INT. J. BIOL. BIOTECH., 3 (1): 113-120, 2006.

CORRELATION OF MULTIPLE STRESS TOLERANCE IN INDIGENOUS BACTERIA

Erum Shoeb and Nuzhat Ahmed

Centre for Molecular Genetics, University of Karachi, Karachi-75270, Pakistan; a_nuzhat@yahoo.com

ABSTRACT

Forty three strains were isolated from metal contaminated sites close to the residential areas of Karachi, Pakistan. These strains were found to tolerate heavy metals and antibiotics. In case of heavy metals 62.8% of the strains have shown tolerance against chromate and copper, and 51.2% against nickel. Eight antibiotics were checked, 97.7% of the strains have shown tolerance against streptomycin, 44.2% against neomycin, 27.9% against ampicillin, 25.6% against tetracycline, 13.9% against chloramphenicol, 9.3% of the strains have shown tolerance against kanamycin and novobiocin, and 2.3% against rifampicin. Attempt was made to find out correlation between heavy metal tolerance and antibiotic tolerance of these strains with the assumption of multiple stress tolerance of bacteria. Statistical analysis has shown positive correlation between heavy metal tolerance and antibiotic tolerance of these strains, t-test has been conducted to test the null hypothesis of no correlation which has been rejected at $t_{.05}$ level of significance. Omegon-transposon containing kanamycin tolerance gene did not hybridize with the strain CMG2K8 which has shown tolerance upto 1500 µg/ml of kanamycin, when colony hybridization was performed through DIG labelled probe, this result has indicated that mechanism of tolerance to kanamycin of the strain CMG2K8 is different from the one found in Omegon-transposon.

Key-words: Enrichment cultures, Heavy metal tolerance, Antibiotics tolerance, Correlation, Multiple stress.

INTRODUCTION

Living organisms are in direct contact with heavy metals since the beginning of life on earth, as in early evolutionary history geochemical events caused the release of heavy metals from the earth's crust into the biosphere. Bacteria, being one of the most primitive life forms on earth, naturally developed tolerance to a wide range of toxic heavy metals including As, Cd, Co, Cr, Cu, Hg, Ni, Sb, Te, and Zn (Silver and Walderhaug, 1992; Silver and Ji, 1994) in its genome. Some of the heavy metals are purely toxic, while other metals are essential for life at low concentration but become toxic at high concentrations (Badar et al., 2000).

Increased industrialization and modern agricultural practices have left persistent toxic heavy metals in the environment, which are of great threat to the eco-system (Badar et al., 2001). Using bacterial tolerance against widespread metal compounds and utilizing the mechanism of accumulation or metal reduction, one can minimize the effect of heavy metal on total biological activity of the ecosystem (Francis and Dodge, 1998; Silver et al., 2001). Unlike many organisms, bacteria share their DNA with one another in exchange unrelated to reproduction (Parker and Hall, 1990). They adapt, and survive, by acquiring genes that allow them to tolerate toxins (Miller, 1993). Recent research has indicated that similar mechanisms for tolerance to a single metal may cross a wide range of bacterial genera, and that related mechanisms of tolerance may apply to different heavy metals (Silver and Walderhaug, 1995). Environment of multiple stress create a selective pressure, which in turn leads to the mutations in microorganisms that will allow them to survive and multiply (Baquero et al., 1998). Thus it is improbable to believe that any organism is fit for survival just by tolerating one of the toxic compounds rather it would be more favorable for the survival of bacterium to acquire multiple stress resistance. Selfish Operon Theory (Lawrence, 2000) explains the importance of useful genes clustering for the organism. Bacterial tolerance to antibiotics and other antimicrobial agents is one of the major issues of our society considering that infectious diseases are becoming more difficult and more expensive to treat. It could be estimated very easily that in an environment with widespread use of heavy metals in the course of industrialization and increased use of antibiotics in health care, high percent of bacteria are expected to be tolerant to metals as well as antibiotics (Calomiris et al., 1984).

