Introduction

Bacterial communities are known to reflect their microenvironmental conditions by readily responding at extremely fast rates to environmental and pollution changes (Bell et al., 2009; Thiyagarajan et al., 2010; Ławniczak et al., 2016; Sydow et al., 2016). However, human activities have dramatically changed the composition and organisation of soils. Among wastes with increased concentrations of contaminants as a result of different anthropogenic activities heavy metals occur frequently (Liu et al., 2005). Moreover, their contribution in overall contamination of soils in Europe is of about 35%, what reveals a greater fraction in comparison to mineral oils (Panagos et al., 2013; Sydow et al., 2017). Although some of heavy metals are necessary in trace amounts for a variety of metabolic processes in the cell but in high concentrations they react to form toxic compounds that can cause its damage (Nies, 1999). Most common heavy metals that were found at contaminated sites are lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu), mercury (Hg), and nickel (Ni) (Gwrtac, 1997; Wuana and Okieimen, 2011; Tóth et al., 2016). Because of their high degree of toxicity, arsenic, cadmium, chromium, lead, and mercury rank among the priority metals that are of public health significance (Tchounwou et al., 2012). Once in the soil, heavy metals are adsorbed by initial fast reactions (minutes, hours), followed by slow adsorption reactions (days, years) and are, therefore, redistributed into different chemical forms with varying bioavailability, mobility, and toxicity (Shiowatana et al., 2001). Although bacteria have been interacting with heavy metals in their natural environments to various extents since their...
early evolutionary history, heavy-metals-contaminated soil for long time caused selective pressure on microorganisms, which are forced to respond to these changes in environment by selection of heavy-metal-resistant bacteria. These bacteria have evolved several mechanisms that regulate metal ion accumulation to avoid heavy metal toxicity for the cell. The best-known mechanisms of heavy-metal resistances include permeability barriers, intra- and extra-cellular sequestration, efflux pumps, enzymatic detoxification, and reduction (Nies, 1999). The most well characterized operons conveying resistance against heavy metals in Gram-negative bacteria are the cze (cobalt-zinc-cadmium resistance) and cnr (cobalt-nickel resistance) operons from Cupriavidus metallidurans CH34 (Mergeay et al., 2003), the ncc (nickel-cobalt-cadmium resistance) and nre (nickel resistance) systems from Achromobacter xylosoxidans 31A (Schmidt and Schlegel, 1994) and cze (cadmium-zinc-nickel resistance) operon from Helicobacter pylori (Salvador et al., 2007). In Gram-positive bacteria, the cad operon from Bacillus and Staphylococcus members has been well studied (Silver and Phung, 1996). In both Gram-positive and Gram-negative bacteria the ars operons from Escherichia coli (Mobley et al., 1983; Saltikov and Olson, 2002) and Staphylococcus strains (Ji and Silver, 1992; Rosenstein et al., 1992), and the mer systems from E. coli (Nascimento and Chartone-Souza, 2003) and Bacillus populations (Bogdanova et al., 1998) have been characterized. In addition, the cyanobacterial smt locus from Synechococcus PCC 7942 also contains a well-characterized heavy metal resistance system (Erbe et al., 1995).

In our previous work, a few of hardly cultivable and previously uncultured bacterial isolates from toxic-metal contaminated soil were cultivated, partly identified and characterised by using a diffusion chamber approach. Obtained results showed that all these isolates were resistant to nickel, cobalt, zinc, copper and cadmium ions and that this resistance in majority of β- or γ-Proteobacteria was mediated via a system of transmembrane metal pumps carried by these bacteria (Remenár et al., 2015). These efflux systems are represented mainly by CBA efflux pumps driven by proteins of the resistance-nodulation-cell division superfamily, P-type ATPases, cation diffusion facilitator and chrome proteins, NreB- and CnrT-like resistance factors. Some of these systems are widespread and serve in the basic defence of the cell against superfluous heavy metals, but some are highly specialized and occur only in a few bacteria (Nies, 2003).

In detail, CBA transporters are three-component protein complexes that span the whole cell wall of Gram-negative bacteria. The most important component of the transporter is an RND protein that is located in the inner membrane. It mediates the active part of the transport process, determines the substrate specificity and is involved in the assembly of the trans-envelope protein complex. The RND protein family was first described as a related group of bacterial transport proteins involved in heavy metal resistance (C. metallidurans), nodulation (Mesorhizobium loti) and cell division (E. coli) (Saier et al., 1994). The RND protein is usually accompanied by the membrane fusion protein (MFP) (Saier et al., 1994) and outer membrane factor (OMF) (Paulsen et al., 1997; Johnson and Church, 1999). OMF and MFP proteins have a rather static function during CBA-mediated trans-envelope efflux. These three proteins form an efflux protein complex that may export its substrate from the cytoplasm, the cytoplasmic membrane or the periplasm across the outer membrane directly to the outside of the cell (Zgurskaya and Nikaido, 1999a; 1999b; 2000a; 2000b). In bacteria and archaea, CBA transporters are involved in transport of heavy metals, hydrophobic compounds, amphiphiles, nodulation factors and proteins (Tseng et al., 1999). In addition, these transport systems could remove cations even before they have the opportunity to enter the cell and could mediate 10 further export of the cation that had been removed from the cytoplasm by other efflux systems (Nies, 2003).

