Revista Brasileira de Ciência do Solo

Division – Soil Processes and Properties | Commission – Soil Biology

Tolerance to and Accumulation of Cadmium, Copper, and Zinc by *Cupriavidus necator*

Rayssa Pereira Vicentin[(1)], Jessé Valentim dos Santos[(1)], Cláudia Regina Gontijo Labory[(2)], Amanda Monique da Costa[(3)], Fatima Maria de Souza Moreira[(4)*] and Eduardo Alves[(5)]

[(1)] Universidade Federal de Lavras, Departamento de Ciência do Solo, Programa de Pós-Graduação em Microbiologia Agrícola, Lavras, Minas Gerais, Brasil.
[(2)] Universidade Federal de Lavras, Departamento de Ciência do Solo, Programa de Pós-Graduação em Ciência do Solo, Lavras, Minas Gerais, Brasil.
[(3)] Universidade Federal de Lavras, Curso de Engenharia Ambiental e Sanitária, Lavras, Minas Gerais, Brasil.
[(4)] Universidade Federal de Lavras, Departamento de Ciência do Solo, Lavras, Minas Gerais, Brasil.
[(5)] Universidade Federal de Lavras, Departamento de Fitopatologia, Lavras, Minas Gerais, Brasil.

ABSTRACT: Preliminary results of *in vitro* experiments with multicontaminated soils and solid media indicated that nodulating diazotrophic bacteria of the genus *Cupriavidus* are promising for the remediation of contaminated environments due to their symbiosis with legumes and metal tolerance. Thus, strains of *Cupriavidus* spp. (LMG 19424^T, UFLA 01-659, UFLA 01-663, and UFLA 02-71) were tested for their ability to tolerate and bioaccumulate cadmium (Cd), copper (Cu), and zinc (Zn) in Luria-Bertani broth. Changes in the growth pattern of *Cupriavidus* strains in the presence or absence of heavy metals were analyzed by scanning electron microscopy and metal allocation by transmission electron microscopy, to clarify the mechanisms of bioremediation. Highest tolerance was detected for strain UFLA 01-659 (minimum inhibitory concentration of 5, 4.95, and 14.66 mmol L^{-1} of Cd, Cu, and Zn, respectively). Among the removal rates of the metals tested (9.0, 4.6, and 3.2 mg L^{-1} of Cd, Cu, and Zn, respectively), the bacterial activity was clearly highest for Cd. The efficiency of strain UFLA 01-659 in removing the heavy metals is associated with its high biomass production and/or higher contents of heavy metals adsorbed and absorbed in the biomass. In response to the presence of heavy metals in the liquid culture medium, the bacteria produced exopolysaccharides and small and aggregated cells. However, these responses varied according to the strains and heavy metals. Regarding allocation, all heavy metals were adsorbed on the cell wall and membrane, whereas complexation was observed intracellularly and only for Cu and Zn. These results indicate the possibility of using *C. necator* UFLA 01-659 for remediation in areas with very high Cd, Cu, and Zn contents.

Keywords: heavy metals, electron microscopy, diazotrophic bacteria, bioremediation, tolerance mechanisms.

*** Corresponding author:**
E-mail: fmoreira@dcs.ufla.br

Received: March 9, 2017

Approved: November 8, 2017

How to cite: Vicentin RP, Santos JV, Labory CRG, Costa AM, Moreira FMS, Alves E. Tolerance to and accumulation of cadmium, copper, and zinc by *Cupriavidus necator*. Rev Bras Cienc Solo. 2018;42:e0170080.
https://doi.org/10.1590/18069657rbcs20170080

INTRODUCTION

In environments contaminated with heavy metals, diverse bacteria are found that are tolerant to high heavy metal concentrations *in vitro* and have a high capacity to remove these metals (Moreira et al., 2008; Chien et al., 2011; Andreazza et al., 2012). This ability of the bacteria is expressed through different mechanisms, such as efflux pumps, thione production, and enzyme activity (Zoropogui et al., 2008; Hynninen et al., 2009), varying according to the strains and metal evaluated.

Efflux pump activity is one of the most important mechanisms of heavy metal tolerance, allowing cells to extract metals taken up through ATP decomposition. Wang et al. (2015) sequenced the genome of *Cupriavidus gilardii* CR3 (bacteria with multiple metal resistance) and identified diverse *operons* that codify efflux pumps, such as *czc* (Cd^{2+}, Zn^{2+}) and *cus* (Cu^{+}, Ag^{+}) linked to heavy metal resistance. Behavioral variations in strains of the same genus were observed by Bianucci et al. (2011), who found that not all the four strains of *Bradyrhizobium* were able to accumulate Cd in the biomass, and that in strains with higher Cd tolerance and biomass contents, the glutathione (antioxidant) levels increased in response to the presence of the metal.

Biofilm formation, another mechanism linked to bioaccumulation, was investigated by Chien et al. (2013), who found that strain EJ01 (*Pseudomonas* sp.) had a greater ability of removing Cd and nickel (Ni) than the mutant strain m-3055, with poor capacity of biofilm formation.

In addition to the direct removal of heavy metals by adsorption and incorporation into plant biomass, some bacteria may contribute indirectly by their relationship with plants grown in contaminated environments. Strains of *Pseudomonas* inoculated on sunflower and corn plants, for example, reduced Cu toxicity and induced greater phytoextraction of this metal in both crops (Li and Ramakrishna, 2011). The greater heavy metal uptake of plants inoculated with *Pseudomonas koreensis* was attributed to solubilization of the metals in the rhizosphere (Babu et al., 2015). Heavy metal tolerance of diazotrophic bacteria was also reported in symbiosis with leguminous plants, where the bacteria can contribute to plant establishment in contaminated soils, favoring an increase of N content in these environments (Mahieu et al., 2011; Ferreira et al., 2013). The genus *Cupriavidus* has shown promising results in various studies on the contribution of nodulating diazotrophic bacteria to phytoextraction of toxic metals. For example, Klonowska et al. (2012) identified *Cupriavidus taiwanensis* isolates with high tolerance to Ni, Zi, and Cr, and high efficiency in biological N_2 fixation (BNF) in symbiosis with *Mimosa pudica*. In another study, inoculation with strain TJ208 (*Cupriavidus taiwanensis)* increased the biosorption efficiency of *Mimosa pudica* plants with regard to Cd, Cu, and lead (Pb) (Chen et al., 2008).

