Doctoral Thesis

Production of (S)-styrene oxide with recombinant bacteria

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PRODUCTION OF (S)-STYRENE OXIDE WITH RECOMBINANT BACTERIA

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree of
Doctor of Natural Sciences

presented by

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Dr. Marcel. G. Wubbolts, co-examiner
Prof. Dr. Amrhein, chairman

Zürich, August 1999
Seite Leer / Blank leaf
Caminante, no hay camino. Se hace camino al andar.
(Antonio Machado)

Meiner Familie - Nina und Lydia
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and those that are mentioned somewhere else. They helped a lot in feeling good here.

And finally thanks to those people who helped me through my ups and downs (of which there were many) as friends or simply by being there at the right time, and to my dear little family, who was always there to support me and to light up my day.

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<td>References</td>
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<td>9.</td>
<td>Curriculum vitae</td>
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A bioprocess which employed recombinant *Escherichia coli* cells as biocatalyst was developed for the production of enantiopure styrene oxide, a versatile building block for the synthesis of biologically active molecules. The development covered the steps from cloning of the genes of a styrene monooxygenase to a pilot scale production process. The styrene monooxygenase of styrene degrading *Pseudomonas* sp. strain VLB120 converts styrene to the chiral building block (S)-styrene oxide with an enantiomeric excess of more than 99%. Its genes *styAB* were used to construct *E. coli* and *P. putida* recombinants that expressed the genes via the octane-responsive *alk* regulatory system of *P. oleovorans* GPo1. Based on the *E. coli* recombinants, a 2 L two-liquid phase process was developed with bis(2-ethylhexyl)phthalate as the organic phase at a phase ratio of 50%. Cells reached a maximum volumetric productivity of 2.2 g of (S)-styrene oxide per liter liquid volume per hour. The process was styrene mass transfer limited when scaled up to 30 L with power input close to that of commercially operating units. Still, the process maintained an average volumetric productivity of approximately 1.2 g of (S)-styrene oxide per liter liquid volume per hour for more than 10 h, which resulted in the production of 388 g of (S)-styrene oxide in only one batch.
ZUSAMMENFASSUNG

**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ATP</td>
<td>adenosinetriphosphate</td>
</tr>
<tr>
<td>BEHP</td>
<td>bis(2-ethylhexyl)phthalate</td>
</tr>
<tr>
<td>BINAP</td>
<td>binaphthyl</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>cal</td>
<td>calory</td>
</tr>
<tr>
<td>cdw</td>
<td>cell dry weight</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>CSTR</td>
<td>continuously stirred tank reactor</td>
</tr>
<tr>
<td>d</td>
<td>days</td>
</tr>
<tr>
<td>DCPK</td>
<td>dicyclopropylketone</td>
</tr>
<tr>
<td>E</td>
<td>enantiomeric ratio</td>
</tr>
<tr>
<td>e.e.</td>
<td>enantiomeric excess</td>
</tr>
<tr>
<td>EMBL</td>
<td>European Molecular Biology Laboratory</td>
</tr>
<tr>
<td>EPRS</td>
<td>electron paramagnetic resonance spectroscopy</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosinetriphosphate</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>Fig.</td>
<td>figure</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilo base pairs</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>L</td>
<td>liter</td>
</tr>
<tr>
<td>$L_{aq}$</td>
<td>liter aqueous phase</td>
</tr>
<tr>
<td>$L_{org}$</td>
<td>liter organic phase</td>
</tr>
<tr>
<td>$L_{tot}$</td>
<td>liter total liquid volume</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>m</td>
<td>milli, 10^-3</td>
</tr>
<tr>
<td>m</td>
<td>meta</td>
</tr>
<tr>
<td>μ</td>
<td>micro, 10^-6</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>n</td>
<td>nano, 10^-9</td>
</tr>
<tr>
<td>NAD(H)</td>
<td>nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>NADP(H)</td>
<td>nicotinamide adenine dinucleotide phosphate (reduced form)</td>
</tr>
<tr>
<td>o</td>
<td>ortho</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>p</td>
<td>para</td>
</tr>
<tr>
<td>P</td>
<td>partition coefficient in the standard octanol/water system</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SD</td>
<td>Shine-Dalgarno</td>
</tr>
<tr>
<td>sp.</td>
<td>species</td>
</tr>
<tr>
<td>std.dev.</td>
<td>standard deviation</td>
</tr>
<tr>
<td>t</td>
<td>tons</td>
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<td>Tab.</td>
<td>table</td>
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<tr>
<td>tert</td>
<td>tertiary</td>
</tr>
<tr>
<td>U</td>
<td>unit, μmol*min^-1</td>
</tr>
<tr>
<td>wt/vol</td>
<td>weight per volume</td>
</tr>
<tr>
<td>vol/vol</td>
<td>volume per volume</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION
1. Introduction

The chemo-, regio-, and enantioselectivity of enzymes makes them valuable catalysts in the production of so-called building blocks, which serve as precursors for the manufacturing of complex organic molecules like drugs. The exploitation of biological systems for the production of large amounts of building blocks is frequently associated with a number of problems, for example poor water solubility of the substrate of a reaction or toxicity of the product. Biochemical reaction engineering has provided solutions to a number of these problems. In some especially useful reactions - as enantioselective epoxidations - process productivities have remained low because the used biocatalysts had low specific activities and were used in low concentrations. The techniques of modern recombinant biotechnology allow to specifically manipulate model microorganisms like Escherichia coli for the overexpression of a desired activity. Moreover, such model microorganisms have been well studied in processes which were performed at high biomass concentrations. This offers a route to eliminate a central bottleneck in setting up highly productive processes. The work described below was undertaken to investigate the potential of E. coli to facilitate the development and improve the productivity of a production process for a toxic and hardly water soluble building block, (S)-styrene oxide.

The objectives of this introduction are
- to highlight the importance of single enantiomer preparations of drugs and agrochemicals
- to introduce into the role that enatiopure epoxides might play in the synthesis of these compounds
- to characterize briefly biological and chemical catalysts that were used to obtain enatiopure epoxides
- to summarize biotechnological process developments for the production of epoxides and identify their drawbacks, and
- to illustrate some future directions of the field.
1.1. CHIRALITY IN ORGANIC CHEMISTRY

1.1.1. CHIRALITY AND BIOLOGICAL FUNCTION

Organic molecules that cannot be superimposed on their mirror image are called chiral molecules (Fig. 1.1A). In other words, a molecule is chiral if it lacks reflectional symmetry (327). One of the reasons for chirality is the presence of a carbon atom with four different substituents, a stereogenic or asymmetric center. One such chiral molecule is styrene oxide, and its formation is the central topic of this work (Fig. 1.1A). The two possible configurations of chiral molecules, called enantiomers, are different in the three-dimensional orientation of some of their atoms, and as a result the two enantiomers might react in different ways with a partner which is also a chiral molecule. This can be the interaction of a chiral drug and the active site of an enzyme or a receptor or a biological macromolecule in general (70). Examples of two enantiomers which differ in their biological effect are shown in Fig. 1.1B. The differences can be quite dramatic. As a consequence, regulatory agencies like the Food and Drug Administration in the USA adopted a critical, although not prohibitive, position to the marketing of drug racemates (equimolar mixtures of enantiomers) (12), which in turn influenced the product development process in the pharmaceutical, agrochemical, and food industry (68).

E.g., of 1200 synthetic drugs that were approved before 1982, only 58 were sold as single isomer preparations, 422 were sold as racemates, and the remaining 720 synthetic drugs were achiral. The situation was completely different with natural and semisynthetic drugs, of which only 6 of 475 approved drugs were achiral, and 461 of the 469 remaining chiral drugs were sold as single enantiomer preparations. Clearly, single enantiomer preparations received little attention in the chemical synthesis of drugs before 1982 (327). In 1990, the picture had changed: of the 20 top-selling drugs, 6 were achiral, 4 were sold as racemates, and 10 were marketed as single isomers. That is the sales of optically pure drugs in 1990 amounted to US$ 12 billion or were 53.4 % of the sales of the 20 top-selling drugs. Achiral drugs amounted to another 30.6 % of the share and racemic drug preparations only to 16 % (327). A more recent survey of the pharmaceutical market shows that (for the period of 1994 to 1996) the share of single enantiomer preparations among
A

\[
\begin{align*}
\text{styrene} & \quad \rightarrow \quad \text{(S)-styrene oxide} \\
\text{H} & \quad \text{O} \\
\text{C} & \quad \text{C} \\
\end{align*}
\]

S styrene oxide \quad R

B

\[
\begin{align*}
\text{HS} & \quad \text{COOH} \\
\text{H}_2 & \quad \text{N} \\
\text{H} & \quad \text{SH} \\
\text{HOOC} & \quad \text{SH} \\
\end{align*}
\]

S antiarthritic \quad R mutagen

\[
\begin{align*}
\text{O} & \quad \text{H} \\
\text{N} & \quad \text{O} \\
\text{H} & \quad \text{N} \\
\text{O} & \quad \text{H} \\
\end{align*}
\]

S teratogen \quad R sedative

\[
\begin{align*}
\text{Cl} & \quad \text{HO}_2 \quad \text{rBu} \\
\text{N} & \quad \text{N} \\
\end{align*}
\]

2R,3R fungicide

\[
\begin{align*}
\text{O} & \quad \text{H} \\
\text{N} & \quad \text{O} \\
\end{align*}
\]

carvone

\[
\begin{align*}
\text{H}_2 & \quad \text{NCOH} \quad \text{C} \quad \text{COOH} \\
\end{align*}
\]

S bitter taste

\[
\begin{align*}
\text{HOOC} & \quad \text{CH}_2\text{CONH}_2 \\
\text{H} & \quad \text{NH}_2 \\
\end{align*}
\]

R sweet taste
newly approved drugs was between 55 and 60%, outnumbering racemic preparations by 5 to 1. The market for sales of single enantiomer drug preparations in the year 2000 was estimated around US$ 90 billion (21).

The agrochemical area is a similar case, although with a less intensive regulatory pressure. Pesticides are frequently distributed on the order of multiple thousands of tons, so distributing racemic mixtures of which only one isomer is effective means putting a considerable, unnecessary, and potentially dangerous burden on the environment. Before 1981, 83 of the 90 applied synthetic chiral pesticides were sold as racemates (out of a total of 550). The 13 natural products that were in use as pesticides, by contrast, were all marketed as single enantiomer preparations (327). In 1991, 9% of a US$ 7.7 billion insecticide market were occupied by single enantiomer preparations. This figure will probably increase to 20% in the year 2000 (68).

All in all, there is a clear tendency to market chiral drugs and agrochemicals as single enantiomer preparations. There is therefore an interest in efficient synthesis routes for these compounds. Their syntheses can be considerably facilitated by providing useful building blocks which already contain at least one asymmetric center. Such building blocks should possess a functional group that allows an easy and enantioselective integration into the overall synthesis.

Epoxides are versatile functional groups. The three-membered ring is strained and thus very reactive, and - with the exception of ethylene oxide - epoxides possess at least one asymmetric center which makes them potentially chiral. The epoxide function can be opened with many different nucleophiles in a regio- and enantioselective manner (115, 357) and thus provides easy access to single enatio-

Fig. 1.1. (A) Formation and chirality of styrene oxide. The asterisk indicates the asymmetric carbon atom. (B) Examples for the different effects of two enantiomers in biological systems. Bu: butyl; tBu: tert. butyl. Adapted from (67, 327).
mer preparations of other chiral compounds (84, 261). For these reasons, methods to obtain enantiomerically enriched or even enantiopure epoxides have been intensively investigated with both chemical and biological methods. Enantiopure styrene oxide has the potential to become such a useful chiral building block. Kaneka has proposed its use for the production of the nematocide levamisole (149) and BASF produces it for internal use (393).

1.1.2. The Sources of Single Enantiomer Preparations

Single enantiomer preparations are available from two types of reactions: asymmetric synthetic reactions and resolutions of racemates. The degree of selectivity of these reactions is expressed in terms of the concentration of the two enantiomers in the product mixture by the enantiomeric excess

\[ e.e. = \frac{[S] - [R]}{[S] + [R]} \]

where \([S]\) is the concentration of the \(S\) enantiomer, \([R]\) of the \(R\) enantiomer, and \(S\) is produced in excess of \(R\). The enantioselective epoxidation of prochiral styrene is one example of asymmetric syntheses; an achiral compound is converted into a chiral compound in a way that of the two thermodynamically equivalent enantiomers only one is formed. Asymmetric synthesis involves either a single enantiomer preparation of a chiral catalyst or an already present asymmetric center in the substrate. This first asymmetric center can then direct the course of the reaction that creates the second asymmetric center.

A special case is an asymmetric synthesis that starts from a \(meso\) compound, which is a compound with at least two asymmetric centers, but which is symmetric and thus not chiral. Here an asymmetric synthesis can either introduce a new asymmetric center or remove one; in both cases the product will be a single enantiomer preparation of a chiral compound.

Alternatively, a racemic mixture of two enantiomers can be produced and later on one enantiomer is selectively removed either physically or kinetically. The physical
resolution of racemates is complicated since enantiomers do not differ in physicochemical properties that can be exploited for classical separation techniques like crystallization, although these techniques still work in exceptional cases (69). Racemates must frequently react with a second chiral compound to produce diastereomers which can then be separated.

In kinetic resolutions one enantiomer reacts considerably faster than the other in the presence of a (chiral) catalyst. The product might well be another chiral compound which is frequently produced with excellent e.e. (69) (in the following, excellent e.e.’s indicate e.e.’s ≥96 %, very good ≥90 %, good ≥80 %, and moderate between 20 and 80 %). However, resolutions have one intrinsic limitation: their chemical yield is limited to 50 %, irrespective of whether one is interested in the remaining enantiomer or (in kinetic resolutions) in the product. The importance of this is clear when the synthesis of the angiotensin converting enzyme (ACE) inhibitor Ramipril, marketed by Hoechst, is considered. It contains 5 asymmetric centers leading to, theoretically, 32 possible stereoisomers. If a racemate was produced and resolved at each step that introduces an asymmetric center, this would reduce the theoretical yield of a production process to around 3 %, which does not even take into account other losses from isolations of intermediates and product or not completely selective reactions.

The limited product yield can frequently be increased if the kinetic resolution can be converted into a dynamic resolution or a deracemization. In a dynamic resolution an additional reaction step racemizes the remaining enantiomer and thus allows for a theoretical yield of 100 % (347). In deracemizations both enantiomers are converted: for example, one enantiomer is enantiospecifically converted to product in a first step (for instance by enzymatic hydrolysis), and the remaining enantiomer is converted to the same product in a second step (e.g. by a controlled acid hydrolysis). The stereoconfiguration at the asymmetric center is maintained in one reaction and inverted in the other (352).

Asymmetric syntheses as well as resolutions can be carried out with chemical as well as biological catalysts. Products from a number of routinely carried out asymmetric syntheses and resolutions together with readily available enantiopure
natural products form the so-called chiral pool, which is a loosely defined term for single enantiomer preparations of compounds that are produced in the range of 100 to 100,000 t per annum at a price between US$ 1 and 150 per kg. The pool consists of amino acids, hydroxy acids, carbohydrates and their derivatives, terpenes, and alkaloids (69).

1.1.3. CHIRALITY AND BIOCATALYSIS

Enzymes are the catalysts of living systems. Their amino acids are entirely of the L configuration (or S, according to the Cahn-Ingold-Prelog convention) and thus provide an intrinsically chiral reaction environment for catalysis. As a consequence, a large number of enzymes either introduce chirality into prochiral molecules or discriminate between the two enantiomers of racemic mixtures. The utilization of enzymes is often associated with a number of advantages over chemical catalysis, which are shown in Tab. 1.1.

This work is concerned with the use of the first enzyme class, oxidoreductases, for asymmetric synthesis, and more specifically with the use of monooxygenases. Oxidoreductases that have found commercial applications so far include dehydrogenases, oxygenases, and peroxidases. Dehydrogenases transfer hydride ions from a substrate to a biological cofactor and back. Oxygenases introduce either one atom (monooxygenases) or both atoms (dioxygenases) of molecular dioxygen into an organic substrate. Peroxidases utilize hydrogen peroxide as an oxidant.

A general feature of the first two enzyme types is that they frequently depend on specific reduced biological cofactors like NADH, which need to be recycled during the progress of the reaction to avoid expensive stoichiometric addition. This can happen in whole cells by the host metabolism, the preferred method when considering industrial applications. Schemes have been developed that allow to use purified enzyme preparations with concomitant recycling of the cofactor in a membrane reactor, for example by the addition of formate and formate dehydrogenase to the reaction medium to provide the necessary reducing equivalents (for a survey, see 99).
Tab. 1.1. Advantages and disadvantages of enzymes (adapted from 99, 327)

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Enzymes are efficient catalysts with $10^8$ and $10^{12}$-fold rate accelerations with excellent chemo-, regio-, and stereoselectivities.</td>
<td>- Enzymes are provided in only one enantiomeric form.</td>
</tr>
<tr>
<td>- Enzymes act under mild conditions (pH 5-8, 20-40°C), reducing undesired side reactions and energy input.</td>
<td>- Enzymes require narrow operation parameters.</td>
</tr>
<tr>
<td>- Enzymes catalyze a broad spectrum of reactions.</td>
<td>- Enzymes display their highest catalytic activity in water.</td>
</tr>
<tr>
<td>- Enzymes are an inexpensive source of complex chiral ligands.</td>
<td>- Enzymes are prone to inhibition phenomena.</td>
</tr>
<tr>
<td></td>
<td>- Particular classes of enzymes need cofactor regeneration, which makes the use of isolated enzymes difficult.</td>
</tr>
</tbody>
</table>

The role of dehydrogenases, peroxidases and oxygenases in the preparation of epoxides will be discussed in sections 1.2.1.1 and 1.2.1.3. However, oxygenases have already an industrial record in asymmetric synthesis: ICI used benzene, toluene, and naphthalene dioxygenases to produce a variety of cis-dihydrodiols from benzene and its substituted derivatives (329).

The most successful and most intensively researched enzymes for biotechnological applications are hydrolases, which function as catalysts for enantiospecific kinetic resolutions (99). Hydrolases are cofactor independent, are frequently excreted by the producing microorganisms into the medium, have a broad substrate spectrum, and frequently function even in nearly anhydrous media. Interesting reactions are the hydrolytic transformation of amide- and ester-bonds, and even more the reverse reactions in low-water reaction media. A comprehensive overview of the potential of hydrolytic enzymes in the production of chiral compounds is given by Faber (99). An example for the industrial potential of hydrolases is the DSM route to the
sweetener α-aspartame: the protease thermolysin is used to couple exclusively L-phenylalaninemethylester to N-protected L-aspartic acid which allows to use the racemic phenylalaninemethylester in the reaction (327). The role of hydrolases for single enantiomer preparations of epoxides is discussed in section 1.2.1.2.

Another class of enzymes in biocatalytic asymmetric synthesis are lyases, although they are considered to be limited by their narrow substrate specificity (99). Examples of commercial use are the fumarase and the ammonia lyase reactions by Tanabe, in which L-malic acid is produced from fumaric acid by the addition of water and L-aspartic acid by the addition of ammonium (327), and the production of L-carnitin from γ-butyrobetaine by LONZA (233, 304). Ligases, transferases, and isomerases have so far had no significant impact on industrial biocatalytic asymmetric synthesis, which does not mean that these enzymes are unimportant; glucose isomerase is used to produce $6\times10^6$ t of high fructose corn syrup per year from corn starch (304).

1.2. Catalysts for the enantioselective production of chiral epoxides

1.2.1. Biological catalysts

1.2.1.1. Single enantiomer epoxide preparations as products in biochemical reactions

The epoxidation of carbon-carbon double bonds by enzymes has been studied intensively for reasons of fundamental interest and application. In the early 1980’s, interest focused on the epoxidation of short chain olefins as a potential substitute for the environmentally unfavorable and complex chemical processes (207). The discovery that the alkane hydroxylase of Pseudomonas oleovorans GPo1 can enantioselectively epoxidize 1,7-octadiene to ($R$)-7-epoxy-1-octene and ($R,R$)-1,7-diepoxyoctane (228, 229) made oxygenases as catalysts in asymmetric synthesis interesting and led to the detailed study of a number of enzymes (for a survey, see 115).

Olefinic compounds are intensively used in investigations of the catalytic mechanisms of oxidative enzymatic reactions, which contributed to the number of
known possible epoxidation reactions without necessarily paying attention to the applicability (113, 297, 314, 372).

Finally, much knowledge about epoxidations derived from investigations on the fate of environmental pollutants in mammalian systems where these pollutants are frequently oxidized (see for example 276, 380).

The following part of the introduction is intended to discuss representatives of the major enzyme groups that produce (frequently enatioselectively) epoxides from prochiral olefins. Kinetic resolution of epoxide racemates (17) is discussed in section 1.2.1.2. Biochemical routes to optically active styrene oxide are summarized in Fig. 1.2 and Tab. 1.2.

1.2.1.1. Oxygenases

Nonheme iron monooxygenases. In order to activate molecular oxygen for incorporation into the organic molecule, biological systems utilize a cofactor, for example iron ions or flavin based cofactors. Two examples are the alkane hydroxylase of *P. oleovorans* GPO1 and the xylene oxygenase of *P. putida* mt-2.

Alkane hydroxylase produces alkanols as the first step of alkane degradation. It consists of an integral membrane protein, the hydroxylase component AlkB, and two soluble proteins, rubredoxin AlkG and rubredoxin reductase AlkT (367, 368). The hydroxylase component AlkB contains probably an active site diiron cluster which is o xo-bridged (323) and might be coordinated by the nitrogen atoms of eight histidines, which together form a typical amino acid sequence motif (324). The spectroscopic properties of AlkB as determined from electron paramagnetic resonance spectroscopy (EPRS) measurements are similar to water soluble proteins of the diiron-carboxylate type that also contain a diiron center but coordinate this via histidines and the carboxyl groups of aspartate or glutamate. This diiron center has been shown spectroscopically to participate in the reactions (72, 256, 389). This suggests that AlkB represents a new type of nonheme diiron enzyme which uses only histidines to coordinate the active iron atoms (205). Furthermore, one to two more iron atoms of so far unknown function were found in AlkB preparations of high specific activity (323), although in another report AlkB was assumed to
Asymmetric synthesis: nonheme monooxygenases; peroxidases; cytochrom P450cam

Fungal dehydrogenase

\[
\text{prochiral } \alpha\text{-haloacetophenone}
\]

\[
\begin{align*}
\text{HO,} & \\
\text{stvrene} & \\
\end{align*}
\]

Asymmetric synthesis: nonheme monooxygenases; peroxidases; cytochrom P450cam

Kinetic resolution: Fungal and microbial hydrolases

MnIII Jacobsen catalyst

Diethylzinc with chiral aminoalcohol

BNAP-LiAl-hydride; alpine boron; boron with oxazaborolidine derived catalyst

Fig. 1.2. Major biological and chemical routes to single enantiomer preparations of styrene oxide. X, halogen atom; rac, racemic. The configuration at the asymmetric center.
contain only two iron atoms (344). An iron-oxo species was proposed as the reactive group which attacks the double bond and creates an intermediate with a partial radical and partial cationic character (59, 112, 113, 181).

Alkane hydroxylase can perform a variety of reactions: hydroxylation of alkanes, epoxidation of terminal alkenes, sulfoxidations, and demethylations have been reported (112). However, the interest for application remained focused on the hydroxylation of unfunctionalized terminal alkyl groups and epoxidation of terminal double bonds (115, 227, 366).

P. putida mt-2 TOL plasmid derived xylene oxygenase catalyzes the hydroxylation of methylsubstituents of benzene nuclei (49). The enzyme consists of a NADH:acceptor reductase component XylA and the membrane located oxygenating component XylM (325, 326). XylM shares 25 % amino acid sequence identity with AlkB (356) including the eight-histidine motif (324). This suggests a similar reaction mechanism (396).

Xylene oxygenase also hydroxylates the methylsubstituents of homo- or heteroaromatic 5- or 6-membered rings (189, 190) and its potential is commercially exploited in the conversion of dimethylpyrazine to 5-methyl-2-pyrazinecarboxylic acid by LONZA (233). It has been suggested to perform the hydroxylation of indole on the way to the microbial formation of indigo (232). Moreover, it enantioselectively epoxidizes the vinyl-substituent of styrene or its substituted derivatives (395, 398). It produced (S)-styrene oxide with an e.e. of 92 % and substituted derivatives with varying e.e.'s: 3-chlorostyrene was epoxidized with an e.e. >96 %, 3- and 4-methylstyrene with an e.e. >95 %, styrene with 92 %, and 4-chlorostyrene with an e.e. of 37 % (395). When the enzyme was presented with methyl- and vinyl-substituent on the same molecule, as in 3- and 4-methylstyrene, the oxidation of the methyl-substituent occurred 5 to 6 times faster than oxidation of the vinyl-substituent.

Alkane hydroxylase and xylene monooxygenase are examples of alkane monooxygenases, which oxidize methyl- as well as vinyl-functions (147). Alkene monooxygenases attack only vinyl-functions. This was shown in studies with the alkene monooxygenases of Nocardia corallina B-276 and Xanthobacter sp. strain Py2.
b. 1.2. Routes to olly active styrene oxide starting either from prochiral styrene or from racemic styrene oxide.

**catalysis**

<table>
<thead>
<tr>
<th>Antimer</th>
<th>Microorganism/Enzyme</th>
<th>e.e. oxide</th>
<th>Yield</th>
<th>e.e. diol</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>xylene oxygenase</td>
<td>92</td>
<td>n.m.</td>
<td>n.a.</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td>styrene monoxygenase</td>
<td>&gt;99</td>
<td>n.m.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>Chapter 2</td>
</tr>
<tr>
<td>P. putida S12</td>
<td>&gt;98</td>
<td>n.m.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>(257)</td>
</tr>
<tr>
<td>Mycobacterium sp. strain E20</td>
<td>98</td>
<td>n.m.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>(257)</td>
</tr>
<tr>
<td>N. corallina B-276</td>
<td>69</td>
<td>n.m.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>(116)</td>
</tr>
<tr>
<td>chloroperoxidase</td>
<td>49</td>
<td>89</td>
<td>n.a.</td>
<td>n.a.</td>
<td>(85)</td>
</tr>
<tr>
<td>cytochrome P450&lt;sub&gt;cam&lt;/sub&gt;</td>
<td>66</td>
<td>n.m.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>(111)</td>
</tr>
<tr>
<td>human myoglobin (F43Y)</td>
<td>92</td>
<td>60</td>
<td>n.a.</td>
<td>n.a.</td>
<td>(210)</td>
</tr>
<tr>
<td>A. radiobacter</td>
<td>&gt;99</td>
<td>n.m.</td>
<td>n.m.</td>
<td>n.m.</td>
<td>(340)</td>
</tr>
<tr>
<td>A. niger</td>
<td>99</td>
<td>28</td>
<td>65</td>
<td>50</td>
<td>(282)</td>
</tr>
<tr>
<td>A. niger</td>
<td>100</td>
<td>32</td>
<td>n.m.</td>
<td>n.m.</td>
<td>(56)</td>
</tr>
<tr>
<td>B. bassiana</td>
<td>98</td>
<td>34</td>
<td>83</td>
<td>45</td>
<td>(282)</td>
</tr>
<tr>
<td>R. glutinis</td>
<td>98</td>
<td>18.5</td>
<td>48</td>
<td>n.m.</td>
<td>(384)</td>
</tr>
<tr>
<td>rabbit liver mEH</td>
<td>&gt;98</td>
<td>22</td>
<td>25</td>
<td>77</td>
<td>(28)</td>
</tr>
</tbody>
</table>

**chemical catalysis**

<table>
<thead>
<tr>
<th>Antimer</th>
<th>Catalyst</th>
<th>e.e. oxide</th>
<th>Yield</th>
<th>e.e. diol</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jacobsen epoxidation</td>
<td>57</td>
<td>75</td>
<td>n.a.</td>
<td>n.a.</td>
<td>(407)</td>
</tr>
<tr>
<td>Jacobsen epoxidation&lt;sup&gt;a&lt;/sup&gt;</td>
<td>86</td>
<td>89</td>
<td>n.a.</td>
<td>n.a.</td>
<td>(273)</td>
</tr>
<tr>
<td>BINAP-porphyrin</td>
<td>48</td>
<td>67</td>
<td>n.a.</td>
<td>n.a.</td>
<td>(131)</td>
</tr>
<tr>
<td>tetraphenylporphyrin</td>
<td>52</td>
<td>90</td>
<td>n.a.</td>
<td>n.a.</td>
<td>(138)</td>
</tr>
<tr>
<td>ruthenium carbonyl porphyrin</td>
<td>70</td>
<td>79</td>
<td>n.a.</td>
<td>n.a.</td>
<td>(30)</td>
</tr>
<tr>
<td>Jacobsen resolution (Co)</td>
<td>98</td>
<td>38</td>
<td>98&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(560)</td>
</tr>
<tr>
<td>Jacobsen resolution&lt;sup&gt;1&lt;/sup&gt; (Cr)</td>
<td>98</td>
<td>24</td>
<td>n.m.</td>
<td>n.m.</td>
<td>(225)</td>
</tr>
</tbody>
</table>

1. Oxygenation; 2. Hydrolysis; b) n.a., not applicable; n.m., not mentioned; c) product: phenylethanediol, only applicable for kinetic resolutions; d) mEH, microsomal epoxide hydrolase; e) modified catalyst structure, different dant; f) after recrystallization
The B-276 enzyme consists of the two-polypeptide epoxygenase component AmoAC, a reductase component AmoD, and a coupling protein AmoB which is necessary but of which the function is not well understood (237, 310). The epoxygenase has two subunits and contains 2 mol iron per mol epoxygenase (237). EPRS measurements indicated a bridged dimuclear iron center (120) and sequence alignment with the α-subunit of the soluble methane monooxygenase suggested that the diiron center is oxo-bridged in its ferric state (FeIII-FeIII) and coordinated by two histidines, four glutamates, and two water molecules. This is similar to the well studied hydroxylase component of soluble methane monooxygenase (72, 121).

While methane monooxygenase usually does not induce significant degrees of optical activities (387), probably due to a less constrained substrate binding site (121), the purified B-276 alkene monooxygenase produces (R)-propylene oxide with an e.e. of 83%. Whole cells of _N. corallina_ B-276 were used to enantioselectively epoxidize a variety of alkenes (for a survey, see (115)).

The alkene monooxygenase of _Xanthobacter_ sp. strain Py2 is a complex enzyme consisting of six different polypeptides in four separable components (337): a monomeric reductase, a ferredoxin, an αβγ2 structured epoxygenase, and a coupling protein is required for full activity. The ferredoxin transfers electrons from the reductase to the epoxygenase component. The latter contains two iron atoms per αβγ protomer. The coupling protein is necessary for full activity but its function is not understood. Sequence comparisons and secondary structure modeling for one polypeptide of the oxygenase component, XamoA, indicated a diiron-carboxylate center probably coordinated via two histidines and four glutamates (408). The enantioselectivity of the enzyme can be inferred from experiments with whole cells of _Xanthobacter_ sp. strain Py2, which oxidized propylene to (R)-propylene oxide with an enantiomeric excess of 94% (135).

In summary, a bridged diiron center occurs frequently as the cofactor to activate molecular oxygen in nonheme enzymes capable of epoxidation reactions. It is interesting to note that the soluble examples of diiron proteins are of the diiron-carboxylate type (iron coordination via glutamate/aspartate and histidine), while the two membrane bound components of the alkane hydroxylases together with a
membrane bound steaoryl-CoA desaturase use a diiron center, which is coordinated only with histidines (323, 324).

**Heme monooxygenases.** If the catalytic iron in the enzyme is part of a protoporphyrin IX tetrapyrrole system, the resulting prosthetic group is designated a heme group. The iron is coordinated by four nitrogen atoms, one from each of the pyrrole rings, and has two coordination sites left. The molecules that occupy (or not) these coordination sites and the surrounding polypeptide chain that defines the electronic and steric environment of the heme group convey the typical catalytic spectrum to the enzymes.

Monooxygenases that use a heme group usually belong to the class of cytochrome P450s. Such cytochrome P450s were found in almost all types of organisms and are thought to be responsible for the majority of biological hydroxylations, epoxidations, heteroatom oxidations, desaturations, and heteroatom dealkylations (286). Because of their importance in the detoxification of drugs, activation of prodrugs, metabolism of environmental pollutants, and in the solubilization of xenobiotic compounds in mammalian cells, they have been studied intensively (133, 164, 339).

Cytochrome P450s use NADPH or NADH for reducing equivalents and need an electron transport protein to deliver them. Enzymes found in mitochondria and bacterial systems employ an FAD flavoprotein and an iron-sulfur protein for electron delivery. Enzymes found in microsomes usually utilize only one electron transfer protein, a NADPH:cytochrome P450 reductase which contains an FAD and an FMN molecule. An exception is the cytochrome P450-BM3 of *Bacillus megaterium* which consists of only one polypeptide chain with a P450 moiety and an electron transport moiety of the microsomal type. Bacterial cytochrome P450s are soluble while the eucaryotic enzymes tend to be linked to membranes (339).

The catalytic cycle of cytochrome P450s was studied in detail (339, 372). For epoxidation reactions two reactive species are discussed: an iron-oxo species (164) or an iron-hydroperoxo species (372).

A cytochrome P450 dependent styrene monooxygenase was found in the black yeast *Exophiala jeanselmei* (65, 66). It converted styrene to styrene oxide, had a broad substrate range and was a part of the organism’s styrene degradation
pathway. The enantioselectivity of the reaction was not investigated. Styrene is converted by mammalian liver cytochrome P450s to styrene oxide with a moderate enantiomeric excess (107, 380). The \textit{P. putida} cytochrome P450\textsubscript{cam} converts styrene to (S)-styrene oxide with an e.e. of 66\% and also epoxidizes cis-\(\beta\)-methylstyrene but the specific activities are low (111). Benzaldehyde is formed as a major byproduct due to uncoupling of the NADH reduction from product formation which leads to the formation of hydrogen peroxide.

Epoxidation reactions were also found with heme containing enzymes other than members of the cytochrome P450 group. Although in a few cases promising e.e.'s were reported, styrene oxide formation by the dioxygen transport protein sperm whale myoglobin and a human myoglobin mutant enzyme were only low (210, 297).

\textbf{Flavin monooxygenases.} Four two-component styrene monooxygenases were recently identified in \textit{Pseudomonas} sp. strains, the amino acid sequences of which have more than 90\% sequence identity to each other (29, 373, Chapter 2)). The amino acids of the epoxidizing components and physiological data from styrene oxidation in cell-free extracts indicated that the enzymes utilized FAD rather than a diiron center or a cytochrome as the cofactor (148, Chapter 2). The sequences of the second components did not indicate the presence of a cofactor as it would be expected for a typical electron transfer protein. However, their presence was necessary for full activity (Chapter 2). The styrene monooxygenase of \textit{Pseudomonas} sp. strain VLB120 epoxidized styrene to (S)-styrene oxide with an e.e. of more than 99\% (Chapter 2). The styrene monooxygenase of \textit{P. putida} S12 produced (S)-styrene oxide with an e.e. of more than 98\% (257) and also epoxidized 4-chloro- and 3-methylstyrene with e.e.'s >98\%. The substrate spectrum of the styrene monooxygenase of \textit{P. fluorescens} ST was investigated after synthesis in \textit{E.coli}; it epoxidized heteroaromatic styrene analogues, \(\alpha\)-methylstyrene, and 2,3-trans-disubstituted styrenes. 1,2-Dihydronaphthalene was epoxidized to a small extent. Product isolation led to the hydrolyzed diols in the case of \(\alpha\)-methylstyrene and 1,2-dihydronaphthalene (86). The obtained epoxides were optically pure, while hydrolysis to diols was not stereospecific.
The amino acid sequences of the epoxidizing components of the styrene monooxygenases of *P. fluorescens* ST (29), *P. putida* S12 (GenBank accession number Y13349), and the one of *Pseudomonas* sp. strain Y2 (373) share between 94.7 and 99.3% sequence identity with the epoxidizing component of *Pseudomonas* sp. VLB120 (Chapter 2). It will be interesting to see if there are differences in the substrate spectrum of the enzymes, and if yes, whether and to what extent the differences can be rationalized by the minor differences in amino acid sequences.

### 1.2.1.1.2. Miscellaneous enzymes

In the majority of cases the prosthetic group of peroxidases consists of an iron-protoporphyrin IX moiety. Their catalytic mode is thought to be similar to that of cytochrome P450s as they also involve the so-called oxoferryl species (365). However, peroxidases oxidize organic substrates to free radicals. This prevents hydroxylation as in the case of the cytochrome P450s (286). Haloperoxidases can add hypohalous acid to an olefinic double bond, which leads to the formation of racemic α-halohydrins. The chloroperoxidase of the fungus *Caldariomyces fumago* has as a fifth heme ligand a cystein-thiolate instead of the histidine imidazol, which is usually found in peroxidases. In agreement with this cytochrome P450-like property it epoxidized styrene in the absence of halogenide ions (123, 270) with an e.e. of 49% and a chemical yield of 89% (85). Chloroperoxidase also epoxidized cis-2,3-disubstituted and 1,1-disubstituted alkenes with e.e.'s between 12 and 95% and chemical yields between 1 and 100% (3, 85). The epoxides of *m*- or *p*-substituted styrene derivatives were of the *R* configuration with moderate e.e.'s and yields, and a number of byproducts were formed because of the aggressive oxidant used (hydrogen peroxide or *tert*-butylperoxide) (61). Furthermore, chloroperoxidase enantioselectively oxidizes thioethers (327).

Horseradish peroxidase and cytochrome *c* peroxidase epoxidized styrene only with low rates (235, 314).

One strategy that might be useful in the long run in asymmetric synthesis is the application of catalytic antibodies. So far, single reactions have been scaled up to
the gram scale (301). One antibody was able to incorporate oxygen into the double bond of a number of aliphatic alkenes with e.e.’s between 67 and >98 %. The oxygen was derived from peroxy carboximidic acid, which had been formed in situ from acetonitrile and hydrogen peroxide. This procedure turned out to be mild enough to maintain the functionality of the antibody (192).

1.2.1.2. Racemic epoxides as substrates for enantiospecific resolutions

Asymmetric synthesis and desymmetrization of meso-compounds represent only a minority of the reports on the production of enantiopure compounds. They are outnumbered by reports on kinetic resolutions by approximately 1:4 (352), which illustrates the attractiveness of this approach. All classes of organisms possess epoxide hydrolases that efficiently kinetically resolve racemic preparations of epoxides to the corresponding vicinal diols (17-19, 106). Vicinal diols are also chiral and the resulting diol product is frequently optically active. Enantiospecific epoxide hydrolysis was also achieved with catalytic antibodies (336).

