Bacillus salexigens sp. nov., a New Moderately Halophilic *Bacillus* Species

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Bacillus salexigens sp. nov. is proposed based on the characteristics of six moderately halophilic, gram-positive, rod-shaped strains isolated from salterns and hypersaline soils located in different geographical areas of Spain. These strains were motile, formed endospores, were strictly aerobic, were catalase and oxidase positive, and contained peptidoglycan of the *meso*-diaminopimelic acid type in their vegetative cell walls. The DNA base compositions of these strains ranged from 36.3 to 39.5 mol%, and these organisms constitute a homology group with levels of DNA-DNA homology ranging from 73 to 100%. The 16S rRNA sequence of strain C-20Mo^T, which was used as the representative strain of these isolates, groups with the 16S rRNA sequences of members of the genus *Bacillus*, and the highest level of similarity is 95.4%. The type strain is strain C-20Mo (= ATCC 700290 = DSM 11483 = CCM 4646).

Moderately halophilic bacteria are microorganisms which grow optimally in media containing 3 to 15% (wt/vol) salt (8). These organisms are widely distributed in different saline habitats. Taxonomically, they are a very heterogeneous group, which includes both gram-positive and gram-negative organisms (24). While gram-positive moderately halophilic cocci have been studied in some detail, to date only the following three species of aerobic, moderately halophilic, gram-positive rods have been described: *Bacillus halophilus* (25), *Halobacillus litoralis*, and *Halobacillus trueperi* (21).

In this study we isolated six moderately halophilic, gram-positive, rod-shaped organisms from hypersaline habitats located in different geographical areas of Spain. These strains were characterized phenotypically, as well as with respect to their DNA base compositions, levels of DNA-DNA homology, and cell wall compositions. In addition, we determined the sequence of the 16S ribosomal DNA (rDNA) of a representative of this group of isolates, strain C-20Mo^T, in order to determine its phylogenetic position. On the basis of the results of these studies, the six isolates exhibited characteristics that were sufficiently different from the characteristics of the aerobic endospore-forming rods described previously to justify their inclusion in a new species of the genus *Bacillus*, for which we propose the name *Bacillus salexigens*.

MATERIALS AND METHODS

Organisms and growth conditions. The six strains studied were isolated from ponds of solar salterns or hypersaline soils located in different areas of Spain (Huelva, Cádiz, Sevilla, and Mallorca). The isolation medium and the method used for isolation have been described previously (27). These strains were cultivated in complex medium containing 8.1% (wt/vol) NaCl, 0.7% (wt/vol) MgCl₂, 0.96% (wt/vol) MgSO₄, 0.036% (wt/vol) CaCl₂, 0.2% (wt/vol) KCl, 0.006% (wt/vol) NaHCO₃, 0.0026% (wt/vol) NaBr, 1.0% (wt/vol) yeast extract (Difco Laboratories, Detroit, Mich.), 0.5% (wt/vol) Proteose Peptone no. 3 (Difco), and 0.1% (wt/vol) glucose. The pH was adjusted to 7.5 with 1 M KOH. When required, the medium was solidified with 2.0% (wt/vol) Bacto-Agar (Difco).

For the phenotypic characterization studies the following reference strains were used: *Bacillus alcalophilus* DSM 485^T, *Bacillus badius* ATCC 14574^T, *Bacillus circulans* PCM 1398, *Bacillus globisporus* DSM 4^T, *Bacillus insolitus* DSM 5^T, *Bacillus laterosporus* DSM 25^T, *Bacillus marinus* DSM 1297^T, *Bacillus megaterium* DSM 32^T, *Bacillus pallidus* DSM 3670^T, *Bacillus pantothenicus* PCM 454, *Bacillus pumilus* ATCC 7061^T, *Bacillus smithii* DSM 4216^T, *B. halophilus* DSM

4771^T, and *Bacillus* sp. strain DSM 578 (= strain 21-1). For the homology studies we used the reference strains shown in Table 1. The recommended growth media were used to grow these organisms.

Phenotypic characterization. Tests for 83 characteristics, including morphological, cultural, physiological, biochemical, and antibiotic susceptibility features, were carried out. The method used has been described previously (2, 5, 19, 26). A total of 95 nutritional tests were performed with the BIOLOG automatic identification system (Biolog, Inc., Hayward, Calif.). Strains were grown on complex medium at 37°C for 24 h and suspended in a sterile solution containing 10% (wt/vol) salts. The cell density was adjusted to an *A*₅₉₀ of 0.34 to 0.39 with a BIOLOG model 21101 photometer. Immediately after the cells were suspended in the salt solution, the suspensions were transferred into sterile multichannel pipetter reservoirs, and BIOLOG GP MicroPlates were inoculated with 125 µl of cell suspension per well by means of an eight-channel repeating pipetter. The inoculated BIOLOG plates were incubated at 37°C for 24 h, and the results were read with a MicroPlate Reader by using MicroLog 3.50 computer software to perform automated reading.

DNA extraction and purification. Cells of all of the strains studied were harvested, washed, suspended in 0.15 M NaCl–0.1 M EDTA buffer (pH 8.0) (5 g [wet weight] in 50 ml of buffer), and lysed with lysozyme (approximately 10 mg) at 37°C and with sodium dodecyl sulfate at a final concentration of 2% (wt/vol) at 60°C. The DNA was extracted and purified by the method of Marmur (11). The purity was assessed from the *A*₂₆₀/*A*₂₈₀ and *A*₂₃₀/*A*₂₆₀ extinction ratios (6).

