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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).

Sequences were aligned with BioEdit software (Hall, 1999) and phylogenetic trees were built with the Neighbour Joining algorithm (Saitou and Nei, 1987) in Mega4 software (Tamura et al., 2007) based on the Kimura two parametric matrix (Kimura, 1980). Quality of phylogenetic trees was assessed by the bootstrapping method.

Communities of resistant isolates were compared with χ^2-test which is based on sequences comparison. Diversity was estimated with Shannon–Wiener index calculated in EstimateS software (Colwell and Coddington, 1994).

Comparison of sequences of clone libraries were compared with Libshuff software (Singleton et al., 2001) based on the Cramer–von Mises test.

4. Results

4.1. Phylogenetic analysis of 16S RNA clone library amplified from empty P. scaber guts

After the amplification of 16S RNA genes from DNA isolated from egested isopod guts ($n = 10$ per location) two-hundred clones per location were picked randomly and analyzed by RFLP (see Lapanje et al., 2008). According to restriction with three different enzymes all clones were divided into 105 RFLP groups (Idrija) and 79 RFLP groups (Radlek).

148 clones from the Idrija location and 147 from Radlek were distributed into 32 (Idrija) and 23 (Radlek) major RFLP groups, giving 73.5% and 74% library coverage, respectively. Only the major RFLP groups, 32 for Idrija and 23 for Radlek, were further analyzed. 36 of the 147 clones from Radlek and 46 of the 148 from Idrija were selected as representatives of RFLP groups and sequenced.

When the 16S rRNA sequences were compared with Libshuff software we observed that 16S rRNA sequences derived from pristine and the Hg-polluted environments differ ($p = 0.008$ for Idrija/Radlek pair and $p = 0.01$ for Radlek/Idrija pair comparison at critical values $p = 0.025 - 95\%$ confidence interval). According to the number of taxa, the diversity of 16S RNA genes was higher in the clone library constructed from the egested guts of animals from the mercury-polluted environment than in the egested guts of animals from the pristine environment (Table 1, Fig. 1).

A large variety of phyla and classes within the clone libraries of microbial 16S rRNA sequences from *P. scaber* egested guts from both environments were observed and included phyla and classes *Planctomycetes*, *Alpha-*, *Beta-*, *Gammaproteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria* (Fig. 1). Representatives of phyla *Planctomycetes* (2% in Radlek) and *Bacteroidetes* (14% in Radlek) are completely absent from the clone library of 16S rRNA genes in guts of animals from Idrija. The most prominent differences were observed among *Proteobacteria*. *Gammaproteobacteria* were present in higher amounts in guts of animals from Radlek (40% of the whole library) than in guts of animals from Idrija (6%) while *Beta-* and *Alphaproteobacteria* were elevated in guts of animals from Idrija (7% and 18% against 22% and 45% in Radlek and Idrija, respectively). 16S rRNA sequences belonging to the phylum *Actinobacteria* were also elevated in guts of animals from Idrija (3% in Radlek and 11% in Idrija) while ratio of 16S rRNA sequences belonging to *Firmicutes* were uniform, at 16% in guts of both groups of animals (Fig. 1, Table 1).

We observed 8 genera or groups present in guts of animals from pristine or polluted environments. Among those genera/groups *Paracoccus*, *Ochrobactrum anthropi* relatives, *Rhizobium/Agrobacterium*, *Achromobacter*, *Staphylococcus succinis* relatives and *Bacillus* were enriched and sequences belonging to *Pseudomonas* and *Listeria* genera were reduced in numbers in guts of animals from the contaminated environment (Fig. 2). Representatives belonging to the genera *Paracoccus* were present in high numbers in egested guts of animals from both environments. We found 16S rRNA sequences belonging to the *Rickettsiella* relatives (2.7% of clone library) only in guts of animals from the Hg-contaminated environment (Fig. 3 – named as Uncultured alpha).

