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ORIGINAL ARTICLE

Occurrence and variability of tobacco rhizosphere and phyllosphere bacterial communities associated with nicotine biodegradation

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Abstract With the aim of learning more about functional bacterial communities in the tobacco rhizosphere and phyllosphere, a total of 96 nicotine-degrading (ND) bacterial strains isolated using nicotine as the sole carbon source—56 from the rhizosphere and 40 from the phyllosphere—were quantified and analyzed phylogenetically. The ND efficiency of 19 phyllosphere strains (47.5 %) and 39 rhizosphere strains (69.6 %) exceeded 90 %. Patterns of phylogenetic relationships based on 16S rRNA gene sequences revealed a high heterogeneity of community composition and suggested the existence of microenvironment-specific communities. The phyllospheric ND bacterial community was distributed over ten genera, of which *Pseudomonas* spp. was the dominant population. However, the rhizospheric ND bacterial community was composed of six genera, of which *Arthrobacter* spp. was the major group. This is the first report of members of genera *Massilia*, *Erwinia*, *Brevundimonas* and *Paenibacillus* capable of degrading nicotine. Diversity indices were calculated provisionally using sequence data obtained from each ND bacterial library. The species richness, diversity and dominance index of the ND bacterial community of the phyllosphere were higher than that of rhizosphere community, while the evenness index of the phyllopheric community was lower compared to rhizospheric ND bacteria. Metabolic intermediate detection showed that the Pseudomonads isolates possessed all four proposed metabolic pathways of nicotine degradation while the *Arthrobacter* strains all had only one pyridine pathway. These results greatly enhance our knowledge of the diversity of ND bacteria and demonstrate that the tobacco-associated micro-environment contain diverse and novel ND bacteria, which might be a valuable biotechnological resource for biodegradation of nicotine.

Keywords Biodegradation · Tobacco · Nicotine · Phyllosphere · Rhizosphere · 16S rRNA

Introduction

Nicotine [1-methyl-2-(3-pyridyl-pyrrolidine), $C_{10}H_{14}N_2$] is the main alkaloid component of cigarettes and the main non-recyclable powdery waste in the tobacco manufacturing process. Nicotine accounts for as much as 3 % of dry leaf mass (Armstrong et al. 1998). The media center of the World Health Organization reported that tobacco accounted for almost 6 million deaths every year (including over 600,000 deaths from exposure to second-hand smoke), and is projected to increase to 8 million by 2030 (http://www.who.int/mediacentre). Tobacco processing creates high nicotine-containing solid and liquid wastes that have been classified as 'toxic and hazardous wastes' by European Union Regulations (Novotny and Zhao 1999). Therefore, decreasing the nicotine content in tobacco waste is an urgent requirement for tobacco product and environmental remediation.

Biological treatment with microbes is an economical and efficient approach to manipulating nicotine content during production and the detoxification of tobacco wastes containing high concentrations of nicotine. The bacterial communities residing in the tobacco-associated microenvironment have presumably adapted to make use of nicotine and have evolved biochemical strategies to metabolize nicotine and its derivatives (Brandsch 2006). From the mid-twentieth century

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onwards, microbial organisms have been used as research materials for nicotine degradation. *Pseudomonas* sp. 41 (Wada and Yamasaki 1954) and *Arthrobacter oxydans* (Sguros 1955) are the two earliest nicotine-degrading (ND) bacterial isolates known. In recent years, significant advances have been made in the field of nicotine biodegradation resources and the metabolic mechanisms of nicotine biotransformation. Many new bacterial genera with ND ability have been reported, such as species of *Ochrobactrum* (Yuan et al. 2005), *Agrobacterium* (Wang et al. 2009), *Rhodococcus* (Gong et al. 2009), *Acinetobacter* (Li et al. 2011), *Shinella* (Jiang et al. 2011) *Sphingobacterium* (Ma et al. 2012) and *Sinorhizobium* (Ma et al. 2012). However, *Pseudomonas* spp. and *Arthrobacter* spp. remain the two most investigated groups for nicotine biodegradation. In the genus *Arthrobacter*, the pyridine pathway of nicotine degradation is thoroughly elucidated and the related enzymes have been well characterized (Gherna et al. 1965; Schenk et al. 1998; Baitsch et al. 2001; Igloi and Brandsch 2003; Sachelaru et al. 2005; Brandsch 2006; Chiribau et al. 2006). In the Gram-negative *Pseudomonas* strains, four degradation pathways (via N-methylmyosmine, cotinine, nicotyrine, and nornicotine, respectively) have been proposed and partially investigated (Tang et al. 2008; Li et al. 2010). Recently, a few successful studies have attempted to investigate the bacterial diversity on tobacco leaves using culture-independent methods (CIMs) (Huang et al. 2010; Su et al. 2011; Su et al. 2012). However, little is known about the difference in distribution and diversity of ND bacteria in the tobacco rhizosphere and phyllosphere.

To examine the impact of the microenvironment on the abundance and diversity of bacterial communities, isolates were identified from the tobacco rhizosphere and phyllosphere with a nicotine cultivation-dependent approach with subsequent phylogenetic analysis based on bacterial 16S rDNA sequencing. Additionally, we were able to identify the potential metabolic pathways of some of the most efficient isolates by detecting intermediate degradation products.

