Available online at www.sciencedirect.com
SCIENCE DIRECT®
Chemie der Erde 65 (2005) S1, 145–156
CHEMIE der ERDE GEOCHEMISTRY
www.elsevier.de/chemer

Isolation and characterization of indigenous copper-resistant actinomycete strains

V.H. Albarracín[a,b], M.J. Amoroso[a,c], C.M. Abate[a,b,c,*]

[a]*Planta Piloto de Procesos Industriales y Microbiológicos (PROIMI), CONICET. Av. Belgrano y Pasaje Caseros, 4000 Tucumán, Argentina*
[b]*Facultad de Ciencias Naturales e Instituto Miguel Lillo, Universidad Nacional de Tucumán, 4000 Tucumán, Argentina*
[c]*Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, 4000 Tucumán, Argentina*

Received 10 January 2005; accepted 17 May 2005

Abstract

Fifty actinomycetes were isolated from copper contaminated and non-contaminated area. Primary qualitative screening assays showed that 100% of the isolated microorganisms of the contaminated area were resistant up to 80 mg L^{-1} of $CuSO_4$. On the other hand, 100% of isolates from non-contaminated area grew at 16 mg L^{-1}, 87.4% at 40 mg L^{-1} and only 19.4% of them were capable of growing at 80 mg L^{-1} of $CuSO_4$. The semiquantitative assay showed that the isolated strains from the sediments of the contaminated site were resistant up to the highest concentration tested (1000 mg L^{-1}) with the exception of AB2C strain; however, the strains isolated from non-contaminated sediments were sensitive to Cu^{2+} concentrations higher than 200 and 400 mg L^{-1}, respectively. Microbial growth of AB0 strain in presence of 39 mg L^{-1} copper showed an inhibition of 32% after 6 days of incubation as compared to the control, and copper residual concentration indicated a reduction in the supernatant of 71.2% after 6 days of incubation: pellet acid digestion proved that copper was accumulated by the cells. 16S rDNA restriction digestion of 1300 bp amplicons with *Cfo*I and *Hpa*II showed only

*Corresponding author. PROIMI, Av. Belgrano y Pasaje Caseros, 4000 Tucumán, Argentina. Tel.: +54 381 4344888; fax: +54 381 4344887.
E-mail address: cabate@proimi.org.ar (C.M. Abate).

0009-2819/$ - see front matter © 2005 Elsevier GmbH. All rights reserved.
doi:10.1016/j.chemer.2005.06.004

one restriction pattern for all the strains and it matched with the control, *Streptomyces coelicolor*.
© 2005 Elsevier GmbH. All rights reserved.

Keywords: Actinomycetes; Copper resistance; Copper biosorption; Bioremediation

1. Introduction

Copper (Cu) is an essential element for all animals and plants and must exist in soil or diets of organisms. However, Cu is toxic at higher concentrations in the soils or diets. Portions of the landscape have been found to be Cu deficient, limiting the survival and reproduction of plant and/or animal species. Other areas are affected by excess environmental concentrations of Cu from natural sources (erosion and run-off of copper bearing minerals and soils that occur in Earth's crust) and from anthropogenic sources, e.g., building and construction materials, automotive parts, domestic products, mining, smelting, power generation, burning of fossil fuels, Cu-based fungicides on agricultural crops and as a constituent of sewage sludge used as a fertilizer (Fairbrother et al., 1999).

Copper cannot be destroyed and tends to be accumulated in soils, plants and animals, increasing their concentrations in the superior level of food chains. This metal has been shown to be directly toxic to vertebrates from dietary sources, usually in the range of 100–1000 mg L^{-1} (Georgopoulus et al., 2002). However, the great impact of Cu on wildlife may be indirect through stronger effects on the plant and soil invertebrate communities that support the entire ecosystem. In Tucumán, Argentina, the main hydrographic river basin is the Salí River. It crosses the entire state, having an influence area of 60,000 ha and receiving effluents from local industries. The analyses of sediments samples collected from the former river basin in Tucumán indicate the presence of metals (cobalt, chromium, copper, manganese, nickel, zinc), pesticides, oils of the rind of lemon and sub-products of the paper processing industry (Romero et al., 1997). On the other hand, near of the Salí River, there is a Filter Plant for copper processing. Its effluents are discharged in a drainage channel that provides water to a sugar cane culture, which is growing around this channel and finally ends in the Frontal Hondo dam (Benimeli et al., 2003).

