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Abstract

The boundary between ice and basalt on Earth is an analog for some near-surface environments of Mars. We investigated neutrophilic iron-oxidizing microorganisms from the basalt-ice interface in a lava tube from the Oregon Cascades with perennial ice. One of the isolates (*Pseudomonas sp. HerB*) can use ferrous iron Fe(II) from the igneous mineral olivine as an electron donor and O_2 as an electron acceptor. The optimum growth temperature is ~12-14^{o}C, but growth also occurs at 5^{o}C. Bicarbonate is a facultative source of carbon. Growth of *Pseudomonas sp. HerB* as a chemolithotrophic iron oxidizer with olivine as the source of energy is favored in low O_2 conditions (*e.g.*, 1.6 % O_2). Most likely, microbial oxidation of olivine near pH 7 requires low O_2 to offset the abiotic oxidation of iron. The metabolic capabilities of this bacterium would allow it to live in near-surface, icy, volcanic environments of Mars in the present or recent geological past, and make this type of physiology a prime candidate in the search for life on Mars.

Introduction

The present day temperature of Mars' surface is mostly below the freezing point of water, the thin atmosphere leaves the surface exposed to UV radiation, and the absence of a magnetic field exposes the surface to ionizing radiation. Because of inhospitable conditions, primary production through photosynthesis is assumed not to occur. Yet, the shallow subsurface of the Red Planet, where temperatures are above freezing, could harbor chemolitho-autotrophic microorganisms. In the recent geological past, Mars' surface could have been above freezing because of residual geothermal heat, orbital forcing, or greenhouse gas effects (Carr, 1995; Fogg, 1996; Abramov and Kring, 2005). Liquid water could have existed on Mars over much of the planet's history, and it may still exist at the rock-ice interface, in rocks and soil as a result of impact events, and in brines (Travis *et al*., 2003; Clifford *et al*., 2010; Fairén, 2010; Samarkin *et al*., 2010). Much of Mars' surface is composed of igneous rocks similar to basalt on Earth (Bandfield *et al*., 2000; Edwards *et al*., 2008). As in terrestrial basalts, a prominent component is Fe(II), which is present in the minerals olivine and pyroxene and in glass (Hoefen *et al*., 2003; Edwards *et al*., 2008).

The Mars-like terrestrial habitat that we have focused on in this study is the rock-ice interface from lava tube caves, which occur frequently in basalt flows. In this type of habitat on Mars, a film of liquid water can exist at the rock's surface, where life would be protected from intense solar irradiation. Yet, because it is exposed to the atmosphere, this habitat also has the benefit of an abundant source of energy in the form of redox disequilibrium between the oxidized surface of Mars and Fe(II)-bearing minerals such as olivine and pyroxene. Although iron oxidation can also occur by phototrophy, the most common process to extract energy from Fe(II) minerals on Earth is with oxidants such as dioxygen (O_2) and nitrate (NO_3^-), (Widdel *et al*., 1993; Kappler and Newman, 2004; Schippers *et al*., 2005; Miot *et al*., 2009; Newman, 2010). On

Mars, electron acceptors for Fe(II) may include putative superoxides and NO_3^- from rock surfaces and atmospheric O_2 (~8-13 μbars).

Microbes can influence (trigger or limit) the dissolution of olivine, pyroxene, or basalt (Santelli *et al*., 2001; Welch and Banfield, 2002; Benzerara *et al*., 2004; Josef *et al*., 2007; Wu *et al*., 2007). Weathering features and chemical signatures that are indicative of life were reported in olivine from Earth, and similar features were also observed in Mars meteorites (Fisk *et al*., 2006). We proposed that some of these features are produced by neutrophilic iron-oxidizing (nFeO) microorganisms that use Fe(II) from olivine (Fisk *et al*., 2006). Neutrophilic iron-oxidizing bacteria (nFeOB) are common in freshwater ecosystems (Straub *et al*., 1996; 2004) and marine basalts (Stevens, 1997; Emerson and Moyer, 2002; Edwards *et al*., 2003a,b; Lehman *et al*., 2004; Bailey *et al*., 2009). The most recognized phylotypes belong to the genera *Gallionella*, *Lepthotrix*, *Sideroxydans*, *Marinobacter*, *Mariprofundus,* and *Sphaerotilus.* Although best studied in bacteria, this physiotype is also present in some archaea such as *Ferroglobus placidus* (Hafenbradl *et al*., 1996). Recently, a diverse collection of α-, γ-, and ζ-Proteobacteria were found that are capable of such activity, although they are not closely related to any previously known nFeOs (Edwards *et al*., 2004; Emerson and Floyd, 2005; Duckworth *et al*., 2009; Wang *et al*., 2009). Even phylogroups that are dominated by heterotrophic species, such as *Pseudomonas* or *Acidovorax*, contain strains that are facultatively or even obligate nFeOs (Kappler *et al*., 2005; Bailey *et al*., 2009). In a recent paper, we reported that bacteria from a basalt subseafloor habitat (Juan de Fuca Ridge) preferentially colonize olivine above all other igneous minerals and that many heterotrophic oligotrophic isolates colonizing basalt minerals and glass are facultative nFeOs (Smith *et al.*, 2011). The presence of olivine in basalts led us

to suspect that nFeOs play an important role in the ecology and biogeochemical cycles of basalt-hosted subsurface ecosystems.

Olivine ($(Mg,Fe)_2SiO_4$) is a class of minerals that has a variable iron to magnesium ratio. The abundance of iron relative to magnesium ($[Fe/(Fe+Mg)]\cdot100$) ranges from 0 % Fe(II) in forsterite to 100 % Fe(II) in fayalite. Most commonly, olivine contains about 10 % Fe(II). Although olivine only contains iron in reduced form (Fe(II)), no strain of nFeO microorganism has ever been reported to have the capacity to use this mineral as a source of energy. Such a finding would be invaluable for the study of olivine bio-weathering; the identification of biosignatures and microfossils; determination of whether life and associated microhabitats were, or are, in existence on Mars; and the search for extraterrestrial life. Here, we report that olivine-oxidizing nFeO bacteria (nFeOB) are present in basalt in cold, near surface, aphotic environments such as caves (*esp.* lava tubes) with permanent ice, and we show the olivine-dependent growth characteristics of one such isolate. The similarity of this environment to environments on Mars suggests that nFeO microorganisms that live at the basalt-ice interface could survive on Mars, or may have thrived on Mars in the past when the temperature, atmospheric pressure, and (possibly) the O_2 partial pressure (P_{O_2}) were higher than they are today and appropriate for olivine-dependent growth of nFeO microorganisms.

