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EVALUATION OF COPPER RESISTANT BACTERIA FROM VINEYARD SOILS AND MINING WASTE FOR COPPER BIOSORPTION

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ABSTRACT

Vineyard soils are frequently polluted with high concentrations of copper due application of copper sulfate in order to control fungal diseases. Bioremediation is an efficient process for the treatment of contaminated sites. Efficient copper sorption bacteria can be used for bioremoval of copper from contaminated sites. In this study, a total of 106 copper resistant bacteria were examined for resistance to copper toxicity and biosorption of copper. Eighty isolates (45 from vineyard Mollisol, 35 from Inceptisol) were obtained from EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária) experimental station, Bento Gonçalves, RS, Brazil (29º09'53.92"S and 51º31'39.40"W) and 26 were obtained from copper mining waste from Caçapava do Sul, RS, Brazil (30º29'43.48"S and 53'32'37.87W). Based on resistance to copper toxicity and biosorption, 15 isolates were identified by 16S rRNA gene sequencing. Maximal copper resistance and biosorption at high copper concentration were observed with isolate N2 which removed 80 mg L^{-1} in 24 h. Contrarily isolate N11 (*Bacillus pumilus*) displayed the highest specific copper biosorption (121.82 mg/L/OD unit in 24 h). GenBank MEGABLAST analysis revealed that isolate N2 is 99% similar to *Staphylococcus pasteuri*. Results indicate that several of our isolates have potential use for bioremediation treatment of vineyards soils and mining waste contaminated with high copper concentration.

Key words: Copper contamination, vineyard soil; mining waste, copper biosorption; bioremediation.

INTRODUCTION

Copper is a very important element. Living organisms require copper as an essential micronutrient. Prior to the recognition of the existence of microorganisms on Earth, the Egyptians, Greeks, Romans, and Aztecs used copper compounds for hygiene and for the treatment of diseases (21). Many fungicides, paints, antimicrobial medicines, oral hygiene products, hygienic medical devices, antiseptics and other products contain copper as an antimicrobial agent (21). However, at high copper concentrations, it is very toxic to most forms of life in addition to microorganisms (5).

Mining activities of modern societies, extensive industrial use of copper and its widespread use as a pesticide in crop production are major sources of copper pollution of soils and water. Toxic heavy metals pose a serious threat to human health, biodiversity and the ecosystem (3). In vineyards, copper pollution negatively impact grape production. Consequently,

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the development of methods to remove toxic heavy metals such as copper from water and soils is currently an area of intensive research (1, 6, 7, 8, 12, 14, 20, 22, 23, 25, 26, 27).

Treatment technologies such as ion exchange adsorption, electrodialyses, precipitation and chemical reduction can be used to remove heavy metals (17). These methods are conversely expensive compared to bioremediation processes. Biological removal of pollutants is attractive in this technology, and it is considered cost-effective and eco-friendly (4, 7, 8, 13, 14, 25). Contaminated environments select resistant microorganisms over pollutants (3). Microorganisms that are resistant to toxic and recalcitrant chemicals can be isolated from polluted sites as well as natural soils and used for bioremediation of environments contaminated with specific chemicals to which they are resistant (6, 7, 15, 27, 28). Biosorption is an important bioremediation process for removal of copper and other toxic heavy metals from the environments. In this study, in search for efficient strains for copper bioremoval, we examined a total of 106 copper resistant bacteria isolated from two copper contaminated vineyard soils and copper mining waste for copper biosorption at high concentration. We employed DNA-based methods to identify promising copper resistant isolates with potential for copper bioremoval from contaminated environments.

MATERIALS AND METHODS

Soil sample

Soil samples were collected from three contaminated soils from South of Brazil. Two of them were collected from copper contaminated vineyards areas of EMBRAPA experimental station, Bento Gonçalves, RS, Brazil (29º09'53.92"S and 51º31'39.40"W). These two soils were classified as Inceptisol and Mollisol. The copper mining waste sample was obtained from the copper mining area of Caçapava do Sul, RS, Brazil (30º29'43.48"S and 53'32'37.87W). Soil samples and copper mining waste were characterized. Table 1 presents the physico-chemical parameters analyzed in the Laboratory of Soil Analysis from Federal University of Rio Grande do Sul.

Enrichment and Isolation of Copper-Resistant Bacteria

Enrichment of copper resistant bacteria was in 100 mL of nutrient broth (NB) (5 g of Peptone and 3 g of Beef extract) in 250 mL Erlenmeyer flasks to which 300 mg L^{-1} of Cu(II) as copper sulfate ($CuSO_4.5H_2O$) was added and pH was adjusted to 7.0. NB was sterilized by autoclaving at 121ºC for 20 min. The soil samples were independently used to inoculate (1%, w/v) sterile medium amended with Cu(II) and incubated for 24 h, with shaking (150 rpm) at 30ºC. Subsequently, 1 mL of enrichment culture was used to inoculate 99 mL of sterile medium amended with Cu(II) and incubated for 24 h, with shaking (150 rpm, 30ºC). This procedure was repeated two times. Cu(II)-resistant bacterial were thereafter purified by repeated streaking on nutrient agar (NA) plates containing Cu(II) (300 mg L^{-1}). The isolates were coded with letter C for Mollisol isolates, letter N for Inceptisol isolates and letter R for waste from copper mining area.

