Distribution of the Oxytetracycline Resistance Determinant Tet 34 among Bacteria Isolated from Diseased Fish

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The distribution of a newly cloned oxytetracycline (OTC) resistance determinant, Tet 34, was examined among bacterial strains isolated from diseased fish. We analyzed 33 OTC-resistant strains isolated from 1998 to 2000 in Kagawa Prefecture. Tet 34 was detected in 3 of the strains, which were grouped in the genus *Vibrio*. Minimum inhibitory concentrations (MICs) of the 3 strains were higher than 500 µg/ml in the presence of MgCl₂, and 125 or 250 µg/ml in its absence. Tet 34 was found in chromosomal DNA in all positive strains. Polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis of 16S rDNA revealed the 3 strains to have the same migration profiles. The sequences of their PCR products were also identical, suggesting that the species were the same. It was concluded that Tet 34 has been present since at least 1998, and the determinant occurs only in *Vibrio* species.

Key words: *Vibrio*, oxytetracycline, resistance, Tet 34, distribution

Antimicrobial drugs such as oxytetracycline (OTC) are widely used in the aquaculture industry to treat fish diseases. Previously, we showed that OTC-resistant bacteria are abundant in the intestines and surroundings of cultured yellowtail (*Seriola quinqueradiata*)¹⁴. We isolated these OTC-resistant bacteria and cloned a new OTC resistance determinant, Tet 34, from a strain of *Vibrio* sp. No. 6 (Antimicrobial Agents and Chemotherapy, in press). This determinant is very similar to the xanthine-guanine phosphoribosyl transferase (XPRT) genes of *Vibrio cholerae*, suggesting that Tet 34 requires Mg²⁺. To date, tetracycline (TC) resistance genes, *tet* (A)–(E), *tet* (G)–(K), *tet* (M), *tet* (O), *tet* (Q), *tet* (W), *tet* (X), *tet* (30) and *tet* (31), have been reported in 39 genera of Gram-negative bacteria including marine environmental isolates⁵.

The Tet series has been reported in a variety of pathogenic and commensal bacteria isolated from humans^{9,11,13,16},

animals^{1,21}, and the environment^{2,4,6,7,8,16,20}, however the distribution of Tet 34 is not known. The objective of the present study is to clarify the distribution of Tet 34 among OTC-resistant, disease-causing bacteria isolated from fish.

Materials and Methods

Bacterial strains and growth conditions

OTC-resistant bacteria were isolated from diseased fish (see Table 1) cultured at 14 aquaculture sites in Kagawa Prefecture, Shikoku Island, Japan, (Fig. 1) between 1997 and 2000. Brain heart infusion (BHI) medium supplemented with 1.5% NaCl was used to isolate the bacteria. Strains that grew on Thiosulfate Citrate Bile Salts Sucrose (TCBS, Nissui, Tokyo) plates were considered to be of the genus *Vibrio*. *Edwardsiella tarda*, *Lactococcus garvieae* and *Photobacterium damsela* subsp. *piscicida* were identified by a slide agglutination test using antisera against each species. As control strains, *Vibrio anguillarum* V-1037 and *Vibrio parahaemolyticus* were incubated in Marine broth 2216 (DIFCO, Detroit, MI) or on the solid medium at 25°C for 24 h, and *Escherichia coli* JM 109 was incubated in LB

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Abbreviations: MIC, minimum inhibitory concentration; TC, tetracycline; OTC, oxytetracycline; BHI, brain heart infusion; PBS, phosphate-buffered saline.

Table 1. The OTC-resistant strains used in this study and the results of dot-blot hybridization.

