A Regulator of the Flagellar Regulon of Escherichia coli, flhD, Also Affects Cell Division

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Received 28 August 1995/Accepted 22 November 1995

The role of an activator of flagellar transcription in Escherichia coli, flhD, was investigated in the regulation of cell division. When grown in tryptone broth, flhD mutant cells divided exponentially until they reached a cell density of 2.5 × 10⁶ cells per ml. Wild-type cells and flhC mutant cells divided exponentially until they reached a cell density of 4 × 10⁶ cells per ml. flhD mutant cells divided 5 times more than wild-type cells before they reduced their cell division rate and reached a cell density 37 times higher than that of wild-type or flhC mutant cultures. In stationary phase, the biomasses of all cultures were similar; however, flhD mutant cells were significantly smaller. Additional tryptone, Casamino Acids, and individual amino acids, added at the beginning of growth, allowed wild-type cells to grow to higher cell densities. Serine was determined to have the greatest effect. In contrast, the addition of Casamino Acids did not exhibit an effect upon flhD mutant cells. flhD mutant cells exhibited normal rates of uptake of serine and other amino acids. In both wild-type and flhD mutant cultures, the concentrations of serine in the media dropped from 140 to 20 μM within the first 2 h of growth. Serine concentrations and cell division rates were highly correlated. Wild-type cells reduced their cell division rate at a medium concentration of 50 μM serine, and the addition of serine at this time caused cells to resume a higher rate of division. We conclude that the reduction of the cell division rate in wild-type cells is caused by the depletion of serine from the medium and that flhD mutant cells seem to be unable to sense this depletion.

The flagellar regulon in Escherichia coli consists of 40 genes in 14 operons that are transcriptionally ordered in a hierarchy. At the top of the hierarchy is the master operon, composed of flhD and flhC. FlhD and FlhC are flagellum-specific transcriptional activators that are required for the expression of all other flagellar genes (2). The second level of the hierarchy includes genes necessary for the early assembly of the flagellum and a flagellum-specific σ factor, fliA, required for the expression of level III genes (18). Level III includes genes for late morphogenesis, energy transduction, and signal processing (for a review of flagellar expression, see reference 19).

FlhD and FlhC act as heterotetramers to bind to level II operons (17). Therefore, flhD and flhC have the same Flα- phenotype.

Flagellar formation is a response to environmental stress. This response appears to take place at the level of flhDC expression. flhDC expression is subject to cyclic AMP (cAMP)-catabolite gene activator protein-mediated catabolite repression (31) and also requires the heat shock proteins DnaK, DnaJ, and GrpE (28). Recently, acetyl phosphate has been shown to be involved in the inhibition of flagellation at elevated temperatures (26). The temperature elevation led to an increase in the acetyl phosphate concentration, which was correlated to an inhibition of flagellar expression. This inhibition now appears to be mediated through phosphorylation of the response regulator of the osmoregulation system, OmpR, which then negatively regulates the expression of flhDC (29).

The flhDC operon has been implicated in the regulation of nonflagellar genes. Kim has shown that the overexpression of flhDC causes an inhibition of cell growth (14), and Connell (5) and Tentler (32) have shown that expression of the microcin B12 (mcbA) promoter was positively regulated by flhD.

Other observations suggest a connection between the expression of flagella and cell division. Nishimura and Hirota found that the expression of flagella by cell division mutants and DNA replication mutants decreased dramatically upon reaching the nonpermissive temperature (23). They postulated that the flagellar regulon is under the control of a cell division regulatory mechanism. This hypothesis is supported by our observation that the expression level of flhD, a level II operon, increases 5 to 10 min before cell division (24). In these cases, the authors propose that the expression of flagella is regulated by the bacterial cell cycle. Regulation of cell division by a component of the flagellar system has not been shown.

In this study, it was demonstrated that flhD but not flhC has a function outside of the flagellar regulon. This function is the involvement in the regulation of cell division in E. coli. flhD mutants grown in tryptone broth (TB) continued to divide at a rate typical for mid-exponential growth and at a cell density at which wild-type and flhC mutant cells started to reduce their cell division rate. flhD mutant cultures had biomasses (optical densities) similar to those of wild-type cultures; however, flhD mutant cells were much smaller as they entered stationary phase. We demonstrated that the reduction of the cell division rate during entry into stationary phase of wild-type cells was correlated to a depletion of serine from the medium.

