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.

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Two moderately halophilic, gram-positive, heterotrophic bacterial strains were isolated from hypersaline sediments of the Great Salt Lake in Utah. These two strains, designated SL-4^T (T = type strain) and SL-5^T, were motile, spore-forming, strictly aerobic rods which contained peptidoglycan of the Orn-D-Asp type in their vegetative cell walls. The guanine-plus-cytosine contents of the DNAs of strains SL-4^T and SL-5^T were 42 and 43 mol%, respectively. A detailed investigation of the phenotypic and phylogenetic characteristics of these organisms revealed that each isolate represents a new species that is closely related to *Sporosarcina halophila*, a moderately halophilic, spore-forming coccus. Phylogenetic data indicate that there is only a distant relationship between *Sporosarcina halophila* and *Sporosarcina ureae*, the type species of the genus *Sporosarcina*. The sequences of the 16S rRNA genes of strain SL-4^T and *Salinicoccus roseus* DSM 5351 were determined. We propose that a new genus, *Halobacillus*, should be created; this genus includes *Halobacillus halophilus* (formerly *Sporosarcina halophila*) as the type species, as well as *Halobacillus litoralis* DSM 10405^T (= SL-4^T) and *Halobacillus trueperi* DSM 10404^T (= SL-5^T).

The Great Salt Lake is a hypersaline lake that is located in Utah and is divided by a railroad causeway into two parts. The two parts differ significantly in their levels of salinity. Since freshwater can enter only from the south, the level of salinity in the north arm has increased to up to 33% (wt/vol) because of evaporation (18). Cultivation studies have revealed that extremely halophilic archaea are the predominant members of the microbial population in the north arm, whereas moderately halophilic bacteria dominate in the south arm (7). Workers have performed several studies to investigate the microbial diversity of the north arm, but there is little information available about the aerobic, moderately halophilic population in the south arm. In general, moderately halophilic microorganisms grow optimally in media containing 3 to 15% (wt/vol) salt (10), and these organisms are distributed among diverse taxonomic groups in the domains *Bacteria* and *Archaea* (26). There have been many descriptions of gram-negative, moderately halophilic species belonging to the domain *Bacteria*, whereas there have been only a few reports about gram-positive, moderately halophilic bacteria (3, 8, 17, 27, 28). In this paper we extend the range of observed phenotypes of aerobic, heterotrophic bacteria found in hypersaline sediments by describing two moderately halophilic, gram-positive, rod-shaped bacterial species.

MATERIALS AND METHODS

Sampling site and isolation procedure. A sample of surface sediment was obtained from the south arm of the Great Salt Lake in Utah near Salt Lake City and was transported within 1 week to a laboratory in a 1-liter plastic bottle. The salinity of the sample was determined to be 18.5% with a refractometer. Aliquots of the sediment were suspended in water containing 20% (wt/vol) NaCl. After the sediment had settled, 0.1-ml portions of supernatant were spread onto isolation medium containing (per liter) 200 g of NaCl, 20 g of MgSO₄ · 7H₂O, 0.1 g of MnCl₂ · 4H₂O, 0.1 g of KCl, 5 g of casein peptone, 3 g of yeast extract, and 15 g of agar. The pH was adjusted to 7.2.

After incubation at 30°C for several days, white- and orange-pigmented colonies began to appear on the agar plates. No colonies with the red pigmentation typical of halophilic archaea were observed. Orange colonies formed by rod-

shaped bacteria were picked and restreaked several times to obtain pure cultures. Two strains were chosen for detailed characterization.

