

Isolation of thermophilic aerobic heterotrophic bacteria from deep-sea hydrothermal vents (Manus Basin, Papua New Guinea)

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A numerical taxonomic study was carried out with thermophilic aerobic heterotrophic bacteria newly isolated from deep-sea hydrothermal vents (Manus Basin, Papua New Guinea) and several thermophilic reference strains representing *Bacillus* and *Thermus* species. The deep-sea isolates consisted of rods, single cells, pairs or filaments of variable lengths and grew aerobically above 65°C and some up to 75°C. Results from unweighted average linkage (UPGMA) clustering applied to a similarity matrix derived from the simple matching (SSM) coefficient showed the formation of a cluster defined at the 83% similarity level whereas 5 isolates remained unclustered.

Keywords: Thermophilic aerobic bacteria, *Bacillus*, *Thermus*, deep-sea, hydrothermal vent, numerical taxonomy.

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1. Introduction

The presently known aerobic heterotrophic thermophilic bacteria are essentially confined to *Thermus* and *Bacillus* genera. Both are present in thermal environments and widely distributed in thermal areas of the world.

The first publication on thermophilic microorganisms was appeared in 1888 and reported characterization of thermophilic spore-former, *Bacillus thermophilus* isolated from the river Seine in Paris [1]. Since then, numerous reports of *Bacillus* strains able to grow at or above 60°C and descriptions of thermophilic organisms have been published.

Bacillus strains can be isolated from a wide range of both thermophilic and mesophilic environments (see for review Allen [2] and Sharp [3]). Thermophilic environments are generally hot springs, solfatara or geothermally heated soils and man-made thermal systems. Most thermophilic microorganisms can only be found in natural thermal systems but *Bacillus* is ubiquitous and can be found in a number of other habitats that are only heated transiently and even in permanently cold places. Thermophilic strains have been isolated from soils, composting vegetation, sewage digesters, river-, lake- and sea-water, deep terrestrial subsurface habitats, canned foods, air and glacier ice (see for review Sharp [3], [4-6]). Several *Bacillus* strains have been isolated from deep ocean basin cores at 1,378 to 2,000m depth [7, 8] and from ocean-bottom mud samples from 50 to 500m [9]. They have also been isolated from shallow water marine hot springs [10] but only recently reported from deep-sea hydrothermal vents [11]. At present, the genus *Bacillus* includes about ten thermophilic species or strains documented to grow at or above 65°C but the list is expanding rapidly.

Thermus strains are normally found in all thermal systems above 55°C and at pH above 5.1 [12] and are probably the most common aerobic non-sporulating heterotrophs [13] present in many natural and man-made thermal systems. Although *Thermus* seems to be mainly found in terrestrial freshwater systems of low salinity it has also been isolated from some shallow water marine hot springs. These springs occur normally on the seashore or at shallow depth and are mostly of terrestrial origin. As for *Bacillus*, the presence of aerobic *Thermus*-

like thermophiles in deep-sea hydrothermal vents has been very recently demonstrated by Marteinsson *et al.* [14].

Several submersible surveys and sampling were conducted in the Desmos caldera, located in the eastern Manus back-arc basin, Papua New Guinea, and particularly at the Onsen site (Desmos hydrothermal site) where chemical characteristics of hydrothermal fluids, mainly discharging from white smoker chimneys and porous sediments, are atypical for sea floor venting: pH, alkalinity, and Ca values are extremely low, whereas H₂S, SO₄²⁻, and Si concentrations are high (Gamo *et al.* [15]).

In this paper we report the isolation and characterization of thermophilic *Bacillus* and *Thermus* strains growing at above 65°C in unusually acidic (pH<2.1) and sulfate-rich hydrothermal fluids venting with a magmatic contribution from a submarine caldera.

We describe also a numerical classification based on phenotypic features to compare the isolates together with several high temperature reference strains (growing at above 65°C) of the genera *Bacillus* and *Thermus*.

2. Materials and Methods

2.1 Sampling and study sites

During the oceanographic cruise BIOACCESS (Japan-France cooperative STARMER project) organized by JAMSTEC (Japan Marine Science and Technology Center) aboard the mothership R/V "NATSUSHIMA", surveys were made by the manned submersible "SHINKAI 2000" within the DESMOS caldera (3° 42' 0" S, 151° 52' 3" E) at Manus Basin (Papua New Guinea) and the samples collected were brought to the surface.

