Mapping of *Escherichia coli* H27-Specific Epitope from H-Specific Polypeptides

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A murine monoclonal antibody (MAb) reactive to H27 flagellin antigen was produced and characterized. Forty-nine partially purified native H-type flagellins were used to evaluate the specificity of the MAb. The *fliC* gene of H27 is 1,464 bp in length (487 amino acids [aa]); 50.88 kDa). The central variable region (CVR) of the H27 flagellin gene was defined by comparison with flagellin sequences derived from H8, H34, and H49. To study the distribution of antigenic epitopes, the CVR covering amino acid residues 70 to 457 (388 aa) was dissected into seven overlapping fragments. Fragments carrying the H-type-specific antigenic determinants were identified by H27-specific antisera. Polyclonal antibodies raised against different H-type flagellin proteins were used to determine the cross-reactive determinants. Three fragments, spanning amino acid residues 240 to 380, which carried the potential H-specific determinants were used for MAb production. A MAb specific to H27 was produced, and the specific epitope was mapped to amino acid residues 330 to 340. In this study, we produced MAbs from predetermined H27-specific polypeptides and used whole flagellin in enzyme-linked immunosorbent assays to circumvent the interference of anti-glutathione S-transferase antibodies. These factors when combined could help to improve the identification of the desired MAb.

The bacterial flagellum is a complex consisting of a basal body, a hook, and a filament (13). The flagellar filament is a homopolymer composed of a repeated protein, flagellin (7), which is *Escherichia coli* is encoded by the *fliC* gene (10). Sequence analysis of the *fliC* genes for different serological specificities reveals highly conserved N termini (150 amino acids) and C termini (100 amino acids) and a central region with considerable variation (10, 16, 17).

The N-terminal and C-terminal conserved regions are essential for the polymerization and secretion of flagellin molecules (4, 6, 8, 11). On the other hand, the central variable region (CVR) encodes major determinants contributing to the different *E. coli* H types (2, 9, 12, 15). Therefore, flagellin carries H-type-specific epitope(s) that are useful for H serotyping. The agglutination test is routinely used for *E. coli* H serotyping. Nevertheless, serological cross-reactions are frequently observed in agglutination tests due to the presence of cross-reactive epitopes on the different H-type flagellins. Therefore, identification of H-type-specific epitopes could help to improve *E. coli* serotyping. Studies of the characterization of H-specific epitopes by using monoclonal antibodies (MAbs) (3, 12, 15, 18) and polyclonal antibodies (17) have been reported. However, the production and characterization of MAbs to different H-type flagellins using whole-flagellin immunization are tedious and time-consuming. Several rounds of screening and confirmation are required to verify the antigenic specificity of each MAb. In this study, we modified the protocol with the following advantages: (i) only polypeptides displaying H specificity were introduced for immunization, (ii) the complete flagellin sequence was obtained, and (iii) cross-reactive-polypeptide information was available to facilitate the production and characterization of the desired MAb. In this study, we combined molecular cloning and gene expression to identify the potential H27-specific polypeptides and used the MAb technique to map the epitope.

**MATERIALS AND METHODS**

*Bacterial culture, plasmid and PCR amplification, and cloning of fliC*. H-type *E. coli* strain cultures purchased from ECRC (*Escherichia coli* Reference Center, Pennsylvania State University, University Park) were grown in Luria-Bertani medium (Becton Dickinson, Paramus, N.J.). Bacterial genomic DNA was prepared as previously described (14). Plasmid pGEX-2T (Amersham Pharmacia Biotech, Uppsala, Sweden) was used for glutathione S-transferase (GST) fusion protein expression. MacVector 6.0.1 (Oxford Molecular Group, Oxford, United Kingdom) was used to analyze the DNA and protein sequences. Oligonucleotide primers were designed based on the published sequence of the *E. coli* K-12 fliC gene (10). The forward primer, comfla-1 (5′-CCGGATCCATGGCACAAGTC ATTA-3′), contained the first 16 nucleotides of the *fliC* 5′ terminus with a BamHI restriction site (underlined), and the reverse primer, comfla-2 (5′-CCGG AAATCTTTAACCTGACTTAAGATCA-3′), contained 18 3′-terminal complementary nucleotides of *fliC* with an EcoRI restriction site (underlined).

