Vol. 8: 171–177, 1990 | DISEASES OF AQUATIC ORGANISMS Dis. aquat. Org. | Published July 12

Drug resistance in a non-hemolytic *Streptococcus* sp. isolated from cultured yellowtail *Seriola quinqueradiata*

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ABSTRACT: The antibacterial activities of 19 chemotherapeutic agents were tested against 370 strains of a non-hemolytic *Streptococcus* sp. pathogenic for a marine fish, the yellowtail *Seriola quinqueradiata*. The strains were isolated from cultured yellowtail in 1986 and 1987. Sixty-two of the tested strains showed resistance to macrolide antibiotics (MLs), lincomycin (LIM), tetracycline (TC), and chloramphenicol (CP). These resistant strains were classified into intermediate- and high-level resistance to MLs, LIM, TC, or CP. Intermediate-level resistance was constitutive and the responsible resistance determinants were not transferred to *S. faecalis* JH2-2 or to *Streptococcus* sp. SSS-1, the latter a yellowtail isolate. On the other hand, high-level resistance to MLs, LIM, and TC and to MLs, LIM, and CP was inducible and transferable to *S. faecalis* JH2-2 and to *Streptococcus* sp. SSS-1. These drug-resistant strains of the non-hemolytic *Streptococcus* sp., capable of transferring their resistance, were first isolated in cultured yellowtail in various areas of Japan.

INTRODUCTION

Epizootics due to a *Streptococcus* sp. in cultured yellowtail *Seriola quinqueradiata* have been observed frequently in various world locations since 1974 and have caused serious economic damage to fish culture operations in Japan. The biochemical characteristics of the non-hemolytic *Streptococcus* sp. causing the infection closely resembled those of *Streptococcus faecalis* and *S. faecium* (Kusuda et al. 1976). However, this single species of *Streptococcus* did not belong to any of the described Lancefield groups (Kitao 1982).

For the treatment of streptococcal infections, the chemotherapeutics commonly used are macrolide antibiotics (MLs, e.g. erythromycin, spiramycin, kitasamycin, and josamycin) and lincomycin. MLs and lincomycin have shown strong antibacterial activity against *Streptococcus* sp. isolated from cultured yellowtail (Aoki et al. 1983). Recently, these antibiotics have on occasion been ineffective in treating the streptococcal infections. This phenomenon suggested the appearance of MLs and lincomycin-resistant *Streptococcus* sp. strains in yellowtail farms.

The resistance mechanism of Gram-positive cocci to MLs in human pathogens is well known (Saito et al. 1969, Lai & Weisblum 1971, Lai et al. 1973, Skinner et al. 1983, Weisblum et al. 1971). The ML resistance of streptococci has been divided into 2 types: inducible and constitutive (Hyder & Streitfeld 1973). ML resistance in Lancefield Group A, B, and D streptococci is transferable from the ML-resistant strains to sensitive ones via conjugation (Clewell 1981).

In the present study, strains of non-hemolytic *Streptococcus* sp. collected from marine fish farms were tested for their sensitivity to various chemotherapeutics, in particular MLs and lincomycin, to determine whether drug resistance explains the growing incidence of treatment failures on yellowtail farms. The drug-resistant strains detected were further studied for the transfer properties of their drug resistance.

MATERIALS AND METHODS

Bacterial strains. Three hundred and seventy strains of a non-hemolytic *Streptococcus* sp., collected in 1986 and 1987 from diseased, cultured yellowtail in various districts of Japan (Fig. 1), were used in the drug susceptibility tests.

Drug-resistant strains of *Streptococcus* sp. (Strains EH8632, EH8702, KG8703, ME8631, and ME8714) were selected at random and used for ML, tetracycline

