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Detection of R Factors in Naturally Occurring *Vibrio anguillarum* Strains

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R factors were detected in *Vibrio anguillarum* strains from vibrio-diseased freshwater fish, ayu (*Plecoglossus altivelis*), in Japan. It was found that 65 out of the 68 random isolates from epidemics of vibrio disease in 1973 carried transferable drug resistance factors. The most common type determined resistance to sulfonamides, streptomycin, chloramphenicol, and tetracycline and belong to the *fi*$^-$ type. The high incidence of R factors in *Vibrio anguillarum* from cultured fish is assumed to be due to selective pressure exerted by chemotherapeutic agents used in fish culturing.

Vibrio disease of eels (*Anguilla anguilla*), rainbow trout (*Salmo gairdneri* f. *irideus*), and ayu (*Plecoglossus altivelis*) has been well studied in fish. The first report of this disease appeared in 1909 (2); the agent of this disease has formerly been called *Vibrio anguillarum* (2) or *Vibrio piscium* var. *japonicus* (4) and more recently has been identified as *V. anguillarum* (3).

Because of its rapid growth and fine taste, the culturing of ayu is very popular in Japan. The use of chemotherapeutic agents to prevent infection in the culturing of ayu has become very common in recent years. From May to August 1973 in Japan, there were large-scale epidemics of vibrio disease in ayu culturing farms (8). A dominant characteristic of these epidemics was the ineffectiveness of the commonly used antibiotics.

MATERIALS AND METHODS

Strains. The strains of *V. anguillarum* used for this study were collected from various districts in Japan (Table 1). *Escherichia coli* RC85 *nal* (a methionine-requiring, nalidixic acid-resistant F$^-$ derivative of *E. coli* K-12) and W3102 *str* (a prototrophic, galactose- and lactose-nonfermenting, streptomycin-resistant F$^-$ derivative of K-12) were used as recipients for conjugal transfer of R factors. *E. coli* W2252 *Hfr str* (a methionine-requiring, streptomycin-resistant *Hfr* derivative of K-12) was used as a host for R factors in studying the fertility inhibition (*fi*) property (11) of the R factors.

Media. The nutrient agar used was composed of 10 g of beef extract (Kyokuto), 10 g of polypeptone (Takeda), and 15 g of agar (Difco) in 1,000 ml of distilled water. BTB-lactose nutrient agar was prepared by adding 0.0045% bromothymol blue and 1% lactose to the nutrient agar. L agar (7) was used for the sensitivity test of male-specific phages f1 and Qβ. All media were adjusted to pH 7.3. Semisynthetic agar medium, as described by Knight (5), supplemented with 0.2% Casamino Acids (Difco) was used to test the sensitivity for sulfonamide, and heart infusion agar (Eiken) and modified Muller-Hinton agar (Nissui) were used to test the sensitivity for other chemotherapeutic agents. The liquid medium used was Penassay broth (Difco). For the cultivation of *V. anguillarum*, 2% NaCl was added to the above media.

Chemotherapeutic agents. Chemotherapeutic agents used were sulfamonomethoxine (Daiichi), chloramphenicol powder (Sankyo), tetracycline hydrochloride (Lederle), streptomycin sulfate (Sankyo), spectinomycin (Upjohn), kanamycin sulfate (Meiji), aminobenzyl penicillin (Meiji), furazolidone, one of the nitrofuran derivatives (Nif; Takeda), and nalidixic acid (Nal; Daiichi).

MICs of various drugs. Each strain of *V. anguillarum* was incubated in Penassay broth supplemented with 2% NaCl at 30 C to middle logarithmic growth phase and diluted in the same medium. A 0.025 ml amount containing about 10^3 bacteria was dropped on 2% NaCl-supplemented plates containing a serial twofold dilution of various chemotherapeutic agents and incubated at 30 C for 48 h. Concentrations of various chemotherapeutic agents which gave less than ten colonies were taken as minimal inhibitory concentrations (MICs). *E. coli* strains were incubated at 37 C in media with the standard NaCl concentrations.

