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# Growth Phase-Coupled Alterations in Cell Structure and Function of *Escherichia coli*

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***Escherichia coli*** **cultures can be fractionated into more than 20 cell populations, each having a different bouyant density and apparently representing a specific stage of cell differentiation from exponential growth to stationary phase (H. Makinoshima, A. Nishimura, and A. Ishihama, Mol. Microbiol. 43:269–279, 2002). The density increase was found to be impaired at an early step for a mutant *E. coli* with the disrupted *rpoS* gene, which encodes the RNA polymerase RpoS (sigma-S) for stationary-phase gene transcription. This finding suggests that RpoS is need for the entire process of cell density increase. In the absence of RpoF sigma factor, the flagella are not formed as observed by electron microscopy, but the growth phase-coupled density increase takes place as in wild-type *E. coli*, confirming that the alteration in cell density is not directly correlated with the presence or absence of flagella. In the stationary-phase cells, accumulation of electron-dense areas was observed by electron microscopic observation of bacterial thin sections. By chemical determination, the increase in glycogen (or polysaccharides) was suggested to be one component, which contributes to the increase in weight-to-volume ratio of stationary-phase *E. coli* cells.**

During the growth transition of *Escherichia coli* culture from the exponential growth to the stationary phase, a number of morphological and physiological changes take place, including cell volume decrease, cell shape change, nucleoid compaction, alteration in cell wall composition, and accumulation of some storage materials (9, 11, 17, 28). In parallel, the pattern of gene expression changes in such a way that the growth-related genes are mostly switched off; instead, the stationary-phase-specific genes are expressed (for reviews, see references 10, and 22). More than 100 stationary-phase-specific genes have been identified, and these genes appear to be expressed sequentially in a defined order (12). These findings, taken together, indicate a time-dependent alteration in the *E. coli* phenotype even after the cessation of cell growth. As an initial step to gain insight into the genetic program underlying the prolonged survival of *E. coli* in the stationary phase, it is important to identify the expression order of the stationary-phase genes.

Exponential-phase cultures are considered to be homogenous even though cell populations differ in their stage of the cell cycle (16). On entry into the stationary phase, however, the heterogeneity of the cell population increases, because the stationary-phase cultures include various types of cell on various pathways toward different cell fates such as dormancy and cell death (9, 11, 17, 28). Accumulation of advantageous mutations in the stationary phase leads to further increases in the cell diversity (6). In addition, it remains unsolved whether entry into the stationary phase begins at a specific stage of the bacterial cell cycle, such as the eukaryotic $G_0$ phase. If bacterial cells enter the differentiation pathway to the stationary phase at any stage of the cell cycle, the heterogeneity in the cell population should further increase in the stationary phase. For detailed characterization at the molecular level of *E. coli* cell alteration during the transition into the stationary phase, it is necessary to use homogenous cell populations. To date, however, the growth phase-coupled changes in the global gene expression have been studied using random cultures.

For synchronization of large-scale *E. coli* cultures, mutants with temperature-sensitive mutations in the DNA replication apparatus have been used, but such an invasive method as heat treatment, which leads to changes in the gene expression pattern, cannot be employed in studies of growth phase-coupled alteration in gene expression. We then made systematic attempts to fractionate random cultures of *E. coli* into homogenous cell populations. By using Percoll gradient centrifugation, we have succeeded, for the first time, in physically separating *E. coli* cultures into more than 20 cell populations (26). The cell separation was based on the difference in buoyant density. Moreover, the density shift was found to take place in a discontinuous manner because each cell population apparently formed a single band on the Percoll gradient. Although we identified some molecular markers characterizing each cell population, we still do not know why the cell density increases in the stationary phase and how the density shift takes place in discontinuous manner.

To gain insight into the molecular basis of the cell density increase in the stationary phase, we have started to identify the gene(s) involved in each step of the density increase. In this

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TABLE 1. Bacterial strains and plasmid used in this study

| Strains and plasmid | Relavant characteristics | Reference or source |
|---|---|---|
| Strains | | |
| W3110 (A) | Wild type | 14 |
| KP7600 | W3110 (A) *lacI*$^q$ *lacZ*ΔM15 *galK2 galK22* | T. Miki (unpublished) |
| JD22323 | KP7600 *rpoS* | T. Miki (unpublished) |
| JD22327 | KP7600 *rpoF* | T. Miki (unpublished) |
| JD24004 | KP7600 *rpoH* | T. Miki (unpublished) |
| Plasmid | | |
| pRpoS01 | *rpoS* sequence in pUC18 | This study |

report, we describe evidence indicating the involvement of the RNA polymerase RpoS sigma subunit, which plays a key role in transcription of at least some stationary-phase genes. We also analyzed morphological characteristics of each population by using different methods of electron microscopy.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The bacterial strains used in this study are listed in Table 1. Cells were grown at 30 or 37°C under aeration in Luria broth (LB) medium. Cell growth was monitored by measuring the turbidity at 600 nm. The culture conditions used in all the experiments were fixed as follows: a few colonies from cultures grown overnight on LB agar plates were inoculated into 2 ml of fresh LB medium, the overnight culture was diluted 1,000-fold into 50 ml of fresh LB medium, and the incubation was carried out at 37°C with shaking at a constant rate (120 rpm).

