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Prevalence of *tet*(B) and *tet*(M) genes among tetracycline-resistant *Vibrio* spp. in the aquatic environments of Korea

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ABSTRACT: Of 24 tetracycline(Tc)-resistant *Vibrio* spp. isolated from different marine sources in Korea between 1993 and 2003, 23 were identified as carrying both *tet*(B) and *tet*(M), while 1 strain carried *tet*(B) only. In conjugation experiments, 3 strains appeared to be able to transfer both *tet*(B) and *tet*(M) to the recipient. Both discriminatory PCR and sequence analysis showed that *tet*(M) genes of *Vibrio* spp. appear to be a single allele containing a specific region of *tet*(M) in Tn*1545*. However, *erm*(B) and *aphA3*, known to be linked to Tn*1545*-like genes, were not detected in Tc-resistant *Vibrio* spp., even in 9 strains resistant to erythromycin. In analysis to examine the relative position of *tet*(B) and *tet*(M), it was shown that *tet*(M) was present at the 3′-end of the insertion sequence IS*10* of Tn*10* carrying *tet*(B). At the junctional region between Tn*10* and *tet*(M), we found a 14 bp sequence of unknown function and the deletion of regulatory sequences reported to be needed for *tet*(M) expression in conjugative transposons. This is the first report of the simultaneous presence of *tet*(B) and *tet*(M), and of the *tet*(M) gene being linked to the 3′-end of Tn*10* in Tc-resistant *Vibrio* spp. in Korea.

KEY WORDS: Tetracycline-resistance genes · PCR · *Vibrio* spp.

INTRODUCTION

Tetracycline (Tc) use in man and aquatic animals over the last 50 yr has influenced the appearance of Tc-resistant bacteria in aquatic environments (Anderson & Sandra 1994, Guardabassi et al. 2000). In bacteria, 3 mechanisms of Tc resistance are known to be mediated by >38 different Tc-resistance determinants (Roberts 2005). The first is mediated by *tet* genes encoding ribosomal protection proteins (RPP-encoding genes), including *tet*(M) and *tet*(O). The second mechanism involves the *tet*(X) gene product from Bacteroides, and is the only known example of enzymatic inactivation of Tc that has been described so far. In the third mechanism, Tc-efflux genes, including those designated *tet*(A) to *tet*(E), *tet*(G) and *tet*(H) in Gram-negative bacteria and *tet*(K), *tet*(L) and *tet*(P) in Gram-positive bacteria, encode membrane-associated proteins that transport Tc out of cells (Chopra & Roberts 2001, Roberts 2005). Many studies have examined the distribution of different *tet* genes encoding efflux pumps in various aquaculture environments (DePaola et al. 1993, DePaola & Roberts 1995, Schmidt et al. 2001, Nonaka & Suzuki 2002, Teo et al. 2002). In previous studies, we have also shown a multiplex PCR to determine and differentiate *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E) and *tet*(G), which are found primarily in Gram-negative bacteria, by the different sizes of the resulting PCR products (Jun et al. 2004). However, few reports have described *tet* genes associated with RPPs in

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aquaculture. Among the RPP-encoding genes, *tet*(M) is the most common and is present in a diverse range of Gram-positive and Gram-negative bacteria and mycoplasma (Chopra & Roberts 2001, Roberts 2005). Recently, *tet*(M) has also been found in *Vibrio* spp., *Lactococcus garviae* and *Photobacterium damselae* (Kim et al. 2004). However, these studies did not examine the presence of other *tet* genes.

The *tet*(M) gene has been associated with the conjugative transposons Tn*916*, Tn*1545* and other related transposons (Rice 1998). Nucleotide sequences of the *tet*(M) genes in Tn*916*, Tn*1545* and *Ureaplasma urealyticum* share 92 to 99% nucleotide identity (Gascoyne-Binzi et al. 1993, Oggioni et al. 1996). Such differences have suggested that a cell can acquire >1 *tet*(M) element via transposons and that these acquired genes may recombine to yield a hybrid element (Rice 1998).

Oxytetracycline is the most widely used antibiotic agent to treat infections in marine-cultured fish (Smith et al. 1994, Nonaka et al. 2000, Rhodes et al. 2000, Tendencia & de la Pena 2001). Thus, knowing the distribution of *tet* genes in Tc-resistant fish pathogens, especially in *Vibrio* spp., which are common in marine environments, might help elucidate the origin of resistance genes and their relationship to human and animal pathogens.

