Distribution of Tetracycline Resistance Gene, tet(M), in Gram-Positive and Gram-Negative Bacteria Isolated from Sediment and Seawater at a Coastal Aquaculture Site in Japan

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We found increased numbers of oxytetracycline (OTC)-resistant bacteria in sediment and seawater around a marine aquaculture site after OTC therapy. Samples were collected at an aquaculture site along the coast of the Seto Inland Sea, Japan in 2004. In April, the percentage of bacteria resistant to 60 µg mL⁻¹ OTC in the surface sediment was 6.8%–20.0%. The percentages increased during OTC therapy in the summer reaching 53.3%–60.7% in September. Ninety-two days after drug cessation, the percentages decreased to below 22.9%. Tet(M)-positive bacteria were detected in the sediment and seawater samples. Tet(M) was evident in both Gram-positive and Gram-negative bacteria from various genera, and was newly identified in Paenibacillus, Sporosarcina, Shewanella, and Pseudoalteromonas. The dominant tet(M)-positive isolates were strains of Vibrio suggesting that this genus is an important reservoir for tet(M) in the marine environment. Two different alleles were found, tet(M)-A and tet(M)-B, each in isolates from five genera. The data suggests drug therapy used in the aquaculture acted as a selective pressure promoting increased numbers of resistant bacteria.

Key words: tetracycline resistance, tet(M), distribution, marine sediment and seawater, aquaculture

The tetracyclines (TCs), discovered in the 1940s, are a family of antibiotics that inhibit protein synthesis by preventing aminoacyl-tRNA from attaching to the ribosome. Oxytetracycline (OTC) is one of the most extensively used drugs in aquaculture for chemotherapy against fish disease in Japan and occurrences of OTC or TC resistant bacteria in fish and shrimp pathogens have been reported[2,5,11,18,35] where resistance is a severe problem in aquaculture production. Several mechanisms of resistance to TCs are known[6,24,25,29] and currently, 38 different TC resistance genes have been reported including 23 coding for the energy-dependent efflux proteins, 11 coding for ribosomal protection proteins (RPPs), and four genes for alternative mechanisms[9,29].

The occurrence of the TC resistance gene at sites where TC has been used for chemotherapy or as a growth promoter has been studied in terrestrial environments. Tet(M) is the most extensively studied antibiotic resistance gene in terms of molecular ecology[9] and has been found among numerous species of Gram-negative and Gram-positive bacteria including not only human pathogens[6,20,22,38] but also bacteria obtained from several terrestrial environments associated with human activity[9] such as in agriculture[33], groundwater[8], and daily diets[14,16,26,39]. Tet(M) is often accompanied by mobile elements such as plasmids and transposons, such associations contributing to the wide distribution of this gene in bacterial genera obtained from different environments[9]. It is suggested that there is a gene pool and TC resistance genes circulate among the microbes in the rumen, gut, and feces of production animals where RPP genes including tet(M) occur[9]. Thus, studying the molecular ecology of the antibiotic resistance gene is required to understand the impact of the use of antibiotics on the dissemination of the resistance gene in the environment.

However, few studies have examined the distribution of resistance genes circulating in the marine environment.
around aquaculture sites\textsuperscript{13,17}. Previously, we reported the occurrence of \textit{tet}(M) among the intestinal microflora (and fish pathogens) isolated from cultured yellowtail (\textit{Seriola quinqueradiata}) and isolates from seawater\textsuperscript{17}; however there is no information about the dissemination of \textit{tet}(M) in the sediment of coastal aquaculture sites. To clarify the reservoir of \textit{tet}(M) in the sediment is necessary for understanding the occurrence and flux of the resistance in the aquaculture environment. Further, the bacterial community in the sediment under the cages may be substantially affected because the drug is administered by mixing it with food for fish and uneaten food and feces end up on the bottom.

Our objectives were to clarify the effect of the administration of OTC on the bacterial community in sediment and seawater of the aquaculture site by determining the increase and decrease OTC-resistant bacteria knowing the drug dose regimen; additionally, to investigate the presence of \textit{tet}(M) in the marine environment; and to clarify the diversity of the reservoir of genes by detecting the bacterial genera possessing \textit{tet}(M) in sediment and seawater from a coastal aquaculture site.

