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Nova Biotechnol Chim (2017) 16(1): 42-47
DOI: 10.1515/nbec-2017-0006

Nova Biotechnologica *et* Chimica

Genetic diversity of *Acinetobacter* spp. adapted to heavy metal polluted environments

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Article info

Article history:
Received: 12th April 2017
Accepted: 12th June 2017

Keywords:
Acinetobacter
Heavy metals
Resistance
Bioremediation
Plasmids

Abstract

Multiple metallotolerant bacterial strains were isolated from soil and drainage water samples collected from three industrially heavy metals polluted areas in Slovakia. Obtained bacterial isolates were identified using MALDI-TOF mass spectrometry and bacterial isolates that belonged to the *Acinetobacter* genus were subjected for the further study. *A. calcoaceticus* was found to be prevalent species among analyzed *Acinetobacter* spp. strains, followed by *A. lwoffii* and *A. johnsonii*. *A. calcoaceticus* strains exhibited higher minimum inhibitory concentration to Mn, Zn, and Cu cations compared to *A. lwoffii* and *A. johnsonii*. On the other hand minimum inhibitory concentration to Co and Ni were identical in all *Acinetobacter* spp. isolates. Genetic analyses demonstrated multiple plasmids presence in *A. lwoffii* and *A. johnsonii* but not in *A. calcoaceticus*. Using ERIC-PCR the presence of two different genotypes of *A. calcoaceticus* was detected in heavy metal polluted environments in Slovakia.

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Introduction

Bacteria classified as members of the genus *Acinetobacter* are gram-negative, strictly aerobic, indole negative, oxidase negative, catalase positive and non-fermenting coccobacilli. The name of this genus is derived from the Greek word "*akinetos*" because of its non-motility. Species of this genus are subjects of interest in various fields of science, e.g. molecular microbiology, environmental microbiology, clinical microbiology, various sectors of biotechnology etc. These strains can be obtained from soil or water often contaminated by human activity – agricultural or industrial production, traffic, waste disposal equipment, mining and quarrying, building industries – and contain increased amounts of heavy metals, phenols, biphenyls or chlorinated biphenyls, crude oil, phosphate, divers dyes or another chemical compounds that have a negative impact to environment and therefore to human and animals health. These bacterial species (pathogenic or non-pathogenic) can be regularly isolated from living organisms as well. Due to its ability to cause nosocomial infections they have been attracting increasing attention in clinical practice. Clinical strains are often multiresistant to a broad spectrum of antibiotics. Besides antibiotic multiresistance

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they are often resistant to classical disinfectants (gluconate, chlorhexidine and phenols) and to desiccation or radiation (Dougrahi *et al.* 2011 and references therein).

Bacterial strains of this genus are typical by a wide range of biotechnological use. Some *Acinetobacter* species (*A. calcoaceticus* RAG-1, *A. radioresistens* KA53, *A. calcoaceticus* BD4) produce bio-emulsifiers which are used in food industry, agrochemical industry, cosmetic industry, pharmaceutical industry. Another application potential is made possible by the ability of several strains to accumulate various types of biopolymers – cyanophycin, wax esters, polyhydroxyalkalonic acids etc (Abdel-El-Haleem 2003).

Species of this genus have the ability to biotransform heavy metals, phenols or other contaminants to less toxic forms. It has been found that *A. calcoaceticus* strain PUCM 1011 reduces hexachloroplatinic acid forming platinum nanoparticles (Gaidani *et al.* 2014); *A. calcoaceticus* PUCM 1005 synthesizes silver nanoparticles (Gaidani *et al.* 2013); *Acinetobacter* sp. strain MemCl4 have ability to biodegrade neurotoxic chlorpyrifos (Pailan *et al.* 2016); *Acinetobacter* spp. such as *A. calcoaceticus* NCIM 2890 could biodegrade diverse textile dyes (Ghodake *et al.* 2009); *A. calcoaceticus* may be involved in degradation of linear alkylbenzosulfonate and sodium dodecyl sulfate (Abboud *et al.* 2007); some *Acinetobacter* spp. representatives are capable of degrading diesel or heating oil (Marín *et al.* 1995).