Detection of R factors. Each strain of drug-resistant *V. anguillarum* was incubated at 30 C in 2% NaCl-supplemented Penassay broth with aeration to an optical density equivalent of about 2×10^8 bacteria/ml. *E. coli* RC85 *nal* was incubated in Penassay broth with aeration at 37 C to an optical density equivalent of about 5×10^8 bacteria/ml. *Vibrio* culture (0.5 ml) was mixed with 0.5 ml of *E. coli* culture in a flat-bottomed tube, incubated with-

out aeration at either 30 or 37 C for 2 h or overnight, and then plated on BTB-lactose nutrient agar containing 25 μg of either tetracycline hydrochloride or chloramphenicol powder per ml, to one of which all *Vibrio* cultures were resistant, with 50 μg of nalidixic acid per ml. If the drug resistance was transferred to *E. coli*, lactose-fermenting colonies would develop. The number of colonies developing were scored and reisolated for purification on a similar selective medium. The purified colonies were then tested for their resistance to other drugs using sensitivity disks (Showa) on modified Muller-Hinton agar. R factors transferred to *E. coli* RC85 *nal* from *V. anguillarum* were retransferred to *E. coli* W3102 *str* by mixed cultivation in Penassay broth at 37 C in a procedure similar to that described above. The R factors were also transferred from original R^+ *Vibrio* strains to *E. coli* W2252 *Hfr str*. Recipient bacteria which received R factors were selected on BTB-lactose nutrient agar containing 1,000 μg of streptomycin sulfate per ml and 25 μg of either tetracycline hydrochloride or chloramphenicol powder per ml depending on their resistance markers of R factors.

Study of fi property of R factor. 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 *E. coli* K-12 (11). The presence of F pili on R factor-carrying male bacteria was determined by testing the sensitivity of these bacteria to male-specific bacteriophages. Each W2252 *Hfr str* strain carrying an R factor was grown in L broth to about 5×10^8/ml, and a drop of this culture was added to 2 ml of melted soft L agar containing 2.5×10^{-3} M $CaCl_2$. This medium was poured on top of L agar containing 2.5×10^{-3} M $CaCl_2$. When the soft agar solidified, a drop of male-specific bacteriophage f1 or Qβ (with a titer higher than 10^9/ml) was spotted on its surface, and the plate was incubated at 37 C overnight. If a lytic zone developed, the R factor was regarded as fi^-; if none developed, it was regarded as fi^+.

RESULTS

MIC of various chemotherapeutic agents on naturally occurring V. anguillarum strains. The distribution of MICs of various chemotherapeutic agents in naturally occurring strains of *V. anguillarum* are shown in Fig. 1. With sulfamonomethoxine, tetracycline hydrochloride, chloramphenicol powder, nitrofuran, and nalidixic acid, the strains formed two apparent groups and indicated the presence of resistant strains for these drugs. The presence of bi- or trimodal distribution of streptomycin-resistant strains suggested that there were at least two different types of resistance. Distribution patterns of kanamycin and aminobenzyl penicillin sensitivities showed the presence of only one group, and all strains were thought to be susceptible to these two drugs. Resistance patterns of these strains are shown in Table 2.

TABLE 1. *V. anguillarum isolated from ayu (Plecoglossus altivelis) in 1973*[a]

Location	No. of strains
Tokushima prefecture	58
Shizuoka prefecture	4
Aichi prefecture	2
Okayama prefecture	2
Tokyo metropolis	2

[a] All strains were isolated from different farms.