In previous studies, the *Cupriavidus necator* strains UFLA 01-659, UFLA 01-663, and UFLA 02-71 (studied here), proved highly tolerant (2.5, 10, 10, and 5 mmol L^{-1} to Cd, Cu, Zn, and Pb, respectively) in solid medium, and reasonably efficient in biological N_2 fixation in symbiosis with the legume species *Mimosa pudica, Mimosa caesalpiniifolia*, and *Leucaena leucocephala* (Ferreira et al., 2012). In experiments with multicontaminated soil, strain UFLA 01-659 induced an increased of N content in shoots of *Mimosa pudica* and *Leucaena leucocephala*, and strain UFLA 02-71 in *Mimosa caesalpiniifolia,* contributing to soil recovery (Ferreira et al., 2013).

Given the promising results of the *C. necator* strains UFLA 01-659, UFLA 01-663, and UFLA 02-71, the objective of this study was to evaluate the heavy metal tolerance and removal capacity of these bacteria in liquid medium, with regard to Cd, Cu, and Zn, and to investigate the allocation of these metals by electron microscopy, deepening the understanding of mechanisms of heavy metal tolerance and removal by the bacteria.

MATERIALS AND METHODS

Strains and inoculum preparation for the experiments in liquid medium

In this study, the strains UFLA 01-659, UFLA 01-663, and UFLA 02-71 of the species *Cupriavidus necator* (Silva et al., 2012) were used, as well as strain LMG 19424^T of the species *Cupriavidus taiwanensis* (Vandamme and Coenye, 2004), as a representative strain of the genus. All strains studied were preserved by lyophilization in the culture collection of the Soil Microbiology Laboratory of the Soil Science Department of UFLA.

To obtain the inoculum, the strains were cultured in Luria-Bertani (LB) agar containing: 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 5 g L^{-1} NaCl (Sambrook et al., 1989), for two days at 28 °C. Thereafter, the cells were suspended in sterile saline solution (0.85 % NaCl). The cell concentration was standardized to an optical density (OD) of 45 % transmittance, corresponding to a density of colony-forming units (CFU) of 6 × 10^8 CFU mL^{-1}. For the experiments of minimum inhibitory concentration and metal removal, a proportion of 1 mL of inoculum to 100 mL of liquid culture medium was adopted.

Minimum inhibitory concentration (MIC)

The strains were cultured in 10 mL LB broth at pH 6, under horizontal shaking (110 rpm), and incubated at 28 °C for 4 days, a period that corresponds to the stationary phase, according to Ferreira (2011). Before this, the culture medium was supplemented with increasing rates of the metals Cd (0-5 mmol L^{-1}), Cu (0-5 mmol L^{-1}), and Zn (0-15 mmol L^{-1}), in three replicates. The following salts were used as sources of the heavy metals: cadmium sulfate octahydrate ($CdSO_4.8H_2O$), pentahydrate copper sulfate ($CuSO_4.5H_2O$), and heptahydrate zinc sulfate ($ZnSO_4.7H_2O$).

To avoid precipitation of the metals, 6.0 g L^{-1} TRIS was added to the culture medium (Mergeay et al., 1985). At the end of the incubation period, the colony-forming units of the liquid medium (CFU per mL) were quantified by the microdrop method (Herigstad et al., 2001).

Heavy metal removal

In this experiment, the strains were cultured in 80 mL LB broth under the same conditions of pH, inoculation, shaking, and incubation time as for MIC determination. The culture medium was supplemented with Cd at 1 mmol L^{-1} and Cu and Zn at 2 mmol L^{-1}, separately, corresponding to 112.41, 127.09, and 130.76 mg L^{-1}, respectively. These concentrations were established based on the results of the MIC experiments, adopting the highest possible metal concentrations at which all strains could be cultured. As a negative control, the strains were also cultured in a metal-free culture medium. All treatments were performed in triplicate.

After the period of incubation, the bacterial broth was centrifuged for 10 min at 10,000 rpm. The supernatant was discarded and the pellet washed twice with sodium phosphate buffer (8 mmol L^{-1} $Na_2HPO_4.12H_2O$, 1.9 mmol L^{-1} $NaH_2PO_4.2H_2O$, and 8 g NaCl with pH 7.3), as described by Moreira et al. (1993) and Pot et al. (1994). The resulting bacterial biomass was weighed and then subjected to nitric-perchloric acid (2:1) wet digestion. After that, the concentration of each based on this result, the rate of metal removal from the culture medium (mg L^{-1}) and the metal concentrations in the biomass (mg g^{-1}) were calculated.

Scanning electron microscopy

Samples of bacterial suspensions from the experiment of heavy metal removal were analyzed by scanning electron microscopy (SEM). Prior to sample preparation,

10 µL poly-L-lysine (0.1 %) was deposited and spread on glass cover slips to form a basis for bacteria adherence on their surface. After the poly-L-lysine had dried, 20 µL of bacterial suspension was placed on the cover slips and left to dry again.

The samples were prepared according to the protocol: fixation in Karnosvsky solution for 36 h, washing with cacodylate buffer (0.02 mol L^{-1}), post-fixation with osmium tetroxide for 1 h, and dehydration in acetone gradient, followed by drying in a critical point apparatus. Gold metallization was performed to increase the electrical conductivity of the samples. Micrographs were taken with a scanning electron microscope LEO EVO 40 XPV.

