31-Jan-2008

Dear Dr. Abate,

I am pleased to inform you that the reviewers have recommended publication of your paper by taking care of their comments. The comments of the referees are included at the bottom of this letter.

Please mark in the revised script where changes have been made and return the paper (see the attached document listing the file requirements for your revision) within TWO WEEKS.

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Thank you for submitting your interesting work to JBM.

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Regards,

Prof. Erika Kothe

Editor-in-Chief, Journal of Basic Microbiology

erika.kothe@uni-jena.de

****

**** Referee(s)' and Editors Comments to Author. (Please note that these comments may have been delivered to us as files. We attach a description at the bottom how to access them.)**

Reviewer: 1

Comments to the Author

"Copper bioaccumulation by the actinobacterium Amycolatopsis sp. AB0"

Albarracin et al. 2007

The authors have presented a manuscript demonstrating that Amycolatopsis sp. AB0 is able to tolerate the presence of high copper concentrations in the culture medium. Copper seems to be bioaccumulated in the cytosolic and cell wall fractions, as well as in an extracellular polymeric substance (EPS). In addition, the authors proposed the involvement of a putative copper P-type ATPase in strain AB0 for the tolerance mechanism.

The results presented in this paper together with previous publications (Albarracin et al. 2005. Chemie der Erde/Geochemistry. 65-S1:7-27) make an interesting story. This group is well established in the field considering other published original papers dealing with this topic. In my opinion this manuscript deserves publication; however, after a minor revision.

Specific comments:

Pag. 9- lines 185-187: The authors stated that "the EPS is characterized of approximately 20 units and it contents only glucose". Considering that a significant amount of copper is sequestered from the medium by the EPS (approx. 40 %), the methodology for the exopolymer analysis should be described in Material and Methods section and not only referenced from the study of other authors.

Reviewer: 2

Comments to the Author

JBM 2007-00360

Copper bioaccumulation by the actinobacterium Amycolatopsis sp. ABO

Albarracin and colleagues describe the accumulation of copper by an actinomycete which according to 16S rRNA belongs to the genus Amycolatopsis. The strain ABO, which was isolated from copper contaminated sediments showed good bioadsorption of the metal when compared to other bacteria reported in the literature. Copper biosorption was determined by a silver staining procedure complemented with subcellular fractioning. Furthermore, a putative copper P-type ATPase sequence was obtained which showed 71% homology a Nocardia strain.

My main question is why the authors did not use a reference microorganism to compare their studies. Are the methods used in the works presented in Table 2 the same as the methods used by the authors for a direct comparison?

The manuscript needs some revision.

Abstract

Page 2, line 34

To our knowledge this is the first report of the presence of copper P-type ATPase genes in the Amycolatopsis genus.

Page 3, line 53: metal is not completely available....

Page 4, line 77: valid technique

Page 9, line 176: inespecific

Page 10, line 199: ....copperis up took? I do not understand this phrase.

Page 238, line 238: However, amplification in the optimized conditions was not detected in the sensitive strain DNA. This sentence is confusing. Which sensitive strain?

Page 13, line 259: these regards?

Review Editor: Vobis, Gernot

Comments to the Author:

I consider, that the article "Copper bioaccumulation by the actinobacterium Amycolatopsis sp. ABO" is a highly relevant contribution to the corresponding subject. The two reviewers recommended a minor revision from different standpoints.

I support the arguments of both to improve the manuscript.

***** Attachment: viewing Reviewers and Editors comments delivered as files*****

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Copper bioaccumulation by the actinobacterium *Amycolatopsis* sp. AB0

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Running headline. Copper bioaccumulation by *Amycolatopsis* sp. AB0.

