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Alicyclobacillus sp. strain CC2, a thermo-acidophilic bacterium isolated from Deception Island (Antarctica) containing a thermostable superoxide dismutase enzyme

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Abstract A gram-positive, rod-shaped, aerobic, thermo-acidophilic bacterium CC2 (optimal temperature 55℃ and pH 4.0), belonging to the genus *Alicyclobacillus* was isolated from geothermal soil collected from "Cerro Caliente", Deception Island, Antarctica. Owing to the harsh environmental conditions found in this territory, microorganisms are exposed to conditions that trigger the generation of reactive oxygen species (ROS). They must have an effective antioxidant defense system to deal with this oxidative stress. We focused on one of the most important enzymes: superoxide dismutase, which was partially purified and characterized. This study presents the first report of a thermo-acidophilic bacterium isolated from Deception Island with a thermostable superoxide dismutase (SOD).

Keywords **Antarctica, thermo-acidophile, SOD, geothermal, Deception Island**

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1 Introduction

Antarctica can be considered the most extreme and pristine continent on the planet owing to its environment, geographical position, isolation from human activities and low pollution[1]. Despite low temperatures and cold habitats, several geothermal sites have been described in Antarctica, including Mount Erebus, Mount Melbourne, Mount Rittmann and Deception Island[2]. Deception Island is an active volcano in the South Shetland Islands. Its geological characteristics include flooded calderas, hot springs, barren volcanic slopes, steaming beaches and ash-layered glaciers, and provide a unique environment for extremophilic microbial growth. So far only a few thermophiles have been isolated from Deception Island including bacteria of genera *Geobacillus* and *Bacillus*[3-4]. The presence of a thermo-acidophilic bacterium has never been reported on this island.

All organisms in Antarctica are exposed to conditions that trigger the generation of reactive oxygen species (ROS). One of these conditions is the high UV radiation present owing to the depletion of the stratospheric ozone layer over the past three decades[5]. Microorganisms found under these conditions must have an effective antioxidant defense system to prevent the oxidative stress generated by ROS. We focused

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on the most important enzymes among the components of the antioxidant system: catalase (CAT) and superoxide dismutase (SOD). Both enzymes play a protective role against oxygen toxicity in aerobic organisms. CAT catalyzes the decomposition of hydrogen peroxide to water and oxygen[6] and SOD catalyzes the dismutation of superoxide radicals to hydrogen peroxide and oxygen[7].

In the present study, we described a thermo-acidophilic bacterium belonging to the genus *Alicyclobacillus*, isolated from the geothermal soils of "Cerro Caliente" in Deception Island, and the description of a partially purified superoxide dismutase enzyme.

2 Materials and methods

2.1 Sample collection

Soil samples were collected during the Antarctic Chilean Expedition 45 (ECA 45), from totally de-glaciated areas from geothermal sites around Cerro Caliente, Deception Island (62°58.045′S, 60°42.609′W). The temperature of the sites ranged from 75℃ to 95℃ and pH was 5.5. All samples were aseptically collected and transferred to sterile vials.

2.2 Isolation and culture conditions

All samples were aerobically grown in LB medium (5 $g \cdot L^{-1}$ yeast etract, 10 $g \cdot L^{-1}$ tryptone and 10 $g \cdot L^{-1}$ NaCl) at 50℃ . pH was adjusted to 5.0 with HCl. Strain CC2 was isolated from these samples using a serial dilution technique and inoculated on solid LB medium, containing 2% Gelrite and 0.75 $g \cdot L^{-1}$ $MgCl_2$.

2.3 Growth measurements

Growth in liquid media was monitored by measuring the optical density with a spectrophotometer (Shimadzu, Japan, UV-VIS) at 600 nm. All experiments were performed in triplicate. The temperature range for growth was determined by incubating the isolate at temperatures ranging from 30℃ to 70℃ . The pH range for growth was determined in LB medium adjusting pH in a range from 2.0–7.0 with HCl. All pH measurements were made at 55℃.

2.4 Phenotypic characterization

Cell morphology was examined using scanning electron microscopy (SEM) (JEOL JSM-T300) and the method of Sonobe et al.[8]. Spore staining was performed using the method of Gordon and Pang[9] and Gram staining determination was carried out using a chemical method[10]. Acid production on different carbon sources was tested in OF liquid media (2.0 $g \cdot L^{-1}$ casein peptone, 3.0 $g \cdot L^{-1}$ K_2HPO_4, 0.3 $g \cdot L^{-1}$ methyl orange, 5.0 $g \cdot L^{-1}$ NaCl and 2.5 $g \cdot L^{-1}$ bacteriological Agar) containing 3.0 $g \cdot L^{-1}$ of the following carbon sources: glucose, maltose, ribose, D-galactose, L-arabinose, lactose, sorbitol or mannitol. Catalase and oxidase activities were determined according to Smibert and Krieg [11]. Nitrate reduction was determined using the method of Lanyi [12].

