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Mutations in the *flhD* gene of *Escherichia coli* K-12 do not cause the reported effect on cell division

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Abstract

The hetero-oligomeric FlhD/FlhC complex is a global regulator of transcription in *Escherichia coli*. FlhD alone, independently of FlhC, has also been reported to control when *E. coli* cells stop dividing and enter stationary phase (Prüß & Matsumura, 1996). This work is frequently cited as evidence that FlhD regulates cell division, however our data indicate that this is not the case. The results presented here show that the previously observed phenotype is not due to the *flhD* locus, but instead is due to differences in the *thyA* alleles present in the *flhD*$^+$ and *flhD*$^-$ strains used in the original studies. We find that when the strains being compared have the same *thyA* allele (wild-type or mutant), *flhD* mutations have no effect on growth.

Introduction

The hetero-oligomeric FlhD/FlhC complex is a global regulator of gene expression in *Escherichia coli*. In addition to controlling transcription of the genes encoding the flagellar apparatus and the chemotaxis machinery (Macnab, 1996), FlhD/FlhC is involved in regulating expression of >20 other operons and genes whose products function in a variety of physiologic pathways, including anaerobic respiration, aerobic respiration, the Enter-Doudoroff pathway, and the synthesis of curli fimbriae (Prüß, *et al.*, 2001, Prüß, *et al.*, 2003).

FlhD by itself, independently of FlhC, has also been reported to regulate cell division in *E. coli* (Prüß & Matsumura, 1996). Cells in *flhD* mutant cultures were observed to continue dividing for several generations after cells in the *flhD*$^+$ parental culture had stopped growing and entered stationary phase. This work is frequently cited as evidence that FlhD regulates cell division (Kaper & Sperandio, 2005, Umehara, *et al.*, 2007, Cui, *et al.*, 2008, Hatt & Rather, 2008, Isalan, *et al.*, 2008), however our data indicate that this is not the case. We reexamined the effects of *flhD* mutations on entry to stationary phase and found that the previously observed phenotype is not due to the *flhD* locus. We show here that the difference in final cell number is due to the *thyA* mutation in the parental *flhD*$^+$ strain, which had apparently reverted in the *flhD*$^-$ mutant strain used in the study. When the strains being compared have the same *thyA* allele (wild-type or mutant), *flhD* mutations have no effect on growth.

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Methods

Strains and phage

The *E. coli* K-12 strains and phage used in this study are listed in Table 1. λWM7 (Mao & Siegele, 1998) is a derivative of λRS45 (Simons, *et al.*, 1987) that carries an operon fusion between the *mcb* operon promoter (positions -344 to +79) and the *lac* operon. Strains lysogenic for λWM7 were isolated by infecting YK410 and YK4131 with λWM7 and screening survivors on medium containing X-Gal where lysogens form blue colonies. Monolysogens were identified by measuring β-galactosidase activity in several independent isolates of each lysogen. Transductions with P1*vir* were performed as described by Miller (Miller, 1972). Hfr mapping was performed as described (Singer, *et al.*, 1989) using as donors the Hfr strains described in that paper.

To facilitate the exchange of *flhD* alleles, derivatives of YK410 (λP*mcb-lacZ*) and YK4131 (λP*mcb-lacZ*) were constructed that carry the linked *uvrC279*::Tn*10* mutation and retain their original *flhD* allele. These are strains DS507 and DS511, respectively, which were used as the donor strains in all subsequent strain constructions. Motility assays (described below) were used to determine whether transductants carried the wild-type or mutant *flhD* allele. Introduction of the *uvrC279*::Tn*10* mutation did not affect expression of the P*mcb-lacZ* fusion (Table 2 and data not shown).

