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Molecular Subtyping of *Salmonella enteritidis* Phage Type 8 Strains from the United States

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***Salmonella enteritidis* is now the most common serotype of the genus *Salmonella* reported in the United States. Bacteriophage typing has been helpful for subdividing *S. enteritidis* strains from different sources in the United States. Most *S. enteritidis* outbreaks reported were egg related, and the majority of them were caused by strains of phage type 8. To determine whether restriction fragment length polymorphism of the rRNA genes (ribotyping) and of the genomic DNAs from two lysogenic phages from *S. enteritidis* could be used to discriminate between *S. enteritidis* phage type 8 strains, we conducted Southern hybridization studies on 24 isolates from different outbreaks and six non-outbreak-associated strains using DNA probes for 16S and 23S rRNA genes and *S. enteritidis* typing phages 1 and 2 from the Ward typing system (L. R. Ward, J. D. H. de Sa, and B. Rowe, Epidemiol. Infect. 99:291–294, 1987). Of seven restriction endonucleases screened with the probe for rRNA genes, *Acc*I provided the best discrimination between strains; six distinct patterns were observed. *Acc*I ribosomal DNA patterns 1 to 6 were detected among 76.7, 3.3, 6.7, 3.3, 3.3, and 6.7% of isolates tested, respectively. Strains of *Acc*I ribosomal DNA pattern 3 could be further subdivided into two additional patterns by using *Sma*I. Epidemiologically related strains had identical patterns. No discrimination between strains was achieved by probes for phages 1 and 2. No sequences homologous to the phage I probe were detected among phage type 8 strains, and all strains tested with six restriction enzymes had the same hybridization pattern with the phage 2 probe. These findings demonstrate that ribotyping with *Acc*I and *Sma*I provides an additional means of discriminating between some phage type 8 strains; however, ribotyping and the phage 2 hybridization results from egg-related outbreak strains support previous findings that these strains are closely related.**

The isolation rate of *Salmonella enteritidis* (*Salmonella enterica* subsp. *enterica* serotype enteritidis) has been increasing in the last 10 years in the United States, particularly in the Northeast. In 1990 it surpassed *Salmonella typhimurium* to become the most common *Salmonella* serotype reported in the United States; 8,591 isolates from human sources and 3,158 isolates from nonhuman sources were reported to the Centers for Disease Control and Prevention (CDC) *Salmonella* Surveillance System; this represented 21.1 and 13.9%, respectively, of all *Salmonella* isolates reported from those sources in that year (4). In a recent study of 573 *S. enteritidis* strains from human, animal, food, and environmental sources, Hickman-Brenner and coworkers (6) reported that phage type 8 strains accounted for 48.2% of the strains tested. Results of phage typing performed at the National *Salmonella* Reference Laboratory (CDC) on isolates involved in outbreaks from 1986 through 1992 also showed that phage type 8 strains are the most common. Phage type 8 strains were isolated from 61.6% (130 of 211) of outbreaks studied during that period. Seventy-two percent (94 of 130) of these phage type 8 outbreaks were epidemiologically linked to contaminated eggs (unpublished data).

Epidemiologic studies of outbreaks of *S. enteritidis* phage type 8 infections have been hampered by the lack of adequate subtyping procedures. In a study of *S. enteritidis* phage type 8 strains from the United States, Stubbs et al. (13) found that most strains were identical by plasmid profile analysis, antibiotic susceptibility testing, biotyping, and additional phage typing systems. Recently, the characterization of restriction fragment length polymorphisms in the genes for rRNA (ribotyping) has been successfully applied to several *Salmonella* serotypes (3, 5, 7, 8). Almeida et al. (2) also successfully applied restriction fragment length polymorphism in prophage DNA to *Vibrio cholerae* isolates. In this report, we describe results of Southern hybridization studies that were done to determine whether ribotyping can be used to subdivide *S. enteritidis* phage type strains from the United States, and we examined these strains for restriction fragment length polymorphisms of genomic DNAs from two lysogenic phages from *S. enteritidis*.