2.5 Gene sequencing and phylogenetic analyses

Genomic DNA was extracted using the chloroform-isoamyl alcohol method[12]. 16S rRNA gene sequences were amplified from genomic DNA by PCR using the bacteria specific primers Bac E334F and E939R[14]. The reaction mix consisted of 2.5 U Taq DNA polymerase, 200 μM each deoxynucleotides (dATP, dCTP, dGTP and dTTP), 1X reaction buffer, 0.75 mM $MgCl_2$ and 0.5 mM of each primer. The following thermal conditions were applied: 95℃ for 45 s, 55℃ for 45 s and 72℃ for 45 s. Each cycle was repeated forty times. A final elongation step of 72℃ for 10 min was added. Amplification reactions were carried out using a Palm Gradient Cycler (Corbett). PCR product was observed on agarose gel (1.5%) with 1×TAE buffer (40 mM Tris-acetate, 10 mM EDTA and 0.5 $\mu g \cdot mL^{-1}$ ethidium bromide) under UV light. PCR product was sequenced using the set of primers described above, analyzed, and manually edited using ChromasPro software (Technelysium Pty Ltd.) for a final sequence extension of 843 bp. ClustalW software was used to align the partial sequence of a 16S rRNA gene from CC2 with selected sequences retrieved from GenBank. For the construction of a phylogenetic tree, *Thermotoga* sp. was used as an outgroup. The software package MEGA4[15] was used for a phylogenetic analysis and a tree was constructed using the Neighbor-Joining method[16] with a bootstrap analysis of 1 000.

2.6 Superoxide dismutase purification

SOD was purified from *Alicyclobacillus* cells at room temperature. A cellular disruption method specially designed for thermophilic microorganisms was used. Cells were suspended in 150 mL of 50 mM Tris-HCl buffer (pH 7.5) containing 15 mM EDTA (pH 8.0), lysozyme (1 $mg \cdot mL^{-1}$) and DNase I (10 $\mu g \cdot mL^{-1}$) and were incubated at 37℃ for 1 h. The sample was sonicated (Branson sonifier 450) on ice for 5 min, cell debris was removed by centrifugation (8 603× *g* for 20 min) and the supernatant was used as crude extract for the purification. The crude extract (145 mL) was loaded onto a column (Pharmacia, C 16/20) of DEAE-Sepharose Fast Flow (Pharmacia Biotech) equilibrated with 10 mM phosphate buffer (pH 7.2). Proteins were eluted with a linear gradient (120 mL) of 0 to 0.5 M phosphate buffer (pH 7.2). The fraction showing SOD activity was eluted when 135 mM of buffer phosphate was applied to the column. The column was controlled by a Pharmacia FPLC system.

2.7 SOD Assays

SOD activity was assayed based on the ability of SOD to inhibit the reduction of nitroblue tetrazolium (NBT) by

superoxide using the method of Winterbourn et al.[17]. Each reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 10 mM EDTA, 130 mM methionine, 2 mM riboflavine, 0.3 mM NBT and enzyme. The reaction mixture was incubated in a light box for 15 min and SOD activity was measured spectrophotometrically at 21℃ by measuring the reduction of NBT at 560 nm. One unit (U) of SOD activity was defined as the amount of enzyme causing one half of the maximum inhibition of NBT reduction. Protein concentration was measured by Bradford method[18] with a commercial assay kit (Bio-Rad) using BSA as the standard.

2.8 Effects of pH on SOD activity

pH dependence of SOD activity was determined at 21℃ using the following buffer: 50 mM MOPS (pH 6.5–7.0), 50 mM Tris-HCl (pH 7.5–8.8).

2.9 SOD thermostability

SOD thermostability was determined by placing the enzyme in small tubes with O-ring-sealed caps and incubating for 0 – 6 h in a dry bath (Major Science, MD-02N-220) at 50℃ and 70℃. Samples were taken at different times and assayed for enzyme activity. Residual activity was determined at 21℃ under the conditions described in the enzyme assays section.

