

Characterisation of a novel aerobic nicotine-biodegrading strain of *Pseudomonas putida*

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Abstract - An aerobic bacterial strain J5 capable of effectively degrading nicotine was isolated from the rhizosphere of tobacco in Yunnan, China. This strain was identified as *Pseudomonas putida* based on morphology, physiological characteristics, and 16S rDNA sequence analysis. The optimum nicotine concentration for the growth of strain J5 was 2.0 g/l. There was no more nicotine detected in the medium containing 3.0 g of nicotine/l after J5 growth for 24 h and less than 0.2% of the nicotine in a medium containing 4.0 g of nicotine/l after J5 growth for 48 h. There was a statistically significant linear relationship between nicotine degradation and biomass of strain J5. When a J5 cell suspension (10^8 CFU/ml) was applied to tobacco leaves, the nicotine concentration was decreased by 11.7%. These data suggest that the novel strain J5 of *P. putida* may be useful for nicotine biodegradation.

Key words: biodegradation, tobacco, nicotine, *Pseudomonas putida*.

INTRODUCTION

Nicotine [1-methyl-2-(3-pyridyl-pyrrolidine), $C_{10}H_{14}N_2$] is the main alkaloid component of cigarettes and represents up to 3% of the dry mass of tobacco leaves (Armstrong *et al.*, 1998). By mediating cholinergic receptors in the central nervous system, nicotine results in addiction. Smokers consumed about 5.53 trillion cigarettes worldwide in 1995 (Novotny and Zhao, 1999), and smoking caused about 9 million deaths in 2000 (Sloan and Gelband, 2007). By 2020, the global burden of tobacco is expected to exceed 9 million deaths annually, with 7 million occurring in economically developing nations (Sloan and Gelband, 2007). Moreover, the tobacco industry produces a great deal of non-recyclable powdery waste with an average nicotine content of 18 g per kg of dry weight (Civilini *et al.*, 1997). Waste is ranked as "toxic and hazardous" by European Union Regulations when the nicotine content exceeds 500 mg per kg dry weight (Novotny and Zhao, 1999). In the United States, nicotine has been included in the Toxics Release Inventory (TRI) list since November 1994 (Civilini *et al.*, 1997).

Chemical-physical treatments have been extensively used in "denicotinization," i.e., in the removal of nicotine from tobacco industry waste (Ireland *et al.*, 1980; Lenkey, 1989); however, biological treatments with micro-organisms also have potential for nicotine degradation (Civilini *et al.*, 1997).

In particular, the bacterial community residing in the tobacco rhizosphere has presumably adapted to use nicotine as a growth substrate and has developed biochemical strategies to decompose this organic heterocyclic compound. *Arthrobacter nicotianae* (Hochstein and Rittenberg, 1958), *Arthrobacter globiformis* (Maeda and Kisaki, 1981), *Cellulomonas* sp. (Gravelly *et al.*, 1978), and *Ochrobactrum intermedium* (Yuan *et al.*, 2005) can degrade nicotine, and *Arthrobacter* spp. were reported to extract carbon, nitrogen, and energy by the breakdown of nicotine (Schenk *et al.*, 1998).

In this paper, we report the isolation and identification of a new strain of the bacterium *Pseudomonas putida*, a strain with substantial potential for degrading nicotine.

MATERIALS AND METHODS

Isolation of nicotine-degrading bacteria. Soil samples were collected from the rhizospheres of tobacco plants at the Yanhe Base of Yunnan Tobacco Research Institute, Yuxi, Yunnan, China. Two grams of soil sample were suspended in 8 ml of sterile distilled water, and 250 μ l of the soil suspension was spread onto nicotine medium (NIM) (Na_2HPO_4 6 g/l, KH_2PO_4 3 g/l, NH_4Cl 1 g/l, NaCl 0.5 g/l, $MgSO_4$ 0.12 g/l, $CaCl_2$ 0.1 g/l, nicotine 0.5 g/l, agar 15 g/l), in which nicotine (Sigma-Aldrich, Germany) was the sole carbon source nicotine. After 72 h at 30 °C, individual colonies were transferred and streaked on new agar plates. This procedure was repeated until pure isolates were

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obtained. Those isolates that produced pigment on the agar were presumed to be *Arthrobacter* spp. and were not studied further because nicotine degradation by *Arthrobacter* spp. has already been described. One isolate, J5, grew well on the nicotine agar but produced no pigment and therefore had potential to be a new strain of nicotine-degrading bacterium.

