Occurrence and diversity of tetracycline resistance gene, *tet*(M), in Korean coastal seawater

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By

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Contents

Abstract

Oxytetracycline (OTC) is a broad-spectrum antibiotic and widely used around the world. Resistant bacteria against antibiotics are recently recognized to be common in the natural environment. In Korea, the amount of veterinary use of antibiotics is about 1.5 times greater than other countries including USA, UK and EU countries. Although OTC is major antibiotics in Korea, the contamination status in Korean coastal sites has not been well understood. Therefore, the present situation of contamination by OTC and OTC resistance genes is needed to estimate the potential risk of antibiotics and the resistance genes in aquatic environment.

Aims of this study are (1) to estimate the OTC concentration in coastal seawater in Korea; (2) to enumerate OTC resistant (OTC^r) bacteria and their profiling of multidrug resistance; (3) to confirm the persistence of *tet*(M) and its genetic diversity in natural assemblage, and (4) to describe the diversity of *tet*(M)-possessing bacteria.

This study focused on one of major OTC^r genes, *tet*(M), which is studied well and known to distribute in natural environments. The *tet*(M) was examined in natural assemblage and isolated OTC^r bacteria. Seawater samples were taken in Yeosu (KYS), Geoje (KGJ) and Wando (KWD) areas. The OTC concentration in all sites was below detection limit (0.1 μg/L) in 2010 and 2011, suggesting that these areas are not contaminated with OTC.

The occurrence rate of OTC^r bacteria against 60μg/L OTC was observed frequency ranged 0-0.35 % in 2010 and 0-10.6 % in 2011, showing no significant difference between years. The OTC^r bacteria is occurred even in non-contaminated environments and present constantly. Isolated OTC^r bacteria showed nine types of multidrug-resistance patterns, among which resistant to OTC-ampicilin-sulfonamide/trimethoplim-erythromycin was

major (8/35), suggesting OTC^r bacteria convey macrolide, sulfonamide and ß-lactam resistance genes also.

Among the isolated OTC^r bacteria, *tet*(M) possessing rate was 66%. Interesting finding is that isolates from KGJ and KYS sites showed low incidence rate, KGJ (0/26 positive) and KYS (0/2 positive), whereas KWD site showed high rate (70/78 positive). This suggests different distribution of *tet*(M) even in neighboring areas.

The relative abundance of *tet*(M) was under detection limit ($<1.5\times10^4$ copies/mL) at most sampling sites in 2010, whereas that in 2011 ranged from 10^{-5} to 10^{-2} copies/mL, indicating that the *tet*(M) distribution is various in seawater.

The genotypes of *tet*(M) showed 99 to 100 % homogeny among the isolates and natural assemblage in KWD, suggesting that the *tet*(M) in KWD is homogenous. All *tet*(M) in KWD were the same as aquaculture origin *tet*(M) in previous studies. On the other hand, KGJ showed different result. The *tet*(M) showed 91.7 to 100 % similarity, indicating diverse in natural assemblage. Since the high homology was obtained with human origin *tet*(M), KGJ site should be received by human and/or animal effluent. This study suggests a possibility of different sources of *tet*(M) in Korean coastal sites.

The result of *tet*(M)-possessing bacteria obtained from KWD revealed that only Gamma proteobacteria, *Vibrio* sp. and *Photobacterium* sp., possessed *tet*(M). In addition, specific group is suggested to be *tet*(M) reservoir when monitored by culture-method. In Gram negative groups, Gamma proteobacteria are frequently isolated as *tet*(M)-reserving bacteria in human and animal sources. This study found that this phylum is a major group possessing *tet*(M) in marine derived bacteria also, suggesting this phylum should be ubiquitous reservoir of *tet*(M) in natural environment.

Through this study, new findings were obtained that (1) the distribution of OTC^r bacteria and *tet*(M) was not related to OTC contamination; (2) different genotypes of

tet(M) were found in Korean coastal sites; and (3) the major reservoir of *tet*(M) was *Vibrio* sp. and *Photobacterium* sp. in *tet*(M)-possessing bacteria. These are the first evidences in Korean environment.

Chapter 1. Background and literature review

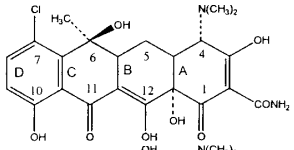
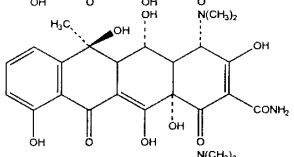
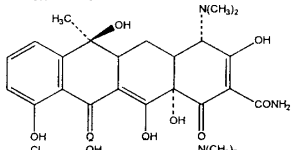
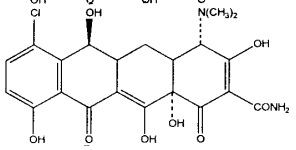
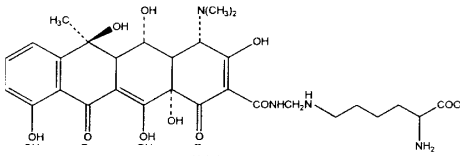
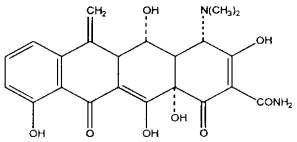
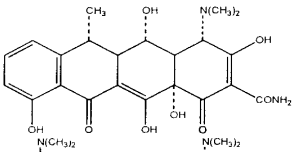
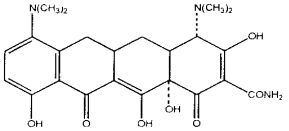
1.1 Tetracyclines (TCs)

The tetracyclines (TCs), discovered in the 1940, are a family of antibiotics that inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site, where the 30S subunit of bacterial ribosome is a target. The TCs are represented in Table 1.1. The TCs can be referred to as first-generation (1948 to 1963, chlortetracycline, oxytetracycline, tetracycline, demethylchlortetracycline, limecycline and clomocycline), second-generation (1965 to 1972, methacycline, doxycycline and minocycline) and third-generation (glycycline) TCs (Chopra and Roberts, 2001).

TCs are broad-spectrum agents, exhibiting activity against a wide range of Gram-positive and Gram-negative anaerobic and aerobic bacteria, atypical organisms such as chlamydiae, mycoplasmas, ricketsiae and protozoan parasites (Chopra and Roberts, 2001). In some countries, including the EU countries, TCs are added at subtherapeutic levels to animal feeds to act as growth promoters, although use as promoters has been banned in EU until 2006 (Castanon, 2007).

Among the TCs, oxytetracycline (OTC) has been used for food animals such as chickens, cows, honeybees, salmons and catfish (Levy, 1992). Total amount of antibiotic use in Korea was 15 times greater than that in Sweden in 2003 (Johansson and Molby, 2006), 2 times greater than that in UK in 2004 (Veterinary Medicines Directorate, 2005) and 1.5 times greater than that in Japan in 2005 (NVRQS, 2005; VMD, 2005). For the veterinary antibiotics, CTC, OTC, sulfamethazine (SMT), sulfathiazole (STZ) and tylosin (TYL) were selected as the greatest priority group in Korea (Seo *et al.,* 2007).

Table 1. 1. Structure of members of the tetracycline class

Chemical name	Structure
7-Chlortetracycline (CTC)	
5-Hydroxytetracycline (OTC)	
Tetracycline (TC)	
6-Demethyl-7-chlortetracycline (DMCT)	
2-*N*-Lysinomethyltetracycline (LMC)	
6-Methylene-5-hydroxytetracycline (methacycline) (MT)	
6-Deoxy-5-hydroxytetracycline (doxycycline) (DTC)	
7-Dimethylamino-6-demethyl-6-deoxytetracycline (minocycline) (MC)	

Chopra and Roberts (2001)

Only 10-20% of antibiotic use can be actively reacted in the animal's body and the rest of these are excreted *via* urine and feces as a form of parent compound with their metabolites (Aminov *et al*., 2001). The residual TCs from human, veterinary animal and agriculture are directly released into the aquatic environment *via* various pathways such as lagoons (Aminov *et al.,* 2001) and wastewater treatment plants (Zhang *et al.,* 2009; Zhang and Zhang, 2011), which are emerging contaminants in aquatic environment, but their effect has not been descried well (Huang *et al.,* 2001).

Consequently, TCs residue should become selective pressure on aquatic bacteria, which results induction, survival, spread and dissemination of resistant bacteria. Environmental OTC concentration reported in Asia is as follows; 44-313 μg/L in seawater from Victoria Harbor in Hong Kong (Minh *et al.,* 2009), 0.39-1.41 μg/L in water and 1.68-3.77 μg/kg in soil along the Hongcheon river in Korea (Ok *et al.,* 2011). In Korea, residual concentrations of TCs are reported as shown in Table 1.2. Ok *et al*. (2011) and Choi *et al.* (2008) reported the concentrations of TCs, at which hot spot was found in animal manure. The river water receiving animal manure was 0.5-1.4 μg/L. Concentration in seawater has not been reported so far in Korea.

Monitoring of released antibiotics including OTC is needed for assessment of aquatic environment in Korea.

