Genetic and Biochemical Analysis of \textit{Salmonella typhimurium} FliI, a Flagellar Protein Related to the Catalytic Subunit of the F$_0$F$_1$ ATPase and to Virulence Proteins of Mammalian and Plant Pathogens

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FliI is a \textit{Salmonella typhimurium} protein that is needed for flagellar assembly and may be involved in a specialized protein export pathway that proceeds without signal peptide cleavage. FliI shows extensive sequence similarity to the catalytic $\beta$ subunit of the F$_0$F$_1$ ATPase (A. P. Vogler, M. Homma, V. M. Irikura, and R. M. Macnab, J. Bacteriol. 173:3564–3572, 1991). It is even more similar to the Spa47 protein of \textit{Shigella flexneri} (M. M. Venkatesan, J. M. Buyse, and E. V. Oaks, J. Bacteriol. 174:1990–2001, 1992) and the HrpB6 protein of \textit{Xanthomonas campestris} (S. Fenselau, I. Balbo, and U. Bonas, Mol. Plant-Microbe Interact. 5:390–396, 1992), which are believed to play a role in the export of virulence proteins. Site-directed mutagenesis of residues in FliI that correspond to catalytically important residues in the F$_1$ $\beta$ subunit resulted in loss of flagellation, supporting the hypothesis that FliI is an ATPase. FliI was overproduced and purified almost to homogeneity. It demonstrated ATP binding but not hydrolysis. An antibody raised against FliI permitted detection of the protein in wild-type cells and an estimate of about 1,500 subunits per cell. An antibody directed against the F$_1$ $\beta$ subunit of \textit{Escherichia coli} cross-reacted with FliI, confirming that the proteins are structurally related. The relationship between these three proteins involved in flagellar assembly (FliI, FliA, and FliP) and homologs in a variety of virulence systems is discussed.

The flagellum of \textit{Salmonella typhimurium} and those of many other bacteria consist of at least three distinct structures: the filament and hook (both of which are completely external to the cell) and the basal body (which spans the cytoplasmic membrane, the periplasmic space, and the outer membrane) (see reference 36 for a review). Thus, many flagellar proteins, following their synthesis inside the cell, need to be exported from the cytoplasm. Only two of these (FlgH and FlgI, from which the outer pair of basal-body rings are constructed) appear to be exported by the conventional signal peptide-dependent pathway (22, 26). The rest do not undergo cleavage of a signal peptide and are presumed to be exported by a unique flagellum-specific pathway (36). The major protein in this class, flagellin, is known to assemble at the distal end of the growing filament (10, 25), and it is presumed that the proteins constituting the other substructures such as the rod and hook are also assembled by distal addition, since they are related to flagellin (20, 23) and the order of assembly of these substructures is from cell proximal to cell distal (first rod, then hook, and finally filament) (27, 31, 48, 49). The physical path for travel of subunits to their destination is almost certainly through the hollow channel that exists in the structure (38, 42). However, there must also be an apparatus, presumably somewhere in the vicinity of the flagellar base, that transports the proteins across the plane of the cell membrane (although not through the bulk membrane itself) into this channel. This export apparatus must be selective in the proteins it recognizes, but a flagellum-specific signal sequence has not yet been identified (20, 23).

Studies with temperature-sensitive mutants revealed several classes that could not regrow sheared filaments when placed at the restrictive temperature, suggesting that the defective gene products might be involved in the export process (52). The deduced sequence of one of these, FliI, was found to have ca. 30% identity to the catalytic $\beta$ subunit of the bacterial F$_0$F$_1$ proton-translocating ATPase and to the equivalent subunits of vacuolar and archaeobacterial ATPases (52). The \textit{Bacillus subtilis} homolog of \textit{S. typhimurium} FliI is also similar to F$_1$ $\beta$ (1). FliI also shows slightly weaker similarity to the regulatory F$_1$ $\alpha$ subunit, which is related to $\beta$.

No further similarities between the other known flagellar proteins (of which there are about 40 [36]) and any of the other subunits of the F$_0$F$_1$ ATPase or other ATPases or between FliI and the components of the primary protein export pathway (Sec) or other proteins responsible for ATP-driven protein or peptide transport were found (but see Discussion regarding similarities to virulence protein export systems, which were described subsequently).

Although the exact function of FliI is not known, the very existence of a flagellar protein that resembles a component of ATP-driven proton translocases is interesting from both functional and evolutionary points of view.

