

Cupriavidus necator strains: zinc and cadmium tolerance and bioaccumulation

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ABSTRACT: Among soil microorganisms, the genus *Cupriavidus* has garnered particular scientific, economic and ecological interest because of its ability to fix nitrogen and tolerate high concentrations of metals. The aim of this study was to analyze four strains of *Cupriavidus necator* for their ability to tolerate and bioaccumulate cadmium and zinc. The tolerance of these strains to these metals was assessed in liquid culture medium containing different concentrations of Zn + Cd and in soil solutions and soils contaminated with multiple elements including Zn, Cd, Cu and Pb. The four strains showed high tolerance to Zn and Cd, both in culture medium and when inoculated into contaminated soil solutions or multi-element contaminated soil. The UFLA02-71 strain displayed the highest ability to bioaccumulate these metals. It was able to accumulate 93.76 $\mu\text{mol g cell}^{-1}$ of Zn and 16.03 $\mu\text{mol g cell}^{-1}$ of Cd when cultured in liquid medium with a total heavy metal concentration of 9,140 $\mu\text{mol L}^{-1}$ (9,000 Zn + 140 Cd) and was able to accumulate 16.98 $\mu\text{mol g cell}^{-1}$ of Cd in the soil solution. An increase in the pH of the culture medium resulting from the growth of the *C. necator* strains reduced the Zn^{2+} and Cd^{2+} ions in the medium and increased the concentrations of the ZnHPO_4 and CdHPO_4 species in solution. Thus, we concluded that they show great potential for use in the bioremediation of HM-contaminated areas.

Keywords: β -proteobacteria, bioremediation, heavy-metal, soil pollution

Introduction

Several microorganisms are capable of colonizing HM-contaminated environments due to a wide range of mechanisms and interactions that allow them to adapt to these environments (Sharaff and Archana, 2015). In general, the main tolerance mechanisms of microorganisms are related to the active transporting of metals across the membrane and out of the cell, resistance plasmids with genes encoding to heavy metal tolerance, adsorption of metals by the cell wall, and biotransformation of the metals directly by specific enzymes or indirectly by cellular metabolites (Gadd, 2004).

Among the soil microorganisms capable of colonizing HM-contaminated environments, nitrogen-fixing bacteria (NFB) that form symbioses with legumes are particularly notable (Vázquez et al., 2006). Among the bacteria that can fix nitrogen in symbiosis with legumes, the species *Cupriavidus taiwanensis* (syn. *Ralstonia taiwanensis*) has been shown to be able to both efficiently nodulate the legume *Mimosa pudica* (Chen et al., 2001, 2003a, b) and accumulate Pb, Cd and Cu (Chen et al., 2008). Other species of the *Cupriavidus* genus that were isolated from tailing pond sediments at a zinc factory have also garnered interest for their potential use in bioremediation, including *Cupriavidus metallidurans*, although this specie does not fix nitrogen. These species are well known for being highly tolerant of Zn, Cd, Co, Ni, Cu, Cr, Hg and Pb (Taghavi et al., 1997; Goris et al., 2001; Mergeay et al., 2003) and for their ability to biodegrade recalcitrant xenobiotic compounds (Louie et al., 2002; Trefault et al., 2004). Notably, *C. taiwanensis*

strains, unlike *C. metallidurans* strains, have been isolated from the nodules of *Mimosa* sp. in uncontaminated environments in Taiwan (Chen et al., 2008), suggesting that the location of origin does not influence metal tolerance in this genus. Recently, the ability of *C. necator* to symbiotically fix nitrogen in legumes (Silva et al., 2012; Ferreira et al., 2013) and to tolerate heavy metals in a solid medium (Ferreira et al., 2012) has been shown. However, there are no studies examining the tolerance of *Cupriavidus necator* under multi-element HM-contamination conditions either in culture medium or using contaminated soils.

Because species of this genus have potential applications for heavy metal biosorption and have the ability to nodulate legumes, the aim of this study was to assess the zinc and cadmium tolerance and bioaccumulation by *Cupriavidus necator* strains in liquid culture medium, soil solutions and multi-element contaminated soil.

Materials and Methods

Sampling and chemical characterization of multi-element contaminated soil

Soil samples contaminated with multiple elements including Zn, Cd, Cu and Pb which were classified as Red-Yellow Latosol (Hapludox), a dystrophic plinthic soil, were collected in the Votorantim Metais (VM) industrial area located in the municipality of Três Marias (18°11'11" S; 45°14'36" W; 557 m altitude) (Minas Gerais, Brazil). These samples were collected during Feb 2009 at a depth of 0-20 cm. In total, 5 composite samples were collected and each composite sample consisted of

5 single samples. Chemical characterization of the soil solution was then performed to determine the available concentrations of Zn and Cd. For this analysis, a saturation extract was produced following the methodology described by Van Raij et al. (2001), which included five replicates. The concentrations of the elements were determined via atomic absorption spectrophotometry (City: Überlingen, State: Baden-Württemberg, Country: Germany).

