Clonal Diversity and Relationships among Strains of *Yersinia enterocolitica*

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Received 25 May 1989/Accepted 28 August 1989

Allelic variation in the chromosomal genome of 81 isolates of *Yersinia enterocolitica* and single isolates of *Yersinia intermedia*, *Yersinia frederiksenii*, *Yersinia mollaretii*, and *Yersinia kristensenii* was assessed by analysis of electrophoretically demonstrable polymorphism in 21 genes encoding metabolic enzymes. Eighteen distinctive multilocus genotypes (electrophoretic types [ETs]) were identified. Clustering of the ETs from a matrix of pairwise genetic distances, based on the 21 enzyme loci, confirmed the genetic distinctness of serogroup 3 isolates of *Y. intermedia*, *Y. frederiksenii*, *Y. mollaretii*, and *Y. kristensenii* and identified another serogroup 3 isolate that was also not a member of *Y. enterocolitica*. The 13 ETs of *Y. enterocolitica* clustered into two groups: cluster A, which included eight ETs represented by isolates of serogroups 1; 2; 3; 5; 27; and 9, and cluster B, which included four ETs represented by isolates of serogroups 8, 13, and 21. Clones of cluster A were found to be distributed worldwide, but those of cluster B were largely restricted to North America. Isolates of genotypes belonging to cluster B were lethal to mice, whereas those of cluster A were not, suggesting an influence of the chromosomal background on the virulence of *Y. enterocolitica*.

Since the 1960s, the importance of *Yersinia enterocolitica* as a pathogen of humans has been increasingly recognized (6, 16). The organism is responsible for a wide spectrum of intestinal and extraintestinal diseases, including gastroenteritis, mesenteric lymphadenitis, reactive arthritis, and occasionally, bacteremia (6, 15, 16). The sources of human infections are not well understood, but it is generally believed that *Y. enterocolitica* is spread via food or water contaminated by human or animal carriers. *Y. enterocolitica* is commonly isolated from animals, food, and the environment, but most strains from these sources are avirulent (14, 16).

Several schemes for classifying strains of *Y. enterocolitica* have been developed on the basis of variation in somatic and flagellar antigens (1, 31), biochemical properties (33), and susceptibility to lytic bacteriophages (18). Species boundaries and relationships have not been well defined by these characteristics, however. Recently, DNA hybridization studies have distinguished several groups of related organisms as new species (2, 4, 5, 7, 29, 32).

The biology of *Y. enterocolitica* is being studied almost entirely within the framework of a classification based on serotyping or other phenotypic characteristics. Nucleotide sequence variation analyses, including restriction endonuclease digestion of the 40- to 50-megadalton (MDa) virulence plasmids (17, 30) and chromosomal DNA (G. Kapperud, T. Nesbakken, K. Dommarsnes, S. Aleksic, and H. H. Mollaret, manuscript in preparation) have recently been used to differentiate between strains, but these methods have not yielded estimates of the extent of variation in the genome as a whole or provided measures of genetic relatedness among strains with different serogroups or biotypes.

We report here the results of a study of the genetic structure of populations of *Y. enterocolitica* based on analysis of electrophoretic protein polymorphism in strains with sero-

groups that are associated with disease in humans and animals. We identified two phylogenetic groups of clones, one of which apparently is endemic to North America. An association between clone group and lethality in mice was indicated.

MATERIALS AND METHODS

Bacterial isolates. A collection of 85 isolates of eight serogroups of *Yersinia* spp. was examined, including 81 isolates received as *Y. enterocolitica* and single isolates of *Y. intermedia*, *Y. frederiksenii*, *Y. mollaretii*, and *Y. kristensenii*, which had reactivity patterns with sera against O antigens similar to those of serogroup 3 *Y. enterocolitica*. The sources of isolation and geographic origins of the strains are given in Table 1.

Phenotypic characterization. Serogroups were determined by slide agglutination with sera against 60 O antigens and 38 H antigens (1). Biotyping was performed by the extended scheme of Wauters et al. (33). Phage types were determined as described by Nicolle (18).

Electrophoresis of enzymes. Isolates were grown in tryptic soy broth with 0.6% yeast extract at 25°C overnight. Methods of cell lysate preparation, starch electrophoresis, and selective enzyme staining were similar to those described by Selander et al. (23). Twenty-one enzymes were assayed. Electromorphs (allozymes) of each enzyme were equated with alleles at the corresponding structural gene locus, and an absence of enzyme activity was attributed to a null allele. Distinctive combinations of alleles over the 21 enzyme loci (multilocus genotypes) were designated as electrophoretic types (ETs) (Table 2).

