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Molecular Characterization of the Gene Encoding H Antigen in *Escherichia coli* and Development of a PCR-Restriction Fragment Length Polymorphism Test for Identification of *E. coli* O157:H7 and O157:NM

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Recent outbreaks of disease caused by *Escherichia coli* O157:H7 have focused much attention on this newly emerged pathogen. Identification of the H7 flagellar antigen is critical for the confirmation of *E. coli* O157:H7; however, clinical isolates are frequently nonmotile and do not produce detectable H antigen. To further characterize nonmotile isolates (designated NM), we developed a PCR-restriction fragment length polymorphism (PCR-RFLP) test to identify and characterize the gene encoding the H antigen (*fliC*) in *E. coli*. The entire coding sequence of *fliC* was amplified by PCR, the amplicon was restricted with *Rsa*I, and the restriction fragment pattern was examined after gel electrophoresis. Two hundred eighty *E. coli* isolates representing serotypes O157:H7 and O157:NM, flagellar antigen H7 groups associated with other O serogroups, and all other flagellar antigen groups were analyzed. A single restriction pattern (pattern A) was identified for O157:H7 isolates, O157:NM isolates that produced Shiga toxin (formerly Shiga-like toxin or verotoxin), and 16 of 18 O55:H7 isolates. Flagellar antigen group H7 isolates of non-O157 serotypes had one of three banding patterns distinct from pattern A. A wide variety of patterns were found among isolates of the other 52 flagellar antigen groups; however, none was identical to the O157:H7 pattern. Thirteen of 15 nonmotile strains that did not produce the A pattern had patterns that matched those of other known H groups. The PCR-RFLP in conjunction with O serogroup determination will be useful in identifying *E. coli* O157:H7 and related strains that do not express immunoreactive H antigen and could be expanded to include other clinically important *E. coli* strains.

Escherichia coli O157:H7 is the causative agent of intestinal disorders ranging from mild infection to severe, bloody diarrhea (hemorrhagic colitis). Complications of infection include hemolytic-uremic syndrome and thrombotic thrombocytopenic purpura. Most isolates associated with these syndromes have been shown to produce Shiga toxins (Stx1, Stx2, or both; formerly referred to as SLTI and SLTII or verotoxins 1 and 2, respectively [6]). Other serotypes of *E. coli* can produce similar toxins, but serotype O157:H7 has been more commonly linked to epidemic and sporadic disease than has any other serotype (13).

Many clinical laboratories now routinely screen stools for this pathogen (5), and there has been a resultant increase in the identification of the organism in stools from patients with bloody and nonbloody diarrhea. *E. coli* O157:H7 is unlike more than 90% of *E. coli* isolates in that most strains ferment sorbitol slowly or not at all. This phenotypic characteristic, along with serogroup O157 identification, is used to presumptively identify *E. coli* O157:H7. Positive identification is made by biochemical confirmation of isolates as *E. coli* and detection of the H7 flagellar antigen by agglutination with H7-specific antiserum. Isolates can be further characterized by detection of stx_1 or stx_2 (formerly *slt*-I and *slt*-II, respectively), the genes encoding Stx1 and Stx2, respectively (6). Additionally, most *E. coli* O157:H7 isolates are deficient in the enzyme β-glucuronidase but possess the gene, *uidA*, that encodes it. *E. coli* O157:H7 isolates can be further characterized by testing for this enzyme (12) or for a polymorphism in *uidA* that appears to be unique to *E. coli* O157:H7 (8, 10).

Some clinical isolates do not fit this textbook definition of *E. coli* O157:H7. In Germany and other parts of Europe, *E. coli* O157 isolates associated with enterohemorrhagic colitis and hemolytic-uremic syndrome fermented sorbitol, produced β-glucuronidase, and were nonmotile (4, 14, 17). A β-glucuronidase-producing strain of *E. coli* O157:H7 was isolated in the United States in 1994 (15). Clinical isolates are sometimes nonmotile or are motile but do not react with the typing sera (termed H undetermined or Hund), making the determination of H flagellar antigen impossible. As a reference laboratory that receives predominantly atypical isolates, the Centers for Disease Control and Prevention has seen O157:NM (NM indicates nonmotile) isolates increase from 6% of the total Stx^+ isolates in the early 1990s to 47% in 1996.

