JOURNAL OF CLINICAL MICROBIOLOGY, May 1993, p. 1108–1110
0095-1137/93/051108-03$02.00/0

Vol. 31, No. 5

Differentiation of *Salmonella* Phase 1 Flagellar Antigen Types by Restriction of the Amplified *fliC* Gene

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Received 28 December 1992/Accepted 8 February 1993

The large antigenic diversity (over 2,300 serotypes) expressed by *Salmonella* strains can probably be observed at the genetic level. The phase 1 flagellin gene *fliC* was amplified, and the amplified fragment was cleaved with a mixture of both endonucleases *Taq*I and *Sca*I. The restriction patterns observed allowed differentiation of flagellar types b, i, d, j, l,v, and z_{10}. Flagellar group g (g,m, g,p, or g,m,s) could be differentiated from the other flagellar types. Flagellar types r and e,h could not be separated, although they could be distinguished from the other flagellar types studied. Practical applications of flagellar gene restriction are the distinction between serotype Gallinarum-Pullorum, which carries a cryptic gene for flagellar type g,m, and nonmotile Vi-negative variants of serotype Typhi, and the tentative assignation of nonmotile variants of *Salmonella* serotypes to a flagellar type.

There are presently 2,324 different *Salmonella* serotypes (5). In practice, only a limited set is really prevalent. For example, 15 serotypes made up 83.2% of the 12,726 clinical isolates studied at the French National *Salmonella* Center in 1991 (2). Serotypes Enteritidis and Typhimurium alone represent 71.9% of the clinical *Salmonella* isolates.

Although serotyping offers a very precise and reliable method for differentiating *Salmonella* strains, identification of the complete set of serotypes is a time-consuming process and requires the use of 167 specific serum samples. With the expanding availability of molecular biology methods, it is tempting to unveil the antigenic diversity where it is coded for, i.e., on the DNA. Most *Salmonella* strains possess two structural genes (*fliC* and *fliB*) coding for flagellins. No more than one of these structural genes is expressed at a time in one bacterial cell. Nonmotile strains generally possess the structural genes but are unable to build up a functional flagellum (4). Early genetic studies have shown the nonmotile serotype Gallinarum-Pullorum to have a structural gene for type gm flagellin (12). Several sequences of the gene coding for phase 1 flagellin (*fliC*) have been published (3, 7–11), and some are under study in our laboratory. The remarkable feature of *fliC* alleles is the sequence conservation of distal parts of the gene, thus making the gene of any serotype suitable for easy amplification.

The purpose of the present study was to differentiate the phase 1 flagellar genes of the major serotypes by a simple method, restriction of the amplified *fliC* gene.

MATERIALS AND METHODS

Bacterial strains. The strains used in the present study are listed in Table 1. FNSC strains were from the French National *Salmonella* Center (located in our laboratory). All other strains were from the Collaborating Center for Reference and Research on *Salmonella*, World Health Organization (also located in our laboratory).

DNA extraction. Bacterial DNA was prepared as described by Brenner et al. (1).

Primers used for amplification. For the amplification of the phase 1 flagellin gene, the following two primers were used (10): AAG GAA AAG ATC ATG GCA (primer 1) and TTA ACG CAG TAA AGA GAG (primer 2).

Amplification of the *fliC* gene. DNA was amplified by the polymerase chain reaction (PCR) (6). A reaction mixture of 100 μl contained 1 μl (1 to 10 μg) of extracted DNA, 50 to 100 pM (each) primer (primers 1 and 2), 200 μM nucleotide mixture (ultrapure deoxynucleoside triphosphate set; Pharmacia Biotechnology LKB, Uppsala, Sweden), 10 μl of 10× *Taq* polymerase buffer (Amersham International, Amersham, United Kingdom), and 2.5 U of *Taq* polymerase (Amersham). The reaction mixture was covered with 75 μl of mineral oil (Sigma Chemical Co., St. Louis, Mo.), and the PCR was performed with a Perkin-Elmer Cetus DNA Ther-

