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Cell-to-cell Contact is Required for Transfer of Tetracycline Resistance Gene *tet*(M) in Marine Bacteria

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Abstract—Tetracycline (TC) resistance gene *tet*(M) is reported to distribute in marine environment, however, how the *tet*(M) transfer occurs in marine bacteria is not clear so far. Objective of this study is to characterize the process of *tet*(M) transfer from marine isolates to recipient cell. *Tet*(M) positive marine bacteria were used as donors, which included *Vibrio* sp. and *Lactococcus garvieae*. *E. coli* JM109 and *Enterococcus faecalis* JH2-2 were used as recipients, which were sensitive to TC. For gene transfer study, filter-mating and filter-separating experiments were done. Result showed that *tet*(M) transfer was occurred from *Vibrio* to *E. coli* and *L. garvieae* to *E. faecalis* only when donor and recipient cells were mixed, but not in the case of filter-separating. The transfer rate in filter-mating examination was 10^{-3} for *Vibrio* and *E. coli* combination and 10^{-5} for *L. garvieae* and *E. faecalis* combination. The transfer of *tet*(M) was confirmed by the detection of *tet*(M) from transconjugant by PCR. Our experiment suggested that the *tet*(M) is transferred from marine bacteria to recipient under cell-to-cell contact condition which is likely due to a conjugation-like mechanism.

Keywords: horizontal gene transfer, tetracycline resistance, *tet*(M), conjugation

INTRODUCTION

It is thought that horizontal gene transfer is one of the important pathways for antibiotic resistance gene transfer in the environment. Horizontal gene transfer plays an important role for evolution and diversification among bacteria (Malik *et al*., 2008). The rapid dissemination of antibiotic resistance genes in bacterial population in various environments are mediated by horizontal gene transfer. In general, there are three processes of horizontal gene transfer in bacteria, which are transformation, transduction, and conjugation. To know spreading of antibiotic resistance gene in environment, gene transfer experiment will help us to understand it.

Tetracycline (TC) is one of the popular antibiotics, which are most extensively used in aquaculture. The occurrence of the TC-resistant bacteria and resistance genes in various marine aquaculture environments were reported (Nonaka *et al*., 2000; Furushita *et al*., 2003; Miranda *et al*., 2003). *Tet*(M) is one of the ribosomal

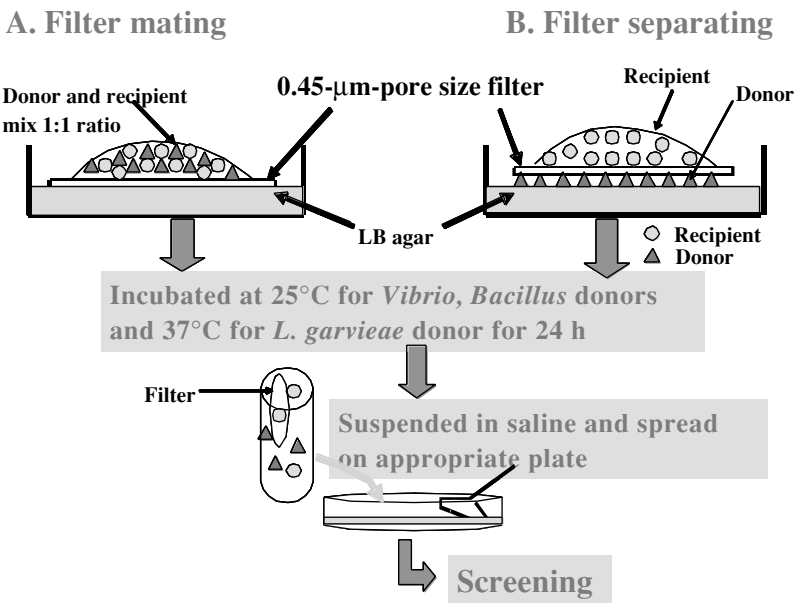

Fig. 1. Gene transfer experiment.

protection protein (RPP) genes, which distributes in marine environment (Kim *et al*., 2004; Nonaka *et al*., 2007). However, the mechanism of *tet*(M) transfer among marine bacteria is still unknown. The objective of this study was to characterize the process of *tet*(M) transfer from marine bacteria to human enteric bacteria. We show here that marine bacteria are capable of transferring *tet*(M) gene into recipient by cell-to-cell contact condition.

MATERIAL AND METHODS

Bacterial strains and culture conditions

Tet(M) positive marine bacteria used as donors were *Vibrio* sp. and *Lactococcus garvieae*. Recipients were *Escherichia coli* and *Enterococcus faecalis*.

