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Characterization of Styrene Catabolic Genes of *Pseudomonas putida* SN1 and Construction of a Recombinant *Escherichia coli* Containing Styrene Monooxygenase Gene for the Production of (*S*)-Styrene Oxide

PARK, MI SO[1,2], JONG WON BAE[1,2], JU HEE HAN[1,2], EUN YEOL LEE[2,3], SUN-GU LEE[1], AND SUNGHOON PARK[1,2]*

[1]*Department of Chemical and Biochemical Engineering, Pusan National University, Busan 609-735, Korea*
[2]*Institute for Environmental Technology and Industry, Pusan National University, Busan 609-735, Korea*
[3]*Department of Food Science and Technology, Kyungsung University, Busan 608-736, Korea*

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Abstract Some *Pseudomonas* species can grow on styrene as a sole carbon and energy source. From the new isolate *Pseudomonas putida* SN1, the genes for styrene catabolism were cloned and sequenced. They were composed of four structural genes for styrene monooxygenase (*styA* and *styB*), styrene oxide isomerase (*styC*), and phenylacetaldehyde dehydrogenase (*styD*), along with two genes for the regulatory system (*styS* and *styR*). All the genes showed high DNA sequence (91% to 99%) and amino acid sequence (94% to 100%) similarities with the corresponding genes of the previously reported styrene-degrading *Pseudomonas* strains. A recombinant *Escherichia coli* to contain the styrene monooxygenase from the SN1 was constructed under the control of the T7 promoter for the production of enantiopure (*S*)-styrene oxide, which is an important chiral building block in organic synthesis. The recombinant *E. coli* could convert styrene into an enantiopure (*S*)-styrene oxide (ee >99%) when induced by IPTG. The maximum activity was observed as 140 U/g cell, when induced with 1 mM IPTG at 15°C.

Key words: (*S*)-styrene oxide, styrene monooxygenase, whole-cell biocatalyst, *Pseudomonas putida* SN1, induction condition

Asymmetric oxidations give versatile chemical building blocks and have been a focus in many organic synthetic researches [7, 22]. The reactions can be conducted by a chemical or biological catalyst. Biological reactions are often more stereo- and regio-specific than chemical reactions, and have been suggested to be useful alternatives to pure chemical approaches [2, 9, 28].

*Corresponding author
Phone: 82-51-510-3049; Fax: 82-51-510-2716;
E-mail: parksh@pusan.ac.kr

Styrene monooxygenase (SMO) that has been found in several *Pseudomonas* species catalyzes the selective conversion of styrene to (*S*)-styrene oxide, an important chiral intermediate for the synthesis of several important pharmaceuticals such as nematocide and levamisole [8, 18]. SMO is a flavin-dependent enzyme complex consisting of two separate enzymes, an NADH-FAD oxidoreductase (StyB) and an FAD-dependent hydroxylase (StyA). Epoxidation of styrene to styrene oxide requires $FADH_2$, which is derived from FAD by oxidation of NADH. Since SMO is composed of two separate enzymes, oxidoreductase and hydroxylase, and its reaction requires continuous regeneration of NADH as a cofactor, the use of whole cell is considered more advantageous than that of the purified enzyme system [4, 5]. Recombinant *Escherichia coli* strains containing the SMO genes or the *Pseudomonas* mutant that lacks the styrene oxide isomerase (SOI) activity have been developed as whole-cell biocatalysts for the SMO reactions [19, 20]. For example, Panke *et al.* [19, 20] have developed a recombinant *E. coli* that harbors a multicopy plasmid containing the *styAB* of *Pseudomonas* sp. VLB120 under the control of the *alk* promoter of *P. oleovorans*. Despite the presence of multicopy *styAB* genes, the specific SMO activity of the recombinant (100 U/g cell) was lower than that of an active wild-type *Pseudomonas* strain (170 U/g cell). The slow rate of NADH regeneration in the recombinant was suggested to be responsible for the low activity. However, the low activity was also suggested to be related to the low transcription efficiency from the *alk* promoter and hence the low expression of SMO enzymes. Although the *alk* promoter is known to be very active in *P. oleovorans* where the promoter is derived, no direct evidence that it is similarly active in *E. coli* is available.

In this study, we characterized styrene catabolic genes of the new isolate *P. putida* SN1 and developed an active whole-cell recombinant biocatalyst for the production of (*S*)-styrene oxide. The styrene catabolic genes of SN1 were cloned and sequenced using the primers designed from the consensus DNA sequences of previously reported styrene-degrading *Pseudomonas* sp. The recombinant biocatalyst was constructed to contain *styAB* of the SN1 strain under the T7 promoter. Since the T7 system generally gives a very high-level expression for many heterologous proteins (up to 50% of total proteins) [3, 10], it was expected to elucidate the effect of SMO expression level on the whole-cell activity. The effect of expression conditions on the SMO activity was also studied.

