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ENVIRONMENTAL MICROBIOLOGY - RESEARCH PAPER

Assessing nickel tolerance of bacteria isolated from serpentine soils

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Abstract

Serpentine soils present unique characteristics such as a low Ca/Mg ratio, low concentration of nutrients, and a high concentration of heavy metals, especially nickel. Soil bacterial isolates from an ultramafic complex located in the tropical savanna known as the Brazilian Cerrado were studied. Nickel-tolerant bacteria were obtained, and their ability to remove nickel from a culture medium was assessed. Bacterial isolates presented higher tolerance to nickel salts than previously reported for bacteria obtained from serpentine environments in other regions of the world. In addition, the quantification of nickel in cell pellets indicated that at least four isolates may adsorb soluble forms of nickel. It is expected that information gathered in this study will support future efforts to exploit serpentine soil bacteria for biotechnological processes involving nickel decontamination from environmental samples.

Keywords Heavy metal resistance · Serpentine soils · Nickel tolerance

Introduction

Serpentine soils are characterized by high concentrations of metals (Ni, Cr, Mn, Co); low availability of N, P, and K; and low Ca/Mg ratios [1, 2]. They are typically inhospitable to most plants; however, several endemic plant species are adapted to the unique chemical characteristics of serpentine soils [3]. Plant-microbe interactions in the rhizosphere may be involved in plant tolerance to metals in serpentine soils [4]. Consequently, bacteria from the rhizosphere of nickel-accumulating plants have been investigated for their potential to improve phytoremediation and phytoextraction in heavy metal–contaminated soils [5–8]. Bacteria presenting high tolerance to metal have been isolated from the rhizosphere of nickel-hyperaccumulating plants; they usually belong to the phyla Actinobacteria or Proteobacteria [9].

On the other hand, there are only a few studies evaluating the microbiota in bulk soil from serpentine soils. Culture-independent studies have shown that bulk soil bacterial communities in serpentine soils from different geographic regions are more similar to each other than to those of surrounding non-serpentine soils [10, 11]. In addition, culture-based studies have demonstrated that serpentine soil bacteria are more tolerant to nickel and zinc than those isolated from non-serpentine soils [12–15].

Bacteria can tolerate metal toxicity by employing one or more of six known metal tolerance mechanisms: metal exclusion by permeability barrier, active transport of the metal from cells, intra- or extracellular sequestration, enzymatic detoxification of the metal to a less toxic form, or reduction in metal sensitivity of cellular targets [16, 17]. Specific mechanisms of metal tolerance exhibited by soil bacteria are dependent on the bacterial species, the particular heavy metal studied, and other

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local environmental factors [18]. Cellular response to metal toxicity is complex, since more than one mechanism for tolerance is often used by each bacterial species [18].

It has been shown that bacteria from several phyla are able to perform biosorption of a variety of heavy metals from aqueous samples [19–24]. Additionally, studies have proposed that the use of bacterial biomass for heavy metal removal from contaminated water environments is more efficient than conventional techniques [19]. Thus, bacteria from serpentine soils may be good candidates for biotechnological processes involving the removal of toxic concentrations of metals under neutral pH conditions [25]. Although many studies have isolated metal-tolerant bacteria from serpentine soils, few have investigated their physiology, with the aim of using them for metal removal from contaminated sites [10].

In this study, the aim was to isolate indigenous bacteria from serpentine soils and evaluate their physiological responses to nickel. More specifically, their capability of growing in increasing concentrations of nickel was investigated, and the nickel content in bacterial cells was measured. It is expected that the information gathered in this study will support future efforts to exploit serpentine soil bacteria for biotechnological processes involving nickel decontamination from environmental samples.

Methods

Soil sampling and characterization

Soil samples were collected from two sites at the ultramafic complex of Barro Alto, Goiás state, Brazil [11]. Soils from site 1, named SAP (15° 06′ 04.4″ S, 49° 00′ 38.4″ W) are loamy Cambisols, mainly composed of saprolites. Soils from site 2, named LAT (15° 06′ 31.1″ S, 49° 01′ 15.0″ W) are sandy clay Oxisols, predominantly composed of laterites [20, 21]. The soil sampling and physicochemical characterization methods employed here have been previously described in Pessoa-Filho et al. [11].

