Subdivision of flagellar region III of the *Escherichia coli* and *Salmonella typhimurium* chromosomes and identification of two additional flagellar genes

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The many genes involved in flagellar structure and function in *Escherichia coli* and *Salmonella typhimurium* are located in three major clusters on the chromosome: flagellar regions I, II and III. We have found that region III does not consist of a contiguous set of flagellar genes, as was thought, but that in *E. coli* there is almost 7 kb of DNA between the filament cap gene, *fliD*, and the next known flagellar gene, *fliE*; a similar situation occurs in *S. typhimurium*. Most of this DNA is unrelated to flagellar function, since a mutant in which 5.4 kb of it had been deleted remained fully motile and chemotactic as judged by swarming on semi-solid agar. We have therefore subdivided flagellar region III into two regions, IIIa and IIIb. The known genes in region IIIa are *fliABCD*, all of which are involved in filament structure and assembly, while region IIIb contains genes *fliEFGHIJKLMNOPQR*, all of which are related to formation of the hook (basal-body)-complex or to even earlier assembly events. We have found that *fliD*, the last known gene in region IIIa, is immediately followed by two additional genes, both necessary for flagellation, which we have designated *fliS* and *fliT*. They encode small proteins with deduced molecular masses of about 15 kDa and 14 kDa, respectively. The functions of *FliS* and *FliT* remain to be determined, but they do not appear to be members of the axial family of structural proteins to which *FliD* belongs.

Introduction

The flagellar gene systems of *Escherichia coli* and *Salmonella typhimurium* (subscripts E and S are used to distinguish *E. coli* and *S. typhimurium* genes or gene products) are closely similar and quite complex, consisting of over 40 genes organized into a regulon with a master operon and at least 14 subsidiary operons whose length varies from one to nine genes (Komeda, 1986; Kutsukake *et al.*, 1988). Extensive mapping studies have established that they are tightly clustered on the

chromosome (Fig. 1) (for references, see Macnab, 1987*a, b*). One major cluster (flagellar region I) is located at around 24 min. The other two major clusters (regions II and III) are located at around 40–43 min and are separated by about 22 kb of DNA (estimated from the restriction data of Kohara *et al.*, 1987) that includes genes unrelated to flagellar function, such as *uvrC*. [In *S. typhimurium* there is also a small cluster of three genes at 56 min, which includes the phase-2 flagellin gene and related control genes (Yamaguchi *et al.*, 1984).]

Within a given region it was believed from detailed genetic analysis that only flagellar, motility and chemotaxis genes exist. This belief has since been supported by molecular analysis in the cases of region I of *S. typhimurium* (Jones *et al.*, 1989; Homma *et al.*, 1990*a, b*; Gillen & Hughes, 1991; K. Kutsukake, personal communication) and region II of *E. coli* (Krikos *et al.*, 1983; Dean *et al.*, 1984; Mutoh & Simon, 1986; Stader *et al.*, 1986; Bartlett *et al.*, 1988; Kim, 1989; Kofoed & Parkinson, 1991), which have been cloned and sequenced in their entirety.

Region III has also been extensively characterized. In

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The nucleotide sequence data reported in this paper have been submitted to GenEMBL (*fliD*_S, accession no. X51740) and GenBank (*fliDST*_E, accession no. M85240; *fliST*_S, accession no. M85241).

Fig. 1. Flagellar and related genes of *E. coli* and *S. typhimurium*, and their organization on the chromosome. They fall into three major regions, I, II and III. Each of these was thought to consist of a contiguous set of genes, all in some way connected with flagellation, motility or chemotaxis, but the present study has shown that region III has a large disruption between *fliD* and *fliE* (vertical arrow). 0/100 (min) represents the arbitrarily assigned start and end of the circular map at the *thr* locus (Sanderson & Roth, 1988; Bachmann, 1990). Operons are shown by overlining, with an arrowhead indicating the polarity where this is known. *S. typhimurium* has a small fourth region, *fliAB hin*; the *tap* gene is absent in *S. typhimurium*, and *fliB* has not been established for *E. coli*.

S. typhimurium it contains 18 genes, *fliABCDEFGHIJ-KLMNOPQR*, in that order. [In *E. coli*, the *fliB* gene (which encodes an enzyme for methylating flagellin; Konno *et al.*, 1976) has not been established.] *fliC_S* (the structural gene for the filament protein, flagellin) was the first flagellar gene to be sequenced (Joys, 1985; Kuwajima *et al.*, 1986), and subsequently *fliA*, *fliB*, *fliD*, and all of the genes from *fliF* through *fliR* were sequenced in at least one of the two species, and in many cases in both (Kuo & Koshland, 1986; Jones *et al.*, 1989; Kihara *et al.*, 1989; Malakooti *et al.*, 1989; Homma *et al.*, 1990a; Ohnishi *et al.*, 1990; Vogler *et al.*, 1991; K. Kutsukake, personal communication; R. M. Macnab, unpublished data; P. Matsumura, personal communication).

This left *fliE* as the last known region III gene to be characterized. In preparing to analyse it, we found that restriction sites for plasmids containing DNA to the right of *fliD* and for plasmids containing DNA to the left of *fliF* – all of which were expected to contain *fliE* information – could not be reconciled. This was true of both *E. coli* and *S. typhimurium*, and raised the question of whether these three genes were in fact contiguous.

The earlier literature contains several observations concerning flagellar region III that could not be fully interpreted with the information available at the time,

but are consistent with there being additional DNA in the vicinity of *fliC–fliE*. These observations include the occurrence of deletion endpoints between known genes (Yamaguchi *et al.*, 1972; Silverman & Simon, 1974), disparate co-transduction frequencies (Silverman & Simon, 1973a), and the unexpectedly large size of a cloned insert of the region (Silverman *et al.*, 1976).

We have therefore re-examined the arrangement of genes within this part of flagellar region III, and found that there is indeed a substantial extent of DNA between *fliD* and *fliE*, most of which can be deleted without affecting flagellar function. We describe these results here, along with a characterization of the region between *fliD* and the start of this non-flagellar region. The detailed characterization of *fliE* has been described elsewhere (Müller *et al.*, 1992).

fliD constitutes a transcriptional unit separate from all other known flagellar genes, and was thought to be monocistronic. However, since it was originally discovered using mutants generated by Mu phage insertion (Komeda *et al.*, 1980), and no complementation analysis of missense mutants was described, the data do not preclude the possibility of additional genes in the operon. We have in fact noted previously (Homma *et al.*, 1990a) that the sequence immediately beyond the 3' end of *fliD_S* suggests the existence of another gene within the same operon. We have now established that *fliD* is followed by two additional flagellar genes located just before the non-flagellar region.

