World Journal of Microbiology & Biotechnology (2005) 21: 1237–1243
DOI 10.1007/s11274-005-1804-0



# Effect of salinity on the tolerance to toxic metals and oxyanions in native moderately halophilic spore-forming bacilli

M.A. Amoozegar[1,*], J. Hamedi[1], M. Dadashipour[2] and S. Shariatpanahi[1]
[1]*Department of Biology, Faculty of Science, University of Tehran, Tehran, Iran*
[2]*Department of Biochemistry, Pasteur Institute of Iran, Tehran, 13164, Iran*
**Author for correspondence: Tel.: +98-21-6112622, Fax: +98-21-6492992, E-mail: alamoze@yahoo.com*

Received 4 October 2004; accepted 4 February 2005

*Keywords:* Chromate, halophilic bacteria, oxyanions, selenite, tellurite, tolerance, toxic metal

## Summary

Ten moderately halophilic spore-forming bacilli were isolated from saline soils in Iran and their intrinsic high-level resistance to chromate, arsenate, tellurite, selenite, selenate and biselenite was identified by an agar dilution method. Minimum inhibitory concentration (MIC) for each oxyanion was determined. All isolates were resistant to higher concentrations of arsenate. The resistance level of the isolates to selenooxyanions was between 10 and 40 mM. Maximum and minimum tolerance against oxyanions was seen in selenite and biselenite, respectively. Although toxic metal resistance in the isolates was not different from non-halophilic bacteria that has been reported, unusual resistance to arsenate (250 mM), sodium chromate (75 mM) and potassium chromate (70 mM) was observed. The results obtained in this study revealed that all isolates were obviously susceptible to silver, nickel, zinc and cobalt, while seven isolates were resistant to lead. Susceptibility to copper and cadmium varied among the isolates. Silver had the maximum toxicity, whereas lead and copper showed minimum toxicity. The impact of salinity on the toxicity of oxyanions was also studied. Our results showed that in general an increase in salinity from 5% (w/v) to 15% (w/v) enhanced tolerance to toxic oxyanions.

## Introduction

High concentrations of toxic metals have caused environmental pollution in agricultural soils, surface and underground water (Nriagu & Pacyna 1988; Kinkle *et al.* 1994). Microorganisms play a major role in bioremediation or biotransformation processes of these toxic elements, converting them to less toxic or non-toxic elements (Kinkle *et al.* 1994). *Acinetobacter*, *Rhodospirillum*, *Pseudomonas*, *Thiobacillus*, *Bacillus* and *Corynebacterium* have been reported as active biotransformers (Kessi *et al.* 1999; McLean & Beveridge 2001). Determining the potential of microorganisms and their tolerance against high concentrations of toxic metals will assist the selection of suitable species for bioremediation and biotransformation of toxic metals (Trevors *et al.* 1985; Burton *et al.* 1987). Halophilic and halotolerant microorganisms are suitable candidates for these processes as they have exceptional properties, that is high concentrations of anions and cations are necessary for the growth of halophilic microorganisms (Ventosa *et al.* 1998), hence they are not only naturally tolerant to some elements that are toxic to other microorganisms, but also they have a requirement for these elements. Furthermore, such information might be desirable, as some of these toxic metal-resistant halophilic bacteria could be used as bioassay indicator organisms in saline polluted environments (Nieto *et al.* 1989). Resistance to toxic metals is a widespread characteristic among bacteria isolated from several environments (Summers 1978, 1985; Trevors *et al.* 1985).

Recently, toxic heavy metal and oxyanion resistance was reported in halophilic microorganisms, e.g. *Halomonas* (Nieto *et al.* 1989; Ventosa *et al.* 1998; Souza *et al.* 2001) and *Pseudoalteromonas* (Rathgeber *et al.* 2002), but there is little work on the resistance of Gram-positive moderately halophilic bacilli to toxic heavy metals and oxyanions. In this study, we have isolated 10 moderately halophilic spore-forming bacilli from various saline soils in Iran, which were not contaminated with toxic oxyanions or heavy metals, and identified their intrinsic high-level resistance against these contaminants. Our aim was to determine resistance levels to toxic metals/oxyanions and the effect of various concentrations of NaCl on the minimum inhibitory concentration (MIC). This is the first report describing resistance to toxic oxyanions in moderately halophilic spore-forming bacilli.