Briefly, three replicate plots were established along a transect line for each site. In each plot, 10 soil samples were collected from the 0- to 10-cm layer using a sterile auger, and these samples were then mixed in a plastic bag to form a composite sample and kept on ice during transport to the laboratory. Samples were sieved through a 4-mm mesh, in order to remove plant debris and roots.

Sites SAP and LAT presented mean Ca/Mg ratios of 0.29 and 0.12, respectively. Soil pH ranged from 6.19 to 6.49. The SAP soil presented higher mean concentrations of DTPA-extractable nickel (603.53 mg kg^{-1}) and soil organic matter (SOM) content (64%) than the LAT soil (nickel,134.66 mg kg^{-1}; SOM, 18%) [11].

Isolation of soil bacteria

For bacterial isolation, two media were used: R2A (BD, USA) [26] and VL-55 [27]. Each medium was adjusted to pH 6.0, since this resembles the indigenous soil pH. The R2A is a standard medium usually used for the isolation of bacteria from serpentine soils [5, 6]. In a previous study, the analysis of bacterial diversity using the 16S rRNA gene revealed a predominance of Acidobacteria belonging to subgroups 4 and 6 in these soils [11]. Since members of the phylum Acidobacteria are difficult to grow on a standard carbon-rich medium [28] such as R2A, VL-55 medium was also used. VL-55 medium is a chemically defined medium that has been used to isolate slow-growing soil bacteria [27].

VL-55 medium consisted of (L^{-1}) 3.9 g of 2-(*N*-morpholino)ethanesulfonic acid, 4 mM of $MgSO_4$, 0.6 mM of $CaCl_2$, 0.4 mM of $(NH_4)_2HPO_4$, 2 mL of a modified Balch's trace elements solution, and 2 mL of Wolfe's vitamin solution [29]. R2A medium consisted of (L^{-1}) 0.5 g of yeast extract, 0.5 g of proteose peptone no. 3, 0.5 g of casamino acids, 0.5 g of dextrose, 0.5 g of soluble starch, 0.3 g of sodium pyruvate, 0.3 g of dipotassium phosphate, and 0.05 g of magnesium sulfate. For solid medium, 1.5% bacto-agar was used as a solidifying agent.

In addition, media were tested with or without the amendment of 5 mM $NiSO_4$; this concentration has been used in previous culture-based experiments found in the literature [14]. The concentration of 5 mM is also an intermediate value between the lowest and the highest environmental concentrations of nickel, estimated to be 2.29 mM in LAT samples and 10.3 mM in SAP [11]. A solution of 1 M $NiSO_4$ was prepared by diluting $NiSO_4{\cdot}6H_2O$ (Sigma-Aldrich) in deionized water. The solution was sterilized by filtering through a 0.22 μm PVDF membrane and properly diluted to the final concentration of 5 mM.

An initial soil suspension was obtained by adding 1 g of soil to 100 mL of the corresponding medium (R2A or VL-55). Soil suspensions were vortexed for 20 min to allow the microorganisms to detach from the soil particles. Serial dilutions were prepared to reach a final dilution of 10^{-6}. Results were expressed in colony forming units (CFU) per gram of wet soil. Plates were incubated at 25 °C, and the number of colonies was recorded on a weekly basis for a period of 5 weeks. Colonies of similar morphological characteristics (morphotypes) were isolated as pure cultures in the same medium in which they were obtained. The criteria for distinguishing morphotypes were colony size, shape, and color. Each morphotype stock was stored in glycerol at − 80 °C.

