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ORIGINAL ARTICLE

High diversity of bacterial mercuric reductase genes from surface and sub-surface floodplain soil (Oak Ridge, USA)

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DNA was extracted from different depth soils (0–5, 45–55 and 90–100 cm below surface) sampled at Lower East Fork Poplar Creek floodplain (LEFPCF), Oak Ridge (TN, USA). The presence of *merA* genes, encoding the mercuric reductase, the key enzyme in detoxification of mercury in bacteria, was examined by PCR targeting *Actinobacteria*, *Firmicutes* or *β/γ-Proteobacteria*. *β/γ-Proteobacteria merA* genes were successfully amplified from all soils, whereas *Actinobacteria* were amplified only from surface soil. *merA* clone libraries were constructed and sequenced. *β/γ-Proteobacteria* sequences revealed high diversity in all soils, but limited vertical similarity. Less than 20% of the operational taxonomic units (OTU) (DNA sequences ⩾95% identical) were shared between the different soils. Only one of the 62 OTU was ⩾95% identical to a GenBank sequence, highlighting that cultivated bacteria are not representative of what is found in nature. Fewer *merA* sequences were obtained from the *Actinobacteria*, but these were also diverse, and all were different from GenBank sequences. A single clone was most closely related to *merA* of *α-Proteobacteria*. An alignment of putative *merA* genes of genome sequenced mainly marine *α-Proteobacteria* was used for design of *merA* primers. PCR amplification of soil *α-Proteobacteria* isolates and sequencing revealed that they were very different from the genome-sequenced bacteria (only 62%–66% identical at the amino-acid level), although internally similar. In light of the high functional diversity of mercury resistance genes and the limited vertical distribution of shared OTU, we discuss the role of horizontal gene transfer as a mechanism of bacterial adaptation to mercury.
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Introduction

Of all the heavy metals, mercury (Hg) is the most toxic and with no biological function (Nies, 1999). Mercury is not abundant in the soil crust, but can be found in ores, the dominant being cinnabar (HgS). Mercury is emitted to the atmosphere by natural sources, for example, soil erosions and volcanic eruptions, and by anthropogenic sources, for example, coal-fueled power plants (Nriagu and Pacyna, 1988). Glacial ice core studies have shown that during the timescale of industrialization in the western world, mercury has increased approximately 20-fold in the ice cores, and anthropogenic activities account for 70% of the last 100 years mercury emissions (Schuster *et al.*, 2002). The atmospheric mercury is transported globally, but deposited locally, and pose a threat to organisms at the top of food webs, due to bioaccumulation of methyl mercury. However, severe mercury contamination of natural environments can often be attributed to inappropriate human activities. Examples include the Minamata Bay in Japan, where the local fish-consuming villagers were exposed and poisoned by high levels of methylmercury in their seafood due to contamination by a nearby acetaldehyde production facility (Eto, 2000; Tomiyasu *et al.*, 2006).

The Y12 nuclear weapons plant facility at Oak Ridge (TN, USA) used mercury for lithium isotope separation during the 1950s and 1960s, and this resulted in an estimated 75–150 tonnes of mercury inadvertently being released to the local environment, including the East Fork Poplar Creek (Han

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et al., 2006). Examination of the lower East Fork Poplar Creek floodplain (LEFPCF) soil revealed total mercury concentrations ranging from 42 to 2400 μg Hg g^{-1} dry weight (Barnett *et al*., 1995). In the LEFPCF studies, the largest fraction of the mercury was estimated to be immobilized as HgS (Revis *et al*., 1989). Although several studies have been conducted at Oak Ridge dealing with mercury effects on higher trophic level organisms like fish (Burger and Campbell, 2004) and mammals (Stevens *et al*., 1997), little has been done to elucidate the effects of mercury on the microbial community in the soil.

Three mechanisms for microbial adaptation to environmental stress have been suggested (reviewed in Sørensen *et al*., 2002): (i) enrichment of populations that carry the required resistance/tolerance traits, (ii) induction of enzymes involved in the detoxification or resistance mechanisms, and (iii) genetic adaptation. The first two are manifested by previously existing sub-populations of resistant strains, while the process of genetic adaptation creates changes in the existing genetic pool of the microbial community and thus the evolution of new capabilities and an increase in the functional diversity of the microbial community. Horizontal gene transfer is a mechanism by which the genetic diversity among bacteria may be enhanced. Thus, it may play an important role in the adaptation and evolution of microbial communities (Sørensen *et al*., 2005). Metal-resistance genes are often carried on plasmids and other mobile genetic elements (Silver and Phung, 1996) and therefore, exchange of these mobile elements through metal-impacted communities may facilitate rapid alleviation of metal toxicity. This is supported by the higher incidence of plasmid DNA among bacteria isolated from polluted environments (Rasmussen and Sørensen, 1998) and by increased degradation by indigenous bacteria after plasmid transfer with relevant catabolic genes (Top *et al*., 2002).

Mercury resistance is found in both *Bacteria* (Barkay *et al*., 2003) and *Archaea* (Schelert *et al*., 2004). Mercury resistance in *Bacteria* is conferred by the *mer* operon. Several *mer* operon-encoded proteins are involved in transport of inorganic oxidized mercury into the cytosol, where the *merA*-encoded mercuric reductase protein, in a NAD(P)H-dependent manner, reduces Hg^{2+} (aq) to volatile, less reactive elemental mercury (Hg^0 (g)) (mercury in the environment and bacterial resistance is thoroughly reviewed in Barkay *et al*., 2003). Mercury resistance has been recognized in many different phyla, including *Firmicutes*, *Actinobacteria* and *Proteobacteria*, of both clinical and environmental origin and it is considered to be an ancient resistance mechanism (Osborn *et al*., 1997). Biochemical and structural studies of the homodimeric mercuric oxidoreductase have shown that there are four cysteine residues in each monomer, that are essential for reducing oxidized mercury to elemental mercury and furthermore that two tyrosine residues are important for optimal functionality (Moore and Walsh, 1989; Distefano *et al*., 1990; Schiering *et al*., 1991; Moore *et al*., 1992). Some mercuric reductase proteins contain a mercury binding N-terminal domain, which gives better protection against oxidized mercury (Ledwidge *et al*., 2005), but this is not essential, as it is not found in the mercuric reductase enzymes of, for example, *Actinobacteria* and *Archaea*.

If there has been long-term selective pressure for mercury-resistant bacteria, all individual bacteria will probably be resistant, and the resistance trait (the *merA* protein) will probably have evolved differently in different bacteria, thus leading to high functional diversity, closing in on 16S rRNA diversity. Alternatively, if the selective pressure by mercury is of a newer date, only resistant bacteria and those bacteria that could adapt to the new selective pressure by, for example, horizontal gene transfer thus receiving the required resistance traits would proliferate. In the latter case scenario, the community diversity would decrease somewhat, and the functional diversity would be low.

Most of the studies dealing with heavy metal contamination in soil have been conducted with surface soils (Rasmussen and Sørensen, 2001; Muller *et al*., 2001a; Muller *et al*., 2001b; Ellis *et al*., 2003), whereas only few studies have looked at sub-surface soils, and most of these have dealt with other metals, for example, uranium (North *et al*., 2004; Abulencia *et al*., 2006) or organic pollutants (Lowe *et al*., 2002; Zhou *et al*., 2004).

The purpose of the present study was to examine the diversity of *merA* genes from surface and sub-surface soil, exposed to mercury for decades, and thus address the role of horizontal gene transfer in bacterial adaptation. It is well known that most soil bacteria are fastidious with regards to cultivation media (Janssen, 2006), and it has been estimated that in 1 g of soil, there might be as many as 10^6 different bacterial species (Gans *et al*., 2005). With the obvious limitations of cultivation of soil bacteria, molecular approaches may assist in deciphering bacterial functional diversity. In the present study, we used a cultivation-independent approach, based on PCR amplification, cloning and partial sequencing of the *merA* gene, enabling determination of *merA* diversity in soil. Bacterial isolates from LEFPCF (Oregaard *et al*., 2007) were also examined with regards to the presence of *merA* genes.

Materials and methods

merA *primer design*

Mercuric reductase (*merA*) sequences were obtained by searching the GenBank nucleotide database at NCBI. Putative mercuric reductase genes were found in the following phyla: *Firmicutes*, *Actinobacteria*, *Deinococcus-Thermus*, *Bacteroidetes*, *Proteobacteria*

Table 1 *merA*-specific primers for different bacterial phyla

Phylum[a]	*Primer*	*Primer sequence*[b]	*Degeneracy*[c]	*Reference*
Act	Act-Fw	5′-CSG AVT TCG TST ACG TCG C-3′	12	This study
Act	Act-Rv	5′-GCC ATG AGG TAS GGG-3′	2	This study
Firm	Fir-Fw	5′-GTT TAT GTW GCW GCY TAT GAA GG-3′	8	This study
Firm	Fir-Rv_{1832}	5′-CCT TCW GCC ATY GTT ARA TAW GG-3′	16	This study
Firm	Fir-Rv_{1892}	5′-CCT GCA CAR CAA GAT AAT TTB GA-3′	6	This study
α	Al-Fw	5′-TCC AAG GCG MTG ATC CGC GC-3′	2	This study
α	Al-Rv	5′-TAG GCG GCC ATG TAG ACG AAC TGG TC-3′	1	This study
β/γ	BG-Fw_{349}	5′-CCA TCG GCG GCA CCT GCG T-3′	1	This study
β/γ	BG-Fw_{698}	5′-TCG AYC GCT GCY TVR TCG CCA C-3′	24	This study
β/γ	#91 (A7s-n.F)	5′-CGA TCC GCA AGT GGC IAC BGT-3′	3	Schaefer[d]
β/γ	#54 (A5-n.R)	5′-ACC ATC GTC AGR TAR GGR AAV A-3′	24	Schaefer
β/γ	BG-Rv_{1349}[e]	5′-CAV GTI GCC ACT TGC GGA TCG-3′	3	This study

[a]The primers were designed to be selective for merA of particular phyla Act, *Actinobacteria*; Firm, *Firmicutes*; α and β/γ, *Proteobacteria* sub-phyla.
[b]The degenerate nucleotides are: S: G/C; V: G/C/A; W: A/T; Y: C/T; R: A/G; B: G/T/C; I: Inosine; M: A/C.
[c]Degeneracy: the number indicates how many primers are present in the primer solution.
[d]Schaefer *et al.*, 2004.
[e]#91 reverse and complimentary.

