Transfer of *Bacillus halodenitrificans* Denariáz *et al.* 1989 to the genus *Virgibacillus* as *Virgibacillus halodenitrificans* comb. nov.

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A Gram-variable, endospore-forming moderately halophilic rod, strain SF-121, was isolated from a marine solar saltern of the Yellow Sea in Korea. The result of 16S rRNA gene sequence analysis showed that strain SF-121 has highest sequence similarity (99.7%) with the type strain of *Bacillus halodenitrificans*. Phylogenetic analyses based on 16S rRNA gene sequences revealed that *B. halodenitrificans* DSM 10037^T and strain SF-121 are more closely related to the genus *Virgibacillus* than to the genus *Bacillus*. Strain SF-121 and *B. halodenitrificans* DSM 10037^T exhibited 16S rRNA gene similarity levels of 95.3–97.5% with the type strains of *Virgibacillus* species and 94.0% with the type strain of *Bacillus subtilis*. DNA–DNA relatedness and phenotypic data indicated that *B. halodenitrificans* DSM 10037^T and strain SF-121 are members of the same species. *B. halodenitrificans* DSM 10037^T and strain SF-121 exhibited DNA–DNA relatedness values of 9–11% with the type strains of *Virgibacillus carmonensis* and *Virgibacillus marismortui*. On the basis of the phenotypic, chemotaxonomic, phylogenetic and genetic data, *B. halodenitrificans* should be reclassified in the genus *Virgibacillus* as *Virgibacillus halodenitrificans* comb. nov.

Innovations in the study of bacterial systematics have aided the reclassification of some bacilli into newly described genera or other related genera (Wisotzkey *et al.*, 1992; Ash *et al.*, 1993; Shida *et al.*, 1996; Heyndrickx *et al.*, 1998; Wainø *et al.*, 1999; Yoon *et al.*, 2001). The genus *Virgibacillus* was first proposed by Heyndrickx *et al.* (1998) for *Bacillus pantothenicus*. Subsequently, a second species, *Virgibacillus proomii*, was included in the genus (Heyndrickx *et al.*, 1999). The genus *Salibacillus* was proposed for a single species assigned to the genus *Bacillus*, *Bacillus salexigens* (Wainø *et al.*, 1999), and *Bacillus marismortui* was later transferred to the genus *Salibacillus* (Arahal *et al.*, 2000). However, Heyrman *et al.* (2003) proposed to combine the two genera *Virgibacillus* and *Salibacillus* in a single genus. According to the Rules of the Bacteriological Code, the two *Salibacillus* species were transferred to the genus *Virgibacillus*. In addition, Heyrman *et al.* (2003) proposed three novel *Virgibacillus* species, *Virgibacillus carmonensis*, *Virgibacillus*

necropolis and *Virgibacillus picturae*. The genus *Virgibacillus* comprises these seven species with validly described names at the time of writing. Recently, a moderately halophilic bacterial strain, SF-121, was isolated from a marine solar saltern of the Yellow Sea in Korea. This organism was found to have closest phylogenetic affiliation to *Bacillus halodenitrificans* from the results of 16S rRNA gene sequence analysis. However, strain SF-121 and *B. halodenitrificans* were found to be phylogenetically more closely related to the genus *Virgibacillus* than to the genus *Bacillus*. Accordingly, the aim of the present study was to establish the exact taxonomic positions of strain SF-121 and *B. halodenitrificans* using a combination of phenotypic properties, phylogenetic analysis based on 16S rRNA gene sequences and genotypic relatedness.

Strain SF-121 was isolated by the usual dilution plating technique on marine agar 2216 (MA; Difco) at 30 °C. *B. halodenitrificans* DSM 10037^T, *Virgibacillus marismortui* DSM 12325^T and *V. carmonensis* DSM 14868^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. Cell morphology was examined by light microscopy (Nikon E600) and transmission electron microscopy. Flagellum type was examined by transmission electron

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strain SF-121 and *Bacillus halodenitrificans* DSM 10037^T are AY543168 and AY543169, respectively.

