BioMetals (2005) 18:529–536
DOI 10.1007/s10534-005-0837-z

Iron Demand by Thermophilic and Mesophilic Bacteria Isolated from an Antarctic Geothermal Soil

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Received 24 January 2005; accepted 13 July 2005

Key words: thermophilic bacteria, geothermal soils, iron bioavailability, continental Antarctica

Abstract

The thermophilic bacterial strain MP4 assigned to a new species, likely of the genus *Alicyclobacillus*, was isolated from geothermal soils on the NW slope of Mount Melbourne, Antarctica. These soils have high iron concentrations and the strain MP4 requires iron additions for growth. Four mesophilic bacterial strains *Paenibacillus validus* MP5, MP8, and MP10, and *P. apiarius* MP7, isolated from the same site, need iron supply for growth depending on the medium. Growth temperature of thermophilic strain ranges from 42 to 70 °C, and that one of mesophiles from 25 to 44 °C. Thermophilic and mesophilic strains shared microenvironments with temperature of 42–44 °C and showed optima of pH values ranging from 5.5 to 6.0. The thermophilic strain MP4 reached values of 10^6 CFU ml^{-1} in aqueous soil extract from the NW slope of Mt. Melbourne, and 10^5 CFU ml^{-1} in water extracts from other geothermal Antarctic areas (Mt. Rittmann and Cryptogam Ridge). Growth of thermophilic bacteria in aqueous extracts of the NW slope of Mount Melbourne soils caused a reduction of 50% of soluble iron content, which was recovered in bacterial biomass. These results suggest a possible involvement of the thermophilic strain MP4 in iron bioavailability in these geothermal soils.

Introduction

Microorganisms require trace elements, which are actively taken up by specific transport systems. The uptake of iron is of special importance because this element may behave as a growth limiting factor (Lengeler *et al*. 1998). In fact, although iron is the fourth most common element in the earth crust (O > Si > Al > Fe), it is not readily available. Under oxic conditions, iron is oxidized to Fe(III), which forms extremely unsoluble oxide hydrates FeO(OH), carbonate $Fe_2(CO_3)_3$, and magnetite Fe_3O_4[iron(II/III) oxide]. These compounds become soluble only at very acidic pH. Therefore most aerobic microorganisms, to overcome low iron availability, produce and secrete Fe^{3+} -complexing organic compounds called siderophores (iron carriers) which transport iron complexes into cells (Winkelmann 1991; Winkelmann & Carrano 1997; Siegel & Siegel 1998). In anaerobic conditions iron is not limiting because Fe^{2+} is soluble and it can be absorbed through simple cation transport systems (Walsh 1979).

In continental Antarctica there are some volcanic sites where fumaroles warm the soil and steam emissions condense to maintain relatively steady moisture supply and well adapted biotic communities (e.g. Broady *et al*. 1987; Bargagli *et al*. 1996). In Victoria Land warm soils occur at high altitude (>2200 m) in three active volcanoes: Mount Erebus, Mount Rittmann and Mount Melbourne. Microbial communities in these soils have been widely investigated. From Mount Erebus geothermal substrata different thermophilic bacterial strains were isolated, and assigned to the genera *Bacillus* and *Clostridium* (Hudson &

Daniel 1988; Hudson *et al*. 1989). At the Mount Rittmann, some thermophilic species belonging to the genus *Alicyclobacillus* were isolated at a temperature of about 60 °C and accepted as a subspecies of *A. acidocaldarius* (*A. acidocaldarius* subsp. *rittmannii*) (Nicolaus *et al*. 1998). In the Mount Melbourne (northern Victoria Land) ground heated by geothermal activity has a constant temperature of about 60 °C at 25 cm depth and in a small area "Cryptogam Ridge", located in the rim of the summit caldera, there are a well developed community of algae (about a dozen species), one species of moss, and one of livewort (Broady *et al*. 1987). First bacteria isolated from warm soils were the thermotolerant–thermophilic *Thermospora* sp., *Streptomyces coelicolor* and different Gram-positive non identified strains (Broady *et al*. 1987). Later, a thermophilic strain belonging to the new species *Bacillus thermoantarcticus* (now *thermantharcticus*) was isolated from Cryptogam Ridge soils, and the strain appeared to resemble *Bacillus* (now *Alicyclobacillus*) *acidocaldarius* (Nicolaus *et al*. 1991; 1996). Recently Logan *et al*. (2000) isolated a new species (*Bacillus fumarioli*) from soils with mosses collected on Cryptogram Ridge, while some sporeformers microorganisms were isolated from warm soil collected on the NW slope of Mount Melbourne, but they were not studied because of difficulties in their cultivation in laboratory.