4.2. Quantification and characterization of mercury-resistant strains

Prior to characterization of isolates we first quantified culturable bacteria on nutrient agar plates (NA) and Hg amended NA plates (NA_{Hg}). We observed greater CFU numbers on the NA_{Hg} plates in all samples from Idrija than in samples from Radlek (Table 2). While we detected similar CFU counts on NA plates in samples of full guts from animals from both environments the elevated total CFU on NA plates were observed in empty guts of animals from the polluted environment. The ratio between Hg-resistant and all bacterial CFU was higher in full guts (Idrija/Radlek ratio = 385) than in empty guts (Idrija/Radlek ratio = 2.51) (Table 2). The ratio of NA_{Hg}/NA CFU between Idrija and Radlek samples was precisely in the order: full guts > food (Of horizon) > egested guts > soil (Ah horizon).

More than 300 colonies per NA plate with added $HgCl_2$ have grown on the plates with soil and food samples from both locations and from full and egested *P. scaber* guts from contaminated location (Idrija) and only representatives of different colony morphotypes were further subcultured ($n = 103$). In contrast, the number of grown colonies of Hg-resistant bacteria was low on samples of full and empty *P. scaber* guts from non-contaminated location (up to 23 colonies per plate). All strains ($n = 56$) were further subcultured.

A proportion of all subcultures, predominantly the *Actinomyceta* group was lost during subcultivation but 130 isolated Hg-resistant strains were maintained. We grouped these strains into 29 groups according to the results of 16S rRNA gene RFLP analysis. All the strains in a given RFLP group were then assigned to the taxonomic group according to the identification of the selected sequenced representative and results in Table 1 are presented for all 130 strains originally included in RFLP analysis.

After sequencing and RFLP analysis of Hg-resistant isolates, we observed statistically different structures of bacterial species in gut samples from polluted or unpolluted environments (egested guts – χ^2 test $p < 1.32 \times 10^{-5}$ and full guts – χ^2 test $p < 3.67 \times 10^{-19}$). In addition, the diversity of isolated Hg-resistant strains is higher in samples from the contaminated area, as measured by the Shannon diversity index (egested guts = 1.17, 1.67, full guts = 1.97, 2.11, food = 2.23, 2.30, soil = 2.35, 2.40 in pristine and polluted sites, respectively).

All of the mercury-resistant strains are distributed among *Gammaproteobacteria*, *Betaproteobacteria*, *Alphaproteobacteria*, *Firmicutes* and *Actinobacteria* phyla with the highest number of *Gammaproteobacteria*. We only isolated mercury-resistant bacteria from all mentioned phyla from the full guts of animals collected in Idrija (Table 1). The highest numbers of Hg-resistant isolates belong to *Gammaproteobacteria* in egested guts of animals from both Radlek and Idrija as well as in full guts of animals from Radlek. In full guts of animals collected in Idrija we observed the same proportion of *Firmicutes* as of *Gammaproteobacteria* species. From food and soil samples we obtained a small number of isolates, distributed among *Gama-*, *Betaproteobacteria*, *Firmicutes* and *Actinobacteria* phyla. *Gammaproteobacteria* are prevalent in soil samples from Radlek, but the bacteria belonging to *Firmicutes* were mostly isolated from Idrija soil samples.

Among Hg-resistant *Gammaproteobacteria* we found the highest abundance of isolates belonged to the *Pseudomonas* genus in egested as well as full guts of animals from polluted environment, and to the *Halomonas*-like group from full guts of animals from pristine environment. The *Halomonas*-like group was the only

Table 1
Comparison of identified 16S rDNA sequences from clone library and from isolated mercury-resistant strains.

