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The Heavy-Metal Resistance Determinant of Newly Isolated Bacterium from a Nickel-Contaminated Soil in Southwest Slovakia

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Abstract

A bacterial isolate MR-CH-I2 [KC809939] isolated from soil contaminated mainly by high nickel concentrations in southwest Slovakia was previously found carrying *ncc*A-like heavy-metal resistance determinant, marked as MR-CH-I2-HMR [KF218096]. According to phylogenetic analysis of short (696 bp) 16S rDNA (16S rRNA) sequences this bacterium was tentatively assigned to Uncultured beta proteobacterium clone GC0AA7ZA05PP1 [JQ913301]. *ncc*A-like gene product was on the same base of its partial (581 bp) sequences tentatively assigned to CzcA family heavy metal efflux pump [YP_001899332] from *Ralstonia picketii* 12J with 99% similarity. In this study the bacterium MR-CH-I2 and its heavy-metal resistance determinant were more precisely identified. This bacterial isolate was on the base of phylogenetic analysis of almost the whole (1,500 bp) 16S rDNA (16S rRNA) sequence, MR-CH-I2 [MF102046], and sequence for *gyr*B gene and its product respectively, MR-CH-I2-gyrB [MF134666], assigned to *R. picketii* 12J [CP001068] with 99 and 100% similarities, respectively. In addition, the whole *ncc*A-like heavy-metal resistance gene sequence (3,192 bp), marked as MR-CH-I2-nccA [KR476581], was obtained and on the base of phylogenetic analysis its assignment was confirmed to MULTISPECIES: cation efflux system protein CzcA [WP_004635342] from Burkholderiaceae with 98% similarity. Furthermore, although the bacterium carried one high molecular plasmid of about 50 kb in size, *ncc*A-like gene was not located on this plasmid. Finally, the results from RT-PCR analysis showed that MR-CH-I2-nccA gene was significantly induced only by the addition of nickel.

Key words: 16S rRNA (16S rDNA), DNA gyrase subunit B, heavy-metal resistance determinant, high molecular plasmid, nickel-contaminated soil, RT-qPCR

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

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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 *czc* (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 *czn* (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 chromate 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 *czc* determinant encodes resistance to Cd^{2+}, Zn^{2+} and Co^{2+} by metal-dependent efflux driven by the proton motive force (Nies, 1995). Ni^{2+} and Co^{2+} are, in some occasions, 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; 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 Sereď (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 (Bohuš 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 (*gyr*B) 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 *gyr*B 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 *Pfx* 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

Table I
Primer sets used in this study.

Probes	Sequence	Description[a]
27F	5' AGAGTTTGATCCTGGCTCAG 3'	16S rDNA universal primers, positions 8–27 and 704–685 and 1512–1492, resp. in the *E. coli* K12 [NC_000913] numbering system; (Lane, 1991)
1492R	5' ACGGCTACCTTGTTACGACTT 3'	
2555nccAF	5' AGCCG (C,G) GA (C,G) AACGG CAAGCG 3'	2536–2555 and 3136–3117 degenerative nccA primers, positions on plazmid p9 in the *Achromobacter xylosoxidans* 31A [L31363] numbering system; (Karelová *et al.*, 2011)
3117nccAR	5' CCGATCACCACCGT (T,C) GC CAG 3'	
nccA9F	5' ACGTATCATTAGTTTCGCCA 3'	1861923–1861904 and 1859057–1859076 *ncc*A primers, positions on chromosome in the *Ralstonia picketii* 12J [CP001068] numbering system; This work
nccA2875R	5' ATCGGATAAACGACAGCATC 3'	
nccA1244F	5' GCTCTCGAAAGAGGAAGGCA 3'	1862989–1862970 and 1861746–1861765 *ncc*A primers, positions on chromosome in the *Ralstonia picketii* 12J [CP001068] numbering system; This work
nccA1244R	5' TTCGGTTTCGAGCGGTGAAT 3'	
nccA642F	5' GCTAGTCTTCACGGGCATT 3'	1859211–1859193 and 1858570–1858589 *ncc*A primers, positions on chromosome in the *Ralstonia picketii* 12J [CP001068] numbering system; This work
nccA642R	5' GCTCTTCGTCATGACACCAC 3'	
nccA923F	5' GGTCGCTTCCATTAACCG 3'	1860996–1860979 and 1860074–1860091 *ncc*A primers, positions on chromosome in the *Ralstonia picketii* 12J [CP001068] numbering system; This work
nccA923R	5' GATCGGATGCAATCTCCG 3'	
nccA-F	5' GTCGCCTTGTTCATCGG 3'	1860425–1860409 and 1860301–1860319 *ncc*A primers, positions on chromosome in the *Ralstonia picketii* 12J [CP001068] numbering system; This work
nccA-R	5 GCAAACGTCAATACAACGG 3'	
gdhA-F	5' CGTACTCAATGAACGAAGGC 3'	388722–388741 and 388866–388850 *gdh*A primers, positions on chromosome in the *Ralstonia picketii* 12D [CP001644] numbering system; This work
gdhA-R	5' TCGATGCCGAGATTGCG 3'	
UP1	5' GAAGTCATCATGACCGTTCTG CA(TC)GC(TCAG)GG(TCAG)GG (TCAG)AA(AG)TT(TC)GA 3'	*gyr*B gene primers, positions 91–104 and 495–509 amino acid residues (the numbering corresponds to that of the *E. coli* K12 protein [(GYRB_ECOLI in the SWISS-PROT database)]) (Yamamoto and Harayama, 1995)
UP2r	5' AGCAGGGTACGGATGTGCGAG CC(AG)TC(TCAG)AC(AG)TC(TC AG)GC(AG)TC(TCAG)GTCAT 3'	
UP-1S	5' GAAGTCATCATGACCGTTCT GCA 3'	
UP-2Sr	5' AGCAGGGTACGGATGTGCG AGCC 3'	