(α-, β-, γ- and δ-) and in *Crenarchaeota* and *Euryarcheota*, the latter two belonging to *Archaea*. The DNA sequences were prepared in FASTA format and subjected to alignment by RevTrans (http://www.cbs.dtu.dk/services/RevTrans/; Wernersson and Pedersen, 2003). In short, the sequences were *in silico* translated to amino acids, aligned and then converted back to DNA sequences, but according to the amino-acid alignment. These multiple DNA alignments were examined manually using *clustalX* software (Chenna *et al.*, 2003). Primers were manually designed, placing them in conserved regions of the multiple alignments. The *merA* of the different phyla were too diverse for primers encompassing them all. Primers intended to be specific for the following phyla were designed; *α-Proteobacteria*, additional *β/γ-Proteobacteria* primers, *Firmicutes* and *Actinobacteria* (Table 1).

The primer pairs were tested in the laboratory on available *merA* genes from *γ-Proteobacteria* (*merA* from *Serratia marcessens* plasmid pDU1358, cloned into pHG103 (Griffin *et al.*, 1987), kind gift of Dr Tamar Barkay, Rutgers University) and *Firmicutes merA* genes. Three different *merA* genes originating from *Staphylococcus aureus* pI258 (Laddaga *et al.*, 1987), *Bacillus* sp. RC607 (Wang *et al.*, 1987) and *Bacillus megaterium* MB1 (Huang *et al.*, 1999), all cloned into *Escherichia coli* vectors (pRAL2, pYW40 and pGB3A, respectively, kind gift of Dr Tamar Barkay, Rutgers University) were used. Primers targeting *Actinobacteria* or *α-Proteobacteria* were tested on mercury-resistant isolates from Oregaard *et al.* (2007). The plasmids containing *merA* genes were extracted from their *E. coli* host cells with QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) after overnight growth in LB broth. Genomic DNA of uncharacterized isolates was extracted from colonies grown on dilute media (Oregaard *et al.*, 2007) or in 10% tryptic soy broth supplemented with 10 μM $HgCl_2$, with High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manual.

Soil

The soil came from LEFPCF, which has a history of mercury contamination. The soil samples were obtained from three depths at the same site: 0–5 cm (surface, soil B), 45–55 cm below surface (soil C) and 90–100 cm below surface (soil D). The soil was stored at 4°C after initial pre-sieving (2 mm mesh).

Soil pre-treatments

The soils were pre-treated with mercury (or water in control treatments) and set up in plastic bags with the equivalent of 20 g dry soil. All pre-treatments were done in triplicate. Pre-treatment with mercury was performed to selectively enrich the soils with mercury-resistant bacteria. A volume of 1 ml autoclaved distilled H_2O was added to the control soils (w). The mercury pre-treatments (Hg) included $HgCl_2$, in a final volume of 1 ml. The amount of mercury was adjusted for each soil type to obtain a start mercury concentration of 10 $\mu g\,g^{-1}$ soil–water. The soil microcosms were stored at 25°C in the dark for 7 days, after which the soil was stored at −80°C.

merA *clone libraries*

Soil DNA was extracted from control and mercury-stimulated soil with Fast DNA Spin for Soil kit BIO 101 Systems (http://www.qbiogene.com).

The *β/γ-Proteobacteria* PCR reactions were performed with 2 μl soil DNA, primers #91 and #54 (0.4 μM final concentration) and water in a total volume of 25 μl with PuReTaq Ready-To-Go PCR beads (GE Healthcare, Hillerød, Denmark). Expected

amplicon size was 288 bp. The PCR reaction consisted of an initial denaturing step at 95°C/3 min; then 45 cycles with two steps: 95°C/10 s and 60°C/1 min. Final extension was at 72°C for 2 min.

The *Actinobacteria* PCR reactions were done with Phusion High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland). The PCR reaction mix consisted of 2 μl soil DNA, Act-Fw and Act-Rv primers (0.5 μM final concentration), 4 μl HF buffer, dNTP (200 μM final concentration), Phusion DNA polymerase (0.02 U μl) and water in a total volume of 20 μl. Expected amplicon size was 391–397 bp. The PCR reaction consisted of an initial denaturing step at 98°C/2 min; then 35 cycles with three steps: 98°C/10 s, 62°C/10 s and 72°C/20 s. Final extension was at 72°C for 2 min.

The *Firmicutes* PCR reactions were performed with Phusion polymerase. The PCR reaction mix consisted of 1 μl soil DNA, Fir-Fw and Fir-Rv_{1892} primers (0.5 μM final concentration), 2 μl HF buffer, dNTP (200 μM final concentration), Phusion DNA polymerase (0.02 U μl) and water in a total volume of 10 μl. Expected amplicon size was 455 bp. The PCR reaction consisted of an initial denaturing step at 98°C/2 min; then 35 cycles with three steps: 98°C/10 s, 64°C/10 s and 72°C/20 s. Final extension was at 72°C for 2 min.

The total PCR reaction volume from each reaction was loaded onto 1.2% agarose gels, gel electrophoresed and stained with ethidium bromide. If *merA* bands of correct size were observed, they were cut out and purified with QIAEX II Gel Extraction Kit (Qiagen). The extracted amplicons obtained with the same primers were pooled before cloning (for example, soil B, stimulated with water, three replicates, all mixed and aliquot of this used for cloning). The *merA* amplicons were cloned with the TOPO TA cloning kit (Invitrogen, Paisley, UK). A volume of 2–4 μl *merA* amplicon extracted from gel was mixed with 1 μl salt solution and 1 μl pCR4-TOPO vector, and water to a final volume of 6 μl. The cloning and transformation was done according to the manual, using competent TOP10 *E. coli* cells. The transformants were grown overnight at 37°C on LB agar with Ampicillin (100 μg ml^{-1}) and re-streaked onto LB agar with Kanamycin (50 μg ml^{-1}).

merA *sequencing, analysis and phylogeny*

The transformants were grown overnight and the cloning vectors (with *merA* inserts) were extracted with Qiaprep Spin Miniprep Kit (Qiagen). The plasmid DNA concentration was determined spectrophotometrically (A_{260}/A_{280}). The sequence reaction mix consisted of 4 μl DYEnamic ET Dye Terminator mix (MegaBACE, Amersham, UK), 1 μl M13 forward primer (10 μM), water and 200–400 ng plasmid DNA in a final volume of 10 μl. The dideoxy nucleotide sequencing (Sanger sequencing) reaction was done in the following way: initial denaturing at 94°C for 3 min followed by 30 cycles consisting of three steps: 94°C/20 s, 50°C/15 s and 60°C/60 s. Subsequently, 30 cycles were done in the same manner but with elongation occurring for 120 s. Finally, there was a single step at 60°C for 5 min. The sequence reaction mix was purified with a Sephadex G-50 plate (96 wells), and the 96-well plates were sequenced on a MegaBACE 1000 Sequenator (Amersham Biotech, Amersham, UK).

The sequences were manually trimmed for vector and primer sequences. *Blastn* (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al.*, 1997) was used to detect probable gap positions in the clone sequences, and *in silico* translation of sequences into functional mercuric reductase protein sequences was done to confirm that the obtained DNA sequence resembled mercuric reductase enzymes. The sequences were subjected to RevTrans alignment as above. The multiply aligned DNA sequences were subjected to DNA distance matrix calculations with the DNADIST of the *Phylogeny Inference Package* (Phylip, version 3.65, Joseph Felsenstein, http://www.evolution.genetics.washington.edu/phylip.html). The *DOTUR* software program (Schloss and Handelsman, 2005) was fed with the DNA distance matrices for determination of operational taxonomic units (OTUs) and calculation of different diversity measures, including Shannon–Weaver diversity index and Chao1 richness estimator. Library coverage was calculated as: $C = [1-(n/N)]100\%$, where n is number of OTUs and N the number of sequences.

Sequences

The sequences obtained in this study have been deposited at NCBI. The *merA* clones of β- and γ-*Proteobacteria* and *Actinobacteria* are associated with accession numbers EF460128–EF460310. The *merA* sequences from the isolates are associated with accession numbers EF455060–EF455079.