Flagellar antigen group H7 is one of 53 flagellar antigen groups described for *E. coli* (9). The variability of the H antigen is found within the flagellar filament, which is a polymer of a single protein, flagellin, the product of the *fliC* gene (16, 19). The N-terminal and C-terminal portions of flagellin are critical for the structure of the flagella and are highly conserved (22). The middle region encodes surface-exposed and antigenically

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TABLE 1. Summary of strains tested and *fliC* RFLP patterns

Group[a]	No. of isolates tested	No. of isolates with the following RFLP pattern[b]:				
		A	B	C	D	Other
O157:H7 (70)						
Stx$^-$	2	2				
Stx1	2	2				
Stx2	12	12				
Stx1 Stx2	54	54				
O157:NM (58)						
Stx$^-$	12	3				9
Stx1	2	2				
Stx2	19	19				
Stx1 Stx2	25	25				
O157:Hund (5)						
Stx$^-$	2					2
Stx1 Stx2	3	3				
Other H7 (42)						
O1:H7	6			5	1	
O18:H7	5			5		
O19:H7	1		1			
O27:H7	2		2			
O50:H7	5			5		
O55:H7	18	16	2			
O128:H7	4		4			
O153:H7	1		1			
Other H group isolates	101					101
Other nonmotile isolates	4					4

[a] Numbers in parentheses are the total number of isolates in each group.
[b] A to D refer to the 4 RFLP patterns observed for flagellar antigen H7. Other refers to the 57 RFLP patterns observed for the remaining 52 flagellar groups.

variable portions of the protein and can be quite variable. In *E. coli*, flagellar filaments have been subdivided into morphotypes based on the surface structure of flagella observed by electron microscopy (20). Flagellar antigen group H7 belongs to morphotype E along with flagellar groups H1, H12, H23, H45, H49, and H51. Flagella from these strains have looped edges suggestive of a helical sheath (20). A common surface structure for morphotype E strains suggests the possibility that they possess sequence similarities not found in other morphotypes.

Because the 5′ and 3′ ends of genes encoding flagellins are highly conserved, they are good targets for generic PCR. The conserved sequences at each end of the gene allow for the amplification of a wide range of alleles with a single set of primers. This approach has been used to characterize flagellin genes from other bacterial species (1, 2, 18, 29). To further characterize nonmotile O157 isolates, a PCR-restriction fragment length polymorphism (PCR-RFLP) test that detects and characterizes *fliC* was developed. A unique RFLP pattern that can be used to characterize nonmotile *E. coli* O157 isolates was identified for *fliC* from *E. coli* serotypes O157:H7 and O55:H7. RFLP pattern matches between nonmotile strains and other H antigenic groups were also seen, indicating that this approach may be useful for the identification of other clinically important *E. coli*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A panel of 280 *E. coli* strains were used in this study, and their characteristics are summarized in Table 1. The Stx$^+$ O157:H7 and O157:NM strains were isolated between 1983 and 1996 and were primarily from the United States; however, 11 isolates from Canada, Europe, or South Korea were tested. Most of the H standard flagellar antigen type strains (9) were included in the panel, and when only one isolate of an H flagellar antigen type was tested, it was the standard strain. All strains except two *E. coli* O157:NM strains from Germany (17) were from the collection of the Foodborne and Diarrheal Diseases Branch, Centers for Disease Control and Prevention. Bacteria were grown on Trypticase soy agar overnight at 37°C for DNA purification.

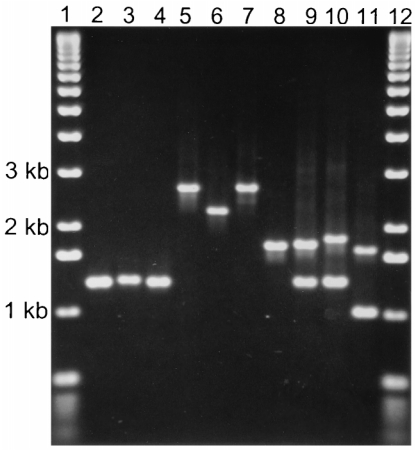

FIG. 1. Representative PCR fragments from the *fliC* PCR. Lanes 1 and 12, 1-kb ladder (Gibco BRL, Gaithersburg, Md.); lane 2, U40-41 (O4:H5); lane 3, HW31 (O133:H29); lane 4, SN3N/1 (O?:H56); lane 5, 5017-53 (O86:H36); lane 6, E408/68 (O148:H53); lane 7, E223/69 (O161:H54); lane 8, 3186-92 (O157:H7); lane 9, TX1 (O78:H12); lane 10, 669-58 (O?:H51); lane 11, 781-55 (O3:H44).