Table 1. Chemical and physical properties of vineyard soils contaminated with copper (Inceptisol and Mollisol) and copper mining wastes (Waste).

Tratamento	pH 1:1	CEC* $cmol_c\ dm^{-3}$	OM** g dm^{-3}	Clay %	Cu	Zn	Mn
					---------- mg dm^{-3} ----------		
Inceptisol	6.3	17.2	2.6	19	207	19	55
Mollisol	6.0	13.9	2.5	29	142	18	35
Waste	7.9	-	0.9	2	576	0.8	2
	Ca	**Al**	**Mg**	**H + Al**	**S**	**P**	**K**
	----------cmolc dm^{-3}----------				---------- mg dm^{-3} ----------		
Inceptisol	10.9	0.0	3.1	2.8	6.1	28	142
Mollisol	7.8	0.0	2.1	3.5	5.9	27	167
Waste	24.2	0.0	1.7	-	12.3	32	32

* CEC - cation exchange capacity.
**OM - organic mater.

Analysis of isolates for Cu(II)-resistance profile and biosorption

Monoculture isolates were evaluated for Cu(II)-resistance and biosorption as follows: Inoculants were prepared by transferring three loops of each isolate to NB medium amended with 300 mg L^{-1} of copper and incubated at 30ºC for 24 h with shaking (150 rpm). After, each inoculum was adjusted with sterile saline solution (0.85%) to optical density of 0.85 (OD_{600}) and 0.1 mL of each inoculum was added into 20 mL of NB medium containing 300 mg L^{-1} of Cu(II) in 50 mL Erlenmeyer and incubated (150 rpm, 24 h, 30ºC). Biomass (cell density) was determined by measuring absorbance at OD_{600} of appropriately diluted cultures. Copper biosorption was determined by measuring copper remaining in the cell-free supernatant, using an atomic absorption spectrophotometer. Briefly, 5 mL of replicate cultures were subjected to centrifugation (10,000 rpm, 10 min). Total copper was analyzed using atomic absorption spectrometer (Perkin-Elmer 2380). Aliquots of culture supernatant (1000 µL aliquots) were diluted 20 times and injected into the atomic absorption spectrometer. Copper biosorption was calculated as the difference in total copper added to the medium and total copper remaining in the medium after different microbial treatments. (CuBiosor = CuTotal added – CuTotal after growth).

DNA based identification of isolates

Isolates were identified by 16S ribosomal RNA gene sequencing as follows. The isolates were grown by streaking on nutrient agar with incubation at 30ºC for 24 h. DNA of each isolate was extracted from colonies forming units pooled from the nutrient agar plate using Promega Wizard Genomic DNA Purification Kit (Promega, Madison, WI) with slight modification. Briefly, cells were re-suspended in 300 µL of nucleic acid lyses solution, incubated at 80ºC for 15 min and allowed to cool at room temperature. RNase solution (1.5 µL) was added and incubated at 37ºC for 60 min. Protein precipitation solution (100 µL) was added and incubated on ice for 5 min. Following centrifugation, the supernatant was transferred to an ice cold tube with 95% ethanol. The precipitate was recovered by centrifugation. The pellet was washed with 70% ethanol at room temperature and re-suspended in sterile nuclease free distilled water. Two primers corresponding to *E. coli* positions 27F (5'-AGATTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGAC TT-3') were used for PCR amplification of the 16S ribosomal RNA (18). The PCR reaction mixture consisted of 12.5 µL of PCR master mix (Promega, Madison, WI), genomic DNA template (0.5 µL), primer 27F (2.5 µL=12.5 pmol), primer 1492R (2.5 µl=12.5pmol) and made up to 25 µl final volume with nuclease-free water. The 16S rRNA gene was amplified using a 35-cycle PCR (initial denaturation, 95ºC for 5 min; subsequent denaturation, 95ºC for 0.5 min; annealing temperature, 50ºC for 1 min; extension temperature, 72ºC for 1 min and final extension, 72ºC for 5 min). The PCR amplification products were analyzed by electrophoresis on a 1% agarose gel. Millipore Montage PCR filter units (Millipore, Billerica, MA) were used to remove primers, salts, and unincorporated dNTPs according to the manufacturer's instructions. DNA cycle sequencing was performed using BigDye terminator kit (Applied Biosystems, Foster City, CA) with sequencing primer 519r (5'-GWATTACCGCGGCKGCTG-3') in independent reactions at the Institute of Integrative Genome Biology (IIGB) of UCR, Riverside, CA.

