A Novel Gene, Encoding 6-Hydroxy-3-Succinoylpyridine Hydroxylase, Involved in Nicotine Degradation by *Pseudomonas putida* Strain S16

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Previous research suggested that *Pseudomonas* spp. may attack the pyrrolidine ring of nicotine in a way similar to mammalian metabolism, resulting in the formation of pseudooxynicotine, the direct precursor of a potent tobacco-specific lung carcinogen. In addition, the subsequent intermediates, 6-hydroxy-3-succinoylpyridine (HSP) and 2,5-dihydroxypyridine (DHP) in the *Pseudomonas* nicotine degradation pathway are two important precursors for drug syntheses. However, there is little information on the molecular mechanism for nicotine degradation via the pyrrolidine pathway until now. In this study we cloned and sequenced a 4,879-bp gene cluster involved in nicotine degradation. Intermediates N-methylmyosmine, pseudooxynicotine, 3-succinoylpyridine, HSP, and DHP were identified from resting cell reactions of the transformant containing the gene cluster and shown to be identical to those of the pyrrolidine pathway reported in wild-type strain *Pseudomonas putida* S16. The gene for 6-hydroxy-3-succinoylpyridine hydroxylase (HSP hydroxylase) catalyzing HSP directly to DHP was cloned, sequenced, and expressed in *Escherichia coli*, and the purified HSP hydroxylase (38 kDa) is NADH dependent. DNA sequence analysis of this 936-bp fragment reveals that the deduced amino acid shows no similarity with any protein of known function.

Nicotine, as one of the most biologically active chemicals in tobacco smoke and smokeless tobacco, is well known to be harmful to human health for its ability of crossing biological membranes and blood-brain barrier easily (33). Recently, Hecht et al. provided definitive evidence for mammalian 2'-hydroxylation of nicotine into pseudooxynicotine, from which 4-(methylamino)-1-(3-pyridyl)-1-butanone (NNK), a potent tobacco-specific lung carcinogen (17, 18), is formed. In addition, during the manufacture and processing of tobacco products, powdery solid or liquid wastes are generated in high concentrations of nonrecyclable nicotine as the main toxic compound (8, 15). Due to its heterocyclic structure, nicotine is soluble in water and can be easily transported to groundwater (11, 22). Considering its harmful effects, nicotine was classified as a “toxic release inventory” chemical by the U.S. Environmental Protection Agency in 1994 (25). Therefore, detoxification of these tobacco wastes by removal of nicotine is a major challenge. Several microorganisms have been used to degrade and detoxify tobacco wastes (11, 39), and the pathway of nicotine degradation in *Arthrobacter* is the most thoroughly analyzed. The pyridine pathway is preferred in *Arthrobacter*, which starts with the hydroxylation in the 6-position of the pyridine ring and subsequently splits the pyrrolidine ring. Related metabolic mechanisms of this strain have been elucidated at the molecular and enzymatic level (1–6, 9, 10, 16, 20, 29, 32). In contrast, it was suggested that the pyrrolidine ring was initially attacked in the *Pseudomonas* pathway. Nicotine was degraded to N-methylmyosmine (P); the pyrrolidine ring was then opened with the formation of pseudooxynicotine and, subsequently, the pyridine ring was hydroxylated at the 6-position (Fig. 1). However, there is relatively little published information on the molecular biology of this process (6, 12, 21). In 1979, Thacker et al. reported that nicotine-nicotinate degradation pathways were based on a transmissible plasmid. The nicotine degradation plasmid could give rise to an independent fertility factor T, which was capable of mobilizing chromosomal genes and a nontransmissible NIC structural gene when the plasmid was transferred from *Pseudomonas convexus* Pc1 to *Pseudomonas putida* PpG1 (37, 38). It is necessary to investigate the genetic mechanism in *Pseudomonas* since the pyrrolidine pathway is different from the pyridine pathway and the genetic and enzymatic mechanisms are distinct.

Previously, a soil-isolated bacterium, *Pseudomonas putida* S16 was reported to be capable of utilizing nicotine as its sole source of carbon, nitrogen, and energy (39). The nicotine degradation pathway of strain S16 was proposed based on the identification of intermediates produced by resting cells and crude extracts (41). Our efforts were directed toward confirmation of the pyrrolidine pathway in strain S16 by comprehensive characterization of the intermediates P, pseudooxynicotine, 3-succinoylpyridine (SP), 6-hydroxy-3-succinoylpyridine (HSP), and 2,5-dihydroxypyridine (DHP) (40, 41). Knowledge of the genes involved in nicotine metabolism in *Pseudomonas* will have applications for detoxification of the tobacco wastes.

