Structural characterization of Tn916-like element in

*Streptococcus parauberis* serotype II strains isolated from
diseased Japanese flounder

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Running title: Tn916-like element in *Strep. parauberis*

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Abstract

**Aims:** To screen for the existence and determine the structure of Tn916-like element in *Streptococcus parauberis* serotype II strains isolated from cultured Japanese flounder in western Japan.

**Methods and Results:** In this study, the structure of Tn916-like element and the flanking regions were characterized by polymerase chain reaction (PCR) and inverse PCR, followed by cloning and sequencing. The Tn916-like element is 18,031 bp in length and composed of 22 ORFs. Southern blot hybridization analysis showed that the *Hinc*II-digested internal structures of Tn916-like elements yielded two patterns among *Strep. parauberis* serotype II strains. The flanking sequences were identical with the corresponding region of *Strep. parauberis* serotype I strain except for the addition of 6-bp coupling sequence (ATCATA) being adjacent to the upstream of the element.

**Conclusion:** The Tn916-like element exhibited high homology (more than 99%) with Tn916 observed in other streptococci and enterococci and was integrated in the same site of chromosome for all of the tested *Strep. parauberis* serotype II strains.

**Significance and Impact of the Study:** The results indicate that the Tn916-like element encoding *tet(M)* gene is present in all of the tested *Strep. parauberis* serotype II strains, which are disseminated in the flounder-culturing areas in western Japan.
Key words: Japanese flounder, Japan, Streptococcus parauberis, serotype, tetracycline resistance, Tn916

Introduction

From the beginning of this century, a streptococcal infection caused by Streptococcus parauberis has emerged in the aquaculture industry of Japanese flounder Paralichthys olivaceus in Japan (Kanai et al. in press). Strep. parauberis was first described as the etiologic agent of bovine mastitis (Williams and Collins 1990) and later caused streptococcosis in cultured turbot Scophthalmus maximus in Spain (Toranzo et al. 1995; Doménech et al. 1996). Currently, Strep. parauberis infection in Japanese flounder has spread across the western districts of Japan and has become a leading cause of economic losses in its aquaculture industry. Japanese isolates of Strep. parauberis were classified into two groups, serotype I and II, based on a serological investigation (Kanai et al. in press).

For the chemotherapeutic treatment of streptococcal infections in Japanese flounder, the usage of tetracyclines is the only way approved by the Ministry of Agriculture, Forestry and Fisheries (http://www.maff.go.jp/j/syouan/suisan/suisan_yobo/pdf/suisan_iyakuhin.pdf). However, the worldwide use of tetracyclines for the control of infections, as prophylactic agents or as growth promoters, has facilitated the emergence and spread of acquired resistance (Roberts 2005). In our preliminary experiments (Meng et al. in press), tetracycline-resistant strains of Strep. parauberis
serotype I and II were found in our culture collections, and, surprisingly, all of serotype II strains derived from divergent aquaculture sites were tetracycline-resistant and harbored \textit{tet}(M), \textit{xis} (excisase) and \textit{int} (integrase) genes which are components of Tn916 family transposons (Clewell et al. 1995). So it is supposed that \textit{Strep. parauberis} serotype II strains possessed Tn916-related genetic elements.

\textit{tet}(M) gene, a tetracycline resistance determinant of ribosomal protection, is the most common in both Gram-positive and Gram-negative bacteria (Roberts 2005). Tn916, which harbored \textit{tet}(M) gene, was first documented as a conjugative transposon detected on the chromosome of \textit{Enterococcus faecalis} DS16 (Franke and Clewell 1981). Since then, Tn916 has undergone intensive investigations that introduced its wide host range (Rice 1998), mechanisms of excision and insertion (Caparon and Scott 1989), as well as conjugal transferability (Clewell et al. 1995) and complete DNA sequence (Flannagan et al. 1994). According to the numerous documents, Tn916 family transposons have acquired additional resistance determinants, such as Tn1545 [bearing \textit{erm}(B) and \textit{aphA}-3], Tn6002 [bearing \textit{erm}(B)] and Tn6003 [bearing \textit{erm}(B), \textit{aadE}, \textit{sat4} and \textit{aphA}-3] (Caillaud et al. 1987; Warburton et al. 2007; Cochetti et al. 2008).