DNA Sequence Similarity and Phylogenetic Analysis

GenBank BLAST (N) was used for homology searches. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.1 (30). Nucleotide sequence similarity searches were conducted by Genbank BLAST (N). The ribosomal RNA gene sequences were submitted to the GenBank database under accession numbers ranging from FJ577657 to FJ577671.

RESULTS

Biosorption of Cu(II) by isolates

Table 2 presents Cu(II) biosorption by 55 bacteria isolated

from Mollisol collected from vineyard soil polluted with copper. Maximum biomass development at high copper concentration (300 mg L^{-1}) was observed with isolate C28, C40, C41 and C44. Cu(II) biosorption was maximal in cultures of isolates C12 (62.21 mg L^{-1} in 24 h) and C14 (61.77 mg L^{-1} in 24 h). Isolate C34 displayed the lowest Cu(II) biosorption (6.48 mg L^{-1} in 24 h) although it grew luxuriantly (1.55 OD_{600}) units at high concentration of Cu(II) (300 mg L^{-1}).

Table 2. Biomass levels and Cu(II) bioremoval in cultures of isolates from vineyard Mollisol incubated in NB medium contaminated with 300 mg L^{-1} of Cu(II) and incubated at 30ºC for 24 h with orbital shaking.

Isolates	Biomass	Cu(II) bioremoval	Specific Cu(II) bioremoval
	---- OD_{600} ----	---- mg L^{-1} ----	--- mg L^{-1}/OD units ---
C1	1.16±0.0643*	12.53±0.6330	11.73±0.096
C2	1.53±0.0464	20.14±1.0338	13.25±0.848
C3	1.71±0.0122	34.91±3.1758	20.41±1.890
C4	1.14±0.0343	18.57±0.3165	15.76±0.712
C6	1.57±0.0328	31.78±2.2971	20.24±1.301
C7	1.16±0.0475	35.36±3.1652	32.10±1.762
C8	1.45±0.0101	15.88±1.5826	11.12±1.146
C9	1.22±0.0250	13.20±0.9496	10.88±0.494
C11	1.31±0.0227	36.03±1.5826	26.66±1.208
C12	1.19±0.0219	62.21±2.5322	53.71±1.773
C14	1.16±0.0250	61.77±2.0675	53.72±2.900
C15	0.83±0.0435	29.09±2.9805	36.83±6.043
C16	0.85±0.0125	16.56±0.6330	19.82±0.490
C17	0.80±0.0195	13.87±1.2661	17.00±1.020
C18	0.64±0.0709	20.14±1.5826	33.07±2.614
C20	0.93±0.0128	27.30±3.7983	28.83±4.064
C21	1.58±0.0554	34.69±1.5826	21.75±0.068
C22	0.89±0.0424	18.79±1.8091	21.13±1.781
C24	1.01±0.0478	29.99±5.4273	29.09±5.062
C28	1.92±0.0080	25.96±0.6330	13.59±0.371
C30	0.84±0.0265	41.40±7.9131	51.04±8.672
C31	1.75±0.0301	17.90±2.5322	10.59±1.560
C34	1.55±0.0242	6.48±0.9496	4.07±0.534
C35	1.59±0.0092	10.74±2.8429	6.71±1.746
C36	1.67±0.0457	19.24±1.3675	11.01±0.139
C37	1.60±0.0168	31.33±1.1265	19.81±0.194
C38	1.03±0.0319	10.29±1.3675	9.91±1.303
C39	1.52±0.1418	8.95±1.1265	5.79±0.223
C40	1.91±0.0269	24.61±2.3260	12.99±1.365
C41	1.89±0.0289	20.59±1.9512	10.90±1.057
C42	1.65±0.1697	17.45±3.6458	10.12±2.210
C43	1.52±0.1072	24.61±6.3305	15.68±2.827
C44	1.91±0.0347	41.40±2.2157	22.13±1.710
C45	1.16±0.0470	15.21±1.8991	12.20±1.367
C46	1.00±0.0246	17.45±3.6458	17.75±3.899
C47	1.24±0.0962	12.98±2.1154	9.27±0.596
C48	1.63±0.0248	15.88±1.5826	9.67±0.454
C49	1.56±0.0204	12.98±2.1154	8.30±1.341
C50	1.39±0.0205	15.21±3.9787	11.32±0.902
C52	1.45±0.1317	18.79±0.2584	13.85±1.607
C53	1.45±0.0654	15.21±3.9787	11.05±3.050
C54	1.63±0.0335	26.40±2.0675	16.12±1.155
C55	1.72±0.0829	33.12±1.5720	19.79±1.941

*Values are means ± standard error of the mean

Forty copper resistant bacteria were isolated from Inceptisol collected from copper contaminated vineyard area (Table 3). Growth of the isolates in media amended with 300 mg L^{-1} was not directly related to copper biosorption. Cell density was highest in cultures of isolate N18 (1.98 OD_{600} units). The highest biosorption of copper was recorded in culture of isolate N2 (80.22 mg L^{-1} in 24 h) while the lowest Cu(II) biosorption (5.85 mg L^{-1} in 24 h) was observed for the isolate N20 although cell density was high (1.41 OD_{600} units in 24 h).

