Conjugative transferability of drug resistances in the fish pathogen, *Photobacterium damselae* subsp. *piscicida*

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Conjugative transferability of drug resistances from the donor *Photobacterium damselae* subsp. *piscicida* strains that exhibited different resistance patterns to the recipient *Escherichia coli* K-12 χ1037 rifanpicin-resistant mutant strain was investigated. The resistances to ampicillin (Ap), chloramphenicol (Cm), erythromycin (Em), kanamicin (Km), sulfamonomethoxine (Su), tetracycline (Tc) and trimethoprim (Tmp) were transferred via the 100 kb plasmid at the transfer frequency of 10^{-3}-10^{-4} and/or the 50 kb plasmid at the frequency of 10^{-6}. Regarding the donors that did not carry or transfer Ap resistance, all transferable drug resistances in each of the donors were transferred via only the 100 kb plasmid. Regarding the donors that transferred Ap resistance, the resistances in each donor were separately transferred via the 50 kb plasmid carrying Ap, Cm, Em and/or Tc resistances and the 100 kb plasmid bearing Km, Su and/or Tc resistances. In this case, the transconjugants selected with Ap, Cm, Em and/or Tc usually harbored the 100 kb plasmid in addition to the 50 kb plasmid, because the frequency of the 100 kb plasmid was very high as compared to that of the 50 kb plasmid. However, all transferable resistances in each donor were also transferred via a plasmid of approximately 100 kb which might have fused with the 100 and 50 kb plasmids. The plasmids observed in the donors were of almost the same size as the 100 kb plasmid but were different from the 50 kb plasmid in the transconjugants.

Key Words: *Photobacterium damselae* subsp. *piscicida*, drug resistance, conjugative transferability, transfer frequency, transferable R plasmid, transconjugant

INTRODUCTION

In Japan, fish culture developed rapidly from about 1950 and fish diseases also increased with the development of fish culture. Chemotherapeutic studies for fish diseases started from about 1955. Presently, various antimicrobial compounds have been widely used as feed additives to farmed fish or administered directly into fish pond water for prevention or treatment of bacterial diseases. The extensive use of drugs has resulted in an increase of drug-resistant strains of bacterial fish pathogens.[1~7] Drug resistance is mostly attributed to the presence of transferable R plasmids that have been regarded to be responsible for the drug resistance of various fish pathogenic bacteria.[8~20] The evolution of multiple drug resistances has made it difficult to prevent bacterial infection by chemoprophylaxis and chemotherapy, which in turn has caused serious economical damage in fish farms. Studies on conjugative transferability of drug resistances in fish pathogenic bacteria are very important for the prudent use of antimicrobial compounds for controlling bacterial fish diseases.

Photobacterium damselae subsp. *piscicida* comb. nov. (formerly, *Pasteurella piscicida*) is well known as the causative agent of pseudotuberculosis in cultured yellowtail *Seriola quinqueradiata* in Japan. The disease is a septicemia in which acute cases exhibit only a few pathological signs. Internally, granulomatous-like deposits may develop on the kidney and spleen. These deposits comprise many grayish-white bacterial colonies of 0.5-1.0 mm^2 in size.[21] Purulent material may accumulate in the abdominal cavity.[22] The disease has become of considerable economic importance, causing significant losses in farmed yellowtail. Since its initial recognition in yellowtails (during the summer of 1969),[23] pseudotuberculosis appears to have spread to other fish species, including red sea bream[24] and black sea bream.[25,26] Transferable drug resistances of *P. damselae* subsp. *piscicida* have been detected in multiple drug resistant strains and a close relationship between

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the increasing number of resistant strains and the extensive use of chemotherapeutics in yellowtail farms has been suggested by Aoki and his coworkers.[14,15,20]

In the previous paper,[27] the conjugative transferability of drug resistances in *P. damselae* subsp. *piscicida* was described from a viewpoint with chroramphenicol resistance. In the present paper, the conjugal transfer of drug resistances, the transfer frequency of drug resistances, transferable R plasmids and drug resistances encoding on the transferable R plasmids in *P. damselae* subsp. *piscicida* are reported in regard to all drug resistances. Moreover, plasmid DNA harboring in *P. damselae* subsp. *piscicida* was analyzed to know the relation with R plasmid DNA transferred from the bacteria.

MATERIALS AND METHODS

Bacteria, media and growth conditions

One hundred and eighty-three isolates of *Photobacterium damselae* subsp. *piscicida* were isolated from diseased yellowtail *Seriola quinqueradiata* in various areas of Japan between 1984 and 1994 (see the previous paper[27] in detail). The transconjugants were obtained from mating with the donor *P. damselae* subsp. *piscicida* strains and the recipient *Escherichia coli* K-12 χ1037 rifampicin-resistant (Rp^r) mutant strain (see the previous paper[27] in detail).

P. damselae subsp. *piscicida* was incubated in brain heart infusion (BHI) broth (Difco, Becton-Dickinson, Sparks, Maryland, USA) containing 2% NaCl at 28℃ and *E. coli* in Luria-Bertani (LB) broth (1% bacto tryptone (Difco), 0.5% bacto yeast extract (Difco), 1% NaCl, pH 7.5) at 37℃. Mueller-Hinton medium (Difco) containing 2% NaCl and 1.5% agar was used for the drug susceptibility test.

Conjugal transfer assay

Each donor strain was incubated under shaking conditions in BHI broth for 9 h and *E. coli* recipient strain in LB broth for 8 h. Aliquots (0.5 mL each) of donor and recipient cultures were mixed in 50-mL Erlenmeyer flask and 4 mL of equal volumes each of BHI and LB broths were added to the flask. Mating was performed at 28℃ for 3 h. 0.1 mL of 10-fold serial dilutions of mating mixture was spread on BTB-lactose agar with Rp 50 μg/mL and each selected drug (exhibited drug resistance in *P. damselae* subsp. *piscicida*): ampicillin (Ap) 100 μg/mL; chloramphenicol (Cm) 25 μg/mL; erythromycin (Em) 100 μg/mL; kanamicin (Km) 100 μg/mL; nalidixic acid (Na) 200 μg/mL; sulfamonomethoxine (Su) 1600 μg/mL; tetracycline (Tc) 25 μg/mL; and trimethoprim (Tmp) 400 μg/mL. In addition, the drug concentrations were decided from MIC values against the *E. coli* recipient strain.[27] Colonies growing in this double-inhibitor-supplemented medium after 24 to 48 h of incubation at 37℃ were scored as presumptive transconjugants, and the frequency of transfer was calculated as the number of transconjugants per initial number of donors.

The transconjugants from mating were also used for isolation of transferable R plasmids.

