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Toward a population genetic analysis of *Salmonella*: Genetic diversity and relationships among strains of serotypes *S. choleraesuis, S. derby, S. dublin, S. enteritidis, S. heidelberg, S. infantis, S. newport,* and *S. typhimurium*

(clones/multilocus genotypes/enzyme polymorphism/antigen profiles)

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ABSTRACT **Variation in the chromosomal genomes of 1527 isolates of eight common serotypes (O and H antigen profiles) of *Salmonella* was assessed by analysis of electrophoretically demonstrable allelic polymorphism at 23 metabolic enzyme loci. Seventy-one distinctive electrophoretic types, representing multilocus genotypes, were identified. A basically clonal population structure was indicated by the presence of strong linkage disequilibrium among enzyme loci, the association of each serotype with a relatively small number of multilocus enzyme genotypes, and the global distribution of certain genotypes. For each of six of the serotypes, 83–96% of isolates were members of a single clone. The occurrence of each of four serotypes (*S. derby, S. enteritidis, S. infantis,* and *S. newport*) in isolates of clones belonging to several evolutionary lineages, some of which are distantly related, suggests that the horizontal transfer and recombination of chromosomal genes mediating expression of cell-surface antigens has been a significant process in the evolution of the salmonellae. Two divergent clone clusters of *S. derby* differ in the relative frequency with which they cause disease in birds versus mammals, and two major lineages of *S. newport* differ in the frequency with which their clones are associated with disease in humans versus animals.**

Enterobacteria of the genus *Salmonella* are presently characterized and classified by their antigenic properties according to a serological scheme instituted by White (1) and elaborated by Kauffmann (2). On the basis of extensive diversity in cell-surface antigens, primarily those of the somatic lipopolysaccharide (O) and flagellar proteins (H), 2200 or more distinctive serotypes (antigen profiles) have been distinguished (3). Most of the serotypes have been designated by Latin trivial names (e.g., *Salmonella typhimurium*) as a consequence of Kauffmann's (4) conviction that each serovar is a distinct species; and, historically, two genera, *Salmonella* and *Arizona*, have been recognized for the salmonellae.

In 1973, studies of chromosomal DNA hybridization demonstrated that the enteropathogenic bacteria then assigned to numerous species in the genera *Salmonella* and *Arizona* are congeneric and, indeed, closely enough related to satisfy the 70% nucleotide-sequence-similarity criterion as a single genospecies (5). Seven groups, variously designated as subgenera or subspecies, have been distinguished on the basis of chromosomal DNA hybridization and variation in certain phenotypic characters (3, 6). Sixty percent of the serotypes belong to group 1, including those of strains responsible for 99% of cases of salmonellosis in humans (7).

Phage subtyping schemes have been developed for strains of several of the common medically important serotypes (8–10), and plasmid profiling (11–15) and several methods of detecting nucleotide sequence variation, including restriction endonuclease digestion of plasmid and chromosomal DNA (12, 16–18), sometimes combined with the use of chromosomal probes (19), have also recently been applied in epidemiological research (20). But these techniques have contributed little to an understanding of the evolutionary relationships of strains and have as yet had no application in population genetics.

For both evolutionary genetics and medical bacteriology, the important question that has not been addressed is, To what degree do the different serotypes (antigen profiles) correspond to meaningful units of population structure? If the population structure of *Salmonella* is clonal, as suggested by recent analyses of the associations of plasmids, outer-membrane proteins, phage types, and other characters (11, 12, 18, 21), the question becomes, Does identity of serotype among isolates indicate clonal identity? A related problem concerns the relative extent of genomic diversity among strains of serotypes with broad host ranges (e.g., *Salmonella enteritidis*, which causes disease in man, other mammals, and birds) versus those that are confined largely or entirely to single host species (e.g., *Salmonella dublin*, in cattle; *Salmonella typhi*, in humans).

We have undertaken a study of molecular population genetics of *Salmonella* to determine the genetic structure of natural populations and the relationships of the extensive surface antigenic variation to that structure. Our ultimate objective is to construct a genetic framework for the genus *Salmonella* within which to study various problems relating to pathogenicity, host specificity, and the evolutionary origins of the organisms causing human enteric (typhoid) fever (especially *S. typhi*).

