Isolation and screening of heavy metal resistant bacteria from wastewater: a study of heavy metal co-resistance and antibiotics resistance

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ABSTRACT

The uncontrolled discharges of wastes containing a large quantity of heavy metal create huge economical and healthcare burdens particularly for people living near that area. However, the bioremediation of metal pollutants from wastewater using metal-resistant bacteria is a very important aspect of environmental biotechnology. In this study, 13 heavy metal resistant bacteria were isolated from the wastewater of wadi El Harrach in the east of Algiers and characterized. These include zinc-, lead-, chromium- and cadmium-resistant bacteria. The metal-resistant isolates characterized include both Gram-negative (77%) and Gram-positive (23%) bacteria. The Minimum Inhibitory Concentration (MIC) of wastewater isolates against the four heavy metals was determined in solid media and ranged from 100 to 1,500 $\mu$g/ml. All the isolates showed co-resistance to other heavy metals and antibiotic resistance of which 15% were resistant to one antibiotic and 85% were multi- and bi-antibiotics resistant. The zinc-resistant species Micrococcus luteus was the much more heavy metal resistant. The results of toxicity tests on Vibrio fischeri showed that the DI$_{50}$ (5 min) as low as 0.1 carried away luminescence inhibition greater than 50%.

Key words | antibiotic resistance, bioluminescence test, heavy metal resistant bacteria, wastewater

INTRODUCTION

Heavy metals are one of the major sources of environmental pollution. They are released due to the discharge of effluent into the environment by a large number of industrial activities such as metal processing, mining, electroplating, leather tanning and pigments production, and the levels of these toxic chemical compounds vary widely in the environment. Wastewater contains a high concentration of heavy metals that are not degraded by the conventional process of wastewater treatment; this has a deleterious impact on aquatic life (Moten & Rehman 1998; Kobyta et al. 2005; Rehman et al. 2008). Meanwhile, many heavy metals like zinc, iron and copper are essential trace elements to cell at low levels but they can exert toxic effects at concentrations encountered in polluted environments. Cadmium is non-essential but poisonous for plants, animals, humans and microbes (Gupta & Gupta 1998, Chovanová et al. 2004). Lead, found in soil, water and air, is a highly toxic and hazardous waste (Low et al. 2000). For chromium, the forms hexavalent (Cr(VI)) and trivalent (Cr(III)) are the most prevalent species of this metal in the natural environment and it is toxic at high concentration (Chung et al. 2006). Heavy metals are present in soluble form and are extremely toxic and too dangerous for any biological function and disturb ecological activity in the aquatic environment. Besides that, they have the ability to form unspecific complex compounds in cells, which leads to toxic effects. In response to toxic concentrations of metal ions, many microorganisms including bacteria can develop resistance and become heavy metal resistant (Kasan & Baecker 1989). Among the various intra- and extracellular mechanisms are bioaccumulation (Blindauer et al. 2002), bio-sorption (Leung et al. 2000), bio-mineralization and precipitation (Podda et al. 2000; Mire et al. 2004), enzymatic oxidation or reduction to a less toxic form (Silver 1996) and efflux heavy metal systems. Stress of heavy metals is known to shift a native bacterial community to a composition in which resistant and tolerant bacteria become numerously dominant. Therefore, much attention has been paid to the removal of metal ions by microorganisms due to its potential applications in environmental protection and recovery of toxic or strategic heavy metals (Zaki & Farag 2010).
Bioluminescence tests using luminescent bacteria are widely used to assess risks related to toxic compounds in wastewaters (Kahru et al. 2000). This bioassay is based on the measurement of a decrease in light emission as a function of sample concentration by the wild-type luminescent bacteria Vibrio fischeri following a short time exposure to the sample (Quershi et al. 1998).

The present work aimed to isolate and characterize heavy metal resistant bacteria from wastewater, to study the co-resistance of the isolates to other metal-salts used and antibiotics, and to measure the toxicity of wastewater by a bioassay using the bioluminescent bacterial strain V. fischeri NRRL.B-11177.

MATERIALS AND METHODS

Site description

Wadi El Harrach is located in El Harrach city at the east of Algiers/Algeria. It’s formed by the confluence of wadi Smar, wadi Ouchayeh and the Littoral Rive Gauche. Wadi El Harrach receives effluent from many local industries like a chemical yeast factory, insect-powder and pipe of wadi Smar, battery factory, paint, paper, plaster, oils, gas-works and tannery of El Harrach and Littoral Rive Gauche. The origin of the domestic and hospital effluent was the local inhabitants military barracks, prison, hospital and polyclinic. It receives also agriculture and urban sewage and discharges them directly into the sea.

