Characterization of environmentally friendly nicotine degradation by *Pseudomonas putida* biotype A strain S16

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Nicotine and some related alkaloids in tobacco and tobacco wastes are harmful to health and the environment, and a major environmental requirement is to remove them from tobacco and tobacco wastes. In this study, an isolated strain, S16, identified as *Pseudomonas putida* biotype A, was used to investigate nicotine degradation. Possible intermediates were identified based on the results of NMR, Fourier-transform (FT)-IR and UV spectroscopy, GC-MS and high-resolution MS (HR-MS) analysis. The pathway of nicotine degradation in *P. putida* was proposed to be from nicotine to 2,5-dihydroxypteridined through the intermediates N-methylmyosmine, 2'-hydroxynicotine, pseudooxynicotine, 3-pyridinebutanal, C-oxo, 3-succinolpyridine and 6-hydroxy-3-succinolpyridine. N-Methylmyosmine, 2,5-dihydroxypteridine and succinic acid were detected and satisfactorily verified for the first time as intermediates of nicotine degradation. In addition, an alcohol compound, 1-butanone, 4-hydroxy-1-(3-pyridyl), was found to be a novel product of nicotine degradation. These findings provide new insights into the microbial metabolism of nicotine and the environmentally friendly route of nicotine degradation.

INTRODUCTION

Nicotine, a major alkaloid synthesized as the L-isomer in tobacco plants, plays a critical role in smoking addiction. In China, 20% of the world’s population (1.2 billion people) consumes 30% of the world’s cigarettes. If current smoking patterns persist, tobacco will kill around 100 million Chinese in the next 50 years (Holden, 2001). In 2000, about 4.9 million smoking-related premature deaths occurred throughout the world. In the USA, tobacco use was responsible for nearly one in five deaths, or an estimated 440,000 deaths per year, in the period 1995–1999. Smoking accounts for at least 30% of all cancer deaths and 87% of lung cancer deaths (American Cancer Society, 2005). Currently, regulatory strategies to control the tobacco-induced disease epidemic are very much focused on nicotine. Reduced-nicotine cigarette products have been advocated to gradually lower the level of nicotine dependence (Benowitz & Henningfield, 1994). The American Medical Association has endorsed a public health strategy in which the nicotine level of tobacco would be forcibly reduced (Henningfield et al., 1998).

Nicotine is also a significant toxic waste product in tobacco production. The tobacco-manufacturing process and all activities that use tobacco produce solid or liquid wastes with high concentrations of nicotine (Novotny & Zhao, 1999). A non-recyclable, powdery, nicotine-containing waste is formed during tobacco production, which has an average nicotine content of 18 grams per kilogram dry weight. This waste is classified as ‘toxic and hazardous’ by European Union regulations when the nicotine content exceeds 500 milligrams per kilogram dry weight (Civilini et al., 1997). Therefore, there is a major environmental requirement to remove nicotine from tobacco wastes.

Nicotine degradation by micro-organisms has received increasing attention in the past 50 years because micro-organisms have the potential to reduce nicotine levels in tobacco and to detoxify tobacco wastes (Civilini et al., 1997; Wang et al., 2004). However, the current understanding of nicotine metabolism in micro-organisms is poor. Some bacteria, such as *Pseudomonas* sp. no. 41 (Wada & Yamasaki, 1954), *Pseudomonas convexa* PC1 (Thacker et al., 1978), *Arthrobacter oxidans* (Sguros, 1955), *A. oxidans* P-34 (Gherma et al., 1965; reclassified as *Arthrobacter ureafaciens* by Kodama et al., 1992), *A. oxidans* pAO1 (Decker & Bleeg, 1965; reclassified as *Arthrobacter nicotinovorans* by Kodama et al., 1992), etc., have been isolated and used to study nicotine degradation. Of these, *P. putida* biotype A, a well-known degrader of xenobiotics, is of special interest because of its ability to degrade many chlorinated and aromatic compounds.
et al., 1992) and *Achromobacter nicotinophagum* (Hylin, 1959), have been proposed to degrade nicotine, mainly via two different pathways (Kaiser et al., 1996). In the genus *Arthrobacter*, the pathway and related metabolic mechanism in the molecular biology of nicotine degradation have been thoroughly elucidated (Gherna et al., 1965; Brandsch et al., 1982; Schenk et al., 1998; Igoli & Brandsch, 2003). However, the nicotine-degradation mechanisms in *Pseudomonas* and other genera are less well documented. In *Pseudomonas* species, N-methylmyosmine, DHP, and succinic acid in nicotine degradation by *Pseudomonas* species has been reported, and no complete pathway could be reliably constructed. Furthermore, although the identification of the intermediates in earlier investigations was probably correct, the techniques used at the time did not conform to current standards of metabolite characterization.

