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Bacillus sp. WW3-SN6, a novel facultatively alkaliphilic bacterium isolated from the washwaters of edible olives

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Abstract A novel Gram-positive facultatively alkaliphilic, sporulating, rod-shaped bacterium, designated as WW3-SN6, has been isolated from the alkaline washwaters derived from the preparation of edible olives. The bacterium is nonmotile, and flagella are not observed. It is oxidase positive and catalase negative. The facultative alkaliphile grows from pH 7.0 to 10.5, with a broad optimum from pH 8.0 to 9.0. It could grow in up to 15% (w/v) NaCl, and over the temperature range from 4° to 37°C, with an optimum between 27° and 32°C: therefore, it is both halotolerant and psychrotolerant. The bacterium is sensitive to a range of β-lactam, sulfonamide, and aminoglycoside antibiotics, but resistant to trimethoprim. The range of amino acids, sugars, and polyols utilized as growth substrates indicates that this alkaliphile is a heterotrophic bacterium. D(+)-glucose, D(+)-glucose-6-phosphate, D(+)-cellobiose, starch, or sucrose are the substrates best utilized. The major membrane lipids are phosphatidylglycerol and diphosphatidylglycerol, with smaller amounts of phosphatidylethanolamine and an unknown phospholipid. During growth at high pH, the proportion of phosphatidylglycerol is increased relative to phosphatidylethanolamine. The fatty acyl components in the membrane phospholipids are mainly branched chain, with 13-methyl tetradecanoic and 12-methyl tetradecanoic acids as the predominant components. The G + C content of the genomic DNA is 41.1 ± 1.0 mol%. The results of 16*S* ribosomal RNA sequence analysis place this alkaliphilic bacterium in a cluster, together with an unnamed alkaliphilic *Bacillus* species (98.2% similarity).

Key words Olive wastes · *Bacillus* · Alkaliphile · Growth characteristics · Lipid composition · Phylogeny · Adaptation

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Introduction

Two categories of bacteria are capable of growing at high pH. Those that generally have an optimum pH for growth of approximately 10 are known as alkaliphiles and include representatives such as *Tindallia magadii*, *Bacillus alcalophilus*, and *Bacillus haloalkaliphilus* (Vedder 1934; Fritze 1996; Duckworth et al. 1996; Kevbrin et al. 1998). A second group, known as alkalitolerants, can tolerate alkaline pH conditions but their optimal growth is at neutrality or just above; these include organisms such as *Synechococcus* sp. (Kroll 1990). The alkaliphiles can be also divided into facultative alkaliphiles, which have an optimum at pH 10.0 or above, but also possess the ability to grow at neutral pH; and obligate alkaliphiles, which grow optimally at pH approximately 10 but are unable to grow below pH 8.5 to 9.0 (Krulwich and Guffanti 1989).

Alkaliphilic bacteria present a wide biodiversity (Horikoshi 1991). They include bacteria such as *Bacillus* spp., which are the main representatives in this category (Duckworth et al. 1996), phototrophic cyanobacteria, purple sulfur bacteria, and Archaea, e.g., *Natronococcus* spp. Alkaliphiles are generally aerobic or facultatively anaerobic, but do include some anaerobes such as *Clostridium* and *Methanobacterium* spp. They can also be divided into psychroalkaliphiles (e.g., *Micrococcus* sp. 207; Kimura and Horikoshi 1988, 1989), mesoalkaliphiles (e.g., *Bacillus firmus* OF4; Guffanti et al. 1986), thermoalkaliphiles (e.g., *Bacillus* sp. TAR-1; Nakamura et al. 1994), haloalkaliphiles (e.g., *Natronococcus occultus* and *Bacillus haloalkaliphilus*; Tindall et al. 1984; Fritze 1996), and haloalkaliphilic methanogens (e.g., *Methanolobus taylorii* GS-16; Ni et al. 1994). The diversity of alkaliphiles is further illustrated by the fact that there are examples of sulfate reducers such as *Desulfonatronovibrio hydrogenovorans* (Zhilina et al. 1997), denitrifiers such as *Halomonas desiderata* (Berendes et al. 1996), and those that produce ethylene, e.g., *Bacillus* sp. ALK-7 (Bae and Kim 1997), examples of alkaliphilic sulfate-reducing, denitrifying, and ethylene-producing bacteria, respectively.

High numbers of alkaliphiles are found in saline alkaline environments such as soda lakes and ponds, such as Lakes Magadi and Nakuru in Kenya, Crimea saline basins in Ukraine, and soda Tuva Lakes (Grant and Tindall 1986; Zhilina and Zavarzin 1994; Khmelenina et al. 1997; Jones et al. 1998). Alkaphiles are also found in Ca^{2+} alkaline environments such as the $Ca(OH)_2$ springs of Oman, and in alkaline springs and soda deserts (Grant and Tindall 1986; Jones et al. 1994, 1998).

Marine environments contain many alkalitolerant bacteria and usually a small number of alkaliphiles, particularly those that are related to the genus *Bacillus* (Duckworth et al. 1996). A few alkaliphilic bacteria isolated from seawater are related to *Pseudomonas* and *Vibrio* spp. (Maeda and Taga 1980), and some alkaliphilic strains related to the actinomycetes group have been isolated from the Mariana Trench at a depth of 10897 m (Takami et al. 1997). Most of the extreme alkaliphiles found in soil are *Bacillus* species, but only a few of these species appear to be present in highly alkaline saline environments as compared to soil samples (Weisser and Truper 1985; Grant and Tindall 1986).

