Precursors are phenylpyruvic acid, 2-phenylethylamine, 2-phenylethanol, and phenylmalonic semialdehyde (Ferrández et al. 1997; Long et al. 1997). This assumption is strengthened by homology search of *styD* genes and gene products to the non-redundant genome database which reveals many high-score hits. However, only a few reports about functionally characterized phenylacetaldehyde dehydrogenases are available so far.

3.4.2 The Regulatory System of the Sty-Operon

The two-component regulatory system StyS/StyR of the *sty*-operon from the strains *Pseudomonas putida* CA-3, *Pseudomonas* sp. Y2, and *Pseudomonas fluorescens* ST was investigated in detail (Velasco et al. 1998; Santos et al. 2000; O'Leary et al. 2001, 2002a; Leoni et al. 2003). With respect to the conserved operon organization among pseudomonads (Fig. 3.3a, b), the described features of this regulatory system are supposed to occur and interact similarly in all functional *sty*-operons of pseudomonads.

The gene products of *styS* and *styR,* which are located proximate upstream to *styABCD* (Figs. 3.3 and 3.4), both show similarities to several two-component transduction systems from prokaryotes and eukaryotes (Reizer and Saier 1997). In fact, they were found to positively affect *sty*-operon transcription (Fig. 3.4) and both genes are most likely expressed in a transcription-coupled fashion (O'Leary et al. 2001). On amino acid level, StyS exhibits similarity to sensor kinase proteins, especially to TodS, TutC, and TutS which regulate toluene catabolism in *Pseudomonas* spp. and *Thaurea* sp. (Coschigano and Young 1997; Lau et al. 1997; Leuthner and Heider 1998). A similar two-component regulator was reported for the biphenyl degradation pathway of *Rhodococcus* sp. M5 (Labbé et al. 1997). Close relationship of StyS to the above regulator proteins could also be demonstrated by the ability of styrene to act as an inducer of toluene degradation (Cho et al. 2000; Mosqueda and Ramos 2000). The sensor kinase StyS consists of five functionally different domains: input-1, histidine kinase-1 (HK-1), receiver, input-2, and histidine kinase-2 (HK-2) (Fig. 3.4). Both input domains contain typical motives of PAS-sensing domains (these are signaling modules for changes of oxygen level, redox potential, light, and small ligand concentrations), and are

