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ACTINOBACTERIA ISOLATED FROM MINERAL ORES IN PERU

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ABSTRACT

A total of twenty-four isolates of actinobacteria from arsenopyrite, pyrite, polymetallic sulfides and magnetite from Peruvian mining zones have been used with the purpose of characterizing them by morphological, physiological and molecular studies for future biotechnological applications. 23 strains were identified as *Streptomyces* and1 as *Actinomadura sp*.95% and 70% of the strains were able to grow at pH 3.5 and pH 2, respectively. Only 19% of them were able to oxidize iron at pH 2. Also, they have been tested by growth on arsenopyrite tailings and *Streptomyces sp.* E1 and *Streptomycesvariabilis* AB5 leached 19.1 % and 15.5 % of arsenic, respectively, while the control without inoculum only showed 2.5% of leaching. Results indicate that these strains show important characteristics for leaching processes.

Keywords: *Streptomyces*, *Actinomadura*, minerals, bacterial leaching, arsenopyrite

INTRODUCTION

There is a wide diversity of microorganisms developing on mineral ores, concentrates and tailings. The microbial composition in these environments has been studied based on cultures, as well as sequencing of 16SrRNA **(Schippers *et al.*, 2010)**. In metagenomic studies of mineral piles, bacteria have predominated against archaea and eukaryotes **(Ding *et al.*, 2009; Mendez *et al.*, 2008; Urbieta*et al.*, 2012)**. In all reports of microbiological studies based on 16SrRNA, the presence of phylum Actinobacteria **(Ding *et al.*, 2009; Korechi*et al.*, 2014; Mendez*et al.*, 2008)** has been determined, which include the order Acidimicrobiales that has 5 validated genera and a candidate genus "Acidithiomicrobium" **(Jones and Johnson, 2015).** The 5 genera have a single species: *Acidimicrobium ferrooxidans*, *Aciditerrimonas ferrireducens*, *Ferrimicrobium acidiphilum*, *Acidithrix ferrooxidans*, *Ferrithrix termotolerans* **(Jones and Johnson, 2015)**, which have been reported in many parts of the world relating them to biohydrometallurgical processes **(Brierley and Brierley, 2013; Schippers *et al.*, 2014)**. Also, the composition of acidic waters **(Bacelar-Nicolau and Johnson, 1999)** and sediments from water from mining activity **(Blöthe*et al.*, 2008; Sanchez-Andrea *et al.*, 2011)** have been evaluated and the presence of sequences of Actinobacteria and sequences belonging to the order *Acidimicrobiales* and *Actinomycetales* were reported **(Bacelar-Nicolauand Johnson, 1999; Blöthe*et al.*, 2008).**

It's been possible to isolate actinobacteria from orders different to acidimicrobiales from tailings or mining concentrates **(Matsubara and Hurtado, 2013; Schippers *et al.*, 2002)**, founding genres such as *Catellatospora* **(Lee *et al.*, 2000a)**, *Saccharothrix* **(Lee *et al.*, 2000b)**, *Pseudonocardia* **(Lee *et al.*, 2001)**, and *Nocardiopsis metallicus***(Schippers*et al.*, 2002)**.

Bacterial leaching or Bioleaching is a process that implies the solubilization of metals using microorganisms and it's determined by the mineralogy and other factors. Bacterial cells can affect the dissolution of metals via the oxidation of iron or sulfur **(Trujillo *et al.*, 2012)**, the generation of organic acids **(Schippers *et al.*, 2014; Vera *et al.*, 2013)**, or processes not yet determined **(Schippers *et al.*, 2014)**. The use of the actinobacteria *Nocardiopsis metallicus* has been demonstrated in the dissolution of metals of alkali silicates **(Vera *et al.*, 2013)**.

The objective of our study was to investigate 24 strains of actinobacteria isolated from minerals from different mining areas of Peru to determine their taxonomic classification and physiological diversity. Likewise, it has been tried to determine the important characteristics for leaching processes and their capacity to grow in arsenopyrite since most of the strains come from arsenopyrite minerals.

