JOURNAL OF BACTERIOLOGY, May 1989, p. 2728–2734
0021-9193/89/052728-07$02.00/0

Vol. 171, No. 5

DNA Sequence Analysis, Gene Product Identification, and Localization of Flagellar Motor Components of *Escherichia coli*

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The *Escherichia coli* operon designated *flaA* contains seven flagellar genes; among them are two switch protein genes whose products are believed to interface with the motility and chemotaxis machinery of the cell. Complementation analysis using several plasmids carrying different portions of the *flaA* operon and analysis of expression of these plasmids in minicells allowed the identification of two flagellar gene products. The MotD (now called FliN) protein, a flagellar switch protein, was determined to have an apparent molecular weight of 16,000, and the FlaAI (FliL) protein, encoded by a previously unidentified gene, had an apparent molecular weight of 17,000. DNA sequence analysis of the *motD* gene revealed an open reading frame of 414 base pairs. There were two possible initiation codons (ATG) for *motD* translation, the first of which overlapped with the termination codon of the upstream gene, *flaAII* (*fliN*). The wild-type *flaAI* gene on the chromosome was replaced with a *flaAI* gene mutated in vitro. Loss of the *flaAI* gene product resulted in a nonmotile and nonflagellated phenotype. The subcellular location for both the MotD and FlaAI proteins was determined; the FlaAI protein partitioned exclusively in the insoluble fraction of a whole minicell sonic extract, whereas the MotD protein remained in both the soluble and insoluble fractions. In addition, we subcloned a 2.2-kilobase-pair DNA fragment capable of complementing the remaining four genes of the *flaA* operon (*flbD* [*fliO*], *flaR* [*fliP*], *flaQ* [*fliQ*], and *flaP* [*fliR*]).

The isolated bacterial flagellar structure has been shown to consist of products of genes found predominantly in flagellar region I (13). Other gene products required for complete flagellar assembly and function are known not to copurify with the basal body structure (1). It is possible that during the isolation of basal bodies, components not firmly attached to the basal body are lost. Biochemical and genetic data suggest that a number of flagellar proteins exist as a complex in the membrane adjacent to the basal body (21). Site-limited incorporation of some of the flagellar membrane proteins suggests that these proteins are components of a larger flagellar substructure (8, 36). Allele-specific suppression between flagellar membrane proteins suggests that these proteins physically interact to form a complex (36). These data have led to the concept of the total motor, consisting of a basal body structure and an underlying membrane substructure known as the switch complex (8, 21). The protein components of this complex identified to date are the *flaAII*, *flaBII*, and *motD* gene products, which are believed to be involved in switching the direction of the flagellar rotation (21). (New nomenclature for flagellar genes is as follows: *fliL* replaces *flaAI*, *fliM* replaces *flaAII*, *fliN* replaces *motD*, *fliO* replaces *flbD*, *fliP* replaces *flaR*, *fliQ* replaces *flaQ*, and *fliR* replaces *flaP* (10). Both the FlaAII and FlaBII proteins have been localized in the cytoplasmic membrane (8; D. H. Bartlett and P. Matsumura, unpublished data), which is consistent with their roles as switch proteins. These proteins also interact with known soluble components of the sensory transduction machinery, CheY and CheZ, which affect flagellar rotation (28).

In this study, we characterized two components of the putative protein substructure of the flagellar motor by DNA sequence analysis, gene product identification, and gene product localization. In addition, we localized the remaining four genes of the *flaA* operon to a 2.2-kilobase-pair (kbp) DNA fragment.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *Escherichia coli* K-12 strains and plasmids used are listed in Table 1. Strains C600, JM103, JC7623, and χ1488 were used as hosts for propagating plasmids and bacteriophage M13 clones, for linear DNA transformation, and as the minicell-producing strain, respectively. L-broth medium (9) was used to grow all strains except JM103, which was grown in M9 minimal medium (22). Bacto-Agar (Difco Laboratories, Detroit, Mich.) was added to L broth at 17.5 g/liter for solid media and at 3.8 g/liter for motility plates. Antibiotic supplements (Sigma Chemical Co., St. Louis, Mo.) were tetracycline (30 μg/ml), penicillin (100 μg/ml), and kanamycin (50 μg/ml).

Complementation, cloning, transformation, and plasmid purification procedures have been described previously (4). Flagellar staining was performed according to the procedure of Leifson (20). Restriction nuclease enzymes were purchased from either Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) or Amersham Corp. (Arlington Heights, Ill.) and used according to the directions of the suppliers. T4 DNA ligase was purified by the method of Pirotta and Bickle (29). Calf intestinal alkaline phosphatase was purchased from P-L Biochemicals, Inc. (Milwaukee, Wis.). Chemicals were obtained from Sigma. Radioisotopes ([α-^{35}S]dCTP for sequencing reactions and [^{35}S]methionine for protein labeling) were purchased from Amersham.