[a] Numbers in parenthesis indicate the GenBank accession number.

(LabCycler, Goettingen, Germany) with the following cycling conditions: 2 min of denaturation at 95°C, 35 cycles of 20 s at 95°C, 30 s at 53°C (16S rRNA) or 58°C (*gyr*B), 1 min 40 s (both, 16S rRNA and *gyr*B) at 68°C, and a final cycle of extension at 68°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 *gyr*B genes respectively, were excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Detection of complete *ncc*A-like gene and its sequencing strategy. DNA extracted from bacterium MR-CH-I2 was used in PCRs with subsequent primer sets:

i) *ncc*A-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 *ncc*A-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 *ncc*A-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 *ncc*A-like gene with nccA923F and nccA923R primers located in earlier known early and terminal parts 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 $MgCl_2$, 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 (i), 58°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 8 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 2,866, 1,244, 642 or 923 bp in size for i) – iv) gene fragments respectively, were excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Sequencing of 16S rRNA (16S rDNA), *gyr*B and *ncc*A-like amplicons. Subsamples of either purified 16S rRNA (16S rDNA), *gyr*B (*gyr*B was sequenced with UP-1S and UP-2Sr primers (Table I)) or all *ncc*A-like amplicons from isolate were sequenced by GATC Biotech, Constance, Germany.

Bacterial strain and *ncc*A-like gene product identifications and phylogenetic analysis. Bacterial strain identification, identification of *ncc*A-like gene products and phylogenetic analysis were performed as described in Karelová *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 (Biometra, 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 *ncc*A-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 (OD_{420}) of 0.5, five heavy metals were added to a previously optimized final concentration of 250 µg/ml Ni^{2+}, 100 µg/ml Cd^{2+}, 50 µg/ml Zn^{2+}, 25 µg/ml Co^{2+} and 10 µg/ml Cu^{2+}, 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 *ncc*A gene primers (nccA-F, nccA-R) or with housekeeping *gdh*A 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 *gyr*B 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 picketii* 12J chromosome 1 [CP001068] with 99 and 100% similarities, respectively (Figs. 1, 2). This

