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Comparison of the Taxonomy, Serology, Drug Resistance Transfer, and Virulence of *Citrobacter freundii* Strains from Mammals and Poikilothermic Hosts

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In this study, the phenotypic, antigenic, and virulence characteristics of 32 *Citrobacter freundii* strains of fish, human, and veterinary origin were comparatively analyzed. In addition, the spread of drug resistance factors by conjugation was investigated. Regardless of the source of isolation, the strains exhibited variable reactions mainly for arginine dihydrolase, ornithine decarboxylase, and fermentation of sucrose, melibiose, amygdalin, and salicin. Total fatty acid methyl ester analysis by gas chromatography proved to be useful for an intratypic differentiation within the *C. freundii* strains studied. In fact, although all of the isolates exhibited similar fatty acid methyl ester profiles, significant differences in the major fatty acids 16:1 and 16:0 and in the 17:0Δ region were observed between the isolates from salmonids and the remaining strains. Serological studies using agglutination tests, analysis of lipopolysaccharides (LPS), and the corresponding immunoblots with 13 antisera indicated a great antigenic diversity among the strains. Common LPS patterns were shared only by some isolates showing high cross-agglutination titers. In contrast, although all strains exhibited very similar surface protein patterns, only two common outer membrane proteins of 54 and 58 kDa were immunologically related. Infectivity trials performed in mice and rainbow trout indicated that all of the *C. freundii* strains were not pathogenic for mice (50% lethal dose of $>5 \times 10^7$). Although the isolates displayed a low degree of virulence for trout, inoculated strains were always recovered from the survivors in pure culture. The detection of plasmid DNA by agarose gel electrophoresis revealed that poikilothermic isolates harbored one or more large-molecular-size plasmid bands ranging from 110 to 25 MDa, whereas practically no plasmids were detected in the isolates from homoiotherms. Although all *C. freundii* strains were multidrug resistant, only the isolates from fish (13 of 14) displayed resistance to tetracycline and oxytetracycline, drugs that are commonly used to treat fish in aquaculture facilities. Mating assays indicated that 7 of 13 strains selected as potential donors possessed transferable R factors to *Escherichia coli* coding mainly for tetracyclines alone or in association with chloramphenicol and streptomycin. Restriction endonuclease cleavage analysis allowed us to determine the genetic similarities among some of the plasmids transferred. The positive results of secondary matings demonstrated the potential dissemination of the conjugative R factors in the environment.

Aquaculture is a rapidly growing industry in many parts of the world. With this development, the interest in aquaculture problems, including its ecological and public health effects, has increased. Although a number of bacteria have been shown to be opportunistic pathogens for both mammals and fish, there are few reports of infections in humans in which the cause was directly related to diseased finfish (25, 28, 50). However, with the environmental deterioration of streams and ocean waters, fish could become infected with bacteria of zoonotic importance and could serve as the source of a pathogen whose spread might result in disease epidemics and/or epizootics. Some potential bacterial pathogens of humans that have been isolated from fish include mainly members of the genera *Aeromonas*, *Vibrio*, *Plesiomonas*, *Edwardsiella*, *Citrobacter*, *Serratia*, *Pseudomonas*, *Streptococcus*, *Staphylococcus*, *Clostridium*, *Mycobacterium*, and *Corynebacterium* (34, 45, 46; reviewed in references 2, 14, 17, 39, and 40).

On the other hand, antibacterial agents are extensively administered to animals therapeutically to combat microbial diseases and subtherapeutically as growth promoters. Potential consequences of the treatment of infected fish and drainage from livestock farms are: (i) the development and dissemination of drug-resistant bacteria, (ii) resistance transfer from bacteria in the environment to pathogenic bacteria, and (iii) reduced efficacy of drug treatment for human and animal diseases caused by resistant pathogens. The importance of these points has been emphasized in numerous reports (1, 8, 12, 13, 21, 22, 24, 30, 31, 36, 44, 45, 48).

Citrobacter freundii is an enterobacterium commonly isolated from soil, water, sewage, and food as well as from different organs of diseased and healthy animals, including mammals, birds, reptiles, and amphibians, in which it is considered to be an opportunistic or secondary pathogen (10, 29). However, during the past few years, the number of reports associating *C. freundii* with fish disease outbreaks has increased (3, 4, 19, 32, 33).

The first objective of this study was to investigate possible intratypic differentiation in a large number of strains of *C. freundii* from different homoiothermic and poikilothermic sources. With this aim, classical taxonomic tests, the API 20E system, and fatty acid methyl ester (FAME) analysis were

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TABLE 1. Virulence for mice and fish, drug resistance profiles, and presence of plasmids in the *C. freundii* strains used in this study