Minicell purification has been described previously (23). For the subcellular localization study, the radiolabeled minicells were centrifuged at 10,000 × *g* for 30 s in an Eppendorf microcentrifuge, suspended in 10 mM Tris hydrochloride (pH 7.9), and subjected to sonication (three 30-s bursts). The sonicated suspension was centrifuged briefly to remove

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TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Relative genotype or phenotype	Source or reference
E. coli strains		
C600	F⁻ *thi thr leu lacY tonA supE*	3
RP4248	*cheC4248*	Parent RP437; 28; J. S. Parkinson, unpublished data
RP3379	*motD4117*	Parent YK410; 12, 28
RP4184	*flbD52*	Parent W3110; 14, 28
JM7623	*flaAI*::Kan	Parent JC7623; this study
JC7623	*recB recC sbcB*	17
χ1488	F⁻ *rpsL hst minA minB purE pdxC his ile met ade ura* r_K m_K^+	24
JM103	F⁺ Δ(*lac pro*) *thi rpsL supE endA sbcB hsdR4 traD proAB lacI*^{q}	26
Plasmids		
pBM2190	Kan^r cassette	M. Berman, unpublished data
pBR322	Cloning vehicle	7
pRL31	Promoter *lac*UV5 expression vehicle	R. Linzmeier, M.S. thesis, University of Illinois, Chicago, 1983
pDV4	Promoter *trp* expression vehicle	D. Vacante and P. Matsumura, unpublished data
pUC18	Promoter *lac* cloning vehicle	35

unbroken cells, and the soluble (cytoplasmic and periplasmic) and insoluble (membrane) fractions were collected by high-speed centrifugation of the supernatant at 140,000 × *g* in a Ti50 rotor for 1 h. These fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at a polyacrylamide concentration of 15% (19).

DNA sequence analysis. *E. coli* JM103 was grown and infected with M13 phage derivatives. Phage-containing inserts were identified, and the double-stranded replicative-form phage DNA (for analysis by gel electrophoresis for detection of correct insert) as well as single-stranded phage DNA (for DNA sequencing) were isolated and purified as described elsewhere (25). To obtain overlapping DNA fragments, the inserted DNA was digested with the restriction enzyme *Hpa*II or *Sau*3A and cloned into the *Acc*I or *Bam*HI site, respectively, of phage M13mp8. Dideoxy-chain termination sequencing reactions were carried out with ^{35}S-substituted deoxycytosine 5-[α-thio]triphosphate (31) and subjected to electrophoresis on acrylamide gradient gels as described previously (6). All sequences were determined for both strands. Dideoxynucleotide triphosphates and deoxynucleotide triphosphates were obtained from P-L Biochemicals, Inc., M13 sequencing primer was purchased from Clontech Laboratories, Inc. (Palo Alto, Calif.), and DNA polymerase I (Klenow fragment) was purchased from Amersham.

RESULTS

Complementation tests. Plasmid pYK3258 is a pBR322 derivative containing a 3.4-kbp *Pst*I flagellar DNA fragment. This plasmid was known to complement a *motD* defect (data not shown). To localize the position of the *motD* gene, a restriction map of this plasmid was constructed, and DNA subfragments were isolated and cloned in high-expression vehicles carrying either the *trp* or *lac*UV5 promoter (Fig. 1). Plasmid pJM2, which carries a 1.9-kbp *Bam*HI-to-*Pst*I fragment, was capable of complementing *flaAII* (*cheC*) and *motD* mutants to the wild-type chemotaxis and motility phenotype (Fig. 1A). Plasmid pJM3, which contains a 703-bp *Pvu*II-*Pst*I fragment, was capable of complementing only a *motD* defect. Because the gene order of *motD* and *flbD* was not clear (8, 11, 12, 15), we used the complementation pattern in Fig. 1A to establish the physical location of *motD* and *flbD*. Neither pJM2 nor pJM3 was capable of complementing the *flbD* defect, but pJM8 and pJM9 complemented the *flbD* defect to wild-type motility and chemotaxis phenotypes (Fig. 1A). This finding indicates that the *motD* gene is the promoter-proximal gene, with the *flbD* gene located downstream of the *Pst*I restriction site. We also subcloned a 2.2-kbp *Pst*I fragment containing the remainder of the *flaA* operon. This clone was capable of complementing *flbD* (Fig. 1), *flaR*, *flaQ*, and *flaP* mutations (data not shown).

***flaAI* null phenotype.** During this study, we discovered a previously unidentified flagellar gene that has also been described by Kuo and Koshland (16). The new gene was designated *flaAI* and sequenced (data not shown). Our *flaAI* and *flaAII* sequences and identification of the gene products agreed with the data of Kuo and Koshland; however, our sequence data showed a T deletion at position −104 upstream of the transcription initiation site at +1 (16).