Results

Full-length mercuric reductase sequences deposited at GenBank (NCBI) were retrieved and analyzed. The four essential cysteine residues (positions 207, 212, 628 and 629 in *Bacillus cereus* RC607 *merA* enzyme, BAB62433) along with the two important tyrosine residues (positions 264 and 605) were used to discriminate mercuric reductase sequences from other similar disulfide oxidoreductases like dihydrolipoamide dehydrogenases. Using these amino acids as criteria for accepting sequences as mercuric reductases, along with general similarity to *merA* sequences found with *blastn*/*blastp* (Altschul *et al.*, 1997; Schaffer *et al.*, 2001), *merA* of different phyla were identified (Table 2). It was apparent, that many sequences annotated as mercuric reductase encoding genes, were probably wrongly annotated, when using these criteria, since they lacked the two

Table 2 Presence of particular amino acids, essential for mercuric reductase activity

Phylum[a]	*No. of sequences*[b]	*Essential amino acids in mercuric reductase enzymes*					
		Cys 207[c]	*Cys 212*	*Tyr 264*	*Tyr 605*	*Cyr 628*	*Cyr 629*
α	14	+	+	+	+	+	+
β/γ	30	+	+	+[d]	+	+	+
δ	4	+	+	+	+	+	+
Dein-Therm	2	+	+	+	+	+	+
Bacteroidetes	1	+	+	+	+	+	+
Firmicutes	11	+	+	+	+	+	+
Actinobacteria	9	+	+	+	+	+	+
Archaea	12	+	+	+	Phe[e]	+	+
Outgroup	4	+	+	−	+[f]	−	−

[a]α, *β/γ* and *δ*, sup-phyla of *Proteobacteria*; *Dein-Therm*, *Deinococcus-Thermus*. Same sequences as used for Figure 1.
[b]Number of sequences retrieved from GenBank and examined for conserved amino acids.
[c]The numbering is from the *Bacillus* sp RC607 mercuric reductase protein (BAB62433).
[d]Isoleucine in CAC80079.
[e]Phenylalanine in position 605 of all *Archaea*.
[f]Phenylalanine in YP_628559.

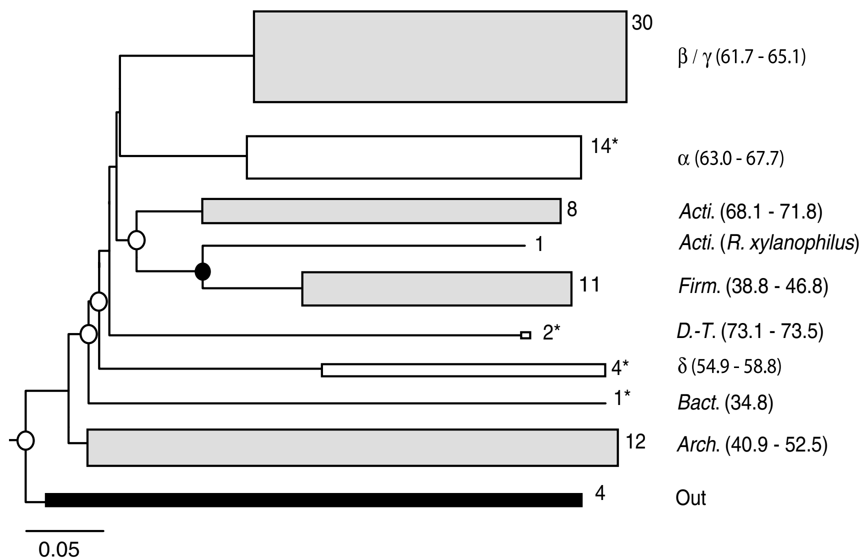

Figure 1 Neighbour-joining tree of mercuric reductase *protein* sequences. The sequences were obtained from GenBank at NCBI. The boxes reflect phyla with more than one sequence. The gray boxes represent phyla with phenotypic mercury resistance, based on mercuric reductase proteins. The asterisk (*) indicates phyla or isolates in which phenotypic mercury resistance has not been recognized. The horizontal length of a box is equivalent to the *maximum length* from node to tip of a particular sequence in that phylum. The number at the right-hand corner of the box reflects the number of proteins included in the alignment. The value in parenthesis is the range of GC content of the *merA* gene of the particular phylum. Black and white circles at nodes reflect bootstrap values higher than 95 and 50%, respectively (out of 1000 bootstraps). The bar at the bottom reflects 5% difference at the amino-acid level. The accession numbers of the proteins used for the neighbour-joining tree were as follows *Archaea*; NP_376996, NP_147957, CAF18529, NP_560142, NP_344015, YP_256307, NP_110770, YP_023042, ZP_00610004, YP_134400, NP_394797 and ZP_00610602; *δ-Proteobacteria*: NP_954464, ZP_01140457, ZP_01388909 and YP_383148; Outgroup: ABC45686, NP_952368, YP_628559 and YP_010258; *Bacteroidetes*: ZP_01060916; *Deinococcus-Thermus*: YP_144419 and YP_004762. The *β/γ-Proteobacteria* and *Actinobacteria merA* protein accession numbers are shown in Figures 2 and 5, respectively. *α-Proteobacteria* and *Firmicutes* accession numbers are shown in Figure 4.

terminal cysteines, Cys628 and Cys629 (data not shown). All mercuric reductase proteins used for primer design by multiple alignments contained all four cysteines and both tyrosines, except one *Pseudomonas* sequence in which Tyr264 had been substituted with isoleucine (CAC80079). All the *Archaea* sequences had a phenylalanine residue at position 605, in contrast to tyrosine in bacteria (Table 2). None of the *Archaea* sequences from the NCBI database used here have been experimentally characterized as conferring mercury resistance to their host. A neighbour joining tree of *merA* proteins of different phyla is shown in Figure 1. The outgroup sequences were four proteins annotated as mercuric reductases, but without the signature Tyr264, Cys628 and Cys629 amino acids (Table 2).

The multiple alignments were used to design primer pairs targeting *merA* from *Firmicutes*, *Actinobacteria* and *α-Proteobacteria*, and additional

primers for *β/γ-Proteobacteria* (Table 1). *In silico* PCR reactions with the primers against the different phyla revealed that they gave amplicons of expected size and were specific to their phyla, except for the *Actinobacteria* primers, which also seemed to equally well amplify *β/γ-Proteobacteria merA*.

Three different *merA* genes originating from *S. aureus* (Laddaga *et al.*, 1987), *Bacillus* sp. RC607

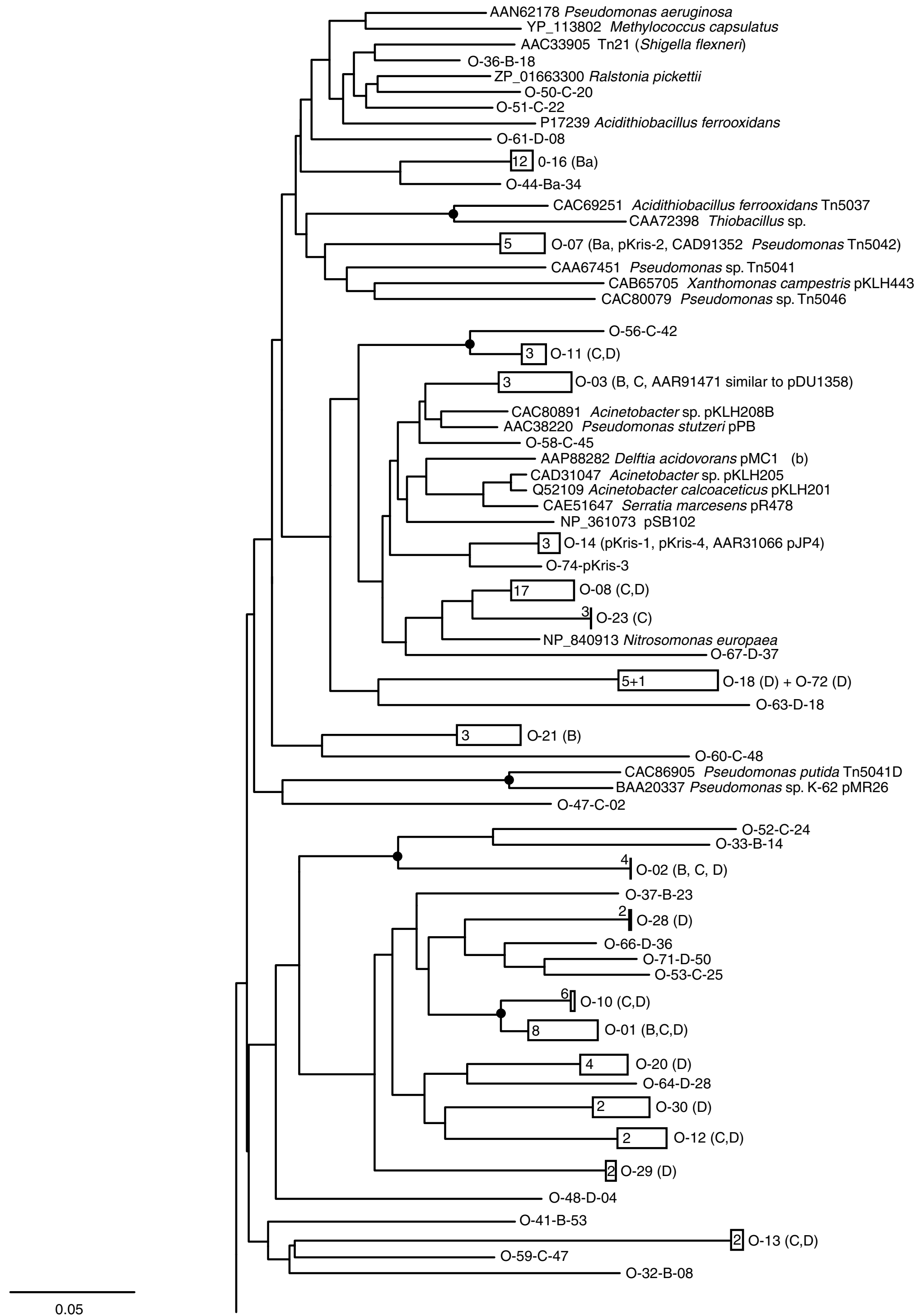

Figure 2 Neighbour-joining tree of *merA* DNA sequences. The sequences were obtained from *β/γ*-type *merA* clone libraries (B, C, D and Ba) or from exogenously isolated plasmids (pKris). The boxes reflect OTUs with more than one sequence. The horizontal length of a box is equivalent to the maximum length from node to tip of the particular OTU. The OTUs were defined by DOTUR. Black circles at nodes reflect bootstrap values higher than 90% (out of 1000 bootstraps). Letters in parenthesis reflect the soil type. The number inside the box, or at the upper left-hand corner of the box reflects the number of sequences within the particular OTU. *Archaea merA* gene from *Haloarcula marismortui* was used as outgroup. The bar at the bottom reflects 5% difference.