TABLE 1. Strains of *Salmonella* included in the present study

Serotype	Strain no.	Phase 1 flagellar antigen
Typhi	Ty2	d
Typhi	E 75 2099	d
Typhi	E 80 2027	j
Typhi	E 285K	j
Enteritidis	64K	g,m
Dublin	65K	g,p
Montevideo	46K	g,m,s
Panama	73K	l,v
Brandenburg	24K	l,v
Bredeney	30K	l,v
Typhimurium	LT2	i
Paratyphi B	8006	b
Hadar	473K	z_{10}
Heidelberg	16K	r
Virchow	41K	r
Bovismorbificans	53K	r
Infantis	158K	r
Newport	12302	eh
Gallinarum-Pullorum	FNSC 4.83	–[a]
Gallinarum-Pullorum	FNSC 51.66	–
Nonmotile O9,12	FNSC 10.90	–
Nonmotile O9,12	FNSC 14.90	–
Nonmotile O3,10	FNSC 5.89	–

[a] –, no flagellar antigen.

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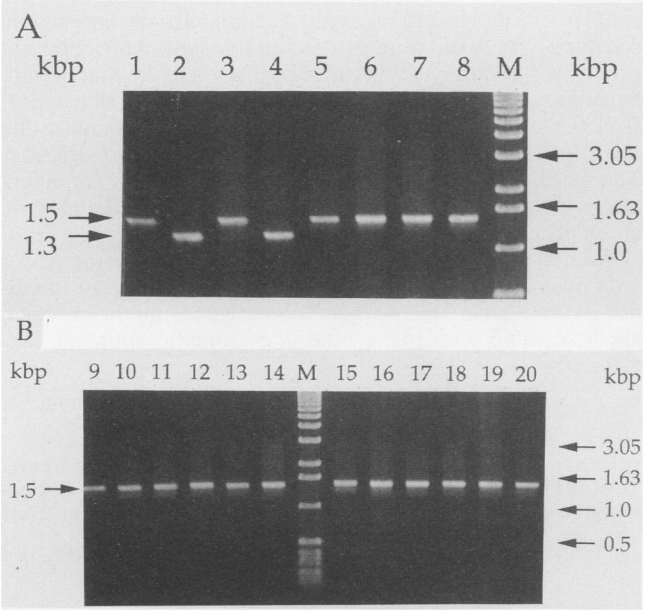

FIG. 1. DNA fragments amplified by using primers 1 and 2. (A) DNA samples were from the following *Salmonella* strains: lane 1, Typhi Ty2 (H1-d); lane 2, Typhi E 285K (H1-j); lane 3, Typhi E 75 2099 (H1-d); lane 4, Typhi E 80 2027 (H1-j); lane 5, Gallinarum-Pullorum 4.83; lane 6, Gallinarum-Pullorum 51.66; lane 7, Enteritidis 64K; lane 8, Panama 73K. (B) DNA samples were from the following *Salmonella* strains: lane 9, Dublin 65K; lane 10, Brandenburg 24K; lane 11, Bredeney 30K; lane 12, Montevideo 46K; lane 13, Typhimurium LT2; lane 14, Paratyphi B 8006; lane 15, Hadar 473K; lane 16, Heidelberg 16K; lane 17, Virchow 41K; lane 18, Bovismorbificans 53K; lane 19, Infantis 158K; lane 20, Newport 12302. Lanes M, 100-bp ladder.

