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Bioremediation and Metal Resistant Bacteria in A Closed, Cold Northern Mine

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Abstract. Heavy metals, e.g. copper and nickel, are released to the environment as a result of mining activities. Heavy metals are required by most living organisms as trace elements, but in excess they are toxic and cause considerable environmental stress. Microbes have developed different strategies to tolerate otherwise toxic conditions. In the surroundings of closed Kotalahti Mine the concentrations of copper and nickel in the water have earlier been highly elevated. In order to decrease the concentrations of heavy metals and increase water pH, manure sludge was added to the flooded mine pit. This *in situ* bioreactor has operated successfully for 15 years after the treatment. The current concentrations of heavy metals are generally low. Nevertheless, resistance genes for copper (*cop*A), cadmium-nickel (*cnr*A), nickel-cadmium-cobalt (*ncc*A) and cadmium-zink (*czc*A) could still be found in the microbial community of the flooding water.

Introduction

Heavy metals, such as copper and nickel, are released from bedrock environments e.g. as a result of mining activities. Most living organisms require these metals at trace concentrations, but excess concentrations can be toxic and harmful to the environment. Microorganisms have developed different strategies to tolerate otherwise toxic conditions, such as efflux pump systems to remove heavy metal ions from the cell. Such systems are e.g. the *cnr* resistance determinant for cobalt-nickel resistance, the *ncc* for nickel-cobalt-cadmium resistance, the cadmium-zinc efflux pump (*czc*) and the P-type ATPase, e.g. copper transporting ATPase, *cop* [1].

Mining wastes are one of the greatest waste streams in Europe. Only in Finland, the number of operational and closed mines exceeds 1000 [2]. Upon mine closure, the mining pits and quarries generally fill with local groundwater and precipitation forming pit lakes. These young pit lakes develop their own microbial flora, which is influenced by the local geology and the interactions between the water and the bedrock of the pit lake and mine tailings. Exposure to both oxygen and water leads to oxidative dissolution of the remaining minerals from the rock material. Microorganisms are able accelerate the weathering of the rock leading to leaching of metals and sulphate into the pit lake water from the exposed rock surfaces. This decreases the pH of the water while increasing the metal and sulfate concentrations leading to increased risk for contamination of the natural water ecosystems in the vicinity of the mining site [3].

Nevertheless, microorganisms not only leach metals from rock surfaces, but e.g. sulfate reducing bacteria (SRB) may be able to reduce the metal content in the water. SRB were used to precipitate harmful metals and improve water quality in Kotalahti Mine, Finland [4]. After mine closure in 1987 the mine filled with water and reached its maximum water level in 1994 with heavily elevated nickel, copper, iron and sufate concentrations. During 1996-1997 a total of 635 m^3 of pigs manure and AIV-effluent was introduced into the Vehkankuilu shaft of the mine as inoculum of SRB and carbon substrate. A rapid decrease in metal concentration and sulfate was observed, and the water quality was maintained for at least 15 years. The volume of the mine is 3.5 million m^3 and its deepest shafts reach a depth of 800 m.

The bacterial community in the water of Kotalahti Mine is diverse and vary between different parts of the mine in response to physicochemical parameters. While most of the detected bacterial lineages belong to Proteobacteria, less than 1% of the bacterial community are SRB [5]. In the

present study, we aimed to investigate the metal resistance strategies of the microbial community in more detail by specifically targeting the *cop*, *cnr* and *ncc* resistance determinants for P-type ATPAse for copper, cobalt-nickel and nickel-cobalt-cadmium resistance, respectively.