The best-characterized CBA transporter is the CzcCBA complex from C. metallidurans CH34. The cze determinant encodes resistance to Cd²⁺, Zn²⁺ and Co²⁺ by metal-dependent efflux driven by the proton motive force (Nies, 1995). Ni²⁺ and Co²⁺ are, in some occasions, exported by the same CBA transporters as Zn²⁺ and Cd²⁺ (for example NccCBA from A. xylosoxidans 31A and CzcCBA from C. metallidurans CH34) (Schmidt and Schlegel, 1994; Legatzki et al., 2003).

Thus, in our studies, we wanted to accurately identify a newly isolated bacterium tentatively assigned to uncultured betaproteobacteria and its heavy-metal-resistance gene product because we expected that such bacterium isolated from extreme environment could serve as a specific soil bacterial strain carrying heavy-metal-resistance gene product that facilitates the cells to survive in soil contaminated with nickel and also containing other metals, such as cobalt, zinc, iron, copper and cadmium.

Experimental

Materials and Methods

Isolation and cultivation of bacterium. Bacterium MR-CH-I2 was isolated by diffusion chamber approach (Kaeberlein et al., 2002) with some modifications (Remenár et al., 2015) from farmland near the town of Sered (48°16’59” N, 17°43’35” E) in southwest
Slovakia. The sampling site was situated near a dump containing heavy-metal-contaminated waste. Investigated field site contained high concentrations of nickel (2109 mg/kg), slightly above the natural occurrence of cobalt (355 mg/kg) and zinc (177 mg/kg), even too low concentration of iron (35.75 mg/kg) for a normal soil and not a toxic amount of copper (32.2 mg/kg) and cadmium (< 0.25 mg/kg). The content of heavy metals in the soil sample was measured using an atomic absorption spectrometer (PerkinElmer model 403, USA) (Karelová et al., 2011). The site is according to environmental monitoring of Slovakia a part of strongly disturbed environment (Bohúš and Klinda, 2010).

Bacterium MR-CH-I2 was cultivated on LB (Luria-Bertani) agar plates aerobically at 30°C for 24 h and independently growing colonies were used for further analysis.

**DNA extraction.** Bacterial DNA from bacterial MR-CH-I2 cells was isolated using the DNeasy purification kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions as described in Karelová et al. (2011). The resulting high-molecular-weight DNA was stored at –20°C and was used as a template in appropriate PCR experiments.

**Detection of 16S rRNA (16S rDNA) and DNA gyrase subunit B (gyrB) genes.** DNA extracted from bacterium MR-CH-I2 was used in PCR either with universal 16S rRNA gene primers (27F, 1492R) or with universal degenerate gyrB primer set (UP-1 and UP-2r) (Table I). Each 20 μl reaction mixture contained 1 μl (10 ng) of the DNA template, 2.0 μl 10× AccuPrime Pf Reaction mix (Invitrogen, USA), 1.25 U AccuPrime Pfx DNA polymerase (Invitrogen, USA) and 0.5 μM of each primer. PCRs were performed in a thermal cycler.
Subsamples of either purified nccA923F and nccA923R primers located in earlier known terminal part and outside of the gene, respectively (Table I); iii) amplification of terminal part of nccA-like gene with nccA642F and nccA642R primers located in earlier known terminal part and outside of the gene, respectively (Table I); iv) amplification of middle part of nccA-like gene with nccA923F and nccA923R primers located in earlier known terminal part and outside of the gene, respectively (Table I).

Each 50 μl reaction mixture contained 1 μl (10 ng) of the DNA template, 5.0 μl 10× Taq buffer (Qiagen, Hilden, Germany), 2.5 U Taq DNA polymerase (HotStar; Qiagen, Hilden, Germany), 1.5 mM MgCl₂, 400 nM of each dNTP and 0.5 μM of each primer. PCRs were performed in a thermal cycler (LabCycler, Goettingen, Germany) with the following cycling conditions: 15 min of denaturation at 95°C, 35 cycles of 1 min at 95°C, 1 min at 55°C (ii), 57°C (iii) or 56°C (iv), 5 min 40 s (i), 2 min 36 s (ii), 1 min 20 s (iii) or 1 min 50 s (iv) at 72°C, and a final cycle of extension at 72°C for 10 min. PCR products were separated by electrophoresis in a 1% (w/v) agarose gel (Merck, Germany) and stained with GelRed Nucleic Acid Gel Stain (Biotium, USA). DNA bands, approximately 1,500 or 1,200 bp in size for the 16S rRNA and gyrB genes respectively, were excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Detection of complete nccA-like gene and its sequencing strategy. DNA extracted from bacterium MR-CH-I2 was used in PCRs with subsequent primer sets: i) nccA-like gene fragment amplification with nccA9F and nccA2875R primers located in early and terminal parts of the gene, respectively (Table I); ii) amplification of beginning of nccA-like gene with nccA1244F and nccA1244R primers located before the gene and in known early part of the gene, respectively (Table I); iii) amplification of terminal part of nccA-like gene with nccA642F and nccA642R primers located in earlier known terminal part and outside of the gene, respectively (Table I); iv) amplification of middle part of nccA-like gene with nccA923F and nccA923R primers located in earlier known terminal part and outside of the gene, respectively (Table I).

