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Similarity of Tetracycline Resistance Genes Isolated from Fish Farm Bacteria to Those from Clinical Isolates

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Tetracycline-resistant (Tet^r) bacteria were isolated from fishes collected at three different fish farms in the southern part of Japan in August and September 2000. Of the 66 Tet^r gram-negative strains, 29 were identified as carrying *tetB* only. Four carried *tetY*, and another four carried *tetD*. Three strains carried *tetC*, two strains carried *tetB* and *tetY*, and one strain carried *tetC* and *tetG*. Sequence analyses indicated the identity in Tet^r genes between the fish farm bacteria and clinical bacteria: 99.3 to 99.9% for *tetB*, 98.2 to 100% for *tetC*, 99.7 to 100% for *tetD*, 92.0 to 96.2% for *tetG*, and 97.1 to 100% for *tetY*. Eleven of the Tet^r strains transferred Tet^r genes by conjugation to *Escherichia coli* HB-101. All transconjugants were resistant to tetracycline, oxycycline, doxycycline, and minocycline. The donors included strains of *Photobacterium*, *Vibrio*, *Pseudomonas*, *Alteromonas*, *Citrobacter*, and *Salmonella* spp., and they transferred *tetB*, *tetY*, or *tetD* to the recipients. Because NaCl enhanced their growth, these Tet^r strains, except for the *Pseudomonas*, *Citrobacter*, and *Salmonella* strains, were recognized as marine bacteria. Our results suggest that *tet* genes from fish farm bacteria have the same origins as those from clinical strains.

Many different kinds of antibiotics have been used as therapeutic agents in aquaculture in Japan. Intensive work was done until the 1980s to develop guidelines for antibiotic usage in fish farms. The guidelines regulated doses and required a period of drug-free rearing before sale of fish and succeeded in keeping the residual antibiotics in cultured fish to nondetectable levels. However, Samuelsen et al. (35) found that antibiotic-resistant bacteria persisted in fish farm sediments for at least 18 months after chemotherapy. Since the products of aquaculture are consumed by humans and since many antibiotic resistance determinants are encoded by transferable plasmids, cultured fish may serve as a vehicle for transmission of antibiotic resistance to bacteria that are commensal or pathogenic to humans (34).

Tetracyclines are among the therapeutic agents most commonly used in human and veterinary treatment. Oxytetracycline is permitted to be mixed with feed for fish, and food sanitation law in Japan permits certain residual levels in fish. Because of the widespread use of tetracycline, resistance to it has been disseminated to many species of marine bacteria (4, 6, 18, 42, 46).

More than 30 different kinds of tetracycline resistance determinants have been published. Resistance genes have been mainly categorized into two major groups, those responsible for proton-dependent efflux of tetracycline (24) and those conferring ribosomal protection by cytoplasmic proteins (9). Dissemination of the proton-dependent tetracycline efflux protein in aquaculture environments has been reported (5, 12, 13, 14, 22, 34, 37). Previous work has identified the relevant genes by using DNA hybridization or PCR methods (4, 12, 13, 14, 15, 16, 27, 28, 34, 37), but the nucleotide sequences of these determinants remain unknown. In the present study, we isolated numerous Tet^r gram-negative fish farm bacteria and determined the DNA sequences of the Tet^r genes. We found that these genes were identical to the Tet^r genes identified in clinical isolates and that some were transferable to a laboratory *Escherichia coli* strain.

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MATERIALS AND METHODS

Isolation of bacteria resistant to tetracycline. Bacteria resistant to tetracycline were isolated from fishes collected at three different fish farms (A, B, and C) in August and September 2000. Fish farm A cultured yellowtail amberjack (*Seriola lalandi*) and northern bluefin tuna (*Thunnus thynnus*) and was located on a coastline facing the Sea of Japan. The culture area is encircled by a breakwater, and no river is present. Fish farm B cultured 1- and 2-year-old yellowtails (*Seriola quinqueradiata*) in a bay with a depth of 100 m. Fish farm C cultured greater amberjack (*Seriola dumerili*) and yellowtail amberjack (*Seriola lalandi*) in a coastal area of the Seto Inland Sea.

