The presence of copper triggers general and specific responses in *Apiotrichum loubieri* M12

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JB and LV conceived and designed research. JB, EC and MP conducted experiments. RG contributed new reagents or analytical tools. All authors analyzed data. JB and LV wrote the manuscript. All authors read and approved the manuscript.

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ABSTRACT

Extreme environments are promising sites to isolate heavy metal-resistant microorganisms. The present work aimed to isolate and study a microorganism capable to remove heavy metals, from sediments of an Acid Mine Drainage-affected environment located in San Luis (Argentina). *Apiotrichum loubieri* M12 was selected for its ability to tolerate heavy metals. The microorganism was able to remove 30-35% of copper from liquid media when faced to 40 μg mL^{-1}Cu(II). The analysis of the biomass exposed to the metal through SEM-EDS showed uniform bioadsorption of the metal on the cell surface (4.09% w/w) and variations in the proportion of other biomass constituent elements. Proteomics revealed that the presence of Cu(II) induces differential expression of intracellular proteins involved in a wide variety of metabolic processes. Interestingly, a specific response to the metal was detected in cell-free supernatants, in which copper binding proteins were identified. A large number of proteins with metal ion binding sites were detected both at the intra and extracellular levels. The readjustment of protein expression is crucial to counteract the metabolic imbalances produced by the metal and to sequester and transport copper, being fundamental to reduce the toxic effects that Cu(II) could exert on the cell.

KEYWORDS: Sediments; *Apiotrichum*; Heavy metal removal; Proteomics; Copper

INTRODUCTION

Soils and sediments are known as reservoir for heavy metals that are released into natural environments from natural sources or as a consequence of anthropogenic activities that usually cause imbalances in the ecosystem (Azarbad et al. 2016). Among the industrial activities that are considered responsible for the release of these xenobiotics into the environment, mining activities occupy one of the first places (Akhtar et al. 2021). Particularly, Acid Mine Drainages (AMDs) are one of the most documented environmental issues associated with mining activities (Méndez-García et al. 2015). The term refers to a complex multi-factor pollution that occurs in active and abandoned mines as a result of chemical, physical and biological interactions (Ighalo et al. 2022).

One of the serious consequences of the formation of AMDs in mining sites lies in the ability of these drainages to enhance the mobility of heavy metals. The acidity of AMDs can exceed up to 10,000 times the values of neutral waters, so they become powerful leaching agents that can dissolve significant amounts of metallic compounds (Gaikwad and Gupta 2008). These metals are mobilized and can easily contaminate near and far soils and sediments (Gao et al. 2019; Ighalo et al. 2022). However, the inherent buffering capacity of soils and sediments when receiving these toxic contaminants has been extensively demonstrated. In these matrices, microorganisms are key biological components for this buffering capacity. Long-term exposure to metal contamination leads to the adaptation of microbial communities that can survive and persist in environments with extreme conditions (Stefanowicz et al. 2008; Shuaib et al. 2021). Likewise, microorganisms in natural environments can mitigate the toxic effects of pollutants through their transformation and/or degradation by employing wide variety of cellular machineries, which respond to both the deficiency and excess of metals (Ma et al. 2009; Solioz et al. 2010; Mishra et al. 2021). For these reasons, the isolation of heavy metal-resistant microorganisms from AMD-affected environments is a promising strategy with a view to future biotechnological applications in environmental remediation (Bonilla et al. 2018 and 2021).

The study of heavy metal-resistant microorganisms requires multidisciplinary approaches and the use of advanced tools. The homeostatic mechanisms that the resistant microorganisms trigger when faced to heavy metals differ among the different genera and/or species and relatively little is known about them at molecular level. Currently, Proteomics is highly employed and appears as an efficient technology for

obtaining information about metabolic responses, tolerance and detoxification mechanisms in microorganisms under metal stress (Mishra et al. 2021; León-Vaz et al. 2021).

Proteins act as effectors of biological responses, are more stable than other macromolecules and possibly confer a comprehensive view of the biological function (León-Vaz et al. 2021). The dynamic population of proteins in the cells reflects the relationship between gene regulation and extracellular influences (Wu y col. 2016). Proteome is a complex cellular structure that refers to all the proteins that are produced by an organism and it is based on the concept of understanding "proteins as the complement of the genome" (Abhilash 2009). The dynamism observed in the cellular protein composition differs from the static information offered by genomic analyses. The proteome is important not only for analyzing the cell behavior, but also for closing the gap between the organisms' genome and metabolites with a holistic perspective.