Drug susceptibility test of transconjugants

Ten or more transconjugants (colonies) selected with individual drugs in mating with each of the donors were picked and tested for antibiotic resistance. The culture was grown overnight in Mueller-Hinton broth and diluted to about 10^6 cells/mL using buffered saline with gelatin (BSG) solution (0.85% NaCl, 0.03% KH_2PO_4, 0.06% Na_2HPO_4, 0.01% gelatin) in accordance with the method of the Japanese Society of Chemotherapy.[28] The diluted culture was inoculated using a microplanter (3 mm in diameter and 4 mL for 1 microplanter, Toyo Sokki Co. Ltd., Japan) on Mueller-Hinton agar with Rp 50 μg/mL and each selected drug mentioned above. Growth was recorded after 24 to 48 h of incubation at 37℃.

Plasmid isolation and gel electrophoresis

Plasmids were extracted according to the method of Kado and Liu[29] and electrophoresed on 0.7% agarose gel. In addition, lysozyme was used in combination with alkaline SDS (sodium dodecyl sulfate) solution for lysis of *P. damselae* subsp. *piscicida*. Approximate molecular sizes of plasmids in the donors and transconjugants were calculated using reference plasmids R27 (167 kb), R100-1 (100 kb), RP4 (56 kb), pLA2917 (22 kb), pMSG-cat (8.4 kb), pBR322 (4.361 kb), pUC119 (3.162 kb) and pHSG398 (2.227 kb).

RESULTS

Transferability of drug resistance

Transfer frequency of drug resistances transferred from the donor *P. damselae* subsp. *piscicida* strains to the recipient *E. coli* K-12 χ1037 Rp^r mutant strain is shown in Table 1. The resistances to Su, Km, Tc, Cm, Ap, Em and Tmp were transferred from the donors to the recipient. However, the transferability of drug resistances was different with the pattern and level of the resistances. The resistances to Su, Km, Tc, Cm, Em

Table 1. Tranfer frequency of drug resistances transferred from *Photobacterium damselae* subsp. *piscicida* strains that showed different resistance patterns to *Escherichia coli* K-12 χ 1037 rifanpicin-resistant mutant strain

Drug resistance pattern of donors		Donor strain	Transfer frequency of drug resistances transferred						
			Su	Km	Tc	Cm	Ap	Em	Tmp
1	Su	PP8513	Not transferred						
2	Su Km Cm	PP8509	Not transferred						
3	Su Na Tmp	PP9301	2.6×10^{-4}	/	/	/	/	/	-
4	Su Tc Cm	PP8401	1.6×10^{-4}	/	1.9×10^{-4}	2.5×10^{-4}	/	/	/
5	Su Km Tc	PP9212	7.3×10^{-4}	7.1×10^{-4}	7.8×10^{-4}	/	/	/	/
6	Su Km Na Tmp	PP9005	4.1×10^{-4}	5.9×10^{-4}	/	/	/	/	-
7	Su Km Tc Tmp	PP9017	1.9×10^{-4}	2.5×10^{-4}	3.1×10^{-4}	/	/	/	-
8	Su Km Tc Cm	PP8702	1.6×10^{-3}	9.7×10^{-4}	2.4×10^{-3}	9.2×10^{-4}	/	/	/
9	Su Km Tc Cm Na	PP9214	3.3×10^{-4}	5.9×10^{-4}	5.4×10^{-4}	2.1×10^{-4}	/	/	/
10	Su Km Tc Cm Ap	PP8808	3.2×10^{-3}	2.6×10^{-3}	2.3×10^{-3}	2.2×10^{-3}	-	/	/
11	Su Km Tc Cm Em	PP9202	3.0×10^{-4}	3.1×10^{-4}	5.4×10^{-4}	2.8×10^{-4}	/	1.8×10^{-4}	/
12	Su Km Tc Cm Na Tmp	PP8906	1.3×10^{-4}	1.0×10^{-4}	2.1×10^{-4}	2.1×10^{-4}	/	/	-
13	Su Km Tc Cm Ap Tmp	PP8836	3.0×10^{-4}	1.8×10^{-4}	3.2×10^{-4}	3.4×10^{-4}	-	/	-
14	Su Km Tc Cm Em Tmp	PP9401	1.1×10^{-4}	1.5×10^{-4}	2.4×10^{-4}	1.9×10^{-4}	/	5.8×10^{-4}	2.2×10^{-4}
15 (1)	Su Km Tc Cm Ap Na	PP8608	8.2×10^{-4}	5.1×10^{-4}	3.6×10^{-4}	6.7×10^{-4}	-	/	/
15 (2)	Su Km Tc Cm Ap Na	PP8511	6.2×10^{-4}	3.8×10^{-4}	6.6×10^{-4}	3.0×10^{-6}	4.3×10^{-6}	/	/
16	Su Km Cm Ap Na Tmp	PP8912	1.2×10^{-4}	2.0×10^{-4}	/	6.7×10^{-6}	6.2×10^{-6}	/	-
17	Su Km Tc Cm Ap Na Tmp	PP9010	2.5×10^{-4}	1.2×10^{-4}	6.7×10^{-6}	1.4×10^{-6}	$4.4. \times 10^{-6}$	/	-
18	Su Km Tc Cm Ap Em Na Tmp	PP9105	2.4×10^{-3}	3.4×10^{-3}	1.4×10^{-6}	3.5×10^{-6}	5.4×10^{-6}	7.6×10^{-6}	-

Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Km, kanamicin; Na, nalidixic acid; Su, sulfamonomethoxine; Tc, tetracycline; Tmp, trimethoprim. Symbols: -, not transferred; /, not determined.

and Tmp were transferred from the donors containing resistances to Tc and/or Na Tmp. However, Tmp resistance transfer was restricted to strains carrying high-level resistance (pattern 14: see the previous paper[27]). The resistance to Ap was transferred from the donors containing resistances to Na Tc and/or Na Tmp (patterns 15(2)-18). However, Ap resistance could not transfer in all the strains bearing resistances to Na Tc (pattern 15(1)). On the other hand, the resistances to Na and Tmp that exhibited intermediate-resistance (see the previous paper[27]) were not transferred from any of the resistant strains. Moreover, none of the resistances transferred from those donors that did not possess resistances to Tc, Na Tc or Na Tmp (patterns 1 and 2).