Identification of the isolated bacteria

Morphotypes were identified by sequence analysis of the 16S rRNA gene, which was amplified by colony PCR [30] using

universal bacterial primers 27F (5′ AGA GTT TGA TCM TGG CTC AG 3′) and 1492R (5′ GGY TAC CTT GTT ACG ACT T 3′) [31]. Each reaction contained 1X of Taq polymerase reaction buffer, 1 U Taq polymerase (Phoneutria Biotecnologia, Brazil), 1.5 mM $MgCl_2$, 0.2 μM of each primer, and 0.2 mM dNTP mix. Amplification was carried out using the following steps: 3 min at 95 °C for denaturation; 30 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min and 40 s; and extension at 72 °C for 7 min. The amplification of the expected fragment was evaluated by agarose gel electrophoresis.

Amplified fragments were purified using the GeneJet PCR Purification Kit (Thermo Scientific) following the manufacturer's instructions and sequenced using a 3130xL Genetic Analyzer (Applied Biosystems). Sequences were edited using BioEdit [32]. The classification was achieved by comparison of the partial 16S rRNA gene nucleotide sequences (600 bp) to sequences in the Ribosomal Database Project (RDP) [33] using the RDP Naive Bayesian rRNA Classifier Version 2.11. A threshold of 90% was used to determine the appropriate taxonomic rank for classification [34]. A total of 92 16S rRNA gene sequences originated from this initial analysis were deposited in GenBank Database with accession numbers MK775368–MK775459.

Thirteen bacterial isolates were selected for further analysis. The nearly full sequences of 16S rRNA gene from these 13 isolates and sequences retrieved from the RDP using the "sequence match" were aligned applying the RDP Aligner [33]. A phylogenetic tree was obtained using MEGA X software [35]. The evolutionary history was inferred using the maximum likelihood method and the Tamura-Nei model [36]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches applying a cutoff value of 80% [37]. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and then selecting the topology with superior log likelihood value [38] and are in the units of the number of base substitutions per site. These sequences were deposited in GenBank Database under the numbers MK774772–MK774784.

Determination of nickel tolerance of soil bacterial isolates

Tolerance to Ni was analyzed on solid media. The 13 preselected isolates were inoculated on plates containing R2A medium amended with the following concentrations of both $NiCl_2$ and $NiSO_4$: 0.5, 1, 2, 4, 8, 16, 32, and 64 mM. The 1 M $NiCl_2$ solution was prepared as described above using $NiCl_2 \cdot 6H_2O$ (Sigma) (Figure S1).

Tolerance to nickel hydroxide ($Ni(OH)_2$), which is an insoluble source of nickel, was evaluated at the following concentrations: 0, 0.464, 0.927, 1.854, and 4.635 g L^{-1} (approximately 0, 5, 10, 15, 20, and 50 mM, respectively). To achieve these concentrations, $Ni(OH)_2$ powder was mixed with the medium before sterilization.

The evaluation of bacterial growth was carried out after 1 week at 30 °C. The highest concentration at which growth was still observed was defined as the maximum tolerable concentration (MTC) [39, 40]. The *Escherichia coli* ATCC11229 strain was used as a nickel-susceptible control.

Nickel quantification by atomic spectroscopy

Isolates SAP B3, SAP F4, SAP E5, LAT A10, LAT C8, and SAP E12 (Table 2) were grown in 100 mL of R2A liquid medium supplemented with 0, 1, or 2 mM of $NiSO_4$. Polycarbonate baffled Erlenmeyer flasks were used to avoid nickel interaction with glassware. Bacterial isolates were grown with shaking at 180 rpm, 30 °C. The experiment was conducted in triplicate and ended when the stationary phase was achieved.

Cells were collected by centrifugation at 10,000*g* for 30 min at 4 °C, and the supernatant was discarded. To remove soluble unbound nickel, the cell pellet fractions were washed twice with sterile R2A medium by resuspending the cells in 10 mL of R2A without nickel, followed by centrifugation as described above. The fraction containing washed cells was lysed using ultrasound, 40% amplitude, 130 W, 20 kHz for 4 cycles of 2 min each. After lysis, the lysed cell fractions were resuspended with 10 mL of R2A medium. Fractions were lyophilized, and their nickel content was quantified. We also evaluated the presence of nickel in the medium without bacterial inoculum. Nickel quantity was expressed as the percentage of nickel from the medium retained in the cell pellet (Figure S1).