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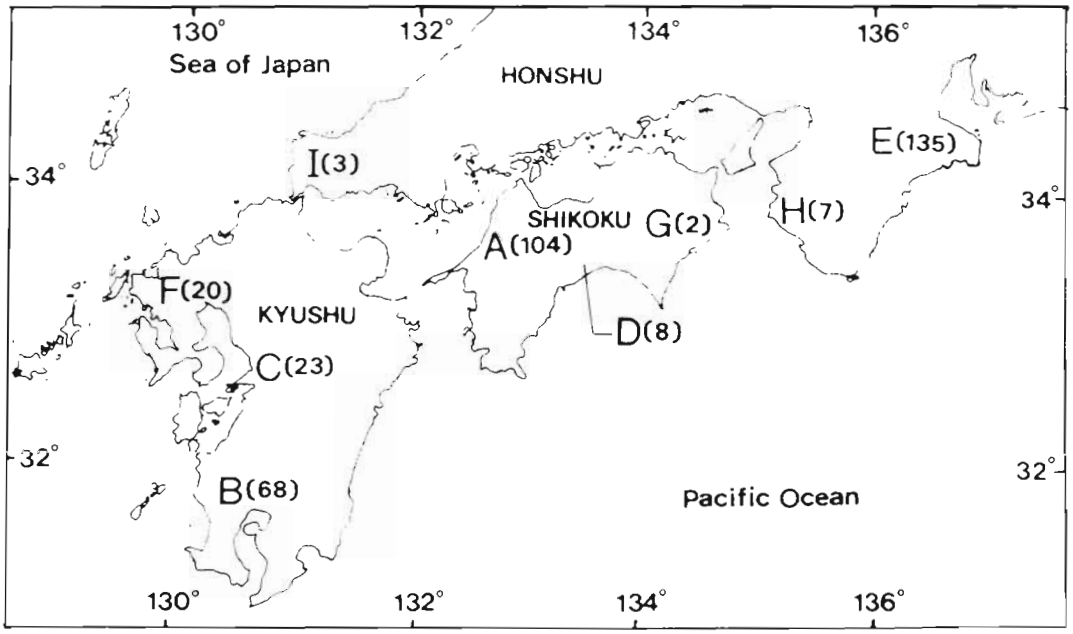

Fig. 1. Map of sampling areas. Letters represent the sampling locations: (A) Ehime; (B) Kagoshima; (C) Kumamoto; (D) Kochi; (E) Mie; (F) Nagasaki; (G) Tokushima; (H) Wakayama; and (I) Yamaguchi. Numbers in parentheses indicate number of isolated *Streptococcus* sp. strains at each location

(TC), and chloramphenicol (CP) induction tests and also as donors to permit estimates of the frequency of transfer of their drug-resistance determinants. *S. faecalis* JH2-2 (Jacob & Hobbs 1974) and *Streptococcus* sp. SSS-1 (a derivative of *Streptococcus* sp. EH8727, highly resistant to streptomycin) were used as recipients for the transfer of drug-resistance determinants. The antibiotic resistance characteristics of the strains used are shown in Table 1.

Media. Heart infusion broth (Nissui) containing 0.2% glucose (HIG broth) and HIG containing 1.5% agar (HIG agar) (Nissui) were employed for the cultivation of the *Streptococcus* sp. and for selecting transconjugant cells.

A sensitivity-disk medium (modified Mueller Hinton agar) (Nissui), supplemented with 0.2% glucose, was used for testing susceptibility to trimethoprim (TMP) and ormethoprim (OMP). HIG agar was used for tests with the remaining antibiotics and furazolidone (NF).

Drug sensitivity test. Minimal inhibitory concentrations (MICs) against *Streptococcus* sp. were determined by the serial 2-fold dilution method in agar standardized by the Japan Society of Chemotherapy (Japan Society of Chemotherapy 1981). The MIC value was determined after incubation for 20 h at 25°C.

The drugs used for the sensitivity tests were erythromycin (EM), oleandomycin (OM), triacethyloleandomycin (TAO), josamycin (JM), kitasamycin (KTM), midecamycin (MDM), spiramycin (SPM), lincomycin (LIM), ampicillin (ABP), cephalexin (CEX), cefazolin (CEZ), chloramphenicol (CP), tetracycline (TC), doxycycline (DOTC), streptomycin (SM), kanamycin (KM), NF, TMP, and OMP.