Materials and methods

We collected ice and rock fragments from the rock/ice interface in South Ice Cave in the Oregon Cascades (Lat 43° 34’ 59’’N, Long 121° 04’ 38’’W). South Ice Cave, a basalt lava tube at an elevation of 1530 m., is the result of an eruption on the southern flank of Newberry Caldera and contains permanent ice. This basalt flow contains ~9.0 % iron as FeO, and its mineralogy is primarily

plagioclase feldspar, pyroxene, and olivine (personal communication Julie Donnelly-Nolan). The rock/ice samples were stored in sterile bags and packed on ice for transportation to the lab. Culture media were inoculated with melted ice and rock fragments within two days of collection. Our overall strategy for isolation of a microorganism capable of olivine-dependent growth is summarized in the flow chart from Fig. 1.

For enrichments, we used test tubes with 5 mL sterile 0.2 micron-filtered cave water and olivine sand with 9 % Fe/(Fe+Mg). The enrichments were incubated at 5^{o}C for about 4 weeks to favor the growth of nFeO microorganisms that are also psychrophilic or psychrotolerant. The enrichments were inoculated by streaking on Tryptone Soy Agar (TSA) organotrophic oligotrophic plates. Colonies that exhibited differing morphologies were picked up, saved in a library, and preserved at -80 ^{o}C in 50 % glycerol.

The mineral medium used for culturing isolates contained per L: 1 mL trace minerals solution, 1 mL vitamins mix, 30 mmol phosphate buffer (pH 7), 20 mmol bicarbonate, 30 mmol nitrate, and 100 g olivine. The trace minerals solution contained: 6.72 mM Na_2EDTA, 5.6 mM H_3BO_3, 1 mM NaCl, 0.54 mM $FeSO_4$, 0.5 mM $CoCl_2$, 0.5 mM $NiSO_4$, 0.39 mM Na_2MoO_4, 0.15 mM $NaSeO_4$, 0.13 mM $MnCl_2$, 0.13 mM $ZnCl_2$, and 0.02 mM $CuCl_2$. The vitamins mix contained per ml: 5 µg p-aminobenzoic acid, 5 µg biotin, 5 µg cyanocobalamin, 5 µg folic acid, 100 µg i-inositol, 100 µg nicotinic acid, 100 µg pyridoxine, 100 µg panthotenic acid, 100 µg riboflavin, and 1 µg thiamine. The vitamins mix was added filter sterilized after autoclavation. All chemicals were reagent grade. Sand (0.2-0.8 mm grain size) composed of 100 % olivine (Fo91) that contained 8 wt % FeO was provided by Unimin Corporation. Tumbled olivine (~Fo_{90} beads 1-3 mm in size, ~24 grains/g) were obtained from a local supplier of minerals and gems. For most experiments (including enrichments), the olivine was washed

with dH_2O and autoclaved in the culture medium. Throughout this work, we used un-inoculated controls and controls that represent media with and without various chemical modifications. The controls for each experiment (whenever applicable) are explained in the Results section.

In some experiments, organics-free olivine was used; this was obtained by heating olivine in a furnace at 500 ^oC for 90 min in air. After cooling, the olivine showed evidence of surface oxidation (uneven small patches with yellow-rusty appearance). Part of the iron oxides were removed by acid dissolution in three 24-hour long washes with occasional stirring at room temperature. The acid washing solution contained 0.25 ml/L H_2SO_4 and 20 mM Na_2SO_4, pH ~2.5, and was used in a proportion of 100 ml solution to about 10 g olivine. We compared the UV spectra of the various washes with the spectra of control unheated olivine and calculated the concentration of Fe(III) relative to a standard. Under these conditions, ferric iron absorbs strongly in the 295-304 nm range, while ferrous iron absorbs mostly in the 220-250 nm range (Steiner and Lazaroff, 1974). In this method, 304 nm peaks in solutions containing Fe(II) are used as evidence of Fe(III). This method allows detection of concentrations of Fe(III) as low as 20 µM even in the presence of high concentrations of Fe(II), because the absorbance of Fe(III) at 304 nm is ~300 times larger than that of Fe(II). After the acid treatment, the olivine was washed with dH_2O and dried in a 55 ^oC oven. Macroscopically, the heated olivine retained a yellow-green appearance with pink-rusty patches. Under a dissecting scope, most oven heated and acid washed olivine sand particles appeared transparent and colorless, while the similarly treated olivine beads appeared pale green and translucent.

For phylogenetic identification, we obtained biomass by growing cells in liquid tryptone soy broth (TSB) medium in aerobic conditions. Cells were separated by centrifugation (14,000 rpm, 2 ^oC, 5 min.), and genomic DNA

(gDNA) was extracted with a Qiagen genomic tip kit and quantified with a NanoDrop1000 instrument. A fragment of the SSU rRNA gene was amplified by PCR with the primers 8F (5'- AGAGTTTGATCCTGGCTCAG) and 1492R (5'-GGTTACCTTGTTACGACTT) (Baker *et al*., 2003). We used 20 µL PCR volumes containing 10 µL Fermentas mix, 0.8 µL of each µM primer, 6.5 µL of dH_2O and 2 µL of 100 ng/µL gDNA. The PCR conditions were as follows: denaturing at 95 ^oC for 5 min., 40 cycles of 94 ^oC for 30 sec., 50 ^oC for 30 sec., and 72 ^oC for 2 min., and final extension at 72 ^oC for 7 min. The size of the PCR products was verified by 0.7 % agarose electrophoresis, and the remaining 15 µL PCR product was cleaned with an UltraClean PCR DNA purification kit (MoBio). The amplicons were sequenced at the DNA Sequencing Core facility of Oregon Health and Sciences University with three primers: 8F, 515F (5'-GTGCCAGCMGCCGCGGTAA), and 1492R (Baker *et al*., 2003) by capillary electrophoresis on an ABI 3130xl instrument. Duplicate sequences were manually aligned, and when differences between duplicates were found, we repeated the PCR and sequencing to compare triplicates for each sequence. The sequences of each isolate were assembled into contiguous DNA fragments and blasted in the Ribosomal Database for phylogenetic identification. Sequences were imported in MEGA 4 (Tamura *et al*., 2007) and aligned versus phylogenetic relatives. The evolutionary history was inferred by using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances (base substitutions per site) were computed by using the Maximum Composite Likelihood method (Tamura *et al*., 2004).