Strain	Origin	Organ	Year	Station ^a	Family or species	MIC on			Hybridization ^c
						NSA ^b		BHI with 1.5% NaCl	
						with Mg ²⁺	without Mg ²⁺		
KIDX-97076	Striped beakperch	Kidney	1997	A	<i>Vibrio</i> sp.	<62.5	<62.5	nd	—
KOHX-98043	Yellowtail	Kidney	1998	A	<i>Vibrio</i> sp.	500	250	nd	—
KHOX-98101	Yellowtail	Kidney	1998	A	<i>Vibrio</i> sp.	500	125	nd	—
KTX-98024	Red sea bream	Liver	1998	G	<i>Vibrio</i> sp.	500	125	nd	+
KIDX-98074	Striped beakperch	Liver	1998	A	<i>Vibrio</i> sp.	<62.5	<62.5	nd	—
KHRX-98123	Gold striped amberjack	Kidney	1998	G	<i>Vibrio</i> sp.	500	250	nd	—
KFX-98118	Tiger puffer	Liver	1998	C	<i>Vibrio</i> sp.	500	<62.5	nd	—
KKAX-99017	Great amberjack	Liver	1999	A	<i>Vibrio</i> sp.	<62.5	<62.5	nd	—
KCSX-99104	Sea bass	Brain	1999	A	<i>Vibrio</i> sp.	<62.5	<62.5	nd	—
KKUX-99102	Schlegel's black rockfish	Kidney	1999	I	<i>Vibrio</i> sp.	<62.5	<62.5	nd	—
KFX-99065	Tiger puffer	Kidney	1999	C	<i>Vibrio</i> sp.	250	125	nd	—
KHX-00105	Yellowtail	Brain	2000	A	<i>Vibrio</i> sp.	<62.5	<62.5	nd	—
KFX-00014	Tiger puffer	Liver	2000	K	<i>Vibrio</i> sp.	<62.5	<62.5	nd	—
KFX-00019	Tiger puffer	Kidney	2000	L	<i>Vibrio</i> sp.	>500	250	nd	+
KRX-00017	Japanese flounder	Liver	2000	K	<i>Vibrio</i> sp.	>500	250	nd	+
KRE-97013	Japanese flounder	Kidney	1997	B	<i>Edwardsiella tarda</i>	nd	nd	250	—
KRE-97068	Japanese flounder	Kidney	1997	C	<i>Edwardsiella tarda</i>	nd	nd	250	—
KRE-97120	Japanese flounder	Kidney	1997	D	<i>Edwardsiella tarda</i>	nd	nd	<31.3	—
KRE-98063	Japanese flounder	Liver	1998	B	<i>Edwardsiella tarda</i>	nd	nd	125	—
KRE-98161	Japanese flounder	Liver	1998	D	<i>Edwardsiella tarda</i>	nd	nd	<31.3	—
KRE-99017	Japanese flounder	Liver	1999	J	<i>Edwardsiella tarda</i>	nd	nd	250	—
KRE-99090	Japanese flounder	Liver	1999	C	<i>Edwardsiella tarda</i>	nd	nd	250	—
KRE-99122	Japanese flounder	Liver	1999	K	<i>Edwardsiella tarda</i>	nd	nd	125	—
KRE-00049	Japanese flounder	Kidney	2000	G	<i>Edwardsiella tarda</i>	nd	nd	250	—
KRE-00115	Japanese flounder	Kidney	2000	C	<i>Edwardsiella tarda</i>	nd	nd	250	—
KHS-97051	Yellowtail	Brain	1997	A	<i>Lactococcus garvieae</i>	nd	nd	250	—
KHS-98032	Yellowtail	Brain	1998	E	<i>Lactococcus garvieae</i>	nd	nd	250	—
KHS-98057	Yellowtail	Brain	1998	F	<i>Lactococcus garvieae</i>	nd	nd	250	—
KHS-99008	Yellowtail	Brain	1999	H	<i>Lactococcus garvieae</i>	nd	nd	250	—
KHS-99047	Yellowtail	Brain	1999	C	<i>Lactococcus garvieae</i>	nd	nd	250	—
KHS-00005	Yellowtail	Kidney	2000	G	<i>Lactococcus garvieae</i>	nd	nd	250	—
KHS-00083	Yellowtail	Brain	2000	A	<i>Lactococcus garvieae</i>	nd	nd	250	—
KHOPA-00030	Yellowtail	Liver	2000	L	<i>Photobacterium damsela</i> subsp. <i>piscicida</i>	nd	nd	<31.3	—
No. 6	Positive control (origin strain of Tet 34)				<i>Vibrio</i> sp.	500	<62.5	125	+
V-1037	Fish origin negative control <i>Vibrio</i> (sensitive to OTC)				<i>Vibrio anguillarum</i>	<62.5	<62.5	<31.3	—
V-1070	Fish origin negative control <i>Vibrio</i> (sensitive to OTC)				<i>Vibrio anguillarum</i>	<62.5	<62.5	<31.3	—
V-1109	Fish origin negative control <i>Vibrio</i> (sensitive to OTC)				<i>Vibrio anguillarum</i>	<62.5	<62.5	<31.3	—
V-1212	Fish origin negative control <i>Vibrio</i> (sensitive to OTC)				<i>Vibrio anguillarum</i>	<62.5	<62.5	<31.3	—
ATCC 17802T	Non-fish strain negative control (sensitive to OTC)				<i>Vibrio parahaemolyticus</i>	<62.5	ng	<31.3	—
NCMB1903T	Non-fish strain negative control (sensitive to OTC)				<i>Vibrio alginolyticus</i>	<62.5	<62.5	<31.3	—

nd, not done. ng, did not grow.

