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Flagellar Transcriptional Activators FlbB and FlaI: Gene Sequences and 5′ Consensus Sequences of Operons under FlbB and FlaI Control

DOUGLAS H. BARTLETT,† BETSY B. FRANTZ,‡ AND PHILIP MATSUMURA*

Laboratory of Cell, Molecular and Developmental Biology, Department of Biological Sciences, University of Illinois at Chicago, P.O. Box 4348, Chicago, Illinois, 60680

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The regulation of the expression of the operons in the flagella-chemotaxis regulon in *Escherichia coli* has been shown to be a highly ordered cascade which closely parallels the assembly of the flagellar structure and the chemotaxis machinery (T. Iino, Annu. Rev. Genet. 11:161–182, 1977; Y. Komeda, J. Bacteriol. 168: 1315–1318). The master operon, *flbB*, has been sequenced, and one of its gene products (FlaI) has been identified. On the basis of the deduced amino acid sequence, the FlbB protein has similarity to an alternate sigma factor which is responsible for expression of flagella in *Bacillus subtilis*. In addition, we have sequenced the 5′ regions of a number of flagellar operons and compared these sequences with the 5′ region of flagellar operons directly and indirectly under FlbB and FlaI control. We found both a consensus sequence which has been shown to be in all other flagellar operons (J. D. Helmann and M. J. Chamberlin, Proc. Natl. Acad. Sci. USA 84:6422–6424) and a derivative consensus sequence, which is found only in the 5′ region of operons directly under FlbB and FlaI control.

Over 3% of the *Escherichia coli* K-12 genome is concerned with the synthesis, assembly, and function of its flagella (21). It is therefore not surprising that the regulation of the assembly of the flagellar apparatus is highly coordinated and controlled at least in part by the sequential expression of known flagellar operons. *fla-lacZ* fusion studies have revealed a hierarchy of transcription beginning with genes whose products are required for the formation of the basal body apparatus and hook, followed by the flagellar filament itself, and ending with the motility and chemotaxis machinery (21).

Expression of the flagellar gene cascade begins with the *flbB* operon, consisting of the *flbB* and *flaI* genes (23, 35, 36). These genes are required for the expression of all the remaining flagellar genes. Hence, the *flbB* operon is believed to serve a master regulatory role (37). There is evidence that other genes may be important for flagellar gene expression farther down the cascade (19, 22).

The production of the flagellum is under positive control from the pleiotropic regulatory protein cyclic AMP (cAMP) receptor protein (CRP) (also called catabolite-activated protein) (1, 41). Therefore, similar to other operons which are catabolite repressed, flagellar genes are not expressed in the presence of glucose (37). Constitutive flagellar synthesis mutations which are insensitive to catabolite repression have been mapped to the *flbB* locus in *E. coli* and to the analogous locus in *Salmonella typhimurium* (24, 36). Presumably, in the presence of sufficient cAMP, a CRP-cAMP complex binds to a region near the *flbB* operon, thereby inducing its transcription. In addition, the flagellar genes are expressed optimally at 34°C and not at 42°C (30). Mutations which result in the synthesis of flagella at high temperatures map to two loci: the *flaD* gene and the *flbB* operon (37).

The regulation of groups of operons in an ordered cascade often involves the participation of an alternate sigma factor which binds to RNA polymerase, displaces the major sigma factor, and changes the sequence specificity of RNA polymerase. Alternate sigma factors are activated in response to specific environmental signals (13, 16). These groups of operons regulated by alternate sigma factors enable the bacteria to cope with environmental stress. The *E. coli* flagellar-chemotaxis system is in the category of specific responses to environmental changes, and the FlbB protein shares similarities with *Bacillus subtilis* σ^{28}, which is the sigma factor responsible for flagellation in *B. subtilis* (15). It has been recently suggested by Helmann and Chamberlin (14) that in *E. coli* and *S. typhimurium* the flagellar-chemotaxis genes are also controlled by an alternate sigma factor.

