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ORIGINAL ARTICLE

Diverse cellulolytic bacteria isolated from the high humus, alkaline-saline chinampa soils

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Abstract Aiming at learning the functional bacterial community in the high humus content, saline-alkaline soils of chinampas, the cellulolytic bacteria were quantified and 100 bacterial isolates were isolated and characterized in the present study. Analysis of 16S-23S IGS (intergenic spacer) RFLP (restriction fragment length polymorphism) grouped the isolates into 48 IGS types and phylogenetic analysis of 16S rRNA genes identified them into 42 phylospecies within 29 genera and higher taxa belonging to the phyla Actinobacteria, Firmicutes and Proteobacteria, dominated by the genera *Arthrobacter*, *Streptomyces, Bacillus*, *Pseudomonas, Pseudoxanthomonas* and *Stenotrophomonas*. Among these bacteria, 63 isolates represent 26 novel putative species or higher taxa, while 37 were members of 17 defined species according to the phylogenetic relationships of 16S rRNA gene. Except for the novel species, the cellulolytic activity was not reported previously in 9 of the 17 species. They degraded cellulose in medium at pH4.5–10.0 or supplied with NaCl up to 9 %. In addition, 84.8 and 71.7 % of them degraded xylan and Avicel, respectively. These results greatly improved the knowledge about the diversity of cellulolytic bacteria and demonstrated that the chinampa soils contain diverse and novel cellulolytic bacteria functioning at a wide range of pH and salinity levels, which might be a valuable biotechnological resource for biotransformation of cellulose.

Keywords Cellulolytic bacteria · Diversity · Phylogeny · Chinampa · Rhizosphere

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Introduction

Cellulose is one of the most common organic compounds in plant litter, and approximately 40 billion tons per year is produced by photosynthesis (Black and Evans 1965) and constitutes between 20 and 30 % of the litter mass (Amann et al. 1996). As a 1,4-β-linked glucan, cellulose is a biodegradable biological macromolecular and provides a significant carbon source to the soil microbial community. Recently, the interest in using cellulose for biofuel production has increased (Kamm and Kamm 2007). In relation to the use of cellulose, cellulolytic bacteria are also being studied in different aspects, including population diversity in different environments, like industrial sugarcane bagasse feedstock piles (Pattanachomsri et al. 2011), sandy and loamy soils with a history of manure application (Ulrich et al. 2008), forestry and agricultural soils in temperate zones (Hatami et al. 2008), landfill of municipal solid waste (Barlaz et al. 1989), gut of termites (Ramin et al. 2009) and rumen of animals (Varel et al. 1991).

In soil, the existence of microbes capable of producing proteases, xylanases, amylases and cellulases is ubiquitous (Ghosh et al. 2007; Zhang et al. 2007), and they may encompass biotechnological applications (Lynd et al. 2002). Although fungi (Berg and Laskowski 2006; Eriksson et al. 1990) and anaerobic bacteria (Tamaru et al. 2010) are important cellulose degraders in many environments, the most efficient decomposition of cellulose occurred in

mesophilic aerobic microorganisms (Black and Evans 1965), which are also easier to handle in the production of efficient enzymes. Since the cellulase genes are highly heterogeneous (Rabinovich et al. 2002), studies on the diversity of cellulolytic bacteria have been performed with culture-dependent methods. The cellulolytic aerobic bacteria belong to very diverse groups, including Firmicutes, Actinobacteria, Bacteroidetes and Proteobacteria (Ulrich et al. 2008). The well-known genera are *Bacillus, Cellulomonas, Streptomyces, Cytophaga, Cellvibrio*, and *Pseudomonas* (Lynd et al. 2002). Great effects of environmental conditions on the abundance and decomposing activity of cellulolytic bacteria have been revealed in the previous studies (Dilly et al. 2001; Hiroki and Watanabe 1996; Ulrich et al. 2008), and therefore distinct populations of cellulolytic bacteria might be detected in different environments.

As an artificial sustainable agro-ecosystem (Altieri 1999) without the necessity of chemical fertilizer supply and irrigation, the construction of chinampas, or floating gardens, has a history of 3,000 years (Albores-Zarate 1998) in Lake Xochimilco, an urban water body in Mexico City, and they are currently used for culture of flowers, vagetables and animal fodder (Albores-Zarate 1998). The chinampas are composed of an enclosure of dead reed-grass and alternating layers of rock, aquatic vegetation, natural waste and sediment from the lake that fill the empty spaces. The anthropogenic origin of chinampas provides them with characteristics differing from other soils by having high moisture and organic matter content, high porosity, and a saline-alkaline condition (Ramos-Bello et al. 2001). Some investigations about the soil characters, such as heavy metal content and inhibition of nematodes have been performed (Mercado et al. 2000; Ramos-Bello et al. 2001, 2002), but there is little information about the microbial communities in the chinampa soils, although they play a central role in soil productivity (Zvyagintsev et al. 1991; Mahmood et al. 2006). Considering that the diversity and distribution of microorganisms in the soil is affected by physical and chemical parameters (Han et al. 2009), and that the types of vegetation and crop residues dramatically change the community composition of cellulolytic microorganisms (Ulrich and Wirth 1999), abundant and diverse celulolytic bacteria in the chinampa soils would be expected, which might be a valuable resource for searching for cellulose-degrading bacteria adapted to the high concentration of humus, high pH and salinity.

In order to learn the abundance and community composition of cellulolytic bacteria in the chinampa soils, as well as their degradation capacity, two representative chinampas cultivated with different crops were selected in this study. The population densities of total mesophilic aerobic bacteria and cellulolytic bacteria in both the rhizosphere and non-rhizosphere soils were estimated. The cellulolytic bacteria were isolated and further analyzed by molecular methods to evaluate their phylogenetic diversity.

Materials and methods

Soil sampling

Since the high salt concentration and pH value decreases the agricultural production, the farmers change the chinampa surface soils every 2 or 3 years by adding decayed or fresh plant materials to maintain the productivity. According to the farmer's experience, the added plant materials are digested completely within 4–6 months. To avoid the effect of adding plant materials, and considering the fact that all the chinampas have very similar soils and environmental conditions, two mature chinampas were chosen as representatives in this study, which have been maintained for more than 100 years according to the owners. These chinampas are located in Canal Apatlaco Paraje Potrero (19°15′N, 99°08′W), Colonia San Juan Moyotepec, Xochimilco in Mexico City, altitude 2,500 m, mean annual temperature 15 °C, mean annual precipitation 709 mm. The place was far from the tourist zone to avoid intensive disturbance by people. Soils of the two sampled chinampas showed a black color due to the high content of organic matter, mainly humic acids with high melanization and high molecular weight (Ramos-Bello et al. 2001). In these chinampas, fertilizer is not used (personal communication with the owners). In chinampa I, alfalfa (*Medicago sativa* L.) and chard (*Beta vulgaris* L.) plants were cultured; while in chinampa II, alfalfa, a grass [*Arrhenatherum elatius* (Linn.) Pressl] and common sorrel (*Rumex crispus* L.) grew at the moment of sampling, from which rhizosphere soils were obtained.

