Diversity of a Chlorine-Resistant *Bacillus* Population Isolated from a Wastewater Treatment Station

Fernanda A. Paes[1], Denise C. Hissa[1], Alysson L. Angelim[1], Natasha W. Pinto[1], Thalles B. Grangeiro[2], Vânia M. M. Melo[1]*

ABSTRACT: This paper describes the phenotypic and genotypic diversity of a Gram-positive, aerobic bacterial population isolated from the chlorine tank of a wastewater treatment plant. A total of 12 spore-forming, rod-shaped isolates were identified using 16S rRNA gene sequencing and biochemical tests. Pairwise genetic comparisons revealed the identity among sequences obtained from isolates varied from 92.6 to 100%. Similarity searches on GenBank showed that five strains were closely related (99 to 100% identity) to *Bacillus subtilis* and two were almost identical (99%) to *B. megaterium* and *B. licheniformis*. Because the five remaining strains were either closely related (97 to 99% identity) or identical to *B. cereus*, *B. thuringiensis*, and *B. anthracis*, they were classified as belonging to the *B. cereus* group. Apart from one strain, all clades in the phylogenetic tree were identical to clusters formed in the dendrogram based on biochemical tests results. According to the biochemical profiles, all isolates were characterized as different strains. In addition to chlorine resistance, all isolates were found to be resistant to at least one of five antibiotics tested. These results identify the potential risk of spreading antibiotic resistance genes in the environment by chlorine-resistant strains of *Bacillus*. *Water Environ. Res.*, **84**, 274 (2012).

KEYWORDS: *Bacillus* spp., chlorine disinfection, wastewater.

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Introduction

Water resource availability is decreasing for much of the world's population as a result of increasing pressures on freshwater sources. Consequently, water reuse has become a sustainable alternative for water supply, especially in arid regions such as northeastern Brazil. Although recycled water is used globally for agriculture irrigation and industry applications, a variety of policies and regulations on water reuse have been established to help protect the environment from potential adverse effects of wastewater discharges.

[1] Laboratório de Ecologia Microbiana e Biotecnologia (LEMBiotech), Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil.

[2] Laboratório de Genética, Departamento de Biologia, Universidade Federal do Ceará, Fortaleza, Ceará, Brazil.

* Laboratório de Ecologia Microbiana e Biotecnologia (LEMBiotech), Departamento de Biologia, Universidade Federal do Ceará, Av. Humberto Monte 2977, Campus do Pici, Bloco 909, Fortaleza, Ceará, Brazil, 60455-970; e-mail address: vmmmelo@ufc.br.

The conventional treatment of wastewater relies on a combination of physical, chemical, and biological methods, with chlorine disinfection as the final step of the treatment process. Chlorine is the most widely used bactericidal agent used in wastewater treatment plants given its well known efficacy against microorganisms, low cost, and ease of use. Although chlorine disinfection is used to destroy or inactivate pathogenic organisms of fecal origin, its use does not guarantee the complete destruction (sterilization) of all living organisms (Mir et al., 1997).

Bacteria belonging to the genus *Bacillus* are aerobic or facultative anaerobic, Gram-positive or variable, and form endospores that exhibit resistance to environmental stress. Members of this genus are important to human activities because of their widespread use in industrial, agricultural, and medical applications. For example, several strains of *Bacillus* are used in the production of antibiotics, enzymes, and solvents (Fekete, 2009); probiotics (Casula and Cutting, 2002); or insecticides (Santos et al., 2009). However, some species such as *B. anthracis* and *B. cereus* constitute a threat to public health and welfare (Shangkuan et al., 2000).

The uniqueness of *Bacillus* lies in its ability to produce spores that can survive desiccation, heat, and cold, and remain able to germinate (Young and Setlow, 2003). Additionally, *Bacillus* species present a high degree of genetic plasticity that enables the colonization of a wide range of habitats, including sewage and collection systems and wastewater (Fekete, 2009). Although most pathogenic microorganisms are destroyed or inactivated during the wastewater treatment process, disinfection-resistant microbes can survive treatment and represent a potential risk when released back to the environment. It has been shown that bacteria can develop resistance to different disinfection agents used in water and wastewater treatment, including chlorine (Ridgway and Olson, 1982) and sodium dichloroisocyanurate (D'Auria et al., 1989). In addition, it has been suggested that chlorine-resistant organisms might be able to produce functional enzymes under conditions in which most known enzymes would be inactivated as a result of environmental stress (Demirjian et al., 2001).

In the literature, microbial communities associated with water and wastewater treatment systems have been studied from two different points of view. From an ecological perspective, researchers are interested in:

(1) Evaluating the effect of recycled water into the environment;
(2) Determining the microbial populations that occur in these systems and their diversity;

(3) Associating these populations with their specific role in the environment;
(4) Determining their resistance to treatment. Second, from a public health perspective, researchers are interested in determining the identity, pathogenicity, and treatment resistance of emerging isolates related to water reuse (Gilbride et al., 2006).

Considering that wastewater treatment systems are gradually selecting and releasing chlorine-resistant microorganisms back to the environment, the main objective of this study was to identify and characterize the chlorine-resistant bacteria population isolated from the disinfection tank of a wastewater treatment plant in northeastern Brazil.

