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Notes

Experimental Transfer of Tetracycline Resistance Genes from Fish-derived Bacteria to *Escherichia coli*

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To determine whether the tetracycline resistance genes *tet*(34), *tet*(M), and *tet*(S) can be transferred among bacteria, we used a filter mating experiment allowing intimate cell-cell contact between donor and recipient. The *tet*(34) gene, conveyed on a chromosome of *Vibrio* species (No. 6 and SW-42) was not transferred to *Escherichia coli* JM109, suggesting that it is not transferred among bacterial species. The *tet*(M) gene was transferred from three *Vibrio* strains (4-E, SW-18, and SW-38) to *E. coli* at frequencies of 8.5×10^{-5} to 2.1×10^{-6}. The *tet*(S) gene was transferred from *Lactococcus garvieae* KHS98032 to *E. coli* at a frequency of 1.8×10^{-6}. Transconjugated recipients showed increased minimum inhibitory concentrations against oxytetracycline. Although the donors possess the Tn*916*-Tn*1545* transposons, they were not detected in transformed recipients, suggesting that the transfer of *tet*(M) and *tet*(S) is mediated by elements or mechanisms. Two ribosomal protect protein genes were also transmissible from marine bacteria to *E. coli*, suggesting gene hopping among marine, terrestrial, and human environments.

Key words: Resistance gene, Tetracycline, *tet*(M), *tet*(S), *tet*(34), *Escherichia coli*

Introduction

Tetracycline (TC) has been widely used in aquaculture, and many studies have reported TC-resistant bacteria pathogenic to fish (Aoki et al., 1977; De-Paola et al., 1988; Ho et al., 2000; Rhodes et al., 2000). The determinants of TC resistance are widespread among bacterial species worldwide (Levy, 1988; Chopra and Roberts, 2001). To date, 40 different tetracycline resistance genes (*tet*), including oxytetracycline (OTC) resistance genes (*otr*), have been registered in GenBank. It is well-known that resistance determinants can be transferred among bacteria by plasmids, transposons, and integrons in aquatic environments (Aoki et al., 1987; Salyers et al., 1995; Chandrasekaran et al., 1998). All of the TC-resistance genes reported from aquatic bacteria are efflux genes, and none are ribosomal protect protein (RPP) genes. The distribution of *tet*(34), *tet*(M), and *tet*(S) genes in bacteria isolated from fish and seawater has been described (Nonaka and Suzuki, 2002; Kim et al., 2003, 2004). A transposon was detected to accompany *tet*(M), suggesting that the transfer of *tet*(M) is mediated by a transposon (Clewell et al., 1995; De Barbeyrac et al., 1996; Rhodes et al., 2000). Gene transfer among marine bacteria may contribute to the maintenance and distribution of resistance genes in the marine environment. TC resistance genes that originated in human clinical and terrestrial bacteria are conveyed on plasmids or transposons, indicating that the resistance genes are mediated by these mobile elements (Clewell et al., 1995; Roberts, 1996). However, little is known about the mechanisms responsible for the transfer of *tet* genes in the marine environment. Here, we describe the possibility for the transfer of the *tet*(34), *tet*(M), and *tet*(S) genes from marine isolates to *Escherichia coli*.

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Materials and Methods

Mating procedure

We performed a conjugation experiment to examine the transfer of TC resistance genes from marine isolates to *E. coli* JM109 using the filter mating method (Sandaa et al., 1992). The *tet* donors