Materials and methods

Chemicals

Nicotine, cotinine, and nornicotine were purchased from Sigma-Aldrich (St. Louis, MO) as standards. Pseudooxynicotine, nicotyrine and 6-hydroxynicotine were obtained from Toronto Research Chemicals (Toronto, ON, Canada). All analytical and high-performance liquid chromatography (HPLC) grade reagents were bought from Merck China (Beijing, China).

Sample preparation and ND-bacteria isolation

Tobacco rhizosphere and leaf samples were collected from the field of Yanhe Base established in 1947 of Yunnan Tobacco Research Institute, Yuxi, Yunnan, China. The study site is located at N24.258°, E102.501° and 1,689 m above sea level and has a mild subtropical highland climate with over 2,300 h sunlight a year in the region. This soil had a pH of 7.3 and contained 0.05 % available nitrogen and 1.5 % organic matter. One-third of the 688-mm annual rainfall occurs from May to September. Triplicate tobacco rhizosphere samples, defined as the adhering soil particles, were taken about 40 m apart and collected by gently shaking the fresh root systems in air and blending as reported previously (Zhang et al. 2007). Rhizosphere soil sample (5 g) was suspended in 45 mL sterile distilled water with glass beads, and shaken at 200 rpm for 30 min. The culture was filtered through four layers of sterile gauze (cotton) to remove the slurry and passed through 0.2 μm filters (Whatman, Dassel, Germany) under low vacuum (−200 to −400 mbar) to collect bacteria. The filter membranes were suspended in 10 mL sterile distilled water and eluted by ultrasonication. The bacteria were pelleted from the elution by centrifugation at 12,000 rpm for 5 min and resuspended in 1 mL sterile distilled water. The final bacterial suspension was spread on nicotine medium (NIM) plates (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, Steinheim, Germany) was the sole carbon source. After 72 h at 30 °C, individual colonies were transferred and streaked on new agar plates. This procedure was repeated until all the colonies in the same plate showed identical morphology. Then a Gram stain was performed and the cellular morphology was observed under a light microscope (Olympus BX51, Tokyo, Japan) to ensure the purity of the isolates. All the pure isolates were stored at −70 °C in LB broth supplemented with 20 % (w/v) of glycerol.

Fresh tobacco leaves were collected from mature plants and those with a superficial injury were excluded. After sampling, leaves were placed in sterile plastic bags, transported to the laboratory in an icebox, and analysed within 24 h. Phyllospheric bacteria were extracted from tobacco leaves according to a previous report with slight modification (Yang et al. 2001; Huang et al. 2010; Su et al. 2011). The leaves were cut into segments of 2–3 cm; 5 g leaves were then immersed in 45 mL sterile phosphate buffer (0.1 M, pH 7.2) and sonicated in an ultrasonic cleaner for 30 min. Supernatant bacterial suspension was recovered by filtration and centrifugation as described above. The final 1 mL phyllosphere bacterial suspension was spread onto NIM. The ND bacteria were screened, purified and stored as described above.

Degradation activity of ND bacteria

Purified single colonies of individual isolates were inoculated in LB liquid medium and cultured to saturation at 30 °C. Cultures (50 μL) were inoculated into 5 mL NIM liquid with 1 g/L nicotine and cultured for 12 h at 30 °C. Then all the samples were centrifuged for 5 min at 12,000 rpm. The nicotine concentrations in the supernatants were determined by high-pressure liquid chromatography (HPLC) (Agilent 1100 Series, Wilmington, DE) using an Agilent C-18 column (5 μm, 4.6×150 mm) as described previously (Wei et al. 2008). All experiments were performed independently at least twice with three replicates.

Sequencing of 16S rRNA gene and phylogenetic analysis

The 16S rRNA gene was amplified using primers 27 F (5′-GAG AGT TTG ATC CTG GCT CAG-3′) and 765R (5′-CTG TTT GCT CCC CAC GCT TTC-3′) (Coombs and Franco 2003). The PCR reaction mixtures contained 0.2 M each dNTP, 25 pM each primer, 100 ng template, 24 and 2 U *Taq* DNA polymerase (Takara, Kyoto, Japan) with a reaction buffer supplied by the manufacturer in a total volume of 100 μL. All reagents were mixed and processed in a PCR thermal cycler (Techgene, Cambridge, UK) under the following conditions: denaturation at 94 °C for 5 min; 35 cycles of 94 °C for 40 s, 55 °C for 40 s, and 72 °C for 1 min; followed by elongation at 72 °C for 10 min. PCR products were visualized in an agarose gel and purified with a QIAquick PCR purification kit (Takara). PCR products were ligated into the pMD18-T vector (Takara) and then transformed into *Escherichia coli* DH5α competent cells. The clone libraries were screened for ampicillin resistance and α-complementation using X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside). The 16S rDNA were sequenced with reverse and forward M13 primers at Shanghai BioAsia Biotechnology (Shanghai, China). About 750 bp of each clone was assembled by the two orientational sequences. Taxonomic assignments were done by comparing the clone sequences with the nucleotide database in GenBank by using BLAST. Sequences were aligned by the CLUSTAL W version 1.8 (Thompson et al. 1994). The phylogenetic trees were constructed using the neighbor-joining (NJ) method (Saitou and Nei 1987) with the Kimura two-parameter model (Kimura 1980). Bootstrap confidence values were obtained with 1,000 resamplings.

