Full Length Research Paper

Isolation and characterization of *Acinetobacter* sp. ND12 capable of degrading nicotine

Hongjuan Li[1], Yanqing Duan[2], Guanghui Ma[1], Liping Lei[3], Ke-Qin Zhang[1] and Jinkui Yang[1]*

[1]Laboratory for Conservation and Utilization of Bio-Resources, and Key Laboratory for Microbial Resources of the Ministry of Education, Yunnan University, Kunming 650091, P. R., China.
[2]Technology Centre of Hongyun Honghe Tobacco (Group) Co, Ltd., Kunming 650202, P. R., China.
[3]Tobacco Agricultural Science Institute of Yunnan Province, Yuxi 653100, P. R., China.

A bacterium strain ND12, was isolated from the tobacco plantation soil and identified as *Acinetobacter* sp. based on its morphology, physiology, and 16S rRNA gene sequence. The isolate could utilize nicotine as the sole source of carbon and nitrogen, and completely degrade 1.0 g/L nicotine within 14 h at 28°C and pH 6.0. The optimum nicotine concentration for the growth of strain ND12 was 2.5 g/L. Strain ND12 grew at a broader pH range than other reported nicotine-degrading bacteria and showed higher nicotine-degrading activity than those bacteria at acidic environment (pH 4.0). The resting cells of *Acinetobacter* sp. ND12 could decompose 90% of the nicotine in upper tobacco leaves after treating for 11 h. Moreover, the nicotine metabolites of strain ND12 were analyzed by high performance liquid chromatography (HPLC) and compared with *Arthrobacter* sp. HJ2-3 and *Pseudomonas* sp. Nic22. Our results suggested that strain ND12 could be useful for reducing nicotine concentration in nicotine-polluted environments and tobacco wastes.

Key words: Nicotine, biodegradation, *Acinetobacter* sp. ND12, resting cells, tobacco leaves.

INTRODUCTION

Nicotine in tobacco plants is produced by the ornithine metabolic pathway and, together with various leaf treatments, is responsible for the smoking proprieties of cigarettes. Nicotine is not a direct cause of most tobacco smoking-related diseases, but it is highly addictive (Benowitz et al., 1999). Smoking accounts for at least 30% of all cancer deaths and 87% of lung cancer deaths. About 80 to 90% of inhaled nicotine is absorbed during smoking as assessed using ^{14}C-nicotine (Armitage et al., 1975). Nicotine can penetrate biological membranes and the blood-brain barrier easily to exert its effects (Schievelbein, 1982). Smoking has enormous negative health consequences worldwide, and unfortunately, the worldwide tobacco consumption is still rising (Mackay et al., 2006).

During the manufacturing and processing of tobacco products, both solid and liquid wastes are generated in high concentrations with nicotine as the main toxic compound. Typically, these wastes cannot be recycled. Nicotine is soluble in water and can be easily transported into groundwater (Civilini et al., 1997). Moreover, in China, upper tobacco leaves are not used for cigarette manufacturing because of their high nicotine content ranging from 3 to 5% (w/w), and form an important source of tobacco waste (Zhang et al., 2003). Therefore, detoxification and reduction of these tobacco waste products by removal of nicotine is a major environmental challenge.

Many bacteria capable of utilizing nicotine have been isolated and characterized (Brandsch, 2006). They include predominantly members of the genera *Pseudomonas* (Chen et al., 2008; Ruan et al., 2005; Tang et al., 2008, 2009; Wang et al., 2005, 2009a) and *Arthrobacter* (Brandsch et al., 1982; Igloi and Brandsch, 2003; Schenk et al., 1998). These microbes are of

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*Corresponding author. E-mail: jinkuiyun@yahoo.com.cn Tel: 86-871-5032538. Fax: 86-871-5034838.