In our previous publication (Wang et al., 2005), a ‘green’ route to HSP from the nicotine of tobacco waste employing whole cells of *Pseudomonas* sp. S16 was developed, and HSP was easily purified with a 43.8% (w/w) yield and characterized. However, other metabolites from nicotine have not yet been completely detected and identified. In this paper, we further describe the isolation, identification and characterization of the nicotine-degrading bacteria S16 and the possible intermediates of nicotine degradation. A proposed pathway for nicotine degradation by strain S16 is also postulated and discussed according to the results of NMR, Fourier-transform IR (FT-IR) and UV spectroscopy, GC-MS, and high-resolution MS (HR-MS) analysis, as is the transformation reaction by the cell-free extract.

**METHODS**

**Isolation and growth of bacteria.** A soil sample (0.5 g, wet weight) from a field under continuous tobacco cropping in Shandong, People’s Republic of China, was incubated with liquid medium containing (per litre) 13.3 g K2HPO4·3H2O, 4 g KH2PO4, 0.2 g MgSO4·7H2O and 0.5 ml trace elements solution. The trace elements solution contained (per litre) 13.3 g K2HPO4·3H2O, 4 g KH2PO4, 0.2 g MgSO4·7H2O and 0.5 ml trace elements solution. The trace elements solution contained (per litre) 0.1 M HCl 0.05 g CaCl2·2H2O, 0.05 g CaCl2·2H2O, 0.008 g MnSO4·H2O, 0.04 g FeSO4·7H2O, 0.1 g ZnSO4·0.1 g Na2MoO4·2H2O and 0.05 g Na2WO4·2H2O. Nicotine (≥99% purity, purchased as the free base using the BLASTN search program.

**Nicotine degradation by resting cells.** Cells were harvested in mid-exponential phase by centrifugation at 6000 g for 15 min at 4°C, and then washed three times with 0.05 M sodium phosphate buffer (pH 7.0). These cells were called resting cells. The degradation experiment was performed in a 5 flasks containing 3.73 mg dry cell weight (DCW) ml−1 of resting cells (OD620 = 6.10 OD620 units = 0.056 mg DCW ml−1). The DCW was produced by transformation reaction by the cell-free extract.

**General analytical methods.** During the course of bacterial growth or resting cell reaction, aliquots of the culture or cell suspension were sampled and the cells removed by centrifugation at 6000 g for 15 min at 4°C. The supernatant was used for GC, HPLC, UV and TLC analysis. GC and HPLC analysis was performed as previously described (Wang et al., 2005). Identification and quantitative data for nicotine, DHP and intermediates were obtained by comparing the retention time and peak areas of the unknown compounds with those of standards (DHP was from SynChem OHG) or of the intermediates of known concentration isolated and purified from this study. The supernatant was also diluted with 0.1 M HCl and scanned with a UV-3100 spectrophotometer (Shimadzu) to record the UV absorption spectra of the metabolites. TLC analysis was carried out as previously described (Wang et al., 2005), with a slight modification, using silica gel HSGF254 0.20 mm plates (Huanghai) and chloroform/ethanol/methanol/0.5 M NaOH (30:15:2:1.5, v/v) for development. The spots of the metabolites were examined under UV light (254 nm).

**Isolation of metabolites SP and HSP.** After incubation of nicotine and resting cells for ~3 h, several possible metabolites were found by TLC analysis (Fig. 1b), and metabolites SP and HSP accumulated. In order to isolate the two metabolites, the reaction was stopped by centrifugation at 12,000 g for 10 min at 4°C to remove the resting cells. A 11 volume of supernatant was evaporated at 50°C under reduced pressure to about 100 ml. To obtain metabolite SP, the condensate was adjusted to pH 4.0 with 1 M HCl, and extracted with chloroform. The chloroform phase was concentrated by evaporation and a white crystalline substance was obtained. To obtain metabolite HSP, the mother liquid after removing metabolite SP was evaporated again to about 50 ml, and 3 M HCl was added until the mixture became cloudy. From this mixture, a brick-red precipitate was obtained by filtration and drying. The two metabolites were purified by recrystallization.