Hot fluid samples and sediment cores collected at the venting site (8 dives at 1,900m depth) were fixed with 15% glycerol for further isolation of thermophilic bacteria in the laboratory and stored at -20°C. A few samples, for isolation of anaerobic microorganisms, were transferred into serum bottles and immediately deoxygenated by filling the bottles with degassed sterile artificial sea water and then reduced by sodium sulfide using reazurin as redox indicator and kept at 4°C before enrichment.

Temperatures of several hot hydrothermal fluids were recorded on the Onsen site (Onsen=hot spring in Japanese) to select the appropriate white smoker. Then, some end-member hot water samples were collected above the vent

using a manifold-pump sampling system [16] fixed to the submersible before placing a Vent Cap equipped with a temperature probe for 3 days on the active vent orifice. Bacterial growth in outflows of smokers can be detected by fitting a vent with a titanium or iron "cap" from which glass microscope slides and other surfaces (pieces of metals: iron, copper or ceramics) for microbiological colonization can be suspended in the emerging hot water.

Unfortunately no sample of the chimney has been collected during the cruise because of the very brittle nature of the diffuser wall at Onsen site and the high technical difficulties in approaching the smokers at Pacmanus site.

2.2 Enrichments and isolation

Enrichments and isolation were performed, in the laboratory, 4 months after storage. About 0.1 to 1g of solid or 1ml of liquid samples from Desmos site were added to 10ml of *Thermus* medium, referred to as 162 [17] supplemented with NaCl at 1% final concentration (designated 162-1) [19], and at two different pH concentrations (pH3 and 5).

After incubation at 65 and 72°C for 7 days, the cultures were centrifuged (3,000 rpm at 4°C for 15 minutes), supernatants carefully discarded and pellets transferred (0.1 to 0.5ml) and spread on agar plates containing the same medium. Finally agar plates were incubated at 65 and 72°C for one week.

All colony types obtained on plates were picked at random and purified by streaking on complementary agar media at least four times.

2.3 Source of other Strains

Thermus reference strains were: *T. aquaticus* strain YT-1 (ATCC 25104), *T. scotoductus* strain X-1 (ATCC 27978) and *T. thermophilus* strain HB-8 (DSM 579).

Bacillus reference strains were: *B. thermodenitrificans* (DSM 466) and *B. thermoleovorans* (DSM 5366).

2.4 Media and culture conditions

The deep-sea vent isolates and reference strains were routinely grown on agar medium 162-1 (1% NaCl) whereas *T. aquaticus* and *T. scotoductus* were kept on the same medium but without salt. The 162-1 medium was used for

all phenotypic tests and all growth tests were performed at 65°C. When clearly visible or confluent growth on agar had occurred after 7 days of incubation, growth was scored as positive. In liquid medium growth was followed by measuring increase in turbidity at 660 nm with a spectrophotometer.

2.5 Physiological tests

Most phenotypic characteristics were examined using the methods of Alfredsson *et al.* [18, 19], Munster *et al.* [20], Hudson *et al.* [21] and Nunes *et al.* [22].

Temperature range for growth was determined by using samples (0.2ml) from overnight cultures as inoculates in 10ml medium. Tubes were incubated and shaken in covered water baths at 65, 72, 75 and 78°C respectively and growth was recorded daily up to 4 days.

Halotolerance was determined by testing strains in 162 liquid media supplemented with 0, 1, 3, 6 and 8% (w/v) NaCl. Cultures tubes were shaken and incubated at 65°C for 4 days.

Utilization of compounds as sole source of carbon was tested on "minimal agar plates" inoculated with automatic multipoint inoculator (Mast sampler, Mast Laboratories, LTD, Liverpool, England) as previously described by Alfredsson *et al.* [18].

The viability of the suspension was checked, in parallel, on inoculation medium 162-1 (positive control) and on "minimal" medium without any carbon source (negative control). Plates were incubated for 7 days at 65°C.

Antibiotic sensitivities were tested with discs (Bio Mérieux, France). Exponentially growing culture (200 μ l) were spread uniformly over the surface of agar plates and the antibiotic discs were then applied automatically with a disc dispenser (8 per plate). Diameters of growth inhibition were measured after 24 h incubation at 65°C.

