JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2003, p. 1033–1040 Vol. 41, No. 3
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.3.1033–1040.2003

Serology and Genetics of the Flagellar Antigen of *Escherichia coli* O157:H7a,7c

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Received 8 July 2002/Returned for modification 3 September 2002/Accepted 23 November 2002

Among *Escherichia coli* strains of the O55:H7 serovar, which is considered the ancestor of Shiga toxin-producing *E. coli* (STEC) O157:H7, two subtypes, H7a,7b and H7a,7c (briefly, H7a,b and H7a,c, respectively), of the H7 flagellar antigen have been described previously [J. Wright and R. Villanueva, J. Hyg. (Camb.) 51:39–48, 1953; Y. A. Ratiner and V. A. Sinelnikova, Zh. Microbiol. Epidemiol. Immunobiol. 3:111–116, 1969). We have now studied 13 STEC O157:H7 strains and 1 O55:H7 strain that were epidemiologically unrelated, that originated from six countries on two continents, and that had different profiles when analyzed by multilocus enzyme electrophoresis, pulsed-field gel electrophoresis, and PCR for *stx* and *eae*. They were all found to possess the H7a,c flagellar antigen. Serum cross-absorption assays confirmed that their H antigens were indistinguishable from each other and from that of *E. coli* O55:H7a,c but differed from the standard H7a,b antigen of *E. coli* H test strain U5/41. It was shown by phage-mediated transduction that the flagellin genes for these two H-antigen subserotypes were alleles of the *E. coli fliC* locus. On the basis of the serological data obtained in this study and the molecular characteristics of *E. coli* $fliC_{H7}$ alleles recently published, it is inferred that H7a,c and H7a,b are the main serological subtypes of the group of *E. coli* H7 flagellins.

Escherichia coli flagella, called the H antigen in classical bacteriology, are fine long tubular structures, the walls of which consist of flagellin subunits. The serological specificities of the H antigens are determined by epitopes that are displayed exclusively on the outer flagellar surface and that are recognizable by the classical method of agglutination of flagellated whole bacterial cells.

Absorbed monovalent H-antigen antisera are highly specific in H-antigen identification (4, 25, 39, 43). The diversity and genetics of H antigens are complex in the natural population of the *E. coli* species. Numerous different flagellar antigens and at least five different flagellin-specifying loci, *fliC* (*fliC′* and *fliC″*), *flkA*, *fllA*, and *flmA*, are distributed at distantly located positions on the chromosomes (23, 26, 27) among the strains of the species. About 15% of the H-antigen test strains possess non-*fliC* or double *fliC* (one strain) flagellin-encoding genes (26). In all, 53 *E. coli* H antigens have been officially registered and named by successive numbers in order of their description: H1 to H12, H14 to H21, H23 to H49, and H51 to H56 (4, 20). However, the variety of flagellar antigens in the *E. coli* natural population is much greater: there are other H antigens serologically unrelated to the 53 reference antigens (24, 27, 28, 31), and fine serological distinctions exist between antigens of the same name (number) in different strains. This concerns many H antigens (for instance, H1, H2, H3, H4, H6, H7, H8, H10, H12, H18, and H34), and special designations such as H7a,7b and H7a,7c (briefly, H7a,b and H7a,c, respectively) have been proposed as designations for the subtypes (2, 17, 18, 19, 21, 29, 30, 32). Thus, some *E. coli* O:H serotypes may consist of several distinct H subserotypes that differ in the fine structures of their H antigens, e.g., O55:H2a,b and O55:H2a,c (30, 32).

E. coli O55:H7 is considered an ancestor of *E. coli* O157:H7 (5, 41, 42). Two variants of the former serotype have been reported; one (O55:H7a,c) possesses an H antigen distinct from the standard H7 antigen (32), and the other (O55:H7a,b) possesses an H antigen identical to the standard H7 antigen (44). However, the H-antigen subtype of O157:H7 has not been described. Exact data on the potential serological diversity and H7 subtypes of this new emerging food-borne pathogen would be helpful in clarifying its phylogeny and the subserotype identity of its ancestor, O55:H7a,b or O55:H7a,c.

The data presented above refer specifically to the outer flagellar surface assayed by classical serological methods. Disrupted flagella and monomeric flagellin subunits studied with monoclonal antibodies and by enzyme-linked immunosorbent assay techniques exhibit less serological specificity than whole flagella (9, 10, 35). Monoclonal antibodies specific for H7 have been found to react with the H7 flagellin of *E. coli* O157 at different intensities than they react with the H7 flagellin of *E. coli* strain U5/41 (O1:K1:H7), the standard H7 test strain (9); however, no other evidence of their probable nonidentity was shown. The restriction fragment length polymorphism (RFLP) assay has revealed remarkable differences between the PCR products (with the term "PCR products" referring here and hereafter to those of the flagellin genes only) obtained from *E. coli* O157:H7 strains and those obtained from the standard H7 test strain (6, 8). Moreover, slight differences between the sequences of the PCR products of different *E. coli* O157:H7 strains isolated in different geographical regions have been reported (40). However, it is not known whether these differ-