In this study, we have made selected mutations and examined their phenotypic consequences. We have overexpressed and purified the FliI protein and examined its properties with respect to ATP binding and hydrolysis.
Finally, we have used antibodies to estimate its cellular stoichiometry.

MATERIALS AND METHODS

Subcloning of \textit{fliI}. \textit{pMH22} (21) is a pBR322-based plasmid containing an \textit{EcoRI-SalI} fragment of \textit{S. typhimurium} DNA that encodes flagellar genes \textit{fliH} and \textit{fliG} plus a 3' fragment of \textit{fliJ} and a 5' fragment of \textit{fliF}. An \textit{Eco47I-Eco47H} fragment from \textit{pMH22} was inserted into the \textit{SalI} site within the multiple cloning region of plasmid pHS398 to yield plasmid \textit{pIK2001}. The fragment of \textit{pIK2001} flanked by the \textit{BamHI} and \textit{EcoRI} sites of the multiple cloning region was then placed into \textit{BamHI-EcoRI}-digested \textit{pET-11a} to yield plasmid \textit{pIK2101} with an in-frame fusion of a short 5' portion of the vector gene-10 and a 3' fragment of \textit{fliH}. The actual DNA sequence and deduced amino acid sequence in the region of the fusion are shown below (linker, sequence from the polylinker region of pHS398); amino acids deriving from flagellar genes are shown in uppercase letters. The construction places the fusion gene and other genes in the same operon, notably \textit{fliI}, under control of the \textit{T7}lac promoter (p\textit{T7lac}).

\textbf{FIG. 1.} Construction of a plasmid for the overproduction of \textit{FliI}. \textit{pMH22} is a pBR322-based plasmid containing an \textit{EcoRI-SalI} insert of \textit{S. typhimurium} DNA that encodes flagellar genes \textit{fliH} and \textit{fliG} plus a 3' fragment of \textit{fliJ} and a 5' fragment of \textit{fliF}. An \textit{Eco47I-Eco47H} fragment from \textit{pMH22} was inserted into the \textit{SalI} site within the multiple cloning region of plasmid pHS398 to yield plasmid \textit{pIK2001}. The fragment of \textit{pIK2001} flanked by the \textit{BamHI} and \textit{EcoRI} sites of the multiple cloning region was then placed into \textit{BamHI-EcoRI}-digested \textit{pET-11a} to yield plasmid \textit{pIK2101} with an in-frame fusion of a short 5' portion of the vector gene-10 and a 3' fragment of \textit{fliH}. The actual DNA sequence and deduced amino acid sequence in the region of the fusion are shown below (linker, sequence from the polylinker region of pHS398); amino acids deriving from flagellar genes are shown in uppercase letters. The construction places the fusion gene and other genes in the same operon, notably \textit{fliI}, under control of the \textit{T7}lac promoter (p\textit{T7lac}).

\textbf{Materials and Methods.} The \textit{FliI} K-188→E (K188E), K188I, and D272N mutations (see Results) were created by the gapped duplex method (30) applied to the \textit{BamHI-EcoRI} fragment of \textit{pIK2001} cloned into M13, by using a kit supplied by Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and the following mutagenic primers: 5'-GAACCGATTC(T→C)ACCAAGCG-3' (K188E), 5'-AGAACCGAT(T→A)TACC AACG-3' (K188I), and 5'-CAAGCGAT(C→T)CATATG AG-3' (D272N). The mutant \textit{fliI} gene on the \textit{BamHI-EcoRI} fragment was then recloned into pHS398.

The Y363S mutation was created by the polymerase chain reaction-directed mutagenesis procedure outlined by Higuchi (19). To generate the two mutagenic fragments, the two primary polymerase chain reactions used either \textit{FliI}-460a (5'-CAAATCGAATCTGCTGG-3', at bp 460 of \textit{fliI}) and \textit{FliI-Y363Sb} (5'-AATGGCGGAT[T→G]TGGCAGG-3') or \textit{FliI-Y363Sa} (5'-CGGGCAGT[A→C]TCGGCAGATT-3') and \textit{FliI-031b} (5'-TGAGTGTTTTTCCAGGC-3', at bp 31 of \textit{fliI}). These two fragments were then allowed to anneal and extend, and the two outside primers, \textit{FliI}-460a and \textit{FliI-031b}, were used to amplify the extended fragment. The mutagenic site was then cloned as a \textit{SalI}-EcoRV fragment (Fig. 1) replacing the corresponding wild-type sequence of \textit{fliI} in plasmid \textit{pIK2001}. All mutations were verified by sequencing using the dyeoxy method (43).

