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***Geobacillus thermoleovorans* subsp. *stromboliensis* subsp. nov., isolated from the geothermal volcanic environment**

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A novel thermophilic, aerobic, endospore-forming bacterium, designated strain Pizzo^T, was isolated from geothermal volcanic environment. Samples were collected from the Pizzo sopra la Fossa site at Stromboli Island (Eolian Islands, south of Italy) at the high altitude of 918 m. Cells of strain Pizzo^T were rod-shaped and stained Gram-positive. Growth was observed between 50 and 75°C (optimum 70°C) and at pH 5.0–8.0 (optimum pH 7.0). NaCl (0.4%, w/v) supported growth and among the hydrocarbons tested none induced growth. The G+C content of the DNA was 54.1 mol% and the sequence analysis of the 16S rRNA gene showed that the new isolate was phylogenetically closely related to the members of the *Bacillus* rRNA Group 5. DNA-DNA hybridization studies revealed a borderline similarity between the new isolate and *Geobacillus thermoleovorans* DSM 5366^T (69.8%) and *Geobacillus kaustophilus* DSM 7263^T (63.4%). On the basis of phylogenetic analysis and physiological traits of the isolate, it should be described as a new member of the *Geobacillus thermoleovorans* species and it is proposed that strain Pizzo^T can be classified as *Geobacillus thermoleovorans* subsp. *stromboliensis*, subsp. nov. (ATCC BAA-979^T; DSM 15393^T).

Key Words—enzymes; Eolian Island; fatty acid; *Geobacillus thermoleovorans*; lipid; thermophiles

Introduction

A number of aerobic thermophiles have been isolated from a variety of geothermal environments such as hot springs, solfataric fields and hydrothermal vents throughout the world. Members of the genus *Bacillus* are probably the most frequently isolated thermophilic aerobes from terrestrial and marine hot-water environments (Caccamo et al., 2000; Gugliandolo et al., 2003; Logan et al., 2000; Maugeri et al., 2002; Nazina et al., 2001; Nicolaus et al., 1996, 2000; Sako et al., 2001; White et al., 1993). The taxonomy of the genus *Bacil-*

lus showed that thermophilic species were members of *Bacillus* rRNA Group 5 (Ash et al., 1991; Rainey et al., 1994). Accordingly to the level of DNA-DNA reassociation values *Bacillus thermoleovorans*, *Bacillus kaustophilus* and *Bacillus thermocatenulatus* should be combined into one species, namely *Geobacillus thermoleovorans* which also included *Bacillus caldolyticus*, *Bacillus caldovelox* and *Bacillus caldotenax* (Nazina et al., 2001; Sunna et al., 1997).

In this study we describe the isolation and characterization of a new thermophilic *Geobacillus* strain from a volcanic geothermal environment at the Pizzo sopra la Fossa site on Stromboli Island (Eolian Islands, Italy). On the basis of morphological, physiological and phylogenetic studies, this new isolate is found to be closely related to the members of *Geobacillus thermoleovorans*.

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Materials and Methods

Description of sampling site. Stromboli is situated in the north of the Eolian Islands (Italy) and lies about 50 km NE of Lipari. The island rises from a depth of about 2,000 m; thus the entire edifice of Stromboli ranks in height next to Etna (about 3,340 m). The highest point of the volcano (I Vancori) lies at 924 or 926 m (according to various sources) above sea-level, whereas the place commonly described as the summit, named Pizzo sopra la Fossa, reaches 918 m elevation.

Normal activity is defined as Strombolian explosions occurring at irregular intervals lasting from 5 min to 1 h, with explosions consisting of short bursts of incandescent lava fragments, ash, or both together. The light-colored material exposed on the left side of Pizzo consists of strongly fumarolized pyroclastic material with hot and acid gases irregularly bedded near vents.

Sampling and isolation. Samples, consisting of ash and geothermal soil, were collected from Pizzo sopra la Fossa (Italy, Eolian Islands, site Stromboli) at a 50 cm depth where temperature and pH measured 75°C and 8.0 respectively.

Samples were incubated in the following media: YN medium (% value are in w/v): yeast extract (0.6%, Oxoid), NaCl (0.4%); Bacto Marine Broth 2216 (Difco); and TH medium (Romano et al., 2004). Cultures were incubated at 70 and 75°C at different pH values. The enrichment of aerobic, thermophilic, heterotrophic bacteria was done into YN medium at 70°C and pH 7.0. Isolation was done in the same medium supplemented with agar (2%, Oxoid). After 1 day of incubation, cream colonies formed on the plates. Well-isolated colonies were picked and the cells were incubated in fresh liquid YN medium at 70°C. To ensure purity, the streaking and isolation steps by using the technique of serial dilutions were repeated at least three times. The first pure culture was designated strain Pizzo^T and was investigated in detail.