A variety of technologies are currently available to treat soils contaminated with hazardous materials (US EPA, 1988). Among them, bioremediation, which involves the use of microorganisms to detoxify and degrade environmental contaminants, has received increasing attention as an effective biotechnological approach to clean up a polluted environment (Boopathy, 2000).

Soil microorganisms play an important role in the environmental fate of toxic metals with a multiplicity of mechanisms affecting transformations between soluble and insoluble forms. These mechanisms are integral components of natural biogeochemical cycles and have a potential for both, in situ and ex situ bioremedial treatment processes, for solid and liquid wastes (Gadd, 2000). Actinomycetes is the

most abundant group of bacteria in soils (90%) and shows primary biodegradative activity, secreting a range of extracellular enzymes that allow them to metabolize recalcitrant molecules (Kieser et al, 2000). Amongst actinomycetes in soil, there are examples of different strategies, from cycles of rapid proliferation and sporulation to the maintenance of populations by prolonged slow growth and scavenging. This metabolic and morphological versatility gives them a great potential to perform bioremediation processes, including metal recovery (Ravel et al, 1998).

There is a lot of information available on copper resistance mechanisms in Gram-negative bacteria such us *Escherichia coli* and *Pseudomonas* sp. and even genetic determinants have been proposed (Munson et al., 2000). Copper metabolism seems to be very much clearer in the Gram-positive bacterium *Enterococcus hirae* (Odermatt et al., 1992). Nevertheless, there is not enough specific information on the mechanisms involved in the resistance to copper by actinomycetes (Erardi et al., 1987).

The objective of this work was to isolate and characterize copper-resistant actinomycete strains from sediments contaminated with this heavy metal. On the basis of ARDRA, we also attempted to characterize these strains taxonomically.

2. Materials and methods

2.1. Samples

Sediment samples were collected, from the water reservoir "El Cadillal" (not contaminated area), and from a drainage channel that receives effluents from a copper filter plant (contaminated area). Both places are located in Tucumán, Argentina. Each sample was aseptically collected using sterile test tubes, and kept at 5 °C until they were dried at 30 °C to constant weight. Samples were diluted with sterile water prior inoculation onto agar plates in duplicate.

2.2. Microorganisms and media

Isolation of microorganisms was carried out in Minimal Medium (MM) containing per liter in grams: L-asparagine, 0.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; glucose, 10.0; agar, 15.0; supplemented with $CuSO_4$ 16 mg L^{-1} (pH 7). The medium was supplemented with 10.0 μg mL^{-1} of nalidixic acid (NA) and cycloheximide to inhibit Gram-negative bacteria and some Gram-positive bacteria and fungi growth, respectively (Ravel et al., 1998). Plates were incubated at 30 °C and colonies were purified by streaking on agar medium without antibiotics. Actinomycete colonies were maintained by monthly transfer to agar slant tubes of MM incubated at 30 °C and stored at 4 °C.

2.3. Qualitative assays of copper resistance

Primary qualitative screening assays were carried out in plates containing MM agar medium supplemented with $CuSO_4$ 16, 40 and 80 mg L^{-1}. One hundred microliters of spore suspensions (1×10^9 SFU/mL) of all the isolates were prepared as stated before by Kieser et al. (2000) and inoculated in the plates. Resistant isolates capable of growing up to the highest

concentrations tested were transferred to a new culture amended with higher $CuSO_4$ concentrations. In this case, rectangular wells were made in the center of Petri dishes by aseptically removing strips of agar which were filled with 500 μL of solutions of $CuSO_4$: 160, 320 and 480 mg L^{-1}. Isolates were inoculated by streaking 100 μL of spore suspension perpendicularly to the wells. Microbial growth close to the well was used as the qualitative parameter of metal resistance according to Amoroso et al. (1998).

2.4. Semi quantitative assays of copper resistance

Fifty microliters of $CuSO_4$ solution at different concentrations (100–1000 mg L^{-1}) were used to fill the wells of Petri dishes culture media previously inoculated with 100 μL of spore suspensions (1×10^9 SFU/mL) of the strain to be tested. The diameter of the growth inhibition was measured after incubation at 30 °C for 4 days. The strains were considered resistant (up to 5 mm), and non-resistant (more than 5 mm) (Amoroso et al.,1998).

