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Regions of *Salmonella typhimurium* Flagellin Essential for Its Polymerization and Excretion

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Immunological methods were used to examine the flagellin production of *Salmonella typhimurium* strains that carried a mutation in one of the two possible genes for flagellin (*H1* or *H2*) and also were incapable of expressing the other gene. Some mutants produced flagellin that was excreted into the culture medium; others accumulated flagellin intracellularly. These two phenotypes were detected in both *H1* and *H2* mutants. The mutation sites were mapped on the corresponding deletion map (consisting of 21 segments in the case of *H1* and 31 segments in the case of *H2*). *H1* and *H2* mutations causing excretion of flagellin were clustered mainly in segment 12 and segment 6 from the proximal end, respectively, suggesting that the corresponding segments of the flagellins play a role in polymerization. Mutations causing accumulation in the cytoplasm were clustered in segments 19 to 21 of the *H1* map and in segments 25 to 29 of the *H2* map, suggesting that an essential region for flagellin transport exists toward the C terminus of flagellin.

A bacterial flagellum consists of three distinct parts: the basal body, the hook, and the filament. The basal body is embedded in the cell surface layers and forms part of the flagellar motor, whereas the hook and the filament extend away from the cell body. In *Salmonella typhimurium*, the filament-hook structure is composed of at least five species of proteins: flagellin, hook protein, and the hook-associated proteins FlaW, FlaU, and FlaV (4, 10). Flagellin and hook protein are the major structural components of the filament and the hook, respectively, and the three hook-associated proteins are minor components essential for filament formation. These five proteins are synthesized in the cell and must be transported across all three layers of the cell surface—cell membrane, peptidoglycan layer, and outer membrane—to their extracellular destination.

Many exported proteins are synthesized as transient precursors with an N-terminal signal peptide (18). However, in the case of the five hook-filament proteins, no evidence for signal peptides has been obtained (6, 14; M. Homma, unpublished data). Furthermore, the N terminus of the sequence of flagellin (predicted from the DNA sequence) does not resemble that of a signal peptide (13, 21).

Flagellar filaments grow at their distal ends (1, 8), with the elongation rate decreasing exponentially with length (9). Structural data show an empty channel in the core of the filament and hook (17, 19). In basal-body-defective mutants, flagellin is not transported to the extracellular space (M. Homma, unpublished data), whereas with several classes of flagellar assembly mutants of *S. typhimurium* (defective in *flaW*, *flaU*, or *flaV*), unpolymerized flagellin is a major protein in the culture medium (3), typically present at about 10 μg/ml in a stationary-phase culture. From the above observations, it appears that flagellin is transported through a central channel in the basal body, the hook, and the filament. The other four proteins, i.e., hook protein and hook-associated proteins, are likely to be transported by the same pathway. In this regard, the basal body structure must be considered not only as a portion of the flagellar motor but also as a protein translocation device. We suspected, therefore, that flagellin might contain a region responsible for its recognition by the device.

In *S. typhimurium* there are two flagellin genes, *H1* and *H2*, which are alternately expressed by a mechanism known as phase variation (11). In this study, we isolated a number of *H1* mutants (in a background in which expression of *H2* was blocked) and analyzed these together with existing *H2* mutants (in a background in which expression of *H1* was blocked). With some of these mutants, we detected defective flagellins which were excreted into the culture medium. With others, we detected unexcreted flagellin in the cell body. A strong correlation was found between mutant phenotype and location of the mutational sites on a deletion map of the flagellin gene.

MATERIALS AND METHODS

Bacterial strains. *S. typhimurium* SJW1103 was used as the parent strain for the isolation of *H1* mutants. This strain is phase-1 monophasic (i.e., *H2* is not expressed) and produces H1 flagellin of serotype i (2). *H2* mutants had previously been isolated from strain SJW806, which is phase-2 monophasic and produces H2 flagellin of serotype enx (2). Strain SJW803 *H1-2187* (S. Yamaguchi, unpublished data) was used as the standard for *H1* complementation. The other strains are described in the text.

