Distribution of an Oxytetracycline Resistance Determinant *tet*(34) among Marine Bacterial Isolates of a *Vibrio* species

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(Received December 13, 2002—Accepted March 14, 2003)

The distribution of *tet*(34) was examined among oxytetracycline (OTC) resistant *Vibrio* strains isolated from Japan, Korea and the Philippines. *tet*(34) was detected in 10 isolates (6 in fish, 4 in seawater) from Japan and Korea, suggesting that it is widely distributed among fish and seawater bacteria in these countries. Ninety-eight percent of the *Vibrio* strains in the Philippines were sensitive to OTC, but none of the 60 isolates from the Philippines. The minimum inhibitory concentration (MIC) of OTC-resistant isolates increased 2 to 8 fold in the presence of Mg²⁺, and MICs were higher in the Japanese and Korean isolates than the Philippine isolates. The MIC for furaltadone (FD) was low in Japanese (23.9%) isolates at over 3.1 µg/ml and high in Korean (50%) and Philippine (56.7%) isolates. This was probably due to the different frequency of use of FD in these countries. Sequences of 16S rDNA of *tet*(34)-positive isolates were 100% identical, suggesting that *tet*(34) is conveyed in a particular *Vibrio* species.

Key words: Oxytetracycline, *tet*(34), *Vibrio*, 16S rRNA, Furaltadone

Oxytetracycline (OTC) is one of the most widely used antibiotics in aquaculture. OTC-resistant bacteria are found in aquaculture sites every year in Japan. A variety of OTC-resistant bacteria have been identified in not only humans and animals but also marine environments^{18,21}. OTC resistance determinants are widespread among bacterial species around the world^{8,10}. To date, 36 different tetracycline resistance genes (*tet*) including OTC resistance genes (*otr*) have been registered in GenBank. In addition, it is well known that the resistance determinants can be transferred among bacteria by plasmids, transposons and integrons in aquatic environments^{1,5,19,24}. In a previous study, a new OTC resistance determinant *tet*(34) gave high resistance to the recipient host *E. coli* against both tetracycline and OTC¹⁵. *tet*(34) is suspected of having a new resistance mechanism, which is associated with xanthine-guanine phosphoribosyl trans-

ferase (XPRT)-like activity¹⁵. *tet*(34) has been cloned from *Vibrio* sp. strain No. 6 isolated from the intestinal content of cultured yellowtail *Seriola quinqueradiata* in Japan¹⁵. *tet*(34) has been found distributed in certain *Vibrio* species but not in other fish-related bacteria such as *Edwardsiella tarda*, *Lactococcus garvieae* and the *Photobacterium damsela* subsp. *piscicida*¹⁶.

The first aim of the present study is to clarify the distribution of *tet*(34) in OTC-resistant bacteria collected from Japan, Korea and the Philippines. Moreover, the genetic relationship is examined among the bacteria possessing *tet*(34) based on the 16S rDNA sequence.

Furthermore, to examine the resistance of the OTC-resistant bacteria to other drugs, we monitored susceptibility to furaltadone (FD) in all isolates. The incidence of individual and multiple resistance to antibiotic agents has been shown to be associated with the history of antibiotic use^{2,14}. The furans are seldom used in Japan, but in Korea and the Philippines, nitrofurantoin, furazolidone and FD are used for bacterial chemotherapy^{3,6,7,17,23}.

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Abbreviations: MIC, minimum inhibitory concentration; TC, tetracycline; OTC, oxytetracycline; FD, furaltadone.