Bacteria in general and *Acinetobacter* spp. can in some cases adapt to various extreme conditions, including high concentrations of heavy-metals. Bacterial adaptation mechanisms in environment are metal sorption, accumulation and uptake, mineralization, precipitation, efflux from the cell, oxidation or reduction to less toxic or nontoxic forms (Nies 1999). Genes encoding proteins involved in the mechanisms of biotransformation of toxic heavy metals to less toxic forms are often present on circular plasmid DNA which may be involved in the processes of horizontal gene transfer (Li *et al.* 2015). Heavy – metal tolerant bacterial strains could be used in bioremediation but before their real incorporation into the real processes we must get as much information about these strains. The present study deals with characterization of metallotolerant and alkali-tolerant bacteria from anthropogenically polluted areas of Slovak Republic.

Experimental

Sampling and identification of bacteria

Bacterial samples were obtained and collected from heavy metal polluted areas of Slovakia: brown sludge dump from aluminium plant near Žiar nad Hronom (48°35′3″N, 18°51′39″E) situated at Žiarska basin, landfill waste from the production of nickel near Sereď (48°17′11″N, 17°44′15″E) situated in the southwest of Slovakia, and tailings impoundment near Slovinky (48°52′43″N, 20°50′38″E) in the East of Slovakia. Samplings were realized in 2010 (Žiar nad Hronom), 2013 (Sereď), and 2014 (Slovinky) resp. Numbers of cultivable bacterial strains were obtained upon cultivation on TSA agar (BD Difco, USA). Individual pure bacterial cultures were identified by MALDI-TOF MS (Matrix – assisted laser desorption ionization time-of-flight mass spectroscopy) method. Bacterial isolates were re-suspended in 300 µL of sterilized distilled water. Resuspended cells were centrifuged with 900 µL of ethanol for 2 min at maximum speed. Ethanol was removed and pellets were centrifuged 2 min again. Pellets were re-suspended in 50 µL of 70 % formic acid and 50 µL of acetonitrile. The tubes were centrifuged 2 min at maximum speed. 1 µL of prepared samples were pipetted on MALDI plate and then 1 µL of MALDI matrix (solution of 50 % acetonitrile, 47.5 % of distilled water and 2.5 % trifluoroacetic acid mixed with HCCA matrix) on them (Ferreira *et al.* 2011). All analyses were performed on Microflex LT (Bruker Diagnostics, Germany) MALDI-TOF MS system. The members of the genus Acinetobacter were subjected to further analysis.

Determination of minimum inhibitory concentration (MIC)

Species from the genus *Acinetobacter* were tested for their heavy-metal tolerance. Analyzed strains

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were inoculated on TSA plates with gradually increasing concentrations (1 mM, 2 mM, 4 mM, 8 mM, 15 mM, 30 mM, 60 mM) of heavy metal salts ($CuCl_2.2H_2O$, $ZnCl_2$, $NiCl_2.6H_2O$, $CoCl_2.6H_2O$, $MnCl_2$) which were filtered through microbial filter first and then added to the sterilized TSA medium (pH=7.3). Inoculated bacteria were incubated at 25 °C for 24 h.

Bacterial ability to grow at alkaline pH

Bacterial strains were inoculated on TSA medium with addition of 100 mM Tris-HCl to control pH (9 and 10.5) and cultivated at 25 °C for 24 h. The growth was evaluated visually.

Plasmid isolation and gel electrophoresis

The presence of plasmids in industrial isolates was determined using alkaline lysis method. Three mL of overnight grown liquid bacterial cultures were centrifuged for 3 min at 25 °C, 13 000 RPM. Bacterial pellets were resuspended in 250 µL of 10 mM EDTA and 50 mM Tris-HCl with addition of lysozyme. Resuspended bacterial cells were incubated in thermostat at 37 °C, 15 min. After incubation, 250 µL of lysis solution (0.2 M NaOH and 1 % SDS) was added to the tubes which were mixed by inverting. Subsequently, 350 µL of 3M potassium acetate (pH=5) was added to the tubes which were mixed by inverting again and incubated 15 min on ice. After 10 min centrifugation at 4 °C and 13 000 RPM supernatants with dissolved double-stranded plasmids were transferred to the new tubes. RNA removal was achieved by 30 min incubation with RNase (1 µL) at 37 °C. Then 1/2 volume of chloroform was added to the tubes which were centrifuged 3 min at 4 °C. The aqueous phases were transferred to the new tubes and 3/4 volume of isopropanol was added to them. The tubes were centrifuged at 4 °C, 10 min. Pellets were washed with 500 µL of 70 % ethanol and centrifuged 5 min. Ethanol was completely removed and the plasmid DNA was dissolved in TE solution (50 µL) of 10 mM Tris-HCl and 1mM EDTA. Electrophoresis on 1 % agarose gel stained with ethidium-bromide was used for plasmids separation and UV light for their visualization. The plasmids sizes were compared with FastLoad 1Kbp DNA Ladder (SERVA).

