# Structural characterization of Tn*916*-like element in *Streptococcus parauberis* serotype II strains isolated from diseased Japanese flounder

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Running title: Tn*916*-like element in *Strep. parauberis*

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**Abstract**

**Aims:** To screen for the existence and determine the structure of Tn*916*-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 Tn*916*-like element and the flanking regions were characterized by polymerase chain reaction (PCR) and inverse PCR, followed by cloning and sequencing. The Tn*916*-like element is 18,031 bp in length and composed of 22 ORFs. Southern blot hybridization analysis showed that the *Hin*cII-digested internal structures of Tn*916*-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 Tn*916*-like element exhibited high homology (more than 99%) with Tn*916* 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 Tn*916*-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, Tn*916*

## 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 *tet*(M), *xis* (excisase) and *int* (integrase) genes which are components of Tn*916* family transposons (Clewell *et al*. 1995). So it is supposed that *Strep. parauberis* serotype II strains possessed Tn*916*-related genetic elements.

*tet*(M) gene, a tetracycline resistance determinant of ribosomal protection, is the most common in both Gram-positive and Gram-negative bacteria (Roberts 2005). Tn*916,* which harbored *tet*(M) gene, was first documented as a conjugative transposon detected on the chromosome of *Enterococcus faecalis* DS16 (Franke and Clewell 1981). Since then, Tn*916* 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, Tn*916* family transposons have acquired additional resistance determinants, such as Tn*1545* [bearing *erm*(B) and *aphA-3*], Tn*6002* [bearing *erm*(B)] and Tn*6003* [bearing *erm*(B), *aadE*, *sat4* and *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 Tn*916*-related element by analyzing its total DNA sequence on the chromosome of a *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 Tn*916*-related element on the chromosome by comparing with the corresponding region of a serotype I strain. Because the Tn*916*-related element was revealed to have a high similarity with Tn*916*, we would like to use Tn*916*-like element instead of Tn*916*-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 Tn*916*

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℃ 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 Tn*916*-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 Tn*916*-like element as a probe and detection system (Roche). Chromosomal DNAs (1.5 $\mu$g) extracted from serotype II strains of *Strep. parauberis* and *Ent. faecalis* CG110 were digested to completion at 37°C with *Hin*cII, *Hin*dIII or

*Sau*3AI (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 Tn*916*-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 Tn*916*-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 $\mu$l) per 10 recipients (500 $\mu$l) was collected on a sterilized membrane filter (0.45 $\mu$m) (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 $\mu$g ml$^{-1}$), rifampicin (25 $\mu$g ml$^{-1}$) and fusidic acid (25 $\mu$g ml$^{-1}$).

**Nucleotide sequence accession number**

The nucleotide sequences of the Tn*916*-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 Tn*916*-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 Tn*916* from *Ent. faecalis* DS16 (Flannagan *et al*. 1994; GenBank Accession No. U09422).

To amplify the total region of the Tn*916*-like element long and accurate (LA) PCR was carried out with the primer pair of Tn*916*-FW and Tn*916*-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 Tn*916*, were obtained for all of the serotype II strains (data not shown).

**Nucleotide Sequence analysis of the Tn*916*-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 Tn*916* were used. The PCR product of each fragment from *Strep. parauberis* NUF1049 was cloned and sequenced. The entire nucleotide sequence of the Tn*916*-like element was found to be 18,031 bp in length and showed high homology with Tn*916* or Tn*916*-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 Tn*916*-like element in *Strep. parauberis* serotype II strains**

All of the serotype II strains showed the same *Sau*3A- and *Hin*dIII-digestion patterns as Tn*916* with DIG-labeled Tn*916*-like element as the probe (data not shown). However, *Hin*cII-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 *Hin*cII fragments like Tn*916*, 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 Tn*916*-like element**

*Hin*dIII-restriction analysis suggested that the Tn*916*-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 Tn*916*-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 Tn*916*-like element did not interrupt any ORFs of chromosomal DNA of *Strep. parauberis* serotype II strains.

To verify whether the Tn*916*-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 Tn*916*-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 Tn*916*-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 Tn*916*-like element.