**Percoll gradient centrifugation of *E. coli* cultures.** Percoll gradient centrifugation of *E. coli* cultures was carried out as described previously (26). In brief, the cells were harvested by centrifugation and resuspended in appropriate volumes of phosphate-buffered saline (PBS). A 0.5-ml cell suspension was layered on top of the preformed 80% Percoll gradient. After centrifugation, the Percoll gradient was fractionated using a Piston gradient fractionator (Biocomp).

**Electron microscopy of negatively stained *E. coli* cells.** Aliquots of cell suspension were placed, without fixation, in a drop of 2% sodium phosphotungstic acid (pH 7.0 or 4.5) on a Collodion-coated grid. After the solution was removed with a filter paper, the grid was air dried. This procedure took only 10 s. The samples were directly observed with a JEM-1200EXII electron microscope (JEOL, Tokyo, Japan). Micrographs were obtained at an accelerating voltage of 80 kV.

**Electron microscopy of thin sections of *E. coli* cells.** Specimens were processed by the method of Beveridge et al. (3). In brief, *E. coli* cells were washed, fixed in 2% glutardialdehyde, and treated with 1% $OsO_4$. After dehydration in increasing concentrations of ethanol, the cells were embedded in Epon capsules containing Poly/Bed 812 (Polyscience, Inc.) at 60°C and thinly sectioned using a type II ultramicrotome (LKB). Specimens were stained with uranyl acetate and viewed at 100 kV and 20 μA under a JEM-100CX electron microscope (JEOL).

**Quantitative immunoblot analysis.** A quantitative Western blot analysis was carried out by standard methods as described previously (26). In brief, proteins were precipitated with 5% trichloroacetic acid on ice and then separated on sodium dodecyl sulfate–7.5 or 15% polyacrylamide gels. Proteins in the gels were directly electroblotted onto polyvinylidene difluoride membranes (Nippon Genetics). The membranes were blocked overnight with 5% skim milk in PBS, probed with polyclonal antibodies against each protein, washed with 0.5% Tween 20 in PBS, and incubated with goat anti-rabbit immunoglobulin G conjugated with hydroxyperoxidase (Cappel). The blots were developed with an enhanced chemiluminescence kit (Amersham-Pharmacia Biotech). The image was analysed with a LAS-1000 Plus lumino-image analyzer and IMAGE GAUGE (Fuji Film).

**Determination of glycogen.** Polysaccharides were determined using the anthrone-sulfuric acid method (8). In brief, cells were treated for 20 min at 95°C in 24% KOH. After extraction with 75% ethanol, anthrone-sulfuric acid solution was added at a final concentration of 0.07% anthrone. After incubation for 20 min at 95°C, the absorbance was measured at 620 nm.

## RESULTS

**Influence of sigma gene disruption on the centrifugation pattern of *E. coli* cells.** RpoS, the recognition factor for some stationary-phase-specific gene promoters, is a critical factor for the switching in the global gene expression pattern during the early phase of transition from exponential growth to the stationary phase. As an initial attempt to examine the possible relationship between the stepwise increase in *E. coli* cell density and the expression of stationary-phase genes, we tested the role of RNA polymerase RpoS in the cell density shift. For this purpose, *E. coli* mutants, each defective in one of the sigma subunit genes, were subjected to Percoll gradient centifugation.