were *Vibrio* species No. 6 and SW-42, which contain the *tet*(34) gene; *Vibrio* species 4-E, SW-18, and SW-38, which contain the *tet*(M) gene; and *Lactococcus garvieae* KHS98032, which contains the *tet*(S) gene. The donor strains were grown on nutrient salt agar (NSA: Nonaka and Suzuki, 2002) plates supple-mented with 1% NaCl and 32 μg/mL oxytetracycline (OTC; Sigma Chemical Co, St. Louis, MO, USA) at 25°C. The recipient *E. coli* JM109 was grown on Luria-Bertani (LB: Difco, Detroit, MI, USA) medium at 37°C. The donors and recipient were grown to the exponential growth phase in 2 mL of NSA without agar, in which the salt concentration was 1%. The cell density of each culture was adjusted to 10^8 cells/mL. Equal volumes of the donor and recipient cultures were mixed; 1 mL of the mixture was spotted on a 47-mm-diameter HA membrane filter (0.45-μm pore size: Millipore, Bedford, MA, USA) placed on NSA medium. After incubation at 25°C for 24 h, *E. coli* that had received the *tet* gene from the donor was selected as follows. 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 solution was diluted, and 100 μL was spread on LB plates supplemented with 20 μg/mL OTC. The plates were incubated at 42°C to suppress the growth of the donor stain. PCR was used to detect the target *tet* gene as described by Kim et al. (2004) to confirm the transfer of the *tet* gene to the recipient *E. coli*.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of oxytetracycline for donors and recipients were measured as follows. The bacterial cell suspension was prepared in phosphate-buffered saline (PBS), and the cell density was adjusted to MacFarland No. 0.5. A 1-μL aliquot of the suspension was spotted on Mueller-Hinton medium (Difco) plates supplemented with 1.5% NaCl containing 15, 30, 60, and 120 μg/mL OT C. The plates were incubated at 25°C for 48 h, and the lowest OTC concentration required for inhibition was recorded.

Results

Mating experiment

We recorded the transfer frequencies of the *tet* genes (Table 1). Transfer of *tet* genes was successful for four of the six donor strains: the three *Vibrio* strains containing the *tet*(M) gene (4-E, SW-18, and SW-38), and *L. garvieae* (KHS98032) containing the *tet*(S) gene. The transfer of *tet*(34) from *Vibrio* species (No. 6 and SW-42) to *E. coli* was not successful. The transfer frequency ranged from 8.5×10^{-5} to 2.1×10^{-6} for *tet*(M), and was 1.8×10^{-6} for *tet*(S). The Tn*916* transposon was not detected in *E. coli* transconjugants (Table 1).

Minimum inhibitory concentrations

The MICs for donor strains were ≥120 μg/mL OTC, but those of recipient strains were <5 μg/mL OTC. However, transconjugants with the *tet*(M) or *tet*(S) genes had higher MICs. The transfer of *tet*(M) to *E. coli* resulted in an MIC ≥120 μg/mL OTC, whereas the transfer of *tet*(S) to *E. coli* showed an MIC of 60 μg/mL OTC (Table 2).

Table 1. Transfer rate in the mating experiment

Donor strain	Origin	Resistance gene	Tn*916*	Recipient strain	After conjugation		Transfer rate
					Detected gene	Tn*916*	
Vibrio No. 6[a]	Yellowtail (I)[b]	*tet*(34)	-	*E. coli* JM109	ND[c]	-	--
Vibrio SW-42	Seawater	*tet*(34)	-	*E. coli* JM109	ND	-	--
Vibrio 4-E	Yellowtail (I)	*tet*(M)	-	*E. coli* JM109	*tet*(M)	-	1.2×10^{-5}
Vibrio SW-18	Seawater	*tet*(M)	-	*E. coli* JM109	*tet*(M)	-	8.5×10^{-5}
Vibrio SW-38	Seawater	*tet*(M)	-	*E. coli* JM109	*tet*(M)	-	2.1×10^{-5}
Lactococcus garvieae	Yellowtail (I)	*tet*(S)	-	*E. coli* JM109	*tet*(S)	-	1.8×10^{-5}

[a]Strain name; [b]I, intestine; [c]ND, not detected.

Table 2. MIC of donors and recipient strain

Donor strain	Resistance gene	MIC (μg/mL OTC)	Transconjugant	MIC (μg/mL OTC)
			E. coli JM109 (negative control)	<5
Vibrio 4-E	*tet*(M)	>120	Trans *E. coli* 4-E	>120
Vibrio SW-18	*tet*(M)	>120	Trans *E. coli* SW-18	>120
Vibrio SW-38	*tet*(M)	120	Trans *E. coli* SW-18	120
Lactococcus garvieae KHS98032	*tet*(S)	>120	Trans *E. coli* KHS98032	60

Discussion

We previously described the distribution of the TC resistance genes *tet*(34), *tet*(M), and *tet*(S) among bacteria isolated from fish and seawater sampled from coastal aquaculture sites in the Philippines, Korea, and Japan between 1997 and 2002 (Kim et al., 2003, 2004). Here, we showed that TC resistance genes *tet*(M) and *tet*(S) can be transferred to *E. coli* from *Vibrio* species and *L. garvieae* under cell-cell contact.

