**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.**

Michael Wainø, B. J. Tindall, Peter Schumann and Kjeld Ingvorsen

**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 &
Well as in hypersaline areas, e.g. Bacillus halophile (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 diposauri (Lawson et al., 1996), Bacillus pantothenticus (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, 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, 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. diposauri could be placed in the same higher taxon as strain NN, and that the species B. salexigens and B. pantothenticus should be removed from the genus Bacillus. Based on the results presented, it is proposed that strain NN, be placed in the genus Gracilibacillus gen. nov., as Gracilibacillus halotolerans sp. nov. It is also proposed that B. diposauri be transferred to the genus Gracilibacillus as Gracilibacillus diposauri 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. pantothenticus to the genus Virgibacillus, as Virgibacillus pantothenticus (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; tryptase peptone (BBL), 2 g; trace metal solution (TMS 3), 2 ml (Ingversen & Jørgensen, 1984); KH2PO4 (50 g l-1), 10 ml; CaCl2, 2H2O (20 g l-1), 10 ml; FeCl3/MnCl2 solution [FeCl3, 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, 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; tryptase 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 FeCl3/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 for scanning electron microscopy was done according to the method of Paerl & Shimp (1973) modified as follows: a 20% (w/v) glutaraldehyde 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 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. 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) Na2SO4, 0.05% (w/v) NH4(2SO4) and 0.02% (w/v) NaH2PO4 as potential electron acceptors. Fermentation of glucose and xylene was tested by incubation in the same medium devoid of inorganic electron acceptors.

**Biochemical tests.** Presence of catalase, oxidase, lecinthinase, phenylalanine deaminase activity and H2S production were all determined according to API 20E (bio-mérieux). 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 NH3 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). Methy1 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; MgSO4.7H2O, 0·50; yeast extract, 0·25. Reduction of NO3 to NO2/N2 was tested in 10 ml test tubes containing anaerobic GSL-2 medium, 10 mM KNO3 and 0·2% (w/v) glucose, and inverted Durham tubes were used (Smibert & Krieg, 1981). Nitrite/nitrate was detected using the Griess-Llosay 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 AZCZ-amylase and Tween 80 agar plates, respectively, while hydrolysis of ascesulin 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 lipquinones and fatty acids included strain NNT (= DSM 11805T), B. pantothenticus DSM 26T, B. saleisogens, DSM 11483T, B. dipsoaui DSM 11125T, Marinococcus albus DSM 20748T. Halobacillus halophilus DSM 2266T, Halobacillus litoralis DSM 10405T and Halobacillus trueperi DSM 10404T. All strains were grown on Bacto Marine Broth and a 1:0.9 (by vol.) aqueous NaCl mixture to

Extraction of respiratory lipquinones and polar lipids. Respiratory lipquinones and polar lipids were extracted from 100 mg freeze-dried cell material using the two-stage method described by Tindall (1990a, b). Respiratory lipquinones were extracted using methanol/hexane (Tindall, 1990a, b) and the polar lipids were extracted by adjusting the remaining methanol/0.3% aqoues 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 pelletted 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 lipquinones. Respiratory lipquinones were separated into their different classes (maquisoquinones and ubiquinones) by TLC on silica gel (Macherey-Nagel) using hexane/tert-butylmethyl ether (9:1, v/v) as solvent. UV-absorbing bands corresponding to maquisoquinones 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 x 125 mm, 3 mm, RP<sub>18</sub>) using methanol as the eluant. Respiratory lipquinones 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 dodecamyloxybdiphosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin (free amino groups), periodate–Schiff (z-glycols), Dragendorff (quaternary nitrogen) and anisaldehyde–sulphuric acid (glyco-lipids).

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 x 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<sup>-1</sup>.

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<sup>T</sup>, 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. saleisogens DSM 11483T, Y1603; Bacillus subtilis NCDO 1769<sup>T</sup>, X60646; B. pantothenticus NCDO 1765<sup>T</sup>, X60627; B. dipsoaui DSM 11125<sup>T</sup>, X82436; Halobacillus halophilus (basonym Sporosarcina halophilia) NCIMB 9251<sup>T</sup>, X62174; Halobacillus litoralis DSM 10405<sup>T</sup>, X94558; and M. albus DSM 20748<sup>T</sup>, X90834.

