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Long-term Hg pollution-induced structural shifts of bacterial community in the terrestrial isopod (*Porcellio scaber*) gut

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Chronic environmental mercury pollution induces bacterial community shifts and presence of elevated number as well as increased diversity of Hg-resistant bacteria in guts of isopods.

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ABSTRACT

In previous studies we detected lower species richness and lower Hg sensitivity of the bacteria present in egested guts of *Porcellio scaber* (Crustacea, Isopoda) from chronically Hg polluted than from unpolluted environment. Basis for such results were further investigated by sequencing of 16S rRNA genes of mercury-resistant (Hg^r) isolates and clone libraries. We observed up to 385 times higher numbers of Hg^r bacteria in guts of animals from polluted than from unpolluted environment. The majority of Hg^r strains contained *merA* genes. Sequencing of 16S rRNA clones from egested guts of animals from Hg-polluted environments showed elevated number of bacteria from *Pseudomonas*, *Listeria* and *Bacteroidetes* relatives groups. In animals from pristine environment number of bacteria from *Achromobacter relatives*, *Alcaligenes*, *Paracoccus*, *Ochrobactrum relatives*, *Rhizobium/Agrobacterium*, *Bacillus and Microbacterium* groups were elevated. Such bacterial community shifts in guts of animals from Hg-polluted environment could significantly contribute to *P. scaber* Hg tolerance.

1. Introduction

Large quantities of different metal ions are introduced into the environment by industrialization and waste disposal, and affect all groups of organisms and ecosystem processes, including those mediated microbially (Babich and Stotzky, 1985; Baath, 1989; Giller et al., 1998). The effects of mercury on biota are among the most difficult to study as a result of factors such as mercury bioaccumulation, transformation and speciation (Nies, 1999; Barkay et al., 2003).

Mercury in the soil alters the genetic structure and functional diversity of bacterial communities as evidenced by cultivation, substrate utilization patterns, phospholipid fatty acid analysis, denaturing gradient gel electrophoresis (DGGE), 16S rRNA gene libraries, ribosomal intergenic spacer pattern analysis, measurements of enzyme activity and utilization of multiple carbon sources (Ranjard et al., 1997, 2000; Ellis et al., 2001, 2003; Müller et al., 2001; Rasmussen and Sorensen, 2001; Feris et al., 2003, 2004). It is well known that soil microbial activity and functional diversity are driven by activity of detritivorous animals (Drake and Horn, 2007) and the process of gut passage is known to substantially increase numbers of soil microbes in the ingested organic matter and thus make a major contribution to cycling of soil nutrients (Brown, 1995; Drake and Horn, 2007). When pollution affects detritivorous animals, it results in perturbation of nutrient cycling in the soil ecosystem (Van Gestel and Van Straalen, 1994).

In this study, the terrestrial isopod *Porcellio scaber* (Crustacea) was used as a model detritivorous organism. *P. scaber* survives well in polluted environments, and its absence in polluted sites is not a limiting factor for such studies (Hopkin, 1989). In addition, the physiology and anatomy of *P. scaber* have been studied extensively; *P. scaber* is one of the terrestrial organisms most frequently used in ecotoxicity studies. Its gut is a well-defined micro-environment which, with its small size and simple anatomy, facilitates sampling (Drobne et al., 2002).

When *P. scaber* is exposed to an environment polluted with mercury, its gut bacterial community changes (Lapanje et al., 2007, 2008). If the pollution is chronic, the *P. scaber* gut bacterial community may develop a pollution-induced community tolerance (PICT) (Lapanje et al., 2008). However, not all members of the gut bacterial community tolerate Hg and this could result in differences in bacterial species richness between animals from Hg-polluted

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and pristine environments (Lapanje et al., 2007). In previous investigations we have been unable to assess reasons for the lower bacterial species richness and high mercury tolerance of community in animals from chronically Hg-polluted environment. We speculate that the reasons for gut bacterial community tolerance might lie in elevated populations as well as in increased diversity of Hg-resistant strains in the guts of animals from the Hg-polluted environment.

