Molecular Evolutionary Genetics of the Cattle-Adapted Serovar Salmonella dublin

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An electrophoretic analysis of allelic variation at 24 enzyme loci among 170 isolates of the serovar Salmonella dublin (serotype 1,9,12[Vi];g,p:--) identified three electrophoretic types (Du 1, Du 3, and Du 4), marking three closely related clones, one of which (Du 1) is globally distributed and was represented by 95% of the randomly selected isolates. All but 1 of 114 nonmotile isolates of serotype 1,9,12:--:-- recovered from cattle and swine in the United States were genotypically Du 1. The virulence capsular polysaccharide (Vi antigen) is confined to clone Du 3, which apparently is limited in distribution to France and Great Britain. For all 29 isolates of Du 3, positive signals were detected when genomic DNA was hybridized with a probe specific for the ViaB region, which contains the structurally determinant genes for the Vi antigen; and 23 of these isolates had been serologically typed as Vi positive. In contrast, all 30 isolates of Du 1 tested with the ViaB probe were negative. These findings strongly suggest that the ViaB genes were recently acquired by S. dublin via horizontal transfer and additive recombination. The clones of S. dublin are closely similar to the globally predominant clone (En 1) of Salmonella enteritidis (serotype 1,9,12:g,m:--) in both multilocus enzyme genotype and nucleotide sequence of the flIC gene encoding phase 1 flagellin. Comparative sequencing of flIC has revealed the molecular genetic basis for expression of the p and m flagellar epitopes by which these serovars are distinguished in the Kauffmann-White serological scheme of classification.

The serovar Salmonella dublin (serotype 1,9,12 [Vi];g,p:--) is most frequently recovered from cattle, to which it is regarded as strongly host adapted (7, 34), although it has shown a tendency to spread to swine and occasionally infects sheep and other domesticated animals (27, 48). In cattle, S. dublin causes enteric fever in both calves and adults and also induces abortion by invading the fetal blood system; and the frequent development of a carrier state, including a condition in which cows shed bacteria in their milk (40), provides the main reservoir of infection (10, 46, 54). Human infections with S. dublin, which are severe and may be fatal, especially in AIDS patients and other immuno-compromised persons (8, 19, 26, 53), are usually associated with the consumption of unpasteurized dairy products (13, 15).

An unusual feature of variation in S. dublin is that some strains express the virulence capsular antigen (Vi antigen), an acetylated polymer of galactosaminuronic acid that forms a coat on the external surface of the bacterial cell (1, 14, 25). The only other bacteria known to express this polysaccharide are the distantly related serovars Salmonella typhi and Salmonella paratyphi C (11, 38, 44) and a few strains of Citrobacter freundii (12, 43). For these bacteria, it has been established that the genes determining the structure of the Vi antigen are located in the ViaB region of the chromosome (20, 22, 44, 45).

Strains of S. dublin are normally motile and monophasic, expressing a phase 1 flagellin of antigenic type g,p; but in recent years, nonmotile strains of serotype 1,9,12:--;-- have been recovered from cattle and other animals with increasing frequency both in North America (14a) and Europe (17).

Although not typeable to serovar in the absence of flagellar antigen expression, many such isolates have provisionally been identified as variants of S. dublin on the basis of their recovery from cattle or swine and, occasionally, also by the profiling and fingerprinting of plasmids (17).

We here report the results of an analysis of the genetic structure of natural populations of S. dublin and the evolutionary relationships between strains of this serovar and those of the serovar Salmonella enteritidis.

MATERIALS AND METHODS

Bacterial isolates. Our study is based primarily on a sample of 170 isolates of S. dublin, most of which were obtained from the collections of the Institut Pasteur, Paris, France; the Institut für Veterinarmedizin des Bundesgesundheitsamtes, Berlin, Germany; and the National Veterinary Services Laboratories, Ames, Iowa. This sample includes 110 isolates of S. dublin previously studied by Beltran et al. (4), together with 53 additional isolates from France and 7 isolates collected in the course of recent outbreaks of disease in humans in California and Washington (49).