The drug resistances in the donors that did not carry (patterns 3-9, 11, 12 and 14) or transfer Ap resistance (patterns 10, 13 and 15(1)) were transferred with almost the same frequencies (10^{-3}-10^{-4}, mostly 10^{-4}), regardless of the differences in the transferred resistances and the tested strains. On the other hand, the transfer frequencies of drug resistances in the donors that transferred Ap resistance (patterns 15(2)-18) were different among individual drug resistances transferred from the same donors. Moreover, the frequencies were also different with the pattern of drug resistances being carried in the donors. That is, in the donors carrying resistances to Na Tc (pattern 15(2)), Su, Km and Tc resistances were transferred at the frequencies of about 10^{-4} and Cm and Ap resistances at those of about 10^{-6}. In the donors carrying resistances to Na Tmp (patterns 16-18), Su and Km resistances were transferred at the frequencies of about 10^{-3}-10^{-4} and Tc, Cm, Ap and/or Em resistances at those of about 10^{-6}.

Drug resistances of transconjugants

Drug resistances of the transconjugants obtained in mating with the donor *P. damselae* subsp. *piscicida* stains that exhibited different resistance patterns and the recipient *E. coli* K-12 χ1037 Rp^r mutant strain are shown in Table 2. Regarding the donors that did not carry (patterns 3-9, 11, 12 and 14) or transfer Ap resistance (patterns 10, 13 and 15(1)), every transconjugant selected with individual drugs in mating with each of the donors exhibited the same resistances, regardless of the drug resistance patterns of the donors. Regarding the donors that transferred Ap resistance (patterns 15(2)-18), respective transconjugants selected with individual drugs in mating with each donor expressed different drug resistances and the resistances also differed with drug resistance patterns of the donors as below.

For the donors carrying resistances to Na Tc (the pattern 15(2); transferred Su, Km, Tc, Cm and Ap resistances), the transconjugants selected with Su, Km

Table 2. Drug resistance of transconjugants obtained in mating with the donor *Photobacterium damselae* subsp. *piscicida* strains that showed different resistance patterns and the recipient *Escherichia coli* K-12 χ 1037 rifanpicin-resistant mutant strain

Drug resistance pattern of donors (Number of strain studied)		Transconjugant selected with respective drugs	Drug resistance of respective transconjugants						
			Su	Km	Tc	Cm	Ap	Em	Tmp
1	Su	Not transferred							
2	Su Km Cm	Not transferred							
3	Su Na Tmp (2)	Su-transconjugant	+	/	/	/	/	/	-
4	Su Tc Cm (1)	Su, Tc, Cm- 〃	+	/	+	+	/	/	/
5	Su Km Tc (15)	Su, Km, Tc- 〃	+	+	+	/	/	/	/
6	Su Km Na Tmp (9)	Su, Km- 〃	+	+	/	/	/	/	-
7	Su Km Tc Tmp (1)	Su, Km, Tc- 〃	+	+	+	/	/	/	-
8	Su Km Tc Cm (39)	Su, Km, Tc, Cm- 〃	+	+	+	+	/	/	/
9	Su Km Tc Cm Na (11)	Su, Km, Tc, Cm- 〃	+	+	+	+	/	/	/
10	Su Km Tc Cm Ap (37)	Su, Km, Tc, Cm- 〃	+	+	+	+	-	/	/
11	Su Km Tc Cm Em (2)	Su, Km, Tc, Cm, Em- 〃	+	+	+	+	/	+	/
12	Su Km Tc Cm Na Tmp (1)	Su, Km, Tc, Cm- 〃	+	+	+	+	/	/	-
13	Su Km Tc Cm Ap Tmp (2)	Su, Km, Tc, Cm- 〃	+	+	+	+	-	/	-
14	Su Km Tc Cm Em Tmp (9)	Su, Km, Tc, Cm, Em, Tmp- 〃	+	+	+	+	/	+	+
15 (1)	Su Km Tc Cm Ap Na (5)	Su, Km, Tc, Cm- 〃	+	+	+	+	-	/	/
15 (2)	Su Km Tc Cm Ap Na (10)	Su, Km, Tc- 〃	+	+	+	-	-	/	/
		Cm, Ap- 〃	-	-	-	+	+	/	/
		Cm, Ap- 〃	+	+	+	+	+	/	/
16	Su Km Cm Ap Na Tmp (1)	Su, Km- 〃	+	+	/	-	-	/	-
		Cm, Ap- 〃	-	-	/	+	+	/	-
		Cm, Ap- 〃	+	+	/	+	+	/	-
17	Su Km Tc Cm Ap Na Tmp (29)	Su, Km- 〃	+	+	-	-	-	/	-
		Tc, Cm, Ap- 〃	-	-	+	+	+	/	-
		Tc, Cm, Ap- 〃	+	+	+	+	+	/	-
18	Su Km Tc Cm Ap Em Na Tmp (2)	Su, Km- 〃	+	+	-	-	-	-	-
		Tc, Cm, Ap- 〃	-	-	+	+	+	-	-
		Tc, Cm, Ap- 〃	+	+	+	+	+	-	-
		Cm, Em- 〃	-	-	-	+	-	+	-
		Cm, Em- 〃	+	+	-	+	-	+	-

Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Km, kanamicin; Na, nalidixic acid; Su, sulfamonomethoxine;Tc, tetracycline; Tmp, trimethoprim. Symbols: +, resistant; -, sensitive; /, not determined.

or Tc (Su, Km or Tc transconjugants, respectively) in mating with each donor were usually resistant to Su, Km and Tc. However, Su and Km or Su, Km and Tc transconjugants very rarely showed resistances to Su and Km or Su, Km, Tc and Cm, respectively, in the confirmatory experiments. On the other hand, the transconjugants selected with Cm or Ap (Cm or Ap transconjugant, respectively) were resistant to Su, Km, Tc, Cm and Ap in large part (for colonies obtained) and to Cm and Ap in small part.

For the donors bearing resistances to Na Tmp (patterns 16-18), Su and Km transconjugants carried resistances to Su and Km, regardless of drug resistance patterns of the donors. On the other hand, the transconjugants other than Su and Km transconjugants bore in large part (for colonies obtained) all resistances that were transferred from the donors and in small part the resistances other than Su and Km resistances.

Plasmid DNA in transconjugants

Agarose gel electrophoresis of plasmid DNA in Su, Km, Tc, Cm, Em, Tmp and Ap transconjugants obtained in mating with the donor *P. damselae* subsp. *piscicida* strains and the recipient *E. coli* K-12 χ 1037 Rp^r mutant strain are shown in Figs.1 to 5, respectively. In addition, the electrophoresis of plasmids are shown for all the strains that harbored different plasmids in molecular size or number in spite of being the same transconjugants.

