Fish Farming Affects the Abundance and Diversity of the Mercury Resistance Gene *merA* in Marine Sediments

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(Received March 1, 2011—Accepted April 11, 2011—Published online May 11, 2011)

Microbes in sediments below fish farming cages are exposed to a variety of compounds used in aquaculture, such as antifoulants, antimicrobials and mercury. Here, we assessed the abundance of the mercury resistance gene *merA* in the sediment of four fish farms and four pristine sites using quantitative PCR, and at three fish farms and two pristine sites by constructing clone libraries and sequencing *merA* amplicons. The abundance of *merA* was consistently greater at fish farm sites than at pristine sites, and the *merA* phylotypes were different between fish farm and pristine sites. Mercury concentrations were too low to cause selection pressure and did not differ significantly among samples. The primers designed specifically for this study were capable of detecting two new potential *merA* clades.

Key words: aquaculture, Baltic sediment, *merA*, mercury, resistance

Fish farms in natural waters are open systems that can influence the chemical and ecological balance of the farming area. Fish feed, pharmaceuticals (for example antibiotics) and anti-foulants (for example copper compounds) used in aquaculture (16) can affect microbial communities in the sediments under the fish cages, increasing the incidence of microbes carrying different resistance genes (3). In addition to additives such as antibiotics, fish feed has been reported to contain considerable amounts of mercury (5).

The mercury resistance (*mer*) operon is widely distributed among microbes and has been found in chromosomes and mobile elements, such as plasmids and transposons (18, 24). The resistance mechanism is based on the reduction of Hg(II) by *merA*-encoded mercury reductase (2) to the volatile form Hg(0), which diffuses passively out of the cell. Culture-independent methods have shown that *merA* resistance genes are highly diverse and are commonly found in mercury-contaminated soils and sediments (4, 17, 21). Previous studies have focused on highly contaminated or mercury pre-treated samples. Furthermore, a few studies have reported on the temporal stability of *merA* (14) or the sequence diversity among different locations (17, 19).

In this study, we studied the abundance and diversity of *merA* in fish farm and pristine sediments. Sediment samples were collected from four geographically distinct fish farms located in the northern Baltic Sea. Specific PCR primers were designed, capable of detecting *merA* genes from Gram-negative bacteria without prior enrichment by mercury and suitable for quantitative PCR. The primers were used to quantify *merA* in farm and pristine sediments and to construct *merA* clone libraries. Potential selection pressure for *merA* was evaluated by measuring total and bioavailable concentrations of mercury in the sediments. Our results show that

the abundance and diversity of *merA* genes is increased in the sediment impacted by fish farming.

Materials and Methods

Sediment sampling

The first set of sediment samples was collected during the summers of 2006–2008 from FIN1 and FIN2 farm sites, two medium-scale fish farms in the northern Baltic Sea in the Turku Archipelago. In addition, SWE1 and SWE2 farm sites corresponding to two small-to-medium scale farms in the Stockholm Archipelago were sampled in August 2007. Pristine reference samples outside the FIN1 farm site were collected at distances of 200, 400, 600, 800 and 1,000 m from the farm. Pristine samples from the FIN2, SWE1 and SWE2 farm sites were collected at a distance of 200 m from each farm. The sampling locations are in shallow water (around 10 meters) in close vicinity to the shore (less than 100 meters). Pristine control samples and gradient samples were taken away from the farm along the shoreline. The Baltic Sea is a eutrophic low-salinity brackish-water sea with no tide. Sampling sites are characterized in Table 1. Surface sediment (upper 5 cm; 0–5 cm) was collected with an Ekman Grab (ENVCO, Romania) during the summer of 2006 and with a Limnos sediment sampler (Limnos, Turku, Finland) during the summers of 2007 and 2008. Sediments were instantly frozen on dry ice and stored at –80°C. Oxygen content, pH and temperature were measured from water immediately above the sediment (Eutech Instruments, Thermo Scientific, USA).