Table 1. 2. Concentration of TCs in literatures from various sources in Korea

Compound	Sources		TCs concentration	References
Chlortetracycline	Livestock manure	Cattle	1.65 mg/kg	Kim *et al.,* (2011)
		Pig	16.56 mg/kg	
		Poultry	15.62 mg/kg	
Chlortetracycline		Wastewater	0.11-0.97 μg/L	Choi *et al.,* (2008)
Chlortetracycline	Swine manure composting facility	Water (River)	0.53-0.76 μg/L	Ok *et al.,* (2011)
Oxytetracycline		Water (River)	0.39-1.41 μg/L	
Tetracycline		Water (River)	0.50-0.58 μg/L	
Chlortetracycline		Soil	0.31-0.89 μg/kg	
Oxytetracycline		Soil	1.68-3.77 μg/kg	
Tetracycline		Soil	0.82-2.94 μg/kg	

1.2. Mechanisms of TCs resistance and TC resistance genes

Bacterial resistance to TCs is occurred through a variety of mechanisms. These mechanisms include, (1) energy-dependent efflux pumps (Chopra and Roberts, 2001; Roberts, 2005), (2) ribosomal protection proteins (RPPs) (Chopra and Roberts, 2001; Roberts, 2005), and (3) enzymatic inactivation (Speer *et al*., 1992; Roberts, 2005).

TC resistance genes are called as *tet* series (Roberts, 2005, 2012). Currently there are 45 *tet* genes are recorded (Roberts, 2005, 2012; Brown *et al.,* 2008). Fig. 1.1. shows the various *tet* genes with each category (Thaker *et al.*, 2010). There are 26 efflux pump genes found in Gram-positive and Gram-negative bacteria. All the *tet* efflux genes code membrane-associated proteins which export TCs from the cell (Chopra and Roberts, 2001). This action reduces the intercellular concentration of TCs and protects the bacterial ribosomes. The efflux proteins exchange a proton for a tetracycline-cation complex against a concentration gradient (Roberts, 2005), and finally pump the antibiotic out the cell (Thaker *et al*., 2010).

Ribosomal protection proteins (RPPs) are a major resistance mechanism, representing a widely distributed class of tetracycline resistance determinants. There are 11 different types of RPPs spanning Gram-positive as well as Gram-negative bacterial genera. Among them, *tet*(M) is an well studied RPP. The host bacteria of the *tet*(M) are found in 42 genera (Roberts, 2005), suggesting that this gene shows wide host range. The reason for the wide host range may in part be due to its association with conjugative transposons that also have a very wide host range. These elements encode putative anti-restriction functions and possess few restriction enzyme cleavage sites. The *tet*(M) has been detected in isolated bacteria from environmental samples in Korea (Jun *et al.,* 2004; Kim *et al.,* 2004; Kim *et al.,* 2007) thus, *tet*(M) could be expected to widely distribute in Korean costal seawater.

Recently, quantitative real-time PCR (qPCR) assay has been frequently employed to study the *tet* genes for environmental samples (Peak *et al.,* 2007; Wu *et al.,* 2010; Tamminen *et al.,* 2011; Zhang and Zhang, 2011). The relatively high level of *tet*(M) is not surprising because it is seen equally in both Gram-positive and Gram-negative bacteria, and it is the most commonly detected *tet* genes among bacterial studies so far (Roberts, 2005). The research on *tet*(M) is entering to new generation with quantitative detection and genome analysis for environmental samples.

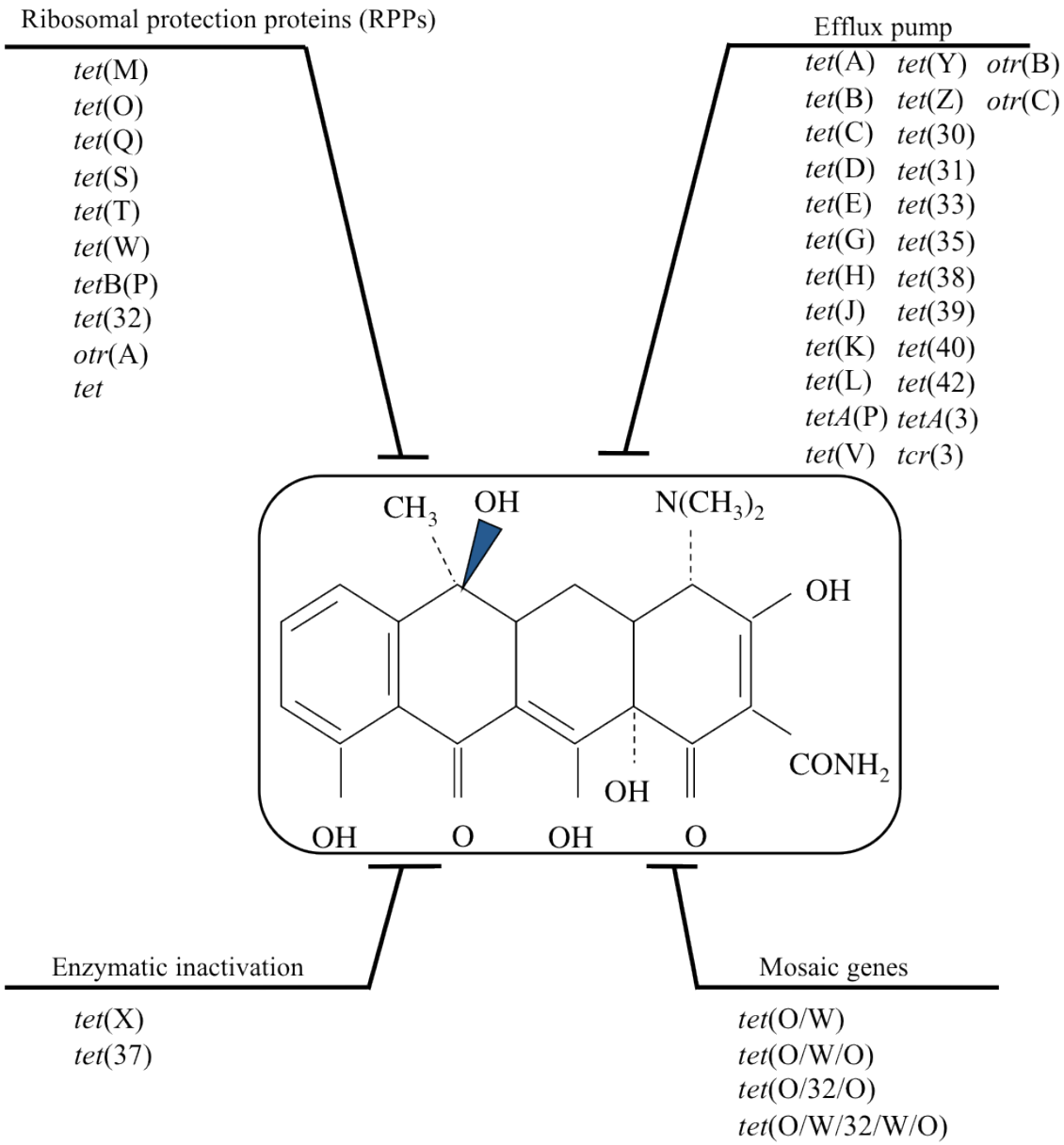


Fig. 1.1. Various *tet* genes found in bacteria (Thaker *et al*., 2010)

1.3. Distribution of *tet*(M) in aquatic environments

The study of ARB has been performed by culture-dependent method, because the effect of antibiotics can only be observed by colony formation. Most of human pathogenic bacteria are cultulable, however, most of aquatic bacteria are non-culturable (Colwell and Grimes, 2000). Thus the culture-independent approach is needed in order to know the reservoirs in aquatic environment. Research on *tet*(M) has been primarily confined in the study of cultured isolates (Kim *et al.,* 2004; Rahman *et al.,* 2007; Kobayashi *et al.,* 2007a). Previous studies investigated *tet*(M) gene in environment by PCR-DGGE method (Aminov *et al.,* 2001). Recent quantitative real-time PCR study (Tamminen *et al.,* 2011) could track *tet*(M). Genomics and qPCR approaches will advance this study area further.

The reports in culturable bacteria in the past showed both Gram-negative and Gram-positive bacteria isolated from healthy and diseased fish as well as from seawater in Japan and Korea possessed *tet*(M) (Kim *et al.,* 2004).

However, as mentioned above, culturable bacteria are represent only a fraction of the actual population, and conventional culture-dependent analysis may be biased toward recognition of a limited part of the genes in isolated OTC^r bacteria and resistance genes.

Several studies have reported a variety of genotypes of the *tet*(M) gene from natural environments (Kobayashi *et al.,* 2007b; Rahman *et al.,* 2008) or resistance isolates (Chee-Sanford *et al.,* 2001; Aminov *et al.,* 2001). Thus, study of the distribution and diversity of *tet*(M) in isolates and natural assemblage is required to understand the origin of *tet*(M) in environment

Chapter 2. Estimation of OTC concentration in Korean coastal seawater

2.1. Introduction

Antibiotics can be metabolized after administration; however, up to 90 % of antibiotics are excreted through urine or feces without complete decomposition, which are still active. Substantial amount of excreted antibiotics are released into the aquatic environment, and thus have been detected in aquatic environments (Huang *et al*., 2001; Choi *et al*., 2008; Managaki *et al*., 2007). The antibiotic contamination increases number of ARB (Park *et al*., 2005; Lee *et al*., 2008).