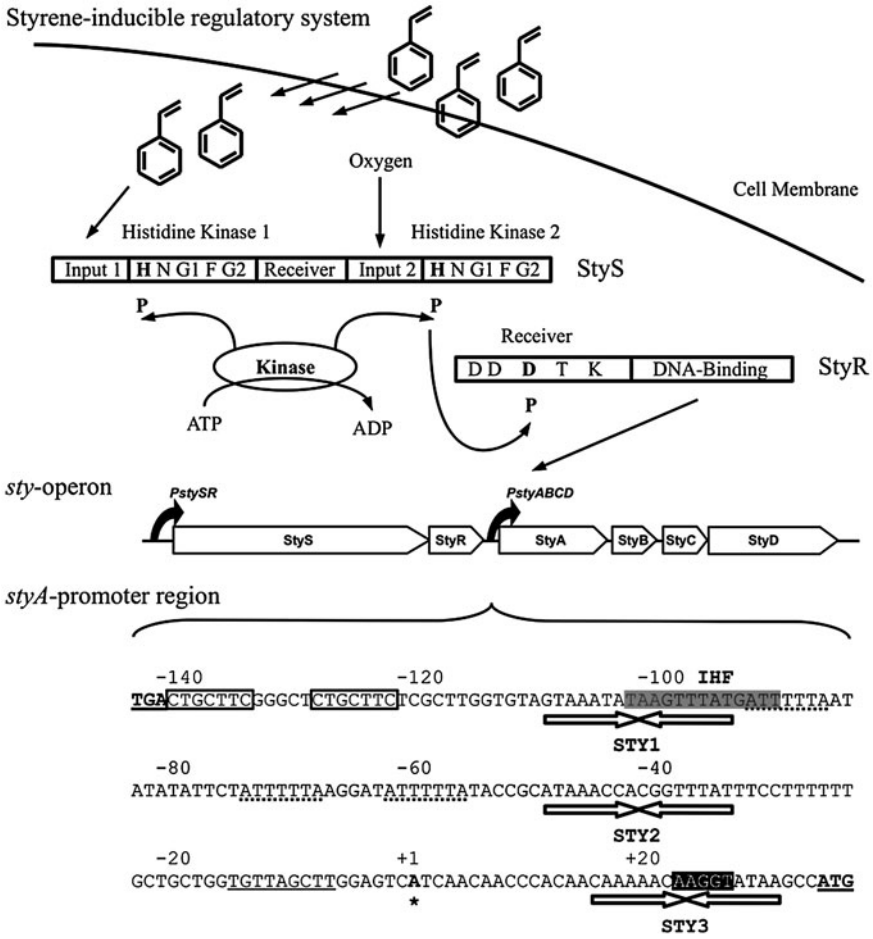


Fig. 3.4 Structure and mechanism of the styrene-inducible regulatory system of the *sty*-operon and a detailed view on the complex *styA*-promoter region (adapted from O'Leary et al. 2002b). The regulatory system (StyS/StyR with conserved motifs, *sty*-operon with indicated promoter sites *PstySR* and *PstyABCD*, and *styA*-promoter region) and its effect on the transcription of styrene-catabolic genes based upon data obtained from styrene-utilizing *Pseudomonas fluorescens* ST. Styrene is sensed by the input 1 domain of StyS and changing oxygen concentrations might be also sensed by another input domain of StyS. Afterwards one of the two histidine kinase domains gets activated by phosphorylation of a histidine residue (*bold 'H'*) and the phosphoryl group is subsequently transferred onto an aspartate residue (*bold 'D'*) of the receiver domain of StyR. This activated response regulator StyR can then bind by its C-terminal DNA-binding domain to one of the three STY-sites of the promoter region. In the presence of styrene as the sole source of carbon, activated StyR binds to STY2 and RNA polymerase and then binds to the promoter region (*underlined*) and initiates transcription of *styABCD*. The complete *styA*-promoter region is outlined, numbered according to the transcriptional start site (+1, *bold 'A'*), and the following elements between StyR-stop and StyA-start codon (*bold faced*) are indicated: the three palindromic sequences STY1, STY2, STY3 (*arrows*), the consensus sequence 5′-WATCAANNNNTTR-3′ (complementary encoded to *styABCD*) for binding of the integration host factor IHF (*gray box*), a directed repeat sequence 5′-CTGCTTC-3′ at the beginning (*boxed*), two 5′-ATTTTTA-3′ motifs (*dotted lines*), and the potential ribosome binding site (*black box*)

expected to sense traces of styrene or degradation intermediates, perhaps as a consequence of an altered redox potential of the cell (Coschigano and Young 1997; Lau et al. 1997; Velasco et al. 1998; Santos et al. 2000). The two histidine kinase domains H←N←G1←F←G2 are highly conserved and harbor a characteristic kinase amino acid motif including a single histidine residue for the phosphoryl group transfer towards the response regulator StyR (Coschigano and Young 1997; Grebe and Stock 1999; Mosqueda and Ramos 2000). Slight differences in their amino acid sequence allow their classification into the kinase superfamilies 1a (HK-1) and 4 (HK-2). The receiver domain belongs to the RA2-receiver superfamily and contains a conserved amino acid motif D←D←S←K typical for bacterial response regulators (Grebe and Stock 1999).

The second half of the *sty*-operon regulatory system is represented by StyR. Like StyS, this protein shows high similarity to two-component regulators, especially those ones of toluene catabolism like TodT and TudC (Lau et al. 1997; Leuthner and Heider 1998). StyR comprises two different domains, an N-terminal regulatory (or receiver) domain (pos. 1 to 127) and a C-terminal DNA-binding domain (pos. 142 to 208), which are joined by a 34-amino acid long Q-linker (O'Leary et al. 2002b). The receiver domain belongs to the RA4-receiver subfamily and harbors the conserved amino acid motif (D←)D←D←T←K (Baikalov et al. 1996; Grebe and Stock 1999), whereas the highly conserved C-terminus shows similarities to family-3 response regulators (Reizer and Saier 1997; Velasco et al. 1998). Thus, StyR belongs to the FixJ/NarL-subfamily of response regulators.

Several studies have demonstrated that StyS-StyR plays a key role within styrene degradation and affects positive as well as negative the expression of catabolic genes (Fig. 3.4) (Panke et al. 1998; Velasco et al. 1998; Santos et al. 2000, 2002; O'Leary et al. 2001, 2002a, b; Leoni et al. 2003, 2005). The presence of styrene is indispensable for the transcription of *styA* and the complete functional *sty*-operon. In most cases, transcripts of the regulatory proteins were found only in presence of styrene (O'Connor et al. 1995; O'Leary et al. 2001). Various carbon sources like phenylacetic acid, glutamate, glucose, and citrate, were found to repress the transcription of catabolic genes even when styrene is present in the culture medium (O'Connor et al. 1995; Santos et al. 2000; O'Leary et al. 2001). Only styrene induces significantly the upper route and phenylacetic acid or its metabolites do not, even at presence of phenylacetic acid in the medium, the *sty*-operon transcription is repressed (O'Leary et al. 2001). Thus, the upper (*sty*-operon) and lower (phenylacetic acid degradation genes) pathway of styrene metabolism are likely to be regulated separately.

Based on identified regulatory elements, the following regulation mechanism was postulated. Styrene is sensed by the sensory input domain of StyS and as a result, one of the two histidine kinase domains is activated by a kinase-catalyzed phosphorylation of conserved histidine residue (H). This phosphoryl group is then transferred onto a conserved aspartic acid residue (D) of the receiver domain of StyR. It was demonstrated that phosphorylated and thus activated StyR binds co-operatively to a palindromic sequence STY2 (Fig. 3.4) of the *styA* promoter region (*PstyABCD*), leading to highly attracted binding of RNA polymerase to a conserved sequence of

the promoter region (5′-TGTTAGCTT-3′). In that case, StyR controls gene transcription of the upper styrene degradation route as an activator, but after translation of catabolic genes, high amounts of phosphorylated StyR may accumulate and act then as a repressor of transcription. The latter effect is caused by binding of activated StyR to a negative regulatory site STY3 within the *styA* promoter region. Another regulatory sequence STY1, located upstream to STY2 and STY3, might affect transcription of catabolic genes positively (presence of styrene) or negatively (presence of glucose or other carbon sources). Additionally, it was demonstrated that an integration host factor (IHF, a small heterodimeric protein) affects *styABCD* transcription due to binding to a consensus sequence in the *styA*-promoter region (5′-WATCAANNNNTTR-3′, complementary encoded to *styABCD*) (Fig. 3.4) (Leoni et al. 2005). A positive role for *PstyABCD* regulation is expected.