Media. Media were described previously (7).

Isolation of *H1* mutants. Spontaneous nonflagellate mutants were isolated by use of the flagellum-specific phage χ as described previously (24). The mutants were subjected to complementation tests with an *H1* mutant (SJW803) by P22(HT)-mediated transduction. Mutants showing no complementation were classified as *H1* mutants. Reversion of each was checked by streaking overnight broth cultures in lines on semisolid-medium plates and examining the plates for spontaneous swarms after 48 h of incubation at 37°C.

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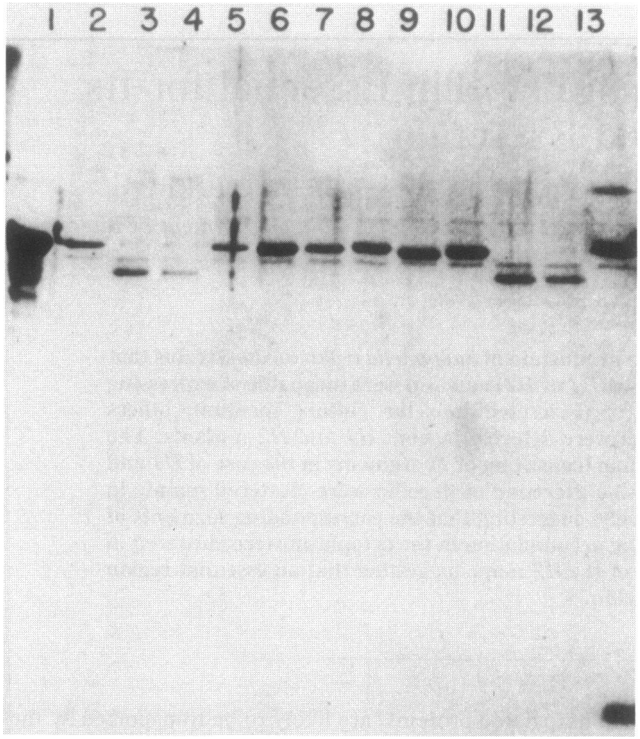

FIG. 1. SDS-PAGE profiles of excreted flagellin. Purified flagellin from the parent strain SJW1103 was loaded in lane 1. Culture medium from the 12 *H1* mutants that excreted flagellin (see Results) was loaded into lanes 2 to 13. Note the shift in mobility of flagellin in lanes 3, 4, 11, and 12.

Usually 0.1 ml of a broth culture (ca. 10^9 cells per ml) was streaked in five lines on a plate. If no swarm was observed on five plates, the mutant was regarded as stable.

Ring test for detection of flagellin in culture medium. A stationary-phase culture of a given mutant was pelleted in an Eppendorf centrifuge tube. A 50-μl sample of the supernatant fluid was carefully overlaid in a small tube (30 by 4 mm) containing 50 μl of an anti-flagellin antibody solution which had been diluted 1/20 with 1% bovine serum albumin in 10 mM phosphate buffer (pH 7.2) containing 0.15 M NaCl. The tube was incubated at room temperature for 30 min and inspected for the presence or absence of an opaque immunoprecipitate at the interface.

Preparation of antiflagellin antibody. H1 flagellin was prepared from strain SJW1103 by the method of Suzuki and Iino (20). H2 flagellin prepared from strain SL23 (20) was kindly supplied by H. Suzuki (Tokyo University, Tokyo, Japan). After flagellin had been separated from contaminants by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the band was cut out from the gel and used directly for immunization as previously described (4).

Electrophoresis. SDS-PAGE was performed by the method of Laemmli (16).

Detection of intracellular flagellin. Cells of a given *H1* or *H2* mutant were analyzed by an immunoblotting procedure (5).

