Detection of Resistance Factors in Fish Pathogen *Aeromonas liquefaciens*

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SUMMARY

Thirty-nine out of 72 drug-resistant strains of *Aeromonas liquefaciens* isolated from cultured fish and soft-shelled turtles (*Trionyx sinensis japonicus*) in various districts of Japan carried transferable drug resistance factors. The fish included eel (*Anguilla japonica*), carp (*Cyprinus carpio*), ayu (*Plecoglossus altivelis*) and goldfish (*Carassius auratus*). The most common type of resistance factors had the markers of resistance to sulphanilamide and tetracycline and all belonged to the *fi*⁻ type. Transferable drug resistance was not found in any of 12 strains of *A. liquefaciens* isolated from wild eels. The high incidence of resistance factors in *A. liquefaciens* from cultured fish is assumed to be due to the selective pressure exerted by chemotherapeutics used in fish culturing.

INTRODUCTION

The use of antibiotics and other synthetic chemotherapeutics as feed additives for farm livestock, including poultry (see Stokstad, 1954; Jukes, 1955) has resulted in an increase in drug-resistant enteric bacteria in these animals, and a majority of these drug-resistant bacteria carry transferable drug resistance factors (or R factors) (Anderson & Lewis, 1965; Anderson, 1968, 1969; Smith, 1969). Various antibiotics and synthetic chemotherapeutics have also been used for cultured fish as feed additives in various countries (Schäperclaus, 1955, 1956; Snieszko, 1957, 1959; Hoshina, 1962; Wolf & Snieszko, 1963; Shimizu & Takase, 1967) to prevent and treat various infections as in other livestock. They have sometimes been administered directly into fish-pond water to control fish infections (Hoshina, 1962; Muroga & Egusa, 1968). There have been several reports which dealt with the drug sensitivities of the fish pathogen *Aeromonas liquefaciens* (synonyms: *A. punctata* and *A. hydrophila*) (Schäperclaus, 1956, 1958; Caselitz & Maass, 1962; Hoshina, 1962; Karasek, 1967; Shimizu & Takase, 1967; Muroga & Egusa, 1968) but other workers have studied rather small numbers of strains.

We had suspected that the use of antibiotics and other chemotherapeutics might have caused an increase in drug-resistant fish-pathogenic bacteria, because of the effects of antibiotic feeding on animal enteric bacteria and also from our knowledge that the administration of drugs to cultured fish has sometimes been ineffective in

recent years. We have indeed shown that a considerable proportion of *Aeromonas liquefaciens* strains isolated from cultured fish and soft-shelled turtles (*Trionyx sinensis japonicus*) are drug-resistant (Aoki & Egusa, 1971). These fish included eel (*Anguilla japonica*), carp (*Cyprinus carpio*), ayu (*Plecoglossus altivelis*) and goldfish (*Carassius auratus*). Seventy-eight of 250 strains of *A. liquefaciens* isolated from fish and soft-shelled turtles were resistant to from one to four drugs excluding aminobenzyl penicillin to which *A. liquefaciens* seems naturally resistant (Aoki & Egusa, 1971).

We suspected from the patterns of drug resistance of some of the drug-resistant *Aeromonas liquefaciens* strains that their drug resistances might be due to R factors. Our preliminary studies with two such strains showed that this was so (Watanabe, Ogata, Aoki & Egusa, 1969). Their drug resistance markers were found transferable as single units to *Escherichia coli* K12 in mixed cultivation and they could be spontaneously lost, as units, as shown by the penicillin screening method combined with replica plating (see Watanabe, 1964). We report here a larger scale investigation using the 72 drug-resistant strains of *A. liquefaciens* isolated previously (Aoki & Egusa, 1971).

METHODS

Strains. The strains of *Aeromonas liquefaciens* isolated in our previous study (Aoki & Egusa, 1971) are listed in Table 1 and the drug resistance patterns of the drug-resistant strains are shown in Table 2. All the strains were isolated from various organs of different individual fish and soft-shelled turtles. Two hundred and forty-four of the 250 strains were isolated from cultured fish or soft-shelled turtles and six strains from diseased wild ayu. We did not include more than one strain isolated from fish from the same fish pond or from fish ponds owned by the same fish culturist. One hundred and fifty-four of the strains studied were from diseased fish or turtles. The other 96 strains were isolated from normal-looking fish.