Biochemical characterization of the wild-type styrene monooxygenase StyA/StyB from *Pseudomonas* sp. VLB120 indicated that the expression level of the oxygenase subunit StyA exceeds by far that of the NADH:FAD oxidoreductase StyB (Otto et al. 2004). This behavior is similar to a 4-hydroxyphenylacetate 3-monooxygenase (Louie et al. 2003) and probably accounts for the fact that StyB has a much higher specific activity than StyA. Moreover, StyA should not be limited by a molar deficit of StyB since reducing equivalents ($FADH_2$) are transferred mainly by diffusion and a general necessity of StyA/StyB contact is not given (Kantz et al. 2005; Otto et al. 2004). Proteome analysis of *Pseudomonas putida* CA-3 pointed to similar results and indicated that StyA and StyD are by far the most abundant proteins of the upper *sty*-operon which exceed StyB and StyC for at least one order of magnitude (Nikodinovic-Runic et al. 2009).

The regulatory elements *styS-styR* from pseudomonads are the only ones hitherto found to be involved in styrene catabolism. Similar elements are lacking in close neighborhood of all other (putative) styrene monooxygenases identified from metagenomes and different Actinobacteria (Fig. 3.3c). However, a distant localization of related two-component regulatory systems cannot be excluded. Interestingly, two other regulatory elements were found in the neighborhood of (hypothetical) styrene monooxygenases from Actinobacteria: a PaaX- and an AraC-like regulator. The first one is typical for phenylacetic acid degradation which suggests a dependency to styrene degradation. The latter one belongs to the diverse group of AraC/XylS-family transcriptional regulators (Gallegos et al. 1997) frequently involved in the regulatory machinery of aromatic hydrocarbon degradation. A functional link of both elements to SMO regulation is still missing.

3.4.3 Genetic Localization of Single Styrene Monooxygenases in Other Organisms

Despite the fact that the identification of complete styrene-catabolic operons is currently limited to members of the genus *Pseudomonas*, there is certain evidence that styrene degradation by side-chain oxygenation is widespread among bacteria.

This assumption can be drawn since several gram-positive and gram-negative isolates were described to utilize styrene as the sole source of carbon and to produce traces of styrene oxide during this degradation process (Table 3.2) (Hartmans et al. 1989; Arnold et al. 1997; Alexandrino et al. 2001; Przybulewska et al. 2006). In addition, several genes in (meta)genomes and in members of Actinobacteria, which encode for functionally proven or hypothetical styrene monooxygenases, were identified (Fig. 3.3c) (Guan et al. 2007; van Hellemond et al. 2007; Tischler et al. 2009). Besides, the congruence of important amino acid motifs in all of them, the biochemically characterized SMOs from the soil metagenome and from *Rhodococcus opacus* 1CP show a similar behavior and substrate pattern as SMOs from pseudomonads (van Hellemond et al. 2007; Tischler et al. 2009, 2010). None of these SMO genes are part of a *styABCD*-like gene cluster and the virtual absence of SOI- and PAD-like genes raised questions on a functional involvement in styrene degradation. Certain evidence for a role in styrene degradation can be drawn from the observation that each of the novel SMO genes is surrounded by at least one ORF, encoding a protein with function in phenylacetic acid metabolism, like a PaaX-like regulator, a CoA-ligase, a CoA-transferase, a CoA-hydroxylase, and a CoA-thioesterase (Olivera et al. 1998; Di Gennaro et al. 2007; Tischler et al. 2009).

The identification of *styA1/styA2B* from *R. opacus* 1CP and corresponding sets of genes in *Arthrobacter aurescens* TC1, *Nocardia farcinica* IFM10152, and *Streptomyces platensis* CR50 pointed to a novel class of two-component monooxygenases with high similarity to SMOs from pseudomonads. Most remarkably, the gene encoding the FAD:NADH oxidoreductase subunit ("*styB*") is fused with that one of a second version of the oxygenase subunit ("*styA2*") and the corresponding protein StyA2B was shown in *R. opacus* 1CP to act as a single-component self-sufficient monooxygenase with (low) styrene-oxygenating activity (Tischler et al. 2009). Later on, addition of the other oxygenase subunit StyA1 was shown to be necessary for full epoxidation activity and evidence for a specific inter-protein communication was provided (Tischler et al. 2010).