In the present study, we used multiplex PCR to carry out an extended analysis of several *tet* genes in Tc-resistant *Vibrio* spp. obtained from aquatic samples during the years 1993 to 2003 at various locations in Korea. In a novel finding, a *tet*(B) gene was identified in isolates of *Vibrio* spp. that also carry the *tet*(M) gene. The genetic characteristics of *tet*(B) and *tet*(M), as well as gene mapping and the results of nucleotide sequence analysis are described herein.

MATERIALS AND METHODS

Bacterial cultures. Twenty-four *Vibrio* spp. isolated between 1993 and 2003 from different diseased fish in marine aquatic farms of Korea were cultured aerobically on tryptose soy broth (TSB; Difco) supplemented with 1% (w/v) NaCl at 25°C for 18 h. In this study, Tc-resistant *Vibrio* spp. were defined as those isolates producing yellow colonies on thiosulfate citrate bile salts sucrose (TCBS; Difco) plates containing 30 µg Tc ml^{-1} (Sigma), but showing no growth on chromocult coliform plates (CC; Merck). Isolates were further identified by an API 20E kit (BioMerieux) and by conventional laboratory methods including Gram stain, O/F test, mobility and catalase test. To compare the genetic features of *Vibrio* spp. with those of other bacteria, 6 reference strains described by Jun et al. (2004) were used (Table 1). The control strain *Streptococcus* sp. CJ, carrying the *tet*(M) gene, was obtained from Tc-resistant isolates maintained in our laboratory after analyzing the nucleotide sequence of the amplicon produced by PCR with the TMF/TMR primer set (Table 2). Another 2 control strains of *Streptococcus* spp., SJ14 and 48, were used as erythromycin (EM)-resistant strains carrying the *erm*(B) gene.

Antibiotic-sensitivity assay. For Tc- and minocycline (Mino)-sensitivity assay, 2-fold dilutions (1 to 512 µg ml^{-1}) were carried out in microplates (Alderman & Smith 2001). Minimum inhibitory concentration for each isolate was recorded after 24 h incubation at 37°C and 25°C for *Escherichia coli* strains and laboratory isolates, respectively. Antibiotic-susceptibility testing was performed by an agar-diffusion assay on Muller-Hinton agar with disks (Becton Dickinson) containing 10 U penicillin G (PE), 30 µg chloramphenicol (CM), 15 µg erythromycin (EM), 2 µg oxolinic acid (OA) or 25 µg kanamycin (KM). Zones of growth inhibition were evaluated after overnight incubation according to NCCLS guidelines (National Committee for Clinical Laboratory Standards 2002).

DNA extraction. Bacterial isolates were grown aerobically on TSB supplemented with 1% (w/v) NaCl at 25°C for 18 h. After culture, cells were harvested by centrifugation at 8000 × *g* for 10 min. The cells were then resuspended in TE buffer (10 mM Tris/HCl [pH 8.0], 1 mM EDTA) and lysed with 5.5% SDS and 0.125 mg Proteinase K ml^{-1} (Boehringer Mannheim). Bacterial nucleic acids were extracted with a phenol/chloroform/isoamyl alcohol mixture (25:24:1 by vol.; Sigma), precipitated with 2 V of an ethanol/0.3 M sodium acetate mixture at –80°C , and resuspended in 50 µl of distilled water. The DNA was stored at –20°C until further use.

Primer design. To obtain different lengths of PCR-generated amplicons corresponding to the 6 different *tet* genes, 6 antisense primers, TAR, TBR, TCR, TDR, TER and TGR, complementary to specific regions of the *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E) and *tet*(G) genes, respectively, were mixed with the sense primer TETF. The latter contained 1 degenerate base at the fifth position, derived from the nucleotide sequence conserved among the 6 different *tet* gene sequences (Table 2). The specificity of these primers for PCR is described by Jun et al. (2004). PCR for the detection of *tet*(M) was carried out using the TMF/TMR primer set, which was derived from the conserved region of the *tet*(M) alleles U09422, X90939, U08812, M21136 and X92947, obtained from the GenBank database. For discrimination of the specific region of *tet*(M) together with the detection of the *tet*(B) gene, the following mixture of 5 different primers was used in discriminatory multiplex PCR: TMF, TMRV1 (antisense primer derived from the specific region of *tet*(M) present in

