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Molecular Characterization of *Yersinia enterocolitica* by Pulsed-Field Gel Electrophoresis and Hybridization of DNA Fragments to *ail* and pYV Probes

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Sixty strains of *Yersinia enterocolitica* from five serogroups (O:3; O:9; O:8; O:5; and O:5,27) and eight non-*Y. enterocolitica* strains, recovered from diverse sources (humans, animals, food, and the environment) in Europe, Argentina, and the United States, were examined by the pulsed-field gel electrophoresis (PFGE) technique of contour clamped homogeneous electric field electrophoresis (CHEF) by using *Not*I and *Xba*I as restriction enzymes. *Not*I and *Xba*I generated 36 and 33 restriction endonuclease digestion profiles (REDP), respectively. By combining the results of both enzymes, 42 unique genomic groups were differentiated. DNA fragments were transferred to nylon membranes and hybridized with digoxigenin-labelled oligonucleotide probes to the *ail* gene and virulence plasmid to determine hybridization patterns and the potential virulence of the strains. The strains were tested for the presence of the plasmid by PFGE-CHEF and phenotypic characteristics encoded for by the virulence plasmid. Thirty of the 60 *Y. enterocolitica* strains tested harbored the virulence plasmid. The specificity of the *ail* and pYV probes was 100% when tested with 68 *Yersinia* strains and 19 different non-*Yersinia* strains. Sixteen selected *Y. enterocolitica* strains were tested for their virulence by lethality in iron- and desferrioxamine-sensitized mice. No correlation between REDP and the virulence of the strains was observed. The observed REDP and the hybridization patterns were very homogeneous within a serogroup and independent of the source of isolation. In addition, PFGE-CHEF was shown to be valuable in identifying and confirming serogroups. Principal component analysis of Dice similarity indices from REDP was an excellent tool for determining genetic relatedness among strains.

The genus *Yersinia* contains 11 species, 3 of which (*Y. pestis*, *Y. pseudotuberculosis*, and some serotypes of *Y. enterocolitica*) are pathogenic for humans. *Y. enterocolitica* is primarily transmitted by foods (2, 6, 27, 42) although contaminated water (14) and blood transfusions (21, 33) have been implicated in several cases, and it has emerged as a significant food-borne pathogen that is associated with a variety of clinical and immunological manifestations (10). *Y. enterocolitica* encompasses 57 different serogroups, not all of which can cause disease (43). Two groups of multiple serogroups are pathogenic for humans: (i) serogroups O:3, O:9, and O:5,27, which are pathogenic but not lethal to mice, and (ii) serogroups O:4,32; O:8; O:13a,13b; O:18; O:20; and O:21, which are highly pathogenic and lethal to mice (10). In addition, O:1,2,3 is pathogenic for chinchillas, and O:2,3 is pathogenic for hares (10). Other *Y. enterocolitica* serogroups have been isolated from healthy humans.

A prerequisite for expression of pathogenicity is a 70-kb virulence plasmid (pYV), which is commonly present in all pathogenic *Yersinia* spp. (12, 44). Plasmid pYV is associated with phenotypic characteristics such as low calcium response at 37°C (16), autoagglutination at 37°C (26), binding of Congo red at 37°C (38), and absence of pyrazinamidase activity (23). Several animal models including the guinea pig conjunctivitis model (Sereny test) (44), mouse intraperitoneal challenge, and mouse diarrhea and splenic infection following oral challenge (3) have also been used in the study of *Yersinia* pathogenicity.

The presence or absence of pYV has often been used to discriminate between virulent and nonvirulent *Y. enterocolitica* strains (22, 24, 29). However, methods based on the detection of the virulence plasmid or the expression of plasmid-encoded properties may lead to false-negative results, because the plasmid is easily lost during repeated subculturing (36, 44). More recently, plasmid-independent properties were shown to be involved in *Y. enterocolitica* pathogenicity (17, 19). The chromosomal gene *ail* (adhesion-invasion locus) (31) was shown to be restricted to strains of serotypes associated with disease (32). Therefore, we used DNA probes homologous to the virulence plasmid and the *ail* gene to determine specific hybridization patterns and the potential virulence of strains.

The present study was undertaken to develop a molecular characterization strategy for *Y. enterocolitica* that provides (i) highly reproducible, discriminatory subtyping, (ii) patterns for determination of the potential virulence, and (iii) an overview of genomic relatedness among phenotypically similar yersiniae.

MATERIALS AND METHODS

Strains. Sixty strains of *Y. enterocolitica* were examined. Strains were isolated from humans, animals, foods, and the environment in Europe, Argentina, and the United States. These strains are from the following serogroups: O:3 (28 strains), O:9 (23 strains), O:8 (3 strains), O:5 (1 strain), and O:5,27 (5 strains). In addition, one strain each of *Y. kristensenii*, *Y. ruckeri*, *Y. rohdei*, *Y. frederiksenii*, *Y. bercovieri*, *Y. mollaretii*, *Y. intermedia*, and *Y. pseudotuberculosis* was examined. Some of these strains, isolated from a locally restricted area (eastern Austria), previously typed by pulsed-field gel electrophoresis (PFGE) (9), were included in this study in order to identify their hybridization patterns and for the determination of the

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overall genomic relatedness among *Yersinia* strains. To determine the specificity of the *ail* and pYV oligonucleotide probes, eight non-*Y. enterocolitica* strains and representative strains of other bacterial species, including *Alcaligenes faecalis*, *Alcaligenes xylosoxydans*, *Bacillus cereus*, *Citrobacter freundii*, *Enterobacter agglomerans*, *Escherichia coli*, *Flavobacterium lutescens*, *Flavobacterium meningosepticum*, *Listeria monocytogenes*, *Listeria innocua*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Shigella dysenteriae*, and *Vibrio vulnificus*, were tested.

Serotyping. Serogroup data were provided by the original strain curators and confirmed by slide agglutination with O serogroup antiserum (Accurate Chemical & Scientific Corp., Westbury, N.Y.).