MATERIALS AND METHODS

Materials

Chemicals were purchased from Sigma (St. Louis, MO, U.S.A.). The enzymes for DNA manipulations were from NEB (Beverly, MA, U.S.A.). Plasmid pET31b(+) and the pGEM-T easy vector system were obtained from Novagen (U.S.A.) and Promega (U.S.A.), respectively. A miniprep kit and a DNA gel purification kit were purchased from Qiagen (Hilden, Germany), and primers were synthesized by Bioneer (Daejeon, Korea).

Isolation and Sequencing of Styrene Catabolic Genes

DNA manipulations were performed according to the procedures described by Sambrook *et al.* [23]. Genomic DNA was prepared from the *P. putida* SN1 and employed as templates for amplifying various genes after partial digestion with KpnI restriction enzyme. The genes, *styAB*, *styCD*, and *stySR*, were amplified by polymerase chain reaction (PCR). The primers were designed based on the consensus sequence of each gene(s) for the previously reported *Pseudomonas* strains, which are available from the GenBank database. Table 1 shows the primers and the PCR conditions used for the amplification. After amplification, PCR products were purified, cloned in pGEM-T easy vector, and sequenced using ABI 3700 (Applied Biosystems). Sequence similarity of DNA and protein was examined by the programs provided by the National Institute for Biotechnology Information such as BLASTP, BLASTN, and BLASTX.

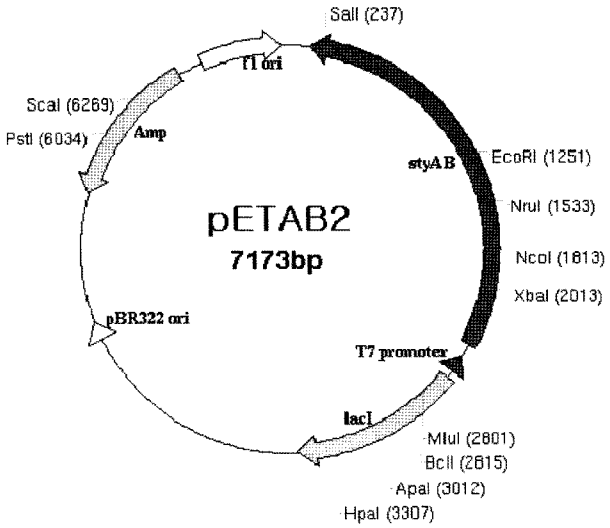

Fig. 1. Gene map of the recombinant plasmid pETAB2 containing *styAB* under the control of the T7 promoter.

Construction of Recombinant *E. coli* and Measurement of SMO Activity

The DNA fragment containing *styAB* genes (1.8 kb), amplified by PCR and cloned in pGEM-T easy vector, was excised by *Nde*I-*Xho*I digestion and inserted into the compatible site of the pET31b(+) vector. The resulting SMO expression vector, pETAB2 (Fig. 1), was then transformed into competent *E. coli* BL21(DE3) cells by heat-shock treatment. The *E. coli* transformants harboring the plasmid were selected on an LB plate containing ampicillin (100 mg/l). Colony PCR was performed with the primers AB1 and AB2 (see Table 1) to confirm the presence of pETAB2 in the transformants.

For measuring the whole-cell SMO activity, the recombinant *E. coli* (pETAB2) was grown overnight at 37°C in an LB medium containing ampicillin (100 mg/l). Then, the cells were inoculated in a fresh M9 mineral salt medium supplemented with glucose (5 g/l), thiamine (0.1 g/l), US* trace (1%, v/v) [20], and ampicillin (100 mg/l), and cultivated at 37°C for

Table 1. Primers and conditions used for the PCR amplification of styrene catabolic genes in *Pseudomonas putida* SN1.

Target genes	Primer name	Sequences (5'-3')	PCR conditions
styAB	AB1	GGAGGAATGAAAAAGCGTATCGGTATTGTTG	Annealing temp.:55°C
	AB2	GAATTCTTCGTTGCGCAATCAATTCAGTGGCA	Extention time:90 s
styCD	CD1	TCTAGACACGGCAACCCGTTGCCACTGAATTGATTG	Annealing temp.:55°C
	CD2	AAGCTTGCCTGGCACGCAGCAATCGCCCAGGACAAA	Extention time:90 s
stySR	SR1	GCTCTAGAGAGACTTCTTTCTGCTGTAG	Annealing temp.:50°C
	SR2	CCCAAGCTTTCAATTCAGTGGCAACGGGTT	Extention time:120 s

3 h. To induce the *styAB* genes, IPTG was added, and the cultivation was continued further for 4 h. After 7 h of total cultivation, the cells were harvested by centrifugation, washed, and resuspended in potassium phosphate buffer (50 mM, pH 7.0). The SMO activity was measured in a 165-ml serum bottle (liquid volume, 15 ml) fitted with a butyl rubber and aluminum cap. Styrene (2 μl) was added in the aqueous phase. The reaction bottle was incubated on a reciprocal shaker bath at 30°C and 180 strokes/min. Gas samples were periodically taken and analyzed for styrene, and liquid samples were analyzed for styrene oxide. When studying the effect of induction conditions on whole-cell SMO activity, cells were initially cultivated at 37°C for 3 h and then shifted to different temperatures (10°C–30°C) with supplementation of various amounts of IPTG (0.1 mM-1.0 mM).