Here we report the results of an analysis of electrophoretically demonstrable allelic variation in 23 enzyme-encoding chromosomal genes in isolates of eight serotypes that are commonly associated with disease in humans and domesticated mammals. Our study has revealed that the genetic structure of natural populations of *Salmonella* is basically clonal and that, for six of the serotypes, most isolates are

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Abbreviation: ET, electrophoretic type.

members of single clones of worldwide distribution. However, horizontal transfer and recombination of chromosomal genes is implicated as a significant factor in the evolution of the salmonellae by the discovery that each of the antigen profiles designated as *Salmonella derby, S. enteritidis, S. newport*, and *S. infantis* occurs in isolates belonging to several distantly related clonal lineages.

MATERIALS AND METHODS

Bacterial Isolates. A collection of 1527 isolates of eight serotypes of *Salmonella* was obtained from the Enteric Bacteriology Section, Centers for Disease Control, Atlanta, GA; the National Veterinary Services Laboratories, Ames, IA; the Institut für Veterinarmedizin des Bundesgesundheitsamtes, Berlin; and the Instituto Nacional de Ciencias y Tecnologia-DIF, Mexico, D.F. Additionally, a small sample of isolates of *S. enteritidis* was provided by the Epidemiology Program of the Connecticut Department of Health, through the courtesy of M. L. Cartter. Serotyping was repeated for isolates that had unusual multilocus enzyme genotypes.

The geographic sources of the isolates are shown in Table 1. Almost all the isolates were recovered from clinical cases of diarrhea in humans and other species of mammals, mostly domesticated. In total, 50% of the isolates were recovered from humans, and 73% were collected in the Americas, mostly in the United States.

Electrophoresis of Enzymes. Methods of lysate preparation, electrophoresis, and selective enzyme staining have been described by Selander *et al.* (22). Twenty-three enzymes encoded by chromosomal genes were assayed: isocitrate dehydrogenase (IDH), aconitase (ACO), carbamylate kinase (CAK), adenylate kinase (ADK), acid phosphatase 1 (AP1), acid phosphatase 2 (AP2), 6-phosphogluconate dehydrogenase (6PG), phosphoglucose isomerase (PGI), nucleoside phosphorylase (NSP), catalase (CAT), hexokinase (HEX), leucylglycylglycine peptidase 1 (LG1), leucylglycylglycine peptidase 2 (LG2), phenylalanylleucine peptidase (PLP), malate dehydrogenase (MDH), glucose-6-phosphate dehydrogenase (G6P), mannose-1-phosphate dehydrogenase (M1P), glucose dehydrogenase (GDH), phosphoglucomutase (PGM), glutamate dehydrogenase (GLU), indophenol oxidase (IPO), mannose-phosphate isomerase (MPI), and glutamic-oxaloacetic transaminase (GOT).

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 electromorphs over the 23 enzyme loci (multilocus genotypes) were designated as electrophoretic types (ETs).

Statistical Analysis. Genetic diversity at an enzyme locus among ETs was calculated from allele frequencies as $h = (1 - \sum x_i^2)[n/(n-1)]$, where x_i is the frequency of the ith allele and n is the number of ETs (22). Mean genetic diversity (H) is the arithmetic average of h values over all loci. Genetic distance between pairs of ETs was expressed as the proportion of enzyme loci at which dissimilar alleles occurred (mismatches), with null alleles ignored.

RESULTS

Multilocus and Single-Locus Diversity. All enzyme loci except adenylate kinase were polymorphic, and the average number of alleles per locus was 4.1 (range, 1–17). Seventy-one ETs, each representing a distinctive allele profile over the 23 enzyme loci assayed, were distinguished among the 1527 isolates of the eight serotypes examined. The number of ETs represented by isolates of each serotype is indicated in Table 2.

Mean genetic diversity per locus (H) for the 71 ETs was 0.255 (Table 2); strains of *S. derby* showed the greatest diversity ($H = 0.258$), and those of *S. heidelberg* were the least variable ($H = 0.092$).