**Recombinant truncated CVR of flagellin.** The CVR of H27 was defined by aligning it with the flagellin amino acid sequences derived from H8, H34, and H49 using the Mac Vector program. The CVR of H27, which comprised amino acid residues from 70 to 457 (388 amino acids [aa]), was dissected into seven truncated fragments by PCR. Oligonucleotide primers with a BamHI site at the 5′ end and an EcoRI site at the 3′ end were designed based on the sequence of the H27 *fliC* gene. The relative position of each truncated fragment was determined (see Fig. 2a). Each fragment was cloned and expressed as a GST fusion protein.

**Preparation of H-specific polyclonal antibodies from guinea pigs.** Native flagella were purified using semisolid medium and ultracentrifugation as described previously (3). Partially purified flagellin was emulsified with the adjuvant MONTANIDE ISA 70 (Seppic, Paris, France) according to the manufacturer’s protocol. Guinea pig anti-H antisera were prepared as described previously (17). Thirty-six *E. coli* H-specific antisera were generated in our laboratory: H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H14, H16, H18, H19, H21, H24, H27, H28, H29, H31, H32, H33, H34, H37, H38, H40, H42, H43, H44, H45, H46, H49, H52, and H54.

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Antigenic specificity of MAb. The antigenic specificity of the MAb H27S1 was confirmed by testing it against different H-type flagellins in ELISA. A panel of 49 partially purified H-type flagellins was used in this study. Each antigen was individually adsorbed onto microtiter wells in triplicate to verify the specificity of the MAb. MAb H27S1 showed a strong reaction to purified H27 flagellin but showed no reaction to other H-type flagellin proteins and had weakly positive reactions to H2, H4, H5, H6, and H38 (Table 1). These H-type flagellins later tested negative to H27S1 in immunoblotting.

Mapping of H27S1 epitope. Fragments 3, 6, and 7, comprising residues 240 to 380 of the H27 CVR, were initially used for MAb production. Therefore, the fragment containing the putative epitope which the MAb derived from was not immediately clear. The preliminary ELISA data showed that the MAb specifically reacted with fragment F3, indicating the location of the epitope (data not shown). With the specificity confirmed, fragment F3 (80 aa) was further dissected into three overlapping fragments, F3-1 (aa 270 to 300), F3-2 (aa 320 to 350), and F3-3 (aa 295 to 325), respectively (Fig. 3a). When incubated with the MAb, only fragment F3-2 (aa 320 to 350) was reactive, suggesting that fragment F3-2 carried the putative epitope of H27S1 (Fig. 3a). The minimal sequence of the H27-specific epitope was defined by serially truncating fragment F3-2 (aa 320 to 350) from both termini (Fig. 3b). Three overlapping constructs varying in length, sF3-1 (aa 325 to 340; 16 residues), sF3-2 (aa 330 to 345; 16 residues), and sF3-3 (aa 330 to 340; 11 residues), were constructed. Interestingly, the MAb reacted indiscriminately with these fragments in an immunoblotting assay, suggesting that all three fragments shared a common sequence which was essential to form an immunogenic epitope. Therefore, the H-type epitope of H27 was found in residues 330 to 340.

DISCUSSION

An agglutination test based on the somatic (O), capsule (K), and flagellin (H) antigens is conventionally used to identify E. coli serotypes. However, the presence of common epitopes in these antigens has resulted in some H-type E. coli isolates not being readily discriminated. In this study, we report a method to map the H27-specific epitope combining molecular cloning, gene expression, MAbs, and polyclonal antibodies. The distribution of both specific and cross-reactive determinants could be readily determined by antisera to different H-type flagellins. In the process of MAb production, only those polypeptides displaying H27 specificity with few antigenic determinants (compared to whole-flagellin or whole-bacteria immunization) were used for MAb production. In the subsequent screening stages, purified H27 flagellin was used in ELISA to enhance the screening efficiency. In conventional H epitope mapping, whole flagellin is used for both MAb production and ELISA screening. Therefore, the lack of flagellin sequence information, the dominance of cross-reactive epitopes, and the undefined potential H-specific polypeptides hinder epitope identification.

