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Unraveling the *Amycolatopsis tucumanensis* copper-resistome

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Abstract Heavy metal pollution is widespread causing serious ecological problems in many parts of the world; especially in developing countries where a budget for remediation technology is not affordable. Therefore, screening for microbes with high accumulation capacities and studying their stable resistance characteristics is advisable to define cost-effective any remediation strategies. Herein, the copper-resistome of the novel copper-resistant strain *Amycolatopsis tucumanensis* was studied using several approaches. Two dimensional gel electrophoresis revealed that proteins of the central metabolism, energy production, transcriptional regulators, two-component system, antioxidants and protective metabolites increased their abundance upon copper-stress conditions. Transcriptome analysis revealed that in presence of copper, superoxide dismutase, alkyl hydroperoxide reductase and mycothiol reductase genes were markedly induced in expression. The oxidative damage of protein and lipid from *A. tucumanensis* was negligible compared with that observed in the copper-sensitive strain *Amycolatopsis eurytherma*. Thus, we provide evidence that *A. tucumamensis* shows a high adaptation towards copper, the sum of which is proposed as the copper-resistome. This adaptation allows the strain to accumulate copper and survive this stress; besides, it constitutes the first report in which the copper-resistome of a strain of the genus *Amycolatopsis* with bioremediation potential has been evaluated.

Keywords *Amycolatopsis* · Copper-resistome · Oxidative stress

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Introduction

The quality of life on Earth is linked to the quality of the environment, which in turn depends on human activities. Nowadays, heavy metal pollution is among the most significant environmental problems; metals as a resource are increasingly scarce, albeit they also threaten human health and ecosystem. Among heavy metals of concern are included Hg, Cr, Pb, Cu, Cd, As, Co; precious metals (Pd, Pt, Ag, Au, Ru) and radionuclides (U, Th, Ra, Am). Some heavy metal ions are essential trace elements, however, essential or not, metals are toxic at high concentrations (Wang and Chen 2009).

In the case of copper, because of its ability to cycle between $Cu^{2+}$and Cu^{+} at biologically relevant redox

potentials, it has become a cofactor for over 30 known enzymes; nevertheless, copper can act as a catalyst in the formation of Reactive Oxygen Species (ROS) causing serious damage of cellular components. To have some physiological or toxic effect, copper does not need to enter the cell (Hong et al. 2012), however, once inside; usually, cells solve this problem by using different resistance mechanisms, e.g.: efflux transporters, intracellular metal-binding chaperones and unspecific chelators such as glutathione (Stoyanov et al. 2001; Rensing and Grass 2003). Recently, it has been demonstrated that an efficient antioxidant mechanism is also imperative to cope with the toxic effects of copper (Dávila Costa et al. 2011b).

Over the past years, environmental biotechnology has gained more importance due to copper pollution occurring all over the world. Understanding the metabolism of heavy metals, including copper, and to use the metabolic functions of microorganisms in bioremediation, is nowadays the pragmatic goal for metal removal from treatable sources (Vidali 2001; Haferburg et al. 2009). Potentially, bacteria for bioremediation processes may be isolated from almost any environmental conditions. Bacteria will be adapted to almost any environmental condition like desert conditions, subzero temperatures or even extreme heat; because of their adaptability, bacteria and other biological systems may be used to bioremediate polluted environments (Benimeli et al. 2007; Polti et al. 2007).

Amycolatopsis tucumanensis DSM 45259 has been isolated from a copper-polluted area in the province of Tucumán, Argentina. This strain was widely studied for its remarkable copper-resistance as well as for its ability to bioremediate copper polluted soil microcosms (Albarracín et al. 2008, 2010a, b). A previous report has given evidence that the cupric reductase activity, detected in *A. tucumanensis*, is positively correlated with its copper resistance. Additionally, an efficient antioxidant mechanism given by high levels of antioxidant enzymes, as well as high contents of metallothioneins was evidenced in this strain (Dávila Costa et al. 2011a, b). Albeit already included into screening procedures to support the hypothesis that *A. tucumanensis* is a promising tool for performing bioremediation strategies, its copper-resistome has not been elucidated.

In line with these statements and highlighting the widespread heavy metal pollution, it is imperative to strengthen the knowledge on the basic principles at work in microorganism species that are able to efficiently clean up the environment. The goal of this study was to elucidate the copper-resistome of *A. tucumanensis*; the results obtained in this manuscript have helped to attain a more detailed picture of how this resistance is achieved and how the bacterium manages the toxic effects of copper.

Materials and methods

Strains, culture media and growth conditions

Amycolatopsis tucumanensis DSM 45259^T a copper resistant strain, and the copper-sensitive collection strain *Amycolatopsis eurytherma* DSM 44348^T were used in this study (Dávila Costa et al. 2011a, b). All assays were carried out in minimal media broth (MM_b in g/L: 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). When indicated, 10 (MM_{b10}), 20 (MM_{b20}) or 30 (MM_{b30}) mg/L of Cu^{2+}, using a stock solution of $CuSO_4 \cdot 5H_2O$ (0.25 M), were added. Cultures without copper (MM_{b0}) were used as controls. One hundred microliters of spore suspensions (1×10^9 CFU/mL) of each strain, prepared as described before (Albarracín et al. 2008), were inoculated in batch cultures (200 ml) of MM_{b0}, MM_{b10}, MM_{b20} or MM_{b30}. The cultures were incubated at 30 °C in orbital shaker and samples were harvested in the exponential growth phase. The cells were centrifuged at $8{,}000 \times g$ for 10 min and washed twice with washing buffer (25 mM Tris buffer pH 7, EDTA 2 mM).