Phenotypic, physiological and biochemical characterisation. The morphology of strain J5 was studied after the bacterium had grown for 48 h on Luria-Bertani (LB) medium (Sambrook and Russell, 1998). King's B medium was used to determine the fluorescence (King *et al.*, 1954). Cell morphology was observed with a transmission electron microscope (Hitachi-H600, Japan) after staining negatively with 1% (w/v) phosphotungstic acid. Conventional physiological and biochemical characteristic assays were determined by the procedures of Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994).

DNA base composition and 16S rDNA determination. The percentage of G+C content in the DNA of strain J5 was measured by the thermal denaturation method (Sambrook and Russell, 1998). DNA from *Escherichia coli* strain K-12 was used as a standard.

Strain J5 was incubated in LB liquid medium at 30 °C for 24 h. The cells were collected by centrifugation and washed twice with sterile distilled water. Total chromosomal DNA was prepared following a standard procedure (Sambrook and Russell, 1998). Oligonucleotide primers 27F (5'-GAGAGTTTGATCCTGGCTCAG-3') and 1494R (5'-CTACGGCTACCTTGTACGA-3') (Weisburg *et al.*, 1991) were used for 16S rDNA amplification. PCR reactions and 16S rDNA cloning were carried out as described previously (Lei *et al.*, 2007). The positive clone was sequenced at Invitrogen Ltd., China.

The 16S rDNA sequence of strain J5 was deposited in GenBank (accession number DQ659138). Twelve strains close to J5 from the GenBank database were aligned using DNAMAN ver. 5.0 (Lynnon Biosoft, Quebec, Canada). A phylogenetic tree was constructed with Mega 3.1 (Kumar *et al.*, 2004).

Optimal growth and nicotine-degradation conditions. Strain J5 was cultured to saturation in LB liquid medium at 30 °C and then diluted (1:1000) into 100 ml of NIM liquid medium containing 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, or 4.0 g of nicotine/l as the sole carbon source. For determining cell density, 3 ml of culture were collected and the absorbance (600 nm) was measured with a spectrophotometer at 2 h intervals. Then all samples were centrifuged for 5 min at 12000 r/min. The nicotine concentrations in the supernatants were determined by high-pressure liquid chromatography (HPLC) (Agilent 1100 Series, USA) using an Agilent C-18 column (5 µm, 4.6 x 150 mm). All experiments were independently performed at least twice and with three replicates each time.

Degradation of nicotine in tobacco leaves. Tobacco seedlings (*Nicotiana tabacum* L. cv. K326) were grown in a greenhouse. When mature, tobacco leaves were harvested, and each leaf was cut into two parts along the main vein with sterile scissors. One part was soaked with bacterial culture (1 x 10⁸ CFU of strain J5/ml LB) for 6 h, and the

other was soaked with cell-free medium. The tobacco leaves were then baked at 60 °C for 6 days, crushed to a powder, and passed through a sieve with 0.25-mm apertures. The tobacco powder was extracted with methanol to determine the concentration of nicotine according to a previous report (Xie *et al.*, 2000). This experiment was performed three replicates.

RESULTS AND DISCUSSION

Identification of strain J5

A total of 154 bacterial strains from tobacco rhizosphere were isolated on NIM agar plates with nicotine as the sole carbon source. Most strains produced blue pigment around the colonies, similar to those produced by *Arthrobacter* sp. (Hochstein and Rittenberg, 1959), a known nicotine-degrading bacterium. Strain J5, however, grew well on nicotine agar but produced no pigment. J5 is an aerobic, Gram-negative, nonspore-forming bacterium. Colonies of strain J5 on nutrient agar were circular, convex, and yellow. Cells were rod-shaped, 0.4-1.0 µm in diameter, and 1.5-4.0 µm long, and were motile with more than one flagellum at one polar (Fig. 1). Strain J5 produced a fluorescent pigment on King's B medium. Other physiological and biochemical characteristics are summarised in Table 1.