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TABLE 1. *E. coli* strains

Strain[a]	Serotype[b]	H-antigen genotype[c]	Source and geographical origin [reference(s)]
RH 1438	O157:H7	Unknown	UK-NEQAS (4157), London, England
RH 1545	O157:H7	Unknown	UK-NEQAS (4462), London, England
RH 3535	O157:H7	Unknown	Institute of Medical and Veterinary Science, Adelaide, Australia
RH 3536	O157:H7	Unknown	Institute of Medical and Veterinary Science, Adelaide, Australia
RH 3537	O157:H7	Unknown	Institute of Medical and Veterinary Science, Adelaide, Australia
RH 3538	O157:H7	Unknown	Institute of Medical and Veterinary Science, Adelaide, Australia
RH 3539	O157:H7	Unknown	Institute of Medical and Veterinary Science, Adelaide, Australia
IH 40962	O157:H7	Unknown	National Public Health Institute, Helsinki, Finland (from a patient who returned from Turkey) (34)
IH 40986	O157:H7	Unknown	National Public Health Institute, Helsinki, Finland (34)
IH 41039	O157:H7	Unknown	National Public Health Institute, Helsinki, Finland (from a patient who returned from Spain) (34)
IH 41285	O157:H7	Unknown	National Public Health Institute, Helsinki, Finland (34)
IH 41905	O157:H7	Unknown	National Veterinarian and Food Research Institute, Helsinki, Finland
IH 53393	O157:H7	Unknown	National Public Health Institute, Helsinki, Finland (from a patient who returned from Spain) (34)
U5/41	O1:K1:H7a,b	Unknown	Standard H test strain; International *Escherichia coli* and *Klebsiella* Reference Centre, Copenhagen, Denmark (20)
A42	O55:H7a,c	Unknown	Mechnikov Research Institute for Vaccines & Sera, Moscow, Russia (32)
B99-H7a,b	O55:H7a,b	Unknown	Artificially constructed; Mechnikov Research Institute for Vaccines & Sera, Moscow, Russia
B99-H7a,c	O55:H7a,c	Unknown	Artificially constructed; Mechnikov Research Institute for Vaccines & Sera, Moscow, Russia
B99-2	O55:H6	$fliC_{H6}^{on}$	Mechnikov Research Institute for Vaccines & Sera, Moscow, Russia (23, 26)
200PS	OR:H48	$fliC_{H48}^{on}$	*E. coli* K-12 derivative (11)
EJ34	OR:NM	$fliC_{i}$ ah-1	*E. coli* K-12 derivative (3)

[a] Strains B99-H7a,b and B99-H7a,c were obtained earlier by transducing the relevant flagellar characteristics to strain B99-2 from strains U5/41 and A42, respectively; similar transductant clones obtained in this study were designated B99:$H7_{U5/41}$ and B99:$H7_{A42}$, respectively.

[b] The detailed flagellar antigen characteristics of strains U5/41 and A42 were investigated earlier (32), whereas those of the O157 strains have not been reported earlier.

[c] The flagellar genotypes of the O157 strains (strains U5/41, A42, B99-H7a,b, and B99-H7a,c) have not been reported earlier. In strain EJ34, native *E. coli* K-12 flagellin gene $fliC_{H48}$ was replaced with *Salmonella* phase 1 flagellin gene $fliC_{i}$ (previously named H1), which is altered by the *ah-1* mutation and which is unable to produce flagellin: therefore, the strain was nonflagellated and nonmotile, although all of the other flagellar genes were intact. The superscript "on" represents the expression of a flagellin gene.

ences are relevant to the serological specificity of the flagella, determined by their outer surface epitopes.

Strict evidence showing that the H7 flagellin gene(s) in fact refers to the *fliC* locus has not been published. In fact, the amplification products of *E. coli* flagellin genes have a priori been associated with the *fliC* locus, in analogy with the findings for *E. coli* K-12, without paying attention to the fact that several *E. coli* strains possess at least two different flagellin-specifying loci encoding different flagellins, with one of the loci (mostly *fliC*) usually being cryptic (23, 26, 27). Therefore, at least some such strains are able to amplify two different PCR products simultaneously if the primers used are complementary to both flagellin genes. On the other hand, if some such strains amplify a single PCR product, it may relate to a cryptic flagellin *fliC* gene, i.e., not to the expressed H antigen. All this may complicate the interpretation of the PCR and RFLP assay results or even lead to their misinterpretation.

On the basis of the background data presented above, the multiplicity of flagellin loci in *E. coli* should be kept in mind when molecular methods for differentiation of *E. coli* flagellin genes are developed and their results are interpreted. The importance of a comparison between the molecular diversity of flagellin genes and the serological subdivision of H antigens has also been emphasized (15). As to *E. coli* O157:H7, the purpose of this study was (i) to find out if its H7-specifying gene is an allele of *fliC*, (ii) to ascertain whether the H antigens of epidemiologically unrelated O157:H7 strains are indistinguishable, and (iii) to determine their antigenic factor formulas in comparison with those of the known variants of the H7 antigen. The results obtained are discussed with respect to the interrelation between H-antigen variants H7a,b and H7a,c and published data on the molecular characteristics of H7-specifying genes in different *E. coli* serovars.

