Bacillus saliphilus sp. nov., isolated from a mineral pool in Campania, Italy

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A haloalkaliphilic Gram-positive bacterium, strain 6AG^T, that grew aerobically at an optimum temperature of 37 °C and at pH 7–10 (optimum 9.0), was isolated from algal mat from a mineral pool located in Malvizza in the Campania region (southern Italy). The isolate tolerated high concentrations of NaCl, up to 25 %. On the basis of 16S rRNA gene sequence similarity, the strain was shown to belong to the genus *Bacillus*. Analysis of the 16S rRNA gene sequence revealed high similarity between strain 6AG^T and an unidentified isolate from Hailaer soda lake (China) (99.9 % identity) and two Kenyan isolates, 3E1 and WE4 (98.3 and 97.8 % identity, respectively). The G + C content of the DNA was 48.4 mol%. The predominant respiratory quinones were MK-7(H₂), MK-7(H₄) and DMK-7(H₂); phosphatidylglycerol and diphosphatidylglycerol were the predominant polar lipids. iC15:0 and aiC15:0 were the major fatty acids. Strain 6AG^T accumulated osmolytes. The phylogenetic distance of strain 6AG^T (= DSM 15402^T = ATCC BAA-957^T) from any recognized species within the genus *Bacillus* allowed it to be classified as the type strain of *Bacillus saliphilus* sp. nov.

The Campania region (Italy) is rich in mineral environments but they have not been explored in any detail regarding their microbial communities. This research was aimed at the screening, isolation and identification of halo-philic, alkaliphilic and haloalkaliphilic micro-organisms from the local ecosystems in Campania, Italy. Recently we have isolated, from a hydrothermal site in Campania, a novel haloalkaliphilic species classified as *Planococcus rifietoensis*, able to hydrolyse 5-bromo-4-chloro-3-indoyl β -D-glucopyranoside (XGlc) (Romano *et al.*, 2003). In 1985, the isolation of an obligately alkaliphilic, extremely halotolerant *Bacillus* strain, WN13, was described, later classified by Fritze (1996) as *Bacillus haloalkaliphilus*. Little effort has been devoted to the study of intracellular ion concentrations in halophilic Gram-positive bacteria. Apparent intracellular cation concentrations as high as those present in the growth medium were reported in *Bacillus haloalkaliphilus* (Weisser & Trüper, 1985). However, this species is now known to produce ectoine as the main osmotic solute (Oren, 2002). Few *Bacillus* species that grow in the presence of high NaCl concentrations and are also alkaliphilic have been described (Fritze, 1996; Arahal & Ventosa, 2002). The significance of haloalkaliphiles has prompted the isolation

and characterization of such strains from a variety of environments.

In this study, a new member of the genus *Bacillus* is proposed on the basis of polyphasic studies. Strain 6AG^T was isolated from a mat of green algae collected from a mineral pool at the Malvizza site (Montecalvo Irpino, Avellino province, Campania region, south Italy). The strain was isolated by the dilution-plating technique from the algal mat (pH 8.0, 18 °C) at the green–red algae boundary in a small pool. Enrichment medium (medium 1) contained the following components (l⁻¹): Na₂CO₃, 3.0 g; KCl, 2.0 g; MgSO₄·7H₂O, 1.0 g; NaCl, 160 g, trisodium citrate, 3.0 g; yeast extract, 10.0; MnCl₂·4H₂O, 0.36 mg; FeSO₄, 50 mg. Na₂CO₃ and NaCl were autoclaved separately. The pH of medium 1 was 9.0. Growth on single carbon sources at 10 g l⁻¹ was tested on liquid medium (medium 2) containing (g l⁻¹) K₂HPO₄, 7.0; KH₂PO₄, 2.0; MgSO₄·7H₂O, 0.1; (NH₄)₂SO₄, 1.0; NaCl, 160; Na₂CO₃, 3.0; with 500 μ l thiamine hydrochloride solution l⁻¹ (100 mg l⁻¹). Solid media were prepared by the addition of 1.8 % agar.

