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Escherichia coli fliAZY Operon

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We have cloned the *Escherichia coli fliAZY* operon, which contains the *fliA* gene (the alternative sigma factor σ^F) and two novel genes, *fliZ* and *fliY*. Transcriptional mapping of this operon shows two start sites, one of which is preceded by a canonical $E\sigma^F$-dependent consensus and is dependent on σ^F for expression in vivo and in vitro. We have overexpressed and purified σ^F and demonstrated that it can direct core polymerase to $E\sigma^F$-dependent promoters. FliZ and FliY are not required for motility but may regulate σ^F activity, perhaps in response to a putative cell density signal that may be detected by FliY, a member of the bacterial extracellular solute-binding protein family 3.

The motility regulon consists of the genes whose products are structural components of flagella plus the genes whose products regulate the expression of these genes or the activity of the completed flagella (reviewed in reference 45). In *Escherichia coli* and *Salmonella typhimurium*, this regulon is subject to complex regulation in which all genes at the first two levels of the three-tiered hierarchy must be functional for subsequent classes to be expressed (35, 36, 40). Similar motility hierarchies are present in many other bacterial species, though details vary. For example, the gram-positive bacterium *Bacillus subtilis* has almost the same set of genes and a similar regulatory framework, but no class I genes have as yet been identified (27, 52).

At the top of the enteric hierarchy is the class I operon, *flhDC* (7). This master operon is thought to be the primary site for the control of the motility regulon. Information is received regarding cell nutrition (through the catabolite repression system [1]), external osmolarity (through OmpR [42, 43, 65]), temperature (through the heat shock system [1, 64]), and other environmental conditions (3, 15, 22, 43, 63). If conditions are appropriate to expend the significant amount of energy necessary for motility (46), the master operon is expressed, and the FlhDC complex induces transcription of the class II genes. FlhDC appears to act by binding upstream of class II promoters and stimulating transcription by $E\sigma^{70}$ (44) (unpublished data).

Class II genes include most of the structural genes for the flagellar hook-basal body complex plus the alternative sigma factor *fliA*. The product of the *fliA* gene, σ^F, is a member of the σ^{28} family of sigma factors and recognizes the consensus promoter $TAAAGTTX_{11}tGCCGATAAc$ (25) (data not shown). This factor directs the transcription of the class III genes (55), which encode the filament protein, the hook-associated proteins, the motor proteins, and various chemotaxis proteins (4, 25). One other class III gene, *flgM*, encodes a negative regulator of σ^F activity (18, 19). When any of the class II structural genes are mutant, FlgM binds to σ^F and blocks the transcription of class III genes (56). If all of these genes are functional, their products assemble into a complete basal body that can pump FlgM out of the cell, allowing the transcription of class III genes (31, 41). FlgM probably also helps control the timing of induction and repression of this regulon during growth of wild-type cells.

Although *fliA* is a critical player in the regulation of this system, it had not previously been cloned from *E. coli*, though it has been cloned from a number of other bacterial species (11, 27, 32, 33, 48, 55, 70). Mutants in *E. coli fliA* were originally isolated by Silverman and Simon in 1973 (68). They were found to map between *fliC* and *uvrC*, at about 42.5 min on the current genetic map of *E. coli*. While the *fliB* locus separates *fliA* from *fliC* in *S. typhimurium* (45), this locus has not as yet been identified in *E. coli* and may have been a recent alteration in *S. typhimurium* mediated by an adjacent IS*200* insertion element. We used this information to tentatively localize the *fliA* gene to λ 341 from the Kohara *E. coli* library (34), possibly expressed from a potential promoter located at the end of the region downstream of *fliC* sequenced by Hanafusa et al. (24).

MATERIALS AND METHODS

Standard molecular biological techniques were used according to Sambrook et al. (61), and genetic techniques generally followed those described by Silhavy et al. (67). Plasmid DNA was purified on QIAGEN columns according to the manufacturer's instructions. His · Bind resin was purchased from NOVAGEN. Polyethylenimine (P-3143), IPTG (isopropyl-β-D-thiogalactopyranoside; I-6758), and actinomycin D (A-1410) were purchased from Sigma.

Bacterial strains and plasmids. The following *E. coli* strains were used in this study. RP437 (wild type for motility, *thr-1 leuB6 his-4 metF159 thi-1 eda-50 ara-14 mtl-1 xyl-5 tonA31 tsx-78*) was provided by the Koshland lab (University of California, Berkeley) (57). YK0410 (wild type for motility, *araD139 lacU169 rpsL thi pyrC46 gyrA thyA his*), YK4104 (an isogenic *fliA* strain) and YK4519 (an isogenic *flhD*::Tn*10* strain) were provided by Robert Macnab (Yale University, New Haven, Conn.) (29, 35, 37). JC7623 [*recBC sbcB sbcC ara-14 leu-6 his-4 thr-1 thiA lac4 mlt-1 xyl-5 galK2 proA2 argE3 rpsL31 tsx-33 sup-37*(Am)] was obtained from the Clark laboratory (University of California, Berkeley) (30). BL21 (DE3)pLysS was purchased from NOVAGEN. XL1-Blue was purchased from Stratagene.

pBLUESCRIPT II-KS$^+$ (pBS II-KS$^+$) was purchased from Stratagene. pET22b was purchased from NOVAGEN. pUC4K was purchased from Pharmacia. pBR322 was a gift of Lillian Hsu (Mount Holyoke College, South Hadley, Mass.). pDNA1 is described in Arnosti and Chamberlin (4). pKK223-3 has been described by Brosius and Holy (8). pRG1 has been described by Griffin and Kolodner (21).

Media and growth conditions. Rich medium was Luria-Bertani medium (10 g of Bacto-tryptone per liter, 5 g of Bacto-yeast extract per liter, 10 g of NaCl per liter), and the minimal medium was Fraser's medium (4.5 g of KH_2PO_4 per liter, 10.5 g of Na_2HPO_4 per liter, 3 g of NH_4Cl per liter, 15 g of Casamino Acids per liter, supplemented to 2.5 mM $MgSO_4$, 0.3 mM $CaCl_2$, 3% glycerol, and any strain growth requirements). Cells were grown with shaking at 200 rpm on a shaker at 30°C. Swarm assays were performed by placing fresh bacteria on semisolid agar ([per liter] 10 g of tryptone, 5 g of NaCl, 3.5 g of Bacto-agar) and incubating the plates overnight.

Oligonucleotides. The oligonucleotides used in this study were synthesized on a Biosearch 8750 DNA synthesizer and were generally purified by polyacryl-

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amide gel electrophoresis, though this proved unnecessary for most applications. Oligonucleotide DSM1 corresponds to nucleotides 207 to 228 in Fig. 2, while DSM3 corresponds to the complement of nucleotides 309 to 290. DSM6 is 5′-GCGACCGAAGTGTCCAATATG, and DSM24 is the complement of nucleotides 1484 to 1465. DSM16 corresponds to nucleotides 989 to 1007, while DSM44 corresponds to the complement of nucleotides 1807 to 1790. DSM33 is 5′-CGGGATCCTCATTATAACTTACCCAGTTTAGTGCGTAACCG, and DSM34 is 5′-GGCGGAATTCGGTACCATGGCATATGGGCAGCAGCCATCATCATCATCATCATGACGACGACGACAAGATGAATTCACTCTATACCGCTGAAGGTGTAATG. TAR is 5′-GCGAATACCCCCAGGACCATTACC. DSM65 is 5′-CTCTGCACCGGAATTCATATGCCGCACTTTAACTTTGACTACC, DSM67 is 5′-CTCTGCACCGGAATTCGATGTAGCGGAGTTGTTTTTGTG, DSM68 is 5′-CTCTGCACCGGAATTCATATGAAATTAGCACATCTGGGACG, DSM72 is 5′-CTCCTCCCCTCGAAAGCTTACAAAAACAACTCCGCTACATC, and DSM73 is 5′-CTCCTCCCCTCGAAAGCTTATTATGCTGATTATTTGGTCACATC.