3.4.4 Mobility of Styrene-Catabolic Genes

The *sty*-operon of pseudomonads was shown to be both, chromosomally encoded in *Pseudomonas* sp. strain Y2 (Velasco et al. 1998) as well as located on a plasmid (designated as pEG) in *Pseudomonas fluorescens* ST (Bestetti et al. 1984), which indicates mobility. The plasmid pEG was found to be 37 kbp in size, circular, and self-transmissible. Uptake of pEG by the styrene-negative, plasmid-free *Pseudomonas putida* strain PaW 340 led to a styrene-degrading phenotype and demonstrated mobility of the upper styrene catabolism (Bestetti et al. 1984). Later studies revealed that unidentified DNA-fragments as well as some transposable elements (IS-elements of class IS*21*-like) are encoded on the chromosome as well as on pEG of *P. fluorescens* ST, and that the complete pEG plasmid or

fragments thereof might transpose into the chromosome (Beltrametti et al. 1997, Ruzzi and Zennaro 1989). This assumption was supported by another study of *P. fluorescens* ST, in which the authors reported about the identification of the complete functional *sty*-operon located next to an IS-element on the chromosome (Marconi et al. 1996). However, the IS-element located adjacent to the *sty*-operon was found to be a copy of IS*1162* which can be found two times on the pEG plasmid. Thus, the transposable elements might be responsible for the construction as well as for a kind of mobility (between plasmid and chromosome) of the styrene-catabolic gene cluster. Transposable elements were identified in close proximity of the SMO genes *styA1/styA2B* from *Rhodococcus opacus* 1CP, too, which might be an indication of mobility of these monooxygenases (Fig. 3.3c) (Tischler et al. 2009).

3.4.5 Genetic Aspects of Styrene Catabolism by Direct Ring Cleavage

Compared to the mechanism of side-chain oxygenation, apparently no information about genes and regulatory elements of the direct ring cleavage route are available (Fig. 3.1). From *Pseudomonas putida* MST (Bestetti et al. 1989), *Rhodococcus rhodochrous* NCIMB 13259 (Warhurst et al. 1994), and *Xanthobacter* sp. 124X (Hartmans et al. 1989), only metabolites and enzyme activities were described. However, *meta*-cleavage is a more general pathway for the aerobic degradation of (alkylated) aromatic compounds and occurs in many microorganisms (Knackmuss et al. 1976; Warhurst et al. 1994; Marín et al. 2010). Warhurst and co-workers observed no significant difference in the enzyme activities of the *meta*-cleavage route for styrene compared to similar pathways like that encoded by the TOL plasmid for toluene and xylenes. It probably evolved like *meta*-cleavage pathways from other microorganisms. Interestingly, toluene-grown cells of *Pseudomonas putida* F1 were able to co-metabolize styrene by enzymes of the *meta*-cleavage route of toluene (Cho et al. 2000). First genetic insights were reported for the genome-sequenced *Rhodococcus jostii* RHA1 which confirmed earlier assumptions (Patrauchan et al. 2008). Patrauchan and co-workers found out that strain RHA1 degrades styrene by direct ring attack and *meta*-cleavage, but no gene cluster involved exclusively in styrene degradation was determined. By means of a proteomic approach and targeted gene disruption, responsible genes and products were identified as part of a biphenyl-catabolic gene cluster. The initial activity towards styrene was attributed to a single biphenyl dioxygenase (BPDO). Relevant genes of that biphenyl-catabolic gene cluster are regulated by a two-component regulatory system (BphS/BphT) (Patrauchan et al. 2008). BphS phosphorylates BphT in response to various aromatic substances and the latter one then promotes the transcription of catabolic genes. A regulatory coupling highly similar to BphS/BphT is responsible for transcription of a gene cluster involved in ethylbenzene degradation, too. In presence of both, biphenyl and ethylbenzene, gene clusters are co-expressed, but styrene was found to induce only genes of biphenyl degradation.

This indicates a substrate-dependent regulation of both the biphenyl- and the ethylbenzene gene cluster.

In summary, styrene degradation by direct ring attack occurs rather by an adapted pathway and has not been specifically evolved for the alkenylbenzene than it is the case for the *sty*-operon from pseudomonads.

3.5 Biotechnological Aspects

Styrene-degrading microorganisms and enzymes of styrene-catabolic pathways were shown to be of relevance for the purposes of white (grey) biotechnology.

3.5.1 Styrene Monooxygenases

Perhaps, the most prominent application derived from styrene-degrading bacteria is the production of enantiopure epoxides by means of highly stereoselective styrene monooxygenases (SMOs) (Fig. 3.5). Optically active oxiranes are high-value building blocks for pharmaceutical and agrochemical industry as well as for fine chemistry (Rao et al. 1992; Badone and Guzzi 1994; Besse and Veschambre 1994; Hattori et al. 1995; Schulze and Wubbolts 1999; Breuer et al. 2004). All styrene monooxygenases investigated so far show a strong preference for the formation of (*S*)-isomer of styrene oxide from styrene (enantiomeric excess >98%) (O'Leary et al. 2002b; Mooney et al. 2006b). An oxygenating activity from ethene-grown cells of *Mycobacterium* sp. E3, which was found to convert styrene into (*R*)-styrene oxide (>98% e.e.), probably cannot be assigned as a "true" styrene monooxygenase due to its expression by a different growth substrates. Even more important styrene biotransformation rates of ethene-grown cells were shown to be a factor 40 lower than those ones of styrene-grown pseudomonads expressing an SMO (Nöthe and Hartmans 1994). In fact, other types of monooxygenases are often able to convert styrene into styrene oxide, but high activities and selectivities of real SMOs are never reached (Archelas and Furstoss 1997; Mooney et al. 2006b; O'Leary et al. 2002b).