Table 1. *Years and sources of the strains of Aeromonas liquefaciens used in the present investigation*

Year	Source*	No. of strains isolated
1963	Eel	1
1964	Eel	4
1965	Carp	1
1966	Eel	21
1967	Ayu	12
	Eel	8
	Goldfish	8
1968	Carp	2
	Eel	52
	Goldfish	3
1969	Carp	24
	Eel	102
	Goldfish	10
	Soft-shelled turtle	2
Total		250

* All the sources of *Aeromonas liquefaciens* strains were cultured fish and soft-shelled turtles except six wild ayu.

Two hundred and twenty-seven strains (97.8 %) of the 232 strains of *Aeromonas liquefaciens* studied were resistant to more than 200 µg. of aminobenzyl penicillin/ml., and 78 out of the 250 total strains isolated were resistant to from one to four other drugs (Aoki & Egusa, 1971) (Table 2). Six of these drug-resistant strains were lost between the previous study and the present investigation. Besides the above drug-resistant strains, 12 *A. liquefaciens* strains isolated from the intestinal contents of wild eels were also studied for the conjugal transferability of their drug resistances. These strains are not included in Tables 1 and 2, because their drug resistances were studied with sensitivity discs (Showa) unlike the other strains whose drug resistances were studied by determining the minimal inhibitory concentrations.

Table 2. *Drug resistance patterns of resistant strains of Aeromonas liquefaciens isolated from fish and soft-shelled turtles*

Source	No. of resistant strains	No. of strains studied	Resistance pattern*	No. of strains
Ayu	1	12	Tc†	1
Carp	14	27	Sm	1
			Su	3
			Tc	1
			Su, Tc	4
			Su, Sm, Tc	1
			Su, Sm, Cm, Tc	4
Eel	59	188	Sm†	1
			Cm	3
			Tc	8
			Tc†	3
			Nf	2
			Su, Tc†	1
			Su	2
			Su, Tc	30
			Sm, Tc	1
			Sm, Nf	1
			Su, Sm, Tc	3
			Su, Sm, Cm, Tc	4
Goldfish	3	21	Tc	3
Soft-shelled turtle	1	2	Su, Tc	1
Total	78	250		78

* Abbreviations: Su, sulphanilamide; Sm, streptomycin; Cm, chloramphenicol; Tc, tetracycline; Nf, nitrofurant.

† The aminobenzyl penicillin sensitivity of these strains was not tested, because they were lost before the test.

93.1 % of the strains listed in this table were resistant to aminobenzyl penicillin.

For methods see text.

Escherichia coli CSH-2 (methionine- and proline-requiring F⁻ derivative of K12) was used as a recipient for the conjugal transfer of R factors from *Aeromonas liquefaciens* strains. *Escherichia coli* W2252/S^r (methionine-requiring, streptomycin-resistant Hfr derivative of K12) was used as a host of R factors for studying the fertility inhibition (*fi*) property (Watanabe, Fukasawa & Takano, 1962; Watanabe, Nishida, Ogata, Arai & Sato, 1964) of the R factors.

Culture media. Nutrient agar was composed of 10 g. beef extract (Kyokuto), 10 g. polypeptone (Takeda), 2 g. Bacto agar (Difco) in 1 l. distilled water. BTB-lactose

nutrient agar was prepared by adding 0.0045 % bromothymol blue and 1 % lactose to nutrient agar, and agar as prescribed by Lennox (1955), all adjusted to pH 7.3. The liquid medium used was Bacto Penassay broth (Difco).

Chemotherapeutic agents. Incorporated into BTB-lactose nutrient agar were: sulphathiazole (Su) (Takeda), 500 µg./ml.; chloramphenicol powder (Cm) (Sankyo), 25 µg./ml.; tetracycline hydrochloride (Tc) (Lederle), 25 µg./ml.; kanamycin sulphate (Km) (Meiji), 50 µg./ml.; dextromycin sulphate (Nm) (Takeda) (identical to neomycin sulphate), 50 µg./ml.; aminobenzyl penicillin (Ap) (Meiji), 25 µg./ml.; or furazolidone (Nf) (Takeda), 12.5 µg./ml.

