Characterization of Drug Resistance in *Streptococcus parauberis* Isolated from Japanese Flounder

Fei Meng¹, Kinya Kanai^{2*} and Kazuma Yoshikoshi²

¹Graduate School of Science and Technology, Nagasaki University, Nagasaki 852-8521, Japan

²Faculty of Fisheries, Nagasaki University, Nagasaki 852-8521, Japan

(Received October 3, 2008)

ABSTRACT—Antimicrobial susceptibility of *Streptococcus parauberis* against nine drugs were investigated in 64 strains isolated from Japanese flounder *Paralichthys olivaceus* at different culture areas of western Japan, and resistance genes were determined by PCR and Southern blot hybridization. Five of the tested strains belonging to *S. parauberis* serotype I (44 strains) were resistant to oxytetracycline and erythromycin and were found to possess both *tet*(S) and *erm*(B) genes. Minimum inhibitory concentrations (MICs) of oxytetracycline and erythromycin were 128 and 512 µg/mL, respectively. Southern blot hybridization analysis showed that the *tet*(S) gene was encoded on an 11-kb conjugative plasmid, while the *erm*(B) gene resided on the chromosomal DNA. All of the examined *S. parauberis* serotype II strains (20 strains) were tetracycline-resistant (MIC = 32 µg/mL) and were found to possess *tet*(M) gene. Both *int* (integrase) and *xis* (excisase) genes were also detected in all of the serotype II strains, suggesting the presence of Tn916-related element in these strains. These results present a threat of difficulty in treatment of *S. parauberis* infection by tetracyclines, especially those caused by serotype II strains.

Key words: *Streptococcus parauberis*, *Paralichthys olivaceus*, Japanese flounder, drug resistance, *tet*(S), *tet*(M), *erm*(B)

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

For the control of streptococcal infections in Japanese flounder in Japan, the usage of tetracyclines is the only way approved by the Ministry of Agriculture, For-

estry 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). 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, 2) an allosteric disruption of tetracycline binding site(s), namely ribosomal protection (RP), and 3) 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 related to Gram-positive species *tet* genes, such as *tet*(K) and *tet*(L) efflux genes and *tet*(M), *tet*(O) and *tet*(S) RP genes (Chopra and Roberts, 2001; Roberts, 2005). Among these *tet*(M) has the widest host range up to 42 genera. The association of *tet*(M), *int* (integrase) and *xis* (excisase) genes characterizes the

* Corresponding author
E-mail: kanai@nagasaki-u.ac.jp

Tn916–Tn1545 family (Clewell *et al.*, 1995).

Investigation of the antimicrobial susceptibility of isolates is needed for the treatment of diseases, particularly susceptibility to tetracyclines in the case of *S. parauberis*. The aim of this study was to analyze the antimicrobial susceptibility of *S. parauberis* strains isolated from Japanese flounder in Japan. We also screened the resistance determinants among resistant strains.

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). Identification of the strains was performed by PCR analysis of *S. parauberis* 23S rDNA (Mata *et al.*, 2004), and serotyping was carried out by slide agglutination test with anti-*S. parauberis* type I and II rabbit antisera (Kanai *et al.*, 2009). Of the 64 strains, 44 and 20 were found to belong to serotype I and II, respectively. *Enterococcus faecalis* FA2-2 (Shiojima *et al.*, 1997) was used as a recipient for conjugation of plasmids, and *E. faecalis* CG110 containing Tn916 (Shimoji *et al.*, 1994) as a positive control for investigating the presence of Tn916 family transposons by PCR and Southern hybridization. *S. parauberis* and *E. faecalis* 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.

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) 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 $\mu\text{g/mL}$ for ABPC and EM and from 0.125 to 512 $\mu\text{g/mL}$ for CP, KM, LCM, OA, OTC, SMMX and TMP.

Screening for antimicrobial resistance and transposon-associated genes

The presence of antimicrobial resistance and transposon-associated genes were screened by PCR. Oligonucleotide primer sets and PCR condition for detection of genes are listed in Table 2. Macrolide- and tetracycline-resistant genes were tested on all resistant strains. They were also examined for the presence of *int*, *xis*, *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). Chromosomal and plasmid DNAs of each strain were prepared using Wizard Genomic DNA Purification kit (Promega) and QIAprep Miniprep kit (Qiagen), respectively, according to the manufacturer's protocols. PCR was performed using 50 ng of chromosomal or plasmid DNA as template, 0.5 μM of each primer, 5 μL of $10 \times \text{Ex Taq}$ buffer (Mg^{2+} plus), 8 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 distilled water. The reaction condition varied according to the primers used and the size of product.