MATERIALS AND METHODS

Bacterial strains. Included in the present study were 30 *S. enteritidis* phage type 8 strains that were previously analyzed by Stubbs et al. (13) for plasmid content, antibiotic susceptibility patterns, biochemical reactions, and lysis patterns by four different phage typing methods. The strains were biochemically and serologically characterized by standard microbiologic methods and were phage typed by the method of Ward et al. (14). The strains were isolated from 18 egg-related outbreaks in the northeastern, mid-Atlantic, and midwestern regions of the United States, two outbreaks in

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TABLE 1. Epidemiological characteristics and rDNA patterns of *S. enteritidis* phage type 8 strains

CDC strain no.	Outbreak code[a]	Geographic origin	Suspected vehicle	Source	rDNA pattern	
					*Acc*I	*Sma*I
C1451	88-001	Connecticut	Pureed diet with eggs	Human stool	2	1
C1454	88-002	Connecticut	Pureed diet with eggs	Human stool	1	1
C1824	88-005	Pennsylvania	Stuffed beef roll with eggs	Human stool	1	1
C1923	88-007	New Jersey	Scrambled eggs	Chicken egg	1	1
C1924	88-007	New Jersey	Scrambled eggs	Human stool	1	1
C1919	88-008	New Jersey	Homemade ice cream with eggs	Ice cream	1	1
C1920	88-008	New Jersey	Homemade ice cream with eggs	Ice cream	1	1
C1674	88-009	Maryland	Lasagna with eggs	Human stool	6	1
C1675	88-009	Maryland	Lasagna with eggs	Human stool	6	1
C1766	88-010	Virginia	Homemade ice cream with eggs	Human stool	1	1
C1752	88-011	Virginia	Homemade ice cream with eggs	Ice cream	1	1
C1755	88-011	Virginia	Homemade ice cream with eggs	Human stool	1	1
C1760	88-012	Virginia	Scrambled eggs	Human stool	3	3
C1759	88-014	Washington, D.C.	Eggs Benedict	Human stool	1	1
C1279	88-015	Maine	Pooled eggs	Human stool	1	1
C1814	88-016	Illinois	Homemade ice cream with eggs	Human stool	1	1
C1479	88-017	Illinois	Diced eggs	Chicken egg	5	4
C1910	88-018	New York	Hollandaise sauce with eggs	Human stool	1	1
D0083	89-002	Maryland	Scrambled eggs	Chicken egg	1	1
D0069	89-006	Tennessee	Hollandaise sauce with eggs	Human stool	1	1
D0214	89-010	Pennsylvania	Pureed dinner with eggs	Human stool	1	1
D0219	89-016	Pennsylvania	Potato filling with eggs	Human stool	1	1
D0116	1	Switzerland	Unknown	Human stool	1	1
D0149	2	Switzerland	Unknown	Human stool	1	1
D0010	—[b]	Pennsylvania	Unknown	Chicken egg	1	1
B1267	—	Ohio	Unknown	Human stool	1	1
B6660	—	North Carolina	Unknown	Chicken egg	3	2
B8509	—	Pennsylvania	Unknown	Chicken egg	1	1
B9967	—	Puerto Rico	Unknown	Swine	4	1
C0002	—	New York	Unknown	Giraffe	1	1
PT 8	[c]	England	Unknown	Human stool	1	1
PT 1	[d]	England	Unknown	Unknown	1	1
D1342	91-028[e]	Louisiana	Hamburger	Human stool	1	1
LT2	[f]				D[g]	D

[a] All the strains with the same outbreak code belong to the same outbreak.
[b] —, non-outbreak-related strain.
[c] *S. enteritidis* phage type 8 reference strain (L. Ward phage typing scheme [14]).
[d] *S. enteritidis* phage type 1 reference strain (L. Ward phage typing scheme [14]).
[e] *S. enteritidis* phage type 13a.
[f] *S. typhimurium* LT2.
[g] D, different rDNA pattern.

