

An Alkane-Responsive Expression System for the Production of Fine Chemicals

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Membrane-located monooxygenase systems, such as the *Pseudomonas putida* mt-2-derived xylene oxygenase, are attractive for challenging transformations of apolar compounds, including enantiospecific 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. Determination of the nucleotide sequence of *alkS*, whose activated gene product positively regulates the transcription of the structural genes *alkBFGHJKL*, on a 3.7-kb *Sall*-*HpaI* OCT plasmid fragment was completed, and the N-terminal amino acid sequence of an AlkS-LacZ fusion protein was found to be consistent with the predicted DNA sequence. 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 per g (dry weight) of cells 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 h per liter of aqueous phase were obtained; these values represented a fivefold improvement compared with previous results.

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 (18, 30, 40). The ability of xylene oxygenase to hydroxylate methyl substituents on substituted benzenes or their heteroaromatic equivalents has made it an important biotechnological enzyme (24, 56). This enzyme consists of a membrane-bound component, XylM, which carries out the oxygenation step (50), and a cytoplasmic NADH:acceptor reductase component, XylA, which supplies reducing equivalents to XylM (45). The potential of this system for biological production of fine chemicals has already been exploited; wild-type cells of *P. putida* mt-2 are used by Lonza to produce heteroaromatic acids on a commercial scale (24). 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 (55) (Fig. 1). *Escherichia coli* recombinants carrying the genes for xylene oxygenase have produced (*S*)-styrene oxide from inexpensive styrene in a 2-liter reactor (54). Unfortunately, so far the productivities displayed by such recombinants have been insufficient to commercially exploit their synthetic potential (17, 55). A number of observations have indicated that expressing the xylene oxygenase genes via the *alk* regulatory system of *Pseudomonas oleovorans* GPo1 might provide suitable biocatalysis strains for two-liquid-phase cultures. *P. oleovorans* GPo1 degrades medium-chain-length alkanes with a set of enzymes encoded by two *alk* gene clusters on the catabolic OCT plasmid (Fig. 2A) (51). The first cluster contains the *alkBFGHJKL* operon, which contains all but one of the struc-

tural genes for conversion of alkanes to the corresponding alkanic acids and coupling of these compounds to coenzyme A (Fig. 2B). The second cluster contains the remaining structural gene, *alkT*, and the gene which encodes the regulatory protein AlkS (11). 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 unrelated inducers, such as dicyclopropylketone (DCPK) (16, 49). 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 (35). This indicated that AlkS, together with its cognate promoter *alkBp* (26), 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* (46, 58), 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 Table 1. We used Luria-Bertani (LB) complex medium (Difco Laboratories, Detroit, Mich.) or M9 mineral medium (44) 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 1 M hydrochloric acid and (per liter) 1.50 g of MnCl₂ · 4H₂O, 1.05 g of ZnSO₄, 0.30 g of H₃BO₃, 0.25 g of Na₂MoO₄ · 2H₂O, 0.15 g of CuCl₂ · 2H₂O, and 0.84 g of Na₂EDTA · 2H₂O. Trace element solution US* also contained (per liter) 4.87 g of FeSO₄ · 7H₂O and 4.12 g of CaCl₂ · 2H₂O. Alternatively, we used M9* mineral medium, which was identical to M9

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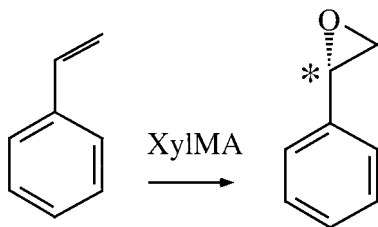


FIG. 1. Conversion of inexpensive styrene to (*S*)-styrene oxide by xylene monoxygenase. The chiral carbon atom is indicated by an asterisk.

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 each mineral medium at a concentration of 0.5% (wt/vol) as a carbon source or to complex media 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 (32, 33). Antibiotics were added at the following concentrations: ampicillin, 150 μ g/ml; kanamycin, 50 μ g/ml; chloramphenicol, 30 μ g/ml; and tetracycline, 12.5 μ g/ml. When necessary, thiamine was added at a concentration of 1 mg per liter. The cloning and DNA modification protocols used have been described elsewhere (44). 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).

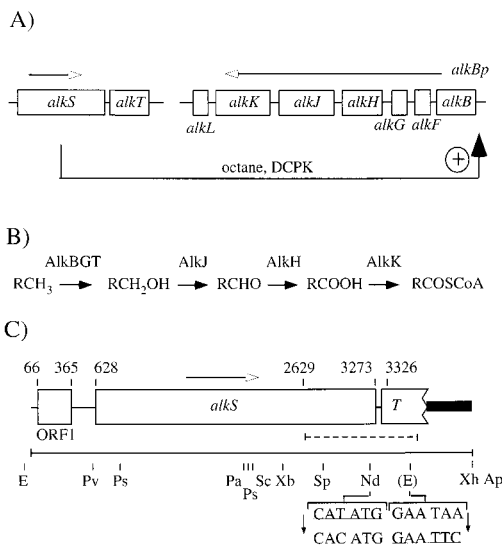


FIG. 2. (A) Organization and regulation of the *alk* genes on the OCT plasmid in *P. oleovorans* GP01. The regulatory protein AlkS is activated by octane or DCPK and induces transcription from the *alkBp* promoter. The directions of transcription are indicated by arrows with open arrowheads. Transcriptional 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 (CoA). R represents pentyl to undecyl residues. The functions of AlkF and AlkL are unclear. (C) Detailed structure of pBG11. The region sequenced is indicated by the solid line, while the previously described sequence is indicated by the dashed line. The coordinates (in the deposited sequence) are indicated at the top together with the direction of transcription of *alkS*. The solid bar indicates the portion of the plasmid that was derived from pJRD158. Restriction sites that were used for cloning in this work and sites which also occur in the pUC18 polylinker are indicated at the bottom. The *EcoRI*, *XhoI*, and *ApaI* sites were derived from the polylinker of pGEM-7Zf(+), which was the basis of pBG11. Two modifications introduced by site-directed mutagenesis are indicated at the bottom; the sequences of newly introduced (in parentheses) or removed restriction sites are underlined. Abbreviations: Ap, *ApaI*; E, *EcoRI*; Nd, *NdeI*; Pa, *PacI*; Ps, *PstI*; Pv, *PvuII*; Sc, *SacI*; Sp, *SphI*; Xb, *XbaI*; Xh, *XhoI*.