Table 1. Geographic sources of 1527 isolates of eight serotypes of *Salmonella*

Serotype	No. (%) of isolates from indicated region			
	Americas	Europe	Other	Total
S. choleraesuis				
Common genotype (Cs 1)	45 (92)	25 (93)	5 (56)	75 (88)
Variant genotypes	4 (8)	2 (7)	4 (44)	10 (12)
Total	49	27	9	85
S. derby				
Division I				
Common genotype (De 1)	64 (94)	0	2 (67)	66 (93)
Variant genotypes	4 (6)	0	1 (33)	5 (7)
Total	68	0	3	71
Division II				
Common genotype (De 13)	161 (61)	0	2 (100)	163 (61)
Variant genotypes	104 (39)	0	0	104 (39)
Total	265	0	2	267
Division III				
Common genotype (De 31)	11 (100)	0	0	11 (100)
Variant genotypes	0	0	0	0
Total	11	0	0	11
S. dublin				
Common genotype (Du 1)	44 (96)	52 (91)	6 (50)	102 (89)
Variant genotypes	2 (4)	5 (9)	6 (50)	13 (11)
Total	46	57	12	115
S. enteritidis				
Common genotype (En 1)	163 (95)	61 (95)	15 (71)	239 (93)
Variant genotypes	9 (5)	3 (5)	6 (29)	18 (7)
Total	171	64	21	257
S. heidelberg				
Common genotype (He 1)	122 (90)	47 (94)	8 (42)	177 (87)
Variant genotypes	13 (10)	3 (6)	11 (58)	27 (13)
Total	135	50	19	204
S. infantis				
Common genotype (In 1)	52 (100)	42 (93)	15 (94)	109 (96)
Variant genotypes	0	3 (7)	1 (6)	4 (4)
Total	52	45	16	113
S. newport				
Division I				
Common genotype (Np 1)	27 (38)	0	0	27 (38)
Variant genotypes	45 (62)	0	0	45 (62)
Total	72	0	0	72
Division II				
Common genotype (Np 11)	27 (84)	0	0	27 (84)
Variant genotypes	5 (16)	0	0	5 (16)
Total	32	0	0	32
Unassigned (Np 6)	1	0	0	1
S. typhimurium				
Common genotype (Tm 1)	180 (87)	50 (76)	19 (70)	249 (83)
Variant genotypes	26 (13)	16 (24)	8 (30)	51 (17)
Total	206	66	27	299

Genetic Relationships Among ETs. Fig. 1 is a dendrogram generated by the average-linkage method (23) for the 71 ETs from a matrix of pairwise coefficients of genetic distance.

Table 2. Genetic diversity among ETs represented by isolates of eight serotypes of *Salmonella*

Serotype	Antigen formula*	No. Isolates	No. ETs	$H^{\dagger}$
S. choleraesuis	6,7:c:1,5	85	6	0.165
S. derby	1,4,12:f,g:-	349	6	0.258
S. dublin	1,9,12:g,p:-	115	4	0.101
S. enteritidis	1,9,12:g,m:-	257	14	0.176
S. heidelberg	1,4,5,12:r:1,2	204	8	0.092
S. infantis	6,7:r:1,5	113	4	0.152
S. newport	6,8:e,h:1,2	105	13	0.149
S. typhimurium‡	1,4,5,12:i:1,2	299	17	0.114
Total		1527	71	0.255

*The formula specifies, in sequence, the O, H1, and H2 antigenic factors occurring in most isolates of a given serotype (3).
†Mean genetic diversity per locus.
‡Includes 56 isolates of *S. typhimurium* variety *copenhagen*.

The six ETs of *S. choleraesuis* form a distinct group consisting of five closely related ETs in cluster I and an allied lineage (J) represented by ET Cs 6.