Alkaliphiles have also been isolated from a number of alkaline wastes formed as by-products of food-processing industries. For example, *Exiguobacterium aurantiacum* has been isolated from potato processing plant effluents (Collins et al. 1983) and a *Bacillus* sp. from edible olive wastewaters, as reported in the present paper. The production of edible olives generally involves an initial stage in which NaOH is used to precipitate a bitter compound of the fruit (Jimenez Araujo et al. 1994; Marsilio et al. 1996). The fresh fruits are placed in specially formed tanks containing sodium hydroxide solutions ("lyes") with concentrations ranging from 1% to 3% (w/v), which penetrate the skin, the flesh, and sometimes the pit, and darken the color of the olives. After a few days the olives are rinsed in water to remove residual sodium hydroxide. Combined with the lyes, these washings produce millions of liters of highly alkaline (pH = 12–13) wastewaters in the Mediterranean region during November and December (Kopsidas 1992).

The disposal of such highly alkaline edible olive washwaters (EWW) pollutes aquatic and terrestrial environments (Kopsidas 1992), particularly in the Mediterranean region (Andrich et al. 1992), causes phytotoxic effects, and has antimicrobial activity because of the polyphenols that are concentrated in the EWW (Saez et al. 1992). However, there is the potential for bioremediation of EWW to make them useful as a fertilizer and growth promoter for commercial crops. This article gives information on the phenotypic and genotypic characteristics of a novel facultatively alkaliphilic bacterium that was isolated from the washwaters of edible olives during an investigation of bacteria capable of the bioremediation of EWW and for the production of novel enzymes. We show that this alkaliphilic *Bacillus* sp. is a novel lineage within the genus, and present data in relation to its adaptive mechanisms for growing in the highly alkaline environment of EWW.

Materials and methods

The facultatively alkaliphilic bacterium was isolated from a sample of fresh EWW provided by Professor C. Balis, Harokopio University, Athens, Greece. The sample had a pH at collection of approximately 13.5, as judged by pH meter and pH paper; this value is likely to be inaccurate because of the difficulty of measuring pH at such high values, but what is not in doubt is that the sample was highly alkaline. Isolation of bacteria involved dilution plating on a solid medium consisting of diluted EWW (50%, v/v, aq.) containing 2% (w/v) bacteriological agar (Oxoid). Single colonies were replated on fresh plates of the same medium to obtain pure isolates. Cultures were maintained and grown routinely using an alkaliphilic medium composed of 0.05 M sodium L-glutamate (Sigma), 0.5% (w/v) yeast extract (Oxoid), and a buffer (Na_2CO_3-K_2HPO_4 or $NaHCO_3$-K_2HPO_4 or Na_2HPO_4-NaH_2PO_4) containing 0.1% (w/v) NH_4SO_4 plus 0.1 mM $MgSO_4$ at the appropriate pH (Quirk et al. 1991).

To investigate the possibility of growth at pH values above 10.5, the pH of the medium containing Na_2CO_3-K_2HPO_4 buffer was adjusted with 5M NaOH. To investigate bacterial metabolism, sodium L-glutamate was replaced with specific sugars, amino acids, or other substrates as the sole carbon source, and the yeast extract concentration was lowered to 0.005% (w/v); no bacterial growth was observed in media containing this yeast extract concentration as the sole carbon energy source. Salt, metabolic substrates, yeast extract, and the buffer were autoclaved separately and mixed aseptically. Solid media were made by adding 2% (w/v) bacteriological agar. Tolerance to salt was investigated by using NaCl concentrations (3% to 20%, w/v) in a medium containing sodium L-glutamate. The effect of temperature on growth was determined on glutamate/yeast extract solid medium using a gradient incubator over the temperature range 4°–39°C. Anaerobic growth was tested using an anaerobic jar (Merck) with sodium L-glutamate medium containing 0.2% (w/v) sodium thioglycollate. The presence of catalase and oxidase was tested as described in Cowan and Steel (1965). Antibiotic sensitivity was investigated by placing antibiotic disks (Oxoid) or a small amount of antibiotic impregnated in a filter paper disk on plates of glutamate/yeast extract medium. Spore formation was checked as described in Cowan and Steel (1965).

Microscopy

Cellular morphology and size were examined by phase-contrast light microscopy of unstained cells and by electron microscopy of negatively stained cells. To view bacteria by electron microscopy, one drop of an exponentially growing culture in glutamate/yeast extract liquid medium was placed on gold-coated grids covered with carbon. The bacteria were allowed to grow on the grids in a humid atmosphere and then fixed with 2.5% (v/v) glutaraldehyde, pH 10.5; the grids were washed with carbonate buffer (vide supra) and

water successively before staining with 2% (w/v) ammonium molybdate at pH 9.0. The bacteria were viewed using a Hitachi H7000 electron microscope (acceleration voltage, 75 kV).

