Bull. Environ. Contam. Toxicol. (2005) 74:219–226

DOI: 10.1007/s00128-004-0573-z

Bulletin of Environmental Contamination and Toxicology

Screening of Copper Tolerant Bacterial Strains and Their Potential to Remove Copper from the Environment

N. Ahmed, A. Nawaz, U. Badar

Center for Molecular Genetics, University of Karachi, Karachi 75270, Pakistan

Received: 20 February 2004/Accepted: 1 November 2004

Industrialization has given mankind the gift of advancement and the bane of environmental pollution. Untreated industrial waste is a major source of pollution in the environment and is basically composed of toxic levels of organic and inorganic compounds. Heavy metal contaminants such as cadmium, copper, chromate, zinc and nickel form a major part of the industrial wastes. Bacteria exposed to high levels of heavy metals in their environment have adapted to this stress by developing various resistance mechanisms. These microorganisms could be utilized for detoxification and removal of heavy metals from polluted environments.

Certain heavy metals are essential for metabolic processes of the bacterial physiology. These are required in trace amounts as micronutrients. Copper is an essential micronutrient for bacteria, which aids in various redox reactions in the organism. But in high concentrations, copper proves to be toxic and may cause membrane destruction and inactivation of certain bacterial enzymes (Trevors et al., 1985). Copper toxicity is based on the production of hydroperoxide radicals (Rodriguez Montelongo et al., 1993) and on its interaction with the cell membrane (Suwalsky et al., 1998). Several bacterial species have been shown to resist copper via uptake and efflux of the heavy metal. The resistance mechanisms and their genetic controls are responsive to the requirement to accumulate cations at trace levels and at the same time reduce cytoplasmic concentrations from potentially toxic levels (Brown et al, 1992). Copper resistance is also mediated by intracellular sequestration via inducible low molecular weight metallothionein-type molecules rich in sulphydryl groups (Hamer, 1986). Plasmid encoded copper resistance has been shown in Grams –ve bacteria like *Pseudomonas* (Cooksey, 1994), *E. coli* (Brown et al., 1994, 1995) and *Enterobacter sp.* (Badar, unpublished data).

The object of this study was to isolate, identify and characterize the bacteria that performed efficient uptake of copper ions for their possible use in removal of copper from polluted environments. The isolated bacteria were then subjected to a number of investigations to determine the mechanism of copper resistance, which included screening for the production of stress proteins under the heavy metal stress via SDS PAGE.

Correspondence to: N. Ahmed

MATERIALS AND METHODS

Bacterial strains were isolated from a soil sample from an oil-contaminated site in Karachi. The sample was inoculated into 100mL of L.B broth and incubated overnight at 37°C. After 24hr of enrichment, the samples were spread on agar plates and two morphologically different colonies were purified and tested for antibiotic and heavy metal resistance. The strains were tested against the following heavy metal salts i.e. Cr(III), Cr(VI), $CdCl_2$, $CoCl_2$, $CuSO_4$, $NiSO_4$ and $ZnCl_2$, which were prepared in stock solutions of 1*M* concentrations. In order to find out the MTC of metal salts, overnight cultures were streaked on tris minimal agar (OXOID) plates containing varying concentrations of metal salts (0.5m*M*-5m*M*). Plates were examined for visible growth after 24-48 hours at 37°C. To determine the maximum tolerable concentration (MTC) of antibiotics such as streptomycin, erythromycin, kanamycin, ampicillin, rifampicin, chloramphenicol and tetracycline, bacterial cultures were streaked on nutrient agar plates containing variable concentration of antibiotic solutions. The plates were incubated at 37°C and growth was observed after 24 hr. Two of the isolates were selected on the basis of their high resistance to multiple antibiotics and heavy metal salts. The selected soil isolates were tentatively identified on visual basis i.e. production of violet zones on GSP agar (Merck) indicator plates. Confirmed identification was performed by partial gene analysis of the 16S rRNA gene of the isolates. Genomic DNA of the soil isolates was isolated using genomic DNA isolation kit (GENTRA, USA). The genomic DNA was used as template DNA in a touchdown PCR reaction (Thermal cycler: Applied Biosystems, GeneAmplification system 2400) using the primers **16S3'** CCCGGGAACGTATTCACCG and **16S5'** GCYTAAYACATGCAAGTCGA, which amplify a sequence of 1.4Kb (approx.). The reaction consisted of 37 cycles i.e. 94°C for 3min. (1 cycle) 94°C for 1min. 60°C for 30sec, 72°C for 3min. (2 cycles) and then each following cycle after every two cycles had a consecutive decrease of 1°C in the primer annealing step upto 50°C. A final extension time was given for 5 min at 72°C. (Scott C., 2002). Reaction mixture was composed of: 2µL of each primer (20pmol), 5µL dNTP (200µ*M*), 5µL NH4 buffer (1X), 2µL $MgCl_2$ (4m*M*), 1µL Taq polymerase (1U/50µl), Template (1µL), Sterilized distilled water (32µL) bringing the total volume up to 50µL.