We used the H type H27 as an example to illustrate the mapping strategy to identify the H27-specific epitope. The CVR of H27 was first defined by aligning it with three sequences (H8, H34, and H49) (unpublished results). Sequence alignment revealed that two regions in the H27 flagellin near

**RESULTS**

**H27 fliC gene analysis and determination of CVR.** A single DNA fragment was amplified from H27 genomic DNA. The PCR fragment was restricted with BamHI and EcoRI and cloned into the pGEX-2T expression vector for sequence analysis. The fliC gene of H27 was found to contain 1,464 bp, and the deduced protein had a relative molecular mass of 50.88 kDa. The CVR of H27, which comprised amino acid residues 70 to 457, was defined by aligning it with the amino acid sequences of fliC genes derived from H8, H34, and H49 using the MacVector program (Fig. 1).

**Construction of overlapping fragments and distribution of antigenic sites on the CVR of H27.** The CVR of H27 was dissected into seven overlapping fragments by PCR, and each fragment was expressed as a GST fusion protein. The relative size and position of each fragment is illustrated in Fig. 2a. These fragments were useful in understanding the distribution of antigenic sites in the H27 CVR. When all the truncated fragments were incubated with anti-H27 flagellin antiserum in immunoblotting, the antigenic sites of H27 were primarily found in fragments 1 (aa 70 to 150), 3 (aa 270 to 350), 4 (aa 370 to 457), 5 (aa 140 to 180), 6 (aa 240 to 280), and 7 (aa 340 to 380) (Fig. 2b).

**Determination of the distribution of cross-reactive determinants by using different H-type antisera.** The CVR of flagellin has been shown to carry both H-type-specific and cross-reactive epitopes. Cross-reactive epitopes can be identified by using different H-type-specific antisera. Truncated H27 CVR fragments were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred nitrocellulose membranes for immunoblotting. Thirty-six antisera raised against different H-type flagellins, excluding anti-H27 flagellin antiserum, were pooled and incubated with all of the truncated H27 CVR fragments. As shown in Fig. 2c, fragment 1 (aa 70 to 150), fragment 4 (aa 370 to 457), and fragment 5 (aa 140 to 180) were positive, suggesting that these fragments carried cross-reactive determinants. Therefore fragments 3, 6, and 7, carrying H27-specific epitopes, were used for MAb production.

**Production of MAb.** Fragments 3, 6, and 7 from the H27 CVR, comprising residues 240 to 380 and showing specificity for H27 flagellin, were used for MAb production. A hybridoma clone, H27S1, showing a positive reaction to partially purified native H27 flagellin, was recovered. This MAb was identified as the immunoglobulin G1 subtype.
FIG. 1. Amino acid sequences of flic genes from H types H8, H27, H34, and H49. All sequences were aligned using MacVector to define the CVR of H27. The CVR of H27 was designed to cover residues 70 to 457 (indicated by the arrows). Identical amino acid residues are boxed and darkly shaded. Gaps inserted to facilitate alignment are denoted by dashed lines.
the N and C termini carried several nonidentical amino acids: region 1, comprising residues 100 to 150 (equivalent to fragment 1 in Fig. 2a), and region 2, comprising residues 540 to 614 (equivalent to fragment 4 in Fig. 2a) (Fig. 1). The functional roles of these nonidentical amino acid residues in sequence conservation and cross-reactive-epitope formation were not clear. Therefore, the H27 CVR was designed to include regions 1 and 2 (comprising residues 70 to 457). There was a slight difference in the conservation of residues according to the published result (16). However, both region 1 (fragment 1) and region 2 (fragment 4) showed strong reactions with non-H27 antisera in an immunoblotting assay (Fig. 2c), suggesting that regions 1 and 2 could be either carrying cross-reactive determinants or sharing conserved sequence with other H-type flagellins.