Lipid analysis

Polar lipids were extracted using the method of Bligh and Dyer as described by Kates (1982). Cellular fatty acid methyl esters obtained by transmethylation of the total lipid extract (Kates 1982) were analyzed by capillary gas chromatography using a Hewlett Packard 5880A series chromatograph equipped with a flame-ionization detector (injector temperature, 250°C; detector temperature, 250°C), fitted with a splitter (ratio, 100:1) and a 30 m × 0.25 mm (i.d.) × 0.2 μm film thickness Supelco SP-2380 fused silica capillary column, operated isothermally at 180°C. Nitrogen was used as the carrier gas with a flow rate of 1 ml/min. Chromatograms were analyzed using a Spectra-Physics SP 4270 Computing Integrator with a Hewlett Packard 5880A series GC terminal. Fatty acids were identified by comparison of their retention times with authentic standards (Supelco), before and after hydrogenation and bromination (Kates 1982). Individual phospholipid and (phospho)glycolipids were separated by one-dimensional thin-layer chromatography using $CHCl_3$:CH_3OH:H_2O:CH_3COOH (85:15:3.5:10 by volume) as the solvent system. Phospholipids and glycolipids were identified by using specific sprays and staining reagents for phosphate (molybdate), glycolipids (α-naphthol), amino groups (ninhydrin), and vicinal hydroxyl groups (Schiff periodate), as described by Kates (1982). Phospholipid composition was quantitated by phosphorus analysis of individual components separated on thin-layer chromatograms (Kates 1982). Quinone analysis was performed using reverse-phase thin-layer chromatography (Merck HPTLC RP $18F_{254}$) and by spectrophotometric analysis of the eluted (with $CHCl_3$) components that had been visualized on thin-layer chromatograms viewed under ultraviolet light (254 nm) as dark spots on a blue fluorescent background (Collins 1985).

16S rDNA sequence determination and phylogenetic analysis

DNA was isolated as described in Maniatis et al. (1982). Primers A (5′-GGAGAGTTAGATCTTGGCTCAG-3′) and C_{rev} (5′-AGAAAGGAGGTGATCCAGCC-3′) were used to amplify the complete 16*S* rDNA sequence. DNA was amplified using a PCR system 9600 (GeneAmp; Perkin Elmer) with a denaturation step of 5 min at 94°C followed by 35 cycles of 1 min denaturation at 94°C, 2 min annealing primer at 55°C, and 1 min DNA chain extension at 72°C, completed by 5 min DNA chain extension at 72°C. The PCR product was purified using Microcon columns (Amicon; Millipore, Bedford, MA, USA).

The amplified product was analyzed using a ABI Prism 310 Genetic Analyser (Perkin Elmer). The sequences of PCR products of each isolate were assembled using the MegAlign, EditSeq, and SeqMan software using a Power Macintosh computer. Homology searches against the NCBI database were carried out using the Basic Local Alignment Search Tool (BLAST) program (Altschul et al. 1990). Percentage sequence similarity between the organism in the present study and the species selected for comparisons were calculated using the BestFit program of the Wisconsin GCG computer package (1996) Version 9. Alignment of the sequences was carried out using the ARB Sequence Database Tools (http://www.mikro.biologie.tu-muenchen.de/).

The phylogenetic trees were generated by maximum-likelihood (DNAml) and parsimony analysis (DNAPARS) [maximum likelihood (Cavalli-Sforza and Edwards 1967) and maximum parsimony (Kluge and Farris 1969)] using the PHYLIP Version 3.55c suite of programs (Kuhner and Felsenstein 1994). Bootstrap values were determined using the SEQBOOT program. TREECON for Windows 95/NT (Version 1.3b) was also used for constructing phylogenetic trees (Van der Peer and De Wachter 1993). In this program, evolutionary distances were calculated using the method of Jukes and Cantor (1969), and the topology was inferred using the "neighbor-joining" method (Saitou and Nei 1987) based on bootstrap analysis of 100 or 1000 trees. *Clostridium paradoxum* was chosen as the outgroup sequence.

DNA base composition

The genomic DNA was isolated and its G + C content was determined by high-performance liquid chromatography of the derived deoxyribonucleosides as described by Tamaoka and Komagata (1984).

Nucleotide sequence accession number

The 16*S* rDNA sequence of strain WW3-SN6 has been deposited in the database NCBI under GenBank accession number AF137020, and the organism has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM 12918).

Sequences incorporated in the present study are under the following accession numbers: *Bacillus* sp. DSM 8714, X76438; *Bacillus* sp. DSM 8717, X76441; *Bacillus clausii* DSM 8716, X76440; *Bacillus gibsonii* DSM 8722, X76446; *Bacillus horikoshii* DSM 8719, X76443; *Bacillus pseudofirmus* DSM 8715, X76439; *Bacillus pseudoalcalophilus* DSM 8725, X76449; *Bacillus alcalophilus*, X60603; *Bacillus alcalophilus* DSM 8724, X76448; *Bacillus agaradhaerens* DSM 8721, X76445; *Bacillus clarkii* DSM 8720, X76444; and *Bacillus vedderi*, DSM 9768 Z48306.

Results

Morphological characteristics

Bacterial isolate WW3-SN6 grew as creamy yellow-orange colonies with diameters of 1–4 mm. The cells were bacilloid

(0.4–0.6 × 1.6–2.8 μm), and they occurred singly, in pairs, or in clusters of up to three bacteria by association along their long axis. Endospores were observed as central oval spores. Cells were nonmotile, and flagella or pili were never observed by either phase-contrast light microscopy of unstained bacteria or electron microscopy of negatively stained cells. The isolate WW3-SN6 gave a purple color in response to Gram staining and negative reactions for the Gram test with 3% KOH and the aminopeptidase and catalase tests, indicating that it is a Gram-positive bacterium. The oxidase test was positive for this bacterial isolate.

