Study on Appearance and Mechanism of Drug Resistance in Pathogenic Strains of Streptococcus parauberis from Japanese Flounder Paralichthys olivaceus

Author(s)
Meng, Fei

Citation
Nagasaki University (長崎大学) 博士 (学術) (2009-03-19)

Issue Date
2009-03-19

URL
http://hdl.handle.net/10069/22352

NAOSITE: Nagasaki University’s Academic Output SITE
http://naosite.lb.nagasaki-u.ac.jp
Study on Appearance and Mechanism of Drug Resistance in Pathogenic Strains of *Streptococcus parauberis* from Japanese Flounder *Paralichthys olivaceus*

December, 2008

Graduate School of Science and Technology

Nagasaki University

MENG Fei
Contents

Abbreviations ........................................................................................................ iv

Introduction ........................................................................................................ 5

Chapter 1. Serological differentiation and antimicrobial susceptibilities of \textit{S. parauberis} strains from Japanese flounder ........................................ 10

  Materials and methods .................................................................................. 12

  Results ............................................................................................................. 14

  Discussion ....................................................................................................... 15

  Conclusion ...................................................................................................... 17

Chapter 2. Screening for antimicrobial resistance and transposon-related genes ...... 22

  Materials and methods .................................................................................. 24

  Results ............................................................................................................. 25

  Discussion ....................................................................................................... 26

  Conclusion ...................................................................................................... 27

Chapter 3. Characterization of resistance determinants in \textit{S. parauberis} serotype I strains ..................................................................................................................... 32

  Materials and methods .................................................................................. 34

  Results ............................................................................................................. 36

  Discussion ....................................................................................................... 39

  Conclusion ...................................................................................................... 41

Chapter 4. Characterization of resistance determinants in \textit{S. parauberis} setotype II strains ..................................................................................................................... 48
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABPC</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>CP</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>EM</td>
<td>Erythromycin</td>
</tr>
<tr>
<td>KM</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>LCM</td>
<td>Lincomycin</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl 1-thio-β-D-galactoside</td>
</tr>
<tr>
<td>LB-broth</td>
<td>Luria-Bertani broth</td>
</tr>
<tr>
<td>MICs</td>
<td>Minimum inhibitory concentrations</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>OA</td>
<td>Oxolinic acid</td>
</tr>
<tr>
<td>OTC</td>
<td>Oxytetracycline</td>
</tr>
<tr>
<td>SMMX</td>
<td>Sulfamonomethoxine</td>
</tr>
<tr>
<td>Tn</td>
<td>Transposon</td>
</tr>
<tr>
<td>TH broth</td>
<td>Todd-Hewitt broth</td>
</tr>
<tr>
<td>TMP</td>
<td>Trimethoprim</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
</tbody>
</table>
Introduction
With increased development of intensive operations, disease has become a significant hurdle to the profitable culture of fish and shellfish. Aquaculture industries in Japan often suffer from severe diseases. For the treatment of bacterial infections in cultured fish, 25 approved chemotherapeutic agents have been used under the guidance of the Ministry of Agriculture, Forestry and Fisheries (Sano, 1998). Because of the extensive usage of chemotherapeutic agents, the prevalence of drug-resistant fish-pathogenic bacteria has caused economic loss in fish culture industry (Aoki, 1988).

Williams and Collins (1990) proposed a new species, *Streptococcus parauberis*, for the genotype II group of *S. uberis* an etiological agent of bovine mastitis. Doménech et al. (1996) was the first to identify *S. parauberis* as a fish pathogen by analyzing of the 16S rRNA of several isolates from turbot *Scophthalmus maximus*. Another group reported that this pathogen caused high economic losses in cultured turbot in Spain (Toranzo et al., 1995). Since then, streptococciosis caused by *S. parauberis* resulted in major losses and limited to the turbot and Japanese flounder *Paralichthys olivaceus* cultured in Spain and Korea, respectively (Toranzo et al., 2005; Baeck et al., 2006). *S. parauberis* has emerged as one of the main pathogenic species in Japanese flounder in Japan since 2002. Based on a serological investigation, two serotypes, I and II, were proposed in Japanese isolates of *S. parauberis* (Kanai et al., 2009).

In Japan, for the control of streptococcal infection in Japanese flounder, the use 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). Tetracyclines as broad spectrum agents have been widely used for disease treatment in aquaculture in Japan (Kusuda and Salati, 1993; Sano, 1998). However, the
worldwide use of tetracycline in the treatment of infections, as prophylactic agents, or as growth promoters, has facilitated the emergence and spread of acquired resistance (Roberts, 2005). The high frequency in detection of tetracycline-resistant bacteria has been reported among various fish pathogens and mariculture environments (Aoki and Takahashi, 1987; Kusuda and Salati, 1993; Sano, 1998; Nonaka and Suzuki, 2002; Kim et al., 2004; Maki et al., 2008). Occurrence of tetracycline resistance among bacterial species is commonly due to three principal mechanisms; 1) efflux of tetracyclines from cells, 2) ribosomal protection (RP) in which the tetracycline-binding site is allosterically disrupted, 3) presence of tetracycline-inactivating enzymes (Chopra and Roberts, 2001; Roberts, 2005). There are currently approximate 40 different tetracycline resistance determinants. In streptococci, the common tetracycline resistance determinants are encoded by the tet genes which are usually found in Gram-positive species. Of these tet genes reported, tet(K) and tet(L) were found to be the genes that code the proteins of efflux pump in cells and tet(M), tet(O) and tet(S) were reported to be responsible for RP (Chopra and Roberts, 2001; Roberts, 2005). Among these tet genes, tet(M) has the widest host range up to 42 genera. The association of tet(M), int (integrase) and xis (excisionase) genes characterizes the Tn916–Tn1545 family (Clewell et al., 1995).

Transferable drug resistances of fish-pathogenic bacteria have been emerged in multi-drug resistant strains. Aoki and his coworkers have suggested a considerable relationship between the increasing number of resistant strains and the indiscriminate use of chemotherapeutics in fish farms (Aoki, 1988). Drug resistance is commonly attributed to the presence of conjugative plasmids and/or conjugative transposons that have been regarded to be responsible for the drug resistance of various bacteria (Levy
and Marshall, 2004). R plasmids have played an important role in the dissemination of antimicrobial resistance genes in the bacterial population with horizontal gene transfer by conjugation. Tn916, which encodes resistance to tetracycline, was first documented as a conjugative transposon detected on the chromosome of Enterococcus faecalis DS16 (Franke and Clewell, 1981). Since then, Tn916 has undergone intensive investigation which 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 current information, Tn916-like elements have acquired additional resistance determinants, as well as have been integrated within larger multiple resistance elements (Caillaud et al., 1987; Rice and Carias, 1998). Tn916-Tn1545 family transposons and Tn916-like elements are currently disseminated with a broad host range (Roberts, 2005), and most contain tet(M) (Rice, 1998).

The occurrence and transferability of drug resistances in S. parauberis have not been performed. It is important to determine the antimicrobial susceptibility of a bacterial pathogen, particularly susceptibility to tetracyclines in the case of S. parauberis, for the treatment of diseases. The aim of this study was to characterize the antimicrobial susceptibilities of S. paraubeirs strains isolated from Japanese flounder in Japan. The resistant strains found were then screened to confirm the resistance determinants.

In the first chapter, the serological differentiation of the Japanese S. paraubeirs isolates were described, and their antimicrobial susceptibilities against nine antimicrobial compounds were also investigated. In the second chapter, based on the results of chapter 1, I detected the antimicrobial resistance genes and transposon-related genes. In the third chapter, I characterized the resistance genes harbored by the
serotype I resistance strains. The conjugal transferabilities of plasmids were also analyzed by filter mating assay. In the forth chapter, the characterization of Tn916-like element harbored by the serotype II strains was described. Since tet genes were observed in conjugative plasmid and transposon, the use of tetracyclines for the control of S. parauberis infection in Japanese flounder could result in the selection of the resistant strains and transfer of the genes to the other pathogens of animals including humans, being threat to public health.
Chapter 1

Serological differentiation and antimicrobial susceptibilities of *S. parauberis* strains from Japanese flounder
*Streptococcus parauberis*, a Gram-positive coccus, is known as a causative agent of mastitis in cows as well as streptococciosis in turbot *Scophthalmus maximus* and Japanese flounder *Paralichthys olivaceus*. It has recently become one of the most important pathogens of cultured Japanese flounder in the western part of Japan.

In this chapter I investigated the serological nature of *S. parauberis* strains from Japanese flounder. It is important to determine the antimicrobial susceptibility of a bacterial pathogen, particularly susceptibility to tetracyclines in the case of *S. parauberis*, for the treatment of diseases. Since it has not been documented the resistance of *S. parauberis* strains in Japan, the minimum inhibitory concentrations (MICs) of nine antimicrobial agents were determined against the *S. parauberis* strains isolated from Japanese flounder.
Materials and Methods

Bacterial strains and culture media

Sixty-four *S. parauberis* strains isolated from Japanese flounder between 2002 and 2007 at commercial aquaculture sites in the western districts of Japan were used in this study (Table 1-1). *S. parauberis* strains were grown in Todd Hewitt (TH, Difco) broth or agar. Stock cultures were stored at −80°C in TH broth containing 10 % (v/v) glycerol.

Identification of *S. parauberis* by PCR

Identification of the *S. parauberis* strains was performed by PCR amplification of the partial 23S rDNA with the primer pair: S.para-F (5' TTTCGTCTGAGGCAATGTT G 3') and S.para-R (5' GCTTCATATATCGCTATACT 3') (Mata *et al*., 2004). PCR reaction mixture for detection of the 23S rDNA of *S. parauberis* contained colony of *S. parauberis*, 0.5 μM of each primer, 5 μL of 10 × *Ex Taq* buffer (Mg²⁺ plus), 4μL of dNTP mixture (0.2 mM each) and 1.25 U of *Ex Taq* DNA polymerase (Hot Start Version, TaKaRa) up to a total volume of 50 μL by adding deionized distilled water. PCR amplification was conducted in a thermal cycler and utilized denaturation for 2 min at 95°C. Then, the PCR mixture was subjected to 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 45 s. A final extension was performed at 72°C for 10 min. The amplified products were analyzed by electrophoresis on a 0.7% agarose gel and stained with ethidium bromide.
Rabbit antisera and serotyping

One milliliter of suspension containing 50 mg formalin-killed cells (FKC) of *S. parauberis* KRS-02083 (serotype I) or KRS-02109 (serotype II) was emulsified with an equal volume of Freund's complete adjuvant (Wako) and injected subcutaneously to a Japanese white rabbit twice with a 2-week interval. Two weeks after the second injection 50 mg FKC without adjuvant was injected intraperitoneally to the rabbit. Total blood was obtained when the agglutination titer rose to an appropriate value. Antisera were heated for 30 min at 56°C and stored at -30°C. Serotyping was carried out by slide agglutination test with anti-*S. parauberis* serotype I and II rabbit antisera.