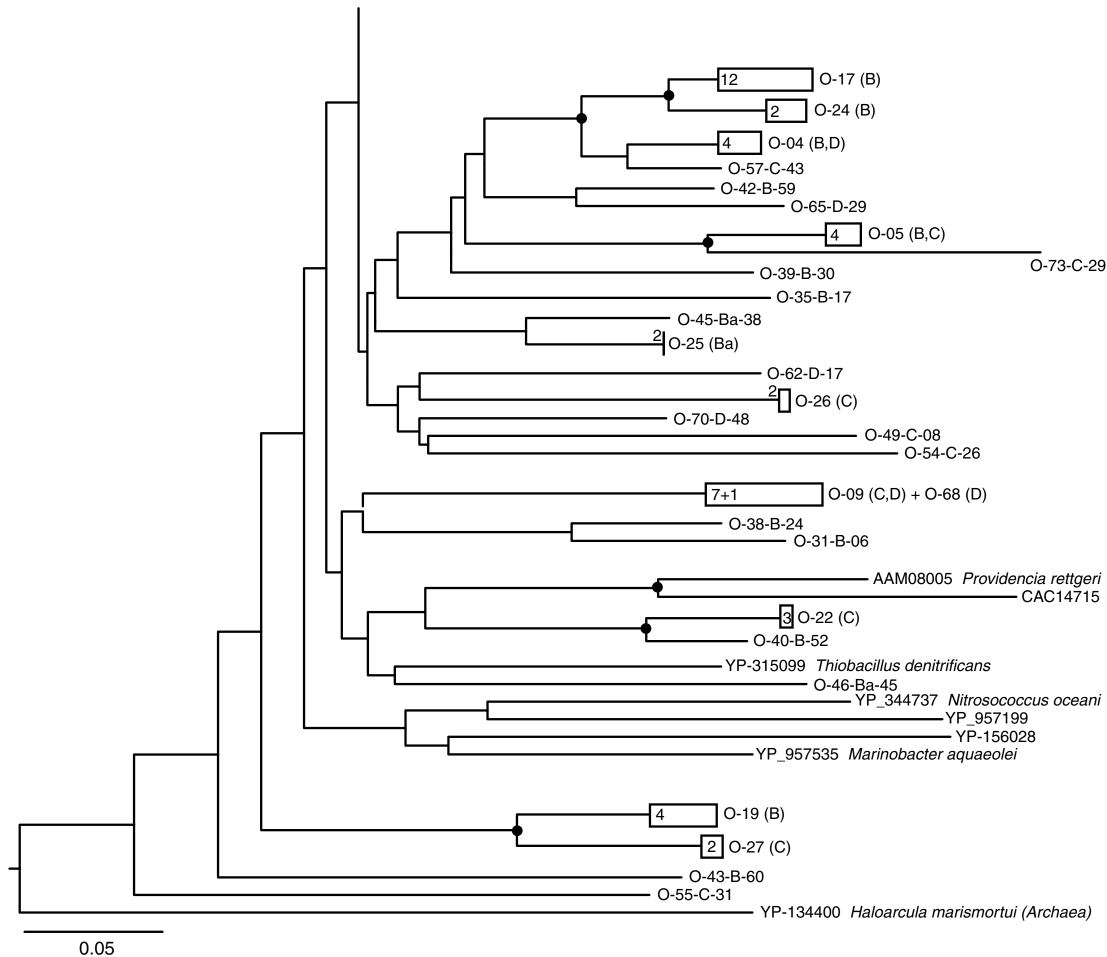

Figure 2 Continued.

(Wang *et al.*, 1987) and *B. megaterium* (Huang *et al.*, 1999) confirmed that *Firmicutes* type *merA* primers, Fi-Fw/Fi-Rv1832 and Fi-Fw/Fi-Rv1892, resulted in amplicons of approximately 395 and 455 bp, respectively (data not shown). The *β*/*γ*-type new primers worked with pDU1358 *merA* (Griffin *et al.*, 1987) (data not shown). To test the applicability of the *Actinobacteria* primers, genomic DNA from the mercury-resistant *Streptomyces* isolate (Is-BDOE1) from soil B (Oregaard *et al.*, 2007) was used in PCR reactions. The amplicon was approximately 400 bp, corresponding to the expected 391–397 bp (data not shown).

In the present study, we were interested in describing the diversity of *merA* genes in surface and sub-surface soils with a history of mercury contamination. Primers targeting *β*/*γ-Proteobacteria* (#91 and #54), *Actinobacteria* (Act-Fw and Act-Rv) and *Firmicutes* (Fi-Fw and Fi-Rv_{1892}) (Table 1) were used in PCR reactions with DNA extracted from soils pre-treated with mercury or control soils pre-treated with water. The *β*/*γ* PCR reactions gave partial *merA* amplicons in surface and both sub-surface soils, and this was seen for both stimulated and control soils. In contrast, partial *Actinobacteria* *merA* genes were only amplified in surface soil (in both control and mercury-stimulated soil). The *Firmicutes*-type *merA* was not detected in any soil. The *merA* amplicons of the *β*/*γ* and the *Actinobacteria* were cloned and transformed. Cloned partial *β*/*γ* *merA* genes, mainly from control soils stimulated with water were sequenced, resulting in 43 sequences from surface soil (B), 49 sequences from sub-surface soil (C) and 51 sequences from deeper sub-surface soil (D). Furthermore, of the 40 sequences amplified with *Actinobacteria* primers, 20 *merA* sequences turned out to be *β*/*γ-Proteobacteria* in origin (Ba). All *β*/*γ-Proteobacteria* *merA* clone sequences (B, C, D and Ba) were aligned along with 30 reference *β*/*γ* *merA* sequences from GenBank. The multiple alignment was subjected to *DOTUR* analysis (Schloss and Handelsman, 2005), allowing determination of OTUs and diversity estimates of the different soils. When discriminating bacterial species from one another based on 16S rRNA sequences, a threshold of 3% is typically used, and for genus separation, 5% are used (Schloss and Handelsman, 2005). A threshold of 5% difference was used in the analysis, obtaining more conservative estimates of diversity, compared to 3% thresh-

old. With the 5% threshold for definition of OTUs, the 163 β/γ *merA* clones fell into 70 OTUs, of which 28 contained more than one sequence. The phylogeny of the β/γ *merA* sequences from the different soils is shown in Figure 2 along with reference sequences from GenBank. Since the cloned sequences were only around 244 bp in length (excluding the primers), all the reference sequences and plasmids were also truncated, to only contain the fragment obtained with primers #91 and #54 primers. All the cloned sequences showed highest similarity to other β/γ *merA* genes at GenBank, as determined by *blastn* or *blastp* search, except clone C-31, see below. It is apparent from Figure 2 that the β/γ *merA* gene is highly diverse. Since all partial *merA* clone sequences were most similar to other *merA* sequences at GenBank, it is unlikely that the high diversity observed was due to non-specific amplification of genes other than *merA*. If such non-specific amplification occurred, it would be most probable that the amplicons had different sizes than the *merA* fragments. Interestingly, all the clones were more than 5% different from the reference sequences, except OTU-03 (B-63 and C-37) grouping with *merA* from pDU1358, and OTU-07 (Ba-39, Ba-44 and Ba-47) grouping with *merA* of plasmid pKris-2 and a *merA* gene from a *Pseudomonas* isolate (Figure 2).

Exogenous plasmid isolation had been conducted on soils B, C and D, resulting in four different plasmids (pKris-1 to -4), as determined by plasmid restriction enzyme analysis (Lipthay *et al.*, 2007). All plasmids conferred mercury resistance to their host (*E. coli* or *Pseudomonas putida*). In the present study, the *merA* gene was amplified with β/γ primers BG-Fw$_{349}$ and #54 and sequenced, obtaining over 1100 nucleotides from each *merA* gene. The phylogenetic relationship of these *merA* genes is as mentioned above, shown in Figure 2. When looking at the 244 bp fragment obtained with primers #91 and #54 (excluding primers), the four plasmids fell into three different OTUs ($\geqslant$95% identity) (Figure 2).