Sampling

Wastewater samples were collected in sterile glass bottles from the surface, at 500 m from the sea where wastewater is discharged into the wadi El Harrach at 10:00 h in the morning in April 2010. Glass bottles containing the sample of wastewater were put in an ice-box and transported to the laboratory immediately where they were analyzed within 8 h of collection.

Heavy metals analysis

The heavy metals concentration was determined according to Dean-Ross & Mills (1989). Samples were acidified with 5 ml of concentrated nitric acid per liter. After overnight incubation at ambient temperature, the resulting liquid was diluted and the concentrations of cadmium, lead, zinc and chromium were determined using an atomic adsorption spectrophotometer (SAA).

Bioassay test

Bioluminescence analysis is one of the most promising express methods of biological monitoring for the environment because the luminescent system is highly sensitive to even micro-quantities of pollutants (Medvedeva et al. 2009). The bioassay was carried out with a Toxtracer system (SKALAR, 2000) following the standard procedure of the European Standard NF EN ISO 11348-1 (1998), using the Gram-negative marine bioluminescent bacteria of the species V. fischeri NRRL.B-11177. To prevent the interference of TSS on the bacteria luminescence, wastewater samples were filtered using 0.45 μm pore size membrane. The lyophilized culture of V. fischeri was reconstituted in HEPES (HEPES: 4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid) and cultured on solid and liquid media. Seven consecutive dilutions of wastewater were prepared (0.5, 0.25, 0.125, 0.062, 0.031, 0.015, 0.07) and tested for their toxic effect. To prepare the first dilution (0.5), 5 ml of wastewater were introduced into 5 ml of sterile physiological solution [NaCl 9‰]. To obtain the second dilution (0.25), 5 ml of the first dilution were transferred into 5 ml of sterile physiological solution, etc. The dilution factor is 1:2. The control used in this bioassay contained 500 μl HEPES/500 μl SCS (standard cellular suspension). Inhibition of bioluminescence was measured at a wavelength of 490 nm with readings after 5 and 15 min of incubation at 15 °C.

Isolation procedures and characterization

Heavy metal resistant bacteria were isolated from wastewater using nutrient-agar (Difco) as basal medium (Rajbhanshi 2008; Rani et al. 2010). Heavy metal solutions were prepared in sterile distilled water and sterilized by autoclaving at 120 °C for 20 min. A measure of 100 μg/ml of each heavy metal (zinc, lead, chromium, cadmium) was incorporated separately in the medium. Wastewater was serially diluted with sterile distilled water and 0.1 ml of each dilution was shown in mass of nutrient-agar. Petri plates were incubated at 30 °C for 24–48 h. The different distinct colonies obtained on the solid media were sub-cultured on the same media for purification. The pure cultures were characterized and identified on the basis of colonies and cells morphology, Gram-coloring, study of some physiological and biochemical
characteristics such as respiratory type determination, catalase, oxidase and nitrate reductase tests, degradation of glucose, lactose, gas and H₂S production on Kligler and Hadjna medium (KIA) using standard methods (Cuppucino & Sherman 1983; Marchal et al. 1987). Api 20E systems were also used to identify the heavy metal resistant bacteria.

**Determination of minimum inhibitory concentration (MIC)**

MIC of the wastewater bacteria against their respective heavy metal, was determined by gradually increasing the concentration of the heavy metal by 50 µg/ml each time on nutrient-agar plate. The initial concentration used for this study was 100 µg/ml. Each plate was spread with overnight culture of the bacterial strains. Petri plates were incubated at 30 °C for 10 days. The culture growing on the last concentration was transferred to the higher concentration by streaking on a new plate. MIC was noted when the strains failed to grow on plates even after 10 days (Shakoori et al. 1998).

**Co-resistance to other heavy metals**

All the isolates resistant to their selective heavy metal were tested for their ability to resist the other metal-salts used in this study. A measure of 100 µg/ml was used as initial concentration of each heavy metal which was gradually increased by 50 µg/ml each time until MIC was obtained.

**Determination of antibiotic resistance**

As heavy metal resistance is linked with antibiotic resistance, the isolates were tested for their resistance to seven antibiotics using Mueller-Hinton agar and the disc diffusion method (Bauer et al. 1996). Standard antibiotic-impregnated discs were placed on freshly prepared lawns of each isolate on Mueller-Hinton plates. After 24–48 h of incubation at 30 °C, the diameter of the inhibition zones was measured and the strains were classified as resistant (R), intermediate (I) or susceptible (S) following the standard antibiotic disc chart. Discs containing the following antibiotics were tested: ampicillin (10 µg), cefalotin (30 µg), gentamycin (10 µg), kanamycin (30µU), amikacin (30 µg), doxycyclin (20 µg), nalidixic acid (30 µg).