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and synthesis of useful products of pharmaceutical importance. To achieve such goals, an in-depth understanding of the molecular biology in nicotine catabolism is required. A key step in the nicotine catabolism by strain S16 is the conversion of HSP into DHP. In this report, we cloned, sequenced, and characterized the novel gene involved in the latter stage of nicotine catabolism, from HSP to DHP, in *P. putida* S16.

**MATERIALS AND METHODS**

**Chemicals.** L-(-)-Nicotine (≥99% purity) was purchased as a free base from Fluka Chemie GmbH (Buchs Corp., Switzerland). DHP was purchased from SynChem OHG (Kassel Corp., Germany). Succinic semialdehyde purchased from Sigma (Germany) was used as a standard. HSP was isolated and purified from the broth of nicotine metabolized by strain S16 and served as a standard in the present study (40, 41). All other reagents were of analytical grade and commercially available.

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in the present study are shown in Table 1.

**Media and culture conditions.** *P. putida* S16 was isolated and cultured as previously described (39, 40, 41). *E. coli* cells were grown at 37°C in Luria-Bertani (LB) medium, and ampicillin or kanamycin was used at appropriate concentrations.

**DNA manipulation and DNA sequence analysis.** Genomic DNA was isolated from strain S16 by using the Wizard Genomic DNA purification kit (Promega Corp., Madison, WI). Restriction endonucleases and T4 DNA ligase were used according to the manufacturer’s instructions (Promega). Purification of PCR products was performed with a Wizard Plus Minipreps DNA purification system (Promega). Isolation of DNA fragments from agarose gels was accomplished with the Qiaex II gel extraction kit (Qiagen Corp., Germany). Digestions with restriction endonucleases, ligations, and transformations were performed according to standard procedures (30). Sequencing was performed by using an ABI sequencer by Shanghai Invitrogen Biotechnology Co., Ltd, China. The sequences were determined by complete sequencing of both strands and analyzed with DNA-Star (version 5) and Vector NTI DNA analytical software (version 8). Homology searches were performed with the BLAST programs at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST.html).

**Genomic library construction and screening.** Strain S16 genomic DNA was partially digested with the restriction enzyme Sau3AI. The products were separated by electrophoresis on a 0.8% agarose gel, and then the DNA fragments in 3 to 6 kb were isolated, purified, and ligated to BamHI-digested pUC19 cloning vector. The ligation mixture was transformed into *Escherichia coli* DH5α cells. White colonies were selected on LB agar plates containing IPTG (isopropyl-D-thiogalactopyranoside; 20 μg ml⁻¹) and X-Gal (5-bromo-4-chloro-3-indolyl-

![FIG. 1. Proposed pathway of nicotine degradation by *P. putida* S16.](image)

### TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Genotype or phenotype <em>a</em></th>
<th>Source or references</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
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<tr>
<td><em>P. putida</em> S16</td>
<td>Wild-type Nic⁺</td>
<td>39, 40, 41</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>F⁻ Δ(b80lacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(ric− mcr−) phoA supE44 thi-1 gyrA96 relA1)</td>
<td>TaKaRa</td>
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<tr>
<td><em>E. coli</em> BL21(DE3)</td>
<td>F⁻ ompT hsdSB(rK− mK−) dcm gal λ(DE3)</td>
<td>Invitrogen</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<td></td>
</tr>
<tr>
<td>pUC19</td>
<td>Ap⁺</td>
<td>TaKaRa</td>
</tr>
<tr>
<td>pMD18-T</td>
<td>Ap⁺</td>
<td>TaKaRa</td>
</tr>
<tr>
<td>pET-27b (+)</td>
<td>Ka⁺</td>
<td>Merck</td>
</tr>
<tr>
<td>pUC19-GTPF</td>
<td>Ap⁺, Nic⁺, pUC19 with BamHI insert of <em>P. putida</em> S16 (4,879 bp)</td>
<td>This study</td>
</tr>
<tr>
<td>pMD18-ORF1</td>
<td>Ap⁺, pMD18-T with EcoRI-PstI insert from ORF1 (2,230 bp)</td>
<td>This study</td>
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<tr>
<td>pMD18-ORF2</td>
<td>Ap⁺, pMD18-T with PstI-EcoRI insert from ORF2 (936 bp)</td>
<td>This study</td>
</tr>
<tr>
<td>pMD18-ORF3</td>
<td>Ap⁺, pMD18-T with EcoRI-SalI insert from ORF3 (581 bp)</td>
<td>This study</td>
</tr>
<tr>
<td>pET27b-hsp</td>
<td>Ka⁺, pET-27b with EcoRI-Xhol insert from ORF2 (936 bp)</td>
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</tr>
<tr>
<td>pMD18-W1R2F</td>
<td>Ap⁺, pMD18-T with EcoRI-PstI insert from pUC19-GTPF (2,840 bp)</td>
<td>This study</td>
</tr>
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*Ap⁺, ampicillin resistance; Ka⁺, kanamycin resistance; Nic⁺, growth on nicotine.*
β-D-galactopyranoside; 20 μg ml−1. Subsequently, recombinants were screened on plates containing nicotine as the sole carbon and nitrogen source.