MATERIALS AND METHODS

***E. coli* strains.** Seventeen strains of three serovars (serovars O157:H7, O55:H7, and O1:K1:H7) were studied for their H7 serological subtypes, and three strains (strains 200PS, EJ34, and B99-2) were used as recipients in transduction experiments (Table 1). The strains used as controls in PCRs for virulence genes were ATCC 35401 (heat-labile enterotoxin positive [LT^+], heat-stable enterotoxin positive [ST^+]), ATCC 43886 (LT^+), ATCC 43890 (stx_1 positive), ATCC 43894 (stx_1, stx_2, and *eae* positive), ATCC 43895 (stx_1, stx_2, and *eae* positive), ATCC 43896 (ST^+), and RH 4260 (enteroaggregative *E. coli* [EAEC] strain 17-2 [1]), and ATCC25922 (negative for all characteristics mentioned above).

Sera, serum absorption, and H-antigen serotyping. Rabbit antisera were used for serological studies of flagellar antigens. H7 antiserum was produced against H7 reference strain U5/41 (O1:K1:H7). To prepare factor-specific H7b and H7c antisera, antisera to strains B99:H7a,b and B99:H7a,c were absorbed by strains B99:H7a,c and B99:H7a,b, respectively. The strains used to produce other antisera are mentioned in the Results section. The production and absorption of antisera and tube H-antigen agglutination were carried out as described previously (4, 22). H-antigen serotyping assays were preformed by slide agglutination and with Rapid Diagnostic H-antigen sera (a set of seven polyvalent preparations which are able to identify all known *E. coli* H antigens by slide agglutination without the use of monovalent H sera) and have been reported previously (25).

TABLE 2. MLEE patterns of *E. coli* O157:H7 strains

Strain(s)	ET morphotype	Allele of the following enzyme loci[a]:									
		ADH	M1P	IDH	6PG	GOT	ADK	βGA	ACO	MPI	PGI
RH 3535, RH 3536, RH 3537, RH 3538, RH 3539, IH 40962	A	3	3	3	3	3	3	3	1	2	2
RH 1438, IH 41285, IH 41039, IH 40986	B	3	3	3	3	4	3	3	1	2	2
RH 1545	C	3	3	3	3	4	4	3	1	2	2

[a] In some loci, alleles numbered 1 and 2 were absent since the alleles assigned these numbers belonged to *E. coli* strains of other O groups which were studied by MLEE in the same batch with the O157 strains. ADH, alcohol dehydrogenase; M1P, mannitol-1-phosphate dehydrogenase; IDH, isocitrate dehydrogenase; 6PG, 6-phosphogluconate dehydrogenase; GOT, glutamic oxalacetic transaminase; ADK, adenylate kinase; βGA, β-galactosidase; ACO, aconitase; MPI, mannose phosphate isomerase; PGI, phosphoglucose isomerase.

PFGE. Pulsed-field gel electrophoresis (PFGE) was carried out as described by Lukinmaa et al. (14), with the following exception. Genomic DNA was prepared from bacteria grown on Luria agar (37°C, 18 h) and suspended in TEN buffer (optical density, about 0.19; depth of cuvette, 10 mm). Restriction endonuclease digestion was performed with 0.1 U of *Xba*I (Boehringer Mannheim GmbH, Mannheim, Germany) per ml according to the recommendations of the manufacturer. The pulse time was increased from 5 to 50 s over 26 h. The PFGE patterns were visualized by ethidium bromide staining. The genomic relatedness or unrelatedness of the strains was assessed as described by Tenover et al. (38).

MLEE. The electrophoretic types (ETs) of 10 enzymes (alcohol dehydrogenase, mannitol-1-phosphate dehydrogenase, isocitrate dehydrogenase, 6-phosphogluconate dehydrogenase, glutamic oxalacetic transaminase, adenylate kinase, β-galactosidase, aconitase, mannose phosphate isomerase, phosphoglucose isomerase) were studied as described by Selander et al. (36), with some modifications. Briefly, supernatants (obtained by centrifugation at 10,000 × *g* for 15 min at 4°C) of sonicated bacterial suspensions (meat-peptone agar cultures washed off with saline) were stored at −70°C until use. Samples were electrophoresed in a nondenaturing polyacrylamide gel (separating gel, 10% acrylamide and 0.26% bisacrylamide; stacking gel, 4% acrylamide and 0.13% bisacrylamide) in a Vertical Mini-PROTEAN II Cell electrophoresis machine (Bio-Rad Laboratories, Hercules, Calif.). Polyacrylamide gel electrophoresis was used, as it has been reported to be more discriminative for multilocus enzyme electrophoresis (MLEE) than starch electrophoresis (7). Results were documented with a DC50 zoom camera (Kodak Digital Science; Eastman Kodak Company, Rochester, N.Y.) or by photocopying. The electromorphs (alleles) were enumerated according to the electrophoretic mobilities of the enzymes (36). If several bands were seen in an electrophorogram, a new gel was prepared without the substrate; if the band appeared it was considered a false-positive result, whereas the absence of a band corresponded to positivity for the enzyme tested.