In this study, we have sequenced the *flbB* operon and identified the gene product of the *flaI* gene. Comparison with the sequence of *B. subtilis* σ^{28} (15) indicates that a carboxy-terminal region of FlbB shares homology with a highly conserved region found in all sigma factors. In addition, we have identified a highly conserved consensus sequence in the 5′ regions of several flagellar operons which have been shown to be under direct positive regulation by the products of the *flbB* operon.

MATERIALS AND METHODS

The principal bacterial strains, bacteriophages, and plasmids used in this study are presented in Table 1. Bacteria were grown in L broth (9). Solid media contained 1.7% agar, except motility medium, which contained 0.38% agar (Difco Laboratories, Detroit, Mich.). Antibiotics were purchased from Sigma Chemical Co., St. Louis, Mo. The antibiotic concentrations used for the selection of resistant transformant colonies were as follows: penicillin, 100 μg/ml; kanamycin, 50 μg/ml; and chloramphenicol, 25 μg/ml. Incubations were carried out at 37°C, except for the complementation of *fla* mutants, which was performed at 30°C (34).

The cloning, transformation, and plasmid purification pro-

* Corresponding author.
† Present address: The Agouron Institute, La Jolla, CA 92037.
‡ Present address: Department of Chemistry, Northwestern University, Evanston, IL 60201.

TABLE 1. *E. coli* K-12 strains, phages, and plasmids used in this study

Strain, phage, or plasmid	Relevant genotype and comments[a]	Source or reference
E. coli K-12		
C600	*lacY*	3
JM103	M13 host	28
YK4131	*flbB*	23
YK4136	*flaI*	23
Phages		
M13mp8	DNA sequencing vector	28
M13mp9	DNA sequencing vector	28
Plasmids		
pMK2004	Cloning vehicle	20
pPM61	*flbB flaI*	This study
pDB20	*flbB flaI*Δ	This study
pMS9	ptrp expression vehicle	29
pRL31	placUV5 expression vehicle	R. Linzmeier[b]

[a] The complete genotypes are given in the references cited.
[b] R. Linzmeier, M.S. thesis, University of Illinois, Chicago, 1982.

cedures have been described previously (4, 27). Restriction endonucleases were purchased from Amersham Corp., Arlington Heights, Ill., or Bethesda Research Laboratories, Inc., Gaithersburg, Md. Exonuclease III and S1 nuclease were purchased from Bethesda Research Laboratories. The procedure for exonuclease III–S1 nuclease-generated deletions has been described previously (4). Minicells were prepared and labeled as previously described (4, 27).

The use of the M13 phage derivatives mp8 and mp9 has been described previously (28). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) were purchased from Sigma. The DNA sequencing protocol was similar to that of Biggin et al. (5). The nucleotide mixes were purchased from P-L Biochemicals, Milwaukee, Wis. [α-^{35}S]dCTP was purchased from New England Nuclear Corp., Boston, Mass. M13 primer and DNA polymerase I (Klenow fragment) were purchased from New England BioLabs, Inc., Beverly, Mass.

RESULTS

Localization of the *flbB* operon. The *flbB* operon has been previously found to reside on the Clarke and Carbon plasmid pLC38-14 (8, 27). This plasmid originates from a chromosome bank of *E. coli* K-12 strain CS520, which has no known flagellar gene defects. It contains the *flbB* and *mochA* operons and is 20 kilobases in size. The *flbB* and *flaI* genes were further localized by subcloning a 2.9-kilobase *Bam*HI-to-*Pst*I fragment from pLC38-14 into the *Bam*HI and *Pst*I sites of the cloning vector pMK2004. The resultant plasmid, designated pPM60, has both *flbB* complementation activity and *flaI* recombination activity. Addition of an adjacent *Pst*I fragment resulted in a plasmid, pPM61, which complemented both *flbB* and *flaI* (Fig. 1). It was later shown by DNA sequence analysis that the *Pst*I site in pPM60 is in the *flaI* coding region. Therefore, pPM60 lacks the last 106 bases of the *flaI* gene. Furthermore, it was shown that the 1.5-kilobase region between *Pst*I and *Hin*dIII was capable of complementing *flbB* mutations (Fig. 1).