The aims of the present study were to clarify the structure of the Tn916-related element by analyzing its total DNA sequence on the chromosome of a \textit{Strep. parauberis} serotype II strain, to compare the structure within serotype II strains and to determine the transferability of the element.
We also characterized the insertion site of the Tn916-related element on the chromosome by comparing with the corresponding region of a serotype I strain. Because the Tn916-related element was revealed to have a high similarity with Tn916, we would like to use Tn916-like element instead of Tn916-related element hereafter.

Materials and methods

Strains, medium and DNAs

Thirty-two Strep. parauberis serotype II strains used in this study were those isolated from diseased Japanese flounder at aquaculture sites located in Kagawa (8 strains), Ehime (9), Kumamoto(1), Oita (10), Kagoshima (1) and Nagasaki (3) Prefectures from 2002 to 2008 in Japan. All the strains were provided by the six prefectural fisheries experimental stations. Identification of the strains was performed by PCR analysis of Strep. parauberis 23S rDNA (Mata et al., 2004), and serotyping was carried out by slide agglutination test in our laboratory (Kanai et al., 2009). Stock cultures were stored at —80℃ in stock broth containing 10 % (v/v) glycerol, 1% Polypeptone (Nissui), 0.5% Yeast Extract (Difco Laboratories) and 0.5% NaCl. Strep. parauberis NUF1003, isolated in Shimane Prefecture in 2003, and NUF1049, isolated in Oita Prefecture in 2007, were used as the representative strains of serotype I and II, respectively. Ent. faecalis CG110 that harbored Tn916
was used as the reference strain (Shimoji et al. 1994) and *Ent. faecalis* FA2-2 as the recipient strain in conjugal transfer experiments (Shiojima et al. 1997). These strains were grown at 28°C in Todd-Hewitt broth (THB) (Difco Laboratories) for 16 h with agitation. Chromosomal and plasmid DNAs were prepared using Wizard Genomic DNA Purification kit (Promega) and QIAprep miniprep kit (Qiagen), respectively.

**PCR and inverse PCR amplification**

The primer pairs used in this study are listed in Table 1, and loci of the primers are shown in Fig. 1. *TaKaRa Ex Taq* Hot start version (Takara Bio) was used for PCR, except for amplifying the total region of the Tn916-like element, for which *TaKaRa LA Taq* Hot start version was used. The Tn-upstream and -downstream regions were amplified by inverse PCR (Ochman et al. 1988). In order to increase the specificity of the inverse PCR products, nested PCR was carried out. The PCR was performed on C1000 Thermal Cycler (Bio-Rad).

**Southern blot hybridization**

Southern blot hybridization was carried out with DIG-labeled Tn916-like element as a probe and detection system (Roche). Chromosomal DNAs (1.5 μg) extracted from serotype II strains of *Strep. parauberis* and *Ent. faecalis* CG110 were digested to completion at 37°C with *Hin*II, *Hind*III or
Sau3AI (Takara Bio). The digested DNAs were electrophoresed on 0.7% agarose gel and stained with ethidium bromide. After depurination, denaturation and neutralization of the gel, DNAs were transferred to Hybond N+ membrane (GE Healthcare) by capillary blotting overnight. Hybridization and washings were under stringent conditions, and chemiluminescent reaction was carried out with AP-labeled anti-DIG antibody and CDP-Star (Roche).

**Sequencing analysis of the Tn916-like element and flanking region**

PCR products (Tn A, B, C and D) and inverse PCR products (Tn-upstream 2 and Tn-downstream 2) from *Strep. parauberis* NUF1049 (serotype II) as well as the DNA sequence of *Strep. parauberis* NUF1003 (serotype I) corresponding to the insertion site of the Tn916-like element were cloned into pGEM-T Easy vector (Promega) and transformed into *E. coli* JM109 competent cells (Promega).