Growth media

For β-galactosidase assays, cultures were grown in TB medium (1% Bacto-Tryptone, 0.5% NaCl (Arber, *et al.*, 1983)) supplemented with $MgSO_4$ (10 mM), thymidine (10 μg ml^{-1}) and thiamine (2 μg ml^{-1}). For plates, 1.3% Bacto-agar (Difco Laboratories) was included. For growth assays, cultures were grown in the TB medium used by Prüß and Matsumura (Prüß & Matsumura, 1996), which contained 1% Bacto-Tryptone and 1% NaCl and was supplemented with thymidine and thiamine, each at 20 μg ml^{-1}. When present, tetracycline was used at 12.5 μg ml^{-1}, kanamycin at 50 μg ml^{-1}, and X-gal at 20 μg ml^{-1}.

Motility assays

To assay motility, fresh overnight colonies were stabbed into TB motility agar and the plates were incubated for 5 to 8 hours at 30° C. TB motility agar contains 1% Bacto-tryptone, 0.5% NaCl, and 0.2% Difco Bacto-agar (Adler, 1966). Motile and non-motile control strains were included on each plate. All transductants were colony purified on selective medium before being tested for motility.

Growth phenotype assays

Overnight cultures were grown in tryptone broth and diluted 1:100 into either 10 ml of the same medium in 125 ml Erlenmeyer flasks or 3 ml of the same medium in 18 × 150 mm test tubes. Cultures in flasks were incubated at 37°C in a shaking water bath at 250 rpm. Cultures in test tubes were grown on a roller drum in a 37°C incubator. At the indicated time points, samples were removed from each culture, serially diluted, and, in most experiments, plated in duplicate to determine CFU per ml. The results shown are the mean of two or more independent cultures of each strain.

β-Galactosidase assays

β-Galactosidase assays were performed as described by Miller (Miller, 1972), using cells permeabilized with SDS and $CHCl_3$. β-Galactosidase specific activity is expressed in Miller units (OD_{420} per min per OD_{600}). To measure β-galactosidase levels, fresh overnight cultures were diluted 1:500 (for stationary phase measurements) or 1:2500 (for exponential

phase measurements) into 250 ml Erlenmeyer flasks containing 25 ml of TB medium supplemented with thiamine and thymine and incubated at 30°C shaking at 250 r.p.m. in a New Brunswick gyratory water bath. Samples were removed at regular intervals throughout growth of the cultures and assayed for β-galactosidase activity. The exponential phase levels of β–galactosidase activity are the mean of two to three samples taken after 5 to 8 generations of growth (OD_{600} between 0.015 and 0.1). The stationary phase levels of β-galactosidase activity are the mean of four to five samples taken at hourly intervals after the onset of stationary phase, which was defined as the point where the OD_{600} of the culture stopped increasing. Two or more independent cultures of each strain were assayed in duplicate.

Results and Discussion

Exchanging *flhD* alleles between YK410 and YK4131 did not affect when cultures entered stationary phase

Upon entry to stationary phase the number of cells per ml in cultures of YK4131 (*flhD4131*) is approximately 10-fold higher than in cultures of YK410 (*flhD*$^+$) or YK4136 (*flhC4136*) (Prüß & Matsumura, 1996). This difference was originally attributed to the difference in the *flhD* alleles present in the strains and FlhD was proposed to control when cells enter stationary phase. To retest this conclusion, we assayed growth of the parental strains YK410 and YK4131 and derivatives where we had exchanged the *flhD* alleles: YK410 *flhD4131* and YK4131 *flhD*$^+$. Motility assays were used to determine whether the wild-type or mutant *flhD* allele was present in the transductants. (Note that all four strains carry the *uvrC279*::Tn*10* marker used in the strain constructions and are lysogenic for λP*mcb-lacZ*; for the sake of clarity the two strain backgrounds will continue to be referred to as YK410 and YK4131.) The results are shown in Fig. 1. The parental strains showed the expected phenotypes. Cultures of YK410 grew to ca. 1×10^8 CFU ml^{-1} and had entered stationary phase by 150 min post-inoculation. Cultures of YK4131 were still growing at 240 min post-inoculation and grew to $>1 \times 10^9$ CFU ml^{-1}. However, the growth phenotypes did not change when the *flhD* alleles were exchanged between the two strains. YK410 *flhD4131* had the same growth phenotype as its *flhD*$^+$ parent and grew to only 1×10^8 CFU ml^{-1}, while the *flhD*$^+$ derivative of YK4131 still grew to $>1 \times 10^9$ CFU ml^{-1}. These results showed that the *flhD4131* mutation was neither necessary nor sufficient for the difference in growth between YK410 and YK4131.