2-DE and in silico analysis of 2-D protein maps

For protein extraction, frozen biomass samples were crashed with liquid nitrogen. After acetone-trichloroacetic acid 50 % precipitation at −20 °C, proteins were suspended in IEF buffer: 8 M urea, 4 % (w/v) CHAPS and 1 % (w/v) 1,4-dithiothreitol and stored at −80 °C until use.

For the first dimension, pH 3–10 and 4–7, 18-cm IPG strips were rehydrated overnight in 340 mL of DeStreak rehydration solution (GE Healthcare, USA), containing 340 μg of sample protein, 0.5 % (w/v) IPG buffer (GE Healthcare) and 1 % (w/v) DTT. Ettan IPG III system (GE Healthcare) was used for protein separation performing: 300 V, 30 min (step); 500 V, 1 h (step);

1,000 V, 1 h (gradient); 3,000 V, 1 h (gradient); 10,000 V, 2 h 30 min (gradient); 10,000 V, 1 h (step). All the steps were performed at 20 °C using 75 μA *per* strip. After IEF, the IGP strips were saturated with an equilibration buffer containing 6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS, 0.05 M Tris–HCl pH 6.8 and 2 % (w/v) DTT for 15 min. Then, thiol groups were blocked by substituting DTT with 2.5 % (w/v) iodoacetamide in the equilibration buffer. The focused proteins were then separated on 12 % polyacrylamide gels (SDS-PAGE) at 10 °C using a maximum setting of 40 μA *per* gel and 120 V; gels were stained using colloidal Coomassie Brilliant Blue G-250.

Image analysis was performed using ImageMaster 2-D platinum version 6.0 (GE Healthcare) according to the manufacturer′s instructions. Spot detection was accomplished automatically using the auto-detection device and verified manually; spot quantification was calculated as the intensity of the spot. To correct differences in gel staining, spot intensities were normalized (%Int) to the sum of the intensity of all spot detected on each gel by the software. Gels were matched using reproducible landmarks. The differential analysis was carried out on the biomasses collected from two parallel cultures, and for each biomass three technical gel replicates were performed for each pH range investigated.

MS analysis and protein identification

Spot from 2-DE were excised from the gel and sent to the Center of Biological and Chemical Studies by Mass Spectroscopy MALDI-TOF (CEQUIBIEM-Buenos Aires-Argentina), for further identification. MASCOT software version 2.2 (Matrix Science, UK) was used to identify spots by peptide mass fingerprint (PMF) or MS/MS Ion Search, searching a NCBI non-redundant database. Candidates with a statistically significant score, which excluded ($\rho < 0.05$) that the protein identified was resulting from a random match, were further evaluated by comparison with *M*r and p*I* experimental values obtained from 2-DE gels.

DNA purification, primer design and PCR amplification of superoxide dismutase (*sod*), mycothiol reductase (*mtr*) and alkyl hydroperoxide reductase (*ahpC*) genes

For DNA preparation, *A. tucumanensis* was grown in MM_{b0}. Total genomic DNA extraction was performed according to the lysozyme treatment, modified for actinobacteria as described previously (Albarracín et al. 2008). Conserved domains of *sod*, *mtr* and *ahpc* were indentified using the NCBI database and MEGA 5 program. Primers were designed using the *Amycolatopsis mediterranei* codon usage table from the codon usage database available on line (www.kazusa.or.jp/codon) (Table 1). Amplifications were performed in 25 μl reaction volumes using an automated thermal cycler (Perkin–Elmer, model 9700, Applied Biosystems). PCR products were visualized using an Image Analyzer Gel Doc BIORAD. The nucleotide sequences reported in this paper have been deposited in Genbank under accession numbers JF957853 (*mtr*), JF957854 (*ahpC*) and JF937111 (*sod*).

RNA isolation, RT-PCR and qRT-PCR

For isolation of total RNA, *A. tucumanensis* was grown in MM_{b0}, MM_{b10} and MM_{b30} until exponential phase (96 h, maximum uptake of copper) (Albarracín et al. 2008). RNA was isolated using the UltraClean Microbial RNA Isolation Kit (Mo Bio Laboratories) according to the manufacturer's protocol. Three independent RNA samples were extracted from liquid cultures for RT-PCR analyses. RNA extracted from MM_{b0}, MM_{b10} and MM_{b30} (2 μg) was reverse transcribed using a GoScript™ Reverse Transcriptase (Promega, USA) according to the manufacturer's instructions for random hexamer-primed reactions. qRT-PCR was accomplished using primer pairs amplifying intragenic regions of *mtr*, *ahpC* and *sod* (Table 1). One microliter of the synthesized cDNA was used as a template in a 20 μl volume of the GoTaq qPCR Master Mix (Promega, USA) containing 0.5 μM of each primer. The reaction conditions were as follows: one cycle at 95 °C for 1 min, followed by 40 cycles of 10 s at 95 °C, 60 s at 59 °C and 32 s at 72 °C. All reactions were performed in triplicate. The *16S* gene encoding ribosomal RNA was used as an internal control to quantify the relative expression of the *mtr*, *ahpC* and *sod* genes and water as a negative control (Livak and Schmittgen 2001).

