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Helical Macrofiber Formation in *Bacillus subtilis*: Inhibition by Penicillin G

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The folding process required for helical macrofiber formation after the outgrowth of *Bacillus subtilis* spores was found to be blocked by very low concentrations of penicillin G (1 to 3 ng/ml). Under such conditions, growth and septation without cell separation resulted in characteristic disorganized multicellular structures. Higher concentrations (4 and 10 ng/ml) were needed to inhibit spore outgrowth and vegetative growth, respectively.

The production and properties of helical *Bacillus subtilis* macrofibers have been described previously (5). These highly ordered multicellular structures are formed when division suppressed (4), autolytic enzyme-deficient (2), or wild-type (12) strains are cultivated in rich media at low cell densities. A number of models have been advanced to account for the helical growth pattern (3, 5, 11), all based on the notion of highly ordered cell wall polymers. Mendelson proposed (5) that the insertion of wall polymers during helical growth introduces stress into the cell surface and that the resulting strain is responsible for the shape deformation.

Direct evidence that the peptidoglycan is involved in helical deformation was recently found during a study of the effects of relevant enzymes on helical cell shape (N. H. Mendelson, M. M. Briehl, and D. Favre, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, K68, p. 147; N. H. Mendelson and D. Favre, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K160, p. 203). The addition of either lysozyme or glucosaminidase from *B. subtilis* to live macrofibers evokes helical turning motions. These are described as "relaxation" motions because the digestion apparently relieves tension in the structures. Study of the dynamics and kinetics of relaxation motions in both right- and left-handed macrofibers suggests that the wall polymers may be organized in a range of conformations that affect both the shape of the cell and its sensitivity to cleavage by such enzymes. This assumption is supported by the finding that the twist of the macrofibers in strain FJ7 varies as a function of the growth temperature and ranges from tight right-handed structures through neutral to tight left-handed structures (N. H. Mendelson, D. Favre, and J. J. Thwaites, Proc. Natl. Acad. Sci. U.S.A., in press).

The fact that peptidoglycan is involved in helical shape maintenance prompted us to examine the effects of inhibitors of peptidoglycan metabolism on helical macrofibers. The observations described here indicate that helical growth is more sensitive to inhibition by penicillin G than is total vegetative growth.

Spores from a number of *B. subtilis* strains were produced in the usual way (9) and stored at 4°C in distilled water. Results are shown only for strain Ni15 (8), a thymine and tryptophan double auxotroph. Appropriately diluted spores were heat-shocked for 10 min at 70°C and incubated at the desired temperature. Various rich media were tested; all were found to support the production of typical structures leading to the formation of macrofibers from outgrowing spores. An enriched minimal medium produced from a defined salts solution (based on 20 mM phosphate buffer supplemented with 0.5% glucose, 1% casein hydrolysate, and the nutritional requirements of the strain) was also able to support macrofiber development. In all cases macrofibers were obtained by using spore preparations diluted to less than 10^3 spores per ml. Typical structures are shown in Fig. 1.

The spore density-dependent concentration of penicillin G required for outgrowth inhibition was determined after 12 h of incubation in TB medium at 37°C (Table 1). At very low spore densities, approximately 4 ng of the drug per ml was sufficient for inhibition. Exponentially growing vegetative cells, however, were killed only by ≥10 ng/ml (data not shown). Outgrowth of spores is therefore more sensitive to penicillin G than is vegetative growth. The development of macrofibers was even more sensitive. Clones produced during germination and outgrowth of spores in medium containing 1 to 3 ng of penicillin G per ml showed extensive cell growth (Fig. 2) but never underwent the folding required for helix formation, and therefore macrofibers did not develop. Instead, disorganized multicellular structures, consisting of loosely organized open aggregates of long cellular filaments, were produced. As the drug concentration approached the growth-inhibitory level, the degree of disorganization became more pronounced. This inhibition of macrofiber formation was, however, fully reversible. Adding penicillinase to such clones resulted in the production of typical helical structures (data not shown). This observation is compatible with the interpretation of relaxation motion experiments, i.e., that cell wall peptidoglycan is responsible for the maintenance of the helical shape deformation. It further suggests that some step in peptidoglycan synthesis is essential for the folding reaction to occur.