Autoagglutination. Strains were tested for the ability to autoagglutinate in RPMI 1640 tissue culture medium (Sigma Chemical Co., St. Louis, Mo.) with 10% calf serum (Sigma) and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (Sigma) (26). Strains were positive if autoagglutination occurred when strains were grown at 37°C but not at 25°C.

Low calcium response. Organisms were grown on brain heart infusion agarose (BHO) which was prepared with agarose type V (Sigma), at a final concentration of 1.2%, and brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.1% magnesium chloride (Difco) (5). When incubated at 37°C for 24 h, plasmid-bearing strains formed pinpoint colonies while calcium-independent strains measured more than 1.5 mm in diameter.

Pyrazinamidase. The pyrazinamidase test was done as described by Kandolo and Wauters (23). Strains were inoculated over the entire slant of pyrazinamide agar, incubated at 25°C for 48 h, and tested with 1 ml of a freshly prepared 1% solution of ferrous ammonium sulfate (aqueous). A positive pyrazinamidase reaction was indicated by a pink to brown color that developed on the slant.

Congo red. Organisms were tested for the ability to bind Congo red when grown on BHI agar (Difco) containing 75 μg of Congo red (Sigma) per ml for 18 h at 37°C and at 25°C (5). Positive strains formed red pinpoint colonies at 37°C, and negative strains were colorless or light orange at both temperatures.

Mouse virulence assay. For mouse infection studies, adult Swiss Webster mice (17 to 20 g; male or female) were obtained from Simonson Labs, Inc., Gilroy, Calif. One day prior to infection, mice were injected intraperitoneally with 5 mg of iron dextran (Fermenta Animal Health Co., Kansas City, Mo.) and 5 mg of desferrioxamine mesylate (Ciba Geigy Inc., Summit, N.J.) in 0.2 ml of sterile physiological saline (39). Five mice were injected intraperitoneally with 0.1 ml of culture containing approximately 10^5 CFU per mouse. Mice were observed for 7 days, and strains which were lethal to four or more mice were considered virulent.

PFGE. Preparation of DNA in agarose plugs, digestion, and fractionation of the resulting fragments were done by using a protocol previously described (9).

Plasmid analysis. DNA for plasmid analysis was prepared in agarose plugs, by using bacterial cultures at a concentration of about 10^9 CFU/ml. The intact, uncleaved genome DNA, was analyzed by the PFGE technique of clamped homogeneous electric fields (CHEF). An electrophoretic regimen of 200 V for 21 h at a temperature of 18°C and a switching time from 1 to 40 s was employed so that the circular plasmid could migrate into the agarose gel. The gel was stained and photographed as described previously (9). To verify that the visible band was pYV, DNA was transferred to a nylon membrane by the method of Southern (41) and hybridized with the pYV probe, as described below.

DNA transfer, probes, labelling, and hybridization. Fractionated DNA restriction fragments were transferred to a positively charged nylon membrane (magnagraph; MSI, Westboro, Mass.) by the method of Southern (41). The DNA was fixed for 2 min under short-wave UV light, and the membrane was wrapped in Saran Wrap and stored at 4°C until used. Two synthetically produced 19-base oligonucleotide probes (National Biosciences, Plymouth, Minn.) were used. The oligonucleotide probe specific for pYV, heretofore referred to as the pYV probe (5'-TGCCGCAGAGACACTGATC-3'), was selected from sequence analysis of the *yopA* gene, which codes for the outer membrane protein YadA (24). The oligonucleotide probe specific for the *ail* gene (*ail* probe) (5'-GAACTC GATGATAACTGGG-3') (25) was selected from the published nucleotide sequence of the *Y. enterocolitica ail* gene (30). The probes were labelled with digoxigenin (Oligonucleotide Tailing Kit; Bohringer, Mannheim, Germany). The Digoxigenin Nucleic Acid Detection Kit (Bohringer) was used for visualizing the hybridized probe. Labelling, hybridization, and detection of digoxigenin-labelled probes were done according to the manufacturer's instructions. Hybridizations were carried out for 18 h at 46°C for the *ail* probe and at 50°C for the pYV probe. For rehybridization, membranes were boiled twice in 1% sodium dodecyl sulfate to remove probe-DNA and then reprobed with the second probe.

Data management. Restriction endonuclease digestion profile (REDP) similarity was determined as described previously (9). Each *Not*I or *Xba*I REDP was characterized by a binary score of 91 or 94 characters, respectively.

Principal component analysis. To more clearly visualize genomic relationships among *Yersinia* strains, principal component analysis of the similarity coefficients of concatenated *Not*I and *Xba*I REDP data was performed by using the Statistical Analysis System (SAS Institute, Cary, N.C.), essentially as described by Chen et al. (11).

RESULTS AND DISCUSSION

Analyses of genomic fingerprints of *Y. enterocolitica* and other *Yersinia* spp. PFGE was used for molecular characterization of yersiniae because it is a reproducible, highly discriminatory typing method (40) that allows subtyping of strains belonging to the same serogroup or biogroup (9, 20). In the present study, 60 strains of *Y. enterocolitica* consisting of five different serogroups and eight other *Yersinia* spp. were examined with the low-frequency cleavage endonucleases *Not*I and *Xba*I (Fig. 1 and 2). *Not*I and *Xba*I produced up to 45 and 47 visible fragments between 2 and 300 kb or 2 and 340 kb in size, respectively. The 60 *Y. enterocolitica* strains yielded 28 and 25 REDPs with *Not*I and *Xba*I, respectively. A total of 34 different genomic groups, defined by the combination of the various *Not*I and *Xba*I REDPs, were observed (Table 1). There was no REDP common among the eight non-*Y. enterocolitica* species and their fingerprints did not match the REDPs displayed by any of the 60 *Y. enterocolitica* isolates analyzed by PFGE-CHEF. No relationship was observed between REDP and strain origin, because strains from different sources and countries shared the same restriction profiles with one or both enzymes.