Phylum or class genera (or higher taxonomic group) of the closest relative	Group[a]	Clone library		Isolated mercury-resistant strains							
		Empty guts		Empty guts		Full guts		Food		Soil	
		Ra[b]	Id[b]	Ra	Id	Ra	Id	Ra	Id	Ra	Id
Planctomycetes											
Planctomyceataceae	2	2									
Gammaproteobacteria											
Xanthomonas/Pseudoxanthomonas	3			3	2	1	1	1		3	
Stenotrophomonas	3								2		1
Vibrio rumoence relat.	2		2								
Pseudomonas	1	**59**	**7**		**13**	5	**8**			2	2
Klebsiella	3				4	1	3				
unidentified gammaproteobacteria II	3			1	3	2			1		
unidentified *Halomonas*-like	3			1	1	**11**					
Betaproteobacteria											
Achromobacter relat.	1	**11**	**22**				**9**	1			
Alcaligenes	2		**7**								
Comamonadaceae	2		3								
Alphaproteobacteria											
Paracoccus I	2	**21**	**33**								
Roseobacter relat.	2		5								
Ochrobactrum anthropi relat.	1	1	**8**				2				
Rhizobium/Agrobacterium	2	4	**15**								
Mesorhizobium	2		1								
Uncultured alphaproteobacteria	2		4								
Unidentified alphaproteobacteria	2		1								
Firmicutes											
Bacillus	1	4	**12**		3		**12**		4		1
Exiguobacterium	1		3					2			
Listeria	2	**15**	2								
Caryophanon	2	4									
Staphylococcus succinis relat.	2	1	2								
Planococcus, *Planomicrobium*	2		3								
Paenibacillus	2		2								
Bacteroidetes											
Cytophaga relat.	2	2									
Flavobacterium relat.	2	2									
Bacteroidetes relat.	2	**16**									
Actinobacteria											
Brevibacterium	1	5				1					
Microbacterium	1		**8**		2		3				
Arthrobacter	1		5								**7**
Agromyces	3				1						
Cellulosimicrobium	3						1				
Rhodococcus	3						2				
Nocardioides	3								1		2
Uncultured *Actinobacteria*	2		3								
ND	3						2	1			1
		147	148	5	29	21	43	5	8	5	14

[a] Group 1 – sequences detected in clones and strains; group 2 – sequences detected in clones only; group 3 – sequences detected in strains only.
[b] Locations: Ra (Radlek; non-contaminated); Id (Idrija; contaminated) numbers in bold represent the groups with high relative abundance (more than 7 clones).

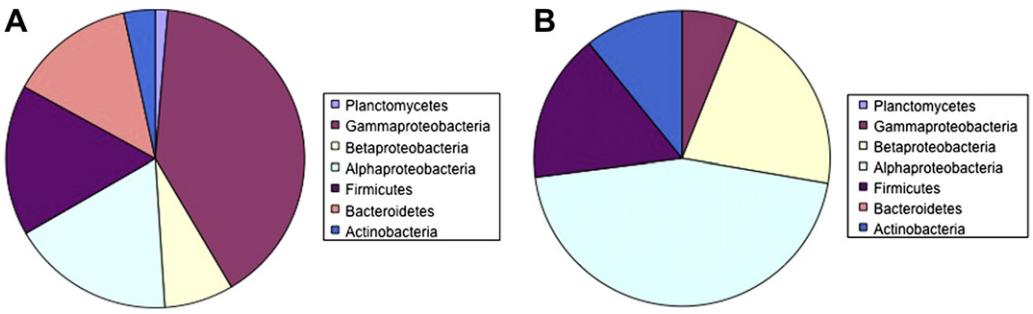

Fig. 1. Ratio of clones distributed among bacterial phyla within the clone libraries of egested guts of *Porcellio scaber* animals collected from polluted and non-polluted environments. A – Radlek, B – Idrija.

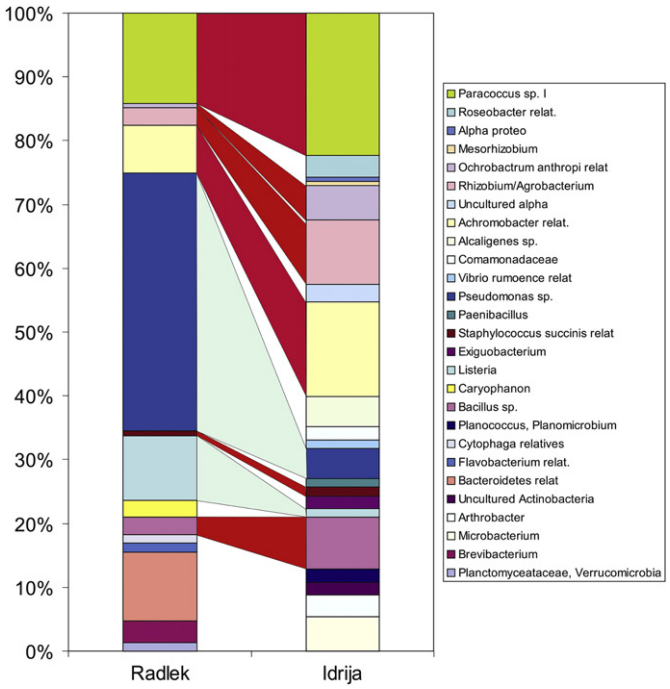

Fig. 2. Proportion of identified taxa within the clone libraries of egested guts of *Porcellio scaber* animals collected from polluted and non-polluted environments. In samples from a polluted environment (Idrija) the prevalence of certain genera either increased (shown in red) or decreased (shown in blue).

group of Hg-resistant bacteria in egested guts of animals from Radlek (all 5 isolates).