In the latter site four soil samples were collected in January 2002 around a warm ground colonised by the moss *Pohlia nutans*, and the thermophilic strain MP4 *Alicyclobacillus* sp. and four mesophilic bacteria *Paenibacillus validus* strains MP5, MP8, and MP10, and *P. apiarius* strain MP7 were isolated (Bargagli *et al*. 2004). As the total iron concentration and bioavailability in soils from this site were higher than in geothermal soils from "Cryptogam Ridge" and the Mount Rittmann, the aim of this study was to investigate if the new thermophilic and mesophilic isolated strains need an iron enriched medium for their growth.

Materials and Methods

Study area

Four surface soil samples (0–5 cm) were aseptically collected in the NW slope of Mt. Melbourne along a thermal gradient: 43, 34, 33, and 25 °C for sample A, B, C, and D respectively. The pH (1:2.5, w/v; in water) ranged from 5.62 to 5.71; the gravel percentage from 32.1 to 36.0%, that of sand from 54.8 to 56.3%, silt 2.5 to 3.6%, clay 6.7 to 8.0%. The bacterial strain MP4 isolated at 55 °C, was chosen to carry out the experimental work together with four mesophilic strains belonging to the genus *Paenibacillus* isolated from the same soil samples (Bargagli *et al*. 2004). The iron concentrations in water extracted soils ranged from 0.97 to 1.08 μg g^{-1} (Bargagli *et al*. 2004).

Growth tests at different temperatures and pH values

Tests with thermophilic strain MP4 and the mesophilic ones MP5, MP7, MP8, and MP10 were carried out in Tryptic Soy Broth (TSB) medium (Difco) containing 17.0 g of Bacto Tryptone, 3.0 g of Bacto Soytone, 2.5 g of Bacto Dextrose, 5.0 g of NaCl, and 2.5 g of K_2HPO_4 per litre of distilled water, sterilized in autoclave at 121 °C for 15 min. Ten millilitres of TSB were added to 18 ml tubes, in duplicate for each strain, and inoculated 1:100. Thermophilic strain cultures were amended with 0.25 mM of iron from a freshly prepared $FeSO_4 \times 7H_2O$ solution, sterilized by using 0.2 μm pore-size sterile filters (Sartorius). Strain MP4 was incubated at temperatures ranging from 37 to 70 °C, while strains MP5, MP7, MP8, and MP10 from 4 to 50 °C. Growth at different pH values was detected in TSB medium adjusted, by adding NaOH 1 M or HCl 1 M, at different pH ranging from 2.0 to 10.0 and sterilized by filtration. Aliquots of 1.0 ml were collected and the optical densities (i.e. the absorbance at 600 nm) measured by an UV-spectrophotometer (Ultraspec 2100 Pharmacia). Blanks of TSB medium at different pH values were always used for UV-spectrophotometer measurement, to correct possible interferences by slight precipitates in TSB medium at high pH values.

Growth in the presence of iron and other elements

Thermophilic and mesophilics strains were tested in different media: FeP w/o iron containing 5.0 g of tryptone (Difco), 0.5 g of D-glucose (BDH), and 0.1 g of $(NH_4)_2SO_4$ per litre of deionised water; Trypticase Soy Broth (TSB); DSMZ 259 containing

3.5 g of $Na_2HPO_4 \times 12H_2O$, 1.0 g of K_2HPO_4, 0.03 g of $MgSO_4 \times 7H_2O$, 0.05 g of NH_4Cl, and 4.0 g of Yeast Extract per litre of distilled water; PCA containing 5.0 g of tryptone (Difco), 2.5 g of Yeast Extract (Oxoid), and 1.0 g of d-glucose per litre of distilled water; LB containing 10.0 g of tryptone (Difco), 10.0 g of NaCl, and 5.0 g of Yeast Extract (Oxoid) per litre of distilled water. All the media were sterilized in autoclave at 121 °C for 15 min. Iron was present in the culture media at the following concentrations: 0.0015 mM in LB medium; 0.0026 mM in FeP w/o Fe; 0.0048 mM in Medium 259; 0.006 mM in TSB; and 0.046 mM in PCA medium. Iron concentration of 0.25 mM was added to the different culture media from a freshly prepared solution of $FeSO_4 \times 7H_2O$, sterilized by using 0.2 μm pore-size sterile filters (Sartorius). For each strain 10 ml of different media with and without iron were distributed in different 18 ml tubes, inoculated (1:100) and incubated at proper temperature. Growth was detected by measuring the absorbance at 600 nm.