The transconjugants harbored each or both of approximately 100 or 50 kb plasmid. The difference in plasmids in molecular size and number was dependent on the transfer frequency of drug resistances, which are encoded on the plasmids. That is, the transconjugants carrying drug resistances that transferred at the frequency of about 10^{-4} (see Table 1) harbored a plasmid of approximately 100 kb; that is, lanes A-Q in Fig. 1 (Su transcongugant), lanes A-O in Fig. 2 (Km transconjugant), lanes A-L in Fig. 3 (Tc transconjugant), A-I in Fig. 4 (Cm transconjugant), A and B in Fig. 5 (Em transconjugant) and E in Fig. 5 (Tmp transconjugant). On the other hand, the transconjugants bearing the resistances that transferred at the frequency of about 10^{-6} (see Table 1) usually possessed a

plasmid of approximately 50 kb or both the 50 and 100 kb plasmids; that is, M-P in Fig. 3 (Tc transconjugant), J-Q in Fig. 4 (Cm transconjugant) and F-M in Fig. 5 (Ap transconjugant). Moreover, another 100 kb plasmid (the presumptive cointegrate which the 50 and 100 kb plasmids might have fused with each other because the 100 kb plasmid exhibited the resistances carried by both of 100 and 50 kb plasmids: see DISCUSSION in detail) was uncommonly harbored in the transconjugants; that is, Q in Fig. 3 (Tc transconjugant), R and S in Fig. 4 (Cm transconjugant), N and O in Fig. 5 (Ap transconjugant). In addition, chromosomal DNA and unknown DNA (found occasionally) banded before and after the 50 kb plasmid, respectively.

Besides, regarding the donors that did not carry (patterns 3-9, 11, 12 and 14) or transfer Ap resistance (patterns 10, 13 and 15(1)), the transconjugants from mating with the donors harbored only the 100 kb plasmid encoding for all resistances transferred from each donor (see also Table 2). On the other hand, regarding the donors that transferred Ap resistance (patterns 15(2)-18), the transconjugants from mating with the donors bore the 50 kb plasmid encoding for Ap, Cm, Em and/or Tc resistances and/or the 100 kb plasmid encoding for Km, Su and/or Tc resistances. In addition, the transconjugants harboring the two plasmids carried all the transferred resistances. In some cases, the transconjugant possessing only another 100 kb plasmid (Q in Fig. 3, R and S in Fig. 4, N and O in Fig. 5) also bore all the transferable resistances in each donor (see also Table 2).

Plasmid DNA in *P. damselae* subsp. *piscicida*

Agarose gel electrophoresis of plasmid DNA isolated from the donor *P. damselae* subsp. *piscicida* strains is shown in Fig. 6. The results obtained from one strain each (exception; from two strains harboring only Su resistance) in the same resistance patterns are presented. The plasmids in the strains expressing only Su resistance (lanes B and C) were different from the strains exhibiting multiple resistances in molecular size

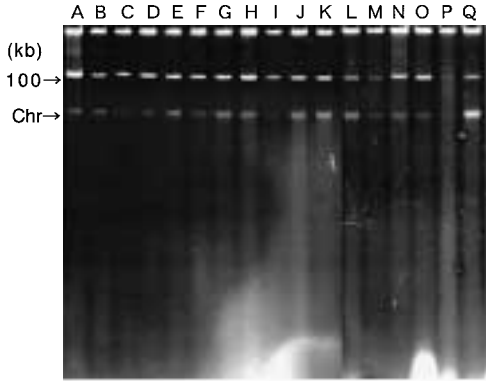

Fig. 1 Agarose gel electrophoresis of plasmid DNA from transconjugants selected with sulfamonomethoxine (Su) in mating with the donor *Photobacterium damselae* subsp. *piscicida* strains and the recipient *Escherichia coli* K-12 χ 1037 rifampicin-resistant mutant strain. Lane A, pPDP9301 (plasmid DNA of the transconjugant obtained from mating with the donor PP9301 strain (pattern 3, Table 1)) with resistance to Su; lane B, pPDP8401 (pattern 4); lane C, pPDP9212 (pattern 5); lane D, pPDP9005 (pattern 6); lane E, pPDP9017 (pattern 7); lane F, pPDP8702 (pattern 8); lane G, pPDP9214 (pattern 9); lane H, pPDP8808 (pattern 10); lane I, pPDP9202 (pattern 11); lane J, pPDP8906 (pattern 12); lane K, pPDP8836 (pattern 13); lane L, pPDP9401 (pattern 14); lane M, pPDP8608 (pattern 15(1)); lane N, pPDP8511 (pattern 15(2)); lane O, pPDP8912 (pattern 16); lane P, pPDP9010 (pattern 17); lane Q, pPDP9105 (pattern 18). Abbreviations: Chr, chromosomal DNA.

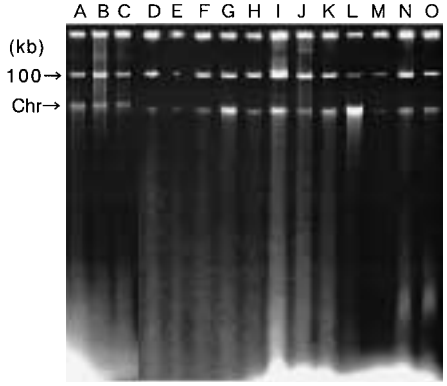

Fig. 2 Agarose gel electrophoresis of plasmid DNA from transconjugants selected with kanamycin (Km) in mating with the donor *Photobacterium damselae* subsp. *piscicida* strains and the recipient *Escherichia coli* K-12 χ 1037 rifampicin-resistant mutant strain. Lane A, pPDP9212 (plasmid DNA of the transconjugant obtained from mating with the donor PP9212 strain (pattern 5, Table 1)) with resistances to Su Km Tc; lane B, pPDP9005 (pattern 6); lane C, pPDP9017 (pattern 7); lane D, pPDP8702 (pattern 8); lane E, pPDP9214 (pattern 9); lane F, pPDP8808 (pattern 10); lane G, pPDP9202 (pattern 11); lane H, pPDP8906 (pattern 12); lane I, pPDP8836 (pattern 13); lane J, pPDP9401 (pattern 14); lane K, pPDP8608 (pattern 15(1)); lane L, pPDP8511 (pattern 15(2)); lane M, pPDP8912 (pattern 16); lane N, pPDP9010 (pattern 17); lane O, pPDP9105 (pattern 18). Abbreviations: Chr, chromosomal DNA.