Southern hybridization

Based on the results of PCR, resistant strains were applied for Southern blot hybridization analysis. Chromosomal DNAs (1.5 μg) or plasmid DNAs (500 ng) extracted from those strains were completely digested

Table 1. Number of *Streptococcus parauberis* strains used in this study

Prefecture	Serotype	Year						Total
		2002	2003	2004	2005	2006	2007	
Kagawa	I	5		3	4			12
	II	8						8
Ehime	I			2	16	1		19
	II							
Oita	I				4	4	5	13
	II		2		3	2	1	8
Kagoshima	I							
	II					1		1
Nagasaki	I							
	II				1	2		3
Total		13	2	5	28	10	6	64

Table 2. Primers used for PCR detection of genes

Target gene	Primer sequences (5' to 3')	PCR fragment size (bp)	Annealing temperature (°C)	Reference
<i>erm</i> (A)	GTTCAAGAACAATCAATACAGAG GGATCAGGAAAAGGACATTTTAC	420	52	Lina <i>et al.</i> , 1999
<i>erm</i> (B)	GTAAACAGTTGACGATATTCTCG CGTACCTTGGATATTCACCG	224*	50	Nagai <i>et al.</i> , 2001
<i>erm</i> (C)	GCTAATATTGTTTAAATCGTCAATTCC GGATCAGGAAAAGGACATTTTAC	359	52	Lina <i>et al.</i> , 1999
<i>mef</i> (A)	AGTATCATTAACTACTAGTGC TTCTTCTGGTACTAAAAGTGG	345	52	Sutcliffe <i>et al.</i> , 1996
<i>tet</i> (K)	TATTTTGGCTTTGTATTCTTTCAT GCTATACCTGTTCCCTCTGATAA	1,159	50	Giovanetti <i>et al.</i> , 2003
<i>tet</i> (L)	ATAAATTGTTTCGGGTCGGTAAT AACCAGCCAATAATGACAATGAT	1,077	50	Giovanetti <i>et al.</i> , 2003
<i>tet</i> (M)	GTAAATAGTGTCTTGGAG CTAAGATATGGCTCTAACAA	657*	57	Aarestrup <i>et al.</i> , 2000
<i>tet</i> (O)	AACTTAGGCATTCTGGCTCAC TCCCACTGTTCCATATCGTCA	515	55	Jeric <i>et al.</i> , 2002
<i>tet</i> (S)	ATCAAGATATTAAGGAC TTCTCTATGTGGTAATC	573*	55	Charpentier <i>et al.</i> , 1993
<i>int</i>	TGACACTCTGCCAGCTTTAC CCATAGGAAGTTGACGTTTCG	579	57	Barbeyrac <i>et al.</i> , 1996
<i>xis</i>	AGCAGACTGACATTCCTA GCGTCCAATGTATCTATAA	193	55	Amezaga <i>et al.</i> , 2002
<i>tnpA</i>	GCTTCCATGGGACTCGGGAC GCTCCCAATTAATAGGAGA	2,133	55	Poyart <i>et al.</i> , 2000
<i>tnpR</i>	CCAAGGAGCTAAAGAGGTCCC GTCCCAGTCCCATGGAAGC	1,547	55	Poyart <i>et al.</i> , 2000

* Also used to obtain a specific probe for hybridization.

with *Hind*III. Digested DNAs were resolved by electrophoresis in 0.7% agarose gel, stained with ethidium bromide and photographed. After depurination, denaturation and neutralization of the gel, DNAs were 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 *tet*(M), *tet*(S) or *erm*(B) probe which was gel-purified PCR fragment (Table 2) 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).

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.

Results

Drug susceptibilities

Fig. 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 ≤0.016 to 0.063 µg/mL and 0.5 to 1 µg/mL, and five strains were low resistant with MIC values of 0.5 to 1 µg/mL and 4 µg/mL, respectively.

For EM and OTC, the MICs of the 64 strains were categorized to three groups, that is, 39 strains were susceptible, 20 strains were intermediately resistant to EM and OTC and five strains were highly resistant to EM and OTC. The highly-resistant strains were ABPC/LCM-low resistant strains mentioned above. The EM/OTC-intermediately-resistant group consisted with sero-

Fig. 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.