SDS-PAGE Analysis

Cells were disintegrated by two passages through a French pressure cell (Thermo Electron Corporation, U.S.A.) at 18,000 psi. The disintegrated cell broth was centrifuged at 12,000 ×*g* and 4°C for 10 min to separate the supernatant from the pellet. The pellet was solubilized in 50 mM Tris buffer (pH 7.5) containing 5 mM DTT and 8 M Urea. Both samples from the supernatant and pellet were applied onto 18% polyacrylamide gel, and the protein bands were stained with Coomassie Brilliant Blue G-250 (Sigma). The intensity of the protein bands in the gel was analyzed by BIO-RAD Gel Doc 2000 (Bio-Rad).

Analytical Methods

Styrene in the gas samples was analyzed by a gas chromatograph (GC; HP6890, Hewlett Packard Inc., U.S.A.) equipped with a flame ionization detector. An HP-530 capillary column (Hewlett Packard Inc., U.S.A., 15 m long, 0.53 mm ID, and 1.5 μm film thickness) coated with cross-linked 5% PH ME siloxane was used for gas separation in the GC. Nitrogen was used as a carrier gas at a flow rate of 1 ml/min. The oven, injector, and detector temperatures were kept at 80°C, 150°C, and 300°C, respectively.

Styrene oxide in the liquid phase was extracted with cyclohexane and analyzed by a GC (M600D, Young-Lin, Korea). A flame ionization detector and a Supelco β-DEX 120 column (fused silica cyclodextrine capillary column, 60 m long, 0.25 mm ID, and 0.25 μm film thickness) were used. The sample was split at the ratio of 1:100 after injection. Helium was used as a carrier gas at 0.5 ml/min. The oven, injector, and detector were at 110°C, 250°C, and 250°C, respectively.

Cell density was measured by a Lambda 20 spectrophotometer (Perkin-Elmer, U.S.A.) at 660 nm. One OD unit corresponded to 0.3 g cell/l [21].

RESULTS AND DISCUSSION

Analysis of the Genes for Styrene Catabolism in *P. putida* SN1

The styrene-degrading strain *P. putida* SN1 was isolated from a biofilter used for treating gaseous styrene [21]. It could grow on styrene or styrene oxide as a sole energy and carbon source, but not on benzene or toluene. The styrene-degrading activity in SN1 was not detected in the cells grown on glucose and also not exposed to styrene. From the growth characteristics and degradation activity on various aromatic compounds, SN1 was suggested to have SMO as the first enzyme for styrene catabolism [21]. Since several previously reported *Pseudomonas* strains having an SMO contain four styrene catabolic genes (*styA*, *B*, *C*, and *D*) and two regulatory genes (*styS* and *R*), cloning and characterization of these genes in *P. putida* SN1 were attempted. The primers for amplifying the putative styrene catabolic genes were designed based on the consensus DNA sequences of each gene available in the GenBank database (Table 1). The PCR products were obtained for two genes at a time; i.e., *styAB*, *styCD*, and *stySR*. They were obtained as single bands (data not shown) and their sizes were estimated to be 1.8 kb, 2.0 kb, and 3.5 kb, respectively. Table 2 shows the characteristics of the six genes deduced from the sequences of the PCR products and their protein products including size, GC contents, and sequence similarities with previously reported styrene-degrading *Pseudomonas* strains. There were some differences in DNA sequences, but the sizes and GC contents of the corresponding genes were quite similar. The sizes of the corresponding proteins were nearly identical, and the similarities of amino acid sequences among the corresponding proteins were over 94%. The promoter-like sequences were identified in the upstream of *styA*, but not in the upstream of *styB*, *styC*, or *styD*. On the other hand, RBS-like sequences and terminator-like sequences were found for all four genes. These results suggest that *styABCD* in SN1 constitutes an operon as in other styrene-degrading *Pseudomonas* strains [27]. The nucleotide sequences reported in this study have been submitted to the GenBank/EMBL databank (accession no. DQ177365).