Bacterial strains and growth media. The newly isolated strains have been deposited in the Deutsche Sammlung von Mikroorganismen as *Halobacillus litoralis* DSM 10405^T (= SL-4^T) (T = type strain) and *Halobacillus trueperi* DSM 10404^T (= SL-5^T). The medium used for maintenance and growth of these strains contained (per liter) 100 g of NaCl, 5 g of MgSO₄ · 7H₂O, 5 g of casein peptone, and 3 g of yeast extract. The pH of this medium was adjusted to 7.5. Solid media were prepared by adding 15 g of agar per liter. Seawater agar was used to observe spore-forming cells (6). This medium contained (per liter) 50 g of NaCl, 10.99 g of MgCl₂ · 6H₂O, 4.06 g of Na₂SO₄, 1.51 g of CaCl₂ · 2H₂O, 0.69 g of KCl, 0.20 g of NaHCO₃, 0.10 g of KBr, 0.1 g of FePO₄ · 4H₂O, 0.042 g of SrCl₂ · 6H₂O, 0.027 g of H₃BO₃, 0.01 g of MnCl₂ · 4H₂O, 0.005 g of Na₂SiO₃ · H₂O, 0.003 g of NaF, 0.002 g of NH₄NO₃, 5.0 g of casein peptone, 1.0 g of yeast extract, and 15.0 g of agar; the pH was adjusted to 7.5.

Salinicoccus roseus DSM 5351¹ and *Sporosarcina halophila* DSM 2266^T were used for comparison; these strains were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. The recommended growth media were used to grow these organisms.

Phenotypic characterization. The cellular morphology of the newly isolated bacteria was studied by using phase-contrast microscopy (Axioplan; Zeiss, Oberkochen, Germany). To obtain photomicrographs of sporulating bacteria, slides were thinly coated with water agar (2%, wt/vol). Flagellation was studied by using the staining methods described by Drews (5) or Heimbrook et al. (9).

Whether the isolates grew in the presence of different salt concentrations was determined by using complex medium containing 0.5 to 30% (wt/vol) marine salts (29). All other growth characteristics were determined by using the maintenance medium. The temperature range for growth was determined by incubating agar plates at temperatures between 5 and 45°C. The pH range for growth was determined by adjusting the final pH of the medium to values between 5.5 and 10.0 with NaOH or HCl. Growth rates were determined by measuring the turbidity of cultures at 660 nm with a UV/Vis spectrophotometer (Pharmacia LKB, Freiburg, Germany).

Physiological and biochemical tests were performed as described previously (19, 29).

Peptidoglycan analysis. The interpeptide bridge in the cell wall peptidoglycans was analyzed by using the methods described by Schleifer and Kandler (22). Total hydrolysates of cell walls were separated by one-dimensional or two-dimensional chromatography on cellulose thin layers (Merck, Darmstadt, Germany). The following amino acids were used as references in the thin-layer chromatography experiments: L-ornithine, L-lysine, and meso-diaminopimelic acid.

DNA base compositions and levels of DNA-DNA similarity. Chromosomal DNA was isolated and purified by the method of Meyer and Schleifer (15). The G+C content of DNA was determined from the midpoint of the thermal denaturation profile obtained with a Gilford model 2600 spectrophotometer (13). The G+C content was calculated by using the equation of De Ley (4) and *Escherichia coli* ATCC 11775 DNA as the reference (G+C content, 51 mol%).

DNA-DNA hybridization experiments were performed by using a modification

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FIG. 1. Phase-contrast micrographs of strains SL-4^T (A) and SL-5^T (B) showing spore-forming cells. Bar = 10 μ m.

of the hydroxyapatite method of Lind and Ursing (11). DNAs were labelled with [α -³²P]dCTP by using the nick translation method (Boehringer, Mannheim, Germany) described by Rossello-Mora et al. (20). The radioactively labelled DNA was reacted with unlabelled DNA under optimal conditions for 16 h. An incubation temperature that was 25°C below the thermal denaturation midpoint of the radioactively labelled DNA was considered optimal, as described by Marmur and Doty (14). The pooled standard deviation for the experiment was 1.8%.

Phylogenetic analysis. In vitro amplification of 16S rRNA genes and direct sequencing of the amplified DNA fragments were performed as previously described (24). The newly determined sequences were added to an alignment of more than 2,000 bacterial 16S rRNA sequences available from rRNA sequence databases (12, 25). Data analyses were performed with the ARB software package as described previously (20, 23). Distance matrix trees were reconstructed by using the neighbor-joining method of Saitou and Nei (21). The trees inferred were compared with the results of a parsimony analysis of the complete data set.