Protein carbonyl

Protein carbonyls were measured in cells from *A. tucumanensis* and *A. eurytherma* (copper-sensitive control) grown in MM_{b0}, MM_{b10}, MM_{b20} and MM_{b30}

Table 1 Primer used in this study

Primer name	Sequence	Description	Product Size (pb)
mtr-1F	ATCCCCACGAAGATGTTCGT	Used to amplify *mtr*	1,034
mtr-1R	CCTGGATGAGCGGCTGGATCA	Used to amplify *mtr*	1,034
mtr-F	GTACGAGCTGAAGCACGTCG	Used to quantify *mtr* transcripts (JF957853)	167
mtr-R	CGATGCCCGCATAATCCTGT	Used to quantify *mtr* transcripts (JF957853)	167
sod-F	AGATCAACGAGCTGCACCACA	Used to amplify *sod*	434
sod-R	GTTCTTGTAGTCCAGGTAGAA	Used to amplify *sod*	434
sod-1F	GTTCGGCTCGTTCGACAAGT	Used to quantify *sod* transcripts (JF937111)	201
sod-1R	TCCAGGTAGAAGGCGTGCTC	Used to quantify *sod* transcripts (JF937111)	201
ahpC-F	TTCACGCTCAACGACTACAAC	Used to amplify *ahpC*	210
ahpC-R	GCCGGACGTGTCGATCAAGAA	Used to amplify *ahpC*	210
ahpC-1F	CTGGTCTTCTACCCGTTCGC	Used to quantify *ahpC* transcripts (JF957854)	123
ahpC-1R	CCTGTGCGGCCAGAAGTC	Used to quantify *ahpC* transcripts (JF957854)	123
16-1F	CTCTTTCGCCAGGGACGAAG	Used to quantify *16S* transcripts	211
16S-1R	CTACCGAACTCAAGCCTGCC	Used to quantify *16S* transcripts	211

until exponential phase, using an OxiSelect Protein Carbonyl ELISA kit following the manufacturer's protocol (Cell Biolabs, San Diego, CA). Briefly, BSA standards or samples were absorbed onto a 96-well plate for 2 h at 37 °C. The protein carbonyls present in the sample or standard were derivatized to DNP hydrazone and probed with an anti-DNP antibody, followed by an HRP-conjugated secondary antibody. The plate was then read at 405 nm by a plate reader. The protein carbonyl content in the sample was determined by comparison with a standard curve that was prepared from predetermined reduced and oxidized BSA standards.

Lipid peroxidation

Lipid peroxidation was measured in cells from *A. tucumanensis* and *A. eurytherma* grown in MM_{b0}, MM_{b10}, MM_{b20} and MM_{b30} until exponential phase, using the OxiSelect TBARS assay kit (Cell Biolabs, San Diego, CA) following the manufacturer's instructions. The thiobarbituric acid-reactive substance (TBARS) assay is a well-established assay for monitoring and screening lipid peroxidation; malondialdehyde (MDA) forms a 1:2 adduct with thiobarituric acid (TBA), and can be measured colorimetrically or fluorometrically using an MDA equivalent standard.

Results and discussion

Effect of copper upon protein abundance

We analyzed the response to copper in order to identify differentially synthesized protein, which might include resistance factors. Total protein was extracted from *A. tucumanensis* biomass collected from MM_{b0} and MM_{b20}; it is important to highlight that in MM_{b20}, the uptake of Cu^{2+} by *A. tucumanensis* is nearly 10 mg Cu/g of cells (Albarracín et al. 2008).

Proteins were analyzed covering pH 3–10 and 4–7 gradients and the resulting 2-DE gel images were used for in silico comparison. Most proteins focused on the pH range 4–7, in agreement with the proteomic analysis performed with *Amycolatopsis balhimycina* and *Streptomyces coelicolor* (Manteca et al. 2006; Gallo et al. 2010). Intensity changes statistically significant (ρ-value <0.05) between spots were considered and analyzed by MALDI-TOF TOF. In the presence of copper, 294 spots were detected; 38 % of these spots were only detected upon copper stress, 27 % increased and 35 % decreased in intensity in a statistically significant manner compared with the spots obtained from non copper-stressed biomass (Fig. 1). The identities of ten regulated proteins by the presence of copper included metabolic pathways, regulation and stress response proteins (Table 2; Fig. 2a).

