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Thermophilic bacteria present in a sample from Fumarole Bay, Deception Island

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Abstract: Deception Island, an active stratovolcano located in the South Shetland Islands, Antarctica, provides excellent conditions for the thermophilic bacteria growth because of high ground temperatures in specific areas, such as Fumarole Bay where the temperatures are above the mesophilic range. Denaturing Gradient Gel Electrophoresis (DGGE) was used with the 16S ribosomal gene to analyse cultures of thermophilic bacteria from a soil sample taken from Fumarole Bay. Nine bands were sequenced and analysed from DGGE and they indicated the presence of bacteria from the genera *Geobacillus*, *Bacillus*, *Brevibacillus*, *Thermus* and uncultured sulphate reducing bacteria. Some of which have been reported in other Antarctic geothermal sites. *Geobacillus*, *Bacillus* and *Brevibacillus* genera were successfully cultivated in an enriched medium. A pure culture of one thermophilic *Geobacillus* bacterium was obtained closely related to *Geobacillus jurassicus*.

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Introduction

Antarctic volcanic activity was a constant phenomenon during the Cenozoic period. Currently geothermal activity has been found in a number of circumpolar islands (South Sandwich Islands, Deception Island) and in few geographical sites on the continent (Mount Erebus, Mount Melbourne and Mount Rittmann).

Measurements on Mount Erebus, Mount Melbourne and Mount Rittmann indicate that in these sites temperatures range from 50°C to 75°C. With extremely low concentrations of N and P and high concentrations of metals (Cu, Zn, Cd, Pb and Hg) the microbial life in these soils is probably constrained not only by the high temperatures but also by low nutrients and high concentrations of toxic metals (Logan & Allan 2007). Our current microbiological knowledge strongly suggests a correlation between the presence of microbial diversity and the geochemical composition of a geographical site.

Deception Island (South Shetland Islands, Antarctica) is a complex stratovolcano with a "horseshoe" shape whose central part has a caldera structure (Fig. 1). This volcanic island has been very active during the last century: fumarolic emissions, thermal springs and hot soils are evidence of Deception Island's continuing activity (Caselli *et al*. 2004). Geothermal anomalies are mainly found from Fumarole Bay to Pendulum Cove. The temperatures of emissions range from 90–110°C at the fumaroles of Fumarole Bay. The soil temperature is highly variable, reaching values between 40–60°C at Whalers Bay, and more than 70°C at Pendulum Cove (Ortiz *et al*. 1992).

Geothermal sites are widely distributed throughout the world and provide ideal environments for the development of thermophilic microorganisms. Currently, a number of thermophilic and hyperthermophilic microorganisms have been isolated from terrestrial and marine hot environments, including shallow and deep marine hydrothermal vents, solfataric fields, hot springs and volcanoes (Barns *et al*. 1994, Sako *et al*. 1996, Reysenbach *et al*. 2000, Casamayor *et al*. 2001). Thermophilic and hyperthermophilic microorganisms grow optimally at temperatures of 50°C to 80°C and above 80°C, respectively (Turner *et al*. 2007). Culture dependent analysis of geothermal soils from McMurdo volcanic sites indicates the presence of aerobic and facultative anaerobic thermophilic species of the genera *Bacillus*, *Brevibacillus*, *Anoxybacillus*, *Aneurinibacillus* and *Alicyclobacillus* among others (Nicolaus *et al*. 1998, 2000, Logan *et al*. 2000, Allan *et al*. 2005, Poli *et al*. 2006) and anaerobic thermophiles related to the genus *Clostridium* (Hudson & Daniel 1988). On the other hand only six thermophilic, Gram-positive, aerobic rods have been described and isolated from geothermal areas of Deception Island (Llarch *et al*. 1997), but none of them from Fumarole Bay.