Total genomic DNA isolation

5 mL of Luria-Bertani liquid cultures of all isolates were centrifuged at 4 °C for 10 min at maximum speed. Bacterial pellets were resuspended in 1 mL of SET solution (25 mM EDTA, 20 mM Tris-HCl, 75 mM NaCl) with lysozyme. Wrapped tubes were incubated at 37 °C for 30 min with stirring first and then with 1/5 volume of 10 % SDS at 55 °C for 15 min statically. After incubation 1/3 volume of 5 mM NaCl and chloroform (1:1) were added to the tubes and were incubated for 30 min at laboratory temperature with stirring. Tubes were centrifuged for 10 min, 10 000 RPM. The top aqueous phases with equal amount of chloroform were centrifuged at laboratory temperature for 10 min, 10 000 RPM. Then the equal amount of izopropanol was added to the aqueous phases, the tubes were incubated at laboratory temperature for 15 min and centrifuged at 4 °C for 10 min, 10 000 RPM. DNA pellets were washed with 200 µL of 70 % ethanol, centrifuged at 4 °C for 5 min, 10 000 RPM, air dried, and DNA was dissolved in 50 µL of TE solution.

Enterobacterial repetitive intergenic consensus polymerase chain reaction (ERIC-PCR) of A. calcoaceticus isolates

An ERIC-PCR reaction (Versalovic *et al.* 1991) was performed in C1000 Thermal Cycler (Bio-Rad Laboratories, Richmond, USA) in 50 µL reaction mixture which consists of 5 µL 10x concentrated High Yield Buffer with 3 mM $MgCl_2$, 200 µM of each dNTP, 1 µM of primer ERIC 1R (5´-ATGTAAGCTCCTGGGGATTCAC-3´) and 1 µM of primer ERIC2 (5´-AAGTAAGTGACTGGGGTGAGCG-3´), 1.25 U of Taq polymerase (Taq Core Kit, Jena Bioscience, Germany), 50 ng of DNA. Thermocycling conditions for ERIC-PCR reaction were: initial denaturation: 95 °C, 15 min; denaturation: 94 °C, 1min; annealing: 48 °C, 2 min;

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elongation: 65 °C, 3 min; final extension: 65 °C, 10 min (37 cycles). PCR products were separated by 1 % agarose gel electrophoresis and visualized by UV light. The PCR products were compared with FastLoad 1Kbp DNA Ladder (SERVA).

Results and Discussion

Currently, in Slovakia environmental loads information database there are 1 963 areas registered: 900 as probable environmental loads, 279 as existing/affirmed environmental loads, 784 as remedied/recultivated environmental loads (http://www.minzp.sk/files/sekcia-geologie-prirodnych-zdrojov/spsez_2016_2021.pdf).

In our work we studied metallotolerant bacteria of the *Acinetobacter* genus naturally present in heavy metal polluted industrial areas near Žiar nad Hronom, Sereď and Slovinky. The main goal of our research was to identify prevalent species, to analyze their genetic organization and to estimate their potential use in bioremediation as an alternative cost- and eco-friendly remediation method.

Due to metal industry activities all analyzed sampling sites are recognized by elevated concentrations of heavy metals. Despite of these extreme environmental conditions numbers of cultivable bacterial strains were isolated. Seventy nine pure bacterial cultures were obtained upon classical cultivation methods on agar plates. Strains were than identified by MALDI-TOF MS. From identified bacteria eleven belong to *Acinetobacter* spp. and eight were analyzed (Table 1).

Table 1. Characterization of *Acinetobacter* isolates analyzed.

Isolate name	Species	Sample		
		Locality	Source	pH
K1	*A. lwoffii*	Žiar nad Hronom	soil	10.2
K6	*A. calcoaceticus*			
K13	*A. calcoaceticus*			
NHL1	*A. calcoaceticus*	Sereď	soil	8.1
NHL4	*A. calcoaceticus*			
NHL11	*A. johnsonii*			
P19	*A. calcoaceticus*	Slovinky	drainage water	7.8
P20	*A. calcoaceticus*			

In similar study no strains belonging to the *Acinetobacter* genus were detected among 24 isolates from nickel sludge disposal site near Sereď (Pristas *et al.* 2015).