Growth characteristics

The bacterial isolate WWW3-SN6 grew in 100% EWW with a maximum cell yield of 2 to 3 × 10^8 cells/ml. It grew at pH values 7.0–10.5, but no growth was observed at pH 6.0 or 10.7, and it had a broad pH optimum of 8.0–9.0. Therefore, this bacterium is a facultative alkaliphile, particularly as the growth rates at pH 7.0 and 10.5 were similar. It could grow over a temperature range from 4° to 37°C with an optimum between 27° and 32°C; no growth was observed at 39°C. This facultative alkaliphile failed to grow under anaerobic conditions, and therefore it is a strict aerobe. It is halotolerant, tolerating up to 15% NaCl, with a stimulation of growth rate at 3% and 5% NaCl, but no further increase in rate up to 15% NaCl; no growth was observed at 20% NaCl.

Growth tests

Several amino acids, sugars, and alkali salts of some organic acids were utilized by the facultatively alkaliphilic bacterium WW3-SN6 (Table 1). Yeast extract could be used as the sole carbon and energy source. D(+)-Glucose, D(+)-glucose-6-phosphate, D(+)-cellobiose, starch, and sucrose were the best growth substrates for this facultative alkaliphile. Amino acids supported only weak growth or none at all (Table 1). Antibiotic sensitivity testing revealed that bacterial isolate WW3-SN6 was sensitive to penicillin G, amoxycillin, ampicillin, carbenicillin, ceftazidime, chloramphenicol, kanamycin, neomycin, streptomycin, and sulphamethoxazole, but was resistant to cephalexin and trimethoprim.

Lipid composition

The major phospholipids of bacterial isolate WW3-SN6 grown at pH 10.5 were phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG), together with a small amount of phosphatidylethanolamine (PE) and a trace of an unidentified phospholipid (PL) (Table 2). When the pH of the growth medium was lowered to 7.0, the proportion of PG decreased by 19% and there was a corresponding increase in the amount of PE to 17% (Table 2).

The predominant fatty acids in the membrane phospholipids were i15:0 and a15:0, together with smaller amounts of i17:0, i17:0, a14:0, 14:0, il6:0, and 16:0 (Table 3).

Table 1. Growth of *Bacillus* sp. strain WW3-SN6 on several substrates

Substrate utilization			
L-Alanine	w	L(+)-Arabinose	+
L-Arginine	–	D(+)-Cellobiose	+
L-Asparagine	w	α-Cellulose	w
L-Aspartate	w	D(−)-Fructose	–
L-Cysteine	–	D(+)-Galactose	w
L-Cystine	w	D(+)-Glucose	+
L-Glutamine	–	D(+)-Glucose-6-P	+
Glycine	–	Lactose	+
L-Histidine	w	Maltose	m
Hydroxy-L-proline	w	Mannose	+
L-Isoleucine	w	D(+)-Melezitose	+
L-Lysine	–	α-D(+)-Melibiose	m
L-Lysine HCl	–	D(+)-Raffinose	+
L-Methionine	–	L(+)-Rhamnose	–
L-Ornithine HCl	–	D(−)-Ribose	+
L-β-Phenylalanine	–	Sucrose	+
L-Proline	w	D(+)-Xylose	+
L-Serine	w	Acetate	–
L-Threonine	w	Ethanol	–
L-Tryptophan	–	Glutamate	w
L-Tyrosine	w	Glycerol	m
L-Valine	–	Malate	w
		D-Mannitol	m
		Myo-inositol	m
		Starch	+
		Sorbitol	m
		Succinate	m
		Trehalose	m

w, weak growth when OD_{500} was in the range 0.04–0.06; m, moderate growth when OD_{500} was in the range 0.1–0.2; +, growth when OD_{500} was more than 0.2; −, no growth
All media contained 0.005% (w/v) yeast extract

Table 2. Effect of growth pH on the phospholipid composition of *Bacillus* sp. strain WW3-SN6

Phospholipid	Composition (wt %)	
	pH 7.0	pH 10.5
DPG	24.1 ± 2.1	22.4 ± 1.0
PE	17.2 ± 3.7*	0.4 ± 0.1*
PL	Trace	Trace
PG	58.4 ± 4.9**	77.1 ± 1.0**

The data values are means ± SEM ($n = 3$) of duplicate analyses of lipid extracted from three independent cultures at each pH; trace, <0.4%
Statistical significance was determined using Student's *t* test for paired samples: *, $P < 0.01$; **, $P < 0.05$

There were many statistically significant differences in the fatty acid compositions of this facultatively alkaliphilic bacterium when grown at its upper (pH 10.5) or lower (pH 7.0) limits of pH (Table 3). Individually, no single pH-dependent change predominated, but there were some overall trends. The amount of branched fatty acid was higher at the upper pH limit, and a decrease in the average chain length occurred at the lower pH limit. The percentages of *iso* compared to *anteiso* and the amount of unsaturated fatty acids were also greater at the upper pH limit. However, the percentage of unsaturated fatty acids was low in comparison with the amounts of straight-chain fatty acids, including the percentage of total branched fatty acids.