Statistical analyses. Genetic diversity at an enzyme locus among ETs was calculated as $h = (1 - \sum x_i^2) / (n/n - 1)$, where x_i is the frequency of the i th allele and n is the number of ETs. Mean genetic diversity (H) is the average of h values over the 21 enzyme loci. Genetic distance between pairs of ETs was expressed as the proportion of loci at which

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TABLE 1. Characteristics of 85 isolates of *Yersinia* spp.

Species, ET, and isolate	Source	Country	Serogroup		Biotype	Phage type
			O	H		
<i>Y. enterocolitica</i>						
1						
YE11.131	Human	Canada	3	bc	4	IXb
YE11.137	Human	Canada	3	abc	4	IXb
YE751	Human	Canada	3	abc	4	IXb
MCH697	Human	Canada	3	abc	4	IXb
MCH700	Human	Canada	3	abc	4	IXb
YE859	Pork	Canada	3	abc	4	IXb
21603a	Pig	Denmark	3	abc	4	VIII
2713-TKS	Human	Finland	3	abc	4	VIII
3668	Human	Finland	3	abc	4	VIII
4147	Human	Finland	3	abc	4	VIII
8265	Human	France	3	abc	4	VIII
PA1400	Human	Japan	3	NM ^a	3	II
PA1472	Human	Japan	3	abc	4	VIII
SW13123	Pig	Japan	3	ac	4	VIII
SW13711	Pig	Japan	3	NM	3	II
M254	Pork	Japan	3	ac	4	VIII
M388	Pork	Japan	3	NM	3	II
29C-33	Human	Norway	3	abc	4	VIII
29C-43a	Human	Norway	3	abc	4	VIII
29C-46	Human	Norway	3	abc	4	VIII
201/86	Human	Norway	3	abc	4	VIII
1084/86	Human	Norway	3	c	4	VIII
1389/86	Human	Norway	3	NM	4	VIII
Y2a	Pig	Norway	3	ac	4	VIII
Y89	Pig	Norway	3	ac	4	VIII
Y310	Pig	Norway	3	abcv	4	VIII
Y763	Pig	Norway	3	NM	4	VIII
Y772	Pig	Norway	3	c	4	VIII
Y325	Pork	Norway	3	abc	4	VIII
8258a	Human	Spain	3	abc	4	VIII
P774	Pig	Sweden	3	abc	4	VIII
IP194	Human	Belgium	3	ab	4	VIII
2 Y764	Pig	Norway	3	abc	4	VIII
3 8246	Human	Yugoslavia	3	abc	4	VIII
4						
YE771	Human	Canada	5,27	bcv	2	Xz
YE873	Pork	Canada	5,27	abc	2	Xz
IP885	Dog	England	5,27	bc	2	Xz
7500	Coypu	France	5,27	abc	2	Xz
PA9436	Human	Japan	5,27	abc	2	Xz
D113	Dog	Japan	5,27	abc	2	Xz
SW14391	Pig	Japan	5,27	abc	2	Xz
IP47	Monkey	The Netherlands	5,27	abc	2	Xz
5						
7877	Human	Belgium	9	ab	2	X3
W827	Pig	Belgium	9	ab	2	X3
W828	Pig	Belgium	9	ab	2	X3
W829	Pig	Belgium	9	ab	2	X3
YE099	Human	Canada	9	abv	2	X3
7OULU	Human	Finland	9	abv	2	X3
7894	Human	France	9	ab	2	X3
8125	Human	France	9	ab	2	X3
PA177	Human	Japan	9	abc	2	X3
3315a	Human	The Netherlands	9	abv	2	X3
3520	Human	The Netherlands	9	ab	2	X3
IP6	Chinchilla	Unknown	1	abc	3	II
IP197	Chinchilla	United States	1	abc	3	II
IP714	Chinchilla	United States	1	abc	3	II
6 IP132	Chinchilla	The Netherlands	1	abc	3	II

