fliU and *fliV*: two flagellar genes essential for biosynthesis of *Salmonella* and *Escherichia coli* flagella

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The possible functions of two recently described flagellar genes, *fliU* and *fliV*, have been examined. Introduction of gene *fliC*, encoding the bacterial flagellin protein, into a number of flagellin-deficient *Salmonella* and *Escherichia coli* strains failed to complement the mutations in these strains, and the FliC flagellin was accumulated in the bacterial cytoplasm. Complementation with *fliU* and *fliV*, which map downstream of *fliC*, restored motility to some of the mutants which became flagellated. After inactivation of either *fliU* or *fliV*, such complementation no longer occurred and the flagellin protein accumulated in the cytoplasm, which suggested that both genes are required for the secretion of flagellin and expression of motility. Expression of these genes from high copy number plasmids resulted in the synthesis of exceptionally long flagella and in detection of the FliV protein on polyacrylamide gels.

Introduction

The bacterial flagellum consists of three distinct parts known as the basal body, the hook and the filament (DePamphilis & Adler, 1971; Macnab, 1987). The filament, which is the main component of the flagellum and is located at the bacterial cell surface, is composed of a major subunit of a single polypeptide called flagellin. Flagellin, like other structural proteins which form the flagellum, has to be translocated from its site of polymerization, within the bacterial cytoplasm, across the cell membrane. The secretion of flagellar proteins via the central channel through the proximal part of the flagellum is regulated, and perhaps even driven, by a specific flagellum exporting pathway (Kuwajima *et al.*, 1989; Homma *et al.*, 1990; Vogler *et al.*, 1991).

Flagella production is subject to catabolite repression (Silverman & Simon, 1974) and has been shown to be co-ordinated with cell division (Nishimura & Hirota, 1989). Genes of flagellar proteins are expressed in a complex transcriptional cascade, which ensures that expression of the genes occurs in the required sequence (Kutsukake *et al.*, 1990; Ohnishi *et al.*, 1990). *lacZ* operon fusions that were used to characterize expression patterns of the flagellar regulon showed that late gene expression is dependent on successful expression of early genes. The

expression of the early genes themselves is dependent on the FlhD and FlhC gene products (for review see Macnab & Parkinson, 1991).

More than 40 genes, grouped at three different chromosomal loci and divided into three transcriptional activity classes (Kutsukake *et al.*, 1990), form the flagellar regulon of *Salmonella typhimurium* and *Escherichia coli*. A fourth locus, comprising operon *fliAB*, is present in biphasic *Salmonella* serotypes (Jones & Macnab, 1990). Class I includes genes *fliH* and *fliC*, which encode positive activators of flagellar gene expression (Komeda *et al.*, 1975). Class II contains basal body and flagellin-specific export pathway genes, in addition to the *fliA* gene, which encodes a flagella-specific sigma factor (Ohnishi *et al.*, 1990) that is responsible for transcribing genes of class III (Kutsukake *et al.*, 1990). Class III contains genes that encode filament structural proteins. These genes are characterized by specific -10 and -35 consensus promoter DNA sequences. Genes of class II, although having diverse -35 promoter DNA sequences, are characterized by the same -10 DNA sequence as genes of class III (Kutsukake *et al.*, 1990).

Gene *fliC*, which maps at 40 min on the *S. typhimurium* chromosome, encodes a flagellin protein of ca. 55 kDa. Recently, we showed that the DNA sequence downstream to gene *fliC* encodes two genes that have been termed *fliU* and *fliV* (Doll & Frankel, 1993). The DNA sequence of the genes suggested that they belong to class II, since their DNA sequences resemble the -10 but not the -35 consensus promoter sequence. In this paper, we

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Table 1. List of bacterial strains and their flagella serotype

Bacterial strain	Strain I.D.	Flagella phenotype (serotype)	Origin/source	Reference
<i>S. dublin</i>	88A5405	+(g.p)	USA	
<i>S. muenchen</i>	88A4490	+(d:1.2)	USA	
<i>S. enteritidis</i>	88A5483	+(g.m)	USA	
<i>S. typhi</i>	EN103	+(j)	Indonesia	Doll & Frankel (1993)
<i>S. typhi</i>	EN108	+(d)	Mexico	Doll & Frankel (1993)
<i>S. dublin</i>	SL5928	—	B. Stocker,	Newton <i>et al.</i> (1989)
<i>S. typhimurium</i> *	SJW1647	—	Dept of	Homma <i>et al.</i> (1987)
<i>S. typhimurium</i> *	SJW2546	—	Medical Microbiology,	Homma <i>et al.</i> (1987)
<i>S. typhimurium</i> *	SJW1728	—	Stanford Univ.,	Homma <i>et al.</i> (1987)
<i>E. coli</i>	CL447	—	CA, USA	Wei & Joys (1985)

* Defined by Homma *et al.* (1987) as export defective mutants having deletions spanning the 3' end of gene *fliC-i*.