Genomic DNA purification. Genomic DNA was prepared according to the method cited by Ausubel et al. (5). Cells (1 ml) were spun down and resuspended in 400 μl of TE (Tris-EDTA buffer) containing 0.5% sodium dodecyl sulfate (SDS) and 10 μg of proteinase K per ml. They were incubated for 1 h at 37°C, and then 85 μl of 4 M NaCl was added. After thorough mixing, 52 μl of 10% cetyl-trimethylammonium bromide–0.7 M NaCl was added, and the mix was incubated for 10 min at 65°C. The samples were extracted once with chloroform and once with phenol-chloroform, and then the DNA was precipitated with a 0.6 volume of isopropanol, washed with 70% ethanol, dried, and resuspended in 20 μl of TE.

Southern blotting. Southern blots were performed according to a modified version of the method of Southern (69). Digested genomic or phage DNA was electrophoresed through a 1% agarose gel. Gels were shaken for 15 min in 0.2 M HCl, washed twice with distilled water, soaked twice for 15 min each in 1.5 M NaCl–0.5 N NaOH, washed twice with distilled water, neutralized twice for 30 min each in 1.5 M NaCl–1 M Tris-Cl (pH 7.4), and transferred to nitrocellulose overnight. The blot was then baked for 60 min in a vacuum dryer at 80°C.

Hybridization was performed by soaking the blot for 2 h in a prehybridization solution (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 5× Denhardt's solution, 0.5% SDS, 0.1 mg of sheared calf thymus DNA per ml) at approximately 5°C below the calculated T_M of the probe and then adding approximately 10 pmol of end-labeled probe with various specific activities (generally 10^5 to 10^6 cpm/pmol). Hybridization was done for 3 h at the prehybridization temperature. The blot was washed for 2 min periods in 500 ml volumes of 0.2% SDS containing, successively, 2× SSC (room temperature), 0.5× SSC (room temperature), 0.5× SSC (37°C), 0.1× SSC (37°C), 0.1× SSC (5°C below the prehybridization temperature), and 0.1× SSC (5°C below the prehybridization temperature for 4 min) and then autoradiographed overnight.

Colony hybridizations. Colonies were transferred to nitrocellulose disks by placing the disks onto fresh plates and allowing the bacteria to adhere. The disks were then placed sequentially on Whatman paper saturated with 10% SDS (3 min), 1.5 M NaCl–0.5 N NaOH (5 min), 1.5 M NaCl–1 M Tris-Cl (pH 7.4; 5 min), and 2× SSC (5 min). They were air dried for 30 min and then baked for 60 min in a vacuum dryer at 80°C. Hybridization was performed as described for the Southern blots.

DNA sequencing. DNA sequencing of supercoiled plasmid or lambda DNA was performed according to the protocol of Del Sal et al. (13) as modified in Mytelka and Chamberlin (53). When compressions occurred, 7-deaza GTP was substituted for GTP or the reaction products were run on gels containing 40% formamide.

PCR. PCR was performed in a Perkin-Elmer Cetus DNA thermal cycler. The conditions employed were based on those suggested by Krummel (38). Template DNA (0.1 pmol), 50 pmol of each primer, and 5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus) were mixed in a volume of 200 μl containing 150 μM each deoxynucleoside triphosphate, 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 2.5 mM $MgCl_2$, and 0.01% gelatin, and the mixture was subjected to 25 cycles of 1 min at 94°C, 1 min at 37 to 55°C (depending on primer T_M), and 1 min at 72°C.

PCR cloning. The *fliA* promoter region was cloned from RP437 by PCR with primers DSM6 (approximately 175 nucleotides upstream of the region sequenced in Fig. 2, between the *Bam*HI and *Kpn*I sites shown in Fig. 1) and DSM3 and digested with *Kpn*I and *Eco*RI. The resulting 335-bp fragment was cloned into pBS II-KS$^+$. The *fliA* gene was cloned from YK4104 by PCR with oligonucleotides DSM6 and DSM24, the product was digested with *Kpn*I and *Dra*I, and the resulting 1.1-kb DNA fragment was amplified into the *Kpn*I-*Sma*I sites of pBS II-KS$^+$.

Construction of plasmids overexpressing *fliZ* and *fliY*. PCR was used to amplify regions and put *Eco*RI and *Hin*dIII sites on the 5′ and 3′ ends, respectively. After digestion with appropriate enzymes, fragments were cloned into the *Eco*RI and *Hin*dIII sites of pKK223-3, to create plasmids expressing the genes from the *tac* promoter. The fragments in pDSM29-pDSM32 are (respectively) *fliZ* (nucleotides 987 to 1606 cloned with DSM65 and DSM72), *fliY* from the first possible start site (1585 to 2472 cloned with DSM67 and DSM73), *fliY* from the second possible start site (1660 to 2472 cloned with DSM68 and DSM73), and *fliZY* (987 to 2472 cloned with DSM65 and DSM73).

Release of periplasmic proteins by osmotic shock. Osmotic shock was performed generally according to the method of Nossal and Heppel (54). Plasmids pDSM30 and pDSM31 (containing *fliY* starting at the first and second possible start sites) and the parental plasmid pKK223-3 were transformed into XL1-Blue, which contains the lactose superrepressor on an F′. Fresh overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.125 in 50 ml of LB and then grown to an OD_{600} of approximately 0.5. Preinduction samples (1 ml) of each culture were removed and cracked in 100 μl of a solution containing 10 mM Tris-Cl (pH 6.8), 1% β-mercaptoethanol, 1% SDS, and 6 M urea per OD_{600}. The rest of each culture was induced with 1 mM IPTG and grown for an additional 2 h. Another sample was cracked as described above, and the rest of the cells were collected by spinning at 1,000 × *g* for 7 min. After being washed twice with 10 ml of ice-cold 10 mM Tris · Cl (pH 7.5)–30 mM NaCl–0.1 mM EDTA, the cells were resuspended in 5 ml of 20% sucrose at room temperature in the same buffer. After 10 min, cells were collected by spinning at 6,000 × *g* for 5 min and then resuspended in 5 ml of ice-cold 0.5 mM $MgCl_2$. After 10 min on ice, the samples were spun for 10 min at 13,000 × *g*. The supernatant from this spin contains released periplasmic proteins. A sample of the resuspended pellet equivalent in the number of cells to the preinduction and induction samples was cracked as described above. Proteins were separated on an SDS–15% PAGE gel with a 5% stacking gel and visualized by Coomassie blue staining.

Disruption of *fliZY*. A disruption plasmid (pDSM28) was created by cloning the Kan^r marker from pUC4K into a plasmid containing the *Cla*I fragment of the *fliAZY* operon (nucleotides 633 to 2232). The Kan^r marker was excised from pUC4K with *Hin*cII and cloned between the *Nru*I site at position 1137 and the (blunt-ended) *Aat*II site at position 1681. The resulting plasmid was digested to separate this region from the plasmid replication origin, recircularized, and used to transform the *recBC* strain JC7623. A Kan^r Amp^s colony had the appropriate disruption based on PCR amplification of the region with DSM16 and DSM44 (1 ml of an overnight culture was washed twice with 1 volume of water, and then 50 μl was incubated for 5 min at 95°C; 50 μl of a 2× PCR mix was added, and PCR was performed as described above).

RNA purification. RNA was prepared according to the method described by Ausubel et al. (5). Cells (10 ml) were spun down at 12,000 × *g* for 10 min at 4°C and then resuspended in 10 ml of protoplasting buffer (15 mM Tris-Cl [pH 8.0], 0.45 M sucrose, 8 mM EDTA) plus 80 μl of a 50-mg/ml lysozyme solution. After 15 min on ice, the cells were spun down at 6,000 × *g* at 4°C and resuspended in 0.5 ml of gram-negative lysis buffer (10 mM Tris-Cl [pH 8], 10 mM NaCl, 1 mM sodium citrate, 1.5% SDS) plus 15 μl of diethyl pyrocarbonate. The viscous solution was transferred to an Eppendorf tube, incubated for 5 min at 37°C, and then chilled for 5 min on ice. A saturated NaCl solution (250 μl) was added, and the solutions were mixed by inversion. After 10 min on ice, the tubes were spun for 10 min at 4°C. The supernatant was transferred to two Eppendorf tubes, and 1 ml of 100% ethyl alcohol (EtOH) was added to each. After 30 min in a dry ice-EtOH bath, the tubes were spun for 15 min at 4°C. The pellets were rinsed with 70% EtOH, dried, and resuspended in a total of 100 μl of TE.