Macrofiber formation involves helical growth of individual cells, suppression of cell separation after septation, folding, and wrapping together of the arms produced by the fold to form a single fiberlike structure (6). Macrofiber growth is accompanied by rotation of the fiber shaft along its long axis. The repeated cycle of growth, rotation, and folding always results in the production of structures in which all the cell

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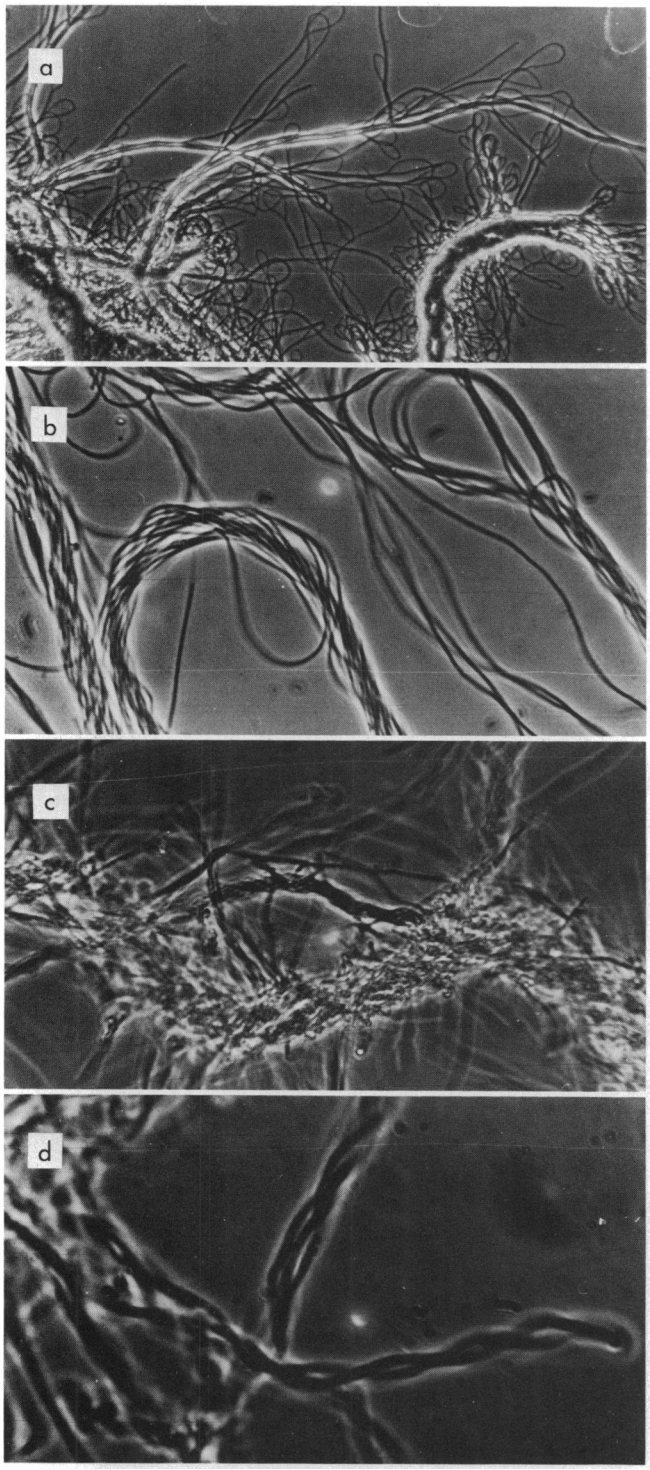


FIG. 1. Helical growth of *B. subtilis* after spore outgrowth. Spores of strain Ni15 were heat-shocked at 70°C for 10 min, diluted to 10^2 to 10^3/ml in either complex medium (TB) (a and b) or an enriched minimal medium (c and d). The cultures were incubated for 4 h at 37°C and then for 4.5 h at 27°C (a), 24 h at 27°C (b), 24 h at 32°C (c), or 24 h at 27°C (d). Live structures were photographed, using a phase-contrast microscope with the condenser adjusted to give either a bright-field phase contrast or a dark-field image.