Cell morphology was determined by phase-contrast microscopy (Zeiss). Colony morphology was analysed on solid medium with a Leica M8 stereomicroscope. Tolerance of NaCl and growth at various temperatures and pH was assessed in medium 1. All growth tests were done at the optimal growth temperature for 3 days. Sensitivity of the strain to antibiotics was tested by using medium 1 with agar

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(1.8 %) and Sensi discs (6 mm; Oxoid); tests were incubated for 48 h. Antibiotic tests were carried out according to Romano *et al.* (1993). Casein and gelatin hydrolysis, oxidase, tyrosinase, aminopeptidase (Bactident-Merck) and catalase were tested in medium 1. For nitrate reduction, medium 1 plus 0.1 % KNO₃ was employed. Hydrolysis of hippurate was assessed in medium 1 plus hippurate (1 %) (Romano *et al.*, 1996). Gram-staining was performed according to Dussault (1955). The KOH test was performed according to Halebian *et al.* (1981). Hydrolysis of *N'*-benzoyl-arginine *p*-nitroaniline (BAPNA) stereoisomers was tested according to Oren & Galinski (1994). The extraction of intracellular solutes, their purification and quantification were done according to Romano *et al.* (2001).

Cell mass for quinone and lipid analysis was obtained from culture in medium 1 at 37 °C at pH 9.0 at optimal NaCl concentration. Lipid analysis was performed as reported by Romano *et al.* (2001). Lipid hydrolysis and identification of fatty acid methyl esters were done as reported by Romano *et al.* (2001). Quinones were analysed by LC/MS on a reverse-phase column, by EI/MS and ¹H NMR spectra.

The G+C content was determined by the method of Tamaoka & Komagata (1984). DNA was hydrolysed and the resultant nucleotides were analysed by HPLC. The DNA was isolated as described previously (Romano *et al.*, 2003).

The total 16S rRNA gene sequence was determined by direct sequencing of the PCR product. Genomic DNA extraction, amplification of the 16S rRNA gene and purification of PCR products were carried out as described previously (Romano *et al.*, 2003). Purified PCR products were

sequenced by the DSMZ using the ABI PRISM Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems) as directed in the manufacturer's protocol. Sequence reactions were electrophoresed using an Applied Biosystems 373A DNA sequencer.

The multiple sequence alignment program CLUSTAL W (Chenna *et al.*, 2003) was used to align the 16S rRNA gene sequence of our isolate with the sequences of representative organisms belonging to the *Bacillus/Staphylococcus* group. Sequences of 16S rRNA genes for comparison were obtained from the EMBL database or RDP. Phylogenetic analysis was performed using the PHYLIP package, version 3.5 (Felsenstein, 1993). Distances (distance options according to Kimura's two-parameter model) and clustering with the maximum-parsimony method were determined by using bootstrap values based on 1000 replications.

16S rRNA gene phylogenetic analysis based on the neighbour-joining method (Saitou & Nei, 1987) showed strain 6AG^T to be placed in the genus *Bacillus* (Fig. 1). In particular, the sequence similarity to any other species within the genus *Bacillus* with validly published names was less than 95.7 %.

16S rRNA gene sequencing of strain 6AG^T showed 99.9 % similarity to an unidentified and not described isolate from Hailaer soda lake (China) named strain T1 (AF275706). Further, two undescribed isolates, strains 3E1 (X92162) and WE4 (X92161), isolated from Lake Elementeita in the Kenyan section of the East African Rift Valley, showed similarity greater than 97 %. The Kenyan isolates were related to but distinct from *Bacillus agaradhaerens* and *Bacillus clarkii* (Duckworth *et al.*, 1996; Nielsen *et al.*, 1995),

Fig. 1. Phylogenetic tree showing the position of strain 6AG^T among related bacteria of the genus *Bacillus* and related genera. The tree was obtained using the neighbour-joining method. Numbers at branching points are bootstrap percentages, based on 1000 resamplings. Bar, 0.01 substitutions per nucleotide position.

while our isolate showed less than 96 and 95 % similarity, respectively, to these two latter species. Phylogenetic analysis based on the neighbour-joining and maximum-parsimony methods showed the same position for strain 6AG^T and the three undescribed isolates among the genus *Bacillus*, grouping together and forming a cluster separated from other species (Fig. 1). The G+C content of the DNA of strain 6AG^T was 48.4 mol%.