Strain	Origin	Virulence (LD_{50})[a]		Plasmid content (MDa)	Drug resistance pattern[b]
		Mice	Trout		
Cultured fish					
HB-761	Bluegill (kidney), USA	$>1.2 \times 10^8$	NT[c]	90	Am, Te, OT, S, E, Sf
RB-750	Goldfish (brain), USA	$>1.5 \times 10^8$	NT	90	Am, Te, OT, S, E, Sf
A-70	Angelfish (kidney), USA	$>7.0 \times 10^7$	8.7×10^6	90, 40	C, Te, OT, S, K, E, Sf, Sxt
A-344	Angelfish (kidney), USA	$>1.2 \times 10^8$	NT	90, 55, 2.7, 2.4, 2.2, 1.3	Am, Te, OT, S, E, OA, Sf, F
A-351	Striped bass (kidney), USA	$>1.2 \times 10^8$	$>6.0 \times 10^7$	65	C, Te, OT, S, K, E, OA, Sf, Sxt, F
A-408	Channel catfish (sore), USA	$>1.2 \times 10^8$	NT	40	Am, C, Te, OT, S, E, Sf, Sxt
A-334	Tilapia (kidney), USA	$>1.2 \times 10^8$	$>1.2 \times 10^8$	90	S, E, Sf
SR-101	Rainbow trout (liver), Spain	$>1.0 \times 10^9$	9.0×10^6	90	Am, C, Te, OT, S, K, E, Sf, Sxt
P-53	Rainbow trout (kidney), Spain	$>8.0 \times 10^7$	$>2.0 \times 10^7$	110, 40, 6, 2.8, 2.3	Am, C, Te, OT, S, E, Sf, F
P-81	Rainbow trout (kidney), Spain	$>2.0 \times 10^8$	$>1.0 \times 10^7$	110, 40, 6, 2.8, 2.3	Am, C, Te, OT, S, E, Sf, F
P-95	Rainbow trout (kidney), Spain	$>6.5 \times 10^7$	$>2.0 \times 10^7$	110, 6, 2.8, 2.3	Am, C, Te, OT, S, E, Sf
TW 45-92	Rainbow trout (kidney), Spain	$>3.5 \times 10^8$	5.0×10^6	95, 8, 4	Am, C, Te, OT, S, K, E, Sf, Sxt
SSO-41	Atlantic salmon (kidney), Spain	$>5.1 \times 10^7$	5.2×10^6	25, 2.5	Am, C, Te, OT, S, K, E, OA, NA, Sf, Sxt, F
A-409	Aquarium water, USA	$>3.5 \times 10^8$	NT	90	Am, Te, OT, S, E, Sf
Mammals and other animals					
CP2-636	Tortoise (intestine), USA	$>4.0 \times 10^8$	9.0×10^6	—[d]	Am, S, E, OA, Sf
CP2-2467	Bovine (intestine), USA	$>5.2 \times 10^8$	NT	—	Am, S, E, Sf
CP2-2835	Bovine (intestine), USA	$>1.3 \times 10^8$	NT	—	Am, S, E, Sf
CP2-3050	Bovine (gallbladder), USA	$>3.7 \times 10^8$	8.3×10^6	—	Am, S, E, OA, Sf
CP1-3772	Equine (intestine), USA	$>3.2 \times 10^8$	NT	—	Am, S, E, OA, Sf
CP2-3007	Equine (intestine), USA	$>2.0 \times 10^8$	NT	—	Am, S, K, E, OA, Sf
CP2-3042	Canine (intestine), USA	$>1.2 \times 10^8$	5.0×10^6	90, 75	Am, S, E, Sf
CP2-3123	Canine (intestine), USA	$>9.8 \times 10^7$	8.0×10^6	105, 32, 6.5, 3.5, 3, 2.4	Am, C, S, K, E, Sf, Sxt
CP2-4250	Feline (intestine), USA	$>2.0 \times 10^8$	NT	—	Am, S, E, Sf, F
CP2-2626	Avian (intestine), USA	$>2.0 \times 10^8$	NT	—	Am, S, E, Sf
CP2-3615	Avian (intestine), USA	$>3.0 \times 10^8$	NT	—	Am, K, E, OA, Sf
CP2-3633	Avian (intestine), USA	$>6.2 \times 10^7$	$>6.0 \times 10^7$	—	S, E, Sf
CP2-3119	Avian (cecum), USA	$>7.4 \times 10^7$	NT	—	Am, S, E, Sf
CP2-5098 A	Human (feces), USA	$>1.2 \times 10^8$	6.5×10^6	11, 5	Am, S, E, Sf
CP2-5098 C	Human (feces), USA	$>1.5 \times 10^8$	4.3×10^6	—	Am, S, E, Sf
A-236	Human (skin ulcer), Spain	$>9.0 \times 10^7$	NT	2.7	Am, S, E, Sf
A-287	Human (feces), Spain	$>2.4 \times 10^8$	6.2×10^6	—	Am, S, E, Sf, F
ATCC 8090	Human (feces), USA	$>1.5 \times 10^8$	NT	—	Am, E, Sf

[a] Expressed as the number of bacterial cells needed to kill the 50% of the inoculated animals. Strains displaying an LD_{50} of <107 were considered virulent. The categories of virulence are defined in Materials and Methods.

[b] Abbreviations: Am, ampicillin; C, chloramphenicol; Te, tetracycline; OT, oxytetracycline; S, streptomycin; K, kanamycin; E, erythromycin; OA, oxolinic acid; NA, nalidixic acid; Sf, sulfafurazole; Sxt, trimethoprim-sulfamethoxazole; F, nitrofurantoin.

[c] NT, not tested.

[d] —, no plasmids detected.

used, and the antigenic relationships among these isolates were determined. In addition, the pathogenic potential for fish and mice as well as the incidence of multiple-drug resistance in *C. freundii* strains and their transferability by conjugation were also evaluated.

MATERIALS AND METHODS

Bacterial strains. The 32 strains of *C. freundii* used in this study, as well as their hosts and geographic origin, are shown in Table 1. The majority of the strains were isolated in our laboratories by streaking the samples directly onto brain heart infusion agar or trypic soy agar (both from Difco Laboratories, Detroit, Mich.) supplemented with NaCl to a final concentration of 1%. The fish isolate TW 45-92 was obtained from F. Sanz, TROW, SA, Cojobar, Burgos, Spain, while the human strains A-236 and A-287 were supplied by J. Llovo, Hospital Provincial de Santiago de Compostela, Santiago de Compostela, Spain. Strain ATCC 8090 from the American Type Culture Collection was included as a reference. Unless otherwise stated, all strains from poikilotherms were routinely cultured in brain heart infusion agar at 25°C, whereas the isolates from homoiothermic animals were incubated at 37°C. Bacteria were maintained in tubes of soft agar (0.1% Casitone [Difco], 0.3% yeast extract [Oxoid Ltd., Basingstoke, Hampshire, England], 1% NaCl, 0.3% agar [pH 7.2]) under mineral oil and frozen at −70°C in tryptic soy broth (Difco) with 15% (vol/vol) glycerol.