Bulk soils were sampled in February 2008 by cross-sampling method (from five points) in each chinampa from two vertical zones: 0–30 cm and 30–60 cm depth, and stored in plastic bags. For sampling rhizosphere soils, five individuals of each plant mentioned above were uprooted together with soil and stored in plastic bags. All samples were transported directly to the laboratory where the five soil samples from each vertical zone of the same chinampa were pooled as a composite sample. The rhizosphere soils were collected from the plant roots as described previously (Sun et al. 2009). All soil samples were kept at 4 °C for 1–7 days until their utilization. The physiochemical features of the soils were determined with conventional methods and are presented in Table 1.

Quantification of mesophilic aerobic and cellulolytic bacteria

For each soil sample, a serial of decimal dilutions was prepared in sterilized NaCl solution (0.89 %). An aliquot of 0.1 mL of the dilutions 10^{-4}, 10^{-5} and 10^{-6} was spread in duplicate in Petri dishes containing PY medium (peptone, 5 g; yeast extract, 3 g; $CaCl_2$, 0.6 g; agar, 18 g; distilled

Table 1 Characteristics of chinampa soils involved in the study

Soil sample	pH	%[a]			mg kg^{-1} soil			Texture
		Humidity	WHC[b]	Organic matter	Total N	Total P	Total K	
Ch I 0–30 cm	8.6	24	120.2	7.5	0.80	22.4	4,468	Sandy clay loam
Ch I 30–60 cm	8.8	44	116.7	4.6	0.56	17.4	3,539	Sandy clay loam
Ch II 0–30 cm	8.7	38	148.0	7.5	0.75	3.7	3,728	Sandy clay loam
Ch II 30–60 cm	8.0	48	112.1	6.4	0.58	19.6	2,054	Sandy clay loam

[a] Mean value of duplicate.

[b] *WHC* Water holding capacity.

water, 1 L; pH8.0) or Congo red agar (K_2HPO_4, 0.5 g; $MgSO_4$, 0.25 g; cellulose powder, 1.88 g; Congo red, 0.2 g; gelatin, 2.0 g; soil extract 100 mL; agar, 18 g; distilled water, 900 mL; pH8.0) (Hendricks et al. 1995). All the plates were incubated at 28 °C and observed during 2–7 days. Colonies of mesophilic aerobic bacteria were counted in PY medium, while the single colonies surrounded by a semitransparent ring in Congo red agar were counted as cellulolytic bacteria. The abundance of both the mesophilic aerobic bacteria and cellulolytic bacteria were calculated from the counting data, as colony forming units (CFU) per gram of dry soil.

Isolation of cellulolytic bacteria

Single colonies surrounded by semitransparent rings on the Congo red agar were selected according to their morphology and picked for further streaking on the same medium (Ulrich and Wirth 1999). Considering the fact that bacteria showing similar colonies may be of different species, two or three colonies were picked for the predominant colony types. 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 to ensure the purity of the isolates. All the pure isolates were stored at −70 °C in PY broth supplied with 20 % (w/v) of glycerol.

Degradation activity of cellulolytic aerobic bacteria

The degradation activity of the isolates was tested on mineral medium (K_2HPO_4, 7 g; KH_2PO_4, 2 g; $MgCl_2{\cdot}7H_2O$, 0.1 g; NH_4Cl, 1 g; NaCl, 5 g; agar, 10 g; distilled water, 1 L; pH7.6), separately supplied with 2 mg mL^{-1} of carboxy methyl cellulose (CMC, amorphous cellulose), xylan (hemicellulose) and avicel (microcrystalline cellululose); and Congo red (CR) was used as indicator (Ghosh et al. 2007). In addition, the degradation of cellulose powder was also determined in the same medium but the pH was adjusted to 4.5, 5.0, 6.0, 9.0, 10.0 and 11.0, respectively, or NaCl was supplied to the final concentration of 1.0, 3.0, 5.0, 7.0, 9.0 and 12.0 % (w/v). Each bacterium was inoculated by pricking in 20 squares of 1×1 cm in a plate. The inoculated plates were incubated 24–120 h at 28 °C, and were checked each 24 h until the clear zones were observed around the colonies, which indicated the decomposition of CMC, xylan or Avicel. Then, diameters of the colony and semitransparent rings were measured to calculate the degradation index (DI) which is presented as the rate of D/d (ring diameter/colony diameter) (Hankin and Anagnostakis 1977; Hendricks et al. 1995; Lu et al. 2005).

RFLP (restriction fragment length polymorphism) analysis of amplified 16S-23S IGS (intergenic spacer)

This analysis was performed to group the isolates into species as suggested in other studies (Rasolomampianina et al. 2005; Yavuz et al. 2004). Genomic DNA was extracted from bacterial culture (in 5 mL of PY broth at 28 °C with agitation, 12 h for bacteria and 48 h for actinomyces) with the procedure of Zhou et al. (1995), and was used as template to amplify 16S-23S rRNA IGS, with primers FGPS6 (5′-GGA GAG TTA GAT CTT GGC TCA-3′) and 23S-38 (5′-CCG GGT TTC CCC ATT CGG-3′), and the procedure described by Rasolomampianina et al. (2005). The PCR products were visualized by agarose gel (1 %, w/v) electrophoresis in 0.5× TE (Wang et al. 1999) and stored at −4 °C for further analysis. For RFLP analysis, an aliquot of 10 μL PCR products was digested separately by 5 U of each of the restriction enzymes *Msp*I, *Hinf*I, and *Hae*III in a final volume of 20 μL at 37 °C for 6 h. Then, restriction products were separated by electrophoresis in 2 % (w/v) agarose gel to visualize restriction patterns. Strains shared the same RFLP patterns were designed as a single IGS type.

Sequencing of 16S rRNA gene and phylogenetic analysis

For each IGS type, one isolate was selected randomly for sequence analysis of 16S rRNA gene. The 16S rRNA gene was amplified using the DNA sample mentioned above as

template in a final volume of 50 μL with the procedure, and using universal primers fD1 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and rD1 (5′-AAG GAG GTG ATC CAG CC-3′), described by Weisburg et al. (1991). Sequencing was conducted under Big Dye™ terminator cycling conditions with the same primers using Automatic Sequencer 3730XL in Macrogen (Korea). All sequences were compared with sequences of the reference organisms by Blast search (Altschul et al. 1990). The sequences were aligned using CLUSTAL X (2.0) software with default settings (Larkin et al. 2007) according to the phylum (Actinobacteria, Firmicutes and Proteobacteria). Minor modifications in the alignment were made using BIOEDIT sequence editor. Maximum likelihood analyses were performed using PhyML [http://atgc.lirmm.fr/phyml/] (Guindon et al. 2010). MODELTEST 3.06 (Posada 2008) was used to select appropriate models of sequence evolution by the AIC (Akaike Information Criterion). The confidence at each node was assessed by 1,000 bootstrap replicates. Similarities among sequences were calculated using the MatGAT v.2.01 software (Campanella et al. 2003). Taxonomic assignment was obtained by using the Roselló-Mora (Roselló-Mora and Amann 2001) prokaryotes criteria. Rarefaction curve was made using EstimateS 8.2.0 program (Colwell 2011). The sequences obtained were submitted to GenBank under the access numbers JN571036–JN571083.

