JOURNAL OF BACTERIOLOGY, Oct. 1993, p. 6238–6244
0021-9193/93/196238-07$02.00/0

Vol. 175, No. 19

Multiple Factors Underlying the Maximum Motility of *Escherichia coli* as Cultures Enter Post-Exponential Growth

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Received 1 June 1993/Accepted 26 July 1993

Motility and chemotaxis allow cells to move away from stressful microenvironments. Motility of *Escherichia coli* in batch cultures, as measured by cell swimming speed, was low in early-exponential-phase cells, peaked as the cells entered post-exponential phase, and declined into early stationary phase. Transcription from the *flhB* operon and synthesis of flagellin protein similarly peaked in late exponential and early post-exponential phases, respectively. The increase in swimming speed between early-exponential and post-exponential phases was correlated with twofold increases in both flagellar length and flagellar density per cell volume. This increased investment in flagella probably reflects the increased adaptive value of motility in less favorable environments. The decrease in speed between post-exponential and stationary phases was correlated with a threefold decrease in torque produced by the flagellar motors and presumably reflects decreased proton motive force available to stationary-phase cells.

Motility and chemotaxis are mechanisms that bacteria use in response to environmental stress. The microclimates inhabited by many types of bacteria are spatially and temporally heterogeneous as a result of a wide variety of factors. Such factors include microheterogeneity in the chemical and physical nature of a substrate, in the metabolic activities of the microbial community, and in the metabolic activities of a host organism (2, 3, 11, 18, 20, 27, 35). An ability to move away from more stressful areas and into microenvironments more favorable for growth and reproduction is clearly of great adaptive value to bacterial cells in nature (20, 26, 39).

Neither motility nor chemical perception alone is sufficient as a response mechanism to environmental stress because a cell needs both to detect environmental heterogeneity and to be able to move into less stressful microclimates. In *Escherichia coli*, the genes for flagellar synthesis (designated *flg*, *flh*, and *fli*), flagellar rotation (designated *mot*), chemotactic membrane receptors (designated *tar*, *tsr*, *tap*, and *trg*), and chemotactic signal transduction (designated *che*) are all part of the same regulon (29, 30). This flagellar regulon consists of over 40 genes in at least 13 operons which are expressed in a hierarchical order. The *flhD* operon is the top level; it is expressed first, and its expression is required for the expression of all other genes in the regulon (23). The second level of the regulon includes genes necessary for flagellar basal body and hook assembly as well as the sigma factor necessary for expression of the third-level operons. This last level includes the *che*, *mot*, and receptor genes as well as some flagellar genes, including *fliC*, which codes for flagellin, the protein which comprises the flagellar filament (29, 30).

The motility-chemotaxis mechanism is a part of several global stress response networks. Expression of the *flhD* operon, and therefore all flagellar genes, is dependent on the presence of cyclic AMP-catabolite gene activator protein (23, 43). This is the regulatory element of the catabolite repression network which also regulates proteins involved with responses to starvation, is an inducer of numerous catabolic enzymes, and in some species is involved with cell division and a variety of other functions (7). Expression of the *flhD* operon is also dependent on the presence of the heat shock response network proteins DnaK, DnaJ, and GrpE (42). In addition, the chemotaxis proteins involved in sensing and transmitting environmental signals are members of the two-component family of homologous signal transduction mechanisms (36, 38, 48). This family includes mechanisms which regulate responses to a wide variety of environmental stresses. These include regulation of nitrogen and phosphate uptake, osmoregulation, oxygen regulation, sporulation, competence, and the production and export of degradative enzymes, exoproteins, and alginate (38, 48). There is a great deal of similarity in the sequences and biochemical functions of the histidine kinase and response regulator components of these systems. Cross-talk, or signaling from one system to another, occurs in vitro and in some systems under physiologically relevant conditions in vivo (38, 48). This provides another possible mechanism for global regulation of motility, chemotaxis, and other stress responses.

