***Gracilibacillus* gen. nov., with description of *Gracilibacillus halotolerans* gen. nov., sp. nov.; transfer of *Bacillus dipsosauri* to *Gracilibacillus dipsosauri* comb. nov., and *Bacillus salexigens* to the genus *Salibacillus* gen. nov., as *Salibacillus salexigens* comb. nov.**

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A Gram-positive, extremely halotolerant bacterium was isolated from the Great Salt Lake, Utah, USA. The strain, designated NN^T (= DSM 11805^T), was strictly aerobic, rod-shaped, motile by peritrichous flagella and spore-forming. Strain NN^T grew at salinities of 0–20% (w/v) NaCl. A distinctive feature of strain NN^T was its optimal growth in salt-free medium. The polar lipid pattern of strain NN^T consisted of phosphatidyl glycerol, diphosphatidyl glycerol and two phospholipids of unknown structure. The G+C content of its DNA was 38 mol%. The morphological, physiological and, particularly, the 16S rDNA sequence data, showed that strain NN^T was associated with 'Bacillus group 1'. However, the organisms showing the greatest degree of sequence similarity to strain NN^T were members of the genus *Halobacillus* and the species *Marinococcus albus*, *Virgibacillus pantothenicus*, *Bacillus salexigens* and *Bacillus dipsosauri*. On the basis of chemotaxonomic data, strain NN^T was shown to be chemically most similar to *B. salexigens* and *B. dipsosauri*, with the greatest degree of similarity being shown to the latter organism. This was consistent with the 16S rDNA sequence data. Members of the genus *Halobacillus* comprise a chemically distinct group and can easily be distinguished from all other organisms of 'Bacillus group 1'. On the basis of the 16S rDNA data, chemotaxonomy and the physiology of strain NN^T, it is proposed that this organism is a member of a new species, within a new genus, for which the name *Gracilibacillus halotolerans* is proposed. It is also proposed that *B. dipsosauri* be transferred to this genus as *Gracilibacillus dipsosauri* comb. nov. and that *B. salexigens* be transferred to the genus *Salibacillus* gen. nov., as *Salibacillus salexigens* comb. nov. Finally, additional data is provided to support the transfer of *Bacillus pantothenicus* to the genus *Virgibacillus*, as *Virgibacillus pantothenicus* Heyndrickx et al. (1998).

Keywords: *Gracilibacillus halotolerans*, *Gracilibacillus dipsosauri*, *Salibacillus salexigens*, *Virgibacillus pantothenicus*, taxonomy

INTRODUCTION

Micro-organisms from extreme environments have been investigated intensively in recent years. Studies of hypersaline environments have revealed the presence

of taxonomically very diverse micro-organisms, which also differ widely in salt requirements and metabolic capabilities. The moderately halophilic as well as halotolerant bacteria found in hypersaline waters may have evolved from their marine relatives (Forsyth *et al.*, 1971; Rodriguez-Valera, 1986). Indeed, aerobic spore-forming obligately halophilic bacteria are commonly found in marine environments (Rüger &

The GenBank accession number for the sequence of strain NN^T (= DSM 11805^T) reported in this paper is AF036922.

Richter, 1979), e.g. *Bacillus halophilus* (Ventosa *et al.*, 1989) and *Bacillus marinus* (Rüger & Richter, 1979), as well as in hypersaline areas, e.g. *Bacillus salexigens* (Garabito *et al.*, 1997), *Halobacillus litoralis* and *Halobacillus trueperi* (Spring *et al.*, 1996). Several halotolerant bacteria, able to grow at salinities found in both marine and hypersaline environments, have also been described, such as *Bacillus dipsosauri* (Lawson *et al.*, 1996), *Bacillus pantothenicus* (Proom & Knight, 1950), *Halomonas elongata* (Vreeland *et al.*, 1980) and *Halomonas meridiana* (James *et al.*, 1990). However, to our knowledge, no extremely halotolerant bacterium that does not require NaCl for optimal growth has been characterized to date. Strain NN^T, isolated from the Great Salt Lake in Utah, USA, is therefore the first extremely halotolerant species described which grows optimally without NaCl in its environment.

Using a combination of genotypic (16S rDNA sequence analysis and G+C content) and phenotypic (polar lipids, respiratory lipoquinones, fatty acids and physiology) characters, it was possible to show that strain NN^T, despite the apparently high degree of 16S rDNA sequence similarity to members of 'Bacillus group 1', could be placed in a novel taxon at the genus and species level. It is generally accepted that the genus *Bacillus* consists of several divergent phyletic lines and should be taxonomically revised (e.g. Ash *et al.*, 1991). During the course of this work, it was shown that the species *B. dipsosauri* could be placed in the same higher taxon as strain NN^T and that the species *B. salexigens* and *B. pantothenicus* should be removed from the genus *Bacillus*. Based on the results presented, it is proposed that strain NN^T be placed in the genus *Gracilibacillus* gen. nov., as *Gracilibacillus halotolerans* sp. nov. It is also proposed that *B. dipsosauri* be transferred to the genus *Gracilibacillus* as *Gracilibacillus dipsosauri* comb. nov. In addition, it is proposed that *B. salexigens* be transferred to the genus *Salibacillus* gen. nov., as *Salibacillus salexigens* comb. nov. Finally, additional data is provided which supports the transfer of *B. pantothenicus* to the genus *Virgibacillus*, as *Virgibacillus pantothenicus* (Heyndrickx *et al.*, 1998).