^a see Fig. 1.^b Nutrient salt agar.^c +, positive; –, negative.

Fig. 1. Location of sampling sites in Kagawa Prefecture, Shikoku Island, Japan.

broth (DIFCO) at 37°C for 12 h.

Screening for OTC resistance

To detect OTC-resistant strains, the following screening was performed. Filter disks containing 30 µg/ml of OTC were placed on agar plates spread with each strain and incubated at 25°C. After 16–18 h, the diameters of the inhibition halos that formed surrounding the filter disks were measured. If the diameter of the halo was greater than 25 mm, the bacteria were considered to be resistant to OTC. A total of 33 OTC-resistant strains were detected, including 15 strains of *Vibrio*, 10 strains of *E. tarda*, 7 strains of *L. garvieae* and 1 strain of *P. damsela* subsp. *piscicida* (Table 1).

Minimum inhibitory concentration (MIC)

The MICs of the OTC-resistant strains were measured as follows. Nutrient salt agar (NSA) plates containing 5 g of polypeptone (Wako, Osaka), 20 g of NaCl per liter of distilled water, and 1 g each of yeast extract (DIFCO), proteose peptone (DIFCO), and beef extract (Wako, Osaka) were used for *Vibrio* sp. To test whether Mg²⁺ was needed for OTC resistance, nutrient salt broths with and without 10 mM MgCl₂ were used. BHI agar plates supplemented with 1.5% NaCl were used to determine the MICs of *E. tarda*, *L. garvieae* and *P. damsela* subsp. *piscicida*. A cell suspension was prepared in phosphate-buffered saline (PBS), and the cell density was adjusted to MacFaland No. 0.5. The suspensions were spotted on agar plates containing 62.5, 125, 250 and 500 µg OTC/ml for *Vibrio* sp. For the other strains,

the agar plates contained 31.3, 62.5, 125, 250 and 500 µg OTC/ml. The plates were incubated at 25°C or 30°C for 24 h, and the lowest concentration needed for inhibition was recorded.

Chromosomal and plasmid DNA extraction

Genomic DNA was extracted from the OTC-resistant strains and control strains as follows. First, a bacterial 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]. It was further extracted two times with a TE-saturated phenol:chloroform:iso-amyl alcohol (25:24:1, v/v/v) solution, and one time with chloroform. The DNA was finally precipitated using ethanol. Plasmid extraction was performed according to the method of Sambrook *et al.*^{19). Purified chromosomal and plasmid DNA was resuspended in 50 µl of TE and stored at –20°C.}

Dot-blot and Southern hybridization

To examine the distribution of Tet 34 (DDBJ accession number; AB061440) among the OTC-resistant strains, dot-blot hybridization was performed. A 2-µg portion of the chromosomal and plasmid DNA of the OTC-resistant strains was incubated at 95°C for 5 min, and then the same volume of 20×SSC was added to the solution. This solution was directly applied onto a nylon membrane using a Bio-Rad Bio-Dot SF microfiltration apparatus (Bio-Rad Laboratories, Hercules, CA). The blotted filters were then denatured by treatment with a solution containing 0.5 N NaOH and 1.5 M NaCl, and neutralized with a solution containing 0.5 M Tris-HCl (pH 7.2), 1.5 M NaCl and 1 mM EDTA. The DNA was fixed with UV-light (254 nm) irradiation for 1 min. The blotted filters were prehybridized with 5 ml of warmed hybridization buffer [0.5 M NaH₂PO₄, 1 mM EDTA and 7% SDS (pH 7.2)] in a heat-sealing bag at 65°C for 5 min. The Tet 34 fragment cleaved out from the plasmid pOV, which was constructed by the ligation of Tet 34 into pUC119, was labeled with the DIG-Chem-Link Labeling and Detection Set (Roche Diagnostics, Mannheim, Germany) in accordance with the manufacturer's directions. The labeled DNA probe was denatured by heating at 95°C for 10 min and rapidly cooled on ice before use for hybridization. A 50-ng aliquot of the digoxigenin-labeled DNA probe was added to the solution, and hybridization was performed at 65°C overnight with shaking. After the hybridization, the membrane was washed three times with washing

solution (40 mM NaH_2PO_4 and 0.1% SDS, pH 7.2) for 5 min each. The digoxigenin-labeled DNA was reacted with nitroblue tetrazolium chloride/ 9-bromo-4-chloro-3-indoyl phosphate toluidine salt (NBT/BCIP), followed by an anti-digoxigenin, antibody-conjugated, alkaline phosphatase (Roche Diagnostics, Mannheim, Germany).

