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Identification of enzyme-producing thermophilic bacilli isolated from marine vents of Aeolian Islands (Italy)

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Abstract - Enzyme-producing thermophilic bacilli were isolated from different thermal sites located in and around Aeolian Islands (Italy) and characterised by both molecular and culture-based methods. Spore-forming bacteria with optimal growth from 45 to 70 °C were isolated from submarine vents and a geothermal well of Aeolian Islands (Italy). Eighteen selected strains were screened for extracellular enzyme presence by using nine substrates: Tween 20, Tween 80, tributyrin, soluble starch, xylan, dextran, carragenan, gelatine and casein. Sixteen isolates were able to grow at pH 9. The isolates were differentiated on the basis of restriction pattern of their amplified 16S rDNA (ARDRA) prior to 16S rRNA gene sequence analysis. On the basis of the most complete sequencing results strain V3 was identified as *Geobacillus thermodenitrificans*, most of isolates (10/14) was similar at high level (≥ 95%) to different reference strains of the species *Bacillus licheniformis*. The remaining isolates, exhibiting sequence similarity below 95%, may represent novel species of the genus *Bacillus*.

Key words: enzyme screening, hydrothermal vents, thermophilic bacilli.

INTRODUCTION

Thermophilic bacteria show optimal growth at temperatures ranging from 45 to 70 °C and can be isolated from both thermophilic and mesophilic environments (Marteinsson *et al.*, 1996). They are source of products for industrial use, such exopolysaccharides or compatible solutes and thermostable enzymes, named thermozymes (Harwood, 1989; Sharp *et al.*, 1992; Rainey *et al.*, 1994; Bruins *et al.*, 2001; Demirjian *et al.*, 2001; Maugeri *et al.,* 2001; Yavuz *et al.*, 2004). Thermal stability enables thermozymes to be active in the presence of chemical denaturants and to resist harsh process conditions (Kristjansson, 1989).

Bacillus and new *Geobacillus* species are considered more useful bacteria in industrial process. *Bacillus subtilis* is the dominant enzyme-producing microorganism. Its cultural characteristics, high growth rate, its capacity to excrete protein into cultivation medium and its security for food industry are good reasons for the choice. Strains of *Bacillus licheniformis* are also used by industry to produce useful enzymes as proteases and amylases.

Strains of *Geobacillus stearothermophilus* are producers of xylanase, pullulanase, utilised in the starch industry, and of α-amylase in beverage production (Pandey *et al.*, 2000; Schallmey *et al.*, 2004).

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Lipases have been isolated and characterised from thermophiles, mainly from *G. thermoleovorans* (Lee *et al.*, 1999) and from *Geobacillus* sp. TW1, a species isolated from a hot spring in China (Li and Zhang, 2005). All *Bacillus* reference species described so far were isolated from compost, mud, air and water of terrestrial hot springs (Sharp *et al.*, 1992; Andersson *et al.,* 1995; Combet-Blanc *et al.*, 1995; Meier-Stauffer *et al.*, 1996; Blanc *et al.*, 1997). Until recently, few were the reports about thermophilic bacilli inhabiting marine hot springs (Hjörleisfdottir *et al.*, 1989; Marteinsson *et al.*, 1996). Members of the genus *Bacillus* are considered the most frequently isolated thermophilic aerobes from terrestrial and marine hot-water environments (Logan *et al.*, 2000; Caccamo *et al.*, 2000; 2001; Maugeri *et al.*, 2002a; 2002b; Gugliandolo *et al.*, 2003). Members of *Bacillus* rRNA group 5 (Ash *et al.*, 1991) have been renamed as *Geobacillus* spp., accordingly to the level of DNA-DNA reassociation values (Nazina *et al.*, 2001).

Shallow, marine thermal vents of Aeolian Islands (Italy) allowed isolating and characterising thermophilic spore-formers that showed a high heterogeneity in respect to known thermophilic bacilli (Maugeri *et al.*, 2001). Genotypic studies allowed to recognise a new *B. licheniformis* strain and five new thermophilic species, *Geobacillus vulcani* (Caccamo *et al.*, 2000), three novel *Geobacillus* spp., strains 1bw, 5-2 and 10-1 (Maugeri *et al.*, 2002a), *Bacillus aeolius* (Gugliandolo *et al.,* 2003).

