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Responses of environmental *Amycolatopsis* strains to copper stress

José Sebastián Dávila Costa [a], Virginia Helena Albarracín [a,b], Carlos Mauricio Abate [a,b,c,*]

[a] Pilot Plant of Industrial and Microbiological Processes (PROIMI), CONICET. Av. Belgrano y Pasaje Caseros, 4000 Tucumán, Argentina
[b] Natural Sciences College and Miguel Lillo Institute, National University of Tucumán, 4000 Tucumán, Argentina
[c] Biochemistry, Chemistry and Pharmacy College, National University of Tucumán, 4000 Tucumán, Argentina

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ABSTRACT

Copper is a redox-active metal, which acts as a catalyst in the formation of Reactive Oxygen Species (ROS) encouraging oxidative stress. Protection against oxidants is intrinsic to every living cell; however, in stress conditions, cells are forced to increase and expand their antioxidative network. In this work, the novel copper-resistant strain *Amycolatopsis tucumanensis* and the copper-sensitive *Amycolatopsis eurytherma* were grown under copper increasing concentrations in order to elucidate the dissimilar effects of the metal on the strains viability, mainly on morphology and antioxidant capacity. Although biosorbed copper encouraged ROS production in a dose-dependent manner in both strains, the increase in ROS production from the basal level to the stress conditions in *A. tucumanensis* is lesser than in the copper-sensitive strain; likewise, in presence of copper *A. eurytherma* suffered inexorable morphological alteration while *A. tucumanensis* was not affected. The levels of antioxidant enzymes and metallothioneins (MT) were all greater in *A. tucumanensis* than in *A. eurytherma*; in addition MT levels as well as superoxide dismutase and thioredoxin reductase activities in *A. tucumanensis,* were higher as higher the concentration of copper in the culture medium. This work has given evidence that an efficient antioxidant defense system might aid microorganisms to survive in copper-stress conditions; besides it constitutes the first report of oxidative stress study in the genus *Amycolatopsis* and contributes to enlarge the knowledge on the copper-resistance mechanisms of *A. tucumanensis*.

1. Introduction

Heavy metals pollution is widespread causing serious ecological problems in many parts of the world. Among heavy metals, copper, commonly generated from mining, smelting, electroplating and painting, cause extensive pollution of soils, water and air; producing further ecotoxicological effects on humans exposed to this contaminant (Georgopoulus et al., 2002). Thus, new technologies to clean up our environment need to be developed.

Conventional physicochemical treatments methods, which have been practiced for several decades for the removal of toxic heavy metals from wastewaters, may be ineffective or very expensive. These disadvantages become more pronounced at metal concentrations less than 100 mg l^{-1} (Kapoor and Viraraghavan, 1995). The potential of using bioaccumulation or biosorption, which have been used for the removal of heavy metal ions by microorganisms, has become an attractive subject over the past decade (Haferburg and Kothe, 2007).

It is the pragmatic goal of current bioprocess research on metal removal from treatable sources to identify species of microorganisms that are able of efficient uptake environmentally and economically important metals such as copper. Therefore, screening for microbes with high accumulation capacities and studying their stable resistance characteristics is an inevitable part of any remediation strategy (Malik, 2004).

Amycolatopsis tucumanensis, the strain of a recently recognized novel species of the genus *Amycolatopsis*, has been studied for its remarkable copper resistance as well as for its ability to bioremediate copper polluted soil microcosms (Albarracín et al., 2008, 2010a, 2010b). Moreover, *A. tucumanensis* has shown a noteworthy cupric reductase activity positively correlated with its copper resistance (Dávila Costa et al., 2011); in contrast, *Amycolatopsis eurytherma*, a termophilic strain, revealed low cupric reductase activity in concurrence with a high sensitivity to the metal (Kim et al., 2002; Dávila Costa et al., 2011).

Currently, we are gaining experimental evidences to support use of *A. tucumanensis* as a promising tool for performing bioremediation strategies (Albarracín et al., 2008, 2010a, 2010b; Dávila Costa et al., 2011), albeit its cupric-resistome has not been completely elucidated; in addition, it might be interesting to understand the main physiological characteristics, which made *A. eurytherma* an extremely copper-sensitive strain. This basic knowledge will be needed to develop efficient bioremediation strategies.

* Corresponding author at: Pilot Plant of Industrial and Microbiological Processes (PROIMI), CONICET, Av. Belgrano y Pasaje Caseros, 4000 Tucumán, Argentina. Fax: +54 381 4344887.
E-mail addresses: jsdavilacosta@gmail.com (J.S. Dávila Costa), viralbarracin@gmail.com (V.H. Albarracín), cabate@proimi.org.ar (C.M. Abate).

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Copper has known physiological functions in biological systems, nevertheless being a redox-active metal, copper can act as a catalyst in the formation of Reactive Oxygen Species (ROS). The production of these oxidants as well as the protection against them is intrinsic to every living cell; in stress conditions; however, normal capacities of these mechanisms are insufficient, triggering cells to increase and expand their antioxidative network (Georgopoulus et al., 2002). In most living organisms superoxide dismutases (SOD) and catalases (CAT) are main enzymes involved in antioxidant mechanisms (Cuypers et al., 2010); in addition, thioredoxin systems are essential for maintaining a cytoplasmatic reductive environment, appropriate DNA synthesis, and transcription regulation in cells (Attarian et al., 2009).

Copper resistance mechanisms widely revised in the literature normally include (1) efflux transporters, (2) intracellular copper-binding chaperones, or (3) unspecific chelators such as glutathione (Petersen and Moller, 2000; Rensing and Grass, 2003; Stoyanov et al., 2001). So far, an efficient copper-induced oxidative stress response was not studied as a possible resistance mechanism, and within actinobacteria some genera such as *Amycolatopsis* lacks of any information. Antioxidative network within actinobacteria was widely studied in *Mycobacterium* genus but mainly with the purpose of attacking its protection system, making these pathogenic cells more vulnerable (Newton et al., 2008; Ung and Av-Gay, 2006).

Accordingly with the former arguments, the goal of this study was to elucidate the dissimilar effects of the metal on viability of *A. tucumanensis* and *A. eurytherma*, copper-resistant and sensitive strains, respectively. ROS production, activity levels of antioxidant enzymes and morphological changes upon copper increasing exposure were evaluated. The results obtained in this manuscript helped to attain a more detailed picture of copper toxic effects and the induced-biological responses in both, resistant and sensitive strains.

2. Materials and methods

2.1. Strains and culture media

A. tucumanensis DSM 45259^T, a copper resistant strain, previously isolated from polluted sediments (Albarracín et al., 2010a) and the sensitive collection strain *A. eurytherma* DSM 44348^T were used in this study. All assays were carried out in Minimal Media broth (MM_b in g l^{-1}: L-asparagine, 0.5; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01 and glucose, 10.0; pH 7) or Minimal Media agar (MM_a; added with 15 mg l^{-1} agar; pH 7). When indicated, MM_a and MM_b were added with 10 (MM_{a10}; MM_{b10}) mg l^{-1}, 20 (MM_{a20}; MM_{b20}) mg l^{-1} or 30 (MM_{a30}; MM_{b30}) mg l^{-1} of Cu(II), using a stock solution of $CuSO_4 \cdot 5H_2O$ (0.25 M). Cultures without copper (MM_{a0}; MM_{b0}) were used as controls.