Mercury-resistant strains in most cases utilize *mer* operon translated proteins in order to detoxify Hg^{2+} ions and organic compounds containing mercury. Mercury ions are detoxified by reduction of Hg^{2+} to Hg^0 by MerA reductase (Summers, 1986). In Hg-resistant bacteria Hg^0 evaporates from the bacterial cells (Barkay et al., 2003) and the mercury content of the environment containing Hg-resistant bacteria becomes progressively lower (Wagner-Dobler et al., 2000). If present, mercury-resistant bacteria in the isopod gut could function as a source of resistant determinants such as *mer* operon and so contribute to the tolerance of the entire bacterial community (Summers, 1986; Silver, 1996; Osborn et al., 1997). Lowering of the mercury concentration in the gut environment by these bacteria (e.g. Wagner-Dobler et al., 2000) might contribute to the animal tolerance of high Hg concentrations in the food. Consequently, whole animal tolerance to Hg, as previously observed by Nolde et al. (2006) and Lapanje et al. (2008), might be the outcome of bacterial and animal tolerance separately.

In this investigation using culture dependent and independent approaches we aimed to (i) show long-term Hg pollution effects on structural shifts of the bacterial community in *P. scaber* guts and (ii) provide evidence of bacterial community tolerance for long-term Hg environmental pollution in animal gut. Bacterial community tolerance is indicated by higher diversity and abundance of mercury-resistant genotypes among cultured bacteria isolated from animal gut.

2. Methods

2.1. Collection of environmental samples

One of the world's largest mercury mines operated in Idrija, Slovenia and with 500 years of documented mining history, this was selected as a contaminated environment. As a control, we chose a non-contaminated area in Radlek, 60 km from Idrija, which has never been the subject of reports of any kind of pollution. At the Radlek site, the level of total Hg determined in soil samples (horizon Ah – mineral horizon near the soil surface enriched with organic matter) was 0.193 μg/g (SD = 0.005, $N = 3$) and in Idrija it was 263 μg/g (SD = 8.528, $N = 3$) (Lapanje et al., 2008).

At each location we collected: (i) 30 isopods belonging to *P. scaber* species, (ii) a food sample (Of horizon – least decomposed organic layer of the soil), which was soil mixed with partially decomposed plant material in the immediate proximity of the animals, and (iii) soil sample (Ah soil horizon) taken from the immediate proximity, within 0.5 m of the location of *P. scaber* colony. After transfer to the laboratory, the soil and food samples were immediately processed to cultivate bacteria on agar plates. From both locations four samples were included in further cultivation of bacteria: soil, food, egested gut and full gut.

The animals were divided into two groups of 15 animals each and guts were aseptically isolated as described previously (Drobne et al., 2002). The first group of animals, from which the guts were isolated immediately, represented a sample of full guts ($n = 15$). The second group was deprived of food for the next 24 h and represented an empty gut sample ($n = 15$). Out of the fifteen guts per group, ten were pooled together and stored at −20 °C in sterile phosphate buffer (0.12 M, pH 8.0) prior to isolation of DNA. The remaining five guts were each separately resuspended in 0.9% NaCl and macerated. Ten-fold dilutions of suspension were put on agar plates to obtain culturable bacteria. Soil samples (Of and Ah horizons) were suspended in 0.9% NaCl and diluted up to 10^{10} times in prior to obtain separated colonies on Hg amended nutrient agar (NA) medium after the incubation.

2.2. Construction of 16S RNA clone library directly amplified from empty guts

The same clone library reported previously (Lapanje et al., 2008) was used in the analysis. We isolated DNA from ten empty guts (see above) from animals from each of two locations by SmartHelix DNA Isolation Kit (IFB d.o.o., Grosuplje, Slovenia). The isolated DNA was aliquoted and stored at −80 °C. 16S rRNA genes were amplified and ligated into pGem-T easy vector (Promega) and ligated plasmids were used for transformation of JM109 *Escherichia coli* cells. Two-hundred clones from either Idrija or Radlek were randomly selected from one agar ampicillin plate. Up to 3 clones with same restriction fragment length polymorphism (RFLP) profiles of 16S rRNA genes were selected for sequencing. Differences in RFLP were detected with GelProAnalyser software (Media Cybernetics, Maryland, USA). The UPGMA (Unweighted pair group method with arithmetic mean) tree was built on the basis of a discrete matrix of presence or absence of bands in agarose gels.