Materials and Methods

Microorganisms. The 12 studied strains (LAMI 002 to LAMI 013) were isolated from the chlorine tank of the wastewater treatment station on Pici Campus at the Federal University of Ceará, Brazil. Briefly, triplicate samples were collected from the water column of the chlorine tank in sterile flasks and immediately transported to the laboratory in an ice box. In order to select the sporulating bacteria, the samples were heated for 10 minutes at 80 °C (Marten et al., 2000). Next, 10-fold serial dilutions were prepared using sterile phosphate buffered saline (pH 7.2) and plated onto Tryptone Soya Agar (TSA; Oxoid, Basingstoke, U.K.). Following incubation at 35 °C for 48 hours, a variety of different types of colonies (colony color, shape, appearance, and size) were selected and cultured on TSA. The colonies were stored at −80 °C for subsequent identification.

Note that all strains evaluated in this study have been retained in the bacterial collection of LEMBiotech at the Federal University of Ceará, Brazil.

Sequencing. Bacterial genomic DNA extraction was performed using a CTAB (cetytrimethyl ammonium bromide)-based protocol (Warner, 1996). The 16S rRNA was amplified by polymerase chain reaction (PCR) using the primers 27F (forward: 5′-AGAGTTTGATCCTGGCTCAG-3′) and 1525R (reverse: 5′-AAGGAGGTGATCCAGCC-3′), as described by Weisburg et al. (1991). Amplification reactions were performed in a final volume of 25 µL containing 300 ng of genomic DNA (template); 20 mM Tris-HCl, pH 8.4; 50 mM KCl; 1.5 mM $MgCl_2$; 200 µM each of 2′-deoxyadenosine-5′-triphosphate, 2′-deoxycytidine-5′-triphosphate, 2′-deoxyguanosine-5′-triphosphate, and 2′-deoxythymidine-5′-triphosphate; 12.5 pmoles of each primer; and 1 unit of *Taq* DNA polymerase. All PCR reactions were conducted using a PTC-200 thermocycler (MJ-Research Inc., Watertown, Massachusetts) programmed for an initial denaturation step (4 minutes at 94 °C) followed by 35 cycles of 1 minute at 94 °C, 1 minute at 55 °C, and 2 minutes at 72 °C. The last cycle was followed by a final extension of 10 minutes at 72 °C. Amplification of DNA was checked by a 1.0% agarose gel electrophoresis. After the specificity of the amplifications was confirmed, the PCR product was purified from the remaining reaction using GFX™ PCR DNA and gel band purification kit (GE Healthcare, Piscataway, New Jersey). The concentration of purified PCR product was determined measuring absorbance at 260 nm. Sequencing of DNA was performed with the DYEnamic™ ET Terminator Cycle Sequencing Kit (GE Healthcare, Piscataway, New Jersey), and both strands were sequenced using the primers 27F and 1525R and 782R (5′-ACCAGGGTATCTAATCCTGT-3′) and 1100R (5′-AGGGTTGCGCTCGTTG-3′). Sequencing reactions were analyzed in a MegaBACE™ 1000 automatic sequencer (GE Healthcare, Piscataway, New Jersey). High quality reads (phred > 20) were used to generate near full-length 16S rRNA gene sequences using the Phred/Phrap/Consed package (Ewing and Green, 1998; Ewing et al., 1998; Gordon et al., 1998). All sequences were deposited in GenBank (accession numbers EU082292 and FJ413043-FJ413053).

Phylogenetic Analysis. Searches for homologous sequences deposited on public databases were performed using the Basic Local Alignment Search Tool (BLAST) program (Altschul et al., 1990). The sequences obtained in this study were aligned to other *Bacillus* 16S rRNA gene sequences retrieved from GenBank.

All GenBank sequences were from the following species (for those derived from genome sequences, the genomic region corresponding to the 16S rRNA gene is shown after the genome accession number): *B. amyloliquefaciens* strains FZB42 (CP000560; 9760-11314), BEH 111 (FN677986), CICC 2383 (GQ 375222), and BCRC 11266 (EF423605); *B. clausii* KSM-K16 (AP006627; 11675-13227); *B. pseudofirmus* OF4 (CP001878; 1584260-1585820); *B. pumilus* SAFR-032 (CP000813; 9570-11120); *B. halodurans* C-125 (BA000004; 22819-24370); *B. cereus* strains ATCC 10987 (AE017194; 9335-10842), ATCC 14579 (AE016877; 9188-10699), E33L (CP000001; 9337-10846), Q1 (CP000227; 9338-10843), and AH820 (CP001283; 9334-10842); *B. thuringiensis* serovar konkukian strain 97-27 (AE017355; 9337-10846); *B. thuringiensis* strain Al Hakam (CP000485; 9309-10762); *B. anthracis* strains Sterne (AE017225; 9336-10845), Ames (AE016879; 9335-10841), A0248 (CP001598; 9233-10741), CDC 684 (CP001215; 9207-10715), and Ames Ancestor (AE 017334; 9335-10841); *B. cereus* subsp. cytotoxis NVH 391-98 (CP 000764; 9424-10967), *B. weihenstephanensis* KBAB4 (CP000903; 9027-10571), *B. pseudomycoides* strains J14 (GU826151), BIHB 337 (FJ859684), 818 (FJ544335), and Nl4 (GU391527); *B. mycoides* strains REG65 (EU647707), 820 (FJ544336), and L2S8 (EU 221418); *B. subtilis* subsp. *subtilis* strains 168 (AL009126; 9810-11364), CICC 10088 (GQ375233), and BCRC 10255 (EF423592); *B. subtilis* subsp. *spizizenii* strains BCRC 10447 (EF433403), BCRC 17366 (EF433402), and ATCC 6633 (AB018486); *B. subtilis* strains S8-18 (EU624423), CCM 1999 (DQ207730), N10 (AF 318900), BHP6-1 (AY162131), B1-33 (EU435361), and BZ15 (AY162133); *B. licheniformis* strains ATCC 14580 (CP000002; 9909-11453), S8-03(EU620411), BCRC 15413 (DQ993676), IM AUB1014 (FJ641027), and BM-Y6 (FJ432006); and *B. megaterium* strains C1 (GQ304786), GMX5 (AM910818), IAM 13418 (D16273), A10-2 (AB244298), and rif200861 (FJ527630).

