UNIQUE MORPHOGENETIC AND CELL DIVISION BEHAVIOR IN POPULATIONS OF *Escherichia coli* TREATED WITH 6-AMIDINOPENICILLANIC ACID

By

HARVEY ANTONIO BLALOCK

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Harvey Antonio Blalock

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A novel physiological system is described in *Escherichia coli* by means of which cellular morphology and division are perturbed in such a way that division occurs repeatedly at non-deterministic sizes and loci for a given exponential rate of mass increase. *E. coli* cells in balanced exponential growth, when treated with the novel penicillin derivative FL-1060 (Mecillinam, 6-amidinopenicillanic acid), assume an osmotically stable spherical configuration associated with retarded division. Under these conditions, dry weight of cells/ml and optical density at 450 nm increase exponentially at the same rate as in untreated cultures. Viable count and total particles per ml increase at a lower rate. This results in the development of a population of spheroid cells which divide symmetrically at non-characteristic volumes and (presumably) ages. Upon removal of excess antibiotic, either by washing of the cells, or by treatment with penicillinase, the anomalous morphology and division patterns persist for at least five divisions.

The generation of a system in which the cycle of cell division is thus separated from the cycle of replication and distribution of

cell constituents as measured by mass increase provides a potentially powerful probe of the control of both morphogenesis and division. One application of this probe is to determine whether or not the rate of synthesis of cellular envelope components is controlled in such a way as to maintain a quantity of envelope consistent with the altered surface-to-volume ratios accompanying a change from a cylindrical to spherical morphology. Experiments were done to measure the uptake and fixation of 2,6-diaminopimelic acid (DPM) into the cell-wall fraction of a culture of E. coli ATCC 13071, an absolute DPM auxotroph, in the presence and absence of FL-1060. It was found that cells in the spherical morphology accumulate labeled DPM at lesser rates than do untreated cells on the basis of mass. This difference does not conform to the surface-to-volume ratios expected.

INTRODUCTION

It has become increasingly clear in the past few years that the bacterial cell cycle is as delicately poised with regard to the segregation and partitioning of cellular components as it is in the orderly synthesis of macromolecular species (4, 11). A key role in the separation as well as in the partitioning of such components is ascribed to the cell envelope (4, 9, 10). In this dissertation, I present a system which offers unique opportunities to probe the behavior of the mechanisms which synthesize the cell envelope under a variety of physiological conditions.

Perhaps the most difficult aspect of studying the cell cycle is that all experimental approaches in some way depend upon perturbing the normal functioning of the cell and examining the effect(s) of the perturbation on a variety of parameters. Whether the perturbation is transient, as in the case of a variety of division-inhibitory compounds, or permanent, as in the case of a variety of division mutants, it is thus far not possible to devise a system by virtue of which only a single cellular function is altered leading to alteration in the division process. In the absence of such a system, there is great difficulty in ensuring that effects observed in systems with altered division are not artifactual and therefore trivial, or even more confusing, parallel sequela to the same common metabolic cause as the division alteration.

In spherical organisms, where all points in the growing envelope are putatively equivalent, minimal coordination of envelope and cytosol biosynthesis requires only temporal regulation. This may be the case in micrococci, but streptococci, which divide along parallel planes, are clearly more regular in their division orientation, while sarcinae, which divide in alternating orthogonal planes show the greatest constraints in the orientation of their divisions.

In bacilliform bacteria, control of the cell cycle is further complicated by the necessity for controlling shape as well as size at division. The rod-shaped organism must regulate the synthesis of its envelope in such a way as to provide for the extension of the cell along the longitudinal axis, punctuating this extension with the formation of septa perpendicular to the extension axis at each division. Indeed, examination reveals that bacilli appear to control the disposition of division loci across generations in a way that superficially seems similar to the way that DNA replication is regulated across generations (12).

The above observation is substantiated by the finding that conditional division mutants growing rapidly accumulate division potential (i.e., nascent loci for septation) when placed at the restrictive temperature (12). De Pedro and Canovas (25) have shown that such mutants may be induced to divide by the inhibition of protein synthesis with chloramphenicol. This finding would argue against the validity of those models which invoke the synthesis of some "dividase" molecule late in the cell cycle. It would also argue against those which involve the dilution of some division-inhibitory species synthesized early in the cell cycle.

What is known about the cell cycle of bacilliform bacteria suggests that the division process and the processes by which the cell maintains its shape are intimately interconnected. For example, in E. coli, growth of cells in balanced exponential cultures is accomplished solely by extension of the cell envelope along the cell's longitudinal axis, the diameter being essentially constant throughout the cycle (22). Models proposed to account for this observation are predicated on a peptidoglycan rigid layer which is anisotropic and easily extensible only in one dimension. Recently, however, reports have appeared (35) which suggest that the diameters of thymine auxotrophs are controlled by the relative rates of DNA synthesis in cells growing at the same mass-doubling rate. It was also found that such organisms have different diameters and lengths at division than do other similar organisms at equivalent growth rates.

That shape of E. coli is genetically determined is demonstrated by the existence of pleiomorphic, filamentous, and even spherical mutants. That spherical mutants have been isolated under conditions varying widely, as elevation of temperature (12), exposure to antibiotics (14), or random nitrosoguanidine mutagenesis argues that a wide variety of biochemical alterations can result in the loss of the ability to differentiate between extension of the cell envelope and septation.

Fan and his co-workers (6, 7) have demonstrated that the end walls of Bacillus subtilis are more resistant to digestion with autolysin than are the side walls of that organism. This suggested that perhaps end walls are conserved and do not turn over in the growth of the cell. They performed an interesting series of

experiments to test this hypothesis in the course of which the cell envelope was density-labeled by growing the organism in medium dissolved in deuterium oxide. The transfer of labeled cells into light-water medium and subsequent growth provided walls which were uniformly intermediate in density, no large differences existing between the more easily digested side walls and the refractory end walls. The adaptive value to the organism of this distinction between end and side walls remains obscure.

Normark and his associates have identified a locus in the chromosome of Escherichia coli K-12 designated envB, which appears to mediate the orientation of the cell's axis of elongation (24, 33). A mutant in this locus, envB1, characteristically produces irregular to spheroid cells which are osmotically stable. These workers were struck by the morphological identity of the mutant organism and that of wild-type cells treated with small amounts of FL-1060. They suggest that the target of the antibiotic and the gene product of the envB gene may be closely linked biochemically.

The foregoing work is suggestive of the subtlety and complexity of the controls operating during the cell cycle. The implications of the non-spheroid shape become even more apparent when we consider that bacilli, by virtue of their geometry, exhibit a primitive sort of structural specialization with regard to such modalities as distribution of flagella and of mesosomes, localization of periplasmic enzymes, and sporulation in the case of at least two genera.

The determination of cell shape, then, is clearly much more than a fortuitous evolutionary remnant, and in a sense, represents

the simplest level at which differentiation can take place. The understanding of the genetic and biochemical bases of morphogenesis is thus central to an understanding of the cellular growth and division cycle in bacteria.

Regulation of Cell Size and Shape in E. Coli. Workers in Maaløe's laboratory demonstrated in the 1950's that Salmonella typhimurium in balanced growth divided at a fixed size related to the growth rate of the organism (18, 22). It was later found that the changes in cell size as a function of age within the cell division cycle could be accounted for by differences in cell length, the diameters apparently remaining constant (22). Recent work with thymine auxotrophs, however, suggests that cell diameter is regulated to the rate of DNA replication while cell length changes to accomodate exponentially differing rates of cytosol synthesis over a single cycle (35). There is some division of opinions on this point (1). Pritchard has proposed a model for the cell cycle which can account for many of the observations above (28).

In this model, Pritchard invokes Previc's notion of an anisotropic peptidoglycan which has preferential extension along the longitudinal axis of the cell suggesting that septation occurs at predetermined sites as a consequence of the replication of a late gene providing for an increase in the capacity to synthesize cell envelope relative to total biosynthetic capacity (27). The various mutant strains of E. coli showing aberrant morphology are seen in this light as mutants having lost the capacity to maintain the orientation of the growth axis (33, 24) or having lost the capacity to cross-link peptidoglycan or otherwise create the anisotropy required for preferential extension.

That peptidoglycan is required for the maintenance of the rod morphology is demonstrated in the work of Schwarz and Leutgeb (31), who were able to induce a spherical morphology in diaminopimelate auxotrophs of E. coli. That alteration of peptidoglycan composition is not in itself sufficient to account for morphogenesis was shown by workers in the same laboratory, who demonstrated that osmotically stable forms could be produced which were spherical but had peptidoglycan chemically indistinguishable from the rod form of the same organism (9). It can be readily seen that shape is controlled concurrently with a variety of other phenotypic traits, as spherical mutants of E. coli have been isolated by selection for resistance to amidinopenicillanic acid (14), and elevated temperature (9, 12) among other selective factors.

Techniques Causing Perturbations of Morphology and Division. A wide variety of techniques have been reported which lead to aberrations of shape and division in E. coli. These can be categorized into two classes: those which lead to a cessation of division without affecting the topological pattern of envelope elongation and thus lead to the formation of filaments, and those which affect both division and morphogenesis, i.e., those in which the characteristic shape is lost as well as the capacity to divide.

Normally rod-shaped E. coli have been shown to assume a spherical morphology under many conditions. Penicillins and cephalosporins in low doses cause the formation of osmotically fragile spheroplasts, as does the removal of meso-2,6-diaminopimelic acid (Dpm) from the growth medium of auxotrophic strains. Both

the above treatments result in osmotically unstable populations of cells which have lost the capacity to divide but which retain the ability to synthesize protein and nucleic acids.

6-Amidinopenicillanic acid (Mecillinam, FL-1060) is a relatively new type of penicillin which differs from previously described penicillins in that the 6-acylamino substituent on penicillanic acid is replaced by an amidino substituent. The result of this alteration in the structure of the molecule is a dramatic change in the spectrum of antimicrobial activity. In contrast to penicillins and cephalosporins, which are generally found to be more effective in preventing the growth of Gram-positive organisms than Gram-negative organisms, FL-1060 has exceptionally high activity against enteric organisms, and indeed, exhibits a stronger effect on Gram-negative organisms than on Gram-positive species (23). Furthermore, ordinary penicillins are known to cause the formation of osmotically fragile spheroplasts, whereas FL-1060 causes the conversion of E. coli to osmotically stable spherical forms (23, 32, 33).

Spratt and Pardee (32) have estimated that FL-1060 binds specifically to about ten molecules per cell of an inner membrane protein associated with the initiation of elongation of the E. coli wall. This inner membrane protein has been shown to be different from the receptor proteins for other beta-lactam antibiotics in that FL-1060 does not inhibit the acylation of periplasmic enzymes by radiolabeled penicillin (23).

Although incubation with penicillinase abolishes the activity of FL-1060, Matsuhashi, et al. reported that peptidoglycan

transpeptidase, D-alanine carboxypeptidase, and endopeptidase (bridge-splitting enzyme), all enzymes which are inhibited by ampicillin, are unaffected by killing levels of the amidino penicillin (23). Similarly, they report that none of the proteins which bind ^{14}C-penicillin G were blocked by prior treatment with FL-1060. From these observations, it is apparent that the mode of action as well as the target of FL-1060 are distinct from those of the better-known penicillins.

Treatment of E. coli with FL-1060 seems to result in the loss of an outer membrane protein, protein G, which has been shown to accumulate in cells treated with nalidixic acid (15). As a result of this work, James has proposed that the antibiotic may act to selectively inhibit the elongation of the cell wall and that the spherical morphology observed in cells treated with FL-1060 represents the growth of the envelope in a fashion typical only of the septation process (15).

Braun and Wolff have found that FL-1060 selectively inhibits the synthesis of peptidoglycan and results in the accumulation of bound lipoprotein (3). They also report that although less than 1.0 μg/ml FL-1060 inhibits peptidoglycan synthesis by 50%, a thousandfold excess of the antibiotic does not further affect biosynthesis of peptidoglycan. Their interpretation, that there exists a second, antibiotic-insensitive pathway to permit the continued synthesis of peptidoglycan is not unequivocal. Simple geometrical considerations (see Appendix I) suggest that if the quantity of envelope is regulated to maintain a relatively constant proportion of envelope to volume (that is, an essentially invariant

thickness of envelope), then the assumption of a spherical morphology may indirectly result in the reduction of peptidoglycan synthesis without a specific enzymatic inhibition occuring. An interesting observation which may ultimately shed some light on the system above is the report of Iwaya et al. who have mapped FL-1060-resistant mutants of E. coli (14). Particularly intriguing was their observation that these mutants were found to be spherical in an exceptionally high percentage of cases.