Table 3. Detection of gene markers in *Streptococcus parauberis* resistant strains

Serotype	Strain no.	MIC to OTC ($\mu\text{g/mL}$)	Gene marker												
			<i>erm</i> (A)	<i>erm</i> (B)	<i>erm</i> (C)	<i>mef</i> (A)	<i>tet</i> (K)	<i>tet</i> (L)	<i>tet</i> (M)	<i>tet</i> (O)	<i>tet</i> (S)	<i>tnpR</i>	<i>tnpA</i>	<i>int</i>	<i>xis</i>
Type I	1	128	—	+	—	—	—	—	—	—	+	—	—	—	—
	2	128	—	+	—	—	—	—	—	—	+	—	—	—	—
	3	128	—	+	—	—	—	—	—	—	+	—	—	—	—
	4	128	—	+	—	—	—	—	—	—	+	—	—	—	—
	5	128	—	+	—	—	—	—	—	—	+	—	—	—	—
Type II	1	32	—	—	—	—	—	—	+	—	—	—	—	+	+
	2	32	—	—	—	—	—	—	+	—	—	—	—	+	+
	3	32	—	—	—	—	—	—	+	—	—	—	—	+	+
	4	32	—	—	—	—	—	—	+	—	—	—	—	+	+
	5	32	—	—	—	—	—	—	+	—	—	—	—	+	+
	6	32	—	—	—	—	—	—	+	—	—	—	—	+	+
	7	32	—	—	—	—	—	—	+	—	—	—	—	+	+
	8	32	—	—	—	—	—	—	+	—	—	—	—	+	+
	9	32	—	—	—	—	—	—	+	—	—	—	—	+	+
	10	32	—	—	—	—	—	—	+	—	—	—	—	+	+
	11	32	—	—	—	—	—	—	+	—	—	—	—	+	+
	12	32	—	—	—	—	—	—	+	—	—	—	—	+	+
	13	32	—	—	—	—	—	—	+	—	—	—	—	+	+
	14	32	—	—	—	—	—	—	+	—	—	—	—	+	+
	15	32	—	—	—	—	—	—	+	—	—	—	—	+	+
	16	32	—	—	—	—	—	—	+	—	—	—	—	+	+
	17	32	—	—	—	—	—	—	+	—	—	—	—	+	+
	18	32	—	—	—	—	—	—	+	—	—	—	—	+	+
	19	32	—	—	—	—	—	—	+	—	—	—	—	+	+
	20	32	—	—	—	—	—	—	+	—	—	—	—	+	+

type 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 $\mu\text{g}/\text{mL}$) than those from Kyushu island (Oita Prefecture) (1 to 2 $\mu\text{g}/\text{mL}$).

Detection of genes

erm(A), *erm(C)* or *mef(A)* was not detected in 25 EM-intermediately- and highly-resistant strains, while the five EM-highly-resistant strains gave the expected 224-bp PCR amplification product of *erm(B)*. *Tn917*-related genes, *tnpA* or *tnpR*, were not detected in any of the EM-resistant strains (Table 3).

Among 25 OTC-resistant strains, *tet(M)* and *tet(S)* genes were detected in 20 serotype II and five serotype I highly-resistant strains, respectively. *int* and *xis* genes of the *Tn916* family were also detected in 20 serotype II strains (Table 3). All of the 20 serotype II strains yielded the same PCR reactions for *int* and *xis* genes as *E. faecalis* CG110, the positive control of *Tn916* (Table 3).

Localization of resistance genes

PCR-amplified products of antimicrobial resistance genes, *tet(S)*, *erm(B)* and *tet(M)*, were used as probes to hybridize with the DNAs 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 kb (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. 2). The *erm(B)* probe hybridized with the chromosomal DNAs of the *tet(S)*-positive strains (Fig. 3). These five strains came from the same prefecture but were isolated at different aquaculture sites. For the 20 OTC-intermediately-resistant serotype II strains, a 7.2-kb *Hind*III-digested fragment of the chromosomal

Fig. 2. *Hind*III-digested plasmid DNAs from EM/OTC-highly-resistant *Streptococcus parauberis* serotype I strains (panel I) and Southern blot hybridization analysis of *Hind*III-digested plasmid DNAs with *tet(S)* probe (panel II). Lane M, DNA size marker (1 kb DNA ladder, New England Biolabs); Lanes 1 to 5, EM/OTC-highly-resistant *S. parauberis* serotype I strains No. 1 to 5.