Nucleotide sequence accession numbers. The newly determined sequences of the 16S rRNA genes of *Halobacillus litoralis* and *Salinicoccus roseus* have been deposited in the EMBL data library under accession numbers X94558 and X94559, respectively.

RESULTS

Cellular and colonial morphology. The cells of newly isolated strains SL-4^T and SL-5^T were similar in morphology. They were gram-positive rods with pointed ends that occurred singly, in pairs, or in short chains. Under optimal conditions the cells of strain SL-4^T were 0.7 to 1.1 by 2.0 to 4.5 μ m, and the cells of strain SL-5^T were somewhat wider (0.7 to 1.4 by 2.0 to 4.5 μ m). The cells of both strains were motile by means of flagella, which occurred predominantly as tufts at both poles of each cell. Lateral flagella were also observed sometimes. Under conditions that were not optimal for growth the majority of the cells were nonmotile and elongated cells up to 20 μ m long occurred. Both strains formed endospores after growth on seawater agar. The spores were ellipsoidal or sometimes spherical (diameter, 0.7 to 1.5 μ m) and were located at a central to subterminal position. The spores survived heating at 75°C for at least 10 min. The frequency of spore-forming cells was higher among strain SL-5^T cells than among strain SL-4^T cells (Fig. 1).

Colonies of both strains were 1 to 2 mm in diameter, circular, smooth, slightly raised, and orange pigmented. The pigment was insoluble in water and nondiffusible. The pigmentation varied with the salt concentration of the medium.

Growth and metabolic properties. Strain SL-4^T grew in media containing 0.5 to 25% (wt/vol) salt, and optimum growth occurred in medium containing around 10% salt. This strain grew at temperatures ranging from 10 to 43°C, and the opti-

um temperature for growth was 35°C. The pH range for growth was pH 6.0 to 9.5, and the optimum pH was 7.5.

Strain SL-5^T grew in media containing 0.5 to 30% (wt/vol) salt, and optimum growth occurred in medium containing around 10% salt. The temperature range for growth of this organism was 10 to 44°C, and the optimum temperature was 35°C. The optimum pH for growth was 7.7; no growth occurred at pH values below 6.0 or above 9.5.

Under optimal conditions both strains grew relatively fast; the doubling times were 0.48 h for SL-4^T and 0.42 h for SL-5^T. Strains SL-4^T and SL-5^T grew only under aerobic conditions and produced catalase and oxidase. Broth cultures of both strains were uniformly turbid. Biochemical and physiological characteristics useful for identifying and differentiating strains SL-4^T and SL-5^T and the type strain of *Sporosarcina halophila* are shown in Table 1.

Peptidoglycan types. The peptidoglycan composition of the cell walls of strains SL-4^T and SL-5^T was similar to that of *Sporosarcina halophila*. The peptidoglycan type was Orn-D-Asp.

DNA base composition and levels of DNA-DNA similarity. The G+C contents of the DNAs of strains SL-4^T and SL-5^T were 42 and 43 mol%, respectively. DNA-DNA hybridization experiments revealed low levels of similarity (18 to 24%) between *Sporosarcina halophila* DSM 2266^T DNA and strain SL-4^T and SL-5^T DNAs. The highest level of similarity found (30%) was the level of similarity between labelled strain SL-4^T DNA and unlabelled strain SL-5^T DNA.

Phylogeny. The almost complete 16S rRNA sequence of strain SL-4^T was determined. Partial sequencing of the 16S rRNA gene of strain SL-5^T showed that the sequence of this gene was indistinguishable from the SL-4^T sequence in a variable part near the 5' end of the 16S rRNA (positions 100 to 450; *E. coli* 16S rRNA numbering). In addition, the sequence of the 16S rRNA gene of *Salinicoccus roseus* was determined because the genus *Salinicoccus* is another genus of gram-positive halophilic bacteria. Table 2 shows the results of a comparison of the 16S rRNA sequence of strain SL-4^T and the 16S rRNA sequences of other gram-positive bacteria. The highest level of similarity found (97.9%) was the level of similarity between the sequences of SL-4^T and *Sporosarcina halophila*, whereas the level of similarity between the strain SL-4^T sequence and the 16S rRNA sequence of *Sporosarcina ureae* was only 91.0%. Among the *Bacillus* species, *Bacillus pantothenicus*, which belongs to rRNA group I, was most closely