In the present study organisms were isolated from contaminated sites of the workshops situated in the residential areas. Metal tolerance and antibiotic tolerance behaviour of these organisms were studied with the aim to identify the correlation. Colony hybridization was also being performed with a probe of transposon carrying kanamycin resistance genes in order to check whether all the kanamycin resistant isolates have the same origin or not.

MATERIALS AND METHODS

Sampling

Bacterial strains were isolated from the metal contaminated sites situated close to metal electroplating and automobile workshops of Gulshan-e-Iqbal, Karachi, Pakistan. Soil samples were collected in sterilized 50 ml.

plastic bottles. 1 gm. of soil was inoculated in 100 ml. of Luria Bertania (LB) broth medium (Maniatis et al., 1982), with constant shaking (100 rpm) at 37° C in 500 ml. flasks. Growth was observed after 24 hours. 1 ml. of culture was inoculated in 100 ml. of fresh LB broth flasks under similar conditions. 10µl. Of 10^{-6} dilution of overnight (O/N) culture equivalent to 0.2 OD_{600} was spreaded on solidified LB agar (1.5%) plates and after incubation of 24 hours at 37°C isolated colonies were picked and inoculated in LB broth flasks to get the pure culture. Pure cultures were characterized on the basis of colonial morphology and Gram reaction. Purified cultures were stored in LB agar (1.3%) as stab cultures in 20% (vol/vol) glycerol (Miller, 1972) at –20°C.

Media and Growth conditions

The isolated strains were routinely grown in Luria Bertania (LB) broth medium at 37°C with agitation in the shaking incubator at 100 rpm. For antibiotic tolerance LB medium supplemented with 1.5% agar, solidified plates were utilized. Metal tolerance was analyzed in Tris minimal media (Fasim et al., 2002) in broth as well as in solidified agar (2%) plates.

Antibiotic Tolerance

Maximum tolerable concentration (MTC) of all the strains against eight antibiotics was checked. Stock solutions of kanamycin (Km; 25mg/ml), tetracycline (Tc, 5mg/ml), rifampicin (Rif; 25mg/ml), ampicillin (Amp; 50mg/ml), chloramphenicol (Cm; 34mg/ml), streptomycin (Sm; 50mg/ml), novobiocin (Nov; 5mg/ml), and neomycin (Neo; 50mg/ml) were used to make dilutions in 25 ml (Sambrook et al., 1989). O/N cultures were streaked on LB agar plates and incubated at 37°C for 48 hours after which growth was observed. Strains which could not grow on 10 µg/ml and more concentration of any antibiotic were considered as sensitive.

Metal Tolerance

All the 43 strains were checked for Metal tolerance. Stock solutions (1M) of hexavalent Chromium (Cr_2O_5), Nickel Chloride ($NiCl_2$), Copper Sulphate ($CuSO_4$) were prepared to make appropriate dilutions (08, 0.1, 0.16, 0.5, 0.75,1.0, 1.25, 1.5, 1.75, and 2mM) in 25 ml. Tris minimal media supplemented with agar plates. Strains which tolerated up to 2mM were further checked. MTC were estimated by streaking colonies from O/N culture on Tris agar plates with appropriate concentration of metal salts and growth was observed after 72 hours of incubation at 37°C. Strains which could not grow on 0.08mM concentration of respective metal were considered as sensitive.

Colony Hybridization

Colonies of all the strains were grown in grids on single LB agar plate for 12 hours and shifted in the refrigerator prior to be lifted by Nylon Membrane [Cat. No. 1699083 (132 mm)] of Roche Molecular Biochemicals. These lifted colonies were Denatured, Neutralized, and Hybridized with Digoxigenin labeled probe of Omegon-Transposon according to The DIG System User's guide Filter Hybridization (Roche Molecular Biochemicals). Colorimetric detection was done using DIG DNA Labelling and Detection Kit (Cat. No. 1093 657).

Correlation

Statistical analysis had been conducted using Microsoft Excel version Microsoft XP Professional. Average values of antibiotic tolerance of each of the strain is taken and correlation with average values of metal tolerance of all the strains was calculated. t-test has also been conducted in order to check whether the correlation coefficient was significant or not.