Materials and Methods

OTC-resistant bacteria isolated in Japan

Table 1 shows the 46 OTC-resistant *Vibrio* strains, including 23 isolates obtained from healthy yellowtail *Seriola quinqueradiata*, 21 isolates from seawater, and 4 isolates from sediment of a farm in Ehime Prefecture, Japan, in 2000. The isolates were identified as *Vibrio* sp. by the Gram stain method, pigment, and results of the oxidation and fermentation (OF) test and cytochrome oxidase test. They were cultured at 25°C in nutrient marine agar plates containing 5 g of Polypepton (Wako Pure chemical industries, Ltd, Osaka), 1 g of yeast extract (Difco, Detroit, MI, USA), 1 g of proteose peptone (Difco), 1 g of beef extract (Wako Pure chemical industries, Ltd, Osaka), 7.5 g of Bacto agar (Difco) and 20 g of NaCl per liter of distilled water. OTC-resistant bacteria classified to the genus *Vibrio* have a minimum inhibitory concentration (MIC) of 62.5 µg/ml or more.

Bacteria isolated in Korea and the Philippines

Table 2 shows the 58 *Vibrio* strains isolated in Korea, including 29 isolates from healthy and diseased fish in farms and 29 isolates from seawater, between 2000 and 2002. Table 3 shows the 60 *Vibrio* strains of isolated from a hatchery in Iloilo, the Philippines in 2001, including 7 isolates from the eggs of mud crab *Scylla* sp. collected at a shrimp farm, 32 isolates from the larval stages of shrimp, 14 isolates from nearshore sediment, and 7 isolates from water used to culture the shrimp. Since the MIC for OTC has not been determined for these strains, susceptibility was tested before the *tet(34)* examination.

Determining the minimum inhibitory concentration (MIC)

The MICs for all strains were measured as follows. Nutrient marine agar plates¹⁵⁾ were used for the *Vibrio* isolates. To test whether Mg²⁺ was needed for the strains to become OTC-resistant, nutrient salt broths with 10 mM of MgCl₂ and broths with no MgCl₂ were used. A bacterial cell suspension was prepared in phosphate-buffered saline (PBS), and the cell density was adjusted to MacFarland No. 0.5. One microliter portions of the suspensions were then spotted on agar plates containing 31.3, 62.5, 125, 250 or 500 µg OTC/ml and 3.1, 9.4 or 18.8 µg/ml of FD. The plates were incubated at 25°C for 48 h, and the lowest concentration needed for inhibition was recorded. OTC and FD were obtained from the Sigma Chemical Co, USA.

Genomic DNA extraction

Genomic DNA was extracted from the OTC-resistant strains and control strains as follows. First, a bacteria cell pellet was suspended in an extraction solution (0.15 M NaCl, 0.1 M EDTA, 0.5 mg/ml RNase A and 0.5% SDS) and incubated at 65°C for 5 min. The DNA was purified by extraction with phenol saturated with TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA]. The DNA was further extracted twice with a TE-saturated phenol:chloroform:iso-amyl alcohol (25:24:1, v/v/v) solution, and once with chloroform. The DNA was finally precipitated using ethanol. Plasmid extraction was performed according to the method of Sambrook and Russell²⁰⁾. Purified chromosome and plasmid DNA was resuspended in 50 µl of TE buffer and stored at -20°C.

Polymerase chain reaction (PCR) amplification

To identify the *tet(34)* in extracted DNA, PCR was performed. The forward primer was 5'-GCTTGCGATTAAT-TGGTTCC-3' and reverse primer was 5'-TTGGGACGC-CATGCAAAATT-3'. This primer set amplified 434 bp from position 314 to 747 of *tet(34)*. The PCR mixture for *tet(34)* (total volume 25 µl) contained 20 pmol of each primer, Ex *Taq* reaction buffer (TaKaRa, Japan), 100 µM of dNTPs and 1.0 U of Ex *Taq* DNA polymerase (TaKaRa, Japan). PCR amplification was performed with a thermal cycler TP-3000 PCR system (TaKaRa, Japan). A single cycle in the amplification program included 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. A total of 25 cycles were performed, and a final extension was performed at 72°C for 5 min. The PCR products were visualized by electrophoresis on a 1.2% agarose gel (Invitrogen, USA) stained with ethidium bromide.