In this study we describe the isolation and characterisation of 18 thermophilic bacilli producers of attractive

enzymatic activity isolated from the thermal environment of Aeolian Islands. Novel thermophilic strains of *Bacillus* and *Geobacillus* genera are very promising producers of enzymes with optimal activity under high temperature and alkaline conditions.

MATERIALS AND METHODS

Sampling and isolation. Water and sediment samples were collected in April and September 2005, from five different shallow, submarine thermal vents located around the Aeolian Islands of Vulcano, Panarea and Lipari (Italy) and from a terrestrial geothermal well at Lipari Island (Table 1). Marine sampling was made by scuba divers using sterile samplers. Temperature and pH of water were immediately recorded by a multiparameter probe (CS304 Greenspan Technology).

Water and sediment samples were spread onto Bacto Marine agar 2216 (MA, Difco) plates and incubated in aerobic conditions at 55 °C for three days. After isolation and purification, strains were routinely cultivated at 55 °C and maintained onto MA slants.

Eighteen field strains, listed in Table 1, were selected for their optimal growth temperature (thermophilic bacteria). They were compared to the following thermophilic strains of rRNA group 5 *G. stearothermophilus* DSM 22^T, *G. thermodenitrificans* DSM 466 and *G. thermoleovorans* DSM 5366^T (included as reference strains), as well as three strains isolated from Aeolian Islands system, *G. vulcani* DSM 13174; *B. aeolius* DSM 15084 and a *B. licheniformis* lab strain, (Aeolian reference strains).

Phenotypic studies. The isolates were Gram stained and observed for cell morphology, spores production and motility. Oxidase and catalase activity was also tested.

Temperature and pH range for growth was determined following incubation of the strains for 3 days at 37, 50, 55, 60, 65 and 70 °C and pH 5.5, 6.0, 7.0, 8.0 and 9.0 in Bacto Marine broth 2216 (MB, Difco). Halotolerance was tested in Bacto Nutrient broth supplemented with 0, 2, 3, 5, 7 and 10% (w/v) NaCl. Optimal growth in MB was evaluated by measuring the increase in turbidity at 600 nm with a spectrophotometer (Ultraspec 3000, Amersham Pharmacia Biotech).

Biochemical characteristics were screened by the miniaturized systems API 20E, API 20 NE, API 50CHB and API ZYM (bioMérieux) according to Maugeri *et al.* (2001). Strips were incubated at 55 °C for 24 h.

Screening for enzymes. Lipase was tested by using Tween 20 (0.5%, v/v) and Tween 80 (0.5%, v/v) on Sierra agar modified (Sierra, 1957). Hydrolysis of tributyrin (1%, v/v), casein (10% skimmed milk), gelatine (0.4%, w/v) and starch (1%, w/v) was performed in mineral medium D (MD) reported in Maugeri *et al*. (2001). Hydrolysis of xylan (0.5, w/v), dextran (0.5, w/v) and carragenan (0.1%, w/v) was tested according to White *et al*. (1993).

DNA extraction and PCR amplification. Genomic DNA from isolates was extracted according to Ausubel *et al*. (1994). The 16S rRNA genes were amplified by PCR using universal bacterial primers 27f (5'-GAGTTTGATCCTGGCTCAG-3'; position 9-27 in *E. coli* numbering) and 1525r (5'-AGAAAGGAGGTGATCCAGCC-3'; positions 1542-1525 in *E. coli* numbering) (Rainey *et al.*, 1994).

PCR reactions were performed with a HotStar*Taq* Master MixKit (Eppendorf) and a PCR Sprint thermal cycler (Hybaid). The reaction mixtures contained (per 50 µl) 1 µl PCR buffer (50 mmol l^{-1} KCl, 10 mmol l^{-1} Tris-HCl pH 8.3, 1.5 mmol l^{-1} $MgCl_2$), each deoxynucleoside triphosphate at a concentration of 200 µmol l^{-1}, each primer at a concentration of 0.5 µM, and 2.5 U of HotStar*Taq* DNA polymerase, and 5 µl of extracted DNA as templates. The temperature profile for the PCR was as follows: initial denaturation at 95 °C for 1 min, followed by denaturation at 95 °C for 40 s, annealing at 50 °C for 1 min, and primer extension at 72 °C for 1 min. After the 35th cycle, the extension step was prolonged for 10 min to complete synthesis of all strands, and then the samples were kept at 4 °C until analysis. Negative and positive controls were included in every experiment. PCR products were detected by gel electrophoresis. Samples (5 µl) of final PCR products were loaded onto 1.2% agarose gels and subjected to electrophoresis in 1X TAE buffer (0.04 mol l^{-1} Tris-acetate, 0.001 mol l^{-1} EDTA) for 60 to 90 min at 70 V. The gels were stained with ethidium bromide and photographed with UV light transillumination.