The geographic sources of the isolates, listed by electrophoretic type (ET) (see Results), are as follows, with the number of isolates included in parentheses: for Du 1 (139), United States (36 from 14 states), Canada (2), Panama (2), Brazil (9), Peru (2), Great Britain (11), France (32), Germany (1), Belgium (5), Switzerland (4), Poland (5), Luxembourg (4), the Netherlands (4), Norway (3), Sweden (4), Yugoslavia (1), Israel (3), Iraq (4), Upper Volta (Burkina Faso) (2), and Austria (5); for Du 3 (29), France (26) and Great Britain (3); for Du 4, United States (2).

We also examined 114 nonmotile salmonellae of serotype 1,9,12:--;-- that were recovered from cattle (n = 111) and...
The total of 585 isolates from France, 30 isolates were specifically selected by us from the collection of the Institut Pasteur because they had previously been serologically typed at that institution as Vi negative, and 23 were selected from the same collection because they had been typed as Vi positive.

Analyzed but not included in our sample of S. dublin are five isolates from Thailand that were received from the Institut für Veterinarmedizin des Bundesgesundheitsamtes as S. dublin and were earlier designated as a distinctive multilocus enzyme genotype (Du 2) of that serovar by Beltran et al. (4). When reserotyped at the Centers for Disease Control and the National Veterinary Services Laboratories, these isolates were reported as S. dublin, but they were recognized as being serologically distinctive in expressing phase 1 flagellar antigen factor m in addition to factors g and p (14a, 29a). Hence, their flagellar antigen profile (g,m,p) is a combination of the factors by which S. dublin (g,p) and S. enteritidis (g,m) are distinguished as different serovars in the Kauffman-White serological scheme of classification (12, 24).

Electrophoresis of enzymes. Methods of lysozyme preparation, protein electrophoresis, and selective enzyme staining have been described by Selander et al. (39) and Beltran et al. (4). Twenty-four metabolic enzymes encoded by chromosomal genes were assayed for all isolates; this was the same panel of enzymes previously assayed by Beltran et al. (4), with the addition of shikimate dehydrogenase (38).

Electromorphs (allozymes) of each enzyme were equated with alleles at the corresponding structural gene locus, and distinctive combinations of alleles (multilocus genotypes) were designated as ETs (39). Genetic distance between pairs of ETs is expressed as the proportion of enzyme loci at which dissimilar alleles occur (mismatches).

Testing for Vi antigen expression. Fifty-three of the isolates obtained from the Institut Pasteur had been serologically tested at that institution for expression of Vi antigen at or near the time of their recovery (variously from 1982 to 1988). In 1990, two isolates of Du 1 and four isolates of Du 3 that had been obtained from the Institut für Veterinarmedizin des Bundesgesundheitsamtes were tested by us for Vi-antigen expression. The serological procedure employed at the Institut Pasteur has been described by Le Minor and Nicolle (25) and that used herein involved slide agglutination with Vi-specific antiserum (45).

DNA probing for Vi antigen genes. An 8.6-kb segment of the ViaB region of the C. freundii chromosome (strain WR7004) that is specific for the structural genes determining the Vi antigen (22, 35) was used as a probe to ascertain whether the failure of most strains of S. dublin to express Vi antigen is a consequence of gene regulation or an absence of the ViaB genes.

Plasmid profile analysis. Plasmid DNA from 59 isolates of S. dublin was prepared by the method of Kado and Liu (21), electrophoresed on agarose gels, and compared with standard plasmids of known molecular weights, as described by Smith and Selander (43).

Nucleotide sequencing of flagellin genes. The fltC gene encoding phase 1 flagellin was amplified by the polymerase chain reaction, and the entire coding region of the gene (1,518 nucleotides) was sequenced in both orientations, by the protocol described by Smith and Selander (43), for five strains: S. dublin RKS 4699 (representing ET Du 1), S. dublin RKS 1595 and RKS 4732 (Du 3), RKS 1550 of serotype 1,9,12:g,m,p-- (from Thailand), and S. enteritidis RKS 53, representing the globally predominant ET En 1 (4).

Nucleotide sequence accession numbers. The sequences reported here have been assigned GenBank accession numbers M84972, M84973, and M84974.