Due to its role in the detoxification of xenobiotics, mammalian liver microsomal epoxide hydrolases were intensively studied. They accept a wide variety of (hetero)alicyclic epoxides as substrates with frequently very good e.e.’s for the remaining epoxide (19). Rabbit liver epoxide hydrolase prefers the R enantiomer of styrene oxide as a substrate. However, in order to obtain excellent e.e.’s for the remaining epoxide, the reaction has to go on to 80 % conversion, which compromises the utility and the e.e. of the resulting diol (28). (±)-cis-β-Methyl styrene oxide was resolved with excellent e.e.’s leaving behind pure (1S,2R)-1-phenylpropylene oxide. (±)-trans-β-Methyl styrene was hydrolyzed considerably less specifically (28). Expanding the methyl group to ethyl and propyl groups still allowed to isolate the remaining epoxides in excellent e.e.’s (27).

Rat microsomal hydrolase was synthesized in E. coli (16, 25). It served as a model to study the reaction mechanism of these enzymes: an aspartic acid residue attacks the epoxide group and forms a covalent ester intermediate, which is resolved by
water. This is facilitated by a mechanism similar to the catalytic triad known from proteases (17).

Obviously, although these enzymes are interesting from a synthetic point of view, mammalian epoxide hydrolases are difficult to obtain in larger quantities unless they become available from recombinant microorganisms, which is so far rarely the case. In contrast, epoxide hydrolases of microorganisms are readily available. Bacterial epoxide hydrolase efficiently resolve racemates of substituted aliphatic epoxides (17, 377) but epoxides with aromatic substituents are frequently bad substrates (17). However, the epoxide hydrolase of Agrobacterium radiobacter obtained from recombinant E. coli resolved various racemic mixtures of styrene oxide derivatives with excellent e.e.'s for the remaining S enantiomer but moderate chemical yield (27-36 %) (340).

Epoxide hydrolases are also widespread in fungi where pairs of hydrolases with opposite enantiospecificities are available frequently from screens of only a moderate number of strains (<100) (19). The resolution of (substituted) styrene oxide was investigated in considerable detail by the group of Furstoss. The two fungi Aspergillus niger and Beauveria bassiana enantiocomplementarily resolve racemic styrene oxide with excellent e.e.'s; the A. niger enzyme leaves the S enantiomer, the B. bassiana enzyme the R enantiomer (282).

The substrate spectra of two hydrolases differ significantly: the A. niger enzyme did not transform racemic epoxymdene, epoxydihydronaphthalene, or β-methylstyrene derivatives with a substitution at the β-carbon atom, the B. bassiana enzyme did (279, 280). Although both preparations readily resolved racemates of para-substituted styrene oxide derivatives, A. niger did so with excellent e.e.'s (>98 %), while the e.e.'s obtained with B. bassiana varied (282). The substrate spectrum of the A. niger enzyme was further investigated and included various glycidyl phenylethers, of which the R enantiomer remained with e.e.'s >99 % (56). The yeast Rhodotorula glutinis resolved racemic styrene oxide leaving behind the S enantiomer with an e.e. of >98 %. The e.e. of the diol was unfortunately low. However, the e.e.'s of both, (1R,2R)-1-phenylpropylene oxide and the corresponding 1R,2S diol were excellent when the racemic α-methylstyrene oxide was used (384).
A number of cases have been reported in which the limited chemical yield of kinetic resolutions could be increased to more than 50% (26, 27, 198, 199, 269). For example, the treatment of racemic p-nitrostyrene oxide with an enzyme preparation of *A. niger* led to (S)-p-nitrostyrene oxide and the corresponding R diol (see above). When the mixture was subjected to controlled acid hydrolysis, the S epoxide was converted into the R diol. Thus, the R diol was obtained in a chemical yield of 94% and 80% e.e. to serve as the basis for the synthesis of the β-blocker Nifénalol (281). The same effect was obtained with the epoxide hydrolases of *A. niger* and *B. bassiana* which transform different styrene oxide enantiomers but produce the same R diol with a chemical yield of 92% and an e.e. of 89% (278).

Although the results obtained with epoxide hydrolases are frequently very good with respect to the e.e. of the remaining epoxide enantiomer, they are sometimes compromised with respect to the e.e. of the resulting diol. Spontaneous hydrolysis of the racemic epoxide might lead to lower e.e.'s than expected. Moreover, the e.e. of the diol that resulted from the attack of the hydrolase does not depend only on the enantiospecificity of the hydrolase (i.e., to what extent it prefers one enantiomer or the other) but also on its regiospecificity (which of the two carbon atoms of the oxirane function is attacked) (236, 243).

Two more reactions are known from bacterial enzymes that use epoxides as the substrate. In bacteria that grew with styrene as the only carbon source via the formation of styrene oxide, the oxide was isomerized to phenylacetaldehyde by a styrene oxide isomerase. This enzyme exhibited only a small preference for the S enantiomer (176, 257, Chapter 2).

*N. corallina* B-276 and *Xanthobacter* sp. strain Py2 used a carboxylation step to transform propylene oxide which was formed when the strains grew with propylene to the corresponding β-ketoacid (4, 6, 338). In *Xanthobacter* sp. strain Py2, this step depended on CO₂, NADPH, and NAD⁺ (7, 358). The epoxide carboxylase consisted of four components, the first of which was assumed to contain the epoxide binding site (5). The second has been hypothesized to function as a pyridine nucleotide-disulfide oxidoreductase that reduces an active site disulfide (7, 358). Components 3 and 4 belong to the group of short-chain dehydrogenases/reductases and were involved in the enantiospecific degradation...
of the two propylene oxide enantiomers; reconstituted enzyme that consisted of components 1, 2, and 3 could carboxylate (R)- but not (S)-propylene oxide, while reconstituted enzyme that consisted of components 1, 2, and 4 carboxylated (S)- but not (R)-propylene oxide (8). A similar system operates in N. corallina B-276 (8). These enzymes are unusual since they form one enzyme that is completed by different components depending on the stereoconfiguration of the substrate.

1.2.1.3. Other ways to enantiopure epoxides

Because of the synthetic potential of single enantiomer preparations of epoxides, a number of alternative approaches were developed. In the center of these approaches is the formation of an enantiopure halohydrin, i.e., a hydroxy group on an asymmetric carbon atom with a halo-substituted carbon atom in α-position. This functional moiety can be easily modified in a stereocontrolled manner in basic conditions, so that the oxygen of the hydroxy group closes the oxirane ring and the halo-substituent leaves the molecule. Single enantiomer preparations of halohydrins can be obtained by enantioselective microbial reduction of prochiral α-haloketones. Pioneering results were provided by Imuta et al. (171), who obtained (R)-styrene oxide with 95% e.e. by reducing the corresponding α-bromoketone with Cryptococcus macerans. The versatility of this method was demonstrated by producing all stereoisomers of β-methylstyrene oxide from 1-phenyl-2-propanone and 1-phenyl-1-propanone after chlorination (31). Kaneka produces the two styrene oxide enantiomers in this way: starting out from α-chloroacetophenone, either Candida humicola or R. glutinis is used to obtain the S or the R halohydrin, respectively, which is then converted, with a chemical yield of 99%, to (S)- or (R)-styrene oxide (393). Unfortunately, no data on yields and productivities are available for this process.

Another dehydrogenase based strategy to obtain a single enantiomer preparation of a halohydrin has been proposed for the production of the HIV protease inhibitor crixivan by Merck. A bromoperoxidase in a crude enzyme extract of Curvularia protuberata MF4500 produced racemic bromoindanol from indene, which was kinetically resolved by a dehydrogenase that was also present in the extract. As a
result, \((1S,2S)-\text{bromoindanol}\) with an e.e. of 80 % was obtained, which could be easily converted to the \(1S,2R\) epoxide, which in turn can serve as a building block for the production of crixivan (406).

BASF uses a lipase based route to obtain a single enantiomer preparation of the chlorohydrin that is used to form \((R)\)-styrene oxide; racemic 2-chloro-1-phenylethanol is kinetically resolved by esterification of the unwanted halohydrin isomer with vinyl propanoate in the presence of an “activated lipase”. The remaining \(R\) enantiomer is then treated with potassium hydroxide to obtain \((R)\)-styrene oxide.

### 1.2.2. Chemical Catalysts

One important reaction in the area of asymmetric oxidation is the Sharpless epoxidation with which a wide variety of allylic alcohols can be converted to epoxides with e.e.’s typically over 90 % and chemical yields between 70 and 90 % (122). A single enantiomer preparation of a titanium-tartrate complex is used as the catalyst with \(\text{tert-butyldi}c\text{holperoxide}\) as oxygen donor in water-free conditions. As both enantiomeric forms of the catalyst are available, both enantiomers of the product can be formed in optically active form. The Sharpless epoxidation is exploited commercially in the production of \((R)\)- and \((S)\)-glycidol by ARCO, of \((2S,3S)\)-epoxyoctanol by Upjohn, and in the synthesis of an intermediate for the insect pheromone disparlure (327).

The presence of an asymmetric center at C1 of the allylic alcohol (the carbon atom that carries the hydroxy-group) is usually enough to interfere with the proper alignment of one of the substrate enantiomers with the Sharpless catalyst, which leads to fundamentally different reaction rates of the two enantiomers. Thus, the Sharpless catalyst can also be used for kinetic resolutions (178).

Of similar synthetic value is the Sharpless dihydroxylation. Osmium tetroxide complexed with cinchona alkaloid-based ligands is used to stereospecifically form a \(\text{cis}\) diol from essentially any type of alkene, again in both enantiomeric forms depending on the chirality of the catalyst (195). The e.e’s vary depending on the substrate between 20 and more than 99 %, for some alkene classes even more than
90% can be reached reliably. Only for 1,2-disubstituted cis-alkenes does the e.e. not exceed 80%. Cyclic alkenes are frequently poor substrates, and good chemical yield with only poor induction of asymmetry is common (195, 348).

The Jacobsen/Katsuki epoxidation is a valuable complementation of the Sharpless epoxidation (407). Chiral manganese(III)-salen complexes are utilized with (in today’s version of the catalytic protocol) sodium hypochlorite as the stoichiometric reagent to donate the oxygen for epoxidation of different alkenes. Very good e.e.’s can be obtained with cis-alkenes conjugated with aryl, acetylenic, or alkenyl groups (19, 177, 348). Styrene is epoxidized with an e.e. of up to 57% (177). However, trans alkenes are bad substrates and for truly excellent e.e.’s the substrates should be conjugated (19).

A major progress was the recent report on hydrolytic kinetic resolution of simple olefins with a modified Jacobsen catalyst, a cobalt(III)-acetate-salen complex. Excellent e.e.’s were obtained for the retained epoxide and the formed diol. Chemical yields were frequently greater than 40% (360). Styrene oxide was resolved and the remaining enantiomer had an e.e. of 98%. Even more important, the procedure gave excellent results for the resolution of synthetically important three carbon units like propylene oxide and epichlorohydrin.

Apart from these major discoveries, a large number of alternative systems play a role in asymmetric oxidations. Most prominent among them are biomimetic approaches; metalloporphyrins for example are used for chemical epoxidations (32). Chemoselectivity in these systems is influenced by the combination of metals with available ligands, enantioselectivity by selecting chiral ligands. Frequently used are binaphthyl (BINAP) and “basket handle” metalloporphyrins, whose nomenclature refers to substituents of the tetrapyrrrol backbone. These substituents should direct access of the substrate to the catalytic center (32). A whole variety of substituent patterns were tried (177). Preferred substrates are cis-alkenes. Other ligands than the tetrapyrrrol system to complex the central metal were investigated, however only with limited success (177, 348).

A number of researchers have adopted indirect ways relying on the same central intermediates as discussed in the biocatalytic section, namely halohydrins. Instead of using enzymes, stoichiometric amounts of an enantiopure chiral reagent or
catalytic amounts of a chiral catalyst are used. Again, a frequently used approach is the enantioselective reduction of an α-haloketone.

Using chiral binaphthol-modified lithium aluminium hydride in stoichiometric amounts, α-bromoacetophenone could be enantioselectively and quantitatively converted to the corresponding bromohydrin from which (S)-styrene oxide was obtained by treatment with NaOH with an e.e. of 95 % and a chemical yield of 97 % (258). Similarly, α-haloacetophenones can be enantioselectively reduced with alpine-boran (obtained from enantiopure α-pinene) with e.e.’s between 86 and 89 % (46). As an alternative, a chlorohydrin leading to (S)-styrene oxide can be obtained starting from α-chloroacetophenone by reduction with borane in the presence of a oxazaborolidine derived chiral catalyst in 96 % yield and with 96.5 % e.e. (62).

Instead of having the halo atom in α-position of the phenyl- and hydroxysubstituted asymmetric carbon atom, it can also be the hydroxygroup in α-position and the halogroup at the asymmetric carbon atom; for example, reaction with diethylzinc in the presence of a chiral β-aminoalcohol allowed to kinetically resolve (already chiral) 2-chlorophenylacetaldehyde, which resulted in (S)-2-chlorophenylethanol, from which (R)-styrene oxide could be obtained, however with only moderate e.e. (152).

Other options are available to introduce chirality into a functional analogue of the halohydrin, which expands the available chemical tool box for indirect routes to enantiopure styrene oxides considerably.

1.3. Process development for oxygenase-based routes to fine chemicals

1.3.1. Epoxide production with whole cells

1.3.1.1. In situ removal techniques

The majority of potentially interesting substrates and products for or from biotransformations is poorly soluble in water and/or toxic to living cells (209, 213, 255, 311). This represents a major challenge for biochemical reaction engineering as
Tab. 1.3. *In situ* product recovery techniques (adapted from 108)

(i) Evaporation via vacuum fermentation, gas stripping, or pervaporation: this is effective mainly for volatile low molecular weight molecules like ethanol, butanol, or short-chain epoxides.

(ii) Size selective permeation based on membrane dialysis.

(iii) Reversible complex formation, based on a chemical reaction with a soluble reagent (e.g. Schiff's base), or biological recognition, leading to soluble or insoluble complexes.

(iv) Product immobilization via adsorption or specific binding onto water-insoluble polymeric carriers.

(v) Extraction into another phase, for example water immiscible solvents, supercritical fluids, or second aqueous phases.

Whole cells are adapted to aqueous environments and process efficiency may be low in the presence of toxic chemicals. The toxicity of the product might be reduced by *in situ* product removal, that is, the toxic product is removed from the aqueous phase before it can accumulate to levels that impair proper functioning of the biocatalyst. The technology depends on the presence of a second phase in the reaction system that receives the product from the aqueous phase in which it is formed. Which technique is suitable for a system depends on the chemical and biochemical characteristics of the product (108).

Of the possibilities shown in Tab. 1.3, only the first and the last technique had a major impact on process development for biochemical epoxide production. Short chain epoxides (ethylene and propylene oxide) are reasonably well soluble in water, can be stripped out of the aqueous reaction medium with the airflow, and can be later collected by washing the gas stream with organic solvents (40).

Epoxides of a higher molecular weight are liquid. Depending on the toxicity of the corresponding olefinic substrates, they can be added as a second liquid phase or have to be diluted into a biologically inert carrier solvent phase (see below). Two-liquid phase technology was chosen for this work as the production method for styrene oxide. It will be discussed in more detail in the next section.
1.3.1.2. TWO-LIQUID PHASE CULTIVATIONS

1.3.1.2.1. TWO-LIQUID PHASE CULTIVATIONS AND CHEMICAL REACTION ENGINEERING

Two-liquid phase systems can consist of an aqueous and an organic or two aqueous phases. In the latter case, each of the two phases has dissolved a different polymer (e.g. dextran and polyethylene glycol). This leads to phase separation (11). For the context of the present work, the term two-liquid phase cultivation is meant to imply an aqueous and an organic phase, as only this system was used in biochemical whole-cell oxidations.

Microbial cells and enzymes are usually surrounded by an aqueous environment to which they are well adapted. However, water is a problematic reaction media for organic synthesis: apart from the fact that most of today’s organic chemicals are poorly water soluble, water has a high heat capacity and a high boiling point which makes product isolation difficult; the synthetic power of hydrolytic equilibrium reactions like esterifications cannot be exploited; and its polar nature makes it a good nucleophile (214). This has led to the investigations of aqueous-organic two-phase systems. Influences of two-liquid phase technology on a bioprocess are multifold. They are summarized in Tab. 1.4.

If the properties of substrate or product make a two-phase process necessary, than the choice of the organic phase is of importance. This choice is influenced by a variety of parameters, such as toxic or inhibitory effects of the solvent on the cells (see below), solvent capacity for substrate and product, partition coefficients of substrate and product, degradability of the solvent by the biocatalyst, flammability and toxicity to operators, density difference to water, boiling point, and, finally, price.

1.3.1.2.2. MICROBIAL CELLS IN THE PRESENCE OF ORGANIC SOLVENTS

In 1975, Buckland *et al.* reported the formation of cholestenone from cholesterol in carbon tetrachloride by a *Nocardia* sp strain (48). Since then, much effort has gone
Tab. 1.4. Advantages and disadvantages of two-liquid phase technology (adapted from 43, 213, 255, 311, 362, 371)

<table>
<thead>
<tr>
<th>Advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) The substrate concentration of an apolar substrate in a reactor can be increased: If a substrate is apolar and not toxic to the cells, it can be added directly to the reactor as a separate second phase, which facilitates process design. If the substrate is toxic, it can be diluted into an organic and nontoxic carrier solvent and then be added as a second liquid phase.</td>
</tr>
<tr>
<td>(ii) The same is true for an apolar product: concentrations of an apolar product that is extracted into an already present organic phase - which might consist of the substrate - will exceed the aqueous solubility of the product.</td>
</tr>
<tr>
<td>(iii) Dilution of a toxic substrate or product into a nontoxic carrier solvent reduces their aqueous concentration and might thus relieve substrate or product inhibition.</td>
</tr>
<tr>
<td>(iv) In equilibrium reactions, the partition behavior of substrate and product can be exploited; a reaction can be pushed or pulled into the product side because the substrate is permanently available in excess (large substrate reservoir in the organic phase) or the product is continuously extracted (organic phase as a product sink).</td>
</tr>
<tr>
<td>(v) The extraction of the product into the organic phase might prevent it from hydrolysis or another attack by polar molecules.</td>
</tr>
<tr>
<td>(vi) <em>In situ</em> product extraction into a separate phase might facilitate downstream processing.</td>
</tr>
<tr>
<td>(vii) Oxygen has a higher solubility in organic solvents than in water, so oxygen transfer of the process might be improved.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Many organic solvents are, at least to a certain degree, toxic to cells. Thus, the typical low molecular weight solvents of organic chemical synthesis cannot be applied.</td>
</tr>
<tr>
<td>(ii) Solvents, the necessary safety measures against exposure of the operator and explosion, and waste disposal or solvent recycling mean additional costs.</td>
</tr>
<tr>
<td>(iii) Downstream processing might be more difficult if large amounts of amphiphilic substances are formed and a stable emulsion is formed.</td>
</tr>
<tr>
<td>(iv) Two-liquid phase cultures might become mass transfer limited.</td>
</tr>
</tbody>
</table>
into understanding the effects of organic solvents on microorganisms and how to exploit such systems.

Different parameters have been used to predict the effect of a particular organic solvent on cells when the solvent formed a separate liquid phase. In a study covering 34 organic solvents, Brink and Tramper concluded that a solvent with a low Hildebrand solubility parameter ($\delta < 8 \text{(cal}^*\text{mL}^{-1})^{0.5}$), which indicates a low solvent polarity and a high molecular weight (>200), has little toxic effect on a *Mycobacterium* strain that epoxidized propylene and 1-butene (41). However, the predictive power of the Hildebrand criterion remained limited (41).

Laane and coworkers established the logarithm of the partition coefficient of a compound in a standard octanol/water system to predict microbial activity retention in two-liquid phase systems with whole cells.

$$\log P = \log_{10} \frac{[\text{compound}]_{\text{octanol}}}{[\text{compound}]_{\text{aqueous}}}$$

Plotting the retention of activity against logP, they found S-shaped curves (203, 293). Solvents with a logP <2 generally inactivated the tested biological systems and solvents with a logP >4 lead to minimal or no inactivation. They also found this relation for the activity of isolated enzymes in a variety of solvent/aqueous buffer systems (204). The logP concept was successful as a rough way to predict the biocompatibility of solvents for whole cell applications; a large data set from previous experiments which involved different classes of solvents and a method to reasonably accurately calculate missing logP values from hydrophobic fragmental constants is available (299).

However, the logP provides only a rough criterion. For a given bacterium, the critical logP value above which solvents can be considered nontoxic might be a function of the applied medium and of the class of solvents tested (e.g. alkenes or alkylbenzenes) (293, 374). One solvent with a logP between 3.0 and 5.0 might have different effects on different microorganisms. In general, Gram-negative bacteria, and there particularly bacteria of the genus *Pseudomonas* (293), appear to be more resistant to such solvents than Gram-positive bacteria (145, 172). Finally,
some solvents are toxic to cells although their logP is greater than 5 which means theoretically that the solvent is not toxic. For these solvents, additional toxicity mechanisms apply (293).

Activity retention of *Morinda citrifolia* plant cell cultures in the presence of organic solvents was also investigated and resulted in the S-shaped curves that were already known from bacterial cells. The logP above of which the solvents were nontoxic was 5 (24).

Two types of toxicity might occur with a separate organic phase. On the one hand, the solvent molecules that are dissolved in the aqueous phase can interfere with the proper functioning of the cell. This aspect is called molecular toxicity.

The presence of a separate organic phase might exert an additional detrimental effect, which is termed phase toxicity (22). Reasons for phase toxicity include depletion of essential nutrients as they are extracted from the broth, blockage of nutrient diffusion from broth to cell because of solvent coating, and cell wall disruption as a result of extraction of some outer cellular components (23). Molecular and phase toxicity effects of a solvent were frequently not separated and the rule of thumb that solvents with a logP > 4 are usually not toxic to most microorganisms reflects the combined influences of both toxicities.

The origin of molecular toxicity was intensively investigated. In some cases, the reasons appear to be rather specific; for example, *E. coli* cannot grow in the presence of tetralin phase, presumably because the transformation product tetralin hydroperoxide is toxic to the cell (105).

More general are aspecific toxic effects such as interactions of hydrophobic organic molecules with the hydrophobic parts of the cell (333, 334). Gram-negative cells, which were more profoundly analyzed with respect to resistance to organic solvents, are surrounded by a rather complex cell envelope (140, 254). The outer membrane consists of an almost completely asymmetric bilayer which is studded with a restricted number of protein species that are present in high copy-number. The inner layer of the outer membrane consists of phospholipids with saturated fatty acids, while the outer layer consists of glycolipids or lipopolysaccharides (LPS). In enterobacteria, LPS is a high-molecular weight, strongly negatively
charged molecule. This membrane shows a relatively low permeability to hydrophobic molecules, probably due to the LPS.

The cytoplasmic membrane consists of a phospholipid bilayer in which integral membrane proteins are inserted. It plays a crucial role in selective uptake and excretion of solutes, maintenance of the energy status of the cell, regulation of the intracellular environment, turgor pressure, and signal transduction. The fluidity of the membrane is an important parameter in the cell’s homeostasis.

The membrane is difficult to penetrate for hydrophilic, polar molecules but easy for lipophilic compounds, which tend to accumulate in the cytoplasmic membrane. The accumulation of lipophilic molecules in the phospholipid bilayer causes changes in the structure of the membrane. Compounds that accumulate in the hydrophobic part of the cytoplasmic membrane interfere with the lipid annulus that surrounds membrane proteins and with the interactions of the acyl chains, which leads to an increase in membrane fluidity, eventually to membrane swelling (381).

Amphiphatic molecules interfere with the outer part of the membrane, lead to membrane invagination, and affect the hydration characteristics of the membrane surface. These structural modifications lead to an increased proton efflux impairing the proton motive force of the cell and, indirectly, various cellular functions that make use of the proton gradient over the cytoplasmic membrane (333). This involves transport processes, energy transduction, ATP charge of the cell, and the maintenance of the intracellular pH. A failure to control internal pH will severely disturb the cell’s ability to function properly. Furthermore, membrane thickness, head group hydration, fluidity, and fatty acid composition regulate the activity of membrane enzymes. In summary, accumulation of (partly) lipophilic compounds impairs the function of the membrane as a matrix for embedded proteins and as a selective barrier (333).

The toxic effects are a function of the actual concentration of the compound in the membrane, which is determined by its aqueous concentration at equilibrium. If the aqueous solubility of a given compound is very low (as, for example for hexadecane), the membrane concentration of this compound in a membrane/aqueous buffer system is low as well. As a consequence, compounds with a logP > 5 usually show no molecular toxicity to bacterial cells because these
compounds do not accumulate to toxic levels in the membrane. Compounds with a logP between 4 and 5 give varying results, and compounds with a logP of less than 4 usually lead to a disrupted cytoplasmic membrane (271, 332).

1.3.2. Process developments with oxygenases

Initial efforts to apply biological epoxidation aimed at substituting the chemical multi-step process for the production of the bulk product propylene oxide. In the chemical process, the treatment of chlorine-containing wastes contributed greatly to the overall costs (207). Bacteria that could epoxidize short chain alkenes were readily isolated (for a survey, see 147) and frequently belonged to the genera Mycobacterium, Nocardia, and Xanthobacter. One particular interesting strain was Nocardia corallina B-276 (119). Isolated with ethylene, it converted a wide range of short to long chain terminal alkenes, probably due to the presence of multiple alkane or alkene monooxygenases (119).

The e.e.'s of the epoxidation reactions were moderate to excellent depending on the strain used (116, 135, 387). Short chain epoxides irreversibly inactivated the epoxidizing enzyme in Mycobacterium sp. strains (137). Providing a cosubstrate, such as ethanol or ethylacetate, to Mycobacterium sp. strains led to increased epoxidation rates and stabilities of these activities (136). Providing methanol as a cosubstrate also increased the propylene oxidation rates of Methylococcus capsulatus (Bath) to 550 µmol*(min*g of cell dry weight)*⁻¹ (equivalent to 550 U*(g cdw)*⁻¹), which is the fastest rate reported to date.

However, when the various strains were used to set up continuous production processes, these processes were inefficient (Tab. 1.5); average activities ranged from 3 to 35 U* mol⁻¹ in various types of reactors with immobilized cells and scales between a few mL and 1.7 L (42, 76, 167, 238). Only for a fed-batch process with N. corallina B-276 considerable oxidation rates were reported; at a cell density of 15 g*aL⁻¹, the bacteria reached maximum activities of 280 U*aL⁻¹ (see Tab. 1.5). Glucose served as the substrate to provide reducing equivalents in this experiment (116). The processes summarized above generated gaseous epoxides. In a typical
experiment in a gas-solid bioreactor, the formed epoxides were removed from the cells with the airflow and collected in an organic phase (42, 76, 139, 167).

A propylene oxide production process was designed by Cetus in the beginning of the 1980s which included the use of partially purified oxidoreductases instead of living cells (250). The action of glucose oxidase on glucose delivered hydrogen peroxide, which was used by the *C. fumago* chloroperoxidase to convert propylene into the corresponding chlorohydrin. A halohydrin epoxidase converted the chlorohydrin to propylene epoxide. The process was never commercialized and no data on its productivity are available. It is the current perception that large scale short-chain epoxide production with biological systems is not competitive (207).

Other efforts concentrated on the production of optically active epoxides as fine chemicals. 1-Octene and 1,7-octadiene served as model substrates for the epoxidation of medium and long chain alkenes, primarily with *P. oleovorans* GPol as the biocatalyst. Following the discovery of the reactions (1, 320) and their enantioselectivity (228, 229), this system was intensively investigated (113, 115, 397). Addition of 20 % (vol/vol) of cyclohexane to an aqueous culture of *P. oleovorans* GPol improved the total yield of epoxides from 1,7-octadiene fivefold because it reduced the concentration of the epoxide in the aqueous phase (321). The complexity of the system was reduced when 1-octene served concomitantly as the substrate of the epoxidation reaction and as the second phase to extract the epoxide (83). In this way epoxide concentrations up to 200 mM in the organic phase were obtained after 60 h (82).

*P. oleovorans* GPol could also epoxidize the terminal double bond in prochiral allylethers, which eventually lead to a commercial route for the production of Metoprolol and Atenolol involving the enantioselective epoxidation step to (S)-arylglycidylethers (179). This reaction was also investigated with ethylene and propylene utilizing bacteria (220). Bacteria grown with ethylene produced (S)-phenylglycidylether with e.e.'s over 93 %. A *Mycobacterium* sp. strain M156 was used to investigate the formation of phenyl glycidyl ether in a two-liquid phase system with hexadecane as the second phase. However, the productivity of the reaction remained poor, partly because the product inactivated the enzyme (290).
*N. corallina* B-276 epoxidized unsubstituted terminal alkenes and 2-methyl-1-alkenes in two-liquid phase cultures with good to excellent e.e.’s on scales up to 3 L (116, 118, 359). (R)-1-Epoxytetradecane was produced from tetradecene that concomitantly served as the second liquid phase in a remarkable yield of 65%. *N. corallina* B-276 produced (R)-styrene oxide with an e.e. of 69% (116) in the presence of hexadecane with an average activity of 7 U*(g cdw)^{-1} over 24 h. However, no data are available on yields.

A potentially very promising approach to two-liquid phase styrene oxide production has been developed with a mutant of the solvent resistant styrene degrader *P. putida* S12. This mutant accumulated styrene oxide from styrene (257). The authors claim that they found high rates of styrene oxidation in small scale experiments. Unfortunately, it is not completely clear how they obtained the data and whether these activities can also be obtained in cultivations at higher cell densities or in two-liquid phase cultures.

Optically active styrene epoxides and its substituted derivatives were also produced with *E. coli* recombinants which synthesized xylene oxygenase (398) and were grown in the presence of an organic solvent (395). This process was developed into a high-cell density fed-batch culture with dodecane as the second phase. The culture reached maximum volumetric productivities of 0.3 g of styrene oxide per liter (394).

The data on the various epoxide production processes is summarized in Tab. 1.5. Only reports that described process development beyond mere biocatalyst characterization and optimization were included. The data show that with one exception, all processes have used wild-type or mutated wild-type strains and no recombinant strains. The exception came from our laboratory. Apart from the propylene oxide production process with *N. corallina* B-276, which did not involve a second liquid phase, no process has been reported the productivity of which exceeded 50 U^*L^{-1}. Either processes were performed with only moderately active cells at low cell dry weights or at higher cell dry weights with low specific activities.

This is not a general property of oxidative two-liquid or single-phase phase bioprocesses that incorporate molecular oxygen, as it can be seen from the second
half of Table 1.5. For example, the production of toluene-cis-glycol by *P. putida* UV4 occurred with a productivity of 875 U*L⁻¹* over a period of 6 h at a cell dry weight of 10.5 g*L⁻¹*, which represents the highest productivity for a two-liquid phase process reported in the literature (216). The toluene was diluted into a tetradecane phase to a concentration of 20 % (vol/vol), and the organic phase made up for 30 % of the total liquid volume.

The production of octanoic acid with *E. coli* recombinants that carried the *alk* gene clusters of *P. oleovorans* GPO1 with octane as a second phase could be developed into a 2 L fed-batch high cell density culture. The culture reached a cell dry weight of 40 g*L⁻¹* and a maximum productivity of 10 mmol*(L⁻¹* *h⁻¹* ) (394). Even more productive was the 6-hydroxynicotinic acid production by *Achromobacter xylosoxydans* that is carried out on an industrial scale by LONZA in a single phase culture. It proceeded at an average productivity of 1000 U*L⁻¹* over 12 h with resting cells that are fed only a carbon source for cofactor regeneration but no further medium components (126). For a comparison, available data on selected oxygenative processes are also shown in Tab. 1.5.

1.4. **FUTURE DEVELOPMENTS IN BIOTRANSFORMATIONS WITH OXYGENASES**

1.4.1. **STRATEGIES TO PROTECT CELLS FROM SOLVENT TOXICITY**

Strategies to protect whole cells from the detrimental effects of a solvent can be directed against phase or molecular toxicity. Strategies against phase toxicity aim at separating the cells from the organic solvent. For example, cells can be immobilized in a gel. This has the advantages that go in general with biocatalyst immobilization, such as easy reuse of the biocatalyst, facilitated continuous operations, and dilution rates higher than wash-out conditions (361). Moreover, only solvent molecules that diffuse into that carrier come into contact with the cells. However, mass transfer limitations might occur (40). By including water soluble cosolvents during immobilization that influence the partition coefficient of the substrate, such limitations may be reduced in some cases (213). Alternatively, the cells might be separated from the organic phase via a membrane, for example the wall of silicon
Tab. 1.5. Microbial epoxidation and hydroxylation processes

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>Microorganism</th>
<th>Scale</th>
<th>2nd liquid phase</th>
<th>Max. spec. activity</th>
<th>Cell dry weight</th>
<th>Average (max.) total activity</th>
<th>Product concentration</th>
<th>Process type</th>
<th>Process time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[U g cdw (^{-1})]</td>
<td>[g dry (\text{L}_{\text{dry}})^{-1}]</td>
<td>[U g (\text{L}_{\text{dry}})^{-1}]</td>
<td>[mM]</td>
<td>A</td>
<td>120 (76)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2ene</td>
<td>C2ox</td>
<td><em>Mycob. Py1</em></td>
<td>1.5 L</td>
<td>n.a.</td>
<td>25</td>
<td>n.m.</td>
<td>13</td>
<td>n.a.</td>
<td>A</td>
<td>120 (76)</td>
</tr>
<tr>
<td>C3ene</td>
<td>C3ox</td>
<td><em>Mycob. E3</em></td>
<td>1.7 L</td>
<td>C16</td>
<td>0.2</td>
<td>29</td>
<td>2.7 (5.8)</td>
<td>16</td>
<td>B</td>
<td>48 (42)</td>
</tr>
<tr>
<td>C3ene</td>
<td>(R)-C3ox</td>
<td><em>N. corallina</em></td>
<td>6 mL</td>
<td>paraffin</td>
<td>7.2</td>
<td>3</td>
<td>8.6 (22)</td>
<td>n.a.</td>
<td>B</td>
<td>150 (238)</td>
</tr>
<tr>
<td>C3ene</td>
<td>(R)-C3ox</td>
<td><em>N. corallina</em></td>
<td>8 mL</td>
<td>n.a.</td>
<td>6.6</td>
<td>0.7</td>
<td>4.7 (2.4)</td>
<td>n.a.</td>
<td>A</td>
<td>70 (390)</td>
</tr>
<tr>
<td>C3ene</td>
<td>(R)-C3ox</td>
<td><em>N. corallina</em></td>
<td>2.6 L</td>
<td>n.a.</td>
<td>20</td>
<td>15</td>
<td>15 (280)</td>
<td>n.a.</td>
<td>C</td>
<td>180 (116)</td>
</tr>
<tr>
<td>C3ene</td>
<td>C3ox</td>
<td><em>Methylos. sp.</em></td>
<td>8.5 mL</td>
<td>n.a.</td>
<td>15</td>
<td>2.4</td>
<td>35</td>
<td>n.a.</td>
<td>A</td>
<td>9 (167)</td>
</tr>
<tr>
<td>C8ene</td>
<td>(R)-C8ox</td>
<td><em>N. corallina</em></td>
<td>85 mL</td>
<td>C16</td>
<td>3.4</td>
<td>7.5</td>
<td>12</td>
<td>100</td>
<td>B</td>
<td>192 (182)</td>
</tr>
<tr>
<td>C8ene</td>
<td>(R)-C8ox</td>
<td><em>P. oleovorans</em></td>
<td>50 mL</td>
<td>C8ene</td>
<td>n.m.</td>
<td>n.m.</td>
<td>33</td>
<td>n.m.</td>
<td>B</td>
<td>2000 (370)</td>
</tr>
<tr>
<td>C8ene</td>
<td>(R)-C8ox</td>
<td><em>P. oleovorans</em></td>
<td>1 L</td>
<td>C8ene</td>
<td>n.m.</td>
<td>n.m.</td>
<td>11</td>
<td>40</td>
<td>D</td>
<td>60 (82)</td>
</tr>
<tr>
<td>1.7-C8ene</td>
<td>(R)-1-C8ox</td>
<td><em>P. putida</em></td>
<td>30 mL</td>
<td>1.7-C8ene</td>
<td>3.3</td>
<td>20</td>
<td>8.3</td>
<td>5.5</td>
<td>E</td>
<td>25 (144)</td>
</tr>
<tr>
<td>1.7-C8ene</td>
<td>(R)-1-C8ox</td>
<td><em>P. oleovorans</em></td>
<td>50 mL</td>
<td>C7</td>
<td>10.7</td>
<td>2.5</td>
<td>30</td>
<td>240</td>
<td>F</td>
<td>1000 (90)</td>
</tr>
<tr>
<td>C14ene</td>
<td>C14ox</td>
<td><em>N. corallina</em></td>
<td>3 L</td>
<td>C14ene</td>
<td>3</td>
<td>15</td>
<td>44</td>
<td>400</td>
<td>C</td>
<td>150 (118)</td>
</tr>
<tr>
<td>APE</td>
<td>(S)-PGE</td>
<td><em>Mycob. M156</em></td>
<td>5 mL</td>
<td>C16</td>
<td>6</td>
<td>2.5</td>
<td>2.5 (7.5)</td>
<td>0.6</td>
<td>D</td>
<td>4 (290)</td>
</tr>
<tr>
<td>2-MH</td>
<td>2-MHox</td>
<td><em>N. corallina</em></td>
<td>20 L</td>
<td>C14</td>
<td>1.5</td>
<td>15</td>
<td>12</td>
<td>50</td>
<td>C</td>
<td>72 (359)</td>
</tr>
<tr>
<td>sty</td>
<td>(S)-stox</td>
<td><em>E. coli</em></td>
<td>1.5 L</td>
<td>C12</td>
<td>5</td>
<td>30</td>
<td>22 (37)</td>
<td>7</td>
<td>C</td>
<td>15 (394)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C8</td>
<td>C7COOH</td>
<td><em>E. coli</em></td>
<td>1.5 L</td>
<td>C8</td>
<td>4</td>
<td>42</td>
<td>125 (167)</td>
<td>4</td>
<td>C</td>
<td>35 (394)</td>
</tr>
<tr>
<td>C8</td>
<td>C7COOH</td>
<td><em>E. coli</em></td>
<td>650 mL</td>
<td>C8</td>
<td>2.5</td>
<td>2.3</td>
<td>58</td>
<td>11</td>
<td>G</td>
<td>200 (102)</td>
</tr>
<tr>
<td>C8</td>
<td>C8OH</td>
<td><em>P. putida</em></td>
<td>600 mL</td>
<td>C8</td>
<td>60</td>
<td>1</td>
<td>20</td>
<td>7.7</td>
<td>H</td>
<td>24 (38)</td>
</tr>
<tr>
<td>2,5-DMP</td>
<td>5-MPCOOH</td>
<td><em>P. putida</em></td>
<td>20 L</td>
<td>n.a.</td>
<td>n.m.</td>
<td>n.m.</td>
<td>45</td>
<td>145</td>
<td>C</td>
<td>54 (189)</td>
</tr>
<tr>
<td>Compound</td>
<td>Source</td>
<td>Supplier</td>
<td>Volume</td>
<td>pKa</td>
<td>t1/2</td>
<td>t50</td>
<td>t90</td>
<td>C</td>
<td>2-PP</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>--------------</td>
<td>------------</td>
<td>--------</td>
<td>-----</td>
<td>------</td>
<td>-----</td>
<td>-----</td>
<td>---</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>toluene</td>
<td>TCG</td>
<td><em>P. putida</em></td>
<td>1.7 L</td>
<td>C14</td>
<td>120</td>
<td>10.5</td>
<td>875</td>
<td>450</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>NAP</td>
<td>NCG</td>
<td><em>P. putida</em></td>
<td>75 mL</td>
<td>C12</td>
<td>104</td>
<td>0.4</td>
<td>25</td>
<td>7.5</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>benzene</td>
<td>BCG</td>
<td><em>Pseudom. sp.</em></td>
<td>0.9 L</td>
<td>n.a.</td>
<td>45</td>
<td>0.3</td>
<td>14</td>
<td>9.6</td>
<td>G</td>
<td></td>
</tr>
<tr>
<td>toluene</td>
<td>TCG</td>
<td><em>E. coli</em></td>
<td>5 L</td>
<td>n.a.</td>
<td>35</td>
<td>2.6</td>
<td>17(40)</td>
<td>7.9</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>F-benzene</td>
<td>FC</td>
<td><em>P. putida</em></td>
<td>2.5 L</td>
<td>n.a.</td>
<td>23a</td>
<td>6</td>
<td>140</td>
<td>117</td>
<td>J</td>
<td></td>
</tr>
<tr>
<td>PGT</td>
<td>PGTox</td>
<td><em>A. ochraceus</em></td>
<td>250 mL</td>
<td>OA</td>
<td>2.1</td>
<td>3</td>
<td>2.8(6.3)</td>
<td>7</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td>CTX</td>
<td>CTXox</td>
<td><em>C. lutea</em></td>
<td>75 mL</td>
<td>C8</td>
<td>n.m.</td>
<td>n.m.</td>
<td>2.2(5.5)</td>
<td>n.m.</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>PAOH</td>
<td><em>A. faecalis</em></td>
<td>n.m.</td>
<td>n.m.</td>
<td>n.m.</td>
<td>280</td>
<td>704</td>
<td>C</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>NA</td>
<td>NAOH</td>
<td><em>A. xylosoxydans</em></td>
<td>n.m.</td>
<td>n.m.</td>
<td>1000</td>
<td>719</td>
<td>719</td>
<td>C</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>2-PP</td>
<td>2-PPOH</td>
<td><em>E. coli</em></td>
<td>2 L</td>
<td>n.a.</td>
<td>5.4</td>
<td>6.5</td>
<td>35</td>
<td>31</td>
<td>J</td>
<td></td>
</tr>
</tbody>
</table>

a C2ene. ethylene; C3ene. propylene; C8ene. 1-octene; 1,7-C8ene. 1,7-octadiene; C14ene. 1-tetradecene; APE. allylphenylether; 2-MH. 2-methylhept-1-ene; sty. styrene; C8. n-octane; 2.5-DMP. 2.5-dimethylpyrazine; NAP. napthalene; F-benzene. fluorobenzene; PGT. progesterone; CTX. cortexolone; PA. picolinic acid; NA. nicotinic acid; 2-PP. 2-hydroxyibiphenyl; C16. n-hexadecane; C7. n-heptane; C12. n-dodecane; OA. oleic acid
b C2ox. ethylene oxide; C3ox. propylene oxide; C8ox. 1-epoxyoctane; 1-C8ox. 7-epoxyoct-1-ene; C14ox. 1-epoxitetradecane; PGE. phenylglycidylether; 2-MHox. 1-epoxy-2-methylhexane; stox. styrene oxide; C7COOH. octanoic acid; C8OH. 1-octanol; 5-MPCOOH. 5-methylpyrazinecarboxylic acid; TCG. toluene-cis-glycol; NCG. napthalene-cis-glycol; BCG. benzene-cis-glycol; FC. fluorocatechol; PGTox. 11α-hydroxy progesterone; CTXox. hydrocortisone; PAOH. 6-hydroxypicolinic acid; NAOH. 6-hydroxynicotinic acid; 2-PPOH. 2,3-dihydroxyibiphenyl
d n.m. not mentioned and not indirectly accessible; n.a. not applicable
e A. immobilized cells in gas-solid reactor; B. immobilized cells in two-liquid phase culture; C. fed-batch process in continuously stirred tank reactor (CSTR), includes resting cells with added carbon source but no other medium components; D. shaking flasks or tubes; E. resting cells (no carbon source added) in CSTR; F. continuous process in membrane reactor; G. continuous culture in CSTR; H. batch mode in CSTR; J. fed-batch mode in CSTR, in situ product removal by a solid phase, either in the reactor or in a separate compartment
tubing through which the substrate and the product but not the solvent permeates (89, 90, see section 1.4.2).