Organic substrates for growth. In an attempt to find organic substrates that could support or stimulate growth of the isolate, various organic substrates were tested using M162 minimal medium (Degryse et al., 1978; Romano et al., 2004). Each of the following substrates was added alone or with 0.06% yeast extract at a concentration of 1% (w/v): D-glucose, mannose, D-xylose, galactose, sucrose, cellobiose, maltose, D-trehalose, D-cellobiose, D-fructose, lactose, Casamino

acids, starch and tryptone peptone (Oxoid), and 0.1 to 1.0% *n*-alkanes (v/v) (from 10 to 16 carbons in length). The cells were pre-cultured in each medium prior to inoculation of the same medium. Unless otherwise stated the metabolic and growth studies were carried out in the enrichment medium YN. The temperature range for growth was determined by incubating the isolate from 37 to 75°C. The pH dependence of growth was tested in the pH range 4.0 to 7.0. NaCl requirement was determined by varying the concentration of NaCl from 0 to 5% (w/v) in YN medium.

All growth tests were done at 70°C and growth was scored positive if the Optical Density at 540 nm was greater than 0.300 after 24 h.

Morphological studies. Cellular morphology was determined by phase-contrast microscopy (Zeiss) and by scanning electron microscopy (SEM). For SEM analysis the samples were fixed for 24 h in 2.5% glutaraldehyde. They were subsequently dehydrated in a graded series of ethyl alcohol, critical point dried, gold coated by sputtering (SEM BALTECMED 020) and observed with a Philips XL 20 ESEM.

Colony morphology was determined with a Leica M8 stereomicroscope, using cultures grown on agar plates for 24 h at 70°C. Gram staining was performed according to Dussault (1955). The KOH test was performed according to Halebian et al. (1981).

Phenotypic studies. Most phenotypic characteristics were examined as applied by Maugeri et al. (2002) and Nicolaus et al. (2002) in YN medium and YN agar medium incubated at 70°C for 3 days.

Starch hydrolysis was tested by flooding cultures on solid enrichment medium YN containing 0.2% (w/v) starch with Lugol's iodine. For the spore formation test, enrichment medium YN plus 0.001% (w/v) MnCl₂·4H₂O was used. Antibiotic tests were performed as reported previously (Nicolaus et al., 2002).

Aminopeptidase was assayed with a Bactident Aminopeptidase Kit from Merck (Germany). Hydrolysis of *N*'-benzoyl-arginine-*p*-nitroanilide (BAPA) stereoisomers was tested according to Oren and Galinski (1994). Catalase activity was determined by the formation of oxygen bubbles with 3% hydrogen peroxide solution. Oxidase activity was determined by the oxidation of 1% (w/v) TMPD (tetramethyl-*p*-phenylenediamine dihydrochloride) solution on filter paper at room temperature using *Thermus thermophilus* HB8^T (ATCC 27634^T) as a positive control. All tests were carried out on cells grown in the enrichment medium YN at 70°C.

Membrane lipid analyses: Cells were harvested in the late exponential growth phase by centrifugation. Freshly harvested cells (5–10 g) were lyophilized and extracted by Soxhlet with $\text{CHCl}_3/\text{MeOH}$ (1 : 1 by vol.) for 5 h at 70°C (Nicolaus et al., 1995).

Lipids were analyzed by thin layer chromatography (TLC) on silica gel (0.25 mm, F254, Merck) eluted with $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ (65 : 25 : 4 by vol.). Lipids were detected by spraying the plates with 0.1% $\text{Ce}(\text{SO}_4)_2$ followed by heating at 100°C for 5 min. Staining tests for complex lipids were performed using specific reagents for phospho-, amino- and glycolipids (Manca et al., 1992).

The total lipid extract was treated with two volumes of *n*-hexane at 30°C for 12 h. The quinone content was analyzed on high-performance liquid chromatography (HPLC) using an RP-18 Lichrospher (250×4 mm) column eluted with *n*-hexane/ethylacetate (99 : 1 by vol.) with a flow rate of 1.0 ml/min. Compounds were identified by ^1H NMR and MS as previously described (Nicolaus et al., 1995).

Lipid hydrolysis was performed by acid methanolysis. GC-MS analyses were performed with an HP5890 series II plus-5989B equipped with an HP-V column with a flux of 45 ml/min. Fatty acid methyl esters were detected using the temperature program of 120°C (1 min), from 120 to 250°C at 2°C/min; the identification of compounds was obtained with standards, and by interpretation of mass spectra.

