Journal of Sciences, Islamic Republic of Iran 18(3): 209-213 (2007) http://jsciences.ut.ac.ir
University of Tehran, ISSN 1016-1104

Isolation and Characterization of a New *Acidothiobacillus ferrooxidans* from the Aliabad Copper Mine in Yazd Using 16s-23s Spacer Gene Nucleotide Sequencing Method

J. Raheb,[1,*] H. Khoshroo,[2] A. Azimi,[2] B. Nasernejad,[3]
M.H. Sanati,[1] S. Naghdi,[1] and M. Arabnezhad[1]

[1]*National Institute for Genetic Engineering and Biotechnology, Tehran, Islamic Republic of Iran*
[2]*National Iranian Copper Industries Company, Tehran, Islamic Republic of Iran*
[3]*Amirkabir University, Tehran, Islamic Republic of Iran*

Abstract

With the financial protection of National Iranian Copper Industries Company, the composition of bacterial populations in Aliabad copper mine was investigated by electron microscopy and by analysis of DNA obtained from bacteria after ores laboratory cultures. This analysis consisted of the characterization of the spacer regions between the 16 and 23s genes in the bacterial rRNA genetic loci after PCR amplification. The size of the spacer region amplified from DNAs obtained from samples, were compared with the sizes of that obtained from culture of the bioleaching bacterial reference (*Acidothiobacillus ferrooxidans DSMZ 583*) and the 16s-23s intergenic spacer regions sequences in database. Further identification of the locale species was achieved by sequencing of the PCR product. The alignment result showed a great amount of similarity between the new bacteria and *Thiobacillus ferrooxidans.*

Keywords: Bioleaching; *Thiobacillus ferrooxidans*; PCR; Molecular identification

Introduction

Bioleaching, a general term refers to the conversion of an insoluble metal (*e.g.* CuS) into a soluble form (usually SO_4^{2-}) by biological oxidation and by applying microbes [1]. Metals for which this technique is mainly employed include copper, cobalt, nickel, zinc and uranium. For recovery of gold and silver the activity of leaching bacteria is applied only to remove interfering metal sulfides from ores bearing the precious metals prior to cyanidation treatment [2].

The use of microbes in bioleaching process has some distinct advantages over traditional physicochemical methods. This process is more environmentally friendly, less consuming energy and useful for the low-grade ores. And so, it is increasingly being used because of its economical advantages [1].

Bioleaching is carried out by astonishing diverse groups of bacteria. Today, at least 11 putative prokaryote divisions can be related to this phenomenon [2]. The most common microorganisms; belong to the genera *Thiobacillus* and *Leptosprillium* which are mesophile, acidophil and chemolithoautotrophe. They obtain energy from oxidation of either ferrous ion to

[*]*E-mail: jam@nrcgeb.ac.ir*

ferric or reduction of sulfur compounds to sulfuric acid [1,2].

The first step in development of industrial bioleaching process in a metal mine is to investigate of its bacterial population. One of the major encountered obstacles in studying the ecology of these organisms is the difficulty involved in isolating, identifying and enumerating individual species and strains from an environment which contains a plethora of strains with similar metabolic requirements [3].

Yates et al., 1986 were the first to suggest the use of genetic probes to identify different species of bioleaching bacteria commonly isolated from biomining operation [4].

In PCR-mediated detection of bioleaching bacteria, the DNA obtained from samples after laboratory cultures was analyzed by PCR of the spacer regions between 16s-23s antigenic spacer[5], 16s rDNA [3,6], or 23s rDNA [5].

In this study for the first time a new strain from Aliabad copper mine was isolated and characterize by 16s-23s spacer gene sequencing. Also, here we report the 16s-23s spacer gene sequence of *Acidothiobacillus ferrooxidans* DSM 583.

Materials and Methods

Strains and Culture Conditions

The strains used in this study were *Acidothiobacillus ferrooxidans* DSM 583, *Pseudomonas aeroginosa* ATCC 9027, *E. coli* (DH5α). The first bacterium cultured in 9k medium contained (per liter): KH_2PO_4 (0.4 gr), $CaCl_2.2H_2O$ (0.2 gr), $MgSO_4.7H_2O$ (0.4 gr), $(NH_4)_2SO_4$ (0.4 gr), $FeSO_4.7H_2O$ (33.3 gr), pH: 1.5-2 at 30°C and 180 rpm. The last two bacteria were grown in LB medium at 37°C. Ore samples treated with H_2SO_4 (0.5 N) for three weeks, then, crushed into small particles (0.2-0.5 mm in diameter). 5 g of the sample incubated in 200 ml of 9k medium. The culture conditions were the same as *A. ferrooxidans* DSM 583. Cultures in 9k medium were incubated for 1 to 8 weeks until growth was observed microscopically or until a chemical change occurred in the medium compared with an uninoculated control. In cultures the growth was accompanied by a characteristics ferric precipitation and orange coloring of medium. Solid media were gelled with 0.6% (w/v) agarose.