The detection of variations in the proteome can serve to discriminate between two biological states in an organism and; therefore, Proteomics provides relevant data regarding the structure and function of the cell, as well as the response mechanisms against different stress situations (Aslam et al. 2017). The application of proteomic techniques can help us to know the molecular mechanisms involved in the response and removal of heavy metals by resistant microorganisms.

The present work aimed to isolate a microorganism resistant to heavy metals from sediments of the AMD-affected environment located in San Luis (Argentina), and to study the removal and resistance mechanisms employing multidisciplinary approaches.

MATERIALS AND METHODS

ISOLATION OF A MICROORGANISM RESISTANT TO HEAVY METALS

The heavy metal-resistant microorganism was isolated from sediments collected from the abandoned gold mine located in San Luis (Argentina) by following the scheme specified in our previous works (Bonilla et al. 2018 and 2021). The metals Fe(II) ($FeSO_4.7H_2O$), Cr(VI) ($K_2Cr_2O_7$) and Cu(II) ($CuSO_4.5H_2O$) were added to the modified Yeast Extract-Glucose Medium (EG*) (g L^{-1}: Glucose 10.0, Yeast Extract 1.0, K_2HPO_4 0.5, KH_2PO_4 0.5, Agar 15.0) from 1 to 5 µg mL^{-1}, acting as selection pressure when employing the sequential enrichment method specified by Perez Silva et al. (2008).

The qualitative metal-tolerance of the isolates was determined in solid media through the agar diffusion technique by adding Fe(II), Cu(II) and Cr(VI) from 100 to 1,000 µg mL^{-1} (Villegas et al. 2004). After selection of the most tolerant microorganism, the Minimum Inhibitory Concentration (MIC) was determined in liquid EG* medium by employing the cell viability test using Resazurin, modified from the one specified by Borra et al. (2009). The metals were added to the liquid medium containing $1x10^4$ cells mL^{-1} in increasing concentrations from 15 to 500 µg mL^{-1} (Rex 2008; Bonilla et al. 2021). The MIC was defined as the interval comprised between the maximum concentration where cell viability was detected and the minimum concentration where viability was not observed. All the assays were carried out in triplicate.

The identification of the microorganism was carried out through PCR amplification and sequencing of the genomic regions ITS1 – 5.8S rDNA complete sequence - ITS2 - 28S rDNA partial sequence (ITS1 F: 5´-TCCGTAGGTGAACCTGCGG-3´; ITS4 R: 5´ TCCTCCGCTTATTGATATGC-3´) and D1/D2, 28S rDNA (NL1 F: 5′-GCATATCAATAAGCGGAGGAAAAG-3´; NL4 R: 5′-GGTCCGTGTTTCAAGACGG-3´). The PCR protocol consisted of a first denaturation cycle at 95 °C for 5 min, followed by 30 cycles of 95 °C for 60 s, 55 °C for 60 s and 72 °C for 90 s, with a final elongation cycle at 72 °C for 7 min. Amplicons were sequenced by Macrogen (www.macrogen.com; South Korea) and edited with Molecular Evolutionary Genetics Analysis (MEGA v7.0). The sequences were analyzed with BLASTn (www.ncbi.nlm.nih.gov) and the Barcode Of Life Data System (Bold Systems v4; www.boldsystems.org).

REMOVAL OF Cu(II) FROM LIQUID EG* MEDIUM

40 µg mL^{-1} Cu(II) were added to 100 mL of liquid EG* medium containing $1x10^6$ cells mL^{-1}. Cultures were incubated at 200 rpm and 30 °C for 120 h. Samples were taken every 24 h and were centrifuged for 10 min at 11,000 *xg*. The copper residual concentration was determined in the cell-free supernatants through inductively coupled plasma mass spectrometry (ICP-Mass ELAN DRC-e). Dry weight techniques were used for growth kinetics studies in the presence and absence of the metal.

ANALYSIS OF MICROBIAL BIOMASS EXPOSED TO COPPER

The surface mapping and the microelemental analysis of the microbial biomass exposed to Cu(II) was performed by Scanning Electron Microscopy coupled to X-Ray Dispersive Energy Spectrometry (SEM-EDS) by following the specified by Bonilla et al. (2021). Briefly, the biomass obtained from cultures in

the presence and absence of copper after 48 h of incubation was mounted on aluminum stubs and dried at room temperature. Then, samples were sputter-coated with carbon (SPI metallizer) and analyzed with Zeiss LEO 1450 VP SEM Scanning Electron Microscope, operated at 20 kV, coupled to a Genesis 2000 energy dispersive spectrometer (EDS).

