

## Engineering of Solvent-Tolerant *Pseudomonas putida* S12 for Bioproduction of Phenol from Glucose

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**Efficient bioconversion of glucose to phenol via the central metabolite tyrosine was achieved in the solvent-tolerant strain *Pseudomonas putida* S12. The *tpl* gene from *Pantoea agglomerans*, encoding tyrosine phenol lyase, was introduced into *P. putida* S12 to enable phenol production. Tyrosine availability was a bottleneck for efficient production. The production host was optimized by overexpressing the *aroF-1* gene, which codes for the first enzyme in the tyrosine biosynthetic pathway, and by random mutagenesis procedures involving selection with the toxic antimetabolites *m*-fluoro-DL-phenylalanine and *m*-fluoro-L-tyrosine. High-throughput screening of analogue-resistant mutants obtained in this way yielded a *P. putida* S12 derivative capable of producing 1.5 mM phenol in a shake flask culture with a yield of 6.7% (mol/mol). In a fed-batch process, the productivity was limited by accumulation of 5 mM phenol in the medium. This toxicity was overcome by use of octanol as an extractant for phenol in a biphasic medium-octanol system. This approach resulted in accumulation of 58 mM phenol in the octanol phase, and there was a twofold increase in the overall production compared to a single-phase fed batch.**

Processes for the “green” production of chemicals from natural renewable resources require multiple steps in whole cells. The rapidly increasing interest in such production methods is driven both by environmental issues (closed carbon cycles) and by a tremendous political drive to be less dependent on fossil resources (30). In our laboratory whole-cell bioprocesses for the production of aromatic compounds from sugar are being developed. Examples of “green” bioprocesses that have been studied for this class of compounds are the production of *p*-hydroxybenzoic acid (7), phenol (19), and cinnamic acid (32). A major drawback in terms of the economics of biobased processes is the susceptibility of host microorganisms to the toxicity of aromatic compounds. We use the solvent-tolerant organism *Pseudomonas putida* S12 as a platform for the production of substituted aromatic compounds. This organism has evolved several mechanisms to deal with toxic solutes, including modifications of the inner and outer membrane and active extrusion of a broad range of compounds, including aromatic compounds (11, 44). Membrane-associated efflux pumps have been detected in several pseudomonads (24, 37). It has been shown that solvent-tolerant pseudomonads can also be successfully used in the production of toxic substituted aromatic compounds from toxic substrates, such as toluene (36). An important issue here is that the use of these strains offers a wider degree of freedom in the choice of solvents for in situ extraction methodologies (21, 38, 45).

Phenol is one of the most economically important hydroxylated aromatic compounds. With an annual production of more than 7 million tons, it is utilized mostly for the production of bisphenol A and phenolic resins. Currently, the main production method for phenol is the chemical oxidation of

cumene (26), which is produced from benzene (25). This process is very energy intensive and produces much toxic waste, and there is a hazardous explosive intermediate. Furthermore, a sharp increase in demand and a dramatic rise in oil prices have prompted us to seriously address the possibility of a “green” production method for phenol. Previously, Gibson et al. (19) described the bioproduction of shikimic acid from glucose, followed by the chemical conversion of shikimic acid to phenol.

The aim of this study was to develop and optimize an entirely biobased production process for the conversion of a renewable substrate (glucose) into the toxic bulk chemical phenol. The conversion of de novo-synthesized tyrosine into phenol in the solvent-tolerant host *P. putida* S12 was achieved by introduction of the *tpl* gene from *Pantoea agglomerans* (23). This gene encodes the pyridoxal 5'-phosphate-dependent enzyme tyrosine phenol lyase (TPL) (EC 4.1.99.2), which catalyzes the formation of phenol, pyruvate, and ammonia from tyrosine (27, 28, 34). We optimized the production host by a combination of targeted genetic alteration, random mutagenesis, antimetabolite selection, and high-throughput screening. A further increase in phenol production was achieved by fed-batch cultivation in a biphasic medium-octanol system.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** Bacterial strains and plasmids used in this study are listed in Table 1. *P. putida* S12 was originally isolated as a styrene-degrading bacterium (20).

pTn-1 was used as the basic expression vector (Table 1). The *tpl* expression vector pNW1 was constructed as follows. The *tpl* gene was amplified from *P. agglomerans* AJ2985 genomic DNA by PCR with primers 5'-GCGGTACCATG AACTATCCTGCCGAGCC-3' (forward) and 5'-GCGGCCGCTTAAATAAA GTCAAAACGCGC-3' (reverse), which were designed from the previously published sequence of *tpl* (GenBank accession no. D13714). Restriction sites for KpnI and NotI were added to the primers for cloning in pTn-1. After A tailing, the PCR fragment was cloned into pGEM-T Easy (Promega) according to the manufacturer's instructions, which yielded pTtpIC. The *tpl* gene on this plasmid