2.5. The effect of Cu supplement on the bacterial growth

Spore suspensions (1×10^9 SFU/mL) of the copper-resistant strains isolated from the contaminated area were inoculated in MM liquid medium (25 mL) supplemented with 80 mg L^{-1} $CuSO_4$ by triplicate. Cultures were incubated in orbital shaker (100 rpm) at 30 °C for 7 days. Biomass was collected by centrifugation at 3000*g* during 10 min, washed twice with distilled water and dried at 105 °C until constant weight. Actinomycete cultures without copper were used as controls.

2.6. Copper analysis

Copper concentrations were determined by atomic absorption spectrometry (Perkin Elmer Aanalyst 100) in the supernatant and the biomass cultures. Total final biomass was first digested with concentrated nitric acid. A similar procedure was carried out with the sediment samples (Standard Methods, APHA, 1992).

2.7. DNA isolation and purification

Selected strains were grown in liquid MM for 4 days. The pellets were collected by centrifugation and washed twice with sterile distilled water. Total genomic DNA extraction was carried out according to the technique described by (Kieser et al., 2000) and modified by Albarracín et al. (2004).

2.8. 16S rDNA PCR amplification

Total genomic DNA were used as templates for PCR amplification. *Streptomyces coelicolor* from DSMZ collection (German Collection of Microorganisms and Cell Cultures) and sterile bidistilled water were used as positive and negative controls in all the assays, respectively. Amplifications were performed in 25 μL reaction volumes using 16S universal oligonucleotide primers: 63 Forward (5′-CAG GCC TAA CAC ATG CAA GTC-3′) y 1389 Reverse (5′-ACG GGC GGT GTG TAC AAG-3′) (Brosius et al, 1978) Amplifications reactions were carried out in an automated thermal cycler (Perkin-Elmer, model 9700, Applied Biosystems). PCR products were run in 1.0% agarose gel, stained with ethidium bromide and then visualized using an Image Analyzer Gel Doc *BIORAD*.

2.9. Restriction analysis

Amplification products were digested by two enzymes: *Cfo*I (Gibco, BRL) and *Hpa*II (Promega). Restriction reactions were carried out in a final volume of 15 μL using 0.1 U of the corresponding enzyme and the corresponding buffer 1 × . Restriction products were run in 2% agarose gel stained with ethidium bromide and then visualized using an Image Analyzer Gel Doc *BIORAD*.

3. Results

3.1. Samples and microorganisms

Sediment is an important sink and reservoir for copper. In pristine areas, sediment generally contains less than 50 μg/g; the level can reached several thousand μg/g in polluted areas (Georgopoulus et al. 2002). Copper concentration was measured in the sediment samples used in this work, the results obtained were 629 μg/g for the sample from drainage channel and only 30 μg/g from the water reservoir "El Cadillal".

Thirty-one actinomycetes were isolated from the non-contaminated area and 19 from the contaminated area. All isolates showed the actinomycetes typical morphology with both a substrate mycelium and aerial pigmented branched hyphae.

3.2. Selection of actinomycete copper-resistant strains

Primary qualitative screening assays (Fig. 1) showed that 100% of the isolated strains of the contaminated area were resistant up to 80 mg L^{-1} of $CuSO_4$.

On the contrary, actinomycetes strains isolated from non-contaminated areas are more sensitive in copper amended media. Nevertheless, 19.4% of these strains are capable of growing with a concentration of 80 mg L^{-1} of $CuSO_4$. This could indicate that copper resistance mechanisms facultatively exist in some cells.

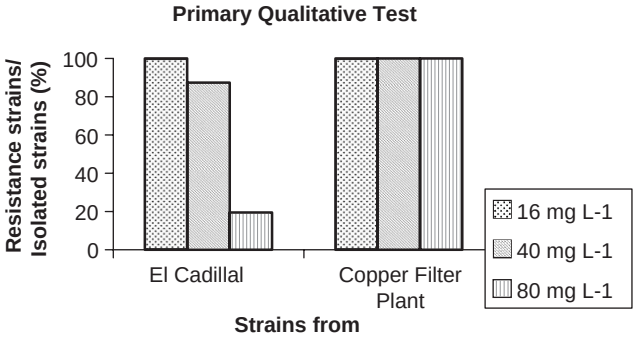

Fig. 1. Qualitative copper resistance of actinomycete strains isolated from a drainage channel (19 strains) and a water reservoir, El Cadillal (31 strains).