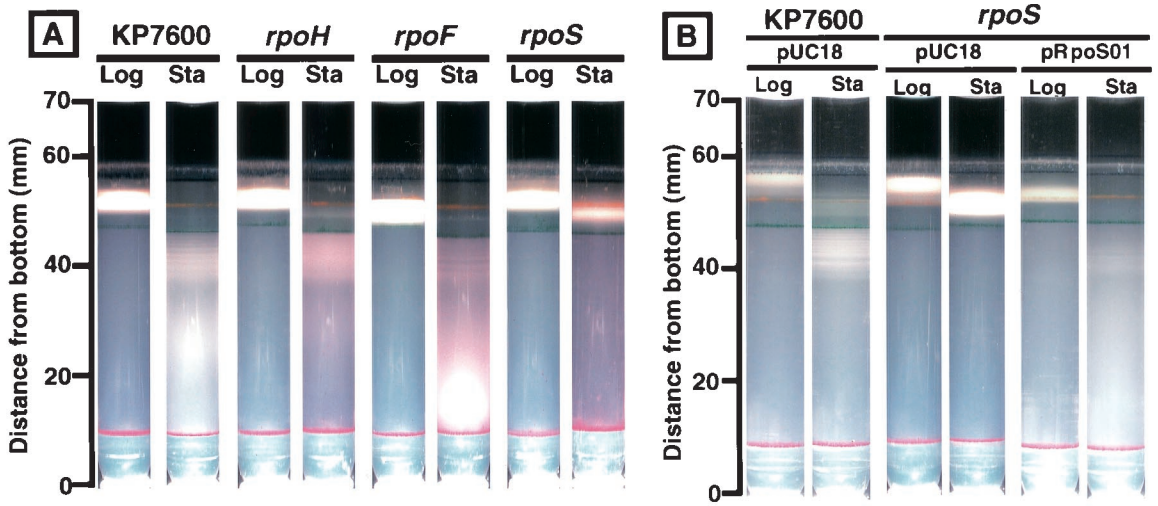


FIG. 1. Percoll gradient centrifugation of *E. coli* mutants lacking sigma factors. (A) Wild-type KP7600 (W3100 derivative) and mutants lacking RNA polymerase sigma subunits (RpoH, RpoF, and RpoS) were grown on LB medium at 37°C. The cultures were subjected to centrifugation on a Percoll gradient (80%) by the established procedure (26). (B) The *rpoS* disruptant carrying the *rpoS* expression plasmid, pRpoS01, was subjected to Percoll gradient centrifugation. As controls, wild-type *E. coli* KP7600 with the vector pUC18 and the *rpoS* disruptant with pUC18 were centrifuged in parallel. Log, logarithmic growth; Sta, stationary phase.

In the exponential phase (6 h after inoculation), the wild type and all mutants formed apparently single bands with low densities (Fig. 1A) (note that the exponential-phase cultures formed multiple bands in high-resolution gradients with low concentrations of Percoll [26]). In the stationary phase (18 h after inoculation), the density of mutants with the disrupted *rpoF* and *rpoH* genes increased, as did that of the wild-type KP7600 strain (Fig. 1A). The patterns were slightly different when compared at a fixed time (18 h after inoculation) of the cell culture. For instance, the *rpoF* disruptant formed cell bands with higher densities than those of the wild type and the *rpoH* disruptant. This difference is, however, attributable to the slight difference in the growth rate between these strains. Under the culture conditions used, the growth of the *rpoF* disruptant was slightly but significantly faster than that of the wild type, reaching the stationary phase earlier than the wild-type cells did, but the *rpoH* disruptant grew as fast as the wild type (data not shown). In fact, the growth phase-dependent transition in the centrifugation pattern was essentially the same among the wild type and the *rpoF* and *rpoH* disruptants (data not shown). RpoF is involved in transcription of the flagellum-chemotaxis regulons (1). As expected, no flagella were found associated with the *rpoF* disruptant (see Fig. 3). This finding excludes the possibility that the cell-cell aggregation that occurs by formation of flagellum networks is involved in the fast sedimentation of stationary-phase cells in Percoll gradient.

In contrast, the *rpoS* disruptant apparently formed a single low-density band even in the stationary phase (after 18 h of culture), and the growth phase-coupled density increase was small (Fig. 1A). Even after prolonged culture, no further increase in the cell density was observed for this *rpoS* disruptant (data not shown). Thus, we concluded that the growth phase-coupled increase in *E. coli* cell density ceased at an early stage for the *rpoS* disruptant. This finding indicates that the RpoS sigma factor is involved in expression of the genes, which are needed at an early step of the cell density increase.

To confirm that the alteration in Percoll gradient centrifugation pattern for the *rpoS* disruptant is due to the lack of RpoS, we introduced the intact *rpoS* gene into the *rpoS* disruptant by using a gene expression vector under the control of its own promoter. The cell density of the *rpoS* mutant with the *rpoS* expression vector increased as much as did that of the wild-type *E. coli* (Fig. 1B), and the RpoS protein was expressed at detectable levels on entry into stationary phase (data not shown). The Percoll gradient pattern of the wild-type *E. coli* did not change after addition of the *rpoS* expression vector (Fig. 1B).

**Decrease in flagellum number for the stationary-phase *E. coli* cells.** The number of flagella in the wild-type *E. coli* strain, as observed by electron microscopy after negative staining without fixation, increased with the increase in cell density (Fig. 2A and B) and reached maximum at the late exponential phase (Fig. 2C). The increase in the number of flagella is in good agreement with the increase in flagellin (FliC) content (see below). The increased number of flagella on cells is also correlated with the level of flagellum tangling. On entry into the late stationary phase, however, the number of flagella decreased again (Fig. 2D and E). The loss of flagella in the late stationary phase may not be the factor which leads to the increase in cell density, because the *rpoF* mutant lacking flagella stays at low densities (Fig. 1).

To test the possible relationship between cell aggregation and the increase in cell density, the cultures were fractionated by Percoll gradient centrifugation and then each Percoll fraction was subjected to electron microscopic observation. No significant difference was observed in the level of cell aggregation between Percoll fractions. Cells recovered in low-density fractions always contained more flagella than did cells with high buoyant densities (data not shown). After prolonged storage, without shaking, of the stationary-phase cells with decreased numbers of flagella, cell aggregates were formed (data not shown). This cell aggregation appears to involve the formation of a network of stationary-phase pili (S. Aizawa, unpublished data). Although the type of pili involved in cell-cell aggregation remains unidentified, the chance of pilus network formation should increase in the absence of flagella on cell surface.

Next we also observed the growth phase-coupled alteration in cell structure in mutants lacking one of the sigma subunits. Figure 3A and B shows the electron micrographs for the *rpoS* mutant defective in sigma S. To our surprise, there were more flagella than in wild-type *E. coli* in both the exponential and stationary phases (compare Fig. 2 and 3). The number of flagella decreased in the stationary phase in wild-type *E. coli* (Fig. 2) whereas it remained at a high level even after an 18-h culture of the *rpoS* mutant (Fig. 3B), suggesting that the shutoff control of flagellum formation does not take place in the *rpoS* mutant. The gene(s) involved in the shutoff of flagellum formation may be under the control of RpoS. Alternatively, the level of functional RpoF for transcription of the flagella-chemotaxis regulon remains high in the absence of RpoS, which otherwise competes with RpoF in binding to the core RNA polymerase (25).