Fishes ranging from 4 to 6 kg were collected and were immediately processed in coastal facilities. About 1 cm^2 of fish skin, 1 g of flesh, and the contents of the rectum were homogenized in 9 ml of sterilized artificial seawater (ASW; 0.3 M NaCl, 0.02 M $MgSO_4$, 0.01 M $CaCl_2$, 0.01 M KCl), and then 0.1 ml of a decimal dilution was spread on modified ZoBell 2216E agar plate medium (47) in which 0.5% polypeptone and 0.1% yeast extract were dissolved in 1,000 ml of ASW. After incubation for 3 days at 25°C, the colonies were counted, and randomly selected colonies were purified with the modified ZoBell 2216E plate. These purified colonies were examined for Gram reaction by the KOH method (33). The gram-negative strains were transferred with a platinum needle to Mueller-Hinton agar plates (Becton Dickinson Microbiology Systems, Sparks, Md.) prepared with a solution containing 1% NaCl and 32 μg of tetracycline ml^{-1}. The bacterial colonies grown on the Mueller-Hinton plates after incubation for 3 days at 25°C were considered to be tetracycline resistant.

Seawater was also collected by using a sterilized Hyroth water sampler (Shibata, Tokyo, Japan) to determine viable counts of heterotrophic bacteria.

Antibacterial susceptibility test. Antibacterial susceptibility was examined according to a standard method recommended by the National Committee for

TABLE 1. PCR primer sets and restriction enzymes used for the detection of tetracycline efflux genes

Target gene	Primers[a] (sequences)	Predicted amplified fragment size (bp)	Restriction enzyme	Restriction fragment sizes (bp)
tetA	tetAC-F (5′-CGCYTATATYGCCGAYATCAC-3′), tetAC-R (5′CCRAAWKCGGCWAGCGA-3′)	417	*Sma*I	267, 150
tetC		417	*Sal*I	216, 201
tetB	tetBDEFHJ-F (5′-GGDATTGGBCTTATYATGCC-3′), tetBD-R (5′-ATMACKCCCTGYAATGCA-3′)	967	*Sph*I	173, 302, 492
tetD		964	*Sph*I	324, 640
tetG	tetGY-F (3′-TATGCRTTKATGCAGGTC-5′), tetGY-R (5′-GACRAKCCAAACCCAACC-3′)	917	*Eco*RI	368, 549
tetY		911	*Sph*I	197, 714
tetE	tetBDEFHJ-F, tetEHJ-R (5′-AWDGTGGCDGGAATTTG-3′)	650	*Nde*II	77, 148, 425
tetH		650	*Nde*II	267, 383
tetJ		650	*Nde*II	117, 297, 236

[a] F, forward; R, reverse.

Clinical Laboratory Standards (30), with Mueller-Hinton II agar (Becton Dickinson Microbiology Systems) and susceptibility test disks (Becton Dickinson Microbiology Systems) that contained ampicillin (10 μg), kanamycin (30 μg), tetracycline (30 μg), chloramphenicol (30 μg), oxytetracycline (30 μg), doxycycline (30 μg), and minocycline (30 μg).

Identification of Tet^r strains. Tet^r bacteria were identified on the basis of the 16S rRNA gene sequence. Isolated bacterial colonies were suspended in 100 μl of sterilized distilled water. The suspensions were boiled and centrifuged, and the supernatants were used as template DNA for PCR. The 16S rRNA gene was amplified by PCR with forward primer 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse primer 1492R (5′-TACGGYTACCTTGTTACGACTT-3′), which are located in *E. coli* at nucleotide positions 8 to 27 and 1492 to 1512, respectively (8). A 100-μl portion of the reaction mixture contained 1.5 U of *Taq* polymerase (Takara, Kyoto, Japan), 20 nmol of each deoxynucleoside triphosphate, 0.1 μl of the template DNA, 100 pmol of each primer, and 10 μl of the reaction buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$). The PCR amplification included 30 thermal cycles of 60 s at 94°C, 45 s at 60°C, and 90 s at 72°C, with an additional extension of 300 s at 72°C.

