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Manila clams from Hg polluted sediments of Marano and Grado lagoons (Italy) harbor detoxifying Hg resistant bacteria in soft tissues

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ABSTRACT

A mechanism of mercury detoxification has been suggested by a previous study on Hg bioaccumulation in Manila clams (*Ruditapes philippinarum*) in the polluted Marano and Grado lagoons and in this study we demonstrate that this event could be partly related to the detoxifying activities of Hg-resistant bacteria (MRB) harbored in clam soft tissues. Therefore, natural clams were collected in six stations during two different periods (winter and spring) from Marano and Grado Lagoons. Siphons, gills and hepatopancreas from acclimatized clams were sterile dissected to isolate MRB. These anatomical parts were glass homogenized or used for whole, and they were lying on a solid medium containing 5 mg l^{-1} $HgCl_2$ and incubated at 30 °C. A total of fourteen bacterial strains were isolated and were identified by 16S rDNA sequencing and analysis, revealing that strains were representative of eight bacterial genera, four of which were Gram-positive (*Enterococcus, Bacillus, Jeotgalicoccus* and *Staphylococcus*) and other four were Gram-negative (*Stenotrophomonas, Vibrio, Raoultella* and *Enterobacter*). Plasmids and *merA* genes were found and their sequences determined. Fluorescence in situ hybridization (FISH) technique shows the presence of *Firmicutes*, *Actinobacteria* and *Gammaproteobacteria* by using different molecular probes in siphon and gills. Bacterial clumps inside clam flesh were observed and even a Gram-negative endosymbiont was disclosed by transmission electronic microscope inside clam cells. Bacteria harbored in cavities of soft tissue have mercury detoxifying activity. This feature was confirmed by the determination of mercuric reductase in glass-homogenized siphons and gills.

1. Introduction

Manila clams (*Ruditapes philippinarum*) are euryhaline species and feed on particulate organic matter (POM) mainly constituted by algal plankton, benthic algae and organic detritus that are carried along the bottom sediments. These species live in sand, sandy-silt or muddy-gravel bottoms from intertidal zone to several meters depth. The natural habitat is located in the Indo-Pacific region Japan, Korea and China (Gosling, 2003), while the coastal lagoons of the Northern Adriatic Sea are among the most worldwide productive locations of Manila clams. Although introduced in Italy in 1983 from the Indo-Pacific, fishing and exploitation of Manila clams increased over time, and Italy has become the leading country in Europe for production of this shellfish (Chiesa et al., 2011). Indeed, Manila clams adapted very well to the environmental conditions of both Tyrrhenian and Adriatic Italian coasts. Marano and Grado lagoons (located in the Northern Adriatic Sea) represent two of the most important sites of Manila clam aquaculture; however, the clams cultivation is threatened by Hg pollution. Hg accumulation in Manila clams is of great interest because they are widely consumed by humans worldwide and this cultivation in a high Hg polluted area such as Marano and Grado lagoons (Northern Adriatic Sea) is a challenge for local fishermen. The sediments of Marano and Grado lagoons are polluted by Hg in the form of cinnabar transported by the Isonzo (Soča) River (Foucher et al., 2009) and from a dismissed chlor-alkali plant (Covelli et al., 2009). A recent survey on total mercury and methylmercury (MeHg) accumulation in soft tissues of reared and wild Manila clams from this region (Giani et al., 2012) revealed the existence of a Hg content higher than that found in the same organisms cultivated in other Italian areas (Trombini et al., 2003; Sfriso et al., 2008) and in other parts of the world (Pan and Wang, 2011), but MeHg/Hg ratio was significantly lower. Overall, bivalves reduce the metal toxicity through different molecular mechanisms including its sequestration in metallothionein (MT) and lipofuscin, forming inert granules in lysosomal vesicles for storage and finally for cell exocytosis (Marigomez et al., 2002). In this study we would like to explore