The G+C content of strain J5 was 61.9 mol% as determined by the thermal denaturation procedure. A phylogenetic tree was constructed based on 16S rDNA sequences (Fig. 2). The sequence similarity of J5 was 99.8% with *Pseudomonas putida* IFO 14671 (D85998), 97.8% with *Pseudomonas stutzeri* ATCC 17591 (U26261), and 96.8% with *Pseudomonas fluorescens* ATCC 49642 (AF094732).

Based on 16S rDNA sequence analysis and on morphological, physiological, and biochemical characteristics, strain J5 was identified as *Pseudomonas putida*. A culture of J5 has been deposited in the China General Microorganism Culture Collection (AS1828).

Nicotine degradation by *Pseudomonas putida* strain J5

A negative correlation was found between cell density and the nicotine content when strain J5 was incubated in NIM medium initially containing 1 g of nicotine/l as the sole carbon source (Figs. 3 and 4). The nicotine concentration was dramatically decreased during the log phase of growth and was not detectable after 10 h of incubation. By that time, the growth of J5 was at the late-log phase (OD₆₀₀ 1.17), indicating that J5 could use nicotine as a carbon source for growth.

Most basic insights into nicotine catabolism have been based on studies using *Arthrobacter* sp. and *Pseudomonas* sp. (Kaiser *et al.*, 1996; Brandsch, 2006). As mentioned in a previous study, *A. nicotinovorans* could convert nicotine to a blue pigment (Hochstein and Rittenberg, 1959). Further experiments demonstrated that the blue-violet water-soluble pigment was a product of the transformation of nicotine to 2,3,6-trihydroxypyridine, which showed rapid spontaneous oxidation in the presence of oxygen (Holmes *et al.*, 1972; Igloi and Brandsch, 2003). Although the mechanism has not been studied, a green pigment was also produced during nicotine degradation by *Pseudomonas* sp. HF-1 (Ruan *et al.*, 2005). Nevertheless, no pigment was detected from the J5 culture with or without nicotine. The failure to produce pigment indicates that strain J5 of *P. putida*

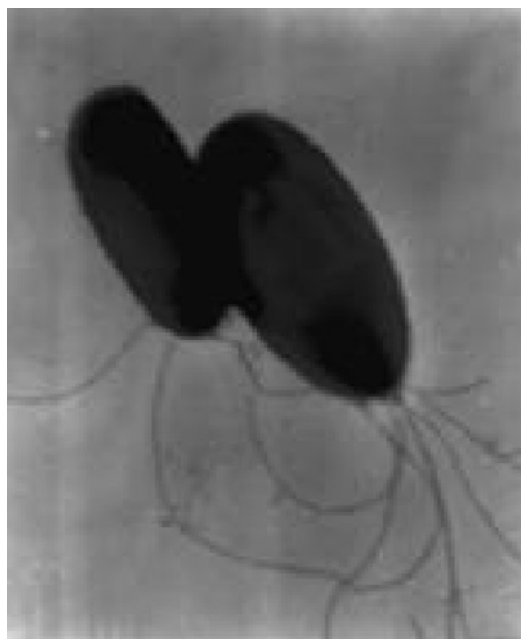


FIG. 1 - Electron micrograph of strain J5 cells (25000x).

TABLE 1 - Physiological and biochemical characters of strain J5

Characteristic	+/-
Fluorescent, diffusible pigment	+
Diffusible non-fluorescent pigments	-
Non-diffusible non-fluorescent pigments	-
Growth at 41 °C	-
Growth at 4 °C	+
Arginine dihydrolase	+
Oxidase reaction	+
Gelatin hydrolysis	-
Starch hydrolysis	-
Levan formation from sucrose	-
Nitrate reduction	+
Utilization of:	
Glucose	+
Sucrose	+
Trehalose	-
Maltose	+
Mannitol	+
Inositol	-
L-Valine	+
L-Proline	+
β-Alanine	+
L-Arginine	+

might degrade nicotine through a different pathway from *Arthrobacter* sp. and *Pseudomonas* sp. HF-1.