Sample Collection

Ore samples were collected from various damp sites in Aliabad mine in November 2005, using polypropylene flasks. Samples maintained at room temperature.

DNA Extraction

200 ml from the cultured media at the end of exponential phase was filtered through a Millipore filter (pore size, 0.22 µm), and washed twice with sterile distilled water. The pellet was resuspended in 100 µl TE (0.01 M Tris, 0.001 M EDTA[PH:7.5]) and DNA was extracted by DNA extraction kit (Cinnagene Co. Cat. No. DN8115C).

PCR

The spacer region between 16s and 23s genes was amplified by PCR using 1.5 ηg of DNA, G1 (GAAGTCGTAACAAGG) and L1 (CAAGGCATCC ACCCGT) primers. G1 oligonucleotide is located about 30 to 40 nucleotide upstream from the spacer boundary and L1 oligonucleotide is located about 20 bases downstream from the spacer boundary [7,8].

PCR was performed according to Pizzaro *et al*. [7], including 25 cycles at 15s at 94°C, 7 min at 55°C and 30 s at 72°C and the one additional cycle was run at 72°C for 10 min. electrophoresis was performed on 1% agarose gel with TAE buffer and the DNA was visualized by staining the gel with ethidium bromide.

Scanning Electron Microscopy

Bacterial samples were fixed on specimen stubs, gold coated by auto spotter coater (BioRad E5200) and examined by a SEM (Cambridje S-360) at 20 kv [8].

Sequencing

The nucleotide sequence of the PCR bands was directly determined by automated sequencing 3700ABI (Gene fanavaran, Macrogene Seoul ,Korea).

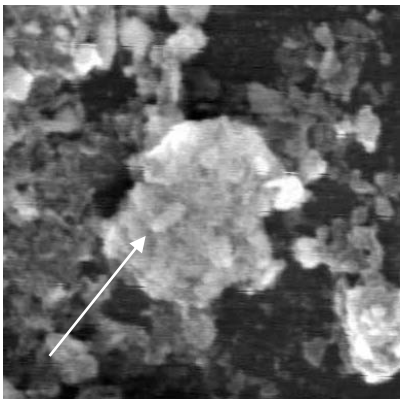
Resualts

A new strain of *Thiobacillus* was isolated after 8 weeks incubation of Aliabad mine copper ores using 9k medium. Since *Thiobacillus* strains are *Lithoautotrophe*, they are not easily culturable in solid medium.

The samples were prepared and examined for light and scanning electron microscopy (Fig. 1).

The primary examination of SEM as a microscopic result and applying the stringent condition of culture medium (pH: 1.5-2) demonstrated that the strain is *Acidothiobacillus* species.

a)



b)

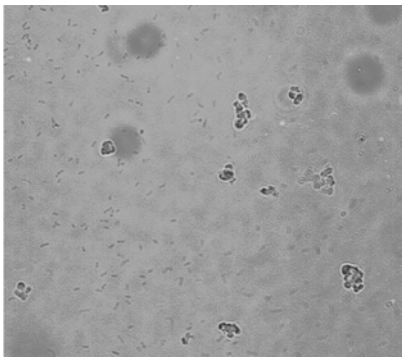


Figure 1. Light (b) and scanning electron microscopy photograph (a) (1000× and 10000×).

Figure 2. PCR amplification product of spacer regions between the 16S and 23S RNA genes of DNA from cultured sample. 1. *Pseudomonas aeroginosa*, 2. *Acidothiobacillus ferrooxidans DSM583*, 3. *E. coli* (DH5α), 4. Aliabad ore culture sample, 5. Molecolar weight marker 100 bp (Roche).

Next step was using the modified PCR technique for amplification of 16s-23s intergenic spacer genes with specific primers, and intense and highly reproducible fragments appeared close to 500 bp molecular weight marker band (Fig. 2). The similarity with 99% identity was demonstrated compared with those *Acidothiobacillus* strains sequences in database. The Figure 3 demonstrated the result of chromatogram for the sequence of NICI1 (Fig. 3).

Finally the strain identified as a new strain of *Acidothiobacillus ferrooxidans* and named NICI1.

In addition, analysis of the growths rate of NICI1 was applied using the Fe^{3+} production which was compared with *Acidothiobacillus ferrooxidans* DSM583 (Fig. 4).

