JOURNAL OF BACTERIOLOGY, Sept. 1997, p. 5585–5588 Vol. 179, No. 17
0021-9193/97/$04.00+0

Negative Feedback from a *Proteus* Class II Flagellum Export Defect to the *flhDC* Master Operon Controlling Cell Division and Flagellum Assembly

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Received 23 April 1997/Accepted 23 June 1997

The *Proteus mirabilis* flagellum class I *flhDC* operon was isolated, and its transcript was shown to originate from a σ^{70} promoter 244 bp 5′ of *flhD* and 29 bp 3′ of a putative cyclic AMP receptor protein-binding site. Expression of this regulatory master operon increased strongly as cells differentiated into elongated hyperflagellated swarm filaments, and cell populations artificially overexpressing *flhDC* migrated sooner and faster. A class II *flhA* transposon mutant was reduced in flagellum class III gene expression, as would be expected from the FlgM anti-σ^{28} accumulation demonstrated in *Salmonella typhimurium*, but was unexpectedly also reduced in cell elongation. Here, we show that levels of *flhDC* transcript were ca. 10-fold lower in this flagellum export mutant, indicating that in cells defective in flagellum assembly, there is additional negative feedback via *flhDC*. In support of this view, artificial overexpression of *flhDC* in the *flhA* mutant restored elongation but not class III flagellum gene transcription.

Proteus mirabilis differentiates and displays multicellular behavior during swarming migration over the surface of a rich solid medium. Short motile vegetative rods with few peritrichous flagella differentiate into multinucleate, nonseptate swarm cells of up to 40-fold vegetative cell length with a more than 50-fold greater density of surface flagella. These migrate rapidly away from the colony as multicellular rafts in cycles of differentiation and consolidation (1). A likely fulcrum of swarm cell regulation is the *flhDC* flagellum class I (master) operon, shown in *Escherichia coli* and *Salmonella typhimurium* to be the apex of the three-tier hierarchy of flagellum biogenesis (19), because not only does FlhDC activate transcription of flagellum class II genes and thus the flagellum gene hierarchy (17), but FlhD has recently been shown to repress cell division in *E. coli* (23). Artificial overexpression of *flhDC* increased elongation and hyperflagellation in the swarming bacterium *Serratia liquefaciens* (7).

In a nonmotile mutant of *Proteus* lacking the class II FlhA flagellum export protein (20), expression of class II and III flagellum genes is suppressed (9, 10). This would be expected as a result of the intracellular accumulation of the class III anti-σ^{28} factor FlgM demonstrated in *Salmonella*, i.e., by post-translational inhibition of σ^{28}, a transcriptional activator of class II and III genes (12, 16, 18, 22). Less expected was our finding (9) that the *flhA* mutant did not elongate beyond vegetative cell length. The cause of this was not clear, because the FlgM-mediated negative feedback to class II and III genes is the only mechanism identified by which the flagellum hierarchy is downregulated in cells defective in flagellum assembly. Here, we provide further evidence for the importance of *flhDC* in swarm cell differentiation and indicate an additional level of negative feedback from defective flagellum assembly to class I *flhDC*.

Isolation and characterization of the *P. mirabilis flhDC* operon. *E. coli* MC1000 *flhD*::Tn*10* is nonflagellated and nonmotile because of a transposon insertion in *flhD* (17). After transformation of this strain with a pBluescript II (Stratagene) library of partial *Sau*3AI fragments (3, 21, 24) from the *P. mirabilis* U6450 chromosome, ca. 0.1% of ca. 10,000 colonies were able to swim through 0.3% Luria-Bertani (LB) agar containing 50 μg of ampicillin and 25 μg of kanamycin per ml. A representative complementing plasmid, pGF111, containing a ca. 6.4-kbp chromosomal insert was taken to derive the similarly complementing pRBF1, which carries a ca. 1.5-kbp *Eco*RV fragment (Fig. 1). Both DNA strands of pRBF1 were sequenced (25) with T3 and T7 vector primers and primers complementary to *flhDC* with the T7 sequencing kit (Pharmacia). The DNA and protein sequences (analyzed by Genetics Computer Group software, Wisconsin package, version 9) (6) revealed two complete open reading frames of 351 and 582 bp (Fig. 1) that encode the class I flagellum master operon proteins FlhD and FlhC, respectively, showing 81 and 88% amino acid similarity (68 and 79% identity) to those of *E. coli* (4). Additional 3′ sequencing revealed *motA*, which is also immediately downstream of *flhDC* in other species (2, 19). The *P. mirabilis* 452-bp *flhDC* upstream region has no detectable open reading frames and no clear σ^{28} promoter consensus sequence.