PCR. Amplifications of the sequences specific for stx_1, stx_2, *eae*, LT, and ST and the sequences of the genes specific for EAEC were performed as described previously (13).

Transduction. Transducing temperature-inducible phage P1clr100Km-3, the method of the transduction of flagellin-specifying genes, selection of flagellar transductants by motility, counterselection of a recipient, investigation of transductants, and curing the transductants when necessary, were as reported previously (23, 26).

RESULTS

To ensure that the epidemiologically unrelated O157:H7 strains studied were genetically different, their PFGE and MLEE patterns as well as the genes for certain virulence factors were investigated.

Strains RH 1545, RH 3535, RH 3536, RH 3537, RH 3538, RH 3539, IH 40986, IH 41039, and IH 41905 were positive for stx_1, stx_2, and *eae*, whereas strains RH 1438, IH 40962, IH 41285, and IH 53393 carried only stx_2 and *eae*. Typical of the serotype O157:H7 strains, none of these strains possessed ST, LT, or the characteristics of EAEC. According to MLEE, the strains belonged at least to three ET morphotypes (Table 2). More diversity among the O157 strains was apparent by PFGE than by MLEE: no identical PFGE patterns were found. Pairs of strains (strains RH 3538 and RH 3535, strains RH 3539 and RH 3537, and strains RH1545 and RH1438) exhibited less than three band differences (a probable close relation, according to the recommendations of Tenover et al. [38]). All the other combinations of the strains exhibited more than seven band differences, thus indicating their epidemiological unrelatedness (38).

It is noteworthy that epidemiological unrelatedness was demonstrated among strains isolated in the same country. Strains received from England (strains RH 1545 and RH 1438) fell in the category of probably closely related by PFGE. However, they should be considered unrelated since they belonged to different ET morphotypes and differed in stx_1. All the strains isolated in Finland should also be considered unrelated by their PFGE patterns. In addition, they differed in the presence of stx_1, strain IH 40962 belonged to a different ET morphotype, and some of them were reported (34) to belong to different phage types. Australian strains fell in three unrelated groups (strains RH 3535 and RH 3538, strains RH 3537 and RH 3539, and strain RH 3536).

All O157:H7 strains and the strain A42 (O55:H7a,c) produced a fast and strong agglutination reaction with the H7c antiserum but failed to react with the H7b antiserum, which reacted strongly with strain U5/41 (O1:K1:H7a,b). Therefore, we named the flagellar antigen of the O157 strains studied H7a,c.

To analyze the flagellar serology of the O157:H7 strains in more detail, antibody cross-absorption assays were carried out, and the H-antigen agglutination tests were performed in tubes. The H antigens of all strains studied were indistinguishable when they were tested with nonabsorbed antisera; i.e., they possessed a common antigenic factor (H7a). The strains differed in the antibody cross-absorption assay, thus demonstrating the presence of specific antigenic factors (Table 3). H-antigen antibodies specific to U5/41 were retained in the homologous antiserum absorbed by strain A42 or any of the O157:H7 strains, meaning that strain U5/41 possesses a specific partial H-antigen factor (i.e., H7b) absent from all other strains tested. On the other hand, the results of the absorption of antiserum to strain A42 or RH 3535 showed that these strains shared a specific partial H-antigen factor (H7c) absent from strain U5/41. The cross-absorption tests justified the designation H7a,c given above to the flagellar antigens of O157:H7 strains, in analogy with the designation for strain A42.

To ascertain which flagellin locus encodes the H7 antigen in

TABLE 3. Comparison of flagellar antigens of the studied strains O157:H7 with flagellar antigens of strains U5/41 (O1:K1:H7a,b) and A42 (O55:H7a,c) by antibody absorption technique

Strain used as vaccine for antiserum production	Strain used for absorption[a]	Titer of H antibodies to strain[b]:		
		U5/41 (O1:K1:H7a,b)	A42 (O55:H7a,c)	O157:H7[a]
U5/41 (O1:K1:H7a,b)	None	51,200	51,200	51,200
	A42 (O55:H7a,c)	800	—	—
	O157:H7	1,600	—	—
	U5/41 (O1:K1:H7a,b)	—	—	—
A42 (O55:H7a,c)	None	25,600	25,600	25,600
	A42 (O55:H7a,c)	—	—	—
	O157:H7	—	—	—
	U5/41 (O1:K1:H7a,b)	—	1,600	1,600 or 3,200
RH 3535 (O157:H7)	None	6,400	6,400	6,400
	A42 (O55:H7a,c)	—	—	—
	O157:H7	—	—	—
	U5/41 (O1:K1:H7a,b)	—	400	400

[a] Each of the 13 O157 strains (see Table 1) was used as the absorbent and as the antigen in the agglutination assay, and all gave the same result.
[b] Reciprocal titer by the tube agglutination assay. The starting dilution of the antiserum was 1:25. —, titer less than 1:25.