Strains belonging to the *Betaproteobacteria* (10 strains) are primarily distributed inside the *Achromobacter* relatives group and 9 isolates of all 10 strains were present in only full guts of animals from the polluted area. The fewest isolates among *Proteobacteria*, only 2 isolates from full guts of animals from Idrija, belong to *Alphaproteobacteria*.

We also cultivated *Firmicutes* (22 strains in Idrija, 0 in Radlek). Of these, isolates belonging to the genus *Bacillus* were predominant (20 strains). From phylum *Actinobacteria* (20 strains) almost all representatives are present only in the guts of animals from polluted environment, the exception being bacteria belonging to the genus *Brevibacterium* (Table 1). Hg-resistant *Actinobacteria* were more diverse in samples from full guts of animals collected in the polluted environment than in samples from full guts of animals from pristine environment. These were distributed among 4 different genera (*Microbacterium*, *Agromyces*, *Cellulosimicrobium* and *Rhodococcus* – Table 1). We isolated bacteria belonging to *Arthrobacter* genus only from Ah soil horizon samples. We isolated Hg-resistant bacteria belonging to *Nocardioides* genus from both Ah and Oh horizons and from both locations.

4.3. Plasmids and merA gene in isolated Hg-resistant strains

The presence of plasmids was tested in 35 out of 39 representative isolates (4 isolates failed to re-grow from storage cultures). Eckhardt gels indicated presence of plasmids in 32 strains (91.4%). The estimated plasmid sizes ranged from 90 kb to 250 kb. Plasmids could not be detected in strains identified as *Exiguobacterium*, *Agromyces* and in some *Bacillus* strains.

The *merA* gene was present in 28 (80%) of the isolated mercury-resistant strains, but the polymorphism was not further studied. The majority of strains with plasmids also amplified *merA* gene with exceptions of some *Pseudomonas*, *Rhodococcus*, *Cellulosimicrobium* and *Ochrobactrum* relatives, whereas some other strains assigned to genera *Exiguobacterium*, *Agromyces* and some *Bacillus* strains had *merA* gene but no detectable plasmids.

5. Discussion

In this study we have focused on changes of the bacterial community resulting from long-term Hg pollution in the *P. scaber* guts. Higher numbers as well as higher diversity of Hg-resistant bacterial isolates were found and in addition we provide evidence gained from use of culture-independent methods on the bacterial community structural shift in the *P. scaber* gut.

Consistent with published data, the enrichment of mercury-resistant bacteria is linked to the selection and promotion of growth of mercury-resistant genotypes on the one hand and horizontal gene transfer (HGT) on the other (Albrechtsen and Christensen, 1994; Hill et al., 1994; Smets et al., 2003). While promotion of growth of selected microbes contributes to the higher numbers of resistant cells (e.g. higher CFU), HGT is mostly responsible for higher diversity of mercury-resistant strains in the polluted environment. Since the selection process is ubiquitous, several factors, reviewed by Smets and Barkay (2005), are required for the HGT to occur. In the light of reports of HGT processes in the arthropod gut described by Hoffmann et al. (1998) (in *Folsomia candida*), Hinnebusch et al. (2002) and Petridis et al. (2006) (both in *Musca domestica*), it is likely that HGT also occurs in *P. scaber* gut.