Bacterial strain and nccA-like gene product identifications and phylogenetic analysis. Bacterial strain identification, identification of nccA-like gene products and phylogenetic analysis were performed as described in Karellová et al. (2011) with following modifications: multiple sequence alignments and phylogenetic trees were constructed with the MEGA software (version 6, Tamura et al., 2011). Maximum likelihood method with 100 bootstrap replications was chosen with Tamura-Nei model of substitutions and the resulting tree was presented with the Tree Explorer of the MEGA package.

Plasmid DNA purification and electroporation. Plasmid DNA from bacterial MR-CH-I2 cells was purified using the Wizard minipreparation kit (Promega, USA) according to the manufacturer's instructions. Plasmid transformations were performed by electroporation on equipment ECM 630 (BTX Harvard apparatus, USA) after one impulse effect (2.5 kV, 200 Ω, 25 μF).

Pulsed-field gel electrophoresis (PFGE). PFGE was performed in equipment ROTAPHOR (Biotest, Germany) as described in Nováková et al. (2013) with the following modifications: electrophoresis ran at 10°C for 18 h with constant interval of about 2 s, under linear angle from 130° to 110° and linear voltage from 130 V to 90 V.

Bacterial cells preparation for nccA-like gene expression analysis. Bacterial culture MR-CH-I2 was grown aerobically in liquid Luria-Bertani (LB) medium in Erlenmeyer flasks in a rotary shaker (90 rpm) at 30°C. When cultures reached an optical density at 420 nm (OD₄₂₀) of 0.5, five heavy metals were added to a previously optimized final concentration of 250 μg/ml Ni²⁺, 100 μg/ml Cd²⁺, 50 μg/ml Zn²⁺, 25 μg/ml Co³⁺ and 10 μg/ml Cu²⁺, respectively (Remenár et al., 2015). Appropriate aliquots of bacterial cultures were withdrawn from culture either before heavy metals addition (control sample) or 0, 2, 4, 6 and 8 h after heavy metal additions.

Total RNA isolation and purification and cDNA preparation. Total RNA from bacterial MR-CH-I2 cells, cultivated in the presence of different heavy metals and without heavy metal additions (control sample), was isolated and purified using the RiboPure Bacteria Kit (Ambion, USA) according to the manufacturer’s instructions. cDNA was prepared from isolated and purified RNA using ImProm-II Reverse Transcription System (Promega, USA) according to the manufacturer’s instructions.

Real-time PCR. cDNA prepared from RNA of bacterium MR-CH-I2 was used in Real-time PCR either with nccA gene primers (nccA-F, nccA-R) or with housekeeping gdhA primer set (gdhA-F and gdhA-R) (Table I) generating approximately 125 bp or 145 bp products in size respectively. Each 20 μl reaction mixture contained 2 ng of the cDNA template, 4.0 μl 5× HOT FIREPol EvaGreen qPCR SuperMix (Solis Bio-
Dyne, Esthonia) and 0.5 μM of each primer. Reaction was performed in a thermal cycler ABI 7900HT FAST Real-Time PCR System (Life Technologies, USA) with the following cycling conditions: 12 min of denaturation at 95°C, 40 cycles of 15 s at 95°C, 20 s at 55°C and 20 s at 72°C. Results were evaluated using SDS software of the ABI 7900HT device.

**Nucleotide sequence accession numbers.** The sequences generated in this study have been deposited in the GenBank database under accession number MF102046 for MR-CH-I2 16S rRNA (16S rDNA) gene, MF134666 for MR-CH-I2-gyrB gene and KR476581 for complete MR-CH-I2-nccA gene of MR-CH-I2.

**Results and Discussion**

**Identification of the specific heavy-metal resistance bacterium.** To identify unequivocally previously isolated heavy-metal resistant bacterium we performed a phylogenetic analysis of almost the whole (1,500 bp) 16S rDNA (16S rRNA) sequence and sequences for gyrB gene and its product, respectively. The results from these analyses showed that the bacterial isolate, marked as MR-CH-I2 [MF102046] and MR-CH-I2-gyrB [MF134666], respectively was assigned to *Ralstonia pickettii* [12] chromosome 1 [CP001068] with 99 and 100% similarities, respectively (Figs. 1, 2).