It has been recognized that only a small fraction of microorganisms found in natural habitats is revealed by using culture-dependent techniques. Cultivating bacteria from thermal sites was often unsuccessful due to difficulties

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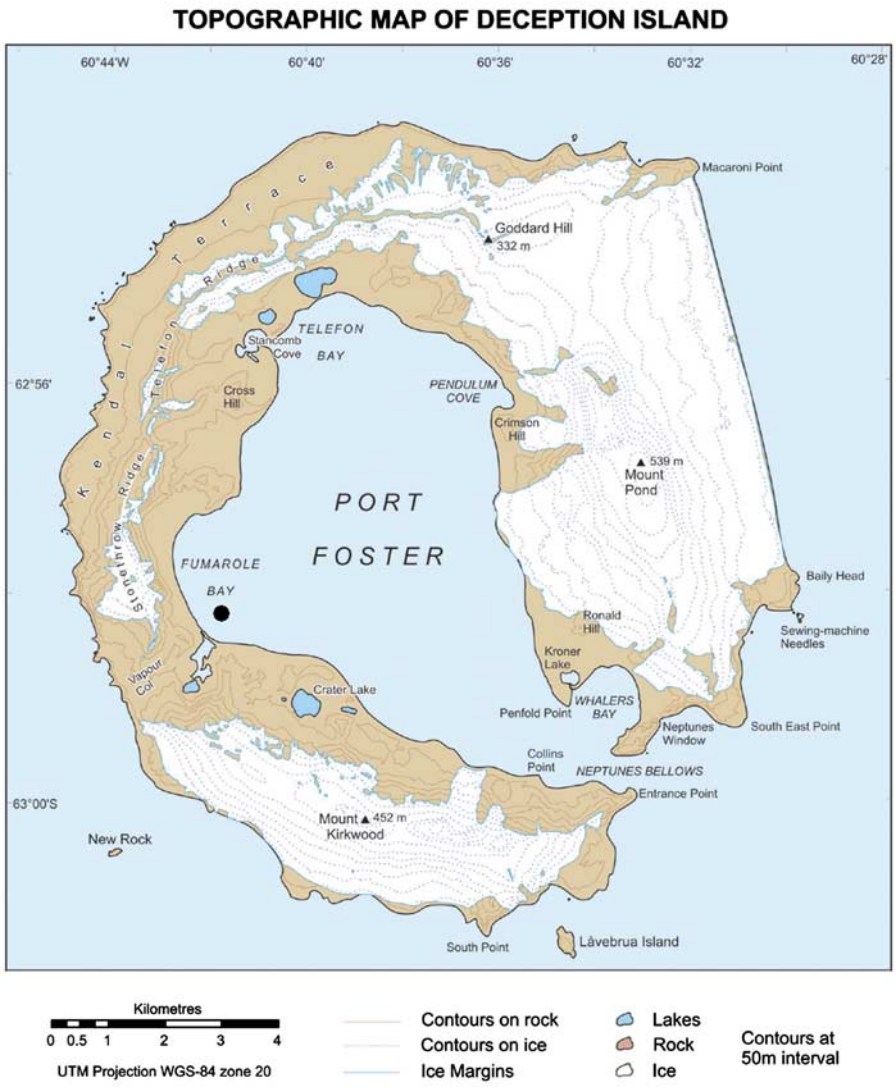


Fig. 1. Map of Deception Island (South Shetland Islands, Antarctica). The black dot indicates the sampling site selected in the present work. Courtesy of British Antarctic Survey.

in reproducing the complex geochemical composition of the environment in the growth media, resulting in limited knowledge of real microbial diversity (Muyzer 1999). Currently, culture-independent methods are considered an important tool in identifying a larger part of the microbial community composition, revealing the existence of a high number of as-yet-uncultured microorganisms (Maugeri *et al*. 2009).