Transmission electron microscope

An aliquot of 1 mL of bacterial broth (only of strain UFLA 01-659) was taken from the treatments of the metal removal experiments, centrifuged for 10 min at 10,000 rpm in a microcentrifuge, and the supernatant discarded. The pellet obtained was fixed in Karnovsky solution, centrifuged again, and polymerized with agarose (2 %) to facilitate preparation. Agarose cubes containing the pellet were removed, followed by washing in cacodylate buffer, and post-fixing in osmium tetroxide for 2 h and uranyl acetate (0.5 %) for 12 h. Dehydration in acetone gradient (25, 30, 40, 50, 60, 70, 75, 80, 90, 95, and 100 %) followed by embedding in Spurr's resin. Ultra-thin sections were cut with an ultramicrotome, followed by contrast staining with uranyl acetate and lead citrate. Sections were examined with a Tecnai G2-12 transmission electron microscope (120 kV).

Statistical analyses

Software R was used for all statistical analyses. The MIC values were predicted by regression equations of the CFU based on increasing rates of each metal ($p<0.05$). The data regarding cell biomass, metal removal rate, and metal content in the biomass were evaluated in a (4 × 4) factorial arrangement; the first factor consisted of four bacterial strains and the second of the LB broth composition (control without metals, broth supplemented with Cd, Cu, and Zn). The means in the analysis of variance were compared by the Tukey test, at a significance degree of 5 %.

RESULTS

Minimum inhibitory concentration

For strain UFLA 01-659, greater tolerance to all metals was observed (MIC of 5.00 mmol L^{-1} Cd, 4.95 mmol L^{-1} Cu, and 14.66 mmol L^{-1} Zn) (Table 1). These values correspond to 562, 314, and 958 mg L^{-1} of the respective metals. A comparison of the metal tolerance ability of the strains studied shows a 2.5 times greater MIC of UFLA 01-659 for Cd than the other strains, while the MIC for Cu and Zn were 1.7 and 3 times greater, respectively.

The MIC for Zn (the most tolerated metal) differed considerably among the strains (in decreasing order: UFLA 01-659 > UFLA 02-71 = UFLA 01-663 > LMG 19424^T). The MIC for Cd and Cu were quite similar among the three strains, with lower tolerance than for Zn.

Removal of heavy metals

With regard to the effect of metals on the strains, a higher biomass production of *C. taiwanensis* LMG 19424^T was observed in the medium cultured with zinc ($p<0.05$), exceeding the control treatment without addition of any metal (Figure 1a). A comparison of biomass production between the Cu and control treatments showed that the presence of the metal did not affect the biomass production of strain UFLA 01-659. For all strains, Cd was the metal most detrimental to biomass production.

Table 1. Minimum inhibitory concentration (MIC) of Cd, Cu, and Zn for the growth of different bacterial strains of the genus *Cupriavidus* spp. in liquid LB culture medium, calculated by quadratic equations

Bacteria	MIC	MIC	Equation	R^2
	mmol L^{-1}	mg L^{-1}		
			Cd	
LMG 19424^T	1.93	216.95	$\hat{y} = 9.7094 - 1.9831x^{**} - 1.5787x^{2**}$	0.9235
UFLA 01-659	5.00	562.05	$\hat{y} = 8.3751 + 1.5312x^{**} - 0.6418x^{2**}$	0.9622
UFLA 01-663	2.02	227.07	$\hat{y} = 6.5478 + 2.2535x^{**} - 2.7258x^{2**}$	0.9910
UFLA 02-71	2.00	224.82	$\hat{y} = 8.4650 + 3.3889x^{**} - 3.7724x^{2**}$	0.9780
			Cu	
LMG 19424^T	2.95	187.46	$\hat{y} = 8.6503 + 3.2183x^{**} - 2.0864x^{2**}$	0.8824
UFLA 01-659	4.95	314.55	$\hat{y} = 8.2845 + 1.8808x^{**} - 0.7183x^{2**}$	0.9364
UFLA 01-663	3.57	226.86	$\hat{y} = 6.783 + 1.6322x^{**} - 0.9879x^{2**}$	0.9843
UFLA 02-71	3.06	194.45	$\hat{y} = 8.199 + 4.048x^{**} - 2.1958x^{2**}$	0.9517
			Zn	
LMG 19424^T	3.59	234.71	$\hat{y} = 9.0996 + 3.2853x^{**} - 1.622x^{2**}$	0.9464
UFLA 01-659	14.66	958.47	$\hat{y} = 9.5031 + 0.2887x^{**} - 0.0639x^{2**}$	0.9579
UFLA 01-663	5.87	383.78	$\hat{y} = 6.8592 + 1.6516x^{**} - 0.4802x^{2**}$	0.9137
UFLA 02-71	5.95	389.01	$\hat{y} = 9.4849 + 1.1051x^{**} - 0.4533x^{2**}$	0.9791

* and **: significant at the level of 5 and 1 %, respectively.

Comparing the biomass production of the strains (Figure 1b), the strain UFLA 01-659 had a higher biomass production when Cd was added to the culture medium, whereas in the presence of Cu, strains UFLA 01-659 and UFLA 02-71 produced more biomass. When the strains were cultured with Zn, strain LMG 19424^T had the highest biomass production.

The metal removal rates of strain UFLA 01-659 were higher (9, 4.6, and 3.2 mg L^{-1} of Cd, Cu, and Zn, respectively) than those of the other strains (Figure 2a). In the treatments with Cu and Zn, strain UFLA 01-659 removed up to six times more than the others and for Cd, the removal was around four times greater.

In relation to the metal concentrations in the biomass (Figure 2b), in the Cd treatment, the highest metal concentrations in the biomass were found in the strains UFLA 01-659 and UFLA 01-663s, and in the treatment with Cu, in strains UFLA 01-659 and LMG 19424^T. In the Zn treatment however, high metal concentrations in the biomass were only found in strain UFLA 01-659.

Electron microscopy of *Cupriavidus* cells cultured with heavy metals

Scanning electron microscopy showed changes in cell growth patterns (size and shape) and in exopolysaccharide production in response to metal exposure (Figure 3 and 4). In the control treatment of strain LMG 19424^T, exopolysaccharide production was not observed (Figure 3a). However, when cultured with Cd (Figure 3b) or Cu (Figure 3c), this strain maintained the same growth pattern, however produced exopolysaccharide. When cultured with zinc, it had small and aggregated cells (Figure 3d).