DNA base composition. The guanine-plus-cytosine (G+C) content of the DNA was determined from the midpoint of the thermal denaturation profile (12) obtained with a model UV-Vis 551S spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.) at 260 nm; this instrument was programmed for temperature increases of 1.0°C/min. The G+C content was calculated from the thermal denaturation temperature by using the equation of Owen and Hill (16). The G+C content of reference DNA from *Escherichia coli* NCTC 9001 was assumed to be 51 mol% (17).

Preparation of ³H-labeled DNA and DNA-DNA hybridization experiments. Strain C-20Mo^T DNA was radioactively labeled by the multiprimer system with a commercial kit (catalog no. RPN 1601Y; Amersham International, Amersham, England) by using [1',2',5-³H]dCTP (Amersham). The average specific activity obtained with this procedure was 8.8 × 10⁶ cpm/µg of DNA. The labeled DNA was denatured before hybridization by heating it at 100°C for 5 min and then placing it on ice. DNA-DNA similarity values were determined by the competition procedure described by Johnson (6). Competitor DNAs were sonicated (Braun Melsungen, Melsungen, Germany) at 50 W twice for 15 s. Membrane filters (HAHY; Millipore Corp., Bedford, Mass.) containing reference DNA (ca. 25 µg cm⁻²) were placed in 5-ml screw-cap vials which contained the labeled, sheared, denatured DNA and the denatured sheared competitor DNA. The ratio of competitor DNA concentration to labeled DNA concentration was at least 150:1. The final reaction mixture concentrations were 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 30% formamide, and the final volume was 140 µl. The hybridization experiments were carried out under optimal conditions with temperatures ranging between 45.3 and 48.0°C, which are within the limits of validity for the filter method (3). The vials were shaken slightly for 18 h in a water bath (Grant Instruments, Cambridge, England); these procedures were performed in triplicate. After hybridization the filters were counted with a liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.), and the levels of homology were calculated as described by Johnson (6). At least two

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TABLE 1. DNA G+C contents and levels of DNA-DNA relatedness for *B. salexigens* and related species

Source of unlabeled DNA ^a	G+C content (mol%)	% Homology with ³ H-labeled DNA from <i>B. salexigens</i> C-20Mo ^T
<i>Bacillus salexigens</i> strains		
C-20Mo ^T	39.5	100
C-29Mo	36.3	81
C-69Mo	37.8	73
C-26Mo	38.2	100
C-41Mo	37.4	ND ^c
C-64Mo	38.9	ND
<i>Bacillus alcalophilus</i> DSM 485 ^T	47.0	0
<i>Bacillus badius</i> ATCC 14574 ^T	46.4	6
<i>Bacillus circulans</i> PCM 1398	50.4	12
<i>Bacillus globisporus</i> DSM 4 ^T	49.0	8
<i>Bacillus halophilus</i> DSM 4771 ^T	51.1 ^b	0
<i>Bacillus insolitus</i> DSM 5 ^T	36.3	22
<i>Bacillus laterosporus</i> DSM 25 ^T	40.2 ^b	0
<i>Bacillus lentus</i> ATCC 10840 ^T	36.3 ^b	15
<i>Bacillus marinus</i> DSM 1297 ^T	36.9	10
<i>Bacillus megaterium</i> DSM 32 ^T	41.9	13
<i>Bacillus pallidus</i> DSM 3670 ^T	48.3	0
<i>Bacillus pantothenicus</i> PCM 454	39.8	15
<i>Bacillus pumilus</i> ATCC 7061 ^T	43.6	0
<i>Bacillus smithii</i> DSM 4216 ^T	44.6	1
<i>Bacillus</i> sp. strain DSM 578	ND	0
<i>Bacillus subtilis</i> ATCC 6051 ^T	42.9 ^b	5

^a DSM, Deutsche Sammlung von Mikroorganismen; ATCC, American Type Culture Collection; PCM, Polish Collection of Microorganisms.

^b Data from references 4 and 25.

^c ND, not determined.

independent determinations were carried out for each experiment, and the means are reported below.

Determination of isomers of diaminopimelic acid. The isomers of diaminopimelic acid in the cell walls of the six strains were determined by using the method of Stanek and Roberts (23). Dried cells (approximately 10 mg) were hydrolyzed with 1 ml of 10 N HCl at 100°C for 20 h in a screw-cap tube, and 1 ml of the hydrolysate was filtered and chromatographed on a cellulose thin layer (E. Merck AG, Darmstadt, Germany) along with a reference amino acid mixture. Methanol-distilled water-10 N HCl-pyridine (80:26:4:10, vol/vol/vol/vol) was used as the solvent for separation. Then the chromatogram was dried at room temperature, sprayed with a 5% ninhydrin-butanol solution, and heated at 100°C for 3 min.

Isolation of genomic DNA and 16S rDNA sequence analysis. Isolation of genomic DNA from strain C-20Mo^T, which was used as a representative of our isolates, PCR amplification of the 16S rRNA gene, and direct determination of PCR-amplified 16S rDNA sequences were carried out by using methods that have been described previously (13).

We used a program set for data entry, editing, sequence alignment, secondary-structure comparison, similarity matrix generation, and dendrogram construction for 16S rRNA data written in Microsoft Quick BASIC for use with IBM PC-AT and compatible computers (18). A similarity matrix was constructed from the aligned sequences by using only those positions at which there were data for 90% of the strains. The similarity matrix was corrected for multiple base changes at single positions by the method of Jukes and Cantor (7). A phylogenetic tree was constructed by using the neighbor-joining method of Saitou and Nei (20).

