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Development of Recombinant *Pseudomonas putida* Containing Homologous Styrene Monooxygenase Genes for the Production of (*S*)-Styrene Oxide

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Abstract Recently isolated, *Pseudomonas putida* SN1 grows on styrene as its sole carbon and energy source through successive oxidation of styrene by styrene monooxygenase (SMO), styrene oxide isomerase (SOI), and phenylacetaldehyde dehydrogenase. For the production of (*S*)-styrene oxide, two knockout mutants of SN1 were constructed, one lacking SOI and another lacking both SMO and SOI. These mutants were developed into whole-cell biocatalysts by transformation with a multicopy plasmid vector containing SMO genes (*styAB*) of the SN1. Neither of these self-cloned recombinants could grow on styrene, but both converted styrene into an enantiopure (*S*)-styrene oxide (e.e. > 99%). Whole-cell SMO activity was higher in the recombinant constructed from the SOI-deleted mutant (130 U/g cdw) than in the other one (35 U/g cdw). However, the SMO activity of the former was about the same as that of the SOI-deleted SN1 possessing a single copy of the *styAB* gene that was used as host. This indicates that the copy number of *styAB* genes is not rate-limiting on SMO catalysis by whole-cell SN1.

Keywords: styrene monooxygenase, (*S*)-styrene oxide, whole-cell biocatalyst, *Pseudomonas putida* SN1, *styABC*-deleted mutant, self-cloning

INTRODUCTION

Bioconversion of styrene using styrene monooxygenase (SMO) is recognized as a promising process for the production of enantiopure aryl epoxides such as (*S*)-styrene oxide, a versatile chiral building block for many pharmaceuticals [1-3]. SMO activity exists in several styrene-degrading *Pseudomonas* strains, the *styABCD* cluster of which is responsible for the catabolism of styrene [4,5]. Among the styrene-catabolic enzymes, SMO is encoded by *styAB*, styrene oxide isomerase (SOI) is encoded by *styC*, and phenylacetaldehyde dehydrogenase is encoded by *styD* (Fig. 1).

The reaction catalyzed by SMO requires the continuous regeneration of NADH as a cofactor [6,7]. Although an *in vitro* system is possible if NADH is regenerated *in situ*, most practical processes with SMO are based on the use of (recombinant) whole cells expressing SMO. In the work of Panke *et al.*, a recombinant *Escherichia coli* strain containing the *styAB* genes of *Pseudomonas* sp. VLB21 has been developed and applied to the pilot-scale production of (*S*)-styrene oxide [8]. They employed a multicopy plasmid and the strong *alk* promoter for the transcription of *styAB*, and could obtain a maximum SMO activity of 70 U/g cdw. We have developed two strains for the SMO reaction, a recombinant *E. coli* containing *styAB* and a *styC*-negative mutant of the styrene-degrading *Pseudomonas putida* SN1. In the former, *styAB* genes were placed in a multicopy plasmid under the control of strong T7 promoter [9]. SMO activity was low in this recombinant strain under most culture conditions due to excessive expression of SMO, resulting in inactive SMO protein aggregates. The *styC*-deleted mutant of *P. putida* SN1 exhibited a high activity of 130 U/g cdw. However, *styAB* genes were present in the chromosome as a single copy downstream of its original promoter, which was inducible by styrene. Subsequent experiments employing a water-bis (2-ethylhexyl)phthalate two-phase system yielded a high level accumulation of (*S*)-styrene oxide (150 mM in the organic phase at 13 h) with an enantiomeric excess greater than 98%.

E. coli is the most popular host organism for the production of recombinant proteins. However, it is not always the most suitable strain for developing recombinant whole-cell biocatalysts. This is especially true when a substrate and/or product is toxic to the cell or insuffi-

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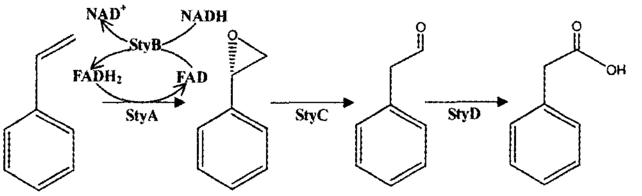

Fig. 1. Pathway for the catabolism of styrene in styrene-degrading *Pseudomonas* species (StyA, styrene monooxygenase; StyB, reductase; StyC, styrene oxide isomerase; and StyD, phenylacetaldehyde dehydrogenase).

ciently soluble in aqueous phase [10-12], requiring the biocatalytic reaction to be performed in the presence of an organic solvent [13,14]. The SMO reaction typically proceeds in a two-phase water-organic solvent system, since styrene and styrene oxide are toxic to most bacterial cells and both are more soluble in organic solvents [15,16]. *Pseudomonas* is more resistant to organic solvents due to the presence of a strong efflux pump that reduces the cellular concentration of organic compounds [17]. It has been suggested to be a better host strain than *E. coli* for whole-cell SMO biocatalysis.

