

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-dihydropyridine through the intermediates *N*-methylmyosmine, 2'-hydroxynicotine, pseudooxynicotine, 3-pyridinebutanal, C-oxo, 3-succinoylpyridine and 6-hydroxy-3-succinoylpyridine. *N*-Methylmyosmine, 2,5-dihydropyridine 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-pyridinyl), 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.

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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* (Sgueros, 1955), *A. oxidans* P-34 (Gherina *et al.*, 1965; reclassified as *Arthrobacter ureafaciens* by Kodama *et al.*, 1992), *A. oxidans* PAO1 (Decker & Blegg, 1965; reclassified as *Arthrobacter nicotinovorans* by Kodama

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Abbreviations: BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; DCIP, 2,6-dichlorophenolindophenol; DCW, dry cell weight; DHP, 2,5-dihydropyridine; ESI, electrospray ionization; FT-IR, Fourier-transform IR; HR-MS, high-resolution MS; HSP, 6-hydroxy-3-succinoylpyridine; SP, 3-succinoylpyridine; TMS, trimethylsilyl.

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence of the 16S rRNA gene of strain S16 determined in this study is AY574282.

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, pseudoxyntine, methylamine, 3-succinoylpyridine (SP) and 6-hydroxy-3-succinoylpyridine (HSP) were detected as the initial catabolic products of nicotine by Wada & Yamasaki (1954) and Tabuchi (1955). HSP has been supposed to be further metabolized by cleavage at the 3 position of HSP with the formation of 2,5-dihydroxypyridine (DHP) and succinic acid (Kaiser *et al.*, 1996). However, until now, no research on the detection or satisfactory characterization of *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 bacterium 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 1 g L⁻¹ (-)-nicotine at 30 °C with shaking at 120 r.p.m. in an incubator. The liquid culture medium was a minimal 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 of 0.1 M HCl) 0.05 g CaCl₂·2H₂O, 0.05 g CuCl₂·2H₂O, 0.008 g MnSO₄·H₂O, 0.004 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 from Fluka) was added to the medium after filter-sterilization. After bacterial growth was observed, the culture was used as an inoculum and transferred twice. The final culture (0.1 ml) was serially diluted and spread onto agar plates containing nicotine. After 2 days, colonies began to appear on plates incubated at 30 °C. The colonies were picked and streaked to purity on nicotine agar plates. One isolate

with higher capacity for nicotine degradation was selected for further study. It was routinely and optimally cultured with 3 g nicotine l⁻¹ at 30 °C and pH 7.0.

Strain identification and characterization. Physiological characteristics were determined according to Palleroni (1984). Cell morphology was observed using a Hitachi S-570 scanning electron microscope. Fatty acids were analysed using the Sherlock microbial identification system, version 4.0 (MIDI) with the TSBA (revision 4.10) database of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). The 16S rRNA gene was amplified by PCR using the oligonucleotides 27f (5'-AGAGTTTGATCMTG-GCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') as primers (Johnson, 1994). The fragment generated was purified by agarose gel electrophoresis and band extraction before sequencing analysis. Related sequences were obtained from the GenBank database 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, 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 l flask containing 3.37 mg dry cell weight (DCW) ml⁻¹ of resting cells (OD₆₂₀ ~6; 1.0 OD₆₂₀ unit=0.56 mg DCW ml⁻¹), 3 g nicotine l⁻¹ and 1 l sterilized 0.05 M sodium phosphate buffer or deionized water (pH adjusted to 7.0 at the beginning of the reaction), at 30 °C with shaking at 120 r.p.m.

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 absorption 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 12000 g for 10 min at 4 °C to remove the resting cells. A 1 l 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 liquor 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