Strain 6AG^T was a Gram-positive non-sporulating coccus measuring 0.8–0.9 µm. It grew as single cells and/or clusters and was non-sporulating, even in the presence of 100 mg MnCl₂ ml⁻¹. Colonies on enrichment medium 1 were usually yellow. The main features of the novel isolate are listed in Table 1.

The isolate required aerobic conditions for growth. It grew in medium 1 containing up to 25 % NaCl and did not grow in the absence of salts. The optimum NaCl concentration in the medium was 16 %. Strain 6AG^T did not have a specific requirement for the Na⁺ ion, as it also grew in the presence of KCl. The optimal temperature for growth was 37 °C and the temperature range was 4–50 °C. The optimal pH for growth was 9.0 and the pH range was 7–10 (Table 1). Strain 6AG^T was able to grow on medium 2 containing glucose. Other carbon sources did not sustain growth. Among the antibiotics tested, strain 6AG^T was sensitive to (µg) vancomycin (30), ampicillin (25), novobiocin (30), fusidic acid (10), streptomycin (25), chloramphenicol (10), erythromycin (5 and 30) and bacitracin (10).

Table 1. Phenotypic properties of strain 6AG^T, *B. agaradhaerens* and *B. clarkii*

Data for reference taxa were taken from Nielsen *et al.* (1995). +, Positive or present; –, negative or absent; NR, not reported; w, weak reaction. All three strains are negative for starch hydrolysis.

Property	6AG ^T	<i>B. agaradhaerens</i> DSM 8721 ^T	<i>B. clarkii</i> DSM 8720 ^T
Sampling site	Algal mat	Soil	Soil
Cell morphology	Cocci	Rods	Rods
Colony colour	Yellow	White and rhizoid with filamentous margin	Cream–white to dark yellow
Spore formation	–	+	+
Motility	–	NR	NR
Gram reaction	+	NR	+
Oxidase	+	NR	NR
Catalase	w	NR	NR
Hydrolysis of:			
Gelatin	+	–	+
Casein	–	–	+
Hippurate	–	–	+
Nitrate reduction	+	+	NR
Tyrosine decomposition	+	NR	NR
Phenylalanine deamination	–	–	NR
NaCl concentration for growth (% w/v):			
Minimum	>0	0.5	0.5
Optimum	16	NR	NR
Maximum	25	16	16
Growth with KCl	+	NR	NR
Growth temperature (°C):			
Range	4–50	10–45	15–45
Optimum	37	30	30
pH for growth:			
Range	7–10	>7.0	>7.0
Optimum	9.0	10.0	>10.0
DNA G+C content (mol%)	48.4	39.5	43.0
Quinone composition	MK-7(H ₄) (15 %); MK-7(H ₂) (70 %); DMK-7(H ₂) (15 %)	NR	NR
Osmolytes	Glycine betaine (81 %), glutamate (4 %), ectoine (12 %), hydroxyectoine (3 %)	NR	NR

The phenotypic features of strain 6AG^T and *B. agaradhaerens* and *B. clarkii* differ in several aspects (Table 1). Strain 6AG^T is a regular non-sporulating coccus, while *B. agaradhaerens* and *B. clarkii* are sporulating rods. The most interesting common feature between the above-mentioned *Bacillus* species and strain 6AG^T is their haloalkaliphilicity; in fact, they grew well at high pH values and in the presence of NaCl and they were able to tolerate NaCl concentrations up to 16–25 %. Carbohydrate utilization profiles showed growth on glucose for 6AG^T and on several sources for *B. agaradhaerens*, while no growth was observed in minimal carbohydrate media for *B. clarkii* (Nielsen *et al.*, 1995).

