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STRUCTURE ANALYSIS AND DIVERSITY OF BACTERIAL COMMUNITY AND THEIR RESISTANCE DETERMINANTS IN A NICKEL-CONTAMINATED SOIL IN SOUTHWEST SLOVAKIA

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Abstract

In this study we aimed to analyse the structure and diversity of overall bacterial community and its resistance determinants from nickel-contaminated soil in Slovakia by both, cultivation-dependent and independent approaches. The phylogeny was reconstructed using partial sequences of 16S rRNA (16S rDNA) and heavy-metal resistance genes from separated isolates and bacterial clones. A total of 518 bacterial sequences obtained from both, isolates and clones, represented 266 species belonging to 8 bacterial phyla: Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Gemmatimonadetes, Proteobacteria (α-, β- and γ-classes), Verrucomicrobia, and one yet unclassified group. In addition, among isolates and clones, 49 different *ncc*A-like genes were found in the final output. Majority of them were assigned to a system of transmembrane metal pumps. Our results demonstrate the fact that the nickel-contaminated soil is able to present very specific heavy-metal resistant bacterial community which can be used in different bioremediation processes.

Key words: bacterial community structure, cultivation-dependent and independent approaches, heavy-metal resistance genes, nickel-contaminated soil, phylogenetic analysis

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INTRODUCTION

Bacteria respond to heavy metals in the environment, either from natural sources or due to anthropogenic activities. Bacteria have been interacting with heavy metals since their early evolutionary history. However, industrial and urban wastes, agricultural applications and also mining activities have resulted in an increased concentration of heavy metals in soils (SHERAMETI & VARMA 2010). Copper, chromium, cadmium and nickel are known to be the most common heavy metals used

and widely spread contaminants of the environment (HUSSEIN et al. 2003; VIRENDER et al. 2010). Different environments contain significant concentrations of heavy metals that are not degraded by the conventional processes in nature, so that heavy metals are accumulated and persist for long time in the environment thus affecting its microbial assemblages (ŠMEJKALOVÁ et al. 2003). In high concentrations they react to form toxic compounds in cells (NIES 1999). Presence of high concentration of toxic heavy metals in environment can cause severe problems to human health (KERAMATI et al. 2011). Nickel is one of the most abundant metals in the earth crust (IYAKA 2011) which is necessary in trace amounts for a variety of metabolic processes but in high concentration causing oxidative stress in the cell. However, microorganisms have evolved several mechanisms that regulate metal ion accumulation to avoid heavy metal toxicity in the presence of this metal. The best known mechanisms of nickel resistance are mediated by efflux pumps such as CnrCBA (cobalt-nickel resistance) from *Cupriavidus metallidurans* CH34, NccCBA (nickel-cobalt-cadmium resistance) and NreB (nickel resistance) from *Achromobacter xylosoxidans* 31A, CznABC (cadmium-zinc-nickel resistance) from *Helicobacter pylori* (SALVADOR et al. 2007).

In general, culture-dependent or independent approaches are often used to study the structure and diversity of microbial communities in different environments, including also extreme, e.g. toxic-metal contaminated environments. Although a vast amount of new approaches based on molecular biology comprise more effective tools for the study of bacterial diversity, cultivation is still indispensable for increasing our understanding of specific organisms (PALLERONI 1997). However, sampling of diverse environments shows that only 0.01 – 1% of cells visible under the microscope will form colonies on a Petri dish (CHO et al. 2004; KELLER & ZENGLER 2004). Thus the remaining majority of cells are "uncultivable" (WAGNER & HORN 2006) and thus uncharacterized (MASON et al. 2012). But, when both approaches are combined, this multi-techniques procedure can provide a more complete picture about the bacterial structure in heavy metal contaminated soils. It seems that development of novel technologies based on enormous progress in next-generation-sequencing, such as single-cell sequencing, gives answers to many questions about functions of individual cells in the environment (SHAPIRO et al. 2013).