The 16S rRNA gene sequences from *Lactobacillus casei* ATCC 334 (CP000423, 259510-261077) and *L. sakei* 23K (CR936503, 306178-307748) were also retrieved from GenBank and used as outgroups. Sequence alignments were produced and manually edited using the programs ClustalW (Thompson et al., 1994) and BioEdit version 7.0.5 (Hall, 1999), respectively. The multiple alignments were then used to generate a phylogenetic tree using the MEGA software, version 4.0 (Tamura et al., 2007). The Neighbor-Joining distance method (Saitou and Nei, 1987) was employed using the Kimura 2-parameter model of sequence evolution to compute the distances (Kimura, 1980). The stability of the recovered clades was assessed by the bootstrap test

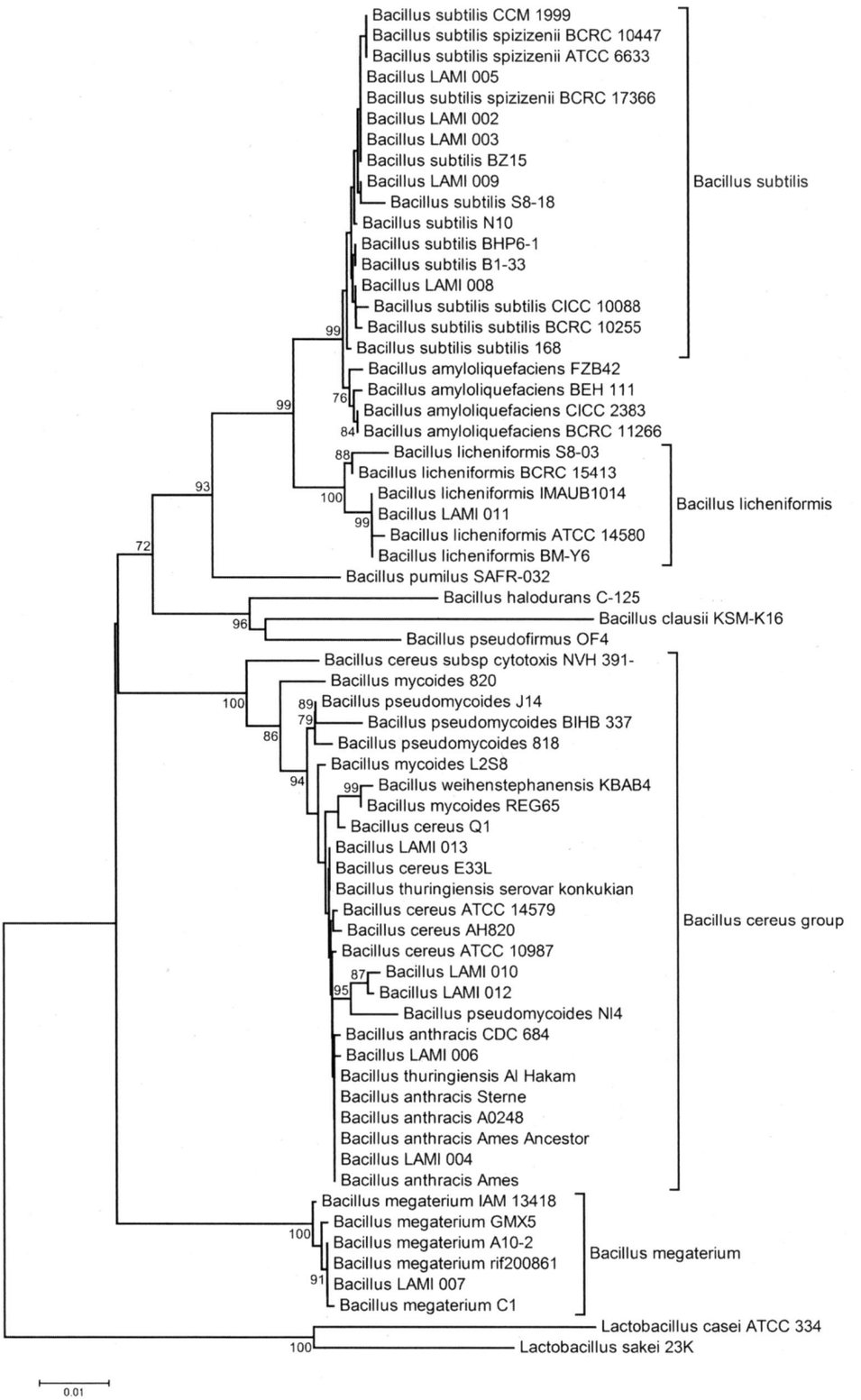

Figure 1—Neighbor-joining tree of the 16S rRNA gene sequences from *Bacillus* species. The optimal tree with sum of branch length (0.44356290) is shown. The percentage of replicate trees in which associated taxa are clustered together in the bootstrap test (1000 replicates) is shown next to the branches (only values > 70% are shown). Note that tree is drawn to scale using branch lengths with the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances computed using Kimura 2-parameter method (Kimura, 1980) and are in number of base substitutions per site (scale bar).