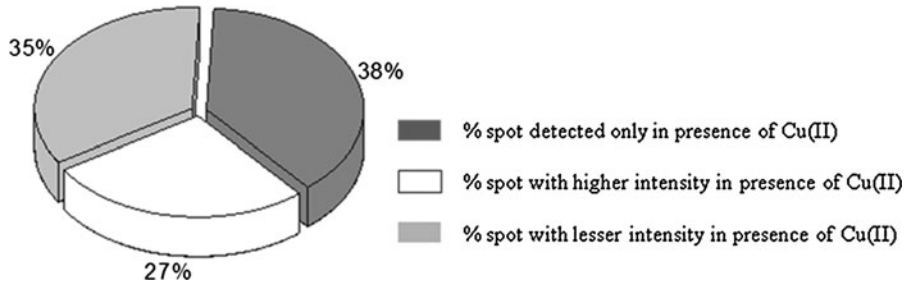

Fig. 1 Distribution of the 294 spots detected by proteomic study, upon copper-stress condition. Differential proteomics analysis revealed that 38 % of these spots were detected only upon copper-stress; 27 % increased and 35 % decreased their %Int in a statistically significant manner related to the spots obtained from non copper-stressed biomass

The abundance of the protein succinyl-CoA synthetase, which participates in the TCA cycle producing ATP, was increased upon copper-stress in *A. tucumanensis* (Table 2; Fig. 2, spot 1). The TCA cycle is a source of ATP needed during heavy metals stress. To circumvent aluminum toxicity, *Pseudomonas fluorescens* elaborates an intricate system involving succinyl-CoA synthetase to produce ATP via substrate level phosphorylation (Lemire et al. 2010). Pyrophosphate-dependent phosphofructokinase, another protein involved in the central metabolism was also identified upon copper-stress (Table 2; Fig. 2, spot 7). In many organisms, the phosphorylation of fructose-6-phosphate (F-6-P) to fructose-1,6-bisphosphate (F-1,6-P2) is catalyzed by an ATP-dependent phosphofructokinase enzyme (ATP-PFK). However, a second type of PFK enzyme, using pyrophosphate (PPi) as a substrate instead of ATP (PPi-PFK) and catalyzing the reversible phosphorylation of F-6-P to F-1,6-P2, exists in plants, some bacteria and archaea (Reeves et al. 1974). In agreement with our results, a PPi-PFK enzyme was also identified in *Amycolatopsis methanolica* and in others actinobacteria (Alves et al. 2001). In *A. tucumanensis*, the expression of the gene encoding a PPi-PFK instead of ATP-PFK might be explained because of the high need for ATP upon copper stress also seen with the increased abundance of the succinyl-CoA synthetase (Seiler et al. 1996). Although there is no record in the genus *Amycolatopsis*, several reports have demonstrated that plants may utilize PPi as an alternative energy donor during ATP deficiency. In contrast to the strong decline in the amount of ATP, PPi content was found to be relatively stable under adverse conditions (Mustroph et al. 2005).

In plants and microorganisms the enzyme acetohydroxyacid synthase (AHAS) catalyzes the first common reaction in the biosynthesis pathways leading to leucine, isoleucine and valine; this protein was also detected in *A. tucumanensis* under copper stress (Table 2; Fig. 2, spot 4). It is well known that copper can exacerbate the production of H_2O_2 in the cell; in turn, extreme levels of H_2O_2 might act disrupting amino acid biosynthetic pathways. A *copA cueO cusCFBA Escherichia coli* mutant was not able to grow in copper-challenged culture, however, its growth was partially restored when adding branched amino acids in the culture medium; it might indicate that this pathway is affected for copper toxicity (Macomber and Imlay 2009). Expression of branched-chain amino acids and genes with functions in oxidative-stress resistance were increased in *Staphylococcus aureus* during exposure to the drug mupirocin (Reiß et al. 2012).

Regulatory functions under copper stress

The mechanisms whereby cells sense environmental signals and transmit that information to changes in gene expression, often involve the participation of transcription factors that respond either directly or indirectly to small molecules or substrates (Seoane and Levy 1995). The MarR and GntR family of transcriptional regulators were identified in *A. tucumanensis* upon copper-stress (Table 2; Fig. 2, spots 2 and 3). The MarR (multiple antibiotic resistance regulator) protein is a prototypical member of the MarR family of transcriptional regulators that are found in bacteria and archaea (Seoane and Levy 1995). The MarR members regulate a variety of biological functions, including resistance to multiple antibiotics and others toxic chemicals such as organic solvents, oxidative stress agents and adaptation to different environments (Alekshun and Levy 1999).

Table 2 List of identified *A. tucumanensis* proteins whose abundance were increased upon copper-stress