Continued on following page

TABLE 1—Continued

Species, ET, and isolate	Source	Country	Serogroup		Biotype	Phage type
			O	H		
7						
IP8	Hare	Unknown	2	abc	5	XI
IP1154	Hare	Belgium	2	abc	5	XI
IP1211	Hare	Belgium	2	abc	5	XI
8 IP1142	Hare	Belgium	2	abc	5	XI
9 2943	Goat	Norway	2	abc	5	XI
10						
YE653	Human	Canada	8	befiv	1B	Xz
YE665	Human	Canada	8	befi	1B	Xz
YE10.121	Human	Canada	8	befiv	1B	Xz
WA	Human	United States	8	befiv	1B	Xz
A2635	Human	United States	8	befi	1B	Xz
1700	Human	United States	8	befi	1B	Xz
1821	Human	United States	8	befiv	1B	Xz
1824	Human	United States	8	befi	1B	Xz
NY81-68	Human	United States	8	befi	1B	Xz
NY81-71	Human	United States	8	befi	1B	Xz
NY81-87	Human	United States	8	befi	1B	Xz
NY81-85	Human	United States	8	befiv	1B	Xz
FRI-YE1	Pig	United States	8	befiv	1B	Xz
FRI-YE3	Pig	United States	8	befiv	1B	Xz
FRI-YE10	Pig	United States	8	befiv	1B	Xz
11 8081	Human	United States	8	befi	1B	Xz
12 YE886	Primate	Canada	13	abi	1B	Xo
13 YE737	Human	Canada	21	befi	1B	Xz
<i>Y. intermedia</i> , 14, 357-36/85	Water	Federal Republic of Germany	3	q	ND ^b	XI
X, 15, 8018	Rodent	Norway	3	abcd	1A	XI
<i>Y. frederiksenii</i> , 16, 176-36/80	Sewage	Federal Republic of Germany	3	p2	ND	XI
<i>Y. mollaretii</i> , 17, 87-36/82	Human	Federal Republic of Germany	3	u	6	XI
<i>Y. kristensenii</i> , 18, Y332	Pork	Norway	3	NM	ND	Xo

^a NM, Nonmotile.^b ND, Not determined.

dissimilar alleles occurred (null alleles excluded). Clustering of ETs on the basis of genetic distances was performed by the average linkage method (28).

RESULTS

Single-locus and multilocus diversity. In the collection of 85 isolates, all 21 enzyme loci were polymorphic for alleles encoding electrophoretically detectable variants. The average number of alleles per locus was 4.4.

A total of 18 distinctive allele combinations (ETs) were identified (Table 2), among which *H* was 0.551. The genetic relationships among the 18 ETs is shown by the dendrogram (Fig. 1). Five ETs (ETs 14 through 18) differed from the other ETs at more than 13 of the 21 loci (genetic distance, >0.62) and had alleles that were not represented in ETs 1 through 13 at 10 or more enzyme loci. These five ETs were distantly related to one another (*H* = 0.757) and differed, on average, at 15.9 loci. ETs 14 through 18 represented the genotypes of the single isolates of *Y. intermedia* (ET 14), *Y. frederiksenii* (ET 16), *Y. mollaretii* (ET 17), and *Y. kristensenii* (ET 18) and of one isolate, 8018, (ET 15) that was received as *Y. enterocolitica* serogroup 3 but was atypical in that it was biotype 1A instead of 3 or 4 and lacked the 40- to 50-MDa virulence plasmid.

When these five isolates were excluded, only 16 of the 21 enzyme loci were polymorphic, and the average number of alleles per locus was 2.2. Genetic diversity among the 13 ETs of *Y. enterocolitica* was 0.357. The dendrogram in Fig. 1 revealed two major lineages, clustering at a genetic distance of 0.55, among the 13 ETs of *Y. enterocolitica*. Cluster A consisted of ETs 1 through 9, which were represented by 62 isolates, and cluster B included ETs 10 through 13, which were represented by 18 isolates.

Genetic structure in relation to serogroup. The 62 isolates of ETs in cluster A were of serogroups 1; 2; 3; 5,27; and 9, and the 18 isolates of ETs in cluster B were of serogroups 8, 13, and 21.

There was little genetic diversity among isolates of the same serogroup. In cluster A, the 34 serogroup 3 isolates of *Y. enterocolitica* belonged to ETs 1, 2, and 3, which were

TABLE 2. Allele profiles at 21 enzyme loci in 18 ETs of *Yersinia* spp.