Vibrio sp. was grown in Marine Broth (MB) (BD, USA) at 25°C and *L. garvieae* was grown in Brain Heart Infusion (BHI) (BD, USA) broth at 37°C. Media was supplemented with 30 μg/ml of TC (Sigma, USA). *E. coli* was grown in Luria-Bertani (LB) broth and *E. faecalis* was in LB with 10 μg/ml of Chloramphenicol (Cm) at 37°C. *E. coli* after transformation (transconjugant) was grown on LB plate contained 20 μg/ml of TC at 42°C for 24 h. *E. faecalis* transconjugant was grown on LB plate contained 20 μg/ml of TC and 10 μg/ml of Cm at 37°C for 24 h. Minimum inhibitory concentration (MIC) was determined by agar dilution method according to the report of Neela *et al*. (2007).

Table 1. Transfer rate of TC resistance by filter-mating and filter-separating methods.

Donor	MIC	*tet*(M)	Recipient	MIC	Transconjugant		
					Transfer rate	MIC	*tet*(M)
Filter-mating							
Vibrio sp. 04Ya311	128	+	*E. coli*	2	$(6.62 \pm 1.61) \times 10^{-3}$	>256	+
			E. faecalis	0.5	$<(4.21 \pm 1.33) \times 10^{-9}$	–	–
L. garvieae KHS-00083	128	+	*E. coli*	2	$<(1.63 \pm 0.76) \times 10^{-9}$	–	–
			E. faecalis	0.5	$(4.18 \pm 0.43) \times 10^{-5}$	>256	+
Filter-separating							
Vibrio sp. 04Ya311	128	+	*E. coli*	2	$<(3.43 \pm 0.44) \times 10^{-9}$	–	–
			E. faecalis	0.5	$<(7.40 \pm 5.13) \times 10^{-9}$	–	–
L. garvieae KHS-00083	128	+	*E. coli*	2	$<(2.44 \pm 1.10) \times 10^{-10}$	–	–
			E. faecalis	0.5	$<(4.70 \pm 4.66) \times 10^{-8}$	–	–

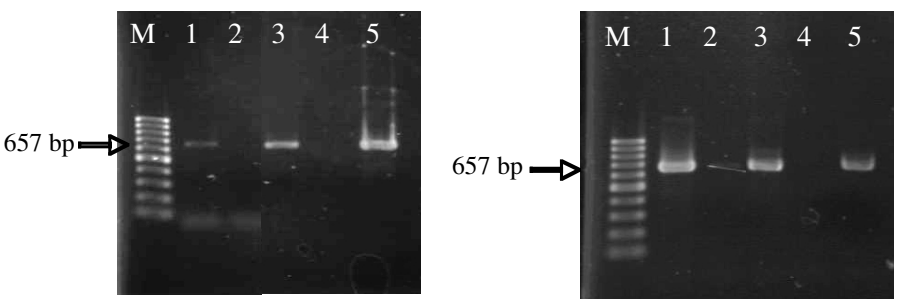

Fig. 2. Detection of *tet*(M) from transconjugant. (A): Lane 1, *Vibrio* sp. 04Ya311 (donor); lane 2, *E. coli* JM109 (recipient); lane 3, *E. coli* transconjugant; lane 4, negative control (DW); lane 5, positive control (10^6 copy number of *tet*(M)) and M, size marker 100 bp DNA Ladder. (B): Lane 1, *L. garvieae* KHS-00083 (donor); lane 2, *E. faecalis* JH2-2 (recipient); lane 3, *E. faecalis* transconjugant; lane 4, negative control (DW); lane 5, positive control (10^6 copy number of *tet*(M)) and M, size marker 100 bp DNA Ladder.

Filter-mating experiment

Filter-mating was carried out according to Sandaa *et al.* (1992) (Fig. 1A). The cells of donor and recipient cells at exponential growth phase were mixed with 1:1 ratio and spotted on a sterile nitrocellulose membrane filter with a 0.45-μm pore size (Millipore, USA) and placed on LB plates; plates were incubated at 25°C for *Vibrio* donor and 37°C for *L. garvieae* donor for 24 h. After incubation, filter was suspended in phosphate buffered saline (PBS), followed by dilution and spreading on selective medium for transconjugants.

Filter-separating experiment

In filter-separating experiment (Fig. 1B), donor in exponential growth phase was spread on LB plates and was covered with a 0.45-μm pore size filter. Recipient cells were spotted on the filter. Incubation was performed according to the filter-mating method. Screening of transconjugant was performed as mentioned above. The transfer rate was calculated by the number of transconjugants/number of recipient cells according to Gevers *et al.* (2003).

Detection of tet(M) by PCR

Genomic DNA was extracted from bacterial cells by the method of Neela *et al.* (2008). Primers used for detection of *tet*(M) were *tet*(M)-1 (5′-GTTAAATAGTGTTCTTGGAG-3′) and *tet*(M)-2 (5′-CTAAGATATGGCTCTAACAA-3′), which gave a 657 bp amplicon (Aarestrip *et al.*, 2000). PCR amplification was performed as describe in Neela *et al.* (2008).