R factor in drug-resistant strains of V. anguillarum. Sixty-five resistant strains of *V. anguillarum* were examined for transfer of resistance to *E. coli*. All strains examined transferred at least some of these drug resistances to *E. coli*. The patterns of transferred resistance are shown in Table 2. Two *V. anguillarum* strains which transferred sulfamonomethoxine, streptomycin sulfate, and chloramphenicol powder resistances as a single unit were also resistant to nalidixic acid and nitrofurans. One strain, which transferred only tetracycline hydrochloride resistance, was also resistant to sulfamonomethoxine, streptomycin sulfate, chloramphenicol powder, nalidixic acid, and nitrofurans. The most common type of transferable resistance (R factors) had the markers of resistance to sulfamonomethoxine, streptomycin sulfate, chloramphenicol, and tetracycline. Nitrofuran and nalidixic acid resistances were not transferred by any strains of *V. anguillarum* examined. Transfer of R factors occurred equally well at 30 and 37 C.

MIC of various chemotherapeutic agents for strains of RC85 nal with R factor from V. anguillarum. The MICs of tetracycline, chloramphenicol, sulfamonomethoxine, streptomycin sulfate, spectinomycin, kanamycin, nitrofurans, and aminobenzyl penicillin for strains of RC85 *nal* with R factors are shown in Fig. 2. The levels of resistance for various drugs conferred by R factors differed in *E. coli* and *V. anguillarum*. The high streptomycin-resistant R factors in *V. anguillarum* (all of those resistant to 200 μg/ml, MIC) are also resistant to high concentrations of streptomycin sulfate (12.5 μg/ml, MIC) and spectinomycin (25 μg/ml, MIC) in *E. coli*. Those R factors determine lower resistance to streptomycin sulfate in *V. anguillarum* and determine lower levels of resistance to streptomycin sulfate and spectinomycin in *E. coli*.

R factors from V. anguillarum in E. coli. R factors were retransferred from RC85 *nal* to *E. coli* W3102 *str* for further examination of transfer between strains of *E. coli* and stability in *E. coli*. All R factors examined were stable in *E.*