microscopy using cells from exponentially growing cultures. Gram reaction was determined using the bioMérieux Gram Stain kit according to the manufacturer's instructions. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on MA and MA supplemented with nitrate that had been prepared anaerobically. The pH range for growth was determined in marine broth (MB; Difco) adjusted to pH values 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5. Growth at various NaCl concentrations was investigated in MB or trypticase soy broth (Difco). Growth in the absence of NaCl was investigated in trypticase soy broth without NaCl. Growth at various temperatures (4–50 °C) was measured on MA. Oxidase activity was determined by oxidation of 1 % *p*-aminodimethylaniline oxalate. Catalase activity was determined by bubble production in a 3 % (v/v) hydrogen peroxide solution. Hydrolysis of aesculin, Voges–Proskauer and methyl red reactions and nitrate reduction were determined as described by Lanyi (1987). Hydrolysis of casein, starch and Tween 80 and urease activity were determined as described by Cowan & Steel (1965). Hydrolysis of hypoxanthine, tyrosine and xanthine was examined on MA with the substrate concentrations described previously (Cowan & Steel, 1965). The API ZYM system (bioMérieux) was used to determine enzyme activity. Acid production from carbohydrates was determined as described by Leifson (1963). Utilization of substrates as sole carbon and energy sources was tested according to the method of Baumann & Baumann (1981) supplemented with 2 % (v/v) Hutner's mineral base (Cohen-Bazire *et al.*, 1957) and 1 % (v/v) vitamin solution (Staley, 1968).

Cell mass for analysis of the cell wall, menaquinones and polar lipids and for DNA extraction was produced in MB at 37 °C. For fatty acid methyl ester analysis, cell mass of strain SF-121 and *B. halodenitrificans* DSM 10037^T was obtained from agar plates after cultivation for 2 days at 37 °C on MA. The isomer type of diaminopimelic acid in the peptidoglycan was determined by the method described by Komagata & Suzuki (1987). Menaquinones were analysed as described previously (Komagata & Suzuki, 1987) using reverse-phase HPLC. Polar lipids were extracted using the procedures described by Minnikin *et al.* (1984) and identified by two-dimensional TLC followed by spraying with appropriate detection reagents (Komagata & Suzuki, 1987). For quantitative analysis of cellular fatty acid compositions, a loop of cell mass was harvested and fatty acid methyl esters were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). Chromosomal DNA was isolated and purified according to the method described previously (Yoon *et al.*, 1996), with the exception that ribonuclease T1 was used together with ribonuclease A. The DNA G+C content was determined by the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reverse-phase HPLC. The 16S rRNA gene was amplified by PCR using two universal primers as described previously

(Yoon *et al.*, 1998). The PCR product was purified with a QIAquick PCR Purification kit (Qiagen). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis was performed as described previously (Yoon *et al.*, 2003). DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained in each sample were excluded and the mean of the remaining three values was taken as the relatedness value.

Strain SF-121 was a Gram-variable rod, approximately 0.6–0.8 × 2.0–4.0 µm after cultivation for 3 days at 37 °C on MA. It was motile by means of a single polar flagellum. Terminal or subterminal ellipsoidal spores were observed in swollen sporangia. Colonies of strain SF-121 were circular to irregular, raised, translucent, cream in colour and 2–3 mm after incubation for 3 days at 37 °C on MA. It grew at 10 and 45 °C with an optimum temperature of 35–40 °C; it did not grow at 4 or 50 °C. Optimal growth was at pH 7.5; it grew at pH 5.0 but not at pH 4.5. Strain SF-121 grew in the presence of 0–23 % NaCl with an optimum of 2–7 % NaCl. It grew anaerobically on MA only when nitrate was present. Strain SF-121 showed catalase and oxidase activities but no urease activity. Hypoxanthine, starch, Tween 80, tyrosine and xanthine were not hydrolysed. It reduced nitrate to nitrite. Strain SF-121 contained *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. The predominant menaquinone found in strain SF-121 was menaquinone-7 (MK-7). Strain SF-121 had a cellular fatty acid profile that consisted mostly of branched fatty acids; the major fatty acids were anteiso-C_{15:0} (54.1 %) and anteiso-C_{17:0} (32.0 %). This profile was most similar to that of *B. halodenitrificans* DSM 10037^T; the major fatty acids detected in *B. halodenitrificans* DSM 10037^T were anteiso-C_{15:0} (56.3 %), anteiso-C_{17:0} (25.5 %) and iso-C_{16:0} (8.7 %). The cellular polar lipids found in strain SF-121 were phosphatidylglycerol and diphosphatidylglycerol. The DNA G+C content of strain SF-121 was 39 mol%. Other phenotypic characteristics are shown in Table 1 or are given in the species description below.