Molecular methods based on 16S ribosomal DNA (rDNA) are widely used to study intrinsic genetic diversity. Denaturing Gradient Gel Electrophoresis (DGGE) of 16S rDNA amplified by PCR is used to obtain insights into the bacterial and/or archaeal community diversity. This technique has been used in different geothermal environments, including hydrothermal vents of Panarea Island, Italy (Maugeri *et al*. 2009), hot springs from Yellowstone National Park, USA (Barns *et al*. 1994), and Uzon Caldera, Kamchatka, Russia (Kublanov *et al*. 2009) to provide more information about the diversity of those sites.

In this study DGGE fingerprinting of 16S rDNA was used to investigate the diversity of thermophilic bacteria in Fumarole Bay, Deception Island.

Materials and methods

Sample collection

A sample of *c.* 10.0 g of soil was taken from the fumarole with the highest temperature at Fumarole Bay, Deception Island during the Chilean Antarctic Scientific Expedition ECA44. Samples consisting of mixtures of sediment, pyroclast and marine water were collected at 40 cm depth in a fumarolic emission. The temperature of the site ranged from 75°C to 95°C and pH was 6.0. The sample was collected aseptically using metal spoons and Whirl-Pak bags (8 x 8 inch) and was transferred to a sterile vial. Approximately 4.0 g of soil we re-stored at -20°C for the genomic DNA extraction. Triplicate samples were collected to assess consistency of DNA extraction, amplification and DGGE methods.

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Enrichment of environmental sample

Three different soil samples of 2.0 g were inoculated into 20 ml of enrichment medium. Enrichment medium (EM) contained (g/l) 5.0 maltose, 5.0 tryptone, 5.0 yeast extract, 1.2 NH_4Cl, 14.0 NaCl, 2.0 $MgSO_4$, 3.5 $MgCl_2$, 0.35 KCl, 0.3 $CaCl_2$, 0.3 KH_2PO_4, 0.06 NaBr, 0.015 H_3BO_3, 0.02 $SrCl_2$, 0.06 sodium citrate, 0.02 KI and 10 ml of trace elements solution (Freier *et al*. 1988). The pH was adjusted to 8.5, 7.0 and 5.5 with H_2SO_4 or NaOH and the cultures were named PCHOC1, PCHOC2 and PCHOC3, respectively. All cultures were incubated aerobically at 65°C for 24 hours.

From the three cultures, one of the enriched samples was transferred to a liquid modified basal (MB) medium containing (g/l) 5.0 peptone, 1.0 yeast extract, 6.0 K_2HPO_4, 2.0 KH_2PO_4, 0.5 $MgSO_4$ and 10 ml of trace elements solution. pH was adjusted to 6.5 with H_2SO_4. A thermophilic bacterium was isolated from the inoculated 20 ml MB medium using serial dilutions, and inoculation on solid MB medium followed by transfer to a liquid MB medium. Incubations were at 65°C and the purity of the isolate was tested microscopically and confirmed by DGGE.

Denaturing Gradient Gel Electrophoresis (DGGE)

Genomic DNA was extracted directly from soil sample, mixed enriched cultures and pure cultures by PowerSoil™ DNA isolation kit (Mo Bio). Total DNA was used to amplify the 16S rDNA. PCR amplification was performed using universal primer 907R (Muyzer *et al*. 1998) and bacterial specific primer 358F (Muyzer *et al*. 1993). A 40-bp GC clamp (5'-CGCCCGCCGCGCCCCGCGCCCGT CCCGCCGCCCCCGCCCG-3') was attached to the 5' end of primer 358F (Muyzer *et al*. 1993). The reaction mix consisted of 2.5 U of Taq DNA polymerase, 200 μM of each deoxy (d) nucleotide (dATP, dCTP, dGTP and dTTP), 1X of reaction buffer, 0.75 mM of $MgCl_2$ and 0.5 μM of each primer.