DNA sequencing was carried out with BigDye Terminator v3.1 Cycle Sequencing kit and ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The sequences were assembled and analyzed using DNASIS program (Hitachi Software Engineering) and BLAST at the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov/BLAST). Alignment program ClustalW2 (www.ebi.ac.uk/Tools/clustalw2) was used for DNA and protein multiple sequence alignment.
**Conjugative transfer experiments**

Filter mating was performed with logarithmic-phase broth cultures of six *Strep. parauberis* serotype II strains (one strain per prefecture) as donors and *Ent. faecalis* FA2-2 as recipient. Mixture of donor and recipient cells at a ratio of one donor (50 µl) per 10 recipients (500 µl) was collected on a sterilized membrane filter (0.45 µm) (Millipore), and the filter was incubated on a 5% horse blood agar plate at 28°C overnight. Transconjugants were selected on TH agar plates containing oxytetracycline (8 µg ml⁻¹), rifampicin (25 µg ml⁻¹) and fusidic acid (25 µg ml⁻¹).

**Nucleotide sequence accession number**

The nucleotide sequences of the Tn916-like element and flanking region of the strain NUF1049 are available in the DDBJ, EMBL and GenBank nucleotide database with the accession number AB468159.

**Results**

**Screening for the Tn916-like element among Strep. parauberis serotype II strains**

Approximately 4.5-kbp PCR products were obtained with the primer pair of *tet*(M)-FW and *int*-RV for all of the serotype II strains. Analysis of the nucleotide sequences of the PCR products from two
strains showed that their sequences were identical to the amplicon tet(M)-int of Tn916 from *Ent. faecalis* DS16 (Flannagan et al. 1994; GenBank Accession No. U09422).

To amplify the total region of the Tn916-like element long and accurate (LA) PCR was carried out with the primer pair of Tn916-FW and Tn916-RV and chromosomal DNAs from serotype II strains as template. Positive amplifications, the sizes of which were the same as that from *Ent. faecalis* CG110, the positive control of Tn916, were obtained for all of the serotype II strains (data not shown).

**Nucleotide Sequence analysis of the Tn916-like element**

Positive amplifications were also obtained for all of the serotype II strains when four primer pairs based on the four overlapped fragments (Tn A to D) of Tn916 were used. The PCR product of each fragment from *Strep. parauberis* NUF1049 was cloned and sequenced. The entire nucleotide sequence of the Tn916-like element was found to be 18,031 bp in length and showed high homology with Tn916 or Tn916-like transposons from *Strep. agalactiae* (Accession No. AE00948, identity=99.96%), *Ent. feacalis* plasmid pCF10 (AY855841, 99.93%), *Ent. faecalis* DS16 (U90422, 99.80%) and *Strep. suis* SC84 (EF432727, 99.37%).
Restriction endonuclease analysis of the Tn916-like element in *Strep. parauberis* serotype II strains

All of the serotype II strains showed the same *Sau*3A- and *Hind*III-digestion patterns as Tn916 with DIG-labeled Tn916-like element as the probe (data not shown). However, *Hinc*II-digestion patterns were diverse, that is, two patterns were observed among serotype II strains (Fig. 2). Only one strain derived from Kagoshima Prefecture out of 32 serotype II strains showed Pattern 1, which consisted of 5.6-, 4.9-, 3.0-, 1.7-, 1.2-, 0.9- and 0.4-kbp *Hinc*II fragments like Tn916, whereas the rest 31 strains showed Pattern 2 that contained 10.5-kbp fragment instead of 5.6- and 4.9-kbp fragments. The nucleotide sequence analysis revealed that *Hinc*II-restriction site of the 31 strains between 5.6- and 4.9-kbp fragments was modified to GTCACC.

