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# Hypervariable Region IV of *Salmonella* Gene *fliC*$^d$ Encodes a Dominant Surface Epitope and a Stabilizing Factor for Functional Flagella

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**To identify the major antigenic determinant of native *Salmonella* flagella of antigenic type *d*, we constructed a series of mutated *fliC*$^d$ genes with deletions and amino acid alterations in hypervariable region IV and in regions of putative epitopes as suggested by epitope mapping with synthetic octameric peptides (T. M. Joys and F. Schödel, Infect. Immun. 59:3330–3332, 1991). The expressed product of most of the mutant genes, with deletions of up to 92 amino acids in region IV, assembled into functional flagella and conferred motility on flagellin-deficient hosts. Serological analysis of these flagella with different anti-*d* antibodies revealed that the peptide sequence centered at amino acids 229 to 230 of flagellin was a dominant B-cell epitope at the surface of *d* flagella, because replacement of these two amino acids alone or together with their flanking sequence by a tripeptide specified by a linker sequence eliminated most reactivity with antisera against wild-type *d* flagella as tested by enzyme-linked immunosorbent assay or by Western immunoblot. Functional analysis of the mutated flagellin genes with or without an insert suggested that amino acids 180 to 214 in the 5′ part of hypervariable region IV (residues 181 to 307 of the total of 505) is important to the function of flagella. The hybrid proteins formed by insertion of peptide sequence pre-S1 12–47 of hepatitis B virus surface antigen into the deleted flagellins assembled into functional flagella, and antibody to the pre-S1 sequence was detected after immunization of mice with the hybrid protein. This suggests that such mutant flagellins containing heterologous epitopes have potential as vaccines.**

The bacterial flagellar filament consists of a single protein named flagellin (14). The flagellin monomer, after synthesis inside the cell, is believed to travel by a central channel through the rod, hook, and filament and to be added to the filament at its tip. The rigid helical structure of flagellar filaments enables them to impart translational movement to the cell when they are driven by rotary motors. This unique property suggests that the primary structure of flagellin may be subject to rigorous constraints to ensure its proper transportation and correct assembly into a functional structure.

The *Salmonella* flagella carry the H antigen, one of the major antigens that elicit immune responses in the infected host (11). The H antigen is highly variable, with various serotypes (5). The antigenic properties of flagella have been studied by selection and genetic analysis of spontaneous, serum-selected flagellar antigen mutants (18–20), with results suggesting that only a small part of the flagellin molecule carries the flagellar antigen determinants. The cloning and sequencing of several *Salmonella* flagellin genes (13, 36, 37) showed highly conserved amino acid sequences at the ends and hypervariable regions at the middle of flagellin, a protein of 505 amino acids (aa). This result suggests that the structural and functional features common to different flagella are determined by the conserved ends of flagellin genes, whereas the serological variety of different flagella is determined by the center part, especially its hypervariable regions IV (aa 181 to 307) and VI (aa 333 to 370) (amino acid residues numbered from the N-terminal alanine of flagellin, encoded by the codon next to the initiation codon [16, 36]). By comparing the nucleotide sequences of *fliC*$^d$ and *fliC*$^j$, which encode two flagellin variants of *Salmonella typhi* with distinct serotypes *d* and *j*, Frankel et al. (6) showed that the gene for flagellar antigen *j* was derived from that for antigen *d* by deletion of 261 bp, which encoded aa 217 to 303 within region IV. This result indicates that the *d* flagellar antigenic determinants reside within region IV. Newton et al. (27) further characterized these determinants by study of selected mutants for their ability to spread in semisolid medium in the presence of a just-immobilizing concentration of anti-flagellar antibodies and an in vitro deletion mutant generated by *Eco*RV digestion and religation; three segments, aa 207 to 222 (encoded by nucleotides 634 to 681, the segment between two *Eco*RV sites), aa 212 to 235 (nucleotides 649 to 720), and aa 257 to 291 (nucleotides 784 to 888), and one amino acid residue, aa 245 (nucleotide 749), were identified as involved in flagellar epitopes. All these segments are within and cover about 50% of region IV.

Recently Joys and Schödel (17) searched the complete amino acid sequence of *d* flagellin from *S. muenchen* for antigenic determinants by use of octameric peptides synthesized on polyethylene pins. Peptides from four regions, all in the middle part of the flagellin gene, reacted with each of several anti-*d*-flagella antisera; this suggests that they are major flagellar antigenic determinants. Two of them were in hypervariable region IV: the octameric peptides 224 (aa 224 to 231) to 230 (aa 230 to 237) and 237 (aa 237 to 244) to 240 (aa 240 to 247); the other two were in regions V and VI. One of the most reactive peptides, Phe-Lys-Asp-Gly-Gln-Tyr-Tyr-Leu (FKDGQYYL), or aa 225 to 232, fell within one of the segments identified by Newton et al. (27), i.e., aa 212 to 235.

To further identify the major antigenic determinants of native flagellin protein, we have constructed a series of mutant flagellin genes with different segments of region IV deleted in

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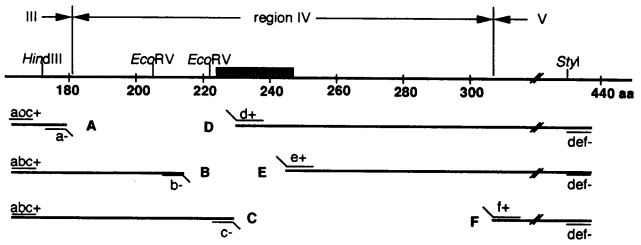


FIG. 1. Six flagellin gene fragments generated by PCR, using cloned *fliC*$^d$ (*S. muenchen*) in pLS405 as template and pairs of oligonucleotides (see Fig. 2) as primers. The hatched bar represents aa 224 to 237 and aa 237 to 247, the two epitopes as determined by synthetic octameric peptides (17). The PCR products were digested with *Hin*dIII and *Bam*HI (for fragments A, B, and C) or *Bam*HI and *Sty*I (for fragments D, E, and F) to create corresponding sticky ends. Hypervariable region IV, comprising aa 181 to 307, shows less than 30% homology among different serotypes (36).