Discussion

The most microorganisms require suitable environmental factors for optimal growth conditions. To isolate a specific strain of bacteria, from its habitat, it is necessary to bring together all sufficient chemical and physical environmental factors in a selective medium. In this investigation variety of medium with the required factors were used to isolate an important native microorganism acid mine drainage and bioleaching environments *Acidothiobacillus ferrooxidans* from Iranian Aliabad ores. A modified 9k medium was specific in identification and isolation of this strain while the other medium were not effective in growth conditions.

The optimal growth environmental factors for most bacteria arrange in specific range; however there are broad variety range of environmental factors in a microorganism as a natural habitat flora due to their structure and chemical combination of ores. The most common type of bacteria grow well in a natural temperature between 25°C and 40°C, while the range for a natural flora is quiet variable. Although the optimal temperature seems to be 28°C, but it must be modified in pilot plan particularly in natural habitat conditions.

The optimal pH for the most bacteria to grow best is located between 6.5 and 2.5.

Since the bacteria isolated from Aliabad ore are acidophil grow in pH range much lower than above range. In addition due to interfere of organic acid with some media ingredients, the external buffer may needed to maintain the correct pH.

In addition to above primary isolation based on environmental factors, PCR amplification of the 16s-23s intergenic spacer region by using the highly conserved flanking sequence was applied. The PCR technique

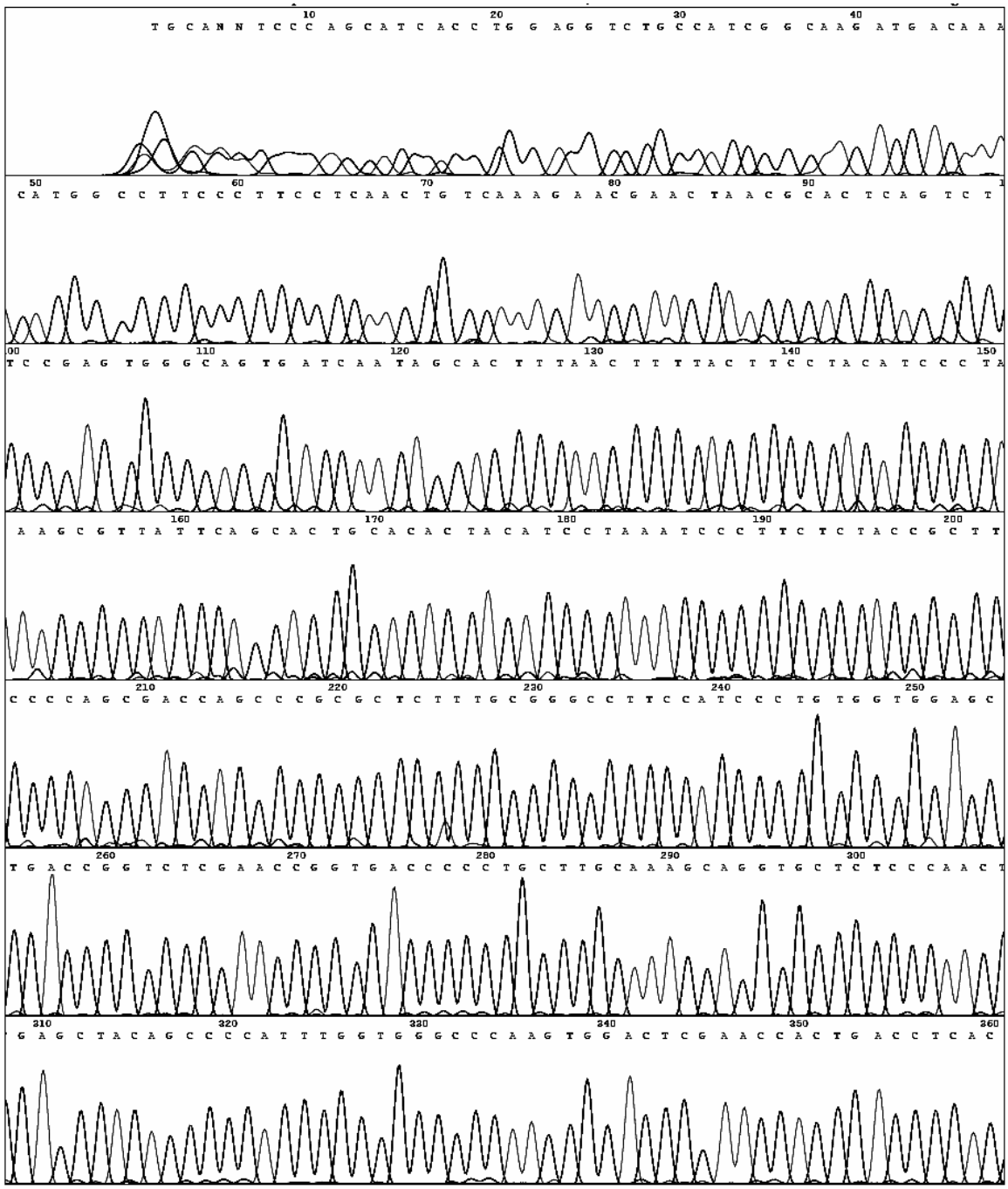


Figure 3. The Thibacillus ferrooxidans NICI1 chromatogram.