DNA extraction

DNA was extracted from 0.5 g fish farm and pristine site sediments using the FastDNA SPIN Kit for Soil and the FastPrep Instrument (MP Biomedicals, Santa Ana, CA, USA), as described by the manufacturer. In an added step, DNA was washed twice with 1 mL of 5.5 M guanidine thiocyanate before transferring the binding matrix suspension to the spin filter tubes to remove humic acids.

merA primer design and PCR

Forward (*merAf*) and reverse (*merAr*) PCR primers targeting *merA* were designed by aligning *merA* genes of Gram-negative bacterial origin from the GenBank nucleotide database using MUSCLE (6). The specificity of the primers for *merA* was

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Table 1. Sampling locations and their characteristics

Sampling site	Farmed fish	Production volume (tons year ⁻¹)	Antibiotic use	Other notes
FIN1 farm site	Common whitefish, rainbow trout	50	Stopped using antibiotics in ~2,000.	
FIN1 200–1000 m sites	None	None	None	Sampled 200–1,000 m from FIN1 farm site at 200-m intervals.
FIN2 farm site	Rainbow trout	50	Stopped using tetracyclines in ~2,000; have since occasionally used florfenicol	
FIN2 pristine site	None	None	None	Sampled 200 m from FIN2 farm site.
SWE1 farm site	Rainbow trout	5	Florfenicol occasionally	
SWE1 pristine site	None	None	None	Sampled 200 m from SWE1 farm site.
SWE2 farm site	Rainbow trout	50	Florfenicol occasionally	
SWE2 pristine site	None	None	None	Sampled 200 m from SWE2 farm site.

assessed by a BLAST search (1) of the primer sequences against the NCBI nucleotide database using the blastn algorithm with settings adjusted for a short input sequence. The primers were tested on DNA extracts from the fish farm sediments. *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA, USA) genomic extract and sterile water were used as a negative control. The PCR reaction mixture consisted of 1 U Platinum *Taq* DNA polymerase (Finnzymes, Espoo, Finland), 1×Platinum *Taq* buffer, 0.2 mM of each dNTP, 1.5 mM MgCl₂, 0.5 µM each of merAf and merAr primers, 1 µL sediment DNA, and water in a total volume of 20 µL. The PCR program was an initial denaturation step of 94°C for 1 min, 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, followed by final extension at 72°C for 10 min. The amplicons obtained by PCR were visualized by electrophoresis on 1% agarose gels and sequenced to confirm the specificity of the primers.

Quantitative PCR of merA and 16S rRNA genes

Quantitative real-time PCR was performed using the *merA*-targeting primers merAf and merAr. 16S rRNA gene copy numbers, determined using universal 16S rRNA gene targeting primers pA (7) and 358R (published as forward primer (12)), were used to normalize the number of *merA* copies in individual sediment samples, allowing bacterial abundance to be monitored and simplifying comparisons among different sediment samples. Serial dilutions of a plasmid constructed from the pDrive vector (Qiagen, Hilden, Germany) and *merA* fragments were used as a standard for *merA* quantification. Serial dilutions of *E. coli* K12 strain genomic DNA, containing seven copies of 16S rRNA gene per chromosome, were used as standards for quantification of the 16S rRNA gene. Real-time PCR was performed using an Applied Biosystems 7500 Real-time PCR System (Applied Biosystems, Carlsbad, CA, USA) with MicroAmp Optical 96-Well Reaction Plates (Applied Biosystems) and a DyNamo Flash SYBR Green qPCR Kit (Finnzymes). The reaction mixture consisted of 1× DyNamo Flash SYBR Green reaction mixture, 0.5 µM each of forward and reverse primers, 1×ROX passive reference dye and 5 µL DNA in a reaction volume of 25 µL. All reactions, including negative controls, were conducted in triplicate. The following cycling conditions were used: initial denaturation at 95°C for 7 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Melting curves for the amplicons were measured by increasing the temperature slowly from 60°C to 95°C while monitoring fluorescence. The results were analyzed using Sequence Detection Software v.1.2.3. (Applied Biosystems, Foster City, CA, USA). The differences in quantitative PCR results between farm and pristine sediments were tested using the Mann-Whitney test provided by R (R Development Core Team, 2010).

merA clone libraries

merA clone libraries were generated by amplification of DNA extracted from fish farm and pristine site sediments using PCR and merAf and merAr primers, as described above. The *merA* amplicons

were cloned using a Qiagen PCR Cloning Plus Kit (Qiagen), and plasmid DNA was extracted with Qiagen Biorobot 3000 (Qiagen). The inserts were re-amplified from the cloning vector using M13-targeting forward and reverse primers (pDrive; Qiagen). The resulting amplicons were purified with MultiScreen 384 PCR plates (Millipore, Billerica, MA, USA) and sequenced using a 3730 DNA Analyzer (Applied Biosystems), a BigDye Terminator v.3.1 Cycle Sequencing Kit and a primer targeting the T7 site in the cloning vector (Qiagen).

merA sequence analyses and phylogeny

The *merA* clone library sequences were trimmed using the Staden Package Trev 1.9 program (25) to isolate the 322-bp region between the *merA*-specific primers merAf and merAr used in cloning. Clone sequences were aligned for analysis by ClustalW (30). A distance matrix was created using an F84 substitution model in DNADIST in the PHYLIP package (8). Phylotype richness was estimated using DOTUR (22).