increasing interest due to their potential to reduce the nicotine content in tobacco leaves during tobacco manufacturing and the disposal of tobacco wastes (Chen et al., 2008; Wang et al., 2005). *Acinetobacter* is a Gram-negative genus of bacteria belonging to the *Gamma-proteobacteria* (Vanbroekhoven et al., 2004). They are important soil organisms where they contribute to the mineralization of, for example, aromatic compounds. However, there is no report of nicotine degradation by *Acinetobacter*. In this study, a novel nicotine-degrading bacterium *Acinetobacter* sp. ND12 was isolated from a tobacco plantation soil and identified. We characterized the range of conditions during its degradation of pure nicotine. Moreover, the biodegradation of nicotine in upper tobacco leaves by the strain was also examined.

MATERIALS AND METHODS

Samples and culture conditions

(S)-Nicotine (>99.0% purity) was purchased from Sigma Company (USA). All other chemicals were of analytical grade. The soil sample was taken from a tobacco plantation soil in Kunming, Yunnan Province, China.

Luria-Bertani medium (LB), the enrichment medium (EM), and an inorganic salt medium (ISM) were prepared according to our recent report (Gong et al., 2009). ISM was used for determining the nicotine-degrading activity of bacteria. The initial pH of ISM was adjusted to 7.0, and a certain amount of nicotine was added to the media after sterilization, according to experimental design. All experimental liquid cultures were aerated at 150 rpm and a fixed temperature designated by the specific test.

Pseudomonas sp. Nic22 was identified in our previous work (Chen et al., 2008). *Arthrobacter* sp. HJ2-3 was isolated from the tobacco plantation soil and its 16S rRNA gene has been submitted to GenBank under accession number HM449827, which showed a high degree of identity (98.8%) to nicotine-degrading bacterium *Arthrobacter nicotinovorans*. *Acinetobacter* sp. strain ND12 isolated in this work had been deposited in China general microbiological culture collection center under accession number CGMCC 3410.

Isolation of a nicotine-degrading bacterium

Two grams of soil was inoculated into 100 ml of EM medium containing 0.5 g/L nicotine, and incubated at 28°C and 150 rpm for 7 days. Enrichment of nicotine-degrading bacterium was performed by transferring 10 ml of the culture to fresh EM medium for 7 days and gradually increasing the concentration of nicotine from 0.5 to 1.0 g/L. After four cycles of enrichment in the EM medium, the culture was transferred into ISM medium (containing 1.0 g/L nicotine), spread onto LB plates using the dilution plate method, and incubated at 28°C for 48 h. Purified colonies were then inoculated into liquid ISM containing 1.0 g/L nicotine to test their nicotine degradation activity. One isolate capable of degrading nicotine was found and named strain ND12. This isolate was selected for further experiments.

Identification of strain ND12

The morphology of strain ND12 was observed under a light microscope (Olympus BX51, Japan). Physiological and biochemical characteristic assays were determined according to the methods of Dong and Cai (2001). The genomic DNA of strain ND12 was extracted using the method described by Wilson (1997). The 16S rRNA gene was amplified by PCR using Taq polymerase and the universal primer pair of 20F (5'-GAGTTTGATCCTGGCTCAG-3') and 1500R (5'-GTTACCTTGTTACGACTT-3') described by Weisburg et al. (1991). The 16S rRNA sequence of strain ND12 and reference sequences obtained from GenBank were aligned using multiple sequence alignment software Clustal_X version 1.83 (Thompson et al., 1997). A phylogenetic tree was constructed using Mega 4.0 (Kumar et al., 2008) based on the 16S rRNA sequences of strain ND12 and other bacteria.

Optimal conditions for growth and nicotine degradation

5 ml of ISM at an initial pH7.0 containing 0.5 to 4.5 g/L nicotine, respectively, were inoculated with 50 µl cell suspension of strain ND12 and incubated at 28°C. The nicotine concentration of 1.0 g/L was subsequently tested with a variety of pH conditions adjusted with 1 M of HCl or NaOH to pH 2 to 11, respectively. In addition 50 µl cell suspension of strain ND12 was transferred into 5 ml of nicotine inorganic salt medium with nicotine concentration of 1.0 g/L and pH 6.0 and incubated at 15 to 37°C, respectively, at 150 rpm for 12 h. The non-inoculated ISM was used as a negative control. Each test was repeated three times.