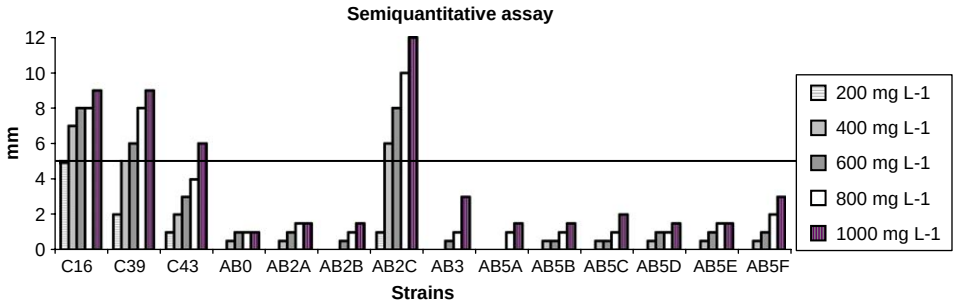

Fig. 2. Semiquantitative resistance at 200, 400, 600, 800 and 1000 mg L^{-1} of Cu^{2+} concentrations measured as inhibition zone in mm. The horizontal line indicates the arbitrary limit used to consider copper-resistant (below) and non-resistant (up) strains. Fifty microliters of various concentrations were placed in a well in the MM agar medium. AB-strains – from sediment of the drainage channel. C-strains – from El Cadillal.

Eleven and three actinomycete strains isolated from contaminated and non-contaminated areas, respectively, were selected because they show resistance up to 80 mg L^{-1} of $CuSO_4$ in evaluating their resistance in MM solid medium with high $CuSO_4$ concentrations (160, 320 and 480 mg L^{-1}). This method was used to give a rapid but qualitative estimation of the copper resistance of these strains as stated by Abbas and Edwards (1989). Seventy-one percent of the selected strains were resistant at all concentrations tested and they belong to the polluted area samples. The remaining test strains showed a pattern of marked inhibition at 320 and 480 mg L^{-1}, most of them were isolated from the non-polluted area sample (data not shown).

Later a semiquantitative assay in agar culture was made (Fig. 2) at $CuSO_4$ concentrations from 200 to 1000 mg L^{-1}. The results showed that the strains isolated from the sediments of the drainage channel (AB: 0, 2A, 2B, 3, 5A, 5B, 5C, 5D, 5E and 5F) were resistant up to the highest concentration tested with the exception of AB2C strain, however, the strains isolated from the sediments of El Cadillal (C: 16, 39 and 43) were sensitive at $CuSO_4$ concentrations higher than 200 (C16) and 400 mg L^{-1} (C: 39 and 43)(Fig. 2).

3.3. The effect of Cu supplement on the bacterial growth

For performing this experiment, we chose the most resistant actinomycete strains belonging to the AB group isolated from the sediments of the Copper Filter Plant and the strain AB2C (the most sensitive strain) was used as a control. The growth expressed as dry weight (mg mL^{-1}) after cultivation in MM liquid medium supplemented with 80 mg L^{-1} Cu^{2+} was determined. Only the strains AB0, AB2A, AB5A and AB2B showed the greatest growth compared to the AB2C-sensitive strain (Fig. 3a). This experiment was carried out in triplicate.

When copper residual was determined in the supernatant of the culture medium (Fig. 3b) the results revealed a diminution of 71% by AB0, 65% by AB2A, 27%