Touchdown PCR was performed on the amplification reaction mix. The following thermal conditions were applied: 95°C for 45 s, 65°C-55°C for 45 s at the ramp of 0.5°C per cycle, 72°C for 45 s, then twenty cycles were carried out at 95°C for 45 s, 55°C for 45 s, 72°C for 45 s; and a final elongation step of 72°C for 5 min. Amplification reactions were carried out using a Palm Gradient Cycler (Corbett).

PCR products were resuspended in 5 μl of sterile distilled water and 5 μl of DGGE loading dye (10 mM Tris-acetate pH 7.8; 1 mM EDTA; 0.1% bromophenol blue). The mixture was loaded on an 8% polyacrylamide gel with a 20% to 80% denaturant gradient (100% denaturant corresponded to 6 M urea and 40% formamide). Electrophoresis was performed for 10 min at 200 V and subsequently for 16 h at a constant voltage of 65 V at 60°C. Gel was stained with ethidium bromide (0.5 μg/ml in Tris-acetate-EDTA pH 8.3) for 25 min and visualized on an UV transilluminator. Most representative bands were cut, amplified and sequenced using the set of primers described above.

Phylogenetic analysis

Clustal W software was used to align the partial sequence of 16S rDNA (~350 bp) from the most representative bands in DGGE with selected sequences retrieved from GenBank. Accession number of the retrieved sequences are indicated in parenthesis: *Geobacillus jurassicus* (FN428697), *Geobacillus stearothermophilus* (FN428694), *Geobacillus thermoleovorans* (Z26923), *Bacillus thermantarcticus* (AJ493665), *Bacillus fumarioli* (AJ250059), *Bacillus licheniformis* (AY052767), *Bacillus bataviensis* (EU334358), *Thermus thermophilus* (X07998), *Thermus aquaticus* (L09663), *Thermus scotoductus* (AF020205), *Thermus* sp (Z15061), *Desulfocapsa sulfoexigens* (Y13672), *Brevibacillus agri* (AB112716), *Brevibacillus brevis* (AB271756), *Brevibacillus borstelensis* (AB116134), *Brevibacillus thermoruber* (AB112722) and uncultured bacteria (AB294274, EF540443, AF050536, EF667749, DQ677861). *Thermotoga* sp. (AF355615) was used as outgroup. The software package MEGA 4.0 (Tamura *et al*. 2007) was used for a phylogenetic analysis and a tree was constructed by using the Neighbor-Joining method with a bootstrap analysis of 1000.

Nucleotide sequence accession numbers

The partial 16S rDNA gene sequences generated from the DGGE and microbial culture analysis have been deposited in the GenBank nucleotide sequences databank under accession numbers: HM449942–HM449950 and GU366067.

Results and discussion

The wide microbiological biodiversity found in thermal environments has been consistently underestimated, due to limited experimental procedures and methodologies to correctly handle samples from extreme environments either from culture dependent or independent approaches (Casamayor *et al*. 2001, Christner *et al*. 2001). Additionally, the selectivity of the cultures enrichment fails to emulate the conditions that particular microorganisms require for their proliferation in their own natural habitats.

In this study, the fact that cultured bacteria represent less than 1% of environmental species produced meant that a further bacterial diversity analysis (from Fumarole Bay) was conducted by DGGE (Sing & Sing 2010). Generated DGGE profile using bacterial primers is presented in Fig. 2. The number of bands obtained in our DGGE analysis is similar to the number of bands amplified from samples of other geothermal sites (Casamayor *et al*. 2001, Maugeri *et al*. 2009). All bands from the DGGE analysis were excised and sequenced. Lane A shows bacterial thermophiles in a soil sample from Fumarole Bay. However, when sequences were