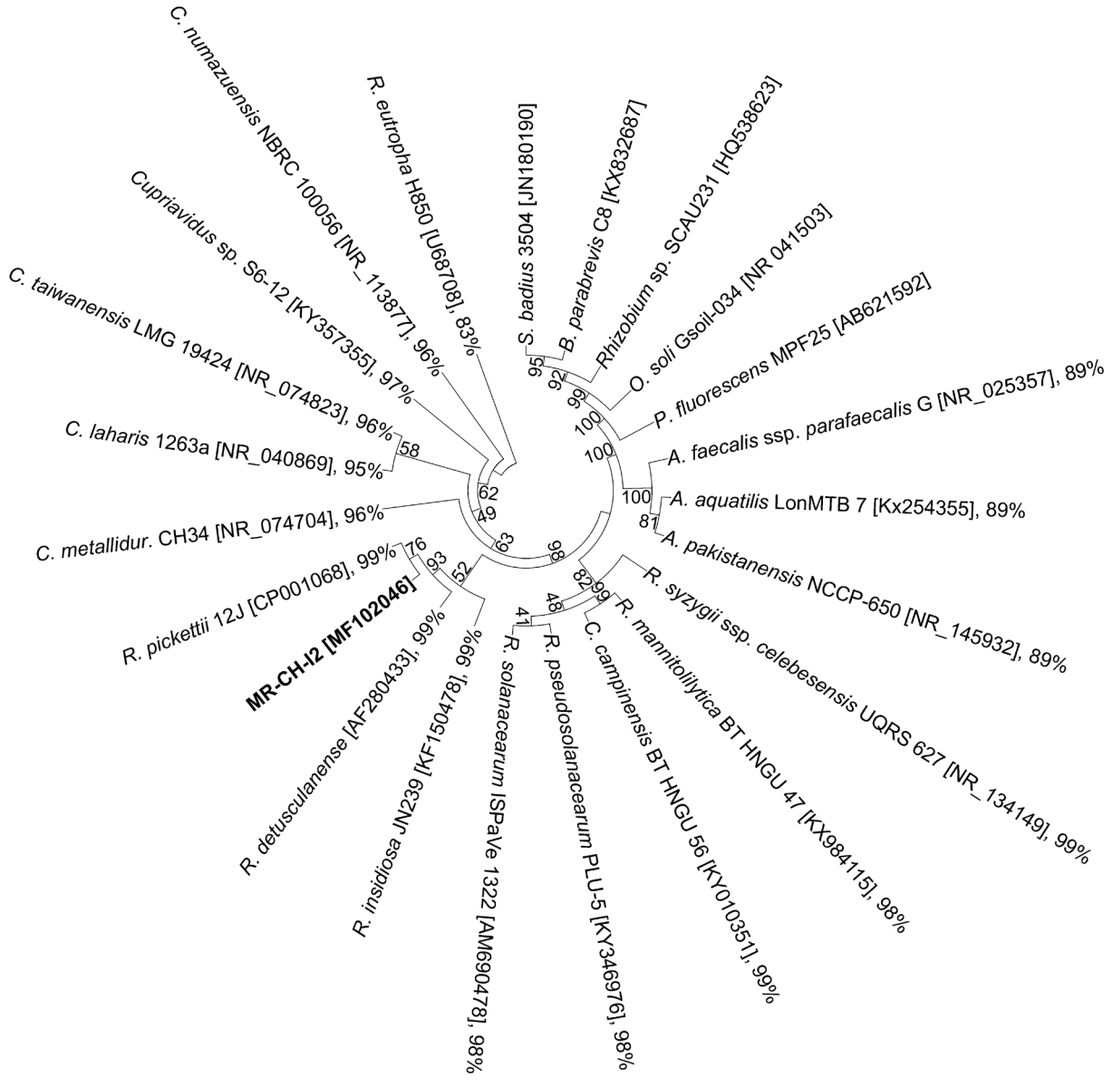

Fig. 1. Unrooted phylogenetic tree obtained by the maximum likelihood method with 100 bootstrap replications showing phylogeny of 16S rRNA (16S rDNA) gene sequences of MR-CH-I2 isolate (in bold) and members of the genera *Ralstonia*, *Cupriavidus* and *Alcaligenes*, respectively. *Rhizobium* sp. SCAU231 [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.