PROTEOMIC ANALYSIS

Cultures in the presence and absence of Cu(II) after 48 h of incubation were centrifuged during 20 min at 4 °C and 4,000 *xg*. For the intracellular proteins collection, pellets were washed twice with phosphate buffered saline (mM: NaCl 124; NaH_2PO_4 10; KH_2PO_4 3) and were broken using liquid N_2 and a mortar and pestle. The powder was recovered with Tris-Sucrose buffer (Sucrose 11.29 g dL^{-1}; Tris-HCl 1.5M pH 8.8 3.33 mL dL^{-1}) and centrifuged during 20 min at 4° and 8,500 *xg* to eliminate the pellet and conserve the supernatants containing the proteins. The extracellular proteins were collected from the cell-free supernatants, which were filtered with nitrocellulose membranes (Microclar 0.2 µm) and were concentrated 20X with Vivaspin Turbo 15 3,000 MWCO, polyethersulfone membrane (PES) (Sartorius).

The protein concentration was determined by Bradford (1976). Then, samples were lyophilized and reconstituted to achieve a final concentration of 50 mM Tris-HCl pH 8.0 and 1 µg $µL^{-1}$ of protein concentration. Finally, samples were reduced with DTT and alkylated with Iodoacetamide. Digestion was carried out with Trypsin and the obtained peptides were concentrated with SpeedVac (Thermo Savant). The tryptic peptides were analyzed by Ultra Performance Liquid Chromatography on a nano scale (nanoUHPLC), coupled to tandem mass spectrometry (nanoUHPLC-ESI-MS/MS) (**Online Resource 1**).

The bioinformatic analysis of the mass data was carried out against Swiss-Prot and NCBI databases, combined with MASCOT v2.5.1 (www.matrixscience.com, local license). The database for *Apiotrichum porosum* NCBI v20190211 (9185 sequences, 4529209 residues) was used to conduct the analysis due to the lack of specific databases at the repository sites. The comparative study was performed using ProteoIQ v2.8 (local license) (Bonilla et al., 2020 and 2021). Additionally, the extracellular protein locations were predicted by the subCELlularLOcalization predictor (CELLO v2.5, http://cello.life.nctu.edu.tw) (Yu et al. 2004 and 2006). All the assays were performed in biological triplicates and analytical duplicates.

RESULTS

ISOLATION OF A HEAVY METAL RESISTANT MICROORGANISM

The microorganism isolated from sediments of the AMD-affected environment located in San Luis (Argentina), by adding iron, chromium and copper to liquid EG* medium as selection pressure, was able to grow in the presence of up to 250 µg mL^{-1} Fe(II), 45 µg mL^{-1} Cr(VI) and 60 µg mL^{-1} Cu(II). Based on these results, the MIC values (µg mL^{-1}) were established in the range of $250 < MIC \leq 375$ for Fe(II), $45 < MIC \leq 60$ for Cr(VI) and $60 < MIC \leq 90$ for Cu(II).

For the identification of the isolate, primers for eukaryotic organisms were selected for PCR amplification based on observations at macro and microscopic levels. After the analysis of the 461 bp sequence (NCBI Accession Number KY596699) corresponding to the ITS1–5.8S rDNA-ITS2-28S rDNA region, and the 583 bp sequence (NCBI Accession Number MN199100) corresponding to D1/D2, 28S rDNA genomic region, the microorganism was identified as *Apiotrichum loubieri* M12.

REMOVAL OF Cu(II) FROM LIQUID EG* MEDIUM

Despite the tolerance showed by *A. loubieri* M12 against Fe(II) and Cr(VI), the microorganism was unable to remove these metals after 120 h when exposed to 50 % of the maximum concentration of each metal in which cell viability was detected for each metal. However, variations of Cu(II) concentration were detected at 120 h of exposure (data not shown). For this reason, the copper-removal capacity was evaluated by facing *A. loubieri* M12 to 40 µg mL^{-1} Cu(II) in liquid EG* medium.

The presence of copper at this concentration affected the growth of *A. loubieri* M12 about 60%, when compared to the control culture in the absence of the metal. The cultures in the presence and absence of the metal reached the stationary phase after 72 h (**Figure 1-A**). Regarding the Cu(II)-removal capacity, **Figure 1-B** shows that the maximum value (32.8 %) was obtained after 48 h of culture and remained constant for the rest of the test. The pH values (pH=6.0) of the cultures kept constant during 120 h in both conditions.

MICROBIAL BIOMASS EXPOSED TO COPPER

The surface mapping and the microelemental composition of *A. loubieri* M12 exposed to Cu(II) during 48 h was evaluated through SEM-EDS. This time was selected to obtain the biomass in the exponential growth phase. In this analysis, the influence of the metal presence on the composition of the biomass was

evaluated, as well as the concentration and distribution of copper in the biomass exposed to the metal. The results are shown in **Figure 2**.