## Materials and methods

### *Bacterial isolation and identification*

Ten Gram-positive spore-forming aerobic moderately halophiles were isolated from various concentrations of saline soils in Gheshm, Karaj, Tehran, Qom and Garmsar of Iran (Figure 1). About 1 g of soil samples was added to a 100 ml Erlenmeyer flask containing 20 ml nutrient broth plus maximum 10% (w/v) NaCl (Amoozegar *et al.* 2003). Inoculated flasks were incubated in an orbital shaker at 34 °C in 150 rev/min. Isolates colonies were taken by culturing the nutrient agar plus 10% (w/v) NaCl. The resistance of isolates to heavy metals was assayed on the nutrient agar plus 5, 10 and 15% (w/v) NaCl. If needed, the pH of the media adjusted at $7.2 \pm 0.1$ with 1 M KOH.

The morphological and physiological tests were conducted on the isolates. Purified isolates were identified by the Persian Type Culture Collection (PTCC), Tehran-Iran.

### *Chemicals*

$Pb(NO_3)_2$, $AgNO_3$, $Cd(NO_3)_2$, $CoCl_2$, $NiCl_2$, $CuSO_4$, $ZnSO_4$, $Na_2CrO_4$, $K_2CrO_4$, $Na_2SeO_3$ and $NaHSeO_3$ were obtained from Merck. $K_2TeO_3$, $Na_2SeO_4$ and $Na_2HAsO_4$ were purchased from Sigma. Stock solutions of chemicals were dissolved in distilled water and sterilized with 0.22-$\mu$m bacteriological filters. Working solutions were stored in the refrigerator for up to 5 days.

### *Determining the toxic metal tolerance*

The agar dilution method was used to determine the bacterial tolerance to toxic metals (Washington & Sutter 1980). Various concentrations of heavy metals (0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 12.0, 14.0, 16.0, 18.0 and 20.0 mM) and various concentrations of toxic oxyanions (0.05, 0.1, 0.3, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0, 50.0, 55.0, 60.0, 65.0, 70.0, 75.0 and 80.0 mM) were examined. Sodium arsenate was an exception that was examined up to 300 mM, because the isolates were highly tolerant to it. Appropriate concentrations of heavy metals/toxic oxyanions were added to 100-ml Erlenmeyer containing 20 ml molten nutrient agar plus 10% (w/v) NaCl. Complete medium was poured in 8-mm glass Petri dishes. Then 10 $\mu$l of the bacterial suspension ($1.5 \times 10^8$ c.f.u./ml) was inoculated and spread on the surface of each plate. The plates were incubated at 34 °C for 72 h. The MIC for each oxyanion was determined. The plates were prepared in triplicate.

Similar experiments were performed by nutrient agar containing 5 (w/v) and 15% (w/v) NaCl. Also, a control medium (without any metal/oxyanion) was used. The inoculated plates were incubated at 34 °C for 72 h. The minimum concentration of the metals/oxyanions that completely inhibited growth of the strain was reported as MIC. The MIC assay was performed in triplicate. It is well known that, unlike antibiotic resistance, which is evaluated with therapeutic doses, there are no standard acceptable metal concentrations used to specify metal or


*Figure 1.* Sampling sites for the isolation of moderately halophilic bacteria.

oxyanion resistance (Nieto *et al.* 1989; McLean & Beveridge 2001; Rathgeber *et al.* 2002). As mentioned above, this is the first report describing resistance to toxic oxyanions in moderately halophilic spore-forming bacilli, therefore, the interpretation of the bacterial resistance to oxyanions was based on the criteria used in previous studies carried out with other bacteria (Nieto *et al.* 1989; McLean & Beveridge 2001; Rathgeber *et al.* 2002). The strain was considered as resistant, when its growth was not inhibited by the resistance limit concentration of any metals/oxyanions. The resistance limit concentrations for arsenate, tellurite, selenate, selenite, biselenite, chromate, cadmium, cobalt, nickel, lead, zinc, silver and copper were 10, 0.5, 20, 20, 10, 5, 1, 1, 1, 1, 1, 1, 1, respectively.