Unlike other stress responses, motility and chemotaxis can be monitored in individual living cells and without genetic or biochemical manipulation. We wish to understand the biochemical and molecular signals which modulate stress responses and have chosen motility as a model. We also hope ultimately to define environmental conditions, physiological factors, and biophysical constraints which affect flagellar function and thereby influence flagellar gene expression. As a step toward these goals, we have developed a technique to quantify one measure of motility and flagellar function, cell swimming speed. We have investigated the temporal relationship between swimming speed, flagellar gene expression, flagellin protein production, flagellar number and density, and the torque produced by the flagellar motor. Expression of a second-level flagellar operon, *flhB*, is low during the early exponential growth phase but increases severalfold to a maximum during late and post-exponential growth. Swimming speed also increases to a maximum as the

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culture enters post-exponential growth because the flagella become longer and more densely packed on the cell surface. Gene expression decreases as cells enter stationary phase, and swimming speeds also decrease. This decrease in swimming speed also correlates with a threefold decrease in torque produced by the flagellar motors.

MATERIALS AND METHODS

Bacterial strains. The chemotactic wild-type *E. coli* K-12 strain RP437 (37) was used in all experiments except measurement of *flhB* operon expression. Expression of the *flhB* operon was measured by using strain MC453, a λ lysogen. This lysogen was constructed by first transcriptionally fusing the *flhB* promoter to a *lacZ* gene in λRZ5 (17), which was then used to lysogenize MC1000 (9).

Bacterial growth conditions. All cells were grown in tryptone broth (1% Bacto Tryptone, 1% NaCl). In all experiments, 50-ml cultures in 300-ml flasks were initiated by addition of 0.5 ml of stationary-phase cells from overnight cultures grown at 37°C. The 50-ml cultures were shaken at 300 rpm and incubated at 30°C. Cell density was measured in Klett units. It was determined that under these growth conditions, cell density (number of cells × 10^8/ml) equals (0.0450 · Klett units) − 0.160.

Bacterial swimming speed determinations. Swimming cells were visualized with aid of a light microscope and phase-contrast optics. Cells were observed as they swam just under the coverglass. It was usually necessary to reduce the concentration of cells from the cultures for microscopic observation. The diluent used was medium withdrawn from the cultures at the same time as the cells to be observed but from which cells had been removed by filtration. To quantitatively measure swimming speed, videotaped images of unstimulated, swimming cells were processed with a Motion Analysis Corp. VP 110 video processor. Distances on the video image were calibrated against a stage micrometer; one pixel equals 0.492 μm. Computer motion analysis programs were developed with Motion Analysis Corp. CellTrak software and Lotus 123 version 3.1 macros. These programs quantitatively and simultaneously measure speed and identify tumbles. Instantaneous swimming speed (calculated from the distance traveled in 0.0667 s) was measured 15 times per s for 2 s in 100 cells at time points throughout the growth curve.

Bacterial tethering. Bacteria were tethered by using a modification of the technique described by Slocum and Parkinson (44). Cells were kept at 30°C or room temperature throughout the procedure and were suspended in tryptone broth from the same cultures but from which cells had been removed by filtration. Tethered cells which were actively spinning were videotaped with the aid of a light microscope within 30 min. Angular velocity, cell length, and the center of rotation were later determined from the video images of 30 cells at each time interval. The resolution of the videotapes was insufficient to distinguish width differences, but cell length-to-width ratios were known from measurements obtained by using electron microscopy, as described below. Consequently, cell widths were calculated from the cell length by using these length-to-width ratios. Rotational drag coefficient was determined with a BASIC computer program developed by S. Block (Rowland Institute) and based on the calculations of Tirado and García de la Tore (52, 53). Torque was calculated from the drag coefficient and angular velocity.

Biochemical determinations. β-Galactosidase activities were monitored throughout the growth curve and assayed as described by Sambrook et al. (40). Flagellin protein production throughout the growth curve was measured on a per-cell basis by quantitative Western blot (immunoblot) analysis. Known quantities of cells were removed from culture at various time intervals and resuspended in 2× Laemmli sample buffer (25). After boiling, aliquots were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then the proteins were transferred to aminophenylthioester cellulose (Schleicher & Schuell, Inc.). These blots were probed with polyclonal anti-CheA antibody, which was then labeled with ^{125}I-protein A. Flagellin protein per cell was quantified by comparison with the amount of purified flagellin protein on the same blots, using a β-particle scanner (AMBIS, Inc.).

Electron microscopy. Cell length and width and the number of flagella per cell were determined at various time points in fresh, unfixed, negatively stained whole cells with the aid of a transmission electron microscope. Cells were washed with 10 mM phosphate buffer (pH 7.0), allowed to stick to a Formvar-coated grid for 1 to 2 min, and then negatively stained with 1% uranyl acetate. Measurements were made from ×8,000 micrograph negatives. Cell volumes (V) were calculated from measured cell length (L) and radius (r) as if cell shape was a cylinder (i.e., $V = \pi r^2 L$). Flagella were counted by the number of filaments arising in the cell body that ended outside the cell body (i.e., did not loop back into the cell).