Deletion of *flbB* and *flaI*. Because it was originally observed that expression of *flbB* and *flaI* from their normal promoter was not sufficient to identify their gene products in radiolabeled minicells (27), *flbB* and *flaI* were cloned into a high-level expression vector. We obtained in vitro-generated deletions with exonuclease III and S2 nuclease to create DNA fragments which could be used for fusing and positioning *flbB* and *flaI* in a high-level transcription vector. The plasmid pDB20 contains the *flbB* gene and the *flaI* gene up to the *Pst*I site and was used for nuclease digestions (Fig. 2A). Figure 2B shows the extent of a family of deletions, the presence or absence of the complete coding region of *flbB*, and the presence or absence of the *flaI* coding region up to the *Pst*I site. A number of these deletions were sequenced so that the exact ends could be mapped.

Identification of the *flaI* gene product. *Eco*RI-*Pst*I fragments of various lengths obtained from the in vitro nuclease treatment were cloned into an expression vector by using the *lac UV5* promoter. One such construction, pDB40, is shown in Fig. 3A. In this plasmid, the *Eco*RI-*Pst*I fragment with deletion endpoint 4 was used. This construction fused the *lac* promoter 36 bases from the translational start of *flaI*. pDB40 and its derivatives were used to program the synthesis of radiolabeled proteins in minicells (Fig. 3B). pDB40 codes for 30- and 18-kilodalton (kDa) proteins. The 30-kDa protein corresponds to the kanamycin resistance protein, and the 18-kDa protein corresponds to a truncated *flaI* coding region. In a *Pvu*II-*Pvu*II deletion which removes *flaI* recombination activity, no protein other than the 30-kDa kanamycin resistance protein is made. A *Pst*I-*Pst*I fragment which restores the 106 bases in the 3′ end of *flaI* and the 5′ end of the ampicillin resistance gene was inserted into pDB40 to create pDB40-P. This plasmid encodes the 30-kDa kanamycin resistance protein, the ampicillin resistance protein, and a new 22-kDa protein. The 22-kDa protein corresponds to the FlaI protein predicted by DNA sequence analysis. Two *Eco*RI-*Pst*I DNA fragments which have endpoints 70 (deletion no. 9) and 167 (deletion no. 10) bases 5′ to the first in-frame start codon were used in an attempt to overexpress *flbB* or *flbB* and *flaI* together. Neither of these constructions resulted in the visualization of the FlbB or FlaI protein with the minicell system.

DNA sequence analysis. Having localized *flbB* and *flaI* by subcloning and complementation analysis, we sequenced the region of interest (Fig. 4). Two open reading frames of 348 and 576 bases were found which corresponded to the *flbB* and *flaI* genes, respectively. The longest possible open reading frame for *flbB* would be 357 bases if a GTG (base 265) was used instead of the first ATG (base 274) as the start codon. A second possible ATG start for *flbB* exists at 370. A good match to the consensus ribosome-binding site (22) is

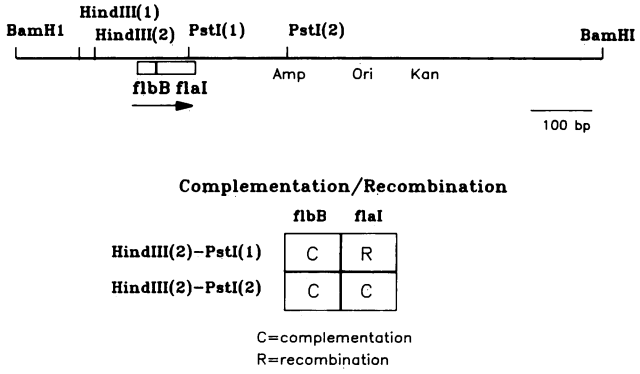
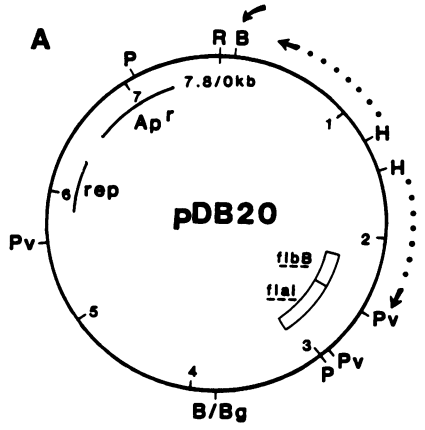


