Oceanobacillus oncorhynchi subsp. *incaldanensis* subsp. nov., an alkalitolerant halophile isolated from an algal mat collected from a sulfurous spring in Campania (Italy), and emended description of *Oceanobacillus oncorhynchi*

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A halophilic, alkalitolerant bacterium, strain 20AG^T, was isolated from an algal mat collected from a sulfurous spring located in Santa Maria Incaldana (Mondragone, Campania Region, southern Italy). The isolate is Gram-positive, ferments several carbohydrates and has motile, rod-shaped cells that do not sporulate. The isolate grows at pH 6.5–9.5 and in 5–20 % NaCl. On the basis of 16S rRNA gene sequence similarity, the strain was shown to belong to the genus *Oceanobacillus*; strain 20AG^T showed 96.6 % 16S rRNA gene sequence similarity to the type strain of *Oceanobacillus iheyensis*, DSM 14371^T, and 99.5 % similarity to *Oceanobacillus oncorhynchi* NCIMB 14022^T. Levels of DNA–DNA relatedness between strain 20AG^T and *O. iheyensis* DSM 14371^T and *O. oncorhynchi* NCIMB 14022^T were respectively 29.4 and 59.0 %. The G + C content of the DNA of strain 20AG^T was 40.1 mol%. The predominant respiratory quinone was MK-7, phosphatidylglycerol and phosphatidylcholine were the predominant polar lipids and minor phospholipids were also detected. ai-C14 : 0, ai-C15 : 0 and i-C15 : 0 were the major fatty acids. Strain 20AG^T accumulated osmolytes and produced exopolysaccharide. On the basis of phenotypic characteristics, phylogenetic data and DNA–DNA relatedness data, isolate 20AG^T should be designated as the type strain of a subspecies of *Oceanobacillus oncorhynchi*, for which the name *Oceanobacillus oncorhynchi* subsp. *incaldanensis* subsp. nov. is proposed. The type strain is 20AG^T (=DSM 16557^T = ATCC BAA-954^T).

The Campania Region of Italy is rich in mineral environments, but they have not yet been explored in any detail regarding their microbial communities. This research was aimed at the screening, isolation and identification of halophilic, alkaliphilic and haloalkaliphilic micro-organisms from local ecosystems in Campania. Recently, we have isolated novel haloalkaliphilic species in the genera *Planococcus*, *Bacillus*, *Salinivibrio* and *Halomonas* from hydrothermal sites in Campania (Romano *et al.*, 2003, 2005a, b). Recent studies have clearly shown that halophilic and alkaliphilic species are good sources of biomolecules of industrial interest (Ito *et al.*, 1998; Margesin & Schinner, 2001; Berkeley *et al.*, 2002), particularly for the production of exopolysaccharides and biosurfactants (Horikoshi, 1999; Oren, 2002a, b) or compatible solutes (Sauer & Galinski, 1998), and enzymes of biotechnological interest, such as

proteases for use in the paper-pulp industry and for inclusion in laundry detergents (Demirjian *et al.*, 2001; Sanchèz-Porro *et al.*, 2003). The isolates from Campania springs possessed enzymes of interest such as glycosidases and proteases and produced endo- and exopolymers of industrial interest (Romano *et al.*, 2003, 2005a, b).

The genus *Oceanobacillus* was created by Lu *et al.* (2001) to allocate some alkaliphilic isolates and a second species was recently accommodated in the genus (Yumoto *et al.*, 2005). In this study, a new member of the genus *Oceanobacillus* is proposed on the basis of polyphasic studies.