DNA sequencing and analysis

To investigate the 16S rDNA of the *tet(34)*-positive strains, a 1451 bp long PCR product was sequenced. The position was between 8 and 1512 (*Escherichia coli* numbering²⁶⁾). The fD1 primer was 5'-AGAGTTTGATCCTGGCT-CAG-3' and rP2 primer was 5'-ACGGCTACCTTGTTAC-GACTT-3'. Forward primers used in this study spanned positions (*E. coli* numbering) as follows; 5'-CCTACGG-GAGGCAGCAG-3' (341 to 357) and 5'-CACAAGCG-GTGGAGCATGT-3' (933 to 1011). Reverse primers included 5'-GGCCCGGAACGTATTACACC-3' (1389 to 1370) and 5'-ATTACCGCGGCTGCTGG-3' (534 to 518). Conditions consisted of 30 cycles of 95°C (2 min), 42°C (30 sec) and 72°C (4 min), plus one additional cycle with a final

Table 1. Minimum inhibitory concentration (MIC) and *tet*(34) detection in *Vibrio* sp. isolated from intestinal content of yellowtail and seawater at Uwajima, Japan, in 2000.

Strain	Origin	Area	Year	MIC (μ g OTC/ml)		MIC (μ g FD/ml)	<i>tet</i> (34)
				without Mg^{2+}	with Mg^{2+}		
1-A	Yellowtail (I)	Uwajima	2000.10	62.5	125	3.1	–
1-C	Yellowtail (I)	Uwajima	2000.10	62.5	250	<3.1	–
1-D	Yellowtail (I)	Uwajima	2000.10	62.5	250	<3.1	–
1-F	Yellowtail (I)	Uwajima	2000.10	62.5	>500	3.1	–
1-G	Yellowtail (I)	Uwajima	2000.10	62.5	>500	3.1	+
1-H	Yellowtail (I)	Uwajima	2000.10	62.5	>500	3.1	–
2-A	Yellowtail (I)	Uwajima	2000.10	62.5	250	<3.1	–
2-H	Yellowtail (I)	Uwajima	2000.10	62.5	125	<3.1	+
2-I	Yellowtail (I)	Uwajima	2000.10	62.5	250	<3.1	+
2-J	Yellowtail (I)	Uwajima	2000.10	62.5	250	<3.1	–
3-A	Yellowtail (I)	Uwajima	2000.10	62.5	250	3.1	–
3-C	Yellowtail (I)	Uwajima	2000.10	62.5	250	3.1	–
3-E	Yellowtail (I)	Uwajima	2000.10	62.5	>500	<3.1	–
3-G	Yellowtail (I)	Uwajima	2000.10	250	250	3.1	–
3-J	Yellowtail (I)	Uwajima	2000.10	250	>500	<3.1	–
4-A	Yellowtail (I)	Uwajima	2000.10	250	>500	<3.1	–
4-B	Yellowtail (I)	Uwajima	2000.10	250	>500	<3.1	–
4-E	Yellowtail (I)	Uwajima	2000.10	250	>500	<3.1	–
4-F	Yellowtail (I)	Uwajima	2000.10	250	>500	<3.1	–
4-G	Yellowtail (I)	Uwajima	2000.10	62.5	125	<3.1	–
4-H	Yellowtail (I)	Uwajima	2000.10	250	>500	<3.1	–
5-A	Yellowtail (I)	Uwajima	2000.10	62.5	125	<3.1	+
5-B	Yellowtail (I)	Uwajima	2000.10	62.5	250	<3.1	–
Sw-1	seawater	Uwajima	2000.10	125	250	<3.1	–
Sw-5	seawater	Uwajima	2000.10	62.5	250	<3.1	–
Sw-6	seawater	Uwajima	2000.10	62.5	250	<3.1	–
Sw-7	seawater	Uwajima	2000.10	62.5	250	<3.1	–
Sw-8	seawater	Uwajima	2000.10	62.5	125	<3.1	–
Sw-11	seawater	Uwajima	2000.10	250	>500	<3.1	–
Sw-14	seawater	Uwajima	2000.10	62.5	>500	<3.1	–
Sw-16	seawater	Uwajima	2000.10	62.5	>500	<3.1	–
Sw-18	seawater	Uwajima	2000.10	250	>500	<3.1	–
Sw-32	seawater	Uwajima	2000.10	250	>500	<3.1	–
Sw-34	seawater	Uwajima	2000.10	62.5	250	<3.1	–
Sw-37	seawater	Uwajima	2000.10	125	>500	<3.1	–
Sw-38	seawater	Uwajima	2000.10	125	>500	<3.1	–
Sw-41	seawater	Uwajima	2000.10	125	>500	<3.1	–
Sw-42	seawater	Uwajima	2000.10	125	>500	<3.1	+
Sw-44	seawater	Uwajima	2000.10	62.5	>500	18.8	–
Sw-48	seawater	Uwajima	2000.10	125	>500	<3.1	–
Sw-50	seawater	Uwajima	2000.10	125	>500	3.1	–
Sw-a	seawater	Uwajima	2000.10	250	>500	3.1	–
Sd-2	sediment	Uwajima	2000.10	62.5	250	<3.1	–
Sd-7	sediment	Uwajima	2000.10	62.5	>500	3.1	–
Sd-8	sediment	Uwajima	2000.10	62.5	125	3.1	–
Sd-18	sediment	Uwajima	2000.10	125	>500	<3.1	–
No. 6	Positive control {origin strain of <i>tet</i> (34)}; <i>Vibrio</i> sp.			125	>500	<3.1	+
ATCC 17802T	Non-fish strain negative control (sensitive to OTC); <i>Vibrio parahaemolyticus</i>			<31.3	<31.3	<3.1	–