Tests for heavy metal tolerance show that *A. calcoaceticus* from all analyzed areas exhibit higher MIC of zinc and manganese compared to *A. lwofii* from Žiar nad Hronom and *A. johnsonii* from Sereď. MIC of copper was higher for *A. calcoaceticus* from Žiar nad Hronom (K6, K13) and for *A. calcoaceticus* from Slovinky (P19, P20) compared to *A. calcoaceticus* (NHL1, NHL4) and *A. johnsonii* (NHL11) from Sereď. The lowest MIC of copper was observed in *A. lwofii* from Žiar nad Hronom (K1). Tolerance to cobalt and to nickel was the same among all studied isolates (Table 2). For comparison, in Guheswori sewage treatment plant *Acinetobacter* spp. strains were found resistant to cadmium (1.33 mM), copper (3.15 mM) and cobalt (3.05 mM) tolerant (Rajbanshi 2008); in South India sewage water was found arsenic (MIC 13 mM), cadmium (4 mM) and chromium (0.7 mM) tolerant strain *A. radioresistens* BC3 (Raja *et al.* 2009); from Saudi Arabia industrial area (Hafar Al Batin) *A. baumanii* HAF-13 strain was isolated exhibiting resistance to arsenic (3.34 mM), chromium (4.81 mM), cadmium (1.78 mM), lead (0.84 mM), and mercury (0.5 mM) (El-Sayed 2016). Some investigations show that several strains of the genus *Acinetobacter* can thrive on mediums with acidic or alkaline pH (Yavankar *et al.* 2007). Tests of bacterial ability to grow at alkaline pH showed that all analyzed isolates are able to grow at pH 9.0 but not at pH 10.5 (Table 2).

Genetic variability in *Acinetobacter* spp. is high and in different species various numbers of genetic orthologs could be observed. The presence of plasmids is one of sources of genetic diversity observed and frequently, heavy metal resistance in acinetobacters is plasmid encoded. In permafrost strains of *A. lwoffii* multiple plasmids encoding resistance to Hg, Chr, Co/Zn/Cd, Ni, and Ars were detected (Midlin *et al.* 2016). In analyzed *Acinetobacter* spp. collection of plasmid DNA were detected in *A. lwoffii* K1 and *A. johnsonii* NHL11 isolates (Fig. 1) but not in *A. calcoaceticus* isolates (data not shown) which show higher tolerance to heavy metals. Strains K1 and NHL11

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Table 2. Bacterial growth at alkaline pH and heavy metals minimum inhibitory concentrations of *Acinetobacter* spp.

Isolate name	Species	Growth at pH		Minimum inhibitory concentration (mM)				
		9	10.5	Mn	Zn	Cu	Ni	Co
K1	*A. lwoffii*	+	-	4	2	2	2	1
K6	*A. calcoaceticus*	+	-	30	15	8	2	1
K13	*A. calcoaceticus*	+	-	30	15	8	2	1
NHL1	*A. calcoaceticus*	+	-	30	15	4	2	1
NHL4	*A. calcoaceticus*	+	-	30	15	4	2	1
NHL11	*A. johnsonii*	+	-	8	4	4	2	1
P19	*A. calcoaceticus*	+	-	30	15	8	2	1
P20	*A. calcoaceticus*	+	-	30	15	8	2	1

contain at least 4 plasmids. Their sizes vary from above 2 kbp to 25 kbp. Currently, in Genome NCBI Database (https://www.ncbi.nlm.nih.gov/genome/), there are 212 complete *Acinetobacter* spp. plasmid sequences available. Plasmid sizes vary from 1.735 Kbp (*Acinetobacter* sp. M131, pM131-11; Peng *et al.* 2014) to 398.857 Kbp (*Acinetobacter johnsonii* XBB1, pXBB1-9; Zong 2014). The putative role of plasmids in our isolates will be further studied.