**Identification of metabolites SP and HSP.** The identification of the metabolites SP and HSP was performed by UV, FT-IR, MS and NMR analysis. The UV spectra were recorded with a UV-3100 spectrophotometer (Shimadzu) in 0.1 M HCl and 0.1 M NaOH. FT-IR
Isolation and HR-MS analysis of metabolite A. In order to obtain metabolite A, the reaction was stopped by centrifugation to remove the resting cells after incubation for ~4 h; at that time metabolite A had accumulated to a maximum (Fig. 1b). The reaction solution was adjusted to pH 11.0 with 1 M NaOH and extracted with chloroform. The chloroform phase was concentrated and applied to preparative TLC on silica gel HSGF254 0.50 mm plates (Huanghai) employing chloroform/ethanol/methanol/0.5 M NaOH (30:15:2:1.5, v/v) for development. The fluorescent spots were detected under a UV light (254 nm). The band of metabolite A on the plate was scraped off and eluted with methanol and ultrason. After concentration, the eluate was analysed by GC-MS (Waters GCT mass spectrometer, coupled to an Agilent HP6890 gas chromatograph) to record the high-resolution mass spectrum of metabolite A. The system was equipped with a J&W DB-5MS column (0.25 mm internal diameter × 50 m length × 0.25 μm film thickness, Folsom). Chromatographic conditions were: 0.5 μl injection volume (splitless injection, 30:1); carrier gas, helium at a constant flow of 1.0 ml min⁻¹; temperature programme 50 °C for 2 min, then to 280 °C at a rate of 10 °C min⁻¹ for 10 min. The ionization energy was 70 eV, and the temperature was 280 °C with a mass-to-charge ratio of 20–600.

**GC-MS analysis for other metabolites.** During the nicotine-degradation experiment, the reaction mixture was sampled hourly. After removing the cells, the samples (25 ml) were evaporated to dryness at 50 °C under reduced pressure and dissolved in 2 ml acetonitrile. The acetonitrile solution (0.2 ml) was transferred to a vial and dried under a stream of nitrogen. The residue was silylated by addition of 100 μl bis(trimethylsilyl)trifluoroacetamide (BSTFA; Sigma-Aldrich) at 70 °C for 3 h. After drying under a stream of nitrogen, the sample was redissolved in acetonitrile. Two controls were silylated by the same procedure. One contained nicotine alone without resting cells, and the other contained resting cells without nicotine. The samples were analysed by GC-MS (GCD 1800C, Hewlett Packard) equipped with a 50 m J&W DB-5MS column (Folsom).

**Transformation of nicotine and its metabolites by a cell-free extract.** Cells were harvested in mid-exponential phase by centrifugation at 6000 g for 15 min at 4 °C, washed three times with 0.05 M sodium phosphate buffer (pH 7.0), then suspended in the same buffer and disrupted by ultrasonification in an ice/water bath. After centrifugation at 20000 g for 30 min at 4 °C, the clear supernatant was used as cell-free extract for degradation of nicotine, and the metabolites SP and HSP. All the reactions were performed in a 1 ml total volume at 30 °C with gentle shaking. Reaction mixtures contained 0.05 M sodium phosphate buffer (pH 7.0), an appropriate volume of cell-free extract (a final protein content of 0.3 mg ml⁻¹) and substrates at 0.3 mg ml⁻¹. The changes in the substrates and the formation of products were determined by UV spectroscopy, GC and HPLC after 30 min (10 min for the HSP degradation reaction).

**RESULTS**

**Characterization and identification of a nicotine-degrading bacterium**

A nicotine-degrading bacterium was isolated from soil samples obtained from a field under continuous tobacco cropping in Shandong, People’s Republic of China, and designated strain S16. It could use nicotine as the sole carbon, nitrogen and energy source. S16 grew rapidly and completely degraded nicotine within 10 h with a maximum biomass of 1.4 mg DCW ml⁻¹ (Wang et al., 2004).

