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Pyrite oxidation and copper sulfide ore leaching by halotolerant, thermotolerant bacteria

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ABSTRACT

Pyrite oxidation was observed in a mixed culture of salt-tolerant, thermotolerant, acidophilic bacteria from warm, acidic, coastal sediments of the island of Milos (Greece). Analysis of 16S rRNA gene sequences cloned from DNA extracted from the mixed culture indicated two species which were related to *Thiobacillus prosperus*. One of the sequences was found previously in warm, sediment samples from the island of Vulcano (Italy). Iron solubilization from pyrite by the Milos culture at 47 °C was most rapid in the presence of NaCl at 30 g l^{-1}. A novel species was isolated from the mixed culture and grew in pure culture on pyrite with 50 g l^{-1} NaCl, but iron solubilization was most rapid at just below 50 °C with 20 g l^{-1} NaCl. Establishment of activity of the halotolerant, thermotolerant bacteria in copper sulfide ore leaching columns was more difficult than with related bacteria growing at lower temperatures.

1. Introduction

Thiobacillus prosperus (Davis-Belmar et al., 2008; Huber and Stetter, 1989; Nicolle et al., 2009) and similar mesophilic bacteria (Norris and Simmons, 2004) have been isolated from close to shallow water hydrothermal vents at Vulcano, Italy. They oxidize ferrous iron and sulfur in the presence of salt (NaCl) concentrations at least twice that of seawater, which indicates potential application in biomining where only saline water is available. One of these bacteria, strain V8, was the dominant ferrous iron-oxidizing strain in pyrite oxidation by a mixed culture (Norris and Simmons, 2004). However, these bacteria were not represented among rRNA genes amplified and cloned (88 clones) from sediment samples from which the bacteria were isolated: a clone bank comprised only sequences of sulfur-oxidizing *Acidithiobacillus* species (Simmons and Norris, 2002). A third of cloned rRNA genes with origin at a nearby 45 °C sample site did indicate another species related to *T. prosperus*, a species therefore potentially more thermotolerant than the previously studied strains. The identification of *T. prosperus*-like rRNA gene sequences in samples from a similar environment of the island of Milos (Greece) is now described, together with the activity of *T. prosperus*-related, thermotolerant cultures from this environment in pyrite oxidation and copper sulfide ore leaching.

2. Materials and methods

2.1. Sampling and enrichment cultures

Sediment samples were from a shallow, acidic pool and geothermal sediments by the shore of Baia di Levante, Vulcano, as described previously (Simmons and Norris, 2002) and from acidic, geothermal sediments of Palaeochori Bay of the island of Milos in the Aegean Sea. The latter samples were from shallow, acidic pools, open to the sea, at the base of sulfur-rich cliffs. The sediment, about 60 °C a few centimetres below its surface, was covered with water at 35 to 40 °C. A water/sediment sample was used to establish a pyrite enrichment culture at 50 °C in a medium which contained (g l^{-1}) $MgSO_4.7H_2O$ (0.5), $(NH_4)_2SO_4$ (0.4), K_2HPO_4 (0.2), NaCl (25) and a pyrite concentrate (10; minus 75 μm particle diameter). The effects of NaCl and the temperature on bacterial growth on pyrite (10 g l^{-1}) were tested with cultures (100 ml) grown in shaken flasks. Isolation of a thermotolerant strains from the enrichment culture was through single colony isolation using ferrous iron-containing Phytagel-solidified medium (Davis-Belmar et al., 2008). Iron in the solution was measured by atomic absorption spectrophotometry.

2.2. 16S rRNA gene analysis

16S rRNA genes were amplified by PCR with universal bacterial primers (f27: 5′-AGAGTTTGATCMTGGCTCAG-3′; r1492: 5′- TACGGYTACCTTGTTACGACTT-3′) or a forward primer designed to be specific for *T. prosperus*-like sequences (5′-TAGCCCGGAAATCCGGAT-3′, used with

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primer r1492). Cloned sequences (TOPO TA Cloning Kit, Invitrogen) were aligned before analysis with PHYLIP programmes (Felsenstein, 2006).

2.3. Ore leaching columns

Laboratory ore leaching columns were operated and monitored essentially as described previously (Davis-Belmar et al., 2008), but with addition of ferric sulfate to the irrigation solution to give an iron concentration of 1 g l^{-1}. This solution at pH 1.7 also contained 25 g NaCl l^{-1}. Each column contained 0.7 kg of copper ore as fragments with a mean weight of 1.3 g. The ore contained 0.6% w/v copper which was mainly present in chalcopyrite, chalcocite and some covellite. The solution flow was 150 ml day^{-1}, corresponding to a surface application rate of approximately 4 l m^2 h^{-1} and there was no solution recycling. Aeration from the base of the columns was at 20 ml min^{-1}. A column at 36 °C was inoculated with a halotolerant, mesophilic mixed culture as described previously (Davis-Belmar et al., 2008); the culture contained *T. prosperus*-like bacteria and salt-tolerant, sulfur-oxidizing *Acidithiobacillus* species. A column at 47 °C was inoculated with a mixed culture of two halotolerant, thermotolerant strains which are related to *T. prosperus* (and which are noted in Section 3.3) and *Acidithiobacillus caldus*. Adjustments to operating conditions are indicated with the results.