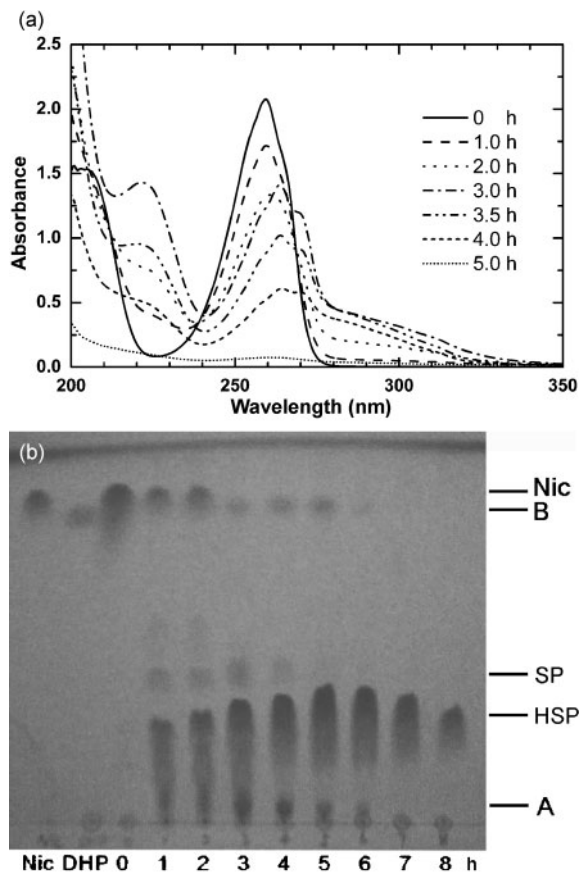


Fig. 1. (a) UV and (b) TLC analysis of nicotine metabolites. Nicotine (3 g l^{-1}) and $3.37 \text{ mg DCW ml}^{-1}$ resting cells were incubated at pH 7.0 and 30°C in 0.05 M sodium phosphate buffer (pH 7.0) (a) and deionized water (pH 7.0) (b). In (a), only nicotine was in the reaction mixture at 0 h (maximum absorbance at 259 nm), and later metabolites (maximum absorbance at ~ 223 , 263, 275 and 310 nm) were formed and then degraded. (b) Nic, nicotine standard; DHP, 2,5-dihydropyridine standard.

spectra were obtained on a Nexus 470 spectrometer (Nicolet). MS analysis was run on an API 4000 mass spectrometer with electrospray ionization (ESI) (Applied Biosystems) using methanol as solvent. NMR spectra were recorded for solutions in DMSO- d_6 on an Avance 600 spectrometer (Bruker) operated at 600 MHz for ^1H and at 150 MHz for ^{13}C . DEPT90, DEPT135, H-H COSY, HMQC and HMBC spectra of metabolite SP were also recorded.

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 ultrasound. 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 \times 50 m length \times 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 $10^\circ\text{C min}^{-1}$ 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 $20\,000 \text{ g}$ 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 optimally with $3 \text{ g nicotine l}^{-1}$, and at 30°C and pH 7.0, and completely degraded nicotine within 10 h with a maximum biomass of $1.4 \text{ mg DCW ml}^{-1}$ (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 ($0.52\text{--}0.65 \times 1.05\text{--}1.43 \mu\text{m}$) 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 ($\geq 99.0\%$) with those of *P. putida* (AB029257.1), *Pseudomonas monteilii* (AF064458.1), *Pseudomonas plecoglossicida* (AB009457.1), *P. putida* KT2440 (AE016774.1) and *P. putida* ATCC 12633^T (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_{\max} (in 0.1 M HCl) = 276.6 (E_M 10246.5), 206.6 (E_M 16298.7), λ_{\min} (in 0.1 M HCl) = 230.4; λ_{\max} (in 0.1 M NaOH) = 304.2 (E_M 20384.7), λ_{\min} (in 0.1 M NaOH) = 240.8; FTIR (KBr), 3428 and 3240 cm^{-1} (OH), 1719 (CO) cm^{-1} . 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_f value (0.04) and exhibited the following mass spectrum [m/z (relative intensity, %)]: [160.0993 (M^+ , 63.1), 159.0914 (100.0), 144.0686 (15.0), 130.0651 (6.2), 119.0601 (26.7),

Table 1. Physical and chemical properties of metabolite SP from nicotine degradation by S16

Property	Value
Appearance	White crystal
Melting point	159–161 °C
Molecular formula	$C_9H_9NO_3$
Molecular mass	179.2
MS (ESI), m/z	180.3 $[M+H]^+$
R_f by TLC (chloroform/ethanol/methanol/0.5 M NaOH 30:15:2:1.5)	0.37
UV absorption	λ_{\max} (0.1 M HCl) = 222.4 (E_M 6289.4), 263.2 (E_M 5373.6), λ_{\min} (0.1 M HCl) = 211.0, 241.4; λ_{\max} (0.1 M NaOH) = 229.8 (E_M 9648.6), 266.6 (E_M 3649.6), λ_{\min} (0.1 M NaOH) = 212.8, 252.4
FTIR (KBr)	3435 and 3358 cm^{-1} (KBr, OH), 1708 cm^{-1} (CO)
¹ H NMR, 600 MHz in DMSO- d_6 (δ , mult. J)	12.20 (brs, 1H, 10-COOH), 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)
¹³ C NMR, 150 MHz in DMSO- d_6 (δ)	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)