2.2. Growth conditions and copper specific biosorption

Time-course growth of both *A. tucumanensis* and *A. eurytherma* were performed in order to establish its exponential growth phase. One hundred microliters of spore suspensions (1×10^9 UFC ml^{-1}) of each strain, prepared as described before (Albarracín et al., 2005), were inoculated in batch cultures (50 ml) of MM_{b0}, MM_{b10}, MM_{b20} and MM_{b30}. The cultures were incubated at 30 °C in orbital shaker at 100 rpm and samples were collected after 48, 96 and 120 h of growth. The cells were centrifuged at 10000 rpm for 10 min and washed twice with Washing Buffer (25 mM Tris buffer pH 7, EDTA 2 mM). The resulting cell pellet was dried at 105 °C until a constant weight for biomass determination. Copper concentration in the free cell supernatant was determined by atomic absorption spectroscopy method (A-Analyst 400, Perkin Elmer). Copper specific biosorption (mg Cu g^{-1} of cells) was calculated as follows: $(X-Y)/Z$,

X: Initial copper concentration in MM_b (mg l^{-1}).
Y: Residual copper concentration in MM_b (mg l^{-1}).
Z: Dry weight of the culture pellet (g l^{-1}).

2.3. Preparation of extracts for ROS and enzymatic activity measurements

All centrifugation procedures were carried out at 4 °C. Exponential phase cells from *A. tucumanensis* and *A. eurytherma* grown in MM_{b0}, MM_{b10}, MM_{b20} and MM_{b30}, were harvested by centrifugation at 10,000 rpm for 10 min and washed twice with Washing Buffer. The pellet was suspended in 50 mM sodium phosphate buffer pH 7.8, containing 0.1 mM PMSF (phenylmethylsulphonylfluoride) as antiproteolytic agent and the cell suspensions were broken in a French press at 20,000 psi. The resultant cell disrupted suspension was centrifuged at 10,000 rpm for 15 min and the supernatant (cellular extract) was used for ROS and enzymatic activities determination. Proteins in the cellular extracts were detected by Bradford reagent.

2.4. ROS determination

ROS were determined as described previously (Henderson and Chappell, 1993) using Dihydrorhodamine (DHR) 123 as fluorescent reagent. DHR was used to a final concentration of 5 μM (λ_{ex}=505; λ_{em}=529 nm) and fluorescence from the own cellular extract was used as blank. Concentration of ROS was expressed as fluorescence intensity per mg of protein.

2.5. Scanning electron microscopy

A. tucumanensis and *A. eurytherma*, were grown in MM_{a0}, MM_{a10}, MM_{a20} and MM_{a30} for 4 days. Agar containing mycelium were hacked to small pieces (3–4 mm in width and 8–10 mm in length) from the culture medium and dissected. The pieces were fixed in 4.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 h at 4 °C and dehydrated in a graded acetone series (Albarracín et al., 2008). The critical drying point was obtained by exchanging the acetone through liquid CO_2. The samples were coated with gold and visualized under vacuum using a Zeiss Supra 55VP (Carl Zeiss NTS GmbH, Germany) scanning electron microscope.

2.6. SOD activity staining and quantification assay

Native PAGE of cellular extracts was performed using 10% polyacrylamide gels with 40 μg protein loaded per lane. Gel staining and quantification activity assays were performed as described by Beauchamp and Fridovich (1971) where the reduction of nitro blue tetrazolium (NBT) by $O_2^{\bullet -}$ has been utilized as the basis of assays for superoxide dismutase, which indicates its presence by inhibiting the reduction of NBT. One unit of enzyme activity (U) was defined as the amount of SOD required to inhibit the reduction of NBT by 50%.

2.7. CAT activity staining and quantification assay

Native PAGE of cellular extracts was carried out under the same conditions as SOD. The activity staining for catalase was performed incubating gels in presence of peroxidase, H_2O_2 and diaminobenzidine as described by Clare et al. (1984); catalase activity quantification was performed using a procedure described by Aebi (1983). One unit of enzyme activity (U) was defined as mM of H_2O_2 consumed per minute and the enzymatic activity was expressed as U mg^{-1} protein.

2.8. Thioredoxin reductase (TrxR) activity assay

Thioredoxin reductase activity was assayed using 5.5′-dithiobis (2-nitrobenzoic acid) (DTNB) as described by Stefanková et al. (2006). The increase in A_{412} was monitored and control reactions in which NADPH was omitted showed no significant increase in absorbance. One unit of enzyme activity (U) was defined as a variation of 0.01 in the absorbance per minute.

2.9. Dot-Blot for thioredoxin reductase (trxb) gen

Isolation of chromosomal DNA from *A. tucumanensis* and *Streptomyces coelicolor* DSM 40783 was carried out according to the lysozyme treatment modified for Actinobacteria as described previously (Albarracín et al., 2008). The 700-pb internal trxb fragment to be used as probe was amplified by PCR from *S. coelicolor* strain DNA using the primers Trxb-Forward (5′-GCT ACA CGG CGG CGC TCT ACA C-3′) and Trxb-Reverse (5′-CAG GTC GAG CTG GCC CTT GAA-3′). Hybridizations were carried out overnight at 55 and 60 °C using the Kit AlkPhos Direct Labeling and Detection System with CDP-Star (Amersham).

2.10. Determination of metallothioneins

Exponential phase cells from the strains grown in MM_{b0}, MM_{b10}, MM_{b20} and MM_{b30}, were harvested by centrifugation at 10,000 rpm for 10 min and washed twice with Washing Buffer. Metallothionein concentration in the cellular extracts was analyzed using a modified procedure, originally optimized for detection of metallothioneins in marine organisms (Viarengo et al., 1997). In this work, the procedure was adapted as follow: Cells were resuspended in three volumes of

Buffer A (0.5 M sucrose, 20 mM Tris–HCl buffer, pH 8.6, with added 0.1 mM PMSF and 0.01% β-mercaptoethanol as a reducing agent) and the cells suspension were broken in a French press at 20,000 psi. The resultant cell disrupted suspension was then centrifuged at 10,000 rpm for 15 min and cell pellet was dried at 105 °C until a constant weight for biomass determination. Aliquots of 1 ml of supernatant containing metallothioneins were then added with 1000 µl of cold (−20 °C) absolute ethanol and 80 µl of chloroform; the samples were then maintained at −20 °C for 1 h and centrifuged at 8000 rpm for 10 min at 0–4 °C. The metallothionein-containing pellet was then washed with 87% ethanol and 1% chloroform in Buffer A, centrifuged at 8000 rpm for 10 min and dried. The pellet was then resuspended in 200 µl 0.25 M NaCl and subsequently 200 µl 1 N HCl containing EDTA 4 mM were added to the sample. A volume of 4200 µl 2 M NaCl containing 1 mM DTNB (5.5-dithiobis-2-nitrobenzoic acid) buffered with 0.2 M Na-phosphate, pH 8 was then added to the sample at room temperature. The sample was finally centrifuged at 5000 rpm for 5 min; the supernatant absorbance was evaluated at 412 nm and metallothionein concentration was estimated utilizing reduced glutathione (GSH) as a reference standard (Viarengo et al., 1997). Metallothionein concentration was expressed as µg of Cysteine g^{-1} of cells (dry weight).