Restriction analysis of 16S DNA. Amplified ribosomal DNA restriction analysis (ARDRA) was used as a pre-screen method for clustering organisms in genetically homogeneous groups before 16S rRNA gene sequencing. Amplified 16S rRNA gene sequences obtained with the two universal

TABLE 1 - Characteristics of hydrothermal sites and related isolates

Aeolian Island	Site	Type*	Depth (m)	T (°C)	pH	Isolate
Vulcano	Porto di Levante-Acque calde 1	V	6.0	48	5.60	V8, V9, V10, V11, V12
	Porto di Levante-Acque calde 2	V	0.3	65	5.20	V1, V3, V4, V5, V6
	Punta Conigliara	V	15.0	45	6.09	Vc1, Vc2, Vc3
Panarea	La Calcara	V	19.8	95	5.10	P1, P3
Lipari	Terme di San Calogero	W	0	46	7.26	Lt1, Lt2
	Inzolfata	V	3.1	30	5.86	Li

* V: shallow submarine vent, W: geothermal well.

primers 27f and 1525r were digested with 3 units of the restriction enzyme *Alu*I (Boheringer Mannheim).

Approximately 20-50 ng of amplified 16S rDNA products were cleaved in a total volume of 20 µl by incubating the reaction mixtures at 37 °C for 3 h. The enzyme was then inactivated by heating the mixtures at 65 °C for 15 min. The reaction products were analysed by agarose gel (2.5% w/v) electrophoresis in TAE buffer (0.04 mol l^{-1} Tris-acetate, 0.001 mol l^{-1} EDTA) stained with ethidium bromide.

16S rDNA sequence determination and analysis. Isolates (14 out of 18) showing unique ARDRA profiles were selected for 16S rDNA sequencing. The PCR products were purified using the Wizard Genomic DNA Purification kit (Promega) according to the manufacturer's protocol and sequenced by an ALFexpress DNA sequencer (Amersham Biosciences). BLAST software (**http://www.ncbi.nlm.nih.gov/blast/**) and the *Ribosomal Database Project* (RDP) version II available at the web site (**http://rdp.cme.msu.edu/index.jsp**) were used to conduct homology searches of the GenBank database (Altschul *et al.*, 1997).

RESULTS AND DISCUSSION

Physico-chemical characteristics of the examined samples are reported in Table 1. The source of the isolates was the initial of their names: V specified for Vulcano isolates, P for Panarea and L for Lipari.

Phenotypic characteristics

Eighteen strains of thermophilic bacteria spore forming, Gram-positive, catalase and oxidase positive were isolated from Aeolian Islands.They fermented ribose, fructose, maltose and grew on sucrose. All strains were able to grow at 37 °C and most from 37 °C until 60 °C. Only the *G. stearothermophilus* DSM 22^T was able to grow from 37 until 75 °C (Table 2). Most of field isolates (16/18) was able to grow at pH 9. Strains P1, P3, Vc1 and Lt1 needed 2% of NaCl for growth. Strains Lt1 and Vc1 were able to grow from 2 to 5% of NaCl. Vc1 exhibits optimum growth at 5% NaCl. Esterase on C4, esterase lipase (C8), alkaline and acid posphatase, and phosphoamidase were the activities more frequently found in reference and field strains essaying by API ZYM system.

All the environmental isolates were able to hydrolyse gelatine. Seventeen isolates hydrolysed xylan, 16 carrageenan, 15 dextran and Tween 20, 12 isolates starch, only 10 hydrolysed tributyrin, casein and Tween 80 (Table 2). V3 and V8 isolates were active on all assayed substrata. The strains V1, V4, V5 and V9 hydrolysed 8 substrata. Seven isolates, V10, V11, Vc1, Vc2, Vc3, Lt1, Lt2, utilised seven substrata as *G. vulcani*. The isolates P1 and V6 hydrolysed six substrata as *B. aeolius*. Li and P3 hydrolysed 5 substrata. Finally, strain V12 hydrolysed only 3 substrata. All *Geobacillus* spp., except *G. vulcani*, an Aeolian strain able to grow on Tween 20, were unable to hydrolyse lipids (Table 2).