Estimates of *merA* diversity in the three different soil microcosms, based on the sequenced clones, are shown in Table 3. The diversity estimates were similar for all three soils. Although all three soils were very similar in *merA* diversity, the actual cloned sequences were generally different as is shown in the Venn diagram in Figure 3. The circles represent different soil clones and plasmids from these soils, as indicated by letters on their side, and reference sequences from GenBank, with the

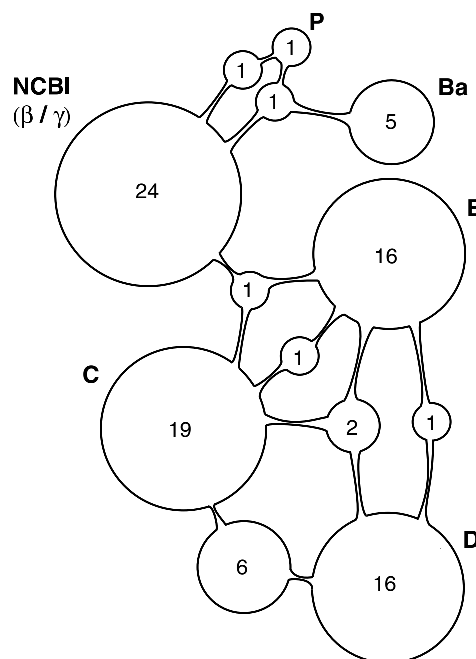

Figure 3 A Venn diagram representing shared and unique β/γ-type *merA* OTUs. The OTUs were defined as being no less than 95% identical at the DNA level. Shared OTUs are interconnected between the relevant circles. The area of the circles reflects the number of OTUs belonging to these. A total of 198 sequences were included in the analysis, of which 30 came from NCBI (27 OTUs), four sequences from exogenously isolated plasmids (three OTUs), 20 Ba sequences (six OTUs), 43 B sequences (21 OTUs), 49 C sequences (29 OTUs) and 51 D sequences (25 OTUs). OTUs, operational taxonomic units.

Table 3 Diversity of cloned β/γ-*Proteobacteria merA* sequences

Soil	*No. of sequences*	*Estimates by DOTUR*			*Coverage %*[c]
		OTU (5%)[a]	*Chao1*[b]	*Shannon–Weaver diversity index*	
B	43	21	52 (29–133)[d]	2.6 (2.3–3.0)	51
C	49	29	58 (38–117)	3.2 (2.9–3.4)	41
D	51	25	43 (30–85)	2.9 (2.6–3.2)	51
Ba[e]	20	6	8 (6–21)	1.3 (0.8–1.7)	70
B-C-D-Ba	163	68	143 (101–239)	3.8 (3.6–3.9)	52

[a]OTU, operational taxonomic unit (DNA$\geqslant$95% identical).
[b]Chao 1: bias corrected Choa1 richness estimator.
[c]Coverage: {1−[OTU/*N*]}100%; *N* = no. of sequences.
[d]Values in parenthesis: 95% confidence interval.
[e]Clones from soil B, amplified with *Actinobacteria* primers Act-Fw and Act-Rv, but showing highest similarity to β/γ-*Proteobacteria merA* DNA.

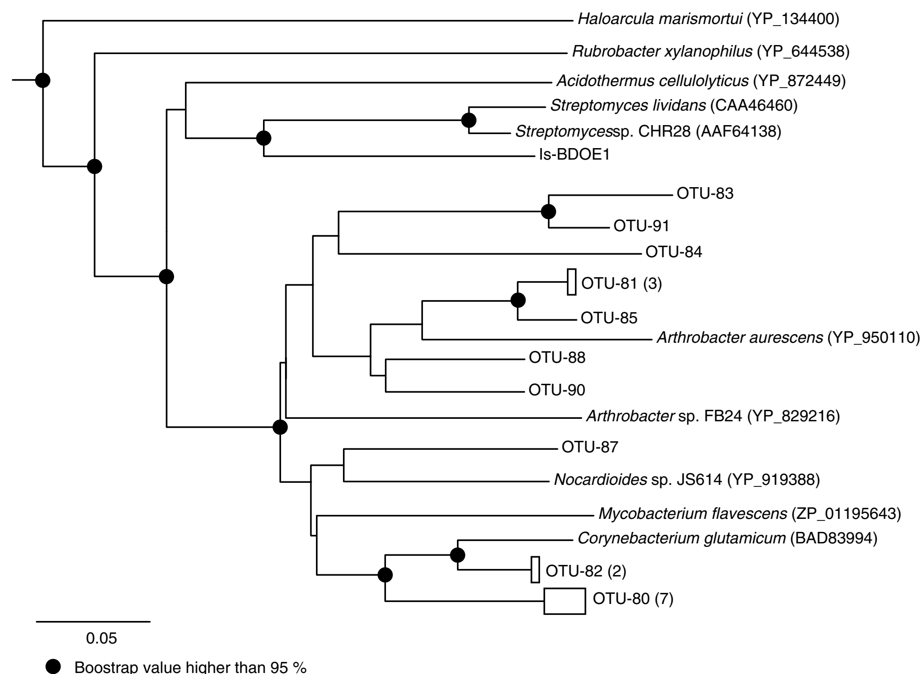

Figure 4 Neighbour-joining tree of *Actinobacteria merA* DNA clone sequences, isolate Is-BDOE1 and reference sequences from GenBank. The boxes reflect OTUs with more than one sequence. The horizontal length of a box is equivalent to the maximum length from node to tip of the particular OTU. The OTUs were defined by DOTUR ($\geqslant$95% similar). Black circles at nodes reflect bootstrap values higher than 95% (out of 1000 bootstraps). Values in parenthesis reflect the number of sequences in the particular OTU. *Archaea merA* gene from *Haloarcula marismortui* was used as outgroup. The bar at the bottom reflects 5% difference. OTU, operational taxonomic unit.

numbers inside indicating the number of OTUs. The smaller interconnecting circles show how many OTUs were shared between the connected circles (environments). Only two OTUs contained sequences from all three soils (OTU-01 and OTU-02, in total 12 sequences) (Figure 2). The OTUs with representatives of different soils seemed fairly equally distributed between B/C (four OTUs), B/D (three OTUs) and C/D (eight OTUs) (Figure 3). Note that only three OTUs were connected to GenBank sequences.

The *Actinobacteria merA* PCR and cloning resulted in 20 sequences showing highest similarity to *Actinobacteria merA* proteins. One of these was discarded, as it seemed chimeric, by high N-terminal similarity to β/γ *merA*, whereas the C-terminal protein region was most similar to *Actinobacteria*. The remaining 19 sequences were aligned against nine *Actinobacteria merA* references and a DNA Neighbour-Joining tree was calculated (Figure 4). The 19 sequences fell into 10 OTUs (5% threshold), of which seven OTUs contained one sequence. It is noteworthy that most of the cloned sequences are quite divergent from the sequences deposited at GenBank.

The C-31 clone sequence mentioned above and obtained with β/γ-type primers did not show similarity to other *merA* genes by *blastn*. When the C-31 clone sequence was virtually translated into 81 amino acids, the protein sequence showed similarity to other putative *merA* proteins from α-*Proteobacteria*, for example, *Xanthobacter autotrophicus* (59 identical amino acids (72%)) (Figure 5). The neighbour-joining tree in Figure 5 includes nine partial *merA* sequences obtained from anoxic river sediments, contaminated with mercury and obtained from PCR reactions using similar primers as in the present study (Ni Chadhain *et al.*, 2006). Note how two of the sequences (SBC-049 and SBC-183) are most closely related to other α-*Proteobacteria merA* sequences. The other seven SBC sequences all seem closer related to α-*Proteobacteria* than to the Gram-positive *Firmicutes*-type *merA* genes.

Several isolates obtained from the same soils as used in this study (Oregaard *et al.*, 2007) were subjected to *merA*-specific PCR with the primers shown in Table 1, and some were partially sequenced (Table 4). The *Streptomyces* (Is-BDOE1) isolate seemed to contain a *merA* sequence diverging from its closest relative by approximately 27% at the amino-acid level (Table 4). The *merA* gene of Is-BDOE1 was amplified on two occasions and sequenced with both primers (Act-Fw and Act-Rv) but only the forward primer resulted in acceptable sequence data. The sequences obtained with the forward primer were identical. The *Dyella*-like isolates had *merA* genes that differed somewhat from the closest match, pSB102, a full-sequenced plasmid from Alfalfa rhizosphere (Schneiker *et al.*, 2001).

Partial α-*Proteobacteria merA* DNA sequences of several different isolates were subjected to both *blastn* and *blastp* search. *Blastn* did not reveal any relatedness to other *merA* genes and did not result in any meaningful hits. *Blastp* search showed

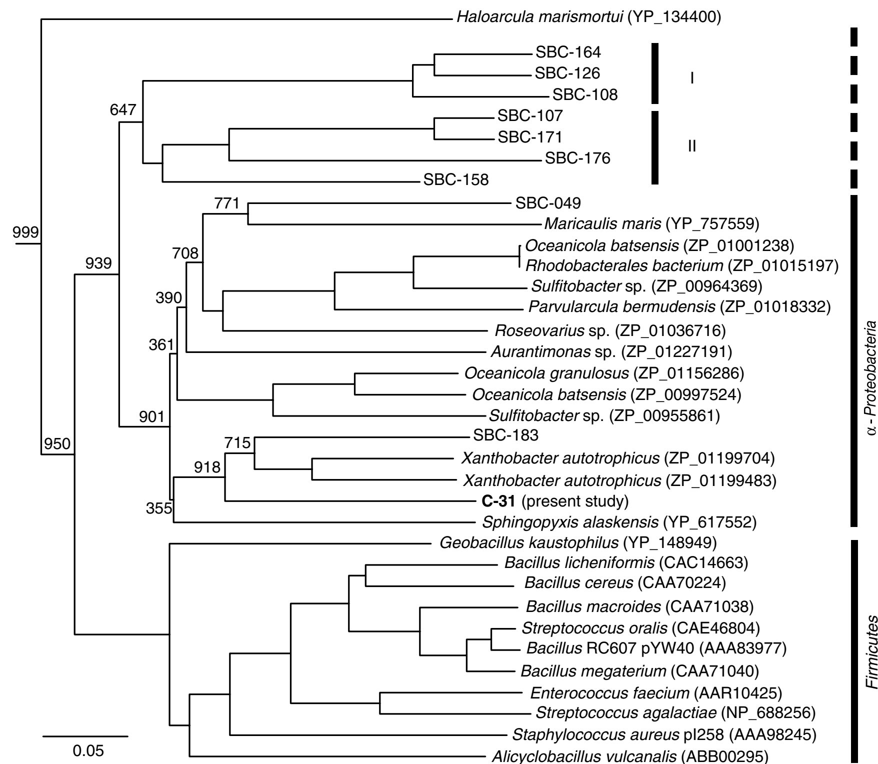

Figure 5 Neighbour-joining tree of *α-Proteobacteria merA* clone sequences obtained in the present study (C-31) and in a study of anoxic sediments (SBC-prefix), see Discussion for details. All the reference sequences from *α-Proteobacteria* are putative *merA* genes. *Firmicutes*-type *merA* DNA is also included. I and II are clades defined in the original paper describing these clones, obtained with similar primers as used for the *β/γ*-type *merA* clone library of the present study. The values to the left at some of the nodes reflect the bootstrap values (out of 1000). Archaea *merA* gene from *Haloarcula marismortui* was used as outgroup. The bar at the bottom reflects 5% difference.