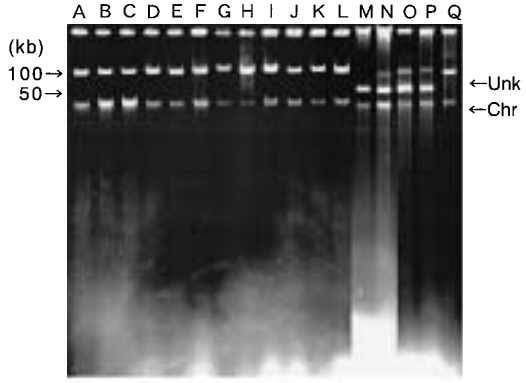

Fig. 3 Agarose gel electrophoresis of plasmid DNA from transconjugants selected with tetracycline (Tc) in mating with the donor *Photobacterium damselae* subsp. *piscicida* strains and the recipient *Escherichia coli* K-12 χ 1037 rifampicin-resistant mutant strain. Lane A, pPDP8401 (plasmid DNA of the transconjugant obtained from mating with the donor PP8401 strain (pattern 4, Table 1)) with resistances to Su Tc Cm; lane B, pPDP9212 (pattern 5); lane C, pPDP9017 (pattern 7); lane D, pPDP8702 (pattern 8); lane E, pPDP9214 (pattern 9); lane F, pPDP8808 (pattern 10); lane G, pPDP9202 (pattern 11); lane H, pPDP8906 (pattern 12); lane I, pPDP8836 (pattern 13); lane J, pPDP9401 (pattern 14); lane K, pPDP8608 (pattern 15(1)); lane L, pPDP8511 strain (pattern 15(2)); lane M, pPDP9010 (pattern 17); lane N, pPDP9105 (pattern 18); lane 0, pPDP9010 (pattern 17); lane P, pPDP9105 (pattern 18); lane Q, pPDP9010 (pattern 17). Abbreviations: Chr, chromosomal DNA; Unk, unknown DNA.

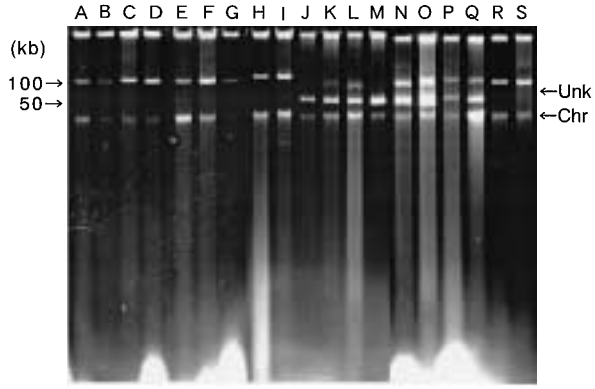

Fig. 4 Agarose gel electrophoresis of plasmid DNA from transconjugants selected with chloramphenicol (Cm) in mating with the donor *Photobacterium damselae* subsp. *piscicida* strains and the recipient *Escherichia coli* K-12 χ 1037 rifampicin-resistant mutant strain. Lane A, pPDP8401 (plasmid DNA of the transconjugant obtained from the donor PP8401 strain (pattern 4, Table 1)) with resistances to Su Tc Cm; lane B, pPDP8702 (pattern 8); lane C, pPDP9214 (pattern 9); lane D, pPDP8808 (pattern 10); lane E, pPDP9202 (pattern 11); lane F, pPDP8906 (pattern 12); lane G, pPDP8836 (pattern 13); lane H, pPDP9401 (pattern 14); lane I, pPDP8608 (pattern 15(1)); lane J, pPDP8511 (pattern 15(2)); lane K, pPDP8912 (pattern 16); lane L, pPDP9010 (pattern 17); lane M, pPDP9105 (pattern 18); lane N, pPDP8511 (pattern 15(2)); lane O, pPDP8912 (pattern 16); lane P, pPDP9010 (pattern 17); lane Q, pPDP9105 (pattern 18); lane R, pPDP8511 (pattern 15(2)); lane S, pPDP9010 (pattern 17). Abbreviations: Chr, chromosomal DNA; Unk, unknown DNA.

and number (lanes D-T). That is, the plasmids banded in the region from approximately 4.2 to 90 kb (the size was ascertained from negative films) concerning the strains that expressed only Su resistance and from approximately 3.3 to 100 kb in strains that exhibited multiple resistances. The plasmid profiles were collectively similar among the strains carrying multiple resistances (lanes D-T) though plasmids differed in molecular size and/or number in some strains. In addition, weak bands of approximately 18 and 33 kb were occasionally observed as seen from lanes J to T but absent from lanes D to I (analyses were individually performed on the former and the latter strains). Moreover, the plasmids mainly showed six bands in the range from 3.3 to 9.0 kb but to date four bands were also observed in our study.

Conjugal transfer of plasmid DNA from *P. damselae* subsp. *piscicida*

Agarose gel electrophoresis of plasmid DNAs in the donor *P. damselae* subsp. *piscicida* strain and Km and Cm transconjugants from mating with the donor is shown in Fig. 7. In addition, the plasmid profiles of Km and Cm transconjugants were the same as those of Su and Ap・Tc transconjugants, respectively. The 100 kb plasmid in the transconjugants was observed in the donor strain but the 50 kb in the transconjugant differed from that in the donor in molecular size.

DISCUSSION

The transferability of drug resistances was different from the resistance patterns in the donors. That is, the drug resistance transfer occurred with the donors when they exhibited resistances to Tc, Na Tc or Na Tmp. Previously, it has been reported that drug resistances in *P. damselae* subsp. *piscicida* strains were not transferred from the strains exhibiting resistances to Ap Cm Tc[20] but were transferred from the strains possessing resistances to Cm Su.[20] This report may see

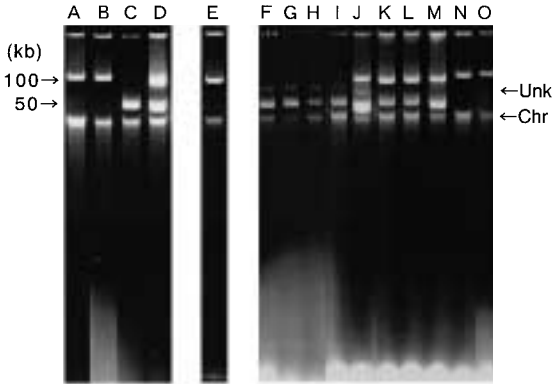

Fig. 5 Agarose gel electrophoresis of plasmid DNA from transconjugants selected with erythromycin (Em) (the left, lanes A to D), trimethoprim (Tmp) (the middle, lane E), or ampicillin (Ap) (the right, lanes F to O) in mating with the donor *Photobacterium damselae* subsp. *piscicida* strains and the recipient *Escherichia coli* K-12 χ1037 rifampicin-resistant mutant strain. Lane A, pPDP9202 (plasmid DNA of the transconjugant obtained from the donor PP9202 strain (pattern 11, Table 1)) with resistances to Su Km Tc Cm Em; lane B, pPDP9401 (pattern 14); lane C, pPDP9105 (pattern 18); lane D, pPDP9105 (pattern 18); lane E, pPDP9401 (pattern 14); lane F, pPDP8511 (pattern 15(2)); lane G, pPDP8912 (pattern 16); lane H, pPDP9010 (pattern 17); lane I, pPDP9105 (pattern 18); lane J, pPDP8511 (pattern 15(2)); lane K, pPDP8912 (pattern 16); lane L, pPDP9010 (pattern 17); lane M, pPDP9105 (pattern 18); lane N, pPDP8511 (pattern 15(2); lane O, pPDP9010 (pattern 17). Abbreviations: Chr, chromosomal DNA; Unk, unknown DNA.