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Hg detoxifying activity depending on mercury-resistant bacteria (MRB) in clams from Marano and Grado lagoons. This Hg-detoxifying activity was already detected in the two lagoons, where MRB represented up to 45% of cultivable aerobic heterotrophic bacteria in sediments (Baldi et al., 2012). In general, MRB possess one or more copy/ies of the *mer* operon that may be located on plasmids, trasposons, and/or chromosomes (Fondi et al., 2010). This operon contains the seven genes *merR*, *merT*, *merP*, *merC*, *merD*, *merA*, and *merB* (Barkay et al., 2003; Nascimento and Chartone-Souza, 2007). The *merA* gene codes for the mercuric reductase (MerA), an enzyme responsible for Hg^{2+} reduction to Hg^0, which is removed from the cytoplasm by passive diffusion out of cells because it is a rather volatile element. MerB, an organomercurial lyase, enables to break down C–Hg bond of MeHg down to methane and ionic mercury is always coupled to MerA, which reduces Hg^{2+} to Hg^0. The passive efflux of Hg^0 from bacterial cells is considered a beneficial adaptation and this process is often reported as one of biotechnologically important processes to reduce Hg contamination in the environment. Since MRB can survive in environments containing high ionic Hg concentrations, they, contribute to the detoxification of the surrounding habitat through Hg efflux. Therefore, the aim of this study was to check the presence of MRB in clam soft tissue and their characterization through a combination of different techniques, including isolation procedures, and identification associated with their MerA activity. In addition to this, we used optical and transmission electron microscopy (TEM), and the technique of fluorescence in situ hybridization (FISH) in order to localize bacteria in gills and siphons, the two anatomical parts mostly influenced by lagoon water flux, if we think that one individual can filter up to 0.6–1.6 l per hour (Gosling, 2003).

2. Materials and methods

2.1. Wild Manila clams sampling

In this study we use wild Manila clams (*Ruditapes philippinarum*), belonging to same pools (3.5–4.0 cm shell length class size), which were previously analyzed for bioaccumulation of total mercury and methylmercury from Marano and Grado lagoons (Giani et al., 2012). Wild clams were harvested in December 2008 in 2 sites: in MA station close to Lignano tidal inlet, where commercial Manila clam farming is currently active and at MB station near the Stella River, a new candidate area for clam farming (Fig. 1). In March 2009 the bivalves were taken in four sites: (i) at MC station, an interdicted area for clam harvesting (Giani et al., 2012), because of Hg contamination due to Aussa River plume affected by the dismissed chlor-alkali plant outflow located at Torviscosa (Covelli et al., 2009; Baldi et al., 2012); (ii) at the edge of Grado lagoon (IGC station) which is known to be polluted by Hg, mainly in the form of cinnabar transported by the Isonzo (Soča) River (Foucher et al., 2009); (iii–iv) clam pools were harvested at two additional stations, M2 and M4, from the less Hg polluted sector of Marano Lagoon, no far from MB and MA stations respectively (Fig. 1).

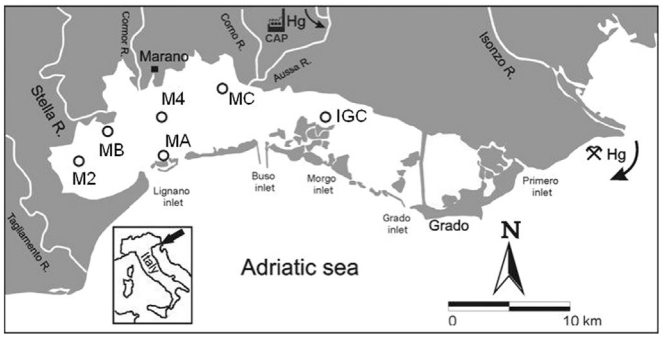

Fig. 1. Sampling sites of harvested Manila clams in winter and springtime at Marano and Grado lagoons in Northern Adriatic sea (Italy).

Sampling was carried out in winter and spring by trawling and/or manual collection; clams were then transported to the laboratory in 2 h, acclimatized for 48 h in artificial sea-water (22 °C, 33% salinity) (Kester et al., 1967).

2.2. MRB isolation from soft tissues

Siphons, gills and hepatopancreas of individual clam of the same class size (3.5–4 cm) were glass homogenized to isolated MRB by streaking the dense suspension on solid Nelson Medium (NeM) at *pH* 7.5 and 30 °C, containing per liter: 2 g D-glucose, 5 g casamino acids, 1 g yeast extract, 10 g NaCl, 2.3 g $MgCl_2 \cdot 6H_2O$, 3 g KCl, and 15 g Bacto-agar (Difco). After autoclaving at 50 °C solid NeM was amended with $HgCl_2$ (5 μg ml^{-1} Hg). Colonies with different shapes, colors and consistency were purified twice on agar plates and single colony was stored in cryovials at −80 °C in 20% glycerol for further analyses. In spring the isolation of MRB was also carried out directly on whole siphons and gills, which were cut off with a scalpel and the tissue was lying on solid NeM, and we wait until colonies were formed on the tissue (Fig. S1). These colonies were isolated and stored as above reported.