## Results

### *Isolation and identification*

All isolates were Gram-positive rod-shaped spore-forming bacteria. The isolates were separated into groups A, B and C, according to the concentration of NaCl needed for growth. The optimum concentration of NaCl for the A, B and C groups was 15, 10 and 5%, respectively. The isolates could not grow in NaCl-deficient medium and thus were halophiles. The characteristics of the isolates are shown in Table 1. The morphological and physiological results obtained and other complementary tests that were done by the Persian Type Culture Collection (PTCC) revealed that all isolates were *Bacillus* sp.

### *Minimum inhibitory concentration of toxic metals*

The MIC of the metals in the media containing 10% NaCl for each isolate is shown in Figure 2. 40% of the isolates were resistant to $Cu^{2+}$ and $Pb^{2+}$ and all strains were sensitive to $Ag^{+}$, $Ni^{2+}$, $Zn^{2+}$, Co and $Cd^{2+}$. The maximum tolerance to $Pb^{2+}$ was seen with the strains named as C1 and C2, although most of the strains were sensitive to $Ag^{+}$, A3, C3 and C4 tolerated up to 0.05 mM $Ag^{+}$.

### *Minimum inhibitory concentration of toxic oxyanions*

The MIC of the oxyanions in the media containing 10% NaCl for each isolate is shown in Figure 3. All of the strains tolerated selenite, selenate, tellurite and arsenate. Maximum tolerance was seen to arsenate (250 mM), although 90% of the strains were resistant to biselenite, sodium chromate and potassium chromate. C5 was sensitive to these oxyanions and tolerated only 4.5 mM of them. In addition, this strain showed minimum tolerance to biselenite and chromate (2.5 mM).

All strains were sensitive to $Ni^{2+}$, $Ag^{+}$, $Zn^{2+}$ and $Co^{2+}$, but the strains named as $A_1$, $B_2$, $C_1$ and $C_2$ were not only resistant to $Pb(NO_3)_2$ and $CuSO_4$, but also showed the highest tolerance to toxic metals and oxyanions.

It must be emphasized that the isolates not only tolerated selenite, biselenite and tellurite, but also reduced these oxyanions. Reduction of tellurite and seleno-oxyanions by these bacteria resulted in black and dark-red colonies on agar plates, due to the accumulation of intracellular crystals of black elemental Te and

*Table 1.* Major characteristics of 10 Gram-positive rod-shaped spore-forming halophilic bacteria.

| Characteristics | Strain | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A1 | A2 | A3 | B1 | B2 | C1 | C2 | C3 | C4 | C5 |
| Pigment production | White | Cream | – | Cream | – | – | – | – | – | Cream |
| Spore location in the cell | Central | Central | Central | Sub-terminal | Central | Central | Central | Central | Central | Central |
| Motility | – | NA | + | – | – | + | – | – | – | – |
| Oxidase reaction | + | + | + | + | + | + | + | + | + | – |
| Growth on 0% NaCl | – | – | – | – | – | – | – | – | – | – |
| Growth on 1% NaCl | – | – | – | + | – | + | – | + | – | – |
| Growth on 25% NaCl | + | + | + | – | + | + | + | + | + | + |
| Optimum concentration of NaCl for growth | 15% | 15% | 15% | 10% | 10% | 5% | 5% | 5% | 5% | 5% |
| Acid production from | | | | | | | | | | |
| Glucose | + | + | + | + | – | + | + | + | + | – |
| Galactose | – | + | – | – | – | + | – | + | – | – |
| Fructose | + | + | + | + | + | + | – | + | + | – |
| Sucrose | + | + | – | – | + | + | + | + | + | + |
| Maltose | + | + | + | + | + | + | + | + | + | + |
| Xylose | + | + | + | – | + | + | + | + | + | + |
| Mannitol | + | + | + | + | + | + | + | + | – | + |
| Hydrolysis of | | | | | | | | | | |
| Gelatin | + | – | + | + | + | + | – | + | + | – |
| Casein | + | + | + | + | + | + | + | + | + | + |
| Starch | – | + | – | + | – | + | – | – | – | – |
| Tween 80 | – | – | – | – | – | – | – | – | – | – |
| DNA | + | + | + | + | + | + | + | + | + | + |
| Nitrate reduction | – | – | – | – | – | – | – | – | – | – |