Gelatin hydrolysis was examined on 162-1 medium without tryptone containing 4g l⁻¹ of gelatin (Sigma). After 3 and 7 d of incubation at 65°C, plates were flooded with 15% HgCl₂ in 20% concentrated HCl. Tests were positive when clear zones appear around colonies.

Starch hydrolysis was tested on 162-1 medium without tryptone and supplemented with 10g l⁻¹ soluble starch (Merck). Hydrolysis was examined by flooding plates with Lugol's iodine solution, after incubation for 7 days

and recorded when diffuse zones appear around the margin of growth.

Hippurate hydrolysis was estimated by resuspending a large loopful of 3 days old culture in 1ml of 1% (w/v) sodium hippurate (Sigma) in 1% (w/v) aqueous NaCl. Cultures were incubated for up to 20 h at 65°C before adding 0.2ml of ninhydrin reagent (3.5g of ninhydrin in 100ml of a 1:1 mixture of acetone and butanol). A positive test was indicated by the formation of a deep purple colour after an additional incubation of 30 min at 65°C.

2.6 Analysis of results

The phenetic data of 39 characters were scored as positive or negative and analysed by using unweighted average linkage analysis (UPGMA), [23]. The similarity coefficients were calculated using the coefficient of Sokal and Michener [24] and results were expressed in the form of a dendrogram. The results of tests that were identical for all of the strains were eliminated from subsequent numerical analysis.

3. Results

A total of 15 deep-sea thermophilic isolates and 5 reference strains were successively subjected to phenotypical characterization and numerical classification.

3.1 Sampling and isolation:

Concentration of liquid cultures and subsequent spreading on agar plates was necessary to obtain the isolates. The time for development of colonies varied: clear colonies usually appeared after three days but sometimes took more days.

Out of 16 samples collected in the Manus Basin, 14 were scored positive for growth and yielded 17 isolates: 15 by concentrating enrichments on agar plates at 65°C and 2 at 72°C; 9 strains were obtained at pH3 and 8 at pH5 (Table 1).

All samples originated from sediment cores or fluids collected in the vicinity of the hydrothermal vent. Sources of positive samples are given in Table 2.

The colonies obtained had tan, pale yellow, white or cream colour. Sizes were highly different and sometimes growth converged all over the agar plate.

All selected strains were rod shaped cells containing spores except isolate B005.

In this study we examined the 14 sporeforming strains along with the only non-sporeforming isolate. All strains were examined for 59 characters but some were excluded from numerical analysis because of poor growth, bad reproducibility or when results were identical for all the strains. The 39 characters finally selected for numerical analysis are listed in Table 3.

Table 1 Number of deep-sea samples and results from their enrichments

Sampling site:	Desmos site (Manus basin, Papua New Guinea)			
No. of total samples:	16			
No. of positive samples:	14			
No. of total isolates:	17			
	65°C		72°C	
	pH3	pH5	pH3	pH5
Spore-forming isolates	8	7		1
Non spore-forming isolates			1	

Table 2 Designation and sources of isolates or reference strains in this study

Strain	No. dive	Source or site of isolate
Phenon		
B001	K3-08	Tube core sediment
B002	916	Tube core sediment
B003	917	Water (bottle n°3)
B004	917	Tube core sediment
B00a	919	Water (above the vent cap)
B006	921	Core sediment (near the vent cap)
B00b	921	Vent cap water and deposits
B00d	924	Water (in Vestimentifera field)
B0e3	924	Water (1m above the vent cap)
B009	923	Core sediment (with mussels)
B0e6	924	Water (1m above the vent cap)
<i>B. thermoleovorans</i>		(DSM 5366)
B007	921	Water (3m above the vent cap)
B00f	924	Water (1m above Vestimentifera)
B010	924	Core sediment
Unclassified strains		
<i>B. thermodenitrificans</i>		(DSM 466)
B005		Core sediment
<i>T. thermophilus</i>		(DSM 579)
<i>T. aquaticus</i>		(ATCC 25104)
<i>T. scotoductus</i>		(ATCC 27978)

Table 3 Percentage of positive results for the clustered strains derived from the SSM analysis