RESULTS

Isolation, Purification and Maintenance

Forty three bacterial strains were purified and analyzed in the present study. They were maintained and preserved in 20% vl/v glycerol at 20°C.

Antibiotic Tolerance

Maximum tolerable concentrations of all the strains against eight different antibiotics has shown that the highest MTC was recorded against Km which was as high as 1500 µg/ml. shown by CMG2K8. 97.7% of the strains have shown tolerance against Sm, 44.2 % have shown tolerance against Neo, 27.9% against Amp, 25.6% against Tc, 13.9% against Cm, 9.3% against Km and Nov, and 2.3% against Rif (Table 1, **Fig. 1**).

Table 1. MTCs of isolated strains against all the antibiotics tested.

Strains Code	Km µg/ml	Tc µg/ml	Rif µg/ml	Amp µg/ml	Cm µg/ml	Sm µg/ml	Nov µg/ml	Neo µg/ml	Average
CMG2K1	0	0	0	0	0	150	0	100	31.25
CMG2K2	0	0	0	0	0	150	0	0	18.75
CMG2K3	0	0	0	0	0	75	0	0	9.375
CMG2K4	375	100	0	125	25	200	0	100	115.625
CMG2K5	0	25	0	10	0	200	0	100	41.875
CMG2K6	40	10	0	25	0	200	0	100	46.875
CMG2K7	425	100	25	100	50	200	0	100	125
CMG2K8	1500	25	0	75	25	75	0	75	221.875
CMG2K9	0	0	0	0	0	75	0	0	9.375
CMG2K10	5	0	0	50	0	75	0	0	16.25
CMG2K11	0	5	0	10	0	75	0	0	11.25
CMG2K12	0	0	0	0	0	500	0	100	75
CMG2K13	0	0	0	0	0	0	0	0	0
CMG2K14	0	0	0	0	0	25	0	0	3.125
CMG2K15	0	0	0	0	0	100	0	0	12.5
CMG2K16	0	0	0	0	0	25	0	0	3.125
CMG2K17	5	5	0	25	10	200	0	100	43.125
CMG2K18	0	0	0	0	0	150	0	75	28.125
CMG2K19	0	0	0	0	0	100	0	0	12.5
CMG2K20	0	0	0	0	0	100	0	0	12.5
CMG2K21	0	0	0	25	0	200	0	100	40.625
CMG2K22	0	0	0	0	0	100	0	0	12.5
CMG2K23	0	0	0	0	0	75	0	0	9.375
CMG2K24	0	0	0	0	0	75	0	0	9.375
CMG2K25	0	0	0	0	0	150	0	10	20
CMG2K26	0	0	0	0	0	100	200	25	40.625
CMG2K27	0	0	0	0	0	100	0	0	12.5
CMG2K28	0	0	0	10	0	100	0	0	13.75
CMG2K29	0	10	0	0	0	500	0	0	63.75
CMG2K30	0	0	0	0	0	100	0	0	12.5
CMG2K31	0	0	0	0	0	200	200	100	62.5
CMG2K32	0	10	0	0	0	10	0	50	8.75
CMG2K33	0	10	0	0	0	200	0	0	26.25
CMG2K34	0	25	0	50	25	25	0	0	15.625
CMG2K35	0	10	0	0	0	100	0	0	13.75
CMG2K36	0	0	0	0	0	100	0	100	25
CMG2K37	0	0	0	0	0	200	200	100	62.5
CMG2K38	0	0	0	0	0	10	0	0	1.25
CMG2K39	0	0	0	0	0	200	0	200	50
CMG2K40	0	0	0	0	0	100	0	0	12.5
CMG2K41	0	0	0	0	0	100	0	0	12.5
CMG2K42	0	0	0	0	0	200	200	200	75
CMG2K43	0	10	0	100	25	100	0	200	54.375
Percentage	9.3%	25.6%	2.3%	27.9%	13.9%	97.7%	9.3%	44.2%	

Table 2. MTCs of isolated strains against all the heavy metals tested.

