Characterization of the *fliE* Genes of *Escherichia coli* and *Salmonella typhimurium* and Identification of the FliE Protein as a Component of the Flagellar Hook-Basal Body Complex

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Within flagellar region III of *Escherichia coli* and *Salmonella typhimurium*, the genomic organization has been largely established. An exception is *fliE*, a gene whose exact location and product function are not well understood. We cloned the *fliE* gene, obtained its DNA sequence, and identified its product. *fliE* was found to be a monocistronic transcriptional unit, adjacent to and divergent from the large *fliF* operon. It is several kilobases distant from the nearest flagellar operon in the other direction, the *fliD* operon, and constitutes the first operon within the newly defined region IIIb, which contains the genes *fliE* through *fliR*. *fliE* encodes a small, moderately hydrophilic protein with a deduced molecular mass of 11,114 Da (*E. coli*) or 11,065 Da (*S. typhimurium*). We identified a protein within the isolated hook-basal body complex as the *fliE* gene product on the basis of its size and comparison of its N-terminal amino acid sequence with that deduced from the gene sequence. From gel electrophoresis and autoradiography of ³⁵S-labeled *S. typhimurium* hook-basal body complexes (C. J. Jones, R. M. Macnab, H. Okino, and S.-I. Aizawa, J. Mol. Biol. 212:377–387, 1990) and the deduced number of sulfur-containing residues in FliE, we estimated the stoichiometry of the protein in the hook-basal body complex to be about nine subunits. FliE does not undergo cleavage of a signal peptide, nor does it show any sequence similarity to the axial components like the rod or hook proteins, which are believed to be exported by the flagellum-specific export pathway. On the basis of this and other evidence, we suggest that FliE may be in the vicinity of the MS ring, perhaps acting as an adaptor protein between the ring and rod substructures.

The flagellar systems of *Escherichia coli* and *Salmonella typhimurium* are encoded by at least 40 genes, which until recently were thought to be organized into three regions on the chromosome (plus, in the case of *S. typhimurium*, the phase 2 flagellin region) (27). Region I contains mostly flagellar structural genes, and region II contains a mixture of flagellar, motility, and chemotaxis genes; for most of the genes in those two regions, the DNA sequences and the functions of their products are known (27).

Region III was known to contain genes *fliA* through *fliR*, which were thought to be contiguous. However, we have found that it is interrupted by a large stretch of DNA unrelated to flagellar function, justifying its subdivision into regions IIIa and IIIb (17). Except for *fliE*, the genomic organization and DNA sequences of all of these region IIIa and IIIb genes in either *E. coli*, *S. typhimurium*, or both were known, with *fliA* through *fliD* belonging to region IIIa and *fliF* through *fliR* belonging to region IIIb (9, 14, 18, 22, 23, 28, 29, 32, 33, 44). We report here that *fliE* lies at the start of region IIIb. It is a monocistronic operon oriented divergently from the large *fliF* operon. We have obtained the DNA

sequence of the gene, shown that its product is a component of the flagellar basal body, and estimated its subunit stoichiometry within that structure.

MATERIALS AND METHODS

Bacterial strains. The following *E. coli* strains were used: for cloning, subcloning, and sequencing, DH5α (GIBCO BRL, Gaithersburg, Md.) and XL1-Blue (Stratagene, La Jolla, Calif.); for minicell production, UH869 (*minA minB*) (12); for complementation analysis, YK1101 (*fliD*) (41), RP4191 [*fliE*79(Am)] (34), MS111 (*fliF*) (38), and RP4501 (*fliG* [*scyB10*]) (34).

S. typhimurium SJW1103 (47) was used for isolation of hook-basal body complexes and analysis of the N-terminal amino acid sequences of their component proteins and as the source of *S. typhimurium* region III DNA for cloning. SJW1381 (*fliE*) (45) was used as the host for transformation with plasmids containing *S. typhimurium* region III DNA.