Northern blotting. RNA was isolated from bacteria grown to an OD_{600} of approximately 0.6 in LB. Approximately 20 μg of RNA in a volume of 11 μl was mixed with 5 μl of 10× MOPS (morpholinepropanesulfonic acid) buffer (0.2 M MOPS [pH 7.0], 50 mM sodium acetate [pH 5.2], 10 mM EDTA)–8.75 μl of formaldehyde–25 μl of deionized formamide and heated for 15 min at 55°C. Loading buffer (10 μl) (50% glycerol, 0.2% xylene cyanol, 0.2% bromphenol blue) was added to each sample, and the samples were loaded on a 1.2% agarose gel containing 1.1% formaldehyde and 1× MOPS buffer. After 3 h of electrophoresis at 80 V, the gel was soaked in 10× SSC for 45 min and then transferred to nitrocellulose overnight in 20× SSC. After the filter was baked, marker lanes were visualized by staining with 0.2% methylene blue, and then the blot was probed by using the same protocol used for Southern blots.

Primer extension assays. Primer extension assays were performed generally according to the method of McKnight and Kingsbury (49). RNA samples were resuspended in an annealing mix containing 250 mM KCl, 2 mM Tris-Cl (pH 8.0), 0.2 mM EDTA, and 1 pmol of end-labeled oligonucleotide (oligonucleotide DSM3 for *fliA*; TAR for *tar*) of various specific activities (generally around 200,000 cpm/pmol). The reaction mixtures were incubated at 55°C for 1 h and then cooled for 15 min at room temperature. The extension mix (40 μl) (for final concentrations of 20 mM Tris-Cl [pH 8.0], 10 mM $MgCl_2$, 100 μg of actinomycin D per ml, 5 mM dithiothreitol, 1 mM each deoxynucleoside triphosphate, and 10 U of avian myeloblastosis virus reverse transcriptase [Life Sciences, Inc., St. Petersburg, Fla.]) was added, and the reaction mixtures were incubated for 1 h at 37°C. The reactions were stopped by the addition of 100 μl of phenol-chloroform, 60 μl of TE was added for volume, and the samples were extracted with phenol-chloroform and then chloroform. The samples were precipitated with 10 μl of 3 M NaOAc and 300 μl of 100% EtOH, washed with 70% EtOH, dried, resuspended in formamide loading buffer (80% deionized formamide, 1× TBE [0.09 M Tris-Borate, 1 mM EDTA], 0.05% xylene cyanol, 0.05% bromphenol blue), and electrophoresed on a 6% polyacrylamide (19:1 acrylamide-bisacrylamide, 7 M urea, 1× TBE) gel until the xylene cyanol reached 20 cm. Quantitation was performed with an AMBIS scanner.

In vitro transcription. Core and Eσ^{70} RNA polymerase were purified by the method of Burgess and Jendrisak (9) as modified by Gonzalez et al. (20) or Hager et al. (23). Eσ^F holoenzyme was purified in our laboratory (50) or as described below. In vitro transcription was performed by mixing polymerase with plasmid template (pDSM1 containing the promoter region of *fliA* or pDNA1 containing the *tar* promoter) in a 50-μl reaction volume containing 40 mM

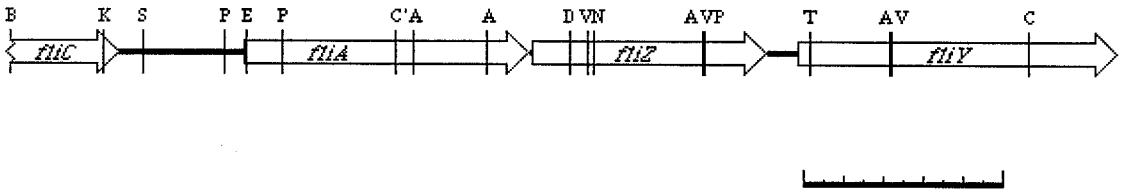

FIG. 1. Restriction map of *E. coli fliA* region. Restriction endonuclease sites: A, *Alu*I; B, *Bam*HI; C, *Cla*I; C′, *Cla*I blocked by overlapping *dam* methylation site; D, *Dra*I; E, *Eco*RI; K, *Kpn*I; N, *Nru*I; P, *Pst*I; S, *Stu*I; T, *Aat*II; V, *Pvu*II. The bar at the bottom right corresponds to a length of 500 bp.

Tris-Ac (pH 8.0), 10 mM β-mercaptoethanol, 4 mM $MgOAc_2$, 20 mM KOAc, 50 mM NH_4OAc, 4 mM spermidine, and in vivo nucleoside triphosphate concentrations (47). The reaction mixtures were incubated for 3 min at 30°C and then phenol-chloroform and chloroform extracted. RNA was precipitated with 0.1 volume of 3 M NaOAc and 3 volumes of ethanol, washed with 70% ethanol, and dried. The RNA present was examined by primer extension, or a radiolabeled nucleotide was added during synthesis and the transcripts were viewed directly.

Overexpressing and purifying σ^F. PCR was performed on the *fliA* gene with the oligonucleotides DSM33 and DSM34, which add an *Nde*I site, six histidine codons, and an enterokinase cleavage sequence on the 5′ end of the gene and a *Bam*HI site on the 3′ end of the gene. The *Nde*I and *Bam*HI sites were used to clone the resulting product into pET22b to make plasmid pDSM10. BL21 (DE3)pLysS containing this plasmid was grown to an OD_{600} of 0.5 in 1 liter of LB and then induced with 1 mM IPTG. After 2 h of induction, the cells were spun down at 5,000 × *g* for 5 min at 4°C. The resulting pellet was resuspended in 40 ml of 1× binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl [pH 7.9]) and then lysed by sonication. Polyethylenimine was added to 0.3% to cross-link DNA, and the extract was incubated for 20 min on ice with occasional pestling. Insolubles were spun out for 20 min at 39,000 × *g* at 4°C. The σ^F protein was extracted by resuspending half of the pellet in 1× binding buffer containing 0.25% Sarkosyl and incubating the mixture for 60 min on ice with occasional pestling, followed by an additional spin at 39,000 × *g*. The resulting supernatant was loaded on a 2.5-ml His · Bind column, which was run according to the manufacturer's specifications and eluted with 1× binding buffer containing 100 mM imidazole.

$E\sigma^F$ was reconstituted by mixing various amounts of core polymerase and a 10-fold excess of purified σ^F in a mixture containing 10 mM Tris-Cl (pH 7.9), 1 mM dithiothreitol, 0.1 mM EDTA, 250 mM KCl, and 100 μg of bovine serum albumine-OAc per ml. After incubation for 10 min at 30°C, the reaction mixture was diluted sixfold to lower the salt concentration and loaded on a phosphocellulose column containing 50% glycerol (20). The column was eluted with a step gradient, and $E\sigma^F$ was recovered in the 160 mM KCl eluate.

Nucleotide sequence accession number. The nucleotide sequence of the *fliAZY* operon has been deposited in GenBank under accession no. U18539.

RESULTS

Cloning *fliA* locus. In order to confirm and supplement existing restriction information on the *fliA* region (60), we designed an oligonucleotide (DSM1) that hybridizes to a sequence downstream of *fliC* which resembles class II promoters and thus could be the promoter for *fliA*. We used DSM1 to probe a Southern blot of DNA from both wild-type *E. coli* and λ 341 from the Kohara *E. coli* library (34). This hybridization confirmed that we had selected a phage that contains an appropriate insert and that it has no major rearrangements, since its restriction pattern mirrored that of genomic DNA (data not shown). It also identified restriction endonuclease sites suitable for cloning this region (Fig. 1).

We used this information to subclone the region from λ 341 into pBS II-KS^+. We initially cloned the *Kpn*I-*Alu*I region into the *Kpn*I-*Sma*I sites of pBS II-KS^+ (plasmid pDSM1), identifying the appropriate clones by colony hybridization with oligonucleotide DSM1, and later cloned a larger fragment extending from the *Bam*HI site to the *Cla*I site, again by colony hybridization. This plasmid, pDSM4, proved to contain the entire *fliA* gene as well as an open reading frame (ORF) downstream of it and most of a third ORF. A third plasmid was subsequently isolated; this plasmid contains the entire region, from the *Kpn*I site to a *Bam*HI site well downstream of the third ORF (pDSM17).