Ten millilitres of liquid medium FeP w/o iron were added to 18 ml tubes, amended with 0.25 mM of the different solutions of $FeSO_4 \times 7H_2O$, $MgSO_4 \times 7H_2O$, $MnSO_4 \times H_2O$, $CaCl_2 \times 2H_2O$, and $CoCl_2$, sterilized by using 0.2 μm pore-size sterile filters (Sartorius). Tubes were inoculated (1:100) with an over-night culture of strain MP4, and incubated in the presence of oxygen at 55 °C. Optical density was measured after 24 h against a control of the medium amended with the different tested solutions.

The thermophilic strain was tested in the presence of different concentrations of Fe^{2+} and Fe^{3+} ranging from 0.0 to 0.5 mM, from freshly prepared solutions of $FeSO_4 \times 7H_2O$, $FeCl_3$, or Fe-citrate, sterilized by using 0.2 μm pore-size sterile filtrers (Sartorius). Experiments were carried out in 18 ml tubes containing 10 ml of FeP w/o liquid medium, inoculated (1:100) with a preculture of the thermophilic strain MP4. The optical density (i.e. the absorbance at 600 nm) was measured after 24 h at 55 °C, against a blank.

Growth of the thermophilic strain MP4 in the original geothermal soils

Aliquots of 0.5 g (dry weight) of soil samples from Mount Rittman, Cryptogam Ridge, and NW slope of Mount Melbourne, were added to 25 ml of deionised water, amended with 0.05% of Yeast Extract as vitamins supply in 50 ml flasks. Strain MP4 was inoculated (1:50) from an overnight culture harvested by centrifugation, washed twice and resuspended in buffer solution (NaCl 0.9%). Experiments were carried out in quadruplicate, and flasks were incubated at 55 °C. A control flask with deionised water and 0.05% of Yeast Extract was inoculated with strain MP4 and incubated as above. Weight of each flask was detected at time zero and, before each sampling, the initial weight was re-established by adding sterile deionised water. At different times aliquots of 1.0 ml were harvested in order to detect pH values (Crison pH-meters) and viable bacteria count as CFU ml^{-1}.

Growth of the thermophilic strain MP4 in aqueous extracts from geothermal soils

Water soil extracts (soil-deionised water, 1:5, w:v) obtained from samples collected on Mount Rittman, Cryptogam Ridge, and NW slope of Mount Melbourne were filtered by using 0.2 μm pore-size sterile filters (Sartorius). Inocula (1:50) were carried out from an overnight culture of strain MP4 harvested by centrifugation and washed twice with buffer solution (NaCl 0.9%). Inoculated cultures were incubated at 55 °C. Weight of each flask with culture were detected at time zero and before each sampling the initial weight was re-established by adding sterile deionised water. At different times aliquots of 100 μl were harvested and plated on solid FeP medium to calculate CFU ml^{-1} content and pH values were measured (Crison pH-meters).

Chemical analyses

Culture supernatants, and aqueous soil extracts were treated with 1.0 ml of concentrated HNO_3 and used for chemical analyses. Soils (about 0.2 g) were mineralised with ultrapure-grade HNO_3 in a Teflon bomb at 120 °C for 8 h. Analytical determinations of macro- and microelements concentrations in acid digested soils and in soil water extracts were performed, before and after bacterial growth, through a combination of spectrometric techniques, including Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) for Al, Mn, and Zn, Flame Atomic Absorption Spectrophotometry (FAAS) for Ca, K, and Fe, and Electrothermal Atomic Absorption with Zeeman

background correction (ZETAAS) for Cd, Cu, Fe and Pb, Flow Injection Atomic Absorption Spectrometry (FIAS) for As (hydride generation). Element concentrations were determined by using the method of standard additions, and replicate determinations were performed to check the sample homogeneity and uncertainties related to mineralization and analysis of samples. Procedural blanks were below the detection limits and the accuracy of digestion and analytical procedures was checked by routine determination of macro- and microelements in standard reference material (SRM No. 2711 "Montana Soil" from NIST, Gaithersburg, USA) with certified element concentrations.