	Cluster	Unclustered strains				
	(16 isolates)	<i>B.thermoden.</i>	B005	<i>T. therm.</i>	<i>T. aqua.</i>	<i>T. scot.</i>
Growth at/in:						
75°C	6	-	-	+	+	+
78°C	0	-	-	+	+	+
81°C	0	-	-	+	-	+
5% NaCl	94	+	+	+	-	-
8% NaCl	6	-	-	-	-	-
Hydrolysis of:						
Casein (7days)	12	-	-	-	+	-
Starch (7days)	75	+	+	-	+	+
Utilization of carbon sources:						
Galactose	94	+	+	-	-	-
Glucose	94	+	+	-	-	-
Lactose	88	-	+	+	-	-
Maltose	94	+	+	-	-	-
Saccharose	100	+	+	-	-	+
Starch	100	+	+	-	-	+
Glycerol	100	+	+	-	+	+
Sorbitol	94	-	-	-	-	-
Malate	100	+	-	-	-	-
Alanine	100	+	+	+	-	-
Arginine	88	+	+	+	+	+
Asparagine	94	+	+	+	+	-
Glutamine	12	+	-	-	-	-
Leucine	100	+	+	+	-	-
Ornithine	100	+	+	+	-	-
Proline	100	+	+	+	-	+
Serine	19	-	-	-	-	-
Threonine	94	-	+	+	+	+
Valine	94	-	-	-	-	-
Acetate	94	+	+	-	-	+
Citrate	88	+	+	-	-	-
Fumarate	100	+	+	+	-	-
Gluconate	100	+	+	-	-	-
Glutamate	100	+	+	+	-	+
Succinate	100	+	+	-	-	-
Antibiotic resistance:						
Fusidic acid (10µg)	100	+	-	-	-	-
Colistin (10µg)	12	-	-	-	-	-
Polymyxin-B (300U)	81	-	-	-	-	-
Rifampicin (30µg)	100	+	+	-	-	-
Sulfamides-G (250µg)	81	+	-	-	-	-
Vancomycin (30 µg)	94	+	+	+	+	+
Spores	100	+	-	-	-	-

3.2 Morphology and Characterisation

Almost all selected strains (except strain B005) were rod-shaped cells (2–10 μm in length, 1–1.5 μm in diameter) which formed endospores and grew aerobically at 65°C or higher temperature.

The non-sporeforming isolate (B005) consisted of smaller cells (0.5–1 $\times$ 2–5 μm) which formed often long filaments, especially in liquid cultures and at temperatures above 70°C, large filaments aggregates and “rotund bodies” (Fig. 1) as described for *Thermus* by Brock and Freeze [25], Kraeplin and Gräevenstein [26] and Becker and Starzyk [27].

NaCl was not required for their growth but all strains grew at 1 and 3% NaCl and some up to 5% NaCl in

media. They generally grew on most of the carbon sources but failed to grow on phenylalanine, glutamate, pyruvate or gelatin. Very few grew on glutamine and serine. They could not hydrolyse hippurate. Antibiotics sensitivity was general among the strains. They were all sensitive to ampicillin (10mg), chloramphenicol (30mg), erythromycin (15mg), gentamycin (10mg), kanamycin (30mg), lincomycin (15mg), meticillin (5mg), penicillin G (10U), streptomycin (10mg) and virginiamycin (15mg) while a few were resistant to fusidic acid (10mg), polymyxin-B (300U), rifampicin (30mg), sulfamides-G (250mg) and vancomycin (30mg). Resistance to colistin (10mg) was high and resistance to nalidixic acid (30mg) was shown by all the isolates.