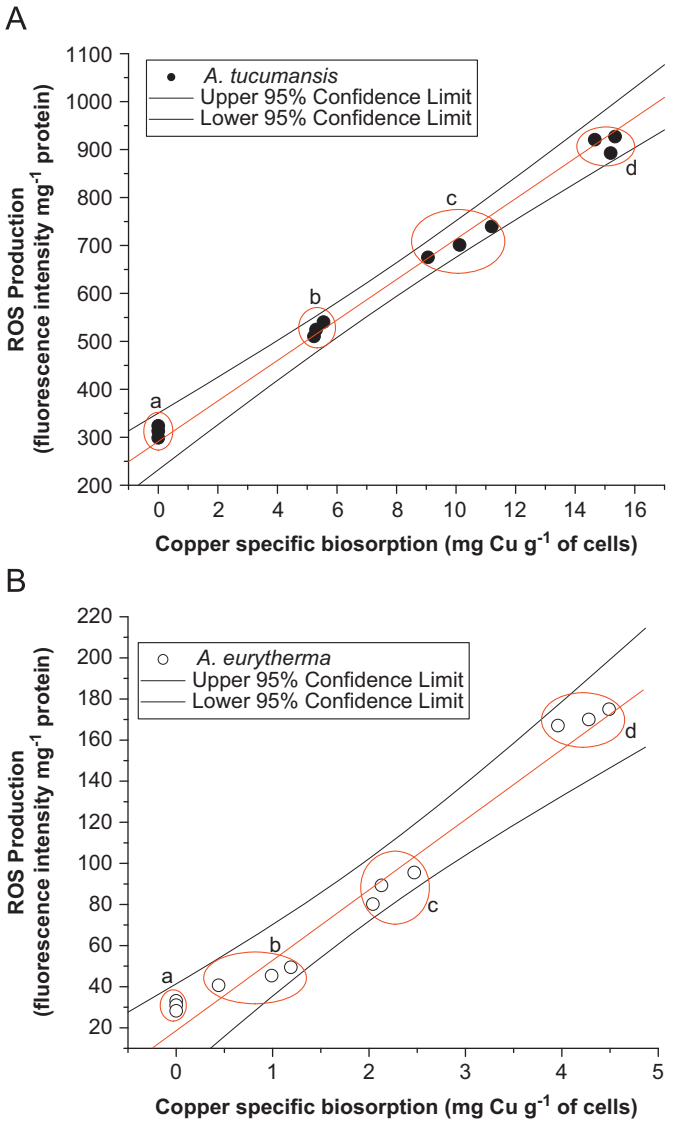

Fig. 1. Linear regression analysis of the ROS production and copper specific biosoption of cells grown until exponential phase in MM_{b0} (a), MM_{b10} (b), MM_{b20} (c) and MM_{b30} (d) ($n=3$, $P<0.01$): (A) *A. tucumanensis* and (B) *A. eurytherma*.

2.11. Statistical analyses

All tests were performed in triplicate. Statistical analyses were conducted using the Microcal Origin Working Model Version 6.0. Paired t-test and variance analysis were used with a probability level of $p<0.05$.

3. Results

3.1. Copper specific biosorption and intracellular ROS production

It is well known that intracellular copper triggers oxidative stress by producing ROS in most living organisms (Baker et al., 2010; Kovácik and Backor, 2008). In order to test this hypothesis, we have determined the pattern of increasing ROS production related to copper specific biosorption in cells from *A. tucumanensis* and *A. eurytherma* grown in MM_{b0}, MM_{b10}, MM_{b20} and MM_{b30}.

ROS levels in both strains detected under control conditions reflected the basal rate production due to aerobic metabolism (Wang et al., 2008), being this level higher in *A. tucumanensis* (ca. 300 fluorescence intensity mg^{-1} protein) (Fig. 1) than in the sensitive control.

ROS production in both strains increased linearly when increasing the copper specific biosorption (SB_{Cu}) as revealed by data regression analysis (Fig. 1); however, the SB_{Cu}/ROS production rate was different according to the strain. For *A. tucumanensis*, the SB_{Cu} was higher than in *A. eurytherma* for all conditions; in MM_{b10}, 6-fold; in MM_{b20}, 5-fold; and in MM_{b30}, 3-fold (Table 1). Nonetheless, upon the same stress conditions, ROS increment from the basal state was higher for *A. eurytherma* than for *A. tucumanensis*; e.g.: in *A. eurytherma* the basal production of ROS was 30 fluorescence intensity per mg of protein (mean value) while after being exposed to 30 mg/L of copper (and copper specific biopsortion of 4 mg Cu/g of cells) the amounts of ROS raised to 170 (mean value), this means ca. 6-fold increase (Table 1). In *A. tucumanensis* the increment is less pronounced; with a similar

Table 1
Copper specific biosorption (mg Cu g^{-1} of cells), dry weight ($g\,l^{-1}$) and ROS production (fluorescence intensity mg^{-1} of protein) of the cultured cells grown until exponential phase. The values shown correspond to the mean value ± SD of three replicated samples. ROS production/ Basal ROS production rate is indicated in brackets next to the absolute values of ROS production.

		MM_b initial copper concentration ($mg\,l^{-1}$)			
		0	**10**	**20**	**30**
Copper specific biosorption (mg Cu g^{-1} of cells)	*A. tucumanensis*	0	5.36 ± 0.16	10.12 ± 1.07	15.06 ± 0.35
	A. eurytherma	0	0.87* ± 0.22	2.21* ± 0.22	4.24* ± 0.26
Dry weight of cells ($g\,l^{-1}$)	*A. tucumanensis*	1.18 ± 0.10	1.08 ± 0.22	1.01 ± 0.13	0.90 ± 0.18
	A. eurytherma	1.21 ± 0.26	0.95 ± 0.32	0.65* ± 0.23	0.57* ± 0.45
ROS production (fluorescence intensity mg^{-1} of protein)	*A. tucumanensis*	311.80 ± 12.60 (1)	525.10 ± 14.96 (1.68)	705.24 ± 32.40 (2.26)	913.48 ± 18.29 (2.93)
	A. eurytherma	30.77 ± 2.45* (1)	45.18 ± 4.37* (1.47)	88.35 ± 7.72* (2.87)	170.67 ± 4.04* (5.55)

* Indicates significant difference of the corresponding values compared to *A. tucumanensis* at the $P<0.05$ level (one-way ANOVA).

amount of biosorbed copper (Table 1) the rise in ROS went from 311 to 525 fluorescence intensity per mg of protein (ca. 1.7-fold increase).

3.2. Morphological study in copper-challenged cultures

The morphological characteristics of hyphal development on copper amended media in *A. tucumanensis* and *A. eurytherma* were analyzed by scanning electron microscopy in order to correlate morphological damage with copper-induced stress previously documented by the drastic raise of ROS production (Fig. 1). With this purpose, both strains were cultured on MM_a with increasing concentrations of copper as described in Section 2.

When growing on MM_{a0}, both strains produced well-developed, branched substrate mycelium as well as a profuse aerial mycelium (Fig. 2A–D); both types of hyphae fragmented into squarish rod-shape elements. The aerial mycelium displayed spore-like structures (0.3×0.8–$1.2 - 1.5$ μm) with smooth surface in long