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Abstract

Amycolatopsis sp. AB0, a copper resistant actinobacterium isolated from polluted sediments, has shown high copper specific biopsortion ability (25 mg g^{-1}). Two approaches were used to confirm metal accumulation in growing cells of *Amycolatopsis* sp. AB0; we performed subcellular fractioning assays which showed that the retained copper was associated with the extra-cellular fraction (exopolymer, 40%), but mainly within the cells. Intracellular distribution of copper was: 86% in the cytosolic fraction, 11% at the cell wall and 3% associated with the ribosome/membrane fraction. Its copper bioaccumulation ability was corroborated by using silver enhanced staining of copper with the Timm´s reagent technique, which has not been used to detect metal deposits in bacteria before. In addition, we constructed specific oligonucleotides for targeting genes coding for copper P-Type ATPases that could be involved in the copper uptake ability of this strain. A 607 bp DNA fragment was amplified and sequenced from *Amycolatopsis* sp AB0. BLAST search analysis showed 71% protein homology of the deduced sequence with a putative cation-transporting ATPase of *Nocardia farcinica* and 65% with a copper translocating ATPase of *Mycobacterium flavescens*. To our knowledge this is the first report of the presence of copper P-type ATPase genes in the *Amycolatopsis* genus.

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Key words: copper bioaccumulation, actinobacteria, *Amycolatopsis*, bioremediation

Introduction

Copper (Cu) is an essential element for all animals and plants required for growth and reproduction [1]. Copper released to the environment, mainly by mining and industrial activities, tends to accumulate in soils, plants and animals, increasing their concentrations at superior levels of food chains [1, 2]. In Argentina, for instance, the legal limit permissible for copper in drinking water is 1 mg L^{-1}, whereas European Union limit is higher, 3 mg L^{-1} [1]. Ecotoxicologically relevant effects have been reported for higher concentrations, including gastrointestinal effects which occurred in patients with whole blood copper levels of 2.9 mg mL^{-1}, whereas whole blood copper levels in excess of 7.9 mg mL^{-1} were observed to cause jaundice, renal dysfunctions, or shock [1].

Microorganisms able to accumulate and immobilize toxic metals are considered key tools for the bioremediation of polluted environments [3]. Recent progress has been made studying metal resistance in actinobacteria isolated from polluted areas [4-12]. However, copper resistance in actinobacteria has been little studied [7, 9, 11, 13]. In previous works, the reported copper resistance levels for actinobacteria were obtained by testing in agar complex media [4-5, 14] but, in this kind of assays, metal is not completely available to the microorganisms which could lead to assume erroneous, high levels of resistance. Moreover, there is not enough specific information on the mechanisms involved in the resistance to copper by actinobacteria as only a mechanism of copper sulphide precipitation was proposed [15]. In a previous screening study, the actinobacterium *Amycolatopsis* sp. AB0 has showed to be the most copper resistant strain among 50 actinobacteria isolated from polluted and non–polluted sediments [9]. Nevertheless, a better understanding of the

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183 mechanisms responsible for its copper resistance phenotype is needed to develop novel
184 bioremediation processes for copper polluted areas [9].
185 For the safe management of copper, prokaryotic and eukaryotic cells have developed
186 various mechanisms. The copper ATPases are a sub-class of the P-type ATPases also
187 called CPx-type ATPases that have been found in homeostatic systems of virtually every
188 organism, from *Escherichia coli* to humans and include homologues that are implicated in
189 the human Menkes and Wilson diseases [16]. The existence of this kind of pumps has not
190 been reported in the genus *Amycolatopsis*.
191 One approach widely used to detect copper accumulation in cells of higher eukaryotes
192 including humans is electron microscopy coupled with histochemical methods [17-18].
193 Nevertheless, these methods have not been used to detect metal accumulation in
194 microorganisms where generally direct visualization of metals by electron microscopy in
195 bacteria has been used [19-20]. In some cases, X-ray microanalysis has been proposed as
196 another approach for visualization of heavy metal deposits in bacteria [21-23]. Among the
197 most common histochemical techniques, the Timm´s reagent histochemical treatment has
198 proved to be the most sensitive procedure for diagnosis of copper accumulation in liver
199 when compared to orcein and rhodanine methods [24]. On the other hand, subcellular
200 fractioning is a very common and valid technique for detecting metal distribution within
201 the cell as it has been successfully applied to prove Cr and Cd accumulation in
202 *Streptomyces*, *Pseudomonas stutzeri* and *Bacillus subtilis*, respectively [8, 25-26].
203 The aim of this work was to demonstrate the copper bioaccumulation ability of
204 *Amycolatopsis* sp. AB0 using silver enhanced staining by the Timm´s reagent cytochemical

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205 method coupled with subcellular fractioning assays in biosorption experiments. In addition,
206 we constructed specific oligonucleotides for targeting genes coding for copper P-Type
207 ATPases that could be involved in the copper uptake ability of this strain.

