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Characterization of the Thermophilic Bacterium *Geobacillus* sp. Strain GWE1 Isolated from a Sterilization Oven

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A gram-positive, rod-shaped, spore-forming, motile thermophilic bacterium was isolated from a sterilization oven. The microorganism GWE1, formally named *Geobacillus wiegelii* identified as a member of the genus *Geobacillus*. GWE1 grew under aerobic conditions of between 60-80ºC (optimum 70ºC), in a pH range of 3.0-8.0 (optimum $pH^{70ºC}$ 5.8), and between 0 and 2 M NaCl (optimum 0.3 M). The membrane polar lipids were dominated by branched saturated fatty acids, which included as the major constituents; iso-15:0 (13.3%), 16:1(ω7) (12.8%), 16:0 (28.5%), iso-17:0 (13.5%) and anteiso-17:0 (12.3%). The DNA G+C content was 47.2 mol% (determined by HPLC). The 16S rRNA gene sequence of GWE1 showed a high similarity with *Geobacillus caldoxylosilyticus* (97%). However, the level of DNA–DNA relatedness was only 58%. These data suggest that GWE1 is probably a novel specie of the genus *Geobacillus*.

Keywords: Thermophilic bacterium, *Geobacillus*, sterilization oven

Introduction

The genus *Geobacillus* was introduced by Nazina *et al.* (2001) [19]. To date, 16 different species of this genus have been reported [4].

Although the searching of thermophiles has been usually performed in 'hot' environments, these thermophilic bacilli have been also found in cool soil environments [1, 15, 16]. In particular, members of genus *Geobacillus* are widely distributed and not restricted to specialized nutritional environments. Oxygen is usually the terminal electron acceptor for aerobic respiration in members of genus *Geobacillus*, however, facultative anaerobes of this genus can replace oxygen by nitrate.

They are Gram-positive, rod-shaped, motile cells, present in single or short chains and includes microorganisms with optimal growth temperature ranging between 37-75°C [19].

Geobacillus have been described as sources of interesting enzymes such as proteases [3], lyases [7], esterases [17], amylase and β-galactosidase and cellulolytic enzymes [24] among others.

This report presents the isolation of *Geobacillus wiegelii* (GWE1), a microorganism isolated from a sterilization oven, an environment where temperature can easily surpass 150ºC. Drastic changes in humidity and periodic cleaning desiccation cycles of the equipment with oxidizing solutions, organic solvents, among others, make this an extremely hostile environment previously thought to be unable to sustain life.

Here we described the isolation and characterization of a a new microorganism *Geobacillus wiegelii* belonging to genus *Geobacillus*.

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Materials and Methods

Sample collection and isolation procedure

GWE1 strain was isolated from samples consisting of a dry, dark brown crust, aseptically collected from the corners and cracks of a sterilization oven. Samples were cultivated in rich liquid modified marine medium containing: 2.5 g/l yeast extract, 2.5 g/l peptone, 0.0025 g/l citrate, 1.5 g/l maltose, 0.6 g/l NH_4Cl, 17.5 g/l NaCl, 1.75 g/l $MgSO_4$, 0.16 g/l KCl, 0.38 g/l $CaCl_2$, 0.25 g/l KH_2PO_4, 0.025 g/l NaBr, 0.0075 g/l H_3BO_3, 0.0038 g/l $SrCl_2$, 0.025 g/l KI, 0.0055 g/l $FeCl_3$, 0.0025 g/l $MnSO_4$, 0.0015 g/l Na_2WO_4 x $2H_2O$, 0.001 g/l $NiCl_2$, 0.0005 g/l $CoSO_4$, 0.0005 g/l $ZnSO_4$, 0.00005 g/l $CuSO_4$, 0.00005 g/l Na_2MoO_4. For colony isolation, medium was supplemented with Gelrite at 1.5% (w/v). GWE1 stands for *Geobacillus wiegelii*. GWE1 has been deposited in DSMZ (DSM 24745).

Morphological and biochemical characterization

Cell morphology was examined by Scanning Electron Microscopy (SEM) using an electronic microscope JEOL JSM-T300 (resolution up to 10 nm). Gram staining was performed [2]. To determine the optimum growth conditions of GWE1, the bacterium was grown at a temperature range from 60-80ºC, pH range from 3.0-8.0 and NaCl concentration range from 0-2.0 M. Growth was monitored in a spectrophotometer at 600 nm.