TABLE 1. Phenotypic characteristics of species belonging to the genus *Halobacillus*

Characteristic	<i>H. halophilus</i> DSM 2266 ^{1a}	<i>H. litoralis</i> SL-4 ^T	<i>H. trueperi</i> SL-5 ^T
Peptidoglycan type	Orn-D-Asp	Orn-D-Asp	Orn-D-Asp
Morphology	Coccoid	Rod shaped	Rod shaped
Spore production	+	+	+
Motility	+	+	+
Pigmentation	Orange	Orange	Orange
Anaerobic growth	—	—	—
Growth in the presence of 0.5% salt	—	+	+
Catalase	+	+	+
Oxidase	+	+	+
Voges-Proskauer test	—	—	—
Nitrate reduction	—	—	—
Acid production from:			
D-Fructose	—	+	+
D-Galactose	—	—	+
Maltose	—	+	+
Sucrose	—	+	+
D-Xylose	—	+	—
D-Glucose	—	+	+
D-Mannitol	—	+	—
D-Trehalose	—	+	+
Hydrolysis of:			
Esculin	—	—	—
Casein	+	—	—
Gelatin	+	+	+
Starch	+	—	—
Pullulan	+	—	+
Tween 80	—	—	—
Tyrosine	—	—	—
Enzyme activities			
DNase	+	+	+
Urease	—	—	—
Phosphatase	+	—	—
Lecithinase	—	—	—
Phenylalanine deaminase	—	—	—
Arginine dihydrolase	—	—	—

^a Data from reference 2.

related to strain SL-4^T. The level of similarity between the 16S rRNA sequences of these organisms was 94.5%.

The phylogenetic tree in Fig. 2 shows the relationship of strain SL-4^T to some representative gram-positive bacteria with low DNA G+C contents. The inferred tree topology was stable even when we used various sets of alignment positions that differed in their degrees of sequence conservation.

DISCUSSION

Newly isolated strains SL-4^T and SL-5^T are moderately halophilic, aerobic, heterotrophic bacteria that were isolated from a hypersaline sediment characterized by changing levels of salinity that depended on the rainfall or snowmelt. The adaptation of these organisms to this kind of habitat is reflected by their tolerance of a wide range of salt concentrations. In contrast to most other microorganisms isolated from samples obtained from the Great Salt Lake in Utah, which have generation times ranging from 6 to 16.9 h (7, 18), strains SL-4^T and SL-5^T grow comparatively fast (generation times, 0.4 to 0.5 h).

The data presented in this paper indicate that strains SL-4^T and SL-5^T are closely related to *Sporosarcina halophila*. The following phenotypic characteristics suggest that these organisms should be placed in the same genus: they are Gram pos-

TABLE 2. Overall levels of sequence similarity between strain SL-4^T 16S rRNA and 16S rRNAs from representative gram-positive bacteria with low DNA G+C contents^a

Organism or group	Strain	Source, accession no., or reference ^b	% Similarity to strain SL-4 ^T
Groups containing <i>Bacillus</i> species and related taxa			
rRNA group I (26 species)		1	90.8–94.5
rRNA group II (6 species)		1	89.3–90.8
rRNA group III (10 species)		1	87.0–92.0
rRNA group IV (2 species)		1	89.6–90.1
rRNA group V (3 species)		1	89.9–91.3
rRNA group VI (9 species)		16	90.6–92.9
<i>Sporosarcina halophila</i>	NCIMB 2269	X62175	97.9
<i>Sporosarcina ureae</i>	NCIMB 9251	X62174	91.0
<i>Planococcus citreus</i>	NCIMB 1493	X62172	91.6
<i>Planococcus kocurii</i>	NCIMB 629	X62173	91.1
<i>Sporolactobacillus inulinus</i>	ATCC 15538	M58838	89.9
<i>Marinococcus halophilus</i>	NCIMB 2178	X62171	89.7
<i>Staphylococcus carnosus</i>	DSM 20501	X66099	89.7
<i>Salinicoccus roseus</i>	DSM 5351	X94559	89.6
<i>Enterococcus faecalis</i>		RDP	88.1
<i>Pediococcus acidilactici</i>	DSM 20284	M58833	85.4
<i>Pediococcus pentosaceus</i>	ATCC 33316	M58834	84.7