Phenotypic tests. All strains tentatively assigned to the *C. freundii* species were subjected to taxonomic analysis using standard morphological, physiological, and biochemical plate and tube tests (10, 16, 29, 35) and by the API 20E system (Biomerieux, Madrid, Spain). Caseinase, gelatinase, phospholipase, lipase, or amylase activity was determined by a radial diffusion method with a basal nutrient agar (4 g of peptone [Difco] per liter, 1 g of yeast extract per liter, and 15 g of agar per liter) containing 1% sodium caseinate (Difco), 1.5% gelatin (Oxoid), 1% (vol/vol) egg yolk emulsion (Oxoid), 1% Tween 80 (Sigma), or 0.4% starch (Merck, Darmstadt, Germany), respectively. Hemolytic activity was evaluated by the standard diffusion method using tryptic soy agar (Difco) supplemented with 5% sheep or trout erythrocytes.

The drug sensitivities of the strains were determined by the disk diffusion method on Mueller-Hinton agar (Oxoid). The following chemotherapeutic agents (at the concentrations [micrograms per disk] in parentheses) were used: ampicillin (10), chloramphenicol (30), oxytetracycline (30), tetracycline (30), streptomycin (10), kanamycin (30), gentamycin (10), erythromycin (15), oxolinic acid (2), nalidixic acid (30), nitrofurantoin (300), sulfafurazole (300), and trimethoprim-sulfamethoxazole (23.75-1.25). All drugs were supplied by Oxoid.

Fatty acid analysis of *C. freundii* strains. FAME were prepared from bacterial cultures essentially as previously described (23). The extracted FAME were analyzed by gas-liquid chromatography on a Shimadzu 14A chromatograph equipped with a fused silica capillary column (30 m by 0.25 mm) containing a 5% phenylmethyl silicone (DB-5) stationary phase (J & W Scientific, Folsom, Calif.) and fitted with an AOC-9 automatic sampler connected to a CR6A data processor (Shimadzu, Columbia, Md.). Helium was used as the carrier gas at a velocity of 37 cm/s at 150°C. The split ratio was approximately 100:1.

The analysis utilized a temperature program of 150°C for the initial 4 min followed by a temperature increase of 4°C/min to a maximum temperature of 240°C. The injector was maintained at 250°C, and the detector was operated at 300°C. Peak identifications were made by calibration of the data processor with the retention times of a standard FAME mix (Matreya, Pleasant Gap, Pa.). The results were subjected to the Student-Newman keuls test in order to discern significant differences among the percentage of FAME among the isolates.

Serological analysis. The serological relationships among all *C. freundii* strains were analyzed by slide agglutination tests as previously described (43), using antisera raised against 13 different isolates. The antisera were obtained from rabbits injected intravenously with saline-washed suspensions (10^9 cells per ml) of formalin-killed bacteria in consecutive daily doses of 0.25, 0.5, 1.0, and 2 ml followed by a single 1.5-ml injection 2 weeks later. The sera were stored in aliquots at −30°C until used. The reactions were performed with the somatic O antigens, prepared by heating the bacterial suspensions at 100°C for 1 h. A strong and rapid agglutination was recorded as positive, and a weak agglutination after 5 min was considered negative. Saline and sera from nonimmunized rabbits were used as negative controls.

Positive strains were subjected to cross-quantitative agglutination tests conducted in microtiter plates, using serial twofold dilutions of 25-μl aliquots of antisera (27). The titer was considered the reciprocal of the highest dilution of the antiserum which gave a positive reaction after incubation with the O antigens overnight at 15°C.

Analysis of LPS and cell membrane proteins. The lipopolysaccharides (LPS) were obtained basically by the method of Hitchcock and Brown (15). Bacteria were grown in brain heart infusion agar at 22°C for 24 h and resuspended in 3 ml of phosphate-buffered saline (PBS). Bacterial suspensions (1.5 ml) were centrifuged, and the pellets were resuspended in 50 μl of 2× sample buffer (0.065 M Tris, 2% sodium dodecyl sulfate [SDS], 10% glycerol, 5% β-mercaptoethanol, 0.001% bromophenol blue [pH 6.8]). After boiling for 10 min, 10 μl of proteinase K (2.5 mg/ml) in 2× sample buffer was added, and the samples were incubated at 60°C for 1 h.

Total and outer membrane proteins (OMP) were extracted as previously described (42). The outer membranes were obtained by treatment of total cell envelopes with 3% (wt/vol) Sarkosyl in 10 mM Tris-HCl (pH 8.0) for 20 min at room temperature.

The LPS samples and the precipitates from total and outer membranes were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (20), using 12.5% acrylamide in the separating gel and 3% acrylamide in the stacking gel. Purified LPS from *Escherichia coli* O128:B12 (Sigma) was included as positive control. The LPS were stained with silver nitrate as described by Tsai and Frasch (49). The proteins were stained with Coomassie brilliant blue R (Sigma), and molecular sizes were determined by comparison with a mixture of commercial markers (Bio-Rad Laboratories, Richmond, Calif.).