RESULTS

Multilocus enzyme genotypes. Three closely similar ETs, corresponding to the three multilocus enzyme genotypes previously designated by Beltran et al. (4) as Du 1, Du 3, and Du 4, were represented among the 170 isolates in our primary sample of S. dublin analyzed for variation at 24 enzyme loci (Table 1). Of the total of 170 isolates, 139 isolates were Du 1, 29 isolates were Du 3, and 2 isolates were Du 4.

In multilocus enzyme genotype, ET Du 3 is distinguishable from Du 1 only on the basis of an allelic difference at the phosphoglucomutase locus (genetic distance, 0.04), and Du 4 differs from Du 1 only at the nucleoside phosphorylase locus.

As shown in Table 1, five isolates of serotype 1,9,12: g,m,p-- from Thailand that have been serotyped as atypical S. dublin (see Materials and Methods) are only distantly related to the ETs of S. dublin (i.e., isolates of serotype 1,9,12:g,p--), from which they differ in having distinctive alleles at three to five enzyme loci (genetic distance, 0.13 to 0.21). In multilocus genotype, the Thailand isolates are more similar to Salmonella agona and to certain strains of S. paratyphi B than they are to strains of S. dublin (35a).

Relative frequency of ETs in natural populations. Excluding both the 53 isolates from France that were specifically selected for study on the basis of their having been typed as either Vi positive or Vi negative and the sample of nonmotile strains from the United States (see Materials and Methods), the total number of randomly selected isolates of S. dublin examined was 117, of which 111 (95%) represent Du 1, 4 (3.4%) are Du 3, and 2 (1.7%) are Du 4. We have examined representatives of Du 1 from 20 countries on six continents, whereas the strains of Du 3 in our sample were recovered only in France and Great Britain, and Du 4 is known only from two isolates from the United States.

Identity of nonmotile isolates. All but 1 of 114 nonmotile isolates of serotype 1,9,12:-- that had recently been recovered from cattle or swine in the United States had the

| TABLE 1. Alleles at five polymorphic enzyme loci in ETs of several serovars | Allele at enzyme locus:* | AP2 | NSP | CAT | PGM | SKD |
|-----------------------------|-------------------------|-----|-----|-----|-----|-----|
| S. enteritidis (1,9,12:g,m:--) | En 1                    | 5   | 3   | 3   | 3   | 5   |
| S. dublin (1,9,12:g,p--)     | Du 1                    | 5   | 3   | 3   | 4   | 1   |
|                             | Du 3                    | 5   | 5   | 3   | 3   | 1   |
|                             | Du 4                    | 5   | 5   | 3   | 4   | 1   |
| Serotype 1,9,12:g,m,p--: "Du 2" | 10 | 3   | 1   | 3   | 5   |

* Enzyme loci abbreviations: AP2, acid phosphatase-2; NSP, nucleoside phosphorylase; CAT, catalase; PGM, phosphoglucomutase; and SKD, shikimate dehydrogenase. Nineteen additional enzyme loci (see Materials and Methods) were monomorphic.

* Designated as an ET (Du 2) of S. dublin by Beltran et al. (4); see the text.
TABLE 2. Properties of isolates of S. dublin ETs Du 1 and Du 3

| ET and source | No. of isolates | Date isolated | Vi-antigen phenotype | Response to ViaB probe | Plasmid profile |
|---------------|-----------------|---------------|----------------------|------------------------|-----------------|
| Du 1 (n = 30) | Bovine          | 10            | 1988                 | +                      | 80-kb virulence |
|               | Beef            | 1             | 1988                 | +                      | 80-kb virulence |
|               | Meat (butcher shop) | 2            | 1988                 | +                      | 80-kb virulence |
|               | Human blood     | 10            | 1988                 | +                      | 80-kb virulence |
|               | Human pus       | 1             | 1988                 | +                      | 80-kb virulence |
|               | Human urine     | 1             | 1988                 | +                      | 80-kb virulence |
|               | Human feces     | 2             | 1988                 | +                      | 80-kb virulence |
|               | Unknown         | 1             | 1988                 | +                      | 80-kb virulence |
|               | Switzerland     | 1             | 1982?                | +                      | 80-kb virulence |
|               | Israel          | 1             | 1982?                | +                      | 80-kb virulence |

