ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1987, p. 1278–1280
0066-4804/87/081278-03$02.00/0

Vol. 31, No. 8

Class D Tetracycline Resistance Determinants of R Plasmids from the Fish Pathogens *Aeromonas hydrophila*, *Edwardsiella tarda*, and *Pasteurella piscicida*

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Received 8 September 1986/Accepted 18 May 1987

Tetracycline resistance determinants of R plasmids from the fish pathogens *Aeromonas hydrophila*, *Edwardsiella tarda*, and *Pasteurella piscicida* were classified as class D by their expression of resistance to tetracycline and minocycline and through their DNA structure.

The application of chemotherapeutics in fish culture ponds has caused an increase in the incidence of drug-resistant bacteria, which have become a major problem in the treatment of bacterial infections in cultured fish populations (5, 7, 20). R plasmids encoding resistance to tetracycline have been isolated with high frequency from the fish pathogens *Aeromonas hydrophila* (4), *Edwardsiella tarda* (5), and *Pasteurella piscicida* (20). We are interested in the origin of tetracycline resistance genes (*tet*^{r}) in R plasmids from fish-pathogenic bacteria.

The tetracycline resistance (Tc^r) determinants of R plasmids from gram-negative bacteria have been divided into two major classes depending upon their resistance levels to tetracycline and minocycline (11). The effect of prior exposure to a subinhibitory concentration of tetracycline also subdivides the group into inducible and uninducible types. Mendez et al. (17) used DNA-DNA hybridization to demonstrate four genetic classes (A, B, C, and D) of Tc^r determinants borne by 25 different plasmids in *Enterobacteriaceae*. Klock et al. (13) have demonstrated heterologous repressor-operator recognition among these four classes of Tc^r determinants and have suggested a close relationship between them. Differences in the expression of resistance to tetracycline analogs can also be correlated with these four classes (17).

In the present study, Tc^r determinants of R plasmids from three fish pathogens, *A. hydrophila*, *E. tarda*, and *P. piscicida*, were classified phenotypically and genotypically.

The sources and properties of R plasmids from *A. hydrophila*, *E. tarda*, and *P. piscicida* are shown in Table 1. Plasmids RP4 for class A, R222 for class B, pBR322 for class C, and RA1 (10) for class D were used to compare resistance levels and DNA structure of the Tc^r determinants (17).

TABLE 1. Sources of R plasmids from fish pathogenic bacteria

R plasmid	Incompatibility group	Detected in	Resistance marker[a]	Isolated in:		Reference
				Area	Yr	
pJA5017	A–C	*A. hydrophila*	Su Tc	Shizuoka	1969	1
pES15	Unclassified	*A. hydrophila*	Cm Km Sm Su Tc	U.S.A.	1974	1
pES41	A–C	*A. hydrophila*	Su Tc	U.S.A.	1974	1
pTW537	A–C	*A. hydrophila*	Su Tc	Taiwan	1979	1
pJA6012	A–C	*E. tarda*	Cm Km Sm Su Tc	Shizuoka	1975	2,3
pJA110	A–C	*E. tarda*	Cm Su Tc	Shizuoka	1976	2
pET13	A–C	*E. tarda*	Su Tc	Tokushima	1977	2
pTW688	A–C	*E. tarda*	Cm Su Tc	Taiwan	1977	2
pJA8001	Unclassified	*P. piscicida*	Cm Km Su Tc	Kochi	1980	6
pJAPS8102	Unclassified	*P. piscicida*	Cm Km Su Tc	Saga	1981	20
pJAPE8225	Unclassified	*P. piscicida*	Ap Cm Km Su Tc	Ehime	1982	20
pJAPN8310	Unclassified	*P. piscicida*	Cm Km Su Tc	Nagasaki	1983	20

[a] Abbreviations used: Resistance to ampicillin (Ap), chloramphenicol (Cm), kanamycin (Km), streptomycin (Sm), sulfonamide (Su), and tetracycline (Tc).

Resistance to tetracycline and its analogs doxycycline, minocycline and oxytetracycline in *Escherichia coli* C600 (F$^-$ *lac leu lonA supE thi thr*) (15) carrying various R plasmids was determined by the method of Aoki et al. (7). *E. coli* strains carrying R plasmids from *A. hydrophila*, *E. tarda*, and *P. piscicida* were resistant to doxycycline, minocycline, oxytetracycline, and tetracycline with MICs of 100, 37.5, 400, and 200 μg/ml, respectively. These resistance levels resembled those of RA1 but differed from those of RP4, R222, or pBR322.