Bacterial strains

The bacterial strains used in this study had been previously isolated from the rhizosphere of the legume *Sesbania virgata* using *Phaseolus vulgaris* and *Leucaena leucocephala* as bait plants to isolate bacteria that nodulate legumes in uncontaminated soil (Florentino et al., 2009). The isolated bacterial strains showed rapid growth, resulting in alkaline culture medium and reduced gum production in medium 79 (Fred and Waksman, 1928). These strains were identified as *C. necator* by Silva et al., 2012 based on phenotypic characterization, 16S rRNA gene sequencing and sequencing of other housekeeping genes and comparison of these sequences with the NCBI database. The four strains (UFLA01-663, UFLA01-659, UFLA02-73 and UFLA02-71) were selected based on the efficiency of their symbioses with legumes that have the potential to revegetate degraded areas and their tolerance of Cd and Zn concentrations in solid culture medium (Ferreira et al., 2012).

Evaluation of growth of *C. necator* strains in LB liquid medium containing Cd and Zn and in a multi-element contaminated soil solution

Starter strains were grown in LB [Luria-Bertani] medium (5 g L⁻¹ NaCl, 5.0 g L⁻¹ yeast extract and 10 g L⁻¹ tryptone) (Sambrook et al., 1989) at pH 6.0 with orbital agitation at 110 rpm (0.68 × g) at 28 °C until they reached an optical density (OD) at 560 nm of 1.0 (1 × 10⁹ CFU [colony-forming units] mL⁻¹). One milliliter of the inoculum was then transferred into 100 mL of LB liquid medium containing HEPES (1.3 mg L⁻¹ de N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid) and MES [1.1 mg L⁻¹ de 2-(N-morpholino) ethane sulfonic acid] (Cole and Elkan, 1973) at pH 6.0. Different concentrations of Zn and Cd were added to this medium, resulting in 9.14 μmol L⁻¹ (9.0 Zn + 0.14 Cd); 91.4 μmol L⁻¹ (90 Zn + 1.4 Cd); 914 μmol L⁻¹ (900 Zn + 14 Cd); and 9,140 μmol L⁻¹ (9,000 Zn + 140 Cd)]. The medium was then autoclaved at 121 °C for 20 min. The concentrations of Zn and Cd were defined based on the levels of Zn and Cd found in the soil solution while maintaining the ratio of 64.3 Zn:Cd found in the multi-element contaminated soil solution.

The evaluation of the growth of *C. necator* in the soil solution was performed as described above except that the Zn:Cd ratio was 32.16 in this solution. In addition, 5 g of NaCl, 5 g of yeast extract and 10 g of tryptone per liter of solution were added to the soil so-

lution. This medium was autoclaved (20 min at 121 °C) and subsequently characterized by ion chromatography (City: Sunnyvale, State: California, Country: United States) and atomic absorption spectrophotometry (City: Überlingen, State: Baden-Württemberg, Country: Germany). The results were used for chemical speciation using the Visual MINTEQ program (Version 3.0) (This version was compiled in Visual Basic 2012 on 1 Oct 2014 by Jon Petter Gustafsson, KTH, Div. of Land and Water Resources Engineering, Stockholm, Sweden.) at pH 6.0 (before growth) and pH 7.86 (after 120 h of growth) to evaluate the distribution of Zn and Cd species in solution.

The cultures were incubated at 28 °C under agitation at 110 rpm. Samples were taken following 3, 6, 12, 24, 48, 72, 96 and 120 h of incubation. At each time point, 100 μL aliquots were collected and diluted in 900 μL of 0.85 % saline to determine the number of viable CFUs as described by Miles and Misra (1938).

Survival of *C. necator* strains in multi-element contaminated soil

The same methods as described above were used to evaluate the survival of strains in soil contaminated with multiple HM. The soil was sieved through 2.0 mm mesh, and 200 g of soil was then transferred to 500 mL Erlenmeyer flasks. These samples were autoclaved three times for 1 h at 121 °C and allowed to stand for 30 days to stabilize. These soils displayed the following chemical characteristics after autoclaving: pH = 6.2; P = 37.6 mg dm⁻³ (Mehlich I); K = 36 mg dm⁻³; Ca = 1.8 cmolc dm⁻³; Mg = 0.3 cmolc dm⁻³; H+Al = 1.5 cmolc dm⁻³; Zn = 12,514 mg dm⁻³; Cd = 12.4 mg dm⁻³; Pb = 18.3 mg dm⁻³; Cu = 1,144.9 mg dm⁻³; and MO = 1.8 dag kg⁻¹. The semi-total concentrations (USEPA 3051A) (USEPA, 1998) of Zn, Cd, Pb and Cu were 47,910, 1,207, 6,488 and 738 mg kg⁻¹, respectively.

Cultures of *C. necator* strains were grown in 20 mL of LB broth to an OD₅₆₀ = 1.0 and transferred to Erlenmeyer flasks containing soil. The flasks were then rotated to ensure that the inoculum was uniformly distributed. Each assay was performed in triplicate. The moisture content of the inoculated soil was maintained at approximately 70 % of the total pore volume (TPV) by weighing the flask and adding autoclaved distilled water. To assess the number of viable bacterial cells, 5 g of soil was added to 45 mL of 0.85 % saline and the number of CFUs was then determined using serial dilutions (Miles and Misra, 1938) at 0, 7, 14, 21, 28, 35 and 42 day intervals following inoculation.