Studies have been done comparing the growth and division of E. coli made spherical with FL-1060 with growth and division of those induced by depriving Dpm auxotrophs of that compound (8) and with those of the envB mutant strain of Normark (24). An interesting insight into the extent of genetic control of shape and division is afforded by the work of Long et al. (21) who transduced a filament-forming strain of genotype lon^- with an envB strain of spherical phenotype and produced populations which formed giant spherical cells upon ultraviolet irradiation of the culture (21). These investigators found that treatment of an irradiated lon^- strain with FL-1060 resulted in essentially identical giant cells. This suggests that FL-1060 and the envB locus may direct similar morphological and physiological behavior through a common control mechanism.

A Unifying Model for Control of Morphogenesis and Division. In 1970, Previc enunciated a model which accounted for the growth and division of bacterial cells partly as a function of the stereochemistry of the linkages formed by amino acids in the third position in the tetrapeptide moiety of various peptidoglycans (27). Since that time, it has become apparent that the determination

of cell shape is a phenomenon distinct from its maintenance and that peptidoglycan, although necessary for shape-maintenance, cannot be shown to be sufficient to its determination (Cf. 2, 10, 31).

A particularly convincing demonstration of the latter point is found in the work of Braun and Leutgeb (2) who used a Dpm auxotroph of E. coli to establish the compositional invariance of the peptidoglycan in two morphologies. The organism was starved for Dpm; when the cells were in the osmotically fragile spherical form induced by the starvation, Dpm was restored. It was found that osmotic stability was rapidly recovered, but that the cells remained round for over two mass doublings. Moreover, there was no difference detectable between the compositions of the peptidoglycans from the stable round form and from the normal rod form.

Pritchard's 1974 review (28) retained the most attractive features of Previc's earlier model, while incorporating data which accounted for both cell division and morphology without requiring the specific alteration of stereochemical aspects of peptidoglycan synthesis throughout the cell division cycle. One particularly appealing feature of this latter work is that it also accounted for hitherto paradoxical findings, such as the stimulation of temperature-sensitive division mutants to divide at the restrictive temperature by inhibitors of protein synthesis and their analogous reversal of the formation of filaments by nalidixic acid (25). A more detailed summary of Pritchard's model is found in Appendix II.

Both Pritchard and Previc in their models accounted for the seeming invariance in cell diameter with age in exponentially growing cells by postulating that the envelope possesses a sort of

anisotropy which favors extension along the cellular axis rather than perpendicularly. The driving force for the extension is thought to be the osmotic pressure caused by the synthesis of protoplasmic constituents. The increase in diameter observed in shifts up (18) is then seen as an accommodation to a rapid increase in turgor pressure which could not occur through the capacity to extend the cell envelope, thus forcing a reduction in the cell's surface-to-volume ratio.

By changing the rate of elongation of DNA without an overall change in the rate of mass increase in thymine auxotrophs Zaritsky and Pritchard have elicited changes in cell length and diameter which are inversely related (35). Under these conditions the mean cell volume remains essentially constant. Thus, an increase in the rate of DNA elongation is accompanied by a decrease in girth and an increase in cell length in their hands (29).

Begg and Donachie, however, have found no such correlation between cell diameter and rate of DNA elongation in a different group of thymine auxotrophs (1). We are then left with a model which has the capability to account for a variety of observations on growth and morphogenesis, but which still suffers from apparently contradictory data.

If spherical E. coli can be induced to divide, Pritchard's model of a sort of non-stereospecific involution of a growing envelope could still account for symmetrical division. Models requiring a particular prepared locus for septation in a straight segment of wall would, however, become untenable. In this study, I report the repeated division of E. coli under conditions of

spherical morphology, thereby providing an opportunity to study the cell division event in isolation from the normal process of elongation. Conversely, this system provides a technique by which the control of envelope synthesis can be observed in juxtaposition with perturbations of morphology.

MATERIALS AND METHODS

Bacterial Strains and Growth Media. E. coli W-7 was obtained from E. W. Goodell. E. coli ATCC 13071 was obtained from the American Type Culture Collection. Growth was in a minimal salts-glucose medium supplemented with meso-2,6-diaminopimelic acid (Dpm) and, in some cases, either L-lysine HCl or Vitamin-free Casamino Acids (Difco) (CAA). The mineral salts medium (MSG) contained per liter: K_2HPO_4, 7.0 g ; KH_2PO_4, 3.0 g ; $(NH_4)_2SO_4$, 1.0 g ; trisodium citrate dihydrate, 0.5 g ; D-glucose, 2.0 g ; and 1.0 M $MgSO_4$, 0.5 ml. Solid media were as above with 1.5% agar added.

Growth Conditions. Care was exercised to utilize cultures in balanced growth, defined as a condition in which the culture mass and numbers increase in parallel and exponentially with time. Inocula were prepared by washing colonies grown on plates of the appropriate medium overnight. The suspensions thus produced were adjusted to the optical density at 450 nm of approximately 0.10, incubated at 37 C and transferred when the O.D. reached 0.6. After this transfer, a balanced state of growth was usually observed. If it was not, further dilution transfers were performed until balanced growth was achieved. Liquid cultures were grown in Erlenmeyer flasks with aeration by agitation in either a New Brunswick Gyratory water bath for smaller cultures or a larger New Brunswick air incubator-shaker for larger flasks. Growth was always at 37 C.

Changes in the mass of all cultures were monitored by measurement of optical density at 450 nm on a Beckman DU-2 spectrophotometer set at a slit width of 0.02 mm and path length of 1.0 cm. Between 0.1 and 0.7 the absorbancy was directly proportional to bacterial mass for exponentially growing cells. For cells in the extremely aberrant large spherical morphology, the dry weight of cultures at known optical densities was measured to ensure the relative accuracy of the technique, and it was established that for these cells, the measurement of optical density at 450 nm results in a slight underestimate of culture mass. This is in accordance with the theoretical considerations discussed by Koch (19).

Cell numbers were determined with a Celloscope electronic particle counter, with a computation module for analyzing population size distributions. The usual conditions for measurement were current, 1; amplification, 48; aperture size, 24 μm. Cell suspensions were counted for thirty or sixty seconds. For sample dilution 0.9% NaCl was used.

Chemicals. Organic solvents, acids, and most common salts were A.C.S. reagent grade obtained from Scientific Products or Fisher Scientific. Amino acids were obtained from Sigma Chemical Company. 1,7-^{14}C-2,6-diaminopimelic acid (Dpm) was obtained from New England Nuclear, and FL-1060 was the kind gift of Dr. F. Lund, Leo Pharmaceuticals.

Preparation of Cells for Photography. Cells in liquid medium were suspended in equal volumes (approx. 1.0 ml) of the same medium containing 2.0% Ionagar #1 (Oxoid) at 45 C and mixed

thoroughly. A drop of the cell suspension was placed on a cover slip and the edges of the slip coated with Corning high-vacuum silicone grease. The cover slip was then placed on a slide and phase contrast photomicrographs taken through a Wild microscope with a Zeiss shutter and 35 mm camera back. By using Ilford HP-5 film, it was possible to accelerate film speed to A.S.A. 4000, permitting the use of shutter speeds of 0.2 seconds.

Viable Plate Counts. To maximize the recovery and quantification of viable cells, the following procedure was used. Cultures grown in supplemented MSG were serially diluted in mineral salts without glucose at room temperature. From the final tube, 0.5 ml duplicate samples were delivered to the surfaces of MSG agar supplemented in the same way as the liquid medium; 3.0 ml of molten half-strength nutrient agar containing 40 μg/ml Dpm was added, mixed with the bacterial suspension by tilting, and allowed to spread to the edge of the plate and solidify. When this layer was solidified, it was overlaid with another 3 ml of molten agar of the same composition. After cooling for approximately ten minutes, the plates were incubated in the inverted position at 37 C for twenty-four hours. Colonies were discrete and relatively uniform in size and appearance using this technique. Dilutions used were chosen to yield between 100 and 300 colonies per plate.

Determination of Dry Weights. Cultures in liquid medium were grown by shaking at 37 C. At thirty minute intervals, samples estimated to contain approximately 1.0 mg of dry weight biomass were removed from the culture and filtered through tared 0.4 μm

pore size Nuclepore filters, washed twice with distilled water, and the filters dried at 60 C for one hour. The dried filters were permitted to equilibrate with ambient air to constant relative humidity overnight, then weighed.

Determination of the Activity of Penicillinase on FL-1060. Cells of E. coli ATCC 13071 in balanced growth were exposed to 0.5 μg/ml FL-1060 which had previously been permitted to react with 1000 units of penicillinase for fifteen minutes at room temperature. A positive control was provided by treatment of a parallel culture with unaltered FL-1060 at 0.5 μg/ml. Optical density was monitored to ensure the viability of the cultures. After growth for one hour, the cells were examined by phase-contrast microscopy to observe morphological changes.

Treatment of Cells with FL-1060. To cultures of E. coli growing exponentially in liquid medium were added freshly made up aqueous solutions of FL-1060 to final concentrations ranging from 0.5 to 5.0 μg/ml. The cultures were monitored by optical density for mass increase and by phase microscopy to observe morphological changes. After cells were seen to be uniformly spherical, excess FL-1060 was removed, either by filtration and resuspension of the cells in warm medium without antibiotic, or by treatment with 1000 units/ml of penicillinase (Difco).

The antibiotic was found to be unstable in aqueous solution; even solutions frozen immediately after being made up were found to have lost activity within a few days. At room temperature, a solution containing 0.1 mg/ml was found to have lost activity when diluted to 0.5 μg/ml after standing for six hours. The practice

was therefore adopted of weighing out only as much FL-1060 as could be used in a single experiment and of making up the aqueous solution no more than fifteen minutes before use. For cultures of 100 ml or less, this led to difficulties in precisely weighing out the sub-milligram quantities of antibiotic required. Fortunately, this work and that of others (23) revealed no significant difference in the effect of the antibiotic over a range of from 0.1 to 5 μg/ml. Thus, experiments were performed using convenient concentrations of FL-1060 in the range 0.1 to 0.75 μg/ml.

Experiments Using ^{14}C-Dpm. 1,7-^{14}C-2,6-diaminopimelic acid of specific activity 10 μCi/umol was added to cultures of E. coli ATCC 13071 in balanced exponential growth. The growth medium for these experiments was mineral-salts-glucose with 1.0 mg/ml L-lysine and 1.2 μg/ml Dpm as supplements. The radiolabeled compound was added at the rate of 0.1 μCi/ml, thus adding 2.0 μg/ml of Dpm to the medium. Immediately after addition of the labeled compound a 2.0 ml sample was removed from the culture flask and mixed with 2.0 ml cold (zero C) 10% trichloroacetic acid (TCA), then allowed to stand in ice for thirty minutes. This process was repeated at five-minute intervals until the end of the particular experiment. After thirty minutes in the cold, the samples were filtered through a 0.45 μm pore size Millipore filter, washed with 4 ml cold 10% TCA, then washed twice with 4-ml aliquots of cold distilled water. The washed filters were dried in an oven at 60 C for thirty minutes, placed in scintillation vials, 10 ml of PCS scintillation fluid added, and the samples were counted on a Beckman scintillation spectrometer, set for ^{3}H + ^{14}C counting. Based upon an internal standard, efficiency was always at least 88%.

After the final sample was taken, the remainder of the culture was placed in a 250-ml polycarbonate centrifuge bottle containing 40 ml ice and centrifuged at 7000 x g for five minutes at 4 C in a Sorvall RC2B refrigerated centrifuge using a GSA rotor. The pellet was resuspended in 20 ml cold distilled water and centrifuged again at 7000 x g for five minutes in 30-ml Corex tubes using an SS-34 rotor in the above centrifuge. The washed pellets were suspended in cold water, passed through a French pressure cell, and the crude particulate (wall) fraction collected by centrifugation at 15,000 x g for three hours in the SS-34 rotor as above. At each stage, 0.1 ml aliquots of supernatants and pellets were placed in scintillation vials with 10 ml of PCS and counted as above. All scintillation spectrometer counts were run to yield a 96% confidence interval no larger than $\pm$ 1%.

RESULTS

Preliminary Observations. Studies in the literature (3, 14, 23) and data from the present study indicate that FL-1060 is effective in causing a spherical morphology in a variety of strains of E. coli. The effect of the antibiotic appeared over a range of dosages from 0.1 to 5.0 μg/ml.