Nucleotide sequence accession number. The sequence of the 16S rRNA gene of *B. salexigens* C-20Mo^T has been deposited in the EMBL data library under accession no. Y11603.

RESULTS

Cellular and colonial morphology. The cells of the six strains which we studied were gram-positive rods that were 0.6 to 0.9 by 1.5 to 3.5 µm and occurred singly, in pairs, or in short chains (Fig. 1). These organisms were motile and produced oval endospores located at subterminal or central positions in swollen sporangia.

The colonies of the strains on 10% (wt/vol) total salts com-

plex medium were nonpigmented, circular, smooth, convex, and entire.

Physiological, biochemical, and nutritional characteristics. The six isolates grew in solid media containing 10 to 20% (wt/vol) total salts, and optimal growth occurred in the presence of 10% salts. They grew at temperatures ranging from 15 to 40°C; the optimum temperature for growth was 37°C. The pH range for growth was 7.0 to 10.0, and optimum growth occurred at pH 7.5.

The organisms were strictly aerobic and catalase and oxidase positive. Acid was produced from D-glucose, but not from dulcitol or D-melibiose. DNA was hydrolyzed; starch, Tween 80, and tyrosine were not hydrolyzed. Indole, Voges-Proskauer, and Simmons citrate tests were negative. Arginine dihydrolase, lysine and ornithine decarboxylases, and phenylalanine deaminase were not produced. Table 2 shows the characteristics which differentiate the six strains which we studied from one another. Other phenotypic characteristics are included below in the species description.

DNA base composition. The G+C contents of the DNAs of the six isolates ranged from 36.3 to 39.5 mol% (Table 1).

DNA-DNA hybridization. The results of the DNA-DNA hybridization experiments are shown in Table 1. The levels of relatedness of labeled DNA from a representative strain to DNAs from the other isolates ranged from 73 to 100%. On the other hand, the levels of relatedness between strain C-20Mo^T, which was used as a representative of our isolates, and the strains belonging to related species used for comparison were low (0 to 22%).

Determination of diaminopimelic acid in the cell wall. The cell walls of the six isolates contained *meso*-diaminopimelic acid in their peptidoglycan. These results were confirmed by N. Weiss (29).

Phylogeny. The almost complete 16S rDNA sequence of strain C-20Mo^T was determined. This sequence was compared with more than 4,000 16S rRNA sequences deposited in databases (10). The results of a comparison of the 16S rRNA

FIG. 1. Phase-contrast micrograph of strain C-20Mo^T, showing spore-forming rods. Bar = 10 µm.

TABLE 2. Characteristics in which the six *B. salexigens* strains differed

Characteristic	No. of strains positive	Reaction of strain C-20Mo ^T
Growth in the presence of:		
5% (wt/vol) salts	2	—
7% (wt/vol) salts	4	—
25% (wt/vol) salts	2	—
Growth at:		
45°C	4	+
50°C	1	—
pH 6	3	+
pH 11	3	+
Hydrolysis of:		
Gelatin	5	+
Casein	3	+
Esculin	3	+
Nitrate reduction	2	—
Phosphatase	4	—
H ₂ S production	5	+
Acid production from:		
D-Galactose	1	—
D-Fructose	5	+
Lactose	2	—
Maltose	3	+
D-Mannitol	4	—
Glycerol	5	+
D-Trehalose	1	—
D-Xylose	2	—
D-Mannose	5	+
L-Rhamnose	1	—
Utilization of:		
α-Cyclodextrin	1	—
β-Cyclodextrin	3	+
Inulin	1	—
Tween 40	1	—
N-Acetyl-D-mannosamine	5	+
L-Arabinose	3	—
D-Arabitol	1	—
Arbutin	4	—
D-Galactose	3	—
α-D-Lactose	1	—
D-Mannitol	5	—
3-Methyl-glucose	2	+
α-Methyl-D-glucoside	5	—
α-Methyl-D-mannoside	5	—
L-Rhamnose	3	—
Sucrose	5	—
D-Trehalose	5	—
Acetic acid	3	—
α-Hydroxybutyric acid	3	—
β-Hydroxybutyric acid	5	—
γ-Hydroxybutyric acid	1	—
α-Ketovaleic acid	1	—
Lactamide	1	—
D-Lactic acid methyl ester	2	—
L-Lactic acid	5	—
D-Malic acid	1	—
L-Malic acid	5	+
Methylpyruvate	5	+
Methylsuccinate	1	—
Succinamic acid	3	—
N-Acetyl-L-glutamic acid	5	+
L-Glutamic acid	5	+
Adenosine	5	+
UMP	1	—
Fructose 6-phosphate	1	—
Glucose 6-phosphate	2	—
DL-α-Glycerolphosphate	1	—
Antibiotic susceptibility:		
Carbenicillin	5	+
Nalidixic acid	1	—
Rifampin	5	+
Penicillin G	5	+
Polymixin	1	—
Bacitracin	5	+
Erythromycin	5	—

sequence of strain C-20Mo^T, the 16S rRNA sequences of some species of the genus *Bacillus*, and the 16S rRNA sequences of other related species are shown in Table 3. The sequence of C-20Mo^T exhibited the highest level of similarity with the 16S rRNA sequence of *B. pantothenicus* (95.4%), which belongs to rRNA group I of the genus *Bacillus* (1). The reference strains used in this study and their 16S rRNA sequence accession numbers are shown in Table 4.