FIG. 1. Localization of *flbB* and *flaI* on plasmid pPM61. Symbols: →, direction of transcription; □□, approximate coding regions for *flbB* and *flaI*.

not found in front of either of the first two in-frame ATG codons. The second open reading frame starts 2 bases past the putative *flbB* coding region. This coding region, contrary to *flbB*, has a good match to the consensus ribosome-binding site. This *flaI* coding region predicts a gene product of the same size (22 kDa) that was found on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

At the 3′ end of the operon, a sequence corresponding to a rho-independent terminator was found 51 bases past the stop codon for *flaI* (10). This region contains a 9-base inverted repeat separated by 6 bases and followed by a string of 8 thymidines. The *mocha* operon is the next adjacent operon and starts 127 bases past the end of *flaI* (10).



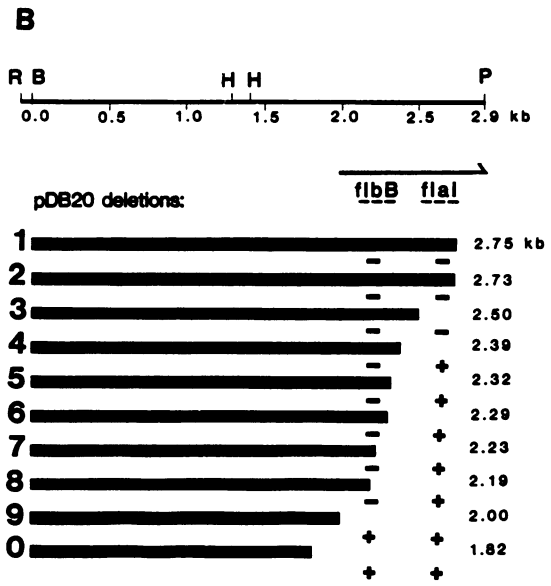
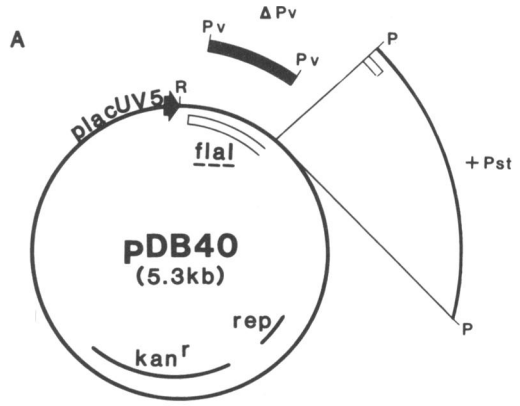


FIG. 2. (A) Strategy for in vitro generation of deletions going up to and into the *flbB* operon. Symbols: ·····, nuclease activity of exonuclease III extending 3′ to 5′ from the two *Hin*dIII sites; →, *Bam*HI site cleaved after the exonuclease III treatment; □□, *flbB* and *flaI*. (B) Representation of in vitro-generated deletions. Symbols: ▬, the amount of DNA removed from pDB20; →, position of the *flbB* operon. The numbers to the right indicate the kilobases of DNA lost. The presence and absence of the complete coding region for *flbB* or the coding region of *flaI* up to the *Pst*I site are indicated by the plus and minus signs, respectively. Abbreviations: R, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; Pv, *Pvu*II; P, *Pst*I; Bg, *Bgl*II.