Strain 20AG^T was isolated by the dilution-plating technique from an algal mat (pH 8.0, 27 °C) collected from a sulfurous spring in the Santa Maria Incaldana site (Mondragone, Caserta Province, Campania Region, southern Italy). Strain 20AG^T was the predominant organism in the enrichment and was the only colony-forming organism at the highest dilutions. The enrichment medium (medium 1) contained

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 20AG^T is AJ640134.

the following components (l^{-1}): Na_2CO_3 , 3.0 g; KCl, 2.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g; NaCl, 100 g; trisodium citrate, 3.0 g; yeast extract, 10.0 g; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.36 mg; FeSO_4 , 50 mg. Na_2CO_3 and NaCl were autoclaved separately. The pH of medium 1 was 9.0. Growth on single carbon sources was tested in a liquid medium (medium 2) containing the following (l^{-1}): K_2HPO_4 , 7.0 g; KH_2PO_4 , 2.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g; $(\text{NH}_4)_2\text{SO}_4$, 1.0 g; NaCl, 100 g; Na_2CO_3 , 3.0 g; thiamine hydrochloride solution (500 μl ; final concentration 100 mg l^{-1}); each tested compound was added at 10.0 g l^{-1} . Solid media were prepared by the addition of 1.8% agar. Growth under anaerobic conditions was examined as previously reported (Jeanthon *et al.*, 1995).

Cell morphology was determined by phase-contrast microscopy (Zeiss). Colony morphology was analysed on solid medium by using a Leica M8 stereomicroscope. Tolerance of NaCl and growth at various temperatures and pH values were determined in medium 1. All growth tests were done at the optimal growth temperature (37 °C) for 3 days. Sensitivity of the strain to antibiotics was tested by using medium 1 with agar (1.8%) and Sensi discs (6 mm; Oxoid); tests were incubated for 48 h. The antibiotic tests were carried out according to Romano *et al.* (1993). Casein (5%) and gelatin (12%) hydrolysis and oxidase, tyrosinase, aminopeptidase (Bactident; Merck) and catalase activities were tested in medium 1. For nitrate reduction, medium 1 plus 0.1% KNO_3 was employed. Hydrolysis of hippurate was examined in medium 1 plus hippurate (1%) (Romano *et al.*, 1996). For the indole test, strain 20AG^T was grown at pH 8.0 in a medium containing all the above-mentioned components without Na_2CO_3 . Gram-staining was performed according to Dussault (1955). The KOH test was performed according to Halebian *et al.* (1981). Hydrolysis of *N*'-benzoylarginine *p*-nitroaniline (BAPNA) stereoisomers was tested according to Oren & Galinski (1994). Extraction of intracellular solutes and their purification and quantification were performed according to Motta *et al.* (2004). Cells for exopolysaccharide studies were grown on minimal medium. For exopolysaccharide recovery, cells were harvested in the stationary growth phase and the supernatants were treated and analysed as reported by Manca *et al.* (1996) and Nicolaus *et al.* (2004).

Cell mass for quinone and lipid analysis was obtained from culture in medium 1 at 37 °C, pH 9.0, at the optimal NaCl concentration (10%). Lipid analysis was performed as reported by Romano *et al.* (2001). Lipid hydrolysis and identification of fatty acid methyl esters were performed as reported by Romano *et al.* (2001). Quinones were analysed by LC/MS on a reversed-phase column by EI/MS and ^1H NMR spectrum analysis. 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 previously described (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 the 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 by the manufacturer. 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 obtained in this study and sequences of organisms belonging to the genus *Bacillus* and related genera showing the highest sequence similarity. Sequences of 16S rRNA genes for comparison were obtained from the EMBL database (<http://www.ebi.ac.uk/fasta33/index.html>) or RDP (<http://www.ebi.ac.uk/Tools/similarity.html>). Phylogenetic analysis based on the 16S rRNA gene sequence by the neighbour-joining method (distance options according to Kimura two-parameter model) was performed using the PHYLIP package, version 3.6 (Felsenstein, 2004). Clustering with the maximum-parsimony method was made by using bootstrap values based on 100 replications. DNA–DNA hybridization was performed by the DSMZ between strain 20AG^T and *O. iheyensis* DSM 14371^T and *O. oncorhynchi* NCIMB 14022^T (kindly supplied by I. Yumoto and grown according to Yumoto *et al.*, 2005). The DNA was isolated as described previously (Romano *et al.*, 2003).

