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Drug Resistance in Fish-Pathogenic Bacteria

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어병 세균으로부터 분리된 R-plasmid의 특성과 그 DNA 구조는 *Aeromonas hydrophila, A. salmonicida, Edwardsiella tarda, Enterococcus seriolicida, Pasteurella piscicida, Vibrio onguillarum*등 세균의 종류에 따라 다르다. 그러나 *A. hydrophila*와 *E. tarda*로부터 그리고 *A. hydrophila*와 *A. salmonicida*로부터 분리된 몇몇 R-plasmid는 같은 내성형을 가지면서 유사한 DNA 구조를 보였다. *V. anguillarum*에서 분리된 R-plasmid는 1977년 이전, 1980년과 1983년 사이 그리고 1989년과 1991년 사이에 분리된 것이 그 DNA 구조에 차이를 보여 각각 그룹 1, 2, 3으로 구분되어졌다. *P. piscicida*의 경우에는 연도와 지역에 관계없이 동일한 DNA 구조를 갖는 R-plasmid가 분리되었다. Macrolide계 항생제(MLs), lincomycin (LM), tetracycline(TC) 그리고 MLs, LIM, chloramphenicol(CP) 내성을 나타내는 R-plasmid를 갖는 *E. seriolicida*가 각 지역의 Yellowtail 어장에 분포되어 있었다.

*P. piscicida*의 R-plasmid에는 type Ⅰ의 chloramphenicol acetyltransferase(CAT)에 의하여 CP 내성을 나타내는 유전자(*cat*)가, 그리고 *E. tarda, A. salmonicida*와 1980년 이후에 분리된 *V. anguillarum*의 R-plasmid에는 CAT type Ⅱ에 해당하는 *cat* 유전자가 분포되어 있었다.

TC 내성 유전자(*tet*)의 경우에는 1977년 이전과 1980년 이후에 분리된 *V. anguillarum*으로부터 class B, G의 *tet* 유전자가 확인되었으나, *E. tarda, P. piscicida, A. hydrophila* 그리고 1989년 이후에 분리된 *V. anguillarum*등 어병세균의 R-plasmid에는 class D의 *tet* 유전자가 널리 분포하고 있는 것으로 나타났다.

Key Words : fish pathogenic bacteria, drug resistance, R plasmid, drug-resistance gene.

Bacterial infectious diseases occur frequently in marine and freshwater farms. Accordingly, various chemotherapeutic agents have been used to treat bacterial infections in cultured fish. These chemotherapeutics (amoxicillin, ampicillin, bicozamycin, colistin, florfenicol, lincomycin, macrolide antibiotics, novobiocin, pyridonecarboxylic acids, sodium nifurstyenate, sulfonamides, sulfamonomethoxine : oremetoprim complex, tetracycline derivatives, and thiamphenicol) were authorized for treatment of bacterial fish diseases in fish farms by the Japanese Fisheries Agency in 1992. The rules of standard chemotherapy in terms of route of administration, dosage, disease treated, and withdrawal time were established by the Fisheries Agency. Almost all chemotherapeutics have been administered by incorporating them in feed pellets. Colistin, oxolinic acid, and sulfamonomethoxine have been administered by dipping the fish in a drug solution(Aoki, 1992a).

Because, chemotherapeutics have been widely used for treating bacterial diseases in fish farms, multiple drug resistant strains of fish-pathogens have increased in fish farms(Aoki, 1992a & 1992b). It has become difficult to treat vibriosis(Aoki *et al.*, 1984), pseudotu-

berculosis(Takashima *et al.*, 1985), and streptococcal infections(Aoki *et al.*, 1990a) with drug-resistant strains using chemotherapeutics. Acquired drug resistance results essentially from the selective pressure exerted on bacteria during the administration of chemotherapeutics. Drug resistance can be determined by genes that reside in the host-cell chromosomes, on plasmids or on transposons(Russell & Chopra, 1990). Resistance to nitrofuran and pyridonecarboxylic acids can be acquired by chromosomal mutations of fish pathogens. R plasmid-determined drug resistance is more common than chromosomal resistance in pathogenic bacteria. R plasmids are widely distributed in the Gram-negative and Gram-positive pathogenic bacteria of humans, domestic animals and fish. R plasmids are extrachoromosomal genetic elements that replicate independently of the chromosome, a circular DNA-conferred drug resistance. As the cell divides, the R plasmids also divide and are inherited by the daughter cells. R plasmids transfer to other bacteria by conjugation of cell-to-cell contact.