Diversity index analysis

Diversity indices, which include the Shannon-Weaner diversity index (H′), species richness (D) and evenness (J), were used to determine ND species composition and their diversity across the different samples according to a previous report (Hill et al. 2003). The H′ index is commonly used to characterize species diversity in a community; it accounts for both abundance and evenness of the species present. The proportion of a species relative to the total number of species (P_i) is calculated and then multiplied by the natural logarithm of this proportion ($\ln P_i$). We estimated the evenness of the numbers of bacterial species in each sample using Pielou's index (J). The formulae were used as follows:

$$H' = -\sum P_i \cdot \ln P_i$$

$$D = 1/\sum P_i^2$$

$$J = H'/\ln S$$

Detection of metabolic intermediates of nicotine biodegradation

In order to analyze the nicotine metabolic intermediates, the selected bacterial strains were cultured in NIM liquid with 1 g/L nicotine and incubated at 180 rpm, 30 °C for 6 h. The cells were removed by being centrifuged twice at 12,000 rpm for 10 min at 4 °C. The intermediate metabolites [CT (cotinine), NN (nornicotine), PN (pseudooxynicotine), NT (nicotyrine) and 6HN (6-hydroxynicotine)] of nicotine in the supernatants were determined by HPLC analysis, comparing the retention times and peak areas with those of standards. HPLC was performed on Agilent 1100 equipped with a Agilent C-18 column. The mobile phase was 85 % methanol at a flow rate of 0.6 mL/min, and the other conditions were adapted from the manufacturer and the method developed before (Xu et al. 2004; Qiu et al. 2012).

Nucleotide sequence accession numbers

The 16S rRNA gene sequences obtained in this study were deposited into the NCBI GenBank database under accession numbers KF429263 to KF429358.

Results and discussion

Isolation of ND bacteria from tobacco rhizosphere and phyllosphere

Over 100 bacterial strains were isolated on NIM where nicotine was the sole carbon source. Isolates with potential for

biotechnology applications would need to be readily cultivable, so isolates that proved difficult to maintain on media were removed from further study. All in all, a total of 96 bacterial strains were confirmed to utilize nicotine distinctly; 56 from the tobacco rhizosphere and 40 from the tobacco phyllosphere. There were 33 phyllosphere bacterial strains

Table 1 Tobacco phyllospheric nicotine-degrading (ND) bacteria

Isolate	Closest GenBank match			Nicotine degradation (%)[a]	Blue pigment[b]	Metabolic intermediates[c]
	Reference species (RS)	Accession no. of RS	Identity (%)			
L1	*Bacillus simplex*	FJ644693	99	17	−	nd
L2	*Pseudomonas putida*	D85999	98	100	−	PN
L3	*Pseudomonas putida*	DQ026520	99	96	−	PN
L4	*Pantoea dispersa*	EU244766	98	34	−	PN
L5	*Pseudomonas agarici*	D84005	98	92	−	PN
L6	*Arthrobacter oxydans*	EU833935	99	99	+	6HN
L7	*Pseudomonas putida*	EF595660	97	97	−	PN
L8	*Pseudomonas stutzeri*	DQ358054	99	100	−	PN
L9	*Pseudomonas putida*	D85999	98	97	−	PN
L10	*Pseudomonas putida*	D85999	98	95	−	PN
L11	*Pseudomonas stutzeri*	X98607	99	96	−	PN
L12	*Pseudomonas putida*	EF595660	97	97	−	PN
L13	*Paenibacillus polymyxa*	EU373421	96	15	−	nd
L14	*Pseudomonas stutzeri*	X98607	99	93	−	PN
L15	*Pseudomonas stutzeri*	DQ211352	99	93	−	PN
L16	*Pseudomonas stutzeri*	DQ518613	99	94	−	PN
L17	*Pseudomonas stutzeri*	DQ211352	99	100	−	PN
L18	*Pseudomonas stutzeri*	DQ211352	100	99	−	PN
L19	*Arthrobacter* sp.	EU710551	100	90	+	6HN
L20	*Arthrobacter* sp.	EU672428	100	92	+	6HN
L21	*Pseudomonas putida*	D85999	98	92	−	PN
L22	*Pseudomonas putida*	FM611294	99	90	−	PN
L23	*Arthrobacter nitroguajacolicus*	FM213395	99	100	+	6HN
L24	*Arthrobacter oxydans*	EU833935	99	100	+	6HN
L25	*Pantoea dispersa*	EU244766	98	38	−	nd
L26	*Massilia timonae*	AM237371	99	32	−	nd
L27	*Pseudomonas putida*	FJ217182	99	100	−	PN
L28	*Sphingomonas* sp.	EF061133	100	79	−	nd
L29	*Pseudomonas putida*	EU258552	98	99	−	PN
L30	*Erwinia persicina*	EU681952	99	54	−	nd
L31	*Pantoea* sp.	FJ646663	99	36	−	nd
L32	*Cellulosimicrobium cellulans*	EU816697	100	62	−	nd
L33	*Pseudomonas stutzeri*	DQ211352	99	89	−	PN
L34	*Pseudomonas stutzeri*	DQ211352	100	93	−	PN
L35	*Brevundimonas vesicularis*	EU862355	99	23	−	nd
L36	*Pseudomonas putida*	FJ217182	99	100	−	PN
L37	*Pseudomonas stutzeri*	DQ211352	100	89	−	PN
L38	*Pseudomonas stutzeri*	DQ358054	99	92	−	PN
L39	*Pseudomonas putida*	FJ472861	100	95	−	PN
L40	*Pseudomonas* sp.	FJ267585	99	99	−	CT