E. coli O157, O55, and O1, transduction of their H-antigen characteristics to recipient strains with known flagellar antigen genetics was carried out (Table 4). Transductants were selected by determination of their motilities in the presence of an H antiserum which immobilized (counterselected) the recipient cells. Motile transductants resulted in all experiments carried out with recipient strains 200PS (with counterselecting H48 antiserum), B99-2 (with counterselecting H6 antiserum), and EJ34 (with no antiserum, since the recipient was nonmotile). In addition, all transductants tested had the H7 antigen, and their ability to react with the factor-specific H7b or H7c antiserum depended on the respective donor strain (Table 4, section A). When the H7 antiserum was used as an additional counterselecting agent, no motile transductants were found, not even with recipient strain EJ34, which is known to become flagellated and motile if any intact flagellin-specifying gene is accepted (3, 26). It follows that no transductants of any other H serotype arose; i.e., no intact genes capable of encoding any other than H7 flagellar antigen were present in the donors. To ensure that just the *fliC* genes of the recipients were replaced (and not cryptically retained) in the transductants, second-step transductions were carried out (Table 4, section B). Three strains of each of three transductant classes, strains 200PS:$H7_{157}$, 200PS:$H7_{U5/41}$, and 200PS:$H7_{A42}$, which were isolated from recipient strain 200PS and which had inherited their flagellar characteristics from donor strains RH 3535, U5/41, and A42, respectively, were used as second-step donors in crosses with recipient strain EJ34. Only H7 transductants and not any H48 transductants resulted. It indicated that in the second-step donors $fliC_{H48}$ of strain 200PS had been replaced by the flagellin gene of a respective first-step donor; i.e., the H7 flagellin-specifying genes in strains RH 3535, U5/41, and A42 were alleles of the *fliC* locus.

The flagellar antigens of the H7 transductants were compared with the flagellar H7 antigens of the parent strains by the antibody absorption test. The serological specificities of the H antigens of the transductants derived from recipient B99-2 were identical to those of the H antigens of the relevant donors (Table 5). Analogous results were obtained with transductants derived from strain 200PS. Thus, the antigenic characteristics of the strains studied depended exclusively on the respective flagellin-specifying genes, and any hypothetical posttranscriptional modification specific for *E. coli* O157:H7 (that one might presume) did not influence the specificities of their H antigens.

DISCUSSION

The serological characteristics of the H7 flagellar antigens of 13 Shiga toxin-producing *E. coli* O157:H7 strains and 1 O55:H7 strain originating from six countries on two continents were studied in detail. According to the MLEE and PFGE patterns obtained, as well as the distribution of stx_1, all O157:H7 strains studied except two pairs of strains (strains RH 3535 and RH 3538 and strains RH 3537 and RH 3539) were confirmed to be epidemiologically unrelated. Previously, two H7 flagellar antigen subserotypes (H7a,b and H7a,c) have been described for *E. coli* (32). However, the O157:H7 strains evaluated in the present study all belonged to the same flagellar subserotype, H7a,c, despite their genetic differences, as revealed by PFGE and MLEE assays as well as by the presence or absence of stx_1. Thus, their flagellar antigens differed from the standard H7 antigen of *E. coli* H-antigen test strain U5/41 (O1:K1:H7a,b) by the presence of specific partial antigenic factor H7c instead of H7b. On the other hand, it was identical to the H antigen of strain A42 (O55:H7), which was originally described as H7a,c (32). Flagellar antigen H7a,c has also previously been described for some other *E. coli* O-antigen groups (30).

In the present study, a serological distinction between the flagellar antigens of *E. coli* O157:H7 strains and those of standard strain U5/41 was initially established by a very simple technique: slide agglutination with factor-specific H7b and H7c antisera. To confirm this finding, the classical approach to the study of fine flagellar serology (partial H-antigen factors), the H antibody cross-absorption and tube agglutination tests, was used. Formalinized highly motile *E. coli* cultures are highly

TABLE 4. Transduction of flagellar antigen specificities

Donor strain[a]	Recipient strain	Counterselecting H antiserum	Production of transductants[b]	Tested transductants	
				No.	H antigen identified
Section A					
RH 3535	200PS	H48	Yes	15	H7a,c
	B99-2	H6	Yes	10	H7a,c
	EJ34	None	Yes	16	H7a,c
	200PS	H48 + H7	No		
	B99-2	H6 + H7	No		
	EJ34	H7	No		
U5/41	200PS	H48	Yes	10	H7a,b
	B99-2	H6	Yes	10	H7a,b
	EJ34	None	Yes	10	H7a,b
	200PS	H48 + H7	No		
	B99-2	H6 + H7	No		
	EJ34	H7	No		
A42	200PS	H48	Yes	10	H7a,c
	B99-2	H6	Yes	11	H7a,c
	EJ34	None	Yes	10	H7a,c
	200PS	H48 + H7	No		
	B99-2	H6 + H7	No		
	EJ34	H7	No		
Section B					
200PS:H7$_{RH3535}$	EJ34	None	Yes	29	H7a,c
	EJ34	H7	No		
200PS:H7$_{U5/41}$	EJ34	None	Yes	30	H7a,b
	EJ34	H7	No		
200PS:H7$_{A42}$	EJ34	None	Yes	30	H7a,c
	EJ34	H7	No		
200PS	EJ34	None	Yes	16	H48

