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Genetic characterization of clones of the bacterium *Listeria monocytogenes* causing epidemic disease

(listeriosis/multilocus enzyme genotypes/clones/serotypes/phage types)

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ABSTRACT **One hundred and seventy-five isolates of the pathogenic bacterium *Listeria monocytogenes* recovered from human clinical (blood and cerebrospinal fluid), animal, and environmental sources in Europe, North America, and elsewhere were analyzed electrophoretically for allelic variation at 16 genetic loci encoding metabolic enzymes. Forty-five distinctive allele profiles (electrophoretic types, ETs) were distinguished, among which mean genetic diversity per locus (*H*) was 0.424. Cluster analysis of a matrix of genetic distances between paired ETs revealed two primary phylogenetic divisions of the species separated at a distance of 0.54. ETs in division I were presented by strains of serotypes 4b, 1/2b, and 4a, whereas strains of ETs in division II were of serotypes 1/2a and 1/2c. Human and animal isolates did not represent distinctive subsets of ETs. The occurrence of linkage disequilibrium between enzyme loci and the widespread distribution of certain ETs indicate that the genetic structure of *L. monocytogenes* is clonal. One clone, marked by ET 1, caused major epidemics of human disease in western Switzerland in the period 1983–1987 and in Los Angeles County, California, in 1985, both of which were attributed to contamination of soft cheese. ET 1 is closely related to the clone (ET 7) that caused two large outbreaks of listeriosis in Massachusetts in 1979 and 1983.**

Bacteria of the genus *Listeria* are widely distributed in the environment and also occur in the intestinal tract of healthy animals and humans (1). Among the several species of the genus, only *Listeria monocytogenes* is commonly pathogenic for humans (2, 3), in which it causes serious invasive disease (septicemia, meningitis, and meningoencephalitis), primarily in the immunologically compromised host, the neonate, and the fetus (1, 2). Listeriosis in adults usually occurs after consumption of contaminated food, and prenatal or perinatal disease follows colonization or infection of the mother, who may be an asymptomatic gastrointestinal carrier or may experience acute listeriosis. The organism can multiply at temperatures as low as 4°C and survives within phagocytic cells, two characteristics that assist transmission and pathogenesis.

Epidemiological studies of several recent large outbreaks of disease caused by *L. monocytogenes* have indicated that commercial dairy products are important vehicles for the transmission of human listeriosis (2, 4–6). This was clearly demonstrated for two epidemics involving contaminated soft cheese and a third large outbreak caused by contaminated vegetable products, specifically coleslaw. In the course of an epidemic in western Switzerland in the period 1983–1987, more than 120 cases of invasive human disease were attributed to organisms cultured from "Vacherin," a regional soft cheese (ref. 7; J.B., unpublished data). In a second epidemic, which occurred in Los Angeles County, California, in 1985, 63 cases of invasive disease, predominantly in pregnant Hispanic women and their offspring and in immunocompromised adults, were traced to the consumption of a Mexican-style soft cheese that was contaminated with a strain of *L. monocytogenes* of the same serotype and phage type as the clinical isolates (6). Another large outbreak of listeriosis occurred in the Maritime Provinces of Canada in 1981 as a result of contamination of vegetables through the use of sheep manure as fertilizer (4). Transmission of *L. monocytogenes* in milk products, vegetables, and seafood has been strongly suspected in other outbreaks occurring in Massachusetts and elsewhere, but without the conclusive evidence provided by isolation of the etiologic agent from food and demonstration of its identity to clinical isolates (2, 4, 6, 8).

Epidemiological research requires methods of analysis that allow effective discrimination of strains and provide a basis for determining degrees of genetic relatedness among strains. A serotyping scheme developed for species of *Listeria* on the basis of variation in the somatic (O) and flagellar (H) antigens (9) is of limited use because most clinical isolates of *L. monocytogenes* represent only three serotypes (4b, 1/2a, and 1/2b) (2, 10). A phage typing scheme is also available (11, 12), but only 60 to 70% of isolates of *L. monocytogenes* are typeable (2, 12); and phage typing does not provide estimates of genetic relatedness among strains.