Majority of the isolates here studied showed lipolytic and amylolytic activities. Microbial lipases have been used as important biocatalysts in biomedical applications. Because of their excellent catalytic performance in a variety of organic solvents, they could be used for the synthesis of compounds of pharmaceutical interest. Lipases are also used for transesterification and for racemization reactions to produce optically pure enantiomers (Muralidhar *et al.*, 2002). Such kinetic resolution processes are very important for example in the preparation of non steroidal anti-inflammatory drugs, anti-tumour agents, alkaloids, antibiotics and vitamins (Pandey *et al.*, 1999).

The genotypic characterisation

The genotypic characterisation by ARDRA shows that the isolates produced 14 distinct restriction patterns, containing from three to six fragments ranging in size from 150 to about 1000 base pairs. The cluster analysis (GelComparII, Applied Maths) allowed to group *G. thermodenitrificans* DSM 466, *G. thermoleovorans* DSM 5366^T and strain V3 in the same cluster. Remaining isolates grouped in another cluster where is possible to distinguish four homology groups (Group 1, 2, 3 and 4) and 9 different profiles. Group 1 contains the isolates V4 and V8, group 2 the isolates V5 and V6, group 3 the isolates V10 and V12, group 4 the isolates P1 and Vc1.

BLAST analysis of the most complete sequencing results revealed the similarity percentages among field isolates and reference strains (Table 3). Homology between the 16S rRNA gene sequences of V3 and *Geobacillus thermodenitrificans* HRO10 (accession no: AJ785764) (Ezeji *et al.*, 2005), was 100%.

The other isolates were associated with different *Bacillus* spp. showing sequence similarities between 93 and 99.9%.

Alignments between our isolates V6, V9 and Vc1 and *B. licheniformis* DSM13T (accession no: CP000002) showed sequence similarity from 99.6 to 99.9%. Isolates V8 and Lt1 exhibited 98% sequence similarity to two different strains of *B. licheniformis* (accession no: AW842874, AY842871). Isolates V1, P3 and Li exhibited 95-97% sequence similarity to three different strains of *B. licheniformis* (accession no: AF440444, AY030337, AY842871). Among the remaining isolates, Vc2 showed 97% sequence similarity to *Bacillus aeolius* DSM 15084 (accession no: AJ504797). Isolate V10 showed more distant relationship (93%) with *B. licheniformis* (accession no: AY536536). Finally, V11, Vc3 and Lt2 showed 95, 94 and 93% sequence identity to *Bacillus* spp., respectively. In Table 3 is enclosed the environmental origin of the closest related reference bacteria present in the NCBI database.

Among the field isolates, the highest sequence similarity (99.8 %) was found between isolate V9 and V6. The last was also similar (98%) to V8 and (97%) to Li, Lt1 and P3. V9 was both similar to Vc1 (99.9%) and Lt1 (97%). Lt1 and Li showed quite high sequence similarity (97%).

Riboprinting analysis

The isolates V6, V9 and Vc1, showing high 16S rDNA sequence similarity each other and to *B. licheniformis* DSM 13^T, but phenotypically different (Table 4), were further analysed by riboprinting using restriction enzyme *Eco*RI (Allerberger and Fritschel, 1999). In Figure 1 are shown the riboprint patterns of field isolates in comparison with *B. licheniformis* DSM 13^T and *B. licheniformis* lab strain. Strain V9 and Vc1 produced the same pattern, their pattern was different from those of the *B. licheniformis* DSM 13^T and the *B. licheniformis* lab strain. V6 exhibited a distinctive pattern in respect to the other strains.