Gelatin degradation was determined by growing the microorganism on media containing 15% gelatin. Oxidase activity was detected by the method of Cowan and Steel [5]. Under anaerobic conditions, nitrate reduction was determined using the method of Lanyi [13]. Growth on sole carbon source was performed by substituting yeast extract, peptone, citrate and maltose from the medium with 0.3% (w/w) of each of the following compounds: glucose, lactose, xylane, arabinose, maltose, xylose, starch, fructose, galactose, gluconate, mannose and cellulose. To identify some of the enzymes produced by GWE1, the qualitative test APYZYM (bioMérieux, Inc.) was performed. All experiments were performed in triplicates.

Analysis of cellular fatty acids

Cellular fatty acids were extracted from GWE1 dry cells by soxhlet extraction for 48 h with chloroform/methanol (1:1, v/v), methylated and then analyzed by using GC/MS following the instructions of the Microbial Identification System (MIDI, Microbial ID Inc.).

Phylogenetic analyses and DNA-DNA hybridization

Genomic DNA was isolated using chloroform-isoamyl alcohol extraction procedure [12]. The 16S rRNA gene was amplified from genomic DNA by PCR using primers 27F [21], E341F, E939R [22, 30] specific for bacteria, and the universal primer 1492R [28].

Obtained sequences were assembled, analyzed, and manually edited using ChromasPro software (Technelysium Pty Ltd.) for a final sequence extension of ~1400 bp. 16S rRNA sequence of strain GWE1 was aligned with sequences from microorganisms belonging to genus *Geobacillus* available in Genbank, using the multiple sequence alignment program ClustalW software [10, 29]. The accession number of GenBank for the 16S rRNA gene of GWE1 is FJ598658.

Phylogenetic analyses were conducted using the software MEGA 4 [27]. The phylogenetic tree was inferred from the multiple-sequence alignments, after the removal of all gaps, by Neighbor-Joining method [23, 26]. The evolutionary distances were computed using the Maximum Composite Likelihood [26]. One thousand bootstrap replicates were used to estimate the reliabilities of the nodes on the phylogenetic trees [8].

The G+C content of GWE1 genomic DNA was determined at the DSMZ (Leibniz Institute, Germany) according to the method of Mesbah *et al.* [18], using a HPLC system (Shimadzu, Japan) and *Bacillus subtilis* (DSM 402), *Xanthomonas campestri* pv. *campestris* (DSM 3586^T) and *Streptomyces violaceoruber* (DSM 40783) as references.

DNA-DNA hybridization was determined at the DSMZ as described by De Ley *et al.* [6], considering the modifications described by Huss *et al*. [11] using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6x6 multicell changer and a temperature controller with *in situ* temperature probe (Varian). We have performed the DNA-DNA hybridization of *Geobacillus wiegelii* (GWE1) against *Geobacillus caldoxylosilyticus* (DSM 12041T), *Geobacillus stearothermophilus* (DSM 22^T) and against *Geobacillus tepidamans* (DSM 16325^T).

Results and Discussion

This report presents the isolation of a microorganism from a sterilization oven, an environment where tempera-

ture can easily surpass 150ºC. Drastic changes in humidity and periodic cleaning desiccation cycles of the equipment with oxidizing solutions, organic solvents, among others, make this an extremely hostile environment previously thought to be unable to sustain life. The isolation of thermophilic bacteria from anthropogenic hot environments has only occurred recently. *Geobacillus wiegelii* (GWE1), a thermophilic member of *Geobacillus* genus, was isolated from samples consisting of a dry and dark brown crust, collected aseptically from the corners and cracks of a sterilization oven. Samples were inoculated in 20 ml of modified marine medium for 22 h at 70ºC and then plated on solid medium. Colonies were pick-up and re-inoculated in fresh medium. Serial dilution was performed on the culture until obtaining an axenic culture with microorganisms that presents similar morphology and growth conditions.