The phylogenetic tree in Fig. 2 shows the relationship between strain C-20Mo^T and some representative *Bacillus* species and other related species with low G+C contents. Strain C-20Mo^T and *B. pantothenicus* are on the same phylogenetic branch, close to *Halobacillus halophilus*, *H. litoralis*, and the recently described species *Bacillus dipsosauri*.

DISCUSSION

The six strains studied in this work have very similar phenotypic, chemotaxonomic, and genotypic characteristics and thus should be placed in the same taxon. On the other hand, these strains are quite different from the moderately halophilic gram-positive rods described previously (9, 21, 25).

The six new strains are gram-positive, motile, endospore-forming rods that are strictly aerobic. On the basis of their phenotypic features, cell wall peptidoglycan (*meso*-diaminopimelic acid type), and DNA G+C contents, these strains can be considered members of the genus *Bacillus* (2). Their salt requirement indicates that they should be included in the group containing moderately halophilic bacteria (8). The only species of the genus *Bacillus* included in this group is *B. halophilus*, an endospore-forming, gram-positive, rod-shaped organism that produces an extracellular nuclease in saline medium (15, 25). However, there are many phenotypic and genotypic differences between *B. halophilus* and our isolates (Table 5), including the high G+C content of *B. halophilus* (51.5 mol%) compared with the G+C contents of our isolates (36.3 to 39.5 mol%). Recently, Lawson et al. (9) described a new halotolerant species, *B. dipsosauri*, that has a DNA G+C content (39.4 mol%) and cell wall peptidoglycan (*meso*-diaminopimelic type) similar to the G+C content and cell wall peptidoglycan of our isolates; however, the two organisms are different phenotypically and phylogenetically.

In a recent study, Spring et al. (21) proposed the creation of the new genus *Halobacillus*. These authors have included in this genus two new species of moderately halophilic gram-positive rods, *H. litoralis* and *H. trueperi*. In addition, *Sporosarcina halophila*, an endospore-forming gram-positive moderately halophilic coccus, has been reclassified in this genus as *Halobacillus halophilus* (21). However, our isolates are phenotypically and chemotaxonomically different from these three species; in fact, *H. litoralis*, *H. trueperi*, and *H. halophilus* have peptidoglycan of the Orn-D-Asp type (Table 5).

The DNA relatedness experiments showed that our six isolates form a single DNA homology group with homology values equal to or greater than 73%. Most authors agree that the levels of DNA homology among strains of the same species should be equal to or greater than 70% (17, 28). Thus, on the basis of DNA relatedness results, our isolates form a group of organisms that are sufficiently related to constitute a genus. On the other hand, low levels of DNA homology were obtained between the representative strain of our isolates and the moderately halophilic, marine, or nonhalophilic bacteria that are members of the genus *Bacillus* which we studied (Table 2).

The comparative sequence analysis of 16S rDNAs revealed that strain C-20Mo^T should be included in the phylogenetic

TABLE 3. Similarity matrix based on 16S rRNA sequence comparisons of *B. salexigens* C-20Mo^T and related species