To determine whether Tet 34 was located on chromosomal or plasmid DNA, Southern blot hybridization was performed. A 10- μg aliquot of chromosomal or plasmid DNA was digested with the endonucleases *Pst* I (TaKaRa, Japan) and *EcoR* I (TaKaRa, Japan), separated on a 0.8% agarose gel, and briefly depurinated with 0.25 N HCl. Denaturation was performed by treatment with a solution containing 0.5 N NaOH and 1.5 M NaCl, and then neutralized with a solution containing 0.5 M Tris-HCl (pH 7.2), 1.5 M NaCl and 1 mM EDTA. The DNA was then transferred to a positively charged nylon membrane (Hybond N+, Amersham, Buckinghamshire, UK) by upward capillary transfer using $20\times\text{SSC}$ for 16–20 h, and fixed with UV-light (254 nm) irradiation for 1 min. The methods used for hybridization with the Tet 34 probe and detection were the same as described above.

Polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE)

DGGE can identify a single point mutation on a gene, so it can be used to separate bacterial species based on the 16S rDNA sequence¹²⁾. To examine whether the Tet 34-positive strains were of the same species or not, 16S rDNA-targeted PCR was performed and the products were analyzed by DGGE. The V6, 7, 8 region of 16S rDNA was amplified with universal primers for the conserved region of the 16S rRNA¹⁰⁾. The PCR product was 455 bp, corresponding to the position from 933 to 1387 (*E. coli* numbering). A hot start and touch down PCR was performed according to a previous report¹⁰⁾. The PCR products were analyzed on 8% polyacrylamide gels with urea-formamide gradients of 40 to 70%. Electrophoresis was performed at 10 V for 10 min and then at 200 V for 4 h at 55°C using the DCode System (Bio-Rad Laboratories, Hercules, CA). After electrophoresis, the gels were stained with ethidium bromide solution and visualized under UV light. The PCR products run on the DGGE gel were sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (PE Biosystems, Tokyo) and ABI PRISM 310 DNA sequencer (PE Biosystems, Tokyo) at the Center for Gene Research, Ehime University.

Results and Discussion

A total of 33 strains of OTC-resistant bacteria were isolated from diseased fish between 1997 and 2000. Three strains (KTX-98024, KFX-00019 and KRX-00017) of the genus *Vibrio* were determined to be Tet 34-positive based on dot-blot hybridization (Table 1). KTX-98024 was isolated in 1998 and the other 2 strains in 2000, suggesting that Tet 34-carrying bacteria have occurred since at least 1998. The other *Vibrio* sp. strains and all strains of *E. tarda*, *L. garvieae* and *P. damsela* subsp. *piscicida* were negative for Tet 34. We isolated *Vibrio* sp. harboring Tet 34 from the intestinal contents of apparently healthy yellowtail in 1999 (unpublished data). Thus, OTC-resistant bacteria possessing Tet 34 are thought to be ubiquitous in cultured fish and their environment. The administration of OTC might apply a selective pressure on the bacteria having Tet 34 in cultured fish.

The Tet 34-positive strains (KTX-98024, KFX-00019 and KRX-00017) showed MICs of $\geq 500 \mu\text{g/ml}$ in the presence of Mg^{2+} . These MICs were two-fold greater than those without MgCl_2 . The resistance of the *Vibrio* sp. No. 6 conferred by Tet 34 increases in the presence of MgCl_2 (Table 1). The results of the present study confirm that the resistance caused by Tet 34 is Mg^{2+} -dependent. Known TC-resistant mechanisms include efflux systems and ribosomal protection. All efflux genes [*tet* (A), *tet* (C), *tet* (D), *tet* (E), *tet* (G) and *tet* (H) and probably *tet* (I)] need Mg^{2+} ⁵⁾. Therefore, the resistant strains without Tet 34 may become OTC-resistant by known Mg^{2+} -dependent mechanisms.