Bioaccumulation of Zn and Cd by the bacterial strains

To evaluate the ability of the strains UFLA01-663, UFLA01-659, UFLA02-73 and UFLA02-71 to accumulate Zn and Cd, 1 mL aliquots of the bacterial starter cultures were transferred into 100 mL of LB medium which contained different concentrations of Zn + Cd metals.

The heavy metal concentrations used in this experiment were 9.14 $\mu\text{mol L}^{-1}$ (9.0 Zn + 0.14 Cd); 91.4 $\mu\text{mol L}^{-1}$ (90 Zn + 1.4 Cd); 914 $\mu\text{mol L}^{-1}$ (900 Zn + 14 Cd); and 9,140 $\mu\text{mol L}^{-1}$ (9,000 Zn + 140 Cd), and 100 mL of a soil solution with concentrations of 7,140 and 222 $\mu\text{mol L}^{-1}$ of Zn and Cd, respectively. The soil solution was extracted as described above. After being incubated at 28 °C for 120 h with agitation, the cultures were centrifuged at 10,000 rpm for 20 min, and the bacterial pellet was subsequently washed with a solution of 0.1 N HCl. The pellet was digested in a tube digester using HNO_3 as described in USEPA 3050B. The concentrations of the elements were determined via atomic absorption spectrophotometry (City: Überlingen, State: Baden-Württemberg, Country: Germany).

The strains' ability to bioaccumulate Zn and Cd was evaluated by calculating their bioaccumulation factor (BF), which is defined as the concentration of the element in the cell biomass compared to the concentration of the element in the culture medium,

$$\text{BF} = \frac{[\text{Metal}]_{\text{cell biomass}}}{[\text{Metal}]_{\text{culture medium}}}$$

Transmission electron microscopy

The UFLA02-71 strain was grown in 50 mL of LB medium supplemented with or without 9,140 $\mu\text{mol L}^{-1}$ HM (9,000 Zn + Cd 140) under agitation at 110 rpm. After 120 h of growth, bacterial biomass was harvested by centrifugation at 10,000 rpm for 5 min. The bacterial pellet was homogenized in melted agar/water medium at approximately 50 °C. Small clusters of cells were soon formed upon solidification of the agar, which facilitated handling of the material. This material was then prefixed in 2.5 % glutaraldehyde and 2.5 % paraformaldehyde in a buffer containing 0.05 mmol L^{-1} cacodylate, pH 7.0 and 0.001 mmol L^{-1} CaCl_2 . After 24 h, the material was washed in a 0.05 mmol L^{-1} cacodylate buffer (3 washes for 10 min) and post-fixed in 1 % osmium tetroxide in a 0.05 mmol L^{-1} cacodylate buffer for 2 h. The material was then washed in distilled water (3 washes for 10 min) and dehydrated in a graded series of acetone, embedded in Spurr epoxy resin and 100 % acetone (1:1) for 5 h and transferred to pure resin for one night. These blocks were then transferred to molds containing resin and incubated in an oven at 70 °C for 48 h. The resin blocks were trimmed and prepared for an ultramicrotome. Ultrathin sections (approximately 70 nm) were collected on nickel grids covered with Formvar, contrasted with uranyl acetate and lead citrate and examined in a transmission electron microscope (City: Oberkochen, State: Baden-Württemberg, Country: Germany).

Statistical analysis

Analysis of variance (ANOVA) (SISVAR) and regression analysis were used to analyze the data generated for these strains at different concentrations of Zn + Cd and for different incubation periods. The Table Curve 2D for Windows program v. 5.03 was used for the analysis.

Results

The growth curves of the *C. necator* strains UFLA01-663, UFLA01-659, UFLA02-71 and UFLA02-73 in LB medium with or without Zn + Cd are presented in Figure 1. The strains' maximum growth rates were determined using the equations in Figure 1. The maximal growth rates were 9.84 and 9.77 $\log \text{CFU mL}^{-1}$ for the UFLA01-663 and UFLA01-659 strains and 9.44 and 9.48 $\log \text{CFU mL}^{-1}$ for the UFLA02-73 and UFLA02-71 strains in culture medium without the metals.

Bacterial growth at concentrations of 9.14, 91.4 to 914 $\mu\text{mol L}^{-1}$ Zn + Cd followed the same growth patterns as the bacterial growth in the medium without metal (Figure 1). At these heavy metal concentrations, these strains grew in a log phase for 24 h and then entered a stationary phase. The maximum growth rates of these strains in the stationary phase at a concentration of 914 $\mu\text{mol L}^{-1}$ of Zn + Cd was 9.73 $\log \text{CFU mL}^{-1}$ for UFLA01-663, 9.67 $\log \text{CFU mL}^{-1}$ for UFLA01-659, 9.83 $\log \text{CFU mL}^{-1}$ for UFLA02-73 and 9.59 $\log \text{CFU mL}^{-1}$ for UFLA02-71. The growth rate of the strains at the concentration of 9,140 $\mu\text{mol L}^{-1}$ Zn + Cd was significantly lower than in the presence of lower concentrations of heavy metals, indicating that high concentrations of Zn and Cd in solution have a toxic effect on the growth of these strains. The strains grown at the highest concentration of heavy metals had a prolonged period of growth but grew at lower rates than the cultures grown in low concentrations of Zn + Cd. The growth of these strains continued until 120 h after inoculation and it was not possible to distinguish the phases of a typical bacterial growth curve. At this concentration of heavy metals, the UFLA02-71 strain exhibited the highest $\log \text{CFU mL}^{-1}$ (8.74) after 120 h.