Plasmids. pIK1001 (see Fig. 1a) is a pBR322-based plasmid with an *E. coli* chromosomal fragment containing the *fliC-F_E* (subscripts E and S are used to distinguish the genes or proteins of *E. coli* and *S. typhimurium*, respectively) region of JA11 (17); the subclones of pIK1001 constructed in this study are described in Results.

pOYA1 (Fig. 1b) is a pUC119-based plasmid with a 6.7-kb *EcoRI* insert of *S. typhimurium* DNA extending from within *fliD_S* through the start of *fliF_S*. Its construction is described in Results.

Chemicals and enzymes. All of the chemicals and enzymes used were obtained from standard commercial sources.

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Antibiotics were used at the following concentrations: ampicillin, 50 to 100 $\mu\text{g/ml}$; kanamycin, 25 $\mu\text{g/ml}$; tetracycline, 10 $\mu\text{g/ml}$.

Manipulation of DNA. Isolation of plasmid DNA, manipulation of DNA, and transformation of host cells were all carried out by standard procedures (30).

DNA sequencing. DNA sequencing of *E. coli* DNA was performed on the *XbaI-PstI* fragment of pVM5 (see Fig. 1a) and subfragments thereof. *Bal* 31 deletions from the *SmaI* site were obtained by digestion first with *SmaI* and then with *Bal* 31 and ligation in the presence of an *EcoRI* linker; the *EcoRI-XbaI* fragments were then subcloned into M13 for sequencing. Sequencing of *S. typhimurium* DNA was performed on a 2.5-kb *EcoRI-BamHI* fragment subcloned from pOYA1 (see Fig. 1b) into M13. Sequencing was done by the dideoxynucleotide method (36) with the modified T7 DNA polymerase (42) Sequenase 2.0 (U.S. Biochemical Corp., Cleveland, Ohio) and commercial and custom-synthesized primers.

Sequence analysis. Sequence manipulations and analysis were carried out as previously described (14). Amino acid sequence comparisons were made against the National Biomedical Research Foundation protein sequence data base (version 25.0; June 1990) by using the FASTA algorithm and against the GenBank DNA data base (version 67.0; March 1991) by using the TFASTA algorithm.

N-terminal amino acid sequencing. Hook-basal body complexes from a 7.5-liter culture of SJW1103 were isolated essentially as previously described (1), subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (26) in two 1-cm lanes of a 1-mm-thick 12.5% polyacrylamide gel, electroblotted for 30 min onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, Mass.), and stained with Coomassie blue by following the protocol described by Matsudaira (31). Bands of interest were then cut out, and amino acid sequence analysis was performed on them as previously described (16).

Nucleotide sequence accession numbers. The *fliE_E* and *fliE_S* sequences have been deposited in the GenBank data base under accession numbers M84992 and M84993.

RESULTS

Cloning of *fliE_E*. While characterizing flagellar region III and establishing that it is interrupted by DNA unrelated to flagellation (17), we had cloned a 14-kb *EcoRI* fragment from the chromosome of *E. coli* JA11, a strain which was wild type for all flagellar genes except flagellin gene *fliC_E* (which had been partially deleted and disrupted with a kanamycin resistance gene cassette). The resulting plasmid, pK1001, contains the following genes: $\Delta fliC_{E::Km^r}$, *fliD_E*, *fliE_E*, and *fliF_E*, with a 5.4-kb region unrelated to flagellar function lying between the *MluI* and *XbaI* sites shown in Fig. 1a. Plasmids such as pVM5, which lack the left-hand part of the insert but retain the right-hand part, failed to complement *fliD_E* mutants in a swarm assay on semisolid agar but complemented *fliE_E* and *fliF_E* mutants. Thus, *fliE_E* and *fliF_E* lie to the right of the intervening nonflagellar region, within flagellar region IIIB.