Table 3. Effect of growth pH on the fatty acid composition of *Bacillus* sp. strain WW-SN6

Fatty acid	Abbreviation	Composition (wt %)	
		pH 7	pH 10.5
11-Methyl dodecanoate	i13:0	0.2 ± 0.0	0.1 ± 0.0
10-Methyl dodecanoate***	a13:0	0.1 ± 0.0	0.05 ± 0.0
12-Methyl tridecanoate**	i14:0	3.8 ± 0.1	2.9 ± 0.2
Tetradecanoate**	14:0	2.4 ± 0.1	0.9 ± 0.1
13-Methyl tetradecanoate**	i15:0	27.7 ± 0.5	32.2 ± 0.6
12-Methyl tetradecanoate**	a15:0	46.3 ± 0.6	39.5 ± 0.2
Pentadecanoate**	15:0	1.7 ± 0.1	0.6 ± 0.1
14-Methyl pentadecanoate*	i16:0	3.4 ± 0.1	4.0 ± 0.2
Hexadecanoate	16:0	6.0 ± 0.5	4.5 ± 0.2
15-Methyl hexadecanoate**	i17:0	2.5 ± 0.4	5.6 ± 0.2
14-Methyl hexadecanoate**	a17:0	4.6 ± 0.3	7.3 ± 0.3
Hexadecenoate	16:1	0.1 ± 0.0	0.2 ± 0.0
Heptadecanoate***	17:0	tr	0.1 ± 0.0
14-Methyl hexadecenoate***	a17:1	0.1 ± 0.0	0.5 ± 0.2
Octadecanoate	18:0	0.5 ± 0.0	0.7 ± 0.2
Octadecenoate	18:1	0.3 ± 0.0	0.3 ± 0.1
ECL		15.15	15.42
%br		88.7	86.4
a/i		1.36	1.21
%UFA		0.5	1.0

tr, trace; ECL, equivalent chain length; %br, percentage of branched-chain fatty acids; a/i, ratio of *anteiso*-branched to *iso*-branched fatty acids; %UFA, percentage of unsaturated fatty acids
The data values are means ± SEM ($n = 3$) of duplicate analyses of lipid extracted from three independent cultures at each pH
Pairs of values for given fatty acid at the two pH values that are significantly different were identified using Student's t test and are shown: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.01$ or 0.05, due to the small values

A putative menaquinone was isolated by preparative TLC (Rf = 0.71; using a nonpolar solvent system of hexane:diethyl ether, 85:15, v/v), eluted, and analyzed by its absorption spectrum characteristics and also by subsequent reversed-phase TLC. Spectral analysis confirmed the existence of menaquinone, and reverse-phase TLC using a solvent system of CH_3COCH_3:H_2O (92:8, v/v) showed that it was probably menaquinone-5 (MQ-5) on the basis of its Rf value relative to the menaquinone-7 (MQ-7) standard isolated from *Bacillus subtilis* (NCIMB 3610) known to contain this menaquinone (Ratledge and Wilkinson 1988). This result is consistent with the finding of Jones et al. (1994), who reported the existence of MQ-5 in some alkaliphilic bacilli.

16*S* rDNA (rRNA) sequence analysis

Analysis of 1507 bases of the 16*S* rDNA gene of the facultatively alkaliphilic bacterium WW3-SN6 showed that it had a closest match (98.2% homology) with that from the alkaliphilic *Bacillus* species DSM 8714 studied by Nielsen et al. (1994, 1995). The next highest similarity was with *Bacillus* spp. DSM 8716 (97.0%) and DSM 8717 (97.0%). These homology similarities were greater than those between the DSM strains themselves, which ranged from 96.2% to 96.8%. Construction of phylogenetic trees using distance matrix methods (Jukes and Cantor 1969) and character-based methods [maximum parsimony (Kluge and Farris 1969) and maximum likelihood (Cavalli-Sforza and Edwards 1967)] placed the facultative alkaliphile WW3-SN6 in a phylogenetic position related to alkaliphilic *Bacillus* species DSM 8714 (Fig. 1).

The G + C content of the genomic DNA of *Bacillus* sp. strain WW3-SN6 was 41.1 ± 1.0 mol%, indicating that this facultative alkaliphile belongs to low G + C content bacteria.

Discussion

Bacterial isolate WW3-SN6 was identified as being a facultatively alkaliphilic *Bacillus* species, with the typical phenotypic characteristics of this genus such as its rod shape and ability to sporulate (Ash et al. 1991; Holt et al. 1994). The fact that *Bacillus* sp. strain WW3-SN6 could grow at low temperatures and salinity up to 15% (w/v) NaCl indicates that this alkaliphile is a psychrotolerant and halotolerant bacterium. Its inability to grow under anaerobic conditions reveals that it is a strictly aerobic bacterium. The utilization of several organic compounds indicates that it is also a chemoorganoheterotroph.

The anionic phospholipids phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG) dominate the membrane lipid composition of *Bacillus* sp. strain WW3-SN6 at all growth pH values. It could be suggested that the domination of anionic lipids in the membrane is an adaptation to the high concentration of cations in the alkaline lakes and artificial habitats such as the washwaters of edible olives, which contain a high concentration of Na^+.

