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Effect of Ca^{2+} on Morphology and Division of *Yersinia pestis*

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Wild-type cells of *Yersinia pestis* are known to exhibit a nutritional requirement for physiological levels of Ca^{2+} (~2.5 mM) at 37 but not 26 C. Upon shift of Ca^{2+}-deficient cultures from 26 (permissive condition) to 37 C (restrictive condition), bacterial mass quadrupled as the organisms doubled in number and then became elongated to about twice their normal size. As shown in thin sections, the resulting static cells contained axial filaments which differed from the typical irregularly lobate nucleoids of normal yersiniae grown under the permissive condition. Following prolonged cultivation under the restrictive condition (12 h), the organisms generally exhibited apparent degenerative changes, including separation or infolding of the cell wall and cytoplasmic membrane, degeneration of deoxyribonucleic acid, and appearance of vacuoles within the cytoplasm. At this time, the cells were unable to reinitiate cell division at 37 C upon addition of Ca^{2+} but divided in partial synchrony after return to 26 C. This observation indicated that, at 37 C, continuous exposure to Ca^{2+} is necessary for yersiniae to maintain normal morphology and the ability to divide.

Ca^{2+} may serve as a cofactor for prokaryotic membrane-bound enzymes and exoenzymes (10, 26) and can exist as a structural component of the cell wall and cytoplasmic membrane (8, 9, 32). Despite these roles, instances of nutritional dependence upon Ca^{2+} are uncommon in microorganisms but, when reported, often relate to morphological aberrations that occur in the absence of the cation (6, 20, 21, 29).

Another example of Ca^{2+} dependence in bacteria is the unique temperature-dependent requirement expressed by *Yersinia pestis*. Wild-type organisms grow readily at 37 C in medium containing physiological levels of Ca^{2+} (~2.5 mM) or at 26 C in the presence or absence of the cation (permissive conditions). However, during cultivation at 37 C in Ca^{2+}-deficient medium (restrictive condition), yersiniae fail to divide or synthesize deoxyribonucleic acid (DNA), but remain viable and continue synthesis of ribonucleic acid (RNA) and protein. The plague virulence, or V and W, antigens are selectively expressed under the restrictive condition; the latter are not produced by Ca^{2+}-independent mutants which, incidentally, are avirulent (3).

Yang and Brubaker (cited by Brubaker [3]) noted that prolonged cultivation under the restrictive condition resulted in loss of ability to divide at 37 C after addition of Ca^{2+}. In contrast, cell division occurred normally after shift to 26 C. Static yersiniae were previously shown to be larger than normal cells (4). One purpose of this report is to extend these observations by presenting a comparison of cell number and mass in cultures shifted to and from the restrictive condition. Another objective is to describe some morphological changes which occurred during the onset of bacteriostasis and during the subsequent period where ability to divide at 37 C, upon addition of Ca^{2+}, was irreversibly lost.

MATERIALS AND METHODS

Bacteria. The Ca^{2+}-dependent, potentially virulent strain EV76 and an isogenic Ca^{2+}-independent mutant were used throughout this study. The latter was selected at 37 C on the magnesium oxalate agar of Higuchi and Smith (15).

Media. Liquid synthetic medium, used with or without added $CaCl_2$ (2.5 mM), has been described (36); in most experiments this medium contained 20.0 mM $MgCl_2$. Blood agar base (Difco) and magnesium oxalate agar were used as routine plating media.

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Cultivation. Liquid synthetic medium (100 ml per 1-liter Erlenmeyer flask) was inoculated (~ 10^8 cells/ml) with organisms previously washed in 0.003 M potassium phosphate buffer (phosphate buffer), pH 7.0, after growth for 2 days at 26 C on slopes of blood agar base. The flasks were aerated at 26 or 37 C for various periods of time on a model BB wrist-action shaker (Burrell Corp., Pittsburgh, Pa.) or a model G-76 rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.). Appropriate dilutions of cultures were routinely plated on magnesium oxalate agar to monitor the incidence of Ca^{2+}-independent mutants; the latter approached 10% of the total population after incubation under the restrictive condition for 40 h.