Strains code	Cr_2O_5 mM	$NiCl_2$ mM	$CuSO_4$ mM	Average
CM2K1	1	0	0.5	0.5
K2	1	0	0.08	0.36
CM2K3	0	0.08	0	0.02
CM2K4	0	10	0.5	3.5
CM2K5	0	0.08	0.5	0.19
CM2K6	0	13	0.5	4.5
CM2K7	0	17	0.5	5.83
CM2K8	0	9	0.5	3.16
CM2K9	1	0	0	0.33
CM2K10	0.1	0.16	0.08	0.11
CM2K11	0	0	0.75	0.25
CM2K12	1	0.08	0.5	0.52
CM2K13	1	0.08	0.08	0.38
CM2K14	1	0	0	0.33
CM2K15	0.1	0.08	0	0.06
CM2K16	0.1	0	0	0.03
CM2K17	0	0	0.5	0.16
CM2K18	1	0	0.08	0.36
CM2K19	1	0.16	0.08	0.41
CM2K20	1	0.08	0.08	0.38
CM2K21	1	0	0.5	0.5
CM2K22	1	0	0	0.33
CM2K23	1	0	0.08	0.36
CM2K24	1	0.16	0	0.38
CM2K25	0	0.16	0.08	0.08
CM2K26	1	0	0.5	0.5
CM2K27	0	0	0	0
CM2K28	0	0.08	0	0.026667
CM2K29	1	0.16	0	0.386667
CM2K30	1	0.08	0	0.36
CM2K31	1	0.08	1	0.693333
CM2K32	0	0	0	0
CM2K33	1	0.08	0	0.36
CM2K34	0	0	0	0
CM2K35	0	0	0	0
CM2K36	1	0	0.08	0.36
CM2K38	0	0.16	0.08	0.08
CM2K39	1	0	0.5	0.5
CM2K40	1	0.08	0.75	0.61
CM2K41	0.5	0	0	0.166667
CM2K42	0.1	0	1	0.366667
CM2K43	0	0	0.75	0.25
Percentage	62.8	51.2	62.8	

Metal Tolerance

Results of the maximum tolerable concentrations of all the strains against different metals have shown that strains such as CMG2K4, CMG2K6, CMG2K7, CMG2K8 tolerated high concentrations of nickel, which was as high as 10, 13, 17, and 9mM respectively. 62.8% of the strains were found to have tolerance against Cr_2O_5 and $CuSO_4$, and 51.2% against $NiCl_2$ (Table 2, Fig. 2).

Table 3. Results of Colony Hybridization through DIG-labelled Omegon-Transposon probe.

Code	Results of colony Hybridization
CMG2K1	Not Hybridized
CMG2K2	Not Hybridized
CMG2K3	Not Hybridized
CMG2K4	Hybridized
CMG2K5	Hybridized
CMG2K6	Not Hybridized
CMG2K7	Not Hybridized
CMG2K8	Not Hybridized
CMG2K9	Not Hybridized
CMG2K10	Hybridized
CMG2K11	Not Hybridized
CMG2K12	Not Hybridized
CMG2K13	Not Hybridized
CMG2K14	Not Hybridized
CMG2K15	Not Hybridized
CMG2K16	Not Hybridized
CMG2K17	Not Hybridized
CMG2K18	Hybridized
CMG2K19	Not Hybridized
CMG2K20	Not Hybridized
CMG2K21	Not Hybridized
CMG2K22	Not Hybridized
CMG2K23	Not Hybridized
CMG2K24	Not Hybridized
CMG2K25	Not Hybridized
CMG2K26	Not Hybridized
CMG2K27	Not Hybridized
CMG2K28	Not Hybridized
CMG2K29	Not Hybridized
CMG2K30	Hybridized
CMG2K31	Not Hybridized
CMG2K32	Not Hybridized
CMG2K33	Not Hybridized
CMG2K34	Not Hybridized
CMG2K35	Not Hybridized
CMG2K36	Not Hybridized
CMG2K37	Not Hybridized
CMG2K38	Not Hybridized
CMG2K39	Hybridized
CMG2K40	Hybridized
CMG2K41	Not Hybridized
CMG2K42	Not Hybridized
CMG2K43	Not Hybridized
Percentage hybridized	16.2%

Colony Hybridization

Results of colony hybridization with Omegon-Transposon have shown that few strains contain homolog sequences of Omegon-Transposon in their genome (Table 3, Figure 3). Only 16.2 % of the strains have shown homology to the DIG – labeled probe. Isolate CMG2K8 has tolerance of kanamycin upto 1500 µg/ml but it did not hybridize with Omegon-Transposon, which has suggested separate origin of tolerance mechanism.