(I); intestinal content

Table 2. Minimum inhibitory concentration (MIC) and tet(34) detection in *Vibrio* sp. isolated from diseased fish and seawater in south Korea.

Strain	Origin	Area	Year	MIC (μ g OTC/ml)		MIC (μ g FD/ml)	tet(34)
				without Mg ²⁺	with Mg ²⁺		
FK0001	Flounder (K)	Yosu	2000. 1	<31.3	<31.3	3.1	–
SK0102	Sea bass (K)	Yosu	2001. 2	<31.3	<31.3	<3.1	–
RFK01041	Rock fish (K)	Yosu	2001. 4	<31.3	<31.3	<3.1	–
RFS01042	Rock fish (S)	Yosu	2001. 4	<31.3	<31.3	<3.1	–
FK01073	Flounder (K)	Wando	2001. 7	125	250	<3.1	+
FK0103	Flounder (K)	Youngkwang	2001. 3	<31.3	<31.3	<3.1	–
FK01071	Flounder (K)	Youngkwang	2001. 7	<31.3	<31.3	<3.1	–
FK01072	Flounder (K)	Youngkwang	2001. 7	62.5	250	9.4	–
FI0203	Flounder (I)	Youngkwang	2002. 2	<31.3	<31.3	<3.1	–
FI0204	Flounder (I)	Youngkwang	2002. 2	62.5	250	3.1	+
FI0205	Flounder (I)	Youngkwang	2002. 2	125	500	<3.1	–
FI0206	Flounder (I)	Youngkwang	2002. 2	62.5	250	3.1	–
FI0207	Flounder (I)	Youngkwang	2002. 2	<31.3	<31.3	18.8	–
FI0208	Flounder (I)	Youngkwang	2002. 2	<31.3	31.3	<3.1	–
FI0209	Flounder (I)	Youngkwang	2002. 2	125	>500	3.1	–
RI0210	Rock bream (I)	Youngkwang	2002. 2	<31.3	125	3.1	–
RI0211	Rock bream (I)	Youngkwang	2002. 2	31.3	125	3.1	–
RI0212	Rock bream (I)	Youngkwang	2002. 2	<31.3	<31.3	3.1	–
FI0213	Flounder (K)	Wando	2002. 2	<31.3	<31.3	<3.1	–
FI0214	Flounder (K)	Jindo	2002. 2	<31.3	<31.3	<3.1	–
FI0215	Flounder (K)	Jindo	2002. 2	<31.3	<31.3	<3.1	–
FI0216	Flounder (I)	Jindo	2002. 2	<31.3	<31.3	<3.1	–
FI0217	Flounder (I)	Jindo	2002. 2	<31.3	<31.3	3.1	–
FI0218	Flounder (I)	Jindo	2002. 2	<31.3	<31.3	3.1	–
FI0219	Flounder (I)	Jindo	2002. 2	<31.3	<31.3	31.3	–
FI0220	Flounder (I)	Jindo	2002. 2	<31.3	<31.3	<3.1	–
FI0221	Flounder (I)	Jindo	2002. 2	<31.3	<31.3	18.8	–
FI0222	Flounder (I)	Jindo	2002. 2	<31.3	62.5	3.1	–