Fig. 3. *Hind*III-digested plasmid (lanes 1–5) and chromosomal (lanes 6–10) DNAs from EM/OTC-highly-resistant *Streptococcus parauberis* strains (panel I) and Southern blot hybridization analysis of *Hind*III-digested plasmid (lanes 1–5) and chromosomal (lanes 6–10) DNAs with *erm(B)* probe (panel II). Lane M, DNA size marker (1 kb DNA ladder, New England Biolabs); Lanes 1 to 5, EM/OTC-highly-resistant *S. parauberis* strains No. 1 to 5.

DNAs showed strong signal with the *tet(M)* probe (data not shown). The reference strain, *E. faecalis* CG110, also yielded the positive signal.

Conjugal transfer of tetracycline-resistant plasmid

Conjugation experiments were carried out on the five OTC-highly-resistant strains with the plasmid-free recipient strain, *E. faecalis* FA2-2. In transconjugants from each donor, the *tet(S)*-positive plasmids were detected, and the 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.

Discussion

In this study MIC values of susceptible strains of *S. parauberis* against ABPC (≤ 0.016 to $0.063 \mu\text{g}/\text{mL}$), EM (≤ 0.016 to $0.063 \mu\text{g}/\text{mL}$) and OTC (0.5 to $2 \mu\text{g}/\text{mL}$) were comparable to those of *S. iniae*, another important pathogen of streptococciosis in Japanese flounder, whose MIC values were reported to be 0.00625 to $0.025 \mu\text{g}/\text{mL}$, $0.05 \mu\text{g}/\text{mL}$ and $0.39 \mu\text{g}/\text{mL}$, respectively (Sako, 1993). It is interesting that MIC values of 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 might result from variations in history of tetracycline consumptions in each geographical area.

In this study multiple drug resistant strains of *S. parauberis* were found among the serotype I strains tested. They were isolated at different aquaculture areas in one prefecture and were highly resistant to EM and OTC. 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 *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* that 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 where the EM/OTC-highly-resistant strains were isolated. 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 suggests that the conjugative plasmid encoding *tet*(S) is conserved and disseminated in western Japan.

In this study *tet*(M), *int* and *xis* genes were detected in all of serotype II strains. These genes are known to associate with Tn916-Tn1545 family transposons (Clewell *et al.*, 1995; Chopra and Roberts, 2001). So it is supposed that Tn916-related element presents on the chromosomes of *S. parauberis* serotype II strains. We are now investigating the genetic structure of the Tn916-related element. All of the serotype II strains also showed intermediately resistant to EM. In streptococci, some of mobile genetic elements carrying *tet*(M) also harbor macrolide resistance gene, *erm*(B), such as Tn1545 and Tn6002 (Cochetti *et al.*, 2008). In this study, however, detection of *S. parauberis* serotype II strains for EM resistance genes was failed. Accordingly, these strains may have different mechanism for EM resistance.

Our results showed the presence of different mechanisms in acquisition of OTC resistance between *S. parauberis* serotype I and II. Serotype I strains contained a conjugative plasmid encoding *tet*(S) gene, while serotype II strains possessed Tn916-related element harboring *tet*(M) gene. Due to the dissemination of the OTC-resistant strains it is likely that the therapy of *S. parauberis* infection in cultured Japanese flounder will become more difficult because of only relying on tetracyclines. In order to control the disease further investigation is needed on the mechanism of transfer and spread of the tetracycline resistance determinants. Likewise, preventive measures aiming at different serotypes of *S. parauberis* should be developed to minimize the usage of tetracyclines, such as immunoprophylaxis.

Acknowledgements

We 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. We also thank staffs of Kagawa Prefectural Fisheries Experimental Station, Ehime Prefectural Fish Disease Control Center, Ehime Prefectural Chuyo Fisheries Experimental Station, Fisheries Research Institute, Oita Prefectural Agriculture, Forestry and Fisheries Research Center and Kagoshima Prefectural Fisheries Technology and Development Center for their providing *S. parauberis* strains.