For phylogenetic analyses, the trimmed sequences sharing 99% similarity were combined with the Staden Package Gap 4.10 program and aligned using ClustalW (30) and MUSCLE (6). BLAST analysis (1) was performed using the blastn algorithm with default settings against the NCBI nucleotide database to identify the sequences. A phylogenetic tree was constructed using a maximum likelihood-based method RAXML-VI-HP (26) with a GTR+MIX model and 1,000 bootstrap samples. A consensus tree was generated using CONSENSE from the PHYLIP package (8).

The sequences were subjected to Bayesian clustering using BAPS (29). Statistical support for Bayesian clustering by sampling site was assessed using the chi-square tests provided by R (20).

Bioavailability of mercury

The presence of bioavailable mercury in sediment samples was determined using bacterial bioreporters. The bioluminescent bioreporter *E. coli* strains MC1061 (pmerRlux) (9), which responds to inorganic mercury, and MC1061 (pmerRBlux) (11), which responds to both inorganic and organic mercury, were used. The *E. coli* MC1061 (pDNlux) strain was used as a non-inducible control (11). This control strain takes into account potential changes in the luminescence of bioreporter bacteria not caused by mercury, including potential interference of the sample matrix (stimulation or quenching of the luminescence) due to the presence of nutrients, toxicants or turbidity of the sample, and changes in luminescence attributable to changes in the physiological state of the cell.

Lyophilized bioreporter bacteria were rehydrated with HMM medium (13) supplemented with 0.5% hydrolyzed casein and 0.4% glucose. Bacteria were incubated at room temperature for 2 hours before bioavailability assays.

Sediment suspensions were prepared by adding 9 mL sterile water to 1 g (wet weight) frozen sediment and then shaking on a rotary shaker at 220 rpm for 24 h. For subsequent measurements, suspensions were diluted up to 300-fold with sterile water. Mercury

standards were prepared by making serial dilutions of HgCl_2 in sterile water. For bioavailability assays, 50 μL mercury solution, sediment suspension or water was plated in 96-well microplates (PerkinElmer, Waltham, MA, US) and mixed with an equal volume of bacterial suspension. Plates were then incubated for 2 h at 37°C , and luminescence was measured using a Victor Wallac 1420 Multilabel Counter (PerkinElmer, Finland). All measurements were obtained from two replicates in each of two independent experiments. Bioreporter induction was reported as an induction coefficient (IC), calculated as $\text{IC} = L_M/L_W$, where L_M is the luminescence in the metal solution and L_W is the background luminescence measured in water. The limit of detection was determined according to the scheme presented by (10).

Chemical analysis of mercury

Geochemical analysis of mercury was performed at the analytical chemistry laboratory of Labtium Oy (Espoo, Finland). The sediment samples were freeze-dried, sieved to obtain the <2-mm fraction, homogenized and digested using hydrofluoric acid and perchloric acid. Mercury was analyzed with an Hg analyzer AMA254 (LECO, St. Joseph, MI, USA) using the pyrolytic method.

Accession numbers of the *merA* sequences

The sequences were deposited in the EMBL nucleotide sequence database under accession numbers FN677991–FN678064 for SWE1 pristine site sequences, FN678065–FN678149 for FIN1 pristine site sequences, FN678150–FN678215 for SWE1 farm site sequences, FN678216–FN678281 for FIN2 farm site sequences and FN678282–FN678351 for FIN1 farm site sequences.

Results

Performance of *merA*-specific primers

Primers were designed to target *merA* genes typical of Gram-negative bacteria because this group had suitable conserved areas in the sequence alignment of 44 sequences (the sequence alignment is available as supplementary material). The designed primers *merAf* (5'-GTG CCG TCC AAG ATC ATG-3') and *merAr* (5'-GGT GGA AGT CCA GTA GGG TGA-3') amplified the expected 360-bp gene fragment from sediment DNA extracts without generating nonspecific products (data not shown). The amplicon of this length is also suitable for quantitative PCR. Clone libraries confirmed that the amplicon was a *merA* gene fragment.