Bacterial genetic determinants of mercury-resistance include the *mer* operon genes (Barkay et al., 2003). The operon could be located on plasmids, transposons or on the chromosome and it is known that it is transferred by HGT to other bacterial groups (Barkay et al., 1993; Coombs and Barkay, 2004). In the *P. scaber* gut, a high percentage (80%) of *merA* positive mercury-resistant isolates confirms the specificity of mercury stress in the mercury-polluted environment. The diversity of strains among *mer*A positive genotypes as well as the presence of plasmids in these strains could be consequences of HGT of *mer* genes in the *P. scaber* gut. The absence of amplification of *mer*A gene in other 20% of Hg-resistant strains belonging to *Pseudomonas*, *Rhodococcus*, *Cellulosimicrobium* and *Ochrobactrum* genera could be due to the mutation of sequences in the primer target sites (Barkay et al., 2003) or utilization of some other mechanisms to deal with Hg toxicity.

The results of CFU enumeration could be explained by interplay of several factors such as food retention time in guts, proportion of culturable cells, quantity of copiotrophic bacteria and differences of bacterial activity in such environments. We observed similar amounts of total CFU of all culturable bacteria in full guts of animals from the polluted and unpolluted environments. However, in egested guts, bacterial CFU were at least two orders of magnitudes higher in animals from the mercury-polluted environment. Such a discrepancy could stem from longer food retention time in egested guts (Drobne and Hopkin, 1995) or alternatively, the animals could be unable to suppress overgrowth of bacteria as a result of pollution-induced disturbance of their immune system, or both (Stewart, 1993; Herbinič re et al., 2005; Sorvari et al., 2007; Snyman and Odendaal, 2009).

The percentage of mercury-resistant culturable strains is very high in full guts (Table 2). This could be due to a cultivation bias which might in turn be a consequence of the larger amounts of copiotrophs (Baath et al., 1998; Edenborn and Sexstone, 2007). In contrast, elevated amounts of unculturable microorganisms in egested guts and smaller amounts of Hg-resistant culturable strains are expected because of the close association with animals (e.g. symbionts) and consequently special nutrient requirements (Kostanjsek et al., 2004a, 2004b; Wang et al., 2004). In agreement