[a] Nicotine degradation (%) at 12 h in NIM with 1.0 g/L nicotine as sole carbon source
[b] + blue pigment detected around the colonies in nicotine medium; − no blue pigment
[c] 6HN, PN, CT and nd represent 6-hydroxynicotine, pseudooxynicotine, cotinine and no detection, respectively

Table 2 ND bacteria from tobacco rhizosphere

Isolate	Closest GenBank match			Nicotine degradation (%)[a]	Blue pigment[b]	Metabolic intermediates[c]
	Reference species (RS)	Accession no. of RS	Identity (%)			
J1	*Arthrobacter* sp.	AB461069	99	100	+	6HN
J2	*Arthrobacter* sp.	AB461069	99	98	+	6HN
J3	*Pseudomonas stutzeri*	DQ211352	99	92	−	PN
J4	*Arthrobacter oxydans*	EU333869	98	100	+	6HN
J5	*Pseudomonas putida*	CP000926	98	100	−	PN
J6	*Enterobacter* sp.	EU438990	100	42	−	nd
N7	*Ensifer adhaerens*	EF198418	99	100	−	nd
J8	*Pseudomonas* sp.	EU563230	99	89	−	NN, NT
J9	*Arthrobacter oxydans*	X83408	98	94	+	6HN
J10	*Bacillus subtilis*	AL009126	100	13	−	nd
J11	*Arthrobacter* sp.	AB461069	99	94	+	6HN
J12	*Arthrobacter globiformis*	EU221373	99	100	+	6HN
J13	*Pseudomonas stutzeri*	DQ211352	99	99	−	PN
J14	*Arthrobacter oxydans*	EU333869	98	100	+	6HN
J15	*Arthrobacter globiformis*	EU221373	99	100	+	6HN
N16	*Sinorhizobium meliloti*	EU603721	99	89	−	nd
J17	*Enterobacter* sp.	EU438990	100	50	−	nd
J18	*Pseudomonas stutzeri*	DQ211352	100	82	−	PN
J19	*Pseudomonas stutzeri*	DQ211352	99	82	−	PN
J20	*Arthrobacter* sp.	EU571174	99	92	+	6HN
J21	*Arthrobacter* sp.	EU710551	100	93	+	6HN
J22	*Arthrobacter oxydans*	EU833935	99	100	+	6HN
J23	*Pseudomonas stutzeri*	X98607	99	87	−	PN
J24	*Arthrobacter oxydans*	EU833935	99	99	+	6HN
J25	*Arthrobacter* sp.	FJ006892	100	97	+	6HN
J26	*Pseudomonas stutzeri*	DQ211352	99	93	−	PN
J27	*Arthrobacter* sp.	EU710551	100	93	+	6HN
J28	*Pseudomonas stutzeri*	X98607	99	96	−	PN
J29	*Arthrobacter* sp.	AB461069	99	95	+	6HN
J30	*Arthrobacter* sp.	EU571174	99	95	+	6HN
J31	*Arthrobacter globiformis*	EU221373	99	99	+	6HN
N32	*Sinorhizobium medicae*	EU445266	98	85	−	nd
J33	*Arthrobacter oxydans*	EU833935	99	100	+	6HN
J34	*Arthrobacter* sp.	DQ129877	99	93	+	6HN
J35	*Pseudomonas rhodesiae*	FJ462694	100	82	−	NN, NT
J36	*Arthrobacter* sp.	EU571174	99	99	+	6HN
J37	*Pseudomonas stutzeri*	DQ211352	99	93	−	PN
J38	*Arthrobacter globiformis*	EU221373	99	99	+	6HN
J39	*Arthrobacter* sp.	EU571174	99	89	+	6HN
J40	*Arthrobacter* sp.	AY439244	99	89	+	6HN
N41	*Sinorhizobium medicae*	EU445266	98	90	−	nd
J42	*Pseudomonas* sp.	FJ598323	97	87	−	CT
J43	*Arthrobacter globiformis*	EU221373	99	100	+	6HN
J44	*Pseudomonas* sp.	FJ598323	97	89	−	NN, NT
J45	*Arthrobacter* sp.	EU710551	99	99	+	6HN
J46	*Arthrobacter* sp.	EU710551	99	93	+	6HN
J47	*Arthrobacter* sp.	EU571174	99	93	+	6HN