**Nicotine bioavailability assay.** E. coli DH5α cells harboring recombinant plasmids were incubated in 500-ml flasks/chant with 100 ml of LB medium containing 30 mg of ampicillin liter−1. After 1 h of vigorous shaking at 37°C, cells were harvested by centrifugation (7,000 × g for 5 min at 4°C) and washed twice with 50 mM phosphate-buffered saline (PBS; pH 7.0). The cells were then resuspended in 10 ml of the same buffer (6.5 g of dry cell weight per liter). A 20-μl aliquot of nicotine stock solution (500 g liter−1) was added to each batch of resting cells. The cell suspension was sampled during the reaction, the cells were removed by centrifugation at 10,000 × g for 10 min at 4°C, and the supernatant was used for analysis.

**General analytical methods.** The qualitative analysis of nicotine, P, SP, HSP, and DHP was performed by using analytical thin-layer chromatography (TLC) according to published procedures (40, 41). Quantitative data of SP, HSP, and DHP were obtained by high-performance liquid chromatography (HPLC) analysis comparing the retention times and peak areas with those of standards. HPLC analysis was performed on an Agilent 1100 series (Hewlett-Packard Corp.) equipped with a KR100-5 C18 column (column size, 150 by 4.6 mm; particle size, 5 μm; Kromasil Corp., Sweden) and a UV detector at 207 nm. The mobile phase was a mixture of methanol–1 mM H2SO4 (25:75 [vol/vol]) at a flow rate of 0.5 ml min−1.

The structural identifications of P, SP, HSP, DHP, succinic semialdehyde, and succinic acid were performed by liquid chromatography-mass spectrometry (LC-MS) or gas chromatography-MS (GC-MS). Samples were thawed and centrifuged to remove insoluble particles. The supernatants were evaporated to dryness under nitrogen, and the residues were dissolved in chloroform. The supernatants were subjected to direct-injection mass spectrometry analysis on an API 4000 LC-MS system (Applied Biosystems, Foster City, CA). MS analysis was performed on an API 4000 mass spectrometer under both negative- and positive-ion turbo ion spray ionization mode. GC-MS analysis of P, SP, HSP, and DHP was performed as previously reported (41).

**Localization and subcloning of the gene for HSP degradation.** A positive transformant designated GTFP contains the nicotine degradation gene cluster from the genomic library of strain S16. Different DNA fragments designated open reading frame 1 (ORF1), ORF2, ORF3, nic3, W1R1F, and W1R2F were amplified from the sequence of GTFP by PCR. These fragments were subcloned into pMD18-T vector (TaKaRa) in both orientations and transformed into E. coli DH5α. Then, HSP (1 g liter−1) was added to the resting cells (6.5 g of dry cell weight liter−1), and the transformants that could transform HSP to DHP were selected and the transformant designated GTPF exhibiting nicotine hydroxylase activity was obtained (Fig. 2) (41).

**Hybridization and PCR analysis.** Primers were designed to incorporate an EcoRI site in the forward primer and a Sall site in the reverse primer. The primers for hsp with restriction sites underlined were as follows: forward, 5'-GGGATCCATGCGAAGAAAGCTT-3' (corresponding to positions 1081 to 1095), and reverse, 5'-TTCATTGCGAGGACACTCTTGT-3' (positions 2016 to 2020). The PCR was performed with rTaq DNA polymerase (TaKaRa Co., Ltd., China).