Viable cells. Samples of cultures were transferred to sterile tubes (20 by 150 mm), briefly treated with a model 60100-05 mixer (Matheson Scientific Co., Chicago, Ill.) to effect even suspension of cells, appropiately diluted in phosphate buffer, and spread in quadruplicate on the surface of plates of blood agar base. The plates were incubated for 2 days at 26 C.

Light microscopy. Cultures were either examined directly under phase contrast with a microscope or else cells in samples of 10 ml were collected by centrifugation (27,000 × *g*) for 10 min and then prepared for viewing by suspension in phosphate buffer. The size of the organisms was determined by inspection of photographs of over 100 cells with a no. 30,169 comparator (Edmund Scientific Co., Barrington, N.J.).

Electron microscopy. After fixation for 6 h in 1% osmium tetroxide as described by Kellenberger et al. (18), the specimens were dehydrated in a graded series of hexyline glycol (50 to 100%) prior to embedding in low-viscosity medium (Polysciences, Warrington, Pa.) by the procedure of Spurr (30). Thin sections, cut with a diamond knife on a model III Ultrotome (LKB Instruments, Inc., Rockville, Md.) were collected, unsupported, on 300-mesh copper grids. The sections were poststained with uranyl acetate and lead citrate and examined with either a Hitachi HUII or Philips 300 electron microscope at acceleration voltages of 75 and 80 kV, respectively.

RESULTS

Wild-type cells, inoculated at similar concentrations in five flasks containing Ca^{2+}-deficient medium, were aerated at 26 C until growth commenced. When the optical density approached 0.1, three of the cultures were shifted to 37 C without addition of Ca^{2+}, the fourth was shifted to 37 C after receiving Ca^{2+}, and the fifth was maintained without change. Logarithmic growth was maintained for about 12 h in the cultures incubated under the two sets of permissive conditions, whereas cell mass quadrupled in 4 h and then failed to increase further in the three cultures shifted to the restrictive condition (Fig. 1). During this time, the number of viable cells in the latter doubled (Fig. 2), suggesting the occurrence of elongation. Static organisms and normal cells grown at 37 C with Ca^{2+} were shown by direct observation to be 5.45 ± 1.15 μm and 2.6 ± 0.5 μm in length, respectively. The increase in width was slight and probably not significant.

Upon return to 26 C after 6 h of bacteriostasis, cell mass again increased at a rate which approximated that of the culture maintained at 26 C. In contrast, the rate of increase at 37 C after addition of Ca^{2+} was less than that observed after return to 26 C (Fig. 1). This difference became more pronounced as the period of bacteriostasis was increased. In fact, organisms maintained for 12 h under the restrictive condition failed to increase in mass (not illustrated) or to divide at 37 C after addition of Ca^{2+} (Fig. 3). Addition of Ca^{2+} at this time, however, did prevent loss of viability, which occurred during further incubation under the restrictive condition. A similar culture was maintained for 12 h under the restrictive condition and then shifted to 26 C; partially synchronized cell division occurred after a short lag (Fig. 3).