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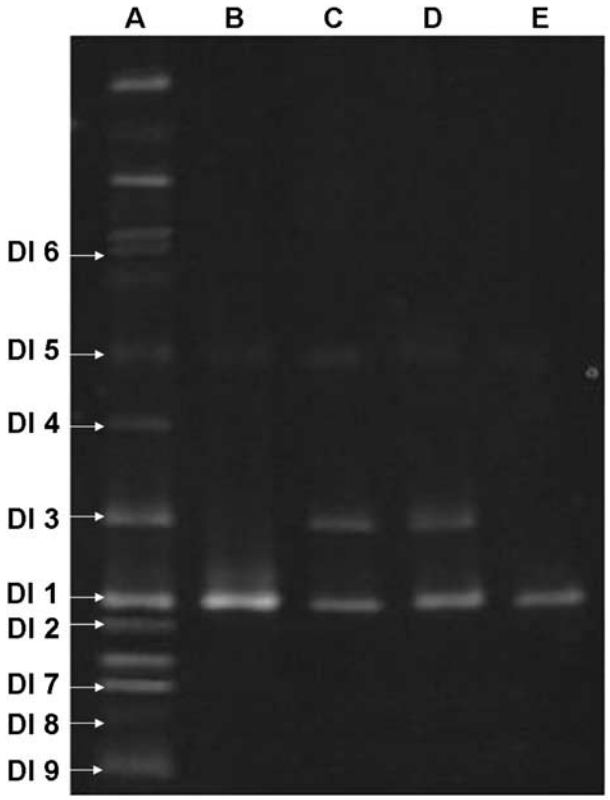


Fig. 2. DGGE profile of PCR amplified 16S rDNA gene of a soil sample (lane A), enriched cultures PCHOC1 (lane B), PCHOC2 (lane C), PCHOC3 (lane D), and pure culture of *G. jurassicus* (Lane E) obtained from Fumarole Bay, Deception Island. All bands were excised and sequenced. The unmarked unnumbered bands did not produce readable sequence data.

analysed by BlastN, only nine bands (named DI 1–9, Fig. 2) matched with hits of 16S rDNA sequences stored in GenBank (Table I). Remaining bands amplified from the soil sample did not give matches in BlastN. DGGE fingerprint represents the cultured and non-cultured bacteria of samples from Fumarole Bay. We have been able to obtain three different enriched cultures, containing species represented in bands DI1, DI3 and DI5 (Lane B–D, Fig. 2).

We used the enrichment medium EM because it has proved to be an excellent medium to obtain different high-density thermophilic heterotrophic microorganisms. In fact, we were able to obtain three mixed cultures using this medium at three different pH values. DGGE analysis indicated that under alkaline conditions (PCHOC1, lane B Fig. 2) it was possible to cultivate *G. jurassicus* and *B. thermoruber*. Additionally, *G. jurassicus*, *B. thermoruber* and *B. fumarioli* were able to grow under neutral (PCHOC2, lane C Fig. 2) and acidic pH (PCHOC3, lane D Fig. 2). *Brevibacillus thermoruber* and *B. fumarioli* have a maximum temperature for growth under 60°C. This indicates that bacteria obtained from these mixed cultures corresponded to more thermophilic strains, and we were able to cultivate the most thermophilic bacterium *G. jurassicus*. The characterization of the bacterium (not shown) indicates that our strain has a range of temperature between 50–72°C. Due to its resistance to high temperatures *G. jurassicus* is a good model for future studies of thermal adaptations of thermophilic Antarctic microorganisms.

Table I shows the nine DGGE bands that match with sequences from GenBank. BlastN analysis indicates that the nine bands match with the 16S rDNA sequences that belong to the Bacteria domain. We used Dinsdale *et al.* (2010) to apply the latest nomenclature for *Geobacillus*. Our results indicate a high likelihood of identity for five bands with thermophilic members of the genera *Geobacillus* (DI1 and DI2), *Bacillus* (DI3 and DI4) and *Brevibacillus* (DI5). The sequence of DI9 band matched with *Thermus* sp. and showed 89% identity with this thermophilic bacterium. Three bands of DGGE (DI6 to DI8) correspond to uncultured sulphate reducing bacteria with a range of identity between 78–87%.