Induction of drug resistance. Five strains of *Strep-*

Table 1 *Streptococcus* sp. Properties of strains used in this study. See text for abbreviations

Strain	Resistance phenotype	Source
Donor strains		
Streptococcus sp. ME8631	EM, OM, SPM, LIM, TC	Yellowtail
Streptococcus sp. ME8714	EM, OM, SPM, LIM, TC	Yellowtail
Streptococcus sp. EH8632	MLs, LIM, TC	Yellowtail
Streptococcus sp. EH8702	MLs, LIM, TC	Yellowtail
Streptococcus sp. KG8703	MLs, LIM, CP	Yellowtail
Recipient strains		
S. faecalis JH2-2	Fusidic acid, rifampicin	Jacob & Hobbs (1974)
Streptococcus sp. SSS-1	SM	Derivative of EH8727

tococcus sp. resistant to MLs, LIM, TC, or CP (Table 1) were cultured separately in HIG broth overnight. One ml of each culture was inoculated into 100 ml of the same medium. After 30 min incubation, 6 ml of each culture were added to 2 ml of fresh HIG broth with or without various concentrations of drugs. The concentration incorporated into HIG were as follows: 0.05 or 0.1 μg ml^{-1} of EM and LIM, and 0.5 or 1.0 μg ml^{-1} of OM, SPM, TC, and CP for the induction of resistance. After 1 h induction with one drug at a time, drug resistance of induced cells was examined in HIG broth containing various concentrations of the appropriate drugs. Mixtures were aerobically incubated at 25 °C and the turbidity measured at 30 min intervals at a wave length of 610 nm (OD_{610}) until control cells (drug-free culture) reached an OD_{610} of 0.5.

Antibiotic-resistant mutants. SM-resistant mutants were isolated by the method of Horodniceanu et al. (1979). *Streptococcus* sp. EH8727, which was isolated from yellowtail, was grown in 100 ml of HIG broth overnight. Cells were harvested by centrifugation and resuspended in 1 ml of HIG broth. The culture was then plated on HIG agar containing 8000 μg ml^{-1} SM. Mutant colonies arose at a frequency of approximately 1×10^{-10} per viable cell and the SM-resistant strain was designated as SSS-1.

Mating procedures. Mating in a liquid medium was performed as described by Dunny & Clewell (1975). Cells of 5 antibiotic-resistant donor strains of *Streptococcus* sp. and cells of the recipient *S. faecalis* JH2-2 and *Streptococcus* sp. SSS-1 strains were cultured separately overnight in HIG broth. Donor and recipient cultures were mixed together at a ratio of 1 donor to 10 recipient cells (0.5 ml) in a test tube. Mixtures with JH2-2 were incubated at 37 °C, and those with strain SSS-1 at 30 °C, for 4 h. Cultures were spread on HIG agar containing antibiotics appropriate for the selection of resistant recipients.

Mating on a membrane filter was done according to Burdett (1980), and 0.5 ml of the donor strain cells was mixed with 4.5 ml of cells of a recipient strain in a test tube. One ml of this mixture was collected on a millipore membrane filter (HAWP, 0.45 μm pore size) and the filter was then placed on HIG agar at 37 or 30 °C for 18 h. After incubation, the cells on the filter were suspended in 5 ml of HIG broth. The suspension was then plated on HIG agar containing antibiotics appropriate for the selection of trans-conjugants.

Antibiotics present in selective media were added at the following levels: EM = 0.4 μg ml^{-1} (donor was ME8631 or ME8714) or 25 μg ml^{-1}; TC = 4 μg ml^{-1} (donor was ME8631 or ME8714) or 10 μg ml^{-1}; CP = 10 μg ml^{-1}; fusidic acid (Fus) = 25 μg ml^{-1}; rifampicin (Rif) = 50 μg ml^{-1}; and SM = 1000 μg ml^{-1}.

The frequencies of transfer were expressed in terms of the number of trans-conjugants per number of donor strain cells.