Strategies to protect cells from molecular toxicity aim at modifications of the microorganism itself. A pioneering report of Inoue and Horikoshi in 1989 on *Pseudomonas* strain IH-2000 isolated in the presence of a toluene phase demonstrated that bacterial strains can resist the presence of a separate phase of a logP of 2.5 (173). Such strains are termed solvent resistant. For this discussion, a strain will be considered solvent resistant if it can grow in the presence of a second organic phase which is as or less hydrophobic than hexane, i.e. with a logP ≤3.7.

The determinants of solvent resistance were investigated only in Gram-negative strains. However, organic solvent resistance was also found with some Gram-positive bacteria (242, 272). Two factors are discussed to contribute to the solvent resistance phenotype in Gram-negative bacteria: specific solvent extrusion systems and changes in the composition of the outer and the cytoplasmic membrane.

Analogous to drug efflux pumps involved in antibiotic resistance, solvent resistant bacteria appear to be able to actively export solvent molecules from the cytoplasmic membrane across the outer membrane (75). When cells of the styrene degrading *P. putida* S12 were exposed to subsaturating levels of nonmetabolizable toluene, preexposed cells accumulated less radioactively labelled toluene than nonpreexposed cells. The effect could be eliminated by addition of compounds that interfered with the respiratory chain or the proton gradient over the cytoplasmic membrane (174). Furthermore, toluene adapted cells of *P. putida* S12 increased their resistance to a variety of structurally unrelated antibiotics 30 to 1000-fold (175). The genes encoding an energy dependent extrusion mechanism could be cloned from transposon mutants of *P. putida* S12 which could no longer grow in the presence of a toluene-saturated gas atmosphere. The genes *srpABC* encoded a three component proton-dependent efflux pump of the resistance/nodulation/cell division family and complemented the solvent sensitive mutant. Transfer of the genes into a solvent sensitive *Pseudomonas* strain increased to a certain extent the solvent resistance of this strain (188). However, the resulting strain was not resistant to a separate toluene phase.
A similar enzyme mechanism was found to be operative in *P. putida* DOT-T1. This strain accumulated in an energy-dependent fashion less 1,2,4-\[^{14}\text{C}\]-trichlorobenzene in the cytoplasmic membrane when adapted to growth on toluene. A transposon-mutant that could no longer resist a separate toluene phase was found to have lost this extrusion property (295). More detailed analysis revealed two such extrusion systems, one of which was present constitutively and one which could be induced. Inactivation of the constitutively synthesized extrusion system by transposon mutagenesis lead to a mutant which had lost resistance to toluene but not to octanol. Sequencing of the knocked-out gene revealed extended areas of identity to one component of a multidrug efflux pump of the RND family (294).

A second line of evidence for the importance of such solvent extrusion system comes from studies with mutated *E. coli* strains that have acquired the ability to grow in the presence of a cyclohexane phase (logP 3.4). Cyclohexane resistant mutants of *E. coli* JA300 displayed resistance against a set of antibiotics. Mutants selected from *E. coli* JA300 for resistance against these antibiotics showed in turn resistance to cyclohexane. The authors speculated that they had isolated *mar* mutants (multiple antibiotic resistance) (15).

In wild-type strains, the *marR* gene product acts as a repressor of the *marRAB* operon and the *marA* gene product serves as a positive regulator of antibiotic resistance. Missense mutations in *marR* lead to synthesis of MarA and expression of the antibiotic resistance phenotype. As a consequence, overexpression of *marA* but also of each of two more regulatory genes *soxS* and *robA* led to cyclohexane resistance (20, 246, 247).

*SoxS* is involved in the regulation of the superoxide response and activates the multiple members of the *sox* regulon. Overexpression of *robA* led to increased resistance to various antibiotics and heavy metals. Furthermore, *robA* function was found to depend on *soxRS* (246).

It was reported recently that the antibiotic efflux pump AcrAB is efficiently synthesized in *marR* deficient strains (266). In addition, previously cyclohexane resistant strains made deficient in *acrAB* had lost their solvent resistant phenotype, irrespective of the overexpression of either of the three regulatory genes. This
observation provided a rationale for the role of the three regulatory proteins SoxS, RobA, and MarA: apparently, overexpression of each of these regulators led to synthesis of the AcrAB efflux system. Further support comes from the observation that elevated amounts of TolC which forms a trans-envelope efflux system together with AcrAB and AcrA were found in the outer membrane of cyclohexane resistant _E. coli_ strains (14).

Another role in solvent resistance was attributed to the composition of the cytoplasmic and the outer membrane. It was known from _E. coli_ K-12 strains growing in aqueous medium in subsaturating but growth-inhibiting amounts of phenol that they have increased the degree of saturation of the fatty acids in the cytoplasmic membrane. Such an effect is supposed to compensate for an increase of membrane fluidity induced by the insertion of the phenol into the membrane (185). An increase in fatty acid saturation after adding an organic solvent was also observed in the solvent resistant strains _P. putida_ S12 (155) and _P. putida_ Idaho (284). Toluene resistant _P. putida_ DOT-T1 increased the relative amount of cardiolipin and decreased that of phosphatidylethanolamin. The observations were interpreted as rigidifying the cytoplasmic membrane (295). An increased amount of total fatty acids was found in _P. putida_ Idaho (284) as a result of an induced increase in the fatty acid biosynthesis rate after addition of o-xylene, suggesting an increased ability of membrane repair (283).

The most intriguing observation on the level of cytoplasmic membrane lipid composition was made on the level of fatty acid configuration; following exposure to sub- or supersaturating amounts of organic solvents _trans_-unsaturated fatty acids were formed. These fatty acids were first observed in phenol-exposed _P. putida_ P8 (154) and not dependent on _de novo_ fatty acid or protein synthesis. _P. putida_ synthesizes fatty acids via the anaerobic pathway. Double bonds in fatty acid synthesis are formed only by a dehydration of the β-hydroxydecanoyl-acyl carrier protein complex. This implies that the subsequent part of the fatty acid synthesis leads to either 16:1(_cis_ 9) or 18:1(_cis_ 11) fatty acids. As a consequence, the occurrence of 16:1(_trans_ 9) and 18:1(_trans_ 11) fatty acids can only be explained by a direct conversion of the corresponding _cis_ unsaturated fatty acids (186). This was confirmed by experimental observations (88, 295).
The formation of this new type of fatty acids as a consequence of the exposure to organic solvents was observed in all *Pseudomonas* strains that were investigated (54, 154, 284, 295, 382). It was argued that this isomerization led to a decrease in membrane fluidity as a compensation for accumulation of solvent molecules in the cytoplasmic membrane. This was supported by the observation that the temperature for the transition from the gel to the liquid-crystalline state was higher for membranes with *trans* unsaturated fatty acids than for membranes with *cis* unsaturated fatty acids (54, 87, 382).

It has to be pointed out, however, that only one study provided some direct functional evidence of a role of *cis-trans* isomerization in solvent resistance: A transposon mutant of *P. putida* DOT-T1 that was unable to carry out this isomerization failed to grow in the presence of 0.1% (vol/vol) toluene. However, no data on the knocked-out gene were given (295). It cannot be excluded that the loss of *cis-trans* isomerization was a secondary effect of the knock-out of another gene, which was actually responsible for the loss of solvent resistance. Another indirect indication was provided by transposon-mutants of *P. putida* P8 that had also lost the ability to carry out the isomerization and failed to grow at elevated temperatures (166).

Interestingly, *cis-trans* isomerization is found with *P. putida* MW1200 and *P. oleovorans* GPo1, which are not resistant to solvents of a logP below that of hexane (3.9) (54, 284). *o*-Xylene adapted cells of *P. putida* Idaho (i.e., with *trans* unsaturated fatty acids) were not less permeable to the hydrophobic antibiotic difloxacin than nonadapted cells (i.e. without *trans* unsaturated fatty acids), although the cells had a much higher minimal inhibitory concentration in general than the not solvent resistant strain *P. putida* MW1200 (284). This suggested, but did not prove, that *trans*-unsaturated fatty acids did not play a role in antibiotic resistance. These observations might indicate that the influence of *cis-trans* isomerization might be less important, relatively speaking.

*cis-trans* Isomerization has been correlated with other environmental stress factors like elevated concentrations of heavy metals and toxic concentrations of solutes like sodium chloride and sucrose (157) and was suggested as an indicator for toxicity and environmental stress in bioremediation (156).
The genes of the responsible enzyme were cloned from \textit{P. putida} P8 and functionally expressed in \textit{E. coli} and \textit{P. putida} P8, but no data on the solvent resistance phenotype of recombinant strains were provided (166). The enzyme probably uses a cytochrome \textit{c} type heme group for the isomerization (165).

In summary, it is so far not convincingly clear whether \textit{cis-trans} isomerization is only a result of the cell's exposure to solvents or whether the modification in the cytoplasmic membrane serves a purpose in solvent resistance. This information, however, would be necessary to assess a potential role of the enzyme in engineering strains for two-liquid phase applications.

The outer membrane of cyclohexane and \textit{p}-xylene resistant \textit{E. coli} strains was reported to have become more hydrophilic, potentially due to an increase in LPS amounts per cell (14). Pinkart \textit{et al.} investigated the LPS profiles of solvent resistant \textit{P. putida} Idaho and the toluene utilizing but \textit{o}-xylene sensitive \textit{P. putida} MW1200 in varying subsaturating amounts of \textit{o}-xylene (284). They found that the nonsolvent resistant strain produced smooth LPS irrespective of the presence of \textit{o}-xylene, while \textit{P. putida} Idaho produced rough LPS. In the latter strain, one high molecular weight band disappeared with increasing \textit{o}-xylene concentrations and a low molecular weight band emerged. However, functional implications of the rough LPS have not been analyzed.

Ramos \textit{et al.} found that knocking out the \textit{P. putida} DOT-T1 outer membrane protein OprL, which plays a role in maintaining the cell envelope integrity (305), led to cells that were hypersensitive to toluene (295).

A toluene resistant mutant of a \textit{P. aeruginosa} PAO1 variant lacked the outer membrane protein OprF, which was reported to function as a diffusion porin. This suggested that a reduction of the outer membrane permeability is involved in bringing about the solvent resistance (211). Synthesis of the porin OmpF was repressed in solvent-resistant \textit{E. coli} mutants, which supported this hypothesis (14).

In summary, resistance to organic solvents is accompanied by many changes in the composition of the inner and outer membrane. However, frequently the functional implications were not demonstrated beyond the point of rationalizing the anticipated effect of the observed change. The picture that might emerge from the data is that a solvent resistance phenotype is the result of a variety of mechanisms,
including energy dependent efflux systems, appropriately structured outer and inner membranes, and biosynthetic capabilities. The relative contributions of each mechanism may differ, as E. coli strains are unable to produce trans unsaturated fatty acids but still can be made resistant to p-xylene. Resistance to solvents with a logP < 3, however, might require the combination of at least some, if not all, of the discussed mechanisms. This observation has implications for recombinant biocatalyst construction: It is difficult to transfer a multi-factor phenotype to a large degree to E. coli cells. It seems more likely that cells that are already solvent resistant could serve as the host for genes that would be advantageous to exploit in two-liquid phase whole-cell cultures. Solvent resistant strains might serve as a source of secreted hydrolytic enzymes that are especially suited for application in low-water enzyme reaction systems (264). Furthermore, these strains appear to display superior bioremediation properties in soils containing supersaturating amounts of toluene (170). They were also suggested for biocatalysis reactions like 3-methylcatechol formation, in which it would be advantageous to extract the transformation product into a solvent with a logP < 4 (75). It might also be possible to use the solvent resistance phenotype of such strains to increase the concentration of a toxic substrate in an inert carrier phase in a two-liquid phase culture. This might be necessary in order to improve mass transfer rates between phases in two-liquid cultures. A specific example is discussed in Chapter 5.

1.4.2. ALTERNATIVE REACTOR AND PROCESS CONFIGURATIONS

High pressure reactor. Two-liquid phase processes that utilize a flammable organic liquid and depend on the delivery of considerable amounts of oxygen to the biocatalyst must operate under special safety measures. Points to consider include flammability of the solvent, oxygen content of the air, and pressure inside the reactor. A way to realize safe operation of a two-liquid phase process is the utilization of an explosion proof reactor that can be operated under internal pressures up to 16 bar (316).
Such a reactor has been developed at our Institute. It allows to realize one of two modes of operation which reduce explosion danger; the reactor can be run at internal pressures close to ambient pressures or at considerably higher pressures. In the first case, the maximum pressure of a potential explosion will remain below 16 bar. In the latter case, the vapor pressure of the flammable liquid will remain under the critical vapor pressure necessary for explosion (316). Combined with dilution of the flammable organic substrate of the biotransformation into an inert carrier solvent, this reactor will allow to apply two-liquid phase cultivations as a routine process tool (315).

**Reactor configuration and down stream processing.** The release of surface active compounds from bacteria into a two-liquid phase culture medium might lead to the formation of a stable emulsion which is difficult to separate (317). In order to keep the aqueous phase separate from the organic phase and thus to avoid the formation of an emulsion, a continuous culture of *P. oleovorans* GPo1 was established which circulated through a membrane module. In this, the growth substrate heptane and the biotransformation substrate 1,7-octadiene permeated into the medium. Concomitantly, the two substrates were added directly into the aqueous phase. The rate was chosen such that no second phase formed and mass transfer limitations were reduced. Although the achieved concentration of 7-epoxy-1-octene in the storage vessel of the organic phase after approximately 50 d was promising (240 mM), overall volumetric productivities were only 30 U*L⁻¹ (90).

Alternatively, strategies can be developed to circumvent the use of organic solvents completely. A suitable alternative might be provided by a combination of controlled addition of the substrate and solid phase extraction of the product. This was suggested for the formation of toxic 2,3-dihydroxybiphenyl from toxic 2-hydroxybiphenyl with an *E. coli* recombinant strain that synthesized 2-hydroxybiphenyl 3-monoxygenase of *P. azelaica* HBP1 (159). Essentially, the substrate was fed at a limiting rate and the culture broth was circulated through the reactor and a second compartment, which contained a solid phase that adsorbed the product. High circulation rates were possible, which in turn should allow to benefit from high volumetric biocatalytic activities without many of the drawbacks of an organic liquid phase.
Enzyme reactors. So far, biochemical processes have been reviewed that used whole cells that synthesized enantioselective monooxygenases (see section 1.3.2.). Recent results indicate that it is also possible to produce optically active epoxides with isolated or enriched enzymes, for example with isolated chloroperoxidase of *C. fumago*. To produce oxindole from indole, a fed batch reactor (25 to 215 mL) was established that contained an aqueous buffered medium with tert-butyl alcohol and initially 3700 U*L⁻¹* of chloroperoxidase. Hydrogen peroxide was fed as an oxidant.

Regulated hydrogen peroxide addition led to maintenance of the activity for at least 3 h and the yield was extrapolated to 113 (g oxindole)⁺(L*ₚ)⁻¹* (590 U*L⁻¹*). Total turnover numbers for chloroperoxidase were in the range of 650'000 (322). The formation of enantiopure (substituted) styrene oxide(s) by kinetic resolutions has also been exploited in enzyme reactors, although so far on a small scale. The *A. niger* epoxide hydrolase was used in a partially purified form to set up 2 to 10 mL enzyme reactors, in which very high average productivities of 5.3 g⁺(L*ₚ*h)⁻¹* (533 U*L⁻¹*) were obtained for the production of (S)-p-nitrostyrene oxide over 4 h. The optimal initial p-nitrostyrene oxide concentration was 330 mM (241, 251). To increase the solubility of the p-nitrostyrene oxide, the aqueous medium was mixed with dimethylsulfoxide (251). When racemic p-nitrostyrene oxide was added as a second phase, the enzyme became inhibited (241).

A similar process was set up for the resolution of p-bromo-α-methylstyrene oxide (58) with the *A. niger* enzyme preparation at 4°C. However, the average activities were only 13.5 U*L⁻¹*.

Although the results with enzyme reactors described here for the hydrolases and in the paragraph above for the chloroperoxidase are very promising, one has to keep in mind several points. The applied protocol involves three steps: Cells are grown first, then enzymes are enriched, and finally the biotransformation is carried out. Furthermore, the reaction scales so far have been on the order of mL. However, the necessary methodology is simple once the enzyme is available in a stable form. This will facilitate the use of epoxide hydrolases to chemical laboratories not familiar with biochemical engineering.
1.4.3. Recombinant biocatalysis

Recent advances in genomic sequencing and understanding enzyme structure-function relations should allow an increase in the pool of available biocatalytic enzymes. There are no less than 20 completely sequenced bacterial genomes publicly available, and 65 more microbial genome projects are in progress (http://www.tigr.org/). Comparative genomics and the ongoing research to reveal and detail the function of single unknown open reading frames will allow the assignment of functions to an ever increasing portion of the available sequence data (57). The genetic information is easily available via PCR reactions and can rapidly be integrated into one of the many available expression systems for E. coli (221). Thus, this huge and expanding pool of sequence data is easy to tap, given that an appropriate search algorithm is available.

This recent source of information is complemented by the already available large amount of sequence information, for example on biodegradative enzymes which have already served as a source of commercially exploited biocatalysts (126, 189, 329).

Much information on potentially interesting oxidative biotransformation reactions was obtained from toxicity studies in mammalian systems. These reports frequently detail substrates, products, and enantioselectivities (see for example (380) for a survey on the fate of styrene in mammalian systems). Such systems are obviously not available for biocatalysis purposes on larger scale. However, a number of recent publications suggests that mammalian cytochrome P450s can be synthesized in E. coli with activities up to 1 U/g (cdw) if a suitable mammalian P450 reductase is concomitantly synthesized (35, 276, 330). If these approaches can be generalized, the number of potentially available enzymes will expand significantly.

The increase in available three-dimensional protein structures over the last ten years has triggered a whole series of attempts to manipulate enzymatic rates, reaction specificities, and regio- and enantioselectivities with very promising results. For example, the substitution of an active site tyrosine by either an alanine (Y96A) or a phenylalanine (Y96F) allowed to increase the styrene oxidation rate per unit enzyme of cytochrome P450cam 9 to 25-fold up to 100 nmol·min⁻¹·nmol P450.
enzyme) without compromising the enantioselectivity of the reaction (>70 %) (252). This value is in the range of the recently isolated styrene monooxygenases (see chapter 2). The Y96F mutant afforded also an increase in reaction rates for the hydroxylation of simple linear and branched alkanes. Moreover, expansion of the substrate spectrum to hydroxylation of phenylcyclohexane was obtained, although a mixture of regio- and stereoisomers of the resulting alcohol was formed (349). Introduction of a second mutation (V247A) essentially limited the products of the reaction to one regioisomer in only stereochemical configuration (180). Unfortunately, no rates were given. The mentioned reports represent only prominent examples out of a growing body of literature on rational protein design of oxygenases.

The engineered (as well as the wild-type) proteins are far from ideal, for instance, due to hydrogen peroxide formation, which leads to byproduct formation even in the case of the modified enzymes (252). However, they do represent examples for the power of rational protein design from which new catalysts are or will become available. This approach is complemented by the directed evolution of enzymes (200, 240) which is currently also applied to oxygenating enzymes (47, 319) and was even used to improve the enantiospecificity of a hydrolysis (298). In summary, the modern techniques of protein engineering are very likely to form a third major source of new catalysts.

These developments should shift the focus of screening for novel reactions from microorganisms to the enzymes themselves. If the screening is concerned with cofactor-dependent reactions, a corresponding process is likely to be realized with whole cells. Given the amount of data that is available on its molecular biology and its cultivation, E. coli is the prime candidate as the standard host for recombinant whole-cell biotransformations. However, biotransformations for fine chemical production involve frequently toxic substrates and products. As a consequence, a highly active recombinant biocatalyst needs to be integrated into a suitable process design that provides favorable conditions for growth and enzyme synthesis.
1.5. Scope of this thesis

The biotechnological production of enantiopure epoxides with oxygenases is hampered by the low productivities of production processes. Recombinant biocatalysis might offer an alternative and more efficient way to biocatalyst construction and subsequent exploitation of the synthetic potential of the catalyst. If successful, this could serve as a model for the future design of whole-cell biocatalytic processes for the production of toxic added-value compounds.

In order to evaluate this potential of recombinant biocatalysis, we investigated its application to the production of enantiopure styrene oxide. Chapter 2 describes the cloning and characterization of the genes of a novel styrene monooxygenase that epoxidizes inexpensive styrene to (S)-styrene oxide with an e.e. of >99 %. In Chapter 3 the construction and performance of a new expression vector based on the alk regulatory system of P. oleovorans GPol is described, which allows high level expression of monooxygenases under a regulation which is suitable for two-liquid phase cultivations. Chapter 4 describes the establishment of an efficient two-liquid phase fed-batch process for the production of (S)-styrene oxide, whose scale-up to 30 L is the topic of Chapter 5. Chapter 6 addresses the question whether it is possible to construct highly active whole-cell biocatalysts by chromosomal integration of target genes to avoid plasmid-related segregational instability. Chapter 7 presents a summary of the results.
CHAPTER 2: TOWARDS A BIOCATALYST FOR (S)-STYRENE OXIDE PRODUCTION: CHARACTERIZATION OF THE STYRENE DEGRADATION PATHWAY OF PSEUDOMONAS SP. VLB120

SVEN PANKE, BERNARD WITHOLT, ANDREAS SCHMID, AND MARCEL G. WUBBOLTS
In order to design a biocatalyst for the production of optically pure styrene oxide, an important building block in organic synthesis, the metabolic pathway and molecular biology of styrene degradation in Pseudomonas sp. strain VLB120 was investigated. A 5.7-kb XhoI fragment, which contained on the same strand of DNA six genes involved in styrene degradation, was isolated from a gene library of this organism in Escherichia coli by screening for indigo formation. T7 RNA polymerase expression experiments indicated that this fragment coded for at least five complete polypeptides StyRABCD corresponding to five of the six genes. The first two genes encoded the potential carboxy-terminal part of a sensor named StySc and the complete response regulator StyR. Fusion of the putative styAp promoter to a lacZ reporter indicated that StySc and StyR together regulate expression of the structural genes at the transcriptional level. Expression of styScR also alleviated a block that prevented translation of styA mRNA when a heterologous promoter was used. The structural genes styA and styB produced a styrene monooxygenase that converted styrene to styrene oxide, which was then converted to phenylacetaldehyde by StyC. Sequence homology analysis of StyD indicated a probable function as a phenylacetaldehyde dehydrogenase. To assess the usefulness of the enzymes for the production of enantiomerically pure styrene oxide, we investigated the enantioselectivities of the reactions involved. Kinetic resolution of racemic styrene oxide by styrene oxide isomerase was studied in E. coli recombinants carrying styC, which converted styrene oxide at a very high rate but with only a slight preference for the S enantiomer. However, recombinants producing styrene monooxygenase catalyzed the formation of (S)-styrene oxide from inexpensive styrene with an excellent enantiomeric excess of more than 99 % at rates up to 180 U (g cell dry weight)$^{-1}$. 
INTRODUCTION

Styrene degradation in microbial systems is of considerable interest for two reasons. As an important monomer in the manufacturing of polymers, styrene frequently enters our natural environment. Styrene itself is toxic to living systems in fairly low amounts mainly due to membrane related effects (37). In addition, the immediate degradation product of styrene in human liver, styrene oxide, is a known carcinogen (107). Furthermore, styrene possesses malodorous properties when present in amounts less than 1 ppm (148). All of this makes efficient removal of styrene from the environment highly desirable, and microorganisms have been found to play a key role in this process (114).

Secondly, reactions typically involved in styrene degradation possess significant potential in synthetic organic chemistry. Rhodococcus rhodochrous NCIMB 13259 was shown to degrade styrene via a dioxygenase attack on the aromatic ring, leading to 3-vinylecatechol after rearomatization (380). 3-Vinyl-1,2-cis-dihydroxycyclohexa-3,5-diene, the transformation product of the dioxygenase reaction, served as the starting point for an enantioselective organic synthesis of (-)-Zeylona, exemplifying a route to cyclohexene oxides with antitumor potential (169). A variety of bacteria from the genus Pseudomonas (148, 223, 262, 380) have been shown to transform styrene through an attack on the vinylic side chain to styrene oxide and subsequently to phenylacetaldehyde. From a biotechnological point of view, this pathway contains at least two potentially useful reactions for the formation of enantiopure styrene oxide, which is known as a valuable building block in the manufacturing of optically active compounds such as pharmaceuticals (115). These reactions could be either the enantiospecific formation of styrene oxide and/or the kinetic resolution of racemic styrene oxide. The enantioselective oxidation of styrene has been observed in a chemical mutant of the styrene degrader P. putida S12 (257), for which no genetic data are available, leading to an enantiomeric excess (e.e.) of more than 98%. Resolution could in theory be catalyzed by styrene oxide hydrolases or styrene oxide isomerases. The latter reaction has been found in bacteria, where styrene oxide is converted to phenylacetaldehyde, but the enantioselectivity of the reaction has not
been reported (29) or was low (257). The former reaction has been described for bacterial and fungal enzymes, which enantiospecifically hydrolyze one enantiomer of racemic styrene oxide and produce an optically active vicinal diol, while the other styrene oxide enantiomer is left behind (278, 341). Until recently, *P. fluorescens* ST has been the only microorganism in which the genes and enzymes that affect styrene degradation have been investigated in more detail (29). In another study, regulatory and structural genes involved in styrene degradation in *Pseudomonas* sp. strain Y2 have been analyzed and found to be very similar to the *P. fluorescens* ST genes (373). Up to now, no data on the enantioselectivities of any of the reactions have been published. Obviously, the concomitant presence of enzymes that produce and consume styrene oxide in one host limits the biotechnological potential. Therefore, we used recombinant strains of *Escherichia coli* producing exclusively either styrene monooxygenase or styrene oxide isomerase. The genes and biotechnological potential of the cognate enzymes involved in styrene degradation in *Pseudomonas* sp. strain VLB120 were identified, and this knowledge was exploited to design recombinant biocatalysts suited to the production of (S)-styrene oxide at high e.e.

**Materials and Methods**

**Media, chemicals, strains, and plasmids.** We used routinely Luria-Bertani (LB) broth (Difco, Detroit, Mich., USA) or M9 mineral medium (312) supplemented with MT trace element solution (289). When necessary, cultures were supplemented with kanamycin at a final concentration of 50 mg·L⁻¹, ampicillin (150 mg·L⁻¹), chloramphenicol (30 mg·L⁻¹), or 1 mM indole. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM to liquid cultures except where otherwise indicated. Cells were routinely grown on horizontal shakers at 200 rpm and 30 or 37°C. Strains and plasmids used in this study are listed in Tab. 2.1. Restriction and DNA modification enzymes were obtained from Boehringer Mannheim (Rotkreuz, Switzerland), NEB (Schwalbach, Germany), Gibco (Basel, Switzerland), or Promega (Zurich, Switzerland). Chemicals
were obtained from Fluka (Buchs, Switzerland) (styrene, >99 %, styrene oxide, >97 %, phenylacetic acid, >99 %) and Sigma (Buchs, Switzerland) (phenylacetaldehyde, >90 %).

Isolation and identification of *Pseudomonas* sp. strain VLB120. *Pseudomonas* sp. strain VLB120 was isolated in the area of Stuttgart, Germany, from forest ground with styrene as the sole substrate. Details on its isolation and physiology will be published elsewhere. Sequencing of the first 470 nucleotides of the 5' end of the 16S rRNA revealed that *Pseudomonas* sp. strain VLB120 is a member of the genus *Pseudomonas* but does not belong to any of the previously described species. It is most closely related to *P. alcaligenes* (LMG 1224-T), *P. pseudoalcaligenes* (LMG 1225-T and DSM 50188-T), *P. oleovorans* (DSM 1045-T), and *P. aureofaciens* (LMG 1245-T). All of these strains have a 16S rRNA sequence identity of 98.7 % to *Pseudomonas* sp. strain VLB120.

Growth and maintenance of *Pseudomonas* sp. strain VLB120. Cells were routinely transferred once per month between M9 mineral medium plates supplemented with MT trace element solution. The plates were stored in an atmosphere saturated with styrene at room temperature. All *Pseudomonas* sp. strain VLB120 cultures were started from such plates. Cells were inoculated into tubes with M9 mineral medium, and styrene was added to a final concentration of 1.7 mM with only the volume of the liquid phase taken into account. From these tubes, larger cultures were started with the same concentration of styrene. Where necessary, the addition of styrene was repeated after 12 h to increase biomass concentration. To test the growth of *Pseudomonas* sp. strain VLB120 on different substrates, cells were streaked onto M9 mineral medium plates and these were incubated at room temperature in an atmosphere saturated with the respective substrate. Phenylacetic acid was added to the plate to 0.1 % (wt/vol).

Construction of a genomic library of *Pseudomonas* sp. strain VLB120 in *E. coli* DH10B. Genomic DNA of *Pseudomonas* sp. strain VLB120 was prepared from styrene grown cells by using the Qiagen kit for isolation of genomic DNA (Qiagen, Basel, Switzerland). This preparation was digested with one of the following restriction enzymes: *SalI, EcoRI, HindIII, BamHI, XhoI*, and *KpnI*. 1 μg of digested
Tab. 2.1: Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VLB120</td>
<td>Wild-type <em>Pseudomonas</em>, styrene prototroph</td>
<td>K.-H. Engesser</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH10B</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; merA Δ(mrr hsdRMS-merBC) Δ80lacZΔM15 ΔlacX74 &lt;br&gt;deoR recA1 araΔ139 Δ(ara leu)7697 galU galK ΔrpsL &lt;br&gt;endA cspG</td>
<td>Gibco BRL</td>
</tr>
<tr>
<td>JM101</td>
<td>supE thi-1 Δ(lac-proAB) F&lt;sup&gt;−&lt;/sup&gt;[traD36 proAB&lt;sup&gt;+&lt;/sup&gt; lacI lacZΔM15]</td>
<td>(312)</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>hsdS gal (λcI857 (Ts) ind1 S7 (Am) mini5 lacUV5-T7 gene 1</td>
<td>(353)</td>
</tr>
<tr>
<td>CC118</td>
<td>Δ(ara-leu) araD ΔlacX74 galE galK spoA20 thi-3 rpsE &lt;br&gt;rpoB argE (Am) recA1</td>
<td>(222)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td></td>
</tr>
<tr>
<td>pZero2.1</td>
<td>lacZα-ccdB, cloning vector, Km&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pUC18Not</td>
<td>lacZα, cloning vector, Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>(161)</td>
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<tr>
<td>pUJ9</td>
<td>lacZ, Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>(79)</td>
</tr>
<tr>
<td>pUC18Not/T7&lt;sup&gt;*&lt;/sup&gt;</td>
<td>pUC18Not with T7 promoter and lacZp in tandem, Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>(162)</td>
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<tr>
<td>pVT33</td>
<td>*E. coli/<em>Pseudomonas</em> shuttle vector, lacFl-orIT. &lt;br&gt;RSF1010 oriV, Km&lt;sup&gt;f&lt;/sup&gt;</td>
<td>(78)</td>
</tr>
<tr>
<td>pCK01</td>
<td>lacZα, pSC101 oriV, Cm&lt;sup&gt;f&lt;/sup&gt;</td>
<td>(104)</td>
</tr>
<tr>
<td>pBG63</td>
<td>pGEM7Zf(+) (Promega) derivative. lacZp xy/MA, Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>(395)</td>
</tr>
<tr>
<td>pPT7T</td>
<td>pUC18Not/T7&lt;sup&gt;*&lt;/sup&gt; with T7 terminator in the HindIII site, Ap&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pSPW1</td>
<td>pZero2.1 carrying a 5.7-kb genomic DNA fragment of &lt;br&gt;<em>Pseudomonas</em> sp. VLB120, lacZp styScRABCD, Km&lt;sup&gt;f&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>pSPW2</td>
<td>lacZp styABCD, deletion derivative of pSPW1, Km&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>pSPW3</td>
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<td>This work</td>
</tr>
<tr>
<td>pSPW4</td>
<td>lacZp styScRABC, deletion derivative of pSPW1, Km&lt;sup&gt;f&lt;/sup&gt;</td>
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</tr>
</tbody>
</table>
pSPW5  \( \text{lacZp styScRAB, deletion derivative of pSPW1, Km}\)  This work

pSPW6  \( \text{lacZp styScRA, deletion derivative of pSPW1, Km}\)  This work

pSPW7  \( \text{lacZp styCD, deletion derivative of pSPW1, Km}\)  This work

p3His  \( \text{pZero2.1 styScRA-H6, Km}\)  This work

pSTFull  \( \text{lacZp styAB, with 163-bp of styRA intergenic region,} \)
\( \text{pUC18Nott derivative, Ap}\)  This work

pSTHalf  \( \text{lacZp styAB, with 77-bp of styRA intergenic region,} \)
\( \text{pUC18Nott derivative, Ap}\)  This work

pT7ST-Sc  \( \text{lacZp T7p stySc, pPT7T derivative, Ap}\)  This work

pT7ST-R  \( \text{lacZp T7p styR, pPT7T derivative, Ap}\)  This work

pT7ST-A  \( \text{lacZp T7p styA, pPT7T derivative, Ap}\)  This work

pT7ST-B  \( \text{lacZp T7p styB, pPT7T derivative, Ap}\)  This work

pT7ST-C  \( \text{lacZp T7p styC, pPT7T derivative, Ap}\)  This work

pT7ST-D  \( \text{lacZp T7p styD, pPT7T derivative, Ap}\)  This work

pT7ST-ScD  \( \text{lacZp T7p styScRABCD, pPT7T derivative, Ap}\)  This work

pT7ST-ADm\(^2\)  \( \text{lacZp T7p styA*BCD, pPT7T derivative, Ap}\)  This work

pT7ST-ADm  \( \text{lacZp T7p styA* B, pPT7T derivative, Ap}\)  This work

pT7ST-Abm  \( \text{lacZp T7p styA* B, pPT7T derivative, Ap}\)  This work

pT7ST-Am  \( \text{lacZp T7p styA* B, pPT7T derivative, Ap}\)  This work

pCKST-Abm  \( \text{lacZp styA* B, pCK01 derivative, Cm}\)  This work

pCKST-B  \( \text{lacZp styB, pCK01 derivative, Cm}\)  This work

pCKST-ScR  \( \text{lacZp styScR, pCK01 derivative, Cm}\)  This work

pCKST-Sc  \( \text{lacZp stySc, pCK01 derivative, Cm}\)  This work

pSPW20  \( \text{styAp fused to lacZ of pUJ9, Ap}\)  This work

pVLST-R  \( \text{tac styR, pVLT33 derivative, Km}\)  This work

\(^5\): H\(_6\) denotes a gene that has been extended by six histidine codons at the 3'end. The asterisk indicates that the translation initiation signals of the ORF or gene have been modified.
DNA was ligated to 50 ng of accordingly digested vector pZero2.1 (Invitrogen, Leek, The Netherlands) (the SalI digest was ligated into the XhoI site of the vector) in 0.5 M KGB buffer (50 mM potassium glutamate, 12.5 mM Tris-acetate (pH 7.5), 5 mM magnesium acetate, 25 μg/mL bovine serum albumin, 0.25 mM β-mercaptoethanol) supplemented with 1 mM ATP at 6°C for 24 h. The ligation mixtures were used to transform E. coli DH10B cells made competent by rubidium chloride. Cells were plated on LB medium supplemented with kanamycin and indole and incubated at 30°C.