A DNA probe was prepared by randomly labeling the PCR product ORF2 with digoxigenin-11-UTP using a DIG DNA labeling and detection kit (Roche Corp., Germany). The genomic DNA of strain S16 was partially digested by Ssa31A1 and completely digested by EcoRI and then separated on a 0.8% agarose gel and transferred to Hybond-N+ nylon membrane (Amersham Corp., United Kingdom). Hybridization was performed according to the suggested protocol of the random primers DNA labeling system (Roche).

**Expression and purification of His6-tagged HSP hydroxylase.** The hsp gene was amplified by PCR from strain S16 genomic DNA with PrimeSTAR HS DNA polymerase (TaKaRa). Primers were designed to incorporate an EcoRI site in the forward primer and an Xhol site in the reverse primer. The primers for hsp, with restriction sites underlined, were as follows: forward, 5'-GGGATCCATGCGAAGAAAGCTT-3' (corresponding to positions 1081 to 1095), and reverse, 5'-TTCATTGCGAGGACACTCTTGT-3' (positions 2016 to 2020). The PCR product of hsp was digested with EcoRI and Xhol and ligated into pET27b (+) (Novagen). Transformed E. coli BL21 (DE3) cells with hsp were incubated in 500-ml flasks/chant with 100 ml of LB medium containing 100 mg of kanamycin liter−1 until an optical density at 600 nm of 0.5 was reached. IPTG was then added to a final concentration of 1 mM, and the culture was incubated for up to 6 to 12 h at 30°C for protein expression.

The induced E. coli cells were washed and resuspended in binding buffer (20 mM PBS, 100 mM NaCl, 10 mM imidazole [pH 7.4]). The protease inhibitor phenylmethanesulfonyl fluoride was added to the cell suspension to a final concentration of 0.1 mM. This suspension was disrupted by sonication in an ice-water bath. Cell debris and unbroken cells were removed by centrifugation (14,000 × g for 20 min at 4°C). The supernatant was applied to a column of Ni-NTA agarose (Qiagen), which had been equilibrated with the binding buffer. After a wash with 50 ml of wash buffer (20 mM PBS, 100 mM NaCl, 20 mM imidazole [pH 7.4]), His6-tagged HSP hydroxylase was eluted from the column with elution buffer (20 mM PBS, 100 mM NaCl, 10 mM imidazole [pH 7.4]). All subsequent chromatographic steps were performed with a fast protein liquid chromatography system (Amersham Corp., Sweden) at 4°C. InVision His tag-in-gel stain (Invitrogen) was used to determine the purity of HSP hydroxylase.

**SDS-PAGE.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method by Laemmli (23) on a Mini-Protein III electrophoresis cell (Bio-Rad, Hemel Hempstead Corp., United Kingdom) using 12.5% polyacrylamide gels. Coomassie blu R-250 was used to visualize proteins.

**Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS analysis of HSP hydroxylase.** In-gel tryptic digestion was performed. Excised gel plugs were placed in 96-well plates. The plugs were stored in 50 mM NH4HCO3 buffer and treated in the following steps: wash with 20 μl of 50% acetonitrile, reduction with 20 μl of 10 mM dithiothreitol at 56°C for 1 h, alkylation with 55 mM iodoacetamide, washing with 50% of acetonitrile, and digestion with trypsin for 10 h at 37°C (Sigma Corp.). Peptides were extracted from the gel plug with 1% formic acid-2% acetonitrile. Digests were spotted onto a target as previously reported (13). Spectra were acquired on a 4700 MALDI TOF/TOF mass spectrometer (Applied Biosystems).

**Enzymatic assay on cell extracts.** Cells of transformants containing pET27b-hsp plasmids were harvested by centrifugation (7,000 × g for 8 min at 4°C), washed with 50 mM PBS (pH 7.4) and resuspended in the same buffer to a 1.87 g of dry cell weight (equivalent) per liter. Cells were disrupted by sonication, and the insoluble fractions were removed by centrifugation (12,000 × g for 20 min at 4°C). HSP (1.0 g liter−1) was added to cell extracts containing HSP hydroxylase, and the enzyme activity was measured based on the increase of DHP by HPLC. HSP (1.0 g liter−1) and NADH (0.5 mM) were incubated with an appropriate quantity of purified HSP hydroxylase at 37°C for 1 h in 20 mM PBS buffer (pH 8.0), and the content of produced DHP was monitored by HPLC. The sample was acidified by the addition of 10 μl of HCl (6 M) and centrifuged to remove insoluble proteins.