The G + C content of DNA from strain NN<sup>T</sup> 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<sup>T</sup> 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
M. Wainø and others

**Fig. 1.** Scanning electron micrograph of strain NN\(^\text{T}\), showing the presence of both rod-shaped and filamentous bacteria. Bar, 1 μm.

**Fig. 2.** Influence of salinity on growth of strain NN\(^\text{T}\) 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\(^\text{T}\) 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\(^\text{T}\) 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\(^\text{T}\) did not require NaCl for growth; the highest cell yields were obtained in salt-free medium, even after six consecutive transfers. Strain NN\(^\text{T}\) 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\(^\text{T}\) 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), carbencillin (115 mg), chloramphenicol (60 mg), erythromycin (78 mg), novobiocin (100 mg), penicillin (5 mg) and rifampicin (30 mg) inhibited growth of strain NN\(^\text{T}\). 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\(^\text{T}\) 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\(^\text{T}\) 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\(^\text{T}\) 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\(^\text{T}\) 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. pantothenticus*. Among these organisms, the greatest degree of sequence similarity was observed between strain NN\(^\text{T}\) and *B. dipsosauri* (96% sequence similarity). A rooted phylogenetic tree in Fig. 3 shows the relationship of strain NN\(^\text{T}\) 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
**Gracilibacillus** and **Salibacillus** gen. nov.

**Table 1.** Morphological and physiological characteristics of strain NN¹, **Bacillus dipsosauri**, **Bacillus salexigens**, **Halobacillus halophilus** and **Virgibacillus pantothenticus**

<table>
<thead>
<tr>
<th>Character</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology</td>
<td>Thin rods &amp; filaments</td>
<td>Thin rods &amp; filaments</td>
<td>Rods, single or in chains</td>
<td>Rods</td>
<td>Rods</td>
</tr>
<tr>
<td>Pigmentation</td>
<td>Creamy white</td>
<td>Ellipsoid</td>
<td>Non-pigmented Oval</td>
<td>Non-pigmented Ellipsoid</td>
<td>Greyish white Ellipsoid or spherical Terminal</td>
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<td>Spherical</td>
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<td>-</td>
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<td>+</td>
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</table>

* 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 NN¹ and related taxa

<table>
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<td>96.0</td>
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<td>90.9</td>
<td>91.4</td>
<td>92.6</td>
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</table>

Phosphatidyl glycerol, diphosphatidyl glycerol and a characteristic glycolipid with an R, 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 NN¹, *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. pantothenticus* were phosphatidyl glycerol, diphosphatidyl glycerol and phosphatidyl ethanolamine, together with an aminophospholipid and a phospholipid. *V. pantothenticus* was the only organism studied which produced large amounts of a glycolipid (Fig. 4). A number of minor polar lipids were also present.
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. pantothenticus* 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 A1γ (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
Gracilibacillus and Salibacillus gen. nov.

Fig. 4. Polar lipid composition of (a) Salibacillus salexigens, (b) Gracilibacillus halotolerans, (c) Gracilibacillus dipsosauri and (d) Virgibacillus pantothenticus 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 phylectic group defined by the genus Halobacillus and allowed a number of quite clear divisions to be made. Strain NN 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. pantothenticus was significantly different from all organisms examined, since it was the only species which contained...
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\textsuperscript{T} and B. dipsosauri, and two further groups comprising the single species V. pantothenticus and B. salexigens. Closer examination of the fatty acid data of B. salexigens, B. dipsosauri, V. pantothenticus and strain NN\textsuperscript{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\textsuperscript{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\textsuperscript{T}, for instance, cannot grow anaerobically, but produces H\textsubscript{2}S 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\textsuperscript{T}, while the other species are either halophilic or moderately halotolerant. Indeed, strain NN\textsuperscript{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. pantothenticus 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\textsuperscript{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\textsuperscript{T} be placed as the type strain of a new species in a new

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**Table 3. Cellular fatty acid composition of strain NN\textsuperscript{T}, Bacillus dipsosauri, Bacillus salexigens and Virgibacillus pantothenticus**