FI0223	Flounder (I)	Jindo	2002. 2	<31.3	<31.3	<3.1	–
SMI0224	Striped mullet (I)	Mokpo	2002. 2	<31.3	<31.3	<3.1	–
SMI0225	Striped mullet (I)	Mokpo	2002. 2	<31.3	<31.3	3.1	–
SW01071	Sea water	Youngkwang	2001. 7	<31.3	31.3	3.1	–
SW01072	Sea water	Youngkwang	2001. 7	125	>500	3.1	–
SW01073	Sea water	Youngkwang	2001. 7	250	>500	31.3	–
SW01074	Sea water	Youngkwang	2001. 7	<31.3	31.3	3.1	–
SW01075	Sea water	Youngkwang	2001. 7	<31.3	<31.3	3.1	–
SW01076	Sea water	Youngkwang	2001. 7	125	>500	3.1	–
SW0201	Sea water	Youngkwang	2002. 2	31.3	250	3.1	–
SW0202	Sea water	Youngkwang	2002. 2	125	250	3.1	+
SW0203	Sea water	Youngkwang	2002. 2	62.5	250	3.1	–
SW0204	Sea water	Youngkwang	2002. 2	<31.3	<31.3	<3.1	–
SW0205	Sea water	Youngkwang	2002. 2	<31.3	<31.3	<3.1	–
SW0206	Sea water	Youngkwang	2002. 2	125	250	3.1	+
SW0207	Sea water	Youngkwang	2002. 2	<31.3	<31.3	<3.1	–
SW0208	Sea water	Youngkwang	2002. 2	<31.3	<31.3	<3.1	–
SW0209	Sea water	Youngkwang	2002. 2	<31.3	31.3	<3.1	–
SW0210	Sea water	Youngkwang	2002. 2	125	250	<3.1	–
SW0211	Sea water	Youngkwang	2002. 2	<31.3	31.3	3.1	–
SW0212	Sea water	Youngkwang	2002. 2	125	250	<3.1	+
SW0213	Sea water	Youngkwang	2002. 2	<31.3	62.5	<3.1	–
SW0214	Sea water	Mokpo	2002. 2	<31.3	<31.3	<3.1	–
SW0215	Sea water	Mokpo	2002. 2	<31.3	<31.3	<3.1	–
SW0216	Sea water	Jindo	2002. 2	62.5	250	<3.1	–
SW0217	Sea water	Jindo	2002. 2	<31.3	<31.3	3.1	–
SW0218	Sea water	Jindo	2002. 2	<31.3	<31.3	<3.1	–
SW0219	Sea water	Yosu	2002. 2	<31.3	<31.3	<3.1	–
SW0220	Sea water	Yosu	2002. 2	<31.3	<31.3	3.1	–
SW0221	Sea water	Yosu	2002. 2	<31.3	<31.3	3.1	–

(K) kidney; (S), spleen and (I), intestinal content

Table 3. Minimum inhibitory concentration (MIC) and *tet*(34) detection in *Vibrio* sp. isolated from crustaceans and the environment at Iloilo, the Philippines, in 2001.