Among the antibiotics, OTC has been extensively used in Korea for treating and preventing of bacterial infectious diseases in veterinary medicine and aquaculture. The consumed OTC is about 1.5 times greater than other countries including USA, UK and Europe countries (Kim *et al.,* 2008; Ok *et al.,* 2011).

Concerning on water environment, the OTC concentration in river water was reported to be 0.39-1.41 μg/L (Ok *et al*., 2011), but there are no reports on seawater. This Chapter describes OTC concentration in seawater in three areas in two years.

2.2. Materials and methods

2.2.1. Sampling area and water sampling

Seawater samples were taken at three sites in Yeosu (KYS-1, 2 and 3), three sites in Geoje (KGJ-1, 2 and 3) and three sites in Wando (KWD-1, 2 and 3) in 9-11, November 2010 and 30, May to 1, June 2011 (Fig. 2.1) in Korean costal sites. These 3 habitats were selected because they were representative of the aquatic environment exposed to various chemical and antibiotics. Briefly, Aquaculture is intensively located in Wando, such as laver (*Porphyra tenera*), abalone (*Haliotis discus*) and olive flounder (*Paralichthys olivaceus*). Yeosu and Geoje are urbanized places and aquacultural industries such as seaweed; rock bream (*Oplegnathus fasciatus*) are closely located in these areas. Therefore, antibiotics could be directly released to aquatic environment.

From all sites, seawater samples were taken by alcohol-sterilized stainless bucket, and stored in a sterilized polypropylene bottle for the bacterial experiment and a glass bottle for antibiotic analysis. All samples were transported on ice and taken to the lab within several hours for the experiment. At each site environment measurements of salinity, pH and temperature was taken by pH/conductivity meter (Horiba D-54, Horiba, Kyoto, Japan). The collected samples were pre-filtered through 50 μm nylon plankton net to remove large particles.

2.2.2. Oxytetracycline (OTC) analysis

OTC was analyzed according to Ye *et al.* (2006). Briefly, 100 mL of samples was passed through 47mm of diameter GF/F filters (Whatman, Maidstone, Kent, UK), and adjusted to pH 3.0 with 1N HCl. OTC was concentrated by solid-phase extraction (SPE) using Sep-Pak plus-2 C18 cartridge (Waters, Milford, MA, USA) after conditioning with

10 mL of methanol and 5 mL of EDTA. After loading the 100 mL sample water, the column was washed with 10 mL of sterilized Milli-Q water. Elution of OTC from the column was performed. The extract was dried under as N_2 stream and dissolved in 1 mL of 1.36 % KH_2PO_4. The cartridges were stored at -20 ˚C until the analysis. The sample solution was injected into a HPLC instrument with fluorescence detector (Hitachi Elite LaChrom; excitation and emission wavelengths: 380 and 520 nm, respectively). The mobile phase solution consisted of 8.0 % methanol in imidazole buffer (1.0 M imidazole, 1.0mM EDTA, and 0.08M Mg-acetate pH 7.2) and the flow rate was 1.2 mL/min. The extracted OTC was separated on a C18 column (Bridge C18, Waters; 4.6 mm diameter, 150 mm length, 5 μm pore size) at 40 ˚C. Concentrations in the samples were calculated by an internal standard method based on the ratio of peak area of OTC, and the linearity of the calibration curve was confined (r^2>0.99). The recoveries of spiked OTC standards were ranged with 108±15, 86.7±7 in 2010 and 2011, respectively. Milli-Q water for procedural blank was analyzed with extraction as controls for laboratory contamination. Limit of quantification (LOQ) was defined as 10 times the procedural blank value, and LOQ of OTC was 0.1 μg/L.

2.3. Results and discussion

2.3.1. OTC concentration in sampling site

Table 2.1 summarizes the environmental conditions of Korean sampling sites in 2010 and 2011. Surface water in KYS, KGJ and KWD showed water temperature was 12.0-15.0 ˚C (2010) and 16.6-20.6 ˚C (2011). Salinity in KYS and KWD was 30-35 in both years. However, KGJ-3 showed salinity of 30-32, indicating freshwater effect in this area.

The OTC concentration was listed in Table 2.1. Most sites showed below detection limit (0.1 μg/L) except for one site in KWD-2 (Wando) 2010, which showed 0.18±0.01 μg/L. This suggests that the OTC contamination was not occurred constantly in all sites. Several studies in Korea reported that antibiotics have been detected in various sources at concentrations of the ng/L to μg/L (Park *et al.,* 2005; Ok *et al*., 2011). In addition, the TC series and sulfonamides ranged 0.11 to 0.97 μg/L and 0.45 to 10.57 μg/L, respectively, in 12 sewage treatment plant (STP) influents and wastewater sampling sites (Choi *et al*., 2008) indicating the excreted antibiotics released to natural environment. The results until now suggest that Korean coastal sites are less contaminated with OTC.

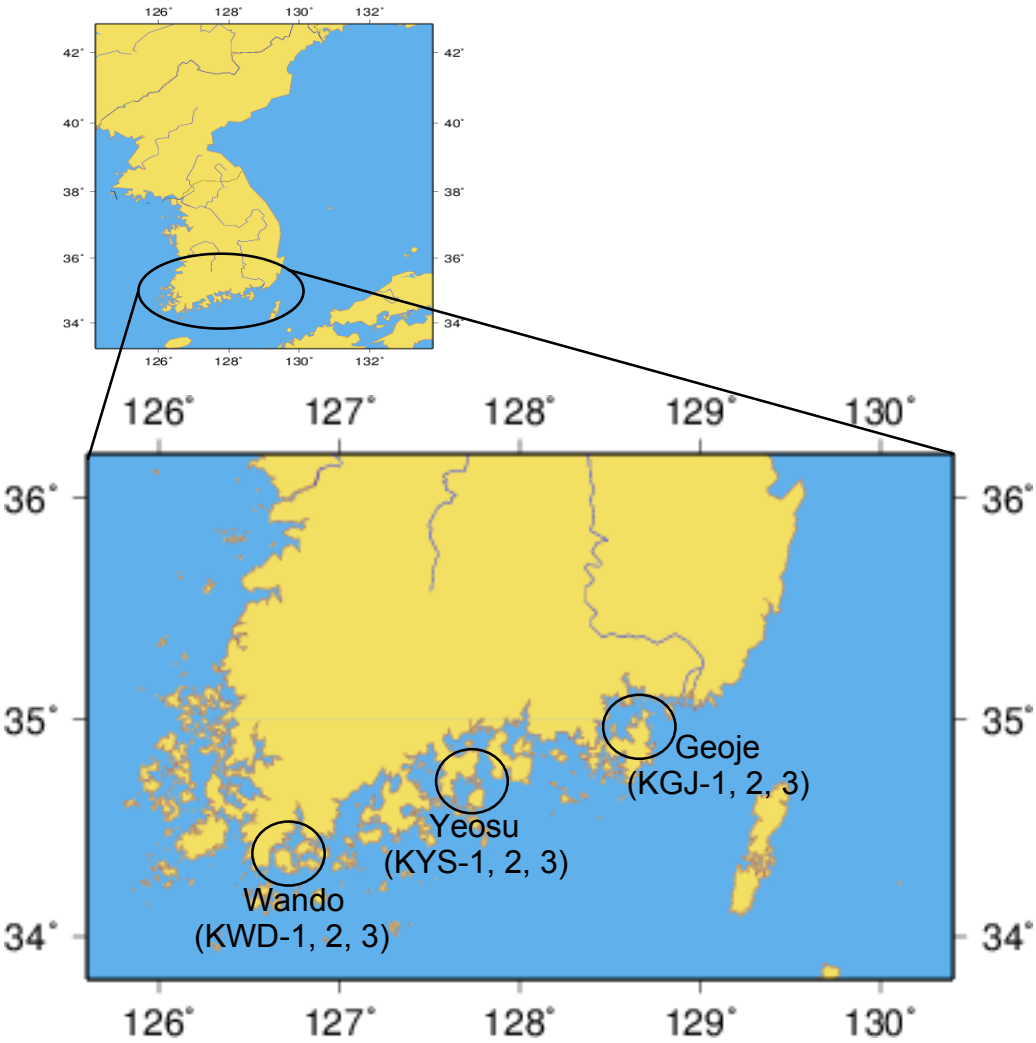


Fig. 2.1. Study area and sampling locations in Korean coastal sites. Circles indicate sampling sites. Seawater samples were collected at nine sampling sites (KYS-1, 2,3, KGJ-1, 2,3 and KWD-1, 2,3).