To evaluate the growth of these bacteria in the soil solution, it was necessary to perform the evaluation process of the soil samples again because this experiment required a larger volume of solution. The concentrations of ions found in soil solution were (mg L^{-1}): F⁻: 30.67, Cl⁻: 3733.53, NO₂⁻: 16.09, NO₃⁻: 310.71, PO₄²⁻: 254.44, SO₄²⁻: 1,433.62, Na⁺: 2,960.83, NH₄⁺: 378.01, K⁺: 448.03, Mg²⁺: 107.32, Ca²⁺: 496.86, Cu²⁺: 0.50, Zn²⁺: 467.00, Cd²⁺: 25.00, Pb²⁺: 0.007, Mn²⁺: 4.40, Fe²⁺: 0.20, total dissolved carbon: 5,850.00 and electrolytic conductivity (mS cm^{-1}): 2.907. In this experiment, the strains grew more slowly in the soil solution due to the high concentrations of zinc (7,140 $\mu\text{mol L}^{-1}$) and cadmium (222 $\mu\text{mol L}^{-1}$) (Figure 2). The behavior of these strains was not similar to a typical microbial growth curve over the 120 h following inoculation. However, at 120 h, the strains had similar numbers of CFU mL^{-1} as were observed in the stationary phase of the experiment using LB medium supplemented with Zn + Cd at concentrations of 9.14, 91.4 and 914 $\mu\text{mol L}^{-1}$ (Figure 1).

When the strains were inoculated into the contaminated soil, there was a 30 % log reduction in the

number of cells at the end of 42 days of incubation compared to the original inoculated culture. Nevertheless, the number of viable cells remained high ($> 10^6$), indicating a moderate ability of the strains to survive in contaminated soil (Figure 2).

Notably, there was an increase in the capacity of *C. necator* strains to bioaccumulate Cd and Zn ions as the doses of these metals increased (Figure 3). The amounts of zinc retained by these strains at the doses of 9,140 and 9.14 $\mu\text{mol Zn} + \text{Cd}$ were on average 0.16 and 81.49 $\mu\text{mol g cell}^{-1}$. The average amounts of Cd absorbed by the strains were 0.012 and 13.84 $\mu\text{mol g cell}^{-1}$ at 9.14 and 9,140 $\mu\text{mol Zn} + \text{Cd}$, respectively. The UFLA02-71 strain had the highest Zn and Cd absorption capacity at the higher concentrations of metals in solution compared with the other strains. This strain bioaccumulated

93.76 $\mu\text{mol g cell}^{-1}$ of Zn and 16.03 $\mu\text{mol g cell}^{-1}$ of Cd. The UFLA02-73 strain also had a high capacity to bioaccumulate Zn (84.48 $\mu\text{mol g cell}^{-1}$) (Figure 3).

In the soil solution extracted from the contaminated soil, the UFLA02-71 strain bioaccumulated the highest amounts of Cd, corresponding to 16.98 $\mu\text{mol g cell}^{-1}$, whereas the UFLA02-73 strain had the highest levels of zinc absorption at 54.53 $\mu\text{mol g cell}^{-1}$ (Figure 4B). There was an increase in Cd absorption by strains in the soil solution compared to the medium supplemented with 9,140 $\mu\text{mol L}^{-1}$ of Zn + Cd because there was a greater amount of this cation present and available in this solution as the ratio of Zn:Cd was 32.16 in this soil solution (Figure 4A). Thus, the higher concentrations of Cd in the solution contributed to the higher levels of Cd absorption by these strains.