Methods

Bacterial strains. *E. coli* strains used in this study are listed in Table 1. JA11, kindly supplied by J. Asaka, is a non-revertible *fliC_E* mutant, in which the 5' half of the *fliC_E* coding region has been deleted and replaced by the kanamycin resistance gene (J. Asaka, personal communication). We refer to this genotype as $\Delta fliC_E::kan$.

Plasmids. pIK1001 and its derivatives are pBR322-based plasmids with an *E. coli* chromosomal fragment containing the *fliC–F_E* region; they are described in Results. pMH83 and its derivatives are pBR322-based plasmids with an *S. typhimurium* fragment containing *fliC_S*, *fliD_S* and about 3 kb beyond (Homma *et al.*, 1985). pOYA1 is pUC119-based and contains a 6.7 kb *EcoRI* fragment of *S. typhimurium* region III chromosomal DNA (Müller *et al.*, 1992). Plasmid pUC4K, carrying a *kan* gene cassette, was obtained from Pharmacia/LKB.

Chemicals and enzymes. All chemicals and enzymes were obtained from standard commercial sources. Antibiotics were used at the following concentrations: ampicillin, 50 µg ml⁻¹; kanamycin, 25 µg ml⁻¹; tetracycline, 10 µg ml⁻¹. *SalI* linker, and *EcoRI* and *XhoI* linkers, were purchased from Pharmacia/LKB and New England Biolabs, respectively. Random primer and [α -³²P]dCTP [6000 Ci µmol⁻¹ (222 TBq µmol⁻¹)] were purchased from Boehringer-Mannheim and Amersham, respectively.

Manipulation of DNA. Isolation of chromosomal and plasmid DNA, manipulation of DNA, and transformation of host cells were all carried out by standard procedures, mainly based on Sambrook *et al.* (1989).

Table 1. *E. coli* strains used in this study

Strain	Relevant genotype	Reference
CES200	F ⁻ <i>recB21 recC22 sbcB15 sbcC hsdR</i>	Wyman <i>et al.</i> (1985)
DH5 α	F ⁻ <i>recA1 fla⁺</i>	Gibco/BRL
JA11	F ⁻ Δ <i>fliC_E::kan hsdR hsdM</i>	J. Asaka, personal communication
MS111	<i>fliF</i> of MS1350	Silverman & Simon (1973b)
RP4191	<i>fliE</i> of W3623(λ)	Kondoh (1977); Parkinson <i>et al.</i> (1983)
RP4501	<i>fliG</i> of RP437	Parkinson <i>et al.</i> (1983)
YK1101	<i>fliD</i> of YK410	Komeda <i>et al.</i> (1980, 1984)
YK4104	<i>fliA</i> of YK410	Suzuki & Komeda (1981)
YK4146	<i>fliC</i> of YK410	Komeda <i>et al.</i> (1980, 1984)

DNA sequencing and sequence analysis. DNA sequencing was performed on fragments cloned into M13 phage using the dideoxy chain-termination method (Sanger *et al.*, 1977). Both strands were sequenced in their entirety. Deletions of restriction fragments were obtained by *ExoIII* digestion using standard protocols (Sambrook *et al.*, 1989). Custom primers were synthesized by a facility in the Department of Chemistry, Yale University. Routine sequence manipulations were carried out using the personal computer package DNANALYZE (Greg Wernke, University of Cincinnati Medical School). Sequence comparisons were made against the NBRF protein database (version 24.0; March, 1990) using the FASTA algorithm and the GenBank DNA database (version 67.0; March, 1991) using the TFASTA algorithm.

Motility assay. Motility of cells was detected as swarming on semi-solid agar plates (containing antibiotics where appropriate) at 30 °C. Motility and flagellation were also observed by high-intensity dark-field light microscopy (Macnab, 1976).

Minicell analysis. Gene products were identified by radiolabelling of minicells carrying plasmids of interest, by the method described by Homma *et al.* (1985).

DNA hybridization. For preparing DNA probes, restriction fragments were isolated, denatured, and labelled with [α -³²P]dCTP by means of random-primed DNA synthesis (Feinberg & Vogelstein, 1983). Chromosomal DNA was isolated, digested with restriction enzyme, subjected to 0.7% agarose gel electrophoresis, and transferred onto nitrocellulose membrane (Sambrook *et al.*, 1989). Hybridization with radiolabelled primer was carried out under stringent conditions (incubated in 0.75 M-NaCl, 75 mM-sodium citrate, Denhardt's reagent, 50% v/v, formamide, 0.1% SDS, 10% dextran sulphate and 0.1 mg salmon sperm DNA ml⁻¹, 42 °C, 12 h; washed in 15 mM-NaCl, 1.5 mM-sodium citrate, 0.1% SDS, 60 °C, 2 h) and detected by autoradiography.

Results

Cloning of the *fliC*-*F* region of *E. coli*

Restriction mapping data and other evidence had led us to suspect that *fliD* and *fliE* might be separated by a considerable distance (see Introduction). We elected to investigate the issue initially in *E. coli* rather than *S. typhimurium*, because it had already been established that the entire set of genes from *fliC_E* through *fliF_E* lay within a single *EcoRI* restriction fragment (Silverman *et al.*, 1976).

As the source of the chromosomal DNA, we used an *E. coli* K12 derivative, JA11, which is wild-type for all flagellar genes except *fliC_E*; that gene has been altered by a 5'-deletion and insertion of a *kan* cassette at the site of the deletion (Δ *fliC_E::kan*) (J. Asaka, personal communication). The DNA was digested with *EcoRI* and ligated into plasmid pBR322 and the recombinant plasmid pool used to transform the *recA* strain DH5 α . An Amp^r Kan^r Tet^r transformant was shown to contain a plasmid with a 14 kb *EcoRI* insert. This plasmid, designated pIK1001 (Fig. 2), complemented *fliD_E*, *fliE_E* and *fliF_E* mutants, but not *fliA_E*, *fliC_E* or *fliG_E* mutants. Restriction analysis of pIK1001 yielded a map (Fig. 2) that was consistent with existing information in the vicinity of *fliD_E* and *fliF_E* (Bartlett & Matsumura, 1984; G. Kuwajima, personal communication), and indicated a large distance between the two genes. We constructed and characterized various derivatives from pIK1001, some of which are shown in Fig. 2; the restriction and complementation data obtained were again consistent with existing information. Therefore we concluded that, as expected, pIK1001 carries a fragment with the complete *fliC*-*F_E* region of the chromosome (except for the deleted 5' portion of *fliC_E*). A derivative plasmid, pIK1007 (described in more detail below), lacked a 5.4 kb *MluI*-*XbaI* fragment but nonetheless complemented *fliD_E*, *fliE_E* and *fliF_E* mutants (Fig. 2). Thus the existence of an intervening region in the vicinity of *fliE_E* was confirmed. Plasmid pIK1004 (which lacked all of the left-hand part of the insert except for a small portion of *fliD_E*, but retained the right-hand part) complemented *fliE_E* and *fliF_E* mutants, proving that *fliE_E* lies close to *fliF_E* to the right of the intervening region. This has now been further proved by a detailed analysis of the *fliE_E* gene (Müller *et al.*, 1992).