METHODS

Isolation procedure. Surface mud obtained from the southern part of the Great Salt Lake was used as an inoculum for the enrichment of halophilic or halotolerant bacteria in liquid GSL-2 medium which consisted of (l⁻¹ distilled water): NaCl, 100 g; MgSO₄·7H₂O, 10 g; KCl, 5 g; NH₄Cl, 2 g; NaHCO₃, 1 g; citric acid, 0.5 g; yeast extract (Difco), 2 g; trypticase peptone (BBL), 2 g; trace metal solution (TMS 3), 2 ml (Ingvorsen & Jørgensen, 1984); KH₂PO₄ (50 g l⁻¹), 10 ml; CaCl₂·2H₂O (20 g l⁻¹), 10 ml; FeCl₂/MnCl₂ solution [FeCl₂·4H₂O (20 g l⁻¹) + MnCl₂·4H₂O (20 g l⁻¹)], 2 ml. Glucose (0.2% w/v) was added as substrate. The pH of the medium was adjusted to 7.4 with NaOH before autoclaving. Pure cultures were obtained by plating 1:10 serial dilutions of enrichment cultures on solid GSL-2 medium (1.5% agar) containing 2 g glucose l⁻¹ as

substrate. Although several strains were isolated, only one of them was shown to consist of Gram-positive, halotolerant micro-organisms, the detailed characterization of which is reported here.

Growth media. A Tris-based medium (Tris-medium) was used for growth experiments and maintenance of strain NN^T, unless otherwise stated. The medium contained (l⁻¹ distilled water): NaCl, 100 g; MgSO₄·7H₂O, 20 g; KCl, 5 g; (NH₄)₂SO₄, 2 g; NaBr, 0.1 g; yeast extract (Difco), 2 g; trypticase peptone (BBL), 1 g; Tris/HCl, 12 g; trace metal solution (TMS 3), 2 ml. The pH was adjusted to 7.8 with NaOH prior to sterilization. After sterilization and cooling to 5 °C, 10 ml sterile phosphate solution (KH₂PO₄, 50 g l⁻¹), 5 ml sterile CaCl₂ solution (CaCl₂·2H₂O, 100 g l⁻¹) and 2 ml sterile FeCl₂/MnCl₂ solution (as above) were added. The final pH of the Tris-medium was 7.5±0.2. A salt-free medium was prepared by omitting NaCl, MgSO₄, KCl and NaBr from the Tris-medium.

Morphological tests. All tests were performed with exponentially growing bacteria, except for spore tests, which were made with stationary phase cultures. Preparation of strain NN^T for scanning electron microscopy was done according to the method of Paerl & Shimp (1973) modified as follows: a 2.0% (w/v) glutardialdehyde solution in 50 mM phosphate buffer was used in the fixation step and the transfer from 100% ethanol to amyl acetate was omitted. Cells were examined with a JEOL JSM-840 scanning microscope. Colony morphology was observed on solid GSL-2 medium after 5 d growth. Gram staining was done according to Dussault (1955), while the Gram test was performed using 3% KOH (Finegold & Baron, 1986). Flagella and endospores were stained according to the methods of Leifsson and Schaeffer-Fulton, respectively (Smibert & Krieg, 1981).

Physiological tests. Growth was determined by measuring either the optical density (OD₆₀₀) or the protein content of the culture according to Bradford (1976). The effect of salinity (at 30 °C) or temperature on the growth of strain NN^T was tested using 0.2% (w/v) lactose as carbon and energy source. The effect of pH on growth was determined at 30 °C using 0.2% (w/v) sodium D-glucuronate as substrate, since glucuronate was a non-acidogenic substrate with strain NN^T. Tris-medium was used for incubation at pH values above 5.0, whereas Tris/HCl was replaced with sodium acetate (8 g l⁻¹) at pH values below 5.0.

Anaerobic growth was tested by incubation in 100 ml rubber sealed screw-cap bottles containing anoxic Tris-medium, in which (NH₄)₂SO₄ was replaced by NH₄Cl and peptone was omitted. The medium further contained either glucose, lactose, acetate or peptone at a final concentration of 0.2% (w/v) as potential substrates, and 0.1% (w/v) KNO₃, 0.02% (w/v) Fe(NH₄)₂(SO₄)₂·6H₂O or 0.02% (w/v) Na₂S₂O₃ as potential electron acceptors. Fermentation of glucose and xylose was tested by incubation in the same medium devoid of inorganic electron acceptors.