208 **Materials and methods**

209 **Strain, media and culture conditions.** *Amycolatopsis* sp. AB0 strain (PROIMI Collection,
210 NCBI accession number: DQ886938) previously isolated from copper polluted sediments
211 [9], was used in this study. 100 µl of spore suspension (1x 10^9 CFU mL^{-1}) prepared as
212 described before [9] were inoculated in batch cultures (30 ml) of Minimal Medium (MM:
213 in grams: L-asparagine, 0.5; K_2HPO_4, 0.5; $MgSO_4$. $7H_2O$, 0.2; $FeSO_4$. $7H_2O$, 0.01; glucose,
214 10.0 per liter; supplemented with Cu (II) 32 mg L^{-1} and pH 7). *Amycolatopsis* sp. AB0
215 cultures without copper were used as controls.

216 The cultures were incubated at 30 ºC for 7 days in an orbital shaker at 100 rpm. Samples
217 were taken at 3, 5 and 7 days; cells were collected by centrifugation at 3,000 *g* for 10 min
218 at 4 ºC and washed twice with distilled water. The resulting cell pellet was used to prepare
219 sections for electron microscopy. Independent duplicate and triplicate cultures were used to
220 perform the metal analysis by nitric acid digestion and biomass determination (105 °C).

221 **Subcellular fractioning.** In order to analyze the copper distribution within the cell,
222 stationary-growth phase culture samples of *Amycolatopsis* sp. AB0 grown in a copper
223 supplemented MM were taken. Aliquots were removed for whole cell copper sorption
224 analyses by nitric acid digestion while the remaining cells were broken in a French pressure
225 cell at 20,000 psi (1,38 x 10^5 KN m^{-2}) for sub-fractional sorption studies by differential

centrifugation as described previously [8]. Samples of each fraction were removed for copper analysis. The exopolymer was processed as previously described [27]. Agregar descripción

Ultrastructural determination of copper deposits. Subcellular localization of copper was examined ultracytochemically using a modified procedure of Timm´s reagent method for metal staining [17]. Pellets of *Amycolaptosis* sp. AB0 cells cultivated with and without copper were fixed in a solution containing 2% para-formaldehyde, 0.1% glutaraldehyde adjusted to pH 7.4 in 0.1 M phosphate buffer for 3 h at 4 ºC. Samples were then placed in 15% trichloroacetic acid (TCA) solution for 15 min, rinsed three times with distilled water and stained using Timm's reagent (18% arabic gum, 2% hydroquinone, 0.1% silver nitrate in 0.03 M citrate buffer pH 3.8 for 30 min). After washing with saline phosphate buffer (PBS), samples were incubated overnight in 1% osmium tetroxide buffered with PBS, dehydrated in a graded ethanol series, exchanged through acetone and embedded in Spurr resin (Pelco, Int., USA). Ultrathin sections stained with uranyl acetate and lead citrate were examined under transmission electron microscope, TEM (Zeiss EM 109). The copper ion precipitates formed by the treatment with silver nitrate (Timm's reagent) were observed as electron opaque granular deposits. Parallel samples cultivated without copper served as controls.

Additionally, AB0 cells cultivated with and without copper were processed without staining: fixation (glutaraldehyde 3% in phosphate buffer 0.1 M pH 7.4) for 3 h at 4ºC followed by osmium tetroxide (1% in the same buffer). Samples were then dehydrated in an alcohol series transferred to acetone and embedded in Spurr resin.

Copper determination. Aliquots of pellets from the subcellular fractions were removed, weighted and wet-digested with 10 N HNO_3 by heating, the HNO_3 concentration of the clear residual liquid was adjusted to 1 N by addition of double-distilled water [9]. The amounts of copper in the residual liquid were analyzed by a PQ3-S (ThermoElemental, Winsford, UK) inductively coupled plasma mass spectrometer, ICP-MS. The relative standard deviations were always lower than 0. 5%.