In solid medium, colonies of GWE1 were white-colored, circular, convex, non-translucent with entire margins of 1.0-2.0 mm in diameter. Cell staining revealed gram-positive bacilli. Electron microscopy showed rod-shaped microorganims with 0.8-1.0 μm width and 8.0 μm length (Fig. 1A, Table 1). The formation of oval shaped terminal endospores was only detected when fresh cultures were exposed at −20°C. No diffusible pigments were produced on any media tested. GWE1 grows optimally at 70ºC at pH 5.8 and 0.2 M of NaCl. Moreover, this bacterium can grow in the range of temperature between 60-80ºC and pH range of 3.0-8.0. GWE1 was able to grow under microaerophilic conditions using nitrate as final electron acceptor. These properties are shared with some members of *Geobacillus* genus. Furthermore, the ability of GWE1 to survive at 150ºC is probably due to a sporulation phenomenon that is triggered at high temperatures, in desiccation conditions, and under exposure to ultraviolet radiation of A, B and C type. Table 1 resumes the characterization of GWE1.

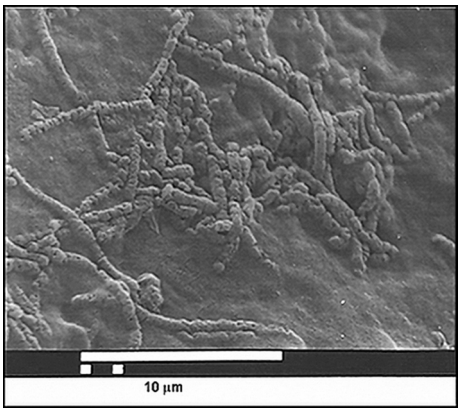

Fig. 1. Electron microscopy of strain GWE1. It shows a rod shaped microorganisms with a length of about 8.0 μm.

GWE1 produced acid from lactose, xylose, galactose, glucose and starch as sole carbon source. Slight acid production was observed in the presence of arabinose. Growth with no acid production was observed in the medium that contained maltose as carbon source. However, no growth was observed on fructose, gluconate, mannose, cellulose

Table 1. Characterization of the strain GWE1.
The symbols represent: +, positive reaction; −, negative reaction; and w, weakly positive reaction.

Characteristic	GWE1
Cell width (μm)	0.8-1.0
Cell length (μm)	8.0
Motility	−
DNA G+C content (mol%)	47.2
NaCl range (M)	0-2.0
pH range	3.0-8.0
Temperature range (ºC)	60-80
Acid production from:	
Lactose	+
Xylose	+
Glucose	+
Galactose	+
Starch	+
Arabinose	w
Xylane	−
Maltose	−
Fructose	−
Gluconate	−
Mannose	−
Cellulose	−
Nitrate reduction to nitrite	+
Enzimatic activity:	
Gelatin degradation	−
Oxidase	−
Esterase (C4)	+
Lipase (C8)	+
α-galactosidase	+
β-galactosidase	+
Alkaline phosphatase	+
Acid phosphatase	+
Leucine arylamidase	+

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and xylan. GWE1 was able to reduce nitrate under anaerobic conditions, suggesting that this bacterium possesses the metabolic machinery to carry out denitrification. Nitrate could also be used by GWE1 as electron acceptor being reduced to nitrite , allowing its growth under anaerobic conditions.

Table 2. Whole-cell fatty acid profile of GWE1.
The profile is given as percentage composition. The double bond position was determined only for C16:1ω7. Abbreviations: ω, double bond position described as the number from the methyl end of the fatty acid.

Fatty acid	GWE1
iso-14:0	0.5
14:0	3.1
iso-15:0	13.6
anteiso-15:0	3.6
15:0	3.2
16:1(ω7)	12.8
16:0	28.5
iso-17:0	13.5
anteiso-17:0	12.3
17:0	2.9
18:1	2.6
18:0	3.5

Enzymatic activities were negative for gelatin degradation and oxidase test. Furthermore, the API ZYM test showed that GWE1 possesses the enzymatic activities: lipase (C8), esterase (C4), α and β-galactosidase, alkaline- and acid- phosphatase and leucine arylamidase. Previous studies revealed the presence of lipase, esterase, β-galactosidase, alkaline- and acid- phosphatase activities from different member of genus *Geobacillus* [14, 17, 25, 31, 32].