Fig. 1. Unrooted phylogenetic tree obtained by the maximum likelihood method with 100 bootstrap replications showing phylogeny of 16S rRNA (16S rDNA) gene sequences of MR-CH-I2 isolate (in bold) and members of the genera *Ralstonia*, *Cupriavidus* and *Alcaligenes*, respectively. *Rhizobium* sp. SCAU231 [HQ538623], *Pseudomonas fluorescens* strain MPF25 [AB621592], *Streptomyces badius* strain 3504 [JN180190], *Olivibacter soli* strain Gsoil 034 [NR_041503] and *Brevibacillus parabrevis* C8 [KX832687] were used as outgroup. Numbers in square brackets indicate the GenBank accession number and similarity to closest relative is shown after the clone designation. Sequences of about 1,500 bp in length were aligned with ClustalW.
bacterium was firstly found inside of bacterial community isolated by using diffusion chamber methods from strongly disturbed environment mainly by high nickel concentrations in southwest Slovakia, and marked as MR-CH-I2 [KC809939] and tentatively assigned to Uncultured beta proteobacterium clone GC0AA7ZA05PP1 [JQ913301] on a β-Proteobacteria branch of phylogenetic tree (Remenár et al., 2015). Several representatives of this class of Proteobacteria are often highly versatile in their degradation capacities and many of them are found in environmental samples, such as waste water or soil (Stackebrandt et al., 1988). But only one of them, *R. pickettii* is able to survive as an oligotrophic organism in areas with a very low concentrations of nutrients (Adley et al., 2007). It was found in moist environments such as soils, rivers and lakes (Coenye et al., 2003). Several strains have shown an ability to survive in environments highly contaminated with metals such as copper (Cu), nickel (Ni), iron (Fe) and zinc (Zn). The ability to persist in these harsh conditions makes *R. pickettii* a unique candidate for bioremediation (Fett et al., 2003). *R. pickettii* can also break down several aromatic hydrocarbons or volatile organic compounds such as cresol, phenol
Bacterial heavy-metal resistance determinant and toluene and it is able to exploit this resource by using the hydrocarbons as both a source of carbon and energy (Adley et al., 2007). Thus, the identification of *R. pickettii* among β-Proteobacteria representatives isolated from heavy-metal contaminated soil by using of diffusion chamber is not surprising.

In addition, pulsed-field gel electrophoresis (PFGE) showed that the bacterium MR-CH-I2 carried a unique high molecular weight plasmid of about 50 kb in size (Fig. 3). It is known that there are two separate strains of *R. pickettii*, 12D and 12J which vary significantly in their entire genome size, 3.5 Mb and 3.0 Mb respectively. In spite of the fact that their rRNA sequences are identical, there are significant differences in their genomic structures (http://genome.jgi-psf.org/ralpd/ralpd.home.html). While both, 12D and 12J strains contain two circular chromosomes 3,647,724 bp and 1,323,321 bp in size and 3,942,557 bp and 1,302,228 bp in size respectively, the 12D strain contains three circular plasmids 389,779 bp, 273,136 bp and 51,398 bp in size whereas the 12J strain has only one circular plasmid that is 80,934 bp in size (http://www.expasy.ch/sprot/hamap/RALP1.html; http://expasy.org/sprot/hamap/RALP1.html). In spite of the fact that there are some discrepancies about the plasmid size between our data and cited one, our bacterium carried only one plasmid, thus this result also endorses the correct assignment of bacterium MR-CH-I2 to *R. pickettii*, strain 12J. In addition, another *R. pickettii* strains were found which possesses a unique plasmid of about 50 kb in size as well (http://genome.jgi-psf.org/ralpd/ralpd.home.html).

**The correct assignment of nccA-like heavy-metal resistance determinant.** Except the presence of one plasmid of a higher molecular weight, MR-CH-I2 has been found to carry nccA-like heavy-metal resistance determinant, firstly marked as MR-CH-I2-HMR [KF218096]. On the base of phylogenetic analysis of its partial (581 bp) sequence it was tentatively assigned to CzcA family heavy metal efflux pump [YP_001899332] from *R. picketti* 12J with 99% similarity (Remenár et al., 2015). According to sequencing strategy of PCR amplicons covering a complete encoding area of nccA-like heavy-metal resistance gene and its neighbouring sequences partly before and partly after of its encoding area (in detail described in “Materials and methods” section) (Fig. 4), the whole nccA-like heavy-metal resistance gene sequence was obtained and marked as MR-CH-I2-nccA [KR476581] of about 3,192 bp in length and of 1,063 amino acids (115,620 Da in molecular weight), respectively. The results from following phylogenetic analysis of complete nccA-like heavy-metal resistance gene have confirmed its assignment to CzcA family heavy metal efflux pump [WP_004635342] from *R. picketti* 12J with 98% similarity (Fig. 5). However, the presence of this heavy-metal resistance gene on the plasmid was not confirmed. WP_004635342 is a new term replacing YP_001899332. Thus previous protein reference sequence YP_001899332 has been replaced by WP_004635342.1.