**Materials and Methods**

**Sediment and water sampling**

Samples were taken at an aquaculture site (Fig. 1) along the coast of the Seto Inland Sea, Japan, on April 20, May 14, June 18, September 17, and December 8, 2004 (Table 1). Sediment cores (5 cm) were collected using a KK-core sampler under or beside the fish cages (Fig. 1). The water depth was approximately 6 m at all sites. The sediment samples were horizontally sectioned at 3 cm intervals. The cut samples were stored in sterile plastic bags and transferred to the laboratory on ice. Surface seawater samples were taken at all sites using a plastic bucket and stored on ice. All microbiological techniques using the sampled sediment and seawater were performed within 9 h.

**OTC Regimen**

Pen culture net cages (4.5 m×4.5 m×2 m depth) were set at the aquaculture site for short-term rearing of juvenile fish. The culture conditions are summarized in Table 1. Juvenile red seabream (\textit{Paragrus major}) were cultured from May 11 to June 1 (22 days, “term-1”). Subsequently, another group of the same species was reared from June 15 to 18 (4 days, “term-2”). Juvenile convict grouper (\textit{Epinephelus septemfasciatus}) were then cultured from August 14 to 16 (3 days, “term-3”); followed by juvenile red spotted grouper (\textit{Epinephelus akaara}) from September 3 to 6 (4 days, “term-4”).

Oxytetracycline (OTC, KOHKIN CHEMICAL CO., LTD, Osaka, Japan) was administered to the fish by mixing the drug with dry and/or moist pellet feed. The total amounts of OTC administered to the aquaculture site during May, June, August and September were 2.0 kg, 0.8 kg, 1.2 kg, and 0.03 kg, respectively (Table 1). The first drug regimen was 14 days of OTC followed by 3 days of flumequine; then the administration of OTC was resumed for 3 days. During the second, third, and fourth regimens, OTC was administered continuously for 4, 3, and 3 days, respectively. There were 13, 26, and 16 days, respectively, between each drug administration period.

**Colony counting and isolation of OTC-resistant bacteria**

The CFU numbers were measured using the plate spread method. Five hundred milligrams of sediment was homogenized in 4.5 ml of phosphate-buffered saline (PBS: 100 mM phosphate buffer, 2.7 mM KCl, and 137 mM NaCl, pH 7.4); and serial 10-fold dilutions were prepared. One hundred microliters of each dilution was spread onto Marine Broth 2216 (MB) (DIFCO Laboratories, Sparks, MD) plus 1.5% agar with 0, 60, 120, 240, and 480 µg mL\textsuperscript{-1} of OTC (Nacalai
Tesque, Kyoto, Japan); and plates were incubated at 25°C for 4 days. Resistant colonies were randomly selected from the plates containing OTC for further experiments.

**DNA purification**

DNA from the cultured bacteria was purified as described previously\(^2\).

**Amplification of tet(M) and 16S rRNA gene by PCR**

The primers used for amplification of tet(M) were tet(M)-1: 5'-GTAAAATAGTTCTTGGAG-3' and tet(M)-2: 5'-CTAAGATATGGCTCACA-3'\(^1\). To determine the bacterial genus, the primers f341 (5'-CCTACGGGAGGCAGCAG-3') and r534 (5'-ATTACCGCGGCTGCTGG-3') were used for the amplification of the V3 variable region of the bacterial 16S rRNA gene. The sizes of the PCR products for the tet(M) and V3 region were 657 bp and 194 bp, respectively. The reaction mixtures for the PCR contained 1×PCR buffer (TaKaRa, Ohtsu, Japan), 0.2 mM of each dNTP, 0.5 μM of each primer, 0.625 U of TaKaRa Ex Taq (TaKaRa), and 20–90 ng of template DNA in a final volume of 25 μl. Amplification reactions were performed using a GeneAmp PCR System 9700 (Applied Biosystems Japan Ltd. Tokyo, Japan). The reaction consisted of a denaturation
at 94°C for 5 min, followed by 30 cycles: denaturing at 94°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 1 min; and a final extension at 72°C for 5 min.

**DNA Sequencing**

Sequencing of the PCR products was performed by Macrogen, Inc. (Seoul, Korea). The same primers used for PCR amplification were used for sequencing the tet(M) and V3 region, respectively.

**Phylogenetic analysis**

The sequence of the 16S rRNA gene was aligned with the nearest reference sequences in the DDBJ/EMBL/GenBank database using BLAST with Clustal X 1.83 software. A phylogenetic tree was constructed using the neighbor joining method. The sequences of tet(M) obtained in this study were compared to the tet(M) sequences deposited in the DDBJ database using BLAST.