RESULTS

Minimal inhibitory concentrations

The distribution of MICs of 19 chemotherapeutic agents against 370 strains of non-hemolytic *Streptococcus* sp. is shown in Fig. 2. The strains could be placed into 3 apparently discrete groups according to their MIC values to EM. These groups were termed sensitive, intermediate-level, and high-level resistant with MIC values ranging from 0.05 to 0.8, 1.6 to 3.1, and > 100 μg ml^{-1}, respectively. Of the 370 strains tested, 308 were sensitive to EM, 40 were intermediate-level, and the remaining 22 strains were high-level resistant to the drug. The strains were also separable into 3 groups of 308 sensitive, 40 intermediate-level, and 22 high-level resistant with regard to the pattern of the MICs of OM. In the case of TAO, JM, KTM, and MDM, the strains fell into 2 groups: 348 strains were sensitive to these chemically related drugs, and the remaining 22 strains were high-level resistant. No strains with intermediate-level resistance to these drugs were found. With SPM and LIM, the strains formed 3 groups: 308 strains were sensitive, 40 strains intermediate, and the remaining 22 were high-level resistant. The MIC values of LIM, EM, and TAO were less than 0.8 μg ml^{-1} for 90 % of strains tested (Table 2). The 40 strains showing intermediate-level resistance to EM, OM, SPM, and LIM were the same, and the 22 strains with high-level resistance to MLs and LIM were also the same.

The MIC values of ABP ranged from 0.2 to 1.6 μg ml^{-1}, and no ABP-resistant strains were detected. CEX was ineffective against the tested strains but CEZ was moderately effective. All strains isolated in 1986 showed sensitivity to CP, and 9 of 219 strains in 1987 were resistant to CP. Thirteen strains had a high resistance to TC, and 40 strains were moderately resistant – their MIC values ranged from 3.1 to 6.2 μg ml^{-1}. The MIC values of DOTC were slightly lower than those of the related compound TC. The MIC values of the aminoglycosides SM and KM ranged from 12.5 to 100 μg ml^{-1}. NF, TMP, and OMP showed little activity against *Streptococcus* sp. (Table 2).

Induction of drug resistance

As shown in Table 3, the resistance levels of ME8631 and ME8714 encoded with intermediate-level resistance to EM, OM, SPM, and LIM were not induced after exposure to EM, OM, SPM, or LIM. High-level resist-