Nucleotide sequence analysis. A 3.7-kb *SalI-HpaI* OCT plasmid fragment containing *alkS* was excised from plasmid pGec258 together with a 340-bp portion of original cloning vector pJRD158 as a *SalI-XhoI* fragment. This fragment was inserted into the *XhoI* restriction site of pGEM-7Zf(+) so that the *XhoI* site that was reconstituted was oriented away from the *lacZp* promoter of the vector, while the destroyed *SalI* site was oriented towards the promoter. The resulting plasmid was designated pBG11. Unidirectional deletions from each end of the cloned fragment in pBG11 were generated by exonuclease III digestion as described previously (21). The collection of clones was completed by *Sau3A* and *TaqI* shotgun clones. The *alkS* sequence was determined by the Sanger dideoxy method. The subsequent data analysis was performed with the DNASTAR software package (DNASTAR, Madison, Wis.), the BlastP algorithm (1) of the GenBank database at the National Center for Biotechnology Information, and the analysis tools (42) available at the ExpASY Molecular Biology Server.

Determination of the N-terminal sequence of AlkS. To construct an AlkS'-*lacZ* fusion protein with the first 75 amino acids of AlkS and the *lacZ* gene product of plasmid pMC1871, we excised the *lacZ* gene as a *PstI* fragment and inserted it into the *PstI* site close to the 5' end of *alkS* in pBG11, which generated the desired fusion in plasmid pBG11*lacZ*. *E. coli* X7026(pBG11*lacZ*) was grown in liquid LB medium and induced by adding 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) in the exponential growth phase. A crude cell extract was obtained 4 h later by sonication, and this extract was clarified by centrifugation. The supernatant was applied to a β -galactosidase affinity column containing *p*-aminobenzyl-1-thio- β -galactopyranoside agarose (Sigma Chemical Co., St. Louis, Mo.) (15) connected to a fast protein liquid chromatography system (Pharmacia, Dübendorf, Switzerland). The retained protein was eluted with 0.1 M sodium borate (pH 10). The fractions with the highest levels of β -galactosidase activity were pooled and desalted by fast protein liquid chromatography by using a Sephacryl column (Sigma). The resulting partially purified protein was subjected to sodium dodecyl sulfate gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes as described previously (31), and the sequence of the first 15 amino acids of the fusion protein was determined by Edman degradation by using an Applied Biosystems automatic protein sequencer.

Site-directed mutagenesis. Site-directed mutagenesis was performed as described previously (29) by using plasmid pGC2, plasmid pGC2Mlu (which is identical to pGC2 except that an additional *MluI* site is inserted by using oligonucleotide 6 [Table 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 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 pHP45 Ω as a *SphI-HindIII* fragment into pUC18. *NdeI* sites were removed from plasmid pBG4 by digestion with *NdeI*, filling in with the Klenow polymerase, and religation leading to pBG4 Δ N, 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 pCCKO4 into its polylinker. The resulting plasmid, pBG4 Δ N Δ , 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 *AscI* 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 *SmaI-HindIII* OCT plasmid fragment with a new *NdeI* site on the ATG at the beginning of the *alkB* gene. The modified promoter was excised as a 273-bp *SmaI-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 *AscI* sites and the terminator.