Table 2 (continued)

Isolate	Closest GenBank match			Nicotine degradation (%)[a]	Blue pigment[b]	Metabolic intermediates[c]
	Reference species (RS)	Accession no. of RS	Identity (%)			
J48	*Bacillus pumilus*	FJ494698	99	20	−	nd
J49	*Arthrobacter* sp.	EU571174	98	93	+	6HN
J50	*Arthrobacter* sp.	FJ378036	98	98	+	6HN
J51	*Pseudomonas* sp.	FJ598323	97	89	−	CT
J52	*Arthrobacter* sp.	EU571174	99	92	+	6HN
J53	*Arthrobacter* sp.	AB461069	99	92	+	6HN
J54	*Arthrobacter globiformis*	EU221373	99	100	+	6HN
J55	*Arthrobacter* sp.	DQ129877	99	97	+	6HN
J56	*Arthrobacter oxydans*	X83408	98	100	+	6HN

[a] Nicotine degradation (%) at 12 h in NIM with 1.0 g/L nicotine as sole carbon source
[b] + blue pigment was detected around the colonies in nicotine medium; − no blue pigment
[c] 6HN, PN, CT, NN, NT and nd represent 6-hydroxynicotine, pseudooxynicotine, cotinine, nornicotine, nicotyrine and no detection, respectively

capable of degrading 50 % nicotine (1 g/L) in 12 h, which represents 82.5 % of ND isolates from the tobacco phyllosphere (Table 1). Moreover, 19 phyllosphere bacterial strains (47.5 %) metabolized more than 90 % of the nicotine and 7 strains (17.5 %) could completely degrade all of the nicotine (Table 1). By contrast, in rhizosphere ND bacteria, the degrading efficiency of 52 strains (92.8 %), 39 strain (69.6 %) and 12 strains (21.4 %) exceeded 50 %, 90 % and 100 %, respectively (Table 2). In addition, 5 phyllospheric isolates and 34 rhizospheric isolates produced blue pigment around the colonies on nicotine medium; such a blue pigment has been reported previously during the degradation of nicotine by *Arthrobacter* spp. (Hochstein and Rittenberg 1959). A blue-violet water-soluble pigment is produced for the metabolization of nicotine to 2,3,6-trihydroxypyridine, which shows rapid spontaneous oxidation in the presence of oxygen (Holmes and Rittenberg 1972). So, it was presumed that the ND isolates producing blue pigment in this study were *Arthrobacter* spp., which will be confirmed by 16S rRNA analysis as below.

Sequence analysis of 16S rRNA genes

The cultivable isolates degrading nicotine were characterized by comparative sequence analysis of the 16S rDNA generated by PCR, obtaining determinations of approximately 750 base pairs of the complete gene sequence. The 16S rDNA sequences were compared with sequences in the NCBI database using BLAST. The results are shown in Tables 1 and 2. All the ND isolates could be assigned with confidence to a bacterial

Table 3 Summary of the phylogenetic assignments of ND bacteria

Major taxon and group	Genus	No. of organisms identified[a]		
		Rhizosphere	Endophytic	Total
High G+C Gram positive	*Arthrobacter*	34 (60.7)	5 (12.5)	39 (40.6)
	Cellulosimicrobium	0 (0)	1 (2.5)	1 (1.0)
Low G+C Gram positive	*Bacillus*	2 (3.6)	1 (2.5)	3 (3.1)
	Paenibacillus	0 (0)	1 (2.5)	1 (1.0)
Alphaproteobacteria	*Sphingomonas*	0 (0)	1 (2.5)	1 (1.0)
	Brevundimonas	0 (0)	1 (2.5)	1 (1.0)
	Ensifer	1 (1.8)	0 (0)	1 (1.0)
	Sinorhizobium	3 (5.4)	0 (0)	3 (3.1)
Betaproteobacteria	*Massilia*	0 (0)	1 (2.5)	1 (1.0)
Gammaproteobacteria	*Pseudomonas*	14 (25.0)	25 (62.5)	39 (40.6)
	Pantoea	0 (0)	3 (7.5)	3 (3.1)
	Erwinia	0 (0)	1 (2.5)	1 (1.0)
	Enterobacter	2 (3.6)	0 (0)	2 (2.1)
Total		56	40	96

[a] Values presented as number of clones (percentage of clones)

genus (≥97 %). The patterns obtained revealed a high heterogeneity of community composition and suggested the existence of microenvironment-specific communities. A higher number of genera (ten) was isolated from the tobacco phyllosphere, while only six genera were found from the tobacco rhizosphere (Table 3). Sequences for two genera, *Pseudomonas* and *Arthrobacter*, were shared across the two communities. Nevertheless, *Pseudomonas* was the dominant population of the phyllospheric ND bacteria, constituting 62.5 % of all isolates, while *Arthrobacter* was the most frequently isolated group of the rhizospheric ND bacteria, comprising 60.7 % of all isolates. All the isolates producing blue pigment on nicotine medium presumed previously were identified as *Arthrobacter* spp. Besides the familiar bacteria, i.e., *Pseudomonas* spp. and *Arthrobacter* spp., a number of other species capable of degrading nicotine were isolated dependent on the microenvironments. Some species of *Brevundimonas*, *Cellulosimicrobium*, *Erwinia*, *Sphingomonas*, *Massilia* and *Pantoea* were isolated specifically from the tobacco phyllosphere (Fig. 1), while members of *Ensifer* and *Sinorhizobium* were isolated from the tobacco rhizosphere (Fig. 2). *Brevundimonas* spp., *Cellulosimicrobium* spp., *Erwinia* spp., *Sphingomonas* spp., *Massilia* spp. and *Pantoea* spp. are widespread epiphyte and commensal bacteria (Innerebner 2011), of which *E. amylovora* infects tobacco and is the causative agent of

