Ecotoxicology and Environmental Safety 140 (2017) 162–169

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Metal resistance mechanisms in Gram-negative bacteria and their potential to remove Hg in the presence of other metals

Patricia Giovanella[a,*], Lucélia Cabral[b], Alexandre Pereira Costa[a], Flávio Anastácio de Oliveira Camargo[c], Clesio Gianello[c], Fátima Menezes Bento[a]

[a] Fuels and Biofuels Biodeterioration Laboratory (LAB-BIO), Departament of Microbiology, Immunology and Parasitology – Institute of Health Science, Federal University of Rio Grande do Sul, 500 Sarmento Leite St, 90050-170 Porto Alegre, RS, Brazil

[b] Research Center for Chemistry, Biology and Agriculture (CPQBA), University of Campinas (UNICAMP), 999 Alexandre Cazelatto St, Caixa Postal: 6171 Betel, 13081-970 Paulínia, SP, Brazil

[c] Departament of Soil Science, Federal University of Rio Grande do Sul, 7712 Bento Gonçalves Ave, 91540-000 Porto Alegre RS, Brazil

ARTICLE INFO

Keywords:
Multi-metal resistant bacteria
Bioremediation
Mercury
Biosorption
Biofilm
Siderophores

ABSTRACT

Contamination of the environment by heavy metals has been increasing in recent years due to industrial activities. Thus research involving microorganisms capable of surviving in multi-contaminated environments is extremely important. The objectives of the present study were to evaluate the removal of mercury alone and in the presence of cadmium, nickel and lead by four mercury-resistant microorganisms; estimate the removal of Cd, Ni and Pb; understand the mechanisms involved (reduction, siderophores, biofilms, biosorption and bioaccumulation) in the metal resistance of the isolate *Pseudomonas* sp. B50D; and determine the capacity of *Pseudomonas* sp. B50D in removing Hg, Cd, Ni and Pb from an industrial effluent. It was shown that the four isolates evaluated were capable of removing from 62% to 95% of mercury from a culture medium with no addition of other metals. The isolate *Pseudomonas* sp. B50D showed the best performance in the removal of mercury when evaluated concomitantly with other metals. This isolate was capable of removing 75% of Hg in the presence of Cd and 91% in the presence of Ni and Pb. With respect to the other metals it removed 60%, 15% and 85% of Cd, Ni and Pb, respectively. In tests with effluent, the isolate *Pseudomonas* sp. B50D removed 85% of Hg but did not remove the other metals. This isolate presented reduction, biosorption, biofilm production and siderophore production as its metal resistance mechanisms. *Pseudomonas* sp. B50D was thus a candidate with potential for application in the bioremediation of effluents with complex metal contaminations.

1. Introduction

Metal contamination of the environment has increased in recent years due to the most diverse industrial activities (Fu and Wang, 2011). The residues generated by the metallurgical, petrochemical, battery, polyvinyl chloride and mining industries can contain multiple toxic metals simultaneously, presenting co-contamination problems (Kadirvelu, 2000). Metals are not biodegradable and present a tendency to bio-magnification of the trophic chain with serious impact on human health and the environment (Li et al., 2013). Amongst the metals, cadmium, mercury, lead and nickel are extremely toxic in low concentrations (Fu and Wang, 2011; Waalkes et al., 1992), thus demanding special attention with respect to the treatment of contaminated effluents and residues before being discarded into the environment.

The remediation of environments contaminated with toxic metal cations has conventionally employed physicochemical techniques that include precipitation, filtration and electrochemical recovery, and also membrane separation, excavation, deposition of residues in sanitary landfills or even the covering up of the contaminated site. These techniques are not very efficient, are expensive and generally produce additional residues with greater contamination potential (Naja and Volesky, 2011).