DNA manipulations. DNA was purified by using the Puregene Kit (Gentra Systems, Minneapolis, Minn.) according to the instructions of the manufacturer, except that approximately 10^9 organisms scraped from an agar plate were used in place of an overnight broth culture. The purified DNA was suspended in 300 μl of water, and the mixture was diluted 1 to 10 for use in the PCR.

***fliC* PCR-RFLP.** The entire coding sequence of the *fliC* gene was amplified by PCR with the primers F-FLIC1 (5′-ATG GCA CAA GTC ATT AAT ACC CAA C-3′) and R-FLIC2 (5′-CTA ACC CTG CAG CAG AGA CA-3′), which are minor modifications of the primers reported by Schoenhals and Whitfield (25). *Taq* polymerase (Perkin-Elmer, Foster City, Calif.) was incubated with TaqStart antibody (Clonetech, Palo Alto, Calif.) according to the specifications of the manufacturer before addition to the reaction mixture. A 100-μl reaction mixture contained 2 μl of template DNA (approximately 50 ng), 1 μM (each) primer, 200 μM (each) deoxyribonucleotide triphosphate, 10 μl of 10× PCR Buffer (Perkin-Elmer), 2.5 U of *Taq* polymerase, and 2.5 U of TaqStart antibody. The standard cycling conditions were 30 s at 95°C, 1 min at 60°C, and 2 min at 72°C for 35 cycles. For some strains, the annealing temperature was reduced to 40 to 55°C to increase the amount of product that was amplified. The PCR products were purified on a Sepharose CL-6B (Pharmacia Biotech, Inc., Piscataway, N.J.) column as described previously (23). Approximately 15 μl of the PCR mixture was then digested with *Rsa*I (Promega, Madison, Wis.) according to the manufacturer's instructions. The restriction fragments were separated on a 3.2% Nu-Sieve 3:1 agarose or 3.0% MetaPhor agarose gel (FMC BioProducts, Rockland, Maine). The other restriction enzymes tested were used according to the instructions of the manufacturer. The BioImage pattern matching image analysis program (BioImage, Ann Arbor, Mich.) was used to help identify related RFLP patterns.

RESULTS

PCR amplification of *fliC*. The TaqStart antibody was used to increase specificity by preventing the occasional amplification of a nonspecific band of about 0.45 kb. Most *E. coli* strains tested produced a single band between approximately 1.3 and 2.6 kb (Fig. 1); the exceptions were one of four H12 isolates (TX1, serotype O78:H12 [26]), all three isolates with flagellar antigen H51, and the one H44 isolate tested. These five strains reproducibly amplified a second band (Fig. 1). Attempts to prevent the amplification of the extra band by the use of more stringent annealing temperatures and reaction conditions in the PCR were unsuccessful. Most strains of a single H type

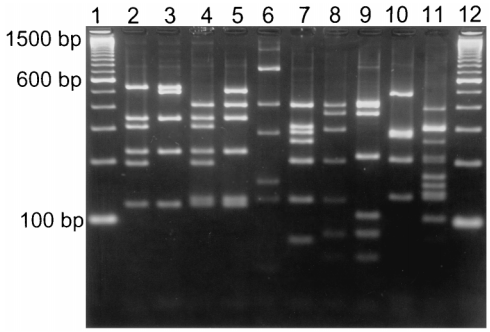

FIG. 2. RFLP patterns for isolates with H flagellar antigens belonging to morphotype E. Lanes 1 and 12, 100-bp ladder (Gibco BRL); lane 2, pattern A, 3186-92 (O157:H7); lane 3, pattern B, 3350-73 (O128:H7): lane 4, pattern C, U5-41 (O1:H7); lane 5, pattern D, 1932-75 (O1:H7); lane 6, pattern E, Bi316-42 (O9:H12); lane 7, 2147-59 (O6:H49); lane 8, H0400-8 (O157:H45); lane 9, 4106-54 (O52:H45); lane 10, HW23 (O45:H23); lane 11, 669-58 (O?:H51).