Determination of minimum inhibitory concentration

Minimum inhibitory concentrations (MICs) of antimicrobial agents were determined by the agar dilution method (Japanese Society of Antimicrobials for Animals, the Committee, 2003) using sensitivity test broth (Nissui, Tokyo, Japan) containing 1.5% agar. The following nine antimicrobial agents were tested: ampicillin (ABPC), chloramphenicol (CP), erythromycin (EM), kanamycin (KM), lincomycin (LCM), oxolinic acid (OA), oxytetracycline (OTC), sulfamonomethoxine (SMMX) and trimethoprim (TMP). The MIC values were determined after incubation at 28°C for 20 h. Final concentrations of drugs were from 0.016 to 512 µg/mL for ABPC and EM and from 0.125 to 512 µg/mL for CP, KM, LCM, OA, OTC, SMMX and TMP.
Results

Identification of *S. parauberis* strains

All of the strains gave the expected 718bp-PCR amplification product of 23S rDNA specific to *S. parauberis*.

Agglutination reactions of the strains

Sixty-four *S. parauberis* strains isolated from Japanese flounder were serologically divided into two groups, designated serotype I and II, by agglutinating reactions against two kinds of antiserum used in this study. Of the 64 strains, 44 and 20 were found to belong to serotype I and II, respectively (Table 1-1).

Drug susceptibilities

Fig. 1-1 shows the susceptibilities of the 64 strains of *S. parauberis* to nine antimicrobial agents. The exhibitions of MIC distributions were termed as susceptible, intermediately resistant and highly resistant. The MICs of all strains were shown to be one group for CP, KM and TMP as susceptible, while for OA and SMMX as resistant. *S. parauberis* strains exhibited an intrinsic resistance to OA and SMMX. The MICs of both ABPC and LCM presented two groups, 59 strains were susceptible with MIC values of $\leq 0.016$ to $0.063 \, \mu g/mL$ and $0.5$ to $1 \, \mu g/mL$, and five strains were low resistant with MIC values of $0.5$ to $1 \, \mu g/mL$ and $4 \, \mu g/mL$, respectively.
The MICs of EM ranged from $\leq 0.063$ to 512 $\mu$g/mL, and the MICs of OTC ranged from 0.5 to 128 $\mu$g/mL, respectively. For EM and OTC, the MICs of the 64 strains presented three groups, that is, 39 strains were susceptible, whereas 20 strains were intermediately resistant and five strains were highly resistant. The highly resistant strains were the ABPC/LCM-low resistant strains mentioned above. The EM/OTC-intermediately resistant group consisted with serotype II strains, while the EM/OTC-highly resistant group consisted with serotype I strains. Additionally, OTC-susceptible strains exhibited two peaks; the strains derived from Shikoku Island (Kagawa and Ehime Prefectures) showed a lower MIC value against OTC (0.5 $\mu$g/mL) than those from Kyushu Island (Oita Prefecture) (1 to 2 $\mu$g/mL).

**Discussion**

*S. parauberis* causes a chronic infection and is associated with large-scale mortality in wild and cultured trout and Japanese flounder (Toranzo et al. 1995; Baeck et al., 2006; Kanai et al., 2009). Clinical signs of the diseased Japanese flounder observed in Japanese cases are hemorrhages in mouth, on the operculum and in the trunk muscle as well as gill necrosis, and internal signs which are the distended abdomen with hepatomegaly (Fig1-2). These signs were similar to those of Korean diagnosis except for bilateral exophthalmia. Toranzo et al. (1995) reported that all of the *S. parauberis* strains from turbot were serologically homogeneous. In this study, 64 Japanese *S. parauberis* isolates derived from Japanese flounder were divided into two serotypes, type I and II, and it was found that the two types were distributed in the western Japan. There are no reports describing the serological nature of Korean *S. parauberis* isolates.
Hence, it is needed to study the serological relationship between Japanese, European and Korean isolates of *S. parauberis*.

MIC values of susceptible strains against ABPC (\( \leq 0.016 \) to 0.063 \( \mu \text{g/mL} \)), EM (\( \leq 0.016 \) to 0.063 \( \mu \text{g/mL} \)) and OTC (0.5 to 2 \( \mu \text{g/mL} \)) were comparable to those of *S. iniae*, another important pathogen of streptococcosis in Japanese flounder, whose MIC values were reported to be 0.00625 to 0.025 \( \mu \text{g/mL} \), 0.05 \( \mu \text{g/mL} \) and 0.39 \( \mu \text{g/mL} \), respectively (Sako, 1993). They not only showed similar MIC values as *S. parauberis* but also exhibit an intrinsic resistance to OA and SMMX. It is interesting that MIC values for the susceptible strains derived from Shikoku Island (Kagawa and Ehime Prefectures) against OTC were somewhat lower than those of Kyushu Island (Oita Prefecture). These differences in susceptibility to OTC may reflect variations in the history of tetracycline consumption in each geographical area.

There has been only a short history of using OTC to control the streptococcal infections caused by *S. parauberis* in cultured Japanese flounder since the outbreak of this disease in the beginning of 21 century. However, this chapter showed that the resistant strains for EM and OTC, which is the only qualified drug in treating of streptococcal infection Japanese flounder, have spread among *S. parauberis* serotype II strains in different sites. The long-term use of OTCs to control other streptococcal infections may have contributed to higher rates of antimicrobial resistance in *S. parauberis*. But in our study the effectiveness of the common drug, such as ABPC, was proved for *S. parauberis* infection therapy. In the previous studies, *S. iniae* strains from Japanese flounder were observed to be susceptible to EM/OTC (Sako, 1993), and 17.8% and 14.3% of 370 *Lactococcus gavieae* isolates from yellowtail *Seriola quinqueradiata* were reported to be intermediately or highly resistant to EM and OTC,
respectively (Aoki et al, 1990). In comparison with them, a serious problem was present in treatment of streptococcal infections caused by serotype II strains, because in the study all of the S. parauberis serotype II strains were intermediately resistant to OTC (Fig. 1-1).

**Conclusion**

1. Japanese S. parauberis isolates from Japanese flounder were divided into two serological phenotypes.
2. S. parauberis has an instinct for resistance to SMMX and OA.
3. Five strains of serotype I had low resistance to penicillin (0.5 to 1 µg/mL) and LCM (4 µg/mL) as well as highly resistance to EM (512 µg/mL) and OTC (128 µg/mL).
4. All of serotype II strains were EM (0.25 to 1 µg/mL) and OTC (32 µg/mL) intermediately resistant.
### Table 1-1. Number of *Streptococcus parauberis* strains used in this study

<table>
<thead>
<tr>
<th>Prefecture</th>
<th>Serotype</th>
<th>2002</th>
<th>2003</th>
<th>2004</th>
<th>2005</th>
<th>2006</th>
<th>2007</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kagawa</td>
<td>I</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Ehime</td>
<td>I</td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>17</td>
<td></td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oita</td>
<td>I</td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td></td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Kagoshima</td>
<td>I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Nagasaki</td>
<td>I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>13</td>
<td>2</td>
<td>5</td>
<td>29</td>
<td>9</td>
<td>6</td>
<td>64</td>
</tr>
</tbody>
</table>
**Fig. 1-1.** MIC distributions of *Streptococcus parauberis*.
Black bar, *S. parauberis* serotype I strains (n=44); Striped bar, *S. parauberis* serotype II strains (n=20). ABPC, ampicillin; CP, chloramphenicol; EM, erythromycin; KM, kanamycin; LCM, lincomycin; OA, oxolinic acid; OTC, oxytetracycline; SMMX, sulfamonomethoxine; TMP, trimethoprim.
Fig.1-2. Gross pathology of Japanese founder suffering from *Streptococcus parauberis* infection; gill necrosis (A) and hemorrhaging in the trunk muscle (B).
Chapter 2
Screening for antimicrobial resistance and transposon-related genes
In the previous chapter, 5 out of the 44 serotype I strains were highly resistant to EM/OTC, while all of serotype II strains were intermediately resistant to EM/OTC. Comparing with *S. iniae* (Sako, 1993), the relative high proportion of the isolates of *S. parauberis* were found to be resistant to tetracycline (39% of the examined isolates).

As broad-spectrum agents, tetracyclines have been extensively used in human and veterinary chemotherapy. Furthermore, tetracyclines are still used as growth promoters in some countries (Roberts, 2005). Conjugative plasmid and/or transposons encoding drug resistance genes usually play important roles for acquisition of new genes in most bacteria (Roberts, 2005). In multiple antimicrobial resistant streptococci, tetracycline-resistant strains in association with these mobile elements sometimes contain macrolide resistance gene. Some of macrolide resistance genes in streptococci can also occur on other mobile elements, such as conjugative plasmids or member of Tn916 or Tn917 family transposons.

In this chapter, the resistance and transposon-related genes were characterized in *S. parauberis* strains.
Materials and methods

Strains, culture and reagents

Five serotype I EM/OTC-resistant strains and all of the serotype II strains (n=20) identified as resistant strains were used for investigation of antimicrobial resistance genes. Enterococcus faecalis CG110 containing Tn916 (Shimoji et al., 1994) was used as a positive control for investigating the presence of Tn916 family transposons by PCR and Southern blot hybridization. S. parauberis and E. faecalis strains were grown in Todd-Hewitt broth (THB; Difco). Escherichia coli JM109 strain was grown in Luria-Bertani (LB) broth. Drugs were used at the following concentrations: 100 µg/mL for ampicillin, 50µg/mL for X-gal and 0.1mM for IPTG. Chromosomal and plasmid DNA of each strain were prepared using Wizard Genomic DNA Purification Kit (Promega) and QIAprep miniprep kit respectively, according to the manufacturer's protocols.