Multilocus enzyme electrophoresis has recently been employed to study the genetic structure and epidemiology of a number of pathogenic bacteria of various genera (13–15). By this method, bacterial isolates are differentiated according to variation in the electrophoretic mobility of a large number of metabolic enzymes. Electromorphs of each enzyme are equated with alleles at the corresponding structural gene locus, and electromorph profiles over a sample of enzymes (electrophoretic types, ETs), which are interpreted as multilocus genetypes representative of the chromosomal genome, provide a basis for estimating levels of genetic diversity and relatedness among isolates from natural populations.

We here report the results of the application of multilocus enzyme electrophoresis to the analysis of the genetic structure of *L. monocytogenes*. We have been particularly interested in determining the genetic relationships among strains recovered from humans, animals, and the environment and from several large outbreaks of listeriosis in Europe and North America. Our results demonstrate that recent epidemics in Switzerland and California associated with contaminated soft cheese were caused by strains of the same multilocus genotype and that two other recent large out-

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Abbreviation: ET, electrophoretic type.

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breaks were caused by organisms of a very closely related clonal genotype.

MATERIALS AND METHODS

Bacterial Isolates. We analyzed 175 isolates of *L. monocytogenes* collected from natural populations in Switzerland and several other European countries, the United States, and New Zealand. They were cultured from humans (n = 114 isolates), animals (n = 17), the environment (n = 7), and dairy products and equipment used in cheese production (n = 33). The sample included 59 isolates recovered in the course of an epidemic of listeriosis in the Lausanne region of western Switzerland (7): 41 isolates recovered from patients, 7 isolates cultured from Vacherin cheese, and 11 isolates cultured from cheese-making tools (brush, grate, board, and strainer) in several cheese cellars near Lausanne.

The collection also included three patient isolates of the phage type (340-2389-2425-2671-3552) that was responsible for the epidemic of invasive disease in California in 1985 (6), two patient isolates from a multiple-hospital outbreak of listeriosis that occurred in Boston in 1979 (8), and a patient isolate of the strain that caused a milk-borne outbreak in Massachusetts in 1983 (5). (These isolates were provided by W. F. Bibb, Centers for Disease Control, Atlanta.) Four reference strains of *L. monocytogenes* from the Culture Collection of the University of Lausanne were also included in the analysis.

Electrophoresis of Enzymes. Methods of lysate preparation, electrophoresis, and selective enzyme staining have been described by Selander *et al.* (13). Sixteen enzymes encoded by chromosomal genes were assayed (see Table 1).

Serotyping and Phage Typing. Serotyping was performed by the method of Seeliger and Hohne (9); some of the strains had been serotyped by H. P. R. Seeliger (Wurzburg, F.R.G.). Phage types were determined with the test phages and methods previously described (11).

Statistical Analysis. Genetic diversity for an enzyme locus among ETs was calculated as $h = (1 - \Sigma x_i^2)\,[n/(n-1)]$, where x_i is the frequency of the ith allele and n is the number of ETs (13). Mean genetic diversity (H) is the arithmetic average of h values for all 16 loci assayed. Genetic distance between pairs of ETs was expressed as the proportion of loci at which dissimilar alleles occurred (mismatches), with the contribution of each locus being weighted inversely by the genetic diversity (h) at the locus among all ETs (13, 16). Clustering of ETs from a matrix of coefficients of pairwise genetic distances was performed by the average-linkage method.

RESULTS

Genic and Genotypic Diversity. Fourteen of the 16 enzyme loci were polymorphic for from 2 to 6 alleles (mean number per locus, 3.6) (Table 1). There were 45 distinctive allele profiles or ETs, among which mean genetic diversity per locus (H) was 0.424.

Genetic Relationships Among ETs. Clustering of the 45 ETs revealed two primary divisions (I and II) separated at a genetic distance of 0.54 (Fig. 1). Division I included ET 1–ET 22, and division II was composed of ET 23–ET 45. For the ETs in divisions I and II, the mean genetic diversity per locus (H) was 0.176 and 0.283, respectively. Notable differences between the two divisions were a lack of sharing of alleles at the L-phenylalanyl-L-leucine peptidase locus and markedly different frequencies of alleles at seven other loci. The coefficient of divergence, G_{ST}, which estimates the ratio of the probability of a mismatch at a locus for 2 ETs chosen randomly from different divisions to the average probability of a mismatch in the pooled sample of ETs (16, 17), was 0.459.