PCR products were purified using QIAquick PCR purification kit (Qiagen, UK) and sequenced. Cycle sequencing was performed using ABI PRISM Big Dye Terminator v3.0 ready reaction cycle sequencing kit. The labeled DNA was sent to Proteonomics and functional genomics lab, University of Birmingham, Edgbaston, Birmingham for automated DNA sequencing. Sequence data obtained were analyzed by using BLAST algorithm (http://www.ncbi.nlmnih.gov/blast/Blast.cgi).

Copper accumulation was quantitatively measured by a spectrophotometric method based on that of L. E. Macaskie, 1995 and described by Qureishi et al., 2001. To 100µL of borate buffer [26.9g of $B(OH)_3$, 2.6g of NaOH in 900mL of

distilled water adjusted to pH 8.1 with 2*M* NaOH and made up to 1L] 20µL of reagent (0.5g of *bis*-cyclohexanone-oxalyldihydrazone in 100mL of 1:1 [vol/vol] water-ethanol, dissolved by heating and cooled and filtered before use) was added, followed by addition of 880µL of aqueous sample (supernatant), and the solution was mixed thoroughly. The colored complex formed was measured at 595nm against a standard copper calibration curve prepared in the same manner (Qureishi et al., 2001). The sensitivity of the assay ranged from 7.98mgL^{-1} down to 0.25mgL^{-1}. Overnight cultures of CMG464 and CMG466 were inoculated in 100mL 0 f 0 .2% t ris gluconate i n 5 00mL flasks. E ach s train w as i noculated i n 0.1mM and 0.5mM of $CuSO_4$. Optical density of hourly samples was taken and each sample was then centrifuged for 3 minutes at 10,000rpm. The supernatant was transferred to sterile eppendorf tubes for copper assay while the pellet was washed with sterile 0.89% saline and stored at –20°C for protein estimation. Both the pellet and supernatant for CMG464 and CMG466 were stored at –20°C. The supernatant was used in the copper assay.

Protein assay was performed by the method described by Ahmed *et al.* (1997) Absorbance was recorded at 555nm using spectrophotometer (Cam spec M302) and assay was calibrated against a standard curve of bovine serum albumin prepared in a similar manner as described.

Over night cultures of CMG464 and CMG466 were inoculated in 25mL of 0.2% tris gluconate with and without copper. After incubation of 72 hours at 37°C in a shaking incubator, cells were harvested in a refrigerated centrifuge (Kokusan model H-200nr) at 6000rpm at 4°C. The pellet was washed with 0.5mL of ice-cold 50m*M* tris-HCl (pH 7.4), centrifuged and then the supernatant was removed. The pellet was resuspended in 25mL of distilled water and 25µl of 2X SDS gel loading buffer. After vortexing for 20 seconds the samples were placed in a boiling water bath for 5 minutes, the cells were then properly lysed by adding 4-5 sterilized glass beads in the tube by vigorous vortexing. The eppendorf tubes were then centrifuged for 10 minutes at room temperature a nd the supernatant was transferred to a new autoclaved eppendorf tube.

12.5% SDS gel was prepared in a vertical gel slab with a stacking gel layered above a running gel according to the method described by Maniatis (Maniatis *et al.*, 1982). 10µL of sample was loaded in the wells and electrophoresed at 120Volts. Protein bands were observed by staining the gel with commasie blue for 24 hr and destaining in a solution of 7mL glacial acetic acid, 50 mL methanol and 43 mL distilled water.