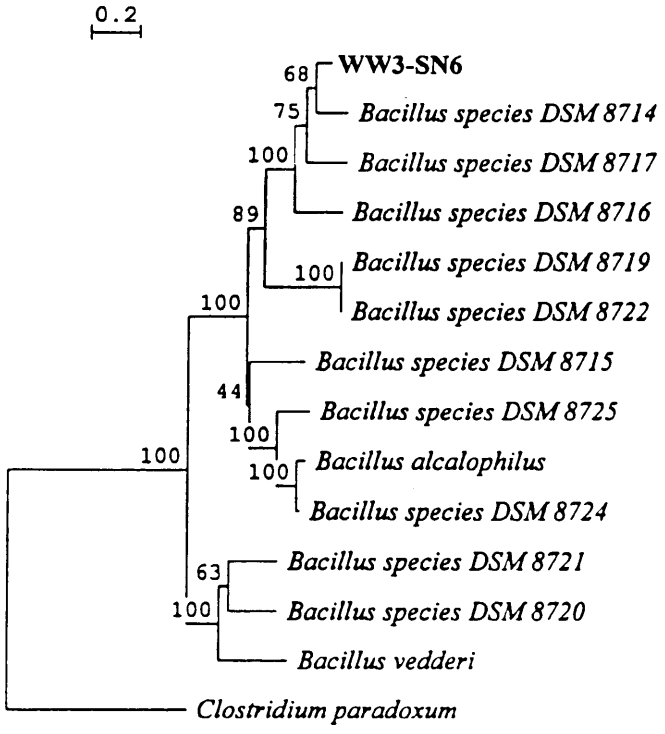

Fig. 1. Phylogenetic tree of the facultatively alkaliphilic *Bacillus* sp. strain WW3-SN6 isolated from edible olive washwaters associated with other members of the *Bacillus* genus. Evolutionary distances were calculated using the method of Jukes and Cantor (1969), and the topology was inferred using the "neighbor-joining" method (Saitou and Nei 1987) based on bootstrap analysis of 100 trees. The *scale bar* represents 0.02 inferred substitutions per nucleotide position. The 16*S* rRNA sequence of *Clostridium paradoxum* was chosen arbitrarily as the outgroup sequence

However, the amount of bacterial surface charge provided by the lipid is relatively small in relation to the external concentrations of cations (Russell 1989). Alternatively, the large proportion of anionic lipids may be an adaptation to preserve membrane bilayer integrity at high pH, because PG is a bilayer-stabilizing lipid compared with unsaturated PE, which forms nonbilayer phases (Russell 1989). If one assumes that there is a greater need to stabilize the bilayer at high pH values, this interpretation is supported by the observation that there is a significant pH-dependent shift in phospholipid composition because phosphatidylethanolamine (PE), which comprises <1% of the total in cells grown near the upper limit of pH (10.5), rises to 17% when cultures are grown at neutral pH.

The observed pH-dependent changes in *Bacillus* sp. strain WW3-SN6 are in contrast to the findings of Clejan et al. (1986), who observed that the alkaliphilic *Bacillus firmus* has the same phospholipid composition whether grown at pH 7.5 or 10.5. Furthermore, we have found that three obligate alkaliphiles which were also isolated from the edible olive washwater (Ntougias 1999) did not alter their lipid composition in a pH-dependent fashion.

Bacillus sp. strain WW3-SN6 also changes the fatty acid composition of its phospholipids in response to growth at neutral or high pH. The changes are complex. Although branched fatty acids dominate at both pH values (86%–89% of the total), at alkaline pH (10.5) there is a decrease in the *anteiso*-branched relative to *iso*-branched fatty acids, with a longer average chain length. In terms of fluidity these are opposing effects, so it is more likely that the reason for the changes is related to structural lipid packing considerations (Russell 1989). In addition, there is a doubling of the amount of unsaturated fatty acid, but the proportion is always <1% so it is unlikely to significantly affect membrane fluidity.

Bacillus sp. strain WW3-SN6 is not closely related to any of the alkaliphiles isolated by Duckworth et al. (1996) from Lake Nakuru, from which it differs in both molecular and phenotypic characteristics. Taxonomically, *Bacillus* sp. strain WW3-SN6 was accommodated in a broad cluster, consisting of a number of alkaliphilic *Bacillus* species, and more specifically within a group consisting of *Bacillus* spp. DSM 8714 and DSM 8717, with which it showed a high 16*S* rDNA homology (98.2% and 97.0%, respectively) (see Fig. 1). *Bacillus clausii* DSM 8716 also has a high homology (97.0%) with this alkaliphilic strain, although it is on a distinct branch. One clade of this cluster included *Bacillus* sp. strain WW3-SN6 and *Bacillus* sp. DSM 8714, and the other contained *Bacillus* sp. DSM 8717. *Bacillus* spp. DSM 8714 and DSM 8717 were 96.8% homologous, and in the study of Gordon and Hyde (1982) were grouped as *Bacillus lentus* type I, although later they were classified as phenotypic group 4 by Fritze et al. (1990) on the basis of their phenotypic characteristics and determination of DNA base composition.

On the basis of their phenotypic characteristics, the G + C content of genomic DNA and their 16*S* rDNA sequences, *Bacillus* spp. DSM 8714 and DSM 8717 were assigned to the same genospecies, although DNA–DNA hybridization revealed a low association (approximately 20%), indicating the taxonomic differentiation of these two bacilli (Nielsen et al. 1994, 1995). Despite the fact that these alkaliphilic bacilli were genotypically distinct according to Nielsen et al. (1995), they displayed some phenotypic differences, so that no names of these taxa were given by these authors until newly related strains could be isolated. The taxon, including *Bacillus* sp. DSM 8714, consisted of strains isolated from soil, whereas the taxon of *Bacillus* sp. DSM 8717 was composed of isolates from human and animal manure.