Immunoblotting of LPS and proteins. The OMP and LPS components fractionated by SDS-PAGE were transferred to nitrocellulose sheets essentially as described by Towbin et al. (47). After transfer, the nitrocellulose membranes were blocked for 1 h with 3% gelatin in Tris-buffered saline (TBS; pH 7.5), washed twice with TTBS (TBS plus 0.05% Tween 20), and incubated for 1 h with different antisera against *C. freundii* (diluted in TTBS with 1% gelatin). After further washing in TTBS, membranes were incubated for 1 h with goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Bio-Rad) diluted 1:3,000. Bands were visualized by incubating the nitrocellulose membranes in 0.1 M carbonate buffer (pH 9.8) containing tetrazolium blue (0.3 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (0.15 mg/ml).

Pathogenicity assays in rainbow trout and mice. The infectivity trials in fish were performed by intraperitoneal injection as previously described (42), using fingerling rainbow trout (*Oncorhynchus mykiss*) (10 g, average weight). Groups of six fishes were inoculated intraperitoneally with 0.1 ml of bacterial doses ranging from 10^2 to 10^8 cells. Fish were maintained at 22°C with aeration during the course of the experiment (2 weeks). Fish surviving challenge were killed, and reisolation of the inoculated strain was attempted to test for possible carrier state. The mouse pathogenicity assay was performed with 5 to 10 BALB/c mice (10 to 12 weeks old, 21 to 25 g), which were inoculated intraperitoneally with doses ranging from 10^4 to 10^8 cells of each *C. freundii* isolate. Control animals received PBS (pH 7.4). The LD_{50} (50% lethal dose) was calculated by the Reed and Müench (26) method. In accordance with standard criteria of degrees of virulence, the strains were considered as high virulent (LD_{50} of $<10^4$ cells), low virulent (LD_{50} of 10^5 to 10^6 cells), or not virulent (LD_{50} of $>10^7$ cells) (reviewed in reference 40).

Plasmid isolation. The plasmid content of the *C. freundii* strains was detected by the technique of Kado and Liu (18) as modified by Toranzo et al. (41) and by the method of Birnboim and Doly (5). Plasmids from *E. coli* V517 (ranging from 35.8 to 1.4 MDa) and *Vibrio anguillarum* 775 (47 MDa) were included in all electrophoresis runs as molecular size reference markers.

Conjugal transfer assays. Multiresistant *Citrobacter* strains harboring high-molecular-mass plasmids (>20 MDa) and exhibiting resistances to chloramphenicol, tetracyclines, and kanamycin but sensitivity to nalidixic acid were tested as potential donors in the mating assays.

Nalidixic acid-resistant (Na^r) *E. coli* K-12 185 (Lac^+) and *E. coli* K-12 711 (Lac^-) strains, as well as the Na^r laboratory derivatives obtained from the fish pathogenic strains *Edwardsiella tarda* E-11-2 (streptomycin resistant [S^r] Na^r), *V. anguillarum* PB-15 (S^r kanamycin resistant [K^r] Na^r), and *Aeromonas hydrophila* 67-P-24 (Na^r), were used as recipients for conjugal transfer of R factors. The wild-type strains of the fish pathogens were kindly supplied by T. Aoki, Department of Fisheries, Faculty of Fisheries, Miyazaki University, Miyazaki, Japan.

The mating assays were performed basically as previously described (44). Briefly, the potential donors and *E. coli*, *E. tarda*, *A. hydrophila*, and *C. freundii* recipient strains were incubated overnight in tryptic soy broth (Difco) at 37°C. In the

TABLE 2. FAME compositions of *C. freundii*[a]

Origin	No. of strains	% of total fatty acid composition[b]													
		12:0	13:0	14:0	15:0	3OH 14:0	ω7c 16:1	16:0	17:0Δ	17:0	18:2	ω9c 18:1	ω7c 18:1	18:0	19:0Δ
Fish															
No salmonids	8	9 (1)	1	12 (2)	5 (2)	6 (1)	25 (4)	25 (2)	8 (3)	1	T	T	8 (1)	T	T
Salmonids	6	4	T	9	3	4	35 (2)	37 (4)	1	1	T	T	5 (1)	1	0
Human	5	6 (2)	1	9 (1.5)	6 (3.5)	5 (1)	23.5 (3)	30 (4)	8 (4)	1.5	T	T	8 (1.5)	0	0
Veterinary	13	9 (3)	2 (1)	10 (2)	6 (4)	7 (2)	23 (2)	24 (6)	9 (2)	1	T	T	8 (3)	0	T

[a] The number before the colon indicates the number of carbon atoms; the number after the colon refers to the number of double bonds. 3-OH indicates the hydroxy group at carbon 3. ω7c and ω9c indicate the double bond position from hydrocarbon end of carbon chain; c represents *cis* isomer. Δ indicates the presence of a cyclopropane ring in the carbon chain.

[b] The first number is arithmetic mean of the relative percentage of the fatty acid. Standard deviations are given in parentheses. The absence of a number in parentheses indicates that the standard deviation was less than 1%. T, trace amount.

case of the *V. anguillarum* recipient strain, the same medium with 1% NaCl added and a temperature of 25°C were the culture conditions used in all steps. Overnight donor and recipient cultures were mixed to a 1:2 ratio in Erlenmeyer flasks, and the matings were performed at 37 or 25°C (depending of the recipient strain). After an 18-h incubation, matings were interrupted and 10-fold serial dilutions of each mixture were spread on plates of tryptic soy agar medium supplemented with 100 μg of nalidixic acid per ml and 25-μg/ml concentrations of the selected drugs to which the potential *Citrobacter* donor strains were resistant (tetracycline, chloramphenicol, and kanamycin). Colonies growing in this double-inhibitor-supplemented medium after 24 to 48 h incubation were scored as presumptive transconjugants and tested for their biochemical characteristics, drug resistance, and plasmid content. Transfer frequency was calculated as the number of transconjugants per initial number of donor cells per milliliter.