Sequencing *fliA* region. The nucleotide sequence of the entire operon was determined by using a modified version of the Sanger (dideoxynucleotide-mediated chain termination) method (53, 62). The sequence was obtained progressively by synthesizing new oligonucleotides based on a previously obtained sequence, starting at the end of the *fliC* region (24). The complete sequence of the region was eventually obtained on both strands and is presented in Fig. 2. The sequence from the *Cla*I site to the end of the operon (Fig. 2, nucleotides 2238 on) was obtained by sequencing one strand directly from λ 341 and was confirmed by sequencing the other strand from pDSM17.

A number of differences were observed between the beginning of this sequence and the short overlapping region previously sequenced by Hanafusa et al. downstream of *fliC* (24). In order to determine whether these differences represented mutations that had accumulated during propagation of the lambda phage, we cloned this region from RP437, a motile *E. coli* strain, and resequenced the region in question. This new sequence proved to be identical to the one obtained from pDSM4, demonstrating that our sequence is correct for at least this strain.

The cloned sequence includes the *fliA* gene. This region of DNA contains three potential ORFs of up to 239, 195, and 285 codons, respectively. In order to determine whether these ORFs are transcribed, we isolated RNA from wild-type *E. coli* and from an *flhD*::Tn*10* mutant strain (YK4519) and examined transcription in this region by Northern (RNA) hybridization. Since *fliA* is a class II gene, we would expect it to be transcribed in the wild-type strain but not in the class I mutant YK4519. We found that a single major transcript of about 2,300 nucleotides is present in the former background only; a fainter smear present at 1,400 nucleotides and below in this strain might represent premature termination after the transcription of the first two genes and degradation of this less stable transcript (Fig. 3). Since this region is preceded by a sequence acting as a strong transcription terminator in vitro (data not shown), the 2,300-nucleotide transcript apparently indicates cotranscription of the three ORFs. Transcription presumably ends at the potential terminator present at nucleotides 2476 to 2506 (Fig. 2), which would give a transcript of approximately 2,270 nucleotides from the start site determined below.

As expected, the first ORF is homologous to the previously sequenced *S. typhimurium fliA* locus (55). The nucleotide sequence is 86% identical to the *S. typhimurium* homolog, and the predicted protein product would be 94% identical. We demonstrated that this ORF is the *fliA* gene in two fashions. First, we used PCR to clone this region from YK4104, a *fliA* mutant strain, and sequenced the ORF. We found that it has a single base pair deletion at position 570, 571, or 572 (indicated by a caret in Fig. 2) that would cause a frameshift disrupting the second half of the predicted protein product. Second, we showed that the cloned gene could complement this lesion. Although the high-copy-number plasmid originally used (pDSM4) failed to complement YK4104, as demonstrated by

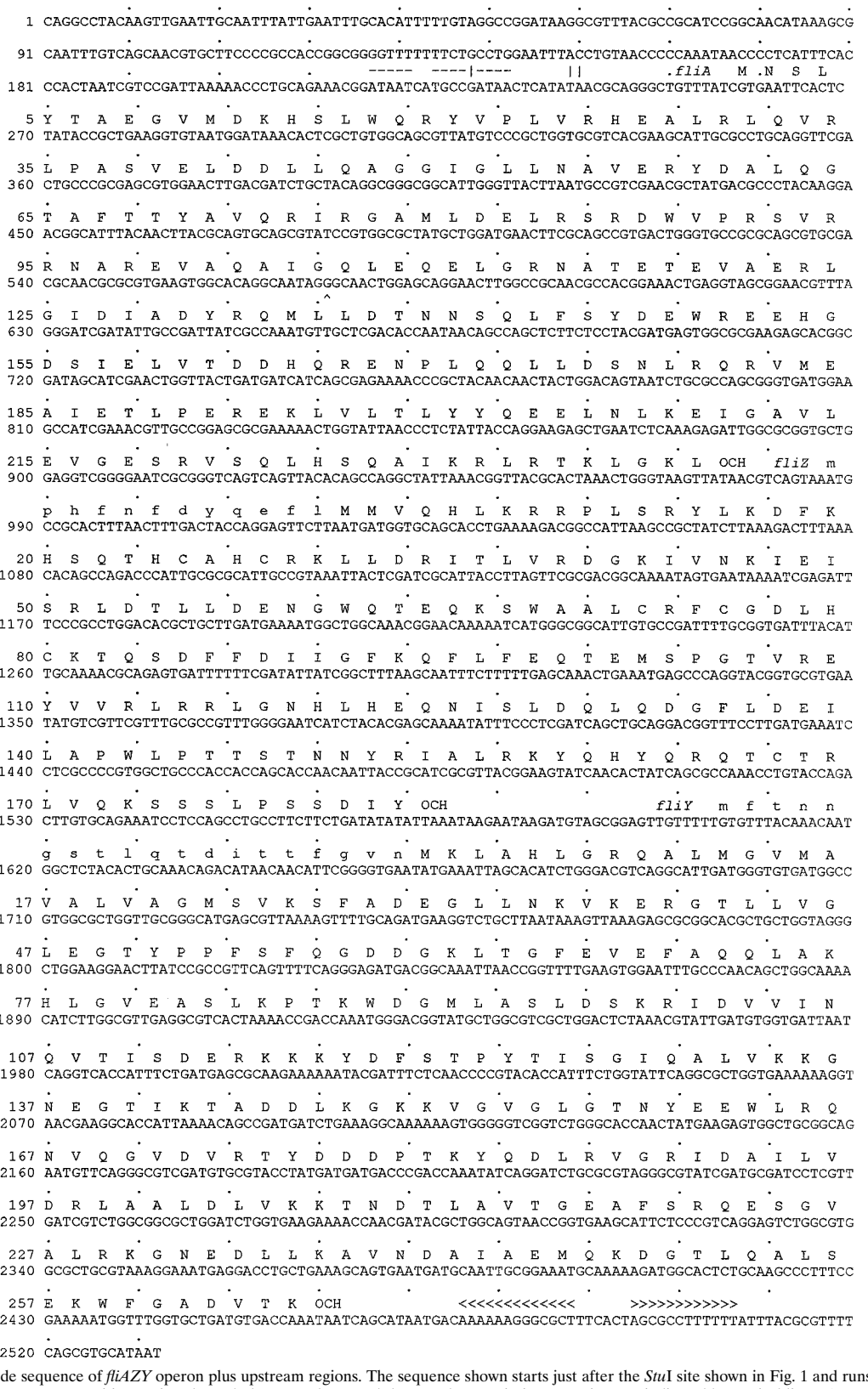

FIG. 2. Nucleotide sequence of *fliAZY* operon plus upstream regions. The sequence shown starts just after the *Stu*I site shown in Fig. 1 and runs to the end of the operon. Potential promoter recognition regions have dashes over them, and the actual transcription start sites are indicated by vertical lines. A caret is under the site of the mutation in strain YK4104, a *fliA* mutant. The potential terminator hairpin is indicated by < and > characters. Amino acids in lowercase are parts of ORFs that are probably not translated (see the text).

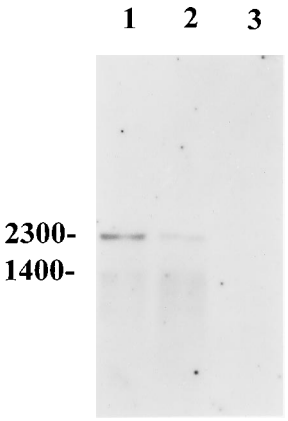

FIG. 3. Northern blot of RNA isolated from exponential phase *E. coli*. RNA from YK0410 (wild type, lane 1), RP437 (another wild type, lane 2), and YK4519 (*flhD*, lane 3) was probed with DSM3, an oligonucleotide complementary to a sequence early in *fliA*. Molecular sizes (in nucleotides) are indicated on the left.

the failure of YK4104 pDSM4 to swarm on semisolid plates and the absence of motile cells when observed microscopically, the same cloned region was able to provide moderate complementation when present on the lower-copy-number plasmid pDSM7, which contains a pBR322 origin (data not shown). Wild-type motility was restored by occasional recombination events between a plasmid and the chromosome, either because of the further reduction in transcription levels caused by the propagation of the mutated plasmid or because of the elimination of polar effects of the chromosomal frameshift mutation on *fliY*, which is present only in a truncated form on the plasmid.