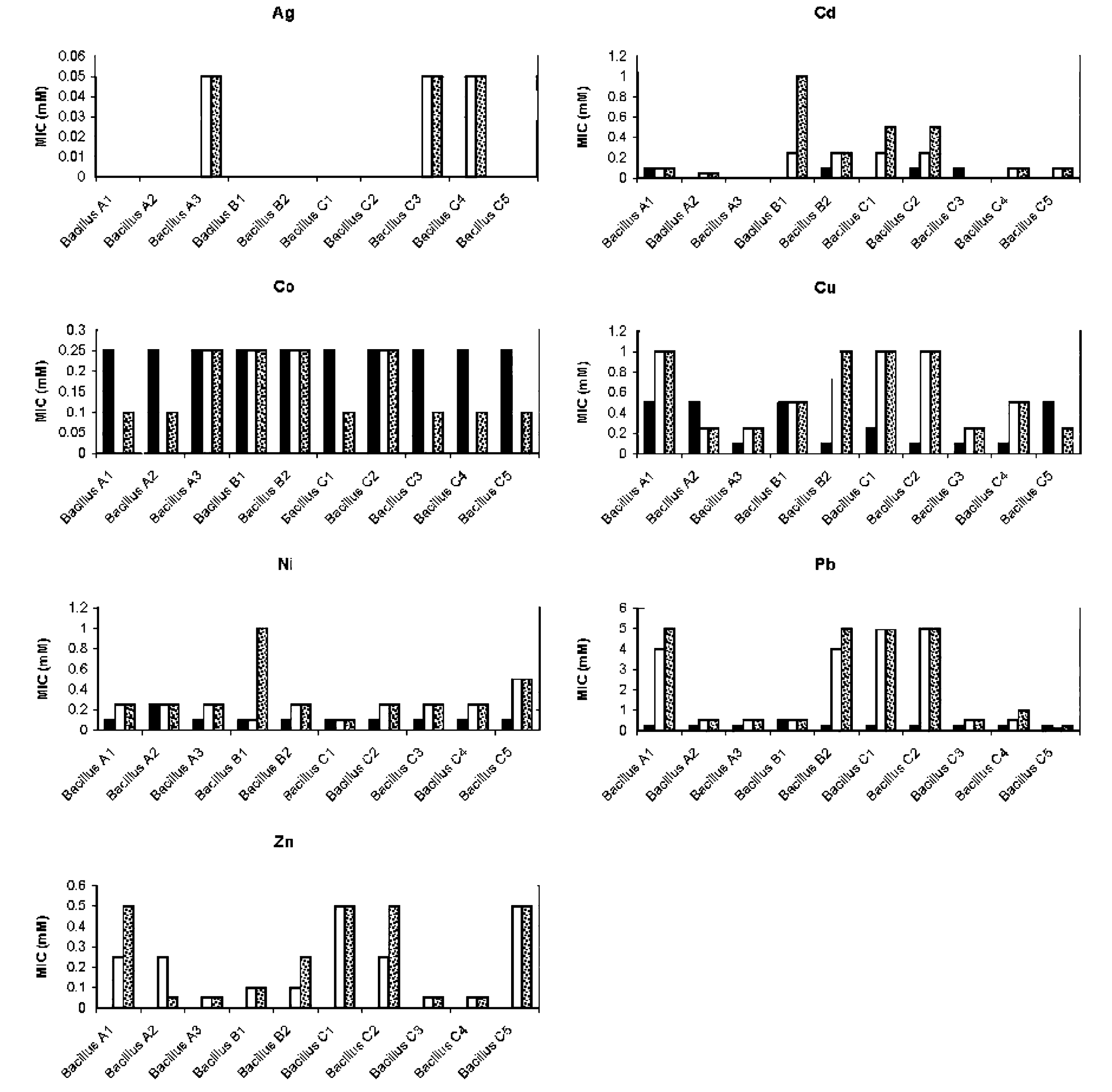


*Figure 2.* Toxic metal resistance of 10 halophilic spore-forming bacilli (expressed as MIC) in culture medium containing ■ 5%, □ 10% and ▨ 15% NaCl.

red, amorphous, elemental Se, respectively (Rathgeber *et al.* 2002).

### *Effect of NaCl concentrations on resistance of the strains to toxic metals and oxyanions*

We examined the effect of the NaCl concentrations on the resistance of the strains to toxic metals and oxyanions and the results are shown in Figures 2 and 3, respectively. Although increasing the concentration of NaCl led to greater tolerance to chromate, selenate, selenite, biselenite and tellurite, the isolates had different tolerances to arsenate. The maximum effect of salt concentration was seen with the chromate anion. NaCl concentration increasing from 5 to 15% enhanced tolerance to sodium chromate and potassium chromate from 5 to 75 mM. The strains named A2 and B1 showed maximum tolerance to sodium chromate in the medium containing 15% NaCl.

With the exception of cobalt, for which the maximum tolerance was seen in 5% (w/v) NaCl-containing medium, generally, when the concentration of NaCl was increased from 5 to 15% (w/v), toxic metal tolerance was enhanced. The maximum effect of the NaCl concentration on the toxic metal resistance was seen in the Pb-containing medium.

As mentioned above, all isolates failed to grow in NaCl-deficient medium, while when some strains, such as B1 and C1, were cultured in the medium plus 30 and 20 mM sodium selenite, respectively, good growth was seen.

