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Nucleoid Condensation and Cell Division in *Escherichia coli* MX74T2 *ts52* After Inhibition of Protein Synthesis

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The reorganization of the bacterial nucleoid of an *Escherichia coli* mutant, MX74T2 *ts52*, was studied by electron microscopy after protein synthesis inhibition by using whole mounts of cell ghosts, ultrathin-sectioning, and freeze-etching. The bacterial nucleoid showed two morphological changes after chloramphenicol addition: deoxyribonucleic acid (DNA) localization and DNA condensation. DNA localization was observed 10 min after chloramphenicol addition; the DNA appeared as a compact, solid mass. DNA condensation was observed at 25 min; the nucleoid appeared as a cytoplasm-filled sphere, often opened at one end. Ribosomes were observed in the center. Giant nucleoids present in some mutant filaments showed fused, spherical nucleoids arranged linearly, suggesting that the tertiary structure of the nucleoid reflects the number of replicated genomes. Inhibitors which directly or indirectly blocked protein synthesis and caused DNA condensation were chloramphenicol, puromycin, amino acid starvation, rifampicin, or carbonyl cyanide *m*-chlorophenyl hydrazone. All inhibitors that caused cell division in the mutant also caused condensation, although some inhibitors caused condensation without cell division. Nucleoid condensation appears to be related to chromosome structure rather than to DNA segregation upon cell division.

The bacterial chromosome of *Escherichia coli* is thought to be a single, circular macromolecule over 1.1 mm in length (2). The long filament of deoxyribonucleic acid (DNA) is tightly packaged in a volume which barely exceeds 1 μm^3 (19). The packaged chromosome, observed in a structural and functional entity by phase-contrast microscopy of living cells (13), must be sufficiently organized to permit DNA replication and chromosome segregation, while maintaining individual genes available for transcription and translation (1, 12, 20). Nucleoid structure has been studied morphologically (5, 9, 18) and, more recently, biochemically (22, 24). The results of these experiments suggest a complex and organized nucleoid structure.

We have studied cell division and nucleoid morphology in an *Escherichia coli* mutant, MX74T2 *ts52* (25). This mutant is conditionally lethal; it gradually stops dividing when transferred from 30 to 40 C in rich medium, but continues to synthesize DNA and protein. Filaments which result from growth of the mutant at 41 C can be induced to divide, even at the high temperature, by some conditions that inhibit peptide bond formation, such as the presence of chloramphenicol (100 μg/ml), amino acid starvation, or rifampicin (200 μg/ml) (25).

In the present study, we examined nucleoid morphology by electron microscopy after protein synthesis inhibition by using whole mounts of cell ghosts, ultrathin-sectioning, and freeze-etching. Inhibitors which directly or indirectly block protein synthesis were found to cause nucleoid condensation in both the mutant and wild-type strains. Not all inhibitors that cause nucleoid condensation, however, permit cell division in filaments of the mutant. The results are consistent with a simple, non-energy-requiring model for nucleoid condensation in which the absence of protein synthesis causes conden-

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sation by reducing the interaction of the DNA with the cytoplasm, allowing the coiled DNA folds to tighten.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* K-12 strains MX74T2 (*thi⁻ lac⁻ arg-1⁻ rel⁻ str^R thy⁻*) (8) and MX74T2 *ts52* (temperature sensitive in rich medium) (25) were used. Fresh, overnight cultures were grown for 3 h at 30 C in supplemented M9 medium containing: 7 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl, 0.2 g of $MgSO_4 \cdot 7\ H_2O$, and 1 liter of water; the medium was supplemented with 4 mg of glucose/ml, 2 μg of thiamine/ml, 4 μg of thymidine/ml, and 50 μg of arginine/ml. Exponential-phase cultures were then supplemented with 0.5% Casamino Acids (vitamin free, Difco) that had been partially purified by mixing with 1% activated carbon and filtered through a membrane filter (0.22 μm). In experiments requiring subsequent amino acid starvation, the cells were resuspended in a defined rich medium containing an amino acid composition similar to the Casamino Acids medium, as described previously (25). After 3 h of incubation at 30 C in the rich media, cultures were shifted to 41 C. After 1 h of growth at the high temperature, inhibitors were added (cell densities were then about 10^8 cells/ml). Arginine starvation was achieved by resuspending the cells in the defined rich medium lacking arginine.

Chloramphenicol, rifampicin, and carbonyl cyanide *m*-chlorophenyl (CCCP) hydrazone were obtained from Calbiochem (Los Angeles, Calif.). Puromycin dihydrochloride was obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio).

Preparation of cell ghosts. Cultures (5 ml) were cooled to 0 C and centrifuged at 5,000 rpm for 10 min. The cell pellets were suspended in 0.2 ml of medium containing 0.5% Casamino Acids. To facilitate subsequent handling and enzyme digestion, the grids placed on the Formvar film on the water surface were picked up directly on a glass microscope slide, and the entire slide was carbon coated and glow discharged. A drop of the concentrated culture was placed on the prepared grids while still attached to the glass slides. The excess liquid was removed, and the culture was allowed to air-dry. The cells were then fixed and digested with ribonuclease (200 μg/ml) and pepsin (200 μg/ml) as suggested by Peters and Wigand (17). The method used previously (25) was modified slightly to reduce deoxyribonuclease (DNase) contamination; the ribonuclease was boiled before use, and the pepsin was preincubated at 37 C for 30 min.

Preparation of ultrathin sections. Cultures (20 ml) were cooled to 0 C and centrifuged at 5,000 × *g* for 10 min. The cells were washed twice with 0.01 M phosphate buffer, pH 7.0. The cells were fixed as pellets in 1% glutaraldehyde in 0.01 M phosphate buffer (pH 7.0) for 90 min at 0 C and were washed four times in 0.01 M phosphate buffer. The cells were then postfixed in 1% OsO_4 in 0.01 M phosphate buffer (pH 7.0) for 3 h at 0 C, stained in mass in buffered 0.5% uranyl acetate for 2 h at room temperature, dehydrated in graded ethanol and propylene oxide, and embedded in Epon 812. The embedded specimens were thin-sectioned with a diamond knife on an LKB III ultramicrotome and stained with uranyl acetate and lead citrate.