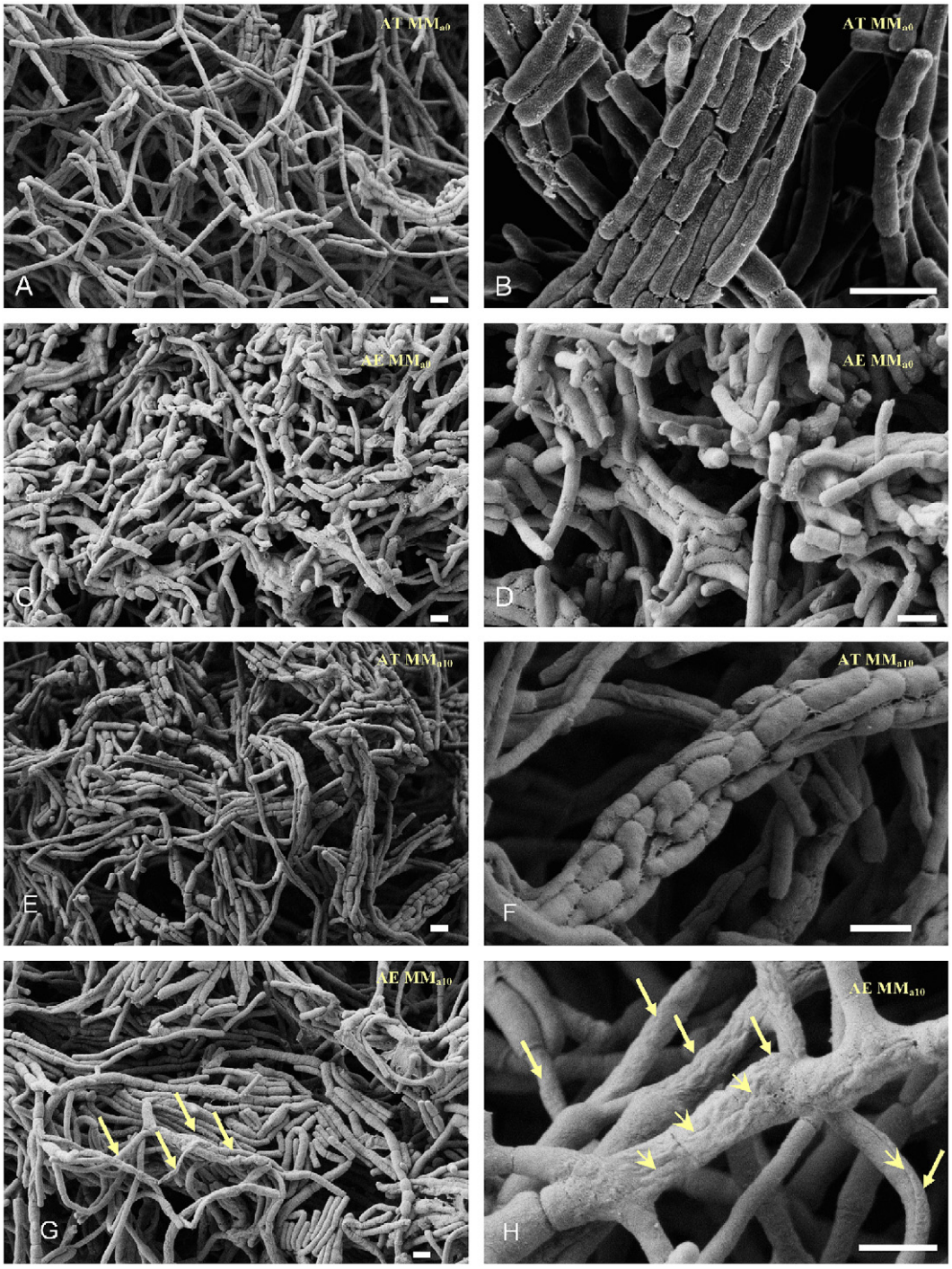

Fig. 2. Changes in morphology upon copper-increasing concentrations in the culture agar medium in *A. tucumanensis* (AT) and the sensitive control, *A. eurytherma* (AE). Scanning electron micrographs of strains grown on MM_{a0} (A–D), MM_{a10} (E–H), MM_{a20} (I–L) and MM_{a30} (M–P). Degenerating hyphae (G–H) of *A. eurytherma* are shown (long solid arrows). Degenerating hyphae initially display a number of discrete depressions along the wall (H; short arrows). As the process continues, the hyphae shrink and collapsed (O; long dashed arrows). Finally, dead hyphae appeared as irregular, tubular-deflated structures (P; double short arrows). Spore chains (L; circles) and aberrant morphologies (P, stars) were also visible. No degenerative hyphae were observed in *A. tucumanensis* at any copper concentrations. Bars: 1 μm.

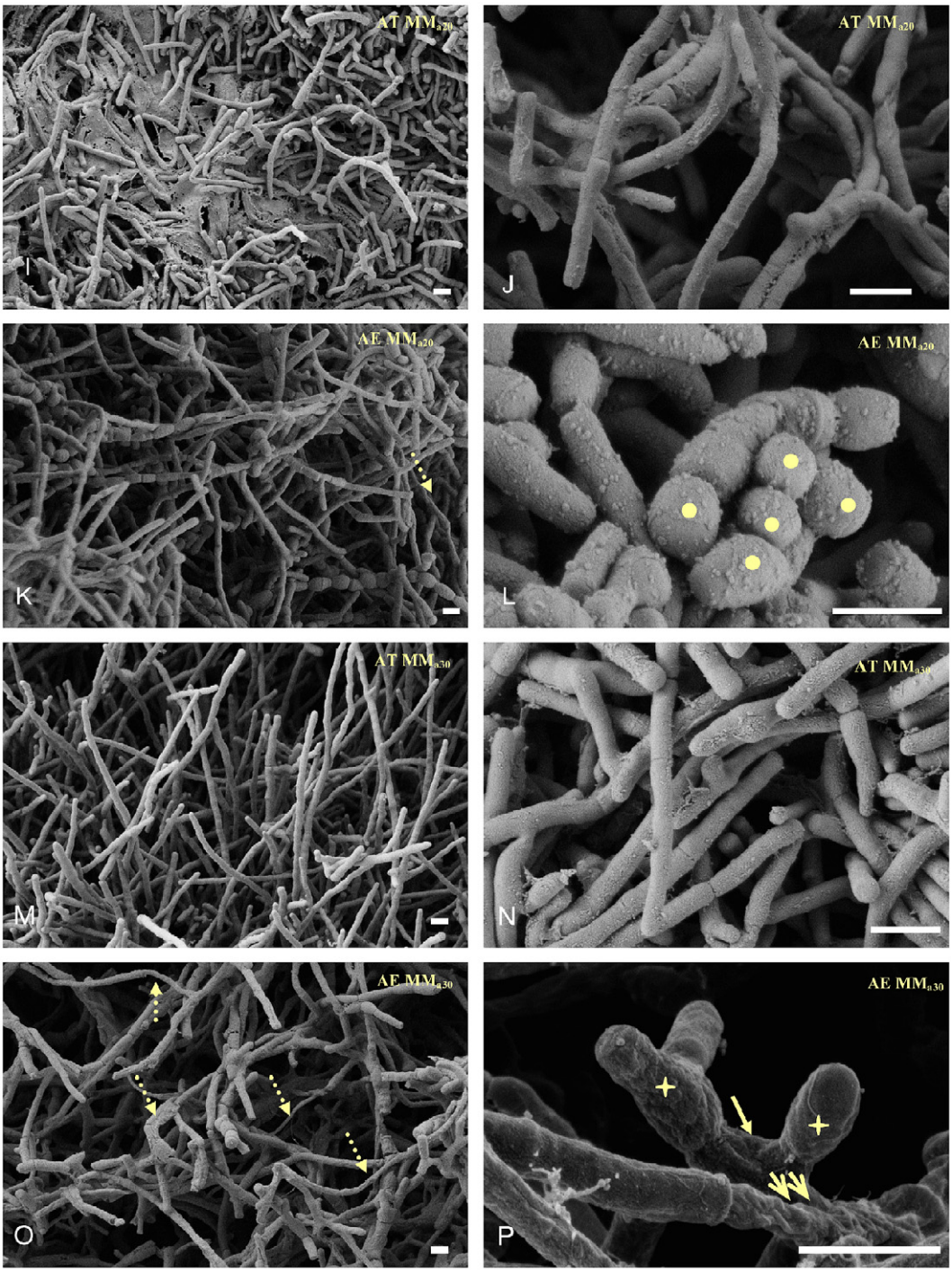

Fig. 2. (*continued*)

straight to flexuous chains (Fig. 2A and C). All of these properties are consistent with the morphological characteristics of the genus *Amycolatopsis* (Albarracín et al., 2010a).

On the contrary, when copper was added to MM_a the morphology of *A. tucumanensis* was slightly modified while *A. eurytherma* displayed a patent morphological alteration (Fig. 2G, H, K, L, O and P). Indeed, it was observed a wide occurrence of hyphae undergoing cellular degeneration and these hyphae could be distinguished by the aberrant shapes they displayed (Fig. 2H and P).

