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Research article

Interactions of the metal tolerant heterotrophic microorganisms and iron oxidizing autotrophic bacteria from sulphidic mine environment during bioleaching experiments

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ABSTRACT

Iron and sulfur oxidizing chemolithoautotrophic acidophilic bacteria, such as *Acidithiobacillus* species, hold the dominant role in mine environments characterized by low pH values and high concentrations of reduced sulfur and iron compounds, such as ores, rocks and acid drainage waters from mines. On the other hand, heterotrophic microorganisms, especially their biofilms, from these specific niches are receiving increased attention, but their potential eco-physiological roles have not been fully understood. Biofilms are considered a threat to human health, but biofilms also have beneficial properties as they are deployed in waste recycling and bioremediation systems. We have analyzed interactions of the metal tolerant heterotrophic microorganisms in biofilms with iron oxidizing autotrophic bacteria both from the sulphidic mine environment (copper mine Bor, Serbia). High tolerance to Cu^{2+}, Cd^{2+} and Cr^{6+} and the presence of genetic determinants for the respective metal tolerance and biofilm-forming ability was shown for indigenous heterotrophic bacteria that included strains of *Staphylococcus* and *Rhodococcus*. Two well characterized bacteria- *Pseudomonas aeruginosa* PAO1 (known biofilm former) and *Cupriavidus metallidurans* CH34 (known metal resistant representative) were also included in the study. The interaction and survivability of autotrophic iron oxidizing *Acidithiobacillus* bacteria and biofilms of heterotrophic bacteria during co-cultivation was revealed. Finally, the effect of heterotrophic biofilms on bioleaching process with indigenous iron oxidizing *Acidithiobacillus* species was shown not to be inhibitory under in vitro conditions.

1. Introduction

Mining processes are producing large amounts of waste materials still containing high amounts of valuable and in some cases toxic metals, that are usually deposited as waste dumps or tailings near mine areas (Schippers et al., 2000). Bioleaching is often a method of choice for remediation of heavy metal contaminations (Hashim et al., 2011). Iron and sulfur oxidizing chemolithoautotrophic acidophilic bacteria, such as *Acidithiobacillus* species, have the dominant role in bioleaching (Schippers et al., 2014). Environments characterized by low pH values and high concentrations of reduced sulfur and iron compounds, such as ores, rocks and acid drainage waters from mines, represent prime niches of iron oxidizing *Acidithiobacillus* species (Kelly and Harrison, 1989). Most of the research on the microbiology of mines and mine tailings is focused mainly on this group of bacteria and their role in these ecological niches. Still, mine environments are inhabited with other indigenous bacteria that also have specific ecological roles. The evidence of the existence of heterotrophs in mines usually comes from the metagenomic studies (Bajkic et al., 2013; He et al.,

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2007; Islam and Sar, 2011). However, metabolic potential and ecological roles of this group of microorganisms has not been examined. Relationships of autotrophs and heterotrophs are reciprocal: iron- and sulfur-oxidizing acidophiles – primary producers in the mine – provide necessary organic compounds to heterotrophic iron oxidizers, while heterotrophs utilize lysates and exudates produced by the autotrophs and remove organic substances toxic to certain autotrophs (Hallberg et al., 2006; Okibe and Johnson, 2004). Heterotrophic microorganisms from these specific niches are receiving increased attention and it was evident from the recent study that microorganisms present in the mine samples are often in the form of biofilms (Johnson, 2014).

Large copper mining and smelting complex Bor, Serbia has been focus of our studies for a while (Beškoski et al., 2009; Savić et al., 1998). In our previous work, both acidophile autotrophs and neutrophile heterotrophs from samples within copper mine Bor, have been isolated offering evidence for their cohabitation in this environment (Avdalović et al., 2015; Bajkic et al., 2013). Isolated neutrophile heterotrophic bacteria from the acidic mine pits of copper mine Bor were highly tolerant to selection of heavy metals (Bajkic et al., 2013). Tolerance of indigenous bacteria to heavy metals was not surprising as mining activities result in the release of large amounts of heavy metals into the environment (Passariello et al., 2002). Given that biofilms, compared to planktonic cultures, exhibit increased tolerance to unfavorable environmental impacts (Harrison et al., 2007) it is not surprising that heterotrophic microorganisms present in the mine samples are often found in the form of biofilms and mats (Ghauri et al., 2007; Hallberg et al., 2006; Johnson, 2014). These biofilms are utilized as biomarkers for monitoring of stream water quality for instance from mine drainage and more recently have been examined for heavy metal adsorption properties (Edwards and Kjellerup, 2013). Biofilm-based bioremediation systems such as biofilters and aerobic and anaerobic granular sludge reactors are widely used today (Leear, 2016). For this reason, an in-depth understanding of biofilm formation and of the specific processes that occur inside biofilms, is mandatory to improve bio-processes depending on microorganisms. Biofilm formation is an innate defense mechanism against metal toxicity and the way in which heterotrophic bacteria biofilms interact with heavy metals and influence natural bioleaching process remains to be clarified. Therefore, the possible effect of heterotrophic heavy metal tolerant bacterial biofilms on bioleaching process with indigenous iron oxidizing *Acidithiobacillus* species was the main aim of this research. We have analyzed interaction and survivability of autotrophic iron oxidizing *Acidithiobacillus* spp. bacteria and biofilms of heterotrophic bacteria during co-cultivation. In the present study, beside indigenous heterotrophic bacteria, we included two well characterized strains: *Pseudomonas aeruginosa* PAO1 (known biofilm former) and *Cupriavidus metallidurans* CH34 (known metal resistant representative). The presence of genetic determinants conferring metal tolerance within this selection of heterotrophic microorganisms was studied and their ability to form biofilms as single cultures and in defined consortia in the presence of metals was quantified.