References

- Aarestrup, F. M., Y. Agerso, P. Gerner-Smidt, M. Madsen and L. B. Jensen (2000): Comparison of antimicrobial resistance phenotypes and resistance genes in phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecium* from humans in the community, broilers, and pigs in Denmark. *Diagn. Microbiol. Infect. Dis.*, **37**, 127–137.
- Amezaga, M. R., P. E. Carter, P. Cash and H. McKenzie (2002): Molecular epidemiology of erythromycin resistance in *Streptococcus pneumoniae* isolates from blood and noninvasive sites. *J. Clin. Microbiol.*, **40**, 3313–3318.
- Aoki, T. and A. Takahashi (1987): Class D tetracycline resistance determinants of R plasmids from the fish pathogens *Aeromonas hydrophila*, *Edwardsiella tarda*, and *Pasteurella piscicida*. *Antimicrob. Agents Chemother.*, **31**, 1278–1280.
- Aoki, T., K. Takami and T. Kitao (1990): Drug resistance in a non-hemolytic *Streptococcus* sp. isolated from cultured yellowtail *Seriola quinqueradiata*. *Dis. Aquat. Org.*, **8**, 171–177.
- Baeck, G. W., J. H. Kim, D. K. Gomez and S. C. Park (2006): Isolation and characterization of *Streptococcus* sp. from diseased flounder (*Paralichthys olivaceus*) in Jeju Island. *J. Vet. Sci.*, **7**, 53–58.
- Barbeyrac, B. D., M. Dupon, P. Rodriguez, H. Renaudin and C. Bebear (1996): A Tn1545-like transposon carries the *tet*(M) gene in tetracycline resistant strains of *Bacteroides ureolyticus* as well as *Ureaplasma urealyticum* but not *Neisseria gonorrhoeae*. *J. Antimicrob. Chemother.*, **37**, 223–232.
- Charpentier, E., G. Gerbaud and P. Courvalin (1993): Characterization of a new class of tetracycline-resistance gene *tet*(S) in *Listeria monocytogenes* BM4210. *Gene*, **131**, 27–34.
- Chopra, I. and M. Roberts (2001): Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.*, **65**, 232–260.
- Clewell, D. B., S. E. Flannagan and D. D. Jaworski (1995): Unconstrained bacterial promiscuity: The Tn916–Tn1545 family of conjugative transposons. *Trends Microbiol.*, **3**, 229–236.
- Cochetti, I., E. Tili, M. Mingoia, P. E. Varaldo and M. P. Montanari (2008): *erm*(B)-carrying elements in tetracycline-resistant pneumococci and correspondence between Tn1545 and Tn6003. *Antimicrob. Agents Chemother.*, **52**,