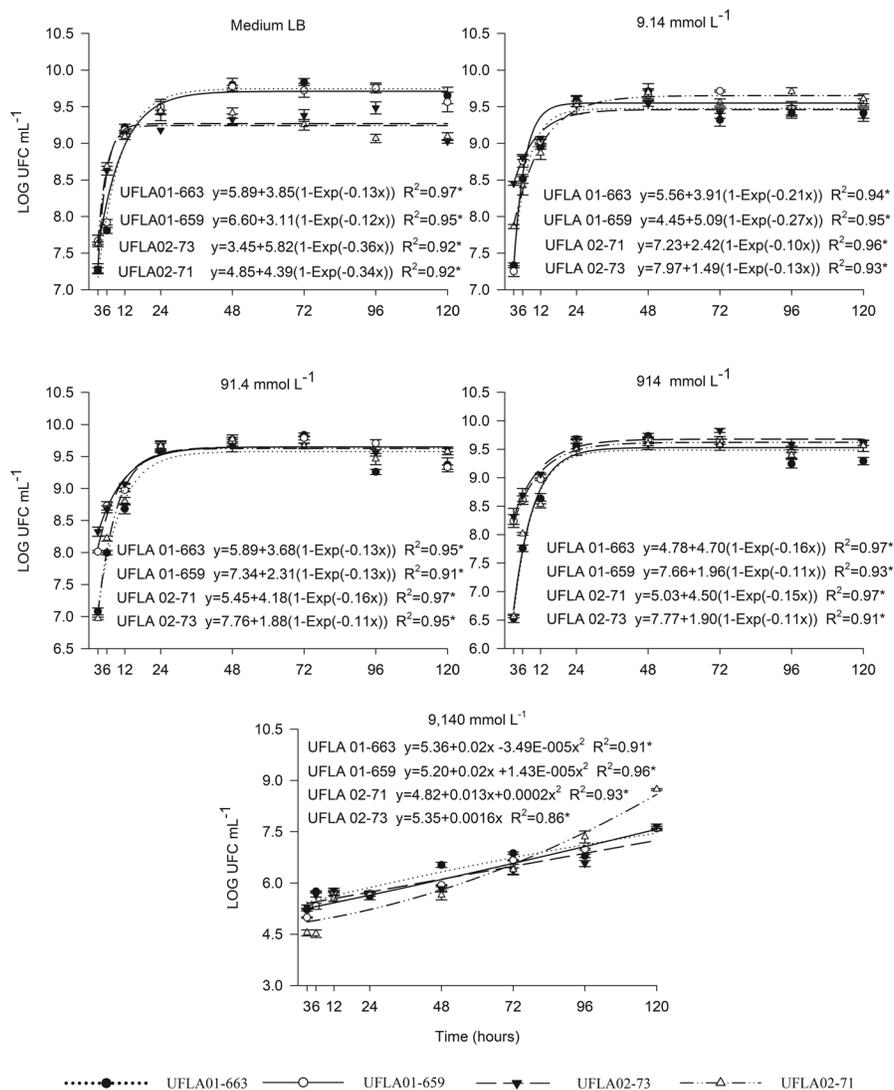


Figure 1 – Number of viable cells of strains UFLA01-663, UFLA01-659, UFLA02-73 and UFLA02-71 in LB liquid medium as a function of time and different concentrations of Zn + Cd in the media. The Zn:Cd ratio for all concentrations was 64.3.

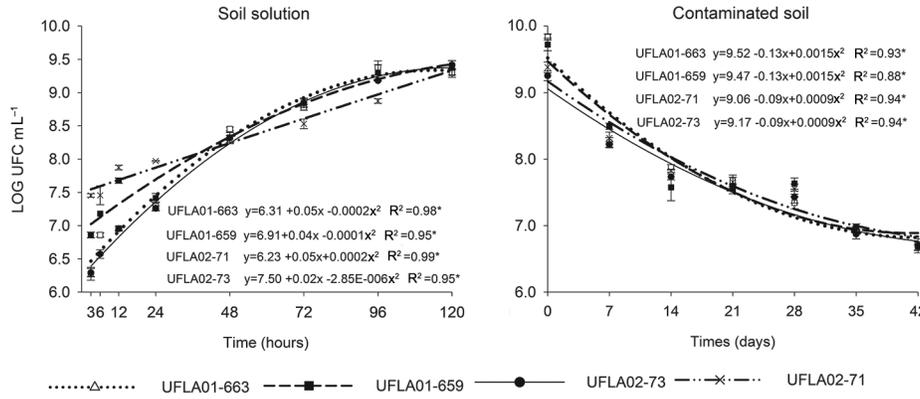


Figure 2 – Numbers of viable cells of the strains UFLA01-663, UFLA01-659, UFLA02-73 and UFLA02-71 in the soil solution and the contaminated soil.

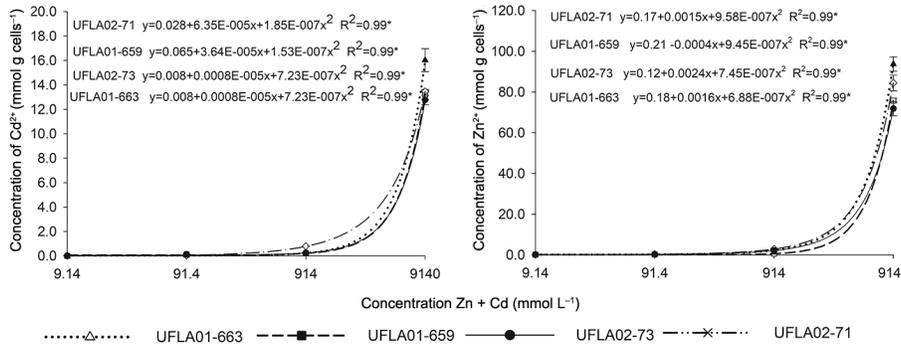


Figure 3 – Concentrations of Zn and Cd absorbed by the *Cupriavidus necator* strains at Zn + Cd concentrations of 9,140 $\mu\text{mol L}^{-1}$ and in the soil solution extracted from contaminated soil.

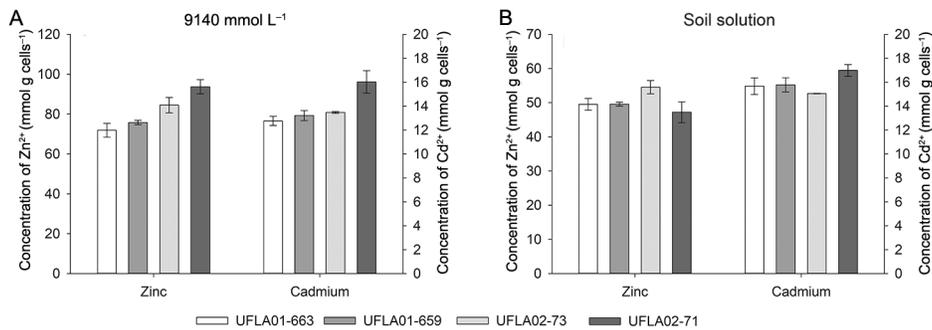


Figure 4 – Concentrations of Zn and Cd per strain of *Cupriavidus necator* grown in the presence of different levels of Zn/Cd after 120 h in 9,140 mmol L^{-1} of Zn + Cd (A) and in the soil solution (B).