TABLE 1. Cell density-dependent concentration of penicillin G required for growth inhibition during spore outgrowth

Penicillin G (ng/ml)	Growth[a] at cell density[b]:							
	1	10	10^2	10^3	10^4	10^5	10^6	10^7
0	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	+	+
2	±	±	±	±	+	+	+	+
3	−	−	±	±	±	+	+	+
4	−	−	−	±	±	+	+	+
5			−	−	−	±	+	+
6				−	−	±	+	+
8						±	+	+
10						±	+	+
20						−	+	+
50						−	±	+
100						−	−	+

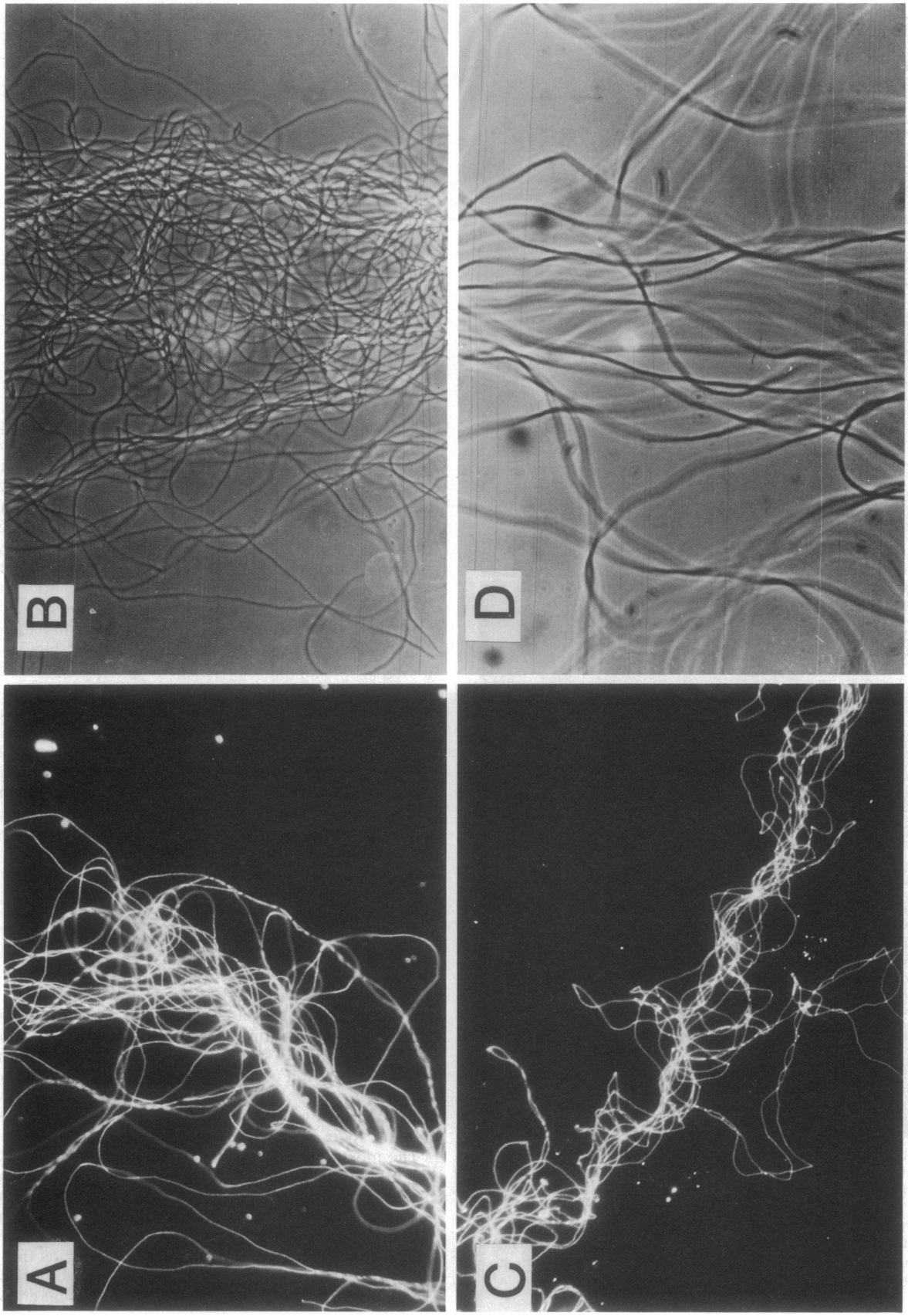
[a] +, Growth equivalent to that of controls; −, no growth detectable; ±, scant growth.
[b] Spores per milliliter.