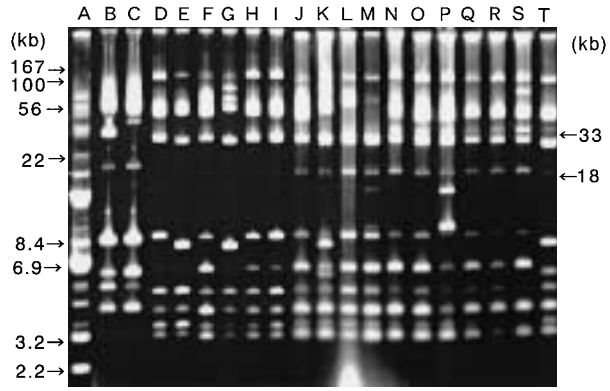

Fig. 6 Agarose gel electrophoresis of plasmid DNA isolated from *Photobacterium damselae* subsp. *piscicida* strains. Lane A: reference plasmids; lane B and C, PP8513 and PP9101 strains (pattern 1); lane D, PP8509 strain (pattern 2); lane E, PP9301 strain (pattern 3); lane F, PP8401 strain (pattern 4); lane G, PP9212 strain (pattern 5); lane H, PP9005 strain (pattern 6); lane I, PP9017 strain (pattern 7); lane J, PP8702 strain (pattern 8); lane K, PP9214 strain (pattern 9); lane L, PP8808 (pattern 10); lane M, PP9202 strain (pattern 11); lane N, PP8906 strain (pattern 12); lane O, PP8836 strain (pattern 13); lane P, PP9401 strain (pattern 14); lane Q, PP8608 strain (pattern 15(1)); lane R, PP8912 strain (pattern 16); lane S, PP9010 strain (pattern 17); lane T, PP9105 strain (pattern 18). See Table 1 or 2 for drug resistances in each strain.

no connection between resistances to Tc, Na Tc and Na Tmp and the transferability of drug resistances. A further study needs to be done on the structure and function of the whole assembly of resistance gene which is attached to other segments containing gene for plasmid replication and transfer function, and on the mechanism of transposition of the resistance genes.

Moreover, all of the resistances to Cm, Em, Km, Su and Tc were transferred from the donors containing Tc resistance but the results of Kim and Aoki[20] were different from our results (In addition, Em resistance transfer are the first findings in *P. damselae* subsp. *piscicida*). Besides, Ap resistance containing these transferable resistances were transferred from most part of the donors carrying resistance to Na Tc and all bearing resistance to Na Tmp, but as per the results of Kim and Aoki[20] resistances to Ap, Cm and Tc had not been transferred from almost all of the donors which showed the same resistance pattern as ours. As per these results, the transferability of resistances to Ap, Cm and Tc were considerably different between our

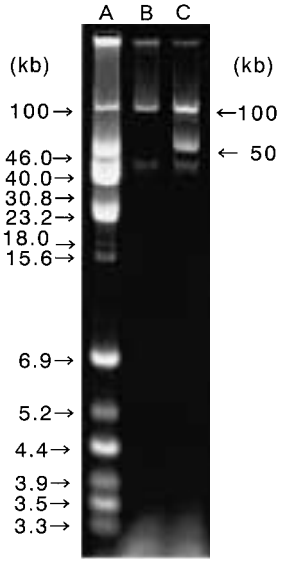

Fig. 7 Agarose gel electrophoresis of plasmid DNA isolated from a donor *Photobacterium damselae* subsp. *piscicida* strain [PP9010 strain (pattern 17), lane A] and the transconjugants selected with Km (lane B) or Cm (lane C) in mating with the donor strain.

results and those of Kim and Aoki.[20] The reason for considerable difference is difficult to understand.

Regarding the donors that transferred Ap resistance, drug resistances in each donor were usually transferred via two plasmids: the 50 kb plasmid carrying Ap, Cm, Em and/or Tc resistances; and the 100 kb plasmid bearing Km, Su and/or Tc resistances. Therefore, transconjugants harboring the two plasmids carried all the transferable resistances in each donor. In some cases, transconjugants possessing only the other 100 kb plasmid also bore all the transferable resistances in each donor, indicating that the 50 and 100 kb plasmids might have fused with each other to be described below. Moreover, the transferability of Ap resistance varied with drug resistance patterns in the donors. These facts suggest that Ap resistance is transferred by the transposition mechanism similar to the Tn3 family transposon as mentioned below. In addition, Km, Su and/or Tc transconjugants usually harbored only the 100 kb plasmid carrying Km, Su and/or Tc resistances; and Ap, Cm, Em and/or Tc transconjugants mostly possessed the 100 kb plasmid in addition to the 50 kb plasmid bearing Ap, Cm, Em and/or Tc resistances, because the transfer frequencies of the 100 kb plasmid were higher than those of the 50 kb plasmid.

Ap resistance transposon Tn3 was the first transposable element encoding antibiotic resistance as described by Hedges and Jacob.[30] Tn3 is very closely related to a whole group of Ap resistance transposons isolated from a range of bacteria.[31] Tn3 encodes β-lactamase that destroys β-lactam antibiotics like penicillin and ampicillin, and the two enzymes: transposase which catalyzes the insertion of transposons into new sites; and resolvase which catalyses resolution by recombination across two copies of Tn3 in a cointegrate. Intermolecular transposition of Tn3 is known to be a sequential two-step process involving, in the first transposition step, replication of the transposon and an initial fusion of the donor and recipient replicon to create cointegrate transposition intermediate. The second site-specific recombination step involves the resolution of cointegrates to the normal transposition end products. Other members of the Tn3 family also appear to have the same replicative transposition mechanism and appear to be unique among transposable elements in encoding their own site-specific recombination systems to convert the cointegrate products of intermolecular transposition into the normally observed transposition end products.[32] Notwithstanding this, in laboratory experiments, intramolecular transposition of Tn3 gives replicative deletions and inversions which occur at frequencies comparable to those for intermolecular transposition.[33] The possibility of retransposition of elements in deleted circular molecules or of homologous recombination between such transposons and homologous sequences in other molecules is always present, and can generate more complex DNA rearrangements. These facts suggest that Ap resistance in *P. damselae* subsp. *piscicida* is transposed by the same transposition mechanism as in the Tn3 family and elucidate why the transfer of Ap resistance differed with drug resistance patterns in the donors; namely, the transposable element is generated by rearrangement of some resistance genes.