Enzymatic activities: Strain Pizzo^T was grown in YN medium at 70°C. Cells were collected during the stationary phase by centrifugation at 9,000×*g* for 30 min. The collected cells washed with isotonic saline solution, lysed by freeze-thawing followed by an ultrasonic treatment (Heat System Instrument) for 4 min and suspended in 20 mM Tris-HCl pH 7.2 (1 : 3, w/v; crude homogenate). The crude homogenate was dialyzed against 20 mM sodium phosphate buffer at pH 7.0, overnight. Enzyme activities were assayed in the same buffer using synthetic substrates (Nicolaus et al., 1999).

Genetic characterization. The total 16S rRNA gene sequence was determined by direct sequencing of PCR-amplified 16S rDNA.

Genomic DNA extraction, PCR mediated amplification of the 16S rDNA and purification of PCR products were carried out as described previously (Rainey et al., 1996). Purified PCR products were sequenced using the ABI PRISM[™] Dye Terminator Cycle Se-

quencing Ready Reaction Kit (Applied Biosystems, Germany) as directed in the manufacturer's protocol. Sequence reactions were electrophoresed using the Applied Biosystems 373A Sequencer. The resulting sequence data from the strain was put into the alignment editor ae2 and compared with representative 16S rRNA gene sequences of organisms belonging to the *Geobacillus* group (Maidak et al., 1999). For comparison, 16S rRNA gene sequences were obtained from the EMBL data base or RDP (Maidak et al., 1999; Nazina et al., 2001).

The % G+C content was determined by the HPLC method (Mesbah et al., 1989; Tamaoka and Komagata, 1984). The calibration was performed with non-methylated λ DNA (Sigma) G+C content 49.85 mol%, and with *Halomonas pantelleriensis* DNA G+C content 65.02 mol%.

The DNA-DNA hybridization (in 2× SSC at 69°C) was performed with DNA from *Geobacillus thermoleovorans* DSM 5366^T, *Geobacillus kaustophilus* DSM 7263^T, *Geobacillus vulcani* DSM 13174^T and with DNA from strain Pizzo^T. For spectroscopic DNA-DNA hybridization, DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). DNA-DNA hybridization was carried out as described previously (De Ley et al., 1970; Huss et al., 1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6×6 multicell changer and a temperature controller with in-situ temperature probe (Varian). ATCC American type collection; DSM Deutsche Sammlung von Mikroorganismen; EMBL European Molecular Bioinformatics Institute (data-base).

Results and Discussion

Morphology and growth parameters

Enrichment cultures in YN medium were from the samples obtained at a 50 cm depth at the site Pizzo sopra la Fossa at 75°C and pH 8.0. The enrichment cultures grown at 70°C consisted of long rod-shaped bacteria and were streaked onto YN agar plates. Most of the colonies were large (2 mm in diameter), cream and rough, although several small (1 mm in diameter), light yellow colonies were observed. A single large, cream colony was purified and designated strain Pizzo^T and investigated in detail.

Cells of strain Pizzo^T were Gram-positive motile

rods, about 5.0–7.0 μm long and 0.5–0.9 μm wide, with terminal cylindrical endospores (Fig. 1). The isolate grew under aerobic culture conditions between 50 and 75°C, reaching the exponential growth phase within 19 h. Its optimal growth occurred at 70°C in media at neutral pH, but it tolerated pH values between 5.5 and 8.0. The isolate did not require NaCl for growth but was able to tolerate NaCl concentration up to 1.5%. Optimum growth conditions were detected at 0.4% NaCl, 70°C and pH 7.0.

Nutrition

The isolate was able to utilize complex organic substrates such as yeast extract, Casamino acids and tryptone as energy and carbon sources. Furthermore, (1%, w/v) sucrose, maltose, cellobiose, lactose and trehalose improved the growth yield of the isolate in the presence of 0.01% yeast extract. However, none of the hydrocarbons used in this study stimulated growth.

Phenotypic characters

Among the tested antibiotics, it was resistant to nystatin (100 μg) and sensitive to bacitracin (10 μg), neomycin (30 μg), tetracyclin (30 μg), chloramphenicol (10 μg), vancomycin (30 μg), erythromycin (30 μg), streptomycin (25 μg), penicillin G (10 μg), ampicillin (25 μg), novobiocin (30 μg) and kanamycin (30 μg). The isolate Pizzo^T was oxidase, nitrate reduction, D-tyrosine decomposition, hydrolysis of hippurate and starch positive, sensitive to lysozyme, and weakly reactive to catalase. Negative results were obtained for hydrolysis of casein and gelatin, D-L phenylalanine deamination, and indole production. Differential phenotypic characters of strain Pizzo^T and *Geobacillus* reference strains are shown in Table 1.