A similar situation exists in *S. typhimurium*. Plasmid pOYA1 (Müller *et al.*, 1992) contains a 6.7 kb *EcoRI* fragment of *S. typhimurium* chromosomal DNA that begins at bp 532 of *fliD_S*, and ends with the complete *fliE_S* gene and the first 74 bp of *fliF_S*. The known extents of the partial *fliD_S* sequence, the complete *fliE_S* sequence, and

Fig. 2. Restriction map and complementation properties of plasmid pIK1001 and deletion derivatives thereof. pIK1001 contains an *EcoRI* fragment of *E. coli* DNA from flagellar region III that spans the genes from *fliC_E* through *fliF_E* and part of *fliG_E*. The approximate location of *fliE_E* close to *fliF_E* was established by the complementation properties of pIK1004; its exact location and its orientation has since been established by sequence analysis (Müller *et al.*, 1992). *fliC_E* has been inactivated by partial deletion and by insertion of a *kan* gene cassette. Restriction sites are indicated as follows: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; M, *Mlu*I; RV, *Eco*RV; Sm, *Sma*I; Sp, *Sph*I; X, *Xba*I; Xh, *Xho*I; multiple instances of a site are indicated by the numbers in parentheses; where a site has been altered, it is shown in brackets. pIK1007 contains a *Sal*I linker. The *Bam*HI-*Mlu*I fragment (hatched box) was used for sequencing the 3' end of *fliD_E* and the region downstream.

the partial *fliF_S* sequence indicate a distance of about 5.0 kb between *fliD_S* and *fliE_S*.

Construction of a mutant with a deletion in the intervening region

At this point, we had established that in region III there was a large distance between two flagellar genes, *fliD* and *fliE*, that had been thought to be adjacent. The next question was whether the intervening DNA consisted of previously unrecognized flagellar genes, or whether it was unrelated to flagellar function. To answer this, we needed to construct by recombination a strain with the region deleted from its chromosome.

We wished to use the kanamycin resistance (*kan*) gene as a selectable marker for the chromosomal replacement. The *kan* insert in *fliC_E* of pIK1001 was first inactivated by excising the DNA from a *Sal*I site within the vector to a *Xho*I site in the *kan* gene, and ligating the compatible cohesive ends. The resulting plasmid pIK1002 (Fig. 2) was then digested with *Mlu*I and *Xba*I, filled with Klenow fragment, and ligated with T4 DNA ligase in the presence of a *Sal*I linker to produce plasmid pIK1007, in which 5.4 kb of the intervening region had been deleted. A 1.3 kb *kan* gene cassette from pUC4K was then inserted into the *Sal*I site to give plasmid pIK1007K (Fig. 3a).

For a control in which a known flagellar gene was deleted and the recombinant strain should therefore be non-flagellate, pIK1003K was constructed as follows. Plasmid pIK1003, derived from pIK1002, lacks a 1.8 kb *Cla*I fragment starting about 0.8 kb after the start of *fliD_E* and as a result fails to complement a *fliD_E* mutant (Fig. 2). An *Acc*I-digested *kan* gene cassette was then inserted into the unique *Cla*I site of pIK1003 to give plasmid pIK1003K (Fig. 3b).

The deleted versions were then transferred onto the *E. coli* chromosome. First, a motile clone of the *rec-BC_E sbcB_E* strain CES200 was selected and named IK20. IK20 was transformed with pIK1007K DNA (Fig. 3a) that had been linearized by cutting at the unique *Eco*RV site, which lies within *fliD_E*. Medium-size, Kan^r colonies were picked and tested for ampicillin resistance and motility. Of 24 Kan^r transformants tested, all were Amp^s, lacked covalently-closed circular DNA when checked by rapid small-scale preparation of plasmid DNA, and swarmed on semi-solid agar plates as rapidly as their wild-type parent, IK20. One clone, IK27, was saved and characterized further. It was found to be well-flagellated and vigorously motile.

IK20 was also transformed with pIK1003K DNA (Fig. 3b) linearized by cutting at the unique *Nde*I site in the vector. Again 24 out of 24 Kan^r clones tested were Amp^s but, as expected from homologous recombination

Fig. 3. Strategy for testing by chromosomal replacement whether the intervening region between *fliD_E* and *fliE_E* is needed for flagellar function. (a) pIK1007K is a *kan* insertion derivative of plasmid pIK1007, which has had about 75% of the intervening region deleted (Fig. 2), from the first *Mlu*I site to the *Xba*I site; it was linearized and used to transform and recombine with the chromosome of strain IK20, to give strain IK27. (b) A similar procedure was used with a control plasmid pIK1003K (a derivative of plasmid pIK1003, which has *fliD_E* and its 3' flanking region deleted) to give strain IK23. Motility of the parental and recombinant strains is shown at the right, and demonstrates that the deleted region in IK27 is unrelated to flagellation and motility.

Fig. 4. DNA hybridization of *Eco*RI digests of chromosomal DNA from strains IK23 and IK27 (Fig. 3). Probes are indicated as: M, chromosomal *Mlu*I fragment in intervening region; C, chromosomal *Cla*I fragment extending from *fliD_E* into intervening region; K, kanamycin resistance gene.

of a defective *fliD_E* allele, they were non-motile. One clone, IK23, was saved for further analysis.

DNA hybridization confirmed that IK23 and IK27 had the desired deletions and *kan* gene insertion. Chromosomal DNA was digested with *Eco*RI and subjected to hybridization using three different radio-labelled DNA probes: (i) probe C, the 1.8 kb *Cla*I(2)–*Cla*I(3) fragment of pIK1002 containing the 3' region of *fliD_E* that had been deleted in pIK1003; (ii) probe M, the 3.5 kb *Mlu*I(1)–*Mlu*I(2) fragment of pIK1002, representative of the intervening region deleted in pIK1007; and (iii) probe K, a 1.3 kb *Eco*RI fragment of pUC4K

containing the *kan* gene. Under stringent hybridization conditions, an *Eco*RI fragment of the IK23 chromosome should be detected by probes M and K but not C, while a fragment of the IK27 chromosome should be detected by probes C and K but not M. The results (Fig. 4) conformed to the expected pattern, demonstrating that the strains had the intended chromosomal replacements.