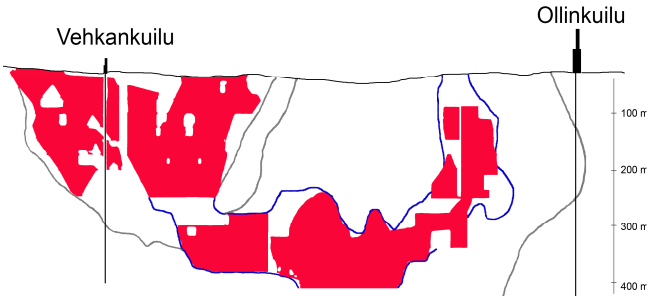

Figure 1. Schematic partial cross section of Kotalahti Mine. The red areas show the excavated parts of the mine, the blue lines outline the ore body and the grey lines the mafic and ultramafic complex. The shafts are situated approximately 1 km from each other. Modified from [4, 6].

Table 1. Groundwater measurements from Vehkankuilu (VK) and Ollinkuilu (OK) samples.

	VK 10m	VK 30m	VK 70m	VK 100m	OK 10m	OK 30m	OK 70m	OK 100m
T°C	5.70	5.70	5.60	5.6	7.7	6.90	6.5	6.9
O_2 mg/L	0	0.34	0.84	0.48	0	0.19	0.13	0
O_2 %	0	2.70	6.70	3.8	0	1.6	1.1	0
pH	7.4	7.4	7	7	9.80	9.80	9.0	9.80
Tot N mg/L	0.16	0.16	0.42	0.39	4.7	4.7	4.7	4.7
Tot P µg/L	31	32	9	9	76	86	46	58
SO_4 mg/L	86	85	480	500	1.1	1.2	<0.5	0.54
Fe mg/L	1	1	1	1	45	61	19	25
Cu µg/L	1.50	1.80	1.10	1.10	17	29	6.60	3.4
Ni µg/L	180	180	1700	1800	290	410	79	140

Materials and methods

The samples used in this study originated from two shafts of the flooded closed Kotalahti Mine (Figure 1), the Vehkankuilu shaft and the Ollinkuilu shaft [5]. Both shaft was sampled from 4 depths (10 m, 30 m, 70 m and 100 m), and two samples (1×100 mL and 1×50 mL) were obtained from each depth. The microbial biomass of the water samples was concentrated on Sterivex filter units and the DNA was extracted using the MoBio PowerWater Sterivex kit [5]. For detection of metal resistance genes in the microbial communities of the mineshaft water the primers *cop*A for copper, *cnr*A3 for cobalt-nickel and *ncc*A for cobalt-nickel-cadmium resistance were used as described by [7]. PCR reactions were prepared in 25 µL reactions using the 2×MiTaq ready mix with appropriate primers and 1 µL of 1:10 diluted DNA extract. The PCR amplification products were purified and cloned using the TOPO TA cloning kit (Invitrogen) using parallel Luria agar plates containing 50µg/mL Kanamycin for each transformation, according to the manufacturer's instructions. Clone libraries were screened by colony PCR of 47 colonies per sample using the M13F and M13R primers. From each gene, 12 clones were chosen for sequencing from each replicate sample at Macrogen Inc., Korea, using the T7 promoter primer. Phylogenetic analyses were performed with GENEIOUS PRO (Biomatters Ltd, New Zeeland). The bacterial community composition of the two deepest samples were analysed by 454 high throughput (HTP) sequencing of the bacterial 16S rRNA genes as described in [8]. The HTP sequence data was analysed using the Mothur software, v 1.34.2 [9] where the sequences were quality checked, adapter sequences and barcodes removed, aligned using the Silva v119 alignment as template [10], clustered into operational taxonomic units (OTUs) according the their sequence similarities and identified using the Silva v119 taxonomy database.

Results and Discussion

Genes for copper (*cop*A) and cadmium-nickel (*cnr*A) resistance were found from both Vehkankuilu and Ollinkuilu shaft water, but nickel-cadmium-cobalt (*ncc*A) resistance genes only from Vehkankuilu (Figure 2). However, the *cnr*A specific primers also detected cadmium-zinc efflux pump (*czc*A) genes in Vehkankuilu. The Cu concentration in the water samples was generally low, with the highest concentration of Cu found in Ollinkuilu at 30 m depth (Table 1). It is thus not surprising that only few *cop*A gene sequences were detected. However, these sequences were not derived from the samples with the highest Cu concentration. *cnr*A genes were obtained from all samples. The different types of *cnr*A genes were distributed among the different samples according to the concentration of Ni detected. In addition, the different shafts had unique *cnr*A gene profiles distinct from each other. *ncc*A genes were restricted to only Vehkankuilu.