Freeze-etching. Cultures were washed as described above and fixed in 2% glutaraldehyde in 0.01 M phosphate buffer (pH 7.0) for 30 min at 0 C and were subsequently washed four times with phosphate buffer (pH 7.0). Cell pellets were processed through graded glycerol solutions of 5, 10, 20, 30, and 40% and then were quenched in a Freon 22 (DuPont) bath. The frozen sample was mounted in specimen holders and fractured at −195 C in a Denton DFE-3 freeze-etching apparatus. The temperature of the cells was raised to −90 C, and they were etched for 1 min. Platinum-carbon replicas were prepared. All of the electron micrographs were taken with a Philips 300 electron microscope, at 60 kV, equipped with an anticontamination device.

RESULTS

Chloramphenicol and certain other inhibitors of protein synthesis were previously shown to induce cell division at the restrictive temperature in filaments of MX74T2 *ts52* (25). Because inhibitors of protein synthesis are also known to cause nucleoid condensation (3, 9, 15), the morphology of the cell division mutant was investigated under various inhibitory conditions to determine whether nucleoid condensation is related to the triggering of cell division. Nucleoid condensation can be rapidly monitored in cell ghosts prepared by digesting bacteria with pepsin and ribonuclease (17). This method was used in a preliminary experiment to study the kinetics of chloramphenicol-related nucleoid condensation in filaments of *ts52* (Fig. 1) and the parent strain MX74T2 (Fig. 2). The untreated nucleoids appeared as loosely packed, uniform puddles of electron-dense material against the almost electron-transparent sacculus. The electron-dense material disappeared after deoxyribonuclease treatment. The comparable longitudinal thin sections of *ts52* revealed that the translucent nucleoid material was largely dispersed with only small, vascular pools of fine fibrils located at random in the cytoplasm (Fig. 3a). The filaments were not uniform in size; longer filaments often contained folds of membrane material near the cell poles (Fig. 3a) (21) and occasionally at parallel sites of peripheral membrane surfaces (Fig. 4).

Addition of chloramphenicol (100 μg/ml) to the growth medium resulted in condensation of the nucleoids and division of the filamentous cells (Fig. 1 and 3). Nucleoid condensation was initiated by DNA localization and began about

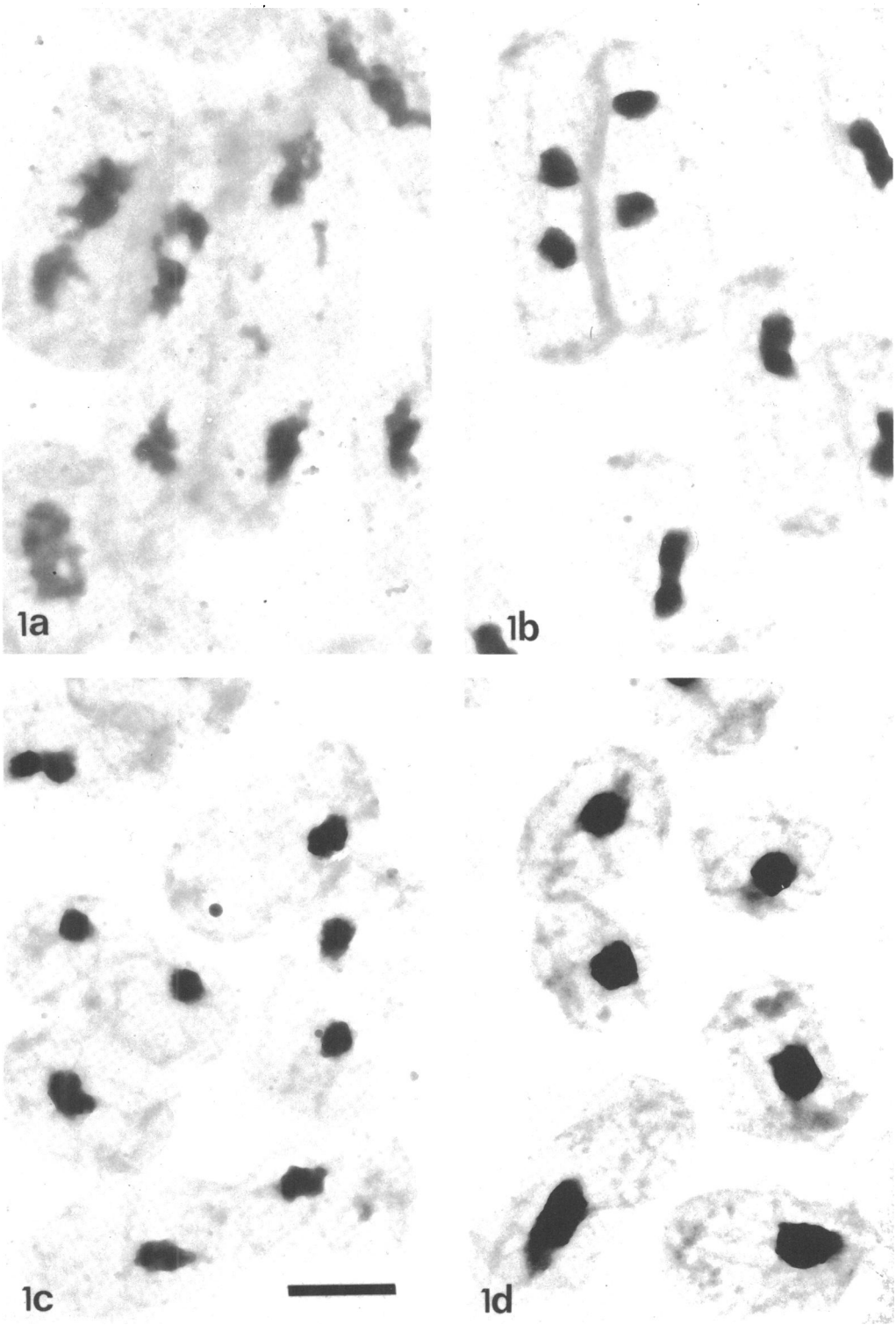


FIG. 1. *Kinetics of cell division and nucleoid condensation in filamentous cultures of E. coli MX74T2 ts52 as observed in cell ghosts. Filamentous cultures of ts52 were prepared by growing cells for 60 min at 41 C in the Casamino Acids medium. Filaments of ts52 were incubated with chloramphenicol (100 μg/ml) for (a) 0 min, (b) 10 min, (c) 20 min, or (d) 60 min. The cells were quickly cooled to 0 C and then were digested with pepsin and ribonuclease, as suggested by Peters and Wigand (17). The nucleoids appear as electron-dense material within the almost transparent sacculus. The shape of the succulus is flattened by this treatment. The bar represents 1 μm.*