**Conjugative transfer of the Tn*916*-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 Tn*916*-like element that exhibited high structural homology with Tn*916* or Tn*916*-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 Tn*916*-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 Tn*916* family transposon.

Tn*916* 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 Tn*916*-like element was failed. Conjugative transposition of Tn*916* 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 squences flanking the transposon in the donor DNA (Scott, 1992). The nucleotide content of the coupling sequences plays a frequency-determining role upon excision of Tn*916* with unknown mechanism (Jaworski and Clewell, 1994). Therefore, conjugative transposition of Tn*916* 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,

1994). In this study, the sequencing results of the chromosomal region where the Tn*916*-like element was integrated provided evidence that the Tn*916*-like element introduced a coupling sequence (ATCATA) from its previous host. Accordingly, the 6-bp sequences adjacent to the Tn*916*-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 Tn*916*-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 Tn*916*-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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**References**


Caillaud, F., Carlier, C. and Courvalin, P. (1987) Physical analysis of the conjugative shuttle transposon Tn*1545*. *Plasmid* **17**, 58-60.

Caparon, M. G. and Scott, J. R. (1989) Excision and insertion of the conjugative transposon Tn*916* involves a novel recombination mechanism. *Cell* **59**, 1027-1034.

Clewell, D. B., Flannagan, S. E. and Jaworski, D. D. (1995) Unconstrained bacterial promiscuity: The Tn*916*-Tn*1545* family of conjugative transposons. *Trends Microbiol* **3**, 229-236.

Cochetti, I., Tili, E., Mingoia, M., Varaldo, P. E. and Montanari, M. P. (2008) *erm*(B)-carrying elements in tetracycline-resistant pneumococci and correspondence between Tn*1545* and Tn*6003*. *Antimicrob Agents Chemother* **52**, 1285-1290.

Doménech, A., Fernández-Garayzábal, J. F., Pascual, C., Garcia, J. A., Cutuli, M. T., Moreno, M. A., Collins, M. D. and Dominguez, L. (1996) Streptococcosis in cultured turbot, *Scopthalmus maximus* (L.), associated with *Streptococcus parauberis*. *J Fish Dis* **19**, 33-38.

Flannagan, S. E., Zitzow, L. A., Su, Y. A. and Clewell, D. B. (1994) Nucleotide sequence of the 18-kb conjugative transposon Tn*916* from *Enterococcus faecalis*. *Plasmid* **32**, 350-354.

Franke, A. E. and Clewell, D. B. (1981) Evidence for a chromosome-borne resistance transposon (Tn*916*) in *Streptococcus faecalis* that is capable of "conjugal" transfer in the absence of a

conjugative plasmid. *J Bacteriol* **145**, 494-502.

Jaworski, D. D. and Clewell, D. B. (1994) Evidence that coupling sequences play a frequency-determining role in conjugative transposition of Tn*916* in *Enterococcus faecalis*. *J Bacteriol* **176**, 3328-3335.

Kanai, K., Yamada, M., Meng, F., Takahashi, I., Nagano, T., Kawakami, H., Yamashita, A., Matsuoka, S., Fukuda, Y., Miyoshi, Y., Takami, I., Nakano, H., Hirae, T., Shutou, K. and Honma, T. (2009) Serological differentiation of *Streptococcus parauberis* strains isolated from cultured Japanese flounder in Japan. *Fish Pathol* **44** (in press). (In Japanese with English abstract)

Mata, A. I., A. Gibello, A. Casamayor, M. M. Blanco, L. Dominguez and J. F. Fernandez-Garayzabal (2004): Multiplex PCR assay for detection of bacterial pathogens associated with warm-water streptococcosis in fish. *Appl. Environ. Microbiol.*, **70**, 3183-3187.

Meng, F., Kanai, K. and Yoshikoshi, K. (2009) Characterization of drug resistance in *Streptococcus parauberis* isolated from Japanese flounder. *Fish Pathol* **44** (in press).

Ochman, H., Gerber, A. S. and Hartl, D. L. (1988) Genetic applications of an inverse polymerase chain reaction. *Genetics* **120**, 621-623.