2. Materials and methods

2.1. Localities for isolation of microorganisms

Microorganisms were isolated from solid and liquid samples taken from copper mine Bor. Two liquid and three solid samples were collected from copper sulphide mine wastewater Lake Robule in Bor, Serbia (44°3.858′N; 22°8.221′E) (Avdalović et al., 2015) as well as two sediment samples from underground mine pit in Bor (44°04.884′N; 022°05.915′E) (Bajkic et al., 2013).

2.2. Microbiological growth media

For isolation of heterotrophic microorganisms from Bor mine sediments minimal TG agar medium (pH 6) containing soil extract and glucose as carbon source were used (Sørheim et al., 1989). For enumeration, characterization and isolation of microorganisms from solid and liquid samples from copper sulphide mine wastewater (Lake Robule) in Bor, Serbia, the following media were used: 9K media (pH 2.5) for isolation and enumeration of iron oxidizing *Acidithiobacillus* sp.: $FeSO_4 \times 7H_2O$ (44.8 g L^{-1}), $(NH_4)_2SO_4$ (3.0 g L^{-1}), K_2HPO_4 (0.5 g L^{-1}), $MgSO_4 \times 7H_2O$ (0.5 g L^{-1}), KCl (0.1 g L^{-1}), $Ca(NO_3)_2 \times 4H_2O$ (0.01 g L^{-1}) H_2SO_4 (0.5 g L^{-1}) (Silverman and Lundgren, 1959); Rodine media (pH 5.0) for enumeration of total sulfure oxidizing bacteria: 10 g L^{-1} $Na_2S_2O_3 \times 5H_2O$, 2 g L^{-1} NH_4Cl, 3 g L^{-1} K_2HPO_4, 0.5 g L^{-1} $MgCl_2 \times 6H_2O$, 0.2 g L^{-1} $CaCl_2$ (Rodina, 1965); Nutrient agar (Sigma-Aldrich) (pH 7.3) was used for enumeration of total aerobic and facultative anaerobic mesophilic chemoorganotrophic bacteria; Malt agar (Sigma-Aldrich) (pH 5.4) was used for enumeration of total yeast and molds.

2.3. Strain selection

2.3.1. Heterotrophic strains

Heterotrophic bacterial strains were isolated from Bor mine sediments, and their growth capability on solid medium was assessed in the presence of elevated concentrations of heavy metals (Bajkic et al., 2013). Based on the growth profiles and high metal tolerance two strains, namely *Staphylococcus* sp. MSI08 and *Staphylococcus* sp. MUI10, were selected for this study. Based on the same criteria, an additional strain from the laboratory collection, *Rhodoccocus* sp. TN113, able to grow in the presence of 100 mM Cu^{2+} on solid medium (Narancic, 2012) was also included. All strains belong to the in-house culture collection of the Laboratory for Molecular Genetic and Ecology of Microorganisms (IMGGE, University of Belgrade, Serbia) and are available upon request. *C. metallidurans* CH34 (ATCC 43123), well known for the presence of a number of genes responsible for tolerance to heavy metals (Monsieurs et al., 2011) and *P. aeruginosa* PAO1 (ATCC 15692), known for the ability to form persistent biofilms (Cotton et al., 2009) were included as defined and well characterized reference strains. During this study a defined bacterial consortium composed of all five strains was also utilized.

2.3.2. Autotrophic strains

For leaching studies, and interaction with heterotrophic microorganisms experiments, iron oxidizing *Acidithiobacillus* sp. B2 that has previously been isolated and taxonomically identified from water samples taken from Lake Robule, from the copper mine Bor, Serbia, was utilized and routinely grown and maintained on 9K medium (Avdalović et al., 2015).

2.4. Growth of selected heterotrophic bacterial strains in the presence of heavy metals

Heavy metal tolerance of selected strains was tested using metal toxicity (MT) medium (Sani et al., 2001), containing: 5.1 g L^{-1} sodium lactate, 2.1 g L^{-1} Na_2SO_4, 0.06 g L^{-1} anhydrous $CaCl_2$, 1 g L^{-1} NH_4Cl, 1 g L^{-1} $MgSO_4$, 0.05 g L^{-1} yeast extract, 0.5 g L^{-1} tryptone and 10.9 g L^{-1} 1.4-piperazinediethanesulfonic acid (PIPES), pH 7. To prepare solid MT medium, high purity agar (Becton, Dickinson and Company, Franklin Lakes, USA) was added to a final concentration of 15 g L^{-1}. Tolerance was tested in the presence of cadmium (Cd^{2+}), chromium (Cr^{6+}) and copper (Cu^{2+}), added to the MT medium in the form of the following salts: $3CdSO_4 \times 8H_2O$,