To characterize the O_2 preference of the isolates, we inoculated tryptone soy semisolid agar gradient tubes containing 0.15 % agar and 2 mg/L resazurin. The capacity of the isolates to grow as microaerophilic iron oxidizers was verified in gradient tubes with semisolid medium (0.15 % agar) and 2 % agar plug

containing 36 mM $FeCO_3$ or olivine sand as Fe(II) sources (modified after: Emerson and Moyer, 1997; Emerson and Floyd, 2005). When growth was seen in a gradient tube, we repeated the inoculation a couple of times from tubes with growth into fresh tubes by using a stabbing needle. Because some cells may grow in $FeCO_3$ gradient tubes by using the agar or agar contaminants as energy sources, when necessary, growth by neutrophilic iron oxidation was also verified in liquid mineral medium with 5 mM soluble ferrous sulfate, at pH 7 and under 1.6 % O_2. When testing for nitrate reduction capabilities, we used a medium with 10 mM NO_3^- at pH 7 (DIFCO Catalog #226810) in culture tubes containing an inverted Durham tube to capture N_2 gas that may have been produced by denitrification. After 5 days of incubation, the cultures were examined for evidence of denitrification and tested for nitrate and nitrite reduction (Leboffe and Pierce, 2010). To verify growth by olivine oxidation, we incubated cells in test tubes with mineral medium with or without olivine.

Growth on TSB medium was monitored by spectrophotometry (Abs600) and microscopy. Media with olivine sand contain suspended mineral particles, which make spectrophotometry readings difficult to interpret. Therefore, growth on olivine-containing media was determined only by microscopy. To study the effect of temperature on growth, we analyzed growth at 2 ^oC, 5 ^oC, 10 ^oC, 15 ^oC, 25 ^oC, 30 ^oC, 37 ^oC, and 40 ^oC. To test for autotrophic growth in olivine-containing mineral media, we incubated cells with various concentrations of HCO_3^- as the sole source of carbon. Incubations in liquids under microaerophilic conditions were done in serum bottles sealed with a 1 cm thick butyl stopper and purged prior to autoclaving with dinitrogen gas containing 1.6 % O_2. The O_2 concentration in the head space was measured by gas chromatography (SRI 310C instrument, Molecular sieve column and TCD detector). The gas pressure was measured with an Omega pressure meter (Omega Engineering, Inc. CT). The

concentration of O_2 in the liquid phase was derived from a saturation of 236 μM O_2 in freshwater with air at 760 mmHg and at 30 °C.

We also verified whether the growth of one of the olivine-using isolates (*Pseudomonas* sp. HerB) may be explained by organic contaminants present on olivine surfaces. In this experiment, we used 5 ml liquid mineral medium (composition shown above) in Hungate tubes, with 20 mM HCO_3^-, 0 mM nitrate (as *Pseudomonas sp*. HerB does not reduce nitrate), pH 7, ~1 g olivine per tube, sealed and crimped, purged with 1.6 % O_2, and autoclaved. A volume of diluted vitamin mixture solution was injected filter sterilized after autoclavation to a proportion of 1 ml/L. We inoculated washed cell pellets, from serial dilutions into tubes containing heat-treated vs. non-heat treated olivine and sand vs. beads, as well as medium without olivine.

Results

Of 29 aerobic heterotrophs that we isolated from South Ice cave, eleven strains showed growth in a mineral medium with olivine as the source of energy (Fig. 2). In these incubations, vitamins were also present and the olivine had not been heated to remove traces of organics. Hence, alternative physiologies could not be excluded (*e.g.*, oligotrophic organotrophs using vitamins or traces of organics from olivine surfaces). Seven of these strains were mesophilic γ-*Proteobacteria* from the genus *Pseudomonas*, two strains were cryophilic *Brevundimonads* (α-*Proteobacteria*), and two strains (also cryophilic) belong with *Acidovorax* (β-*Proteobacteria*) (Fig. 2). One of these strains, *Pseudomonas sp*. HerB, was selected for further work because among all isolates it reached the highest density while growing in a mineral medium with 10 % w:v olivine sand, 20 mM HCO_3^-, 1 mL vitamins mix per L, pH 7, and 30 °C. Starting from ~10^3 cells/ml, this strain reached ~$5 \cdot 10^7$ cells/ml in one week.

Cells of *Pseudomonas sp*. HerB are aerobic under heterotrophic conditions. While on TSA plates and TSB tubes, growth was faster in air than in a 1.6 % O_2 atmosphere. However, in mineral media with olivine sand as the source of energy and at pH 7, growth was very slow at 21 % O_2, better at ~5 % O_2, and best at ~1.6 % O_2 where cultures reached densities of ~$3 \bullet 10^7$ cells/mL after seven days of incubation. Figure 3(a) shows the effect of olivine on the growth of *Pseudomonas sp*. HerB in mineral medium (with/without olivine, and with/without NO_3^-). No growth occurred when olivine was absent, but in the presence of olivine the culture reached $2.5 \bullet 10^7$ cells/mL without NO_3^- and $3.7 \bullet 10^7$ cells/mL with NO_3^-. The difference between with/without NO_3^- was within one standard deviation (based on triplicates), and thus significant statistical difference between these treatments could not be confirmed. Verifying nitrate-reducing capabilities by using the protocol shown in Smith et al. (2011), we found that *Pseudomonas sp*. HerB was not capable of such activity (results not shown). Regarding O_2 consumption, 10 mL culture of *Pseudomonas sp*. HerB used ~180 µmol O_2 in 20 days (Fig. 3(b)).

The ability of *Pseudomonas sp*. HerB to grow as a neutrophilic iron oxidizer was also seen in gradient tubes with iron carbonate and in liquid mineral medium with 5 mM Fe(II) at pH 7 and under 1.6 % O_2. Growth was also observed during serial inoculation of gradient tubes that contained only inorganic media. We compared growth on a mineral medium with 10 % w:v olivine sand relative to growth in the same mineral medium with 5 mM soluble Fe(II) initial concentration, 20 mM HCO_3^-, pH 7, 1.6 % O_2, incubated at 30 ^oC. The extent of growth was similar for these two media. The cultures containing olivine reached ~$8 \bullet 10^6$ cells/mL in 10 days.