The *alkS** gene was obtained as a 3.5-kb *EcoRI* fragment from plasmid pBG11E Δ N and was inserted into pUC18N Δ N, a pUC18Not derivative whose internal *NdeI* site had been removed as described above for pBG4 Δ N. 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 *SmaI-HindIII* fragment containing the *alkBp* promoter and the T4 transcriptional terminator of pBGPalk, which resulted in plasmid pSPZ2Not. An equivalent manipulation sequence based on the cloning vector pUC18Sfi resulted in 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 *rrnBT1* transcriptional terminator (4) 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 *SmaI* fragment of mini-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Source or reference
<i>E. coli</i> strains		
X7026	F ⁻ Δ(<i>gpt-lac</i>)5 <i>supE44 relA1 spoT1</i>	23
JM101	<i>supE thi-1 Δ(lac-proAB) F'</i> [<i>traD36 proAB⁺ lacI^s lacZΔM15</i>]	44
CJ236	<i>dut-1 ung-1 thi-1 relA1/pCJ105</i> (Cm ^r F')	29
Plasmids		
pUC18	<i>lacZα</i> , cloning vehicle, Ap ^r	57
pUC18Not	<i>lacZα</i> , cloning vector, polylinker flanked by <i>NotI</i> sites, Ap ^r	22
pUC18Sfi	<i>lacZα</i> , cloning vector, polylinker flanked by <i>SfiI</i> sites, Ap ^r	22
pHP45Ω	Source of the T4 transcriptional terminator, Sm ^r Ap ^r	39
pGEM-7Zf(+)	<i>lacZα</i> , cloning vector, phage <i>flori</i> , Ap ^r	Promega
pGEM-7Zf(-)	Same as pGEM-7Zf(+) but <i>f1 ori</i> is inverted, Ap ^r	Promega
mini-Tn5Km	Source of kanamycin interposon, Ap ^r Km ^r	8
pBR322	Cloning vehicle, Ap ^r Tc ^r	3
pGC2	pBR322 derivative with M13 <i>ori</i> , Ap ^r	34
pMC1871	pBR322 derivative with promoterless <i>lacZ</i> , Tc ^r	6
pCKO4	Source of <i>xylN</i> , Cm ^r	37
pBG63	Source of <i>xylMA</i> , <i>flori</i> , Ap ^r	55
pGEc258	pBR322/pJRD158 derivative, source of <i>alkS</i> , Ap ^r	11
pGEc286	Promoter probe vector with <i>alkBp-CAT</i> fusion, Ap ^r	25
pGC2Mlu	pGC2 with additional <i>MluI</i> site, Ap ^r	This study
pBG11	pGEM7-Zf(+) with <i>alkS</i> , Ap ^r	This study
pBG11 <i>lacZ</i>	pBG11 derivative with <i>alkS'</i> - <i>lacZ</i> fusion, Ap ^r	This study
pBG4	pUC18 with T4 transcriptional terminator of pHP45Ω, Ap ^r	This study
pBG4ΔN	pBG4 without <i>NdeI</i> site, Ap ^r	This study
pBG4NΔN	pBG4ΔN with <i>xylN</i> of pCKO4, Ap ^r	This study
pBGL	pBG4NΔN with small new polylinker, Ap ^r	This study
pGEMPalkN	Cloning vector with <i>alkBp</i> promoter and <i>NdeI</i> site on the ATG of <i>alkB</i> , Ap ^r	This study
pBGPalk	pBGL with <i>alkBp</i> of pGEMPalkN, Ap ^r	This study
pBG11EΔN	pBG11 with an additional <i>EcoRI</i> site and removed <i>NdeI</i> site internal to <i>alkS</i> , Ap ^r	This study
pUC18NΔN	pUC18Not without <i>NdeI</i> , Ap ^r	This study
pUC18NS*	pUC18NΔN with <i>alkS*</i> of pBG11EΔN, Ap ^r	This study
pSPZ1(+)	pGEM-7Zf(+) with a new polylinker, Ap ^r	This study
pSPZ1(-)	Same as pSPZ1(+) but from pGEM-7Zf(-), Ap ^r	This study
pSPZ2Not	pUC18NS* with <i>alkBP</i> and <i>T4t</i> of pBGPalk, contains <i>alkS* alkBp T4t</i> as a <i>NotI</i> cassette, Ap ^r	This study
pSPZ2Sfi	Same as pSPZ2Nde but <i>SfiI</i> sites instead of <i>NotI</i> sites, Ap ^r	This study
pBG63N	pBG63 with <i>NdeI</i> site in <i>xylM</i> removed and new <i>NdeI</i> site on the ATG of <i>xylM*</i> , Ap ^r	This study
pSPZ2MA	pSPZ2 with <i>xylM*A</i> of pBG63N under <i>alkBp</i> control, Ap ^r	This study
pBRNS	pBR322 with <i>rrnBT1</i> terminator and additional <i>NotI</i> and <i>SfiI</i> restriction sites, Ap ^r	This study
pBRNSKm	pBRNS with kanamycin resistance of mini-Tn5Km replacing the <i>bla</i> gene, Km ^r	This study
pBRNSKmΔ	pBRNSKm without remnants of the <i>tet</i> gene, Km ^r	This study
pSPZ3	pBRNSKmΔ with <i>alkS* alkBp xylM*A</i> of pSPZ2MA, Km ^r	This study

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 *rrnBT1* terminator from pBRNS). The resulting plasmid was designated pBRNSKm. The remaining portions 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, pBRNSKmΔ, was made blunt by T4 DNA polymerase treatment. An identically treated *NotI*-*AseI* 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 an 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 the appropriate time (see below) and were subjected to a whole-cell assay for styrene oxide formation under optimized conditions as described elsewhere (38). 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 of activity was defined as the amount of activity that produced 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. 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 liters. Seals and O-rings were made out of solvent-resistant Viton. The reactor contained 1.025 liters of a mineral medium which contained (per liter) 8.82 g of KH₂PO₄, 10.85 g of K₂HPO₄, 8.82 g of Na₂HPO₄, 1.0 g of NH₄Cl, 0.5 g of NaCl, 0.49 g of MgSO₄ · 7H₂O, 14.7 mg of CaCl₂ · 2H₂O, 2.78 mg of FeSO₄ · 7H₂O, 5 g of glucose, 100 μl of polypropylene glycol 2000 (Fluka, Buchs, Switzerland), 0.5 ml of trace element solution US, thiamine, and kanamycin. The pH was maintained at 7.1 by adding 25% NH₄OH and 25% phosphoric acid. The NH₄OH also served as the source of nitrogen. After inoculation with 100 ml of the preculture, the reactor was aerated at a rate of 1 liter per min and was stirred at 1,500 rpm for ca. 12 h (overnight), which resulted in a culture in the stationary phase which contained approximately 2.2 g (dry weight) of cells per liter. The medium was then supplemented with 6 mg of thiamine per liter, 8 ml of trace elements solution US per liter, and 8.2 mg of FeSO₄ · 7H₂O per liter. 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 MgSO₄ · 7H₂O. One hour later, 375 ml of a mixture containing hexadecane, 1% (vol/vol [organic phase]) octane