Table 2. 1. General information of sampling sites and OTC concentration

Sample ID	Date	Latitude	Longitude	Surface water (˚C)	pH	Salinity	OTC concentration (μg/L)
KYS-1	9, Nov. 2010	N 34°38′14.6″	E 127°48′24.5″	12.0	7.98	33	BLD*
	30, May 2011	N 34°40′08.0″	E 127°44′38.6″	20.6	7.83	32	BLD*
KYS-2	9, Nov. 2010	N 34°43′13.6″	E 127°42′14.5″	12.0	8.08	34	BLD*
	30, May 2011	N 34°43′24.9″	E 127°42′37.9″	20.3	7.89	31	BLD*
KYS-3	9, Nov. 2010	N 34°51′33.5″	E 127°43′14.6″	14.4	7.89	35	BLD*
	30, May 2011	N 34°51′35.4″	E 127°43′04.3″	20.4	8.05	30	BLD*
KGJ-1	10, Nov. 2010	N 34°54′33.6″	E 128°30′49.2″	14.7	8.02	31	BLD*
	31, May 2011	N 34°54′23.9″	E 128°30′18.4″	18.0	7.84	32	BLD*
KGJ-2	10, Nov. 2010	N 34°54′33.6″	E 128°34′34.4″	14.5	8.0	32	BLD*
	31, May 2011	N 34°53′44″	E 128°36′50″	16.9	7.85	30	BLD*
KGJ-3	10, Nov. 2010	N 34°53′44.8″	E 128°36′51.2″	15.0	7.92	30	BLD*
	31, May 2011	N 34°49′11.4″	E 128°30′17.7″	16.7	7.66	34	BLD*
KWD-1	11, Nov. 2010	N 34°19′32.7″	E 126°45′0.7″	14.5	8.08	34	BLD*
	1, June 2011	N 34°19′04.2″	E 126°45′07.8″	16.6	7.84	30	BLD*
KWD-2	11, Nov. 2010	N 34°20′24.2″	E 126°44′17.4″	14.7	7.88	34	0.18±0.01
	1, June 2011	N 34°20′24.2″	E 126°44′16.8″	17.2	7.71	30	BLD*
KWD-3	11, Nov. 2010	N 34°20′28.5″	E 126°47′1.2″	14.4	8.04	34	BLD*
	1, June 2011	N 34°20′28.8″	E 126°47′01.3″	17.7	7.69	32	BLD*

BDL*; below detection limit (0.1 μg/L).

Chapter 3. Occurrence of OTC resistant (OTC^r) bacteria and profiling of multidrug resistance (MDR) in OTC^r isolates

3.1. Introduction

Antibiotic resistant bacteria (ARB) are recently known to be common in natural environment, especially aquatic habitats (Kümmerer, 2009a; 2009b). Antibiotic resistance genes (ARGs) are reserved by such ARB, which are recognized as "biological pollutants" (Pruden *et al*., 2006; Baquero *et al*., 2008). Furthermore, recent evidences show that the selection occurs even under low concentration than the inhibition dose (Gullberg *et al*., 2011). In addition, the development of multidrug resistant (MDR) bacteria in aquatic environment is of great concern because the bacteria could be transported back to human through water utilization and fisheries products.

Most studies about MDR have only been performed for important pathogenic bacteria such as *Escherichia coli* (Ryu *et al*., 2012), *Edwardsiella tarda* (Jun *et al*., 2004), *Enterococci* (Aarestrup *et al*., 2000) and *Aeromonas* spp. (Kim *et al.,* 2011). As OTC^r bacteria, fish pathogens are known (Kim *et al*., 2004; Nonaka *et al*., 2007), because OTC is frequently used in aquaculture. Among the OTC^r bacteria, MDR plasmid from the *Photobacterium damsela* is recently isolated and sequenced (Nonaka *et al*., 2012). Several previous studies have shown that the consumption of antibiotics in aquatic environment can also play important role in the selection and dissemination of ARB in the environment. However, the situation of OTC^r bacteria in natural seawater has not been well understood. In previous study (Chapter 2), OTC contamination was not occurred in all sites monitored in this study. However, as mentioned above, ARB are occurred even in non-contaminated environment, this Chapter aimed to enumerate the OTC^r bacteria and examined MDR profile in isolates.

3.2. Materials and methods

3.2.1. Total bacterial counts

For total bacterial cell count was performed under fluorescence microscopy. To 1 mL of sample water, 2.0 % of formalin was added. This fixed samples was filtrated with a polycarbonate filter (0.2 μm pore size, Millipore). The filter was stained with 4'-6-diamidino-2-phenylindone (DAPI) (final concentration 0.1 μg/L) for 7 min. The bacteria were enumerated by epifluorescene microscopy (BX51, Olympus, Tokyo, Japan). More than 300 cells in minimum 20 fields were counted.

3.2.2. Culturable bacterial number

The colony forming units (CFUs) were measured using the plate spreading methods. Seawater samples of 0.5 mL were mixed in 4.5 mL of phosphate buffer saline (PBS, pH 7.4) and serial 10-fold dilutions was prepared. The dilutions were spread on nutrient broth (Difco Laboratories, Detroit, MI, UK) plus 1.5 % Bacto agar and 2.0 % NaCl. The colony number was counted after incubation at 20 ˚C for 4 days.

3.2.3. Enumeration of OTC^r bacteria

The same sample for CFU counting was employed for OTC^r bacterial counting. Nutrient Broth (Difco Laboratories, Detroit, MI, UK) plus 1.5 % Bacto agar and 2.0 % NaCl plate was supplemented with 60 μg/mL of OTC, and incubated at 20˚C for 4 days in duplicates.

3.2.4. MDR testing

A total of 35 OTC^r isolates obtained in 2010 were examined for MDR profile by Etest strip according to the instructions of the manufacturer (AB Biomeriúx, Solna, Sweden). The antibiotics tested were ampicillin (ABPC), ciprofloxacin (CI), tetracycline (TC), erythromycin (EM), sulfamethoxazol/trimethoprim (SMX/TS) and streptomycin (SM). A bacterial cell suspension was prepared in PBS and the cell density was adjusted to McFarland No. 0.5. The suspension was spread on a Mueller Hinton agar (Difco Laboratories, Detroit, MI, USA) plates supplemented with 2.0 % NaCl. Etest strips were then placed on the agar plate and incubated at 20 ˚C for 24 h. The minimum inhibitory concentration (MIC) values were determined by the concentration on strip scale. Antimicrobial resistance was assessed using breakpoints recommended by the Clinical and Laboratory Standard Institute (CLSI) guidelines (2008).

3.3. Results and discussion

3.3.1. Occurrence of OTC resistant (OTC^r) bacteria

Seawater samples were collected from coastal seawater in November 2010 and May to June 2011 in Korea. This season was not active for aquaculture. As shown Fig. 3.1, total cell counts (DAPI counts) were 9.17×10^5 to 1.67×10^6 cells/mL, 6.78×10^5 to 1.24×10^6 cells/mL, whereas colony count was $0.26 \pm 0.14 \times 10^3$ to $5.00 \pm 2.8 \times 10^5$ CFU/mL, $1.0 \pm 0.01 \times 10^3$ to $1.95 \pm 0.26 \times 10^5$ CFU/mL in the seawater 2010 and 2011, respectively. OTC^r bacteria were accounted for 0-0.35 % in 2010, and 0-10.6 % in 2011 (Fig.3.1). The tetracycline-resistant (TC^r) bacterial rate in the sediment under the fish cages were reported to be 2.7-60.7 % and 4.7-64.8 % in seawater, in aquaculture site in Japan (Neela *et al.,* 2007) and that in open ocean sediment was 0.79-14.7 % (Rahman *et al.,* 2008). Compare to those, the present data showed lower rate, although the sampling area were coastal and received human activity. Difference of the OTC^r rate between years was not significantly different, but KWD showed tendency to be higher in the rate. Source of the OTC^r bacteria might be different among the areas.

3.3.2. Profiling of multidrug resistance (MDR)

To know the MDR patterns in OTC^r isolates, the susceptibility of the 35 OTC-resistant strains against other antibiotics was examined. Eight strains (23%) out of 35 isolates showed resistance to 3 drugs, ABPC, SMX/TS and EM (Table 3.1), followed by cross-resistance to EM and ABPC. Neela *et al*. (2007) showed in Japanese coastal aquaculture site that water column isolates of OTC^r were cross-resistant to ABPC-mecillinam (26%) and ABPC-EM-mecillinam (22%), whereas sediment isolates were cross-resistant to ABPC-EM (42%). Furthermore, it is found that the seawater strains

showed low occurrence rates to ABPC-EM (Neela *et al.,* 2007). Present result in Korea showed similar trend. The OTC^r determinants might be linked to ABPC and EM resistance determinants, but not to SM and CI in examined area. Correlation between OTC^r and resistance to ABPC- analogous ß-lactams and macrolides was known to be occurred by multi-drug efflux system (Putman *et al.,* 2000). This study showed nine types of resistance patterns. The highest resistance patterns along with OTC^r were OTC-ABPC-SMX/TS-EM, followed by OTC-EM and OTC-ABPC. This suggests that OTC^r bacteria in examined areas possess genes responsible for OTC-macrolide-sulfonamide-ß-lactam. Sayah *et al*. (2005) reported that the occurrence rate of ß-lactam (ABPC and MPC) resistance was high (86%) in seawater strains. Furthermore, single drug exposure can lead the cross-resistance to other unrelated drugs (George, 1996). The result in the present study indicated presence of OTC^r and multidrug-resistant bacteria in OTC-free environment, especially to ABPC-EM combination, which suggested the antibiotics such as ampicillin and erythromycin could be released in sampling area. Further monitoring is needed in various seasons when aquaculture is actively performed.

