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Salmonella typhimurium Mutants Defective in Flagellar Filament Regrowth and Sequence Similarity of FliI to F_0F_1, Vacuolar, and Archaebacterial ATPase Subunits

ALFRIED P. VOGLER, MICHIO HOMMA,† VERA M. IRIKURA, AND ROBERT M. MACNAB*

Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06511

Received 14 December 1990/Accepted 13 March 1991

Many flagellar proteins are exported by a flagellum-specific export pathway. In an initial attempt to characterize the apparatus responsible for the process, we designed a simple assay to screen for mutants with export defects. Temperature-sensitive flagellar mutants of *Salmonella typhimurium* were grown at the permissive temperature (30°C), shifted to the restrictive temperature (42°C), and inspected in a light microscope. With the exception of switch mutants, they were fully motile. Next, cells grown at the permissive temperature had their flagellar filaments removed by shearing before the cells were shifted to the restrictive temperature. Most mutants were able to regrow filaments. However, *flhA*, *fliH*, *fliI*, and *fliN* mutants showed no or greatly reduced regrowth, suggesting that the corresponding gene products are involved in the process of flagellum-specific export. We describe here the sequences of *fliH*, *fliI*, and the adjacent gene, *fliJ*; they encode proteins with deduced molecular masses of 25,782, 49,208, and 17,302 Da, respectively. The deduced sequence of FliI shows significant similarity to the catalytic β subunit of the bacterial F_0F_1 ATPase and to the catalytic subunits of vacuolar and archaebacterial ATPases; except for limited similarity in the motifs that constitute the nucleotide-binding or catalytic site, it appears unrelated to the E_1E_2 class of ATPases, to other proteins that mediate protein export, or to a variety of other ATP-utilizing enzymes. We hypothesize that FliI is either the catalytic subunit of a protein translocase for flagellum-specific export or a proton translocase involved in local circuits at the flagellum.

The bacterial flagellum is a complex structure comprising intracellular, envelope-spanning, and extracellular components. In *Salmonella typhimurium*, it is encoded by about 40 genes (39).

Of those flagellar proteins located within the cell envelope or outside the cell, only the outer-ring (P and L) proteins appear to be exported by the primary signal peptide-dependent pathway (33, 36, 42). The rest (four rod proteins, a hook protein, three hook-associated proteins, and the filament protein, flagellin) are thought to be exported by a unique, flagellum-specific pathway (30, 34, 38, 59), travelling through the hollow core of the nascent structure (70) and assembling at its distal end (19, 37) (Fig. 1). The process must be a highly organized one, in which protein subunits are incorporated into the growing flagellum in the correct order and stoichiometry (45). The export apparatus obviously must be selective in the proteins it recognizes for export. It may also have to supply energy for the process. Given that the physical path for export is through the nascent structure, it seems likely that the export apparatus is located at the flagellar base (Fig. 1).

Essentially nothing is known about this apparatus; specifically, its genetic origin and biochemical composition are completely unknown. We describe here initial attempts to identify its components genetically by use of temperature-sensitive mutants. From these experiments, we have identified three, possibly four, *S. typhimurium* genes that may be involved in the export process.

For two of these (*fliN* and, in *Escherichia coli*, *flhA*), the DNA sequences are known (50, 51, 65). We present here the gene sequences and deduced amino acid sequences of the other two, *fliH* and *fliI*. The FliI protein displays significant sequence similarity to components of proton-translocating F_0F_1 ATPase and other related enzymes.

MATERIALS AND METHODS

Bacterial strains. Most of the temperature-sensitive flagellar mutants of *S. typhimurium* were spontaneous mutants of wild-type strain ST1 (43); others were kindly supplied by S. Yamaguchi and are derivatives of wild-type strain SJW1103. They are listed in Table 1, along with the functions and locations of the known gene products.

Temperature shift and shearing protocols. A given mutant was allowed to swarm on a soft tryptone agar plate at 30°C (the permissive temperature). It was then grown in tryptone broth at 30°C to the early log phase; typically, more than 80% of the cells were motile. The culture was placed on ice, passaged 50 times through a hypodermic needle (26 gauge, 3/8 in. [1 in. = 2.54 cm]) to shear off the flagellar filaments, and immediately placed at 42°C (the restrictive temperature) along with an unsheared control culture. As a further control, a portion of the sheared cells was placed at 30°C. After 25 min, both cultures were examined and the percentages of motile cells were scored.

Plasmids. The plasmids used to sequence the *fliH*, *fliI*, and *fliJ* genes of *S. typhimurium* were pMH21 and pAMH3 and deletion derivatives (32, 50).

DNA sequencing. DNA sequencing was carried out on fragments cloned into bacteriophage M13 by using the dideoxy-chain termination method (75) and conventional protocols as previously described (42). Deletions of restriction fragments were obtained by the single-strand sequential

* Corresponding author.
† Present address: Laboratory of Medical Mycology, Research Institute for Disease Mechanism and Control, Nagoya University, Showa-ku, Nagoya 406, Japan.