No visible modifications in *C. necator* strain UFLA 01-659 were detected by SEM at the metal concentrations tested (Figures 3e, 3f, 3g, and 3h). In all treatments, strain UFLA 01-663 showed characteristic biomass production. In the control treatment (Figure 4a) and in the presence of Cu (Figure 4c), exopolysaccharide production was observed. However, this effect was absent in the presence of Cd (Figure 4b) or of Zn (Figure 4d). In the presence of Cd (Figure 4f) and Cu (Figure 4g), the *C. necator* UFLA 02-71 samples had similar growth patterns and visibly greater exopolysaccharide production than in

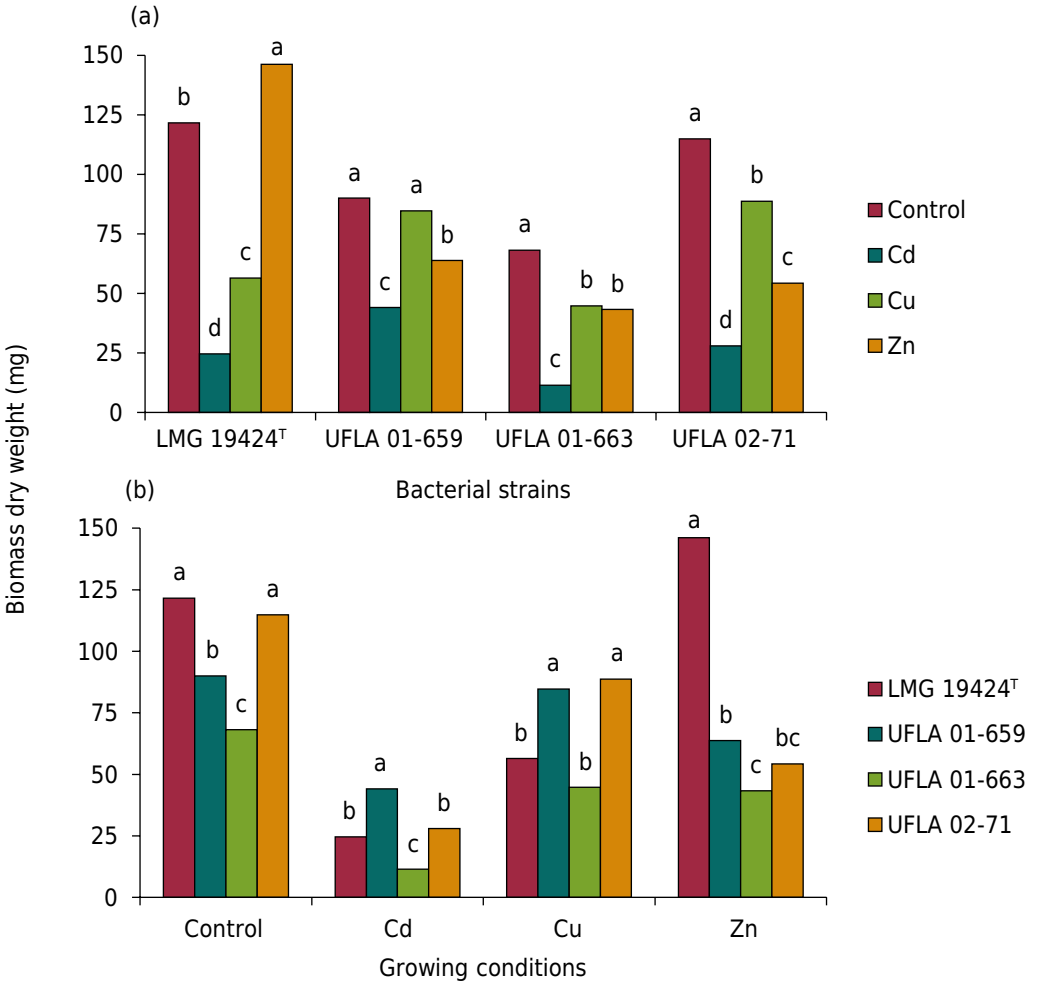

Figure 1. Biomass of bacterial strains (LMG 19424^T, UFLA 01-659, UFLA 01-663, and UFLA 02-71) cultured in the absence of metals (control) and in the presence of the metals Cd (1 mmol L^{1}) and Cu and Zn (2 mmol L^{-1}) in liquid LB culture medium. Mean values followed by the same letter do not differ from each other by the Tukey test ($p<0.05$).

the control treatment (Figure 4e). In the presence of Zn, this strain maintained the same cell shape, though with high exopolysaccharide production (Figure 4h).

In the images taken with the transmission electron microscope (TEM), an accumulation of all metals can be seen (Figure 5b, 5c, and 5d), both in the cytoplasmic membrane and the cell wall. The metals Cu (Figure 5c) and Zn (Figure 5d) were also accumulated within the cells of strain UFLA 01-659.

DISCUSSION

The literature reports values for MIC, metal removal rate, and metal concentration in the bacterial biomass for heavy metal-resistant strains. the MIC values registered for Cd were 0.75 - 3 mmol L^{-1} for *Pseudomonas, Enterobacter* sp., *Acinetobacter* sp., and *Cupriavidus metallidurans* CH34; the last mentioned is considered highly tolerant (Chien et al., 2008; Chien et al., 2011; Klonowska et al., 2012). With regard to accumulation in biomass, Bianucci et al. (2011), studied *Bradyrhizobium* strains and observed Cd concentrations in the biomass of 7.7 mg g^{-1}.

The *C. necator* strain UFLA 01-659 in this study had MIC values of approximately 5.00 mmol L^{-1} Cd in liquid medium, a removal rate of 9.00 mg L^{-1} Cd in culture medium, and a high concentration of 16.3 mg g^{-1} of the metal in the biomass. These values are higher than those previously reported in the literature.