We conclude that the region between *Mlu*I(1) and *Xba*I of pIK1001 does not contain any flagellar genes. IK27 grew in minimal medium at the same rate as its parent, even after serially subculturing for 7 d; thus the deleted region does not contain any genes that are essential for viability under these conditions. Normal growth was also observed with a *S. typhimurium* mutant, SJW1673 (Kihara *et al.*, 1989), which has a large deletion from *fliC_S* through *fliE_S* and so has lost not only several flagellar genes, but also all of the non-flagellar region.

Characterization of the region immediately beyond *fliD*

The results described thus far allowed us to say that most of the DNA between *fliD* and *fliE* was not needed for flagellation, but left the DNA immediately beyond *fliD* uncharacterized in that regard.

The recombinant mutant IK23 (Fig. 3b) has a chromosomal deletion that extends from within *fliD_E* to beyond the *Mlu*I site that defines the left boundary of the known non-flagellar region. It could therefore be used as a host in complementation experiments in which various plasmids containing the homologous region of *S. typhimurium* DNA were used as the donor, and transformants were checked for their ability to swarm on semi-solid agar plates.

Fig. 5. Ability of various plasmids containing *S. typhimurium* inserts to complement *E. coli* mutants defective in *fliC_E*, *fliD_E* or *fliD_E* and beyond (strain IK23, see Fig. 3b), the purpose being to test whether or not the DNA beyond *fliD* is flagellar-related (*fli?*). Complementation was judged by swarming ability and flagellation. *SalI** represents the site of deletion of a small *SalI*-*SalI* fragment during the original construction of pMH83. The *HindIII*-*SalI** fragment (hatched box) was used for sequencing the region beyond *fliD_S*.

Plasmid pMH83 (Fig. 5) contains about 6 kb of contiguous *S. typhimurium* DNA between its two *SalI* sites; the second site, indicated as *SalI**, represents a deletion of a small *SalI*-*SalI* fragment after which the insert continues for another 1.3 kb. The region up to the *SalI** site contains *fliC_S*, *fliD_S* and 1.7 kb beyond the 3' end of *fliD_S*. Plasmids pMH821 and pMH823, derived from pMH83, retain *fliC_S* and *fliD_S* intact but have had material downstream deleted. The deletion in pMH821 begins at a *MluI* site about 750 bp beyond the terminus of *fliD_S*, while that in pMH823 begins at the *HindIII* site just beyond *fliD_S*. Plasmid pMH822 has an even larger deletion starting at the first *EcoRI* site within *fliD_S*.

pMH83 was able to complement not only mutants with defects in *fliC_E* and *fliD_E*, but also strain IK23 (Fig. 5). In contrast, pMH821 and pMH823 were able to complement the *fliC_E* and *fliD_E* mutants, but not IK23. As expected, pMH822 complemented the *fliC_E* mutant, but not the *fliD_E* mutant. High-intensity dark-field light microscopy (Macnab, 1976) showed that lack of swarming was a consequence of lack of flagellation. Thus the *S. typhimurium* information necessary to restore flagellation to IK23 includes not only *fliD_S* itself but DNA extending beyond it by at least 750 bp. Since the complementation analysis was between *S. typhimurium* as donor and *E. coli* as recipient, we concluded that information necessary for flagellation exists beyond *fliD* in both species, and

therefore proceeded to characterize this part of the chromosome in more detail.

Characterization of *fliD* and its downstream region in *E. coli*

Only a partial sequence of *fliD_E* exists, obtained by Hanafusa *et al.* (1989) in the course of a study of the adjacent *fliC_E* operon; the sequence terminates at the *BamHI* site at bp 460 (Fig. 2). To characterize *fliD_E* and the flagellum-related DNA beyond it, we cloned the 2.3 kb *BamHI*-*MluI* fragment from pIK1001 into M13 phage. Using *ExoIII* deletion derivatives, the fragment was sequenced in its entirety in both directions. We also used a synthetic primer to sequence through the *BamHI* site and verify that our sequence was contiguous to that of Hanafusa *et al.* (1989).

The open reading frame continues for 950 bp beyond the *BamHI* site (Fig. 6) and, on the basis of comparison with *fliD_S* (Homma *et al.*, 1990a), clearly corresponds to *fliD_E*. Following the *fliD_E* coding region there is a gap of 24 bp before an open reading frame of 408 bp (ORF1), a TGATG overlap, a second open reading frame of 363 bp (ORF2), a gap of 77 bp, and a partial open reading frame, [ORF], that extends to the limit of the sequenced fragment. Following ORF2 and extending slightly into [ORF] there is a pronounced inverted repeat sequence.

Fig. 6 (facing page). DNA sequence in the vicinity of the *fliD* operon of *E. coli*, and deduced amino acid sequences of the *fliD_E*, *fliS_E*, and *fliT_E* products. In the text, *fliS* and *fliT* are initially referred to as ORF1 and ORF2. The sequence prior to the *BamHI* site derives from Szekely & Simon (1983) and Hanafusa *et al.* (1989). Start and stop codons, putative -35 and -10 promoter sequences, putative ribosome binding sites (S-D, Shine-Dalgarno sequence) and restriction sites referred to in the text are shown with underlining. Arrows below the non-coding region beyond *fliT_E* mark an inverted repeat that may be a transcription termination sequence for the *fliD_E* operon. [ORF] is a partial open reading frame, unrelated to flagellation, that extends to the *MluI* site in Fig. 2(a).