Transferable R plasmids have been detected in the following drug-resistant strains of fish-pathogens : *Aeromonas hydrophila*(Akashi and Aoki, 1986), *A. salmonicida*(Aoki *et al.*, 1971), *Edwardsiella tarda*(Aoki *et al.*, 1986a), *Enterococcus seriolicida*(Aoki *et al.*, 1990a), *Pasteurella piscicida*(Takashima *et al.*, 1985), *Pseudomonas fluorescens*(Aoki *et al.*, 1977), marine *Vibrio* sp.(Aoki *et al.*, 1973), *Vibrio anguillarum*(Aoki *et al.*, 1984), and *Yersinia ruckeri*(De Grandis *et al.*, 1985).

In this paper, the drug resistance of fish pathogens carrying R plasmids, the properties of the R plasmids, and drug resistant genes coding the R plasmids are discussed.

1. Properties of R plasmids from fish-pathogens

Hemorrhagic septicemia caused by *A. hydrophila* in warmwater and freshwater fish. The properties of R plasmids detected in *A. hydrophila* are shown in Table 1. The most common type of R plasmid have markers resistant to sulfonamide(SA), tetracycline(TC) and to chloramphenicol(CP), SA, and streptomycin(SM) (Akashi & Aoki, 1986). The R plasmids detected, by location and year, and encoded with resistance to SA and TC, were classified into incompatibility(Inc) group A-C. The R plasmids with resistance to SA and TC were highly homologous. R plasmids with resistance to CP, SA and SM showed homology within a specific species and with R plasmids from *A. salmonicida*. We demonstrated that *A. hydrophila* strains carrying R plasmids having identical DNA structures are widely distributed in freshwater fish culture ponds in various areas(Akashi and Aoki, 1986).

Drug resistant strains of *A. hydrophila* carrying R plasmids have been found in France, Ireland, Taiwan, and the U. S. A.(Aoki, 1992b). Recently, these strains were isolated from cultured snakehead fish(*Ophicephalus striatus*) in Thailand(Aoki *et al.*, 1990b). The DNA structure of R plasmids found in Japan and Thailand were identical.

Aeromonas salmonicida is a commercially important pathogen, causing systemic furunculosis in salmonids. Infections with drug resistant strains were first observed in 1957 in salmonids farms in the U. S. A.(Aoki *et al.*, 1971). A transferable R plasmid with resistance to SA and TC was detected in one of these strains. Infections with drug resistant strains occurred later in salmonid farms in Japan. Almost all transferable R plasmids with resistance to CP, SA, and SM have been detected in *A. salmonicida* strains isolated from several areas(Table 1)(Aoki *et al.*, 1986b). All of these were classified into Inc group U(Bradley, 1982).

Table 1. Properties of R plasmids detected from fish-pathogens

Fish-pathogen	Resistance markers of R plasmids	Incompatibility group
Aeromonas hydrophila	SA TC*	A-C
	CP SA SM	U
Aeromonas salmonicida	CP SA SM	U
	TC	?
Edwardsiella tarda	CP SA TC	A-C
	SA TC	A-C
Pasteurella piscicida	CP KM SA TC	new group
Vibrio anguillarum		
(isolated in 1973~1977)	CP SA SM TC	A-C, E
	CP SA TC	A-C, E
(isolated in 1980~1983)	AP CP SA SM TC TMP	A-C
(isolated in 1989~1991)	CP KM SA SM TC TMP	?
Enterococcus seriolicida	LCM MLs CP	?
	LCM MLs TC	?

* Abbreviations : AP, ampicillin ; CP, chloramphenicol ; KM, kanamycin ; SA, sulfamonomethoxine ; SM, streptomycin ; TC, tetracycline and TMP, trimethoprim.

The R plasmids with resistance to CP SA, and SM from different sources were highly homologous.

Non-transferable R plasmids encoded with TC have been detected in *A. salmonicida* strain isolated in fish farms(Aoki and Takahashi, 1986 ; Aoki, 1992b). The isolation of drug resistant strains of *A. salmonicida* carrying R plasmids has been reported in France, Ireland, and also the U. K(Aoki, 1992b).