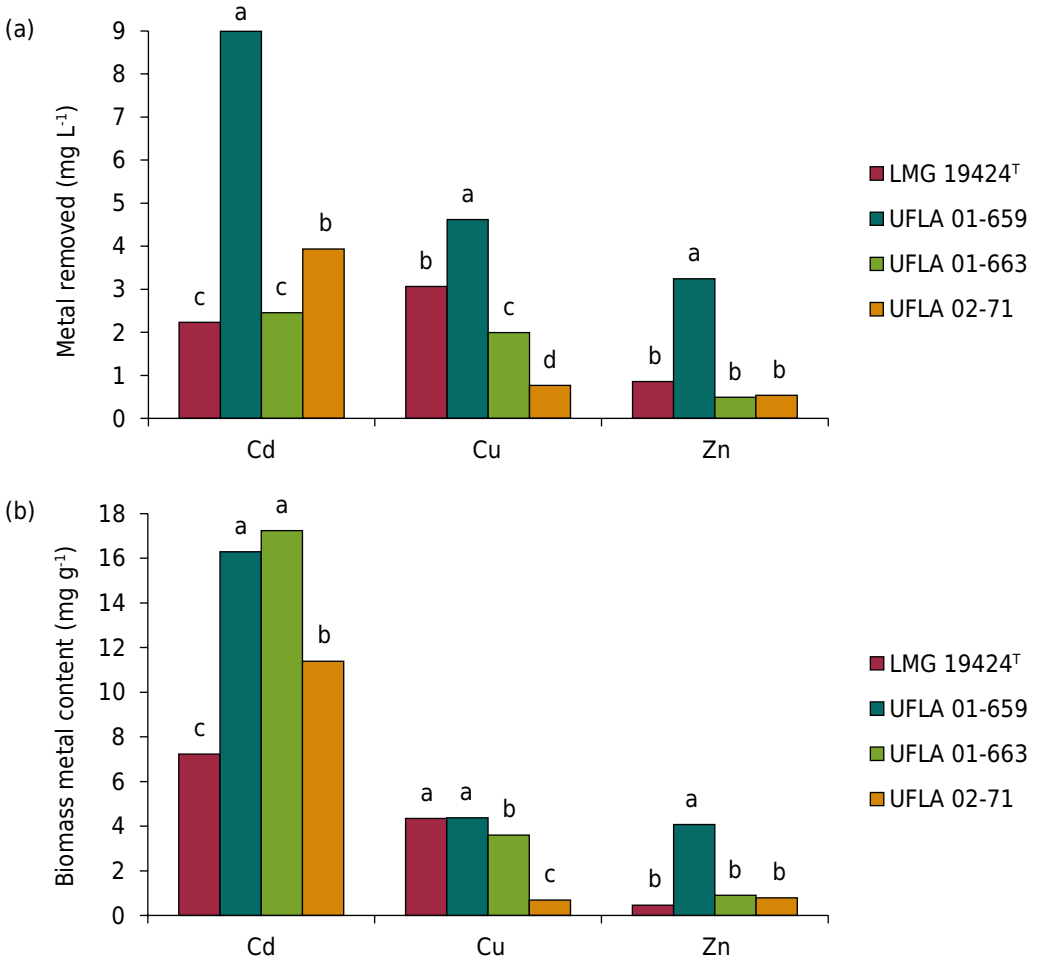

Figure 2. Metal removed from the liquid LB culture medium supplemented with Cd (1 mmol L^{-1}), and Cu and Zn (2 mmol L^{-1}) by bacterial strains (LMG 19424^T, UFLA 01-659, UFLA 01-663, and UFLA 02-71), and heavy metal contents in the biomass of these strains.

In relation to Cu, MIC values of 5.00 mmol L^{-1} for *Pseudomonas*, *Cupriavidus taiwanensis*, and endophytic bacteria, and concentrations of 0.838 mg g^{-1} in the biomass of *Pseudomonas sp.* are registered (Chen et al., 2008; Li and Ramakrishna, 2011; Luo et al., 2011). In our studies, we registered a MIC value similar to that already reported (5.00 mmol L^{-1}); however, the indices of removal rates of 4.6 mg L^{-1} and Cu content in the biomass of 4.4 mg g^{-1} in strain UFLA 01-659 were considerable.

Strains of *Pseudomonas* were isolated from lake sediments by Li and Ramakrishna (2011), with MIC values of 6 mmol L^{-1} Zn in solid medium and a content of 15.877 mg g^{-1} Zn in the biomass. For some *Pseudomonas* isolates, Klonowska et al. (2012) obtained MIC values of 15 mmol L^{-1} Zn, while in the same study, strain LMG 19424^T exhibited a high MIC index (10 mmol L^{-1}), much higher than the values found in this study (3.59 mmol L^{-1}). This low tolerance in our experiments may be due to differences in composition of the medium because we used LB broth supplemented with TRIS (buffer), which prevents precipitation of the compound by changing the pH and, consequently, reducing its bioavailability. In the study of Klonowska et al. (2012) however, YM medium was used, without buffer. In our study, the tolerance of strain UFLA 01-659 to Zn was also high (14.66 mmol L^{-1}), while the indices of removal (3.24 mg L^{-1}) and content (4.1 mg g^{-1}) of Zn in the biomass were less marked.

The superiority of strain UFLA 01-659 in tolerating the metals studied was also noted in the images taken by in SEM (Figure 3), in which no changes were observed in the development pattern (cell size and exopolysaccharide production) in a comparison of