TABLE 1. Temperature-sensitive flagellar mutants of *S. typhimurium* used in this study

Strain[a]	Genotype	Location of gene product	Reference
MY617	*fliF*	Basal-body M ring	43
MY629	*flgC*	Basal-body rod	43
MY609	*flgG*	Basal-body rod	44
MY601	*flgE*	Hook	44
MY648	*flgK*	Hook-filament junction	43
MY638	*flhA*	Unknown	43
MY654			43
SJW2201			89
SJW2257	*fliE*	Basal body	89
MY671	*fliH*	Unknown	44
MY644	*fliI*	Unknown	43
SJW2197	*fliP*	Unknown	89
MY621	*fliO*	Unknown	44
MY630	*fliG*	Flagellar switch	43
MY650			43
SJW2198			89
SJW2221			89
SJW2284	*fliN*	Flagellar switch	89

[a] MY strains are derivatives of strain ST1 (3), and SJW strains are derivatives of SJW1103 (23). Both parental strains are wild type for motility at the permissive and restrictive temperatures used.

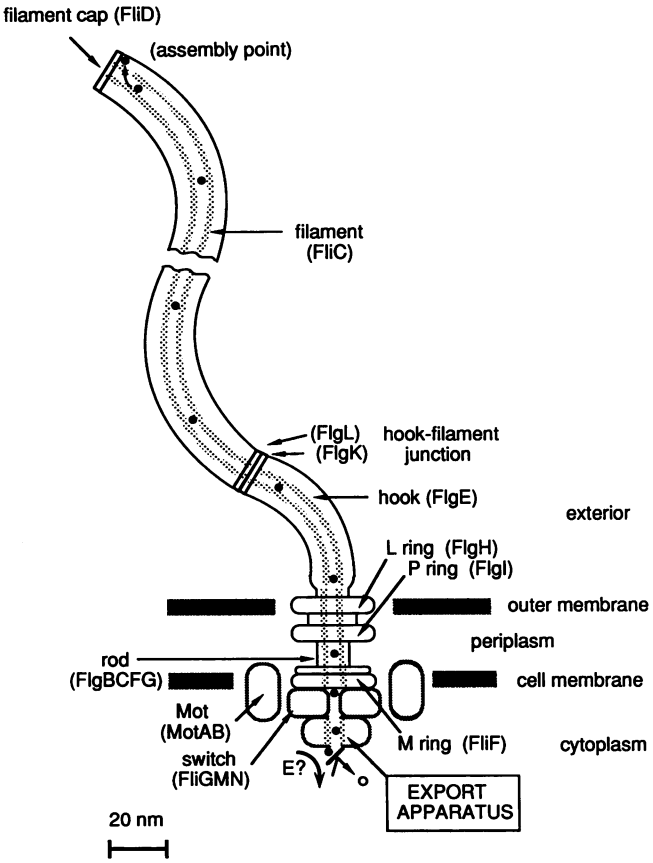

FIG. 1. Diagram of the flagellum of *S. typhimurium*. Many flagellar proteins, including those that make up the rod, hook, hook-filament junction, filament, and filament cap, are external to the cell membrane and (except for the rod) the outer membrane. They are exported by a flagellum-specific pathway which does not involve signal peptide cleavage. The protein subunits are believed to travel down a hollow core in the nascent structure itself before they assemble at the distal end. The export apparatus is very poorly understood but is likely to be at the flagellar base, must be able to distinguish flagellar proteins that are to be exported (solid circles) from other molecules that are not (open circle), and may require energy (E). Known gene products located in various substructures are shown. Structures that have been identified by electron microscopy are indicated by solid outlines; those that are known on genetic and biochemical grounds only are indicated by shaded outlines.

method (11) by using the CYCLONE I Biosystem (International Biotechnologies, Inc., New Haven, Conn.); both strands were sequenced in their entirety. Sequence analysis and comparisons were carried out by using the personal computer package DNANALYZE (Gregory Wernke, University of Cincinnati Medical School). Comparisons with the NBRF protein data base (version 25.0, June 1990) and extractions of DNA sequences from the GenBank data base (version 64.0, June 1990) for translation into the corresponding protein sequences were carried out at the Biomedical Computing Unit at Yale University.

Nucleotide sequence accession number. The DNA sequence reported in this study has been deposited in GenBank under accession no. M62408.

RESULTS

Proposed protocol for detection of export-defective mutants. Many mutants (representing at least 21 different genes) have no detectable flagellar structure by electron microscopy (43, 85, 86). This makes it difficult to distinguish between defects in early structural components and defects in the assembly of later components. For example, if a mutant were unable to assemble the basal body, one would be unable to test its ability to export the components of the major external structures, namely, the hook and filament. If, however, all of the early components were already in place one might be able to make such a test. We decided, therefore, to examine our collection of temperature-sensitive mutants (Table 1) for evidence of defective export. The protocol (Fig. 2) involved growing cells at the permissive temperature, shearing their filaments off, shifting them to the restrictive temperature, and monitoring whether the cells were able to regrow their filaments and recover motility (Table 2). Shearing left the cells completely immotile, and the cells possessed no filaments of sufficient length to be detected by high-intensity dark-field light microscopy (61). At the permissive temperature, the first signs of motility were evident within about 5 min, indicating that it was a result of filament regrowth rather than de novo synthesis of the entire organelle and that the shearing protocol did not cause irreparable damage to the flagellar structure.