In our previous study, we have shown that the SMO activity of *styC*-deleted SN1 is high and stable during bioreactor experiments. However, the specific activity of the *styC*-deleted SN1 cannot be higher than in the wild-type strain. Since the biocatalyst concentration in the bioreactor can be reduced, the availability of highly-active biocatalysts is very important for the development of the SMO biocatalysis process. In a continuing effort to develop a *Pseudomonas*-based, active whole-cell SMO biocatalyst, the present study aims at developing recombinant strains of *P. putida* SN1 containing the homologous *styAB* in a multicopy plasmid and examining the gene dosage effect of *styAB* in the *Pseudomonas* recombinants. Two different host strains, one lacking SOI and another lacking both SMO and SOI, were developed by transformation with a multicopy plasmid vector containing *styAB* of the SN1 gene and tested for SMO reaction activity.

MATERIALS AND METHODS

Materials

The enzymes for DNA manipulations were acquired from Boehringer Mannheim GmbH (Mannheim, Germany), and the pGEM-T easy vector system was procured from Promega (WI, USA). A Miniprep kit and DNA gel purification kit were purchased from Qiagen (Mannheim, Germany), and DIG labeling and detection kits were acquired from Roche (Mannheim, Germany). Plasmid pUCP19, shuttle vector for *E. coli*, and *Pseudomonas* sp., was obtained from H. P. Schwiezer [15]. Plasmid LITMUS28 was secured from NEB (Beverly, MA, USA), and primers were synthesized at Bioneer (Daejeon, Korea). All other chemicals used in this study were purchased from Sigma (St. Louis, MO, USA).

Microorganisms and Culture Conditions

P. putida SN1 was isolated from a styrene biofilter used in a gas treatment plant [19]. The knockout mutant *styC*$^-$ was developed previously [20], while *styABC*$^-$ was developed for the present study. *P. putida* SN1, its knockout mutants *styC*$^-$ and *styABC*$^-$, and the recombinant *P. putida* SN1 were cultured in M9 mineral salts medium supplemented with 0.5 g/L glucose and 1 g/L yeast extract (M9+ medium, hereafter) at 30°C. When necessary, 50 mg/L kanamycin, 50 mg/L tetracycline, or 100 mg/L ampicillin was added to the medium. Before harvesting the cells, SMO activity was induced by adding styrene (1 mM) for 4 h. Citrate at 0.05% (w/v) was also added as a carbon substrate during the induction period. Recombinant *E. coli* BL21 (DE3) containing the multicopy plasmid pETAB2 for *styAB* [9] was grown in the M9 medium supplemented with glucose (0.5 g/L), yeast extract (1 g/L), thiamine (0.1 g/L), US* trace (1%, v/v), and ampicillin (100 mg/L) at 37°C. To induce expression of the *sty*AB genes, 1 mM IPTG was added 4 h before harvest. Microorganisms and plasmids used in this study are presented in Table 1.

Construction of *styABC*-deleted Mutant of *P. putida* SN1

DNA manipulations were performed according to the procedures described by Sambrook *et al.* [21]. The pGEM-T easy vector system was used for PCR product cloning, and the DIG labeling and detection kits were used for Southern blot hybridization. DNA fragments were amplified by PCR (GeneAmp PCR System 2400, PerkinElmer Inc., USA) and purified using a Qiagen kit.

The disruption of *styABC* in *P. putida* SN1 was conducted by homologous recombination. The *styABC* disruption vector, pLSM2 (km^R, amp^R), was constructed by integrating two flanking regions of *styABC* into plasmid LITMUS28/Km [20]. The two flanking regions consisted of *styRstyS* 3′ (1 kb) containing truncated *styS* and complete *styR*, and *styD* 5′ (1 kb) containing truncated *styD*. These were amplified using the genomic DNA of *P. putida* SN1 as a template after a partial digestion with *Kpn*I. The primers and restriction enzymes used for vector construction are given in Table 2. The disruption of the *styABC* genes in *P. putida* SN1 was achieved by double crossover according to the procedures reported by Padda *et al.* [22] (Fig. 2). In short, pLSM2 (km^R, amp^R) was transformed into *P. putida* SN1 by electroporation, km^R mutants were obtained, and *styABC*-negative mutants were selected by Southern blot hybridization from among the km^R mutants. Before electroporation, SN1 cells harvested at mid-log growth phase were washed three times with ice-cold 10% glycerol to induce competency. Two gene probes that were developed previously were employed for Southern blot hybridization: styBA3′ (1 kb) from the *styAB* DNA sequences, and styC (0.3 kb) from the *styC* DNA sequences [20].