Switzerland, and six non-outbreak-associated sources. The geographic origins, suspected vehicles, and sources of the strains are given in Table 1. Also included were reference strains of *S. enteritidis* representing Ward phage types 1, 8, and 13a and *S. typhimurium* LT2. All strains were stored both frozen at −70°C and in nutrient agar stab cultures at room temperature. Strains were subcultured onto Trypticase soy agar (TSA; BBL Microbiology Systems, Cockeysville, Md.) or blood agar when needed.

Preparation of purified bacterial genomic DNA. Bacterial cells grown overnight on TSA were harvested in TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA). Whole-cell DNA was extracted with guanidium thiocyanate as described previously (9).

Preparation of purified phage DNA. Phages 1 and 2, kindly supplied by Linda Ward, Central Public Health Laboratory, London, England, were propagated on the *S. enteritidis* phage type 1 reference strain by an agar overlay method described by Adams (1). No phages from the strain used to propagate phages 1 and 2 were inducible by mitomycin C (0.5 μg/ml). Phage DNA was isolated and purified as described by Silhavy et al. (11) and was stored at 4°C until needed.

Preparation of DNA probes. *Acc*I-restricted DNAs from phages 1 and 2, lambda DNA, and 1-kb ladder DNA (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and cDNAs of 16S and 23S rRNAs from *Escherichia coli* (Boehringer Mannheim, Indianapolis, Ind.) were labeled by random priming with digoxigenin dUTP as described previously (10).

Bacterial and phage DNA restriction endonuclease digestion and Southern hybridization analysis. Purified DNAs from phages 1 and 2 were digested with *Acc*I, *Ava*I, *Bgl*II, *Bam*HI, *Cla*I, *Eco*RI, *Eco*RV, *Hin*fI, *Hin*dIII, *Mbo*I, *Pvu*II, *Sal*I, and *Sma*I (New England Biolabs, Inc., Beverly, Mass.), according to the manufacturer's instructions, to identify enzymes for hybridization analysis that would generate four or more restriction fragments. Total genomic DNAs from *S. enteritidis* isolates representing three outbreaks (isolates C1454, C1479, C1752, and C1755) and two non-outbreak-associated isolates (isolates B6660 and B9967) were digested with *Acc*I, *Ava*I, *Cla*I, *Eco*RI, *Eco*RV, and *Pvu*II to hybridize with the phage 1 probe and with *Acc*I,