Detection of R factors. Each strain of drug-resistant *Aeromonas liquefaciens* was incubated in Penassay broth with aeration at 28° to an extinction equivalent of about 5×10^8 bacteria/ml. *Escherichia coli* CSH-2 was incubated in Penassay broth with aeration at 37° to an equal density. One ml. *Aeromonas* culture was mixed with 1 ml. *E. coli* CSH-2 culture in a test tube and incubated overnight without aeration at either 28° or 37° and then plated on BTB-lactose nutrient agar containing each of the drugs to which the *Aeromonas* strain was resistant. If the drug resistance was transferred to *E. coli* CSH-2, lactose-fermenting colonies would develop among the lactose-non-fermenting growth of *Aeromonas* colonies. Some strains of *A. liquefaciens* ferment lactose slowly but none formed yellow colonies on the selective media after overnight incubation when plated alone. Each of the colonies of CSH-2 which received drug resistance was re-isolated for purity on a similar selective medium. The purified colony was then tested for resistance to other drugs using sensitivity discs (Showa). In determining drug sensitivities with the discs, R⁻ CSH-2 strain was tested simultaneously as a control.

When the drug resistance of *Aeromonas liquefaciens* was found transferable to CSH-2, resistance was transferred from *A. liquefaciens* to *Escherichia coli* w2252/s^r by mixed cultivation either directly or by way of CSH-2. The procedure used for transferring the drug resistance from *A. liquefaciens* to w2252/s^r was the same as that used for CSH-2. When the drug resistance was to be transferred from CSH-2 to w2252/s^r, the mixed culture of CSH-2 (R⁺) and w2252/s^r, after incubation overnight, was plated on BTB-lactose nutrient agar containing 1000 µg. of Sm/ml. plus each of the other drugs. Since the levels of Sm resistance conferred by R factors on *E. coli* K12 strains are in the range of 10 to 50 µg./ml. (Watanabe, 1963*b*) in contrast to chromosomal Sm resistance, which is higher than 1000 µg./ml., the donor bacteria do not grow with 1000 µg./ml. of Sm, even if they carry Sm resistance R factors. Only the recipient clones which received the R factors form colonies on such selective media.

Method for study of fi property of R factors. R factors can be classified into *fi*⁺ and *fi*⁻ types depending on the presence and absence of fertility inhibition or the inhibition of the formation of F pili by the sex factor F of *Escherichia coli* K12 (Watanabe *et al.* 1962; Watanabe, 1964; Meynell, Meynell & Datta, 1968). The *fi*⁺ and *fi*⁻ R factors are probably equal to the F-like and I-like R factors, respectively (Novick, 1969). We use our old terms here because we have so far studied only the fertility inhibition property by testing the sensitivity of the R factor-carrying male bacteria to male-specific bacteriophages (Watanabe *et al.* 1962) and we have not shown whether they actually produce F pili or I pili.

Each R factor-carrying w2252/s^r strain was grown in Penassay broth to about 5×10^8 /ml. and a drop of this culture was added to 2 ml. of melted soft Lennox agar

kept at 48° and containing M/400 CaCl₂; this medium was poured on the top of ordinary Lennox agar containing M/400 CaCl₂. When the soft agar was solidified, a drop of male-specific bacteriophage f1 or f2 (with a titre higher than 10⁹/ml.) was spotted on its surface and the plate incubated at 37° overnight. If a lytic zone developed, the R factor was regarded as *fi*⁻, otherwise as *fi*⁺.

RESULTS

R factors in drug-resistant strains of Aeromonas liquefaciens. Ap resistance was not transferred by any of the *Aeromonas liquefaciens* studied and so Ap resistance is not included in Table 3. Table 3 shows that two out of 23 one-drug-resistant strains of *A. liquefaciens* transferred their Su resistance to *Escherichia coli* CSH-2 by mixed cultivation. Thirty-two out of 37 two-drug-resistant strains transferred their Su, Tc resistance and one of the two-drug-resistant strains transferred its Tc resistance alone. One out of four Su, Sm, Tc-resistant strains could transfer its Su, Tc resistance. Two out of eight Su, Sm, Cm, Tc-resistant strains transferred their Su, Tc resistance, and another strain transferred its Su, Cm, Tc resistance. Transfer of R factors occurred equally well at 28° and at 37°.