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence
1.....	5' <u>TTG</u> TTC CCA TGT GAT TCC ACG 3'
2.....	5' TTT TCC TCA <u>TGT</u> GCC ACT TTA 3'
3.....	5' GTG TCC ATA <u>TGT</u> CCA CCT ACT 3'
4.....	5' GAG AAC ACC <u>ATA</u> TGC TTG AGA 3'
5.....	5' ATG GTA ATA TTG <u>GAA</u> TTC GTA TAA AA 3'
6.....	5' CTG GCA CGC <u>GTT</u> GGA CGC GCA 3'
7.....	5' TAT GTT AAC GGC GCG CCC ATG 3'
8.....	5' GGG CGC GCC GTT AAC A 3'
9.....	5' AAT TCA TAA AAC GAA AGG CTC AGT CGA AAG ACT GGG CCT TTC GTT TTA TCT GTT GTT TGC GGC CGC GGC CGC CTA GGC C 3'
10.....	5' AGC TGG CCT AGG CGG CCG CCG CCG CAA ACA ACA GAT AAA ACG AAA ACG AAA GGC CCA GTC TTT CGA CTG AGC CTT TCG TTT TAT G 3'
11.....	5' GGC GCG CCT GCA GAA TTC TCG AGA AGC TTC CCG GGA TCC TAG GCG CGC CAT GCA 3'
12.....	5' TGG CGC GCC TAG GAT CCC GGG AAG CTT CTC GAG AAT TCT GCA GGC GCG CCC GCC 3'

^a Oligonucleotides 1 to 6 were used as mutagenesis primers during site-directed mutagenesis (the mutagenic nucleotide[s] in each oligonucleotide is underlined). Oligonucleotides 7 to 12 were used for construction of the following polylinkers: oligonucleotides 7 and 8, *NdeI-HpaI-AscI*; oligonucleotides 9 and 10, *EcoRI-rmB T1-NorI-SfiI*-kill *HindIII*; oligonucleotides 11 and 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).

(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 2,500 rpm and the aeration rate was reduced to 0.67 liter per min 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/liter) 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 μ M in order to compensate for the reduced amount of yeast extract compared to our previous experiments (13, 54); the nonheme iron monooxygenase iron requirements, which were around 3 μ mol per g (dry weight) of cells (47), were taken into account. The preparation started to produce large amounts of foam within 1 h after induction; the foam was controlled by repeatedly adding silicone oil-based antifoam agent 289 (Sigma). Cultivation was carried out twice, and 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 concentrations 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 (38). 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. The 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.

RESULTS

Gene for the AlkS regulatory protein. Previous experiments showed that the regulatory protein AlkS is encoded on a 3.7-kb *Sall-HpaI* OCT plasmid fragment (11, 12). We determined the

rest of the sequence of this fragment on plasmid pBG11 and found that it contained two open reading frames (ORFs). ORF 2, from coordinate 628 to coordinate 3276 of the sequence deposited in the database (Fig. 2C), encodes a protein having a molecular mass of 99,833 Da, which is very similar to the estimated molecular mass of AlkS, 99 kDa (11), and contains the previously determined 3' part of the nucleotide sequence (12). Consequently, *alkS* was assigned to ORF 2. To verify the N terminus of the encoded protein, we constructed a '*lacZ*' gene fusion to *alkS* on plasmid pBG11*lacZ* and found that the first 15 amino acids of the partially purified AlkS'-*LacZ* fusion protein were in perfect agreement with the amino acids predicted from the nucleotide sequence (MKIINNDPVAKVG). The hydropathy profile of AlkS indicated that it is a soluble protein (41, 42).

Assembling the *alk* regulatory elements for expression. We developed an easily excisable cassette containing the *alkS* regulatory gene and the *alkBp* promoter (Fig. 3B). Since it has been found that the untranslated *alkB*-mRNA leader structure affects the efficiency of gene expression (25), 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* sites 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 *AscI* site. The whole segment was flanked either by *NorI* 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 (8, 9, 22, 28).

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

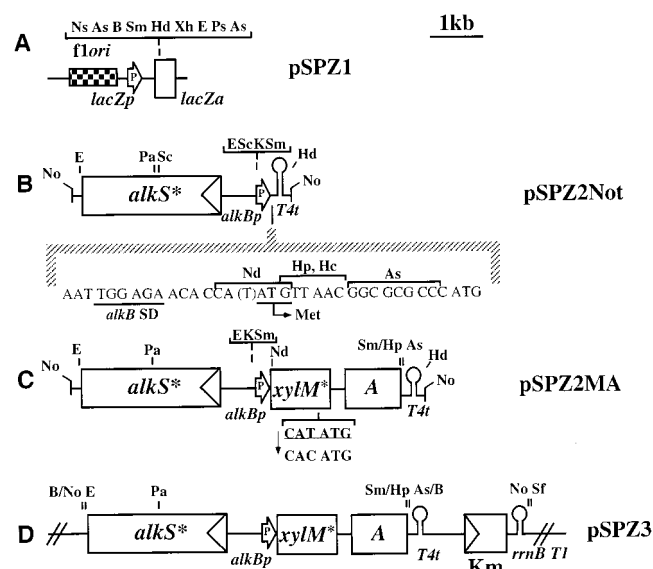


FIG. 3. Structure of the central genetic elements constructed in this study. (A) pSPZ1 helper plasmid with polylinker flanked by *AscI* sites. (B) pSPZ2Not. We assembled in the *NotI*-flanked polylinker of pUC18Not (i) the 2.7-kb OCT plasmid fragment with *alkS** as an *EcoRI* fragment, (ii) the 275-bp OCT fragment with *alkBp* from the upstream *SmaI* site to the transcription start site of *alkB*, (iii) an artificial polylinker, and (iv) the 272-bp *SphI-HindIII* pHP45 Ω 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 (*alkB* SD) sequence and the translation start site. For a more detailed restriction map of *alkS** see Fig. 2C. (C) pSPZ2MA carrying the xylene oxygenase expression cassette. The modification of *xylM* (to *xylM**) which removed the *NdeI* 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*; Hc, *HincII*; Hd, *HindIII*; Hp, *HpaI*; K, *KpnI*; No, *NotI*; Ns, *NsiI*; Sm, *SmaI*; Xh, *XhoI*; E, *EcoRI*; Ps, *PstI*; Pa, *PacI*; Sc, *SacI*; Nd, *NdeI*; Sf, *SfiI*.