**Fig. 3.** Pulsed-field gel electrophoresis analysis of high molecular plasmids from bacterium MR-CH-I2.

Legends: Lane 1 = mass standard (Lambda Ladder PFGE Marker); lane 2 = plasmid from isolate MR-CH-I2; lane 3 = control sample (without plasmid); lane 4 = mass standard (1 Kb DNA Ladder). The arrow indicates the band of about 50 kb in size.

**Fig. 4.** The whole MR-CH-I2-nccA [KR476581] gene sequencing strategy of MR-CH-I2 isolate (cf. Detection of complete nccA-like gene and its sequencing strategy in section Materials and methods and Table I).

Legends: Numbers in bold indicate positions of the MR-CH-I2-nccA gene (dark-skinned grey arrow) and its neighbourhood areas (light grey arrow) on chromosome in the *Ralstonia pickettii* 12J [CP001068] numbering system; thin arrows indicate positions of appropriate primers on the MR-CH-I2-nccA gene and its neighbourhood areas; nccA1244F and nccA1244R primers were used for sequencing of the beginning of the MR-CH-I2-nccA gene; nccA9F and nccA2875R primers were used for sequencing of the beginning and terminal parts of the middle area of the MR-CH-I2-nccA gene; nccA923F and nccA923R primers were used for sequencing of the middle parts of the middle area of the MR-CH-I2-nccA gene; nccA642F and nccA642R primers were used for sequencing of terminal part of the MR-CH-I2-nccA gene.
is 100% identical to WP_004635342.1 for “MULTISPECIES: cation efflux system protein CzcA [Burkholderiaceae]” over its full length. It is known that NCBI nonredundant RefSeq protein (WP_) can be annotated on large numbers of bacterial genomes that encode that identical protein (https://www.ncbi.nlm.nih.gov/protein/YP_001899332). The czc determinant encodes resistance to Cd$^{2+}$, Zn$^{2+}$ and Co$^{2+}$ by metal-dependent efflux driven by the proton motive force of the Czc-CBA complex (Nies, 1995). This system is the best-characterized CBA transporter from C. metallidurans CH34. Possession of this system makes a bacterium heavy metal resistant (Nies, 2003). Really, the isolate MR-CH-I2 showed relatively high level of resistance against nickel, cobalt, zinc and cooper and markedly higher level of resistance against cadmium. However, it did not carry resistance against any of investigated antibiotics and inhibited the growth of any of investigated bacteria (Remenár et al., 2015).

Expression of nccA-like heavy-metal resistance gene. Finally, RT-PCR experiments were done to search for expression of MR-CH-I2-nccA [KR476581] in the presence of different concentrations of five heavy metals (Ni = 250 µg/ml, Cd = 100 µg/ml, Zn = 50 µg/ml, Co = 25 µg/ml, Cu = 10 µg/ml). RNA was isolated either from these five heavy metal-induced MR-CH-I2 bacte-
The results suggest that we have obtained a new R. pickettii strain MR-CH-12 [MF102046] carrying MR-CH-12-nccA [KR476581] heavy-metal resistance gene which is specific for particular contaminated sites, is cultivable, and has high pollutant-degradation activity.

Acknowledgements

This work was supported by the project entitled “New microbial isolates containing the genes of catabolic and detoxification pathways and their use in biotechnology” under the project code ITMS-26240200010 within the frame of the support programme Research and Development from the European Regional Development Fund.

Literature

Abdelatey L.M., W.K.B. Khalil, T.H. Alit and K.F. Mahrous. 2011. Heavy metal resistance and gene expression analysis of metal resistance genes in Gram-positive and Gram-negative bacteria present in Egyptian soils. J. Appl. Sci. Environ. Sanit. 6: 201–211.

Adley C., J. Pembroke and M. Ryan. 2007. Ralstonia pickettii in environmental biotechnology potential and applications. J. Appl. Microbiol. 103: 754–764.

Bell C.W., V. Acosta-Martinez, N.E. McIntyre, S. Cox, D.T. Tissue and J.C. Zak. 2009. Linking microbial community structure and function to seasonal differences in soil moisture and temperature in a Chihuahuan desert grassland. Microb. Ecol. 58: 827–842.

Bogdanova E.S., I.A. Bass, L.S. Minakhin, M.A. Petrova, S.Z. Mindlin, A.A. Volodin, E.S. Kalysheva, J.M. Tiedje, J.L. Hobman, N.L. Brown and V.G. Nikiforov. 1998. Horizontal spread of mer operons among Gram-positive bacteria in natural environments. Microbiology 144: 609–620.

Bohus P. and J. Klinda. 2010. Environmentálna regionalizácia Slovenskej republiky. Bratislava: MŽP SR, Banská Bystrica: SAŽP , 2010, s. 9–21 (In Slovak).