Characterization of the flanking regions and termini of the Tn916-like element

*Hind*III-restriction analysis suggested that the Tn916-like element of serotype II strains was inserted in the same chromosomal location with the same direction, since 15.2- and 7.2-kbp fragments were obtained from all the serotype II strains (32 strains) (data not shown). Sequence analysis of the flanking regions showed that the insertion site of the Tn916-like element was an A-T rich region (Fig. 3). GenBank ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and homology search revealed that the amino acid sequences coded by putative CDSs (coding sequences) of ORFs Sp-O1,
-O2 and -O3 have 68%, 72% and 84% identity to transcriptional regulator and dihydroxyacetone kinase (Accession No.CP000262) and threonyl-tRNA synthetase (AE014074) from Strep. pyogenes, respectively. Insertion of the Tn916-like element did not interrupt any ORFs of chromosomal DNA of Strep. parauberis serotype II strains.

To verify whether the Tn916-like element was inserted in the same site among serotype II strains PCR was conducted with the left and right terminus primer sets (Table 1). The same-sized PCR products were obtained respectively at the left and right terminus regions for all of serotype II strains, indicating that the Tn916-like element was inserted in the uniform location in these strains.

To determine the structure of the insertion site the corresponding region of the representative serotype I strain, NUF1003, was amplified with the flanking region primer set and sequenced. The result showed that 6-bp sequence, ATCATA, was generated in the serotype II strain adjacent to the left end of the Tn916-like element in the integrated state (Fig. 3). The 6-bp sequence, TATATA, at the right side represented the initial target sequence for the insertion of the Tn916-like element.

**Conjugative transfer of the Tn916-like element**

In conjugative transfer experiment no transconjugants were obtained when six Strep. parauberis serotype II strains from different prefectures were used as donors and Ent. faecalis FA2-2 as the recipient. Transfer frequency was calculated to be less than 10⁻⁹.
Discussion

The present study demonstrated that the Tn916-like element that exhibited high structural homology with Tn916 or Tn916-like transposons from the other streptococci and enterococci were observed in all of examined Strep. parauberis serotype II strains isolated from diseased Japanese flounder. Restriction endonuclease analysis suggested the presence of some diversity in the nucleotide sequence within the strains, but the Tn916-like element was integrated in the same site of chromosome for all of the strains. To our knowledge it is the first case that all the strains of a particular group of bacterial species harbored a Tn916 family transposon.

Tn916 family conjugative transposons have been found in a broad variety of Gram-positive and Gram-negative organisms and become important vehicles for disseminating antimicrobial resistance through cell to cell contact (Rice 1998). In this study, however, horizontal transfer of the Tn916-like element was failed. Conjugative transposition of Tn916 is via an excision-insertion mechanism with three stages; excision, conjugal transfer and integration (Caparon et al. 1989). Excision begins with generating single strands of 6-bp termed coupling sequences flanking the transposon in the donor DNA (Scott, 1992). The nucleotide content of the coupling sequences plays a frequency-determining role upon excision of Tn916 with unknown mechanism (Jaworski and Clewell, 1994). Therefore, conjugative transposition of Tn916 occurred at widely differing frequencies ranging from less than $10^{-8}$ to greater than $10^{-4}$ per donor depending on the coupling sequences (Jaworski and Clewell,
In this study, the sequencing results of the chromosomal region where the Tn916-like element was integrated provided evidence that the Tn916-like element introduced a coupling sequence (ATCATA) from its previous host. Accordingly, the 6-bp sequences adjacent to the Tn916-like element (ATCATA and TATATA) may result in its extreme low transfer frequency.

Although serotypes were not documented, a high rate of tetracycline resistance due to tet(M) was also reported in Korean isolates of Strep. parauberis (Park et al. in press). According to our findings it is likely that the prevalence of the tet(M)-positive serotype II strains in Japan are due to clonal dissemination of a original strain that acquired the Tn916-like element from another bacterial species rather than selection of tet(M)-positive strains by frequent use of tetracyclines. This hypothesis is supported by the fact that all of the tested serotype II strains were also low resistant to erythromycin (Meng et al. in press). It is thinkable that excision of the Tn916-like element from these strains is an extremely rare event resulting in all of the serotype II isolates possessing the element.