Copper biosorption and biomass levels in cultures of 30 bacterial isolates from copper mining waste are presented in Table 4. In general, no direct relationship was observed between amount of biomass in culture and biosorption of Cu(II) by the isolates. Bacterial cell density was highest in cultures of isolates R27 (1.20 OD_{600} units in 24 h), R17 (1.15 OD_{600} units in 24 h) and R8 (1.09 OD_{600} units in 24 h). Maximal Cu(II) biosorption occurred in cultures of isolates R17 (70.47 mg L^{-1} in 24 h) and R4 (68.34 mg L^{-1} in 24 h).

Table 3. Biomass levels, Cu(II) bioremoval, and specific copper bioremoval in cultures of isolates from vineyard Inceptisol incubated in NB medium contaminated with 300 mg L^{-1} of Cu(II) and incubated at 30ºC for 24 h with orbital shaking.

Isolates	Biomass ---- OD_{600} ----	Cu(II) bioremoval ------ mg L^{-1} ------	Specific Cu(II) bioremoval --- mg L^{-1}/OD units ---
N1	1.46±0.0195*	32.79±1.4768	22.01±0.957
N2	1.45±0.0426	80.22±2.5696	53.40±2.950
N3	1.36±0.0046	32.16±2.3257	23.75±1.776
N4	1.43±0.0231	51.38±1.3427	36.02±1.280
N5	1.13±0.0424	27.15±2.9239	23.65±1.838
N6	1.20±0.0861	30.91±4.1770	27.84±5.232
N7	1.41±0.0288	35.92±3.0121	25.31±1.773
N8	1.38±0.0202	32.16±0.8861	23.14±0.030
N9	1.33±0.0290	22.56±2.4116	16.97±1.805
N10	1.40±0.0150	30.91±0.8980	22.11±0.234
N11	0.67±0.1021	67.25±1.0070	121.83±0.900
N12	1.44±0.0358	29.66±6.4979	21.20±4.386
N13	1.50±0.2895	43.86±4.3875	33.68±2.649
N14	1.61±0.0315	38.01±3.5030	23.53±2.093
N16	1.61±0.0718	44.69±0.5907	27.22±1.822
N17	1.46±0.0396	36.34±4.2869	24.97±2.920
N18	1.98±0.3887	52.21±4.7257	26.10±1.003
N20	1.41±0.0268	5.85±0.5000	4.17±0.112
N22	1.43±0.0478	31.54±2.0675	22.54±2.503
N23	1.40±0.2701	37.80±0.2954	26.96±0.419
N24	1.38±0.2681	37.17±5.3165	26.44±2.927
N25	1.38±0.2647	32.16±7.0886	23.27±5.036
N26	1.33±0.0146	32.16±4.3609	23.96±3.019
N27	1.30±0.0100	19.21±5.5151	14.64±4.152
N28	1.31±0.0070	29.66±6.0242	22.75±4.735
N29	1.36±0.0166	38.01±2.9338	28.10±2.380
N30	1.41±0.0031	16.50±5.0211	11.72±3.553
N32	1.68±0.0959	32.58±5.2559	18.68±2.279
N33	1.44±0.0201	35.30±2.0675	25.07±1.261
N34	1.39±0.0210	7.10±0.5907	4.98±0.366
N35	1.31±0.0177	21.72±5.0356	16.28±3.634
N36	1.29±0.0122	30.91±1.5060	23.92±1.201
N38	1.26±0.0187	35.09±4.6576	27.56±3.245
N39	1.39±0.0181	41.77±1.7390	30.03±1.059
N40	1.18±0.0657	38.84±2.8432	33.48±2.701

*Values are means ± standard error of the mean.

Specific copper removal capacity was generally high in isolates from mining waste. Isolate N11 from vineyard Inceptisol contaminated with copper, however, displayed the highest specific copper bioremoval (121.82 mg/L/OD unit) in 24 h (Table 3). This was followed by isolates R4, R5, R17 and R3 with specific copper bioremoval capacities of 79.70, 67.21, 61.46 and 64.61 mg/L/OD unit in 24 h respectively (Table 4). Statistical evaluation of copper removed by each isolate based on remaining copper in culture supernatant, however, showed that isolate N2 from copper contaminated vineyard Inceptisol was significantly higher than others isolates. Isolates N11, R4 and R17 removed similar levels of copper from the culture.