Statistical analyses. Statistical significances of differences between means of time interval samples for swimming speeds, flagellin protein per cell, flagellar number and density, and cell length, width, and volume were determined. Each analysis was performed by using the statistical software package SAS (41). One-way analysis of variance (SAS Analysis-of-Variance or General Linear Models procedure) was used to determine treatment effects. Results from analysis-of-variance tests are presented as the value for the F distribution, with degrees of freedom and error terms as subscripts (i.e., $F_{df,\ error}$ = value). The Ryan-Einot-Gabriel-Welsh multiple-range (REGWQ) test was used with the SAS analysis-of-variance procedure to determine differences between means (choice of specific test based on reference 13). A three-way analysis of variance (SAS General Linear Models procedure) with time interval as a fixed factor and tumble frequency and replicate experiment as random factors was also performed with the swimming speed data to determine the interactive effects of tumble frequency and sample date on speed. Significance of difference between means of torque calculations for 5- and 10-h-old cells was determined by using the SAS TTEST procedure.

RESULTS

Swimming speed. Swimming speed determinations were begun when the cultures were 2.75 h old (early to mid-exponential phase of growth) because the majority of cells were nonmotile before this time. Swimming speeds were significantly affected by tumble frequency (maximum speed, $F_{5,\ 5007} = 18.77$ [$P < 0.0005$]; mean speed, $F_{5,\ 5007} = 176.9$ [$P < 0.0001$]); therefore, all speed data were sorted by tumble frequency for further analyses. Mean swimming speed increased throughout exponential growth, peaked as the cultures entered post-exponential growth, and then decreased as the cells entered stationary phase (Fig. 1). This same pattern was observed in maximum swimming speeds (data not shown). The pattern was apparent in all cells, regardless

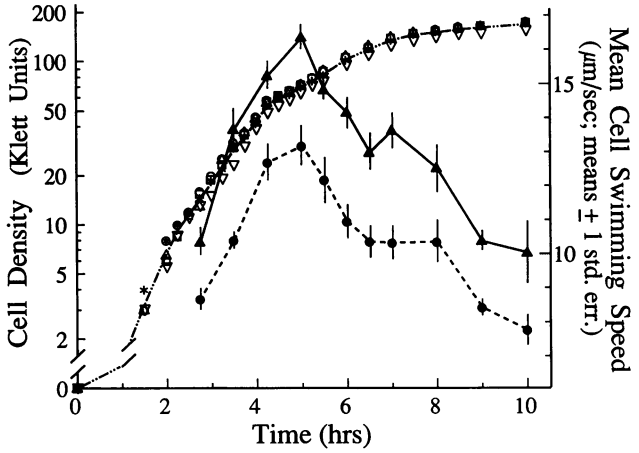

FIG. 1. Mean cell swimming speed and cell density as functions of time after culture initiation. Mean cell swimming speed indicates that the mean of 30 instantaneous swimming speeds recorded over 2 s of sampling was calculated for each cell and used in the analyses. Filled triangles indicate 0 tumbles s^{-1} (no tumbles during the 2 s of observation) and filled circles indicate 0.5 tumble s^{-1} (one tumble during the 2 s of observation). Sample size is five replicate experiments, with the value for each replicate representing the mean of observations on the swimming speed over a 2-s interval for 16 to 54 cells per tumble frequency (mean number of cells per sample = 41.3 ± 0.7). The open symbols represent cell density expressed in Klett units from five replicate experiments. The dashed-dotted line connects the mean Klett value for each time point. Analysis of variance indicated statistically significant effects of culture age (time) on swimming speed for both 0 tumbles s^{-1} ($F_{10, 42} = 16.77$, $P < 0.0001$) and 0.5 tumbles s^{-1} ($F_{10, 42} = 14.32$, $P < 0.0001$). At both tumble frequencies, the peak in swimming speeds at 4 to 5 h was significantly different from the initial (2.75 h) and final (10.0 h) swimming speeds (REGWQ test, $P < 0.01$).