Choudhary S. and P. Sar. 2016. Real-time PCR based analysis of metal resistance genes in metal resistant Pseudomonas aeruginosa strain J007. J. Basic Microbiol. 56: 688–697.

Coene Y., P. De Vos, J. Goris and P. Vandamme. 2003. Classification of Ralstonia picketti-like isolates from the environment and clinical samples as Ralstonia insidiosa. Int. J. Syst. Evol. Microbiol. 53: 1075–1080.

Erbe J.L., K.B Taylor and L.M. Hall. 1995. Metalloregulation of the cyanobacterial smt locus: Identification of SmtB binding sites and direct interaction with metals. Nucleic Acids Res. 23: 2472–2478.

Table II

| Time (h) | Nickel | Cadmium | Cobalt | Copper | Zinc |
|---------|--------|---------|--------|--------|------|
|         | ∆∆Ct  | RQ      | ∆∆Ct  | RQ      | ∆∆Ct | RQ  |
| 0       | 0.00   | 1.00    | 0.00   | 1.00    | 0.00 | 1.00 |
| 2       | 2.00   | 0.67    | 0.63   | 0.63    | 6.13 | 0.01 |
| 4       | 4.00   | 0.53    | 0.69   | 3.23    | 0.11 | 0.06 |
| 6       | 6.00   | 0.51    | 0.02   | 5.22    | 0.03 | 0.03 |
| 8       | 8.00   | 0.67    | 0.63   | 7.61    | 0.005 | 0.005 |

* Standardization of gene expression according to the house-keeping gene after heavy metal additions; Ct = threshold cycle; 
\[ \Delta\Delta C_t = \Delta C_t - \Delta C_t(gdh) \]
\[ RQ = 2^{-\Delta\Delta C_t} \]

gdh – the gene encodes glutamate dehydrogenase (house-keeping gene).

Nickel, Cadmium, Cobalt, Copper and Zinc were added to the medium in 0 h and 2, 4, 6, 8 h after heavy metal additions. The expression gene pattern was normalised according to the reference gene gdhA. The results from RT-PCR analysis showed that only nickel after 2 h from its addition to the medium has significantly affected MR-CH-12-nccA gene expression, it increased up to 16-times. The addition of remaining heavy metals did not significantly affect the MR-CH-12-nccA gene expression (Table II). In fact, MR-CH-12-nccA gene was identified as CzcA family heavy metal efflux pump [WP_004635342] from R. picketti 12 with 98% similarity (Fig. 5). However, this gene mediates inducible resistance to cobalt, zinc, and cadmium in A. eutrophus (Nies et al., 1987; Nies, 1992; Kunito et al., 1996). In addition, significant upregulation of czcA gene in Pseudomonas aeruginosa strain was found upon exposure only to low concentrations of zinc and cadmium for short duration of their influences on bacterium (Choudhary and Sar, 2016). Similarly, Abdelatey et al. (2011) have also confirmed the nccA-like gene inductions by cobalt and cadmium additions to the medium in Pseudomonas sp. and Bordetella sp. strains isolated from heavy-metal contaminated soils. Thus, significant Ni-induction of MR-CH-12-nccA gene is surprising because the function of the combined nickel-cobalt-cadmium resistance is mediated by the CzCB2A-related NccCBA efflux system from A. xylosoxidans (Schmidt and Schlegel, 1994). But, in some occasions, Ni\(^{2+}\) and Co\(^{2+}\) are exported by the same CBA transporters as Zn\(^{2+}\) and Cd\(^{2+}\) (for example NccCBA from A. xylosoxidans 31A and CzcCBA from C. metallidurans CH34) (Schmidt and Schlegel, 1994; Legatzi et al., 2003). This suggestion could explain MR-CH-12-nccA gene induction only after nickel addition even because the natural environment of this bacterium strain was contaminated mainly by high nickel concentrations. These results suggest that we have obtained a new...
Fett J., K. Konstantinidis, N. Isaacs, D. Long and T. Marsh. 2003. Microbial diversity and resistance to copper in metal-contaminated lake sediment. *Microbiol. Ecol.* 45: 191–202.

Govtac. 1997. Remediation of metals-contaminated soils and groundwater. *Tech. Evaluation. Rep.* 97-53.

Gi G. and S. Silver. 1992. Regulation and expression of the arsenic resistance operon from *Staphylococcus aureus* plasmid p258. *J. Bacteriol.* 174: 3684–3694.

Johnson J.M. and G.M. Church. 1999. Alignment and structure prediction of divergent protein families: periplasmic and outer membrane proteins of bacterial efflux pumps. *J. Mol. Biol.* 287: 695–715.

Kaeberlein T., K. Lewis and S.S. Epstein. 2002. Isolating “uncultivable” microorganisms in pure culture in a simulated natural environment. *Science* 296: 1127–1129.