Strain S16 was deposited at the China Center for Type Culture Collection (CCTCC; accession no. M 205038). It was a Gram-negative, mobile, rod-shaped bacterium (0.52–0.65 × 1.05–1.43 μm) with one or two flagella at one pole (data not shown). Its physiological characteristics (data not shown) were identical to those of *Pseudomonas putida* (Holt et al., 1994). S16 could grow on glucose, xylose, arabinose, citrate, galactose, rhamnose, glycerol, valine,
arginine, alanine, fructose, mannose, creatine and ethanol. However, it could not grow on lactose, sucrose, mannitol, sorbitol, trehalose, raffinose or inositol. Fatty acid analysis of S16 gave a 0.881 similarity index with *P. putida* biotype A in the TSBA (revision 4.10) database. The partial 16S rRNA gene sequence of S16 (1453 nt; GenBank accession no. AY574282) was determined. BLASTN search analysis revealed that the sequence showed high homology (≥99.0%) with those of *P. putida* (AB029257.1), *Pseudomonas monteilii* (AF064458.1), *Pseudomonas plecoglossicida* (AB009457.1), *P. putida* KT2440 (AE016774.1) and *P. putida* ATCC 12633T (AF094736.1). The results suggested that strain S16 was closely related to the genus *Pseudomonas*. Based on the comparative 16S rRNA gene sequencing, chemotaxonomy, and morphological and physiological data, we concluded that strain S16 belonged to the species *P. putida* biotype A.

**Nicotine degradation by resting cells of S16**

To detect the products of nicotine degradation, we carried out the experiments in two different media using resting cells of S16. When the degradation experiment was performed in 0.05 M sodium phosphate buffer (pH 7.0), nicotine was fully degraded in 5 h (Fig. 1a), while it took more than 8 h to completely decompose nicotine in deionized water (pH adjusted to 7.0 at the beginning of the reaction; see Fig. 1b). As nicotine was degraded in deionized water, the pH of the suspension dropped, and the low pH value of the mixture inhibited the reaction from proceeding. This suggested that acidic metabolites were formed by the reaction. Moreover, there were substantial changes in both the intensity and the wavelength of the absorption maximum of the UV absorption spectra of the reaction mixture in the degradation experiments (Fig. 1a). The altered UV absorption suggested the formation of metabolites. Furthermore, several possible metabolites were also found in the TLC analysis (Fig. 1b).

**Isolation and identification of metabolites SP and HSP**

Two principal metabolites, SP and HSP, were produced during the incubation reaction (Fig. 1b), and they were isolated and identified according to the description in Methods. The physical and chemical properties of SP are summarized in Table 1. The NMR data of HSP have been presented in a previous publication (Wang *et al.*, 2005). Other properties of HSP determined in this study were: melting point, 290–293°C; UV absorption, *λ*<sub>max</sub> (in 0.1 M HCl) = 276.6 (E<sub>M</sub> 10246.5), 206.6 (E<sub>M</sub> 16298.7), *λ*<sub>min</sub> (in 0.1 M HCl) = 230.4; *λ*<sub>max</sub> (in 0.1 M NaOH) = 304.2 (E<sub>M</sub> 20384.7), *λ*<sub>min</sub> (in 0.1 M NaOH) = 240.8; FTIR (KBr), 3428 and 3240 cm<sup>−1</sup> (OH), 1719 (CO) cm<sup>−1</sup>. These results confirmed that the metabolites were SP and HSP. The structures of SP and HSP are shown in Fig. 4 (SP is VII and HSP is VIII).

**Isolation and identification of metabolite A by TLC and GC-HR-MS analysis**

Metabolite A was produced in the early phase and later disappeared during nicotine degradation by the resting cells of S16 (Fig. 1b). It was partially purified by preparative TLC and analysed by GC-HR-MS. The metabolite was unstable and its content in the methanol elution decreased several hours later with the formation of other compounds, even though the sample was refrigerated. Accordingly, it was difficult to obtain a pure sample. Metabolite A had a low *R*<sub>f</sub> value (0.04) and exhibited the following mass spectrum [m/z (relative intensity, %)]: [160.0993 (M<sup>+</sup>^+, 63.1), 159.0914 (100.0), 144.0686 (15.0), 130.0651 (6.2), 119.0601 (26.7),

