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Tetracycline resistance gene *tet*(M) of a marine bacterial strain is not accumulated in bivalves from seawater in clam tank experiment and mussel monitoring

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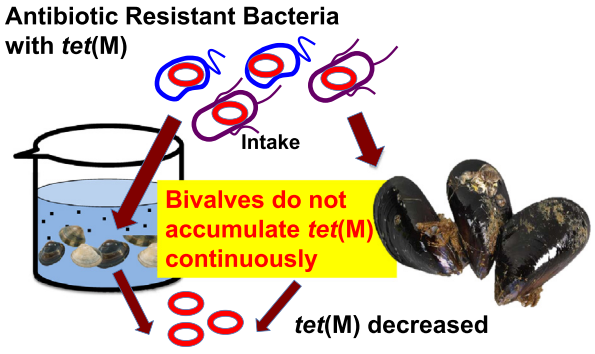
HIGHLIGHTS

- Japanese littleneck clams quickly took up *tet*(M)-possessing bacteria.
- *tet*(M) level in clams remained constant for 96 h with bacteria addition every 24 h.
- Mediterranean mussels collected in the field did not show accumulation of *tet*(M) from seawater.
- This study suggests no continuous accumulation of *tet*(M) from natural bacterial communities.

GRAPHICAL ABSTRACT



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ABSTRACT

Antibiotic resistance genes (ARGs) are found in marine as well as terrestrial bacteria. Bivalves are known to accumulate chemical pollutants and pathogenic microbes, however, the fate of ARGs in bivalves after the intake of ARG-possessing bacteria is not known. Here we show that the copy number of oxytetracycline resistance gene *tet*(M) increased rapidly in the clam digestive tract by filtering water, then remained constant over 96 h in a tank experiment even with the addition of *tet*(M)-possessing bacteria every 24 h. >99.9% of the added *tet*(M) was decomposed, reaching a balanced state. Environmental sampling of mussel digestive tract and seawater supported the hypothesis that *tet*(M) was decomposed in bivalves as *tet*(M) was present in seawater from April to October at a concentration of 10^{-5} to 10^{-6} copies/16S, whereas *tet*(M) in mussels was mostly below the detection limit. Two (April) and three (July and October) individual mussels were positive for *tet*(M) with a concentration equivalent to that of seawater. We therefore conclude that bivalves do not accumulate *tet*(M) from seawater.

1. Introduction

The World Health Organization proposed the "One Health Approach" to tackle the antibiotic resistance issue (WHO, 2015), which integrates health of human, animal and environment. Not only pathogenic bacteria but also environmental bacteria are a target of this approach.

Antibiotic resistant bacteria (ARB) are developed by gene mutation or acquisition of antibiotic resistance genes (ARGs) through horizontal gene transfer. We reported that the tetracycline resistance gene *tet*(M) could be transferred from marine γ-Proteobacteria to *E. coli* and

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fish pathogen *Lactococcus* to human *Enterococcus* (Neela et al., 2009). It is reported that *tet*(M) widely distributed in aquatic environments including seawater (Suzuki, 2010). This example suggests that certain ARGs, such as *tet*(M), can be transferred among bacterial communities in various environments (Smets and Barkay, 2005; Suzuki and Hoa, 2012). Horizontal gene transfer among human and natural environments is probable hypothesis (Vaz-Moreira et al., 2014; Manaia, 2017). In order to prevent exposure to and the spread of ARB, attention should be paid to potential reservoirs and vectors in the aquatic environment as well as in clinical settings. The potential spread of resistances from aquatic bacteria to human pathogens is still debated, and the consequent risk for human health is unclear. Thus, there is a need to examine both risk evaluation and control of ARB and ARGs in the marine environment from both clinical and environmental viewpoints. Seafood consumption (Alderman and Hasting, 1998) and recreation in the sea (Leonard et al., 2015) are main routes of exposure to marine ARGs.

The coastal sea environment and microflora in seawater are closely related to and affected by human life, and vice versa. Chemical pollution (Islam and Tanaka, 2004) and gene contamination (Marti et al., 2013) are known to occur in coastal areas, and contamination can be monitored by biological indicators. Bivalves are frequently used as biological indicators for contaminants such as metals (Wang et al., 2005). The "mussel watch" is a well-known monitoring campaign that utilizes the Mediterranean mussel (*Mytilus galloprovincialis*) (Goldberg, 1986) and was conducted around the world (Cantillo, 1998). Bivalves filter large quantities of water, for example, clearance rate of mussel *Mytilis coruscus* was 0.3–2.3 L/h/g (Sui et al., 2016) and thus concentrate the components in water, making them useful for monitoring. Concentration occurs for not only chemical pollutants but also pathogenic microbes including bacteria (Ripabelli et al., 1999) and viruses (La Bella et al., 2017), and as bivalves can remove *E. coli* (Ismail et al., 2016), they play both roles of accumulation and decomposition.