Table 1. Bacterial strains and plasmids

Strain and plasmid	Characteristics	Source or reference
Strains		
P. putida SN1	Wild-type *Pseudomonas*; styrene prototroph	Park *et al.* (2005)
P. putida SN1 *styC*$^-$ mutant	Mutant *Pseudomonas*; *styC* gene (–)	Han *et al.* (2006)
P. putida SN1 *styABC*$^-$ mutant	Mutant *Pseudomonas*; *styABC* gene (–)	This study
E. coli DH10B	F- *mcrA* △*(mrr hsdRMS-mcrBC)* △*80dlacZ* △*M15 DlacX74 deoR recA1 ara* △*139* △*(ara leu)7697*	Gibco BRL
Plasmids		
pGEM-T easy	*lacZ*α; cloning vector; pGEM 5zf(+) derivative; 3'T-overhang; Ap^r	Promega
LITMUS28/Km	*lacZ*α; cloning vector; LITMUS28 derivative; Ap^r, Km^r	Han *et al.* (2006)
pLSM2	*lacZ*α; 1 kb *styRstyS* 3' and 1 kb *styD* 5'; LITMUS28/Km derivative; Ap^r, Km^r	This study
pUCP19	*lacZa*, *Pseudomonas-E. coli* shuttle vector; pUC19 derivative; Ap^r; ColE1 *ori*	H. P. Schweizer (1991)
pUCP19/Tet	*Pseudomonas-E. coli* shuttle vector; pUCP19 derivative; Tet^r, Ap^r	This study
pPSAB5	*styAp*; 2 kb *styAB*; pUCP19 derivative; Tet^r, Ap^r	This study

Table 2. Primers for amplification of styrene catabolic gene. Underlined sequences indicate restriction sites given in the last column

Target genes	Primers	Sequence (5′-3′)	Restriction enzymes
stySR	H1	AAATCTAGACGGATCTTCGAACCGTTT	*Xba*I
	H2	AAAAAGCTTGCCTGCACCAACAATACC	*Hind*III
styD	H3	AAACTCGAGAGGAGCCTAACCATGAACAGTTCT	*Xho*I
	H4	GGAAGATCTTCCACCGGAACCAACCACGATGCTTTC	*Bgl*II
tet	Tet1	AAATCTAGAAGGCCCTTTCGTCTT	*Xba*I
	Tet2	AAAGGTACCAGTTCTCCGCAAGAA	*Kpn*I
styAB	ApstyAB1	AAATCTAGAAGGATATTTTTATACCGG	*Xba*I
	ApstyAB2	AAAAAGCTTCTTCCGGTGATCGGCACA	*Hind*III

Construction of Multicopy Plasmid and Transformation into *styC*$^-$ and *styABC*$^-$ Strains of *P. putida* SN1

The DNA fragment containing *styAB* genes (1.8 kb) and their promoter region (170 bp upstream to the translation start codon) were amplified by PCR using primers ApstyAB1 and ApstyAB2 (Table 2). After cloning in pGEM-T easy vector, the DNA fragment was excised by *Xba*I-*Hind*III digestion and inserted into pUCP19/Tet vector to obtain the SMO expression vector, pPSAB5 (Fig. 3). Vector pUCP19/Tet was constructed from an *E. coli-Pseudomonas* shuttle vector (pUCP19) and the *Eco*RI fragment of pBR322 containing a tetracycline-resistant (*tet*^{R}) gene. Since the cloning host, *styC*-deleted or *styABC*-deleted *P. putida* SN1, was Km^R and Amp^R, the *tet*^{R} gene was introduced as a differential selection marker for the recombinant plasmid. The resulting SMO expression vector, pPSAB5, was then transformed into competent *styC*-deleted and *styABC*-deleted *P. putida* SN1 cells by electroporation. The SN1 transformants harboring the plasmid (self-cloned *styC*$^-$ SN1 and *styABC*$^-$ SN1, hereafter) were selected on a LB plate containing tetracycline (100 mg/L) and kanamycin (50 mg/L). Colony PCR was performed with the primers ApstyAB1 and ApstyAB2 to confirm the presence of pPSAB5 in the transformants.