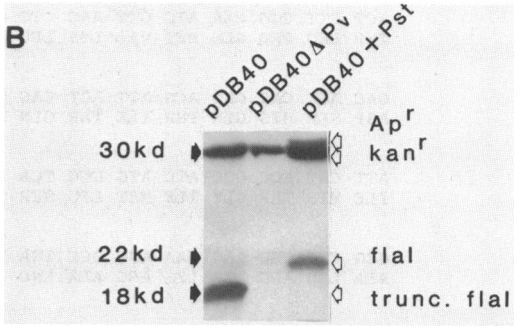


FIG. 3. Overexpression and identification of the *flaI* gene product. (A) pDB40 is represented by the complete circle. Symbols: ▬, extent of the *Pvu*II deletion which generated pDB40ΔPv (the *Pst*I fragment which reconstructed the 3′ portion of the *flaI* gene is also shown); □, coding region of the *flaI* gene. (B) Autoradiogram. The solid arrows indicate the estimated protein molecular masses, and the open arrows distinguish specific labeled protein bands.

At the 5′ end of the operon, a potential CRP-cAMP binding site was identified. This sequence is centered approximately 40 bases in front of the first in-frame ATG codon and contains the highly conserved GTGA sequence found in other known CRP-cAMP binding sites (11).

5′ DNA sequence of flagellum-related operons. It has been previously noted that a conserved sequence is found in front of all flagellar operons that have been sequenced (14). We obtained the sequences of the 5′ regions of three more operons, *flaB*, *flaG*, and *flbB* (Fig. 5). Both the *flaB* and *flaG* operons are in transcription level 3 of the flagellar expression cascade (Fig. 6) and therefore are under direct *flbB* and *flaI* control (21). These two operons also display the consensus sequence previously described by Helmann and Chamberlin (14) at the putative −10 region (GCCGATAA), but not at the putative −35 region (TAAA). The *flbB* operon is in level 1, but also displayed the single putative −10 region consensus sequence. The addition of the two transcription level 3 operons to the previously sequenced level 3 operon, *flaA* (26), allowed the identification of a larger 13-base consensus sequence, TT(A/T)GCCGATAACG, which was found only in level 3 operons, and the *meche* operon, which has not been assigned to a transcription level. This consensus sequence was positioned 29 or 30 bases in front of the putative start codons.

DISCUSSION

The assignment of the *flbB* and *flaI* genes to the specific sequences is based upon several criteria. (i) The plasmid

