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Clonal Relationships among *Shigella* Serotypes Suggested by Cryptic Flagellin Gene Polymorphism

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The presence of cryptic *fliC* alleles in the genomes of 120 strains representative of the four *Shigella* species was investigated. One fragment was obtained by PCR amplification of *fliC*, with a size varying from 1.2 to 3.2 kbp, depending on the species or serotype. After digestion with endonuclease *Hha*I, the number of fragments in patterns varied from three to nine, with sizes of between 115 and 1,020 bp. Patterns sharing most of their bands were grouped to constitute an F type. A total of 17 different F types were obtained from all strains included in this study. A unique pattern was observed for each the following serotypes: *Shigella dysenteriae* 1, 2, 8, and 10 and *S. boydii* 7, 13, 15, 16, and 17. On the contrary, *S. dysenteriae* serotype 13 and *S. sonnei* biotype e were each subdivided into two different F types. *S. flexneri* serotypes 3a and X could be distinguished from the cluster containing *S. flexneri* serotypes 1 to 5 and Y. *S. flexneri* serotype 6 clustered with *S. boydii* serotypes 1, 2, 3, 4, 6, 8, 10, 11, 14, and 18 and *S. dysenteriae* serotypes 4, 5, 6, 7, 9, 11, and 12. Two other clusters were outlined: one comprising *S. dysenteriae* serotypes 3, 12, 13 (strain CDC598-77), 14, and 15 and the other one joining *S. boydii* serotypes 5 and 9. None of the 17 *fliC* patterns was found in the *fliC Hha*I pattern database previously described for *Escherichia coli*. Overall, this work supports the hypothesis that *Shigella* evolved from different ancestral strains of *E. coli*. Moreover, the method outlined here is a promising tool for the identification of some clinically important *Shigella* strains as well as for confirmation of atypical isolates as *Shigella* spp.

Shigella species and serotypes, except for *Shigella boydii* serotype 13, show more than 73% DNA relatedness to *Escherichia coli* K-12 (4). Because of this close genetic relationship, *Shigella* strains can be considered *E. coli* clones which are much less biochemically active, are host restricted, and carry a plasmid encoding invasiveness. However, for historical reasons, nonmotile, anaerogenic *Enterobacteriaceae* causing dysentery are classified in the genus *Shigella*, which comprises four species: *S. dysenteriae, S. flexneri, S. boydii*, and *S. sonnei* (11).

Since *Shigella* strains produce neither flagella nor capsular antigens, their antigenic characterization relies exclusively on the properties of their somatic antigens (O antigens) (3, 17). *S. dysenteriae, S. boydii*, and *S. flexneri* have been subdivided into 15, 18, and 6 serotypes, respectively. *S. flexneri* contains two additional variants (X and Y), and serotypes 1 to 5 are further subdivided into subserotypes. Only one serotype has been described for *S. sonnei*, but strains of this species can be divided into five biotypes.

In contrast to *Shigella* strains, *E. coli* strains are often motile and produce a structural complex flagellum consisting of three main structural regions: the basal body, the hook, and the filament (22, 23). The flagellar filament is composed of many thousands of copies of a single protein subunit, flagellin (18), which carries antigenic determinants of the H (flagellar) antigen. The variability of the H antigen is associated with the flagellin amino acid sequence and its structural gene (*fliC*). The N- and C-terminal portions of flagellin are important for the structure of flagella and are highly conserved (25). The middle region can be quite variable and contains portions of the protein that are surface exposed and H type specific.

Several sequences of the gene encoding flagellin (*fliC*) are now available (33), allowing restriction of amplified *fliC* genes to be used for the identification of H types in different bacteria (1, 2, 9, 20, 36, 37).

Recently, Machado et al. (21) demonstrated that *Hha*I restriction of the amplified *fliC* gene could be used for a flagellar identification system fitting all *E. coli* serovars. The correlation between F types (*fliC*-RFLP types) and H types allowed the deduction of H types from F types. Furthermore, F types of nonmotile isolates could be identified. Actually, nonmotile strains generally possess the structural gene but are unable to build up a functional flagellum (23). In another study, it was found that all *E. coli* O157:NM strains producing Shiga toxin carried the gene encoding H7 (12, 13).

Although *Shigella* has long been described as a nonflagellated organism, intact, but cryptic, flagellin genes have been detected for *S. flexneri* and *S. sonnei* strains (35). Furthermore, the production of flagella by prototypic strains of all four *Shigella* species has been demonstrated by electron microscopy (14).