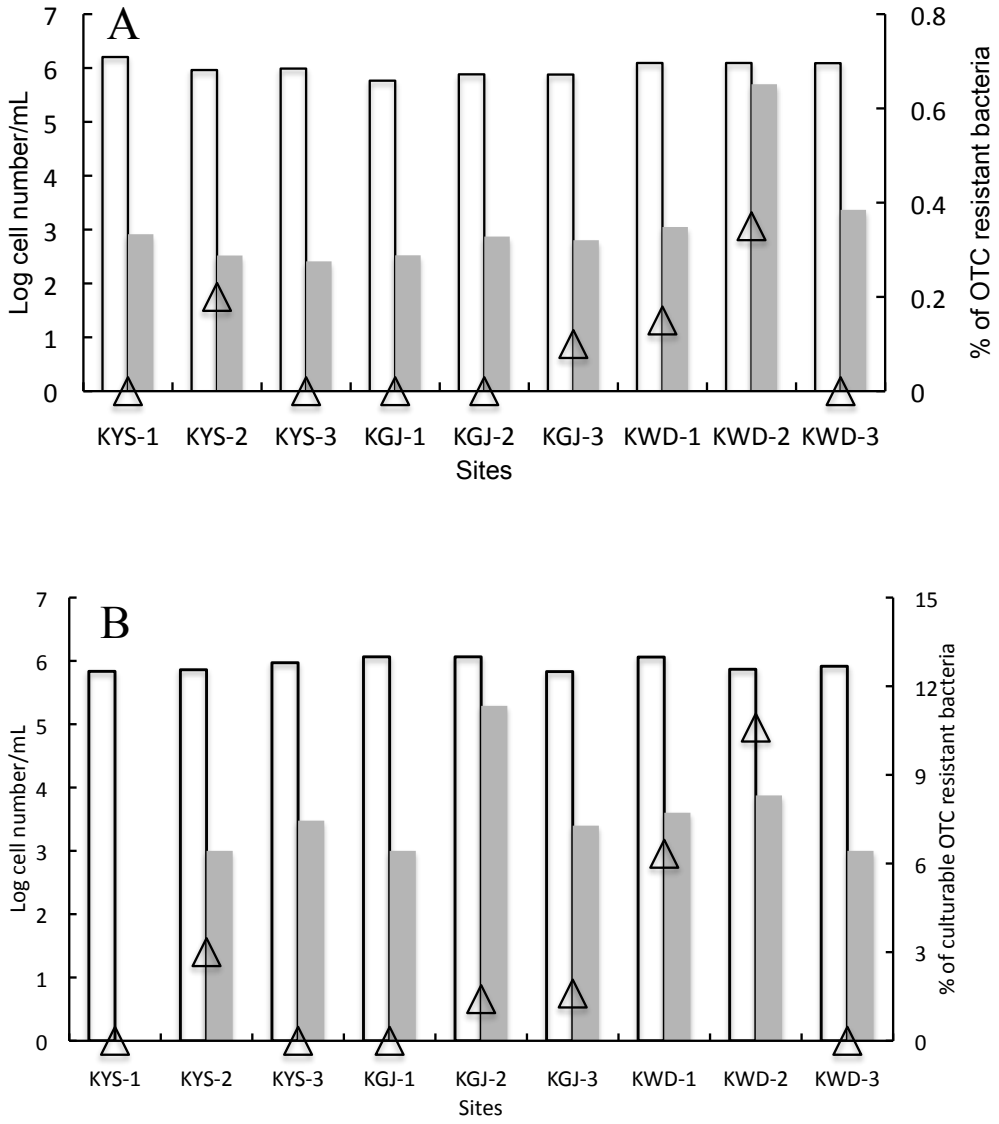


Fig. 3.1. Total cell counts by DAPI staining (open bar, unit is left axis), viable counts (closed bar, unit is left axis) and occurrence rate (%) of OTC^r bacteria (triangle, unit is right axis). A, 2010 and B, 2011. It should be paid attention that unit scale of % in A and B is different.

Table 3.1. Multidrug resistant patterns in 35 isolated OTC^r strains in 2010

Resistance to	Occupation number (%)
OTC	7 (20%)
OTC-ABPC-SMX/TS-EM	8 (23%)
OTC-ABPC	6 (17.1%)
OTC-EM	6 (17.1%)
OTC-ABPC-EM	2 (5.7%)
OTC-EM-SMX/TS	2 (5.7%)
OTC-EM-SM	1 (2.85%)
OTC-ABPC-SMX/TS	1 (2.85%)
OTC-ABPC-SMX/TS-EM-CI	1 (2.85%)
OTC-ABPC-SMX/TS-EM-CI-SM	1 (2.85%)

ABPC: ampicillin; CI: ciprofloxacin; OTC: oxytetracycline; SM: streptomycin; SMX/TS: sulfamethoxazol/trimethoprim; EM: erythromycin

Chapter 4. Persistence and diversity of *tet*(M)

4.1. Introduction

To date, more than 40 genes have been described as tetracycline resistance determinant (*tet*) (Roberts, 2012). In Korean aquatic environment, *tet*(M) and *tet*(S) were detected in *Vibrio* sp. from healthy/diseased fish (Kim *et al*., 2004) and *tet*(A) and *tet*(B) were detected in *E. tarda*., *E. coli.* and *Aeromonas* sp. from fish farms (Jun *et al*., 2004) and fish (Kim *et al*., 2011; Ryu *et al*., 2012).

It is reported that most marine bacteria are non-culturable on agar plate (Colwell and Grimes, 2000). Therefore, not only culture-dependent method but also culture-independent method is needed to examine real situation of reserving antibiotic resistance genes (ARGs). Recent study in Philippines reported the comparison between the two methods, showing quite different profiles of sulfonamide resistance genes (Suzuki *et al*., 2013). They suggest that the marine bacterial community has specific potential reservoir.

Among the *tet* genes, *tet*(M) is a well studied and widely distributed gene (Roberts, 2005). The *tet*(M) is only one *tet* with evolutional evidence (Kobayashi *et al.*, 2007a). Thus the *tet*(M) is an appropriate gene to chase the environmental dynamics and genetically diversity. To analyze diversity of *tet* genes will be useful to reveal complex situation of antibiotic resistance genes (ARGs) in coastal seawater. The quantitative real-time PCR (qPCR) assay has recently been employed to study ARGs (Tamminen *et al.,* 2011; Chen *et al.,* 2013). Several studies have reported a variety of genotypes of the *tet*(M) from natural environment (Kobayashi *et al*., 2007b; Rahman *et al*., 2008) and in isolated bacteria also (Aminov *et al*., 2001; Kim *et al*., 2004; Nonaka *et al*., 2007). This Chapter describes the diversity of *tet*(M) detected in isolates and natural assemblage.

4.2. Materials and methods

4.2.1. OTC^r bacteria

Morphologically different colonies were isolated from the agar plates with OTC for the samples from Korean coastal sites. 35 isolates were obtained in 2010, and 71 isolates in 2011. The isolates include 23 isolates from KGJ-3, 20 isolates from KWD-1 and 28 isolates from KWD-2. After checking possession of *tet*(M) by PCR specific for this gene (Kobayashi *et al.*, 2007b), *tet*(M)–possessing isolates were obtained.

4.2.2. DNA extraction from natural assemblage and isolated bacteria

To concentrate natural bacterial assemblage, seawater samples (100 mL to 200 mL) were filtered through 0.2 μm pore size polycarbonate filter (Millipore, Eschborn, Germany), and then were stored at -20 ˚C until use.

Extraction of DNA from the filter was performed according to a modified version of the cethyltrimethy-lammonium bromide (CTAB)-method (Wilson, 1987). Briefly, thawed filters were dipped in TE buffer (10 mM Tris-HCl [pH8.0], 1 mM EDTA) containing sodium dodecyl sulfate (SDS, 0.5 %), Proteinase K (0.1 mg/mL, Takara, Otsu, Japan) and RNase (0.05 mg/mL, SIGMA-ALDRICH, ST. Louis, MO, USA). The filter was incubated at 37 ˚C for 1h. To remove polysaccharides, CTAB/NaCl solution (10 % CTAB and 0.7 M NaCl) was added, and the samples were incubated at 65 ˚C for 10 min. The freezing thawing was repeated three cycles at -80 ˚C and at 65 ˚C to increase the recovery of DNA from bacterial cells. Subsequently, an equal volume of phenole-chloroform-iso amyl alcohol (25:24:1) was added, and the tubes were inverted and centrifuged at 2,100×g at 4 ˚C for 10 min. The supernatant was collected into a new 1.5 mL tubes, and an equal volume of chloroform-iso amyl alcohol (24:1) was added. The

tubes were centrifuged at 21,600×g for 10 min at 4 ˚C, and supernatant was collected in another 1.5 mL tube. The samples were precipitated with an addition of 0.1 volume of 3 M sodium acetate and then a 0.6 volume of iso-propanole. The precipitated pellets were dried under vacuum and dissolved in 50 μL of sterilized Milli-Q water. The recovered DNA were quantified by ultraviolet absorption meter (DU640, BECKMAN COUNTER, Orange Country, CA, USA), and the quality of the DNA was checked by electrophoresis on 1.0 % agarose gel with GelRed™ staining (Biotium Inc., Hayward, CA, USA) and visualized on an Epi-Light UV FA1100 system with a Luminous Imager version 2.0 (Aisin Cosmos R&D, Aichi, Japan). The extracted environmental DNA was examined for detecting of 16S rRNA gene and *tet*(M) by quantitative real-time PCR (qPCR).

For DNA isolation of cultured bacteria, bacterial suspension were boiled in 50 μL of sterilized Milli-Q water at 94 ˚C for 10 min to obtain total DNA from the cells.