To evaluate the potential spreading of the R^+ factors, the transconjugants obtained in the primary matings (R^+ *C. freundii* × *E. coli* K-12 Na^r) were used as donors in secondary matings with ampicillin-resistant (Am^r) *E. coli* K-12 as the recipient strain. Transconjugants from these retransfer assays were selected on medium containing 100 μg of ampicillin per ml and 25 μg of chloramphenicol, tetracycline, or kanamycin per ml.

Restriction endonuclease cleavage analysis. To determine the possible genetic homology of the conjugative resistance factors present in some *C. freundii* strains, plasmids from different drug-resistant transconjugant clones of *E. coli* K-12 obtained in the mating assays were extracted by the method of Birnboim and Doly (5) and subjected to cleavage with the restriction enzymes *Eco*RI, *Bam*HI, *Hin*dIII, and *Sal*I (from Boehringer, Mannheim, Germany) under the manufacturer's recommended conditions. Reactions were stopped by the addition of 5 μl of a mixture containing 7% SDS, 33% glycerol, and 0.07% bromophenol blue. The samples were electrophoresed in 1.0% agarose gels at 70 V and stained with ethidium bromide. Digests of λ DNA with the same enzymes were used as controls. As comparative purposes, some nonconjugative high-molecular-weight plasmids extracted from the *Citrobacter* strains were also subjected to restriction analysis.

RESULTS AND DISCUSSION

Characterization of the *Citrobacter* isolates. On the basis of classical plate and tube phenotypic tests, the *Citrobacter* strains were identified as *C. freundii*. Regardless of the geographic origin or source of isolation, the strains showed variable reactions mainly with respect to arginine dihydrolase, ornithine decarboxylase, and fermentation of sucrose, melibiose, amygdalin, and salicin. However, these reactions are already known to be variable within this species (10, 29). Moreover, all strains failed to ferment D-adonitol, which is an important differentiating trait among *C. freundii*, *C. diversus*, and *C. amalonaticus* (10, 29). Only a few isolates exhibited unusual reactions in one of the following tests: citrate utilization (five negative strains), indole (five positive strains), and H_2S production (six negative strains). However, exceptional strains failing in some of these reactions have been isolated (3, 19, 33).

All of the strains showed similar ranges of growth temperature (4 to 10°C to 44°C) and tolerated a salinity up to 8% NaCl. Regarding enzymatic activities, all *C. freundii* isolates lacked the capacity to produce gelatinase, caseinase, lipase, phospholipase, amylase, and urease. However, with rare exceptions (three isolates from fish and one from mammals), the strains exhibited weak hemolytic activities against trout and sheep erythrocytes.

In general, the API 20E system was useful for the rapid presumptive identification of *C. freundii* isolates, since the results in the majority of the reactions were in agreement with those obtained in the conventional tests. In only some isolates, urease and citrate tests showed false-positive and negative reactions, respectively, in the API 20E system. However, the code numbers generated by these isolates were also included in the commercial API code index as belonging to strains of *C. freundii*.

The analysis of the strains by gas-liquid chromatography revealed that independently of the source of isolation, all strains exhibited similar FAME profiles (Table 2). However, significant differences ($P < 0.001$) in the major fatty acids 16:1 and 16:0 as well as in the 17:0Δ region were observed between the isolates from salmonids and the remaining strains of fish, human, or veterinary origin, which can indicate an adaptation of the bacteria to the particular lipid composition of the salmonid hosts. Therefore, in agreement with Haldeman and Amy (11), FAME analysis can prove useful for distinguishing between isolates belonging to the same species or biotype, especially in environmental studies.

Serological and antigenic relationships among the isolates. Agglutination assays using the thermostable O antigens demonstrated a great serological diversity among the strains (Table 3). Whereas some of the 13 anti-whole-cell sera used were specific, giving high titers only with the homologous strains, other antisera showed cross-reactions with different isolates regardless of their origin, which indicates that these strains are serologically related.

These results were supported by the LPS analysis because although different electrophoretic patterns were exhibited by the *C. freundii* isolates, common LPS profiles were shared only by the isolates showing high cross-agglutination titers. The

TABLE 3. Cross-agglutination titers among *C. freundii* isolates showing positive results in the slide agglutination test with at least one antiserum

Source of O antigen[b]	Cross-agglutination titer[a] with antiserum												
	RB-750	A-408	P-53	SSO-41	CP2-636	CP2-3050	CP2-3007	CP2-3042	CP2-4250	CP2-3633	CP2-5098C	A-287	ATCC 8090
Fish strains													
RB-750	1,024												
A-344										64			
A-351		8					8						
A-408		1,024											
A-334													
SR-101		32											
P-53			8,192							8			8
P-81		8	8,192						16	8			8
P-95			8,192										32
SSO-41				4,096						8			
Strains from mammals and other animals													
CP2-636					512								
CP2-2467		128											
CP2-2835						32							
CP2-3050						512							
CP2-3007							128						
CP2-3042								512					
CP2-4250									256	64			
CP2-2626		128											
CP2-3633									32	512			
CP2-3119									32			256	
CP2-5098C						64					1,024		
A-287												1,024	
ATCC 8090													

[a] Reciprocal of the highest dilution of antiserum that gave a positive reaction after an overnight incubation with the antigen at 15°C. The absence of a number indicates a titer of less than 2.