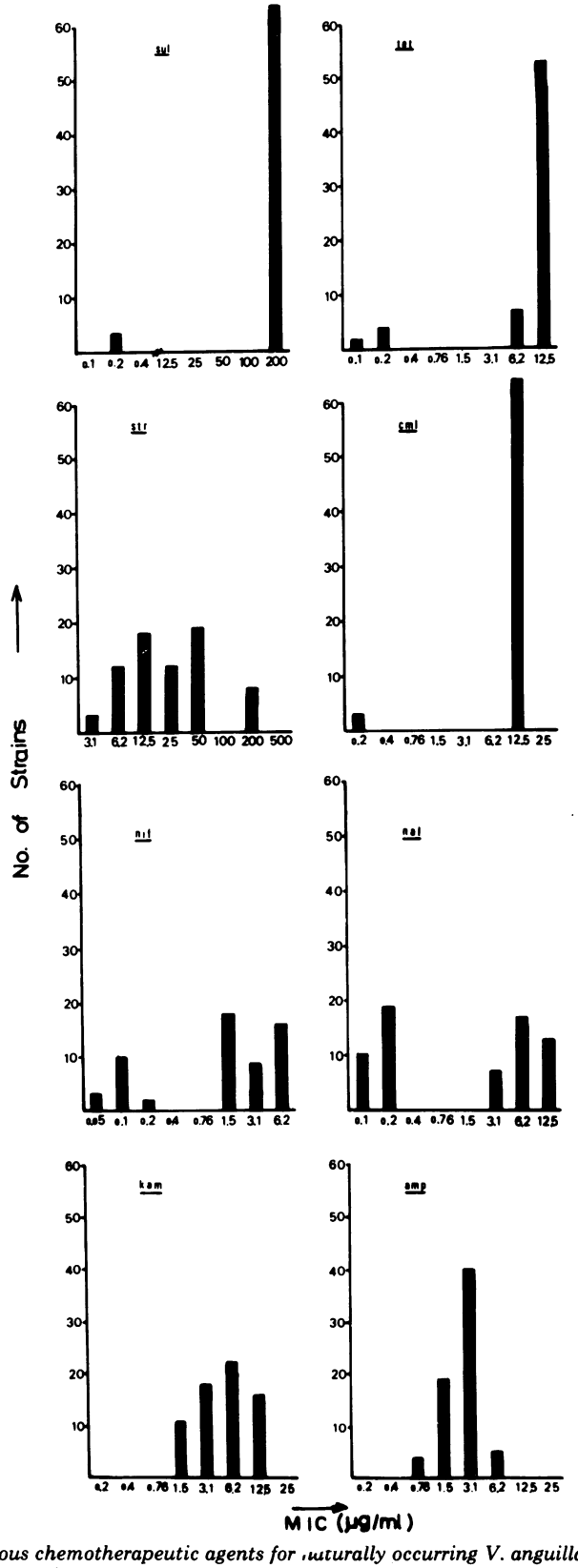

FIG. 1. *MICs of various chemotherapeutic agents for naturally occurring V. anguillarum strains. Abbreviations: sul, sulfamonomethoxine; str, streptomycin; tet, tetracyline; cml, chloramphenicol; nif, furazolidone; kan, kanamycin; nal, nalidixic acid; amp, aminobenzyl penicillin.*

TABLE 2. *Resistance patterns and R factors of V. anguillarum isolated from ayu (Plecoglossus altivelis)*[a]

Resistance pattern of original strains	R^+ strains/ strains studied	Resistance pattern of R factor
Sul, str, cml, tet	13/13	Sul, str, cml, tet
Sul, str, cml, tet, nal	2/2	Sul, str, cml, tet
Sul, str, cml, tet, nif	14/14	Sul, str, cml, tet
Sul, str, cml, nal, nif	2/2	Sul, str, cml
Sul, str, cml, tet, nal, nif	1/1	Tet
Sul, str, cml, tet, nal, nif	33/33	Sul, str, cml, tet
Sensitive strains	0/3	

[a] Sul, str, cml, tet, nal, and nif: sulfonamide, streptomycin, chloramphenicol, tetracycline, nalidixic acid, and nitrofuran derivative resistance, respectively.

coli not only as whole units but also in their resistance markers. That is, the spontaneous loss of total R factors, as well as the spontaneous loss of transferred resistance markers in *E. coli*, was less than one per several hundred clones.

Fi property of R factors from V. anguillarum. R factors were transferred directly from original strains of *V. anguillarum* or indirectly from *E. coli* RC 85 *nal* to W2252 *Hfr str* for the study of *fi* property of these R factors. All R^+ clones of W2252 which received any of these R factors were found to be sensitive to male-specific phages f1 and Qβ. This result indicated that these R factors all belong to fi^- type.

DISCUSSION

There has been no report so far of the detection of R factors in naturally occurring *Vibrio*