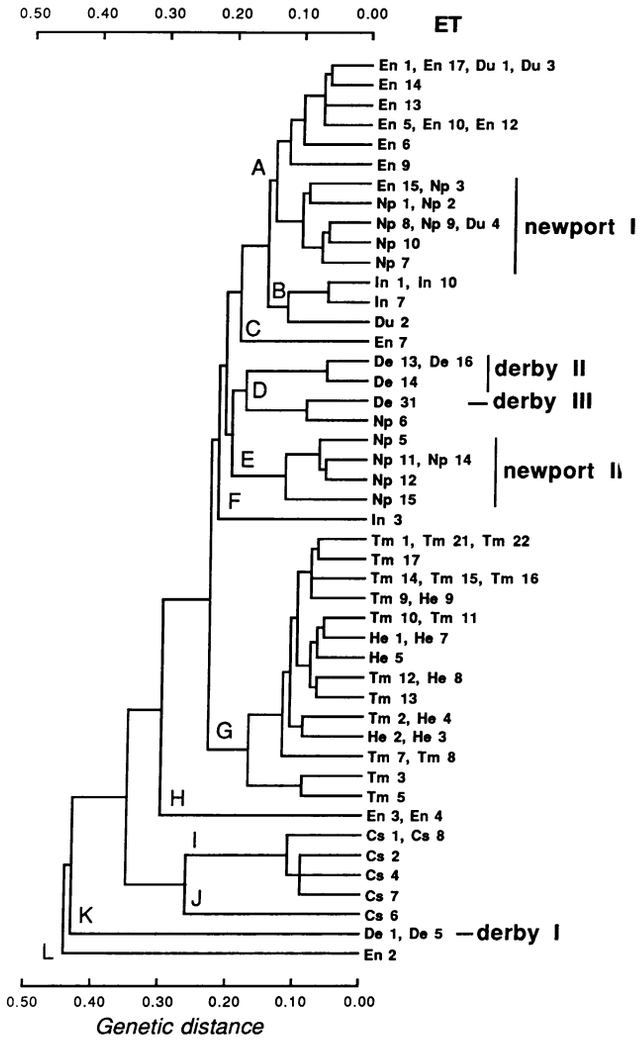

FIG. 1. Dendrogram showing genetic relationships of 71 ETs of eight serotypes of *Salmonella*. The lineages were truncated at a genetic distance of 0.04, which reflects a single locus difference between ETs. Major lineages and clusters are designated by letter. ETs of the eight serotypes are designated as Cs (*S. choleraesuis*), De (*S. derby*), Du (*S. dublin*), En (*S. enteritidis*), He (*S. heidelberg*), In (*S. infantis*), Np (*S. newport*), and Tm (*S. typhimurium*).

Cluster G includes the 25 ETs of isolates serotyped as *S. typhimurium* or *S. heidelberg*, which are closely similar in antigen profile (Table 2). The common genotypes (Tm 1 and He 1) of strains of these two serotypes differ at only one enzyme locus, and all the ETs of the *S. heidelberg* isolates are bracketed in the dendrogram by ETs of *S. typhimurium*. Tm 3 and Tm 5 form a divergent group differing from ETs in the main part of cluster G at an average of 4.4 loci.

Of the 299 isolates of *S. typhimurium* examined, 56 had been serosubtyped as *S. typhimurium* variety *copenhagen*, which differs antigenically from the standard *S. typhimurium* only in lacking O factor 5. The five ETs to which they were assigned (Tm 1, Tm 2, Tm 7, Tm 8, and Tm 10) do not cluster apart from other ETs of "typical" *S. typhimurium*; and 87% of the *S. typhimurium* var. *copenhagen* isolates shared the same genotype (Tm 1) with the great majority of *S. typhimurium* isolates.

Three of the four ETs of *S. infantis* are in cluster B, but the fourth ET, In 3, forms lineage F.

Ten of the 14 ETs of *S. enteritidis* isolates (En 1, En 5, En 6, En 9, En 10, En 12–15, and En 17) fall in cluster A, but other ETs of this serotype occur in three additional lineages. En 7 forms lineage C; En 3 and En 4, which constitute lineage H, are only distantly related to the ETs in cluster A and lineage C; and En 2 (lineage L), represented by an isolate from Brazil, is even more divergent.

Three of the four ETs of *S. dublin* (Du 1, Du 3, and Du 4) are in cluster A and are very similar to ETs of *S. enteritidis* (with which they share O and H1 antigens; Table 2); the common ET of *S. dublin* (Du 1) differs from the common ET of *S. enteritidis* only in having a null allele at the phosphoglucomutase locus. However, Du 2, represented by five isolates from Thailand, is more closely related to certain ETs of *S. infantis*, in cluster B.

The six ETs of *S. derby* isolates belong to two very distantly related lineages, D and K. Among the four ETs of cluster D, De 31 is sufficiently distinct to be regarded as a third lineage. We have designated these three divisions as *S. derby* I (De 1 and De 5, in cluster K), *S. derby* II (De 13, De 14, and De 16, in cluster D), and *S. derby* III (De 31, also in cluster D). The common ETs of *S. derby* I (De 1) and *S. derby* II (De 13) differ at 9 of the 23 enzyme loci assayed.

There are two distinctive groups of *S. newport* ETs: division I, consisting of the seven ETs in the lower part of cluster A; and division II, consisting of the five ETs that form cluster E. In addition, one ET, Np 6, falls in cluster D. The common ETs of *S. newport* I (Np 1) and *S. newport* II (Np 11) differ at five enzyme loci.