4.2.3. Detection of *tet*(M) in isolates and natural assemblages

The *tet*(M) were determined by PCR using the primer set: *tet*(M)-1 (5'-GTTAAATAGTGTTCTTGGAC-3') and *tet*(M)-2 (5'-CTAAGATATGGCTCTAACAA-3') (Aarestrup *et al.,* 2000), which generated a 657 bp amplicon. The PCR program was run on a GeneAmp PCR system 9700 (Applied Biosystems, Foster, City, Ca, USA) thermal cycler and utilized 30 cycles [denaturation at 95 ˚C for 30s, annealing at 56 ˚C for 30s and extension at 72 ˚C for 1min 30s] and final extension at 72 ˚C for 7 min. PCR products on 1.2 % agarose gel were stained with GelRed (Biotium Inc., Hayward, CA, USA) and visualized on an Epi-Light UV FA1100 system with a Luminous Imager version 2.0 (Aisin Cosmos R&D, Aichi, Japan).

4.2.4. Quantitative PCR (qPCR) in natural assemblage DNA

To investigate the persistence of *tet*(M) gene in natural seawater, the qPCR was performed in a 96 well format in the Bio-Rad CFX96 Real Time System (BioRad, Laboratories, Hercules, CA, USA). Reaction mixture included 1ng of DNA from filter as template, 10 µM of each primer, 1× Sso Fast EvaGreen SuperMix (BioRad, Laboratories, Hercules, CA), which responds to increases in double-stranded DNA with an increase in fluorescence and sterile H_2O in a total reaction volume of 20 µL. All reaction, including negative control (sterile H_2O), were conducted is duplicate. Serial 1:10 dilutions of the plasmid were used as standards (STD) for quantification. The following plasmids were used as standard: pGEM-*tet*(M), pGEM carrying the *tet*(M) gene from pFD310 (Smith *et al.,* 1992); pGEM-16S rRNA, pGEM carrying the 16S rRNA gene from *E. coli.* K12 strain. The DNA extracted from filter was diluted to avoid inhibition by the environmental substances. Standard deviation values of the measurement were determined from the duplicate. All assays included a melting curve analysis for which all samples displayed single peaks for each primer pairs. Primers were 16S rRNA gene, F-5'-CGGTGAATACGTTCYCGG-3', R-5'-GGWTACCTTGTTACGACTT-3' and *tet*(M) gene, F-5'-GCAATTCTACTGATTTCTGC-3', R-5'-CTGTTTCATTACAATTTCCGC-3' (Tamminen *et al.,* 2011). The program consisted of an initial denaturation of 30s at 95 ˚C and 40 cycles of 5s at 95˚C (denaturation) and 10s at 50˚C for *tet*(M) and 10s at 95˚C and 20s at 57 ˚C for 16S rRNA gene, respectively. Melting curves for the amplicons were measured by raising the temperature slowly while monitoring fluorescence. The copy numbers of *tet*(M) was normalized by dividing by the 16S rRNA gene copy number at the respective time point to take into account any temporal variation in bacterial cell numbers.

4.2.5. Detection of other tetracycline resistance genes than *tet*(M) in isolated bacteria

A total 71 OTC^r isolates in 2011, were investigated for efflux genes, *tet*(A) and (B) (Furushita *et al.*, 2003) and ribosomal protection protein genes, *tet*(S) and *tet*(W) (Kobayashi *et al*., 2007b).

The primers and PCR conditions used for *tet*(A) were described by Aerstrup *et al.* (2003), for *tet*(B) were as described in Ng *et al.* (1999), while those for *tet*(S) and *tet*(W) were described by Aminov *et al.* (2001). Bacterial suspension were boiled in 50 µL sterilized PBS at 95 ˚C for 10 min to obtain total DNA from the cells. Amplification reaction was carried out in 50 µL reaction mixture containing 0.25 pmol each primer, 0.25 mM dNTP, 5X Ex buffer, 2.5 U of Ex *Taq* DNA polymerase (TaKaRa, Kyoto, Japan). One µL of bacterial suspensions including whole cell DNA were used as template for PCR amplification. A total 30 amplification cycles were carried out. Each cycle consisted of 30s at 95 ˚C, 30s at 50˚C [*tet*(S)], 55˚C [*tet*(B)], 62 ˚C [*tet*(A)] or 64 ˚C [*tet*(W)] and 45s at 72 ˚C. PCR products on 1.2 % agarose gel were stained with GelRed™ (Biotium Inc., Hayward, CA, USA) and visualized on an Epi-Light UV FA1100 system with a Luminous Imager version 2.0 (Aisin Cosmos R&D, Aichi, Japan).

4.2.6. Sequencing analysis

PCR products were cloned by using a TOPO-TA Cloning kit (Invitrogen, Carlsbad, Calif.) according to the manufacturer's instructions.

White colonies of ampicillin-resistant transformants were screened for the presence of *tet* fragments by PCR with the same primer set used for amplification. And sequence analysis of *tet*(M) was performed by Macrogen Inc., (Seoul, Korea, http://www.macrogen.com).

Similarity search for the nucleotide sequence was carried out with BLAST at the National Centre of Biotechnology Information website (http:// www.ncbi.nml.nih.gov./BLAST).

For phylogenetic analyses of the *tet*(M), evolutionary analyses were conducted in MEGA5 (Tamura *et al*., 2011) and the *tet*(S) gene *of Lactococcus lactis* (clone GMCZ-T4) was chosen as outgroup. The phylogenetic tree (Fig. 4.2) for *tet*(M) was performed using the maximum likelihood with the best model: TN92+G+I (Tamura-3 parameter).

To test the confidence of *tet*(M) and 16S rRNA gene tree topologies, the heuristic search was performed using the neighbor-joining tree as the starting tree and bootstrap values were calculated by Maximum Likelihood (100 replicates).

The *tet*(M) sequences from isolates and natural assemblage are deposited to GenBank database, which can be accessed by the accession numbers KG408148 to KF408178.

4.3. Results and discussion

4.3.1. Relative abundance of *tet*(M) in natural assemblage

Relative abundance of *tet*(M) in both years is shown in Fig. 4.1. The relative abundance of *tet*(M) in 2011 ranged from 10^{-5} to 10^{-2} copies/mL. The highest was in KWD-2 site ($1.91\pm0.57\times10^{-2}$ copies/mL), followed by KYS-2 ($1.51\pm0.13\times10^{-2}$ copies/mL) and KGJ-3 ($6.45\pm0.28\times10^{-3}$ copies/mL) KWD-1 ($4.32\pm0.57\times10^{-3}$ copies/mL), KGJ-2 ($3.87\pm0.68\times10^{-3}$ copies/mL), KWD-3 ($2.32\pm0.48\times10^{-3}$ copies/mL) and KYS-3 ($1.29\pm0.89\times10^{-3}$ copies/mL), whereas KGJ-1 ($9.97\pm0.96\times10^{-5}$ copies/mL) and KYS-1 ($4.46\pm0.55\times10^{-5}$) were lower. The results of this study showed that *tet*(M) is highly persisted among the sampling site without OTC contamination in 2011.

To date, the quantitative analysis of *tet* genes was reported in past-aquaculture sediment (Tamminen *et al*., 2011), indicating *tet*(M) was at a level of 10^{-4} to 10^{-2}/16S rRNA gene in Swedish and Finnish aquaculture farms where the aquaculture has been ceased for 10 years. Another Chinese aquaculture data shows 10^{-6} to 10^{-4}/16S rRNA gene in sediment (Gao *et al*., 2012), *tet*(M) was prevalent in soils adjacent to swine feedlots at a range of 10^{-5} to 10^{-2}/16S rRNA (Wu *et al.,* 2010) and 10^{-1} to 10^{-2}/16S rRNA in sewage treatment plants (Zhang and Zhang, 2011). The results suggested that *tet* genes widely persisted in natural environments, although aquaculture sites were lower than terrestrial environments.

