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

Farzana Ashrafi Neela, Nobumasa Nagahama and Satoru Suzuki

Center for Marine Environmental Studies (CMES), Ehime University, Bunkyo-cho 3, Matsuyama 709-8577, Japan

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Abstract—Tetracycline (TC) resistance gene \textit{tet}(M) is reported to distribute in marine environment, however, how the \textit{tet}(M) transfer occurs in marine bacteria is not clear so far. Objective of this study is to characterize the process of \textit{tet}(M) transfer from marine isolates to recipient cell. \textit{Tet}(M) positive marine bacteria were used as donors, which included \textit{Vibrio} sp. and \textit{Lactococcus garvieae}. \textit{E. coli} JM109 and \textit{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 \textit{tet}(M) transfer was occurred from \textit{Vibrio} to \textit{E. coli} and \textit{L. garvieae} to \textit{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 \textit{Vibrio} and \textit{E. coli} combination and $10^{-5}$ for \textit{L. garvieae} and \textit{E. faecalis} combination. The transfer of \textit{tet}(M) was confirmed by the detection of \textit{tet}(M) from transconjugant by PCR. Our experiment suggested that the \textit{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, \textit{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). \textit{Tet}(M) is one of the ribosomal
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).
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.

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

<table>
<thead>
<tr>
<th>Donor</th>
<th>MIC</th>
<th>tet(M)</th>
<th>Recipient</th>
<th>MIC</th>
<th>Transconjugant</th>
<th>MIC</th>
<th>tet(M)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Filter-mating</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vibrio</em> sp.</td>
<td>128</td>
<td>+</td>
<td><em>E. coli</em></td>
<td>2</td>
<td>(6.62 ± 1.61) × 10^-3</td>
<td>&gt;256</td>
<td>+</td>
</tr>
<tr>
<td>04Ya311</td>
<td></td>
<td></td>
<td><em>E. faecalis</em></td>
<td>0.5</td>
<td>(4.21 ± 1.33) × 10^-9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>L. garvieae</em></td>
<td>128</td>
<td>+</td>
<td><em>E. coli</em></td>
<td>2</td>
<td>(1.63 ± 0.76) × 10^-9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>KHS-00083</td>
<td></td>
<td></td>
<td><em>E. faecalis</em></td>
<td>0.5</td>
<td>(4.18 ± 0.43) × 10^-5</td>
<td>&gt;256</td>
<td>+</td>
</tr>
<tr>
<td><strong>Filter-separating</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vibrio</em> sp.</td>
<td>128</td>
<td>+</td>
<td><em>E. coli</em></td>
<td>2</td>
<td>&lt;(3.43 ± 0.44) × 10^-9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>04Ya311</td>
<td></td>
<td></td>
<td><em>E. faecalis</em></td>
<td>0.5</td>
<td>&lt;(7.40 ± 5.13) × 10^-9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>L. garvieae</em></td>
<td>128</td>
<td>+</td>
<td><em>E. coli</em></td>
<td>2</td>
<td>&lt;(2.44 ± 1.10) × 10^-10</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>KHS-00083</td>
<td></td>
<td></td>
<td><em>E. faecalis</em></td>
<td>0.5</td>
<td>&lt;(4.70 ± 4.66) × 10^-8</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

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-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′-GTAAATAGTGGTTCTTGAG-3′) and tet(M)-2 (5′-CTAAGATATGGCTCTAAACA-3′), which gave a 657 bp amplicon (Aarestrup et al., 2000). PCR amplification was performed as described 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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F. A. Neela, N. Nagahama and S. Suzuki (e-mail: ssuzuki@agr.ehime-u.ac.jp)