(Felsenstein, 1985) using 1000 replicates. All positions containing gaps and missing data were eliminated from the dataset.

Phenotypic Characterization. Phenotypic characterization of bacterial strains was achieved using typical microbiological methods provided in *Bergey's Manual of Systematic Bacteriology* (Claus and Berkeley, 1986). The following phenotypic criteria were considered: colony morphology (size, shape, and pigmentation); cell morphology (Gram stain, size, shape, and presence of endospore) and motility; oxidase and catalase tests; oxygen requirement for growth; carbohydrate fermentation; indole production, Voges-Proskauer test; use of citrate as carbon source; salt tolerance (growth in the presence of up to 10% NaCl); pH range for growth (2 to 12); temperature tolerance (5 to 55 °C); and hypochlorite resistance (growth in the presence of up to 10 000 mg/L of sodium hypochlorite). All isolates were tested for their ability to produce amylase, gelatinase, and caseinase (Collins and Lyne, 1989); esterase (Gudelj et al., 1998) and alcohol dehydrogenase (Conway et al., 1987); lipase (Kouker and Jaeger, 1987); chitosanase (Piza et al., 1999); hemolysin (Garcia et al., 2002); and biosurfactants (Iqbal et al., 1995). Sensitivity to five antibiotics (amoxacillin, chloramphenicol, erythromycin, tetracycline, vancomycin) was also tested (Bauer et al., 1966).

A cluster analysis of biochemical data was performed using Euclidian similarity coefficient of Paleontological Statistics Software (PAST; Hammer et al., 2001).

Results and Discussion

The selected bacterial population isolated from the chlorine tank of the wastewater treatment station studied in this paper was composed mainly of Gram-positive, aerobic, and spore-forming rods. The near full-length 16S rRNA gene sequences were determined for all strains and ranged from 1255 base pairs (LAMI 007 strain) to 1481 base pairs (LAMI 008 strain). Pairwise comparisons excluding the sites with gaps revealed that the sequence identity varied from 92.6% (sequences from strains LAMI 003 and LAMI 012) to 100% (between the sequences from strains LAMI 002 and LAMI 005). Similarity searches on GenBank using BLAST showed that the sequences from five strains (LAMI 002, 003, 005, 008, and 009) were most related (99 to 100% identity) to 16S rRNA gene sequences from different strains of *Bacillus subtilis*. On the other hand, the 16S rRNA gene sequences from the strains LAMI 007 and LAMI 011 were almost identical (99%) to homologous sequences from *B. megaterium* and *B. licheniformis* strains, respectively. The 16S rRNA gene sequences from the five remaining strains (LAMI 004, 006, 010, 012, and 013) were either closely related (97 to 99% sequence identity) or identical to the corresponding sequences from strains of *B. cereus*, *B. thuringiensis*, and *B. anthracis*. Therefore, the 12 strains were classified as belonging to *B. megaterium* (LAMI 007), *B. licheniformis* (LAMI 011), *B. subtilis* (LAMI 002, 003, 005, 008, and 009), and the *B. cereus* group (LAMI 004, 006, 010, 012, and 013) (Table 1). Moreover, the Neighbor-Joining tree (Figure 1), derived from the alignment of 65 16S rRNA gene sequences, shows 4 main clusters grouping the sequences from *B. subtilis*, *B. licheniformis*, *B. megaterium*, and *B. cereus sensu lato* (bootstrap values = 99 to 100%).

The study results show that 16S rRNA characterization alone was not sufficient to identify all *Bacillus* isolates. The strains related to the *B. cereus* group are believed to have evolved from a common ancestor and have 16S rRNA gene sequences that are virtually identical with each other, which encumbers the molecular characterization of the *Bacillus* strains assessed in this study. Based on their close genotypic similarity, the *B. cereus* group includes *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. pseudomycoides*, *B. weihenstephanensis*, and *B. mycoides* (Bavykin et al., 2004; Guinebretiere et al., 2008). To date, taxonomy of these species has been based on analysis of morphological, biochemical, physiological, and immunological characteristics, despite their unreliability (Bavykin et al., 2004). Among these species, *B. thuringiensis* has been traditionally distinguished from *B. cereus* by the production of a plasma-encoded toxin (Aronson, 1993), whereas anthrax toxin components are used to discriminate *B. anthracis* from *B. cereus* and *B. thuringiensis* (Hadjinicolaou et al., 2009). Although it is known that some plasmids can be lost through laboratory culturing (Bavykin et al., 2004), González et al. (1982) previously demonstrated the transference of toxin-coding plasmids into *B. cereus* strains cultivated with *B. thuringiensis*, confirming the possibility of gene transference between different *Bacillus* species. Researchers continue to improve discrimination among *B. cereus* group bacteria (Tourasse and Kolstø, 2008; Tourasse et al., 2006).

By analyzing the biochemical and physiological profiles, the 12 isolates were classified as different strains. The overall phenotypic characteristics are showed in Table 2 and Table 3. According to these results, the strains that were more closely related by the 16S rRNA gene sequences analysis exhibited

Table 1—16S rRNA gene sequence analysis of bacteria isolated from chlorine tank of a wastewater treatment station, based on BLAST (BLAST = Basic Local Alignment Search Tool).