Despite their high regio- and enantioselectivity, SMOs show a somewhat broad substrate specificity. Substituted styrenes, like monohalogenated derivatives (fluoro-, chloro-, bromostyrene), monomethylstyrenes, and *trans*-β-methylstyrene as well as structurally similar compounds like pyridine analogs, dihydronaphthalene, (substituted) allylbenzenes, indole, and indene are converted to the corresponding epoxides (Bernasconi et al. 2000; Hollmann et al. 2003; van Hellemond et al. 2007; Tischler et al. 2009; Lin et al. 2010). Enantiopure indene oxide is by the way hardly available by chemical synthesis and serves as a precursor of *cis*-1*S*,2*R*-aminoindanol, an intermediate in the production of the anti-HIV-1 drug Crixivan® (indinavir). Furthermore, SMOs are able to oxidize phenylalkyl sulfides

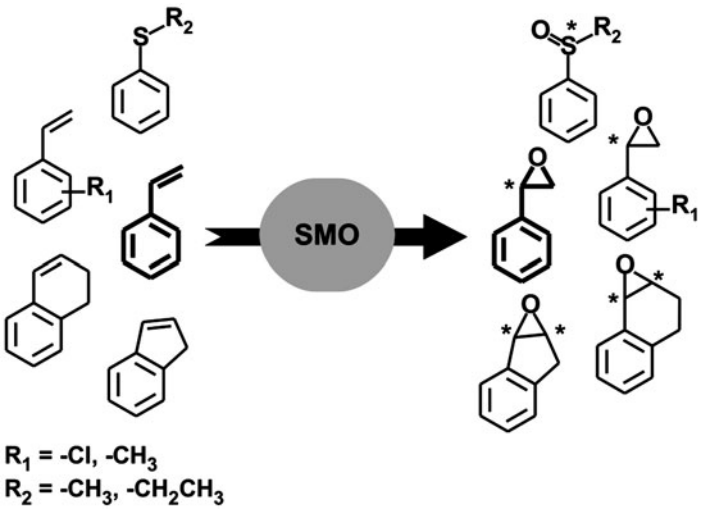


Fig. 3.5 Enantioselective oxygenations catalyzed by styrene monooxygenases (SMOs). *Stars* indicate optically active centers (Tischler et al. 2010)

to sulfoxides and depending on the host, different enantioselectivities are achieved. An SMO from a metagenome was found to yield predominantly the (*R*)-enantiomer of phenylmethyl sulfoxide (>75% e.e.) (van Hellemond et al. 2007), whereas a strong preference of the (*S*)-enantiomer (>99% e.e.) was shown for StyA1/StyA2B from *R. opacus* 1CP (unpublished).

Since the application of SMOs allows the production of a wide range of valuable epoxides, a first pilot-scale biotransformation process was established (Panke et al. 2002). Due to cofactor dependence (NADH, FAD), a whole-cell biotransformation is favored allowing the regeneration of expensive NADH. StyA/StyB from *Pseudomonas* sp. VLB120 were recombinantly expressed in *E. coli* during a 30-L fed-batch to produce enantiopure (*S*)-styrene oxide (307 g, 40% yield). A two-phase extractive fermentation was chosen and subsequently optimized in order to maximize volume productivity and process stability (Panke et al. 2000, 2002; Park et al. 2006a, b). In addition, biocatalysts were modified by approaches of protein engineering (Gursky et al. 2009; Qaed et al. 2010) and different expression systems were analyzed for their suitability (Panke et al. 1999; Han et al. 2006; Park et al. 2006b; Bae et al. 2008, 2010). An evaluation of the optimized process indicated a high degree of competitiveness towards conventional methods of chemical synthesis (Kuhn et al. 2010). The usage of toxic and environmentally harmful bis(2-ethylhexyl)phthalate as organic phase was found to be the major drawback of this type of process.

The use of biofilms instead of two-phase systems may be another approach in order to minimize toxic effects of substrate and product during biotransformations and was demonstrated for a SMO-harboring *Pseudomonas* strain (Gross et al. 2007).

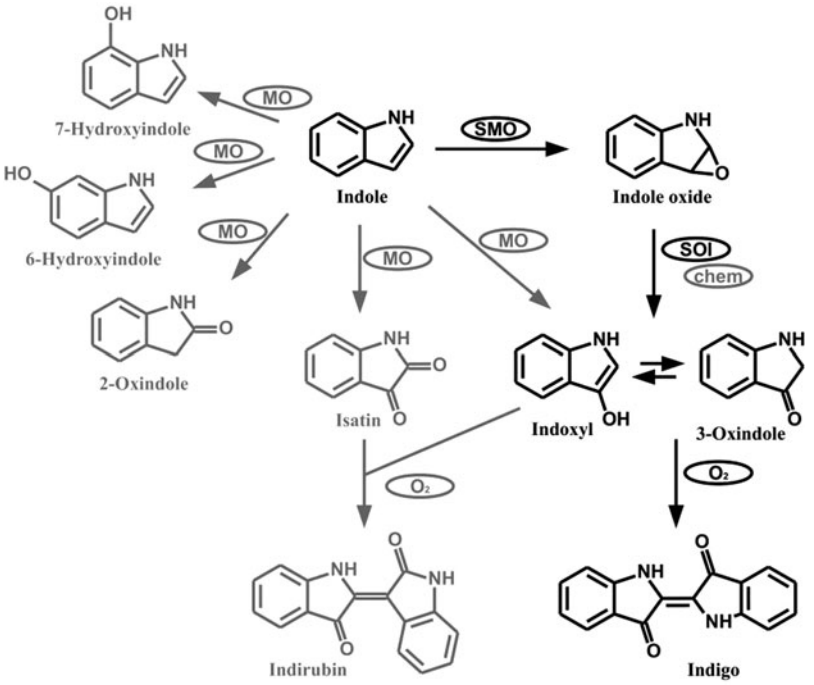


Fig. 3.6 Oxo functionalization of indole and the formation of indigoid dyes (from McClay et al. 2005). SMO/SOI-based oxygenation (*black*) and oxidation of indole (*grey*) catalyzed by various oxygenases (e.g. P450- or toluene monooxygenases; MO) can result in the formation of indigo and indirubin. Indoxyl can be yielded slowly by chemical rearrangement (chem) or faster by SOI activity