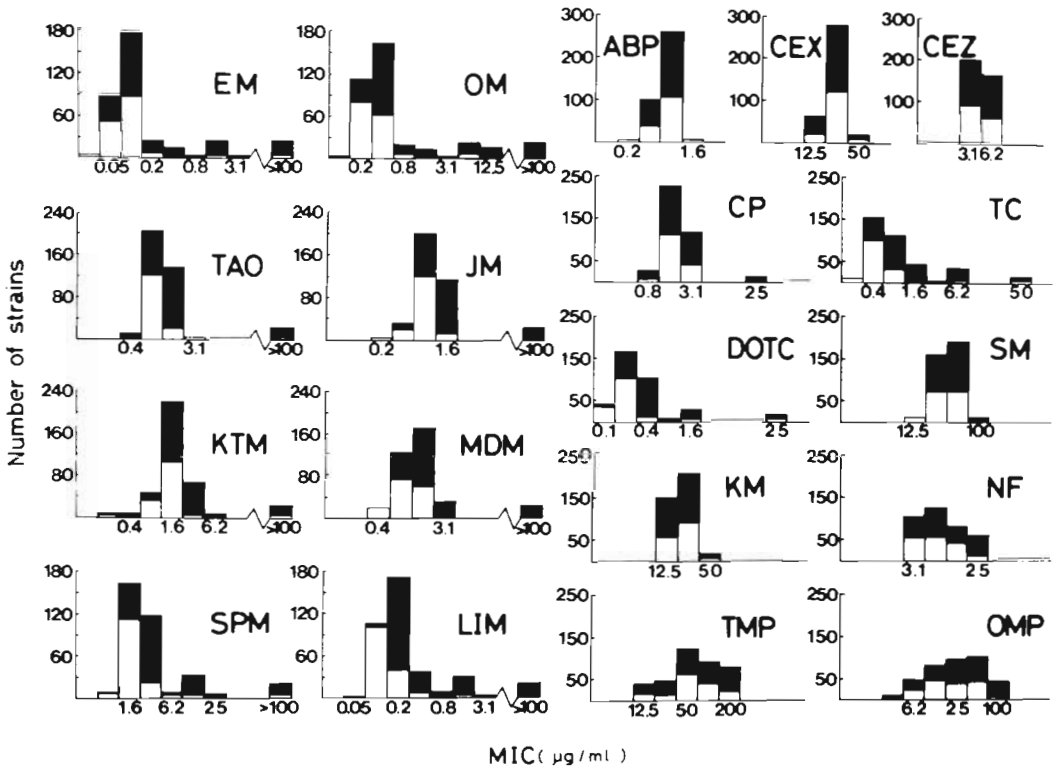

Fig. 2. *Streptococcus* sp. Minimal inhibitory concentrations (MICs) of various chemotherapeutics against naturally occurring non-hemolytic strains. *Streptococcus* sp. strains isolated in 1986 (□) and 1987 (■). Abbreviations are: (EM) erythromycin; (OM) oleandomycin; (TAO) triacetyloleandomycin; (JM) josamycin; (KTM) kitasamycin; (MDM) midecamycin; (SPM) spiramycin; (LIM) lincomycin; (ABP) ampicillin; (CEX) cephalexin; (CEZ) cefazolin; (CP) chloramphenicol; (TC) tetracycline; (DOTC) doxycycline; (SM) streptomycin; (KM) kanamycin; (NF) furazolidone; (TMP) trimethoprim; (OMP) ormethoprim

Table 2. *Streptococcus* sp. Minimal inhibitory concentrations (MICs) of chemotherapeutics for strains used in this study. See Fig. 2 for abbreviations. MIC for inhibition of 50 and 90 % of the isolates (22 high-level resistant strains not included)

Drug	Range	MIC (μg ml^{-1}) 50 %	90 %
EM	0.025– 3.1	0.32	0.36
OM	0.1–12.5	1.47	1.64
TAO	0.4– 3.1	0.67	0.74
JM	0.2– 1.6	1.01	1.12
KTM	0.2– 6.2	1.85	2.05
MDM	0.4– 3.1	1.38	1.53
SPM	0.8–25	4.15	4.61
LIM	0.05– 3.1	0.30	0.33
ABP	0.2– 1.6	0.70	0.78
CEX	12.5–50	24.29	26.99
CEZ	3.1– 6.2	4.51	5.01
CP	0.8– 3.1	2.02	2.24
TC	0.2– 6.2	1.27	1.41
DOTC	0.1– 1.6	0.36	0.40
SM	12.5–100	39.63	44.03
KM	12.5– 50	20.88	23.20
NF	3.1– 25	9.60	10.67
TMP	12.5–200	86.86	96.51
OMP	3.1–100	34.25	38.06

Table 3. *Streptococcus* sp. Type of induction of macrolide and lincomycin resistance. See Fig. 2 for abbreviations. (I: inducible, C: constitutive)

Strain	Inducer EM	OM	SPM	LIM
EH8632	I	I	I	I
EH8702	I	I	I	I
KG8703	I	I	I	I
ME8631	C	C	C	C
ME8714	C	C	C	C

ance in EH8632, EH8702, and KG8703 to MLs and LIM was induced after exposure to EM, OM, SPM, or LIM.

The TC resistance levels of ME8631 and ME8714 were not increased after induction with 0.5 μg ml^{-1} of TC. On the other hand, the resistance levels of EH8632 and EH8702 were increased by exposure to concentrations of TC equal to between 0.5 and 1.0 of the TC MIC value. Fig. 3 shows the results for Strains ME8714 and EM8702. The CP resistance of Strain KG8703 was increased to its resistance level by exposure to 1.0 μg ml^{-1} CP (data not shown).