Preparing of resting cells and nicotine degradation

Resting cells cultured in both ISM and LB media were prepared according to the reported methods (Gong et al., 2009; Wang et al., 2005). The degradation experiment were performed in a 250-ml flask with the cell concentration of OD_{600} at 0.5, 1.0 nicotine (g/L), and 50 ml sterilized 0.05 M sodium phosphate buffer (pH 7.0) at 28°C with a constant shaking at 150 rpm. Degradation of nicotine in tobacco leaves was carried out in a 250-ml flask containing 5 ml resting cells, 2 g tobacco leaves (Baoshan, Hongda, B_3F) and 30 ml sterilized sodium phosphate buffer (0.05 M, pH 6.0) with a constant shaking at 150 rpm at 28°C.

Analytical methods

Cell growth was determined using a Nucleic Acid and Protein Analyzer (Beckman DU 640, USA). After the cells in the samples were removed by centrifugation at 12,000 g for 10 min at 4°C, the supernatant was used for nicotine content analysis. The nicotine and related metabolites were analyzed by high-performance liquid chromatography (HPLC, Agilent 1200 Series, USA), equipped with an SB C-18 column (5 mm, 4.6 × 250 mm) and an ultraviolet detector operating at 230, 259 and 295 nm.

Comparison of nicotine metabolites by strain ND12, *Pseudomonas* sp. Nic22 and *Arthrobacter* sp. HJ2-3

Resting cells of strain ND12, *Pseudomonas* sp. Nic22, and *Arthrobacter* sp. HJ2-3 were prepared and degradation experiments were performed as previously described. The cultures were sampled at 2, 4, 6, and 8 h, respectively, and nicotine and related metabolites in the culture were determined by HPLC.

Table 1. Partial biochemical characteristics of *Acinetobacter* sp. ND12.

Characteristic	Strain ND12
Morphology	Short rod
Gram staining	-
Growth at 4°C	-
Growth at 40°C	-
Aerobic growth	+
Oxidase	-
Catalase	+
Nitrate reduction	+
Indole production	+
Methyl red test	+
Voges-Proskauer test	-
Phenylalanine deaminase	-
Hydrolysis of	
Gelatin	-
Starch	-
Fermentation/oxidation of	
Glucose	+
Mannitol	-
D-xylose	+
Sucrose	-
Lactose	–
Ethanol	+
Citrate utilization	+
Origin	soil

+, Positive; -, Negative.

RESULTS

Isolation and identification of a nicotine-degrading bacterium

A nicotine-degrading Gram-negative bacterium, strain ND12, was isolated from a tobacco plantation soil. This rod-shaped bacterium could use nicotine as the sole source of carbon, nitrogen and energy for its growth. Colonies of strain ND12 incubated on LB plates for 48 h were typically hoar, opaque, circular, low-convex, about 0.5 to 1 mm in diameter. The physiological and biochemical characteristics of strain ND12 are summarized in Table 1. These morphological and biochemical properties of strain ND12 were consistent with bacteria of the genus *Acinetobacter*.

The 16S rRNA gene sequence of strain ND12 (comprising 1435 nucleotides) was determined and deposited in the GenBank database under accession number GU176412. A phylogenetic tree was constructed based on 16S rRNA gene sequences (Figure 1). Strain

ND12 was related to the *Acinetobacter* sp. lineage, and closely clustered with *Acinetobacter lwoffii* (GenBank accession no. AY176770). Therefore, phylogenetic analysis further confirmed that strain ND12 belongs to the genus *Acinetobacter*.