Strain 6AG^T possessed complex lipids based on fatty acids. The total lipid content accounted for 9–11 % of the total dry weight of cells grown under optimal growth conditions in medium 1. Under these conditions, phosphoethanolamine, diphosphatidylglycerol and phosphatidylglycerol were the main lipids (approx. 96 %), identified by ¹H and ¹³C NMR spectra. The chemical shift values of signals present in spectra were similar to those reported by Romano *et al.* (2001), with the exception of the absence of the signals due to double bonds in the fatty acid chains. The assignments were done by comparison with commercial standards and by distortionless enhanced polarization transfer (DEPT) experiments. Glycolipids were not visualized; this was probably due to their absence or occurrence in very small amounts.

The fatty acid composition determined from cells grown under standard growth conditions was characterized by the abundance of branched saturated acyl chains; in particular, aiC15:0 reached 89 % of total fatty acid methyl esters.

LC/MS as well as EI/MS analyses of the quinone content of strain 6AG^T showed three major peaks, assigned to MK-7(H₄), MK-7(H₂) and demethylated MK-7(H₂) [DMK-7(H₂)] in a relative ratio of 1.5:7.0:1.5.

Little effort has been devoted to the study of osmoprotectants accumulated by halophilic Gram-positive bacteria; under optimal growth conditions, strain 6AG^T accumulated glycine betaine, hydroxyectoine, ectoine and glutamate.

On the basis of these results, we suggest that strain 6AG^T represents a novel species, *Bacillus saliphilus* sp. nov. This affiliation is preliminary, in that the creation of a new genus based on a single strain makes it impossible to assess the limits of variability of the taxon. Finally, it is well known that the genus *Bacillus* is clearly heterogeneous, and many contributions have been made to the development of *Bacillus* systematics (Berkeley *et al.*, 2002).

Description of *Bacillus saliphilus* sp. nov.

Bacillus saliphilus (sal.i.phi'lus. L. n. *sal* salt; Gr. adj. *philos* loving; N.L. masc. adj. *saliphilus* loving salt).

Alkalitolerant and halotolerant. Cells are Gram-positive, regular cocci, 0.8–0.9 µm in diameter, appearing singly

and/or in clumps. Colonies are yellow. Mesophilic, exhibiting optimum growth at 37 °C, but is able to grow between 4 and 50 °C and at pH 7.0–10.0 (optimum pH 9.0). Grows at salinity of 1–25 % (w/v), with optimum growth at 16 % (w/v) salts. Able to grow on glucose as a sole carbon source. Positive for oxidase, gelatinase, tyrosine decomposition and nitrate reduction but negative with respect to starch, casein, hippurate and phenylalanine hydrolysis. Catalase reaction is weak. Possesses α-glucosidase activity. Menaquinones are MK-7(H₂) (70 %), MK-7(H₄) (15 %) and DMK-7(H₂) (15 %) and the predominant polar lipids are phosphatidylglycerol and diphosphatidylglycerol. iC15:0, aiC15:0 (89 %) and C17:0 are the main cellular fatty acids. Accumulates glycine betaine as the major osmoprotectant and hydroxyectoine, ectoine and glutamate as minor components. The G+C content of DNA is 48.4 mol%. Phylogenetically related to three uncharacterized microorganisms and to *B. agaradhaerens* (95.4 % similarity) on the basis of 16S rRNA gene sequences. The following antibiotics inhibit growth (µg unless stated): chloramphenicol (10), erythromycin (5, 30), bacitracin (10), vancomycin (30), ampicillin (25), fusidic acid (10), streptomycin (25) and novobiocin (30). Grows in the presence of penicillin G (2, 10 IU), tetracycline (30, 50), kanamycin (30), neomycin (30) and gentamicin (30).

The type strain, strain 6AG^T (= DSM 15402^T = ATCC BAA-957^T), was isolated from an algal mat around a small, bubbling mineral pool at the Malvizza site (Campania, Italy).

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