The 28 isolates of *Y. enterocolitica* serogroup O:3 revealed 11 and 9 different REDPs with *Not*I and *Xba*I, respectively. The 23 *Y. enterocolitica* serogroup O:9 isolates produced 10 different REDPs with *Not*I and 9 with *Xba*I (Fig. 1, 2, and 3A and C). Three REDPs each were generated by both enzymes with

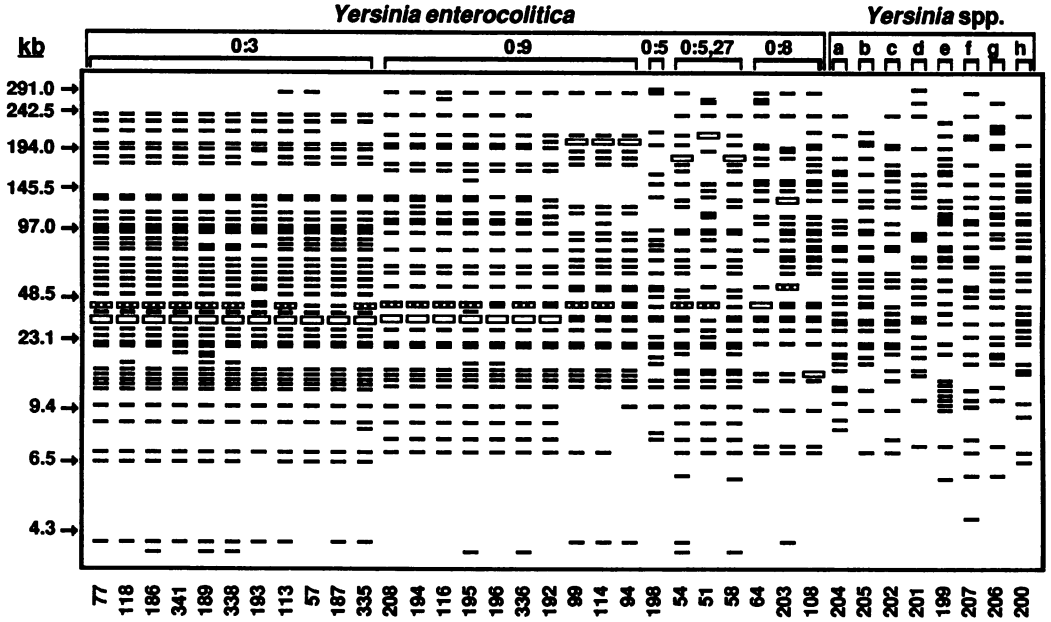

FIG. 1. Diagram of 36 representative *Not*I REDPs of 60 *Y. enterocolitica* strains and 8 other *Yersinia* spp. and their hybridization patterns. The strain numbers (below each lane) are identified in Tables 1 and 2. Lanes: a to h, *Y. kristensenii*, *Y. ruckeri*, *Y. rohdei*, *Y. frederiksenii*, *Y. bercovieri*, *Y. mollaretii*, *Y. intermedia*, *Y. pseudotuberculosis*, respectively. ▭, *ail* probe; ▪▪, pYV probe. Size markers are indicated on the left.

the five strains of *Y. enterocolitica* serogroup O:5,27 and the three serogroup O:8 strains. The one strain of *Y. enterocolitica* serogroup O:5 exhibited an unique profile with each enzyme, distinctively different from all the other REDPs observed (Fig. 1 and 2).

A clear correlation between serogroups and REDPs was observed, in agreement with previous findings for yersiniae (9, 20) and other bacterial pathogens such as *Leptospira* (18) and *Listeria* (8) species, but was not observed for *Streptococcus pneumoniae* (28). These results are consistent with the results of a study by Andersen and Saunders (1), who found restriction fragment length polymorphism types of *Y. enterocolitica* to be serogroup specific. Strains of species other than *Y. enterocolitica* exhibited distinct REDPs.

Principal component analysis. To define genetic relatedness among strains, a Dice similarity matrix was determined (data not shown) by pairwise comparison of concatenated REDP data from representative strains of the 34 genomic groups within *Y. enterocolitica* and the 8 genomic groups within the other *Yersinia* spp. and used for conducting a principal component analysis. The first and second components (Fig. 4, *x* and *y* axes, respectively) of the principal component analysis ac-

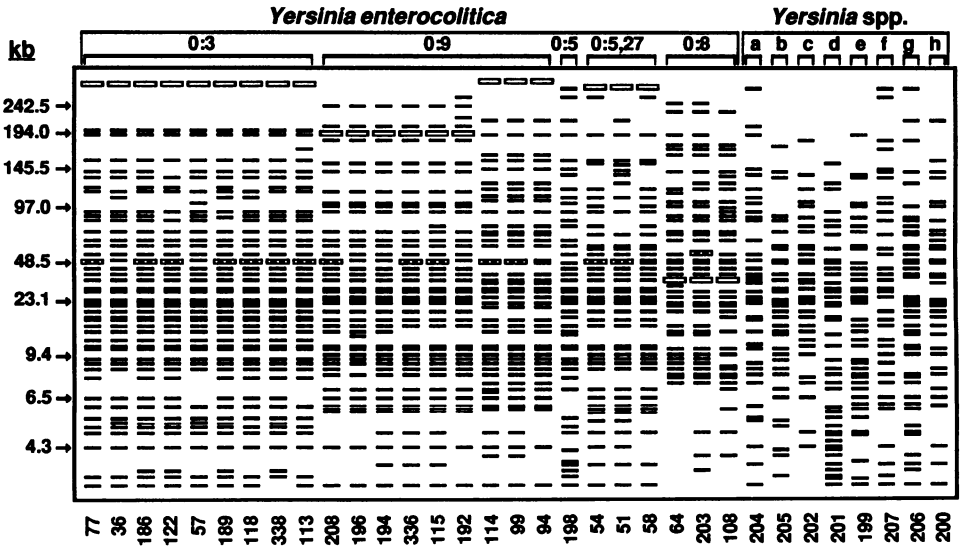

FIG. 2. Diagram of 33 representative *Xba*I REDPs of 60 *Y. enterocolitica* and 8 other *Yersinia* spp. and their hybridization patterns. The strain numbers (below each lane) are identified in Tables 1 and 2. Lanes: a to h, *Y. kristensenii*, *Y. ruckeri*, *Y. rohdei*, *Y. frederiksenii*, *Y. bercovieri*, *Y. mollaretii*, *Y. intermedia*, *Y. pseudotuberculosis*, respectively. ▭, ail probe; ▪▪, pYV probe. Size markers are indicated on the left.