Since the genes for StyA and StyB are to be cloned for the production of chiral styrene epoxide, they were examined in more details. Figure 2 shows the alignment of protein sequences of StyAB among various *Pseudomonas* species together with those of a hydroxylase and oxidoreductase in hydrocarbon catabolic pathways. In addition to the high similarity with several StyA of *Pseudomonas*, the putative StyA of SN1 also exhibited a close relationship with flavin-containing aromatic hydroxylases such as *p*-hydroxybenzoate hydroxylase (PobA) from *P. fluorescense* and salicylate hydroxylase (NahG) from *P. putida* [27]. The overall

Table 2. Comparison of genes and proteins of styrene catabolic ORFs in styrene-degrading strains.[a]

ORF	Gene/ Protein		Strains			
			P. putida SN1	*P. fluorescens* ST	*P.* sp. VLB120	*P.* sp. Y2
styS	Gene	GC content (%)	57	58	57[b]	57
		Similarity (%)	-	88	89[b]	98
	Protein	Amino acid residue	982	983	258[c]	982
		Similarity (%)	-	94	95[c]	99
styR	Gene	GC content (%)	53	54	53	53
		Similarity (%)	-	97	93	99
	Protein	Amino acid residue	207	208	207	207
		Similarity (%)	-	97	98	100
styA	Gene	GC content (%)	59	59	58	59
		Similarity (%)	-	92	94	94
	Protein	Amino acid residue	415	415	415	415
		Similarity (%)	-	96	97	97
styB	Gene	GC content (%)	59	58	58	57
		Similarity (%)	-	92	91	91
	Protein	Amino acid residue	170	170	170	170
		Similarity (%)	-	95	95	95
styC	Gene	GC content (%)	57	57	56	56
		Similarity (%)	-	92	97	97
	Protein	Amino acid residue	169	169	169	169
		Similarity (%)	-	95	97	97
styD	Gene	GC content (%)	60	60	60	60
		Similarity (%)	-	96	98	97
	Protein	Amino acid residue	496	502 or 496[d]	496	502
		Similarity (%)	-	98	98	99
Reference			This study	[1]	[17]	[25]

[a]Sequences included in this table: StyS, sensor histidine kinase; StyR, response regulator; StyA, styrene monooxygenase unit; StyB, NADH-flavin oxidoreductase; StyC, styrene oxide isomerase; StyD, phenylacetaldehyde dehydrogenase in the styrene catabolic pathway, respectively. Similarities of gene and amino acid sequences are related to *P. putida* SN1.

[b]Genetic properties of *stySc* (777 bp of truncated 3' part of a *styS*).

[c]Protein properties of StySc (258 aa residues of carboxylic part of StyS).

[d]*styD* in *P. flourescens* ST shows two possible start codons. The second ATG codon encoding a 496-aa protein seems to be led by a better ribosomal binding site.

sequences among an individual hydroxylase and styrene monooxygenase were quite different, but some specific domains exhibited a high sequence similarity. For instance, all the enzymes had three conserved glycine residues (GXGXXG) (between 9 and 14 amino acids in StyA) in the amino-terminal β-α-β unit (between 5 and 31 amino acids in StyA) whose role is associated with the binding of the ADP portion of FAD [1]. All the oxygenases possess a cofactor, a transition metal, flavin, or pteridine that interacts with dioxygen. The cofactor is required to overcome the restriction by spin forbidden reactions between molecular oxygen and carbon in organic compounds [6]. All these features confirm that the putative StyA of SN1 is very similar to other known StyA and belongs to a flavin adenine dinucleotide (FAD)-binding monooxygenase.

Figure 2 also shows that the putative StyB of SN1 has a high similarity with StyB or StdB of several *Pseudomonas* sp. and NADH:FMN oxidoreductase such as NtaB in nitrilotriacetate catabolic pathways of *Agrobacterium tumefaciens*. In addition, there were several conserved amino acid sequences in StyB and the coupling protein (HpaC) acting as flavin:NADH reductase of the two-component 4-hydroxyphenylacetate-3-monooxygenase from *Klebsiella oxytoca* [27]. These results suggest that the putative StyB protein of SN1 is the small subunit of the two-component SMO, which functions as flavin:NADH oxidoreductase. Although StyB has no catalytic activity in a styrene epoxidation reaction, StyB is essential for the maximal activity of SMO [18].