linker consisting of hybridized oligonucleotides 11 and 12, which resulted in a new polylinker flanked by *AscI* sites (Fig. 3A). *AscI* 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 with 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-AscI* fragment and inserted into pSPZ2Not 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-SmaI* 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. 3C). We placed the cassette containing the desired elements on a medium-copy-number pBR322 derivative, which resulted in plasmid pSPZ3 (Fig. 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

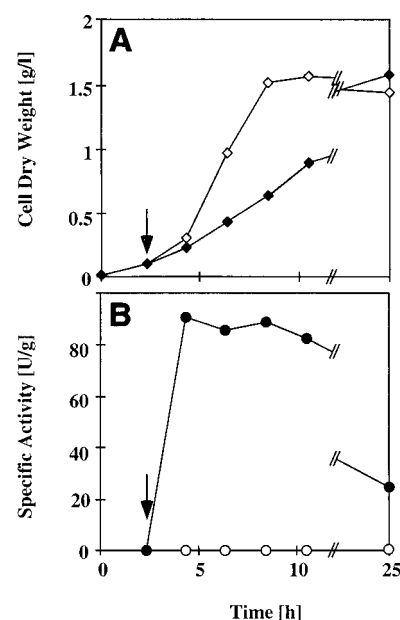


FIG. 4. Induction kinetics of styrene monooxygenase activity in *E. coli* JM101(pSPZ3) in shaking flask experiments. (A) Growth curves with and without addition of DCPK at a cell density of approximately 0.09 g per liter. (B) Styrene monooxygenase activities with and without DCPK. Symbols: ◇ and ○, no DCPK added; ◆ and ●, DCPK added at the time indicated by the arrow.

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 (27). Two additional restriction sites, a *NotI* site and an *SfiI* site, were introduced, and these sites allow further easy modification of the plasmid (for instance, introduction of plasmid maintenance functions [14] or proteins that extend the range of pMB1 plasmids to other bacterial genera [52]).

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 [dry weight] of cells⁻¹) (Fig. 4). Induction was rapid, and the growing cells exhibited the maximal specific xylene oxygenase activities 2 h after the inducer was added. Glucose did not repress translation from the *alkBp* promoter in the growth phase, which is consistent with previous observations (46, 58).

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 experiments performed with xylene oxygenase (54). *E. coli* JM101(pSPZ3) was first grown in a 3-liter reactor containing 1.025 liters of aqueous phase in batch mode in a mineral medium containing glucose as the carbon source. After the carbon source was 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 was added, a sharp increase in the DOT indicated a glucose limitation. To verify this, 10 ml of 50% glucose was added, which resulted in an

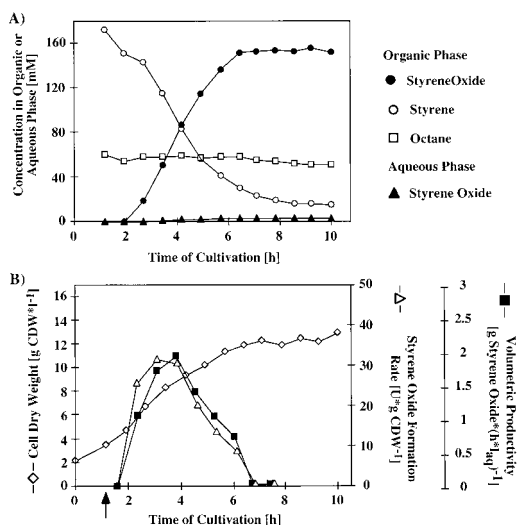


FIG. 5. Production of (*S*)-styrene oxide by *E. coli* JM101(pSPZ3). The culture was grown in a two-liquid-phase medium which contained 25% (vol/vol) hexadecane containing 1% (vol/vol) octane as an inducer and 2% (vol/vol) styrene as the substrate. The organic phase was added 1 h after initiation of feeding. (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 and development of productivities. Styrene oxide formation was calculated by determining the total rate of styrene oxide formation per gram (dry weight) of cells (CDW). Volumetric productivity was calculated by determining the mass of styrene oxide formed per hour per liter of aqueous phase (l_{aq}).

immediate decrease in the DOT; this indicated that glucose had indeed been limiting. Approximately 1.5 h after the glucose was 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 (dry weight) of cells per liter of aqueous phase at the end of the experiment.

As shown in Fig. 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. 4). Styrene oxide was formed at rates between 20 and 35 $\text{U} \cdot \text{g}$ (dry weight) of cells $^{-1}$ for 3 h, during which most of the styrene was consumed (Fig. 5B). The calculated bioconversion rate corresponded to a peak volumetric productivity of 2 g of styrene oxide per liter of aqueous phase per h, which was equivalent to a total transformation activity of at least 270 U per liter of aqueous phase. 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 the 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. Figure 6A shows the effect of adding an organic phase which contained 0.5% (vol/vol) styrene instead of the 2% (vol/vol) styrene used