To gain insight into the mechanism of the growth phase-coupled change in flagellum number, we measured the level of RpoF and FliC (flagellin subunit of the flagella) for both the wild-type and the *rpoS* mutant *E. coli* strains. In wild-type *E. coli,* the level of RpoF relative to RpoA (RNA polymerase alpha subunit) (note that the level of RpoA stays constant throughout the growth phase [10, 12]) decreased in the stationary phase to about one-third of the maximum level, which is found at the late exponential phase (Fig. 4). In parallel, the level of FliC decreased to about half of the exponential-phase level. In contrast, the levels of RpoF and FliC in the *rpoS* mutant slightly increased on entry into the stationary phase. Thus, we conclude that in wild-type *E. coli*, the level of RpoF decreases concomitantly with the production of stationary-phase proteins, which are under the control of RpoS. In the absence of RpoS, these stationary-phase proteins are not produced and the level of RpoF remains high, leading to continued flagellin synthesis and flagellar formation.

In the absence of flagella, the cell surface pili could be easily identified (Fig. 3). From the morphological characteristics, type I fimbriae appear to be associated with the stationary-phase cells (Aizawa, unpublished).

**Increase in the levels of storage materials in the stationary-phase *E. coli* cells.** To check for possible changes in the intracellular structure of *E. coli* cells during the increase in cell bouyant density, we investigated thin sections of *E. coli* cells by

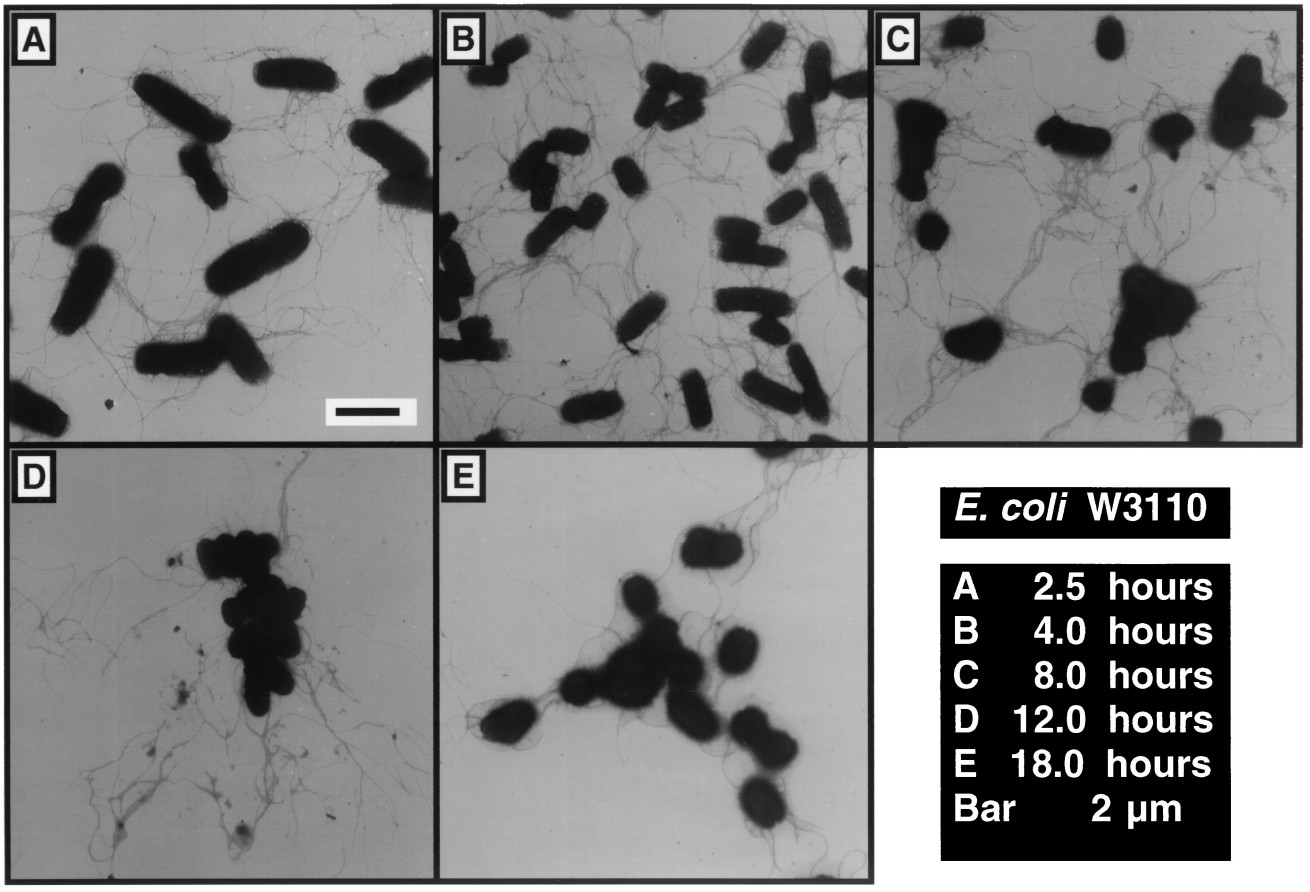


FIG. 2. Electron micrograph of wild-type *E. coli*. Wild-type *E. coli* W3110 was grown in LB medium for 2.5 h (A), 4 h (B), 8 h (C), 12 h (D), and 18 h (E). A cell suspension was directly placed on a Collodion-coated grid. The specimens were negatively stained with 2% sodium phosphotungstic acid and examined under a transmission electron microscope. Bar, 2 μm in all the micrographs.