Construction of Recombinant *E. coli* Overexpressing Styrene Monooxygenase

The SMO expression plasmid pETAB2 was constructed by inserting a *styAB* gene into pET 31b(+) and transformed into *E. coli* BL 21(DE3). Many transformants were obtained on an LB plate containing ampicillin (100 mg/l), and the colony PCR performed with the primers AB1 and AB2 (see Table 1) confirmed the presence of pETAB2 in the

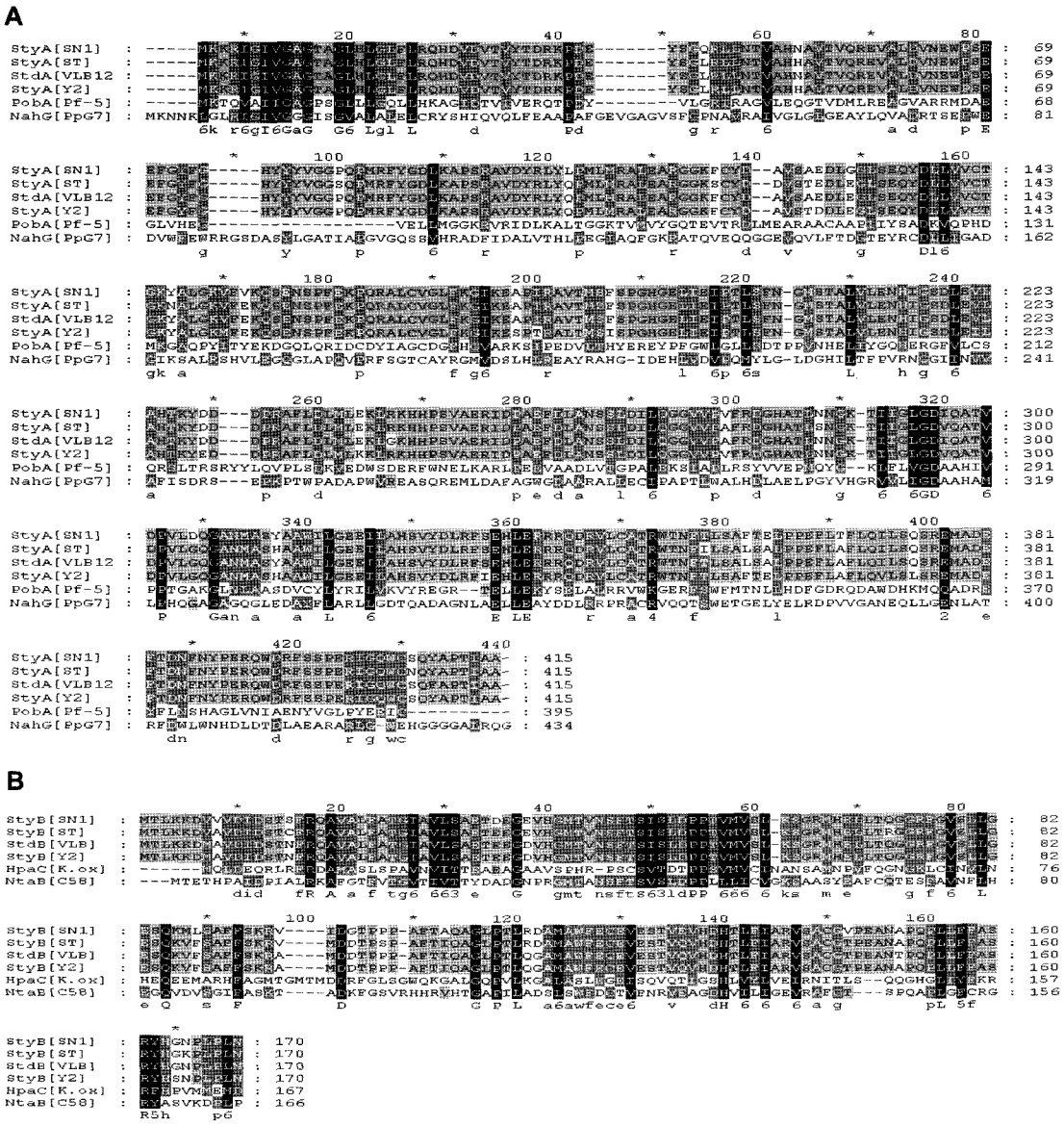

Fig. 2. Multiple sequence alignment of amino acids of StyAB and related proteins by the BLAST search.
A. StyA or StdA, styrene monooxygenase of *P. putida* SN1, *P. fluorescence* ST, *P.* sp. VLB120, and *P.* sp. Y2; PobA, 4-hydroxybenzoate 3-monooxygenase of *P. fluorescence* Pf-5; NahG, salicylate 1-monooxygenase of *P. putida* PpG7. **B**. StyB or StdB, reductase of styrene monooxygenase of *P. putida* SN1, *P. fluorescence* ST, *P.* sp. VLB120, and *P.* sp. Y2; HpaC, Flavin:NADH reductase of 4-hydroxyphenylacetate 3-monooxygenase of *Klebsiella oxytoca*; NtaB, NADH:FMN reductase of the nitrilotriacetate monooxygenase of *Agrobacterium tumefaciens* C58. Conserved sequences are shaded in three levels. Gaps in the alignments are indicated as (-).