A hybridoma clone, H27S1, reactive to purified H27 flagellin in both ELISA and immunoblotting, was produced by immunizing BALB/c mice with H27-specific polypeptides. The specificity of the MAb was confirmed by reacting it with a panel of 49 partially purified flagellins. Most of the flagellins gave readings equivalent to that of the negative control except H types H2, H4, H5, H6, and H38 (Table 1). Flagellin extracted from H types H2, H4, H5, H6, and H38 were weakly reactive in an ELISA screen, which was later proven to be nonspecific interaction in an immunoblotting assay (data not shown). In order to determine the smallest fragment sequence encoding the

![FIG. 2. Truncation of H27 CVR. These fragments were used to study the distribution of H27-specific and cross-reactive determinants. See Results for details. (a) The 388-aa variable region (amino acid residues 70 to 457) of flIC from H27 was dissected into seven overlapping fragments (F1 to F7). (b) Recombinant peptides reacted with anti-H27 antiserum, showing the distribution of immunogenic sites. (c) Thirty-six antisera were pooled to identify the cross-reactive determinants in the CVR of H27. An arrow denotes each truncated flagellin.](image-url)

| H-type flagellin<sup>a</sup> | OD<sub>490</sub><sup>b</sup> | Immunoblotting result |
|-----------------------------|-------------------------|----------------------|
| H2                          | 0.107 ± 0.007           | −                    |
| H4                          | 0.134 ± 0.055           | −                    |
| H5                          | 0.223 ± 0.079           | −                    |
| H6                          | 0.135 ± 0.037           | −                    |
| H38                         | 0.11 ± 0.0136           | −                    |
| H27                         | 1 ± 0.170               | +                    |
| H-types<sup>c</sup>         | 0.073 ± 0.01            | −                    |

<sup>a</sup> Partially purified H-type flagellin

<sup>b</sup> ELISA results are expressed as means of three OD determinations ± standard deviations.

<sup>c</sup> H-type flagellin tested in ELISA: H1, H3, H7, H8, H9, H10, H11, H12, H14, H15, H16, H17, H18, H20, H21, H23, H24, H25, H26, H28, H29, H30, H31, H32, H33, H34, H36, H37, H39, H40, H41, H42, H43, H44, H45, H46, H47, H48, H49, H51, H52, H53, and H54.
H27-specific epitope, three overlapping polypeptides with the core sequence KAATASDLDLN, comprising residues 330 to 340, were constructed. All of the polypeptides, sF3-1, sF3-2, and sF3-3, reacted indiscriminately with the MAb (Fig. 3b). Therefore, the H27-specific epitope was mapped to amino acid residues 330 to 340. The epitope sequence was submitted to GenBank for comparison. A candidate flagellin gene, STEC-O26:H11 (19), which has one amino acid different from the H27 epitope at residue 334, was identified from GenBank. An alanine (A) at residue 334 of the H27 flagellin was replaced by a leucine (L) at the same position in the H11 flagellin. However, ELISA and immunoblotting repeatedly showed that there was no cross-reaction between these two H types and ruled out the possibility that the identified region could be an immunodominant region in other serotypes, such as H type H11.

We also tried to predict the putative H-specific epitope by comparing flagellin amino acid sequences derived from several H-type E. coli strains. However, no significant conclusion was made based on the sequence alignment. In addition, no consensus sequence was defined due to the highly variable CVR. Moreover, it has been shown that a single-amino-acid change in the CVR is sufficient to determine a single serotype in E. coli (12). The role of the primary sequence of flagellin in determining the specificity and cross-reaction of H-antigen is clear, but more flagellin sequences are needed in order to identify the H-specific epitope through sequence analysis.

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