DNA sequencing. The DNA sequence of a 5.7-kb XhoI genomic fragment of Pseudomonas sp. strain VLB120 was determined from deletion clones of pSPW1 or from subclones in pUC18Not by using fluorescently labeled -40 forward and reverse primers (MWG Biotech, Ebersberg, Germany) and a Sequenase kit with 7-deaza-dGTP (Amersham, Zurich, Switzerland), according to a cycle sequencing protocol. The reaction mixes were separated and analyzed on a LICOR sequencer with BaseImagIR version 2.3 software (MWG Biotech, Ebersberg, Germany). Subsequent data analysis was performed with the DNASTar software package (DNASTar, Madison, Wisconsin, USA), the BlastP algorithm (9) of the GenBank database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), and the FOLD program of the Genetics Computer Group (GCG) software package for mRNA analysis (GCG, Madison, Wisconsin, USA).

DNA manipulation and constructs. Plasmids for the deletion analysis of sty genes were obtained from pSPW1 by digestion with the enzymes indicated in Fig. 2.1C and by religation. For efficient labeling of translation products, the T7 terminator of pUJ9 was introduced as a 0.4-kb HindIII fragment into the HindIII site of pUC18Not/T7* to yield pPT7T. This plasmid possesses two promoters in tandem (lacZp and the T7 promoter) pointing towards the polylinker of pUC18Not. Either the whole XhoI fragment or isolated parts of it, as indicated in Fig. 2.1D, were inserted into the polylinker of pPT7T.

Plasmid p3His contains a modified styA gene with six histidine codons added to its 3' end. To achieve this, PCR was performed with pSPW1 as the template (primer P1, 5' CCAGGGGCGGCAAGTTCTGCTACGAC 3'; primer P2, 5' CGCGCGTCTAGA
TTAGTGATGGTGATGGTGATGGGCCGCGATAGTGGGTGCGAACTGACTG
CAC 3') P1 annealed upstream of the BgIII site internal to styA. Sequences identical to pSPW1 DNA are underlined. P2 annealed at the end of styA and introduced six histidine codons shown in boldface type before the stop codon of styA and a new XbaI site (in italics) after the stop codon. In the PCR, annealing took place at 57°C for 90 s, extension took place for 1 min at 72°C, and denaturation took place for 45 s at 94°C (for annealing sites, see also Fig. 2.1B). Plasmid pSPW1 was then digested with BgIII and XbaI eliminating styABCD but retaining stySc (which encodes the carboxy-terminal part of a sensor, StySc) and most of styR, and ligated to the amplified fragment digested with the same enzymes. This led to a construct that lacked the 0.6-kb BgIII fragment containing the start of styA. Introduction of this fragment in the right orientation led to p3His.

Plasmid pT7ST-ADm contains styA*, that is, styA with its upstream sequences replaced by a sequence containing a new Shine-Dalgarno (SD) sequence. To construct this plasmid, a second PCR was performed with pSPW1 as the template (primer P3: 5' CCCACGAATTCTAAAAGGAGGAACArArGAAAAAGCGTATCGGTATTTGTTGGTGCG 3'; primer P4: 5' CCAGTGCACACAACCAGCAGATCGTAC 3'). P3 annealed at the ATG codon of styA and introduced a new SD sequence (in boldface type) and new EcoRI and Ndel sites (in italics). P4 was identical to pSPW1-DNA and primed downstream of the BgIII site in styA. A 450-bp fragment was amplified (with annealing for 1 min at 55°C and extension and denaturation as described above), digested with EcoRI and BgIII, and ligated into plasmid pT7ST-AD (see Fig. 2.1D), which had been digested with the same enzymes. The resulting construct was pT7ST-ADm. The open reading frame (ORF) with the altered ribosome binding site was called ORF 3*. pT7ST-ADm served as the source of styA* in various constructs: From this plasmid, styA* and styB were excised as a 2.0-kb EcoRI/SmaI fragment and inserted into pPT7T to yield pT7ST-ABm or into pCKO1 to yield pCKST-ABm. Plasmid pT7ST-Am consists of styA* as a 1.6-kb EcoRI/PstI fragment from pT7ST-ABm inserted into pPT7T. For construction of pCKST-B, the 0.8-kb EcoRI/HindIII fragment with styB from pT7ST-B (see Fig. 2.1D) was inserted into pCKO1.
To study the regulation of styrene degradation, the 1.9-kb EcoRI/SnaBl fragment of pSPW1 containing \textit{styScR} was introduced into EcoRI/HincII digested vector pCKO1 to yield pCKST-ScR. Plasmid pCKST-Sc, containing only \textit{stySc}, was constructed by introducing the 0.9-kb EcoRI/Sall fragment of pSPW1 into pCKO1. Transfer of the 2.0-kb FspI fragment of pSPW1 containing only \textit{styR} into the HincII site of the polylinker of pVLST33 led to pVLST-R. To obtain pSPW20, containing a translational fusion of the 5' part of \textit{styA} to \textit{lacZ}, pUJ9 was digested with SmaI and ligated to the 0.7-kb HincII fragment of pSPW1 containing the putative promoter region. Furthermore, the 2.2-kb SaeI/Smal fragment of pSPW1 containing \textit{styAB} was ligated into SaeI/Smal digested pUC18Not, leading to pSTFull. Partial digestion of pSTFull with NcoI and EcoRI, treatment with T4 polymerase, and religation yielded pSTHalf, which is similar to pSTFull but has lost the 5' terminal 86-bp of the insert.

\textbf{[\textsuperscript{35}S]methionine labeling of translation products.} Plasmids carrying genes under control of the T7 promoter (Fig. 2.1D) were introduced into \textit{E. coli} BL21(DE3) together with plasmid pVLT33, which provided a \textit{lacI} gene to prevent early expression of the T7 RNA polymerase gene of \textit{E. coli} BL21(DE3). Cells from an overnight culture were transferred to fresh LB medium supplemented with antibiotics and grown at 30°C to an optical density at 600 nm of 0.8. A volume of 2 mL of the culture was centrifuged and cells were washed in 1.5 mL of M9 medium and resuspended in 1 mL of the same medium supplemented with 0.2 % glucose and 0.02 % methionine assay medium (Difco). The cells were incubated for 60 min at 30°C, IPTG was added to 0.6 mM, and the cultures continued to grow for 60 min at 30°C. Subsequently, rifampicin was added to 600 mg\textsuperscript{-1}L\textsuperscript{-1} and the cultures were transferred to 42°C for 45 min and then to 30°C for 30 min. Afterwards, cells were incubated for 5 min with 10 \textmu Ci of [\textsuperscript{35}S]methionine (Hartmann Analytic, Braunschweig, Germany), collected by centrifugation, washed, and resuspended in sample buffer (150 mM Tris (pH 6.8), 3.5 % sodium dodecyl sulfate (SDS), 25 % glycerol, 0.06 % bromphenolblue, 0.12 % \textbeta-mercaptoethanol). After heating to 95°C, proteins were separated on an 12 % SDS-12 % polyacrylamide gel which was subsequently fixed in 25 % isopropanol/10 % acetic acid/65 % water, stained for 10 min with Coomassie
brilliant blue (Bio-Rad, Glattbrugg, Switzerland), and destained (312), dried, and analyzed by exposing X-ray film to it.

Detection of metabolites and determination of enzyme activities in whole cell assays. For β-galactosidase assays, E. coli CC118 was transformed with the plasmids under study. Whole cell assays for investigating biotransformations were carried out with E. coli JM101. Recombinant strains containing the plasmids under study were incubated in 50 or 100 mL of medium in the presence of the respective antibiotics. At an optical density at 450 nm of 0.3 to 0.4, cells were induced by the addition of IPTG and the incubation was continued for 4 h, during which the cells entered stationary phase. Cells were then either subjected to a β-galactosidase assay, as described elsewhere (234), or used for determination of specific activities. In the latter case, cells were harvested and resuspended to a certain cell dry weight (cdw) in 100 mM potassium phosphate buffer, pH 7.4, containing 1 % (wt/vol) glucose. Aliquots of 1 mL were distributed in Pyrex tubes and incubated horizontally on a rotary shaker at 200 rpm. After 3 min, substrate was added to a final concentration of 1.5 mM from a 20-fold stock solution in ethanol. The reaction continued for 5 min in the shaker and was then stopped by incubating the sample on ice and by immediately adding 1 mL of ice-cold ether containing 0.1 mM 1-dodecanol as an internal standard. After addition of saturating amounts of sodium chloride, the water phase was extracted by vigorous shaking for 5 min at 30°C and the phases were separated by centrifugation. The organic phase was dried over anhydrous sodium sulfate and analyzed by gas chromatography.

For separation of the compounds we used a Chrompack Cp-Sil5CB column (Chrompack, Bergen op Zoom, The Netherlands) with splitless injection and a temperature profile from 40 to 140°C at 10°C min⁻¹ with hydrogen as the carrier gas. Alternatively, a Supelco Beta-DEX 120 column (fused silica capillary column, 30 m, 0.25 mm inner diameter, 0.25 μm film thickness; Supelco, Buchs, Switzerland) was used with split injection (20:1) and an isothermal oven temperature profile at 90°C for separation of styrene oxide enantiomers. Compounds were detected by a flame ionization detector. We usually had recoveries above 80 % with respect to the amount of substrate added with this protocol. Substances were identified by
retention times with commercially available standards or, in the case of 2-phenylethanol, via gas chromatography-mass spectrometry (results not shown). As a rule, 2.5 (g cdw)$^{-1}$ were applied in an assay. The amount of cells was reduced to 1 g$^{-1}$ when productivity numbers for styrene oxide formation exceeded 80 U*(g cdw)$^{-1}$ or to 0.028 g$^{-1}$ and fractions thereof for the determination of specific activities of styrene oxide isomerase.

One unit (1 U) is defined as the activity that produces 1 µmol of a product from a given substrate in 1 min. Specific activities were calculated as an average activity based on the amount of product formed in a given, constant time per unit with an awareness of the possibility that the substrate conversion is not linear in time during the assay period. Experiments were repeated at least three times independently.

**Determination of e.e. and enantiomeric ratio.** The e.e. was determined from the concentrations of the styrene oxide stereoisomers according to the following equation e.e. = |(S - R)|(S + R)$^{-1}$ with S and R representing the concentrations of the two stereoisomers. To calculate the selectivity of the isomerase reaction, the extent of phenylacetaldehyde formation from racemic styrene oxide was determined and corrected for formation of phenylacetaldehyde due to the injector temperature. Where necessary, the assay time was shortened to ensure that at least 30% of the starting material was left. Under the assumption of treating an irreversible reaction, the enantiomeric ratio, E, for the kinetic resolution of racemic styrene oxide was derived from the e.e. of the remaining substrate and the extent of conversion, as described elsewhere (52).

**N-terminal sequencing of StyA.** A hexahistidine affinity tag was added to the C terminus of StyA to allow preparation of a protein fraction enriched in a modified StyA-H6. *E. coli* JM101(p3His) was grown and induced as described for whole cell assays, harvested by centrifugation, and treated according to the protocol for nondenaturing isolation of hexahistidine tagged proteins on nickel-nitrilotriacetic acid spin columns (Qiagen). In short, proteins were loaded onto a Ni-affinity column in the presence of 20 mM imidazole to prevent unspecific binding and eluted with 250 mM imidazole. This protocol yielded a fraction which contained approximately
90% StyA-H6. Separation of proteins in the resulting enriched fraction by SDS-PAGE and transfer of the proteins to polyvinylidene difluoride membranes was performed as described previously (226). The N-terminal amino acid sequence of StyA-H6 was determined on an Applied Biosystems automatic protein sequencer by automated Edman degradation.

**Nucleotide sequence accession number:** DNA sequences were deposited into publicly accessible databases (the 5.7-kb XhoI fragment was assigned GenBank accession no. AF031161; the 16S rRNA sequence was assigned EMBL accession no. AJ224883).

**Results**

**Growth of Pseudomonas sp. strain VLB120 on different substrates.** Pseudomonas sp. strain VLB120 was able to use styrene, styrene oxide, phenylacetaldehyde, and phenylacetic acid as growth substrates when plated on M9 mineral medium. The strain did not grow on toluene, benzene, ethylbenzene, 1-phenylethanol, 2-phenylethanol, or 1,2-phenylethandiol. This growth substrate profile strongly suggests that epoxidation of the vinylc side chain was the first step in styrene degradation.

**Selection of clones carrying styrene degradation genes from an E. coli gene library.** It is well documented that some mono- and dioxygenases can convert indole in E. coli to indoxyl with subsequent spontaneous formation of indigo (97, 232, 263). Xylene oxygenase from the TOL plasmid is a monooxygenase that catalyzes the oxidation of toluene to benzylalcohol (142). In addition, it oxidizes styrene to styrene oxide (398) and initiates indigo formation in E. coli probably by converting indole to indoxyl (232). In analogy to this, we reasoned that E. coli clones carrying styrene oxidation genes from Pseudomonas sp. strain VLB120 might be able to produce indigo and turn blue when grown on LB medium plates containing indole. Of the six different genomic libraries of Pseudomonas sp. strain VLB120 that we prepared in E. coli DH10B, three yielded
Tab. 2.2. Properties of ORFs and their predicted gene products

<table>
<thead>
<tr>
<th>ORF Position(^a)</th>
<th>SD</th>
<th>Size (no of amino acids)</th>
<th>Predicted molecular wt</th>
<th>App. molecular mass (kDa)(^d)</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 47-778</td>
<td>-</td>
<td>244</td>
<td>27030</td>
<td>-</td>
<td>StySc</td>
</tr>
<tr>
<td>2 842-1465</td>
<td>AAGGAG</td>
<td>208</td>
<td>23245</td>
<td>27</td>
<td>StyR</td>
</tr>
<tr>
<td>3 1646-2893</td>
<td>AAGGTA</td>
<td>416</td>
<td>46351</td>
<td>47</td>
<td>StyA</td>
</tr>
<tr>
<td>4 2948-3460</td>
<td>GGTGGA</td>
<td>171</td>
<td>18364</td>
<td>19</td>
<td>StyB</td>
</tr>
<tr>
<td>5 3530-4039</td>
<td>AAGAGG</td>
<td>170</td>
<td>18050</td>
<td>18</td>
<td>StyC</td>
</tr>
<tr>
<td>6 4054-5544</td>
<td>AAGGAG</td>
<td>497</td>
<td>52658</td>
<td>55</td>
<td>StyD</td>
</tr>
</tbody>
</table>

\(^a\) Relative to the start of the 5.7-kb XhoI fragment

Fig. 2.1. (A) Proposed reaction sequence mediated by enzymes encoded on the 5.7-kb XhoI fragment of *Pseudomonas* sp. strain VLB120. Compounds, from left to right: styrene, styrene oxide (the asterisk indicates the chiral carbon atom), phenylacetaldehyde, and phenylacetic acid. (B) Restriction site map and genetic structure of the XhoI fragment in pSPW1. Stippled boxes indicate vector pZero2.1 derived sequences. The lacZ\(p\) promoter is provided by the vector. The putative sty promoter is denoted styAp. The restriction sites external to the two XhoI sites, and therefore external to the insert, were derived from the pZero2.1 polylinker. Arrows indicate annealing sites for PCR primers P1 to P4 (see Materials and Methods). (C) Analysis of the XhoI fragment with different deletion derivatives of pSPW1. The sites used for deletions are indicated (and also presented in panel B). The lacZ\(p\) promoter remained unaffected by the deletions. Designations of the plasmids resulting from the deletions are indicated on the right. *E. coli* recombinant strains carrying such plasmids were analyzed for formation of styrene oxide (So) from styrene or of phenylacetaldehyde and 2-phenylethanol (Pl) from styrene oxide or production of indigo (In) after overnight growth on LB medium. Plus signs indicate quantitative conversion of the substrate or blue color for indigo; minus signs indicate no conversion or no blue color in the culture medium. The plus sign in parentheses indicates an at least 12-fold-lower styrene oxide productivity value than that indicated by a plus sign without parentheses (conversion was not complete). (D) pPT7T derived constructs for T7 RNA polymerase based labeling of translation products. The fragments were obtained by digestion with the indicated restriction enzymes and introduced into the pPT7T polylinker with either the same sites in the polylinker or sites indicated additionally. The position of the T7 promoter (T7\(p\)) and the T7 terminator (T7\(t\), stem-loop structure) are given only for one construct but are present in all plasmids. The names of the resulting plasmids are given on the right. Restriction sites relevant for constructions: A, Apal; Bg, BglII; C, Cid; E, EcoRI; F, FspI; H, HincII; Ne, Neol; Nr, NraI; P, PstI; Sl, SauI; Sc, SacI; Sm, SmaI; Sn, SnaBI; St, Stul; Xb, XbaI; Xh, XhoI.
A) 

\[
\begin{align*}
\text{StyAB} & \rightarrow \text{StyC} & \rightarrow \text{StyD} \rightarrow \text{StyE} \\
\end{align*}
\]

B) 

\[
\text{pSPW1}
\]

C) 

\[
\begin{align*}
\text{Sc} & \rightarrow \text{R} & \rightarrow \text{A} & \rightarrow \text{B} & \rightarrow \text{C} & \rightarrow \text{D} \\
\end{align*}
\]

D) 

\[
\begin{align*}
\text{Sc} & \rightarrow \text{R} & \rightarrow \text{A} & \rightarrow \text{B} & \rightarrow \text{C} & \rightarrow \text{D} \\
\end{align*}
\]
Tab. 2.3. Homologs of *s*ty gene-encoded proteins

<table>
<thead>
<tr>
<th>ORF Homolog</th>
<th>Organism</th>
<th>Function</th>
<th>Sequence identity (%)</th>
<th>ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TutC</td>
<td><em>Thauera</em> sp. strain T1</td>
<td>His kinase</td>
<td>42.6&lt;sup&gt;b&lt;/sup&gt; (64)</td>
</tr>
<tr>
<td></td>
<td>NwsA</td>
<td><em>B. japonicum</em></td>
<td>His kinase</td>
<td>41.4&lt;sup&gt;b&lt;/sup&gt; (130)</td>
</tr>
<tr>
<td></td>
<td>NodV</td>
<td><em>B. japonicum</em></td>
<td>His kinase</td>
<td>40.2&lt;sup&gt;b&lt;/sup&gt; (127)</td>
</tr>
<tr>
<td></td>
<td>TodS</td>
<td><em>P. putida</em> F1</td>
<td>His kinase</td>
<td>36.9&lt;sup&gt;b&lt;/sup&gt; (206)</td>
</tr>
<tr>
<td></td>
<td>FixL</td>
<td><em>B. japonicum</em></td>
<td>His kinase</td>
<td>33.2&lt;sup&gt;b&lt;/sup&gt; (13)</td>
</tr>
<tr>
<td>2</td>
<td>TutB</td>
<td><em>Thauera</em> sp. strain T1</td>
<td>Transcriptional regulator</td>
<td>52.4 (64)</td>
</tr>
<tr>
<td></td>
<td>NodW</td>
<td><em>B. japonicum</em></td>
<td>Transcriptional regulator</td>
<td>49.0 (127)</td>
</tr>
<tr>
<td></td>
<td>NwsB</td>
<td><em>B. japonicum</em></td>
<td>Transcriptional regulator</td>
<td>46.2 (130)</td>
</tr>
<tr>
<td></td>
<td>TodT</td>
<td><em>P. putida</em> F1</td>
<td>Transcriptional regulator</td>
<td>45.7 (206)</td>
</tr>
<tr>
<td></td>
<td>FixJ</td>
<td><em>B. japonicum</em></td>
<td>Transcriptional regulator</td>
<td>41.0 (13)</td>
</tr>
</tbody>
</table>

<sup>a</sup> In the cases of both ORF 1 and ORF 2, many homologs with more than 20% sequence identity were found; only the top 5 are shown. <sup>b</sup>: Sequence identity was calculated only for the C-terminal part of the listed protein to correspond to StySc (see text).<sup>c</sup>: *Bradyrhizobium japonicum*

blue colonies on LB-indole plates after 2 days of incubation (*XhoI, BamHI, and KpnI* gene libraries). Of these, the library based on *XhoI* fragments yielded one blue colony per approximately 500 transformants. Plasmid DNA was isolated, and 85% of the 48 colonies contained a 5.7-kb insert in pZero2.1, in the majority of cases together with one of a variety of second inserts. Of the plasmids isolated from these transformants, four conveyed the ability to convert styrene to phenylacetaldehyde to *E. coli* DHB10 grown in liquid culture with or without induction by IPTG. They all harbored only the 5.7-kb *XhoI* fragment. One such plasmid was named pSPW1 and served as the basis for further studies. Transformants from the *BamHI* and the *KpnI* libraries did not yield clones that showed the desired transformation ability, nor did the restriction patterns of plasmids from such clones match the 5.7-kb *XhoI* fragment.
Sequence of the 5.7-kb XhoI fragment: The sequence of both DNA strands was determined. Computer analysis of the sequence revealed six ORFs (ORFs 1 to 6) in the same direction as the lacZp promoter (Fig. 2.1B). ORF 1 did not possess a SD sequence similar to the known consensus sequence (331). For ORF 6, we reasoned that the translational start with the best SD sequence was most likely the actual translation start (AAGGAGN7ATG). The properties of the ORFs and their predicted gene products are listed in Tab. 2.2. We compared the deduced amino acid sequences of the first two ORFs to entries in the GenBank database by using the BlastP algorithm (9), and homologs with more than 20% sequence identity are listed in Tab. 2.3. Homologs to proteins encoded by ORFs 3 to 6 have been discussed elsewhere (see Discussion) (29).

Radioactive labeling of proteins encoded on the 5.7-kb XhoI fragment. DNA fragments covering either the complete XhoI fragment or the single predicted ORFs were placed under the control of a T7 RNA polymerase driven promoter (Fig. 2.1D). [³⁵S]methionine labeling of translation products showed that at least five proteins were encoded on the XhoI fragment (Fig. 2.2). The vector-only control indicated that transcription termination by the T7 terminator was not complete (Fig. 2.2, lane 1), since three bands were detected. As the bla gene encoding β-lactamase in pPT7T is transcribed from the same strand of DNA as the insert under study, it is likely that these bands correspond to the complete bla gene transcript at 31.6 kDa, the mature β-lactamase immediately under it, and a putative degradation product at approximately 27 kDa. An identical pattern of bands derived from the β-lactamase primary transcript has been found elsewhere (91). While a plasmid carrying ORF 1 did not yield an identifiable product other than that encoded by the vector-only control (not shown), plasmids carrying ORFs 2 to 6 did. Furthermore, for each ORF a translation product that was in good agreement with protein sizes predicted from the DNA sequence and with proteins synthesized from the original XhoI fragment were identified (Fig. 2.2, lane 2). Based on this, we considered ORFs 2 to 6 to represent the genes styRABCD with their cognate protein products. ORF 1 was tentatively designated stySc (see below). Interestingly, expression of the sty genes from the XhoI fragment led to prominent bands for StyR, StyB and/or StyC, and StyD, but only to a faint band for StyA. Consistent with this, the StyA band was
very weak in a recombinant carrying pT7ST-A (Fig. 2.2, lane 4). However, from previous SDS-PAGE analysis it was likely that StyA could be expressed to significantly higher levels in *E. coli* JM101(pSPW1) (data not shown). To investigate whether sequences upstream of *stx* were involved in determining the weak expression of *stx* from pT7ST-A, we modified the DNA sequences upstream of the translation start of *stx*. First, the original translation start of StyA was verified by N-terminal sequencing. It read MKKRIIGVAGTAGLHLGLF and was identical to the sequence predicted from GREET. Subsequently, an EcoRI site together with a modified SD sequence was inserted in front of the ATG codon of *stx*, removing at the same time the original upstream sequences, resulting in pT7ST-Am carrying *stx* (Fig. 2.3A). The sequence exchange led to a very prominent StyA band with the same apparent size as StyA synthesized from pT7ST-A in labeling experiments (Fig. 2.2, lanes 4 and 5).

Additional bands appeared for constructs carrying *stx* and *sty*. These could be explained by taking into account that the fragments used for cloning contained truncated flanking ORFs. Inspection of the DNA sequences of pT7ST-A, pT7ST-Am, and pT7ST-C revealed truncated ORFs coding for proteins that matched the sizes of the additional bands visible in Fig. 2.2, lanes 4, 5, and 7. In the constructs carrying *stx* or *stx*, a second artificial ORF from the start of *sty* to the T7 terminator would be expected to encode a gene product of 14.3 kDa, which matches the band observed at 15 kDa reasonably well. In case of pT7ST-C, an ATG codon with a reasonable SD sequence (AGAGGGN13ATG) lies within the truncated *sty* sequence between the T7 promoter and the predicted translation start of *sty*. This predicted artificial 14.0 kDa protein matches the observed band at 14 kDa.

**Deletion analysis of the 5.7-kb *XhoI* fragment.** To correlate the information derived from the DNA sequence for *stx*ScRABC with functions of the proteins, we performed a deletion analysis of the 5.7-kb *XhoI* fragment in *E. coli* JM101 (Fig. 2.1C). From the nucleotide sequence, we assumed that *stx*ABCD were structural genes for conversion of styrene to phenylacetic acid, with *stx*AB encoding the components of a styrene monooxygenase. In partial agreement with this, we found the minimum DNA element needed for indigo formation in *E. coli* to
Fig. 2.2. [\(^{35}\text{S}\)]methionine labeling of T7 RNA polymerase based translation products. *E. coli* BL21(DE3) carrying pPT7T without insert (lane 1), with the complete 5.7-kb *XhoI* fragment (lane 2), or with fragments containing only one complete ORF (lanes 3 to 8), was induced with IPTG and proteins synthesized under control of the T7 promoter were labeled with [\(^{35}\text{S}\)]methionine. Protein extracts were separated on an SDS-12% polyacrylamide gel. Lanes: 1, pPT7T; 2, pT7ST-ScD; 3, pT7ST-R; 4, pT7ST-A; 5, pT7ST-Am; 6, pT7ST-B; 7, pT7ST-C; 8, pT7ST-D.
Tab. 2.4. Functional analysis of StyA, StyB, and StyC in E. coli recombinants

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Plasmids</th>
<th>Expressed gene(s)</th>
<th>Copy no.</th>
<th>Substrate [rel. retention time]</th>
<th>Product [rel. retention time]</th>
<th>Avg. specific activity ± SD [U/g cdw]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pCKO1</td>
<td>L</td>
<td>styrene</td>
<td>ND</td>
<td>styrene oxide</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td></td>
<td>pT7ST-B</td>
<td>H</td>
<td>styrene</td>
<td>ND</td>
<td>styrene oxide</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td>2</td>
<td>pCKO1</td>
<td>L</td>
<td>styrene</td>
<td>ND</td>
<td>styrene oxide</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>3</td>
<td>pCKO1</td>
<td>L</td>
<td>styrene</td>
<td>(0.657 ± 0.007)</td>
<td>(1.086 ± 0.002)</td>
<td>7.0 ± 2.5</td>
</tr>
<tr>
<td>4</td>
<td>pCKST-B</td>
<td>L</td>
<td>styrene</td>
<td>(0.657 ± 0.007)</td>
<td>(1.087 ± 0.002)</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>5</td>
<td>pCKST-ABm</td>
<td>L</td>
<td>styrene</td>
<td>(0.657 ± 0.007)</td>
<td>(1.088 ± 0.002)</td>
<td>79 ± 5</td>
</tr>
<tr>
<td>6</td>
<td>pCKST-ABm</td>
<td>H</td>
<td>styrene</td>
<td>(0.658 ± 0.007)</td>
<td>(1.090 ± 0.002)</td>
<td>&lt;5</td>
</tr>
<tr>
<td>7</td>
<td>pT7ST-C</td>
<td>H</td>
<td>styrene oxide</td>
<td>(1.090 ± 0.002)</td>
<td>phenylacetaldehyde</td>
<td>21,000 ± 3300</td>
</tr>
</tbody>
</table>

L, low-copynumber (below 10); H, high-copy-number (above 100).

Retention times are given relative to the retention time of the internal standard dodecanol. Relative retention times of authentic standards: styrene, 0.654 ± 0.003; styrene oxide, 1.090 ± 0.006; phenylacetaldehyde, 1.018 ± 0.006; ND, not detected.

Consist only of styA; cells carrying pSPW2 or pSPW6 produced indigo while those carrying pSPW3 did not. Given our hypothesis that the reactions that convert indole and styrene are performed by the same enzyme, this would attribute the oxidation activity to StyA. However, to detect styrene oxidation activity in whole cell assays, elements upstream of styA had to be present as in E. coli JM101(pSPW6). Since indigo formation and accumulation over time is a much more sensitive indication of styrene monooxygenase activity than styrene oxide formation in whole cell assays, this indicates that elements upstream of styA can increase specific styrene monooxygenase activity but are not necessarily a structural part of the enzyme. The same conclusion resulted from comparison of the transformation abilities of E. coli JM101(pSPW1), which was able to transform...
styrene to phenylacetaldehyde, and *E. coli* JM101(pSPW2), which was not. However, inspection of the results obtained with *E. coli* JM101 carrying pSPW5 and pSPW6 indicated that StyB was necessary for maximal activity of StyA in the presence of the elements upstream of styA, as it increased the rate of styrene oxide formation at least 12-fold.

The analysis also allowed a correlation between the formation of phenylacetaldehyde and 2-phenylethanol from styrene oxide and the expression of styC. This activity was present in *E. coli* JM101 carrying pSPW7 or pSPW4 but not in *E. coli* JM101 carrying pSPW6. Deletion of the region upstream of styC did not lead to loss of this activity, and deletion of a part of styD did not impair either of the above mentioned activities.

**Functional analysis of StyB and StyC.** To further investigate the role of StyB, we carried out a series of experiments using plasmids carrying styA*, thus eliminating all of the upstream sequences interfering with styAB expression (see above). Genes styA* and styB were provided on the same or different plasmids, and expression from both genes was driven from the lac promoter. Tab. 2.4 shows clearly that in *E. coli* JM101, the presence of styA* alone was sufficient to oxidize styrene (experiment 3) while expression of styB alone did not lead to detectable styrene oxide formation (experiment 2). Nevertheless, to achieve high levels of activity, the presence of styB was also required (experiment 4). These results confirmed that gene products other than those of styAB do not play a structural role in styrene monooxygenase function. As shown in experiments 5 and 6 (Tab. 2.4), providing styAB on a high-copy-number plasmid increased styrene oxide formation rates relative to those achieved with a low-copy-number plasmid. However, the correct molar ratio of the two proteins appeared to play a role in the efficiency of styrene oxide formation (experiment 4 and 5 (Tab. 2.4)). Although these data qualitatively confirm results obtained for equivalent experiments with genes of *P. fluorescens* ST (29), it is important to note that in contrast to the earlier study, the data presented in this report were derived from genetic elements containing only the genes of interest, i.e., styA* and styB, without major portions of upstream DNA.

The results from the deletion analyses also indicated that StyC was involved in the conversion of styrene oxide to phenylacetaldehyde. This observation was
supported by the fact that JM101(pT7ST-C) was able to convert styrene oxide to phenylacetaldehyde while JM101(pPT7T) was not (experiment 7 and 8 (Tab. 2.4)). No 2-phenylethanol was formed in this experiment.

**Enantioselectivity of styrene oxide formation:** Having clarified the functions of the various structural genes, we determined the enantioselectivity as a key parameter of the cognate enzymes to evaluate their usefulness for biocatalysis. Using *E. coli* JM101(pSPW5), which carries styScRAB under lacZp promoter control, we have investigated the e.e. of styrene oxide formed in whole cell biotransformations and found it to be greater than 99 % (Fig. 2.4C) by chiral gas chromatography. (S)-Styrene oxide produced by *E. coli* JM101(pBG63) expressing the genes for TOL plasmid derived xylene oxygenase served as a reference (Fig. 2.4B) (395). The enantiomer formed by styrene monooxygenase eluted at the same time as the major enantiomer formed by xylene oxygenase, indicating that the product consisted of at least 99.5 % (S)-styrene oxide.

**Enantiomeric ratio of styrene oxide consumption.** *E. coli* JM101(pT7ST-C) recombinants synthesizing styrene oxide isomerase from a high-copy-number plasmid showed a very high reaction rate (experiment 8 (Tab. 2.4)). However, the enzyme demonstrated only a slight preference for the transformation of the S enantiomer of racemic styrene oxide. From the enantiomeric excess of remaining styrene oxide and the extent of conversion when terminating whole cell assays before the complete consumption of styrene oxide, we determined the enantiomeric ratio E of styrene oxide conversion to be 1.4.

**Expression of the styrene monooxygenase structural genes.** To clarify why we could not obtain measurable styrene oxide formation by *E. coli* JM101(pSPW2), we constructed two high-copy-number plasmids carrying styAB under lacZp promoter control using either the SacI site 163-bp upstream (pSTFull) or the NcoI site 77-bp upstream (pSTHalf) of the translational start of styA (Fig. 2.3A) and compared the resulting styrene monooxygenase expression to that from pT7ST-ABm. The latter construct contained the same modified ribosome binding site as pT7ST-Am (see above). As shown in the left panel of Fig. 2.3B, we were unable to detect formation of styrene oxide with applying either of the first two constructs but specific activities were very high with applying the construct in which all of the upstream
Fig. 2.3. Production of (S)-styrene oxide by StyAB. (A): Separation of 1.5 mM racemic styrene oxide on a chiral cyclodextrin column. The peak at 18.7 min is phenylacetaldehyde, which can be formed due to the injector temperature. (B) (S)-Styrene oxide with an e.e. of 93 % formed by E. coli JM101(pBG63), which produces xylene oxygenase and served as a reference. (C) (S)-Styrene oxide is formed with an e.e. of > 99 % by E. coli JM101(pSPW5), which produces styrene monooxygenase. Columns were intentionally overloaded to obtain a signal for the least abundant enantiomer in panels B and C.
DNA sequence had been eliminated. Furthermore, SDS-PAGE analysis of whole cell extracts from IPTG induced *E. coli* JM101 carrying the respective plasmids showed a prominent band at 47 kDa corresponding to the predicted size of StyA with either the original plasmid pSPW1 or pT7ST-ABm (data not shown). This excludes the possibility that the new SD sequence is the sole cause of the differences of translation efficiency between pSTFull or pSTHalf and pT7ST-ABm. No band at 47 kDa was visible with either pSTFull or pSTHalf (results not shown). Apparently, the presence of the 163-bp of wild-type DNA upstream of *styA* leads to a lack of detectable styrene oxide formation in recombinant strains.

**Regulatory functions encoded by *stySc* and *styR***. Amino acid sequence similarities for *styScR* encoded assumed or observed proteins suggested that the gene products of these two ORFs represent a two component regulatory system. With the results obtained so far in mind, it appeared likely that the two proteins together form a positive control system for styrene degradation that also alleviates the block in styrene monoxygenase synthesis, as observed in *E. coli* JM101(pSPW2). To investigate this further, we repeated the experiments with pSTFull and pSTHalf in the presence of *styScR* under *lacZp* promoter control on the low-copy-number plasmid pCKO1 (Fig. 2.3B, right panel). When *styScR* were expressed, the production of styrene oxide by StyAB from pSTFull was clearly measurable, while it was not detectable when pSTHalf was present. Part of this effect was also observed with a translational fusion of *styA* including the upstream region from the *SacI* site to a *lacZ* reporter gene. The β-galactosidase activity derived from the reporter gene was increased 21-fold in *E. coli* CC118(pCKST-ScR, pSPW20) containing *styScR* compared to *E. coli* CC118(pCKO1, pSPW20) without the regulatory genes (Fig. 2.5B, columns 1 and 2). We investigated the functions of *styScR* in more detail by providing *stySc*, *styR*, and the *styA-lacZ* fusion on different plasmids (Fig. 2.5B, columns 3 to 6). Basal activity of the reporter protein was found when only *stySc* was expressed (450 Miller units). However, expression of *styR* increased β-galactosidase activity 38-fold and expression of *stySc* and *styR* at the same time increased the activity 115-fold, relative to when *stySc* was expressed only.
Fig. 2.4. Effect of shortening or removing the 163-bp sequence upstream of styA in the presence or absence of the regulatory genes styScR. (A) Structure of the investigated plasmids. Drawing is not to scale. The stippled and hatched boxes represent the wild-type SD sequence and altered SD sequences of styA, respectively. The latter was inserted to provide translational signals. (B) Left panel: styrene oxide formation in E. coli JM101 cells carrying one of the plasmids 1 to 3, shown in panel A, measured 4 h after IPTG induction. Right panel: styrene oxide formation in E. coli JM101 cells carrying pSTFull and pSTHalf in the presence of low-copy-number plasmid pCKST-ScR expressing styScR from lacZp, measured 4 h after IPTG induction.
**DISCUSSION**

**Biotechnological potential of styrene oxide monooxygenase.** Enantioselective biotransformations of styrene to styrene oxide or kinetic resolution of racemic styrene oxide and its derivatives have received a lot of attention in recent years (241, 257, 278, 280, 384, 395). *P. putida* S12 and *Mycobacterium* sp. strain E3 were shown to produce the two possible stereoisomers of styrene oxide with enantiomeric excesses greater than 98 %, albeit with different reaction rates: 200 U*(g cdw)*-1 for a mutant *P. putida* S12 and 5 U*(g cdw)*-1 for *Mycobacterium* sp. strain E3 (257). Interestingly, *E. coli* recombinants carrying *styAB* of *Pseudomonas* sp. strain VLB120 produce the same enantiomer as the *P. putida* S12 mutant and *E. coli* recombinants carrying *xylMA* from the TOL plasmid upper pathway, the *S* enantiomer (143, 395). The enantiomeric excess of the reaction catalyzed by StyAB is more than 99 % (Fig. 2.4) and is thus significantly better than that of the reaction catalyzed by xylene oxygenase (395). Furthermore, reaction rates could be increased 8-fold compared to *E. coli* recombinants synthesizing xylene oxygenase (Fig. 2.3, Tab. 2.4) (395). These results make recombinants synthesizing StyAB promising biocatalysts for future applications.