In addition to the above mentioned substrates, styrene monooxygenases show a common feature to convert indole to indole oxide. Indole oxide then rearranges spontaneously or by the action of an SOI to indoxyl, followed by spontaneous dimerization to indigo under aerobic conditions (Fig. 3.6) (O'Connor et al. 1997). The expression of SMO/SOI either by styrene-degrading wild-type hosts or by recombinant strains is thus a strategy to produce indigoid dyes. Compared to other oxygenase-dependent transformations as well as to chemical syntheses, the combination of SMO and SOI is more specific and less by-products like hydroxyindole, 2-oxoindole, isatin, or indirubin are formed (McClay et al. 2005), leading to production of highly pure indigo.

3.5.2 Styrene Oxide Hydrolases and Styrene Oxide Isomerases

As mentioned before, epoxide hydrolases (EHs) can be involved in styrene metabolism (Fig. 3.1). More frequently, these enzymes play a role in the

detoxification process of xenobiotics with epoxide-like structures, especially in mammalians. EHs are enantioselective and show a broad substrate spectrum.

Styrene oxide is a model substrate for this enzyme class and most representatives convert solely one enantiomer of racemic styrene oxide into a chiral phenylethan-1,2-diol. Thus, EHs can be used to produce either enantiopure epoxides or enantiopure 1,2-diols (Archer 1997; Archelas and Furstoss 1997; Orru and Faber 1999; Breuer et al. 2004; Kim et al. 2006; Liu et al. 2006). Especially EHs from fungi were subject of considerable research during the past decades because these enzymes can be produced in high amounts and allow the formation of enantiopure compounds with a high value for pharmaceutical industry. Exemplary, the company 'Merck' screened various fungi for enantioselective hydrolase activity on racemic indene oxide to produce (1*S*,2*R*)-indene oxide, which serves as a precursor for the HIV protease inhibitor indinavir (Crixivan®) (Archer 1997). The industrial application of EHs enables 'Merck' to produce this pharmaceutical in high quality.

Compared to styrene oxide hydrolases, less information is available on styrene oxide isomerases (SOIs) which convert styrene oxide to phenylacetaldehyde (Hartmans et al. 1989; Itoh et al. 1997a). In addition, SOIs from *Pseudomonas* species are able to convert various substituted styrene oxides into the corresponding aldehydes or ketones (Miyamoto et al. 2007). During the production of indigo by SMOs, the presence of SOIs yields an almost complete conversion of indole oxide (SMO product) into indoxyl. The absence of isatin prevents the formation of the unwanted by-product indirubin, which is usually formed, if other less specific oxygenases are applied (Fig. 3.6).

However, two major drawbacks of SOI representatives identified so far have to be addressed: (i) SOIs are membrane-bound enzymes and seem to be expressed at very low levels in wild-type microorganisms as well as in recombinant hosts (Hartmans et al. 1989; Itoh et al. 1997a; Nikodinovic-Runic et al. 2009), and (ii) SOIs convert only epoxides with styrene oxide-analogous structure and are exhibiting low enantioselectivity (Itoh et al. 1997a; Miyamoto et al. 2007).

3.5.3 Phenylacetaldehyde Reductases

Phenylacetaldehyde reductases (PAR) from styrene-assimilating bacteria are highly interesting biocatalysts for production of chiral alcohols from various prochiral ketones, *β*-ketoesters, and aldehydes (Itoh et al. 1997b, 2002, 2007; Makino et al. 2007). Itoh and coworkers reported a system utilizing a recombinantly expressed PAR from *Rhodococcus* sp. ST-10 (formerly described as *Corynebacterium* sp. ST-10). On the one hand, it converts ketones and ketoesters to valuable products at the expense of NADH, and on the other hand, it regenerates that reduced cofactor in the presence of 2-propanol. Thus, an additional NADH-regeneration system as for example formate dehydrogenase is not necessary.

Further, enzyme engineering yielded mutants with broader substrate spectrum which are less affected by high amounts of substrates (e.g. 2-propanol).

3.5.4 Production of Substituted Catechols

Substituted *ortho*-diphenols are useful building blocks for dyes, polymers, and pharmaceuticals. Certain isomers are only hardly available from conventional chemical syntheses and hence biocatalytic preparation strategies are becoming of increasing interest. Catechols are important intermediates of the peripheral degradation of aromatic hydrocarbons typically by the action of a dioxygenase and a dihydrodiol dehydrogenase. Their further metabolization is achieved during the central *ortho*- and *meta*-cleavage pathway by intradiol-acting- (C12Os) or extradiol-acting dioxygenases (C23Os), respectively. It is well known that C23Os are specifically inhibited by 3-fluoro- and 3-chlorocatechol by a so-called "suicide inactivation", leading to an accumulation of catechols by the action of initial dioxygenases/dehydrogenases (Bartels et al. 1984; Mars et al. 1997). Taking into consideration the toxic behavior of accumulated catechols for the host, this phenomenon represents a simple route to produce various catechol derivatives.