Spot	Protein	Mass (kDa)	pI	Microorganisms	Identification technique[a]	Peptide	MASCOT score[b]
1	Succinyl-CoA synthetase subunit alpha	31.75	5.50	*Streptomyces coelicolor* A3(2)	MS/MS	K.SGTLTYQMMYELR.D	47
2	Transcriptional regulator, GntR family	26.96	5.91	*Streptomyces violaceusniger* Tu 4113	PMF	MAFGDQPAYLR.V	70
						R.GGFHAAGEATPFR.Q	
						R.QEQADER.V	
						R.VLGSWDAHSEQEEAGAETAGR.L	
						R.TPVMLPEEGPLAGR.G	
						R.MAALDLVVDNVAEEVGARPGR.A	
						R.TYFAGGRPVETADVVVLAER.Y	
3	Transcriptional regulator, MarR family	24.10	5.00	*Streptosporangium roseum* DSM 43021	PMF	R.LTYFPQLAAER.L	75
						K.MLVAVGPCSQQTLSEELR.I	
						R.SVMVNLCDDLER.A	
						R.LLTEAETSVPEFLDR.T	
						R.DQLTVLLGK.L	
						K.LLDVHPPASGPR	
4	Acetohydroxyacid synthase	18.12	5.00	*Mycobacterium avium*	PMF	R.VAALFSRR.G	56
						R.RGFNIESLAVGATEQK.D	
						K.DMSRMTIVVSAEETPLEQITK.Q	
						K.LINVIK.I	
						K.LINVIKIVELEDGNSVSR.E	
						R.ADAGTRSQVIEAVNLFR.A	
						R.AKVIDVSPEALTIEATGDR.G	
						K.VIDVSPEALTIEATGDRGK.I	
						R.GKIEALLR.V	
						R.VLEPSVSVRSSNR.E	
						R.EWCRCPGPR.G	
5	Anti-sigma regulatory factor/ serine, threonine protein kinase	35.62	5.10	*Conexibacter woesei* DSM 14684	PMF	R.GFEPTTELPDDLLQAGGGR.G	62
						R.QHAEALPAREEAAPDGTFGK.E	
						K.ESFLRALVVQLAQAVEANEGPDAAAAA VAQVGADVGGR.M	
						R.VEGITGELSPMQMADLYVR.L	
						R.LKAAIDGDFYVIEADEER.I	
						R.ACPFGDVVR.R	
						R.APGLCRMTSSVFGGIAAR.N	

Table 2 continued

Spot	Protein	Mass (kDa)	pI	Microorganisms	Identification technique[a]	Peptide	MASCOT score[b]
6	Putative two-component system response regulator	22.5	6.10	*Saccharopolyspora erythraea* NRRL 2338	MS/MS	R.VLVAEDEALIR.L R.IAPVVILTAFSQR.D	72
7	Pyrophosphate-dependent phosphofructokinase	36.95	6.25	*Amycolatopsis methanolica* NCIB 11946	MS/MS	K.GIEAHGWEIVGFR.S R.VGVLTGGGDCPGLNAVIR.A	99
8	4-methyl-5-β-hydroxyethylthiazole kinase	28.81	5.01	*Carboxydothermus hydrogenoformans* DSM 6008	PMF	K.IANAVVINIGTLHSR.Q K.YQKPLLLDPVGLGATTYR.N R.NETTFELLNSGNFTLIR.G K.GVDSQTSDFAAENLTEVAK.R K.ISAGPGSFLVNFIDSLYNLTK.E	57
9	Fatty acid-binding protein-like protein	26.69	6.07	*Mycobacterium marinum* ATCC BAA-535	PMF	MTSDASQDGPDAVAGSGDR.A R.NIPTFDDLPLPADTANLR.H R.HGANLHDALLALLPLVGVWR.G R.FGQQIVVSHDGGDYLNWEAR.S R.LDEDGQYEEPGLR.E	58
10	Fe/Mn-containing superoxide dismutase	18	5.21	*Streptomyces netropsis*	MS/MS	K.GANDTLDQLAEAR.D	47

[a] *PMF* Peptide mass fingerprinter, *MS/MS* MS/MS ion search

bPMF protein scores >55 were significant ($\rho < 0.05$). *MS/MS* individual ions scores >39 indicate identity or extensive homology ($\rho < 0.05$)

The GntR family of transcriptional regulators showed to act as environmental sensors, whereby might be also involved in response to copper stress (Hillerich and Westpheling 2006).

Anti-sigma regulatory factor/serine, threonine protein kinase and a putative two-component (Table 2; Fig. 2, spots 5 and 6) hint at different regulatory pathways involved in copper stress. Sevcikova et al. (2010) demonstrated that sigma factor σ^H has a role in regulation of the osmotic stress response in *Streptomyces coelicolor* A3(2). In addition, the expression of the genes encoding TonB-dependent transporters proteins that bind and transport ferric chelates, is regulated inter alia by σ/anti-σ factor systems (Noinaj et al. 2010). Bacterial two component systems (Stock et al. 2000) generally consist of a response regulator and a sensor kinase; a previous report has demonstrated that the two component system CusR/CusS which is involved in the regulation of transcription from the *cusCFBA* genes play a central role in copper and silver resistance in *E. coli* (Gudipaty et al. 2012).

Antioxidants and protective metabolites

Thiamine (vitamin B1) is an essential cofactor whose biosynthesis involves a phosphorylation catalyzed by 4-methyl-5-β-hydroxyethylthiazole kinase, producing the biologically active form, thiamine pyrophosphate (THI-PP) (Jeyakanthan et al. 2009). In copper-challenged culture from *A. tucumanensis*, this enzyme was induced (Table 2; Fig. 2, spot 8). Madeo et al. 2012, have given evidence that THI-PP plays a critical role in the acid-stress tolerance of *Listeria monocytogenes*. In addition, *Yersinia kristensenii* improved its resistance to divalent metal ions and antibiotics, when thiamine was amended in the culture medium (Hustavová and Havraneková 1997). In *A. tucumanensis*, the increase in the abundance of the protein 4-methyl-5-β-hydroxyethylthiazole kinase might be related to a higher requirement of THI-PP, in order to cope with the toxic effects of copper (Hustavová and Havraneková 1997; Madeo et al. 2012).