filament layers are wrapped together in the same helix hand (6). Modeling the forces required for such a morphogenetic process (7) revealed that negative twist must be involved. Two sets of forces must therefore be acting in opposition to one another during macrofiber formation: one oriented in the direction of the helix hand of the entire structure, and the second oriented in the opposite direction. At the cellular level, it is postulated that these forces reside in the cylindrical portion of the cell and in the septa, respectively (7).

The presence of septa in clones during the initial stages of spore outgrowth before the first fold that leads to a double-stranded helical structure concords with this hypothesis (Fig. 3). We searched for septa in clones blocked from folding by being grown out in 1 to 3 ng of penicillin G per ml, as inhibition of cell division has been reported in *Escherichia coli* at very low drug concentrations that do not interfere with balanced growth (10), and a similar finding would support our hypothesis. The long unfolded *B. subtilis* filaments produced in the presence of penicillin G contained evenly spaced septa (Fig. 4). This finding raises the possibility that the septa may not be the structural source of the negative twist. Unfortunately, it is not currently possible to determine whether these septa are deficient in some way that affects the biomechanics, whether the drug affects the development of positive twist associated with growth of the cylindrical portion of the cell, or whether penicillin G affects the interaction between positive and negative twist.

The shape of cellular filaments in clones grown but unable to fold in the presence of penicillin G suggests helical growth. Present information does not, however, permit quantitative comparison of the twist with that of normal helical growth. Kinetic studies of growth in low concentrations of penicillin G should provide some information about the rate of rotation as a function of elongation, but such data are not easy to obtain because of the technical difficulty of producing high-quality microcinematographic records of individual spore outgrowth clones in fluid.