Deletion mapping. Deletion mapping was performed according to the method of Yamaguchi et al. (22).

Test for complementation and recombination. Complementation and recombination between pairs of mutants were examined by P22-mediated transduction. The donor phage suspension (ca. 10^{10} PFU/ml) was mixed with an equal volume of the recipient bacterial culture (ca. 10^9 cells per ml). The transduction mixture was streaked in five lines (ca. 20 μl per line) on a semisolid-medium plate. Production of trails (abortive transductants) and production of swarms (complete transductants) on the plates were used as the criteria for complementation and recombination, respectively.

RESULTS

Excretion of defective flagellin in *H1* mutants. From a pool of nonflagellate mutants of SJW1103, we identified 149 *H1* mutants. Among them, 34 were stable and presumed to be multisite or deletion mutants; the remaining 115 were revertible and presumed to be missense, nonsense, or frameshift mutants.

To determine whether the mutants excreted defective flagellin into the culture medium, we used the ring test described in Materials and Methods. This test is a simple and rapid procedure whose sensitivity is sufficient to detect flagellin at ca. 10 μg/ml. In such tests, the culture medium of 12 *H1* mutants produced a ring of precipitation near the interface. The culture medium from these strains were then analyzed by SDS-PAGE to determine the molecular weight of the excreted flagellin (Fig. 1). The major protein in the culture medium was expected to be flagellin; eight of the mutants excreted a major protein with the mobility of wild-type flagellin, and the remaining four excreted a major protein of significantly higher mobility which, by immunoblotting (data not shown), was shown also to be flagellin. The apparent molecular weight of wild-type flagellin is 52,000 (15), and the molecular weight of these smaller flagellins was estimated as 45,000; the mapping study described below established that these four mutants were deletion mutants.

Production of unexcreted flagellin in *H1* mutants. We attempted to detect intracellular flagellin in the *H1* mutants that did not excrete it. After the proteins from a late-log culture of each *H1* mutant had been separated by SDS-PAGE, they were transferred onto a nitrocellulose sheet and incubated with antiflagellin antibody. Bound antibody was labeled with goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase and detected by the color development reagent 4-chloro-1-naphthol. Based on the intensity of the color development and the position of defective flagellin on the nitrocellulose sheet (Fig. 2), H1 mutants were classified as follows: class I, strong color development and same molecular weight as wild-type flagellin (13 mutants); II, strong color development and decreased molecular weight (6 mutants); III, weak color development and decreased molecular weight (20 mutants); IV, weak color development and increased molecular weight (1 mutant); V, nondetectable color development (the remaining 97 mutants). By adding as class VI the 12 flagellin-excreting mutants described in the previous section, *H1* mutants were placed into six classes.

Correlation between mutational sites and phenotypes for *H1* mutants. To investigate the relationship between site of mutation and phenotype, deletion mapping of the *H1* gene was carried out by two series of transductional crosses: (i) recombination tests among all pairwise combinations of stable mutants and (ii) recombination tests among stable and revertible mutants. The *H1* gene was divided into 21 segments on the basis of the overlapping pattern of deletions (Fig. 3), with the numbering starting from the end adjacent to the *flaV* gene, where the *H1* promoter is located (6, 23).