| Primer | Sequence |
|---|---|
| abc+ | 5'-TAGCTCTAAAACACTGGGACT-3' |
| a- | 3'-ACAGGTCCTACGGATGTGGCCTAGGGC-5' *Bam*HI |
| b- | 3'-GTTTGACGTTAACCGCCACCTAGGGC-5' *Bam*HI |
| c- | 3'-CCCGACTATAGTTTAAATTTCTACCTAGGGC-5' *Bam*HI |
| d+ | 5'-CGGGATCCCCCGGGTATTTAGATGTTAAAGGCGGTGC-3' *Bam*HI *Sma*I |
| e+ | 5'-CGGGATCCCCCGGGGCCACTTATGATGAAACTACAAAG-3' *Bam*HI *Sma*I |
| f+ | 5'-CGGGATCCCCCGGGGCCGATAAGGACAATACT-3' *Bam*HI *Sma*I |
| def- | 3'-ACGGAACCGTGTCCAACTAA-5' *Sty*I |

FIG. 2. PCR primers for generating flagellin gene fragments. Symbols: +, upstream primers; −, downstream primers (see Fig. 1).

vitro. Serological analysis of these genetically engineered flagellins indicated a dominant surface epitope centered at aa 229 to 230. When a foreign peptide sequence derived from the pre-S1 region of the surface antigen of hepatitis B virus (HBV) was inserted into or replaced this flagellar epitope, the hybrid flagellins were assembled into functional flagella which were immunogenic and elicited antibody to the viral antigen when used to immunize mice.

## MATERIALS AND METHODS

**Bacterial strains.** *Escherichia coli* CL447 (C600 *hag* = *fliC*) (25), *S. dublin* SL5928 (*fliC*$^i$::Tn*10*) (26), and *S. typhimurium* LB5000 ($r^-$ $m^+$ *flaA66*) (2, 30) have been described previously. *S. dublin* SL7276 (32) was derived from SL5928 by selection for tetracycline sensitivity by the method of Bochner et al. (1); it is presumed to have a Tn*10*-derived deletion or inversion, preventing reversion to *fliC*$^+$ by "clean excision" but not extending into the adjacent genes *fliD* or *fliA*, since a *fliC*$^+$ gene (in pLS408 [27]) introduced into SL7276 makes it motile in a semisolid medium. (The relationship of two newly discovered genes reported to be essential for motility, *fliU* and *fliV* [3], between *fliC* and *fliA*, to the secondary mutation of SL7276 has not been investigated.) All bacterial hosts with plasmids were grown in Luria broth (LB) containing 50 μg of ampicillin per ml.

**Construction of recombinant flagellin genes.** To construct the series of deleted flagellin genes, the 3.5-kb *Eco*RI fragment carrying the wild-type *d* flagellin gene *fliC* of *S. muenchen* was first moved from pLS405 (26) to the *Eco*RI site of pUCH, a modified pUC19 vector in which the *Sma*I-*Hin*dIII portion of the polylinker has been removed. The new plasmid, named pUCHF, has a unique *Hin*dIII site at nucleotide 526 of *fliC* and no *Bam*HI site. Six DNA fragments were synthesized by PCR (12) (Fig. 1), using pairs of oligonucleotides (Fig. 2) as primers and the wild-type *fliC*$^d$ gene in pLS405 as a template. These PCR products were digested with *Hin*dIII and *Bam*HI (for fragments A, B, and C) or *Bam*HI and *Sty*I (for fragments D, E, and F) to generate cohesive termini and were ligated with plasmid vectors. By serial subcloning, each of fragments A, B, and C was combined with one of fragments D, E, and F and used to replace the *Hin*dIII-*Sty*I segment of *fliC* on pUCHF, generating a total of nine plasmids, pXSH501 through pXSH509 (Table 1). To construct the recombinant flagellin genes with an insert encoding the peptide sequence pre-S1 12–47 of HBV surface antigen, we synthesized the insert DNA fragment by PCR with the oligonucleotides 5′-CGGGATC CATGGGGATGAATCTTTCTG-3′ and 5′-TCCCCCGGGG TTGAAGTCCCAATCTG-3′ as primers and plasmid pBR322-adw$_2$, which carries a head-to-tail dimer of the cloned HBV genome (31), as the template. The PCR product was digested with *Bam*HI and *Sma*I to generate corresponding ends and ligated with vectors pXSH501 through pXSH509, cut with the same restriction enzymes at the *Bam*HI-*Sma*I linker introduced into the recombinant flagellin genes carried by these vectors. All the cloning procedures were performed with *E. coli* JM109 as the host. The nucleotide sequences of all the PCR-generated DNA fragments were verified by sequencing. Correctly constructed plasmids were transformed into the flagellin-negative *E. coli* CL447 or into *S. typhimurium* LB5000 ($r^-$ $m^+$) and then into the flagellin-negative *S. dublin* SL5928 or SL7276.

**Antibodies.** Rabbit antiserum U121 (kindly provided by R. Brey, Praxis Biologics, Rochester, N.Y.) was raised against a purified protein expressed from a fusion flagellin gene with a frameshift at the join of the heterologous gene to the *fliC* gene cut by *Eco*RV, resulting in truncation by a stop codon a little way downstream of the join. U116 is a rabbit antiserum against wild-type *d* flagella of *S. typhi*, obtained from the National Bacteriological Laboratory, Stockholm, Sweden. U173 is a rabbit antiserum against wild-type *d* flagella of *S. muenchen*, kindly provided by T. M. Joys. U168 is a pool of mouse antisera against wild-type *d* flagella of *S. muenchen*. U117 is a monoclonal antibody against *d* flagella of *S. typhi* (28), kindly provided by A. Qadri. F2 is a pool of mouse antisera against flagella made of a recombinant *d* flagellin with aa 215 to 244 replaced by Ser-Pro-Gly. E2 is a pool of mouse antisera against the synthetic peptide of pre-S1 12–47 of HBV surface antigen, raised by immunizing BALB/c mice with the peptide conjugated to thyroglobulin, a gift from A. Judd. Peroxidase- or alkaline phosphatase-labeled secondary antibodies were from Boehringer Mannheim or Sigma.