To determine the null phenotype of the *flaAI* gene, the wild-type *flaAI* gene on the chromosome was replaced with a *flaAI* gene mutated in vitro. Mutagenesis was achieved by insertional inactivation of the *flaAI* gene cloned in plasmid pYK3258 (Fig. 2). Plasmid pYK3258 was partially digested with the restriction enzyme *Bam*HI. A 1.4-kbp DNA fragment containing the kanamycin resistance gene from plasmid pMB2190, which is flanked with multiple restriction enzyme sites, was inserted into the *Bam*HI site of pYK3258. The orientation of insertion (kanamycin resistance) was counterclockwise with respect to the direction of transcription of the *flaAI* gene (Fig. 2). This construction was designated pYK3258-K. Next, plasmid pYK3258-K was digested with the restriction enzyme *Eco*RI, which cuts the plasmid in a single site outside of the flagellar DNA, and treated with calf intestinal alkaline phosphatase. The linear DNA was used to transform *E. coli* JC7623 carrying *recBC* and *sbcB* mutations. The *sbcB* mutation prevents exonuclease degradation of linear DNA and suppresses the Rec phenotype of *recBC* mutations; therefore, homologous recombination can take place through an alternative pathway (17). Kanamycin-resistant colonies were screened for tetracycline sensitivity to ensure that the plasmid DNA had been lost. The constructed (JM7623) *flaAI* mutant was analyzed by Southern blot DNA-DNA hybridization (34), using a 326-bp nick-translated *Hpa*II DNA fragment that spans the *Bam*HI site in the wild-type *flaAI* gene (Fig. 3). As expected, the probe specifically hybridized to the 3.4-kbp *Pst*I fragment purified from pYK3258 (Fig. 3, lane 2) and the same fragment from the genomic digest of a *flaAI* wild-type strain (Fig. 3, lane 3). Since the kanamycin resistance determinant inserted in the *Bam*HI site was flanked by two *Pst*I sites, the genomic digest of the mutant strain produced two fragments, of 1.5 and 1.9 kbp, which hybridized to the 326-bp *Hpa*II probe (Fig. 3, lane 4). These results verified that strain JM7623 contained a disrupted *flaAI* gene.