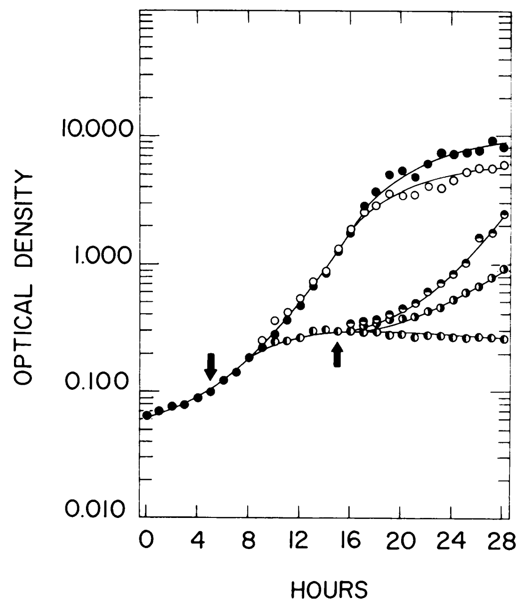


FIG. 1. *Effect of Ca^{2+} and temperature on the increase in mass of cells of Y. pestis strain EV76. A series of Ca^{2+}-deficient cultures was inoculated and aerated at 26 C; one culture remained under this condition throughout the experiment (●). At the point shown by the first arrow, another culture received $CaCl_2$ (2.5 mM) and was shifted to 37 C (○); the remainder were shifted to 37 C without addition of Ca^{2+} (◐). One of the latter was maintained under this conditon and, at the point shown by the second arrow, another was shifted to 26 C without addition of Ca^{2+} (◓); the final culture was retained at 37 C after addition of Ca^{2+} (2.5 mM) (◑).*

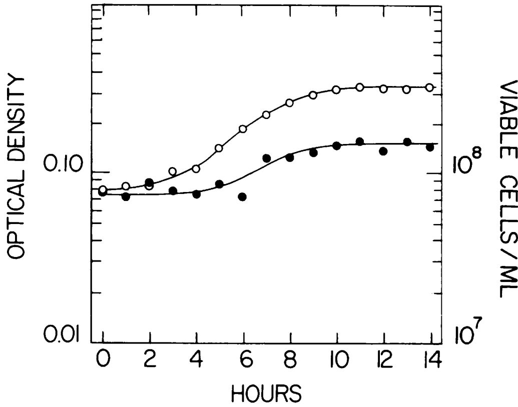


FIG. 2. *Increase of viable cells (●) and optical density (○) in a Ca^{2+}-deficient culture of Y. pestis strain EV76 after shift from 26 to 37 C.*

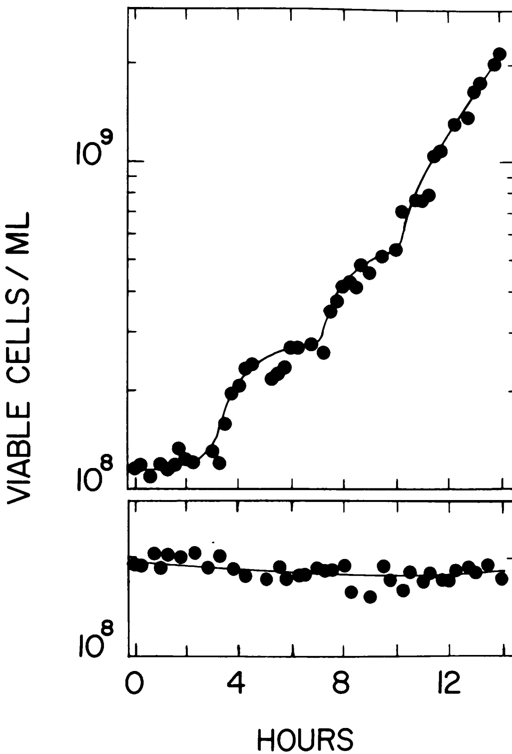


FIG. 3. *Growth of wild-type cells of Y. pestis strain EV76 after incubation at 37 C for 12 h in Ca^{2+}-deficient medium after (bottom) addition of Ca^{2+} (2.5 mM) and maintenance at 37 C, or (top) shift to 26 C without addition of Ca^{2+}.*

Thin sections were examined in an attempt to detect aberrant septae or other structures in static cells suggestive of a defect in the process of division. Other than size, the organization of nucleoids was the only obvious distinction noted between normal cells and those cultivated under the restrictive condition for 12 h or less. Nucleoids in cells grown under permissive conditions exhibited a typical irregularly lobate appearance as opposed to the axial filaments observed within static organisms (Fig. 4).