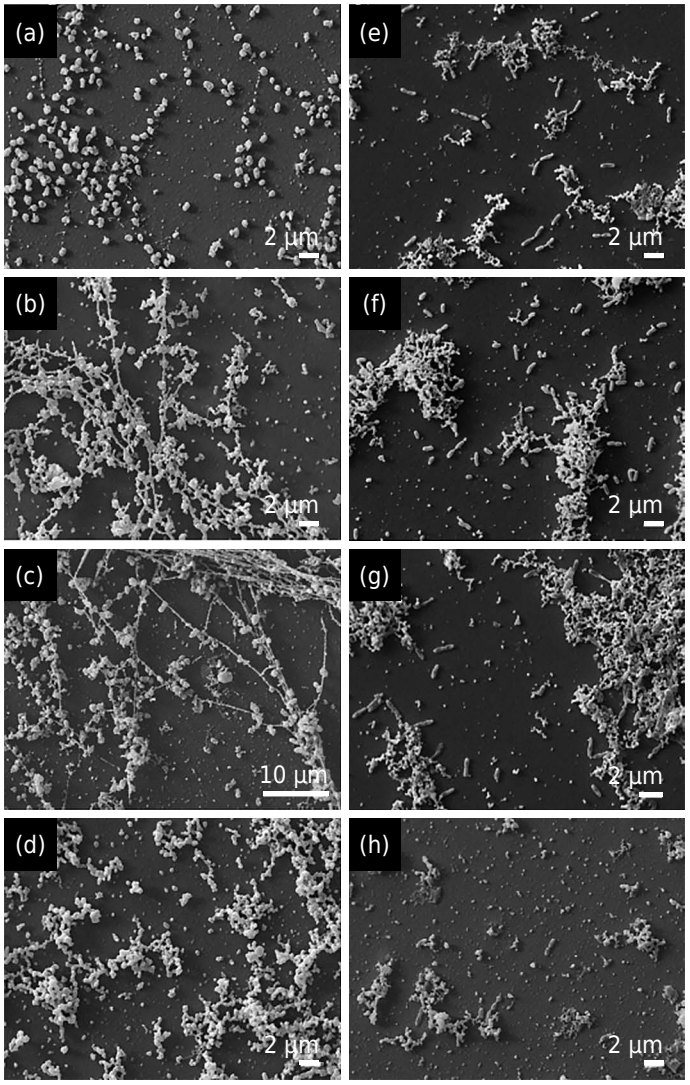

Figure 3. Scanning electron micrograph of the microbial biomass of the strains LMG 19424^T (a, b, c, and d) and UFLA 01-659 (e, f, g, and h) cultured in liquid LB medium in the following treatments: control – absence of metals (a and e), Cd at 1 mmol L^{-1} (b and f), Cu at 2 mmol L^{-1} (c and g), and Zn at 2 mmol L^{-1} (d and h).

the cultures with metals and the control. Their high efficiency in removing metals from the culture medium was expressed either through higher biomass production (observed in the Cd and Cu cultures), or by higher metal content in the biomass (when cultured with Zn) (Figures 1 and 2). According to Babu et al. (2015), the efficiency in heavy metal removal from the culture medium is due to the ability of the strains in increasing the cell density and to the saturation of metal adsorption sites on the cell surface.

Increased exopolysaccharide production by bacterial strains with the presence and increase in Cd and Ni concentrations in culture medium was reported by Chien et al. (2013). Nevertheless, no difference in exopolysaccharide production was observed between the strains with the highest and lowest metal removal rates. This confirmed that greater removal may be linked to the composition of these exopolysaccharides and their capacity of metal adsorption.

In our study, the presence of metals in the culture medium induced exopolysaccharide production in the strains LMG 19 424^T and UFLA 02-71 (Figure 3 and 4); however, these strains had a lower removal rate. In relation to tolerance mechanisms, Babu et al. (2015) confirmed that multimetal complexes (As, Cd, Cu, Pb, and Zn) were found on the surface of and outside the cells, and attributed an important role to exopolysaccharide

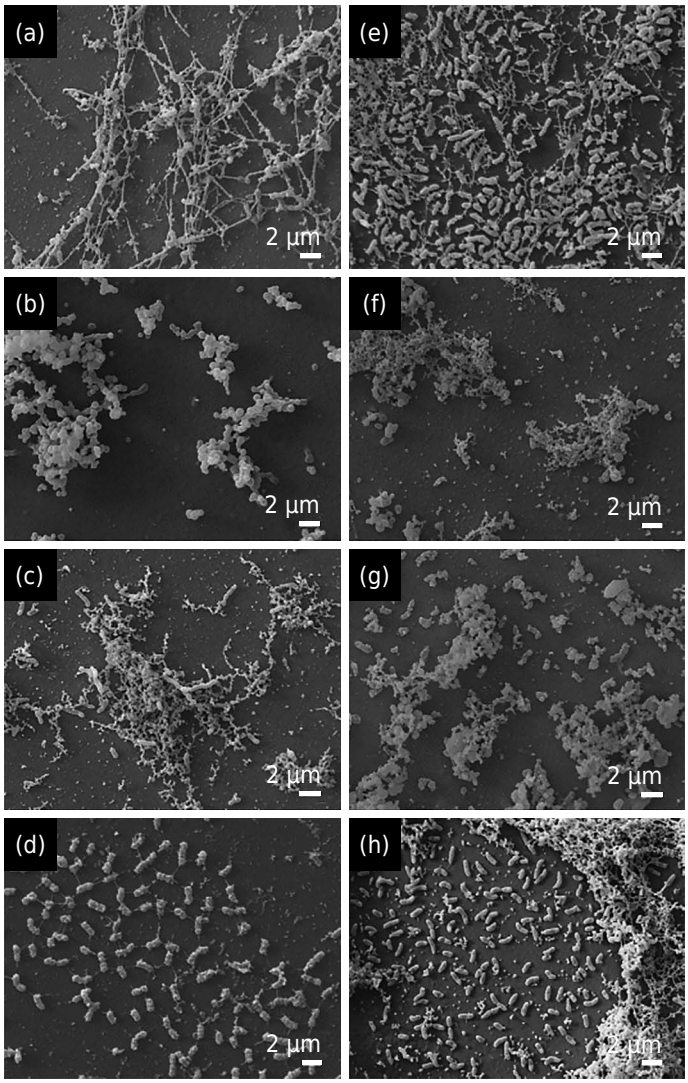

Figure 4. Scanning electron micrograph of the biomass of the strains UFLA 01-663 (a, b, c, and d) and UFLA 02-71 (e, f, g, and h) cultured in liquid LB medium in the following treatments: control - absence of metals (a and e), Cd at 1 mmol L^{-1} (b and f), Cu at 2 mmol L^{-1} (c and g), and Zn at 2 mmol L^{-1} (d and h).