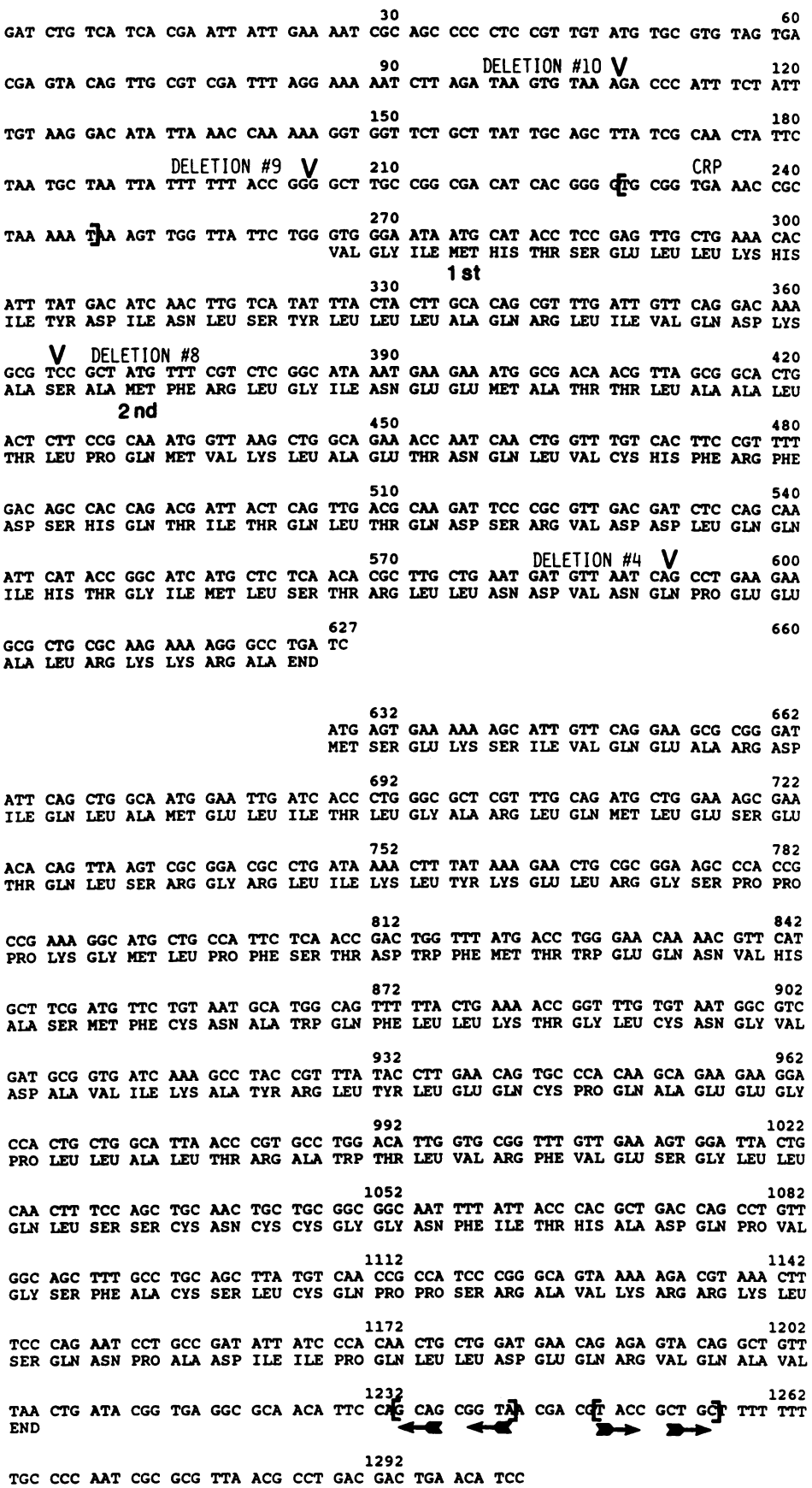


FIG. 4. DNA sequence of the *flbB* operon. Along with the DNA sequence, the derived amino acid sequence of the FlbB and FlaI proteins is presented. The translated products are shown for both the *flbB* and *flaI* gene products as initiating at the first possible ATG or GTG. The deletion endpoints for pDB20 deletions 4, 8, 9, and 10 are indicated. The first and second possible *flbB* translation initiation sites are also indicated. A possible CRP-cAMP binding site is shown in brackets. An inverted repeat, indicating a possible termination signal, is shown with arrows and brackets.

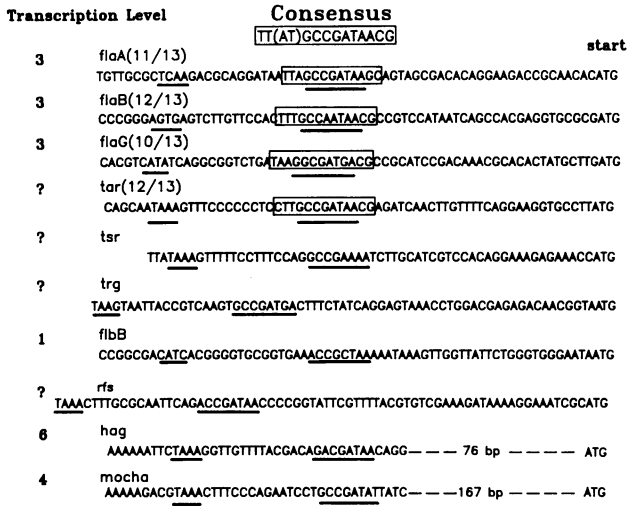


FIG. 5. DNA sequence comparison of the 5′ portion of *E. coli* flagellum-related operons. Sequences preceding the proposed coding regions are aligned from the first in-frame ATG start codon. The boxed sequences in the 5′ regions indicate similarity to the boxed consensus sequence. The 8-base underlined sequences are similar to −10 sequences of operons regulated by σ^{28} of *B. subtilis*. The 4-base underlined sequences are separated from the 8-base underlined sequences by 15 bases. Sequence data were adapted from the following references: *flaA* from reference 28 and J. Malakooti and P. Matsumura, unpublished results; *flaB* from B. Frantz and P. Matsumura, unpublished results; *flaG* from M. Cho and P. Matsumura, unpublished results; *tar* from reference 27; *tsr* from reference 7; *trg* from reference 6; *mocha* from reference 11; *rfs* and *hag* from reference 40; and *flbB* from this study.