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics <sup>a</sup>	Reference(s) and/or source
<b>Strains</b>		
<i>Pseudomonas putida</i> S12	Wild type	20, 43a
<i>Pantoea agglomerans</i> AJ2985	Source of <i>tpl</i>	32a
<i>Escherichia coli</i> DH5 $\alpha$	<i>supE44</i> $\Delta$ <i>lacU169</i> $\phi$ 80 <i>dlacZ</i> $\Delta$ <i>M15</i> <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>grrA96</i> <i>thi-1</i> <i>relA1</i>	39
<i>E. coli</i> TnAroF-1	Transposon donor strain, contains pTnAroF-1	This study
<i>E. coli</i> RK2013	Mobilizing strain for triparental mating	15a
<i>P. putida</i> S12TPL	<i>P. putida</i> S12 containing plasmid pNW1	This study
<i>P. putida</i> S12TPL1	TnAroF-1 transposon mutant derived from <i>P. putida</i> S12TPL	This study
<i>P. putida</i> S12TPL2	Derived from <i>P. putida</i> S12TPL1 by NTG mutagenesis and <i>m</i> -fluoro-DL-phenylalanine selection	This study
<i>P. putida</i> S12TPL3	Derived from <i>P. putida</i> S12TPL2 by NTG mutagenesis and <i>m</i> -fluoro-L-tyrosine selection	This study
<b>Plasmids</b>		
pTn-1	Ap <sup>r</sup> Gm <sup>r</sup> , basic expression vector containing the salicylate-inducible <i>NagR/pNagAa</i> promoter	21a, 32
pNW1	Ap <sup>r</sup> Gm <sup>r</sup> , pTn-1 containing the <i>tpl</i> gene under control of the <i>NagR/pNagAa</i> promoter	This study
pJWDAHP1	Ap <sup>r</sup> Gm <sup>r</sup> , pTn-1 containing the <i>aroF-1</i> gene from <i>P. putida</i> S12 under control of the <i>NagR/pNagAa</i> promoter	32
pTnModKmO	Km <sup>r</sup> , basic modular plasmid	13
pTnAroF-1	Km <sup>r</sup> , derived from pTnModKmO, contains <i>aroF-1</i> gene under control of the <i>NagR/pNagAa</i> promoter	This study
pGEM-T Easy	Ap <sup>r</sup> , used for cloning PCR fragments	Promega
pTtpIC	pGEM-T Easy containing the <i>tpl</i> gene from <i>P. agglomerans</i>	This study

<sup>a</sup> Ap<sup>r</sup>, Gm<sup>r</sup>, and Km<sup>r</sup>, ampicillin, gentamicin, and kanamycin resistance, respectively.

was sequenced and shown to contain the correct sequence. The *tpl* fragment was then excised with KpnI and NotI and ligated into pTn-1 to obtain pNW1 (Table 1).

Plasmid pTnAroF-1 was constructed as follows. The *aroF-1* expression cassette, containing the *NagR/pNagAa* promoter, the *aroF-1* gene, and the *Tn-1* terminator, was amplified from pJWDAHP1 by PCR with primers 5'-GCACT AGTGCACAAGACCAGTCGCATGGGAGAAC-3' (forward) and 5'-CTGGT GAGACATGGGAAGCGGCC-3' (reverse). The restriction site for SpeI was added to the forward primer for insertion into plasmid pTnModKmO (13). The amplified fragment was digested with AvrII and SpeI and ligated in plasmid pTnModKmO, which was digested with SpeI and dephosphorylated, to obtain pTnAroF-1.

*P. putida* S12TPL was obtained by transforming *P. putida* S12 with pNW1. *P. putida* S12TPL1 was created by introducing transposon TnAroF-1 into *P. putida* S12TPL by triparental mating using *Escherichia coli* RK2013 as the mobilizing strain and established procedures (15). *P. putida* S12TPL2 and *P. putida* S12TPL3 were obtained by successive *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) mutation rounds after antimetabolite selection and screening for enhanced phenol production (Table 1). *E. coli* strain DH5 $\alpha$  was used for transformation and amplification of recombinant plasmids by standard methods (39).

**DNA methods.** Plasmid DNA was isolated with a Qiaprep spin miniprep kit (QIAGEN). DNA fragments for cloning were isolated from 0.8% agarose gels with a QIAEXII gel extraction kit (QIAGEN). Genomic DNA was isolated with a DNeasy tissue kit (QIAGEN). DNA digestion and ligation were carried out with enzymes from Fermentas GmbH used according to the manufacturer's instructions. PCRs were performed with an Accuprime Pfx Taq polymerase kit (Invitrogen) according to the manufacturer's instructions. For Southern blots of transposon mutants, genomic DNA was digested with KpnI, separated by agarose gel electrophoresis, and transferred to nylon filters by using standard procedures (39). Probes were synthesized with a PCR digoxigenin probe synthesis kit (Boehringer) used according to the manufacturer's instructions. Blots were probed with part of the Km<sup>r</sup> gene, stripped, and reprobed with part of the *tnp* gene of the pTnAroF-1 plasmid to check for proper integration of the transposon. Hybridizations were done using a digoxigenin DNA labeling and detection kit (Boehringer) according to the manufacturer's recommendations. Chromosomal DNA flanking the transposon was isolated as described by Ausubel et al. (5). Oligonucleotide synthesis and DNA sequencing were performed by MWG Biotech AG. Nucleotide sequence analysis was carried out with the National Center for Biotechnology Information BLAST server (3).