In an experiment described in the Materials and Methods section, it was confirmed that the effect of the antibiotic was abolished by penicillinase, as had been reported (23). Incubation of the antibiotic with penicillinase for fifteen minutes completely abolished activity while cells treated with unreacted antibiotic became spherical.

It was then decided to attempt the development of a system utilizing Dpm auxotrophs in different morphologies. The rationale was that such auxotrophs would provide an ideal system in which to study the control of the synthesis of peptidoglycan under a variety of physiological conditions.

A review of the literature revealed a paucity of Dpm auxotrophs, and even greater scarcity of organisms auxotrophic for both Dpm and lysine. It was possible that the high turnover rate of the diaminopimelate decarboxylase found in E. coli would lead to a large quantity of any Dpm label administered being decarboxylated and incorporated into cytoplasmic protein as lysine (34). Because of these considerations, E. coli W-7 was chosen as the organism for

many of the experiments in this study. E. coli W-7 is reported to have both the Dpm^- and lys^- phenotype (3).

In early experiments using ^{14}C Dpm it became clear that E. coli W-7 did not effectively transport Dpm. A review of the literature revealed that there is a class of Dpm auxotrophs which are characterized by both a low rate of transport of Dpm and a large internal pool of the compound (20). This finding made the organism almost useless for the above purposes; thus a search was immediately begun for another which would transport Dpm at a rate typical of that found in the case of other amino acids. In Figure 1 it can be seen that Dpm is quickly transported into E. coli ATCC 13071, and, in fact that it resembles the uptake curves one finds in the literature for other amino acids. Comparison of Figure 1 with the data reported by Leive and Davis (20) places this organism in the group they designated rapid transporters of Dpm. Although the uptake of Dpm does not level off in the time of the experiment, the curve can be seen to be hyperboloid, and one would presume that on an intensive basis, that is, uptake per unit of biomass, the uptake of Dpm does, in fact, level off. This will later be shown to be the case.

To obviate the possibility of heavily labeling cytoplasmic protein via lysine the organism was grown in the presence of a large concentration of L-lysine. Experiments to determine the tolerance of E. coli ATCC 13071 to relatively large concentrations of L-lysine revealed not only that the organism grows without adverse effects in MSG supplemented with up to 1.0 mg/ml of the amino acid, but also that the presence of the lysine resulted in a Dpm-sparing

effect. The data in Figure 2 clearly demonstrates that 1 μg/ml of Dpm supports a greater quantity of mass increase in the presence of lysine than in its absence, and further, that increases in the concentration of lysine enhance the increment of mass. Turning again to the literature, one finds that a tenfold excess of lysine over Dpm inhibits the diaminopimelate decarboxylase of E. coli by 50% in vitro (34). It would appear from the data of Figure 2 that the 500- to 1000-fold excess of L-lysine used here has a similar effect in vivo. Further, it has been reported that the enzyme is repressible by L-lysine (17). The practice was therefore adopted of performing all labeling experiments using E. coli ATCC 13071 grown in a medium containing 1.0 mg/ml L-lysine and ca 1.5 μg/ml Dpm.

Induction of Morphogenesis by FL-1060. Although the effects of FL-1060 on morphology have been well-documented, there are as yet no definitive studies on the sequence of events leading to the conversion of a rod-shaped organism to a spherical one under the influence of this antibiotic. There is also no data in print on possible strain-specific variances in the range of effective doses. It was therefore necessary to establish that the Dpm auxotrophs used in this study were capable of being affected by FL-1060 and to determine working ranges of antibiotic concentration.

Cultures of E. coli W-7 or E. coli ATCC 13071 were propagated in balanced exponential growth as described in the Materials and Methods section. At an optical density of approximately 0.15 to 0.25, varying quantities of FL-1060 were introduced into replicate culture flasks. Optical density was followed on all cultures and microscopic examinations performed to determine.

morphogenetic effects. Figure 3 demonstrates the results of one such experiment using E. coli W-7 growing in MSG supplemented with 0.2% CAA and 100 μg/ml Dpm. Figure 4 is the result of a similar experiment with E. coli ATCC 13071 growing in MSG supplemented with 1.0 mg/ml L-lysine and 1.6 μg/ml Dpm treated with 0.75 μg/ml FL-1060. In both cases, it can be seen from the figures that the rate of increase of optical density is little affected by treatment with the antibiotic. Microscopic observations were made to monitor the morphogenetic processes occuring during the experiments.

Approximately one generation equivalent time after treatment, cells of both strains treated with FL-1060 were observed to be swollen. By one and one-half doubling times, essentially all the cells in the culture were rounded. Figures 5 and 6 are typical of the normal and rounded morphologies observed. The striking differences between these figures are actually less dramatic than those seen as the spheres grow larger over time. If one follows the culture for a very long time, one observes very large spheres beginning to assume squared ends, and later sees the formation of very large cells similar in shape to epithelial cells. Ultimately, these give rise to colonies of cells with normal morphology.

Cultures of E. coli B and E. coli CSH 74 (K-12) were also treated with FL-1060. Both strains responded by becoming spherical without an alteration in the rate of increase of the optical density of cultures. That these four strains of widely different provenance all react essentially identically when treated with FL-1060 is a strong indication that the effect is a valid probe of the physiology of a wide range of strains.

Figure 1. Uptake of ^{14}C-diaminopimelate by E. coli ATCC 13071.

The organism was placed in a condition of balanced growth as described in the Materials and Methods section. Cells were grown in MSG supplemented with 1.0 mg/ml L-lysine and 2.0 µg/ml Dpm. At the time it was judged appropriate to start the labeling process, 20 ml of a culture at O.D. 0.6 was rapidly passed through a 0.45 µm pore size millipore filter, then rapidly resuspended in 50 ml of warm medium containing 2 µg/ml ^{14}C Dpm representing 0.2 µCi/ml.

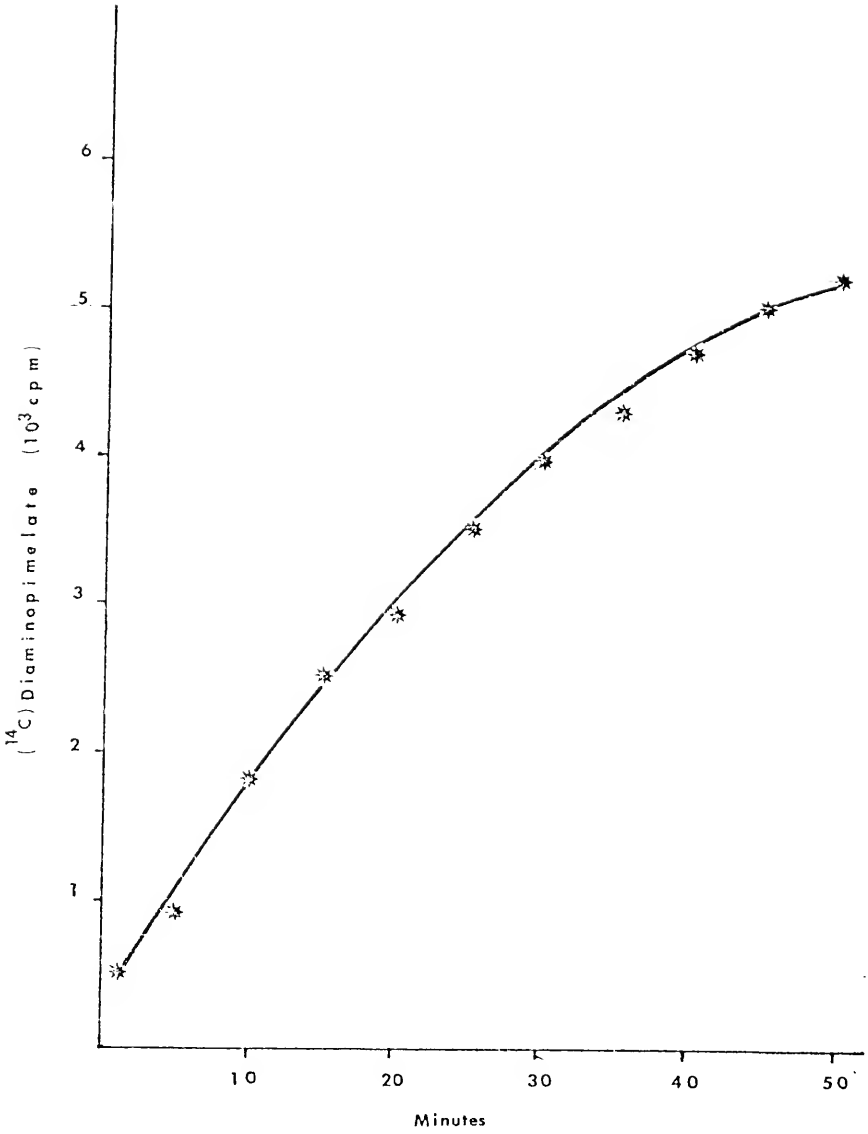

6
5
4
3
2
1
(^{14}C) Diaminopimelate (10^3 cpm)
10
20
30
40
50
Minutes

Figure 2. Diaminopimelate-sparing effect of L-lysine in *E. coli* ATCC 13071.

Cells in balanced growth were diluted into media containing various amounts of L-lysine. The original culture was grown in MSG supplemented with 10 μg/ml Dpm. Dilutions were 1:10 into medium containing 1.0 μg/ml Dpm and 0 (✱), 100 (✲), and 1000 (✶) μg/ml L-lysine HCl.

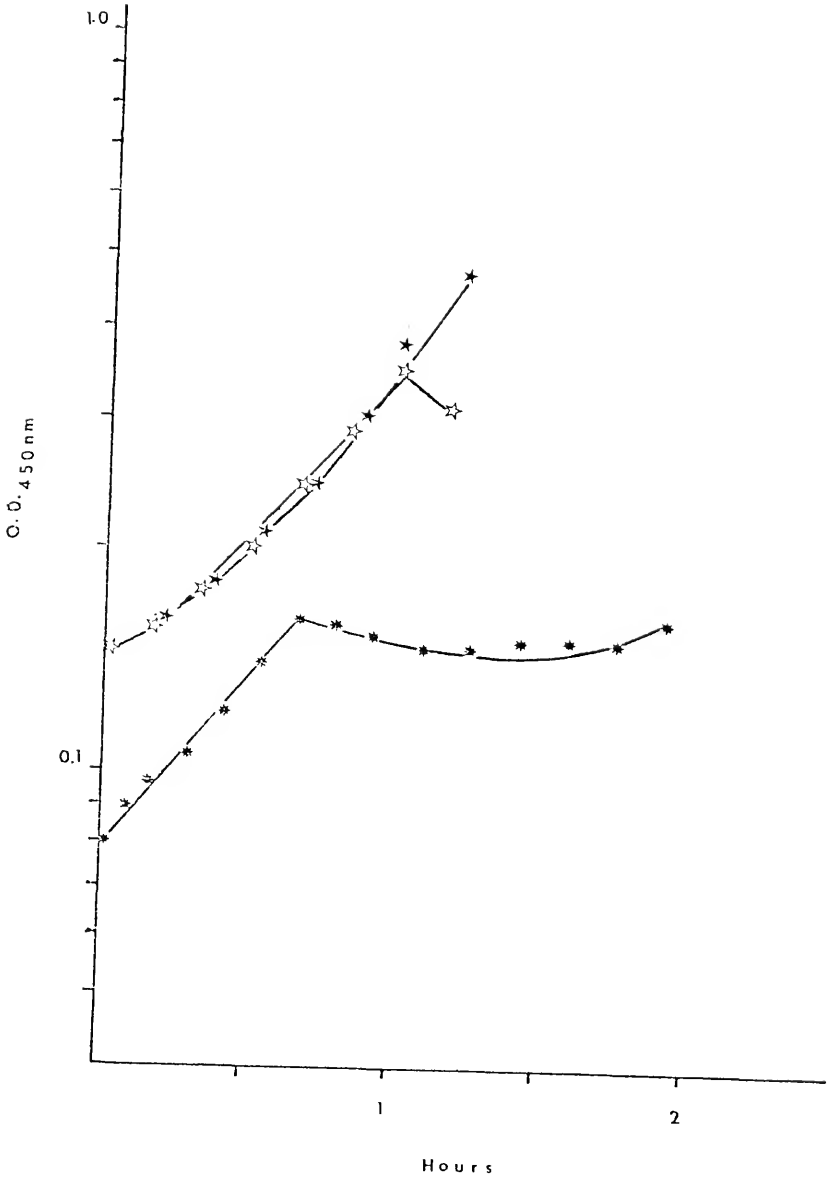

1.0
O. D. 450nm
0.1
1
2
Hours

Figure 3. Effect of FL-1060 on optical density of growing cultures of E. coli W-7.