Biochemical tests. Presence of catalase, oxidase, lecithinase, phenylalanine deaminase activity and H₂S production were all determined according to Smibert & Krieg (1981). Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase activities were tested by the Falkow method (Skerman, 1967), adding 2.50% (w/v) NaCl and 0.05% (w/v) MgCl₂·6H₂O to the test medium. Urease activity was tested by measuring NH₄⁺ concentrations in a culture grown in the presence of 2% (w/v) urea. Presence of alkaline phosphatase was tested using an API ZYM test (bio-

Mérieux). Methyl Red and Voges–Proskauer tests were performed as described previously (Smibert & Krieg, 1981), with the following constituents added to the medium (% w/v): NaCl, 10.0; KCl, 0.20; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.50; yeast extract, 0.25. Reduction of NO_3 to NO_2/N_2 was tested in 10 ml test tubes containing anaerobic GSL-2 medium, 10 mM KNO_3 and 0.2% (w/v) glucose, and inverted Durham tubes were used (Smibert & Krieg, 1981). Nitrite/nitrate was detected using the Griess–Llosvay reagents (Skerman, 1967). Indole production was tested with Ehrlich's reagent (Smibert & Krieg, 1981) in Tris-medium containing tryptophan, devoid of yeast extract and peptone. Hydrolysis of starch and Tween 80 was tested with AZCL-amylose and Tween 80 agar plates, respectively, while hydrolysis of aesculin and gelatin was tested with an API 20NE test (bioMérieux). Hydrolysis of casein was tested with Azocasein (Morikawa *et al.*, 1994). Antibiotic resistance was examined by inoculating bacterial suspensions on agar plates of Tris-medium containing 0.2% (w/v) glucose and applying Neo-Sensitabs (Rosco Diagnostica). Inhibition zones were measured after 10 d incubation and interpreted according to the manufacturer's manual.

Strains used for chemical analysis. Strains used in the chemical analysis of the polar lipids, respiratory lipoquinones and fatty acids included strain NN^T (= DSM 11805^T), *B. pantothenicus* DSM 26^T, *B. salexigens*, DSM 11483^T, *B. dipsosauri* DSM 11125^T, *Marinococcus albus* DSM 20748^T, *Halobacillus halophilus* DSM 2266^T, *Halobacillus litoralis* DSM 10405^T and *Halobacillus trueperi* DSM 10404^T. All strains were grown on Bacto Marine Broth and 10% MH (moderate halophile) medium, described by Garabito *et al.* (1997), at 37 °C with shaking for 24 h. Cells were harvested by centrifugation, freeze-dried and stored at –20 °C until further analysis.

Extraction of respiratory lipoquinones and polar lipids. Respiratory lipoquinones and polar lipids were extracted from 100 mg freeze-dried cell material using the two-stage method described by Tindall (1990a, b). Respiratory lipoquinones were extracted using methanol/hexane (Tindall, 1990a, b) and the polar lipids were extracted by adjusting the remaining methanol/0.3% aqueous NaCl phase (containing the cell debris) to give a chloroform/methanol/0.3% aqueous NaCl mixture (1:2:0.8, by vol.). The extraction solvent was stirred overnight and the cell debris pelleted by centrifugation. Polar lipids were recovered into the chloroform phase by adjusting the chloroform/methanol/0.3% aqueous NaCl mixture to a ratio of 1:1:0.9 (by vol.).

Analysis of respiratory lipoquinones. Respiratory lipoquinones were separated into their different classes (menaquinones and ubiquinones) by TLC on silica gel (Macherey-Nagel) using hexane/*tert*-butylmethylether (9:1, v/v) as solvent. UV-absorbing bands corresponding to menaquinones or ubiquinones were removed from the plate and further analysed by HPLC. This step was carried out on a LDC Analytical HPLC (Thermo Separation Products) fitted with a reverse phase column (Macherey-Nagel, 2 mm × 125 mm, 3 µm, RP₁₈) using methanol as the eluant. Respiratory lipoquinones were detected at 269 nm.

Analysis of polar lipids. Polar lipids were separated by two-dimensional silica gel TLC (Macherey-Nagel). The first direction was developed in chloroform/methanol/water (65:25:4, by vol.), and the second in chloroform/methanol/acetic acid/water (80:12:15:4, by vol.). Total lipid material and specific functional groups were detected using dodecamolybdophosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin (free amino

groups), periodate–Schiff (α-glycols), Dragendorff (quaternary nitrogen) and anisaldehyde–sulphuric acid (glycolipids).

Fatty acid analysis. Fatty acids were analysed as methyl ester derivatives prepared from 10 mg dry cell material. Cells were subjected to differential hydrolysis in order to detect ester-linked and non-ester-linked (amide-bound) fatty acids (B. J. Tindall, unpublished). Fatty acid methyl esters were analysed by GC using a 0.2 µm × 25 m non-polar capillary column and flame ionization detection. The run conditions were: injection and detector port temperature 300 °C, inlet pressure 60 kPa, split ratio 50:1, injection volume 1 µl, and temperature programme of 130–310 °C at a rate of 4 °C min^{–1}.

Cell wall analysis. The cell wall peptidoglycan was purified as described by Schleifer & Kandler (1972). The structure of the peptidoglycan was determined by analyses of amino acids and peptides of cell wall hydrolysates using two-dimensional TLC and GC as described by Groth *et al.* (1996) and by using electrospray mass spectrometry (Schumann & Ihn, 1996).

16S rDNA sequence analysis and DNA base composition. Extraction of genomic DNA from strain NN^T, PCR-mediated amplification and purification of the PCR products were carried out as described previously (Rainey *et al.*, 1996) at the DSMZ, Braunschweig, Germany. Purified PCR products were sequenced with the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) as directed in the manufacturer's protocol. Sequence reactions were electrophoresed using the Applied Biosystems 373A DNA Sequencer.