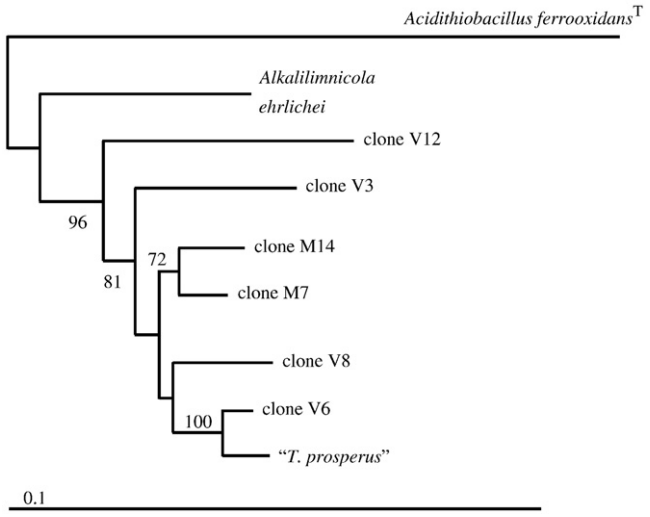

Fig. 1. A phylogenetic distance tree of *Thiobacillus prosperus*-related 16S rRNA gene sequences which were cloned from environmental DNA samples or enrichment cultures. The sequences from *T. prosperus*, from the *Acidithiobacillus ferrooxidans* type strain (as an out-group sequence) and from an *Alkalilimnicola* species (see text) are also included. Bootstrap values greater than 70 from 100 replicates are shown. The scale bar indicates 0.1 substitutions per site.

3. Results and discussion

3.1. T. prosperus-like bacteria in acidic samples from Milos and Vulcano

Using universal bacterial primers, 16 S rRNA genes were amplified from DNA extracted from three water/sediment samples from the base of sulfur-rich cliffs of Milos. Cloned sequences with different RFLP patterns were analyzed. 29% (of 52), 12% (of 49) and 3% (of 73) of clones from the three samples were derived from rRNA genes with sequence identity to a sequence designated as clone type V3 when it was found previously in environmental DNA from a Vulcano sample (Simmons and Norris, 2002). No other *T. prosperus*-like sequences were obtained from two of the Milos samples while 44% (of 73 clones) of the third clone bank represented a novel *T. prosperus*-like sequence, designated clone M14. The majority of cloned sequences from sample two were similar to that of *Sulfobacillus thermosulfidooxidans*, while the other clone banks mainly comprised sequences related to those of heterotrophic, marine bacteria. A small clone bank (20 clones) was constructed using primers designed to be specific for the *Acidithiobacillus* genus and comprised only *A. caldus* sequences. Primers designed to be specific for the *T. prosperus* group were also used with DNA from the first two samples. The first of these specific clone banks comprised sequences of clone V3 (96% of clones), clone V6 which represents *T. prosperus* (2%), and clone M14 (2%). The second group specific clone bank comprised clones V3 (98%) and V6 (2%). Strain V8, which was previously found to dominate 35 °C pyrite enrichment cultures (Norris and Simmons, 2004), was not represented in the clone banks, but PCR with strain V8 16 S rRNA gene-specific primers amplified strain V8 rRNA sequences from all three samples (not shown).

Universal primers were used in PCR amplification of 16 S rRNA genes from a 45 °C sediment sample from Vulcano which had been stored frozen since a previous analysis (Simmons and Norris, 2002). As before, clone type V3 sequences comprised over 30% of the clones, while a *T. prosperus*-like sequence not recorded previously, and now designated clone type V12, represented 12% of the clones.

The phylogenetic relationships of the cloned *T. prosperus*-like sequences, including a novel clone type from the Milos pyrite enrichment culture (clone M7, see Section 3.2.), are shown (Fig. 1). The sequences are phylogenetically closer to sequences from many alkaliphiles (*Alkalilimnicola* sp. is shown as an example) than to those from the most studied pyrite-oxidizing acidophile, *Acidithiobacillus ferrooxidans*.

3.2. Pyrite oxidation in thermotolerant, halotolerant enrichment cultures

A pyrite enrichment culture of a Milos sample was maintained through many serial sub-cultures at 47 °C in medium with NaCl (25 g l^{-1}). One sequence in a clone bank (29 clones) of 16 S rRNA genes amplified from DNA extracted from the tenth serial sub-culture represented clone type V3. The other clones represented a novel *T. prosperus*-like sequence, designated clone type M7, which was not seen in the analysis of the environmental samples.

The effect of temperature on the dissolution of pyrite during growth of the thermotolerant enrichment culture was compared to the effect on an enrichment culture which had been originally established with samples from Vulcano (Simmons and Norris, 2002) and had been maintained at 30 °C. The original Vulcano culture oxidized the pyrite extensively at 40 °C but did not survive incubation at 45 °C (Fig. 2A). Analysis of this mesophilic culture revealed *T. prosperus*-like 16S rRNA gene sequences corresponding to the strains V6 and V8, while no V3, M7 or M14 clone-type sequences were found. Dissolution of pyrite by the 47 °C enrichment culture was not inhibited at 50 °C (Fig. 2B). It grew at 47 °C with NaCl at 50 g l^{-1}, while most iron was solubilized and remained in solution with NaCl at 30 g l^{-1} (Fig. 2C).

3.3. Isolation of thermotolerant strains and pyrite oxidation in pure culture

Pure cultures of thermotolerant strains from the Milos enrichment culture were obtained from colonies on solid medium. Two strains were isolated with 16S rRNA gene sequences that corresponded to the cloned V3 and M7 sequences (Fig. 1). Both strains oxidized ferrous iron, sulfur and tetrathionate with some differences in their capacities for autotrophic growth (data not shown).

The solubilization of iron from pyrite during growth of strain M7 is shown (Fig. 3). Iron in solution did not increase significantly in a sterile control at 50 °C over the same period (not shown). The effect of temperature on the growth of strain M7 was measured with an inoculum grown at 47 °C. The maximum rate of solubilization occurred