Bacterial strain	GenBank accession number	Species most closely related in GenBank	Identity over 1000 base pairs (%)
LAMI 002	FJ413043	*Bacillus subtilis*	99
LAMI 003	FJ413044	*Bacillus subtilis*	99
LAMI 004	FJ413045	*Bacillus thuringiensis/Bacillus anthracis/Bacillus cereus*	100
LAMI 005	FJ413046	*Bacillus subtilis*	100
LAMI 006	FJ413047	*Bacillus cereus/Bacillus thuringiensis/Bacillus anthracis*	99
LAMI 007	FJ413048	*Bacillus megaterium*	99
LAMI 008	EU082292	*Bacillus subtilis*	99
LAMI 009	FJ413049	*Bacillus subtilis*	100
LAMI 010	FJ413050	*Bacillus thuringiensis/Bacillus anthracis/Bacillus cereus*	98
LAMI 011	FJ413051	*Bacillus licheniformes*	99
LAMI 012	FJ413052	*Bacillus cereus/Bacillus thuringiensis/Bacillus anthracis*	97
LAMI 013	FJ413053	*Bacillus cereus/Bacillus anthracis/Bacillus thuringiensis*	100

Table 2—Biochemical characterization of *Bacillus* spp. isolated from chlorine tank of a wastewater treatment station.

	Strain											
	B. subtilis					*B. cereus* group					*B. megaterium*	*B. licheniformis*
Reaction	002	003	005	008	009	004	006	010	012	013	007	011
Indole production	−	−	−	−	−	−	−	−	−	−	−	−
Voges-Proskauer (V−P)	+	+	+	+	+	−	+	−	−	+	−	+
V−P (pH < 6)	−	−	−	−	−	+	+	+	+	+	−	+
V−P (pH > 7)	+	+	−	+	+	−	−	−	−	−	−	−
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	−	+	+	−	+	+	+
Gelatinase	+	+	+	+	+	+	−	−	+	+	−	−
Caseinase	+	+	+	+	+	+	+	+	+	+	+	+
Chitosanase	−	−	−	−	−	+	−	−	−	+	−	−
Esterase	+	+	+	−	+	−	+	−	+	+	−	+
Lipase	+	+	+	+	−	−	+	−	−	−	−	+
Alcohol-desidrogenase	+	+	+	+	−	−	+	−	−	+	−	+
Hemolysin	−	+	+	−	−	−	−	+	+	+	−	−
Emulsification activity	+	+	+	+	+	+	−	+	+	+	+	−
D-glucose fermentation	−	−	−	−	+	+	+	+	+	+	+	+
D-mannitol fermentation	−	+	−	+	−	−	+	−	−	−	−	+
L-arabinose fermentation	−	−	−	−	−	−	+	−	−	−	+	+
D-xilose fermentation	−	+	−	−	−	−	+	−	−	−	−	−
Citrate as carbon source	−	−	−	−	−	−	−	−	−	−	−	−

different biochemical and physiological profiles. However, apart from LAMI 006, the clades formed in the 16S rRNA gene sequences phylogenetic tree (Figure 1) were identical to clusters formed in the dendrogram based on biochemical profiles of the strains (Figure 2). This finding shows that the *B. cereus* group and *B. megaterium* share some similarities and belong to a distinct cluster from that formed by *B. subtilis* and *B. licheniformis*.

This study also shows that conventional chemical disinfection of wastewater with chlorine is not suitable for decontamination of spore-forming bacteria, including potentially pathogenic *Bacillus* (Morrow et al., 2008). Bacterial spores are the most resistant of bacterial forms to antiseptics, disinfectants, and preservatives because of the presence of outer spore layers that present a barrier to the intracellular penetration of many biocides. In addition, the environment in the spore core, including low water content and low pH, might also contribute to their resistance (Cortezzo and Setlow, 2005).

As a result of wastewater treatment, most potentially pathogenic bacteria are removed or inactivated; however, microorganisms that are resistant to disinfection can persist and disseminate into the aquatic environment. Further, many of these organisms carry antibiotic-resistance genes and, once such a gene is generated, it can be released to natural bacterial ecosystems where non-pathogenic bacteria could serve as a reservoir of resistance genes (Baquero et al., 2008). In this study, the strains LAMI 004, LAMI 005, LAMI 006, LAMI 010, LAMI 011, and LAMI 012 were resistant to at least one of the five antibiotics tested (Table 4). Although it is essential to consider the dissemination of chlorine- and antibiotic-resistant organisms by wastewater treatment systems, it is also appealing to consider the applicability of these resistant bacteria or their products in biotechnology. Microorganisms are a potential source for the development of highly optimized enzymes, which are suited to perform optimally under the physicochemical conditions of the microorganism habitats (Owusu et al., 1991; Pace, 1997).

Table 3—Profile of resistance to temperature, pH, osmotic pressure, and hypochlorite of the *Bacillus* spp. isolated from chlorine tank of a wastewater treatment station.