Incubation under the restrictive condition for periods longer than 12 h generally resulted in gross morphological changes associated with loss of viability. After preparation for electron microscopy, such cells often exhibited disorganized DNA and contained vacuoles at the poles (Fig. 5). Also observed were electron-dense granules of undetermined composition which were deposited within the cytoplasm and longitudinal invaginations of the cell wall and cytoplasmic membrane. The latter structure sometimes became disconnected at the poles, and the resulting space appeared to contain ribosomes (not illustrated).

Observation of large numbers of static cells disclosed an aberrant form which was evidently arrested in the process of cell division. In this case, the cytoplasmic membrane but not the cell wall had undergone septation (Fig. 6). For purposes of comparison, cells arrested in division during growth under permissive conditions are shown in Fig. 7.

DISCUSSION

Yersiniae can grow within phagocytic cells (3), despite the fact that mammalian intracellular fluid lacks detectable Ca^{2+} (22). This apparent inconsistency between in vivo and in vitro responses may, as in the case of *Coxiella* (2) or brucellae (11), reflect intracellular residence within phagocytic vacuoles where a significant level of Ca^{2+} might be maintained. Alternatively, cultivation in Ca^{2+}-deficient media might induce regulatory changes necessary for metabolism or survival within the intracellular environment but which would be unsuitable for growth in vitro. Bacteriostasis of yersiniae within a simulated intracellular environment may, therefore, reflect a block similar to that known to exist in obligate intracellular parasites during their attempted cultivation in the absence of living cells (24, 25, 36). Accordingly, an understanding of why *Y. pestis* requires Ca^{2+} to divide at 37 C may permit a better understanding of the general phenomenon of host-cell dependence.

Results of others (14) showed that wild-type yersiniae could be cultivated at 37 C for at least 23 generations in the presence of Ca^{2+}. It is now evident, however, that growth at 37 C is dependent upon the continued presence of this

cation. Bacteriostasis induced by 12 h of cultivation under the restrictive condition could not be reversed at 37 C by addition of Ca^{2+}, athough the ability to divide (in synchrony) was regained after shift to 26 C. The occurrence of two cycles of synchronous division after the return of static cells to 26 C was unexpected and implies the existence of chromosomal alignment and uniformity of cell mass (13, 23). Nevertheless, the quality of synchrony was generally poor and appeared to depend upon a number of variables, including the rate of growth prior to shift to the restrictive condition and the duration of bacteriostasis (unpublished data). The potential to divide in synchrony, therefore, is not an intrinsic property of static cells but is contingent upon control of factors which, in addition to regulating the state of mass and DNA, may also influence the accumulation and decay of division potential (19, 27). Perhaps of more significance than the demonstration of synchrony was the observation that prolonged cultivation under the restrictive condition results in irreversible loss of ability to divide at 37 C.

After 12 h of cultivation under the restrictive condition, the cells were about twice as long as those incubated with Ca^{2+}. Static yersiniae are known to be highly resistant to the action of penicillin (36), suggesting a deficiency of murine hydrolase and synthetase activities (28). The expression of these enzymes is known to be correlated with chromosome replication (28). As already noted, little or no DNA was synthesized during cultivation under the restrictive condition (36). Further study will be necessary to determine if elongation during the onset of bacteriostasis reflects residual synthesis of normal murine or cleavage of cross-linkages of peotidoglycan, as occurs upon introduction of *Chlamydia psittaci* to the mammalian intracellular environment (34). In this context, it is significant that W antigen, expressed optimally under the restrictive condition, is a lipoprotein and may thus originate from some surface component of the bacterium (3).