In our previous works, a phylogenetic analysis was performed either to determine the structure and diversity of cultivable (KARELOVÁ et al. 2010, 2011), hardly cultivable and previously uncultured bacterial isolates by using a diffusion chamber (REMENÁR et al. 2015) or non-cultivable (HARICHOVÁ et al. 2012) fractions of bacterial assemblages in the same heavy-metal contaminated farmland soil in southwest Slovakia using 16S rRNA (16S rDNA) and heavy-metal resistance genes. Thus, the aim of the present study was to characterise overall bacterial assemblage from the same toxic-metal contaminated soil by using a combination of data from both approaches.

MATERIAL AND METHODS

The soil samples, down to 10 cm depth, were collected from farmland situated nearby a dump containing heavy-metal-contaminated waste (48º16'59''N, 17º43'35''E) in southwest Slovakia. They were transported in autoclaved bags, placed in an icebox and stored at 4 ºC in a refrigerator until use. The content of heavy metals in the soil sample was measured using an atomic absorption spectrometer (PerkinElmer model 403, USA) (KARELOVÁ et al. 2011).

A 10 g portion (wet weight) of the soil was mixed in a sterile 250 mL Erlenmeyer flask with 90 mL of a 0.85% (w/v) salt solution and incubated at 30°C on a shaker incubator at 90 rpm for 2 h (KARELOVÁ et al. 2011).

Bacteria were cultivated and isolated by using both, traditional cultivation techniques (KARELOVÁ et al. 2011) and diffusion-chamber-based approach (REMENÁR et al. 2015). As cultivation medium

either soil-extract agar medium (SEA) or Nutrient agar No. 2 (Biomark, India) was used (KARELOVÁ et al. 2011). The diffusion chambers were prepared according to KAEBERLEIN et al. (2002) with some modifications (REMENÁR et al. 2015). The plates (from both, traditional cultivation and diffusion-chamber) were incubated aerobically at 30°C for either 24 – 48 h or 1 – 2 weeks. The numbers of CFUs were repeatedly counted to ensure that, at the time of isolation, the appearance of new colonies had dropped off. Independently growing colonies from both approaches were selected on the basis of their morphology for further analysis.

Bacterial DNA from soil isolates was isolated using the DNeasy purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions as described in KARELOVÁ et al. (2011). The total DNA from soil samples was isolated with PowerSoil DNA Kit (MO BIO Laboratories, Inc., Carlsbad, Canada) according to the manufacturer's instructions as described in HARICHOVÁ et al. (2012). The resulting high-molecular-weight DNA obtained from both, isolates and soil samples, was used as a template in PCR either with universal 16S rRNA gene primers or with non-specific degenerated *ncc*A primer sets (Tab. 1) as described either in KARELOVÁ et al. (2011) or HARICHOVÁ et al. (2012).

In case when the total soil DNA was used in PCR, the relevant 16S rRNA and *ncc*A-like amplicons were separately pooled, after purification ligated into the pDrive Cloning Vector, and transformed into QIAGEN EZ competent cells using the PCR Cloningplus Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Clones containing the potential inserts either of 16S rRNA or *ncc*A-like genes were screened by PCR with primers M13 (Tab. 1) as described in HARICHOVÁ et al. (2012).

Subsamples of either purified 16S rRNA (16S rDNA) correct PCR amplicons of approximately 696 bp from isolates (from diffusion chamber and Petri-dishes) and soil clones or *ncc*A-like correct PCR products of approximately 581 bp, were sequenced by GATC Biotech, Constance, Germany.

The sequences generated or used in this study have been deposited in the GenBank database under accession numbers as follows: a) for bacterial 16S rRNA (16S rDNA) genes from (i) isolates – from GU935266 to GU935334 (KARELOVÁ et al. 2011), from KC809922 to KC809958, from KJ510963 to KJ511005 and from KJ811542 to KJ811562 (REMENÁR et al. 2015), and (ii) clones – from HM038047 to HM038080 (HARICHOVÁ et al. 2012), from JQ756459 to JQ756488 and from JQ772510 to JQ772513 (this study); b) for *ncc*A-like genes from (i) isolates – from GU935257 to GU935265 (KARELOVÁ et al. 2011), from KF218087 to KF218094 and from KF218096 to KF218099 (REMENÁR et al. 2015), and (ii) clones – from HM038081 to HM038096 (HARICHOVÁ et al. 2012) and from JQ916035 to JQ916046 (this study).