| Du (n = 29)  | Bovine          | 15            | 1982-1988            | +                      | 80-kb virulence |
|             | Bovine calf     | 1             | 1985                 | +                      | 80-kb virulence |
|             | Animal food     | 1             | 1986                 | +                      | 80-kb virulence |
|             | Human blood     | 1             | 1986                 | +                      | 80-kb virulence |
|             | Human blood     | 1             | 1986                 | +                      | 80-kb virulence |
|             | Human blood     | 5             | 1986-1988            | +                      | 80-kb virulence |
|             | Unknown         | 1             | -                    | -                      | 80-kb virulence |
|             | Great Britain   | 3             | 1982?                | +                      | 80-kb virulence |

a Except as otherwise indicated, the isolates were recovered in France. 
b See Table 3 for the description of plasmid profiles. 
c —, unknown.

multilocus enzyme genotype Du 1 of S. dublin. The exceptional strain had a genotype unrelated to those of the ETs of S. dublin (data not shown).

Distribution of Vi antigen. All 30 isolates of Du 1 tested failed to hybridize with the ViaB-specific probe (Table 2), and for none of these isolates or those of Du 4 is there evidence of the expression of Vi antigen. In contrast, positive signals were detected from all 29 isolates of Du 3 in our sample that were hybridized with the ViaB-specific probe, and 23 of them had been serologically typed, at or near the time of their recovery, as Vi positive. Of the six remaining isolates of Du 3, two from France had earlier been typed as Vi negative, and one from France and three from Great Britain had been typed as Vi negative by us in 1990, several years after the time of their recovery (Table 2).

Plasmid profiles. In a survey of plasmid content in 56 strains of S. dublin from five continents, Helmuth et al. (18) found that 50% (89%) of them carried an 80-kb virulence plasmid (9, 23, 47, 50, 51). However, Beninger et al. (6) reported that a single Vi positive isolate of S. dublin from Great Britain lacked an 80-kb plasmid but, instead, carried a 60-kb plasmid.

To extend these observations, we determined the plasmid content of all 29 of our Du 3 strains and 30 isolates of Du 1 (Table 3). All but 1 of these 59 strains carried a large plasmid that, by comparison with plasmids of known size, was identified as the 80-kb virulence plasmid, and two strains of Du 1 had a 3-kb plasmid in addition to the 80-kb plasmid. Surprisingly, 25 of the 29 strains of Du 3 carried a medium-sized plasmid in addition to the 80-kb virulence plasmid. In 22 of these 26 Du 3 isolates, the medium-sized plasmid was 40 kb in size, but in 3 isolates it was 50 kb. Of the remaining four isolates of Du 3, one isolate (RKS 4732) had only a 40-kb plasmid, and three isolates carried only the 80-kb plasmid.

Because of the association of medium-sized plasmids with the Vi-antigen phenotype, plasmid DNA from a French isolate of Du 3 (RKS 4717), which had both 80-kb and 40-kb plasmids, and one Du 1 strain (RKS 4699) were tested by dot blot analysis with the ViaB-specific probe to determine whether the ViaB genes are plasmid borne. As a control, EcoRI-digested total DNA (plasmid and chromosomal) from both strains was tested with the probe in the same experiment. Hybridization with the ViaB probe was detected only in the EcoRI-digested total DNA of the Du 3 strain (data not shown), thus indicating that the ViaB region in this strain is located on the chromosome.

Nucleotide sequences of the phase 1 flagellin gene. The only serological distinction between the serovars S. dublin (1,9,12,g,p:–) and S. enteritidis (1,9,12,g,m:–) is the expression of phase 1 flagellar antigens g,p and g,m, respectively (12). To determine the molecular basis for this difference, we undertook a comparative study of the sequence of the fliC gene, which encodes phase 1 flagellin (41, 42). Variation in the sequence of the 1,518-nucleotide-coding region of the fliC gene among one isolate of S. enteritidis, three isolates of S. dublin, and an isolate of serotype 1,9,12,g,m,p:– is shown in Table 3. Only five (0.33%) of the nucleotide sites were polymorphic, and the maximum difference between pairs of sequences was 4 nucleotides (0.26%). At the five polymorphic sites, which occurred in five codons, two of the substitutions were synonymous and three were nonsynonymous. All three amino acid substitutions were located in a 99-codon segment (codons 220 to 318) in the central part of the gene.