The elements C, O, P, S, K and Ca were found as common for samples obtained in the presence and absence of the metal. However, a four-fold decrease in K intensity was detected in the biomass exposed to copper (0,66% w/w), when compared to the biomass obtained in the absence of the metal (2.53% w/w). The signals of N (5.29% w/w), Mg (0.23% w/w) and Cu (4.09% w/w) were detected only in the biomass grown in the presence of Cu(II). The metal was found uniformly distributed in the cells, which presented an average width of 3.29 µm in both conditions.

PROTEOMIC ANALYSIS

INTRACELLULAR PROTEOMIC ANALYSIS

The differential protein expression at the intracellular level when the microorganism was exposed to copper was analyzed by performing a shotgun proteomic analysis. In this analysis, from a total of 247 identified proteins, 72 were detected as exclusive to the group exposed to the metal while 47 appeared as proteins related to the cells obtained in the absence of copper. Likewise, 128 proteins were detected as common for both conditions (**Figure 3**). In this group, only 18 proteins showed significant differences when analyzing the relative abundance using Label-Free Quantification (LFQ), taking into account the normalized spectral counts (SpCs) (**Figure 3-B**), being 13 the proteins over-expressed and five down-regulated in the presence of the metal (**Table 1**). According to their cellular function obtained from Gene Ontology, the proteins over-expressed were grouped in proteins involved in protein biosynthesis (Bars 1, 2, 4 to 7, 12 and 13), carbohydrate metabolism (3) and redox stress (11). It is important to highlight that, in this group, Isocitrate dehydrogenase, Ketol-acid reductoisomerase and ATP-citrate synthase present metal ion binding sites, mainly for +2 ions, such as Mg (II).

Proteins identified only in the group exposed to copper are shown in **Table 2** and were grouped in: **i)** Protein biosynthesis (17 proteins), including ribosomal proteins, translation factors, mRNA splicing proteins, and folding proteins; **ii)** Oxidation-reduction processes (6); **iii)** Degradation proteins (7), such as proteases, and proteasome and ubiquitination proteins; **iv)** Nucleic acid binding proteins (9); **v)** Oxidative stress indicator proteins, such as Heat shock proteins ; **vi)** Kinases/Phosphatases (4), which can be important for the enzymatic activation or inactivation, and for the intracellular signaling pathways;

vii) Carbohydrate metabolism proteins (10), mainly involved in the glycolytic process and the Krebs cycle; **viii)**Proteins involved in intracellular protein transport (4); and **ix)** Proteins responsible for post-translational modifications (PTMs) (2) (**Figure 4**).

A significant number of the proteins detected in the cells exposed to the metal present metal ion binding sites, such as 3-isopropylmalate dehydrogenase, which is capable to bind Mn(II) or Mg(II), as well as metalloendopeptidases such as Aminopeptidase 2, Methionine aminopeptidase, E3 ubiquitin-protein ligase and mitochondrial processing protease EHS24_00808. Among proteins that bind nucleic acids, Zn(2)-C6 fungal-type domain-containing protein, Lactose regulatory protein lac9, and Cellular nucleic acid-binding protein are capable of binding Zn(II) and show important functions in the DNA transcription process. Other identified proteins with bivalent ion binding sites include Inositol hexakisphosphate and diphosphoinositol-pentakisphosphate kinase, Serine/threonine-protein phosphatase pp1, Pyruvate carboxylase, Aconite hydratase mitochondrial, Protein transport protein SEC23, Ubiquitin-protein ligase peroxin 12, Translocase tim8, Fimbrin and a RING-type domain-containing protein.

EXTRACELLULAR PROTEOMIC ANALYSIS

The shotgun proteomic analysis was also carried out in concentrated cell-free supernatants of *A. loubieri* M12 after 48 h of incubation in the presence and absence of copper in the culture medium. The analysis allowed us to identify 60 proteins in the cell-free supernatants obtained without copper and 106 proteins in the presence of the metal. As mentioned before, the location of the identified proteins was predicted by CELLO v2.5. From this prediction, only nine proteins in the absence of the metal and 12 proteins in the presence of cooper where found to be probably secreted into the extracellular space (**Table 3**). The rest of the identified proteins are assumably present in the extracellular space as result of lysis or cell disruption, common processes in microbial cultures.