Measurement of SMO Activity

Cells were harvested by centrifugation, washed twice with a potassium phosphate buffer (50 mM, pH 7.0), and resuspended in the same buffer. The cell suspension (15 mL) was placed in a 165-mL serum bottle and citrate or glucose was added as a carbon source for the regeneration of intracellular NADH at a concentration of 3 g/L. Liquid styrene (2 μL) was added to the liquid phase of the bottle sealed with a butyl rubber and an aluminum cap. The reaction was allowed to proceed in a reciprocally-shaking water bath at 30°C and 180 strokes/min. During the reaction, gas samples were measured periodically for styrene and liquid samples were measured for styrene oxide. One unit (U) of SMO activity was defined as the activity that degraded 1 μmol of styrene or pro-

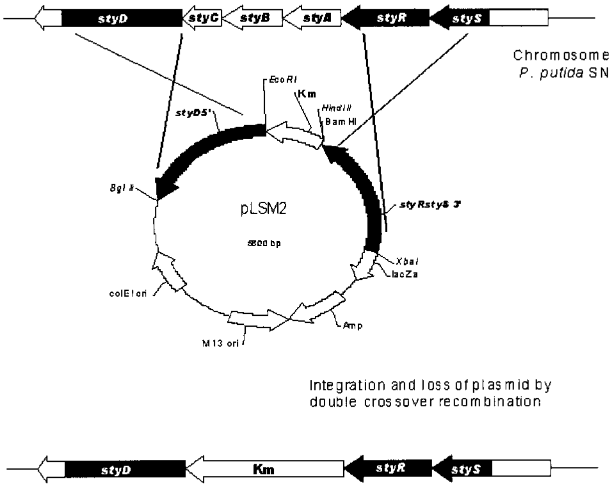

Fig. 2. Construction of pLSM2 for disruption of styABC in *P. putida* SN1. The homologous regions in the circular DNAs and chromosome of *P. putida* are shaded.

duced 1 μmol of styrene oxide in 1 min.

SDS-PAGE Analysis

Cells were disintegrated by two passages through a French pressure cell (Thermo Electron Corporation, USA) at 18,000 psi. The disintegrated cell broth was centrifuged at 5,000 g and 4°C for 10 min to remove cell debris. The resulting supernatant was applied onto 18% polyacrylamide gel for electrophoresis. The protein bands obtained were stained with Coomassie Brilliant Blue G-250 (Sigma). The intensity of the protein bands in the gel was measured by a BIO-RAD Gel Doc 2000 (Bio-Rad).

Production of Styrene Oxide in Shaking Flask

Shake-flask experiments with self-cloned SN1 were conducted using 250 mL capped flasks according to two stages: batch cell growth (12 h), and styrene oxide production in a two-phase water-organic system (18 h). In the first stage, the self-cloned recombinants were grown at 30°C for 12 h in 15 mL of M9 minimal medium supplemented with 2 g/L glucose and 4 g/L yeast extract. Tetracycline was added at 50 mg/L, or not added at all. Without tetracycline, the recombinant plasmid was not maintained stably. On the other hand, its presence significantly reduced cell growth and SMO activity. Inoculum was grown in M9+ medium containing 50 mg/L tetracycline at 30°C for 12 h. At 12 h, the second stage was commenced by adding 15 mL of bis(2-ethylhexyl)phthalate (BEHP) containing 2% (v/v) styrene, corresponding to 174 mM in the organic phase. A carbon source (citrate or glucose) was also added to bring the mixture to a concentration of 0.2% (w/v of aqueous phase). The reaction temperature was 30°C and the flask shaking speed was 250 rpm. The styrene oxide concentration in the organic phase was analyzed over time. For comparison, experiments with the *styC*$^-$ SN1 were also conducted in the same way except for the addition of tetracycline to the culture medium.

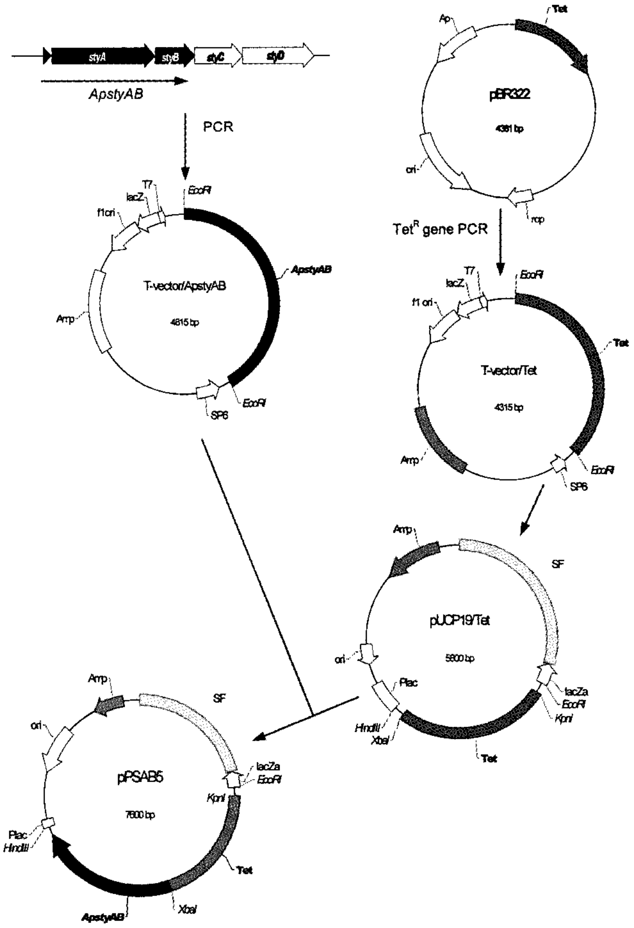

Fig. 3. Construction of the expression vector (pPSAB5) for the production of (*S*)-styrene oxide in *P. putida* SN1.