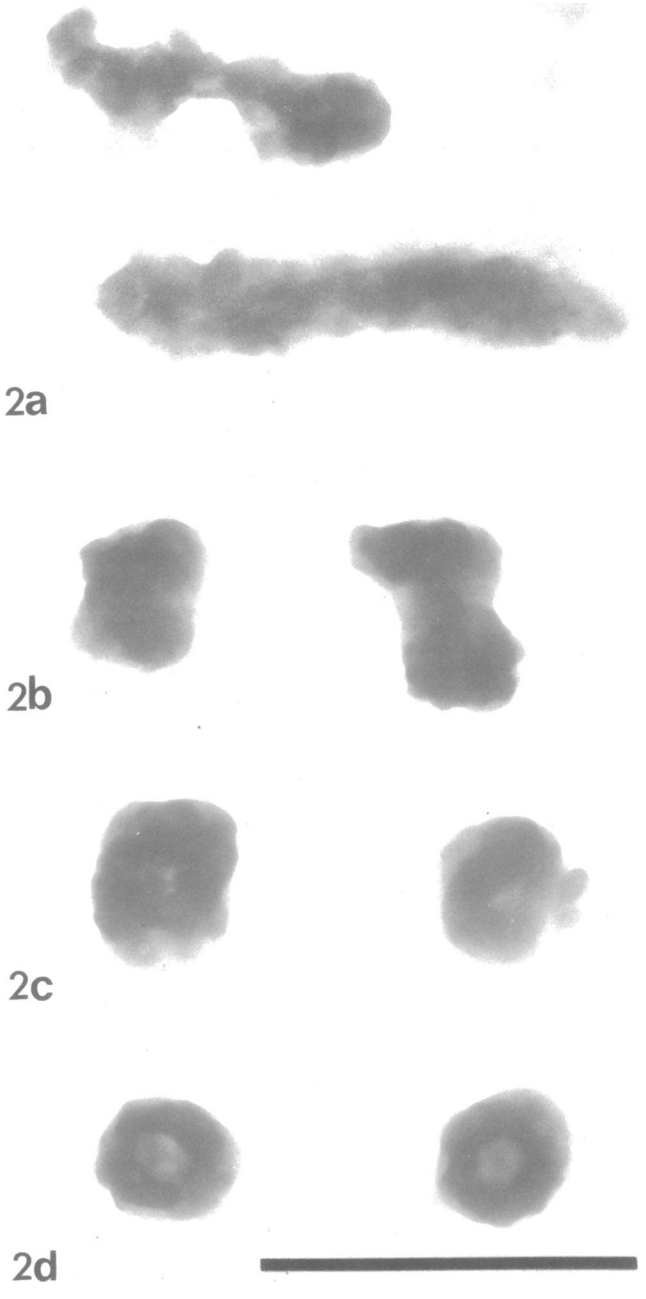


FIG. 2. *Nucleoid condensation in E. coli MS74T2 as observed in cell ghosts. A culture was grown at 41 C in the Casamino Acids medium. Chloramphenicol (100 μg/ml) was added to the culture, and cells were removed at intervals, chilled, and digested as described in Fig. 1. Because of the very high density of the nucleoids in comparison with the sacculus, changes in the organizational state of the DNA are most apparent when the micrograph negatives are printed to only show the nucleoids. The bar represents 1 μm.*

10 min after chloramphenicol addition. The nucleoids of the digested cell ghosts of wild-type and mutant cells at this time appeared as compact spheres with an irregular surface and having a diminished nucleoid area of increased electron density (Fig. 1b and 2b). Thin sections

of *ts52* show the DNA fibrils organized into discrete areas, often having a coiled appearance (Fig. 3b). The change in nucleoid morphology is observed in every cell, although some minor variations in the time sequence were seen.

The nucleoids continued to condense in the presence of chloramphenicol until about 25 min after its addition; after this time, no change in nucleoid appearance was observed for at least 1 to 2 h. The nucleoids of the digested wild-type cell ghosts appeared as regular, compact spheres, with an area of lesser density in the center (Fig. 2). Thin sections of *ts52* reveal that the DNA is organized into a ring of coiled fibrils around a "core" (Fig. 3c; 5). The three-dimensional configuration of the condensed nucleoid appeared to be a hollow sphere which may be opened at one end, for both longitudinal and cross sections showed closed, and occasionally, open rings (Fig. 5, 6). Such a nucleoid structure was first proposed by Kellenberger (9) on the basis of his studies of aureomycin (4 μg/ml) treated *E. coli*. The fractured planes of freeze-etched *ts52* cells also showed ring-shaped or "C"-shaped nucleoids (Fig. 7). The "C"-shaped nucleoids indicate that the cytoplasmic material (ribosomes) is continuous with the "core" material.

It is difficult to estimate the change in volume of the DNA after chloramphenicol addition on the basis of the electron micrographs. Besides the usual problem of comparing different ultrathin sections, the nucleoids vary in DNA content, depending on the amount of DNA replication in different cells. After chloramphenicol addition, the DNA content of some nucleoids will increase in a heterogeneous manner inversely related to cell age and previous replication.