Most of the extracellular proteins detected in the control group in *A. loubieri* M12 are involved in carbohydrate metabolism. Likewise, extracellular proteins with peptidase activity and proteins with no characterized functions were detected. When analyzing the proteins of the group exposed to the metal, proteins capable to sequester bivalent ions were identified, such as Glyoxal Oxidase, a mononuclear oxidase with the ability to complex Cu(II) and responsible for the hydrogen peroxide production. This substance can contribute to the oxidative stress that the microorganism demonstrates when facing the metal. Other proteins capable of binding bivalent metal ions are LigB domain containing proteins,

Extracellular metalloproteinase MEP5 and DUF1996 domain-containing proteins, which are involved in the stress response. Other five proteins with no characterized functions were detected in the extracellular space in the presence of the metal.

Additionally, when analyzing those proteins identified in the cell-free supernatants obtained in the presence of copper with no probability to be secreted into the extracellular space, according to CELLO v2.5, some of them present bivalent metal ions binding sites. Interestingly, a specific response to the copper presence was detected in cell-free supernatants of *A. loubieri* M12 exposed to the metal. Some of these proteins are involved in the transport of copper ions into and/or out of the cell, such as Cytosolic copper metallochaperone (A0A427Y9X1), Mitochondrial copper homeostasis protein (A0A427XGC2), and Copper chaperone (A0A427Y1Y0), which play a fundamental role in the detoxification metabolism against reactive oxygen species. Another interesting detected protein is the High-affinity iron permease/copper-dependent ferroxidase (A0A427XKW9), which may be involved in the transport of Cu(II). Additionally, other identified proteins capable of binding bivalent cations are Methionine-synthesizing 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase(A0A427XJJ3), Oxidored_q6 domain-containing protein (A0A427XCV8), and Ca(+2)-dependent cysteine protease (A0A427Y694). These results demonstrate that proteins located in the extracellular space fulfill essential functions regarding the sequestration, transport and uptake of Cu(II) ions specifically in *A. loubieri* M12.

DISCUSSION

ISOLATION OF A HEAVY METAL-RESISTANT MICROORGANISM

In this study, *Apiotrichum loubieri* M12 was isolated from sediments of the AMD-affected environment located in San Luis (Argentina), characterized by low pH values and high concentrations of heavy metals and sulfate ions, when compared to unaffected environments (Bonilla et al. 2018). The microorganism showed tolerance to high concentrations of Fe(II), Cu(II) and Cr(VI) and was able to remove copper from liquid culture medium. In the literature, there are many works that demonstrate the ability of *Apiotrichum* genus members to tolerate and remove heavy metals (Bajgai et al. 2012), being Cu one of the most documented metals. Among these works, we can mention the one carried out by Vadkertiová and Sláviková (2006) who analyzed the tolerance of *A. pullulans* against Zn, Cu, Ni and Cd. Georgieva et al. (2010) reported the *A. cutaneum* R57 tolerance to Cu(II), Cd(II) and Cr(VI) and they showed that this microorganism removed 42% of 0.05 mM Cu(II) from aqueous solutions after 24 h. Our results showed

that *A. loubieri* M12 was able to grow in the presence of 0.6 mM Cu(II) (40 µg mL^{-1}), a concentration 12 times greater than that used by the authors, and showed a removal capacity close to 35% after 48 h. Regarding growth analyses in the presence of heavy metals, Lazarova et al. (2014) showed that the presence of 3 mM Cu(II) enhanced the growth of *A. cutaneum* R57 (167%). By contrast to these results, we observed a 60% growth decrease in *A. loubieri* M12 when it was exposed to 0.6 mM Cu(II).

Other works also relate *Apiotrichum* genus members to contaminated sites. For example, Muñoz et al. (2012) isolated microorganisms with heavy metal-biosorption capacity in Spain. Among the isolated microorganisms tolerant to Pb(II), Zn(II) and Ag(I), they identified three *Apiotrichum* genus members: *Apiotrichum* sp. 1L1, *Apiotrichum*sp. 4S3 and *Apiotrichum* sp. 4L2. Furthermore, Vadkertiová et al. (2016) isolated and identified *A. porosum* and *A. laibachii* from an abandoned mining area and studied their tolerance to chemical compounds, including heavy metals such as Cd and Zn. In parallel, Ramos-Garza et al. (2016) isolated *Apiotrichum* members from soils contaminated with heavy metals. The microorganisms isolated by these authors were sensitive to the Cu(II) presence, tolerating a maximum of 0.5 mM of this metal.