DNA preparation. *Amycolatopsis* sp. AB0 was grown in liquid MM for 4 days. The pellets were collected by centrifugation at 3,000 *x g* for 10 min at 4ºC and washed twice with distilled water. Total genomic DNA extraction was carried out according to the lysozyme-treatment modified for actinobacteria as previously described [28].

Primer design and PCR amplification. Primers were designed using the Workbench Biology online program Version 3.2 (San Diego Super Computer Center) and DNAMAN software 5.2 (Lynnon Biosoft, Quebec, Canada) to target copper P-types ATPases conserved domains. Proteins and their respective accession numbers used to create the oligonucleotides are shown (Table 1).

Total genomic DNA was used as templates for PCR amplification. Copper sensitive *Streptomyces* sp. AB2C from PROIMI Collection [9] and sterile distilled water were used as negative controls. Amplifications were performed in 25 µL reaction volumes using primers: PT-2F Forward (5´-GAC AAG ACC GGC ACC -3´) and PT-3R Reverse (5´-GTC GTT SAT GCC GTC GCC GAC- 3´). Primer PT-2F targets a conserved DKTGT(L/I)T signature sequence associated with the phosphorylation of bacterial P-type ATPases [20].

269 Amplifications reactions were carried out in an automated thermal cycler (Perkin-Elmer,
270 model 9700, Applied Biosystems). PCR products were run in 1.0% agarose gel, stained
271 with ethidium bromide and then visualized using an Image Analyzer Gel Doc BIORAD.
272 The amplified fragments were purified with Prep-A-Gene DNA Purification Systems (Bio-
273 Rad Laboratories, Hercules, CA, USA), according to the manufacturers protocol. The 1 Kb
274 DNA Ladder (Promega, Madison, WI, USA) was used as a molecular weight marker.
275 **DNA sequencing and analysis.** DNA sequencing on both strands was performed using the
276 dideoxy chain termination method with an ABI prism DNA Analyzer 3730xl using the ABI
277 Prism Big Dye Terminator Cycle Sequencing Ready Reactions kit (Applied Biosystems,
278 Foster City, CA, USA). The sequences were analyzed using the Workbench Biology online
279 program Version 3.2 (San Diego Super Computer Center) and DNAMAN software 5.2
280 (Lynnon Biosoft, Quebec, Canada).
281 **Nucleotide sequence accession numbers.** The nucleotide sequences reported in this paper
282 have been deposited in Genbank under accession numbers DQ886938 and EF177831.
283 **Statistical analyses.** Statistical analyses were conducted using the Microcal™ Origin
284 Working Model Version 6.0. Paired *t*-test and variance analysis were used with a
285 probability level of $p<0.05$.
286 **Results and discussion**
287 **Biosorption of copper by growing cells of *Amycolatopsis* sp. AB0.**
288 *Amycolatopsis* sp. AB0 isolated from sediments polluted with copper from a drainage
289 channel could tolerate high copper concentrations (1000 mg L^{-1}) when testing by a
290 semiquantitative assay [9]. In this study, its growth in liquid MM supplemented with 32 mg

291 L^{-1} of copper was studied with regard to metal absorption in biomass from the culture
292 supernatant. Copper specific biosorption of the tested strain increased with incubation time
293 up to a maximum level at day 5, when apparently an equilibrium phase is produced (Fig.
294 1). *Amycolatopsis* sp. AB0 showed the highest biosorption value for growing cells (25 mg
295 g^{-1}) among other microorganisms previously proposed for bioremediation processes (Table
296 2). Microbial dead biomass from the compared microorganisms evidence higher
297 biosorption values than growing cells of *Amycolatopsis* sp. AB0 (Table 2). But in these
298 cases, copper retention from the culture medium is only dependent on the physical
299 characteristics of the cell wall or envelopes of the dead cells and in this sense, the process
300 is inespecific and may not guaranteed the stabilization of the toxic; moreover, lower
301 efficiencies have been observed when this kind of bioabsorbents were applied to
302 decontamination strategies [34]. In contrast, a bioremediation strategy using AB0 strain
303 growing cells may take advantage of its copper resistance and cellular metabolism to
304 continuously uptake and immobilize the bioavailable toxic in a specific-permanent manner.