Edwardsiella tarda, which causes edwardsiellosis, is an important bacterial pathogen of both freshwater fish, such as eel and tilapia, and marine fish, such as flatfish and seabream. Infections with multiple drug-resistant strains of *E. tarda* have occurred frequently in eel culture ponds in Japan as well as in Taiwan (Aoki *et al.*, 1986a). Transferable R plasmids detected in drug-resistant strains were encoded with resistance to SA, TC and CP, SA, and TC, and classified into Inc group A-C(Table 1). The DNA structures of these R plasmids were highly homologous. The DNA structures of R plasmids with resistance to SA, TC and CP, SA, and TC from *E. tarda* were similar to those with resistance to SA and TC from *A. hydrophila*. These results show that drug-resistant strains of *E. tarda* carrying R plasmids with the same DNA structures have prevailed in eel ponds(Aoki *et al.*, 1986b).

Pasteurella piscicida is the etiological agent of psudotuberculosis, which causes severe economic losses for those who culture yellowtail. Drug-resistant infections

with *P. piscicida* started to occur in yellowtail farms in 1980. The following year, the infection spread to all districts of Japan. R plasmids encoded with resistance to combinations of CP, kanamycin(KM), SA and/or TC were classified into a new Inc group(Table 1). There was high homology of these R plasmids independent of their geographical origin, drug resistance marker and year of isolation(Takashima *et al.*, 1985 ; Kim and Aoki, 1993). In 1992, we isolated strains of *P. piscicida* carrying R plasmid resistance to florfenicol (FF)(Kim *et al.*, 1993). Florfenicol had been an excellent therapeutic agent for treatment of pseudotuberculosis and has been widely used in yellowtail farms since 1990. It was surprising that FF-resistant strains appeared so quickly in fish farms.

Vibrio anguillarum is an important pathogen of fish in which it is the etiological agent of vibriosis. This is the most serious disease in the ayu culture industry. An epidemic of drug-resistant strains of *V. anguillarum* has been spreading in ayu farms in Japan since 1973 (Aoki *et al.*, 1984). The detected transferable R plasmids were resistant to ampicillin(AP), CP, SA, SM, TC, and/or trimethoprim(TMP) and were clssified into Inc groups A-C and E(Table 1). These infections with drug-resistant strains have been observed in ayu farms up to the present. The R plasmids, which were detected in *V. anguillarum* isolated between 1989 and 1991, were resistant to CP, KM, SA, SM, TC, and TMP(Zhao *et al.*, 1992). R plasmids detected in *V. anguillarum* can be classified into three main groups by their DNA structure : one group detected before 1976, a second from 1980 to 1983 and a third from 1989 to 1991.

Enterococcus seriolicida is a gram-positive pathogen that causes streptococcicosis in yellowtail. This is one of the most important diseases of yellowtail cultured in Japan. Infections with drug-resistant strains of *E. seriolicida* have occurred in yellowtail farms since 1986. Transferable R plasmids were first detected by high-level resistance to macrolide antibiotics(MLs), lincomycin(LIM) and TC, and to MLs, LIM, and CP from *E. seriolicida*(Table 1). These drug-resistant strains carrying R plasmids with the same DNA structure were widely distributed in yellowtail farms(Aoki *et al.*, 1990a).

2. Drug-resistant genes encoding R plasmids from fish-pathogens

R plasmids consist of one replication, one transfer, and one drug-resistance gene. The R plasmids detected in fish-pathogens are encoded with resistance to antibiotics, sulfonamide, and trimethoprim. In particular, chloramphenicol-resistance(*cat*) and tetracycline-resistance(*tet*) determinants have been detected in R plasmids of all kinds of fish-pathogens. It is of interest to consider the origin and structure of these resistant determinants of R plasmids from fish-pathogens.

Resistance to tetracycline in gram-negative bacteria is mostly mediated by determinants encoding active effux of the drug from the resistant cell. The *tet* determinants have been categorized into six classes(A, B, C, D, E, F and G)(Table 2)(Levy *et al.*, 1989). These share extensive sequence homology in their structural genes. *Tet* determinants of plasmids from *A. hydrophila*, *E. tarda*, and *P. piscicida* are grouped into Tet class D(Aoki & Takahashi, 1987). The *tet* determinant of class D is widely distributed in R plasmids from fish-pathogens.

The *tet* determinant of the non-transferable R plasmid from *A. salmonicida* was classified as class C. This *tet* determinant has been transposed into the cosmid pKY96(Aoki and Takahashi, 1986 ; Aoki, 1988).

Table 2. Classification of chloramphenicol-resistant determinants of R plasmids in fish-pathogens.