The purposes of this work were to (i) detect the cryptic flagellar gene in all *Shigella* serotypes, (ii) report on the distribution of flagellar restriction patterns among serotypes, and (iii) compare these patterns with those of *E. coli* serotypes.

MATERIALS AND METHODS

Bacterial strains. A total of 120 reference, collection, or clinical strains representing all recognized *Shigella* serotypes and biotypes were included in this study (Table 1).

Amplification of the *fliC* gene and restriction of PCR products. Cell lysis and DNA extraction were done as previously described (16). The amplification of flagellin genes by PCR, digestion of the amplified product with endonuclease *Hha*I, and electrophoresis for fragment separation were performed following the

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TABLE 1. List of strains and their F types

F type	Size of the amplified fragment (kbp)	Species and serotype or biotype	Strain[a]
P1	2.3	*Shigella dysenteriae* 1	NCDC1007-71
		Shigella dysenteriae 1	UE 31127
		Shigella dysenteriae 1	3116-95 EK1
		Shigella dysenteriae 1	3125-5 EK1
		Shigella dysenteriae 1	3090-2 EK1
		Shigella dysenteriae 1	3095-10 EK1
P2	2.2	*Shigella dysenteriae* 2	NCDC4106-65
		Shigella dysenteriae 2	UE 9-88
P3	1.6	*Shigella dysenteriae* 3	NCDC3690-75
		Shigella dysenteriae 3	UE 13-88
		Shigella dysenteriae 3	UE 30-86
		Shigella dysenteriae 3	UE 60-85
		Shigella dysenteriae 3	Polska 38-4064
		Shigella dysenteriae 12	UE 3-89
		Shigella dysenteriae 12	UE 40-85
		Shigella dysenteriae 13	CDC598-77
		Shigella dysenteriae 14	CDC576-83 (E22383)
		Shigella dysenteriae 15	CDCE23507
P4	1.6	*Shigella dysenteriae* 4	CIP 52-30
		Shigella dysenteriae 4	CIP 67-59
		Shigella dysenteriae 4	UE 21-87
		Shigella dysenteriae 4	UE 29-90
		Shigella dysenteriae 4	UE 40-89
		Shigella dysenteriae 5	NCDC853-58
		Shigella dysenteriae 5	CIP 57-42
		Shigella dysenteriae 5	CIP 58-26
		Shigella dysenteriae 6	CIP 52-32
		Shigella dysenteriae 6	UE 16-89
		Shigella dysenteriae 7	NCDC4788-55
		Shigella dysenteriae 7	CIP 67-60
		Shigella dysenteriae 7	CIP 52-123
		Shigella dysenteriae 7	UE 6-87
		Shigella dysenteriae 9	NCDC2860-74
		Shigella dysenteriae 11	NCDC3873-50
		Shigella dysenteriae 12	NCDC3341-55
		Shigella boydii 1	NCDC6310-65
		Shigella boydii 1	UE 47-90
		Shigella boydii 1	UE 52-89
		Shigella boydii 2	NCDC2854-67
		Shigella boydii 2	UE 2-87
		Shigella boydii 2	UE 51-89
		Shigella boydii 3	NCDC67
		Shigella boydii 3	CIP 52-50
		Shigella boydii 3	UE 13-87
		Shigella boydii 4	NCDC871-74
		Shigella boydii 4	UE 42-89
		Shigella boydii 6	NCDC3467-56
		Shigella boydii 6	UE 2162 (Ljublian)
		Shigella boydii 8	NCDC3073-50
		Shigella boydii 8	UE 14-88
		Shigella boydii 10	NCDC05-74
		Shigella boydii 10	UE 41-89
		Shigella boydii 11	UE 24-90
		Shigella boydii 14	NCDC2770-51
		Shigella boydii 14	UE 18-86
		Shigella boydii 14	UE 44-89
		Shigella boydii 18	Ewing 10163
		Shigella boydii 18	UE 20-87
		Shigella boydii 18	UE 42-90
		Shigella boydii 18	UE 98-8059
		Shigella flexneri 6	NCDC2924-71
P5	1.5	*Shigella dysenteriae* 8	NCDC599-52
		Shigella dysenteriae 8	CIP 53-134
		Shigella dysenteriae 8	UE 9-86
P6	1.6	*Shigella dysenteriae* 10	NCDC2050-52
		Shigella dysenteriae 10	CIP 58-27
		Shigella dysenteriae 10	CIP 58-28
P7	2.9	*Shigella dysenteriae* 13	CDC2489-78
P8	1.6	*Shigella boydii* 5	NCDC5393-79
		Shigella boydii 5	UE 88-10
		Shigella boydii 9	NCDC73
		Shigella boydii 9	UE 17-87
P9	2.5	*Shigella boydii* 7	NCDC2954-72
		Shigella boydii 7	UE 52-54
P10	1.6	*Shigella boydii* 12	UE 38-87
		Shigella boydii 12	UE 98-0297
		Shigella flexneri 1	NCDC1921-71
		Shigella flexneri 1	CIP 52-37
		Shigella flexneri 1a	UE London Sc529
		Shigella flexneri 1b	NCDC4173-66
		Shigella flexneri 2	UE 140-80
		Shigella flexneri 2	UE 324-89
		Shigella flexneri 2a	NCDC2747-71
		Shigella flexneri 2b	NCDC97-68
		Shigella flexneri 3	UE 137-80
		Shigella flexneri 3b	NCDC2146
		Shigella flexneri 3c	NCDC2479
		Shigella flexneri 4	UE 89-80
		Shigella flexneri 4	UE 303-89
		Shigella flexneri 4a	NCDC6603-63
		Shigella flexneri 4b	NCDC1242
		Shigella flexneri 5	NCDC6154-61
		Shigella flexneri 5a	NCDC1170-74
		Shigella flexneri 5b	UE Sc541
		Shigella flexneri Y	NTCC 4839
P11	1.2	*Shigella boydii* 13	NCDC1610-55
		Shigella boydii 13	UE London SL624
P12	1.9	*Shigella boydii* 15	NCDC965-58
		Shigella boydii 15	CIP 58-19
		Shigella boydii 15	UE Dakar80
P13	3.2	*Shigella boydii* 16	NCDC3610-54
P14	3.2	*Shigella boydii* 17	Ewing 3615-53
P15	1.6	*Shigella flexneri* 3a	NCDC2783-71
		Shigella flexneri X	UE London 667
P16	1.5	*Shigella sonnei* a	UE 6-86
		Shigella sonnei a	UE 11359
		Shigella sonnei a	UE 11362
		Shigella sonnei a	UE 11363
		Shigella sonnei a	UE 98-8725
		Shigella sonnei d	CIP 66-4
		Shigella sonnei d	UE 60-85
		Shigella sonnei e	UE 238-87
		Shigella sonnei f	UE 68-89
		Shigella sonnei f	UE 155-87
		Shigella sonnei g	UE 23-88
		Shigella sonnei g	UE 250-88
		Shigella sonnei g	UE 285-89
P17	1.7	*Shigella sonnei* e	UE 55-87
		Shigella sonnei e	UE 94-2558
NA[b]	—[c]	*Shigella boydii* 12	NCDC266-59