To localize *fliE_E* in more detail, various deletion derivatives of pVM5 were made and tested for the ability to complement a *fliE_E* mutant. The results (Fig. 1a) indicated that *fliE_E* was located within a 0.6-kb *AccI-SmaI* fragment (plasmid pVM511). This was confirmed by loss of complementation when a small *PvuII* deletion was made within this fragment (plasmid pVM512) or when a small extent of *Bal* 31

FIG. 1. Plasmids used to characterize the *E. coli* and *S. typhimurium* *fliE* genes and the surrounding sequences. (a) Plasmid pK1001 was the parent plasmid used to study regions IIIa and IIIB in *E. coli* (17). It has a large insert consisting of (i) a partially deleted and *Km^r*-disrupted allele of *fliC_E* and the wild-type allele of *fliD_E*, both within region IIIa, (ii) the intervening nonflagellar region between regions IIIa and IIIB (hatched line), and (iii) *fliE_E* and *fliF_E* of region IIIB. The complementation properties of a derivative plasmid, pVM5, in swarm assays established that *fliE_E* lies to the right of the nonflagellar region. Further derivatives placed it between the *AccI* and *SmaI* sites. Restriction and sequence analysis had established previously that the DNA between the *SmaI* and *EcoRI* sites contains the *fliF_E* promoter, the *fliF_E* gene, and the 5' end of *fliG_E* (32). Plasmid pVM3 (not shown) was subjected to *Bal* 31 digestion around the *SmaI* site and ligated with an *EcoRI* linker to give plasmid pVM3-2. nt, not tested. (b) Plasmid pOYA1 contains an insert of *S. typhimurium* DNA extending from *EcoRI* sites within *fliD_S* (in flagellar region IIIa) and *fliF_S* (in flagellar region IIIB). The rightward *BamHI-EcoRI* fragment was subcloned for sequencing of the *fliE_S* gene.

digestion was allowed to proceed from the *SmaI* site (plasmid pVM3-2). The inferred location of *fliE_E* is consistent with the facts that the *SmaI* site lies just before the putative *fliF_E* promoter and that the *PstI* site lies just within the 5' end of the *fliF_E* coding region (2, 32).

DNA sequence analysis of the *fliE_E* gene. We sequenced both strands of the *PstI-XbaI* fragment of pVM5 in their entirety by using various restriction and deletion derivatives. The sequence of the *PstI-AccI* portion of the fragment is shown in Fig. 2a.

The sequence between the *PstI* and *SmaI* sites, which contains the promoter of the *fliF_E* operon and the first five codons of the *fliF_E* gene (Fig. 2a), is identical to that obtained by B. Frantz and P. Matsumura, who have sequenced the *fliF_E* gene (32).

Between the *SmaI* and *AccI* sites, there were no reasonable candidates for open reading frames with the same orientation as *fliF_E*. In the orientation opposite to that of *fliF_E*, there was an open reading frame extending from positions 231 to 542 (Fig. 2a). This open reading frame was assessed as a coding region at the 96% confidence level by

(a) *E. coli*

6
1

FliF start
A A T A N M
CTGCAGTCGC ATTCATCGCG CACCTCGTGG
PstI S-D

31 CTGATTATGG ACGGCGTTAT TGGCAAAGTG GAACAAGACT CACTCCCCGGG TCAGCAAAT CGAAAAATTG ACGTCCCAT TATTTGTGCT TTCGTTTTT
fliF -10 SmaI

131 TCTATCCGCC AATAAACCCG TTTTITTTGTT GCTATTAGC GCCTTTGTCT TATTGACTTA CTGGTAGGCT TTGCTACCAG AAATTATCCG GGAGACGAGA
fliE -10 S-D

FliE start

1 M S A I Q G I E G V I S Q L Q A T A M S A R A Q E S L P Q P T I S F
231 ATGTCAGCGA TACAGGGGAT TGAAGGGGTT ATCAGCCAGT TACAGGCTAC GGCATGAGT GCGCGTGC GCAGTCACT GCCGCAACCG ACCATTAGTT
35 A G Q L H A A L D R I S D T Q T A A R T Q A E K F T L G E P G V A
331 TTGCCGGGCA GCTGCACGCC GCGCTCGATC GCATTAGTGA TACACAAACA GCTGCCCGCA CGCAGGCAGA AAAATTCAGT CTCGGTGAAC CCGGCGTGGC
PvuII PvuII