Table 1. Genetic diversity (h) at 16 enzyme loci among ETs of *L. monocytogenes*

Enzyme locus	No. of alleles	h in indicated group of ETs			G_{ST}
		Total	Div. I	Div. II	
6PG	3	0.603	0.000	0.466	0.613
ADK	2	0.510	0.000	0.087	0.915
PLP	3	0.534	0.091	0.000	0.915
ACP	5	0.676	0.468	0.300	0.432
PGI	3	0.127	0.000	0.245	0.032
MPI	6	0.714	0.177	0.664	0.411
GP1	3	0.527	0.091	0.312	0.618
CAT	5	0.612	0.260	0.755	0.171
FUM	5	0.643	0.255	0.423	0.472
ALD	3	0.394	0.567	0.000	0.280
NSP	1	0.000	0.000	0.000	0.000
LDH	6	0.213	0.260	0.170	−0.008
GD2	3	0.167	0.091	0.237	0.018
IPO	1	0.000	0.000	0.000	0.000
G6P	4	0.647	0.173	0.423	0.539
IDH	5	0.417	0.385	0.439	0.011
Mean	3.6	0.424	0.176	0.283	0.459*

Div., division; 6PG, 6-phosphogluconate dehydrogenase; ADK, adenylate kinase; PLP, L-phenylalanyl-L-leucine peptidase; ACP, acid phosphatase; PGI, phosphoglucose isomerase; MPI, mannose phosphate isomerase; GP1, NAD-dependent glyceraldehyde-3-phosphate dehydrogenase; CAT, catalase; FUM, fumarase; ALD, alanine dehydrogenase; NSP, nucleoside phosphorylase; LDH, lactate dehydrogenase; GD2, NADP-dependent glutamate dehydrogenase; IPO, indophenol oxidase; G6P, glucose-6-phosphate dehydrogenase; IDH, isocitrate dehydrogenase.

*Calculated as $G_{ST} = (H_T - H_S)/H_T$, where H_T = 0.424 is the mean genetic diversity per locus among all 45 ETs and H_S = 0.229 is the mean diversity among ETs in divisions I and II.

Sources of Isolates. The sources of isolates of the various ETs are indicated in Fig. 1. Six ETs (ETs 1, 3, 7, 31, 32, and 35) were represented by isolates from both humans and animals. This observation and the widespread distribution in the dendrogram of ETs represented by animal isolates indicate that human and animal isolates do not belong to distinctive subsets (lineages) of ETs.

Some ETs are represented by isolates from several countries in diverse parts of the world. Isolates of ET 1 were recovered in Switzerland, France, the Federal Republic of Germany, Italy, the United States, and New Zealand; and ET 7 was represented by isolates from seven European countries, the United States, and New Zealand.

Distribution of Serotypes. All isolates of serotypes 4a, 4b, and 1/2b were of ETs in division I, whereas the isolates of all ETs in division II were of serotypes 1/2a and 1/2c. In division II, 1/2a was the common serotype, and strains of the minority serotype 1/2c were not confined to ETs in one lineage or cluster of lineages. Similarly, in division I, the minority serotype 1/2b occurred in isolates of ETs belonging to several lineages. ET 7 was represented by 21 isolates of serotype 4b and 5 isolates of serotype 1/2b. Hence, there was no indication that the distribution of serotypes within either division reflects the overall genetic relationships of the ETs.

Distribution of Phage Types. Phage type was determined for 109 of the 175 isolates examined. Phage type A is defined by sensitivity to the test phages 30, 47, 108, 2389, 2425, 2671, and 3274; and phage type B includes strains lysed by phages 47, 108, 340, and 2389. Other phage types are considered derivatives of A or B if they do not differ from A by more than two reactions and from B by more than one reaction (10).

Isolates of the same ET may be of different phage types. For example, 36 (45%) and 28 (35%) of the 83 isolates of ET 1 were phage types A and B (or their derivatives), respec-