The phylogenetic affiliation of each clone group within the UPGMA tree was determined by partial 16S RNA sequencing of inserts in plasmids of selected clone(s). Sequences of 16S rRNA genes of two clone libraries were compared with the Libshuff software (http://whitman.myweb.uga.edu/libshuff.html).

2.3. Isolation and characterization of mercury-resistant bacterial strains

To create selective plates containing mercury, $HgCl_2$ was added to the nutrient agar (NA) reaching a final concentration of 8.4 μg/ml (Sadhukhan et al., 1997). Samples of 5 full guts, 5 empty guts, all each inoculated separately, and 0.5 g of soil and food samples separately, were suspended in 9 ml of phosphate buffer and vortexed for 1 min. Ten-fold dilutions were inoculated on two NA/Hg plates and incubated at 30 °C for 7 days.

From the plates growing bacteria from soil and food samples from both locations, as well as full *P. scaber* guts from the contaminated location where CFU (colony forming units) were numerous only representatives of each morphotype were subcultured, whereas all colonies from empty and full guts from non-contaminated location (Radlek) were isolated in pure cultures. Subculturing was done on NA/Hg plates. Strains were Gram stained and then stored at −80 °C in a liquid nutrient broth (NB) medium augmented with 15% glycerol.

2.4. Analysis and identification of isolated mercury-resistant strains

Isolated strains were further analyzed with restriction fragment length polymorphism (RFLP) of amplified 16S RNA gene. Crude DNA prepared with Chelex 100 (Biorad) was used according to the protocol reported by Giraffa et al. (2000). The ribosomal 16S gene was amplified as described below, products were cut with *Hae*III and the length of the restriction fragments was determined with GelProAnalyser software (Media Cybernetics, Silver Spring, MD, USA). A matrix with the restriction fragment lengths and presence/absence of the fragment in each strain was constructed and used for calculation of UPGMA tree using the Jaccard coefficient in FreeTree software (http://www.natur.cuni.cz/~flegr/programs/freetree).

Correct groupings produced by the FreeTree UPGMA tree construction method were checked back with inspection of the patterns on the agarose gels. From each group in the UPGMA tree obtained, one to three isolates were selected for further analysis which included identification by partial 16S RNA sequencing, plasmid isolation and amplification of *merA* gene.

2.5. Plasmid detection

The presence of plasmids in isolated Hg-resistant strains was analyzed by cell lysis in Eckhardt gels (Eckhardt, 1978). From the isolates where plasmid was detected in Eckhardt gels, isolation of the plasmid was performed with alkali lysis from 20 ml overnight culture (Sambrook et al., 1989). Isolated plasmid DNA was cut with *Eco*RI and *Pst*I and from restriction fragments the plasmid size was estimated.

3. PCR protocols for amplification of selected genes

The *merA* genes were detected in all isolated mercury-resistant strains with PCR amplification using primers A1f and A5r and PCR conditions described by Liebert et al. (1997).

For amplification of 16S rRNA genes from isolates as well as from the DNA isolated from environmental samples, primers 27f and 1495rev described by Bianciotto et al. (1996) were used. The PCR programme began with 4 min denaturation at 94 °C, followed by 5 cycles of 30 s at 94 °C, 30 s at 60 °C and 4 min at 72 °C, another 5 cycles of 30 s at 94 °C, 30 s at 55 °C and 4 min at 72 °C and 30 cycles of 30 s at 94 °C, 30 s at 50 °C and 4 min at 72 °C.

3.1. Sequencing of 16S rRNA gene and identification of sequenced 16S rRNA genes

The amplified 16S rRNA genes from isolates or cloned 16S RNA genes were partially sequenced with primer 27f in the Seqlab (Goettingen, Germany). The closest relatives for given sequences were determined by using BLAST tool in the GenBank sequences databank and SeqMatch in RDPII database (Cole et al., 2009).