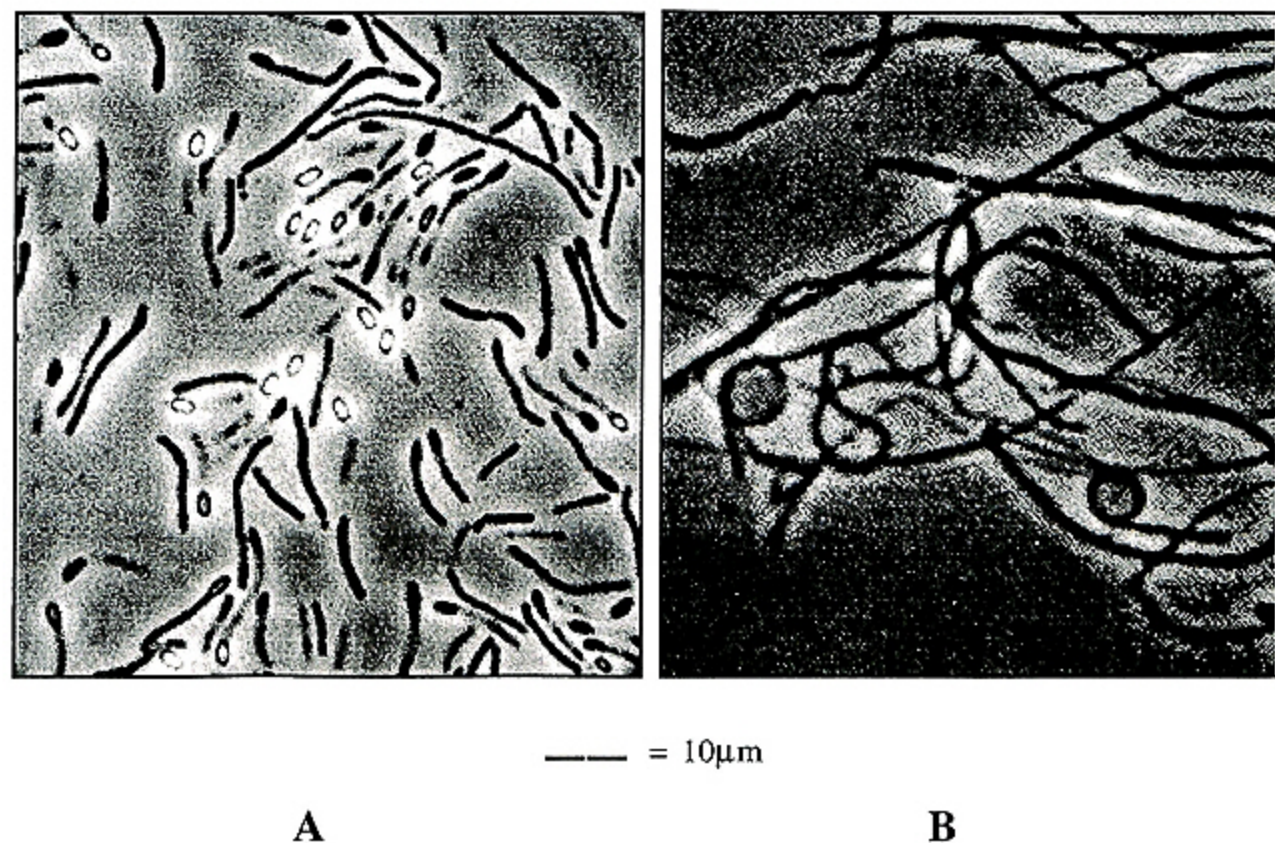


Fig. 1 Photomicrographs of *Bacillus* (A) and *Thermus*-like (B) bacteria observed through phase-microscopy. (Bar=10 μm).

3.3 Clustering of isolates

A phenogram derived from UPGMA-SSM analysis is shown in Fig. 2 and a list of different characteristics of the isolates is given in Table 3. Only one important group which consisted of 14 wild isolates and a reference strain *B. thermoleovorans* was defined at 83% similarity level while four reference strains (*T. scotoductus*, *T. aquaticus*, *T. thermophilus* and *B. thermodenitrificans*) and a deep-sea isolate (B005) formed single membered clusters.

The reference strain *B. thermodenitrificans* grouped with the cluster at 81% similarity and the isolate B005 remained apart and joined this formed group at 77%. The 3 *Thermus*-reference strains (*T. scotoductus*, *T. aquaticus* and *T. thermophilus*) matched with all others strains at only 40%.

In the cluster, entirely composed of wild deep-sea isolates and of the reference strain *B. thermoleovorans*, all strains (15) were able to grow at 72°C and only one at 75°C. 14 strains grew at 5% and only 1 at 8% NaCl. Isolates were able to metabolize almost carbon sources (except glutamine and serine) but unable to hydrolyse casein (except 2 isolates); only 2 of them were resistant to colistin (10mg).