**Culture conditions.** LB broth (39) was used as the complete medium. Transposon mutants were selected on pseudomonas isolation agar (Difco) containing gentamicin and kanamycin. Mineral salts medium was prepared as described by Hartmans et al. (20), using 37 mM phosphate buffer at pH 7.0. D-Glucose (22 mM) was used as the carbon source unless indicated otherwise. Solid media contained 1.5% (wt/vol) agar. Ampicillin (100 mg/liter) and kanamycin (50 mg/liter) were added to maintain plasmids in *E. coli*. Kanamycin (50 mg/liter) was used in the selection for *P. putida* S12TPL1 transposon mutants. Gentamicin was used to maintain pNW1 in all *P. putida* S12TPL strains at a concentration of 25 mg/liter for all types of culture media except liquid mineral medium, in which a concentration of 10 mg/liter was used. When needed, 0.1 mM sodium salicylate was added as an inducer for expression of *tpl* or *aroF-1*.

*P. putida* S12 was grown at 30°C, *E. coli* was grown at 37°C, and *P. agglomerans* was grown at 26°C. Liquid cultures were grown in shake flasks in a horizontal rotary shaker at 180 rpm unless indicated otherwise. Batchwise phenol production experiments were performed in 10 ml medium in airtight 250-ml Boston bottles with Mininert valves (Alltech). Cultures were inoculated from an overnight preculture so that the optical density at 600 nm (OD<sub>600</sub>) was approximately 0.2.

Fed-batch cultivation was performed in a BioFlo IIc fermentor (New Brunswick Scientific) by using a working volume of 2.5 liters. Pure oxygen was supplied in the headspace at a rate of 300 ml/min and was mixed into the culture medium by stirring with a double impeller at the bottom of the reactor. During cultivation, pH 7.0 was maintained by automatic addition of 4 M KOH, and the dissolved oxygen tension was kept at approximately 20% saturation by automatic adjustment of the impeller speed. The initial batch phase (1.5 liters) was started with washed cells from an overnight culture in 50 ml mineral medium with glucose (MMG). Feeding was started when no more increase in biomass was observed. The feed rate was adjusted based on the biomass in the fermentor; it was 4 ml/h when cell dry weight (CDW) was less than 3 g/liter, 9 ml/h when the CDW was between 3 and 4.5 g/liter, and 20 ml/h when the CDW was more than 4.5 g/liter. Samples were taken at regular intervals, and phenol, ammonium, and glucose concentrations were measured. Since *P. putida* S12 converts extracellular glucose to gluconic acid and 2-ketogluconic acid, these compounds were also measured. The compositions of the culture media were as follows. Batch medium contained (per liter) 30 mmol K<sub>2</sub>HPO<sub>4</sub>, 20.5 mmol NaH<sub>2</sub>PO<sub>4</sub>, 25 mmol D-glucose, 15 mmol NH<sub>4</sub>Cl, 1.4 mmol Na<sub>2</sub>SO<sub>4</sub>, 1.5 mmol MgCl<sub>2</sub>, 0.5 g yeast extract, 10 ml trace solution 1, 10 mg gentamicin, and 0.1 mmol salicylate. Feed medium contained (per liter) 750 mmol D-glucose, 225 mmol NH<sub>4</sub>Cl, 21 mmol Na<sub>2</sub>SO<sub>4</sub>,

7.4 mmol  $\text{MgCl}_2$ , 13 mmol  $\text{CaCl}_2$ , 0.5 g yeast extract, 100 ml trace solution 2, 10 mg gentamicin, and 1 mmol salicylate. Trace solution 1 contained (per liter) 4 g EDTA, 0.2 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 1.5 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02 g  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.2 g  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.04 g  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , and 0.1 g  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ . Trace solution 2 contained (per liter) 4 g EDTA, 0.2 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 6.5 g  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02 g  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.2 g  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.04 g  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.1 g  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.024 g  $\text{H}_3\text{BO}_3$ , and 0.02 g  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ .

Two-phase fed-batch fermentation was performed by using a method similar to the method used for single-phase fed-batch fermentation, with the following exceptions. The initial batch phase was allowed to proceed until the  $\text{OD}_{600}$  of the culture reached approximately 1, after which 400 ml octanol was pumped into the fermentor over the course of 1 h. Ammonium concentrations were measured periodically during the batch phase, and feeding was started when ammonium was depleted. The feed rate was kept at 4 ml/h for the first 10 h of the feeding phase, after which it was increased to 9 ml/h. Effluent gas  $\text{CO}_2$  concentrations were determined and used as a measure of biological activity, since the emulsified octanol hindered accurate determination of the biomass.