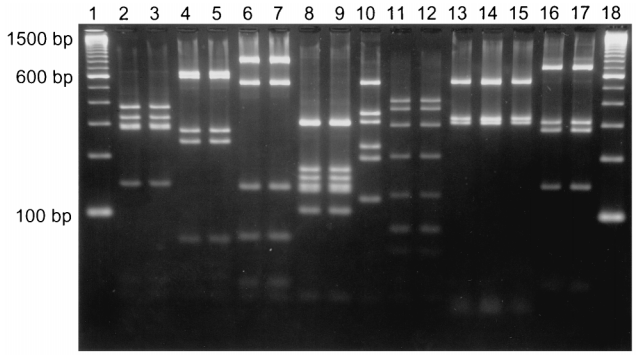

FIG. 3. Representative RFLP pattern matches for isolates of *E. coli* O157:NM, O157:Hund, and O111:NM with known H groups. Lanes 1 and 18, 100-bp ladder; lane 2, F8316-41 (O6:H16); lane 3, 3588-90 (O157:NM); lane 4, A18d (O9:H19); lane 5, 3002-89 (O157:Hund); lane 6, HW27 (O131:H26); lane 7, 3279-88 (O157:NM); lane 8, P9b (O69:H38); lane 9, 3319-95 (O157:NM); lane 10, 3186-92 (O157:H7); lane 11, H0400-8 (O157:H45); lane 12, G7258 (O157:NM); lane 13, Bi623-42 (O11:H10); lane 14, 6154-72 (O151:H50); lane 15, 3333-95 (O157:NM); lane 16, AP302c (O2:H8); lane 17, H0245 (O111:NM).

produced bands of very similar sizes on a 0.8% agarose gel; the exceptions were two strains with flagellar antigen H35, which produced PCR fragments of very different sizes (approximately 1.4 versus 2.3 kb [data not shown]).

Analysis of H7 flagellar antigen strains. A single RFLP pattern (pattern A; Fig. 2, lane 2) was identified for all O157:H7 isolates and for 16 of 18 O55:H7 isolates (Table 1). In an attempt to differentiate *E. coli* O157:H7 from the O55:H7 isolates that had the A pattern, the PCR fragments from three isolates of each of these groups were restricted with 11 other enzymes. All isolates gave identical patterns with each enzyme (data not shown). Three other *Rsa*I RFLP patterns (patterns B, C, and D; Fig. 2, lanes 3 to 5, respectively) were observed for the remaining 26 isolates with H7 flagellar antigens (Table 1). Pattern B was observed for 2 of the 18 serotype O55:H7 isolates and for all isolates of serogroups O19, O27, O128, and O153. Pattern C was observed for all isolates of serogroups O18 and O50 and for five of six isolates of serogroup O1. One serogroup O1 isolate possessed the D pattern. Pattern C is consistent with the published sequence for *fliC* from an O1:H7 isolate (25).

To further characterize the four alleles that encoded the H7 flagellar antigen, two isolates each representing patterns A, B, and C and the one isolate with pattern D were analyzed with additional restriction enzymes (data not shown). Those isolates that were indistinguishable with *Rsa*I were also indistinguishable with the other enzymes tested; however, the other enzymes were less discriminatory. *Alu*I and *Hpa*II divided the H7 isolates into two groups (isolates with the A or B pattern were indistinguishable, and isolates with the C or D pattern were indistinguishable), and *Hae*III differentiated them into three groups (strains with the C or D pattern were indistinguishable). All seven strains were indistinguishable when the PCR fragments were restricted with *Hin*fI.

Analysis of O157:NM and O157:Hund strains. All O157:NM or O157:Hund strains that produced Shiga toxin (Stx1, Stx2, or both) produced the A pattern that was identified for *E. coli* O157:H7 (Table 1). Additionally, 3 of 12 O157:NM isolates that were Stx^- also produced the A pattern (Table 1). The remaining nine NM and two Hund isolates that were Stx^- had any of seven patterns (Fig. 3). Three isolates matched the pattern observed for H19 (lanes 4 and 5), two isolate each matched the patterns identified for H45 (lanes 11 and 12) and H16 (lanes 2 and 3), and one isolate each matched the patterns observed for H26 (lanes 6 and 7), H38 (lanes 8 and 9), and H10 and H50 (lanes 13 to 15; see below). One O157:NM isolate had a unique pattern that did not match that of any strain with a known H type.