Phenotypic characteristics of each unclustered strain could be examined separately for discrimination in Table 3. Some important differences could be noted between strains of the cluster and *B. thermodenitrificans*. This reference strain is able to grow on glutamine but not on lactose, sorbitol, threonine or valine and is resistant to polymyxin-B (300U). The deep-sea isolate B005 presented large phenotypic similarities with *B. thermodenitrificans* and the cluster, with respect to growth and utilization of almost carbon sources (excepted lactose, sorbitol, malate, glutamine, threonine and valine), whereas several other characters such as growth on malate or glutamine and resistance to fusidic acid or sulfamides-G were exclusively common with the 3 *Thermus*-reference strains.

4. Discussion

While the existence of aerobic, heterotrophic thermophiles in shallow marine thermal vents is well-known [7, 8, 9, 10, 18, 22, 28, 29] their existence and characterization in deep-sea hydrothermal vents has been only recently reported [11,14].

In this study, the unusual isolation method successfully used for the isolation of two non-pigmented *Thermus* strains from hot tap water in London and from Icelandic hot springs [30] was preferred to the filtration method more generally used for isolation of aerobic thermophiles species [10, 12, 19, 28]. Almost all samples yielded positive growths after enrichments in liquid media and concentrations on agar plates whereas, on the contrary, no growth was observed by the filter method. These results indicate that bacteria were in very low numbers in the samples and confirm the recent observations [11] suggesting that free-living aerobic bacteria discharged by the hot thermal fluid are rapidly diluted around the outlet by huge amounts of cold sea-water and strong deep-sea currents. Furthermore, the fact that we could isolate aerobic heterotroph thermophilic bacteria from some reduced samples collected with the intention of isolating anaerobic microorganisms could indicate that these bacteria may be resistant even when stored under anaerobic conditions.

Enrichments were carried out on different media and the richest 162-1 medium (without salt for *T. aquaticus* and *T. scotoductus*) giving the highest number of positive growths was used for phenotypic tests. All enrichments obtained in liquid medium, at 65 or 72°C and pH 3 or 5 were purified by several streaking on complementary agar medium but preservation of viable cultures during the last steps of purification or later when subculturing from liquid to solid media or vice versa proved to be very difficult. Moreover the large differences observed in the release and in the intensity of growth responses among the isolates caused difficulties in obtaining synchronous exponential cultures. This loss of activity could be due to a lack or deficiency of trace elements in the culture medium and/or to conditions of culture, since the obligate aerobic strains described here have a maximum temperature range from 65 to 75°C and have been isolated at pH3 or 5 while all tests were performed at 65°C and pH7.

Despite these difficulties and the preliminary nature of the conclusions drawn from the numerical taxonomy dendrogram, it is obvious that the phenotypic diversity among *Bacillus* isolates is not substantial.

By taking into account the variations in optimum growth temperature, morphology and other physiological