We verified the growth of *Pseudomonas sp*. HerB in a mineral medium containing olivine at six pH values (Fig. 4(a)). No growth was observed at pH

4.5 and pH 8.5, and the largest cell density (after one week of incubation) was seen at pH 7. Fig. 4(b) shows the effect of HCO_3^- on the growth of *Pseudomonas sp*. HerB in mineral media containing olivine. No growth was observed without HCO_3^-, ~$1 \bullet 10^7$ cells/mL at 10 mM HCO_3^- and little variation in cell density above 10 mM HCO_3^-. In this experiment, we also incubated cells in controls without olivine and (similar to above) no measurable growth was seen.

We compared growth rates at different temperatures, using the slopes of the exponential growth phases (Fig. 5(a) and (b)). We found the following temperatures for olivine growth: ~4-5°C minimum, ~12-14°C optimum, and ~30-31°C maximum (Fig. 5(b)).

To determine whether the olivine surface is a limiting factor in the growth of *Pseudomonas sp*. HerB, we compared growth in the presence of olivine sand *vs*. olivine beads. In these experiments, we also compared growth at 5°C *vs.* 20°C (Fig. 6). We expected to find higher cell density with olivine sand than with beads and higher cell density at 20 °C relative to 5 °C. Olivine beads with 3 mm diameter have about 120 mm^2/g, while olivine sand with 0.4 mm particle diameter have about ~7,000 mm^2/g, (*i.e*., about a 55-fold increase in surface:mass ratio). After seven days of incubation, we found only about a two-fold increase in cell density on sand ($4.6 \bullet 10^7$ cells/mL) *vs*. beads ($2.1 \bullet 10^7$ cells/mL), and no significant differences in cell density between the 5 °C and 20 °C treatments.

Because pseudomonads can metabolize a wide variety of organic molecules, excess of vitamins in the culture media can represent an additional source of carbon and energy. Fig. 7 shows the growth of *Pseudomonas sp*. HerB in mineral media with olivine and various concentrations of vitamins. In the olivine-containing media, good growth (>10^7 cells/mL) was seen in all treatments. In the olivine-absent media, growth was not observed when the vitamins mix was ≤ 1 mL/L. We also found that *Pseudomonas* sp. HerB grew in olivine freed of

organics, and that no significant growth differences existed (within +/- 1 SD) between heat treated- and non-heat-treated olivine (Fig. 8).

Discussion

nFeOB have been reported to inhabit seawater, freshwater, groundwater, terrestrial basalts, subseafloor basalt, hydrothermal systems, iron oxyhydroxide mats, and the surface of glass and Fe(II)-containing minerals from a wide variety of sources (Emerson and Moyer, 2002; Kappler *et al*., 2005; Edwards *et al*., 2003a;b; Gronstal *et al*., 2009; Miot *et al*., 2009). Our finding extends the palette of environments where nFeOB exist to the basalt/ice boundary habitat in a lava-tube ice cave and to olivine minerals as a source of energy. The properties of this habitat (near 0 ^{o}C, dark, oligotrophic, circumneutral pH, and at the interface between basalt, and ice near an oxidized atmosphere) makes it a terrestrial analogue for a near-surface aphotic environment on Mars, where life may exist today and could have thrived in the past when the atmospheric pressure and surface temperature of Mars were higher than today.

Of eleven strains of putative nFeOB we isolated, we report the olivine-dependent growth characteristics of one: *Pseudomonas sp*. HerB. Regarding the source of carbon, this microorganism is a heterotroph and facultative autotroph with the capacity to use CO_2 as a sole source of carbon. Regarding the source of energy, it is an organotroph and facultative chemolithotroph, capable of neutrophilic iron oxidation with Fe(II) from olivine as the sole electron donor and O_2 as the electron acceptor. The optimum pH for growth by olivine oxidation was ~7, but we did not verify to what extent this optimum was due to competition with iron autooxidation or to metabolic preference for circumneutral pH. Regarding temperature preference, *Pseudomonas sp*. HerB is sub-mesophilic. The optimum growth in TSB medium was about 14-15^{o}C, but slow growth was

seen at temperatures as low as ~5 ^{o}C. Based on the growth profile, we predict that the minimum temperature for growth is ~4 ^{o}C. The growth rate vs. temperature profile is skewed toward higher temperatures (Fig. 5b), a profile that is difficult to explain with the data at hand. Regarding O_2 tolerance, this strain is microaerophilic and facultative aerobe, and the growth in olivine-containing mineral medium is faster at low O_2 concentration (1.6 %) than at higher O_2 concentrations (5 % and 21 %). The fact that most of our isolates were from the genus *Pseudomonas* is not unexpected. Pseudomonads are versatile and show all metabolic capabilities presented above. Some strains of *Pseudomonas* were shown to be nFeO (Bailey *et al*., 2009). Pseudomonads are important denitrifiers in soil (Chan *et al*., 1994; Smil, 2000), albeit *Pseudomonas sp*. HerB strain is not a denitrifier and cannot reduce nitrate. We did not study the source of nitrogen used by *Pseudomonas sp*. HerB for assimilation. The pathway for carbon fixation in *Pseudomonas sp*. HerB is unknown, though some facultative autotrophic pseudomonads have already been shown to fix CO_2 by using RuBisCo (Mahmood *et al*., 2009; Morikawa and Imanaka, 1993; Yuliar, 1997).

The mechanism of dissolution of olivine during the activity of *Pseudomonas sp*. HerB is unclear. Iron-oxidizing bacteria are known to dissolve a variety of Fe(II)-containing minerals, including pyrite, iron monosulfides, magnetite, siderite, and vivianite at rates usually controlled by the solubility of the phase (Schippers and Jorgensen, 2002; Kappler and Newman, 2004; Miot et al., 2009). Organic ligands such as citrate, oxalate, malonate, gallate, salicylate, and phthalate (some of which are known metabolic byproducts of pseudomonads) were shown to dissolve basalt (Neaman et al., 2005). To obtain iron, many bacteria produce siderophores, which also promote mineral dissolution (Buss et al., 2007; Luo and Gu, 2011). Pseudomonads are known to produce a wide diversity of siderophores (Cornelis and Matthijs, 2002), but siderophores

transport iron mostly in Fe(III) form (Martínez et al., 2000), while the iron from olivine is valuable as an energy source to *Pseudomonas sp*. HerB in the Fe(II) form. If a specialized mechanism to extract Fe(II) from olivine crystals does not exist, then the growth of the olivine-using *Pseudomonas sp*. HerB should be controlled predominantly by the rate of olivine dissolution and by the kinetics of chemical Fe(II) oxidation (which are probably low). The growth of olivine-oxidizing nFeOB is probably favored by low temperature, low O_2, and the presence of HCO_3^- or other iron-binding agents. Low temperature and low O_2 decrease the rate of iron oxidation while increasing the availability of soluble Fe(II). The role of low temperatures in controlling the growth of nFeOB was little studied. The fact that nFeOB prefer low O_2 conditions is well known; it is due to competition between microbial iron oxidation and iron autooxidation (Edwards *et al*., 2003b). The pH may also play an important role in the olivine-dependent growth of *Pseudomonas sp*. HerB. Because final mineral products vary significantly with the pH, the redox potential (E^o) of the Fe^{3+}/Fe^{2+} couple is pH-dependent, taking more positive values in acidic conditions (Thauer *et al*., 1977). Iron oxidation is more exergonic at neutral pH than at acidic pH.