\textbf{Complementation analysis.} Mutant alleles of \textit{fliI} were tested for complementation using \textit{Escherichia coli} YK4178 (\textit{fliI}) (4) as the host. Semisolid tryptone agar plates containing chloramphenicol were used to examine the motility of transformed cells at 30°C. Motility and flagellation were observed by high-intensity dark-field light microscopy (35).

\textbf{Construction of plasmids for overexpression of wild-type and mutant alleles of \textit{fliI}.} Plasmid p\textit{ET-11a} (8) has a \textit{T7}lac promoter-operator region, an optimally placed ribosome binding sequence, and a \textit{BamHI} site shortly after the start of the natural open reading frame (phase gene-10). \textit{pIK2001} was digested with \textit{BamHI} and \textit{EcoRI}, and the fragment containing the insert was isolated and ligated with \textit{pET-11a}, which had been digested with the same enzymes. The resulting construction, \textit{pIK2101} (Fig. 1), contained an operon under control of the \textit{T7} promoter, starting with an in-frame fusion of the 5' end of gene 10 and the 3' end of \textit{fliH}; this fusion was followed by the intact \textit{fliI} gene in its normal translational relationship to \textit{fliH} and a truncated version of \textit{fliJ} terminated by a stop codon just beyond the \textit{SalI} site of the insert. Derivatives of \textit{pIK2101} were constructed as follows: \textit{pIK2102} contained an out-of-frame internal \textit{BssHII}-\textit{BssHII} deletion resulting in a truncated \textit{FliI} product with a molecular mass of 21 kDa, \textit{pIK2103} contained an in-frame internal \textit{MluI}-\textit{BssHII} deletion resulting in a \textit{FliI} product with a molecular mass of 43 kDa, and \textit{pIK2105} contained an \textit{EcoRV-XbaI} deletion (constructed by using an XbaI site in the polylinker region of the vector) religated with an \textit{EcoRV-XbaI} linker and resulting in a truncated \textit{FliI} product with a molecular mass of 39 kDa.

\textbf{Overproduction and purification of \textit{FliI}.} Plasmid \textit{pIK2101} was used to transform \textit{E. coli} MG77 (33), which has a chromosomally encoded copy of \textit{T7} RNA polymerase. Cells were grown overnight at 37°C in 10 ml of Luria medium containing ampicillin at 200 μg ml⁻¹. The overnight culture was used to inoculate a 1-liter volume of the same medium, and the cells were grown at 37°C to an optical density of 600 nm of 0.7. At this point, 10 ml of 100 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and growth was continued for a further 6 h. The cells were harvested by centrifugation (10,000 × g, 15 min), and the pellet was resuspended to a volume of 40 ml in 10 mM Tris-HCl, pH 7.5, and stored at −50°C until needed.

Frozen cells were thawed and divided into 5-ml batches, plated on ice, and sonicated (Heat Systems model W140D; three 1-min cycles, 40 W). The sonicated samples were pooled and centrifuged (2,000 × g, 5 min) to pellet intact cells, and the supernatant was carefully decanted and centrifuged (17,500 × g, 20 min). The pellet was resuspended in 10 ml of a buffer containing 100 mM Tris-HCl (pH 7.3), 100 mM NaCl, 5 mM MgCl₂, and 1 mM phenylmethylsulfonil fluoride. An equal volume of 6 M guanidinium-HCl was
added with constant stirring, and the sample was incubated on ice for 20 min. It was then centrifuged for 30 min at 17,500 × g, and the supernatant was dialyzed overnight (Spectra/Por 2 membrane) at 4°C against 1 liter of 0.5 M K-glutamate–0.1 M NaCl, with two changes; during this process, FliI came out of the solution as a flocculent precipitate. The dialysate was centrifuged for 20 min at 31,000 × g, and FliI was recovered in the pellet, resuspended in 10 ml of buffer containing 100 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 5 mM MgCl₂, and divided into aliquots that were stored at −50°C. Further purification was achieved by ion exchange chromatography (LCC-500 system; Pharmacia LKB Biotechnology, Piscataway, N.J.) on a Mono S (16/10) fast protein liquid chromatography (FPLC) column. The column was first equilibrated in 50 mM Tris-HCl (pH 7.5)–50 mM K-glutamate–3 mM guanidine-HCl at a flow rate of 2 ml min⁻¹. A 1-ml frozen sample of the partially purified FliI was thawed, and the flocculent precipitate was gently pelleted in a clinical centrifuge (150 × g, 10 min) and resuspended in the same buffer as that used to run the column. A 0.1-ml sample was loaded onto the column, and the protein concentration of the eluate was monitored spectrophotometrically at 280 nm, by using an extinction coefficient of 3.1 × 10⁴ M⁻¹ cm⁻¹ on the basis of the known Trp+Tyr content of FliI (52). The proteins were eluted in three peaks, the middle one of which was by far the largest and was found to contain FliI in >95% purity. The separation procedure was repeated several times with further crude samples in order to accumulate purified material.