Strain	Origin	Area	Year	MIC (μ g OTC/ml)		MIC (μ g FD/ml)	<i>tet</i> (34)
				without Mg ²⁺	with Mg ²⁺		
OIS2 ₃	Egg: <i>Scylla serrata</i>	Iloilo	2001. 5	<31.3	<31.3	9.4	–
OIT1 ₁	Egg: <i>Scylla tranquebarica</i>	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
OIT1 ₂	Egg: <i>Scylla tranquebarica</i>	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
OIT1 ₃	Egg: <i>Scylla tranquebarica</i>	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
S4 ₁	Egg: <i>Scylla serrata</i>	Iloilo	2001. 5	<31.3	<31.3	3.1	–
O ₅	Egg: <i>Scylla olivacea</i>	Iloilo	2001. 5	<31.3	<31.3	3.1	–
U	Egg: Unablated <i>Scylla serrata</i>	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
Z1-1	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
Z1-2	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
Z2-1	Zoea in crustacean hatchery	Iloilo	2001. 5	31.3	125	3.1	–
Z2-2	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
Z3-1	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	3.1	–
Z3-2	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	9.4	–
Z4-1	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
Z4-2	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
ZT3-1	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
ZT3-2	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
ZT4-1	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
ZT4-2	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
ZT6-1	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
ZT6-2	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
ZT11-1	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
ZT11-2	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
Z1-1	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
Z1-2	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
Z2-1	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
Z2-2	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
Z3-1	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
Z3-2	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	3.1	–
Z4-1	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	3.1	–
Z4-2	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
ZT3-1	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
ZT3-2	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	3.1	–
ZT4-1	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	9.4	–
ZT4-2	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	3.1	–
ZT6-1	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	3.1	–
ZT6-2	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	3.1	–
ZT11-1	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
ZT11-2	Zoea in crustacean hatchery	Iloilo	2001. 5	<31.3	<31.3	3.1	–
Sdt1	Nearshore sediment	Iloilo	2001. 5	<31.3	<31.3	3.1	–
SW1 ₁	Nearshore sediment	Iloilo	2001. 5	<31.3	<31.3	3.1	–
SW1 ₂	Nearshore sediment	Iloilo	2001. 5	<31.3	<31.3	3.1	–
SW1 ₃	Nearshore sediment	Iloilo	2001. 5	<31.3	<31.3	3.1	–
Sdt2 ₁	Nearshore sediment	Iloilo	2001. 5	<31.3	<31.3	3.1	–
Sdt2 ₂	Nearshore sediment	Iloilo	2001. 5	<31.3	<31.3	3.1	–
SW2 ₁	Nearshore sediment	Iloilo	2001. 5	<31.3	<31.3	3.1	–
SW2 ₂	Nearshore sediment	Iloilo	2001. 5	<31.3	<31.3	3.1	–
Sdt3 ₁	Nearshore sediment	Iloilo	2001. 5	<31.3	<31.3	3.1	–
Sdt3 ₂	Nearshore sediment	Iloilo	2001. 5	<31.3	<31.3	3.1	–
Sdt3 ₃	Nearshore sediment	Iloilo	2001. 5	<31.3	<31.3	<3.1	–
Sdt3 ₄	Nearshore sediment	Iloilo	2001. 5	<31.3	<31.3	3.1	–
Sdt3 ₅	Nearshore sediment	Iloilo	2001. 5	<31.3	<31.3	3.1	–
Sdt3 ₆	Nearshore sediment	Iloilo	2001. 5	<31.3	<31.3	3.1	–
WL1	Water from zoea	Iloilo	2001. 5	<31.3	<31.3	3.1	–
WL2	Water from zoea	Iloilo	2001. 5	<31.3	<31.3	3.1	–
WP2	Water from 48-day postlarva	Iloilo	2001. 5	<31.3	<31.3	3.1	–
WP8	Water from 48-day postlarva	Iloilo	2001. 5	<31.3	<31.3	3.1	–
WP9	Water from 48-day postlarva	Iloilo	2001. 5	<31.3	<31.3	3.1	–
WP11	Water from 48-day postlarva	Iloilo	2001. 5	<31.3	<31.3	3.1	–
WP14	Water from 48-day postlarva	Iloilo	2001. 5	<31.3	<31.3	3.1	–

