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Frequencies of heavy metal resistance are associated with land cover type in the Upper Mississippi River

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HIGHLIGHTS

- Resistance to Cd, Cr, and Mn was associated with developed land cover.
- Resistance to Hg and Zn was associated with forested land cover.
- Nutrient concentrations and bacterial orders were poorly related to resistance.
- Bacterial resistance to Cd^{2+}, Cr^{3+}, Cu^{2+}, Hg^{2+}, Mn^{2+}, and Zn^{2+} was low.
- Land cover type is a main driver of heavy metal resistance patterns.

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ABSTRACT

Taxonomic compositions of freshwater bacterial communities have been well-characterized via metagenomic and amplicon-based approaches, especially next-generation sequencing. However, functional diversity of these communities remains less well-studied. Various anthropogenic sources are known to impact the bacterial community composition in freshwater riverine systems and potentially alter functional diversity. In this study, high-throughput functional screening of large (~10,000 clones) fosmid libraries representing communities in the Upper Mississippi River revealed low frequencies of resistance to heavy metals in the following order: $Mn^{2+} > Cr^{3+} > Zn^{2+} > Cd^{2+} > Hg^{2+}$. No resistance to Cu^{2+} was detected. Significant, but weak, correlations were observed between resistance frequencies of Cd and Cr with developed land cover ($r^2 = 0.08$, $P = 0.016$ and $r = 0.07$, $P = 0.037$, respectively). While discriminant function analyses further supported these associations, redundancy analysis further indicated associations with forested land cover and greater resistance to Hg and Zn. Nutrient and metal ion concentrations and abundances of bacterial orders were poorly correlated with heavy metal resistance, except for an association of *Pseudomonadales* abundance and resistance to Hg and Zn. Taken together, results of this study suggest that allochthonous bacteria contributed from specific land cover types influence the patterns of metal resistance throughout this river.

1. Introduction

Over the last several decades, many studies have assessed the sensitivity and tolerance of environmental microbial communities to heavy metals in water, soils, and the rhizosphere (Hassen et al., 1998; Abou-Shanab et al., 2007). It has been well-established that high concentrations of heavy metal pollutants in the environment are associated with declines in bacterial diversity, particularly among rare taxa (Gans et al., 2005; Ancion et al., 2010; Hemme et al., 2010). However, not all metals are equally toxic, and several (e.g., Mn, Fe, Mo, and Zn) are known to be critical for cellular functions (Seiler and Berendonk, 2012), while others (e.g., Cd, Al, and Hg) are known to form highly toxic complexes (Nies, 1999). Nevertheless, even biologically important trace elements can be toxic at elevated concentrations. Furthermore, environmental conditions, such as pH, that affect the valence state and solubility of metal ions are also important factors affecting metal toxicity (Nies, 1999; Seiler and Berendonk, 2012).

Taxonomic structures in bacterial communities in lotic systems (rivers and streams) in both the water and sediment have been shown to be influenced by surrounding land cover type, resulting from both the

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introduction of non-indigenous bacteria as well as other pollutants that cause variations in community structure (Wang et al., 2011; Gibbons et al., 2014; Staley et al., 2014a). Heavy metals are among the contaminants contributed to the environment resulting from a variety of anthropogenic practices, including agriculture (Han et al., 2000), aquaculture (Burridge et al., 2010), and the discharge of industrial and municipal effluents (Ahluwalia and Goyal, 2007). A recent study also indicated that bacterial biofilms can capture and retain these metals and potentially transfer them to higher trophic levels, representing a concern for human health (Ancion et al., 2010).

In general, mechanisms of heavy metal resistance fall into one of three types. Heavy metal ions may be incorporated into complexes for sequestration, toxicity of intracellular ions may be lessened by reduction of metal ions to less toxic valence states, and/or toxic ions may be removed from the cell via efflux pumps (Nies, 2003; Seiler and Berendonk, 2012). Several bacteria, such as *Ralstonia metallidurans*, have naturally adapted mechanisms for chromosomally-encoded heavy metal resistance to survive in highly metal-rich habitats (Mergeay et al., 2003), and the functions and distribution of these genes have been well reviewed (Silver and Phung, 1996; Nies, 2003). However, anthropogenic pollution, in the form of increased heavy metal ion concentrations or other pollutants, imposes a selective pressure in favor of these resistance mechanisms and has resulted in their incorporation onto mobile genetic elements (e.g., plasmids and conjugative transposons), enabling horizontal gene transfer (Silver and Phung, 1996; Nies, 2003). Of particular concern is the spread of resistance mechanisms that also confer resistance to other antimicrobial compounds, including antibiotics (Chapman, 2003; Nies, 2003) which are often linked.

Monitoring of the microbial community has been proposed over the last several decades as a biological indicator of soil health (Pankhurst et al., 1995; Yakovchenko et al., 1996), with some suggesting that bacterial community responses may be detectable in advance of detectable changes in abiotic, edaphic parameters (Pankhurst et al., 1995). Similarly, in a recent study we have shown that changes in the structure of the microbial community in the Upper Mississippi River may be indicative of more subtle variations in chemical contaminants as a result of anthropogenic practices (Staley et al., 2014a). However, a number of studies have suggested that bacterial functional responses, rather than taxonomic community structure, are better indicators of perturbation (Comte and del Giorgio, 2009; Burke et al., 2011; Steffen et al., 2012). In response specifically to metal contamination, for example, an earlier study indicated that Cu contamination resulted in a decrease in photosynthetic potential among a phototrophic community (Massieux et al., 2004). More recently, in the Upper Mississippi River, we have found that while the distribution of functional traits is highly conserved, slight but significant variations in the distribution of functional traits are also linked with surrounding land cover using inference-based and whole genome shotgun approaches (Staley et al., 2014b).