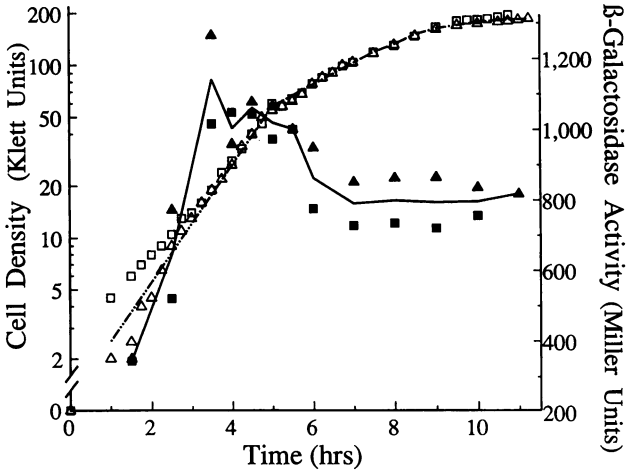

FIG. 2. *flhB* operon expression and cell density as functions of time after culture initiation. Gene expression is measured as β-galactosidase activity and expressed in Miller units. Filled triangles and squares represent β-galactosidase activities from two different replicate experiments, with the solid line connecting mean activities. Open triangles and squares represent cell density expressed in Klett units from each replicate experiments. The dashed-dotted line represents the average growth curve and was fit by eye.

of tumble frequency, but the sample sizes for tumble frequencies of >0.5 s^{-1} were not large enough to allow meaningful statistical analyses (data for tumble frequencies of >0.5 s^{-1} are not shown).

***flhB* operon expression.** Gene expression was low in early-exponential-phase cells and then increased sharply with a peak at mid- to late exponential phase (Fig. 2). Expression declined slowly during post-exponential growth to a relatively high, steady level as the cultures approached stationary phase (Fig. 2). Data from two replicate experiments with similar growth curves are presented in Fig. 2. The experiment was repeated under several different growth conditions, with qualitatively identical results (data not shown). The growth curve of the lysogen MC453 (Fig. 2) was slightly different from that observed in the wild-type strain during swimming speed determinations (Fig. 1). Wild-type cultures grown simultaneously with the lysogens always followed the same pattern as shown in Fig. 1; consequently, the differences observed in the growth of MC453 represent inherent differences in the strains and not differences in growth conditions. In preliminary experiments, MC453 was shown to have the same general swimming speed pattern as RP437, with low swimming speeds in early exponential phase, a peak near early post-exponential phase, and a decline as the cells entered stationary phase (data not shown).

Flagellar synthesis. Variation in flagellin synthesis in wild-type cells closely mirrored changes in swimming speeds (Fig. 3). Cell-specific flagellin contents were low at 2.75 h after culture initiation, when the cells were just becoming motile, peaked at 4 to 5 h (early post-exponential phase), and then declined until the last sample at 10 h, when the cells had entered stationary phase (Fig. 3). The number of flagella per cell increased slightly from 2.75 h to 5.0 h (Table 1), but cell size decreased by 45% during this interval (Table 1; Fig. 4), resulting in a >2-fold increase in the density of flagella per unit cell volume (Table 1). The number of flagella per cell decreased by half between 5.0 h and 10.0 h, but the cells also

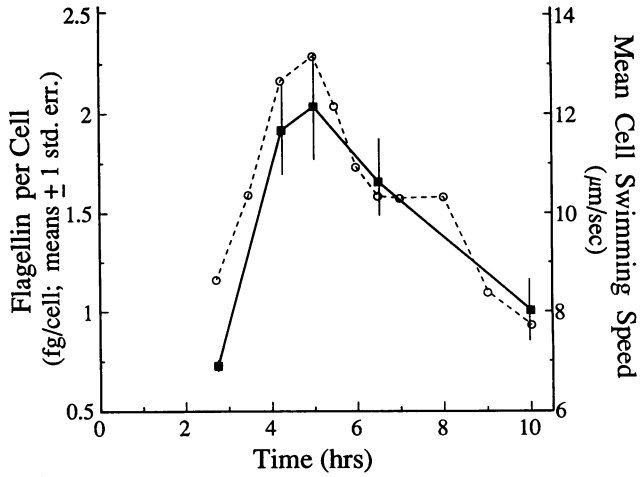

FIG. 3. Flagellin protein per cell as a function of time after culture initiation. The mean cell swimming speed data (open circles) are the same as in Fig. 1 for 0.5 tumble s^{-1}. They are presented for comparison with the flagellin data. Flagellin per cell (filled squares) indicates the amount of flagellin per cell in femtograms. Sample size was three replicate experiments with growth curves similar to those presented in Fig. 1. Analysis of variance indicated statistically significant effects of culture age (time) on flagellin per cell ($F_{4, 10} = 8.82$, $P < 0.0026$). The peak in flagellin per cell at 4 to 5 h was significantly different from the initial (2.75 h) and final (10.0 h) levels (REGWQ test, $P < 0.05$).