3 Results and discussion

Only two members of genus *Alicyclobacillus* have been isolated from geothermal sites in Antarctica: *A. pohliae*[19] and *A. acidocaldarius*, subsp. *rittmannii*[20]. The presence of members of this genus have not been previously reported from Deception Island. In this work, a thermo-acidophilic bacterium designated as CC2 was isolated from "Cerro Caliente", Deception Island during Scientific Expedition ECA 45. Vegetative cells of CC2 were long rods (0.5–1.0 mm in diameter and 8.0 mm long), non-motile, gram-positive and spore-forming. On solid media, colonies were white, opaque, convex and circular with an 0.8–1.0 mm diameter after 3 d of incubation at 55℃ . The bacterium grew aerobically at temperatures between 30 ℃ and 70 ℃ with an optimum temperature of 55℃ , and in the pH range of 2.0–7.0 with an optimum at 4.0. Growth incubation time was 24 h with a doubling time (t_d) of 39 min. CC2 was able to reduce nitrate under anaerobic conditions, is oxidase negative and has weak catalase activity. It was able to grow under anaerobic and microaerophilic conditions, but its optimal growth was detected under aerobic conditions. Figure 1 shows the phylogenetic relationship of the bacterium CC2 in a 16S rRNA based tree. The thermophilic strain CC2 belongs to genus Alicyclobacillus and it forms a cluster with *A. acidocaldarius* subsp. *acidocaldarius*, *A. sendaiensis*, *A. vulcanis* and *A. acidocaldarius*, subsp. *rittmannii*, but is separate from *A. pohliae*. Strain CC2 appears to be more similar to *A. acidocaldarius* subsp. *acidocaldarius*. In comparison to the other two species from the genus isolated from Antarctica, CC2 was slightly different in its growth conditions and biochemical characteristics (Table 1).

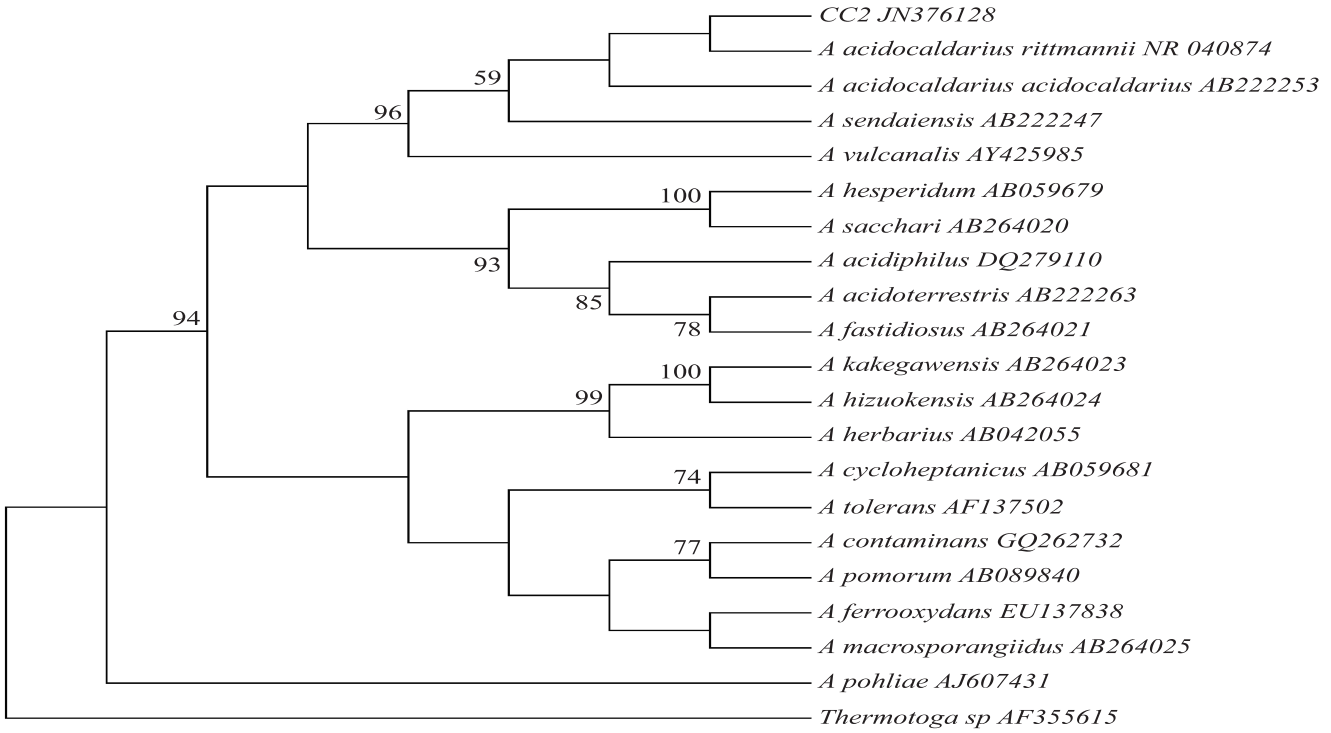

Figure 1 Strain CC2's 16S rDNA gene sequence compared with species from the genus *Alicyclobacillus*. A *Thermotoga* sp. was used as an outgroup. The phylogenetic tree was inferred using the Neighbor-joining method with a bootstrap test of 1 000.