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305 **Subcellular distribution of biosorbed copper in *Amycolatopsis* sp. AB0**

306 Subcellular fractioning showed the distribution of biosorbed copper in *Amycolatopsis* sp.
307 AB0 after 7 days of growth (Table 3). Interestingly, copper initially sequestered from the
308 culture medium has showed to be complex intracellularly, and also associated with an
309 exopolymer fraction (Table 3). The exopolymer, that was characterized as an
310 exopolysaccharide (EPS) of approximately 20 units and constituted only by glucose, has
311 been observed during the growth of *Amycolatopsis* sp. AB0 in MM cultures produced after
312 7 days. Moreover, the EPS harvested from a copper culture has shown to be green while

313 the control culture EPS is white or cream colored. This indicates a copper binding property
314 of the EPS as has been proposed previously for other microorganisms [35-36]. Within the
315 cell, copper accumulation was found mainly in the cytosol (86%) with lower copper
316 content deposit at cell wall (11%) or membranes (3%) (Table 3). These results are in
317 accordance with intracellular copper deposits stained by the cytochemical method (Fig. 2).
318 The requirement of living systems to both, acquire and reject metals, has led to the
319 selection of a whole repertoire of mechanisms of interaction which ensure the adaptation of
320 microorganisms to a changing and frequently hostile environment [37]. In this way,
321 *Amycolatopsis* sp. AB0, isolated from highly copper polluted sediments (600 mg Kg^{-1}) [9]
322 seems to cope with copper in two ways. The observed EPS may act as an extra-cellular
323 biosorbent [35-36] but also it is evident from the subcellular assay, that copper is uptaken
324 with its further bioaccumulation within the cell. These findings concurred with the copper
325 biopsortion behaviour observed before (Fig. 1). Copper bioaccumulation in a non-toxic
326 way within the cell can be achieved by copper-binding proteins such as metallochaperones
327 [38]. Preliminary work used gel filtration to detect intracelullar storage compounds in
328 *Amycolatopsis* sp. AB0 [39]. A completely different pattern of proteins was observed from
329 cytosol for cells grown with or without copper in the MM. Moreover, high copper
330 concentrations were found in the obtained fractions [39]. This may indicate that copper
331 specific binding-proteins, like the ones mentioned above, are differentially expressed for
332 storage and detoxification in *Amycolatopsis* sp AB0. Further work dealing with the
333 screening of this kind of proteins in *Amycolatopsis* sp. AB0 will be needed to elucidate
334 those mechanisms.

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335 **Ultracytochemical detection of copper deposits in *Amycolatopsis* sp. AB0**

336 Detection of heavy metal binding sites in cells was facilitated only after suitable
337 histochemical methods were adapted. Timm´s technique was modified and adapted to serve
338 the requirements of electron microscopy [17] and, in combination with biochemical
339 determinations of heavy metal content, has served to precisely detect the distribution of
340 these elements in cells of higher eukaryotes [18, 24]. This method has not been used to
341 detect metal deposits in unicellular organisms even though microbial metal resistance
342 processes and their application for bioremediation technologies has been a major focus in
343 biotechnology for the past two decades [3, 37]. In this work, this low cost and high
344 sensitivity staining method was applied for the first time in bacteria to detect heavy metal
345 accumulation with no need of expensive X-ray microanalysis equipment.

346 The precipitated copper formed by silver nitrate treatment was detected as electron opaque
347 granular deposits in cells of *Amycolatopsis* sp. AB0. After 3 days of growth, staining was
348 observed mainly at the cell wall, with scarce intracellular granules (Fig. 2A) and some
349 reaction deposits associated with the EPS. At days 5 and 7, the copper accumulation was
350 significantly detected with cells full of granular deposits (Fig. 2B and C, respectively).
351 Cells cultivated in a copper-free medium did not show clear deposits (Fig. 2D). It is
352 important to notice that even when cells were full of copper deposits (Fig. 2C), it was not
353 detected any cellular abnormal morphology or lyses processes, thus, confirming the copper
354 resistance ability of this strain [9]. The ultracytochemical observations agree with the
355 results obtained in the biosorption (Fig. 1) and subcellular assays (Table 3).