[b] Two other antigens tested, from A-334 (fish) and ATCC 8090 (other), were autoagglutinating.

majority of the strains showed profiles constituted by core and O side chains in a ladder-like pattern (smooth forms) with bands ranging mainly between 20 and 150 kDa (Fig. 1a). A few isolates contained core LPS only. The LPS of some *Citrobacter* strains were highly concentrated in the medium- and high-molecular-mass range. Therefore, as is well known, when these bands are not clearly individualized, overstain artifacts (blurs) are usually originated (Fig. 1).

As expected from the serological results cited above and the LPS profiles, the immunoblot analysis demonstrated an antigenic similarity only among strains showing strong cross-agglutination reactions as well as similar LPS profiles (Fig. 1b and c). Therefore, the detected low cross-agglutination titers (Table 3) can be due to common thermostable polysaccharidic capsular material remaining in the preparation of the O antigens.

The serological heterogeneity found in the *C. freundii* strains, even within isolates from the same source, is in agreement with the results obtained by Chart et al. (7) comparing the structures and antigenic properties of LPS of *C. freundii* isolated from different food samples and of distinct clinical origins.

The analysis of total cell envelope and OMP revealed very similar profiles among all of the *Citrobacter* strains (Fig. 2). However, when the corresponding Western blotting (immunoblotting) was performed, only two common major OMP of about 54 and 58 kDa were immunologically related (data not shown).

Pathogenicity assays. To assess the possible pathogenic potential of *C. freundii* for mammals and poikilothermic hosts, infectivity trials were conducted in mice and trout (Table 1). The results indicated that under laboratory conditions, all strains were not pathogenic for mice (LD_{50} of $>5 \times 10^7$) even when high doses (10^8 and 10^9 cells) were used. Similarly, regardless of the source of isolation, the strains were not virulent or displayed a low degree of virulence for fish (LD_{50} of about 10^6 cells). However, in all cases, the inoculated strains were recovered in pure culture from the internal organs of survivor rainbow trout, which indicates that the fish can harbor these bacteria in a carrier state. Therefore, any stress impaired by a decrease of the immunological defense mechanism of the host, environmental factors, or a combination of such effects could give rise to significant fish mortalities. This hypothesis is supported by the fact that the majority of isolations of *C. freundii* from fish were associated with high levels of water pollution (sewage, organic matter, etc.) in the aquaculture facilities or were recovered in mixed infections with other bacterial or viral agents (4, 19, 32, 33, 46).

All of these findings reinforce the importance of *C. freundii* as an opportunistic bacterial fish pathogen which can be easily disseminated to other geographic areas not only directly through the water or carrier fish but also through potential vectors such as parasites which swim between different hosts (9) or fish-eating birds. In fact, various fish pathogens such as *Aeromonas* spp., *Plesiomonas* spp., *E. tarda*, *E. ictalurii*, and *Yersinia ruckeri* have been isolated from the intestinal tract of cranes, egrets, herons, cormorants, and gulls around culture facilities (38, 51, 52).

Drug resistance, plasmid content, and mating assays. Determination of resistance patterns to 13 antibiotics and chemotherapeutic agents revealed that although all *C. freundii* strains were multiresistant, only the isolates from fish (13 of 14)

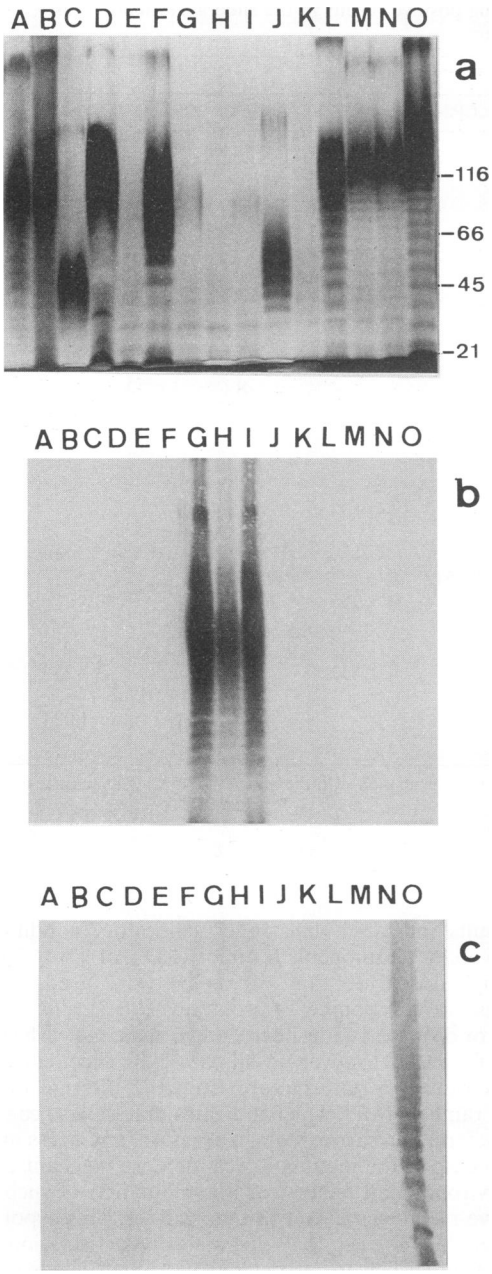

FIG. 1. Silver-stained SDS-PAGE profiles of LPS prepared from strains of *C. freundii* strains (a) and the corresponding immunoblots, using antisera raised against the fish isolate P-53 (b) and the bovine strain CP2-3633 (c). Lanes: A, A-70; B, A-344; C, A-351; D, A-408; E, A-334; F, SR-101; G, P-53; H, P-81; I, P-95; J, TW 45-92; K, SSO-41; L, A-409; M, CP2-2467; N, CP2-2626; O, CP2-3633. Numbers on the right indicate molecular size markers in kilodaltons. The overstained zones in the medium- and high-molecular-mass ranges in some gel lanes are originated by the high LPS content present in closed individual bands.