Nickel quantification was performed as follows: lyophilized fractions were diluted in a mixture of 5 mL each of distilled nitric and hydrochloric acids in a 30-mL PTFE vessel. They were placed in an analytical microwave (Multiwave 3000, Anton Paar), and a three-step microwave program was applied. First, pressure was kept at 20 Bar and temperature was increased to 120 °C over a period of 5 min and maintained for 55 min. Then, the temperature and pressure were further decreased to room temperature and atmospheric pressure, respectively. Following digestion, samples were transferred to a 25-mL volumetric flask and diluted with ultrapure water. Blank samples and the working standards for calibration were prepared in the same concentration of nitric and hydrochloric acids as for the sample.

Nickel quantification was performed by inductively coupled plasma optical emission spectroscopy (ICP-OES, Thermo Scientific, iCAP 6000), with the operational

conditions RF power 1150 W, flush pump rate 50 rpm, auxiliary gas flow 1 L min^{-1}, and plasma gas fixed 12 L min^{-1}. The analytical line of Ni 231.6 nm was selected. Working standard solutions were prepared from a standard Ni^{2+} metal stock solution (1000 mg L^{-1} Fluka) at the same concentration of nitric and hydrochloric acids as for the sample. A standard plot of ICP-OES signal versus Ni^{2+} was linear between 0 and 68.15 μM of Ni^{2+} ($R^2 = 0.9979$). The limit of detection (LOD) and the limit of quantitation (LOQ) of Ni^{2+} were estimated by analyzing 10 replicate aliquots of calibration blanks after microwave digestion. The LOD (0.008 μM of Ni^{+2}) was calculated on the basis of threefold the standard deviation of the blank control divided by the slope of the calibration curve, and the LOQ (0.027 μM of Ni^{+2}) as tenfold the standard deviation of the blank control [41].

To check the statistical significance of differences observed in the percentage of nickel retained in the pellet, the Kruskal-Wallis test was performed, with Dunn's post hoc test. Normality was tested with the Shapiro-Wilk test. We considered a confidence threshold of $p < 0.05$. Statistical analysis was performed in R, using the package FSA.

Results

Bacterial isolation and identification

Treatments without nickel amendment presented the highest average CFU per gram of wet soil: SAP soils (presenting 603.53 mg kg^{-1} of DTPA-extractable Ni) yielded an average of 2.9×10^9 CFU g^{-1} wet soil, whereas LAT (presenting 134.66 mg kg^{-1} of Ni) derived 4.5×10^8 CFU g^{-1} wet soil (Table 1). The amendment of 5 mM $NiSO_4$ to the culture medium resulted in a decrease in the number of colonies to 6.5×10 [6] CFU g^{-1} wet soil for SAP and 6.4×10 [6] CFU g^{-1} wet soil for LAT (Table 1).

A total of 105 isolates were classified by 16S rRNA gene sequencing; these belong to the phyla Actinobacteria, Proteobacteria, Bacteroidetes, Firmicutes, and Acidobacteria (Fig. 1). Only one colony belonging to the phylum Acidobacteria was identified, but it was not stable under the culture conditions used.

Overall, the phylum Actinobacteria was predominant, ranging from 34 to 79% of the identified isolates (Fig. 1). Among the Actinobacteria obtained, the genus *Arthrobacter* was detected in all the treatments (Table S1). Members of classes' alpha, beta, and gamma of the phylum Proteobacteria were also obtained. However, the classes Gamma Proteobacteria and Bacilli (Phylum Firmicutes) were only identified in treatments without nickel (Fig. 1).

Table 1 Number of CFU/g wet soil in two serpentine soil samples in media amended with 5 mM $NiSO_4$ (+Ni) or without the amendment of $NiSO_4$ (−Ni)

Sample	CFU/g soil	
	−Ni	+Ni
SAP	2.9×10^9	6.5×10^6
LAT	4.5×10^8	6.4×10^6

Nickel tolerance in bacterial isolates

Thirteen strains isolated from the nickel-amended treatment were randomly selected for further analysis. The selection was based on two criteria: the isolates should grow well on R2A medium, and at least one isolate from each phylum should be selected to provide a wide spectrum of bacterial species. Although the genus *Arthrobacter* was identified in all treatments, none of the isolates grew well on R2A medium. A phylogenetic analysis using the 16S rRNA gene sequences indicated that these isolates were different from each other, and isolates of the same genus are likely to be of different species (Figure S2).