1 ATTCCCCTA TTAATAAAAA CAATTAACG TAAACTTTGC GCAATTGAG CCGATAACCC CGGTATTCGT TTTACGTGTC GAAAGATAAA AGGAATCGC
 FliD start *fliD-35* *fliD-10* S-D
 1 M A S I S S L G V G S G L D L S S I L D S L T A A Q K A T L T P I S
 101 ATGGCAAGTA TTTTCATCGCT GGGAGTCGGG TCAGGTCTGG ATTAAAGTTC CATCCTTGAT AGCCTCACC CCGCGCAAAA AGCGACGCTA ACCCCCATTT
 35 N Q Q S S F T A K L S A Y G T L K S A L T T F Q T A N T A L S K A
 201 CAAATCAGCA ATCGTCGTTT ACCGCTAAAC TTAGCGCCTA CGGTACGCTG AAAAGCGCGC TGACGACTTT CCAGACCGCC AATACTGCAT TGCTAAAGC
 68 D L F S A T S T T S S T T A F S A T T A G N A I A G K Y T I S V T
 301 CGATCTTTTT TCGGCCACCA GCACCACCAG CAGCACCACC GCGTTTCAGT CCACCACTGC GGGTAACGCC ATCGCCGGGA AATACACCAT CAGCGTCACC
 101 H L A Q A Q T L T T R T T T D D T K T A I A T S D S K L T I Q Q G G
 401 CATCTGGCGC AGGCGCAAAC CCTGACCAGC CGCACCACCA CAGACGATAC GAAACGCGC ATCGCCACCA GCGACAGTAA ACTCACCATT CAACAAGGCG
 135 D K D P I T I D I S A A N S S L S G I R D A I N N A K A G V S A S
 501 GCGACAAAGA TCCGATTACC ATTGATATCA GCGCGGCTAA CTCATCGTTA AGCGGATCC GTGATGCCAT CAACAACGCA AAAGCAGGCG TAAGCGCAAG
BamHI
 168 I I N V G N G E Y R L S V T S N D T G L D N A M T L S V S G D D A
 601 CATCATTAAC GTGGTAACG GTGAATATCG TCTGTACGTC ACATCAAATG ACACCGGCTT TGATAATGCG ATGACACTCT CGGTACAGCG TGATGATGCG
 201 L Q S F M G Y D A S A S S N G M E V S V A A Q N A Q L T V N N V A I
 701 CTACAAAGTT TTATGGGCTA TGACGCCAGT GCCAGCAGCA ACGGTATGGA GGTCTCGGTT GCCGCCGAGA ATGCGCAGCT GACAGTCAAC AACGTCGCCA
 235 E N S S N T I S D A L E N I T L N L N D V T T G N Q T L T I T Q D
 801 TCGAGAACAG CAGCAACACC ATCAGCGACG CGCTGGAAAA CATCACCTG AACCTGAACG ATGTCACCAC GGGCAACGAG ACCTAACCA TCACTCAGGA
 268 T S K A Q T A I K D W V N A Y N S L I D T F S S L T K Y T A V D A
 901 CACCTCCAAA GCACAACCG CGATTAAGA CTGGGTGAAT GCCTACAAC CGCTAATAGA TACCTTCAGC AGCCTGACCA AATACACCGC CGTAGATGCG
 301 G A D S Q S S S N G A L L G D S T L R T I Q T Q L K S M L S N T V S
 1001 GGAGCTGATA GCCAGAGTTC TAGCAATGGT GCACTGCTCG GCGACTCCAC GCTGCGGACG ATTCAGACGC AGTTGAATC GATGCTGAGT AATACCGTCA
ClaI
 335 S S S Y K T L A Q I G I T T D P S D G K L E L D A D K L T A A L K
 1101 GTTCTTCCAG CTATAAACG TTGGCGCAGA TTGGTATCAC GACCGATCCC AGCGATGGA AACTGGAAT GGATGCCGAC AAACCTACCG CTGCACTGAA
 368 K D A S G V G A L I V G D G K K T G I T T T I G S N L T S W L S T
 1201 AAAAGATGCC AGCGGCGTAG GTGCATTGAT TGTGGGCGAT GGTAAAAA CCGGCATCAC GACCACCATC GGCAGCAACC TGACGAGTTG GCTTTCGACA
 401 T G I I K A A T D G V S K T L N K L T K D Y N A A S D R I D A Q V A
 1301 ACGGGCATT TTAAGCCGC TACCGATGCG GTTAGTAAGA CCCTGAATAA ATTAATAA GACTACAAC CCGCCAGCGA TCGCATTGAT GCGCAGGTCG
 435 R Y K E Q F T Q L D V L M T S L N S T S S Y L T Q Q F E N N S N S
 1401 CTCGCTACAA AGAACAATTT ACCCAACTGG ACGTTTAAAT GACCTCGTTA AACAGCACCA GCAGCTACTT AACGCAGCAG TTCGAAAACA ACAGTAATTC
 FliD end *fliS* start
 468 K * S-D M Y A A K G T Q A Y A Q I G V E S A V M S A S
 1501 CAAGTAAGCA ATATTCATCG GGAGACAGGT CATGTACGCG GCAAAAGGCA CCCAGGCCA TGCACAAAT GGCCTCGAAA GCGCCGAAT GAGCGCCAGC
 24 Q Q Q L V T M L F D G V L S A L V R A S L F M Q D N N Q Q G K G V S
 1601 CAGCAGCAGC TGGTCACCAT GCTATTGAT GGAGTCTGA GCGACTGGT TAGAGCGAGC CTGTTTATGC AGGACAACAA TCAGCAAGGC AAAGGCGTCT
 58 L S K A I N I I E N G L R V S L D E E S K D E L T Q N L I A L Y S
 1701 CTTTGTCAAA AGCGATCAAC ATCATTGAGA ACGGACTGCG GGTGAGTCT GATGAAGAGA GCAAAGACGA ACTAACCCAA AACTTGATTG CTCTTTATAG
 91 Y M V R R L L Q A N L R N D V S A V E E V E A L M R N I A D A W K
 1801 CTATATGGTC AGGCGCTTGC TGCAAGCCAA TTTACGCAAC GATGTCTCCG CAGTCGAAGA AGTGGAAGCA TTAATGCGCA ATATTGCCGA TGCTGGAAA
 1 S-D *fliS* end/*fliT* start
 124 E S L L S P S L I Q D P V * M N H A P H L Y F A W Q Q L V E K S Q L
 1901 GAGTCGTTAC TCTCCCTTC TTTGATTGAG GACCCAGTCT GATGAACCAT GCACCGCATT TATATTTGCG CTGGCAACAA CTCGTCGAAA AAAGCCAGCT
 21 M L R L A T E E Q W D E L I A S E M A Y V N A V Q E I A H L T E E
 2001 CATGTTACGC CTGGCAACGG AAGAACAATG GGACGAATC ATCGCCAGCG AAATGGCGTA TGTGAATGCG GTGCAGGAGA TTGCACATTT GACTGAAGAG
 54 V D P S T T M Q E Q L R P M L R L I L D N E S K V K Q L L Q I R M D
 2101 GTTGACCCGT CCACCACGAT GCAGGAGCAG CTCCGCCGGA TGCTGCGCCT GATTCTGAC AACGAAAGCA AGGTAAAGCA GTTATTACAG ATTCGGATGG
 88 E L A K L V G Q S S V Q K S V L S A Y G D Q G G F V L A P Q D N L
 2201 ATGAACCTGGC GAAACTGGTC GGTACGTCAT CGGTGCAAAA ATCGGTGTTA AGTGCCTATG GCGATCAGGG CGGCTTTGTG CTGGCTCCCG AGGATAACCT
 FliT end [ORF] start
 121 F * M R N P T L
 2301 CTTTGAATC TGAATGAGTC GATGGCTCGC GAATAATCCG ATTACGGCTA CGCTTCTAAT GTTCCCTTGG AATGGAGTCG AAGAATGCGT AATCCACGC
 -----> ----> --> <--- S-D <---<-----

Fig. 7. DNA sequence in the vicinity of *fliS* and *fliT* of *S. typhimurium*, and deduced amino acid sequences of their products. The complete sequence of *fliD_S*, and the sequence beyond it to the *Hind*III site shown, was known (Homma *et al.*, 1990a). Start and stop codons, putative -35 and -10 promoter sequences, putative ribosome binding sites (S-D, Shine-Dalgarno sequence) and restriction sites referred to in the text are shown with underlining. Arrows below the non-coding region beyond *fliT_S* mark an inverted repeat that may be a transcription termination sequence for the *fliD_S* operon. [ORF] is a partial open reading frame, unrelated to flagellation and closely similar to [ORF] of *E. coli* (Fig. 6), that extends to the *Sal*I* site in Fig. 5.