Fluorescence spectroscopy. Spectra were taken by using an SLM 8000 C spectropho fluorimeter (Aminco, Urbana, Ill.). The fluorescence of 2'(3')-O-(2,4,6-trinitrophenyl)-ATP (TPN-ATP) (Molecular Probes, Eugene, Oreg.) was measured at 30°C in 2 ml of 10 mM Tris-HCl (pH 7.5)–20% glycerol by using a TPN-ATP concentration of 2.5 μM. The excitation wavelength was set at 410 nm (slit width, 2 nm), and the emission wavelength was scanned from 480 to 640 nm (slit width, 2 nm). FliI at 7 mg ml⁻¹ (140 μM) in 3 M guanidine-HCl was diluted to a final concentration of 0.3 μM. For quantitative estimates of fluorescence as a function of TPN-ATP concentration, a fixed emission wavelength of 550 nm was used. Fluorescence enhancement was defined as the observed fluorescence in the presence of FliI minus the fluorescence of TPN-ATP alone at the same concentration.

The intrinsic tryptophan fluorescence of FliI was measured at 30°C in the buffer described above by using a sample volume of 2 ml and a FliI concentration of 6 μM. The excitation wavelength was set at 295 nm (slit width, 4 nm), and the emission wavelength was scanned from 310 to 400 nm (slit width, 2 nm).

ATPase assay. ATPase activity was assayed in 0.5 ml of a reaction mixture containing 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid-KOH (pH 7.8), 5 mM MgCl₂, 5 mM ATP, 5 mM phosphoenolpyruvate, and 50 μg of pyruvate kinase ml⁻¹. Samples of FliI (0.05, 0.1, and 0.15 mg) were incubated at 30°C for different times ranging from 5 s to 60 min and assayed for release of Pi by the method of Fiske and Subbarow (13); Saccharomyces cerevisiae plasma membrane ATPase, generously provided by Carolyn Slayman (Yale University), was used as a control enzyme for the assay.

Antibodies. Overproduced FliI, partially purified by solubilization of inclusion bodies in 3 M guanidine-HCl and precipitation by dialysis against 0.5 M K-glutamate plus 0.1 M NaCl, was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels (45). The FliI band was excised from the gels (0.75 mm thick, 12 10-mm-wide lanes) and used for production of polyclonal rabbit antibody (BABco, Berkeley, Calif.). The initial injection corresponded to 125 μg of FliI, and subsequent boosts of the same amount were carried out every 21 days; blood was collected 1 week after each injection and clotted, and the crude antiserum was reserved for use. Polyclonal rabbit antibody directed against the β subunit of the E. coli F₆F₅ ATPase was a gift from Alan Senior, University of Rochester.

Immunoblotting. Proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose for 5 h (50). After staining (0.5% Ponceau S, 7.5% trichloroacetic acid; 10 min) and destaining with 7.5% acetic acid for 15 min with three changes, the sheets were marked and blocked with 3% gelatin in H₂O for 1 h. Anti-FliI and anti-F₆F₅ β antiserum were used at 1:1,000 and 1:5,000 dilutions, respectively, in 1% gelatin in 100 mM Tris-HCl (pH 7.5)–0.9% NaCl–0.1% (vol/vol) Tween 20. The nitrocellulose sheets were then processed by using the Vectastain ABC-AP kit (Vector Laboratories, Burlingame, Calif.).

RESULTS

Site-directed mutations in FliI. F_I β has been subjected to extensive mutational analysis (14, 46), and so we sought to use the information obtained with it to aid us in our understanding of FliI.

Some of the F_I β mutations involve residues that are important for assembly of the multisubunit enzyme. We did not consider these residues further, since (i) a flagellar-assembly apparatus would presumably have a very different structure than a proton translocase and (ii) no sequence similarity to the remaining F₆F₅ subunits among any of the other flagellar proteins has been found.