Analysis of the two downstream ORFs. The two downstream ORFs have tentatively been named *fliZ* and *fliY*, since they are transcribed as part of the class II *fliA* operon. We attempted to learn more about these two sequences by using the programs BLASTN and BLASTP at the National Center for Biotechnology Information) (2) to search for homologies in the National Institutes of Health databases of nucleic acid and protein sequences, respectively. Both proved to be novel genes, though a portion of *fliZ* had previously been isolated in a search for *E. coli* sequences that can function as upstream activating sequences in yeast cells (73).

The protein encoded by the *fliY* gene proved to be similar to members of the extracellular solute-binding protein family 3 (Fig. 4). This alignment suggests that the second possible start codon of *fliY* (the ATG at nucleotides 1662 to 1664) may well be the actual start codon, rather than the GTG just after the end of *fliZ* at 1605 to 1607. This hypothesis is supported by the fact that all members of this family are exported to the periplasmic space, and the region following the second possible start site would form a good export signal (initial region with a positive-charge, hydrophobic core, potential signal peptidase cleavage site [71]), while the region following the GTG would not (data not shown). However, the GTG is preceded by a better match to the canonical ribosome binding site.

In order to determine whether FliY is actually exported, we osmotically shocked cells to release proteins in the periplasmic space. We performed this procedure with cells potentially overexpressing FliY from both possible start sites (XL1-Blue pDSM30), with cells overexpressing FliY from the second possible start site only (XL1-Blue pDSM31), and with control cells containing the parental plasmid (XL1-Blue pKK223-3). We found that both overexpressing strains produced a cell-bound protein with a size of about 30 kDa, which was apparently cleaved to produce a mature 27-kDa periplasmic version (Fig. 5). Since the FliY products are the same sizes in the two overexpressing strains, FliY must be translated from the second possible start site, since the first start site is not present in pDSM31.

FliZ is not significantly homologous to any other protein, but it has some weak similarity to several ATP-binding proteins. In particular, it has some similarity to *B. subtilis rbsA*, a member of the transport ATPase family that interacts with extracellular solute-binding proteins in small molecule import. However, the members of this family are all lodged in the cytoplasmic membrane, and FliZ does not appear to have a signal sequence or a potential transmembrane segment. In addition, FliZ does not have an obvious ATP-binding region, as was determined with the motif-finding program PROSITE (6). Thus, it appears unlikely that FliZ provides this function for FliY.

The FliZ protein could begin either at the ATG immediately after the end of *fliA* (nucleotides 987 to 989) or at the next ATG (1023 to 1025). The second possible start site has a much better match for the ribosome binding site, so we examined the intervening region to see whether it is similar to the short comparable region that has already been sequenced in *S. typhimurium*. We found that the region after the second possible start site is very similar in the two species, but that the region between the two possible start sites diverges substantially. This suggests that the second possible start site is the major start site for FliZ translation.

We used two methods to examine the functions of FliZ and FliY. We first overexpressed the genes in a wild-type background, to see whether they would adversely affect motility. We transformed plasmids overexpressing *fliZ* (pDSM29), *fliY* (pDSM30), and *fliZY* (pDSM32) into RP437 pRG1 and compared their motilities with those of the strain containing the parental plasmid pKK223-3. The plasmid pRG1 (21) contains the *lacI*^{q} gene on a compatible plasmid to prevent overexpression toxicity, particularly problematic in strains producing an exported protein; when IPTG was added to 10 μM, overexpressed proteins became visible on Coomassie-stained SDS-PAGE gels, indicating the presence of about 10,000 copies per cell, but the cultures' doubling times were not significantly affected (data not shown). Under these conditions, we did not see any major change in motility, either microscopically or on swarm plates.

We next attempted to delete the two genes. We replaced most of *fliZ* and the signal sequence of *fliY* by a disruption cassette conferring kanamycin resistance. We performed this disruption in the *recBC* strain JC7623, which was unknown mutations that render it nonmotile. When we moved the disruption into the wild-type strain RP437 by P1 transduction, we found that three of nine transductants were motile. The remaining six were nonmotile, presumably because of cotransduction of some other mutation from JC7623. This cotransduction frequency suggests that JC7623 has a mutation about 12.5 kb away (67), presumably in *fliE* or *fliF*. The three motile transductants were demonstrated to have only the disrupted version of the operon by PCR. They form significantly smaller swarms than wild-type cells on semisolid agar and have over 99% nonmotile cells in liquid cultures inoculated from single colonies (compared with almost none in cultures from wild-type cells). This effect was not reversed by the *fliZY*-expressing plasmid pDSM32 but could be reversed by a recombination event replacing the *fliAZY* region by the wild-type sequence from the plasmid pDSM17.