Consideration of the sequence beyond *fliD_E* will be postponed until the corresponding sequence of *S. typhimurium* has been described in the following section.

Characterization of the region downstream of *fliD* in *S. typhimurium*

The sequence of *fliD_S* has already been described (Homma *et al.*, 1990a), along with a small amount of 3' flanking sequence up to the *Hind*III site shown in Fig. 5. We have now subcloned the *Hind*III-*Sal*I* fragment of pMH83 and sequenced it, and also sequenced through the *Hind*III site of pMH83 to establish contiguity.

The DNA following *fliD_S* (Fig. 7) exhibits a similar pattern to that for *E. coli*: a gap of 14 bp following the *fliD_S* coding region, an open reading frame of 405 bp (ORF1), a TAATG overlap, a second open reading frame of 366 bp (ORF2), a gap of 71 bp containing a pronounced inverted repeat sequence, and a partial open reading frame, [ORF], that extends to the limit of the sequenced fragment.

The deduced amino acid sequences of the partial open reading frame, [ORF], in the two species are very similar (14% difference for the N-terminal 139 residues for which sequence is available in both species vs. 23% difference at the nucleotide level; data not shown), arguing that it does indeed correspond to the 5' portion of a gene. Since its disruption does not impair flagellation or motility, it is presumably not part of the flagellar gene system, and is being investigated separately (M. Raha and R. M. Macnab, unpublished data).

Identification of ORF1 and ORF2 as flagellar genes

In both species, ORF1 and ORF2 have strong potential ribosome binding sites, and were assessed as coding regions at the 96% confidence level by the program TESTCODE (Fickett, 1982). The fact that the deduced amino acid sequences for corresponding open reading frames in the two species (i.e. ORF1_E vs. ORF1_S, and ORF2_E vs. ORF2_S) show significant identity (see below)

Fig. 8. Identification of FliS_S and FliT_S produced in radiolabelled minicells transformed with the plasmids shown, which contain a *SalI* fragment that covers the *S. typhimurium* *fliCDST* region. pVM500 contains the wild-type version, pVM501 and pVM502 have in-frame linker insertions into *fliS*_S (to generate products FliS' and FliS*), and pVM503 has *fliT*_S truncated. Flagellar proteins and molecular mass markers are indicated.

further supports the conclusion that they are authentic coding regions.

We already knew that ORF2 played a role in flagellar structure or assembly, based on the complementation properties of pMH821 (Fig. 5), in which it is truncated at the *MluI* site (Fig. 7). However, the complementation behaviour of pMH823 did not permit any conclusion concerning ORF1, since ORF2 was also lacking. We wished therefore to disrupt ORF1 without affecting ORF2. This was done by cloning the *SalI* fragment of pMH83 into pBR322 and inserting an in-frame *XhoI* linker into the *Eco47III* site in ORF1 to give pVM702. We also generated a plasmid, pVM703, in which ORF2 was truncated as a result of deletion from the *MluI* site in ORF2 through to a *SphI* site in the vector. pVM702 and pVM703 both complemented mutants JA11 ($\Delta fliC_E::kan$) and YK1101 (*fliD_E*), but were unable to complement IK23. Failure to complement as judged on swarm plates was subsequently shown by high-intensity dark-field light microscopy to stem from failure to restore flagellation.

We conclude that both ORF1 and ORF2 are necessary for flagellation and show below that they are expressed genes. In the revised nomenclature for the flagellar genes of *E. coli* and *S. typhimurium* (Iino *et al.*, 1988), genes in former region III were assigned the symbol *fli* followed by extensions *A*, *B*, etc. The last known *fli* gene being *fliR*, we have named the two newly discovered genes *fliS* and *fliT*, respectively.

Identification of the *fliS* and *fliT* gene products

We next wished to verify that *fliS* and *fliT* were expressed and to identify their products. Plasmid pVM500 is a pUC18-based plasmid containing the *SalI* insert of *S. typhimurium* DNA from pMH83, which includes *fliCDST_S*. From it, we constructed three derivatives: pVM501 and pVM502 with in-frame *EcoRI* and *XhoI* linkers, respectively, inserted into the *Eco47III* site in *fliS_S*; and pVM503 with *fliT_S* truncated at the *MluI* site. These plasmids were used to identify the products of *fliS_S* and *fliT_S* in a minicell expression system (Fig. 8). Proteins corresponding to FliC_S and FliD_S were detected with all four plasmids as a poorly resolved doublet at an apparent molecular mass of about 52 kDa. With pVM500, two further proteins were seen with apparent molecular masses of about 12 and 11 kDa, respectively. With pVM501 and pVM502, the 12 kDa band was replaced by one with a higher apparent molecular mass; with pVM503, the 11 kDa band was absent. We conclude that *fliS_S* and *fliT_S* were being expressed, with the 12 kDa and 11 kDa bands corresponding to FliS_S and FliT_S, respectively.

Deduced sequences of the FliS and FliT proteins

FliS of *E. coli* and *S. typhimurium* show a moderate degree of conservation (60% identity) uniformly distributed throughout the sequence (Fig. 9b). FliT is rather

Fig. 9. Comparison of the sequences of (a) FliD, (b) FliS and (c) FliT proteins in *E. coli* and *S. typhimurium*. Bars indicate positions of identity. Colons indicate conservative substitutions, defined as G/A, I/L/F/M/V, F/W/Y, Q/N, S/T, D/E and H/R/K. Residue numbers apply to the *E. coli* sequence. In (a), the arrow above R154 of FliD_E indicates the start of the sequence obtained in the present study; the N-terminal sequence up through residue 153 is from Szekely & Simon (1983) and Hanafusa *et al.* (1989). The FliD_S sequence is from Homma *et al.* (1990a). Vertical arrows in the C-terminal region indicate hydrophobic heptad repeats that had been noted previously in the *S. typhimurium* sequence and are conserved in the *E. coli* sequence.

weakly conserved by comparison with other flagellar proteins whose sequences have been described in both species, with an overall identity of only 34% (Fig. 9c). The N-terminus is especially divergent, with the first 71 residues showing only 23% identity.