Table 4. Biomass levels and Cu(II) bioremoval in cultures of isolates from copper mining waste incubated in NB medium contaminated with 300 mg L^{-1} of Cu(II) and incubated at 30ºC for 24 h with orbital shaking.

Isolates	Biomass ---- OD_{600} ----	Cu(II) bioremoval ----- mg L^{-1} -----	Specific Cu(II) bioremoval --- mg L^{-1}/OD units ---
R1	0.81±0.0009*	35.06±1.1920	43.45±1.528
R2	0.92±0.0106	34.22±0.6438	37.48±1.236
R3	0.94±0.0705	56.55±7.7480	36.84±0.900
R4	0.80±0.0151	68.35±4.6992	79.70±5.918
R5	0.84±0.0012	53.60±5.2693	67.21±6.448
R6	0.94±0.0045	44.33±1.3547	53.06±3.562
R7	0.82±0.0002	44.75±3.1631	49.54±1.826
R8	1.09±0.0403	40.75±1.4900	28.98±0.993
R9	0.89±0.0042	31.27±0.4214	35.46±2.351
R10	0.89±0.0024	31.69±2.0789	53.35±3.408
R11	0.84±0.0092	47.28±2.7094	62.70±3.122
R12	0.85±0.0219	52.34±2.4331	52.10±1.854
R17	1.15±0.0422	70.46±0.7299	76.49±3.448
R18	0.84±0.0226	36.74±3.8007	44.77±5.862
R19	0.73±0.0012	29.58±0.9733	40.79±1.439
R20	0.86±0.0415	34.64±1.3547	41.18±3.019
R21	0.82±0.0019	31.69±1.4800	38.70±1.845
R22	0.86±0.0099	39.69±1.2875	45.96±1.089
R23	0.67±0.1426	40.54±1.7032	44.00±1.299
R24	0.90±0.0250	27.89±0.8773	31.30±0.851
R25	0.85±0.0257	37.17±2.1212	44.01±3.490
R26	0.80±0.0073	30.42±1.7546	38.03±2.596
R27	1.20±0.0193	36.32±0.8429	30.26±0.718
R28	0.78±0.0061	30.84±1.9003	39.43±2.634
R29	0.79±0.0125	27.89±0.9733	36.38±0.935
R30	0.85±0.0047	23.68±1.1150	27.96±1.447

*Values are means ± standard error of the mean.

Identity of selected isolates based on 16S rRNA gene sequence

Fifteen bacterial isolates selected on the basis of copper biosorption and resistances to high copper concentration were identified by 16S rRNA gene sequence analysis. Nucleotide sequences in the range of 471-506 nucleic acid bases (Table 5) were used for Genbank blast analysis and construction of phylogenetic tree. Most of the isolates were identified as *Bacillus* species in the phylum Firmicutes. Nine isolates (C28; C44; C12; C40; C41; N11; N16; R4 and R6) were identified as *Bacillus pumilus*. Two isolates (C45 and N14) were identified as *Bacillus thuringiensis* and three (N18; R3 and R16) as *Bacillus* sp. One isolate was identified as *Staphylococcus pasteuri* (N2). Blast analysis revealed that isolates C44, C41, C40, N11, N2, R4 and R16 were 99% similar to the Genbank match. Isolates C28, C45, C12, N16, N14, N18 and R3 were

98% similar to the Genbank match and 97% similarity was observed for isolate R6. Figure 1 presents the phylogenetic relationship among selected isolates from three different copper contaminated areas in study.

Table 5. DNA-based identification of isolates from different contaminated soils vineyard Mollisol (C), vineyard Inceptisol (N) and copper mining waste (R).

Isolates	Source	16S rDNA Nucleotides	GenBank Submition	GenBank Match	Identity (%)
C28	Mollisol (Vineyard)	494	FJ577657	EU102277.1	*B. pumilus* (98)
C45	Mollisol (Vineyard)	472	FJ577658	EU037097.1	*B. thuringiensis* (98)
C44	Mollisol (Vineyard)	476	FJ577659	EF528292.1	*B. pumilus* (99)
C12	Mollisol (Vineyard)	478	FJ577660	DQ412563.1	*B. pumilus* (98)
C41	Mollisol (Vineyard)	471	FJ577661	FJ032017.1	*B. pumilus* (99)
C40	Mollisol (Vineyard)	474	FJ577662	AY792029.1	*B. pumilus* (99)
N11	Inceptisol (Vineyard)	503	FJ577663	EU102277.1	*B. pumilus* (99)
N16	Inceptisol (Vineyard)	506	FJ577664	EU855197.1	*B. pumilus* (98)
N14	Inceptisol (Vineyard)	502	FJ577665	EU037097.1	*B. thuringiensis* (98)
N18	Inceptisol (Vineyard)	505	FJ577666	EU821778.1	*Bacillus* sp. (98)
N2	Inceptisol (Vineyard)	494	FJ577667	EU373331.1	*S. pasteuri* (99)
R3	Mining Waste	502	FJ577668	EU821778.1	*Bacillus* sp. (98)
R4	Mining Waste	495	FJ577669	EU855197.1	*B. pumilus* (99)
R6	Mining Waste	495	FJ577670	FM179663.1	*B. pumilus* (97)
R17	Mining Waste	472	FJ577671	DQ122328.1	*Bacillus* sp. (99)