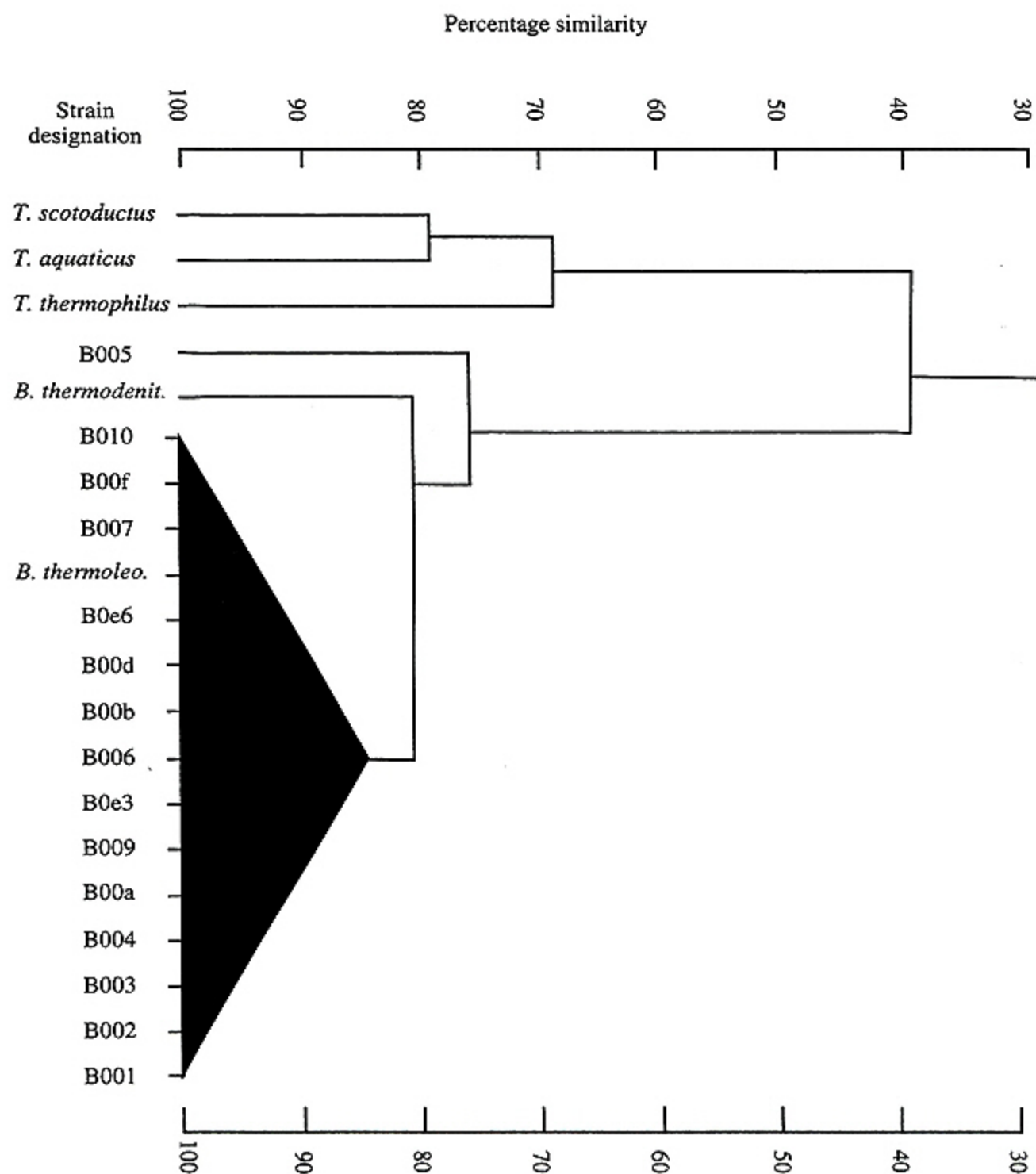


Fig. 2 Simplified phenogram showing the phenotypic relationships based on simple matching coefficient (SSM) and the unweighted average pair group method with averages (UPGMA) clustering for 15 deep-sea isolates and 5 reference strains. *B. thermoleovorans* was classified in the cluster.

traits reported for different *Thermus* isolates [25, 31, 32, 33] it is possible to conclude that at least the strain (B005) isolated at 72°C and at pH 3 presented (though less thermophile than reference strains) all the classical features of the genus *Thermus*. The fact that its resistance to antibiotics was very similar to that of the reference strains and that its utilization of carbon sources was largely different could imply this particular *Thermus*-like deep-sea isolate represented new species. Of course, additional DNA base composition and recombination data are needed before these strains (*Bacillus* and *Thermus*) can be proposed as new species.

This study confirms once again the existence of aerobic thermophiles in the immediate vicinity of deep-sea vents but describes for the first time the presence of thermophilic *Bacillus* and *Thermus* strains in very unusually acidic and sulfate-rich hydrothermal fluids. Because of the lack of samples from the chimney itself, it is not possible at present to precise their precise location (at the microbe scale) within the Onsen site or their abundance.

The future work envisaged will endeavour to identify their specific niches by conducting more accurate sampling (in and along chimney walls in particular) and compare these new environmental isolates with numerous other strains already isolated from three geographically separate deep-sea hydrothermal vents (Snakepit: Mid-Atlantic Ridge, Guaymas Basin: Gulf of California and Lau Basin: SW Pacific).

Acknowledgements

We are grateful to Prof. S. Ohta (Ocean Research Institute, University of Tokyo, Japan), chief scientist of BIOACCESS cruise organised by JAMSTEC, for inviting one of us to participate in the expedition. Many thanks are also due to the operation team of "SHINKAI 2000" and the crew members of the R/V "NATSUSHIMA" for their collaboration.

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(Manuscript received 9 July 1998)