The affiliation of isolates was mainly based upon the grouping results in the phylogenetic tree, taking the suggested threshold (97, 95, 90 and 80 % 16S rRNA gene sequence similarities for species, genus, family and phylum, respectively) (Schloss and Handelsman 2004) as reference.

Statistic analysis

This analysis was performed to evaluate the difference on the degradation ability (ID) among the isolates for CMC, xylan and Avicel at different pH values and different concentration of NaCl. The significant differences among the isolates were estimated from three replications (n=3) by the analysis of variance (ANOVA) and Tukey post hoc (Tukey Honest Significant Difference) in the R 2.14 package (R Development Core Team 2011).

Results

Abundance of aerobic mesophilic and cellulolytic bacteria

The abundance of total cultivable mesophilic aerobic bacteria (MAB) and cellulolytic bacteria is presented in Fig. 1. In bulk soils, the abundance of total bacteria (10^7CFU g^{-1}) and cellulolytic bacteria (10^6CFU g^{-1}) was similar in the two chinampas at both depths (0–30 and 30–60 cm). The growth

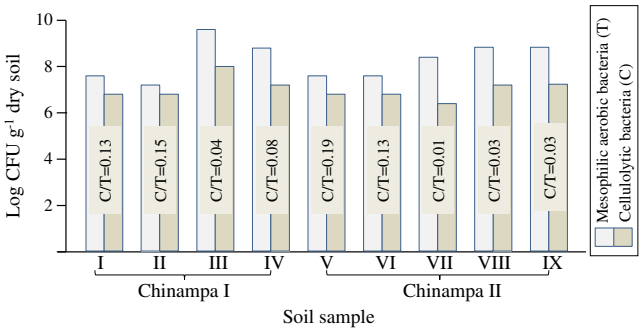

Fig. 1 Abundance of mesophilic aerobic bacteria and cellulolytic bacteria in the chinampa soils. Soil samples *I* (0–30 cm), II (30–60 cm), *III* (alfalfa rhizosphere), *IV* (chard rhizosphere) were from chinampa I; *V* (0–30 cm), VI (30–60 cm), *VII* (alfalfa rhizosphere), *VIII* (sorrel rhizosphere) and IX (grass rhizosphere) were from Chinampa II. C/T Cellulolytic bacterial density/total bacterial density

of plants apparently enhanced the bacterial density in rhizosphere, and this rhizosphere effect was greater for the mesophilic aerobic bacteria than for the cellulolytic bacteria (Fig. 1). The R/S ratio (bacteria density in rhizosphere/in bulk soil, where the bacterial density of bulk soil was average of the data from soil samples 0–30 and 30–60 cm) ranged between 74.2 (alfalfa rhizosphere in chinampa I) and 7.4 (in chard rhizosphere) for MAB (average 24.3), and between 20.4 (alfalfa rhizosphere in chinampa I) and 0.79 (alfalfa rhizosphere in chinampa II) for cellulolytic bacteria (average 6.4). On the other hand, the proportion of cellulolytic bacteria in the total MAB (C/T ratio) was much greater in bulk soils (average 0.15) than in rhizosphere (average 0.04) (Fig. 1).

IGS-RFLP of cellulolytic bacteria

A total of 100 isolates representing all the colony morphotypes were selected from the nine soil samples, including 41 Gram-negative rods and 59 Gram-positive rods. In IGS amplification, all isolates produced single bands about 2,500 bp. According to RFLP patterns, 48 IGS types were defined (Table 2).

Rarefaction analysis showed that the 48 genotypes defined among a sample size of 100 isolates represented 80.8 % of the total richness expected with confidence of 95 %. The diversity of population gives a Shannon index of 3.48.

Sequence analysis of 16S rRNA genes

All the 48 representative isolates produced single bands of about 1,500 bp in amplification and the obtained sequences had molecular length of 1,360–1,490 bp. For phylum Actinobacteria, the GTR + I + G model (α=0.3170 for the gamma distribution; A=0.2271, C=0.2484, G=0.3223, T=0.2022;

Table 2 ITS-RFLP groups and taxonomic affiliation of cellulolytic bacteria isolated in this study

Isolate and taxonomic affiliation[a]	ITS group[b]	Origin of soil sample[c]	Related species[d]	Similarity (%)[e]
Class Bacilli				
Bacillus sp. I A1, A3, A13, P3, **P18**, CH12, H8	1	IV, V, VI, IX	*Bacillus thuringiensis* (AF290545)	95.9
Bacillus sp. II **C11**, L5	2	I, VIII	*Bacillus thuringiensis* (AF290545)	96.8
Bacillus sp. III **C1**,C10,CH5	3	I, V	*Bacillus vietnamensis* (AB697709)	96.5
Genus B of Bacillales **H9**	4	VI	*Bacillus boroniphilus* (FJ544338)	89.0
Genus A of Bacillales **H17**	6	VI	*Staphylococcus epidermidis* (D83363)	86.8
Class Actinobacteria				
Streptomyces sp. F2, F5, SN, **AF14,** P15, P16, CH17, H1	19	III, V, VI, VII, IX	*Streptomyces anulatus* (NR043489)	97.9
Streptomyces anulatus **C4B**	22	I	*Streptomyces anulatus* (NR043489)	99.8
Actinomycetales Genus C, sp. I C12, S2, **S3**	20	I, II	*Streptomyces europaeiscabiei* (HQ441827)	84.7
Actinomycetales Genus C, sp. II **C2**	21	I	*Streptomyces europaeiscabiei* (HQ441827)	85.8
Arthrobacter sp. V A2, **A4,** A6, A11, AF12, AF10, L10, P1, P5, P14, CH6	7	IV, V, VII, VIII, IX	*Arthrobacter nicotianae* (JQ071518)	98.9
Arthrobacter sp. I **P6**	8	IX	*Arthrobacter scleromae* (JF505938)	96.1
Arthrobacter sp. II **AF6**	9	VII	*Arthrobacter arilaitensis* (JN592607)	93.3
Arthrobacter sp. III **CH19b**	10	V	*Arthrobacter scleromae* (JF505938)/ *Arthrobacter polychromogenes* (AB167181)	98.6
Arthrobacter sp. IV **P2,** P10	11	IX	*Arthrobacter nicotianae* (JQ071518)/ *Arthrobacter protophormiae* (AB210984)	98.7
Microbacterium oxydans **A5**	12	III	*Microbacterium oxydans* (HQ202812)	99.6
Microbacterium sp. I **AF7**	13	VII	*Microbacterium thalassium* (JQ071522)	97.3
Microbacterium natoriense **CH2**	14	V	*Microbacterium natoriense* (NR042983)	99.3
Microbacterium sp. II **CH4**	15	V	*M. hydrocarbonoxydans* (EU714368)	98.1
Knoellia subterranea **CH16**	16	V	*Knoellia subterranea* (EU867301)	99.6
Leucobacter komagatae A8, **A9**	17	IV	*Leucobacter komagatae* (JF792093)	99.6
Leucobacter sp. **L1**	18	VIII	*Leucobacter luti* (NR042425)	97.0
Corynebacterium callunae **L2**	23	VIII	*Corynebacterium callunae* (NR037036)	97.9
Corynebacterium amycolatum **L9**	24	VIII	*Corynebacterium amycolatum* (NR026215)	97.9
Nocardioides kongjuensis **P9**	27	IX	*Nocardioides kongjuensis* (NR043651)	99.6
Agromyces sp. **L6B**	25	VIII	*Agromyces cerinus* (AM410681)	96.1
Promicromonospora umidemergens F11, **AF13**	26	III, VII	*Promicromonospora umidemergens* (JN180227)	99.9
Cellulosimicrobium cellulans **S4**	28	II	*Cellulosimicrobium cellulans* (X79455)	98.8
Class Alphaproteobacteria				
Agrobacterium rubi **L11**	29	VIII	*Agrobacterium rubi* (AB680384)	97.5
Hyphomicrobiaceae genus I. **H13**	30	VI	*Hyphomicrobium zavarzinii* (NR026429)	92.3
Sphingomonadaceae genus I **L13**	31	VIII	*Sphingobium xenophagum* (NR026304)	91.4
Sphingopyxis sp. **F10**	32	III	*Sphingopyxis chilensis* (NR024631)	95.9
Class Betaproteobacteria				
Alcaligenes sp. AF15a, CH11, **CH14**	33	V, VII	*Alcaligenes faecalis* (HQ143627)	96.9
Delftia acidovorans **A7**	34	IV	*Delftia acidovorans* (AB680449)	99.1
Class Gamaproteobacteria				
Aeromonas media **H7D**	35	VI	*Aeromonas media* (EU488684)	100
Stenotrophomonas maltophilia C3, C5, C7, C13, C8, **C9**, CH3, CH19r	36	I,V	*Stenotrophomonas maltophilia* (AJ293464)	98.5
Pseudomonadales family II CH1, CH13, **CH22**	37	V, IX	*Acinetobacter psychrotolerans* (AB207814)	89.0
Pseudoxanthomonas mexicana CH7, CH20, **CH21**, H10, H15	38	V, VI	*Pseudoxanthomonas mexicana* (AB681338)	97.7
Xanthomonadaceae genus I **H3,** H2	39	VI	*Pseudoxanthomonas ginsengisoli* (JN637330)	91.8
Pseudomonadales Family I A12, **CH8**	40	IV, V	*Pseudomanas gessardii* (NR024928)	86.5