68 L N D V M T D M Q K A S V S M Q M G I Q V R N K L V A A Y Q E V M
431 GTTAAACGAT GTGATGACCG ATATGCAAAA AGCCTCAGTT TCTATGCAAA TGGGGATTCA GGTGCGTAAT AAGCTGGTGG CCGCGTATCA GGAAGTGATG

FliE end

101 S M Q V *
531 AGCATGCAGG TGTAGTTTT GTAACCTGTT GTTAATTACA TCCGATCTCA CCGCGTGGGG CATGGATGGG GCAAATCAC TCAATTTCTG GTTTAAATG
SphI

631 GATACCTGAT CCTGAACGTT TTCTGCCATC CACGATCCGT ATAC
AccI

(b) *S. typhimurium*

FliF start

27 P I R P N A R L R N L W E L P K P Q T A T S A T A S M
1 GAATT CGGGGATTCG CGCGTAGGCG ATTAAGCCAC TCAGAGGTT TAGGTTGGT TGCGATCGAT GCAGTCGCAC TCATCTCGCA CCTCGTGACT
EcoRI S-D

96 GATCGTGGAC GCGGTTTTTT GCAAAGTGA ACAAGACTCA CTCCCCGGTC AGCAAATCG AAAAATTGAC GGTCTCATT TTTGTGGTT CGAATTTTTT
fliF -10 SmaI

196 TATGCGCTAA AAACCGAGGT TTTTATCGT TATTTAACGC CTTTATCTTA TTGACAACT GGTAACTCT GCTGCGTAAA AAATCATCCA GGGGAATAGC
fliE -10 S-D

FliE start

1 M A A I Q G I E G V I S Q L Q A T A M A A R G Q D T H S Q S T V S F
296 ATGCGAGCAA TACAGGGGAT TGAAGGGGTT ATTAGCCAGT TACAGGCGAC GGCATGGCC GCGCGCGGGC AAGACACGCA CTCGCACTCG ACGGTGAGTT
SalI

35 A G Q L H A A L D R I S D R Q A A A R V Q A E K F T L G E P G I A
396 TTGCCGGCCA GCTACATGCT GCGCTGGATC GTATTAGCGA CAGACAAGCG GCGCGCGCGG TTCAGGCGGA AAAATTCAGT CTGGGTGAGC CGGGCATTGC

68 L N D V M A D M Q K A S V S M Q M G I Q V R N K L V A A Y Q E V M
496 GCTTAATGAC GTGATGCGCG ATATGCAAAA AGCGTCCGTC TCTATGCAAA TGGGGATTCA GGTGCGCAAC AAGCTGGTGG CCGCGTATCA GGAAGTCATG

FliE end

101 S M Q V *
596 TCCATGCAGG TTTAAGCCAG GGCTGACAAC ACATCCGAG CGAACTCCCG GG
SmaI

FIG. 2. DNA sequences of the *fliE* genes of *E. coli* and *S. typhimurium* and the deduced sequences of their products. (a) Sequence of the *PstI*-*AccI* fragment of pVM5 (Fig. 1a), which contains the *fliE_E* gene and its control regions. Also shown is the *fliE_E* promoter region and the start of the *fliF_E* gene, which is oriented opposite to *fliE_E*. (b) Sequence of a portion of the rightward *EcoRI*-*Bam*HI fragment of pOYA1 (Fig. 1b), which contains the *fliE_S* gene and its control regions, and the start of the *fliF_S* gene. The start and stop codons, putative -10 promoter sequences, putative ribosome-binding sites (S-D [Shine-Dalgarno sequence]), and restriction sites referred to in the text are underlined.