**Biotechnological potential of styrene oxide isomerase.** A second route to optically pure styrene oxide utilizes kinetic resolution of racemic mixtures of styrene oxide. This route has been investigated especially for fungal epoxide hydrolases, where it was proven to produce excellent optical yields (278). Bearing in mind that the first step of styrene transformation in VLB120 is enantioselective and that microbial systems for the enantiospecific conversion of epoxides had been described previously (385, 386), we investigated whether the second step, conversion of the epoxide, was enantiospecific. Our results suggest that this is not the case for the *Pseudomonas* sp. strain VLB120 derived enzyme StyC. Both styrene oxide enantiomers are converted, with only slightly different reaction rates. The poor enantiomeric ratio E of 1.4 makes styrene oxide isomerase from *Pseudomonas* sp. strain VLB120 a poor enzyme for kinetic resolution of styrene oxide enantiomers. A similar observation was made for *P. putida* S12 grown on
Fig. 2.5: Involvement of stySc and styR in transcriptional regulation of sty structural genes. (A) Plasmids used for analysis. The basic replicon determining the plasmid copy-number is indicated in brackets. The hatched elements represent the styAp promoter and the part of styA that has been fused to the lacZ gene of pUJ9. Drawing is not to scale. (B) Transcriptional activity of IPTG-induced E. coli CC118 carrying different plasmid combinations. Left panel, experiment with styScR present on one plasmid; right panel, experiments with stySc and styR present on two different plasmids. Minus signs indicate that the vector without insert was present as a control; plus signs indicate the presence of the plasmid shown on the left.
phenylacetic acid, which converted (S)-styrene oxide slightly faster than (R)-styrene oxide (257).

**Genetics of styrene degradation in Pseudomonas sp. strain VLB120.**

Combination of the sequence data and biotransformation studies suggests that *Pseudomonas* sp. strain VLB120 transforms styrene to styrene oxide through the action of a two-component styrene monooxygenase, StyAB, in which StyA is the epoxidizing component and StyB might function as an enhancer of monooxygenase activity, either as a provider of reducing equivalents whose action might be substituted for to a small degree by unspecific reductases of the *E. coli* host or in an up to now poorly understood mode similar to the action of the HpaC protein of *E. coli* W (291). As a second step, styrene oxide is isomerized to phenylacetaldehyde by the styrene oxide isomerase StyC, and phenylacetaldehyde is very likely to be converted by the phenylacetaldehyde dehydrogenase StyD to phenylactic acid. Recently, a similar system from *P. fluorescens* ST has been analyzed at the sequence and functional levels (29). In the regions of the structural genes, the systems have a high degree of sequence identity (between 91.8% and 96.6%). The equivalent enzymes appear to catalyze identical reactions. To this end, it is noteworthy that although the specific activity of phenylacetaldehyde formation was high in *E. coli* JM101(pT7ST-C) (experiment 8 (Tab. 2.4)), we did not observe a specific protein band by SDS-PAGE (not shown) indicating a high specific activity of the enzyme. We assume that formation of small amounts of 2-phenylethanol, which was observed in deletion analyses, is not part of the activity of StyC. We did not detect this reaction product when *E. coli* JM101(pT7ST-C) was added to whole cell assays under conditions of excess styrene oxide. Possibly, 2-phenylethanol formation is catalyzed by enzymes of the host. The rate of 2-phenylethanol formation remained thus below the detection level when small amounts (5.6 mg* L*⁻¹) of cell material were provided but not when cells were added to 2.5 g* L*⁻¹ as was done in the deletion analyses. In agreement with this, *E. coli* JM109 was found to convert phenylacetaldehyde to 2-phenylethanol (29). Furthermore, *P. putida* S12 also appears to convert styrene oxide to phenylacetaldehyde with no formation of 2-phenylethanol (257).
**Regulation of styrene degradation.** The protein homologs of StySc and StyR (Tab. 2.3) suggest that the proteins constitute a two component regulatory system. StySc possesses sequence motifs that are typical for signal transmitting domains of sensors, especially the H-box, containing the catalytic histidine typical for its function as a histidine kinase, as well as the N, D, F, and G boxes (351). Given the small size of the protein, which leaves no space for a signal receiving domain, stySc is likely to be the truncated 3' part of a larger gene, stxS.

StyR displays high similarities over its entire length to a number of putative or proven response regulators which act at the transcriptional level (Tab. 2.3). In particular, an aspartate residue (D55 in StyR) is conserved; this residue is assumed or has been shown for the homologs of StyR to receive the phosphate residue from the cognate histidine kinases (64, 130, 206, 219). Furthermore, StyR possesses all of the 10 amino acids that are highly conserved for topological and functional reasons in the CheY superfamily of signal-receiving domains in response regulators (375). Consensus sequences for three classes of transcriptional regulators have been proposed (275), and StyR coincides at 17 of 21 positions spread over the DNA binding domain with the consensus sequence for class 3 transcriptional regulators.

**Functional analysis of the regulatory genes.** No StySc protein was detected in T7 expression experiments. Given the predicted size of StySc (27 kDa), it is possible that the band corresponding to the degradation product of β-lactamase comigrates with the respective band in the T7 expression experiment. Alternatively, the signal emitted from StySc could be too low, relative to the intensity of the β-lactamase related bands, to be detected. On the other hand, it appears reasonable to assume that stySc encodes a functional polypeptide that is produced in E. coli. It has been shown that liberated domains of transcriptional regulators in two component regulatory systems may retain their activity in a constitutive fashion (10, 239), and expression of stySc augmented transcriptional activity of the putative styA promoter when styR was expressed at the same time (Fig. 2.5).

Nevertheless, expression of StyR alone suffices to initiate transcription. In this respect, the sty system behaves similarly to the tod system of P. putida F1 (206). In the sty system as well as in the tod system, this observation implies that there is activation of the transcriptional regulator by either a noncognate histidine kinase or
by a chemical phosphorylating agent such as acetyl phosphate (231, 379). The differences in β-galactosidase activities between the experiment with \( \text{styScR} \) on one plasmid and the experiment with \( \text{stySc} \) and \( \text{styR} \) on separate plasmids (Fig. 2.5, columns 1 and 6) might be explained by differences in the copy-numbers of the vectors and promoter strength, leading to the assumption that the level of \( \text{StyR} \) is higher in cultures of recombinants carrying \( \text{pVLST-R} \) than of those containing \( \text{pCKST-R} \) (73, 151, 308). Taken together, functional and DNA sequence data suggest that \( \text{StyR} \) is a response regulator that may be transformed into its active state by phosphorylation catalyzed by intact \( \text{StyS} \) in \emph{Pseudomonas} sp. strain VLB120.

During isolation of \( \text{pSPW1} \) from the genomic library, 41 clones that contained the 5.7-kb \( \text{XhoI} \) fragment were obtained, but only 4 of them transformed styrene to phenylacetaldehyde. In the light of the results mentioned before, we assume that providing a heterologous promoter for the transcription of \( \text{styScR} \) is essential for achieving styrene monooxygenase levels sufficient for the detection of styrene oxide formation in a whole-cell assay. This implies that plasmids that contained the fragment under study in the reverse orientation relative to \( \text{pSPW1} \) or where a second insert was inserted between the 5.7-kb \( \text{XhoI} \) fragment and the \( \text{lacZp} \) promoter provided by the vector did not lead to detection of styrene oxide formation but did lead to sufficient basal activity to transform indole to indigo in the much more sensitive screening assay on LB-indole agar plates.

**Analysis of the region between \( \text{styR} \) and \( \text{styA} \).** Genes \( \text{styR} \) and \( \text{styA} \) are separated by 180-bp, which apparently prevents the use of heterologous promoters for the synthesis of considerable levels of styrene oxygenase (Fig. 2.3). From the transcriptional activity assays involving the \( \text{HincII} \) fragment containing this region (Fig. 2.5), we postulate that \( \text{StyR} \) binds to this region and initiates transcription of the downstream genes, thus constituting the \( \text{styAp} \) promoter.

To observe specific activity levels of biotechnological interest for styrene monooxygenase, we either had to present \( \text{styScR} \) together with the \( \text{styAp} \) promoter sequence or eliminate the latter completely and provide a heterologous promoter (Fig. 2.3). Interestingly, expression of the complete 5.7-kb \( \text{XhoI} \) fragment from the T7 promoter produced prominent protein bands for \( \text{sty} \) proteins whose genes
surround \textit{styA} but not for \textit{StyA} itself, indicating an inhibition of translation rather than transcription. These observations can be explained by the occurrence of predicted stable mRNA loop structures in the region from the \textit{SacI} site to the \textit{HindIII} site 78-bp downstream of the ATG codon of \textit{styA} or from the \textit{NcoI} site to the \textit{HindIII} site (Fig. 2.6). The corresponding mRNA is very likely to be produced when a heterologous promoter is present upstream of the \textit{styAp} promoter (71). Various stem-loop structures, that might interfere with initiation of translation of \textit{styA}, but not of more downstream genes, were predicted. Removal of this sequence would eliminate such structures and should allow efficient synthesis of \textit{StyAB}, which is what we have observed (Fig. 2.3, Tab. 2.4).

Production of \textit{StyAB} is also triggered when \textit{styR} is expressed. Centered around a position 75-bp upstream of \textit{styA} is an inverted repeat that includes a sequence identical to the \textit{tod} box involved in the regulation of toluene utilization in \textit{P. putida} F1 (206). Reinvestigating the similarity of the potential helix-turn-helix DNA binding domain - a subset of the DNA binding domain mentioned earlier - of TodT to that of \textit{StyR}, we found 65\% sequence identity in the core region of 43 amino acid residues that is assumed to be DNA binding (Fig. 2.6C) (275). The similarity between TodT and every other member of the \textit{StyR} homology group shown in Tab. 2.3 (ORF 2) is significantly lower (between 51.2\% sequence identity for \textit{FixJ} and 44.2\% for \textit{TutB}). Furthermore, if the \textit{tod} box like sequence is involved in \textit{StyR} binding, then deletion of a part of it should decrease transcriptional activity of the promoter in the presence of \textit{StyR}. We found that deletion of the 5' half of the \textit{tod} box-like sequence by using the \textit{NcoI} site present in the box leads to complete abolishment of styrene monooxygenase activity when \textit{styScR} are expressed in the same strain (Fig. 2.3B). We conclude that it is likely that \textit{StyR} binds to this box and induces mRNA synthesis downstream, avoiding the mRNA secondary structures. Synthesis of styrene monooxygenase from a heterologous promoter requires complete removal of this promoter sequence.

Recently, the regulatory and structural genes involved in styrene degradation in \textit{Pseudomonas} sp. strain Y2 have been cloned and analyzed (373). They display a high degree of sequence identity to the genes of \textit{Pseudomonas} sp. strain VLB120 (e.g., 90 and 96\% identity for \textit{stySc} and \textit{styR}, respectively). In particular, this study
Fig. 2.6. Analysis of the region between styR and styA. A) Schematic representation of the region as it is present in pSTFull, which contains the complete 163-bp between the SacI site and the ATG codon of styA, including the vector derived lacZp. Vector- and insert-derived sequences are indicated. Sequence length numbers refer to the two segments for which mRNA secondary structures were predicted. A putative LacZα fusion peptide is terminated by an insert derived in-frame stop codon. Known (+1, for lacZα-mRNA) or assumed (for styA-mRNA) mRNA synthesis starts are indicated. The inverted repeats of the tod box are indicated by repetitive arrow tips, while the larger repeats present in the sty system are indicated by open arrows. B) Predicted mRNA secondary structures when either the region between the SacI site upstream of the 163-bp sequence and the HindII site downstream of the ATG of styA or the region between the Ncol and the HindII site is transcribed. Free energies for the entire structures are given. Relevant sequences are boxed. The tod box sequence is printed in boldface type and the AUG codon of styA mRNA is, additionally, italicized. (C) Amino acid sequence comparison of the putative protein regions involved directly in DNA binding for StyR and TodT, with a postulated consensus sequence for the equivalent region of class 3 response regulators derived from the alignment of 19 proteins (see text). Boxed letters indicate identical residues between StyR and TodT, italicized letters indicate identical residues in all three sequences.
supports our hypothesis that \textit{stySc} represents the truncated version of a larger gene \textit{stdS}, as the equivalent gene in \textit{Pseudomonas} sp. strain Y2 consists of 982 amino acids. Furthermore, the starting point for mRNA synthesis of the structural genes in this strain was mapped between a DNA sequence box nearly identical to the \textit{tod} box and the start of the styrene monooxygenase-epoxidizing component, indicating that the assumptions we have made to explain the inhibitory effect of the \textit{styA} upstream region in heterologous systems are correct.

In the present work, we have demonstrated that \textit{E. coli} recombinants carrying cloned genes of styrene monooxygenase from \textit{Pseudomonas} sp. strain VLB120 convert styrene to (S)-styrene oxide with excellent e.e. at high reaction rates. The results presented here could significantly facilitate the development of an efficient biotransformation process for the production of enantiomerically pure styrene oxide.

\textbf{Acknowledgments}

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CHAPTER 3: AN ALKANE-RESPONSIVE EXPRESSION SYSTEM FOR THE PRODUCTION OF FINE CHEMICALS

Sven Panke, Bernard Without, and Marcel G. Wubbolts
Applied and Environmental Microbiology, 1999, 65(6):2324-2332
Summary

Membrane-located monooxygenase systems, such as the *Pseudomonas putida* mt-2 derived xylene oxygenase, are attractive for challenging transformations of apolar compounds, including enantioselective epoxidations, but are difficult to synthesize at levels that are useful for application to biotechnological processes. In order to construct efficient biocatalysis strains, we utilized the alkane-responsive regulatory system of the OCT plasmid-located *alk* genes of *Pseudomonas oleovorans* GPO1, a very attractive system for recombinant biotransformation processes, in which the activated positive regulator protein AlkS initiates transcription of the cognate *alkBp* promoter. The *alkS* gene and the *alkBp* promoter were assembled into a convenient alkane-responsive genetic expression cassette which allowed expression of the xylene oxygenase genes in a recombinant *Escherichia coli* strain at a specific activity of 91 U*(g cdw)*\(^{-1}\) when styrene was the substrate. This biocatalyst was used to produce (S)-styrene oxide in two-liquid phase cultures. Volumetric productivities of more than 2 g of styrene oxide per hour and liter of aqueous phase were obtained; these results represented a fivefold improvement compared with previous results.

Introduction

*Pseudomonas putida* mt-2 derived xylene oxygenase is encoded in the catabolic TOL plasmid pWW0 upper pathway operon, and together with a set of other enzymes it forms a catalytic cluster which degrades toluene and xylene to (substituted) benzoic acids (143, 224, 296). The ability of xylene oxygenase to hydroxylate methyl substituents on substituted benzenes or their heteroaromatic equivalents has made it an important biotechnological enzyme (190, 398). This enzyme consists of a membrane-bound component, XylM, which carries out the oxygenation step, (356) and a cytoplasmic NADH:acceptor reductase component, XylA, which supplies reducing equivalents to XylM (326). The potential of this system for the biological production of fine chemicals has already been exploited;
Fig. 3.1. Conversion of inexpensive styrene to (S)-styrene oxide by xylene oxygenase. The asymmetric carbon atom is indicated by an asterisk.

Fig. 3.2. (A) Organization and regulation of the alk genes on the OCT plasmid in *P. oleovorans* GPo1. The regulatory protein AlkS is activated by octane or DCPK and induces transcription from the alkBp promoter. The directions of transcription are indicated with open arrow heads. Transcriptional regulation of alkT has not been described. (B) Alkane degradation by enzymes encoded by alk genes. Alkanes are converted to alkanols, alkanals, and the corresponding carboxylic acids, which are coupled to coenzyme A. R indicates pentyl- to undecyl-residues. The functions of AlkF and AlkL are unclear.

Wild-type cells of *P. putida* mt-2 are used by Lonza to produce heteroaromatic acids on a commercial scale (190). Furthermore, xylene oxygenase is selective for the si-face of prochiral vinyl functions on aromatic ring systems, which leads to the formation of optically active epoxides, such as (S)-styrene oxide (395) (Fig. 3.1). *Escherichia coli* recombinants carrying the genes for xylene oxygenase have produced (S)-styrene oxide from inexpensive styrene in a 2L reactor (394). Unfortunately, so far the productivities displayed by such recombinants have been insufficient to commercially exploit their synthetic potential (142, 395).
A number of observations have indicated that expressing the xylene oxygenase genes via the alk regulatory system of *P. oleovorans* GPol might provide suitable biocatalysis strains for two-liquid phase cultures. *P. oleovorans* GPol degrades medium-chain-length alkanes with a set of enzymes encoded by two *alk* gene clusters on the catabolic OCT plasmid (Fig. 3.2A) (368). The first cluster contains the *alkBFGHJKL* operon with all but one of the structural genes for conversion of alkanes to the corresponding alkanoic acids and coupling of these compounds to coenzyme A (Fig. 3.2B). The second cluster contains the remaining structural gene, *alkT*, and the gene which encodes the regulatory protein AlkS (96). Expression of the genes in the first cluster is under control of *alkBp*, the *alk* promoter, and is initiated in the presence of functional AlkS and alkanes or other, structurally nonrelated inducers, such as dicyclopentylketone (DCPK) (132, 350). DCPK is water soluble and hence is a convenient inducer in aqueous cultures, while alkanes are useful inducers in two-liquid phase cultures which contain an organic phase. Expression of the *alk* genes in *E. coli* W3110 via the *alk* regulatory system from the low-copy-number RK2 derivative pGEC47 led to accumulation of membrane located AlkB, until it accounted for up to 10% of the total cell protein (253). This indicated that AlkS, together with its cognate promoter *alkBp*, (194) could be a powerful general system to direct synthesis of recombinant proteins. In addition, the *alk* regulatory system is not subject to catabolite repression in *E. coli* (343, 403), which allows convenient utilization of cheap carbon sources, such as glucose, in cultures of recombinant strains. Because of these attractive features of the *alk* regulatory system, we developed its components into an expression system for general use. In this paper we describe this system and its potential for efficient synthesis of xylene oxygenase for biotransformation of styrene to (S)-styrene oxide at high enantiomeric excess in a two-liquid phase biotransformation system.

**MATERIALS AND METHODS**

**Strains, media, and cultivation conditions.** The strains and plasmids used and constructed in this study are shown in Tab. 3.1. We used Luria-Bertani (LB)
complex (Difco, Detroit, Mich.) or M9 mineral medium (312) supplemented with 1 mL of one of two trace element solutions per liter; when necessary the medium was solidified by adding 1.8% agarose (Difco). Trace element solution US contained per liter 1 M hydrochloric acid 1.50 g of MnCl$_2$·4H$_2$O, 1.05 g of ZnSO$_4$, 0.30 g of H$_3$BO$_3$, 0.25 g of Na$_2$MoO$_4$·2H$_2$O, 0.15 g of CuCl$_2$·2H$_2$O, and 0.84 g of Na$_2$EDTA. Trace element solution US* also contained per liter an additional 4.87 g of FeSO$_4$·7H$_2$O and 4.12 g of CaCl$_2$·2H$_2$O. Alternatively, we used mineral medium M9*, which was identical to M9 mineral medium except that it contained three times more phosphate salts to increase the buffer capacity and did not contain calcium chloride. Glucose was added to mineral medium at a concentration of 0.5% (wt/vol) as a carbon source or to complex medium at a concentration of 1% (wt/vol) to bring about carbon catabolite repression of the lacZp promoter of cloning vectors and/or carbon catabolite repression of the synthesis of the tryptophanase of E. coli, which is involved in the formation of indole on complex media; indole can subsequently be converted to indigo by xylene oxygenase (230, 232). Antibiotics were added at the following concentrations: ampicillin, 150 μg·mL$^{-1}$; kanamycin, 50 μg·mL$^{-1}$; chloramphenicol, 30 μg·mL$^{-1}$; and tetracycline, 12.5 μg·mL$^{-1}$. When necessary, thiamine was added to a concentration of 1 g·L$^{-1}$. The cloning and DNA modification protocols used have been described elsewhere (312). Cultivation for cloning procedures was carried out at 37°C. Cultures and precultures of the E. coli strains used to determine enzyme activities or for production were grown at 30°C. Unless mentioned otherwise, cultures were induced by adding 0.05% (vol/vol) DCPK (Aldrich, Buchs, Switzerland).

**Site directed mutagenesis.** Site directed mutagenesis was performed as described previously (202) by using plasmids pGC2, plasmid pGC2Mlu (which is identical to pGC2 except that an additional MluI site is inserted by using oligonucleotide 6, (Tab. 3.2)), or one of the pGEM-7Zf plasmids as the source of the phage f1 origin. Successful mutagenesis was demonstrated by restriction analysis and functioning of the affected gene when possible. Silent mutations which removed NdeI sites were introduced into xylM and alkS by using oligonucleotides 1 and 2; the modified genes were designated xylM* and alkS*, respectively. New NdeI sites
Tab. 3.1. Bacterial strains and plasmids

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<td>pJM88</td>
<td>Suicide plasmid carrying parA, oriR6K, Ap'</td>
<td>(197)</td>
</tr>
<tr>
<td>RK600</td>
<td>oriColE1, RK2-Mob', RK2-Tra', Cm'</td>
<td>(80)</td>
</tr>
<tr>
<td>pBR322</td>
<td>Cloning vehicle, Ap', Tc'</td>
<td>(36)</td>
</tr>
<tr>
<td>pGC2</td>
<td>pBR322 derivative with M13ori, Ap'</td>
<td>(245)</td>
</tr>
</tbody>
</table>
pCKO4 Source of xylN, Cm°

pBG63 Source of xylMA, f1ori, Ap°

pGEC286 Promoter probe vector with alkBp-CAT fusion, Ap°

pGC2Mu pGC2 with additional MluI site, Ap°

pAM1 pUC18Not with phage T4 terminator of pHPI5Ω, Ap°

pAM2 pAM1 with new polylinker, Ap°


pJMSalkBp pJMS10 with alkBp-CAT of pAM2P in NorI site Km°, Ap°

pBG4 pUC18 with T4 transcriptional terminator of pHPI5Ω, Ap°

pBG4ΔN pBG4 without Ndel site, Ap°

pBG4ΔNΔ pBG4ΔN with xylM of pCKO4, Ap°

pBGL pBG4ΔNΔ with small new polylinker, Ap°


pBG11EAN pBG11 with an additional EcoRI site and removed Ndel site internal to alkS, Ap°

pUC18NΔN pUC18Not without Ndel, Ap°

pUC18NΔS pUC18NΔN with alkS° of pBG11EAN, Ap°

pSPZ1(+) pGEM-7Zf(+) with a new polylinker, Ap°

pSPZ1(-) Same as pSPZ1(+) but from pGEM-7Zf(-), Ap°

pSPZ2Not pUC18NΔS with alkBp and T4t of pBGpalk, contains alkS° alkBp T4t as a NorI cassette, Ap°

pSPZ2Sti Same as pSPZ2Not but SfiI sites instead of NorI sites, Ap°

pBG63N pBG63 with Ndel site in xylM removed and new Ndel site on the ATG of xylM°, Ap°


pBRNS pBRS22 with rnmB1 terminator and additional NorI and SfiI restriction sites, Ap°

pBRNSKm pBRNS with kanamycin resistance of mini-Tn5Km replacing the ble gene, Km°

pBRNSKmΔ pBRNSKm without remnants of the tet gene, Km°

pSPZ3 pBRNSKmΔ with alkS° alkBp xylM°A of pSPZ2MA, Km°

pSPZ4 pSPZ3 with PacI AvrII Ascl linker, Km°
Tab. 3.2. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>No.</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5' TTG TTC CCA TGT GAT TCC ACG 3'</td>
</tr>
<tr>
<td>2</td>
<td>5' TTT TTC TCA TGT GCC ACT TTA 3'</td>
</tr>
<tr>
<td>3</td>
<td>5' GTG TCC ATA TGT CCA CTT ACT 3'</td>
</tr>
<tr>
<td>4</td>
<td>5' GAG AAC ACC ATA TGC TTG AGA 3'</td>
</tr>
<tr>
<td>5</td>
<td>5' ATG GTA ATA TTG GAA TTC GTA TAA AA 3'</td>
</tr>
<tr>
<td>6</td>
<td>5' CTG GCA CGC GTC GTA GGA CGC GCA 3'</td>
</tr>
<tr>
<td>7</td>
<td>5' TAT GTT AAC GCC GCG CCC ATG 3'</td>
</tr>
<tr>
<td>8</td>
<td>5' GGG CGC GCC GTT AAC A 3'</td>
</tr>
<tr>
<td>9</td>
<td>5' AAT CTA TAA AAC GAA AGG CTC AGT CGA AAG ACT GGG CCT</td>
</tr>
<tr>
<td>10</td>
<td>5' AGC TGG CCT AGG CGG CGG CCC AAG ACA AAA GAT AAA</td>
</tr>
<tr>
<td>11</td>
<td>5' GCC GCG CCT GCA GAA TIC TCG AGA AGC TTC CCG GGA TCC</td>
</tr>
<tr>
<td>12</td>
<td>5' TGG CGC GCC TAG GAT CCC GGG AAG CTT CTC GAG AAT TCT</td>
</tr>
</tbody>
</table>

Oligonucleotides 1 to 6 were used as mutagenic primers during site-directed mutagenesis (the mutagenic nucleotide(s) is underlined). Oligonucleotides 7 to 12 were used for construction of the following polynucleotides: 7/8: NdeI/HpaI/AscI; 9/10: EcoRI/Irn4BT1/Nor/VsfI/kill HindIII; 11/12: kill ApaI/AscI/PstI/EcoRI/XhoI/HindIII/SmaI/BamHI/AvrII/AscI/NsiI. “Kill” indicates that there were compatible cohesive ends which did not regenerate the restriction site indicated.

were placed on the ATG codons of *xylM* and *alkB* by using oligonucleotides 3 and 4, and an additional *EcoRI* site was engineered 250-bp after the stop codon of *alkS* with oligonucleotide 5.

**Construction of pSPZ2 plasmids.** Plasmid pBG4 was constructed by inserting the 272-bp phage T4 transcriptional terminator of pH45Ω as an *SphI/HindIII* fragment into pUC18. The *NdeI* site was removed from plasmid pBG4 by digestion with *NdeI*, filling in with the Klenow polymerase, and religation leading to pBG4ΔN, and the plasmid received a segment that provided a new *NdeI* site necessary for subsequent cloning steps by transferring a 2.2-kb *SalI*SphI fragment spanning *xylN* of pCK04 into its polynucleotide. The resulting plasmid, pBG4ΔN, was digested with *NdeI* and *SphI*, which left the terminator intact but removed the 3'-part of *xylN*. The larger of the two fragments was ligated to a linker consisting of
hybridized oligonucleotides 7 and 8, which introduced additional HpaI and AseI sites, eliminated the SphI site between the NdeI site and the terminator, and resulted in construct pBGL.

Plasmid pGEMPalkN contained the alkBp promoter as a 2.3-kb Smal/HindIII OCT plasmid fragment with a new NdeI site on the ATG codon at the beginning of the alkB gene. The modified promoter was excised as a 273-bp Smal/NdeI fragment and was inserted into pBGL digested in the same manner; this resulted in pBGPalk, from which the xylN gene was completely removed and in which the alkBp promoter pointed towards the small polylinker with NdeI, HpaI, and AseI sites and the terminator.

The alkS\* gene was obtained as a 3.5-kb EcoRI fragment from plasmid pBG11E\DeltaN and was inserted into pUC18N\DeltaN, a pUC18Not derivative whose internal NdeI site had been removed as described above for pBG4\DeltaN. The resulting construct was designated pUC18NS\* and contained the regulator gene in the orientation opposite that of the lacZp promoter of the vector. Plasmid pUC18NS\* received the Smal/HindIII fragment containing the alkBp promoter and the T4 transcriptional terminator of pBGPalk, which resulted in pSPZ2Not. An equivalent manipulation sequence based on the cloning vector pUC18Sfi resulted in the construction of plasmid pSPZ2Sfi, which was identical to pSPZ2Not except that the segment of interest was flanked by SfiI sites rather than NotI sites.

Construction of expression plasmid pSPZ3. Plasmid pBRNS was constructed by providing pBR322 with an rrnBTl transcriptional terminator (45) and two new restriction sites by inserting a linker consisting of hybridized oligonucleotides 9 and 10 between its EcoRI and HindIII sites. In the second step, the bla gene was replaced by the kanamycin resistance gene from the kanamycin interposon of mini-Tn5Km; plasmid pBRNS was digested with EcoRI, filled in with T4 DNA polymerase, redigested with DraI, and ligated to the Smal fragment of mini-Tn5Km with the kanamycin resistance gene (which eliminated one of the two phage T4 transcriptional terminators of the interposon) in such an orientation that the new resistance gene was flanked by a different transcriptional terminator on each side (the T4t terminator from the interposon and the rrnBTl terminator from pBRNS). The resulting plasmid was designated pBRNSKm. The remaining portion of the tet
gene of pBR322 were removed by digestion of pBRNSKm with EcoRV and AvaI, T4 DNA polymerase treatment, and religation. The remaining unique BamHI site upstream of the resistance gene (derived from the fragment containing the kanamycin resistance gene) in the resulting plasmid, pBRNSKmA, was made blunt by T4 DNA polymerase treatment. An identically treated NotI/Ascl fragment of pSPZ2MA (see below) carrying alkS*, alkBp, and xylM*A, but not the T4t terminator of pSPZ2MA was inserted, which yielded plasmid pSPZ3.

**Determination of enzyme activities.** To determine enzyme activities by whole cell assays, cells were grown in M9* mineral medium precultures supplemented with glucose, kanamycin, thiamine, and trace element solution US* and then inoculated into larger cultures growing in identical medium. At an optical density at 450 nm of ca. 0.3, the cells were induced with 0.05 % (vol/vol) DCPK; the cells were harvested by centrifugation at an appropriate time (see below) and were subjected to a whole-cell assay for styrene oxide formation under optimized conditions as described elsewhere (Chapter 2). Briefly, cells were resuspended to a dry biomass concentration between 2 and 5 g per liter in 100 mM potassium phosphate buffer (pH 7.4) containing 1 % (wt/vol) glucose and incubated for 5 min with 1.5 mM styrene, and the reaction was stopped by adding ice-cold ether. The biomass concentration was selected so that at least 0.3 mM styrene was left after the transformation. One unit (1 U) of activity was defined as the amount of activity that produced production of 1 μmol of styrene oxide from styrene in 1 min. Specific activities were calculated from the measured transformation rates per unit of cell dry weight (cdw). The results given below are the averages based on three independent experiments.

**Production of styrene oxide in two-liquid phase cultures.** Freshly transformed *E. coli* JM101 cells harboring plasmid pSPZ3 were inoculated into a 5 mL LB medium preculture supplemented with kanamycin and 1 % glucose, grown overnight at 30°C, and diluted 100-fold with 100 mL of M9 mineral medium supplemented with 0.5 % glucose, kanamycin, and thiamine. The resulting culture was incubated for approximately 10 h (during which time the cells entered the stationary phase) on a horizontal shaker at 30°C and then was used as an inoculum for the biotransformation reaction mixture. The biotransformation reaction was
carried out in a stirred tank reactor with two Rushton turbine impellers, four baffles, and a total volume of 3 L. Seals and O-rings were made out of solvent-resistant Viton. The reactor contained 1.025 L of a mineral medium which contained per liter 8.82 g of KH$_2$PO$_4$, 10.85 g of K$_2$HPO$_4$, 8.82 g of Na$_2$HPO$_4$, 1.0 g of NH$_4$Cl, 0.5 g of NaCl, 0.49 g of MgSO$_4$·7H$_2$O, 14.7 mg of CaCl$_2$·2H$_2$O, 2.78 mg of FeSO$_4$·7H$_2$O, 5 g of glucose, 100 μL of polypropylene glycol 2000 (Fluka, Buchs, Switzerland), 0.5 mL of trace elements solution US, thiamine, and kanamycin. The pH was maintained at 7.1 by adding 25 % NH$_4$OH and 25 % phosphoric acid. NH$_4$OH also served as the source of nitrogen. After inoculation with 100 mL of the preculture, the reactor was aerated at a rate of 1 L·min$^{-1}$ and was stirred at 1500 rpm for ca. 12 h (overnight) which resulted in a culture in the stationary phase which contained approximately 2.2 (g cdw$^{-1}$). The medium was then supplemented with 6 mg·L$^{-1}$ thiamine, 8 mL·L$^{-1}$ of trace element solution US, and 8.2 mg·L$^{-1}$ of FeSO$_4$·7H$_2$O. Subsequently, the culture was fed at a rate of 10 mL·h$^{-1}$ with an aqueous solution containing per liter 450 g of glucose, 50 g of yeast extract (Difco), and 9 g of MgSO$_4$·7H$_2$O. One hour later, 375 mL of a mixture containing hexadecane, 1 % (vol/vol (organic phase)) octane (Sigma, Buchs, Switzerland), and 2 % (vol/vol (organic phase)) styrene (Fluka) was added to the reactor; this mixture constituted an organic second liquid phase that was dispersed in the aqueous phase. Concomitantly the stirrer speed was increased to 2500 rpm and the aeration rate was reduced to 0.67 L·min$^{-1}$ to limit foam formation. The time when the organic phase was added to the reactor was the point of induction. Furthermore, before air entered the reactor, it was passed through a wash flask containing 280 mL of the organic phase. In additional experiments, the styrene concentration in the organic liquid added varied between 0.5 and 2 % (vol/vol). Iron (9 mg·L$^{-1}$ of FeSO$_4$·7H$_2$O) was added 2.5 h after induction as described above. The dissolved oxygen tension (DOT) was always kept above 40 % saturation by varying the aeration rate. Iron sulfate was added in pulses during cultivation until the concentration was 58 μM in order to compensate for the reduced amount of yeast extract compared to our previous experiments (103, 394); the nonheme iron monooxygenase iron requirements, which were around 3 μmol·(g cdw)$^{-1}$ (344), were taken into account. The preparation started to produce large amounts of foam
within 1 h after induction; the foam was controlled by the repeatedly adding silicone oil-based antifoam agent 289 (Sigma). Cultivation was carried out twice; nearly identical results were obtained with the two preparations; the results obtained with one of the preparations are shown below.

**Analytic procedures.** The DOT was determined with an autoclavable amperometric probe (Mettler Toledo, Greifensee, Switzerland). Cell dry weights and octane, styrene, and styrene oxide concentrations in the organic phase, as well as the styrene oxide concentration in the aqueous phase of the reactor, were monitored over time. To do this, 10 mL samples were withdrawn from the reactor at regular intervals and centrifuged to separate the phases. The position of the interphase was marked, and the organic phase was removed, dried over sodium sulfate, diluted 50-fold with diethyl ether supplemented with dodecane as an internal standard, and analyzed by gas chromatography to determine the styrene, styrene oxide, and octane contents as described previously (Chapter 2). The aqueous supernatant of each centrifuged sample was also completely removed and extracted with an equal volume of diethyl ether supplemented with dodecane. The ether phase was then dried and analyzed as described above. The remaining cell pellet was resuspended in aqueous 25 mM MgSO₄, and aliquots were distributed into preweighed reaction tubes and centrifuged. Supernatants were discarded, and the tubes were incubated at 80°C until the weights were constant. The observed volumetric productivities and the observed styrene oxide formation rates were calculated as averages for intervals between two data points, taking into account volume corrections for sampling and feeding; the term “observed” refers to the fact that the activities which we measured in these biotransformation preparations were limited by substrate availability in some cases. As a result, the transformation rates given below may be underestimates of the available intrinsic enzyme activity.

**Nucleotide sequence accession numbers.** The DNA sequence of the 3.7-kb OCT plasmid fragment has been deposited in the GenBank database under accession no. X52935. The sequences of plasmids pSPZ1(+), pSPZ2Not, and pSPZ3 have been deposited under accession no. AF118920, AF118921, and AF118922, respectively.
Assembling the *alk* regulatory elements for expression. We developed an easily excisable cassette containing the *alkS* regulatory gene and the *alkBp* promoter (Fig. 3.3B). Since it has been found that the untranslated *alkB*-mRNA leader structure affects the efficiency of gene expression (193), we designed the system as an ATG expression vector with an *NdeI* site on the *alkB* start codon which allowed precise insertion of a recombinant gene. Using restriction enzyme *NdeI* (instead of the frequently used enzyme *NcoI*) left the second codon of an inserted gene unaffected, while the leader mRNA sequence remained nearly unchanged (CAA ATG changed to CAT ATG). This cassette was assembled into the polylinkers of plasmids pUC18Not and pUC18Sfi, from which the internal *NdeI* site had been removed previously; this resulted in plasmids pSPZ2Not and pSPZ2Sfi, each of which contained an *alkS* gene lacking internal *NdeI* sites, the *alkBp* promoter, and a phage T4 transcriptional terminator to prevent readthrough to regions outside the cassette. Recombinant genes could be inserted because of the *NdeI* site and two additional unique restriction sites, an *HpaI* site and an *Ascl* site. The whole segment was flanked either by *NotI* sites or by *SfiI* sites, which made the cassettes compatible with a variety of cloning and transposon vectors that have been developed over the past decade (79, 80, 161, 197).

Construction of pSPZ1 helper plasmids. To facilitate the introduction of a particular gene into the polylinker of one of the pSPZ2 plasmids, we constructed the helper plasmids pSPZ1(+) and pSPZ1(-) by digesting pGEM-7Zf(+) and pGEM-7Zf(-) with *ApaI* and *NsiI* and ligating them to a linker consisting of hybridized oligonucleotides 11 and 12, which resulted in a new polylinker flanked by *Ascl* sites (Fig. 3.3A). *Ascl* is a rarely cutting restriction enzyme with an octameric recognition sequence and is readily available from commercial sources. Thus, as an intermediate step, any fragment can be introduced into one of the pSPZ1 plasmids into the polylinker, be subjected to the necessary modifications with the phage f1 origin of replication provided by the pGEM vectors, and subsequently be excised as an *NdeI/Ascl* fragment and be inserted into pSPZ2Not.
Fig. 3.3. Structure of the central genetic elements described in this chapter. (A) pSPZ1 helper plasmid with polylinker flanked by AscI sites. (B) pSPZ2Not. We assembled in the NorI flanked polylinker of pUC18Not (i) the 2.7-kb OCT plasmid fragment with alkS* as an EcoRl fragment, (ii) the 275-bp OCT fragment with alkBp from the upstream Smal site to the transcription start site of alkB, (iii) an artificial polylinker, and (iv) the 272-bp Sphl/HindIII pHP45∆ fragment with the phage T4 transcriptional terminator. The mutated base pair is in parentheses. Useful restriction sites are indicated, as are the alkB Shine-Dalgarno sequence and the translation start site. (C) pSPZ2MA carrying the xylene oxygenase expression cassette. The modification of xylM (to xylM*) which removed the Ndel site (underlined) is shown at the bottom. The T4t transcriptional terminator is indicated by the stem-loop structure. (D) pSPZ3. Part of the xylene oxygenase expression plasmid is shown. The kanamycin resistance gene is flanked by two different transcriptional terminators. Abbreviations: As, AscI; B, BamHI; E, EcoRI; Hc, HincII; Hd, HindIII; Hp, Hpal; K, KpnI; Nd, Ndel; No, NorI; Ns, NsiI; Pa, Pael; Sc, SacI; Sm, SmaI; Xh, XhoI.
or pSPZ2Sfi. Alternatively, the desired gene can be directly amplified by PCR by using extended primers that introduce the necessary restriction sites.