Table 2 (continued)

Isolate and taxonomic affiliation[a]	ITS group[b]	Origin of soil sample[c]	Related species[d]	Similarity (%)[e]
Pseudomonas-related bacterium **AF3**[f]	44	VII	*P. pseudoalcaligenes* (JF439302)	99.1
Pseudomonas pseudoalcaligenes **H3b**	42	VI	*Pseudomonas pseudoalcaligenes* (JF439302)	99.4
P. pseudoalcaligenes **H7C**, H7	43	VI	*Pseudomonas pseudoalcaligenes* (JF439302)	98.0
P. pseudoalcaligenes H14, **H16**	45	VI	*P. pseudoalcaligenes* (JF439302)	97.4
P. pseudoalcaligenes **F4,** F7A	46	III	*P. pseudoalcaligenes* (JF439302)	99.4
P. pseudoalcaligenes **F6**	5	III	*P. pseudoalcaligenes* (JF439302)	99.1
Luteimonas sp. I **AF4**	47	VII	*Luteimonas aestuarii* (NR044343)	94.7
Luteimonas aestuarii **AF11**	48	VII	*Luteimonas aestuarii* (NR044343)	97.5

[a] One isolate per RFLP was selected for construct phylogenetic trees (in bold)

[b] ITS groups were defined according to restriction profiles of amplified ITS digested with *Hae* III, *Hinf* I and *Msp* I

[c] Soil samples *IIV* were from chinampa I: *I* surface soil (0–30 cm); *II* deep soil (30–60 cm); *III* soil of lucerne rhizosphere; *IV* soil of chard rhizosphere. *VIX* were from chinampa II: *V* surface soil (0–30 cm); VI deep soil (30–60 cm); *VII* soil of lucerne rhizosphere; *VIII* soil of sorrel rhizosphere; *IX*. Rhizosphere soil of grass

[d] Sequences of related species of GenBank access numbers are presented in parentheses

[e] Putative assignment is based on similarities to the closest genera determined by similarity analysis with MatGAT . program

[f] This strain generated a short sequence and its relation with defined species was uncertain

P-inv=0.0080) was selected for the tree search. While the GTR + G model was selected for the phyla Firmicutes (α= 0.6750 for the gamma distribution; A=0.2386, C=0.2548, G=0.3223, T=0.3064) and Proteobacteria (α=0.4480 for the gamma distribution; A=0.2295, C=0.2448, G=0.3124, T=0.2132).

By comparison with 16S rRNA genes in GenBank database and relationships in the phylogenetic trees (Fig. 2), 14 isolates were grouped in the phylum Firmicutes as three putative new species of *Bacillus* and two putative new genera (Table 2); 44 isolates were clustered in the phylum Actinomyces as ten genera (*Agromyces, Arthrobacter, Cellulomonas, Corynebacterium, Knoellia, Leucobacter, Microbacterium*, *Nocardioides, Promicromonospora, Streptomyces*) and a putative new genus for the clade with C2 and S3 (Table 2) in Actinomycetales; 42 isolates were found in the phylum Proteobacteria as four genera (*Agrobacterium, Sphingopyxis* and two novel genera in Sphingomonadaceae and Hyphomicrobiaceae) in Alphaproteobacteria, two genera (*Delftia* and *Alcaligenes*) in Betaproteobacteria and seven genera (*Aeromonas, Stenotrophomonas, Xanthomonas, Luteimonas, Pseudomonas, Pseudoxanthomonas* and a novel genus in Xanthomonadaceae) and two novel families of Pseudomonadales in Gammaproteobacteria.

The relationships between IGS types and the phylogenetic groups are summarized in Table 2. Genera *Pseudomonas, Arthrobacter, Bacillus* and *Microbacterium* were the most diverse groups containing seven, five, three and four IGS types, respectively; *Corynebacterium, Leucobacter, Streptomyces,* and *Luteimonas* had two IGS types; the other genera contained only one IGS type in each (Table 2). The data in Table 2 also show that the most abundant phylogenetic group was *Arthrobacter* (16 isolates), followed by *Bacillus* (12 isolates), *Pseudomonas* (11 isolates), *Streptomyces* (n9ine isolates), and *Stenotrophomonas* (8 isolates). *Streptomyces* and *Bacillus* have the most universal distribution: *Streptomyces* was found in soil samples I, II, III, V, VI, VII, VIII and IX, whereas *Bacillus* was found in soil samples I, II, IV, V, VI, VIII and IX.

Combining the phylogenetic relationships (Fig. 2) and the IGS types, the taxonomic affiliation was determined for each isolate (Table 2). The 14 Firmicutes isolates were defined as three novel *Bacillus* species and two novel genera. The Actinobacteria was the most divergent and abundant celullolytic bacterial group in the chinampa soils which covers 44 isolates corresponding to 22 species, in which only 12 isolates were assigned as 10 defined species and the others represented 12 novel species, including a novel genus.