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Franke, A. E. and Clewell, D. B. (1981) Evidence for a chromosome-borne resistance transposon (Tn916) in *Streptococcus faecalis* that is capable of "conjugal" transfer in the absence of a


**Figure 1** Sequencing strategy of Tn916-like element (A) and flanking regions (B) in HindIII-digested fragments. Arrows indicate the position and direction of primers.
Figure 2 Southern blot hybridization analysis of the Tn916-like element. Chromosomal DNAs were digested with *Hinc*II. The digests on the membrane were hybridized with DIG-labeled Tn916-like element probe. Lane 1, Pattern 1 of serotype II strain; Lane 2, Pattern 2 of serotype II strain; Lane 3, *Ent. faecalis* CG110 (positive control of Tn916).
Figure 3 (A) Genetic structure of the chromosomal region where the Tn916-like element was integrated. The gray areas indicate identical structure between *Strep. parauberis* NUF1003 (serotype I) and NUF1049 (serotype II). (B) Flanking sequences adjacent to the Tn916-like element. Coupling sequence is indicated by boldface and underline.
<table>
<thead>
<tr>
<th>Amplicon</th>
<th>Primer designation</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Source</th>
<th>Annealing temperature (°C)</th>
</tr>
</thead>
</table>
| tet(M)-int * | tet(M)-FW  
int-RV | GTAAATAGTGTTCTTGGAG  
CCATAGGAACCTTGCAGTTCG | GenBank (U90422) | 57 |
| Tn916 * | Tn916-FW  
Tn916-RV | CTGGCCAGGATAAAGTCTGTCAGCG  
CAAACTAGGGATTGATGTGTGTCG | GenBank (U90422) | 68 |
| Tn A | Tn916-FW  
Tn2-AR | CTGGCCAGGATAAAGTCTGTCAGCG  
CGTCCACGCTACCATACATCATCAC | GenBank (U90422) | 56 |
| Tn B | TnB-1F  
Tn2-BR | CTTCATAGCCGAACTCAATC  
GAGTGATCTGCTTGTTGGTGCAG | GenBank (U90422) | 61 |
| Tn C | TnC-1F  
TnC-1R | CGATTCTCAAARGTGGGAAAG  
CCAAAGAATTTATATCTTC | GenBank (U90422) | 61 |
| Tn D | tet(M)-FW  
TnE-1R | GTAAATAGTGTTCTTGGAG  
GTACCTGCTATATGCTGTC | GenBank (U90422) | 65 |
| Tn-upstream 1† | Tn-10F  
Tn-12R | CATCCATACGCAAGCCAGTGAACCTTC  
GCTGGCAAACTTACGATCAGC | this study | 50 |
| Tn-upstream 2‡ | Tn-11F  
Tn-5R | GTATCGTCAAGATGGGTATACCTGCAG  
TTCTCCGCTGAAACCTACCTGGCAG | this study | 63 |
| Tn-downstream 1† | Tn-1F  
Tn-4R | GACCGAATCTAGCAAGCAGCCAAAGGTTCTTG  
CATTCACCTTCCCCAAGGGGTGATAC | this study | 50 |
| Tn-downstream 2‡ | Tn-2F  
Tn-3R | GCCACACATCACTCCACATAGTTTGCGACA  
TTCCGCAAAGATTCTGACGACCTCGATGTG | this study | 60 |
| Left terminus | Tn-11F-4F  
Tn-5R | GAAATACACCAATAATCTCG  
TTCTCCGCAGATGCTTACCTGGCAG | this study | 60 |
| Right terminus | Tn-2F  
Tn-3R-2R | GCCACACATCAGCAGCATATGTGGTCGA  
TTCCGCAAAGATTCTGACGACCTCGATGTG | this study | 60 |
| Flanking region | Tn-11F-4F  
Tn-3R-2R | GAAATACACCAATAATCTCG  
TTCCGCAAAGATTCTGACGACCTCGATGTG | this study | 56 |

* Also used to obtain a specific probe.
† Primers used for inverse PCR.
‡ Primers used for nested PCR.