Relative Frequencies and Geographic Distributions of ETs. For each of the serotypes *S. choleraesuis*, *S. dublin*, *S. heidelberg*, *S. infantis*, and *S. typhimurium*, 83–96% of isolates represented the same ET (Table 1). Similarly, 93% of the isolates of *S. derby* I had the same multilocus genotype (De 1). But isolates of *S. derby* II were of two common genotypes, De 13 and De 14, which were distinguished by alleles at the isocitrate dehydrogenase locus and were represented by 61% and 39% of the isolates, respectively.

In *S. newport* I there are two common ETs, Np 1 and Np 8, which were represented by 38% and 26% of the 72 isolates in that division. The common ET of *S. newport* II, Np 11, was represented by 27 (84%) of the 32 isolates.

The common ETs of each serotype apparently are global in distribution. For example, our collection includes isolates of *S. typhimurium* ET Tm 1 from 34 states, Mexico, Panama, Brazil, seven countries in Europe, Senegal, Mongolia, Malaysia, Thailand, and Guam. And even some of the uncommon clones are widespread; for example, the three isolates of Tm 13 were collected in Australia, Mongolia, and Panama.

For *S. typhimurium*, a larger proportion (87%) of isolates from the Americas were of the common genotype (Tm 1) than

in the case of isolates from Europe (76%) or from other regions of the world (70%) (Table 1; $\chi^2_{(2)} = 9.09$, $P < 0.05$). For each of the serotypes *S. choleraesuis*, *S. dublin*, *S. enteritidis*, and *S. heidelberg*, the common ET occurs in approximately equal frequency in the Americas and Europe, but in each case the limited data suggest that it is less common in other parts of the world.

In the case of *S. derby*, strains of ETs in divisions I and III occur in higher frequencies relative to those of ETs in division II in the Americas than in Europe, and the relative frequencies with which isolates of the two common ETs of division II (De 13 and De 14) are recovered also varies geographically (data not shown).

Host Distribution of ETs. The two major divisions of *S. derby* (I and II) differ statistically in the host distribution of their isolates (Table 3): 21% of isolates of ETs of *S. derby* I were recovered from avian hosts (mostly chickens and turkeys), whereas only 7% of isolates of *S. derby* II were obtained from birds. And 32% of *S. derby* II isolates versus 9% of *S. derby* I isolates were recovered from swine. The frequencies of occurrence in humans were approximately equal for isolates of the two divisions.

Three-quarters of the isolates of ETs in *S. newport* division I were recovered from humans, whereas isolates of ETs in division II were derived primarily (63%) from swine and other domesticated animals (Table 3).

DISCUSSION

Genetic Structure of Populations. The occurrence of strong nonrandom associations (linkage disequilibrium) among metabolic enzyme loci and the association of each of the cellular antigen profiles with one or a small number of lineages, together with the global distribution of the common multilocus genotypes, clearly indicate that the genetic structure of *Salmonella* populations is basically clonal. This conclusion was previously reached by Helmuth *et al.* (12) on the basis of observed nonrandom associations of plasmid profiles and electrophoretic patterns of the outer-membrane proteins. Thus the genetic structure of *Salmonella* is similar to that of *Escherichia coli* (24), and, as in the case of that species, multilocus enzyme genotypes, which quantitatively index variation in chromosomal genomes (25), are considered to mark clones.

O Antigen Groups. The profile of the major O antigen or antigens has been used to assign the serotypes of *Salmonella* to several groups (3), and this classification has been used as a basis for assessing possible associations of phenotypic characters with virulence (26). However, these groups are only partially cognate with the clonal lineages revealed by our population genetic analysis (Fig. 1). Both *S. enteritidis* and *S. dublin* are assigned to the same group (D_1) because they express the 9 and 12 O antigens (Table 2), and our analysis revealed close overall genomic relatedness between the common clones of each of these serotypes (En 1 and Du 1) (Fig. 1). Similarly, clones of *S. typhimurium* and *S. heidelberg*, which are in antigen group B (characterized by the presence of the 1 and 4 O antigens), are very closely related (cluster G, Fig. 1). But *S. derby* is also a member of antigen group B, although strains of this serotype are genotypically very divergent from those of both *S. typhimurium* and *S. heidelberg*. And the similarity in the antigenic profiles of clones of *S. infantis* and *S. choleraesuis*, both of which have the 6 and 7 O antigens, which places them in O antigen group C_1, and the 1 and 5 H2 flagellar antigens, is most surprising in view of the fact that strains of these serotypes (which are distinguished by different H1 antigens) have very different multilocus enzyme genotypes.