LAMI strain	Hypochlorite concentration (mg/L)/time of contact (hours)	Temperature range (°C)	pH range	NaCl (%)
002	5.000/1	30–50	5–12	2–10
003	1.000/1	5–55	3–12	2–10
004	500/24	5–40	7–12	2–7
005	500/1	5–50	5–12	2–7
006	10/1	5–50	7–12	2–10
007	5/1	10–40	7–12	2–10
008	500/1	10–50	5–12	2–10
009	50/1	5–50	7–12	2–7
010	100/24	10–40	7–12	2–5
011	100/24	5–50	5–12	2–10
012	100/1	5–40	5–12	2–7
013	5/1	30–40	7–11	2–5

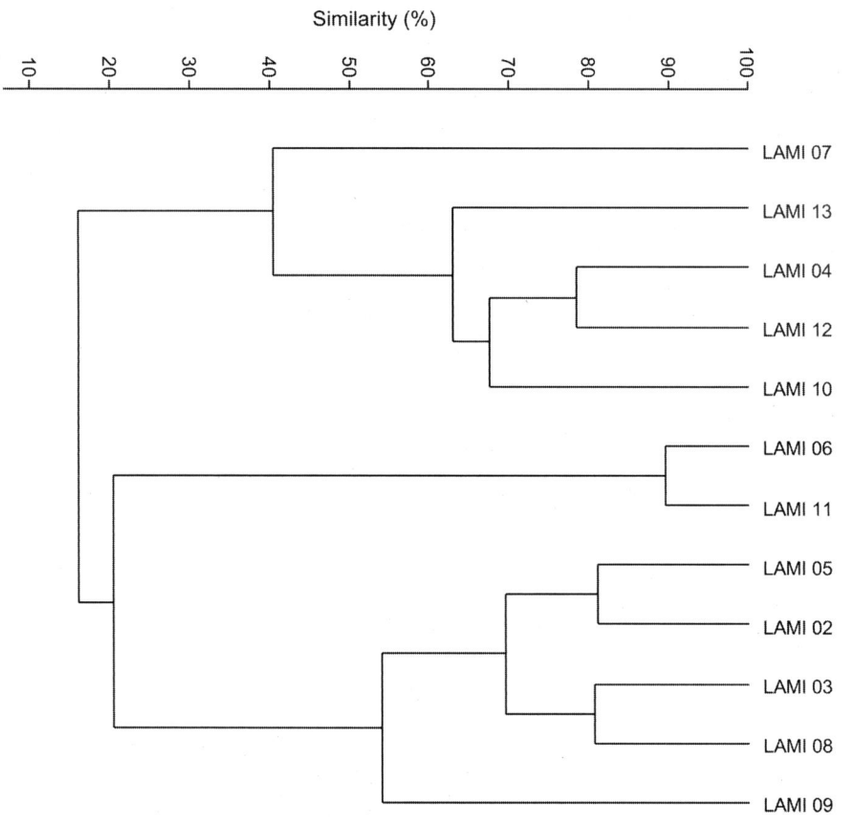

Figure 2—Cluster analysis of *Bacillus* spp. based on their biochemical profiles.

The *Bacillus* strains studied in this paper were able to grow in a wide range of temperatures (5 to 50 °C), pH (3 to 12), NaCl concentrations (2 to 10%), and supported hypochlorite concentrations up to 5000 mg/L. In addition, some strains were able to produce biosurfactants, which are surface-active compounds with great applicability in the food, agrochemical, cosmetic, and pharmaceutical industries. With the exception of LAMI 006 and LAMI 011, all *Bacillus* strains showed significant emulsification activity. In this regard, the potential application of LAMI 008 for the production of surfactin using cashew apple juice as substrate, and for the degradation of petroleum hydrocarbon using bacterial spores entrapped in chitosan beads, has been demonstrated (Barreto et al., 2010; Rocha et al., 2009).

Conclusions

This study shows that both human and animal *Bacillus* pathogens can survive conventional wastewater chlorine disinfection, hindering the use of this water for certain purposes such as agricultural irrigation without additional treatment. The main risk involves spreading pathogenic organisms and antibiotic-resistant

Table 4—Antibiotic susceptibility of the *Bacillus* spp. isolated from chlorine tank of a wastewater treatment station (R = resistant; S = sensitive).

	Antibiotic				
LAMI strain	**amoxacillin**	**chloramphenicol**	**erythromycin**	**tetracycline**	**vancomycin**
002	S	S	S	S	S
003	S	S	S	S	S
004	R	S	S	S	R
005	R	S	S	S	R
006	R	S	R	S	S
007	S	S	S	S	S
008	S	S	S	S	S
009	S	S	S	S	S
010	R	S	S	S	S
011	R	R	S	S	S
012	R	S	S	S	S
013	S	S	S	S	S

genes into the environment. The isolates studied in this paper encompass a diverse group of *Bacillus* with distinctive biochemical profiles, although some shared genetic similarity. In addition, the isolates showed biotechnology potential for use in the production of industrial enzymes and biosurfactants.

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References

Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J. (1990) Basic Local Alignment Search Tool. *J. Mol. Biol.*, **215** (3), 403–410.

Aronson, A. I. (1993) The Two Faces of *Bacillus thuringiensis*: Insecticidal Proteins and Post-Exponential Survival. *Mol. Microbiol.*, **7** (4), 489–496.

Baquero, F.; Martínez, J.-L.; Cantón, R. (2008) Antibiotics and Antibiotic Resistance in Water Environments. *Curr. Opin. Biotechnol.*, **19** (3), 250–265.

Barreto, R. V. G.; Hissa, D. C.; Paes, F. A.; Grangeiro, T. B.; Nascimento, R. F.; Rebelo, L. M.; Craveiro, A. A.; Melo, V. M. M. (2010) New Approach for Petroleum Hydrocarbon Degradation Using Bacterial Spores Entrapped in Chitosan Beads. *Bioresour. Technol.*, **101** (7), 2121–2125.