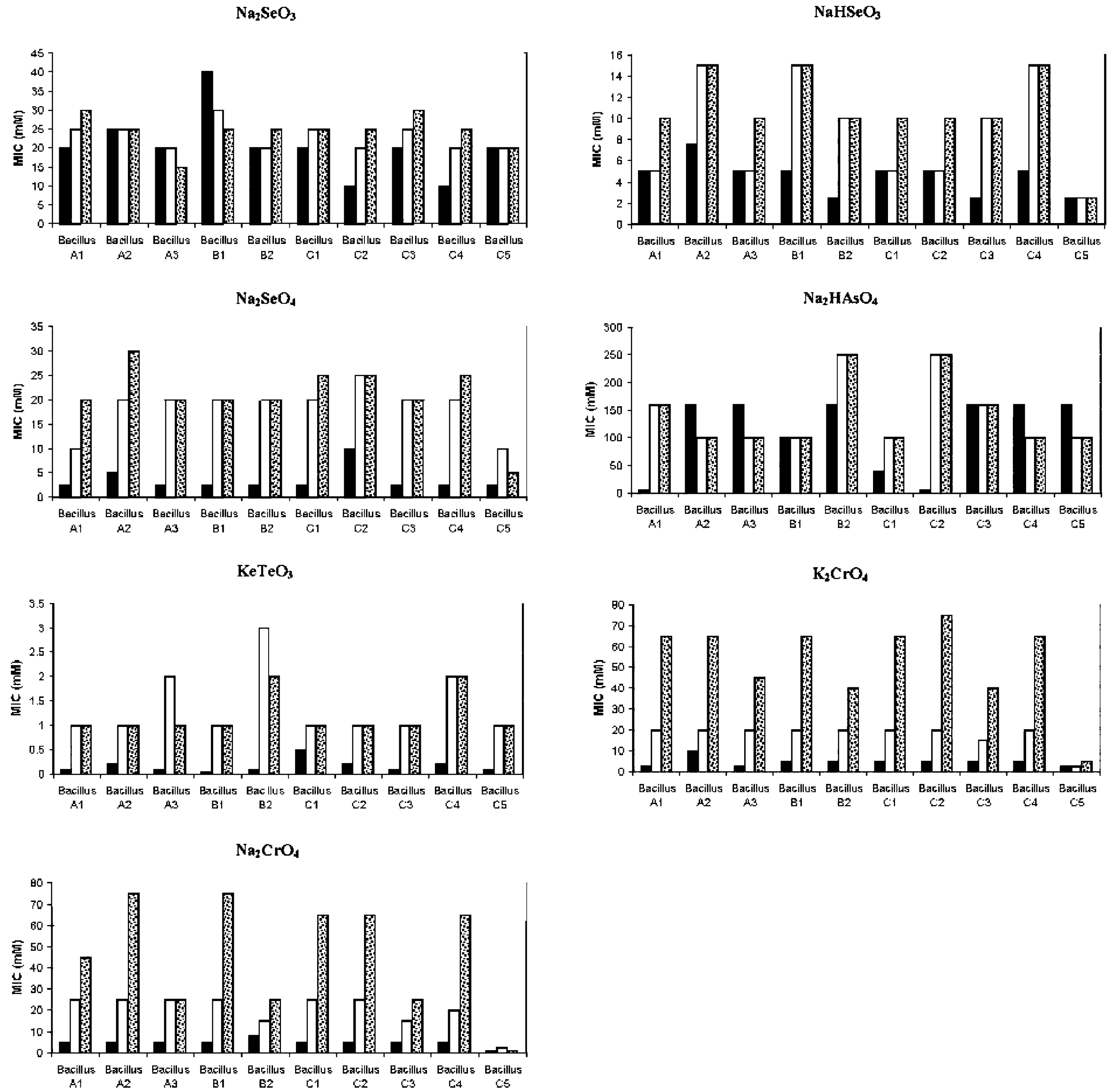


*Figure 3.* Toxic oxyanion resistance of 10 halophilic spore-forming bacilli (expressed as MIC) in culture medium containing ■ 5%, □ 10% and ▩ 15% NaCl.

## Discussion

Moderately halophilic or halotolerant spore-forming Gram-positive rod-shaped bacilli are a diverse phylogenetic group of bacteria which have adapted to life at the lower range of salinities and with the possibilities of rapid adjustment to changes in the extreme salt concentration (Ventosa *et al.* 1998). Useful information has been published on the ecology and physiology of these bacteria (Oren 2002; Venosa 2004). These bacteria may be used for bioremediation of toxic metals from polluted environments; therefore, we studied the toxic metal and oxyanion tolerance in these bacteria.

The results obtained in this study showed that there were two patterns for tolerance in moderately halophilic bacilli. Although the spore-forming halophilic bacilli isolated had relatively low toxic metal tolerance (Figure 2), their resistance to toxic oxyanions was high (Figure 3). It seems that the presence of Na/K in chemical structure of the oxyanions studied was one of the reasons for the high tolerance to oxyanions. Sodium and potassium are necessary elements for the activity of enzymes and pumps in halophiles (Ventosa 1998); therefore, it seems that these elements enhanced the toxic metal tolerance.