**RESULTS AND DISCUSSION**

**Heavy metal analysis**

Analysis of wastewater collected from wadi El Harrach revealed that heavy metal concentration of the sample from which the bacteria were isolated was measured as: 0.616 mg/l chromium, 0.846 mg/l lead, 0.943 mg/l zinc and 0.548 mg/l cadmium. The results revealed that lead and zinc concentrations values were relatively important and they were considered as major pollutants.

**Bioassay test**

Results obtained from the bioassay were expressed in percentage of sample dilutions and luminescence inhibition and showed that the bioluminescence of the luminescent-marine bacteria used in this study decreased with increasing the dilution sample. We observed that the wastewater effect on V. fischeri is important after 5 min of exposure with DI₅₀ = 0.10, r = 0.96, n = 7 and the bioluminescence inhibition reached 51.31%. After 15 min, DI₅₀ = 0.13, r = 0.95 and n = 5 with a bioluminescence inhibition value of 46.87%. The DI₅₀ is the wastewater dilution which inhibited 50% of the bioluminescence of V. fischeri and «n» the number of points.

**Isolation and characterization of wastewater bacteria**

In total, 40 heavy metal resistant bacteria were isolated from wastewater of wadi El Harrach. Based on their high level of metal resistance, 13 isolates were selected and studied extensively. The heterotrophic metal-resistant strains showed wide varieties of species including Gram-negative (77%) and Gram-positive (23%) bacteria. The wastewater bacteria were: cadmium-resistant Aeromonas sp., Alcaligenes sp. and Escherichia coli; chromium-resistant Pseudomonas aeruginosa, Bacillus sp., Pasteurella sp., Pseudomonas sp. and E. coli; lead-resistant Proteus sp., Enterobacter sp. and Klebsiella sp.; and zinc-resistant Micrococcus luteus and Staphylococcus sp. Among them, five species were a member of the family Enterobacteriaceae (38%). The metal-resistant bacteria characterized pertain to eight different families and four bacterial classes.

**Co-resistance to other heavy metals and antibiotic sensitivity**

Wastewater isolates showed high resistance for their relative heavy metal with MIC ranging from 100 to 1,500 µg/ml (Table 1). Lead and chromium bacteria
were resistant to higher concentrations than cadmium and zinc isolates. All the bacteria were tested for their co-resistance to other metal-salts used in this study (Table 2). It is interesting to note that nine isolates (69%) were multi-resistant to heavy metals (Figure 1). Among the tested bacteria, five were co-resistant to the three other metals used: zinc-resistant *M. luteus*, chromium-resistant *P. aeruginosa* and *Bacillus* sp. and lead-resistant *Proteus* sp. and *Klebsiella* sp. All the cadmium-resistant strains were resistant to two other metals tested. Based on the results of co-resistance study, the strain zinc-resistant *M. luteus* was identified as a very efficient isolate which was resistant to higher concentrations than the other strains with the respective values: 1,000 μg/ml for chromium, 1,400 μg/ml for lead and 300 μg/ml for cadmium. The much more heavy metal resistant strain was selected for further studies. We have studied the ability of this species to accumulate cadmium and lead. Most studies reported that *M. luteus* immobilized large quantities of lead when it was grown in a media containing lead salts (Hong et al. 1995). It also showed a significant accumulating ability of cadmium in very high concentration (Doyle et al. 1998). It is capable of concentrating cadmium and the amount taken up is directly proportional to the concentration of heavy metals present initially (Hart & Scife 1997).