The fliC sequences of the Du 1 strain and two Du 3 strains were identical and differed from the sequence of En 1 in having nonsynonymous substitutions in three codons (numbers 220, 315, and 318). The sequence of the Thailand strain shared codon GCG at position 318 with Du 1 and Du 3 and codons GCA and ACT at positions 220 and 315, respectively, with En 1; and, uniquely, it had synonymous substitutions in codons 60 and 138.

DISCUSSION

Genotypic variation and genetic structure of populations. The genetic structure of populations of S. dublin, like that of the salmonellae in general (4, 32, 37, 38), is clonal, as evidenced by the repeated recovery of isolates of the same multilocus enzyme genotype in widely separated geographic regions and over extended periods. Of the 117 isolates randomly selected from natural populations, 95% were representatives of a globally distributed clone, Du 1, and 3.4% were clone Du 3, which apparently is confined in distribution to France and Great Britain.

Nonmotile isolates of S. dublin. Franklin et al. (17) tentatively identified five nonmotile serotype 1,9,12:–:– isolates

TABLE 3. Plasmid profiles of 30 Du 1 and 29 Du 3 isolates of S. dublin

| Plasmid profile | Plasmid size (kb) | No. of isolates | ET | Geographic source(s) |
|-----------------|------------------|----------------|----|----------------------|
| Du 1            |                  |                | 28 | Du 1 (n = 27), Switzerland (n = 1) |
|                 |                  |                | 3  | Du 3 France          |
|                 |                  |                | 2  | Du 1 France, Israel  |
|                 |                  |                | 22 | Du 3 France          |
|                 |                  |                | 3  | Du 3 Great Britain   |
|                 |                  |                | 1  | Du 3 France          |
recovered from cattle in Sweden as *S. dublin* on the basis of the observation that they carried a plasmid that was identical in size and in *EcoRI* and *HindIII* restriction patterns to the plasmids of 21 normal (i.e., motile) strains of *S. dublin*. We have determined that almost all nonmotile 1,9,12:: isolates recovered from cattle and, occasionally, from swine in the United States are indistinguishable in multilocus enzyme genotype from *S. dublin* isolates of clone Du 1. Very rarely, such nonmotile isolates become motile when grown on broth (14a), but nothing is known of the biochemical or molecular genetic basis for the loss or gain of motility.

**Clonal distribution of Vi antigen.** The absence of the Vi antigen in serovars of *Salmonella* that are closely related to *S. dublin* and our discovery that the ViaB genes in *S. dublin* are confined to clone Du 3, which is restricted in distribution to France and Britain, strongly suggest that these genes were very recently acquired by *S. dublin* via horizontal transfer and additive recombination (36). The association of Vi antigen with only a minor segment of the total *S. dublin* population contrasts with the situation in both *S. typhi* and *S. paratyphi* C, in which virtually all isolates of all clones carry the ViaB genes and express Vi antigen (38). The distribution of the ViaB genes in *C. freundii* has not been well studied, but available data suggest that they occur in low frequency in natural populations (34a).

The presence of the ViaB region in several distantly related groups of bacteria is most easily explained by horizontal transfer (38), but neither the ultimate source of these genes nor the proximate source from which they were acquired by *S. dublin* is known.

**Possible role of Vi antigen in pathogenicity.** The name “virulence antigen” notwithstanding, a casual relationship of this polysaccharide to virulence has not been convincingly demonstrated, even for *S. typhi*, in which it has been extensively studied (12, 33). When tested in the mouse model, several Vi-positive strains of *S. dublin* have been found to be no more virulent than Vi-negative strains (6, 29). For the strains in our sample, we do not have the clinical information that would permit us to address the question of whether strains of Du 1 and Du 3 differ in the type or severity of infection caused in humans or animals.