Considerable difference was noted between the configuration of nucleoids in static and normal cells. Axial filaments, similar to those observed within static yersiniae, were induced in *Escherichia coli* by treatment with rifampin (7), an antibiotic known to inhibit the initiation of transcription. Accordingly, RNA polymerase may maintain the structure of the nucleoid and its attachment to the cytoplasmic membrane (7). Although there was no significant difference in the relative rates of RNA synthesis in static and normal cells (36; R. V. Little and R. R. Brubaker, unpublished data), differences in the conformation of the DNA template might be expected to influence the ratio of transcription of ribosomal RNA to messenger RNA (33). Results obtained with rifampin-treated yersiniae indicate that a stable species of RNA is preferentially synthesized by static organisms (R. V. Little, Ph.D. dissertation, Michigan State University, 1974). This situation may again be analogous to that in *C. psittaci* where introduction into the mammalian intracellular environment results in increased size of the nucleoid and appearance of new ribosomes (24).

Apparent degenerative changes associated with loss of viability were observed after incubation for 12 h under the restrictive condition. These alterations in morphology were presumably an effect of some primary block in cell division rather than a direct cause of bacteriostasis. The rare instance of septation of the cytoplasmic membrane, but not the cell wall, superficially resembles that illustrated for wild-type *Escherichia coli* grown at 45 C (31). This aberrant form may represent a cell which had reached some critical stage of division upon shift to the restrictive condition. Minicells, filaments, phage-like structures, and rhaphidisomes were never observed, and, as reported by others (5, 17), capsular substance was lost during preparation for electron microscopy.

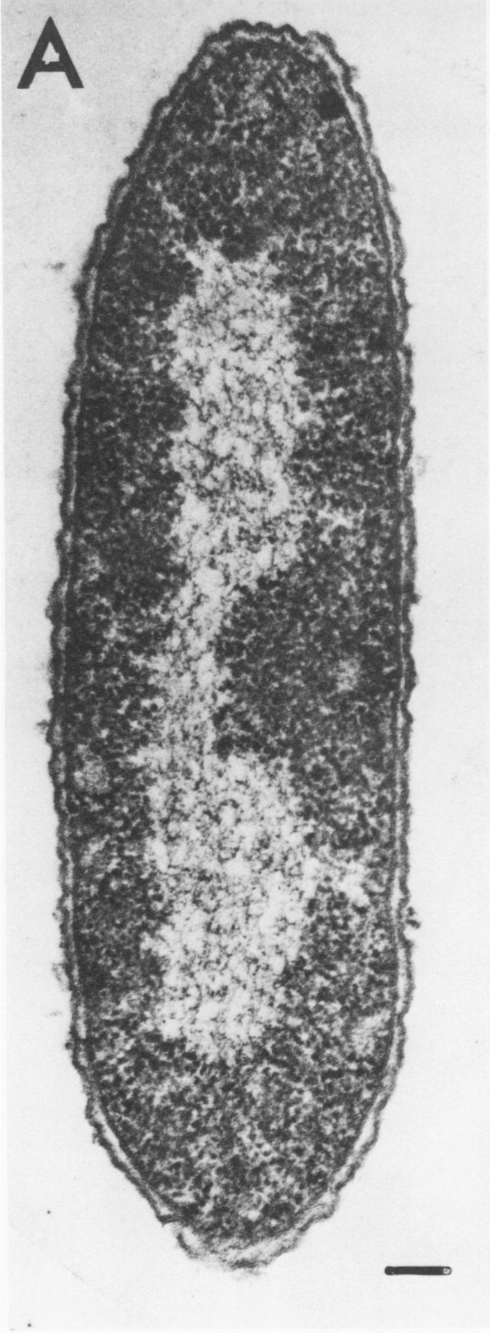
These findings indicate that the block induced in yersiniae by starvation for Ca^{2+} is distinct from those in division mutants typified by *E. coli* isolates BUG-6, P678-54, or CRT 257, which can form nucleated filaments (27), generate anucleated minicells (1), or become enlarged with particulate material (16), respectively. Static yersiniae do resemble *dnaA* or

FIG. 4. *Thin sections of wild-type cells of Y. pestis strain EV76 grown at 37 C in synthetic medium in the (A) presence (2.5 mM) and (B) absence of added Ca^{2+}; bars, 0.1 μm.*