Results

Growth temperature of thermophilic strain MP4 ranged from 42 to 70 °C, with an optimum at 60 °C, and from 25 to 44 °C for the mesophilic ones. Specific growth rates (i.e. the measure of the number of generations occurring per unit of time) were detected for the bacterial strains sharing a common area from 42 to 44 °C. Values of pH ranged from 5.0 to 7.0 for strains MP5, MP8, MP9, and MP10, with a maximum of growth at pH 6.0. Strain MP7 showed growth at pH values ranging from 6.0 to 10.0. The thermophilic strain MP4 showed a pH range from 4.5 to 7.5, with a maximum of growth at pH 5.5.

The thermophilic strain MP4 grew in all the different tested media only if iron was amended at concentrations of 0.25 mM (Figure 1a). Mesophilic bacterial strains showed different behaviours with respect to the iron supply. The mesophilic strain MP5 grew in all the different media in the presence of iron, excluding LB medium. Without iron additions the growth of MP5 strain was detected in all the media but with lower biomass values (reported as percentage of growth; Figure 1b). Iron addition to strain MP7 cultures allowed growth in PCA and in LB media, otherwise not possible. However the best growth values were reached in TSB medium without iron (Figure 1c). The strain MP8 showed a rather similar behaviour with high growth values on TSB medium without iron additions (Figure 1d). The strain MP10 showed a distinctive behaviour among mesophilc strains because it grew in all the tested media, and biomass values were high in almost all growth conditions (Figure 1e).

The thermophilic strain MP4 *Alicyclobacillus* sp. showed the same behaviour during growth in the presence of different iron concentrations added as Fe^{2+} or Fe^{3+}. Iron concentrations stimulate growth of the strain MP4 (Figure 2). In cultures without iron addition, growth of the thermophilic strain increase after the amendment of different solutions containing cations as Mg^{2+}, Mn^{2+}, and Ca^{2+}. On the contrary the addition of Co^{2+} did not allow growth of strain MP4.

The three cultures arranged with the thermophilic strain MP4 in the presence of different geothermal soils (NW slope of Mount Melbourne, Mount Rittman, and Cryptogam Ridge) showed similar growth and pH values. Total Fe concentrations in soils before and after growth of the thermophilic strain MP4 and in the liquid cultures did not show statistically significant variations (Table 1). Only a slight increase of iron concentration was measured in soils at the end of the incubation (ranging from 4.0% to 7.0% of total content). Iron concentrations in the bacterial cell biomasses resulted higher in samples from Critpogam Ridge soils (Table 1). Concerning the other analysed elements, a depletion in Al and Pb concentrations (6.0% and 1.5%, respectively) was detected in samples from the NW slope of Mount Melbourne after growth of the thermophilic strain MP4. The concentrations of Mn and Zn showed a small increase (7.4% and 5.7%, respectively) in the latter geothermal soil, no changes were detected for As, Cd, and Cu concentrations.

The thermophilic strain MP4 showed higher values of biomass (1.4×10^6 CFU ml^{-1}) in water soil extract from the NW slope of Mount Melbourne, whereas values one order of magnitude lower were obtained in aqueous soil extracts from Mount Rittmann and Cryptogam Ridge.

Iron concentrations in the aqueous soil extract from the NW slope of Mount Melbourne decreased about 50% after eight days of *Alicyclobacillus* sp. strain incubation (Figure 3). Almost all the iron lost from the aqueous soil extract was detected in the bacterial biomass; a similar pathway was found in the Cryptogam Ridge aqueous soil extract (Figure 3). On the contrary iron concentrations in the aqueous soil extract from the Mount Rittmann did not change after the growth of bacterial strain MP4 (Figure 3).