TABLE 1. Quantitation of flagellar synthesis and motor torque

Time (h)	n	Cell volume (μm^3; mean ± SE)[a]	No. of flagella/cell (mean ± SE)[b]	No. of flagella/μm^3 (mean ± SE)[c]	fg of flagellin/ flagellum	Motor torque (dyne · cm; mean ± SE)[d]
2.75	37	5.34 ± 0.24	5.57 ± 0.38	1.12 ± 0.10	0.131	
5.0	37	2.91 ± 0.13	6.83 ± 0.51	2.41 ± 0.17	0.298	
10.0	49	1.93 ± 0.08	3.47 ± 0.20	1.97 ± 0.15	0.290	
2.75						ND[e]
5.0	30					$17.5 \times 10^{-12} \pm 1.25 \times 10^{-12}$
10.0	30					$5.87 \times 10^{-12} \pm 0.558 \times 10^{-12}$

[a] The value at each time point is statistically different from the value at each other time point, as determined by analysis of variance ($F_{2, 120} = 133.33$, $P < 0.0001$ in each) and REGWQ test ($P < 0.05$).

[b] The value of each time point is statistically different from the value at each other point as determined by analysis of variance ($F_{2,120} = 23.43$, $P < 0.0001$) and REGWQ test ($P < 0.05$).

[c] The value at each time point is statistically different from the value at each other point as determined by analysis of variance ($F_{2,120} = 18.15$, $P < 0.0001$) and REGWQ test ($P < 0.05$).

[d] Values at 5.0 and 10.0 h are statistically different ($T = 8.63$, $P < 0.0001$).

[e] ND, not determined.

became 33% smaller, resulting in a 20% decrease in flagellar density (Table 1; Fig. 4). It was apparent in preparations for electron microscopy that flagellar length was also variable, with shorter flagella at 2.75 h than at 5.0 and 10.0 h (Fig. 4). However, it was not possible to quantify this difference in electron micrographs because of twisting and breakage of the flagella. Consequently, the amount of flagellin per flagellum was calculated from data presented in Fig. 3 as a quantitative estimation of this change in length. An average flagellum at 5.0 or 10.0 h contained more than twice as much flagellin as did those at 2.75 h (Table 1). This calculation ignores intracellular pools of soluble flagellin, if any. However, the differences in protein per flagellum corresponded with the electron microscopic observations. It is not known how long flagellin monomers remain within the cell before export (30).

Flagellar function. Although video recordings of tethered cells were made at 2.75, 5.0, and 10.0 h, torque calculations were possible only at 5.0 and 10.0 h because the larger cells in 2.75-h-old cultures could not be attached in a manner that allowed them to rotate freely. The flagellar motors of 5.0-h cells generated threefold more torque than did those of 10.0-h cells (Table 1).

DISCUSSION

Adaptive value of motility. As a batch culture grows, metabolically generated changes in the chemical composition of the growth medium can be used as a model of chemical changes in a more natural environment (10, 51). A young culture with a low density of cells has a high concentration of nutrients and a low concentration of toxic metabolic by-products. Clearly, this is a very favorable environment for growth and reproduction. Under these conditions in nature there would be no adaptive advantage gained by moving from such a microenvironment. It is estimated that there are significant metabolic costs of the synthesis of flagella (30), and it is not surprising that cells from the lag and early exponential phases of growth in culture were primarily nonmotile. As the culture grows, however, there is a progressive decrease in available nutrients and a simultaneous increase in the concentration of metabolic by-products (34, 47, 54). The environment of the culture is, therefore, becoming decreasingly advantageous and increasingly stressful. In analogous microenvironments in nature, there would be an increasing likelihood that motility would be adaptively advantageous. It has been known for many years that motility increases between lag and exponential growth phases (1). Correspondingly, we observed a rapid increase in swimming speed during exponential growth, with a peak during the early post-exponential phase (Fig. 1). Functionally, we believe that swimming speed is increased in the post-exponential cells both by having longer flagellar and by having more flagella per unit biomass (Fig. 4; Table 1).