Screening for antimicrobial resistance and transposon-related genes

Antimicrobial resistance and transposon-related genes were screened by PCR. Oligonucleotide primer sets and PCR condition for detection of each gene are listed in Table 2-1. Macrolide and tetracycline resistance genes were tested on all resistant strains. They were also examined for the presence of int (integrase), xis (excisionase), tnpA (transposase) and tnpR (resolvase); the former two genes were associated with conjugative transposons Tn916-Tn1545 family and the latter two were associated with Tn917 (Shaw and Clewell, 1985). PCR was performed using 50 ng of chromosomal or plasmid DNA as template, 0.5 µM of each primer, 5 µL of 10 × Ex Taq buffer (Mg²⁺ plus), 4 µL of dNTP mixture (0.2 mM each) and 1.25 U of Ex Taq DNA polymerase (Hot Start Version, TaKaRa) up to a total volume of 50 µL by adding deionized and distilled water. The reaction condition varied according to the primers used and the size of product.
Sequence analysis

Various (nested) PCR products were cloned into the pGEM®-T Easy vector (Promega) and transformed into E.coli JM109 Competent Cells (Promega). Plasmid DNA was purified with QIAprep Spin Miniprep Kit (QIAGEN). DNA sequencing was carried out with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and performed on the ABI 3100 DNA sequencer (Applied Biosystems) in Center for Frontier Life Sciences, Nagasaki University. Sequenced data were then assembled and analyzed using DNASIS program (Hitachi Software) and BLAST at the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov./BLAST). Alignment program ClustalW2 was used for DNA and protein multiple sequence alignment (Larkin et al., 2007).

Results

Detection of genes

\textit{erm}(A), \textit{erm}(C) or \textit{mef}(A) was not detected in 25 EM-intermediately and highly resistant strains, while the five EM-highly resistant strains gave an expected 224 bp PCR amplification product of \textit{erm}(B) (Fig. 2-1). Tn917-related genes, \textit{tnpA} or \textit{tnpR}, were not detected in any of the EM-resistant strains (Table 2-2).

Among 25 OTC-resistant strains, \textit{tet}(M) and \textit{tet}(S) genes were detected in 20 serotype II and five serotype I highly resistant strains, respectively. \textit{int} and \textit{xis} genes of the Tn916 family were also detected in 20 serotype II strains (Fig. 2-1; Table 2-2). All of the 20 serotype II strains yielded the same PCR reactions for \textit{int} and \textit{xis} genes as \textit{E. faecalis} CG110 strain, the positive control of Tn916.

Sequencing analysis

Sequence analysis revealed that \textit{tet}(S) and \textit{erm}(B)gene from \textit{S. parauberis} serotype I shares 99.4% and 99.9% sequence homology with that of plasmid pKL0018 from \textit{Lactococcus garvieae} (accession number AB290882.1). \textit{tet}(M) gene from \textit{S. parauberis}
serotype II shares 100% identity to that from *S. agalactiae* (accession number AE009948).

**Plasmids detection from serotype I resistant strains**

Plasmids about 11 kbp were detected in 5 EM/OTC-resistant strains. PCR products of *tet(S)* were also obtained when plasmids were used as template.

**Discussion**

Streptococci acquiring OTC resistance commonly according to two mechanisms, including efflux of tetracyclines from cells [coding by *tet(L)* gene] and ribosomal protection [coding by *tet(M)* and *tet(S)* gene] (Roberts, 2005). In this chapter, although *tet(S)* gene was detected in serotype I resistant strains and *tet(M)* gene was detected in all the serotype II strains, both of them shared the same resistant mechanism. Besides *tet(M)* gene, both *int* and *xis* genes were also detected in all the serotype II strains. These genes are known to be components of Tn916-Tn1545 family transposons (Clewell *et al*., 1995; Chopra and Roberts, 2001). So it is suggested that Tn916-related element presents in the *S. parauberis* serotype II strains.

Macrolides are composed of 14 (erythromycin and clarithromycin)-, 15 (azithromycin)-, or 16 (jasamycin, spiramycin, and tylosin)-membered lactones to which amino and/or neutral sugars are attached via glycosidic bonds (Roberts *et al*., 1999). EM was introduced in 1952 as the first macrolide antibiotic. Unfortunately, within a year, EM-resistant staphylococci from the United States, Europe, and Japan were described (Roberts, 2008). The binding site in the 50S ribosomal subunit for EM overlaps the binding site of the newer macrolides, as well as the structurally unrelated lincosamides and streptogramin B antibiotics. The modification by methylase reduces the binding of all three classes of antibiotics, which results in resistance against macrolids, lincosamides, and streptogramin B antibiotics (MLS). Two distinct mechanisms of macrolide resistance in bacteria have been mainly described, including target site modification of the 23S rRNA by methylation of an adenine residue (MLS phenotype) related to *erm* (designated from erythromycin ribosome methylation) class.
methylated genes \([erm(A), (B)\) and (C)] and efflux pump (M phenotype, resistance to 14- and 15-membered ring macrolide) related to \(mef\) genes \([mef(A)]\) (Roberts, 2008). In this chapter, both \(erm(B)\) and \(tet(S)\) genes were detected in 5 serotype I resistant strains and mediated highly resistance to EM and OTC. While \(tet(M)\) gene was found in all serotype II strains and mediated intermediately resistance to OTC. Additionally, in streptococci, some mobile genetic elements carrying \(tet(M)\) also harbor macrolide-resistance \(erm\) genes, such as Tn1545, Tn6002 and Tn6003 (Fig.2-2) (Cochetti et al., 2008). In this study, however, the detection of EM-resistant genes in \(S. parauberis\) serotype II strains was failed. So these strains may have different mechanisms for EM resistance.

Both the MIC values and types of resistance genes detected suggested a possible association between the serotype and the level of the resistance to EM and OTC. Domelier et al. (2008) mentioned that the differences in the bacterial capsule and/or cell wall composition between geno-groups could result in different degrees of susceptibility to transformation or transposition involved in the horizontal transfers of erythromycin resistance genes. Such a mechanism could be applicable to the acquisition of EM and OTC resistance in two types of \(S. parauberis\).

**Conclusion**

1. \(tet(S)\) and \(erm(B)\) genes were detected among five \(S. parauberis\) serotypt I resistant strains.
2. Plasmids that contained \(tet(S)\) gene were observed in those five \(S. parauberis\) serotypt I strains.
3. \(tet(M)\) gene was detected among all of \(S. parauberis\) serotype II strains.
4. \(int\) and \(xis\) genes relating with Tn916 family were also detected among all of \(S. parauberis\) serotypt II strains, suggesting the existence of Tn916-related element.
<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequences (5′ to 3′)</th>
<th>PCR production size (bp)</th>
<th>Annealing temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>erm(A)</td>
<td>GTTCAAGAAACATCAATACAGAG</td>
<td>420</td>
<td>52</td>
<td>Lina et al., 1999</td>
</tr>
<tr>
<td></td>
<td>GGATCAGGAAAAGGACATTAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>erm(B)</td>
<td>GGATACCTTGGATATTCAC</td>
<td>224*</td>
<td>50</td>
<td>Nagai et al., 2001</td>
</tr>
<tr>
<td></td>
<td>GTAAACAGTTGAGCATATTTCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>erm(C)</td>
<td>GCTATATGTTTAAATCGTAATTCC</td>
<td>359</td>
<td>52</td>
<td>Lina et al., 1999</td>
</tr>
<tr>
<td></td>
<td>GGATCAGGAAAAGGACATTAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mef(A)</td>
<td>AGTATCATTAAATCCTAGTGC</td>
<td>345</td>
<td>52</td>
<td>Sutcliffe et al., 1996</td>
</tr>
<tr>
<td></td>
<td>TCTCTCTGTTAATATGTTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tet(K)</td>
<td>TATTTGGGTATTCTCTTATCAT</td>
<td>1,159</td>
<td>50</td>
<td>Giovanetti et al., 2003</td>
</tr>
<tr>
<td></td>
<td>GTATACCTGTCTCTCTGATATAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tet(L)</td>
<td>ATAAATGTTCGGGTCGTTAAT</td>
<td>1,077</td>
<td>50</td>
<td>Giovanetti et al., 2003</td>
</tr>
<tr>
<td></td>
<td>AACCAGGCGACATCAATGACATGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tet(M)</td>
<td>GTTAAATAGTTCTTTGGAG</td>
<td>657*</td>
<td>57</td>
<td>Aarestrup et al., 2000</td>
</tr>
<tr>
<td></td>
<td>CTAAGATATGCGCTTAAACA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tet(O)</td>
<td>AACTTAGGCATTCTGCTCAC</td>
<td>515</td>
<td>55</td>
<td>Jeric et al., 2002</td>
</tr>
<tr>
<td></td>
<td>TCCACTGTCCATATGCTCA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tet(S)</td>
<td>ATCAAGATATATGAGC</td>
<td>573*</td>
<td>55</td>
<td>Charpentier et al., 1993</td>
</tr>
<tr>
<td></td>
<td>TTCTCTATGTGTTAAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>int</td>
<td>TGACACTCTCGAGCTTTTAC</td>
<td>579</td>
<td>57</td>
<td>Barbeyrac et al., 1996</td>
</tr>
<tr>
<td></td>
<td>CCAATGGCAACTGACGTTCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>xis</td>
<td>AGCAGACTGACATTCCTA</td>
<td>193</td>
<td>55</td>
<td>Amezaga et al., 2002</td>
</tr>
<tr>
<td></td>
<td>GCGTCAATATGATCTTAA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tnpA</td>
<td>GCTTCCATGGGACTCGGGGCA</td>
<td>2,133</td>
<td>55</td>
<td>Poyart et al., 2000</td>
</tr>
<tr>
<td></td>
<td>GCTCCCAATTTAAAGGAGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tnpR</td>
<td>CCAAGGAGCTAAAGAGGTCCC</td>
<td>1,547</td>
<td>55</td>
<td>Poyart et al., 2000</td>
</tr>
<tr>
<td></td>
<td>GTCCCGAGTCCCATGGAAGC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Also used to obtain a specific probe for hybridization.
<table>
<thead>
<tr>
<th>Serotype</th>
<th>Strain no.</th>
<th>MIC to OTC (µg/mL)</th>
<th>erm(A)</th>
<th>erm(B)</th>
<th>erm(C)</th>
<th>mef(A)</th>
<th>tet(K)</th>
<th>tet(L)</th>
<th>tet(M)</th>
<th>tet(O)</th>
<th>tet(S)</th>
<th>tnpR</th>
<th>tnpA</th>
<th>int</th>
<th>xis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>1</td>
<td>128</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>128</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>128</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>128</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>128</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Type II</td>
<td>1</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>32</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Fig. 2-1. PCR amplifications of resistance genes.
Lane M, DNA size marker (1kbp DNA ladder, New England Biolabs); Lane 1, \textit{int} PCR product (579 bp); Lane 2, \textit{tet}(M) PCR product (657 bp); Lane 3, \textit{tet}(S) PCR product (573 bp); Lane 4, \textit{erm}(B) PCR product (224bp).
**Fig. 2-2.** The ORF maps of Tn917, Tn916 and Tn916 family transposons Tn6002, Tn6003 and Tn1545. White arrows indicate Tn916 ORFs other than tet(M) (striped). Spotted arrows indicates the ORFs from Tn917 other than erm(B) (checkered). Black arrows indicated ORFs from the ca. 2.8 kbp erm(B) element, except for erm(B) (checkered). Light gray arrows indicate ORFs from the ca. 4.2 kbp macrolide-aminoglycoside-streptothricin element from Tn6003, except for erm(B) (checkered) and a putative IS1239 transposase (vertical lined).
Chapter 3
Characterization of resistance determinants in *S. parauberis* serotype I strains
In many bacteria, drug resistance is carried on transferable R plasmids. R plasmids have been detected in drug resistant strains of various fish-pathogenic bacteria: *Aeromonas hydrophila, Aeromonas salmonicida, Edwardsiella tarda, Pseudomonas fluorescens, Photobacterium damselae subsp. piscicida, Vibrio anguillarum*, and marine *Vibrio* (Aoki, 1988).