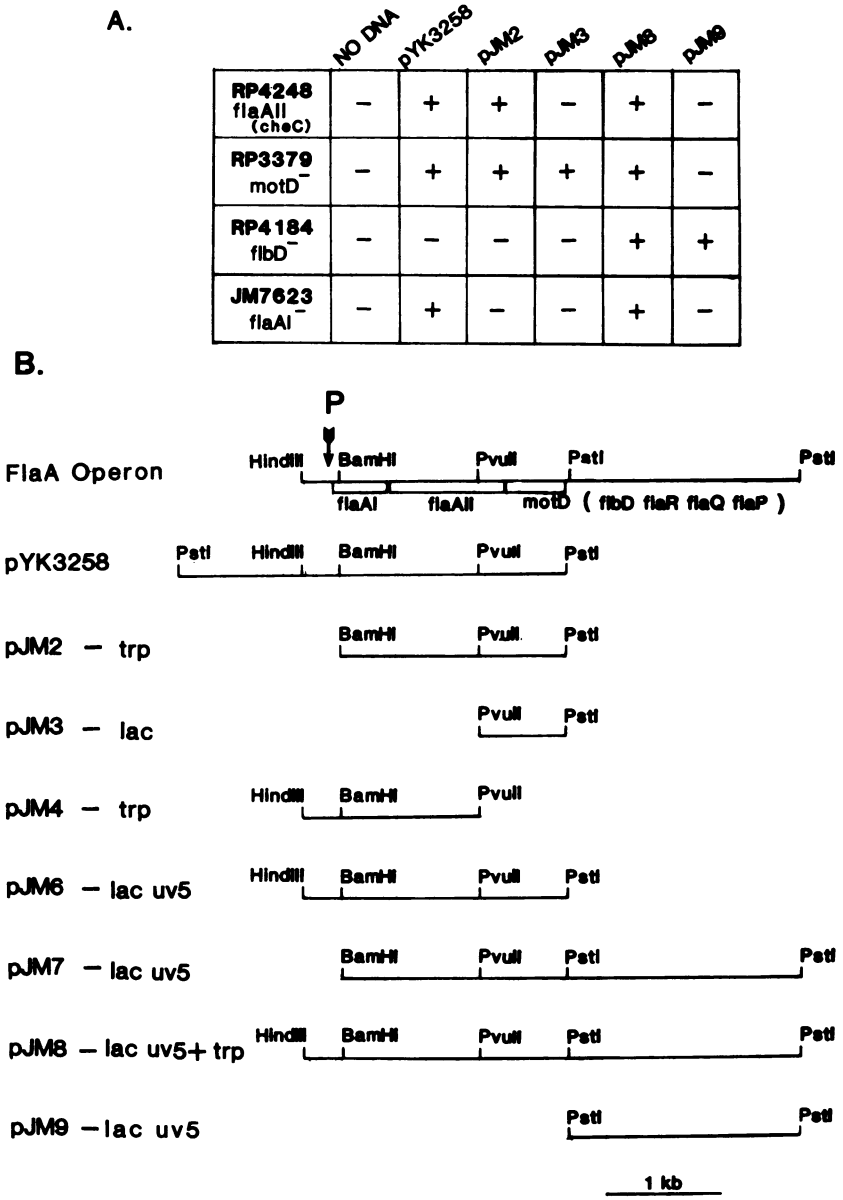

FIG. 1. Complementation analysis and plasmid construction. (A) Complementation for wild-type motility was measured by assaying the swarming ability of mutant strains transformed with the plasmids listed in panel B. + and −, Complementation and no complementation, respectively. (B) Plasmids used in complementation tests. At the top is shown the physical organization of the *flaA* operon. Symbols: ——, insert flagellar DNA contained in each plasmid (these fragments were placed under transcriptional control of high-level expression vehicles carrying high-level promoters such as *lac*UV5, *lac*, and *trp*, as indicated after the names of the plasmids); ↓, location of the promoter of the *flaA* operon; ▭, positions and lengths of the genes. Positions of restriction sites are indicated.

The mutant strain, JM7623, was nonmotile and Kan^r. The *flaAI*-defective and wild-type cells were stained with flagellum-specific stain and viewed under a light microscope. No flagella were observed on strain JM7623, whereas the wild-type strain exhibited numerous flagella. Therefore, the null phenotype of *flaAI* is Fla$^-$. Insertion of the kanamycin resistance determinant normally has a polar effect on the expression of downstream genes. Insertion of the kanamycin resistance determinant in the *motA* gene of the *mocha* operon exhibited a polar effect on expression of the downstream *motB* gene (P. Matsumura, unpublished data). However, the kanamycin resistance determinant insertion was not polar on the expression of downstream genes of the *flaA* operon. The lack of polarity has been shown to be due to promoter activity within the coding region of a number of genes within this operon (J. Malakooti and P. Matsumura, manuscript in preparation). Therefore, the kanamycin cassette insertion removed only *flaAI* complementation activity.

Sequence of the *motD* gene. The complementation data showed that the *motD* gene was contained in the *Pvu*II-to-*Pst*I DNA fragment (Fig. 1). Therefore, the nucleotide sequence of that fragment was determined. An open reading frame composed of 414 bp corresponding to the *motD* gene was identified (Fig. 4). There were two possible in-phase initiation codons (ATG), which were separated by 6 nucleotides. The first start codon overlapped with the termination codon of the preceding gene, *flaAII*. The 3′ end of the *flaAII*