Plasmid DNA was isolated by the method as described by Brinboim and Doly (1979). Electrophoresis was performed on horizontal 0.7% agarose gel slab using TAE buffer. 20µL of the sample was loaded and electrophoresed at 100Volts for 1.5hr. DNA bands were observed after staining the gel with 10µgm/mL solution of ethidium bromide and destained in distilled water. The bands were visualized

using an UV transilluminator at 320nm. Standard DNA markers were also run with the samples.

RESULTS AND DISSCUSION

Two bacterial strains designated as CMG464 and CMG466 were isolated and purified from a sample of petroleum-contaminated soil. Both the strains were identified as of belonging to the *Pseudomonas sp.* by production of violet zones on GSP agar indicator plates. The 16S rRNA gene analysis confirmed that both the strains were of the *Pseudomonas* species. The partial 16S rRNA gene sequence data for CMG464 (Genbank accession number AY59958) and CMG466(Genbank accession number AY599587) was analyzed using NCBI BLAST 2.2.8, where CMG464 showed a 97% homology and was designated as a *Pseudomonas sp.*. While CMG466 showed a 99% homology and was identified as *Pseudomonas aeruginosa*. Morphologically, CMG464 appeared as coccobacilli while CMG466 was observed as short thin rods. The cellular morphology revealed a reduction in size and unstained regions in the cells when growing in presence of copper salt. When stained for spores, no spores were revealed.

This study was undertaken to isolate bacterial strains from an oil contaminated soil sample and were tested for multiple metal resistance against copper, cadmium, chromate etc. Both the strains showed a very high resistance to cadmium chloride i.e. 2 m*M* in minimal media. CMG464 showed resistance to 0.5m*M* of copper sulfate while for CMG466 resistance was recorded upto 1.0m*M* of copper sulfate. The two strains were also resistant to Cr(III), Cr(VI), Cobalt chloride and zinc chloride. Both CMG464 and CMG466 were sensitive to lead acetate and nickel sulfate (table 1).

Tris minimal media substituted with 0.2% gluconic acid as the sole carbon source was used. This minimal medium was selected because enriched media tend to complex heavy metal salts and hence make them less available in the medium.

Table 1. Maximum tolerable concentration of heavy metal salts for CMG464 and CMG466.

Bacterial strains	Maximum tolerable concentration of heavy metal salts (m*M*)						
	$CdCl_2$	$CuSO_4$	Cr(VI)	$CoCl_2$	$NiSO_4$	$ZnCl_2$	$CrCl_3$
CMG464	2.0	0.5	0.5	0.5	-	1.5	1.5
CMG466	2.0	1.0	0.5	0.5	-	1.5	1.5

-, Sensitive at 0.5m*M*.

The bioavailability or toxicity of a metal ion depends upon the chemical constitution of the medium and availability of complexing ligands (Hughes and Poole, 1991). Both the strains were grown in 0.1m*M* and 0.5m*M* $CuSO_4$ and disappearance of copper was checked with time. At 0.1m*M* Conc. of $CuSO_4$, both the strains showed efficient removal of the metal salt from the medium i.e.