ET	Reference isolate	No. of isolates	No. of alleles at indicated enzyme loci ^a																				
			MD	ME	6P	G6	GO	P2	FU	ID	PI	AC	GD	AK	CK	PM	I1	I2	ES	A1	A2	P3	P4
1	SW13711	32	2	2	2	2	3	3	2	1	4	9	6	2	1	5	1	2	3	1	2	3	3
2	Y8246	1	2	2	2	2	3	3	2	1	4	0	6	2	1	5	1	2	3	1	2	3	3
3	Y764	1	0	2	2	2	3	3	2	1	4	9	6	2	1	5	1	2	3	1	2	3	3
4	D113	8	2	2	2	2	3	3	2	1	4	7	6	2	1	5	1	2	3	1	2	3	3
5	PA177	14	2	2	2	2	3	3	2	2	4	5	6	2	1	5	1	2	3	1	2	3	3
6	IP132	1	2	2	2	2	3	3	2	2	4	5	6	2	1	4	1	2	3	1	2	3	3
7	IP1154	3	2	2	2	2	3	3	2	2	4	5	4	2	3	5	1	2	3	1	0	3	3
8	IP1142	1	2	2	2	2	3	3	2	2	4	5	4	2	3	6	1	2	3	1	0	3	3
9	2943	1	2	2	2	2	3	3	2	2	4	5	4	2	3	4	1	2	3	1	0	3	3
10	YE653	15	2	2	2	2	2	5	2	2	4	6	6	1	1	3	3	2	5	2	1	2	2
11	8081	1	2	2	2	2	2	5	2	2	7	7	6	2	1	3	3	2	5	2	1	2	2
12	YE886	1	2	2	2	2	2	3	2	2	4	5	6	1	1	3	3	2	5	2	1	1	1
13	YE737	1	2	2	2	2	2	5	2	1	4	6	6	1	1	2	3	2	5	2	3	1	1
14	357-36/85	1	2	2	4	2	3	2	2	2	2	2	3	4	3	3	5	2	4	4	2	4	3
15	8018	1	2	1	1	2	1	2	2	1	5	8	5	0	1	1	3	2	4	0	2	1	2
16	176-36/80	1	2	2	3	2	1	1	1	2	1	3	5	0	2	3	4	3	3	0	3	1	3
17	87-36/82	1	1	3	3	1	1	2	1	3	3	4	2	2	3	4	3	2	2	3	1	4	1
18	Y332	1	3	1	3	1	1	4	3	2	6	1	1	3	3	3	2	2	1	2	2	2	3

^a Abbreviations: MD, malate dehydrogenase; ME, malic enzyme; 6P, 6-phosphogluconate dehydrogenase; G6, glucose 6-phosphate dehydrogenase; GO, glutamic oxaloacetic transaminase; P2, phenylalanyl-leucine peptidase; FU, fumarase; ID, isocitrate dehydrogenase; PI, phosphoglucose isomerase; AC, aconitase; GD, glutamate dehydrogenase; AK, adenylate kinase; CK, carbamate kinase; PM, phosphoglucomutase; I1 and I2, two indophenol oxidases; ES, esterase; A1 and A2, two alkaline phosphatases; P3 and P4, two leucyl-glycyl-glycine peptidases.

distinguishable solely on the basis of the occurrence of null alleles at the aconitase and malate dehydrogenase gene loci in ETs 2 and 3, respectively. The eight serogroup 5,27 isolates were identical in the multilocus genotype (ET 4),

which differed from ET 1 of the serogroup 3 isolates only at the aconitase locus. All 11 serogroup 9 isolates in our collection were ET 5, as were 3 of the 4 serogroup 1 isolates. The remaining serogroup 1 isolate differed from the others at a single locus, phosphoglucomutase. Five serogroup 2 isolates were ETs 7, 8, and 9, which differed from one another solely in alleles of the phosphoglucomutase locus. All five serogroup 2 isolates were characterized by allele 4 at the glutamate dehydrogenase locus and a null allele at one alkaline phosphatase locus.

In cluster B, 15 of the 16 serogroup 8 isolates had the same genotype, ET 10. The remaining one (8081, representing ET 11) differed from isolates of ET 10 at three loci.

Genetic structure in relation to other properties of the strains. All but 5 of the 80 isolates of *Y. enterocolitica* expressed one or more flagellar antigens. The five isolates lacking H antigens were all ET 1, in cluster A (Table 1). Among the other 27 isolates of ET 1, six H serotypes were represented.