It was previously reported (Prüß & Matsumura, 1996) that transformation of YK4131 with a plasmid carrying the *flhDC* genes, pXL27, complemented the delayed entry to stationary phase phenotype; the strain with the plasmid grew to only 1×10^8 CFU ml^{-1}. We obtained pXL27 and found that the final growth yield (measured as CFU ml^{-1}) of both YK410 and YK4131 was decreased by 78±2.0% compared to the same strains without the plasmid, indicating that the plasmid is deleterious to growth regardless of the *flhD* allele present on the chromosome.

Introduction of *flhD* mutations into another motile strain did not affect growth yield

Because of the possibility that the genotypes of YK410 and YK4131 could have changed since the original growth studies were performed, we tested the effect of *flhD* mutations on growth of RP437, which is another highly motile K-12 strain commonly used in studies of motility and chemotaxis (Parkinson & Houts, 1982). In contrast to YK410, cultures of RP437 grew to about 1×10^9 CFU ml^{-1} in TB medium before entering stationary phase. We then introduced *flhD*::Tn*10* into RP437 by P1*vir* transduction and assayed motility and growth of the transductants. As expected, introduction of the *flhD*::Tn*10* mutation caused a non-motile phenotype, however, it did not affect when cultures entered stationary phase.

RP437 grew to 1.2 ±0.3 × 10^9 CFU ml^{-1}, while RP437 *flhD*::Tn*10* grew to 1.3±0.2 × 10^9 CFU ml^{-1}. Identical results were seen when *flhD*::*kan* or *flhD4131* were introduced into RP437 (data not shown).

The difference in growth phenotype is due to the *thyA* mutation in YK410

In addition to RP437, another *E. coli* K-12 strain, MG1655, was shown to have the same final growth yield as YK4131, 1-2 × 10^9 CFU ml^{-1}. The fact that MG1655, RP437, and YK4131 all grew to 1-2 × 10^9 CFU ml^{-1} suggested that strain YK410 carried an uncharacterized mutation that was responsible for the early entry into stationary phage. To map the mutation we used Hfr mapping with YK410 as the recipient strain and screened for recombinants that grew to 1 × 10^9 CFU ml^{-1}.

An Hfr cross between YK410 and EA1005, an Hfr that transfers counter-clockwise, with the origin of transfer at 84.5 min, and a *srl*::Tn*10* marker at 60.9 min, gave rise to Tet^R Nal^R recombinants that grew to 10^9 CFU ml^{-1} before entering stationary phase. We found that all of the recombinants with the wild-type growth phenotype were also Thy^+, presumably having received the $thyA^+$ allele from the donor strain. (The *thyA* gene is located at 63.8 min.) At the same time, we discovered that the isolate of YK4131 we had received did not have the *thyA* mutation listed in its genotype, but was apparently a Thy^+ revertant.

These findings prompted us to check whether the difference in Thy phenotype was responsible for the growth difference between YK410 and YK4131. We found that YK410 grew to 1.2 × 10^9 CFU ml^{-1} when the medium was supplemented with additional thymidine (200 μg ml^{-1}), while the growth of YK4131 was unaffected. Four independent spontaneous Thy^+ revertants of YK410 were isolated and shown to grow to 1.3±0.2 × 10^9 CFU ml^{-1} before entering stationary phase, while in the same experiment, YK410 grew to only 2.7±0.2 × 10^8 CFU ml^{-1}. Identical results were obtained when a $thyA^+$ allele was introduced into YK410 by transduction selecting for a linked marker (data not shown).