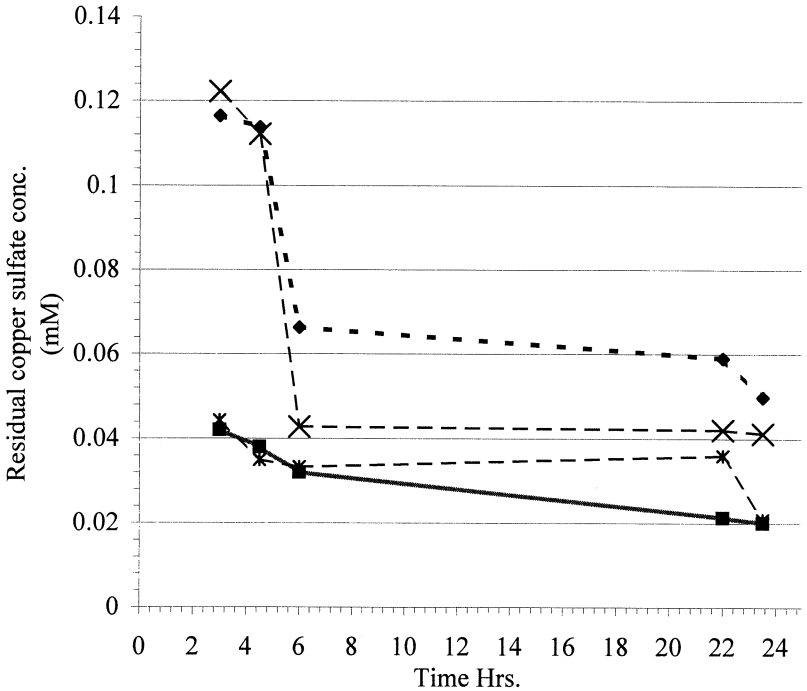

Figure 1. Copper sulfate uptake profile of CMG464 and CMG466 in tris gluconate (◆) CMG466 in 0.5m*M* Cu, (■) CMG466 in 0.1m*M* Cu, (✕) CMG464 in 0.5m*M* Cu, (∗) CMG464 in 0.1m*M* Cu.

CMG464 removed 77% of the total copper from the medium while CMG466 removed 78%. At 0.5m*M* $CuSO_4$, both the strains showed 91% removal of $CuSO_4$ from the medium (Figure 1).

Quantitative protein analysis revealed that at 0.1m*M* of $CuSO_4$, both CMG464 and CMG466 have similar protein concentrations as that in absence of the metal salt. At 0.5m*M*, an increased protein concentration i.e. 1.04μgm/mL for CMG466 was observed after 78hr of incubation for the soil isolate when compared to its respective control which was 0.3405μgm/mL at 78hr (Ahmed *et al.*, 1994).

At time of maximum copper removal i.e. 3-4.5 hr of incubation, the protein was observed to be less as compared to no copper conditions. CMG464 gave a protein concentration of 0.053μgm/mL in contrast to 0.43μgm/mL under normal conditions, giving an 8.11 fold decrease in protein concentration as compared to control; while CMG466 gave a protein concentration of 0.045μgm/mL under copper stress while in no copper condition it gave a protein concentration of 0.31μgm/mL, giving a 6.88 fold decrease in the protein concentration as compared to control.

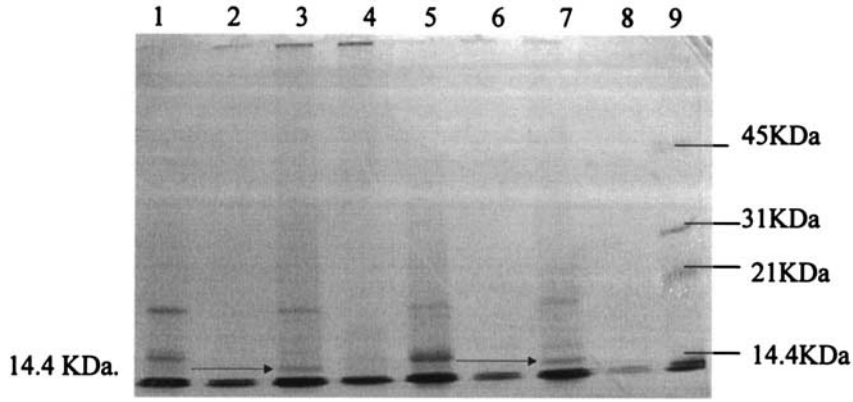

Figure 2.Qualitative protein profile of CMG464 and CMG466 under normal and copper stress conditions in 0.2%tris gluconate. lane 1 & 5 CMG464 and CMG466, protein was extracted from unstressed cells, lanes 2, 3, 4, 6, 7 & 8, protein was extracted from metal stressed cells, lane 2(CMG464 under cadmium stress), lane 3(CMG464 under copper stress), lane 4 (CMG464 under chromate stress), lane 6 (CMG466 under cadmium stress), lane 7 (CMG466 under copper stress), lane 8 (CMG466 under chromate stress), lane 9 (Standard marker).

The removal of copper, as estimated from the growth pattern of the bacteria under copper stress, starts at the early log phase of the bacterial growth and continues till the late log phase which spans till 28 hr of incubation for both the strains. This shows that removal of copper is associated with the log phase or the rapidly dividing phase of the bacterial growth, in other words we can say that these two processes are directly proportional to each other i.e. the number of bacterial cells and copper removal. The copper removal is stopped at the onset of the stationary phase.