Analysis

Styrene and styrene oxide were analyzed by a HP6890 gas chomatograph (Hewlett Packard Inc., USA) equipped with a flame ionization detector. Styrene in the gas phase was determined using an HP-530 capillary column coated with cross-linked 5% PH ME siloxane with the following dimensions: 15 meters in length, 0.53 mm id, and 1.5 μm film thickness (Hewlett Packard Inc.). Nitrogen gas was used as the carrier and applied at a flow rate of 1 mL/min. The oven temperature was maintained at 80°C, the injector was kept at 150°C, and the detector temperature was set to 300°C. Liquid-phase samples were analyzed for styrene oxide. During the two-liquid-phase experiments, liquid samples (1 mL) were withdrawn and centrifuged to separate the phases. An aliquot of the organic phase was diluted with the same volume of cyclohexane and analyzed by gas chromatography. The column used was a Supelco β-DEX 120 fused silica cyclodextrine capillary column 60 meters long with 0.25 mm id and 0.25 μm film thickness. The split ratio of the injection was 100:1. Helium was used as carrier gas at a flow rate of 0.5 mL/min. The temperature was maintained at 110°C in the oven, 250°C in the injector, and 250°C in the detector. Cell density was measured at 660 nm by a Lambda 20 spectrophotometer (Perkin-Elmer, USA). One OD unit corresponded to 0.30 g cdw/L.

RESULTS AND DISCUSSION

Construction of Recombinant *styC*-Deleted and *styABC*-Deleted *P. putida* SN1 Expressing Self-Cloned Styrene Monooxygenase

In order to examine the gene dosage effect of *styAB* on whole-cell SMO activity, two self-cloned *P. putida* SN1 were developed. Both *styC*$^-$ SN1 and *styABC*$^-$ SN1 were used as host strains for expressing the recombinant SMO. The difference between the two recombinant strains is the presence of an additional copy of the *styAB* gene in the chromosome of the first recombinant.

The *styC*$^-$ mutant has been developed previously [20]. For developing a *styABC*$^-$ mutant, disruption vector (pL SM2; *km*^{R}, *amp*^{R}) was inserted into *P. putida* SN1 by electroporation. Among over 1,000 colonies formed on Km plates, the desired *styABC*$^-$ mutants were selected based on Southern blot hybridization. While the colonies of the single-crossover mutants were hybridized with both the *styC* and *styBA3'* probes, those of the double-crossover, *styABC*$^-$ mutants were not hybridized with either of those probes (data not shown). The mutant strain was also tested for cell growth capability in M9 minimal salts medium. The wild-type SN1 or the single-crossover mutants grew on glucose or styrene as a sole carbon source, but the *styABC*$^-$ mutant grew only on glucose (data not shown).

The SMO expression plasmid pPSAB5 was constructed by introducing *styAB* genes into pUCP19/Tet and transformed into the *styC*-negative and *styABC*-negative *P. putida* SN1 by electroporation. Many transformants were obtained on LB plates containing tetracycline (50 mg/L). Five colonies with either the *styC*-negative or *styABC*-negative *P. putida* SN1 were arbitrarily selected, and colony PCR was performed with primers ApstyAB1 and ApstyAB2 (see Table 1). All the colonies tested had the SMO expression vector pPSAB5 (data not shown).

Enzymatic activity of self-cloned recombinants was examined with styrene as a reactant. The cells were grown to a stationary phase on the M9+ medium and gene expression was induced with 1 mM styrene for 4 h. The cells were then washed and resuspended in 50 mM phosphate buffer. When the resting cells were incubated with styrene, (*S*)-styrene oxide accumulated in the reaction mixture (data not shown). No (*R*)-styrene oxide was detected. Neither (*S*)-styrene oxide nor (*R*)-styrene oxide was detected from the wild-type SN1 containing *styABC* or the *styABC*$^-$ host strain without the plasmid pPSAB5. Therefore, it could be concluded that the *styAB* of *P. putida* SN1 was successfully cloned in the *styC*-negative and *styABC*-negative mutants of *P. putida* SN1.