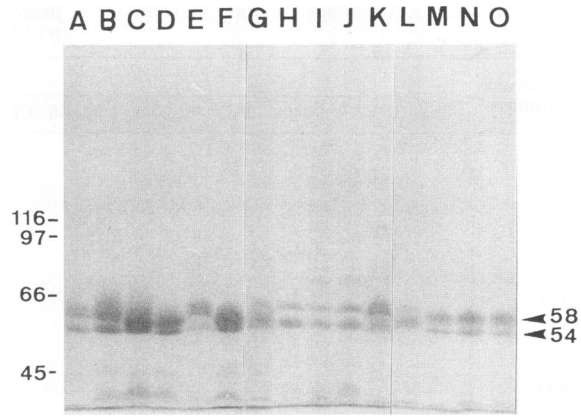

FIG. 2. SDS-PAGE profiles of OMP of *C. freundii* strains isolated from different sources. Lanes: A, A-70; B, A-344; C, A-351; D, A-408; E, A-334; F, SR-101; G, P-53; H, P-81; I, P-95; J, TW 45-92; K, SSO-41; L, A-409; M, CP2-2467; N, CP2-2626; O, CP2-3633. Numbers on the left indicate molecular size markers in kilodaltons. Arrowheads shows the two immunologically related OMP of 54 and 58 kDa.

exhibited resistance to tetracycline and oxytetracycline. These are drugs commonly used to treat fish diseases because they are approved compounds in the aquaculture legislation of the majority of countries. Despite the restrictive use of chloramphenicol, the number of strains resistant to this antibiotic was clearly higher in fish isolates (9 of 14) than in the strains from the other sources (1 of 18) (Table 4). Correlations in the development of drug resistance following medication in fish farms have been demonstrated not only in bacteria isolated from fish (1, 13, 21, 22, 36, 44, 45) but also in bacterial populations recovered from sediments (6, 12, 36) and wild fauna (6, 30) around aquaculture facilities.

Regardless of the source of isolation, the majority of the *C. freundii* isolates displayed resistances to ampicillin, erythromycin, streptomycin, and sulfafurazole, which seems to indicate that this pattern is characteristic of the species.

The analysis of plasmid content by agarose gel electrophoresis revealed that all poikilotherm *C. freundii* possessed one or more high-molecular-mass plasmid bands ranging from 110 to 25 MDa (Table 4). In addition, both large and small plasmids were shared by several of the fish isolates. In contrast, plasmid bands were detected in only four strains from homoiothermic animals.

The analysis carried out to detect transmissible R factors in the *C. freundii* strains indicated that 7 of the 13 strains tested as potential donors (Na^r strains harboring high-molecular-weight plasmids) exhibited drug resistance transfer to *E. coli*. The most common type of R factor determined resistance to tetracycline and oxytetracycline alone or in association with chloramphenicol and streptomycin resistance (Table 4). Only one *C. freundii* isolated from mammals, CP2-3123, showed an ability to transfer resistance to kanamycin. The transfer frequencies to *E. coli* 185 Na^r ranged from 10^{-3} for chloramphenicol-resistant transconjugant clones from P-95 strain to 10^{-7} for K^r and Te^r transconjugant clones from isolates CP2-3123 and TW 45-92, respectively. Similar frequencies were obtained when *E. coli* 711 Na^r was used as the recipient strain (data not shown). However, no transconjugants were obtained in matings performed with *E. tarda*, *A. hydrophila*, and *V. anguillarum* as recipients, regardless of the selected drug markers.

Analysis of the plasmids of donors and transconjugants allowed us to associate a specific plasmid with the resistance factor transferred in five of the isolates (Table 4; Fig. 3). Conjugative plasmids of 90 and 110 MDa were shared, respectively, by two and three *C. freundii* strains isolated from fish. Mobilization of nonconjugative small-molecular-size plasmids was detected in only one isolate (TW 45-92). Interestingly,

TABLE 4. Conjugal transfer of drug resistances of *C. freundii* strains to *E. coli* K-12 185 Na^r and characterization of R plasmids[a]

Donor strains	Resistance patterns[b]	Plasmid content of donors (MDa)	Selective resistance marker	Transfer frequency[c]	Resistance pattern of R factor	Size of plasmid(s) transferred (MDa)
CP2-3123	Am, C, S, K, E, Sf, Sxt	105, 32, 6.5, 3.5, 3, 2.4	C	1.5×10^{-6}	C, K, Sxt	105, 32
			K	1.4×10^{-7}		
HB-761	Am, Te, OT, S, E, Sf	90	Te	5.0×10^{-6}	Te, OT	90
RB-750	Am, Te, OT, S, E, Sf	90	Te	2.5×10^{-5}	Te, OT	90
P-53	Am, C, Te, OT, S, E, Sf, F	110, 40, 6, 2.8, 2.3	C	4.3×10^{-4}	C, Te, OT, S	110
			Te	4.5×10^{-4}		
P-81	Am, C, Te, OT, S, E, Sf, F	110, 40, 6, 2.8, 2.3	C	6.0×10^{-4}	C, Te, OT, S	110
			Te	5.2×10^{-4}		
P-95	Am, C, Te, OT, S, E, Sf	110, 6, 2.8, 2.3	C	1.3×10^{-3}	C, Te, OT, S	110
			Te	2.0×10^{-6}		
TW 45-92	Am, C, Te, OT, S, K, E, Sf, Sxt	95, 8, 4	C	3.0×10^{-5}	C, Te, OT, S	95, 8, 4
			Te	1.0×10^{-7}		
			K	—[d]		

[a] The remaining *Citrobacter* strains tested as potential donors (A-70, A-344, A-351, A-408, SR-10.1, and A-409) were negative in the mating assays.
[b] See Table 1, footnote *b*.
[c] Expressed as the number of transconjugants per initial donors cells per milliliter after 18 h at 37°C.
[d] —, No transconjugants recovered.

restriction endonuclease cleavage analysis of the conjugative plasmids by using different enzymes demonstrated the genetic homology of the R plasmids exhibiting similar sizes and drug resistance patterns (Fig. 4). In addition, the restriction profiles showed by these plasmids were different from those displayed by the nontransferable high-molecular-weight plasmids present in several isolates (data not shown).