TABLE 2 - Growth characteristics and enzymatic production on the nine selected substrates of isolates and reference strains

Strain	Growth T (°C)	Optimum T (°C)	Growth pH	Optimum pH	Growth NaCl (%)	Optimum NaCl (%)	Gelatine	Carragenan	Starch	Casein	Xylan	Dextran	Tributyrin	Tween 20	Tween 80
V1	37-60	55	5.5-9	6	0-5	0	+	+	+	–	+	+	+	+	+
V3	37-70	65	5.5-9	8	0-2	2	+	+	+	+	+	+	+	+	+
V4	37-60	50	5.5-9	5.5	0-2	2	+	+	+	+	+	+	–	+	+
V5	37-60	50	5.5-9	8	0-7	0	+	+	+	+	+	+	+	–	+
V6	37-60	50	5.5-9	7	0-7	2	+	+	+	+	–	–	–	+	+
V8	37-60	50	5.5-9	8	0-2	2	+	+	+	+	+	+	+	+	+
V9	37-60	50	7.5-9	9	0-2	2	+	+	+	+	+	+	+	+	–
V10	37-60	55	5.5-9	7	0-5	2	+	+	+	+	+	+	–	+	–
V11	37-60	55	5.5-9	6	0-2	0	+	+	+	+	+	–	+	+	–
V12	37-60	55	5.5-9	8	0-5	0	+	+	–	–	+	–	–	–	–
Vc1	37-55	50	7-7.5	7.5	2-5	5	+	+	+	+	+	+	+	–	–
Vc2	37-60	50	5.5-9	5.5	0-2	0	+	+	–	+	+	+	–	+	+
Vc3	37-60	50	5.5-9	8	0-5	2	+	+	–	–	+	+	+	+	+
P1	37-60	50	7-9	7	2	2	+	+	–	–	+	+	+	+	–
P3	37-55	50	7-9	7	2	2	+	+	–	–	+	+	–	+	–
Li	37-60	50	6-9	7	0-2	2	+	–	–	–	+	+	+	+	–
Lt1	37-55	50	5.5–8	5.5	2-5	2	+	+	+	–	+	+	–	+	+
Lt2	37-60	50	6-9	7	0-2	2	+	+	+	–	+	+	–	+	+
Aeolian reference strains															
Geobacillus vulcani	37-72	60	5.5-9	6	0-3	2	+	+	+	–	+	+	+	+	–
Bacillus aeolius	37-65	55	7-9	8	0.5-5	2	+	+	+	+	+	–	+	–	–
Bacillus licheniformis lab strain	37-65	55	7-9	7	2-3	2	+	+	–	–	–	–	+	–	–
DSM reference strains															
Geobacillus stearothermophilus 22^T	37-75	60	5.5-9	7	0-3	0	+	+	–	+	–	+	–	–	–
Geobacillus thermodenitrificans 466	37-70	55-65	5.5-9	6-9	0-5	0-2	+	+	+	–	–	–	–	–	–
Geobacillus thermoleovorans 5366^T	37-70	55-70	77	6-7.5	0-2	0	+	+	+	–	+	+	–	–	–

TABLE 3 - Similarity percentage (%) based on the most complete 16S rDNA gene sequences of isolates to their closest bacteria present in the NCBI database and environmental origin of reference strains.

Isolate	Sequence similarity (%)	Affiliation	Reference strain	GenBank accession number	Enviromental origin
V1	95.0	*Bacillus licheniformis*	WED 106	AF440444	Temperate estuarine waters
V3	100	*Geobacillus thermodenitrificans*	HRO10	AJ785764	Soil
V6	99.9	*Bacillus licheniformis*	DSM 13	CP000002	Soil
V8	98.0	*Bacillus licheniformis*	CICC10093	AY842874	Soil
V9	99.9	*Bacillus licheniformis*	DSM 13	CP000002	Soil
V10	93.0	*Bacillus licheniformis*	OWS-F3	AY536536	Temperate estuarine waters
V11	95.0	*Bacillus* sp.	DCA-5	DQ238044	China soil
Vc1	99.6	*Bacillus licheniformis*	DSM 13	CP000002	Soil
Vc2	97.0	*Bacillus aeolius*	DSM 15084	AJ504797	Shallow hydrothermal vents, Italy
Vc3	94.0	*Bacillus* sp.	SD-B2	AB189317	Diverse habitats, Japan
P3	97.0	*Bacillus licheniformis*	KL-185	AY030337	Jet Propulsion laboratories, USA
Li	97.0	*Bacillus licheniformis*	CICC 10181	AY842871	China soil
Lt1	98.0	*Bacillus licheniformis*	CICC 10181	AY842871	China soil
Lt2	93.0	*Bacillus* sp.	SD-B2	AB189317	Soil

TABLE 4 - Phenotypic and chemo-taxonomic properties of *B. licheniformis* isolates compared with the closest related type strain.