Table 1 Phenotypic characteristics of strain CC2 and some species of the genus *Alicyclobacillus*. 1, CC2 (data from the present study); 2, *A. acidocaldarius* subsp. *acidocaldarius* ATCC 27009T [23]; 3, *A. acidocaldarius* subsp. *rittmannii* DSM 11297 [20]; 4, *A. pohliae* CIP 109385T [19]. (+, positive; −, negative; NR, not reported)

Characteristics	1	2	3	4
pH for growth				
Range	2.0−7.0	2.0−6.0	2.5−5.0	4.5−7.5
Optimum	4.0	4.0	4.0	5.0
Growth temperature/°C				
Range	30−70	45−70	45−70	42−60
Optimum	55	60	63	55
Oxidase	−	−	−	−
Catalase	+	+	−	−
Utilization of carbon source				
L-Arabinose	+	+	NR	+
Ribose	−	NR	NR	+
D-Galactose	+	+	+	+
Mannitol	+	+	NR	−
Sorbitol	−	−	NR	−
Maltose	+	+	+	+
Lactose	+	+	NR	+
Glucose	+	+	+	+

The presence of thermophilic long bacilli has been previously described in Deception Island. Muñoz et al.[4] and Llarch et al.[3] reported the existence of thermophiles from the genera *Bacillus, Geobacillus* and *Brevibacillus*. None of the previously described bacteria are capable of growing under acidic conditions like the isolate CC2.

Analysis of the phenotypic and phylogenetic results obtained from the characterization of CC2 indicate that CC2 shares several characteristics with the species of the genus *Alicyclobacillus* previously described from Antarctica.

Enzymatic defense mechanisms have not been previously described in the genus *Alicyclobacillus*. We focused on SOD activity owing to its important role in dealing with oxygen toxicity. Molecular analysis of the complete genome from *A. acidocaldarius* subsp. *acidocaldarius*[21] revealed the presence of an SOD enzyme in its genome, however its activity has not been previously assayed nor reported. CAT and SOD activities were detected in crude extract from CC2. CAT activity was weak compared with SOD activity. Because of this, SOD from CC2 was partially purified and characterized to describe one of the mechanisms that contributes to its antioxidant defense. Optimal activity of the SOD enzyme occurred at pH 7.4, when the temperature for the assay was 21℃ (Figure 2a), suggesting that the microorganism has adaptation mechanisms to maintain a neutral internal pH. The optimal temperature registered for SOD enzymes from CC2 was 50 ℃. Some enzymes have shown optimal temperatures slightly different from the growth temperature of the organism [22]. This could explain the results obtained from the thermostability studies at 50 ℃ and 70℃ (Figure 2b). The enzyme maintained more than 80% of its relative activity when it was incubated for 6 h at 50℃ , but was inactivated at 70 ℃. It lost 50% of its initial activity after 30 min of incubation and showed no activity after 2 h of incubation at 70℃ (Figure 2b).

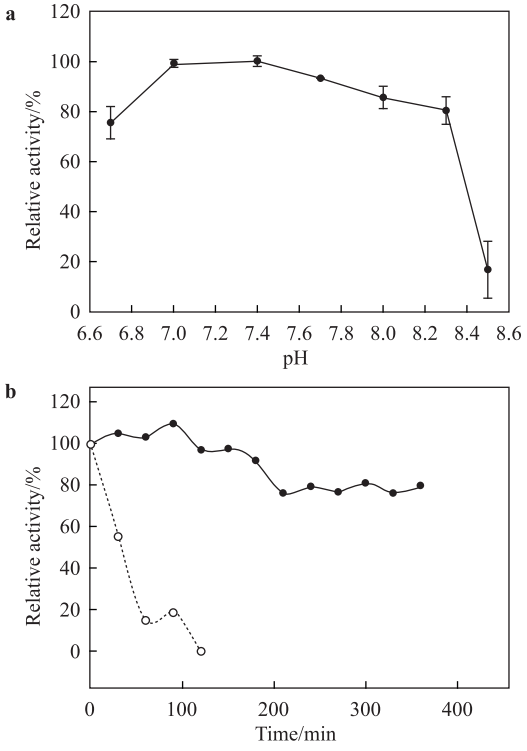

Figure 2 Characterization of SOD activity from CC2. **a**, The optimal pH for SOD activity at 21℃. Assay buffers were 50 mM MOPS (pH 6.5–7.0), 50 mM Tris-HCl (pH 7.0–8.8). **b**, Thermal inactivation profile of SOD at 70℃ (○) and 50℃ (•).

Our results show that strain CC2 is closely related to the genus *Alicyclobacillus*, and has a SOD as part of its antioxidant defense system that allows it to survive under extreme conditions, avoiding the effects of oxidative stress in Antarctica. The extreme environmental conditions present in Antarctica, could act as a selection pressure, activating SOD enzymes from the antioxidant defense system in CC2, as one of its survival mechanisms.

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