**Evolutionary origin of *S. dublin*.** As shown in Table 1, the three ETSs of *S. dublin* are closely similar in multilocus enzyme genotype to ET En 1 of *S. enteritidis*, which was represented by 93% of the 257 isolates examined by Beltran et al. (4). Indeed, the only difference between Du 3 and En 1 is the occurrence of the 1 and 5 alleles, respectively, at the shikimate dehydrogenase locus (genetic distance, 0.04). This degree of genotypic differentiation is no greater than that among the clones of *S. dublin* or those of *S. enteritidis* or *S. typhimurium* (4, 5).

Further evidence of a close relationship between *S. dublin* and *S. enteritidis* is provided by the nucleotide sequences of their *flc* genes, which differ only by three nonsynonymous substitutions, all of which occur in a limited region of the central part of the gene. From a comparison of the sequence data shown in Table 4, it can be deduced that the presence of an alanine codon at position 318 is responsible for expression of the phase 1 flagellar epitope p in *S. dublin*. It is also apparent that the absence of the m epitope in *S. dublin* and its presence in *S. enteritidis* are attributable to substitution at one or both of two codon positions: valine versus alanine at position 220 and isoleucine versus threonine at position 315. The strain of serotype 1,9,12:g,m,p:: from Thailand shares the p epitope with *S. dublin* because of the presence of alanine at codon position 318 and shares the m epitope with *S. enteritidis* because of the presence of alanine and threonine at positions 220 and 315, respectively. Because all five isolates sequenced express the g epitope, its molecular genetic basis cannot be determined from the data presented in Table 4.

The virtual uniformity of the multilocus enzyme genotype and the identity of the *flc* gene sequences among isolates of *S. dublin* indicate that the clones of this serovar have had a recent origin from a common ancestral cell. That there is some variation among strains in fermentative and other biochemical properties (3, 52) is not incompatible with this conclusion, because biotype characters in the salmonellae may change very rapidly (2, 30, 31).

Given the close phylogenetic relationship between *S. dublin* and *S. enteritidis*, it is noteworthy that their virulence plasmids are quite different both in size and restriction pattern (31), although they are, to some extent, functionally interchangeable (6). It remains to be determined whether one or both of these plasmids experienced exceptionally rapid evolution in the course of divergence of these two serovars or whether one of the plasmids represents a replacement of the ancestral plasmid via horizontal transfer from another source.

Although *S. enteritidis* is most frequently associated with fowl, it has a broad host range that includes cattle and other mammals (27). Apart from minor changes in multilocus enzyme genotype, the evolutionary derivation of *S. dublin* from an *S. enteritidis*-like ancestor would have involved modification of the phase 1 flagellar antigen profile (loss of epitope m and acquisition of epitope p), changes in the structure of the virulence plasmid, and the development of a close association with cattle. Restriction to a narrow niche subsequently led to biochemical specialization, including auxotrophy for nicotinic acid (3, 16). It is problematical whether Du 3, which is marked by possession of the ViaB genes and the 3 allele at phosphoglu-
comutase, is also a recent localized derivative of Du 1 or a relict population of a clone that was formerly more widespread in distribution. In any event, it is not surprising that we found no variation in the flic sequences of these clones. Whereas variation in flic in S. dublin was assessed by sequencing the gene in only three isolates, recognition of clonal diversity was based on an assessment of allelic variation in 24 structural gene loci in 170 isolates by application of an electrophoretic technique that has the power to detect a large proportion of amino acid substitutions (36). There is also a source of bias here, inasmuch as strains possessing flic mutations that modify the flagellar antigen profile from g,p are likely to have been classified as serovars other than S. dublin and, hence, would not have been included in our study. A case in point is the serovar Salmonella rostock (1,9,12:g,p,u:-), which is identical in multilocus enzyme genotype to Du 1 and has a flic sequence differing from that of Du 1 by only a single nonsynonymous substitution (mediating the replacement of alanine by glycine at codon position 314), which is responsible for expression of the u flagellar epitope (26a). Maynard Smith (28) has recently interpreted a similar lack of sequence variation in a 250-bp central segment of the flic gene among five ETs of S. typhimurium (41, 42) as evidence of horizontal transfer and recombination of the gene, but in view of the considerations outlined above, this hypothesis is neither warranted nor necessary.

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