Lipid composition of GWE1 was obtained (Table 2). The cellular polar lipids of GWE1 were identified as branched saturated fatty acids, the double bond position was determined only for C16:1ω7 (Table 2). The major cellular fatty acids were iso-15:0, iso-16:0 and iso-17:0, as described for the genus, but they are present in different percentage. In GWE1 the major fatty acids represent 13.6, 28.5 and 13.5%, indicating that GWE1 possesses differences in its lipid composition regarding other members of genus *Geobacillus*. It was demonstrated that at higher temperatures the percentage of iso-15:0, iso-17:0 is increased, due to

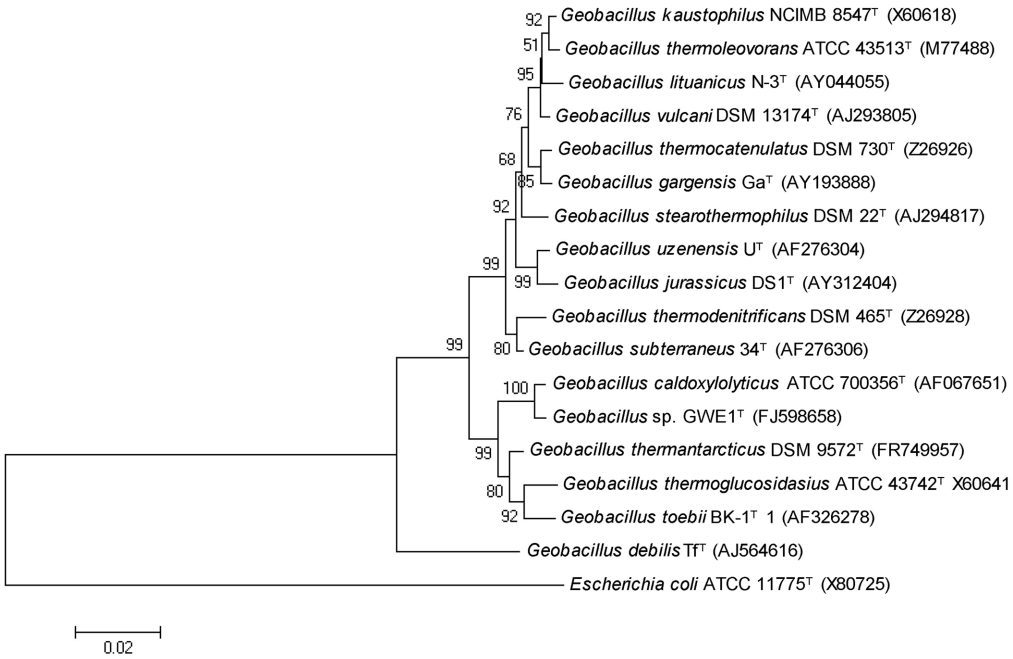

Fig. 2. Phylogenetic position of GWE1 with other validly described species of the genus *Geobacillus* based on 16S rRNA gene. *Escherichia coli* was used as outgroup. Phylogenetic tree was inferred by Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the tree branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The nucleotide sequence accession numbers are indicated in the tree.

high melting points (52.2ºC and 60.5ºC respectively). The contribution of iso-16:0 appears to be strain and specie specific. For example in Thermus aquaticus, iso-16:0 is present in higher percentage than in Thermus thermophilus [20].

Phylogenetic analysis based on the 16S rDNA reveal that GWE1 belongs to the genus *Geobacillus* and it is closely related with *G. caldoxylosilyticus* forming a well supported cluster with this bacterium. The phylogenetic distance between them was 0.97% (Fig. 2).

The obtained data were well correlated with the biochemical and microbiological data for members of the genus *Geobacillus.*

The genomic DNA G+C content of GWE1 does not show a notable difference with other members of *Geobacillus* genus. GWE1 possesses 47.2 mol% of G+C content, while this content for members of *Geobacillus* genus is 48.2-58 mol% [19].

Furthermore, in order to unequivocally determine the species status of the new isolate, DNA-DNA hybridization experiments were performed. The reassociation values with strain *Geobacillus caldoxylosilyticus* DSM 12041^T was well below the threshold for species identity (58%), indicating that GWE1 is a new specie. This strain was deposited in DSMZ (DSM 24745) in a protected way, due to its biotechnological potential.

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