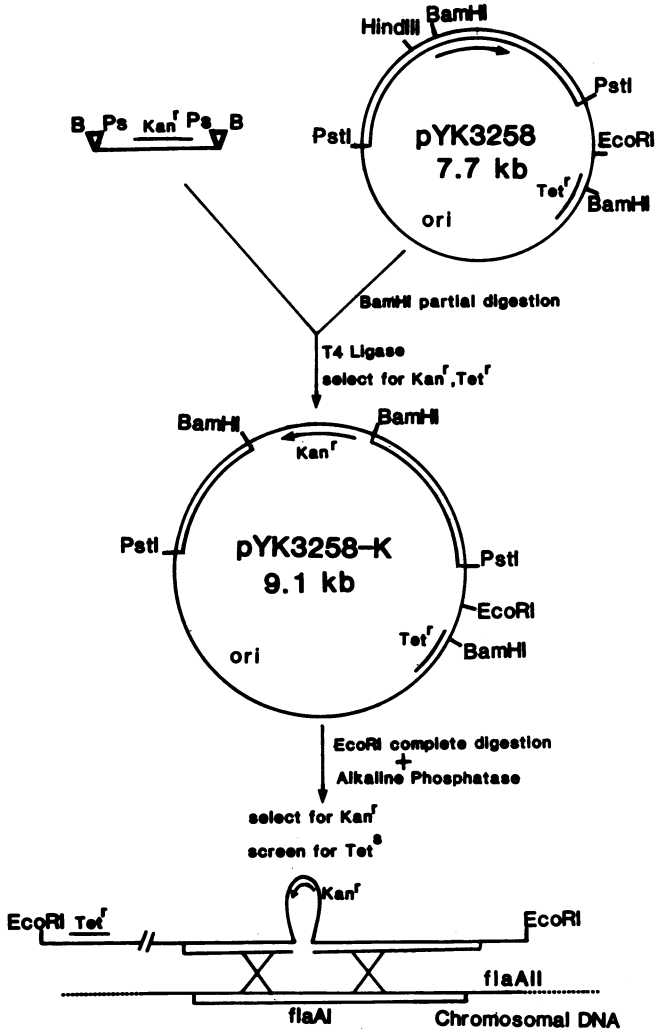

FIG. 2. Strategy for constructing the *flaAI* chromosomal mutation. The kanamycin resistance determinant flanked by the *Bam*HI restriction site was cloned into the *Bam*HI site of the *flaAI* gene in plasmid pYK3258. A linear *Eco*RI DNA fragment containing the disrupted *flaAI* gene and its flanking region was used to transform strain JC7623. The transformed cells were plated on L-agar plates containing kanamycin, and recombinants exhibiting the Kan^r Tet^r phenotype were isolated. Abbreviations: Ps, *Pst*I site; B, *Bam*HI site; ×, crossover site. Arrows within circles show direction of the kanamycin resistance gene or *flaA* operon transcription.

gene also provided a potential ribosome-binding site for translation initiation of the MotD protein. The highly conserved AGGA ribosome-binding site was located 9 bp upstream of the first ATG and showed good complementarity to the 3′ end of the 16S rRNA of *E. coli* (33), whereas upstream of the second ATG there was no homology to the consensus ribosome-binding site. By using the first start codon, the predicted molecular weight of MotD was calculated to be 14,853. The next open reading frame started 2 bp downstream of the *motD* termination codon (Fig. 4).

The hydropathy profile (18) of MotD showed that the hydrophobic segments of the protein, with an average calculated value of 0.72, were located in the last 70% of the polypeptide. The overall hydrophobicity (18) of MotD was −0.074, which is higher than the hydrophobicity of the average soluble protein (−0.4). These values were calculated by the method of Kyte and Doolittle (18).

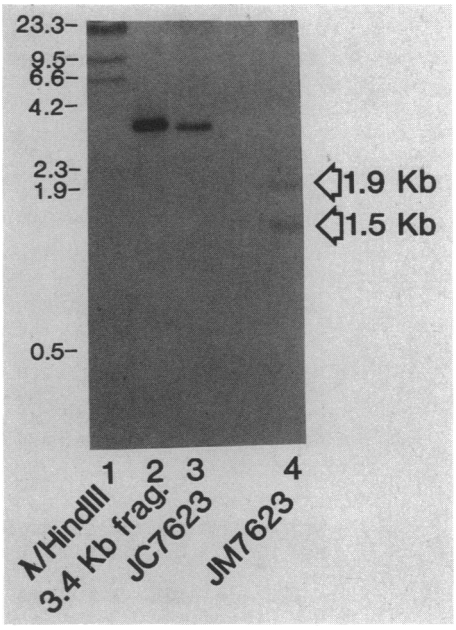

FIG. 3. Southern DNA-DNA hybridization analysis of strain JM7623. Chromosomal DNA was isolated from strains JC7623 (wild type) and JM7623 (FlaAI$^-$), digested with *Pst*I, and probed with a 326-bp nick-translated *Hpa*II fragment spanning the *Bam*HI site of the *flaAI* gene. Lanes: 1, lambda phage DNA digested with *Hin*dIII used as marker DNA (sizes of fragments in kilobase pairs are shown on the left); 2, 3.4-kbp *Pst*I flagellar DNA from pYK3258; 3, DNA from the wild-type strain JC7623; 4, mutant strain JM7623 chromosomal DNA digested with *Pst*I.

Identification of gene products. To correlate genes and gene products, the minicell-producing strain χ1488 was transformed with plasmids pJM2, pJM6, and pJM4 (Fig. 1B). Minicells were purified, and plasmid-encoded proteins were expressed and labeled with [^{35}S]methionine. The radiolabeled proteins were resolved on 15% polyacrylamide-SDS gels and autoradiographed. The proteins encoded by pJM6 and pJM2 (Fig. 1) are shown in Fig. 5 (lanes 2 and 3). In plasmid pJM2, DNA sequences corresponding to the first 40 amino acids of the *cheY* gene were fused to a 1.9-kbp *Bam*HI-*Pst*I fragment of the parental plasmid pYK3258. pJM2, in addition to expressing the tetracycline resistance gene product of about 37 kilodaltons (kDa) and FlaAII protein of 38 kDa (the 37-kDa tetracycline resistance gene product comigrated with the 38-kDa FlaAII band), directed the expression of two more proteins, with sizes of 16 and 14 kDa. To assign one of these bands to the MotD protein, plasmid pJM6 was constructed (Fig. 1). This plasmid contained the same flagellar DNA insert as did pJM2 but had an additional 308-bp *Hin*dIII-*Bam*HI fragment located upstream of the *Bam*HI site on the chromosome. Plasmid pJM6, as predicted, encoded the Kan^r protein and the *flaAI* (see below), *flaAII*, and *motD* gene products. In comparison with pJM2, pJM6 synthesized a protein that comigrated with the 16-kDa band of pJM2, whereas the 14-kDa band was not present in pJM6. On the basis of these findings, we concluded that the 16-kDa protein was the *motD* gene product. Furthermore, DNA sequence analysis of the fusion point in pJM2 (data not shown) revealed an open reading frame that could code for a 13-kDa protein. This construction was an out-of-phase fusion between *cheY* and *flaAI*.