Conditions for optimal growth and nicotine degradation

To determine the optimal conditions of nicotine utilization as sole source of carbon and nitrogen, the growth of strain ND12 was assayed under different conditions of pH, temperature and nicotine concentration. Strain ND12 could grow and degrade nicotine at the temperature ranging from 15 to 37°C, with an optimal growth rate at 28°C, and a sharp decrease in growth rate was observed above 33°C. Therefore, 28°C was used to culture the bacterium and in all subsequent work. Growth of strain ND12 with nicotine as the sole carbon and nitrogen source was observed from pH 3 to 10, with an optimum between pH 4 and 6. Little growth was observed at pH 2 or 11. Growth of strain ND12 was observed with a nicotine concentration ranging from 0.5 to 4.0 g/L. The optimal nicotine concentration was 2.5 g/L, while a sharp decrease in growth rate was observed at concentrations above 3.0 g/L, and no growth was observed at concentration above 4.5 g/L. Under the optimal temperature and pH conditions (28°C, pH 6.0), the complete degradation of 1.0 g/L nicotine was observed in a shaker-incubator (150 rpm) after 14 h (Figure 2). Growth of strain ND12 started after a short lag phase of about 6 h, and a significant correlation was observed between the increase in optical density and a decrease in nicotine concentration in the medium, indicating that degradation of nicotine was mainly dependent on the growth of strain ND12 (Figure 2).