[a] NCDC, National Centers for Disease Control, Atlanta, Ga.; CIP, Collection de l'Institut Pasteur, Institut Pasteur, Paris, France; UE, Unité des Entérobactéries, Institut Pasteur.

[b] NA, not applicable.

[c] —, PCR negative.

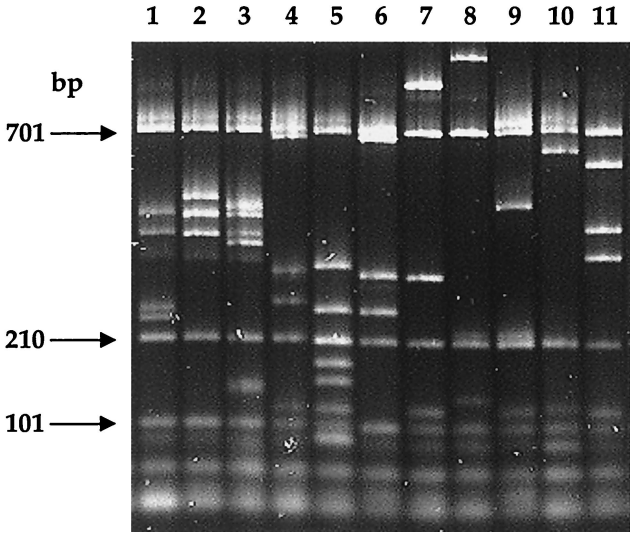

FIG. 1. Restriction length polymorphisms of the *Shigella fliC* gene after *Hha*I digestion. Lane 1, F type P4; lane 2, F type P3; lane 3, F type P7; lane 4, F type P10; lane 5, F type P5; lane 6, F type P8; lane 7, F type P15; lane 8, F type P16; lane 9, F type P12; lane 10, F type P11; lane 11, F type P6. Arrows indicate internal marker fragments.