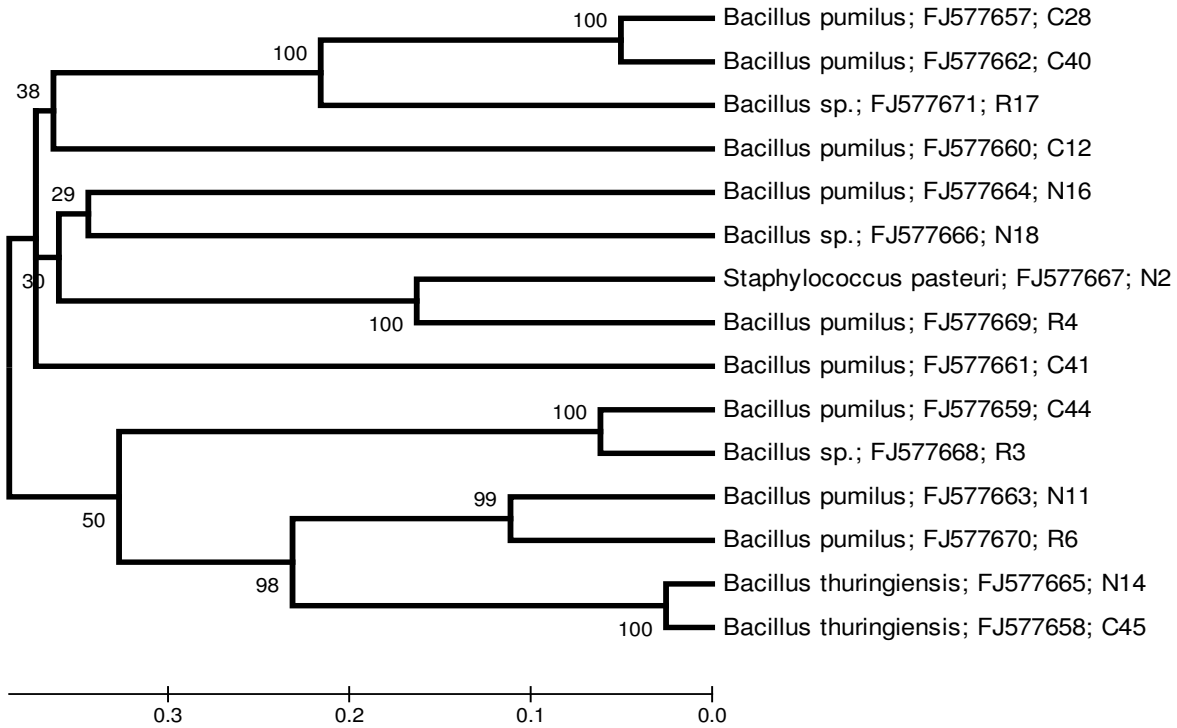

Figure 1. Phylogenetic tree showing evolutionary distance among selected isolates from three copper contaminated areas based on 16S rRNA gene sequence. The number at each node is the bootstrap from 100 replicates. The scale is the evolutionary distance value.

DISCUSSION

Copper is one of the toxic heavy metals of concern in the environment. Toxicity of heavy metals is largely due to their presence in aqueous systems in ionic forms, which are easily absorbed by living organisms (2, 3). There is increasing interest in the use of microbial biomass for biosorption of heavy metals from the environment. Biosorption of heavy metals involve accumulation of the metals in microbial biomass with subsequent recovery and remediation through bioremediation or chemical technologies. Copper resistant microorganisms with the capacity to adsorb copper on biomass can be used as bioremediation tools to remove copper from contaminated terrestrial and aquatic environments (1).

Several bacteria isolated in this study demonstrated tolerance to extremely high copper concentration. An *Agrobacterium tumefaciens,* strain CCNWRS33-2 grew in 2.0 mM or 127 mg L^{-1} of copper in TY liquid medium (5 g tryptone, 3 g yeast extract, and 0.7 g $CaCl_2 \cdot 2H_2O$ per liter), (YMA) (29) but our isolates resisted up to 300 mg L^{-1} of Cu(II) in nutrient broth medium, in which they have almost the same composition. The isolate *A. tumefaciens* CCNWRS33-2 removed 6.35 mg L^{-1} of Cu(II) after 36 h (29), and our best isolate (N2) removed 80 mg L^{-1} after 24 h, being much more efficient on copper bioremoval. One isolate was identified as *Staphylococcus pasteuri*. There is little or no published information on copper biosorption by the genus *Staphylococcus*. However, *Staphylococcus warneri* was isolated from sediment slurry contaminated with Se (24). Microbial communities are affected by environmental pressures which decrease natural populations and select microbes resistant to contaminants (3). Selenate reducing *Bacillus* species were abundant in sediment slurries from an evaporation pond heavily polluted with Se and salt (24). *Bacillus* is an important bacterial genus for bioremediation of heavy metals in different heavy metal contaminated areas (13, 16).