MATERIALS AND METHODS

Bacterial strains. All strains were derivatives of E. coli K-12. YK410 (15) was used as background strain (F− araD139 ΔlacU169 strA thi proC46 naldA thyA his). YK4159 (16) contains a Tn10 transposon, and MC1000 flhD::Kn (20) contains an insertion of the kanamycin gene in flhD. Both mutations inhibit the expression of flhD and flhC. The kanamycin gene was transduced into YK410 by generalized P1 transduction (30) to yield BP67. YK4131, YK4136, and YK4104 contain point mutations in flhD, flhC, and fliA (16).

Bacterial growth conditions. TB consisted of 1% tryptone and 1% NaCl (22), and thymine and thiamine were added at concentrations of 20 μg/ml in some
cases we used 0.5% (0.5%) or 2% (2×) tryptone or supplemented the broth with Casamino Acids, serine, aspartate, or glutamate at the concentrations indicated below. Cells were inoculated from an overnight culture in the same broth and grown at 37°C, and the optical densities at 600 nm were monitored.

Synchronization of cells. Cells were synchronized with the bacterial “baby machine” modified for E. coli K-12 strains (11). A filter unit consisting of a 0.22-μm-pore-size nitrocellulose filter (Millipore Corp., Bedford, Mass.) upon a filter screen was used and was flanked by rubber gaskets on both sides to prevent leaking. Cells and media were loaded onto the membrane with a funnel on both sides fixed to a plastic ring that clamped the two funnels together. The membrane was coated with 50 ml of 50 μg of poly-D-lysine per ml to allow K-12 strains to attach to the membrane, and then it was washed with 100 ml of H2O at a speed of 5 ml/min. Cells (100 ml) grown in tryptone broth for 5 h were loaded onto the membrane at a speed of 2 ml/min. The whole filter unit was inverted, and warm TB was pumped through at a speed of 1 ml/min; eluted recently divided baby cells were collected on ice. Cells were counted and checked under the microscope for homogeneity of size. Cells were diluted in TB.

Determination of the cell number. Cells were visualized with the aid of a light microscope and phase-contrast optics (1). Cells were counted by the MID-Kova system (Madaus Diagnostik, Cologne, Germany), which contains 10 counting units on one slide. One square has the volume of 0.012 μl; typically 4 to 8 squares were counted, and the means of the populations were determined. Standard errors were less than 20%. Total cell numbers were expressed as cells per milliliter of bacterial culture.

Protein determination. Lysates were prepared as previously described (3), and a 10-μl aliquot of the extract was assayed for its protein concentration with the Coomassie Protein Assay reagent (Pierce, Rockford, Ill.) by following the manufacturer’s instructions. Bovine serum albumin was used as a standard, and the protein concentration was expressed as milligrams of protein per cell.

Determination of the concentration of amino acids. Bacteria were grown in tryptone as described above, and 1-ml aliquots were taken at appropriate intervals and filtered through a 0.22-μm-pore-size filter. Twenty microliters was de-vivared with 20 μl of o-phthalaldehydereagent (Sigma, St. Louis, Mo.) for 1 min and analyzed by high-pressure liquid chromatography (HPLC) (Rainin, Woburn, Mass.) by following the manufacturer’s instructions. The concentration of amino acids was expressed in micromolar units.

Electron microscopy. Bacteria were grown as described above for 4 h, harvested, and resuspended in 50 mM NaCl. Twenty microliters of the bacterial suspension was loaded on a 200 hex mesh Ni grid with a carbon-coated Parlodion support film. The suspension was allowed to air dry. Grids were placed in a JEOL 1200 EX transmission electron microscope and photographed at a magnification of 20,000 (8).