MATERIAL AND METHODS

Strains and growth medium

Twenty-four strains of actinomycetes were used, which were isolated from minerals, tailings and concentrates from various mining areas of Peru (Table 1). A total of13 isolates of arsenopyrite, 9 of pyrite, 1 of polymetallic sulphide and 1 of magnetite were used. In a previous study, physiological characteristics of 6 of the 24 strains were reported **(Matsubara and Hurtado, 2013)**.To perform the tests, Casein-Starch medium (AAC)and Glycerol-Yeast Extract medium (XGAL)**(Matsubara and Hurtado, 2013)** and sometimes 10% dilutions of these media were used: AAC 1/10 and XGAL 1/10. ISP4 medium Agar-starch-mineral salts was also used **(Shirling and Gottlieb, 1966)**.

Morphological characterization

Macromorphological characteristics were determined in each growth medium of the 5 media used, as aerial mycelium color, mycelium substrate, diffusible pigment production in the medium and texture of the morphological colonies of the strains were observed following the methods used in The International *Streptomyces* Project (ISP) **(Shirling and Gottlieb, 1966)**. The cultures were incubated at 28°C and revised weekly, up to 30 days.

Molecular analysis

The 24 strains of actinobacteria were cultured in XGAL 1/10 broth. Genomic DNA was extracted from 1.5 mL of culture. Briefly, the bacteria were centrifuged and resuspended in lysis solution (1% SDS, 1mM EDTA 10mM and TRIS-HCl, pH 8). Bacteria were lysed using Fast Prep FP 120 with 100 µm Zirconium beads. The DNA was purified by a chloroform wash and precipitated with isopropanol. Genomic DNA was eluted in 10 mM TRIS-HCl, pH 8 **(Kumar *et al.*, 2010; Kumar *et al.*, 2013)**. The complete sequence of the 16S rRNA gene was amplified using primers 16S 8F (5'AGA GTT TGA TCM TGG CTC AG3') **(Okibe*et al.*, 2003)** and 16S 1492R (5'GGT TAC CTT GTT ACG ACT T3') **(Paramasivam*et al.*, 2011)**. The amplified product size was approximately 1500 bp. The amplified product was sequenced in three overlapping segments using 6 primers. The sequences of the forward primers were: 16S 8F, 16S 337F (5'GAC TCC TAC GGG AGG CWG CAG3') **(Jarice*t al.*, 2009)** and 16S 785F (5'GGA TTA GAT ACC CTG GTA3')**(Jarice*t al.*, 2009)**. The reverse primers were: 16S

1492R, 16S 518R (5'GTA TTA CCG CGG CTG CTGG3') **(Jaric*et al.*, 2009)**, and 16S 907R (5'CCGTCA ATTCCT TTR AGT TT3') **(Jaric*et al.*, 2009)**. The sequences were assembled using the online program MERGER. The complete sequence result was evaluated in the online program BLASTn. The sequences with more than 99% identity were considered as identified isolate.

Table 1 Origin of Actinobacterias isolates

Strain	Origin	pH	Zone	Reference
Y16	Pyrite tailing	6.0	Arequipa,Peru	**Hurtado, 1984**
YA4	Pyrite tailing	6.0	Arequipa, Peru	**Hurtado, 1984**
K1A	Pyrite concentrate 1	1.8	Arequipa, Peru	**Hurtado, 1984**
K1B	Pyrite concentrate 1	1.8	Arequipa, Peru	**Hurtado, 1984**
K2	Pyrite concentrate 1	1.8	Arequipa, Peru	**Hurtado, 1984**
K4	Pyrite concentrate 1	1.8	Arequipa, Peru	**Hurtado, 1984**
AB5	Pyrite concentrate 1	1.8	Arequipa. Peru	**Hurtado, 1984**
AB7	Pyrite concentrate 1	1.8	Arequipa, Peru	**Hurtado, 1984**
11-1	Pyrite concentrate 2	4.0	Arequipa, Peru	**Hurtado, 1984**
X	Magnetite mineral	4.0	Tacna, Peru	**Hurtado, 2003**
21	Polymetallic Sulfide mineral	3.0	Lima, Peru	**Matsubara, 2013**
C2	Arsenopyrite tailing	1.5	Lima, Peru	**Hurtado *et al.*, 1990**
Frep13	Arsenopyrite tailing	1.5	Lima, Peru	**Hurtado *et al.*, 1990**
Frep14	Arsenopyrite tailing	1.5	Lima, Peru	**Hurtado *et al.*, 1990**
Frep15	Arsenopyrite tailing	1.5	Lima, Peru	**Hurtado *et al.*, 1990**
F1	Arsenopyrite tailing	2.0	Lima, Peru	**Hurtado *et al.*, 1990**
F	Arsenopyrite tailing	2.0	Lima, Peru	**Hurtado *et al.*, 1990**
E1	Arsenopyrite tailing	2.0	Lima, Peru	**Hurtado *et al.*, 1990**
Fe5	Arsenopyrite tailing	2.0	Lima, Peru	**Hurtado *et al.*, 1990**
6B	Arsenopyrite concentrate	2.5	Trujillo, Peru	**Matsubara, 2013**
6C	Arsenopyrite concentrate	2.5	Trujillo, Peru	**Matsubara, 2013**
6D	Arsenopyrite concentrate	2.5	Trujillo, Peru	**Matsubara, 2013**
6E1	Arsenopyrite concentrate	2.5	Trujillo, Peru	**Hurtado, 2003**
6E3	Arsenopyrite concentrate	2.5	Trujillo, Peru	**Hurtado, 2003**