As a culture progresses from post-exponential to stationary phase, it follows from the arguments presented above that the environment becomes more stressful and that the adaptive value of motility should increase or at least remain constant. However, motility decreased during this interval (Fig. 1), as has been noted by others (1). In contrast to the increases in swimming speed between early and post-exponential phases, this decrease was not correlated with large changes in flagellar length or density; length remained constant, and although the absolute number of flagella per cell decreased, cell volume decreased as well, resulting in only a 20% decrease in density (Fig. 4; Table 1). The torque produced by the flagellar motors, however, decreased threefold during this interval (Table 1). Consequently, the decrease in speed appears to be a physiological response and likely reflects a decrease in the overall energy available to stationary-phase cells rather than in the adaptive benefits of motility. The rotary, flagellar motor is powered by proton motive force (PMF) (reviewed in references 5, 31, and 50). Swimming speed increases with increasing PMF to a saturation point, above which changes in PMF do not affect swimming speed (21). Since medium viscosity was constant in our experiments and the load per flagellum (inverse of number of flagella per cell volume) increased only slightly, the decrease in torque during this period means that there would be a decrease in the angular velocity of flagellar rotation in swimming cells (5). The number of protons required per flagellar rotation is fixed (32). Functionally then, the decreased swimming speed between 5.0 and 10.0 h was likely the result of PMF falling below the saturation point for maximal rotation and a decrease in the rate at which protons passed through the flagellar motor. (Note that increases in motor torque could have contributed to the increases in swimming speed between early and post-exponential phases, but this could not be measured in early-exponential cells, as described above.)