RESULTS

flhD mutants but not flhC mutants nor wild-type cells keep dividing as they enter stationary phase. Wild-type (strain YK410), flhDC mutant (strains YK4519 and BP67), flhD mutant (strain YK4131), flhC mutant (strain YK4136), and fltA mutant (strain YK4104) cells were grown in TB for 4 h. The growth rates, measured as optical density at 600 nm, and the numbers of cells were determined at distinct time intervals (Fig. 1). The mutations did not have any major effect upon the optical density, although flhD mutant cells grew to a slightly higher optical density than wild-type cells (Fig. 1A). The cell number of flhD mutant cells, in contrast, differed significantly from that of wild-type cells (Fig. 1B). All cultures grew exponentially for 1.5 h until they reached a cell density of 2.7 × 107 cells per ml and an optical density of 0.15. Upon reaching this point, wild-type, flhC mutant, and fltA mutant cells reduced their cell division rate, flhDC and flhD mutant cells increased their cell division rate, reaching cell densities of 7 × 108 cells per ml after 4 h.

In addition, the flhD mutant strain (YK4131) was transformed with a plasmid that expresses flhD. This plasmid restored the wild-type phenotype, while the parent plasmid (lacking flhD) did not affect the FlhD phenotype (data not shown). Also, the Tn10 transposon in flhD in strain YK4519 and the kanamycin insertion of strain MC1000 flhD::Km was transduced into the unrelated background JM109 (33), yielding strains BP65 and BP66 (data not shown). Strain BP66, containing flhD::Km, behaved like YK4131, and JM109 behaved like YK410. Strain BP65, containing flhD::Tn10, also exhibited a phenotype similar to that of YK4131.

At this point it was concluded that flhD has a negative regulatory effect upon cell division and that this effect was not dependent on the kind of mutation or the genetic background. flhD-deficient cells are much smaller in stationary phase. With synchronized cultures of wild-type (strain YK410) and flhD mutant (strain YK4131) cells, the length of each cell cycle throughout growth was determined. Synchronized cells were collected by the membrane filtration technique and grown in TB at 37°C until stationary phase. The lengths of the cell cycles were determined and plotted against the numbers of cells after each cell division (Fig. 2A). Even though cultures started at different cell densities, wild-type cells slowed down to a cell division time of 60 to 90 min at a cell density of (6.75 ± 0.5) × 107 cells per ml, while flhD mutant cells stopped rapid division at a cell density of (2.55 ± 0.1) × 107 cells per ml, which is a 37-times-higher cell density than those of wild-type cultures. To enhance the presentation of these data, we omitted the first cycle, which was typically 70 min.

In addition, the protein concentrations of cell lysates were determined and plotted as milligrams per cell versus the numbers of cells after the corresponding cell division (Fig. 2B). In both strains, the protein concentration after each division de-
The protein concentration in wild-type cells stopped declining at the time at which cells reduced their cell division rate, reaching a protein concentration of \((0.8 \pm 0.14) \times 10^{-9}\) mg of protein per cell. The protein concentration in \(flhD\) mutant cells continued to decrease and reached a value of \((0.03 \pm 0.003) \times 10^{-9}\) mg of protein per cell. This concentration is 27 times less than that of wild-type cells.

To document the differences in sizes, electron photomicrographs of both types of cells are presented (Fig. 3). Wild-type (YK410 and \(flhD\) mutant (YK4131) were grown in TB for 4 h. At this time the cell densities of \(flhD\) mutant cultures were about 10 times higher than the cell densities of wild-type cultures. Cells were photographed (Fig. 3A) at a magnification of 20,000, and the size distribution of the cells was determined for a total of 30 cells each (Fig. 3B). \(flhD\) mutant cells are visibly smaller than wild-type cells. The average length of a wild-type cell was 2.27 ± 0.1 µm, and the average length of a \(flhD\) mutant cell was 0.85 ± 0.05 µm.