We could observe a gradual pattern of hyphal destruction upon increasing copper-concentrations in the culture medium (0–30 mg l^{-1}) in *A. eurytherma*, which was not detected in *A. tucumanensis*. With only 10 mg l^{-1} of copper added to the media, the aerial, as well as the substrate mycelium of the sensitive strain started to show clear symptoms of cellular degeneration such as discrete depressions along the wall together with hyphae shrinkage and collapse (Fig. 2G and H). At higher concentrations, it was observed an intricate network of substrate hyphae in different stages of cellular degeneration. In the aerial mycelium, however, a fraction of the hyphal population metamorphosed into chains of spores (Fig. 2L), while the remainder (nonsporulating hyphae) degenerated and died. The later appeared as irregular, tubular-deflated structures (Fig. 2P).

On MM_{a30}, *A. eurytherma* mycelium consisted mostly of dead hyphae and mature spores (Fig. 2O). Moreover, many hyphae displayed aberrant morphologies (Fig. 2P). In *A. tucumanensis*, there was no change on morphology along the copper-gradient (Fig. 2A, B, E, F, I and J).

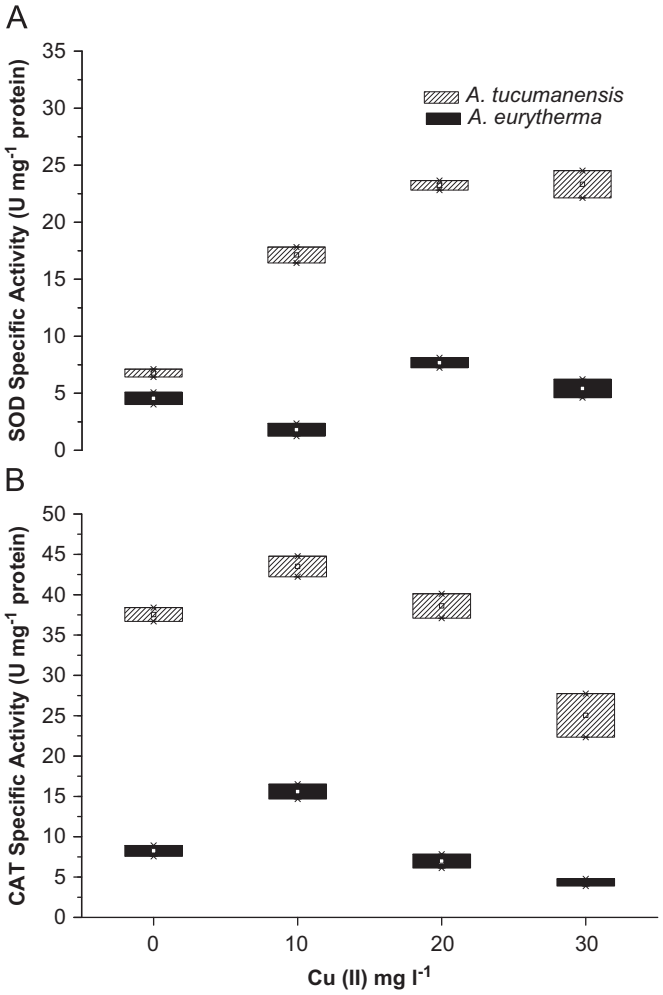

Fig. 3. Superoxide dismutase (A) and Catalase (B) activities in exponential phase cells (cellular extract) from *A. tucumanensis* and *A. eurytherma* grown in MM_{b0}, MM_{b10}, MM_{b20} and MM_{b30}. Box plot represent the median value with 100% of data falling within the box ($n=6$).

3.3. Effect of copper on the levels of SOD and CAT

Superoxide anion is formed by a single electron transfer to molecular oxygen and it is further reduced to hydrogen peroxide either chemically or by the action of superoxide dismutase; subsequently hydrogen peroxide is detoxified by catalase. Generally, the first line of antioxidant defense in the cells is comprised of both enzymes (Kim et al., 1996; Villegas et al., 2009). Taking this into account, we have determined the SOD and CAT activities as well as their electrophoresis profiles in cells from both strains grown in MM_{b0}, MM_{b10}, MM_{b20} and MM_{b30}.

SOD activity in *A. tucumanensis* increased together with the concentration of copper in a range from 0 to 20 mg l^{-1} of Cu(II) in MM_b; however, no significant differences between activities from cells grown in MM_{b20} and MM_{b30} were found (Fig. 3A). In addition, *A. tucumanensis* showed values of SOD activities in MM_{b10}, 15-fold; in MM_{b20}, 4-fold; and in MM_{b30}, 5-fold higher than *A. eurytherma* (Fig. 3A).

Regarding CAT activity in MM_{b10}, *A. tucumanensis* displayed ca. 1.3 times higher levels when compared to the control (MM_{b0}); besides this activity was ca. 2-fold more elevated than that one in *A. eurytherma* (Fig. 3B). CAT activity for both strains decreased in MM_{b20} and MM_{b30} (Fig. 3B).

SOD and CAT were also tested by activity staining. Whereas only a single band with SOD activity was seen in *A. tucumanensis* at normal culture conditions, in copper-challenged cultures this strain exhibited a second band with SOD activity (Fig. 4AI). On the contrary, a unique band with this activity was detected in *A. eurytherma* at all assayed conditions (Fig. 4AII). Electrophoresis profiles of CAT activity in *A. tucumanensis* and *A. eurytherma* showed two bands with this activity whether cells were treated with copper or not (Fig. 4BI and II).

3.4. Thioredoxin reductase activity

Thioredoxin system is formed by thioredoxin reductase (TrxR) and its substrate, the redox active protein thioredoxin (Trx) (Stefanková et al., 2006). Several reports have highlighted the significance of this system in the oxidative stress response, suggesting that loss of thioredoxin reductase results in a cellular damage (Ritz et al., 2000; Trotter and Grant, 2002). Hence, we have

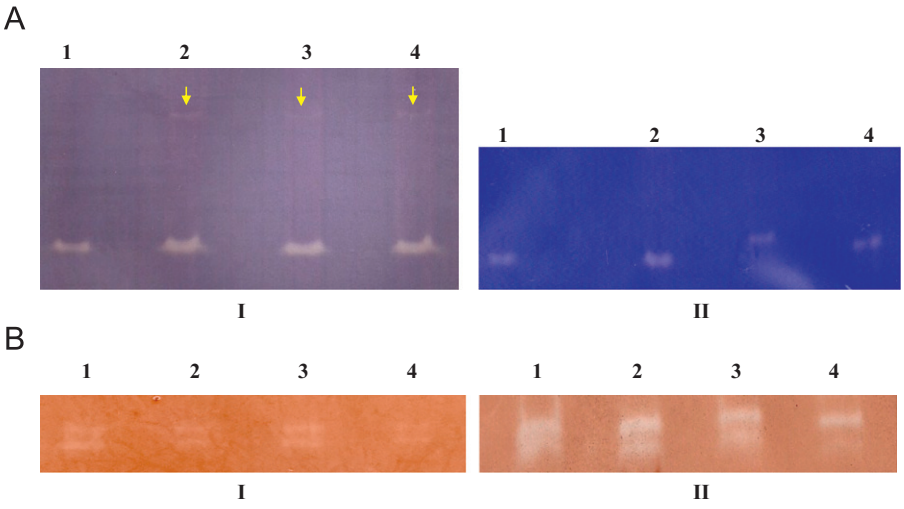

Fig. 4. Staining for Superoxide dismutase (A) and Catalase (B) activities of exponential phase cells from *A. tucumanensis* (AI, BI) and *A. eurytherma* (AII, BII) grown in MM_{b0} (lane 1), MM_{b10} (lane 2), MM_{b20} (lane 3) and MM_{b30} (lane 4).

tested thioredoxin reductase activity in cells grown at different copper concentrations.