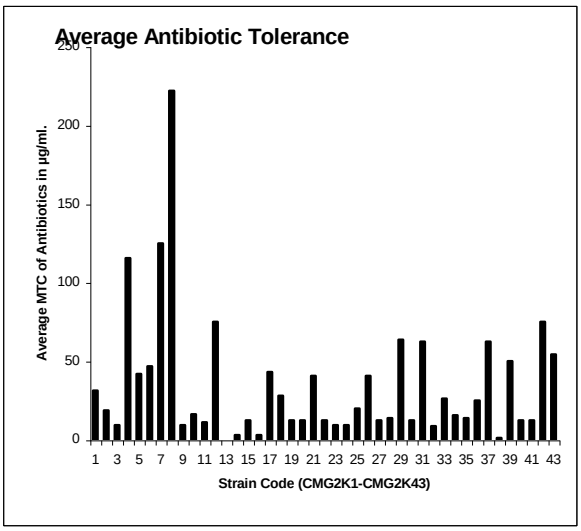

Fig. 1. Bar graph plotted for all the strains versus their average Maximum tolerable concentrations against eight antibiotics Km, Tc, Rif, Amp, Cm, Sm, Nov, Neo.

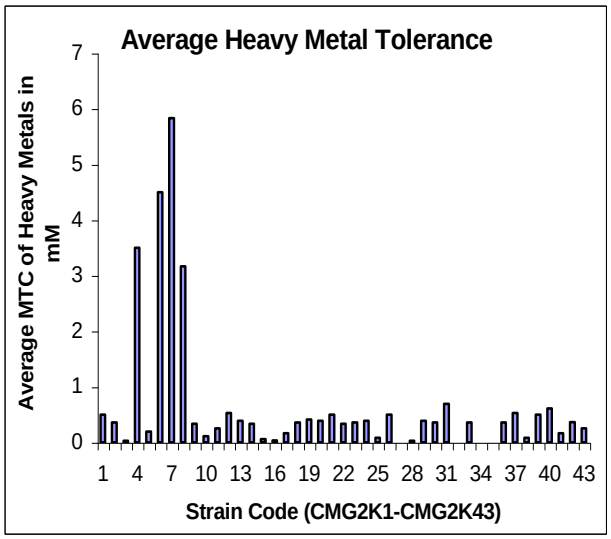

Fig.2. Bargraph plotted for all the strains versus their maximum tolerable concentrations against three heavy metals Cr_2O_5, $NiCl_2$, $CuSO_4$