Filaments of *ts52* grown under the restrictive conditions sometimes contain nucleoids which fail to segregate normally on completion of DNA replication (25). The replicated DNA accumulates to form giant nucleoids. Longitudinal sections of these chloramphenicol-treated filaments show a linear arrangement of fused, spherical nucleoids (Fig. 8). The amount of DNA organized around each core (as measured by the area of longitudinal sections) appeared in many sections to be approximately equal. The ratio of DNA area to core area in these sections remained about 3.5 ± 0.8, even in nucleoids containing enormous amounts of DNA. It is of interest that these giant nucleoids were never observed to show DNA-membrane attachment, which suggests that their abnormality may be related to a membrane defect.

The population of the smaller *ts52* daughter cells increased at the end of 25 min of chloramphenicol treatment. The condensed nucleoids of these cells appeared to be identical to those of undivided cells incubated in chloramphenicol for the same length of time. Daughter cells lack many of the membrane folds observed in some untreated filamentous cells.

The effect of inhibitors on nucleoid condensation was studied in cell ghosts of strains MX74T2 and MX74T2 *ts52*. The results are summarized in Table 1. Both direct and indirect inhibitors of protein synthesis caused condensation. In contrast, DNA inhibition by thymidine starvation did not effect nucleoid morphology; thymidine starvation did not interfere with the chloramphenicol-related nucleoid changes. Condensation even occurred in the presence of CCCP, a potent uncoupling agent for oxidative phosphorylation (6). Thus, nucleoid condensation appears to have resulted from the absence of protein synthesis and to have occurred even under conditions of energy starvation.

Cell division in filaments of *ts52* has been shown previously to be induced by the addition of inhibitors of peptide bond formation (25). Unlike nucleoid condensation, however, inhibitors of protein formation such as puromycin, or inhibitors of energy production like CCCP, do not induce division (Table 1). The presence of CCCP prevents chloramphenicol-induced cell division, but does not prevent nucleoid condensation.

Filaments of *ts52* showed nucleoid condensation within 10 min of chloramphenicol addition, usually before the onset of septation. All septating cells observed after chloramphenicol addition, in fact, showed condensed nucleoids. The inhibitors that cause condensation do not necessarily cause division at the restrictive temperature (Table 1): puromycin and CCCP both cause condensation, but no division. It is interesting that no inhibitor was found that will induce division in the absence of condensation in the mutant, although this is the normal observation in exponential-phase cells.

DISCUSSION

The most striking result observed in these studies is the unique structure of the condensed multiple nucleoids after chloramphenicol addition to cultures of *ts52*. Under these conditions, the DNA appears as electron-dense spheres of coiled fibrils surrounding a cytoplasmic core. Filaments showing abortive nucleoid segregation maintain the basic structure of the condensed nucleoid as linear polymers having a

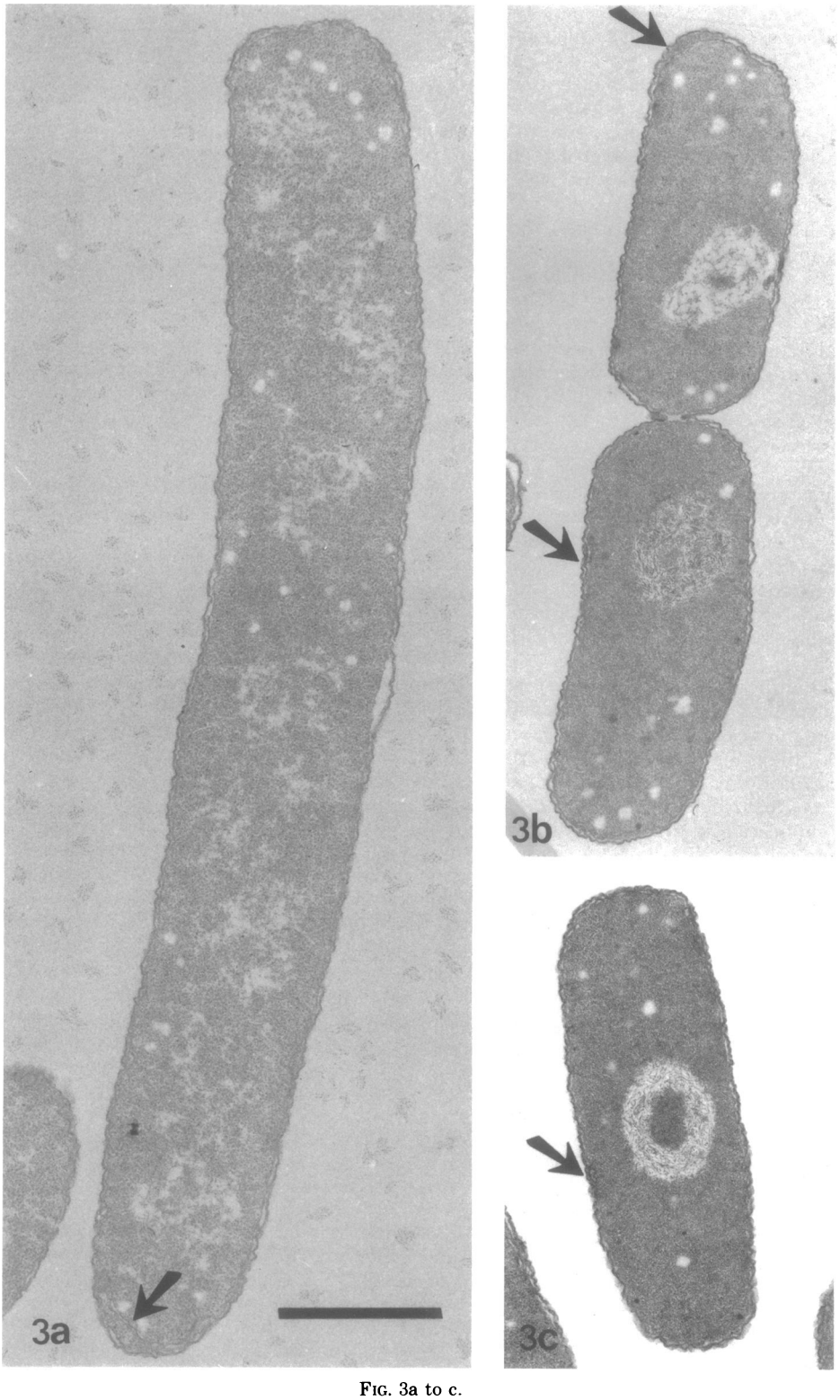
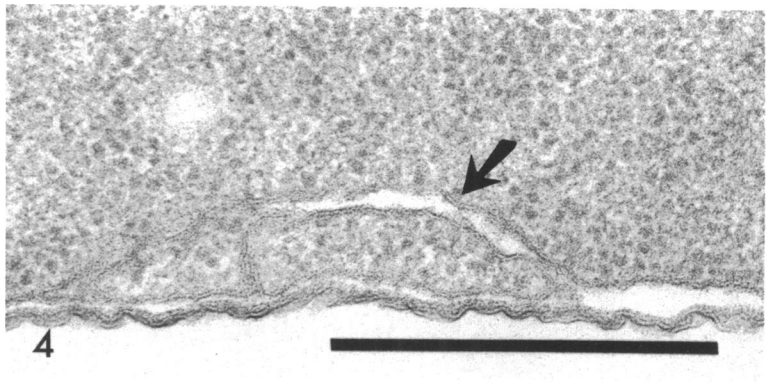