Table 2. List of plasmids and origin of the *fliC* locus

Plasmid	Encoded gene/s	Mutated gene	Flagellar phenotype*	Origin
pLS408	<i>fliC</i> , <i>fliU</i> , <i>fliV</i>	None	+	<i>S. muenchen</i> (ATCC8388)
pHJ103	<i>fliC</i>	None	—	<i>S. typhi</i> (EN103)
pHJ104	<i>fliC</i> , <i>fliU</i> , <i>fliV</i>	None	+	<i>S. typhi</i> (EN103)
pHJ105	<i>fliC</i> , <i>fliV</i>	<i>fliU</i>	—	<i>S. typhi</i> (EN103)
pHJ106	<i>fliC</i> , <i>fliU</i>	<i>fliV</i>	+ / —	<i>S. typhi</i> (EN103)
pHD108	<i>fliC</i>	None	—	<i>S. typhi</i> (EN108)
pHD109	<i>fliC</i> , <i>fliU</i> , <i>fliV</i>	None	+	<i>S. typhi</i> (EN108)

* In *S. dublin* SL5928 and *E. coli* CL447.

report further investigation of these genes. It appears that both gene products are required for secretion of the flagellin monomer from the cell cytoplasm to the tip of the growing flagellum, although the precise role these gene products play in flagellum biosynthesis is still not clear.

Methods

Bacterial strains and plasmids. The bacterial strains and plasmids that were used in this study are listed in Tables 1 and 2, respectively.

Transformation of plasmids to the different strains was achieved by the standard CaCl_2 method (Maniatis *et al.*, 1982). Whenever plasmids were moved from *E. coli* to any of the *Salmonella* strains, *S. typhimurium* strain LB5000 (restriction-negative and modification-proficient) (Bullas & Ryu, 1983) was used as mediator.

Media and motility assay. Luria broth (LB) medium and 1.5% solid agar medium were prepared as described (Maniatis *et al.*, 1982). Motility medium contained 0.2% Difco agar, 8% gelatin (BDH) and 2.1% Oxoid nutrient broth no. 2 in 17% LB. Plates (5 cm) containing solidified motility medium (kept at 4 °C) were incubated with the tested bacteria in the centre overnight at 37 °C. Motility was indicated by the ability of the bacteria to spread through the semi-solid medium.

Electron microscopy. Drops from overnight bacterial cultures, grown at 37 °C without shaking, were applied to carbon films that had been mounted directly on grids; the suspensions were then allowed to settle for 1 min before the excess medium was removed using a filter paper. The grids were stained for 1 min with 1% uranyl acetate and then

rinsed individually with distilled water and allowed to dry. Grids were examined using a Jeol 100S transmission electron microscope.

PAGE and immunoblotting. Bacterial protein extracts and culture supernatants were subjected to 10 or 20% PAGE (Laemmli, 1970). The gels were either stained with Coomassie blue or blotted onto nitrocellulose filters. The filters were blocked overnight with phosphate-buffered saline (PBS) containing 10% low fat milk. Polyclonal antibodies, raised against monomeric flagellin, were added and the filters were incubated for 2 h. After three washes with PBS–milk solution, peroxidase-conjugated goat antibodies to rabbit IgG in PBS–milk were added, and incubation was allowed to proceed for an additional hour. The filters were washed three times with PBS before substrate solution (0.05% diaminobenzidine.HCl in 50 mM-Tris/HCl, pH 7.6 and 0.01% H_2O_2) was added. The reaction was carried out for several minutes at room temperature before the filters were washed with water and dried.

Mutagenesis. Restriction and modifying enzymes were purchased from Boehringer Mannheim. Gene *fliU* was mutated by cleavage of pHJ104 with restriction endonuclease *KspI* (see Fig. 6) and removal from the linear plasmid of the protruding 3' terminal nucleotides by T4 DNA polymerase (Maniatis *et al.*, 1982). The blunt-ended plasmid was recircularized by T4 DNA ligase, generating plasmid pHJ105 (Table 2). Gene *fliV* was mutated by deleting the *SalI* fragment, generated after cleavage of the sites present in *fliV* and the pUC9 polylinker (see Fig. 6). The plasmid DNA was extracted from low melting point agarose gel (Maniatis *et al.*, 1982) and recircularized by T4 DNA ligase, generating plasmid pHJ106 (Table 2). The presence of the mutations was confirmed by DNA sequencing of the corresponding regions by the dideoxy chain termination technique (USB Sequenase kit) of Sanger *et al.* (1977).