the program TESTCODE (5). We conclude that it is the *fliE_E* gene on the basis of the following evidence. (i) The *AccI*-*SmaI* insert of pVM511 was able to complement a *fliE_E* mutant (Fig. 1a). (ii) Restriction mapping had indicated that the insert had a single *SphI* site, which can be seen at position 532 in Fig. 2a, and a *PvuII* site (which turned out to be two closely spaced sites) at positions 339 and 379. Deletion of either the *SphI* fragment (plasmid pVM53, in which the last two amino acids of the gene product are replaced by 19 foreign residues deriving from the pBR322 sequence) or the *PvuII* fragment (plasmid pVM512) resulted in loss of *fliE_E* complementation (Fig. 1a). (iii) *Bal* 31 deletion plasmid pVM3-2, which was unable to complement the *fliE_E* mutation (Fig. 1a), was shown to have lost all of the open reading frame sequence upstream of position 347. (iv)

The deduced amino acid sequence of the N terminus agreed with the experimentally determined sequence of a flagellar protein (see below).

Thus, the sequences of *fliE_E* and the intergenic region between *fliE_E* and *fliF_E* have now been determined. The extent of the intergenic region is 214 bp. The presumed promoter for *fliE_E* is GCCAATAA, which represents a seven-of-eight match to the flagellum-specific -10 consensus sequence (GCCGATAA) described by Helmann and Chamberlin (7). The region upstream of this shows no sequence homologous to the flagellum-specific -35 consensus (TAAA). The presumed ribosome-binding site (GGAG) is strong and well placed (7 bp upstream) with respect to the initiation codon. The codon adaptation index (37) for *fliE_E* is 0.32, suggesting a fairly low level of synthesis. There is no

number of sulfur atoms contributed by the protein that we now know to be FliE_S (13) and dividing by the number of sulfur-containing residues per subunit of the mature protein (seven), we estimated that there are 9.3 ± 1.6 subunits of FliE_S in the hook-basal body complex.

DISCUSSION

This study had as its goal the characterization of the poorly understood *fliE* gene and its product. We established the genomic organization of *fliE* and obtained its gene sequence in both *E. coli* and *S. typhimurium*. We showed that FliE is a basal body protein, estimated its stoichiometry in that structure, and formed some tentative conclusions as to its location within the structure.

Characterization of the *fliE* gene and its location in flagellar region IIIb. Until recently, all of genes *fliA* through *fliR* of *E. coli* and *S. typhimurium* had been thought to be a single contiguous flagellar region, region III. We have, however, established that a nonflagellar region of the chromosome separates the *fliA*_E, *fliC*_E, and *fliD*_E operons from the *fliF*_E and *fliL*_E operons, so that region III is in fact two regions, IIIa and IIIb (17).

The exact location of the *fliE* gene in relation to the nonflagellar region and to the *fliD* and *fliF* genes was not known. We have now shown that it lies immediately adjacent to and divergent from the *fliF* operon (which contains the genes *fliF* through *fliK*). There is no indication of any gene other than *fliE* within the *fliE* operon, consistent with the genetic conclusion that it is monocistronic (20). The orientation of *fliE* had not been established before, whereas the orientation of the multigene flagellar operons was already known from genetic analysis of polarity. Most of the flagellar genes are located in multicistronic operons. The fact that *fliE* is an independent transcriptional unit may reflect a unique structural or functional role.

The pattern of expression of the *fliE* operon (19, 24) places it within class 2, the intermediate class in the transcriptional hierarchy of the flagellar regulon (24). Its putative promoter conforms to this classification (24), since it contains the -10 consensus that is characteristic of flagellar promoters of both classes 2 and 3, but not the -35 consensus that is specific for those of class 3. The gene appears to be expressed at quite low levels, judging from its usage of rare codons and the fact that we were unable to detect the product of the cloned gene consistently in minicell experiments.

We found no evidence for further flagellar operons between *fliE*_E and the *Xba*I site that defines the start of the known nonflagellar region in *E. coli*. It is likely, therefore, that the 3' end of *fliE* represents the start of flagellar region IIIb. From published and unpublished DNA sequence data (14, 18, 22, 28, 29, 32, 44), it is known that region IIIb then continues uninterrupted through to *fliR*. The organization of genes in region IIIb in *E. coli* and *S. typhimurium* is summarized in Fig. 4.