Nicotine degradation by resting cells of strain ND12

Nicotine degradation by resting cells of strain ND12 prepared from both ISM (containing nicotine) (induced conditions) and LB medium (non-induced conditions) was investigated. As shown in Figure 3A, induced resting cells (prepared from ISM growth medium) were able to degrade nicotine completely within 13 h, while resting cells prepared from LB were able to degrade about 83.3% nicotine, but at a much slower rate. Moreover, resting cells prepared from ISM (containing 1.0 g/L nicotine) also degraded nicotine in leaves from the top part of the tobacco plants (henceforth called upper tobacco leaves). As shown in Figure 3B, after treatment by resting cells for 11 h, 90% of nicotine in upper tobacco leaves was degraded. However, the nicotine concentration was basically unchanged in the control sample.
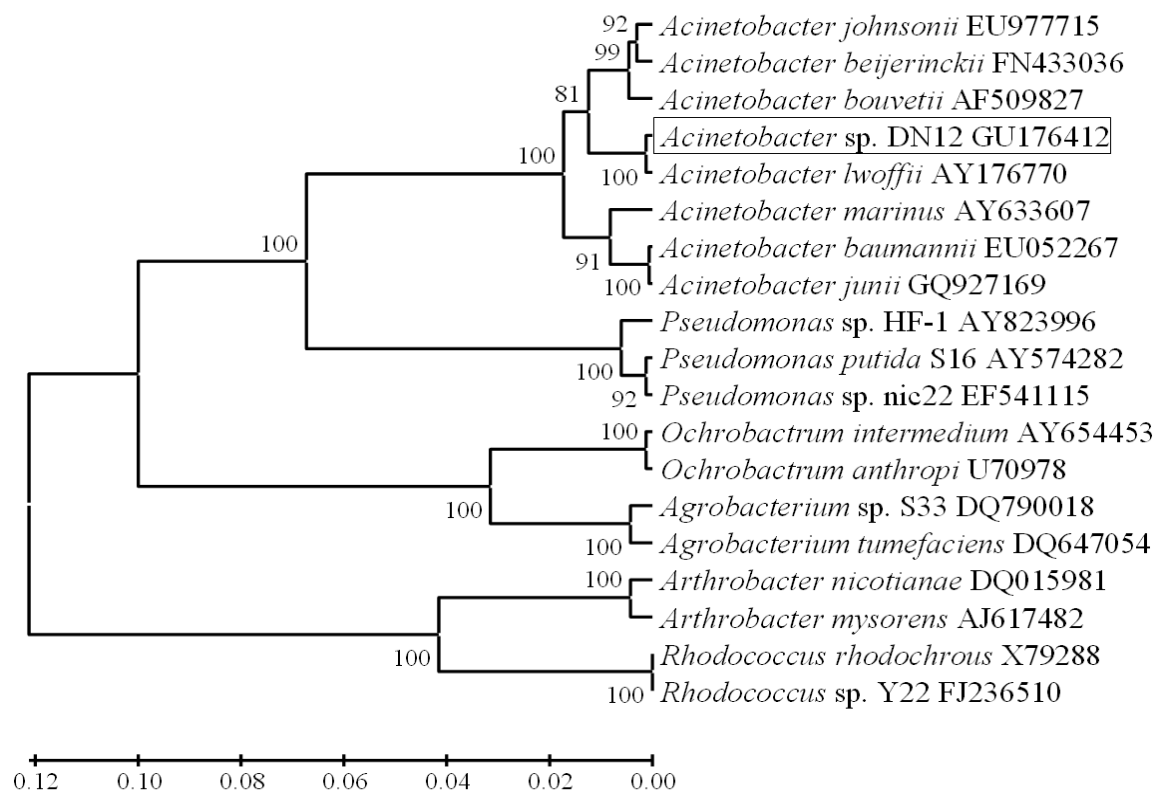
Figure 1. Phylogenetic tree based on 16S rRNA sequences using the unweighted pair group method with arithmetic mean (UPGMA) method in the MEGA 4.0 program. Nucleotide sequences were aligned using Clustal_X version 1.83 with default parameters. Confidence in the topology of phylogenetic trees was evaluated by performing 1000 bootstrap replicates in the program. Scale indicates percent of substitutions per nucleotide position.
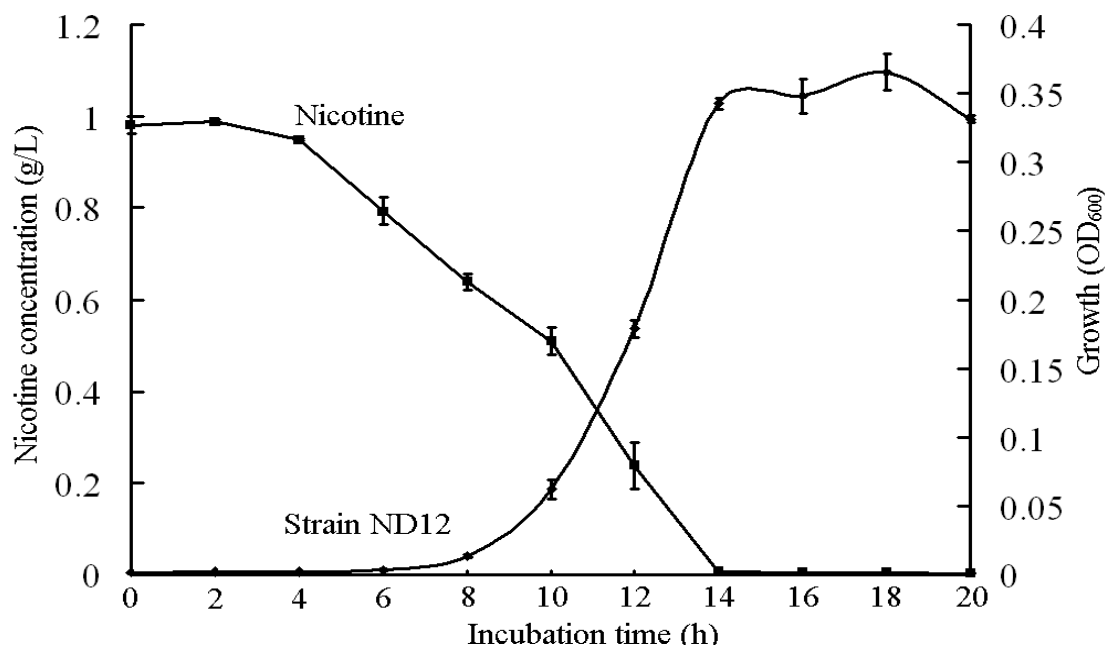

