Detection by polymerase chain reaction-amplification and sequencing of an archaeon in a commercial-scale copper bioleaching plant

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Abstract

An archaeon was detected in the leaching solution from a commercial copper production plant and in copper sulfide ores leached with the solution. The leaching solution in this plant contains a high concentration of sulfate salts. Analysis of the microbial population by polymerase chain reaction-amplification of archaeal 16S rDNAs indicated the presence of a single sequence type. Comparison of the nucleotide sequence of the polymerase chain reaction product with available reference sequences suggested that this archaeon corresponds to a new species of a novel genus and family within the order Thermoplasmales. This archaeon grows in synthetic media but it has not been possible to obtain isolates free of chemolithotrophic bacteria.

Keywords: Archaea; 16S rRNA gene; Bioleaching

1. Introduction

Bioleaching of copper involves the acid-leaching of the metal from copper sulfides after oxidation enhanced by acidophilic autotrophic bacteria [1,2]. Analyses of the microbial community compositions in leaching solutions, using traditional enrichment cultivation in different media and by detection of specific DNA sequences, revealed the presence of a number of bacterial species, including the commonly found autotrophs Thiobacillus ferrooxidans, Thioba-

cillus thiooxidans [3,4] and Leptospirillum ferroxi-
dans [5]. Other iron-oxidizing heterotrophic acidophiles and heterotrophs belonging to the genus Acidiphilium [6] and several fungi of the Fungi imperfecti [7] have been also detected after incubation in culture media, although their relevance in copper leaching is doubtful. These studies were extended to investigate the potential presence of Archaea in samples obtained from a commercial plant operating with leaching solutions containing 120–150 g of sulfate per liter [8,9]. In commercial leaching plants, recycling of the leaching solution, after recovery of copper and replenishment of the sulfuric acid that reacted with salts in the ore [2], causes a continuous enrichment of non-copper sulfate salts [8]. This study
presents the detection of Archaea in a commercial scale copper bioleaching plant.

2. Materials and methods

2.1. Bioleaching, samples and DNA preparation

Agglomerated ore and leached, and cultures were prepared as described previously [10,11]. Total DNA was extracted from ores and cultures as described previously [10,11].

2.2. PCR amplification, label and sequence determination of 16S rDNA

Archaeal 16S rDNA was specifically amplified by PCR using the primer pair Arch21F/Arch958R, as described by DeLong [12], with the following modifications: DNA was denatured in the reaction mixture at 94°C for 3 min without Taq polymerase and the enzyme was added after cooling to 55°C; thermal cycling was begun with the 55°C primer annealing step, primer-extension was repeated for 30 cycles, and the last extension step was increased to 5 min. PCR-DNAS were purified and sequenced as described previously [13]. Radioactive labeling was performed by PCR amplification as described above, except that concentration of deoxynucleoside triphosphate was 30 µM, and the mixture additionally contained 0.6 µCi of [α-32P]dATP per µl.

2.3. Culture conditions

Cultures were prepared in both low salt solution (MS9b) and high salt solution (HSS). MS9b contains the following salts in grams per liter: NH₄SO₄, 0.1; K₂HPO₄·2H₂O, 0.04; MgSO₄·7H₂O, 0.25; FeSO₄·7H₂O, 16.5. HSS contains the following salts in grams per liter: CuSO₄·5H₂O, 3.24; Al₂(SO₄)₃·18H₂O, 136.8; MgSO₄·7H₂O, 123.88; MnSO₄·H₂O, 6.6; NH₄SO₄, 0.1; K₂HPO₄·2H₂O, 0.04; MgSO₄·7H₂O, 0.25; FeSO₄·7H₂O, 16.5. Cultures containing the archaeon were grown by inoculation of 50 ml with either 1 ml of effluent leaching solution from the commercial plant or 0.5 g of agglomerated ore leached with either HSS or the leaching solution from the plant. Incubation was performed at 30°C in an orbit Environ-shaker at 150 rpm. Growth in elemental sulfur or chalcopyrite was performed in the same salt solution containing, respectively, 0.5% sublimed sulfur or 1% chalcopyrite, instead of ferrous iron. Successive subcultures were performed by transfer of 1 ml of the grown culture into 25 ml of medium. The initial culture took 25 days to oxidize the available ferrous iron, while subsequent subcultures grew faster, within 13–18 days.