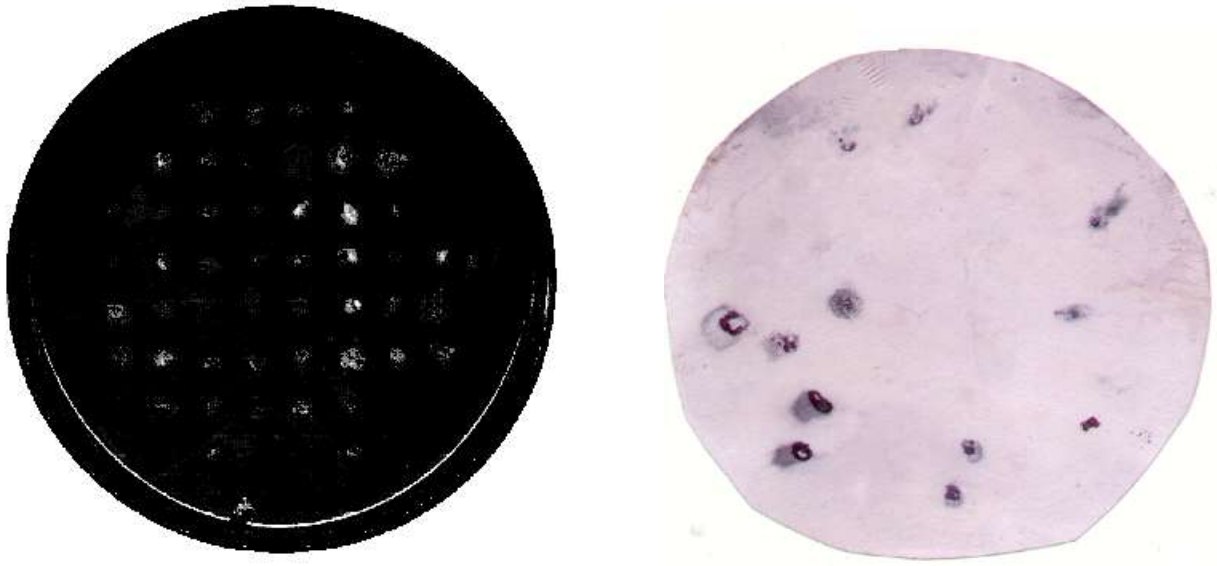

Fig. 3. Showing Results of Colony Hybridization through DIG-labelled Omegon-Transposon probe.
3a) Solidified agar plate having all the colonies. 3b) Nylon Membrane showing positive results.

Correlation

CMG2K4, CMG2K6, CMG2K7, and CMG2K8 have shown highest MTC against nickel and these strains have also shown tolerance against most of the antibiotics.

Statistical analysis has been conducted and the strains have indicated positive linear correlation with respect to average metal tolerance and average antibiotic tolerance. A t-test has been performed to test the null hypothesis of no correlation at $t_{0.05}$ level of significance, and the null hypothesis has been rejected as the calculated value is found to be highly significant.

DISCUSSION

Metal contaminated sites are the potential source of metal tolerant bacteria. Such environments encourage adaptation and selection for heavy metal tolerance (Clausen, 2000). Keeping in mind the aim of the present study i.e., to estimate the correlation between metal tolerance and antibiotic tolerance sampling was done from the workshops surrounded by residential area. Four strains have shown remarkable tolerance against nickel and these four strains have also indicated the antibiotic tolerance. Statistical analysis was conducted which has suggested positive correlation, therefore favoring the concept of evolving microorganisms with multiple stress tolerance which is an alarming threat to the mankind. Since if it is imagined that a pathogenic organism having multiple stress with the capability to adapt itself to the changing environment, one can easily expect difficulty in treating the infections. One of the most commonly used antibiotics in health care is streptomycin, which seems to be tolerated by almost all the strains. Results of colony hybridization have not indicated any homology of Omegon-Transposon with strain CMG2K8 which can tolerate kanamycin up till 1500 µg/ml, which has suggested that there are different mechanisms involved in kanamycin tolerance of microorganisms. It has been reported that different mechanisms of tolerance to toxic compounds operate in microbes (Nies, 1999; Chen et al., 1986) it can be speculated that organisms do not necessarily inherit the genes involved in tolerance but when exposed to stresses, evolve different mechanisms whichever are required for survival. It has been witnessed during the present studies as well, since CMG2K8 despite of having kanamycin tolerance did not show any homology to Omegon transposon. This is where survival of the fittest is proved, and microorganisms are being converted into more and more perfect organisms to survive, by being exposed with high concentrations of toxic compounds including heavy metals and antibiotics.

This study has shown that microorganisms have adapted to tolerate high concentrations of toxic metals in some cases, which could be useful; as such bacteria could be used to clean up metal-contaminated sites. But the presence of metal tolerance mechanism can also contribute to the antibiotic resistance by sharing the genes for multiple stress resistance and by getting the best chance for survival. It is therefore important to realize that what is put into the environment it can cause serious effects on humans as well as on the microbial community and eventually on the whole eco-system.