**NTG mutagenesis and selection of phenol-overproducing mutants.** NTG mutants were obtained by cultivating bacteria overnight in 100 ml MMG. The resulting stationary-phase culture was washed and incubated in citrate-phosphate buffer (170 mM) at pH 6.0 containing 0 to 250 mg/liter NTG for 30 min at 30°C (1). Survival rates were determined by plating dilutions on LB agar plates and counting the CFU. After this, cells were suspended in LB medium with 20% glycerol and stored at  $-80^\circ\text{C}$ .

For mutant screening, bacteria were plated on bioassay trays (Nunc) containing solid mineral medium with 22 mM fructose, 25 mg/liter gentamicin, and either 100 mg/liter *m*-fluoro-DL-phenylalanine (MFP) or 10 to 100 mg/liter *m*-fluoro-L-tyrosine (MFT) (16, 22). Mutant libraries of the resulting analogue-resistant colonies were prepared in 96-well microwell plates using a Versarray colony-picking and arraying system (Bio-Rad). These mutant libraries were screened for enhanced phenol production as described below.

**Analytical methods.** Spectroscopic measurements of cell density at 600 nm were obtained with a Unicam Helios  $\alpha$  spectrophotometer (Fischer Scientific) using plastic cuvettes (Greiner). Alternatively,  $\text{OD}_{600}$  measurements were obtained with a  $\mu$ Quant MQX200 universal microplate spectrophotometer (Biotek) using flat-bottom 96-well microplates (Greiner).

To quickly assess phenol concentrations in *P. putida* cultures, the following ingredients were added to 0.5 ml of culture supernatant: 0.5 ml distilled water, 0.2 ml 1.4% (wt/vol)  $\text{NaHCO}_3$ , 0.2 ml 0.68% 4-aminoantipyrine, and 0.1 ml 5.4%  $\text{K}_3\text{Fe}(\text{CN})_6$  (31). Phenol concentrations were determined by measuring the  $\text{OD}_{500}$  after 1 h of color development at room temperature. For screening of mutant libraries, the reagents were added at the same proportions to complete cell cultures, and red coloration was assessed by eye.

In two-phase cultivations, the total phenol concentration was calculated as follows (45):  $c_{\text{tot}} = (c_{\text{aq}} \cdot V_{\text{aq}} + c_{\text{oct}} \cdot V_{\text{oct}}) \cdot V_{\text{tot}}^{-1}$ , where  $c_{\text{tot}}$ ,  $c_{\text{aq}}$ , and  $c_{\text{oct}}$  are the phenol concentrations in the total system, the aqueous phase, and the octanol phase, respectively, and  $V_{\text{tot}}$ ,  $V_{\text{aq}}$ , and  $V_{\text{oct}}$  are the volumes of the total system, the aqueous phase, and the octanol phase, respectively.

Phenol concentrations were also determined by high-performance liquid chromatography HPLC (Waters). An Alltech Alltima  $\text{C}_8$  column (length, 250 mm; inside diameter, 4.6 mm; particle size, 5  $\mu\text{m}$ ) was used along with a Chromopak UV detector (detection at 268 nm). Samples (10 to 50  $\mu\text{l}$ ) were injected in a mobile phase consisting of 50% acetonitrile and 50%  $\text{KH}_2\text{PO}_4$  (0.05 M) at a flow rate of 0.8 ml/min. Phenol dissolved in octanol was measured after dilution with an equal volume of acetonitrile using a mobile phase consisting of 50% acetonitrile and 50% water at a flow rate of 0.6 ml/min. Gluconic acid and 2-ketogluconic acid concentrations were detected at 210 nm using an Aminex HDP-87H (Bio-Rad) column with an eluent consisting of 0.008 N  $\text{H}_2\text{SO}_4$  at a rate of 0.6 ml/min. Glucose concentrations were analyzed using an Aminex HDP-87N (Bio-Rad) column with 0.01 M  $\text{Na}_2\text{HPO}_4$  as the eluent, along with a Waters 2414 refractive index detector. Ammonium concentrations were determined by cation-exchange chromatography (Dionex). Effluent gas  $\text{CO}_2$  concentrations were determined online with an ADC 7000 gas analyzer.

**Assay of tyrosine phenol lyase in cell extract.** Cell extracts were obtained as follows. Cells were resuspended in 50 mM potassium phosphate buffer (pH 8.0) and sonicated on ice with a Branson Sonifier in the pulse mode three times for 45 s with 15-s intervals. The sonicated suspension was then centrifuged, desalted in a PD10 desalting column (Amersham), and filter sterilized with a 0.2- $\mu\text{m}$  filter. Protein concentrations were determined with Bradford reagents (Sigma-Aldrich) used according to the manufacturer's instructions.