Park, Y. K., Nho, S. W., Shin, G. W., Park, S. B., Jang, H. B., Cha, I. S., Ha, M. A., Kim, Y. R., Dalvi, R. S., Kang, B. J. and Jung, T. S. (2008) Antibiotic susceptibility and resistance of *Streptococcus iniae* and *Streptococcus parauberis* isolated from olive flounder (*Paralichthys olivaceus*). *Vet*

*Microbiol* (in press).

Rice, L. B. (1998) Tn*916* family conjugative transposons and dissemination of antimicrobial resistance determinants. *Antimicrob Agents Chemother* **42**, 1871-1877.

Roberts, M. C. (2005) Update on acquired tetracycline resistance genes. *FEMS Microbiol Lett* **245**, 195-203.

Scott, J. R. (1992) Sex and the single circle: conjugative transposition. *J Bacteriol* **174**, 6005-6010.

Shimoji, Y., Yokomizo, Y., Sekizaki, T., Mori, Y. and Kubo, M. (1994) Presence of a capsule in *Erysipelothrix rhusiopathiae* and its relationship to virulence for mice. *Infect Immun* **62**, 2806-2810.

Shiojima, M., Tomita, H., Tanimoto, K., Fujimoto, S. and Y. Ike (1997) High-level plasmid-mediated gentamicin resistance and pheromone response of plasmids present in clinical isolates of *Enterococcus faecalis*. *Antimicrob Agents Chemother* **41**, 702-705.

Toranzo, A. E., Cutrín, J. M., Núñez, S., Romalde, J. L. and Barja, J. L. (1995) Antigenic characterization of *Enterococcus* strains pathogenic for turbot and their relationship with other Gram-positive bacteria. *Dis Aquat Org* **21**, 187-191.

Warburton, P. J., Palmer, R. M., Munson, M. A. and Wade, W. G. (2007) Demonstration of in vivo transfer of doxycycline resistance mediated by a novel transposon. *J Antimicrob Chemother* **60**, 973-980.

Williams, A. M. and Collins, M. D. (1990) Molecular taxonomic studies on *Streptococcus uberis* types I and II. Description of *Streptococcus parauberis* sp. nov. *J Appl Bacteriol* **68**, 485-490.