Figure 2. Utilization of nicotine as sole carbon source for growth by *Acinetobacter* sp. ND12 (medium of ISM containing 1.0 g/L nicotine, 28°C, pH 6.0, 150 rpm). Error bars indicate standard error (n = 3).
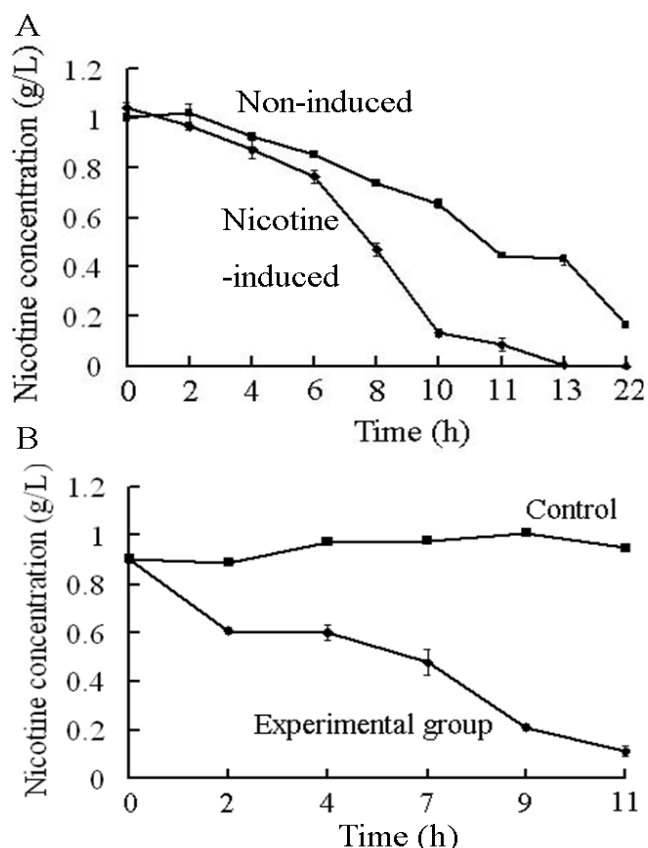
Figure 3. Nicotine degradation by resting cells of strain ND12. (A) Efficiency of nicotine degradation by nicotine-induced resting cells and by non-induced resting cells. (B) Degradation of nicotine in upper tobacco leaves by nicotine-induced resting cells of strain ND12 and control (non-induced resting cells).

Comparison of nicotine metabolites by strain ND12, *Pseudomonas* sp. Nic22 and *Arthrobacter* sp. HJ2-3

Nicotine and related metabolites of three bacteria were analyzed by HPLC at different time points, and the retention times of metabolites from three bacteria were compared. Only one peak was determined in culture of *Arthrobacter* sp. HJ2-3 before 4 h, while three peaks appeared between 6 and 8 h. interestingly, peaks with same retention time and UV absorption spectra were found in the cultures of strain ND12 and *Pseudomonas* sp. Nic22, which suggested that they may have similar metabolic intermediates and nicotine degradation pathways.

DISCUSSION

Bacteria of the genus *Acinetobacter* are involved in the biodegradation of a number of pollutants such as chlorobiphenyl (Shields et al., 1985), aniline (Wyndham,

1986) and phenol (Hoyle et al., 1995). In this study, a novel nicotine-degrading bacterium, strain ND12, was isolated from tobacco plantation soil and identified as *Acinetobacter* sp. ND12. Nicotine-degrading bacteria were broadly distributed in tobacco plants and in the soil, and belonged to different genera (Figure 1). Aside from the two common genera, *Arthrobacter* (Brandsch et al., 1982; Igloi and Brandsch, 2003; Schenk et al., 1998) and *Pseudomans* (Ruan et al., 2005; Wang et al., 2005, 2007; Wei et al., 2008, 2009), several novel nicotine-degrading bacteria such as *Ochrobactrum intermedium* (Yuan et al., 2007), *Rhodococcus* sp. Y22 (Gong et al., 2009) and *Agrobacterium* sp. S33 (Wang et al., 2009b) were recently reported.