The 42 isolates in Proteobacteria were defined into three classes. Four isolates in Alphaproteobacteria represented *Agrobacterium rubi* and three novel species (genera), while the four isolates in Betaproteobacteria represented *Delftia acidovorans* and a novel *Alcaligenes* species. The 34 isolates in Gammaprotobacteria were divided into five defined species and five novel taxa (species, genera or family).

Degradation activity of the cellulotic bacteria

In this analysis, all isolates showed activity on CMC-CR agar plates (100 %), a lower proportion (84.8 %) showed activity on xylan-CR agar plates and only 71.7 % hydrolyzed Avicel (detailed information available in Supplementary Table A).

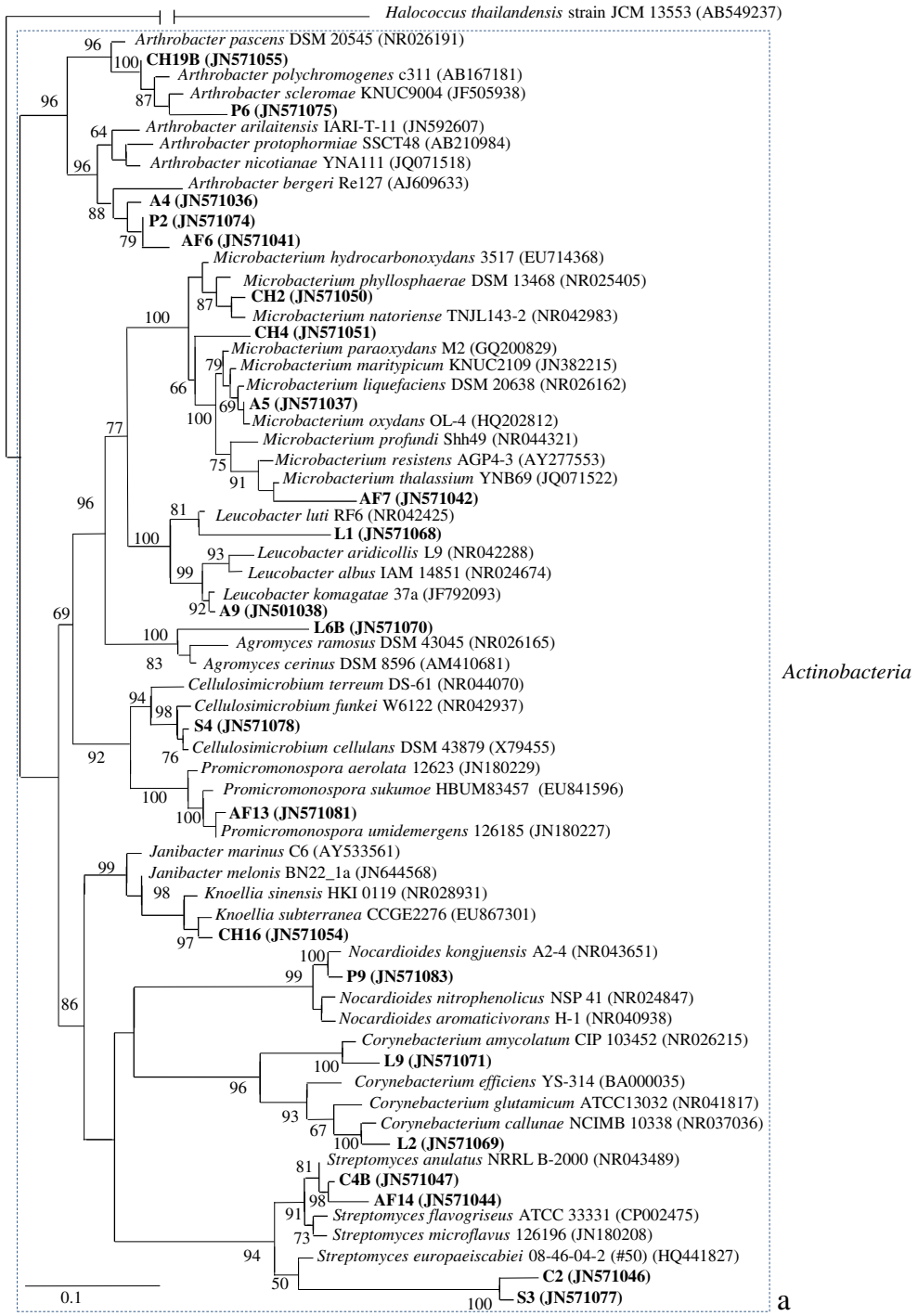

Fig. 2 Phylogenetic trees of 16S rRNA genes showing the diversity of cellulolitic bacteria isolated from chinampa soils: **a** phylum Actinobacteria; **b** phylum Firmicutes; **c** phylum Proteobacteria. The trees were constructed with the maximum likelihood method (Guindon et al. 2010) and Hasegawa-Kishino-Yano model (Posada 2008). *Halococcus thailandensis* JCM 13553 (AB549237) was included as the outgroup. Bootstrap values obtained from 1,000 pseudoreplicates greater than 50 % were indicated at the nodes. *Scale bar* represents 10 % substitution of the nucleotide

Most of the isolates were capable of degrading the substrate within 24 h. The DI ranged from 1 to 6 at 120 h. No significant difference was obtained in distribution and degradation activities for the isolates originated from the different chinampas. Based upon the statistic analysis (Supplementary Table A), there are significant differences in the degradation ability among the isolates at different pH and different salinity for the same carbon source, and also differences for the three substrates (CMC, xylan and Avicel).

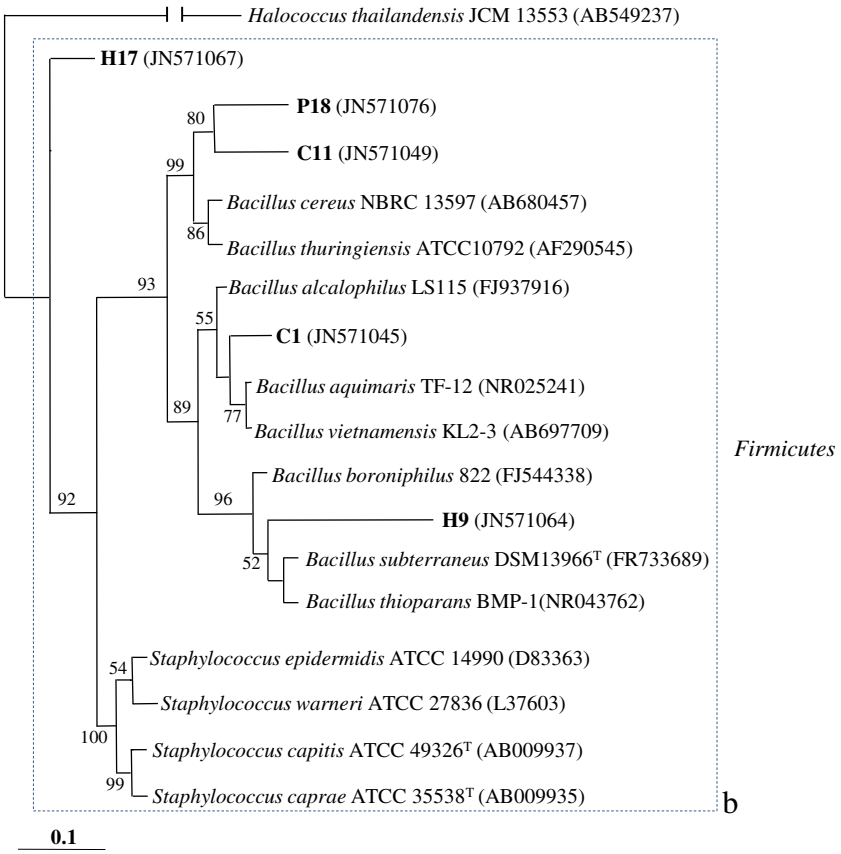

Fig. 2 (continued)