Phylogenetic analysis by using Neighbor-Joining method for the partial 16S rDNA sequences of thermophilic bacteria from Fumarole Bay showed three main clades (Fig. 3). These clades include thermophilic members of the order Bacillales, *Thermus* and uncultured sulphate reducing bacteria. Thermophilic members of the order Bacillales clade can be divided into three groups composed by the genera *Geobacillus*, *Bacillus* and *Brevibacillus*. Members of this clade are closely related, but they show a variety of phenotypically heterogeneous species exhibiting a wide

Table I. Affiliation of 16S rDNA gene from thermophilic bacteria with sequenced bands from DGGE.

Band	GenBank accession number	Result of Blastn (Accession number)	% identity
DI1	HM449942	*Geobacillus jurassicus* (FN428697)	95
DI2	HM449943	*Geobacillus thermoleovorans* (FN666246)	97
DI3	HM449944	*Bacillus fumarioli* (AJ250059)	95
DI4	HM449945	*Bacillus thermantarcticus* (AJ493665)	89
DI5	HM449946	*Brevibacillus thermoruber* (HM011262)	95
DI6	HM449947	Uncultured bacterium (EF540443)	87
DI7	HM449948	Uncultured bacterium (EF667749)	87
DI8	HM449949	Uncultured bacterium (EF667749)	78
DI9	HM449950	*Thermus* sp. (Z15061)	89

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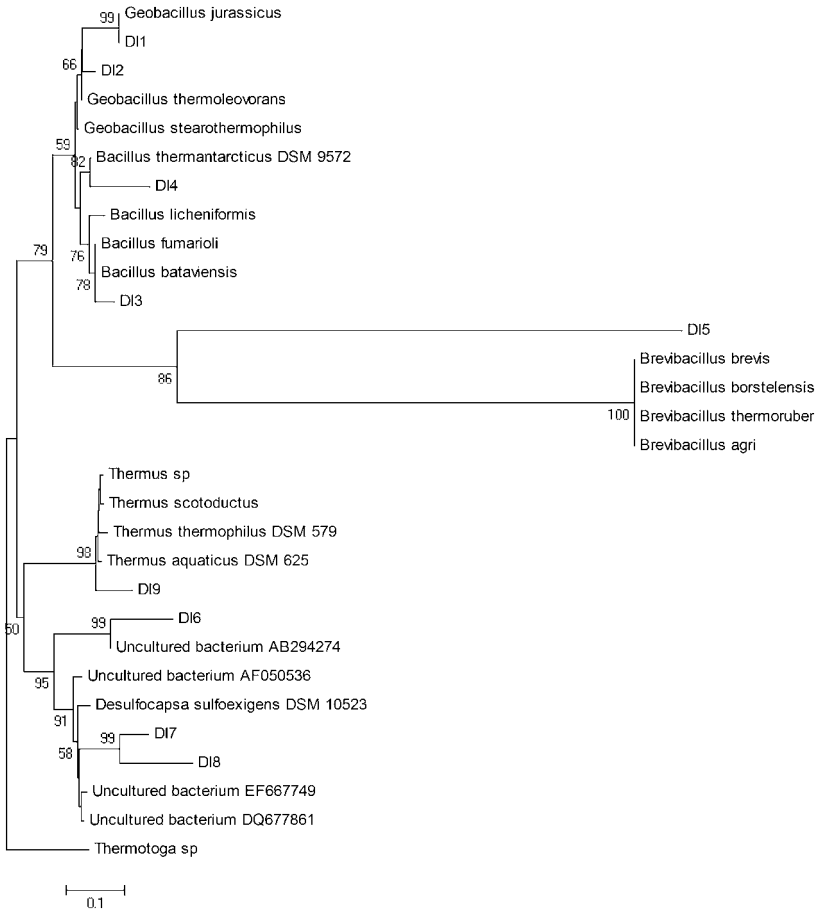