Bacillus sp. strain WW3-SN6 has a broad optimum of pH 8–9 with a rate of growth and cell yield at pH 10.5 that are similar to those at pH 7.0. Therefore, this strain can be characterized as being a facultative alkaliphile. *Bacillus* spp. WW3-SN6 and DSM 8717 could grow at 15% (w/v) NaCl, whereas *Bacillus* sp. DSM 8716 failed to grow in the same salt concentration. In contrast with *Bacillus* spp. DSM 8717 and 8716, *Bacillus* spp. WW3-SN6 and DSM 8714 were unable to grow above 40°C. On the basis of its inability to utilize L-arabinose and fructose, *Bacillus* sp. WW3-SN6 is distinguishable from *Bacillus* sp. DSM 8716. It can also be distinguished from the other two bacilli, because *Bacillus* sp. DSM 8714 could not utilize L-arabinose, ribose, D-xylose, inositol, sorbitol, lactose, fructose, or starch,

whereas *Bacillus* sp. DSM 8717 could not metabolize lactose, fructose, or sorbitol.

The high 16*S* rRNA homology and phylogenetic topology cannot lead to a secure assignment of WW3-SN6, although some phenotypic differences are observed, specially bearing in mind that *Bacillus* spp. DSM 8714 and 8717, which have very few phenotypic differences, represent two distinct taxa even if their homology is also high (96.8%).

The facultatively alkaliphilic bacterium *Bacillus* sp. WW3-SN6 has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM 12918).

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References

Ash C, Farrow JAE, Wallbanks S, Collins MD (1991) Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small subunit ribosomal RNA sequences. Lett Appl Microbiol 13:202–206

Altschul SF, Gish W, Miller M, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Andrich G, Balzini S, Zinnai A, Silvestri S, Fiorentini R (1992) Effect of olive oil waste water irrigation on olive plant products. Agric Medit 122:97–100

Bae M, Kim M-Y (1997) A new alkalophilic bacterium producing ethylene. J Microbiol Biotechnol 7:212–214

Berendes F, Gottshalk G, Heine-Dobbernack E, Moore ERB, Tindall BJ (1996) *Halomonas desirata* sp. nov., a new alkaliphilic, halotolerant and denitrifying bacterium isolated from a municipal sewage works. Syst Appl Microbiol 19:158–167

Cavalli-Sforza LL, Edwards AWF (1967) Phylogenetic analysis models and estimation procedures. Am J Hum Genet 19:233–257

Clejan S, Krulwich TA, Mondrus KR, Seto-Young D (1986) Membrane lipid composition of obligately and facultatively alkalophilic strains of *Bacillus* spp. J Bacteriol 168:334–340

Collins MD (1985) Isoprenoid quinone analysis in bacterial classification and identification. In: Goodfellow M, Minnikin DE (eds) Chemical methods in bacterial systematics. Academic Press, London, pp 267–287

Collins MD, Lund BM, Farrow JAE, Schleifer KH (1983) Chemotaxonomic study of an alkalophilic bacterium, *Exiguobacterium auntiacum* gen. nov., sp. nov. J Gen Microbiol 129:2037–2042

Cowan ST, Steel KJ (1965) Cowan and Steel's manual for the identification of medical bacteria. Cambridge University Press, Cambridge

Duckworth AW, Grant WD, Jones BE, Van Steenbergen R (1996) Phylogenetic diversity of soda lake alkaliphiles. FEMS Microbiol Ecol 19:181–191

Fritze D (1996) *Bacillus haloalkaliphilus* sp. nov. Int J Syst Bacteriol 46:98–101

Fritze D, Flossdorf J, Claus D (1990) Taxonomy of alkaliphilic *Bacillus* strains. Int J Syst Bacteriol 40:92–97

Gordon RE, Hyde JL (1982) The *Bacillus firmus–Bacillus lentus* complex and pH 7.0 variants of some alkalophilic strains. J Gen Microbiol 27:256–262

Grant WD, Tindall BJ (1986) Alkaline saline environment. In: Herbert RA, Codd GA (eds) Microbes in extreme environments. Academic Press, London. pp 22–54

Guffanti AA, Finkelthal O, Hicks DB, Falk L, Sidhu A, Garro A, Krulwich TA (1986) Isolation and characterization of new facultatively alkalophilic strains of *Bacillus* species. J Bacteriol 167:766–773

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994) Bergey's manual of determinative bacteriology, 9th edn. Williams and Wilkins, Baltimore

Horikoshi K (1991) Microorganisms in alkaline environments. Kodansha, Tokyo

Jimenez Araujo A, Labavitch JM, Heredia Moreno A (1994) Changes in the cell wall of olive fruit during processing. J Agric Food Chem 42:1194–1199

Jones BE, Grant WD, Collins NC, Mwatha WE (1994) Alkaliphiles: diversity and identification. In: Priest FG, Ramos-Cormenzana A, Tindall BJ (eds) Bacterial diversity and systematics. Plenum Press, New York, pp 195–230

Jones BE, Grant WD, Duckworth AW, Owenson GG (1998) Microbial diversity of soda lakes. Extremophiles 2:191–200

Jukes TH, Cantor CR (1969) Evolution of protein molecules. In: Munro HN (ed) Mammalian protein metabolism. Academic Press, New York, pp 21–132