electron microscopy (Fig. 5). On entry into the stationary phase, the nucleoid became more compact for both the wild type and the *rpoS* mutant, with a concomitant increase in the area of cytoplasm. The density of the cytoplasm as observed by electron microscopy increased for the stationary-phase cells compared with the exponential-phase cells, and this increase in electron density was greater for the wild-type cells. The increase in electron density is due to the appearance of electron-dense areas of about 10 nm in diameter. Time course experiments indicated the growth time-dependent increase in the number of these electron-dense areas (data not shown). The electron micrograph is not consistent with the notion that the volume of cytoplasm decreases in the stationary phase. The cell wall became thicker, with an increase in the amount of amorphous peptideglycan-like structure (Fig. 5), in agreement with previous observations (9, 17, 28). At present, however, it cannot be ruled out that the apparent increase in cell wall width arose during sample preparation such as cell fixation.

Accumulation of storage materials, including trehalose, glycine betaine, glycogen, polyhydroxyalkanoic acids, and polyphosphate, has been recognized for stationary-phase bacterial cells (11, 12); of these, glycogen, polyhydroxyalkanates (27), and polyphosphate (7) are known to form inclusion bodies. We measured the intracellular concentrations of DNA, RNA, protein, carbohydrate, and lipid in *E. coli* W3110 at various stages of cell growth. A significant increase was observed only for glycogen (or polysaccharides) (Fig. 6). This finding suggests that at least one component which contributes to the increase in cell density is glycogen. The size of glycogen inclusion bodies stays constant, but their number in a cell increases. Thus, the discontinuous transition of the cell density may be due to abrupt formation of glycogen inclusion bodies when the intracellular glycogen reaches a threshold level. The level of polysaccharides reached a maximum at 12 h of culture (Fig. 6). Since the cell density continued to increase, an additional factor(s) may be involved in the increase in cell density after this time point.

## DISCUSSION

**Increase in the cell buoyant density for stationary-phase *E. coli* cells.** The size of *E. coli* cells has been reported to decrease in the stationary phase (reviewed in references 9, 17, and 28). An augument against this concept has been proposed, which states that the decrease in cell volume is due to artifacts that arose after cell fixation for microscopic observation (for instance, see reference 5). Accordingly, attempts to separate *E. coli* cells on the basis of cell size difference were unsuccessful; instead, we succeeded in separating *E. coli* cell populations by