After 120 h of incubation in LB medium supplemented with metals, the bioaccumulation factor (BF) was calculated for all strains at each concentration tested (Table 1). The BF was higher for Cd than for Zn for all strains, ranging from 65.72 to 787.71 $\mu\text{mol L}^{-1}$ for Cd and 3.04 to 133.49 $\mu\text{mol L}^{-1}$ for Zn. These results indicate that these strains have a high capacity for accumulating Cd inside the cell.

In the experiment using the soil solution, the zinc and cadmium species were measured before growth at pH 6.0 and after growth at pH 7.86 to determine the available forms of these metals in solution (Table 2). The free ions Zn^{2+} , Cd^{2+} , Zn-DOM1 and Cd-DOM1 decreased as the pH increased following bacterial growth, which might have reduced the toxic effects of the metals. Conversely, the $\text{ZnHPO}_4(\text{aq})$

Table 1 – Capacity of bioaccumulation for the *Cupriavidus necator* strains exposed to increasing concentrations of Zn + Cd in culture medium.

Strains	Concentrations de Zn + Cd ($\mu\text{mol L}^{-1}$)				
	9.14 (9.0 + 0.14)	91.4 (90.0 + 1.4)	914 (900 + 14)	9140 (9000 + 140)	
	Factor accumulation				
Zn	UFLA02-73	73.39 \pm 1.86*	23.88 \pm 0.40	19.19 \pm 1.70	30.10 \pm 2.31
	UFLA02-71	101.47 \pm 1.74	22.47 \pm 3.64	20.69 \pm 1.59	41.90 \pm 0.53
	UFLA01-659	63.37 \pm 2.77	10.02 \pm 0.22	3.04 \pm 0.01	34.37 \pm 1.89
	UFLA01-663	133.49 \pm 13.05	28.10 \pm 2.35	15.85 \pm 0.57	30.86 \pm 2.38
Cd	UFLA02-73	787.71 \pm 15.32	180.69 \pm 17.53	333.94 \pm 21.02	370.17 \pm 2.84
	UFLA02-71	539.63 \pm 14.31	230.21 \pm 2.49	137.16 \pm 31.77	459.92 \pm 19.28
	UFLA01-659	171.27 \pm 6.37	378.48 \pm 5.51	65.72 \pm 4.14	385.60 \pm 30.71
	UFLA01-663	378.71 \pm 11.8	678.71 \pm 7.78	115.70 \pm 22.99	352.07 \pm 11.64

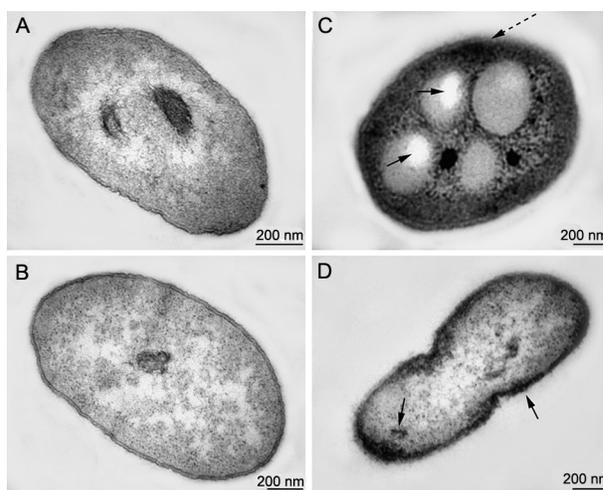
*The standard deviation was calculated based on three replicates.

Table 2 – Percentage distribution of the chemical species of Zn and Cd in the soil solutions extracted from the tailings of Votorantim Metals both before bacterial growth (pH 6.0) and after (pH 7.86).

Zinc	pH		Cadmium	pH	
	6.0	7.86		6.0	7.86
Zn ²⁺	8.412	7.877	Cd ²⁺	8.337	7.675
Zn DOM1	83.422	81.423	Cd DOM1	52.163	50.057
ZnF ⁺	0.072	0.070	CdF ⁺	0.057	0.053
ZnCl ⁺	0.747	0.710	CdCl ⁺	24.499	22.61
ZnCl ₂ (aq)	0.039	0.041	CdCl ₂ (aq)	5.420	5.043
ZnSO ₄ (aq)	5.175	4.920	CdSO ₄ (aq)	5.495	5.189
Zn(SO ₄) ₂ ²⁻	1.517	1.414	Cd(SO ₄) ₂ ²⁻	2.496	2.286
ZnNH ₃ ²⁺	0.011	0.836	CdNH ₃ ²⁺	0.024	1.768
ZnNO ₂ ⁺	0.018	0.016	CdNO ₂ ⁺	0.259	0.229
ZnNO ₃ ⁺	0.139	0.127	CdNO ₃ ⁺	0.174	0.146
ZnHPO ₄ (aq)	0.440	2.007	CdHPO ₄ (aq)	1.070	4.801
ZnOH ⁺	—	0.286	CdOH ⁺	—	0.022
Zn(OH) ₂ (aq)	—	0.145	Cd(NH ₃) ₂ ²⁺	—	0.113
Zn(NH ₃) ₃ ²⁺	—	0.015	—	—	—
Zn(NH ₃) ₂ ²⁺	—	0.103	—	—	—