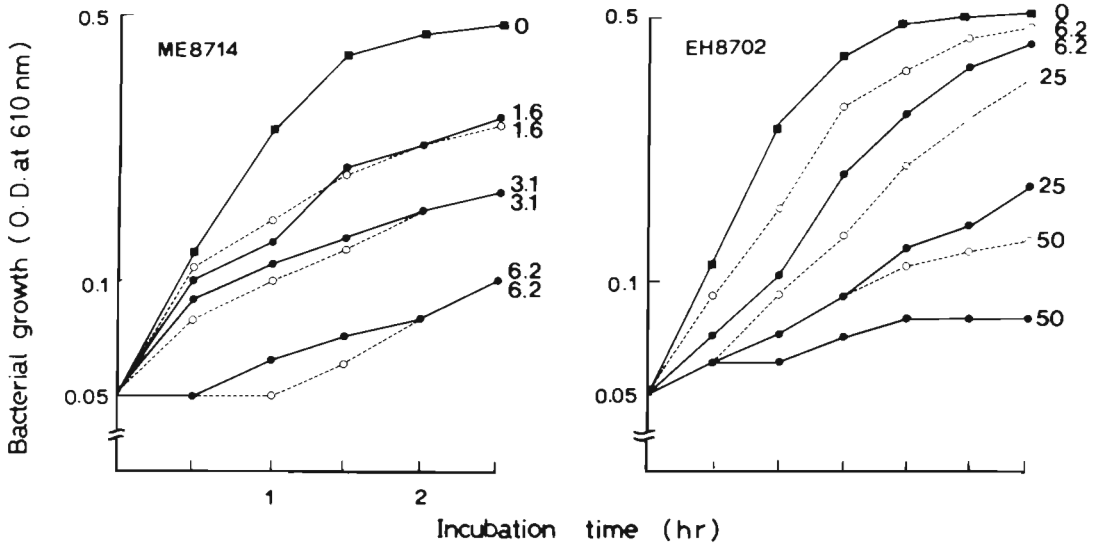

Fig. 3. *Streptococcus* sp. Induction of tetracycline resistance in Strain ME8714, but not in Strain EM8702. Cells were grown in HIG broth at 25°C with (----) or without (——) inducing antibiotics (0.5 or 1.0 μg tetracycline ml^{-1}) for 1 h and were then challenged with various concentrations of tetracycline (0, 1.6, 3.1, 6.2, 25, and 50 μg ml^{-1})

Transferability by conjugation

Determinants of middle-level resistance to MLs, LIM, and TC were not transferred from ME8631 and ME8714 to recipient strains of *Streptococcus faecalis* JH2-2 and *Streptococcus* sp. SSS-1 (Table 4). On the other hand, 13 strains exhibiting high resistance to MLs, LIM, and TC transferred their drug resistance to JH2-2 and SSS-1 by mixed cultivation. Transferable drug resistance was found in 9 strains that showed high resistance to MLs, LIM, and CP. It was found that all the drug resistance markers of MLs, LIM, TC, and MLs, LIM, CP were transferable together.

The transfer frequencies of drug-resistant determinants from *Streptococcus* sp. EH8632, EH8702, and KG8703 strains to the recipients JH2-2 and SSS-1 ranged from 10^{-6} to 10^{-9} using the liquid medium mating method, and from 10^{-3} to 10^{-5} using the membrane filter method. The frequencies of transfer of drug resistance determinants to *Streptococcus* sp. SSS-1 were higher than those to *S. faecalis* JH2-2.

DISCUSSION

This is the first report documenting the occurrence of drug resistance in strains of pathogenic *Streptococcus* sp. isolated from cultured yellowtail *Seriola quinqueradiata*. The drug-resistant Gram-positive strains described here appeared first in marine fish farms and their appearance is likely attributable to the extensive use of chemotherapeutics in fish farms. A similar situation has already been reported on with respect to the Gram-negative fish pathogens which were found at high frequency in fish farms in Japan (Aoki et al. 1977). Due to this increased resistance to antibiotics it is likely that streptococcal infections in cultured yellowtail will become more difficult to combat using chemotherapy.

The drug resistance of the non-hemolytic *Streptococcus* sp. in this study was classified into 2 types: (1) an intermediate-level resistance to MLs, LIM, and TC whose determinants were constitutive and non-transferable; and (2) a high-level resistance to MLs, LIM, and TC or CP whose determinants were inducible and transferable. These 2 types of drug-resistance determinants in *Streptococcus* sp. were found in various areas of Japan. The difference in the resistance mechanisms of intermediate level resistance and high-level resistance to MLs and LIM in the genus *Streptococcus* sp. has become a subject of considerable interest to us. The determinants of middle-level resistance in *Streptococcus* sp. strains were easily deleted by culturing continuously in a drug-free medium (unpubl. data). This loss in resistance also occurred with strains in which the MLs resistance was induced in vitro (unpubl. data). Studies on the mechanism of middle-level resistance to MLs, LIM, and TC are currently underway.