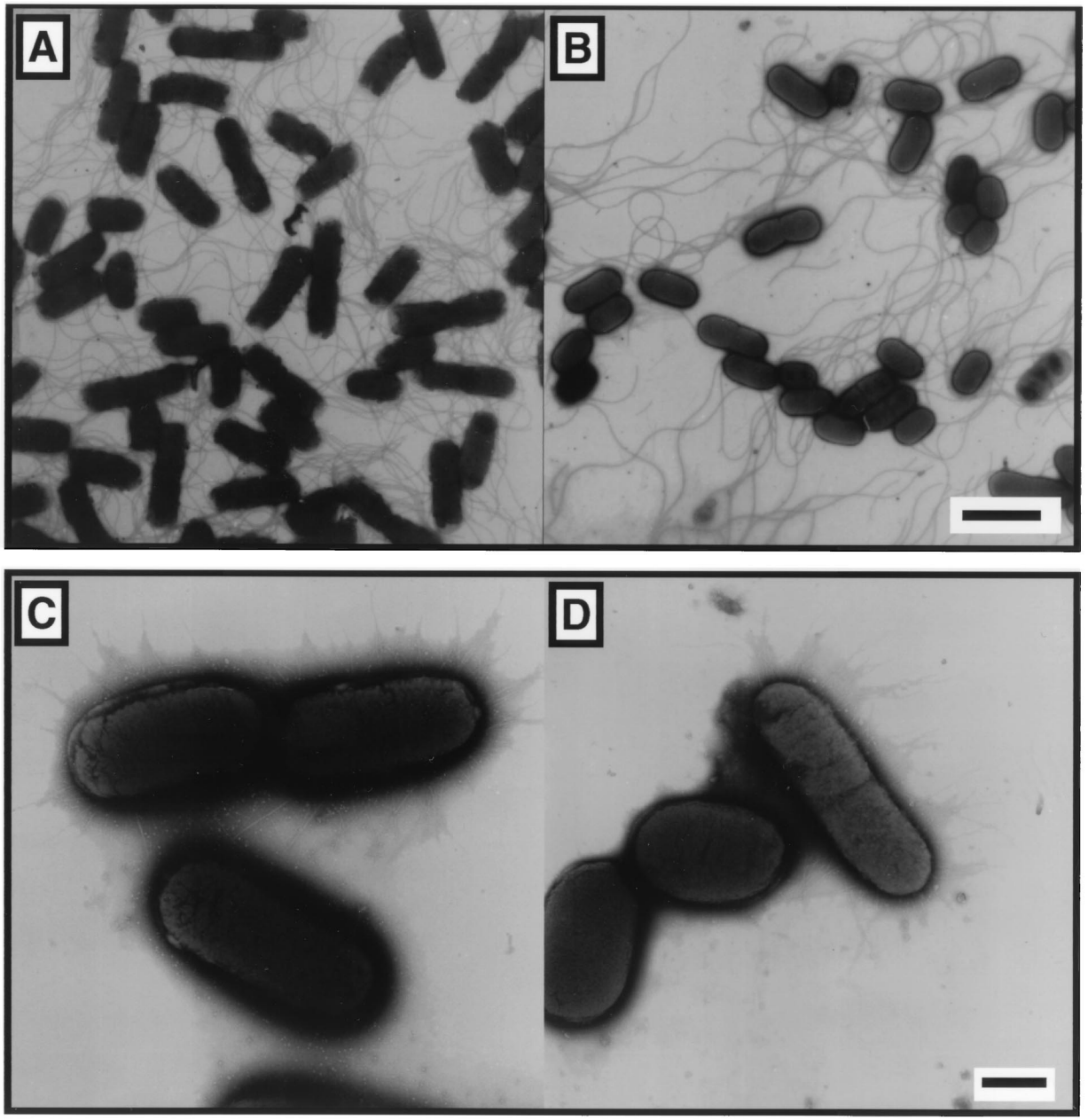


FIG. 3. Electron micrograph of *E. coli rpoS* and *rpoF* disruptants. Wild-type *E. coli rpoS* (A and B) and *rpoF* (C and D) disruptants were grown in LB medium for 4 h (A and C) and 18 h (B and D). Electron micrographic observation was carried out as in the experiment in Fig. 2. Bars, 2 μm (A and B) or 0.5 μm (C and D).

Percoll density gradient centrifugation (26) (Fig. 1). Percoll consists of colloidal silica that is coated with polyvinylpyrrolidone to protect the cells from toxic effects. Since the viscosity is lower than that of other reagents that give the same densities, materials as large as marker beads and bacterial cells quickly sediment to positions characteristic of their densities. Thus, the major factor in cell separation by this newly developed method is the difference in cell buoyant density. This finding agrees with the fact that the weight-to-volume ratio increases from exponentially growing to stationary-phase cells, as measured by transmission electron microscopy and densitometric image analysis, although the bacterial biomass is more variable than was previously assumed from volume-based measurements (23).

Changes in the chemical composition should influence the cell buoyant density or the weight-to-volume ratio, including the increase in the levels of membrane cyclopropyl derivatives as a result of replacing unsaturated fatty acids, the increase in the levels of compatible solutes with osmoprotection activity such as trehalose and glycine betaine, the accumulation of storage compounds such as glycogen and polyphosphate, the decrease in the levels of polyamines, and the decrease in free water content (9, 11, 17, 28). In addition, the spatial rearrangement of cell components within the stationary-phase *E. coli*