Karelóva E., J. Harichová, T. Stojnev, D. Pangallo and P. Ferrand. 2011. The isolation of heavy-metal resistant culturable bacteria and resistance determinants from a heavy-metal-contaminated site. *Biologia (Bratislava)* 66: 18–26.

Kino T., T. Kusano, H. Oyaizu, K. Senoo, S. Kanazawa and S. Matsumoto. 1996. Cloning and sequence analysis of ccc genes in *Alicyclobacillus* sp. strain CT14. *Biocsi. Biotechnol. Biochem.* 60: 699–704.

Lane D.J. 1991. 16S/23S rRNA sequencing, pp. 115–148. In: Stackebrandt E. and M. Goodfellow (eds). *Nucleic acid techniques in bacterial systematic.* John Wiley & Sons, New York.

Lawniczak L., A. Syguda, A. Borkowski, P. Cyplik, K. Marcin- kowska, Ł. Wolko, T. Praczyk, Ł. Chrzanowski and J. Pernak. 2016. Influence of oligomeric herbicidal tonic liquids with MCPA and Dicamba anions on the community structure of autochthonous bacteria present in agricultural soil. *Sci. Total Environ.* 563–564: 247–255.

Legatzi A., S. Franke, S. Lucke, T. Hofmann, A. Anton, D. Neu- mann and D.H. Nies. 2003. First step towards a quantitative model describing Ccc-mediated heavy metal resistance in *Ralstonia metallidurans*. *Biodegradation* 14: 153–168.

Liu H.Y., A. Probs and B. Liao. 2005. Metal contamination of soils and crops affected by the Chenzhou lead/zinc mine spill (Hunan, China). *Sci. Total Environ.* 339: 153–166.

Mergey M., S. Monchy, T. Vallaes, V. Auquier, A. Benotmane, P. Bertin, S. Taghavi, J. Dunn, D. Van der Lelie and R. Watteez. 2003. *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals: towards a catalogue of metal-responsive genes. *FEMS Microbiol. Rev.* 27: 385–410.

Mobley H.L., C.M. Chen, S. Silver and B.P. Rosen. 1983. Cloning and expression of R-factor mediated arsenite resistance in *Escherichia coli*. *Mol. Gen. Genet.* 191: 421–426.

Nascimento A.A.M. and E. Chartone-Souza. 2003. Operon mer: bacterial resistance to mercury and potential for bioremediation of contaminated environments. *Genet. Mol. Res.* 2: 92–101.

Nies D., M. Mergeay, B. Friedrich and H.G. Schlegel. 1987. Cloning of plasmid genes encoding resistance to cadmium, zinc, and cobalt in *Alcaligenes eutrophus* CH34, *J. Bacteriol.* 169: 4865–4885.

Nies D.H. 1992. Resistance to cadmium, cobalt, zinc and nickel in microbes. *Plasmid* 27: 17–28.

Nies D.H. 1995. The cobalt, zinc, and cadmium efflux system CcsABC from *Alcaligenes eutrophus* functions as a cation-proton antiporter in *Escherichia coli*, *J. Bacteriol.* 177: 2707–2712.

Nies D.H. 1999. Microbial heavy metal resistance. *Appl. Microbiol. Biotechnol.* 51: 730–750.

Nies D.H. 2003. Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiol. Rev.* 27: 313–339.

Nováková R., R. Knirschová, M. Farkaškovský, E. Fecková, A. Reháková, E. Mingyar and J. Kormanec. 2013. The gene cluster aurf for the angucycline antibiotic aurfurin is located on a large linear plasmid pSA3239 in *Streptomyces aureofaciens* *CCM 3239*. *FEMS Microbiol. Lett.* 342: 130–137.

Panagos P., M. Van Liedekerke, Y. Yigini and L. Montanarella. 2013. Contaminated sites in Europe: Review of the current situation based on data collected through a European network. *J. Environ. Public Health.* 2013: 11 pp. ID 158764. http://dx.doi.org/10.1155/2013/158764.

Paulsen I.T., J.H. Park, P.S. Choi and M.H.J. Saier. 1997. A family of Gram-negative bacterial outer membrane factors that function in the export of proteins, carbohydrates, drugs and heavy metals from Gram-negative bacteria. *FEMS Microbiol. Lett.* 156: 1–8.

Remeníár, M., E. Karelóva, J. Harichová, M. Žámocký, A. Kamlárová and P. Ferrand. 2015. Isolation of previously uncultivable bacteria from a nickel contaminated soil using a diffusion-chamber-based approach. *Appl. Soil Ecol.* 95: 115–127.

Rosenstein R., A. Peschel, B. Wieland and F. Götz. 1992. Expression and regulation of the antimonite, arsenite, and arsenate resistance operon of *Staphylococcus xylosus* plasmid pSX267. *J. Bacteriol.* 174: 3676–3683.

Saier M.H.J., R. Tam, A. Reizer and J. Reizer. 1994. Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport. *Mol. Microbiol.* 11: 841–847.