**Xylene oxygenase expression controlled by the alk regulatory system.** Plasmid pBG63N is a pBG63 derivative with a 2.3-kb SmaI/HindIII fragment of the catabolic TOL plasmid pWW0 spanning the xylene oxygenase genes, which have been engineered to remove an NdeI site internal to xylM and to place another NdeI site on the ATG of xylM*. xylM*A was excised from this plasmid as an NdeI/Smal fragment and was inserted into NdeI/HpaI digested pSPZ2Not, which yielded pSPZ2MA. Thus, the whole DNA segment of interest, which consisted of alkS*, alkBp, and xylM*A plus the terminator, was available as a NotI fragment (Fig. 3.3C). We placed the cassette containing the desired elements on a medium-copy-number pBR322 derivative, which resulted in plasmid pSPZ3 (Fig. 3.3D). This plasmid contained a kanamycin resistance gene which was shielded by two different transcriptional terminators in order to limit elements of symmetry. The alkBp promoter pointed towards the phage T4 terminator, and the kanamycin resistance gene pointed towards the rrnB T1 terminator. Parts of the tetracycline resistance gene of pBR322 critical to segregational plasmid stability were removed (196). Two additional restriction sites, a NotI site and a SfiI site, were introduced, and these sites allow further easy modification of the plasmid (for instance introduction of plasmid maintenance functions (124) or proteins that extend the range of pMB1 plasmids to other bacterial genera (388)).

*E. coli* JM101 recombinants carrying plasmid pSPZ3 grown on mineral medium containing glucose as the carbon source accumulated xylene oxygenase at high specific enzyme activities (91 U*g (cdw)^{-1}, (Fig. 3.4)). Induction was rapid, and the growing cells exhibited the maximal specific xylene oxygenase activities 2 h after the inducer had been added. Glucose did not repress translation from the alkBp promoter in the growth phase, which is consistent with previous observations (343, 403).

**Production of (S)-styrene oxide by *E. coli* JM101(pSPZ3) in two-liquid phase cultures.** To investigate the efficacy of *E. coli* JM101(pSPZ3) as a biocatalyst under production conditions, we used this strain to produce (S)-styrene oxide in a two-liquid phase fed-batch cultivation experiment analogous to our previous
Fig. 3.4. Induction kinetics of styrene monooxygenase activity in *E. coli* JM101(pSPZ3) in shaking flask experiments. (A) Growth curves with and without addition of DCPK at a cell density of approximately 0.09 g*L⁻¹. (B) Styrene monooxygenase activities with and without DCPK. Open symbols, no DCPK added; closed symbols, DCPK added at the time indicated by the arrow.

Experiments performed with xylene oxygenase (394). *E. coli* JM101(pSPZ3) was first grown in a 3 L reactor containing 1.025 L of aqueous phase in batch mode in a mineral medium containing glucose as the carbon source. After the carbon source had been exhausted, we initiated a linear feed which provided (per hour) 4.5 g of glucose and 0.5 g of yeast extract. One hour later, 375 mL of the organic second liquid phase was added; this phase consisted of the carrier *n*-hexadecane, 2 % (vol/vol) styrene, and 1 % (vol/vol) *n*-octane (an inducer). Approximately 2.5 h after the organic phase had been added, a sharp increase in the DOT indicated a glucose limitation. To verify this, 10 mL of 50 % glucose were added, which resulted in an immediate decrease in the DOT; this indicated that glucose had indeed been limiting. Approximately 1.5 h after the glucose had been added, the DOT level started to increase again, and it kept increasing in an irregular fashion until the end of the experiment. Adding any of the components of the medium could not change
this tendency. The biomass concentration was about 13 (g cdw)L⁻¹ at the end of the experiment.

As shown in Fig. 3.5A, growth continued after the organic phase was added without any significant interruption. Styrene oxide formation began about 2 h later, which confirmed that the short induction period also observed in the shaking flask experiments was valid (Fig. 3.4). Styrene oxide was formed at rates between 20 and 35 U*(g cdw)⁻¹ for 3 h, during which most of the styrene was consumed (Fig. 3.5B). The calculated bioconversion rate corresponded to a peak volumetric productivity of 2 (g styrene oxide)(L₉q⁻¹h⁻¹), which was equivalent to a total transformation activity of at least 270 U*L⁻¹. At the end of the experiment approximately 15 mM styrene was left in the organic phase, while the styrene oxide concentration was 155 mM. The styrene oxide concentration in the aqueous phase reached 3.1 mM during cultivation, while styrene level in the aqueous phase decreased from 150 µM at the beginning of cultivation to levels below the detection level (5 µM).

In order to investigate the reasons for the decrease in the rate of styrene oxide formation towards the end of the experiment, we carried out several additional experiments, in which organic material was added twice to the cultures. First, styrene was added together with 1 % (vol/vol) octane and hexadecane. Later, fresh styrene without octane and hexadecane was added after nearly all of this substrate had been consumed. Fig 6A shows the effect of adding an organic phase which contained 0.5 % (vol/vol) styrene instead of the 2 % (vol/vol) styrene as used for the experiment shown in Fig. 3.5. As Fig. 3.6A shows, cells were rapidly induced, as expected, but the rate of styrene oxide formation was lower and decreased as the styrene concentration in the organic phase decreased. The second addition of styrene, which resulted in a cumulative concentration of 2 % (vol/vol) in the organic phase 4 h after induction, led to an immediate increase in the rate of product formation from 3.5 to 27.5 U*(g cdw)⁻¹, which indicated that the system was substrate limited when the styrene concentration fell below 100 mM in the organic phase and 91 µM in the aqueous phase. After the second substrate addition the styrene oxide formation rate followed the same pattern as the pattern observed after first addition; it decreased from the maximal value (ca. 28 U*(g cdw)⁻¹) to ca.
0.5 U*(g cdw)^{-1} as the organic phase styrene concentration decreased to 15 mM and the aqueous styrene concentration decreased to 14 μM. The aqueous styrene oxide concentration after the pulse increased from 0.7 to 3.2 mM. A similar effect was observed when styrene was added to a cumulative concentration of 3 % (vol/vol) 6 h after transformation had been started by the first addition of the organic phase containing 2 % (vol/vol) styrene (Fig. 3.6B). In this case, the rate of styrene oxide formation increased from 7 to 15.5 U*(g cdw)^{-1} and then decreased. The aqueous styrene oxide concentration increased from 2.1 to 3.2 mM. In both experiments, cell growth remained very similar to the cell growth in the first culture for the first 6 h after induction. When 3 % (vol/vol) styrene was added, the cells ceased to grow after the second addition of styrene.

**DISCUSSION**

*alk regulatory system on pSPZ3 as an expression system.* Previous studies did not identify failed to deliver highly active recombinant biocatalysts that synthesize xylene monooxygenase. One reason for this might be that the biocatalysts used were inadequately designed; high-copy-number plasmids like pGEM-derived pBG63 have been shown to have detrimental effects on host physiology and are particularly unstable when inefficient antibiotic selection is used (34, 309, 394), and heat shock induction might interfere with protein function and plasmid stability (160, 398). Consequently, we designed plasmid pSPZ3 in order to avoid detrimental influences on gene expression based on predictable structural or segregational instability while a simple and effective regulatory system for gene expression is utilized. Our procedure included the use of transcriptional terminators to shield transcriptionally active regions (53, 346), elimination of elements of symmetry in the transcriptional terminators, deletion of known critical regions from the plasmid (196), and utilization of a kanamycin resistance gene instead of the ampicillin resistance gene (150). Use of a medium-copy-number plasmid based on pBR322 in combination with an efficient regulatory system should allow construction of a highly active biocatalyst while the physiological burden of multicopy plasmid DNA
replication is reduced. The *alk* regulatory system has been used previously to express the membrane component of alkane hydroxylase, AlkB, at levels equivalent to up to 10% of total cell protein (253). It appeared that this regulatory system could also work well for functional expression of the *xylMA* genes. Furthermore, induction by octane is easy to achieve in a two-liquid phase culture, and the system is not subject to catabolite repression by glucose in *E. coli*, which is why we used this regulatory system for expression. The resulting plasmid, pSPZ3, could be used very efficiently in shaking flask experiments for xylene oxygenase synthesis; we could express *xylM*A in *E. coli* JM101 hosts at a specific activity of 91 U*(g cdw)*⁻¹ on a minimal medium containing glucose as the carbon source (Fig. 3.4), which is a substantial improvement over the values for recombinant strains reported previously (395).

**Kinetic properties of xylene oxygenase.** The decreases in the observed rates of styrene oxidation as the styrene concentrations in the organic phases of the 1.5 L two-liquid phase cultures decreased were due at least in part to kinetic properties of the xylene oxygenase rather than to physiological limitations of the biocatalysis strain. We reached this conclusion on the basis of the increase in styrene oxide formation when styrene was added (Fig. 3.6). In principle, this could have been due to one (or both) of two reasons; either the half-saturation constant of xylene oxygenase for styrene was rather high, or accumulation of product in the aqueous phase led to reversible product inhibition. Based on the experiments whose results are shown in Fig. 3.5 and 3.6, the organic styrene concentration at which product formation proceeded at the half maximal rate appeared to be on the order of 45 mM. Since the partition coefficient for styrene in our organic phase-aqueous medium system was approximately 1100 at the beginning of cultivation, this suggests that the half saturation constant value was approximately 40 μM. For comparison, *m*-xylene grown *P. putida* mt-2 cells synthesizing xylene oxygenase have been reported to have a half-saturation constant value of 8 μM for toluene. They have also been reported to exhibit product inhibition during conversion of *m*-xylene to 3-methylbenzylalcohol (95). Given that the aqueous styrene oxide concentrations at the times that styrene was added in the experiments shown in Fig. 3.6A and B were 0.7 and 2.1 mM, the possibility that reversible product
Fig. 3.5. Production of (S)-styrene oxide by *E. coli* JM101(pSPZ3). The culture was grown in a two-liquid phase medium which contained 25% (vol/vol) hexadecane containing 1% (vol/vol) octane as an inducer and 2% (vol/vol) styrene as the substrate. The organic phase was added 1 h after initiation of feeding (arrow). (A) Concentrations of styrene, octane, and styrene oxide in the hexadecane phase and of styrene oxide in the aqueous phase. (B) Formation of *E. coli* JM101(pSPZ3) biomass (open symbols) and development of productivities (solid symbols). Styrene oxide formation was calculated by determining the total rate of styrene oxide formation per g cdw. Volumetric productivity was calculated by determining the mass of styrene oxide formed per hour per liter aqueous phase (L_{aq}).
Fig. 3.6. Behavior of *E. coli* JM101(pSPZ3) in two-liquid phase cultures which received styrene pulses. Styrene was added at the times indicated by the arrows. Samples were taken immediately before and after the additions. (A) Culture started with 0.5 % (vol/vol) styrene in the organic phase. The cumulative concentration of styrene was 2 % (vol/vol). (B) Culture started with 2 % (vol/vol) styrene in the organic phase. Enough styrene was added so that the cumulative concentration was 3 % (vol/vol).
inhibition occurred cannot be eliminated. However, it appears likely that toxification of the biocatalyst eventually also started to play a role in limiting the specific activity of the biocatalyst, as cell dry weight ceased to increase after the concentration of styrene added reached a cumulative amount of 3 % (vol/vol) in the experiment shown in Fig. 3.6B, indicating that the combined influence of styrene and styrene oxide impaired cell growth.

**Recombinant whole cells which synthesize xylene oxygenase as a suitable biocatalyst in two-liquid phase cultures.** Expression of the xylene oxygenase genes with the *alk* regulatory system in recombinant strains resulted in whole-cell biocatalysts that had a very high specific activity, more than 90 U*(g cdw)*⁻¹, in shaking flask experiments, which is a remarkably high value for a recombinant monooxygenase. Furthermore, the activity was easy to induce during growth of the recombinants on inexpensive carbon sources like glucose. By studying the effects of improvements, we developed a simple two-liquid phase fed-batch process for production of (S)-styrene oxide which converts 90 % of the supplied styrene, and the observed volumetric productivity is more than 1 g of (S)-styrene oxide per liter of aqueous phase over a period of at least 4 h. Cells produced styrene oxide at a rate of at least 30 U*(g cdw)*⁻¹ when the culture contained 9 (g cdw)*L⁻¹. This led to a volumetric activity that was fivefold higher than the activity reported previously (394).

Two-liquid phase bioprocesses, such as the one described here, represent a feasible technology with considerable economic potential for production of hydrophobic compounds in reactions when either the substrate or the product is toxic to cells (391). The expression tools described here will help to integrate new challenging enzymes into these processes, thus resolving bottlenecks due to low biocatalyst activity.

**ACKNOWLEDGMENT**

This work was supported by the Swiss Priority Program Biotechnology.

We are indebted to Víctor de Lorenzo for providing strains and ideas, to Fernández Rojo for sharing results prior to publication, to Andreas Schmid and Birgit Kessler
for helpful discussions, and to Hans-Jürgen Feiten, Andrew Schmid, and Martina Röthlisberger for help with cultivation and sequencing.

APPENDIX TO CHAPTER 3

A GENERAL PLASMID BASED SYSTEM FOR EXPRESSION OF HETEROLOGOUS GENES WITH THE alk REGULATORY SYSTEM

SVEN PANKE, ANDREAS MEYER, MARCEL G. WUBBOLTS, AND BERNARD WITHOLT

The alk regulatory system of P. oleovorans GPo1 has been used to express a number of genes. Apart from the results on xylene oxygenase presented in this chapter and the well investigated very efficient expression of the membrane located AlkB, it has been used to successfully direct the synthesis of the styrene monooxygenase of Pseudomonas sp. strain VLB120 (see Chapter 4) and the PHA polymerase C1 of P. oleovorans GPo1 (300). Together with the structural genes whose transcription it usually regulates, the alk regulatory system has been extensively used for biotechnological applications like bioplastic and fine chemical production (38, 81, 101, 102, 153, 183, 288, 289, 307, 366, 394). Recently, some insight into the molecular details of the interaction of the positive regulator protein AlkS with its cognate promoter alkBp have become available (50, 343, 403). Taken together, the alk regulatory system is developing into a well understood and biotechnologically useful expression system. This prompted us to provide a number of tools that should facilitate the further study and utilization of the alk system in E. coli.
Materials and Methods

Strains, media, and cultivation conditions. Strains and plasmids used and constructed in this study are included in Tab. 3.1. Protocols were followed as described already in this Chapter. Selection with strains that carried an alkBp-CAT fusion in the chromosome was performed at a chloramphenicol concentration of 15 μg⋅mL⁻¹.

Construction of pSPZ4. Expression plasmid pSPZ3 was digested with *PacI* (inactivating *alkS*) and *BamHI* and the resulting fragment was ligated to a small polylinker (*PacI*/AvrII/Ascl/kill BamHI, 5′ TAACCTAGGGGCAGCGGCCCTCCCTAGGTTAAT) that maintains the *PacI* site inside *alkS* next to an *AvrII* site and an *Ascl* site and destroys the *BamHI* site. This *BamHI* site had been created in the process of ligating the blunt-ended *NotI*/*Ascl* fragment of pSPZ2MA into pBRKmA (see above). *PacI*, like *Ascl*, is a rarely cutting restriction enzyme with a recognition sequence of 8 nucleotides and is also readily available from commercial sources. The resulting construct was called pSPZ4 (Fig. 3.7A).

Strains to select for successful construction of pSPZ4 expression plasmids. Plasmid pUC18Not was equipped with the phage T4 transcriptional terminator as in pBG4 to give pAM1 and provided with a new polylinker (kill EcoRI/SmaI/HpaI/kill *PstI*) by hybridizing an *EcoRI/PstI* digest of pAM1 to a linker of two hybridized oligonucleotides (5’ AATTTCCCGGGTTAACTTGCA 3’ and 5’ AGTTAACCCGGGA 3’) leading to pAM2. An alkBp-CAT fusion which covers a 185-bp OCT plasmid *SmaI/TaqI* fragment of the *alkBp* was excised from plasmid pGEc286 by *XmnI* digestion and inserted into the *SmaI* site of pAM2 to yield pAM2P. The fusion was reexcised as a *NotI* fragment and inserted into the *NotI* site of mini-Tn5 delivery plasmid pJMS10. The resulting plasmid was called pJMSalkBp. Mini-Tn5 transposon mutagenesis with *E. coli* CC118pir(pJMSalkBp) as the donor, *E. coli* JM101 as the recipient, and *E. coli* HB101(RK600) as the helper strain was performed as described elsewhere (80, 197). This protocol led to JM101 exconjugants which were resistant to kanamycin and chloramphenicol and sensitive to ampicillin, presumably due to the presence of the helper plasmid RK600 in the exconjugants. To lose this plasmid, we transduced the alkBp-CAT fusion
together with the kanamycin resistance gene by phage P1 transduction into fresh JM101 or CC118 cells (234). The kanamycin resistance was subsequently removed from the resulting strains JM101alkBp-CAT and CC118alkBp-CAT as described by Kristensen et al. (197). In short, plasmid pJMSB8 with the parA gene encoding the RP4 res site-specific resolvase was conjugated from E. coli S17-1λpir into the target strains and the resolvase mediated the deletion of the resistance gene flanked by res sites from the chromosome. The new strains JM101alkBp-CAT2 and CC118alkBp-CAT2 could be used to select in ligation mixtures for pSPZ4 derived plasmids carrying a reconstituted alkS gene. Transformations were plated onto solidified LB containing only chloramphenicol. Octane was provided as an inducer via the gas phase. Optimum transformation results were obtained when 0.02% DCPK (vol/vol) was added to the cell suspension 30 min after the heat shock and incubation at 37°C continued for another hour before selective plating.

RESULTS

A general plasmid based system for expression of heterologous genes through the alk regulatory system. Encouraged by the promising results with xylene oxygenase expression, we developed plasmid pSPZ3 into a more general system so that it could accommodate fragment that have been cloned into pSPZ2Not or pSPZ2Sfi. We digested pSPZ3 with PacI (inactivating alkS) and BamHII and ligated the resulting fragment to a small polylinker that maintained the PacI site inside alkS next to an AvrII site and an AscI site (Fig. 3.7B). PacI, like AscI, is a rarely cutting restriction enzyme with a recognition sequence of 8 nucleotides, and it is also readily available from commercial sources. The resulting construct was called pSPZ4. A gene can conveniently be excised from pSPZ2Not or pSPZ2Sfi as a PacI/AscI fragment (provided these two sites are not present in the to-be excised fragment, which is unlikely as the enzymes are 8-cutters) and inserted into identically treated pSPZ4 directly. To facilitate the necessary selection steps during cloning, we constructed strains especially useful for cloning of a functional alkS gene: a fusion of the alkBp promoter to the chloramphenicol resistance gene of
Fig. 3.7. A selection system to construct \textit{alk}-regulatory system based expression vectors for \textit{E. coli} recombinants. (A) Map of helper plasmid pSPZ4. Stem and loop structures represent transcriptional terminators. Important restriction sites are indicated. The \textit{alkS}\textsuperscript{*} gene is not complete, only the 3' part of it is present on the vector. (B) System to select for expression plasmids. A \textit{PacI}/\textit{Ascl} fragment covering the 5' part of \textit{alkS}\textsuperscript{*} and the \textit{geneX} is excised from a pSPZ2Not based plasmid and inserted into pSPZ4, where it reconstitutes the \textit{alkS}\textsuperscript{*} gene. Thus functional, the \textit{alkS}\textsuperscript{*} gene allows transcription of the chromosomally located chloramphenicol resistance from the \textit{alkBp} promoter. Transformants with a complete expression vector are resistant to kanamycin and chloramphenicol in the presence of an inducer of the \textit{alk} system. For abbreviations, see Fig. 3.3.
plasmid pKK232.8 (44) was inserted into the chromosome of *E. coli* CC118 and *E. coli* JM101 via the mini-Tn5 transposon delivery plasmid pJMSalkBp. In these strains, one can easily select for successful insertion of the desired gene into plasmid pSPZ4 by exposing the cells to octane in the gas phase during selection after transformation, because only plasmids with a reconstituted *alkS*, indicating insertion of the desired gene, allow activation of the *alkBp* promoter for chloramphenicol resistance (Fig. 3.7B). As the resistance gene of the transposition has been removed again, this selection system is not limited in its capacity to support various antibiotic resistance plasmid markers, but chloramphenicol. To prove the efficiency of the selection, we excised the *xylE* gene from plasmid pSPZE (Chapter 6) as a *PacI/AscI* fragment and ligated the fragment without isolation to equally digested pSPZ4. Successful insertion of the fragment into pSPZ4 was selected for in the presence of chloramphenicol, kanamycin, and octane. Resulting colonies were sprayed with catechol. 95% of the transformants turned yellow, and of a set of ten, all harbored the desired plasmid construct pSPZ12.
CHAPTER 4: PRODUCTION OF ENANTIOPURE STYRENE OXIDE BY RECOMBINANT *ESCHERICHIA COLI* SYNTHESIZING A TWO-COMPONENT STYRENE MONOOXYGENASE

SVEN PANKE, MARCEL G. WUBBOLTS, ANDREAS SCHMID, AND BERNARD WITHOLT

SUBMITTED
**Summary**

A whole cell biocatalytic process was developed to enable the efficient oxidation of styrene to chiral (S)-styrene oxide with an enantiomeric excess better than 99%. Recombinant *Escherichia coli* cells were employed to express the genes *styAB* encoding the styrene monooxygenase of *Pseudomonas* sp. strain VLB120 from an expression plasmid utilizing the *alk* regulatory system of *P. oleovorans* GPol. The strains reached specific activities of up to 70 U/(g cell dry weight) in shaking flask experiments with glucose as the carbon source. An efficient two-liquid phase fed-batch process was established for the production of (S)-styrene oxide with hexadecane as an apolar carrier solvent and a nutrient feed consisting of glucose, magnesium sulfate, and yeast extract. Engineering of the phase ratio and the composition of organic phase and feed led to a 2 L-scale process with maximal volumetric productivities of 2.2 g (S)-styrene oxide per liter liquid volume equivalent to a total transformation activity of 300 U per liter. This optimized process was based completely on defined medium and used bis(2-ethylhexyl)phthalate as the apolar carrier solvent, which together with substrate and inducer consisted of 50% of the total liquid volume. Using this system, we were able to produce per liter liquid volume 11 g of enantiopure (S)-styrene oxide in 10 h.

**Introduction**

Asymmetric oxidations have been a focus of organic synthetic research for the past decade (60, 177, 260, 328) with epoxides as one group of desirable targets since they are versatile chemical building blocks (115). This is reflected in the widespread use of the Sharpless procedure for optically active epoxides from allylic and homoallylic alcohols, and in the Jacobsen/Katsuki procedure starting from unfunctionalized alkenes (348), which frequently yield enantiomeric excesses (e.e.'s) over 90% (32, 177). The stereospecificity of enzymatic reactions has often been suggested to provide useful alternatives to purely chemical approaches due to
One attractive route to access enantiomerically pure epoxides (e.e. >99 %) in a one-step reaction with a theoretical yield of 100 % is the epoxidation of double bonds by monooxygenases (268). This is best realized with whole cells to facilitate cofactor regeneration. However, this approach has to overcome a number of challenges: The epoxide product might be toxic to the cell as well as inhibitory to
the enzyme (117, 137, 290). Also, the substrate of the reaction might be toxic to the cell (395). Furthermore, it has proven difficult to reach satisfying specific activities and as a consequence total volumetric productivities (268), although very impressive rates of up to 500 μmol×min⁻¹×g (cell dry weight)⁻¹ (U×(g cdw)⁻¹) have been reported in isolated cases for short conversion times in the range of minutes for the formation of racemic propene oxide (345). Finally, product catabolism by the cell reduces product yields (76, 134, 168, 268, 357).

In situ product recovery by an organic second liquid phase has been suggested as a solution to product toxicity (108, 268, 392). This strategy also circumvents substrate toxicity by serving as a substrate reservoir while the aqueous concentration remains low (117, 395). Low specific activities and product consumption might be addressed by using recombinant microorganisms which overexpress enzymes of choice. We have found Escherichia coli to be an interesting host for such purposes, since given the detailed knowledge of its genetics, it has been possible to synthesize multicomponent monooxygenases to attractive specific activities (Chapter 3). In addition, the intestine-adapted metabolic capabilities of E. coli make it unlikely that organic products of interest will be subject to significant further utilization and consequent loss (101).

An interesting chiral oxidation product is (S)-styrene oxide, a useful building block used in the synthesis of the nematocide levamisole (149). We have produced enantiomerically enriched (S)-styrene oxide utilizing the Pseudomonas putida mt-2 derived xylene oxygenase in a two-liquid phase process (394). Further improvement of product e.e. and system performance remain necessary however. We have recently isolated the genes styAB encoding a novel two-component styrene monooxygenase from Pseudomonas sp. strain VLB120 that produces (S)-styrene oxide from styrene with an enantiomeric excess of over 99 % (Chapter 2), thus permitting biocatalytic synthesis of enantiopure styrene oxide (Fig. 4.1A). Furthermore, we could establish an expression vector that allowed the expression of the xylene oxygenase genes to levels resulting in 90 U×(g cdw)⁻¹ in shaking flask experiments in mineral medium indicating that it is possible to construct recombinant strains that synthesize even complex monooxygenases to high specific activities (Chapter 3). In this report, we investigated the possibility of
integrating the various elements into a two-liquid phase process for the production of enantiopure (S)-styrene oxide.

**Materials and Methods**

**Genetic procedures.** We followed standard protocols (312). To construct expression plasmid pSPZ10 (Fig. 4.1B), the genes *styAB* were transferred as an *EcoRI/Smal* fragment from plasmid pT7ST-ABm (Chapter 2) to plasmid pSPZ1(+) and from there as an *NdeI/AscI* fragment into plasmid pSPZ2Not (Chapter 3). There, the *styAB* genes were part of an expression cassette which contained the regulatory gene *alkS* and the cognate promoter *alkBp* controlling transcription of *styAB*. This cassette was excised from the resulting plasmid pSPZ2AB as a *NotI/AscI* fragment, made flush, and inserted into *BamHI* digested and then blunt-ended pBRNSKmΔ (Chapter 3) to yield pSPZ10. The *P_{tac}* promoter of plasmid pSPZ11 (Fig. 4.1B) was obtained as a *BglII/BamHI* fragment via PCR from plasmid pVLT33 (78) (using the primers P1

\[5'\ CCAAGATCTCGACTGCACGGTGTCACCAATGCTTCTG 3' \]

introducing a new *BglII* site (underlined) upstream of the promoter and P2

\[5'\ CAAGCTTGCATGCCTGCAGGTCGACTCTAG 3' \]

completely homologous to a region of pVLT33 downstream of the polylinker) and inserted into the *BamHI* site of pBRNSKmΔ. This plasmid, pBRPtac, received *styAB* as an *EcoRI/HindIII* fragment from plasmid pT7ST-ABm and thus resulted in pSPZ11. *E. coli* JM101 (*supE thi Δ(lac-proAB) F'[traD36 lacIq Δ(lacZ)M15 proAB]*) (312) was transformed with either of the two plasmids and the resulting strains were used in our experiments.

**Shaking flask experiments.** Details such as the composition of M9* medium and US* trace element solution have been described (Chapter 3). All experiments apart from cloning procedures were carried out at 30°C. Where necessary, thiamine hydrochloride was added to a final concentration of 10 mg*L⁻¹* and kanamycin to a final concentration of 50 mg*L⁻¹*. Inocula were grown in 5 mL of Luria-Bertani broth (312) supplemented with kanamycin and glucose at 1 % (wt/vol, to prevent
indigo formation) and diluted 100-fold into 100 mL of M9 mineral medium with glucose as the carbon source at 0.5 % (wt/vol), 100 µL of US solution, thiamine, and kanamycin in 500 mL baffled Erlenmeyer flasks. The resulting preculture was grown to the stationary phase and diluted into flasks with 100 mL of medium with an identical composition. The initial OD$_{450}$ was adjusted to 0.1 corresponding to a cell dry weight concentration of 0.03 g*L$^{-1}$. Cultures were grown in a shaker at 200 rpm to an OD$_{450}$ of 0.4 and induced, where necessary, by the addition of 0.05% (vol/vol) dicyclopropylketone (DCPK, Aldrich, Buchs, Switzerland) or 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). At regular intervals, aliquots were withdrawn and the cells were subjected to a whole-cell styrene oxide formation assay (Chapter 2). In short, the cells were centrifuged and resuspended to around 2.5 (g cdw)*L$^{-1}$ in a buffer containing 1% glucose and incubated for 5 min with 1.5 mM styrene. The reaction mixture was extracted with ether and analyzed by gas chromatography.

**Two-liquid phase cultures.** A stirred tank reactor with a total volume of 3 L (for details, see Chapter 3) was used in all reactor experiments. The aqueous medium in the cultivations consisted of per liter aqueous medium 8.82 g KH$_2$PO$_4$, 10.85 g K$_2$HPO$_4$, 8.82 g Na$_2$HPO$_4$, 1.0 g NH$_4$Cl, 0.5 g NaCl, 0.49 g MgSO$_4$*7H$_2$O, 5 g glucose, 100 µL polypropylenglycol 2000 (Fluka, Buchs, Switzerland), 1 mL of the US solution of trace elements, thiamine, and kanamycin. The pH was maintained at 7.1 by addition of 25% NH$_4$OH and 25% phosphoric acid. The addition of NH$_4$OH provided sufficient nitrogen after the initial ammonium had been used up. Usually, 1.025 L of this medium were inoculated with a 100 mL preculture (see above). Incubation continued for around 12 hours (overnight) at a stirrer speed of 1500 rpm and an aeration of 1 L*min$^{-1}$. Under these conditions, the culture grew to a density of about 2 (g cdw)*L$^{-1}$ in approximately 7.5 h and remained in the stationary phase for the rest of the time. Then, the culture was supplemented with (per liter) 4 mL of US* trace element solution and 4 mL of a 1% (wt/vol) thiamine solution. A feed was initiated at 10 mL*hr$^{-1}$ consisting of 45% (wt/vol) glucose and 9 g*L$^{-1}$ MgSO$_4$*7H$_2$O, adjusted to pH 3 with hydrochloric acid. Alternatively, the feed contained an additional 50 g*L$^{-1}$ yeast extract (Difco, Detroit, USA). One hour later, 375 mL of the organic phase resulting in a phase ratio of 0.25 (volume of the
organic phase relative to the total liquid volume) were added. The organic phase consisted of n-hexadecane (99 % pure, Sigma, Buchs, Switzerland) or bis(2-ethylhexyl)phthalate (BEHP, 97 %, Fluka, Buchs, Switzerland) as a carrier solvent, which contained 1 % (vol/vol of organic phase) n-octane as the inducer of the alk regulatory system and 2 % (vol/vol of organic phase) styrene (99 %, Fluka) as the substrate. Concomitantly, the stirrer speed was increased to 2500 rpm and the air flow was reduced to 0.7 L*min⁻¹ to reduce foam formation. Addition of the organic phase to the reactor served as the time point of induction. Foam formation was limited by the addition of antifoam 289 (Sigma, Buchs, Switzerland). When the culture was run at a phase ratio of 0.5, the volume of the aqueous phase was 1 L at the start of the experiment and 1 L of organic phase was added.

**Process analytics.** Details of the reactor analytics and the chemical analytics have been described previously (Chapter 3). In short, in the reactor, dissolved oxygen tension (DOT) was measured and pH, temperature, and stirrer speed were regulated automatically. For chemical analysis, aliquots were withdrawn from the reactor at regular intervals and aqueous and organic phases were separated. Styrene, styrene oxide, octane, and 2-phenylethanol concentrations of the organic phase and styrene, styrene oxide and 2-phenylethanol concentrations in the aqueous phase were followed by gas chromatographic analysis of the organic phase or of ether extracts of the aqueous phase. Cell dry weight concentrations were determined gravimetrically after drying cell pellets in preweighed tubes until the weight was constant. Productivities and activities were determined from the changes in styrene oxide concentrations between two sampling points and represent an average value for this time period. Where necessary, the presence of phenylacetaldehyde in samples was investigated by HPLC (see below). The identity of 2-phenylethanol was established by gas chromatography-mass spectrometry. Enantiomeric excess of the produced styrene oxide was determined as described (Chapter 2).

**Determination of partition coefficients.** Varying concentrations of styrene or styrene oxide in n-hexadecane or BEHP were incubated with M9* medium and allowed to equilibrate under vigorous shaking. The phases were separated and the organic phase was diluted into ether and analyzed by gas chromatography. To the aqueous phase, the same volume of acetonitrile was added and the mixture was
analyzed by HPLC on a Merck/Hitachi system equipped with a C18 HD column (250 mm*4 mm inner diameter, Machery-Nagel, Oensingen, Switzerland) connected to a UV detector set to 210 nm. A mixture of 59.9 % acetonitrile, 0.1 % phosphoric acid, and 40 % of 10 mM potassium phosphate buffer (pH 7.5) served as the eluent at a flow rate of 1 mL*min⁻¹. The corresponding concentrations in the two phases were determined and the partition coefficient was calculated for the concentration range showing a constant quotient of the two concentrations.

RESULTS

The *alk* regulatory system provides an efficient expression system for styrene monooxygenase. *E. coli* JM101(pSPZ10) accumulated styrene monooxygenase to a specific activity of 70 U*(g cdw)*⁻¹ in shaking flask experiments with glucose as the carbon source after induction by DCPK (Fig. 4.2D). Whereas addition of the inducer to cultures of *E. coli* JM101 hardly influenced the growth rate (Fig. 4.2C), it was significantly reduced when the cells expressed the recombinant genes (Fig. 4.2A and B). Styrene oxide formation remained below our detection limit of 0.05 U*(g cdw)*⁻¹ in cultures without addition of DCPK (Fig. 4.2D and F). Maximal levels of activity were as high as when *styAB* was expressed from the *P_{lac}* promoter of pSPZ11 (Fig. 4.2E), which is a standard promoter used for overexpression of recombinant genes (221), but the activity of uninduced cultures was also significantly higher. The two plasmids use the same origin of replication, so it is unlikely that these results are influenced by plasmid copy-number effects. Thus, the use of an *alk* based system allows a very clear on/off response to inducers. Moreover, *E. coli* JM101 recombinants carrying pSPZ10 maintained a useful level of activity over a period of 6 h including a period after growth had ceased, while the level of styrene monooxygenase activity decreased rapidly in recombinants carrying pSPZ11. Consequently, we used the *alk* system based expression plasmid pSPZ10 for styrene monooxygenase for further experiments.
**E. coli JM101(pSPZ10)** efficiently produces styrene oxide in two-liquid phase cultures with hexadecane as organic carrier solvent. *E. coli JM101(pSPZ10)* was grown in 1.125 L mineral medium with 0.5% glucose as the carbon source to 2 (g cdw)·L·1 before a feed of glucose, yeast extract and magnesium sulfate was initiated. After one hour, 375 mL organic phase consisting of hexadecane, 2% (vol/vol of the organic phase) styrene, and 1% (vol/vol) *n*-octane as an inducer of the *alk* system were added, resulting in foaming of the culture which was kept at an acceptable level (around 0.5 L) by the addition of silicone oil based antifoam. Cells continued to grow linearly over the addition of the organic phase to a final
Tab. 4.1. Summary of characteristic process parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>organic phase feed composition phase ratio</td>
<td></td>
</tr>
<tr>
<td>hexadecane yeast extr. 0.25</td>
<td>hexadecane no yeast extr. 0.25</td>
</tr>
<tr>
<td>final [Sty]$_{org}$</td>
<td>mM</td>
</tr>
<tr>
<td>final [Stox]$_{aq}$</td>
<td>mM</td>
</tr>
<tr>
<td>final [Oct]$_{aq}$</td>
<td>mM</td>
</tr>
<tr>
<td>final [2Pe]$_{org}$</td>
<td>mM</td>
</tr>
<tr>
<td>final [Stox]$_{aq}$</td>
<td>mM</td>
</tr>
<tr>
<td>final [2Pe]$_{aq}$</td>
<td>mM</td>
</tr>
<tr>
<td>final [cdw]$_{aq}$</td>
<td>g/L$_{aq}^{-1}$</td>
</tr>
<tr>
<td>max. spec. activity</td>
<td>U*(g cdw)$^{-1}$</td>
</tr>
<tr>
<td>max. total activity</td>
<td>U* L$_{aq}^{-1}$</td>
</tr>
<tr>
<td>max. vol. product.$^a$</td>
<td>g/L$_{aq}^{-1}$</td>
</tr>
<tr>
<td>average activity.$^b$</td>
<td>U* L$_{tot}^{-1}$</td>
</tr>
<tr>
<td>total liquid volume.$^c$</td>
<td>L$_{tot}$</td>
</tr>
<tr>
<td>prod.$^d$ styrene oxide</td>
<td>g</td>
</tr>
<tr>
<td>yield $^e$</td>
<td>%</td>
</tr>
<tr>
<td>transformation time</td>
<td>h</td>
</tr>
</tbody>
</table>

(from induction on)

Sty: styrene; stox: styrene oxide; Oct: octane; 2Pe: 2-phenylethanol; cdw: cell dry weight. Subscripts: org: organic phase; aq: aqueous phase. BEHP: bis(2-ethylhexyl)phthalate. n.d.: not determined. $^a$: productivity; $^b$: determined for the time from induction on. $^c$: volume at the time of induction. $^d$: produced concentration of 8.8 (g cdw)*L$_{aq}^{-1}$ (Fig. 4.3A). Cell growth ceased around 4 h after addition of the organic phase, corresponding to an increased tendency of the culture to foam and an increase in DOT suggesting that cells suffered from the presence of at least one of the various organic compounds. Styrene oxide formation commenced rapidly after induction, resulting in a styrene oxide formation of over 40 U*(g cdw)$^{-1}$ and maximal volumetric productivities of 2.4 (g styrene oxide)*L$_{aq}^{-1}$ corresponding to total activities of 340 U* L$_{aq}^{-1}$ or 255 U* L$_{tot}^{-1}$ (Fig. 4.3A) and an
Fig. 4.3: Two-liquid phase fed-batch culture of *E. coli* JM101(pSPZ10) with n-hexadecane as the organic carrier solvent containing 2% (vol/vol) styrene and 1% (vol/vol) octane at a phase ratio of 0.25. Addition of organic phase occurred at the time indicated by the arrow. Symbols are explained in the figure. A) Feed with yeast extract, glucose, and magnesium sulfate. B) Feed without yeast extract. Upper panel: Concentrations of styrene, styrene oxide, and octane in organic phase. Middle panel: Concentrations of styrene oxide, 2-phenylethanol, and cell dry weight, in aqueous phase. Lower panel: Styrene oxide formation rate and volumetric productivity (proportional to total activity).
average activity of 133 U*L⁻¹*h⁻¹. The styrene oxide formation remained high for three hours and then decreased abruptly, yielding a final styrene oxide concentration of 137 mM in the organic phase. No styrene was left in the organic phase at the end of the experiment, indicating that the abrupt decrease was probably due to limited styrene availability. Conversion of styrene took maximally 4.3 h after addition of the organic phase. The final concentrations of substrates and (by-)products are summarized in Tab. 4.1. Different from experiments with xylene oxygenase (Chapter 3), 2-phenylethanol accumulated in the aqueous phase to 6.5 mM (the organic phase was not investigated for 2-phenylethanol in this set of experiments).