Primer extension was carried out on 50 μg of RNA isolated by the hot phenol method (14) from wild-type cells harvested 1.5, 2.0, and 3.5 h after seeding (9). Oligonucleotides DC7 (5′-CCTCTGCGCCAAAAGCAAATACGATAAA), complementary to nucleotides 42 to 69, and DC9 (5′-GACTCGTA GTAACACTACCTTGTATTACTTG-3′), complementary to nucleotides −143 to −113, from the predicted translational start of FlhD, were 5′ end labelled with [γ-^{32}P]ATP (Amersham) by T4 polynucleotide kinase and extended with Moloney murine leukemia virus reverse transcriptase (Boehringer Mannheim) (24). Extension and sequence reaction products, generated with 5 pmol of primers on pRBF1, were separated on a 7 M urea–6% acrylamide gel and visualized by autoradiography. The results show that the *flhDC* transcript begins 237 bp 5′ of the predicted translation initiation codon (Fig. 2), suggesting transcription from a σ^{70} promoter located 6 bp upstream of the transcription start. There is a putative cyclic AMP (cAMP)-cAMP receptor protein (CRP) binding consen-

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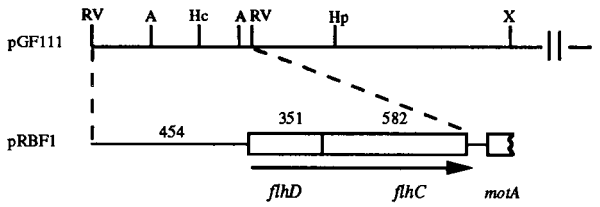
(i)



(ii)