117.0561 (11.6), 90.0432 (3.8), 78.0350 (2.2), 63.0246 (2.7), 42.0376 (1.7)]. This mass spectrum was identical to the results of analysis by low-resolution GC-MS (Table 2, group B II). The molecular mass determined (160.0993) was in good agreement with that calculated from the molecular formula $C_{10}H_{12}N_2$ (160.1000) within 4.7 p.p.m. error. With the help of the exact mass measurement by GC-HR-MS (within 5 p.p.m. error) 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 R_f 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 pseudooxynicotine (IV) and 1-butanone,4-hydroxy-1-(3-pyridinyl) (VI) according to the

Table 2. Mass spectra of products, or their TMS derivatives, from the metabolism of nicotine by resting cells of S16

Product/TMS derivative	<i>m/z</i> of major ion peaks (relative intensity, partially proposed composition)
Group A*	
Succinic acid (IX)	247 (10.2), 172 (3.4), 147 (100), 129 (5.5), 75 (20.5), 73 (50.7), 55 (8.5), 45 (7.0)
DHP (X)	255 (12.7), 240 (100), 168 (7.6), 112 (10.9), 84 (13.8), 73 (40)
Lactic acid (XI)	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)
3-Hydroxybutyric acid (XII)	233 (7.2), 191 (17.0), 147 (100), 133 (7.9), 130 (8.6), 117 (44.8), 88 (15.7), 75 (29.3), 73 (93.8), 66 (11.7), 59 (7.7), 45 (20.0)
Group B†	
<i>N</i> -Methylmyosmine (II)	160 (68.7, M^+); 159 (100, $[M-H]^+$); 144 (15.6, $[M-H-CH_3]^+$); 130 (6.4); 119 (40.9); 117 (17.9); 106 (7.2); 92 (15.7); 84 (28.4); 78 (20.9, $[M-H-C_5H_8N]^+$); 65 (14.1); 51 (15.7); 42 (24.1); 39 (16.7)
Pseudooxynicotine (IV)	178 (13.9, M^+); 124 (12.0); 106 (100, $[M-C_3H_6NHCH_3]^+$); 78 (80.4, $[M-COC_3H_6NHCH_3]^+$); 51 (34.6)
1-Butanone,4-hydroxy-1-(3-pyridinyl) (VI)	237 (3.1, M^+); 222 (45.6, $[M-CH_3]^+$); 204 (3.5); 194 (15.6, $[M-(CH_2)_2-CH_3]^+$); 148 (36.0, $[M-OSi(CH_3)_3]^+$); 130 (11.5); 121 (25.5); 116 (30.2, $[C_3H_6OSi(CH_3)_2]^+$); 106 (39.6, $[M-C_3H_6OSi(CH_3)_3]^+$); 78 (50.1, $[M-COC_3H_6OSi(CH_3)_3]^+$); 75 (100); 73 (54.6, $[Si(CH_3)_3]^+$); 51 (14.1)
SP (VII)	251 (14.5, M^+); 236 (100, $[M-CH_3]^+$); 208 (76.7, $[M-(CH_2)_2-CH_3]^+$); 162 (13.0, $[M-OSi(CH_3)_3]^+$); 134 (30.2, $[M-CO-OSi(CH_3)_3]^+$); 106 (93.0, $[M-(CH_2)_2-CO-OSi(CH_3)_3]^+$); 78 (54.6, $[M-CO-(CH_2)_2-CO-OSi(CH_3)_3]^+$); 73 (23.8, $[Si(CH_3)_3]^+$); 51 (24.8)
HSP (VIII)	339 (49.4, M^+); 324 (100, $[M-CH_3]^+$); 296 (15.9, $[M-(CH_2)_2-CH_3]^+$); 242 (48.1); 194 (63.8, $[M-(CH_2)_2-CO-OSi(CH_3)_3]^+$); 151 (8.1); 134 (38.4); 119 (31.3); 91 (26.7); 77 (23.9); 73 (29.0, $[Si(CH_3)_3]^+$)

*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.