- 1285–1290.
- Doménech, A., J. F. Fernández-Garayzábal, C. Pascual, J. A. García, M. T. Cutuli, M. A. Moreno, M. D. Collins and L. Dominguez (1996): Streptococcosis in cultured turbot, *Scophthalmus maximus* (L.), associated with *Streptococcus parauberis*. *J. Fish Dis.*, **19**, 33–38.
- Fukuda, Y. (2003): Drug susceptibility of fish-pathogenic bacteria isolated from cultured marine fishes in Oita prefecture from 1990 to 2001. *Bull. Oita Inst. Mar. Fish. Sci.*, **4**, 25–50. (In Japanese)
- Giovanetti, E., A. Brenciani, R. Lupidi, M. C. Roberts and P. E. Varaldo (2003): Presence of the *tet*(O) gene in erythromycin- and tetracycline-resistant strains of *Streptococcus pyogenes* and linkage with either the *mef*(A) or the *erm*(A) gene. *Antimicrob. Agents Chemother.*, **47**, 2844–2849.
- Japanese Society of Antimicrobials for Animals, the Committee (2003): Revision of the determination method of the minimum inhibitory concentration (MIC) of antimicrobials against bacteria isolated from animals. *Fish Pathol.*, **39**, 58–67.
- Jeric, P. E., H. Lopardo, P. Vidal, S. Arduino, A. Fernandez, B. E. Orman, D. O. Sordelli and D. Centron (2002): Multicenter study on spreading of the *tet*(M) gene in tetracycline-resistant *Streptococcus* group G and C isolates in Argentina. *Antimicrob. Agents Chemother.*, **46**, 239–241.
- Kanai, K., M. Yamada, F. Meng, I. Takahashi, T. Nagano, H. Kawakami, S. Matsuoka, Y. Fukuda, I. Takami, H. Nakano, T. Hirae, K. Shutou and T. Honma (2009): Serological differentiation of *Streptococcus parauberis* strains isolated from cultured Japanese flounder in Japan. *Fish Pathol.*, **44**, 10–16. (In Japanese with English abstract)
- Kim, S. R., L. Nonaka and S. Suzuki (2004): Occurrence of tetracycline resistance genes *tet*(M) and *tet*(S) in bacteria from marine aquaculture sites. *FEMS Microbiol. Lett.*, **237**, 147–156.
- Kusuda, R. and F. Salati (1993): Major bacterial diseases affecting mariculture in Japan. *Ann. Rev. Fish Dis.*, **3**, 65–85.
- Lina, G., A. Quaglia, M. E. Reverdy, R. Leclercq, F. Vandenesch and J. Etienne (1999): Distribution of genes encoding resistance to macrolides, lincosamides, and streptogramins among staphylococci. *Antimicrob. Agents Chemother.*, **43**, 1062–1066.
- Maki, T., I. Hirono, H. Kondo and T. Aoki (2008): Drug resistance mechanism of the fish-pathogenic bacterium *Lactococcus garvieae*. *J. Fish Dis.*, **31**, 461–468.
- Mata, A. I., A. Gibello, A. Casamayor, M. M. Blanco, L. Dominguez and J. F. Fernandez-Garayzábal (2004): Multiplex PCR assay for detection of bacterial pathogens associated with warm-water streptococcosis in fish. *Appl. Environ. Microbiol.*, **70**, 3183–3187.
- Nagai, K., Y. Shibasaki, K. Hasegawa, T. A. Davies, M. R. Jacobs, K. Ubukata and P. C. Appelbaum (2001): Evaluation of PCR primers to screen for *Streptococcus pneumoniae* isolates and beta-lactam resistance, and to detect common macrolide resistance determinants. *J. Antimicrob. Chemother.*, **48**, 915–918.
- Nonaka, L. and S. Suzuki (2002): New Mg²⁺-dependent oxytetracycline resistance determinant *tet* 34 in *Vibrio* isolates from marine fish intestinal contents. *Antimicrob. Agents Chemother.*, **46**, 1550–1552.
- Poyart, C., G. Quesne, P. Acar, P. Berche and P. Trieu-Cuot (2000): Characterization of the Tn916-like transposon Tn3872 in a strain of *Abiotrophia defectiva* (*Streptococcus defectiva*) causing sequential episodes of endocarditis in a child. *Antimicrob. Agents Chemother.*, **44**, 790–793.
- Roberts, M. C. (2005): Update on acquired tetracycline resistance genes. *FEMS Microbiol. Lett.*, **245**, 195–203.
- Sako, H. (1993): In vitro and vivo susceptibility of *Streptococcus iniae*, isolated from diseased fish, to antimicrobial agents. *SUISANZOSHOKU*, **41**, 397–404. (In Japanese with English abstract)
- Sano, T. (1998): Control of fish disease, and the use of drugs and vaccines in Japan. *J. Appl. Ichthyol.*, **14**, 131–137.
- Shaw, J. H. and D. B. Clewell (1985): Complete nucleotide sequence of macrolide-lincosamide-streptogramin B-resistance transposon Tn917 in *Streptococcus faecalis*. *J. Bacteriol.*, **164**, 782–796.
- Shimoi, Y., Y. Yokomizo, T. Sekizaki, Y. Mori and M. Kubo (1994): Presence of a capsule in *Erysipelothrix rhusiopathiae* and its relationship to virulence for mice. *Infect. Immun.*, **62**, 2806–2810.
- Shiojima, M., H. Tomita, K. Tanimoto, S. Fujimoto and Y. Ike (1997): High-level plasmid-mediated gentamicin resistance and pheromone response of plasmids present in clinical isolates of *Enterococcus faecalis*. *Antimicrob. Agents Chemother.*, **41**, 702–705.
- Sutcliffe, J., T. Grebe, A. Tait-Kamradt and L. Wondrack (1996): Detection of erythromycin-resistant determinants by PCR. *Antimicrob. Agents Chemother.*, **40**, 2562–2566.
- Toranzo, A. E., J. M. Cutrín, S. Núñez, J. L. Romalde and J. L. Barja (1995): Antigenic characterization of *Enterococcus* strains pathogenic for turbot and their relationship with other Gram-positive bacteria. *Dis. Aquat. Org.*, **21**, 187–191.
- Toranzo, A. E., B. Magariños and J. L. Romalde (2005): A review of the main bacterial fish diseases in mariculture systems. *Aquaculture*, **246**, 37–61.
- Williams, A. M. and M. D. Collins (1990): Molecular taxonomic studies on *Streptococcus uberis* types I and II. Description of *Streptococcus parauberis* sp. nov. *J. Appl. Bacteriol.*, **68**, 485–490.