Polymerase chain reaction (PCR). Chromosomal DNA was extracted as previously described (Frankel *et al.*, 1989). DNA amplification with the various primers (Fig. 6; Table 3) was obtained after 30 cycles of denaturation (95 °C, 20 s) annealing (42 °C, 45 s) and DNA elongation (72 °C, 45 s) (Mullis & Faloona, 1987). Products (10 µl) of the amplification reaction were analysed by agarose gel electrophoresis.

Results

Motility assays of *Salmonella* strains transformed with either pHJ103 or pLS408

Plasmid pLS408 contains a 3.35 kb insert, from *S. muenchen* strain ATCC8388, that includes the *fliC-d* flagellin gene and its promoter, together with genes *fliU* and *fliV* and further downstream sequences (Newton *et al.*, 1989; Doll & Frankel, 1993). Plasmid pHJ103 is a

pUC9 containing the natural *fliC-j* gene and its promoter (1438 bp) of *S. typhi* strain EN103 (Fig. 1). In order to test the ability of pHJ103 to complement the *fliC* lesion

Fig. 1. Structure of plasmids pHJ103 and pHJ104.

Fig. 2. Motility assays of *S. dublin* (SL5928), *S. enteritidis* (88A5483) and *S. muenchen* (88A4490) harbouring plasmids pLS408 and pHJ103. The swarm zone diameter reflects the motility of each strain.

Fig. 3. Electron micrograph of *S. dublin* (88A5404) (a), SL5928 (b), SL5928(pLS408) (c) and SL5928(pHJ103) (d).

in *S. dublin* SL5928, this strain was transformed with either pHJ103 or pLS408. SL5928, which is immobile, became motile if it harboured pLS408. However, SL5928(pHJ103) was found to remain immobile (Fig. 2). Since production of flagellar filaments by mixed flagellins, i.e. by flagellins of two different serotypes, may result in immobilization of the bacteria, both plasmids (pHJ103 and pLS408) were also used to transform *S. enteritidis* (flagellar serotype g.m), *S. muenchen* (flagellar serotype d:1:2) and *S. dublin* (flagellar serotype g.p) wild-type strains. Indirect indication that pHJ103 directs the synthesis of flagellin was evident from motility assays showing that both pHJ103 and pLS408 inhibited the

spread through semi-solid medium of wild-type *S. enteritidis* (Fig. 2) and *S. dublin* (not shown) strains. In contrast, neither plasmid had an influence on the motility of the *S. muenchen* strain (Fig. 2). Electron microscopic examination (Fig. 3a–d) revealed that all motile strains were flagellated. Bacteria harbouring pLS408 had exceptionally long flagella (Fig. 3c), while immobilized strains had no flagella (Fig. 3b, d).

Localization of the pHJ103-encoded flagellin

Bacterial cell extracts and culture supernatants of SL5928, with and without plasmids (either pHJ103 or

Fig. 4. Whole-cell extracts of SL5928 and culture supernatants analysed by PAGE-Coomassie blue staining (a), and by Western blotting using polyclonal antibodies against monomeric flagellin (b). Lanes 1 and 2, SL5928; lanes 4 and 5 SL5928(pHJ103); lanes 6 and 7, SL5928(pLS408). Lanes 1, 4 and 6 contain cell extracts; lanes 2, 5 and 7 contain culture supernatants. Lane 3 contains purified flagellin [sample of large scale flagellin preparation from SL5928(pLS408)].

pLS408), were analysed by immunoblotting after electrophoretic separation in polyacrylamide gels (Fig. 4). No flagellin was observed associated with SL5928, but flagellin was present in both the bacterial cell extract and culture supernatant of SL5928(pLS408). In SL5928-(pHJ103), flagellin of the size expected from gene *fliC-j* was seen only in the bacterial extract but not in the supernatant (even when concentrated). The FliC-j flagellin protein (encoded by pHJ103) migrates faster than FliC-d of pLS408 because of an 87-amino-acid deletion in its hypervariable region (Frankel *et al.*, 1989). Similar results were obtained with SL5928(pHD108). pHD108 (insert of 1699 bp) contains a similar insert to pHJ103 (*fliC-j*), except that its *fliC* gene encodes a different flagellin determinant, antigen d (not shown).