When the strain Pizzo^T was grown at 70°C, the major cellular fatty acids were iso-C15:0 (41%), iso-C16:0 (12.8%), normal-C16:0 (3.0%), iso-C17:0 (31.3%) and anteiso-C17:0 (11.4%). Other fatty acids occurred in trace amounts. Menaquinone-7 was the major respiratory quinone. Five phospholipids were detected, one aminophospho, one glycopospho and three phospholipids.

Isolate Pizzo^T was negative for xylanase, α and β -glucosidase, α -mannosidase, esterase and β -galactosidase activities, and it was strongly positive for α -galactosidase and α -amylase activities.

Fig. 1. Scanning electron microscopy of strain Pizzo^T.

After a dehydration procedure the sample is coated with Au-Pd alloy. The coating provides the entire sample surface with a homogeneous layer of metal (18 nm). Finally the sample morphology is observed by using a SEM Philips XL 20 series microscope.

Phylogenetic analyses

The G+C mol% content of DNA of strain Pizzo^T was 54.1, falling into the range values determined for members of *Geobacillus thermoleovorans* (Nazina et al., 2001; Sunna et al., 1997; Zarilla and Perry, 1987) to which strain Pizzo^T belongs phylogenetically. The results of the almost complete 16S rRNA gene sequence (1,508 nt) indicated that *Geobacillus* strain Pizzo^T shared more than 99% sequence similarity to the type strains *G. thermoleovorans* DSM 5366^T (99.6%), *Bacillus vulcani* DSM 13174^T (99.3%) recently reclassified as *Geobacillus vulcani* (Nazina et al., 2004), and *Bacillus caldotenax* DSM 406 (99.2%), and showed 98% similarity with *G. kaustophilus* DSM 7263^T (98.6%), *B. caldolyticus* DSM 405 (98.6%), *B. caldovelox* DSM 411 (98.8%) and *G. lituanicus* DSM 15325^T strain N-3 (98.8%). (Table 2). These data clearly indicated that strain Pizzo^T was phylogenetically closely related to the members of *Geobacillus thermoleovorans* (Nazina et al., 2001). Previous studies have shown that all these thermophilic bacilli named above belong to Group 5 and this group was a phenotypically and phylogenetically coherent group displaying very high similarity among their 16S rRNA gene sequences (Nazina et al., 2001; Rainey et al., 1994). These data supported the genotypic homogeneity of this group.

DNA-DNA hybridization experiments between strain

Table 1. Phenotypic and chemo-taxonomic properties of *Geobacillus* strain Pizzo^T and related thermophilic microorganisms.

	<i>Geobacillus</i> strain Pizzo ^T	<i>Geobacillus thermoleovorans</i> DSM 5366 ^{Ta}	<i>Geobacillus vulcani</i> DSM 13174 ^T	<i>Geobacillus kaustophilus</i> DSM 7263 ^T
G+C mol% DNA	54.1	51–56	53.0	51.0
Catalase	w	v	—	+
Oxidase	+	v	—	—
Growth at 75°C	+	+	—	+
Optimal temperature	70°C	55–65°C	60°C	65°C
Growth with 3% NaCl	—	—	+	—
Anaerobic growth	—	v	—	—
Formation of acetoin	+	—	+	—
Indole	—	—	nd	—
Utilization citrate	—	v	+	+
<i>n</i> -Alkanes	—	+	nd	+
Nitrate reduction	+	+	—	+
Denitrification	—	v	—	+
Hydrolysis of casein	—	+	—	—
Tween 20	—	—	+	nd
Gelatine	—	—	—	+
Starch	+	+	nd	+
α -Glucosidase	—	+	—	nd

Legend: w, weak reaction; v, variable; nd, not determined.

^a Showed the phenotypic characters of the group of *B. thermoleovorans*, *B. caldolyticus* DSM 405, *B. caldotenax* DSM 406, *B. caldovelox* DSM 411 and *B. thermocatenulatus* DSM 730, which were combined in one species, *B. thermoleovorans*, DSM 5366^T (Sunna et al., 1997). Data were obtained from the present study or from Caccamo et al. (2000); Nazina et al. (2001); or Sunna et al. (1997).