The amplified 16S rRNA gene was purified by agarose gel electrophoresis. The partial 479-bp nucleotide sequences of the PCR products were determined with forward primer 27F and reverse primer 517R (5′-GTATTACCGCGGCTGCTGGC-3′). The Tet^r bacteria were identified by referring to the most similar sequence found in the Ribosomal Database Project database (26).

The ability of an isolated strain to produce acid was examined on glucose with Hugh-Leifson medium (20). Oxidase production was examined by using a cytochrome oxidase test strip (Nissui, Tokyo, Japan). Motility was examined with hanging-drop preparations under a phase-contrast microscope. Catalase production was examined with 3% H_2O_2 solution by the method of Taylor and Achanzar (44).

Identification of Tet^r genes. Tetracycline efflux genes were amplified by PCR with the primers listed in Table 1. According to structural identities, the genes were clustered into four groups: (i) *tetA* and *tetC*, (ii) *tetB* and *tetD*, (iii) *tetG* and *tetY*, and (iv) *tetE*, *tetH*, and *tetJ*. Four different degenerate primer pairs, tetAC-F and tetAC-R for *tetA* and *tetC*, tetBDEHJ-F and tetBD-R for *tetB* and *tetD*, tetGY-F and tetGY-R for *tetG* and *tetY*, and tetBDEHJ-F and tetEHJ-R for *tetE*, *tetH*, and *tetI*, were designed based on regions showing high homology in each group. The predicted sizes of the PCR-amplified products were 417 bp for the *tetA* and *tetC* product, 967 or 964 bp for the *tetB* and *tetD* product, 917 or 911 bp for the *tetG* and *tetY* product, and 650 bp for *tetE*, *tetH*, and *tetJ* product (Table 1). GenBank accession numbers of the *tet* genes referred to in this experiment are summarized in Table 2.

The PCR protocol was basically the same as that used for the 16S rRNA gene, but the annealing temperature of the PCR was set at 50°C, except for the tetAC primer pair, where it was set at 55°C. The PCR-amplified products were analyzed by restriction fragment length polymorphism (RFLP) with the restriction enzymes listed in Table 1.

Sequence analysis of Tet^r genes. For determination of entire nucleotide sequences, *tetBCDGY* were amplified by PCR with the primers listed in Table 3.

The PCR protocol was basically the same as that for the 16S rRNA gene, but the annealing temperature was set at 50°C for *tetB* and *tetC* and at 55°C for *tetD*, *tetG*, and *tetY*. Nucleotide sequences of the PCR products were determined with a dye terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.).

Conjugation. The ability of bacteria to transfer the Tet^r gene to *E. coli* HB-101 was examined by a conjugation experiment using a filter mating method (36). The Tet^r strains were grown on tryptic soy broth (Difco, Detroit, Mich.) supplemented with 1% NaCl–32 μg of tetracycline ml^{-1} and incubated at 25°C. *E. coli* HB-101 was grown on Luria-Bertani (LB) medium and incubated at 37°C. Exponential-growth phase cultures of the Tet^r bacteria and the recipient were mixed in 2 ml of tryptic soy broth supplemented with 1% NaCl. Cell densities of both the Tet^r bacteria and the recipient were on the order of 10^8 cells ml^{-1}. The mixed cell suspension was collected on a 0.45-μm-pore-size HA membrane filter (Millipore). The filter was then transferred onto a tryptic soy agar (Difco) plate supplemented with 1% NaCl. After incubation for 24 h at 25°C, the filter was transferred into 2 ml of A-14 buffer (39.3 mM Na_2HPO_4, 22.0 mM KH_2PO_4, 68.5 mM NaCl, 0.8 mM $MgSO_4$, pH 7.2) and stirred. The 100 μl of the appropriate dilution was spread on an LB plate supplemented with 200 μg of streptomycin ml^{-1}, 20 μg of tetracycline ml^{-1}, 25 μl of 4% X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and 0.1 M IPTG (isopropyl-β-D-thiogalactopyranoside) and incubated at 40°C, at which the growth of the donor bacteria was suppressed.

Isolation of plasmids. The extraction of large plasmids was performed according to the method of Kado and Liu (21). The large plasmids were confirmed by an agarose gel electrophoresis.