produced amplification products for this strain in our data base. This was generated by using a couple of modified primers with several PCR modifications. Sequence of 16s-23s spacer region was identical to the other bacteria of the same group using nucleotide blast software.

The rRNA genes PCR, have been extensively characterized by the investigators such as Moriera and his colleagues [5], although in some cases weak and variables secondary amplification products in the different size range were generated. Variable region surrounded by highly conserved stretches facilitate the

sequencing with the necessary data for specific sequences with potential target sites for specific primers.

PCR amplification of the 16s, 23s [5] and 5s region were used to bacterial identification, by investigators.

However, the design of intergenic spacer targeted primers has the important advantage of the higher variability of the intergenic spacer sequences which greatly facilitates the identification of specific target sequences.

On the basis of the results obtained, PCR amplification of 16s-23s spacer region demonstrated that the significant promise as a tool for the identification of strains belonging to a wider range of bacteria.

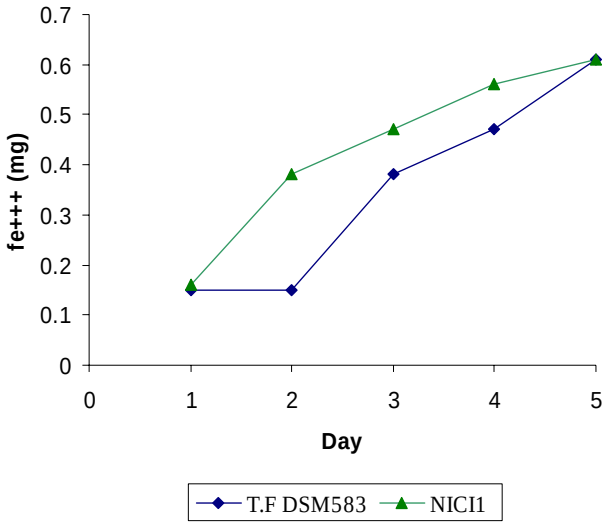


Figure 4. Fe^{3+} production rate in 9k medium.

Acknowledgement

This work was supported by National Iranian Copper Industries Corporation.

References

1. Rawlings D.E. Heavy metal mining using microbes. *Annual Review Microbiology*, **56**: 65-91 (2002).
2. Rohwerder T., Gehrke T., Kinzler K., and Sand W. Bioleaching review part A: progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation. *Applied Microbiology and Biotechnology*, **63**(3): 239-248 (2003).
3. Goebel B.M. and Stackebrandt E. Cultural and phylogenetic analysis of mixed microbial populations found in natural and commercial bioleaching environment. *Applied and Environmental Microbiology*, **60**: 1614-1621 (1994).
4. Yates J.R., Lobos J.H., and Holmes D.S., The use of genetic probes to detect microorganisms in biomining operations. *Journal of Industrial Microbiology*, **1**: 129-135 (1986).
5. Moreira D. and Amils R. PCR-mediated detection of chemolithotrophic bacterium *Thiobacillus cuprinus* using 23s rDNA- and 16s/23s intergenic spacer region targeted oligonucleotide primer. *FEMS Microbiology Letter*, **142**: 289-293 (1996).
6. Gonzalez-Toril E., Liobet-Brossa E., Casamayor E.O., Amann R., and Amils R. Microbial ecology of an extreme acidic environmental. the Tinto river. *Applied and Environmental Microbiology*, **69**(8): 4853-4865 (2003).
7. Pizzaro J., Jedlicki E., Orellana O., Romero J., and Espejo R.T., Bcterial populations in samples of bioleached copper ore as revealed by analysis of DNA obtained before and after cultivation. *Ibid.*, **62**: 1323-1328 (1996).
8. Nicolette J. Coram and Douglas E. Rawlings. Molecular relationship between two groups of the genus *Leptospirillum* and the finding that *Leptospirillum ferriphilum* sp. nov. dominates south African commercial biooxidation tanks that operate at 40°C. *Ibid.*, **68**: 838-845 (2002).