An exponential, balanced growth culture of the organism was subcultured into four flasks containing MSG supplemented with 0.2% Casamino acids and 20 µg/ml Dpm. To the cultures were added 0 (☆), 0.1 (✱), 0.4 (✶), and 0.5 (□) µg/ml FL-1060. After all cultures except the control were observed to contain exclusively spherical cells, they were diluted into fresh warm medium supplemented as above at O.D. ca. 0.1 and optical density followed.

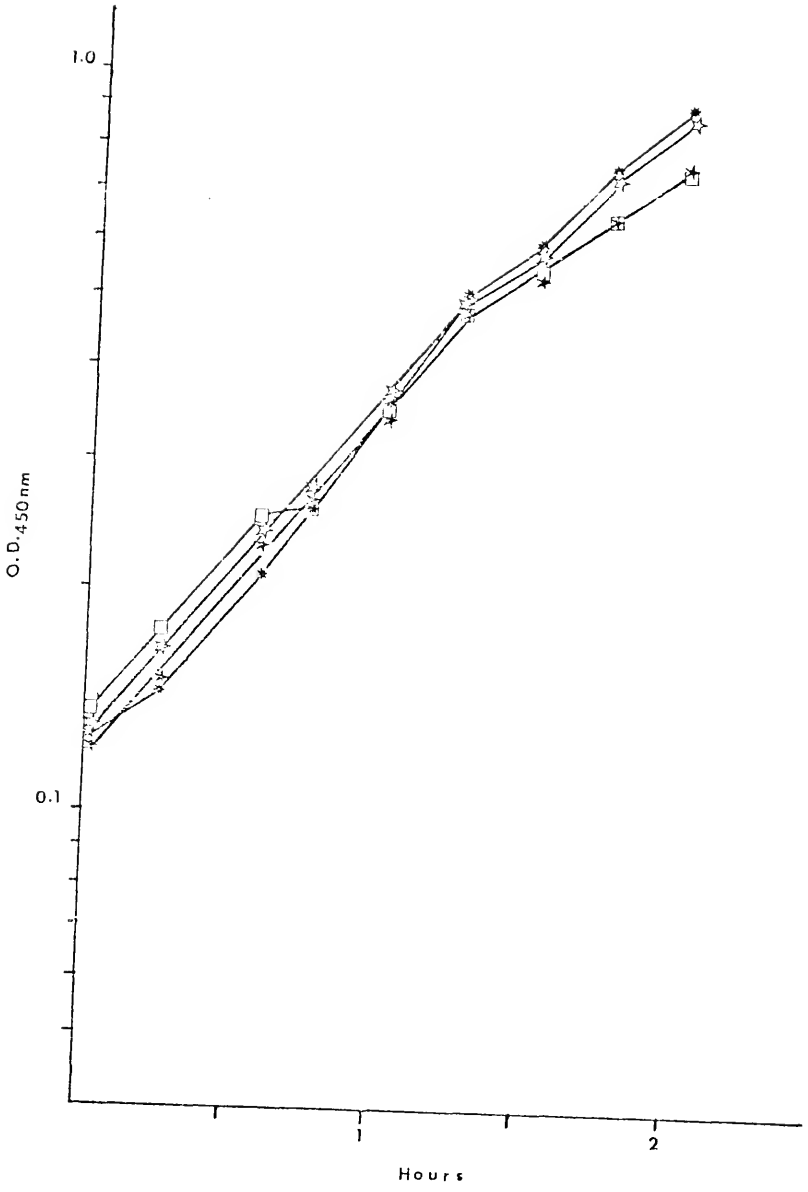

1.0
0.1
O.D.450nm
1
2
Hours

Figure 4. Effect of FL-1060 on optical density of growing cultures of E. coli ATCC 13071.

Cells in balanced exponential growth were diluted into flasks containing MSG supplemented with 1.0 mg/ml L-lysine and 2.0 μg/ml Dpm. One flask also contained 0.75 μg/ml FL-1060 (✶) and the other none (☆).

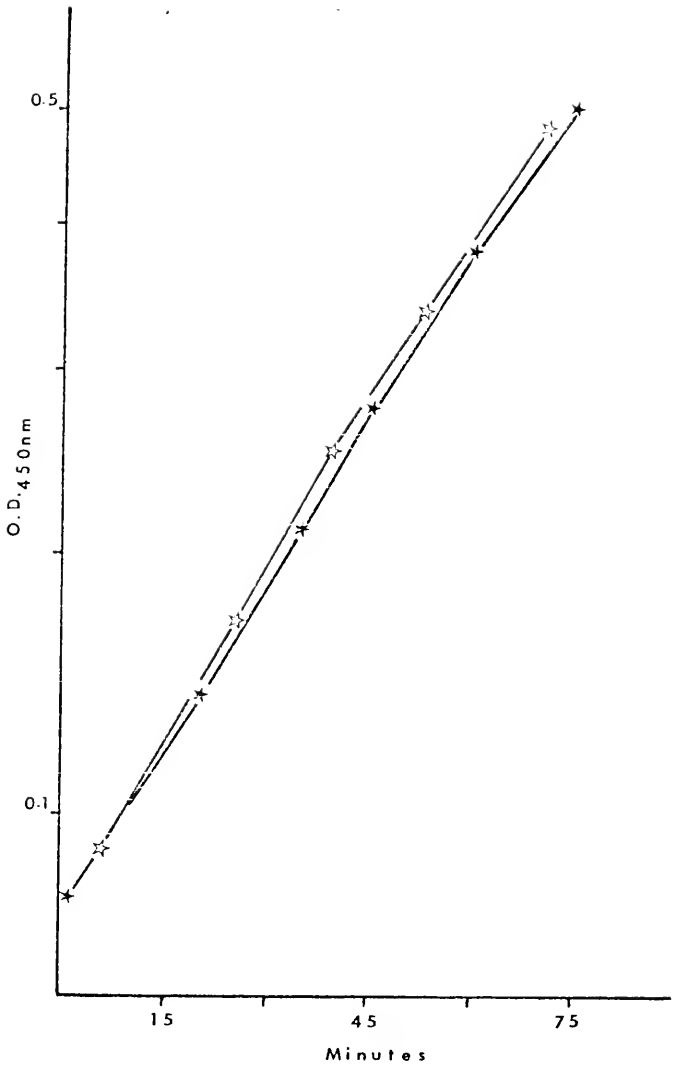

0.5
0.1
O.D.450nm
15
45
75
Minutes

Figure 5. Appearance of E. coli in the normal morphology.

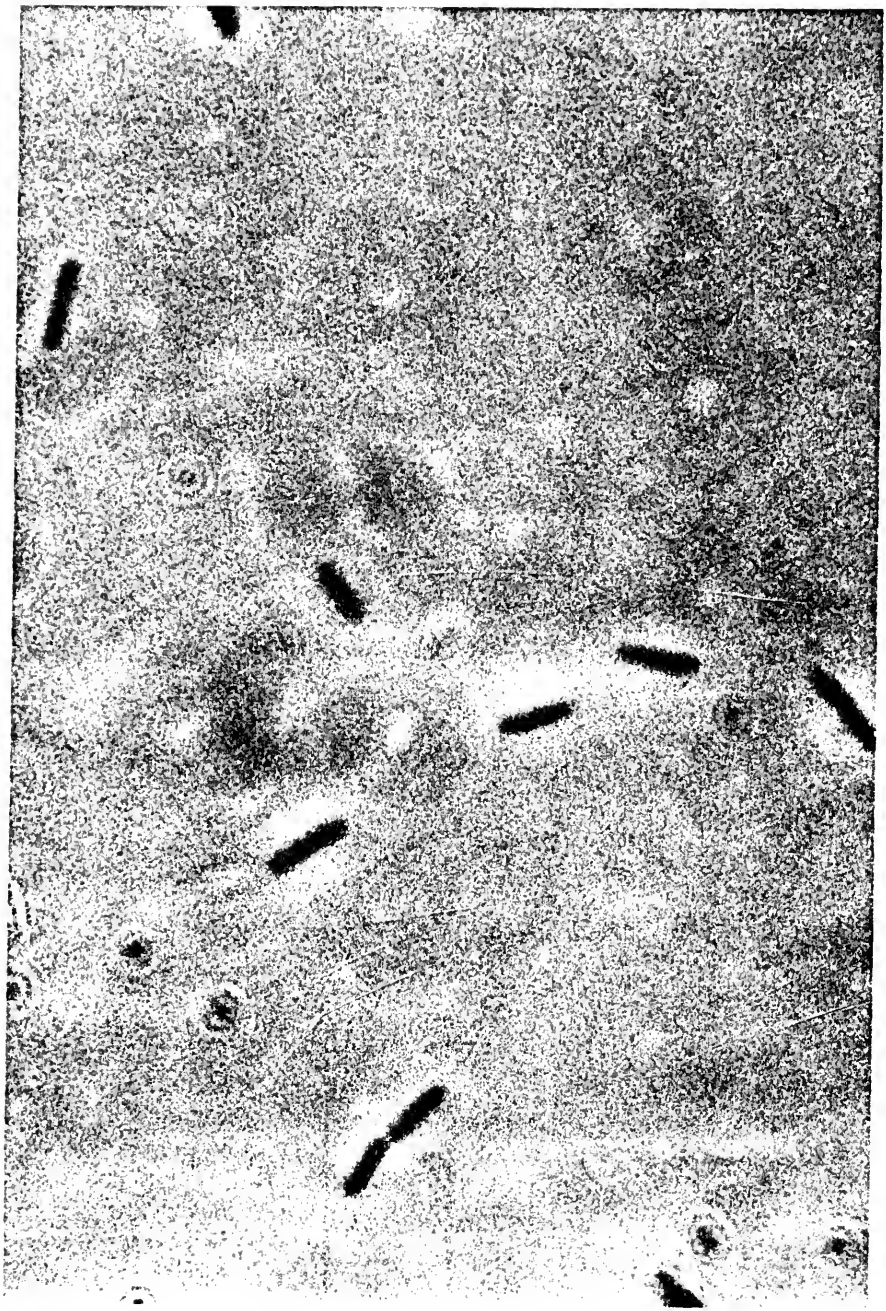
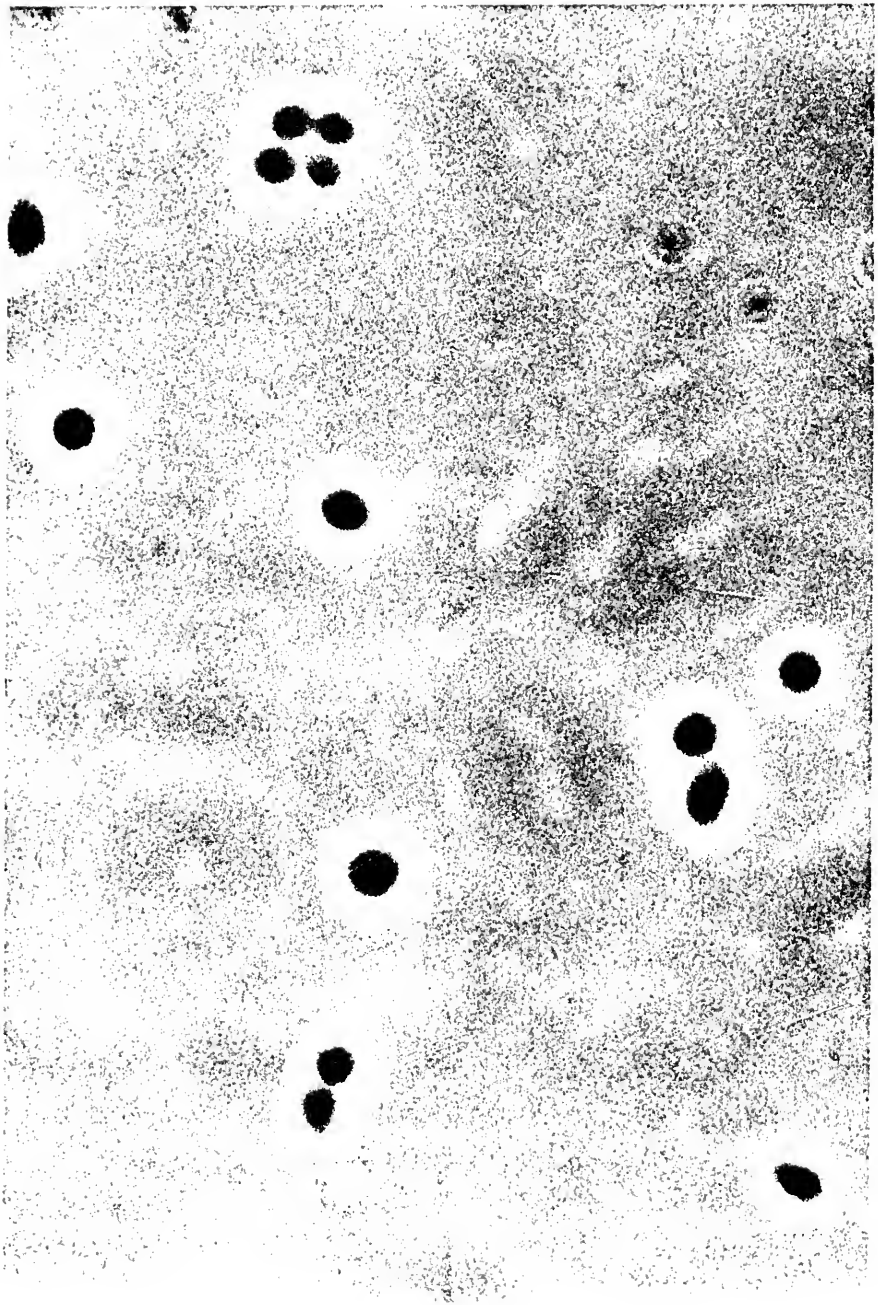