Both, bacterial strain and clones identification and 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 5.1, 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.

Diversity of bacterial assemblages was estimated on the base of the number and frequency of bacterial taxon occurrences in the nickel-contaminated soil (HARICHOVÁ et al. 2006).

RESULTS AND DISCUSSION

In this study our research is oriented on the structure and diversity determinations of overall bacterial assemblages and their heavy-metal resistance determinants in nickel-contaminated soil sample by using combining of data obtained from both, on cultivation dependent and independent approaches.

Investigated field site is situated nearby a dump containing heavy-metal-contaminated waste in southwest Slovakia. This area is according to environmental monitoring of Slovakia a part of strongly disturbed environment (BOHUŠ & KLINDA 2010) which 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). In this area black land soil type predominates which emerged on carbonate fluvial sediments. These sediments include a sufficient amount of basic cations in substrate (for Mg in range from 1.52 to 2.51% and for Ca from 3.84 to 7.2%) as well as adequate amount of high-quality organic compounds (NEŠŤÁK et al. 2007). These soil characteristics suggest that such soil system is rich in buffer abilities and it is able to preserve the near-optimal pH of soil environment (pH/KCl = 7.37, NEŠŤÁK et al. 2007). However, in spite of the fact, that these soil characteristics suggested that the heavy metal bio-availabilities of investigated soil sample cannot be so high, the deleterious effect mainly of nickel, (2109 mg/kg), on micro-organisms could be expected.

A total of 518 sequences (isolates and clones) were divided into 266 species belonging to 8 bacterial phyla and one unclassified group: Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Gemmatimonadetes, Proteobacteria (α-, β- and γ-classes) and Verrucomicrobia (Fig. 1a, 2). We demonstrate here that mainly Actinobacteria, Proteobacteria and Firmicutes predominate and the presence of representatives assigned to Bacteroidetes and Acidobacteria, respectively is a common phenomenon in heavy-metal contaminated soils. In addition, the highest abundance level of γ-Proteobacteria inside of the pool of Proteobacteria is not surprising as well. However, higher abundance of β-Proteobacteria in the pool of this phylum in comparison to α-Proteobacteria is evident (Fig. 1a). According to the fact that the phylum Proteobacteria represented mainly isolates (Fig. 1b), distribution of individual classes is more related to use of cultivation media (KARELOVÁ et al. 2011) and/or cultivation conditions (REMENÁR et al. 2015). It is generally known that the representatives of β-Proteobacteria are isolated in larger numbers by the methods mimicking bacterial natural environment (FERRARI et al. 2005; HOHN et al. 2004), because these methods often prefer the cultivation of slowly-growing k-strategists which are able to live in nutrient-poor conditions (FERRARI et al. 2005; WATVE et al. 2000). Similarly to our results, a few of previous studies using both approaches refer to assignment of isolates to same phyla (KOEPKE et al. 2005; ZHANG et al. 2007; WU et al. 2012). In these types of soils also the representatives of Gemmatimonadetes are often included in bacterial structures (ZHANG et al. 2007; WU et al. 2012). The phylum Verrucomicrobia contains only a few described species, e. g. *Verrucomicrobium spinosum*. Evidence suggests that Verrucomicrobia are abundant within the environment and important, especially to soil cultures (CHO et al. 2004). Furthermore, Cyanobacteria are arguably the most successful group of microorganisms on earth. They are the most genetically diverse; they occupy a broad range of habitats across all latitudes, widespread in freshwater, marine, and terrestrial ecosystems, and they are found in the most extreme niches such as hot springs, salt works, and hypersaline bays. Cyanobacteria fulfil vital ecological functions in the world's oceans, being important contributors to global carbon and nitrogen budgets (STEWART & FALCONER 2008). However, the significance of Actinobacteria within the microbial communities of soils contaminated with heavy metals is unresolved (BAMBOROUGH & CUMMINGS 2009). In contrast, other studies on the impact of heavy metal contamination on bacterial community structure have reported a significant decline in the contribution of Actinobacteria to the bacterial community in a forest soil contaminated with cadmium, copper, zinc and lead (FREY et al. 2006). Similarly, VIVAS et al. (2008) found high dominance indices in the other more polluted soils including heavy metals, indicating the supremacy of populations that may be metabolically more active due to