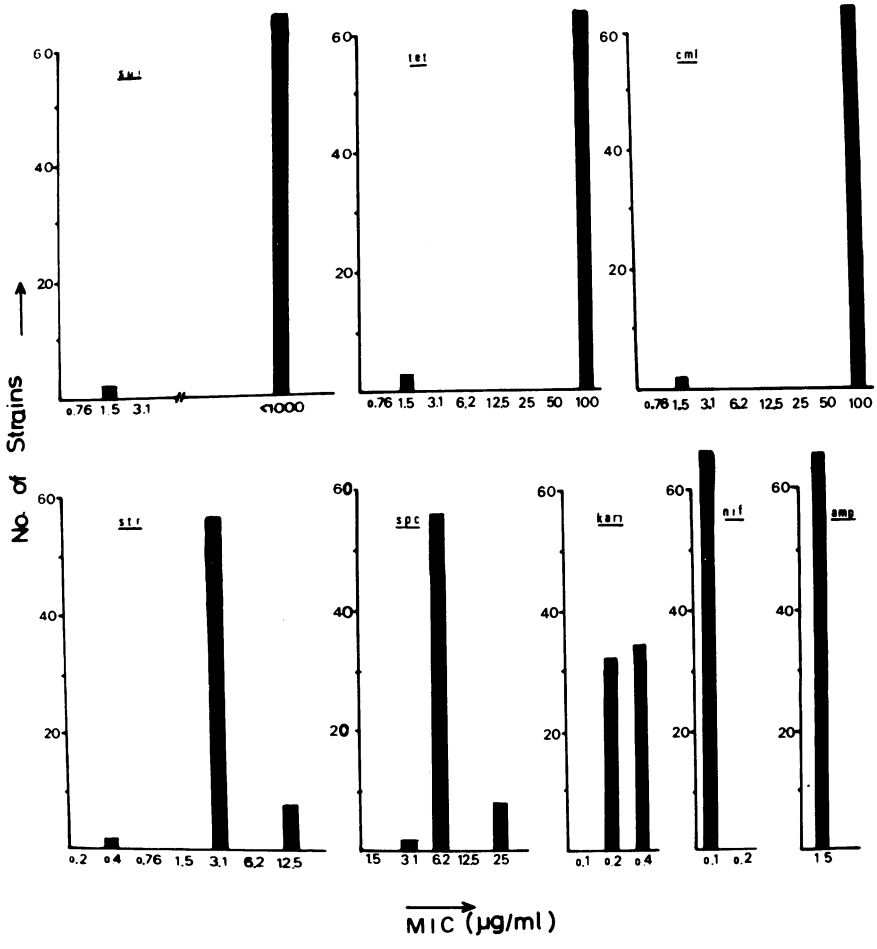

FIG. 2. *MICs of various chemotherapeutic agents for E. coli RC85 nal carrying R factors from V. anguillarum. Abbreviation: spc; spectinomycin.*

strains, except for our report on a few R factors in unidentified marine *Vibrio* (1). Because of the high numbers of identified R factors and the reliability of the bacterial origin, this is the first report of detection of R factors from identified *Vibrio* strains. A characteristic of the R factors from *V. anguillarum* is that a majority determines resistances to sulfonamide, streptomycin, chloramphenicol, and tetracycline and that all are the fi^- type. It is interesting that these characteristics are also common of R factors detected in unidentified marine *Vibrio* (1). Since these R factors from *V. anguillarum* are stable in *V. cholerae* (manuscript in preparation) as well as in *E. coli*, they are different from R factors detected in *E. coli* and *Shigella* isolated from human diseases, which, although they also have the commonly determined sulfonamide, streptomycin, chloramphenicol, and tetracycline resistances (1, 12) and are transferable to *V. cholerae*, are unstable even under the presence of selective drugs (6, 13). It should be emphasized that the existence of the R factors reported in this paper may cause serious problems for the treatment of diseases caused by *Vibrio*, including *V. cholerae*.

It is known that the levels of drug resistance conferred by R factors differ in different hosts (11), and the MICs for tetracycline, chloramphenicol, and sulfonamides are lower in *Vibrio* than in *E. coli*, but the MIC for streptomycin is far higher in *Vibrio* than in *E. coli*. It is also interesting that there are two types of R factors from *V. anguillarum* which give their host bacteria different levels of streptomycin resistance. According to Ozanne et al. (9), there are two kinds of inactivation enzymes for streptomycin, an adenyltransferase and a phosphotransferase. The adenyltransferase inactivates not only streptomycin but also spectinomycin, whereas the phosphotransferase inactivates only streptomycin. Since these R factors were found to give streptomycin and spectinomycin resistances, they are suspected of determining an adenyltransferase, although they were divided into two groups depending on their resistance levels to streptomycin and spectinomycin. Each group of these R factors was always found in different districts.

It is not clear whether drug-resistant *V. anguillarum* strains were present in Japan before 1972. All strains of *V. anguillarum* examined by us before 1972 were sensitive to all chemotherapeutic agents tested. The use of chemotherapeutic agents for fish culturing has been common in Japan for the past 10 years. The high incidence of R factors from *V. anguillarum* in the epidemics of vibrio disease of ayu in 1973 were assumed to be due to the selective pressure exerted by chemotherapeutic agents used in fish culturing, but the cause of the sudden outbreak of the resistant *V. anguillarum* strains is still unknown.

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