Growth in the presence of Na^+. The Tet^r strains were grown on tryptic soy agar supplemented with 1% NaCl. Bacterial colonies were suspended in saline solution, and the turbidity of each cell suspension was adjusted to that of the McFarland 0.5 barium sulfate standard. One loopful of the cell suspension was transferred to Mueller-Hinton agar with or without NaCl (1%). After incubation for 10 to 18 h at 25°C, bacteria that showed faster growth in the presence of NaCl were considered to be marine bacteria.

Nucleotide sequence accession numbers. The tetracycline resistance gene sequences determined in this study were submitted to the GenBank and have been given accession numbers AB089585 to AB089608.

RESULTS

Distribution of Tet^r genes. Bacteria resistant to tetracycline were isolated from fishes collected at three different fish farms (A, B, and C) located in the southern part of Japan. The viable counts of heterotrophic bacteria in seawater of the culture area were on the order of 10^2 ml^{-1} for fish farms A and B and 10^3 ml^{-1} for fish farm C.

We randomly isolated 57 gram-negative strains from the fishes at fish farm A, 90 from farm B fishes, and 218 from farm C fishes and examined susceptibility to tetracycline. Of these

TABLE 2. Sequence data used in this study and sequence identity of *tet* genes

Gene	Element	Source	Accession no.	Reference(s)[a]	Identity[b] (%)
tetB	Tn*10*	*E. coli*	J01830	10, 19	99.3–99.6
	pGBG1	*E. coli*	AJ277653	39	99.3–99.6
	pHCM1	*Salmonella* serovar Typhi CT18	AL513383	32	99.5–99.8
		Shigella flexneri 2a	AF326777	25	99.5–99.8
	R100	*Shigella flexneri* 2b	AP000342	UP (SM)	99.6–99.9
	pHA162	Vector	AY048738	17	99.3–98.9
tetC	pRAS3.2	*Aeromonas salmonicida*	AY043299	23	100
	pSC101	*Salmonella* serovar Typhimurium	X01654	7	99.9
	pBR329	Cloning vector	L08859	UP (G)	99.8
	Lambda TXF97	Cloning vector	U37692	43	99.8
	pMTet1	Positive selection vector	AF438204	29	99.7
	pUCP26	Cloning vector	U07168	40	99.7
	Mini-CTX-lacZ	Integration vector	AF140579	UP (HKBS)	99.6
	pBSL193	Cloning vector	U35135	2	99.6
	pALTER<R>-Ex2	Cloning vector	U47103	UP (M)	99.7
	pRAS3.1	*Aeromonas salmonicida*	AY043298	23	98.2
tetD	pRA1	*Aeromonas* sp.	L06798	45	99.9–100
		Shigella sonnei 119	AF467074	UP (HEIL)	99.9–100
		Shigella flexneri Gibbon32055	AF467077	UP (HEIL)	99.9–100
		Salmonella Ordnez	X65876	3	99.7–99.8
tetG		*Salmonella* serovar Typhimurium DT104	AF071555	31	96.2
	pSTG2	*Pseudomonas* sp.	AF133140	38	96.2
	pJA8122	*Vibrio* anguillarum	S52437	46	96.0
	pSTG1	*Pseudomonas* sp.	AF133139	38	92.0
tetY	pIE1120	IncQ-like plasmid	AF070999	41	97.1–100

[a] UP, unpublished data; SM, G. Sampei and K. Mizobuchi; G, W. Gilbert; HKBS, T. T. Hoang, A. J. Kutchma, A. Becher, and H. P. Schweizer; M. J. Miles; HEIL, A. B. Hartman, I. I. Essiet, D. W. Isenbarger, and L. E. Lindler.
[b] Sequence identity to the *tet* genes isolated from fish farm bacteria.

strains, 21 in fish farm A, 6 in farm B, and 39 in farm C were resistant to tetracycline. The total number of Tet^r bacterial strains was 66.

Tet^r genes of the strains were amplified by PCR with four different sets of primers targeting tetracycline efflux genes (Table 1). PCR products were obtained for 51 Tet^r strains. Restriction fragment patterns of the PCR products of 43 Tet^r strains were identical to the patterns of the tetracycline efflux genes *tetB*, *tetC*, *tetD*, *tetG*, and *tetY*, while the patterns of the other 8 Tet^r strains were not identical to those of any of the genes. No PCR product was obtained for the other 15 strains.