<table>
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<tr>
<td>Molecular formula</td>
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<td><em>R</em>&lt;sub&gt;f&lt;/sub&gt; by TLC (chloroform/ethanol/methanol/0.5 M NaOH: 30 : 15 : 2 : 1.5)</td>
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<td>UV absorption</td>
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<td>FTIR (KBr)</td>
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<td>1H NMR, 600 MHz in DMSO-d&lt;sub&gt;6&lt;/sub&gt; (δ, mult. J)</td>
<td>12.20 (brs, 1H, H-10), 9.14 (s, 1H, H-2), 8.80 (d, J = 4.9 Hz, 1H, H-6), 8.31 (d, J = 7.8 Hz, 1H, H-4), 7.56 (dd, J = 4.9, 7.8 Hz, 1H, H-5), 3.29 (t, J = 6.2 Hz, 2H, H-8), 2.60 (t, J = 6.2 Hz, 2H, H-9)</td>
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<td>13C NMR, 150 MHz in DMSO-d&lt;sub&gt;6&lt;/sub&gt; (δ)</td>
<td>198.22 (7-CO), 173.71 (10-COOH), 153.50 (C-6), 149.17 (C-2), 135.43 (C-4), 131.87 (C-3), 123.97 (C-5), 33.59 (C-8), 27.87 (C-9)</td>
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The mass spectrum was identical to the results of analysis by low-resolution GC-MS (Table 2, group B). The molecular mass determined was in agreement with that calculated from the molecular formula C_{12}H_{12}N_{2} (160.1000) within 4.7 p.p.m. error. With the help of the exact mass measurement by GC-HR-MS and the interpretation of ion fragments, we were able to elucidate the chemical structure (Debrauwer, 2000; Ishikawa et al., 2004; Cai et al., 2002). In this way, metabolite A was identified as N-methylmyosmine, and the structure is shown as II in Fig. 3.

**Formation of DHP in the degradation mixture**

When the degradation experiment was performed in deionized water (the initial pH value was adjusted to ~7.0), a spot (metabolite B) was found by TLC analysis to show blue fluorescence under 300 nm UV light, and to change to a visibly brown colour after several hours' exposure to air (Fig. 1b). Its Rf value (0.84) was the same as that of the DHP standard in TLC analysis. Furthermore, HPLC analysis of the sample obtained from the reaction performed in 0.05 M sodium phosphate buffer (pH 7.0) showed the same retention time (4.17 min) as that of the authentic DHP standard (Fig. 2). Further GC-MS analysis of the reaction sample after silylation with BSTFA indicated that mass spectra of the metabolite were identical to those of the DHP standard (Table 2). Thus it was confirmed that metabolite B was DHP, an important intermediate of nicotine degradation by S16.

**Identification of other metabolites by GC-MS analysis**

Unlike the two major metabolites SP and HSP, other metabolites from nicotine degradation were difficult to isolate and purify because either only small amounts were produced or they were unstable. By GC-MS analysis, the structures of some metabolites could be identified by comparing their mass spectra with those from the standard GC-MS spectral library (Wiley275), especially after silylation with BSTFA (Fig. 3). The metabolites were succinic acid (IX), lactic acid (XI) and 3-hydroxybutyric acid (XII) (Table 2, group A, Fig. 4).

For those compounds whose mass spectra could not be well matched in the standard GC-MS library, the structures were identified according to both their molecular ions and their fragment ions (Table 2, group B, Fig. 4). They were tentatively suggested to be pseudoxy nicot ine (IV) and 1-butanone,4-hydroxy-1-(3-pyridinyl) (VI) according to the