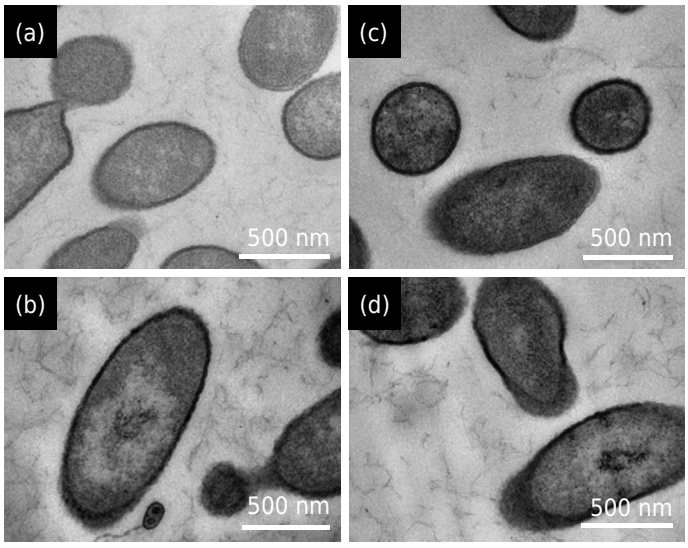

Figure 5. Transmission electron micrograph of the *C. necator* strain UFLA 01-659 cultured in liquid LB medium in the following treatments: control - absence of metals (a), Cd at 1 mmol L^{-1} (b), Cu at 2 mmol L^{-1} (c), and Zn at 2 mmol L^{-1} (d). Arrows indicate the locations of clearest contrasts of metal accumulation.

production with regard to extracellular metal complexation. However, in experiments with isotherms for Zn adsorption by *Pseudomonas aureofaciens* biomass, greater metal adsorption by the strain with low exopolysaccharide production was observed as of the concentration of 0.40-0.45 mmol L^{-1} Zn in equilibrium solution (Drozdova et al., 2014). Considering the cited studies and what we found in the images taken by SEM, it may be said that exopolysaccharide production is a response to the presence of metals, but does not always contribute to heavy metal removal from the environment.

The presence of Cd associated with bacterial cells of *Stenotrophomonas maltophilia* by SEM coupled with energy-dispersive X-ray spectroscopy (EDX) was detected by Pages et al. (2008); however, an exact determination of metal allocation was not possible by this technique. For these authors, the presence of Cd redirects the bacterial metabolism to cysteine production for later use as a precursor in formation of cadmium sulfate particles (CdS). For Wang et al. (2015), the high resistance of *Cupriavidus gilardii* CR3 to various metals is mainly due to the efficient system of ion efflux and metal complexation and reduction, and indirectly, to its self-repair ability.

Thus, it may be inferred that metal allocation is determined by the tolerance mechanisms of each bacterial strain. Allocation of Cu and Zn within the cells of UFLA 01-659 may indicate that the strain has low capacity of metal regulation by ion efflux pumps and/or that it complexes metals, forming particles intracellularly. The presence of Cd only in the cytoplasmic membrane and in the cell wall may indicate that there is efficient extraction of this metal and that its high removal rate is mainly due to physical phenomena of adsorption.

CONCLUSIONS

The greatest capacity of tolerating and removing Cd was detected in *C. necator* strain UFLA 01-659, due to the high biomass production and adsorption to the cell membrane and wall.

Efficiency in Cu and Zn removal by *C. necator* UFLA 01-659 is expressed by its ability of intracellular complexation and adsorption to the cell membrane and wall.

The presence of metals in the culture medium induces exopolysaccharide production of the strains LMG 19424^{T} and UFLA 02-71, which contribute little to metal removal.

ACKNOWLEDGMENTS

The authors would like to thank the Microscopy Center of *the Universidade Federal de Minas Gerais* (http://www.microscopia.ufmg.br), the Brazilian Innovation Agency (Finep), State of Minas Gerais Research Foundation (Fapemig), Brazilian National Council for Scientific and Technological Development (CNPq), and Coordination for the Improvement of Higher Education Personnel (Capes) for providing equipment and technical support for the experiments involving electron microscopy. We are also indebted to CNPq for the scholarships of research productivity of the professors Dr. Eduardo Alves and Dr. Fatima Maria de Souza Moreira, and to Capes for the doctoral scholarship of Dr. Rayssa Pereira Vicentin.

REFERENCES

Andreazza R, Okeke BC, Pieniz S, Camargo FAO. Characterization of copper-resistant rhizosphere bacteria from *Avena sativa* and *Plantago lanceolata* for copper bioreduction and biosorption. Biol Trace Elem Res. 2012;146:107-15. https://doi.org/10.1007/s12011-011-9228-1

Babu AG, Shea PJ, Sudhakar D, Jung I-B, Oh B-T. Potential use of *Pseudomonas koreensis* AGB-1 in association with *Miscanthus sinensis* to remediate heavy metal(loid)-contaminated mining site soil. J Environ Manage. 2015;151:160-6. https://doi.org/10.1016/j.jenvman.2014.12.045

Bianucci E, Fabra A, Castro S. Cadmium accumulation and tolerance in *Bradyrhizobium* spp. (Peanut microsymbionts). Curr Microbiol. 2011;62:96-100. https://doi.org/10.1007/s00284-010-9675-5

Chen W-M, Wu C-H, James EK, Chang J-S. Metal biosorption capability of *Cupriavidus taiwanensis* and its effects on heavy metal removal by nodulated *Mimosa pudica*. J Hazard Mater. 2008;151:364-71. https://doi.org/10.1016/j.jhazmat.2007.05.082

Chien C-C, Jiang M-H, Tsai M-R, Chien C-C. Isolation and characterization of an environmental cadmium- and tellurite-resistant Pseudomonas strain. Environ Toxicol Chem. 2011;30:2202-7. https://doi.org/10.1002/etc.620

Chien C-C, Kuo Y, Chien C, Hung C, Yeh C, Yeh W. Microbial diversity of soil bacteria in agricultural field contaminated with heavy metals. J Environ Sci. 2008;20:359-63. https://doi.org/10.1016/S1001-0742(08)60056-X

Chien C-C, Lin B-C, Wu C-H. Biofilm formation and heavy metal resistance by an environmental *Pseudomonas* sp. Biochem Eng J. 2013;78:132-7. https://doi.org/10.1016/j.bej.2013.01.014

Drozdova OY, Pokrovsky OS, Lapitskiy SA, Shirokova LS, González AG, Demin VV. Decrease in zinc adsorption onto soil in the presence of EPS-rich and EPS-poor *Pseudomonas aureofaciens.* J Colloid Interf Sci. 2014;435:59-66. https://doi.org/10.1016/j.jcis.2014.08.025

Ferreira PAA. Tolerância de *Cupriavidus necator* a cádmio e zinco e sua eficiência simbiótica em leguminosas [tese]. Lavras: Universidade Federal de Lavras; 2011.