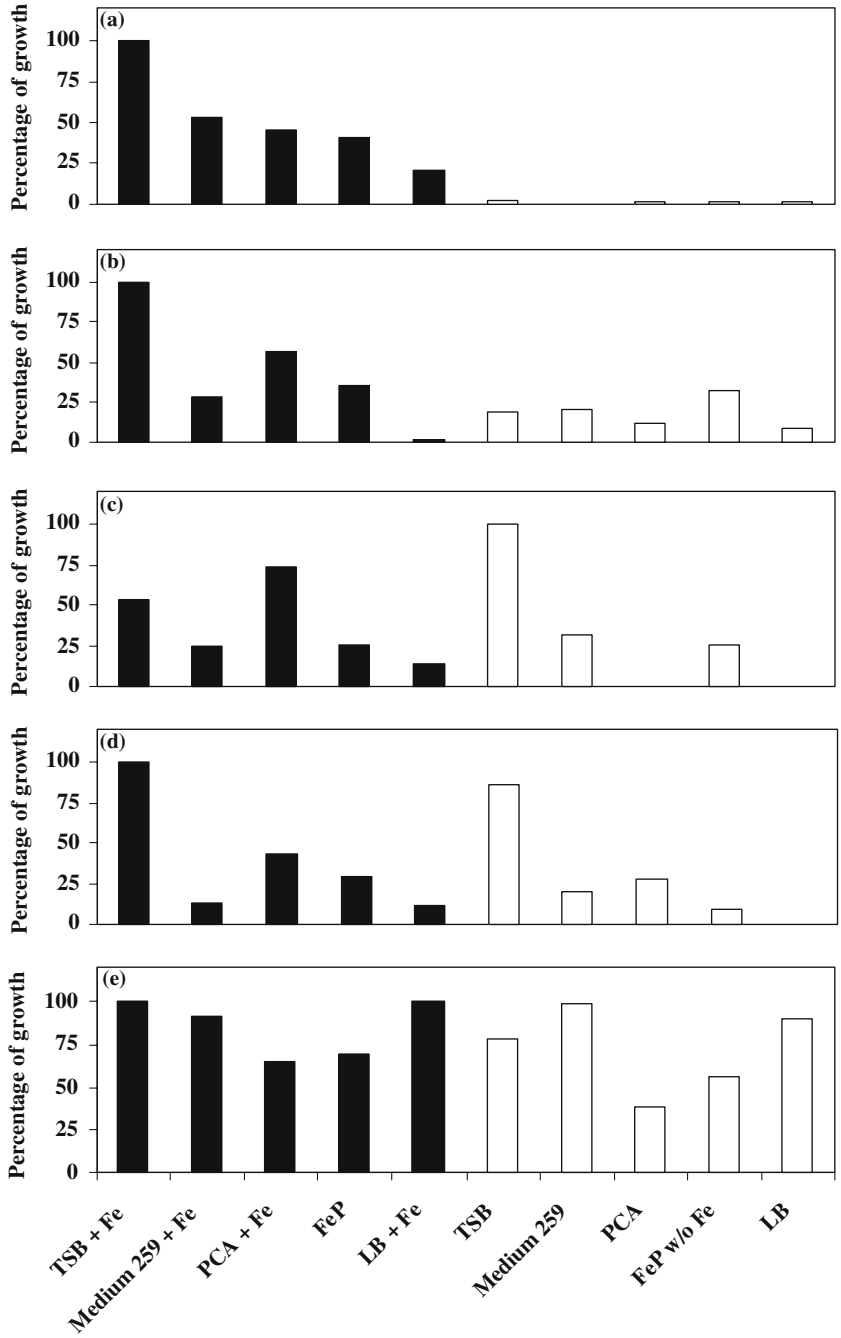

Figure 1. Growth of thermophilic strain MP4 *Alicyclobacillus* sp. (a) and mesophilic strains *Paenibacillus* spp. MP5 (b), MP7 (c), MP8 (d), MP10 (e), isolated from Antarctic geothermal soil samples, expressed as percentages of growth in different media, with and without iron added. Incubation temperature was adapted to each strain. Growth was detected by measuring the absorbance at 600 nm.

Zn, Ca, Cu, and K concentrations in aqueous soil extracts did not change after growth of the thermophilic bacterial strain MP4, while those of Al decreased (about 10%) in bacterial cultures arranged with aqueous soil extracts from Cryptogam Ridge and the NW slope of Mount Melbourne soils.