Table 1. Tetracycline-resistant *Vibrio* spp. and reference strains used in this study. *V. alginolyticus*: YV1, JV12, JV18; *V. vulnificus*: KT1, KT2; *Photobacterium damselae*: JE1, RV5, RV16, YV5, JV13, JV15, M1, GV2; unidentified strains: RV11, YV4, JV14, JV17, M3, M4, GV3, KT4, KT5, KT7, C35. Tc: tetracycline; Mino: minocycline; OA: oxolinic acid; EM: erythromycin; PE: penicillin G; CM: chloramphenicol; KM: kanamycin; ○: susceptible (minimum inhibitory concentration given for Tc and Mino, in µg ml^{-1}); ●: resistant

Strain	Source	Area	Year	Susceptibility:							*tet* gene	*Int-Tn* gene	*erm* gene	Remark
				Tc	Mino	OA	EM	PE	CM	KM				
JE1	Flounder	Namhae	1993	128	8	●	●	●	●	○	B,M	–	–	*Vibrio* isolates
RV5	Seabass	Hadong	1993	64	8	○	●	●	○	○	B,M	–	–	used in the
RV11	Flounder	Namhae	1993	64	16	○	○	○	○	○	B,M	–	–	present study
RV16	Flounder	Pohang	1993	64	8	○	○	●	○	○	B,M	–	–	
YV1	Flounder	Yeosu	1994	128	16	○	●	●	○	○	B,M	–	–	
YV4	Flounder	Yeosu	1994	64	16	●	●	●	○	○	B,M	–	–	
YV5	Flounder	Yeosu	1994	128	32	○	●	●	○	○	B,M	–	–	
JV12	Seaperch	Pohang	2001	64	8	○	●	●	○	○	B,M	–	–	
JV13	Flounder	Namhae	2001	64	8	●	○	●	○	○	B,M	–	–	
JV14	Mullet	Yeosu	2001	64	8	○	○	●	○	○	B,M	–	–	
JV15	Seaperch	Yeosu	2001	128	16	●	○	●	○	○	B,M	–	–	
JV17	Seawater	Jeju	2002	64	16	○	○	●	○	○	B,M	–	–	
JV18	Flounder	Jeju	2002	128	16	○	○	●	●	○	B,M	–	–	
M1	Flounder	Tongyeong	2002	64	32	●	●	●	○	○	B,M	–	–	
M3	Seawater	Tongyeong	2002	64	32	○	○	○	○	○	B,M	–	–	
M4	Flounder	Tongyeong	2002	64	32	●	●	●	○	○	B,M	–	–	
GV2	Seaperch	Geoje	2003	64	16	●	○	●	○	○	B,M	–	–	
GV3	Seaperch	Geoje	2003	64	8	○	○	●	●	○	B,M	–	–	
KT1	Red seabream	Geoje	2003	256	16	○	○	●	○	○	B,M	–	–	
KT2	Seaperch	Geoje	2003	256	16	○	○	●	○	○	B,M	–	–	
KT4	Red seabream	Geoje	2003	256	16	○	○	●	○	○	B,M	–	–	
KT5	Seaperch	Geoje	2003	64	32	●	●	●	○	○	B,M	–	–	
KT7	Seaperch	Geoje	2003	64	32	●	○	●	○	○	B,M	–	–	
C35	Flounder	Pohang	2003	64	8	○	○	●	○	○	B	–		
Streptococcus sp. CJ	Flounder	Jeju	2004	64	8	●	○	○	○	○	M	+	–	Reference
Streptococcus sp. SJ14	Flounder	Tongyeong	2003	64	8	●	●	○	○	○	S	–	B	strains for *tet*(M)
Streptococcus sp. 48	Flounder	Gijang	2005	64	8	●	●	○	○	○	S	–	B	and *erm*(B)
Edwardsiella tarda RE1											A			Reference
Escherichia coli C600 R222											B			strains for *tet*
Escherichia coli HB101 pBR322											C			genes (Jun et al.
Escherichia coli HB101 pPT3											D			2004)
Aeromonas hydrophila HA											E			
Escherichia coli C600 pJA8122											G			

Table 2. PCR primers used in this study. Position is given from the first base of the start codon of each *tet* gene. Primer designs were based on the relevant *tet* gene reference sequence from the GenBank database