A mix of amino acids causes wild-type but not \(flhD\) mutant cells to grow to higher cell densities. Wild-type (strain YK410) or \(flhD\) mutant (strain YK4131) cells were grown in broth containing 0.5× tryptone, 1× tryptone, and 2× tryptone (Fig. 4). Optical densities of the cultures and the numbers of cells were determined. The optical density profiles of wild-type and \(flhD\) mutant cultures were similar in all media tested (data not shown). Wild-type cells grew at the same rate in all three media to a cell density of \(1.3 \times 10^7\) cells per ml (Fig. 4A). At that point, cells grown in 0.5× TB reduced their cell division rate, cells grown in 1× TB increased their cell division rate until they reached a cell density of \(3.75 \times 10^7\) cells per ml, and cells grown in 2× TB continued dividing rapidly until they reached a cell density of \(1.6 \times 10^8\) cells per ml. \(flhD\) mutant cells grew at the same rates until they reached a cell density of \(1.3 \times 10^8\) cells per ml (Fig. 4C). At that point, cells grown in 0.5× TB reduced their cell division rate, cells grown in 1× TB continued dividing rapidly until they reached a cell density of \(1 \times 10^9\) cells per ml, and cells grown in 2× TB continued dividing rapidly until they reached a cell density of \(1.2 \times 10^{10}\) cells per ml.

The experiment was repeated, supplementing the broth with Casamino Acids. Wild-type (strain YK410) and \(flhD\) mutant (strain YK4131) were grown in 0.5× TB unsupplemented or supplemented with 0.2 or 0.4% Casamino Acids. The numbers of cells were determined. Wild-type cells responded to the addition of Casamino Acids by growing to higher cell densities in a way similar to that observed with tryptone-supplemented broth.
medium (Fig. 4C). \( \text{flhD} \) mutant cells grew at the same rate in Casamino Acid-supplemented media during the whole growth curve and grew to a cell density of \( 1.8 \times 10^8 \) cells per ml, before reducing their cell division rate (Fig. 4D).

Two effects can be distinguished. One is an effect of Casamino Acids upon wild-type but not \( \text{flhD} \) mutant cells; the other is an effect of some component in tryptone which affects both wild-type and \( \text{flhD} \) mutant cultures. The cause of this second effect was not determined.

**Wild-type and \( \text{flhD} \) mutant cells consume all amino acids equally.** The concentrations of amino acids in the medium for wild-type (strain YK410) and \( \text{flhD} \) mutant (strain YK4131) cultures during growth (Fig. 5) were measured. The numbers of cells in the cultures and the concentrations of amino acids in the medium were determined in cultures grown at 37°C. Only the amino acid concentrations that changed during the investigated period of 4.5 h were plotted. The amino acid composition of the medium was the same for wild-type and \( \text{flhD} \) mutant cultures during the entire period measured. In both strains, the concentration of serine dropped over the first 2 h of growth. The concentration of aspartate dropped between 1.5 and 3 h of growth, and the concentration of glutamate dropped between 2 and 4.5 h.

Since no difference between wild-type and \( \text{flhD} \) mutant cells with respect to amino acid consumption was observed, it was concluded that the inability of \( \text{flhD} \) mutant cells to reduce their cell division rate as they enter stationary phase is not due to a defect in uptake. Rather, it is due to the cells' inability to sense either the depletion of one or more amino acids or the accumulation of one of their degradation products.

**Serine causes wild-type cells to grow to higher cell densities.** Wild-type cells (strain YK410) were grown in 0.5× TB and in 0.5× TB supplemented with 0.1 or 0.5 mM serine (Fig. 6A), aspartate (Fig. 6B), or glutamate (Fig. 6C). The numbers of cells in the cultures were determined. Wild-type cells grew at the same rate in all media to a cell density of \( 1.3 \times 10^7 \). At that point, serine caused cells to grow to higher cell densities, reaching a maximal cell density of \( 6 \times 10^7 \) cells per ml. Cells

**FIG. 4. Growth of wild-type (strain YK410 [A and B]) and \( \text{flhD} \) mutant (strain YK4131 [C and D]) cells on mixed amino acids.** Cells were grown in 0.5× TB (squares), 1× TB (diamonds), or 2× TB (stars or asterisks) (A and C) or in 0.5× TB unsupplemented or supplemented with 0.2% (diamonds) or 0.4% (stars or asterisks) Casamino Acids (B and D). The growth rates were measured as cell numbers determined by microscopy. The experiment was done in duplicate, and the means of the populations were determined. Standard errors were less than 10%.
grown in 0.5× TB supplemented with 0.1 or 0.5 mM aspartate or glutamate grew at about the same rate as those grown in unsupplemented 0.5× TB during the first 2 h of growth. At that point, cells responded to the addition of aspartate or glutamate by growing to higher cell densities ($3.5 \times 10^7$ and $4 \times 10^7$ cells per ml, respectively).