Bacterial growth was evaluated under increasing concentrations of $NiSO_4$, $NiCl_2$, or $Ni(OH)_2$ (Table 2). Colony morphology was altered by the presence of nickel. Aerial mycelia were not observed on Actinobacteria isolates, which indicates interference in sporulation. The presence of nickel also altered the color of Actinobacteria colonies on solid media (data not shown).

All serpentine soil isolates were able to maintain growth on medium containing nickel concentrations above 1 mM, a concentration which inhibited the growth of *Escherichia coli*, a nickel-susceptible control. The highest tolerance to Ni salts was observed in Actinobacteria belonging to the genera *Kitasatospora*, *Streptomyces*, and *Nocardia*, which were able to grow in the presence of up to 64 mM $NiSO_4$ and/or $NiCl_2$ (Table 2). In general, the MTC for both nickel salts (sulfate or chloride) was similar when tested on a solid culture medium, except for two Actinobacteria isolates and one isolate belonging to the genus *Variovorax*.

Tolerance to insoluble nickel was evaluated using $Ni(OH)_2$, and the isolates showed a greater tolerance to nickel. All the isolates from Barro Alto tolerated 50 mM of $Ni(OH)_2$, which was the highest concentration tested, whereas for *E. coli*, growth was observed only up to 20 mM (Table 2).

Evaluation of nickel content in cells

To investigate if these isolates were able to remove nickel from the culture medium and retain it in their cells, nickel associated with cell pellet after growth on media containing $NiSO_4$ was evaluated. The isolates *Mucilaginibacter* sp. SAP B3, *Nocardia* sp. SAP F4, *Nocardia* sp. SAP E5, LAT *Streptomyces* sp. LAT A10, *Bradyrhizobium* sp. LAT C8, and *Variovorax* sp.

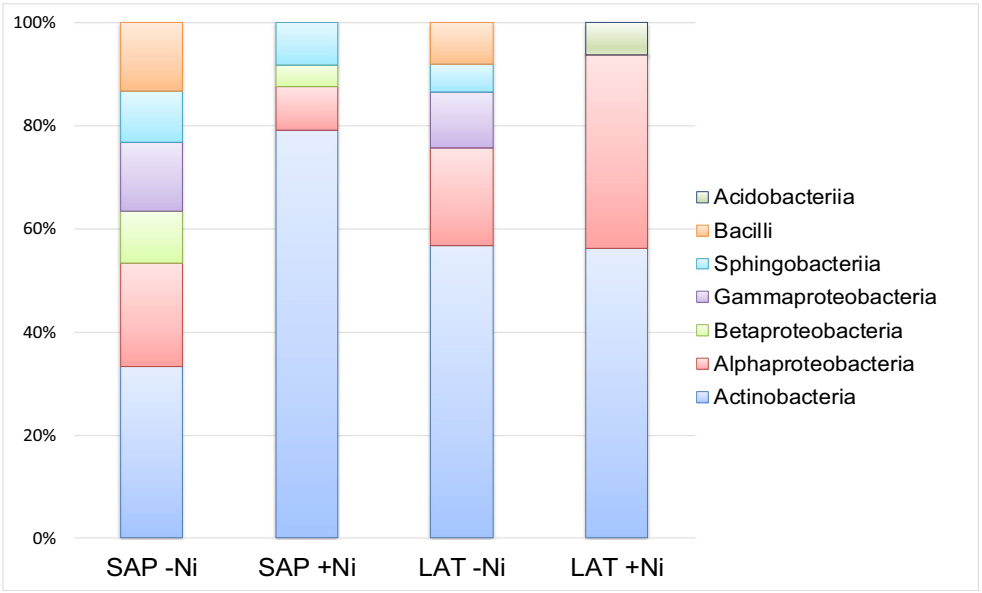

Fig. 1 Percentage of classified 16S rRNA sequences at phylum level of bacteria ($n = 105$) isolated from Brazilian lateritic (LAT) or saprolitic (SAP) serpentine soils on media with and without the amendment of nickel. Classification was achieved using the classifier tool in the Ribosomal Database Project using 90% threshold

SAP E12 were selected. The choice of isolates was based on their ability to grow well on R2A liquid medium.