Mutants with defects in hook basal-body genes. Mutations in various known hook–basal-body genes all produced similar phenotypes (Table 2): unsheared control cells remained motile when placed at the restrictive temperature, and

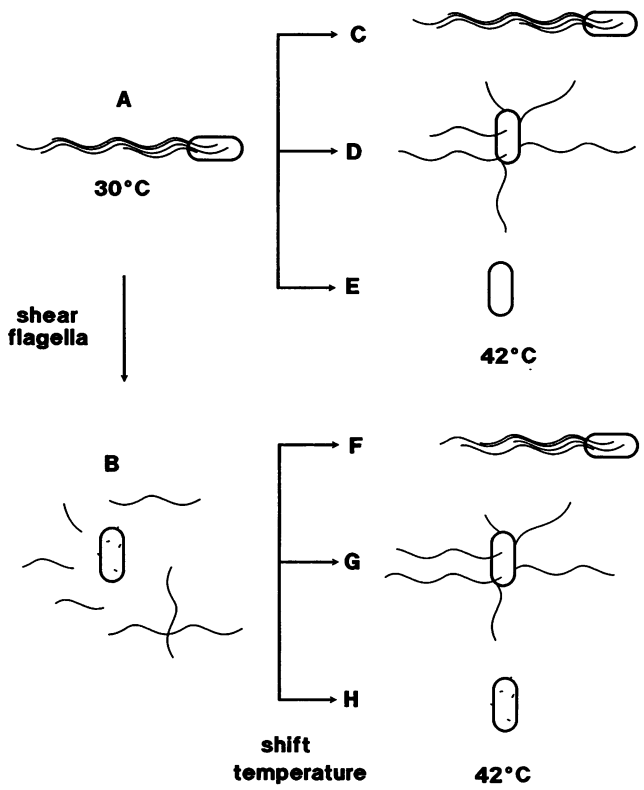

FIG. 2. Protocol used to screen temperature-sensitive flagellar mutants for defects in flagellum-specific export. Cells grown at the permissive temperature of 30°C (A) were flagellated and motile. A portion of the cells were simply shifted to the restrictive temperature of 42°C and inspected to see whether they remained motile (C), became paralyzed (D), or lost their flagella (E). Another portion of the cells (B) had their flagella sheared immediately prior to the temperature shift. After 25 min, they were inspected to see whether they had regrown flagella and were motile (F), had regrown flagella but were paralyzed (G), or had failed to regrow flagella (H). Genes giving rise to category H behavior may be involved in the flagellum-specific export pathway.

sheared cells recovered motility within the 25-min period. This phenotype was displayed by mutants defective in the M-ring gene (*fliF*), rod genes (*flgC* and *flgG*), hook gene (*flgE*), and a gene (*fliE*) that encodes a basal-body protein (68) whose location in that structure is not known. A partial exception was an *flgK* mutant strain in which less than half of the cells recovered motility; *flgK* encodes a protein at the hook-filament junction. We did not have a temperature-sensitive mutant defective in the flagellin gene but presume that such a mutant would be unable to regrow its filaments.

Mutants defective in genes of unknown function. Of the flagellar genes whose function is currently unknown, we had temperature-sensitive mutants representing *flhA*, *fliH*, *fliI*, *fliP*, and *fliO*. Unsheared cells of all of these mutants remained fully motile when placed at the restrictive temperature. Sheared cells of *fliH* and *fliI* mutants exhibited greatly reduced recovery of motility. Sheared cells of two *flhA* mutants remained completely immotile after incubation at the restrictive temperature; a third *flhA* mutant recovered motility normally, indicating that different alleles can vary substantially in thermolability. Sheared cells of *fliP* and *fliO* mutants recovered motility normally at the restrictive temperature.

TABLE 2. Motility of temperature-sensitive mutants after shift to the restrictive temperature

Strain	Genotype	Motility[a]	
		Unsheared	Sheared
MY617[b]	*fliF*	++++	++++
MY648	*flgK*	++++	+++
MY638, MY654	*flhA*	++++	−[c]
SJW2201	*flhA*	++++	++++
SJW2257	*fliE*	++++	++++
MY671	*fliH*	++++	++
MY644	*fliI*	++++	+
SJW2197	*fliP*	++++	++++
MY621	*fliO*	++++	++++
MY630[d]	*fliG*	−[e]	−[f]
SJW2284	*fliN*	−[g]	−[c]

[a] Flagella were either sheared immediately before the temperature shift or not sheared. Motility was recorded 25 min after the temperature shift. ++++, at least 50% and usually more than 80% motile cells; +++, 25 to 50% motile cells; ++, 10 to 25% motile cells; +, ca. 10% motile cells; −, no motile cells.

[b] Results similar to those obtained with the *fliF* mutant were obtained with mutants in other genes encoding hook–basal-body components like the rod (*flgC* and *flgG*) and hook (*flgE*).

[c] Flagella did not regrow.

[d] Similar results were obtained with three other *fliG* mutants (listed in Table 1).

[e] Flagella became paralyzed (temperature-sensitive Mot⁻ phenotype). If the culture was reset to permissive conditions, most cells recovered motility within 2 min.

[f] Flagella regrew but were paralzyed (temperature-sensitive Mot⁻ phenotype). If the culture was reset to permissive conditions, most cells recovered motility within 2 min.

[g] Flagella became paralyzed (temperature-sensitive Mot⁻ phenotype). If the culture was reset to permissive conditions, the cells remained immotile indefinitely.