**Preparation of flagella.** To prepare the partially purified flagella for immunizing mice, the flagellin-negative strain *S. dublin* SL7276 harboring plasmids with recombinant flagellin genes was grown on agar plates at 37°C overnight. The cells were washed off the agar surface with phosphate-buffered saline (PBS), transferred into capped centrifuge tubes, and

TABLE 1. Mutated flagellin gene constructs

| Plasmid | Gene | Construct[a] | | | Deletion (no. of residues) |
|---|---|---|---|---|---|
| | | (A, B, C) + | linker | + (D, E, F) | |
| pXSH501 | *fliC*$^{d}\Delta$(180-306) | A + | Gly-Ser-Pro | + F | 127 |
| pXSH502 | *fliC*$^{d}\Delta$(215-306) | B + | Ser-Pro | + F | 92 |
| pXSH503 | *fliC*$^{d}\Delta$(229-306) | C + | Ser-Pro | + F | 78 |
| pXSH504 | *fliC*$^{d}\Delta$(180-230) | A + | Gly-Ser-Pro-Gly | + D | 51 |
| pXSH505 | *fliC*$^{d}\Delta$(180-244) | A + | Gly-Ser-Pro-Gly | + E | 65 |
| pXSH506 | *fliC*$^{d}\Delta$(215-230) | B + | Ser-Pro-Gly | + D | 16 |
| pXSH507 | *fliC*$^{d}\Delta$(215-244) | B + | Ser-Pro-Gly | + E | 30 |
| pXSH508 | *fliC*$^{d}\Delta$(229-230) | C + | Ser-Pro-Gly | + D | 2 |
| pXSH509 | *fliC*$^{d}\Delta$(229-244) | C + | Ser-Pro-Gly | + E | 16 |

[a] Each mutated flagellin gene construct is a combination of one of the PCR fragments A, B, and C with one of the fragments D, E, and F (Fig. 1) and hence has a DNA segment replaced by a *Bam*HI-*Sma*I linker sequence GGATCCCCCGGG encoding Gly-Ser-Pro-Gly, which was introduced through the PCR primers. Primers b−, c−, and f+ (Fig. 2) were designed in such a way that the Gly codon of the linker sequence overlapped their adjacent flagellin residues Gly-214, Gly-228, and Gly-307; therefore, the number of foreign residues encoded by the linker is not identical in all the constructs.

vortexed at the highest speed for 10 min to shear off the flagella. The bacterial suspensions were centrifuged at 12,000 × *g* for 10 min to remove the cells. The cell-free flagellum suspensions were then ultracentrifuged at 110,000 × *g* for 2 h. The pelleted flagella were resuspended in PBS and examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To prepare the purified flagellin as the antigen for immunoassay of anti-flagellin antibody, we used the procedure of Ibrahim et al. (10) with minor modifications.

**Immunization of mice.** Groups of three or four female BALB/c mice (6 weeks old) were immunized by intraperitoneal injection with 15 μg of the partially purified flagella mixed with MPL+TDM emulsion (Ribi ImmunoChem Research, Inc.) containing 50 μg of each adjuvant, MPL and S-TDCM, per 0.2 ml (a mouse dose). At 3 weeks later the mice were boosted with another intraperitoneal injection of the same dose of antigen without adjuvant. Blood was collected 3 weeks after the second injection.

**Enzyme-linked immunosorbent assay (ELISA) of recombinant flagellins.** *Salmonella* cultures grown to saturation in L broth at 37°C with vigorous agitation were centrifuged to remove cells. Aliquots of each cell-free supernatant were added to wells of Immulon II microtiter plate (Dynatech Laboratories, Inc.) and held at 4°C overnight. After being washed with PBS, the wells were filled with 5% skim milk in PBS, incubated at 37°C for 2 h, and washed with PBS. Antibodies diluted in 3% bovine albumin in PBS were added to each well and incubated at 37°C for 3 h. The plate was again washed with PBS, incubated with peroxidase-labeled secondary antibodies at room temperature for 1 h, washed with PBS, and developed by incubation with a substrate solution containing hydrogen peroxide and *o*-phenylenediamine dihydrochloride tablet (Sigma). The reaction was stopped after 30 min by addition of 3 N HCl, and the optical density at 492 nm ($OD_{492}$) was read.

## RESULTS

**Construction of deleted flagellin genes and test of their function.** To define the antigenic determinants of region IV, we used PCR to create a series of mutated flagellin gene. The PCR primers were designed with the following goals in mind: (i) to examine the effect of amino acid substitutions within aa 225 to 232, the most reactive peptide FKDGQYYL, as determined by epitope mapping (16); (ii) to test the effect of deletion of parts or the whole of region IV; and (iii) to construct new vectors for insertion of foreign epitopes into flagellin. Eight oligonucleotides (Fig. 2), each a complement to a specific sequence of the flagellin gene and, where appropriate, containing a linker sequence of *Bam*HI (downstream primers a−, b−, and c−) or *Bam*HI-*Sma*I (upstream primers d+, e+, and f+) restriction sites at its 5′ end, were thus designed and synthesized. Using the cloned wild-type *d* flagellin gene *fliC* of *S. muenchen* in pLS405 as the template and combinations of the oligonucleotides as primers, we performed PCR (Fig. 1) followed by restriction enzyme digestion, generating six DNA fragments with cohesive termini. By using the combination of these six fragments to replace the *Hin*dIII-*Sty*I segment, or nucleotides 526 to 1286 (36), of *fliC*, a total of nine mutant flagellin genes were created (Table 1), each with a deleted DNA segment of different length replaced by a *Bam*HI-*Sma*I linker of 12 bp specifying Gly-Ser-Pro-Gly (GSPG). These nine mutant genes, carried by a pUC19-based plasmid vector, were designated pXSH501 through pXSH509: pXSH501 has 127 aa deleted (including the whole region IV); pXSH502, pXSH503, pXSH504, and pXSH505 have different segments ranging from 51 to 92 residues within region IV deleted; pXSH508 has residues QY (aa 229 to 230) within the most antigenic peptide FKDGQYYL replaced by residues SPG encoded by the linker sequence; pXSH507 has a segment of 30 residues (aa 215 to 244) deleted (including the two putative epitopes aa 224 to 237 and aa 237 to 247), while pXSH506 and pXSH509 have the N-terminal or C-terminal 16 residues of this 30-aa segment (aa 215 to 244) deleted, respectively.