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1    AAAAAATCGTATAAAATAAAAAATTATCATTAATATTTTAATTTGGGTAGATTCGCTTAT
61   TAATTCTCAGCAAGTTTTATTACTAATATTACTTGAATAGTTAATGAACTAATTCCATAA
121  TAATGATTATCTTTTTGTGATGTAACTCACATAGTATTTAAATTAGACTACTTTATCCGT
          -35                  -10
181  TGAATAACTTAATCATTAAGGAGTAAAGTTATCTCGTTAGGATTATTCTTAACGTGATAT
                                  *
241  ATTGGCGAGCATTTAAAATCCTTTTTAATTTAAATGTTTTTCTCGCTACTTTTTGAAATG
301  ATCGTAATCAAGTAATACAAGGTAGTGTTACTACGAGTCATATTCATAAAATGGGGCCTG
361  GAAATTCAATATACATTTATATTAATTTCCCTCGGGATCTTACTTTAAACAAGATCCTCT
421  TTGTTCAATATAATCGGACGGGATGTAAAGAGATGAGTACGGTTGAATTGCTTAAGCATA
                                  M  S  T  V  E  L  L  K  H  I
481  TTTATGACATAAATTTATCGTATTTGCTTTTGGCGCAGAGGTTAATTAACCAAGAAAGG
       Y  D  I  N  L  S  Y  L  L  L  A  Q  R  L  I  N  Q  E  K  A
541  CTTCCGCAATGTTTAGACTGGGTATCAGCGATTCTATGGCTGATGCACTAAAAGAGCTAA
       S  A  M  F  R  L  G  I  S  D  S  M  A  D  A  L  K  E  L  T
601  CATTGCCTCAATTGGTTAAGTTGGCTGAAACAAACCAACTAATCTGTAATTTCCGGTTTG
       L  P  Q  L  V  K  L  A  E  T  N  Q  L  I  C  N  F  R  F  E
661  AAGACAGCGAAACAATAGAACAACTCACTAAAGAATCCAGAGTGGATGATTTGCAACAAA
       D  S  E  T  I  E  Q  L  T  K  E  S  R  V  D  D  L  Q  Q  I
721  TTCATACTGGTATCCTTCTTTCTTCTAACTTGTTCCGTCAACTATCGGAACATGATACCT
       H  T  G  I  L  L  S  S  N  L  F  R  Q  L  S  E  H  D  T  S
781  CTGCTACAAAGAAACGGGCATAACAATGAGTGAGAAAAGTATCGTCCGAGAGGCGAAAGA
       A  T  K  K  R  A  *     M  S  E  K  S  I  V  R  E  A  K  D
841  TATTCGCCTAGCAATGGAATTAATCACCTTAGGTGCTCGCTTACAAATGCTTGAAAGCGA
       I  R  L  A  M  E  L  I  T  L  G  A  R  L  Q  M  L  E  S  E
901  AACGCAATTAAGTCGAGGGCGTTTAATTAAATTATACAAAGAATTAAGAGGAAGCCCTCC
       T  Q  L  S  R  G  R  L  I  K  L  Y  K  E  L  R  G  S  P  P
961  TCCTAAAGGTATGCTACCTTTTTCAACAGATTGGTTTATGACATGGGAACAAAATATCCA
       P  K  G  M  L  P  F  S  T  D  W  F  M  T  W  E  Q  N  I  H
1021 TTCTTCAATGTTTTATAATGCTTACCGCTTTTTATTAAAAAGCGGGGGGAGCGTAGGTAT
       S  S  M  F  Y  N  A  Y  R  F  L  L  K  S  G  G  S  V  G  I
1081 TGAAGCTGTTGTAAAAGCCTATCGTTTATATTTAGAACAATGTCCACCCGTTAAAGATCA
       E  A  V  V  K  A  Y  R  L  Y  L  E  Q  C  P  P  V  K  D  Q
1141 AGAACCTATTTTAGCACTAACACGAGCATGGACATTAGTTCGTTTTGTTGAAAGTGGTAT
       E  P  I  L  A  L  T  R  A  W  T  L  V  R  F  V  E  S  G  M
1201 GTTACAACTTTCTGTATGTACCAAATGTAATGGTTCATTTATTACACACGCACATCAGCC
       L  Q  L  S  V  C  T  K  C  N  G  S  F  I  T  H  A  H  Q  P
1261 TGCAAGTAATTATGTTTGTAGTCTTTGTCAGCCTCCATCTCGGGCAATAAAAAAACGTAA
       A  S  N  Y  V  C  S  L  C  Q  P  P  S  R  A  I  K  K  R  K
1321 ACTTTCCGCCAATCCTGCCGATATTAACTTACAACTGTTGGATGGTCTTGAACAGTTTCG
       L  S  A  N  P  A  D  I  N  L  Q  L  L  D  G  L  E  Q  F  R
1381 AATGTGATTCGAGATTGAAAGTTAAATATCAGGTTATAGATTAAAGACTCTATAACCTGA
       M  *
1441 GTTGTTTCCTCTTCCCCTCCTATCTCTTGCGGTTATGTT
```

FIG. 1. (i) *Proteus mirabilis flhDC* locus on pGF111. A, *Ava*I; RV, *Eco*RV; Hc, *Hin*cII; Hp, *Hpa*I; X, *Xba*I. (ii) Nucleotide sequence and deduced amino acid sequences of the 1,479-bp pRBF1 insert. *, transport start. The putative Shine-Dalgarno ribosome binding site, the −35 and −10 putative promoter elements, and the putative consensus for CRP binding are all underlined. The GenBank accession number is U96964.

sus sequence 29 bp upstream of the *flhDC* promoter. The positive effect of cAMP on *E. coli* flagellum biosynthesis is well characterized (13, 15, 26).