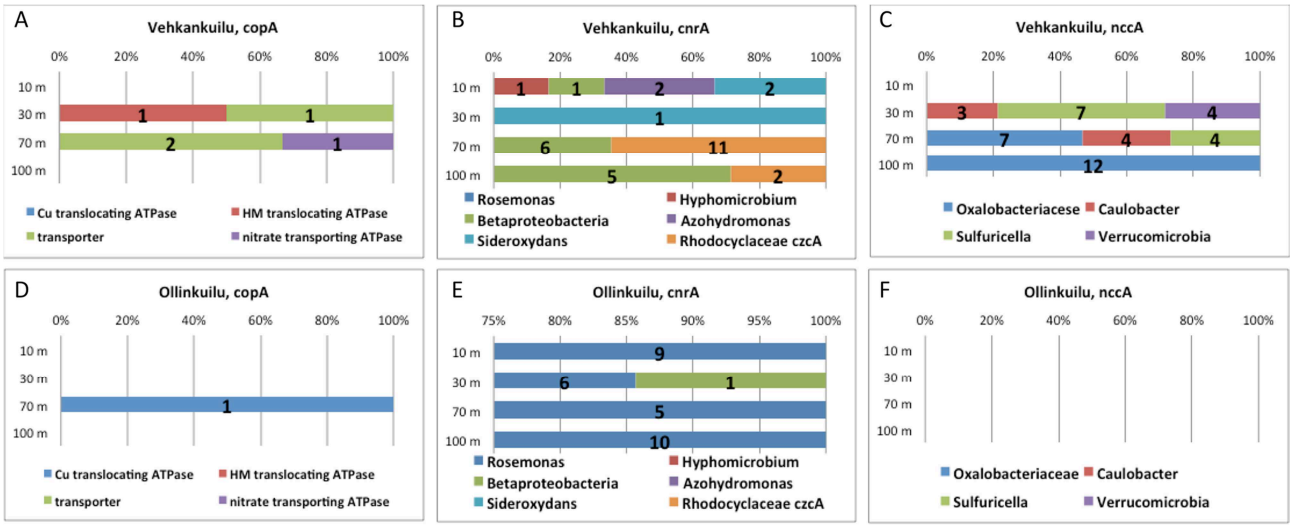

Figure 2. Types of metal resistance genes identified from Vehkankuilu (A-C) and Ollinkuilu (D-F). A and D present genes for enzyme types, B-C and E-F present taxonomic similarities of genes of known bacterial taxa. The number in the chart series indicate the number of clones belonging to each group out of 47 tested clones/sample and gene. The sequences have been submitted to ENA (https://www.ebi.ac.uk/ena) under Webin ID Hx2000047974.