Physiological characterization

The following characteristics were evaluated: 1. growth at different pH (2, 3.5, 5 and 7) and different levels of salinity (1.5, 5, 7 and 10); 2. Growth and oxidation of ferrous sulfate (1 g/L) at different pH (2, 4 and 7) and; 3. Growth and oxidation of sodium thiosulfate (1 g/L) at pH 4 and pH 7.

To perform all the tests the 24 strains were cultured in XGAL 1/10 broth at 30°C for 07 days, and an inoculum with a density corresponding to $5x10^8$bact/mL was prepared. 0.5 mL of the inoculum was seeded into 10 mL XGAL 1/10 tubes. For the tests at different pH, the XGAL 1/10 was previously taken to the final pH by the addition of H_2SO_4 1N. For the other tests, NaCl, ferrous sulfate or sodium thiosulfate were added in the required concentrations. In the tubes with sodium thiosulphate, orange methyl was added as an indicator. All tests and controls were performed in duplicate. They were incubated at 30°C for up to 30 days.

The pellet formation was evaluated as growth indicator every 3 days, using a qualitative scale and,at the end of the test the purity of the culture was checked by microscopic observations and growth in solid media. The oxidation of ferrous ion was evaluated qualitatively by the formation of a red brick color that shows the formation of ferric ion. The oxidation of sodium thiosulfate was considered positive when there was a pink coloration by the turn of the indicator.

Leaching of Arsenopyrite

The following tests were performed: 1. growth of 24 strains on 5% arsenopyrite (pH2 and pH7) and; 2. Test of leaching of arsenopyrite. The arsenopyrite tail had the following composition: As 0.99% and Fe 5.45%.

To perform the growth tests, the 24 strains were grown in AAC1/10 broth at 30°C for 7 days, and an inoculum with a density corresponding to $5x10^8$bact/mL was prepared. 0.5 mL of the inoculum was seeded in Falcon tubes with 30 mL of XGAL 1/10 medium and 1.5 g of arsenopyrite tail. For the tests at pH 2 and pH 7, the solution was previously brought to the final pH by addition of H_2SO_4 1N.Cultures were maintained at 28°C for 30 days. Growth was evaluated macroscopically and microscopically.

For the leaching tests, 25 g of sterile arsenopyrite tail and 500 ml of medium at pH7 were placed in Erlenmeyer flasks. They were inoculated with 5 mL of cultures of strains AB5 and E1; and then shaken for 30 days at 28°C. Control experiments were carried out in the same manner, but without inoculum and under sterile conditions. All tests were run in duplicate. Growth was evaluated periodically by microscopic and macroscopic means. The solution was filtered and the arsenic content in solution was determined in the filtrate by atomic absorption.