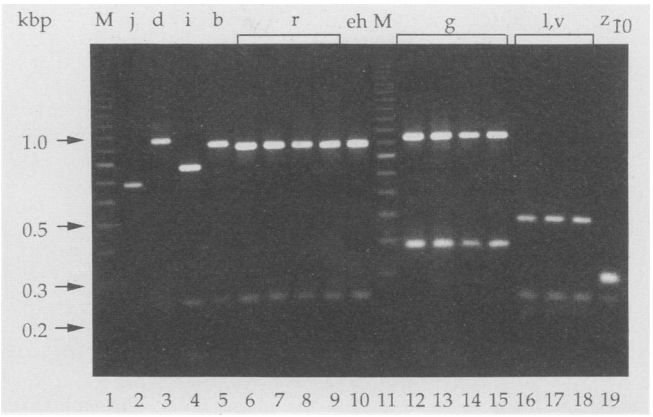

FIG. 2. Restriction profiles of the phase-1 flagellin gene from different serotypes after double restriction with endonucleases *Taq*I and *Sca*I. The DNAs in the lanes were from the following strains: lane 1, 100-bp DNA ladder; lane 2, Typhi E285K; lane 3, Typhi Ty2; lane 4, Typhimurium LT2; lane 5, Paratyphi B 8006; lane 6, Heidelberg 16K; lane 7, Virchow 41K; lane 8, Bovismorbificans 53K; lane 9, Infantis 158K; lane 10, Newport 12302; lane 11, 100-bp DNA ladder; lane 12, Gallinarum-Pullorum 51.66; lane 13, Enteritidis 64K; lane 14, Dublin 65K; lane 15, Montevideo 46K; lane 16, Panama 73K; lane 17, Brandenburg 24K; lane 18, Bredeney 30K; lane 19, Hadar 473K. The phase 1 flagellar antigens are indicated at the top, and lane numbers are indicated on the bottom.

mal Cycler 480 (Perkin-Elmer Corporation, Norwalk, Conn.). After an initial denaturation step of 5 min at 94°C, 25 cycles were performed. Each cycle comprised the following steps: 1 min at 94°C (denaturation), 1 min at 50°C (primer annealing), and 2 min at 72°C (elongation). PCR products (5 μl) were analyzed on a 1% agarose gel.

Purification of PCR products. Because amplification was carried out under stringent conditions, PCR yielded only one amplification product, which could be purified by precipitation (adding LiCl to a final concentration of 0.5 M, 150 μg of glycogen [Boehringer, Mannheim, Federal Republic of Germany], and 2 volumes of absolute ethanol, and incubating for 10 min at −80°C).

Restriction profiles. The amplified and purified *fliC* gene was cleaved with different restriction enzymes according to the manufacturers' instructions. These enzymes were *Pst*I (Amersham), *Eco*RII (GIBCO BRL Life Technologies, Renfrewshire, United Kingdom), *Hpa*I (Pharmacia), *Hin*cII (Amersham), *Taq*I (New England Biolabs, Beverly, Mass.), and *Sca*I (Pharmacia).

Restriction fragments were separated by electrophoresis on a 2% agarose gel.

RESULTS AND DISCUSSION

Restriction profile of the phase 1 flagellin gene (*fliC*). The phase 1 flagellin gene of the 23 *Salmonella* strains studied could be amplified with primers 1 and 2. The size of the PCR product was 1.24 kb for serotype Typhi expressing phase 1 flagellar antigen j (H1-j) and 1.5 kb for all other strains (Fig. 1). The patterns obtained with different restriction enzymes were compared at first for serotype Typhi Ty2 (H1-d), serotype Typhi E285K (H1-j), serotype Gallinarum-Pullorum 4.83 (H1-g,m), and serotype Enteritidis 64K (H1-g,m) representing the phase 1 flagellin types d, j, g,m, and g,m, respectively. In preliminary experiments, the following enzymes were tested: *Pst*I, *Eco*RII, *Hpa*I, *Hin*cII, and *Taq*I, with *Taq*I giving the best results (data not shown). Endonuclease *Taq*I allowed a clear distinction among phase 1 flagellin types d, j, and g,m. Restrictions of the phase 1 flagellin genes of further serotypes were performed with the enzyme *Taq*I, which resulted in specific restriction patterns for the phase 1 flagellin types j, d, z_{10}, and l,v, whereas the serotypes possessing the phase 1 flagellin types i, b, r, and e,h showed a single restriction pattern. One profile was obtained for serotypes containing the epitope g on their flagellins, thus comprising phase 1 flagellin types g,m, g,p, and g,m,s. Finally, a double restriction (Fig. 2) with the enzymes *Taq*I and *Sca*I proved to be efficient for differentiating the phase 1 flagellin type i from the phase 1 flagellin type r. The fragment sizes were consistent with the sizes calculated on the basis of restriction maps, which were obtained from the published sequences of serotype Typhimurium (H1-i) (9) and serotype Rubislaw (H1-r) (11). Further differentiation could be obtained when the restriction fragments were separated on a 2% agarose gel for a longer period of time (overnight at 30 V). The longer migration time made visible the size difference between the largest fragments of phase 1 flagellin types b and r. The phase 1 flagellin types r and e,h could not be distinguished by double restriction of the *fliC* gene with *Taq*I and *Sca*I. To increase the range of specific restriction profiles of phase 1 flagellar antigen, further restriction enzymes must be tested. It also may be of interest to sequence more *fliC* genes to establish restriction maps for the selection of useful enzymes. The experiments proved that the method is easily applicable and therefore can be used in identification in the future.