Primer	Direction	Sequence (5′–3′)	Position	Reference sequence	Purpose	Expected size
TETF[a]	Sense	GCGCTNTATGCGTTGATGCA	148–168		Detection of	
TAR	Antisense	ACAGCCCGTCAGGAAATT	534–517	X0006	*tet*(A) to *tet*(G)	387 bp
TBR	Antisense	TGAAAGCAAACGGCCTAA	318–301	J01830		171 bp
TCR	Antisense	CGTGCAAGATTCCGAATA	778–761	J01749		631 bp
TDR	Antisense	CCAGAGGTTTAAGCAGTGT	631–613	X65876		484 bp
TER	Antisense	ATGTGTCCTGGATTCCT	246–230	L06940		246 bp
TGR	Antisense	ATGCCAACACCCCCGGCG	950–933	S52437		803 bp
TMF	Sense	GAATCTGAACAATGGGAT	511–528	U09422	Detection of	1099 bp
TMR	Antisense	CTAACAATTCTGTTCCAGC	1609–1591		*tet*(M) gene	
TMRV1	Antisense	CGTTTGCAGCAGAGGGAGG	1044–1026	X92947	Discrimination of	534 bp
TMRV4	Antisense	CATTCCACTTCCCAACGG	1374–1357	X90939	*tet*(M) alleles with TMF sense primer	864 bp
Int1	Sense	TGACACTCTGCCAGCTTTA	759–778	X61025	Detection of	579 bp
Int2	Antisense	CCATAGGAACTTGACGTTCG	1318–1337		*Int-Tn* gene	
Em(A)F	Sense	GTTCAAGAACAATCAATACAGAG	4751–4773	K02987	Detection of	421 bp
Em(A)R	Antisense	GGATCAGGAAAAGGACATTTTAC	5171–5149		*erm*(A), (B) and	
Em(B)F	Sense	CCGTTTACGAAATTGGAACAGGTAAAGGGC	892–921	U35228	(C)[b]	359 bp
Em(B)R	Antisense	GAATCGAGACTTGAGTGTGC	1251–1232			
Em(C)F	Sense	GCTAATATTGTTTAAATCGTCAATTCC	8522–8548	NC_007792		572 bp
Em(C)R	Antisense	GGATCAGGAAAAGGACATTTTAC	7998–7976			
Km(3)F	Sense	GAAGGAATGTCTCCTGCTAAG	1026–1047	U51474	Detection of	603 bp
Km(3)R	Antisense	GCAGAAGGCAATGTCATACC	1609–1628		*aphA3* gene	

[a]TETF used a common sense primer for detection of *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E) and *tet*(G), as described by Jun et al. (2004)
[b]See Lina et al. (1999)

Tn*1545*), TMRV4 (antisense primer derived from the specific region of *tet*(M) present in Tn*916*), TETF and TBR (Table 2). All of the PCR primers were designed with gene alignment (MACAW Program Version 2.0.5; National Center for Biotechnology Information, National Institutes of Health) of the different *tet* gene sequences retrieved from the Entrez database. All primers were synthesized using an automated DNA synthesizer (Bioneer) and the phosphoramidite method.

Identification of the Tc-resistance determinant by PCR. PCR amplification was carried out in a 50 µl reaction mixture containing the extracted bacterial nucleic acid (100 ng of total nucleic acid isolated from each bacterial isolate), 10 mM Tris/HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% w/v gelatin, 0.5% Tween-20, 200 µM of each dNTP, 100 pM of each sense and antisense primer (all primers except TETF and TMF in the detection of *tet*(A) to (E) and (G), and discriminatory PCR, respectively) or 200 pM (for TETF and TMF), and 1.25 U AmpliTaq DNA polymerase (Perkin-Elmer). For multiplex PCR, 2 µM sense and 1 µM of each of the 6 specific antisense primers were used in the same PCR reaction mixture as described above. The PCR parameters were 30 cycles of denaturation at 94°C for 30 s and annealing at 55°C for 30 s, followed by a final extension step at 72°C for 30 s. The reaction was performed in 0.2 ml thin-walled tubes and using a Perkin-Elmer 2400 thermal cycler. Amplicons were visualized by agarose gel electrophoresis, as described by Sambrook et al. (1989).

Conjugal transfer assay. Transferability of resistance markers was examined by using filter mating and CC agar plates as a selective and differential medium. Donor and recipient (*Escherichia coli* HB101) cells were grown overnight in 4 ml of TSB. The donor cell culture (0.2 ml) was added to 1.8 ml of the recipient cell culture and mixed. The mixture was passed through a nitrocellulose 0.45 µm filter (Millipore). The filter was placed with the bacterial cells touching the surface of a TSA plate. After incubation at 30°C for 24 h, the filter was removed and mixed with 10 ml of TSB. The mating mixture was spread onto CC agar plates containing Tc. After incubation at 30°C for 24 h, the colors of the colonies were observed as described by Yoo et al. (2003).