transformants (data not shown). Expression of SMO was examined by SDS-PAGE and enzymatic activity. Figure 3 shows the results of SDS-PAGE for the cell extracts. Both recombinant and host cells of *E. coli* were examined before and after induction (1 mM IPTG; 37°C). The soluble fraction of the recombinant after induction exhibited a strong band at the 47-kDa region, corresponding to the size expected for StyA (panel A). On the other hand, uninduced transformant or the *E. coli* host lacking the plasmid pETAB2 did not show the 47-kDa band. The *styB* gene product (MW, 16 kDa) was expected to be shown in the SDS-PAGE, but the induced transformant did not show any band more intense than the uninduced transformant or the *E. coli* host. This was attributed to the lower expression of StyB than StyA or the presence of too many proteins with a similar size as StyB in the cell extract. The SDS-PAGE analysis was also conducted for the particulate fraction of the cell extracts (panel B). The particulate fraction was solubilized with 8 M urea before application onto the PAGE gel. As with the supernatant fraction, a strong StyA band was observed at the 47-kDa region only for the recombinant cells after induction with IPTG.

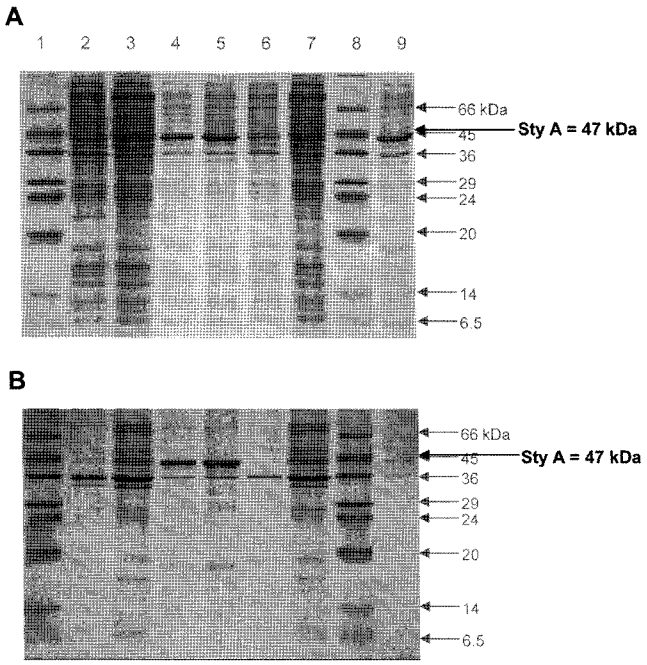

Fig. 3. SDS-PAGE analysis for the soluble (**A**) and particulate (**B**) fractions of the host and recombinant *E. coli*. Cells were induced at 37°C, except for the one at lane 9, which was induced at 15°C.

Lanes 1 and 8, marker protein; lane 2, host cell (4 h); lane 3, host cell (8 h); lane 4, induced recombinant cell (4 h); lane 5, induced recombinant cell (8 h); lane 6, uninduced recombinant cell (4 h); lane 7, uninduced recombinant cell (8 h); lane 9, induced recombinant cell (8 1h; 15°C).

Enzymatic activity of the recombinant *E. coli* was examined with styrene as a reactant. Only the recombinant after induction could produce styrene oxide from styrene. The (*S*)-form was obtained almost exclusively, with an ee value over 99% (data not shown). The host or the recombinant without induction did not produce styrene oxide from styrene. Therefore, it could be concluded that the *styA* and *styB* of *P. putida* SN1 are the genes encoding SMO, and they were successfully cloned in *E. coli*.

Effect of IPTG Concentration and Temperature on Whole-Cell SMO Activity

The whole-cell SMO activity of this recombinant was estimated to be about 10–20 U/g cell, five to ten times lower than the activity of the wild-type *P. putida* SN1 or the previously reported recombinant *E. coli* expressing SMO under the *alk* promoter [19–21]. Since overexpression of a recombinant protein often results in misfolding of the target protein and loss of the protein activity, different induction conditions were studied to increase the recombinant activity by reducing the expression level of the T7 system and the misfolding of the recombinant SMO.

Figure 4 shows the effect of IPTG concentration on cell growth and whole-cell SMO activity. The temperature was fixed at 37°C. Cell growth was not affected significantly