Table 4 LEFPCF soil isolates from which partial *merA* sequences were obtained

Isolate[a]	*Genus*	*Phylum*[b]	*DNA sequences*[c]	*GC %*	*Blastp*		
					Ident. aa (%)[d]	*Accession*[e]	*Genus*
Is-BDOE1	*Strepytomyces*	Actino.	252	69.4	73	AAF64138	*Streptomyces* sp CHR28
Is-D308	*Bradyrhizobium*	α	785	63.8	66	ZP_01199704	*Xanthobacter*
Is-B203a	*Rhizobium*	α	781	64.0	66	ZP_01199704	*Xanthobacter*
Is-C091	*Sphingomonas*	α	783	63.9	66	ZP_01199704	*Xanthobacter*
Is-B040	*Rhizobiales-like*	α	719	63.5	62	ZP_01199704	*Xanthobacter*
Is-D310	*Cupriavidus*	β	537	65.9	100	CAC86905	*Pseudomonas putida*
Is-C065	*Ralstonia*	β	683	64.8	94	CAA70190	*Alcaligenes* sp
Is-D282	*Variovorax*	β	396	64.3	99	CAD10785	*Pseudomonas putida*
Is-D134	*Dyella*	γ	435	59.7	92	NP_361073	*pSB102*
Is-D136	*Dyella*	γ	771	64.2	85	NP_361073	*pSB102*
Is-BDOE2	*Pseudomonas*	γ	673	66.8	97	ZP_01294748	*Pseudomonas aeruginosa*
pKris-1 (plasmid)			1165	64.8	97	CAC80891	*Acinetobacter* sp
pKris-2 (plasmid)			1135	65.9	96	CAD91352	*Pseudomonas fluorescens*
pKris-3 (plasmid)			1150	65.1	96	AAR91471	*Klebsiella pneumoniae*
pKris-4 (plasmid)			1159	66.0	99	AAR31066	*pJP4*

[a]Isolates and plasmids were obtained in previous studies.
[b]Phylum of isolates, Actino., *Actinobacteria*; α, β, γ sub-phylum within *Proteobacteria*. The plamids were exogenously extracted from the soil with *γ-Proteobacteria*.
[c]The length of the sequences (not full-length *merA*).
[d]Identical amino acids in percentage (the sequences were virtually translated to amino acids and subjected to *blastp* search).
[e]Accession numbers of closest related *merA* protein.

similarity to all the putative *α-Proteobacteria merA* protein sequences used in the phylogenetic tree in Figure 5. The *α-Proteobacteria merA* fragments of the *Bradyrhizobium*, *Rhizobium* and *Sphingomonas* isolates (Table 4) were identical. The *merA* of the *Rhizobiales*-like isolate was somewhat different.

The partial *merA* sequence of the *Rhizobiales*-like isolates showed similarity to other putative *merA* genes of *α-Proteobacteria*, ranging from 62% to 49% identical amino acids. Mercuric reductases from *Firmicutes* were second in similarity to the *Rhizobiales*-like *merA* gene, the highest at 45% identity (YP_148949, *Geobacillus kaustophilus*). These are the first (partial) *merA* sequences of *α-Proteobacteria* isolates, which have a mercury-resistant phenotype.

Discussion

The diversity of mercury resistance traits—*merA*—was high in all soil samples tested in this study. There were no significant differences in diversity between surface and sub-surface samples. The *merA* diversity estimators were nevertheless lower than the similar estimators calculated based on 16S rRNA gene clone libraries from the same soil samples (unpublished results, Oregaard, de Lipthay and Sørensen). This difference could indicate recent horizontal spread of the mercury-resistance trait among the soil bacteria in response to the metal contamination. This is corroborated by the findings of identical *merA* genes in different genera of *α-Proteobacteria*, isolated from different depths (see below), and that conjugative plasmids conferring resistance to mercury have been isolated from LEFPCF soils (Lipthay *et al.*, 2007). The adaptation of the bacterial community in the soil has probably not been solely due to horizontal gene transfer of few broad host-range mercury-resistance plasmids, since many different *merA* genes are present in the soil. A combination of horizontal gene transfer and selection of resistant subpopulations seems like a plausible explanation for the observations in the present study.

Even though the soils were very similar in *merA* diversity, the composition of *merA* genes was very different, and most OTUs were unique to the depth at which they were found. It has been argued that environmental factors exerting selective pressure on particular traits will probably not be effective at the phylum level, but more so at genus or species level (Janssen, 2006), and in a recent study of approximately 1900 sequenced 16S rRNA gene clones from three agricultural soils experiencing different management treatments, highly similar diversity values were found, but the bacterial community compositions were different in the three soils (Hartmann and Widmer, 2006). The authors argue that to understand the effects of a particular environmental stimulus, community composition analysis is required, since diversity indices are often insensitive to changes inferred by the stimulus (Hartmann and Widmer, 2006). In the present study, *merA* composition at the examined depths seemed very distinct, and less than 20% (11/65; BCD) of the OTUs were shared between different soil compartments, indicating that these vertically adjacent bacterial soil communities only experience limited mixing. This would mean that horizontal gene transfer would only have limited effect on dissemination of adaptive traits between the vertically separated soil communities.

Of the 62 OTUs obtained with the *β/γ-Proteobacteria* primers (Figure 3), only one is ⩾95% similar to a GenBank reference sequence. This highlights that the actual functional diversity in natural environments is vast compared to what is recognized in culture collections. The group by Dr Janssen has demonstrated elegantly that successful cultivation of *hard to culture* recalcitrant soil bacteria can be achieved by long-term incubation on solid media with complex carbon sources (Janssen *et al.*, 2002; Davis *et al.*, 2005). In a parallel study of the soils used in the present study, several mercury-resistant bacteria were obtained by long-term incubation (Oregaard *et al.*, 2007). Although most of the isolates are similar to previously cultivated bacteria, many of the *merA* genes are novel, and when virtually translating them to protein sequences and comparing with GenBank *merA* proteins, they are less than 95% identical at the amino-acid level (Table 4).

To the best of our knowledge, this is the first time that *merA* genes have been shown in mercury-resistant *α-Proteobacteria* (soil) isolates. The first known mercury-resistant *α-Proteobacteria* was a marine *Rhizobiales*-like isolate from a hydrothermal vent plume in the Pacific ocean, at approximately 2.5 km depth (Vetriani *et al.*, 2005). The authors showed that it grew on artificial seawater medium supplemented with 2 μM $HgCl_2$ and that it volatilized oxidized Hg(II) to elemental Hg(0), but did not confirm the presence of a *merA* gene in this isolate. In a recent study with the same soils as used in the present study (Oregaard *et al.*, 2007), bacterial isolates showing high 16S rRNA gene similarity to *Azospirillum*, *Bradyrhizobium*, *Rhizobium*, *Sphingomonas* and *Rhizobiales* were isolated on dilute agar media amended with $HgCl_2$ and could subsequently be cultured in liquid 10% tryptic soy broth (TSB) supplemented with 20–50 μM $HgCl_2$, except the *Rhizobiales*-like isolate, which resisted growth in 10% TSB. In the present study, manually designed primers were obtained by examination of multiple alignments of *α-Proteobacteria* genomic *merA* sequences. *merA* PCR amplicons (approximately 812 bp) were obtained from all five different isolates mentioned above. The partial *merA* sequences were identical in the *Bradyrhizobium*, *Rhizobium* and *Sphingomonas* isolates, whereas the *Rhizobiales*-like *merA* gene was somewhat different (the *Azospirillum merA* fragment was not sequenced). The three different isolates, *Rhizobium*, *Sphingomonas* and *Bradyrhizobium*, were isolated from surface, intermediate and deep sub-surface soils. We think this is a strong indication that horizontal gene transfer plays a role in dissemination of adaptive traits between related bacteria. The genome-sequenced *α-Proteobacteria*, containing

putative *merA*, are all originating from marine environments, except the *Xanthobacter autotrophicus* Py2, originating from sludge. None of these bacteria have physiologically been characterized with regards to mercury resistance. The deep branching of the *merA* of the isolates compared to the reference sequences (data not shown) can be due to very different environments, from which they originate.