In general, the isolates from the copper mining area showed stronger capacity for copper biosorption. Isolates from copper mining waste area, R4, R17, R3 and R6 removed as much as 70 mg L^{-1} from liquid medium in 24 h. A *P. putida* CZ1 (6) and *Pseudomonas* sp. NA (1) removed from liquid medium 20 to 25 mg L^{-1} in 24 h. *Staphylococcus pasteuri* N2 isolated in our work from vineyard soil polluted with Cu(II) displayed the highest copper biosorption capacity and removed as high as 80 mg L^{-1} of Cu(II) in 24 h. On the contrary, isolate N11 (*B. pumilus*) showed the highest specific rate of copper biosorption calculated relative to cell density. This parity in copper removal capacity of the two isolates indicates that copper biosorption can be directly related to biomass as observed with *S. pasteuri* N2.

High sorptive capacity of prokaryotic (29) and eukaryotic (19) microorganisms are due to components of cell walls that offer an array of functional groups with metal binding capacity. Metals can also be accumulated in the cell cytoplasm (3), but some studies show that higher copper concentrations are linked to the cell wall than the cytoplasm in one prokaryote specie (29). Copper is one of the metals with great potential for bioremoval from contaminated environments through biosorption. In a comparative study (11) on selective binding of different metals to the cell wall of *Pseudomonas* sp., Cu(II) had much more affinity than other heavy metals like Ni(II), Co(II) and Cd(II) when evaluated together.

In summary, we evaluated copper biosorption by several environmental isolates of copper resistant bacteria. DNA-based methods were employed to characterize selected highly copper resistant isolates. Our results showed that several of the isolates have good potential for copper bioremoval from complex environments contaminated with copper.