Identification of the *flaAI* gene product is shown in Fig. 5 (lane 4). Deletion of DNA sequence downstream of the

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          10          20          30          40          50          60
          * S.D.      *           *           *           *           *
TCT CTG AAC GAG GAA CAG CCC AAA TGA
  (flaAII)  *****                 ATG AGT GAC ATG AAT AAT CCG GCC GAT GAC AAC AAC
                                  Met Ser Asp Met Asn Asn Pro Ala Asp Asp Asn Asn

          70          80          90          100         110         120
          *           *           *           *           *           *
GGC GCA ATG GAC GAT CTG TGG GCT GAA GCG TTG AGC GAA CAA AAA TCA ACC AGC AGC AAA
Gly Ala Met Asp Asp Leu Trp Ala Glu Ala Leu Ser Glu Gln Lys Ser Thr Ser Ser Lys

         130         140         150         160         170         180
          *           *           *           *           *           *
AGC GCT GCC GAG ACG GTG TTC CAG CAA TTT GGC GGT GGT GAT GTC AGC GGA ACG TTG CAG
Ser Ala Ala Glu Thr Val Phe Gln Gln Phe Gly Gly Gly Asp Val Ser Gly Thr Leu Gln

         190         200         210         220         230         240
          *           *           *           *           *           *
GAT ATC GAC CTG ATT ATG GAT ATT CCG GTC AAG CTG ACC GTC GAG CTG GGC CGT ACG CGG
Asp Ile Asp Leu Ile Met Asp Ile Pro Val Lys Leu Thr Val Glu Leu Gly Arg Thr Arg

         250         260         270         280         290         300
          *           *           *           *           *           *
ATG ACC ATC AAA GAG CTG TTG CGT CTG ACG CAA GGG TCC GTC GTG GCG CTG GAC GGT CTG
Met Thr Ile Lys Glu Leu Leu Arg Leu Thr Gln Gly Ser Val Val Ala Leu Asp Gly Leu

         310         320         330         340         350         360
          *           *           *           *           *           *
GCG GGC GAA CCA CTG GAT ATT CTG ATC AAC GGT TAT TTA ATC GCC CAG GGC GAA GTG GTG
Ala Gly Glu Pro Leu Asp Ile Leu Ile Asn Gly Tyr Leu Ile Ala Gln Gly Glu Val Val

         370         380         390         400         410         420
          *           *           *           *           *           *
GTC GTT GCC GAT AAA TAT GGC GTG CGG ATC ACC GAT ATC ATT ACT CCG TCT GAG CGA ATG
Val Val Ala Asp Lys Tyr Gly Val Arg Ile Thr Asp Ile Ile Thr Pro Ser Glu Arg Met

         430         440         450         460         470         480
          *           *           *           *           *           *
CGC CGC CTG AGC CGT TAG TG ATG AAT AAC CAC GCT ACT GTG CAA TCT TCC GCG CCG GTT
Arg Arg Leu Ser Arg ---    START ORF