<table>
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<tr>
<th>Product/TMS derivative</th>
<th>m/z of major ion peaks (relative intensity, partially proposed composition)</th>
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<tr>
<td><strong>Group A</strong></td>
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<tr>
<td>Succinic acid (IX)</td>
<td>247 (10.2), 172 (3.4), 147 (100), 129 (5.5), 75 (20.5), 73 (50.7), 55 (8.5), 45 (7.0)</td>
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<td>DHP (X)</td>
<td>255 (12.7), 240 (100), 168 (7.6), 112 (10.9), 84 (13.8), 73 (40)</td>
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<td>Lactic acid (XI)</td>
<td>219 (5.4), 191 (14.6), 190 (15.3), 147 (100), 133 (8.1), 117 (82.1), 88 (6.5), 73 (95.4), 66 (10.2), 59 (6.8), 45 (15.3)</td>
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<tr>
<td>3-Hydroxybutyric acid (XII)</td>
<td>233 (7.2), 191 (17.0), 147 (100), 133 (7.9), 130 (8.6), 117 (44.8), 88 (15.7), 75 (93.8), 66 (11.7), 59 (7.7), 45 (20.0)</td>
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<td><strong>Group B</strong></td>
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<tr>
<td>N-Methylmyosmine (II)</td>
<td>160 (68.7, M+); 159 (100, [M-H]+); 144 (15.6, [M-H-CH3]+); 130 (6.4); 117 (17.9); 106 (7.2); 92 (15.7); 84 (28.4); 78 (20.9, [M-H-C2H4N]+); 65 (14.1); 51 (15.7); 42 (24.1); 39 (16.7)</td>
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<tr>
<td>Pseudoxy nicot ine (IV)</td>
<td>178 (13.9, M+); 124 (12.0); 106 (100, [M-C3H7NHCH3]+); 78 (80.4, [M-COC2H4NHCH3]+); 51 (34.6)</td>
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<tr>
<td>1-Butanone,4-hydroxy-1-(3-pyridinyl) (VI)</td>
<td>237 (3.1, M+); 222 (45.6, [M-CH3]+); 204 (3.5); 194 (15.6, [M-(CH2)2-CH3]+); 148 (36.0, [M-OSi(CH3)2]+); 130 (11.5); 121 (25.5); 116 (30.2, [C2H4OSi(CH3)2]+); 106 (39.6, [M-C3H7OSi(CH3)2]+); 78 (50.1, [M-COC2H4OSi(CH3)2]+); 75 (100); 73 (54.6, [Si(CH3)3]+); 51 (14.1)</td>
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<tr>
<td>SP (VII)</td>
<td>251 (14.5, M+); 236 (100, [M-CH3]+); 208 (76.7, [M-(CH2)2-CH3]+); 162 (13.0, [M-OSi(CH3)2]+); 134 (30.2, [M-CO-Osi(CH3)2]+); 106 (93.0, [M-(CH2)2-CO-Osi(CH3)2]+); 78 (54.6, [M-CO-(CH2)2-CO-Osi(CH3)2]+); 73 (23.8, [Si(CH3)3]+); 51 (24.8)</td>
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<tr>
<td>HSP (VIII)</td>
<td>339 (49.4, M+); 324 (100, [M-CH3]+); 296 (15.9, [M-(CH2)2-CH3]+); 242 (48.1); 194 (63.8, [M-(CH2)2-CO-Osi(CH3)2]+); 151 (8.1); 134 (38.4); 119 (31.3); 91 (26.7); 77 (23.9); 73 (29.0, [Si(CH3)3]+)</td>
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*Group A products were identified according to the standard spectra from the standard library (Wiley275) or standard compounds. Group B products were tentatively identified according to their molecular ions and fragment ions, whose mass spectra could not be well matched in the standard library of GC-MS.*
mass spectra and interpretation of ion fragments. For compound VI, the high-resolution mass spectrum $[m/z$ (relative intensity, %)] $[237.1196 (M^{+}, 3.7), 222.0953 (100.0), 204.0822 (7.2), 194.1000 (24.3), 148.0758 (42.1), 130.0647 (9.0), 121.0531 (21.2), 117.0717 (25.7), 116.0656 (34.1), 106.0289 (27.7), 78.0343 (18.4), 75.0265 (43.1), 73.0475 (22.2), 51.0240 (3.6)]$ was also determined and was identical to the results of analysis by low-resolution GC-MS (Table 2, group B, VI). The molecular mass determined (237.1196) was in good agreement with that calculated from the molecular formula C$_{12}$H$_{19}$NO$_2$Si (237.1185), within 4.6 p.p.m. error. Moreover, the trimethylsilyl (TMS) derivatives of SP and HSP were also detected in the reaction mixture.

**Transformation of nicotine and its metabolites by cell-free extract**

Nicotine and its metabolites SP and HSP could be transformed into related products by the cell-free extract of S16 (Table 3). By GC, HPLC and UV analysis, the products of the enzymic reactions could be detected. Nicotine was transformed into N-methylmyosmine (II) and SP. SP and HSP were converted to HSP and DHP, respectively. The results showed that the metabolic sequence of these compounds in the presence of cell-free extract was from nicotine to SP, HSP and DHP via N-methylmyosmine.