We are the first to show high tolerance to the chromate anion in halophilic bacteria (75 mM). Tolerance to chromate was reported in *Pseudomonas* CRB5 (10 mM) (Mclean & Beveradge 2001), *Salvibrio* (20 mM) (Ríos *et al.* 2001), *Arthrobacter* (100 mg/l) (Mgharaj *et al.* 2000) and *Corynebacterium hoagii* (22 mM) (Viti *et al.* 2003). Isolation of chromate-resistant, moderately halophilic bacilli provides additional bacterial models for studying the mechanisms of chromate resistance. In addition, these strains may have potential for bioremediation of chromate-contaminated saline soil, sediments and waste streams. The potential for biological treatment of chromate-contaminated waste is limited, as most microorganisms lose viability in the presence of high concentrations of chromate (Pattanapipitpaisal *et al.* 2001). Genes encoding chromate resistance in these strains may be useful for introduction into related halophilic bacteria used to degrade other pollutants in order to produce strains capable of bioremediation of mixed wastes. On the other hand, chromate-sensitive strains may be interest for future genetic works.

All isolates were resistant to arsenate and the highest resistance to this oxyanion was seen in the B2 and C2 strains (250 mM). Suresh *et al.* (2004) reported the maximum tolerance to 20 mM arsenate anion in a new bacterium, *Bacillus indicus*.

Our isolates had a relatively high tolerance to selenite, biselenite and tellurite oxyanions. This tolerance was comparable to that previously published (Kinkle *et al.* 1994; Souza *et al.* 2001; Rathgeber *et al.* 2002). The tolerance to these oxyanions was followed by reduction of toxic selenite or tellurite to their less toxic elemental forms. The reduction of soluble selenooxyanions and tellurite to elements could be an important mechanism for the removal of these elements from polluted hypersaline environments. Therefore, these strains may be good candidates for bioremediation of highly polluted effluents from industrial and mining operations. Conventional chemical methods for removing toxic oxyanions are expensive and require high energy or large quantities of chemical reagents, while microbial reduction of these toxic oxyanions is low in price and provides green technology. In addition, bacteria capable of reducing tellurite and selenite could be useful in the applied biometallurgy of tellurite and selenite, rare and expensive metals used extensively for their properties as semiconductors (Rathgeber *et al.* 2002).

Increasing industrial activities cause pollution of soil, water and atmosphere by chemicals such as toxic metals and oxyanions. Bioremediation is a good choice for removing the industrial pollution from the environment. There are two different strategies for bioremediation: (1) screening/development of potent and powerful microorganisms and (2) invention/optimization of bioremediation methods. In both approaches, high yield processes will be obtained by employing tolerant microorganisms. It seems that moderately halophilic spore-forming rod bacteria are useful candidates, because they are widely distributed in soil and resistant to such harsh environmental conditions. In this study, these bacteria were introduced for further studies as highly tolerant reducers of toxic metals and oxyanions such as chromate, arsenate and tellurite.

## Acknowledgements

We are grateful to Persian Type Culture Collection of Microorganisms supervisor, Dr. F. Mohseni, and her colleagues for their technical assistance for identification of the isolates.

## References

Amoozegar, M.A., Malekzadeh, F., Malik, K.A., Schumann, P. & Spröer, C. 2003 *Halobacillus karajensis* sp. nov., a novel moderate halophile. *International Journal of Systematic and Evolutionary Microbiology* **53**, 1059–1063.

Burton, G.A., Gidding, T.H., Debrine, P. & Fall, R. 1987 High incidence of selenite-resistant bacteria from a site polluted with selenium. *Applied and Environmental Microbiology* **53**, 185–188.

Kessi, J., Ramuz, M., Wehrli, E., Spycher, M. & Bachofen, R. 1999 Reduction of selenite and detoxification of elemental selenium by the phototrophic bacterium *Rhodospirillum rubrum*. *Applied and Environmental Microbiology* **65**, 4734–4740.

Kinkle, B.K., Sadowsky, M.J., Johnstone, K. & Koskinen, W.C. 1994 Tellurium and selenium resistance in Rhizobia ands its potential use for direct isolation of *Rhizobium meliloti* from soil. *Applied and Environmental Microbiology* **60**, 1674–1677.