Other mutations in F₁ and other related ATPases, however, involved residues that are highly conserved and catalytically important. For example, mutation of either Lys-155 within the A motif or Asp-242 within the B motif of the E. coli F₆F₅ β subunit results in greatly reduced ATP hydrolysis rates (2, 39). A third highly conserved residue is Tyr-331, which plays a role in ATP hydrolysis but apparently is not directly involved in the bond making-breaking step of catalysis (55), since phenylalanine can be substituted for tyrosine without substantial loss of activity (whereas mutation to other amino acids, including those with polar side chains, results in an inhibited or unstable enzyme).

We therefore carried out site-directed mutagenesis at the corresponding positions in FliI, namely Lys-188, Asp-272, and Tyr-363, in order to see whether they were important for flagellar assembly. The mutations we generated were K188E and K188I (to test change to side chains of approximately the same size, but negatively charged or hydrophobic), D272N (to test change to the related polar but uncharged side chain), and Y363S (to test loss of aromatic character but retention of the polar OH group). When an E. coli fliI mutant host, YK4178, was transformed with plasmids containing these mutant alleles of fliI, no swimming on semisolid agar plates was detected (Fig. 2); high-intensity dark-field light microscopy (35) established that the cells were nonflagellate rather than paralyzed or nonchemotactic (data not shown). Host cells transformed with plasmid pIK2001 (containing the wild-type fliI insert) were capable of swimming (Fig. 2).

Wild-type S. typhimurium LT2 transformed with a plasmid bearing the K188I mutant allele of fliI had the same motility on swarm plates as did cells transformed with the
equivalent plasmid containing the wild-type flII insert (data not shown), establishing that the mutant form of FilI was not dominant.

**Overproduction and characterization of FilI.** FilI was overproduced under control of the T7 promoter by using plasmid pIK2101 (see Materials and Methods). After 6 h of growth under inducing conditions, SDS-PAGE revealed an intense band at the position expected for FilI on the basis of both previous analysis of radiolabeled material in a minicell expression system (21) and the molecular mass (49 kDa) deduced from its gene sequence (52). When induced cells were sonicated and fractionated by medium-speed centrifugation, this protein was by far the major one in the pellet fraction and was present in a much smaller amount in the supernatant fraction (Fig. 3, lanes 7 and 8), indicating that it had formed inclusion bodies.

That the overproduced protein was FilI was confirmed by constructing derivatives of pIK2101 with either deletions or truncations (see Materials and Methods) and verifying that the electrophoretic mobility was altered in the predicted manner (Fig. 3, lanes 1 to 6). Verification that pIK2101 directs expression of a functional copy of flII was obtained by transforming YK4178, a flII mutant of *E. coli*, and showing that it had gained the ability to swarm on soft-agar plates; the same strain transformed with the vector pET-11a or with plasmids containing the truncation or deletion versions of flII remained nonflagellate (data not shown).

The purification procedure we used (see Materials and Methods) included the solubilization of the inclusion bodies with 3 M guanidinium-HCl and dialysis against 0.5 M K-glutamate. This treatment resulted in formation of a flocculent precipitate and substantial purification of the protein (ca. 85%). Further purification (>95%) for fluorescence studies was achieved by ion exchange chromatography at low concentrations of guanidinium-HCl.

We next attempted to find conditions under which the protein would be soluble in the absence of guanidinium-HCl. When the protein in 3 M guanidinium-HCl was added slowly to 10 mM Tris-HCl, pH 7.5, until it had been diluted 20-fold, it still remained in solution, and its concentration was ca. 100 μg ml⁻¹. At this point, it remained in solution even after dialysis against buffer to remove the remaining guanidinium-HCl. However, when the protein was concentrated by ultrafiltration or solvent evaporation, it started to aggregate and precipitate. Interestingly, this precipitate consists of microscopic crystals, and efforts are under way to obtain larger crystals for X-ray diffraction (3).

**Fluorescence measurements of ATP binding.** Since FilI contains motifs A and B (described by Walker et al. [53]), which in F₁β are believed to make major contributions to the nucleotide-binding site of the β subunit, we wished to examine whether FilI would bind ATP. Two different spectroscopic methods were employed. In the first, we used the fluorescent nonhydrolyzable analog, TNP-ATP. Free TNP-ATP in solution shows a characteristic fluorescence spectrum with a maximum at 550 nm; upon binding of TNP-ATP to protein, the fluorescence is enhanced and shifted to the blue (16). This was the effect we observed when FilI was added to a solution of TNP-ATP (Fig. 4). Lysozyme, used as a control protein with no nucleotide-binding ability, caused neither enhancement nor a shift of the fluorescence. The mutant form of FilI produced by pIK2103 has an internal deletion that results in the loss of 62 residues surrounding nucleotide-binding motif A, with motif B remaining intact; it still caused significant fluorescence enhancement of TNP-ATP, but less than that observed when wild-type FilI was used (Fig. 4).