The above experiments demonstrate that *fliZ* and *fliY* are not essential for motility but suggest that they may have a

```
FliY     1 VFTNNGSTLQTDITTFGVNMKLAHLGRQALMGVMAVALVAGMSVKSFADEGLLNKVKERGTLLVG  65
GlnH     1                                        MKSVLKVSLAALTLAFAVSSHAADKKLVVA  30
HisJ     1                                      MKKLALSLSLVLAFSSATAAFAAIPQKIRIG  31
ArgT     1                                      MKKTVLALSLLIGLGATAASYAALPQTVRIG  31
NocT     1                                  MKFFNLNALAAVVTGVLLAAGPTQA--KDYKSITIA  34
OccJ     1                                     MKLKTILCAALLLVAGQAAAQEKSITIA  28
PheC     1                              MPKSFRHLVQALACLALLASASLQAQESRLDRILESGVLRVT  42

Consensus ------------------------------------KLVL--SL-L-A---A---AA--K-LRIG


FliY    66 LEGTYPPFSF-QGDDGKLTGFEVEFAQQLAKHLGVEASLKPTKWDGMLASLDSKRIDVVINQVTI 129
GlnH    31 TDTAFVPFEFKQGD--KYVGFDVDLWAAIAKELKLDYELKPMDFSGIIPALQTKNVDLALAGITI  93
HisJ    32 TDPTYAPFES-KNAQGELVGFDIDLAKELCKRINTQCTFVENPLDALIPSLKAKKIDAIMSSLSI  95
ArgT    32 TDTTYAPFSS-KDAKGEFIGFDIDLGNEMCKRMQVKCTWVASDFDALIPSLKAKKIDAIISSLSI  95
NocT    35 TEGSYAPYNF-KDAGGKLIGFDIDLGNDLCKRMNIECKFVEQAWVGIIPSLTAGRYDAIMAAMGI  98
OccJ    29 TEGGYAPWNF-SGPGGKLDGFEIDLANALCEKMKAKCQIVAQNWDGIMPSLTGKKYDAIMAAMSV  92
PheC    43 TTGDYKPFSY-RTEEGGYAGFDVDMAQRLAESLGAKLVVVPTSWPNLMRDFADDRFDIAMSGISI 106

Consensus TEGTYAPFSF-KGA-GKL-GFDIDLAN-LCKRL---C--VP--WDGIIPSL-AKRIDAIMA--SI


FliY   130 SDERKKKYDFSTPYT-------ISGIQAL---------------VKKGNEGTIKTAD--DLKGKK 170
GlnH    94 TDERKKAIDFSDGYY-------KSGLLVM---------------VKANNNDVKSVKD--LDGKVV 134
HisJ    96 TEKRQQEIAFTDKL--------YAADSRL---------------VVAKNSDIQPTVA--SLKGKR 135
ArgT    96 TDKRQQEIAFSDKL--------YAADSRL---------------IAAKGSPVQPTLE--SLKGKH 135
NocT    99 QPAREKVIAFSRPYLLTPMTFLTTADSPLLKTQVAIENLPLDNIAPEQKAELDKFTK--IFEGVK 161
OccJ    93 TPKRQEVIGFSIPYAAGINGFAVMGDSKLAEMPGLGETYSLDSQADAAKKAIADISS--FLNGTT 155
PheC   107 NLERQRQAHFSIPYL-------RNSKTPI---------------TLCSEEARFQTLEQIDQPGVT 149

Consensus TDERQK-IAFSDPY----------GDS-L---------------V-A-N-----T-----LKGK-


FliY   171 VGVGLGTNYEEW-LRQNV--QGVDVRTYDDDPTKYQDLRVGRIDAILVDRLAALDLVKKTNDTL- 231
GlnH   135 AVKSGTGSVDYA-KANIKTKDLRQFPNIDNAYMELGTNRADAVLHDTPNILYFIKTAGNGQFKAV 198
HisJ   136 VGVLQGTTQETF-GNEHWAPKGIEIVSYQGQDNIYSDLTAGRIDAAFQDEVAASEGFLKQPVGKD 199
ArgT   136 VGVLQGSTQEAY-ANDNWRTKGVDVVAYANQDLIYSDLTAGRLDAALQDEVAASEGFLKQPAGKE 199
NocT   162 FGVQAGTSHEAF-MKQMM--PSVQISTYDTIDNVVMDLKAGRIDASLASVSFLKPLTDK-PDNKD 222
OccJ   156 VGVQGSTTASTFLDKYFK--GSVDIKEYKSVEEHNLDLTSGRLDAVLANATVLAAAIEK-PEMKG 217
PheC   150 AIVNPGGTNEKF-ARANL--KKARILVHPDNVTIFQQIVDGKADLMMTDAIEARLQSRLHPELC- 210

Consensus VGV--GTT-E-F----N---KGVDI--YD--D-IY-DLTAGRIDA-L-D--AA-----K-P--K-


FliY   232 ------AVTGEAFSRQESGVALRKGNEDLLKAVNDAIAEMQKDGTLQALSEKWFGADVTK      285
GlnH   199 GDSLEAQQYGIAFPKGSDELRDKVNGALKTLRENGTYNEIYKKWFGTEPK                 248
HisJ   200 YKFGGPAVKDEKLFGVGTGMGLRKEDNELREALNKAFAEMRADGTYEKLAKKYFDFDVYGG      260
ArgT   200 YAFAGPSVKDKKYFGDGTGVGLRKDDTELKAAFDKALTELRQDGTYDKMAKKYFDFNVYGD      260
NocT   223 LKMFGP-RMTGGLFGKGVGVGIRKEDADLKALFDKAIDAAIADGTVQKLSQQWFGYDASPKQ     283
OccJ   218 AKLVGP-LFSGGEFGV-VAVGLRKEDTALKADFDAAIKAASEDGTIKTLSLKWFKVDVTPQ     276
PheC   211 ------AVHPQQPFDFAEKAYLLPRDEAFKRYVDQWLHIAEQSGLLRQRMEHWLEYRWPTAHGK  268

Consensus -K--GPAV-----FG-G-GVGLRKED--LKAAFDKAI-EA--DGT--KLS-KWF--DV------
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FIG. 4. Comparison of *fliY* to members of bacterial extracellular solute-binding protein family 3. Residues identical to the *E. coli* FliY protein are indicated by bold letters. GlnH is part of the glutamine permease system from *E. coli* (GenBank accession no. X14180). HisJ is the *S. typhimurium* periplasmic histidine-binding protein (SWISS-PROT P02910). ArgT is the Lysine-Arginine-Ornithine binding protein from *S. typhimurium* (SWISS-PROT P02911). NocT is the nopaline-binding protein from *A. tumefaciens* (SWISS-PROT P35120). OccJ is the octopine-binding protein from *A. tumefaciens* (SWISS-PROT P35121). PheC is cyclohexadienyl dehydratase from *P. aeruginosa* (SWISS-PROT Q01269). According to the NCBI BLAST server, the probability that these matches occurred by chance are, respectively, 2.0e-39, 2.7e-35, 1.7e-33, 7.0e-32, 1.1e-23, and 2.1e-17.

regulatory role. Unfortunately, it is difficult to separate the effects of the deletion from the effects of the apparent *fliA* mutation that was presumably cotransduced from JC7623. Subsequent attempts to repeat these experiments in *fliA*$^+$ strains generated only direct repeats of the wild-type and deleted versions of these genes. This suggests that the deletion is deleterious or lethal in a strain expressing a wild-type copy of the *fliA* gene. If this is the case, then one or both of the *fliZY* products may negatively regulate *fliA*.

***fliA* is expressed from two overlapping promoters.** Our next goal was to determine where transcription of the *fliA* operon initiates. An examination of the region immediately upstream of the *fliA* gene reveals sequences similar to both a class II Eσ^F promoter (P_F) and the -10 box of an Eσ^{70} promoter (P_A). We used an oligonucleotide complementary to a region downstream of these (DSM3) to probe for start sites in a primer extension assay. When we examined transcripts isolated from late-exponential-phase wild-type RP437 or YK0410 cells, we found that RNA molecules initiated about seven nucleotides downstream of each of these promoters (Fig. 6, lanes 1 and 2). As expected, the P_F promoter was not used in the *fliA* mutant YK4104 (Fig. 6, lane 3), while the other promoter was, though at a fivefold-lower rate. Neither promoter was active in an *flhD* strain (Fig. 6, lane 4).

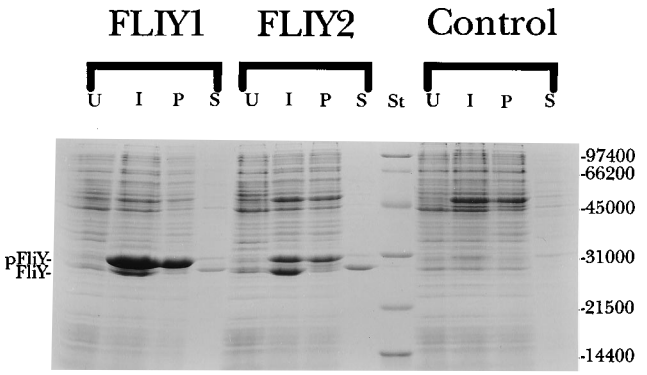