In the degradation analysis, no isolate grew in medium with pH11.0 or supplied with 12 % of NaCl, while the isolates showed cellulose degradation in the medium with pH4.5–10.0 or supplied with 1–9 % of NaCl (Supplementary Table A). The highest proportion of isolates capable of degrading cellulose and the highest degrading efficiency was detected at pH6.0 (96.97 %, DI=1.6–7.4) and pH9.0 (90.91 %, DI=1.3–7.8), respectively (Fig. 3). At pH5.0 and 10.0, 53.54 and 54.55 % of the isolates could degrade cellulose with lower efficiency (DI=1.5–6.3 and 1.2–6.0, respectively). The proportion of isolates capable to degrade cellulose was decreased from 100 to 5.05 % when the NaCl concentration was increased from 1 to 9 % (Fig. 3).

In the phylum Firmicutes, most of the 14 isolates showed higher degradation ability at neutral or acid condition (pH 6.0), with average DI of 3.78; in comparison, 13 isolates showed degradation ability at pH9.0 with lower average DI (2.86). At both pH6.0 and 9.0, the highest DI, 7.4 and 4.4, was detected in isolate CH12 of *Bacillus* sp. I.

In the phylum Actinobacteria, nine isolates were capable of degrading cellulose at pH4.5; 22 at pH5.0; 42 at pH6.0; 38 at pH9.0; and 27 at pH10, demonstrating that a rather high proportion of actinobacteria adapted to the alkaline conditions. In these isolates, *Microbacterium oxydans* A5, *Streptomyces anulatus* AF14 and *Cellulomonas cellulans* S4 showed higher degrading capacity in alkaline conditions (DI=5.9, 7.8, 6.5). *Arthrobacter nicotianae* A4, A11, *Leucobacter luti* L1, *Streptomyces anulatus* F2 and C4B showed degradation at pH4.5 –10.0.

Four isolates in each of the Alphaproteobacteria and Betaproteobactereia were analyzed. All of them showed degradation at pH6.0, and numbers of isolates with degradation ability was reduced to 6, 5, 4 and 4 at pH9.0, 5.0, 10.0 and 4.5, respectively. The highest efficiency (DI=5.0) was detected in isolate *Agrobacterium rubi* L11 at pH4.5, while isolates *Sphingobium*-bacterium L13 and *Alcaligenes* sp. AF15a showed degradation at all the pH levels (pH4.5 – 10.0).

A total of 34 isolates in Gammaproteobacteria were analyzed and all of them showed degradation at pH9.0 (DI= 1.3–6.1, average 3.14); 33 were able to degrade cellulose at pH6.0 (DI=1.8–6.2, average 3.16); 16 at pH10 (DI=1.5–5.4, average 2.71); 18 at pH5.0 (DI=1.8–6.3, average 3.62); 12 at pH4.5 (DI=2.3–5–1, average 3.91). The highest degradation efficiency was detected in isolate *Pseudomonas pseudoalcaligenes* H14 (DI=6.3) at pH5.0, *Stenotrophomonas maltophilia* C3 (DI=6.2) at pH6.0 and *Pseudomonas mendocina* S5A (DI=6.1) at pH9.0. Nine strains showed degradation ability through all the pH levels (pH4.5–10.0).

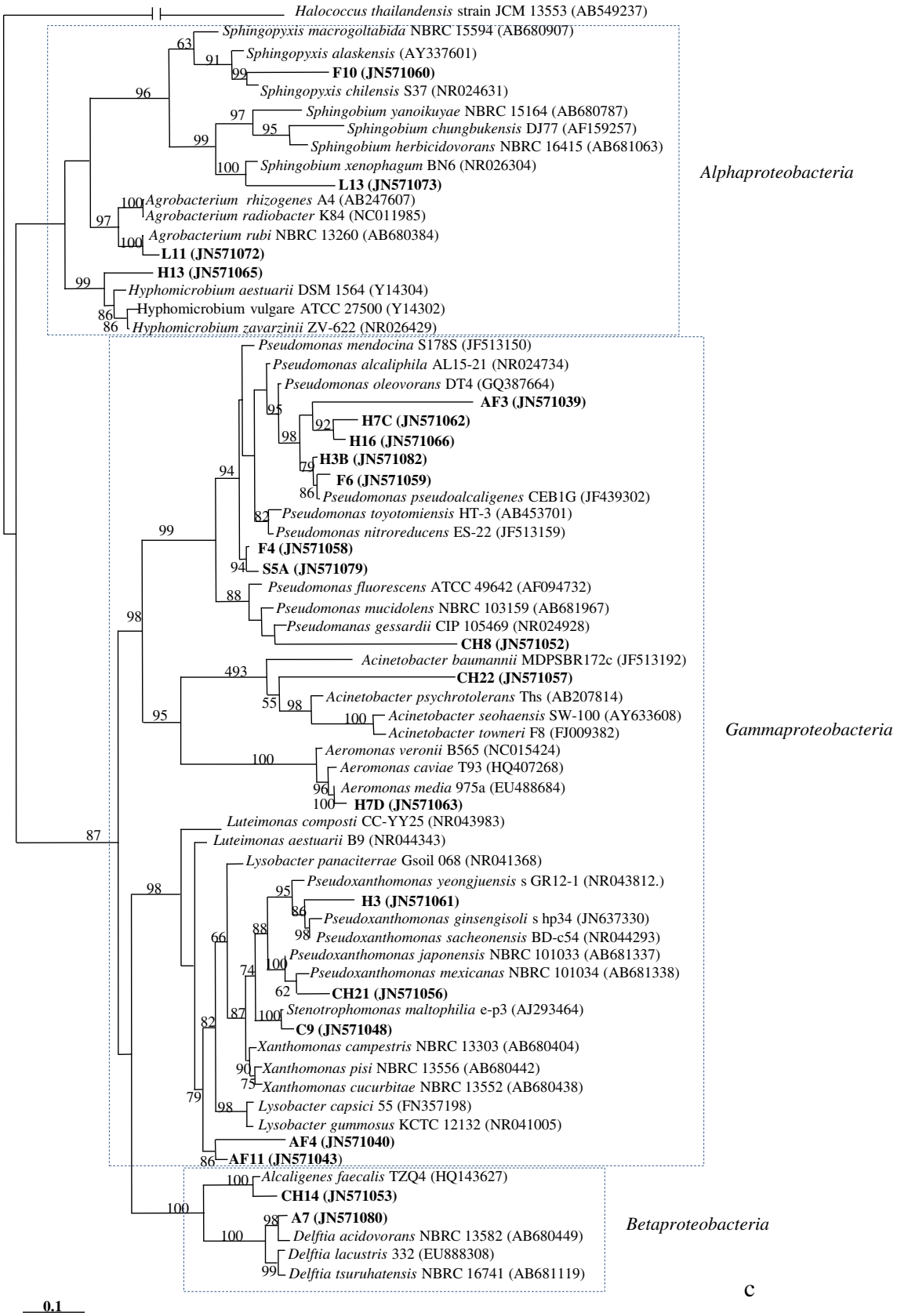

Fig. 2 (continued)