**Nucleotide sequence accession numbers**

The sequences of tet(M) determined in this study were submitted to the DDBJ database and have been given serial accession numbers from AB267750 to AB267805.

**Results**

**CFU numbers**

The number of CFU in the 0–3 cm and 4–6 cm sediment cores was $1.0 \times 10^5$ to $1.0 \times 10^6$ CFU per g during April to December (Fig. 2). The CFU count of the seawater was $3.5 \times 10^3$ to $4.3 \times 10^5$ per ml during the same period. The percentage of OTC-resistant bacteria at 60 µg mL$^{-1}$ in the surface core (0–3 cm) increased from 20.0% to 60.7% (Site 1) and 6.8% to 56.3% (Site 2) from April to September. Similarly, an increase from 10.0% to 53.3% was observed at a depth below 4–6 cm in the sediment. The probabilities in all samples decreased to below 22.9% in December. A similar pattern was observed using 120 µg mL$^{-1}$ OTC; however, the percentage of resistant bacteria was lower. On 60 µg mL$^{-1}$ OTC plates, the percentage of resistant bacteria from the seawater samples was highest in September at 39.1% and 64.8% at site 1 and 2, respectively. In December, the percentage decreased to 4.7% and 20.7%. The percentages of resistant bacteria on the 120, 240, and 480 µg mL$^{-1}$ OTC plates were also highest in September. At site 3, a control site where no OTC was used, no significant changes were observed over the period of the experiment.

Fig. 2. Incidence of OTC-resistant bacteria. A, B, C, sediment (0–3 cm layer), D, E, F, sediment (3–6 cm layer) and G, H, I, seawater: 60 µg mL$^{-1}$ (diamond), 120 µg mL$^{-1}$ (square), 240 µg mL$^{-1}$ (triangle), and 480 µg mL$^{-1}$ (cross). Gray bars show the total CFU per g sediment.
Detection of tet(M) in the isolates from sediment and seawater

Detection of tet(M) using PCR showed the presence of tet(M)-positive bacteria in almost all sediment and seawater samples during April to September (Table 2).

The tet(M)-possessing bacteria in sediment and seawater

To determine the diversity of tet(M)-possessing bacteria at the aquaculture site, sequencing of the V3 region of the 16S rRNA gene of 88 OTC-resistant isolates from sediment and 25 from seawater was performed (Fig. 3). Seventy-five of the 88 strains isolated from sediment were grouped in Vibrio-Photobacterium; four strains were grouped in Shewanella, four in Bacillus, and two in Pseudoalteromonas. A single strain each of Lactococcus, Paenibacillus, and Sporosarcina were found. Thus, in the sediment samples, both Gram-positive and Gram-negative bacteria were identified as tet(M)-positive bacteria.

Twenty-two of 25 strains isolated from seawater were grouped in Vibrio-Photobacterium; a single isolate was Pseudomonas; and two strains were Pseudoalteromonas. Thus, tet(M)-positive bacteria from seawater were entirely Gram-negative bacteria. The sequence of the 16S rRNA gene showed that Vibrio-Photobacterium fell into 19 phylogenetic groups (V1–V19), Shewanella into four (S1–S4), and Bacillus into three (B1–B3). Each group of Pseudomonas (P), Pseudoalteromonas (A), Lactobacillus (LA), Paenibacillus (PE), and Sporosarcina (SP) showed a single phylogenetic group. Among the tet(M)-positive strains from sediment and seawater respectively, a dominant group emerged in each. Of the 88 tet(M)-positive strains from sediment, 35 (75%) were found to be V1, and of the 25 tet(M)-positive strains obtained from seawater, 15 (68%) were found to be V14.

Alleles of tet(M)

 Sequencing of the PCR product of the tet(M) gene was performed to determine the diversity of the tet(M) alleles. Forty-eight and eight of the PCR products obtained from sediment and seawater strains, respectively, were sequenced. An analysis of the expected 617 bp PCR amplicon excised from the primer region showed there were two alleles of tet(M) defined as “tet(M)-A” and “tet(M)-B”. The nucleotide level homology of tet(M)-A and tet(M)-B was 91.7%. Comparing these sequences with those in the databases, tet(M)-A was found to be identical to the tet(M) in both Streptococcal agalactiae (AE014233) and Clostridium perfringens (AF329848) while the closest sequence to tet(M)-B was found in the Streptococcal conjugative transposon (X04388).