The role of FliE in flagellar structure and in the flagellar assembly process. From comparison of the deduced amino acid sequence of FliE and the experimentally determined sequence of a flagellar protein of previously unassigned genetic origin, we have demonstrated that FliE is an 11-kDa protein located in the hook-basal body complex. This represents a further step towards a complete definition of that structure.

We do not know where FliE is located within the hook-basal body complex or precisely what its role is, but several lines of evidence suggest that it is a relatively early compo-

FIG. 4. Organization of the genes in flagellar region IIIb of *E. coli* and *S. typhimurium*. There are three operons, *fliE*, *fliFGHIJK*, and *fliLMNOPQR*, oriented as shown. Gene sizes are approximately to scale. The functions of gene products, when known or suspected (8, 21, 34, 35, 39, 43, 44, 46; this study), are indicated below.

nent in the assembly process. Suzuki and colleagues found that the *fliE* gene was necessary for the earliest structure they could detect, the so-called rivet consisting of the MS ring plus the flagellar rod (Fig. 5) (40, 41). Biochemical analysis of partial structures (21) has since revealed that the rivet contains FliE as well as the MS-ring protein FliF (14, 43) and the four rod proteins FlgB, FlgC, FlgF, and FlgG (11). In a study of the assembly pathway using temperature-sensitive mutants and a differential radiolabeling protocol (15), the labeling pattern of FliE in various mutant backgrounds indicated that FliE was assembled into the basal body after the M ring and at about the same time as the proximal rod (13). Finally, the chromosomal location of *fliE* is suggestive: *fliE* lies in region IIIb along with genes for early structures such as the MS ring and the switch, whereas the rod and later components are all encoded by genes in either region I (genes for the rod, hook, hook-filament junction proteins, and outer rings) or region IIIa (genes for the filament and filament capping protein).

We can also consider the location of FliE in the context of the processes necessary for export of components that are external to the cell membrane. It clearly is not exported by the primary cellular pathway, since it does not have a cleaved signal peptide; among flagellar proteins, only the P- and L-ring proteins appear to employ this pathway (10, 14). Nor does its sequence have the characteristics shared by those of the axial family of proteins that are believed to be exported by the flagellum-specific export pathway (9, 11). The available evidence therefore suggests that FliE is not exported at all. If this is the case, it must be either a peripheral membrane component or a component that actu-

FIG. 5. The hook-basal body complex of *E. coli* and *S. typhimurium*, shown in relation to the cell surface. Morphological features are shown along with the proteins from which they are constructed. A substructure called the rivet (hatched area), first described by Suzuki and coworkers (40, 41), consists of the rod and the MS ring. FliE, the subject of the present study, is a hook-basal body component and is probably located within the rivet (21).

ally spans the membrane. Its fairly hydrophilic sequence seems to argue against the latter possibility, but it should be remembered that the algorithms for prediction of membrane proteins are not necessarily well suited to identification of components of multisubunit complexes such as the flagellum, in which a protein might span all or part of the membrane yet not be in direct contact with lipid.

A poorly understood feature of the basal body has been the S ring, and until recently it would have been tempting to suggest that FliE is responsible for that structure. This possibility is ruled out by the discovery that the M and S rings are in fact constructed from subunits of a single protein, FliF, whose domain structure apparently gives rise to the double-ring morphology (43).

The estimated stoichiometry of about nine FliE subunits per basal body is higher than that for the proximal-rod proteins FlgB, FlgC, and FlgF (about 6 subunits each) but lower than that for the MS-ring protein FliF (about 27 subunits each) (16). This suggests FliE is incorporated into a different type of structure than either the MS ring or the rod. The MS ring has annular symmetry, whereas it seems likely that the rod (like the hook and filament) has helical symmetry (11). A possible role for FliE might then be as an adaptor between these structures.

Although we feel that overall the data are most consistent with a location for FliE near the MS ring, further work is needed to establish positively its location and its functional role in the flagellar basal body.

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