Table 2. % 16S rRNA gene sequence similarity values for strain Pizzo^T and related thermophilic microorganisms.

Strains	1	2	3	4	5	6	7	8	9
1. Strain Pizzo ^T	—								
2. <i>Geobacillus thermoleovorans</i> CCR 11	99.6	—							
3. <i>Geobacillus vulcani</i> DSM 13174 ^T	99.3	99.5	—						
4. <i>Geobacillus thermoleovorans</i> DSM 5366 ^T	99.2	99.7	99.6	—					
5. <i>Bacillus caldotenax</i> DSM 406	99.1	99.5	99.3	99.7	—				
6. <i>Bacillus caldolyticus</i> DSM 405	98.7	99.3	99.1	99.5	99.5	—			
7. <i>Bacillus caldovelox</i> DSM 411	98.8	99.0	98.9	98.9	99.0	99.1	—		
8. <i>Geobacillus lituanicus</i> N-3 DSM 15325 ^T	98.9	99.0	98.1	98.5	98.2	98.2	98.2	—	
9. <i>Geobacillus kaustophilus</i> DSM 7263 ^T	98.7	99.3	98.3	98.5	98.5	98.1	98.2	98.9	—

Accession numbers: *Geobacillus thermoleovorans* CCR 11, AJ536599; *Geobacillus vulcani* DSM 13174^T, AJ293805; *Geobacillus thermoleovorans* DSM 5366^T, Z26923; *Bacillus caldotenax* DSM 406, Z26922.1; *Bacillus caldolyticus* DSM 405, Z26924.1; *Bacillus caldovelox* DSM 411, Z26925; *Geobacillus lituanicus* N-3 DSM 15325^T, YA044055; *Geobacillus kaustophilus* DSM 7263^T, X60618.

Pizzo^T and those thermophilic bacilli strains showing more than 99% 16S rRNA gene similarity values, revealed borderline reassociation values between the new isolate and *G. thermoleovorans* DSM 5366^T (69.8%) and *G. kaustophilus* DSM 7263^T (63.4%)

(Wayne et al., 1987). A lower DNA reassociation value was found between strain Pizzo^T and *G. vulcani* DSM 13174^T (54.3%).

Strain Pizzo^T differed both genotypically and phenotypically from the recognized species of the genus

Geobacillus. The strain utilized sucrose and lactose but not citrate or *n*-alkanes; it did not hydrolyze casein or gelatin. Moreover optimal growth temperature, NaCl requirement, oxidase and catalase tests, nitrate reduction, citrate utilization and Tween 20 hydrolysis allowed us to differentiate strain Pizzo^T from *Geobacillus kaustophilus* and the representatives of the genus *Geobacillus* (Table 1).

On the basis of these results, we propose that thermophilic *Geobacillus* strain Pizzo^T is a novel subspecies of *Geobacillus thermoleovorans*, for which the name *Geobacillus thermoleovorans* subsp. *stromboliensis* is proposed.

Description of *Geobacillus thermoleovorans* subsp. *stromboliensis* subsp. nov. *Geobacillus thermoleovorans* subsp. *stromboliensis* (strom. bo. lien'. sis; M.L. gen. n. adj. *stromboliensis* pertaining to Stromboli—Eolian Islands where the type strain was isolated).

Cells are Gram-positive, motile rods, 5.0–7.0 µm long × 0.5–0.9 µm wide, with cylindrical endospores. Colonies are whitish and 2 mm in diameter. Aerobic, thermophilic, neutrophilic, heterotroph. Growth occurs at temperatures of 50–75°C (optimum 70°C) at pH 5.5–8.0 (optimum 7.0) and in presence of 0–1.5% NaCl (optimum 0.4% NaCl). Oxidase positive, catalase weak reaction. The major fatty acids are iso-C15:0 (41%), iso-C16:0 (12.8%), normal-C16:0 (3.0%), iso-C17:0 (31.3%) and anteiso-C17:0 (11.4%). Menaquinone-7 was the major respiratory quinone. Carbon sources supporting growth: sucrose, maltose, cellobiose, lactose and trehalose. Growth occurs in complex organic substrates such as yeast extract and tryptone-peptone. Positive results were obtained for α-amylase and α-galactosidase activities, nitrate reduction, starch and hippurate hydrolysis. The DNA base composition of the type strain Pizzo^T is 54.1 mol%. The type strain is Pizzo^T (=DSM 15392^T=ATCC BAA-979^T). The 16S rDNA sequence of this strain is deposited at EMBL under accession number AJ704828. Isolated from Stromboli Island, Eolian Islands, Italy.

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