Fluorescence enhancement by FilI measured as a function of TNP-ATP concentration indicated saturation binding (Fig. 5). The data did not conform to a simple hyperbolic binding curve, but half-maximal enhancement occurred at around 4 μM, indicating an apparent dissociation constant of about that value, which is comparable to the value of 5 μM that Garboczi et al. obtained for binding of TNP-ATP to F₁β (17).
The fluorescence of tryptophan residues is sensitive to their microenvironment and is commonly used to monitor protein conformational changes. We have used this property to detect ATP binding to wild-type FliI, which contains three tryptophan residues (Trp-8, Trp-425, and Trp-442). Figure 6 shows that the tryptophan fluorescence is significantly quenched by addition of 200 μM ATP, as has been observed with other ATP-binding proteins, such as the F1 β subunit of the yeast Schizosaccharomyces pombe (7).

**Attempted measurement of ATP hydrolysis by FliI.** Having demonstrated ATP binding, we assayed purified FliI for ATP hydrolysis, using the method of Fiske and Subbarow (13). No hydrolysis was detected.

![Fluorescence spectra of TNP-ATP](image)

**FIG. 4.** Fluorescence spectra of TNP-ATP. The assay medium contained 50 mM Tris-HCl, pH 7.5, and 2.5 μM TNP-ATP, and proteins were added at 17 μg ml⁻¹. Spectra were measured at 30°C by using an excitation wavelength of 410 nm. ——, wild-type FliI from pIK2101; ——, deletional mutant FliI from plasmid pIK2103; ——, lysozyme; —— (boldface), medium without protein.

![Fluorescence enhancement of TNP-ATP by FliI](image)

**FIG. 5.** Fluorescence enhancement of TNP-ATP by FliI as a function of TNP-ATP concentration. The FliI concentration was held constant at 17 μg ml⁻¹ (ca. 0.3 μM), and the concentration of TNP-ATP varied as shown. Fluorescence spectra were taken as for the results in Fig. 4, at a fixed emission wavelength of 550 nm.

![Intrinsic tryptophan fluorescence of wild-type FliI](image)

**FIG. 6.** Intrinsic tryptophan fluorescence of wild-type FliI at 6 μM (——) and its quenching by addition of 200 μM ATP (· · ·).

**Estimation of the wild-type level of FliI.** In order to estimate the level of FliI in wild-type cells, we raised a polyclonal antibody against the purified protein and performed immunoblotting. Figure 7 shows the results with various amounts of purified FliI as standards (lanes 1 to 4), cells of wild-type strain SJW1103 (lane 5), and cells of an flhCD deletion mutant, SJW1368 (32), which does not express any flagellar genes (lane 6). At the position of the FliI standards, a band was present in the case of the wild-type cells but not in the case of the mutant. By comparing the intensity of the band produced by wild-type cells with the intensities of the bands produced by the set of standards, we estimate that the wild-type lane contains ca. 15 ng of FliI, which corresponds to a content of ca. 1,500 subunits per cell.

**Immuochemical cross-reactivity between FliI and F1 β.** Given the degree of amino acid similarity between FliI and E. coli F1 β, we were interested in testing for possible recognition of FliI by anti-β antibody and of β by anti-FliI antibody. Purified FliI was clearly recognized by a poly-

![Detection of FliI in wild-type cells and estimation of its level](image)

**FIG. 7.** Detection of FliI in wild-type cells and estimation of its level. Various amounts (49, 35, 24, and 17 ng) of purified FliI as calibration standards (lanes 1 to 4, respectively), whole cells (1.2 × 10⁸) of a wild-type (WT) strain SJW1103 (lane 5), and an flhCD deletion (ΔCD) strain SJW1368 (lane 6) were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with anti-FliI antibody. The amount of FliI in lane 5 was estimated at ca. 15 ng, leading to an estimate of ca. 1,500 subunits per cell. The absence of a signal in the case of the flhCD strain, which has the entire flagellar regulon inactivated and thus does not synthesize FliI, established that the antibody does not appreciably cross-react with F1 β, which migrates at essentially the same position as FliI. Lane 7, 70 ng of purified FliI probed with a polyclonal antibody raised against the β subunit of the E. coli F1Fβ ATPase; this antibody, as can be seen, cross-reacts with FliI.
clonal anti-β antibody at serum dilutions of 1:5,000 in immunoblots (Fig. 7, lane 7). Interestingly, the cross-reactivity was not reciprocal, as can be seen from the absence of a band at the relevant position (essentially the same as for Flil) in lane 6 (where Flil is absent because of the flhCD mutation). The lack of cross-reactivity, incidentally, meant that no correction was necessary in our estimate of the cellular content of Flil in wild-type cells.