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the presence of pollutants. Thus, this picture about structure of overall bacterial community in nickel-contaminated soil is not surprising.

However, the part of isolates and clones on overall bacterial community is different. Firstly, while cultivable part of the bacterial community was assigned only to four phyla, uncultured fraction was assigned to seven bacterial phyla (Fig. 1b). This result is in accordance with another study using on cultivation dependent approach in which smaller count of the phyla was obtained but with a high level of their abundances (BOLLMANN et al. 2007). Secondly, the majority of the bacteria (74.9%) were obtained exclusively from the Petri dish material in comparison to 25.1% from the uncultured material, and any one of 16S rRNA (16S rDNA) sequences was obtained using both approaches. This result suggests that the bulk of bacterial microflora from both approaches was unique to their used techniques. In addition, most of clones assigned to same phyla as isolates, i. e. Actinobacteria, Bacteroidetes and Proteobacteria respectively, have created an autonomous part of the common phylogenetic tree together with isolates. Only few of them were found inside of common group with isolates although all these clones were situated on the separate branches of the tree (Fig. 2). Moreover, while among isolates Firmicutes predominated, among clones Actinobacteria. The predominance of Actinobacteria in non-cultivable fraction of bacterial community is not surprising, but the absence of Firmicutes and also very low abundance of Proteobacteria in this fraction of bacterial community was not expected with regard to previous data (KARELOVÁ et al. 2011; REMENÁR et al. 2015). On the other hand, the absence of representatives from Acidobacteria, Gemmatimonadetes, Cyanobacteria and Verrucomicrobia, respectively in cultivable fraction of bacterial community is not surprising as well, because only a few individuals from these phyla were isolated up to now (Fig. 1b). These results underlined the fact that only a minor part of bacteria inside of whatever environment is able to form colonies on cultivation media.

Furthermore, although there were only minor discrepancies in diversity level between these two fractions of bacterial community expressed by diversity indices, the diversity of the overall bacterial community exceeded that obtained from individual fractions of bacterial assemblages (Tab. 2).

These differences in the nearest relatives of the 16S rRNA (16S rDNA) genes between isolates and clones even more emphasized the necessity to use multi-technique approach in order to study the bacterial community structures.

All these bacteria which represent the structure of bacterial community in nickel contaminated soil demonstrated metabolic activities mediated via the different heavy-metal resistance genes. Therefore we aimed also on the analysis of heavy-metal resistance genes carried by bacteria occupied this extreme environment. However, we used only degenerative primer set for *ncc*A gene (nickel, cobalt and cadmium resistance) designed only for conserved gene fragments from Gram-negative bacteria (Tab. 1).

49 different *ncc*A-like genes were found in the final output. Corresponding protein sequences were assigned to 15 clusters on phylogenetic tree representing 6 different types with various level of similarity (Fig. 3). Majority of *ncc*A-like genes (36) were after their translation assigned either to cation efflux system protein or heavy metal efflux pump CzcA which pose very similar system of transmembrane metal pumps, one of two basic strategies for a microbe to survive in metal-contaminated environment (NIES 2003). The remaining 13 *ncc*A-like gene products revealed certain homology either to the AcrB/AcrD/AcrF family proteins, or to the two component transcriptional regulator, or to the transcriptional regulator, LysR family, or to the aspartate-semialdehyde dehydrogenase, respectively. These results suggested that such genes could be involved in active protection against heavy metals and also referred to the relatively high degree of variability among resistance determinant products. Similarly, the *czc*+ and/or *ncc*+ strains were detected in a variety of