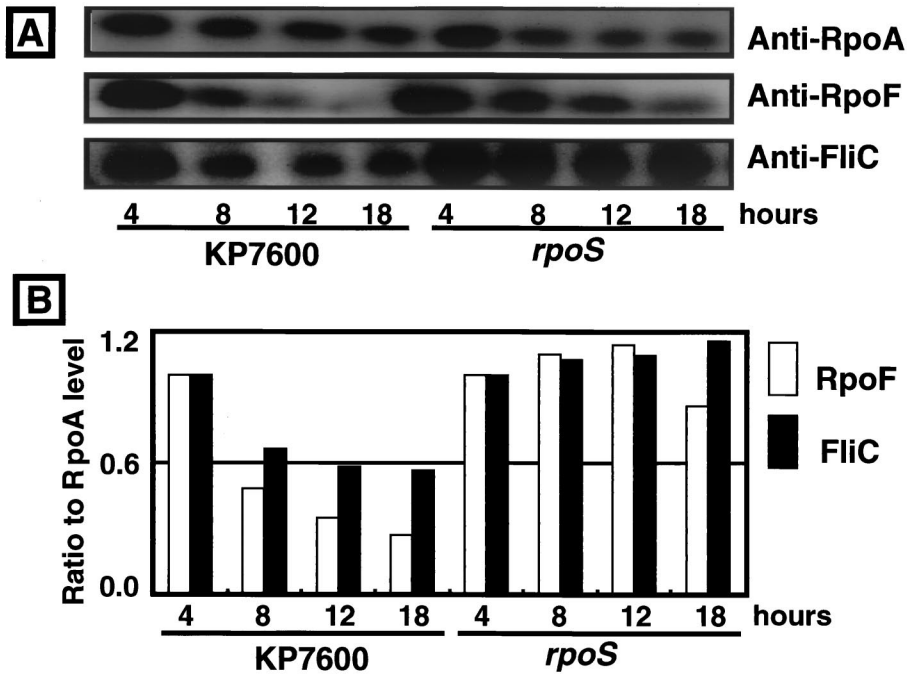


FIG. 4. Determination of the RpoF and FliC levels. (A) Wild-type *E. coli* KP7600 and its *rpoS* disruptant were grown in LB medium for the times indicated. Crude cell extracts were prepared as described by Jishage et al. (15), and the amounts of RpoA, RpoF, and FliC proteins were determined by quantitative immunoblotting by the method of Makinoshima et al. (26). (B) The intensity of immunostained filters was measured with LAS (Fuji), and the sigma-F (RpoF) and flegellin (FliC) levels are shown as values relative to the level of the RNA polymerase alpha subunit (RpoA).

cells, such as the compaction of nucleoid by replacing associated proteins (30), the decrease in DNA superhelicity (13, 19), and the decrease in the levels of associated polyamines (31), might result in the change in the cell density.

The electron microscopic observations using conventional methods indicated the decrease in cell volume in stationary-phase *E. coli* (for reviews, see references 9, 17, and 28). Conventional methods for the preparation of bacterial cells for electron microscopy result in artifacts due to cell fixation, staining, and dehydration (for instance, see reference 5). Direct observation of *E. coli* cells without fixation (Fig. 2) indicates that the growth phase-coupled alteration in cell size is not as great as that observed after cell fixation. By electron microscopic observation of *E. coli* thin sections and chemical determination of cell components, we identified glycogen as one factor that affects the increase in cell density. However, a number of additional factors may be involved in the density shift. For instance, the content of polyphosphate bodies (PPB) in bacteria increases in the stationary phase (5). As well as being a storage material for excess ATP (18) and a regulator for RNA polymerase sigma functions (20), PPB has an as yet unidentified function, possibly in detoxification by sequestering heavy metals. If this is the case, the electron-dense granule observed for the stationary-phase wild-type *E. coli* may also be the metal-sequestered PPBs. Polyhydroxyalkanoic acids are carbon and energy storage polymers that accumulate in inclusion bodies in many bacteria in response to environmental conditions. The PHA inclusion bodies not only are a source of carbon and energy but also act as an anchoring platform for hydrophilic proteins, such as PhaP, a bacterial storage protein (27).

**Growth phase-coupled variation in flagellum number.** The motility and chemotaxis of bacteria allow cells to move away from stressful environments. The motility of *E. coli* in batch cultures, as measured by cell swimming speed, is low in early-exponential-phase cells, peaks in late exponential phase, and declines after the transition to early stationary phase (2). The increase in swimming speed may be correlated with the increase in the number of flagella per cell (and the flagellar length). The decrease in speed may also be correlated with the decrease in torque produced by the flagellar motors, reflecting a decrease in proton motive force. In this study, we found that the growth phase-coupled change of *E. coli* motility is indeed correlated with the number of flagella per cell. The reduction in flagellar density during the growth transition into the stationary phase is related to the decrease in the level of FliC flagellin protein.

The expression, synthesis, assembly, and function of flagella and motility requires the expression of more than 50 genes, constituting a large and coordinately regulated flagellar regulon (24). Within the flagellar regulon, the genes are divided into three temporally regulated, hierarchical transcription classes (21). The class 1 genes encode the master flagellum regulator FlhDC, which activates the transcription of class 2 genes, including those encoding the RpoF sigma factor (or FliA) and anti-sigma factor FlgM for RpoF (4). Transcription of the *fliC* gene is under the control of the RNA polymerase holoenzyme containing RpoF. On reaching a certain cell density at the late exponential phase, the two-component system, QseBC, monitors the quorum-sensing signal and activates transcription of the *flhDC* operon (29). The increase in flagellum number in the late exponential phase is, at least in part,