The deduced molecular masses of the proteins are 14933 Da (FliS_E), 14670 Da (FliS_S), 13813 Da (FliT_E) and 13689 Da (FliT_S), in reasonable agreement with the apparent molecular masses found in the minicell analysis. Neither FliS nor FliT are predicted to have a preponderance of any single secondary structural motif, nor do their hydropathy profiles suggest that they are integral membrane proteins (data not shown). Both are moderately acidic. Although their general properties are similar to each other, they do not appear to be related to each other at the sequence level. Nor are they similar to any of the other flagellar proteins whose sequence is known. Finally, no obvious homologues emerged in comparisons against the NBRF protein database or the translated GenBank database.

FliD is a member of the family of proteins that constitute the axial structure and are exported by the flagellum-specific export pathway (Macnab, 1990). The sequences of the proteins in this family have a number of features in common (Homma *et al.*, 1990a, b). None of these were evident in FliS or FliT.

Comparison of FliD of *E. coli* and *S. typhimurium*

The deduced amino acid sequence of FliD_E and the previously published sequence of FliD_S (Homma *et al.*, 1990a) are compared in Fig. 9(a). Overall the two are moderately similar (52% identity), with the termini showing the highest degree of similarity. The central region is quite divergent, except for a short region of identity around residue 158.

FliD_S has various properties, which are thought to be important in terms of inter-subunit interactions in the axial structure (Homma *et al.*, 1990a, b). These are shared by FliD_E. (i) Unlike the other axial proteins, FliD lacks a consensus ANNLAN motif in its N-terminal region. (ii) A partial match (SNL) to a consensus SGL motif near the N-terminus of other axial proteins was seen in FliD_S and is seen as the full consensus in FliD_E. (iii) The N-terminal region lacks the hydrophobic heptad repeats that are seen in the other axial proteins. (iv) The N-terminal region contains proline residues, unlike the other axial proteins. (v) The central region contains proline residues, as is the case with the other axial proteins. (vi) The C-terminal region lacks proline residues, as is also true of the other axial proteins. (vii) The C-terminal region contains hydrophobic heptad repeats, as is true of the other axial proteins; the repeats

are in phase when the FliD_E and FliD_S sequences are aligned by the algorithm of Lipman & Pearson (1985).

Discussion

In spite of the large amount of classical genetics that has gone into an understanding of the flagellar gene system, analysis at the molecular level can still produce surprises. The results of the present study illustrate this point, since they require that the long-standing view of one of the major flagellar regions of the chromosomes of both *E. coli* and *S. typhimurium*, region III, be substantially modified.

Sub-division of flagellar region III

Region III of the flagellar regulon of *E. coli* and *S. typhimurium* contains a large intervening region, between *fliD* and *fliE*, that had not been recognized until now. Most of it is unnecessary for flagellar structure, assembly or function, since a mutant with a 5.4 kb deletion was still well-flagellated and motile. The mutant also swarmed normally, indicating that chemotaxis was unimpaired. In view of this, we have redefined flagellar region III as two regions, IIIa and IIIb. Region IIIa contains the genes *fliABCD* and also two newly discovered genes, *fliS* and *fliT*; region IIIb contains the genes *fliEFGHIJKLM-NOPQR* (Fig. 10).

The 5.4 kb *MluI*-*XbaI* fragment of *E. coli* must constitute almost the entire non-flagellar region, which probably begins immediately after the end of *fliT* (about 500 bp before the *MluI* site), and can extend no further beyond the *XbaI* site than 700 bp, where the 3' end of the leftward facing *fliE_E* gene is found (Müller *et al.*, 1992). Thus its total extent is probably about 6.6 kb. In *S. typhimurium*, the corresponding estimate is lower, about 4.2 kb; the reason for this difference is not clear.

Regions IIIa and IIIb, though separated, are still very close together on the overall scale of the chromosome. We suspect that they (and possibly region II also) originally constituted a contiguous region and that chromosomal rearrangements, which must have occurred prior to the divergence of *E. coli* and *S. typhimurium*, resulted in their interruption.

It is interesting that regions IIIa and IIIb differ in the types of genes and operons they contain. The four previously known genes in region IIIa (Fig. 10) are all, in one way or another, related to the flagellar filament protein, flagellin: *fliA* encodes a flagellum-specific sigma factor for the flagellin operon and other late operons (Ohnishi *et al.*, 1990); *fliB_S* (not established in *E. coli*) encodes an enzyme that post-translationally modifies flagellin (Konno *et al.*, 1976); *fliC* encodes flagellin itself (Silverman & Simon, 1973a; Horiguchi *et al.*, 1975); and

Fig. 10. Current knowledge concerning the *fliC-fliF* portion of former flagellar region III of *E. coli*, which on the basis of this study has been subdivided into regions IIIa and IIIb with the *fliD* and *fliE* operons separated by a large distance; a similar situation exists for *S. typhimurium*. Operons are indicated by arrows, with gene boundaries within them indicated by vertical bars. In region IIIa, *fliC* is preceded by *fliA* and (in *S. typhimurium*) *fliB*; in region IIIb, *fliF* is followed by *fliGHIJKLMNOPQR* (cf. Fig. 1). *fliS* and *fliT* are newly discovered flagellar genes, which represent a continuation of the *fliD* operon. The first gene in region IIIb is *fliE*, whose precise location and polarity has been determined recently (Müller *et al.*, 1992). [ORF] is a partial open reading frame that appears to be unrelated to flagellar function and extends to the limit of the sequenced fragment at the *MluI* site shown. Between the *MluI* and *XbaI* sites there is 5.4 kb of DNA, which can be deleted without affecting flagellar structure or function. It represents the minimum extent of the non-flagellar region, which probably extends from the 3' end of *fliT* to the 3' end of *fliE* (hatched line).

fliD encodes the filament capping protein (Homma *et al.*, 1985), which is essential for filament assembly. The two newly discovered region IIIa genes, *fliS* and *fliT*, are of unknown function but apparently belong to the same operon as *fliD* (see below) and so may be functionally related.

In contrast, the 14 genes in region IIIb are involved in structures assembled earlier than the flagellar filament: For example, *fliE* encodes a basal-body protein (Müller *et al.*, 1992); *fliF* encodes the basal-body M-ring protein (Homma *et al.*, 1987); *fliG*, *fliM* and *fliN* encode the flagellar switch proteins (Parkinson *et al.*, 1983; Yamaguchi *et al.*, 1986); and *fliI* is an ATPase homologue that may be involved in the process of export and assembly of flagellar proteins (Vogler *et al.*, 1991). Although the specific function of many of the other region IIIb genes is unknown, the mutant phenotype is generally a lack of any detectable flagellar structure.

Thus one can broadly distinguish region IIIa genes (*fliABCDST*) and region IIIb genes (*fliEFGHIJKLMNOPQR*) in a functional sense, as being related to the filament and to structures preceding the filament, respectively.