In parallel experiments, a *thyA*::Tn*10* mutation was introduced into YK4131. The YK4131 *thyA*::Tn*10* transductants grew to only 1.0±0.4 × 10^8 CFU ml^{-1} before entering stationary phase, which was approximately 10% of the final growth yield of YK4131, which grew to 1.2±0.0 × 10^9 CFU ml^{-1}. The *thyA*::Tn*10* mutation was also introduced into strains MG1655 and RP437 with comparable results. Cultures of these *thyA*::Tn*10* transductants entered stationary phase when the number of CFU per ml was only 20±1.0% of the number present in the $thyA^+$ parent.

Mutations in *flhD* did not affect expression of P*mcb-lacZ*

We started these studies on *flhD* because of our interest in the stationary-phase induced *mcb* operon promoter (Hernández-Chico, *et al.*, 1986, Connell, *et al.*, 1987). It had been reported that the level of stationary-phase expression of a P*mcb-lacZ* reporter in YK4131 was only 10% the level seen in YK410 (Connell, 1989). We had previously isolated deletion and point mutations in the *mcb* operon promoter (P*mcb*) that identified promoter regions required for full promoter activity and stationary-phase regulation (Mao & Siegele, 1998). To determine whether of any of these promoter mutations altered interactions with FlhD, we first introduced a P*mcb-lacZ* operon fusion into YK410 and YK4131 by lysogenization with λWM7 (Mao & Siegele, 1998). The *flhD*::Tn*10* mutation was transduced from YK4159 into YK410 (λP*mcb-lacZ*) to produce strain DS478 [YK410 (λP*mcb-lacZ*) *flhD*::Tn*10*].

P*mcb* promoter activity was assayed by measuring β-galactosidase levels throughout growth (Table 2). In stationary phase, YK4131 (λP*mcb-lacZ*) had only 20% the level of β-galactosidase activity as the $flhD^+$ parental strain. In contrast, stationary phase β-galactosidase activity levels in the newly constructed mutant strain YK410 (λP*mcb-lacZ*)

flhD::Tn*10* were almosts identical to those in the parental strain. Introduction of the *flhD4131* allele into YK410 (λP*mcb-lacZ*) by transduction with phage P1*vir* also did not change the levels of β-galactosidase expression.

The difference in P*mcb-lacZ* expression between YK410 and YK4131 is not dependent upon the *thyA* allele present (data not shown). Using Hfr mapping, we have localized the region in YK4131 that is responsible for decreased stationary-phase of activity of P*mcb* to between 9 and 36 min on the *E. coli* chromosome. Our results suggest that more than one mutation may be needed for the phenotype as we recover three classes of exconjugants. In addition to recombinants that have the expected high and low levels of β-galactosidase activity, we recovered recombinants with intermediate levels of β-galactosidase activity. We plan to sequence the genomes of YK410 and YK4131 in order to identify the mutation(s).

In addition to the *mcb* operon, five *E. coli* genes or operons have been reported to be regulated by FlhD independently of FlhC (Prüß, *et al.*, 2003). Because these genes were identified using YK410, YK4131, and YK4136 (an *flhC* derivative of YK410), the observed effects on gene expression may also be due to the same unidentified mutation(s) in strain YK4131 that affects expression from P*mcb*. Further study is needed to answer this question.

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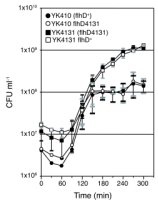

Fig. 1. Exchange of *flhD* alleles does not alter growth phenotypes

Cultures were grown and CFU ml^{-1} determined as described in the Methods. Growth of YK410 (*flhD*$^+$) is shown by closed circles and of YK4131 (*flhD4131*) by closed squares. Growth of YK410 *flhD4131* is shown by open circles and of YK4131 *flhD*$^+$ by open squares. (Note that all four strains also carry *uvrC279*::Tn*10* and are lysogenic for λP*mcb-lacZ*.) The data shown are the mean and standard deviation of 3 independent cultures of each strain (except for the 270 and 300 min time points, which were assayed only twice). The standard deviations for YK410 (*flhD*$^+$) and YK410 *flhD4131* are shown as short, horizontal black or grey lines, respectively. The standard deviations for YK4131 (*flhD4131*) and YK4131 *flhD*$^+$ are shown as long, horizontal black or grey lines, respectively.