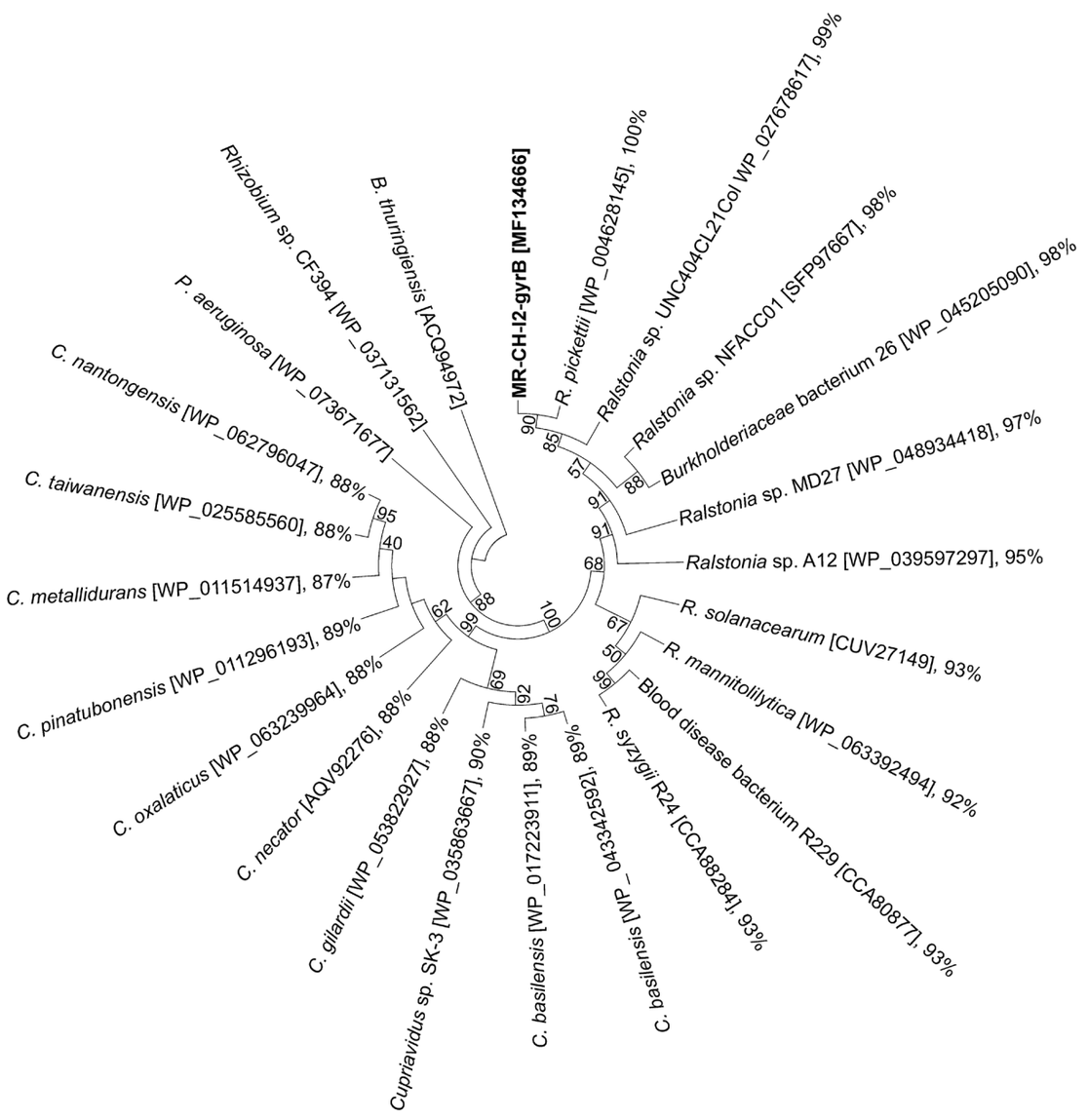

Fig. 2. Unrooted phylogenetic tree obtained by the maximum likelihood method with 100 bootstrap replications showing phylogeny of partial *gyr*B sequences based on 399 presented amino acid sites translated from DNA of MR-CH-I2 isolate (in bold) and members of the genera *Ralstonia*, *Cupriavidus* and family Burkholderiaceae, respectively. *Rhizobium* sp. CF394 [WP_037131562], *Pseudomonas aeruginosa* [WP_073671677] and *Bacillus thuringiensis* [ACQ94972] served as outgroup. Numbers in square brackets indicate the GenBank accession number and similarity to closest relative is shown after the clone designation. Sequences 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. picketti* 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

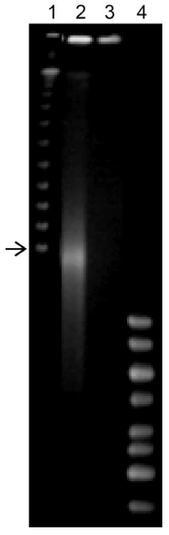

Fig. 3. Pulsed-field gel electrophoresis analysis of high molecular plasmids from bacterium MR-CH-I2.

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.