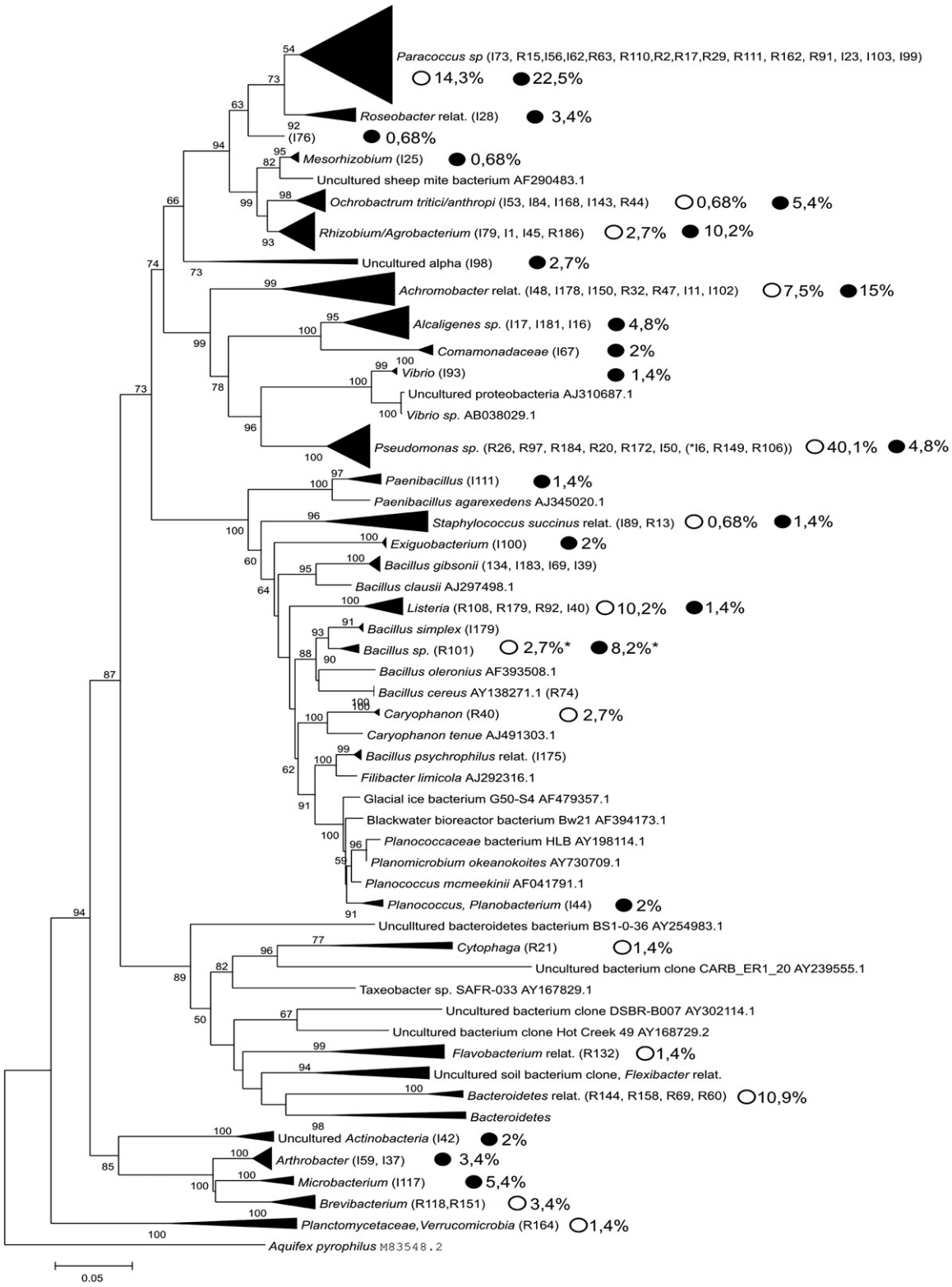

Fig. 3. Taxonomic relationship of 16S RNA clones from egested *Porcellio scaber* guts with the closest relatives in GenBank. Open circles represent clones from non-polluted environment and black filled circles clones from mercury-polluted environment. Sequenced clones were selected from 16S RNA RFLP groups and relative abundance of clones identical to the sequenced one is shown in %. In brackets are written names of clones – clones beginning with R are from unpolluted site and I from polluted. Tree was built with Neighbour Joining method based on Kimura two parametric matrix. Tree branches were tested with bootstrap test. Bootstrap values above 50 are presented on the branches.

with several reports of higher activity of bacteria in the guts of detritivorous animals compared to the adjacent soil environment (Pedersen and Hendriksen, 1993; Brown, 1995; Drake and Horn, 2007) and higher activity in Ah than Oh horizon (Dail et al., 2001; Rogers and Tate, 2001), we observe the highest Radlek/Idrija CFU quantity ratio of Hg-resistant bacteria in full guts (1:385), followed by egested guts and, soil Oh and Ah horizon samples, respectively (see the column "ratio Id/Ra" in Table 2). In our

Table 2
Estimated CFU numbers from four different microniches (sample) grown without (NA) and with mercury selection (NA_{Hg}). In brackets bellow CFU numbers are shown percentage of CFU between Hg-resistant and whole culturable bacteria (NA_{Hg}/NA). Numbers in the last column give the ratio of CFU of resistant bacteria between Idrija (Hg-polluted environment) and Radlek (unpolluted environment). Ra – Radlek, Id – Idrija.

Sample	Location	CFU		Ratio CFU-NA_{Hg} Id/Ra
		NA	NA_{Hg}	
Full guts	Non-polluted (Ra)	1.12 E+05 (100%)	2.41 E+02 (0.002%)	385
	Polluted (Id)	1.1 E+07 (100%)	8.52 E+04 (0.77%)	
Egested guts	Non-polluted (Ra)	3.78 E+03 (100%)	4.8 E+01 (1.27%)	2.51
	Polluted (Id)	7.06 E+05 (100%)	2.26 E+04 (3.2%)	
Oh horizon (food)	Non-polluted (Ra)	2.35 E+08 (100%)	7.0 E+02 (0.00029%)	5.66
	Polluted (Id)	1.66 E+11 (100%)	2.80 E+06 (0.0017%)	
Ah horizon (soil)	Non-polluted (Ra)	1.12 E+09 (100%)	1.5 E+04 (0.0014%)	1.45
	Polluted (Id)	4.44 E+11 (100%)	8.64 E+06 (0.0019%)	