FIG. 3a to c.



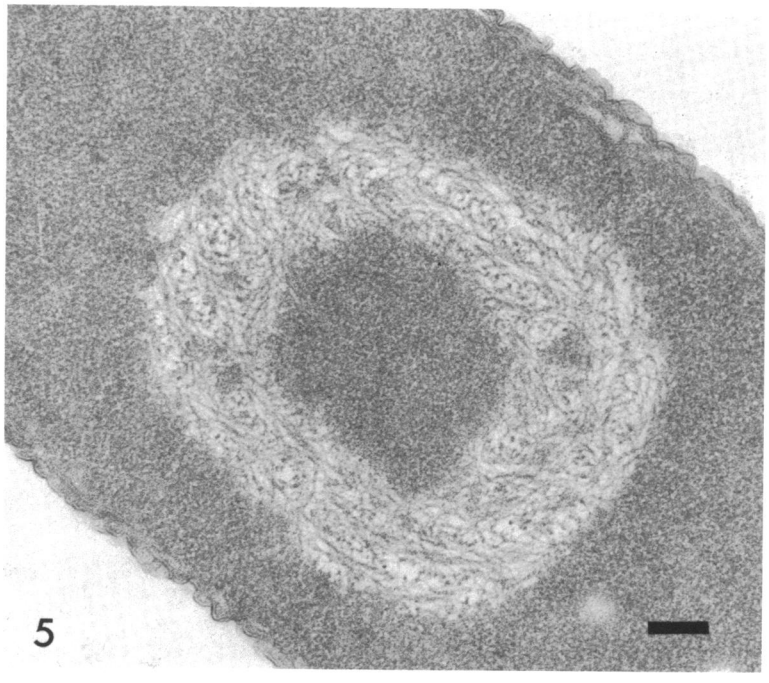


FIG. 4. *Detail of a longitudinal thin section of an E. coli MX74T2 ts52 filament showing peripheral membrane folds (arrow). The bar represents 0.5 μm.*

FIG. 5. *Detail of a longitudinal section of a condensed nucleoid. A filamentous culture was prepared as described in Fig. 1, except that this culture was treated with chloramphenicol for 60 min. The bar represents 100 nm.*

FIG. 3. *Electron micrographs of longitudinal thin sections of E. coli MX74T2 ts52 taken from filamentous cultures before and after chloramphenicol treatment. Filamentous cultures of ts52 were prepared by growing cells for 60 min at 41 C in the Casamino Acids medium. Folds of membrane material appear near the cell poles and occasionally along the cell periphery. (arrows) (a) Filament of ts52 showing the translucent nucleoid material dispersed throughout the cell. (b) Filament incubated in the presence of chloramphenicol (100 μg/ml), for 10 to 15 min at 41 C, showing septation and organized nucleoids. The nucleoid material of one daughter cell appears to be membrane bound. (c) Daughter cell incubated in the presence of chloramphenicol for 25 min showing the completion of cell division and further condensation of the nucleoid material. The compact nucleoid usually has a thick, ring-shaped appearance in longitudinal sections. The core material contains ribosomes. The bar represents 1 μm.*

constant ratio of DNA mass to core mass (Fig. 8). Thus, the tertiary structure of the nucleoid appears in some way to reflect the number of replicated genomes, indicating a possible link between a chromosomal site(s) and nucleoid organization.

Stonington and Pettijohn (22) suggested that a ribonucleic acid (RNA)-DNA-protein link is