FIG. 5. *Cell of Y. pestis strain EV76 after 16 h of cultivation at 37 C in Ca^{2+}-deficient medium; bar, 0.1 μm.*

FIG. 6. *Atypical division pattern in cell of Y. pestis strain EV76 cultivated for 12 h at 37 C in Ca^{2+}-deficient synthetic medium; (A) oblique section (bar, 0.1 μm) and (B) higher magnification of area of atypical septation (bar, 0.001 μm).*

FIG. 7. *Dividing cells of Y. pestis strain EV76 cultivated at 37 C in synthetic medium; (A) wild-type cell grown with added Ca^{2+} (2.5 mM), (B) Ca^{2+}-independent mutant grown with added Ca^{2+}, and (C) Ca^{2+}-independent mutant grown without Ca^{2+}. Bars, 0.1 μm.*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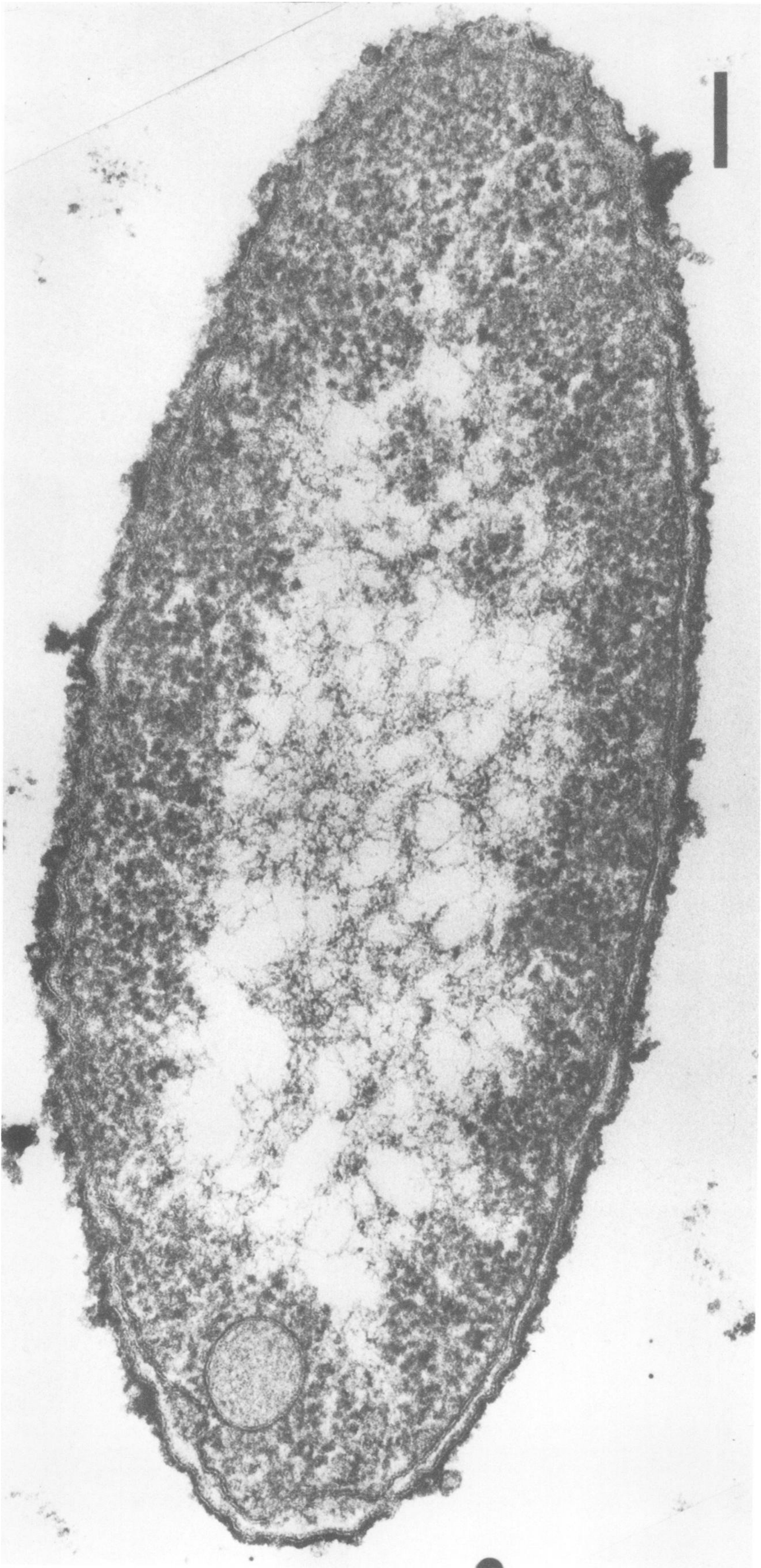