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/RALPJ.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. picketii* 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 assigment of *ncc*A-like heavy-metal resistance determinant. Except the presence of one plasmid of a higher molecular weight, MR-CH-I2 has been found to carry *ncc*A-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. picketii* 12J with 99% similarity (Remenár *et al.*, 2015). According to sequencing strategy of PCR amplicons covering a complete encoding area of *ncc*A-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 *ncc*A-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 *ncc*A-like heavy-metal resistance gene have confirmed its assignment to CzcA family heavy metal efflux pump [WP_004635342] from *R. picketii* 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. The sequence YP_001899332 for "CzcA family heavy metal efflux pump [*R. picketii* 12J]"

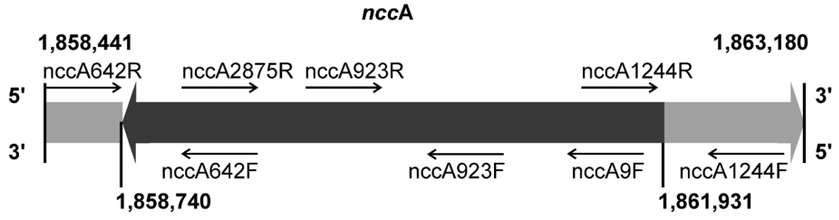

Fig. 4. The whole MR-CH-I2-nccA [KR476581] gene sequencing strategy of MR-CH-I2 isolate (cf. Detection of complete *ncc*A-like gene and its sequencing strategy in section Materials and methods and Table I).

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 picketii* 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.