Since *R. rhodochrous* NCIMB 13259 was shown to degrade styrene by direct ring attack and *meta*-cleavage (Fig. 3.1), the inhibitory effect of 3-fluorocatechol was exploited by Warhurst and co-workers to obtain 3-vinylcatechol (Warhurst et al. 1994). The initial styrene dioxygenase and dihydrodiol reductase of a styrene-grown culture of strain NCIMB 13259 both exhibit a broad substrate tolerance allowing the conversion of toluene and ethylbenzene into 3-methylcatechol and 3-ethylcatechol, respectively.

3.5.5 Formation of p-Hydroxystyrene

The introduction of genes encoding a phenylalanine/tyrosine ammonia lyase (from *Rhodotorula glutinis*) and a *p*-coumaric acid decarboxylase (from *Lactobacillus plantarum*) into *E. coli* WWQ51.1 recently allowed the construction of a pathway for the formation of *p*-hydroxystyrene from the central metabolite tyrosine during growth of that strain on glucose (Qi et al. 2007). Product toxicity was overcome by a two-phase fermentation system in which the biotransformation takes place in the aqueous phase and *p*-hydroxystyrene accumulates in the organic phase. Later on, the host was changed from *E. coli* to a more solvent-tolerant *P. putida* strain lacking styrene monooxygenase and feruloyl-coenzym A synthethase, yielding an increased product formation (Verhoef et al. 2009). *p*-Hydroxystyrene is a valuable target for chemical industry (especially in polymer production) and as a potential substrate for styrene-catabolic enzymes, it might serve as a source for other interesting compounds, like *p*-hydroxystyrene oxide and *p*-hydroxyphenylacetaldehyde. A combination of the above pathway with certain activities of initial

styrene metabolism would allow the formation of these compounds starting from glucose and avoiding the use of toxic styrene.

3.5.6 Formation of Polyhydroxyalkanoates

Many bacteria produce polyhydroxyalkanoates (PHAs) as carbon-based storage compounds under conditions of unbalanced nutrient supply (Anderson and Dawes 1990; Madison and Huisman 1999). Styrene is used as a carbon source in that respect and can be utilized by *P. putida* CA-3 in order to produce PHAs during nitrogen starvation (Ward et al. 2005). Yields of up to 23% of the cell dry weight were obtained and the polymer was shown to consist of the monomers 3-hydroxyhexanoate, 3-hydroxyoctanoate, and 3-hydroxydecanoate in a ratio of 3:22:75. Two other pseudomonads were shown to produce PHAs in lower yields under similar conditions too. Combination of this microbial ability with a process of thermal polystyrene cleavage yielding crude styrene monomer may represent an attractive strategy for the production of biodegradable polymers from recalcitrant polystyrene wastes (Ward et al. 2006).

3.5.7 Biological Waste Gas Treatment

Since a few decades, the principle of biological waste gas treatment has been used in order to remove harmful volatile organic compounds (VOCs) from gas streams. Especially for the removal of low concentrations of biodegradable VOCs from large volumes of waste gases, the application of biofilters, trickling biofilters, and bioscrubbers represent a cost-effective alternative to methods of physico-chemical treatment as for example active carbon filters and waste gas combustion (Deshusses 1997; Delhomenie and Heitz 2005; Malhautier et al. 2005).

All biofiltration techniques, of which the biofilter is the most simple and widely distributed one, depend on the degradative potential of a complex microbial consortium. The degradative capability is either a result of prolonged adaptation of the available microflora (present on the surface of the organic packing material) or may be supported by the addition of microorganisms of certain metabolic properties. Several studies showed the removal of readily biodegradable volatile organic compounds i.e. styrene (Table 3.3) by a simple adaptation and enrichment of naturally available microbial consortia (Corsi and Seed 1995; Arnold et al. 1997; Juneson et al. 2001). However, the application of specialized strains of bacteria or fungi with styrene-catabolic activities was found to be advantageous for the treatment of waste gases containing this kind of hydrocarbon as a major component (Cox and Deshusses 1999; Jang et al. 2004, 2006; Jung and Park 2005; Cox et al. 1997; René et al. 2010).