RESULTS

Detection of Tc-resistant genes

Twenty-four Tc-resistant *Vibrio* spp. isolated from various marine sources in Korea were collected between 1993 and 2003. All these strains were Mino-resistant but KM-sensitive (Table 1). Of the 24 isolates, 22 were multidrug resistant and 9 were EM resistant. In discrim-

inatory multiplex PCR for the *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E) and *tet*(G) genes, all 24 Tc-resistant *Vibrio* spp. were found to carry the *tet*(B) gene, as confirmed by the production of an amplicon with the expected length of 171 bp. In contrast, *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E) and *tet*(G), either as single genes or as a multiple of *tet* genes together with *tet*(B), were not found in any of the Tc-resistant *Vibrio* spp. The isolates were then screened further for the major *tet* gene encoding RPPs, *tet*(M), by PCR using the specific primer set TMF/TMR. Interestingly, in 23 of the 24 *Vibrio* spp., *tet*(M)-specific amplicons with the expected length of ca. 1.1 kb were identified (data not shown).

Characterization of *tet*(M)

The sequence of the control strain *Streptococcus* sp. CJ, corresponding to the TMF/TMRV4 primer set used in this study, was identical to that of *tet*(M) in Tn*916*. The newly determined nucleotide sequence was deposited in GenBank under Accession Number DQ868544.

To characterize the *tet* genes found in Tc-resistant *Vibrio* spp., we designed a discriminatory multiplex PCR using the mixed primers TMF/TMRV1/TMRV4 and TETF/TBR (Table 2). This approach allowed the detection of *tet*(B) and discrimination of specific *tet*(M) alleles as amplicons of 864 and 534 bp, depending upon the specific region derived from *tet*(M) in Tn*916* and Tn*1545*, respectively. As shown in Fig. 1, all Tc-resistant *Vibrio* spp. from this study contained the *tet*(M) allele producing a 534 bp amplicon, indicating the presence of a specific part of the *tet*(M) found in Tn*1545* rather than in Tn*916*.

The control strain, *Streptococcus* sp. CJ, carrying a specific part of the *tet*(M) of Tn*916*, only produced a single 864 bp amplicon and indicated the specificity of this discriminatory multiplex PCR. Although not all 23 amplicons were sequenced, the sequences of 534 bp PCR products of 5 different randomly chosen strains (*Vibrio* spp. JE1, RV5, RV16, YV1 and M4) were identical, except for a 1 base substitution (A to T) at Position 148 (numbered from the 5′ end of TMF primer) in 1 strain (*Vibrio* sp. M4). In these 5 isolates, the sequences of the *tet*(B)-specific amplicons obtained with TETF/TBR primers also proved to be indistinguishable from that of the corresponding structural *tet*(B) gene from Tn*10* (GenBank Accession Number J01830). Thus, the results of both experiments, discriminatory PCR and DNA sequencing, suggested that most of our Tc-resistant *Vibrio* spp. possessed a single type of *tet*(M) allele with a *tet*(B) gene.

Other Tn*1545*-like associated genes, *erm*(B) and the *int-Tn* gene of Tn*1545*, were not detected in 24 Tc-resistant and *tet*(M)-positive *Vibrio* spp., including