Evidence of genes coding for a copper P-type ATPases in *Amycolatopsis* sp. AB0. The copper bioaccumulation phenotype displayed by *Amycolatopsis* sp. AB0 may be related to the presence of specific copper P-type ATPases in the cell membrane as was observed for other resistant microorganism [16]. Using primers specifically designed to target copper P-type ATPases conserved domains, a 607 bp DNA fragment was amplified and sequenced from *Amycolatopsis* sp. AB0 (Accession number: EF177831). However, amplification in the optimized conditions was not detected in the *Streptomyces* sp. AB2C (sensitive strain) DNA. The protein sequence was deduced and compared to the protein database. BLAST [40] search analysis showed 71% homology of the deduced protein of *Amycolatopsis* sp. AB0 with the protein sequence of a putative cation-transporting ATPase of *Nocardia farcinica* IFM 10152 (GenBank accession number YP121974), 64% with a probable copper-exporting ATPase of *Rhodococcus* sp. RHA1 (GenBank accession number YP709015) and 65% with a E1-E2 type: copper - heavy metal translocating P-type ATPase of *Mycobacterium flavescens* PYR-GCK (GenBank accession number ZP01192042), suggesting that this kind of pumps are present in *Amycolatopsis* sp. AB0.

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Concluding remarks

In this work, a copper bioaccumulation phenotype involving metal biopsortion by EPS and intracellular accumulation has been revealed in the actinobacterium *Amycolatopsis* sp. AB0 by several approaches. Intracellular copper bioacccumulation has been corroborated by the Timm´s reagent cytochemical technique which has not been used to detect copper accumulation in unicellular organisms before. In addition, we tried to identify the putative molecular determinants responsible for this phenotype by DNA sequencing of a PCR

product and as a result, we hypothesized about the existence of a copper P-type ATPase in *Amycolatopsis* sp. AB0 which may be used by the cell for up taking copper. This is the first report of such genes in the genus *Amycolatopsis*.

These findings represent an important contribution to the area of copper resistance in actinobacteria and their potential biotechnological use since so far, little information on this subject has been accounted. *Amycolatopsis* sp. AB0 bioaccumulation capacity can be used as an alternative for the removal and recovery of copper from contaminated wastes. The particular morphological characteristics of this strain such us forming high branched substrate filaments and resistant spores may help to concentrate and immobilize the metal within the cell. The final result will be a decrease in the overall metal bioavailability in the copper polluted soil or effluent.

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Further work dealing with the molecular characterization of the EPS and intracellular copper binding proteins involved in the copper bioaccumulation ability of *Amycolatopsis* sp. AB0 will be needed for applying this actinobacterium to copper polluted soils or effluents as safe and effective biotechnologies.

Acknowledgements.

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The authors gratefully acknowledge assistance of Lic. Manuel Siñeriz Louis, Mr. Guillermo Borchia, Dr. Fitzsimons and Dr. Dirk Merten. We also thank Dr. G. Castro for advice and useful discussions.

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512 **Table and figure legends**

513 **Figure 1.** Copper specific biosorption (mg Cu g^{-1} of dry weight) of *Amycolatopsis* sp. AB0
514 cells grown for 7 days in MM supplemented with Cu (II), 32 mg L^{-1}.

515 **Table 1.** Proteins and their respective accession numbers used to create the
516 oligonucleotides. Protein sequences were aligned and conserved aminoacids were detected.
517 Primers were designed using the *Amycolatopsis methanolica* codon usage table from the
518 codon usage database available on line (*www.kazusa.or.jp/codon*).

519 **Table 2.** Copper biosorption/uptake efficiency of growing cells of *Amycolatopsis* sp. AB0
520 and of dead or growing cells of other microorganisms.

521 **Table 3.** Distribution of biosorbed copper in cells of *Amycolatopsis* sp. AB0 grown for 7
522 days in MM supplemented with Cu (II), 32 mg L^{-1}. SD: standard deviation.

523 **Figure 2.** Micrographs obtained from electron microscopy of *Amycolatopsis* sp. AB0
524 grown with Cu (II), 32 mg L^{-1}, in the MM at 3 (**A;** 140,600x), 5 (**B**; 82,640x) and 7 days
525 (**C**; 82,640x) using Timm´s reagent staining method [17]. Arrows indicate copper deposits
526 at the cell wall. **D**. Control cell cultivated in MM without copper (234,300x).

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