**Nucleotide sequence accession number.** The nucleotide sequence reported in the present study has been deposited in the NCBI database under accession number DQ988162.

**RESULTS**

**Identification of genomic cluster containing nicotine degradation genes.** Nicotine degradation genes were isolated by transforming a genomic library of wild-type P. putida S16 into E. coli DH5α. Ampicillin-resistant colonies were selected and subsequently screened for nicotine degradation on plates containing nicotine as the sole carbon and nitrogen source (Fig. 2). A positive transformant designated GTFP exhibiting nicotine degradation activity was obtained (Fig. 2). The DNA fragment (4,879 bp) from the clone designated the nic gene cluster, which was supposed to encode enzymes for nicotine degradation, was isolated and sequenced. Resting cells of GTFP were performed to certify the ability of degrading nicotine and HSP. After 9 h, the initial 2.75 g of nicotine liter−1 present was broken down completely, and the intermediates P, SP, HSP, and DHP, which were shown to be identical to those produced by the wild-type strain S16, were detected (Fig. 3) (41). In a similar reaction, HSP was completely degraded in 10 h and, importantly, the subsequent product of DHP was also detected during the reaction (see Fig. S1 in the supplemental material). A resting cell reaction of E. coli DH5α (harboring pUC19 plasmids) was also subjected to the same conditions as a control. After 20 h, the concentration of nicotine or HSP in the mixture remained constant, and no product was detected.

**Mapping of nic gene cluster containing the gene encoding HSP hydroxylase.** After the nucleotide sequence of the 4,879-bp insert of pUC19 was determined, three large ORFs
(ORF1, ORF2, and ORF3) were proposed by computer analysis, which were linked in tandem as shown in Fig. 4. All three ORFs started with either valine or methionine and were more than 100 amino acids in length. ORF2 (936 bp) began with the start codon ATG at 1081 position and ended with the stop codon TAG at position 2017. The Shine-Dalgarno sequence (35) was found in the upstream region of the putative start codon of ORF2 (TGGAGTGCACAAAAAATG). The deduced amino acid sequence of ORF2 showed no identity with any protein of known function according to the results of BLASTx program. Immediate upstream of ORF2 was ORF3 (582 bp), and downstream of ORF2 was ORF1 (1,853 bp). No significant homology of the three ORFs with other degradative genes was observed. Therefore, subcloning reactions were conducted to identify the function of the degradation genes. Based on the prediction of ORFs and restriction enzyme digestions of nic

FIG. 2. (A) Transformant grown on a nicotine plate containing ampicillin and nicotine. Quadrants 1 and 2 showed the growth of transformant GTPF, and quadrant 3 was streaked with E. coli (pUC19) as a control. (B) Utilization of nicotine as the sole carbon and nitrogen source for growth by transformant GTPF and E. coli (pUC19). Symbols: ▲, cell growth of GTPF; ◼, cell growth of E. coli (pUC19); ■, nicotine concentration in the medium for transformant GTPF growth; □, nicotine concentration in the medium for E. coli DH5α (pUC19) growth as control. The values are means of three replicates, and the error bars indicate the standard deviations.

FIG. 3. (A) Degradation of nicotine by the resting cells of transformant GTPF and E. coli DH5α (pUC19) at 37°C in aqueous phase. Symbols: ■, removal of nicotine by transformant GTPF; ▲, removal of nicotine by E. coli DH5α (pUC19) as a control. The values are means of three replicates, and the error bars indicate the standard deviations. (B) TLC analysis of the products formed by incubation of nicotine and the whole cells of transformant GTPF at pH 7.0 from 0 to 8 h. M (marker), nicotine, SP, and HSP (1 g liter⁻¹) were the standards.
gene cluster, six subclones containing ORF1, ORF2, and ORF3 were ligated into pMD18-T vector in both orientations and transformed into E. coli DH5α (Fig. 4). The resting cells of these subclones were prepared to screen for their ability of degrading HSP. E. coli DH5α transformants individually containing pMD18-nic3, pMD18-W1R1F, and pMD18-ORF2 plasmids could degrade HSP in the culture (data not shown). For further investigation, the resting cells of transformant pMD18-ORF2 could degrade 1.0 g of HSP liter⁻¹ completely in 12 h, and DHP was produced in the mean time (data not shown). Therefore, we concluded that, in the gene cluster, ORF2 (hsp) was responsible for catalyzing HSP to DHP.