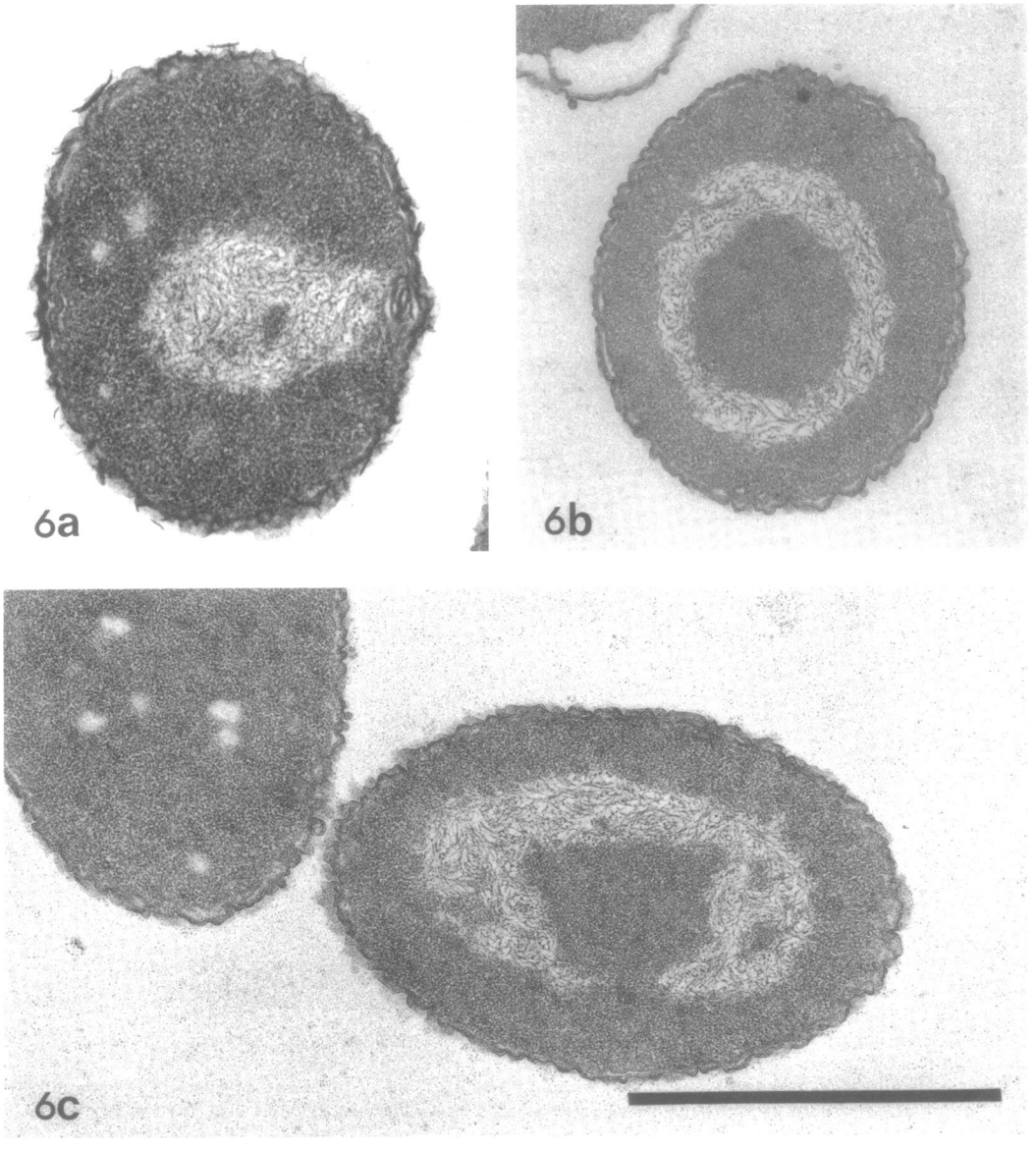


FIG. 6. *Electron micrographs of cross sections of E. coli MX74T2 ts52 after chloramphenicol treatment. A filamentous culture was treated with chloramphenicol (100 μg/ml) for 25 min. The nucleoid material is already condensed and appears as either (a) a thick closed ring, (b) a closed circle, or (c) an open ring. The bar represents 1 μm.*

responsible for maintaining nucleoid structure. They were able to isolate, intact, the folded genome of *E. coli*. The low-viscosity and high-sedimentation rate of the isolated complex suggested a very tightly folded conformation. The complex was unfolded by sodium dodecyl sulfate, pancreatic ribonuclease, or heat. These results were confirmed and extended by Worcel and Burgi (24). They showed by ethidium bromide binding and DNase kinetics that isolated nucleoids contain about 50 loops of supercoiled DNA, each independently bound to an RNA-containing matrix. They calculated that each loop is about 20 μm long and contains about 200 negative superhelices. The condensation observed in chloramphenicol-treated cells may therefore be coil tightening of the DNA loops within the constraints of the RNA-protein matrix. The constraints on the outer surface of the nucleoid may be more limiting, creating a