Results listed in Table 2 revealed that metal-resistant isolates were resistant also to the different drugs. Meanwhile, cadmium-resistant *E. coli* and chromium-resistant *P. aeruginosa* were resistant to five antibiotics between

### Table 1 | Heavy metals resistant bacteria isolated from wastewater

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Resistant to</th>
<th>MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Micrococcus luteus</em></td>
<td>zinc 500</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus sp.</em></td>
<td>250</td>
<td></td>
</tr>
<tr>
<td><em>Alcaligenes sp.</em></td>
<td>100</td>
<td></td>
</tr>
<tr>
<td><em>Aeromonas sp.</em></td>
<td>cadmium 150</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>150</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas sp.</em></td>
<td>900</td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>1,500</td>
<td></td>
</tr>
<tr>
<td><em>Pasteurella sp.</em></td>
<td>chromium 650</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus sp.</em></td>
<td>1,000</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>900</td>
<td></td>
</tr>
<tr>
<td><em>Klebsiella sp.</em></td>
<td>600</td>
<td></td>
</tr>
<tr>
<td><em>Enterobacter sp.</em></td>
<td>lead 1500</td>
<td></td>
</tr>
<tr>
<td><em>Proteus sp.</em></td>
<td>900</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2 | Results of heavy metal co-resistance and antibiotic resistance of wastewater bacteria

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Metal co-resistance (μg/ml)</th>
<th>Antibiotic resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Zinc-resistant bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Micrococcus luteus</em></td>
<td>Cr²⁺(1,000), Pb²⁺(1,400), Cd²⁺(300)</td>
<td>K (R), Amp (S), Dox (S), NA (S), AN (S), CF (S), G (S)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>Pb²⁺(1,200)</td>
<td>Amp (R), Dox (R), K (R), G (S), CF (S), AN (S), NA (S)</td>
</tr>
<tr>
<td><strong>Cadmium-resistant bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Alcaligenes sp.</em></td>
<td>Pb²⁺(700), Cr²⁺(400)</td>
<td>Amp (R), Dox (R), K (R), CF (R), G (S), AN (S), NA (S)</td>
</tr>
<tr>
<td><em>Aeromonas sp.</em></td>
<td>Pb²⁺(500), Cr²⁺(400)</td>
<td>Amp (I), G (S), AN (S), NA (S), CF (S), K (S), Dox (S)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Pb²⁺(1,200), Zn²⁺(250)</td>
<td>Amp (R), Dox (R), K (R), G (R), NA (R), CF (S), AN (S)</td>
</tr>
<tr>
<td><strong>Chromium-resistant bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas sp.</em></td>
<td>Pb²⁺(1,200)</td>
<td>Amp (R), K (R), G (R), NA(R), CF (S), Dox (S), AN (S)</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>Pb²⁺(1,200), Zn²⁺(250), Cr²⁺(150)</td>
<td>Amp (R), Dox (R), K (R), G (R), NA (R), CF (S), AN (S)</td>
</tr>
<tr>
<td><em>Pasteurella sp.</em></td>
<td>Cd²⁺(100)</td>
<td>K (R), G (I), NA (R), CF (S), AN (S), Amp (S), Dox (S)</td>
</tr>
<tr>
<td><em>Bacillus sp.</em></td>
<td>Pb²⁺(1,200), Cd²⁺(300), Zn²⁺(250)</td>
<td>Amp (R), K (R), Dox (S), AN (S), CF (S), NA (S), G (S)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Pb²⁺(1,200)</td>
<td>K (R), G (R), NA (I), CF (S), AN (S), Amp (S), Dox (S)</td>
</tr>
<tr>
<td><strong>Lead-resistant bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Klebsiella sp.</em></td>
<td>Zn²⁺(200), Cd²⁺(150)</td>
<td>Amp (R), K (R), G (R), NA (S), CF (S), NA (S)</td>
</tr>
<tr>
<td><em>Enterobacter sp.</em></td>
<td>Cr²⁺(150), Zn²⁺(250), Cd²⁺(150)</td>
<td>Amp (R), G (R), CF (S), NA (S), AN (S), Dox (S), K (S)</td>
</tr>
<tr>
<td><em>Proteus sp.</em></td>
<td>Cr²⁺(700), Zn²⁺(150), Cd²⁺(300)</td>
<td>K (R), Dox (R), Amp (S), NA (S), AN (S), G (S), CF (S)</td>
</tr>
</tbody>
</table>

R: resistant, I: intermediate, S: susceptible.
seven tested, followed by *Alcaligenes* sp. and *Pseudomonas* sp. which were resistant to four antibiotics. *M. luteus* which was resistant to higher concentration of metals and cadmium-resistant *Aeromonas* sp. appeared to be the most susceptible to antibiotics (Figure 2).