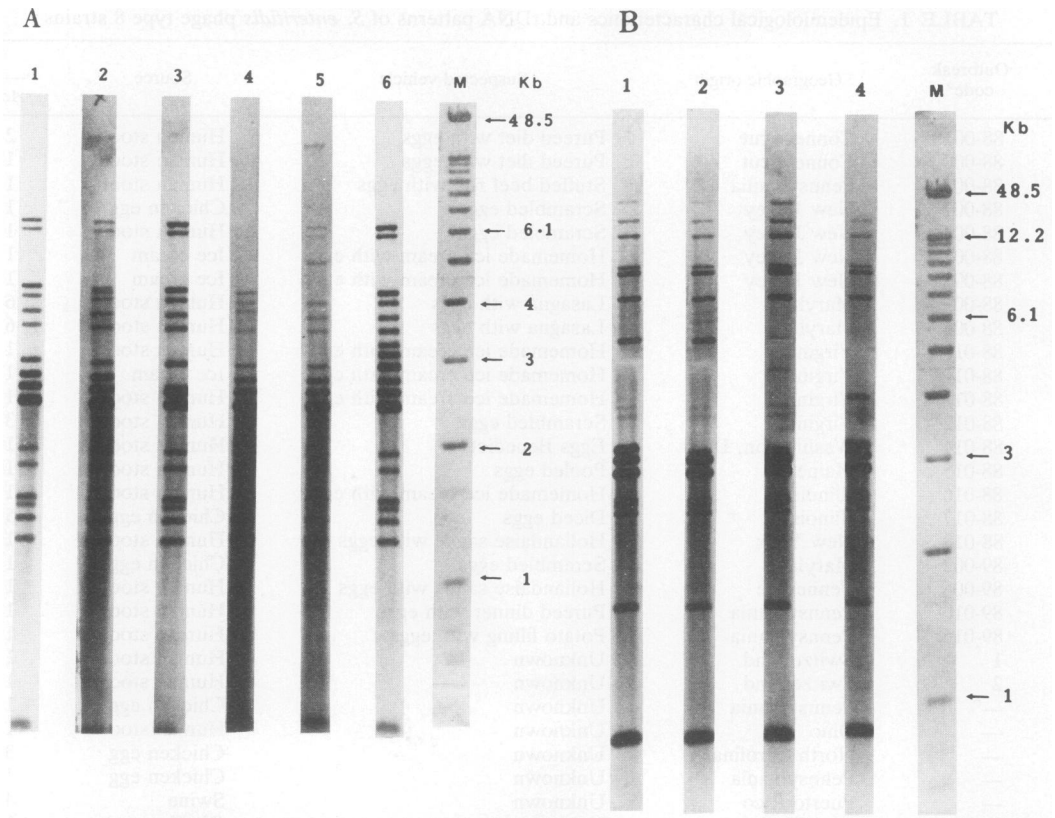

FIG. 1. (A) *Acc*I rDNA patterns. Lanes 1 through 6, patterns 1 through 6, respectively; lane M, 1-kb DNA ladder (GIBCO BRL). (B) *Sma*I rDNA patterns. Lanes 1 through 4, patterns 1 through 4, respectively; lane M, 1-kb DNA ladder (GIBCO BRL).

*Bgl*II, *Cla*I, *Eco*RV, *Hin*fI, and *Pvu*II to hybridize with the phage 2 probe. Restriction fragments were separated by electrophoresis on 0.8% horizontal agarose gels, stained with ethidium bromide, and photographed under UV light. The separated fragments were transferred to nylon membranes (Magnagraph; Micron Separation Inc., Westboro, Mass.) by the method of Southern (12). The membranes were then hybridized overnight at 68°C with digoxigenin-labeled probes, washed, and revealed by procedures described by Popovic et al. (10).

Total genomic DNAs from the six previously selected strains were digested with *Acc*I, *Cla*I, *Eco*RV, *Pvu*II, *Sma*I, *Sph*I, and *Stu*I and were hybridized with the probe for rRNA genes as described above to identify enzymes that would reveal the higher number of ribosomal DNA (rDNA) patterns.

To test the reproducibilities of the rDNA patterns obtained, all the strains were tested at least twice (a mean of 2.8 times), and strains C1923 and C1924 were subcultured 20 times (20 cycles of 12 h at 37°C in nutrient broth) and ribotyped.

RESULTS

Ribotyping. For five of the seven enzymes evaluated, the test strains had the same patterns or were not digested. After digestion with *Acc*I and *Sma*I, four and three different patterns, respectively, were detected among the test strains. A single difference in the numbers or sizes of bands hybridizing with the probe for rRNA genes was scored as a different rDNA pattern. From these preliminary findings, *Acc*I and *Sma*I were selected for ribotyping of the remaining strains listed in Table 1.