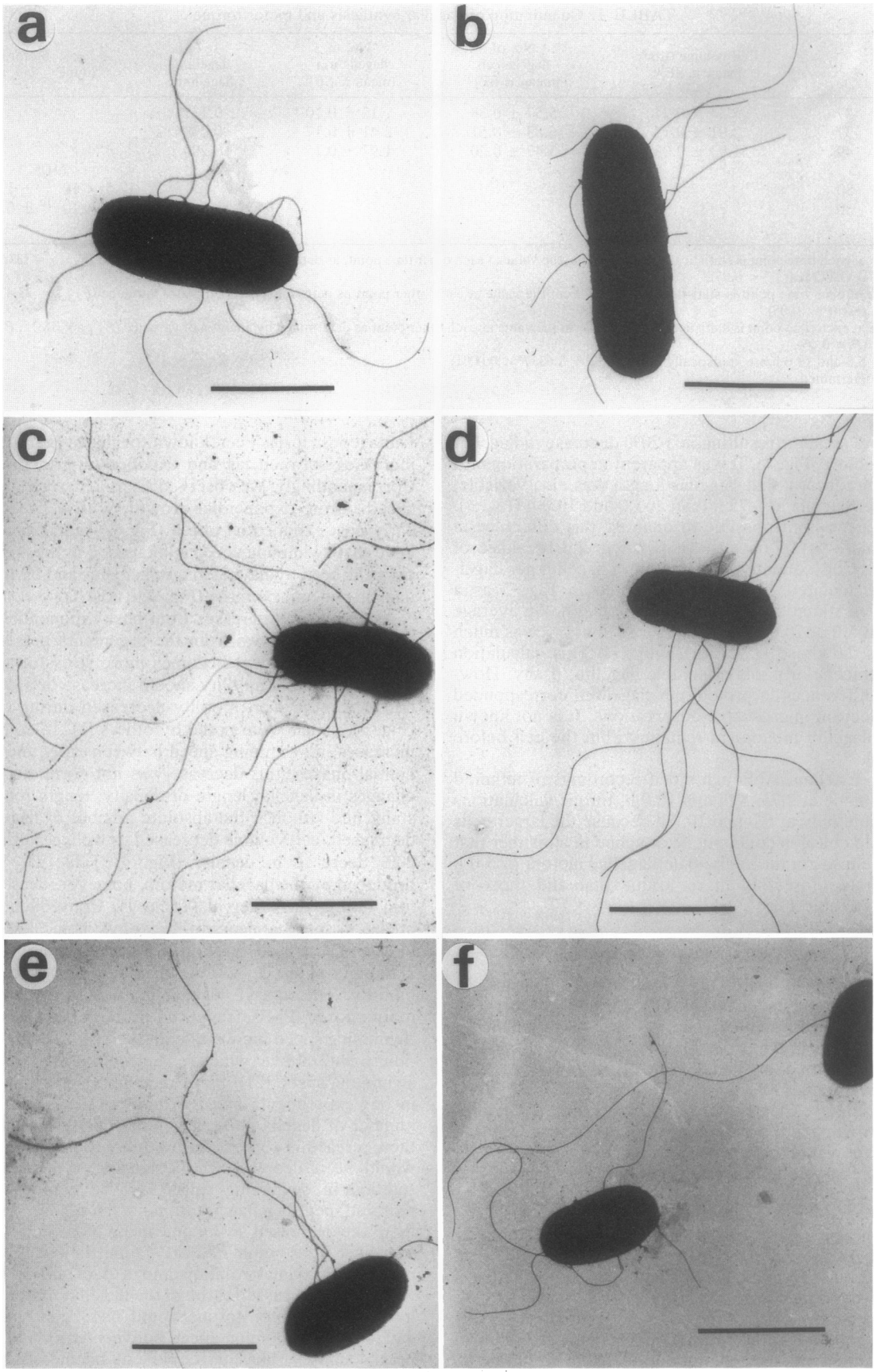

FIG. 4. Electron micrographs of unfixed, negatively stained whole cells. Times after culture initiation: 2.75 h (early exponential phase) (a and b); 5.0 h (early post-exponential phase) (c and d); 10.0 h (early stationary phase) (e and f). Scale bars = 2 μm.

Motility is obviously a necessary component of chemotaxis, and genes for the sensory and signal transduction chemotactic components are part of the flagellar regulon, as noted above. Therefore, products of the *che* and membrane receptor genes will be expressed in response to environmental stress with other genes in the regulon. Cells respond chemotactically to increasingly beneficial but not increasingly detrimental conditions; their behavior is modified by increasing attractant concentrations and decreasing repellent concentrations but not by decreases in attractants or increases in repellents (4, 6). Hence, the machinery necessary for motility and chemotaxis is made in response to stress, but behaviorally the cells react only to decreasingly stressful changes in their microenvironments.

Growth phase-related gene expression. A variety of stress response genes are expressed maximally in post-exponential- or stationary-phase cells. This is consistent with the hypothesis that these culture conditions are analogs of stressful environments in nature. Such growth phase-related modulation is best documented for a variety of genes involved with sporulation in *Bacillus subtilis* (28, 45) but has also been observed in genes or proteins involved with responses to carbon, nitrogen, and phosphate limitation (8, 12, 15, 16, 22, 46), oxygen stress (49), osmotic stress (49), and antibiotic production (14, 19) and in transcription of the *B. subtilis* flagellin gene (33). Consistent with these other stress responses, we observed that transcription of the *flhB* operon and synthesis of flagellin protein were low in late-lag/early-exponential cells but increased rapidly as the culture proceeded into mid- and late exponential phases, with a peak in flagellin per cell in early-post-exponential cells (Fig. 3 and 4). Komeda and Iino (24) reported that transcription of *fliC*, the flagellin gene, increased from lag and early exponential phases to late exponential phase but did not report on later growth phases.

It is possible that there is growth phase-related modulation of gene expression within the flagellar regulon in addition to the overall modulation of expression from the regulon as measured by *flhB* operon expression and flagellin protein production. The amount of flagellin protein per flagellum increases as flagellar length increases during exponential growth (Fig. 4; Table 1). Presumably, increases in flagellar length do not necessitate increases in basal body components or other products of the remaining flagellar genes, and unless these are made in excess (and wasted) throughout post-exponential and stationary phases, there must be modulation of the relative levels of expression of *fliC* and all other genes in the regulon.

ACKNOWLEDGMENTS

We thank S. Block for helpful discussions and for providing computer software which aided torque calculations, D. Reed and P. Raimondi for helpful discussions, and M. Hulett and B. Bouck for use of equipment. Electron microscope facilities were provided by the Electron Microscope Facility of the UIC Research Resources Center.

This work was supported by Public Health Service grants AI 18985 (P.M.) and AI 08547 (C.D.A.) from NIH.

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