Recently greater emphasis has been given to biological metal remediation methods, due to their low operational costs, great removal efficiency, good performance with low metal concentrations (Teemu et al., 2008) and because they are ecologically correct alternatives (Mishra and Malik, 2013). In this context, some microorganisms have developed mechanisms to adapt these contaminants and can be promising candidates since they have metal-resistant systems (Nies, 1999). Amongst the factors related to their metal resistance, some, such as bioaccumulation, reduction (Nies, 1999), biosorption (Quintelas

* Corresponding author.
E-mail address: patricia.giovanella@hotmail.com (P. Giovanella).

http://dx.doi.org/10.1016/j.ecoenv.2017.02.010
Received 3 October 2016; Received in revised form 6 February 2017; Accepted 8 February 2017
Available online 08 March 2017
0147-6513/

et al., 2009, 2013), siderophore production (Braud et al., 2009b; Schalk et al., 2011) and the formation of biofilms (Taga and Bassler, 2003; Von Bodman et al., 2003) could be explored for the development of clean technologies and the control of metal pollution with a view to promoting mitigation of the environmental impacts.

Mercury is persistent in the environment. For this reason, effective remedial methods need to be applied in heavily mercury-polluted aquatic systems (Wang et al., 2004). Many works have shown the capacity of bacteria to remove mercury from environments contaminated with this metal (Von Canstein et., al, 1999; Wagner-Döbler et al., 2000; Von Canstein et al., 2002; Mahbud et al., 2017) but little research has been done to remove mercury from environments co-contaminated with other metals.

Within the perspective of research involving metals, the study of microorganisms with different resistance mechanisms is urgent, in order to discover potential candidates for the bio-removal of contaminants of diverse origins from the environment. Thus the objectives of the present study were to evaluate the removal of mercury alone or in the presence of Cd, Ni and Pb by four mercury-resistant microorganisms, and also to estimate the removal of Cd, Ni and Pb and determine the mechanisms involved (reduction, siderophores, biofilms, biosorption and bioaccumulation) in metal resistance of the isolate *Pseudomonas* sp.B50D and its ability to remove Hg, Ni and Pb from industrial effluents.

2. Material and methods

2.1. Bacterial strains and culture conditions

The bacteria *Pseudomonas putida* C50A, *Pseudomonas* sp. B50D, *Alcaligenes faecalis* U21 and *Brevundimonas* sp.U22 were isolated in previous studies using the methodology of Giovanella et al., (2016a) as from metal contaminated residues and effluents.

All strains were stored at −80 °C with 15% (*v/v*) of sterile glycerol as cryopreservative. For routine cultivation the strains were grown in Petri dishes containing Agar Nutrient (3 g L^{-1} meat extract, 5 g L^{-1} bacteriological peptone and 15 g L^{-1} agar) supplemented with 10 μM of Hg as $HgCl_2$. The culture was grown aerobically at 30 °C for 24 h and kept at 4 °C until use.

2.2. The PCR detection of merA and merB genes

The bacterial genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, WI).

The PCR reactions contained: 5 μL of PCR buffer, 1.5 μL of 25 mM $MgCl_2$, 1 μL of 10 mM dNTPs, 0.02 U of Taq DNA polymerase (Denville, NJ), 0.5 μL of 20 pmol μL^{-1} of each primer (forward and reverse), 16.75 μL of ultrapure water and 1 μL of template DNA. The primers A1 F (ACC ATC GGC GGC ACC TGC GT) and A5 R (ACC ATC GTC AGG TAG GGG AAC) were used to detect the *merA* gene (Liebert et al., 1997). The amplification was performed in a thermal cycler (MJ Research Inc., Watertown, MA, USA), and the program consisted of 1 cycle at 95 ºC for 5 min, followed by 29 cycles at 95 ºC for 1 min, 64 ºC for 2 min and 90 ºC for 3 min, and a final cycle of 5 min at 72 ºC. To detect the *merB* gene the primers IA34 F (TTGGATCCATGAAGCTCGCCCCAT) and IA35 R (TTGGTACCCTAGATGACATGACAT) were used (Santos-Gandelman et al., 2014). The amplification program consisted of 1 cycle of 5 min at 95 ºC, 40 cycles of 1 min at 94 ºC, 1 min at 65 ºC and 1 min at 72 ºC, and a final cycle of 10 min at 70 ºC.

The PCR products were submitted to gel electrophoresis in 1% agarose and the bands visualized by ultraviolet illumination using a Gedoc 2000 system (Bio-Rad, CA).