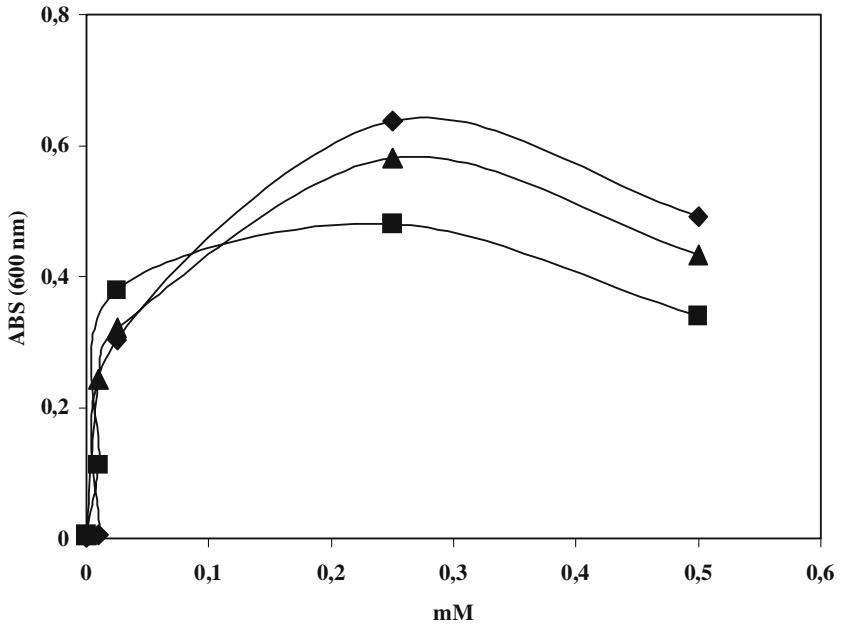

Figure 2. Growth of the thermophilic strain MP4 *Alicyclobacillus* sp., in the presence of different concentrations of Fe added as $Fe(SO_4)_2$ (♦); $FeCl_3$ (■), and Fe-citrate (▲). Growth was detected by measuring the absorbance at 600 nm, after 24 h of incubation at 55 °C.

Table 1. Total concentrations (%) of Fe detected before and after growth of the bacterial strain MP4 *Alicyclobacillus* sp., and in liquid bacterial cultures. ±: standard deviation.

	NW slope of Mount Melbourne	Mount Rittmann	Cryptogam Ridge
	Fe (%)		
Soil before bacterial growth	1.77	1.35	1.62
Soil after bacterial growth	1.83 ± 0.17	1.38 ± 0.11	1.69 ± 0.04
Liquid bacterial culture	0.016 ± 0.006	0.023 ± 0.007	0.37 ± 0.012

Discussion

The above reported results indicate that the thermophilic strain MP4 (likely a new species of the genus Alicyclobacillus) needs iron supplementation for growth.

Bacteria require micromolar levels (0.4–1.0 μM) of bioavailable iron for optimal growth, because this element is an essential cofactor of many enzymes with redox activity (Guerinot 1994). Iron content of growth media used for the experiment conducted with thermophilic and mesophilic strains, ranged from 1.5 to 46 μM, allowing growth only for the mesophilic strains. These bacterial strains use this iron source and are able to grow with different behaviours as respect to iron added, depending on the media, and strain MP10 is growing without addition of the element, likely showing a high affinity for iron in solution. Whereas the thermophilic bacterial strain seems to require an higher iron concentration, reaching the demand of an excess of iron. The different bacterial strains probably have a different behaviour as respect to the use of iron in the same substrata.

Although 0.25 mM of iron necessary for the growth of the aerobic and heterothrophic thermophilic strain MP4 *Alicyclobacillus* sp., may represent an excess of this element, iron-rich culture media were used in the experiments because the formation of insoluble iron-complexes in the rich media can likely decrease the iron availability. There is evidence that the soil bacterium *Bacillus*