[a] Strains 200PS:H7$_{RH3535}$, 200PS:H7$_{U5/41}$, and 200PS:H7$_{A42}$ are the designations of transductant classes obtained from recipient strain 200PS with donor strains RH 3535, U5/41, and A42, respectively. Three transductants of each class were used as donors in experiments with EJ34 as the recipient; and 9 to 10 second-step transductants from each cross, if any resulted, were tested.

[b] Yes, transductants resulted; no, no transductants were found.

specific and sensitive indicators of H antibodies, while O and K antibodies (if present in the serum) do not interfere (43). The specificity of the results was further confirmed by studying flagellar transductants. The recipient strains did not react with any of the H antisera used, whereas the behaviors of the transductants were identical to those of the relevant parental donor (wild) strains. This indicated that the serological results referred to the properties controlled by the flagellin genes.

The transduction experiments also showed that the genes encoding flagellar antigens H7a,b and H7a,c in fact were alleles of the *E. coli* flagellin *fliC* locus since they substituted for *fliC*$_{H6}$ as well as for *fliC*$_{H48}$ in recipient strains B99-2 and 200PS, respectively. This is important knowledge in view of the multiplicity of flagellin-specifying loci in *E. coli*, even though the presence of multiple loci may result in difficulties in flagellar typing by molecular methods. For instance, the ability of each of three *E. coli* standard H-antigen test strains (strain P12b with the H17 antigen, strain 781/55 with the H44 antigen, and strain E223-69 with the H54 antigen) to amplify two distinct PCR products simultaneously has been reported previously (15), but the basis of this finding has not been discussed. Accordingly, the results may be due to the presence of two flagellin genes in both P12b and 781/55 (*fliC′*$_{H4}$ and *fliC″*$_{H17}$ in P12b, *fliC*$_{H4}$ and *fllA*$_{H44}$ in 781/55) but in which only one of them is expressed: *fliC″*$_{H17}$ and *fllA*$_{H44}$ in P12b and 781/55, respectively (26). In strain E223-69, the flagellar antigen expressed, H54, is encoded by another flagellin gene, *flmA*$_{54}$ (26), and the possibility that this strain also possess a cryptic *fliC* gene cannot be excluded. The primers used may have had affinities to both flagellin genes in each of those three strains, thus resulting in two PCR products. This supposition seems to be most reliable because the two-band phenomenon was produced exclusively by strains known to possess non-*fliC* or double *fliC* genes, although some other reasons, for instance, mispriming, cannot be excluded. On the other hand, *E. coli* strain Bi7327/41 (the H3-antigen test strain) is known to be diphasic and possess two flagellin genes, *fliC*$_{H16}$ and *flkA*$_{H3}$; the latter is expressed, and the former is cryptic (23). However, this strain amplified only a single PCR product (15). It means that the primers used were complementary to only one of the two flagellin genes, probably the cryptic *fliC*$_{H16}$ gene. On the other hand, there also might have been two distinct PCR products (*fliC*$_{H16}$ and *flkA*$_{H3}$) indistinguishable from each other by their sizes. Thus, the PCR product obtained could not be

TABLE 5. Comparative study of flagellar antigens of transductants and donor wild strains by antibody absorption technique

Strain used as vaccine for antiserum production	Strain used for absorption	Titer of H antibodies to strain[a]:						
		U5/41	B99:H7$_{U5/41}$	A42	B99:H7$_{A42}$	RH 3535	B99:H7$_{RH3535}$	B99-2[b]
U5/41	None	51,200	51,200	51,200	51,200	51,200	51,200	—
	B99:H7$_{U5/41}$	—	—	—	—	—	—	NT
	B99:H7$_{A42}$	1,600	1,600	—	—	—	—	NT
	B99:H7$_{RH3535}$	800	1,600	—	—	—	—	NT
A42	None	25,600	25,600	25,600	25,600	25,600	25,600	—
	B99:H7$_{U5/41}$	—	—	1,600	1,600	1,600	1,600	NT
	B99:H7$_{A42}$	—	—	—	—	—	—	NT
	B99:H7$_{RH3535}$	—	—	—	—	—	—	NT
RH 3535	None	6,400	6,400	6,400	6,400	6,400	6,400	—
	B99:H7$_{U5/41}$	—	—	200	400	400	400	NT
	B99:H7$_{A42}$	—	—	—	—	—	—	NT
	B99:H7$_{RH3535}$	—	—	—	—	—	—	NT
B99:H7$_{U5/41}$	None	25,600	25,600	25,600	25,600	25,600	25,600	—
	U5/41	—	—	—	—	—	—	NT
	A42	800	1,600	—	—	—	—	NT
	RH 3535	800	800	—	—	—	—	NT
B99:H7$_{A42}$	None	25,600	25,600	25,600	25,600	25,600	25,600	—
	U5/41	—	—	3,200	1,600	3,200	3,200	NT
	A42	—	—	—	—	—	—	NT
	RH 3535	—	—	—	—	—	—	NT
B99:H7$_{RH3535}$	None	12,800	12,800	12,800	12,800	12,800	12,800	—
	U5/41	—	—	800	1,600	800	800	NT
	A42	—	—	—	—	—	—	NT
	RH 3535	—	—	—	—	—	—	NT