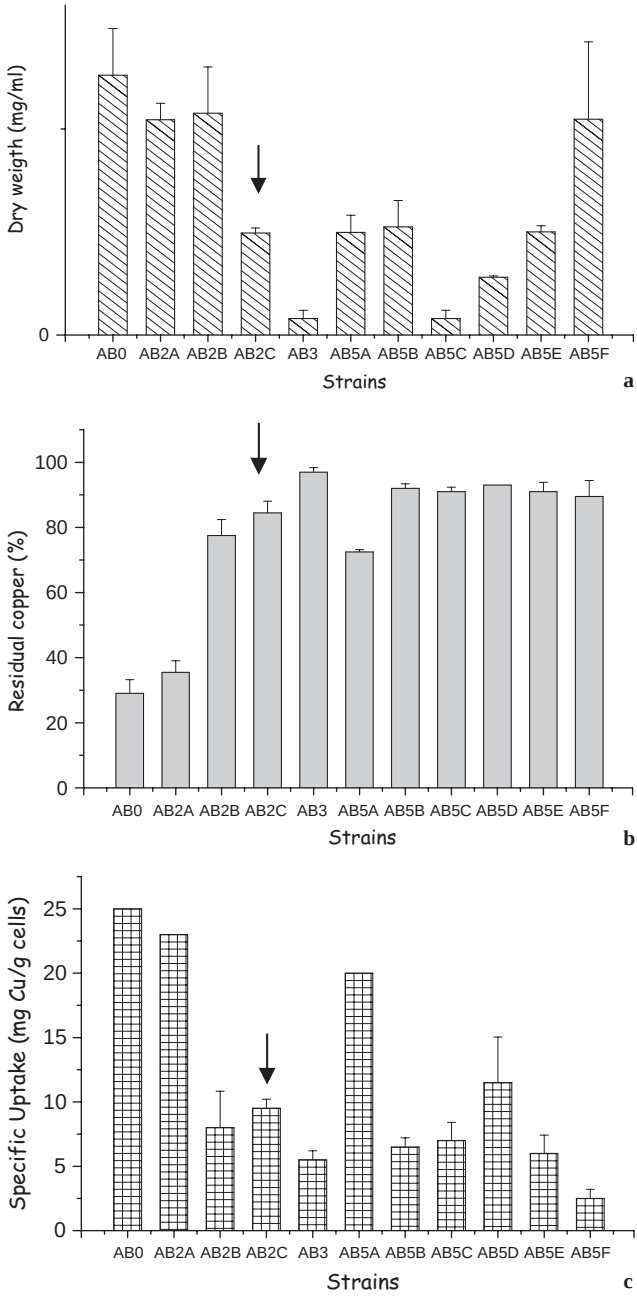

Fig. 3. (a) Biomass as dry weight ($mg\,mL^{-1}$) in MM liquid medium supplemented with $80\,mg\,L^{-1}$ of $CuSO_4$; (b) residual copper concentration ($mg\,L^{-1}$) in the supernatant after 7 days of growth; (c) copper biosorption (mg of Cu/g of cells) of 11 selected actinomycete strains. The control sensitive strain, AB2C, is indicated with an arrow (↓).

AB5A and 23% by AB2B culture strains. Copper biosorption related to the cell growth (Fig. 3c) was also calculated and as expected, the two strains (ABO and AB2A) that had the lowest copper residual had the highest biosorption value. They look promising to study and perform copper bioremediation processes.

3.4. Copper batch culture of AB0 strain

The time course of microbial growth of AB0 strain in the presence of 39 mg L^{-1} copper in individual flasks of 25 mL is shown in Fig. 4. A growth inhibition of 18–28% was obtained after 48 h of cultivation and 32% after 6 days as compared to the control without copper. Copper residual determination indicated a reduction of 71.2% after 6 days of incubation.

Microbial growth was exponential until 120 h. It is important to notice that during the first 48 h the copper initial concentration in the medium did not change appreciably. In contrast, during the period between 48 and 120 h, there was a drastic decrease of residual copper (38–11 mg L^{-1}) that is correlated with the log growing phase. Between 120 and 144 h there was depletion on growing with and without copper that was coincident with no more uptake of copper from the medium by the strain.

It is also important to remark that after 72 h, the culture medium as well as the strain suffered a color change, from blue to light green and from white to light green, respectively. This color change interestingly coincided with the maximal disappearance of metal from the medium (data not shown).

After 7 days of growth, 10 mg of accumulated copper was detected in the acid digested biomass. It corresponds to the expected value taking into account the losses

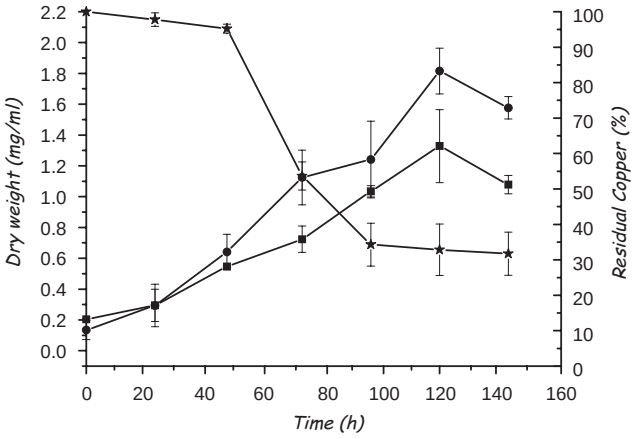

Fig. 4. AB0 actinomycete strain growth in the MM medium, without (●) and with (■) copper measured as dry weight of biomass. Copper depletion from the MM culture medium, during 144 h of incubation (*).

due to sampling, residual biomass left behind in the batch recipient and tubes and also loss or partial digestion during the process.