After *Acc*I digestion, six rDNA patterns, designated *Acc*I rDNA patterns 1 through 6, were detected among the 30 strains listed in Table 1. The number of fragments hybridizing with the probe for rRNA genes ranged from 16 to 19 (Fig. 1A), which is a high number of bands compared with those obtained from most ribotyping schemes. The high number of bands is probably because *Acc*I can cut multiple times within the 16S and 23S operon. *Acc*I rDNA pattern 1 was observed among 76.7% (23 of 30) of the strains tested. *Acc*I rDNA patterns 2 through 6 were detected among only 3.3% (1 of 30), 6.7% (2 of 30), 3.3% (1 of 30), 3.3% (1 of 30), and 6.7% (2 of 30) of the strains tested, respectively. *Acc*I rDNA pattern 1 was detected among strains from 14 outbreaks in the United States as well as among strains from 2 outbreaks in Switzerland. There were four outbreaks in which two isolates were included; those isolates from the same outbreak had the same pattern. Among the controls, phage type 8 strain and two strains with different phage types (phage types 1 and 13a) had *Acc*I rDNA pattern 1 and *S. typhimurium* LT2 had a completely different pattern (data not shown) (Table 1).

After *Sma*I digestion, four rDNA patterns, designated *Sma*I rDNA patterns 1 through 4, were detected among the 30 strains listed in Table 1. The number of fragments hybridizing with the probe for rRNA genes ranged from 15 to 16 (Fig. 1B). *Sma*I rDNA pattern 1 was detected among 90% (27 of 30) of the strains tested. *Sma*I rDNA patterns 2

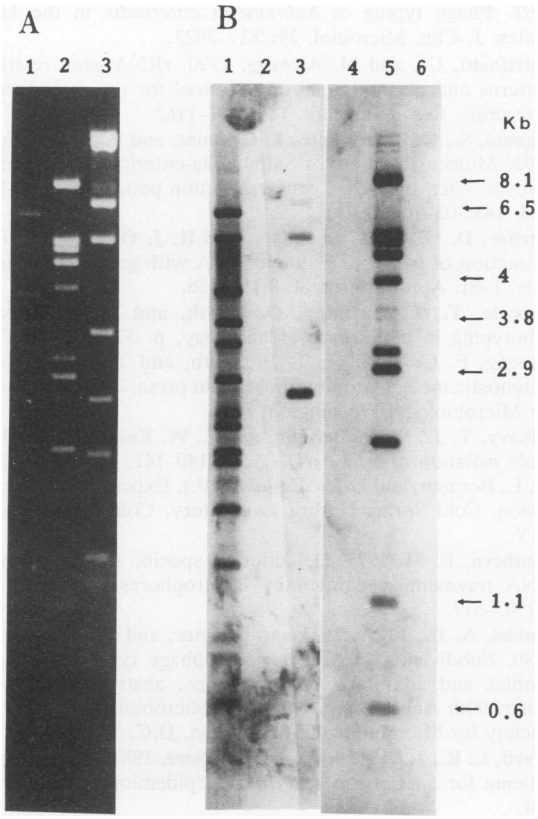

FIG. 2. (A) *Acc*I restriction analysis of DNAs from *S. enteritidis* phage 1 (lane 1), phage 2 (lane 2) (L. Ward phage typing scheme [14]), and bacteriophage lambda (lane 3). (B) Southern hybridization analysis of *Acc*I-restricted DNA from phage 1 (lane 1), phage 2 (lane 2), and bacteriophage lambda (lane 3) with phage 1 probe and from phage 1 (lane 4), phage 2 (lane 5), and bacteriophage lambda (lane 6) with phage 2 probe.

through 4 were each detected among 3.3% (1 of 30) of the strains (Table 1 and Fig. 2). Among the controls, the phage type 8 strain and two strains with different phage types (phage types 1 and 13a) had *Sma*I rDNA pattern 1, and *S. typhimurium* LT2 had a completely different pattern (data not shown) (Table 1).

The reproducibilities of the *Acc*I and *Sma*I rDNA patterns were demonstrated by the identities of the results every time each strain was tested, including three different subcultures (subcultures 10, 15, and 20) of strains C1923 and C1924.

Characterization and hybridization of phage 1 and phage 2 DNA probes. Phage 1 and phage 2 DNAs were unrelated by restriction endonuclease analysis and did not hybridize with each other under the conditions described above (Fig. 2). Phage 1 DNA hybridized to a limited extent with bacteriophage lambda DNA; phage 2 DNA did not. The reciprocal hybridization of lambda DNA to DNA from phages 1 and 2 was not performed. The estimated genomic sizes of phages 1 and 2 were 40 and 42 kb, respectively.