FIGURE LEGEND

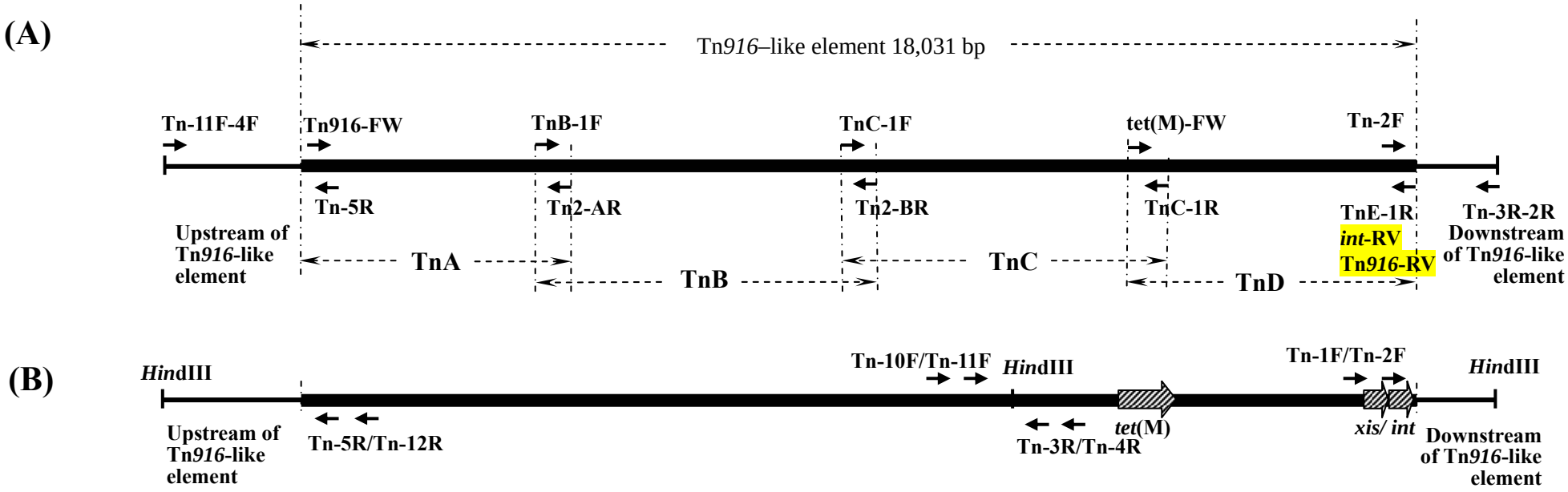


**Figure 1** Sequencing strategy of Tn*916*-like element (A) and flanking regions (B) in *Hin*dIII-digested fragments. Arrows indicate the position and direction of primers.

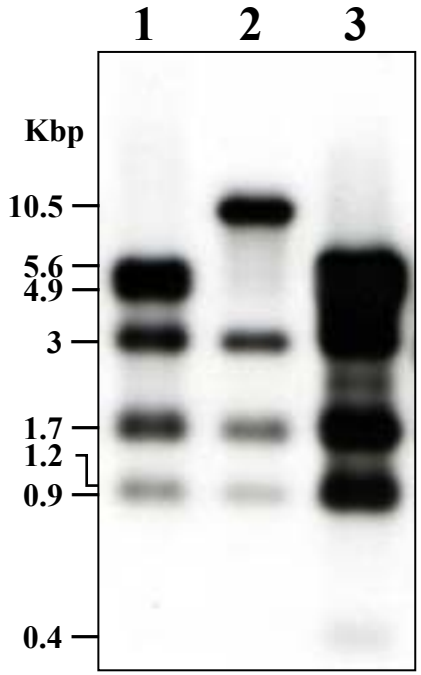


**Figure 2** Southern blot hybridization analysis of the Tn*916*-like element. Chromosomal DNAs were digested with *Hin*cII. The digests on the membrane were hybridized with DIG-labeled Tn*916*-like element probe. Lane 1, Pattern 1 of serotype II strain; Lane 2, Pattern 2 of serotype II strain; Lane 3, *Ent. faecalis* CG110 (positive control of Tn*916*).