Culture-dependent methods have been suggested to better represent the physiological state of bacteria than culture-independent methods (Ellis et al., 2003), due to discrepancies between genes present versus those expressed in the environment. Functional screening of large, metagenomic fosmid libraries has been recently proposed as a method by which to characterize functional traits in a microbial community (Martínez and Osburne, 2013), while at the same time circumventing the lack of culturability of >99% of bacterial species in the laboratory (Amann et al., 1995). This method has been recently used to characterize antibiotic resistance frequencies in river sediments (Amos et al., 2014).

In the current study, we performed functional metagenomic screening of fosmid libraries constructed from water samples collected throughout the Upper Mississippi River in 2011 and 2012 to determine frequencies of resistance to the metals Cd^{2+}, Cr^{3+}, Cu^{2+}, Hg^{2+}, Mn^{2+}, and Zn^{2+}. We previously taxonomically characterized the bacterial community at these sites (Staley et al., 2014a), and we hypothesize that factors influencing taxonomic community structure, such as total carbon and nitrite/nitrate concentrations, as well as major land cover types, will also be associated with resistances to specific metals. Results of this study elucidate the interrelationships between physicochemical parameters, taxonomic variation, and resistances of environmental communities to heavy metals in a major riverine ecosystem.

2. Materials and methods

2.1. Sample collection and processing

Surface water samples (40 l) were collected from the shore in sterile, 20 l carboys from 11 sites along the Upper Mississippi River in Minnesota and major contributing rivers from near the headwaters at Lake Itasca to the southern border near La Crescent, MN, as previously described (Staley et al., 2014a) (Supplementary Fig. S1). Water samples were transported to the lab and either processed immediately or stored at 15 °C overnight and processed the following day. Samples were strained through sterile cheesecloth and filtered through a P5 pre-filter (Whatman Inc., Piscataway, NJ) to remove aggregate bacteria prior to concentrating larger, planktonic bacterial cells on a 0.45-μm-pore-size polyethanesulfonate filter as described previously (Staley et al., 2014a). We have previously reported that this pore size was necessary to efficiently filter this large volume of water (Staley et al., 2013). Cells were elutriated from filters by vortexing in pyrophosphate buffer (0.1% sodium pyrophosphate, 0.2% Tween 20, pH 7) and cell pellets, six per site, representing approximately 6–7 l of water, were stored at −80 °C until used.

2.2. Fosmid library preparation

One cell pellet per site, per year was shipped on dry ice to the Clemson University Genomics Institute (CUGI) [http://www.genome.clemson.edu/] where DNA extraction and fosmid library construction were performed. Briefly, DNA from each of the pellets was extracted using the Metagenomic DNA Isolation Kit for Water (Epicentre Biotechnologies, Madison, WI) followed by end-repair/phosphorylation according to the manufacturer's instructions. DNA fragments between 35 and 50 kb were size selected by pulsed-field gel electrophoresis and were subsequently ligated into pCC2FOS (Epicentre Biotechnologies). Ligated fosmids were transferred into *Escherichia coli* DH10B. Fosmid libraries for each site contained a minimum of 50,000 clones and were shipped back to the laboratory on dry ice as glycerol stocks.

Stock fosmid libraries were diluted to 2.5 CFU μl^{-1} and 1 ml aliquots were plated on 20 × 20 cm Luria Bertani (LB) agar plates containing 12.5 μg ml^{-1} chloramphenicol. Colonies (approximately 10,000 per library, Table 1) were transferred to 384-well plates containing Hogness modified freezing media (HMFM) (Yan et al., 2007) with 12.5 μg ml^{-1} chloramphenicol using the Qbot colony-picking robot (Genetix, Sunnyvale, CA). Fosmid libraries were stored at −80 °C.

2.3. Heavy metal resistance screening

Six metals (Cd^{2+}, Cr^{3+}, Cu^{2+}, Hg^{2+}, Mn^{2+}, and Zn^{2+}) were selected for metal resistance screening based on a wide range of expected toxicities and suspected presence in environmental samples. Chloride salts were used to make stock solutions at the following concentrations: 0.1 M $CdCl_2$, 1 M $CrCl_3$, 1 M $CuCl_2$, 0.1 M $HgCl_2$, 2 M $MnCl_2$, and 1 M $ZnCl_2$. Stock solutions were made fresh weekly and filter-sterilized through a 0.22 μm pore-size filter. Inhibitory metal concentrations were experimentally determined by plating a control strain of *E. coli* DH10B containing a fosmid without insert, grown overnight in LB supplemented with 7 μg ml^{-1} chloramphenicol, in triplicate over a range of concentrations from 0.050 mM to 20 mM (at 0.05 mM increments). The inhibitory concentration was selected as the lowest concentration which completely inhibited growth of all three replicates. Nutrient agar plates containing 7 μg ml^{-1} chloramphenicol were used for all plating, and inhibitory concentrations were determined as 0.25 mM Cd, 2 mM Cr, 1 mM Cu, 0.075 mM Hg, 20 mM Mn, and 0.50 mM Zn.