The potential spreading of the conjugative resistance factors present in the *C. freundii* strains was evaluated by secondary matings. The results showed that drug resistance transfer to *E. coli* Am^r consistently occurred with frequencies similar that those obtained in the primary conjugal assays.

In general the transfer frequencies of R factors in these laboratory experiments are higher than those expected to occur in nature. We recognize that in the development of a

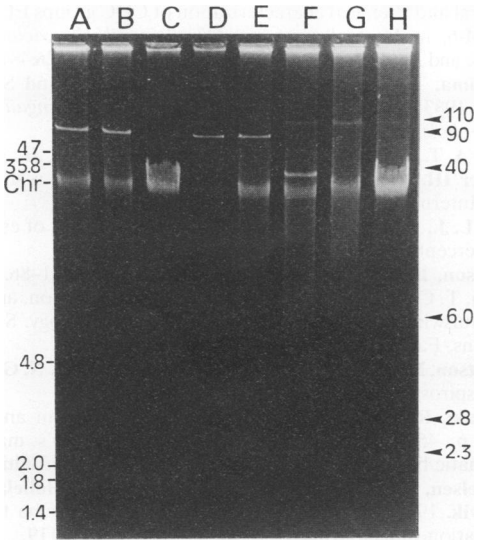

FIG. 3. Detection of plasmid DNA by agarose gel electrophoresis in some *C. freundii* strains carrying transferable R factors and in the corresponding transconjugants in *E. coli* K-12. Lanes: A, donor HB-761; B, tetracycline- and oxytetracycline-resistant (Te^r OT^r) transconjugant; C, donor RB-750; D and E, Te^r OT^r transconjugants; F, donor P-53; G and H, C^r Te^r OT^r S^r transconjugants. Numbers on the left indicate molecular sizes (in kilodaltons) of the reference plasmids present in *E. coli* V517 and *V. anguillarum* 775. Chr, chromosome. Numbers on the right show molecular sizes (in kilodaltons) of the plasmid bands in donors and transconjugants.

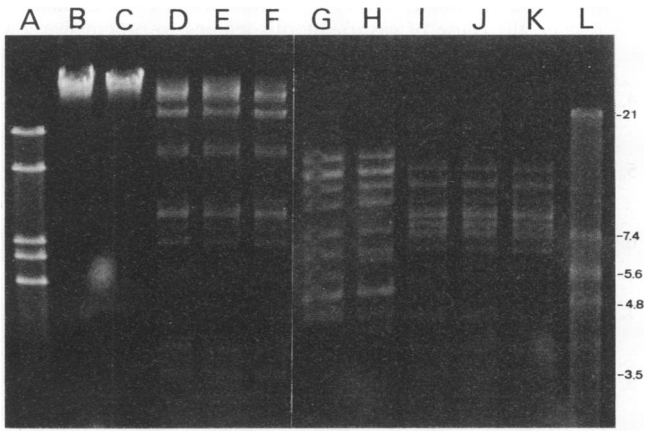

FIG. 4. Homology of conjugative resistance plasmids in some *C. freundii* strains isolated from fish by restriction endonuclease cleavage analysis with the enzymes *Bam*HI (lanes B to F) and *Eco*RI (lanes G to K). Lanes: B, C, G, and H, digestion of the 90-MDa plasmids harbored by strains HB-761 and RB-750 coding for Te^r OT^r; D to F, and I to K, digestion of the 110-MDa plasmids harbored by strains P-53, P-81, and P-95 coding for C^r Te^r OT^r S^r; A and L, digestion of λ phage with *Bam*HI and *Eco*RI, respectively. Sizes of the standard fragments in kilobase pairs are indicated on the right.

resistant clone, the initial amount of donor cells containing R plasmids in a natural situation would be less than in our mating mixtures. However, the antimicrobial pressure exercised by the use of chemotherapeutic agents in fish farms as feed additives or administered directly into rearing water enhances the selection for strains resistant to these drugs, which ultimately become the predominant organisms in the bacterial population. In addition, it is known that in nature, parameters such as bacterial density and nutrient availability affect gene transfer (37). Therefore, the laboratory conditions are similar to those found in environments such as the sediments beneath fish farms that are rich in nutrients and contain large bacterial numbers (10^7 to 10^9) usually attached to particles or forming aggregates which favors contact between cells. In fact, elevated frequencies of bacteria carrying R plasmids in fish farm sediments have been reported (6, 12, 31).

In conclusion, the wide and persistent use of antimicrobial agents in aquaculture systems gives rise to an increase in drug-resistant bacteria during or after fish treatment, causing therapeutic difficulties in subsequent bacterial infections. These drug-resistant microorganisms are released from fish farms either accidentally or deliberately into an environment, which constitutes a risk of dissemination of R factors among a diversified bacterial population. Moreover, the transfer of resistance to bacteria that could develop in other animals, or even in humans, could become a public health hazard.

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