Analysis of other morphotype E strains. The RFLP patterns observed for morphotype E strains (flagellar antigen groups H1, H12, H23, H45, H49, and H51) are presented in Fig. 2, lanes 6 through 11. With the exception of one isolate (TX1) which amplified a second fragment in the PCR, a single pattern (pattern E; lane 6) was observed for isolates with the H1 and H12 flagellar antigens (10 and 4 isolates tested, respectively). Isolate TX1 produced a pattern that was identical to the E pattern, but it had two additional bands whose combined molecular weight corresponded to that of the extra fragment amplified from this strain (data not shown). The finding of a single *Rsa*I restriction pattern for the H1 and H12 flagellar antigen groups conflicts with the *fliC* sequences that were reported by Schoenhals and Whitfield (25), in which H1 and H12 *fliC* sequences are different by one *Rsa*I site. However, H1 and H12 were differentiated by *Alu*I, *Hae*III, and *Hpa*II, as predicted from the published DNA sequences (data not shown). Two patterns were observed among four isolates of H45 (lanes 8 and 9). A single but unique pattern was observed for the three isolates each of H23 and H51 (lanes 10 and 11, respectively). The pattern obtained for the H51 isolates was produced from the two fragments that were amplified from these strains. Only one H49 isolate was tested, but it too had a unique pattern (lane 7). The *Rsa*I restriction pattern for the H49 isolate is consistent with the pattern predicted by the *fliC* sequence reported in GenBank (accession number Z36877).

Analysis of other H flagellar antigen groups and NM strains. The remaining H flagellar antigen groups were not analyzed as closely as those described above; however, several observations were made. None of the isolates tested matched the A pattern that was observed for serotype O157:H7. *E. coli* K-12 strains, which are nonmotile, have been reported to belong to the H48 flagellar antigen group (20), and two K-12 isolates had the same pattern as the H48 strain tested. Four additional nonmotile isolates were tested; one each matched the banding patterns observed for flagellar groups H6, H8, and H33, while the fourth nonmotile isolate had a unique pattern. The isolate that matched the flagellar group H8 pattern was an O111:NM, Stx^+ isolate (Fig. 3, lanes 16 and 17).

For 18 of the 53 H groups of *E. coli*, more than one isolate was tested in the PCR-RFLP. Eleven H groups were repre-

sented by a single pattern (the number of isolates tested ranged from 2 to 10), six H groups were represented by two patterns (the number of isolates tested ranged from 2 to 4), and H7 was represented by four patterns (112 isolates were tested). Three sets of H groups were observed to share the same pattern. Groups H1 and H12 had the same pattern (see above). The one isolate each of groups H10 and H50 had the same pattern. Flagellar antigen groups H2, H35, and H47 also shared a RFLP pattern, while another isolate from each of these H groups had a unique pattern. The serotype was confirmed for all isolates for which multiple patterns were observed for one H group or when an RFLP pattern was shared by two H groups.

DISCUSSION

We developed a PCR-RFLP test for the characterization of the *fliC* sequences from *E. coli* isolates that allowed for the differentiation of flagellar antigen groups. The restriction enzyme *Rsa*I was chosen because, on the basis of published sequences for five *fliC* alleles (GenBank accession numbers L07387, L07388, L07389, M14358, X17440, and Z36877 [accession numbers M14358 and X17440 are for two sequences from *E. coli* K-12 that are identical]), it gave a wide range of band sizes and differentiated the closely related flagellar antigens H1 and H12 (97% DNA sequence homology). In retrospect, *Rsa*I was a good but perhaps serendipitous choice. It was the most discriminatory of all the enzymes that we tried for flagellar antigen group H7, dividing H7 isolates into four groups and allowing us to differentiate O157:H7 from most other H7 group isolates. However, *Rsa*I did not differentiate between flagellar groups H1 and H12, as expected from the published sequences, but had we known that at the start, we may not have tested it.