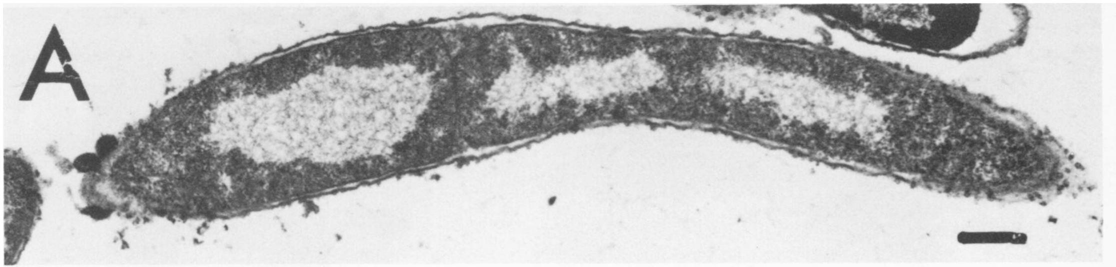
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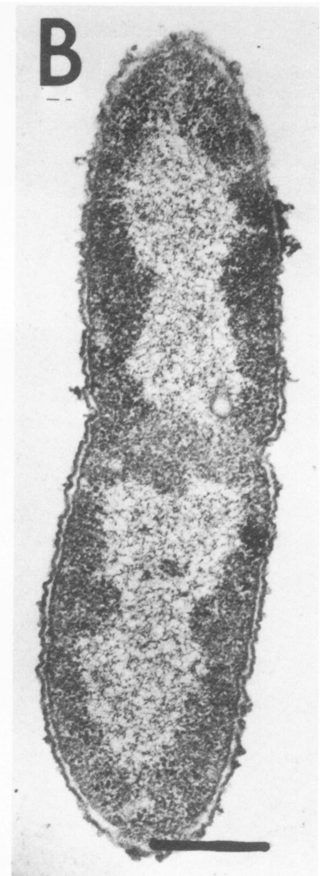
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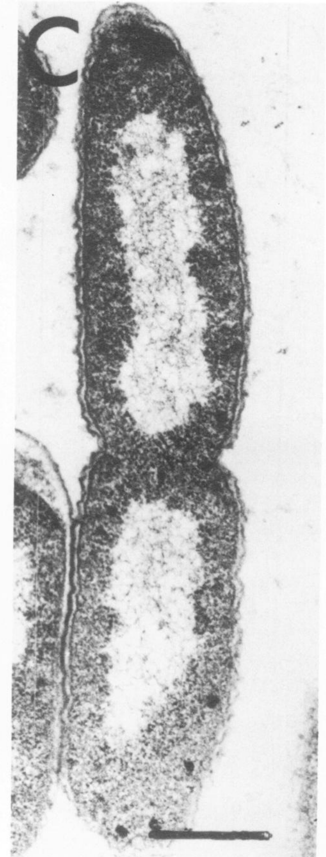
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dnaC mutants of *E. coli* which, under restrictive conditions, are unable to initiate chromosomal replication (12). Further evidence indicating the presence of a block at the initiation of DNA synthesis will be presented in a subsequent communication.

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