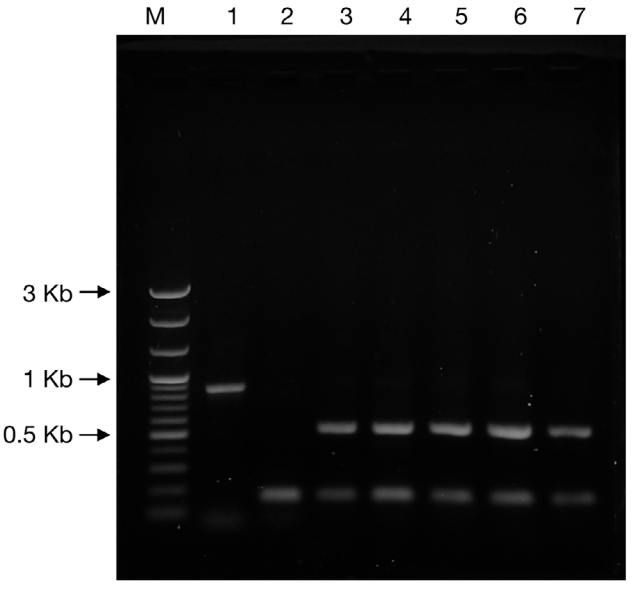

Fig. 1. Agarose gel electrophoresis of amplicons generated by multiplex PCR for the detection of *tet*(B) and the discrimination of *tet*(M) alleles. Lane M: 100 bp DNA ladder; Lane 1: *Streptococcus* sp. CJ; Lane 2: *Vibrio* sp. C35; Lane 3: *Vibrio* sp. RV16; Lane 4: *Vibrio* sp. RV5; Lane 5: *Vibrio* sp. JE1; Lane 6: *Vibrio* sp. M4; Lane 7: *Vibrio* sp. YV1

9 EM-resistant strains, in PCR with the primers specific to these elements. Other EM-resistance genes, *erm*(A) and *erm*(C), were not detected in Tc-resistant *Vibrio* spp. either. However, among 3 control strains of *Streptococcus* spp., EM- and Tc-resistant strains of SJ14 and 48 were found, which carried *tet*(S) rather than *tet*(M), and *erm*(B). There was no *erm*(B) in the Tc-resistant and EM-sensitive CJ strain carrying *tet*(M) with the *int-Tn* gene. All isolates were negative for *aphA3*, another Tn*1545*-like associated gene, and matched with the observed results showing it to be KM sensitive in antibiotic-susceptibility tests.

Location of *tet*(B) and *tet*(M)

To determine the genetic organization of *tet* genes in *Vibrio* spp. carrying both *tet*(B) and *tet*(M), PCR was carried out with different combinations of primers for the 2 genes. In these PCRs, the TETF/TMR primer set produced amplicons of 5326 bp in various *Vibrio* spp. The newly determined nucleotide sequence of the DNA fragment was deposited in GenBank under Accession Number DQ886586. Production of an amplicon in this PCR suggested that *tet*(B) and *tet*(M) genes in Tc-resistant *Vibrio* spp. are located very close to each other and in the same direction (Fig. 2).

The transferability of *tet* genes was examined for 24 strains. As summarized in Table 3, 3 strains were able

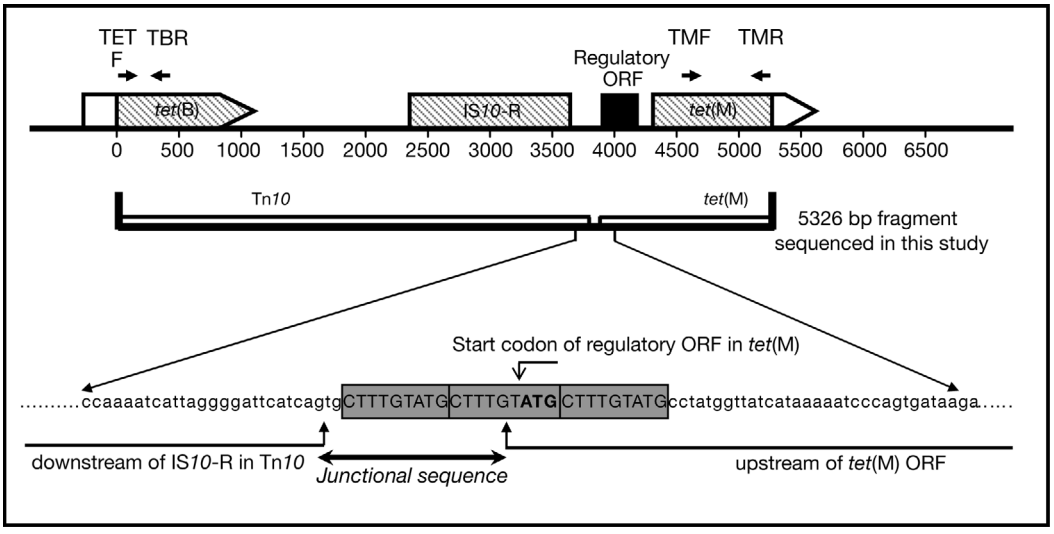

Fig. 2. Schematic illustration of the location of *tet*(B) and *tet*(M). Amplicon produced in PCR with the TETF/TMR primer set (see Table 2) was designated the '5326 bp fragment'. Arrows at the top indicate the direction of the primers used. Junctions consisting of 14 bp and 3 repeating sequences formed with sequences from the 5′-end flanking region of *tet*(M) are enclosed in gray

to transfer their Tc, Mino and EM resistance to *Escherichia coli* HB101 in conjugation experiments. All of the transconjugants showed resistance to those 3 antibiotics. Although the isolates studied did not contain one of the *erm*(A), (B) and (C) analyzed, it was found that *tet*(B)-, *tet*(M)- and EM-resistance genes were able to be transferred to the recipient (Table 3). Both donors and transconjugants produced the same amplicons of 5326 bp in PCR with the TETF/TMR primer set.