Induction of tetracycline resistance was performed by the method of Foster and Walsh (11). *E. coli* C600 carrying each R plasmid was cultured at 37°C for 30 min in Penassay broth (Difco) containing 1 μg of tetracycline per ml. After induction of resistance, the induced and uninduced cells were cultured in fresh Penassay broth containing 100, 50, 25, 12.5,

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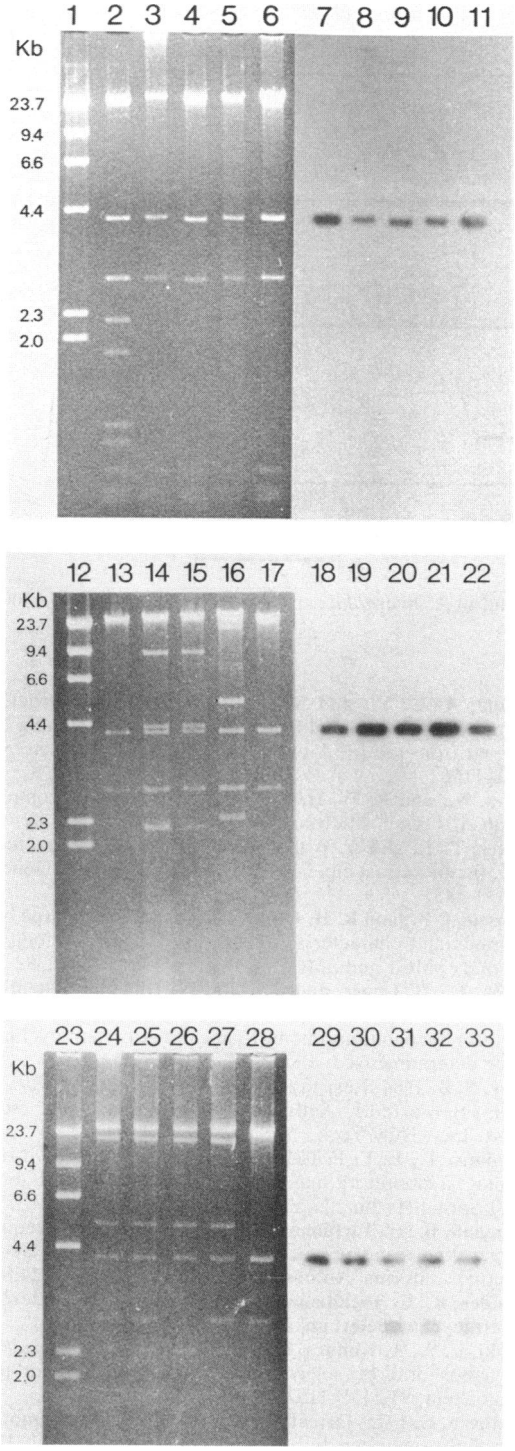

FIG. 1. *Pst*I digestion of R plasmids from *A. hydrophila*, *E. tarda*, and *P. piscicida* and hybridization with a class D *tet*^{r} probe. The left panel in each group shows the agarose gel electrophoretic profiles of R plasmid DNAs digested with *Pst*I restriction endonuclease. The right panel is an autoradiogram of a nitrocellulose filter blotted with the DNA from the left panel and probed with a ^{32}P-labeled *Hin*dIII-*Pst*I fragment of RA1 encoding resistance to tetracycline. λ DNA (lanes 1, 12, and 23) digested with *Hin*dIII was used as a molecular size standard. Plasmids pJA5017 (lanes 2 and 7), pES15 (lanes 3 and 8), pES41 (lanes 4 and 9), and pTW537 (Lanes 5 and 10) were found in *A. hydrophila*. Plasmids pJA6012 (lanes 13 and 18), pJA110 (lanes 14 and 19), pET13 (lanes 15 and 20), and pTW688 (lanes 16 and 21) were found in *E. tarda*. Plasmids pJA8001 (lanes 24 and 29, pJAPS8102 (lanes 25 and 30), pJAPE8225 (lanes 26 and 31), and pJAPN8310 (lanes 27, and 33) came from *P. piscicida*. RA1 is in lanes 6, 11, 17, 22, 28, 33.

and 0 μg of tetracycline per ml, and the optical densities were measured at 30-min intervals. R plasmids from *A. hydrophila*, *E. tarda*, and *P. piscicida* were inducible for Tc^r, as was RA1.

Plasmid DNA was prepared by the method of Birnboim and Doly (8) or that of Hansen and Olsen (12). The DNA was digested with restriction endonucleases *Bgl*II, *Hin*dIII, or *Pst*I (Takara and Nippon Gene Cos., Ltd.) according to the conditions specified by the manufacturer. Digested fragments were resolved by electrophoresis in 0.8% agarose and transferred to nitrocellulose filters as described by Southern (19). The *tet*^{r} probes used were a 0.75-kilobase (kb) *Sma*I fragment of RP4 for class A, a 2.8-kb *Bgl*II fragment of R222 for class B, a 1.4-kb *Ava*I-*Hin*dIII fragment of pBR322 for class C, and a 3.05-kb *Hin*dIII-*Pst*I fragment of RA1 for class D. Probe DNA was labeled with [^{32}P]dCTP (New England Nuclear Research Products) by nick translation (15). Hybridization reactions and autoradiography procedures were carried out as described previously (15).