This is reflected also in the patterns of gene expression. Kutsukake *et al.* (1990) have described the flagellar regulon as a hierarchy, with operons at three levels. Of the four operons in region IIIa, two (*fliC* and *fliD*) definitely belong to class 3 (late flagellar operons) and a third (*fliB*) probably does also, considering that its product is an enzyme that covalently modifies flagellin. The fourth operon (*fliA*), though itself belonging to class 2 (middle flagellar operons), serves to regulate expression of class 3 operons. The three operons in region IIIb, containing *fliE*, *fliF-fliK*, and *fliL-fliR*, respectively

(Komeda, 1986; Kutsukake *et al.*, 1988), all belong to class 2.

The fliD operon and the functions of FliS and FliT

fliD is transcribed separately from other known flagellar genes (Komeda *et al.*, 1980; Kutsukake *et al.*, 1988) and is preceded by a strong flagellar-specific promoter (Szekely & Simon, 1983; Hanafusa *et al.*, 1989; Homma *et al.*, 1990a). The available evidence argues strongly that *fliD*, *fliS* and *fliT* constitute an operon: (i) the three genes are closely spaced or even overlapping; (ii) there are no recognizable promoter sequences prior to *fliS* or *fliT*; (iii) there are no recognizable transcription termination sequences following *fliD* or *fliS*; (iv) there is a substantial non-coding region following *fliT*, and it contains a pronounced inverted repeat sequence, which is likely to represent the transcription termination sequence of the *fliD* operon; (v) disruption of the open reading frame following *fliT* does not affect flagellar function. Detailed analysis of the transcription of the *fliD* operon is in progress elsewhere (K. Kutsukake, personal communication).

The *fliD* gene product is known to be a protein located at the distal end of the flagellar filament, which enables exported subunits of flagellin to assemble rather than being lost to the medium (Homma *et al.*, 1984, 1985; Ikeda *et al.*, 1987).

What about the roles of FliS and FliT? Two lines of evidence suggest that they may be regulatory rather than structural. First, the genes appear to belong in the same operon as *fliD* and should therefore be subject to the same transcriptional control. One would therefore expect their products to be needed at a similar (late) place in the

assembly pathway as FliD (Homma *et al.*, 1984). Yet their deduced amino acid sequences show no resemblance to FliD or to any other members of the axial family (Homma *et al.*, 1990*a, b*). Nor are there any proteins with the molecular masses of FliS or FliT in the external portion of the flagellum, which has been characterized carefully (Ikeda *et al.*, 1987). Thus the evidence argues against *fliS* and *fliT* being structural genes.

Second, the *fliD* operon exerts a poorly understood regulatory effect, such that defects cause over-expression of other late operons (Kutsukake *et al.*, 1990); a repressor-like activity, RflA, has therefore been attributed to this operon. It is not clear how *fliD* itself, as a structural gene, could exert such an effect. If, however, the operon contained regulatory genes, the phenomenon would be easier to understand, at least in principle. Interestingly, we noted that plasmids with *fliD* and *fliS* intact but with *fliT* truncated produced motile transformants on *fliD* hosts much more rapidly than when *fliT* was intact on the plasmid; conversely, if *fliS* was disrupted, transformants appeared much more slowly. This could be a reflection of positive and negative regulatory activities associated with *fliS* and *fliT*, respectively. However, the situation must be more complex than this, since the mutant phenotype associated with *fliT* is non-flagellate.

Defined mutations in *fliD*, *fliS* and *fliT* and an examination of their effects on the levels of expression of late operons are needed to further understand the regulatory role of the *fliD* operon; experiments along these lines are in progress (K. Kutsukake, personal communication). Once the relative contributions of each of these genes to regulation has been established, mechanistic studies can be initiated.

FliD as a member of the axial family of proteins

We have recently analysed the members of a structurally related family of flagellar proteins of *S. typhimurium*, namely those that comprise the flagellar rod, hook, hook-filament junction, filament and filament cap (Homma *et al.*, 1990*a, b*). Of these, the protein of the filament cap, FliD_S, distinguished itself from the rest in that its N-terminal region contained prolines, lacked hydrophobic heptad repeats, and showed little sequence similarity to any of the other members. Its C-terminal region conformed better to the general description provided by the family in that it contained hydrophobic heptad repeats. The sequence of FliD_E reinforces these conclusions.

We had suggested, based on our own results and those of others (Namba *et al.*, 1989; Aizawa *et al.*, 1990;

Vonderviszt *et al.*, 1990), that side-by-side interactions of amphipathic α -helices formed from N-terminal and C-terminal sequence might be important in inter-subunit interactions in the axial structure. We also noted that the unique location of FliD, at the tip of the axial structure, might be the reason for the nonconformity of its N terminal sequence to the patterns seen with all of the other axial proteins. A corollary of this would be that the C-terminal sequence of a given axial protein subunit would be involved primarily in interactions with subunits on its proximal side (i.e. closer to the cell surface) and, conversely, the N-terminal sequence would be involved in interactions with subunits to the distal side (i.e. closer to the tip of the organelle).

The information that was available at the time concerning FliD_E (the N-terminal 153 residues) indicated a rather high degree of divergence from FliD_S, and we postulated this might be because the N-terminus was not constrained by inter-subunit interactions. However, both termini are now seen to be more divergent than in the case of the other two axial proteins where the sequence is known in both species, namely, flagellin (Joys, 1985; Kuwajima *et al.*, 1986) and hook protein (Homma *et al.*, 1990*a*; J. Rosenberg, I. Kawagishi, D. DeRosier & R. Macnab, unpublished data). Thus while it remains true that the N-terminus of FliD has different properties from those of the other axial proteins, it is not significantly less conserved between the two species than is the C-terminus, which does have the characteristics of the other axial proteins.

The internal portion of the sequence of FliD is the most divergent. This has emerged as a general feature of the axial proteins, and is thought to reflect a domain at the outer radius of the axial structure.

Function of the non-flagellar region

Unlike most eukaryotic cells, bacteria have an extremely compact genome and so a region of about 6 kb seems too large to be merely a spacer, and presumably contains several genes. One indication that this is the case is the observation that, shortly beyond the 3' end of *fliT*, there is a partial open reading frame that appears to be the 5' end of an authentic gene, since the deduced amino acid sequences in *E. coli* and *S. typhimurium* show a high level of identity. Investigation of this putative gene and of the remainder of the non-flagellar region is in progress, with results that suggest that at least part of the region is involved in polysaccharide metabolism (M. Raha & R. M. Macnab, unpublished data). It will be interesting to compare the region in *E. coli* and *S. typhimurium* to establish what features have been conserved in the time since the two species diverged.

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