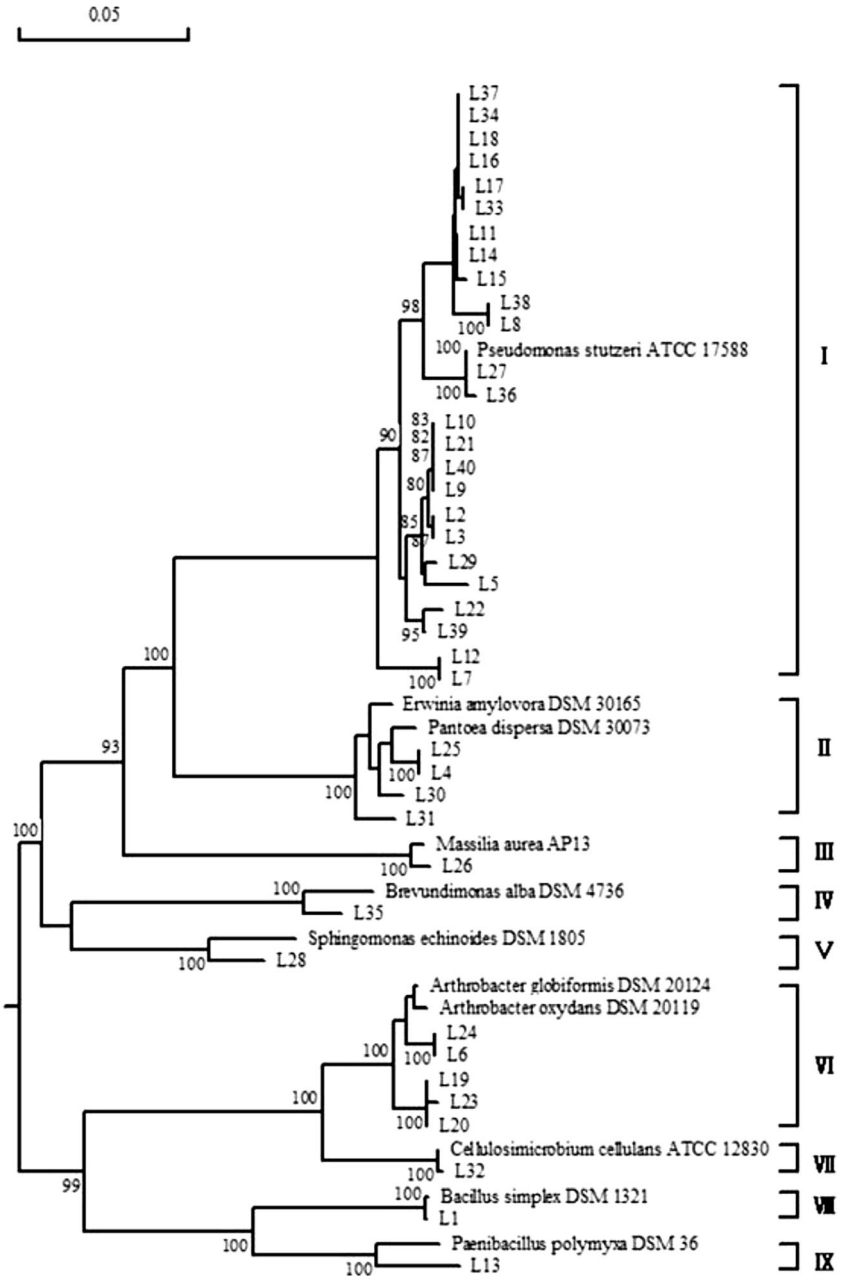

Fig. 1 Phylogenetic tree showing the relationship of 16S rRNA gene sequences of tobacco phyllospheric nicotine-degrading (ND) bacteria to those of representatives of genus. *I Pseudomonas*, *II Pantoea* and *Erwinia*, *III Massilia*, *IV Brevundimonas*, *V Sphingomonas*, *VI Arthrobacter*, *VII Cellulosimicrobium*, *VIII Bacillus*, *IX Paenibacillus*. *Bar* 5 % sequence divergence

fire blight (Smits et al. 2010), and *Sphingomonas* spp. and *Pantoea* spp. are phyllosphere indigenous isolates known as plant beneficial bacteria (Innerebner 2011). As known to all, *Ensifer* spp. and *Sinorhizobium* spp. are two genera of itrogen-fixing bacteria (rhizobia) colonizing in and around the roots resulting in symbiotic relationships within the plant (Hayat et al. 2010). Moreover, the members of genera *Massilia*, *Erwinia*, *Brevundimonas* and *Paenibacillus* are