The plasmid vectors carrying the nine deleted flagellin genes or the wild-type flagellin gene were introduced into the flagellin-negative strain *S. dublin* SL5928 by transformation. The expressed products present in the supernatant of vigorously shaken broth culture were analyzed by SDS-PAGE (Fig. 3). A single major Coomassie blue-stained band was seen at the position expected for proteins encoded by the mutant or wild-type flagellin genes. The yield of each mutated flagellin in the supernatant was two to three times greater than that of the wild-type flagellin as judged by band intensity. When whole-cell lysates of these strains were analyzed by SDS-PAGE and Coomassie blue staining, bands were seen at the same positions (see Fig. 5). Thus all the deleted genes are expressed correctly and at least some of their products are transported out of the cells.

The motility of the flagellin-negative strains *E. coli* CL447 and *S. dublin* SL5928 carrying the mutated flagellin genes was tested on soft agar plates (4) (Table 2). All the deleted flagellins rendered *S. dublin* SL5928 motile, as indicated by

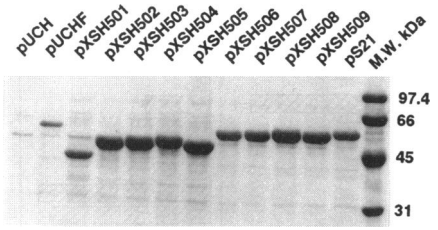


FIG. 3. Expression of deleted flagellin genes in *Salmonella* spp. Cultures of flagellin-negative strain SL5928 carrying plasmids with different flagellin genes were grown at 37°C for 7 h with vigorous shaking. Cells were removed by centrifugation. Proteins in the cell-free supernatants were precipitated with 75% ammonium sulfate, resuspended in PBS, and analyzed by SDS-PAGE. The gel was stained with Coomassie blue. Protein equivalent to the amount in 100 μl of cell-free supernatant was loaded in each lane. pUCH is a plasmid vector without the flagellin gene. pUCHF is the pUCH vector carrying the wild-type *d* flagellin gene. pS21 is a pUC19 vector carrying a wild-type flagellin gene with an insert replacing the sequence between its *Eco*RV sites (see text for details). M.W.kDa, molecular mass in kilodaltons.

spreading growth in soft agar, except that encoded by pXSH501, which made the bacteria marginally motile; the *E. coli* hosts harboring pXSH506, pXSH507, pXSH508, or pXSH509 were motile; those with pXSH502 or pXSH503 were marginally motile; whereas those with pXSH501, pXSH504, or pXSH505 were nonmotile. Thus hypervariable region IV (aa 181 to 307) is not essential for motility, but removal of this entire region impairs motility. Residues 180 to 214, which are missing in pXSH501, pXSH504, and pXSH505 but present in all the other constructs, appear to be required for motility in *E. coli* CL447. The lesser motility conferred by the deleted flagellin genes in *E. coli* compared with *Salmonella* strains may be due to the greater motility of wild-type *Salmonella* spp. compared with *E. coli* and/or to lower expression of these genes of *Salmonella* origin in *E. coli* (data not shown).

**The peptide sequence centered at aa 229 to 230 is the dominant surface epitope.** The reactivity of wild-type and deleted flagellin proteins in their native conformation with different anti-*d* sera and monoclonal antibody was tested by an antibody capture ELISA, in which the wells of a microtiter plate were coated with cell-free supernatant of saturated *Salmonella* culture which contained flagella released or broken off from the cells. The plastic-bound flagella, not exposed to any denaturing or depolymerizing conditions, will capture any antibodies against the epitopes on their surface. After incubation with diluted antibody solutions, the anti-flagellin antibodies bound in the wells were detected with an enzyme-labeled secondary antibody. When a flagellum-containing supernatant serially diluted with a flagellum-free supernatant was used, the $OD_{492}$ signal was proportionally reduced (data not shown), so that the $OD_{492}$ value measures the quantity of flagellar protein bound in the wells.

TABLE 2. Motility of bacteria expressing recombinant flagellin genes

| Plasmid | Gene | Motility[a] of strain: | | |
|---|---|---|---|---|
| | | Without insert | | With pre-S1 12–47 insert (SL5928) |
| | | CL447 | SL5928 | |
| pXSH501 | *fliC*$^d$Δ(*180–306*) | − | ± | ± |
| pXSH502 | *fliC*$^d$Δ(*215–306*) | ± | + | + |
| pXSH503 | *fliC*$^d$Δ(*229–306*) | ± | + | + |
| pXSH504 | *fliC*$^d$Δ(*180–230*) | − | + | ± |
| pXSH505 | *fliC*$^d$Δ(*180–244*) | − | + | ± |
| pXSH506 | *fliC*$^d$Δ(*215–230*) | + | + | + |
| pXSH507 | *fliC*$^d$Δ(*215–244*) | + | + | + |
| pXSH508 | *fliC*$^d$Δ(*229–230*) | + | + | + |
| pXSH509 | *fliC*$^d$Δ(*229–244*) | + | + | + |

[a] Symbols: +, motile (spreading in soft agar detectable within a 12-h incubation); ±, marginally motile (spreading in soft agar detectable after a 24-h incubation); −, nonmotile (no spreading after 24-h incubation).