To examine the location of Tet 34, Southern hybridization was performed for the positive strains. KTX-98024, KFX-00019 and KRX-00017 possessed several plasmids, and the plasmid profiles differed among the strains (Fig. 2). We could not clearly show whether the chromosomal DNA preparation contains mega plasmid DNA, because the plasmid extraction method used in this study was for the small plasmid not for the mega plasmid. A positive signal was detected in the chromosomal DNA at approximately 1 kbp (Fig. 3) but not in the plasmid DNA, and the distribution was restricted in the *Vibrio* species, suggesting that Tet 34 was not associated with mobile elements. Most *tet* genes are associated with mobile plasmids, transposons, conjugative transposons and integrons^{15,17,18)}. These mobile elements allow the *tet* genes to move to other species. Scott *et al.*²²⁾ reported that a transmissible TC resistance element was found in the chromosomal DNA of an obligatory anaerobic rumen bacterium. Moreover, *tet* (G) discovered in the fish patho-

Fig. 2. Plasmid profiles of the *Vibrio* spp. possessing Tet 34. Lane 1, size marker (kbp); lane 2, *Vibrio* sp. KTX-98024; lane 3, *Vibrio* sp. KFX-00019; lane 4, *Vibrio* sp. KRX-00017; lane 5, *Vibrio* sp. No. 6 and lane 6, *E. coli* JM 109 as a plasmid-free control. Chr. indicates chromosome.

Fig. 3. Southern hybridization analysis of chromosomal DNA extracted from OTC-resistant *Vibrio* spp. Lane 1, DNA of KTX-98024 digested with *Pst* I and *Eco*R I; lane 2, DNA of KTX-98024; lane 3, DNA of *Vibrio* sp. No. 6 digested with *Pst* I and *Eco*R I; lane 4, DNA of *Vibrio* sp. No. 6; lane 5, pOV digested with *Pst* I and *Eco*R I and lane 6, non-digested pOV. The hybridization probe was the cloned Tet 34. Chr. and 1kbp at the left indicate chromosome and the size of the positive band, respectively. OC and CC at the right indicate open circular and closed circular plasmid pOV, respectively.

gen *Vibrio anguillarum*^{3, 23}) was found in 10 human and 8 non-human isolates other than *Vibrio*¹³). This suggests that tet (G) is widely distributed in both aquatic and human environments. Rhodes *et al.*¹⁶) reported that a TC resistance-encoding plasmid was disseminated between different

Fig. 4. Profile of DGGE analysis of 16S rDNA amplicons from OTC-resistant strains of *Vibrio* spp. carrying Tet 34. Lanes 1 and 5, *Vibrio* sp. No. 6 used as a positive control; Lanes 2 to 4, OTC-resistant *Vibrio* sp. strains KTX-98024, KFX-00019 and KRX-00017, respectively. Lane 6, *Vibrio anguillarum* V-1037; lane 7, *Vibrio parahaemolyticus* ATCC 17802T and lane 8, *Escherichia coli* JM 109.

Aeromonas species and *E. coli* that occur in distinct human and aquaculture environments. Further study is needed to determine how Tet 34 is transferred among bacterial communities.

A DGGE profile is shown in Fig. 4. The PCR products of the strains KTX-98024, KFX-00019, and KRX-00017, and of *Vibrio* sp. No. 6 showed the same migration profiles. This indicates that the strains had the same 16S rDNA sequence and suggests a very close genetic relation. Furthermore, the sequence of the PCR products of these three strains showed 100% identity, which confirmed that these strains were the same. *Tet* (K), *tet* (M), *tet* (O), *tet* (Q) and *tet* (W) occur among numerous Gram-negative bacterial genera, whereas *tet* (H), *tet* (I), *tet* (J), *tet* (Y), *tet* (30) and *tet* (31) are carried by a restricted few⁵). Our results suggest that Tet 34 occurs only in *Vibrio* species, although further work is required to determine all the hosts. It is worth noting that the 3 species that tested positive for Tet 34 were isolated from diseased fish in 1998 and 2000, whereas the original strain, *Vibrio* sp. No. 6, was isolated from an apparently healthy yellowtail in 1999. This suggests that KTX-98024, KFX-00019, KRX-00017 and *Vibrio* sp. No. 6 may be opportunistic fish pathogens in that they occur normally in host fish but cause disease under certain conditions.

Animals, plants and various environments may contain such opportunists acting not only as pathogens, but as reservoirs of antibiotic resistance genes²⁾. In the future, we plan to examine the distribution and transferability of Tet 34 to clarify its dynamics in the marine environment.

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