Table 3. Host distribution of *Salmonella* isolates

Serotype and division	No. of isolates	% from indicated host			$\chi^2_{(2)}$
		Birds	Human	Swine, other mammals	
S. derby I	53	20.8	69.8	9.4	16.07*
S. derby II	206	7.3	60.7	32.0	
S. newport I	72	6.9	75.0	18.1	20.78*
S. newport II	30	6.7	30.0	63.3	

*$P < 0.001$, comparing distributions of isolates between divisions of a serotype.

Host Distribution. We have shown previously for several other species of pathogenic bacteria that virulence, host range, and some other aspects of natural history may vary among clonal lineages (27, 28). In *Salmonella* as well, divergent clone families with identical serotypes differ in various phenotypic traits, including pathogenic potential and host range. The *S. derby* antigen profile occurs in isolates of clones belonging to widely divergent lineages that are to some degree preferentially associated with different hosts. And in the case of *S. newport*, one of the two major clonal lineages occurs predominantly in humans, while the other is associated primarily with domesticated mammals. But for the other serotypes studied, we found no evidence of genetic differentiation among strains infecting a wide variety of avian and mammalian hosts, including humans. This finding is consistent with other lines of evidence indicating that strains causing salmonellosis in humans are usually acquired from animal sources (14).

Origin of Clusters of Closely Related Clones. For each serotype except *S. derby* and *S. newport* there is one predominant, worldwide clone that is associated with a cluster of genotypically similar clones differing at only one or a few loci. It is most probable that these minor clones are relatively recent mutant derivatives of the common clone.

Occurrence of Same Serotype in Isolates of Distantly Related Clones. We have identified three cases (*S. choleraesuis*, *S. heidelberg*, and *S. typhimurium*) in which a specific antigen profile is confined to a single cluster of closely related chromosomal genotypes and five cases (*S. enteritidis*, *S. dublin*, *S. infantis*, *S. newport*, and *S. derby*) in which isolates of widely divergent clonal lineages have the same serotype. The latter situation theoretically could be accounted for in part by the retention of ancestral antigen profiles in otherwise divergent lineages, but the concept of conservation of antigen-determining genes over long periods of evolutionary time is difficult to reconcile with the abundant antigenic diversity exhibited by extant populations. Consequently, evolutionary convergence of antigen phenotype and the horizontal transfer and recombination of antigen genes between lineages are more plausible hypotheses. And because convergence in antigen profile seems unlikely in view of the considerable number of mutational events that would, in many cases, be required, the hypothesis of horizontal genetic transfer seems the more attractive.

The Kauffmann–White serotyping scheme is based primarily on variation in the somatic (O) and flagellar (H) antigens, which are coded for by three chromosomal genes or clusters of genes. The genes for the biosynthesis and assembly of the O lipopolysaccharide are largely in the *rfb* cluster, at map position 42 (29–31). Strains of most serotypes of *Salmonella* produce two classes of flagellar antigens (designated as phase 1 and phase 2), which are encoded by the H1 and H2 genes, at map positions 40 and 56, respectively (29). (Strains of a few serotypes produce only one flagellar antigen.)

Relevant to the hypothesis of horizontal transfer, it may be significant that the *rfb* region and the H1 gene are separated by only 3 map units. A segment of chromosome of this size could be transferred by conjugation [although not by viral transduction (32)], and only a few recombinational events

would be required to account for the observed distribution of serotypes. Three of the four antigen profiles occurring in divergent clonal lineages (*S. derby, S. dublin*, and *S. enteritidis*) do not include phase 2 antigens, so that modification of the antigen profile would, in these cases, involve only those genes controlling the O and H1 antigens.

Occasional recombination of chromosomal genes could occur without completely eroding the linkage disequilibrium that is generated in populations with a basically clonal structure (33). The significant observation is that many of the antigen profiles of *Salmonella* are widely distributed among clonal lineages, as would be expected if the horizontal exchange of chromosomal genes among lineages is relatively frequent or extensive.

In sum, population genetic analysis of allelic variation in chromosomal structural genes has demonstrated that strains of *Salmonella* of the same serotype are genotypically heterogeneous and, in some cases, represent two or more highly divergent phylogenetic lineages. The extent to which the apparent antigenic identity (*e.g., S. newport* I and II) or similarity (*S. infantis* and *S. choleraesuis*) of strains belonging to distantly related lineages can be attributed to horizontal gene transfer or to convergence of antigen profile phenotype can be determined only by interstrain comparison of the nucleotide sequences of the genes mediating the structure of the lipopolysaccharide and flagellar protein antigens.

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