The antibodies used were U121, a rabbit antiserum against a recombinant *d* flagellin truncated a little way downstream of its *Eco*RV site, hence retaining the 206 N-terminal amino acid residues of wild-type flagellin; U173, a rabbit antiserum against wild-type *d* flagella of *S. muenchen*; U168, a pool of mouse antisera against wild-type *d* flagella of *S. muenchen*; U116, a rabbit antiserum against wild-type *d* flagella of *S. typhi*; U117, a monoclonal antibody against wild-type *d* flagella of *S. typhi* (28), whose epitope specificity has not previously been defined; and F2, a pool of mouse antisera against flagella formed by the expression product of the deleted flagellin gene *fliC*$^d$Δ(*215–244*) of pXSH507, which has aa 215 to 244 replaced by the tripeptide Ser-Pro-Gly. A total of 12 *Salmonella* strains were tested with these antibodies (Fig. 4), 9 of them harboring mutated flagellin gene vectors pXSH501 through pXSH509, 1 carrying the wild-type gene *fliC*$^d$ in pUCHF, 1 carrying the recombinant flagellin gene vector pS21 (38) with the 16 residues between the two *Eco*RV sites (aa 207 to 222) replaced by 26 residues derived from the pre-S2 region of HBV surface antigen, and 1 carrying plasmid pUCH without a flagellin gene, hence not producing any flagellin. As shown in Fig. 4A, the anti-truncated-flagellin serum U121 reacted with all the deleted flagellins (pXSH501 to pXSH509) and with wild-type flagellin carrying or not carrying an insert between the *Eco*RV sites (pS21 and pUCHF); this is presumably because they all have an N-terminal segment, aa 1 to 206, in common with the truncated flagellin used to raise this rabbit antiserum, with the exception that pXSH501, pXSH504, and pXSH505 lacked aa 180 to 206. Antibodies U173, U116, U117, and F2 reacted differently with the wild-type and mutated flagella. To compare the reactivity of different mutants on the basis of equal amounts of antigen protein, the $OD_{492}$ reading with the anti-truncated-flagellin antiserum U121 of each strain (except those carrying pXSH501, pXSH504, and pXSH505) was used to normalize the quantity of flagellar protein in the wells. In this way we defined the specific reactivity of each mutant (or wild-type) flagellum with a given serum as the ratio of the OD with that antibody to the OD with the anti-truncated-flagellin serum. The specific reactivity of each construct is divided by that of the wild-type flagella (pUCHF) to give the relative reactivity (Fig. 4B) for all sera used except F2, for which all specific reactivities were compared with that of pXSH507, which carried the mutated gene encoding the immunogen used to raise this antibody.

Figure 4B shows that the replacement of aa 229 to 230 (QY) of wild-type *d* flagella with SPG (pXSH508) reduced reactivity with the anti-*d* (*S. muenchen*) serum U173 and anti-*d* (*S. typhi*) serum U116 by 80 and 96% respectively, whereas the additional deletion of 14 adjacent residues on either side (pXSH506 and pXSH509) or both sides (pXSH507) of aa 229 to 230 caused only a slight further reduction in reactivity. The pooled mouse anti-*d* (*S. muenchen*) serum U168 gave the same result as rabbit anti-*d* (*S. muenchen*) serum U173 (data not shown). These results suggest that aa 229 to 230 is involved in the major antigenic determinant(s) at the surface of flagella. The result with monoclonal antibody U117 further suggested that the peptide sequence centered at aa 229 to 230 contains a

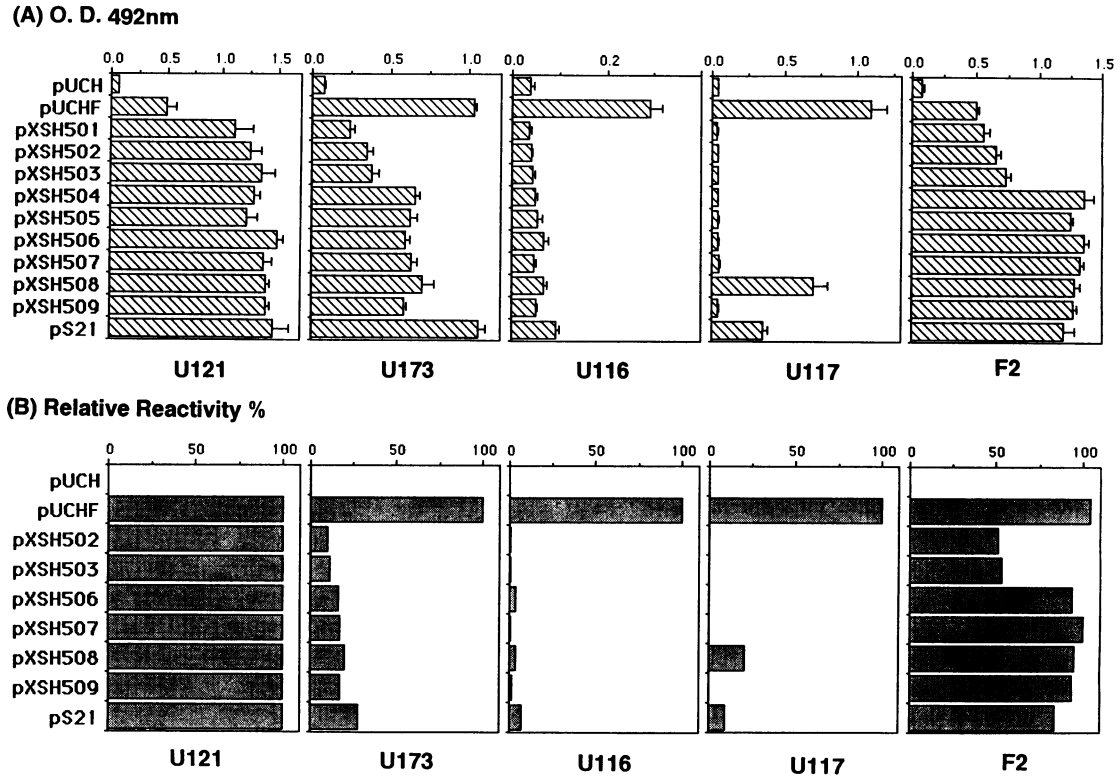


FIG. 4. (A) ELISA of recombinant flagellins in the cell-free supernatant. Wells of microtiter plates were coated with supernatants of saturated cultures of *S. dublin* SL5928 each carrying a flagellin gene and incubated with antibodies diluted as following: U121 (antiserum against truncated flagellin), 1:2,000; U173 (antiserum against *d* flagella of *S. muenchen*), 1:1,000; U116 (antiserum against *d* flagella of *S. typhi*), 1:250; U117 (monoclonal antibody against *d* flagella of *S. typhi*) in the form of reconstituted ascite, 1:2,000; F2 (pool of mouse antisera against flagella formed by expression product of the mutated flagellin gene of pXSH507), 1:500. The diluted antisera U173 and F2 were preadsorbed with acetone powder (8) of SL5928(pUCH). pUCH is a plasmid vector without the flagellin gene. pUCHF is the pUCH vector carrying the wild-type gene *fliC*$^{d}$ of *S. muenchen*. pS21 is a pUC19 vector carrying wild-type *fliC*$^{d}$ with an insert replacing the sequence between its *Eco*RV sites. pXSH501 through pXSH509 are deleted flagellin gene vectors. See the text for a detailed description of these constructs. The $OD_{492}$ was read and is displayed in the figure. Each bar is the mean of readings from four wells. (B) Relative reactivity of the recombinant flagella. The $OD_{492}$ value for each flagellum with a given antibody was divided by the $OD_{492}$ value for the flagellum with U121 to give its specific reactivity with the antibody. The $OD_{492}$ value for the flagellin-negative strain (pUCH) has been deducted from those of other strains as the background reading. To calculate the relative reactivity shown in this graph, the specific reactivity of each recombinant flagellum was divided by that of the wild-type *d* flagellum (pUCHF) for all the antibodies except F2, for which the specific reactivity of pXSH507 was used as the reference to calculate the relative reactivity of other constructs.