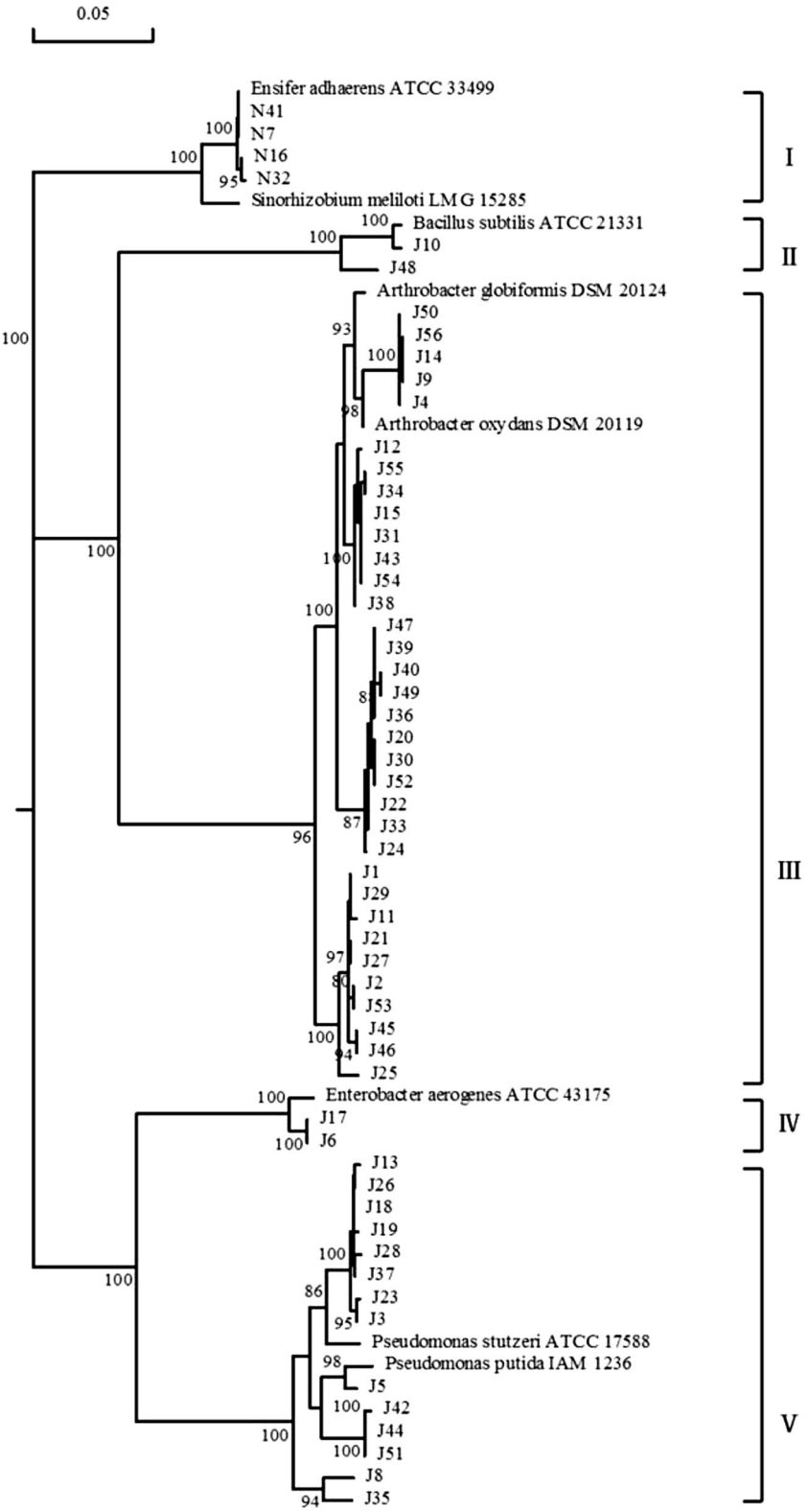

Fig. 2 Phylogenetic tree showing the relationship of 16S rRNA gene sequences of tobacco rhizospheric ND bacteria to those of representatives of genus. *I Ensifer* and *Sinorhizobium*, *II Bacillus*, *III Arthrobacter*, *IV Enterobacter*, *V Pseudomonas*. *Bar* 5 % sequence divergence

reported here for the first time as being capable of degrading nicotine.

In this study, diversity indices were calculated initially by using sequence data obtained from each ND bacteria library. Although we did not analyze the diversity indices of the three individual samples from each sampling niche, preliminary results indicated that the composition and richness of ND bacterial species was microenvironment-dependent. The species richness, diversity and dominance index of the phyllospheric ND bacterial community were higher than those of the rhizosphere community, while the evenness index was lower compared to rhizospheric ND bacteria (Table 4). The term phyllosphere refers to the above-ground plant parts coined by Ruinen in the 1950s by analogy to the rhizosphere (Ruinen 1956). The microflora diversity of the phyllosphere can be enhanced by non-resident colonizers dispersed by wind and rain and via animal feces. Hirano and Upper (2002) summarized this concept, stating that phyllosphere communities generally contain either only a few taxa but with a relatively large number of individuals or numerous taxa with only a small number of individuals for each. It is interesting to explore ND bacterial diversity in different ecological niches. Our preliminary research represents a valuable step in this direction. However, we need to analyze more samples and more sequence data, and include statistical significance analysis, in order to comprehensively understand the ecological adaptability of ND bacteria.