The high-level resistance to MLs, LIM, and TC, and to MLs, LIM, and CP was transferred simultaneously to the recipient strains *Streptococcus faecalis* JH2-2 and *Streptococcus* sp. SSS-1, and drug resistance was stable in these recipient cells. Transfer frequencies of drug resistance determinants from resistant strains of *Streptococcus* sp. to *S. faecalis* JH2-2 and *Streptococcus* sp. SSS-1 were nearly the same value, independent

Table 4. *Streptococcus* sp. Transfer frequencies of the drug resistance determinants in various strains. See Fig. 2 for abbreviations. Values in parenthesis are no. strains with both drug resistance markers/no. tested strains

Donor	Recipient	Drug	Frequencies of transfer	
			Liquid mating	Filter mating
ME8631	JH2-2[a]	EM	$<5.8 \times 10^{-9}$	$<6.0 \times 10^{-8}$
		TC	$<5.8 \times 10^{-9}$	$<6.0 \times 10^{-8}$
	SSS-1[b]	EM	$<7.4 \times 10^{-9}$	$<1.4 \times 10^{-8}$
		TC	$<7.4 \times 10^{-9}$	$<1.4 \times 10^{-8}$
ME8714	JH2-2	EM	$<1.1 \times 10^{-9}$	$<6.0 \times 10^{-8}$
		TC	$<1.1 \times 10^{-9}$	$<6.0 \times 10^{-8}$
	SSS-1	EM	$<8.8 \times 10^{-9}$	$<1.0 \times 10^{-8}$
		TC	$<8.8 \times 10^{-9}$	$<1.0 \times 10^{-8}$
EH8632	JH2-2	EM	8.5×10^{-9} (20/20)	3.1×10^{-5} (100/100)
		TC	7.4×10^{-9} (20/20)	3.7×10^{-5} (100/100)
	SSS-1	EM	1.7×10^{-7} (20/20)	3.6×10^{-3} (100/100)
		TC	1.8×10^{-7} (20/20)	4.8×10^{-3} (100/100)
EH8702	JH2-2	EM	4.1×10^{-9} (20/20)	5.8×10^{-4} (100/100)
		TC	3.1×10^{-9} (20/20)	7.7×10^{-4} (100/100)
	SSS-1	EM	1.8×10^{-7} (20/20)	3.9×10^{-3} (100/100)
		TC	2.6×10^{-7} (20/20)	5.3×10^{-3} (100/100)
KG8703	JH2-2	EM	9.8×10^{-8} (30/30)	4.1×10^{-4} (100/100)
		CP	3.3×10^{-7} (100/100)	3.5×10^{-4} (100/100)
	SSS-1	EM	4.7×10^{-7} (50/50)	4.1×10^{-3} (100/100)
		CP	3.4×10^{-6} (100/100)	4.0×10^{-3} (100/100)

[a] *Streptococcus faecalis* strain
[b] Non-hemolytic *Streptococcus* sp. strain

of the antibiotics selected for each donor strain. The drug-resistant determinants might be located on the R-plasmid or on a transposon. A transferable R-plasmid in streptococci was first detected in a human pathogenic strain of *S. faecalis* in 1974 (Jacob & Hobbs 1974). *S. faecalis* strain JH1 harbored 2 transferable plasmids, pJH1 and pJH2, which encoded for multiple drug resistance and for hemolysin-bacteriosin, respectively (Jacob et al. 1975).

On the other hand, the conjugative transfer of antibiotic resistance determinants can occur in the absence of plasmid DNA when the resistance genes are associated with conjugative transposons (Clewell 1981, Franke & Clewell 1981). The conjugative transposons transfer in filter mating between a wide range of streptococcal species and other Gram-positive bacteria (Nida & Cleary 1983, Kathariou et al. 1987, Weiser & Rubens 1987).

Further work is needed to determine the transfer mechanism of drug resistant determinants in these fish pathogens and to relate it to the mechanisms described for *Streptococcus* species causing human diseases.

Acknowledgements. We thank Dr T. Miyazaki of Mie University, and the staff of the Prefectural Experimental Fisheries Stations for kindly supplying the strains of non-hemolytic *Streptococcus* sp. We also thank Dr D. B. Clewell (University of Michigan, Michigan, USA) for providing the *S. faecalis* JH2-2 strains.

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Responsible Subject Editor: Dr T Evelyn, Nanaimo, B. C., Canada

Manuscript first received: October 27, 1988
Revised version accepted: May 15, 1990