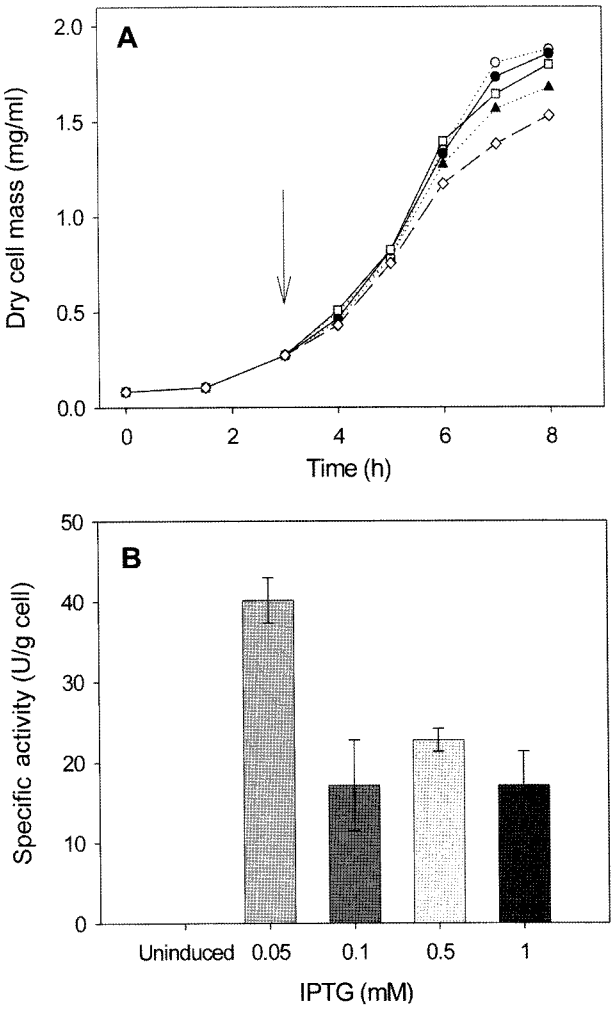

Fig. 4. Effect of IPTG concentration on cell growth (**A**) and whole-cell SMO activity (**B**).

IPTG was added at 3 h (arrow in A) at 0.00 mM (▲), 0.05 mM (○), 0.1 mM (●), 0.5 mM (□), and 1 mM (◇).

by the addition of IPTG, except for the case of 1.0 mM (Fig. 4A). Specific growth rate of the cell before and after the addition of IPTG was about the same at 0.62±0.05 h^{-1}. Final cell density at 8 h was about 1.6±0.2 g/l. Whole-cell activity was determined for the cells harvested at 7 h (Fig. 4B). The cells induced by 0.05 mM IPTG exhibited the highest activity of 55±5.0 U/g cell. The cells induced at higher IPTG concentrations of 0.1–1.0 mM showed a lower activity of 17±8.0 U/g cell.

Figure 5 shows the effect of induction temperature on cell growth and SMO activity with a fixed IPTG concentration of 1.0 mM. The cells were grown at 37°C for the initial 3 h, IPTG was added, and further cultivated at different temperatures for 4 h. Cell growth rate was greatly reduced when the temperature was lowered below 20°C (Fig. 5A). At low temperatures, final cell densities at 8 h were also very low at 0.75 g/l at 20°C, 0.60 g/l at 15°C, and

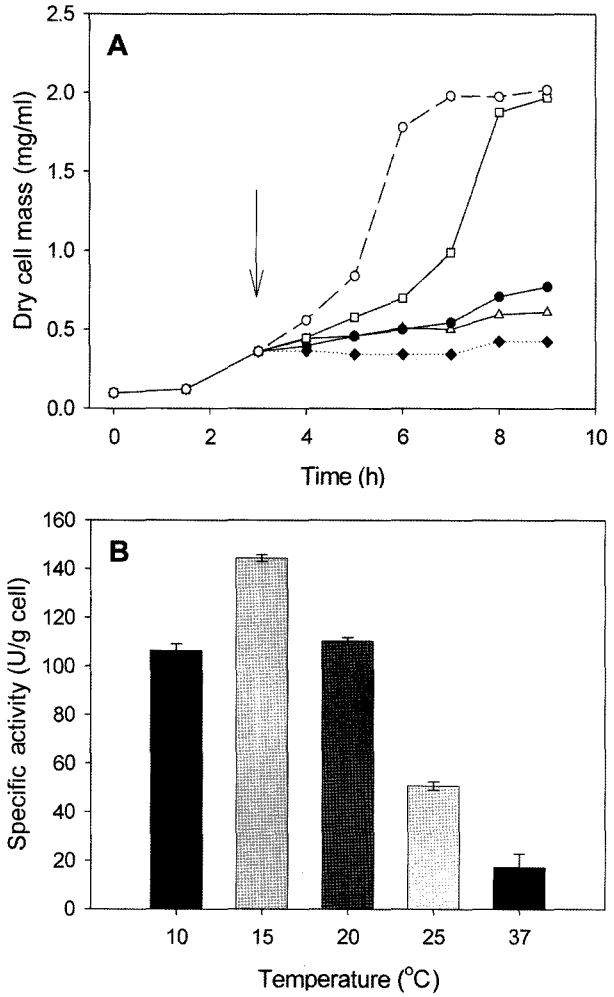

Fig. 5. Effect of induction temperature on cell growth (**A**) and whole-cell SMO activity (**B**).
At 3 h (arrow in A), IPTG was added at 1 mM, and temperature was shifted from 37°C to 10°C (◆), 15°C (△), 20°C (●), 25°C (□), and 37°C (○), respectively.