soil samples highly contaminated by heavy-metals (WUERTZ & MERGEAY 1997; BRIM et al. 1999). However, majority of *ncc*A-like gene products showed only a low level of similarity (40 – 93%) to known proteins encoded by *ncc*A genes (Fig. 3). These sequences may represent new heavy-metal-resistance protein types.

Similarly to bacteria, the products of the resistance determinants from clones and isolates have created autonomous parts of the common phylogenetic tree, expect of three products (EK-I64-hmr, JH-S23-hmr, JH-S44-hmr) of resistance genes either from isolate EK-I64 or clones JH-S23-hmr and JH-S44-hmr, respectively which were found either inside of common group with clone products or inside of common group with isolate products, respectively although all these isolate and clone products were situated on the separate branches of the tree (Fig. 3). It seems that between isolate and clone resistance gene products have not been found any significant sequential similarity, even if (i) all resistance gene products as from isolates as from clones have originated from Gramm-negative bacteria, except of EK-I46-hmr, which was originated from *Arthrobacter chlorophenolicus*, a representant of Gram-positive bacteria (KARELOVÁ et al. 2011) and (ii) the majority of *ncc*A-like gene product from isolates and clones was assigned to very similar system of transmembrane metal pumps, i. e. cation efflux system protein and/or heavy metal efflux pump CzcA, respectively (Fig. 3). In fact, the tested isolates were clustered either to β-Proteobacteria-cluster or γ-Proteobacteria-cluster which were represented either by *Ralstonia* or *Pseudomonads* on phylogenetic tree (KARELOVÁ et al. 2011; REMENÁR et al. 2015). In addition, both, *Ralstonia* and *Pseudomonas* are known to carry heavy-metal-resistance determinants, first of all against nickel, cobalt, zinc and cadmium (MERGEAY et al. 2003; NIES 2003).

Our results exhibit a relatively high degree of bacterial diversity and of variability among resistance determinant products carried by Gram-negative bacteria. A nickel-contaminated soil is able to present very important reservoir for the new and until now partly unknown bacteria, partly heavy-metal-resistance determinants and their products. Microorganisms able to survive in high concentrations of heavy metals are of great interest as bioremediation agents because they can be used in different transformation and immobilization processes.

However, for more realistic study of the bacterial occupation of heavy-metal-contaminated soil and the occurrence of heavy-metal-resistance determinants originated from bacteria in such contaminated environment will be necessary to realize a metagenomic sequencing and/or novel sequencing-based technologies such as single-cell genomics which will uncover cell lineage relationships in more details (SHAPIRO et al. 2013) as well as the function of a single cell inside of indigenous microbial assemblages (MASON et al. 2012).

CONCLUSION

A nickel-contaminated soil presented very specific heavy-metal resistant bacterial community which exhibits important metabolic activities mediated via the different heavy-metal resistance genes carried by bacteria. However, there is a problem how to establish complex bacterial assemblage. Our results suggested that the use of combination on cultivation-dependent and independent approaches could help to resolve this problem. While use of appropriate cultivation techniques enables us to obtain higher numbers not only of previously uncultivable bacteria but also potentially “new” species or genera carrying different heavy-metal resistance genes, the number of bacterial phyla exceeded that obtained from cultured material. However, it is worth mentioning that a metagenomic sequencing and single-cell genomics will be necessary for more realistic study of the bacterial occupation of heavy-metal contaminated soil and the occurrence of heavy-metal resistance determinants originated from bacteria in the environment contaminated as described in the present study. These results also uncover

the advantage to obtain new strains which are specific for particular contaminated sites, are cultivable, and have high pollutant-degradation activity for their possible use in various biotechnologies.

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