Table 4 summarizes the characteristics of the 43 Tet^r strains. The *tetB* gene was found in 31 strains of the Tet^r bacteria. All of the *tetB* strains were motile rods and oxidase positive. These strains showed fermentative metabolism of glucose in Hugh-Leifson medium, while only TC69 showed oxidative metabolism. The *tetC* gene was found in four strains, which all showed oxidative reactions in the Hugh-Leifson medium, except for TA80, which was unable to grow in the medium. The *tetD* gene was found in three fermentative strains and one oxidative strain. The *tetY* gene was found in six strains, four fermentative strains and two strains that showed no color change in Hugh-Leifson medium. Strain TA57 was identified as carrying two different genes, *tetC* and *tetG*, while strains TC33 and TC34 carried both *tetB* and *tetY*.

Sequences of Tet^r genes. To examine the identity of genes, entire DNA sequences of 12 *tetB* genes (including those of oxidative strains TC33 and TC34), three *tetC* genes (those of TA57 and two others), four *tetD* genes, one *tetG* gene, and five *tetY* genes were examined. The identity between sequences of *tet* genes isolated from fish farm bacteria and published sequences is summarized in Table 2. The sequences of *tetB* genes from the fish farm bacteria were 99.3 to 99.9% identical to the sequences reported for clinical bacteria (10, 19). There was

TABLE 3. PCR primer sets for determination of nucleotide sequences of tetracycline efflux genes

Target gene	Primers[a] (sequences [5′–3′])	Amplified fragment size (bp)	Reported size of *tet* gene (bp)
tetB	tetB-F (CTAATCTAGACATCATTAATTCC), tetB-R (TTTGAAGCTAAATCTTCTTTAT)	1,397	1,206
tetC	tetC-F (ATGAAATCTAACAATGCGC), tetC-R (TCAGGTCGAGGTGGCCCGG)	1,191	1,191
tetD	tetD-F (ATGAATAAACCCGCTGTCATCGC), tetD-R (AATGCCGTCCACCATACC)	1,232	1,185
tetG	tetG-F (AGTTTCAGGTGCGCAGC), tetG-R (CCAAWTCGCCATGACTMAAT)	1,232	1,176
tetY	tetY-F (ATGTCAAAATCACTTATAACCGC), tetY-R (TCTCCCGCCAAGATTTTA)	1,191	1,176

[a] F, forward; R, reverse. Amplified fragments included the full lengths of *tet* genes, and some also included up- and/or downstream regions.

TABLE 4. Tetracycline resistance gene and general characteristics[a] of the tetracycline-resistant strains

Strain(s)	Fish farm	Origin	Gene type(s)[b]	Metabolism	Oxidase	Motility	Catalase
TA5, TA45	A	Flesh	B	F	+	+	+
TA49, TA51	A	Rectum	B	F	+	+	+
TB61	B	Rectum	B	F	+	+	+
TC16–23, TC65, TC74	C	Rectum	B	F	+	+	+
TC26–31, TC35–38	C	Flesh	B	F	+	+	+
TC68, TC71, TC76	C	Skin	B	F	+	+	+
TC69	C	Flesh	B	O	+	+	+
TC33, TC34	C	Flesh	B, Y	F	+	+	+
TC32	C	Flesh	Y	F	+	+	+
TC75	C	Skin	Y	F	+	+	+
TC72, TC73	C	Flesh	Y		+	−	+
TA3	A	Skin	D	F	−	+	+
TA6	A	Flesh	D	F	−	+	+
TA55	A	Skin	D	O	+	+	+
TC67	C	Rectum	D	F	−	+	+
TA58	A	Flesh	C	O	+	−	+
TA59	A	Flesh	C	O	−	+	+
TA80	A	Flesh	C	NO	+	+	+
TA57	A	Flesh	C, G	O	−	+	+

[a] +, positive; −, negative; F, fermentative; O, oxidative; NO, no growth.
[b] B, *tetB;* Y, *tetY;* D, *tetD;* C, *tetC;* G, *tetG.*