Sequencing the amplified fragment revealed that it encompassed the entire right portion of Tn*10* of *Shigella flexneri* (GenBank Accession Number AF162223), from the middle of *tet*(B) to the intervening sequence-right (IS*10*-R) of Tn*10*, and a part of the *tet*(M) gene of Tn*1545*. At the joint region consisting of the 3′-end of IS*10*-R of Tn*10* and the *tet*(M) element, we found an additional 14 bp followed by 103 bp of the 5′-end flanking sequences found in the *tet*(M) of Tn*1545* of *Enterococcus faecalis* (GenBank Accession Number X04388). At the junction between Tn*10* and the adjacent *tet*(M) gene, 3 repeated sequences of 9 bp (CTTTGTATG) were detected (Fig. 2).

DISCUSSION

The present study examined the distribution of several *tet* genes in Tc-resistant *Vibrio* spp. isolated from different marine sources in Korea between 1993 and 2003. Unexpectedly, both *tet*(B) and *tet*(M) were detected in 23 out of 24 Tc-resistant *Vibrio* spp. These results partially agreed with those of Furushita et al. (2003), who analyzed the *tet* genes *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(Y) and *tet*(H) in 43 Tc-resistant, heterogeneous Gram-negative strains isolated from fish-farm bacteria and found *tet*(B) to be prevalent in 31 of the strains. Additionally, in another study on the distribution of *tet*(M) and *tet*(S), *tet*(M) was the most commonly occurring *tet* gene in high-level Tc-resistant strains isolated from healthy and diseased fish, as well as from seawater at coastal aquaculture sites in Japan and Korea between

Table 3. Antimicrobial susceptibility of transconjugant strains and results of conjugation experiment. See Table 1 for abbreviations/symbols. Susceptibility to Tc and Mino given as minimum inhibitory concentration, in μg ml^{-1}

Donor strains	*tet* gene	Susceptibility of transconjugant strains							Transferred *tet* gene
		Tc	Mino	OA	EM	PE	CM	KM	
Photobacterium damselae JE1	B,M	128	8	○	●	●	●	○	B,M
Vibrio alginolyticus YV1	B,M	128	8	○	●	●	○	○	B,M
Photobacterium damselae YV5	B,M	128	8	○	●	●	○	○	B,M
Escherichia coli HB101	–	<0.5	<0.5	○	○	○	○	○	–

1997 and 2002 (Kim et al. 2004). In that study, all the Gram-negative isolates that carried *tet*(M) were *Vibrio*/*Aeromonas* strains (Kim et al. 2004). Unfortunately, neither of those 2 studies analyzed the distribution or simultaneous presence of *tet*(B) and *tet*(M) genes. Thus, our study is the first to show that *tet*(B) and *tet*(M) are present simultaneously in Tc-resistant *Vibrio* spp. of Korea and are the dominant *tet* genes.

To determine whether various types of *tet*(M) alleles are carried by Tc-resistant *Vibrio* spp. in Korea, we developed a discriminatory multiplex PCR using primers that detect and discriminate a specific *tet*(M) allele based on amplicons of different sizes, depending upon the specific region of *tet*(M) in either Tn*916* or Tn*1545* (Fig. 1), and found that all of the *tet*(M) alleles present in the 23 *Vibrio* spp. contained a specific part of the *tet*(M) found in Tn*1545*, based on the presence of a 534 bp PCR amplicon (Fig. 1). Although the multiplex PCR technique developed in this study is limited in that it allows the detection of genomic variations only in 2 specific regions of *tet*(M), it nonetheless provides a rapid and simple method to obtain critical information related to the possible mosaic structure of *tet*(M) in many samples without the necessity for nucleotide sequence comparisons.