The global phylogenetic positions of the strains investigated were analysed using the ARB database (Ludwig & Strunk, 1996). 16S rDNA sequences were compared with the existing 16S rDNA database for members of the group classically defined as the genus *Bacillus*. A greater degree of resolution among closely related taxa was performed using the ae2 editor (Maidak *et al.*, 1997). A phylogenetic dendrogram was reconstructed using the treeing algorithm of De Soete (1983), based on similarity values which had been transformed into phylogenetic distance values that compensate for multiple substitutions at any given site in the sequence (Jukes & Cantor, 1969). The neighbour-joining and maximum-likelihood programs contained in the PHYLIP package (Felsenstein, 1993) were used in the construction of phylogenetic dendrograms (Saitou & Nei, 1987).

The sequences used for determining the greatest degree of resolution were obtained from the EMBL/EBI database and were: *B. salexigens* DSM 11483^T, Y11603; *Bacillus subtilis* NCDO 1769^T, X60646; *B. pantothenicus* NCDO 1765^T, X60627; *B. dipsosauri* DSM 11125^T, X82436; *Halobacillus halophilus* (basonym *Sporosarcina halophila*) NCIMB 9251^T, X62174; *Halobacillus litoralis* DSM 10405^T, X94558; and *M. albus* DSM 20748^T, X90834.

The G + C content of DNA from strain NN^T was determined by HPLC according to Mesbah *et al.* (1989) and was carried out in the DSMZ, Braunschweig, Germany.

RESULTS

Morphology

Cells of strain NN^T were predominantly rod-shaped, 0.4–0.6 by 2–5 µm in size. However, filamentous forms were present throughout the growth cycle (Fig. 1). The

Fig. 1. Scanning electron micrograph of strain NN^T , showing the presence of both rod-shaped and filamentous bacteria. Bar, 1 μm .

Fig. 2. Influence of salinity on growth of strain NN^T in Tris-medium containing 0.2% (w/v) lactose. Inoculum used for growth in 0% salinity was washed in sterile demineralized water before transfer to the medium.

cells were Gram-positive and motile by means of peritrichous flagella. Ellipsoid endospores were produced terminally, causing swelling of the sporangia. Cell morphology was not affected by the salt concentration of the growth medium. Colonies of strain NN^T were circular, creamy white, non-transparent and approx. 2 mm in diameter on GSL-2 agar medium after 5 d growth at 30 °C. They had an entire margin, convex elevation, and a shiny, glistening surface.

Physiological, biochemical and metabolic properties

Strain NN^T exhibited extreme halotolerance, being able to grow in Tris-medium containing 0–20% (w/v) NaCl at 30 °C (Fig. 2). Duration of the lag phase and generation time increased with increasing NaCl concentrations. Importantly, strain NN^T did not require NaCl for growth; the highest cell yields were obtained in salt-free medium, even after six consecutive transfers. Strain NN^T grew in the presence of 16% (w/v) NaCl but not in 20% (w/v) NaCl at 40 °C (data not shown). Growth was observed at temperatures of

6–50 °C, but no growth was observed at 55 °C. Optimal growth temperature was 47 °C with a generation time of 3.0 h in Tris-medium containing 10% (w/v) NaCl. Spores suspended in Tris-medium survived heating at 90 °C, but not at 95 °C, for 5 min. Growth occurred at pH 5–10 with optimal growth close to pH 7.5 with glucuronate as substrate.

The cells were obligately aerobic and showed good growth on a large variety of sugar compounds with concomitant acidification of the medium. Growth was only observed on a few alcohols, carboxylic acids and amines. No growth occurred with amino acids, amides, acetone or cellulose. Yeast extract was required for growth whereas trypticase peptone was not, although it stimulated growth. Strain NN^T was resistant to ampicillin (33 mg), gentamicin (40 mg), kanamycin (100 mg), nalidixic acid (130 mg), neomycin (120 mg) and tetracycline (80 mg). Bacitracin (40 units), carbenicillin (115 mg), chloramphenicol (60 mg), erythromycin (78 mg), novobiocin (100 mg), penicillin (5 mg) and rifampicin (30 mg) inhibited growth of strain NN^T . Susceptibility to the antibiotics was tested at 10% (w/v) NaCl. However, as reported by Merkel (1972), the susceptibility may vary with salinity due to the effect of salts on antibiotic action. Other characteristics differentiating strain NN^T from related species are either shown in Table 1 or included in the species description of *Gracilibacillus halotolerans*.

DNA base composition and phylogeny

DNA base composition and 16S rDNA sequence analysis were carried out at the DSMZ, Braunschweig, Germany. The G+C content of strain NN^T was 38 mol%. Approximately 95% of the 16S rDNA sequence was determined and subsequent analysis of the sequence indicated that, based on a global comparison with sequences in the ARB database, strain NN^T belonged within the phyletic group classically defined as the genus *Bacillus* (data not shown). Although, based on further restriction of the sequences used for comparison (data not shown), strain NN^T was shown to be associated with 'Bacillus group 1' as defined by Ash *et al.* (1991), those organisms showing the greatest degree of 16S rDNA sequence similarity (Table 2) were members of the genus *Halobacillus* and the species *M. albus*, *B. salexigens*, *B. dipsosauri* and *V. pantothenicus*. Among these organisms, the greatest degree of sequence similarity was observed between strain NN^T and *B. dipsosauri* (96% sequence similarity). A rooted phylogenetic tree in Fig. 3 shows the relationship of strain NN^T to only those organisms having the greatest degree of sequence similarity which are listed above and in the Methods.