Hybridization and PCR analysis. Southern blot and PCR analysis were used to examine nic gene cluster encoding the nicotine-degrading enzymes derived from strain S16. After agarose electrophoresis and blotting, the hsp probes (ORF2 fragment) hybridized to the corresponding genomic DNA fragments of strain S16, and the DNA fragment with the gene cluster was ~5 kb in size (data not shown). The primers were then designed according to the full-length DNA fragments (1 to 4,879 bp), and the PCR products of strain S16 appeared as an ~5-kb fragment on agarose gels (data not shown). These results confirmed that the DNA inserts of pUC19 are derived from strain S16, and the genes are located in the 4,879-bp cluster.

Expression and purification of recombinant HSP hydroxylase. Expression experiments and enzyme assays were undertaken to study the function of hsp gene. Cellular proteins were analyzed by SDS-PAGE to be the predicted sizes when the pET27b-hsp constructs were expressed in E. coli BL21(DE3). A large amount of recombinant HSP hydroxylase was found in the cell lysate when the pET27b-hsp construct was expressed in E. coli upon IPTG induction. An ~38-kDa protein band was observed in the extract of the IPTG-induced E. coli BL21 (DE3) cells containing pET27b-hsp, whereas no activity or enhanced 38-kDa protein band was detected in controls where the expression vector contained no insert (Fig. 5A). The molecular mass of this protein was similar to the deduced molecular mass of expressed ORF2 (38 kDa). His₆-tagged HSP hydroxylase was purified with Ni-NTA affinity columns (Qiagen, Ltd., Crawley, United Kingdom) under non-denaturing conditions. The purity of the enzyme was confirmed by SDS-PAGE, and a single band was observed close to 40 kDa (Fig. 5B and C). The purified HSP hydroxylase was also confirmed by MALDI-TOF/TOF MS by using the peptide mass fingerprinting techniques on an array of peptide masses from enzymatic digestion of the protein isolated from an SDS-PAGE gel. The two tryptic digested peptides LGVVVTIPSG GANR (which had a Mascot ion score 56.1807) and EHLNS GELAAWTKPR (which had a Mascot ion score 36.0279) matched with the translation product of hsp gene.

Activity of recombinant HSP hydroxylase. The IPTG-treated cells containing pET27b-hsp plasmids were used to convert HSP. After sonication of the cells, the solution was centrifuged, and HSP was added to the supernatant to a final concentration of 1.0 g liter⁻¹. The incubation was carried out in 20 mM PBS (pH 7.0) with shaking at 37°C, DHP was detected by TLC and HPLC after 1 h, and then HSP was fully degraded in 20 h (see Fig. S2 and S3 in the supplemental material). Another transformation for HSP was performed on the purified HSP hydroxylase at an appropriate concentration in 20 mM PBS (pH 8.0) buffer. No enzyme activity or weak enzyme activity was observed without NADH, and HSP hydroxylase became active immediately upon the addition of NADH (Fig. 6), which indicated that the enzyme activity was NADH dependent.

DISCUSSION

The metabolic pathway of nicotine by strain P. putida S16 has been previously proposed based on the results of nuclear magnetic resonance, Fourier-transform infrared and UV spectroscopy, GC-MS, and high-resolution MS analyses (41). However, the pathway was not characterized at the genetic level. We report here the isolation and identification of the gene cluster for nicotine degradation in P. putida S16. HSP and DHP in the Pseudomonas pathway are two important precursors for synthesis of drugs such as analogues of epibatidine, an
extremely effective analgesic molecule, and tetrapyrroles with widespread application in cancer diagnosis and therapy. Bio-transformation with Pseudomonas sp. is a promising strategy to convert nicotine into valuable compounds, such as HSP and DHP, in efficient and environmentally friendly ways (24, 27, 34, 36, 40). The genetic organization of nicotine degradation gene cluster of strain S16 is distinctive and contains three ORFs. When nicotine, SP, or HSP was added to the resting cells of wild-type E. coli, the concentration of the starting material was constant, and no product was produced, which leads to the conclusion that the wild-type E. coli never contained genes for enzymes restoring the nicotine degradation pathway. Based on the analysis of pathway, the process began with an enzyme-catalyzed dehydrogenation, followed by spontaneous hydrolysis and then a repeat of the enzyme-catalyzed dehydrogenation to produce SP. Similar cases were found in Arthrobacter for L-6-hydroxynicotine oxidase or D-6-hydroxynicotine oxidase in the nicotine degradation pathway (5) and a monooxygenase catalyzing sequential dechlorinations of 2,4,6-trichlorophenol by oxidative and hydrolytic reactions (42). SP might then be hydroxylated at position 6 of the pyridine ring to generate HSP by a monooxygenase, and HSP was transformed to DHP by HSP hydroxylase.