By PCR-RFLP analysis of *fliC*, four different banding patterns were observed for flagellar antigen group H7. The four patterns could be accounted for by variability of two *Rsa*I sites, at nucleotides 411 and 1414 of the *fliC* coding sequence reported by Schoenhals and Whitfield (25). Both restrictions sites fall within what are considered to be the conserved regions of the *fliC* gene. Furthermore, comparison of the banding patterns obtained with the additional enzymes tested to the published sequence suggested five more variable restriction sites, all of which were also located in the conserved regions of the gene. These observations suggest that the internal region of *fliC*, encoding the H7 epitope that is detected by serotyping, may be conserved within flagellar antigen group H7.

With three exceptions (H1 and H12; H10 and H50; and H2, H35, and H47), each RFLP pattern was observed for only one H group. The H1 and H12 antigens are cross-reactive serologically (9), the DNA sequences for *fliC* from H1 and H12 are very similar (25), and these two groups were differentiated with other restriction enzymes. H2, H35, and H47 all belong to different morphotypes, while the morphotype of H50 was not determined (20). The relationship between the isolates of these H groups that were tested here remains to be determined. Flagellar antigen group H35 was also problematic in that the two strains of this H group that were tested gave two PCR fragments with very different sizes (1.4 versus 2.3 kb). This is not unprecedented, since isolates of flagellar antigen group H48 have also been reported to have *fliC* PCR fragments of different sizes (25), although the difference was much smaller. The two H35 isolates did have three *Rsa*I fragments that appeared to be the same size, suggesting that the PCR fragments may share some sequence homology, despite their different sizes.

The characteristic banding pattern, pattern A, that was observed for serotype O157:H7 was seen for all of the O157:NM and O157:Hund isolates that were Stx^+, consistent with the idea that these strains belong to the O157:H7 lineage (11, 27). This group included two German isolates that fermented sorbitol and produced β-glucuronidase and that have been considered to be a unique clone of *E. coli* O157 (17). Fourteen of 16 O55:H7 isolates also had the A pattern. We were unable to differentiate the O55:H7 isolates from the O157:H7 isolates with additional restriction enzymes. A common *fliC* allele for O55:H7 and O157:H7 is not unexpected because O55:H7 is the closest genetic relative of O157:H7 on the basis of multilocus enzyme typing (28). In a clinical setting, the scheme for the identification of *E. coli* O157:H7 includes the determination of the O serogroup as the first step; therefore, the inability to differentiate O55:H7 isolates from O157:H7 isolates should not be a problem for the diagnostic use of this test.

The characterization of *fliC* by PCR followed by RFLP should be a useful diagnostic tool for the characterization of nonmotile isolates of *E. coli* O157. By this method, it is no longer necessary to perform multiple passages of an isolate through motility medium to induce motility so that traditional serotyping can be done. Alternatively, the PCR-RFLP procedure can be used as a rapid test to genetically determine H antigen status while motile isolates are sought. Additionally, the *fliC* RFLP pattern can be used, along with determination of Shiga toxin status and the *uidA* PCR, to demonstrate that O157:NM isolates are genetically O157:H7. DNA sequence analysis of the three additional H7 alleles identified here and the development of a PCR test specific for the O157:H7 *fliC* allele will further simplify this determination.

Fifteen of the nonmotile isolates or isolates with undetermined H types produced a *Rsa*I banding pattern other than that observed for *E. coli* O157:H7. For 13 of these nonmotile isolates, we were able to identify a match between the banding pattern observed for the nonmotile isolate and the banding pattern from an isolate with a known H antigen. Two of the O157:NM isolates had an RFLP pattern that matched flagellar group H45, and O157:H45 has been reported to be an enteropathogenic *E. coli* serotype (24). The pattern for an Stx^+ O111:NM isolate matched the pattern obtained for flagellar group H8, and serotype O111:H8 is considered to be an enterohemorrhagic serotype (3, 7, 21). These observations suggest that we should be able to associate characteristic banding patterns to other groups of nonmotile strains, allowing us to expand this approach to include other clinically important *E. coli* strains. Given the very small number of strains that were tested for some flagellar antigen groups, the fact that we were able to identify matches for the majority of the nonmotile isolates suggests that the sequence of *fliC* within an H group may be fairly well conserved.

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