100% identity in *tetB* between one fish farm B strain of bacteria (TB61) and four strains of fermentative fish farm C bacteria. The *tetC* nucleotide sequences of the three fish farm strains were 98.2 to 100% identical to the sequences found in *Aeromonas salmonicida* (23) and *Salmonella enterica* serovar Typhimurium (7). The *tetD* sequences were 99.7 to 100% identical to those of *Shigella sonnei* (AF467074), *Shigella flexneri* (AF467077), and pRA1 harbored by *Aeromonas* (45). The *tetG* nucleotide sequence of TA57 was 92.0 to 96.2% identical to the sequences reported for *Salmonella* serovar Typhimurium DT104 (31), pSTG2 and pSTG1 of *Pseudomonas* spp. (38), and pJA8122 of *Vibrio anguillarum* (46). Identity of the *tetY* genes to the sequence of IncQ-like plasmid pIE1120 (41) ranged from 97.1 to 100%.

Sequences for the PCR products of the eight strains whose *tet* gene restriction fragment patterns showed no identity to those of known *tet* genes by PCR-RFLP were also determined; the sequences did not show any identity to those of the known *tet* genes.

Conjugation. The transferability of Tet^r determinants was examined for TC43. As summarized in Table 5, 11 strains were able to transfer their tetracycline resistance to *E. coli* HB-101 in conjugation experiments. According to analyses of the 16S rRNA gene, these 11 included 2 strains of a *Photobacterium* sp. that carried *tetB*, 2 strains of a *Photobacterium* sp. that carried both *tetB* and *tetY*, and 2 strains of a *Citrobacter* sp. that carried *tetD*. The other strains included one each of a *Vibrio* sp. (*tetB*), a *Pseudomonas* sp. (*tetB*), a *Photobacterium* sp. (*tetY*), an *Alteromonas* sp. (*tetD*), and a *Salmonella* sp. (*tetD*). There was no difference in the PCR-RFLPs of Tet^r genes between the nine transconjugants and their donor strains. Although *Photobacterium* sp. strains TC33 and TC34 were identified as carrying both *tetB* and *tetY*, only the *tetY* gene was transferred to the recipient.

The antibacterial susceptibilities of the donor and the transconjugant strains were determined by a disk diffusion method. As shown in Table 6, all the donor strains were resis-

TABLE 5. Results of conjugation experiment

Donor strain	Genus	Gene(s)[a]	Transconjugant strain	Transferred gene[a]
TA51	*Photobacterium*	B	E-TA51	B
TC21	*Photobacterium*	B	E-TC21	B
TC68	*Vibrio*	B	E-TC68	B
TC69	*Pseudomonas*	B	E-TC69	B
TC33	*Photobacterium*	B, Y	E-TC33	Y
TC34	*Photobacterium*	B, Y	E-TC34	Y
TC32	*Photobacterium*	Y	E-TC32	Y
TA3	*Citrobacter*	D	E-TA3	D
TA6	*Citrobacter*	D	E-TA6	D
TA55	*Alteromonas*	D	E-TA55	D
TC67	*Salmonella*	D	E-TC67	D

[a] B, *tetB;* Y, *tetY;* D, *tetD.*

TABLE 6. Antimicrobial susceptibility[a] of donor strains

Strain	Genus	Gene(s)[b]	Susceptibility to:						
			Tetracycline				ABPC	KM	CM
			TC	OXY	DOXY	MINO			
TA51	*Photobacterium*	B	R	R	R	I	R	S	R
TC21	*Photobacterium*	B	R	R	R	S	R	S	R
TC68	*Vibrio*	B	R	R	R	R	R	S	R
TC69	*Pseudomonas*	B	R	R	I	S	S	S	R
TC33	*Photobacterium*	B, Y	R	R	R	S	R	S	S
TC34	*Photobacterium*	B, Y	R	R	R	S	R	S	S
TC32	*Photobacterium*	Y	R	R	R	S	R	S	S
TA3	*Citrobacter*	D	R	R	R	R	S	R	R
TA6	*Citrobacter*	D	R	R	R	R	S	R	R
TA55	*Alteromonas*	D	R	R	S	S	S	R	I
TC67	*Salmonella*	D	R	R	R	I	S	S	R