RESULTS

Morphological characterization

All strains were able to grow in solid and liquid medium of ISP4, AAC and XGAL. Three isolates were slow growing bacteria (more than 3 weeks). Sometimes, to obtain the growth in complete medium (XGAL or AAC), they had to be previously planted in dilute medium XGAL 1/10. Strains 11.1 and Fe5 formed colonies smaller than 1 mm, unlike all of the others that form colonies of 5 mm. The E1 strain has a red pigment in the colony; the 6C strain is dark brown in color and the Frep 13 and Frep 15 strains are light brown in color. All have aerial mycelium, most of them gray or white. Also, most produce diffusible green or orange pigments in the medium.

Molecular analysis

Analysis of the 16S rDNA gene sequence, with more than 99% identity, identified 23 isolates within the genus *Streptomyces* and one isolated within the genus *Actinomadura* (Table 2).

Physiological characterization

The strains more sensible to NaCl were Fe5 and 11.1, which were able to grow only at 1.5% NaCl, and a total of 8 strains grew at 10% NaCl(Figure 1A).

All strains showed capacity of growthat pH 5 and pH 7, (Figure 1B). But not all of them oxidize iron (Figure 1C). All results of growth and oxidation in XGAL 1/10 + 1 g/L ferrous iron at different pH levels are shown in Figure 1.

Growth in the presence of sodium thiosulfate1 g/Lat pH 7 and 4 was observed in all strains, but no oxidation was obtained.

Table 2 Identification of isolates from different mining areas of Peru based on 16S rDNA gene sequencing.

Strain	Identification
Y16	*Streptomyces heilongjiangensi Y16*
YA4	*Streptomyces albidoflavus YA4*
K1A	*Streptomyces variabilis K1A*
K1B	*Streptomyces thermocarboxydus K1B*
K2	*Streptomyces sp*. K2
K4	*Streptomyces matensis K4*
AB5	*Streptomyces variabilis AB5*
AB7	*Streptomyces sp. AB7*
11-1	*Streptomyces ginglanensis 11-1*
X	*Streptomyces variabilis X*
21	*Streptomyces variabilis 21*
C2	*Streptomyces sp.C2*
Frep13	*Streptomyces champavatii Frep 13*
Frep14	*Streptomyces heilongjiangensis Frep 14*
Frep15	*Streptomyces peruviensis Frep 15*
F1	*Streptomyces sp. F1*
F	*Streptomyces variabilis F*
E1	*Streptomyces sp. E1*
Fe5	*Actinomadura sp. Fe5*
6B	*Streptomyces heilongjiangensis 6B*
6C	*Streptomyces sp. 6C*
6D	*Streptomyces sp. 6D*
6E1	*Streptomyces sp*. 6E1
6E3	*Streptomyces sp. 6E3*

Leaching of Arsenopyrite

Of the 24 strains, only isolates E1 and AB5 were able to develop in arsenopyrite tailings at pH 7 (Table 3). None of the two strains was able to develop at pH 2. The E1 strain formed circular aggregates, which showed the characteristic red color of the strain, they were in the solution and adhered to the wall of the flask. The AB5 strain grew to a lesser extent and formed small grey aggregates. The leached solutions ofE1 and AB5 showed an extraction of 19.1% and 15.5% of the arsenic present in the arsenopyrite tailings. The control showed an arsenic solubilization of 2.5%.

DISCUSSION

All the 24 strains grew in the three medium used, showing different macroscopic morphology. In microscopic observations, aerial mycelium is observed with chains of spores typical of *Streptomyces* **(Kämpfer*et al.*, 2015; Shirling and Gottlieb, 1966)**. The strain Fe5 presented morphology that correlates with the genus *Actinomadura* **(Trujillo *et al.*, 2012)** and it was confirmed by molecular identification. The isolation of *Streptomyces* has been reported from uranium mines **(Lorenz *et al.*, 2007)** and polish black shale ores **(Suto *et al.*, 2007)**. However, there is no a previous report of *Actinomadura* from mineral ores.

Streptomyces isolates do not show correlation by the characteristics of the origin, pH of the sample or the zone of collection. For example, the group formed by Y16, 6B and Frep14, identified as *Streptomyces heilongjiangensi* were collected from pyrite tailings, arsenopyrite concentrate and arsenopyrite tailings, at different pHs and in different regions of Peru (Arequipa, Lima and Trujillo). Results show the heterogeneity of species within the same collection area which would reflect the presence of a high diversity of strains within *Streptomyces* genus.