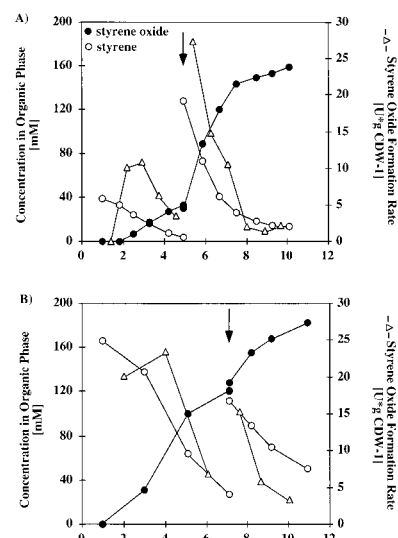


FIG. 6. Behavior of *E. coli* JM101(pSPZ3) in two-liquid-phase cultures which received styrene pulses. Styrene was added at the times indicated by the arrows. Samples were taken immediately before and after the additions. (A) Culture started with 0.5% (vol/vol) styrene in the organic phase. The cumulative 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). CDW, cell dry weight.

for the experiment shown in Fig. 5. As Fig. 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 $\text{U} \cdot \text{g}$ (dry weight) of cells $^{-1}$, 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 the first addition; it decreased from the maximal value (ca. 28 $\text{U} \cdot \text{g}$ [dry weight] of cells $^{-1}$) to ca. 0.5 $\text{U} \cdot \text{g}$ (dry weight) of cells $^{-1}$ 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 until the cumulative concentration was 3% (vol/vol) 6 h after transformation had been started by the first addition of the organic phase containing 2% (vol/vol) styrene (Fig. 6B). In this case, the rate of styrene oxide formation increased from 7 to 15.5 $\text{U} \cdot \text{g}$ (dry weight) of cells $^{-1}$ 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 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

Sequence of the *alkS* regulatory gene. Previous AlkB induction experiments localized *alkS*, which encodes the positive regulator of the *alkBFGHJKL* operon, on a 3.7-kb *SalI-HpaI* OCT plasmid fragment (11). Induction occurred irrespective of the orientation of a somewhat larger 4.9-kb *SalI* fragment

harboring the smaller fragment in pJRD158, suggesting that a transcriptionally autonomous unit was present. Furthermore, a 99-kDa translation product was attributed to the 3.7-kb fragment in minicell experiments (11). Sequencing of a region upstream of the *alkT* gene, which was also present on the 4.9-kb *SalI* fragment, had revealed the presence of the 3'-terminal part of *alkS* (12). These data were confirmed by the presence of a large ORF on the 3.7-kb *SalI-HpaI* subfragment harboring a 2,649-bp gene encoding a protein with a predicted molecular weight of 99,833. The 3' part of this ORF contains the sequence upstream of *alkT* which had already been determined. In addition, in previous experiments Owen mapped the mutations of a number of pleiotropic Tn7S insertion mutants and nitrosoguanidine mutants and found that they are located at or very close to the location of this ORF (36). Given the limited accuracy of traditional mapping procedures, we believe that the large ORF 2 represents the gene, *alkS* which encodes the positive regulator of the *alkBFGHJKL* operon. The translation start of the gene could be verified by N-terminal sequencing of a partially purified fusion protein. A recent report localized the *alkSp* promoter at a position approximately 100 nucleotides upstream of the *alkS* initiation codon, suggesting that the DNA fragment used for cloning contains all of the necessary elements for *alkS* function (5).

***alk* regulatory system on pSPZ3 as an expression system.**

Previous studies did not identify 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 (2, 43, 54), and heat shock induction might interfere with protein function and plasmid stability (20, 56). 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 (7, 48), elimination of elements of symmetry in the transcriptional terminators, deletion of known critical regions from the plasmid (27), and utilization of a kanamycin resistance gene instead of the ampicillin resistance gene (19). 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 (35). 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 (dry weight) of cells⁻¹ on a minimal medium containing glucose as the carbon source (Fig. 4), which is a substantial improvement over the values for recombinant strains reported previously (55).

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-liter two-liquid-phase cultures decreased were due at least in part to kinetic proper-

ties 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. 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. 5 and 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 1,100 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 (10). Given that the aqueous styrene oxide concentrations at the times that styrene was added in the experiments shown in Fig. 6A and B were 0.7 and 2.1 mM, the possibility that reversible product 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. 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 (dry weight) of cells⁻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 (dry weight) of cells⁻¹ when the culture contained 9 g (dry weight) of cells per liter of aqueous phase. This led to volumetric activity that was fivefold higher than the activity reported previously (54).

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 (53). The expression tools described here should help integrate new challenging enzymes into these processes, thus resolving bottlenecks due to low biocatalyst activity.