These results evidently show that the AB0 strain has the ability to uptake copper from the medium. Further investigations should focus on the nature of this uptake system.

3.5. 16S PCR amplification and restriction analysis

Amplicons of approximately 1300 bp were obtained for all the strains studied previously. After digestion with *Cfo*I and *Hpa*II, only one restriction pattern was observed for all the strains and it matched with the control, *Streptomyces coelicolor*.

*Cfo*I restriction analysis pattern consisted of three bands: 460, 290 and 130 bp while *Hpa*II restriction analysis pattern was: 270, 140 and 40 bp (Fig. 5). With this methodology it was not possible to establish differences among the actinomycete strains, even so, they present pattern differences in morphology and physiology (copper resistance levels). Their pattern correlated with *Streptomyces coelicolor* restriction profiles which may indicate that all strains belong, at least, to the same genus: *Streptomyces*.

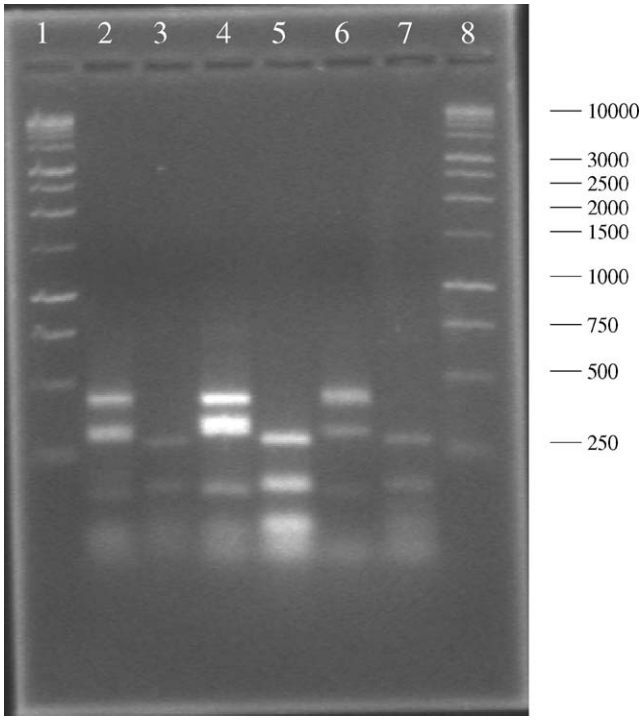

Fig. 5. ARDRA of AB5E and AB0 strains. Lanes 1 and 8. 1 kb Ladder. Lanes 2 and 4 AB5E and AB0 cut with *Cfo*I. Lanes 3 and 5 AB5E and AB0 cut with *Hpa* II. Lanes 6 and 7. *Streptomyces coelicolor* (control) cut with *Cfo*I and *Hpa*II, respectively.

4. Discussion

Living organisms have been exposed to heavy metals released into the environment by geochemical processes (Brown et al., 1998). Since the age of industrialization and enhanced mining activities, this exposure has been dramatically increased by human pollution, then, it is not surprising to find that copper resistance ability is widespread among actinomycetes from both contaminated and non-contaminated soils, as it was demonstrated in this paper. Moreover, strains isolated from copper polluted soil in this work have been proved to be considerably more resistant than strains from non-polluted areas which may firmly indicate an induction mechanism towards copper resistance.

Copper-resistant levels found in the tested strains are highly superior in comparison with the results obtained by Abbas and Edwards (1989) using *Streptomyces californicus*, who showed a relative growth of 50% in a complex liquid medium amended with $10\,mg\,L^{-1}$ Cu^{2+}, and when using a higher concentration ($50\,mg\,L^{-1}$), there was no growth at all. Similar results were obtained by Abbas and Edwards (1990) for *S. coelicolor* with a growth inhibition of 50% after 6 days in starch–yeast extract broth supplemented with $3\,mg\,L^{-1}$ of Cu^{2+}. Amoroso et al. (1998) showed that the actinomycete strain R25 had an inhibition of 40% after 48 h of growing in a minimal liquid medium amended with $32\,mg\,L^{-1}$ of Cu^{2+}. The results obtained in this work may suggest that indigenous actinomycete strains isolated from copper polluted soils should have acquired physiological and genetic mechanisms that allow them to survive in adverse environments and that may give them competitive behavior when growing in polluted culture media like the results presented by Boopathy (2000).