Antigen a was detected in 52 of the 62 (84%) isolates of cluster A, but in only 1 (6%) of the isolates of cluster B. Antigen b was expressed by all isolates of cluster B and by 82% of those of cluster A. Antigen c was represented in 74% of cluster A isolates but in none of the isolates of cluster B. Antigens e, f, and i were restricted to isolates of cluster B; and all but one isolate possessed all three antigens. There was no sharing of H-antigen combinations between isolates of ETs in clusters A and B.

The 80 isolates of *Y. enterocolitica* represented five biotypes, 1B, 2, 3, 4, and 5. Variation in biotype was observed among isolates of ET 1 (29 biotype 4 isolates and 3 biotype 3 isolates) and among those of ET 5 (11 biotype 2 isolates and 3 biotype 3 isolates). All isolates of cluster B were biotype 1B, regardless of their serogroup or source, and none of the isolates of cluster A was biotype 1B.

Seven phage types were represented in the collection of isolates: II, VIII, IXb, XI, X3, Xz, and Xo. Isolates of ET 1 varied in phage type, with three types (II, VIII, and IXb) being represented; and isolates of ET 5 were of phage types II and X3. Isolates of ETs in cluster B were Xz (17 isolates)

FIG. 1. Genetic relationships among 18 ETs of *Yersinia* spp. The dendrogram was generated by the average linkage method of clustering from a matrix of coefficients of genetic distance based on 21 enzyme loci.

or Xo (1 isolate), but the eight isolates of ET 4 in cluster A were also phage type Xz.

Distribution of clones among host species. Four of the five ETs that were represented by multiple isolates were recovered from two or more host species (Table 1).

Geographic distribution of ETs. Some ETs have had a wide, even intercontinental, distribution. Isolates of ET 1 were recovered from humans and pigs (or pork) in seven European countries, Canada, and Japan; and isolates of ET 4 were obtained from humans, pigs (and pork), dogs, a coypu, and a monkey in Canada, England, France, The Netherlands, and Japan. In contrast, isolates of ETs 11 through 13, forming cluster B, were recovered (from humans, pigs, and a primate) only in North America.

DISCUSSION

Although plasmid genes specifying metabolic properties have been identified in *Y. enterocolitica* (8, 9), it is likely that all 21 enzymes assayed are coded by chromosomal rather than plasmid-borne genes, because all the enzymes were expressed in strain IP47, in which no plasmids were detected.

Genetic relatedness among isolates. For several species of bacteria, estimates of relatedness obtained by enzyme electrophoresis and those derived from DNA hybridization experiments have been shown to be strongly correlated (25). It is, therefore, not surprising that our study distinguished four isolates (representing ETs 14, 16, 17, and 18) that resembled *Y. enterocolitica* in somatic antigenic properties but which have recently been recognized as four distinct species on the basis of total DNA reassociation experiments. We also identified another strongly differentiated serogroup 3 isolate (8018 [ET 15], which was recovered from a rodent in Norway) that was received as *Y. enterocolitica* but that represents still another species, which may or may not have been described previously.

Clonal structure of populations. Allelic variation detected by electrophoresis of enzymes has been used to measure genetic relatedness among strains and to identify clones of numerous species of bacteria (24, 25). Because evolutionary convergence to the same multilocus genotype is highly improbable, isolates with identical allelic profiles are considered descendants of a common cell line.

The recovery of the same multilocus genotype from diverse geographic sources and the restriction of certain cellular antigens with one or a small number of related ETs indicate that the genetic structure of populations of *Y. enterocolitica*, like that of most pathogenic species of bacteria (25), is basically clonal. This was suggested earlier by the occurrence of nonrandom associations of specific serogroups, biotypes, and phage types (1, 3, 16) and by the similarity in nucleotide sequence of the 40- to 50-MDa plasmid in isolates from various origins, as determined by restriction endonuclease fragment analyses (17). Because of the nucleotide sequence similarity among plasmids, Nesbakken et al. (17) suggested that isolates of serogroups 3 and 9 represent two clones of recent origin that have achieved extensive geographic distributions. The virtual absence of diversity in multilocus enzyme genotype and the close similarity of restriction fragment patterns of the whole chromosome (Kapperud et al., in preparation) among isolates of each of these serogroups confirm this interpretation.