2.3. Preparation of the cells for the trials

The bacterial inoculants for the trials in the different culture media were standardized as described below. Each mercury-resistant isolate was inoculated into culture media: Luria Bertani Broth (LB) (bacteriological tryptone 10 g L^{-1}, yeast extract 5 g L^{-1} and NaCl 10 g L^{-1}) or Nutrient Broth (3 g L^{-1} meat extract and 5 g L^{-1} bacteriological peptone) containing Hg (10 μM) as $HgCl_2$ and incubated for 24 h at 30 ± 2 ºC with agitation at 130 rpm. Each initial inoculant was standardized at 10^7 CFU mL^{-1} by turbidimetry, analyzing the optical density at 600 nm and the corresponding CFU mL^{-1} in the plates (Koch, 1994). Flasks containing non-inoculated culture media served as the negative control.

2.4. Metal removal assays using isolates in LB medium

Approximately 10^7 CFU mL^{-1} of each isolate were inoculated into LB medium containing 10 μM of Hg as $HgCl_2$ and Hg removal evaluated after 24 h of incubation at 30 ºC. In addition, other experiments were carried out using LB medium containing 10 μM of Hg whit the addition of cadmium, lead or nickel. The concentrations tested were: Cd (0.1; 0.2; 0.3 and 0.4 mM) as $CdCl_2$, Ni (0.25; 0.5; 1 and 1.5 mM) as $NiCl_2$ and Pb (0.25; 0.5; 1 and 1.5 mM) as Pb $(NO_3)_2$. After incubation at 30 ºC for 24 h, samples were removed to determine pH, biomass (OD 600 nm), and remaining mercury, cadmium, nickel and lead.

2.5. Measurement of the removal of mercury, cadmium, lead and nickel

The concentrations of Cd, Ni and Pb remaining in the culture medium were evaluated using the methodology of Li et al. (2013) with modifications. Two mL of culture medium were centrifuged for 3 min at 5000 rpm and the supernatant digested with nitric acid (20%) and perchloric acid (10%). The metal concentration in the diluted solution was measured by plasma induced optical emission spectrometry (Perkin Elmer optima 7000 DV).

The concentrations of mercury remaining in the inoculated and non-inoculated media were determined following digestion as proposed by EPA method 7471B (US EPA, 2007). All the glassware used during the Hg (II) analyses was whashed in 30% HNO_3 and rinsed several times in ultrapure water prior to use. Initially a 100 μL aliquot was removed from the homogenized bacterial culture sample and treated with 5 mL of H_2SO_4 (98%), 2 mL of HNO_3 (65%) and 10 mL of a $KMnO_4$ solution (70 g L^{-1}). This mixture was then autoclaved at 121 ºC and 1.5 atm. for 15 min. Fifty (50) mL of deionized water and 6 mL $ClNH_2OH_2$ (100 g L^{-1}) were then added to the mixture and finally the mercury was converted to the Hg^0 form by treatment with a solution of $SnCl_2$ (50 g L^{-1}). The elemental mercury formed was analyzed in an atomic absorption spectrophotometer measuring the absorbance at 253.7 nm.

2.6. Metal removal assays by Pseudomonas sp. B50D from effluents

The industrial effluent used in these assays was obtained from the industrial residue managing company UTRESA in *Estância Velha* – Brazil. The effluent originated from the decomposition of solid residues contained in landfills, and was submitted to primary and secondary treatments before being sent to the stabilization lakes from where the samples were collected. The chemical characterization of the wastewater was carried out as described in the Standard Methods for the Examination of Water and Wastewater (American Public Health Association et al., 2005) (Table S1).

The assays for the removal of Hg and other metals from effluent were carried out in 125 mL conical flasks containing 50 mL effluent, pH 6, with the addition of 5% LB culture medium. The effluent was artificially contaminated concomitantly with 10 μM of Hg, 0,25 mM of Ni, 0.1 mM Cd and 0.25 mM of Pb and then inoculated with *Pseudomonas* sp. B50D with an initial cell concentration of 2×10^7 CFU mL^{-1}. The inoculated effluent was incubated at 30 ºC for 72 h, and the residual mercury, cadmium, nickel and lead was determined after 0, 24, 48 and 72 h.