16S rRNA gene phylogenetic analysis, based on the neighbour-joining method (Saitou & Nei, 1987), showed the position of strain 20AG^T in the genus *Oceanobacillus* (Fig. 1). In particular, the sequence similarity to any other species with validly published names within the genera *Bacillus*, *Virgibacillus*, *Halobacillus* and *Gracibacillus* was less than 96%. The G + C content of the DNA was determined to be 40.1 mol%.

Characteristics of strain 20AG^T are reported in subspecies description below. It was able to grow on medium 2 containing several saccharides; disaccharides were better substrates for growth than monosaccharides, in particular α -linked disaccharides such as trehalose and maltose.

Among the antibiotics tested, strain 20AG^T was sensitive to (μg) erythromycin (5), penicillin G (2), chloramphenicol (10), bacitracin (10), tetracycline (50), lincomycin (15), vancomycin (30), ampicillin (25), novobiocin (30) and streptomycin (25) and resistant to kanamycin (30) and tetracycline (30).

The total lipid content of strain 20AG^T accounted for 9–11% of the total dry weight of cells grown under optimal growth conditions in medium 1 and, under these conditions, phosphatidylglycerol and phosphatidylcholine were the main lipids (approx. 95%), identified by ^1H and ^{13}C NMR. The chemical shifts of signals present in spectra were similar to those reported by Romano *et al.* (2003), with the exception of the absence of signals

Fig. 1. Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequence comparison showing the relationship of strain 20AG^T and related strains. Numbers next to nodes indicate numbers of bootstrap samples, derived from 100 samples, which supported the internal branches. The sequence of *Lactobacillus delbrueckii* has been included to serve as an outgroup. Bar, 0.01 K_{nuc} unit.

due to double bonds in the fatty acid chains. Assignments were made by comparison with commercial standards and by DEPT (distortionless enhancement by polarization transfer) experiments. Glycolipids were not visualized.

The fatty acid composition determined for cells grown under standard growth conditions was characterized by the abundance of branched saturated acyl chains; in particular, ai-C14, i-C15:0 and ai-C15:0 made up about 90 % of total fatty acid methyl esters. i-C14:0, C15:0, C16:1, C17:0 and ai-C18:0 each made up less than 0.5 %, while C16:0, i- and ai-C17:0 and i-C18:0 constituted about 9.0 % of the total fatty acids. The high level of branched fatty acids present in strain 20AG^T was similar to results for the other two species of the genus *Oceanobacillus* and members of *Virgibacillus*, *Halobacillus* and *Bacillus* (Heyrman *et al.*, 1999, 2003; Lu *et al.*, 2001).

LC/MS as well as EI/MS analyses of the quinone content of strain 20AG^T showed a major peak assigned to MK-7, as was found in *O. iheyensis* DSM 14371^T (Lu *et al.*, 2001).

Little effort has been devoted to the study of osmoprotectants accumulated by halophilic Gram-positive bacteria; under optimal growth conditions, strain 20AG^T accumulated glycine betaine and glutamate. In minimal medium containing trehalose (10 g l⁻¹), strain 20AG^T produced 9 mg exopolysaccharide l⁻¹.

The two species of the genus *Oceanobacillus* are halotolerant and alkaliphilic. Strain 20AG^T could be considered as

moderately halophilic and alkalitolerant, requiring at least 5 % NaCl in the medium for growth and 10 % NaCl for optimal growth, while the existing species of the genus can grow in the absence of NaCl and the optimal NaCl concentration was 3.0 and 7.0 % for *O. iheyensis* DSM 14371^T and *O. oncorhynchi* NCIMB 14022^T, respectively. *O. oncorhynchi* NCIMB 14022^T is an obligate alkaliphile in that it is not able to grow at pH below 9.0, while isolate 20AG^T can grow at a slightly acidic pH (6.5). Among the differential phenotypic characteristics of strain 20AG^T and the closely related *O. oncorhynchi* NCIMB 14022^T was their different behaviour when the strains were grown under anaerobic conditions; *O. oncorhynchi* NCIMB 14022^T was able to grow under anaerobiosis, while 20AG^T was not. Other differential properties were morphology, in that cells of strain 20AG^T are longer than those of *O. oncorhynchi* NCIMB 14022^T, the absence of spore formation in 20AG^T, the ability of 20AG^T to hydrolyse *p*-nitrophenyl α -D-glucopyranoside (PNPG) and the fatty acid composition (20AG^T possesses C14 instead of C17 as one of the main components of the fatty acid mixture). Characteristics that differentiate strain 20AG^T and *O. oncorhynchi* NCIMB 14022^T are summarized in Table 1.