A mixture of 1.5 ml of cell extract, 0.89 mM L-tyrosine (sodium salt), and 10  $\mu\text{M}$  pyridoxal 5'-phosphate was incubated at 30°C. Samples (200  $\mu\text{l}$ ) were taken

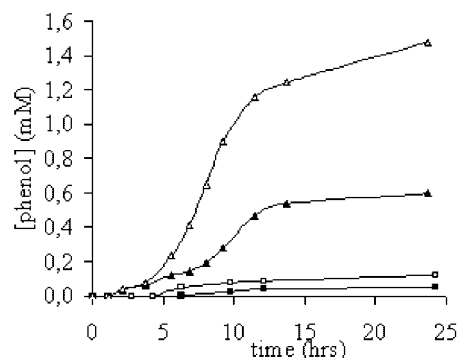


FIG. 1. Phenol production by different *P. putida* S12TPL strains in shake flask cultures in MMG with salicylate. ■, S12TPL; □, S12TPL1; ▲, S12TPL2; △, S12TPL3. The data points are the averages of duplicate experiments. The maximum variation between duplicates was less than 10%.

at regular intervals for colorimetric determination of phenol concentrations. The specific activity of TPL was determined by determining the quantity of phenol formed per gram of protein per minute.

## RESULTS

### Cloning and functional expression of *tpl* in *P. putida* S12.

The gene encoding TPL was cloned into expression vector pTn-1 under the transcriptional control of the salicylate-inducible promoter *NagR/pNagAa*. The resulting plasmid, pNW1, was transformed into *P. putida* S12 to obtain strain *P. putida* S12TPL. Enzyme assays were performed with cell extract of *P. putida* S12TPL to check for functional expression of the *tpl* gene. The specific activity in the first 15 min of the assay was  $1.5 \mu\text{mol g protein}^{-1} \text{ min}^{-1}$ .

After verification that there was functional expression of *tpl* in *P. putida* S12TPL, cultures of growing cells were assayed for phenol production from glucose. In this case production of phenol completely depended on de novo-synthesized tyrosine as the substrate for TPL. The strain was cultured in shake flasks in MMG with and without 5 mM tyrosine in order to determine the effect of tyrosine availability on phenol production. In the absence of tyrosine, *P. putida* S12TPL produced approximately 50  $\mu\text{M}$  phenol with a maximum specific activity ( $Q_{p, \text{max}}$ ) of  $0.1 \mu\text{mol g (dry weight)}^{-1} \text{ min}^{-1}$  (Fig. 1 and Table 2). However, in the presence of tyrosine, 330  $\mu\text{M}$  phenol ac-

TABLE 2. Production parameters for different phenol-producing *P. putida* S12TPL cultures

Strain	Method	Total phenol concn (mM)	Yield (mol/mol)	Maximum sp act ( $\mu\text{mol g [dry wt]}^{-1} \text{ min}^{-1}$ )
S12TPL	Shake flask	0.05	0.23	0.1
S12TPL1	Shake flask	0.13	0.60	0.38
S12TPL2	Shake flask	0.60	2.69	1.9
S12TPL3	Shake flask	1.48	6.67	2.65
S12TPL3	Fed batch	5.01	2.76	2.24
S12TPL3	Biphasic fed batch	9.20	ND <sup>a</sup>	ND <sup>b</sup>

<sup>a</sup> ND, not determined due to octanol consumption.

<sup>b</sup> ND, not determined.

accumulated in the medium, indicating that tyrosine availability in *P. putida* S12TPL indeed limited phenol production.

**Overexpression of *aroF-1* and generation and screening of a library of transposon mutants for enhanced phenol production.** The expression of *aroF-1*, a key metabolic gene in the tyrosine biosynthetic pathway, was increased in order to improve tyrosine availability in *P. putida* S12TPL. The *aroF-1* gene from *P. putida* S12 exhibited 99% sequence similarity to the *aroF-1* gene of *P. putida* KT2440. It presumably encodes a 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase (EC 2.5.1.54) that is sensitive to feedback inhibition by tyrosine. The gene was placed under control of the *NagR/pNagAa* promoter and conjugated to *P. putida* S12TPL on the transposon donor pTnAroF-1 by triparental mating. This procedure yielded a library of 11,000 mutants that carried the TnAroF-1 transposon integrated at random sites in the genome, as confirmed by Southern analysis of 20 randomly selected mutants (data not shown). These 20 mutants were also tested for phenol production in MMG with salicylate. Eighteen of the mutants accumulated on average 40% more phenol than the parent, *P. putida* S12TPL, accumulated.

Obviously, the site of integration of the transposon can greatly influence the biosynthesis of tyrosine. Elimination of side reactions connected to the tyrosine biosynthetic pathway could have a positive effect on the metabolic flux toward tyrosine. Also mutations that (partially) eliminate tyrosine degradation could potentially arise.