**DISCUSSION**

Earlier research has suggested that *Pseudomonas* species are able to oxidize nicotine into II, IV, VII (SP), VIII (HSP), IX and X (DHP), but II, IX and X (DHP) have either never been detected or not been satisfactorily characterized with confirmatory evidence in these studies (Wada & Yamasaki, 1954; Tabuchi, 1955; Kaiser et al., 1996). In this study, one important metabolite, SP, was purified and characterized thoroughly by NMR, FT-IR, UV and MS analysis. To our knowledge, this is the first time that certain physical and chemical properties of SP have been investigated in detail. DHP was also detected and identified for the first time as a nicotine metabolite by TLC, HPLC and MS analysis by comparison with the standard compound. N-Methylmyosmine was partially purified and identified by preparative TLC and GC-HR-MS analysis. The structures of other metabolites (IX, XI and XII) were identified by comparing their mass spectra with those from the standard GC-MS spectral library (Wiley275). For the other two compounds whose mass spectra could not be well matched in the standard GC-MS library, the structures were identified according to both their molecular ions and fragment ions. They were suggested to be pseudooxynicotine (IV) and 1-butanone,4-hydroxy-1-(3-pyridinyl) (VI). In addition, the mechanism of the L-6-hydroxynicotine oxidase in the nicotine degradation pathway of *Arthrobacter* species (Decker & Dai, 1967; Dai et al., 1968) could be helpful to understand the initial attack upon nicotine at the pyrrolidine ring by S16. The flavoprotein converts L-6-hydroxynicotine to 6-hydroxy-N-methylmyosmine in the presence of oxygen. The overall process consists of the enzyme-catalysed dehydrogenation of L-6-hydroxynicotine to produce 6-hydroxy-N-methylmyosmine and hydrogen peroxide, and the spontaneous hydrolysis of 6-hydroxy-N-methylmyosmine to form 6-hydroxypseudooxynicotinic. A similar mechanism has been described for 2,4,6-trichlorophenol 4-monooxygenase in *Ralstonia eutropha* JMP134, which catalyses the sequential dechlorination of 2,4,6-trichlorophenol to 6-chlorohydroxyquinol by oxidative and hydrolytic reactions (Xun & Webster, 2004). Hecht et al. (2000) have reported that nicotine can be metabolized through 2’ or 5’ hydroxylation by cytochrome P450 2A6 and human liver microsomes by two different pathways: 2’-hydroxynicotine is further decomposed into IV and VII (Fig. 4), while 5’-hydroxynicotinic is converted to cotinine. Because IV and VII were produced in considerable amounts
from nicotine degradation by S16 in this study, the highly unstable compound 2'-hydroxynicotine was considered to have a transient existence as the product of hydrolysis in the biochemical reactions that lead to the breaking of the C–N bond.

From the known reaction mechanism and general chemical considerations, it can be proposed that the initial attack upon nicotine by S16 is as follows: nicotine (I)→N-methylymosmine (II)→2'-hydroxynicotine (III)→pseudooxynicotine (IV). Analogically, pseudooxynicotine (IV) could be converted into 3-pyridinebutanal,C-oxo (V) by the same or a similar amine oxidase, while 3-pyridinebutanal,C-oxo (V) could easily be oxidized to 3-succinoyl-pyridine (VII) by aldehyde dehydrogenases. However, we did not find compound V, possibly because it is a transient intermediate in cells.