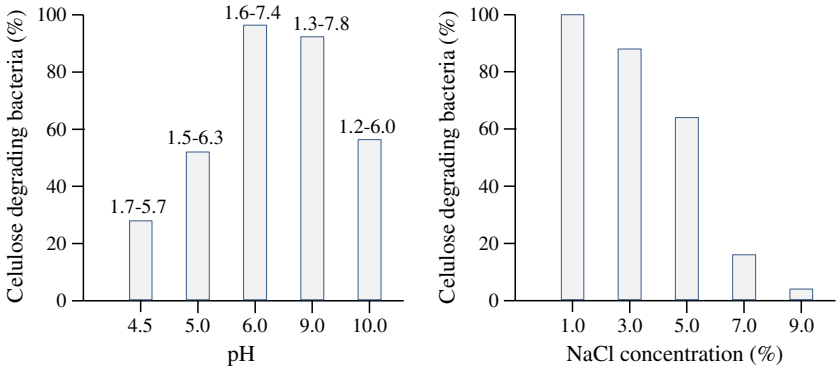

Fig. 3 Proportion of bacterial isolates degrading CMC on medium with different pH and concentration of NaCl. The degradation of CMC in medium at pH7.6 was taken as positive control and 100 % of the isolates could degrade it, while 84.8 % of the isolates degraded xylan and 71.7 % hydrolyzed Avicel (detailed information available in Supplementary Table A)

Several isolates showed simultaneously tolerance for wide ranges of both pH and salinity, such as CH12 (*Bacillus* sp. I), L13 (*Sphingobium*-related bacterium), *Pseudomonas pseudoalcaligenes* H14 and *Streptomyces anulatus* AF4 degraded cellulose at pH4.5–10.0 and NaCl concentration of 1–9 % (Supplementary Table A).

Discussion

According to the Mexican Norm of Soils (NOM-021 SEMARNAT 2000), the soil samples collected from the two chinampas were rich in organic matter, total nitrogen and total potassium, while the total phosphorous content was low to moderately rich (Table 1). The intermediately alkaline and saline characters were similar to the previous report (Ramos-Bello et al. 2001). The high content of organic matter, alkaline-saline feature and high humidity made the chinampa soils different from the other soils or samples used previously for cellulolytic bacteria studies.

In modern microbial ecology studies, the investigation of microbial communities is carried on in most cases with culture-independent molecular-based strategies. However, a traditional culture-dependent approach has been used in this study, because the cellulolitic bacteria are very diverse in phylogeny and the cellulolitic enzymes are also very diverse, and therefore it was not possible to develop a convenient molecular method (such as a common gene for PCR-based sequencing analysis) to investigate their diversity (Ohrmund and Elrod 2010).

It was clear that the Actinobacteria and Proteobacteria were the main cellulose-degrading groups in the chinampa soils. However, *Bacillus* sp. I, *Streptomyces* sp., *Arthrobacter* sp. V, and *Stenotrophomonas maltophilia* were the dominant species (Table 2). To date, several studies on the diversity of cellulolytic bacteria isolated from different soils or environments have been performed (Hatami et al. 2008; Sun et al. 2009; Ulrich and Wirth 1999; Ulrich et al. 2008). The cellulolytic bacterial community in the chinampa soils was similar to those of previous studies as the cellulolytic bacteria were mainly identified into the phyla Actinobacteria, Proteobacteria and Firmicutes (Table 2). However, relative abundances of these bacteria in the chinampa soils were different from those in other soils (Ulrich and Wirth 1999; Ulrich et al. 2008), with lower abundance of *Streptomyces* (13 %) and *Cellulomonas* (1 %) (Ulrich and Wirth 1999), and higher *Arthrobacter* (16 %) and Firmicutes (14 %). In addition, *Burkholderia* and *Fulvimonas* (Ulrich et al. 2008) as the main genera in other soils were not found in chinampa soils, but instead there were *Pseudomonas* (10.75 %) and *Stenotrophomonas* (7.53 %) (Table 2). The cellulolytic bacteria community in chinampa soils was also distinct from that of refuse of a landfill, where Bacillaceae and the genera *Cellulomonas, Microbacterium* and *Lactobacillus* were predominant. These differences may be related to the soil characteristics; thus, the soils of Ulrich et al. (2008) were slightly acid (pH5.6–6.75), poor in organic maters (0.62–2.27 %) and in total nitrogen (0.05–0.19 %). These differences confirmed that the soil pH and salinity are determinants for the biogeography of free-living bacteria (Fierer and Jackson 2006).

As shown in Table 2, all the isolates in Firmicutes and 32 of the 44 isolates in Actinobacteria were novel bacteria. Among the 10 defined species within Actinobacteria, degradation of cellulose was only reported in *Microbacterium oxydans* originated from a defined cellulolytic and xylanolytic bacterial consortium (Okeke and Lu 2011), in *Promicromonospora umidemergens* isolated from indoor wall material (Martin et al. 2010), and in *Cellulosimicrobium cellulans* (Schumann et al. 2001). *Microbacterium natoriense* was defined as a novel D-aminoacylase-producing bacterium isolated from soil (Liu et al. 2005), and *Knoellia subterranea* was isolated from a cave (Groth et al. 2002). *Leucobacter komagatae* is reported as an aerobic Gram-positive, nonsporulating bacterium with 2,4-diaminobutyric acid in the cell wall (Takeuchi et al. 1996). *Corynebacterium amycolatum* was recorded as an agent to cause endocarditis (Dalal et al. 2008). *Corynebacterium callunnae* is a producer of α-1,4-D-glucan phosphorylase and could degrade starch (Weinhäusel et al. 1997). *Nocardioides kongjuensis* was described as an N-acylhomoserine lactone-degrading bacterium (Yoon et al. 2006). Therefore, most of the Gram-positive bacteria found in this study were new

cellulolytic bacteria. Furthermore, most of the defined species were described with one or two strains, and the isolation of members related to them in the chinampa soils enlarged their nature habitats and their potential ecological function.