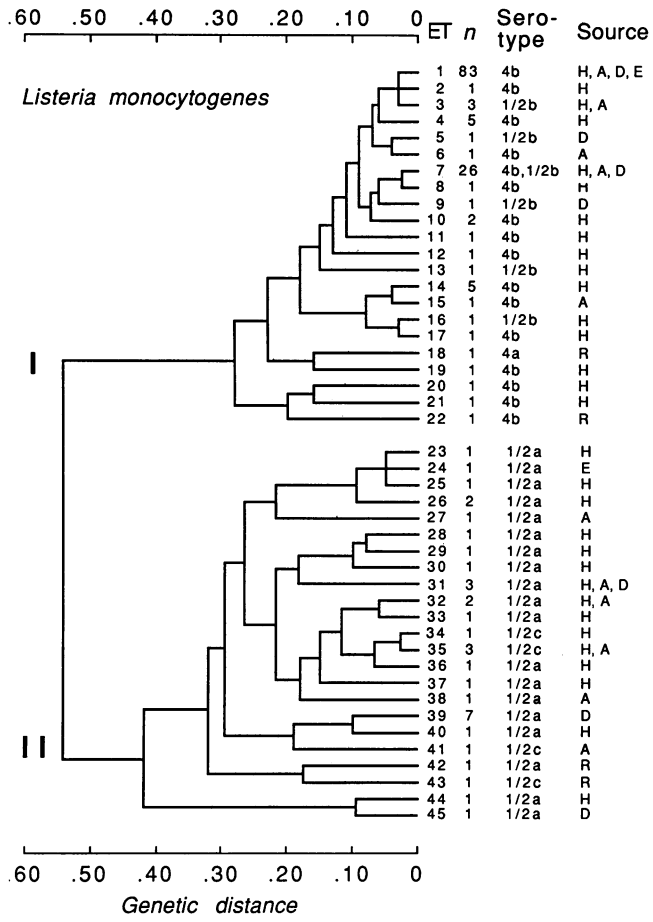

FIG. 1. Genetic relationships among 45 ETs of *L. monocytogenes*. The dendrogram was generated by the average-linkage method of clustering from a matrix of pairwise coefficients of weighted genetic distance, based on electrophoretically demonstrable allelic variation at 16 enzyme loci. *n*, number of isolates; H, human; A, animal; D, dairy products or related sources; E, environment; R, reference strain.

tively. The phage types of the remaining 20% of the ET 1 isolates assayed were distinctive.

ETs Responsible for Epidemics of Listeriosis. Both the strain that caused an epidemic in western Switzerland in the period 1983–1987, through contamination of a regionally produced soft cheese (Vacherin cheese), and the strain that was responsible for an epidemic in Los Angeles County, California, in 1985, through contamination of a Mexican-style soft cheese, are ET 1. Moreover, the dominant phage type (47-108-340-2389-2425-2671-3274) of the 59 epidemic Swiss isolates in our collection is similar to the phage type (340-2389-2425-2671-3552) of isolates from the California epidemic.

We have examined a patient isolate of a strain that caused an epidemic of listeriosis in Massachusetts, in 1983, that is believed to have been propagated by contaminated milk (5) and two patient isolates from a multiple-hospital outbreak that occurred in Boston, in 1979 (8): these three isolates are ET 7.

DISCUSSION

Genetic Structure of Populations. Our data are consistent with the hypothesis that recombination of chromosomal genes is an infrequent event in natural populations of *L. monocytogenes*. Three lines of evidence may be cited. First is the lack of sharing of serotypes between isolates of ETs assigned to the two primary divisions (I and II) of the species (Fig. 1). Second is the occurrence of linkage disequilibrium (nonrandom association of alleles) among ETs for many pairs of the enzyme loci assayed (data not shown). Third is the finding that genetically indistinguishable (by our analysis) isolates have been recovered in diverse parts of the world (for example, isolates of ET 1).

In the following discussion, we have assumed that the genetic structure of natural populations of *L. monocytogenes* is basically clonal and that ETs mark clones.

Host Distribution of Clones. For *Bordetella bronchiseptica* and several other species of pathogenic bacteria, host range and other characteristics of natural history have been shown to vary among clonal lineages (18–20). However, our analysis of *L. monocytogenes* revealed no evidence of genetic differentiation among strains infecting a variety of mammalian hosts, including humans, sheep, rabbits, and horses. This finding is consistent with other lines of evidence indicating that strains causing listeriosis in humans are usually acquired from animals or environmental sources, including foodstuffs (1, 2, 4–8).

Genetic Diversity Within Serotypes. Strains of *L. monocytogenes* are presently classified by their antigenic properties according to a serological scheme instituted by Paterson (21) and modified by Donker-Voet (22) and Seeliger (23, 24). On the basis of diversity in somatic (O) and flagellar protein (H) antigens, 12 serotypes (antigenic profiles) have been distinguished (25), and this classification has been used to examine possible associations of phenotypic characters with virulence (26). However, these serologic groups are only partially cognate with the two major phylogenetic divisions of the species and with the clonal lineages within divisions revealed by our population genetic analysis (see Fig. 1). It is clear that identity of serotype among isolates does not necessarily indicate clonal identity.