Qualitative estimation of protein from both the strains, under normal and stress conditions was performed by SDS-PAGE. Upon comparison of protein samples from control and stressed conditions, of both strains, production of a stress protein was confirmed. An extra low molecular weight protein, approx. 14,400 Daltons, was seen in both CMG464 and CMG466 under copper stress (Figure 2).

To determine whether these heavy metal resistance traits were chromosomally mediated or plasmid mediated, plasmid isolation was performed for CMG464 and CMG466 according to the method described by *Brinboim and Doly* (1979). No plasmid was isolated, even upon repeated trials. Hence the genes for heavy metal resistance may be located on the chromosome. To further confirm the absence of a plasmid DNA, the two strains were repeatedly sub cultured in enriched media to cure the plasmid (if present). After a week of sub culturing, replica plating onto different selection plates showed no loss of any character.

The evidence of the production of stress protein reflects the probable resistance mechanism of CMG464 and CMG466 against toxic heavy metal like copper. Both the isolates showed efficient removal of copper sulfate from the medium and these potential bacteria can be exploited in future for removal of metals salts from industrial effluents.

Acknowledgments. We thank L. E. Macaskie, School of Life Sciences, University of Birmingham and Foaud M. Qureshi, Center for Molecular Genetics, University of Karachi for the 16S rRNA gene sequencing of the bacterial strains. We are grateful to Dr. Fehmida Fasim, Center for Molecular Genetics, University of Karachi for her guidance with the technique of SDS PAGE.

REFERENCES

Ahmed N, Badar U, Hassan MT and Raihan S. (1997). Isolation of local copper tolerant bacteria in Pakistan for biofilm formation on various supports and subsequent biosorption. Res Environ Biotechnol 2:65-72.

Bender CA and Cooksey DA. (1987). Molecular cloning of copper resistance genes from *Pseudomonas syringae pv. Tomato*. J Bacteriol 169:470-474.

Brinboim, HC and Doly J (1979). A rapid alkaline extraction procedure for screening recombinant DNA. Nuc Acid Res 7:1513-1523

Brown NL, Barrett SR, Camakaris J, Lee BTO and Rouch DA (1995). Molecular genetics and transport analysis of the copper resistance determinant (*pco*) from *Escherichia coli* plasmid pRJ1004. Mol Microbiol 17:1153-1166.

Brown NL, Lee BTO and Silver S (1994). Bacterial transport of and resistance to copper. In: Sigel, H. and Sigel, A. (Eds.), Metal ions in biological systems, Vol. 30. Marcel Dekker, New York, pp. 405-435.

Brown NL, Rouch DA and Lee BTO (1992). Copper resistance determinants in bacteria. Plasmid 27:41-51.

Cooksey DA (1994). Molecular mechanisms of copper resistance and accumulation in bacteria. FEMS Microbiol Rev 14:381-386.

Hamer DH (1986). Metallothionein. Ann Rev Biochem 55:913-951.

Hughes MN, Poole RK (1991). Metal speciation and microbial growth-the hard (and soft) facts. J Gen Microbiol 137:725-734.

Macaskie LE. (1995) Copper tolerance, phosphatase activity and copper uptake by heavy metal-accumulating *Citrobacter* species. Microbios 84:137-153.

Maniatis T, Sambrook J and Fritsch EF (1982). Molecular cloning and Laboratory manual, Cold Spring Harbour, New York, Cold Spring handbook laboratory.

Qureshi FM, Badar U, and Ahmed N (2001). Biosorption of copper by bacterial biofilm on PVC flexible conduit. App Environ Microbiol 67 (9), 4349-4352

Rodriguez Montelongo L, de la Cruz Rodriguez LC, Farias RN, Massa EM (1993). Membrane-associated redox cycling of copper mediates hydroperoxide toxicity in Escherichia coli. Biochim Biophys Acta 1144:77-84

Scott Cameron. (2002). PhD thesis titled: Phenotypic and Genotypic Investigations into fluoroquinolone resistance in the Genus *Acinetobacter* University of Dundee, Dundee, Scotland.

Suwalsky M, Ungerer B, Quevedo L, Aguilar F, Sotomayer CP (1998). Cu^{2+} ions interact with cell membranes. J Inorg Biochem 70:233-238.