Also, it is important to remark at this point, that the use of minimal medium for the growth of actinomycetes assures us that the supplemented metal does not form complexes with components of the medium, and that all the metal is available for the cells (Amoroso et al.,1998). Previous works used complex media to investigate the capacity of strains to grow in higher concentrations of copper (Abbas and Edwards, 1989, 1990), but some sequestration of the metal is expected, for instance, with proteins, specially the ones containing amino acids as cysteine, methionine or hystidine that are proven to bind copper (Koch et al., 1997).

AB0 actinomycete strain has shown the ability to remove more than 50% of copper from the culture medium ($39\,mg\,L^{-1}$) in only 72 h indicating that it is a suitable agent for bioremediation of soils or effluents with high concentration of copper. This copper removal and retention ability observed in the AB0 strain may involve a fully integrated system of uptake, storage and distribution of the metal, present in actinomycetes strains. Recent progress in understanding the mechanisms of heavy metal resistance has indicated that similar mechanisms for resistance to a single metal may occur across a wide range of bacterial genera, and that related mechanisms of resistance may apply to different heavy metals (Brown et al., 1998).

Taking this into account, we can infer that copper resistance mechanisms of actinomycetes could be similar to that encountered in other bacteria such as the *Pco*ABCDRS system of *E. coli* or its homologue *Cop*ABCDRS of *Pseudomonas* sp.

and *Xanthomonas campestris* (Nies, 1999). There are also copper resistance mechanisms of a Gram-positive bacterium, *Enterococcus hirae*. These bacteria have a *cop* operon with two structural genes encoding *P*-type ATPases: *Cop*A and *Cop*B (35% of identity with *Cop*A from *Pseudomonas* (Nies, 1999). These kinds of homologue pumps have been found in *Saccharomyces cerevisae*, *E. hirae*, *Synechococcus*, *Helicobacter pylori*, *Escherichia coli*, *Listeria monocytogenes*, *Caenorhabditis elegans*. In man, defects in the function or expression of copper-transporting P-type ATPases are responsible for Menkes and Wilson hereditary diseases (Nies, 1999; Rensing et al., 2000). The conservation of this kind of copper pumps along evolution may indicate that uptake, reduction or efflux of copper in actinomycetes could be also due to P-type ATPases. Further investigations may deal with the screening of genes coding for these proteins in actinomycetes.

Clarification of copper resistance mechanisms both, at physiological and genetic levels, in actinomycetes is important in many ways. The understanding of the genetic and physiological basis of copper resistance increases the ability to use these microorganisms in environmental applications such as bioremediation or biosensors as proposed before (Brown et al., 1998). On the other hand, full awareness of simple models of copper resistance in microorganisms provide a comprehensive mechanistic understanding of the components involved in Cu transport in higher organisms (Koch et al., 1997). As an example we can quote the *S. cerevisae* Cup1 metallothionein. It contains a single domain that has a structure resembling the ß-domain of mammalian metallothioneins, and has served as a useful model for understanding Cu (I) coordination in Cu detoxification and signaling proteins.

The results presented in this work were obtained when Minimal Medium was used as in vitro experiments. However, it could be possible to improve the Cu-remediation in situ situation experiments, because organic matter present in the natural environment could complex Cu and reduce its availability.

Future works must deal with the molecular nature of these widespread copper resistance mechanisms and with the elucidation of the copper uptake mechanism observed in these actinomycete strains.

Acknowledgements

The authors gratefully acknowledge the financial support of CIUNT and CONICET, Argentina and technical assistance of Mr. G. Borchia, Eng. R. Luque and Dr. A. Sales.

References

Abbas, A., Edwards, C., 1989. Effects of metals on a range of *Streptomyces* species. Appl. Environ. Microbiol. 55, 2030–2035.

Abbas, A., Edwards, C., 1990. Effects of metals on *Streptomyces coelicolor* growth and actinorhodin production. Appl. Environ. Microbiol. 56, 675–680.