previous publication, we used culture-independent methods and observed lower species richness in the 16S rRNA clone library of the gut bacterial community of animals from Idrija than in animals from Radlek (Lapanje et al., 2007), but we could not detect the differences in the TTGE (temporal temperature gradient gel electrophoresis) profiles of amplified 16S rRNA genes. In the work presented here, which is based on 16S rRNA sequencing, we show that in egested guts *Alpha-*, *Betaproteobacteria*, some *Firmicutes* and *Actinobacteria* prevail in samples from the polluted environment, while *Bacteroidetes* are associated only with animals from non-polluted environments. Only a few clones of *Gammaproteobacteria* were detected in the polluted environment, but their relative abundance was higher in samples from the unpolluted environment. Within *Firmicutes*, *Bacillus* representatives are strongly associated with polluted environment and *Listeria* with non-polluted environments. Among *Alphaproteobacteria* we obtained 16S rRNA genes of uncultured *Alphaproteobacteria* (2.7% of whole clone library) only in guts of animals from the polluted environment. The sequences of these *Alphaproteobacteria* are similar to the *P. scaber* symbiont – *Hepatincolla porcellionum* (Wang et al., 2004). However, all of known symbionts belonging to this genus are associated with arthropod diseases and occurrence of *H. porcellionum* might be as a consequence of diminished immune responses (Sorvari et al., 2007) or could be simply prevalent in those animals from Idrija.

When we compared culture dependant and molecular methods *Bacillus*, *Pseudomonas*, and *Actinobacteria* were abundant among isolates and clones from the polluted environment. These three groups prevailed also in other studies of mercury-resistant populations (Sabaté et al., 1994; Ellis et al., 2003; Narita et al., 2004). Cultivation-dependent and cultivation-independent methods used on the same environmental sample cannot be compared (Ellis et al., 2003). These two approaches cannot be compared directly in our study as well, because the entire community (without mercury selection) was considered in the construction of clone library, whereas we cultivated only resistant strains. Additionally the growth, as in all culturing approaches, was biased by the selection of medium and cultivation conditions. With this in mind, the bacterial groups detected were subdivided into three sub-groups (1, 2, 3; Table 1) defining whether representatives were detected in strains only, clones only or in both. Because they represent the unselected population and higher detection limit of molecular methods a large proportion of bacterial genera or groups were obtained in cloned sequences only (group 2 in Table 1). A number of bacterial groups were represented in both, 16S rRNA clone library and among mercury-resistant isolated strains (group 1 in Table 1). They were largely the groups prevalent in samples from the polluted environment (Id column; Table 1), such as *Achromobacter* relatives, *Bacillus*, *Micrococcus* and *Arthobacter*. This is consonant with their previously described high tolerance to Hg (Osborn et al., 1997; Benyehuda et al., 2003; Ellis et al., 2003). Group 3, detected only among isolated mercury-resistant strains, includes *Gammaproteobacteria* and *Actinobacteria*. The number of cultivated *Actinobacteria* is low, though many strains from this group were also lost during isolation of pure cultures, and the absence of molecular detection could perhaps be explained on the basis of their low proportion within the entire community. The *Gammaproteobacteria* include the highest number of isolated resistant strains, but the representatives were almost never detected in the clone library of empty guts. This discrepancy was not due to the poor colonization properties of *Gammaproteobacteria*, because the strains were isolated from empty and full guts. A possible explanation is that diversity within *Gammaproteobacteria* 16S rRNA sequences in the clone library was high and the sequences were not distributed into the main RFLP groups further selected for sequencing. Interestingly, *Gammaproteobacteria* are almost the only group which includes resistant strains from an unpolluted environment. Some authors have reported isolation of resistant *Bacillus* strains from non-polluted soils (Osborn et al., 1993; Narita et al., 2004), but in our study this was not the case.

In conclusion, this study shows that isopods in a chronically polluted environment have in their guts a different bacterial community than is possessed by animals whose environment is generally unpolluted. The bacterial community in guts of animals from a polluted environment contains higher amount of mercury-resistant bacteria, which might contribute to physiological resilience of the bacterial community present in the guts of animals. A higher incidence of bacteria containing *merA* genes could also contribute to the higher tolerance of animals as a result of reductase activity of MerA protein. Such activity could lower the concentration of mercury in the animal gut and their micro-environment.

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