The other transposition mechanism of Ap resistance may also be inferred from the facts to be described below. The evolution of multiple-resistance plasmids is made easily by the ability of transposons to recombine without homology and thus to gather together in a single plasmid. The naturally occurring complex plasmid R1 evolved from transposons encoding resistance to Cm and Km, and it also contains the intact transposon Tn4 carrying resistances to Ap, Sm and Su. Tn4 itself includes a separate and independent transposon, Tn3, which is responsible only for the resistance to Ap. The whole assembly of resistance genes (an r-determinant) is attached to other segments containing genes for plasmid replication and for transfer functions, which promote conjugal contact of the host bacterial cell with other bacteria, thus allowing the plasmid to be transferred readly between cells.[34] From these facts, it can be presumed that transferable resistances in *P. damselae* subsp. *piscicida* as donor are carried as a complex plasmid and transferred to the recipients by getting divided into two plasmids which include the Tn3 family or the other transposon. In the future, DNA sequence analyses of R plasmids in the donors and the transconjugants need to be done for resolving the transposition mechanism of drug resistances involving Ap resistance.

It has been reported that most isolates of *Pasteurella piscicida* (*P. damselae* subsp. *piscicida*) possessed one large plasmid of 110 kb and two small plasmids of 3.5 and 5.1 kb.[35] Similar results have also been reported by Toranza *et al.*[36] In our experiment, the small plasmids of 3.5 and 5.1 kb (5.2 kb in our experiment) were detected but the large plasmid of 110 kb was not detected in the results from gel electrophoresis. However, the 110 kb plasmid may correspond to the 100 kb plasmid in our experiment because the molecular size of the 100 kb plasmid was 105.3 kb in the

electron microscopic observation (unpublished data). In addition, the migration distance of plasmids in gel electrophoresis was 2 mm between R27 (167 kb) and R100-1 plasmids (100 kb) and 6 mm between R100-1 and RP4 plasmids (56 kb) in 10 cm of the total distance migrated; hence, it is likely that considerable error is estimated for the molecular sizes determined by gel electrophoresis. Moreover, the size also remarkably varied in the width of the plasmid band.

The plasmid of almost the same size as the 100 kb plasmid in the transconjugants was observed in the donors but the 50 kb plasmid in the transconjugants was different from that in the donors in size. These facts suggest that the harboring R plasmids differ between the donors and the transconjugants. Also, there were some cases where either Ap resistance was transferred or not transferred in mating with the donors of resistance pattern 15. Moreover, in the mating with the donors that transferred Ap resistance, drug resistances in the transconjugants were different even in the experiments that were repeatedly performed. These facts suggest that the transferability of drug resistances is changeable with respect to *P. damselae* subsp. *piscicida* of resistance pattern 15. A detailed study for drug resistances transferability in *P. damselae* subsp. *piscicida* needs to be done in the future.

In addition, the transfer frequency of drug resistances was different with growth conditions for mating assay. For example, all the transferable drug resistances in PP9401 strain (pattern 14) were transferred in the frequencies of about 10^{-1}, and in PP9105 strain (pattern 18) Km and Su resistances were transferred in the frequencies of about 10^{0} and Ap, Cm, Em and TC resistances in those of about 10^{-4}, in mating assay at different incubation time (9 h in *P. damselae* subsp. *piscicida*).

REFERENCES

1) Watanabe T, Aoki T, Ogata Y, Egusa S. R factors related to fish culturing. *Ann. N. Y. Acad. Sci.*, **182,** 383-410 (1971).

2) Aoki T, Egusa S, Ogata Y, Watanabe T. Detection of resistance factors in fish pathogen *Aeromonas liquefaciens*. *J. Gen. Microbiol.*, **65**, 343-349 (1971).

3) Aoki T, Egusa S, Yada C, Watanaba T. Studies of drug resistance and R factors in bacteria from pond-cultured salmonids. I. Amago (*Oncorhynchus rhodurus macrostomus*) and yamame (*Oncorhynchus masou ishikawae*). *Japan. J. Microbiol.*, **16**, 233-238 (1972).

4) Aoki T, Watanabe T. Studies of drug resistance and R factors in bacteria from pond-cultured salmonids. II. Rainbow trout (*Salmo gairdneri f. irideus*). *Fish Pathol.*, **8**, 83-89 (1973).

5) Aoki T, Watanabe T. Studies of drug-resistant bacteria isolated from eel-pond water and intestinal tracts of the eel (*Anguilla japonica* and *Anguilla anguilla*). *Nippon Suisan Gakkaishi*, **39**, 121-130 (1973).

6) Aoki T, Egusa S, Watanabe T. Detection of R^{+} bacteria in cultured marine fish, yellowtails (*Seriola quinque,adiata*). *Japan. J. Microbiol.*, **17**, 7-12 (1973).

7) Aoki T. Studies of drug-resistant bacteria isolated from water of carp-ponds and intestinal tracts of carp. *Nippon Suisan Gakkaishi*, **40**, 247-254 (1974).

8) Aoki T, Egusa S, Arai T. Detection of R factors in naturally occurring *Vibrio anguillarum* strains. *Antimicrobial agents and chemotherapy*, **6**, 534-538 (1974).

9) Aoki T, Arai T, Egusa S. Detection of R plasmids in naturally occurring fish-pathogenic bacteria, *Edwardsiella tarda*. *Microbiol. Immunol.*, **21**, 77-83 (1977).

10) Aoki T, Kitao T. Drug resistance and transferable R plasmids in *Edwardsiella tarda* from fish culture ponds. *Fish Pathol.*, **15**, 277-281 (1981).

11) Aoki T, Kitao T, Kawano K. Changes in drug resistance of *Vibrio anguillarum* in cultured ayu, *Plecoglossus altivelis* Temminck and Schlegel, in Japan. *J. Fish Dis.*, **4**, 233-230 (1981).

12) Aoki T, Kitao T, Iemura N, Mitoma Y, Nomura T. The susceptibility of *Aeromonas salmonicida* strains isolated in cultured and wild salmonids to various chemotherapeutics. *Nippon Suisan Gakkaishi*, **49**, 17-22 (1983).