**DISCUSSION**

*flil* is a gene that lies within one of the flagellar operons, the *flil* operon, which contains genes of known function (such as *flif*, the structural gene for the basal-body MS ring) as well as several genes of poorly understood or unknown function. In an earlier study (52), we had established that *flil* encodes a protein whose sequence shows significant similarity to that of the catalytic β subunit of the F_{0}F_{1} and related ATPases. We have now examined Flil genetically and biochemically, with results that further support the hypothesis that it is a flagellum-related ATPase.

**Genetic evidence that Flil is an ATPase involved in flagellar assembly.** If Flil is involved in flagellar assembly in a process that requires ATP hydrolysis, then residues that are essential for hydrolysis should also be essential for flagellar assembly. Since several residues essential for ATP hydrolysis in F_{0}F_{1} β have already been identified (14, 46), we performed site-directed mutagenesis of the corresponding residues in Flil. In all cases, this resulted in failure to assemble flagella, a result that supports the hypothesis that Flil-mediated ATP hydrolysis is essential for flagellar assembly.

**ATP binding and hydrolysis properties of Flil.** Flil was found to bind ATP by two independent criteria: Flil-induced enhancement of TNP-ATP fluorescence and quenching of the intrinsic tryptophan fluorescence of Flil by ATP. Further evidence for nucleotide-binding ability comes from the fact that Flil is retarded on a Cibacron Blue column and can be displaced by excess ATP (54).

In spite of the fact that sequence comparisons, site-directed mutagenesis, and ATP binding measurements all indicate that Flil is likely to be an ATPase, attempts to detect ATP hydrolysis were unsuccessful. This might be because the protein, having been denatured by guanidinium- HCl, was unable to fold into the fully native conformation (although it was capable of binding ATP and ATP analogs with a fairly high affinity). Another possibility is that Flil is active as an ATPase only if properly incorporated into a multisubunit complex of which it is just one component. This is true of, for example, the β subunit of the F_{0}F_{1} ATPase, which shows negligible activity unless present in a complex with the α and γ subunits (9).

**The high copy number of Flil.** We estimate the number of copies of Flil in a wild-type *S. typhimurium* cell to be about 1,500, much greater than the number of flagella (ca. 5 to 10). It seems extremely unlikely that there could be several hundred copies of the protein physically associated with a single flagellum at the same time. What then could be the reason for the high copy number? A trivial explanation would be that the cell actually uses a much smaller number (say, one per flagellum) but does not tightly control the amounts synthesized. However, there are other intriguing possibilities.

One is that Flil is part of a structure that is needed for flagellar assembly but is not part of the flagellum per se and that this structure is present in high copy number. The difficulty with this model is that it implies that flagellar assembly is controlled from a remote location without providing any insight as to how this could occur.

Another, perhaps more attractive, possibility is that Flil does interact with the flagellum, but in a dynamic fashion. For example, there could be a reaction cycle involving (i) binding to the flagellar base of both the substrate protein that is to be exported (say, flagellin) and ATP-Flil, (ii) ATP hydrolysis accompanied by protein translocation into the axial channel, and (iii) release of the ADP-Flil.

Two observations tend to support the idea that Flil may be in a labile rather than a fixed association with the flagellum. The first is that temperature-sensitive *flil* mutants lost function (the ability to outgrow filaments) when shifted from the permissive to the restrictive temperature, indicating that the protein was not highly constrained by another structure, whereas several other classes of mutants, including *flif* (MS ring) mutants, permitted not only filament outgrowth but also normal motor function. The second is that *flil* mutations were not dominant, which is not the result one would expect if the protein was an integral part of the flagellar structure.

Chaperones are one class of molecules that act in labile association with their protein substrates and with the apparatus for exporting them (and are present in the cell in large amounts [18]). The F_{1} α subunit of mitochondria appears to be a heat shock protein (34), and it has been argued, on the basis of some limited sequence motifs, that it may act as a chaperone. These motifs are less evident in *E. coli* α, less still in *E. coli* β, and even less in Flil. At this point, we do not think there is any significant evidence to suggest that Flil is a chaperone, although we do not rule this out as a possibility.