Figure 6. Appearance of E. coli in the spherical morphology induced by FL-1060.



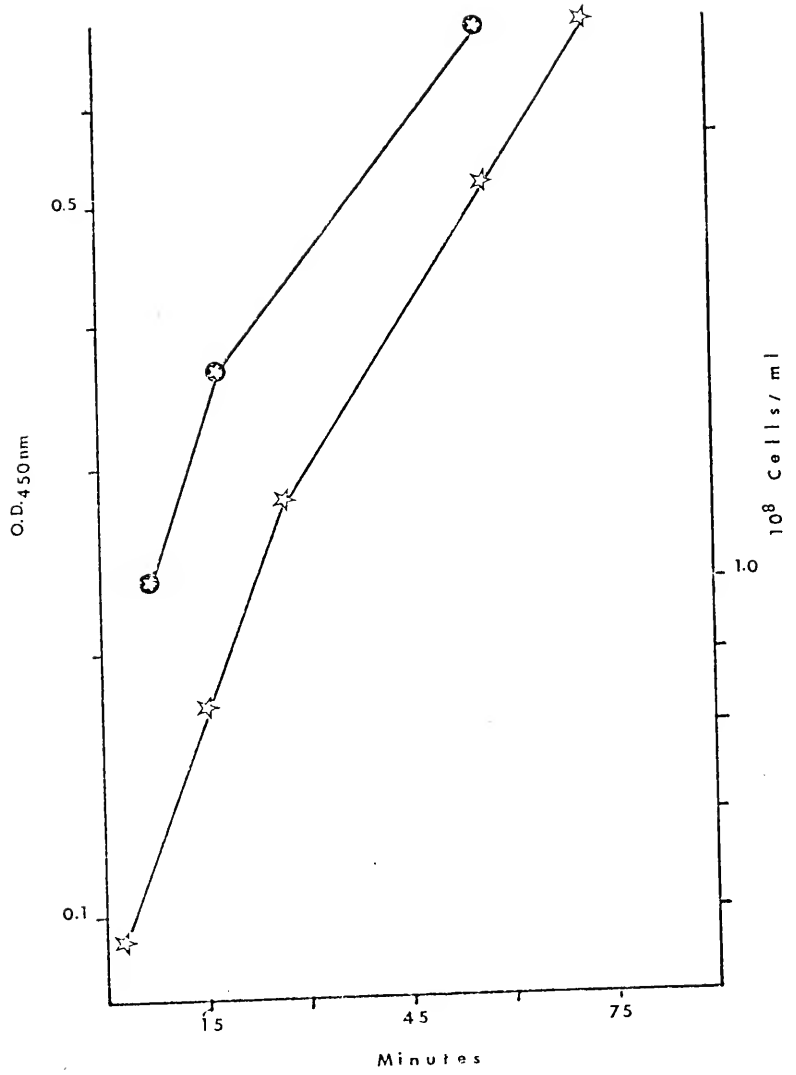
It has been reported that E. coli in the spherical condition induced by FL-1060 are incapable of dividing (16). Much to this author's surprise, E. coli W-7 cells treated with 0.5 μg/ml FL-1060 were not only viable, but also were clearly capable of dividing over a four-generation treatment time during the latter half of which all cells observed microscopically were spherical. Figure 7 demonstrates the relationship found between mass increase and viable count in the latter half of one such experiment. Note here that the rate of increase of viable counts is lower than the rate of increase in optical density. This suggests a system in which division is retarded rather than inhibited, i.e., in which cells are dividing at a size which is increasingly becoming larger. Alternatively, one could envision a condition in which a small proportion of cells remained unaffected and continued to grow and divide normally. This possibility was discounted for two reasons:

1. The viable counts indicated far too great an increase to be accounted for by a very small fraction of the population, and
2. There were no rod-shaped organisms seen under the microscope.

To more clearly quantitate the effects of the FL-1060 treatment on size and division, experiments were performed in which cells were treated with ca 0.5 μg/ml FL-1060 and allowed to become spherical, after which the excess FL-1060 was removed and the cells allowed to grow. During the course of these experiments, samples were taken for size distribution analyses using the Celloscope electronic particle counter. Figure 8 displays the size distribution of a normal population of E. coli W-7 grown in MSG supplemented with 0.2% CAA and 20 μg/ml Dpm. Note that the

Figure 7. Relationship between numbers of viable cells and optical density of cultures of E. coli treated with FL-1060.

Cells in balanced growth were diluted into warm MSG supplemented with 0.2% Casamino acid and 20 µg/ml Dpm. FL-1060 was added at the rate of 0.5 µg/ml and the optical density followed. When the culture was found to contain only spherical cells by microscopic examination, it was diluted into fresh warm medium also containing FL-1060 at O.D. near 0.1 and viable counts determined as in Materials and Methods. (●) Viable cells/ml (10^8 cells) (☆) optical density at 450 nm.


0.5
0.1
O.D.450nm
1.0
10^8 Cells/ml
15
45
75
Minutes

size distribution is invariant with time. This is what one would expect of a population of organisms in balanced exponential growth. Note also that the areas under the curves represent the relative numbers of cells counted in each case. Because the dilution factors were increased to accommodate the exponential increase in numbers experienced, the rate of increase in numbers is even greater than that reflected by the relative areas under the curves.

Figure 9 displays the size distribution analyses of a parallel culture to that of Figure 8 treated with 0.6 μg/ml FL-1060. Here it can be seen that at each succeeding measurement, the overall distributions of sizes are spreading with a strong trend toward the higher-numbered channels which represent the larger volumes. Even more striking is the observation that if one looks at the normal distribution, the largest cells are found in channels 50 to 70; if one looks at Figure 9, it is apparent that these channels represent the smaller cells in the distribution taken 155 minutes after treatment. Here is even clearer evidence that there is no population of small viable cells producing the increase in viable counts.

In Figure 10 can be found the total particles per ml calculated by summing the individual channel counts of Figures 8 and 9 and then multiplying by the dilution factor used in preparing the individual samples for counting. Similarly, a correction was introduced for the tenfold dilution into penicillinase-containing medium. It is clear that the total numbers of particles are increasing in a regular fashion. This situation, is to this writer's knowledge, unique.

Figure 8. Size distribution of cells of E. coli W-7 growing in balanced exponential growth in MSG supplemented with 0.2% Casamino acids and 20 μg/ml Dpm.

Samples were diluted into 0.9% NaCl so as to yield an estimated final density of 10^7 cells/ml. Times of sampling were: 0 (□), 95 minutes after initial sample (✱), and 120 minutes after initial sample. ✰

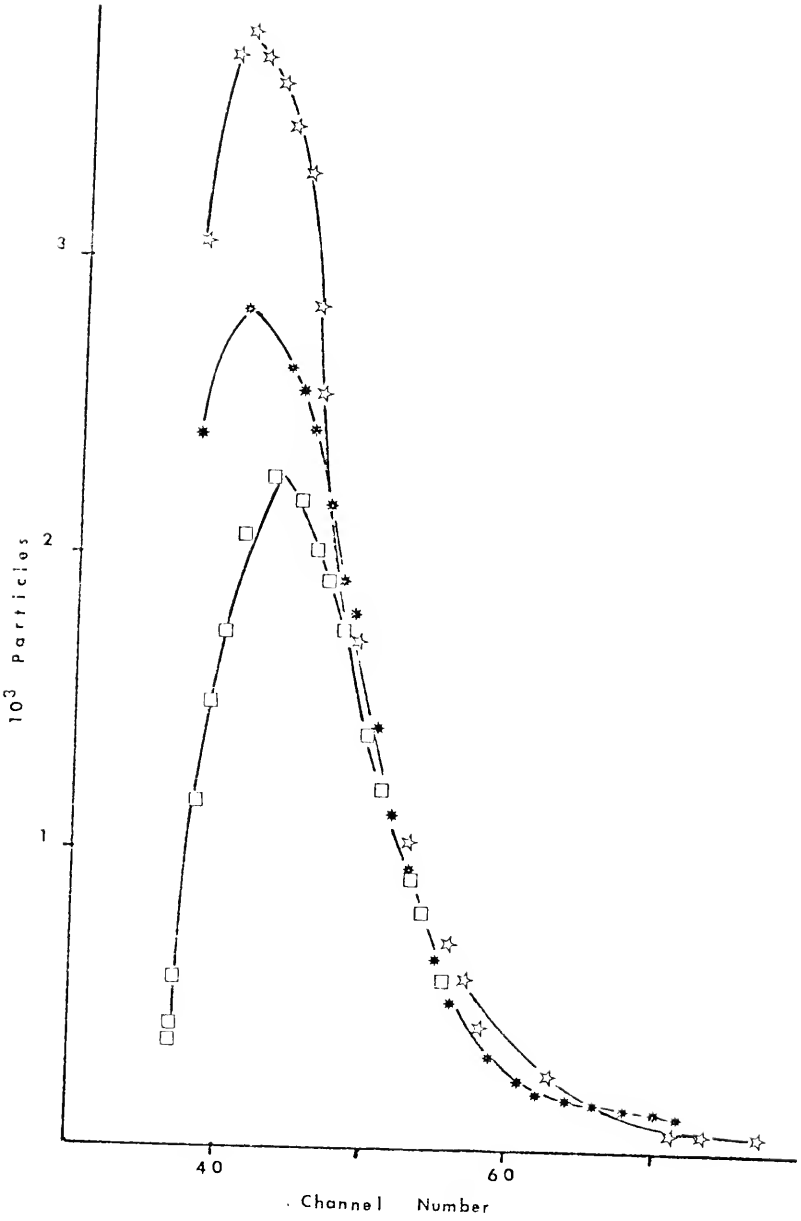

3
2
1
10^3 Particles
40
60
Channel Number

Figure 9. Size distribution of cells of E. coli W-7 growing in the spherical morphology induced by FL-1060.

To a culture in balanced growth, 0.6 μg/ml FL-1060 was added. When the culture density reached 0.8 cells were diluted into medium (MSG plus Casamino acids) containing 1000 units penicillinase. Samples were taken for counting 15 minutes after treatment with FL-1060 (☆), 55 minutes after treatment (○), 95 minutes after treatment (✱), and 155 minutes after treatment (□). Area under the curves represents population density.