Effect of nicotine concentration on degradation activity and cell growth

When strain J5 of *P. putida* was incubated at 30 °C in NIM liquid medium with different initial concentrations of nicotine as the sole carbon source, the J5 cell density (OD₆₀₀) increased as initial nicotine concentration increased from 0.5 to 2.0 g/l. However, the growth of J5 during the first 12 h of incubation was less when the initial nicotine concen-

tration exceeded 2.0 g/l (Fig. 5). The results suggested that the 2 g of nicotine/l was optimal for J5 growth. No nicotine was detected by HPLC when strain J5 was cultured for 24 h culture in the medium containing 3.0 g of nicotine/l, and only 0.8% of the initial nicotine was detected after 48 h in the medium containing 4.0 g of nicotine/l (Table 2). These results suggest that strain J5 of *P. putida* has a higher nicotine-degrading efficiency than other nicotine-degrading bacteria previously studied: *O. intermedium* DN2 required about 36 h to completely degrade 0.5 g of

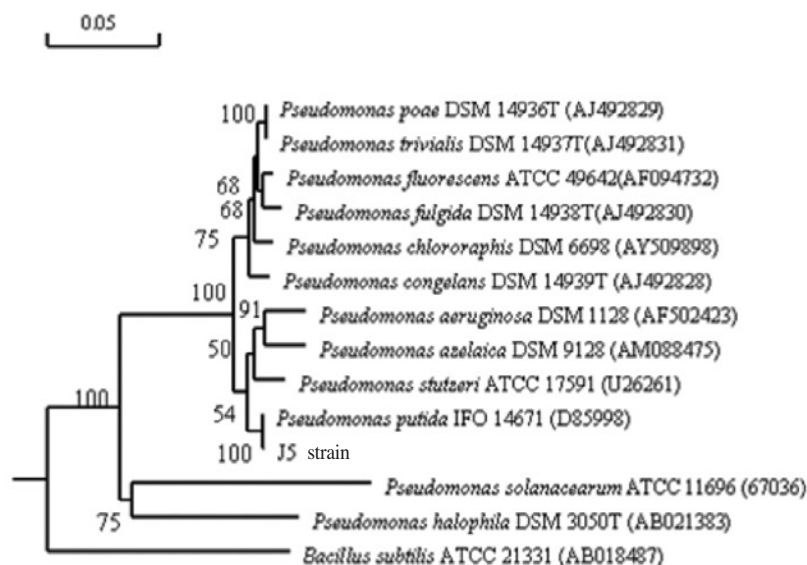


FIG. 2 - Phylogenetic tree based on a comparison of the 16S rRNA gene sequences of strain J5 and some close relatives. The tree was rooted using *Bacillus subtilis* ATCC 21331 (AB018487) as an outgroup. Bootstrap values, expressed as a percentage of 1000 replications, are given at the branching points. Bar indicates 5% sequence divergence.