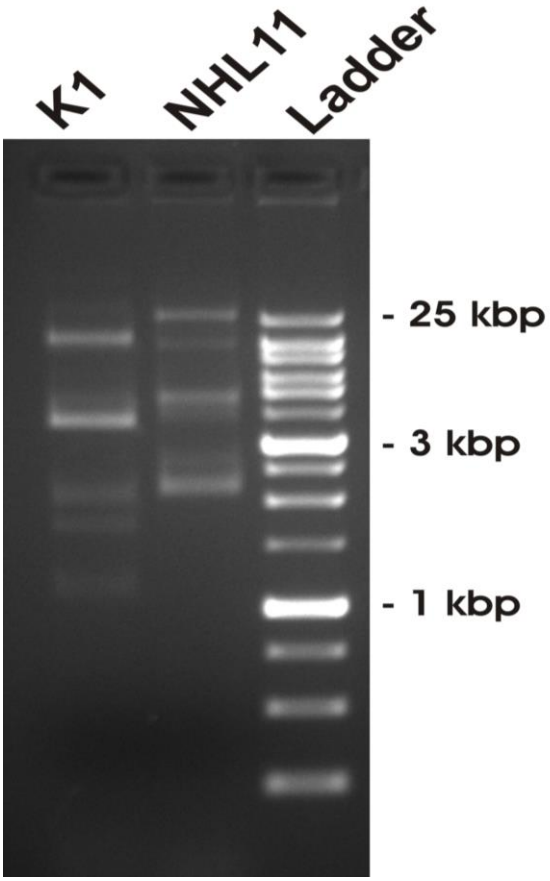

Fig. 1. Plasmid DNA from *Acinetobacter* spp. isolates: *A. lwoffii* (K1) and *A. johnsonii* (NHL11).

To assess genetic diversity among *A. calcoaceticus* isolates genome fingerprinting using ERIC-PCR with the primers ERIC 1R and ERC2 was used. ERIC-PCR is widely used for study of genetic variability in wide spectrum of organisms, including bacteria (Versalovic *et al.* 1991). Despite different origin of *A. calcoaceticus* isolates two genotypes were detected among tested strains (Fig. 2). While unique genotypes were detected in red and brown mud waste disposal site near Žiar nad Hronom and nickel sludge landfill, in mine tailing Slovinky the presence of both aforementioned genotypes was detected, indicating low genetic variability of *A. calcoaceticus* isolates from heavy metals polluted environments in Slovakia.

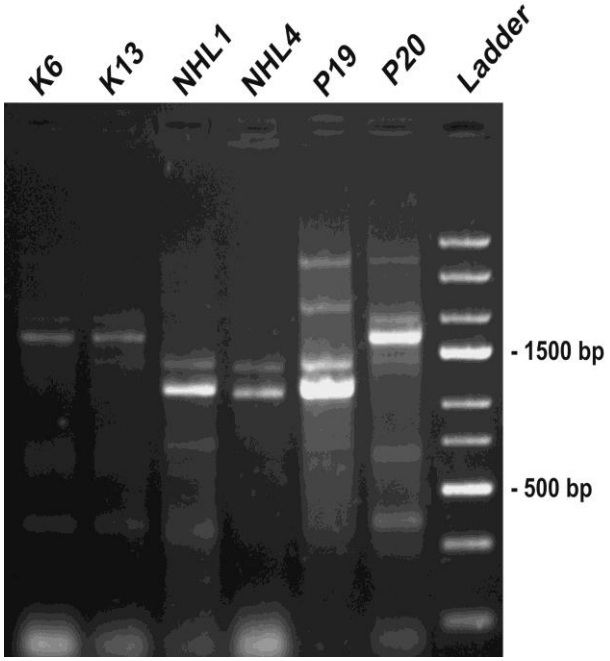

Fig. 2. ERIC-PCR profiles of *A. calcoaceticus* isolates K6, K13 (Žiar nad Hronom); NHL1, NHL4 (Sereď); P19, P20 (Slovinky).

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Conclusions

Acinetobacter spp. represent significant part of cultivable bacteria from heavy metals polluted environments in Slovakia. *A. calcoaceticus* was found to be prevalent species among isolated strains, followed by *A. lwoffii* and *A. johnsonii*. Genetic analyses demonstrated multiple plasmids presence in *A. lwoffii* and *A. johnsonii* but not in *A. calcoaceticus*. Despite genetic diversity among *A. calcoaceticus* detected using ERIC-PCR all *A. calcoaceticus* strains have similar characteristics and increased tolerance to heavy metals compared to *A. lwoffii* and *A. johnsonii*. Heavy metal industry waste disposal sites could be a good source of extremophilic bacteria widely used in modern biotechnologies.

Acknowledgements

This work was supported by a research grant from the Slovak Grant Agency (VEGA 1/0229/17 and VVGS-PF- 2017-270).

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