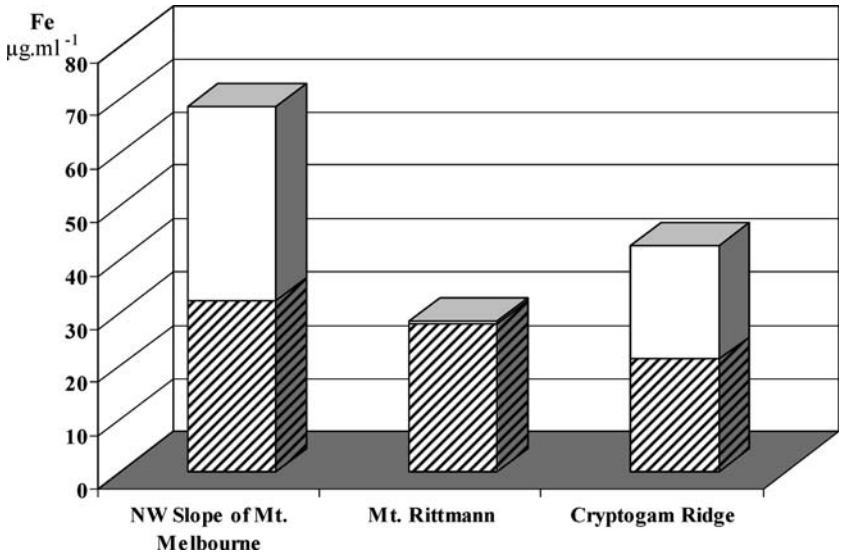

Figure 3. Total concentrations of Fe expressed as μg 10 ml^{-1} in the three aqueous soil extracts from geothermal soils after growth of the thermophilic strain MP4. The heights of the istograms represent the total Fe concentrations before bacterial incubation; the white areas of the istograms represent Fe content detected in the bacterial biomass; and the grey areas of the istograms represent Fe content residue in the aqueous soil extracts after bacterial incubation at 55 °C.

subtilis requires high iron levels in the presence of high salinity which causes iron limitation (Hoffmann *et al*. 2002). Stress due to the high salt concentrations is balanced by providing cells with an excess of iron, adding 250 μM of iron as $FeCl_3$. This improvement in growth is substantial, since it is similar to that caused by the potent osmoprotectant glycine betaine (Boch *et al*. 1994; Hoffmann *et al*. 2002).

An excess of iron in the presence of oxygen is undesirable for the cell because it stimulates the formation of toxic hydroxyl radicals via the Fenton reaction which then damage DNA and membranes (Storz & Zheng 2000). A tight genetic control regulate iron homeostasis by the central iron regulatory protein Fur (Escolar *et al*. 1999). Freshly prepared $FeSO_4 \times 7H_2O$ solution, as well as solutions of the divalent forms of Mn, Co, and Cd activate Fur protein, and Fur also require Mg^{2+} in millimolar concentrations, in conjunction with micromolar amounts of one of the above metals. Aluminium, a trivalent cation, did not enhance binding of Fur to the promoter (Braun *et al*. 1987). In the case of the thermophilic strain MP4 growth is improved by iron addition, or alternatively by additions of divalent forms of Mg, Mn, and Ca; on the contrary Co seems to have a negative effect on bacterial growth. These elements could play a role in the iron use by the strain, probably competitive in the case of Co.

The thermophilic strain MP4 is able to grow on different geothermal soils and on their aqueous extracts. Basically no differences among element concentrations in soils before, and after bacterial growth have been detected. Elements present in the soils were probably not available and possibly bacteria were not able to absorb and/or to uptake iron in these conditions.

An iron lowering in the solution of aqueous soil extracts of NW slope of Mount Melbourne and "Cryptogam Ridge" after bacterial growth was detected, likely as consequence of iron uptake or adsorption by bacterial cells. In fact, the amount of iron lost from the solution was recovered on the biomass of the thermophilic strain. A different behaviour was detected in the culture arranged with Mount Rittmann aqueous soil extract, where iron detected in the biomass represented a little percentage, probably because this substrate was not optimal for thermophilic strain MP4 growth and activity.

The iron demand suggests a possible adaptation of thermophilic strain to geochemical features of geothermal soils, in particular to those of the NW slope of Mount Melbourne, which have higher total and bioavailable iron concentrations

than soils from the other two geothermal areas (Bargagli *et al*. 1996; 2004). Extracted soil of NW slope of Mount Melbourne probably promoted an activity of the thermophilic strain MP4 in iron chelating or iron uptake. It cannot be excluded that in the NW slope of Mount Melbourne the metabolism of the thermophilic strain MP4 may change chemical forms of iron, playing a role in the biogeochemical cycle of the metal. For instance an *Alicyclobacillus* sp. playing an active role in the Fe^{2+} oxidation was isolated from mine soils in Collie (northern Australia) (Kinnunen *et al*. 2003).

Other elements as Mg, Zn, and Ca did not show the same behaviour, suggesting that interaction with iron could represent a specific activity of the thermophilic bacteria, and it is not an unspecific mechanism. In this context bacterial production of iron-chelating molecules (siderophores), could play a role in the bacterial growth in soils and in their aqueous extracts (Chakraborty *et al*. 2003; Weaver & Kolter 2004). Further studies are necessary to understand iron uptake and/or absorption mechanisms in *Alicyclobacillus* sp. strain MP4.

Acknowledgments

This work was supported by funds from PNRA (Programma Nazionale di Ricerche in Antartide).

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