Motility assay and PAGE analysis of SL5928(pHJ104)

Plasmid pHJ104 contains a 2.74 kb insert, from *S. typhi* strain EN103, that includes the *fliC-j* flagellin gene and its promoter together with genes *fliU* and *fliV* (Doll &

Frankel, 1993). SL5928(pHJ104) (Fig. 1) had a similar motile ability and immunoblot pattern (not shown) as that seen with SL5928(pLS408). Coomassie blue staining of whole bacterial lysates separated in 20% polyacrylamide gels revealed an intense protein band of about 20 kDa, which was present in SL5928(pLS408) (Fig. 5a), SL5928(pHJ104) (Fig. 5b) and SL5928-(pHD109) (not shown), but not in the parental strain (Fig. 5a) or in SL5928(pHJ103) (Fig. 5b) or SL5928(pHD108) (not shown). pHD109 (insert of 2999 bp) contains a similar insert to pHJ104, except that its *fliC* gene encodes flagellar antigen d.

Motility assays of bacteria transformed with either pHJ105 or pHJ106 – PAGE and PCR analysis

Plasmid pHJ104 was used to generate mutations in either the *fliU* or the *fliV* genes. The mutated plasmids, pHJ105 and pHJ106 (Table 2) respectively, were used to transform *S. dublin* SL5928. Motility assays revealed that, while inactivation of gene *fliU* (pHJ105) led to

Fig. 5. (a) Electrophoresis on 20% polyacrylamide gels of whole-cell extracts of SL5928 (lane 1) and SL5928(pLS408) (lane 2). The 20 kDa protein, suspected to be the product of gene *fliV*, is arrowed. (b) 20% PAGE of whole-cell extracts: SL5928(pHJ104) (lane 1), SL5928(pHJ103) (lane 2), SL5928(pHJ105) (lane 3), SL5928(pHJ106) (lane 4). The protein which correlates with the presence of plasmid-encoded *fliV* is arrowed.

immobilization of the bacteria, the mutation generated in gene *fliV* (pHJ106) had no effect on motility (not shown). PAGE analysis showed that the 20 kDa protein, seen in SL5928(pHJ104) (Fig. 5b), is present in SL5928(pHJ105) but not in SL5928(pHJ106) cells (Fig. 5b), and therefore is presumably the *fliV* product. Characterization of the mutation present in *S. dublin* SL5928 by PCR, using primers 5 & 6 and primers 7 & 8

(Fig. 6; Table 3), indicated that the DNA sequence of gene *fliV* is present in the bacterial chromosome but gene *fliU* is deleted (Fig. 7a). Other primer pairs (1 & 2, 1 & 3, 4 & 6 and 7 & 8; Fig. 6; Table 3) consistently gave amplification products using wild-type *S. dublin* DNA as template but *S. dublin* SL5928 DNA was successfully amplified using primers 1 & 2 and 7 & 8 only. Accordingly, we mapped the 5' end of the deletion between bp 69 (primer 2) and 406 (primer 3) of *fliC* and its 3' end to the spacer between genes *fliU* and *fliV*.

Motility assays of *E. coli* CL447

E. coli CL447 (also termed *E. coli* C600 *hag*) has a deletion that spans the *fliC* locus (Wei & Joys, 1985). Although the deletion itself has not been defined exactly, transforming this strain with the different plasmids, described above (Table 2), revealed that both genes *fliU* and *fliV* are required for motility, since only pHJ104 and pLS408 (pHD109 was not tested) were capable of complementing the deletion mutation (not shown).

Motility assays and PCR analysis of export-defective *S. typhimurium* mutants transformed with either pHJ103 or pHJ104

pHJ103 failed to complement the deletion mutation of the 3' end of gene *fliC-i* (strain SJW2546) mapped by Homma *et al.* (1987). The plasmid also failed to complement deletions that included downstream DNA sequences (strains SJW1647 and SJW1728), since all strains remained immobilized after transformation. pHJ104 complemented only the deletion of strain SJW1647, but not the deletion of the other two mutants. PCR analysis, using primers 4 & 6 (Fig. 6), revealed that in the three *S. typhimurium* mutants the 3' end of gene *fliC-i* is present, since an amplified fragment of the expected size was generated by all strains, except for the control *S. dublin* SL5928 strain (Fig. 7b).

Fig. 6. Restriction enzyme map of pHJ104 insert and the relative location of primers used for PCR along the DNA sequence.