Switch gene (*fliG* and *fliN*) mutants. Different mutations in switch genes can give rise to a nonflagellate (Fla⁻), paralyzed (Mot⁻), or nonchemotactic (Che⁻) phenotype; the mutants used here were of the temperature-sensitive Fla⁻ phenotype (43). Unsheared cells of these mutants became immotile when shifted to the restrictive temperature (Table 2); i.e., pre-existing flagella exhibited the temperature-sensitive Mot⁻ phenotype. Abrupt loss of motility upon shifting to the restrictive temperature has been noted previously for a temperature-sensitive Mot⁻ *fliG* mutant (12).

In the case of *fliG* mutants, sheared cells incubated at the restrictive temperature were immotile but rapidly (in <2 min) acquired full motility as the culture cooled on a microscope slide. Thus, the lack of motility at the restrictive temperature was a consequence of motor paralysis, not failure to regrow filaments (cf. reference 12). Sheared cells of *fliN* mutants, however, remained immotile indefinitely and lacked filaments as judged by high-intensity dark-field microscopy (61); they therefore resembled the export-negative phenotype of the mutants described above but also shared with the *fliG* mutants the feature of temperature-sensitive paralysis.

Sequence analysis of the *fliH*, *fliI*, and *fliJ* genes. The genes that yielded the phenotype of temperature-sensitive filament regrowth (*flhA*, *fliN*, *fliH*, and *fliI*) have been cloned, and their products have been identified (32, 51, 65).

For two of them (*fliN* and *E. coli flhA*), the gene sequences are also known (50, 51, 65). The other two (*fliH* and *fliI*) are adjacent members of an operon that begins with *fliF* and continues with *fliGHIJK*. The sequences of the first two genes in this operon in *S. typhimurium* have been reported in

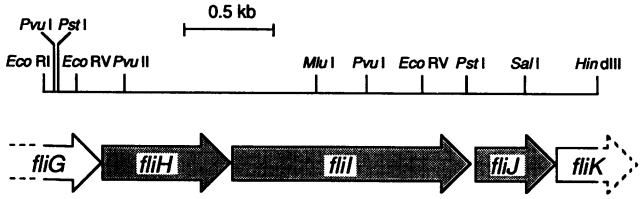

FIG. 3. Restriction site and gene map of the insert of *S. typhimurium* DNA in plasmid pMH21, based on the DNA sequence analysis described in the text (cf. the map based on restriction digestion and complementation given as Fig. 1 of reference 32). The insert contains *fliH*, *fliI*, and *fliJ* as the only intact genes.

the context of studies of the flagellar ring and switch structures, respectively (42, 50). Here we describe the sequences of *fliH*, *fliI*, and *fliJ*. (The sequence of *fliK*, which encodes a protein involved in hook length control [72, 81], will be the subject of a separate communication [48].)

Complementation data have shown that the only intact *S. typhimurium* genes in plasmid pMH21 are *fliH*, *fliI*, and *fliJ* (32), and it is known that the sequence of *fliG* ends about 330 bp after the *Eco*RI site at the start of the insert (50). A restriction map of the insert is shown in Fig. 3. Sequence analysis of the DNA beyond *fliG* revealed the following (Fig. 4). Overlapping with the 3′ end of *fliG* (*ATG*TC*TAA*) is an open reading frame, ORF1, of 705 bp; ORF1 is overlapped at its 3′ end (*TGATG*) by a second open reading frame, ORF2, of 1,368 bp; after a gap of 21 bp, ORF2 is followed by a third open reading frame, ORF3, of 441 bp; ORF3 is overlapped at its 3′ end (*ATGA*) by a fourth open reading frame, ORF4, which continues through the *Hin*dIII site at the end of the insert. We identified ORF1 to ORF3 as *fliH*, *fliI*, and *fliJ*, respectively, by the following criteria. (i) The order and polarity of the genes correspond to those determined previously (57). (ii) The gene immediately upstream of ORF1 has been shown to be *fliG* (50), in agreement with the known gene order. (iii) The sizes of the deduced products are in good agreement with apparent values determined by electrophoresis (see below). (iv) Restriction site and complementation data (Fig. 1 of reference 32) are, with one exception, consistent with the sequence reported here (Fig. 4) in that there are *Eco*RI, *Pst*I, and *Eco*RV sites upstream of ORF1, within *fliG*; a *Pvu*II site within ORF1 at position 236; *Mlu*I, *Pvu*I, *Eco*RV, and *Pst*I sites within ORF2 at positions 1307, 1590, 1906, and 2143; a *Sal*I site within ORF3 at position 2479; and a *Hin*dIII site downstream of ORF3, within ORF4. (The location of the first *Pvu*I site reported on the restriction map of pMH21 [Fig. 1; reference 32] appears to be incorrect, since there is no site in the *fliH* sequence and re-examination of the restriction patterns failed to reveal one at that location; there is, however, a *Pvu*I site upstream of *fliH*, within *fliG* [Fig. 3; reference 50; see bp 729 of Fig. 6].) (iii) The open reading frames were all assessed by the TESTCODE program (22) as authentic coding regions at a confidence level of 96% (ORF2) or 99% (ORF1 and ORF3).

We have also identified ORF4 as *fliK* (48).