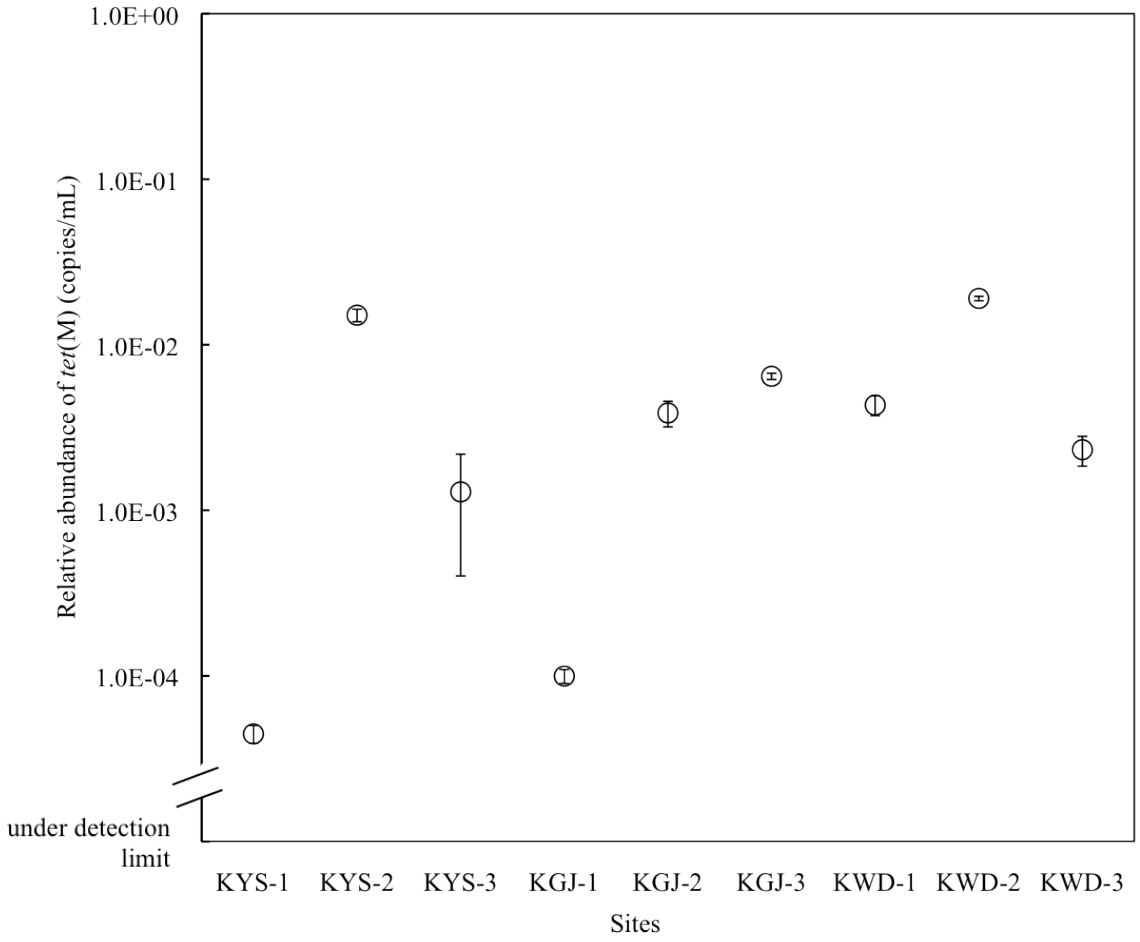


Fig. 4.1. Relative abundance of *tet*(M) (copies/mL) from natural assemblage in 2011. SD from triplicate experiments is shown with vertical bar with symbol. Under detection limit represents less than 1.5×10^{-4}.

4.3.2. Possession of *tet* genes in isolates

As mentioned above, *tet*(M) is persisted in aquaculture site even under non-contaminated areas for long time (Nonaka *et al*., 2007; Tamminen *et al*., 2011).

Twenty three OTC^r isolates were obtained from KGJ-3, 20 isolates from KWD-1 and 28 isolates from KWD-2 in 2011, which were used to detect *tet*(M) and other *tet* genes, *tet*(A), *tet*(B), *tet*(S) and *tet*(W). Table 4.1 shows the detection of targeted *tet* gene in isolated OTC^r bacteria in 2011. *Tet*(S) and *tet*(W) were negative for all isolates. *Tet*(M) gene was found in most isolates in KWD, whereas *tet*(M) was not detected in all isolates in KGJ. *Tet*(A) was detected in only 2 isolates in KGJ. Both *tet*(A) and (B) were simultaneously detected in only 1 isolate in KGJ, whereas all KWD isolates did not show positive result for all *tet*(A) and (B).

Two OTC^r isolates were obtained from KYS-2, 3 isolates from KGJ-3, 1 isolate from KWD-1, 25 isolates from KWD-1 and 4 isolates from KWD-3 in 2010 were used to detected only *tet*(M) in this study. Thirty isolates were obtained from KWD, which were positive for *tet*(M). Overall, 70 isolates (66%) were positive for *tet*(M), indicating the specific distribution of *tet* genes in neighboring areas.

Previous studies in Korea reported presence of *tet*(A), (B), (S), (W) and (M) in isolates collected from fish farm (Jun *et al*., 2004), aquaculture sites (Kim *et al*., 2004; Kim *et al*., 2007) and commercial fish and seafood (Ryu *et al*., 2012). These evidences suggest that various *tet* genes are ubiquitous in various Korean environments and seafood. Fish and seafood might be carriers of ARGs between seawater and human environment.

In this study found the different regional distribution of *tet* genes between KGJ and KWD sites, suggesting *tet*(M) other the tested genes are responsible for the OTC^r in KWD.

Table 4.1 Detection of targeted *tet* genes in isolated OTC^r bacteria from Korean coastal seawater

		Targeted *tet* genes						
Site ID	Isolated OTC^r bacteria	*tet*(S)	*tet*(W)	*tet*(A)	*tet*(B)	*tet*(A)+(B)	*tet*(M)	Unknown
KYS-1	ND*	-	-	-	-	-	-	-
KYS-2	ND*	-	-	-	-	-	-	-
KYS-3	ND*	-	-	-	-	-	-	-
KGJ-1	ND*	-	-	-	-	-	-	-
KGJ-2	ND*	-	-	-	-	-	-	-
KGJ-3	23	ND*	ND*	2	ND*	1	ND*	20
KWD-1	20	ND*	ND*	ND*	ND*	ND*	12	8
KWD-2	28	ND*	ND*	ND*	ND*	ND*	28	-
KWD-3	ND*	-	-	-	-	-	-	-

ND*: not detected

4.3.3. Diversity of *tet*(M) in natural assemblage

The 49 *tet*(M) PCR products from natural assemblage DNA was sequenced. Result of phylogenetic analysis is shown in Fig 4.2. BLAST searches of the GenBank database confirmed that all of the sequenced clones were *tet*(M) gene.

The sequence of natural assemblage *tet*(M) in KWD sites was highly homogenous. The genotype showed 99 to 100 % among clones. This suggests that *tet*(M) in KWD in natural assemblage is homogenous. Isolates from KWD also showed the same sequence to natural *tet*(M), suggesting that *tet*(M) in KWD is homogenous among non-culturable and culturable bacterial community members. The *tet*(M) in KWD was the same to known *tet*(M) from fish and aquaculture (Kim *et al*., 2007; Nonaka *et al*., 2012). The origin of *tet*(M) in KWD might be aquaculture.

On the other hand, KGJ-3 showed different result. The genotypes of *tet*(M) showed variation from 91.7 to 100 % homogeny in natural assemblage, suggesting that the *tet*(M) in KGJ-3 were diverse.

The sequence of *tet*(M) in KGJ was similar to *tet*(M) reported in *Enterococcus*, *Staphylococcus* sp. and *Streptococcus* sp. derived from human and animal (Agero *et al*., 2006; de Varies *et al*., 2009; Sadowy *et al*., 2010). KGJ might receive *tet*(M) from land and/or human environment.

Thus the genetic diversity of *tet*(M) in KWD and KGJ were different, suggesting that each area has specific source of *tet*(M) even in neighboring area. Other reports showed various genotypes of the *tet*(M) could be found in human and animal pathogens (Aminov *et al*., 2001), Mekong river sediment (Kobayashi *et al*., 2007b) and coastal aquaculture sites in Japan (Nonaka *et al*., 2007).

Fig. 4.2. Molecular phylogeny of *tet*(M) in isolates (green letter) and natural assemblages (red letter, KGJ; blue letter, KWD). The phylogenetic tree was obtained by maximum likelihood method based on a 95 sequences dataset (length 701 bp). For the analysis, the Tamura-3 parameter evolutionary model was chosen, with the proportion of invariable sites (I=0.12) and gamma distribution shape parameter (G=0.64). Bootstrap values were inferred from maximum likelihood with 100 replicates. Cul, isolates and Env, natural assemblage are indicated.

Chapter 5. Diversity of *tet*(M) possessing bacteria

5.1. Introduction

The *tet*(M) has been detected in isolated bacteria from Korean environmental samples (Jun *et al.,* 2004; Kim *et al.,* 2004; Kim *et al.,* 2007; Park *et al.,* 2009). Present study detected *tet*(M) from isolated bacteria and natural assemblage (Chapter 4). These studies indicated that *tet*(M) is widely distributed in aquatic environments even at sites without OTC contamination. Furthermore, KWD and KGJ sites in this study showed that different profiles of *tet*(M) genotypes between the two sites. The *tet*(M) is found in various bacterial species including Firmicutes and proteobacteria in human pathogens (D'Hondt *et al.,* 2004). Also from aquatic proteobacteria especially Gamma proteobacteria representing *Vibrio* sp., *Aeromonas* sp., and *Edwardsiella* sp. are known to harbor *tet*(M) (Kim *et al*., 2004; Nonaka *et al*., 2007; Akinbowale *et al*., 2007; Kim *et al*., 2011). These are isolates relating to aquaculture and fish disease. To know *tet*(M) distribution in various phylogenetic groups in natural surface water in coastal areas without OTC contamination, this Chapter describes diversity of *tet*(M)-possessing bacteria among the isolates obtained from KWD sites based on 16S rRNA gene sequence.

5.2. Materials and methods

5.2.1. 16S rRNA gene sequence of isolates

As the isolated OTC^r bacteria were mentioned in Chapter 3, total 106 isolates from 35 isolates in 2010 and from 71 isolates in 2011 were employed for this study.