Species	% Similarity or % difference ^a																								
	<i>Bacillus stearothermophilus</i>	<i>Bacillus alcalophilus</i>	<i>Bacillus insolitus</i>	<i>Bacillus pasteurii</i>	<i>Bacillus globisporus</i>	<i>Sporosarcina ureae</i>	<i>Halobacillus litoralis</i>	<i>Halobacillus halophilus</i>	<i>Marinococcus albus</i>	<i>Bacillus dipsosauri</i>	<i>Bacillus salexigens</i> sp. nov.	<i>Bacillus pantothenicus</i>	<i>Bacillus subtilis</i>	<i>Bacillus cereus</i>	<i>Bacillus megaterium</i>	<i>Bacillus cohnii</i>	<i>Bacillus coagulans</i>	<i>Bacillus brevis</i>	<i>Bacillus laterosporus</i>	<i>Bacillus aneurinolyticus</i>	<i>Paenibacillus amylolyticus</i>	<i>Paenibacillus larvae</i>	<i>Paenibacillus polymyxa</i>	<i>Paenibacillus macerans</i>	<i>Alicyclobacillus acidocaldarius</i>
<i>Bacillus stearothermophilus</i>		91.5	91.6	91.7	91.7	93.3	92.8	91.7	91.3	91.2	90.7	92.6	91.7	92.9	92.9	93.5	92.4	91.0	90.3	92.3	89.8	89.1	89.7	91.2	88.0
<i>Bacillus alcalophilus</i>	9.0		93.4	92.7	92.9	94.2	93.2	92.3	92.2	92.7	92.5	94.0	93.1	93.7	93.7	94.3	93.6	91.3	90.3	91.5	91.5	91.1	91.4	92.3	86.4
<i>Bacillus insolitus</i>	8.9	6.9		95.9	96.3	96.9	92.7	91.9	92.0	91.5	91.9	94.3	92.8	93.8	94.6	94.8	92.6	90.5	90.4	90.9	91.0	90.6	90.6	91.0	86.4
<i>Bacillus pasteurii</i>	8.8	7.6	4.2		96.7	96.9	92.4	91.5	91.0	91.4	91.0	93.0	91.6	92.9	93.2	93.3	93.0	91.1	90.5	91.7	89.9	90.8	90.2	91.7	86.5
<i>Bacillus globisporus</i>	8.8	7.5	3.8	3.4		97.8	92.6	92.0	91.5	91.8	91.5	93.1	92.2	92.7	93.8	93.7	93.0	91.0	90.1	91.7	90.0	90.2	90.7	91.2	86.6
<i>Sporosarcina ureae</i>	7.1	6.0	3.2	3.2	2.3		93.8	93.6	93.1	92.7	92.7	94.9	93.9	94.6	95.0	95.0	94.0	91.8	91.3	92.7	91.4	91.3	91.4	92.2	87.3
<i>Halobacillus litoralis</i>	7.5	7.1	7.7	8.0	7.8	6.4		97.7	96.4	94.8	94.2	95.8	93.4	93.2	93.5	93.6	93.4	91.3	91.1	92.6	91.0	90.4	90.2	91.3	86.8
<i>Halobacillus halophilus</i>	8.8	8.1	8.6	9.0	8.5	6.7	2.3		96.0	93.2	92.4	94.9	91.8	91.9	92.7	92.7	91.9	90.0	90.1	91.6	90.0	89.6	89.4	90.2	86.5
<i>Marinococcus albus</i>	9.2	8.2	8.4	9.6	9.0	7.2	3.7	4.1		92.9	92.2	94.0	91.7	91.8	92.1	92.6	91.4	90.0	89.4	91.0	90.1	89.1	89.3	90.1	86.1
<i>Bacillus dipsosauri</i>	9.4	7.7	9.0	9.2	8.7	7.7	5.4	7.1	7.5		92.9	94.2	93.0	92.4	93.3	93.0	93.6	90.8	90.4	91.3	89.9	89.2	89.4	90.6	85.3
<i>Bacillus salexigens</i> sp. nov.	9.9	7.9	8.6	9.6	9.0	7.6	6.1	8.0	8.2	7.4		95.5	92.8	92.9	93.1	93.1	92.8	89.2	89.2	91.2	89.9	88.9	89.4	90.4	84.9
<i>Bacillus pantothenicus</i>	7.8	6.3	5.9	7.4	7.2	5.3	4.4	5.2	6.3	6.0	4.6		94.1	94.3	94.5	94.8	93.8	90.7	90.6	92.7	91.8	91.2	90.6	92.1	86.5
<i>Bacillus subtilis</i>	8.8	7.3	7.5	9.0	8.2	6.3	6.9	8.7	8.8	7.4	7.6	6.1		94.6	94.5	94.8	94.0	90.5	89.6	91.2	91.1	89.5	89.6	90.4	84.7
<i>Bacillus cereus</i>	7.5	6.5	6.5	7.5	7.6	5.6	7.1	8.6	8.7	8.0	7.5	5.9	5.6		96.3	95.9	94.0	90.7	91.1	91.2	90.9	89.9	90.4	91.4	86.3
<i>Bacillus megaterium</i>	7.5	6.5	5.6	7.1	6.4	5.2	6.8	7.7	8.4	7.0	7.2	5.7	5.7	3.8		97.1	94.7	91.8	91.9	92.0	91.5	90.5	90.5	91.1	86.4
<i>Bacillus cohnii</i>	6.8	5.9	5.4	7.0	6.6	5.1	6.7	7.6	7.7	7.4	7.3	5.4	5.3	4.2	3.0		94.4	92.0	92.1	92.9	92.1	90.8	91.0	91.7	86.9
<i>Bacillus coagulans</i>	8.0	6.7	7.8	7.4	7.3	6.3	6.9	8.6	9.1	6.6	7.6	6.5	6.3	6.2	5.5	5.8		91.3	91.0	92.1	90.6	89.9	90.7	92.3	86.7
<i>Bacillus brevis</i>	9.6	9.3	10.1	9.4	9.5	8.7	9.2	10.7	10.8	9.8	11.7	10.0	10.2	9.9	8.7	8.4	9.3		97.0	92.7	90.8	90.7	90.5	91.0	86.2
<i>Bacillus laterosporus</i>	10.4	10.4	10.3	10.1	10.6	9.3	9.5	10.6	11.4	10.2	11.7	10.0	11.2	9.5	8.5	8.3	9.6	3.1		92.0	90.9	90.5	90.7	91.3	85.6
<i>Bacillus aneurinolyticus</i>	8.1	9.0	9.7	8.8	8.8	7.7	7.8	9.0	9.6	9.2	9.3	7.6	9.3	9.3	8.5	7.5	8.3	7.6	8.4		91.4	91.1	90.7	91.4	87.1
<i>Paenibacillus amylolyticus</i>	11.0	9.0	9.6	10.9	10.8	9.1	9.6	10.8	10.6	10.9	10.8	8.6	9.4	9.7	9.1	8.3	10.1	9.8	9.6	9.1		94.7	94.6	95.5	86.2
<i>Paenibacillus larvae</i>	11.8	9.5	10.0	9.9	10.5	9.2	10.3	11.2	11.7	11.7	12.0	9.3	11.3	10.8	10.2	9.8	10.8	9.9	10.1	9.5	5.5		94.3	94.8	87.4
<i>Paenibacillus polymyxa</i>	11.1	9.1	10.1	10.4	9.9	9.1	10.5	11.4	11.5	11.4	11.4	10.1	11.2	10.3	10.1	9.6	9.9	10.1	10.0	10.0	5.6	5.9		96.4	87.0
<i>Paenibacillus macerans</i>	9.4	8.2	9.6	8.8	9.3	8.2	9.3	10.4	10.7	10.1	10.3	8.3	10.3	9.1	9.5	8.8	8.2	9.5	9.3	9.2	4.7	5.4	3.7		87.6
<i>Alicyclobacillus acidocaldarius</i>	13.0	15.0	15.0	14.9	14.8	13.9	14.5	14.8	15.3	16.4	16.8	14.9	17.1	15.2	15.0	14.5	14.6	15.3	16.0	14.2	15.2	13.8	14.2	13.5	

^a The values on the upper right are percentages of similarity, and the values on the lower left are percentages of difference corrected for multiple base changes by the method of Jukes and Cantor (7).