Many of the strains showed growth at pH levels lower than 5.The growth and oxidation tests of ferrous iron were performed in a diluted medium **(Matsubara and Hurtado, 2013)**, with a final concentration of 0.05%glycerol and yeast extract of 0.02%. This concentration of yeast extract is adequate so that heterotrophic acidophilic bacteria can grow without inhibition **(Bacelar-Nicolau and Johnson, 1999)**. At low pH, the molecules of organic acids in the growth medium are in their undissociated form and without charge. This kind of molecules can easily cross the cell membrane and act as decouples, producing toxic compounds (**Koschorreck, 2008**) and inhibiting the growth. It has been reported that different groups of *Streptomyces* have been isolated from acid soils with an average pH of 4.5. The minimum pH with growth reported is 3 **(Kämpfer *et al.*, 2015; Vera *et al.*, 2013; Willscher and Bosecker, 2003),** however 16 of 23 isolated Streptomyces were able to grow at pH 2.

The peruvian isolates *Streptomyces peruviensis* (Frep15), *Streptomyces ginglanensis* (11.1) and *Actinomadura sp*. (Fe5) were considered as bacteria with slow or difficult growth because sometimes takes more than 20 days to obtain growth.

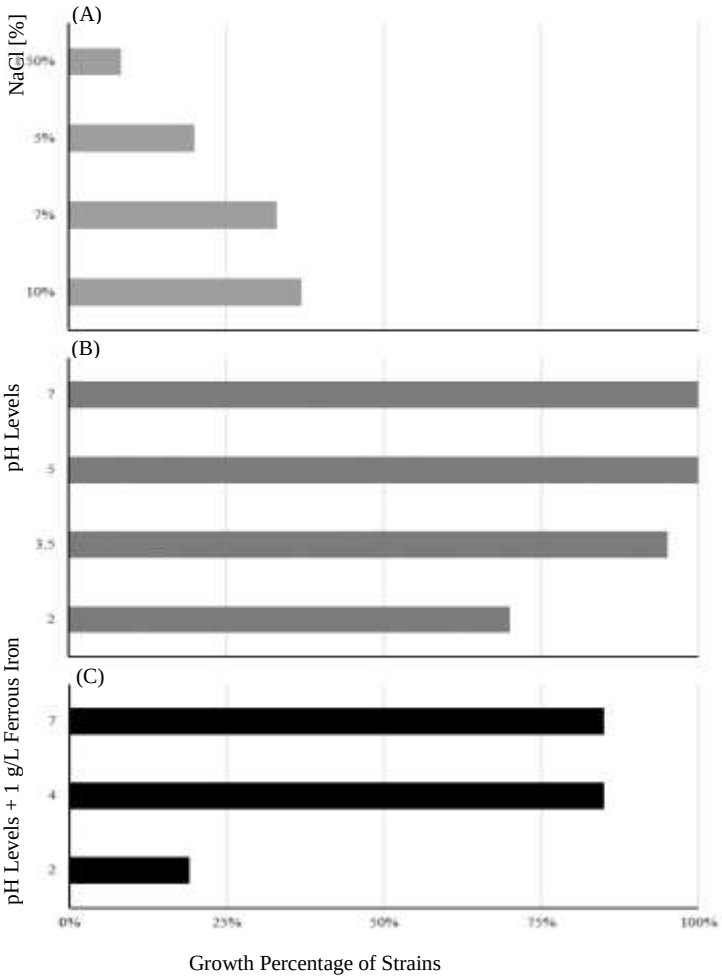

Figure 1 Growth Percentage of Strains at: A. Different levels of salinity; B. different pH levels; C. different pH levels with 1 g/L ferrous iron.