Translational initiation sites of *fliH*, *fliI*, and *fliJ*. *fliH*, *fliI*, and *fliJ* are internal to the *fliF* operon and do not reveal features associated with initiation or termination of transcription. They all have strong, well-placed consensus ribosome-binding sequences (Fig. 4). The overlap between *fliG*-*fliH*, *fliH*-*fliI*, and *fliJ*-*fliK* is a feature that is commonly seen within the flagellar regulon (63) and may reflect translational coupling. Between the termination codon of *fliI* and the start

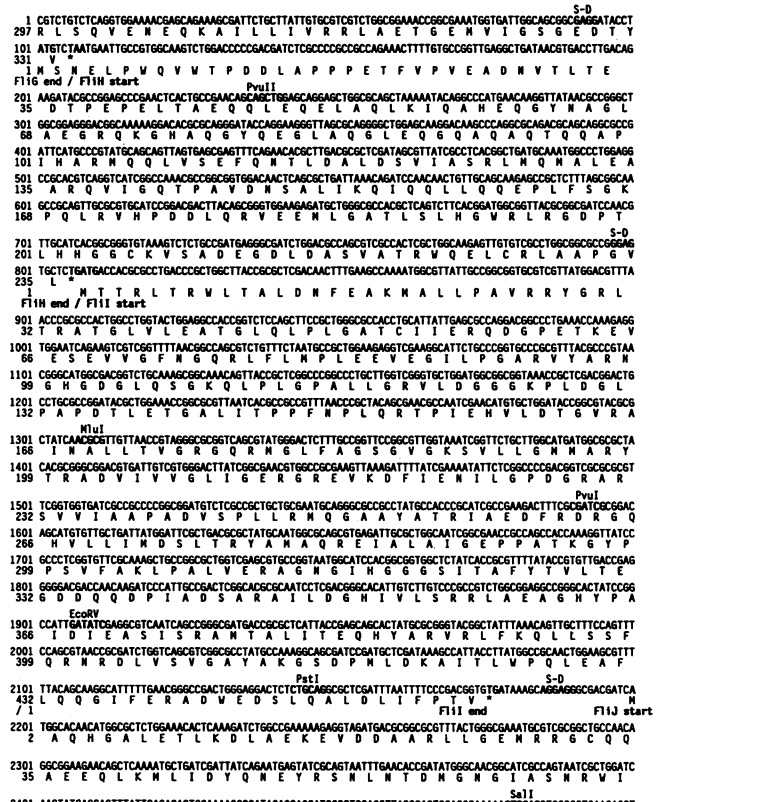

FIG. 4. DNA sequence of the region of the *S. typhimurium* chromosome containing the *fliH*, *fliI*, and *fliJ* genes and deduced amino acid sequences of the gene products. The overlaps with the preceding gene (*fliG*; reference 50) and the following gene (*fliK*; reference 48) are also shown. The restriction sites shown are discussed in the text. S-D, potential ribosome-binding site (Shine-Dalgarno sequence). The asterisks indicate stop codons.

of *fliJ*, there is a noncoding sequence of 21 bp but it contains no symmetry elements (unlike the sequence between the *flgG* and *flgH* genes of the *flgB* operon, which contains a pronounced transcription terminationlike sequence [42]).

The codon usage indices (79) were 0.27, 0.32, and 0.37 for *fliH*, *fliI*, and *fliJ*, respectively, comparable to those seen for other flagellar proteins (e.g., references 30, 34, 42, and 50) and indicating low-to-moderate levels of expression.

Properties of the FilH, FliI, and FliJ proteins deduced from the gene sequences. The deduced sequences of FliH, FliI, and FliJ contain 235, 456, and 147 residues, respectively, and yield molecular masses of 25,782 49,208, and 17,302 Da, respectively. These values are in good agreement with the apparent molecular masses (28, 48, and 15 kDa, respectively) determined by gel electrophoresis (32).

The complete gene sequence of *E. coli fliH* and a partial (5′) sequence for *E. coli fliI* have been determined by Matsumura and colleagues (66). The deduced sequence of *E. coli* FliH shows 82% identity to that of the *S. typhimurium* protein, with the only substantial region of difference in the region from residues 30 to 56 of the *S. typhimurium* sequence, which contains eight additional residues compared

with the *E. coli* sequence; the partial *E. coli* FliI sequence (N-terminal 74 residues) shows 92% identity to the *S. typhimurium* sequence.

The sequences of FliH, FliI, and FliJ are all fairly hydrophilic and give no evidence for any membrane-spanning segments when analyzed by the algorithm of Engelman et al. (20).

FliH is moderately acidic. The N terminus is especially so, with the first 50 residues containing 13 D+E residues and no basic residues. It is also proline rich (8 of the first 39 residues). The protein is predicted (9) to have a considerable amount of α structure but little β structure.

FliI has about equal numbers of acidic and basic residues distributed fairly evenly throughout the sequence. The secondary structure is predicted to contain considerable amounts of both α helix and β sheet.

FliJ has a high proportion of charged residues (D+E+K+R+H = 32 mol%) fairly uniformly distributed throughout, with a slight excess of basic charge. Its glycine and proline contents are low (five and one residues, respectively). Its secondary structure is predicted to be predominantly α helical.

The charge properties of the three protein sequences are consistent with their electrofocussing properties, with FliH focussing at a fairly acidic pH, FliI at a close-to-neutral pH, and FliJ at a fairly alkaline pH (32).