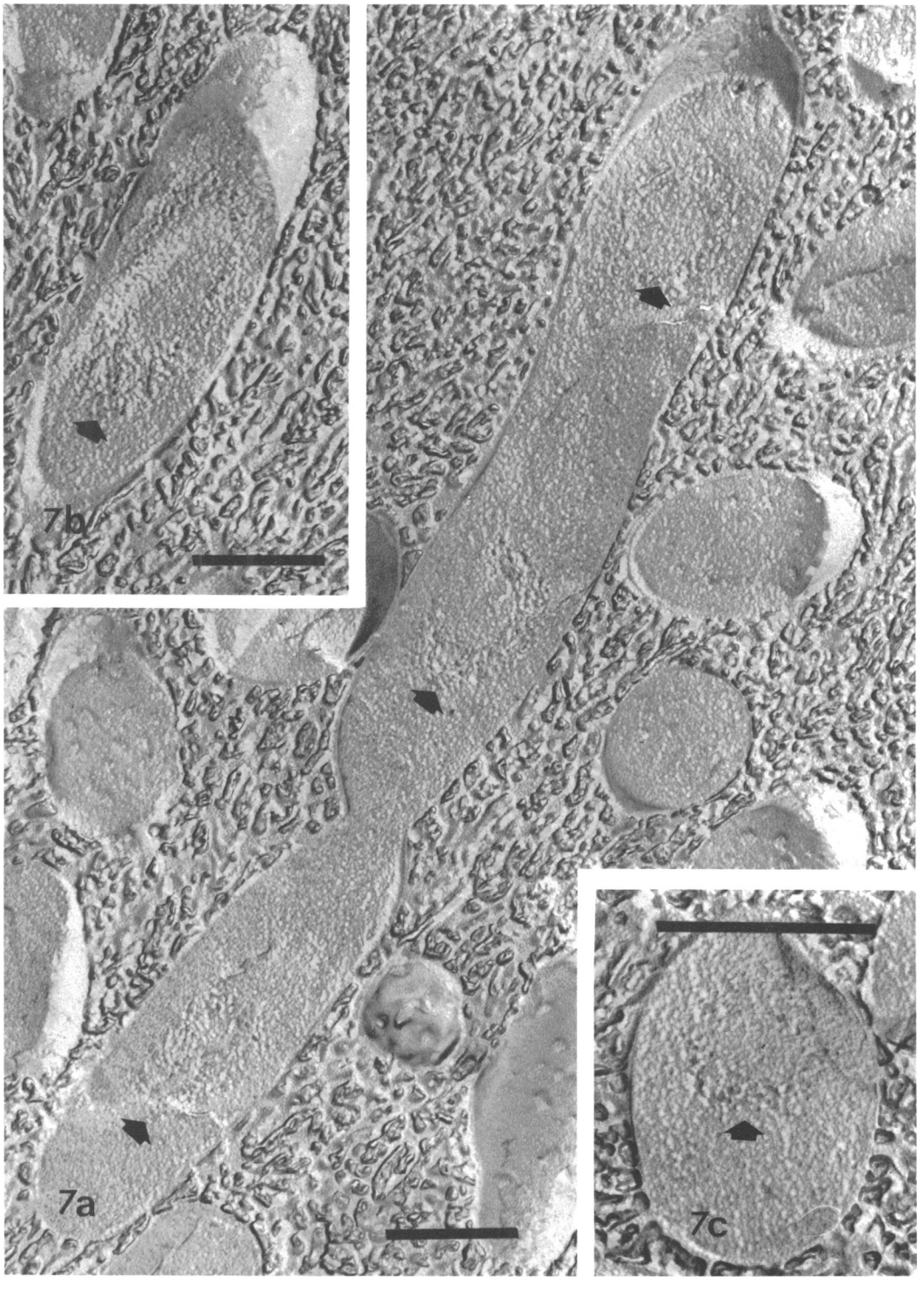


FIG. 7. *Freeze-etching of filaments of E. coli MX74T2 ts52 after chloramphenicol treatment. A filamentous culture was treated with chloramphenicol (100 μg/ml) for 25 min and then was prepared for freeze-etching as described in Materials and Methods. The nucleoids are indicated by arrows. (a) Longitudinal fracture of a dividing filament, (b) longitudinal view of a daughter cell, (c) a cross-sectional fracture showing the nucleoid region. The bars represent 1 μm.*

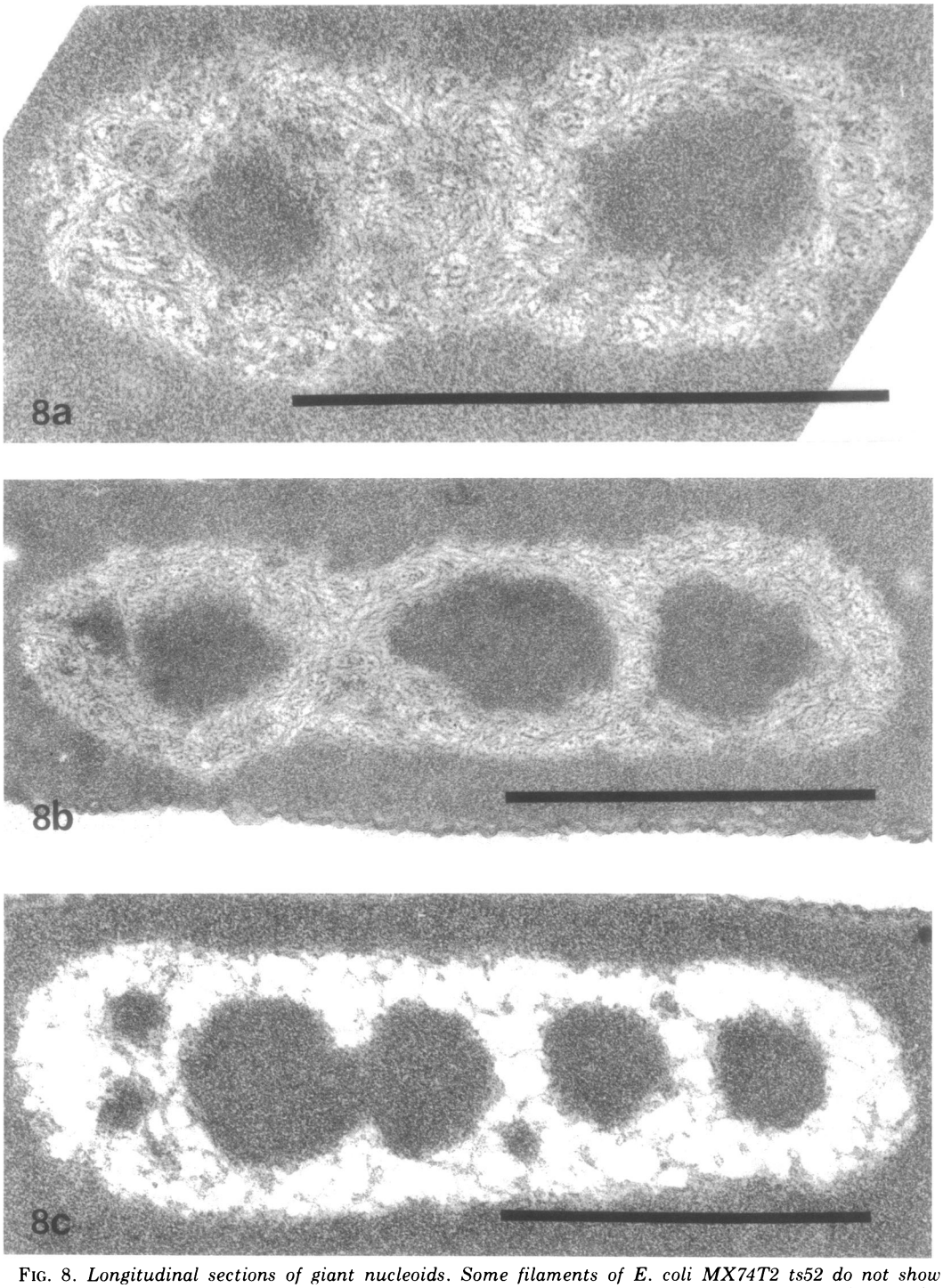


FIG. 8. *Longitudinal sections of giant nucleoids. Some filaments of E. coli MX74T2 ts52 do not show segregation of nucleoids upon completion of chromosome replication. In these filaments, the nucleoid material condenses to form chains after the addition of chloramphenicol (100 μg/ml) for 25 min at 41 C. Longitudinal sections of these giant nucleoids show (a) two, (b) three, (c) four or more thick rings of coiled fibrils fused linearly; fixation did not preserve the DNA fibers and left an empty nucleoid region. Scale markers = 1 μm.*