Optimization of process parameters: feed composition. To investigate the effect of yeast extract on the performance of the culture, we repeated the two-liquid phase culture detailed above omitting the yeast extract in the feed. As shown in Fig. 4.3B and Tab. 4.1, the results are similar to the data presented above, although the development of styrene oxide formation rates occurred somewhat irregularly with a peak ca. 3 h after induction and at a slightly lower average activity over the transformation period. Small amounts of 2-phenylethanol accumulated in the organic phase and the culture still showed a strong tendency to foam. As in the previous culture, the DOT level began to increase approximately 4 h after induction. However, this did not correlate with the cessation of growth. We concluded that addition of yeast extract in the feed was not necessary and conducted further cultivations without it.

Composition of the organic phase. In order to select a carrier solvent that would lead to smaller aqueous styrene oxide concentrations, we determined the partition coefficients for styrene and styrene oxide in n-hexadecane/M9* and BEHP/M9* systems. The results are shown in Tab. 4.2. For a particular styrene oxide concentration in the organic phase, the aqueous concentration should be 2.5-fold lower in a mixture with BEHP than with n-hexadecane. The aqueous styrene concentration should also be 1.4-fold lower. Conducting the culture without yeast extract in the feed and with BEHP instead of hexadecane at a phase ratio of 0.25 (Fig. 4.4A and Tab. 4.1), we did indeed observe a drop of the aqueous concentrations of styrene oxide and 2-phenylethanol, which remained below 1 and
Tab. 4.2. Styrene and styrene oxide partition coefficients in systems with M9* mineral medium and an organic phase

<table>
<thead>
<tr>
<th>Organic phase</th>
<th>Compound</th>
<th>Styrene</th>
<th>Styrene oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-hexadecane</td>
<td>1980 ± 75</td>
<td>42 ± 2</td>
<td></td>
</tr>
<tr>
<td>Bis(2-ethylhexyl)phthalate</td>
<td>2810 ± 102</td>
<td>108 ± 5</td>
<td></td>
</tr>
</tbody>
</table>

1.6 mM, respectively. The concentration of 2-phenylethanol in the organic phase rose to 11 mM indicating that the partition coefficient for this compound was higher for a mixture of aqueous medium with BEHP than with hexadecane. The tendency of the culture to foam had significantly decreased and addition of antifoam to the culture could be omitted altogether. The dissolved oxygen tension did not display the irreversible tendency to rise as in earlier experiments. Instead, it leveled off towards the end of the cultivation. Cells continued to grow during the entire cultivation. The maximal styrene oxide formation rates increased to 48 U*(g cdw)^{-1} and leveled off less abruptly towards the end of the cultivation. Average activities increased to 132 U*L_{tot}^{-1} and total transformation time to 5 h reflecting an increase in yield to 91 % or 7.2 g styrene oxide, up from a yield of 75 % with hexadecane as the apolar carrier phase.

**Increase of the phase ratio Φ.** In order to capitalize on the less pronounced effect of the BEHP-based organic phase on the culture, we increased the phase ratio of the two-liquid phase culture to 0.5. The improved foam characteristics also allowed for an increase in working volume from 50 to 66 % (or 2 L) of the reactor volume. The total amount of styrene dissolved in the organic phase was now 2.7 times greater than used in the earlier experiments. This led to an increase in transformation time from 5 to 10.1 h during which the cells grew from 2 to 14.7 (g cdw)*L_{aq}^{-1} (Fig. 4.4B). Styrene oxide formation rates remained at around 50 U*(g cdw)^{-1}, which translated into continuously increasing levels of total activities as the dry biomass concentration increased. Maximal total activities of
around 600 U*L<sub>aq</sub><sup>-1</sup> or 4.3 (g styrene oxide)*L<sub>aq</sub><sup>-1</sup> were obtained 6 hours after induction. The high total activities towards the end of the cultivation at higher cell dry weight concentrations caused an increase in the average activity to 152 U*L<sub>tot</sub><sup>-1</sup>. The octane concentration in the organic phase decreased during the cultivation from over 60 to 36 mM suggesting that the increased phase ratio led to an increased loss of volatile compounds with the off-gas, which also explains the reduced styrene oxide yield of 71%. Styrene oxide accumulated to 184 mM in the organic phase for a total of 22.2 g of styrene oxide formed in a 3 L reactor in 10 h. The product was found to consist of more than 99.5% of the (S)-enantiomer. Styrene was not completely consumed as consumption rates leveled off towards the end of the cultivation (see below). At the end of the extended transformation, the aqueous and organic concentrations of 2-phenylethanol were disproportionately higher than seen in the earlier experiment.

6.5 h after induction, styrene oxide formation activities dropped for the first time from 460 to 320 U*L<sub>tot</sub><sup>-1</sup>. Addition of fresh styrene (equivalent to 1% (vol/vol) of the organic phase) led to an immediate increase in rates back to the same specific styrene oxide formation rate of around 50 U*(g cdw)<sup>-1</sup>. This observation suggests that the culture had been substrate limited before the second addition of styrene. Styrene limitation apparently occurred at a styrene concentration in the organic phase somewhere between 9 and 35 mM (Fig. 4.4B). The DOT signal had reached a level of around 25% saturation at an airflow rate of 1 L*min<sup>-1</sup> when dry biomass concentrations exceeded 9 g*L<sub>aq</sub><sup>-1</sup> 4 h after induction. Afterwards, the DOT increased until we added styrene, which caused an immediate decrease in DOT from 44 to around 28%. The signal remained at this level, although irregularly, for another 1.5 h before it began to increase again. Towards the end of the cultivation, the total activity decreased to around 60 U*L<sub>aq</sub><sup>-1</sup>, due to limited styrene availability and perhaps other factors. One of them is likely to be a toxification of the culture as suggested by the increased DOT over the last two hours of the transformation and the increased foam formation, which indicated increased release of surface active compounds from the cells, for example by cell disintegration (318).
Fig. 4.4: Two-liquid phase fed-batch culture of *E. coli* JM101(pSPZ10) with defined feed and BEHP as the organic carrier solvent containing 2 % (vol/vol) styrene and 1 % (vol/vol) octane. Addition of organic phase occurred at the time indicated by the black arrow. A) Phase ratio of 0.25 B) Phase ratio of 0.5. Upper panel: Concentrations of styrene, styrene oxide, and octane in organic phase. Middle panel: Concentrations of styrene oxide, 2-phenylethanol, and cell dry weight, in aqueous phase. Lower panel: Styrene oxide formation rate and volumetric productivity (proportional to total activity). Open arrow indicates addition of fresh styrene.
Assemblage of the *styAB* genes in expression vector pSPZ10 under control of the *alk* regulatory system allowed efficient synthesis of styrene monooxygenase in *E. coli* JM101 to specific activities of around 70 U\(^g*(g \text{ cdw})^{-1}\). Remarkably, the specific activity of *E. coli* JM101(pSPZ10) could be maintained in shaking flasks for around 6 hours and into the stationary growth phase, which was different from cells that transcribed *styAB* from the *P_{lac}* promoter. Together with the results obtained earlier with the genes for xylene oxygenase, which was synthesized to 90 U\(^g*(g \text{ cdw})^{-1}\) utilizing the same regulatory system (Chapter 3), this indicates that the basic expression vector might be useful as a more general tool for the expression of monooxygenase genes.

**Two-liquid phase culture with hexadecane.** Subsequent utilization of this whole-cell biocatalyst in a two-liquid phase fed-batch cultivation with *n*-hexadecane as the apolar carrier solvent (Chapter 3) allowed the production of 140 mM (S)-styrene oxide in the organic phase. Styrene oxide formation rates remained around 40 U\(^g*(g \text{ cdw})^{-1}\) for 3 h until shortly before the end of the transformation. This was not the case when xylene oxygenase was used instead of styrene oxygenase in previous experiments with hexadecane (Chapter 3). There, the styrene oxide formation rate leveled off less abruptly, which was attributed to either a high half saturation constant or product inhibition. Clearly, the kinetics of styrene oxide formation were improved in the styrene monooxygenase based process, allowing the process to run more efficiently with hexadecane even in situations where the styrene concentration in the organic phase dropped to levels between 20 and 60 mM.

**Effect of the two-phase medium composition.** The system performance was only slightly influenced by the omission of yeast extract from the feed. Yeast extract has been shown before to improve product formation and cell growth rates when recombinant *E. coli* cells were synthesizing the monooxygenase alkane hydroxylase (103). However, later reports have attributed most of the beneficial effects to an increased supply of iron, needed by cells producing the hydroxylating component AlkB (344), which contains a diiron center (323). The DNA sequence
of styrene monooxygenase gave no indication of the typical diiron-center sequence motif (Glu-X-X-His motif for soluble and varying His-(X)$_{2-4}$-His motifs for membrane bound enzymes (256, 324)), suggesting that leaving out the yeast extract from the feed might have only minor effects on process productivity, which is in agreement with the experimental findings.

Foam formation and increase in DOT towards the end of the transformation in hexadecane-based cultures led us to select an organic solvent with a larger partition coefficient for styrene and styrene oxide, which are both known for their toxicity to *E. coli* (117, 394), to reduce the aqueous concentrations of these compounds relative to those seen for a two-liquid phase system with hexadecane. BEHP appeared to be a good choice. It has high partition coefficients for styrene and styrene oxide (Tab. 4.2) and aqueous concentrations of styrene oxide and 2-phenylethanol were reduced significantly in the cultivations (Fig. 4.4). Despite higher cell densities, there was clearly less foaming; in the cultivation that was run at a phase ratio of 0.25, no antifoam agent at all had to be added, while in the cultivation with a phase ratio of 0.5, antifoam agent was necessary only towards the end of the culture. Furthermore, cells appeared to show a higher specific activity in the presence of a BEHP-based organic phase than in the presence of a hexadecane-based phase, which might also be due to the reduced damage caused by the organic phase. However, we cannot exclude differences in *styAB* expression levels due to altered concentrations of the inducer octane in the aqueous phase or due to other effects of the BEHP/octane mixture on cell physiology, compared to the hexadecane/octane phase. Predictably, the rate of styrene oxide formation decreased more gradually than in the hexadecane-based system towards the end of the cultivation: to reach a given styrene concentration (for instance, the biocatalyst's half saturation value) in the aqueous phase, the styrene concentration in the BEHP phase has to be higher than in the hexadecane phase due to the partition coefficient. Consequently, the styrene oxide formation rate starts to decrease at a higher styrene concentration in the organic phase. Moreover, substitution of BEHP for hexadecane at the same phase ratio led to an increased product yield, suggesting that there was less styrene mass transfer to the off-gas than observed in the hexadecane-based system. Finally, in addition to its low cost
(ca US$ 1.60/kg), BEHP has a high boiling and flash point (386 and 199°C, respectively), which is beneficial considering the intrinsically flammable nature of the organic phase/airflow system.

**Optimization of (S)-styrene oxide production in a BEHP-based two-phase system.** To increase space-time yields, both the phase ratio and the cell densities were increased. Larger amounts of styrene were added to the reactor by raising the phase ratio to 0.5, slightly lower than needed for phase inversion, which generally occurs between phase ratios of 0.4 and 0.7 (215). Together with the possibility of using a larger portion of the reactor due to reduced foam formation, these two effects allowed an increase of the organic phase from 0.375 to 1 L while the volume of the aqueous phase was changed only from 1.125 to 1 L.

The beneficial effects observed in the BEHP-based cultivation at a phase ratio of 0.25 were also observed at a phase ratio of 0.5. The biomass concentration increased throughout the cultivation, while the specific activity of the biocatalyst remained high leading to maximal total activities of around 300 U*L_{tot}^{-1} (600 U*L_{aq}^{-1}), corresponding to volumetric productivities of 2.2 g (S)-styrene oxide per liter liquid volume (4.3 g*L_{aq}^{-1}). Addition of antifoam agents was only necessary towards the end of the culture. The second addition of styrene led to a considerable decrease in DOT suggesting an increased need for oxygen, due at least in part to the increased reaction rate: a culture at 600 U*L_{aq}^{-1} consumes 600 μmoles of oxygen per minute only for the reaction or 6 % of the total amount of oxygen that passed through the reactor at an airflow of 1 L*min^{-1}, which means that a sudden large increase in volumetric productivity due to fresh substrate should be identifiable at the DOT signal.

**Formation of 2-phenylethanol.** The formation of 2-phenylethanol represents a complication of a potential downstream processing by fractionated distillation under reduced pressure, which is exacerbated as this compounds partitions to a larger extent into BEHP than into hexadecane. 2-Phenylethanol has been found to inhibit growth of *E. coli* at concentrations above 25 mM (63). This suggests that the low concentrations encountered in our experiments (between 2 and 6 mM) had rather little effect on the growth behavior of the biocatalyst. More experiments will be necessary to identify the reason for the 2-phenylethanol formation.
Effectiveness of recombinant epoxide production. Previous attempts to produce optically active epoxides by epoxidation of olefinic double bonds have been hampered mainly by low specific activities of the utilized biocatalysts (42, 76, 144, 290, 394), exacerbated by diffusion limitations in the cases where cells where immobilized (40). Reported activities range from 2.7 U*L_{tot}^{-1} (42) to 45 U*L_{tot}^{-1} (118). The enzymes that are involved in epoxidation reactions are usually complex multi-component enzymes with cofactors (218, 237, 337, 356) and difficult to synthesize to high levels of activity, let alone purify. Consequently, formation of enantiopure epoxides via a nonoxidative, preferably hydrolytic route like kinetic resolution of epoxide racemates is a very attractive option and initial attempts have been made for the production of (S)-para-nitrostyrene oxide. Using partially purified enzyme preparations of Aspergillus niger to obtain high total volumetric activities, the quantitative formation of around 160 mM enantiopure (S)-para-nitrostyrene oxide in 5 h corresponding to an average activity of 533 U*L_{tot}^{-1} (5.3 g*(L_{tot}*h)^{-1}) on a 2 to 10 mL scale was reported (241). This approach requires first synthesizing and purifying the epoxide-substrate, which is not necessary when using monooxygenases that directly use the (substituted) styrene as the substrate. (S)-Styrene oxide formation with E. coli JM101(pSPZ10) with BEHP as the carrier solvent of the second phase proceeded at an average of 152 U*L_{tot}^{-1} and a maximum of 300 U*L_{tot}^{-1}. Clearly, integration of process elements based on recombinant whole-cell biocatalysts, a process-compatible regulatory system, and in situ product recovery via an organic phase led to highly effective systems to exploit the biological potential of monooxygenases for asymmetric epoxidations. This suggests that the efficiency of asymmetric epoxidation utilizing recombinant whole cells - as presented in this report - is of the same order of magnitude as an equivalent hydrolytic reaction carried out with an enriched enzyme preparation.

ACKNOWLEDGMENT

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CHAPTER 5: PILOT-SCALE PRODUCTION OF (S)-STYRENE OXIDE FROM STYRENE BY RECOMBINANT ESCHERICHIA COLI SYNthesIZING THE STYRENE MONOOXYGENASE OF PSEUDOMONAS SP. STRAIN VLB120

SVEN PANKE, MARCEL G. WUBBOLTS, ANDREAS SCHMID, HANS-JÜRGEN FEITEN, AND BERNARD WITHOLT
SUMMARY

Recombinant *Escherichia coli* JM101(pSPZ10) cells synthesize the styrene monooxygenase of *Pseudomonas* sp. strain VLB120, which oxidizes styrene to (S)-styrene oxide at an enantiomeric excess larger than 99%. This biocatalyst was used to produce 388 g (S)-styrene oxide in a two-liquid phase 30 L fed-batch cultivation. The average volumetric activity was 170 U per liter aqueous and organic phase over a period of more than 10 h equivalent to mass transfer rates of 10.2 mmoles per liter per hour at a phase ratio of 0.5. At this transfer rate, the culture appeared to be substrate mass transfer limited, which suggested an upper productivity limit for the production of enantiopure styrene oxide that corresponded to 10.7 tons of (S)-styrene oxide per cubic meter per year. The reactor had an estimated power input in the order of 5 W*L⁻¹*, which is close to values typically obtained with commercially operating units. Together with the recently reported 300 L scale production of 2,3-dihydroxybiphenyl by an *E. coli* recombinant synthesizing 2-hydroxybiphenyl 3-monooxygenase of *P. azelaica* HBPI, these are striking examples for the potential of applied recombinant biocatalysis.

INTRODUCTION

The reaction-, regio-, and enantioselectivity of enzymes makes them potentially powerful synthesis tools, either in partially purified form or as whole cell biocatalysts, for example when regeneration of reduced cofactors is required as in reactions catalyzed by monooxygenases (99). These enzymes possess considerable potential in the area of asymmetric oxidation (32, 115, 348), a major field of research in organic synthesis (60, 177, 260).

We believe that the preferred host of whole cell biotransformations will change from (mutated) wild-type cells to one of a small group of model organisms like *Escherichia coli* as established routes to developing new biocatalysts are challenged by the opportunities of modern molecular biotechnology. No less than
20 completely sequenced microbial genomes are currently publicly available (http://www.tigr.org/), and 65 more microbial genome projects are underway. Obviously, this ever growing set of information represents a huge, largely untapped reservoir for new biocatalytic activities, the discovery of which depends essentially on the availability of appropriate search algorithms for the genes of the corresponding enzymes. Moreover, progress is being made with the expression in model microorganisms of oxygenases of eucaryotic origin like the cytochromes P450 in model microorganisms (35, 128, 217, 265, 276, 330). Ultimately, this will open the wealth of data from investigations on the fate of drugs and xenobiotics in mammalian systems to a reevaluation for biotransformation purposes. Major opportunities in biocatalyst selection arise from the modification of the substrate spectrum of existing enzymes, either rationally by site directed mutagenesis (129, 180, 252, 267) or randomly by techniques of guided evolution (47, 200). These developments have in common that they aim at one or more novel genes that need to be expressed predictably in a process compatible fashion to produce high specific activities and high volumetric productivities. The preferred host will in many cases be *E. coli*; its molecular biology is extremely well understood (249), process data for media formulation and high cell density cultivations are abundantly available (208, 303, 402), induction of gene expression can be achieved with one of a number of expression systems that not only permit optimal enzyme synthesis (221), but often also allow tailoring of the biocatalyst for optimal process performance, and finally, given the ecological niche of *E. coli*, most products of interest are unlikely to be consumed (101). These advances in biocatalyst construction need to be complemented by improvements in process design. The majority of potentially interesting substrates for and products from biotransformations are poorly soluble in water and/or toxic to living cells (209, 213, 255, 311). In order to capitalize on the advantages of model organisms, one needs some sort of a standardized growth environment for the cells with minimum concentrations of potentially disturbing compounds. Typically, this is achieved by regulating substrate addition and recovering the product *in situ* (108). For example, the recent development of an on-line solid phase extraction unit (159) has resulted in the kilogram scale production of toxic 2,3-dihydroxybiphenyl from the toxic
substrate 2-hydroxybiphenyl (Held, pers. communication). Alternatively, an inert apolar solvent added to the culture can serve as a substrate reservoir and a product sink in processes where the substrate or the product are toxic and partition into the organic phase (117, 395). The latter approach has been taken for the production of toxic (S)-styrene oxide from toxic inexpensive styrene (394, 395), which we used to demonstrate the potential of recombinant *E. coli* not only for small scale experiments, but also for the production of bulk amounts of fine chemicals. In previous experiments, we have constructed an *E. coli* biocatalyst that efficiently synthesizes the styrene monooxygenase of *Pseudomonas* sp. strain VLB120 (Chapter 4). This strain, *E. coli* JM101(pSPZ10), allowed for the set-up of a 2 L-scale process with maximum volumetric productivities of 300 U per liter aqueous and organic phase (300 U *L*$_{\text{tot}}^{-1}$) in a two-liquid phase system that used bis(2-ethylhexyl)phthalate as the second phase. This resulted in the production of 22 g of (S)-styrene oxide (enantiomeric excess >99 %) in 10 h (Chapter 4). In the experiment reported here, we investigated the behavior of this system on a 30 L scale where power input is close to that of commercially operating units and mass transfer limitations are frequent.

**Materials and Methods**

Construction of the plasmid pSPZ10, which contains the genes *styAB* for the styrene monooxygenase of *Pseudomonas* sp. strain VLB120 under the control of the *alk* regulatory system of *P. oleovorans* GO1, has been described (Chapter 4). *E. coli* JM101 (supE thi Δ(lac-proAB) F$^{'}$[traD36 lacI $^{q}$ Δ(lacZ)M15 proAB]) (312) was transformed with pSPZ10, and the resulting strain served as the biocatalyst in the experiment. All growth steps were carried out at 30°C. Selection of transformants occurred on Luria-Bertani broth (312), which contained 50 μg·mL$^{-1}$ kanamycin and 1% (wt/vol) glucose to repress the expression of the tryptophanase, which otherwise led to the formation of indole from tryptophan (230), which in turn was converted to indigo by the action of styrene monooxygenase (263). Freshly transformed cells were used as an inoculum for a
5 mL LB culture supplemented with glucose and kanamycin as before, which was diluted 100-fold with 100 mL of M9\# medium supplemented with 100 µL of US\# trace element solution (Chapter 3), 0.5 % (wt/vol) of glucose as the carbon source, 50 µg·mL\(^{-1}\) of kanamycin to, and 100 µL of a 1 % (wt/vol) solution of thiamine hydrochloride. After cells had entered stationary phase, they were transferred into a stirred tank reactor with a working volume of 3 L that contained 1.4 L of mineral medium of identical composition as the culture before. The details of the reactor system and its instrumentation have been described (Chapter 3). The pH of the culture was kept at 7.0 by the addition of 25 % (wt/vol) ammonium water and 30 % phosphoric acid. Again, cells were grown into stationary phase before they were used as the inoculum for the large scale cultivation.

The 30 L scale cultivation was carried out in a 42 L stirred tank reactor with an internal diameter of 26.3 cm and regulated temperature, pH, stirrer speed, and internal pressure (New MBR, Zurich, Switzerland). Maintenance of pH was achieved as described above. Fittings were made of Keltan (Angst & Pfister, Zurich Switzerland). Data collection occurred every 30 sec with the Caroline II software (PCS, Wetzikon, Switzerland) on an OS/9 operating system. Mixing was achieved with 2 six bladed Rushton turbine impellers (diameter 8.8 cm). Before sterilization, the reactor contained salts equivalent to 13.5 L of M9\# medium dissolved in 12.5 L of water. After sterilization at 121 °C for 40 min, the reactor contents were supplemented aseptically with 150 mL of a 50 % (wt/vol) glucose solution, 60 mL of US\# trace element solution, 30 mL of 1 M magnesium sulfate, 15 mL of 1 % (wt/vol) thiamine hydrochloride, 15 mL of 50 mg·mL\(^{-1}\) kanamycin, and 1 mL of polypropylene glycol 2000 (Fluka, Buchs, Switzerland). The volume was then adjusted to 13.5 L with water. After the inoculum had been pumped into the reactor, the cultivation continued as a batch culture at a stirring speed of 400 rpm and an aeration of 15 L·min\(^{-1}\) for around 8 h. After 4 h, the DOT decreased to 0 %. A sharp increase in DOT after 5 h indicated that the cells had entered stationary phase. The cells remained in this phase for the next 3 h, after which the production phase began.

The production phase was started by initiation of an aqueous feed consisting of 50 % (wt/vol) glucose and 10 g·L\(^{-1}\) MgSO\(_4\)·7H\(_2\)O, adjusted to pH 3 with
hydrochloric acid. The feed was pumped at a rate of 4.5 \( \text{g glucose} \cdot \text{L}^{-1} \text{h}^{-1} \) equivalent to around 135 mL\text{h}^{-1} for the entire aqueous phase. One hour later, 14.8 L of organic phase were added to the reactor. This phase consisted at the beginning of bis(2-ethylhexyl)phthalate (BEHP, 97%, Fluka, Buchs, Switzerland) as the apolar carrier solvent, 2% (vol/vol organic phase) styrene (99%, Fluka), and 1% (vol/vol organic phase) \( n \)-octane (Acros, Geel, Belgium) as the inducer of the \( \text{alk} \) regulatory system. At regular intervals, samples of around 30 mL were withdrawn and the contents of the aqueous phase (cell dry weight (cdw) and phenylacetaldehyde concentrations) and of the organic phase (concentrations of styrene, octane, styrene oxide, and 2-phenylethanol) were analyzed as described before (Chapter 3). Changes in the volume of the phases due to feeds, three pulses of 150 mL of fresh styrene, and sampling were taken into account. Foam formation towards the end of the cultivation was limited by the addition of polypropylene glycol. Specific activities were calculated as average values from the amount of styrene oxide produced between two sampling points divided by the average biocatalyst cell dry weight over the time between the two samplings.

The cultivation was terminated by harvesting the contents of the reactor and separating the two phases by centrifugation for 30 min at 8400\( \text{g} \) in a Haereus-Christ Cryofuge 8000 centrifuge (Haereus, Zurich, Switzerland). The organic phase was aspirated, added with dry sodium sulfate, and stored at 4°C. The enantiomeric excess of the produced (S)-styrene oxide was analyzed as described (Chapter 2).

**Results**

**Cultivation procedure and growth.** *E. coli* JM101(pSPZ10) was first grown to 2.2 \( \text{g cdw} \cdot \text{L}^{-1} \) in batch mode with glucose as the carbon source. An aqueous feed containing concentrated glucose and magnesium sulfate was then started, which provided 4.5 \( \text{g glucose} \cdot \text{L}^{-1} \text{h}^{-1} \). The addition of the organic phase served as the time of induction. Cell growth did not appear to be disturbed by the addition (Fig. 5.1). The period from starting the feed until the end of the cultivation will be termed the *production period*, while the period from addition of the organic phase
until the end of the cultivation will be called the \textit{transformation period}. Four stages of growth could be observed: a rapid linear growth for the first 4.4 h of the production period to 8.6 g$^\text{g dry biomass}\cdot\text{L}_{\text{aq}}^{-1}$, a period of less rapid but still linear growth for the next 4 h to 10.8 g$^\text{g dry biomass}\cdot\text{L}_{\text{aq}}^{-1}$, a period of slow increase to the maximum dry biomass concentration of 12.2 g$^\text{g dry biomass}\cdot\text{L}_{\text{aq}}^{-1}$ after 13.6 h, and a period of slow decrease in the dry biomass concentration until the end of the cultivation to 10.7 g$^\text{g dry biomass}\cdot\text{L}_{\text{aq}}^{-1}$. The first growth period is accompanied by a rapid reduction of the oxygen concentration in the aqueous phase, the DOT falling to 0 \% in the first hour. We therefore increased stepwise the airflow from 15 to 50 L$^\text{min}^{-1}$ and the stirrer speed from 400 to 950 rpm (Fig. 5.1). Nevertheless, the DOT decreased to below 5 \% after 4.0 h. The DOT started to increase again 2 h later and then continued to increase until the end of the experiment unless the airflow was readjusted or fresh styrene was added (see below). In the second half of the experiment, we reduced the airflow to minimize losses of the substrate styrene with the off-gas. From here on, times refer to the transformation period.

\textbf{Production of (S)-styrene oxide.} The formation of (S)-styrene oxide commenced rapidly after the organic phase had been added (Fig. 5.2). Its concentration in the organic phase rose approximately linearly from 3.2 h after the start of the transformation period until 13.5 h, when the rate of formation started to level off. The final styrene oxide concentration in the organic phase was around 270 mM. Assessment of the specific activity of the biocatalyst was only possible for the first 3.2 h after induction and the last 2.5 h of the cultivation due to a mass transfer limitation over the rest of the period (see Discussion). In these periods, the specific activity rose to around 35 U$^\text{U}\cdot(\text{g cdw})^{-1}$ after induction and decreased to approximately 7 U$^\text{U}\cdot(\text{g cdw})^{-1}$ towards the end of the cultivation. This suggested that it was possible to maintain substantial specific activities even in the presence of low amounts of dissolved oxygen (Fig. 5.3) (see period between 2.1 and 3.2 h after induction, calculated specific activity of 34 U$^\text{U}\cdot(\text{g cdw})^{-1}$, DOT between 7 and 0 \%). The formation of the byproduct 2-phenylethanol started shortly after the formation of styrene oxide. It accumulated in the organic phase at approximately 10 \% of the rate of styrene oxide formation. This resulted in a final concentration of 25 mM in
Fig. 5.1. Parameter and aqueous phase data of a 30 L scale two-liquid phase cultivation of *E. coli* JM101(pSPZ10) in the presence of an organic phase of BEHP, 2% styrene, and octane. Open arrow: addition of organic phase, induction and start of transformation time. Closed arrows: addition of fresh styrene.

Fig. 5.2. Concentrations of compounds dissolved in the organic phase during transformation of styrene to styrene oxide and 2-phenylethanol in *E. coli*. Closed arrows indicate addition of fresh styrene, which contributed to the cumulative concentration of the styrene that had been added up to this time. Measured concentrations of styrene are also indicated. The styrene concentrations immediately before the addition of new styrene were not determined.
Fig. 5.3. Development of volumetric productivity over the transformation time compared to growth and dissolved oxygen tension (DOT). DOT is subject to steep changes due to modifications in stirrer speed and airflow (compare Fig. 5.1). All data refer to the aqueous phase. Open triangles represent measured values of produced styrene oxide and the dashed line represents a linear approximation of the data for the period from 3.2 to 13.5 h after induction to calculate the average volumetric productivity. Note that the horizontal axis is based on transformation time instead of production time and that the left vertical axis refers to the formed amount of styrene oxide per volume aqueous phase which takes into account volume corrections.

Fig. 5.4. Losses of octane and styrene and the compounds formed from styrene over the transformation period. Losses were calculated relative to the total amount theoretically available.
the organic phase. The route of its formation is unclear and its appearance has been discussed (Chapter 4).

**Styrene and octane concentrations in the organic phase.** During the cultivation, the concentration of the inducer octane decreased from the initial value of 61.5 mM to 18.4 mM (Fig. 5.2). The styrene concentration also fell rapidly from the initial value of 174 mM to 61.5 mM 4.6 h after induction, although the styrene oxide concentration rose to only 63 mM. In order to avoid substrate depletion, 150 mL of styrene were added to the culture 5.9 h after the start of the transformation phase. Again, the styrene concentration fell rapidly, prompting us to add styrene twice more after 8.4 h and after 11.0 h. After the third addition of styrene, the culture developed foam, which was contained by adding polypropylene glycol. However, about 8% of the culture was lost through the off-gas tubing. After each of the three styrene additions, the DOT value decreased between 4 and 6%, suggesting an increased oxygen demand.

**Process termination.** Phase separation of the two-phase mixture was efficiently achieved by centrifugation at 8400×g for 30 min. No attempts were made to further optimize this step. 12 L of organic phase were recovered from the reactor (Tab. 5.1), or 80% of the organic phase originally added to the reactor. The recovered 12 L of organic phase contained a total of 388 g of (S)-styrene oxide (equivalent to formation of 450 g in the absence of volume loss). The results are summarized in Tab. 5.1. Analysis of the enantiomeric composition of the styrene oxide with chiral gas chromatography showed an enantiomeric excess of greater than 99%, confirming earlier reports on the stereospecificity of the styrene monooxygenase of *Pseudomonas* sp. strain VLB120 (Chapter 2).

**DISCUSSION**

a) 30 L scale (S)-styrene oxide production process

**Overall performance.** *E. coli* JM101(pSPZ10), which synthesized the styrene monooxygenase of *Pseudomonas* sp. strain VLB120, produced 388 g of
(S)-Styrene oxide in 12 L of organic phase in a total transformation time of 16 h. This corresponded to an average volumetric activity of 140 U*L_{tot}^{-1} or an average volumetric productivity of 1 g*L_{tot}^{-1} of (S)-styrene oxide. Its formation started rapidly after induction, accelerated for the first 3.2 h of transformation time, and then remained approximately constant for the next 10.3 h, after which it decreased. The concentration of the inducer octane in the organic phase decreased by two thirds during the transformation period (Fig. 5.2). We have never observed such a substantial loss of the inducer in previous experiments (Chapter 3, Chapter 4). However, the explanation is straightforward: In previous experiments, airflow rates have usually not exceeded 1 volume air per volume aqueous medium per minute (vvm_{aq}). For the pilot scale reactor, airflow was generally higher and for around 6 h exceeded 3 vvm_{aq}. The airflow was reduced to 2 vvm_{aq} and 1 vvm_{aq} when styrene was added for the second and third time, respectively (Fig. 5.1). Our results suggest that a major portion of the volatile octane was lost with the off-gas. The case was different for styrene and the products formed from it. Plotting the relative amounts of the organic compounds found back in the organic phase (i.e. octane only on the one hand and the sum of styrene, styrene oxide, and 2-phenylethanol on the other) against the transformation time (Fig. 5.4), the combined amounts of substrate and (by-)product fell rapidly for the first 4.6 h and then stabilized. Several factors contributed to this phenomenon. A major portion of the volatile substrate was converted into the less volatile product (and byproduct). Together with the reduced airflow, the additions explain why the relative recovery of substrate and (by-)product rose towards the end of the transformation. The different stages in the growth curve suggest that the second linear stage of growth was influenced by the oxygen limitation. Later growth stages might have been more and more influenced by the detrimental impact of the organic compounds, specifically of the styrene oxide, as indicated by the rise in DOT (Fig. 5.1). This has been shown before to accumulate in the aqueous phase (Chapter 3, Chapter 4).

**Process productivities.** Fed-batch processes in 2 L bench-top reactors with a power input in the order of 60 W*L^{-1} (318) have allowed (S)-styrene oxide formation at volumetric activities of up to 36 mmol*(L_{aq}*h)^{-1} (Chapter 4). The increase of the volumetric productivity during the cultivation paralleled the
increase in cell dry weight in these experiments. This corresponded to an approximately constant specific activity of 50 U*(g cdw)^{-1}. Moreover, dissolved oxygen concentrations did not fall under 20%. In the experiment reported here, the volumetric productivity of the cultivation remained approximately constant at an average rate of 20.4 mmol*(L_{aq} * h)^{-1} over a period of 10.3 h (Fig. 5.3), at least with the present data point resolution (see below). In previous experiments, where alkane mass transfer rates were determined from the rates of alkane utilization by *P. oleovorans* GPol in batch and continuous cultures, rates between 13 and more than 45 mmol*(L_{aq} * h)^{-1} were observed for the transfer of decane or octane and heptane, respectively, to the cells when these solvents were present as a bulk second phase in low-power input experiments (<5 W*L^{-1}) (318). In high-power input experiments (>60 W*L^{-1}), octane mass transfer rates to the cells became limiting when octane was diluted below 3 % (vol/vol) in hexadecene and were in the order of 30 mmol*(L_{aq} * h)^{-1}. One conclusion of the authors was that some of the octane that was used for biomass formation had to reach the cells by direct contact between the organic phase and the cells (318). For the system reported here, a maximum power input in the order of 5 W*L^{-1} can be estimated (318). The average substrate transfer to the cells of at least 20.4 mmol*(h*L_{aq})^{-1} (or 10.2 mmol*(h*L_{aq})^{-1}) is strikingly close to the numbers reported by Schmid et al. (318). However, this comparison might be misleading as the investigated systems differ substantially; phase ratio, organic carrier solvents and substrates are different, indicating potential differences in the values for interfacial area, mass transfer resistance across the interface, and the equilibrium concentration of the substrate in the aqueous stagnant film at the interface. As most of these data have so far not been available to us for the styrene/BEHP system, the similarity of the figures may well be coincidental.