Similarity between FliI and the β and α subunits of the F_0F_1 proton-translocating ATPase. We compared the deduced sequences of the FliH, FliI, and FliJ proteins with protein sequences in the NBRF data base and with sequences of all flagellar and chemotaxis proteins of *S. typhimurium* and *E. coli* to which we had access. The latter, consisting of 43 independent sequences, were FlgBCEFGHIJKL, FlhEAB CD, FliACDSTEFGHIJKLMNOPQR, MotAB, CheAB RWYZ, and a representative receptor, Tar (4, 13, 30, 34, 42, 46, 49, 50–52, 56, 58, 64–66, 69, 71, 73, 82–84; this study); the list excludes only three known flagellar proteins (FlgAD and FliB) and includes two that have not been described in the literature (FliS and FliT; reference 49).

Only one significant similarity was found in these comparisons: FliI showed significant similarity (Fig. 5) to both the β and the α subunits of the ATP-hydrolyzing F_1 portion of the F_0F_1 proton-translocating ATPase from *E. coli* (26, 47, 76) and a variety of other cells and organelles, such as *Bacillus* spp., bovine mitochondria, and maize chloroplasts; F_1 β and α are themselves related (87), although not highly so (26% identity). The optimized scores for the alignments (60) of FliI with F_1 β and α were about 350, while the next nearest scores were about 50 (essentially the noise level).

The similarity to F_1 β and α was first noted by A. Albertini for the deduced product of a gene (currently termed *flaAorf4*) that is almost certainly the functional homolog of *S. typhimurium* FliI, since it has 48% amino acid identity and lies within a cluster of flagellar genes (2).

Alignment and similarity of *S. typhimurium* FliI was closer to the catalytic β subunit of F_1 than to the α subunit (29% versus 25% identity). More significantly, optimal alignment to α required insertion of 13 residues at position 313 (similar to that required for optimal alignment of the β subunit to the α subunit), whereas no major insertion or deletion was required in the alignment of β (Fig. 5).

More detailed considerations provide further support for the significance of the similarities. (i) The residues in the two clusters that are believed to constitute the nucleotide-binding site of the β subunit (termed motifs A and B by Walker et al. [87]) are strongly conserved in FliI (Fig. 5). (ii) There

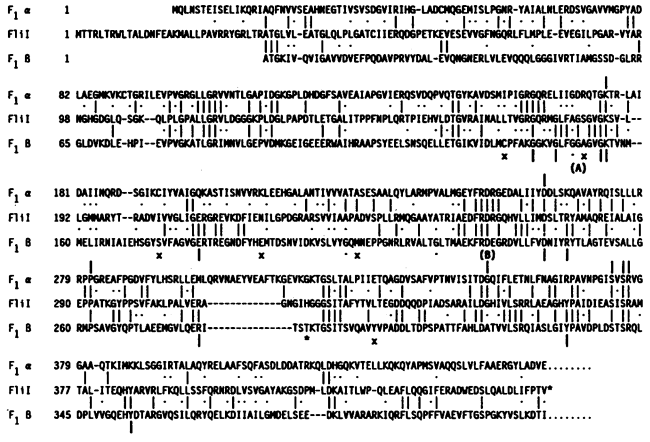

FIG. 5. Alignment of the sequence of FliI with the α and β subunits of the F_1 portion of the F_0F_1 proton-translocating ATPase of *E. coli*. Numbering of the β subunit is for the mature protein after cleavage of the N-terminal methionine; FliI ends at V456, whereas the sequences of the two F_0F_1 ATPase subunits continue. Identities are indicated by vertical lines, and conservative changes (among I-L-V-M-F, F-Y, A-G, D-E, H-K-R, N-Q, or S-T are indicated by dots. (A) and (B) refer to the nucleotide-binding motifs noted by Walker et al. (87). Residues in the α and β subunits that have been implicated in catalysis, either by mutation or by binding of the ATP analog pyridoxal 5′-triphospho-5′-adenosine, are indicated along with the degree of conservation at the corresponding position in FliI, as identical (vertical lines), conserved (dots), or nonconserved (exes). The position indicated by the asterisk is, in the E_1E_2 ATPases, an aspartate residue and is phosphorylated in the catalytic cycle. In α and β, it is not an aspartate, nor does phosphorylation occur; in FliI also it is not an aspartate.

are a number of residues in F_1 α and β that have been shown to affect ATPase activity or to be the target of covalent attachment of the ATP analog pyridoxal 5′-triphospho-5′-adenosine; there are 27 such residues (in α, β, or both) cited in references 24 and 78. For these residues, there is 63% identity among FliI, α, and β.

The results obtained with the F_0F_1 ATPase prompted us to look for similarities to other ATP-driven ion translocases. The vacuolar and archaebacterial proton translocases are fairly closely related to each other and more distantly related to the F_0F_1 ATPase (5, 6, 14, 15, 41). FliI showed significant similarity to the catalytic subunit of both of these types of ATPase, with the regions of highest similarity corresponding to those seen in the comparison with F_1 β. The degree of similarity was slightly lower than that to F_1 β: for example, 28% identity to the Vma-1 protein of the bread mold *Neurospora crassa* (6) and 24% identity to the α subunit of the archaebacterium *Sulfolobus acidocaldarius* (14). Because the noncatalytic and catalytic subunits of these enzymes are themselves related (5, 15), there is also similarity between FliI and the noncatalytic subunits.