The 16S rRNA gene sequences of strain SF-121 and *B. halodenitrificans* DSM 10037^T determined in this study comprised 1521 nt, approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. The 16S rRNA gene sequences of strain SF-121 and *B. halodenitrificans* DSM 10037^T were 99.7 % similar (5 nt differences). The phylogenetic tree based on the neighbour-joining algorithm showed that strain SF-121 and *B. halodenitrificans* DSM 10037^T fell within the radiation of the cluster comprising *Virgibacillus* species, not *Bacillus* species (Fig. 1). Similar tree topology was also found in the tree generated by maximum parsimony (data not shown). Strain SF-121 and *B. halodenitrificans* DSM 10037^T exhibited highest 16S rRNA gene similarity values (97.2–97.5 %) with the type strains of *V. carmonensis* and *V. marismortui*, and 16S rRNA gene

Table 1. Phenotypic characteristics of *Virgibacillus* species and *B. halodenitrificans*

1, *Virgibacillus pantothenicus*; 2, *V. proomii*; 3, *Virgibacillus salexigens*; 4, *V. marismortui*; 5, *V. carmonensis*; 6, *V. necropolis*; 7, *V. picturae*; 8, *B. halodenitrificans*. +, Positive; –, negative; w, weakly positive; v, variable; ND, not determined. Data in columns 1–7 are from Arahall *et al.* (1999, 2000), Heyndrickx *et al.* (1999), Wainø *et al.* (1999) and Heyrman *et al.* (2003). Data in parentheses are for the type strain. All species are rods, motile, positive for catalase and negative for growth on D-arabinose and D-xylose.

Character	1	2	3	4	5	6	7	8
Spore shape*	S/E	S/E	E	E	E/S	E	E/S	E†
Spore position‡	T/ST	T/ST	C/ST/T	T/ST	ST	C/ST/T	T	T/ST†
Gram stain	+	+	+	+	+	+	+	v
Anaerobic growth	+	+	–	–	–	–	–	+
Growth in the presence of:								
0.5 % NaCl	+	w	–	–	–	w	w	v (–)
25 % NaCl	–	–	–	–	–	–	–	v (+)
Temperature range (°C)	15–50	15–50	15–45	15–50	10–40	10–40	5–40	10–45
Nitrate reduction	v	–	–	+	+	+	+	+
H ₂ S production	v	–	+	–	–	–	–	–
Hydrolysis of:								
Aesculin	+	+	+	+	w	–	w	v (–)
Casein	+	+	+	+	+	+	w	+
Gelatin	+	v	+	+	–	w	v	+
Growth on:								
D-Glucose	w	w	w	–	–	+	v	+
Lactose	–	w	+	+	–	w	w	+
D-Fructose	–	–	–	–	–	–	–	v (+)
Sucrose	–	+	+	+	+	+	v	+
Acid production from:								
D-Galactose	–	+	w	–	–	–	w	+
D-Glucose	–	+	w	+	–	w	w	+
D-Fructose	–	+	w	+	–	w	w	+
D-Mannose	–	+	w	+	–	w	w	+
D-Melibiose	–	–	–	–	–	–	w	–
L-Rhamnose	+	v	–	–	–	–	–	–
D-Trehalose	+	+	–	–	–	w	v	+
D-Mannitol	–	–	–	–	–	–	w	v (+)
Polar lipids§	PG, DPG, PE	PG, DPG, PE	PG, DPG	PG, DPG, PE	PG, DPG	PG, DPG	PG, DPG	PG, DPG
DNA G + C (mol%)	36.9–38.3	36.8–37.0	36.3–39.5	39.0–42.8	38.9	37.3	39.5–40.0	38.0–39.0

*E, Ellipsoidal; s, spherical.

†Data for strain SF-121.

‡C, Central; ST, subterminal; T, terminal.

§DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

similarity values of 95.3–96.7 % with the type strains of other *Virgibacillus* species. Levels of 16S rRNA gene similarity between strain SF-121 and *B. halodenitrificans* DSM 10037^T and other species used in the phylogenetic analysis were lower than 95.3 %.

Strain SF-121 and *B. halodenitrificans* were found to have similar phenotypic properties (Table 1). The mean value of DNA–DNA relatedness between strain SF-121 and *B. halodenitrificans* DSM 10037^T was 87 % when their DNA was used individually as a labelled DNA probe for cross-hybridization. This value indicated that they are members of the same genomic species (Wayne *et al.*, 1987). Phylogenetic

analyses based on 16S rRNA gene sequences indicated that *B. halodenitrificans* is more phylogenetically affiliated to the genus *Virgibacillus* than to the genus *Bacillus* (Fig. 1). The predominant menaquinone and fatty acid profiles of *B. halodenitrificans* were similar to those of the genus *Virgibacillus*, although there are few clear-cut differences in chemotaxonomic characteristics between the genera *Bacillus* and *Virgibacillus* (Spring *et al.*, 1996; Wainø *et al.*, 1999; Yoon *et al.*, 2002; Heyrman *et al.*, 2003). *B. halodenitrificans* is also phylogenetically related to the genera *Lentibacillus* and *Oceanobacillus*. However, *B. halodenitrificans* and the genus *Virgibacillus* are clearly differentiated from the genus *Lentibacillus* by differences in fatty acid