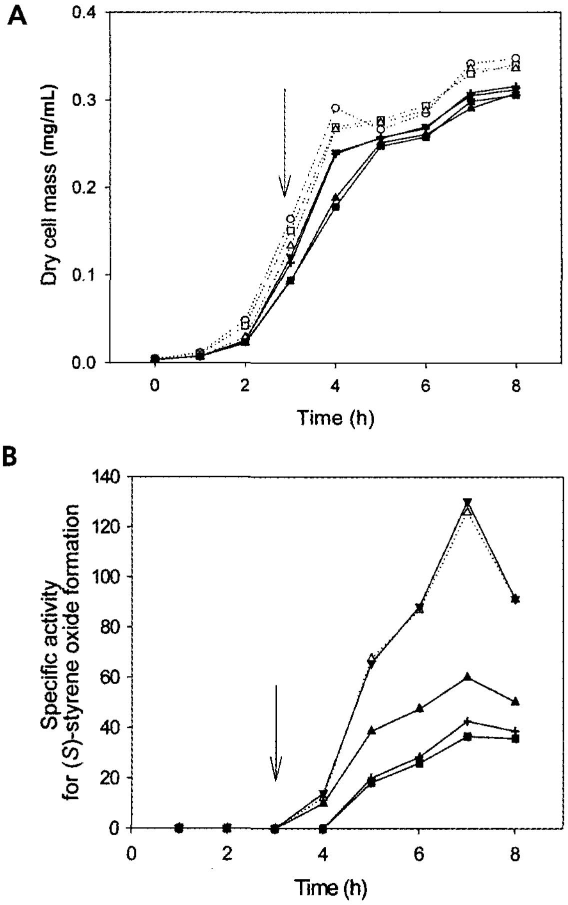

Fig. 4. Cell growth (A) and whole-cell SMO activity (B). Arrow indicates the addition of styrene (1 mM) and citrate (0.5 g/L) at 3 h. For clarity, SN1 and hosts (*styC*$^-$ and *styABC*$^-$) are represented as broken lines. Symbols: (○), SN1; (△), *styC*$^-$; (□), *styABC*$^-$; (▲), *styC* (pPSAB5)/tetracycline added; (▼), *styC*$^-$ (pPSAB5)/tetracycline free; (■), *styABC*$^-$ (pPSAB5)/tetracycline added; (+), *styABC*$^-$(pPSAB5)/tetracycline free.

Characterization of Recombinant *styC*- and *styABC*-Deleted *P. putida* SN1 Expressing Self-Cloned Styrene Monooxygenase

Characteristics of the recombinant SN1 was investigated according to cell growth, SDS-PAGE analysis of protein production, and whole-cell enzymatic activity of SMO. The cells were grown in M9+ medium and induced for SMO activity by styrene and citrate at 3 h (Fig. 4). There were some differences in growth rate and final density (Fig. 4A). The specific growth rates were, in descending order: wild-type, *styC*$^-$ and *styABC*, *styC*$^-$ (pPSAB5), and *styABC*$^-$ (pPSAB5) without tetracycline, and *styC*$^-$(pPSAB5) and *styABC*$^-$(pPSAB5) with tetracycline. The cells could be divided into two groups according to their final cell densities at 8 h: a high-density group and a low-density group. The high-density group included wild-type cells, *styC*$^-$, and *styABC*$^-$. The low-density group consisted of the recombinants containing pPSAB5.

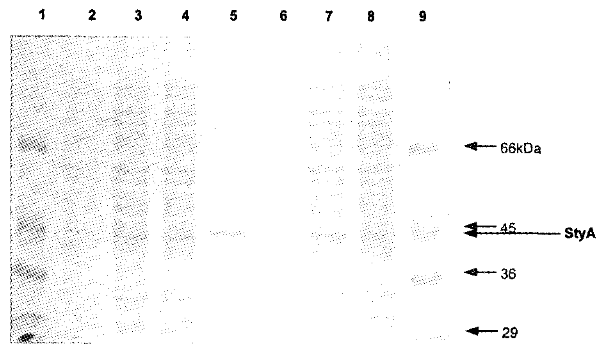

Fig. 5. SDS-PAGE analysis for the soluble fraction of recombinants. SMO expression was induced at 30°C in *P. putida* and at 15°C in *E. coli* (lane 5). Lanes 1 and 9, marker protein; lane 2, *styABC*$^-$; lane 3, *styABC*$^-$ (pPSAB5) tetracycline added; lane 4, *styABC*$^-$ (pPSAB5) tetracycline free; lane 5, recombinant *E. coli* (8 h; 15°C); lane 6, *styC*$^-$; lane 7, *styC*$^-$ (pPSAB5) tetracycline added; lane 8, *styC*$^-$ (pPSAB5) tetracycline free.