The TrxR activity profiles obtained from *A. tucumanensis* were significantly higher (ca. twofold) than those showed for *A. eurytherma* at all assayed concentrations of copper (Fig. 5). Additionally, there was no significant difference between the values obtained in *A. tucumanensis* when comparing cells untreated or treated with 10 and 20 mg l^{-1} of Cu(II); however, TrxR activity in presence of 30 mg l^{-1} of Cu(II) increased 2.5-fold relative to the control without copper (Fig. 5). Furthermore, thioredoxin reductase (*trx*b) gene was confirmed in *A. tucumanensis* by Dot-Blot using a 700-bp PCR probe constructing from amplification of the *trx*b gene from *S. coelicolor*. The hybridization probes were positives at 55 as well as 60 °C (data not shown).

3.5. Metallothionein concentration

The name metallothionein (MT) is a generic term, applied to cysteine rich low-molecular-weight proteins or polypeptides whose functions have been linked to protection against high concentrations of heavy metals, metal homeostasis and oxidative stress (Palmiter, 1998; Robinson et al., 2001; Suhy et al., 1999). Considering this and in order to obtain more detailed information on the anti-oxidant mechanism, we have determined the concentration of metallothioneins (expressed as μg of Cys g^{-1} of cells) in untreated cells and treated with 10, 20 and 30 mg l^{-1} of Cu(II).

A. tucumanensis grown in MM_{b10}, MM_{b20} and MM_{b30} had significantly higher MT concentration than *A. tucumanensis* grown without copper; MT concentration from cultured cells in MM_{b30} was lower that the corresponding value obtained in MM_{b20}, although this depletion was not statistically significant (Fig. 6). In addition, levels of MT in untreated and copper-treated cells from *A. tucumanensis* were on average 3.5-fold more elevated compared with *A. eurytherma* (Fig. 6).

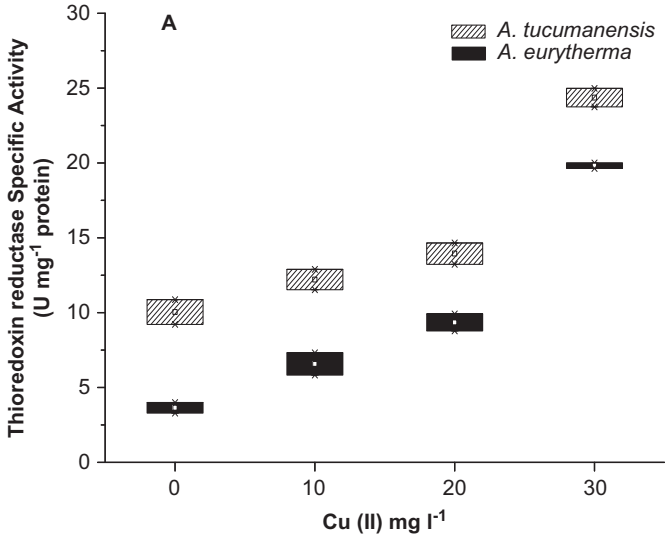

Fig. 5. Thioredoxin reductase activity in exponential phase cells (cellular extract) from *A. tucumanensis* and *A. eurytherma* grown in MM_{b0}, MM_{b10}, MM_{b20} and MM_{b30}. Box plot represent the median value with 100% of data falling within the box ($n=6$).

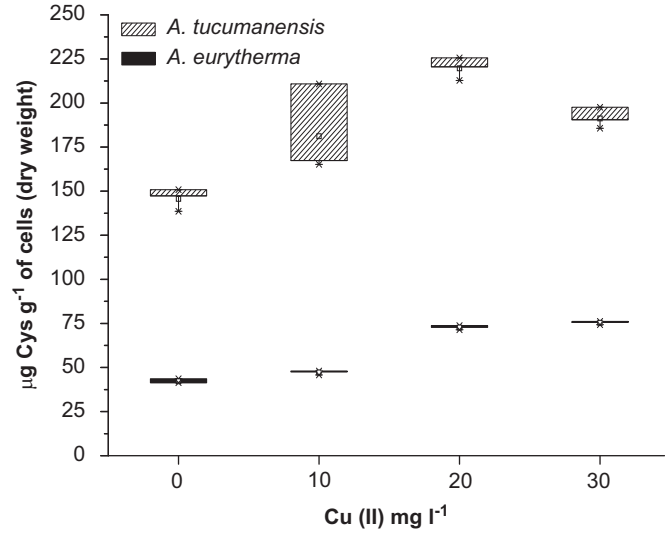

Fig. 6. Metallothionein concentration in exponential phase cells from *A. tucumanensis* and *A. eurytherma* grown in MM_{b0}, MM_{b10}, MM_{b20} and MM_{b30}. Box plot represent the median of three different assays carried out by duplicate.

4. Discussion

It is a well known fact that intracellular copper toxicity relies mostly on its participation in Fenton-type reactions, thereby generating toxic radicals (Magnani and Solioz, 2005); it was not surprising then to observe that in the tested strains the biosorbed copper induced ROS production although in a strictly linear dose-dependent manner (Fig. 1A and B). In agreement with this, Wang et al. (2008) observed in Cu(II)-treated cells from *Vibrio fischeri*, that ROS formation increased relative to untreated controls. Rico et al. (2009) analyzed ROS production in protozoa exposed to different heavy metals and observed that in cells treated with cytotoxic metal concentrations, ROS production was accordingly increased.

In turn, it was not expected to find an outstandingly high basal level of ROS production in *A. tucumanensis* (ca. 10-fold of that in *A. eurytherma*). On this fact, one should consider the dissimilar environmental origin from which these strains were isolated, while *A. eurytherma* was isolated from a non-polluted soil (Kim et al., 2002), our resistant strain was isolated from riverside sediments with 600 mg Cu(II) kg^{-1} (Albarracín et al., 2010a); normal Cu(II) concentration is up to 50 mg Cu(II) kg^{-1} (Georgopoulus et al., 2002). The high basal level of ROS production found in *A. tucumanensis* might be inherent to this strain in order to keep well-adapted antioxidant machinery, which was essential to survive in a copper-challenged environment; a similar behavior was observed in ciliated strains isolated from metallic polluted and non-polluted areas (Rico et al., 2009). This likely adaptive trait has been conserved by *A. tucumanensis*, doing this strain more appropriate to resist increasing copper concentration in the culture media.

It was noteworthy that ROS increment from the basal state to the maximal stress condition – 30 mg l^{-1} of Cu(II) – was higher for *A. eurytherma* (ca. 6-fold) than for *A. tucumanensis* (ca. 3-fold; Table 1). In fact, the drastic raise of ROS production in *A. eurytherma* triggered its inexorable morphological alteration (Fig. 2) whereas *A. tucumanensis* morphology was not appreciably affected. It did not show any change on morphology along the copper-gradient, thus, confirming its copper-resistance phenotype and suggesting an efficient oxidative stress response.

In view of the potential of copper to produce ROS (Fig. 1), it was important to establish whether the biosorbed copper in *A. tucumanensis* (Table 1) cause a stimulatory or detrimental effect on the enzymes involved in the detoxification process. Our results proved SOD activity in *A. tucumanensis* was highly stimulated by the presence of copper and this fact was highlighted against the sensitive strain (Fig. 3A). Previous reports also proved that this activity was increased upon stress conditions in microorganisms; a two-fold increased in SOD activity was induced by 0.5 mM of

Cu(II) in copper-resistant yeast (Villegas et al., 2009) whereas, genes of *sodF* and *sodN* were upregulated by acidic pH shock in *S. coelicolor* (Kim et al., 2008). SOD activity in cells from *Cryptococcus* sp. N6 was markedly stimulated by 10 mM of Cu(II), further study showed that this strain has two distinct SOD activity bands, one band appeared when the cells were grown without Cu(II), and the other band appeared when the cells were grown in presence of 10 mM of Cu(II) (Miura et al., 2002). Likewise, two bands with SOD activity were detected in *A. tucumanensis* in presence of Cu(II), in turn *A. eurytherma* showed a unique band. We could assume that the two SODs from *A. tucumanensis* might be FeSOD and MnSOD on the basis of the observation that bacteria generally contain those two types (Kim et al., 1996; Schmidt et al., 2007). This fact also gives evidence that *A. tucumanensis* is properly prepared to survive under extreme conditions compared with *A. eurytherma*. Further research is being conducted at the moment for revealing *A. tucumanensis* proteomic profiles with or without copper.