In Pseudomonas species, HSP was proposed to be further metabolized by cleavage at the 3-position of HSP with the formation of DHP more than four decades ago (14, 22). Since then, however, a corresponding DHP-forming enzyme or its gene has been neither studied nor isolated. In the present study, we successfully cloned the hsp gene encoding HSP hydroxylase from P. putida S16. Since the nucleotide sequence of hsp showed no homology with any protein of known function, we scanned for any possible known motifs in this sequence (http://hits.isb-sib.ch/cgi-bin/PFSCAN) (Fig. 7). The following sites with known functions were predicted: three putative protein kinase C phosphorylation sites (amino acids 67 to 69, 72 to 74, and 238 to 240), one suspicious cAMP- and cGMP-dependent protein kinase phosphorylation site (amino acids 235 to 238), and one amidation site (amino acids 233 to 236). Interestingly, we also found a possible GGDEF domain (E-value = 0.014, pfam–fs), shown to be

FIG. 5. SDS-PAGE of expressed and purified His6-tagged HSP hydroxylase in E. coli BL21(DE3) on a 12.5% gel. (A) Lane M, protein weight marker (MBI); lanes 1 to 5, cell extracts of E. coli (pET27b–hsp) obtained at 4, 6, 8, 10, and 12 h, respectively, after IPTG induction; lane 6, cell extracts of E. coli containing the pET-27b(+) plasmid. Marker sizes are indicated on the left in kilodaltons. The molecular mass of the expressed protein (indicated by the boldfaced arrow) is about 38 kDa. (B) Coomassie stains of markers and the purified protein (lane 1, purified 38-kDa His6-tagged fusion protein). (C) Gel visualized with Invision His tag in-gel stain and imaged on a UV transilluminator equipped with a video camera (lane 1, purified 38-kDa His6-tagged fusion protein). The molecular mass markers are BenchMark His-tagged protein standard (Invitrogen) also indicated (in kilodaltons).

FIG. 6. Transformation of HSP to DHP by purified HSP hydroxylase. Symbols: ●, DHP concentration in the buffer containing only enzyme; ■, CK:DHP concentration in the buffer alone containing only substrates containing HSP and NADH; ▲, DHP concentration in the buffer containing enzyme and NADH.
homologous to the catalytic domain of adenylyl cyclase, which belongs to the alpha and beta proteins. Even though the catalytic domain has been found in many prokaryotic proteins, its function is still unknown in most of them. GGDEF-domain-containing proteins have been implicated in bacterial signal transduction and synthesis of the second messenger molecule cyclic-di-GMP, and a number of GGDEF proteins are involved in controlling the formation of extracellular matrices (26).

Generally, hydroxylase is a group of enzymes that catalyze oxidation reactions in which one of the two atoms of molecular oxygen is incorporated into the substrate and the other is used to oxidize NADH or NADPH (1, 19, 24). NADH was necessary for the cleavage of HSP, and this mechanism was similar to Hirschberg's and Nakano's reports. In conclusion, HSP hydroxylase might catalyze certain hydration process by cleavage at the 3-position of HSP with the formation of DHP and succinic semialdehyde (22, 41). Succinic semialdehyde could be then converted to succinic acid easily by succinic semialdehyde dehydrogenase, which is widely found in Pseudomonas (7, 28, 31). These reasons provide possible explanations for the sole detection of succinic acid in the resting cell experiments of P. putida S16.

In summary, our study is the report of a gene cluster involving in pyrrolidine pathway for nicotine degradation by a gram-negative Pseudomonas. The details of cloning, sequencing, and characterizing the novel gene participating in the latter stage of nicotine catabolism from HSP to DHP were also discussed. This study provides the basic knowledge for further insights of nicotine degradation in Pseudomonas. The function of the overall gene cluster has been under investigation in our group.

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