Partial 16S rRNA gene was amplified by PCR using a primer set of f341 (5'-CCTACGGGAGGCAGCAG-3') and 907r (5'-CCGTCAATTCMTTTGAGTTT-3') (Muyzer *et al*., 1998), which product length was 566 bp. The reaction mixture for PCR contained 5μL of 10× PCR buffer, 4 μL each of 0.2 mM dNTP, 2.5 U of Ex *Taq* DNA polymerase (TaKaRa, Kyoto, Japan), 50 pmol of each primer and 25-90 ng of template DNA in a final volume of 50 μL. PCR cycle consisted of denaturing at 95 ˚C for 1min, annealing at 58 ˚C for 1 min, and elongation at 72 ˚C for 1 min 30s. The reaction was performed 30 cycles and the final extension was 7 min. Ten μL of PCR product was separated and visualized following the same method descried above.

The PCR products of 16S rRNA gene were sequenced by using the BigDye Terminator cycle sequencing ready reaction kit mix (version 3.1; Applied Biosystems, Foster City, CA, USA) and analyzed with the ABI 3100 Genetic Analyzer at the Center for Gene Research, Ehime University. Similarity search for the nucleotide sequence was carried out with BLAST at the National Centre of Biotechnology Information website (http:// www.ncbi.nml.nih.gov./BLAST).

5.2.2 Phylogenetic analysis

For phylogenetic analyses based on the 16S rRNA gene, sequences obtained from the isolated OTC^r bacteria were added with available sequences from GenBank in 2013 to make a large dataset. There were a total of 136 sequences and 518 unambiguously aligned

base positions in the final dataset. *Ketogulonicigenium vulgare* (strain WSH-001) was used as outgroup as in Suzuki *et al.* (2013). The phylogenetic tree (Fig. 5.1) was inferred by maximum likelihood (ML) using the GTR+G+I model, which was chosen as the best model according to the Akaike Information Criterion of MEGA5 and Modeltest 3.7. To test the confidence of both tree topologies, the heuristic search was performed using the neighbor-joining tree as the starting tree and bootstrap values were calculated by Maximum Likelihood (100 replicates).

The 16S rRNA gene sequences were deposited in GenBank database under the accession number KF408184 to KF408218.

5.3. Results and discussion

5.3.1. Diversity of *tet*(M) possession bacteria

Total 70 isolates of the 106 OTC^r isolates were confirmed to possess *tet*(M). As shown in Fig. 5.1. The *tet*(M)-possessing isolates belong to *Vibrio* and *Photobacterium* were the genus. Neela *et al*. (2007) reported that most of the OTC^r bacteria, some of which showed MDR, from aquaculture environment were *Vibrio* sp. Other studies also showed *tet*(M) were conveyed by *Vibrio* sp. (Kim *et al*., 2004; Kim *et al.*, 2011) and *Photobacterium* sp. (Nonaka *et al*., 2007). Present finding is consistent with these studies. This is possibly due to that this genus could be cultured on nutrient-rich medium. Most of the information of *tet*(M)-possessing human pathogenic bacteria were culturable proteobacteria and Firmicutes (D'Hondt *et al.*, 2004). Recent report suggests marine non-culturable assemblage possesses *sul*3 (Suzuki *et al*., 2013), which gene could not be found in culturable one. Further study with culture-independent method is needed to clarify the reality of ARGs possessing bacteria in environment.

Present study and Kim *et al*. (2007) strengthen a possibility that genus *Vibrio* is a general member of the *tet*(M) reservoir in Korean seawater. The *tet*(M) could be transferred within pathogenic and non-pathogenic bacteria (Neela *et al*., 2007) that might disseminate the spread of *tet*(M) in the aquatic environment. Previous report showed that *tet*(M) can be transferred from marine origin bacteria to human enteric bacteria (Agerso *et al.*, 2002; Neela *et al*., 2007). Whether the marine *Vibrio* is a risk to carry the ARGs to human should be examined further.

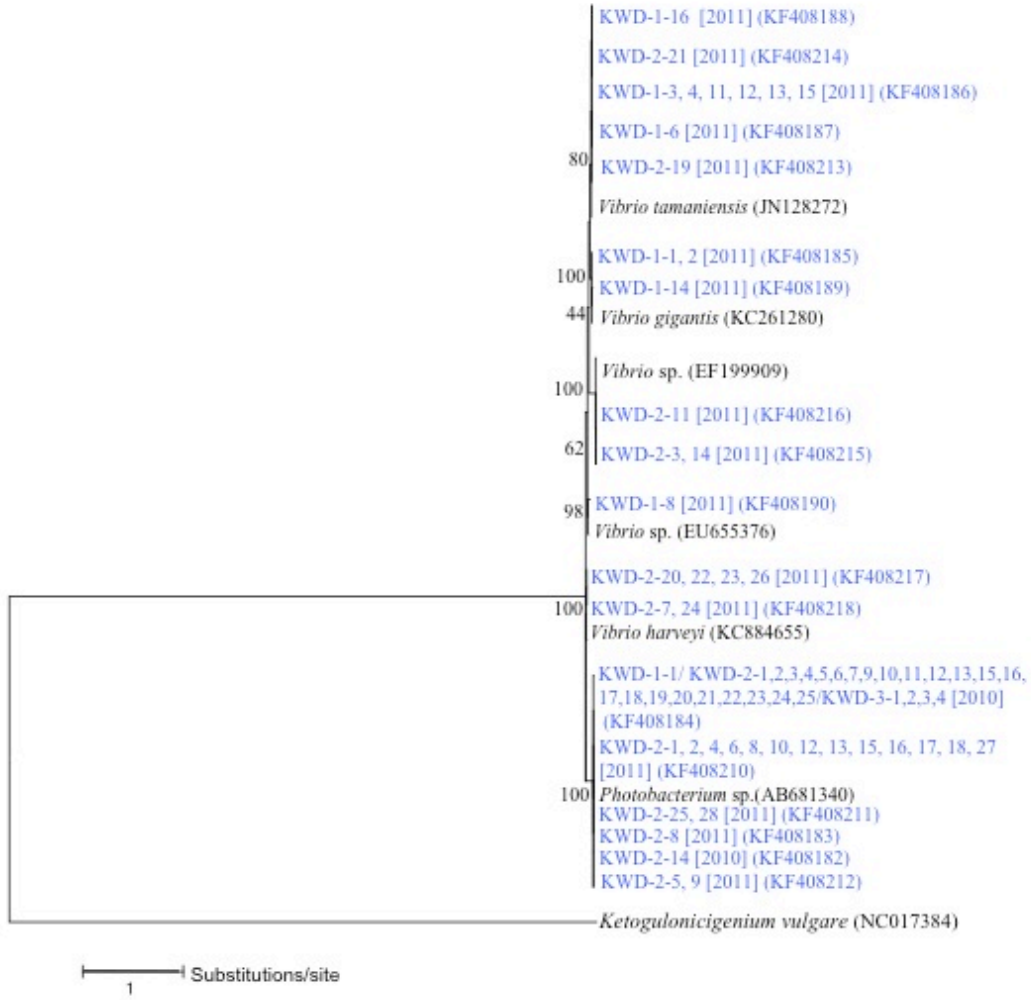


Fig. 5.1 Diversity of *tet*(M) possessing bacteria based on 16S rRNA gene. Seventy *tet*(M)-positive isolates were classified. Parenthesis represents the isolates in 2010. Bracket represents the isolates in 2011. For the analysis, the General Time Reversible (GTR) model was chosen, with the proportion of invariable sites (I=0.45) and gamma distribution shape parameter (G=0.25). Bootstrap values were inferred from maximum likelihood with 100 replicates.

Chapter 6. Conclusion

In Korea, OTC is commonly used in human, animal medicine and aquaculture, thus might be released into the aquatic environment. However, the contamination status of antibiotics and ARB in coastal areas has not been well understood. This study aimed, 1) to estimate the OTC concentration in Korean coastal seawater 2) to enumerate the occurrence of OTC^r bacteria and *tet* genes and 3) to evaluate the diversities of *tet*(M) gene and the *tet*(M)-possessing bacteria.

The concentration of OTC was below detection limit (0.1 μg/L) in all sites, suggesting that these sites are not contaminated with OTC. The occurrence rate of OTC^r bacteria was 0–0.35 % and 0-10.6 % in 2010 and 2011. This result did not show significant difference. These results suggest that the OTC^r bacteria in water column were occurred independent with OTC contamination, and the resistance rate was lower than other previous reports. The occurrence of OTC^r bacteria is not correlated to selection by OTC.

Quantification of the *tet*(M) in coastal seawater resulted that *tet*(M) is persisted in seawater in 2011. The *tet*(M) abundance is higher than occurrence rate of OTC^r bacteria, which suggests that non-culturable bacteria possess *tet*(M) in seawater. The diversity of *tet*(M) showed the genotype was different among areas. The *tet*(M) in Wando was homogenous, however that in Geoje was diverse. This suggests the spatial diversity of *tet*(M) and their origin was different among the sampling sites in Korea. I found that the majority of reservoir of *tet*(M) was *Vibrio* sp and *Photobacterium* sp. Taken together with past reports, this group should be a major reservoir of *tet*(M) in seawater bacteria.

Through this study, the following new findings were obtained; (1) the distribution of OTC^r bacteria and *tet*(M) gene were not related to OTC contamination, (2) genotypes of *tet*(M) showed the regional differences, which might be due to different origin of *tet*(M),

and (3) the majority of *tet*(M) reservoir was belonged to *Vibrio* sp and *Photobacterium* sp. These are the first to show unique evidence of OTC^r bacteria and *tet*(M) in Korean coastal seawater.

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