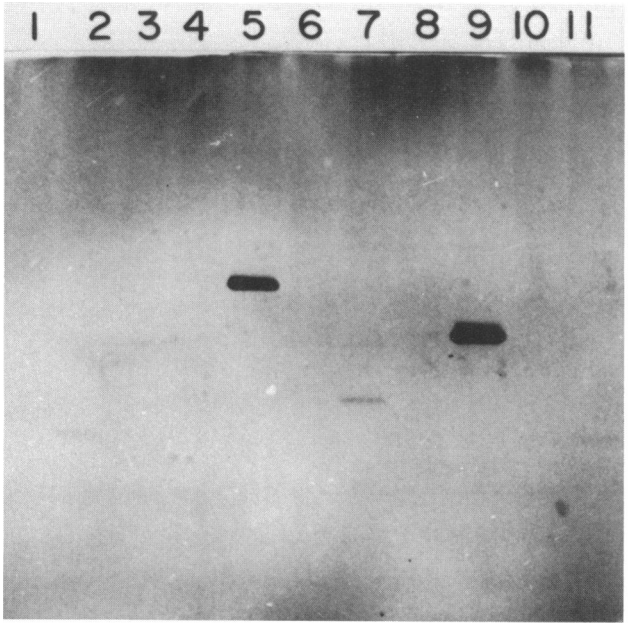

FIG. 2. Detection by immunoblotting of intracellular flagellin from *H1* mutants. After the total-cell proteins of *H1* mutants were separated by SDS-PAGE and transferred onto nitrocellulose, flagellin was detected by antiflagellin antibody and horseradish peroxidase-conjugated second antibody. Mutants were classified according to the intensity and position of the flagellin band, as described in the text. Class I is exemplified by lane 5, class II is exemplified by lane 9, class III is exemplified by lanes 2, 7, and 11, and class V is exemplified by lanes 1, 3, 4, 6, 8, and 10; there are no class IV mutants in this set.

The locations of the mutational sites of the mutants are shown in Fig. 4A; examples were found in most segments. However, the mutations that resulted in excretion of flagellin or its retention in the cell were confined to a quite limited set of sites in the gene. Among 12 mutants excreting defective flagellin into the culture medium (class VI mutants), 6 were shown to carry a mutation in segment 12 and 4 were shown to carry a deletion covering segment 12; the other 2 mutants carried a mutation in segment 4 or 9. Among mutants that retained flagellin intracellularly (class I and II mutants), all of the mutational sites clustered at the distal end of the gene, in segments 19, 20, and 21.

Correlation between mutational sites and phenotypes in the *H2* gene. A deletion map of the *H2* gene, containing 31 segments, has been reported, and a large number of mutants have been isolated and located within this map (22). We examined the defective flagellin of these mutants by the methods described above. To avoid unnecessary screening of chain termination mutations, we chose only nonpolar mutations (with respect to the distal gene *rh1*, which codes for a repressor of *H1* expression). The 41 mutants tested were classified by the same phenotypic designations used with the *H1* mutants. Among them, 12 mutants excreted defective flagellin into the culture medium (class VI), and 7 accumulated defective flagellin intracellularly (2 class I, 5 class II); there were 1, 0, and 21 examples of mutants from classes III, IV, and V, respectively. On the map of the *H2* gene (Fig. 4B), most of the sites (8 of 12) of mutants excreting defective flagellin clustered at neighboring segments 5 and 6. Of the remaining sites, one mapped to segment 29 and three were deletions covering segments 9 and 10, 26 and 27, and 30 and 31, respectively. In contrast, the sites of all seven mutants accumulating defective flagellin clustered at the distal region of the gene, in segments 25 to 29.

DISCUSSION

Flagellin is thought to be transported through a central channel in the flagellar structure, in which case specific recognition and transport events should be required for proper flagellin localization. To clarify the mechanism of flagellin translocation, we isolated mutants making defective flagellin and examined whether the defective flagellin was exported from the cell. The products of both flagellin genes, *H1* and *H2*, were examined in this regard.

Defective flagellins that were excreted into the culture medium were detected by immunoprecipitation. A total of 12 *H1* mutants and 12 *H2* mutants, placed in class VI, were found that make this kind of defective flagellin, which presumably does not differ greatly from intact flagellin with respect to its synthesis, antigenicity, or transportability, but which is unable to polymerize. An example of this type of polymerization-defective mutant has been reported previously (12).