The transposon library was colorimetrically assayed for the production of phenol in MMG with salicylate in microwell plates. Using this approach, 50 mutants that showed a significant increase in red color formation were obtained. Phenol production was verified by HPLC, and chromosomal integration of the TnAroF-1 transposon was demonstrated by Southern blotting. One mutant, *P. putida* S12TPL1, was selected for further analysis. Chromosomal DNA flanking the transposon was sequenced in order to determine the site of integration of the TnAroF-1 transposon in *P. putida* S12TPL1. It was found that the transposon disrupted a gene with 97% sequence similarity to the *oprB* gene of *P. putida* KT2440. Apparently, the combined introduction of *aroF-1* and disruption of *oprB* contributed greatly to productivity.

*P. putida* S12TPL1 accumulated 134  $\mu\text{M}$  phenol during shake flask cultivation in MMG with salicylate, with a  $Q_{p, \max}$  of  $0.38 \mu\text{mol g (dry weight)}^{-1} \text{min}^{-1}$  (Fig. 1 and Table 2). This production is more than double the production of *P. putida* S12TPL. However, in MMG with salicylate and 5 mM tyrosine, *P. putida* S12TPL1 accumulated 617  $\mu\text{M}$  phenol in the culture medium, indicating that tyrosine availability still limited phenol production in this strain. Furthermore, it was observed that addition of 5 mM phenylalanine had a similar effect. It is generally known that phenylalanine is converted into tyrosine in pseudomonads (4).

**Further enhancement of phenol production by NTG mutagenesis combined with antimetabolite selection.** In order to enhance de novo synthesis of phenylalanine and tyrosine, a combination of NTG mutagenesis and selection on the toxic analogue MFP or MFT was used. Previously, the use of these so-called antimetabolites has facilitated selection of mutants with increased metabolic flux toward phenylalanine and tyrosine (16, 32).

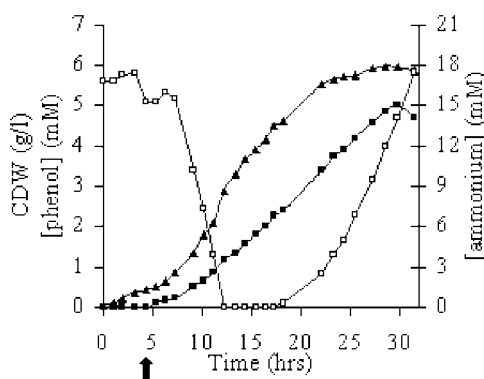


FIG. 2. Phenol production during typical fed-batch cultivation of *P. putida* S12TPL3. Glucose was used as the sole carbon source. The arrow indicates the time that feeding was started.  $\blacktriangle$ , cell concentration;  $\blacksquare$ , phenol concentration;  $\square$ , ammonium concentration.

*P. putida* S12TPL1 cells were treated with NTG, which resulted in a 20% survival rate. Mutants obtained in this way were preselected on mineral medium with fructose containing 100 mg/liter MFP or 10 mg/liter MFT. A library of approximately 2,500 MFP-resistant mutants and 2,500 MFT-resistant mutants was colorimetrically screened for enhanced phenol production in MMG with salicylate in microwell plates. Fifteen mutants were selected, and phenol production in shake flask cultures in MMG with salicylate was monitored by HPLC analysis. Mutant *P. putida* S12TPL2 accumulated the highest levels of phenol (0.6 mM), with a  $Q_{p, \max}$  of  $1.9 \mu\text{mol g (dry weight)}^{-1} \text{min}^{-1}$  (Fig. 1 and Table 2). This mutant came from the MFP-resistant library, but it was also resistant to 10 mg/liter MFT.

Subsequently, *P. putida* S12TPL2 was subjected to another round of NTG mutagenesis (survival rate, 50%) and preselection on fructose medium with 100 mg/liter MFT. The resulting library of 8,000 mutants was screened by using a method similar to the method used for the library described above, which resulted in selection of strain *P. putida* S12TPL3, which produced 1.5 mM phenol in MMG with salicylate with a  $Q_{p, \max}$  of  $2.65 \mu\text{mol g (dry weight)}^{-1} \text{min}^{-1}$  (Fig. 1 and Table 2). This strain converted glucose to phenol with a yield (mol/mol) of 6.7%.

**Phenol production by *P. putida* S12TPL3 during fed-batch cultivation.** *P. putida* S12TPL3 was grown in fed-batch cultures with glucose as the sole carbon source in order to monitor phenol production in a more controlled fashion. The feed medium composition and feed rate were chosen so that nitrogen limitation occurred during fermentation. Carbon was present in the fermentor during the entire course of the fermentation, in the form of glucose, gluconate, or 2-ketogluconate. As reported previously, the latter two compounds were formed by oxidation of glucose (32). After 30 h of cultivation 5 mM phenol had been produced, and the total yield was 2.8% (Fig. 2 and Table 2). Ammonium started to accumulate in the culture fluid when the phenol concentration reached approximately 2.4 mM, despite a medium composition that should have resulted in nitrogen limitation. Phenol in the fermentor became increasingly toxic at concentrations above 2.4 mM, and at a final concentration of 5 mM growth was completely halted