Bauer, A. W.; Kirb, W. M. M.; Sherris, J. C.; Turck, M. (1966) Antibiotic Susceptibility Testing by Standardized Single Disk Method. *Amer. J. Clin. Pathol.*, **45** (4), 493–496.

Bavykin, S. G.; Lysov, Y. P.; Zakhariev, V.; Kelly, J. J.; Jackmen, J.; Stahl, D. A.; Cherni, A. (2004) Use of 16S rRNA, 23S rRNA, and *gyrB* Gene Sequence Analysis to Determine Phylogenetic Relationships of *Bacillus cereus* Group Microorganisms. *J. Clin. Microbiol.*, **42** (8), 3711–3730.

Casula, G.; Cutting, S. M. (2002) *Bacillus* Probiotics: Spore Germination in the Gastrointestinal Tract. *Appl. Environ. Microbiol.*, **68** (5), 2344–2352.

Claus, D.; Berkeley, R. C. W. (1986) *Bacillus* Cohn 1872. In *Bergey's Manual of Systematic Bacteriology*; Sneath, P. H. A., Ed.; Williams and Wilkins: Baltimore, Maryland; Vol. 2, pp 1105–1140.

Collins, C. H.; Lyne, P. M. (1989) *Microbial Methods*. Butterworth: London.

Conway, T.; Sewell, G. W.; Osman, Y. A.; Ingram, L. O. (1987) Cloning and Sequencing of the Alcohol Dehydrogenase II Gene from *Zymomonas mobilis*. *J. Bacteriol.*, **169** (6), 2591–2597.

Cortezzo, D. E.; Setlow, P. (2005) Analysis of Factors that Influence the Sensitivity of Spores of *Bacillus subtilis* to DNA Damaging Chemicals. *J. Appl. Microbiol.*, **98** (3), 606–617.

D'Auria, F. D.; Simonetti, G.; Strippoli, V. (1989) Antimicrobial Activity Exerted by Sodium Dichloroisocyanurate (Engl. Transl.). *Ann. Ig.*, **1** (6), 1445–1458.

Demirjian, D. C.; Morís-Varas, F.; Cassidy, C. S. (2001) Enzymes from Extremophiles. *Curr. Opin. Chem. Biol.*, **5** (2), 144–151.

Ewing, B.; Green, P. (1998) Base-Calling of Automated Sequencer Traces Using *phred*. II. Error Probabilities. *Genome Res.*, **8**, 186–194.

Ewing, B.; Hillier, L.; Wendl, M. C.; Green, P. (1998) Base-Calling of Automated Sequencer Traces using *phred*. I. Accuracy Assessment. *Genome Res.*, **8**, 175–185.

Fekete, T.M.D. (2009) *Bacillus* Species (not *anthracis*). *Clin. Microbiol. Newslett.*, **31** (12), 87–92.

Felsenstein, J. (1985) Confidence Limits on Phylogenies: an Approach using the Bootstrap. *Evolution*, **39** (4), 783–791.

Garcia, D. O.; Timenetsky, J.; Martinez, M.B.; Francisco, W.; Sinto, S.I.; Tanaguita, R. M. (2002) Proteases (Caseinase and Elastase), Hemolysins, Adhesion and Susceptibility to Antimicrobials of *Stenotrophomonas maltophilia* Isolates Obtained from Clinical Specimes. *Braz. J. Microbiol.*, **33** (2), 157–162.

Gilbride, K. A.; Lee, D.-Y.; Beaudette, L. A. (2006) Molecular Techniques in Wastewater: Understanding Microbial Communities, Detecting Pathogens, and Real-Time Process Control. *J. Microbiol. Methods*, **66** (1), 1–20.

González, J. M.; Brown, B. J.; Carlton, B. C. (1982) Transfer of *Bacillus thuringiensis* Plasmids Coding for Delta-Endotoxin among Strains of *B. thuringiensis* and *B. cereus*. *Proc. Natl. Acad. Sci.*, **79** (22), 6951–6955.

Gordon, D.; Abajian, C.; Green, P. (1998) Consed: a Graphical Tool for Sequence Finishing. *Genome Res.*, **8**, 195–202.

Gudelj, M.; Valinger, G.; Faber, K.; Shwab, H. (1998) Novel *Rhodococcus* Esterases by Genetic Engineering. *J. Mol. Catal., B Enzym.*, **5** (1–4), 261–266.

Guinebretiere, M.-H.; Thompson, F. L.; Sorokin, A.; Normand, P.; Dawyndt, P.; Ehling-Schulz, M.; Svensson, B.; Sanchis, V.; Nguyen-The, C.; Heyndrikx, M.; Vos, P. (2008) Ecological Diversification in the *Bacillus cereus* Group. *Environ. Microbiol.*, **10** (4), 851–865.

Hadjinicolaou, A. V.; Demetriou, V. L.; Hezka, J.; Beyer, W.; Hadfield, T. L.; Kostrikis, L. G. (2009) Use of Molecular Beacons and Multi-Allelic Real-Time PCR for Detection of and Discrimination between Virulent *Bacillus anthracis* and Other *Bacillus* Isolates. *J. Microbiol. Methods*, **78** (1), 45–53.

Hall, T. A. (1999) BioEdit: a User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.*, **41**, 95–98.

Hammer, Ø.; Harper, D. A. T.; Ryan, P. D. (2001) PAST: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontol. Electronica*, **4** (1), 1–9.