[a] Susceptibility to antibiotics was identified as resistant (R), intermediate (I), or sensitive (S), according to guidelines of the National Committee for Clinical Laboratory Standards (30). Abbreviations for antibiotics: TC, tetracycline; OXY, oxytetracycline; DOXY, doxycycline; MINO, minocycline; ABPC, ampicillin; KM, kanamycin; CM, chloramphenicol.
[b] B, *tetB;* Y, *tetY;* D, *tetD.*

TABLE 7. Antimicrobial susceptibility of transconjugant strains

Strain	Gene[b]	Susceptibility[a] to:						
		Tetracycline				ABPC	KM	CM
		TC	OXY	DOXY	MINO			
E-TA51	B	R	R	R	R	S*	S	R
E-TC21	B	R	R	I	I	R	S	R
E-TC68	B	R	R	R	R	S*	S	R
E-TC69	B	R	R	R	R*	S	S	R
E-TC33	Y	R	R	R	I	R	S	R*
E-TC34	Y	R	R	R	I	R	S	R*
E-TC32	Y	R	R	R	I	R	S	R*
E-TA3	D	R	R	R	R	S	R	R
E-TA6	D	R	R	R	R	S	R	R
E-TA55	D	R	R	R*	R*	S	R	R
E-TC67	D	R	R	R	R	S	S	R
HB-101		S	S	S	S	S	S	S

[a] All abbreviations are as defined for Table 6. *, susceptibility different from that of the donor strain.
[b] B, *tetB;* Y, *tetY;* D, *tetD.*

tant to tetracycline and oxytetracycline but susceptible to minocycline except for *Vibrio* (TC68) and *Citrobacter* (TA3 and TA6) strains. Strains of *Photobacterium* (TA51, TC21, TC32, TC33, and TC34), *Vibrio* (TC68), *Citrobacter* (TA3 and TA6), and *Salmonella* (TC67) showed resistance to doxycycline, while an *Alteromonas* strain (TA55) was susceptible.

All of the transconjugants were resistant to tetracycline and oxytetracycline (Table 7). Although the *Alteromonas* strain (TA55) was susceptible to doxycycline and minocycline, the recipient (E-TA55) became resistant to these antibiotics after conjugation. The recipient of *tetY* also became resistant to chloramphenicol, whereas the donor was susceptible. Interestingly, some of the transconjugants showed different susceptibilities to ampicillin from those of the donor strains. All transconjugants acquired one or two large plasmids (Fig. 1).

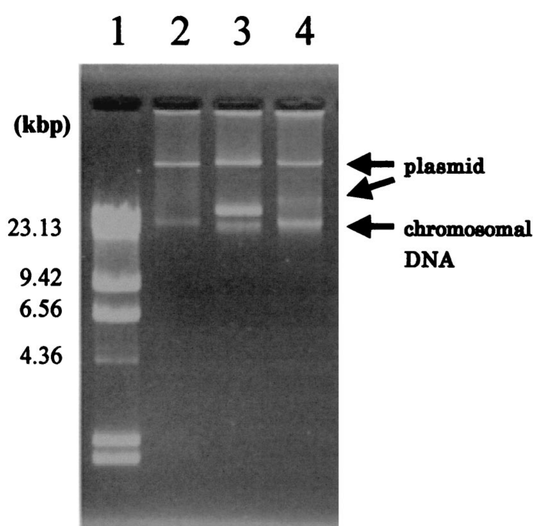

FIG. 1. Gel electrophoresis of large plasmids of a transconjugant strain. Lane 1, λ*Hin*dIII, DNA size marker; lane 2, E-TC68; lane 3, E-TA3; lane 4, E-TC33.

DISCUSSION

From the fishes collected at the three fish farms, 66 gram-negative Tet[r] bacterial strains were isolated. The percentages of resistant strains were 36.8% for fish farm A, 6.7% for fish farm B, and 17.9% for fish farm C. These values may be affected by the history of antibiotic usage at each fish farm, but no reliable data on antibiotic use were obtained from the fish farmers. However, we note that fish farm B was located in far deeper water (100 m) than fish farms A and C.