^a In addition to species of the genus *Bacillus*, most of the strains compared were halophilic or halotolerant bacteria. Ranges of similarity values are given for the six rRNA groups containing *Bacillus* species and related taxa as defined by Ash et al. (1) and Nielsen et al. (16).

^b The accession numbers are the EMBL accession numbers of the sequences analyzed. For each group of organisms, the reference is a reference in which the accession numbers are given. The sequence of *Enterococcus faecalis* has no accession number and was obtained from the Ribosomal Database Project (RDP) (12).

itive, spore forming, moderately halophilic, and motile and possess peptidoglycan of the Orn-D-Asp type. However, these organisms are phenotypically and chemotaxonomically different from *Bacillus halophilus*, the only moderately halophilic, obligately aerobic, spore-forming, gram-positive, rod-shaped organism described previously. In fact, *B. halophilus* has peptidoglycan of the *meso*-diaminopimelic acid type in its cell wall, and its G+C content is 51.5 mol% (27). A comparative sequence analysis of 16S rRNA genes revealed that strain SL-4^T and *Sporosarcina halophila* are closely related. The type species of the genus *Sporosarcina*, *Sporosarcina ureae*, is not closely

FIG. 2. Unrooted phylogenetic tree showing the relationship of *H. litoralis* SL-4^T and *H. halophila* (formerly *Sporosarcina halophila*) to some other gram-positive bacteria. The tree was derived from a distance matrix based on a selection of 16S rRNA sequences of moderately halophilic and nonhalophilic gram-positive bacteria with low DNA G+C contents. Scale bar = 10% estimated sequence divergence.

related to this group of bacteria. The level of similarity between the 16S rRNA sequences of *Sporosarcina ureae* and *Sporosarcina halophila* is only 91.0%. Moreover, *Sporosarcina ureae* is not halophilic and has a peptidoglycan of the Lys-Gly-D-Glu type. Therefore, we propose that the new genus *Halobacillus* should be created for new strains SL-4^T and SL-5^T and *Sporosarcina halophila*.

Several phenotypic characteristics allowed us to differentiate *Sporosarcina halophila* from strains SL-4^T and SL-5^T (Table 1), and the low levels of DNA similarity among the three organisms (maximum level of similarity, 30%) justify placing them in three different species. Thus, we propose that SL-4 should be the type strain of *Halobacillus litoralis* sp. nov. and SL-5 should be the type strain of *Halobacillus trueperi* sp. nov. *Sporosarcina halophila* should be transferred to the genus *Halobacillus* as *Halobacillus halophilus*.

Description of *Halobacillus* Spring, Ludwig, Marquez, Ventosa, and Schleifer gen. nov. *Halobacillus* (Ha.lo.ba.cil'lus. Gr. n. hals, salt; L. n. bacillus, rod; M.L. masc. n. *Halobacillus*, a salt[-loving] rod). Gram-positive, spore-forming, rod-shaped or spherical to oval cells. Motile by means of flagella. Endospores resist heating at 75°C for at least 10 min. The cell wall contains peptidoglycan of the Orn-D-Asp type. Moderately halophilic, strictly aerobic, and chemoorganotrophic. Catalase and oxidase are produced; DNase positive and urease negative. Nitrate is not reduced to nitrite. The Voges-Proskauer reaction is negative. Colonies form an orange nondiffusible pigment. The G+C content of the DNA ranges from 40 to 43 mol% (as determined by the thermal denaturation method). The type species is *Halobacillus halophilus*.