single dominant epitope of wild-type flagellin, since replacement of these two amino acids (QY) with SPG reduced the reactivity with this monoclonal antibody by 75% whereas absence of an additional 14 residues on either side of aa 229 to 230 totally destroyed reactivity. The replacement of the 16 residues between two *Eco*RV sites (aa 207 to 222) with the 26 residues from the pre-S2 sequence of HBsAg (pS21) also reduced but did not eliminate their reactivity with U173, U116, and U117, perhaps by masking of the dominant epitope by the inserted foreign epitope.

Figure 4 also shows the result with the pooled mouse antiserum F2 against deleted flagellin encoded by pXSH507, with a deleted segment of 30 residues (aa 215 to 244) covering the dominant epitope. The reactivity of the deletion constructs pXSH502 and pXSH503 with serum F2 (ca. 50%) is significantly lower than those of the constructs with shorter deletions, pXSH506, pXSH507, pXSH508, and pXSH509; this suggests that the aa 245 to 306 segment missing from pXSH501 to pXSH503 but present in the other mutants contains some secondary flagellar epitopes.

The reactivity of deleted flagellins with various antibodies was further examined by Western immunoblot (Fig. 5), which should detect linear epitopes of denatured flagellin antigen. The anti-truncated-flagellin serum U121 recognized all the deleted flagellins as well as the wild-type flagellin. The polyclonal anti-*d* (*S. typhi*) serum U116 recognized the wild-type flagellin well and gave a weak but consistent signal with all the mutant flagellins including pXSH501 in which the whole of region IV is missing; this suggests that U116 reacts only weakly with epitopes other than that centered at aa 229 to 230, inside or outside region IV. Monoclonal antibody U117 reacted strongly with the wild-type flagellin, reacted poorly with the 2-residue deletion mutant pXSH508, and did not recognize any of the mutants with a longer deletion. These results confirmed that the peptide sequence centered at aa 229 to 230 is the major antigenic determinant of flagella.

**Flagellins with an insert replacing the dominant flagellar epitope can assemble into stable flagellar filament.** To test whether a foreign epitope replacing the major determinant of flagellar antigen *d* was immunogenic, the peptide sequence pre-S1 12–47 of HBV surface antigen, which appears to be involved in the attachment of the virus to cells (23), was

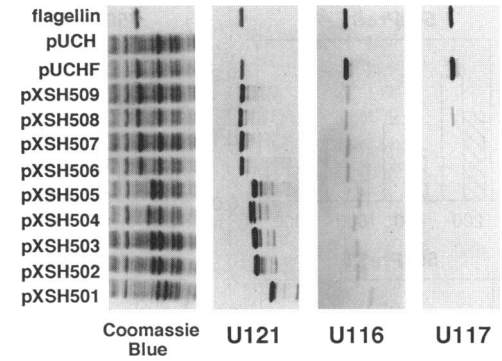


FIG. 5. Western blot analysis of recombinant flagellins. Whole-cell lysates of *S. dublin* SL5928 carrying different plasmids were analyzed by SDS-PAGE along with purified wild-type flagellin. After electrophoresis the gels were either stained with Coomassie blue or electroblotted onto nitrocellulose membranes and incubated with the following antibodies (8): U121, antiserum against truncated flagellin; U116, antiserum against *d* flagella of *S. typhi*; and U117, monoclonal antibody against *d* flagella of *S. typhi*.

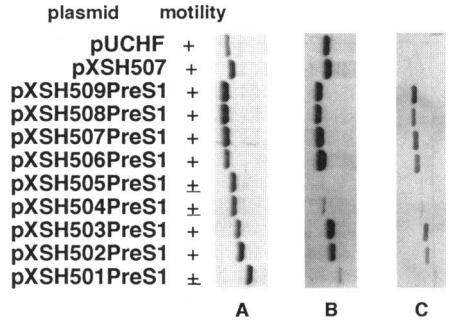


FIG. 6. Formation of functional flagella by flagellin—pre-S1 12–47 fusion protein. (A) Proteins in the cell-free supernatants of vigorously shaken cultures of the flagellin-negative strain *S. dublin* SL5928 carrying different flagellin–pre-S1 hybrid genes were precipitated with 75% ammonium sulfate and analyzed by SDS-PAGE. Protein equivalent to the amount in 40 μl of supernatant was loaded in each lane. The gel was stained with Coomassie blue. (B and C) The cell-free supernatants prepared from *Salmonella* cultures on agar plates (see Materials and Methods) were ultracentrifuged at 110,000 × *g* for 2 h to pellet polymerized flagellum filaments. The pellets were resuspended in PBS, analyzed by SDS-PAGE, and stained with Coomassie blue (B) or electroblotted onto nitrocellulose membrane and incubated with E2, a pool of mouse antisera against preS1 12-47 synthetic peptide (C) (8).