The *tet* genes of 43 Tet[r] gram-negative strains of bacteria were identified. Thirty-one contained *tetB*, and the other 12 contained *tetC*, *tetD*, *tetG*, or *tetY*. The genes of 23 Tet[r] strains were not identified. The prevalence of *tetB* in the fish farms was higher than that previously reported for *tetB* in clinical strains (15, 27, 28) or in phylloplane bacteria in an apple orchard (38). In place of *tetB*, *tetA* and *tetE* have been reported as prevalent in fish farms and marine environments (1, 4, 12, 13, 14, 34, 37). Although neither *tetA* nor *tetE* was amplified by PCR in this experiment, the number of the strains that produced no PCR product was only 15 and did not exceed the number of *tetB* strains.

The nucleotide sequences of *tetB* from fish farm bacteria were very similar to those reported for clinical strains, but the identity to the *tetB* sequence of *Shigella flexneri* was even higher. High identity to clinical strains was also found for *tetC*, *tetD*, and *tetY* from fish farm bacteria. The sequences of *tetC* were 100% identical to those of pRAS3.2 of clinical strains. The sequences of *tetD* of TC67 and *tetY* of both TC72 and TC73 were 100% identical to the sequences from *Shigella* spp. and pIE1120, respectively. These high identities suggest that *tet* genes from fish farm bacteria have the same origins as those from clinical strains.

tetG has been found previously in *Vibrio* spp. (46), *Pseudomonas* spp. (38), and *Salmonella* spp. (31). Since the *tetG* identified here has only 92.0 to 96.2% identity to the previously identified *tetG*, the gene may be a new *tetG* variant.

In the present study, the *tet* genes of 11 strains of fish farm bacteria were transferred to *E. coli* in conjugation experiments. The 11 strains of fish farm bacteria included strains of *Photobacterium*, *Vibrio*, *Alteromonas*, and *Pseudomonas.* The transferred genes were *tetB*, *tetD*, and *tetY.* Higher growth rates in the presence of Na^+ suggested that the strains of *Photobacterium*, *Vibrio*, and *Alteromonas* are marine bacteria. Hence we suggest that the Tet[r] gene of marine bacteria can be transferred to human intestinal bacteria, if the marine Tet[r] bacteria are ingested together with fish flesh. Since many human commensal bacteria carry the same *tet* genes, plasmids, transposons, and integrons as disease-causing bacterial species (11), our results suggest that marine Tet[r] bacteria can also transfer *tet* genes to disease-causing bacteria.

In the conjugation experiments, we observed interesting phenomena. Although *Photobacterium* sp. strains TC33 and TC34 carried both *tetB* and *tetY*, only *tetY* was transferred to the recipient. The difference in genotype is possible if each determinant was loaded on a different vehicle. Although no published work provides evidence that this process occurs, Smalla et al. (41) reported that the IncQ-like plasmid pIE1120 carries *tetY* but not *tetB*. Another interesting phenomenon is the difference in resistance between *Alteromonas* sp. strain

TA55 and its transconjugant, although no difference in the type of *tet* gene was found. An alternate explanation may be that expression of antibiotic resistance may vary among different host strains. Phenotypic differences reported by Sandaa et al. (36) include the appearance of novobiocin resistance after conjugation between fish farm bacteria and *E. coli* HB-101.

In this study, we found that each of three *Photobacterium* strains carried two different Tet^r genes, both *tetB* and *tetY* or both *tetC* and *tetG* (Table 4). These bacteria accounted for about 4.5% of all Tet^r strains. This value was higher than those previously reported for lactose-fermenting coliforms (27), *Salmonella* serovar Typhimurium (28), *Salmonella* serovar Hadar (16), *Aeromonas*, and *Enterobacteriaceae* isolated from catfish, catfish ponds, and rainbow trout farms (12, 13, 14, 37). Although environmental factors have been reported to affect the number of resistance genes carried by a single cell (11), the difference is more likely to be caused by a difference in the number of PCR primers utilized. Previous studies have examined only two to five resistance determinants, while nine different resistance genes were examined in this study.

Although the resistance caused by the *tetY* gene has not yet been well characterized, we found that transconjugants with the *tetY* gene became resistant to all tetracyclines. Hence, *tetY* may cause problems in future clinical treatment.

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