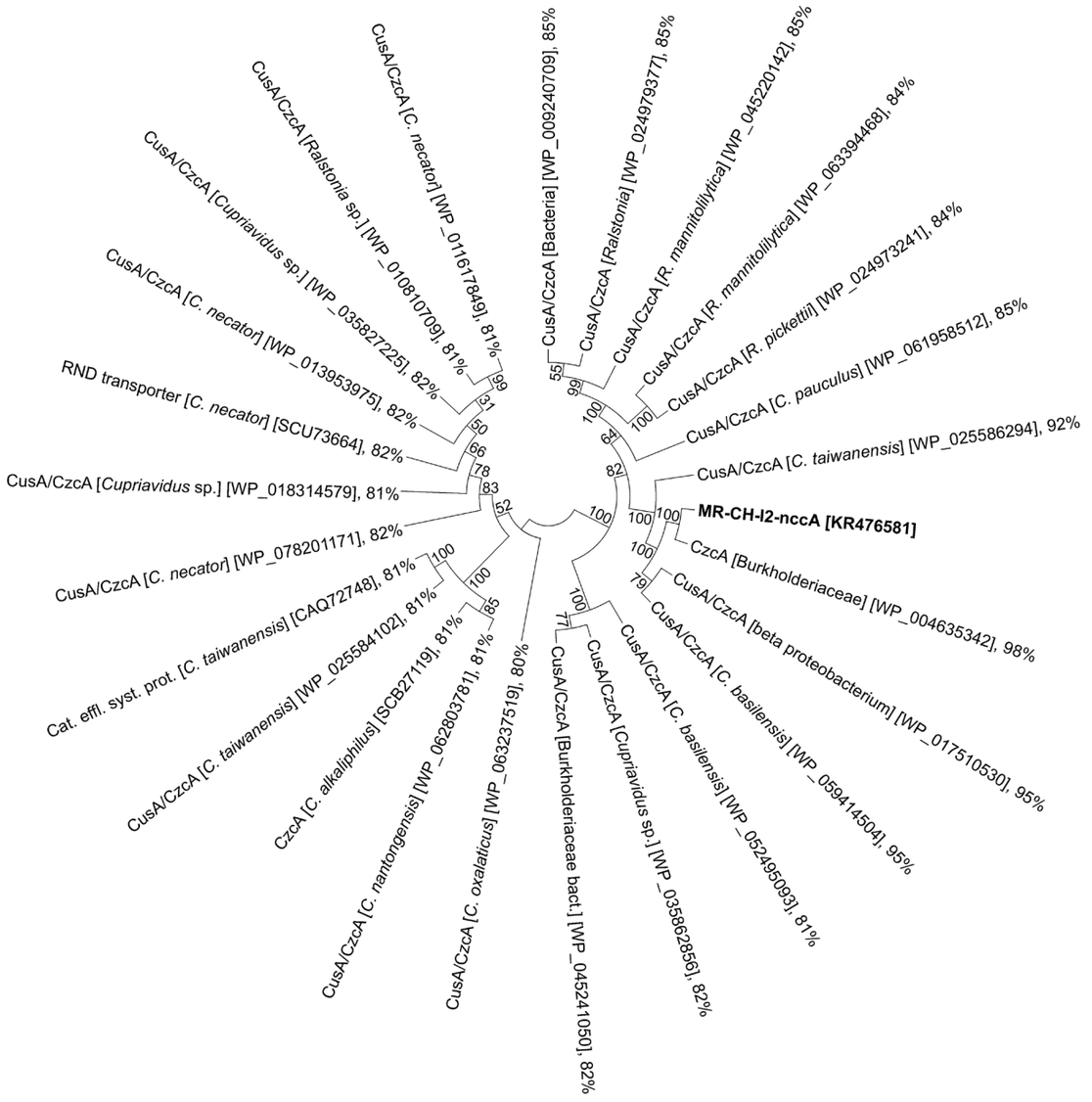

Fig. 5. Unrooted phylogenetic tree obtained by the maximum likelihood method with 100 bootstrap replications showing phylogeny of whole *ncc*A-like sequences based on 1,063 presented amino acid sites translated from DNA of the MR-CH-I2 isolate (in bold). Numbers in square brackets indicate the GenBank accession number and similarity to closest relative is shown after the clone designation. Sequences were aligned with ClustalW.

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 *ncc*A-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-

Table II
Expression of MR-CH-I2-nccA [KR476581] gene after heavy metal additions to the medium.

Time (h)	Nickel[a]		Cadmium[a]		Cobalt[a]		Copper[a]		Zinc[a]	
	ΔΔCt	RQ	ΔΔCt	RQ	ΔΔCt	RQ	ΔΔCt	RQ	ΔΔCt	RQ
0	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00
2	−4.08	16.91	0.53	0.69	3.23	0.11	5.50	0.02	1.81	0.29
4	−0.17	1.13	6.32	0.01	5.18	0.03	6.61	0.01	3.17	0.11
6	−0.51	1.42	5.82	0.02	5.22	0.03	9.38	0.002	3.65	0.08
8	0.67	0.63	6.13	0.01	7.61	0.005	7.52	0.005	2.81	0.14

[a] Standardization of gene expression according to the house-keeping gene after heavy metal additions;
Ct – threshold cycle;
$\Delta\Delta Ct = \Delta Ct^1 (Ct_{nccA} - Ct_{nccA-Control}) - \Delta Ct^2 (Ct_{gdhA} - Ct_{gdhA-Control})$;
$RQ = 2^{-\Delta\Delta Ct}$;
*ncc*A – MR-CH-I2-nccA [KR476581] (nccA-like heavy-metal resistance gene) of MR-CH-I2 bacterium;
*gdh*A – the gene encodes glutamate dehydrogenase (house-keeping gene).

rial cells or from control sample (without heavy metal additions) in 0 h and 2, 4, 6 and 8 h after heavy metal additions. The expression gene pattern was normalised according to the reference gene *gdh*A. The results from RT-PCR analysis showed that only nickel after 2 h from its addition to the medium has significantly affected MR-CH-I2-nccA gene expression, it increased up to 16-times. The addition of remaining heavy metals did not significantly affect the MR-CH-I2-nccA gene expression (Table II). In fact, MR-CH-I2-nccA gene was identified as CzcA family heavy metal efflux pump [WP_004635342] from *R. picketii* 12J with 98% similarity (Fig. 5). However, this gene mediates inducible resistance to cobalt, zinck, and cadmium in *A. eutrophus* (Nies *et al.*, 1987; Nies, 1992; Kunito *et al.*, 1996). In addition, significant upregulation of *czc*A 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 *ncc*A-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-I2-nccA gene is surprising because the function of the combined nickel-cobalt-cadmium resistance is mediated by the CzcCB2A-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; Legatzki *et al.*, 2003). This suggestion could explain MR-CH-I2-nccA gene induction only after nickel addition even because the natural environment of this bacterial strain was contaminated mainly by high nickel concentrations. These results suggest that we have obtained a new *R. picketii* strain MR-CH-I2 [MF102046] carrying MR-CH-I2-nccA [KR476581] heavy-metal resistance gene which is specific for particular contaminated sites, is cultivable, and has high pollutant-degradation activity.

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