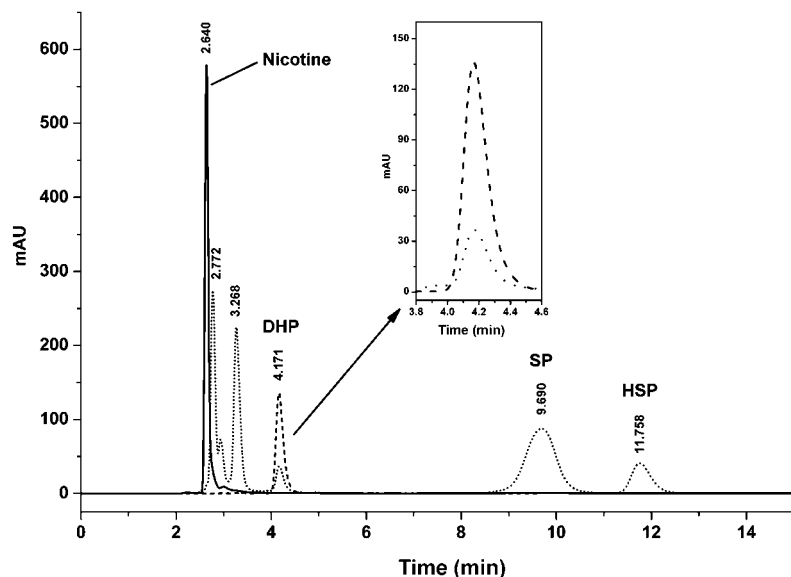


Fig. 2. HPLC profile of the metabolites of nicotine degradation by resting cells of strain S16 at pH 7.0 and 30 °C in 0.05 M sodium phosphate buffer (pH 7.0). The reaction mixture at 0 h (solid line) contained nicotine (2.5 g l^{-1} , 15.43 mM) alone, and at 3.5 h (dotted line) contained DHP (0.06 g l^{-1} , 0.54 mM), SP (1.31 g l^{-1} , 7.32 mM), HSP (0.24 g l^{-1} , 1.23 mM) and other metabolites. The dashed line represents the DHP standard. mAU, milliabsorbance unit.

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 $C_{12}H_{19}NO_2Si$ (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 pseudoxyonicotine (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-hydroxypseudoxyonicotine 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-hydroxypseudoxyonicotine. 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'-hydroxynicotine is converted to cotinine. Because IV and VII were produced in considerable amounts