2.4. Heteroduplex analysis

Hybridizations were performed by mixing the aliquots of amplified 16S rDNAs in 10 µl of 1× renaturation buffer [14] and subsequent denaturation at 96°C for 2 min and annealing at 72°C for 3 min in a thermal cycler. Electrophoresis was performed at 150 V in 7% acryl-bis-acrylamide gels prepared in a chamber Hoefer SE 250 (7×8×0.75 cm). Staining was performed with silver nitrate [15].

3. Results and discussion

Archaeal 16S rDNA was detected initially in plant leaching-solution after cultivation in the same solution. This solution contained approximately 3 g l⁻¹ of ferrous iron that was oxidized during incubation (data not shown). Amplification of archaeal 16S rDNA was observed subsequently in DNA extracts from copper sulfd ore leached with the plant solution and from cultures in high salt solution (HSS) inoculated with this ore. Archaeal 16S rDNA was not detected in total DNA extracts from ore leached with the low salt solution, MS9b, (Fig. 1A). These samples of leached ore previously have been shown to contain a community of populations of *T. ferrooxidans, T. thiooxidans* and *L. ferrooxidans* species ([10] and unpublished observations). The presence of these bacteria could be observed after PCR-amplification with primers Eubac27F/1492R [12] (Fig. 1B). DNA from *T. thiooxidans* ATCC 19377 (lanes 1) and *Sulfolobus acidocaldarius* BC65 (lanes 2) (strain kindly provided by C. Jerez who obtained it from Paul Norris) were used to demonstrate the specificity of amplification. The products observed with the primers for bacterial 16S rDNA in the amplification
of *S. acidocaldarius* BC65 DNA might be due to contamination. The relative growths of Archaea and Bacteria were compared by PCR-amplification of 16S rDNAs using specific primers for Bacteria and Archaea concomitantly. Fig. 1C shows the relative amounts of amplified 16S rDNAs from the leached ores and from an enrichment culture in HHS with ferrous iron as energy source.

The sequence homogeneity of the amplified archaeal 16S rDNA was indicated by the absence of heteroduplexes (data not shown) after denaturation and renaturation [16,17]. When fragments differing in nucleotide sequence are subjected to this treatment, they generate, besides homoduplexes, heteroduplexes which, according to their degrees of dissimilarity, show reduced electrophoretic mobilities in polyacrylamide gels. The possible phylogenetic relationship of the putative archaeon with the phenotypically similar acidophilic chemolithotrophic archaeon *S. acidocaldarius* [18], was explored by measuring the electrophoretic migration of the heteroduplex formed between the detected 16S rDNA and 16S rDNA amplified from DNA extracted from cultures obtained after the incubation of leached ore in HSS with ferrous iron, elemental sulfur or chalcopyrite as energy sources.

Since the 16S rDNA amplified from leached ore or cultures apparently consisted of a single sequence
type quite different from that of *S. acidocaldarius* 16S rDNA. The amplification products from both, ore leached with high leaching solution and the fourth subculture in high sulfate salt solution with ferrous iron were sequenced. The determined nucleotide sequence confirmed the results observed by heteroduplex formation in that it consisted of a single sequence quite different from that of *S. acidocaldarius*. The phylogenetic position of this archaeon, called Aglo120 for identification purposes, was estimated by comparison of 930 nucleotide positions (Fig. 3). Aglo120 clustered most closely with *Picrophilus oshimae* (88.1%) and *Thermoplasma acidophilum* (86.3%), in the taxonomic order *Thermoplasmales*. These two species belong to the two families distinguished within this order. Aglo120 seems to represent a new species within a third, new family of the order *Thermoplasmales* [19,20]. The nucleotide sequence has been deposited in the EMBL Data Library under accession number AJ003138.

As stated above, all cultures contained Bacteria as well as the archaeon. Numerous attempts to isolate Aglo120 into pure culture were unsuccessful. Cultivation in the presence of the antibiotics kanamycin (up to 150 μg ml⁻¹), chloramphenicol (up to 20 μg ml⁻¹) and rifampicin (up to 4 μg ml⁻¹) did not inhibit bacterial growth. Incubation at 45°C, inhibited both Bacteria and Archaea. Supplementation with yeast extract did not increase the proportion of Aglo120. The failure to obtain this archaeon in pure culture has impeded its physiological characterization and hence estimation of its potential role in metal bioleaching. In the last 10 years molecular techniques have enabled the analysis of microbial communities without the necessity for isolation and cultivation, such studies allow an ‘overview’ of the total population in a given sample and provide insight into the physiological potential of the population. However, in this case, it is not possible to infer phenotypic properties of this archaeon due to its distant genotypic relation with well-known Archaea.

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References