Polar lipids and quinones

Members of the genus *Halobacillus* and *M. albus* had a very similar polar lipid pattern which comprised

Table 1. Morphological and physiological characteristics of strain NN^T, *Bacillus dipsosauri*, *Bacillus salexigens*, *Halobacillus halophilus* and *Virgibacillus pantothenicus*

Strain: 1, NN^T (*G. halotolerans* DSM 11805^T); 2, *B. dipsosauri* (*G. dipsosauri* DSM 11125^T); 3, *B. salexigens* (*S. salexigens* DSM 11483^T); 4, *Halobacillus halophilus* DSM 2266^T; 5, *V. pantothenicus* DSM 26^T. +, Positive; –, negative; ND, not determined; U, unspecified for the type species.

Character	1	2*	3†	4‡	5§
Morphology	Thin rods & filaments	Thin rods & filaments	Rods, single or in chains	Rods	Rods
Pigmentation	Creamy white	White	Non-pigmented	Non-pigmented	Greyish white
Spore shape	Ellipsoid	Spherical	Oval	Ellipsoid	Ellipsoid or spherical
Spore position	Terminal	Terminal	Central or sub-terminal	Central	Terminal
Anaerobic growth	–	+	–	–	+
NaCl range (%)	0–20	0–15	8–16	2–24	0–10
Temperature range (°C)	6–50	28–50	15–45	15–50	28–45
Nitrate reduction	+	+	–	–	U
H ₂ S production	+	–	+	–	ND
Acid production from:					
D-Glucose	+	+	+	+	+
D-Xylose	+	ND	–	+	+
D-Mannitol	+	+	–	–	–
Hydrolysis of:					
Gelatin	+	+	+	–	+
Casein	–	–	+	–	+
Starch	+	+	–	–	+
Aesculin	+	+	+	+	ND
Urea	+	–	ND	+	–

* Data from Deutch (1994) and Lawson *et al.* (1996).

† Data from Garabito *et al.* (1997).

‡ Data from Claus *et al.* (1983) and Spring *et al.* (1996).

§ Data from Proom & Knight (1950), Sneath (1986) and Heyndrickx *et al.* (1998).

Table 2. 16S rRNA gene similarity values (%) for strain NN^T and related taxa

Strain: 1, NN^T (*G. halotolerans* DSM 11805^T); 2, *B. dipsosauri* (*G. dipsosauri* DSM 11125^T); 3, *Halobacillus litoralis* DSM 10405^T; 4, *Halobacillus halophilus* NCIMB 9251^T; 5, *M. albus* DSM 20748^T; 6, *B. salexigens* (*S. salexigens* DSM 11483^T); 7, *V. pantothenicus* NCDO 1765^T; 8, *B. subtilis* NCDO 1769^T.

Strain	1	2	3	4	5	6	7	8
1	–							
2	96.0	–						
3	94.3	94.0	–					
4	91.9	91.5	96.4	–				
5	92.6	92.2	95.8	94.3	–			
6	92.1	91.8	93.3	90.9	91.2	–		
7	93.3	93.0	94.3	92.4	92.6	94.3	–	
8	92.2	92.3	92.7	90.1	90.9	91.4	92.6	–

phosphatidyl glycerol, diphosphatidyl glycerol and a characteristic glycolipid with an *R_f* value similar to that reported for the glycolipid from *Salinicoccus roseus* (Ventosa *et al.*, 1992). The lipid pattern from this group of organisms was, however, clearly different from all the other organisms examined. Strain NN^T, *B. salexigens* and *B. dipsosauri* all contained phosphatidyl glycerol, diphosphatidyl glycerol and two phospholipids of unknown structure as the major polar lipids. Phosphatidyl ethanolamine was not detected (Fig. 4). In addition to the major phospholipids, a number of minor components were detected in these strains. *B. dipsosauri* also produced small amounts of two glycolipids. The major polar lipids present in *V. pantothenicus* were phosphatidyl glycerol, diphosphatidyl glycerol and phosphatidyl ethanolamine, together with an aminophospholipid and a phospholipid. *V. pantothenicus* was the only organism studied which produced large amounts of a glycolipid (Fig. 4). A number of minor polar lipids were also present.

Fig. 3. Rooted phylogenetic tree showing the relationship of strain NN^T to members of the genus *Halobacillus*, *Marinococcus albus* and related organisms. Only those species which, on the basis of 16S rDNA similarity values, were most closely related to strain NN^T were included in the tree. The tree was rooted with *Bacillus subtilis*. Scale bar, 10% sequence divergence.

Examination of the respiratory lipoquinone content of all strains examined showed that menaquinones were the only respiratory lipoquinones detected, with MK-7 predominating in all strains. Although MK-7 was the major component, it was interesting to note that both *V. pantothenicus* and strain NN^T synthesized 5–15% MK-8 when grown at higher salinity.

Fatty acids

Due to the distinct nature of the polar lipids detected in members of the genus *Halobacillus* and *M. albus*, which separates these organisms from the other organisms investigated (see above), the fatty acid patterns of the former organisms were not examined. Since the strains examined could grow over a wide range of salinities, they were grown on both Bacto Marine Broth and 10% MH (moderate halophile) medium. The major fatty acids present in all strains were iso- and anteiso-fatty acids. Among these fatty acids anteiso-15:0 and anteiso-17:0 accounted for more than 40% of the fatty acids (see Table 3). In *B. dipsosauri* and *B. salexigens* iso-15:0 accounted for about 20–30% of the fatty acids. However, a number of differences could be seen in the distribution of the fatty acids present in smaller amounts, i.e. those accounting for less than 10% of the total.