20 min chain elongation. All of the *tet(34)*-positive strains (1-G, 2-H, 2-I, 5-A, Sw-42, FK01073, FI0204, SW0202, SW0206 and SW0212) were analyzed. Sequencing was performed using the DNA sequencing Kit (Applied Biosystems, Tokyo) and the ABI PRISM 310 DNA sequencer (Perkin Elmer, Tokyo) at the Center for Gene Research, Ehime University. Searches for similar nucleotide sequences of 16S rDNA of *tet(34)*-positive strains and other strains (*Vibrio* sp. strain No. 6; GenBank accession No. AB089204, *V. scopthalmi*; GenBank accession No. U46579, *V. ichthyenteri*; GenBank accession No. AJ437192 and *V. vulnificus*; GenBank accession No. X76334) were performed using GENETYX-WIN (Ver. 5.0) software.

Results

MIC for OTC and FD

The MICs of 46 OTC-resistant *Vibrio* strains collected from a fish farm in Japan are shown in Table 1. The MIC for OTC was 62.5 to 250 µg/ml when Mg²⁺ was absent, and 2 times higher when Mg²⁺ was present. The MIC for FD was generally 3.1 µg/ml (21.7%) or less (76.1%). One strain had an MIC of 18.8 µg/ml.

The MICs of 58 *Vibrio* strains isolated from marine fish farms in Korea and 60 *Vibrio* strains isolated from a shrimp hatchery in the Philippines are shown in Table 2 and Table 3, respectively. In the Korean strains, 15 strains had MICs of 61.5 µg/ml or higher for OTC, and MICs of 31.3 µg/ml or less when Mg²⁺ was absent. The MIC of most of the sensitive strains with an MIC of <31.3 µg/ml did not increase even when Mg²⁺ was added. Most MICs for FD were 3.1 µg/ml (41.3%) or less (50%). Five strains had MICs of 9.4 to 31.3 µg/ml.

Most of the Philippine strains (98.3%) were sensitive to OTC. Only one strain had an MIC of 31.3 µg/ml. Addition of Mg²⁺ increased the MIC of strain Z2-1 to 125 µg/ml, but caused no increase in the other strains (Table 3). Most MICs for FD were 3.1 µg/ml (51.6%) or less (43.3%). Three strains had an MIC of 9.4 µg/ml. The distribution of the MICs for FD is summarized in Fig. 1.

Detection of *tet(34)*

Five of the Japanese strains tested positive for *tet(34)* (Table 1). Four strains (1-G, 2-H, 2-I and 5-A) were isolated from the intestinal contents of yellowtail, and one strain (Sw-42) was isolated from seawater. The MICs of all *tet(34)*-positive strains were >125 µg/ml in the presence of Mg²⁺ (Table 1).

Fig. 1. Occupation rate of minimum inhibitory concentration (MIC) for furaltadone (FD) in Japanese, Korean and Philippine strains.

In the Korean isolates, 6 *tet(34)*-positive strains were identified: strain FK01073 from the kidney of diseased flounder, strain FI0204 from the intestinal contents of healthy flounder, and 3 strains (SW0202, SW0206 and SW0212) from seawater (Table 2). These 6 strains had MICs of 62.5 to 250 µg/ml in the presence of Mg²⁺. None of the Philippine strains tested positive for *tet(34)* (Table 3).

Sequences of 16S rDNA of *tet(34)*-positive strains

The 16S rDNA of 10 *tet(34)*-positive strains were sequenced. All were 100% identical to *Vibrio* sp. strain No. 6, which is an origin strain of *tet(34)* (Fig. 2).

Discussion

The tetracycline (TC) series are effective against a wide range of Gram-positive and Gram-negative bacteria^{8,11}), so they have been used extensively as antimicrobial agents in aquaculture. However, bacteria resistant to these drugs are now occurring more frequently^{1,21,23}).