Quantification of *merA*

The *merA* genes were quantified by quantitative PCR using the *merAf* and *merAr* primers. *merA* was detected in sediments under the farms at all time points (Fig. 1). Outside the farms, *merA* could be quantified at least once at every sampling location, but often the amount was below the limit of detection (2.0×10^4 copies g^{-1} wet weight sediment). The levels of *merA* genes were normalized to those of 16S rRNA genes, quantified using primers pA and 358R, to allow comparison of *merA* amounts in different sediments. At every sampling time, the levels of *merA* genes and the *merA*/16S rRNA ratio were higher at the fish farms than at the respective pristine sites sampled at a distance of 200–1,000 m from the FIN1 farm site and 200 m from FIN2, SWE1 and SWE2 farm sites. The level of 16S rRNA genes remained relatively constant among different locations. Our results showed that *merA* genes were present at both farm sites and the pristine locations, but the amount of *merA* was increased in fish farm sediments compared with those of pristine locations over the surveillance period of three successive summers. According to the Mann-Whitney test, the increase was significant at $P < 0.001$.

Sequencing effort and diversity estimates

Libraries of *merA* gene fragments were constructed and sequenced from the first samplings collected in 2006 from the FIN1 farm site, the FIN1 pristine site (200 m from the FIN1 farm site) the FIN2 farm site, and the SWE1 farm and

Table 2. Number of sequences, OTU numbers at a unique distance and Shannon diversity estimates of the *merA* clones

Site	Number of sequences	Values by DOTUR	
		OTUs	Shannon diversity index (95% confidence interval)
FIN1 farm site	70	27	2.5 (2.2–2.8)
FIN1 pristine site (200 m)	85	31	1.9 (1.5–2.3)
FIN2 farm site	66	34	2.9 (2.6–3.2)
SWE1 farm site	66	29	2.5 (2.1–2.8)
SWE1 pristine site	74	22	1.6 (1.1–2.0)

Fig. 1. Mercuric reductase-encoding *merA* and 16S rRNA gene copy numbers and their ratios in fish farm and pristine site sediments. Bars indicate the ratio of *merA* and 16S rRNA genes. Error bars indicate standard deviations of three replicates in two independent measurements. Symbol ○ indicates 16S rRNA genes and symbol ● *merA* genes.

Fig. 2. Observed richness (A) and Shannon diversity (B) curves of *merA* clone libraries from DOTUR at different sampling locations using an evolutionary distance of 0 (unique). The sampling locations are indicated as follows: ■ FIN1 farm site, ▲ FIN2 farm site, ● SWE1 farm site, □ FIN1 pristine site and ○ SWE1 pristine site.

pristine sites to observe the phylotype diversities of the *merA* gene at these locations. Library sequencing yielded 358 sequences (Table 2), ranging in length from 315 to 322 bp. Random sampling methods in DOTUR were applied to the sequence data to estimate the sufficiency of the sequencing effort. The diversity of different *merA* phylotypes at unique distances was adequately represented by the sequences obtained, as indicated by rarefaction and Shannon collector curves that reached a plateau at the shortest (unique) evolutionary distance (Fig. 2). The Shannon diversity index can therefore be reliably used to estimate the *merA* phylotype diversity at the sampling locations (Table 2). The Shannon diversity index showed greater *merA* diversity at fish farms than at pristine sites.

BAPS analysis

To organize the sequences of different *merA* phylotypes, we clustered the sequences according to sequence similarities using the BAPS package. BAPS uses Bayesian inference to investigate the nucleotide sequences of *merA* genes to identify the most likely partition of the sequences into discreet clusters. BAPS identified a partition with seven clusters that had posterior probability close to 1 over multiple estimation runs. The distribution of BAPS clusters in different sampling locations was visualized in Fig. 3. The statistical significance of the differences in BAPS cluster distribution between the sampling locations was estimated using pairwise chi-square tests. The chi-square analyses revealed that the Bayesian clustering pattern differed significantly ($P < 0.001$) among sampling sites. This overall difference reflects a significant difference among farms ($P < 0.001$) and between farm and pristine sites ($P < 0.001$); however, the two pristine sites were not significantly different ($P = 0.16$). Fish farming thus appears to have an effect on the phylotype distribution pattern of *merA* genes.

Fig. 3. Percentages of BAPS clusters at different sampling locations. The distribution of BAPS clusters was significantly different among fish farms (chi-square $P < 0.001$), whereas there were no significant differences among pristine sites (chi-square $P = 0.16$). When grouped together, farm and pristine site clusters differed significantly (chi-square $P < 0.001$). BAPS clusters are indicated as follows: ○ cluster 1, △ cluster 2, + cluster 3, × cluster 4, ◇ cluster 5, ■ cluster 6, ● cluster 7.

