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Assessment of Plasmid Profile, Exoenzyme Activity, and Virulence in Recent Human Isolates of *Yersinia enterocolitica*

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We examined a group of 23 recent clinical isolates of *Yersinia enterocolitica* recovered from symptomatic patients residing in the New York, N.Y. area. These isolates were tested for the presence of plasmids, exoenzyme activity, mouse lethality, and phenotypic properties postulated to correlate with virulence. Of the 23 isolates, 17 harbored a 60- to 65-kilobase (kb) plasmid. Six isolates were lethal for white mice, showed the phenotypic markers of autoagglutination and calcium dependence for growth at 37°C, and contained a 60- to 65-kb plasmid. Restriction endonuclease analysis with several different enzymes revealed the presence of three distinct plasmid profiles in these isolates. Isolates with a single plasmid of 60 to 70 kb, typical for this species, were detected, but these were of three distinct types as judged from restriction enzyme digestion. One strain was unusual among clinical isolates of *Y. enterocolitica* in that it contained at least four distinct plasmids. In addition, this nontypable strain showed exoenzymatic activity similar to that of serogroup O8 isolates, was not lethal to mice, and did not require calcium for growth at 37°C.

Much of the current interest in *Yersinia enterocolitica* derives from the discovery of virulence-associated properties such as the presence of plasmids and their phenotypic expression of autoagglutination (10), calcium dependency for growth at 37°C (3), adherence (13), and animal lethality (9). Strains studied for these parameters have been either long-standing reference or stock strains (e.g., strain WA, originally isolated in 1974 from a septicemic patient [3]; strain CDC 2635, recovered in 1976 from the chocolate milk incriminated in the Holland Patent foodborne outbreak of gastroenteritis [3]); isolates largely recovered outside of the United States (12), or strains of *Y. enterocolitica* isolated in the United States and collected over a 10-year period (9). In the present study, we assessed the plasmid profile, virulence-associated phenotypic markers, and exoenzyme activities of 23 recent clinical isolates of *Y. enterocolitica* recovered from 23 different symptomatic patients residing within the New York, N.Y. area. These isolates were additionally tested for animal pathogenicity. Before testing, each strain was subcultured to tryptic soy agar and incubated for 18 h at 25°C.

Table 1 shows the sources, serogroups (courtesy of S. Toma, Ministry of Health, Toronto, Canada), biotypes, plasmid-associated phenotypic markers of autoagglutination and growth inhibition at 37°C in the presence of magnesium oxalate (MOX), plasmid species present, mouse lethality, and exoenzyme profiles of the isolates. All of the strains harboring the 62- or 65-kilobase (kb) plasmids were positive for autoagglutination at 37°C but not at 26°C when incubated overnight in Earle minimum essential medium (Flow Laboratories, Inc., Rockville, Md.) (10) and showed calcium dependency, as determined by a lack of growth on MOX agar at 37°C but not at 26°C or on blood agar base incubated at either temperature.

When exoenzyme activity was examined, most isolates showed serogroup but not temperature-dependent specificity. None of the serogroup O3 or O5,27 isolates produced lecithinase, lipase, protease, or elastase. Serogroup O8 isolates produced lecithinase, esterase, and lipase but not protease or elastase. Lecithinase activity, originally noted by Wauters (15), serves as the basis for delineating biotype 1 isolates of *Y. enterocolitica*. Esterase activity was present in 22 of 23 isolates, whereas DNase was serogroup variable. *Y. enterocolitica* strain 23, the nontypable sputum isolate that contains four plasmid types, is unusual in that it has an exoenzyme profile similar to that of serogroup O8 (lecithinase and lipase positive) but is strongly proteolytic.

Six of the strains were lethal to white mice upon intraperitoneal injection of 10^6 organisms. Note that the strains positive for mouse lethality did not grow on MOX agar at 37°C, were positive for autoagglutination, and possessed a plasmid. These data are consistent with the presence of V (protein) and W(lipoprotein) antigenic complex originally described in *Y. pestis* (6) and subsequently in *Y. enterocolitica* (2). It can be seen, however, that the presence of a plasmid and the phenotypic responses in the autoagglutination and MOX agar tests do not reliably predict mouse lethality. Recently Bolin and co-workers (1) showed that a temperature-inducible (37°C) outer membrane protein (protein 1) that might be identical to the W antigen is associated with strains of *Y. enterocolitica* and *Y. pseudotuberculosis* harboring a 63-kb plasmid. These strains showed calcium dependency at 37°C and were virulent to Swiss albino mice upon oral infection. In our study, no correlation existed between virulence and exoenzyme activity.