Upregulation of *Proteus flhDC* during swarm cell differentiation. Eberl et al. (7) have shown that the elongation and hyperflagellation characteristics of *Serratia liquefaciens* swarm cell differentiation are increased by artificial overproduction of *flhDC*. This was confirmed in *Proteus* cells carrying *flhDC*, which initiated swarming ca. 40 min earlier than wild-type cells (with or without the plasmid vector alone) and over the first 2 h achieved a surface velocity across 1.5% agar ca. 60% greater than that of the wild type (not shown).

Differentiation in the absence of migration was assessed by spreading high densities of stationary-phase cells onto parallel LB agar plates, resulting in synchronously differentiating cells (9). Northern blotting was carried out on total formamide–formaldehyde-denatured RNA (6 μg), isolated from wild-type cells collected at 0.5-h intervals after seeding, which had been separated electrophoretically in 1.5% agarose formaldehyde gels (24) and transferred to nitrocellulose filters (Hybond C; Amersham) by vacuum blotting (Pharmacia LKB). Following prehybridization in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.4])–5× Denhardt's solution–0.5% sodium dodecyl sulfate containing 100 μg of denatured herring sperm DNA per ml, the RNA was hybridized to a 0.8-kbp *Ava*I probe spanning *flhDC* and labelled with [α-^{32}P]dATP by random priming (Boehringer Mannheim). After stringent washing (0.2% SSPE–0.1% sodium dodecyl sulfate at 50°C), autoradiography revealed a single *flhDC* transcript of about 1,150 nucleotides (Fig. 3) that increased greater than 30-fold at 3 to 4 h after seeding, representing peak differentiation, and that returned to near vegetative levels at 5.5 h. Probing the same RNAs with a 0.6-kbp *Hin*cII coding fragment of the class III gene *fliC* (5) showed the kinetic characteristics of a gene upregulated during swarm differentiation.

Downregulation of *flhDC* in the class II *flhA* mutant. Parallel Northern blots of RNA from the *flhA*Tn*phoA* class II mutant of U6450 (9) identified the same *flhDC* transcript, which increased and decreased in parallel with the parent. However, the level of transcript was ca. 10-fold lower throughout the differentiation cycle. Three repetitions with different RNA samples showed reductions of between 7- and 12-fold.

Overexpression of *flhDC* restores elongation in the *flhA* mutant. The *flhA* class II export mutant was transformed with plasmid pBAD18DC (11), which was constructed by placing *flhDC*, including 60 bp upstream, under the control of the

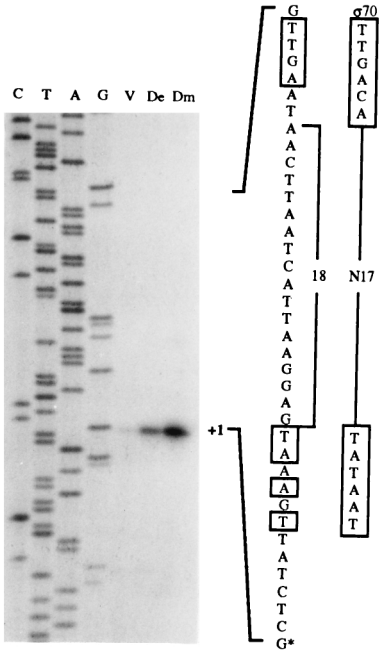

FIG. 2. Primer extension mapping of the 5′ end of the *flhDC* transcript in 50 μg of total RNA. Wild-type early, maximally differentiated, and vegetative cells (De, Dm, and V, respectively) were isolated from seeded LB agar plates at 2, 3.5, and 1.5 h, respectively. C, T, A, and G indicate the sequence lanes. The putative promoter sequence (transcription start is +1) is compared with the consensus σ^{70} promoter.

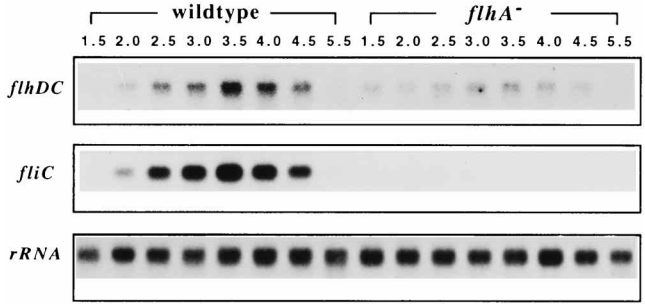

FIG. 3. Northern hybridization of *flhDC* and *fliC* probes to RNA from the wild type and an *flhA* mutant of *P. mirabilis* U6450, sampled over a differentiation cycle. A 16S ribosomal probe cloned as a 0.5-kbp *Hin*dIII-*Eco*RI fragment (data not shown) confirmed that equal quantities of RNA had been loaded in each lane. A 281- to 6,583-base RNA ladder (Promega) was used to determine transcript size. Autoradiographs were photographed with a Kodak DC40 camera, and transcripts were quantified with Kodak Digital Science 1D software.