Tetracycline-resistant streptococci are frequently isolated from human pathogens, and resistances are most conferred by genes encoding ribosomal protection proteins (Roberts, 2005). Aoki (1988) reported that the indiscriminate use of antibiotic by fish farmers led to the emergence of drug-resistant strains and could pose a threat to the public health problems, because some streptococcal species are the pathogens of zoonosis. In the previous chapters, the tetracycline resistance conferred by *tet*(S) gene as well as plasmid were detected among the EM/OTC-resistant strains of *S. parauberis* serotype I. *tet*(S) , a member of genes encoding ribosomal protection protein, showed 79% amino acid identity with Tet(M). *tet*(S) gene responsible for plasmid-mediated resistance was initially found on a 37-kbp conjugative plasmid from clinic isolates of *Listeria monocytogenes* (Charpentier et al., 1993). In this chapter, the relationship between the plasmids and resistance genes found in *S. parauberis* serotype I strains, as well as the conjugal transfer of the plasmids, was investigated.
Materials and Methods

Bacterial strains and culture media

Five EM/OTC-highly resistant *S. parauberis* strains were used in this study. *Enterococcus faecalis* FA2-2 (Shiojima et al., 1997) was used as a recipient for conjugation of plasmids. They were grown in Todd-Hewitt broth (THB; Difco). *Escherichia coli* JM109 was grown in Luria-Bertani (LB) broth. Drugs were used at the following concentrations: 100 µg/mL for ampicillin, 50 µg/mL for X-gal and 0.1 mM for IPTG. Chromosomal and plasmid DNA of each strain were prepared using Wizard Genomic DNA Purification Kit (Promega) and QIAprep miniprep kit, respectively, according to the manufacturer's protocols.

Conjugal transfer of plasmid

Filter mating was carried out by using broth cultures of donor and recipient cells in logarithmic phase. Mixture of the donor and *E. faecalis* FA2-2 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 (30 µg/mL), rifampicin (25 µg/mL) and fusidic acid (25 µg/mL) and incubated at 37°C overnight. Transfer frequencies were calculated as the number of transconjugants per donor cell. Twenty transconjugants were picked up for testing antibiotic resistance and some of them were used for plasmid DNA purification.
Southern blot hybridization

Based on the results of chapter 2, resistant strains were applied for Southern blot hybridization analysis. Chromosomal DNA (1.5 µg) or plasmid DNA (500 ng) extracted from those strains were completely digested with \textit{HindIII}. Digested DNA were resolved by electrophoresis in 0.7 % agarose gel, stained with ethidium bromide and photographed. After depurination, denaturation and neutralization of the gel, DNA was transferred to Hybond N$^+$ membrane (GE Healthcare) by capillary blotting overnight. The membrane was rinsed in 5×SSC, fixed at 120°C for 30 min and prehybridized at 55°C for 30 min in DIG Easy Hyb (Roche). The membrane was then hybridized at 55°C with DIG-labelled \textit{tet}(S) or \textit{erm}(B) probe (Roche) which was gel-purified PCR product (Table 2-1) labeled with digoxigenin-11-dUTP by random priming using DIG-High Prime (Roche). Washings were under stringent conditions, and signals were detected by exposure of the membrane to Hyperfilm ECL (GE Healthcare).

PCR and Inverse PCR amplification

The primer pairs used in this study are shown in Fig. 3-3. TaKaRa \textit{Ex Taq} Hot start version (Takara Bio) was used for PCR. The flanking regions of \textit{erm}(B) were amplified by inverse PCR (Ochman et al. 1988). Briefly, 5µg of genomic DNA was mixed with 12 U of \textit{HindIII} in the specified buffer applied by the supplier (Wako, NIPPON GENE, Japan) in a volume of 50 µL and completely digested at 37°C for 16 h. Digested DNA was extracted twice with phenol equilibrated in TE (pH 8.0) and once with chloroform.
The digested template DNA was precipitated by adding 0.1 volume of 3 M sodium acetate and 2.5 volumes of pre-cold 99% ethanol. The mixture was incubated on ice for 10 min and precipitated DNA was recovered by centrifugation. The precipitated DNA was washed with 200 µL pre-cold 70 % ethanol and dissolved in 30 µL of sterile deionized and distilled water. Ligation mixture comprised 10 µL of cleaved template DNA solution and the equal volume of ligation solution (DNA Ligation Kit, Mighty Mix, TaKaRa). Ligation reaction was then performed at 4°C for 16h and the ligated DNA was then extracted by phenol and chloroform. The inverse PCR was performed using 2 µL of a serial 10-fold dilution of the circularized DNA, 0.5 µM of each primer, 10× Ex Taq buffer (Mg²⁺ plus), 4µL of 0.2mM of dNTP mixture and 1.25U of Ex Taq DNA polymerase (Hot Start Version, TaKaRa) up to a total volume of 50µL by adding deionized and distilled water. In order to increase the specificity of the products of inverse PCR, nested PCR was carried out. The PCR was performed on C1000 Thermal Cycler (Bio-Rad).

**Sequence analysis**

DNA sequencing was carried out as described in the chapter 2.

**Results**

**Conjugal transfer of tetracycline-resistant plasmid**

Conjugation experiments were carried out on the five EM/OTC-highly-resistant
strains with the plasmid-free recipient strain, *E. faecalis* FA2-2. In transconjugants from each donor, the *tet*(S)-positive plasmid was detected, and its size and *Hind*III restriction profile were the same as that in the respective donor strain. From these results it is concluded that the plasmid is a conjugative plasmid. The transfer frequency of the plasmid from each donor to *E. faecalis* FA2-2 was calculated at about 10⁻⁸ per donor cell by filter mating.

**Localization of resistance genes**

PCR-amplified products of antimicrobial resistance genes, *tet*(S) and *erm*(B) were used as probes to hybridize with the DNA from resistant strains. Plasmid profiling of the five EM/OTC-highly-resistant strains showed that these strains possessed one plasmid with an approximate size of 11 kbp (data not shown). *Hind*III digestion patterns of the plasmids from the five strains were identical, and *tet*(S) was detected on the plasmids (Fig.3-1). The *erm*(B) probe hybridized with the chromosomal DNA of those *tet*(S)-positive strains (Fig. 3-2). Those five strains came from the same prefecture but isolated at different aquaculture sites.

**Sequencing analysis of the 2677bp *erm*(B) gene region**

The complete nucleotide sequence of the 2677bp *erm*(B) gene region from *S. parauberis* serotype I strain NUF927 was determined. ORF1 (96bp) is identical to the leader peptide of *erm*(B) gene, whereas ORF2 (738bp) is identical to *erm*(B) gene (Fig. 3-3A). ORF3 (94bp) shows significant homology to the corresponding region in
plasmid pKL0018 from Lactococcus garvieae (accession number AB290882) and share 100% amino acid identity to those of pKL0018. The known nucleotide sequence (531bp) of ORF4 shares 98.1% identity to that of the ORF298 (531/897bp) in Clostridium difficile (accession number AJ294529). A 331bp sequence on the upstream of ORF1 (the _erm_ (B) leader peptide) was homologous to another 331bp sequence on the downstream of the ORF3 (Fig. 3-3B). Located downstream of the both 331bp repeat sequences were 42 and 63bp palindromic sequences, respectively (Fig3-3A). the first palindromic sequence locationg on the upstream, consisted of four direct repeats and two inverted repeats of the 7bp sequence 5'-A/TATCACA/T-3', whereas the second palindromic sequence locating on the downstream, consisted of six direct repeats and 3 inverted repeats (Fig. 3-3A).

**Multiple DNA alignment of 2677bp _erm_(B) gene region sequences and phylogenetic analysis**

The complete nucleotide sequence of the 2677bp _erm_(B) gene region from _S. parauberis_ NUF927 were aligned with those of _Lactococcus lactis_ plasmid pI1o8 (accession number AJ549242), _E. faecalis_ plasmid pRE25 (accession number X92945), _L. garvieae_ plasmid pKL0018 (accession number AB290882), and _Pediococcus acidilactici_ plasmid pEOC01 (accession number DQ220741) generated using the ClustalW software(Fig.3-4A). Herein, this fragment revealed strong sequence homology and identity with related regions from other gram-positive bcteria species (Fig.3-4B).

Phylogenetic tree for the 2677bp _erm_(B) gene region shows _S. parauberis_ closing as expected with _L. garvieae_ (Fig. 3-4C). The _S. parauberis_ _erm_(B) gene region is more
similar to *L. garvieae* plasmid pKL0018 than to *Lactococcus lactis* plasmid pI1o8, *E. faecalis* plasmid pRE25 and *Pediococcus acidilactici* plasmid pEOC01.