We hypothesized that bivalves are candidate ARG reservoirs, which might be exposure risks to consumers of fisheries food. This is not clarified so far. Present study examines whether the bivalves accumulate ARGs from surrounding seawater. To achieve this trial, we used *tet*(M) gene as a target factor, because the *tet*(M) is a widely distributed gene among *tet* series (Roberts et al., 2012) including aquatic bacteria (Suzuki, 2010). Since the *tet*(M) distributes among fish disease bacteria in coast (Kim et al., 2004), defined experiment using the *tet*(M) and the host bacterium *Photobacterium damselae* subsp. *damselae* is a rationale combination for this study. Some reports are available on the detection of antibiotic resistant *Vibrio parahaemolyticus* (Letchumanan et al., 2015) and *E. coli* (Grevskott et al., 2017) from bivalves; however, quantitative evidence of specific ARG accumulation in natural bivalves has not been reported to date. In this study, we examined the fate of *tet*(M) after uptake into bivalves in not only tank experiments with clams but also in environmental monitoring of mussels.

2. Materials and methods

2.1. Tank experiments with clams

ARB *Photobacterium damselae* subsp. *damselae* 04Ya311 strain (Nonaka et al., 2007) was used for the tank experiments with Japanese littleneck clams *Ruditapes philippinarum*. The 04Ya311 strain is multi-drug resistant bacteria isolated from seawater. For use in tank experiments, the 04Ya311 strain was cultured in Marine Broth (MB, Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 10 μg/mL of oxytetracycline (OTC) at 25 °C for 24 h, and cells were collected by centrifugation at 10,000 ×*g* for 10 min and suspended in autoclaved artificial seawater. Colony forming unit (cfu) was measured with agar plate of MB. Cells were adjusted to a final concentration of 10^5 cfu/mL in the tank. One cell of this strain harbors single copy of pAQU1 (Nonaka et al., 2012; Bien et al., 2015); this plasmid shows multi-drug resistance and has seven ARGs, including *tet*(M), a widely detected tetracycline resistance gene (Roberts et al., 2012). Since single copy of *tet*(M) is coded on the pAQU1, quantitation of *tet*(M) reflects the *tet*(M) and pAQU1 number in the 04Ya311 strain population in the tank. Thus, we quantified *tet*(M) as a factor of pAQU1.

For the tank experiments, 26 clams were reared in each glass tank with autoclaved seawater (volume, 2 L) with aeration at 20 °C. Although the clearance rate of *R. philippinarum* is not known, the clearance rate was assumed to be approximately 14 mL/min per gram of clam weight based on data on a similar species (*Mercenaria mercenaria*) (Gainey Jr., 2007). The clams used in this study had a shell length (mean±SD) of 3.1 ± 0.4 cm with average wet weight of 6.6 g. The experimental design (Fig. 1) comprises four treatment groups (each group in a single tank): 1) clams plus bacteria, 2) clams only, 3) bacteria only and 4) autoclaved seawater. Seawater in each tank was exchanged every 24 h, at which time bacteria were added to groups 1 and 3. Before the experiment, it was confirmed that *tet*(M) was not detected in the clams and autoclaved seawater. Four clams (n = 4) were taken in each sampling performed before the start of the experiment (sample B), immediately after the first addition of bacteria (within several minutes, sample 0 h) and every 24 h (samples 24, 48, 72 and 96 h). At the same time, 200 mL of water was taken from each tank for DNA extraction to quantify *tet*(M).

From each clam, the digestive tract was taken by dissection, and DNA was extracted. The average weight of the digestive tract was 0.31 ± 0.11 g. Bivalves have an open blood-vascular system, allowing bacteria circulation in other organs. Since it is difficult to take DNA quantitatively due to leaking out of body fluid and contamination of inhibitor from hepatopancreas, we selectively took digestive tract.

In order to evaluate the degradation of *tet*(M) in seawater containing clam excreta, water from the tank with 26 clams reared in autoclaved seawater for 48 h was divided into two treatments (26 living clams and no clams), and the 04Ya311 strain (10^5 cfu/mL) was added. Separately, to examine bacteria adsorption on shells, autoclaved seawater was allocated to three treatments (26 autoclaved empty shells, 26 living clams and no clams), and the 04Ya311 strain was added. All treatments along with autoclaved seawater were incubated for 24 h and *tet*(M) was quantified in the water at time points B, 0 and 24 h.

2.2. Environmental mussel monitoring

Eleven Mediterranean mussels (*Mytilus galloprovincialis*) were sampled on April 22 and July 21, and twelve mussels were sampled on October 18 in 2016 from the same colonizing group on a pier in Mori Harbor, Iyo City, Ehime, Japan (33°74′51.73″ N, 132°67′93.17″ E). Surface seawater (1 L) around the pier was sampled with sterile bucket in triplicate at the time of collection. Mussels and seawater were transported on ice to the laboratory, and DNA extraction was performed within a few hours by the method as follows.

2.3. DNA extraction

Total DNA from clams and mussels was purified with an ISOFECAL kit (Nippon Gene, Toyama, Japan). Basically, intestine samples (45–50 mg) were homogenized in 500 μL of 1% SDS solution followed by heating at 65 °C for 1 h. After centrifugation at 12,000 ×*g* for 5 min, hexadecyltrimethylammonium bromide (CTAB) was added to the supernatant (300 μL) at a final concentration of 2%. Chloroform extraction was performed, and DNA was precipitated with a precipitation solution included in the kit. DNA was further purified and concentrated by ethanol precipitation and dried.

For DNA purification from seawater, 100 mL of tank seawater (clam tank experiment) or 200 mL of natural seawater (environmental mussel monitoring) was filtered through a 47 mm diameter filter with 0.2 μm pore size (Nuclepore filter, Whatman, GE Healthcare, Buckinghamshire, UK). The filter was cut into strips and suspended in CTAB buffer