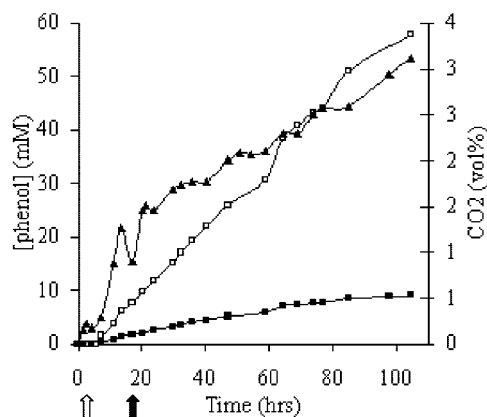


FIG. 3. Phenol production during typical biphasic fed-batch cultivation of *P. putida* S12TPL3 under nitrogen-limiting conditions. The solid arrow indicates the time that feeding was started, and the open arrow indicates when octanol addition was started. □, phenol concentration in the octanol phase; ■, total phenol concentration, calculated as described in Materials and Methods; ▲, percentage of CO<sub>2</sub> (vol/vol) in the effluent gas.

(Fig. 2). Accumulation of ammonium and inhibition of growth did not occur when *P. putida* S12TPL3 was cultured under similar conditions in the absence of the inducer salicylate, when no phenol was produced (data not shown). Apparently, the toxicity of phenol hampered its production.

#### Phenol production in a biphasic medium-octanol system.

From the results described above we concluded that for efficient production, removal of phenol from the culture is imperative. This led to fed-batch cultivation of *P. putida* S12TPL3 in a biphasic system in which octanol was an extractant. The conditions were comparable to the conditions used for the single-phase fed-batch cultivation, with the following exception. The extractant was introduced during the batch phase by pumping 400 ml octanol into the fermentor at a constant rate for 1 h. This resulted in a lag phase of 2 h, which was likely caused by the toxic effects of octanol (Fig. 3). Glucose, gluconate, or 2-ketogluconate was present in the fermentor throughout the fermentation. In a separate batch experiment we noted that octanol was degraded by *P. putida* S12 in the presence of glucose, implying that octanol may also have been a source of carbon in this fed-batch culture. The total phenol concentration in this experiment reached 9.2 mM. The final phenol concentration in the octanol phase was 58 mM, while 1.7 mM phenol accumulated in the water phase (Fig. 3 and Table 2). These results suggested that the partition coefficient between octanol and the aqueous medium was 34.1, which is in good agreement with the logarithm of the partition coefficient of phenol for an octanol-water system ( $\log P_{o/w}$ ) reported previously for phenol (29). During the feeding phase, no ammonium was present in the culture, indicating that the toxic effects of phenol and octanol were not limiting production.

## DISCUSSION

In this study, a *P. putida* S12 derivative was constructed that efficiently converts glucose into phenol through the conversion of de novo-synthesized tyrosine by the enzyme tyrosine phenol

lyase. Both the availability of tyrosine in the production host and product inhibition were critical issues that had to be addressed for optimization of this process.

Overexpression of *aroF-1* resulted in an increase in phenol production in strain *P. putida* S12TPL1. DAHP synthases have been used successfully for overproduction of other products of the tyrosine biosynthetic pathway (6, 12, 16). In most of these cases, a mutated form of the enzyme that was insensitive to feedback inhibition by aromatic amino acids was used. In this study, the native form of the enzyme was used since *P. putida* S12TPL was capable of scavenging excess tyrosine by converting it into phenol. Additionally, *P. putida* utilizes tyrosine as a sole carbon source (4; data not shown) and thus is capable of rapidly degrading any excess tyrosine. These two factors keep intracellular tyrosine concentrations low, thus minimizing feedback inhibition of the DAHP synthase.

Tyrosine biosynthesis in *Pseudomonas* strains is very complex, and it is tightly regulated to prevent overproduction of tyrosine (9, 18). This puts a heavy constraint on the availability of tyrosine for phenol production. In *E. coli*, the phenylalanine and tyrosine biosynthesis pathways have been extensively studied, which allowed an approach based solely on targeted genetic alteration (12, 17). For *P. putida*, however, such information is not available. This led us to use an unprejudiced strategy for strain optimization by means of random mutagenesis and high-throughput screening. Due to the nature of the mutations introduced by NTG, millions of mutants would have had to be screened in order to find one mutant with increased phenol production. Selection on aromatic amino acid analogues meant that only several thousand mutants had to be screened, showing that these fluoro analogues are highly suitable for selection of mutants with increased flux through the tyrosine biosynthetic pathway. Growth inhibition by aromatic amino acid analogs in pseudomonads is very dependent on the type of carbon source used (10). This is why antimetabolite selection took place on mineral medium with fructose. Also, we found that the ability of *P. putida* S12TPL3 to utilize tyrosine as a sole carbon source was disrupted (data not shown), indicating that this screening method also affected the tyrosine catabolic pathway.