**Discussion**

Macrolide antimicrobials are not approved for administrating to Japanese flounder in Japan, but they have been used for treating streptococciosis causing by *Lactococcus garvieae* in yellowtail *Seriola quinqueradiata* for many years. Several published works showed that *L. garvieae* strains isolated from yellowtail carried transferable R-plasmid on which both *erm*(B) and *tet*(S) were integrated (Aoki *et al.*, 1990; Maki *et al.*, 2008). In addition, *L. garvieae* is also one of the pathogens causing streptococciosis in cultured Japanese flounder (Fukuda, 2003). Taken together, horizontal spread of resistance genes between streptococci and lactococci might have occurred in aquaculture environment. During preparing this manuscript one serotype I strain of *S. parauberis* which was highly resistant to OTC but susceptible to EM was found in newly collected strains. This strain was isolated in a prefecture different from the prefecture in which above-mentioned plasmids were detected. From the strain one plasmid was detected, the size and *Hind*III-digested pattern of which were the same as those of plasmids mentioned above (data not shown). This suggested that the same conjugative plasmid encoding *tet*(S) was conserved and disseminated in the western Japan.

In streptococci, macrolide, licosamide and streptogramin B (MLS) resistance is usually due to an *erm*(B) resistance gene carried by R plasmid or conjugative transposon (Roberts *et al.*, 1999). *erm*(B) gene has been found in numerous bacterial
genera, including *Enterococcus, Streptococcus, Lactobacillus* and *Clostridium* and *Escherichia coli* (Roberts et al., 1999). The results presented in this study support the hypothesis for the recent horizontal transfer from a *L. garviaea* origin to *S. parauberis* plasmid. Two palindromic sequences comprising of several direct and inverted 7bp repeats with sequence 5'–A/TATCACA/T–3' were identified in the upstream and downstream of *erm*(B) gene. This particular sequence, usually present in R plasmids coding *erm*(B) (e.g. pI1o8, pRE25, pKL0018 and pEOC01) (Fig. 4-4), may have played a role in the insertion of the *erm*(B) gene region into the chromosomal DNA of *S. parauberis* (Cochetti et al., 2007). Some studies have suggested that the similar palindromic sequence may act as hot spots for plasmid cointegrate formation and resolution (Langella et al., 1993).

Analysis of the known amino acid sequence of ORF4 shares 98.1% identity to that of the ORF298 which had similarity to proteins such as δ from pediococcal plasmid pEOC01 (accession number DQ220741), ParA from bacteriophage P1 (accession number YP_006528), pTAR from *Agrobacterium tumefaciens* (accession number AAF45023), RepB from the enterococcal plasmid pAD1 (accession number AAB00504), SopA from the F plasmid (accession number BAA97916), and IncC from plasmid RK2 (accession number BVECIC). These proteins belong to a superfamily of ATPase and have been proposed or demonstrated to be associated with plasmid or chromosomal partitioning. Since the *erm*(B) determinant is located on the nontransferable element in *S. parauberis* serotype I, it is possible that the putative ORF4 (or ORF298) protein may be associated with the partitioning of the original plasmid coding *erm*(B) gene. These results provide evidence that the *erm*(B) gene region may have been transferred from R plasmid.
Further investigations will be carried out to better characterize the *erm*(B) gene region and conjugative plasmid detected in this study and their relevance in *S. parauberis* serotype I epidemiology.

**Conclusion**

1. *Hind*III digestion patterns of the plasmids from the five EM/OTC resistant serotype I strains were identical.

2. *tet*(S) gene was detected in the conjugative plasmid.

3. *erm*(B) probe hybridized with the chromosomal DNA of the strains harboring the conjugative plasmid.

4. The transfer frequency of the conjugative plasmid from each donor to *E. faecalis* FA2-2 was calculated at about $10^{-8}$ per donor cell by filter mating assay.

5. Two palindromic sequences were comprised of several direct and inverted 7bp repeats with sequence $5'-A/TATCACA/T-3'$ were identified in the upstream of ORF1 and downstream of ORF3.
Fig. 3-1. HindIII-digested plasmid DNAs from EM/OTC highly resistant *S. parauberis* strains (panel I) and Southern blot hybridization analysis of HindIII-digested plasmid DNAs with *tet* probe (panel II). Lane M, DNA size marker (1kbp DNA ladder, New England Biolabs); Lane 1 to 5, EM/OTC highly resistant *S. parauberis* strains No. 1 to 5.
Fig. 3-2. HindIII-digested plasmid (Lane 1-5) and chromosomal (Lane 6-10) DNAs from EM/OTC highly resistant S. parauberis strains (panel I) and Southern blot hybridization analysis of HindIII-digested plasmid (Lane 1-5) and chromosomal (Lane 6-10) DNAs with *erm*(B) probe (panel II). Lane M, DNA size marker (1kbp DNA ladder, New England Biolabs); Lane 1 to 5 and 6 to 10, EM/OTC highly resistant *S. parauberis* strains No. 1 to 5.
Fig. 3-3. (A) The DNA and amino acid sequences of the 2677bp *erm* (B) gene region from *S. parauberis* serotype I resistance strain (NUF927). The primer sets used for inverse and nested PCRs are also indicated. The grey regions indicate the 331bp repeat sequences. The 7bp repeats and their relative orierrtations are indicated by arrows below the nucleotide sequences. Stop codon is indicated by dot. * Primers used for inverse PCR. ** Primers used for nested PCR. (B) Chromosomal location of *S. parauberis* *erm* (B) resistance gene. A checkered arrow indicates *erm* (B) and dotted arowdes indicates ORFs. A small white box indicates a 331bp repeat on the upstream and downstream of the *erm* (B) gene.
A

ORF1 erm(B) leader peptide start

ORF2 erm(B) leader peptide stop

orf2 stop

orf1 repeat

orf2 repeat stop

orf1 start

orf2 start

orf2 stop
### B

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>%identity</th>
<th>%similarity (vs. <em>S. parauberis</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. acidilactici</em></td>
<td>93.3</td>
<td>93.4</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>93.0</td>
<td>93.1</td>
</tr>
<tr>
<td><em>L. garvieae</em></td>
<td>92.9</td>
<td>93.0</td>
</tr>
<tr>
<td><em>L. lactis</em></td>
<td>92.0</td>
<td>92.0</td>
</tr>
</tbody>
</table>

### C

![Phylogenetic tree](image)

**Fig. 3-4.** (A) Alignment of the nucleotide sequences of the 2677bp *erm(B)* gene region from *S. parauberis* NUF927 with those of *Lactococcus lactis* plasmid pI1o8 (accession number AJ549242), *E. faecalis* plasmid pRE25 (accession number X92945), *L. garvieae* plasmid pKL0018 (accession number AB290882), and *Pediococcus acidilactici* plasmid pEOC01 (accession number DQ220741) generated using the ClustalW software. The asterisk (*) shows sequences that are the same as those of the *erm(B)* gene regions. The organism is given on the left of each sequence lane. The sequence number is given on the right of each sequence lane. (B) Degree of homology of the *erm(B)* gene region in *S. parauberis* NUF927 to the related region in other bacteria. (C) Phylogenetic trees of the *erm(B)* gene region drawn by the maximum likelihood method using MEGA (version 4.0) software.
Chapter 4
Characterization of resistance determinants in
*Streptococcus parauberis* setorype II strains
In chapter 1, all of the serotype II strains were EM (MICs=0.25-1 µg/mL) and OTC (MICs=32µg/mL) intermediately resistant. Furthermore, in chapter 2, although positive amplifications were obtained with Tn916-family specific primers for tet(M) (tetracycline resistance), xis (excisionase) and int (integrase) in these strains, the EM resistance genes could not be detected. It means these strains may have different mechanisms for EM resistance. According to the numerous documents, Tn916 family have acquired additional resistance determinants, such as Tn1545 [EM and KM resistances], Tn6002 [EM resistance] and Tn6003 [EM, KM and aminoglycosides resistances] (Caillaud et al., 1987; Warburton et al., 2007; Cochetti et al., 2008). A considerable relationship between Tn916-like element and EM resistance gene might exist in serotype II because no plasmid was detected. In another word, the undetected EM resistance determinant coexisting with Tet(M) might be mediated by the Tn916-like element.

Based on this result, primers according to the sequences of Tn916 (accession no. U09422) in databases were designed to amplify the full length of Tn916-like element. The aim of this chapter is to characterize the Tn916-like element of S. parauberis by comparing the total DNA sequence with that of Tn916 from E. faecalis and the insertion site of the Tn916-like elements in the chromosomes of serotype II strains. The relationship between serotype I and II strains concerning the insertion site was also characterized.
Materials and Methods

Strains, medium and DNA

Thirty-two *S. parauberis* serotype II strains used in this study were isolated at flounder aquaculture sites located in Kagawa (8 strains), Ehime (9), Kumamoto (1), Oita (10), Kagoshima (1) and Nagasaki (3) Prefectures in 2002 to 2008. *S. parauberis* NUF1003 and NUF1049 were used as the representative strains of serotype I and II, respectively. *E. faecalis* CG110 which harbored Tn916 was used as the reference strain (Shimoji *et al*., 1994) and *E. faecalis* FA2-2 as the recipient strain in conjugal transfer experiments (Shiojima *et al*., 1997). These strains were grown in Todd-Hewitt broth (THB; Difco Laboratories). Chromosomal and plasmid DNA of each strain 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 4-1. TaKaRa Ex Taq Hot start version (TaKaRa Bio) was used for PCR, except for Tn916, for which TaKaRa LA (Long and accurate) Taq Hot start version was used. The Tn-upstream and Tn-downstream regions were amplified by inverse PCR (Ochman *et al*. 1988). In order to reduce the contamination in products, nested PCR was carried out to amplify secondary targets within the inverse PCR products. The loci of primers are shown in Fig. 4-1. The PCR was performed on C1000 Thermal Cycler (Bio-Rad).

Southern blot hybridization

Southern blot hybridization analysis was carried out with DIG-labeled probes [\(\text{tet(M)-int}\) and Tn916-like element] (Table 4-1) and detection system (Roche). Chromosomal DNA (1.5 µg) extracted from serotype II strains of *S. parauberis* and *E.
faecalis CG110 were digested to completion at 37°C with HincII, HindIII or Sau3AI (TaKaRa Bio). The digested DNA were electrophoresed on 0.7% agarose gel and stained with ethidium bromide. After depurination, denaturation and neutralization of the gel, DNA 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 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 *S. parauberis* NUF1049 and the relative flanking region of Tn916-like element from *S. parauberis* NUF1003 were cloned into pGEM-T Easy vector (Promega) and transformed into *E.coli* JM109 Competent Cells (Promega). DNA sequencing was carried out with Big Dye Terminator v3.1 Cycle Sequencing kit and ABI Prism 3100 Genetic Analyzer (Applied Biosystems). 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 was used for DNA and protein multiple sequence alignment (Larkin *et al.*, 2007).