inserted into mutated flagellin proteins. This was done by inserting a DNA fragment encoding this peptide synthesized by PCR from a cloned HBV genome (subtype $adw_2$) (31) into each of the deleted flagellin genes, taking advantage of the unique *Bam*HI and *Sma*I sites therein, to form nine plasmids carrying the hybrid flagellin genes, pXSH501-PreS1 to pXSH509-PreS1. The majority of the hybrid flagellin genes conferred motility to the flagellin-negative *Salmonella* strain, but pXSH501-PreS1, pXSH504-PreS1, and pXSH505-PreS1 conferred only marginal motility (Table 2; also as recorded in Fig. 6). When the cell-free supernatants of the *Salmonella* cultures were ultracentrifuged and the pellets were analyzed by SDS-PAGE, a single major protein band of comparable abundance was seen for those carrying the wild-type or deleted flagellin genes without insert (pUCHF and pXSH507) and for the hybrid flagellin genes except pXSH501-PreS1, pXSH504-PreS1, and pXSH505-PreS1, which gave very low levels of pelleted flagella (Fig. 6B). However, when the whole-cell lysate (results not shown) or the total protein precipitated with ammonium sulfate from the cell-free supernatants was analyzed by SDS-PAGE (Fig. 6A), no significant difference was seen between the hybrid clones. This suggests that the pre-S1–flagellin hybrid proteins derived from most of the mutant flagellin genes can polymerize into stable flagellum filaments which can be pelleted by ultracentrifugation and that the hybrid proteins confer motility to the host, whereas the pre-S1 insert destabilized the flagellum structure derived from pXSH504 and pXSH505. This result again indicates that aa 180 to 214, present in all the mutants except pXSH501, pXSH504, and pXSH505, may contain amino acid residues which are important to the function of flagella.

Western blot of the hybrid flagellins containing pre-S1 12–47 inserts with a polyclonal antibody against the pre-S1 12–47 synthetic peptide (Fig. 6C) indicated that the pre-S1 sequence was correctly expressed in the context of all the deleted flagellins, including pXSH501, pXSH504, and pXSH505.

**The pre-S1 12–47 peptide inserted at the dominant flagellar epitope is immunogenic.** The pelleted hybrid flagella produced from the recombinant flagellin genes pXSH503-PreS1, pXSH507-PreS1, pXSH508-PreS1, and pXSH509-PreS1 were used to immunize BALB/c mice in parallel with flagella without insert derived from pXSH507. Sera were collected after two intraperitoneal injections and tested by ELISA for antibodies against purified flagellin and HBV particles (Fig. 7). All the mice developed high titers against flagellin, whereas all but those immunized with flagella not carrying an insert developed lower but clearly detectable titers against the native HBV antigen. The binding of those antisera against hybrid flagella to HBV particles was inhibited by addition of the synthetic peptide pre-S1 12–47, compared with a control peptide pre-S2 120–145. In contrast, the binding of the antiserum against deleted flagellin (construct 507) was not affected by addition of the peptides (Fig. 8). This result indicates that the pre-S1 epitope inserted at or replacing the major flagellar epitope is immunogenic, in the sense that it induced production of antibodies that specifically bound the native virus antigen.

## DISCUSSION

We have constructed a series of mutated flagellin genes with deletions ranging from 2 to 127 aa in hypervariable region IV. Serological analysis of native flagella formed by these mutant flagellins suggests that there is a dominant surface epitope centered at aa 229 to 230, because substitution at these two amino acid residues eliminates most of the reactivity with anti-*d* antisera.

Although the amino acid sequences of the *d* flagellins of *S. typhi* and *S. muenchen* are highly homologous, there are one (aa 198) and two (aa 351 and aa 363, based on the corrected sequence of $fliC^d$ [16]) amino acid differences in their hypervariable regions IV and VI, respectively (7, 16, 36). This is in agreement with the minor difference we detected when anti-*d* (*S. typhi*) or anti-*d* (*S. muenchen*) sera were used in our antibody capture ELISA: the residual reactivities of the various mutated flagellins with the anti-*d* (*S. muenchen*) sera U173 (Fig. 4B) and U168 (data not shown) are consistently higher than those with the anti-*d* (*S. typhi*) serum U116 (Fig. 4B), an example showing the value of this technique, which enables a quantitative analysis of the flagellar antigens and anti-flagellum antibodies.

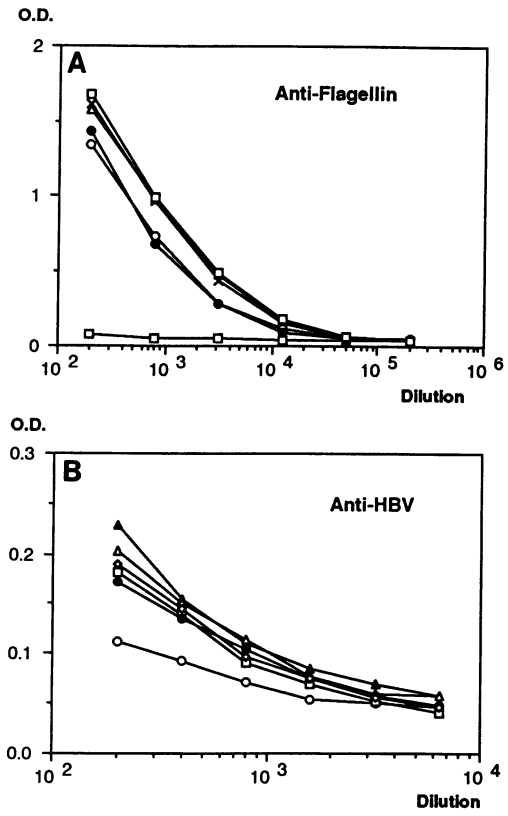


FIG. 7. ELISA of sera from mice immunized with different hybrid flagella against flagellin and HBV. Blood was collected 3 weeks after the second injection. Sera from each group of three or four mice immunized with the same antigen were pooled for this assay. 507 (○) is the flagellum formed by recombinant flagellin encoded by pXSH507, which does not have an insert. 503-PreS1 (▲), 507-PreS1 (●), 508-PreS1 (△) and 509-PreS1 (◇) are four flagella formed by different mutant flagellins carrying a preS1 12-47 insert. pre-S1 (□) is the pre-S1 12–47 synthetic peptide conjugated with thyroglobulin. The ELISA was performed as described previously (8). The microtiter plates were coated with either purified wild-type *d* flagellin (A) or HBV particles (supplied by R. Cheung) (B) and incubated with the serially diluted mouse sera and then with peroxidase-labeled goat antibody against mouse immunoglobulins G and M (Boehringer Mannheim).