In summary, serine seems to have the largest effect upon the cell division rate of wild-type cells; however, aspartate and glutamate also enable cells to grow to higher cell densities. Since aspartate and glutamate are not major metabolites during the first 1.5 h of growth in TB, we believe that in TB the cell division rate is reduced largely because of the depletion of serine.

Serine concentration and cell division rate of wild-type cells are correlated. Wild-type cells (YK410) were grown in TB, and 1 mM serine was added after 2 h (Fig. 7). This concentration is approximately seven times higher than the starting concentration, and after 2 h of growth the number of cells was about seven times higher than the starting number of cells. The number of cells and the concentration of serine in the medium were monitored during growth. Between 30 min and 1.5 h of growth, cells divided at a rate of 2.4 divisions per h, and then they reduced their cell division rate to 0.5 divisions per h. Immediately after the addition of serine, cells started to divide at a rate of 4.2 divisions per h for 30 min, reaching a maximal cell density of $3.5 \times 10^8$ cells per ml. They then reduced their cell division rate again. This growth behavior was in direct correlation with the concentration of serine. During the first 1.5 h of growth, the concentration of serine declined from 137 to 20 μM, and upon the addition of serine, the concentration increased to 1,200 μM and then declined again rapidly during the following 30 min to 66 μM. In both parts of the experiment, cells reduce their cell division rate at a serine concentration of 40 to 60 μM.

DISCUSSION

These studies establish a correlation among the regulation of flagellar and chemotaxis gene expression, cell division, and the central metabolic pathways of cells.

$flhD$ mutants exhibited an interesting phenotype. Mutant cells kept dividing rapidly as they entered stationary phase, while wild-type cells reduced their cell division rate. This occurred without an increase in the optical density of the culture. Mutant cells divided exactly 5 times more often than wild-type cells, reaching 37-times-higher cell densities, had 27 times less protein per cell, and showed a fourfold reduction in length.

The addition of tryptone, as well as Casamino Acids, allowed wild-type cells to grow to higher cell densities. It was concluded that the reduction of the cell division rate as cells enter sta-
...cationary phase was due to nutritional depletion. Since wild-type and \textit{flhD} mutant cells consumed all amino acids identically, it is believed that \textit{flhD} mutant cells are unable to sense depletion of amino acids, especially serine.

In an earlier publication, we demonstrated that \textit{E. coli} metabolized serine first when grown on mixed amino acids (25). This indicates a possible role for serine in the regulation of cell division. In agreement with this assumption, the reduction of the cell division rate in wild-type cells could be suppressed by the addition of excess serine. Adding serine at a time when cells had already reduced their cell division rate resulted in another increase in the cell division rate. We concluded that the reduction of the cell division rate might be due to the depletion of serine from the medium.

Serine depletion, itself, may not be the signal for cells to reduce their cell division rate. It may be that one metabolite which occurs predominantly during serine metabolism, but also during the degradation of certain other amino acid carbon sources, is the signal.

Grown on mixed amino acids, \textit{E. coli} organisms excrete large amounts of acetate as they consume serine (25). When there is an imbalance between the flux of the carbon source into the central metabolic pathways and the flux required for biosynthesis and oxidation to CO$_2$, \textit{E. coli} excretes acetate (for a review, see reference 12). Glutamate and aspartate would be expected to be degraded via the tricarboxylic acid cycle. If the level of uptake of the carbon source is higher than necessary to balance the flux through the tricarboxylic acid cycle, oxaloacetate can be converted to phosphoenolpyruvate (phosphoenolpyruvate carboxykinase), pyruvate (pyruvate kinase), and acetyl-coenzyme A (pyruvate dehydrogenase). Holms discussed this excretion of acetate as an advantage, because it permits a higher growth rate (12). On the basis of this information, it is possible that the reduction of the cell division rate by wild-type but not by \textit{flhD}-deficient cells, when grown on mixed amino acids, might be related to the conversion of serine to acetate.