In the remaining mutants, defective flagellin accumulated intracellularly was tested for by immunoblotting. Several types of defects were found. With 19 *H1* mutants and 7 *H2*

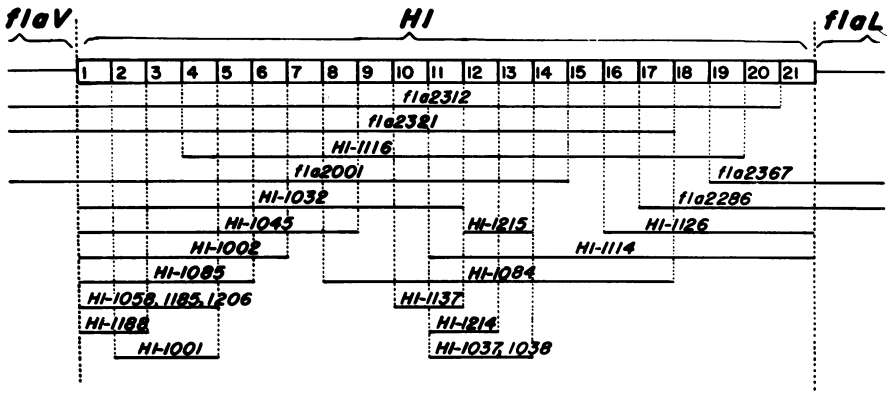

FIG. 3. Deletion map of the *H1* gene (serotypic allele i). Bar lines indicate the extents of the deletions, which were used to define the segments. Numbering is from the promoter-proximal end of the gene. The segments are arbitrarily shown as being of equal length.