FIG. 5. Identification of proteins released by osmotic shock. FLIY1, FLIY2, and control indicate proteins isolated from XL1-Blue pDSM30, XL1-Blue pDSM31, and XL1-Blue pKK223-3, respectively. U, uninduced cells; I, induced cells; P, pellet (nonperiplasmic proteins); S, supernatant (proteins released by osmotic shock). Molecular sizes (in daltons) are indicated on the right.

In order to determine what polymerases are responsible for transcribing these promoters, we tried to transcribe them in vitro and determine the start sites by primer extension. In the presence of $E\sigma^F$, transcription initiated at the P_F, as expected (Fig. 6, lane 6). However, no transcription occurred when $E\sigma^{70}$ was used (Fig. 6, lane 5), which is not surprising since recent data have demonstrated that FlhD and FlhC are necessary for $E\sigma^{70}$ to transcribe from this region (44 and unpublished data).

Overexpression and purification of σ^F. In order to simplify characterization of σ^F, we overexpressed and purified it. We accomplished this by first using PCR to attach a run of six histidine codons at the 5′ end of the gene. We then placed the gene behind a strong T7 promoter with a good ribosome binding sequence to create plasmid pDSM10. After sequencing the plasmid to confirm that no mutations had been introduced by PCR, we introduced it by transformation into the strain BL21 (DE3)pLysS, which contains the T7 RNA polymerase gene. Upon induction, the σ^F protein becomes highly expressed, eventually constituting approximately 50% of the total cellular protein (data not shown).

We attempted to purify this protein on a NOVAGEN His · Bind column under standard conditions, but most of the protein sedimented with the insoluble fraction after cell lysis (presumably as inclusion bodies). However, the protein could be solubilized by breaking this pellet up in a buffer containing 0.25% Sarkosyl (17). When the solubilized protein was run over the column, a fraction with greater than 95% pure σ^F protein could be eluted with 100 mM imidazole. This fraction proved to have σ^F activity, on the basis of its ability to direct core polymerase to transcribe from the class III *tar* promoter (Fig. 7).

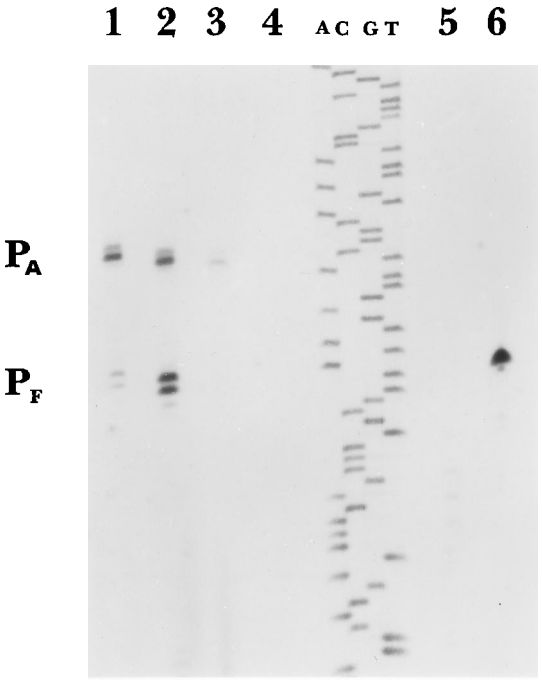

FIG. 6. In vivo and in vitro transcription of *fliA* operon. RNA was isolated from exponential-phase *E. coli* (lanes 1 to 4) or synthesized in vitro (lanes 5 and 6), and the start sites were identified by primer extension with oligonucleotide DSM3. The in vivo RNA was isolated from wild-type cells (RP437, lane 1; YK0410, lane 2), *fliA* cells (YK4104, lane 3), and *flhD* cells (YK4519, lane 4). In vitro RNA was transcribed with $E\sigma^{70}$ (lane 5) or $E\sigma^F$ (lane 6). The sequencing ladder was made with the same primer.

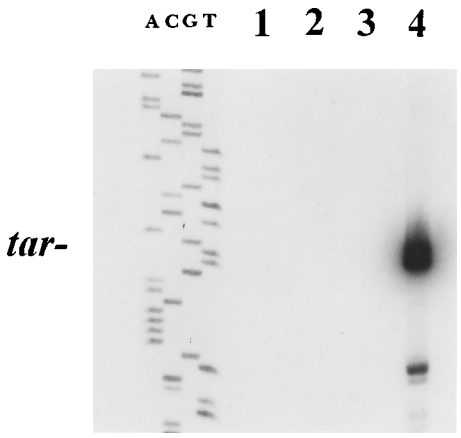

FIG. 7. Transcription of P_F at *tar* gene by using overexpressed, purified σ^F. RNA from in vitro transcription reactions was reverse transcribed with the TAR oligonucleotide as a primer. The lanes contain $E\sigma^{70}$ (lane 1), core polymerase (lane 2), peak σ^F fraction (lane 3), and core plus peak σ^F fraction (lane 4).

DISCUSSION

In this study, we present the sequence of the *fliAZY* operon. This operon encodes the alternative sigma factor σ^F plus two other presumptive motility proteins of 183 and 266 amino acids, respectively. The last gene of this operon encodes a protein (FliY) that may be involved in transporting some molecule or molecules into the cell. On the basis of its closest homologs, FliY probably binds to a polar amino acid or amino acid analog. However, much of the similarity between FliY and its closest homologs is found towards the C terminus of the proteins, which includes regions implicated in binding to the transport ATPases (28). When just the first halves of these proteins are compared, FliY is actually most similar to PheC (data not shown). This makes it less clear what is bound by FliY but suggests that it does interact with a transport ATPase.

The presence of such a gene in a class II operon is somewhat puzzling. All known receptors involved in motility (those governing chemotaxis) are encoded by class III genes or are secondary functions of transporters that are not part of the motility regulon; there does not seem to be any obvious need for small molecule import in conjunction with basal body assembly. One possible explanation would be that *fliY* encodes a

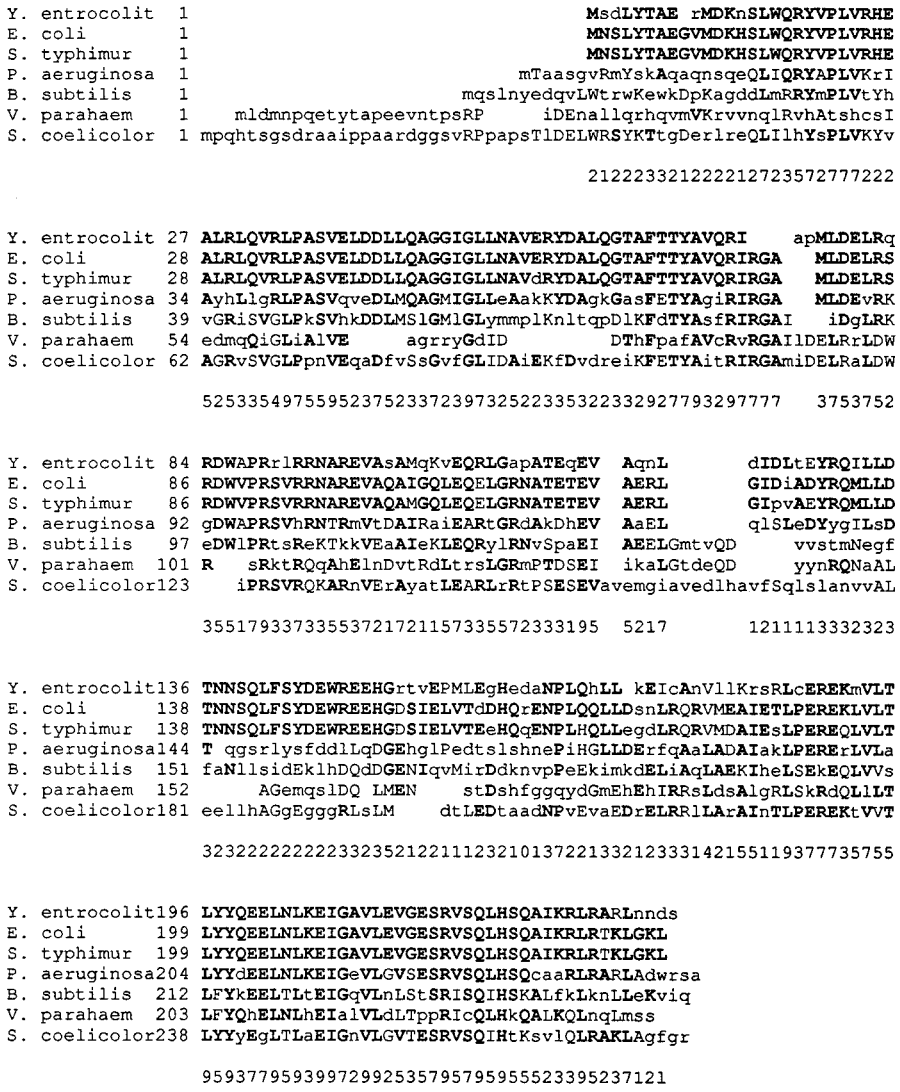

FIG. 8. Comparison of protein sequence of members of σ^{28} family. The most common residue at each position is indicated in bold. Residues which match at least one other sequence are indicated in capital letters. The score indicated is a measure of the conservation at that position (0, all residues differ; 1, two identical residues; 2, three identical residues; 3, four identical or three pairs of identical residues; 4, four identical residues; and another three identical residues; 5, five identical residues; 7, six identical residues; 9, all seven identical residues). Other weighting schemes gave very similar results. Y. entrocolit, *Y. enterocolitica*; S. typhimur, *S. typhimurium*; V. *parahaem*, *V. parahaemolyticus*.

protein that is important for the regulation of class III transcription; it would then be sensible to cotranscribe it with a known regulatory gene, *fliA*. In a number of other bacterial species, it has been found that small amino acid analogs can act as autoinducers: their concentration increases in the medium as the cells become more crowded, until some critical threshold is reached and a pathway is induced (10, 58, 59, 74). That such an inducer might exist in the motility pathway is suggested by the work of Mirel, who has seen that *B. subtilis* grown in rich medium induces or represses flagellin synthesis in response to some factor secreted into the medium or depleted from it (51). He also saw that Casamino Acids in particular have a slight repressive effect, supporting the view that some amino acid analog (which might be present at a low concentration in Casamino Acids or might be poorly replaced by a naturally occurring amino acid) is the repressing signal. FliY may prove to be a receptor that recognizes this signal and helps initiate a corresponding change in the transcription of motility genes.