MICROELEMENTAL COMPOSITION OF MICROBIAL BIOMASS EXPOSED TO Cu(II)

The analysis of the biomass exposed to copper through SEM-EDS revealed the presence of the metal at 4.09 % uniformly distributed in the cells and, at the same time, important variations in the microelemental composition of the biomass. In line with the exposed in this work, SEM-EDS has been applied for heavy metal-removal studies in competent microorganisms. For example, SEM-EDS analyses showed uniform distribution of chromium in *Aspergillus niger*, being the cell wall the main cellular portion involved in the accumulation of the metal (Srivastava and Thakur 2006). Shin et al. (2012) also demonstrated the presence of Pb in the biomass of *Bacillus* sp. MN3-4. In the same line of research, Damodaran et al. (2013) analyzed *Galerina vittiformis* biomass exposed to Pb, Cu, Zn and Cd, but only detected Cd peaks through SEM-EDS. From those results, the authors proposed intracellular bioaccumulation of Pb, Cu and Zn rather than adsorption of the cell surface. In this sense, the authors affirmed that detection of metal peaks in an indicator for cell surface adsorption. Another example of SEM-EDS application is the work carried out by Syed and Chinthala (2015), who demonstrated the adsorption of Cd, Cu and Pb on the surface of three *Bacillus* strains. Vicentin et al. (2018) demonstrated by SEM-EDS the *Cupriavidus*

293 *necator* ability to remove Cd, Cu and Zn through adsorption on the cell surface and also showed
294 intracellular uptake in the case of Cu and Zn.

295 The present work also showed the influence of copper on the microelemental of *A. loubieri* M12 biomass,
296 which presented a lower proportion of K, while N and Mg were detected. Some authors affirm that ion
297 exchange mechanisms on the cell surface can be involved in the heavy metal-removal from aqueous
298 solutions in resistant organisms. For example, Shinde et al. (2012) proposed that the Ni(II) removal by
299 *Yarrowia lipolytica* involves an ion exchange mechanism on the yeast cell surface. The disappearance of
300 the K peaks was reported by Xu et al. (2012) when studying the Cd biosorption in *Penicillium*
301 *chrysogenum* and they related this disappearance with an ion exchange mechanism as well.

302 In *Saccharomyces cerevisiae* against Cu, Sun et al. (2015) applied SEM-EDS and found an important
303 influence of the metal on the microelemental biomass´ composition, since K peak was undetectable in the
304 biomass exposed to the metal and N appeared in the presence of copper. The authors proposed Cu
305 adsorption coupled to K release while N detection was associated with the possible complexation between
306 Cu(II) and compounds of the culture medium. In the present work, an important Cu:K relation is observed
307 in *A. loubieri* M12 as well as the appearance of the N peak, which can be perfectly related to the stated by
308 these authors. Sheng et al. (2016) published the Cd accumulation capacity in the *Lactococcus lactis*
309 membrane, indicating that the electronegativity of elements such as N can be responsible for the observed
310 biosorption. In this case, the presence of Mg was also detected in the spectra, in agreement with the
311 observed in the present study. Contrary to our work, Sivaperumal et al. (2018) detected K but not Mg in
312 *Nocardiopsis* sp. 13H during adsorption of Cs.

313 **PROTEOMIC ANALYSIS**

314 The present study applied proteomic techniques at the intra and extracellular levels to analyze the
315 metabolic response of *A. loubieri* M12 when faced with 40 μg mL^{-1} Cu(II) after 48 h of culture, a time
316 correspondent to the exponential growth phase. As strategy to solve the lack of specific databases at the
317 repository sites for the microorganism, we used available databases for orthologous microorganisms
318 (Bonilla et al. 2020 and 2021). It is important to highlight that proteomic databases robustness are in the
319 center of discussion in different fields of study (Subba et al. 2019).

DIFFERENTIAL EXPRESSION OF INTRACELLULAR PROTEINS IN THE PRESENCE OF Cu(II)

The presence of copper induced differential expression of intracellular proteins involved in wide variety of metabolic processes in *A. loubieri* M12. In concordance with our work, the carbohydrate metabolism and the protein biosynthesis have been affected by the presence of heavy metals in *Cyanothece* sp. CCY 0110 (Mota et al. 2015) and *Oidiodendron maius* (Chiapello et al. 2015). Through proteomic studies, an increased expression of redox stress-related proteins has also been reported as a common response to the presence of heavy metals (Chiapello et al. 2015; Guo et al. 2015; Chen et al. 2015; Ruas et al. 2019). Similar to our results, Ilyas et al. (2016) showed higher expression of proteins involved in energy metabolism and degradation of proteins in *Rhodotorula mucilaginosa*.