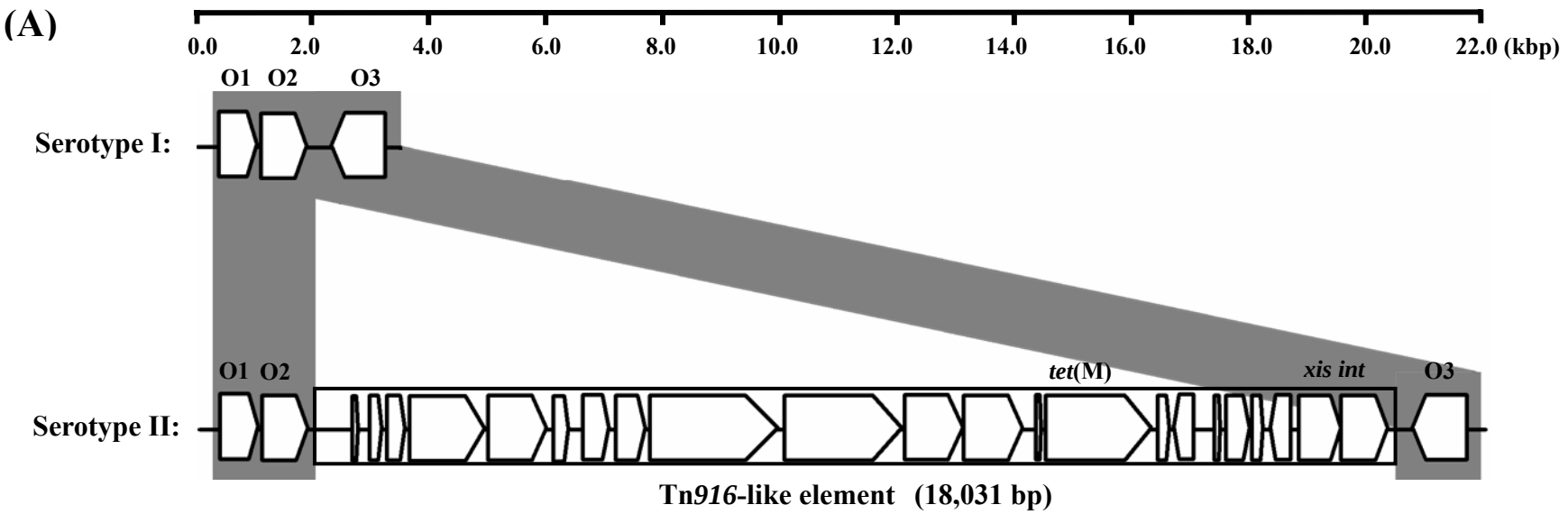


**Figure 3 (A)** Genetic structure of the chromosomal region where the Tn*916*-like element was integrated. The gray areas indicate identical structure between *Strep. parauberis* NUF1003 (serotype I) and NUF1049 (serotype II). **(B)** Flanking sequences adjacent to the Tn*916*-like element. Coupling sequence is indicated by boldface and underline.

**Table 1** Oligonucleotides used for PCR

| Amplicon | Primer designation | Primer sequence (5′ to 3′) | Source | Annealing temperature (°C) |
|---|---|---|---|---|
| *tet*(M)-*int* * | *tet*(M)-FW<br>*int*-RV | GTTAAATAGTGTTCTTGGAG<br>CCATAGGAACTTGACGTTCG | GenBank (U90422) | 57 |
| Tn*916* * | Tn*916*-FW<br>Tn*916*-RV | CTGGCGAGGATAAAGTCGTTCAGCG<br>CAAACTATGTGGAGTGATGTGTGGC | GenBank (U90422) | 68 |
| Tn A | Tn*916*-FW<br>Tn2-AR | CTGGCGAGGATAAAGTCGTTCAGCG<br>CGTGCCACGTCATACATATCATCAC | GenBank (U90422) | 56 |
| Tn B | TnB-1F<br>Tn2-BR | CTTCCATTGGCGAACTCAATC<br>GAGTGATCTGCTTGTGTCCTTGCAG | GenBank (U90422) | 61 |
| Tn C | TnC-1F<br>TnC-1R | CGATTCTCAAAGTGTGGGAAG<br>CCAAGAACACTATTTAACTTC | GenBank (U90422) | 61 |
| Tn D | *tet*(M)-FW<br>TnE-1R | GTTAAATAGTGTTCTTGGAG<br>GTCATGGCTATATTAGCATGTC | GenBank (U90422) | 65 |
| Tn-upstream 1† | Tn-10F<br>Tn-12R | CTATCCTACAGCGACAGCCAGTGAACTTTC<br>GCTGGCAGGAATACTTACTTGAATCATGCG | this study | 50 |
| Tn-upstream 2‡ | Tn-11F<br>Tn-5R | GTATCGCTGACAGTGGAGTATATCGACCAG<br>TTCTTCGCTGAACGACTTTATCCTCGCCAG | this study | 63 |
| Tn-downstream 1† | Tn-1F<br>Tn-4R | GACGCAATCTAGCGTCGCCAAAGGGTCTTG<br>CATTCCACTTCCCAACGGAAGCGGTGATAC | this study | 50 |
| Tn-downstream 2‡ | Tn-2F<br>Tn-3R | GCCACACATCACTCCACATAGTTTGCGACA<br>TTCCGCAAAGTTCAGACGGACCTCGATGTG | this study | 60 |
| Left terminus | Tn-11F-4F<br>Tn-5R | GAACATATCACCAATAATCTC<br>TTCTTCGCTGAACGACTTTATCCTCGCCAG | this study | 60 |
| Right terminus | Tn-2F<br>Tn-3R-2R | GCCACACATCACTCCACATAGTTTGCGACA<br>TTCCGCAAAGTTCAGACGGACCTCGATGTG | this study | 60 |
| Flanking region | Tn-11F-4F<br>Tn-3R-2R | GAACATATCACCAATAATCTC<br>TTCCGCAAAGTTCAGACGGACCTCGATGTG | this study | 56 |

* Also used to obtain a specific probe.

† Primers used for inverse PCR.

‡ Primers used for nested PCR.