In addition, production of an amplicon in PCR with the primer set, sense and antisense primer derived from *tet*(B) and *tet*(M) sequence, respectively, allowed us to determine that *tet*(B) and *tet*(M) genes in Tc-resistant *Vibrio* spp. are located very close to each other. No previous reports have described the use of PCRs to discriminate different alleles of *tet*(M) or to determine the relative location of *tet*(B) and *tet*(M). In attempts to confirm the possibility of conjugal transfer, it was found that both *tet*(B) and *tet*(M) carried by 3 donor strains out of 24 isolates were transferred to the recipient. These 3 donor strains appeared to be those of 9 EM-resistant isolates that also transferred EM resistance to recipients together with *tet*(B) and *tet*(M) (Table 3).

A strong association of the *erm*(B) and *tet*(M) genes with Tn*1545*-related elements was found in human oral streptococci and animal enterococci (Leener et al. 2004, Martel et al. 2005). However, *erm*(B) or even *erm*(A) and *erm*(C) genes were not found in 9 EM-resistant *Vibrio* spp., including 3 strains that transferred EM resistance to recipients in conjugation experiments. Interestingly, the control strains EM-resistant *Streptococcus* spp. SJ14 and 48 carrying *tet*(S) appeared to carry *erm*(B), but not the CJ strain carrying *tet*(M). None of the studies showing the relationship between *tet*(S) and *erm*(B) have included *Streptococcus* spp.

Determination of the nucleotide sequence of the PCR amplicon obtained with the TETF/TMR (registered in GenBank under Accession Number DQ886586) primer set allowed us to confirm the presence of Tn*10* elements, including the insertion sequence, as a possible genetic element in the dissemination of *tet*(B) and *tet*(M) in Tc-resistant *Vibrio* spp. However, the *int-Tn* sequence, which is essential for the excision and integration of Tn*1545* into the chromosome, was not detected in isolates carrying both *tet*(B) and *tet*(M) under the PCR conditions used that were specific to the region (Table 1) (Kim et al. 2004). Kim et al. (2004) detected the *int-Tn* sequence in 4 of 8 *tet*(M)-positive *Vibrio* spp., which suggested that acquisition of *tet*(M) in various environments occurs by different routes of *tet*(M) transmission in *Vibrio* spp.

Nucleotide sequences upstream of the *tet*(M) open reading frame (ORF) have been reported to comprise a region that is important for Tc resistance. The *tet*(M) gene detected in *Vibrio* spp. did not have a complete 5′-end flanking region, as in Tn*1545* or Tn*916* (Gascoyne-Binzi et al. 1993). The 14 bp of unknown sequence at the junction between Tn*10* and *tet*(M) elements was followed only by 103 bp at the 5′-end flanking region of the structural *tet*(M) gene, in contrast to the much longer region in Tn*1545* (Barbeyrac et al. 1996).

Therefore, the flanking region of *tet*(M) found in the *Vibrio* spp. in this study does not include a ribosome-binding site (RBS) for the regulatory ORF in *tet*(M) or inverted repeat sequences, required for transcriptional regulation of *tet*(M) by an attenuation mechanism (Su & Clewell 1992). Tc-resistant isolates containing only a *tet*(M) determinant without regulatory regions have also been observed in Streptococci and Enterococci (Bentorcha et al. 1992, Huang et al. 1997). In the Tn*916* Tc-resistance system of *Vibrio* spp., a RNA polymerase pausing site has been found to be involved in *tet*(M) expression (Su & Clewell 1992). An inverted repeat sequence (5′-GCC TAT GGT TAT GCA TAA AAA TCC CAG TGA TAA-TTA TCA CTG GGA TTT TTA TGC-3′) present in the shorter 5′-end flanking region of the sequence determined in this study could potentially form a stem-loop structure that might affect expression in a similar manner. Although it is necessary to determine whether the *tet*(M) gene in Tc-resistant *Vibrio* spp. used in this study is functional, the absence of *int-Tn* sequences of Tn*1545* may reflect the indispensable function of the genetic elements in Tn*10*, rather than those in Tn*1545*, for the wide dissemination of *tet*(M) in *Vibrio* spp. In conclusion, this study has shown an association between *tet*(B) with Tn*10* elements and *tet*(M) without Tn*1545*-like associated genes in Tc-resistant *Vibrio* spp. Further research is needed to confirm whether Tn*10* or Tn*1545*-like genes act as a vehicle for the simultaneous dissemination of these 2 determinants in nearly all Tc-resistant *Vibrio* spp.

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