Iqbal, S.; Khalid, Z. M.; Malik, K. A. (1995) Enhanced Biodegradation and Emulsification of Crude Oil and Hyper Production of Biosurfactant by Gamma Ray-Induced Mutant of *Pseudomonas aeruginosa*. *Lett. Appl. Microbiol.*, **21** (3), 176–179.

Kimura, M. (1980) A Simple Method for Estimating Evolutionary Rate of Base Substitutions through Comparative Studies of Nucleotide Sequences. *J. Mol. Evol.*, **16** (2), 111–120.

Kouker, G.; Jaeger, K. E. (1987) Specific and Sensitive Plate Assay for Bacterial Lipases. *Appl. Environ. Microbiol.*, **53** (1), 211–213.

Marten, P.; Smalla, K; Berg, G. (2000) Genotypic and Phenotypic Differentiation of an Antifungal Biocontrol Strain Belonging to *Bacillus subtilis*. *J. Appl. Microbiol.*, **89** (3), 463–471.

Mir, J.; Morato, J.; Ribas, F. (1997) Resistance to Chlorine of Freshwater Bacterial Strains. *J. Appl. Microbiol.*, **82** (1), 7–18.

Morrow, J. B.; Almeida, J. L.; Fitzgerald, L. A.; Cole, K. D. (2008) Association and Decontamination of *Bacillus* Spores in a Simulated Drinking Water System. *Water Res.*, **42** (20), 5011–5021.

Owusu, R. P.; Makhzoum, A.; Knapp, J. (1991) The Thermodynamic Stability of Lipases and Proteases from Psychrotrophic Bacteria. *Food Chem.*, **39** (2), 187–195.

Pace, N. R. (1997) A Molecular View of Microbial Diversity and the Biosphere. *Science (Washington, D.C., U.S.)*, **276** (5313), 734–740.

Piza, F. A. T.; Siloto, A. P.; Carvalho, C. V.; Franco, T. T. (1999) Production, Characterization and Purification of Chitosanase from *Bacillus cereus*. *Braz. J. Chem. Eng.*, **16** (2), 185–192.

Ridgway, H. F.; Olson, B. H. (1982) Chlorine Resistance Patterns of Bacteria from Two Drinking Water Distribution Systems. *Appl. Environ. Microbiol.*, **44** (4), 972–987.

Rocha, M. V. P.; Barreto, R. V. G.; Melo, V. M. M.; Gonçalves, L. R. B. (2009) Evaluation of Cashew Apple Juice for Surfactin Production by *Bacillus subtilis* LAMI008. *Appl. Biochem. Biotechnol.*, **155** (1–3), 366–378.

Saitou, N.; Nei, M. (1987) The Neighbor-Joining Method: a New Method for Reconstructing Phylogenetic Trees. *Mol. Biol. Evol.*, **4** (4), 406–425.

Santos, K. B.; Neves, P.; Meneguim, A. M.; Santos, R. B.; Santos, W. J.; Boas, G. V.; Dumas, V.; Martins, E.; Praça, L. B.; Queiroz, P.; Berry, C.; Monnerat, R. (2009) Selection and Characterization of the *Bacillus thuringiensis* Strains Toxic to *Spodoptera eridania* (Cramer), *Spodoptera cosmioides* (Walker) and *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae). *Biol. Control*, **50** (2), 157–163.

Shangkuan, Y.-H.; Yang, J.-F.; Lin, H.-C.; Shaio, M.-F. (2000) Comparison of PCR-RFLP, Ribotyping and ERIC-PCR for Typing *Bacillus anthracis* and *Bacillus cereus* Strains. *J. Appl. Microbiol.*, **89** (3), 452–462.

Tamura, K.; Dudley, J.; Nei, M.; Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. *Mol. Biol. Evol.*, **24** (8), 1596–1599.

Thompson, J. D.; Higgins, D. G.; Gibson, T. J. (1994) CLUSTAL W: Improving the Sensitivity of Progressive Multiple Sequence Alignment through Sequence Weighting, Position-Specific Gap Penalties and Weight Matrix Choice. *Nucleic Acids Res.*, **22** (22), 4673–4680.

Tourasse, N. J.; Helgason, E.; Økstad, O. A.; Hegna, I. K.; Kolstø, A.-B. (2006) The *Bacillus cereus* Group: Novel Aspects of Population Structure and Genome Dynamics. *J. Appl. Microbiol.*, **101** (3), 579–573.

Tourasse, N. J.; Kolstø, A.-B. (2008) SuperCAT: a Supertree Database for Combined and Integrative Multilocus Sequence Typing Analysis of the *Bacillus cereus* Group of Bacteria (including *B. cereus*, *B. anthracis* and *B. thuringiensis*). *Nucleic Acids Res.*, **36** (1), D461–D468.

Warner, S. A. J. (1996) Genomic DNA Isolation and Lambda Library Construction. In *Plant Gene Isolation: Principles and Practice*; Foster, G. D.; Twell, D., Eds.; Wiley & Sons: West Sussex, U.K., pp 51–73.

Weisburg, W. G.; Barns, S. M.; Pelletier, D. A.; Lane, D. J. (1991) 16S Ribosomal DNA Amplification for Phylogenetic Study. *J. Bacteriol.*, **173** (2), 697–703.

Young, S. B.; Setlow, P. (2003) Mechanisms of Killing of *Bacillus subtilis* Spores by Hypochlorite and Chlorine Dioxide. *J. Appl. Microbiol.*, **95** (1), 54–67.