The lower growth rates of recombinants *styC*$^-$(pPSAB5) and *styABC*$^-$(pPSAB5) than those of *styC*$^-$ and *styABC*$^-$ indicate that the presence of the multicopy vector pPSAB5 puts a metabolic burden on its host strains. Tetracycline was necessary for the stable maintenance of pPSAB5 in the SN1 recombinant strains. When tetracycline was deleted from the M9+ medium, about 10% of the cells for both *styC*$^-$ (pPSAB5) and *styABC*$^-$ (pPSAB5) were observed to be plasmid-free after 8 h of cultivation (data not shown). However, the presence of this antibiotic in the culture medium significantly reduced the growth rate of the *tet*^{R} recombinants by more than 20%. In general, the *tet*^{R} gene from pBR322 is known to confer tetracycline resistance to its host by producing a membranous protein which works as a proton-dependent efflux pump. The protein helps maintain a low intracellular tetracycline concentration through a reduced uptake rate and/or an enhanced efflux rate of the drug [23], but its action to maintain low tetracycline concentration inside the cell has been suggested to be responsible for decreased cell growth rates.

Fig. 4B shows the profile of whole-cell SMO activity of the self-cloned SN1. The activity of the *styC*$^-$ mutant is also given for comparison. When induced with styrene at 3 h, the SMO activity gradually increased and reached its maximum values about 4 h after the addition of styrene. The maximum activity was, in descending order: *styC*$^-$ (pPSAB5) without tetracycline and *styC*$^-$, *styC*$^-$ (pPSAB5) with tetracycline, *styABC*$^-$ (pPSAB5) without tetracycline, and *styABC*$^-$ (pPSAB5) without tetracycline. It was observed that whole-cell SMO activity was not improved by self-cloning of *styAB* genes in *Pseudomonas*. However, we noticed several important facts related with the development of a whole-cell SMO biocatalyst. First, the SMO activity was not increased by simple augmentation of the *styAB* gene on the multicopy plasmid. Second, the recombinants using *styC*$^-$ as host exhibited a higher activity than those using *styABC*$^-$ as a host. Third, the presence of tetracycline always reduced the whole cell SMO activity by more than 50%.

Fig. 5 shows the expression of SMO protein as investigated by SDS-PAGE analysis of whole-cell extracts. Both recombinant and host cells of the *styC*$^-$ SN1 and *styABC*$^-$ SN1 were examined before and after induction. For comparison, data from the wild-type SN1 and a recombinant *E. coli* containing *styAB* were also presented. Only the recombinant *E. coli* cell extracts exhibited a strong protein band at the position of StyA (47 kDa). The *styB* gene product (M.W., 16 kDa) was completely absent for all cases, probably because of a low level of expression of StyB or the presence of too many proteins similar in size to StyB in the cell extract [9,24]. It is not possible to estimate from SDS-PAGE results exactly how much the expression level of StyAB proteins has been increased by self-cloning. However, it is evident that the SMO expression level in the self-cloned SN1 is not too high. Therefore, the possibility is eliminated that the low SMO activity was a result of the excessive SMO expression seen in the previous *E. coli* recombinant [9].

Production of (*S*)-Styrene Oxide Using the Recombinant *P. putida* SN1 with Self-Cloned Styrene Monooxygenase in Two-Phase Reaction

Since (*S*)-styrene oxide is toxic to microbial cells and inhibits SMO activity, it is important to keep its concentration low in the reaction mixture. We proved that a two-phase water-organic solvent system was efficient for this purpose [20]. The styrene oxide was produced in the aqueous phase and was continuously extracted to the organic phase during the reaction. After cultivating the cells in M9 salts medium containing glucose and yeast extract for 12 h, BEHP containing 2% (v/v) styrene was added to make an aqueous/organic two phase system with a volume ratio of 5:3. A 0.2% carbon source (w/v of aqueous phase) was added at the beginning of the reaction and a carbon source plus yeast extract (0.3% each w/v of aqueous phase) was added after 8 h of the two-phase reaction (see Materials and Methods). As shown in Fig. 6, there was some lag time initially between the introduction of styrene and the induction of SMO activity in all five cell types. Increases in styrene oxide production were almost linear for up to 18 h, indicating that the volumetric SMO activity remained constant during this period. Among the five strains tested, *styC*$^-$ SN1 and *styC*$^-$ (pPSAB5) without tetracycline exhibited the best yield of styrene oxide, producing concentrations as high as 84 mM after 18 h of incubation. When tetracycline was included in the culture broth during cell growth, the production of (*S*)-styrene oxide was only 40 mM at 18 h. These results, along with the results shown in Fig. 4, indicate that tetracycline resistance should be avoided as a selection marker in developing whole-cell biocatalysts. From *styABC*$^-$ (pPSAB5), the accumulation of (*S*)-styrene oxide was as low as 15 mM (without tetracycline) or 14 mM (with tetracycline). The e.e. values of (*S*)-styrene oxide for all recombinants were above 99% when determined for the samples taken at 18 h. The yield of styrene oxide was above 0.97 mol styrene oxide/mol sty-