Of the various deleterious effects of ROS, oxidation of polyunsaturated fatty acids and phospholipids in membranes generate a family of lipid-derived reactive aldehydes with the most common being malondialdehyde and 4-hydroxy-2-nonenal (Sayre et al. 2006). Fatty acid binding proteins (FABPs) bind non-covalently hydrophobic ligands. FABPs are implicated in fatty acid intracellular uptake and transport, regulation of lipid metabolism and protection from the deleterious action exerted by free long chain fatty acids (Hellberg et al. 2010); in *A. tucumanensis* high level of FABP was detected upon Cu^{2+} stress (Table 2, Fig. 2, spot 9). A work published elsewhere showed that *Mycobacterium tuberculosis* depends on host lipid sources for its survival and persistence; in turn, the ability of *M. tuberculosis* to up-regulate the expression of genes encoding FABPs provide a mechanism to promote the survival of this pathogen under adverse conditions (Shepard et al. 2007).

Copper exacerbates ROS production in *A. tucumanensis* which is counter-acted by enhanced superoxide dismutase (SOD) activity (Dávila Costa et al. 2011b). Here, we could prove that *A. tucumanensis* encodes a Fe/Mn-SOD, whose abundance was increased in the presence of copper (Table 2; Fig. 2, spot 10).

Expression profiles of antioxidant coding genes

The identified proteins seem relevant to allow *A. tucumanensis* to survive under copper stress. However the copper resistance and accumulation ability might be also supported by high expression profile levels of key genes involved in the antioxidant network. To test this hypothesis, using primers specifically designed, the antioxidants genes *sod*, *mtr* and *ahpC* were amplified and sequenced from *A. tucumanensis* (Accession number: JF937111 (*sod*), JF957853 (*mtr*) and JF957854 (*ahpC*)) (Table 1). To determine the expression patterns of these genes by qRT-PCR new specific primer were designed (Table 1) using 16S rDNA as a control, since the ratio of *16S* transcripts to total RNA was almost constant throughout all assayed conditions (MM_{b0}, MM_{b10} and MM_{b30}). The cycle number at which the reaction crossed an arbitrarily placed threshold (C_T) was determined for each gene and the relative expression of each gene to 16S rDNA was estimated using the equation $2^{-\Delta CT}$ (Livak and Schmittgen 2001).

In MM_{b10}, the increase in expressions was 1.61-fold for *sod*, 1.11-fold for *mtr* and 1.35-fold for *ahpC*, higher than in MM_{b0}. In MM_{b30} genes expressions were to *sod*, 2.4-fold; to *mtr* 2.85-fold; and to *ahpC*, 2.2-fold; higher as compared to MM_{b0}. Additionally, the increase in the genes expression levels in MM_{b30} was statistically significant when comparing to the expression levels in MM_{b10} (Fig. 3).