**Similarity between Flil and virulence proteins of mammalian and plant pathogens.** Subsequent to the publication of the Flil sequences of *B. subtilis* and *S. typhimurium* (1, 52), two reports have appeared of proteins that are not related to flagellation but that are even more similar to Flil than they (or Flil) are to F_{1} β.

In the first of these studies, Venkatesan et al. (51) cloned from the mammalian pathogen *Shigella flexneri* a chromosomal region called *spa*, which is involved in the surface presentation of invasion plasmid antigen. The latter are proteins that (like flagelin and other flagellar proteins) are exported by a pathway that does not involve signal peptide cleavage. One of the genes in this region, *spa47*, encodes a protein that is about 40% identical to the Flil proteins of both *S. typhimurium* and *B. subtilis* (and slightly less than 30% identical to *E. coli* F_{1} β).

In the second study, Fenselau et al. (12) cloned from the plant pathogen *Xanthomonas campestris* a region (*hrp*) that is needed for the basic pathogenic response of normal plants and the hypersensitive response of resistant plants. The region contains a gene, *hrpB*, whose deduced product is even more strongly related to Flil (about 50% identity) than is *spa47*; again the resemblance to F_{1} β is lower (about 30% identity). Spa47 and HrpB6 are about as similar to each other (45% identity) as they are to Flil.

**A superfamily of protein export systems?** The connection between the flagellar assembly system and various virulence systems is not restricted to Flil and the Spa47/HrpB6 class of proteins. There appear to be at least two other such connections.

FlhA is a protein that, along with Flil, emerged from the filament regrowth assay as a candidate for the flagellum-specific export apparatus. The *flhA* gene has been sequenced in *Caulobacter crescentus* (41), *B. subtilis* (6), and *E. coli* (29), yielding pairwise amino acid sequence identities of about 40 to 45%. The sequence of FlhA indicates a two-
domain protein, with the N-terminal half being very hydrophobic and predicted (11) to be capable of crossing a membrane about six times and the C-terminal half being quite hydrophilic and likely to represent a water-soluble domain. At the level of both sequence and hydropathy comparisons, FlhA is very similar to an ever-growing family of proteins involved in virulence, such as the LcrD protein of Yersinia pestis (40), the InvA protein of S. typhimurium (15), the MxiA (also known as VirH) protein of S. flexneri (44 [quoted in reference 15]), (51) (GenBank accession no. M91664), whose gene is close to spa47 (51), and the HrpC2 protein of X. campestris (12). There is evidence in both the Y. pestis lcr system and the S. flexneri mxi-vir-spa system that the process involves export of proteins (the Yop and Ipa proteins, respectively) by a mechanism that does not involve signal peptide cleavage.

Yet another family of proteins spanning the flagellar and virulence systems has been recognized recently (47): the S. flexneri Spa24 protein (51) and the X. campestris ORF2 protein reported by Hwang et al. (24), which are closely related to each other, are also related to a flagellar protein, FliP, which exists as a strongly conserved family in B. subtilis (5), E. coli (37), and S. typhimurium (28). The sequence of FliP suggests that it is an integral membrane protein. It is needed quite early in the flagellar assembly process (27, 48, 49), with recent work by Kubori et al. (31) indicating that (like FlhA and FliI) it participates after assembly of the cytoplasmic-membrane MS ring and before the first external structure, the rod. However, a temperature-sensitive fliP mutant (in contrast to temperature-sensitive flhA and fliI mutants) was able to regrow flagellar filaments at the restrictive temperature. This result does not necessarily preclude a role for FliP in export: the protein might simply retain its function at the restrictive temperature, provided it is incorporated at the permissive temperature.

Whatever the exact functions of the various flagellar proteins (FliI, FlhA, and FliP) and their homologs among virulence proteins (Spa47/HrpB6, the LcrD group, and Spa24/ORF2), it seems safe to conclude that they are components of a superfamily of systems that are used in a variety of ways to control and enable processes of export or assembly, or both, of proteins that lie beyond the cell membrane. Probably other components of these systems remain to be identified. For example, there are several other flagellar proteins that are of unknown function but are needed in early stages of the assembly pathway, and the genetic composition of virulence regions has not yet been fully worked out. For now, we are left with two fascinating connections: (i) that between the export-assembly systems for flagellar and virulence proteins and (ii) that between proteins that function as an ATPase responsible for translocating protons and proteins that are suspected to function as an ATPase responsible for translocating proteins.

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