2.3. Analysis of plasmid content

Analytical amounts of plasmid DNA were obtained from 1.5 ml bacterial cultures using either the alkaline lysis method (Sambrook et al., 1989) or the commercial Kit Plasmid Miniprep (Qiagen) according to the manufacturer's instructions. The presence of plasmid molecules was analyzed by agarose gel (0.8% w/v) electrophoresis in TAE buffer (0.04 M Tris-Acetate, 0.01 M EDTA) containing 0.5 μg ml^{-1} (w/v) of ethidium bromide.

2.4. Preparation of cell lysates or genomic DNA and amplification of 16S rRNA genes from bacterial isolates

For preparation of cell lysates, bacterial colonies grown overnight at 28 °C on LB (Luria Broth) plates were resuspended in 20 μl sterile distilled water, heated to 95 °C for 10 min, and cooled on ice for 5 min. Genomic DNA extraction was carried out using the CTAB (hexadecyltrimethylammonium bromide) method as previously described (Giovannetti et al., 1990).

Two μl of each cell lysate were used for the amplification *via* polymerase chain reaction (PCR). Amplification of 16S rRNA genes was performed in a total volume of 50 μl containing 1 × Reaction Buffer, 150 μM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 250 μM, and 2.0 U of Polytaq DNA polymerase (all reagents obtained from Polymed, Florence, Italy) and 0.6 μM of each primer [P0 5′ GAGAGTTTGATCCTGGCTCAG, and P6 5′ CTACGGCTACCTTGTTACGA] (Grifoni et al., 1995). A primary denaturation treatment of 90 s at 95 °C was performed and amplification of 16S rRNA genes was carried out for 30 cycles consisting of 30 s at 95 °C, 30 s at 50 °C and 1 min at 72 °C, with a final extension of 10 min at 72 °C.

Amplification of *merA* gene was carried out using the following primer sets, set 1 (merAgram-forward CCGTCCAAGATCATGAT merAgram-reverse, GGRTCGGTRA-ACACCAC) and set 2 (merAgram+forward GGAAGAAMACCRAATAC merA-gram+reverse, CCTTCWGHCATTGTTA), for Gram-negative (Gram−) and Gram-positive (Gram+) bacteria, respectively. The two primer sets were designed as follows: for both Gram positive and Gram negative bacteria all the *merA* sequences available in database were retrieved. Then, a multialignment using a reduction set of the retrieved sequences was constructed in order to check for the presence of highly conserved regions that might represent the target sites for ad hoc designed primer. Once the conserved regions were identified, two primer sets were designed (one for the Gram positive and the other for the Gram negative bacteria) and the presence of the identified target sequence was checked in each sequence of the complete multialignment. The amplification profiles were as follows; for PCR employing the primer set 1 a primary denaturation at 95 °C for 2 min was followed by 30 cycles consisting of 95 °C for 30 s, 51 °C for 45 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. For PCR employing the primer set 2 a primary denaturation at 95 °C for 2 min was followed by 30 cycles consisting of 95 °C for 30 s, Ta (48, 45, 43) °C for 45 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The expected size of the amplicon was about 930 and 540 bp for Gram negative and Gram positive bacteria respectively, corresponding to a *merA* region spanning from nt 451 and 1381 and nt 1330 and 1870 for Gram negative and Gram positive bacteria, respectively.

2.5. Sequencing of 16S rRNA and merA genes

Amplicons corresponding to the 16S rRNA or *merA* genes (observed under UV, 312 nm) were excised from the gel and purified using the "QIAquick" gel extraction kit (QiAgen, Chatsworth, CA, USA) according to manufacturer's instructions. Direct sequencing was performed on both DNA strands using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and the chemical dye terminator (Sanger et al., 1977). Each sequence was submitted to GenBank and assigned the accession number.