Description of *Halobacillus litoralis* Spring, Ludwig, Marquez, Ventosa, and Schleifer sp. nov. *Halobacillus litoralis* (li.to.ra'lis. L. adj. *litoralis*, of the shore). In addition to the characteristics given above in the description of the genus, *H. litoralis* has the following characteristics. Rod-shaped cells are 0.7 to 1.1 by 2.0 to 4.5 µm and occur singly, in pairs, or in short chains. Sometimes cells up to 20 µm long are present. Motile by means of several flagella, which are inserted at both poles of each cell or laterally. Endospores are ellipsoidal or sometimes spherical and 0.7 to 1.5 µm in diameter; they are located at a central to subterminal position. Acid is produced from D-fructose, maltose, sucrose, D-xylose, D-glucose, D-mannitol, and D-trehalose, but not from D-galactose. Gelatin is hydrolyzed, but casein, starch, pullulan, Tween 80, and tyrosine are not hydrolyzed. Phosphatase is not produced. Growth occurs in media containing 0.5 to 25% (wt/vol) salt, and the optimum salt concentration is around 10%. The temperature range for growth is 10 to 43°C, and the optimum temperature is 35°C. The pH range for growth is pH 6.0 to 9.5; the optimum pH is 7.5. The doubling time is 0.48 h under optimal conditions. The G+C content of the DNA is 42 mol%. Isolated from sediment obtained from the southern part of the Great Salt Lake in Utah. The type strain is strain SL-4, which has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, as strain DSM 10405.

Description of *Halobacillus trueperi* Spring, Ludwig, Marquez, Ventosa, and Schleifer sp. nov. *Halobacillus trueperi* (true'per.i. M.L. gen. n. *trueperi*, of Trueper, in honor of Hans G. Trüper, who has made important contributions to our knowledge of the ecophysiology and taxonomy of halophilic bacteria). In addition to the characteristics given above in the description of the genus, *H. trueperi* has the following characteristics. Rod-shaped cells are 0.7 to 1.4 by 2.0 to 4.5 µm and occur singly, in pairs, or in short chains. Sometimes cells up to 20 µm long are present. Motile by means of several flagella, which are inserted at both poles of each cell or laterally. En-

dospores are ellipsoidal or sometimes spherical and 0.7 to 1.5 µm in diameter; they are located at a central to subterminal position. Acid is produced from D-fructose, D-galactose, maltose, sucrose, D-glucose, and D-trehalose, but not from D-xylose and D-mannitol. Gelatin and pullulan are hydrolyzed, but casein, starch, Tween 80, and tyrosine are not hydrolyzed. Phosphatase is not produced. Growth occurs in media containing 0.5 to 30% (wt/vol) salt, and the optimum salt concentration is around 10%. The temperature range for growth is 10 to 44°C, and the optimum temperature is 35°C. The pH range for growth is pH 6.0 to 9.5; the optimum pH is 7.5. The doubling time is 0.42 h under optimal conditions. The G+C content of the DNA is 43 mol%. Isolated from sediment obtained from the southern part of the Great Salt Lake in Utah. The type strain is strain SL-5, which has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, as strain DSM 10404.

Transfer of *Sporosarcina halophila* to the genus *Halobacillus* as *Halobacillus halophilus* comb. nov. *Halobacillus halophilus* (ha.lo'phi.lus. Gr. n. hals, salt; Gr. adj. *philus*, loving; M.L. masc. adj. *halophilus*, salt loving). Physiological, chemotaxonomic, and phylogenetic data indicate that *Sporosarcina halophila* is more closely related to *H. litoralis* and *H. trueperi* than to *Sporosarcina ureae*, the type species of the genus *Sporosarcina*. The original description of the species given by Claus et al. (2) is unchanged.

ACKNOWLEDGMENTS

We thank N. Weiss for confirming the results of our cell wall analysis, W. Liebl for testing the ability of the strains which we studied to decompose polymers, and R. Rossello-Mora for help with the DNA-DNA hybridization experiments.

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