In the present study, 62 different OTUs were found in soils B, C and D (Figure 3) after sequencing 143 clones obtained with β/γ-specific *merA* primers. In a study by Barkays group, mercury-contaminated anoxic sediments from Berry's Creek, a tributary of the Hackensack river (NJ, USA), were analyzed for the presence of *merA* of the β/γ type (Ni Chadhain *et al.*, 2006). The reverse primer was the same as used in the present study (#54), whereas the forward primer was shifted three nucleotides downstream, resulting in 285 bp long amplicons. The authors found a high diversity, and with a 5% discrimination threshold between phylotypes, as used in this study, the Chao1 richness estimator gives around 92 phylotypes. The 68 OTUs in the present study were obtained from three different soils depths. If these are all considered as originating from the same site, that is 163 *merA* sequences found within 1-m depth from surface, the Chao1 richness estimator gives 143 phylotypes with a 5% threshold (Table 3), indicating that these soils are more diverse with regards to *merA* than the anoxic river sediments at Berry's Creek.

When comparing the *merA* sequences found in the present study, with the sequences from Berry's Creek sediment, and using a 5% discrimination threshold, only three OTUs contain similar sequences (OTU-01 and WBC-054; OTU3 and SBC-204 + WBC-007; OTU10 and SBC-116). This very low similarity between these sites could be due to the anoxic nature of the freshwater sediments, with selection of different *merA* genes, but this is mere speculation and more studies are required to elucidate this difference.

Interestingly, three of the clades (I–III) found in the study cited above, grouped closely to *Firmicutes*-type *merA* of *S. aureus* pI258 and *Bacillus* RC607 (Ni Chadhain *et al.*, 2006) (Figure 5). We find it probable that these sequences are of α-*Proteobacteria* origin, due to high *blastp* similarity to α-type *merA* and by high bootstrap values (95%) at the branch point of α-type and *Firmicutes*-type *merA* in the neighbour-joining tree (Figure 5). Clades I–III (Ni Chadhain *et al.*, 2006) and the genomic α *merA* seem like a monophyletic group, distinct from the *Firmicutes*. The phylogenetic relationship between α-type SBC clones and α-type isolates sequenced in this study cannot be determined, since their sequences do not overlap. An attempt to amplify *merA* of the α isolates in the present study with primers Al-Fw and #54 was unsuccessful.

The *Actinobacteria merA* clones showed that the functional diversity was not unique to the β/γ-*Proteobacteria*, but also occurring within the *Actinobacteria* phylum. With only 19 sequences, 10 different OTUs were found, and all of these were more than 5% different at the DNA level to all reference *merA* genes retrieved from GenBank. Seven of the OTUs were most closely related to Arthrobacter-like genes. The functional importance of these bacteria in the soils with regards to mercury is unclear, since it is thinkable that the primers are biased. When using the *merA* primers targeting *Actinobacteria*, we found that amplification of *merA* from Is-BDOE3, an *Arthrobacter*-like strain from Oregaard *et al.* (2007), resulted in a slightly larger *merA* fragment than obtained from Is-BDOE1. Sequencing revealed that the amplicon was most similar to a ferrochelatase-encoding gene (data not shown). A second PCR and sequencing confirmed this result. The ferrochelatase gene was quite different from all the *Actinobacteria merA* clones. We find it most probably that isolate Is-BDOE3 contains a *merA* gene, since it grows well on 10% tryptic soy broth agar supplemented with 25 μM $HgCl_2$. The primers targeting *merA* of *Actinobacteria* are less than optimal due to false-negative amplification (inability to amplify *merA* of isolate Is-BDOE3) and false-positive amplification (ability to amplify *merA* of β/γ-Proteobacteria, the Ba clones; see Figures 2 and 3). The fact that the *Arthrobacter*-like isolate did not result in a proper *merA* amplicon also hints that the seven OTUs clustering around the *Arthrobacter merA* genes might be of different origin than *Arthrobacter*.

Firmicutes-type *merA* was not amplified from control soil nor mercury stimulated soil. Initial testing of the *Firmicutes merA*-specific primers on three different *merA* genes of *Firmicutes* origin gave proper size amplicons (data not shown). It is therefore most likely that potential *Firmicutes* bacteria of these soils were not mercury-resistant, or that their DNA was extracted insufficiently, thus leading to no amplification. Insufficient DNA extraction could be due to either low abundance of *Firmicutes*-type bacteria or due to inefficient lysis/extraction.

Searching the NCBI databases for putative mercuric reductase proteins led to the definition of eight (monophyletic) groups, consisting of *Firmicutes*, *Actinobacteria*, α-, β/γ- and δ-*Proteobacteria*, *Bacteroidetes*, *Deinococcus-Thermus* and *Archaea* (Figure 1). The criterion for accepting putative *merA* genes as likely mercuric reductase proteins was the occurrence of essential cysteine and tyrosine residues (Table 2). Apart from differences in the actual sequence, the *merA* genes vary in length (according to whether they contain zero, one or two Hg-binding domains at the N terminus) and GC content. The putative mercuric reductase proteins of α- and δ-*Proteobacteria*, along with the *Deinococcus-Thermus* all lack the heavy metal associated (HMA)-domain, found in β/γ and *Firmicutes*-type *merA*. The HMA domain is not essential for resistance to

mercury (Moore and Walsh, 1989; Ledwidge *et al.*, 2005), and both *Actinobacteria* and *Archaea* isolates resistant to mercury do not have this region.

The genome-sequenced *Salinibacter ruber* DSM 13855 (Mongodin *et al.*, 2005) belonging to the *Bacteroidetes* phylum has two genes annotated as mercuric reductases (YP_446491 and YP_444230), but both lack the C-terminal cysteines, and also the tyrosine at position 264 and are thus most unlikely *merA* proteins. The outgroup in Figure 1 consisted of wrongly annotated *Salinibacter merA* gene YP_446491 along with three *δ-Proteobacteria* sequences (NP_952368, YP_628559 and YP_010258) annotated as *merA*, but without the required amino acids described above.

Interestingly, the newly genome-sequenced marine isolate *Leeuwenhoekiella blandensis* (Pinhassi *et al.*, 2006) belonging to the *Bacteroidetes* seems to contain a *merA* gene, although the particular gene is not annotated as such (ZP_01060916). The *Leeuwenhoekiella* putative *merA* also contains a heavy metal binding domain at the N-termini, an important feature of the mercury-resistance mechanism (Ledwidge *et al.*, 2005). The *Leeuwenhoekiella*-putative *merA* gene is to the best of our knowledge the first likely mercuric reductase, found in the *Bacteroidetes* phylum. We have found that the *L. blandensis* isolate grows on marine broth agar supplemented with 30 μM $HgCl_2$, but not with 40 μM $HgCl_2$ (unpublished results, Oregaard and Sørensen) corroborating that this marine *Bacteroidetes* isolate is mercury-resistant.

In conclusion, we have shown that *merA* genes from mercury-contaminated soil environments are very diverse. The three soil communities seemed to share only few OTUs, and most clones were less than 95% identical at the DNA level to *merA* sequences deposited at GenBank. Several *merA* genes of isolates obtained from the soils used in the present study were partially sequenced, and many were less than 95% identical at the amino-acid level to GenBank protein sequences. The *α-Proteobacteria merA* genes are the first evidence of a similar resistance mechanism in this sub-phylum, as observed in *β/γ-Proteobacteria*. However, nothing is known about the *mer* operon structure of the *α-Proteobacteria* isolates, and whether these genes are chromosomally or plasmid encoded. Future work will focus on these issues. With many new genomes added to the NCBI database every month, analysis of the genomes may allow design of primers targeting *merA* of *hard to culture* bacteria from phyla with only few cultivable representatives, thus allowing assessment of their importance in mercury contaminated environments.

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References

Abulencia CB, Wyborski DL, Garcia JA, Podar M, Chen W, Chang SH *et al.* (2006). Environmental whole-genome amplification to access microbial populations in contaminated sediments. *Appl Environ Microbiol* **72**: 3291–3301.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W *et al.* (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389–3402.

Barkay T, Miller SM, Summers AO. (2003). Bacterial mercury resistance from atoms to ecosystems. *FEMS Microbiol Rev* **27**: 355–384.

Barnett MO, Harris LA, Turner RR, Henson TJ, Melton RE, Stevenson RJ. (1995). Characterization of mercury species in contaminated floodplain soils. *Water Air Soil Pollution* **80**: 1105–1108.

Burger J, Campbell KR. (2004). Species differences in contaminants in fish on and adjacent to the Oak Ridge Reservation, Tennessee. *Environ Res* **96**: 145–155.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG *et al.* (2003). Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* **31**: 3497–3500.

Davis KER, Joseph SJ, Janssen PH. (2005). Effects of growth medium, inoculum size, and incubation time on culturability and isolation of soil bacteria. *Appl Environ Microbiol* **71**: 826–834.

Distefano MD, Moore MJ, Walsh CT. (1990). Active site of mercuric reductase resides at the subunit interface and requires Cys135 and Cys140 from one subunit and Cys558 and Cys559 from the adjacent subunit: evidence from *in vivo* and *in vitro* heterodimer formation. *Biochemistry* **29**: 2703–2713.

Ellis RJ, Morgan P, Weightman AJ, Fry JC. (2003). Cultivation-dependent and -independent approaches for determining bacterial diversity in heavy-metal-contaminated soil. *Appl Environ Microbiol* **69**: 3223–3230.

Eto K. (2000). Minamata disease. *Neuropathology* **20**(Suppl): S14–S19.

Gans J, Wolinsky M, Dunbar J. (2005). Computational improvements reveal great bacterial diversity and high metal toxicity in soil. *Science* **309**: 1387–1390.