**Conjugative transfer experiments**

Filter mating assays was performed by using logarithmic-phase broth cultures of six *S. parauberis* serotype II strains (one strain per prefecture) as donors and *E. 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), rifampicin (25 µg/mL) and fusidic acid (25 µg/mL).
**Nucleotide sequence accession number**

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

**Results**

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

The 32 serotype II strains obtained single PCR products [tet(M)-int] of around 4.5 kbp with the primer pair tet(M)-FW−Int-RV (Table 4-1). In Southern blot hybridization analysis, the DIG-labelled tet(M)-int was found to uniformly hybridize to 7.2 kbp HindIII-digested fragments of the chromosomal DNA of the serotype II strains (Fig. 4-2). The results suggested that resistance to tetracycline in S. parauberis serotype II strains is due to the presence of one copy of a chromosomally borne element structurally related to Tn916.

Analysis of the nucleotide sequences of the tet(M)-int from two strains chosen at random showed that their sequences were identical. Comparison of the sequences between tet(M)-int and the corresponding region of Tn916 of E. faecalis DS16 (Flannagan et al., 1994; accession number U09422) also showed that they were identical.

Based on this similarity, primer pair of Tn916-FW and Tn916-RV (Table 4-1) was designed from the database to amplify the full length of Tn916-like element. Positive amplifications of the whole length were obtained when chromosomal DNA from serotype II strains were used as template for LA PCR. The size of amplifications was the same as those from E. faecalis CG110, the positive control of Tn916 (data not shown). These evidences indicate that the resistance elements harbored by serotype II strains are closely related to Tn916.
**Nucleotide Sequence analysis of Tn916-like element**

Positive amplifications were also detected when four primer pairs [Table 4-1, TnA-D] based on the four overlapped fragments of Tn916 were used. Locations of PCR amplifications are shown in Fig. 4-1 (A). Tn916-like element harbored in serotype II strains were constructed by these four fragments which could span the Tn916-like element. PCR products of these four fragments were cloned into pGEM-T Easy vector (Promega) and sequenced. The size of entire Tn916-like element was found to be 18,031 bp in length. Analysis and comparison of the sequencing result showed that the element was 99.8% identical to that of Tn916 original from *E. feacalis* DS16. Tn916-like element encodes 22 ORFs as Tn916.

**Multiple DNA alignment of Tn916 sequences**

The nucleotide sequences of *S. parauberis* NUF1049 Tn916-like element were aligned with those of *S. agalactiae* 2603V/R Tn916-like element, *E. faecalis* plasmid pCF10 Tn925, *E. faecalis* DS16 Tn916, and *S. suis* Tn916, using the ClustalW2 software (Fig. 4-3). All the sequences possess the genetic characterizations for Tn916: *tet* (M), *int* and *xis*. However, *S. parauberis* Tn916 is more similar to Tn916-like element of *S. agalactiae* than to *E. faecalis* DS16 Tn916, *E. faecalis* plasmid pCF10 Tn925 and *S. suis* Tn916.

**Structural comparison of the Tn916-like element harbored by the S. parauberis serotype II strains**

All of the serotype II strains showed the same *Sau3AI* and *HindIII* restriction patterns as Tn916 by Southern blot hybridization analysis. This strongly suggested that all of the serotype II strains harbored complete copies of Tn916-like element and the element existed in the same chromosomal location of all of serotype II strains (Fig. 4-4). However, *HincII* hybridizing patterns of serotype II strains were diverse, namely two patterns existed among serotype II strains (Fig. 4-5). Only one strain derived from
Kagoshima Prefecture out of 32 serotype II strains showed pattern I which consisted of 5.6-, 4.9-, 3.0-, 1.7-, 1.2-, 0.9- and 0.4-kbp \textit{HincII} fragments like Tn916, whereas the rest strains showed pattern II that contained a 10.5-kbp fragment instead of 5.6 kbp and 4.9 kbp fragments. In these two patterns, \textit{tet}(M) gene located in 10.5 and 4.9 kbp \textit{HincII} fragments, respectively.

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

Restriction analysis of the Tn916-like element constructs with \textit{HindIII} had shown that the Tn916-like element insertions were in the same orientation since 15.2 and 7.2kbp fragments had been obtained uniformly among all of serotype II strains. This further proved that a single \textit{HindIII} restriction site exist in the element. Cloning of left and right flanking fragments adjacent to Tn916-like element was accomplished through circularization of those two \textit{HindIII} restriction digests [Fig. 4-1 (B)]. Inverse and nested PCR amplifications were carried out by using primer pairs listed in Table 4-1. Inverse and nested PCR of the left flanking region were expected to provide 4.7 and 4.5kbp fragments, respectively. Furthermore, 4.5kbp and 2.7kbp fragments of the related right flanking region were also obtained. Sequence analysis of the flanking regions of the Tn916-like element showed that insertion site of Tn916-like element of the host chromosome is the A-T rich regions. 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 ORF Sp-O1, -O2 and -O3 have 68%, 72% and 84% identity to transcriptional regulator, dihydroxyacetone kinase (accession number CP000262) and threonyl-tRNA synthetase (accession number AE014074) from \textit{S. pyogenes}, respectively [Fig.4-6(A)]. It is interesting that the insertion of the Tn916-like element did not interrupt any ORF of chromosomal DNA of \textit{S. parauberis} serotype II strains.

Based on the above results, the supposition that the Tn916-like element inserted in the similar sites among serotype II strains was testified. Two primer pairs were chosen, one is the left terminus primer pair, Tn-5R located on the left internal position of
Tn916-like element and Tn-11F-4F located on the upstream of Tn916-like element, and the other is the right terminus primer pair, Tn-2F located on the right internal position of Tn916-like element and Tn-3R-2R located on the downstream of Tn916-like element [Table 4-1; Fig. 4-1 (A)]. The PCR amplification results proved that the Tn916-like element located in the uniform location in all of serotype II strains tested.

Since Tn916-like element exists in all of chromosomal DNA of serotype II strains at the similar locations, it is necessary to clarify the relationship between serotype I and II strains concerning the insertion sites of Tn916-like elements. The primers Tn-11F-4F located on the left side and Tn-3R-2R located on the right side of the flanking region were selected for amplify the corresponding region of serotype I strains [Table 4-1, Fig. 4-6 (A)]. Sequencing analysis of the related flanking region from a serotype I strain showed that the insertion site was generated 6 bp [ATCATA, underlined in Fig. 4-6 (B)] and adjacent to the left end of Tn916-like element in the integrated state in serotype II strain. The 6bp sequence [TATATA, emphasized by the dot line box in Fig. 4-6 (B)] represented the initial target sequence for the insertion of Tn916-like element.

Conjugative transfer of Tn916-like element

Six strains isolated from different prefectures were used as donor in filter mating experiments. No detectable transfer of Tn916-like element from any donor was obtained by using E. faecalis FA2-2 as the recipient.

Discussion

This study elucidates the complete organization and structure of the Tn916-like element observed in S. parauberis serotype II strains examined. Therefore, our data provide evidence that Tn916-like element with tet(M) gene plays as an important selective factor that provides considerable advantages for the emergence and spread of S. parauberis serotype II. The widespread use of tetracycline in cultured Japanese flounder could provide the selective pressure for clone amplification and spreading, thus contributing to the outbreak of serotype II through Tn916-like element.
Tn916-like element is homology with Tn916 but differing from it in structure detected in almost all the strains (31/32) studied here. It is worthy to note that the sizes of internal HincII fragments of the Tn916 restriction map are 5.6, 4.9, 1.7, 1.2 and 0.4 kbp, respectively (Senghas et al., 1988). tet(M) gene is located on 4.9 kbp fragment which is present in Tn916 and in most Tn916-like structures (Bentorcha et al., 1992). However, this Tn916 structural character could be observes only in one strain NUF1048 isolated from Kagoshima Prefecture (Fig. 4-5-Lane 1). The difference is due to a nucleotide change of the restriction site located in ORF14 from GTCAAC to GTCACC for the 31 strains. It is interesting that the similar structure was also detected on the chromosomes or plasmids in enterococcal strains (Bentorcha et al., 1992). These results suggested that the diverse structures of Tn916-like element exist among serotype II isolates.

int and xis genes are involved in the conjugative transposition mechanism in Tn916 family (Rice, 1998). In this study, our PCR results, sequencing data and Southern hybridization results of the Tn916-like element have revealed the presence of these two genes in all of serotype II strains. Based on these results, the element would be predicted to be conjugal transfer. However, horizontal transfer of Tn916-like element was not detected from serotype II strains to recipient by filter mating assays. 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). Conjugative transposition of Tn916 is via an excision-insertion mechanism with three stages: excision and circularization, conjugal transfer and integration (Caparon and Scott, 1989). Excision and insertion of conjugative transposons began with generating staggered nicks of 6 bp (Scott, 1992). The ligated staggered nicks yield heteroduplex in both circular intermediate and target site. The coupling sequence is the 6bp junction sequences comprising heteroduplex which will be resolved by replication. The nucleotide content of the coupling sequences (6bp) flanking the inserted transposon plays a frequency-determining role (Jaworski and Clewell, 1994). Therefore, transposition from different donors into the same target site occurs at widely differing frequencies ranging from less than $10^{-8}$ to greater than $10^{-4}$ per donor. According to this mechanism, our sequence data of serotype I and II provided evidence that the
Tn916-like element introduced a coupling sequence (ATCATA) derived from its previous host target sequence. Analysing the coupling sequence adjacent to Tn916-like element (ATCATA, adjacent to the left end of element; TATATA, adjacent to the right end of element) in a serotype II strain demonstrated that the coupling sequences might result in the extreme low transfer frequency in the second-generation transposition events.

In conclusion, this chapter reported that all of S. parauberis serotype II strains from Japanese flounder harbored Tn916-like element. These transposons expressed tetracycline resistance conferred by tet(M) gene and inserted into an A-T rich region of chromosomal DNA of serotype II strains. With the complete DNA-sequencing of the Tn916-like element and flanking regions, studies of spreading mechanism of this element are possible to further elucidate the molecular interaction between Tn916-like element and the host. Furthermore, future studies on the presence of Tn916-like element or its variants in a broader range of strains of S. parauberis should be enhanced, since it is important to understand the prevalence and transposon-mediated resistance to tetracycline for the reason of clinical importance.