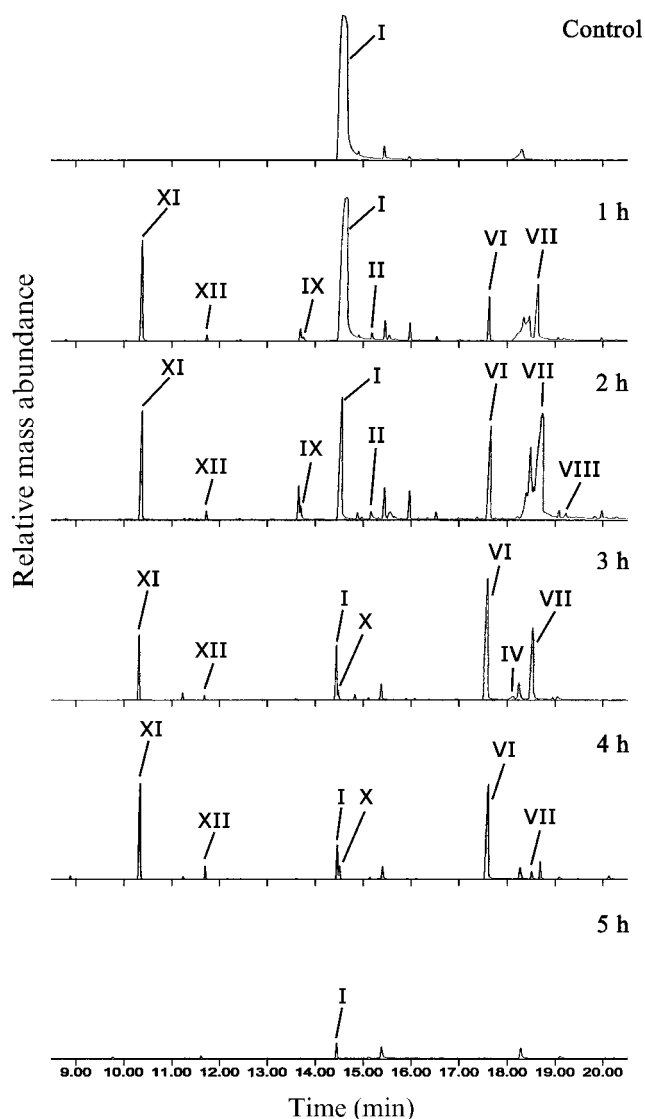


Fig. 3. GC-MS chromatograms of the products, or their TMS derivatives, of nicotine degradation by resting cells of strain S16. The compounds were obtained from the residue of samples evaporated to dryness, and derivatized by silylation. The process of sample treatment was not fully quantitative. I, nicotine (14.5 min); II, *N*-methylmyosmine (15.2 min); IV, pseudooxynicotine (18.2 min); VI, 1-butanone,4-hydroxy-1-(3-pyridinyl) (17.6 min); VII, SP (18.6 min); VIII, HSP (19.2 min); IX, succinic acid (13.7 min); X, DHP (14.6 min); XI, lactic acid (10.3 min); XII, 3-hydroxybutyric acid (11.7 min).

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

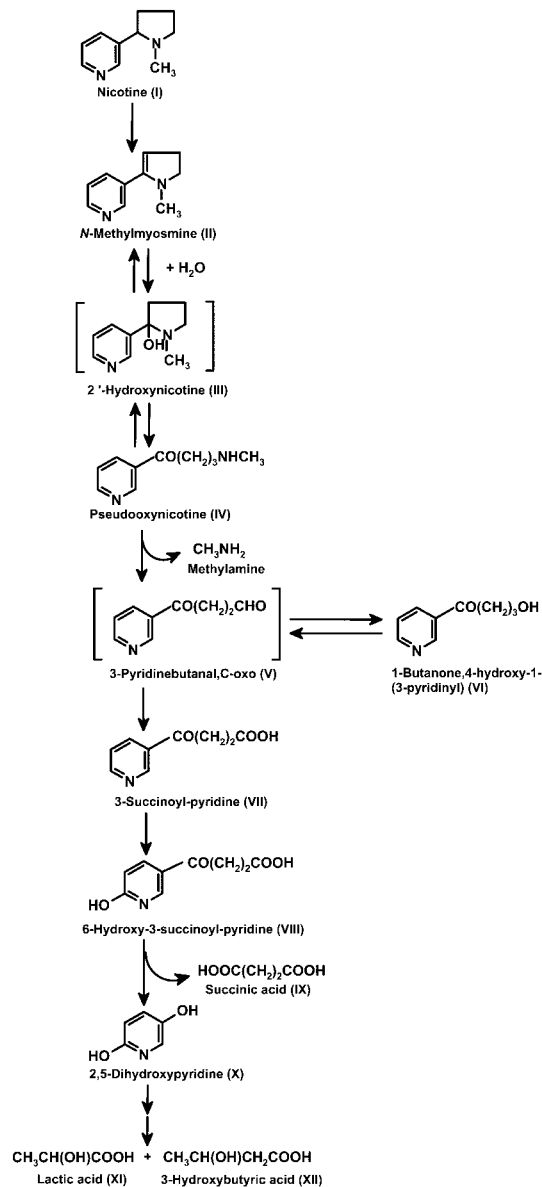


Fig. 4. Proposed pathway of nicotine degradation in *P. putida* S16. Bracketed compounds were not detected.

upon nicotine by S16 is as follows: nicotine (I)→*N*-methylmyosmine (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

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

Values are the means \pm SD of three independent tests, in mg ml⁻¹.

Substrate (0.3 mg ml ⁻¹)	Addition (μ M)	Decrease of substrate concentration	Product
Nicotine	DCIP (0.25)	0.17 \pm 0.019	II (0.05 \pm 0.024), SP (0.07 \pm 0.021)
SP	NAD (200)	0.16 \pm 0.021	HSP (0.09 \pm 0.020)
HSP	NADH (300)	0.19 \pm 0.016	DHP (0.05 \pm 0.017)

and 6-hydroxynicotinate 3-monoxygenase (Behrman & Stainier, 1957; Hurh *et al.*, 1994; Nakano *et al.*, 1999). DHP is further degraded by 2,5-dihydroxypyridine 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) \rightarrow HSP (VIII) \rightarrow 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 non-toxic 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-Trihydroxypyridine 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.

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