The most abundant penicillin-binding protein in *B. subtilis*, PBP V, has been shown to correspond to D-alanine carboxypeptidase (1), but the killing target(s) for each of the penicillins has not yet been identified. Moreover, neither cylindrical growth nor septation in *B. subtilis* is known to be related to specific penicillin-binding proteins, as they are in *E. coli*. Our findings show that there is a sensitive penicillin G target in *B. subtilis* that is involved in helical growth and macrofiber formation but not in septation. Genetic and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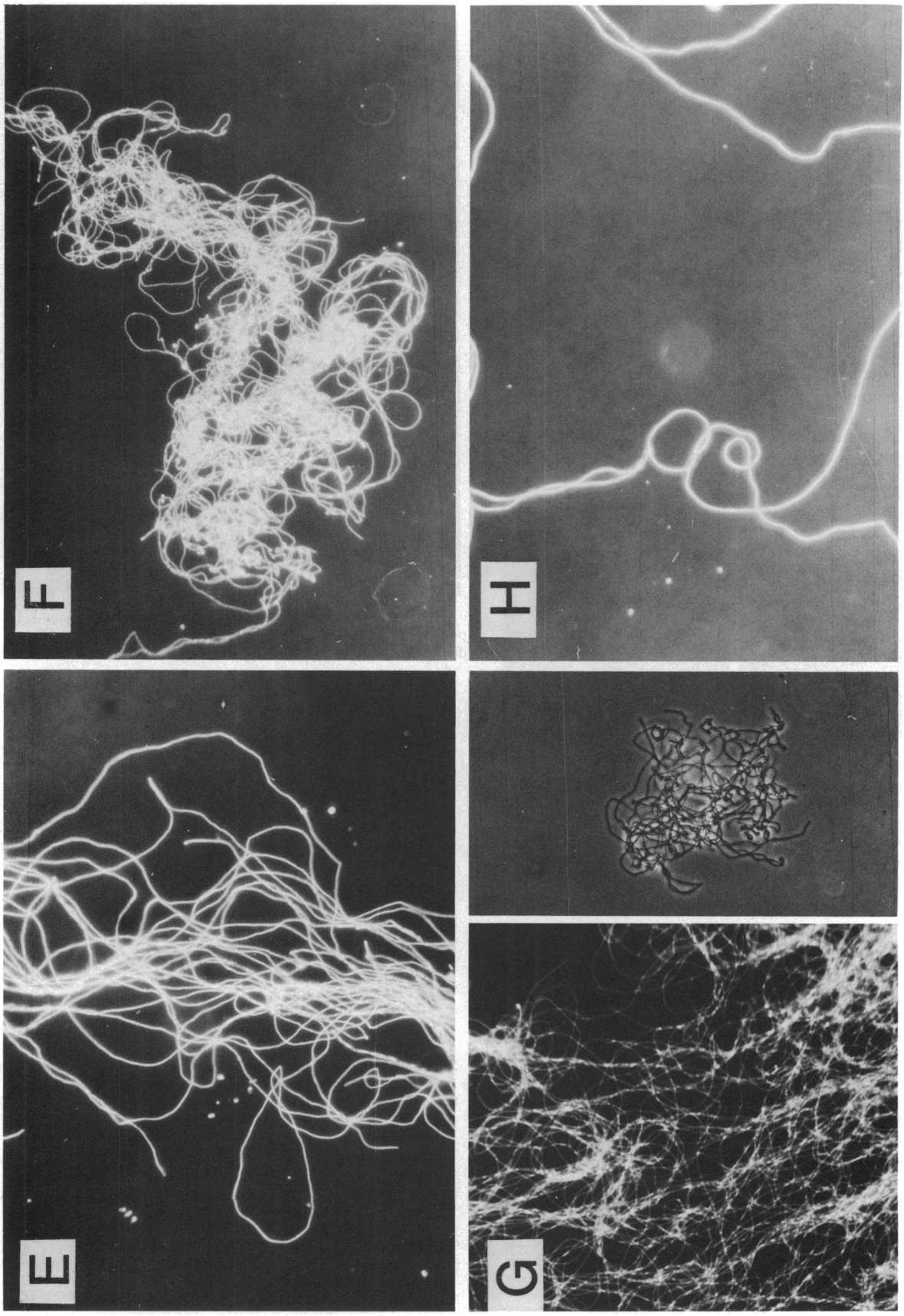

FIG. 2. Penicillin G inhibition of helical macrofiber formation in *B. subtilis*. Spores of strain Ni15 were germinated in complex medium as described in the legend to Fig. 1 with 1 (A and B), 2 (C through F), 3 (G and inset), or 4 (H) ng of penicillin G per ml. Live structures were photographed, using a phase-contrast microscope.