ACKNOWLEDGEMENT

The authors acknowledge the enabling role of the Higher Education Commission, Islamabad, Pakistan and appreciate its financial support through Indigenous Ph.D. Scholarship given to Ms. Erum Shoeb.

REFERENCES

Badar, U., R. Abbas and N. Ahmed (2001). Characterization of copper and chromate resistant bacteria isolated from Karachi tanneries effluents. In: *Industrial and Environmental Biotechnology*. (Ahmed N., Qureshi F.M., Khan O. Y. eds). Horizontal Scientific Press UK, 43-54.

Badar, U., N. Ahmed, A.J. Beswick, P. Pattanapipitpaisal and L.E. Macaskie (2000). Reduction of chromate by microorganisms isolated from metal contaminated sites of Karachi, Pakistan. *Biotechnology letters*, 22: 829-836.

Baquero, F., M.C. Negri, M.I. Morosini and J. Blazquez (1998). Antibiotic-selective environments. *Clinical Infectious Diseases*, 27: S5-S11.

Brown, N.L., D.A. Rouch and B.T.O. Lee (1992). Copper resistance determinants in bacteria. *Plasmid*, 27: 41-51.

Calormiris, J., J.L. Armstrong and R.J. Seidler (1984). Association of metal tolerance with multiple antibiotic resistance of bacteria isolated from drinking water. *Appl. Environ. Microbiol*., 47: 1238-1242.

Chen, C.M., T.K. Misra, S. Silver and B.P. Rosen (1986). Nucleotide sequence of the structural genes for an anion pump: the plasmid-encoded arsenical resistance operon. *J. Biological Chemistry*, 261: 15030-15038.

Clausen, C.A. (2000). Isolating metal-tolerant bacteria capable of removing copper, chromium, and arsenic from treated wood. *Waste manage. Res*., 18: 264-268.

Fasim, F., N. Ahmed, R. Parsons and G.M. Gadd (2002). Solubilization of zinc salts by a bacterium isolated from the air environment of a tannery. *FEMS Microbiology Letters*, 213: 1-6.

Francis, A.J. and C.J. Dodge (1998). Remediation of soils and wastes contaminated with uranium and toxic metals. *Environ Sci Technol*., 32: 3933-3998.

Lawrence, J.G. (2000). Clustering of antibiotic resistance genes: beyond the selfish operon. *ASM News*, 66: 281-286.

Maniatis, T., E.F. Fritsch and J. Sambrook (1982). *Molecular cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Miller, J.H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Miller, R.V. (1993). Genetic stability of genetically engineered microorganisms in the aquatic environment. In: *Aquatic Microbiology: An ecological approach*. (Timothy Edgcumbe Ford ed). Boston: Blackwell Scientific Publications, 483-511.

Nies, D.H. (1999). Microbial heavy metal resistance. *Appl Microbiol Biotechnol*., 51: 730-750.

Parker, L.L. and B.G. Hall (1990). Characterization and nucleotide sequence of the cryptic cell operon of *Escherichia coli* K12. *Genetics*, 124: 455-471.

Sambrook J., E.F. Fritsch and T. Maniatis (1989). *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Silver, S. and G. Ji (1994). Newer systems for bacterial resistance: New surprises. *Annu. Rev. Microbiol*., 50: 753-789.

Silver, S., L.T. Phung J.F. Lu and A. Gupta (2001). Toxic Metal Resistance: Molecular Biology and the potential for bioremediation. In: *Industrial and Environmental Biotechnology*. (Ahmed N., Qureshi F.M., and Khan O.Y. eds). Horizon Scientific Press UK: 33-41

Silver, S. and M. Walderhaug (1992). Gene regulation of plasmid- and chromosome- determined inorganic ion transport in bacteria. *Microbiol. Rev*., 56: 195-228.

Silver, S. and M. Walderhaug (1995). Bacterial plasmid-mediated resistances to mercury, cadmium and copper. In: *Toxicology of Metals. Biochemical Aspects* (R.A. Goyer and M.G.Cherian eds). Springer Verlag, Berlin , 435-458.

(*Accepted for publication December 2005*)