Both of the tet(M) alleles were distributed among several genera of bacteria with tet(M)-A present in 10 groups of five genera (Vibrio, Shewanella, Bacillus, Lactobacillius, and Pseudoalteromonas) and tet(M)-B, in 21 groups of five genera (Vibrio, Photobacterium, Shewanella, Sporosarcina, and Paenibacillus) (Table 3).

Discussion

In this study, to determine the effect of OTC on the bacterial community in sediment and seawater, the occurrence of OTC-resistant bacteria was examined before, during, and after the administration of OTC to fish.

A time course investigation to determine the prevalence of OTC-resistant bacteria at an aquaculture site was performed knowing the drug therapy schedule used at the site. At site 1 and site 2, OTC was administered with fish feed for approximately 20, 4, 3, and 3 days in May, June, August, and September, respectively. We found that the percentage of OTC-resistant bacteria in the sediment increased during May to September at these sites and decreased by December; whereas no such increase was observed at the control site 3, 120 m from the aquaculture site. This suggests that repeated administration of OTC at the aquaculture site put selective pressure on the micro flora in the environment and/or in the fish intestine and could be the cause of the observed increase in the percentage of resistant bacteria.

Table 2. Detection of tet(M) in isolates obtained from sediment and seawater

<table>
<thead>
<tr>
<th>Month</th>
<th>Sampling site</th>
<th>Positive number/examined number of OTC-resistant isolate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sediment</td>
<td>Seawater</td>
</tr>
<tr>
<td>April</td>
<td>1</td>
<td>6/13 (46.1)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2/6 (33.3)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>12/15 (80.0)</td>
</tr>
<tr>
<td>May</td>
<td>1</td>
<td>15/19 (78.9)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>18/20 (90.0)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8/10 (80.0)</td>
</tr>
<tr>
<td>June</td>
<td>1</td>
<td>3/16 (18.8)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0/11 (0.00)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2/2 (100)</td>
</tr>
<tr>
<td>September</td>
<td>1</td>
<td>8/12 (66.7)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>11/12 (91.7)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4/5 (80.0)</td>
</tr>
</tbody>
</table>
Fig. 3. Phylogenetic tree of the sequenced bacterial 16S rRNA gene (160 bp long) from the isolates possessing tet(M). Strains in shaded boxes are from sediment and those in open boxes, from seawater. The value in parentheses is the number of isolates. Bootstrap values are shown for frequencies per 1,000.
In September, 11 days after the day the administrations ceased, the percentage of OTC-resistant bacteria was still high indicating the effect of OTC extended at least 11 days. Moreover, the low percentage of resistant bacteria in December suggests that 92 days was long enough to reduce the effect of OTC in the sediment.

Previous reports show that the use of OTC results in an increase in OTC-resistant bacteria in sediment or seawater. Samuelsen et al. report\textsuperscript{32} that the percentage of OTC-resistant bacteria reached 100% at the end of a 10 day OTC administration period and decreased to 20% and 10% at 72 days and 150 days, respectively, after the administration. Similarly, on a salmon farm\textsuperscript{19}, OTC-resistant bacteria in seawater increased for 16 days after 12 days of OTC administration. Additionally, DePaola et al.\textsuperscript{12} reported the occurrence of OTC-resistant bacteria in freshwater after drug administration in a catfish pond and found a decrease in resistant bacteria 21 days after the end of the administration period. Thus, these studies suggest that the OTC-resistant bacteria were selected for by administering OTC and caused the observed increase in the percentage of the resistant bacteria at the aquaculture site.

The selection of resistant bacteria may occur on and in the sediment because of the residue of OTC in the sediment. This has been reported in many studies measuring the concentration of the drug at an aquaculture site after OTC administration\textsuperscript{34}. The OTC is most likely derived from uneaten feed or fish feces. Because excess feeding of fish is usual in aquaculture, uneaten feed passes through the cage and will be deposited on the sediment\textsuperscript{27}. In a digestive experiment, 40% or 15% of OTC was excreted at 16°C and 24°C, respectively via the feces\textsuperscript{27}. Further, another study suggests a large portion of the drug passes unabsorbed through the intestine of fish and reaches the environment where most of the drug (99.9%) was recovered from the water and sediment in a concrete pond after medication of eels by feeding\textsuperscript{37}. Therefore, a substantial amount of OTC id deposited in the sediment as this is the possible fate of OTC administred to cultured fish; however, the bioavailability of OTC in the sediment may be reduced by the interaction of OTC with multivalent cations\textsuperscript{21} or humic acid\textsuperscript{15}.