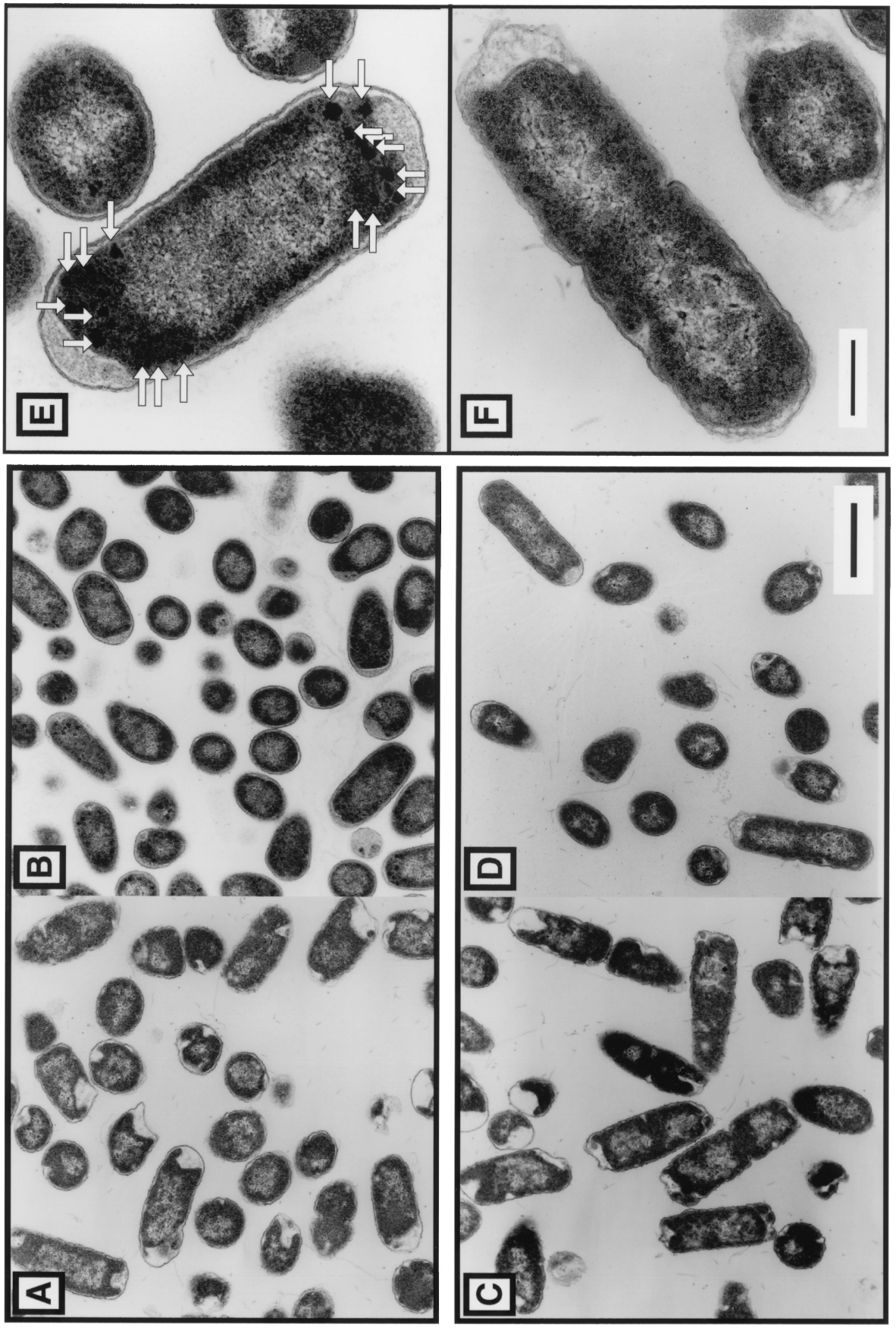


FIG. 5. Electron micrograph of thin sections of wild-type and *rpoS* mutant *E. coli* cells. Wild-type *E. coli* KP7600 (A and B) and its *rpoS* disruptant (C and D) were grown in LB for 4 h (A and C) and 18 h (B and D). Thin sections were prepared and observed with an electron microscope as described in Materials and Methods. (E and F) Expanded micrographs of 18-h cultures of the wild type (B) and *rpoS* mutant (D). Bars, 1 μm (A to D) or 0.33 μm (E and F). Arrows in panel E show some of the stationary-phase-specific electron-dense areas.

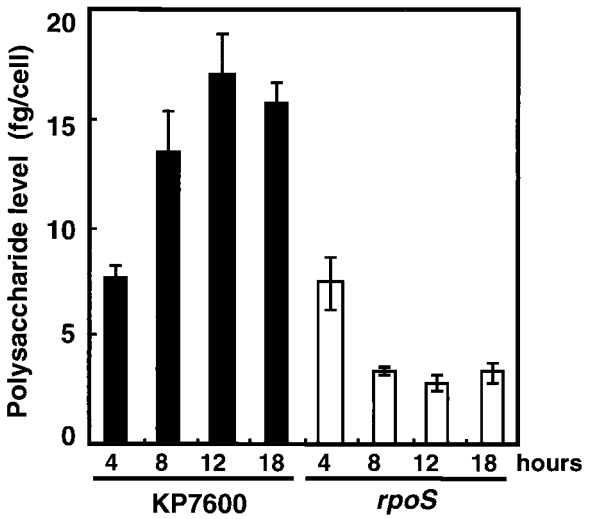


FIG. 6. Intracellular level of glycogen. Wild-type *E. coli* 7600 and its *rpoS* disruptant were grown in LB medium for the indicated times. The glycogen level was determined by the anthrone-sulfuric acid method (8).

due to the increase in the RpoF level. Activation of the pre-existing RpoF by dissociation of its anti-sigma factor FlgM may also be involved in the increase in flagellum production.

The decrease in flagellar numbers in the stationary phase is correlated with the decrease in the level of RpoF (Fig. 4). Furthermore the decrease in the level of RpoF apparently occurs in parallel with the increase in the level of RpoS, which is needed for transcription of some stationary-phase-specific genes. Among the seven sigma factors in *E. coli*, two minor sigma factors, RpoN and RpoF, are present, besides the major RpoD sigma, in exponential-phase cells (15). However, RpoF appears to be stored as an inactive form as a complex with FlgM, indicating that the level of functional sigma-F is controlled by both the synthesis and the activity of RpoF protein. The decrease in the level of FliC protein in the stationary-phase *E. coli* cells may be attributed to the decrease in both protein level and activity of RpoF.

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