and CdHPO₄(aq) species significantly increased as the pH increased from 6 to 7.84. The species ZnOH⁺, Zn(OH)₂(aq), Zn(NH₃)₃²⁺, Zn(NH₃)₂²⁺, CdOH⁺ and Cd(NH₃)₂²⁺ were observed only as the pH increased to 7.84.

Transmission electron microscopy was used to determine the location of the metal accumulation inside the cells of the UFLA02-71 strain (Figure 5A, B, C and D). The control cells (no metal) displayed a homogeneous cytoplasm without electron-dense granules or PHB (poly- β -hydroxybutyrate) granules (Figure 5A and B). Cells grown in medium with high concentrations of Zn + Cd had both PHB accumulation and granular deposits in their cytoplasm (Figure 5C). There were no apparent signs of cell lysis or destruction after 120 h of cultivation in the presence of heavy metals, which is consistent with the observations that this bacterium is able to bioaccumulate toxic elements such as Cd and Zn in the interior of its cells (Figure 5D).

**Figure 5** – Transmission electron microscopy of sections of control *Cupriavidus necator* UFLA02-71 cells (A and B) and *Cupriavidus necator* UFLA02-71 cells cultured in 9,140 $\mu\text{mol L}^{-1}$ of Zn + Cd (C and D). Dashed arrow indicates a possible accumulation of metals at these points. Continuous arrows indicate the production of PHB.

Discussion

The ability of certain microorganisms to grow in the presence of high metal concentrations may result from intrinsic or induced tolerance mechanisms or other environmental factors of the medium (i.e., pH or redox potential) which may also reduce the toxicity of these metals (Leedj arv et al., 2008; Xiao et al., 2010). Mechanisms have been demonstrated to allow bacterial tolerance to various metals, and these mechanisms vary little between uncontaminated and contaminated environments (Barkay and Schaefer, 2001; Matsuda et al., 2002). This may explain the ability of the strains analyzed in this study to tolerate high concentrations of metals despite being isolated from uncontaminated environments.

In the present study, a relationship was established between the metal tolerance of *C. necator* strains

in LB medium supplemented with Zn + Cd and the metal tolerance in the soil solutions. Under all conditions tested, the strains displayed the same number of viable cells. However, there was a reduction in the number of cells when these strains were inoculated into contaminated soil. Nevertheless, the number of cells remained relatively high given that the metal concentrations in this soil were very high. This reduction in the number of *C. necator* cells in contaminated soil may be due to the exhaustion of carbon sources provided by the culture medium upon inoculation and by the high electrical conductivity of the soil tested (1.60 mS cm^{-1}). These data indicate that it may be possible for these strains to establish a symbiosis with legumes that grow in areas contaminated with heavy metals because of their high tolerance.

Working with a similar soil that was contaminated with different proportions of metals, Matsuda et al. (2002) assessed the growth of strains of genera *Bradyrhizobium* and *Azorhizobium* and observed a 20 % survival rate of *Bradyrhizobium* cells in soil containing 1,250, 206, 67.5 and 192.5 dm^{-3} (Mehlich-I) of Zn, Cu, Cd and Pb, respectively. Conversely, when the survival of the *Azorhizobium* strains was evaluated, only a small percentage (approximately 2 %) of cells survived at lower metal concentrations, in this case 750, 111, 22.1 and 65.1 mg dm^{-3} of Zn, Cu, Cd and Pb, respectively. In another study, a strain of *Mesorhizobium metallidurans* was inoculated into mining tailings that displayed Zn and Cd levels of 35,000 and 16.5 mg kg^{-1} , and these strains maintained a constant growth rate of 10^4 CFU per gram of soil over the 45 days of the experiment (Mahieu et al., 2011). The high tolerance of the *C. necator* strains evaluated in the present study for heavy metals is even more evident than the tolerances seen in previous studies, making the strains examined here potentially useful in revegetation programs for contaminated soils.

Other studies related to the tolerance of bacterial species and the ability of bacterial species to accumulate heavy metals have been published in recent years (Vullo et al., 2008; Chen et al., 2008; Velásquez and Dussan, 2009). However, there are currently no studies in the literature which have a practical focus that consider bacterial growth in soil solutions extracted from contaminated soils. Furthermore, studies examining the ability of *C. necator* to tolerate metals have seldom been performed to date. In a study that examined the *C. taiwanensis* strain M2, which was isolated from soils contaminated with metals in Argentina, the authors reported maximum bioaccumulation levels of 230 and $125 \mu\text{mol L}^{-1}$ for Cd and Zn when the strain was grown in medium containing 0.5 mmol L^{-1} of these elements (Vullo et al., 2008). The *C. taiwanensis* strain TJ208, which was isolated from *M. pudica* nodules, showed an estimated maximum adsorption capacity of 19.6 mg g^{-1} cell for Cd at a concentration of 100 mg L^{-1} ($893 \mu\text{mol L}^{-1}$) (Chen et al., 2008). These values show a highest metal accumulation capacity compared to the accumulation capacities of the strains evaluated in this study.