*Pst*I digestion patterns of the four R plasmids from *A. hydrophila* were almost identical to that of RA1, with common fragments of molecular sizes 4.2, 2.78, 0.92, 0.82, and 0.78 kb (Fig. 1). The 3.05-kb probe from RA1 reacted strongly with 4.2-kb fragments of *A. hydrophila* R plasmid (Fig. 1). Conversely, R-plasmid DNAs from *A. hydrophila* were not homologous with the Tc^r determinants of RP4 (class A), R222 (class B), or pBR322 (class C).

There were common fragments also in the digests of R plasmids from *E. tarda* and RA1 (Fig. 1). The 3.05-kb probe from RA1 hybridized strongly with the 4.2-kb fragment from the R plasmids of *E. tarda*. A common fragment with a molecular weight of 4.2 kb was found as well in the digests of R plasmids from *P. piscicida* and RA1 (Fig. 1). With these results, the *tet*^{r} regions of the R plasmids detected from *A. hydrophila*, *E. tarda*, and *P. piscicida* were classified as class D.

The *tet*^{r} regions of pJA5017 from *A. hydrophila*, pET13 from *E. tarda*, pJA8001 from *P. piscicida*, and RA1 were cloned in the vectors pACYC177 (9) and pKY2700 (18). Recombinant plasmids were transformed into *E. coli* strains HB101 [F$^-$ *hsdS*(r_B^- m_B^-) *ara gal lac mtl pro recA rpsL supE xyl*] and KS2120 (*endA* recA SupII$^+$, supplied by K. Shimada; 18). The cloned regions were analyzed using *Hin*cII, *Hin*dIII, *Pst*I, *Pvu*II, *Sal*I, and *Xho*I (Takara and Nippon Gene Cos., Ltd.). Similar restriction sites were observed on the linear restriction maps of pJA5017, pET13, pJA8001, and RA1 (Fig. 2).

In lactose-fermenting coliforms, Marshall et al. (16) detected *tet* class B, most frequently followed by class A and C, but found no class D. A class D Tc^r determinant has been found in a tetracycline-resistant strain of *Vibrio cholerae* (14). Our results demonstrate that class D Tc^r determinants are common in fish pathogens. The R plasmids from *P. piscicida* have been demonstrated to have similar structures, and such plasmids have been found widely distributed in yellowtail (*Seriola quinqueradiata*) marine fish culture farms in various areas (20). R plasmids having identical DNA structure were also detected in *A. hydrophila* strains and *E. tarda* strains which have been isolated in eel (*Anguilla japonica*) culture ponds (1, 2). These facts indicate that the

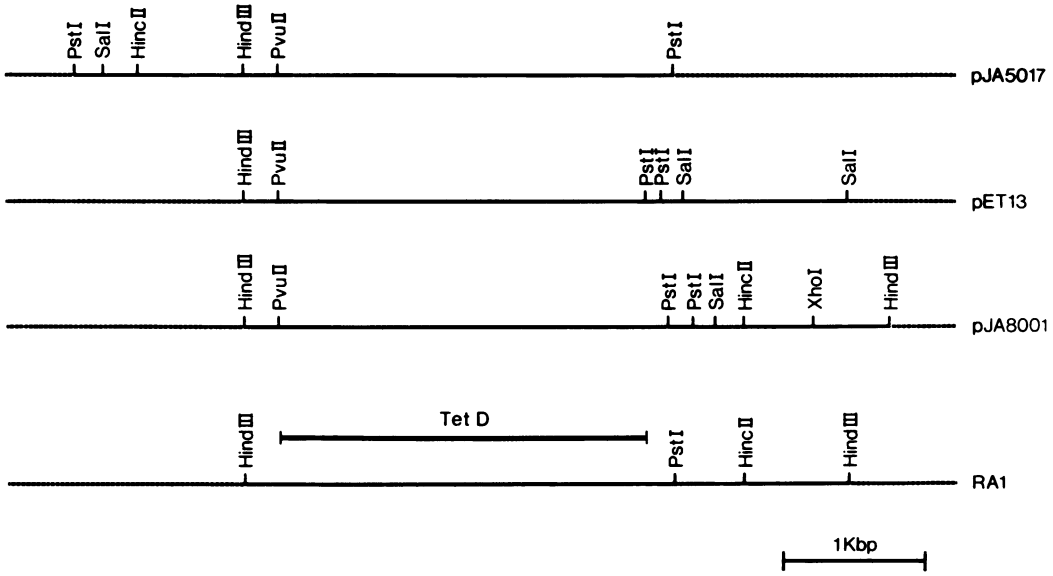

FIG. 2. Restriction map of tetracycline resistance regions. pJA5017 was found in *A. hydrophila*, pET13 in *E. tarda*, and pJA8001 in *P. piscicida*.

tet^r gene of class D is widely distributed in R plasmids from marine and freshwater fish pathogenic bacteria.

We thank T. Kitao for his useful suggestions and discussions.

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