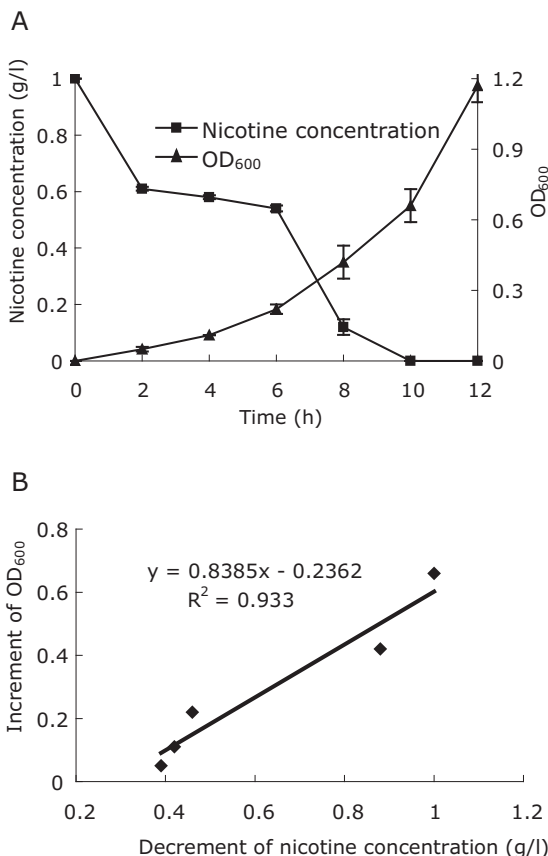


FIG. 3 - (A) Temporal relationship between strain J5 growth rate (change in cell density as determined by spectrophotometry) and nicotine concentration. (B) Regression analysis of cell density vs. nicotine concentration during logarithmic growth of strain J5. Bars indicate ± 1 SE.

nicotine/l (Yuan *et al.*, 2005) and *Pseudomonas* sp. HF-1 required about 25 h to degrade 99.6% of nicotine at 1.0 g/l (Ruan *et al.*, 2005).

Degradation of nicotine in tobacco leaves

Treatment with strain J5 reduced the concentration of nicotine in tobacco leaves by 11.72% (from 23.9 ± 0.8 to 21.1 ± 0.1 mg of nicotine/g leaf). In a previous study, *Debaryomyces nicotianae* and *Micrococcus nicotianae* reduced the nicotine content of tobacco leaves by only 0.45

TABLE 2 - Nicotine degradation in strain J5 cultures at different time

Initial nicotine concentration (g/l)	Nicotine degradation (%)			
	12 h	24 h	36 h	48 h
0.5	100	100	100	100
1.0	100	100	100	100
1.5	100	100	100	100
2.0	81	100	100	100
2.5	74	100	100	100
3.0	53	100	100	100
3.5	44	59	67	99.2
4.0	46	62	84	99.2

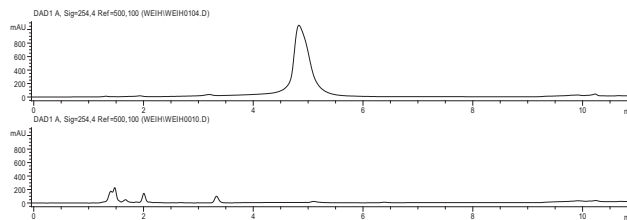


FIG. 4 - Determination of nicotine from strain J5 cultures by HPLC. The analysis was performed using the Agilent column ($5 \mu\text{m}$ 4.6×150 mm), with the mobile phase 40-50% methanol 5 min, 50% methanol 2 min, 100% methanol 4 min, 40% methanol 5 min, 1 ml/min flow rate, and determined by 254 nm ultraviolet. The retention time was 4.9 min. The arrow represents the nicotine peak.

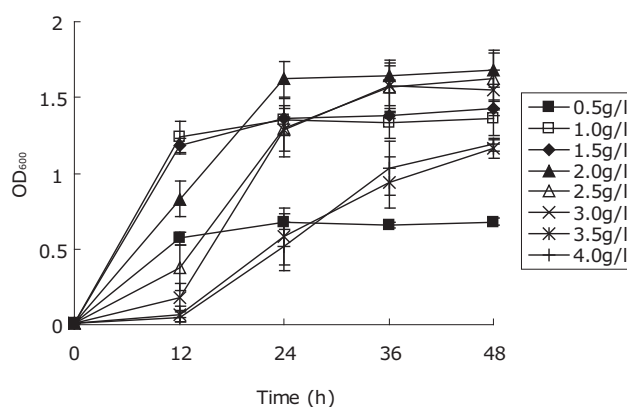


FIG. 5 - Effect of nicotine concentration on growth of strain J5 in a liquid medium. Bars indicate ± 1 SE.

and 0.83%, respectively (Giovannozzi-Sermanni, 1947). Our results suggest that J5 can quickly degrade nicotine in tobacco leaves. Ongoing research in our laboratory is optimising the production process of strain J5 and application measures to improve its degrading efficiency.

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