The 23 isolates were tested for the presence of plasmid DNA (Fig. 1). All samples were subjected to electrophoresis on 0.7% agarose gels with and without digestion with restriction endonuclease *Pst*I. Plasmids were detected in 17 of 23 of

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TABLE 1. Correlation of plasmid profile and exoenzyme activity with mouse lethality

Isolate no.	Source	Serogroup	Biotype	AA[a]	Mox agar[b]	Plasmid species (kb)[c]	Mouse lethality	Exoenzyme[d]					
								Pr	Dn	Es	Le	Li	El
1	Stool	O3	4	+	0	62	0	0	+	+	0	0	0
2	Stool	O3	4	+	0	62	0	0	+	0	0	0	0
3	Stool	O3	4	+	0	62	+	0	+	+	0	0	0
4	Stool	O3	4	+	0	62	0	0	+	+	0	0	0
5	Stool	O3	4	+	0	62	+	0	+	+	0	0	0
6	Stool	O3	4	+	0	62	0	0	+	+	0	0	0
7	Stool	O3	4	+	0	62	0	0	+	+	0	0	0
8	Stool	O3	4	+	0	62	ND[e]	0	+	+	0	0	0
9	Stool	O3	4	+	0	62	0	0	+	+	0	0	0
10	Stool	O3	4	+	0	62	+	0	+	+	0	0	0
11	Stool	O3	4	+	0	62	0	0	+	+	0	0	0
12	Stool	O3	4	+	0	62	0	0	+	+	0	0	0
13	Blood	O3	4	+	0	None	0	0	+	+	0	0	0
14	Blood	O3	4	+	0	62	0	0	0	+	0	0	0
15	Appendix	O8	1	+	0	62	+	0	0	+	+	+	0
16	Abscess	O8	1	0	+	None	0	0	0	+	+	+	0
17	Stool	O8	1	0	+	None	0	0	0	+	+	+	0
18	Stool	O8	1	+	0	65	+	0	+	+	+	+	0
19	Blood	O5,27	2	+	0	62	+	0	+	+	0	0	0
20	Stool	O5,27	2	0	+	None	0	0	+	+	0	0	0
21	Blood	O5,27	2	0	+	None	0	0	+	+	0	0	0
22	Stool	O5,27	2	0	+	None	0	0	0	+	0	0	0
23	Sputum	ND	1	+	+	60, 55, 37, 4	0	+	0	+	+	+	0

[a] Autoagglutination.
[b] Growth on MOX agar 37°C.
[c] Note that 1 kb = 0.66 megadalton.
[d] Pr, Protease; DN, DNase; Es, esterase; Le, lecithinase; Li, lipase (Tween 80); El, elastase. Methods were as previously described (7).
[e] ND, Not determined.

these isolates. It should be noted that we (unpublished observations) and others (e.g., 11) have observed the spontaneous loss of *Y. enterocolitica* plasmids during laboratory cultivation. These isolates were obtained from 1981 to 1982 and were stored frozen at −70°C after initial isolation and subculturing. As in all studies of this type, the possibility that plasmids might have originally been present for the strains that score negative for this property cannot be excluded.