Saltikov C.W. and B.H. Olson. 2002. Homology of *Escherichia coli* R773 arsA, arsB, and arsC genes in arsenic-resistant bacteria isolated from raw sewage and arsenic-enriched creek waters. *Appl. Environ. Microbiol.* 68:280–288.

Salvador M., G. Carolina and E. Jose. 2007. Novel nickel resistance genes from *Staphylococcus* sp. strain CT14. *J. Bacteriol.* 189(4): 1193–1199.

Silver S. and L.T. Phung. 1996. Bacterial heavy metal resistance: New surprises. *Ann. Rev. Microbiol.* 50: 753–789.

Stackebrandt E., R.G.E. Murray and H.G. Trüper. 1988. *Proteobacteria* classis nov., a name for the phylogenetic taxon that includes the „Purple Bacteria and Their Relatives”. *Int. J. Syst. Bacteriol.* 38: 321–325.

Sydow M., O. Wissian, Z. Sczepaniak, G. Framski, B.F. Smets, L. Lawniczak, P. Lisiecki, A. Szulc, P. Cyplik and L. Chrzanowski. 2016. Evaluating robustness of a diesel-degrading bacterial consortium isolated from contaminated soil. *N. Biotechnol.* 33: 852–859.

Sydow M., L. Chrzanowski, N. Cedergreen and M. Wissian. 2017. Limitations of experiments performed in artificially made OECD standard soils for predicting cadmium, lead and zinc toxicity towards organisms living in natural soils. *J. Environ. Manage.* 198(Pt 2): 32–40.

Tamura K., D. Peterson, N. Peterson, G. Stecher, M. Neie and S. Kumar. 2011. *MEGA5*: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28: 2731–2739.

Tchounwou P.B., C.G. Yedjou, A.K. Patlolla and D.J. Sutton. 2012. Heavy metals toxicity and the environment. *EXS* 101: 133–164.

Thiyagarajan V., S. Lau, M. Tooi, W. Zhang and P.Y. Qian. 2010. Monitoring bacterial biodiversity in surface sediment using termini restriction fragment length polymorphism analysis (T-RFLP): Application to coastal environment, pp. 151–163. In: Ishimatsu A. and H.-J. Lie (eds). *Coastal environmental and ecosystem issues of the East China sea*. TERRAPUB & Nagasaki University.

Toth G., T. Hermann, M.R. Da Silva and L. Montanarella. 2016. Heavy metals in agricultural soils of the European Union with implications for food safety. *Environ. Int.* 88: 299–309.

Tsong T.-T., K.S. Grattick, J. Kollman, D. Park, D.H. Nies, A. Goffeau and M.H.J. Saier. 1999. The RND superfamily: an
ancient, ubiquitous and diverse family that includes human disease and development proteins. *J. Mol. Microbiol. Biotechnol.* 1: 107–125.

Wuana R.A. and F.E. Okieimen. 2011. Heavy metals in contaminated soils: A review of sources, chemistry, risks and best available strategies for remediation. *ISRN Ecology* 2011: 20 pp. ID 402647. doi:10.5402/2011/402647

Yamamoto S. and S. Harayama. 1995. PCR amplification and direct sequencing of gyrB genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. *Appl. Environ. Microbiol.* 61: 1104–1109.

Zgurskaya H.I. and H. Nikaido. 1999a. Bypassing the periplasm: Reconstitution of the AcrAB multidrug efflux pump of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 96: 7190–7195.

Zgurskaya H.I. and H. Nikaido. 1999b. AcrA is a highly asymmetric protein capable of spanning the periplasm. *J. Mol. Biol.* 285: 409–420.

Zgurskaya H.I. and H. Nikaido. 2000a. Multidrug resistance mechanisms: drug efflux across two membranes. *Mol. Microbiol.* 37: 219–225.

Zgurskaya H.I. and H. Nikaido. 2000b. Cross-linked complex between oligomeric periplasmic lipoprotein AcrA and the inner-membrane-associated multidrug efflux pump AcrB from *Escherichia coli*. *J. Bacteriol.* 182: 4264–4267.

“*Ralstonia pickettii*” JGI Genome Portal – Home. Web. 2010. http://genome.jgi-psf.org/ralpd/ralpd.home.html.

“HAMAP: *Ralstonia pickettii* (strain 12D) complete proteome.” ExPASy Proteomics Server. Swiss Institute for Bioinformatics. Web. 2010. http://expasy.org/sprot/hamap/RALP1.html.

“HAMAP: *Ralstonia pickettii* (strain 12J) complete proteome.” ExPASy Proteomics Server. Swiss Institute for Bioinformatics. Web. 2010. http://expasy.org/sprot/hamap/RALPJ.html.

https://www.ncbi.nlm.nih.gov/protein/YP_001899332

This article is published in Open Access model and licensed under a Creative Commons CC BY-NC-ND 4.0, licence available at: https://creativecommons.org/licenses/by-nc-nd/4.0/