TABLE 1. Designations, sources, and relevant characteristics of *Y. enterocolitica* strains

FRIK strain	Other designation[a]	Serovar	Biogroup	Source of isolate	Country of isolation	CRB[b]	CAD[c]	AA[d]	PYZ[e]	CHEF plasmid[f]	REDP		Hybridization	
											*Xba*I	*Not*I	*ail*	pYV
34	JBL 1307	9	2	Environment	United States	−	−	−	−	−	5	8	+	−
36	JBL 1309	3	4	Environment	United States	−	−	−	+	−	20	1	+	−
51	E 659	5,27	2	NK[g]	United States	+	+	+	−	+	30	33	+	+
52	E 705	9	2	NK	United States	+	+	+	−	+	7	11	+	+
53	E 675	3	4	NK	United States	+	+	+	−	+	23	23	+	+
54	30118	5,27	3	NK	United States	+	+	+	−	+	29	32	+	+
55	IP 383	9	2	Human	Belgium	−	−	−	−	−	25	27	+	−
56	E y.e. 66	5,27	3	Animal	NK	−	−	−	−	−	31	34	+	−
57	C-122-76	3	4	NK	NK	−	−	−	−	−	22	26	+	−
58	E y.e. 70	5,27	3	Animal	NK	−	−	−	−	−	31	34	+	−
60	E y.e. 117	5,27	2	Animal	NK	−	−	−	−	−	31	34	+	−
64	E y.e. 10	8	1B	Animal	United States	−	−	−	−	−	32	35	+	−
77	J 25146	3	4	Human	Austria	+	+	+	−	+	1	1	+	+
78	J 25322	3	4	Human	Austria	+	+	+	−	+	1	1	+	+
79	J 27181	3	4	Human	Austria	+	+	+	−	+	1	1	+	+
80	J 21466	3	4	Human	Austria	+	+	+	−	+	1	1	+	+
81	J 27932	3	4	Human	Austria	+	+	+	−	+	1	1	+	+
93	IP 23144	3	4	Human	France	+	+	+	−	+	1	1	+	+
94	IP 23140	9	2	Human	France	−	−	−	+	−	28	31	+	−
99	IP 23121	9	2	Human	France	+	+	+	−	+	27	29	+	+
103	IP 23131	3	4	Human	France	+	+	+	−	+	1	1	+	+
104	IP 23135	3	4	Human	France	+	+	+	−	+	2	1	+	+
108[h]	ATCC 23715	8	1B			−	−	−	−	−	33	36	+	−
109	IP 21404	9	2	Food	Argentina	+	+	+	−	+	7	12	+	+
110	IP 21412	9	2	Food	Argentina	−	−	−	−	−	5	8	+	−
111	IP 22348	9	2	Food	Argentina	−	−	−	−	−	5	8	+	−
112	IP 22370	9	2	Food	Argentina	−	−	−	−	−	5	8	+	−
113	IP 21474	3	4	Food	Spain	+	+	+	−	+	24	25	+	+
114	IP 23137	9	2	Human	France	+	+	+	−	+	26	30	+	+
115	IP 19917	9	2	Food	Argentina	−	−	−	−	−	8	8	+	−
116	IP 22349	9	2	Food	Argentina	−	−	−	+	−	5	28	+	−
117	IP 22352	9	2	Food	Argentina	−	−	−	−	−	5	8	+	−
118	IP 20660	3	4	Food	France	−	−	−	−	−	23	23	+	−
119	IP 22373	9	2	Food	Argentina	−	−	−	−	−	5	8	+	−
120	IP 22374	9	2	Food	Argentina	−	−	−	−	−	5	8	+	−
121	IP 21055	3	4	Food	France	−	−	−	−	−	1	1	+	−
122	IP 20931	3	4	Food	Spain	+	+	+	−	+	21	1	+	+
186	238	3	4	Human	Austria	+	+	+	−	+	2	2	+	+
187	1195	3	4	Human	Austria	−	−	−	+	−	1	6	+	−
188	1701	9	2	Human	Austria	−	−	−	−	−	5	8	+	−
189	1765	3	4	Human	Austria	+	+	+	−	+	3	4	+	+
190	1985	3	4	Human	Austria	−	−	−	−	−	1	3	+	−
191	1990	9	2	Human	Austria	−	−	−	−	−	5	8	+	−
192	1987	9	2	Human	Austria	−	−	−	−	−	9	10	+	−
193	2009	3	4	Human	Austria	−	−	−	−	−	1	5	+	−
194	2011	9	2	Human	Austria	+	+	+	−	+	25	27	+	+
195	2012	9	2	Human	Austria	+	+	+	−	+	8	9	+	+
196	2031	9	2	Human	Austria	−	−	−	−	−	6	11	+	−
197	2033	3	4	Human	Austria	−	−	−	−	−	1	1	+	−
198	110550	5	1A	Human	Austria	−	−	−	+	−	10	13	−	−
203	H 162-36	8	1B	Human	United States	+	+	+	−	+	11	14	+	+
208	H 961-36	9	2	Human	Germany	+	+	+	−	+	5	8	+	+
209	H 469-36	3	4	Human	Germany	+	+	+	−	+	1	1	+	+
335	109930	3	4	Human	Austria	+	+	+	−	+	7	12	+	−
337	117679	3	4	Human	Austria	+	+	+	−	+	1	1	+	+
338	120086	3	2	Human	Austria	+	+	+	−	+	4	24	+	+
339	120660	3	4	Human	Austria	+	+	+	−	+	1	1	+	+
341	125663	3	4	Human	Austria	+	+	+	−	+	2	3	+	+
342	126835	3	4	Human	Austria	+	+	+	−	+	1	1	+	+

[a] JBL, J. B. Luchansky Culture Collection, Food Research Institute, Madison, Wis.; E and C, J. Schoeni, Food Research Institute, Madison, Wis.; J, Hygiene Institute Culture Collection, Graz, Austria; IP, Institute Pasteur Culture Collection, Paris, France; ATCC, American Type Culture Collection, Rockville, Md.; H, Hygienisches Institut Culture Collection, Hamburg, Germany.