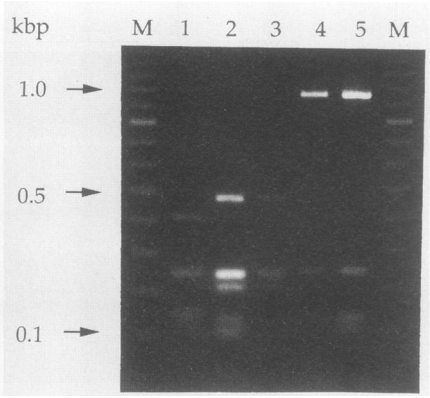

FIG. 3. Restriction profiles of the phase 1 flagellin gene from different serotypes after double restriction with endonucleases *Taq*I and *Sca*I. The DNAs in the lanes were from the following strains: lane 1, nonmotile E1 5.89 (O3,10); lane 2, Panama 73K (O9,12:Hl,v); lane 3, nonmotile D1 14.90 (O9,12); lane 4, nonmotile D1 10.90 (O9,12); lane 5, Typhi Ty2 (O9,12:Hd); lane M, 100-bp DNA ladder.

Restriction profile of the *fliC* gene from nonmotile strains. Both strains of serotype Gallinarum-Pullorum yielded a *fliC* restriction profile identical to those of the g,m and g,p types. This is compatible with the knowledge that serotype Gallinarum-Pullorum has a cryptic *fliC* gene coding for phase 1 antigen g,m (12).

Three nonmotile *Salmonella* strains of unknown serotype (isolates 14.90, 10.90, and 5.89) were examined by amplification and restriction of *fliC*. The restriction patterns obtained were directly compared with the profiles of serotype Typhi (H1-d) and serotype Panama (H1-l,v) on an agarose gel (Fig. 3), which permitted identification of strain 14.90 as a nonmotile variant of a serotype possessing the *fliC* gene encoding phase 1 flagellin type l,v. The phase 1 flagellin gene of strain 10.90 could be determined as type d. These results were consistent with those obtained by ribotyping (1a), which identified strain 14.90 as serotype Panama and strain 10.90 as serotype Typhi. The restriction pattern that was obtained for the third strain (strain 5.89) could not be identified with any of the serotypes included in the present study.

The use of restriction patterns of *fliC* genes proved to be a useful method for facilitating the identification of strains that have lost their flagellar antigens but that have a nonexpressed *fliC* gene. The first important application of the method was the distinction of serotype Gallinarum-Pullorum from nonmotile, Vi-negative variants of serotype Typhi. Although the two serotypes have different habitats (serotype Gallinarum-Pullorum strictly infects poultry, whereas serotype Typhi is adapted to humans), they can be found in the environment or in contaminated food. The present biochemical methods cannot distinguish between serotype Gallinarum-Pullorum and nonmotile, Vi-negative strains of serotype Typhi.

To establish the restriction patterns of *fliC* genes as a tool for identifying the major phase 1 flagellar antigens on a genetic basis, it is necessary to examine the restriction profiles of more strains with a complete or incomplete serotype. The validation of the amplification-restriction approach will require a few years of work to ensure that no rare flagellar types mimic commonly encountered flagellar types. It is too early in the development process to compare the relative costs of serotyping and amplification-restriction. The material is obviously more expensive on the amplification-restriction side, but the labor costs can be reduced by automation.

We can foresee with excitement the day when the major *Salmonella* serotypes are identified by restriction of amplified genes which code for flagellar antigens and those which are associated with the synthesis of somatic antigenic factors.

ACKNOWLEDGMENTS

Thanks are due to Michel Y. Popoff of the Collaborating Center for Reference and Research on *Salmonella*, World Health Organization, for reference strains and Francine Grimont and Catherine Dauga for helpful discussions.

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