It was previously demonstrated that acetyl phosphate, the intermediate that occurs during the conversion of acetyl-coenzyme A to acetate, was correlated to the expression of flagellar and chemotaxis genes at elevated temperatures (26). We postulated that the low concentration of acetyl phosphate in stationary phase might allow the formation of flagella at this time. Shin and Park reported that the inhibition of flagellar expression by acetyl phosphate was mediated by OmpR, the response regulator of the osmoregulation system (29). They report that a mutation in \textit{ompR} caused the insensitivity of \textit{flhDC} expression to mutations in \textit{ack} and \textit{pta}, encoding the acetate activation enzymes acetate kinase and phosphotransacetylase. They concluded that the phosphorylation of OmpR by acetyl phosphate leads to an inhibition of \textit{flhDC} expression.

We believe that during the transition into stationary phase, formation of flagella and the reduction of the cell division rate are controlled by the same regulatory system that informs the cell about its nutritional situation. During early exponential phase, cells degrade serine and synthesize acetyl phosphate, which can function as a phospho donor to phosphorylate OmpR. This phosphorylation may lead to a repression of \textit{flhDC}, the master operon of flagellar expression. As a consequence, the flagellar regulon is repressed and the cells divide rapidly. As serine is depleted, \textit{flhD} and \textit{flhC} are expressed and the cells reduce their cell division rate.

This hypothesis is supported by several observations. (i) McCleary et al. believe that acetyl phosphate is a global signal which signals to the cell an imbalance in carbon flow through glycolysis and into the tricarboxylic acid cycle. It might be the signal that cells are in the intestinal tract (21). (ii) Shin and Park demonstrated that OmpR is able to bind to \textit{flhDC}. They also footprinted two regions where OmpR can bind to the \textit{flhDC} promoter (29). This is supported by our observation that cells deficient for \textit{ompR} divided at a lower rate than is typical for cells in exponential phase. Throughout the whole growth curve, they divided at a constant rate of 0.6 to 1 generation per h (24).

A similar connection between flagellar expression and cell division has been shown in \textit{Caulobacter crescentus} (for a review on cellular differentiation in \textit{C. crescentus}, see reference 10; for a review on hierarchal expression of \textit{C. crescentus} flagellar genes, see reference 4). Some of the level II genes required for flagellum assembly are also involved in cell division. Mutations in these genes lead to long, filamentous cells (6, 34). The major difference between this system and the one just presented for \textit{E. coli} is that the level II genes in \textit{C. crescentus} induce cell division, whereas in \textit{E. coli}, \textit{flhD} inhibits cell division. Another function of \textit{flhD} outside the flagellar regulon has been shown in \textit{Serratia liquefaciens}, in which \textit{flhD} also regulates phospholipase expression (9). This raises the question of whether FlhD as a global regulatory protein in the cell may be involved in many stationary phase processes.

One of the most interesting results of this study is that FlhD alone, without the interaction with FlhC, has a function. To activate transcription of flagella, FlhD and FlhC act as heterotetramers (17), and to regulate cell division, FlhD acts either alone or with a different protein. This could be the first example in which a bacterial transcriptional activator can have different specificities, depending on its complex formation, a phenomenon that is seen in eucaryotic systems.

Comparisons between procaryotic and eucaryotic cell division machineries have been made before. The \textit{E. coli} cell division protein FtsA has similarity with \textit{Saccharomyces cerevisiae} CDC28 and \textit{Schizosaccharomyces pombe} CDC2 (27), as does Fic, which is involved in cell filamentation induced by cAMP (13). Another cell division protein, FtsZ, has been discussed to be a procaryotic homolog of tubulin (7). These three
proteins could be possible targets for a regulation of cell division by FlhD.

ACKNOWLEDGMENTS

We thank C. E. Helmstetter (Department of Biological Sciences, Florida Institute of Technology) for his advice in developing the baby cell machine and J. Gibbons (Department of Biology, University of Illinois at Chicago) for technical help with the electron microscope. We also thank U. Prüß for providing the microscope slides, V. Carr-Brendel and S. Chang for technical assistance, and Peggy O’Neill for a critical reading of the manuscript.

This work was supported by grant AI 18985 from the National Institutes of Health.

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