Detectable concentrations of nickel in the pellet of four of the six tested isolates (Fig. 2) were observed. *Mucilaginibacter* sp. SAPB3, *Variovorax* sp. SAPE12, *Streptomyces* sp. LATA10, and *Nocardia* sp. SAPE5 retained 1.92 to 8.99% of the amount of nickel amended in the medium.

Discussion

Biotechnological processes that use microbes to remove metals from the environment or to improve mining processes have been successfully used in copper and gold mining. The potential of serpentine soil bacteria for biotechnological applications was evaluated in the present study. Our previous work on soil bacterial communities of the ultramafic complex of Barro Alto, Brazil, showed

Table 2 Identification and maximum tolerable concentration (MTC) for $NiSO_4$, $NiCl_2$, and $Ni(OH)_2$ for bacteria isolated from Barro Alto serpentine soils

Isolate	Taxonomic classification		MTC (mM)		
	Genus	Phylum	$NiCl_2$	$NiSO_4$	$Ni(OH)_2^+$
LAT C8	*Bradyrhizobium*	α-Proteobacteria	16	16	> 50
SAP B2	*Bradyrhizobium*	α-Proteobacteria	16	16	> 50
SAP H2	Bradyrhizobiaceae*	α-Proteobacteria	16	16	> 50
SAP E12	*Variovorax*	β-Proteobacteria	8	4	> 50
SAP B3	*Mucilaginibacter*	Bacteroidetes	16	16	> 50
LAT H3	*Streptomyces*	Actinobacteria	64	64	> 50
SAP C1	*Streptomyces*	Actinobacteria	16	16	> 50
LAT A10	*Streptomyces*	Actinobacteria	8	8	> 50
LAT C2	*Streptomyces*	Actinobacteria	32	32	> 50
SAP E5	*Nocardia*	Actinobacteria	64	64	> 50
SAP F4	*Nocardia*	Actinobacteria	32	16	> 50
SAP B12	*Kitasatospora*	Actinobacteria	32	64	> 50
SAP E9	*Kitasatospora*	Actinobacteria	16	32	> 50
*E. coli***			0.5	0.5	20

*All isolates were identified using 100% confidence-at-genus level, except SAP H2, which could only be classified at the family level. **Control strain. $^+$ Concentration in mM is an approximation, since this nickel form is not completely dissociated, as is the case for $NiCl_2$ and $NiSO_4$ (see "Methods" section)