$$Fe^{2+} + ½O_2 + 2H^+ \rightarrow Fe^{3+} + H_2O \qquad \text{Rxn.1}$$

at pH 2 the E^o of Fe^{3+}/Fe^{2+} = + 0.77 V and ΔG^o = - 8.7 kJ per mol Fe

$$Fe^{2+} + ½O_2 + 2H_2O \rightarrow Fe^{3+}(OH)_3 + H^+ \qquad \text{Rxn.2}$$

at pH 7 the E^o of Fe^{3+}/Fe^{2+} = + 0.20 V and ΔG^o = - 63.7 kJ per mol Fe

According to Rxn. 2 and the iron content of the olivine we have used, 180 µmol of O_2 used by a 10 mL culture of *Pseudomonas sp*. HerB in 20 days is equivalent to oxidizing the iron from ~290 mg olivine.

No direct evidence was ever found of olivine-related microbial activity on Mars, and we did not analyze secondary minerals produced by *Pseudomonas sp*. HerB while growing on olivine. Yet, potential martian habitats that contain

secondary minerals (such as iddingsite) produced by olivine weathering in the presence of water (Swindle et al., 2000) are important candidates to search for evidence of such microbial activity.

Conclusions

We report for the first time that a strain of nFeOB from the genus *Pseudomonas* is able to grow by using the mineral olivine as a source of energy. We propose that such microbes are common in nature, and that microenvironments that support olivine-dependent growth have to satisfy a couple of specific requirements. Some of the most important are circumneutral pH, low P_{O2}, low temperature, and low organic load. On Earth, such conditions can be encountered at basalt-ice interfaces where liquid water is also present. This finding is important for astrobiology because the environmental conditions in the recent geological past of Mars (higher pressure and temperature than today) would have allowed such microbes to thrive near the surface in lava tubes, under the ice sheet, and in the basalt subsurface where cells are protected from harmful ionizing radiation and UV radiation, yet still benefit from the oxidants of Mars's surface. Orbital and surface observations of Mars have confirmed that igneous rocks are exposed over significant areas (Edwards *et al*., 2008; Bandfield *et al*., 2000), and that some martian areas are dominated by olivine-bearing rocks (Hoefen *et al*., 2003; Edwards *et al*., 2008). In addition, skylights interpreted as entrances to lava tubes (a physical environment similar to South Ice Cave) have been observed on the flanks of martian volcanoes (Cushing *et al*., 2007). Subsurface environments and martian caves, which could contain permanent ice (Williams *et al*., 2010), have been proposed as astrobiology target sites (Boston *et al*., 1992; Boston, 2010; Northup et. al., 2011).

Calculations of autotrophic energy-producing reactions likely to occur on Mars suggest that the oxidation of Fe(II) by O_2 or NO_3^- could drive microbial

ecosystems (Jepson *et al.*, 2007). It has been proposed that the P_{O2} on Mars is sufficient to support microaerophiles (Fisk and Giovannoni, 1999). Applying $\Delta G = \Delta G^{o\prime} + TR\ln Q$ to Rxn.2 (for $T \approx 0$ °C), it can be shown that this reaction is exergonic (ΔG = -4.2 kJ/mol) even at P_{O2} = 0.1 mbars. Notably, the P_{O2} on Mars (derived from ~7 mbars total pressure and ~0.13 % O_2) is ~ 10 µbars (Seiff and Kirk, 1977). Therefore, the oxidation of olivine and other iron bearing silicates by nFeO microorganisms is possible on Mars if the atmospheric pressure increases ~10 fold (to about 70 mbar).

A key requirement for Earth-colonizing cellular life (including nFeOB) is the presence of liquid water. Even the present day subsurface and the sub-ice conditions on Mars may harbor such microbes because thin films of water exist in soil, even below freezing (Anderson and Tice, 1973). Low-temperature brines (maintaining liquid water at temperatures as low as -20 °C) could have existed over much of Mars's history (Fairén, 2010). Multiple lines of evidence indicate that Mars had liquid water at the surface in the past (Carr, 1995; Head *et al.*, 2003; Carr and Head, 2010; Warner *et al.*, 2010). Thus, some areas of the shallow subsurface of past Mars satisfy two requirements for nFeO-based cellular life: liquid water and redox energy in the form of olivine Fe(II) in disequilibrium with oxidized chemicals from the planet's surface. In the event of increases in temperature and pressure on the surface of Mars (such as during terraformation activities, orbital forcing, or release of greenhouse gas from buried hydrates), olivine-using nFeO microorganisms would be some of the first colonists and important primary producers of the newly formed Mars ecosystems.

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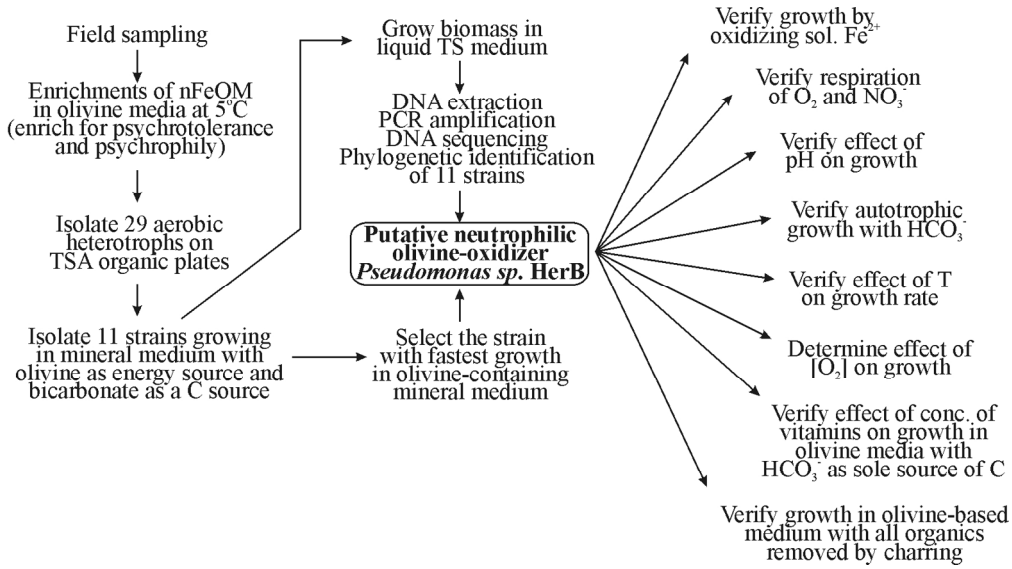