RESULTS AND DISCUSSION

The TC-resistant *Vibrio* and *L. garvieae* containing *tet*(M) gene were used for transfer experiment of TC resistance to *E. coli* and *E. faecalis* by filter-mating and filter-separating methods. As shown in Table 1, transformation rate in filter mating of *E. coli* was 10^{-3} when *Vibrio* was donor, whereas that in *E. faecalis* was 10^{-5} when *L. garvieae* was donor. Positive transconjugants could not be obtained in the case of *Vibrio* with *E. faecalis* and *L. garvieae* with *E. coli*. In filter-separating method, the transfer of TC resistance was not observed in any combinations. The transconjugants showed MIC of >256 μg/ml. The *tet*(M) was detected from transconjugants of *E. coli* and *E. faecalis* by PCR (Fig. 2).

This study indicated that the transfer of *tet*(M) gene from marine bacteria to human enteric bacteria occurred by a conjugation-like mechanism rather than transformation or transduction. Transfer occurred only when the donor and recipient cell contacted. Conjugation is well studied mechanism and responsible for the spread of antibiotic resistance genes among various bacterial species (Kruse and Sørum, 1994; Wolska, 2003). R plasmid transfer was reported in combination of *E. coli* and marine *Vibrio* sp. by only cell-to-cell contact (Paul *et al.*, 1992). Transfer vector in this study is not known, which should be cleared in the next step.

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REFERENCES

Furushita, M., T. Shiba, T. Maeda, M. Yahata, A. Kaneoka, Y. Takahashi, K. Torii, T. Hasegawa and M. Ohta (2003): Similarity of tetracycline resistance genes isolated from fish farm bacteria to those from clinical isolates. *Appl. Environ. Microbiol.*, **69**, 5336–5342.

Gevers, D., G. Huys and J. Swings (2003): In vitro conjugal transfer of tetracycline resistance from

Lactobacillus isolates to other Gram-positive bacteria. *FEMS Microbiol. Lett.*, **225**, 125–130.
Kim, S.-R., L. Nonaka and S. Suzuki (2004): Occurrence of tetracycline resistance genes *tet*(M) and *tet*(S) in bacteria from marine aquaculture sites. *FEMS Microbiol. Lett.*, **237**, 147–156.
Kruse, H. and H. Sørum (1994): Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. *Appl. Environ. Microbiol.*, **60**, 4015–4021.
Malik, A., E.-K. Celik, C. Bohn, U. Böckelmann, K. Knobel and E. Grohmann (2008): Detection of conjugative plasmids and antibiotic resistance genes in anthropogenic soils from Germany and India. *FEMS Microbiol. Lett.*, **279**, 207–216.
Miranda, C. D., C. Kehrenberg, C. Ulep, S. Schwarz and M. C. Roberts (2003): Diversity of tetracycline resistance genes in bacteria from Chilean salmon farms. *Antimicrob. Agents Chemother.*, **47**, 883–888.
Neela, F. A., L. Nonaka and S. Suzuki (2007): The diversity of multi-drug resistance profiles in *tet*racycline-resistance *Vibrio* species isolated from coastal sediments and seawater. *J. Microbiol.*, **45**, 64–68.
Neela, F. A., L. Nonaka, M. H. Rahman and S. Suzuki (2008): Transfer of chromosomal encoded tetracycline resistance gene *tet*(M) from marine bacteria to *Escherichia coli* and *Enterococcus faecalis*. *World J. Microbiol. Biotechnol.* (submitted).
Nonaka, L., T. Isshiki and S. Suzuki (2000): The occurrence of oxytetracycline resistant bacteria in the fish intestine and the seawater environment. *Microbes Environ.*, **15**, 223–228.
Nonaka, L., K. Ikeno and S. Suzuki (2007): Distribution of tetracycline resistance gene, *tet*(M), in Gram-positive and Gram-negative bacteria isolated from sediment and seawater at a coastal aquaculture site in Japan. *Microbes Environ.*, **22**, 355–364.
Paul, J. H., J. M. Thurmond, M. E. Frischer and J. P. Cannon (1992): Intergeneric natural plasmid transformation between *E. coli* and a marine *Vibrio* species. *Mol. Ecol.*, **1**, 37–46.
Sandaa, R.-N., V. L. Torsvik and J. Goksøyr (1992): Transferable drug resistance in bacteria from fish-farm sediments. *Can. J. Microbiol.*, **38**, 1061–1065.
Wolska, K. I. (2003): Horizontal DNA transfer between bacteria in the environment. *Acta Microbiol. Pol.*, **52**, 233–243.

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