Although there is no record regarding catalases involved in oxidative stress in the genus *Amycolatopsis*; the actinobacteria, *S. coelicolor* produces three distinct catalases (CatA, CatB and CatC) to cope with oxidative and osmotic stresses. CatA is induced by H_2O_2 and it is the main catalase required for efficient growth of mycelium and resistance against H_2O_2. CatB is induced by osmotic stress or at the stationary phase, and is required for proper differentiation and osmoprotection of the cell; and CatC is expressed transiently at late exponential to early stationary phase. In our study, both strains showed two bands with catalase activity, it might suggest that these bands correspond to CatA and CatB, taking into account that our cultures were grown until mid-exponential phase (Hahn et al., 2000).

Peroxiredoxins as well as CAT are responsible for H_2O_2 detoxification and it has now been realized that peroxiredoxins may be the most important H_2O_2-removal systems in animals, bacteria and plants (Rhee et al., 2005). Peroxiredoxins are slow at catalyzing H_2O_2 removal, albeit the large amounts present in the cytosol and their low K_m for H_2O_2 (< 20 μM) can compensate for this. Peroxiredoxins are readily inactivated by H_2O_2, being the eukaryotic ones more susceptible to this than bacterial ones (Georgiou and Masip, 2003; Halliwell, 2006). Genes encoding OxyR (*oxyR*) and Peroxiredoxin system (*ahpC* and *ahpD*) have been isolated from *S. coelicolor* A3, the *ahpC* and *ahpD* genes constitute an operon transcribed divergently from the *oxyR* gene and overproduction of OxyR conferred resistance against H_2O_2 (Hahn et al., 2002). Catalase activity in *P. putida* and *B. megaterium* was inhibited at 20 and 40 mM of H_2O_2 (Bucková et al., 2010). In peroxiredoxins, H_2O_2 oxidizes an –SH group to a sulfenic acid (cys-SOH), this reacts with another –SH on the protein to give a disulphide that is then reduced by Trx. TrxR is the enzyme that keeps Trx in the reduced state for peroxiredoxins. Hence, TrxR activity should be in a close relationship with peroxiredoxin activity; indeed, in order to maintain the redox balance of the cells, enzymes and antioxidant molecules work all together in face of changing conditions (Halliwell, 2006). In *A. tucumanensis*, our results demonstrated a remarkable TrxR activity (Fig. 4) coupled with a significant depletion in the CAT activity (Fig. 3B), especially in MM_{b20} and MM_{b30}; this might suggest that *A. tucumanensis* is able to use peroxiredoxins rather than CAT to detoxify H_2O_2, i.e. CAT activity could be offset by peroxiredoxin activity at high concentrations of copper. However, this fact needs further clarification and current research is being conducted at the molecular level to reveal expression of genes from peroxiredoxins and catalases.

So far, only few metallothioneins have been detected in bacteria; however, Schmidt et al. (2010) predicted candidate metallothioneins and metallohistins in actinobacteria by way of an *in silico* approach, suggesting that these molecules also play a key role in these bacteria. The elevated levels of MT detected in *A. tucumanensis* (Fig. 5) are in accordance with the previous observation of copper-binding proteins present in the citosol fraction of this strain cell material (Albarracín et al., 2008). Gold et al. (2008) identified a new MT from a Gram-positive bacterium to which a function of binding Cu(I) and protecting the cell from copper toxicity was assigned; in work reported elsewhere *A. tucumanensis* showed a high cupric reductase activity (Dávila Costa et al., 2011), suggesting that probably the MT from *A. tucumanensis* might bind copper after its reduction.

5. Conclusion

In summary, our results clearly demonstrated that biosorbed copper induced ROS production in a dose-dependent manner in both strains; nonetheless, it was noteworthy that the increase in ROS production from the basal level to the stress conditions in *A. tucumanensis* is lesser than in the copper-sensitive strain. In fact, the drastic raise of ROS production in *A. eurytherma* triggered its inexorable morphological alteration whereas *A. tucumanensis* morphology was not appreciably affected. In addition, detected levels of antioxidants enzymes and metallothioneins in *A. tucumanensis* were significantly high relative to *A. eurytherma*. We also proved that MT levels as well as SOD and TrxR activities in *A. tucumanensis*, increased together with the rise of copper in the MM_b, suggesting that this gradual increase are in accordance with the level of copper-induced oxidative stress. Additionally, although the same pattern of activity was not observed for CAT, the elevated TrxR activity detected in *A. tucumanensis*, might support the hypothesis that in presence of high concentrations of copper it is able to use peroxiredoxin systems rather than CAT to detoxify H_2O_2; however, this will need to be further clarified by another approaches.

This work has given evidence that an efficient antioxidant defense system might aid micoorganisms to survive in copper-stress conditions. Besides this work constitutes the first report of oxidative stress study in the genus *Amycolatopsis* and contributes to enlarge the knowledge on the copper-resistance mechanisms of *A. tucumanensis*. Further work in this direction will enable us to obtain new tools to modify genetically this strain to be used in large-scale soil bioremediation strategies.

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References

Aebi, H.E., 1983. Catalase. 3rd edition In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis, 3. Verlag Chemie, Weinheim, pp. 273–286.

Albarracín, V.H., Alonso-Vega, P., Trujillo, M.E., Amoroso, M.J., Abate, C.M., 2010a. *Amycolatopsis tucumanensis* sp. nov., a novel copper resistant actinobacterium isolated from polluted sediments. Int. J. Syst. Evol. Microbiol. 60, 397–401.

Albarracín, V.H., Amoroso, M.J., Abate, C.M., 2005. Isolation and characterization of indigenous copper resistant actinomycete strains. Chem. Erde-Geochem. 65, 145–156.

Albarracín, V.H., Amoroso, M.J., Abate, C.M., 2010b. Bioaugmentation of copper polluted soil by *Amycolatopsis tucumanensis* to disminish phytoavailable copper for *Zea mays* plants. Chemosphere 79, 131–137.

Albarracín, V.H., Winik, B., Kothe, E., Amoroso, M.J., Abate, C.M., 2008. Copper bioaccumulation by the actinobacterium *Amycolatopsis* sp. AB0. J. Basic Microbiol. 48, 323–330.

Attarian, R., Bennie, C., Bach, H., Av-Gay, Y., 2009. Glutathione disulfide and S-nitrosoglutathione detoxification by *Mycobacterium tuberculosis* thioredoxin system. FEBS Lett. 583, 3215–3220.

Baker, J., Sitthisak, S., Sengupta, M., Johnson, M., Jayaswal, R.K., Morrissey, J.A., 2010. Copper stress induces a global stress response in *Staphylococcus aureus* and Represses *sae* and *agr* expression and biofilm formation. Appl. Environ. Microbiol. 76, 150–160.

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44, 276–287.

Bucková, M., Godocíková, J., Zámocký, M., Polek, B., 2010. Screening of bacterial isolates from polluted soils exhibiting catalase and peroxidase activity and diversity of their responses to oxidative stress. Curr. Microbiol. 61, 241–247.