Ferreira PAA, Bomfeti CA, Silva Júnior R, Soares BL, Soares CRFS, Moreira FMS. Eficiência simbiótica de estirpes de *Cupriavidus necator* tolerantes a zinco, cádmio, cobre e chumbo. Pesq Agropec Bras. 2012;47:85-95. https://doi.org/10.1590/S0100-204X2012000100012

Ferreira PAA, Lopes G, Bomfeti CA, Longatti SMO, Soares CRFS, Guilherme LRG, Moreira FMS. Leguminous plants nodulated by selected strains of *Cupriavidus necator* grow in heavy metal contaminated soil amended with calcium silicate. World J Microbiol Biotechnol. 2013;29:2055-66. https://doi.org/10.1007/s11274-013-1369-2

Herigstad B, Hamilton M, Heersink J. How to optimize the drop plate method for enumerating bacteria. J Microbiol Meth. 2001;44:121-9. https://doi.org/10.1016/S0167-7012(00)00241-4

Hynninen A, Touzé T, Pitkänen L, Mengin-Lecreulx D, Virta M. An efflux transporter PbrA and a phosphatase PbrB cooperate in a lead-resistance mechanism in bacteria. Mol Microbiol. 2009;74:384-94. https://doi.org/10.1111/j.1365-2958.2009.06868.x

Klonowska A, Chaintreuil C, Tisseyre P, Miché L, Melkonian R, Ducousso M, Laguerre G, Brunel B, Moulin L. Biodiversity of *Mimosa pudica* rhizobial symbionts (*Cupriavidus taiwanensis, Rhizobium mesoamericanum*) in New Caledonia and their adaptation to heavy metal-rich soils. Fems Microbiol Ecol. 2012;81:618-35. https://doi.org/10.1111/j.1574-6941.2012.01393.x

Li K, Ramakrishna W. Effect of multiple metal resistant bacteria from contaminated lake sediments on metal accumulation and plant growth. J Hazard Mater. 2011;189:531-9. https://doi.org/10.1016/j.jhazmat.2011.02.075

Luo S-l, Chen L, Chen J-l, Xiao X, Xu T-y, Wan Y, Rao C, Liu C-b, Liu Y-t, Lai C, Zeng G-m. Analysis and characterization of cultivable heavy metal-resistant bacterial endophytes isolated from Cd-hyperaccumulator *Solanum nigrum* L. and their potential use for phytoremediation. Chemosphere. 2011;85:1130-8. https://doi.org/10.1016/j.chemosphere.2011.07.053

Mahieu S, Frérot H, Vidal C, Galiana A, Heulin K, Maure L, Brunel B, Lefèbvre C, Escarré J, Cleyet-Marel J-C. *Anthyllis vulneraria/Mesorhizobium metallidurans,* an efficient symbiotic nitrogen fixing association able to grow in mine tailings highly contaminated by Zn, Pb and Cd. Plant Soil. 2011;342:405-17. https://doi.org/10.1007/s11104-010-0705-7

Mergeay M, Nies D, Schlegel HG, Gertis J, Charles P, Van Gusegem F. *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J Bacteriol. 1985;162:328-34.

Moreira FMS, Gillis M, Pot B, Kersters K, Franco AA. Characterization of rhizobia isolated from different divergence groups of tropical Leguminosae by comparative polyacrylamide

gel electrophoresis of their total proteins. Syst Appl Microbiol. 1993;16:135-46. https://doi.org/10.1016/S0723-2020(11)80258-4

Moreira FMS, Lange A, Klauberg-Filho O, Siqueira JO, Nóbrega RSA, Lima AS. Associative diazotrophic bacteria in grass roots and soils from heavy metal contaminated sites. An Acad Bras Cienc. 2008;80:749-61. http://dx.doi.org/10.1590/S0001-37652008000400014

Pages D, Rose J, Conrod S, Cuine S, Carrier P, Heulin T, Achouak W. Heavy metal tolerance in *Stenotrophomonas maltophilia.* PLoS ONE. 2008;2:e1539. https://doi.org/10.1371/journal.pone.0001539

Pot B, Vandamme P, Kersters K. Analysis of electrophoretic whole organism protein fingerprints. In: Goodfellow M, O'Donnell AG, editors. Chemical methods in prokaryotic systematics. Chichester: John Wiley & Sons; 1994. p. 493-521.

Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989.

Silva K, Florentino LA, Silva KB, Brandt E, Vandamme P, Moreira FMS. *Cupriavidus necator* isolates are able to fix nitrogen in symbiosis with different legume species. Syst Appl Microbiol. 2012;35:175-82. https://doi.org/10.1016/j.syapm.2011.10.005

Vandamme P, Coenye T. Taxonomy of the genus *Cupriavidus*: a tale of lost and found. Int J Syst Evol Micr. 2004;54:2285-9. https://doi.org/10.1099/ijs.0.63247-0

Wang X, Chen M, Xiao J, Hao L, Crowley DE, Zhang Z, Yu J, Huang N, Huo M, Wu J. Genome sequence analysis of the naphthenic acid degrading and metal resistant bacterium *Cupriavidus gilardii* CR3. PLoS ONE. 2015;10:e0132881. https://doi.org/10.1371/journal.pone.0132881

Zoropogui A, Gambarelli S, Covès J. CzcE from *Cupriavidus metallidurans* CH34 is a copper-binding protein. Biochem Bioph Res Co. 2008;365:735-9. https://doi.org/10.1016/j.bbrc.2007.11.030