We noticed that, after prolonged electrophoresis (data not shown), one of the isolates (strain 18) contained a single plasmid species that migrated slightly more slowly than the most common type present in 17 of these strains. The sizes of these two plasmid types, as estimated by comparison with uncut control plasmids of known size and by summing the restriction fragments obtained with four restriction enzymes, was approximately 62 kb for the majority type and 65 kb for the unique plasmid in strain 18. Another isolate (strain 23) contained multiple plasmid species of 60, 55, 37, and 4 kb (Fig. 1). The restriction enzyme fragments obtained with *Pst*I were indistinguishable on agarose gels for the 15 isolates with the 62-kb plasmid, whereas the other two isolates with plasmids (strains 18 and 23) gave unique *Pst*I digestion products. Several additional restriction enzymes were used to characterize these plasmid types, including *Hin*dIII (Fig. 2), *Eco*RI, and *Bam*HI (data not shown). It can be seen (Fig. 2) that each plasmid type gave a distinct restriction fragment pattern, although some fragments of approximately equal size are present. Thus, three distinct profiles were seen for the 17 isolates with plasmids. The majority (15 of 17 isolates) contained a 62-kb plasmid, one contained a 65-kb plasmid

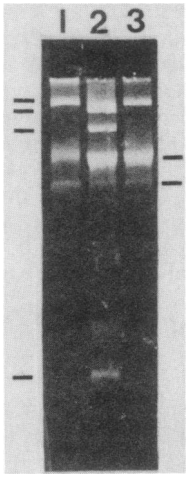

FIG. 1. Detection of plasmids in clinical isolates of *Y. enterocolitica*. Plasmid preparations from strains 8, 23, and 18 were loaded in lanes 1, 2, and 3, respectively, on a 0.7% agarose gel and subjected to electrophoresis for 18 h at 40 V. The bars on the left indicate plasmid species (the upper bar indicates plasmids present in lanes 1, 2, and 3, and the lower bars indicate plasmids present in lane 2), and the bars on the right refer to chromosomal DNA present in these preparations (as judged from control preparations from strains without plasmids). The plasmid sizes, based on comparison with molecular weight markers and summation of restriction fragment sizes (see text), are 62 kb for strain 8 (lane 1); 60, 55, 37, and 4 kb for strain 23 (lane 2); and 65 kb for strain 18 (lane 3). The methods used for gel electrophoresis and restriction enzyme purification have been described previously (4, 5).

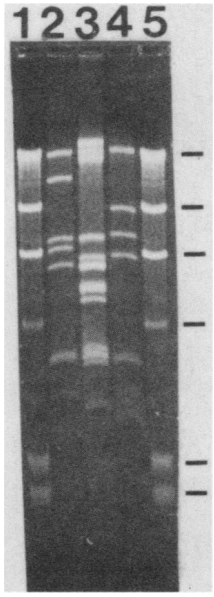

FIG. 2. Restriction endonuclease *Hin*dIII digestion of plasmids obtained from *Y. enterocolitica*. The bars on the right indicate molecular weight markers (lanes 1 and 5) of known sizes obtained by digestion of phage λ DNA with *Hin*dIII. The sizes, from top to bottom, are 21,225, 9,419, 6,559, 4,355, 2,322, and 2,023 base pairs, respectively. Plasmid DNA digested with *Hin*dIII was loaded in lane 2 (strain 8, containing a 62-kb plasmid), lane 3 (strain 23, containing several plasmids), and lane 4 (strain 18, containing a 65-kb plasmid).

distinguishable from the 62-kb type by size and restriction enzyme fragment pattern, and one contained plasmid species of 60, 55, 37, and 4 kb.

Y. enterocolitica strains possess a family of related plasmids of approximately 60 to 120 kb which are associated with lethality in gerbils, cytopathic effects in tissue culture, calcium dependence, and the temperature-dependent presence of three outer membrane proteins (8, 11). It is likely that some or all of the plasmids present in the strains characterized here are similar to those described by other investigators (8, 10, 12, 14, 16). However, isolate 23, which contained four plasmid types, is unusual among naturally occurring strains of clinical *Y. enterocolitica* isolates that have been reported to date. Our report confirms a recent report (14) of the existence of strains with such a large number of plasmids, but the significance of this phenomenon is unknown. Isolate 23 autoagglutinated at 37°C, was avirulent for mice, and did not require calcium for growth. This finding also confirms that not all in vitro markers sometimes associated with mouse virulence, such as lack of growth on MOX agar, correlate with mouse virulence in *Y. enterocolitica* (9). Further studies will be required to determine the role of plasmids present in this isolate upon its exoenzyme profile and its growth on MOX agar and the possibility of relationships at the nucleotide sequence levels of the various plasmid types present in this group of clinical isolates.

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