Zou et al. (2015) reported intracellular transport proteins in *Sargassum fusiforme* exposed to copper. Likewise, they also detected proteins related to carbohydrate metabolism and oxidation-reduction processes, which were also detected in *A. loubieri* M12 exposed to Cu(II). In our work, Cytochrome c oxidase was identified in the presence of Cu(II). This protein has been related to the Cu(II) resistance in a work published by Inesi (2017).

Shu et al. (2019) stated that the degradation of misfolded proteins can be important for the resistance showed by *Viola baoshanensis* against cadmium. These authors also found a higher expression of trehalose-6-phosphate phosphatase, which participates in the biosynthesis of trehalose in the sucrose metabolism pathway. In our work, trehalose-6-phosphate phosphatase was found to be over-expressed in the presence of copper in *A. loubieri* M12. Trehalose, the product of this protein, has also been related to the Cu(II) resistance, since it acts as an antioxidant agent in excess of ROS and inhibits metal uptake (Benaroudj et al. 2001; Mostofa et al. 2015).

A study carried out on *R. mucilaginosa* AN5 highlights the importance of some of the protein groups that have been found in the present work to deal with the toxicity of heavy metals such as carbohydrate metabolism (glycolytic pathway and Krebs cycle), and regulation proteins involved in replication, transcription and translation for protein synthesis (Kan et al. 2019). *Streptomyces* sp. MC1 exposed to Cr(VI) also presented higher expression of proteins related to protein biosynthesis, oxidation-reduction processes and chaperones (Bonilla et al. 2020).

Some authors have arrived to the conclusion regarding the negative effect of heavy metals for the correct folding of new synthesized proteins, even affecting the refolding of denatured proteins (Chiapello et al. 2015). As stated in previous works, the cells would be forced to degrade the defective proteins, promoting an increased expression of chaperones and degradation proteins (Steurer et al. 2018; Kan et al. 2019; Bonilla et al. 2021). The synthesis of new proteins, with all the biosynthetic cellular machinery involved, becomes necessary to maintain normal cellular functions. The oxidative stress caused by the presence of metals seems to be faced by proteins related to stress response and oxidoreductases. Kinases and phosphatases are also important to deal with the activation or inactivation of enzymes when the cells are exposed to heavy metals (Tiwari and Lata 2018).

As highlighted before, many of the proteins identified in *A. loubieri* M12 in the presence of copper possess metal ion-binding sites. Metalloproteins, which bind transiently to metal ions, have been proposed as key for the tolerance and sequestration of heavy metals (Maret 2010; Lancaster et al. 2014). Chiapello et al. (2015) identified Cetol-acid reductoisomerase as an important protein in tolerance to heavy metals. This protein, which has the capacity to bind +2 metal ions, was over-expressed in the presence of Cu(II) in *A. loubieri* M12. Our work provides new evidence for the role of Cetol-acid reductoisomerase in heavy metal-resistance.

Guo et al. (2015) found that most of the over-expressed proteins in *S. pneumoniae* in the presence of copper presented metal-binding motifs. Proteins with metal-binding sites show high affinity for wide variety of metal ions and the promiscuity of the binding sites can be key for the tolerance to heavy metals, either affecting or not their original activity (Izrael-Živković et al. 2018; Farcasanu and Ruta 2018; Fein et al. 2019). The large number of proteins with metal-binding sites found in the present work in *A. loubieri* M12 exposed to copper agree with the studies previously presented, which expose and discuss the importance of these proteins in the tolerance and/or resistance of different organisms against heavy metals.

DIFFERENTIAL EXPRESSION OF EXTRACELLULAR PROTEINS IN THE PRESENCE OF Cu(II)

In this work, the proteins identified in cell-free supernatants obtained in the presence of copper, which show probability to be secreted into the extracellular space according to CELLO v2.5 (Yu et al. 2004 and

2006), showed proteolytic and oxidoreductase activities, highlighting that many of them present bivalent metal ion binding sites.

To the best of our knowledge, there are only few studies related to identified the differential expression of extracellular proteins when cells are faced with heavy metals (Giner-Lamia et al. 2016). In *A. loubieri* M12 against copper, a hypothetical protein with domains with unknown function 1996 (DUF 1996) was detected. Tong et al. (2016) studied the function of three hypothetical proteins with DUF1996 in *Beauveria bassiana* and demonstrated the importance of the DUF in the tolerance to metals and in the adaptation to environmental changes. Another interesting enzyme detected in the presence of copper in *A. loubieri* M12 is Glioxal oxidase, which has the ability to chelate Cu(II) and is responsible for the production of hydrogen peroxide (Crutcher et al. 2019).