Fig. 3. Phylogenetic position of DGGE bands sequences analysed. The sequenced bands were compared to 16S rDNA gene of species from the genus *Geobacillus*, *Bacillus*, *Brevibacillus*, *Thermus* and uncultured bacteria. *Thermotoga* sp. was used as outgroup. Phylogenetic tree was inferred by using the Neighbor-joining method with a bootstrap test of 1000.

range of nutritional requirements, physiology, metabolic diversity and DNA base composition (Xu & Côté 2003). Several species of this clade have been isolated from geothermal sites in Antarctica including thermophilic members of the genera *Bacillus*, *Alicyclobacillus*, *Brevibacillus*, *Aneurinibacillus* and *Anoxybacillus* (Nicolaus *et al*. 1998, Bargagli *et al*. 2004, Allan *et al*. 2005, Poli *et al*. 2006). The sequences of DI3 and DI4 showed a high percentage of identity with thermophilic rods previously isolated from Mount Melbourne, *Bacillus fumarioli* and *Bacillus thermantarcticus*, respectively (Table I), indicating that these bacteria are also present in Fumarole Bay.

DI9 band clustered within the *Thermus* clade indicating the presence of this type of thermophilic bacterium in Deception Island. The presence of *Thermus* has been previously reported in the McMurdo Dry Valleys. The analysis of the small subunit of rDNA gene and the sequences obtained form an independent clade within the *Thermus*/*Deinococcus* bacterial group (de la Torre *et al*. 2003). Additionally, *Thermus* phylogeny based on 16S rDNA sequence analysis displays a geographic clustering (Saul *et al*. 1993). In this way, distant geothermal sites can be considered as isolated sites with barriers for the dispersal of microorganisms, providing the opportunity to study speciation of *Thermus* species in Antarctica.

Three different bands (DI6–DI8) obtained in DGGE analysis from soil samples of Fumarole Bay shore correspond to uncultured sulphate reducing bacteria. Thermophilic sulphate reducing bacteria have been previously reported to belong to the genus *Desulfotomaculum*. Thermophilic members of this genus like *Desulfotomaculum geothermicum* and *Desulfotomaculum hydrothermale* have been isolated from geothermal sites (Daumas *et al*. 1988, Haouari *et al*. 2008). These bacteria are probably implicated in the mineralization of organic carbon derived from primary production by the benthic community. Organic mineralization in sediments is carried out by a joint activity of aerobic bacteria and sulphate reducing bacteria. The contribution of sulphate reduction to aerobic mineralization is greater in coastal sediments and declines in deeper water (Walker 2005). The existence of sulphate reducing bacteria at the seashore of Fumarole Bay might have been expected but thermophilic sulphate reducing bacteria have not been previously described from Deception Island.

In this work a culture-independent method was used to identify the diversity of thermophilic bacteria in soil samples. Additionally, we were able to isolate a thermophilic bacterium using a modified basal medium. Blastn analysis of the 16S rDNA sequence in GenBank revealed that the isolated

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bacterium showed a high correlation with *G. jurassicus* (FN428697). The thermophilic bacterium obtained was a pure culture as indicated by DGGE where a single band correlation (Lane E, Fig. 2), indicated the purity of the isolate. Geobacilli are widely distributed and have been successfully isolated from several geothermal areas. In addition, *Geobacillus* species have also been recovered from artificial hot environments. Members of this genus are good examples for studies of thermal metabolic adaptations. In fact, the isolation of *G. jurassicus* could let us understand mechanisms for growth at high temperatures.

In conclusion, the thermophilic bacterial diversity found at Fumarole Bay is at least composed of members of the genera *Geobacillus*, *Bacillus*, *Brevibacillus*, *Thermus* and uncultured sulphate reducing bacteria. However, thermophilic members of these bacterial genera are widely distributed in Antarctica, and the natural barriers that separate geothermal sites in this continent make this area an ideal scenario to study how thermophilic bacteria evolve in different and distant Antarctic sites.

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