The other major class of ion-translocating ATPases is the E_1E_2 class, exemplified by the prokaryotic K^+ translocase and the eukaryotic Na^+/K^+, Ca^{2+}, and plasma membrane H^+ translocases, which show extensive similarity to each other (28) but very little to the F_0F_1 ATPase or the vacuolar and archaebacterial ATPases. FliI was unrelated to the E_1E_2 ATPases, except for a small amount of similarity at a position (GSIT at residues 320 to 323 of FliI versus GTIT in the Kdp protein of the *E. coli* K^+ translocase; 28) where F_1 β and α also show similarity to the E_1E_2 ATPases. In the

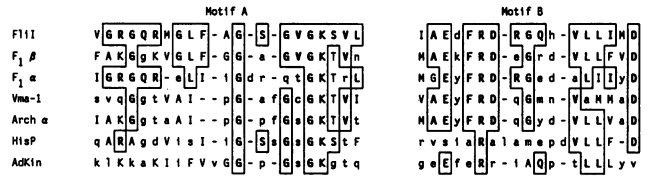

FIG. 6. Alignment of FliI with various ATP-binding or -utilizing proteins in the regions that correspond to nucleotide-binding motifs A and B noted by Walker et al. (87). Residues that are identical in FliI and another protein are in boldface, uppercase letters and are boxed; residues that are conserved (I-L-V-M-F, F-Y, A-G, D-E, H-K-R, N-Q, or S-T) between FliI and another protein are in uppercase; residues that are not conserved between FliI and another protein are in lowercase. F1 β, β subunit of F_0F_1 ATPase; F1 α, α subunit of F_0F_1 ATPase; Vma-1, catalytic subunit of vacuolar ATPase of *N. crassa*; Arch α, α, or catalytic subunit of the archaebacterium *S. acidocaldarius*; HisP, ATP hydrolyzing subunit of the histidine uptake system of *S. typhimurium*; AdKin, bovine adenylate kinase. The latter two proteins were chosen as illustrative examples of ATP-utilizing enzymes generally, in contrast to the four above, which are related proteins within ATP-driven proton translocases.

E_1E_2 ATPases, there is an aspartate three residues upstream which is phosphorylated during the catalytic cycle. FliI, like F_1 β, does not have an aspartate at that position.

Variants of motifs A and B are present in many ATP-utilizing enzymes. However, the degree of agreement between these motifs in FliI and the F_0F_1 and related ATPases is generally stronger than that between FliI and ATP-utilizing proteins generally, as can be seen from the explicit sequence comparisons in Fig. 6. We were unable to achieve significant overall alignments between FliI and any of several such proteins we examined. These included bovine cyclic AMP-dependent protein kinase (80), bovine adenylate kinase (53), *E. coli* homoserine kinase (10), *E. coli* RecA (74), *S. typhimurium* HisP (29), and *E. coli* CheA (a protein kinase in the chemotaxis sensory transduction system) and its substrates CheB and CheY (27). We conclude that FliI is specifically related to subunits of the F_0F_1, vacuolar, and archaebacterial ATPases and not just generally related to nucleotide-binding or nucleotide-utilizing proteins as a whole.

The F_0F_1 ATPase is a large multienzyme complex, with the F_1 component consisting of three α subunits, three β subunits, and one each of the γ, δ, and ε subunits and the F_0 component consisting of one *a* subunit, two *b* subunits, and around 10 *c* subunits. We compared all known flagellar protein sequences with the sequences of these subunits but failed to find any similarities strong enough to be considered significant. Specifically, although F_1 α and β are related, no flagellar protein showed sufficient similarity to either FliI or F_1 α to suggest that it is a homolog.

Given the indications that FliI is involved in export of flagellar proteins, we examined the sequences of the components of the primary cellular pathway for protein export; neither FliI nor any of the other flagellar proteins showed significant similarity to SecA, SecB, SecE, or SecY (7, 16, 55, 77). Nor did they resemble a group of other proteins which have been implicated in ATP-driven protein or peptide transport: the HlyB protein of *E. coli* (21), the McbF protein of *E. coli* (25), the STE6 protein of *Saccharomyces cerevisiae* (54, 67), or the human MDR1 protein (8).

DISCUSSION

Pre-existing flagella still function following temperature shift. With most of the temperature-sensitive mutants we tested, the flagella remained intact and continued to rotate following a temperature shift. The mutant genes included several that encode known components of flagellar structure, such as the M-ring, rod, hook, and hook-filament junction proteins. Together, they make up a large multisubunit complex known from biochemical studies to be able to withstand extreme conditions (1). Apparently, temperature-sensitive components of this complex maintain structure and function at the restrictive temperature once incorporated.

The only proteins that differed from the above description were switch proteins FliG and FliN, which no longer permitted flagellar rotation at the restrictive temperature (cf. reference 12). On the basis of intergenic suppression data (90), the flagellar switch is believed to be a structure peripheral to the cell membrane and may therefore be relatively unconstrained by other flagellar components, with the result that a shift to the restrictive temperature causes loss of its ability to enable flagellar rotation.

***flhA*, *fliH*, *fliI*, and *fliN* mutations affect filament regrowth.** As was described above, mutations in these genes totally or partially prevented regrowth of flagellar filaments at the restrictive temperature. This loss of function therefore indicates that the gene products are involved at some stage in the overall process of assembly of external components.