pPM61 will complement both *flbB* and *flaI* in a *recA* background. (ii) The *Pst*I-*Bam*HI fragment in pPM60 complemented *flbB* only. (iii) The size of the *flaI* gene product which has been overproduced by using the *lacUV5* promoter agrees with the size predicted on the basis of its DNA sequence. The conclusion that FlaI is the gene product which has been overproduced is based upon the fact that a *Pvu*II deletion within *flaI*, which prevented the overexpression plasmid from recombining with *flaI* mutants, also prevented the expression of the truncated FlaI protein. Restoring the remaining 3′ terminus of the *flaI* gene to pDB40 resulted in the production of a larger protein. The increase in size corresponded to the extent predicted by the DNA sequence of the *flaI* region. (iv) Slocum and Parkinson (38) have previously reported the existence of *Pst*I, *Pvu*II, and *Sma*I sites within the *flaI* gene. All of these sites have been found in our *flaI* DNA sequence as well. (v) The adjacent *mocha* operon has been sequenced (10), and the start of the first gene, *motA*, lies just 126 bases downstream from the terminator codon for *flaI*. (vi) There are no significant open reading frames upstream from *flbB*. We have sequenced over 300 bases upstream from the *flbB* open reading frame and found stop codons in all three frames. The largest open reading frame is only 120 bases in length.

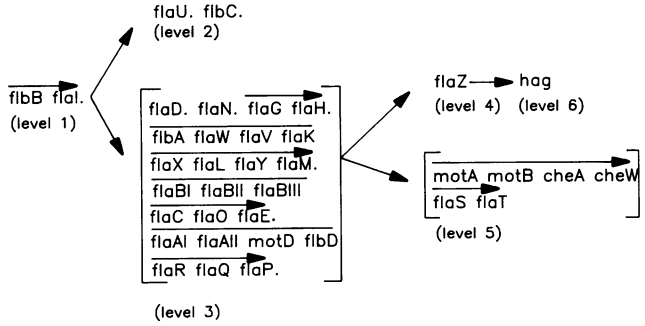


FIG. 6. Transcription map described by Komeda (21). Arrows over genes indicate direction of transcription. The periods indicate the extent of the transcription unit. Arrows between transcription units indicate that transcription of one level requires transcription of another level.

The CRP that participates in the transcription of many other genes (11) also is required for the expression of the *flbB* operon (36). A possible CRP-cAMP-binding site lies upstream from *flbB*. From the known CRP-cAMP-binding sites, the consensus AANTGTGANNTNNNNCANATT has been deduced by de Crombrugghe et al. (11). Of this consensus, the GTGA sequence at positions 5 to 8 is the most invariant and is found at a position 43 bases in front of the first in-frame start codon for *flbB* (Fig. 4).

Ikemura has noted that a strong positive correlation exists between tRNA content in *E. coli* and the occurrence of their respective codons (18). Furthermore, the frequency with which genes contain codons corresponding to the most abundant tRNA species is closely correlated with the amount of gene product produced. Using the codon preferences cited by Ikemura, we have found the frequency of optimal codon usage for *flbB* (from bases 274 to 621) and for *flaI* (from bases 627 to 1202) to be 0.55 and 0.66, respectively. This places the *flbB* gene product in the low-level category and the *flaI* gene product in the middle-level category. The *flbB* optimal codon frequency is between that of the *araC* and *trpR* genes, which also encode products that regulate gene transcription (12, 18, 39). Because the *flaI* gene begins only 2 bases beyond the termination of *flbB*, the possibility exists that the two genes are translationally coupled (2, 31). In this way, despite the better codon usage found in *flaI*, both *flbB* and *flaI* could be expressed in equimolar amounts.