Fig. 1. Flow chart of activities used to isolate, identify, select and characterize a neutrophilic iron-oxidizing microorganism (nFeOM), that is facultative organotroph, psychrotolerant or psychrophilic, using O_2 as an electron acceptor, and capable of growing with bicarbonate as a sole source of carbon and olivine-iron(II) as a sole source of energy. The strain selected for this study is *Pseudomonas sp*. HerB.
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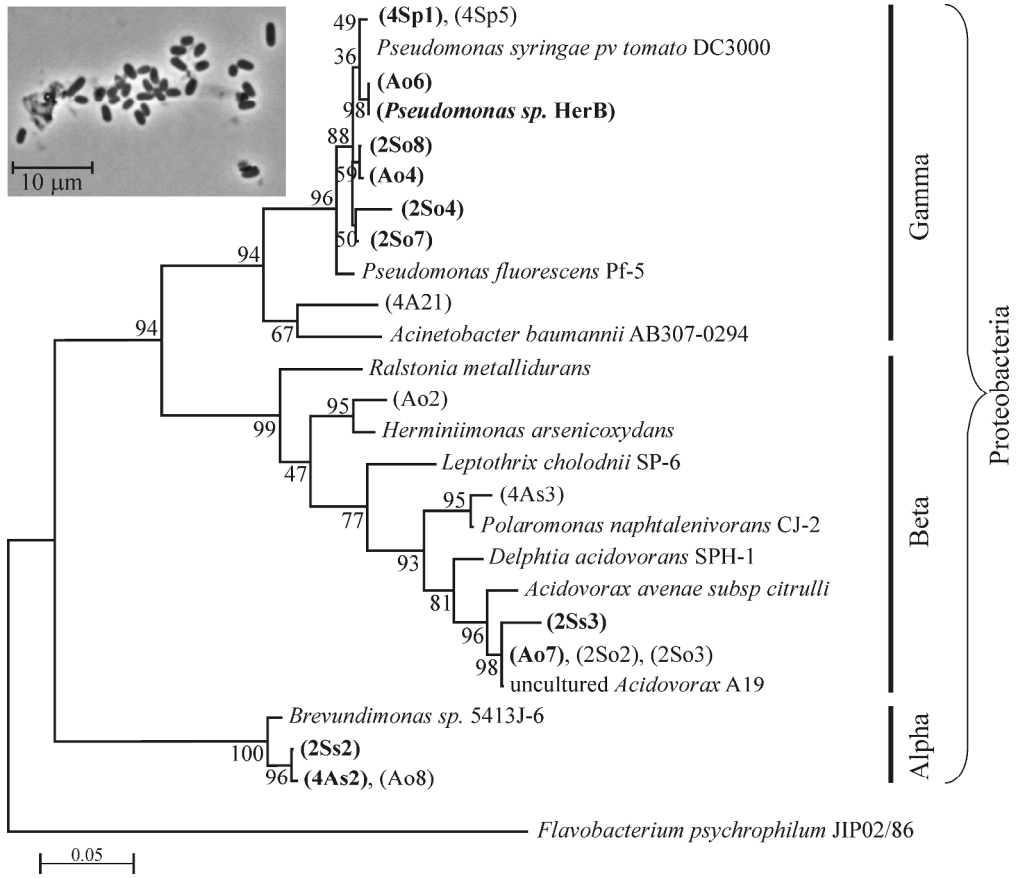

Fig. 2. Tree indicating the phylogenetic position of 29 aerobic heterotrophic strains, in parentheses, isolated from South Ice Cave. Eleven of these strains, in bold font (7 *Pseudomonas*, 2 *Acidovorax* and 2 *Brevundimonas*) also showed growth when incubated in a mineral medium with olivine as the sole source of reducing power. The sequences of these eleven isolates were submitted to GenBank under the accession numbers: JN399075 through JN399085. The evolutionary history was inferred based on partial 16S rRNA gene sequences using neighbor-joining analysis. Bootstrap percentages above 50 %, based on 500 replicates (Felsenstein, 1985) are shown next to the branches. Bar, 5 substitutions per 100 nucleotides. The insert image shows cells of *Pseudomonas sp*. HerB grown in R2A medium seen by phase contrast optical microscopy at 1000x. The same cell shape and size was seen when HerB cells grew in $FeCO_3$ gradient tubes and in olivine-containing liquid mineral medium.