A temperature-sensitive mutation in the structural gene for an exported component that had been sheared off would certainly be expected to result in failure of regrowth. Thus, a temperature-sensitive flagellin would presumably fail to assemble into a filament at the restrictive temperature even if exported successfully. However, this cannot be the explanation for the failure of regrowth in the *flhA*, *fliH*, *fliI*, and *fliN* mutants, since their products are not present in the external flagellar structure, which has been carefully characterized in terms of protein composition and genetic origin (40). Nor can the results be explained in terms of the process of incorporation of flagellin into a filament, since the only proteins needed for this process are the three hook-associated proteins (31, 35, 40), which are not encoded by the genes in question.

The process of flagellar assembly has been analyzed in terms of which genes are necessary to reach a substructure of a given complexity. *flhA*, *fliH*, and *fliI* were all found by Suzuki and coworkers (85) to be needed for the earliest detected flagellar structure, an inner-ring–rod complex (*fliN* mutants were not included in the study). A recent study (43) has shown further that *flhA*, *fliI*, and *fliN* participate after M-ring assembly and before assembly of the first external structure, the rod (*fliH* mutants were not included in that study). Thus, the position of these genes in the assembly pathway is consistent with a role in the process of flagellum-specific export.

The export apparatus, like the switch complex, is likely to be a peripheral structure and to be less constrained for that reason. The thermolability of the FlhA, FliH, FliI, and FliN proteins could involve a relatively subtle structural change or one as drastic as dissociation from the flagellar apparatus. Whatever the nature of the event, it affects only the process of filament regrowth and not (except for switch mutants) other aspects of motor structure and function, which were normal.

In the case of *fliN*, we suspect that the role in enabling filament regrowth is an indirect one. Mutant phenotype and

intergenic suppression data (90, 91) clearly suggest a function in motor energizing and switching and a location exposed to the cytoplasm. FliN may physically interact with the export apparatus and, if defective, may affect its structural integrity.

This study has provided the first clues concerning the genes that may be involved in the flagellum-specific export process. However, with the exception of *fliI* (see below), it is premature to speculate on what specific roles these genes and their products play.

The sequence of FliI indicates that it is a subunit of an ATPase. The sequence similarity between FliI and the F_0F_1 ATPase β subunit and other related proteins is clearly significant, especially when one takes into account the location of the regions of highest similarity, which include the two elements of the nucleotide-binding site and other residues that are important in catalysis. FliI, F_1 β, and the catalytic subunits of the vacuolar-archaebacterial ATPases are roughly equally related, suggesting that they all diverged from a common ancestral protein at about the same time. The degree of similarity is not high enough to justify consideration of the enzymes as members of a common class. It seems more appropriate to regard them as falling into three different but related classes, all quite remote from the E_1E_2 ATPases.

Since the proteins that FliI resembles are all subunits of ATP-driven proton pumps (or, in the reverse direction, proton-driven ATP synthases), it seems likely that FliI participates in some related type of process and not in a totally different kind of ATP-requiring process.

What might the role of FliI be? If it is a component of an ATP-driven proton translocase, then it must be one which is intimately linked to the flagellum, since the phenotype of *fliI* mutants is nonflagellate. There is a large body of evidence that indicates that the flagellar motor is driven by the cell's proton motive force (62). However, Eisenbach and Wolf (18, 88) have found that cells can rotate their flagella in the absence of a proton motive force, provided respiration or ATPase activity from endogenous energy sources is possible. They suggest that there might be dedicated electron transport chains and ATPases that are capable of injecting protons directly into the flagellar motor; in support of this hypothesis, they report that preparations of vesicles with flagella associated with them have higher respiratory and ATP hydrolysis rates than the vesicle population generally. Conceivably, FliI might be part of such a flagellum-specific ATP-driven proton translocase whose function is to rotate the flagellar motor. Recall, however, that the *fliI* mutant could rotate its flagella normally at the restrictive temperature. Thus, there is no evidence that FliI plays a role in motor rotation, at least under our conditions.

In view of the finding, described in the first part of this report, that a defect in FliI blocks filament regrowth, we consider it possible that FliI is part of an ATP-driven protein translocase responsible for export of flagellar proteins via the flagellum-specific export pathway. A related hypothesis would be that FliI is part of an ATP-driven proton translocase (or proton-driven ATP synthase) that is directly associated with the flagellum and that the interconverted energy is used for the flagellum-specific export process.

Regardless of its function, FliI is probably (like F_1 β) only one component of a multisubunit complex. The failure to find any obvious homologs to the other components of the F_0F_1 ATPase could mean that there are homologs but the sequences have diverged too far for similarities to be recognized. Alternatively, the subunit composition of the FliI-containing complex may be entirely different; for example, it may not need the participation of an α-type subunit. And of course one would not expect to find any close homolog of the F_0 component of the proton translocase if FliI were in fact part of a protein translocase.

In the absence of clues based on sequence, other means of identifying components that interact with FliI will be needed, such as intergenic suppression analysis or coimmunoprecipitation. Also, although the sequence similarities seem compelling, it is obviously necessary to test experimentally whether FliI binds nucleotides and hydrolyzes them. It may not be possible to demonstrate catalytic activity of FliI in isolation; in the case of the F_0F_1 ATPase, for example, the minimal structure capable of appreciable ATP hydrolysis rates is not β but the $\alpha_3\beta_3\gamma$ complex (17). Assuming that ATP hydrolytic activity can be shown for FliI or a FliI-containing system, the next challenge will be to determine what the energy generated is used for.

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