TABLE 4. Bacterial strains and accession numbers of sequences used in the 16S rRNA analysis

Species	Strain ^a	EMBL nucleotide sequence accession no.
<i>Bacillus alcalophilus</i>	ATCC 27647 ^T	X60603
<i>Bacillus insolitus</i>	DSM 5 ^T	X60642
<i>Bacillus pasteurii</i>	NCIMB 8841 ^T	X60631
<i>Bacillus globisporus</i>	NCIMB 11434 ^T	X60644
<i>Sporosarcina ureae</i>	NCIMB 9251 ^T	X62174
<i>Halobacillus litoralis</i>	DSM 10405 ^T	X94558
<i>Halobacillus halophilus</i>	ATCC 35676 ^T	X62174
<i>Marinococcus albus</i>	DSM 20748 ^T	X90834
<i>Bacillus dipsosauri</i>	NCFB 3027 ^T	X82436
<i>Bacillus pantothenicus</i>	ATCC 14576 ^T	X60627
<i>Bacillus subtilis</i>	NCDO 1769 ^T	X60646
<i>Bacillus cereus</i>	ATCC 14579 ^T	X55060
<i>Bacillus megaterium</i>	ATCC 14581 ^T	X60629
<i>Bacillus cohnii</i>	DSM 6307 ^T	X76437
<i>Bacillus coagulans</i>	NCDO 1761 ^T	X60614
<i>Bacillus stearothermophilus</i>	ATCC 12980 ^T	X60640
<i>Bacillus brevis</i>	NCIMB 9372 ^T	X60612
<i>Bacillus laterosporus</i>	NCDO 1763 ^T	X60620
<i>Bacillus aneurinolyticus</i>	NCIMB 10056 ^T	X60645
<i>Paenibacillus amylolyticus</i>	NCIB 8144 ^T	X60606
<i>Paenibacillus larvae</i>	ATCC 9545 ^T	X60619
<i>Paenibacillus polymyxa</i>	ATCC 842 ^T	X60632
<i>Paenibacillus macerans</i>	ATCC 8244 ^T	X60624
<i>Alicyclobacillus acidocaldarius</i>	DSM 446 ^T	X60742

^a ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; NCDO, National Collection of Dairy Organisms; NCIMB, National Collection of Industrial and Marine Bacteria; NCFB, National Collection of Food Bacteria.

group containing the low-G+C-content gram-positive bacteria and is closely related to *Bacillus* species belonging to phylogenetic group I (Fig. 2) described by Ash et al. (1).

The levels of sequence similarity between strain C-20Mo^T and the species included in the phylogenetic analysis are shown in Table 3. Most of these levels of similarity were less than 94%; the only exceptions were the levels of similarity with *B. pantothenicus* (95.4%) and *H. litoralis* (94.2%). Recently, Stackebrandt et al. (22) stated that levels of similarity between 16S rRNA sequences that are less than 97% suggest that strains do not belong to the same species. The results of our phylogenetic analysis agree with the DNA homology results and, overall, clearly show that the new isolates are not closely related at the species level to previously described *Bacillus* species.

Strain C-20Mo^T was most closely related to *B. pantothenicus*, which belongs to phylogenetic group I of the genus *Bacillus* as defined by Ash et al. (1), and these organisms were on the same phylogenetic branch and are closely related to *H. halophilus*, *H. litoralis*, and *B. dipsosauri*. Although these bacteria are phylogenetically closely related to the *Bacillus* species belonging to rRNA group I, which includes *Bacillus subtilis* (Fig. 2), *B. pantothenicus*, and our isolates, they could represent a phylogenetic group in the genus *Bacillus* that is different from the six phylogenetic groups currently recognized (1, 14).

All of our results confirmed that our isolates should be classified in a new species of the genus *Bacillus*, for which we propose the name *Bacillus salexigens*.

Description of *Bacillus salexigens* Garabito, Arahall, Mellado, Márquez, and Ventosa sp. nov. *Bacillus salexigens* (sal.ex'i. gens. L. n. sal, salt; L. v. exigo, to demand; M. L. part. adj. salexigens, salt demanding). Gram-positive rods that are 0.3 to 0.6

by 1.5 to 3.5 µm and occur singly, in pairs, or in short chains. Motile. Oval endospores are produced at subterminal or central positions in swollen sporangia. Colonies are nonpigmented, circular, smooth, convex, and entire.

Growth occurs in the presence of 7 to 20% (wt/vol) total salts, and optimal growth occurs in the presence of 10% (wt/vol) total salts. No growth occurs in the absence of salts.

Growth occurs at temperatures between 15 and 45°C (optimal temperature, 37°C) and at pH values between 6.0 and 11.0 (optimal pH, 7.5).