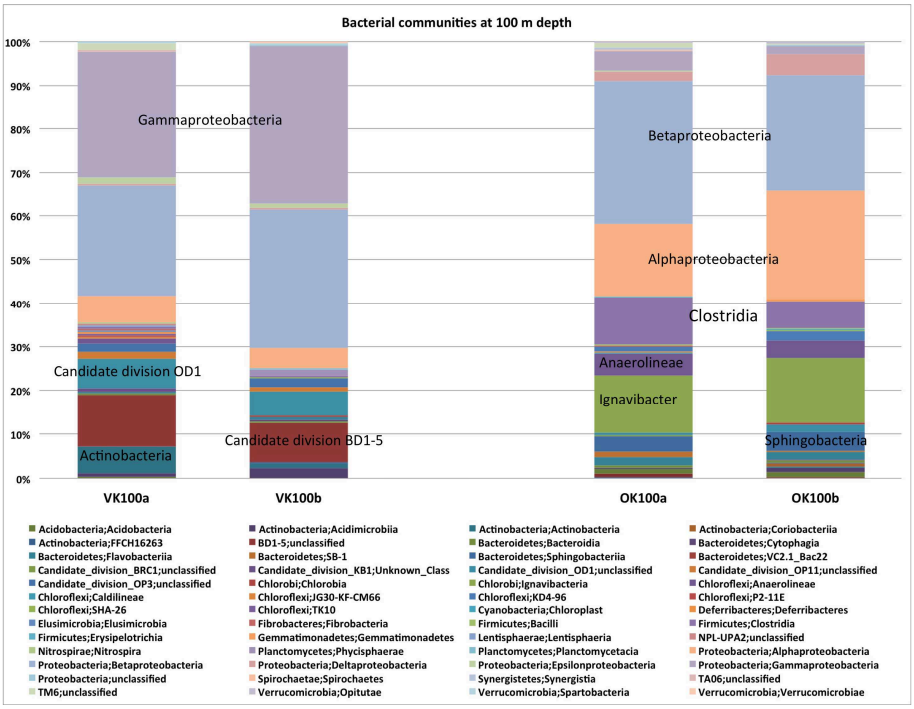

Figure 3. The bacterial community at 100 m in Vehkankuilu (VK) and Ollinkuilu (OK) shafts resolved by 454 pyrosequencing from two parallel samples. The sequences have been submitted to ENA (https://www.ebi.ac.uk/ena) under accession numbers ERS739677-ERS739680)

The bacterial community at 100 m depth of each shaft was diverse and different between the shafts (Figure 3). In

Vehkankuilu the major bacterial groups belonged to Gamma- and Betaprotepbacteria, Actinobacteria and Candidate divisions (Cd) OD1 and BD1-5. In Ollinkuilu the major bacterial groups belonged to Beta- and Alphaproteobacteria, Clostridia, Anaerolineae, Ignavibacter and Sphingobacteria. In addition many other minor groups were present in both shafts. In accordance with [5], only few SRB taxa were detected. Nevertheless, from a depollution point of view, many species of e.g. Gammaproteobacteria have been shown to exert heavy metal resistance [7, 11]. However, the role of the so far uncultured and uncharacterized bacterial groups, such as the Cd OD1 or BD1-5, in AMD environments or detoxification of polluted water is not yet known. The different heavy metal resistance determinants found in the bacterial communities indicate a potential for adaptation to changing environmental condition, such as increased heavy metal stress. Heavy metal resistance genes are often found on mobile genetic elements, such as plasmids [1], which may have the capacity to transfer between microbial species and thus add to the adaptation capacity of the microbial community. Such mobile elements present new opportunities for biotechnological applications, such as metal recovery or removal from industrial and mining waters.

Conclusions

A great bacterial diversity was observed in the water of closed Kotalahti Mine and only a minority of the bacteria were SRB. Many bacterial taxa represented yet uncultured and uncharacterized groups, whose role in AMD and detoxification of polluted sites is still not known. Heavy metal resistance determinants *cop*, *cnr* and *ncc* were detected indicating adaptability of the microbial community to heavy metal stress. Nevertheless, these genes were found in water with only low heavy metal concentration and were even absent at higher concentrations of heavy metals, for example no *cop*A were detected in Ollinkuilu water, which had the highest copper concentrations. The heavy metal resistance determinants searched for here are only a few of the many possible systems employed by natural bacterial communities. The presence of specific genes is probably also determined by the species composition of the microbial community as well as the level of heavy metal stress exerted on the microbial communities. For a complete study of heavy metal resistance in microbial communities in closed mine environments many more determinants should be searched for by e.g. isolating new heavy metal resistant microbial strains, targeting new heavy metal resistance genes by PCR and by metagenomic analysis of microbial communities in heavy metal contaminated environments.

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