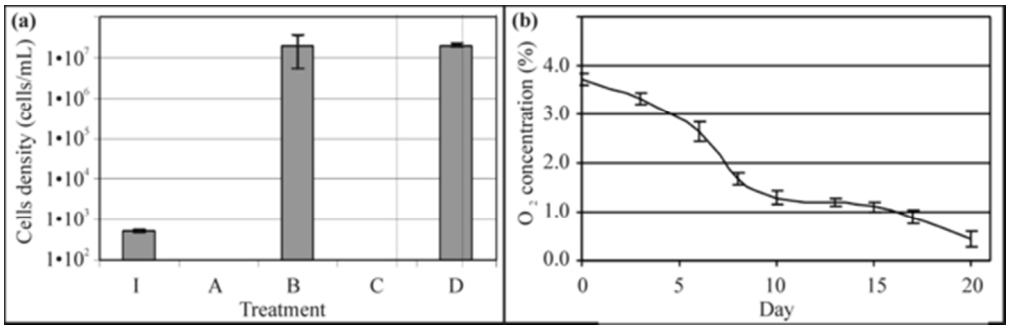

Fig. 3. (a) The growth of *Pseudomonas sp*. HerB with olivine as a source of energy. Incubation in Hungate tubes with 10 mL medium, 20 mM HCO_3^-, 1 mL/L vitamins mix, pH 7, 1.6 % O_2 at 30°C. In this experiment we compared growth with/without 10 % w:v olivine sand, and with/without 10 mM NO_3^-. I = Initial cell density (~$7.7 \bullet 10^2$ cells/mL). A = No olivine, no NO_3^-. B = with olivine, no NO_3^-. C = No olivine, with NO_3^-. D = with olivine, with NO_3^-. Cell counts are based on triplicates and were determined by microscopy after seven days of incubation. Error bars are 1SD from triplicates. (b) Evolution of the O_2 concentration in gas phase during the growth of *Pseudomonas sp*. HerB in a mineral medium with olivine (subtracted from an un-inoculated control). Incubations were in 140 mL serum bottles with 10 mL mineral medium, 10 % w:v olivine sand, 20 mM HCO_3^-, 1 mL/L vitamins mix, pH 7, and ~1.1 bar initial pressure at 30°C. The error bars are 1SD based on triplicate readings of one bottle.
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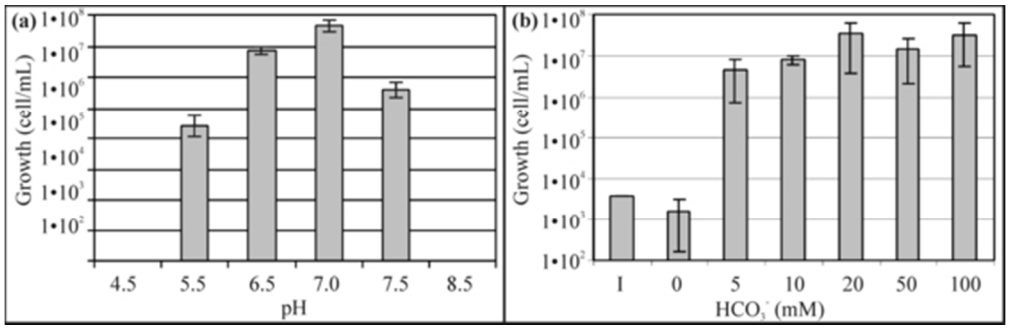

Fig. 4. (a) The growth of *Pseudomonas sp*. HerB in olivine-containing mineral media at different pHs. The media contained 20 mM HCO_3^-, 10 % w:v olivine sand and 1 mL/L vitamins mix, and incubation occurred under 1.6 % O_2 at 30°C for seven days. (b) Growth in the same mineral medium at pH 7 and with various concentrations of HCO_3^-, incubated for 14 days at 20°C. I = Initial cell density (~$3.8 \bullet 10^3$ cells/mL). The error bars are 1SD from triplicates.
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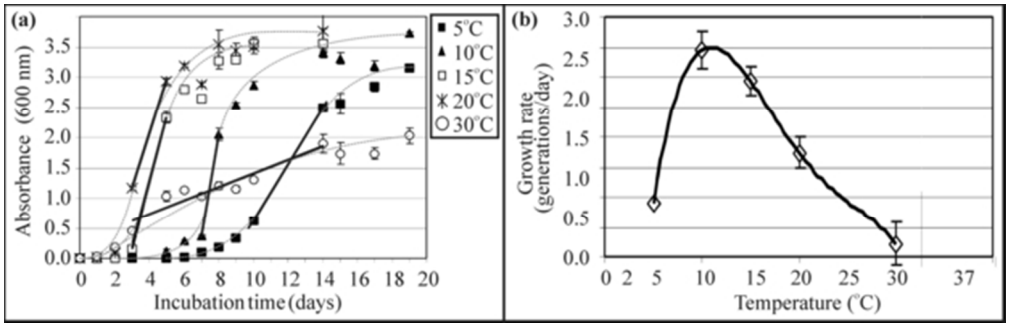

Fig. 5. (a) Growth profiles of *Pseudomonas sp*. HerB in TSB medium at five temperatures (5°C; 10°C; 15°C; 25°C and 30°C). No growth was seen at 2°C, 37°C and 40°C. The values shown are averages of triplicates and the errors bars equal 1SD. The interrupted lines are hand drawn and help observe the general trend of each set of data. The straight lines are linear regression slopes for the data points situated near and opposite sides of the inflexion point of the polynomial fit, in the part of the curve that represents the exponential growth phase. All cultures started from ~10^3 cells mL^{-1} and were incubated in 18 mm diameter test tubes with 10 mL medium. (b) The effect of temperature on the growth rate, calculated based on the slope of the exponential phase shown in (a). The error bars from (b) are 1SD of the expected variation in the slope of exponential growth in (a) based on +/-1SD of cell density.
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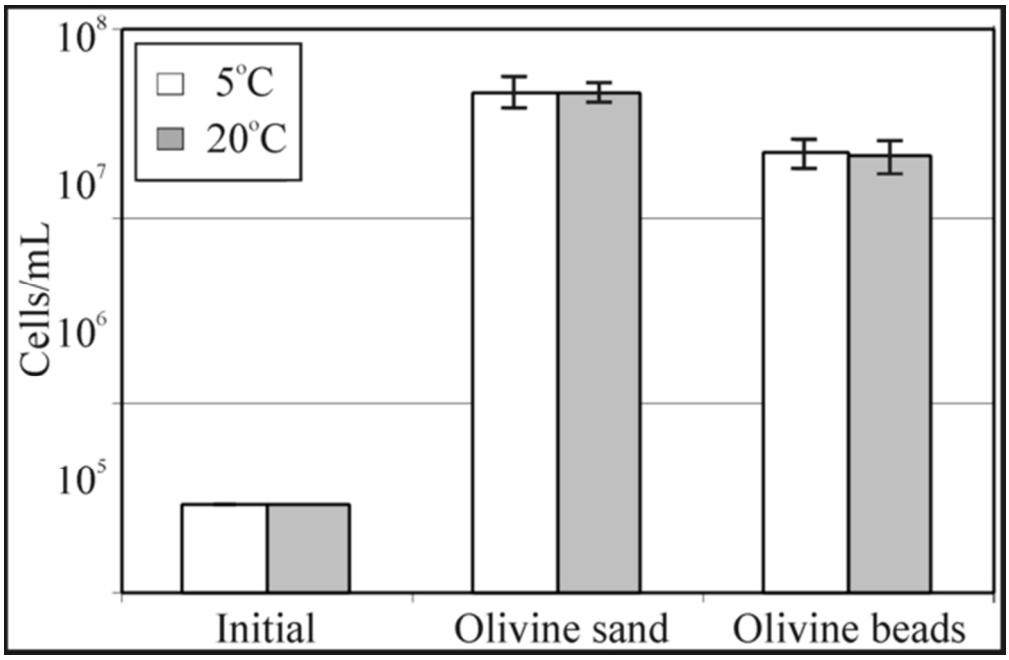