The *tet*(34) gene, however, was not transferred to *E. coli*. A previous 16S rDNA analysis showed that all *tet*(34)-positive strains were 100% identical (Kim et al., 2003). Nonaka and Suzuki (2002) reported that *tet*(34) was detected in certain *Vibrio* species, but not in other fish-related bacteria, such as *Edwardsiella tarda*, *L. garvieae*, or *Photobacterium damsela* subsp. *piscicida*. The *tet*(34) gene occurs on a chromosome of *Vibrio* species No. 6 (Nonaka and Suzuki (2002). Thus, evidence suggests that *tet*(34) is a specific gene in certain *Vibrio* species and is not transferred to other bacterial species. However, the *tet*(34) gene was recently detected from species of *Pseudomonas* and *Serratia* (Miranda et al., 2003). Moreover, it can be transferred from *Pseudomonas pseudoalcaligenes* and *Serratia liquefaciens* to *E. coli* at frequencies of 5.0×10^{-5} and 1.3×10^{-6} per recipient (Miranda et al., 2003). Because Miranda et al. (2003) did not sequence the *tet*(34) gene, we do not know if it is the same *tet*(34) gene identified by Nonaka and Suzuki (2002), who reported that *tet*(34) is similar to xanthine-guanine phosphoribosyl transferase (XPRT), an enzyme involved in GTP synthesis. Miranda et al. (2003) also detected other genes similar to XPRT.

The *tet*(M) gene is often associated with conjugative chromosomal elements such as transposons (Clewell et al., 1995; Salyers et al., 1995). However, we could not detect the Tn*916* transposon in the transconjugant. All the donors we used carry the *tet* gene on a chromosome. We could not determine the mechanism of *tet*(M) transfer. The Tn*916* family consists of Tn*916*, Tn*918*, Tn*919*, Tn*920*, Tn*925*, Tn*1545*, Tn*3702*, Tn*3703*, Tn*3704*, Tn*5251*, Tn*5381*, Tn*5383*, Tn*5397*, and others. All of these elements may accompany the *tet*(M) gene on a chromosome (Clewell et al., 1995; De Barbeyrac et al., 1996; Rice, 1998). Polymerase chain reaction (PCR) for Tn can detect the Tn*916* and Tn*1545* transposons because they are similar and have a similar *Int-Tn* gene encoding the integrases of the transposon. However, other transposon series could not be detected using PCR analysis. Therefore, conjugative transposons other than Tn*916* and Tn*1545* may mediate the transfer of *tet*(M).

The *tet*(S) gene has been detected in *Listeria monocytogenes* BM4210, where it is carried by self-transferable plasmids (Charpentier et al., 1994; Charpentier and Courvalin 1999). *Lactococcus lactis* also carries *tet*(S) on a conjugative plasmid (Perreten et al., 1997). In *Enterococcus faecalis*, *tet*(S) is integrated into the chromosomal DNA (Charpentier et al., 1994; Francois et al., 1997). We found that *tet*(S) was transferred from *L. garvieae* to *E. coli* at a frequency of 1.8×10^{-6}. We did not find any plasmids in either the donor or transconjugants, suggesting that *tet*(S) is encoded on a chromosome of *L. garvieae* and transferred by unknown transfer elements.

This is the first demonstration that the *tet*(M) and *tet*(S) genes can be transferred from marine bacteria to *E. coli*. This suggests that the flow of TC resistance genes can occur between marine bacteria and terrestrial or human intestinal bacteria. Future studies should clarify the mechanisms of gene transfer among marine bacteria and between marine and terrestrial bacteria for the various TC resistance genes.

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