          490         500
           *           *
TCT CGT GCG CCA CTG CTG CAG
```

FIG. 4. Nucleotide sequence of the *motD* gene. Only the strand equivalent to mRNA is shown. Nucleotides 1 to 28 are the 3′ end of the *flaAII* gene. The overlap between the translation termination codon of the *flaAII* gene product and the initiation codon of the *motD* gene product is indicated. The deduced amino acid sequence of the MotD protein is shown beneath the nucleotide sequence. The sequence showing homology to the consensus sequences of ribosome-binding sites (S.D.) is indicated. Some sequences of the downstream open reading frame (ORF) are also shown.

*Pvu*II site (Fig. 1) resulted in the loss of *cheC* complementation activity. The 34-kDa band (lane 4) corresponded to a truncated *flaAII* gene product in which 231 bp of DNA was deleted from the 3′ end of the gene. The 30- and 32-kDa bands were processed and unprocessed β-lactamase gene products. pJM4 also encoded a 17-kDa protein. Since this plasmid carried a DNA insert corresponding only to *flaAI* (the first open reading frame in the *flaA* operon) and a truncated *flaAII* gene, the 17-kDa protein must be the *flaAI* gene product. Because of the very similar molecular weights of the *flaAI* and *motD* gene products, these proteins were not resolved by SDS-PAGE when plasmid pJM6 or other plasmids harboring both genes were expressed. The 17-kDa molecular size of the FlaAI protein was in agreement with the predicted size derived from the amino acid sequence of the gene product.

Subcellular localization of the MotD and FlaAI proteins. To localize the *motD* and *flaAI* gene products, minicells containing pJM2 and pJM4 were labeled and disrupted by sonication. Soluble and insoluble fractions were separated by centrifugation and subjected to SDS-PAGE (Fig. 6). The *motD* gene product appeared in both the soluble (cytoplasmic-periplasmic) and insoluble fractions (Fig. 6A, lanes 2 and 3). A membrane location for the MotD protein is consistent with the hydropathy profile of this protein as deduced from the amino acid sequence. The presence of the MotD protein in both soluble and insoluble fractions may reflect a site-specific incorporation of this protein into specific sites in the membrane which, upon overexpression, are saturated, resulting in excess MotD protein partitioning into the soluble fraction. However, this procedure cannot differentiate between membrane-associated and insoluble proteins.

In contrast to the MotD protein, the overexpressed *flaAI* gene product (Fig. 6B) was found exclusively in the insoluble fraction (lane 4). The truncated 34-kDa FlaAII protein appeared in both the whole-cell soluble and insoluble fractions (lane 3 and 4). A membrane location for FlaAII has been established in cell envelopes devoid of cytoplasmic components (30). Only the fully processed β-lactamase remained in the soluble fraction, whereas the membrane fraction contained both processed and unprocessed polypeptides.

DISCUSSION

The DNA sequence of the *motD* gene of *E. coli* was determined, and the corresponding gene product was iden-

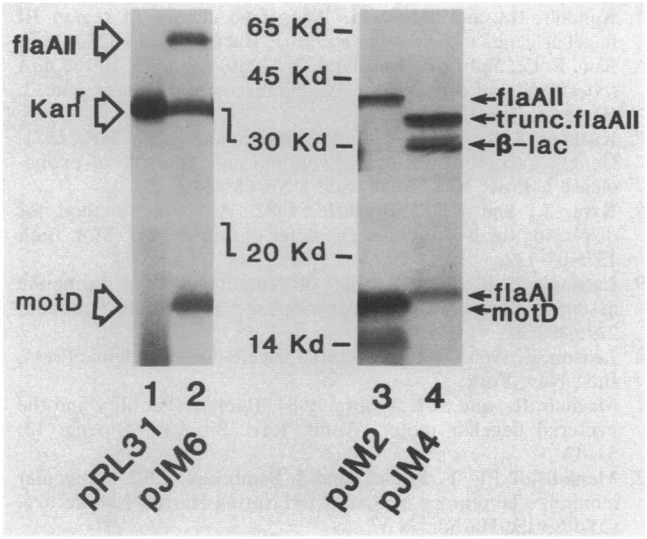

FIG. 5. Identification of the *motD* and *flaAI* gene products. Plasmids used to identify of the MotD and FlaAI proteins are shown in Fig. 1B. Plasmid-encoded proteins were studied in a minicell expression system, and ^{35}S-labeled proteins were analyzed by electrophoresis through an SDS-15% polyacrylamide gel. Lane 1, Proteins encoded by the expression vehicle pRL31 (this plasmid was used as the vector in constructing pJM6 and codes for a 30-kDa Kan^r gene product); lane 2, proteins encoded by pJM6 (FlaAI at 17 kDa, MotD at 16 kDa, FlaAII at 38 kDa, and Kan^r at 30 kDa). Positions of molecular size standards: 65 kDa, bovine serum albumin; 45 kDa, chicken ovalbumin; 30 kDa, carbonic anhydrase; 20 kDa, trypsin inhibitor; 14 kDa, lysozyme. Lane 3, Proteins encoded by pJM2. The 38- and 16-kDa bands represent FlaAII and MotD, respectively. The tetracycline resistance gene product encoded by this plasmid is faintly visible below the 38-kDa band. Lane 4, pJM4-encoded proteins. In addition to the β-lactamase proteins at 30 kDa, this plasmid synthesized a truncated FlaAII (34 kDa) and the *flaAI* gene product (17 kDa).