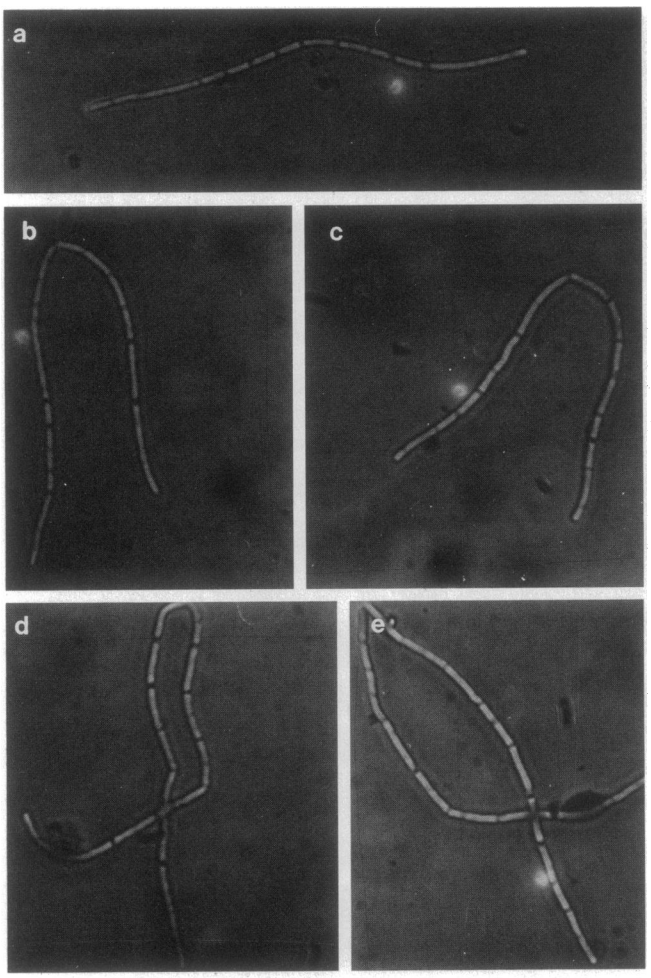

FIG. 3. Septa in clones of strain Ni15 after spore outgrowth. Samples from populations grown in complex medium at 37°C for 8 h with shaking (a and b) or for 4 h (c through e), as described in the legend to Fig. 1, were taken during the initial period of helix formation, fixed by air drying, and stained with crystal violet. Photographs were taken with a phase-contrast microscope.

biochemical studies are under way to determine whether this target is one (or more) of the five known penicillin-binding proteins. In addition, we hope eventually to be able to define the peptidoglycan alteration, resulting from drug inhibition, that is responsible for blocking the folding.

A high degree of septation synchrony was observed along the filaments during the early stages of outgrowth (Fig. 3). The spore outgrowth system used for this study will be used in another context to examine the quantitative relationship between septation and folding. The system may also be applied to studying the loss of synchrony and consequent randomization of divisions in a bacterial clone grown unrestrictedly.

This communication also describes the composition of a defined medium that supports the development of Lyt$^-$ phenocopies. With this medium it should now be possible to perform nutritional control experiments and to examine processes such as the energetics of helical growth and macrofiber formation.

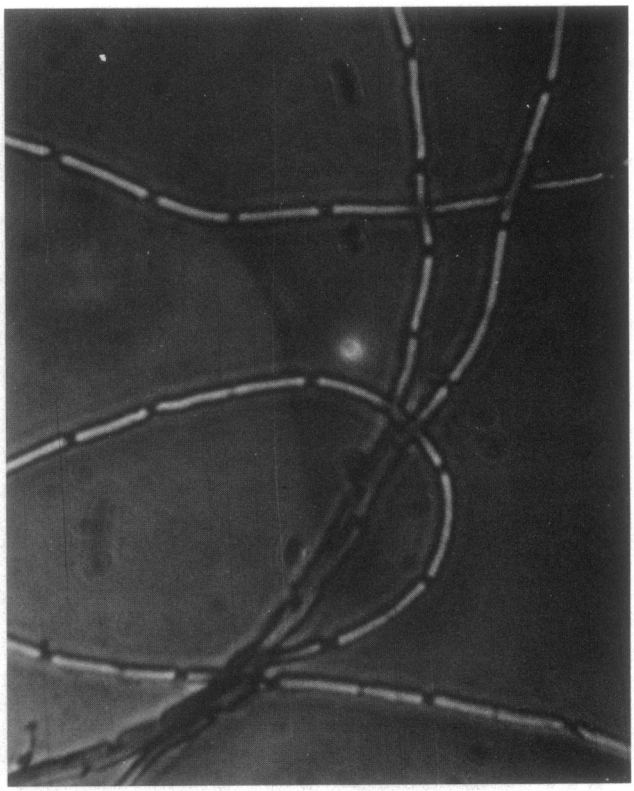

FIG. 4. Septa in clones produced after spore outgrowth in the presence of 2 ng of penicillin G per ml; see the legend to Fig. 3 for details.

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