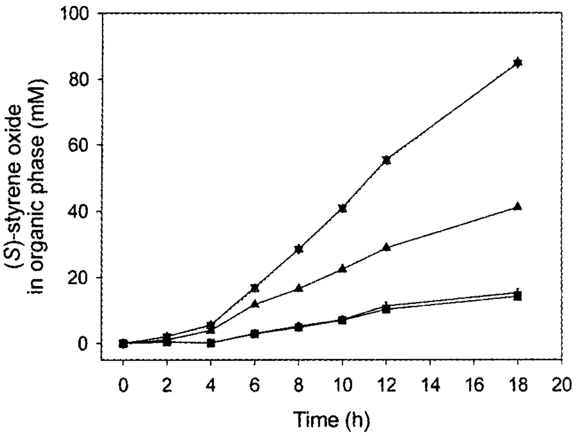

Fig. 6. Time course profile of (*S*)-styrene oxide production in two-liquid-phase shake flask experiments. For clarity, *styC*⁻ is represented as a broken line. Symbols: (△), *styC*⁻; (▲), *styC*⁻ (pPSAB5)/tetracycline added; (▼), *styC*⁻ (pPSAB5)/tetracycline free; (■), *styABC*⁻ (pPSAB5)/tetracycline added; (+), *styABC*⁻ (pPSAB5)/tetracycline free.

rene for all five of the bacteria tested.

Figs. 4 and 6 clearly indicate that the whole-cell SMO activity of the recombinant SN1 having multi-copy *styAB* genes is not higher than that of *styC*⁻ SN1 with a single copy of the *styAB* gene. According to SDS-PAGE analyses shown in Fig. 5, the self-cloned SN1 do not seem to give rise to a much higher level of *styAB* expression than the *styC*⁻ SN1. However, the other possibility, that the expression levels of *styAB* genes in the self-cloned SN1 are lower than those in the *styC*⁻ SN1, is also not expected. This is because the self-cloned SN1 have several times more *styAB* gene copies than the *styC*⁻ SN1 and because the gene regulatory circuit for *styAB* is exactly the same for both strains. The differences in activity between *styC*⁻ (pPSAB5) and *styABC*⁻ (pPSAB5) is another enigma. The only difference between the two recombinants is the presence of the extra copy of the *styAB* gene in the chromosome in the former and it is not reasonable to assume that the gene expression level can be much different between the two.

If the difference in the *styAB* gene expression level is not responsible for the different SMO activity, other factors affecting whole-cell SMO activity should be considered. It requires more extensive study, but, among numerous things, a lowered cell growth rate and/or reduced transport rate of styrene through cell membranes can be possible causes of this phenomenon. The lowered cell growth rate of the recombinant SN1 shown in Fig. 4A may have slowed down the cellular metabolism of *styC*⁻ SN1 and *styABC*⁻ SN1 at the global level, which includes the NADH regeneration rate. Fast NADH regeneration is important for high SMO activity since NADH is a cosubstrate of the SMO reaction (Fig. 1). The slower cellular metabolism in *styC*⁻ (pPSAB5) or *styABC*⁻ (pPSAB5) might offset any enhancement of SMO activity resulting from the increased expression of *styAB* genes. The membrane transport of styrene is associated with the expression of *styE*, the fifth structural gene of the styrene catabolic operon. According to a very recent study [25], *styE* encodes for a membrane protein responsible for the active transport of styrene through cell membranes. When *styE* was up-regulated, the styrene degradation rate of a *Pseudomonas* strain was greatly improved. However, the styrene degradation rate was decreased when *styE* was deleted or down-regulated. In our development of the *styC*⁻ or *styABC*⁻ mutant, *styC* gene or *styABC* genes were removed from the polycistronic styrene operon with *styE* being left to be co-transcribed with *styABD* or *styD* from the styrene promoter, P_{sty} [25,26]. The deletion was conducted carefully so as not to disrupt or modify the styrene promoter and the open reading frame of *styD* and *styE*. However, the possibility still remains that StyE level has decreased due to the removal of those upstream genes and more studies should be conducted.

CONCLUSION

We have developed recombinant strains of *P. putida* SN1 containing homologous *styAB* genes in a multicopy plasmid. The recombinants could produce an enantiopure (*S*)-styrene oxide from styrene but their activities were not improved compared to that of the *styC*-knockout mutant of SN1 with a single copy of the *styAB* genes. The use of tet^R as a selection marker is not recommended since the presence of tetracycline in the culture broth greatly reduced cell growth rate as well as whole-cell SMO activity in the recombinants. Further studies to develop a more efficient SMO biocatalyst based on *P. putida* SN1 are in progress.

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