Strictly aerobic. Catalase and oxidase are produced. Acid is produced from D-glucose, D-fructose, maltose, D-mannitol, glycerol, and D-mannose. Acid is not produced from dulcitol, D-galactose, D-melibiose, lactose, D-trehalose, D-xylose, and L-rhamnose. DNA is hydrolyzed; starch, Tween 80, and tyrosine are not hydrolyzed. Indole, Voges-Proskauer, Simmons citrate, phenylalanine deaminase, arginine dihydrolase, and lysine and ornithine decarboxylase tests are negative.

The following compounds are utilized as sole carbon and energy sources: pyruvic acid, D-xylose, dextrin, N-acetyl-D-glucosamine, cellobiose, D-fructose, D-glucose, maltose, maltotriose, D-mannose, D-psicose, D-ribose, salicin, turanose, L-lactic acid, N-acetyl-glutamate, glycyl-L-glutamic acid, L-alanyl-glycine, glycerol, adenosine, 2'-deoxyadenosine, inosine, thymidine, and uridine.

p-Hydroxyphenylacetic acid and succinic acid are not utilized as sole carbon and energy sources.

Susceptible to chloramphenicol, carbenicillin, rifampin, novobiocin, cephalothin, penicillin G, and ampicillin. Resistant to nalidixic acid and polymyxin.

Other phenotypic features are shown in Table 1.

The DNA base composition ranges from 36.3 to 39.5 mol%, as determined by the thermal denaturation method.

FIG. 2. Unrooted phylogenetic tree derived from the analysis of the 16S rRNA sequences of *B. salexigens* C-20Mo^T, some other *Bacillus* species, and other related gram-positive bacteria having low DNA G+C contents.

TABLE 5. Characteristics useful for distinguishing *B. salexigens* from other gram-positive endospore-forming rods

Characteristic	<i>H. litoralis</i> ^a	<i>H. trueperi</i> ^a	<i>B. halophilus</i> ^a	<i>B. pantothenicus</i> ^a	<i>B. salexigens</i>
Pigmentation	Orange	Orange	None	ND ^c	None
Spore shape ^b	E/S	E/S	E	v	E
Spore position ^b	C/ST	C/ST	C	ND	ST/C
Sporangium swollen	ND	ND	—	+	+
Optimal growth in the presence of 10% salts	+	+	—	—	+
Anaerobic growth	—	—	—	+	—
H ₂ S production	ND	ND	—	ND	+
Hydrolysis of:					
Gelatin	+	+	—	+	+
Starch	—	—	—	+	—
Casein	—	—	—	D	+
Esculin	—	—	+	ND	+
Acid production from:					
D-Galactose	—	+	ND	ND	—
Maltose	+	+	—	ND	+
D-Mannitol	+	—	—	—	—
D-Trehalose	+	+	+	ND	—
D-Xylose	+	—	+	—	—
Cell wall type	Orn-D-Asp	Orn-D-Asp	<i>m</i> -Dpm ^d	<i>m</i> -Dpm	<i>m</i> -Dpm
DNA G+C content (mol%)	42.0	43.0	51.1	36.9	39.5

^a Data from references 2, 21, and 25.^b E, ellipsoidal; S, spherical; C, central; ST, subterminal; v, strain instability.^c ND, not determined; +, positive; —, negative; D, differs among strains.^d *m*-Dpm, *meso*-diaminopimelic acid.

The cell wall contains peptidoglycan of the *meso*-diaminopimelic acid type.

The habitats are solar salterns and saline soils.

The type strain is strain C-20Mo (= ATCC 700290 = DSM 11483 = CCM 4646).

Description of the type strain. The description of the type strain is the same of that of the species, except that the type strain grows in the presence of 10 to 20% (wt/vol) total salt and does not produce acid from D-mannitol. The base composition of its DNA is 39.5 mol%, as determined by the thermal denaturation method. Other characteristics of this strain are shown in Table 2.