Class	Prototype	Origin
CAT I	pBR325	*Enterobacteriaceae*
		Pasteurella piscicida
CAT II	RSa	*Enterobacteriaceae*
		Aeromonas salmonicida
		Edwardsiella tarda
		Vibrio anguillarum(1980~1983)
CAT III	R387	*Enterobacteriaceae*
CAT IV	pJA4318	*Vibrio anguillarum*(1973~1977)

The *tet* determinants of the R plasmids from *V. anguillarum* were classified into two groups. One of the R plasmids detected before 1977 belonged to the Tet B class(Aoki, 1988). However, the *tet* determinant of the R plasmids detected from 1981 to 1983 were classified into class G(Zhao an Aoki, 1992b). These *tet* genes have only been found in the fish-pathogen, *V. anguillarum*. Recently, we classified the *tet* determinant of R plasmids detected in *V. anguillarum* during 1992, into class D(unpublished data). It difficult to determine why the *tet* gene of the R plasmid from *V. anguillarum* changed. There is currently great interest as to how the R plasmid of *V. anguillarum* is constructed naturally.

Bacterial resistance to chloramphenicol is most commonly mediated by production of the enzyme chloramphenicol acetyltransferase, which catalyzes the O-acetylation of chloramphenicol by acetyl coenzyme A. Four distinguishable types(CAT I, II, III and IV) of chloramphenicol acetyltransferase have been found in gram-negative bacteria by analysing their enzymic, biochemical and immunological properties(Table 3) (Shaw, 1983 ; Zhao and Aoki, 1992a). The gene structures of the chloramphenicol-resistance determinants differed depending on each enzyme type. The *cat* determinant of the R plasmid from *P. piscicida* was classified as CAT type I (Aoki, 1988). The *cat* determinant of the R plasmids from *E. tarda* and *A. salmonicida* were classified as CAT II(Aoki, 1988). The *cat* determinant of the R plasmids from *V. anguillarum* was classified into two types. One type, from an R plasmid detected before 1977 was classified as CAT IV type(Zhao and Aoki, 1992a). This type has never been detected in any R plasmids from human or domestic animal pathogens. The other type of R plasmid detected since 1980 was classified as CAT II.

Table 3. Classification of tetracycline-resistant determinants of R plasmids in fish-pathogens.

Class	Prototype	Origin
Tet A	RP4	*Pseudomonas aeruginosa*
		Enterobacteriaceae
Tet B	R222	*Enterobacteriaceae*
		Vibrio anguillarum(1973~1977)
Tet C	pBR322	*Salmonella*
		Aeromonas salmonicida
Tet D	RA 1	*Aeromonas hydrophila*
		Edwardsiella tarda
		Psasteurella piscicida
		Vibrio anguillarum(1989~1991)
Tet E	pSL1456	*Escherichia coli*
Tet F	pGAT400	*Bacteroides fragilis*
Tet G	pJA8122	*Vibrio anguillarum*(1980~1983)

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The properties and DNA structures of R plasmids differ depending on the species of the fish-pathogens *Aeromonas hydrophila, A. salmonicida, Edwardsiella tarda, Enterococcus seriolicida, Pasteurella piscicida* and *Vibrio anguillarum*. However, some R plasmids with the same resistance markers in similar DNA structures were found in *A. hydrophila* and *E. tarda*, as well as in *A. hydrophila* and *A. salmonicida*. R plasmids from *V. anguillarum* were classified into three groups according to their DNA structures. The first group was detected before 1977, the second from 1980 to 1983, and the third from 1989 to 1991. R plasmids have been retained within *P. piscicida* having the same DNA structures and detected at

various locations and times. *E. seriolicida* strains carrying the same R plasmids, which were encoded with resistance to macrolide antibiotics(MLs), lincomycin(LIM), and TC, and to MLs, LIM, and CP, were distributed in yellowtail farms in various districts.

The chloramphenicol-resistance(*cat*) gene of the R plasmids of *P. piscicida* was classified as CAT type I. The *cat* of the R plasmids of *E. tarda, A. salmonicida* was classified as type II. The *cat* of R plasmids of *V. anguillarum* was classified into two types. One type detected before 1977, was classified as CAT IV and the other type, detected after 1980, was classified as CAT II. Tetracycline-resistance (*tet*) *V. anguillarum*, isolated before 1977 and after 1981, was classified as Tet B and Tet G, respectively. The class D *tet gene* was widely distributed in R plasmids from fish-pathogens *A. hydrophila, E. tarda, P. piscicida,* and also *V. anguillarum* isolated after 1989.