[b] CRB, Congo red binding at 37°C.

[c] CAD, low calcium response at 37°C.

[d] AA, Autoagglutination at 37°C.

[e] PYZ, ability to produce pyrazinamidase.

[f] CHEF plasmid, determination of the presence of plasmid pYV by PFGE-CHEF.

[g] NK, not known.

[h] Reference strain.

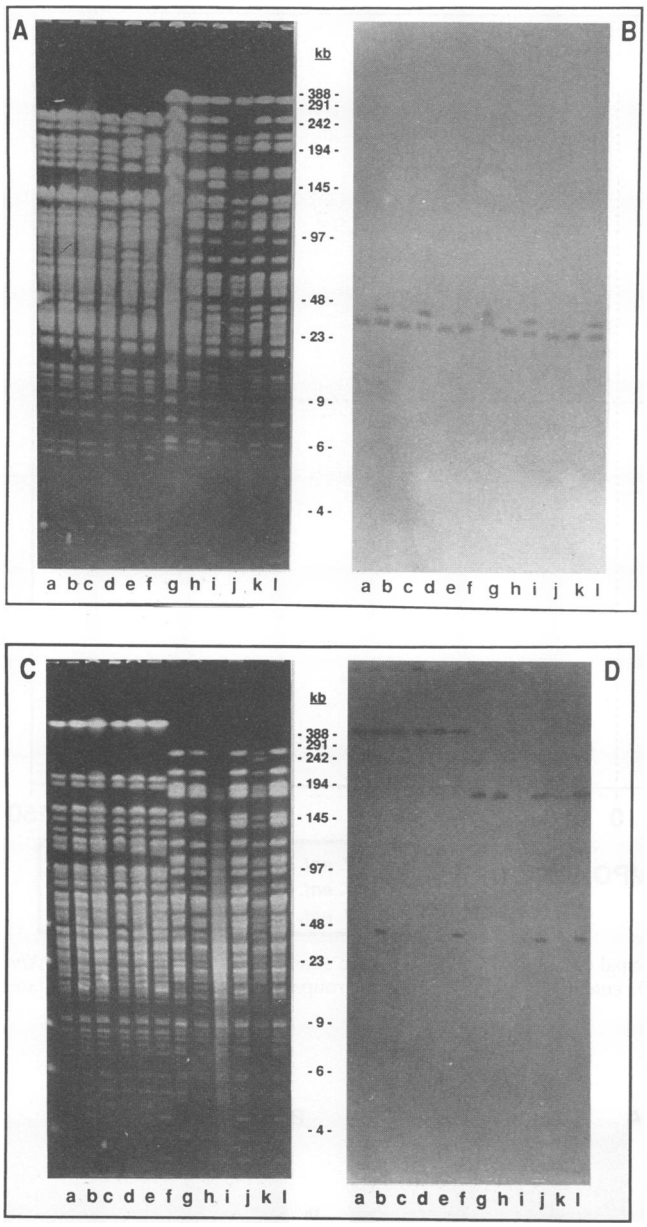

FIG. 3. REDPs of *Not*I (A) and *Xba*I (C) digests of six *Y. enterocolitica* serogroup O:3 and six serogroup O:9 isolates; (B and D) hybridization with *ail* and pVY probes. (A and B) Lanes: a, FRIK 197; b, FRIK 186; c, FRIK 190; d, FRIK 189; e, FRIK 193; f, FRIK 187; g, FRIK 188; h, FRIK 191; i, FRIK 195; j, FRIK 192; k, FRIK 196; l, FRIK 336. (C and D) Lanes: a, FRIK 197; b, FRIK 186; c, FRIK 190; d, FRIK 193; e, FRIK 187; f, FRIK 189; g, FRIK 188; h, FRIK 196; i, FRIK 191; lane j, FRIK 195; lane k, FRIK 192; lane l, FRIK 336. Molecular size markers are indicated between the panels.

counted for 57 and 27% of the total variance, respectively. The first principal component established a clear genomic division between strains of *Y. enterocolitica* serogroup O:3 and all other serogroups of *Y. enterocolitica* and other *Yersinia* spp. Similar results were reported by Blumberg et al. (7), who found that ribotyping differentiated *Y. enterocolitica* O:3 from serogroup O:9; O:1,2,3; O:20; and O:5,27 strains. In addition, our findings are consistent with results obtained with multilocus enzyme electrophoresis conducted on 244 *Yersinia* strains of which strains of *Y. enterocolitica* biotype 4 (serogroup O:3) clustered in one electrophoretic type (13). In contrast to results published by Dolina and Peduzzi (13) that strains of biogroups 4 and 2 are closely related, our results showed that strains belonging to serogroups O:3 (biogroup 4) and O:9 (biogroup 2) are genomically clearly separated. Visual comparison of REDPs, as well as the principal component analysis, showed distinct differences between serogroup O:3 (biogroup 4) and O:9 (biogroup 2) strains (Fig. 1, 2, and 4).