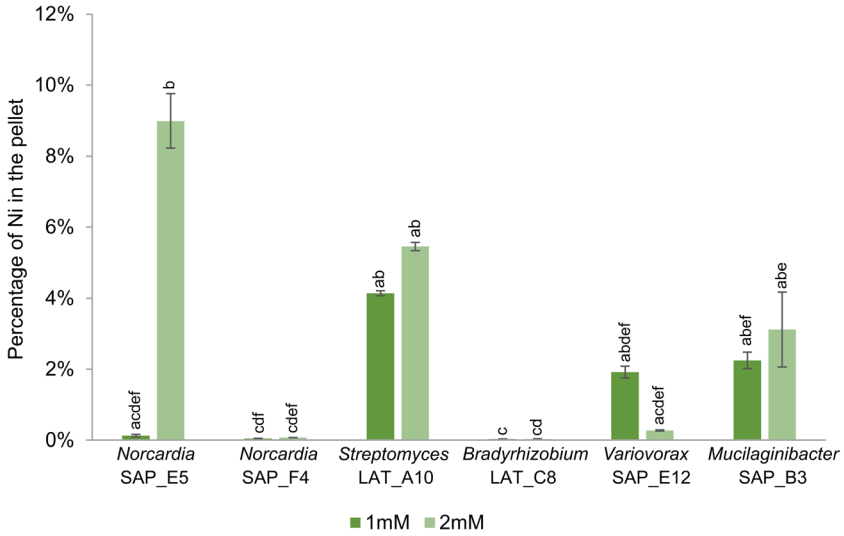

Fig. 2 Average percentage of nickel retained in the pellet fractions of six bacteria isolated from serpentine soils in Brazil. Nickel quantification was obtained with ICP-OES; the limit of detection was 0.008 μM of Ni^{+2}. Error bars indicate standard deviation. Samples sharing the same letter are not significantly different (p value > 0.05, Dunn's test)

that soil microbiological functioning was not affected by the high nickel content [11]. No differences in OTU-based richness and diversity estimates were found between sites. There was an indication, however, that relative abundance of bacterial phyla was different between samples originated from lateritic or saprolitic areas. In this study, bacterial members of these communities were isolated and the tolerance to nickel was evaluated. In addition, isolates were further investigated for a potential ability to remove nickel from the culture medium by retaining this metal associated with the cell components.

The number of colony forming units (CFU) observed on media amended with nickel was lower than on media not amended with nickel. Bacteria from serpentine soils were expected to exhibit a natural tolerance to nickel, but the results indicated that the concentration of 5 mM of $NiSO_4$ is harmful to some of the serpentine soil bacteria. This result may be explained by the difference of toxicity of nickel salts used in culture media compared with the availability of nickel found in the serpentine soils. For instance, bioavailable nickel in serpentine soils in India ranged between 397 and 912 mg kg^{-1} [42], which is greater than the range observed in our samples (134–603 mg kg^{-1}). However, most of the studies that isolated nickel-resistant bacteria from serpentine soils did not provide DTPA-extractable nickel concentrations of sampled sites, which makes a comparison between studies difficult.

The identification of cultured bacterial species from Barro Alto revealed a prevalence of Actinobacteria, especially of the genus *Arthrobacter*, which was obtained from all culture treatments. Actinobacteria was the most commonly isolated phylum from serpentine soils in the USA [43], Italy [14], Turkey [44], and New Caledonia [45–48]. Conversely, soils from Barro Alto yielded bacteria from the genus *Mucilaginibacter* (Bacteroidetes) and from the phylum Acidobacteria, which were not observed in previous studies [43, 44, 49]. It is likely that these differences were due to the chosen culture media. In the present work, the media VL and R2A were used, whereas LB and TSA were used in previous studies [14, 44, 50].

In this work, most of the isolates were able to tolerate Ni salt concentrations above 1 mM, which is considered toxic to *E. coli*. The highest tolerated concentration observed was 64 mM of $NiSO_4$ or $NiCl_2$, for isolates belonging to Actinobacteria. This concentration was greater than the concentration of 15 mM $NiSO_4$ [14, 51] or 20 mM $NiCl_2$ [12, 15, 42, 52] tolerated by bacteria isolated from other serpentine soils worldwide. On the other hand, when the resistance of the present isolates is compared to a well-known nickel-resistant strain like *Acidithiobacillus ferrooxidans*, the resistance observed in the present isolates is low, since these bacteria have been used for bioleaching and can tolerate up to 1 M of nickel [53].

The tolerance exhibited by the Barro Alto isolates was higher on medium containing $Ni(OH)_2$. Nickel hydroxide is insoluble in water, unlike Ni sulfate and chloride salts. As dissociated nickel salts are not common in soil, nickel hydroxide tolerance results may reflect a realistic evaluation of the interaction between environmental bacteria and nickel in serpentine soils and must be further evaluated. To the authors' knowledge, this was the first time an insoluble nickel source was tested for effects on bacterial growth in culture.