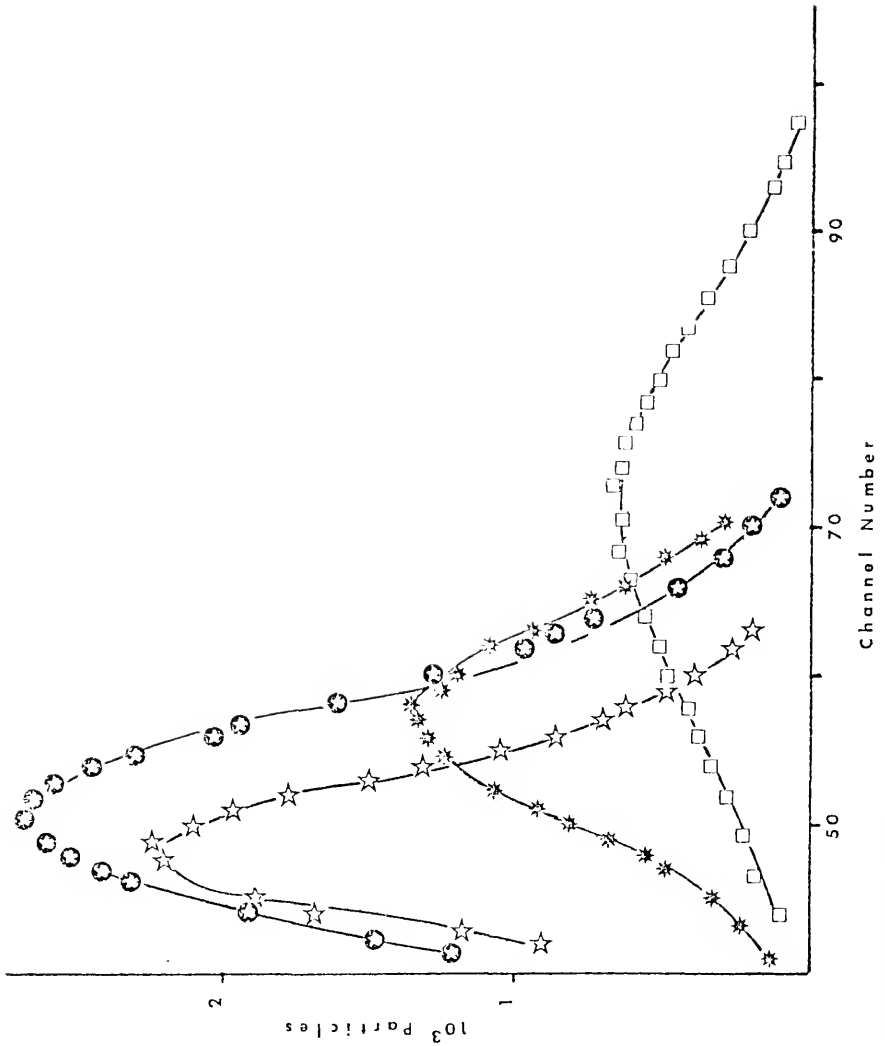
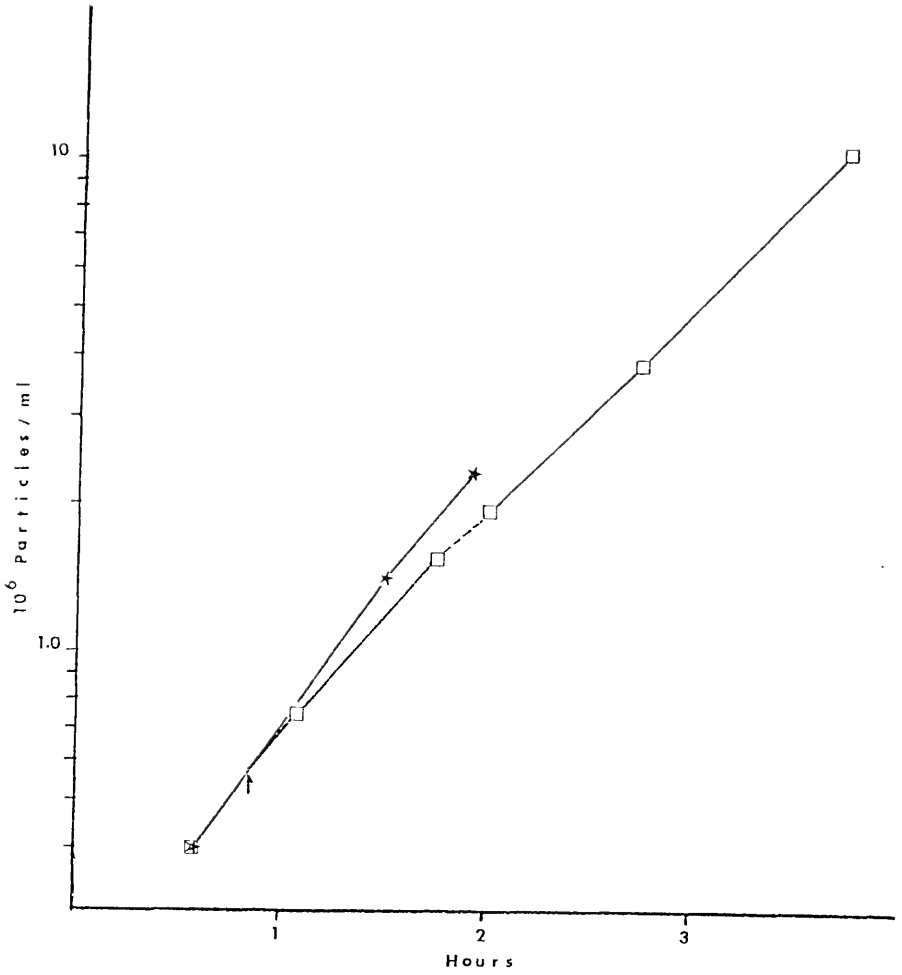

2
1
10^3 Particles
50
70
90
Channel Number

Figure 10. Total particles per ml in culture treated with FL-1060.

Numbers of particles in each channel were totaled and multiplied by the dilution recorded for that sample. The arrow indicates the time of treatment with FL-1060 and the dotted line the time of dilution 1:10 into warm medium containing 1000 units/ml penicillinase. (✱) normal cells; (□) treated cells.


10
1.0
10^6 Particles / ml
1
2
3
Hours

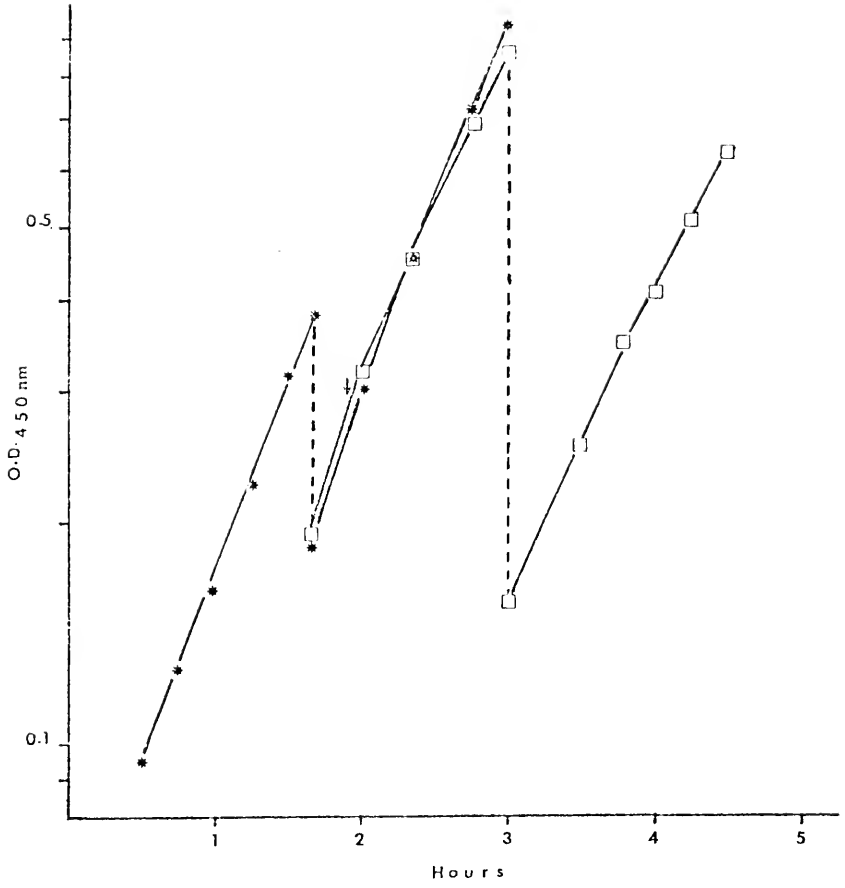
It is apparent from the data that division is occuring at a substantial rate under conditions where there are essentially no cells in the shape and of the size one would expect from the rate of increase in optical density observed in Figure 11, which records that variable for the experiment described. Careful examination of that figure reveals essentially no difference in the rate of increase of optical density of normal cells and those treated with antibiotic. Indeed, one might expect more variation than is seen merely from the altered light-scattering properties of the larger spherical organisms (19).

The final datum required to make the interpretation unequivocal was confirmation that optical density was a good measure of mass throughout the above experiments. As mentioned above, one might expect large spheres to exhibit a markedly different light-scattering behavior than rods. Figure 12 displays the relationship found between dry weight and optical density for a culture of E. coli W-7 treated with 0.5 μg/ml FL-1060 and permitted to grow. It is apparent from the figure that for at least three generation equivalents, the optical density is a good estimator of biomass. To emphasize the significance of this finding, recall that from Figure 9, we know that by two and one-half hours after treatment, the cells have become quite enlarged. Thus it can be asserted with confidence that mass has, in fact been measured by monitoring optical density.

Studies on the Synthesis of Cell Wall. The model which Pritchard has presented for the growth, shape, and cell division of bacteria presents an accounting for many disparate observations. The system described above would seem to be a powerful probe of the

Figure 11. Optical density at 450 nm of the culture of Figure 9.

E. coli W-7 cells were diluted to 0.2 as described in the text. The arrow indicates the time of addition of 0.5ug/ml FL-1060 to one flask. The treated culture was diluted into medium containing 1000 units/ml penicillinase one hour after treatment with FL-1060. (✱) normal cells, (□) treated cells.


0.5
0.1
O·D· 450 nm
1
2
3
4
5
Hours

control of morphogenesis and division. One question which came to mind was whether or not cells might regulate the quantity of cellular envelopes produced at different shapes so as to maintain a wall of the same relative thickness. Henning, et al. have reported this to be the case for peptidoglycan in a temperature-sensitive rod mutant (9).

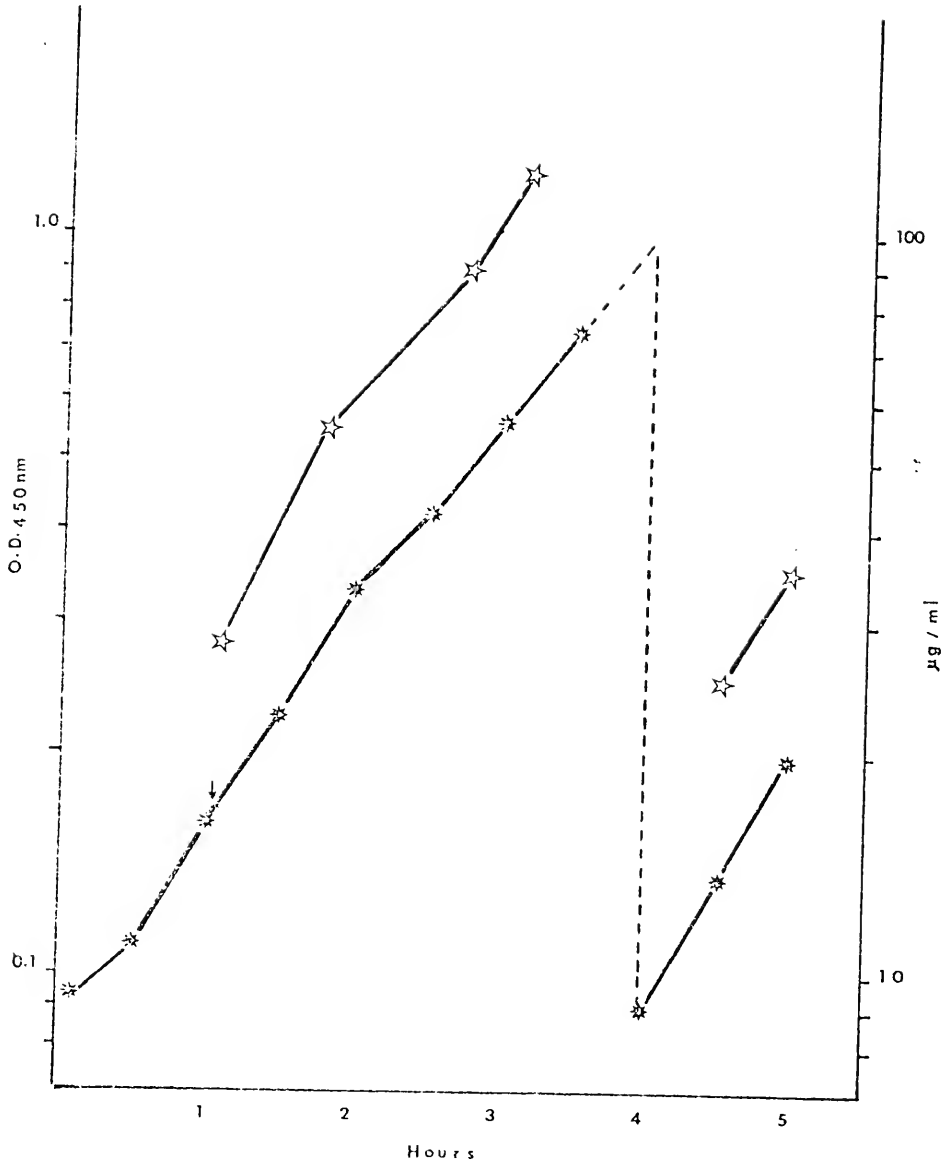
Because cells in the FL-1060 system are both enlarged and spherical, it was interesting to calculate the relative quantity of wall required to enclose a rod of unit volume, a sphere of the same unit volume, and a sphere of volume four times the unit. Appendix II demonstrates one method for estimating mass-volume relationships. Such geometric considerations permitted me to calculate that on the basis of square micrometers of envelope or wall per mg dry weight of cells (assuming constant density of the cytoplasm), the large spheres would require less than one half as much envelope. It was therefore decided that this was an ideal system in which to study the quantity of wall produced.