A close relationship between *Y. enterocolitica* serogroups O:9 and O:5,27 (Fig. 4), consistent with their biochemical characteristics, was found. *Y. enterocolitica* strains of serogroups O:9 and O:5,27 displayed only one RFLP type (1). Our results underscore the close relatedness of these two serogroups but also show the discriminatory power of PFGE-CHEF, which was able to differentiate among REDPs of strains of serogroups O:9 and O:5,27 (Fig. 1 and 2).

Strains of *Yersinia* spp. and *Y. enterocolitica* serogroup O:5 which are not involved in human disease comprise a group distant from *Y. enterocolitica* serogroups O:3, O:9, and O:5,27. Likewise, strains of *Y. enterocolitica* serogroup O:8 are separate from the other *Y. enterocolitica* strains tested. The results from principal component analyses established clear groupings, which correlated significantly with phenotypic characteristics of the strains, in particular with serogroup and biogroup data.

Plasmid screening. Another aim of this study was to use PFGE-CHEF to screen strains for the presence of pYV, since conventional methods for extracting large plasmids are tedious. Baril et al. (4) and Ferdows and Barbour (15) reported that small circular plasmids (<50 kb) display aberrant mobilities in PFGE gels while large circular molecules do not migrate into the agarose gel. We demonstrated that pYV of *Y. enterocolitica*, which has a size of approximately 70 kb, can be detected when highly concentrated and cleaved genomic DNA is subjected to PFGE-CHEF. Because of its aberrant mobility in the circular form, it was detected at the 400-kb size range for linear DNA molecules (Fig. 5A). Hybridization with the pYV probe confirmed that the visible band was the virulence plasmid (Fig. 5B). All 30 strains which exhibited phenotypic properties encoded for by pYV (autoagglutination, low calcium response, and Congo red binding) were positive for the plasmid by PFGE-CHEF and pYV hybridization. Strains of *Y. ruckeri* and *Y. intermedia* each carried one plasmid that was larger (apparent mobility of 450 kb) and *Y. frederiksenii* carried two plasmids that were smaller (apparent mobilities of 250 and 300 kb) than pYV of *Y. enterocolitica* (Table 2). The plasmids found in other *Yersinia* spp. did not hybridize with the pYV probe.

Phenotypic virulence characterization. All strains were tested for autoagglutination at 37°C, for low calcium response at 37°C, for Congo red binding at 37°C, and for the ability to produce pyrazinamidase at 25°C. Of the 60 *Y. enterocolitica* strains, 30 were positive for autoagglutination, low calcium response, and Congo red binding, and 30 were negative (Table 1). In the pyrazinamidase assay, which has been correlated with the ability of the strain to harbor the virulence plasmid and not with the presence of the virulence plasmid itself (23), 54 of the 60 *Y. enterocolitica* strains were negative and 6 were positive (Table 1). All eight strains of the other *Yersinia* spp. were negative for autoagglutination, low calcium response, and Congo red binding (Table 2); however, *Y. ruckeri*, *Y. frederiksenii*, and *Y. intermedia* were positive in the pyrazinamidase assay, while *Y. kristensenii*, *Y. rohdei*, *Y. bercovieri*, *Y. mollaretii*, and *Y. pseudotuberculosis* were negative (Table 2).

In vivo virulence characterization. Sixteen selected strains belonging to three different serogroups, O:3 (eight strains),