The resistance mechanisms of the bacteria are known based on the chemical properties of the drug. The TC has a number of potential metal-binding sites, and they form a complex with magnesium and calcium^{4,9,12,13}). Lunestad and Goksoyr¹²) reported that only about 5% of the OTC exists in its free form. The MIC value of OTC was approximately 10 times higher when Mg²⁺ or Ca²⁺ was in the media, indicating that OTC that has formed a complex with either of these ions has a reduced ability to cross lipid-rich biological membranes. Seawater contains many divalent cations, which cause the activity of OTC to decrease in seawater. MIC values for OTC were 2 to 8 times higher than when there was no Mg²⁺ (Table 1, 2 and 3). These results confirm

Fig. 2. Phylogenetic tree based on 16S rDNA of *tet*(34)-positive isolates (unweighted pair group method with arithmetic mean, UPGMA). The number in parentheses indicates the GenBank accession number.

that most OTC resistance in marine bacteria could be enhanced by an Mg^{2+} associated system^{15,22}. Nonaka and Suzuki¹⁵ proposed new resistance mechanism involved in the association of Mg^{2+} with the *tet*(34) product.

Tendencia and de la Peña²³ reported that 4.7% of bacteria from a shrimp farm in the Philippines were OTC-resistant and the MICs for OTC were 30 μ g/disc. They reported that the occurrence of OTC resistance was much higher than the occurrence of FD, oxolinic acid and chloramphenicol resistance. In our study, the OTC resistance rate was much lower (1.6%) than the occurrence rates of FD (57%) and erythromycin (93.3%, data not shown). The mechanisms of bacterial resistance to erythromycin include the modification of ribosomal targets by a methylase associated with transmissible genes of the *erm* family, various antibiotic efflux mechanisms, and point mutations in the peptidyl-transferase region of 23S rRNA genes²⁵. This difference might be caused by culture conditions or the drug treatment history of the shrimp.

A total of 26% of the OTC-resistant bacteria from Japan were resistant to FD, whereas 46% of the Korean strains and 57% of the Philippine strains were resistant to FD (Fig. 1). This suggests that resistance to antibiotics is affected by the history of antibiotic use^{2,14}. In Korea and the Philippines, the wide used of FD for various bacterial diseases may have caused this increased resistance. FD is not used in Japan, where FD resistance was lower.

tet(34)-positive strains were detected from the *Vibrio* strains isolated from both healthy and diseased fish, and

from seawater. This suggests that *tet*(34) was widely spread among fish and seawater.

tet(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E) and *tet*(G) occur in the *Vibrio* species and other Gram-negative bacteria, suggesting that these genes can be transferred to different bacterial species. This transfer is mediated by many natural mobile elements, including transposons and conjugative plasmids in aquatic ecosystems^{1,5,8,19}. The 16S rDNA in all *tet*(34)-positive strains was 100% identical to that of *Vibrio* sp. strain No. 6, suggesting that *tet*(34) is present in specific species of *Vibrio*. Our results clearly show that this bacterium is widely distributed in fish intestines and seawater in Japan and Korea. It was found that *tet*(34) is conveyed on a chromosome of *Vibrio* sp. No. 6¹⁶, suggesting that *tet*(34) does not transfer to other bacterial species. *tet*(34) might differ from the other known *tet* series in terms of gene transfer. The deduced product of *tet*(34) is XPRT, which is found in many species such as *E. coli*. However, *E. coli* was not resistant to OTC, which suggests that the expression of *tet*(34) occurs more frequently in the *tet*(34)-positive *Vibrio* species than in other bacteria. The molecular properties of *tet*(34) and the distribution of *tet*(34)-positive *Vibrio* sp. will be studied further in future. Since *Vibrio* sp. strain No. 6 is genetically very similar to *V. scopthalmi* and *V. ichthyenteri*, it might be indigenous and an opportunistic pathogen in fish.

Acknowledgements

This study was partly supported by Grants-in-Aid from JSPS and MEXT, Japan. We thank Dr. J. Bower, Hokkaido University, for his critical reading of this manuscript.

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