protocol of Machado et al. (21). Three DNA fragments (101, 210, and 701 bp) obtained by *Hha*I restriction of the amplified *fliC* gene from *E. coli* O4:H5:K3 (strain U4/41) were added to all gel lanes and used as internal fragment size standards (21). Gels were stained with ethidium bromide, and images of band patterns were electronically captured using a charge-coupled device video camera interfaced to a microcomputer (Genomic, Collonges-sous-Saleve, France). Tagged image file format (TIFF) images were transferred to a Macintosh computer (Apple Computers, Cupertino, Calif.) for further analysis.

DNA fragment size determination. The Taxotron package (Taxolab; Institut Pasteur, Paris, France) was used for searching lanes and bands in the TIFF images and for interpretation of patterns (6).

The algorithm of Schaffer and Sederoff (32) was used to derive an experimental function relating molecular size to electrophoretic migration distances of the standard fragments and to interpolate the sizes of the other restriction fragments.

In pattern comparisons, the percentage of tolerated variation (allowed error) was set to 3.5% for fragment size, indicating that two fragments were considered identical when their sizes did not differ by more than this percentage. Fragments of less than 100 bp were discarded, since error was larger in this range.

Comparison of *Shigella* restriction patterns against a previously published *E. coli* database. The *Hha*I restriction patterns of *fliC* (F types) obtained for *Shigella* in this study were compared with the 62 previously published patterns for *E. coli* (21).

RESULTS

One fragment was obtained by PCR amplification of *fliC* from each strain tested, except for the reference strain *S. boydii* serotype 12 NCDC266-59, which failed to give a PCR product. The size of the amplified *fliC* fragment varied from 1.2 to 3.2 kbp (Table 1).

The number of bands in patterns varied from three to nine, with sizes of between 115 and 1,020 bp (Fig. 1 and 2). Patterns sharing most of their bands were grouped to constitute an F type. Seventeen different F types were obtained from all strains. Some F types contained variable bands that were not always present, depending on the strain (bands represented as dotted

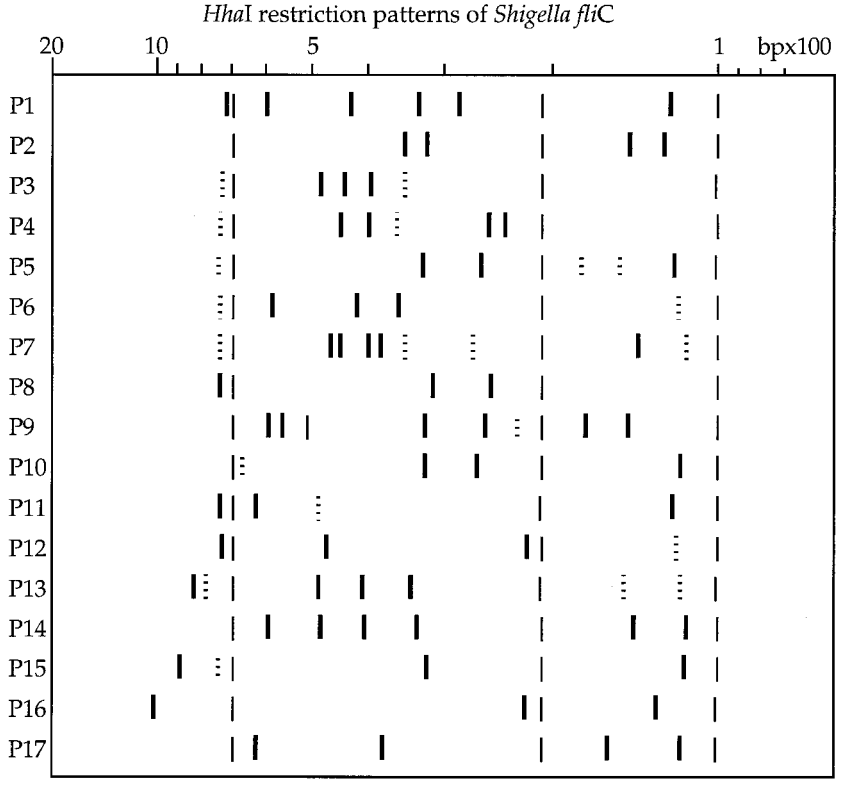

FIG. 2. Schematic representation showing the different *Shigella* F types. Dotted lines indicate variable bands that were not always present, depending on the strain. Thin lines indicate internal marker bands (101, 210, and 701 bp).

lines in Fig. 2). Common bands in each F type (thick lines in Fig. 2) allowed F type identification.