Variation in phenotypic properties in relation to genotype. In Europe and Canada, serogroup 3 isolates of *Y. enterocolitica* are biotype 4, but serogroup 3 isolates recovered in Japan may be either biotype 4 or biotype 3 (27). Isolates of

biotype 3 differed from those of biotype 4 in four biochemical characters (negative reaction in the Voges-Proskauer test at 25°C, inability to ferment sorbose, ability to ferment xylose, and the production of oxidative acid from lactose), in lacking flagellar antigens, and in being phage type II instead of phage type VIII. In spite of these extensive differences in phenotypic properties, biotypes 3 and 4 of serogroup 3 isolates from Japan belonged to the same clone, ET 1. The finding that the 44-MDa plasmids of biotypes 3 and 4 of serogroup 3 isolates had identical restriction endonuclease profiles (17, 27) provides additional evidence of a recent common origin.

Clusters of closely related clones. Our analysis demonstrated that the genotypes of serogroup 1; 2; 3; 5,27; and 9 isolates are closely related ($H = 0.168$) and form a single lineage, cluster A. Close similarity in the chromosomal genome among serogroup 3; 5,27; and 9 isolates was not unexpected in view of the similarity in their 40- to 50-MDa plasmids (12, 17).

Isolates of serogroups 8, 13, and 21 belonged to a very distinctive lineage of slightly more diverse genotypes ($H = 0.246$), cluster B. All isolates of ETs belonging to cluster B were characterized by biotype 1B and by the presence of the flagellar antigen i, which did not occur in any isolates of ETs in cluster A. Heesemann et al. (13) showed that the 42-MDa plasmid of serogroup 8 strain WA-314 has only 75% sequence homology to those of serogroup 3 and 9 isolates, and restriction enzyme analysis of chromosomal DNA has shown that isolates of serogroups 3; 5,27; and 9 have similar fragment patterns which differ significantly from those exhibited by isolates of serogroups 8, 13, and 21 (Kapperud et al., in preparation).

Host distribution. Pork has been implicated as a source of human infections caused by serogroup 3 and 9 organisms. Multilocus genotype analysis did not differentiate isolates recovered from humans with disease, carrier pigs, and pork, supporting the hypothesis that pigs and pork are a route of transmission of pathogenic strains to humans (14). Although serogroup 8 strains apparently do not colonize pigs to the same extent as do those of serogroups 3 and 9 (22), the three serogroup 8 strains from pigs examined here, which were obtained from a unique survey in which isolation from swine was successful (10), were identical to many of those causing infection in humans.

Geographic distribution. Whereas isolates of cluster A were collected in 10 European countries, Canada, the United States, and Japan, all 18 isolates in cluster B were collected in Canada and the United States. This circumstance, together with the fact that serogroup 8 isolates are rarely recovered in Europe and Asia (16) and were not reported in a study of yersiniosis from Brazil (11), suggests that there are endemic North American animal reservoirs of clones of cluster B. Little is known of the occurrence of *Y. enterocolitica* in North American wildlife, apart from a report of the recovery of pathogenic serogroup 8 isolates from a grey fox and a porcupine in New York State (26).

Lethality in mice in relation to genotype. Plasmid-containing serogroup 3; 5,27; and 9 isolates of clones in cluster A differ from those in cluster B (serogroups 8, 13, and 21) in that they are unable to produce lethal disease in mice (17, 19–21). Because virulence plasmids of serogroup 8 isolates have less DNA homology to those of serogroups 3; 5,27; and 9 than plasmids of those clones have to one another (13, 17), differences in the virulence of clones of the two clusters may have been a consequence of variation in plasmid structure. However, Heesemann et al. (12) demonstrated the contribution of chromosomal genes to the mouse lethality character

by showing that a plasmid-cured derivative of a serogroup 8 strain regained the mouse lethality trait after receipt of a plasmid from an isolate that was not lethal to mice. The finding that isolates that are able to produce lethal disease in mice belonged to a distinctive lineage of clones (cluster B) emphasizes the importance of the chromosomal genotype for this particular trait.

ACKNOWLEDGMENTS

We thank I. Bölin, S. G. Christensen, J. Devenish, M. P. Doyle, H. Fukushima, B. Hurvell, J. Lassen, C.-J. Lian, T. Nesbakken, J. Oosterom, T. J. Quan, M. Shayegani, C. Sundqvist, B. Swaminathan, S. Toma, and G. Wauters for providing strains and Carolyn Fleming for technical assistance.

This research was supported by grant 13.48.07-026 from the Norwegian Council for Science and Humanities (to D.A.C.), Public Health Service grant AI24631 from the National Institutes of Health (to R.K.S.), and collaborative grant 86/0721 from the North Atlantic Treaty Organization (to D.A.C. and R.K.S.).

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