Fig. 6. The growth of *Pseudomonas sp*. HerB in mineral medium with olivine of two particle sizes (olivine sand and olivine beads) and at two temperatures (5°C and 20°C). These two olivine samples are different with regard to size (and thus surface area) but are similar in composition. Incubation occurred for seven days in Hungate tubes, 5 mL mineral medium, 10 % w:v olivine, 20 mM HCO_3^-, pH 7, 1.6 % O_2, and 1 mL/L vitamins mix. The error bars are 1 SD from triplicates.
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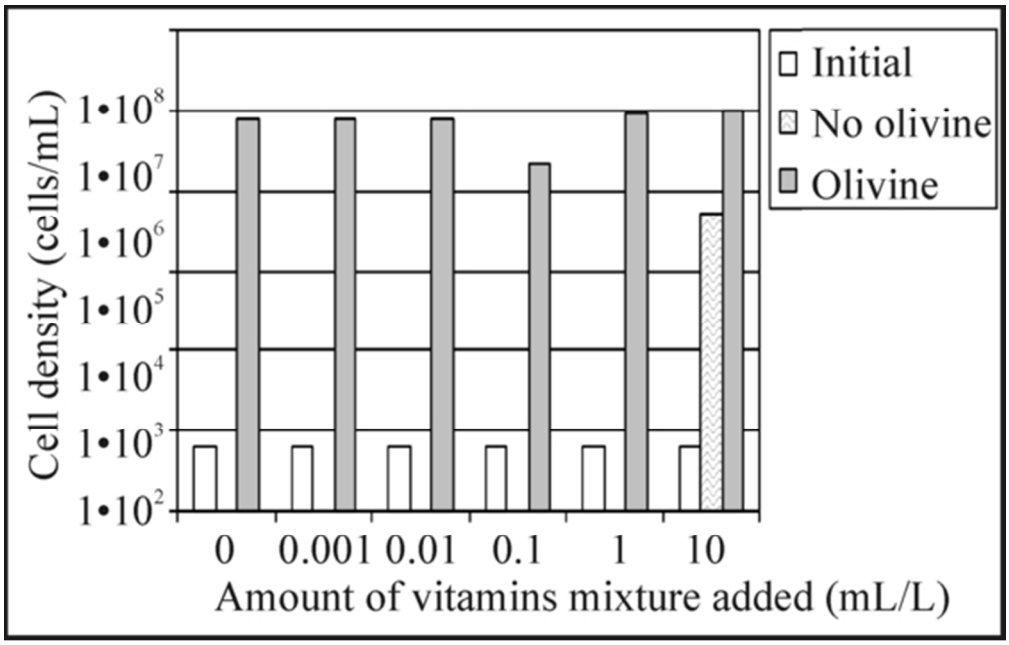

Fig. 7. Growth of *Pseudomonas sp*. HerB in mineral medium (with/without olivine) and various abundances of vitamins mix. Incubation occurred in test tubes with 5 mL mineral medium, 10 mM HCO_3^-, with/without 20 olivine beads (~840 mg olivine per tube), and at pH 7. All cultures started from ~$5.3 \bullet 10^3$ cells/mL. The graph shows cell densities after 7 days at 1.6 % O_2 and 20°C.
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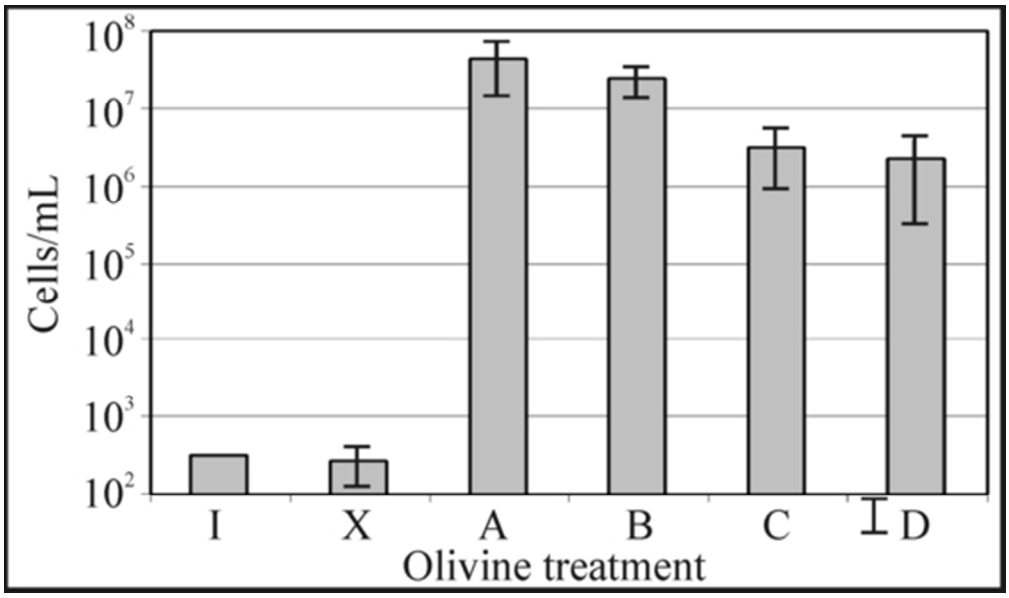

Fig. 8. The growth of *Pseudomonas sp*. HerB in mineral medium with olivine sand and olivine beads after removing traces of organics by heating the crystals at 500°C. Incubation occurred for 14 days in Hungate tubes, with 5 mL mineral medium, 10 % w:v olivine, 20 mM HCO_3^-, pH 7, 1 ml/L vitamins mix and 1.6 % O_2. The treatments shown in the graph are: I = initial cell density; X = no olivine present; A = non heat treated olivine sand; B = heat-treated olivine sand; C = non-heat-treated olivine beads; and D = heat-treated olivine beads. The values shown are averages of triplicates and error bars equal 1 SD.
47x28mm (300 x 300 DPI)