[a] Reciprocal titer by the tube agglutination assay. Strains B99:H7$_{U5/41}$, B99:H7$_{A42}$, and B99:H7$_{RH3535}$ were transductants isolated from recipient B99-2, which inherited their H antigens from wild donor strains U5/41, A42, and RH 3535, respectively. The starting dilution of the antisera was 1:25. —, titer less than 1:25; NT, not tested.

[b] B99-2 was the recipient O55:H6 strain.

inferred to represent the flagellar serotype H3 antigen that is expressed. These examples demonstrate the significance of combined knowledge of the genetics and serology of *E. coli* flagella. With regard to *E. coli* O157:H7, our results validate the common assumption that the PCR products of *E. coli* H7 strains indeed refer to those of the H7 flagellin genes.

It is noteworthy that no monoclonal antibodies specific exclusively to the flagellin of *E. coli* O157:H7 or to that of *E. coli* O1:K1:H7 have hitherto been reported. The reason might be that the investigators have focused on looking for antibodies active against the H7 flagellins of different strains. However, monoclonal antibodies that reacted with different intensities with flagellins of these two *E. coli* serotypes were described (9), suggesting a probable difference between the flagellins. However, this was not sufficient proof that the antigens contain serologically unrelated epitopes. The data presented in this article reveal the presence of unique serologically unrelated antigenic epitopes in the H7 flagellins of different *E. coli* strains, and these are referred to as partial antigenic factors H7b and H7c.

We wondered if there was some regular correlation between the serology of *E. coli* H7 antigens and the molecular diversity of *fliC*$_{H7}$ recently reported. Gannon et al. (8) detected three *Rsa*I digestion profiles for the amplified *fliC*$_{H7}$ alleles: the first occurred with serotype O157:H7 and O55:H7 strains, and the second occurred with O1:K1:H7 strain U5/41 (the standard H7 test strain). The difference concerned only the largest fragments of the digestion profiles: the fragment was larger for the first two strains than for the last strain. The largest fragment in the third *Rsa*I digestion profile (*E. coli* O19:H7), however, was similar to that in the first profile involving O157 and O55 strains, and the differences in fragment sizes were for two smaller fragments. The flagellar antigen of *E. coli* O19:H7 was earlier reported to be H7a,c (30). Therefore, the expression of different antigenic factors (epitopes)—H7c on the flagella of the O157, O55, and O19 strains, on the one hand, and H7b on the flagella of the O1 strains (namely, U5/41), on the other—appears to correlate exclusively with the presence of one of the two largest fragments in the respective digestion profiles.

Fields et al. (6) reported four *Rsa*I digestion profiles (profiles A, B, C, and D) of *E. coli fliC*$_{H7}$ PCR products; profiles A, C, and B were the same as the first, second, and third profiles described by Gannon et al. (8), respectively. The smaller fragments of profile D (a single strain of the O1 group) were similar to those of profile B. However, in contrast to profiles A, B, and C, there were two larger fragments; one of them was similar to the largest fragment of profile A, and another was similar to the largest fragment of profile C. Interestingly, 16 of 18 O55:H7 strains exhibited profile A (typical of H7a,c), while the 2 remaining strains showed profile B. As noted above,

profile B may correspond to flagellar subserotype H7a,c. The close relationship between the flagellins of profiles A and B and between the flagellins of profiles C and D was confirmed by the data of Fields et al. (6). Namely, digestion with *Alu*I or *Hpa*II produced only two restriction profiles; in each case, one of them referred to strains with *Rsa*I digestion profiles A and B (H7a,c) and one referred to strains with *Rsa*I digestion profiles C and D (H7a,b). Thus, the published diversity determined by PCR (*fliC*$_{H7}$)-RFLP analysis does not seem to be chaotic but seems to be consistent with the idea that H7a,b and H7a,c are the main serological subserotypes of the set of H7 antigens within *E. coli*. It is noteworthy that the H7 antigens of 39 *E. coli* strains belonging to 15 different O groups other than O157 and O55 have been reported to react with either the factor-specific antiserum H7b or the factor-specific antiserum H7c, thus falling into two distinct H subserotypes (30).