Of the enzymes screened for hybridization analysis with the phage probes, *Acc*I, *Ava*I, *Cla*I, *Eco*RI, *Eco*RV, and *Pvu*II were selected for testing with phage 1; *Acc*I, *Bgl*II, *Cla*I, *Eco*RV, *Hin*fI, and *Pvu*II were selected for testing with phage 2 because of their ability to produce potentially useful restriction patterns from the phage genome. For the six test strains digested with the enzymes listed above, the phage 1 probe hybridized only weakly or not at all. An additional 13 strains digested with *Acc*I were also tested by using the phage 1 probe, with similar results. Fragments carrying sequences homologous to the phage 2 sequence were detected in all six test strains digested with the enzymes specified above; however, no differences in restriction patterns among the test strains digested with these enzymes or an additional 13 strains digested with *Acc*I were observed.

DISCUSSION

Phages 1 and 2 were isolated directly from lysogenic strains of *S. enteritidis* by Ward et al. (14). Since neither of these phages lysed phage type 8 strains, we hypothesized that they may exist as prophages and could potentially serve as markers for subdividing strains on the basis of restriction fragment length polymorphisms within their genomes and within flanking sequences adjacent to their integration sites in the bacterial chromosome. For phage 1, the lack of detectable homologous sequences within *S. enteritidis* phage type 8 strains suggests that this phage is not lysogenic among these strains. Factors other than lysogenic immunity, such as the lack of a receptor(s) on the bacterium that is required for infection, are probably responsible for the failure of phage 1 to lyse these strains.

Restriction fragments homologous to phage 2 were detected among all strains tested, and the DNA patterns obtained with this phage were highly conserved between strains, which support the hypothesis that *S. enteritidis* phage type 8 strains from egg-related outbreaks in the United States are closely related.

The rDNA patterns for the seven enzymes studied showed a considerable amount of nucleotide sequence conservation. With the two enzymes that revealed polymorphisms (*Acc*I and *Sma*I), most rDNA patterns varied from each other by only a few fragments. Among the 30 strains tested, six *Acc*I and four *Sma*I rDNA patterns were observed. Using a combination of *Acc*I and *Sma*I for ribotyping, the U.S. phage type 8 strains could be subdivided into seven groups. Further studies with a greater number of isolates from different geographic areas and epidemiologic origins will probably give a higher number of patterns. *Sma*I was also reported by Martinetti and Altwegg (7) to be useful for subdividing *S. enteritidis* strains from Switzerland (phage types not specified); they reported two *Sma*I rDNA patterns (7) and observed the greatest discrimination among *S. enteritidis* strains with the enzyme *Sph*I; we did not evaluate these strains because of inconsistent restriction digests with that enzyme.

Eighteen of 20 outbreak-related strains included in the present study had only a single plasmid of approximately 55 kb (13). Strains with this plasmid pattern could be subdivided by ribotyping with *Acc*I into four groups; however, the majority of strains (15 of 20) had identical plasmid profiles and rDNA patterns, providing evidence for the similarities of these strains. Since most strains selected for the present study were isolated from egg-related outbreaks in the northeastern and mid-Atlantic regions of the United States, a study of *S. enteritidis* phage type 8 strains from different sources and geographic areas will be necessary to evaluate the extent to which these related strains have spread to other areas in the United States. Interestingly, two phage type 8 strains from outbreaks in Switzerland had the same rDNA patterns and plasmid profiles (13) as the predominant out-

break-related strains in the United States, suggesting that the distribution of these related strains may be widespread.

We conclude that ribotyping with *Acc*I and *Sma*I is a useful and reproducible method for discriminating between *S. enteritidis* phage type 8 strains and should facilitate epidemiologic studies to assess the relatedness of these strains from the United States and other countries.

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