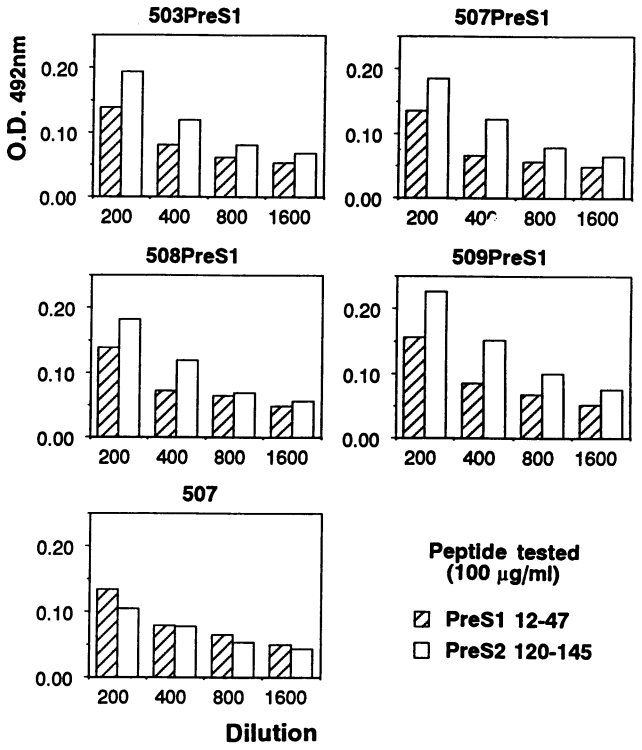


FIG. 8. The peptide pre-S1 12–47 inhibits binding of mouse antibodies to HBV particles. The ELISA was performed as described previously (8). The wells of microtiter plates were coated with HBV particles. Sera from mice immunized with flagella formed by different flagellin–pre-S1 hybrid proteins (503PreS1, 507PreS1, 508PreS1, and 509PreS1) or flagella without an insert (construct 507) were serially diluted with 100 μg of synthetic peptides pre-S1 12–47 or pre-S2 120–145 per ml in 3% bovine albumin before addition to the wells.

Using products of the mutated flagellin genes, we were able to determine the specificity of monoclonal anti-*d* antibody U117 (28), which we found to be for the major epitope centered at aa 229 to 230. Sadallah et al. (29) also isolated four monoclonal antibodies against *d* flagella of *S. typhi*. All of them were later found to bind the same octameric peptides around aa 229 to 230, previously identified by using polyclonal anti-*d* sera (17), although the affinity of these four monoclonal antibodies for this epitope differed somewhat (15). These results are in agreement with our conclusion that the epitope centered at aa 229 to 230 is the single dominant B-cell epitope of flagellar antigen *d*.

The activity of the pooled F2 antisera raised against the mutated flagellin encoded by pXSH507, with the dominant epitope deleted (Fig. 4B), suggested that the sequence at aa 245 to 306, which overlaps two of the subfactors identified by Newton et al. (27), contains flagellar epitopes too. According to Joys and Schödel (17), this region does contain several peptides which react weakly with some anti-*d* sera. However, our data suggest that in the wild-type flagella these epitopes are much less active than the major epitope around aa 229 to 230, because by ELISA the anti-*d* (*S. typhi*) serum U116 does not recognize them, while the anti-*d* (*S. muenchen*) sera U173 and U168 recognize them poorly.

By analyzing the motility of *Salmonella* strains carrying the mutated flagellin genes, with or without a pre-S1 12–47 insert, we showed that a segment of region IV, aa 180 to 214, is important to the function of flagellin, as measured by bacterial motility. This is in agreement with the following observation: whereas aa 207 to 222 between the two *Eco*RV sites, which partially overlaps aa 180 to 214, can be deleted without having a dramatic effect on flagellin function (27), replacement of these 16 residues with foreign sequences frequently destroyed flagellin function; in some cases, even though flagella were seen by electron microscopy, the bacteria were still nonmotile (9, 35, 38). Hydropathicity analysis of flagellin indicates that aa 207 to 222 form one of several relatively hydrophobic regions of flagellin protein (data not shown). These residues may be buried inside the filament structure and have more interaction with other residues, hence contributing to the overall stability of flagellar filament.

The N- and C-terminal regions of flagellins of different *Salmonella* serotypes are very similar in their amino acid sequences (13, 36) and are believed to be important in the formation of the flagellar filament. Image analysis of electron micrographs of flagella (33) identified an outer domain corresponding to the central stretch of the primary amino acid

sequence, perhaps nonessential for flagellar motility (34). In another three-dimensional structure model based on X-ray fiber diffraction of flagella, the innermost domain 1 is responsible for most of the filament formation and is formed by the constant N- and C-terminal parts of flagellin and the outermost or surface domain 3 is assigned to the variable middle part of flagellin, which carries the antigenic determinants (22). Kuwajima (21) reported a minimum-size functional flagellin of *E. coli* in which 187 aa were deleted from the middle part of flagellin. The bacteria expressing this flagellin were still motile. However, previous observations, as well as this work, show that relatively minor alterations in the hypervariable region may change flagellin function drastically. Therefore, the middle part of the flagellin protein, although highly heterologous among different flagellins, is also important to its function, in the sense that changes in the amino acid sequence of this area may cause loss of function. The series of mutant flagellin genes described in this paper should be useful for studies of the structure-function relationship of flagellin.

*Salmonella* flagella are known to be the carriers of H antigen, one of the major antigens that elicits immune responses after infection or vaccine administration. *Salmonella* vaccine strains expressing protective antigens as fusion proteins with flagellin have been used as live vaccines against heterologous infectious agents (25, 26, 38). Flagellin protein is transported from within the bacterial cell to be polymerized into flagellar filaments, which are relatively easily purified and are expected to be more immunogenic than soluble protein. It should therefore be possible to use the purified hybrid flagellar filaments carrying foreign epitopes as vaccines. In this work we have identified the dominant surface epitope of *d* flagella. It will be interesting to see whether foreign epitopes will be more immunogenic when inserted into or at the site of the dominant epitope of this flagellin rather than elsewhere in the molecule. We have inserted the pre-S1 12–47 epitope of the surface antigen of HBV, which has been shown to elicit protective antibodies against HBV infection in chimpanzees (24), at the site of the major flagellar epitope, to form hybrid flagellin. These hybrid proteins were assembled into functional flagella and were capable of inducing antibody response to the pre-S1 insert when given to mice. Hence the mutated flagellin genes constructed in this work can be used as convenient expression vectors of foreign epitopes with potential for vaccine development.

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