McLean, J. & Beveridge, T.J. 2001 Chromate reduction by a Pseudomonad isolated from site contaminated with chromated copper arsenate. *Applied and Environmental Microbiology* **67**, 1076–1084.

Mgharaj, M., Avudainayagam, S. & Naidu, R. 2003 Toxicity of hexavalent chromium and its reduction by bacteria isolated from soil contaminated with tannery waste. *Current Microbiology* **47**, 51–54.

Nieto, J.J., Fernandez-Castillo, R., Marquez, M.C., Ventosa, A., Quesada, E. & Ruiz-Berraquero, F. 1989 Survey of metal tolerance in moderately halophilic eubacteria. *Applied and Environmental Microbiology* **55**, 2385–2390.

Nriagu, J.O. & Pacyna, J.M. 1988 Quantitative assessment of worldwide contamination of air, water and soils by trace metals. *Nature* **333**, 134–139.

Oren, A. 2002 *Halophilic Microorganisms and their Environments*, Dordrecht, The Netherlands: Kluwer Academic Publishers ISBN 1402008295.

Pattanapipitpaisal, P., Brown, N.L. & Macaskie, L.E. 2001 Chromate reduction and 16S rRNA identification of bacteria isolated from a Cr(VI)-contaminated site. *Applied Microbiology and Biotechnology* **57**, 257–261.

Rathgeber, C., Yurkova, N., Stackebrandt, E., Beatty, J.T. & Yurkov, V. 2002 Isolation of tellurite- and selenite-resistant bacteria from hydrotermal vents of the Juan de Fuca Ridge in the pacific ocean. *Applied and Environmental Microbiology* **68**, 4613–4622.

Ríos, M., García, M.T., Arahal, D.R. & Ventosa, A. 2001 *Salinivibrio kushnerii* sp. nov., a new moderately halophilic species tolerant to chromium. In *Proceedings of an International Conference on Halophilic Microorganisms held at Sevilla, Spain*. p. 9.

Souza, M.P., Amini, A., Dojka, M.A., Pickering, I.J., Dawson, S.C., Pace, N.R. & Terry, N. 2001 Identification and characterization of bacteria in a selenium contaminated hypersaline evaporation pond. *Applied and Environmental Microbiology* **67**, 3785–3794.

Summers, A. 1978 Microbial transformations of metals. *Annual Review of Microbiology* **32**, 637–672.

Summers, A. 1985 Bacteria resistance to toxic elements. *Trends in Biotechnology* **3**, 122–125.

Suresh, K., Prabagaran, S.R., Senqupta, S. & Shivaji, S. 2004 *Bacillus indicus* sp. Nov. an arsenic resistant bacterium isolated from an aquifer in West Bengal, India. *International Journal of Systematic and Evolutionary Microbiology* In press.

Trevors, J.T., Oddie, K.M. & Belliveau, B.H. 1985 Metal resistance in bacteria. *FEMS Microbiology Reviews* **32**, 39–54.

Ventosa, A., 2004 *Halophilic Microorganisms*, Berlin, Heidelberg, New York: Springer-Verlag ISBN 3540009264.

Ventosa, A., Nieto, J.J. & Oren, A. 1998 Biology of moderately halophilic aerobic bacteria. *Microbiology and Molecular Biology Reviews* **62**, 504–544.

Viti, C., Pace, A. & Giovannetti, L. 2003 Characterization of Cr (VI)-resistant bacteria isolated from chromium contaminated soil by tannery activity. *Current Microbiology* **46**, 1–5.

Washington, J.A. & Sutter, V.L. (1980). Dilution susceptibility test: agar and macro-broth dilution procedures, Manual of Clinical Microbiology. In E.H. Lennette, A. Balows, W.J. Hausler Jr., & J.P. Truant (Eds.), *Manual of Clinical Microbiology* (pp. 453–458). 3rd edition, American Society for Microbiology, Washington, D.C.ISBN: 1555812554.