On the basis of DNA–DNA relatedness of less than 70 % between 20AG^T and *O. oncorhynchi* NCIMB 14022^T, the new isolate could be considered to belong to a different species, according to Wayne *et al.* (1987). However, their nearly identical 16S rRNA gene sequences (99.5 % similarity), DNA–DNA relatedness above 50 % and the paucity of phenotypic differences did not allow us to assign strain

Table 1. Differential properties of strain 20AG^T and *O. oncorhynchi* NCIMB 14022^TData were obtained from Yumoto *et al.* (2005) and present study.

Property	20AG ^T	NCIMB 14022 ^T
Cell size (µm)	0.5–0.8 × 1.2–2.0	0.4–0.6 × 1.1–1.4
Colony colour	Cream–beige	White
Spore formation	–	+
Anaerobic growth	–	+
Nitrite reduction	+	–
NaCl range for growth (%)	5–20	0–22
NaCl optimum (%)	10	3
Growth temperature range (°C)	10–40	15–40
Optimal growth temperature (°C)	37	30–36
pH range	6.5–9.5	9.0–10.0
DNA G + C content (mol%)	40.1	38.5

20AG^T to a novel species of the genus *Oceanobacillus*; instead, we propose to assign it as a subspecies of *O. oncorhynchi*, on the basis of recent recommendations about the species concept for prokaryotes (Rosselló-Mora & Amman, 2001; Stackebrandt *et al.*, 2002).

The genus *Oceanobacillus* was created by Lu *et al.* (2001, 2002) and at the time of writing contains only two species, *O. iheyensis* (whose entire genome sequence has been determined and is now available; Takami *et al.*, 2002) and *O. oncorhynchi*, the description of which was published online while this paper was under review (Yumoto *et al.*, 2005). Recently, Caton *et al.* (2004) found several *Oceanobacillus* strains in the Salt Plains National Wildlife Refuge in Oklahoma, USA. In particular, one of them, strain GSP73 (GenBank accession number AY553089), showed 99.9 % 16S rRNA gene sequence similarity to strain 20AG^T. Some general phenotypic features were reported on the Salt Plains Microbial Observatory database (<http://www.okstate.edu/artsci/SPMO/>). This isolate, like the two described species of *Oceanobacillus*, is not halophilic but halotolerant in that it was able to grow in the absence of NaCl and required 8.0 % NaCl for optimal growth. Finally, in the EMBL database, the sequence AB189328 of an unidentified and undescribed isolate *Oceanobacillus* sp. JM-Ob showed 100 % 16S rRNA gene sequence similarity with strain 20AG^T. Strains GSP73, JM-Ob and 20AG^T could be classified as members of the same subspecies after further studies. Strain 20AG^T should be designated as the type strain of a novel subspecies, for which the name *Oceanobacillus oncorhynchi* subsp. *incaldanensis* subsp. nov. is proposed. According to Rule 40b of the Bacteriological Code, the creation of this subspecies automatically creates the subspecies *Oceanobacillus oncorhynchi* subsp. *oncorhynchi* subsp. nov.

Emended description of *Oceanobacillus oncorhynchi* Yumoto *et al.* 2005

Oceanobacillus oncorhynchi (on.co.rhyn'chi. N.L. gen. n. *oncorhynchi* of *Oncorhynchus*, named after the rainbow

trout, *Oncorhynchus mykiss*, from which the type strain was isolated).