arabinose-inducible promoter in the vector pBAD18 (8). Overexpression of *flhDC* was induced on 1.5% LB agar containing 0.2% arabinose, resulting in elongation equivalent to or greater than that of wild-type *Proteus* swarm cells (Fig. 4), while the mutant carrying the vector alone generated only short cells. Increased levels of *flhDC* transcript were confirmed by Northern blots (Fig. 4), and hybridization of the RNA to the *fliC* probe demonstrated that this gene, and thus flagellin levels, remained repressed, as would be expected in the presence of FlgM feedback to σ^{28}.

Conclusions. The differentiation of *Proteus* vegetative cells into swarm cells is characterized by the upregulation of flagellar synthesis and assembly and the suppression of cell septation so that long filaments are formed. It seemed likely that *flhDC* would provide an important fulcrum in effecting this change, because in addition to FlhDC controlling transcription of the flagellum hierarchy (17), FlhD has been shown to have a role in the inhibition of cell division (23). By isolating the master operon from *Proteus*, we could show that *flhDC* is indeed strongly upregulated during the change to swarm cells, and we confirmed the observation made with *Serratia* (7) that artificial overexpression enhances hyperflagellation and cell elongation. *Proteus* strains overexpressing *flhDC* also had a shorter lag before migration and a higher migration velocity. Our data strengthen the view that *flhDC* is central to the differentiation process.

We were also able to indicate a novel negative feedback to *flhDC* expression that would explain the loss of cell elongation seen in the class II *flhA* flagellum export mutant. Our finding that we could restore elongation but not the upregulation of the class III flagellum gene, *fliC*, by overexpression of *flhDC* in *trans* is compatible with this view. This novel feedback has not been seen during studies of *Salmonella* flagellum gene expression (15), but it may be less obvious in vegetative cells, as is indeed the case in *Proteus*. Primer extension of the *Proteus flhDC* revealed a transcription start 237 bp 5′ of *flhD*, indicating a σ^{70} promoter 29 bp 3′ of a potential CRP-binding regulatory site. This would suggest that σ^{28} is not involved in the feedback to *flhDC* from defective flagellum assembly.

Nucleotide sequence accession number. The GenBank number for *flhDC* is U96964.

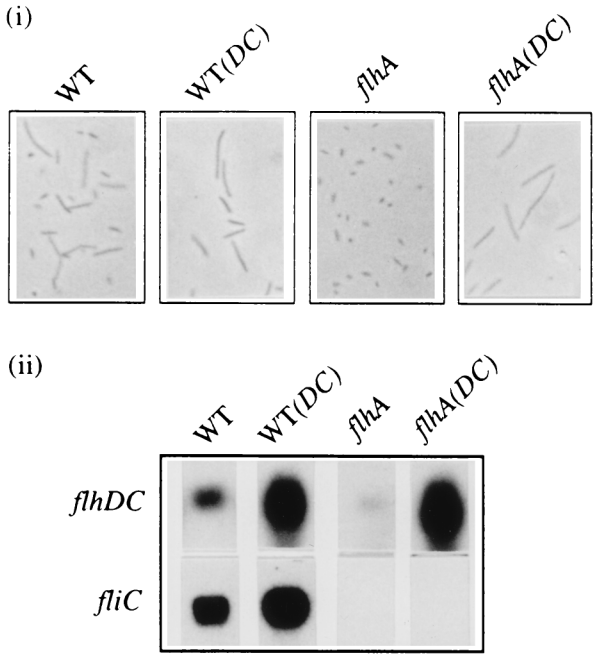

FIG. 4. (i) Complementation of the elongation defect in the class II *flhA* export mutant. Following growth on seeding plates containing 0.2% arabinose for 3.5 h, cells were washed from the surface and photographed at a magnification of ×320. (ii) Northern hybridization with *flhDC* and *fliC* probes to RNA from the wild type and an *flhA* mutant of *P. mirabilis* U6450 containing the pBAD18 plasmid vector or pBAD18DC *flhDC*-overexpressing construct at peak (3.5 h) differentiation. WT, wild type with pBAD18; WT(*DC*), wild type with pBAD18DC; *flhA*, *flhA* with pBAD18; *flhA*(*DC*), *flhA* with pBAD18*flhDC*.

We thank Daniel Gygi and Alain Dufour for helpful discussions and P. Matsumura (University of Illinois) for the *E. coli flhDC* mutant.

This work was supported by a Programme grant from the Wellcome Trust (C.H.) and Medical Research Council studentships (R.F. and G.F.).

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