Sequence analysis of the flagellin genes is by nature more discriminating than RFLP assay of the PCR product. Wang et al. (40) reported that the *fliC*$_{H7}$ genes of strains of different O groups have 10 alleles and divided them into two groups on the basis of fairly significant differences in their nucleotide sequences: U5/41 (O1:K1:H7) and related strains were lumped into one group, whereas other strains including strains of the O157 and O55 groups were lumped into the other group. Thus, the two groups established by Fields et al. (6) conform to the two groups established by Wang et al. (40). The subdivision into two main molecular groups is in agreement with the serological diversity of H7 antigens. However, one cannot exclude the existence of further finer serological varieties of the H antigens in each of the two main groups, especially regarding strains of different O serovars.

Although there are only a few publications concerning molecular studies of the H7 flagellin genes, they have included data on O157 and O55 isolates from different countries. It was reported that all 116 strains studied (70 O157:H7 strains and 46 Shiga toxin-positive O157:NM [nonmotile] strains), including strains from the United States, Canada, Europe, and Asia, exhibited the same pattern (which can now be called the H7a,c pattern) by PCR-RFLP analysis, and this pattern was also found for 16 of 18 O55:H7 strains (6). No contradicting data are known. Since some Australian strains were included in our study, it is noteworthy that the sequence patterns of the flagellin genes were reported to be the same for Australian and European O157:H7 strains, as well as an American O55:H7 strain (40). Thus, the published data suggest that the flagellin genes of the O157:H7 strains are identical (or at least indistinguishable), despite their different geographical origins. However, the identity of the H antigens by PCR-RFLP analysis does not definitely mean that they are identical, because some restriction enzymes may insufficiently discriminate between some sequences. On the other hand, in some strains flagellins may undergo a posttranscriptional modification, controlled by a gene located elsewhere (37). For instance, it was reported that *E. coli* strains Bi623/42 (O11:K10:H10) and 880-67 (O151:H10) exhibited PCR-RFLP patterns indistinguishable from each other (6, 15), even though the partial (factor) structures of their H antigens were known to be different (29). Therefore, we compared by the serum cross-absorption test the H7 antigens of O157 strains that originated from different countries, that were epidemiologically unrelated, and that were genetically distinct (defined by phage types[34], PFGE, MLEE, and the presence or absence of *stx*$_1$) and found that they were indistinguishable from each other.

Contrary to published data on the identities of PCR-RFLP patterns and our data on the H-antigen identities of the O157:H7 strains, sequence differences between the H7 flagellin genes of some O157 strains at one or two nucleotide positions (corresponding to the replacement of one or two amino acids in the flagellin, respectively) have been reported (33, 40). However, it is impossible to exclude the possibility that such diversity might appear during prolonged storage under laboratory conditions without natural (possibly niche-specific) selection. This is potentially reflected by discrepant sequence data obtained in different laboratories for 11 nucleotides of the H7 flagellin gene of standard strain U5/41 (40), which was originally isolated more than 60 years ago and which was subsequently stored and cultured for decades in different laboratories.

On the basis of the identities of the H7a,c antigens of O157 and O55 strains, as shown by serum cross-absorption tests, one can assume that it is more likely that serotype O55:H7a,c rather than serotype O55:H7a,b is the ancestor of O157:H7a,c. The O55:H7a,b serotype is probably rare because no O55:H7 strains exhibiting H7a,b-like PCR-RFLP patterns have been reported.

Those involved with the modern identification of the *E. coli* flagellar types are leaning to the view that phenotypic serological methods can be replaced by various genetic approaches, especially PCR-RFLP assay. No doubt, the PCR-RFLP assay has advantages, for example, the ability to identify all flagellar antigens or all known H antigens in nonflagellated or poorly flagellated cells. However, investigations that use the PCR-RFLP assay need to be carefully fitted to the serological classification of the H antigens (15) and their genetics. Otherwise, the results may contradict serological data in some cases, in particular, when *fliC*$_{H7}$ is detected (12). The knowledge of the genetics and serology of the *E. coli* flagellar antigens, including that from the data presented here, is still far from complete, as exemplified by the complexity of the H7 antigen. Therefore, serological typing has not lost its significance, and its importance in the development of a reliable molecular method of flagellar typing has recently been emphasized by Machado et al. (15).

Prediction of a general victory of molecular methods over other methods for flagellar typing is premature. A new principle for serological detection and/or identification of flagellated *E. coli* in pure cultures or even directly in samples was recently reported (16). This method is simpler, easier, and quicker than PCR-RFLP assay and can be adapted to the detection of motile *E. coli* strains of any O:H serotype, provided that relevant highly specific somatic and flagellar antibodies, especially factor-specific antibodies, are available. Our results will empower the production of factor-specific antibodies to H7b and H7c for the detection of strains of the respective H7 antigenic subserotypes.

ACKNOWLEDGMENTS

We are grateful to P. Helena Mäkelä for critical reading of the manuscript and to Tarja Heiskanen for technical assistance.

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