13) Aoki T, Kitao T, Watanabe S, Takeshita S. Drug resistance and R plasmids in *Vibrio anguillarum* isolated in cultured Ayu (*Plecoglossus altivelis*). *Microbiol. Immunol.*, **28**, 1-9 (1984).

14) Aoki T, Kitao T. Detection of transferable R plasmids in strains of the fish-pathogenic bacterium, *Pasteurella piscicida*. *J. Fish Dis.*, **8**, 345-350 (1985).

15) Takashima N, Aoki T, Kitao T. Epidemiological surveillance of drug-resistanct strains of *Pasteurella piscicida*. *Fish pathol.*, **20**, 209-217 (1985).

16) Takashi A, Aoki T. Characterization of transferable R plasmids from *Aeromonas hydrophila*. *Nippon Suisan Gakkaishi*, **52**, 649-655 (1986).

17) Aoki T, Sakaguchi T, Kitao T. Multiple drug-

resistant plasmids from *Edwardsiella tarda* in eel culture ponds. *Nippon Suisan Gakkaishi*, **53**, 1821-1825 (1987).

18) Aoki T, Takami K, Kitao T. Drug resistance in a non-hemolytic *Streptococcus* sp. Isolated from cultured yellowtail *Seriola quinqueradiata*. *Dis. Aquat. Org.*, **8**, 171-177 (1990).
19) Zhao J, Kim E, Kobayashi T, Aoki T. Drug resistance of *Vibrio anguillarum* isolated from ayu between 1989 and 1991. *Nippon Suisan Gakkaishi*, **58**, 1523-1527 (1992).
20) Kim E, Aoki T. Drug resistance and broad geographical distribution of identical R plasmids of *Pasteurella piscicida* isolated from cultured yellowtail in Japan. *Microbiol. Immunol.*, **37**, 103-109 (1993).
21) Kusuda R, Yamaoka M. Etiological studies on bacterial pseudotuberculosis in cultured yellowtail with *Pasteurella piscicida* as the causative agent-I. On the morphological and biochemical properties. *Nippon Suisan Gakkaishi*, **38**, 1325-1332 (1972).
22) Lewis DH, Grumbles LC, McConnell S, Flowers AI. Pasteurella-like bacteria from a epizootic in menhaden and mullet in Galveston Bay. *J. Wildlife Diseases*, **6**, 160-162 (1970).
23) Kubota M, Kimura M, Egusa S. Studies on bacterial pseudotuberculosis in cultured larval yellowtail-I.Symptoms of a disease and pathological histology-I. *Fish Pathol.*, **4**, 111-118 (1970) (in Japanese).
24) Yasunaga N, Hatai K, Tsukahara J. *Pasteurella piscicida* from an epizootic of cultured red seabream. *Fish Pathol.*, **18**, 107-110 (1983).
25) Moroga K, Sugiyama T, Ueki N. Pasteurellosis in cultured black seabream (*Mylio macrocephalus*). *J. Fac. Fish. Ani. Hus., Hiroshima Univ.*, **16**, 17-21 (1977).
26) Ohnishi K, Watanabe K, Jo Y. *Pasteurella* infection in young black seabream. *Fish Pathol.*, **16**, 207-210 (1982).
27) Morii H, Hayashi N, Uramoto K. Cloning and nucleotide sequence analysis of the chloramphenicol resistance gene on conjugative R plasmids from the fish pathogen, *Photobacterium damselae* subsp. *piscicida*. *Dis. aquat. Org.*, **53**, 107-113 (2003).
28) Japanese Society of Chemotherapy. The revised standard method for determining MIC [minimal inhibitory concentration]. *Chemotherapy*, **29**, 76-79 (1981) (in Japanese).
29) Kado CI, Liu ST. Rapid procedure for detection and isolation of large and small plasmids. *J. Bact.*, **145**, 1365-1373 (1981).
30) Hedges RW, Jacob AF. Transposition of ampicillin resistance from RP4 to other replicon. *Mol. Gen. Genet.*, **132**, 31-40 (1974).
31) Heffron F, McCarthy BJ, Ohtsubo H, Ohtsubo E. DNA sequence analysis of the transposon Tn3: three genes and three sites involved in transposition of Tn3. *Cell*, **18**, 1153-1164 (1979).
32) Sherratt D. Tn3 and related transposable elements: Site-specific recombination and transposition. In: Douglas EB, Martha MH (eds). *Mobile DNA*. American Society for Microbiology, Washington, D.C., 1989, pp. 163-184.
33) Bishop R, Sherratt D. Transposon Tn1 intramolecular transposition. *Mol. Gen. Genet.*, **196**, 117-122 (1984).
34) Watson JD, Hopkins NH, Roberts JW, Steitz JA, Weiner AM. Molecular biology of the gene. The Benjamin/Cummings Publishing Company, Inc., Menlo Park etc., 1987, pp. 313-338.
35) Zhao J, Aoki T. Plasmid profile of *Pasteurella piscicida* and use of a plasmid DNA probe to identify the species. *J. Aqua. Animal Health*, **4**, 198-202 (1992).
36) Toranzo AE, Barja JL, Colwell RR, Hetrick FM. Characterization of plasmids in bacterial fish pathogens. *Infection and Immunity*, **39**, 184-192 (1983).

魚病原細菌*Photobacterium damselae* subsp. *piscicida*の薬剤耐性の接合伝達

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マニッシュ スレンドラ バラドワジュ

Photobacterium damselae subsp. *piscicida*の薬剤耐性のうちアンピシリン（Ap），クロラムフェニコール（Cm），エリスロマイシン（Em），カナマイシン（Km），サルファモノメトキシン（Su），テトラサイクリン（Tc）およびトリメトプリム（Tmp）耐性が，伝達頻度10^{-3}～10^{-4}の100kbプラスミドと伝達頻度10^{-6}の50kbプラスミドにより伝達された。Ap耐性を保持あるいは伝達しない菌株では，各菌株から伝達される薬剤耐性のすべてが100kbプラスミド1つにコードされて伝達された。Ap耐性を伝達した菌株では，各菌株から伝達される薬剤耐性のうちKm，Suおよび（または）Tc耐性は100kbプラスミドに，またAp，Cm，Emおよび（または）Tc耐性は50kbプラスミドにコード伝達された。従って，この両プラスミドを保持した接合伝達体では伝達された薬剤耐性のすべてを保持した。しかし，100kbプラスミド1つを保持した接合伝達体でも各菌株から伝達された薬剤耐性のすべてを保持する場合があり，両プラスミドの融合が示唆された。他方，伝達された100kbプラスミドは供与菌でも確認されたが，50kbプラスミドは確認できなかった。