Identification of intermediates from nicotine degradation

Arthrobacter nicotinovorans and *Pseudomonas putida* are two major ND bacterial species whose biochemical pathways responsible for nicotine degradation have been characterized (Brandsch 2006; Wang et al. 2007; Li et al. 2010). In *A. nicotinovorans*, nicotine is first attacked at the pyridine ring (named pyridine pathway) to produce 6-hydroxynicotine. In some *P. putida* strains the pyrrolidine ring is oxidized first (named pyrrolidine pathway) and converted to N-methylmyosmine, followed by spontaneous hydrolysis of N-methylmyosmine to generate Pseudooxynicotine (Wang et al. 2007; Tang et al. 2008). Compared to the pyrrolidine pathway, the specific and apparent feature of the pyridine pathway is that a deep blue pigment (called "nicotine blue") is produced spontaneously from 2,3,6-trihydroxypyridine during nicotine degradation, which is the oxidative product of 2,6-dihydroxypyridine (Holmes and Rittenberg 1972). We already determined that isolates producing this kind of blue pigment were *Arthrobacter* spp. Besides the known pyrrolidine pathway, three more potential pathways in *Pseudomonas* spp. were proposed recently (Li et al. 2010) based on recent studies on nicotine biodegradation (Ruan et al. 2005; Chen et al. 2008; Sun et al. 2008). Therefore, we selected all of the *Psuedomonas* spp. and *Arthrobacter* spp. to check for known metabolic intermediates of the distinct nicotine degradation pathways. Each of the *Arthrobacter* spp. isolates produced a deep blue pigment, which is in accordance with the generation of 6-hydroxynicotine—a key intermediate of the *Arthrobacter* pyridine degrading pathway. The pyrrolidine pathway was the main metabolic pathway of the isolated Pseudomonads. Pseudooxynicotine was found from 84.6 % of Pseudomonads isolates, indicating that the pyrrolidine pathway of nicotine degradation is the most prevalent type. The other typical intermediate compounds of the three potential pathways were also individually or simultaneously detected from some Pseudomonads isolates, which suggested that a rich ND resource was obtained in this study. Cotinine, but not pseudooxynicotine, was detected from the nicotine cultures of strain L40, J42 and J51. Similar results, i.e., that *Pseudomonas* sp. Nic22 and *Pseudomonas* sp. CS3 could decompose nicotine via the cotinine pathway, have been reported (Chen et al. 2008; Wang et al. 2012). Furthermore, nornicotine and nicotyrine were both detected in the cultures of strain J8, J35 and J44, which is similar to nicotine metabolism of *Pseudomonas* sp. HF-1 (Ruan et al. 2005). In a recent study, a novel ND bacterium, *P. plecoglossicida* TND35 was reported and four new intermediate metabolites, N-methylmyosmine, 4-hydroxy-1-(3-pyridyl)-1-butanone, 3,5-bis (1-methylpyrrolidin-2-yl) pyridine, 2,3-dihydro-1-methyl-5-(pyridin-3-yl)-1H-pyrrol-2-ol and 5-(pyridin-3-yl)-1H-pyrrol-2(3H)-one were isolated and identified (Raman et al. 2013). A novel nicotine biodegradation pathway employed by strain TND35 was also proposed in that study. As standards of those four intermediates were not available to us, we were unable to evaluate whether any of our isolates use this pathway.

Table 4 Diversity indices and community richness of ND bacteria

Site	Strains (*N*)	Community richness (S)	Shannon Index (H′)	Pielou Evenness (J)	Simpson Index (D)
Rhizosphere	56	6	1.1162	0.6230	0.5124
Endophytic	40	10	1.3935	0.6052	0.5838

Conclusion

A total of 96 ND bacterial strains (56 from the tobacco rhizosphere and 40 from the tobacco phyllosphere) were isolated using nicotine as the sole carbon source. The species richness, diversity and dominance index of the phyllosphere ND bacterial community were higher than those of the rhizosphere community, while the evenness index was lower than in the rhizosphere. *Pseudomonas* spp. was the dominant ND

bacteria in the tobacco phyllosphere while *Arthrobacter* spp. was the main ND bacteria in the tobacco rhizosphere. The members of genera *Massilia*, *Erwinia*, *Brevundimonas* and *Paenibacillus* were reported for the first time as being capable of degrading nicotine. Metabolic intermediate analysis showed that the Pseudomonads isolates had one of four proposed metabolic pathways for nicotine degradation, while all of the *Arthrobacter* strains had only the pyridine pathway. The distinct and new ND isolates with novel metabolic mechanisms provide a wide microbial resource and are potentially worth further studied for applications in the future.

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