Table 3. *Patterns of drug resistance and their conjugal transferability in Aeromonas liquefaciens isolated from fish and soft-shelled turtles*

No. of resistance markers	No. of strains	Resistance pattern*	No. of strains	No. of R ⁺ strains	Resistance pattern of R factor
1	23	Su	5	2	Su
		Sm	1	0	
		Cm	2	0	
		Tc	13	0	
		Nf	2	0	
2	37	Su, Tc	35	32	Su, Tc
		Sm, Tc	1	1	
		Sm, Nf	1	0	
3	4	Su, Sm, Tc	4	1	Su, Tc
4	8	Su, Sm, Cm, Tc	8	2	Su, Tc
				1	Su, Cm, Tc
Total	72		72	39	

* Abbreviations: Su, sulphanilamide; Sm, streptomycin; Cm, chloramphenicol; Tc, tetracycline; Nf, nitrofurant.

95.8% of the strains listed in this table were resistant to aminobenzyl penicillin.

All the R factors found in *Aeromonas liquefaciens* strains were identified as *fi*⁻ type.

Transferability of the drug resistance of strains of Aeromonas liquefaciens isolated from wild eels. Three out of 12 strains of *Aeromonas liquefaciens* isolated from wild eels were resistant to Tc and Ap, three others were resistant to Tc, Nf and Ap, five resistant to Tc, Nm, Nf and Ap, and one was resistant to Tc, Km, Nf and Ap. None transferred their resistance to *Escherichia coli* by mixed cultivation.

DISCUSSION

It is characteristic for the R factors from *Aeromonas liquefaciens* that a majority of them have Su, Tc resistance markers and that all of them belong to *fi*⁻ type, unlike most R factors from *Escherichia coli* and *Shigella*. Even an R factor with Cm resistance

marker detected in a fish was identified as belonging to fi^- type. The R factors detected in *E. coli* and Shigella in Japan mostly have the markers of resistance to Su, Sm, Cm and Tc and belong to fi^+ type (see Watanabe *et al.* 1962; Watanabe, 1963*a, b*). R factors can be transferred *in vitro* to every genus of Enterobacteriaceae as well as to Vibrio and Pasteurella (see Watanabe, 1963*a*) and also to Aeromonas of human origin (Abe, Goto & Kuwahara, 1966). Thus it is not surprising that in the present investigation R factors have been detected in strains of *A. liquefaciens*. It should be emphasized, however, that the R factors reported here were found in naturally occurring strains of fish pathogen *A. liquefaciens* isolated from fish and soft-shelled turtles.

A majority of the strains of *Aeromonas liquefaciens* were highly resistant to Ap despite the fact that no penicillin derivative has been used in fish culturing in Japan. None could transfer Ap resistance to *Escherichia coli* in mixed cultivation. Thus the Ap resistance of *A. liquefaciens* may be regarded as 'natural resistance'. It is interesting to note that the R factors found in *A. liquefaciens* all belonged to fi^- type and that most of them had the markers of resistance to Su and Tc and neither Sm nor Cm markers. The latter finding is particularly interesting, because Cm has been used rather extensively for fish culturing in Japan. The cause of this paradoxical finding may be ascribed to the genetic instability of the Sm and Cm markers of R factors in Aeromonas (Abe *et al.* 1966; and our unpublished data).

The increase of R factor-carrying enteric bacteria in animal intestines as a result of antibiotic feeding has been a subject of much dispute because of its potential public health hazards; R factors from animals may be dangerous to man either directly, in infections with resistant pathogens such as Salmonella of animal origin (see Anderson, 1968, 1969), or indirectly, if R factors are transferred to human pathogens by way of non-pathogenic bacteria such as *Escherichia coli* (see Watanabe, 1963*a*). The prevalence of R factor-carrying bacteria among fish pathogen *Aeromonas liquefaciens* may add another problem to this discussion. Our discovery of R factor-carrying bacteria in fish seems to have particular importance in Japan because of the importance of fish-culturing industry in Japan and also because of the Japanese custom of eating raw fish.

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