tified as a membrane protein. Identification of gene products was based on genetic complementation data and expression of the plasmid-encoded protein in minicells. The DNA sequence of a 703-bp *Pvu*II-*Pst*I fragment (which by complementation analysis was inferred to contain the complete *motD* gene) revealed an open reading frame of 414 nucleotides that could be translated into a protein of 137 amino acids (M_r, 14,853). This value is in close agreement with the apparent molecular size of 16 kDa revealed by SDS-PAGE. An interesting feature of this coding region was the potential overlap of the start codon of this gene and the stop codon of the preceding gene (*flaAII*). This sort of compact arrangement of genes clustered in an operon has been reported for several other operons (2, 5, 27, 32). In such cases, equimolar amounts of the gene products are expected for coupled genes (32) when transcription is initiated upstream of the first coding sequence. However, scanning of the autoradiograph of a plasmid expressing both *flaAII* and the *motD* genes in minicells revealed that the relative amount of the MotD protein was considerably higher than that of the FlaAII protein (data not shown). The higher levels of MotD expression could result from either enhanced transcriptional or enhanced translational efficiency. In translationally coupled genes, the coupled downstream gene usually contains a less efficient ribosome-binding site than does the upstream gene (32). In the case of the *flaAII* and *motD* genes, sequences upstream of the start codon of *flaAII* (16) have shown no homology to the conserved ribosome-binding site (33); 9 bp preceding the first ATG codon of the *motD* gene, however, there was a fairly good match to the consensus ribosome-binding sequence. It is possible that an already bound ribosome transversing the *flaAII* mRNA continues the translation of the next gene (*motD*) without being released from the message (32). Perhaps the presence of the ribosome-binding site upstream of the *motD* gene attracts additional ribosomes, which could result in higher efficiency of translation of the *motD* gene. Also, examination of the DNA sequence of the *Bam*HI-*Pst*I fragment (Fig. 1) revealed the presence of a potential internal promoter, which also might account for the higher expression of the *motD* transcript and, subsequently, higher levels of translation (data not shown). The significance of the internal promoter in this region is now under investigation.

Fractionation of the proteins synthesized by minicells showed that although a considerable portion of the overexpressed MotD protein sedimented with the particulate fraction, some still remained in the soluble fraction. This finding may reflect a site-limited incorporation of this protein into specific sites on the membrane. The hydropathy profile of the *motD* gene showed that this polypeptide is hydrophobic, with hydrophobic segments residing mostly in the carboxy terminus. Specifically, the presence of a segment 37 amino acids long (from amino acids 80 to 117), with an average hydropathy value of 0.72, supports the idea that this segment is membrane associated rather than an internal hydrophobic stretch (18). Site-limited incorporation has also been reported for FlaAII, which is another switch protein (8). The MotD protein is believed to be one of the three multifunctional proteins (FlaAII, FlaBII, and MotD) that are involved in energy transduction, assembly, and flagellar rotation (37). On the basis of intergenic suppression of the homologous genes of *Salmonella typhimurium*, it has been suggested that these gene products interact directly with each other (36) and may form the switch complex. The complex is thought to be assembled in the inner membrane in such a way that, via interactions with cytoplasmic components of the sensory transduction apparatus (CheY and CheZ), it determines the clockwise or counterclockwise direction of flagellar rotation

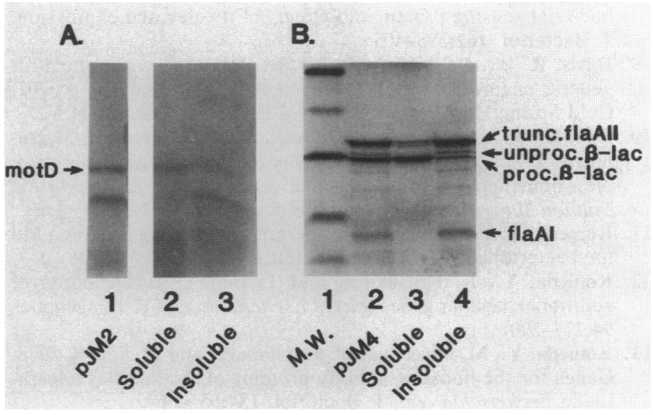

FIG. 6. Subcellular localization of the MotD (A) and FlaAI (B) proteins. (A) Lanes: 1, proteins encoded by pJM2 in whole minicells; 2 and 3, soluble and insoluble fractions, respectively, of the pJM2 expression products. (B) Lanes: 1, molecular size standards (see legend to Fig. 5); 2, pJM4-encoded proteins in whole minicells (bands represent the 34-kDa truncated FlaAII protein, the 32- and 30-kDa unprocessed and processed β-lactamase proteins, and the 17-kDa FlaAI protein); 3 and 4, soluble and insoluble fractions, respectively, of the pJM4 expression products.

(21). As proposed by Yamaguchi et al. (36), the switch complex may be positioned directly under the M ring.

Defects in some flagellum-related genes can cause any combination of the Fla, Che, and Mot phenotypes. By using insertional inactivation of the *flaAI* gene on the chromosome, the null phenotype of the defective strain was determined to be Fla^-. The lack of flagella in the mutant cells was established by testing the swarming behavior of the cells on semisolid agar plates, by flagellar staining, and by examining cells for chemotactic behavior under a light microscope. The product of this gene was localized exclusively to the membrane fraction. This finding and the loss of flagella in the mutant strain suggest that the FlaAI protein plays a role in either the synthesis or assembly of the components of the flagellum. Identification of the FlaAI and MotD proteins will facilitate further delineation of the interactions within the switch complex and therefore of bacterial behavior.

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