Table 3. DNA sequences of primers used for DNA amplification and their location along the *fliCUV* DNA sequence described previously (Doll & Frankel, 1993)

Primer no. (Fig. 6)	Sequence	Location (bp)
1	5' TTTGCAAAAATAATGC 3'	133-148
2	5' GTTCAGGTTATTCTGGG 3'	354-370
3	5' GACTTTCACGCCGTTGAA 3'	514-531
4	5' CAGGCCGGTACCTCCGTTTC 3'	1763-1781
5	5' CTGCCTTCGGCGCTGGGGA 3'	2125-2143
6	5' CAACTCCTGCGCCAACA 3'	2574-2591
7	5' TCATTAGCGCGCTGTTTAC 3'	2775-2793
8	5' AGCCCAACCCGAACAAA 3'	3114-3130

Fig. 7. Genetic characterization, by PCR, of *Salmonella* mutants included in this study. (a) Amplification of genes *fliU* (primers 5 & 6; lanes 2 and 3) and *fliV* (primers 7 & 8; lanes 4 and 5) using as templates DNA extracted from *S. dublin* (88A5404) (lanes 3 and 5) and SL5928 (lanes 2 and 4). (b) PCR analysis of the three *S. typhimurium* flagellin-export mutant strains (SJW1647, SJW2546 and SJW1728, respectively shown in lanes 2-4) using primer 4 & 6. SL5928 was used as a negative control (lane 1).

Discussion

In the present study, we have shown that introduction of a cloned flagellin gene (pHJ103) into the flagellin-deficient *S. dublin* strain SL5928 failed to complement the deletion mutation, as was the case in all other bacterial mutant strains included in this study. Evidence that pHJ103 can direct the synthesis of flagellin came from both immunoblots and motility assays with various wild-type *Salmonella* strains. pHJ103 and pLS408 inhibited motility of *S. enteritidis* (*fliC-g.m*) and *S. dublin* (*fliC-g.p*). This might be due to failure to assemble the mixed flagella that would be generated by both types of flagellins. The fact that neither plasmid had an effect on the motility of *S. muenchen* (*fliC-d*) can be explained by the close antigenic structure of all three flagellins and their ability to copolymerize to functional flagella.

fliU and *fliV* are two new genes of the flagellar regulon (encoding proteins of 19 and 20 kDa, respectively) presumably belonging to class II (Doll & Frankel, 1993). We found no homology between these genes and any other known protein listed in the different databanks. By using the flagellin-deficient *E. coli* strain CL447, we have shown that both *fliU* and *fliV* are needed for the biosynthesis of flagella. We identified the product of gene *fliV* after PAGE as a stained band. However, the band was not detected in either wild-type *Salmonella* strains or in *S. dublin* SL5928 (that contains intact *fliV* gene). This is probably due to the fact that, whenever the *fliV* gene product was observed, it was carried on a high copy number plasmid. In contrast, the product of gene *fliU* was not seen by PAGE. In flagellin-deficient bacterial strains harbouring a plasmid bearing flagellin genes (pLS408, pHJ104 and pHJ109), the flagella that were observed by electron microscopy were exceptionally long. This is probably due to a high concentration of expressed flagellin in the cytoplasm.

S. dublin SL5928 was thus far believed to have the flagellin gene inactivated by Tn10 (Newton *et al.*, 1989). We now suggest that, although the strain is still resistant to tetracycline, part of its flagellin gene and downstream DNA sequence are deleted and that Tn10 may not be present within the flagellin gene itself (no PCR product was obtained using forward primer 1 and a reverse primer derived from the IS sequence of Tn10; results not shown). Some *S. typhimurium* mutants have been reported to have deletions in *fliC-i*, but the cells still synthesize full-size flagellin that remains as a cytoplasmic protein. These mutants were termed transport- or export-defective by Homma *et al.* (1987). We have shown by PCR, complementation and motility assays that such mutants do in fact contain the 3' end of the *fliC-i* gene, and therefore might have deletions spanning *fliV* downstream sequences that might encode for additional flagellar export functions.

Flagellar filaments grow at their distal ends (Iino, 1969), with elongation rates decreasing exponentially with length (Iino, 1974). Structural studies have revealed a central canal in the rod, hook and filament through which the flagellin, hook and hook-associated proteins travel to the flagellar tip (Trachtenberg & Derosier, 1987; Namba *et al.*, 1989). A flagellum-specific export apparatus controls this process and, presumably, selects the proteins that are allowed to pass. Since in basal-body-defective mutants, flagellin is not transported to the extracellular space, the products of genes *fliU* and *fliV* may either be part of the basal body structure or might have a role in flagellin processing or flagellin export. The exact function and location of FliU and FliV in the process of flagellar biosynthesis is now being studied.

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