In nicotinic acid degradation by *Pseudomonas fluorescens* N-9, hydroxylation at carbon 6 is the first step in the pathway, and this is followed by an oxidative decarboxylation to yield DHP, which is catalysed by nicotinic acid dehydrogenase.
and 6-hydroxynicotinate 3-monoxygenase (Behrman & Stainer, 1957; Hurh et al., 1994; Nakano et al., 1999). DHP is further degraded by 2,5-dihydroxyxypidine oxygenase into maleamic acid and formic acid (Gauthier & Rittenberg, 1971a, b), which are used as nutrients and energy sources to synthesize new cell compounds. In this study, SP and HSP could be transformed by the cell-free system or the resting cells (data not shown) of S16 into HSP and DHP, respectively, so a reaction similar to nicotinic acid metabolism in P. fluorescens is believed to happen in nicotine degradation by S16: SP (VII)→HSP (VIII)→succinic acid (IX)→DHP (X). Furthermore, as shown in Fig. 2, most of the nicotine (58.9 %, molar conversion) was metabolized into SP, HSP and DHP. However, lactic acid (XI) and 3-hydroxybutyric acid (XII) were detected in this study, and not maleamic acid and formic acid, which might be a result of the rapid reaction caused by the high degradation activity of the cells and an insufficient oxygen supply. It is interesting that a newly found compound, 1-butanone,4-hydroxy-1-(3-pyridinyl) (VI), was produced during nicotine degradation by the resting cells of S16, and then utilized completely (Fig. 3). At the same time, lactic acid showed similar behaviour, which indicated that oxygen supply was initially insufficient during nicotine degradation by the resting cells of S16. It can be assumed that a limited oxygen supply makes the degradation reaction slower and leads to accumulation of NAD(P)H and metabolites. However, a high level of NAD(P)H and metabolites is toxic to cells, and it is necessary to convert NAD(P)H and toxic metabolites into NAD(P)⁺ and nontoxic compounds. In nicotine degradation, the aldehyde compound 3-pyridinebutanal, C-oxo (V), which is usually thought of as a toxic compound, is believed to be transformed into the corresponding alcohol, 1-butanone,4-hydroxy-1-(3-pyridinyl) (VI), accompanied by oxidation of NAD(P)H. Alcohol dehydrogenases, which catalyse the reversible conversion of aromatic and heterocyclic aldehydes to their corresponding alcohols, have been reported in many micro-organisms (Bradshaw et al., 1992a, b; Hummel, 1999; Mee et al., 2005; Peng et al., 2006; Tasaki et al., 2006). When the oxygen supply is adequate, 1-butanone,4-hydroxy-1-(3-pyridinyl) and lactic acid can be oxidized by corresponding dehydrogenases and further decomposed. From another point of view, the formation of the alcohol and the acid SP also verified that the aldehyde compound 3-pyridinebutanal, C-oxo (V) was an important intermediate of nicotine degradation.

Based on our investigations and the known reaction mechanisms, we propose the pathway for nicotine degradation in P. putida S16 shown in Fig. 4.

The pathways of nicotine metabolism vary in different bacteria. In the Gram-positive Arthrobacter genus, nicotine is hydroxylated at the 6 position before the pyrrolidine ring is opened. The opposite occurs in the Gram-negative Pseudomonas genus, and the further metabolic pathways also differ considerably. 2,3,6-Trihydroxyxypidine and DHP are produced, respectively, in these two bacterial genera after the removal of the side chain which is formed by opening the pyrrolidine ring.

In summary, nicotine and some of its metabolites are harmful to health and the environment; this study provides new insights into the nicotine metabolism of micro-organisms and into an environmentally friendly route of nicotine degradation (Wang et al., 2004, 2005). It is notable that compound IV is the direct precursor of the tobacco-specific lung carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Hecht, 1999; Hecht et al., 2000). The present study suggests that nicotine and its metabolites such as compound IV might be eliminated in later reactions by the Pseudomonas genus when the bacteria are employed to reduce the nicotine content in tobacco and to detoxify tobacco wastes. Moreover, nicotine can be transformed into renewable functionalized pyridines by biocatalytic processes that are difficult to achieve by chemical means (Schmid et al., 2001). We believe that this is a promising strategy to convert nicotine in tobacco and tobacco wastes into valuable compounds by means of biotechnology. Nicotine could be transformed by P. putida S16 (Fig. 4) into valuable compounds such as HSP and DHP, which are precursors for the synthesis of drugs and insecticides (Spande et al., 1992; Roduit et al., 1997; Nakano et al., 1999). Preliminary bioconversion processes with S16 have indicated that they are capable of transforming nicotine into HSP with high yields (Wang et al., 2005). However, the enzymes and genes involved in nicotine degradation by S16 and other Pseudomonas bacteria are still unclear, so future work in our group will focus on the molecular biology of nicotine biodegradation.

### Table 3. Transformation of nicotine and its metabolites by cell-free extract of S16

<table>
<thead>
<tr>
<th>Substrate (0.3 mg ml⁻¹)</th>
<th>Addition (µM)</th>
<th>Decrease of substrate concentration</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>DCIP (0.25)</td>
<td>0.17 ± 0.019</td>
<td>II (0.05 ± 0.024), SP (0.07 ± 0.021)</td>
</tr>
<tr>
<td>SP</td>
<td>NAD (200)</td>
<td>0.16 ± 0.021</td>
<td>HSP (0.09 ± 0.020)</td>
</tr>
<tr>
<td>HSP</td>
<td>NADH (300)</td>
<td>0.19 ± 0.016</td>
<td>DHP (0.05 ± 0.017)</td>
</tr>
</tbody>
</table>
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