In the phylum Proteobacteria, species *Agrobacterium rubi, Stenotrophomonas maltophilia, Pseudoxanthomonas mexicana* and *Pseudomonas pseudoalcaligenes* have been recorded as cellulolytic bacteria in the gut of *Holotrichia parallela* larvae (Huang et al. 2012), in rumen (Rismani-Yazdi et al. 2007), or in a soda lake (Borsodi et al. 2005). Our results in the present study enlarged their natural habitat. *Aeromonas media* is an aquatic bacterium (Allen et al. 1983); *Delftia acidovorans* is a bacterium in soils and sediments, using many aromatic compounds as carbon source (Wen et al. 1999); and *Luteimonas aestuarii* has been found in tidal flat sediment (Roh et al. 2008). The latter three species were novel recorded cellulolytic bacteria. At the species level, *S. maltophilia* (8 isolates), *P. mexicana* (5 isolates) and *P. pseudoalcaligenes* (8 isolates) were the dominant groups.

All the analyses above demonstrated that our results greatly improved the knowledge about the diversity of cellulolytic bacteria and demonstrated that the chinampa soils were valuable resource for searching novel cellulolytic bacteria, especially those adapted to the alkaline-saline soils with high humus.

In the present study, the high pH value, high contents of organic material, total nitrogen and potassium in the two chinampa soils were similar to the data reported previously for other chinampas (Ramos-Bello et al. 2001), implying that these two chinampas were good representatives for the chinampas. In the bulk chinampa soils, the abundances of total cultural bacteria and of cellulolytic bacteria (Fig. 1) were similar to those in an agricultural catchment (Ulrich and Wirth 1999), and in sandy and a loam soil after long-term manure application (Ulrich et al. 2008), but much higher than the values (145.2/37.4 and 326.5/138.6 CFU g^{-1} dry soil) obtained from the forest and farming soils in the north of Iran (Hatami et al. 2008). However, the proportion of cultivable cellulolytic bacteria to the total population of bacteria in chinampa soils (0.13 to 0.19) (Fig. 1) was lower than those in the agricultural catchment (0.17 to 0.4) (Ulrich and Wirth 1999) and in Iran soils (34.7 % and 52.4 %) (Hatami et al. 2008). These data might be related to the fact that the chinampa soils were mature and the bacteria community had reached equilibrium or a stable stage. Previously, Pourcher et al. (2001) have reported that there were more cellulolytric bacteria in 1-year-old refuse samples than in 5-year-old ones.

It is interesting that the abundance of total cultural mesophilic aerobic bacteria in bulk soils of chinampas (Fig. 1) was the same as that in the soils containing low concentrations of organic matter (Ulrich et al. 2008; Ulrich and Wirth 1999). In general, the high content of organic matter enhances the biomass, including the bacterial abundance in soil. The low number of mesophilic aerobic bacteria in chinampa soils might demonstrate the possibility that the high content of organic carbon in chinampa soils (Table 1) was mainly humus, which cannot be used as a carbon source by the bacteria.

Previously, effects of vegetation on the diversity of cellulolytic bacteria have been reported (Rabinovich et al. 2002). In the present study, it was clear that the cultured plant species enhanced the density of both the total bacteria and cellulolytic bacteria in the rhizosphere (Fig. 1), but this enhancement was greater for the total bacteria than for the cellulolytic bacteria, as shown by the values of R/S and T/C ratios (Fig. 1). These results demonstrated that cellulolytic bacteria were not the biophysical group selected by the rhizosphere.

The R/S ratios obtained in the present study also demonstrated that different plants had distinct effects on both total bacteria and cellulolytic bacteria (Fig. 1) in the rhizosphere. In addition, the interaction of soil types and cultured plants affects the rhizosphere microbiota, since the density and R/S values of the alfalfa rhizosphere apparently varied in chinampa I and chinampa II, while the five isolates of *Pseudoxanthomonas mexicana* were only isolated from non-rhizosphere soils. In our study, the P and K contents in chinampa I were greater than those in chinampa II (Table 1), which might be an explanation for the difference of bacteria in the rhizosphere of alfalfa grown in the two chinampas, as reported for soybean rhizobia (Han et al. 2009). The humidity or oxygen concentration in soil might also be a determinant for the horizontal and vertical distribution of cellulolytic bacteria in the chinampas, since all eight isolates of *Stenotrophomonas maltophilia* were only isolated from surface soils of the two chinampas.

Previously, Lu et al. (2005) found DI (D/d) values ranging from 4.2 to 10.4 in mesophilic aerobic bacteria belonging to the genera *Bacillus*, *Halobacillus*, *Aeromicrobium* and *Brevibacterium* isolated from compost, in which *Bacillus* showed higher cellulolytic capacity. In our study, the DI ranged between 1.6 (*Cellulosimicrobium* sp. S4) and 6.5 (*Bacillus cereus* P3) for CMC; between 1.7 (*Pseudomonas* sp. S5B; *Streptomyces* sp. S2) and 7.5 (*Pseudomonas* sp. F7A) for xylan; and between 1.6 (*Stenotrophomonas* C13) and 6.5 (*Pseudomonas* sp. S5B) for avicel (Supplementary Table A). These data demonstrated that many isolates have the ability to degrade different polymers, but the degradation efficiency varied. Cruz et al. (2009) found that *Penicillum* and *Streptomyces* were indicative for xylan degradation, while *Aspergillus* and *Streptomyces* showed great cellulolytic activity in agricultural wastes of the Bogota Plateau. However, our results indicated that the degradation efficiency varied dramatically among the isolates within a single genus.

By comparing the cellulose, xylan and avicel degradation of isolates, we found that: (1) not all the cellulolytic bacteria (CMC-degraders) could degrade xylan and avicel; (2) the

xylan-decomposers were more common than avicel-decomposers; and (3) all of them were ubiquitous in the soil samples. These could be explained by the fact that xylan and cellulose are commonly coexisting in the vegetable materials, but crystal cellulose is not so common in nature and is rather difficult to degrade. Previously, the relationship between the structure and function of cellulolytic communities in soil has not been widely studied. In the present study, the cellulolytic potentials of the isolates were highly variable and did not show a relationship between degradation activity and bacterial taxa (group). Similarly, Ulrich et al. (2008) found that a correlation between community structure and metabolic function did not exist.

The degradation data of the isolates at different pH and salinity revealed that the cellulolytic bacteria isolated from the alkaline-saline soils adopted a wide range of pH and salinity (NaCl concentration) levels. Some strains could degrade cellulose at pH range of 4.5–10.0 and up to 9 % of NaCl. These features might be related to the fact that the surface soils of chinampa are alkaline-saline, but acid microhabits may exist based upon the soil structure.

Conclusively, the community of cellulolytic bacteria in the chinampa soils was unique, characterized by the dominance of *Streptomyces, Arthrobacter*, *Bacillus*, *Pseudomonas* and *Stenotrophomonas* and the existence of many putative novel species, genera or families. The community composition of the cellulolytic bacteria was affected by both the soil types and the vegetation type, although the cellulolytic bacteria were not the biophysical group selected by rhizosphere. The data of this study greatly improve the knowledge about diversity of cellulolytic bacteria and imply that the chinampa soils are an important resource for searching for new cellulolytic bacteria with possible biotechnology applications.

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