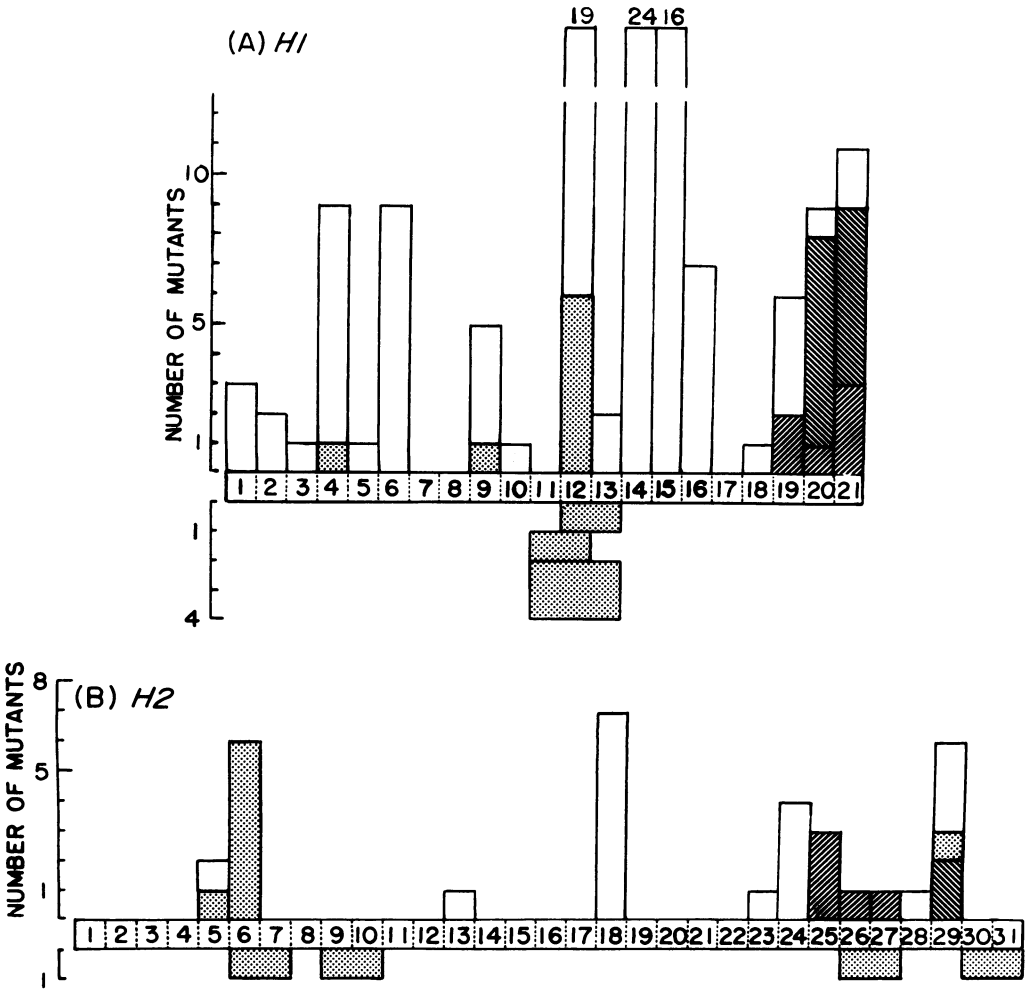

FIG. 4. Correlations among mutational sites within the gene coding for flagellin and the properties of the defective flagellin. Mutations above the strip that represents the gene are single-site mutations; those below it are deletions covering more than one deletion segment. The segments are arbitrarily shown as being of equal length. (A) Mutations in *H1* assigned to segments of the deletion map of Fig. 3. (B) Mutations of *H2* assigned to segments of the deletion map described by Yamaguchi et al. (22). Symbols: ▨, excreted, polymerization-defective flagellin (class VI mutations, see text); ▧, unexcreted flagellin with same molecular weight as intact flagellin (class I mutations); ▨, unexcreted flagellin with decreased molecular weight (class II mutations); □, all other single-site mutations.

mutants, levels of synthesis and antigenicity appeared to be normal, but the flagellin was retained in the cell. In this study, we did not examine whether the flagellin was capable of assembling into filaments.

With both *H1* and *H2*, most of the polymerization-defective mutations were found within a single segment of the gene (Fig. 4). It seems reasonable to assume that the relevant segment of these two serologically different flagellins represents a region of homologous function. In the case of *H2*, the relevant region is segment 6 of a 31-segment deletion map; recombination data (22) indicate that it is located about 20% from the proximal end of the gene. The phenotypically equivalent location within the *H1* gene is segment 12 of a 21-segment map. However, a genetic deletion map does not correspond exactly in scale to the physical map; recombination frequency data (not shown) in fact suggests that segment 12 is located aproximately 20 to 25% from the proximal end of the gene. The DNA sequence is available for several wild-type *H1* alleles of different antigenicity (13, 21), and it reveals a high degree of homology within the proximal 40% of the gene. The first 60 bases of an *H2* sequence have been published (25) and are highly homologous to the *H1* sequences. More extensive sequence data on *H1* should establish whether the phenotypically similar segments of the two genes are homologous and in the same position within the genes.

H2 mutations causing failure of polymerization of flagellin occur at essentially the same positions in the gene as those that cause abnormal filament shape (22). This result is not surprising, since at the sites of flagellin-flagellin interaction a small change might affect the precise quaternary interaction (and hence filament shape [22]), whereas a more drastic change might prevent polymerization entirely. Polymerization-defective mutations and filament-shape mutations are found toward both ends of the deletion map, suggesting that regions at both the N-terminal and C-terminal regions of flagellin are important for polymerization and the determination of the mode of assembly. Three-dimensional reconstruction data (19) suggest that the flagellin subunit within the assembled filament makes contacts important for quaternary structure toward inner radii, while the region of the subunit that is at outer radii—and presumably therefore is

antigenically most important—is relatively free. The structural and genetic data taken together suggest that the subunit may, roughly speaking, be a hairpin, with both the N terminus and the C terminus toward the interior of the filament determining assembly and the central portion of the sequence forming an antigenic domain toward the outside.