Figures given in the Table refer to the ratio of the fatty acid to the total fatty acids. Strain: 1, NN\textsuperscript{T} (G. halotolerans DSM 11805\textsuperscript{T}); 2, B. dipsosauri (G. dipsosauri DSM 11125\textsuperscript{T}); 3, B. salexigens (S. salexigens DSM 11453\textsuperscript{T}); 4, V. pantothenticus DSM 26\textsuperscript{T}. MB, Bacto Marine Broth; 10\% MH, 10% moderate halophile medium (Garabito et al., 1997); tr, trace amount.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>MB 10% MH</td>
<td>MB 10% MH</td>
<td>MB 10% MH</td>
<td>MB 10% MH</td>
</tr>
<tr>
<td>Iso-14:0</td>
<td>4.12 8.96</td>
<td>tr tr</td>
<td>3.03 3.90</td>
<td>tr 1.67</td>
</tr>
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<td>14:0</td>
<td>tr tr</td>
<td>tr tr</td>
<td>tr tr</td>
<td>tr –</td>
</tr>
<tr>
<td>Iso-15:0</td>
<td>8.23 4.19</td>
<td>27 76</td>
<td>17 57</td>
<td>22 24 26.01</td>
</tr>
<tr>
<td>Anteiso-15:0</td>
<td>40.94 56.15</td>
<td>29 73</td>
<td>39 12</td>
<td>30 14 33 77</td>
</tr>
<tr>
<td>15:0</td>
<td>6 61 tr</td>
<td>2 84 tr</td>
<td>tr tr</td>
<td>tr tr</td>
</tr>
<tr>
<td>Iso-16:0</td>
<td>7 12 7 05</td>
<td>3 27 4 67</td>
<td>8 13 11 22</td>
<td>4 82 9 44</td>
</tr>
<tr>
<td>16:1</td>
<td>2 98 tr</td>
<td>– –</td>
<td>– –</td>
<td>– – –</td>
</tr>
<tr>
<td>16:0</td>
<td>14 90 9 05</td>
<td>16 35 7 21</td>
<td>19 45 1 67</td>
<td>9 73 –</td>
</tr>
<tr>
<td>Iso-17:0</td>
<td>– –</td>
<td>6 75 7 25</td>
<td>5 41 6 15</td>
<td>7 63 1 97</td>
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<td>Anteiso-17:0</td>
<td>9.91 14.59</td>
<td>13 29 24 19</td>
<td>11 60 17 29</td>
<td>30 81 31 91</td>
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<tr>
<td>Isomers of 18:1</td>
<td>5 18* tr</td>
<td>– –</td>
<td>– –</td>
<td>– – – –</td>
</tr>
<tr>
<td>18:0</td>
<td>tr tr</td>
<td>tr tr</td>
<td>tr tr</td>
<td>1 50 –</td>
</tr>
</tbody>
</table>

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

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 pantothenticus (basonym Bacillus pantothenticus), was created (Heyndrickx et al., 1998). We present further evidence, i.e. chemotaxonomic data, which supports the transfer of B. pantothenticus to V. pantothenticus.

Description of Gracilibacillus (Waino, 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. Endospores 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 A17). 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 μm 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 susceptible 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): amylase, dt-arabinose, D-cellobiose, D-fructose, D-galactose, glycogen, inulin, lactose, maltose, D-mannose, D-melibiose, D-melibiose, raffinose, stach, D-trehalose, D-xylene, glycerol, L-ascorbic acid, D-galacturonic acid, D-glucuronic acid, D-glucuronic acid, l-malate, oxoglutaric acid, N-acetylglucosamine, trimethylamine and Tween 80. No growth occurs on fucose, butanol, ethanol, methanol, propanol, D-sorbitol, acetate, adipic acid, anisic acid, benzoxate, 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-threonyne, tryptophan, acetamide, benzamide, sulfinilamide, 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 NN₇, isolated from the Great Salt Lake, Utah, USA, has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) as strain DSM 11805T.

Description of Gracilibacillus dipsosauri (Waino, 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 DD1T (= NCFB 3027T = DSM 11255T).

Description of Salibacillus (Waino, 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, Arahal, Mellado, Márquez and Ventosa 1997)]

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

Additional data of value in delineating the genus *Virgibacillus*

The characteristics of the genus are those given by Proom & Knight (1950) for *Bacillus pantothenticus* and by Heyndrickx et al. (1998) for *Virgibacillus pantothenticus*. 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 pantothenticus*.

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REFERENCES