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REFERENCES

- Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**:403–410.
- Birnbaum, S., and J. E. Bailey. 1991. Plasmid presence changes the relative levels of many host cell proteins and ribosome components in recombinant *Escherichia coli*. *Biotechnol. Bioeng.* **37**:736–745.
- Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**:95–113.
- Brosius, J., T. J. Dull, D. D. Sleeter, and H. F. Noller. 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. *J. Mol. Biol.* **148**:107–127.
- Canosa, I., L. Yuste, and F. Rojo. 1999. Role of the alternative sigma factor σ^S in the expression of the AlkS regulator of the *Pseudomonas oleovorans* alkane degradation pathway. *J. Bacteriol.* **181**:1748–1754.
- Casadaban, M. J., A. Martinez-Arias, S. K. Shapira, and J. Chou. 1983. β -Galactosidase gene fusions for analyzing gene expression in *Escherichia coli* and yeast. *Methods Enzymol.* **100**:293–308.
- Chen, J., and D. A. Morrison. 1987. Cloning of *Streptococcus pneumoniae* DNA fragments in *Escherichia coli* requires vectors protected by strong transcriptional terminators. *Gene* **55**:179–187.
- de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis. 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J. Bacteriol.* **172**:6568–6572.
- de Lorenzo, V., and K. N. Timmis. 1994. Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn5- and Tn10-derived mini-transposons. *Methods Enzymol.* **235**:386–405.
- Duetz, W. A., B. Wind, J. G. van Andel, M. R. Barnes, P. A. Williams, and M. Rutgers. 1998. Biodegradation kinetics of toluene, *m*-xylene, *p*-xylene and their intermediates through the upper TOL-pathway in *Pseudomonas putida* (pWW0). *Microbiology* **144**:1669–1675.
- Eggink, G., H. Engel, W. G. Meijer, J. Otten, J. Kingma, and B. Witholt. 1988. Alkane utilization in *Pseudomonas oleovorans*. Structure and function of the regulatory locus *alkR*. *J. Biol. Chem.* **263**:13400–13405.
- Eggink, G., H. Engel, G. Vriend, P. Terpstra, and B. Witholt. 1990. Rubredoxin reductase of *Pseudomonas oleovorans*: structural relationship to other flavoprotein oxidoreductases based on one NAD and two FAD fingerprints. *J. Mol. Biol.* **212**:135–142.
- Favre-Bulle, O., and B. Witholt. 1992. Biooxidation of *n*-octane by a recombinant *Escherichia coli* in a two-liquid-phase system: effect of medium components of cell growth and alkane oxidation activity. *Enzyme Microb. Technol.* **14**:931–937.
- Gerdes, K. 1988. The *parB* (*hok/sok*) locus of plasmid R1: a general purpose plasmid stabilization system. *Bio/Technology* **6**:1402–1405.
- Germine, J., J. Gray, H. Charbonneau, T. V. Vanaman, and B. Bastia. 1983. Use of gene fusions and protein-protein interactions in the isolation of a biologically active regulatory protein. The plasmid replication initiator protein of plasmid R6K. *Proc. Natl. Acad. Sci. USA* **80**:6848–6852.
- Grund, A., J. Shapiro, M. Fennewald, P. Bacha, J. Leahy, K. Markbreiter, M. Nieder, and M. Toepfer. 1975. Regulation of alkane oxidation in *Pseudomonas putida*. *J. Bacteriol.* **123**:546–556.
- Harayama, S., R. A. Leppik, M. Rekkik, N. Mermod, P. R. Lehrbach, W. Reineke, and K. N. Timmis. 1986. Gene order of the TOL catabolic plasmid upper pathway operon and oxidation of both toluene and benzyl alcohol by the *xyIA* product. *J. Bacteriol.* **167**:455–461.
- Harayama, S., M. Rekkik, M. Wubbolts, K. Rose, R. A. Leppik, and K. N. Timmis. 1989. Characterization of five genes in the upper-pathway operon of TOL plasmid pWW0 from *Pseudomonas putida* and identification of the gene products. *J. Bacteriol.* **171**:5048–5055.
- Hasenwinkle, D., E. Jervis, O. Kops, C. Liu, G. Lasnicki, C. A. Haynes, and D. G. Kilburn. 1997. Very high-level production and export in *Escherichia coli* of a cellulose binding domain for use in a generic secretion-affinity fusion system. *Biotechnol. Bioeng.* **55**:854–863.
- Hellmuth, K., D. J. Korz, E. A. Sanders, and W.-D. Deckwer. 1994. Effect of growth rate on stability and gene expression of recombinant plasmids during continuous and high cell density cultivation of *Escherichia coli* TG1. *J. Biotechnol.* **32**:289–298.
- Henikoff, S. 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* **28**:351–359.
- Herrero, M., V. de Lorenzo, and K. N. Timmis. 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bacteriol.* **172**:6557–6567.
- Kahn, P. L. 1968. Isolation of high-frequency recombining strains from *Escherichia coli* containing the V colicinogenic factor. *J. Bacteriol.* **96**:205–214.
- Kiener, A. 1992. Enzymatic oxidation of methyl groups on aromatic heterocycles: a versatile method for the preparation of heteroaromatic carboxylic acids. *Angew. Chem. Int. Ed. Engl.* **31**:774–775.
- Kok, M. 1988. Ph.D. thesis. Rijksuniversiteit, Groningen, The Netherlands.
- Kok, M., R. Oldenhuis, M. P. G. van der Linden, P. Raatjes, J. Kingma, P. H. van Lelyveld, and B. Witholt. 1998. The *Pseudomonas oleovorans* alkane hydroxylase gene: sequence and expression. *J. Biol. Chem.* **264**:5435–5441.
- Kolot, M. N., M. V. Kashlev, A. I. Gragerov, and I. A. Khmel. 1989. Stability of the pBR322 plasmid as affected by the promoter region of the tetracycline-resistance gene. *Gene* **75**:335–339.
- Kristensen, C. S., L. Eberl, J. M. Sánchez-Romero, M. Givskov, S. Molin, and V. de Lorenzo. 1995. Site-specific deletions of chromosomally located DNA segments with the multimer resolution system of broad-host-range plasmid RP4. *J. Bacteriol.* **177**:52–58.
- Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**:367–382.
- Marqués, S., and J. L. Ramos. 1993. Transcriptional control of the *Pseudomonas putida* TOL plasmid catabolic pathways. *Mol. Microbiol.* **9**:923–929.
- Matsudaira, P. 1987. Sequence from picomole quantities of proteins electrophoretically onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262**:10035–10038.
- Mc Fall, E., and E. B. Newman. 1996. Amino acids as carbon sources, p. 358–379. In F. C. Neidhardt (ed.), *Escherichia coli and Salmonella*, vol. 1. ASM Press, Washington, D.C.
- Mermod, N., S. Harayama, and K. N. Timmis. 1986. New route to bacterial production of indigo. *Bio/Technology* **4**:321–324.
- Meyers, R. M., L. S. Lerman, and T. Maniatis. 1985. A general method for saturation mutagenesis of cloned DNA fragments. *Science* **229**:242–247.
- Nieboer, M., J. Kingma, and B. Witholt. 1993. The alkane oxidation system of *Pseudomonas oleovorans*: induction of the *alk* genes in *Escherichia coli* W3110(pGEc47) affects membrane biogenesis and results in overexpression of alkane hydroxylase in a distinct cytoplasmic membrane subfraction. *Mol. Microbiol.* **8**:1039–1051.
- Owen, D. J. 1986. Molecular cloning and characterization of sequences from the regulatory cluster of the *Pseudomonas* plasmid *alk* system. *Mol. Genet.* **203**:64–72.
- Panke, S., J. M. Sánchez-Romero, and V. de Lorenzo. 1998. Engineering quasi-natural *Pseudomonas putida* strains for metabolism of toluene through an *ortho*-cleavage degradation pathway. *Appl. Environ. Microbiol.* **64**:748–751.
- Panke, S., B. Witholt, A. Schmid, and M. G. Wubbolts. 1998. Towards a biocatalyst for (*S*)-styrene oxide production: characterization of the styrene degradation pathway of *Pseudomonas* sp. strain VLB120. *Appl. Environ. Microbiol.* **64**:2032–2043.
- Prentki, P., and H. M. Krisch. 1984. In vitro insertional mutagenesis with a selectable DNA fragment. *Gene* **29**:303–313.
- Ramos, J. L., S. Marqués, and K. N. Timmis. 1997. Transcriptional control of the *Pseudomonas* TOL plasmid catabolic operons is achieved through an interplay of host factors and plasmid-encoded regulators. *Annu. Rev. Microbiol.* **51**:341–373.
- Rao, M. J. K., and P. Argos. 1986. A conformational preference parameter to predict helices in integral membrane proteins. *Biochim. Biophys. Acta* **869**:197–214.
- Rost, B., P. Fariselli, and R. Casadio. 1996. Topology prediction for helical transmembrane proteins at 86% accuracy. *Protein Sci.* **5**:1704–1718.
- Ryan, W., and S. J. Parulekar. 1991. Recombinant protein synthesis and plasmid instability in continuous cultures of *Escherichia coli* JM103 harboring a high copy number plasmid. *Biotechnol. Bioeng.* **37**:415–429.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Shaw, J. P., and S. Harayama. 1992. Purification and characterization of the NADH:acceptor reductase component of xylene monooxygenase encoded by the TOL plasmid pWW0 of *Pseudomonas putida* mt-2. *Eur. J. Biochem.* **209**:51–61.
- Staijen, I. E., R. Marconelli, and B. Witholt. 1999. The $P_{alkBFCUHL}$ promoter is under carbon catabolite repression control in *Pseudomonas oleovorans* but not in *Escherichia coli alk⁺* recombinants. *J. Bacteriol.* **181**:1610–1617.
- Staijen, I. E., and B. Witholt. 1998. Synthesis of alkane hydroxylase of *Pseudomonas oleovorans* increases the iron requirement of *alk⁺* bacterial strains. *Biotechnol. Bioeng.* **57**:228–237.
- Stassi, D. L., and S. A. Lacks. 1982. Effect of strong promoters on the cloning in *Escherichia coli* of DNA fragments from *Streptococcus pneumoniae*. *Gene* **18**:319–328.
- Sticher, P., M. C. M. Jaspers, K. Stemmler, H. Harms, A. J. B. Zehnder, and J. R. van der Meer. 1997. Development and characterization of a whole-cell bioluminescent sensor for bioavailable middle-chain alkanes in contaminated groundwater samples. *Appl. Environ. Microbiol.* **63**:4053–4060.
- Suzuki, M., T. Hayakawa, J. P. Shaw, M. Rekkik, and S. Harayama. 1991. Primary structure of xylene monooxygenase: similarities to and differences from the alkane hydroxylation system. *J. Bacteriol.* **173**:1690–1695.
- van Beilen, J. B., M. G. Wubbolts, and B. Witholt. 1994. Genetics of alkane oxidation by *Pseudomonas oleovorans*. *Biodegradation* **5**:161–174.