Results cited above revealed that a wastewater dilution as low as 0.10 and 0.13 (DI50) have carried away 50% luminescence inhibition of *V. fischeri* NRRL.B-11177 used in the bioassay. As wadi El Harrach collects all the domestic, hospital, urban as well as industrial wastewater of wadi Smar, wadi Ouchayeh, Littoral Rive Gauche and El Harrach, the toxic effect can be linked to diverse and complex chemical compounds which directly affect the luminescent system. The molecules involved can be organic or inorganic such as pharmaceuticals, herbicides and heavy metals (Medvedeva et al. 2009; Rosal et al. 2010). The mechanisms underlying the toxic effects of chemicals may involve interactions with cell surface receptors, disruption of cell membrane functions and chemical reactions with cellular components or inhibition/competition of enzyme systems (Mariscal et al. 2003). Hence, we can assume that in wastewater of wadi El Harrach the concentration of substances reducing a bacterial luminescence by 50% is very important. Wastewater is also the appropriate environment where the microorganisms can develop resistance to heavy metals and antibiotics. In the present, a study high degree of heavy metals resistance associated with multiple antibiotic resistances was detected in wastewater bacteria. In most of the studies, metal resistance has been reported to hold an association with antibiotic resistance (Verma et al. 2001; Rani et al. 2010). Earlier literature revealed that there is an interrelationship between the antibiotic and heavy metal resistance capacities of all the microbes (Harnett & Gyles 1984). Most results obtained from study on many clinical isolates also reveal that heavy metal and antibiotic resistance are often closely associated (Timoney et al. 1987).

Under conditions of metal stress, metal and antibiotic resistance in microorganisms possibly helps them to adopt faster by the spread of resistant factors than by mutation and natural selection (Silver & Misra 1988).

The bacterial resistance to heavy metals is attributed to a variety of mechanisms developed by resistant bacteria such as metal efflux, binding with bacterial cell envelopes, intracellular sequestration and metal reduction. These mechanisms can be encoded in plasmid genes facilitating the transfer of toxic metal resistance from one cell to another. Richard et al. (2002) related the resistance of bacteria to lead, that this metal binds to materials on the cell surface. The precipitation and mineralization of lead in an insoluble form has also been evoked (Roane 1999). Chromium-resistance mechanisms in bacteria, however, have been reported to include reduction, methylation, precipitation at the cell surface, blocking cellular uptake by alerting the uptake pathway and removal from the...
cytoplasm by efflux pumps (Lovley & Pillips 1994). Still, chromium reducing bacteria have been isolated from a number of environments and a wide variety of bacterial cultures have been reported for reducing capacities (Kamnan et al. 2012). Frequently, bacteria reduce chromium using NADH or glutathione as enzyme co-factors. The enzymatic reduction of chromium involves a soluble cytosolic chromate reductase under aerobic conditions or a membrane-bound chromate reductase during anaerobic respiration where chromate acts as the terminal electron acceptor. However, microbes of genera Pseudomonas, Escherichia, Acinetobacter and Bacillus have been reported to reduce chromium through soluble chromate reductase and without accumulating chromium inside the cell (Elangovan et al. 2006). Both sequestration and ion efflux mechanisms (exclusion or active secretion) have been demonstrated for microbial resistance and protection against cadmium and zinc (Beveridge et al. 1997).

Bibliographic study reported that bacteria of the genera Pseudomonas and Alcaligenes are often isolated from environments polluted by heavy metals and used for cleaning metal-polluted water (Diels & Mergeay 1990; Godockov et al. 1998; Wais et al. 2011). Nevertheless, representatives of the genus Klebsiella, Enterobacter, Micrococcus, Aeromonas, Staphylococcus, Bacillus and E. coli are not directly linked with the presence of heavy metals, but they were frequently isolated from the wastewater (Fulthorpe et al. 1993; Basu et al. 1997; Rajbhanshi 2008; Rani et al. 2010). Based on their important potential, the use of metal-resistant microorganisms in the treatment of heavy metal contaminated wastewater has become more important (Shakibaie et al. 2008; Raja et al. 2009; Jabbari Nezhad Kermani et al. 2010), and many efforts have been devoted to the isolation of heavy metal resistant microorganisms during recent years.

**CONCLUSION**

Wadi El Harrach receives domestic, urban and hospital sewage. In addition to that, it receives effluents containing heavy metals like mercury, lead, zinc and chromium from some local industries. The wastewater has carried away more than 50% luminescence inhibition of V. fischeri at a DI_{50} as low as 0.1 after 5 min of exposure. The heavy metal resistant bacteria characterized, revealed the existence of a wide variety of microbial species distributed in eight families and four bacterial classes at different ratios. Among 13 metal-resistant isolates, eleven (85%) were multi-antibiotic resistant. The isolate zinc-resistant *M. luteus* was considered as the more heavy metal resistant strain and selected for further studies.

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**REFERENCES**


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