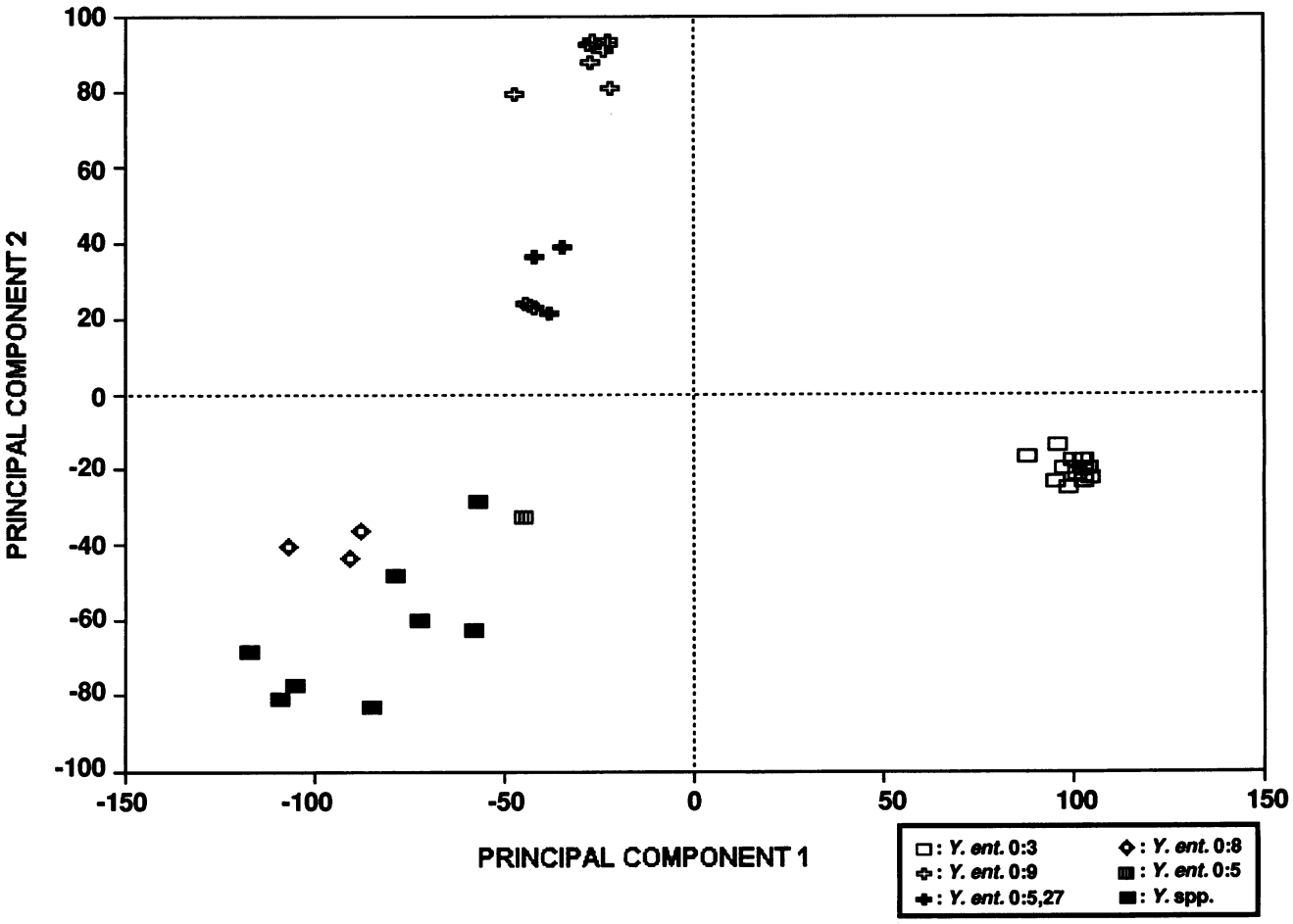

FIG. 4. Plots of first and second principal components obtained by principal component analysis of Dice similarity data of concatenated *Xba*I and *Not*I REDP data from representative strains of 34 genomic groups of *Y. enterocolitica* and 8 genomic groups of non-*Y. enterocolitica* isolates.

O:9 (six strains), and O:5,27 (two strains), and 10 different REDP groups were subjected to a mouse virulence assay. Nine of the tested strains were negative for all plasmid-dependent properties but positive for hybridization with the *ail* probe; eight of these strains were negative and one was positive in the pyrazinamidase assay. All nine strains showed no virulence in the in vivo test. Seven of the tested strains were positive for pYV and hybridization with the *ail* and pYV probes but negative in the pyrazinamidase assay. Five of these strains were virulent in the mouse assay; two were avirulent (Table 3). No correlation between REDPs or REDP groups and the virulence of the strains was observed. For example, three REDP group *Xba*I1-*Not*I1 strains were virulent to mice and two were avirulent. The three plasmid bands in the REDP are not easily discernible because of comigrating fragments, which explains why pYV-positive and pYV-negative strains are classified in the same REDP group. These results confirm that pYV is required for virulence (12, 35, 44) as all of the mouse-virulent strains were positive for pYV and other plasmid-related properties (Table 3). However, strain Food Research Institute Kaspar (FRIK Culture Collection) 114 was positive for both pYV and Congo red binding prior to injection but mouse avirulent. These results could be explained by a defect in pYV or a cell surface receptor for the iron-iron chelator complex. Also, strain FRIK 122 was *ail* and pYV positive but mouse

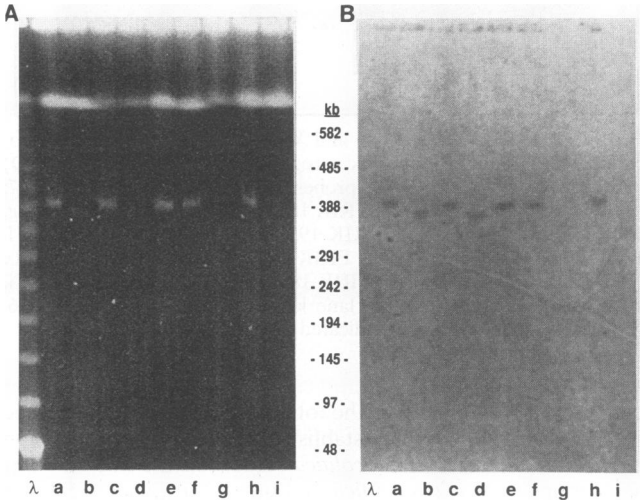

FIG. 5. (A) Virulence plasmid of *Y. enterocolitica* separated from chromosomal DNA by CHEF-PFGE; (B) hybridization with pYV probe (B). Lanes: λ, lambda concatemers; a, FRIK 93; b, FRIK 104; c, FRIK 80; d, FRIK 77; e, FRIK 79; f, FRIK 103; g, FRIK 121; h, FRIK 78; i, FRIK 34.

TABLE 2. Designations, sources, and relevant characteristics of various *Yersinia* spp.

Species	FRIK strain	Other designation[a]	CRB[b]	CAD[c]	AA[d]	PYZ[e]	CHEF plasmid[f]	REDP		Hybridization	
								*Xba*I	*Not*I	*ail*	pVY
Y. kristensenii	204	H 87-36/80	−	−	−	+	−	12	15	−	−
Y. ruckeri	205	H 34-36/85	−	−	−	−	+[g]	13	16	−	−
Y. rohdei	202	H 297-36/86	−	−	−	−	−	14	17	−	−
Y. frederiksenii	201	H 14-36/84	−	−	−	+	+[h]	15	18	−	−
Y. bercovieri	199	H 822-36/89	−	−	−	+	−	16	19	−	−
Y. mollaretii	207	H 286-36/86	−	−	−	−	−	17	20	−	−
Y. intermedia	206	H 29-36/84	−	−	−	+	+[g]	18	21	−	−
Y. pseudotuberculosis	200	H 141-36/84	−	−	−	−	−	19	22	−	−

[a] H, Hygienisches Institut Culture Collection, Hamburg, Germany.
[b] CRB, Congo red binding at 37°C.
[c] CAD, low calcium response at 37°C.
[d] AA, autoagglutination at 37°C.
[e] PYZ, ability to produce pyrazinamidase.
[f] CHEF plasmid, determination of the presence of plasmid pYV by PFGE-CHEF.
[g] One plasmid larger in size than the virulence plasmid of *Y. enterocolitica* was detected.
[h] Two plasmids smaller in size than the virulence plasmid of *Y. enterocolitica* were detected.

avirulent. However, this strain was negative for Congo red binding just prior to injection, indicating that strain FRIK 122 lost its plasmid during transport or subculturing.