Peptidoglycan type

The cell wall analysis revealed that strain NN^T showed the peptidoglycan type A1 γ (Schleifer & Kandler,

1972) (directly cross-linked *meso*-diaminopimelic acid).

DISCUSSION

Examination of the 16S rDNA sequence of strain NN^T indicated that the organism was a member of the phyletic group classically defined as the genus *Bacillus*, a feature consistent with the fact that it was an aerobic, Gram-positive, spore-forming rod. However, such a definition, which verges on a monothetic definition of the taxon, is now widely accepted as being in need of modification. This is particularly appropriate when one considers that members of the genus *Staphylococcus*, which are neither rod-shaped nor spore-forming, are clearly associated with this phyletic group. While the presence or absence of spores and the cellular morphology of the organism may be a useful indicator, in addition to other parameters, in defining a genus, it is obvious that a wider spectrum of parameters (both genotypic and phenotypic) is needed to provide a polythetic definition of genera in this phyletic assemblage (the classical 'genus *Bacillus* and its relatives') which reflects the evolutionary status quo within the group. To approach this problem and to provide additional data of potential taxonomic value, we have adopted a combined and integrated evaluation of the genotypic and phenotypic properties of the organisms studied. In addition to the placement of strain NN^T in the phyletic group of the 'genus *Bacillus* and its relatives', the 16S rDNA indicated that the greatest degree of similarity (above 93%) was shared between strain NN^T, *B. salexigens*, *B. dipsosauri*, *M. albus*, *Halobacillus litoralis* and *Halobacillus halophilus*. In view of the fact that *Halobacillus halophilus* was originally described as *Sporosarcina halophila*, the taxonomic placement of strain NN^T revolves around the questions of whether the creation of the genus *Halobacillus* is justified and whether strain NN^T and its relatives should be placed in the genus *Halobacillus*.

Chemotaxonomy

On the basis of the 16S rDNA data, chemotaxonomic studies were undertaken on those organisms which showed the greatest degree of 16S rDNA sequence similarity in order to test the hypothesis that they belonged within the same higher taxon.

The predominance of MK-7 in all the organisms examined does not contradict their association with 'Bacillus group 1' based on 16S rDNA analysis. However, since MK-7 is relatively widely distributed within the genus *Bacillus*, as well as in other major phyletic divisions, such as the 'Flavobacterium–Cytophaga–Bacteroides group' and the δ -subclass of the *Proteobacteria*, the presence of MK-7 in the organisms examined does not further elucidate their taxonomic position.

On the contrary, the polar lipid composition of the organisms examined showed that there were clearly a

Fig. 4. Polar lipid composition of (a) *Salibacillus salexigens*, (b) *Gracilibacillus halotolerans*, (c) *Gracilibacillus dipsosauri* and (d) *Virgibacillus pantothenicus* grown in 10% MH medium. The polar lipids were identified as follows: 1, diphosphatidyl glycerol; 2, phosphatidyl glycerol; 3, 4, 11 and 12, unidentified phospholipids; 5, phosphatidyl ethanolamine; 6, aminophospholipid; 7, phospholipid; 8, 9 and 10, glycolipids.

number of groupings, which correlated well with the 16S rDNA sequence data. The polar lipid composition indicated that *Halobacillus litoralis*, *Halobacillus trueperi*, *Halobacillus halophilus* and *M. albus* appear to form a monophyletic group. This group is clearly defined by the presence of phosphatidyl glycerol, diphosphatidyl glycerol, and a single glycolipid (see Results), a pattern not found in the other strains examined, suggestive of the fact that these four organisms constitute a distinct higher taxon. Although it would be tempting to transfer *M. albus* to the genus *Halobacillus*, we do not feel that sufficient data are

currently available to wholly ignore the fact that this species does not produce spores. The polar lipid composition of all the other strains examined was distinct from the phyletic group defined by the genus *Halobacillus* and allowed a number of quite clear divisions to be made. Strain NN^T and *B. dipsosauri* were the most similar based on the polar lipid patterns, while *B. salexigens* showed some similarity to these two species, although there were a number of differences (Fig. 4). The polar lipid pattern of *V. pantothenicus* was significantly different from all organisms examined, since it was the only species which contained

Table 3. Cellular fatty acid composition of strain NN^T, *Bacillus dipsosauri*, *Bacillus salexigens* and *Virgibacillus pantothenicus*

Figures given in the Table refer to the ratio of the fatty acid to the total fatty acids. Strain: 1, NN^T (*G. halotolerans* DSM 11805^T); 2, *B. dipsosauri* (*G. dipsosauri* DSM 11125^T); 3, *B. salexigens* (*S. salexigens* DSM 11483^T); 4, *V. pantothenicus* DSM 26^T. MB, Bacto Marine Broth; 10% MH, 10% moderate halophile medium (Garabito *et al.*, 1997); tr, trace amount.