**Conclusion**

1. All of serotype II strains possessed the Tn916-like element harboring tet(M) gene.
2. The S. parauberis Tn916 shares 99% homology with that from E. faecalis DS16 strain (accession number U09422).
3. Insertion site of Tn916-like element was the region with high A-T content and generated 6 bp (ATCATA) attaching at the upstream end of Tn916-like element.
<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>
<tbody>
<tr>
<td>tet(M)-int</td>
<td>tet(M)-FW Int-RV</td>
<td>GTAAAATAGTGTCTTTGGAG CCATAGGAACCTGACGTTCG</td>
<td>GenBank (U90422)</td>
<td>57</td>
</tr>
<tr>
<td>Tn916*</td>
<td>Tn916-FW Tn916-RV</td>
<td>CTGGCGAGGATAAAGTCGTTCAAGCG CAAACTATGTGGAGTGTGTGGC</td>
<td>GenBank (U90422)</td>
<td>68</td>
</tr>
<tr>
<td>Tn A</td>
<td>Tn2-FW Tn2-AR</td>
<td>CTGGCGAGGATAAAGTCGTTCAAGCG CGTGCCACGTCATACATCATCAC</td>
<td>GenBank (U90422)</td>
<td>56</td>
</tr>
<tr>
<td>Tn B</td>
<td>TnB-1F Tn2-AR</td>
<td>CTGGCGAGGATAAAGTCGTTCAAGCG CGTGCCACGTCATACATCATCAC</td>
<td>GenBank (U90422)</td>
<td>61</td>
</tr>
<tr>
<td>Tn C</td>
<td>TnC-1F TnC-1R</td>
<td>CGATTCTCAAAGTGTTGGGAAG CCAAGAACACTATTTAACTTC</td>
<td>GenBank (U90422)</td>
<td>61</td>
</tr>
<tr>
<td>Tn D</td>
<td>tet(M)-FW TnE-1R</td>
<td>GTAAAATAGTGTCTTTGGAG GTCATGGCTATATTAGCATGTC</td>
<td>GenBank (U90422)</td>
<td>65</td>
</tr>
<tr>
<td>Tn-upstream 1†</td>
<td>Tn-10F Tn-12R</td>
<td>CTATCCCTACAGGCAGCACAGCCAGTTACTTTC GCTGGCGAAGATACTTAGATGCG</td>
<td>this study</td>
<td>50</td>
</tr>
<tr>
<td>Tn-upstream 2‡</td>
<td>Tn-11F Tn-5R</td>
<td>CTTCATCGCTGACATCGAGATCTATCGAAGCAACTTATCCTGAGGAAGTTCAGACGGACCTCGGATGTG</td>
<td>this study</td>
<td>63</td>
</tr>
<tr>
<td>Tn-downstream 1†</td>
<td>Tn-1F Tn-4R</td>
<td>GACGCAATCTAGCTGTCGCCAAAGGGGTCTTG CATTTCCAAGAACTCAGGACCTCGGATGTG</td>
<td>this study</td>
<td>50</td>
</tr>
<tr>
<td>Tn-downstream 2‡</td>
<td>Tn-2F Tn-3R</td>
<td>GCCACACATCCTACCTCCCATAGTGGGCAGA TCCCAAGAACTCAGGACCTCGGATGTG</td>
<td>this study</td>
<td>60</td>
</tr>
<tr>
<td>Left terminus</td>
<td>Tn-11F-4F Tn-5R</td>
<td>GAACATATCAACCAATAATCTC TTCTCGCTGAGACATTTATCCTCGACCG</td>
<td>this study</td>
<td>60</td>
</tr>
<tr>
<td>Right terminus</td>
<td>Tn-2F Tn-3R-2R</td>
<td>GCCACACATCCTACCTCCACATAGTTGGCGAC TTTCGCAAGAATCTCAGGACCTCGGATGTG</td>
<td>this study</td>
<td>60</td>
</tr>
<tr>
<td>Flanking region</td>
<td>Tn-11F-4F Tn-3R-2R</td>
<td>GAACATATCAACCAATAATCTC TTTCGCAAGAATCTCAGGACCTCGGATGTG</td>
<td>this study</td>
<td>56</td>
</tr>
</tbody>
</table>

* Also used to obtain a specific probe.
† Primers used for inverse PCR.
‡ Primers used for nested PCR.
Fig. 4-1. Sequencing strategy of Tn916-like element (A) and flanking regions (B). Lengths and directions of solid arrows represent the genes. Arrows show the positions and directions of primers.
Fig. 4-2. Southern hybridization analysis of Tn916-like element. Chromosomal DNAs were digested with Hind III. The digests on the membrane were hybridized with DIG-labeled Tn916-like element probe. Lane C: E. faecalis CG110 (positive control); Lane 1-20: S. parauberis serotype II strains tested in this study.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Sequence</th>
<th>Length</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. suis</td>
<td>S. suis</td>
<td>13199</td>
<td></td>
</tr>
<tr>
<td>S. agalatiae</td>
<td>S. agalatiae</td>
<td>13198</td>
<td></td>
</tr>
<tr>
<td>E. faecalis</td>
<td>E. faecalis</td>
<td>13199</td>
<td></td>
</tr>
<tr>
<td>S. parauberis</td>
<td>S. parauberis</td>
<td>13198</td>
<td></td>
</tr>
<tr>
<td>S. suis</td>
<td>S. suis</td>
<td>13079</td>
<td></td>
</tr>
<tr>
<td>E. faecalis</td>
<td>E. faecalis</td>
<td>13079</td>
<td></td>
</tr>
<tr>
<td>S. parauberis</td>
<td>S. parauberis</td>
<td>13078</td>
<td></td>
</tr>
<tr>
<td>S. suis</td>
<td>S. suis</td>
<td>12959</td>
<td></td>
</tr>
<tr>
<td>E. faecalis</td>
<td>E. faecalis</td>
<td>12959</td>
<td></td>
</tr>
<tr>
<td>S. parauberis</td>
<td>S. parauberis</td>
<td>12958</td>
<td></td>
</tr>
<tr>
<td>S. suis</td>
<td>S. suis</td>
<td>12839</td>
<td></td>
</tr>
<tr>
<td>E. faecalis</td>
<td>E. faecalis</td>
<td>12839</td>
<td></td>
</tr>
<tr>
<td>S. parauberis</td>
<td>S. parauberis</td>
<td>12838</td>
<td></td>
</tr>
<tr>
<td>S. suis</td>
<td>S. suis</td>
<td>12719</td>
<td></td>
</tr>
<tr>
<td>E. faecalis</td>
<td>E. faecalis</td>
<td>12719</td>
<td></td>
</tr>
<tr>
<td>S. parauberis</td>
<td>S. parauberis</td>
<td>12718</td>
<td></td>
</tr>
<tr>
<td>S. suis</td>
<td>S. suis</td>
<td>12599</td>
<td></td>
</tr>
<tr>
<td>E. faecalis</td>
<td>E. faecalis</td>
<td>12599</td>
<td></td>
</tr>
<tr>
<td>S. parauberis</td>
<td>S. parauberis</td>
<td>12598</td>
<td></td>
</tr>
<tr>
<td>S. suis</td>
<td>S. suis</td>
<td>12479</td>
<td></td>
</tr>
<tr>
<td>E. faecalis</td>
<td>E. faecalis</td>
<td>12479</td>
<td></td>
</tr>
<tr>
<td>S. parauberis</td>
<td>S. parauberis</td>
<td>12478</td>
<td></td>
</tr>
<tr>
<td>S. suis</td>
<td>S. suis</td>
<td>12359</td>
<td></td>
</tr>
<tr>
<td>E. faecalis</td>
<td>E. faecalis</td>
<td>12359</td>
<td></td>
</tr>
<tr>
<td>S. parauberis</td>
<td>S. parauberis</td>
<td>12358</td>
<td></td>
</tr>
<tr>
<td>S. suis</td>
<td>S. suis</td>
<td>12239</td>
<td></td>
</tr>
<tr>
<td>E. faecalis</td>
<td>E. faecalis</td>
<td>12239</td>
<td></td>
</tr>
<tr>
<td>S. parauberis</td>
<td>S. parauberis</td>
<td>12238</td>
<td></td>
</tr>
<tr>
<td>S. suis</td>
<td>S. suis</td>
<td>12119</td>
<td></td>
</tr>
<tr>
<td>E. faecalis</td>
<td>E. faecalis</td>
<td>12119</td>
<td></td>
</tr>
<tr>
<td>S. parauberis</td>
<td>S. parauberis</td>
<td>12118</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** The sequences are represented in a tabular format with columns for the organism, the sequence details, length, and location.
Fig. 4-3. Alignment of the nucleotide sequences of Tn916 from S. parauberis NUF1049 (accession number AB468159) with those of S. agalactiae 2603V/R Tn916-like element (accession number AE009948), E. faecalis plasmid pCF10 Tn925 (accession number AY855841), E. faecalis DS16 Tn916 (accession number U09422), and S. suis SC84 Tn916 (accession number EF432727) generated using the ClustalW software. The asterisk (*) shows sequences that are the same as those of Tn916 sequence. The organism is given on the left of each sequence lane. The sequence number is given on the right of each sequence lane.
Fig. 4-4. Southern hybridization analysis of Tn916-like element. Chromosomal DNAs were digested with Sau3AI. The digests on the membrane were hybridized with DIG-labeled Tn916-like element probe. Lane M: *E. faecalis* CG110 (positive control); Lane 1-20: *S. parauberis* serotype II strains tested in this study.
Fig. 4-5. Southern blot hybridization analysis of Tn916-like element. Chromosomal DNAs were digested with HiII. The digests on the membrane were hybridized with DIG-labeled Tn916-like element probe. Lane 1, Pattern I of serotype II strain; Lane 2, Pattern II of serotype II strain; Lane 3, E. faecalisl CG110 (positive control of Tn916).
Fig. 4-6. (A) Structural analysis of *S. parauberis* serotype II strains (NUF1049) and *S. parauberis* serotype I strains (NUF1003) in the areas of the Tn916-like element ORF and the related flanking regions ORF (white arrow). The gray area indicates homology areas of *S. parauberis*. (B) Flanking sequences of the Tn916-like element in *S. parauberis*. Coupling sequences are indicated by boldface and underline.
General discussion
At the beginning of 21 century, sporadic streptococcal infections were identified in Kagawa, Ehime, Oita and Nagasaki Prefectures. The disease signs differed from those of *S. iniae* infection. The causative agent of the new outbreaks was identified as *S. parauberis*. Currently, the spread of *S. parauberis* has been across the western districts of Japan and emerged as a leading cause of streptococcal infection in Japanese flounder.