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequence data showing the phylogenetic positions of *B. halodenitrificans* DSM 10037^T and strain SF-121, *Virgibacillus* species and some other related taxa. Bootstrap values (1000 replications) are shown as percentages at each node only if they are 50% or greater. Bar, 0.01 substitutions per nucleotide position.

profiles, particularly in the proportion of the fatty acid iso-C_{16:0} (Yoon *et al.*, 2002; Heyrman *et al.*, 2003). In the neighbour-joining phylogenetic tree, the genus *Oceanobacillus* joined the type strain of *V. picturae* (Fig. 1). There are not enough phenotypic, particularly chemotaxonomic, differences between *Virgibacillus* and *Oceanobacillus* (Lu *et al.*, 2001; Heyrman *et al.*, 2003). Accordingly, the taxonomic relationship between *Virgibacillus* and *Oceanobacillus* may have to be re-evaluated. Both phylogenetic and phenotypic data indicated that *B. halodenitrificans* is a member of the genus *Virgibacillus* rather than the genus *Bacillus*. *B. halodenitrificans* DSM 10037^T and strain SF-121 were differentiated from the type strains of *V. carmonensis* and *V. marismortui* by DNA–DNA relatedness values of 9–11% and from other *Virgibacillus* species by 16S rRNA gene similarity levels of <96.7% (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). *B. halodenitrificans* was also differentiated from other *Virgibacillus* species by some phenotypic properties (Table 1). Therefore, on the basis of the data presented, it is proposed that *B. halodenitrificans* Denariet *et al.* 1989 be transferred to the genus *Virgibacillus* as *Virgibacillus halodenitrificans* comb. nov.

Description of *Virgibacillus halodenitrificans* (Denariet *et al.* 1989) comb. nov.

Virgibacillus halodenitrificans [ha.lo.de.ni.tri'fi.cans. Gr. n. hals salt; L. v. denitrifico to denitrify; N.L. part. adj. halodenitrificans salt (-requiring) denitrifying].

Basonym: *Bacillus halodenitrificans* Denariet *et al.* 1989.

Cells are rods, 0.6–0.8 × 2.5–4.0 μm. Gram variable. Motile by means of single or peritrichous flagella. Terminal or subterminal ellipsoidal spores are observed in swollen

sporangia (observed only in strain SF-121). Colonies are circular to slightly irregular, raised, translucent, cream coloured and 2–3 mm after incubation for 3 days on MA. Growth occurs between 10 and 45 °C with an optimum of 35–40 °C (38 °C for the type strain). Optimal pH for growth is around pH 7.4–7.5. The pH range for growth is between 5.8 and 9.6 (for the type strain). Growth occurs in the presence of 2–23% NaCl (2–25% for the type strain); optimal growth occurs in the presence of 3–7% NaCl. Growth occurs under anaerobic conditions on MA only in the presence of nitrate. Oxidase positive. Urease negative. Starch, Tween 80 and tyrosine are not hydrolysed. Voges–Proskauer and methyl red reactions are negative. Indole is not produced. H₂S is not produced. Nitrate is reduced to nitrite. Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are absent. When assayed with the API ZYM system, alkaline phosphatase, esterase (C₄), esterase lipase (C₈), naphthol-AS-BI-phosphohydrolase and α-glucosidase are present, but lipase (C₁₄), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase are absent. β-Galactosidase and N-acetyl-β-glucosaminidase are variable (positive for the type strain). Acid is produced from maltose, D-ribose and sucrose. Acid is not produced from L-arabinose, D-cellobiose, D-melezitose, D-raffinose, D-xylose, adonitol, myo-inositol or D-sorbitol. Acid production from lactose is variable. The cell wall peptidoglycan contains meso-diaminopimelic acid. The predominant menaquinone is MK-7. The major fatty acids are anteiso-C_{15:0} and anteiso-C_{17:0}. The G + C content is 38–39 mol% (38 mol% for the type strain).

The type strain, ATCC 49067^T (= DSM 10037^T = LMG 9818^T), was isolated from a solar evaporation pond (saltern) in France. Further descriptive information for the type strain is given by Denariet *et al.* (1989) and in Table 1.

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