Kates M (1982) Techniques of lipidology. Elsevier, Amsterdam

Kevbrin VV, Zhilina TN, Rainey FA, Zavarzi GA (1998) *Tindallia magadii* gen. nov., sp. nov.: an alkaliphilic anaerobic ammonifier from soda lake deposits. Curr Microbiol 37:94–100

Khmelenina VN, Kalyzhnaya MG, Starostina NG, Suzina NE, Trotsenko YA (1997) Isolation and characterization of halotolerant alkaliphilic methanotrophic bacteria from Tuva Soda Lakes. Curr Microbiol 35:257–261

Kimura T, Horikoshi K (1988) Isolation of bacteria which can grow at both high pH and low temperature. Appl Environ Microbiol 54:1066–1067

Kimura T, Horikoshi K (1989) Production of amylase and pullulanase by an alkalopsychrotrophic *Micrococcus* sp. Agric Biol Chem 53:2963–2968

Kluge AG, Farris JS (1969) Quantitative phyletics and the evolution of the anurans. Syst Zool 18:1–32

Kopsidas GC (1992) Wastewater from the preparation of table olives. Water Res 26:629–631

Kroll RG (1990) Alkalophiles. In: Edwards C (ed) Microbiology of extreme environments. Open University Press, Milton Keynes, pp 55–92

Krulwich TA, Guffanti GA (1989) Alkalophilic bacteria. Annu Rev Microbiol 43:435–463

Kuhner MK, Felsenstein J (1994) A simulation comparison of phylogeny algorithms under equal and unequal evolutionary rates. Mol Biol Evol 11:459–468

Maeda M, Taga N (1980) Alkalotolerant and alkalophilic bacteria. Mar Ecol Prog Ser 2:105–108

Maniatis T, Frintsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor, New York

Marsilio V, Lanza B, De Angelis M (1996) Olive cell components: physical and biochemical changes during processing. J Sci Food Agric 70:35–43

Nakamura S, Nakai R, Wakabayashi K, Ishiguro Y, Aono R, Horikoshi K (1994) Thermophilic alkaline xylanase from newly isolated alkaliphilic and thermophilic *Bacillus* sp. strain TAR-1. Biosci Biotechnol Biochem 58:78–81

Ni S, Boone JE, Boone DR (1994) Potassium extraction by the moderately halophilic and alkaliphilic methanogen *Methanococcus taylorii* GS-16 and homeostasis of cytosolic pH. J Bacteriol 176:7274–7279

Nielsen P, Rainey FA, Outtrup H, Priest FG, Fritze D (1994) Comparative 16*S* rDNA sequence analysis of some alkaliphilic bacilli and the establishment of a sixth rRNA group within the genus *Bacillus*. FEMS Microbiol Lett 117:61–66

Nielsen P, Fritze D, Priest FG (1995) Phenetic diversity of alkaliphilic *Bacillus* strains: proposal for nine new species. Microbiology 141: 1745–1761

Ntougias S (1999) Alkaliphilic bacteria from edible olives. PhD thesis, University of London

Quirk PG, Guffanti AA, Plass RJ, Clejan S, Krulwich TA (1991) Protonophore resistance and cytochrome expression in mutant strains of the facultative alkaliphile *Bacillus firmis* OF4. Biochim Biophys Acta 1058:131–140

Ratledge C, Wilkinson SG (1988) Microbial lipids. Academic Press, London

Russell NJ (1989) Adaptive modification in membranes of halotolerant and halophilic microorganisms. J Bioenerg Biomembr 21:93–113

Saez L, Perez J, Martinez J (1992) Low molecular weight phenolics attenuation during simulated treatment of wastewaters from olive oil mills in evaporation ponds. Water Res 26:1261–1266

Saitou N, Nei M (1987) The neighbor-joining method – a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Takami H, Inoue A, Fuji F, Horikoshi K (1997) Microbial flora in the deepest sea mud of the Mariana Trench. FEMS Microbiol Lett 152:279–285

Tamaoka J, Komagata K (1984) Determination of DNA base composition by reverse-phase high-performance liquid chromatography. FEMS Microbiol Lett 25:125–128

Tindall BJ, Ross HNM, Grant WD (1984) *Natronobacterium* gen. nov. and *Natronococcus* gen. nov., two new genera of haloalkaliphilic archaebacteria. Syst Appl Microbiol 5:41–57

Van der Peer Y, De Wachter R (1993) TREECON: a software package for the construction and drawing of evolutionary trees. Comput Appl Biosci 9:177–182

Vedder A (1934) *Bacillus alcaliphilus* n. sp.; benevens enkele ervaringen met sterk alcalische voedingsbodems. J Microbiol 1:143–147

Weisser J, Truper HG (1985) Osmoregulation in a new halophilic *Bacillus* from the Wadi Natrum. Syst Appl Microbiol 6:7–11

Wisconsin package–program manual (1996) Version 9. Genetics Computer Group, Madison, WI

Zhilina TN, Zavarzin GA (1994) Alkaliphilic anaerobic community at pH 10. Curr Microbiol 29:109–112

Zhilina TN, Zavarzin GA, Rainey FA, Pikuta EN, Osipov GA, Kostrikina NA (1997) *Desulfonatronovibrio hydrogenovorans* gen. nov., sp. nov., an alkaliphilic, sulfate-reducing bacterium. Int J Syst Bacteriol 47:144–149