The expression of the flagellar operons and the assembly of the flagellar components has been shown by Iino to be a highly organized sequence of events in which the hierarchical expression of these operons is paralleled by the assembly of the products of these regulated operons (17). Komeda has demonstrated a transcriptional hierarchy which defines the order of positive regulation in this regulon (21, 22). In this scheme, there are six levels of positive regulation (Fig. 6). The *flbB* operon is the first (level 1) to be expressed, and the *hag* gene is the last (level 6). Level 2 and 3 operons require the expression of the *flbB* operon, and level 4 and 5 operons are positively regulated by a factor from the level 3 operons.

The accompanying manuscript (15) and the previous observation of the similarity between the 5′ regions of operons regulated by FlbB-FlaI and *B. subtilis* σ^{28} (14) present a very persuasive argument for a sigmalike role for FlbB. Since FlbB has homology to σ^{28} in a region thought to be a DNA-binding region for all sigma factors and since the 5′ regions of operons under the control of FlbB-FlaI and σ^{28} are very similar, it seems likely that at least FlbB is an example of a sigmalike regulator. The homology unique to FlbB and σ^{28} and not found in the other sigma factors may reflect the similarity in their putative DNA target sites (Fig. 4) (15). The FlaI protein may function together with FlbB as an alternate sigma factor as suggested by Helmann et al. (15), although it is still possible that FlaI has a different mode of regulation.

We have expanded the list of sequences of 5′ regions of

operons which are under the direct control of FlbB and FlaI to include the *flaB* and *flaG* operons. These operons include the conserved region at the putative −10 region pointed out by Helmann and Chamberlin (14), but not the TAAA consensus at the putative −35 region. Since the putative −35 regions of the FlaB and FlaG operons do not conform to the TAAA consensus, they may represent a regulatory subclass in the group of level 3 operons. Interestingly, a six-of-eight match with the putative −10 region consensus was found in the 5′ region of the *flbB* operon, but no match was found at the putative −35 region. This observation raises the possibility that FlbB and/or FlaI feedback regulate the *flbB* operon. Also worth noting is the position of the match to the putative −10 and putative −35 regions in the *mocha* operon. These matches are within the coding region for the *flaI* gene and are separated from the beginning of the *mocha* operon by the stem-loop structure found at the end of the *flbB* operon.

To explore the possibility that FlbB and FlaI have specificity for operons directly under FlbB-FlaI control, we looked for a consensus sequence specific to operons in the level 3 operons defined by Komeda (21). We found the consensus TT(A/T)GCCGATAACG to occur only in the three operons in level 3 for which we have information and not in operons known to be in other levels. In these three operons, this consensus was always found to begin at a position 28 or 29 bases in front of the putative start codon. Although a subset of this consensus is present in other flagellar operons, only the level 3 operons and the *tar* operon, which has not yet been assigned to a level in the hierarchy, share the 13-base consensus, TT(A/T)GCCGATAACG. The specificity of the larger consensus sequence for only level 3 operons may indicate that FlbB and FlaI recognize this consensus as a target. Another possibility is that FlbB and FlaI bind to all flagellar operons (14), whereas other regulatory proteins bind to the larger consensus and are responsible for the sequential expression. The observation of Helmann and Chamberlin (14) that most, if not all, flagellar operons share a consensus sequence of TAAA at the putative −35 region and GCCGATAA at the putative −10 region supports this second possibility.

In addition to the *flbB* and *flaI* genes, other flagellar genes are candidates for regulators of different levels of control of flagellar gene expression. *flaD* and *flaU* appear to be positive and negative regulators, respectively, for the expression from the *mocha* operon (19, 22), and *flaZ* is required for expression of the *hag* gene (21). It will be interesting to see whether 5′ regions of the operons regulated by these gene products also display consensus sequences.

ACKNOWLEDGMENTS

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