Genetic Structure in Relation to Pathogenicity. In bacterial species with clonal population structure, the nonrandom associations of alleles at different genes (linkage disequilibrium) may result in certain clones or families of clones being more highly pathogenic than others or preferentially associated with certain diseases and clinical syndromes (18–20, 27). Although our analysis revealed that a large number of multilocus genotypes of *L. monocytogenes* can cause invasive disease in both humans and animals, strains of only two closely related genotypes, ET 1 and ET 7, accounted for two-thirds of the cases of disease. This discovery may be interpreted as evidence that the genes mediating virulence in *L. monocytogenes* are in linkage disequilibrium with genes of the multilocus enzyme genotypes we have identified.

Strains of the two clones marked by multilocus genotypes ET 1 and ET 7 were responsible for four large and widely publicized epidemics of listeriosis occurring in three widely separated geographic regions (California, Massachusetts, and Switzerland) in this decade. And either ET 1 or ET 7 probably also was responsible for an outbreak of 41 cases of invasive disease in the Maritime Provinces of Canada in 1981 (F. Ashton, personal communication). Moreover, the association of strains of serotype 4b with several other outbreaks (10) and their frequent recovery from endemic cases of invasive disease, together with the restriction of this serotype to isolates of ETs in primary division I, strongly suggest that there are major ecological and epidemiological differences between clones in division I and II.

An important question for research in epidemiology, pathogenesis, and evolutionary genetics is why, given the extensive clonal diversity of natural populations of *L. monocytogenes* (our analysis identified 45 clones), the clone marked by ET 1 was the etiological agent in epidemics in California and Switzerland involving contamination of soft cheese. There are several possibilities: (*i*) Clone ET 1 may be particularly well adapted for growth and/or survival in

Table 2. Numbers of clones of various bacterial species commonly causing disease

Species	No. of clones identified	No. of clones commonly recovered from disease episodes	% disease caused by common clones	Ref.
Bordetella bronchiseptica	21	3	87	18
Bordetella pertussis	2	2	100	27
Bordetella parapertussis	1	1	100	27
Hemophilus pleuropneumoniae	32	2	47	28
Hemophilus influenzae serotype b	182	9	81	19*
Yersinia ruckeri	4	1	89	29
Legionella pneumophila	50	5	52	16
Neisseria meningitidis				
Serogroups B and C	192†	7†	85	30
Serogroup A	50	7	—	31
Shigella sonnei	1	1	100	32
Escherichia coli (neonatal invasive)	18†	5†	63	33
Salmonella spp. (8 serotypes)	71	11	61–100	20

*And J.M.M. (unpublished data).
†Clone families composed of several or many very closely related clones.

cheese. (*ii*) ET 1 may be relatively abundant everywhere in the environment and, therefore, likely to be the agent in any episode of disease involving contamination of foodstuffs. (*iii*) ET 1 may have an unusually high level of pathogenicity. This issue cannot be resolved until more information on variation in the physiological properties (including virulence) of the various clones and their geographic distributions and relative abundances in the environment is available. But the data suggest that there is something distinctive about the physiology or ecology of the ET 1 clone that makes it likely to occur as a contaminant of soft cheese and cause outbreaks of serious human disease. The same may be true of ET 7 with respect to milk, and it is perhaps significant that ET 7 is closely related to ET 1.

The information available for outbreaks of listeriosis caused by *L. monocytogenes* is consistent with a general pattern that has recently emerged from studies of the genetic structure and clonal composition of populations of several other pathogenic bacteria [i.e., that most disease is caused by a small fraction (often one or a few) of the existing clones (Table 2)]. Although much work has been done to identify and characterize virulence genes in *L. monocytogenes* (34–39), these studies have concentrated on strains of serotype 1/2a and 1/2c, which we have shown to be genetically quite distinct from strains responsible for recent epidemic episodes and, probably, in the aggregate, for most cases of human listeriosis. Our findings suggest that the study of *L. monocytogenes* pathogenesis would profit from comparative investigations of clones in divisions I and II.

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