A unique F type was observed for each of the following serotypes: *S. dysenteriae* 1, 2, 8, and 10 and *S. boydii* 7, 13, 15, 16, and 17. On the contrary, *S. dysenteriae* serotype 13 and *S. sonnei* biotype e were each subdivided into two different F types. *S. flexneri* serotypes 3a and X could be distinguished from the cluster containing *S. flexneri* serotypes 1 to 5 and Y. *S. flexneri* serotype 6 clustered with *S. boydii* serotypes 1, 2, 3, 4, 6, 8, 10, 11, 14, and 18 and *S. dysenteriae* serotypes 4, 5, 6, 7, 9, 11, and 12. Two other clusters were outlined: one comprising *S. dysenteriae* serotypes 3, 12, 13 (strain CDC598-77), 14, and 15 and the other one joining *S. boydii* serotypes 5 and 9 (Table 1).

None of the patterns in the present study had been described in the previously published database containing 62 F types of *E. coli* (21).

DISCUSSION

The notion that flagellar antigens could be an indicator of ancient evolutionary divergences was first suggested by Ørskov et al. (27) to explain the two H types detected among strains of nine enteropathogenic serotypes of *E. coli* from different origins. In the present study, cryptic *fliC* was found to be highly conserved among *Shigella* species, revealing the clonal structure of this genus. *Shigella* clusters with the same F types are described here, and these data are in agreement with the results of previous studies.

A previous analysis of esterase electrophoretic polymorphisms distinguished five clusters among *Shigella* serotypes: (i) *S. dysenteriae* serotype 1, (ii) *S. flexneri* serotypes 1 to 5, (iii) *S. flexneri* serotype 6 and *S. boydii* serotypes 2 and 4, (iv) *S. sonnei*, and (v) *S. boydii* serotype 13; these clusters were closely related to those of *E. coli* (15). These data are in accordance with the results of the present study, except for the close relationship of these clusters to those of *E. coli*, which was not observed with the method used here.

Clusters of *Shigella* have been distinguished by ribotyping. In an earlier study, Rolland et al. (31), using a ribotyping system based on restriction with two endonucleases (*Eco*RI and *Hin*dIII), showed that *S. sonnei* and *S. flexneri* were easily distinguishable from *S. boydii* and *S. dysenteriae*, whereas distinction between *S. boydii* and *S. dysenteriae* was less clear. Strains of *S. boydii* serotype 13, which belong to a discrete DNA hybridization group (5), were clearly distinguished from the other *Shigella* strains. Rolland et al. (31) could not demonstrate the close taxonomic relationship between *S. flexneri* serotype 6 strains and *S. boydii* strains, but this association was proven on the basis of antigenic, chemical, and genetic data (10, 29, 30, 34). Another ribotyping system based on restriction with endonuclease *Mlu*I was proposed (8). Results were correlated to serotyping results and, in many cases, the serotypes of clinical strains could be predicted from their ribotypes. Again, some clusters of serotypes were observed within each species. Moreover, *S. flexneri* serotype 3 and *S. boydii* serotype 12 had the same ribotype.

Serotyping (28), biotyping (24), and isoenzyme analysis (26) suggested that *S. sonnei* is genetically homogeneous. However, distinct clones within *S. sonnei* have been revealed by more discriminating molecular biology techniques (19, 31). Strains UE 94-2558 and UE 55-87 of *S. sonnei* biotype e had the same *Mlu*I ribotype (8). That pattern did not correspond to the one expected for *S. sonnei*. These two strains were also determined to be different from other *S. sonnei* strains by *Mbo*II restriction of the amplified chromosomal region coding for the enzymes responsible for O-antigen synthesis (*rfb*-RFLP technique) (7).

The results of this work support the hypothesis that there was no single primordial *Shigella* ancestral strain (15). Groups of *Shigella* serotypes may represent different lineages.

However, some clinically important serotypes had unique F types (Table 1), e.g., *S. dysenteriae* serotype 1. In practical terms, analysis of the *Hha*I restriction patterns of *fliC* is a promising tool for the identification of some clinically important *Shigella* strains as well as for confirmation of atypical isolates as *Shigella* spp.

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