In order to affect class III transcription, FliY must act with a transport ATPase or a signal transducer. Though it is possible that some other product of the motility regulon fills this function, this seems unlikely. Most transport ATPases that act with a single solute-binding protein are cotranscribed with that solute-binding protein, and neither FliA nor FliZ appears capable of filling this role. The only other class II proteins known to traverse the cytoplasmic membrane are components of the basal body. While it is possible that FliY mediates transport of some small inducer through this pore (perhaps only until export of flagellin through the pore indicates that class III transcription has initiated and blocks further induction), it seems more likely that FliY interacts with some transmembrane protein that is not a part of the motility regulon.

The sequence of FliZ yields little insight into its function. The same argument cited above for FliY suggests that FliZ may also help regulate class III transcription. If it acts as an internal sensor for whatever molecule FliY binds, the two

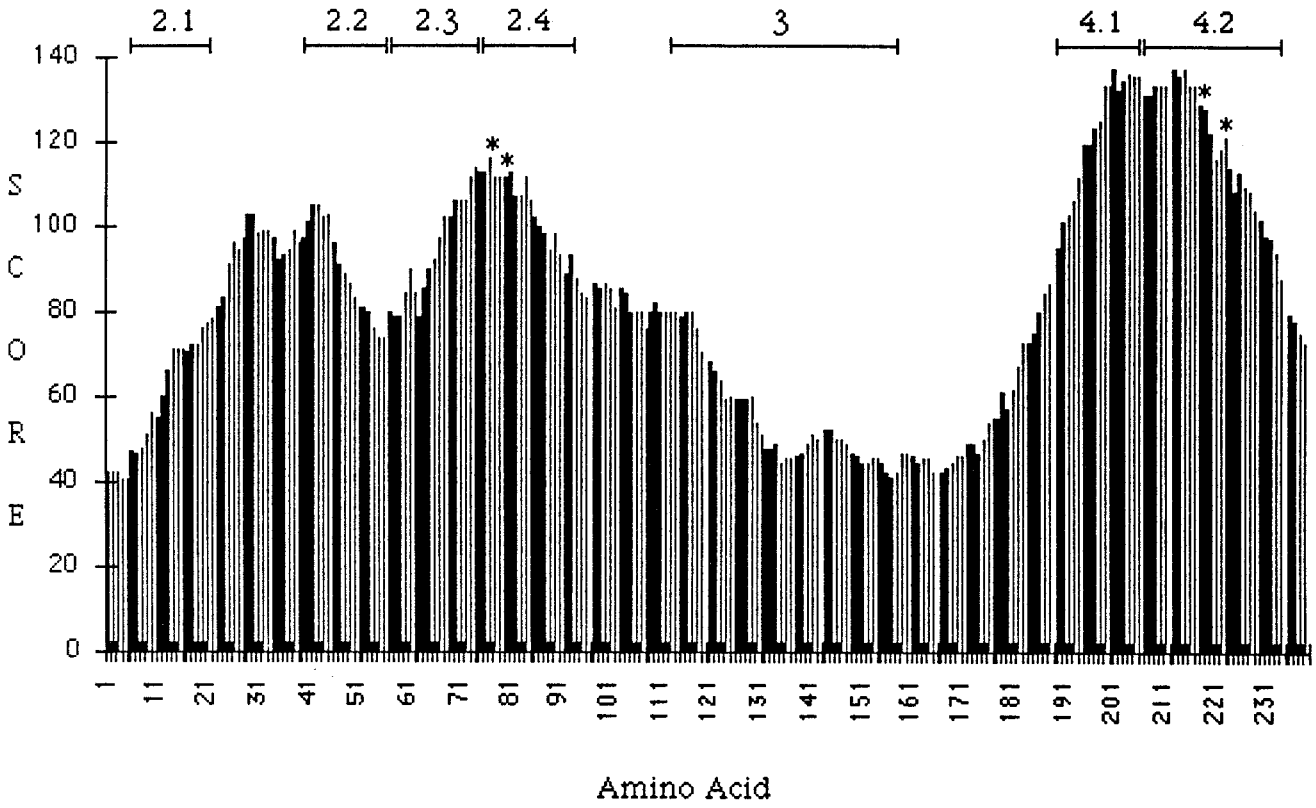

FIG. 9. Regions of similarity among members of σ^{28} family. Scores for each position of the *E. coli* sequence are plotted by adding identity scores over a 21-residue window (see the text). The numbers above the figure indicate conserved regions of sigma factors (26), and the asterisks indicate positions homologous to ones at which mutations in σ^{70} have altered binding site specificity.

proteins would probably have significant homology; they do not. The other likely possibility is that FliZ interacts with either σ^F or FlgM to modulate transcription of class III genes. Further work on this operon should help elucidate its role.

The alternative sigma factor σ^F, encoded by *fliA*, is a member of a group of alternative sigma factors called the σ^{28} family. Members of this family have been sequenced from a number of different bacterial species, including *B. subtilis*, *S. typhimurium*, *Yersinia enterocolitica*, *Vibrio parahaemolyticus*, *Pseudomonas aeruginosa*, *Streptomyces coelicolor*, and now *E. coli* (11, 27, 32, 33, 48, 55, 70). An alignment of these seven protein sequences by using GENALIGN of the INTELLIGENETICS package is shown in Fig. 8. Since all of these proteins appear to recognize similar promoter sequences, they should all have very similar DNA binding regions. In order to quantitatively address this issue, we scored each position in the *E. coli* sequence on the basis of how similar the sequences were at the corresponding positions in this alignment. Each position was initially given a score between zero and nine based on similarity at that position alone and then given a regional score summing the 21 residues centered at that position. Thus, scores could range from 0 (if all seven sequences were different at every position in the 21-residue window) to 189 (if all of the sequences were identical over the entire window). These results are graphed in Fig. 9. They show two peaks of maximum similarity among the seven peptides, which correspond to the regions previously identified in other sigma factors as probable DNA binding regions, based on allele-specific suppression of promoter mutations (12, 16, 66, 72) and relatively high sequence conservation (26). Region 2.2, which may be involved in core binding (26), is also very highly conserved; it may affect the structure of the adjacent DNA binding region as well. Curiously enough, the *Y. enterocolitica* sequence, which is identical to the *E. coli* sequence for the previous 61 residues, diverges in the middle of highly conserved region 2.4; one would predict that this sigma factor probably recognizes a somewhat different −10 sequence.

It has recently become evident that class II operons in *S. typhimurium* and *E. coli* are transcribed at lower levels in *fliA* mutants but at higher levels in *flgM* mutants (39). This work suggests two novel models to help explain these observations. One possibility is that this may primarily be an effect of *fliZY* rather than *fliA*. It is likely that *fliZ* and *fliY* exist in *S. typhimurium* as well, since the published *fliA* sequence from that species includes some downstream sequence that is homologous to *fliZ* (55) (data not shown). Since many of the *fliA* mutants that have been used (including the mutation in YK4104 and any insertion into *fliA*) would be polar on these downstream genes, it is possible that it is their absence that prevents the full induction of class II genes; FliZ and FliY may have positive as well as negative regulatory effects.

While it is possible that the above system would work through FlgM, this seems an unlikely explanation for why a *flgM* mutant would express class II genes at a higher level. A more direct possibility would be that the P_F present in at least this class II gene (and maybe *fliL* and others) is important for its regulation. While the simplest explanation would be that $E\sigma^F$ transcribes all class II genes and boosts their transcription over a certain basal level, it is clear that the expression from the *fliA* P_A is also substantially reduced in an *fliA* mutant (Fig.

6), though $E\sigma^F$ does not appear to transcribe from this promoter. Since σ^F has been shown to bind weakly to P_Fs (14), it is possible that it could bind to the P_F upstream of *fliA* and somehow stimulate transcription from the other promoter. Alternatively, $E\sigma^F$ could bind but have difficulties initiating transcription. The presence of FlgM may then lock σ^F or $E\sigma^F$ in a conformation that blocks transcription from the P_A.

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