Table 3.3 Styrene-metabolizing consortia and single representatives of the microflora of various biofilters for VOC removal

Microflora	Filter: carrier material	Reference
Mixed cultures, *Gordonia* sp. strain D7, *Pseudomonas* sp. strain D26	Biofilter: crushed wood and bark compost, 74% water content	Alexandrino et al. (2001)
Inoculated with activated sludge: *Tsukamurella* sp., *Pseudomonas* sp., *Sphingomonas* sp., *Xanthomonas* sp., γ-Proteobacteria	Biofilter: peat, about 10% burned clay and plastic filling; low-soluble nutrient mix and lime added to adjust nutrient content and pH	Arnold et al. (1997)
Biofilm dominated by the fungus *Exophiala jeanselmei*	Biofilter: Perlite	Cox et al. (1997)
Inoculum: garden soil in 0.015% (v/v) Tween-80	Biofilter: six types of activated carbon, polyurethane, and Perlite	Cox et al. (1993)
Long-term adapted mixed microbial culture	Biofilter: Perlite	Weigner et al. (2001)
Activated sludge	Trickle-bed air biofilter: coal particles	Lu et al. (2001)
Pseudomonas sp. IS-3	Biofilter: cubic polyurethane, minimal salt medium supplied	Kim et al. (2005)
Pseudomonas sp. SR-5	Biofilter: peat and/or ceramic, nutrient solution supplied	Jang et al. (2004, 2006)
Pseudomonas putida ST201	Packed tower biofilter: ceramic	Okamoto et al. (2003)
Rhodococcus pyridinovorans PYJ-1	Biofilter: compost	Jung and Park (2005)
Rhodococcus rhodochrous AL NCIMB 13259	Biofilter: peat and glass beads, water content and pH regulated	Zilli et al. (2003)
Sporothrix variecibatus	Biofilter: sieved Perlite	René et al. (2010)

3.5.8 Biosensors

Biosensor systems for a broad range of toxic aromatics including BTEX compounds are available in order to measure the relative bioavailability of these hydrocarbons in contaminated groundwater, soil, and air, as well as to assess the degree of bioremediation and removal. The *xylR*-, *xylS*-, or *tod*- regulators in combination with different reporter systems have been frequently used for these purposes showing a somewhat broad detection specificity (Keane et al. 2002; Rodriguez-Mozaz et al. 2006). A construction, in which the styrene-inducible regulator gene *styR* was combined with the reporter gene of beta-galactosidase (*lacZ*), recently allowed the formation of a styrene-sensitive biosensor (Alonso et al. 2003b). However, styrene oxide, phenylacetaldehyde, 2-phenylethanol, and toluene were shown to act as alternative inducers.

3.6 Conclusions

Despite of an extensive industrial usage of styrene which brings about significant anthropogenic releases in the environment, the ecological threat of this hydrocarbon is considerably limited due to its high volatility, its distinct chemical reactivity, and a pronounced susceptibility against biological transformations. In mammals and human, these transformations involve an initial cytochrome P450-mediated oxygenation yielding a mutagenic epoxide which, as a proven carcinogen, causes the most hazardous risk of chronic styrene exposure. Among pro- and eukaryotic microorganisms, styrene-catabolic activities were found to be wide-spread under aerobic and anaerobic conditions. Dioxygenation of the aromatic nucleus, followed by an extradiol-cleavage pathway of the resulting catechol as well as side-chain oxygenation of styrene to the central intermediate phenylacetic acid were found to be the two major mechanisms of mineralization, allowing microorganisms to utilize this hydrocarbon as the sole source of carbon and energy. Especially the latter pathway could be elucidated in members of the genus *Pseudomonas* on biochemical and genetic level. In addition, regulation of the so called *sty*-operon comprising the genes of a styrene monooxygenase StyA/StyB, a styrene oxide isomerase StyC, and a phenylacetaldehyde dehydrogenase StyD was characterized in detail. More recent studies have indicated a phylogenetically more diverse distribution of styrene monooxygenases among Actinobacteria. Interestingly, these enzymes differ from those ones typically found in pseudomonads in respect of an unusually fused structure as well as an absence of neighbored genes of the *sty*-operon. StyA1/StyA2B of *Rhodococcus opacus* 1CP is the first biochemically characterized representative of this group of flavin-dependent monooxygenases and evidence was provided for a novel transfer mechanism of reduced FAD between FAD:NADH oxidoreductase- and epoxidizing monooxygenase subunit.

In contrast to the mechanism of side-chain oxygenation, styrene catabolism by initial ring dioxygenation and *meta*-cleavage is a less specific degradation route since it is frequently observed for the mineralization of other (alkylated) aromatic hydrocarbons like benzene, toluene, xylene, and biphenyl. A degradation pathway for the latter compound enables *Rhodococcus jostii* RHA1 to grow on styrene.

Knowledge on the mechanisms and the distribution of styrene-catabolic pathways is not only interesting from a scientific point of view, but also relevant in respect of a biotechnological applicability. Styrene is an important volatile organic component of industrial waste gases and hence styrene-degrading microorganisms are of relevance in the field of biological waste gas treatment. Several styrene-catabolic enzymes like styrene monooxygenase, styrene oxide hydrolase, and styrene oxide isomerase are attractive biocatalytic tools. They can be applied to the highly enantioselective epoxidation of styrene into (*S*)-styrene oxide, to the resolution of racemic styrene oxide coupled with the preparation of enantiopure vicinal diols, and to the preparation of pure aromatic aldehydes, respectively.

Despite the amount of available information on microbial styrene degradation, a number of questions and speculations remain unanswered. In order to face two of them: (i) it seems that important mechanisms of aerobic styrene catabolism, e.g. by sequential monooxygenation of the aromatic nucleus, have not been traced out till yet, and (ii) it should not wonder if abundance of the *sty*-operon could be shown for others members of the class of *Gammaproteobacteria* than pseudomonads since a mobility of these genes has already been shown. Elucidation of these questions is not only interesting from an evolutionary point of view, but also it may lead to the identification of novel biocatalysts and further biotechnological applications.

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