A. loubieri M12 also showed expression of extracellular proteins capable of binding metal ions, such as Extracellular metalloproteinase MEP5 and LigB domain-containing protein. Taking into consideration the importance of proteins with metal-binding sites, Sharma et al. (2018) emphasized the study of copper metalloproteins in *P. syringae* pv. *lapse*. These authors found that of the 45 metalloproteins identified, only five showed probabilities to be secreted into the extracellular space. The five proteins exhibited stress response, transport, protein folding, and proteolysis functions.

Exoproteomics has been defined as the study of all those proteins present in the extracellular space, even if they are secreted by cells or are present in the extracellular space as a result of cell lysis processes (Desvaux et al. (2009). In this way, the exoproteomics recognize the importance that all proteins may have in the tolerance and resistant to heavy metals. It is important to highlight that many of the identified proteins with no probability to be secreted by *A. loubieri* M12 in the presence of Cu(II) possess metal ion binding sites, as occurred in the intracellular space.

Specifically, in the extracellular space of *A. loubieri* M12 in the presence of Cu(II), we detected the presence of two exclusive copper metallochaperones. Copper metallochaperones have the ability to sequester the metal in order to maintain its concentrations at extremely low levels (Bravo-Gómez et al. 2015). One of the functions of these metallochaperones is to deliver the metal to the secretory pathway for the activation of enzymes destined for the cell surface or the extracellular space. They also deliver copper to the mitochondria for the activation of Cytochrome oxidase, a protein that was detected over-expressed in the intracellular space. In the extracellular space and in the presence of metals, proteins

related to heavy metals have been identified, such as copper chaperone CopM or iron-binding protein FutA2. It has even been shown that some metal chaperones respond specifically to the presence of different metals. Copper chaperones have been related to the resistance to this heavy metal, mainly emphasizing the ability to sequester the metal in the extracellular space (Giner-Lamia et al. 2015). Cytosolic copper chaperone has also been suggested as part of the copper resistance system in *S. lividans* (Straw et al. 2018). Most of the proteins detected in the exoproteome fulfill intracellular functions. However, their detailed functions in the extracellular space remain unknown.

CONCLUSIONS

The multidisciplinary approach of environmental issues allows deepening the study of competent biological systems for the remediation of sites affected by anthropogenic activities. In this work, sediments of an AMD-affected environment located in Argentina were used as interest microbial niche for the isolation of the copper-resistant microorganism *A. loubieri* M12. The results obtained in the present work indicate that, when facing the stress caused by abiotic factors, such as the presence of heavy metals, the microorganism is able to uptake copper ions, mainly through the cellular surface. Likewise, the microorganism responds not only by adjusting the intracellular protein expression, but also by adjusting the expression of proteins in the extracellular space. The readjustment of the intracellular protein expression helps the microorganism counteract the metabolic imbalances produced by heavy metal toxicity. Likewise, a differential expression of proteins in the extracellular space may be crucial for the sequestration and transport of the metal, fundamental to reduce the toxic effects that Cu(II) could exert on the cell.

Beside the general responses trigger in the copper presence by the readjustment of the protein profiles, the detection of copper metallochaperones in the extracellular space evidences a specific response of *A. loubieri* M12 to the metal presence. The study of organisms tolerant to high concentrations of metals is important not only for our mechanistic understanding of selective incorporation and/or immobilization, but also for efforts to harness these abilities for bioremediation processes.

Data Availability Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with Ethical Standards

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Conflict of Interest: JOB declares that he has no conflict of interest. EAC declares that he has no conflict of interest. MDP declares that she has no conflict of interest. RAG declares that he has no conflict of interest. LBV declares that she has no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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607 **CAPTIONS**

608 **Fig. 1** Growth of *A. loubieri* M12 in the presence and absence of 40 µg mL^{-1} Cu(II) in the culture medium
609 (**A**). Copper removal capacity of *A. loubieri* M12 exposed to the metal (**B**)

610 **Fig. 2** Surface mapping and microelemental analysis of *A. loubieri* M12 in the absence **(A)** and in
611 presence **(B)** of Cu(II) through SEM-EDS operated at 20kV

612 **Fig. 3** Intracellular proteins identified in *A. loubieri* M12 in the presence (blue) and in absence (red) of
613 Cu(II); the proteins identified as common for both conditions are shown in violet **(A).** Relative abundance
614 using Label-Free Quantification (LFQ) of intracellular proteins detected in the presence (blue) and in
615 absence (red) of Cu(II) **(B)**

616 **Fig. 4** Cellular function of proteins identified in *A. loubieri* M12 exposed to Cu(II)

617 **Online Resource 1** Protein identification by mass spectrometry analysis