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REFERENCES

1. Andreazza, R.; Pieniz, S.; Wolf, L.; Lee, M.; Camargo, F.A.O.; Okeke, B.C. (2010). Characterization of copper biosorption and bioreduction by a highly copper resistant bacterium isolated from copper-contaminated vineyard soil. *Sci. Total Environ*., 408:7:1501-1507.
2. Arica, M.Y.; Bayramoglu, G. (2005). Cr(VI) biosorption from aqueous solutions using free and immobilized biomass of *Lentinus sajor*-caju: preparation and kinetic characterization. *Physicochem. Eng. A*, 253:203-211.
3. Atlas, R.M.; Bartha, R. (1997). Microbial Ecology: Fundamentals and Applications. 4th edn. Benjamin/Cummings Science Publishing, USA, 656 pp.
4. Camargo, F.A.O.; Bento, F.M.; Jacques, R.J.S.; Roesch, L.F.W.; Frankenberger, W.T. (2007). Uso de microrganismos para a remediação de metais. *Tópicos Especiais em Ciência do Solo*, 5:467-496.
5. Cervantes, C.; Gutierrez-Corona, F. (1994). Copper resistance mechanisms in bacteria and fungi. *Microbiol. Rev*., 14:121-138.
6. Chen, X.C.; Wang, Y.P.; Lin, Q.; Shi, J.Y.; Wu, W.X.; Chen, Y.X. (2005). Biosorption of copper (II) and zinc (II) from aqueous solution by *Pseudomonas putida* CZ1. *Colloid. Surface B*., 46:101-107.
7. Chen, H.; Yang, B.; Chen, X. (2007). Identification and characterization of four strains of *Acidithiobacillus ferrooxidans* isolated from different sites in China. *Microbiol. Res*., doi:10.1016/j.micres.2007.09.002.
8. Chen, X.C.; Wu, W.X.; Shi, J.Y.; Xu, X.H.; Wang, H.; Chen, Y.X. (2007). Adsorption of copper and zinc on *Pseudomonas putida* CZ1: Particle concentration effect and adsorption reversibility. *Colloid. Surface B*., 54:46-52.
9. Chen, Y.Q.; Ren, G.J.; An, S.Q.; Sun, Q.Y.; Liu, C.H.; Ahuang, J.L. (2008). Changes of bacterial community structure in copper mine tailings after colonization of reed (*Phragmites communis*). *Pedosphere*, 6:731-740.
10. Chen, X.C.; Hu, S.P.; Shen, C.F.; Dou, C.M.; Shi, J.Y.; Chen, Y.X. (2009). Interaction of *Pseudomonas putida* CZ1 with clays and ability of the composite to immobilize copper and zinc from solution. *Bioresource Technol*., 100:330-337.
11. Choudhary, S.; Sar, P. (2009). Characterization of a metal resistant *Pseudomonas* sp. isolated from uranium mine for its potential in heavy metal (Ni^{2+}, Co^{2+}, Cu^{2+}, and Cd^{2+}) sequestration. *Bioresource Technol*., 100:2482-2492.
12. Cojocaru, C.; Diaconu, M.; Cretescu, I.; Savic, J.; Vasic, V. (2009). Biosorption of copper(II) ions from aqua solutions using dried yeast biomass. *Colloid. Surface A*., 335:181-188.
13. Desai, C.; Jain, K.; Madamwar, D. (2008). Evaluation of In vitro Cr(VI) reduction potential in cytosolic extracts of three indigenous *Bacillus* sp. isolated from Cr(VI) polluted industrial landfill. *Bioresource Technol.*, 99:6059-6069.
14. Freitas, O.; Delerue-Matos, C.; Boaventura, R. (2009). Optimization of Cu(II) biosorption onto *Ascophyllum nodosum* by factorial design methodology. *J. Hazard. Mater*., 167:1:449-454.
15. He, Z.; Gao, F.; Sha, T.; Hu, Y.; He, C. (2009). Isolation and characterization of a Cr(VI)-reduction *Ochrobactrum* sp. strain CSCr-3 from chromium landfill. *J. Hazard. Mater*., 163:869-873.
16. Jiang, C.; Sun, H.; Sun, T.; Zhang, Q.; Zhang, Y. (2009). Immobilization of cadmium in soils by UV-mutated *Bacillus subtilis* 38 bioaugmentation and NovoGro amendment. *J. Hazard. Mater*., 167:1170-1177.
17. Krishna, K.R.; Philip, L. (2005). Bioremediation of Cr(VI) in contaminated soils. *J. Hazard. Mater*., 121:109-117.
18. Lane, D. (1991). 16S/23S sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial sistematics. John Wiley and Sons, New York, USA, 171-244 p.
19. Macfie, S.M.; Welbourn, P.M. (2000). The cell wall as a barrier to uptake of metal ions in the unicellular green alga *Chlamydomonas reinhardtii* (Chlorophyceae). *Arch. Environ. Contam. Toxicol*., 39:413-419.
20. Melgar, M.J.; Alonso, J.; García, M.A. (2007). Removal of toxic metals from aqueous solutions by fungal biomass of *Agaricus macrosporus*. *Sci. Total Environ*.; 385:12-19.
21. Michels, H.T. (2006). Anti-microbial characteristics of copper. Accessed in July 29th of 2009, <http://www.astm.org/SNEWS/OCTOBER_2006/ michels_oct06.html>.
22. Özer, A.; Gürbüz, G.; Çalimli, A.; Körbahti, B.K. (2009). Biosorption of copper(II) ions on *Enteromorpha prolifera*: Application of response surface methodology (RSM). *Chem. Eng. J*., 146:377-387.
23. Sari, A.; Mendil, D.; Tuzen, M.; Soylak, M. (2009). Biosorption of palladium(II) from aqueous solution by moss (*Racomitrium lanuginosum*) biomass: Equilibrium, kinetic and thermodynamic studies. *J. Hazard. Mater*., 162:874-879.
24. Siddique, T.; Zhang, Y.; Okeke, B.C.; Frankenberger, W.T. (2006). Characterization of sediment bacteria involved in selenium reduction. *Bioresource Technol*., 97:1041-1049.
25. Uzel, A.; Ozdemir, G. (2009) Metal biosorption capacity of the organic solvent tolerant *Pseudomonas fluorescens* TEM08. *Bioresour. Technol*., 100:542-548.
26. Vilar, V.J.P.; Martins, R.J.E.; Botelho, C.M.S.; Boaventura, R.A.R. (2009). Removal of Cu and Cr from an industrial effluent using a packed-bed column with algae *Gelidium*-derived material. *Hydrometallurgy*, 96:42-46.
27. Umrania, V.V. (2006). Bioremediation of toxic heavy metals using acidothermophilic autotroph. *Bioresour. Technol*., 97:1237-1242.
28. Voss, M.; Thomas, R.W.S.P. (2001). Sorção de cobre e manganês por bactérias rizosféricas do trigo. *Ciência Rural*, 31:947-951.
29. Wei, G.; Fan, L.; Zhu, W.; Fu, Y.; Yu, J.; Tang, M. (2009). Isolation and characterization of the heavy metal resistant bacteria CCNWRS33-2 isolated from root nodule of *Lespedeza cuneata* in gold mine tailings in China. *J. Hazard. Mater*., 162:50-56.
30. Tamura, K.; Dudley, J.; Nei, M.; Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol*. 24:1596-1599.