TABLE 1. *Effect of inhibitors on exponential phase MX74T2 and filaments of MX74T2 ts52*[a]

Inhibitor	MX74T2		MX74T2 *ts52*	
	Nucleoid condensation	Cell division	Nucleoid condensation	Cell division
Inhibitors of protein synthesis				
None	−	+	−	−
Chloramphenicol (100 μg/ml)	++	−	++	+
Puromycin (500 μg/ml)	+	−	+	−
Puromycin (500 μg/ml) + chloramphenicol (100 μg/ml)	++	−	++	+
Arginine starvation[b]	++	−	+	+
Other inhibitors				
Rifampicin (200 μg/ml)	++	−	++	+
Thymidine starvation	−	−	−	−
Thymidine starvation + chloramphenicol (100 μg/ml)	++	−	++	+
Carbonyl cyanide *m*-chlorophenyl hydrazone (2×10^{-3} M)	++	−	++	−
Carbonyl cyanide *m*-chlorophenyl hydrazone (2×10^{-3} M) + chlorophenicol (100 μg/ml)	++	−	++	−

[a] Cultures of MX74T2 and MX74T2 *ts52* were grown for 60 min at 41 C in the Casamino Acids-supplemented medium. Subcultures were then incubated in the presence of the inhibitory conditions listed. After 30 min of incubation, cell number was determined and portions were prepared for electron microscopy. (++), 90 to 100% of cells; (+), 50 to 60% of cells.

[b] Cultures for this experiment were grown in a defined rich medium as described previously (25). Arginine starvation was achieved by suspending the cultures in the defined medium lacking arginine.

cytoplasmic space within the spherical nucleoids. The swirls of nucleoid material observable in Fig. 3b are very suggestive of the tightened-coil model. The more dispersed exponential-phase nucleoid may contain the same organization as the chloramphenicol-treated nucleoid, although its structure is masked by loose interaction of the DNA coils with the cytoplasm. Daneo-Moore and Higgins (3) and Higgins and Shockman (7) found that the normal and condensed nucleoids of *Streptococcus faecalis* do have the same interconnected structure when examined in thin serial sections.

The condensation of bacterial nucleoids by drugs and environmental conditions has been reported previously in several laboratories (3, 4, 9, 11, 14–16, 23). Kellenberger (9) observed that the addition of auromycin (4 μg/ml) to an exponential-phase culture of *E. coli* caused the "nuclear vacuole" to become globular after 30 to 40 min; the nucleoid frequently contained a central core as evidenced by ring-shaped "nuclear vacuoles" in thin sections (10). Morgan et al. (15) studied nucleoid condensation after addition of chloramphenicol (40 μg/ml). They noted an "island of cytoplasm" within the nuclear matrix in many sections. Thus, the appearance of a cytoplasmic core is not unique to chloramphenicol or the mutant *ts52;* however, the appearance of giant multiple nucleoids in the mutant (Fig. 8) is significant, because it suggests that nucleoid structure is related to the number of genomes. The presence of this spherical nucleoid structure is observed by employing thin-sectioning and freeze-etching. Because freeze-etching techniques require the least handling of material, this structure is most likely the real form and not an artifact.

Blocking protein synthesis with chloramphenicol, puromycin, arginine starvation, rifampicin, or CCCP caused subsequent condensation of the nucleoid material (Table 1). These results are in excellent agreement with the basic model of Daneo-Moore and Higgins (3) for nucleoid condensation in *S. faecalis*. They suggest that the replicating bacterial chromosome is normally dispersed in the cytoplasm because of the interaction of the DNA with the RNA polymerase-polysome transcription and translation machinery. Inhibition of protein synthesis would therefore lead to localization of nucleoid fibrils. This model suggests that nucleoid condensation is a passive reorganization of the DNA based on reduced interaction with the cytoplasm and the inherent tightly organized tertiary structure (24) of the DNA. The data presented support this model: energy starvation with CCCP actually causes condensation; inhibitors of peptide bond formation, either direct (chloramphenicol or arginine starvation) or indirect (rifampicin), cause condensation. On the other hand, puromycin, an inhibitor which induces premature termination of tiny peptides, is somewhat less efficient. Inhibiton of DNA synthesis by thymidine starvation did not change nucleoid appearance; however, the chloramphenicol related morphological changes were independent of DNA synthesis. This observation is in conflict with the results of Daneo-Moore and Higgins, but is in agreement with Morgan et al. (15). It is possible that *E. coli* differs in this respect from *S. faecalis*.

The relationship between nucleoid condensation and cell division is not very clear. While eucaryote chromosomes are known to condense at the onset of mitosis, the bacterial nucleoid in exponential-phase cells usually appears to be dispersed, even during division. The "dis-

persed" nucleoid, however, may contain the same level of organization and coiling as the chloramphenicol-condensed nucleoid; the major difference then may be in the level of interaction of the DNA fibrils with the cytoplasm. Segregation of the bacterial chromosome during division is, perhaps, best explained by membrane-DNA attachment (19) (Fig. 3b). If this hypothesis is correct, then the study of condensation is a useful technique for clarifying the tertiary structure of the exponential-phase nucleoid, but not for looking at a special premitotic state.

Filaments of *ts52* grown at the restrictive temperature for longer time periods are often observed to show membrane folds, in parallel, at regular intervals along the cell periphery. These membrane folds are not seen as frequently after chloramphenicol addition, and may, therefore, be presepta. This anomalous membrane material may be responsible for the increased amounts of one envelope fraction observed after acrylamide gel electrophoresis (25). If this protein proves to be a normal septum component, it would be the first such protein identified.

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