Fatty acid	1		2		3		4	
	MB	10% MH	MB	10% MH	MB	10% MH	MB	10% MH
Iso-14:0	4.12	8.96	tr	tr	3.03	3.90	tr	1.67
14:0	tr	tr	tr	tr	tr	tr	tr	—
Iso-15:0	8.23	4.19	27.76	17.57	22.24	26.01	11.21	5.15
Anteiso-15:0	40.94	56.15	29.73	39.12	30.14	33.77	34.30	49.86
15:0	6.61	tr	2.84	tr	tr	tr	tr	—
Iso-16:0	7.12	7.05	3.27	4.67	8.13	11.22	4.82	9.44
16:1	2.98	tr	tr	—	—	—	—	—
16:0	14.90	9.05	16.35	7.21	19.45	1.67	9.73	—
Iso-17:0	—	—	6.75	7.25	5.41	6.15	7.63	1.97
Anteiso-17:0	9.91	14.59	13.29	24.19	11.60	17.29	30.81	31.91
Isomers of 18:1	5.18*	tr	—	—	—	—	—	—
18:0	tr	tr	tr	tr	tr	tr	1.50	—

* Sum of two different isomers which are resolved as two components (2.73 and 2.45 %).

phosphatidyl ethanolamine, together with a number of glycolipids.

The fatty acid patterns of the strains examined were typical of members of the phyletic group of the 'genus *Bacillus* and relatives', based on the fact that iso-15:0, anteiso-15:0 and anteiso-17:0 comprised the dominant fatty acids. However, as in the case of the polar lipids, a closer examination of the fatty acid composition indicated that there were differences which may be taken into account for taxonomic purposes. On the basis of the polar lipid data, it is possible to place each of the organisms studied into at least four different groups. One group comprises members of the genus *Halobacillus* and *M. albus*, another strain NN^T and *B. dipsosauri*, and two further groups comprising the single species *V. pantothenicus* and *B. salexigens*. Closer examination of the fatty acid data of *B. salexigens*, *B. dipsosauri*, *V. pantothenicus* and strain NN^T indicate that each of the four organisms are distinctive (Table 3). The interpretation of such data is made difficult by the fact that few organisms (i.e. species) in this phyletic group are available for study and those that are available show no more than 96% 16S rDNA sequence similarity. In such cases, the simple explanation that these differences in genotypic and phenotypic data are indicative of different taxonomic rank, is often discarded in favour of more complex evolutionary theory which cannot be easily substantiated. In the present paper, we favour the interpretation of the data in support of several new taxa.

Morphology and physiology

The placement of both strain NN^T and *B. dipsosauri* in a new genus, *Gracilibacillus*, is also supported by several shared morphological and physiological characters, e.g. the formation of thin rods and filaments, growth in media without NaCl and nitrate reduction (see Table 1). However, the two strains also exhibit clear differences. Strain NN^T, for instance, cannot grow anaerobically, but produces H₂S and urease supporting the genotypic and chemotaxonomic data in the separation of the strains in two distinguishable entities. An interesting physiological character is the extreme halotolerance exhibited by strain NN^T, while the other species are either halophilic or moderately halotolerant. Indeed, strain NN^T does not even require NaCl for optimal growth. Although there are several halotolerant bacteria which do not require NaCl for growth, among these *B. dipsosauri*, *V. pantothenicus* and species of the genus *Halomonas* (Vreeland *et al.*, 1980; Hebert & Vreeland, 1987; James *et al.*, 1990), they required at least 1% NaCl for optimal growth. Hence, strain NN^T is, to our knowledge, the first extremely halotolerant bacterium described, which does not require NaCl for optimal growth.

Taxonomy

On the basis of the genotypic and phenotypic data presented in this paper, it is proposed that strain NN^T be placed as the type strain of a new species in a new

genus, *Gracilibacillus halotolerans* gen. nov., sp. nov. It is also proposed that *B. dipsosauri* be transferred to this genus as *Gracilibacillus dipsosauri* comb. nov. In addition to the creation of the genus *Gracilibacillus*, we also propose that *Bacillus salexigens* be transferred to the genus *Salibacillus* gen. nov., as *Salibacillus salexigens* comb. nov.

During the course of this research the genus *Virgibacillus*, comprising the single species *Virgibacillus pantothenicus* (basonym *Bacillus pantothenicus*), was created (Heyndrickx *et al.*, 1998). We present further evidence, i.e. chemotaxonomic data, which supports the transfer of *B. pantothenicus* to *V. pantothenicus*.

Description of *Gracilibacillus* (Wainø, Tindall, Schumann and Ingvorsen) gen. nov.

Gracilibacillus (Gra.ci.li.ba.cil'lus. L. adj. *gracilis* slender; Gr. n. *baktron* rod; M.L. masc. *Gracilibacillus* the slender bacillus/rod).

Gram-positive, motile, spore-forming rods or filaments. Endospores are terminal and swell the sporangia. Colonies are circular. Chemo-organotrophic. Grows on glucose, mannitol and sucrose. Requires yeast extract for growth. Catalase- and oxidase-positive. Nitrate is reduced to nitrite. Voges-Proskauer test and indole production are negative. Hydrolyses gelatin, starch and aesculin. No hydrolysis of casein. Produces β -galactosidase. Does not produce arginine dihydrolase, ornithine decarboxylase or lysine decarboxylase. Cells are resistant to ampicillin but susceptible to chloramphenicol. The G + C content is 38–39 mol %. The major polar lipids are phosphatidyl glycerol, diphosphatidyl glycerol and two phospholipids of unknown structure. The major cellular fatty acid is anteiso-15:0. The main menaquinone type is MK-7. The peptidoglycan contains *meso*-diaminopimelic acid and is directly cross-linked (peptidoglycan type A1 γ). The type species of the genus is *Gracilibacillus halotolerans* sp. nov.