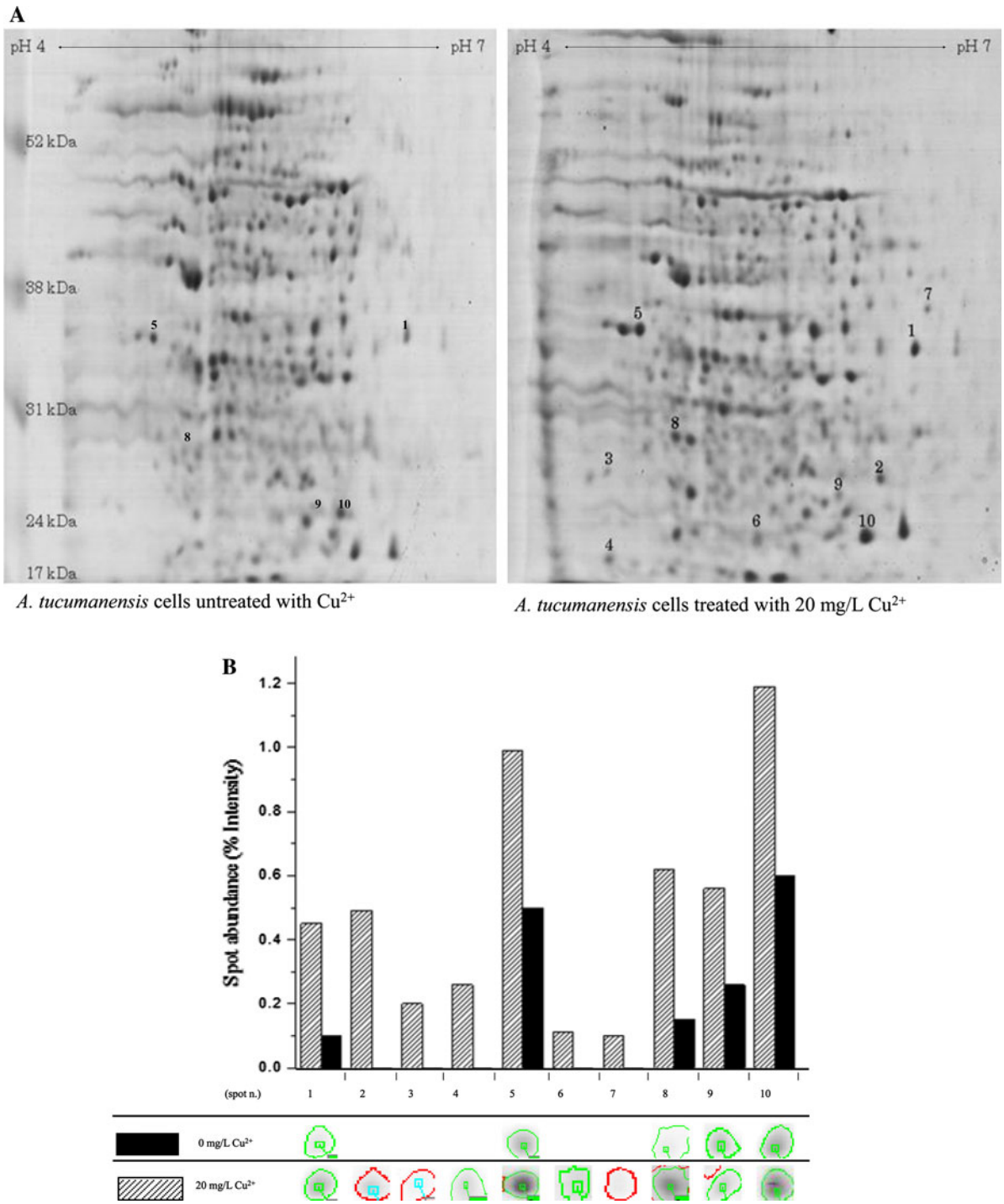

Fig. 2 **a** 2-D gel images of *A. tucumanensis* cells treated with 20 mg l^{-1} Cu^{2+} or untreated. *Spots* showing statically significant up-regulation ($\rho < 0.05$) or absence in control condition were considered as differentially expressed. *Spots*: 1-Succinyl-CoA synthetase; 2-Transcriptional regulator, GntR family; 3-Transcriptional regulator, MarR family; 4-Acetohydroxyacid synthase; 5-Anti-sigma regulatory factor/serine, threonine protein kinase; 6-Putative two-component system response regulator; 7-Pyrophosphate-dependent phosphofructokinase; 8-4-methyl-5-β-hydroxyethylthiazole kinase; 9-Fatty acid-binding protein; 10-Fe/Mn-containing superoxide dismutase. **b** Zoomed 2-DE image and abundance (% Intensity) of the identified proteins in *A. tucumanensis*

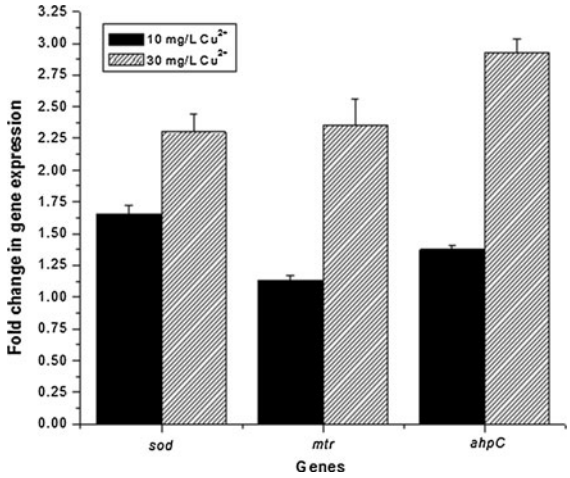

Fig. 3 Fold change in the gene expression levels of superoxide dismutase (*sod*), mycothiol reductase (*mtr*) and alkyl hydroperoxide reductase (*ahpC*) in cells grown in presence of 10 and 30 mg/L Cu^{2+}, compared with those grown without copper. The cycle number at which the reaction crossed an arbitrarily placed threshold (C_T) was determined for each gene and the relative expression of each gene to 16S rDNA was estimated using the equation $2^{-\Delta CT}$

It has been demonstrated that copper enhanced ROS production and superoxido dismutase activity in *A. tucumanensis* (Dávila Costa et al. 2011b). Superoxide dismutase is one of the main enzymes involved in antioxidant response and as we expected, the expression level of *sod* was higher when increasing the concentration of copper in the culture medium (Fig. 3). This confirms the result obtained in our proteomic study (Fig. 2; Table 2), where an increase in the abundance of Fe/Mn-containing SOD was detected in presence of copper. Similar results were obtained when *Streptomyces coelicolor* was exposed to heavy metal stress (Ahn et al. 2006).

Expression levels of *mtr* and *ahpC* in *A. tucumanensis* also increased upon higher concentration of copper (Fig. 3). Alkyl hydroperoxide reductase (*ahpC*) is not only responsible for hydroperoxide detoxification but also affords protection against reactive nitrogen intermediates. Mycothiol reductase (*mtr*) catalyzes the NADPH-dependent reduction of the unique, low molecular weight disulfide mycothiol, which also plays a key role in the antioxidant protection of actinobacteria (Guimara et al. 2005; Hamilton et al. 2009). Several reports have demonstrated an over-expression of *aphC* and *mtr* under stress conditions; within actinobacteria, the expression of these genes were widely studied in *Mycobacterium* genus but mainly with the purpose of searching for new drug target and make these pathogenic cells more vulnerable (Hillas et al. 2000). To our knowledge this is the first report where *aphC* and *mtr* expression levels were studied under copper-stress conditions in *Amycolatopsis*.