**Limitations to productivity.** Volumetric productivity in the 30 L cultivation remained approximately constant from 3.2 h after addition of the organic phase until 13.5 h. Over the same period, the cell dry weight concentration rose from 8.6 to around 12 g*L_{aq}^{-1}, while the DOT level increased from 0 to around 60 %. This indicates that the cells were oxygen limited only for the first 30 % of the period of constant styrene oxide formation. Moreover, the total productivity per unit volume
Tab. 5.1. 30 L cultivation for (S)-styrene oxide production

<table>
<thead>
<tr>
<th>Added organic phase</th>
<th>14.8 L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Styrene pulses</td>
<td>0.45 L</td>
</tr>
<tr>
<td>Removed organic phase (sampling)</td>
<td>0.3 L</td>
</tr>
<tr>
<td>Final</td>
<td>15.05 L</td>
</tr>
<tr>
<td>Recovered</td>
<td>12 L (79.8 %)</td>
</tr>
</tbody>
</table>

Provided styrene: 675.9 g
Recovered in 12 L organic phase:
- Styrene: 49.3 g
- 2-Phenylethanol: 35.9 g
- Styrene oxide: 388 g

Yield per L reaction volume: 15.2 g L<sup>-1</sup>
Yield per L reactor volume: 12.1 g L<sub>reactor</sub> L<sup>-1</sup>
Product yield before purification: 49.7 %

did not increase with the biomass concentration during the period, as it had in the 2 L scale experiments (Chapter 4). This suggested that of the three fluxes that provide reduced cofactors, molecular oxygen, and styrene as substrates to the biocatalyst, most probably the styrene mass transfer was limiting. This may have been due to the comparatively limited power input of approximately 5 W L<sup>-1</sup> in the pilot scale reactor. In such two-liquid phase systems, the driving force of the substrate mass transfer is the concentration gradient between the equilibrium concentration of the apolar compound in the aqueous stagnant film at the interface and the actual concentration in the bulk aqueous phase (215). However, this gradient decreases as the styrene is consumed from the organic phase. This implies that the styrene oxide formation rate is very likely not a straight line as suggested by the data of Fig. 5.3. As the time interval between sampling points was rather long (1.2 h) compared to that between two styrene additions (around 3.6 h), it is entirely possible that resolution of our measurements was not sufficient to detect subtle changes of the styrene oxide formation rates.
b) Implications for styrene oxide production via the monooxygenase route

The bottleneck of (S)-styrene oxide production has shifted away from the specific activity that is available in the biocatalyst (394, Chapter 3, Chapter 4), to what is probably a styrene mass transfer limitation. This implies that future efforts to improve process productivity in a stirred tank reactor can be directed either at increasing the power input into the reactor or the styrene concentration gradient that drives mass transport. This would eventually shift the bottleneck back to specific biological activity. However, E. coli has been shown to tolerate styrene up to 2.5 mM when provided in a single aqueous phase, it has not been found to tolerate styrene concentrations in the organic phase higher than 2% (vol/vol) in octane (395). This suggests that when using BEHP as the carrier solvent, there will be a similar limit to the maximum possible concentration of styrene in the organic phase. To circumvent this problem, at least two approaches can be imagined: First, the cells could be protected from direct contact with the organic phase and therefore from phase toxicity by immobilization (22) or by a membrane (90). However, this approach frequently substitutes the inter-phase mass-transfer limitation by a diffusional limitation inside the immobilized biocatalyst particle (40) or by a mass transport limitation over the membrane (90). Alternatively, biocatalysts could be selected that tolerate higher styrene concentrations in the organic phase (75). Much progress has lately been made in understanding the key mechanisms of extraordinary solvent resistance in single Pseudomonas strains which tolerate the presence of bulk phases of solvents with a logP of 2.5 (75, 158, 188, 295). A key factor appears to be the presence of a solvent efflux system that might help the cell to keep internal solvent concentrations low (188, 294). Styrene has a logP of 3 (204), so it appears to be a promising option to either transfer solvent resistance genes to E. coli or eventually establish such a solvent resistant Pseudomonas as a new model system for fine chemical production.
c) Implications for recombinant biocatalysis

Attempts to use recombinant *E. coli* strains to produce fine chemicals have in general thus far concentrated on the production of dihydrodiols (363, 376, 399, 405), alkanoic acids (101, 102, 307, 394), and poly-3-hydroxybutyrate (PHB) (378), or on pathway engineering requiring the composition of multiple reaction steps in a host cell (92-94, 109, 277). The latter approach has led to a process on the 30 m³ scale for the production of indigo utilizing - among other enzymes - recombinant naphthalene dioxygenase, which is probably the first commercial process development involving a recombinant oxygenase. Unfortunately, data on yields and activities are not available (33). Attempts to produce alkanoic acids were developed to a scale of 2 L with two-liquid phase high cell density cultures of up to 50 (g cdw)*L*eq⁻¹, which resulted in total transformation activities of around 125 U*L*eq⁻¹ (394). PHB was produced to a yield of 104 g*L⁻¹ in high cell density fed-batch cultures (378). Furthermore, L-tryptophan production has been attempted starting from indole dissolved in the organic phase of a two-liquid phase system, although the achieved productivities remained low (302), as was also the case for the recombinant production of dihydrodiols derived from toluene (376). These reports, which have either a direct connection to existing commercial bioprocesses (as in the dihydrodiol case) or have a clear applied focus in themselves, are complemented by the vast literature on genes encoding enzymes with potential links to biocatalysis, which were cloned, functionally expressed, and frequently characterized to some extent in recombinant *E. coli* hosts on a small scale (e.g, 39, 86, 184).

In the light of these data, the results reported here represent significant progress. Earlier experiments have demonstrated the feasibility in principle of our approach on the test-tube scale (398, Chapter 2) and the 2 L scale (394, Chapter 3, Chapter 4), but such experiments were carried out in optimized environments with respect to power input and mass transfer of oxygen and styrene. On a 30 L scale with limited power input, oxygen and styrene transfer limitations can be expected to exert their effect on process productivity (318), but styrene oxide was still formed.
at an average rate of 10.2 mmol\(^{(\text{S})}\)(L\(_{\text{tot}}\)·h\(^{-1}\)) over the period of the assumed styrene mass transfer limitation, equivalent to 1.2 g\(^{(\text{S})}\)(L\(_{\text{tot}}\)·h\(^{-1}\)) of (S)-styrene oxide. Of similar significance is the recent production of the toxic compound 2,3-dihydroxybiphenyl with *E. coli* JM101(pIV61), which expressed the genes for the 2-hydroxybiphenyl 3-monooxygenase of *P. azelaica* HBPI. The toxicity of substrate and product were circumvented by developing a solid phase adsorption module for the continuous removal of the product and adding the substrate in a controlled and limited fashion (159). This process was scaled up to 300 L and produced 1.2 kg of the desired product (Held, pers. communication). Essentially, these two results suggest that bulk production of fine chemicals with recombinant *E. coli* whole cell biocatalysts is feasible. The development of recombinant biocatalysts that synthesize monooxygenases to high specific activities and the ability to provide process means to establish an appropriate growth environment for the recombinant biocatalyst eliminates two fundamental hurdles that have hampered the application of recombinant *E. coli* biocatalysis up to now. Thus, recombinant biocatalysis has “come of age” and this development will shift the focus of interest. There will be screening for new or better enzymes, not for the basic biocatalyst.

d) Conclusion

Two-liquid phase technology is a suitable option for the production of fine chemicals at pilot-scale. Recombinant *E. coli* cells can be engineered such that the bottleneck in the volumetric productivity of a corresponding process need no longer be the specific activity of the whole-cell biocatalyst. More specifically, at least 388 g of (S)-styrene oxide could be produced in a two-liquid phase 30 L pilot-scale cultivation, which appeared to be styrene mass transfer limited at an average of 10.2 mmol\(^{(\text{S})}\)(h·L\(_{\text{tot}}\))\(^{-1}\) over most of the transformation period. Such an upper limit corresponds to a production of 10.7 tons per cubic meter per year of an added value chemical, which might serve as an important chiral building block in the production of biologically active molecules for the pharmaceutical and agrochemical industry.
ACKNOWLEDGMENT

This work was supported by the Swiss Priority Program Biotechnology.
The authors are indebted to Andrew Schmid for valuable discussions.

APPENDIX TO CHAPTER 5

CONSTRUCTION OF A STYRENE OXIDE ISOMERASE-negative, (S)-STYRENE OXIDE ACCUMULATING MUTANT OF SOLVENT RESISTANT PSEUDOMONAS SP. STRAIN VLB120

SVEN PANKE AND BERNARD WITTHOLT

Apparently, in low-power input reactor systems the two-liquid phase production of (S)-styrene oxide by *E. coli* JM101(pSPZ10) becomes styrene mass transfer limited (see above). There are two principal ways to further improve process productivity in such a stirred tank reactor two-liquid phase system: higher power input and higher substrate concentrations in the organic phase. This would either increase the total area of the interface or increase the substrate flux over the interface. While the first option cannot be excluded, more immediate results might be expected from the latter approach. Taking the reaction from styrene to styrene oxide as an example, substrate and product exert their toxic effects in, broadly speaking, two ways. Styrene as the substrate has a logP of 3 (204). This means that it is likely to accumulate to significant concentrations in the cell membrane of the biocatalyst, where it can interfere with membrane integrity and ultimately prevents the membrane from functioning properly (332, 381). This mode of action is based on the physicochemical properties of the molecule. Styrene oxide with its reactive
epoxide function reacts rapidly with nucleophiles in the cellular environment, thereby ultimately impairing cell function by its reactivity. While this latter aspect of the process cannot be completely avoided, means are available to biologically protect a biocatalyst to a significant extent from the effects of organic molecules with a logP between 2.5 and 4 (75). Beginning with the first report on a *Pseudomonas* strain that could grow in the presence of a bulk phase of toluene with a logP of 2.5 (173), the phenomenon of tolerance to solvents with a logP of less than 4 has been intensively investigated (see Introduction of this thesis). Recently, molecular pumps that are homologous in nucleotide sequence to the well studied multidrug resistance pumps have been discovered in a number of *Pseudomonas* strains (188, 191, 212, 294), where they are supposed to keep the intracellular concentration of the solvent low (75). In particular, the ability of the styrene degrader *P. putida* S12 to grow in the presence of a bulk organic phase of a logP of 2.5 (383) has been found to depend on such a pump (188), suggesting that it might be possible to utilize this system to the benefit of a bioprocess for the production of (S)-styrene oxide. There are two ways to capitalize on these observations: either the genes encoding the molecular pumps are transferred to *E. coli* or the mutated wild-type strain is utilized directly. *Pseudomonas* sp. strain VLB120 has been shown to grow with styrene added to a concentration of 1042 mg L\(^{-1}\) which exceeds the aqueous solubility limit (2). This makes this strain a potential candidate for use as a whole cell biocatalyst in two-liquid phase processes that apply styrene concentrations higher than 2 % (vol/vol) in the organic phase. The strain utilizes styrene via epoxidation of the vinyl side chain and a subsequent isomerization of the resulting (S)-styrene oxide by styrene oxide isomerase, which is encoded by the *styc* gene (Chapter 2). The wild-type strain is not useful for the production of styrene oxide, as this compound is part of the styrene assimilation route. Consequently, we set up experiments to rationally construct a mutant strain that cannot produce the styrene oxide isomerase anymore.
MATERIALS AND METHODS.

We followed standard protocols for molecular biology methods (312). Antibiotics were added to the following concentrations for *E. coli* (in parentheses numbers for *Pseudomonas*): kanamycin, streptomycin, and spectinomycin 50 μg·mL⁻¹ (100 μg·mL⁻¹); ampicillin 150 μg·mL⁻¹ (piperacillin 50 μg·mL⁻¹). Gene replacement vectors carried an origin of transfer and the counterselectable *sacB* gene, which conveyed sensitivity to sucrose to the host. They were conjugated from mobilization proficient *E. coli* S17-1pir (335) into *Pseudomonas* sp. strain VLB120 (Chapter 2). Cells of each strain were taken from overnight LB cultures grown at 30°C. The cells were washed with 1 % (wt/vol) aqueous sodium chloride to remove antibiotics, and an aliquot equivalent to 100 μL of the overnight cultures was spotted on a filter (Type HA, Millipore, Bedford, MA). These filters were placed on LB-agar plates and incubated for 3 h at 30°C. The cells were recovered, washed again in sodium chloride solution, and plated on M9 mineral medium agar plates that contained 0.2 % (wt/vol) citrate as the carbon source, streptomycin, and spectinomycin. Double homologous recombination events were selected for by plating on plates that were prepared as described above and supplemented with 5 % (wt/vol) sucrose. For shaking flask experiments we used a mineral medium which contained M9* salts, 1 mL·L⁻¹ of US* trace element solution, and 0.5 % (wt/vol) glucose as the carbon source. Inocula for shaking flask experiments were obtained from mineral medium cultures of the same medium composition. The flasks were incubated at 30°C on a rotary shaker. To induce the styrene oxidation activity, cells received a cell dry weight concentration of 0.1 g·L⁻¹ styrene from a 1 M stock in ethanol to a concentration of 1 mM. To ensure continuous styrene supply via the vapor phase, we placed at the same time a sterile tube inside the flask that contained 1 mL of styrene. When strains were investigated qualitatively for their ability to consume styrene, the cells were harvested 4 h after induction and subjected to a styrene oxide formation assay as described (Chapter 2). For more detailed kinetic studies, aliquots were withdrawn at regular intervals and also analyzed for styrene oxide formation.
RESULTS

Plasmid pSPW1 (Chapter 2) carries the sty genes involved in styrene degradation in Pseudomonas sp. strain VLB120 including styC, which encodes the styrene oxide isomerase, on a 5.7-kb XhoI fragment. The 2.0-kb Smal fragment from plasmid pH45 contains the Ω-interposon coding for streptomycin resistance (287). This fragment was inserted into the two Smal sites of styC on plasmid pSPW1, which resulted in plasmid PSPW1Sm. From there, the Ω-interposon was reexcised together with 1.9 and 1.6-kb of flanking sty DNA as a 5.6-kb HinclII fragment and inserted into the gene replacement vector pEX100T. This resulted in plasmid pEXsty. First attempts to construct a styC knock-out mutant of Pseudomonas sp. strain VLB120 using this plasmid led to exconjugants that were for unknown reasons sensitive to both sucrose and piperacillin. Consequently, we substituted the bla resistance gene on pEXsty by the kanamycin resistance gene of pSPW1 by inserting the 4.0-kb NdeI/EcoRV portion of pSPW1, which spanned the 3’ part of styD, the fl origin of replication, and the kanamycin resistance gene, into NdeI/SspI digested pEXsty. The resulting vector was designated pKOsty (Fig. 5.5). It was conjugated into Pseudomonas sp. VLB120 and single recombination events were selected for by plating the cells from the mating on mineral medium plates containing citrate, streptomycin, and spectinomycin. Resulting colonies were purified, grown overnight in LB broth, incubated for 14 days at 4°C, inoculated into a fresh LB-tube, and plated onto mineral medium plates prepared as described above and supplemented with sucrose to select for the second homologous recombination event. The resulting colonies were analyzed for resistance to kanamycin. 98% of sucrose resistant strains were sensitive to kanamycin. Five such strains were after purification inoculated into mineral medium cultures with glucose as the carbon source and analyzed for their ability to accumulate styrene oxide. In these experiments, the wild-type consumed styrene and did not accumulate any styrene oxide (results not shown). In contrast, all kanamycin sensitive and sucrose resistant strains did accumulate styrene oxide. One of these strains, termed Pseudomonas sp. strain VLB120AC, was then investigated for its styrene oxygenase induction kinetics in mineral medium that contained glucose as the
Fig. 5.5. Gene replacement vector pKOsty. Elements that are indicated outside the circle represent elements of the sty gene cluster of *Pseudomonas* sp. strain VLB120. The streptomycin resistance gene that has been inserted into the styC gene separates it into a 5’ part (styC) and a 3’ part ('styC), which together do not reconstitute the complete styC gene.

Fig. 5.6. Growth profile and styrene monooxygenase induction kinetics of *Pseudomonas* sp. strain VLB120ΔC in mineral medium after induction with styrene via the vapor phase and by adding styrene to 1 mM into the aqueous phase.

- **induced cultures**
- **uninduced cultures**
carbon source (Fig. 5.6). The strain reached a maximum specific activity of 67 U*(g cdw)^{-1}, whereas activities in uninduced cultures remained between 0.2 and 0.7 U*(g cdw)^{-1}.

**Discussion**

Utilization of solvent resistant strains has been suggested as a method to improve existing bioprocesses or to realize processes which thus far have not been feasible. In general, such strategies aim at utilization of solvents with logP values lower than 4 to improve the partitioning of rather polar toxic molecules like catechols. These need to be removed from the aqueous phase that contains the biocatalyst, but since they are in themselves comparatively polar, traditional two-liquid phase solvents with a logP greater than 4 like medium chain length alkanes do not lead to substantial improvements (75). However, it is easily conceivable that strains that tolerate bulk amounts of styrene might serve as production strains in two-liquid phase systems when the organic phase consists of a nontoxic carrier solvent and styrene at concentrations higher than 2%. To allow future investigations into whether the potential of such a strain can be realized, we constructed a mutant of the strain *Pseudomonas* sp. strain VLB120 that was deficient in the styrene oxide isomerase and consequently accumulated styrene oxide. This mutant synthesized styrene monooxygenase to levels of 67 U*(g cdw)^{-1} with glucose as the carbon source. This is in agreement with earlier reports on the styrene degradation inducer profile of *P. putida* CA-3. Cells of this strain synthesized styrene degradation enzymes after induction with styrene when grown with glucose. Compounds such as for example citrate and glutamate acted as repressors (262).

The styrene degrader *P. putida* S12 oxidized styrene with rates of up to 200 U*(g cdw)^{-1} after growth on styrene. This is significantly higher than the value found for the VLB120AC mutant that grew on glucose. However, growth on styrene - for which one might expect maximal induction of the pathway - is no option for a styrene oxide production process, so the data obtained under the conditions reported here appear more suitable to evaluate the potential of
*Pseudomonas* sp. strain VLB120ΔC as a production strain. In this light, the combination of a cheap carbon source such as glucose with substantial activities in the order of 70 U*(g cdw)*^−1^ and the solvent resistance phenotype appears very promising. Tools are available to convert *Pseudomonas* strains into highly active whole-cell biocatalysts with mini-Tn5 transposons carrying expression cassettes based on the *alk* regulatory system of *P. oleovorans* GPo1 (see Chapter 6). Thus, the mutant (or wild-type) strain might be interesting for the construction of recombinant biocatalysts for processes that produce compounds other than (S)-styrene oxide and could benefit from an increased solvent resistance of the biocatalyst.
CHAPTER 6: ENGINEERING OF A STABLE WHOLE-CELL BIOCATALYSTS CAPABLE OF (S)-STYRENE OXIDE FORMATION FOR CONTINUOUS TWO-LIQUID PHASE APPLICATIONS

SVEN PANKE, VÍCTOR DE LORENZO, ARNÉ KAISER, BERNARD WIT bolt, AND MARCEL G. WUBBOLTS
SUBMITTED
**SUMMARY**

Recombinant strains of *Pseudomonas putida* KT2440 carrying genetic expression cassettes with xylene oxygenase and styrene monooxygenase encoding genes on the chromosome could be induced to specific activities that rivaled those of multicopy plasmid based *E. coli* recombinants. Such strains maintained the introduced styrene oxidation activity in continuous two-liquid phase cultures for at least 100 generations, although at a lower level. The data suggest that placement of target genes on the chromosome might represent a suitable route for the construction of segregationally stable and highly active whole-cell biocatalysts.

Biotransformations provide access to asymmetric oxidations which are difficult to achieve with purely chemical methods (32, 348). The required reduced cofactors can best be generated in whole cell biocatalysts, and we have described several *Escherichia coli* recombinants equipped with *Pseudomonas* monooxygenases that carry out regio- and enantioselective oxidations of aromatic and aliphatic compounds (101, 159) including processes for the production of (S)-styrene oxide, a potentially important chiral building block in organic synthesis, from styrene (394, Chapters 3 and 4). This has resulted in recombinant biocatalysts that produce (S)-styrene oxide at average rates between 120 U per liter liquid volume with *P. putida* mt-2 TOL plasmid derived xylene oxygenase (Chapter 3) and 150 U per liter with *Pseudomonas* sp. strain VLB120 derived styrene monooxygenase (Chapter 4). To reduce the toxicity of styrene and styrene oxide in these processes, they were dissolved in an apolar carrier phase (117, 395). The availability of whole cell recombinant biocatalysts that exhibit high specific activities is crucial. This has been achieved by expression of target genes in recombinant *E. coli* via pBR322-derived multicopy plasmid vectors based on the *alk* regulatory system of *P. oleovorans* (Chapters 3 and 4), where the positive regulator protein AlkS activates transcription from the cognate promoter *alkBp* (Fig. 6.1A) (368). Cultures for styrene oxide production were set up as fed-batch experiments, in which the plasmid encoded activity could be stably maintained. However, from an economical
as well as from a physiological point of view, a continuous culture would be advantageous, because it would eliminate periods of low productivity and prevent the drop in the number of viable cells (and consequently a drop in volumetric productivities) when a two-liquid phase culture enters stationary phase (101, 102). Among the challenges in setting up such continuous cultures is the frequently limited genetic stability of highly active biocatalysts (102, 110, 125, 201, 404). Plasmid located recombinant genes in growing cells can be subject to structural or segregational instability. The latter aspect has been addressed intensively and a number of elegant possible solutions have been proposed (55, 124, 187, 248, 285). One way to eliminate the possibility of segregational instability completely might be to place the recombinant genes on the chromosome of a suitable host strain, for example via mini-Tn5 transposons (161). As the transposase is lost during transposition with this system, the recombinant gene remains stably integrated, which has made this system a very attractive model system to engineer microorganisms for environmental, medical, and metabolic engineering applications (77, 98, 244, 292, 306, 354, 355, 364). Furthermore, tools are available to efficiently remove (antibiotic) selection markers (197), thereby facilitating commercial utilization of the resulting strain and biomass disposal. Since such recombinants carry only one to a few gene copies (141), it remains to be shown whether such strains - lacking the opportunity to capitalize on gene dosage effects - produce sufficient activities for application as practical biocatalysts in the production of fine chemicals. In this report, we investigated whether placing genetic cassettes that contain the elements of the alk regulatory system together with monooxygenase genes onto the chromosome of E. coli JM101 or P. putida KT2440 led to stable whole cell biocatalysts with high specific activities.

**Analysis of E. coli mini-Tn5 mutants.** The 6.4-kb NotI fragment of pSPZ2MA containing alkS, alkBp, xylM (the asterisk indicates genes where internal NdeI sites have been removed) (Fig. 6.1B) (Chapter 3) was transferred to mini-Tn5 delivery plasmid pJMS11 (Fig. 6.1C) (274) and the resulting mini-transposon was delivered to E. coli JM101 cells (312) by a tripontal mating with E. coli HB101 (RK600) as the helper as described previously (197). We could readily isolate strains that were resistant to kanamycin but sensitive to ampicillin,
Fig. 6.1. Function of the alk regulatory system and structure of the genetic elements used in this study. A) The regulator protein AlkS is activated by interaction with DCPK or n-octane and initiates transcription from alkBp. B) *Nol* fragments of plasmids pSPZ2MA, pSPZ2AB, and pSPZ2E for the synthesis of xylene oxygenase, styrene monooxygenase, and catechol-2,3-dioxygenase. Drawing is to scale. Asterisks indicate that the wild-type gene has been engineered to eliminate internal *NdeI* sites. Arrows indicate heterologous promoters, triangles within boxes indicate homologous promoters. C) Plasmids that received the *Nol* cassettes shown in B). Drawing in the upper part is to the same scale as in B). The open arrow indicates the direction of transcription of the promoterless *xylE* gene. The two cassettes shown are inserted in the *SalI* site of the basic vector and give rise to the two mini-transposon delivery plasmids pJMS11 and pUT-Km. The grey boxes represent the two *res* sequences that are substrate of the RP4 resolvase. The minitransposon is defined by the I and O ends (black boxes).

indicating true transposition events (80). The transconjugants were grown in 100 mL of M9*- mineral medium supplemented with 100 μL of US*- trace element solution, 100 μL of a 1% (wt/vol) thiamine hydrochloride solution, and 0.5% (wt/vol) glucose as the carbon source as described previously (Chapter 3). The cultures were induced at an OD*<sub>450</sub>* of around 0.4 by the addition of 0.05% (vol/vol) dicyclopropylketone (DCPK; Aldrich, Buchs, Switzerland) and at regular intervals subjected to a whole cell styrene oxide formation assay as described (Chapter 2). However, we failed to detect any styrene oxide formation activity with these strains in our assay (detection limit 0.1 U*(g of cell dry weight)<sup>-1</sup> (U*(g cdw)<sup>-1</sup>), one unit (U) being defined as the enzymatic activity that forms 1 μmole of styrene oxide in 1 min). Previous attempts to produce xylene oxygenase in *E. coli* JM101 from pBR322-derived expression plasmid pSPZ3 had resulted in maximum specific activities of 91 U*(g cdw)<sup>-1</sup> (Chapter 3), suggesting that the more than 900-fold reduced level of specific activity in the present experiment might be - at least in part - a function of the vastly lower gene dosage in the *E. coli* transconjugant.

**Efficient alk-based expression of oxygenases in *E. coli* depends on copy-number.** To investigate this, we constructed a 5.1-kb genetic cassette that was identical to the one above but carried *xylE*<sup>*</sup> instead of *xylM*<sup>A</sup> (Fig. 6.1B), so that transconjugant strains would produce catechol-2,3-dioxygenase which is easy to assay in vitro even in small amounts (259). To obtain a *xylE* gene devoid of internal
Ndel sites but with a Ndel site on the start codon which was compatible with the alk-based expression plasmids (Chapter 3), we performed a first PCR with one primer that annealed at the 5' end of the gene and introduced the Ndel site on the start codon together with a new BamHI site further upstream (5' CATGAGGATCCAAAGAGGTGACCATAATGAAACAAAGGTG 3', BamHI site underlined, Ndel site in italics, xylE start codon in boldface type), and a second primer that primed inside xylE and thereby silently mutated the internal Ndel site (5' GGCACAGCCATAACGCCATCAGATC 3', mutagenic nucleotide underlined). The resulting 300-bp fragment served as the first primer of a second PCR together with a primer which annealed at the end of the xylE gene and introduced an EcoRI site (5' AAAAAAGAATTCCCATCAGACGACCGGTACATGAATCG 3', EcoRI site underlined, xylE stop codon in boldface type). The resulting 940-bp fragment was digested with BamHI and EcoRI and inserted into pSPZ1(+), reexcised as an Ndel/Ascl fragment, and inserted into pSPZ2Not along the lines outlined earlier (Chapter 3). In the resulting plasmid pSPZ2E the xylE* gene was available as a NotI flanked alkS* alkBp-xylE* cassette analogous to the one carrying xylM*A (Fig. 6.1B). This cassette was transferred to pUT-Km (Fig. 6.1C) (79) for mini-transposon delivery and to pVLT31N (Fig. 6.1C) for expression in multicopy. Plasmid pVLT31N is a derivative of the broad host range vector pVLT31 (78) with an additional NotI site introduced by digesting pVLT31 with HpaI and SmaI and ligating the resulting fragment to an octameric DNA linker with the NotI recognition sequence. This also led to the loss of the lacP/P_{lac} expression system present on pVLT31. E. coli JM101 transconjugants carrying the xylE* mini-transposon on the chromosome were readily isolated, grown in mineral medium with glucose as the carbon source as described above, and harvested 4 h after induction. All strains tested produced catechol-2,3-dioxygenase to levels measurable in cell-free extracts, up to a maximum of 0.9 U*(mg protein)^{-1}. E. coli JM101 transformants carrying the xylE* cassette in multicopy on pVLT31N were grown in parallel in the presence of 12.5 µg*mL^{-1} tetracycline and produced after induction catechol-2,3-dioxygenase to 72 U*(mg protein)^{-1} activity in cell-free extracts. This suggested that in E. coli the specific activity of recombinant strains carrying our genetic cassettes was indeed a function of gene dosage, although the
ratio of *in vitro* activities found for the catecho-2,3-dioxygenase is in the order of 80, which does not fully explain our inability to obtain *E. coli* transconjugants with xylene oxygenase activity.

**Analysis of *P. putida* mini-Tn5 mutants.** In wild-type *P. oleovorans* GPo1, the alk regulatory system is located on the low-copy-number catabolic OCT plasmid and membrane located AlkB protein is still synthesized to 2% of total cell protein after induction (342). This raised the possibility that in *Pseudomonas* strains alk-based monocopy constructs might give significantly more active strains. We used the *NotI* cassettes of pSPZ2MA and pSPZ2AB (a 6.1-kb cassette carrying the genes of the styrene monooxygenase of *Pseudomonas* sp. strain VLB120, Fig. 6.1B) (Chapter 4) on pJMS11 to construct *P. putida* KT2440 transconjugants. On pJMS11, the selection markers of the mini-transposons are flanked by *res* sites that are the substrate of the RP4 resolvase. After successful construction and analysis of the biocatalyst (Fig. 6.3A), they can be easily excised by expressing the resolvase gene from a suicide plasmid (197). The transconjugants were used in shaking flask experiments as described above but with 0.5% (wt/vol) citrate as the carbon source. *P. putida* SMA, a *P. putida* KT2440 derivative with alkS* alkBp-xylM* in the chromosome, synthesized xylene oxygenase to levels of 41 U*(g cdw)*⁻¹ (Fig. 6.2B). *P. putida* SAB, with alkS* alkBp-styAB in the chromosome, synthesized styrene monooxygenase to specific activities of 86 U*(g cdw)*⁻¹ (Fig. 6.2C), which was even more than the 70 U*(g cdw)*⁻¹ found for *E. coli* JM101 recombinants expressing the styrene monooxygenase genes from the same cassette on multicopy plasmid pSPZIO (Chapter 4). The same activities were also achieved with the strains *P. putida* SMAΔ and *P. putida* SABΔ, which differed from their parent strains only in that they had removed the transposon selection markers, the kanamycin and the xylE gene, by expressing the RP4 resolvase gene from a suicide plasmid as described (197) (data not shown). To investigate whether these numbers could be increased by increasing the copy-number of the genetic cassettes, analogous to the situation in *E. coli*, we inserted the *NotI* cassettes with the genes for styrene monooxygenase and xylene oxygenase into pVLT31N. The resulting plasmids were conjugated into *P. putida* SMA and *P. putida* SAB using *E. coli* S17-1λpir as the host (335), after control experiments with the plasmids in
unmodified *P. putida* KT2440 had shown that both plasmids were functional (data not shown). Generally, the maximum specific activities of the resulting strains were not higher (Fig. 6.2B and C). These results were in agreement with data reported by Yuste *et al.*, who obtained similar results with an *alkBp-lacZ* fusion (403). Remarkably, although the specific activities did not change significantly upon providing the cassettes in multicopy, the growth behavior of the strains did: strains carrying the cassettes in multicopy grew more slowly, while the strains carrying only the chromosomal copy showed only little influence of induction on growth (Fig. 6.2). Although this leaves open the question of the maximum achievable rate of styrene oxide formation in *Pseudomonas* and the nature of the bottleneck in the development of specific activity, the results indicate that by choosing a host different from *E. coli* it is indeed possible with an *alk*-regulatory based system to construct a whole cell biocatalyst that carries only one copy of the target genes and still can synthesize the encoded enzyme to a high specific activity. The results do not indicate whether the higher activities are due to an increased specific activity of the *Pseudomonas* derived monooxygenases in a recombinant *Pseudomonas* host or due to an increased amount of formed protein, for example because transcription from the *alkBp* promoter is more efficient in a *P. putida* than in an *E. coli* host.

**Continuous two-liquid phase cultivation with *P. putida* SMA.** To obtain a first indication whether the potential for activity and stability could be realized with our transconjugants, we performed a continuous two-liquid phase culture with *P. putida* SMA (Fig. 6.3B). The details of the reactor system have been described previously (Chapter 3). In short, a 3 liter stirred tank reactor with temperature (30°C), pH (7.1, titration with 30% phosphoric acid and 4 M sodium hydroxide), and stirring speed (1500 rounds per minute) regulation and an airflow manually adjusted to 1 liter per minute was placed on a balance (Bioengineering, Wald, Switzerland) which regulated an effluent pump that was activated above a predefined weight of the reactor. This pump then removed liquid from the reactor at around ten-fold the rate of the medium feed, leading to an oscillation in weight (and consequently of the dilution rate) of around 40 g (4%). A stationary phase *P. putida* SMA preculture in 100 mL M9* mineral medium supplemented with US* trace elements, and 0.5% (wt/vol) citrate was pumped into the reactor which
Fig. 6.2. Shaking flask experiments on mineral medium and citrate as the carbon source with recombinant strains synthesizing xylene oxygenase or styrene monooxygenase. Upper panel: growth behavior. Lower panel: Development of the specific activity after induction with 0.05 % (vol/vol) DCPK. Point of induction is indicated by the arrow.

A) Influence of inducer on the growth of parent strain P. putida KT2440. No styrene oxidation activity was observed for this strain irrespective of the induction.

B) Expression of chromosomally located (left panel) and chromosomally and plasmid located (right panel) xylene oxygenase genes. The structure of the gene cassette is indicated on top.

C) Expression of chromosomally located (left panel) and chromosomally and plasmid located (right panel) styrene monooxygenase genes.
contained 900 mL of M9* mineral medium with 2 mL of US* solution per liter, and 0.5% citrate. The working volume was fixed to 1 liter and mineral medium of identical composition was fed into the reactor at a dilution rate of 0.2 h\(^{-1}\). After 50 h, an organic feed was started that pumped an organic phase consisting of 1% (vol/vol of organic phase) \(n\)-octane (Acros, Geel, Belgium) as the inducer of the \(alk\) regulatory system and 1% (vol/vol of organic phase) styrene (99%, Fluka, Buchs, Switzerland) as the substrate for the xylene oxygenase dissolved in AL240 (Chemische Fabrik Schweizerhall, Switzerland) as the carrier solvent at a dilution rate of 0.02 h\(^{-1}\). AL240 is a mixture of iso-, cyclo-, and linear alkanes with a chain length of at least 13 carbon atoms and has no effect on bacterial growth (318). The aqueous feed was reduced to 0.18 h\(^{-1}\) and the weight limits for the effluent pump where adjusted accordingly. This led after equilibration to a volume portion of the organic phase of 10%. Analysis of the liquid phases in the reactor has been described previously (Chapter 3). In the presence of the organic phase, cell dry weight stabilized at around 1.2 g per liter aqueous phase and styrene oxide accumulated in the organic phase to around 16 mM, which translated into a styrene oxide formation rate of 6 U per liter liquid volume or 5 U\(\text{g cdw}\)^{-1} (Fig. 6.3). This activity was maintained until the end of the experiment 100 generations (350 h) after induction. In order to investigate whether the smaller specific activities in the continuous culture were obtained with cells that had lost the ability to synthesize xylene monooxygenase to the high levels observed in the shaking flask experiments, we removed aqueous phase from the reactor at three times (50, 136, and 325 h after induction) and plated it on LB-agar plates. Sets of five of the resulting colonies served as the start of new shaking flask cultures in mineral medium with citrate as the carbon source as described above. In all three cases, the accumulated averaged specific activities 4 h after induction were within 10% of the original strain (results not shown), indicating that the relatively low activities were not due to genetic instability.

One clear change in the experimental protocol from shaking flask experiments to the continuous culture is the presence of a second phase and the mode of induction. While cells were induced by DCPK addition without an organic phase in shaking flask experiments, induction in the continuous culture went via octane
Fig. 6.3. Continuous two-liquid phase culture with *P. putida* SMA. A) Genotype of *P. putida* SMA. The mini-transposon of pJMS-SMA (determined by the I- and O-end, black boxes) contained the *alk* expression cassette for the synthesis of xylene oxygenase and the selection marker *npt* for kanamycin resistance and *xylE* for easy colorimetric selection. The selection marker is flanked by *res* sites (grey boxes) which are the substrate of the RP4 resolvase. B) Continuous culture (\(D = 0.2 \text{ h}^{-1}\)) of *P. putida* SMA on mineral medium with 0.5 % (wt/vol) citrate as the carbon source. The arrow indicates the start of the organic feed of a mixture of long-chain alkanes containing 1 % octane and 1 % styrene, which accounted after equilibration of the system for 10 % of total liquid volume.
dissolved in a carrier solvent of longer chain alkanes. While 1 % (vol/vol) octane was sufficient to induce fed-batch E. coli cultures efficiently (Chapters 3 and 4), this is not necessarily true for cultures with recombinants based on P. putida KT2440. It is also possible that the presence of the organic phase interfered with the accumulation of high specific activities. Although Pseudomonas strains in general are considered to be tolerant to organic solvents with a logP higher than 4 (293, 374) and exceptional solvent resistances have been reported (75), the behavior of P. putida KT2440 in two-liquid phase cultures has never been investigated in detail.

Taken together, the data presented here indicate that chromosomal integration of genes under a suitable regulatory system represents a very useful route to construct a whole-cell biocatalyst that is able to synthesize rather complex monooxygenases to high specific activities and that can maintain a constant activity for extended periods of cultivations in the presence of an organic phase. Further work will address the exploitation of the maximum specific activities of such recombinant strains in continuous cultivations.
CHAPTER 7: SUMMARY AND OUTLOOK

SVEN PANKE
The potential of recombinant biocatalysis was demonstrated by the development of a whole-cell process for the production of enantiopure styrene oxide. Process development included the steps from the isolation of the genes of a novel monooxygenase to the scale-up of a two-liquid phase process to 30 L. *Pseudomonas* sp. strain VLB120 degraded styrene by oxidation to styrene oxide, isomerization to phenylacetaldehyde, and dehydrogenation to phenylacetic acid. The corresponding genes were cloned from a genomic library in *Escherichia coli* and characterized. The two genes *styAB* encoded a styrene oxygenase which converted styrene to (S)-styrene oxide with an enantiomeric excess of >99%. Expression vectors with the genes for styrene monooxygenase and xylene oxygenase were constructed based on the octane responsive *alk* regulatory system of *P. oleovorans* GPo1. In shaking flask experiments with glucose as the carbon source, *E. coli* recombinants that carried these expression plasmids reached maximum specific activities of approximately 90 U*(g cdw)*⁻¹ for xylene oxygenase and 70 U*(g cdw)*⁻¹ for styrene monooxygenase. 22 g of (S)-styrene oxide were produced from inexpensive styrene in a 2 L two-liquid phase fed-batch culture with the styrene monooxygenase synthesizing *E. coli* strain. A bis(2-ethylhexyl)phthalate phase at a phase ratio of 0.5 served as a reservoir of the substrate and as a sink for the product, which accumulated to a final concentration of 184 mM. Maximum (S)-styrene oxide productivities of 2.2 g*(h*L*<sub>org</sub>)*⁻¹ were achieved. This process was scaled-up to 30 L of two-liquid phase medium. In this system, power input was close to commercially operating units. Although the system became rapidly depleted of dissolved oxygen, an average (S)-styrene oxide productivity 1.2 g*(h*L*<sub>org</sub>)*⁻¹ was maintained over more than 10 h, which resulted in a chemical yield of at least 388 g of (S)-styrene oxide. At this stage, the culture appeared to be styrene mass transfer limited. This suggested that the bottleneck of (S)-styrene oxide production resided no longer in the available amount of biological activity.

To further increase the styrene mass transfer rates, higher concentrations of styrene in the organic phase are necessary, which is likely to require a bacterial strain that is exceptionally resistant to styrene and styrene oxide. *Pseudomonas* sp. strain VLB120 might be such a strain. To allow future investigations of its potential,
a mutant *Pseudomonas* sp. strain VLB120AC was constructed that was deficient in the styrene oxide isomerase gene and allowed accumulation of styrene oxide in two-liquid phases with elevated levels of styrene.

One question that will be important in the future investigations of monooxygenase-based bioprocesses is for how long can the high activities be maintained that one can obtain with recombinant strains. Genetic tools were constructed to insert oxygenase genes into the chromosome of Gram-negative bacteria. *P. putida* KT2440 recombinants with high specific activities were thus constructed that maintained xylene oxygenase activity in a continuous two-liquid phase culture for at least 100 generations. This strategy might be extended to other *Pseudomonas* strains like *Pseudomonas* sp. strain VLB120AC.

The results clearly illustrate that it is possible to efficiently use recombinant model hosts as whole-cell biocatalysts for the production of toxic added-value products from toxic substrates, provided an efficient *in situ* product recovery system is available. With the development of a high pressure reactor for the operation of aerobic two-liquid phase processes, the advances in process development are complemented by the appropriate safe equipment, which will allow to regard two-liquid phase technology more and more as a routine technology. Future work on process improvement needs to aim at integrating solvent resistance properties for the biocatalyst or of a novel *in situ* product removal process, for example solid-phase extraction with a high circulation rate for the reaction medium, which might facilitate down stream processing without compromising the volumetric productivity.
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