Hybridization. PFGE-CHEF and hybridization with the *ail* and pYV probes were used to gain information on the locations of virulence-specific sequences on the genome. By assigning the two virulence-related DNA probes to specific macrorestriction fragments within the generated REDPs, hybridization patterns for *Y. enterocolitica* were determined (Fig. 1 and 2).

Southern blots of *Not*I and *Xba*I digests with the *ail*-specific oligonucleotide probe identified serogroup-specific fragments, with the exception of the three serogroup O:8 strains. In all *Y. enterocolitica* serogroup O:3 strains, the *ail*-specific sequence was located in ca. 35- and >350-kb fragments for *Not*I and *Xba*I digests, respectively (Fig. 1, 2, and 3). For serogroup O:9 strains, two groups of closely related REDPs were generated by *Not*I and *Xba*I. Strains with highly related REDPs yielded comparable hybridization results. In one group, the *ail* sequence was found in fragments with sizes of ca. 35 and 194 kb for *Not*I and *Xba*I digests, respectively. In the second group, a 200-kb *Not*I fragment and >350-kb *Xba*I fragment hybridized with the *ail* probe (Fig. 1 and 2). In serogroup O:5,27 strains, the *ail* probe hybridized with fragments with sizes of ca. 194 or 210 kb with *Not*I digests and of ca. 310 kb with *Xba*I digests. Within the three serogroup O:8 strains tested, the *ail* sequence was located in fragments with sizes of ca. 45, 140, or 15 kb (*Not*I digests) and 35 kb (*Xba*I digests) (Fig. 1 and 2). Miller et al. (32) reported that hybridization with radioactive *ail* polynucleotide probes is restricted to strains of serogroups associated with disease. We obtained positive hybridization signals with all 59 *Y. enterocolitica* strains belonging to serogroups associated with disease. No hybridization was observed with DNA from *Y. enterocolitica* serogroup O:5, the eight other *Yersinia* spp., and non-*Yersinia* spp. tested (Tables 1 and 2). The specificity of the *ail* probe for identifying strains of *Y. enterocolitica* associated with disease was 100%.

These results are in agreement with those obtained with ^{32}P-labelled *ail*-specific polynucleotide probes (31, 32) and those obtained by PCR (25). Thus, the *ail* gene seems to be a

TABLE 3. Virulence of 16 *Y. enterocolitica* strains in iron- and desferrioxamine-sensitized mice

FRIK strain	Serovar	Biogroup	CRB[a]	PYZ[b]	CHEF plasmid[c]	Hybridization		REDP		Mouse virulence[d]
						ail	pYV	*Xba*I	*Not*I	
51	5,27	2	+	−	+	+	+	30	33	+
77	3	4	+	−	+	+	+	1	1	+
79	3	4	+	−	+	+	+	1	1	+
99	9	2	+	−	+	+	+	27	29	+
209	3	4	+	−	+	+	+	1	1	+
114	9	2	+	−	+	+	+	26	30	−
122	3	4	−	−	+	+	+	21	1	−
34	9	2	−	−	−	+	−	5	8	−
36	3	4	−	+	−	+	−	20	1	−
56	5,27	3	−	−	−	+	−	31	34	−
119	9	2	−	−	−	+	−	5	8	−
120	9	2	−	−	−	+	−	5	8	−
121	3	4	−	−	−	+	−	1	1	−
190	3	4	−	−	−	+	−	1	3	−
192	9	2	−	−	−	+	−	9	10	−
197	3	4	−	−	−	+	−	1	1	−

[a] CRB, Congo red binding at 37°C.
[b] PYZ, ability to produce pyrazinamidase.
[c] CHEF plasmid, determination of the presence of pYV by PFGE-CHEF.
[d] Death of four or more mice injected with iron- and desferrioxamine (~10^5 CFU).

stable marker for each serogroup of *Y. enterocolitica* and a reliable marker for the identification of potentially virulent strains.

The pYV probe hybridized with a 45-kb fragment in *Not*I digests and a 49-kb *Xba*I fragment, with the exception of one serogroup O:8 strain (FRIK 203) in which the pYV-specific sequence hybridized with a 50-kb fragment in both *Not*I and *Xba*I digests (Fig. 1 and 2). All 30 strains harboring pYV, as determined by phenotypic characterization and by PFGE-CHEF, hybridized with the pYV probe, whereas all strains lacking the pYV were negative (Tables 1 and 2). No hybridization was observed with DNA from *Yersinia* strains other than *Y. enterocolitica* and the 16 other bacterial species tested. Kapperud et al. (24) obtained similar results with a ^{32}P-labelled pYV-specific oligonucleotide probe used in a colony blot hybridization assay. pYV of *Y. enterocolitica* contains three cutting sites for *Xba*I, resulting in fragments with sizes of approximately 5, 12, and 50 kb (37). The pYV probe used in this study hybridized to the 50-kb fragment (Fig. 2).

The hybridization patterns of different strains of *Y. enterocolitica* revealed highly homogeneous hybridization patterns within strains of the same serogroup (Fig. 1, 2, and 3B and D). In *Not*I digests, the hybridization patterns of serogroup O:3 strains and one REDP group of serogroup O:9 strains were identical (Fig. 1). Only within strains of serogroup O:8 did the DNA probes hybridize to fragments with different sizes (Fig. 1). Additionally, *Y. enterocolitica* strains isolated from animals, the environment, food, and humans yielded the same hybridization patterns. Thus, hybridization patterns appear to be independent of their sources of isolation. Similar results were obtained for hybridization patterns of *E. coli* in which strains isolated from humans and animals did not differ significantly (34).

PFGE-CHEF and hybridization patterns with virulence-associated gene probes proved to be a reproducible, discriminatory method for subtyping strains belonging to the same serogroup and providing information on the potential virulence of *Y. enterocolitica* isolates. The genetic relatedness of strains as determined by principal component analyses was in agreement with the phenotypic characteristics of *Y. enterocolitica*, such as serogroups, biogroups, and phenotypic virulence properties.

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