	Geobacillus	*Geobacillus thermodenitrificans*	*Bacillus licheniformis isolates*			*Bacillus licheniformis*
	strain V3	DSM 465	strain V6	strain V9	strain Vc1	DSM 13^T
G+C mol (%) DNA	50.6	50.3	44.9	44.8	45.1	44.7
Growth at/with:						
37 °C	–	–	+	–	–	+
70 °C	+	+	+	+	–	–
pH 5.5	+	+	–	–	–	+
pH 9	+	–	+	+	–	–
NaCl (0%)	+	+	+	+	–	–
NaCl (3%)	–	+	–	–	+	+
Denitrification	+	w	nd	nd	nd	–
Anaerobic growth	+	+	nd	nd	nd	nd
Hydrolysis of:						
Carragenan	+	nd	+	+	+	nd
Dextran	+	nd	–	+	+	nd
Xylan	+	nd	–	+	+	nd
Casein	–	w	–	+	+	+
Tributyrin	+	nd	–	+	+	nd
Starch	+	+	–	+	+	+
Gelatine	+	+	+	+	+	+
Tween 20	+	–	+	+	–	+
Tween 80	+	–	+	+	–	+
Utilization of:						
Citrate	–	–	+	+	+	nd
Glucose	–	+	–	–	–	–
Ramnose	–	–	–	–	–	nd
Lactose	–	+	–	–	+	–
Cellobiose	–	+	–	+	–	–
Galactose	–	+	–	–	–	+
Xylose	–	+	–	–	–	–
Ribose	–	+	+	+	+	nd
Arabinose	–	+	–	–	–	–

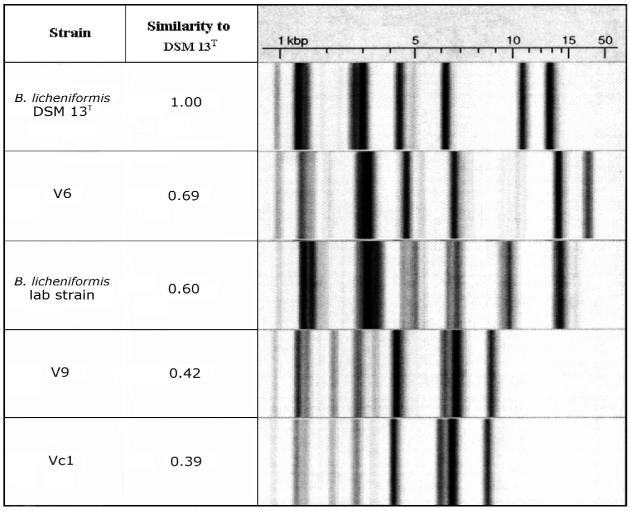

FIG. 1 - Riboprint pattern of strains V6, V9, Vc1, *Bacillus licheniformis* lab strain and *B. licheniformis* DSM 13^T generated with *Eco*RI.

All isolates of the present study displayed at least three enzymatic activities and most of them were able to grow at pH 9. Thus it might be suggested that these strains may be good sources of novel industrial application (Yu *et al.*, 1987; Mamo and Gessesse, 1999).

CONCLUSIONS

The new isolates from shallow thermal system of Aeolian Islands have been identified as strains of *Geobacillus* or *Bacillus* genera. Only one strain, the isolate V3, revealed 16S rRNA gene sequence identity to *G. thermodenitrificans* HRO10 a thermophilic bacillus producing α-amylase and α-glucosidase (Ezeji *et al.*, 2005). Most of isolates (10/14) was similar at high level (≥ 95%) to different reference strains of the species *Bacillus licheniformis*. As it has been proposed that sequence similarity must be below 95% to qualify a novel species (Fogel *et al.*, 1999), we can suppose that V10, Vc3 and Lt2 may represent novel species (Table 3).

The studied bacilli are similar at different percentage by alignment of their 16S rRNA gene sequencing to other deposited bacilli of different environmental origin. Some phenotypic characteristics are also different in respect to the type strains more similar at genetic level. Then the phenotypic characterisation of isolates appears useful to provide additional information on the diversity of microorganisms in nature and to clarify the mechanism of adaptation of microorganisms to their unusual environment. Our strains, phylogenetically related to known species but phenotypically divergent from these, may represent potential sources of new thermostable enzymes.

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