As was reported above, it was quickly apparent that E. coli W-7 was not a suitable organism for this purpose. Therefore the key experiments described in the section on FL-1060 morphogenesis were repeated using E. coli ATCC 13071. After confirming that it reacted to the antibiotic in the same way as W-7, an abbreviated series of experiments was performed to estimate the synthesis of peptidoglycan in the two morphological states.

In Figure 1 is found the rate of uptake of ^{14}C-Dpm by a control culture of E. coli ATCC 13071 grown in MSG supplemented with 1 mg/ml L-lysine and 1.6 μg/ml Dpm. Note the rapid uptake typical of the

Figure 12. Relationship between culture mass and optical density of E. coli W-7 treated with FL-1060.

Cells were grown in MSG supplemented with Casamino acids (0.2%) and Dpm (20 µg/ml). After treatment with 0.5 µg/ml FL-1060, samples were taken for measurement of optical density (✱), and dry weight (☆) as described in the Materials and Methods section. Dilution was into the medium as described above supplemented with 1000 units/ml penicillinase.


1.0
0.1
O.D. 450 nm
100
10
µg / ml
1
2
3
4
5
Hours

transport of a required amino acid and the reduction in intrinsic rate of uptake after thirty minutes or so.

Figure 13 summarizes the results of an analogous experiment using E. coli ATCC 13071 which had been induced to form spheres and allowed to double in mass prior to the introduction of the radio-labeled compound. Both figures are rectangular hyperbolas, typical of a system actively transporting a subtrate. There are obvious differences in the rate of equilibration of internal and external concentrations of ^{14}C-Dpm, presumably reflecting saturation of peptidoglycan-synthesizing pathways at levels typical of the two different physiological states. If the difference is related to a direct effect of the FL-1060 on peptidoglycan synthesis, a previously unreported point at which control of this macromolecular species is exerted might be determined.

To more precisely follow the incorporation of Dpm, the data from Figures 1 and 13 was recalculated on the basis of specific activity of labeled compound and the optical density of the cultures. This provides an intensive measure of the rate of fixation of Dpm into TCA-insoluble material as a function of mass. The data appear in Figure 14. Profound differences are seen between this figure and the previous figures suggesting that peptidoglycan is either not synthesized at an equivalent rate in the presence and absence of FL-1060, that its turnover is decreased by the antibiotic, or both. These results are not inconsistent with other reports in the literature, about which more will be said in the Discussion to follow.

Figure 13. Uptake of ^{14}C-diaminopimelate by E. coli ATCC 15071 in the spherical morphology induced by treatment with FL-1060.

To a balanced-growth culture growing in MSG supplemented with 1.0 mg/ml L-lysine and 2 µg/ml Dpm was added 0.75 µg/ml FL-1060. After cells were all spherical by microscopic observation, 25ml of the culture was rapidly passed through a 0.45 µm pore size Millipore filter, and the cells washed with warm mineral salts without glucose. The cells were then resuspended in warm MSG supplemented as above except that the Dpm contained 0.2 µCi/ml ^{14}C.

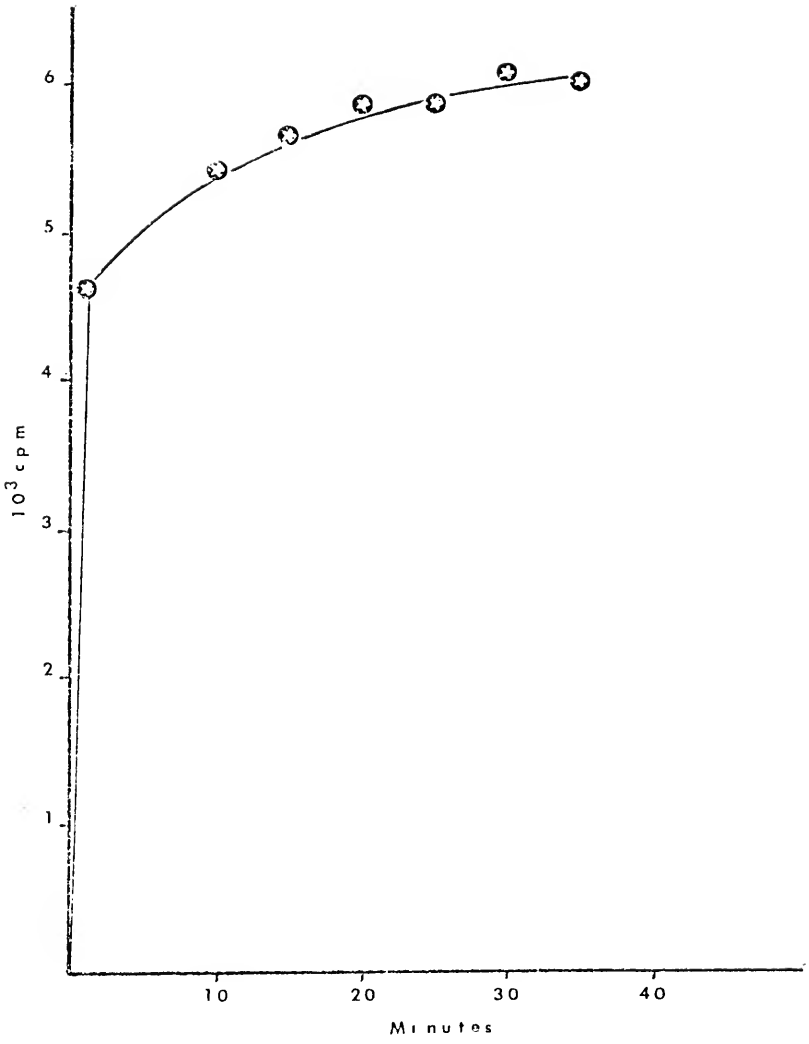
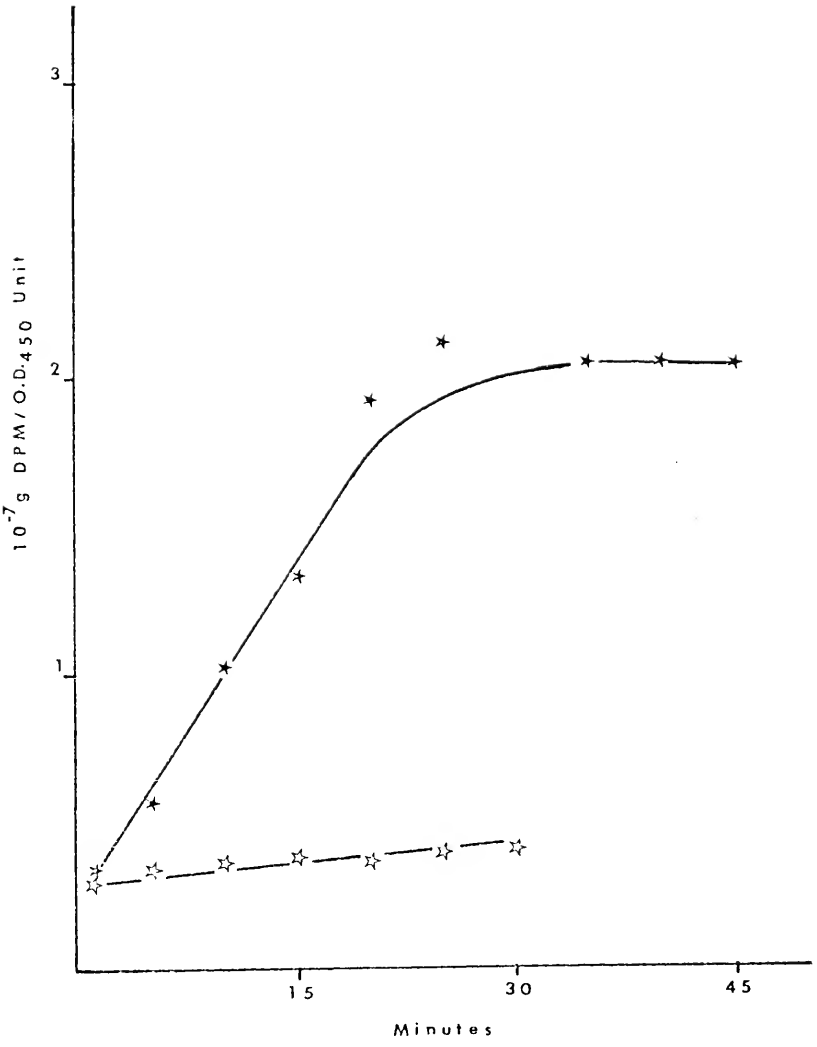

6
5
4
3
2
1
10^3 cpm
10
20
30
40
Minutes

Figure 14. Uptake of diaminopimelate by E. coli ATCC 13071 as a function of culture biomass.

Data was gathered as in Figures 1 and 12. The amount of Dpm fixed into TCA-insoluble material was calculated and then normalized to the optical density of the culture at the time of sampling. This was done for normal cells (★), and for cells in the spherical morphology (✡).


3
2
1
10^{-7}g DPM/O.D.450 Unit
15
30
45
Minutes

DISCUSSION

A system has been described in which one finds cell division occurring repeatedly without regard to the definitive size or shape we have learned to expect of an organism in balanced exponential growth at the time of division. Reports that E. coli which divide at sizes which are either too large or too small for a particular growth rate correct this by accelerating or delaying the subsequent division (5, 12) lead one to suspect that that component of the cell's integrative function which couples morphogenesis and division has been profoundly affected.

The observation that only a very small quantity of FL-1060 per cell is needed to induce the morphological change is not a new one. The persistence of this disturbance for several mass doublings after the FL-1060 activity remaining in the medium or on the cell surface has presumably been lost, either by physical removal through washing and dilution, or by degradation through the action of penicillinase is shown in this study. Thus, as the cells grow and divide, the active FL-1060 present must be distributed to progeny, continuously being diluted by the increasing mass of cytoplasm. This corroborates the notion that an exquisitely sensitive control point is involved.

FL-1060 has been shown to have an effect on the accumulation of certain outer membrane proteins in E. coli (15). One of these

in particular is associated with the round morphology and with cell division in normal cells. The mechanism by which FL-1060 affects the synthesis or degradation of outer membrane proteins has not been determined. It is therefore impossible to say whether the compositional changes in the outer membrane attendant on the morphological changes are causally related.