With H1 flagellin, we did not detect polymerization-defective mutations at the distal region of the gene. However, filament-shape mutations have been detected in that region (S. Yamaguchi and H. Fujita, unpublished results), and polymerization-defective mutations might be detected if more mutants were examined.

Failure of flagellin to polymerize in vivo can be caused by mutations not only in the flagellin genes but also in the hook-associated protein genes *flaW*, *flaU*, and *flaV* (3). Although hook-associated protein mutants produce intact flagellin normally, the flagellin is unable to polymerize onto the hook and is excreted into the culture medium (5). Therefore, some of the flagellins that fail to polymerize may have a defective interaction with hook-associated proteins rather than with other flagellin subunits.

Transport-defective mutations were located at the distal end of the flagellin genes, in segments 19 to 21 of *H1* and in segments 25 to 27 and 29 of *H2* (Fig. 4). However, a deletion mutation, *H2-274*, covers segments 26 and 27, yet the mutant is polymerization defective with apparently normal transport ability. On the other hand, transport-defective H2 flagellins, with mutations mapping to segments 25 to 27, had decreased molecular weights, indicating that the mutations may be either small deletions or single-site changes that have led to premature chain termination. Therefore, we believe that the actual region important for transport is downstream from segment 27. This conclusion is supported by mutations in segment 29 that result in synthesis of transport-defective flagellin with the wild-type molecular weight. By a similar argument, we suggest that in the *H1* flagellin gene a region essential for transport exists in the distal segments 20 and 21.

The evidence from two serologically distinct flagellins strongly suggests that a region essential for transport of flagellin exists near its C terminus. However, this study does not address what the role of this region is in the transport process. It may function to maintain a structure that is capable of passing through the central channel of the flagellum, or it may provide a recognition sequence that targets flagellin to be guided into the central channel. If the latter is true, homologous sequences may be found in hook protein and hook-associated proteins.

Based on the DNA sequence of the genes for three serologically different flagellins (serotypes i, c, and d), the amino acid sequence of the N-terminal and C-terminal regions are highly conserved (13, 21). The locations of transport-defective and polymerization-defective mutational sites may fall within these highly conserved regions.

In this study, we did not detect defective flagellin either intracellularly or extracellularly in many of the strains that were expected to contain single-site mutations. The immunological methods used would not have detected defective flagellin if there was a large change in antigenicity; nonsense mutations could cause such changes, as could the formation of large-scale aggregates. Some of the mutants may synthesize defective flagellin that is rapidly degraded. Others may have low transcriptional or translation levels for flagellin; there is an extremely high degree of conservation of parts of the nucleotide sequence (rather than simply amino acid sequence) among different serotypic alleles (21), which argues for internal regulatory sites within the flagellin gene.