Albarracín, V.H., Benito, J.M., Siñeriz Louis, M., Amoroso, M.J., Abate, C.M., 2004. Identification of copper resistant microorganisms by PCR. In Environmental Microbiology Methods and Protocols, Series "Methods in Biotechnology", vol 6: 243–248. Editors: J.F. T. Spencer y A. Ragout Spencer. Humana Press. Inc., New Jersey, USA.

Amoroso, M.J., Castro, G.R., Carlino, F.J., Romero, N.C., Hill, R.T., Oliver, G., 1998. Screening of heavy metal-tolerant actinomycetes isolated from the Salí River. J. Gen. Appl. Microbiol. 44, 129–132.

Benimeli, C.S., Amoroso, M.J., Chaile, A.P., Castro, G.R., 2003. Isolation of four aquatic streptomycetes strains capable of growth on organochlorine pesticides. Bioresourc. Technol. 89 (2), 133–138.

Boopathy, R., 2000. Factors limiting bioremediation technologies. Bioresourc. Technol. 74 (1), 63–67.

Brosius, J., Palmer, J.L., Kennedy, H.P., Noller, H.F., 1978. Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. Proc. Natl. Acad. Sci. USA 75, 4801–4805.

Brown, N.L., Lloyd, J.R., Jakeman, K., Hobman, J.L., Bontidean, I., Mattiasson, B., Csöregi, E., 1998. Heavy metal resistance genes and proteins in bacteria and their application. Biochem. Soc. Trans. 26, 662–664.

Erardi, F.X., Failla, M.L., Falkinham, J.O., 1987. Plasmid-encoded copper resistance and precipitation by *Mycobacterium scrofulaceum*. Appl. Environ. Microbiol. 53, 1951–1954.

Fairbrother, A., Bennet, R.S., Kapusta, L.A., 1999. Effects of Copper in Terrestrial Systems: A Literature Summary. International Copper Association Ltd., New York, USA.

Gadd, G.M., 2000. Bioremedial potential of microbial mechanisms of metal mobilization and immobilization. Curr. Opin. Biotechnol. 11, 271–279.

Georgopoulus, P.G., Roy, A., Opiekun, R.E., Yonone-Lioy, M.J., Lioy, P.J., 2002. Environmental Dynamics and Human Exposure to Copper: Environmental Dynamics and Human Exposure Issues, vol. 1. International Copper Association Ltd, New York, USA pp 109–128.

Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., Hopwood, D.A., 2000. Preparation and analysis of genomic and plasmid DNA. In: Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., Hopwood, D.A. (Eds.), Practical *Streptomyces* Genetics. The John Innes Foundation, Norwich, England, pp. 161–210.

Koch, K.A., O Peña, M.M., Thiele, D.J., 1997. Copper-binding motifs in catalysis, transport, detoxification and signaling. Chem. Biol. 4, 549–560.

Munson, G.P., Lam, D.L., Outten, F.W., O'Halloran, T.V., 2000. Identification of a copper-responsive two-component system on the chromosome of *Escherichia coli*. J. Bacteriol. 182, 5864–5871.

Nies, H.D., 1999. Microbial heavy-metal resistance. Appl. Microbiol. Biotechnol. 51, 730–750.

Odermatt, A., Suter, H., Krapf, R., Solioz, M., 1992. An ATPase operon involved in copper resistance by *Enterococcus hirae*. Ann. NY Acad. Sci. 671, 484–486.

Ravel, J., Amoroso, M.J., Colwell, R.R., Hill, R.T., 1998. Mercury resistant actinomycetes from Chesapeake Bay. FEMS Microbiol. Lett. 162, 177–184.

Rensing, C., Fan, B., Sharma, R., Mitra, B., Rosen, B.P., 2000. CopA: an *Escherichia coli* Cu(I) – translocating P-type ATPase. Proc. Natl. Acad. Sci. 97 (2), 652–656.

Romero, N.C., Amoroso, M.J., Tracanna de Albornoz, B., 1997. Estudio de la carga orgánica y bacteriana en el embalse de Río Hondo (Tucumán-Santiago del Estero, Argentina). Miscelánea 103, 3–10.

Standard Methods, APHA, 1992. Standard Methods for the Examination of Water and Wastewater, 18th ed. American Public Health Association, Washington, DC.

US Environmental Protection Agency, 1988.Technology Screening Guide for Treatment of CERCLA Soils and Sludges, Publ. EPA/540/5-90/005a, Washington, DC.