Braun and Wolff (3) have reported that in E. coli W-7, FL-1060 inhibits the uptake of radiolabeled Dpm and its fixation into peptidoglycan by 50%. Their experimental conditions, however, differed from those described here in several ways. Because E. coli W-7 transports Dpm only slowly, the minimum time for equilibration of internal pools with external label was over 100 minutes (3). For short-term labeling, such as they performed, the interpretation becomes quite difficult. Secondly, in order to get enough label into the cells in a short time, they starved the cells for Dpm. In view of recent reviews that show synthesis of peptidoglycan to be under stringent control (13, 30) it would seem inadvisable to starve the organism. In addition, uptake of such a critical component as Dpm under non-balanced growth conditions cannot be interpreted unambiguously. The data here are obtained under conditions less physiologically compromising and support their finding in general; that Dpm is fixed into peptidoglycan at a lower rate in the presence of FL-1060 than in its absence.

In the case of the present experiments, the difference is too great to be accounted for by the former model; hence it can safely be assumed there is some direct biochemical influence of FL-1060 on the synthesis of peptidoglycan. This speculation can be tested

by use of such techniques as the density-label turnover studies of Fan (6, 7) as well as by direct measurement of the incorporation of Park nucleotides into peptidoglycan. Further, one would hope to establish whether cells in the round morphology reduce the actual uptake of Dpm or simply do not fix it into TCA-precipitable forms. In the latter case, one should be able to detect leakage of labeled Dpm from a cell grown in radioactive medium, then washed, and placed in medium containing non-radioactive Dpm. Alternatively, one could look for the excretion of peptidoglycan subunits such as disaccharide-pentapeptide units from the periplasmic space.

A particularly intriguing possibility is that FL-1060 exerts its effect on peptidoglycan synthesis at the same point in the pathway as does the stringent control phenomenon. It is known that in the latter case, it is the incorporation of subunits into the cell's sacculus that is inhibited, and not the prior steps in their synthesis (30). If, in fact, FL-1060 acts in this fashion, then a whole realm of investigation into control of periplasmic physiological activities becomes open to investigation.

Additional work needs to be done to characterize the macromolecular composition of cells in the spherical morphology. Normark (24) has reported that the original spherical mutants isolated in his lab with genotype envB1, sloB1 were not only round, but also were resistant to FL-1060. He found, moreover, that the ratio of DNA to protein in that strain was unusually high. It would be interesting to determine whether any such alterations in the pattern of macromolecular synthesis might be found in the case of cells in the FL-1060-induced round state.

Finally, one would wish to utilize the system developed for measurement of the fixation of Dpm to measure the effects on synthesis of peptidoglycan of a variety of division-inhibitory agents. Such chemical inhibitors of division which lead to the formation of filaments as diazouracil and nalidixic acid might be ideal probes of the mechanism of control of the synthesis of septal peptidoglycan, and thus could well provide a critical test of the Pritchard hypothesis.

In summary, a system has been demonstrated which has unique capabilities as a probe of the control of morphogenesis and cell division. In its application to a question already addressed by other techniques, some clarification has been achieved.

APPENDIX I

Surface-to-Volume Ratios of Bacterial Cells

Consider a sphere of radius r. Its volume is $4/3\pi r^3$, and its surface area $4\pi r^2$. One can combine these equations and write an expression for surface area as a function of volume, thus: $S_s = 3V/r$, where the subscript refers to the spherical configuration.

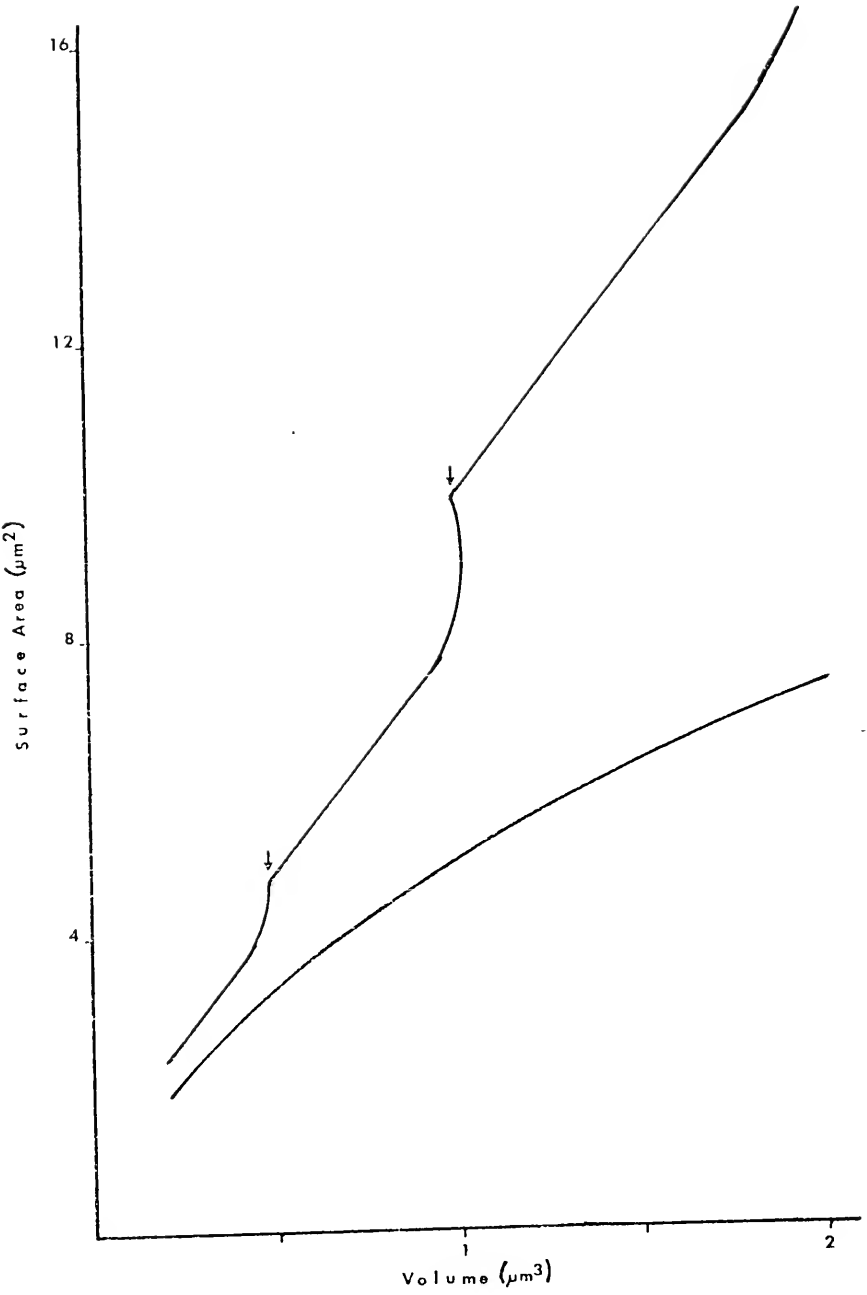
Now consider a right cylinder with hemispherical ends. Let us picture the cylinder growing only by extension of the cylindrical portion throughout most of its replication, then a pair of hemispherical ends being inserted to accomplish the division event. We will calculate the volume and ends of such a solid by simply summing the volumes of the ends and cylindrical portions. This approach avoids dealing with the small, but real, nonlinearities associated with the division event.

The total volume of the pair of hemispherical ends is just $4/3\pi(r')^3$ where r' is the radius of curvature of the cylinder and ends. The volume of the cylinder is $L\pi(r')^2$ where L is the length of the straight-sided portion. The total volume can be shown to be $V_c = \pi(r')^2(\frac{4r'}{3} + L)$, where the subscript refers to the cylindrical configuration. In like fashion we show the total surface area of the cylindroid form to be $S_c = 2\pi(r')(2r' + L)$. The algebraic equation describing surface area as a function of volume then can be shown to be $S_c = \frac{2Vc}{r'} + \frac{4Vc}{4r' + 3L}$.

Figure 15 shows the rate of increase of surface area to volume for a spherical organism which is not dividing and the accumulation of surface in a normally-dividing rod-shaped organism where r' = 0.25 μm.

Figure 15. Surface-volume relationships of rod-shaped and spherical bacteria.

Surface areas were calculated for a non-dividing growing sphere (lower curve) and a rod with radius 0.25 um and length at division 2.0 um dividing normally (upper curve). Note the periods of increased envelope synthesis at division, indicated by the arrows.


16
12
8
4
Surface Area (μm^2)
1
2
Volume (μm^3)

APPENDIX II

The Pritchard Model for Bacterial Cell Division and Morphogenesis

Consider a bacterial cell in balanced growth. During the cell cycle the chromosome is replicated, sequentially duplicating genes from origin to terminus. In the case of most, if not all genes, the degree of repression and hence the rate of synthesis of gene product per gene, will remain constant if the system is in balanced growth. The result of this pattern of growth is that the cell's mass increases exponentially as the summation of the individual gene doublings. For an individual gene, however, the concentration of gene product will decrease steadily as mass increases. At the time of the doubling of the gene in question, the concentration of its gene product will once again reach the initial level.

If the rate of synthesis of cellular envelope were to be limited by the product of an unregulated gene located at the chromosome terminus, then the result would be a stepped linear pattern of envelope synthesis and an exponential pattern of mass synthesis. Thus, synthesis of envelope and of total mass would always be disproportionate. As mass increased, there would be a corresponding increase in turgor pressure. If we picture envelope to be synthesized at a central locus, growing in annular fashion, then the hydrostatic pressure can be visualized to force the nascent envelope to extend, lengthening the cell.

At the doubling of the limiting gene, the capacity to synthesize new envelope would transiently exceed the rate of mass increase. At this time, turgor pressure would drop, and the growing annulus of nascent envelope would be allowed to constrict, resulting in the formation of a septum. Thus, division would be controlled solely by the relative rates of synthesis of envelope and cytoplasm.

The rod shape of bacilliform bacteria can be accounted for by only a slight anisotropy in the cellular envelope, resulting in a small preference for longitudinal extension over increases in girth. This property would also account for the increase in diameter found in shifts-up of cells between balanced growth rates. The immediate consequence of such a shift is an increase in the rate of mass synthesis. This cannot be accommodated by the capacity to synthesize envelope without a reduction in surface-to-volume ratio, which can only be accomplished by an increase in the diameter of the cell. The new diameter is maintained constant as the relative rates of mass and envelope synthesis are fixed by the growth rate under conditions of balanced exponential growth.

This model accounts elegantly for the paradoxical observations that inhibition of division is released by treatment with chloraphenicol in the cases of temperature-sensitive mutants, or cells treated with nalidixic acid. The lon^- mutation is accounted for as providing for a variety of cell envelope having unusually high anisotropy, thus being unable to accommodate physiological conditions which in a normal organism would lead to an increase in diameter. In agreement with this concept is the relative decrease

in cell diameter which follows an increase of rate of DNA elongation in cultures at fixed rates of mass increase (step-up or-down experiments).

This model invokes no specific division signal during the cycle, and we see no fundamental differences between crosswall formation and extension in length. Termination is likely, however, to be needed for cells to undergo the transition from extension to septum formation. Division can only occur when the amount of envelope accumulated in a given interval of time is sufficient not only for accommodation of the mass increment over the same interval, but also leads to a reduction in hydrostatic pressure.

LITERATURE CITED

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BIOGRAPHICAL SKETCH

Harvey A. Blalock was born August 29, 1944, in Asuncion, Paraguay. In June, 1962, he was graduated from South Dade High School, Homestead, Florida. In August, 1974, he received the degree of Bachelor of Science with a major in chemistry from the University of Florida. Since that time he has pursued work toward the degree of Doctor of Philosophy in the Department of Microbiology and Cell Science.

Mr. Blalock has held a number of positions in science and education, having been employed as a chemist in industry, as administrator of an environmental pollution control agency, and as an instructor in environmental science and chemistry at Santa Fe Community College in Gainesville, Florida, where he is currently employed as Director of Technical Education and Public Service Programs.

Harvey A. Blalock is a member of the American Society for Microbiology, The American Institute of Biological Sciences, The American Association for the Advancement of Science, the Florida Pollution Control Association, and Phi Kappa Phi scholarship honorary fraternity. He has served on advisory task forces for the State of Florida Department of Environmental Regulation and the National Science Foundation.

Harvey A. Blalock is married to the former Carol Haines Douglass and has three daughters, ages six, eight, and ten.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Edward P. Previc, Chairman
Associate Professor of Microbiology and Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Arnold S. Bleiweis
Professor of Microbiology and Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

James F. Preston
Associate Professor of Microbiology and Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Dinesh Shah
Professor of Chemical Engineering and Anesthesiology

This dissertation was submitted to the Graduate Faculty of the Department of Microbiology and Cell Science in the College of Arts and Sciences and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Dean, Graduate School