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REFERENCES

- Ash, C., A. E. Farrow, S. W. Allbanks, and M. D. Collins. 1991. Phylogenetic heterogeneity of the genus *Bacillus* revealed by small-subunit-ribosomal RNA sequences. *Lett. Appl. Microbiol.* **13**:202–206.
- Claus, D., and R. C. W. Berkeley. 1986. Genus *Bacillus* Cohn 1872, p. 1105–1139. In P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), *Bergey's manual of systematic bacteriology*, vol. 2. Williams & Wilkins, Baltimore, Md.
- De Ley, J., and R. Tijtgat. 1970. Evaluation of membrane filter methods for DNA-DNA hybridization. *Antonie Leeuwenhoek* **36**:461–474.
- Fahmy, F., J. Flossdorf, and D. Claus. 1985. The DNA base composition of the strains of the genus *Bacillus*. *Syst. Appl. Microbiol.* **6**:60–65.
- Garcia, M. T., A. Ventosa, F. Ruiz-Berraquero, and M. Kocur. 1987. Taxonomic study and amended description of *Vibrio costicola*. *Int. J. Syst. Bacteriol.* **37**:251–256.
- Johnson, J. L. 1994. Similarity analysis of DNAs, p. 655–682. In P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), *Methods for general and molecular bacteriology*. American Society for Microbiology, Washington, D.C.
- Jukes, T. H., and C. R. Cantor. 1969. Evolution of protein molecules, p. 21–132. In H. N. Munro (ed.), *Mammalian protein metabolism*, vol. 3. Academic Press, Inc., New York, N.Y.
- Kushner, D. J., and M. Kamekura. 1988. Physiology of halophilic eubacteria, p. 109–140. In F. Rodriguez-Valera (ed.), *Halophilic bacteria*, vol. 1. CRC Press, Boca Raton, Fla.
- Lawson, P. A., C. E. Deutch, and M. D. Collins. 1996. Phylogenetic characterization of a novel salt-tolerant *Bacillus* species: description of *Bacillus dipsosauri* sp. nov. *J. Appl. Bacteriol.* **81**:109–112.
- Maidak, B. L., G. J. Olsen, N. Larsen, R. Overbeek, M. J. McCaughey, and C. R. Woese. 1996. The Ribosomal Database Project (RDP). *Nucleic Acids Res.* **24**:82–85.
- Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J. Mol. Biol.* **3**:208–218.
- Marmur, J., and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J. Mol. Biol.* **5**:109–118.
- Mellado, E., E. R. B. Moore, J. J. Nieto, and A. Ventosa. 1995. Phylogenetic inferences and taxonomic consequences of 16S ribosomal DNA sequence comparison of *Chromohalobacter marismortui*, *Volcaniella eurihalina*, and *Deleya salina* and reclassification of *V. eurihalina* as *Halomonas eurihalina* comb. nov. *Int. J. Syst. Bacteriol.* **45**:712–716.
- Nielsen, P., F. A. Rainey, H. Outtrup, F. G. Priest, and D. Fritze. 1994. Comparative 16S rDNA sequence analysis of some alkaliphilic bacilli and the establishment of a sixth rRNA group within the genus *Bacillus*. *FEMS Microbiol. Lett.* **117**:61–66.
- Onishi, H., T. Mori, S. Takeuchi, K. Tani, T. Kobayashi, and M. Kamekura. 1983. Halophilic nuclease of a moderately halophilic *Bacillus* sp.: production, purification, and characterization. *Appl. Environ. Microbiol.* **45**:24–30.
- Owen, R. J., and L. R. Hill. 1979. The estimation of base compositions, base pairing and genome size of bacterial deoxyribonucleic acids, p. 217–296. In F. A. Skinner and D. W. Lovelock (ed.), *Identification methods for microbiologists*, 2nd ed. Academic Press, Inc. (London), Ltd., London, United Kingdom.
- Owen, R. J., and D. Pitcher. 1985. Current methods for estimating DNA base composition and levels of DNA-DNA hybridization, p. 67–93. In M. Goodfellow and D. E. Minnikin (ed.), *Chemical methods in bacterial systematics*. Academic Press, Inc. (London), Ltd., London, United Kingdom.
- Paster, B. J., and F. E. Dewhirst. 1988. Phylogeny of campylobacters, wolfinellas, *Bacteroides gracilis*, and *Bacteroides ureolyticus* by 16S ribosomal ribonucleic acid sequencing. *Int. J. Syst. Bacteriol.* **38**:56–62.
- Quesada, E., A. Ventosa, F. Ruiz-Berraquero, and A. Ramos-Cormenzana. 1984. *Deleya halophila*, a new species of moderately halophilic bacteria. *Int. J. Syst. Bacteriol.* **34**:287–292.
- Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**:406–425.
- Spring, S., W. Ludwig, M. C. Marquez, A. Ventosa, and K.-H. Schleifer. 1996. *Halobacillus* gen. nov., with descriptions of *Halobacillus litoralis* sp. nov. and *Halobacillus trueperi* sp. nov., and transfer of *Sporosarcina halophila* to *Halobacillus halophilus* comb. nov. *Int. J. Syst. Bacteriol.* **46**:492–496.
- Stackebrandt, E., C. Koch, O. Gvozdiak, and P. Schumann. 1995. Taxo-

- nomie dissection of the genus *Micrococcus*: *Kocuria* gen. nov., *Nesterenkonia* gen. nov., *Kytococcus* gen. nov., *Dermacoccus* gen. nov., and *Micrococcus* Cohn 1872 gen. emend. Int. J. Syst. Bacteriol. **45**:682–692.
23. Staneck, J. L., and G. D. Roberts. 1974. Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. Appl. Microbiol. **28**:226–231.
24. Ventosa, A. 1994. Taxonomy and phylogeny of moderately halophilic bacteria, p. 231–241. In F. G. Priest, A. Ramos-Cormenzana, and B. J. Tindall (ed.), Bacterial diversity and systematics. Plenum Press, New York, N.Y.
25. Ventosa, A., M. T. García, M. Kamekura, H. Onishi, and F. Ruiz-Berraquero. 1989. *Bacillus halophilus* sp. nov., a moderately halophilic *Bacillus* species. Syst. Appl. Microbiol. **12**:162–166.
26. Ventosa, A., E. Quesada, F. Rodríguez-Valera, F. Ruiz-Berraquero, and A. Ramos-Cormenzana. 1982. Numerical taxonomy of moderately halophilic Gram-negative rods. J. Gen. Microbiol. **128**:1959–1968.
27. Ventosa, A., A. Ramos-Cormenzana, and M. Kocur. 1983. Moderately halophilic Gram-positive cocci from hypersaline environments. Syst. Appl. Microbiol. **4**:564–570.
28. Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, W. E. C. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Truper. 1987. Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. Int. J. Syst. Bacteriol. **37**:463–464.
29. Weiss, N. 1996. Personal communication.