The mechanisms by which microorganisms interact with heavy metals include mechanisms that are independent of or dependent on cellular metabolism. The metabolism-independent mechanisms occur via physicochemical interactions between the metal and cell wall constituents, exopolysaccharide constituents or other materials associated with the external face of the membrane (Naik et al., 2012). These mechanisms are metabolism-independent because energy expenditure by the microbial cell is not required for the absorption of metal ions. Metal removal in this case can occur in both living and dead cells (Gadd, 2004). The transport of metal ions across the cell membrane and their intracellular accumulation is metabolism-dependent and only occurs in living cells capable of generating energy. The removal of these metal ions via this type of mechanism is usually slower than the physicochemical adsorption mechanism. In contrast, larger amounts of metals can be accumulated with these mechanisms (Gadd, 2004), as has been reported for *Pseudomonas aeruginosa*, *Cupriavidus metallidurans* and *Bacillus sphaericus* (Velasquez and Dussan, 2009). Thus, bacterial mechanisms which tolerate heavy metals (HM) already mentioned, can explain the variation in the ability of bioaccumulation by *C. necator*, depending on the HM concentration in culture medium (Table 1). Metabolic mechanisms allow reductions in metallic ions when *C. necator* is submitted to lower concentrations ($9.14 \mu\text{mol L}^{-1}$) and they do not limit the bioaccumulation ability. On the other hand, mechanisms related to intracellular transport, which reduce bioaccumulation, may occur when toxic concentrations are higher than $91.4 \mu\text{mol L}^{-1}$ (Vullo et al., 2008).

The pH of the metal solution plays a very important role in the absorption of metals by microorganisms as it influences the chemical species of metals in solution as well as the surface properties of bacterial cells (López et al., 2000; Pardo et al., 2003). After 120 h of bacterial growth in LB medium containing heavy metals, a pH reading was performed and demonstrated that there was an overall pH increase compared to the initial pH, which was adjusted to 6.0 for all experiments. At the concentrations 9.14, 91.4 and $914 \mu\text{mol L}^{-1}$ of Cd + Zn, the bacterial strains increased the pH by an average of 2.2 pH units, whereas in the medium with $9,140 \text{ mol L}^{-1}$ of Zn + Cd, there was an average increase of 1.9 pH units following bacterial growth. Thus, the strains were able to produce alkalization in the medium capable of reducing the solubility and activity of the metals (Alexander, 1977). An increase in pH promotes the dissociation of hydroxyl groups (OH) from the medium components that have a predominantly negative charge. The resulting species can then bind to metals, decreasing their availability, and consequently their toxicity.

The toxicity of zinc and cadmium at pH 6.0 for *Escherichia coli* and *Arthrobacter* sp. has commonly been associated with the presence of more bioavailable metal species, specifically Cd^{2+} and Zn^{2+} (Worden et al., 2009; Moberly et al., 2010). However, other species

such as CdOH^+ and $\text{ZnHPO}_4(\text{aq})$ in solution may also have a toxic effect on microorganisms. Moberly et al. (2010) reported that the concentration of $\text{ZnHPO}_4(\text{aq})$ increased as the pH increased, favoring a higher absorption of this species into microorganisms due to inorganic phosphate-specific transporters in *Arthrobacter* sp. In the present study, there was an increase in $\text{ZnHPO}_4(\text{aq})$ and $\text{CdHPO}_4(\text{aq})$ concentrations in solution as the pH increased, which may have contributed to the increase in absorption of these metals by the *Cupriavidus necator* strains (Figure 4B) for other bacterial species as described above by Moberly et al. (2010).

This ability to intracellularly accumulate toxic metals has been reported in members of various other bacterial genera such as *Pseudomonas*, *Bacillus*, *Sphingomonas*, *Acidithiobacillus*, *Arthrobacter* and *Microbacterium* (Kazy et al., 1999; Sar and D'Souza, 2001; Suzuki and Banfield, 2004; Nedelkova et al., 2007; Merroun and Selenska-Pobell, 2008). In these studies, sequestration of these metals was associated with a variety of survival strategies developed by the microorganisms.

In addition to the ability of the *C. necator* strains examined here to bioaccumulate metals, these strains were able to produce PHB in the presence of 9,140 $\mu\text{mol L}^{-1}$ of Cd + Zn (Figure 5C and D). Increased production of PHB by *Azospirillum brasilense* in the presence of high doses of copper seems to protect bacterial cells from the stress caused by metals (Kamnev et al., 2002; Kamnev et al., 2012).

The genus *Cupriavidus* is known to have biodegradation abilities and tolerance mechanisms for organic and inorganic pollutants, sparking interest in these microorganisms in the field of environmental biotechnology. Based on the evaluation in this study, the *C. necator* strains analyzed here could be potential tools for the future recovery of areas polluted by mining activities. This tolerance to heavy metals combined with their ability to absorb these metals demonstrates that certain strains of *C. necator* have the potential to decontaminate environments with excess zinc and copper.

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