Table 1

E. coli K-12 strains and phage used in this study.

Strain or phage	Genotype	Source (Reference)
DS380	YK410 (λWM7)	This study.
DS381	YK4131 (λWM7)	This study.
DS395	MC1000 *flhD*::*kan*	P. Matsumura
DS478	DS380 *flhD*::Tn*10*	DS380 × P1*vir* (YK4159)
DS507	DS380 *uvrC279*::Tn*10*	DS380 × P1*vir* (MM5008)
DS511	DS381 *uvrC279*::Tn*10*	DS381 × P1*vir* (DS507) (non-motile transductant)
DS513	DS380 *flhD4131 uvrC279*::Tn*10*	DS380 × P1*vir* (DS511) (non-motile transductant)
DS514	DS380 *uvrC279*::Tn*10*	DS380 × P1*vir* (DS511) (motile transductant)
DS817	RP437 *flhD*::Tn*10*	RP437 × P1*vir* (YK4159)
DS843	RP437 *thyA*::Tn*10*	RP437 × P1*vir* (SK089)
DS846	MG1655 *thyA*::Tn*10*	MG1655 × P1*vir* (SK089)
DS850	DS380 *thyA*::Tn*10*	DS380 × P1*vir* (SK089)
DS852	DS381 *thyA*::Tn*10*	DS381 × P1*vir* (SK089)
EA1005	KL228 *srl*::Tn*10*	R. Young
MG1655	F^- λ^- *rph-1*	C. Gross
MM5008	F^- *thr-1(Am) leuB6 his-4 metF159(Am) thi-1 eda-50 rpsL3156 lacY1 ara-14 xyl–5 mtl-1 tonA31 tsx-78 uvrC279*::Tn*10*	M. Manson
RP437	F^- *thr-1(Am) leuB6 his-4 metF159(Am) thi-1 eda-50 rpsL3156 lacY1 ara-14 xyl–5 mtl-1 tonA31 tsx-78*	M. Manson (Parkinson & Houts, 1982)
SK089	*araD139* Δ*(ara,leu)7697* Δ*lacX74 galU galK hsdR strA thyA*::*Tn*10 F′128 *lacI*^{q}*lacZ*::*Tn5*	J. Hu
YK410	F^- *araD139* Δ*lacU169 strA thi pyrC46 nalA thyA his*	P. Matsumura (Komeda, *et al.*, 1980)
YK4131	YK410 *flhD4131* (A32D) Thy^+ revertant	P. Matsumura (Komeda, et al., 1980, Prüß, et al., 2003)
YK4159	YK410 *flhD*::Tn*10*	P. Matsumura (Komeda, *et al.*, 1980)
λWM7	λRS45::P*mcb mcbA*(-344 to +79)::*lacZYA*	(Mao & Siegele, 1998)

Table 2

Expression of a single-copy P*mcb-lacZ* operon fusion in exponential and/or stationary phase.

		β-Galactosidase Activity in Miller Units*	
Strain	**Relevant genotype**	**Exponential phase**	**Stationary phase**
DS380	YK410 (λP*mcb*::*lacZ*)	40±8 (1.0)	900±5 (1.0)
DS381	YK4131 (λP*mcb*::*lacZ*)	30±6 (0.8)	180±4 (0.2)
DS478	YK410 (λP*mcb*::*lacZ*) *flhD*::Tn*10*	40±1 (1.0)	1000±106 (1.1)
DS514	YK410 (λP*mcb*::*lacZ*) *uvrC279*::Tn*10* (still *flhD*$^+$)	40±5 (1.0)	1000±120 (1.1)
DS513	YK410 (λP*mcb*::*lacZ*) *flhD4131 uvrC279*::Tn*10*	40±6 (1.0)	970±250 (1.0)

*Cultures were grown and β-galactosidase assays were performed as described in the Methods. β-Galactosidase activity is expressed in Miller units (OD_{420} per min per OD_{600}). The values shown are the mean and standard deviation of two or more independent cultures. The values in parentheses show activity relative to the parental *flhD*$^+$ strain assayed on the same day.