Since TPL is inhibited by relatively low concentrations of phenol ( $K_i$ , 36  $\mu$ M) (28), it is essential to keep the concentration of phenol inside the cell below the inhibitory concentrations. The solvent-tolerant properties of *P. putida* S12, specifically the active secretion of solvents, should prevent accumulation of intracellular phenol. Genetic analysis of the first-generation mutant *P. putida* S12TPL1 showed that there is a mutation in a locus homologous to the *oprB* gene of *P. putida* KT2440. This gene encodes porin B, a glucose transport porin (40) which is part of a high-affinity glucose uptake system (2, 47). As suggested by Wylie and Worobec (48), this uptake system may, apart from glucose, transport other compounds that contain a hydroxyl group. Thus, phenol may reenter the cell via porin B and hence contribute to TPL inhibition. This mechanism should be absent from mutant *P. putida* S12TPL1 and its derivatives.

Besides the specific inhibition of TPL, the bacteria also have to cope with the general toxicity of phenol. *P. putida* S12 and *P. putida* S12TPL3 are equally tolerant to phenol and grow in mineral medium in the presence of 12 mM phenol in shake

flasks (data not shown). In fed-batch culture, under phenol-producing conditions *P. putida* S12TPL3 tolerated only 5 mM phenol. This apparent diminished tolerance could have been due to the altered conditions in the fermentor. By virtue of its log  $P_{o/w}$  phenol accumulates to high concentrations in the bacterial membrane (43), which causes the bacteria to be more sensitive to shear stress caused by the agitation in a fermentor. Also, it could be argued that phenol produced within the cell results in a faster increase in the intracellular phenol concentration than externally added phenol.

Octanol was applied as a second phase to counteract the inhibitory effects of phenol. Previously, the scavenging of product from the aqueous phase has been used effectively to increase production by decreasing product toxicity (11, 14, 33, 42, 46). Octanol has favorable physicochemical properties, and phenol (log  $P_{o/w}$ , 1.46) partitions readily into this solvent. Octanol, in spite of its toxicity, was previously shown to be a suitable extractant for solvent-tolerant *P. putida* S12 production hosts (45). However, octanol is not tolerated by most bacteria. Even solvent-tolerant species have been reported to cope poorly with this compound in a biphasic system (38).

In the biphasic fed-batch cultivation system used in this study, production proceeded for a long time, since the phenol concentration in the aqueous phase remained relatively low. The total concentration of phenol produced in the biphasic system was almost twice as high as the concentration in the single-phase fed-batch culture; 58 mM phenol accumulated in the octanol phase. Here, the limits of the system were not reached. Based on the results for the single-phase system, we anticipate that accumulation of phenol to a concentration of at least 2.4 mM in the aqueous phase and thus a phenol concentration of 82 mM in the octanol phase are feasible. This high concentration should greatly facilitate downstream processing.

Octanol was degraded by *P. putida* S12TPL3 at a rate of approximately 0.3 mmol g (dry weight)<sup>-1</sup> h<sup>-1</sup> in a batch culture in MMG supplemented with octanol. In the same culture, glucose was degraded at a rate of 5.7 mmol g (dry weight)<sup>-1</sup> h<sup>-1</sup>. This indicated that approximately 7% of the phenol produced came from octanol rather than from glucose. Extrapolating these data to the biphasic fed-batch system, the estimated yield for phenol is 4.7% (C mol of phenol per C mol of total carbon source).

The process described here is one of the first processes involving "green" production of a bulk chemical as toxic as phenol. Although the current production rates and yield are not high enough that the process is economically feasible, the experiments demonstrated that the bioconversion of glucose into a compound as toxic as phenol is feasible. In order to further improve production, the mutants obtained in this study will be analyzed by comparative transcriptomics. This should provide insight into the changes brought about by the optimization process and should offer key leads for further targeted optimization of the production host. The analysis of *P. putida* S12TPL3 also could enable construction of production hosts for a wide range of other, more valuable chemicals, such as coumaric acid (35) and *p*-hydroxystyrene (8). Since the *tpl* gene in *P. putida* S12TPL3 is plasmid borne, it could be easily replaced by other genes that can convert tyrosine into other compounds of interest. The production of such chemicals

could lead to an economically viable process in a short time (41).

In short, our results indicate that *P. putida* S12 can be used as a biocatalyst for the conversion of glucose into phenol. The combination of targeted genetic modification, random mutagenesis, antimetabolite selection, and high-throughput screening was effective for optimizing the production host. The use of the biphasic medium-octanol system greatly reduced the toxic effects of phenol, increasing production and facilitating downstream processing. This study shows that the use of solvent-tolerant pseudomonads is clearly advantageous for the production of toxic chemicals in biphasic biotransformation processes. In the future, this work may pave the way for "green" production of a whole new range of chemicals that can be of great economic, as well as environmental, benefit.

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