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LITERATURE CITED

1. **Emerson, S. U., K. Tokuyasu, and M. I. Simon.** 1970. Bacterial flagella: polarity of elongation. Science **169**:190–192.
2. **Fujita, H., S. Yamaguchi, T.Taira, and T. Iino.** 1981. A simple method for the isolation of flagellar shape mutants in *Salmonella*. J. Gen. Microbiol. **125**:213–216.
3. **Homma, M., H. Fujita, S. Yamaguchi, and T. Iino.** 1984. Excretion of unassembled flagellin by *Salmonella typhimurium* mutants deficient in hook-associated proteins. J. Bacteriol. **159**:1056–1059.
4. **Homma, M., and T. Iino.** 1985. Locations of hook-associated proteins in flagellar structures of *Salmonella typhimurium*. J. Bacteriol. **162**:183–189.
5. **Homma, M., and T. Iino.** 1985. Excretion of unassembled hook-associated proteins by *Salmonella typhimurium*. J. Bacteriol. **154**:1370–1372.
6. **Homma, M., K. Kutsukake, and T. Iino.** 1985. Structural genes for flagellar hook-associated proteins in *Salmonella typhimurium*. J. Bacteriol. **163**:464–471.
7. **Homma, M., K. Kutsukake, T. Iino, and S. Yamaguchi.** 1984. Hook-associated proteins essential for flagellar filament formation in *Salmonella typhimurium*. J. Bacteriol. **157**:100–108.
8. **Iino, T.** 1969. Polarity of flagellar growth in *Salmonella*. J. Gen. Microbiol. **56**:227–239.
9. **Iino, T.** 1974. Assembly of *Salmonella* flagellin *in vitro* and *in vivo*. J. Supramol. Struct. **2**:372–384.
10. **Iino, T.** 1985. Structure and assembly of flagella, p. 9–37. *In* N. Nanninga (ed.), Molecular cytology of *Escherichia coli*. Academic Press, Inc., (London), Ltd., London.
11. **Iino, T., and K. Kutsukake.** 1983. Flagellar phase variation in *Salmonella*: a model system regulated by flip-flop DNA inversions, p. 395–406. *In* K. Mizobuchi, I. Watanabe, and J. D. Watson (ed.), Nucleic acid research: future developments. Academic Press, Inc., New York.
12. **Ikeda, T., R. Kamiya, and S. Yamaguchi.** 1983. Excretion of flagellin by a short-flagella mutant of *Salmonella typhimurium*. J. Bacteriol. **153**:506–510.
13. **Joys, T. M.** 1985. The covalent structure of the phase-1 flagellar filament protein of *Salmonella typhimurium* and its comparison with other flagellins. J. Biol. Chem. **260**:15758–15761.
14. **Komeda, Y., M. Silverman, and M. Simoh.** 1978. Identification of the structural gene for the hook subunit protein of *Escherichia coli* flagella. J. Bacteriol. **133**:364–371.
15. **Kondoh, H., and H. Hotani.** 1974. Flagellin from *Escherichia coli* K12: polymerization and molecular weight in comparison with *Salmonella* flagellins. Biochim. Biophys. Acta **336**:117–139.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
17. **O'Brien, E. J., and P. M. Bennett.** 1972. Structure of straight flagella from a mutant *Salmonella*. J. Mol. Biol. **70**:133–152.
18. **Pugsley, A. P., and M. Schwartz.** 1985. Export and secretion of proteins by bacteria. FEMS Microbiol. Rev. **32**:3–38.
19. **Shirakihara, Y., and T. Wakabayashi.** 1979. Three-dimensional image reconstruction of straight flagella from a mutant *Salmonella typhimurium*. J. Mol. Biol. **131**:485–507.
20. **Suzuki, H., and T. Iino.** 1973. In vitro synthesis of phase-specific flagellin of *Salmonella*. J. Mol. Biol. **81**:57–70.
21. **Wei, L.-N., and T. M. Joys.** 1985. Covalent structure of three phase-1 flagellar filament proteins of *Salmonella*. J. Mol. Biol. **186**:791–803.
22. **Yamaguchi, S., H. Fujita, K. Sugata, T. Taira, and T. Iino.** 1984. Genetic analysis of *H2*, the structural gene for phase-2 flagellin

in *Salmonella*. J. Gen. Microbiol. **130**:255–265.
23. **Yamaguchi, S., H. Fujita, T. Taira, K. Kutsukake, M. Homma, and T. Iino.** 1984. Genetic analysis of three additional *fla* genes in *Salmonella typhimurium*. J. Gen. Microbiol. **130**:3339–3342.
24. **Yamaguchi, S., T. Iino, T. Horiguchi, and K. Ohta.** 1972. Genetic analysis of *fla* and *mot* cistrons closely linked to *H1* in *Salmonella abortusequi* and its derivatives. J. Gen. Microbiol. **70**:59–75.
25. **Zieg, J., and M. Simon.** 1980. Analysis of the nucleotide sequence of an invertible controlling element. Proc. Natl. Acad. Sci. USA **77**:4196–4200.