0.42 g/l at 10°C. However, the whole-cell SMO activity measured with the cells harvested at 7 h was high for the ones induced in the range of 10 to 20°C: The maximum activity of 144±2 U/g cell was observed in the cells induced at 15°C, which was about 9-folds higher than the activity of the cells induced at 37°C. This SMO activity is comparable to that of the wild-type *P. putida* SN1 or the previously reported recombinant *E. coli* expressing SMO under the *alk* promoter [19–21].

The results on the induction conditions shown in Figs. 4 and 5 indicate that high temperature or high IPTG concentration, the conditions that gave rise to high expression of the recombinant SMO, resulted in low whole-cell SMO activity in the recombinant *E. coli*. In order to elucidate the relationship between the whole-cell activity and the location and/or the amount of SMO in the cell, the amounts of SMO for the cells induced with 1 mM IPTG at 37°C and 15°C were quantified and compared on SDS-PAGE (Fig. 3). Table 3 indicates that the total protein in the cells was much higher when induced at 15°C; however, the total amount of StyA per cell was quite similar regardless of induction temperature. It should be noticed that StyA appeared mostly in the soluble fraction when induced at 15°C, whereas it appeared equally between soluble and particulate fractions when induced at 37°C. This suggests that the enzyme in the particulate fraction does not contribute to the whole-cell SMO activity, and that the activity of the recombinant SMO is dependent on the amount of the enzyme present in the soluble fraction rather than the total amount of the enzyme produced. It is well documented that, to improve the whole-cell enzymatic activity, expression of an enzyme in an active form is much more important than the mere improvement of the protein production level. However, it should be pointed out that the different expression levels of StyA in the soluble fraction induced at 37°C and 15°C do not fully explain the difference in SMO activity shown in Fig. 5: The difference in the amount of soluble StyA is about 2-fold, but the difference in whole-cell SMO activity is about 9-fold. It is speculated that some other factors in addition to the amount of soluble StyA have an influence on the whole-cell activity. For example, the folding efficiency of StyA protein, which was not detected by SDS gel analysis, or the stability of SMO could also give rise to the difference of SMO activities in the cells induced at different conditions. Another factor could be the expression level of StyB: On the SDS-PAGE analysis shown in Fig. 3, the StyB protein was not detected whereas the StyA protein showed a strong band intensity. The possibility that the level of active StyB could be much lower in the cells, when induced at a high temperature, should be examined.

Further optimization of the induction conditions has also been attempted by combining different temperatures and IPTG concentrations (data not shown). Specifically, the effect of IPTG concentration at a fixed temperature of 30°C was

Table 3. Comparison of StyA produced under different induction temperatures in *Pseudomonas putida* SN1.[a]

Induction temperature (°C)	Total protein (mg/mg cell)	Total StyA (mg/mg cell)	Soluble StyA (mg/mg cell)	Particulate StyA (mg/mg cell)
15	0.61	0.18	0.15	0.03
37	0.45	0.16	0.07	0.09

[a]The amounts of StyA were estimated from band intensities of SDS-PAGE analyses.

examined, and we found that the cell growth rate and final cell density were maintained at a moderately high level. However, the results were not satisfactory, since independent of IPTG concentration, the activity was not higher than 80 U/g cell. This suggests that, with the present recombinant, it might be necessary to grow the cells at a rate below 0.125 h^{-1} to obtain high whole-cell SMO activity. For a whole-cell biocatalyst to be economically useful, the cell should be able to grow fast to a high concentration. This indicates that the potential of the present recombinant is rather limited as an industrial SMO biocatalyst.

One of the major objectives of the present study was to have an answer to the question of whether the whole-cell SMO activity can be improved by increasing the expression level of SMO higher than that in the wild-type *P. putida* SN1 or in the previously reported recombinant *E. coli* expressing SMO under the *alk* promoter. It has previously been reported that the StyA levels in the recombinant *E. coli* expressing SMO under the *alk* promoter and in the wild-type *Pseudomonas* sp. VLB120 were about 137.5 mg/g cell (25% of total protein) and 27.5 mg/g cell (5% of total protein), respectively, when total protein was assumed to be 55% of dry cell mass [16]. For the present recombinant induced at 15°C, total StyA was about 180 mg/g cell (29.5% of total protein) and soluble StyA was 150 mg/g cell (Table 3). This comparison indicates that the expression level of StyA was successfully increased, although the activity was not correspondingly increased. Further attempts to elucidate other factors possibly affecting the whole-cell SMO activity and development of highly active whole-cell biocatalysts are under progress.

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