The isolates *Bradyrhizobium* LAT C8 and *Nocardia* SAP F4 were not able to retain nickel in the cell fraction of the culture in any of the tested concentrations. However, these isolates tolerated concentrations of nickel between 16 and 64 mM, indicating the presence of other types of tolerance mechanisms. In fact, a *Bradyrhizobium* strain isolated from serpentine soils in New Caledonia tolerates up to 15 mM $NiCl_2$, and the tolerance to nickel is achieved by efflux pumps

encoded by genes such as *cnr* and *nre* [54]. This tolerance is similar to the 16 mM MTC observed in LAT C8.

Other isolates from Barro Alto were able to retain nickel associated with their cell pellets. Although the mechanism was not evaluated in the present study, some strains of the genera *Variovorax*, *Streptomyces*, and *Nocardia* have been previously reported to remove metals by bioadsorption.

The isolate *Nocardia* SAP E5 retained 8% of nickel present in the medium when amended with 2 mM of nickel. However, when 1 mM was added, no detectable quantities of nickel were observed in the pellet. This indicates that *Nocardia* SAP E5 employs different mechanisms of tolerance to different toxicity levels of nickel. *Nocardia* strains that are able to attach heavy metals to their cells were previously reported [20, 55]. In active sludge, *Nocardia* strains increased metal sorption by the sludge because of the increased surface area their filaments form [55]. Another *Nocardia* strain was able to remove 90% of toxic heavy metals from wastewater [20]. This is consistent with the observation of nickel associated with the pellet of SAP E5. Even though *Nocardia* SAP F4 belongs to the same genus as SAP E5, no cell-associated nickel was observed, showing that different species of the same genus present distinct mechanisms to tolerate nickel.

The isolate *Variovorax* SAP E12 presented greater absorption when growth was performed at 1 mM of nickel sulfate compared with that at 2 mM. This may be related to the effects of nickel toxicity on bacterial growth, since decreased bacterial biomass was seen when this isolate was grown in increasing concentrations of $NiSO_4$. A *Variovorax paradoxus* strain was previously reported as being able to perform biosorption of zinc from the culture medium [19]. Another *V. paradoxus* strain, isolated from the rhizosphere of nickel hyperaccumulator *Alyssum murale*, did not possess genes encoding for *ncc* efflux pumps [12], indicating that other mechanisms may be involved.

Strains of *Streptomyces* isolated from a former uranium mine produced a dark pigment to resist toxic concentrations of various heavy metals [56]. *Streptomyces* LAT A10 also produced a dark coloration when exposed to nickel. This pigmentation may not be related to the adsorption of nickel in the cell pellet observed in this isolate, since pigments are generally associated with extracellular chelation of heavy metals [57–59]. However, this pigment may have precipitated the nickel in the medium, which was probably recovered in the centrifugation steps.

The isolate *Mucilaginibacter* SAP B3 retained up to 3% of nickel in the cell fraction after growing in the presence of nickel sulfate. This genus is relatively unknown [60] and there is no mechanism of heavy metal resistance described for its members. The adsorption observed in our isolate may be related to the large amounts of extracellular polymeric substances (EPS) that it produces. It is known that EPS of different chemical compositions are able to sequester heavy metals [61, 62]. In addition, isolate *Mucilaginibacter* SAP B3 grew copiously in our culture conditions, but further studies will be necessary to characterize its mechanisms for nickel tolerance.

Immobilization of heavy metals in aqueous environments allows their transformation into insoluble or chemically inert forms, which are less toxic than their soluble forms [21]. Furthermore, bacterial biomass is used for heavy metal removal in heavy metal–contaminated environments [19–24]. With this in mind, the isolates able to retain nickel from the supernatant in their pellets may be useful in future biotechnological applications in bioremediation of heavy metal–contaminated water environments.

Conclusions

The results presented here indicate that bacterial isolates from the ultramafic complex of Barro Alto present different nickel tolerance mechanisms. Some of these might be of biotechnological interest. Biosorption/accumulation was observed in four isolates, which might be candidates for the deployment of prototypical processes for the removal of Ni^{+2} ions from contaminated water. Genomic sequencing of selected isolates should pave the way for the discovery of genes involved in heavy metal tolerance.

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