Description of *Gracilibacillus halotolerans* sp. nov.

Gracilibacillus halotolerans (ha.lo'to.le.rans. Gr. n. *hals* salt; L. adj. *tolerans* tolerating; M.L. adj. *halotolerans* salt-tolerating).

In addition to those characteristics given above for the genus, the following features are characteristic for *G. halotolerans*. Cells are rod-shaped, 0.4–0.6 by 2–5 μ m and motile by peritrichous flagella. Endospores are ellipsoid. Colonies are creamy white. Obligately aerobic and extremely halotolerant. Grows at 0–20 % (w/v) NaCl, with optimal growth at 0 % NaCl. Growth occurs at temperatures of 6–50 °C (optimum 47 °C). The pH range for growth is 5–10 (optimum about pH 7.5). H₂S is produced. Tween 80 and urea are hydrolysed. Produces alkaline phosphatase. Does not produce phenylalanine deaminase, chitinase or lecithinase. Cells are resistant to gentamicin, kanamycin, nalidixic acid, neomycin and tetracycline, but suscep-

tible to bacitracin, carbenicillin, erythromycin, novobiocin, penicillin and rifampicin. The following compounds (0.2 %, w/v) are utilized as growth substrates in Tris-medium (10 % NaCl): amylose, DL-arabinose, D-cellobiose, D-fructose, D-galactose, glycogen, inulin, lactose, maltose, D-mannose, D-melibiose, D-melizitolose, raffinose, L-rhamnose, starch, D-trehalose, D-xylose, glycerol, L-ascorbic acid, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, L-malate, oxoglutaric acid, N-acetylglucosamine, trimethylamine and Tween 80. No growth occurs on fucose, butanol, ethanol, methanol, pentanol, propanol, D-sorbitol, acetate, adipic acid, anisic acid, benzoate, butyrate, caproic acid, caprylate, citrate, formate, fumarate, glutaric acid, glycolate, glyoxylate, lactate, nicotinate, picolinic acid, propionate, pyruvate, succinate, valerate, L-alanine, L-arginine, L-aspartate, betaine, L-cysteine, L-glutamate, L-lysine, L-methionine, L-ornithine, L-phenylalanine, L-proline, L-serine, L-threonine, tryptophan, acetamide, benzamide, sulfanilamide, ethanolamine, methylamine, coconut oil, oil of cedar wood, acetone, cellulose or crab shell chitin. Does not ferment glucose or xylose. The G + C content is 38 mol %. The polar lipids are phosphatidyl glycerol, diphosphatidyl glycerol and two phospholipids of unknown structure. The type strain, strain NN^T, isolated from the Great Salt Lake, Utah, USA, has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) as strain DSM 11805^T.

Description of *Gracilibacillus dipsosauri* (Wainø, Tindall, Schumann and Ingvorsen) comb. nov. [basonym *Bacillus dipsosauri* (Lawson, Deutch and Collins 1996)]

The original description of the species is given by Lawson *et al.* (1996). Additional chemical characters found in this study are as follows. The major polar lipids are phosphatidyl glycerol, diphosphatidyl glycerol and two unknown phospholipids of unknown structure, together with two glycolipids. The major cellular fatty acids are iso-15:0, anteiso-15:0 and anteiso-17:0 and the main menaquinone type is MK-7. Type strain is strain DD1^T (= NCFB 3027^T = DSM 11125^T).

Description of *Salibacillus* (Wainø, Tindall, Schumann and Ingvorsen) gen. nov.

Salibacillus (Sal.i.ba.cil'lus. L. masc. *sal* salt; Gr. n. *baktron* rod; M.L. masc. *Salibacillus* the salt bacillus/rod).

The characteristics of the genus are those given by Garabito *et al.* (1997) for *Bacillus salexigens*. Additional chemical characters found in this study are as follows. The major polar lipids are phosphatidyl glycerol, diphosphatidyl glycerol and two phospholipids of unknown structure. The major cellular fatty acids are iso-15:0 and anteiso-15:0 and the main

Description of *Salibacillus salexigens* (Wainø, Tindall, Schumann and Ingvorsen) comb. nov. [basonym *Bacillus salexigens* (Garabito, Arahá, Mellado, Márquez and Ventosa 1997)]

Species description is as given above for the genus. Type strain is strain C-20Mo^T (= ATCC 700290^T = DSM 11483^T = CCM 4646^T).

Additional data of value in delineating the genus *Virgibacillus*

The characteristics of the genus are those given by Proom & Knight (1950) for *Bacillus pantothenicus* and by Heyndrickx *et al.* (1998) for *Virgibacillus pantothenicus*. Additional chemical characters found in this study are as follows. The polar lipids are phosphatidyl glycerol, diphosphatidyl glycerol, phosphatidyl ethanolamine and a glycolipid. The major cellular fatty acid are anteiso-15:0 and anteiso-17:0. The main menaquinone type is MK-7. The type species of the genus is *Virgibacillus pantothenicus*.

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