It has been demonstrated that all strains were incapable of oxidizing thiosulfate, althoughthey were able to growth in the presence ofthiosulfate 1 g/L. Many Streptomyces are able to use the thiosulfate pathway to produce cysteine **(Wink *et al.*, 2017)** and also in some actinobacteria it has been demonstrated thiosulfate oxidation **(Anandham *et al.*, 2008).**

Ferrous iron oxidation was observed mostly at pH 7 and 4, and in less proportion at pH 2. The oxidation of ferrous iron, in all three pH levels, occurred after pellet formation, demonstrating that the iron oxidation it is not related to growth and suggesting that the strains of *Streptomyces* did not obtain energy for growth through the oxidation of ferrous iron. All 24 strains did not grow only with a source of iron (data not shown). The iron oxidation is widely present in bacteria and archea and in many cases it is not related to energy generation **(Ilbert *et al.*, 2013)**.Also, some of them catalize a dissimilatory oxidation of ferrous iron **(Bryan and Jhonson, 2008; Ilbert *et al.*, 2013)** .

Most of Streptomyces strains (17) were able to growth and oxidize iron at pH 7 and 4 (Figure 2).Only *Streptomyces sp.* (strains C2, 6E1 and 6E3) and *S.heilongjiangensis 6B* were able to growth and oxidize iron at pH 2 and tolerate more than 5% sodium chloride.

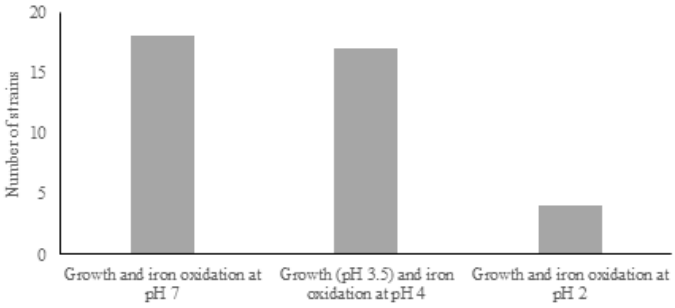

Figure 2 Number of strains showing growth and iron oxidation at different pH levels.

Tolerance was observed at different levels of salinity from 1.5% to 10% NaCl. This tolerance to high concentrations of sodium chloride is due to the fact that the osmotic pressure of the actinomycete cells is quite high, which allows survival of the actinomycetes in soils with high salinity **(Bacelar-Nicolau and Johnson, 1999)**. Moreover, in the case of *Streptomyces* isolates would be related to the high concentration of metal ions present in the minerals from which they have been isolated.

Streptomyces sp. E1 showed the best extraction of arsenic from the arsenopyrite tailing, followed by *Streptomyces variabilis* AB5 (Table 3). *Streptomyces sp.* E1 has been isolated from arsenopyrite, which could explain its ability to oxidize arsenopyrite differently from *Streptomyces variabilis* AB5 which was isolated from pyrite. Both of them were able to oxidize iron at pH 4 and higher. The leaching of arsenopyrite at acidic pH produces arsenic and iron in solution **(Schippers *et al.*, 2014)**, the arsenic is mostly found as As III or As V **(Brierley and Brierley, 2013; Shirling and Gottlieb, 1966; Trujillo *et al.*, 2012)**. At pH greater than 2.5, the iron ion tends to form hydroxides and to precipitate, whereas As III remains in solution at higher pH **(Bacelar-Nicolau and Johnson, 1999)**, for that reason arsenic is found in solution in the leaching test.

Table 3 Characteristics of strains able to leach arsenopyrite.

Strain	Growth		Iron (II) Oxidation			Growth on NaCl [%]
	pH		pH			
	2	3.5	2	4	7	
Streptomyces variabilis AB5	+	+	-	+	+	10
Streptomyces sp. E1	+	+	-	+	+	10

Legend: +, growth or oxidation; -, no growth or no oxidation

CONCLUSION

In this research, it has been possible to identify and characterize23 *Streptomyces* and 1 A*ctinomadura* present in different peruvian mineral ores, being the first report of *Actinomadura* in minerals. Also, it has been demonstrated the capacity of *Streptomyces sp.* E1 and *Streptomyces variabilis* AB5 to grow and leach arsenopyrite. *Streptomyces sp.* (strains C2, 6E1 and 6E3) and *S.heilongjiangensis 6B* were able to grow and oxidize iron at pH 2, which it might allow to be used in acidic environments. All these results further expand the diversity of microorganisms that can be used as leaching agents in biohydrometallurgical and in other biotechnological applications.

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