The large amount of OTC in fish feces\textsuperscript{27} suggests that the fish intestines play an important role in the selection of resistant bacteria. However, the increase of resistant bacteria in the intestine after the administration of OTC was found only in the freshwater fish\textsuperscript{3,12}, there is no evidence of such an increase in saltwater fish\textsuperscript{18,23}. The diversity of the species of fish cultured during this study may have affected the diversity of bacteria found resistant to OTC. Further study is needed to determine the role of the intestine for sea-water fish in the selection of OTC-resistant bacteria.

There are many reports showing that tet(M) is distributed in different terrestrial environments\textsuperscript{8,9,26,39} and suggesting the flow or transfer of the antibiotic resistance gene in the environment. In contrast, there is no information about the distribution of tet(M) in the marine environment, though our previous study provided only evidence that tet(M)-possessing bacteria are present in the intestines of cultured yellowtail (Seriola quinqueradiata), among fish pathogenic bacte-
ria, and in seawater\textsuperscript{17}. Here we describe that tet(M) was detected in bacteria from sediment and seawater in an aquaculture environment and clearly show that tet(M) has been spread at the aquaculture site. We suggest that tet(M) had been in the environment because it was detected in sediment and seawater in April, and the tet(M)-positive bacteria may have been selected for by the administration of OTC and contributed to the increase in the resistant bacteria seen during April to September.

A phylogenetic analysis based on the sequencing of the 16S rRNA gene of tet(M)-positive bacteria indicated the diversity of the tet(M)-possessing bacteria to be high, with tet(M) distributed in eight genera of bacteria. This is the first report to identify tet(M) in *Shewanella*, *Pseudoalteromonas*, *Paenibacillus*, and *Sporosarcina*. Previously, 46 genera were known to show tet(M)\textsuperscript{29}, but now 50 genera including Gram-positive and Gram-negative bacteria are known to carry the element. Further, the group *Vibrio-Photobacterium* has 19 sequence patterns of the 16S rRNA gene and most of the tet(M)-positive bacteria were classified into this group indicating *Vibrio-Photobacterium* play an important role as a reservoir of tet(M) in the coastal aquaculture environment. However, the uncultivable fraction was not studied as a possible reservoir for the resistance gene.

The analysis of tet(M) showed the existence of only two alleles, tet(M)-A and tet(M)-B, each were identified in five genera of bacteria each including Gram-positive and Gram-negative bacteria. This indicates that each allele is distributed among many phylogenetically distantly related bacterial genera and those bacteria share an identical tet(M) sequence. This suggests that the transfer of tet(M) happened recently at this site. Tet(M) is frequently associated with a mobile element such as Tn916 which mediates its transfer between different bacterial species\textsuperscript{9,31} including both Gram-positive and Gram-negative bacteria\textsuperscript{3}, phylogenetic analysis suggests that RPPs including tet(M) have an ancient origin\textsuperscript{30}, and it is unlikely that each tet(M) evolved respectively in each host without mutations. Chee-Sanford et al.\textsuperscript{8} also suggested that the fact that several species of *Enterococcus* possessed the same allele of tet(M) indicated the likelihood of the transfer of tet(M) among species. Although we did not measure the concentration of OTC in this study, Baul et al.\textsuperscript{5} demonstrated that low concentrations of tetracycline promote the transfer of tet(M) mediated by Tn916. The administered drug may not only apply selective pressure to retain the resistance gene but also may force the transfer of the resistance gene in the aquaculture environment. Further, one of the alleles tet(M)-A was identical to a sequence found in both *Streptococcus agalactiae*\textsuperscript{36} and *Clostridium perfringence*\textsuperscript{28,30}, suggesting the existence of gene flow not only between strains in the aquaculture environment but also between strains from the terrestrial and coastal environment.

The mechanism for the appearance of the resistance is still unclear despite that resistance has been a serious problem in the aquaculture industry for a long time. We showed the spread of tet(M) in the aquaculture environment. This suggests that the tet(M) reservoir among the bacteria is contained in sediment and seawater. The detection of tet(M) at the control site during April and September suggests that tet(M) is ubiquitous in coastal sediment. Davies\textsuperscript{10} proposed the existence of a gene pool in the environment that is readily accessible to bacteria when they are exposed to the selective pressure resulting from antibiotic usage. The tet(M)-possessing bacteria in the sediment and seawater might function as a gene pool themselves. Further studies are needed to determine the mechanism of the appearance and dissemination of tet(M) in isolates in aquaculture environments.

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