52. West, S. E. H., H.-P. Schweizer, C. Dall, A. K. Sample, and L. J. Runyen-Janecky. 1994. Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* **128**:81–86.
53. Witholt, B., M.-J. de Smet, J. Kingma, J. B. van Beilen, R. G. Lageveen, and G. Eggink. 1990. Bioconversions of aliphatic compounds by *Pseudomonas oleovorans* in multiphase bioreactors: background and economic potential. *TIBTECH* **8**:46–52.
54. Wubbolts, M. G., O. Favre-Bulle, and B. Witholt. 1996. Biosynthesis of synthons in two-liquid-phase media. *Biotechnol. Bioeng.* **52**:301–308.
55. Wubbolts, M. G., J. Hoven, B. Melgert, and B. Witholt. 1994. Efficient production of optically active styrene epoxides in two-liquid phase cultures. *Enzyme Microb. Technol.* **16**:887–893.
56. Wubbolts, M. G., P. Reuvekamp, and B. Witholt. 1994. TOL plasmid-specified xylene oxygenase is a wide substrate range monooxygenase capable of olefin epoxidation. *Enzyme Microb. Technol.* **16**:608–615.
57. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**:103–109.
58. Yuste, L., I. Canosa, and F. Rojo. 1998. Carbon-source-dependent expression of the *PalkB* promoter from the *Pseudomonas oleovorans* alkane degradation pathway. *J. Bacteriol.* **180**:5218–5226.