Protein oxidation and lipid peroxidation

In oxic conditions, copper can catalyze Fenton-like reactions that may cause lipid peroxidation and protein damage (Dupont et al. 2011). Protein oxidation is defined as the covalent modification of a protein induced either directly by ROS or indirectly by reaction with secondary by-products of oxidative stress. The most common products of protein oxidation in biological samples are the protein carbonyl derivatives of Pro, Arg, Lys, and Thr. These derivatives are chemically stable and serve as markers of oxidative stress (Conrad et al. 2001). Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), natural by-products of lipid peroxidation (Joshi et al. 2011).

To assess the effectiveness of the *A. tucumanensis* copper-resistome, MDA and protein carbonyl concentrations were determined in MM_{b0}, MM_{b10}, MM_{b20} and MM_{b30} compared to the copper-sensitive strain *A. eurytherma* (Fig. 4a, b).

Amycolatopsis eurytherma showed values of protein carbonyl in MM_{b10}, 1.3-fold; in MM_{b20}, 3.2-fold; and in MM_{b30}, 2.9-fold higher than *A. tucumanensis* (Fig. 4a). It has been widely demonstrated that oxidative damage to proteins contribute substantially to the killing of cells exposed to toxic conditions, therefore it is imperative for the cells to keep the integrity of its proteins (Krisko and Radman 2010). Our results clearly demonstrated that copper did not cause serious damage in *A. tucumanensis* proteins, confirming the protective role of its copper-resistome.

Surprisingly, there was no statistical difference in MDA concentration between *A. tucumanensis* and *A. eurytherma* grown in MM_{b10} (Fig. 4b) while MM_{b20} and MM_{b30} did show 1.8 and 3.1-fold higher levels in *A. eurytherma*, respectively (Fig. 4b). In turn, in *A. tucumanensis*, MDA levels were similar in all assayed copper conditions (Fig. 4b). This may be

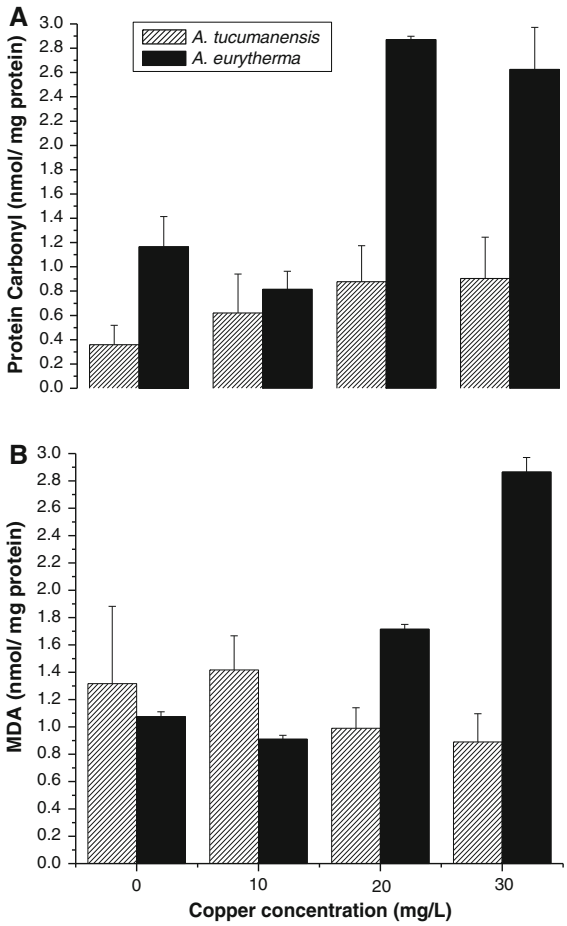

Fig. 4 Protein carbonyl (**a**) and malondialdehyde (MDA) (**b**) formation, as indication of ROS damage, in cells from *A. tucumanensis* and *A. eurytherma* grown in MM_{b0}, MM_{b10}, MM_{b20} and MM_{b30}. *Bars* represent the median of three different assays carried out by duplicated

explained by the finding of the fatty acid binding protein whose abundance was higher in presence of copper, as this protein has been studied for its protective role against lipid peroxidation in eukaryotic cells (Wang et al. 2005). The role of this protein in the protective network of *A. tucumanensis* will be an important area for future investigation.

Conclusion

It is well documented that copper pollution is a growing problem mainly caused by industrialization, which concerns particularly developed countries. The knowledge of the effects produced by copper accumulation in microorganisms with potential application in bioremediation strategies is essential. Reports published elsewhere showed that *A. tucumanensis* is a promising tool for cleaning up copper-contaminated soils. Herein we have identified proteins which were up-regulated upon copper-stress, suggesting that they play a key role in the protective mechanism of *A. tucumanensis*. Transcriptome analysis revealed that, genes from key antioxidant proteins like superoxide dismutase, alkyl hydroperoxide reductase and mycothiol reductase were markedly induced in the presence of copper, confirming that this metal triggers an antioxidant response. The negligible damage in proteins and lipids from *A. tucumanensis* after copper exposure, support the hypothesis that our strain efficiently handled the toxic effects of copper by virtue of a copper-resistome.

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