Cells are Gram-positive, peritrichously flagellated, straight rods, 0.4–0.8 × 1.1–2.0 µm. Colonies are circular and white or beige. Ability to produce ellipsoidal spores is variable. Obligate or facultative alkaliphile; obligate or facultative halophile. Ability to grow under anaerobic conditions is variable. Growth occurs at 10–40 °C with optimum growth at 30–37 °C. Growth occurs at 0–22 % NaCl with optimum growth at 7–10 %. Nitrate may be reduced to nitrite. The DNA G + C content is 38.5–40.1 mol%. Able to produce β-glycosidase and exopolysaccharide. Branched fatty acids from C14:0 up to C18:0 are the major components; traces of unsaturated fatty acids are also found. The type strain is strain R-2^T (=JCM 12661^T=NCIMB 14022^T).

Description of *Oceanobacillus oncorhynchi* subsp. *incaldanensis* subsp. nov.

Oceanobacillus oncorhynchi subsp. *incaldanensis* (in.cal.da.nen'sis. N.L. masc. adj. *incaldanensis* pertaining to the Incaldana site, southern Italy, where the type strain was isolated).

Alkalitolerant, moderately halophilic and obligately aerobic. Cells are regular, motile, non-sporulating rods, 0.5–0.8 × 1.2–2.0 µm. Colonies are beige. Mesophilic, exhibiting optimum growth at 37 °C, but able to grow at 10–40 °C and at pH 6.5–9.5 (optimum pH 9.0). Grows at salinity of 5–20 % (w/v), with optimum growth at 10 % (w/v) NaCl. Able to grow on D-glucose, D-galactose, D-fructose, D-mannose, D-arabinose, D-sorbose, D-ribose, D-xylose, sucrose, maltose, cellobiose, D-lactose, trehalose, glycerol, sodium acetate and trisodium citrate as sole carbon sources. Positive for the KOH test, oxidase, catalase and reduction of nitrate to nitrite, and negative with respect to aminopeptidase test, hydrolysis of starch, casein and gelatin, urease and indole production. The main menaquinone type is MK-7 and the predominant polar lipids are phosphatidylglycerol and phosphatidylcholine. ai-C14:0 (21 %), i-C15:0

(22.8%) and ai-C15:0 (46.5%) are the main cellular fatty acids; minor components are i-C14:0 (0.4%), C15:0 (0.5%), C16:0 (2.8%), C16:1 (0.5%), i-C17:0 (2.0%), ai-C17:0 (1.9%), C17:0 (0.15%), i-C18:0 (1.1%) and ai-C18:0 (0.1%). Accumulates glycine betaine and glutamate as major osmoprotectants. Produces an exopolysaccharide when grown in minimal medium with trehalose. The following antibiotics (μg) inhibit growth: penicillin G (2), tetracycline (50), chloramphenicol (10), erythromycin (5), bacitracin (10), vancomycin (30), ampicillin (25), streptomycin (25), novobiocin (30) and lincomycin (15). Grows in the presence of kanamycin (30) and tetracycline (30). The G+C content of DNA of the type strain is 40.1 mol%. DNA–DNA relatedness of the type strain is 29.4% with *O. iheyensis* DSM 1431^T and 59% with *O. oncorhynchi* NCIMB 14022^T.

The type strain, strain 20AG^T (=DSM 16557^T=ATCC BAA-954^T), was isolated from an algal mat collected from a sulfurous spring in the Santa Maria Incaldana site (Mondragone, Caserta Province, Campania, southern Italy).

Description of *Oceanobacillus oncorhynchi* subsp. *oncorhynchi* subsp. nov.

The creation of *Oceanobacillus oncorhynchi* subsp. *incaldanensis* automatically creates the subspecies *Oceanobacillus oncorhynchi* subsp. *oncorhynchi*. The description is the same as that given for *Oceanobacillus oncorhynchi* by Yumoto *et al.* (2005). The type strain is strain R-2^T (=JCM 12661^T=NCIMB 14022^T).

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