Strain ND12 grew well in medium containing 2.5 g/L nicotine and could tolerate a high concentration of nicotine up to 4.0 g/L. Moreover, strain ND12 grew at a broader pH range than other nicotine-degrading bacteria. For example, *Agrobacterium* sp. S33 showed nicotine degradation activity at pH 5.5 to 8.0 (Wang et al., 2009b); *Pseudomonas* sp. S16 (Wang et al., 2005) and *Pseudomonas* sp. nic22 (Chen et al., 2008) at pH 5.5 to 7.5; and *Pseudomonas* sp. HF-1 at a pH range from 5.5 to 9.0 (Ruan et al., 2005). While the strain ND12 was observed to grow from pH 3 to 10, its optimum was at pH 4 to 6, which suggest that strain ND12 prefers an acidic environment and showed higher nicotine-degrading activity at low pH condition. Interestingly, a slight decline of pH in ISM was determined after reaction, suggesting that some acidic substances were generated during nicotine degradation. Furthermore, many species of *Acinetobacter* are found in activated sludge communities (Barberio and Fani, 1998). Therefore, *Acinetobacter* sp. ND12 might also be able to survive in such conditions and could potentially serve as an ideal candidate for bioremediation of environments polluted by tobacco wastes.

During the degradation of nicotine by *Acinetobacter* sp. ND12, a green to yellow pigment was observed in the ISM liquid medium containing nicotine, but not observed in the ISM medium without nicotine. The phenomena have been described in other nicotine-degrading bacteria. The culture supernatant turned blue after nicotine was degraded by *A. nicotinovorans* (Brandsch et al., 2006) and *Rhodococcus* sp. Y22 (Gong et al., 2009), and a virescent pigment was produced by *Pseudomonas* sp. HF-1 (Wang et al., 2005), while culture supernatant changed initially into bright green, then greenish blue and blue gray during nicotine degradation by *Agrobacterium* sp. S33 (Wang et al., 2009b). These different pigments produced by bacteria indicate that nicotine might be processed via different pathways and produce different metabolites. At present, the pathway of nicotine degradation to produce a water-soluble pigment by strain *Acinetobacter* sp. ND12 remains unknown. In the reported nicotine-degrading bacteria, only *Pseudomonas* sp. Nic22

(Chen et al., 2008) and HF-1 (Ruan et al., 2005) could produce green pigment. Combined with the analysis of nicotine degradation by *Acinetobacter* sp. ND12 and *Pseudomonas* sp. Nic22, we proposed that the nicotine metabolic pathway of strain ND12 may be similar to that of members of the genus *Pseudomonas*. Recently, significant advances have been achieved on nicotine biotransformation by *Pseudomonas* spp. (Ruan et al., 2005; Tang et al., 2008, 2009; Wang et al., 2005, 2009a; Wei et al., 2008, 2009). However, the nicotine metabolisms by *Pseudomonas* spp. were complex, and four putative catabolic pathways have been proposed (Li et al., 2010). Therefore, further study on nicotine degradation by strain ND12 is necessary.

Although, there are several reports of biodegradation by *Acinetobacter* sp., this is the first report on *Acinetobacter* sp. regarding its activity of degrading nicotine as sole carbon and energy source. Our results suggest that strain ND12 could be an effective agent for reducing nicotine concentration in upper tobacco leaves and wastes with a high nicotine concentration.

ACKNOWLEDGMENTS

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