Clare, A., Duong, M.N., Darr, D., Archibald, F., Fridovich, I., 1984. Effects of molecular oxygen on detection of superoxide radical with nitroblue tetrazolium and on activity stains for catalase. Anal. Biochem. 140, 532–537.

Cuypers, A., Plusquin, M., Remans, T., Jozefczak, M., Keunen, E., Gielen, H., Opdenakker, K., Ravindran Nair, A., Munters, E., Artois, T.J., Nawrot, T., Vangronsveld, J., Smeets, K., 2010. Cadmium stress: an oxidative challenge. Biometals 23, 927–940.

Dávila Costa, J.S., Albarracín, V.H., Abate, C.M., 2011. Cupric Reductase Activity in Copper-Resistant *Amycolatopsis tucumanensis*. Water Air Soil Poll 216, 527–535.

Georgiou, G., Masip, L., 2003. Biochemistry: an overoxidation journey with a return ticket. Science 300, 592–594.

Georgopoulus, P.G., Roy, A., Opiekun, R.E., Yonone-Lioy, M.J., Lioy, P.J., 2002. Environmental dynamics and human exposure to copper. In: Georgopoulus, P.G., Roy, A., Opiekun, R.E., Yonone-Lioy, M.J., Lioy, P.J. (Eds.), Environmental Dynamics and Human Exposure Issues. International Copper Association.

Gold, B., Deng, H., Bryk, R., Vargas, D., Eliezer, D., Roberts, J., Jiang, X., Nathan, C., 2008. Identification of a copper-binding metallothionein in pathogenic mycobacteria. Nat. Chem. Biol. 4, 609–616.

Haferburg, G., Kothe, E., 2007. Microbes and metals: interactions in the environment. J. Basic Microbiol. 47, 453–467.

Hahn, J.S., Oh, S.Y., Chater, K.F., Cho, Y.H., Roe, J.H., 2000. H_2O_2-sensitive fur-like repressor CatR regulating the major catalase gene in *Streptomyces coelicolor*. J. Biol. Chem. 49, 38254–38260.

Hahn, J.S., Oh, S.Y., Roe, J.H., 2002. Role of OxyR as a peroxide-sensing positive regulator in *Streptomyces coelicolor* A3(2). J. Bacteriol. 184, 5214–5222.

Halliwell, B., 2006. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol 141, 312–322.

Henderson, L.M., Chappell, J.B., 1993. Dihydrorhodamine 123: a fluorescent probe for superoxide generation? Eur. J. Biochem. 3, 973–980.

Kapoor, A., Viraraghavan, T., 1995. Fungal biosorption: an alternative treatment option for heavy metal bearing wastewaters. A review. Bioresour. Technol 53, 195–206.

Kim, B., Sahin, N., Tan, G.Y., Zakrzewska-Czerwinska, J., Goodfellow, M., 2002. *Amycolatopsis eurytherma* sp. nov., a thermophilic actinomycete isolated from soil. Int J Syst Evol Microbiol. 52, 889–894.

Kim, E.J., Kim, H.P., Hah, Y.C., Roe, J.H., 1996. Differential expression of superoxide dismutases containing Ni and Fe/Zn in *Streptomyces coelicolor*. Eur. J. Biochem. 241, 178–185.

Kim, Y.J., Moon, M.E., Song, J.Y., Smith, C.P., Hong, S.K., Chang, Y.K., 2008. Acidic pH shock induces the expressions of a wide range of stress-response genes. BMC Genomics 9, 604.

Kovácik, J., Backor, M., 2008. Oxidative status of *Matricaria chamomilla* plants related to cadmium and copper uptake. Ecotoxicology 17, 471–479.

Magnani, D., Solioz, M., 2005. Copper chaperone cycling and degradation in the regulation of the cop operon of Enterococcus hirae. BioMetals 18, 407–412.

Malik, A., 2004. Metal bioremediation through growing cells. Environ. Int. 30, 261–278.

Miura, T., Abe, F., Inoue, A., Usami, R., Horikoshi, K., 2002. Superoxide dismutase is involved in high tolerance to copper in the deep-sea yeast, *Cryptococcus* sp N6. Biotechnol. Lett. 24, 1069–1074.

Newton, G.L., Buchmeier, N., Fahey, R.C., 2008. Biosynthesis and functions of mycothiol, the unique protective thiol of *Actinobacteria*. Microbiol. Mol. Biol. Rev. 72, 471–494.

Palmiter, R.D., 1998. The elusive function of metallothioneins. Proc. Nat. Acad. Sci. USA 95, 8428–8430.

Petersen, C., Moller, L., 2000. Control of copper homeostasis in *Escherichia coli* by a P-type ATPase, CopA, and a MerR-like transcriptional activator, CopR. Gene 261, 289–298.

Rensing, C., Grass, G., 2003. *Escherichia coli* mechanisms of copper homeostasis in a changing environment. FEMS Microbiol. Rev. 27, 197–213.

Rhee, S.G., Chae, H.Z., Kim, K., 2005. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. Free Radical Biol. Med. 38, 1543–1552.

Rico, D., Martín-González, A., Díaz, S., De Lucas, P., Gutierrez, J.C., 2009. Heavy metals generate reactive oxygen species in terrestrial and aquatic ciliated protozoa. Comp. Biochem. Physiol. 149, 90–96.

Ritz, D., Patel, H., Doan, B., Zheng, M., Aslund, F., Storz, G., Beckwith, J., 2000. Thioredoxin 2 is involved in the oxidative stress response in *Escherichia coli*. J. Biol. Chem. 275, 2505–2512.

Robinson, N., Whitehall, S., Cavet, J., 2001. Microbial metallothioneins. Adv. Microb. Physiol. 44, 183–213.

Schmidt, A., Schmidt, A., Haferburg, G., Kothe, E., 2007. Superoxide dismutases of heavy metal resistant *streptomycetes*. J. Basic Microbiol 47, 56–62.

Schmidt, A., Hagen, M., Schütze, E., Schmidt, A., Kothe, E., 2010. In silico prediction of potential metallothioneins and metallohistins in actinobacteria. J. Basic Microbiol. 50, 562–569.

Stefanková, P., Perečko, D., Barák, I., Kollárová, M., 2006. The thioredoxin system from *Streptomyces coelicolor*. J. Basic Microbiol. 46, 47–55.

Stoyanov, J., Hobman, J., Brown, N., 2001. CueR (YbbI) of *Escherichia coli* is a MerR family regulator controlling expression of the copper exporter CopA. Mol. Microbiol. 39, 502–512.

Suhy, D.A., Simon, K.D., Linzer, D.I., O′Halloran, T.V., 1999. Metallothionein is part of a zinc-scavenging mechanism for cell survival under conditions of extreme zinc deprivation. J. Biol. Chem. 274, 9183–9192.

Trotter, E.W., Grant, C.M., 2002. Thioredoxins are required for protection against a reductive stress in the yeast *Saccharomyces cerevisiae*. Mol. Microbiol. 46, 869–878.

Ung, K.S.E., Av-Gay, Y., 2006. Mycothiol-dependent mycobacterial response to oxidative stress. FEBS Letters. 580, 2712–2716.

Viarengo, A., Ponzano, E., Donderob, F., Fabbrih, R., 1997. A Simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to mediterranean and antarctic molluscs. Mar. Environ. Res. 44, 69–84.

Villegas, L.B., Amoroso, M.J., Figueroa, L.I., 2009. Responses of *Candida fukuyamaensis* RCL-3 and *Rhodotorula mucilaginosa* RCL-11 to copper stress. J. Basic Microbiol. 49, 395–403.

Wang, W., Lampi, M.A., Huang, X.D., Gerhardt, K., Dixon, D.G., Greenberg, B.M., 2008. Assessment of mixture toxicity of copper, cadmium, and phenanthrenequinone to the marine bacterium *Vibrio fischeri*. Environ. Toxicol. 24, 166–177.