Phylogeny and BLAST analyses

A phylogenetic tree of the *merA* sequences was constructed using maximum likelihood analysis with 1,000 bootstrap samples. The phylogenetic tree strongly supported the division between the phylotypes that were prominent at the fish farms and those that predominated at pristine sites (Fig. 4). Pristine sites were dominated by *merA* clones with low

The whole-cell bacterial bioreporters *E. coli* MC1061 (pmerRlux) and *E. coli* MC1061 (pmerRBlux) were used to detect the bioavailable fraction of mercury in sediment samples collected at fish farms FIN1 and FIN2 and the pristine site from June and September 2006. No bioavailable mercury was detected in any of the samples (the limits of detection for inorganic and organic mercury were 2.8 and

Our results show that *merA* genes are present in all sediment samples, although only low amounts of mercury and no bioavailable mercury were detected. The levels of *merA* and the *merA*/16S rRNA ratio were greater at fish farms than at pristine sites during the 3-year surveillance period (Fig. 1). To our knowledge, the natural background of *merA* in pristine environments has been reported in only one previous study

(17). The primers used in most studies are unable to detect *merA* present in low quantities in uncontaminated environments, and therefore these previous studies have concentrated on mercury-contaminated environments or samples that were enrichment-cultivated with added mercury.

Because the selection pressure caused by mercury was low or nonexistent in all sediments, we first investigated co-selection as the explanation for the elevated *merA* amount. Selection could take place with antibiotic or other resistance genes (15, 31) because most farms treat fish with antibiotics at least occasionally. Co-selection with other antibiotic resistance genes was unlikely at the FIN1 farm site, where no antibiotics have been used since 2000, but it was possible at the other farms. We have previously studied the diversity of the tetracycline resistance genes *tetC* and *tetM* in the same sediment samples (27) and found that the diversity of these genes was much lower than the diversity of *merA*. Furthermore, because the levels of *tetC* and *tetM* genes at the farm sites were 10–100-fold higher than the levels of *merA* (28), it is unlikely that the increase in the copy number of *merA* genes could be explained solely by co-selection with tetracycline resistance; however, the results do not exclude the possibility that some co-selection could take place between *merA* and some other antibiotic or metal resistance genes.

The increase in the number of *merA* genes can potentially be caused by a continuous supply of *merA* genes to fish farm sediments in fish feces or uneaten feed. Fish feces apparently contribute to the microbial community under the farms. Although the fish gut is not a well-studied environment, the animal gut is generally considered a hotspot for horizontal transfer and enrichment of resistance genes (23). Fish feed often contains significant amounts of mercury (5); this is because it is made from predator fish species that accumulate mercury from the environment due to their relatively high location in the food chain. We therefore assume that the selection for *merA*-positive bacteria could take place in the farm-fish gut, where the mercury-containing fish feed is in close contact with a dense and diverse microbial community. Alternatively, selection may have occurred within the predator fish used as a component in feed manufacturing.

We here report two previously undiscovered putative *merA* clades that are prominent in pristine Baltic Sea sediments (Fig. 4). The nucleotide similarity of these phylotypes to existing database sequences was much lower than the nucleotide similarity of the farm-type phylotypes. The tyrosine residue essential for *merA* function (position 264 in the reference *merA* sequence of *Bacillus cereus*, BAB622433) (17) was present in all sequences. The four cysteines and one remaining tyrosine residue essential for *merA* function were located outside our amplicon; therefore, it was not possible to determine whether the genes translate into functional MerA proteins. Molecular studies have previously succeeded in discovering novel *merA* phylotypes (4, 17); however, the present study revealed the highest proportion of novel phylotypes yet discovered, likely reflecting the use of primers with wide specificity. New potential variants of *merA* genes can be found using culture-independent methods, but further research is needed

to confirm whether these genes are functional or even truly related to mercury resistance.

In conclusion, we have used a novel primer set to show that the levels and diversity of *merA* genes are increased in fish farm sediments with an apparent lack of selection pressure from mercury. We have furthermore revealed a previously undetected diversity of *merA* phylotypes, some dominant at fish farming sites and some more typical at pristine locations. The PCR primers described in this study may be applicable for different mercury-contaminated and uncontaminated sample types in future studies to identify new potential *merA* clades.

Acknowledgements

The study was financially supported by the Maj and Tor Nessling foundation, EnSTe Graduate School and the Academy of Finland.

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