Griffin HG, Foster TJ, Silver S, Misra TK. (1987). Cloning and DNA-sequence of the mercuric-resistance and organomercurial-resistance determinants of plasmid pDU1358. *Proc Natl Acad Sci USA* **84**: 3112–3116.

Han FX, Su Y, Monts DL, Waggoner CA, Plodinec MJ. (2006). Binding, distribution, and plant uptake of

mercury in a soil from Oak Ridge, Tennessee, USA. *Sci Total Environ* **368**: 753–768.

Hartmann M, Widmer F. (2006). Community structure analyses are more sensitive to differences in soil bacterial communities than anonymous diversity indices. *Appl Environ Micorbiol* **72**: 7804–7812.

Huang CC, Narita M, Yamagata T, Endo G. (1999). Identification of three merB genes and characterization of a broad-spectrum mercury resistance module encoded by a class II transposon of *Bacillus megaterium* strain MB1. *Gene* **239**: 361–366.

Janssen PH, Yates PS, Grinton BE, Taylor PM, Sait M. (2002). Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*. *Appl Environ Microbiol* **68**: 2391–2396.

Janssen PH. (2006). Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl Environ Microbiol* **72**: 1719–1728.

Laddaga RA, Chu L, Misra TK, Silver S. (1987). Nucleotide sequence and expression of the mercurial-resistance operon from *Staphylococcus aureus* plasmid pI258. *Proc Natl Acad Sci USA* **84**: 5106–5110.

Ledwidge R, Patel B, Dong A, Fiedler D, Falkowski M, Zelikova J *et al.* (2005). NmerA, the metal binding domain of mercuric ion reductase, removes Hg2+ from proteins, delivers it to the catalytic core, and protects cells under glutathione-depleted conditions. *Biochemistry* **44**: 11402–11416.

Lipthay JRd, Rasmussen LD, Oregaard G, Simonsen K, Bahl MI, Kroer N *et al.* (2007). The adaptive potential of subsurface microbial communities to mercury. *FEMS Microbiol Ecol* (submitted).

Lowe M, Madsen EL, Schindler K, Smith C, Emrich S, Robb F *et al.* (2002). Geochemistry and microbial diversity of a trichlorethane-contaminated superfund site undergoing intrinsic *in situ* reductive dechlorination. *FEMS Microbiol Ecol* **40**: 123–134.

Mongodin EF, Nelson KE, Daugherty S, Deboy RT, Wister J, Khouri H *et al.* (2005). The genome of *Salinibacter ruber*: convergence and gene exchange among hyperhalophilic bacteria and archaea. *Proc Natl Acad Sci USA* **102**: 18147–18152.

Moore MJ, Walsh CT. (1989). Mutagenesis of the N- and C-terminal cysteine pairs of Tn501 mercuric ion reductase: consequences for bacterial detoxification of mercurials. *Biochemistry* **28**: 1183–1194.

Moore MJ, Miller SM, Walsh CT. (1992). C-terminal cysteines of Tn501 mercuric ion reductase. *Biochemistry* **31**: 1677–1685.

Muller AK, Rasmussen LD, Sørensen SJ. (2001a). Adaptation of the bacterial community to mercury contamination. *FEMS Microbiol Lett* **204**: 49–53.

Muller AK, Westergaard K, Christensen S, Sørensen SJ. (2001b). The effect of long-term mercury pollution on the soil microbial community. *FEMS Microbiol Ecol* **36**: 11–19.

Ni Chadhain SM, Schaefer JK, Crane S, Zylstra GJ, Barkay T. (2006). Analysis of mercuric reductase (merA) gene diversity in an anaerobic mercury-contaminated sediment enrichment. *Environ Microbiol* **8**: 1746–1752.

Nies DH. (1999). Microbial heavy-metal resistance. *Appl Microbiol and Biotechnol* **51**: 730–750.

North NN, Dollhopf SL, Petrie L, Istok JD, Balkwill DL, Kostka JE. (2004). Change in bacterial community structure during *in situ* biostimulation of subsurface sediment cocontaminated with uranium and nitrate. *Appl Environ Microbiol* **70**: 4911–4920.

Nriagu JO, Pacyna JM. (1988). Quantitative assessment of worldwide contamination of air, water and soils by trace metals. *Nature* **333**: 134–139.

Oregaard G, Sørensen SJ. (2007). Cultivation of mercury resistant bacteria from mercury contaminated soil (Oak Ridge, Tennessee, USA). *FEMS Microbiol Ecol* (submitted).

Osborn AM, Bruce KD, Strike P, Ritchie DA. (1997). Distribution diversity evolution of the bacterial mercury resistance (mer) operon. *FEMS Microbiol Rev* **19**: 239–262.

Pinhassi J, Bowman JP, Nedashkovskaya OI, Lekunberri I, Gomez-Consarnau L, Pedros-Alio C. (2006). *Leeuwenhoekiella blandensis* sp. nov., a genome-sequenced marine member of the family Flavobacteriaceae. *Int J Syst Evol Microbiol* **56**: 1489–1493.

Rasmussen L, Sørensen SJ. (2001). Effects of mercury contamination on the culturable heterotrophic, functional and genetic diversity of the bacterial community in soil. *FEMS Microbiol Ecol* **36**: 1–9.

Rasmussen LD, Sørensen SJ. (1998). The effect of longterm exposure to mercury on the bacterial community in marine sediment. *Curr Microbiol* **36**: 291–297.

Revis NW, Osborne TR, Holdsworth G, Hadden C. (1989). Distribution of mercury species in soil from a mercury-contaminated site. *Water Air Soil Pollution* **45**: 105–113.

Schaefer JK, Yagi J, Reinfelder JR, Cardona T, Ellickson KM, Tel-Or S *et al.* (2004). Role of the bacterial organomercury lyase (MerB) in controlling methylmercury accumulation in mercury-contaminated natural waters. *Environ Sci Technol* **38**: 4304–4311.

Schaffer AA, Aravind L, Madden TL, Shavirin S, Spouge JL, Wolf YI *et al.* (2001). Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements. *Nucleic Acids Res* **29**: 2994–3005.

Schelert J, Dixit V, Hoang V, Simbahan J, Drozda M, Blum P. (2004). Occurrence and characterization of mercury resistance in the hyperthermophilic archaeon *Sulfolobus solfataricus* by use of gene disruption. *J Bacteriol* **186**: 427–437.

Schiering N, Kabsch W, Moore MJ, Distefano MD, Walsh CT, Pai EF. (1991). Structure of the detoxification catalyst mercuric ion reductase from *Bacillus* sp. strain RC607. *Nature* **352**: 168–172.

Schloss PD, Handelsman J. (2005). Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl Environ Microbiol* **71**: 1501–1506.

Schneiker S, Keller M, Droge M, Lanka E, Puhler A, Selbitschka W. (2001). The genetic organization and evolution of the broad host range mercury resistance plasmid pSB102 isolated from a microbial population residing in the rhizosphere of alfalfa. *Nucleic Acids Res* **29**: 5169–5181.

Schuster PF, Krabbenhoft DP, Naftz DL, Cecil LD, Olson ML, Dewild JF *et al.* (2002). Atmospheric mercury deposition during the last 270 years: a glacial ice core record of natural and anthropogenic sources. *Environ Sci Technol* **36**: 2302–2310.

Silver S, Phung LT. (1996). Bacterial heavy metal resistance: new surprises. *Annu Rev Microbiol* **50**: 753–789.

Sørensen SJ, Bailey M, Hansen LH, Kroer N, Wuertz S. (2005). Studying plasmid horizontal transfer *in situ*: a critical review. *Nat Rev Microbiol* **3**: 700–710.

Sørensen SJ, de Lipthay JR, Müller AK, Barkay T, Hansen LH, Rasmussen LD. (2002). Molecular methods for assessing and manipulating the diversity of microbial populations and processes. In: Burns RG, Dick RP (eds). *Enzymes in the Environment*. Marcel Dekker Inc.: New York, pp 363–390.

Stevens RT, Ashwood TL, Sleeman JM. (1997). Mercury in hair of Muskrats (*Ondatra zibethicus*) and Mink (*Mustela vison*) from the US Department of Energy Oak Ridge Reservation. *Bull Environ Contaminat Toxicol* **58**: 720–725.

Tomiyasu T, Matsuyama A, Eguchi T, Fuchigami Y, Oki K, Horvat M *et al.* (2006). Spatial variations of mercury in sediment of Minamata Bay, Japan. *Sci Total Environ* **368**: 283–290.

Top EM, Springae Dl, Boon N. (2002). Catabolic mobile genetic elements and their potential use in bioaugmentation of polluted soils and waters. *FEMS Microbiol Ecol* **42**: 199–208.

Vetriani C, Chew YS, Miller SM, Yagi J, Coombs J, Lutz RA *et al.* (2005). Mercury adaptation among bacteria from a deep-sea hydrothermal vent. *Appl Environ Microbiol* **71**: 220–226.

Wang Y, Mahler I, Levinson HS, Halvorson HO. (1987). Cloning and expression in *Escherichia coli* of chromosomal mercury resistance genes from a *Bacillus* sp. *J Bacteriol* **169**: 4848–4851.

Wernersson R, Pedersen AG. (2003). RevTrans: multiple alignment of coding DNA from aligned amino acid sequences. *Nucleic Acids Res* **31**: 3537–3539.

Zhou J, Xia B, Huang H, Palumbo AV, Tiedje JM. (2004). Microbial diversity and heterogeneity in sandy subsurface soils. *Appl Environ Microbiol* **70**: 1723–1734.