In this study, two points have been first reported. One is that *S. parauberis* were divided into two serotypes according to serological differentiations. The other one is that all of the serotype II strains (used in this study) harbored Tn916-like element with *tet*(M) resistance gene.

Serotyping of bacteria has been widely used as a tool in bacterial epidemiology. Based on the serological investigation in this study, the structurally and immunologically different capsular polysaccharides exist in the Japanese isolates of *S. parauberis*. So with the rabbit antisera reacting specifically with *S. parauberis* serotype I and II, serological methods such as slide agglutination could be conveniently used for clinical diagnosis of *S. parauberis* infections in Japanese flounder.

Comparison of MIC values of *S. parauberis* susceptible strains with those of *S. iniae* not only showed similar MIC values but also exhibit an intrinsic resistance to OA and SMMX. Unfortunately, the resistant determinants for OTC, that is the only qualified drug in treating Japanese flounder streptococcal infection, have spread among *S. parauberis* in different sites. But in our study the effectiveness of the common drugs, such as ABPC and EM, were proved for *S. parauberis* infection therapy.

The worldwide use of tetracyclines in the treatment of infections, as prophylactic agents, and as growth promoters, has facilitated the emergence and spread of acquired resistance (Roberts, 2005). Furthermore, resistant bacteria may rapidly appear in the host or environment after antimicrobial use, but they are slow to be lost, even in the absence of the selecting antimicrobial (Levy and Marshall, 2004). The PCR and hybridization results implied that acquisitions of OTC resistance in *S. parauberis* occur through different mechanisms. *S. parauberis* serotype I strains were detected highly resistant to OTC mediated by a conjugative R-plasmid encoding *tet*(S), while all the serotype II strains were intermediately resistant mediated by the Tn916-like element harboring *tet*(M). The data support the contention that Tn916-like element with *tet*(M) acts as an important selective factor that provides considerable advantages for the
emergence and spread of \textit{S. parauberis} serotype II.

Five EM/OTC-resistant serotype I and six serotype II strains, chosen as representative of the different prefectures, was used as donors in mating experiments. \textit{tet(S)} was the sole that transferred to the recipient from all of the \textit{tet(S)}-positive serotype I donors (only when OTC was used for selection), suggesting no linkage of the two resistance genes [\textit{erm(B)} and \textit{tet(S)}] in different genetic elements. Likewise, hybridization experiments indicated that the chromosomal band hybridized only with an \textit{erm(B)}-specific probe. The variabilities of the resistant genes insertions suggested the involvement of a number of different genetic elements carrying \textit{tet(S)} and \textit{erm(B)}, respectively, in serotype I strains. In conjugative transfer experiment no transconjugants were obtained when six \textit{S. parauberis} serotype II strains from different prefectures were used as donors and \textit{E. faecalis} FA2-2 as the recipient. Transfer frequency was calculated to be less than $10^{-9}$.

Based on the cloning and sequencing the \textit{erm(B)} gene containing region in serotype I resistant strains, it was found to show high homology with other known \textit{erm(B)} gene containing regions in plasmids. Moreover, the important structural feature found conserved in the relative regions was present in serotype I resistant strains.

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 \textit{S. 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 \textit{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, 1994). 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 S. 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 in chapter I. 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.
References


Rice, L. B. (1998): Tn916 family conjugative transposons and dissemination of


Infect. Immun., 62, 2806-2810.


Acknowledgements

I would like to express my deep and sincere gratitude to my supervisor Prof. Kinya Kanai, whose help, stimulating suggestions and encouragement helped me throughout my study. His great knowledge and logical way of thinking have been of great value not only for this period of research, but also for the future of my life.

I am grateful to Prof. Kazuma Yoshikoshi for giving valuable suggestions, helpful comments and extending all possible help whenever needed.

I am deeply indebted to Prof. Hideaki Morii and Associate Prof. Kentaro Kasama for their excellent guidance and invaluable advice.

I wish to express my sincere appreciation to Prof. Kenji Hara and Prof. Kiyoshi Osatomi for their invaluable support and helpful comments.

I would like to thank Prof. Yasuyoshi Ike (Graduate School of Medicine, Gunma University, Japan) and Prof. Takashi Aoki (Graduate School of Marine Science and Technology, Tokyo University of Marine Science and Technology, Japan) for providing *E. faecalis* FA2-2 and useful instructions.

I would like to give my special thanks to all my friends and lab mates for their help and cooperation

I would like to extend my very special thanks to the Japan Rotary Yoneyama Memorial Scholarship (Rotary Yoneyama Memorial Foundation, inc) for the scholarship assistance.

I would like to specially express my gratitude to Dr. Yue Xu who helps me in any time.

Last but not the least; I am very grateful for my parents and my special Japanese friends of Tanaka family for their love and moral support during my study abroad.

MENG Fei
要旨
ヒラメ由来 *Streptococcus parauberis*における薬剤耐性菌の出現と耐性化機構に関する研究

*Streptococcus parauberis* はウシの乳房炎の原因菌として知られ、魚類ではターボット *Scophthalmus maximus* のレンサ球菌症の原因菌としてヨーロッパではじめて報告された。ヨーロッパ以外では、韓国において眼球突出と鰓の壊死を病徵とするヒラメ *Paralichthys olivaceus* の *S. parauberis* 感染症が報告されている。わが国では 2000 年代に入ってヒラメの *S. parauberis* 感染症の発生が確認され、その後発生地域が拡大して発生率および被害率が高い傾向が各地で見られている。

現在、ヒラメのレンサ球菌症の治療薬としてはテトラサイクリン系の抗生物質のみが承認されているが、*S. parauberis* の薬剤感受性および耐性菌の出現についての報告はない。本研究では、日本各地で分離された *S. parauberis* 株について、代表株で作製したウサギ抗血清と凝集性およびテトラサイクリンを含む主要な抗菌剤に対する感受性を調べた。そして薬剤耐性株については、耐性化機構について分子生物学的検討を行った。

(第一章) 2002 年から 2007 年にかけて西日本各地のヒラメ養殖場で分離された *S. parauberis* 64 株は、すべて I 型（44 株）あるいは II 型（20 株）に分類された。血清型別した 64 株を供試菌株として、アンピシリン (ABPC)、カナマイシン (KM)、エリスロマイシン (EM)、リンコマイシン (LCM)、塩酸オキシテトラサイクリン (OTC)、クロラムフェニコール (CP)、オキソリン酸 (OA)、スルファモノメトキシン (SMMX)、トリメトプリム (TMP) の 9 種類の抗菌剤の最小発育阻止濃度 (MIC) を測定した。その結果、OA および SMMX については高い MIC 値を示したことから、*S. parauberis* が本来両薬剤に耐性であると考えられた。また、血清型が I 型の 44 株中 5 株が OTC と EM に高度耐性で、II 型は 20 株すべてが OTC 中等度耐性を示した。

(第二章) 見つかった OTC および EM 耐性株から、耐性遺伝子および耐性遺伝子をコードするトランスポゾンに関連する遺伝子の検出を試みた。その結果、I
型耐性株5株からはOTC耐性遺伝子tet(S)とEM耐性遺伝子erm(B)が検出され、
II型20株からはOTC耐性遺伝子tet(M)およびトランスポゾン916（Tn916）の
挿入酵素遺伝子intと切出し酵素遺伝子xisが検出された。このことから、II
型株にはTn916様の配列の存在が示唆された。

（第三章）血清型I型株から検出された耐性遺伝子のゲノム上の位置をサザン
ハイブリダイゼーションで調べた。OTC/EM耐性株からは約11kbpのプラスミド
が検出され、各耐性遺伝子をプローブとしたサザンハイブリダイゼーションの
結果、tet(S)はプラスミド上に、erm(B)は染色体DNA上にコードされているこ
とが判明した。I型耐性株5株に検出されたプラスミドはすべて受容菌
Enterococcus faecalisFA2-2に伝達され、HindIIIによる切断パターンは同じで
あった。このことから、プラスミドは伝達性であり、同じプラスミドであると
考えられた。染色体DNA上のerm(B)を含むHindIII断片をクローニングし、塩基
配列を調べたところ、その断片はerm(B)の上流と下流に331bpのリピート配
列を有し、4つのORFからなる断片であり、その塩基配列は他の球菌のプラス
ミドの配列と92％以上の相同性を示した。したがって、このerm(B)を含む配列
はプラスミド由来と推察された。

（第四章）血清型II型株にTn916様のトランスポゾンの存在が示唆されたこと
から、GenBankから取得したTn916の塩基配列に基づいて設計したプライマーを
用いて、II型株の染色体DNAを錬型にTn916の4つの部分に相当する配列のPCR
を試みた。その結果、予想される長さの増幅産物がすべてのII型株から得られ、
本Tn916様配列がTn916と極めて類似する遺伝子構造を有することが推察され
た。代表株について得られたPCR産物の塩基配列を調べたところ、他の細菌の
Tn916とはほぼ同じ配列であった。なお、Tn916様配列をプローブとしてサザンハイ
プリダイゼーションを行ったところ、SauIIIおよびHindIIIの切断ハイプリダイ
ゼーションパターンはII型株すべて同一であったが、HincIIの切断パターン
は2種類認められた。したがって、S. parauberisII型株由来のTn916様配列に
は配列に多様性があると考えられた。また、Tn916様配列に隣接する塩基配列を
調べたところ、Tn916様配列の挿入部位はA/Tリッチな場所であり、Tn916様配

92
列を持たない I 型株の同じ部位の塩基配列との比較から、Tn916 様配列には ATCATA の配列が付加されていることが判明した。本研究から、S. parauberis 血清型 I 型株には伝達性プラスミドにコードされた OTC 耐性遺伝子が、II 型株にはトランスポソンに保持された OTC 耐性遺伝子があることが明らかになった。ヒラメのレンサ球菌症ではオキシテトラサイクリン系薬剤が唯一使用できる治療薬であることから、耐性株の存在は S. parauberis 感染症の防除対策を考える上で重要な問題である。I 型株ではプラスミドによって耐性が広まる懸念があり、II 型株ではすべての株が耐性である可能性があることから治療は困難である。今後はテトラサイクリンに代わる治療薬の開発あるいはワクチン等の予防法の確立を目指すことが必要と思われるが、耐性化を助長しないよう、ヒラメ養殖におけるテトラサイクリン系薬剤の使用は慎重に行わなければならない。