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Antibacterial Action of Colistin (Polymyxin E) Against *Mycobacterium aurum*

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Mycobacterium aurum **was susceptible to the antibiotic colistin (polymyxin E), which had an MIC of 5 μg/ml and an apparent bactericidal effect at concentrations above 50 μg/ml. Treatment of actively growing cells with sublethal concentrations of colistin (15 μg/ml) resulted in synchronized cell division once the antibiotic was removed. Under conditions of synchronized cell growth, one cycle of DNA replication lasted 120 min and one cycle of cell division lasted about 180 min. Although the antibiotic treatment during synchronization experiments did not produce apparent changes in the bacterial envelope, it was accompanied by the accumulation of a polysaccharide-like substance in the bacterial cytoplasm which gradually decreased after the removal of antibiotic and by an increase in the number of mesosomes at 3 h after antibiotic removal. This step was closely linked to the doubling time of bacteria. Lethal concentrations of colistin of 50 and 100 μg/ml, which caused about 90 and 99% cell death, respectively, produced significant cytoplasmic membrane injuries, patchy appearance of the cell wall outer polysaccharide layer, and little cell lysis. These data indicate that the cytoplasmic membrane is a site of action of colistin and raise a question as to whether an outer bilayer exists in mycobacteria, at least functionally.**

The antibiotic colistin (polymyxin E) exerts its antibacterial action by interacting with membrane bilayers (1a, 11, 21, 23). In gram-negative bacteria, which are surrounded by an outer membrane bilayer, the antibiotic causes characteristic deformities in the outer membrane, probably binding to lipopolysaccharides (7, 8). Direct evidence of its interaction with the cytoplasmic membrane, however, has been more difficult to obtain (20). As condensation of the bacterial genome is an early event during the action of the antibiotic (20) and, moreover, the bacterial genome is attached to the plasma membrane (3, 18), which is probably necessary for its replication (6), we wondered whether treatment of cells with sublethal concentrations of colistin would result in a reversible detachment from the membrane. If this is the case, the removal of colistin would permit a simultaneous reattachment of the genome in all the cells, leading to synchronized cell division.

To examine the mode of action of colistin and to test the above hypothesis about synchronized cell division, we selected *Mycobacterium aurum*, as it is susceptible to low concentrations of colistin (2). Like that of other mycobacteria (12–16, 19), the cytoplasmic membrane of *M. aurum* has an asymmetrical profile, only the outer layer of which is specifically stained with the Thiéry cytochemical method for electron microscopy (13, 19). This observation facilitated the direct examination of the cytoplasmic membrane as a possible site of action of colistin. Moreover, *M. aurum* forms a polysaccharide outer layer (POL) which is homogenously distributed on the bacterial cell surface and can be revealed by ruthenium red (RR) staining (11a). Recently, it was also hypothesized that covalently bound mycolic acids on the mycobacterial cell surface must form a coherent outer monolayer, and free complex lipids might insert in it as sealers to give a functional bilayer (9). This hypothesis was further supported because two major wall cleavage planes were observed by freeze-fracture studies of mycobacteria (1). For these reasons, we also decided to examine the *M. aurum* POL during the lethal action of colistin.

MATERIALS AND METHODS

Organism and growth. *M. aurum* CIPT 141210005 was from our own culture collection. Bacteria were grown in shake culture in RVB_{10} liquid medium (17) at 37°C and harvested by centrifugation in the exponential phase of growth.

Antibacterial action of colistin. The MICs and MBCs of colistin (Sigma Chemical Co., St. Louis, Mo.) were determined by the broth dilution method. For this purpose, different concentrations of colistin (ranging from 2.5 to 100 μg/ml; membrane filter [pore size, 0.45 μm] sterilized) were added to tubes containing 5 ml of RVB_{10} liquid medium which were then inoculated with 0.1 ml of an actively growing culture of *M. aurum* (containing about 10^8 bacilli per ml). After 5 days of incubation at 37°C, the growth was estimated by turbidity measurements at 650 nm with a Colemen Junior spectrophotometer. For surface plate counts, appropriate dilutions were plated on Lowenstein-Jensen medium, and the CFU were enumerated after 1 week of incubation at 37°C.

To examine the lethal effect of the drug, we treated actively growing cell cultures containing about 10^8 bacilli per ml with 5, 15, 50, and 100 μg of colistin per ml at 37°C, and the surface plate counts were made after regular intervals ranging from 45 min to 24 h.

Synchronization studies. For synchronization studies, a shake culture of *M. aurum* was grown to the exponential phase of growth in RVB_{10} liquid medium at 37°C. Colistin (15 μg/ml) was then added, and after 5 h of treatment at 37°C, the antibiotic was completely washed away and the cells were resuspended in new RVB_{10} medium at 37°C. At 1-h intervals, appropriate dilutions were plated on Lowenstein-Jensen medium for surface plate counts as described above. An *M. aurum* culture without antibiotic treatment served as a control.

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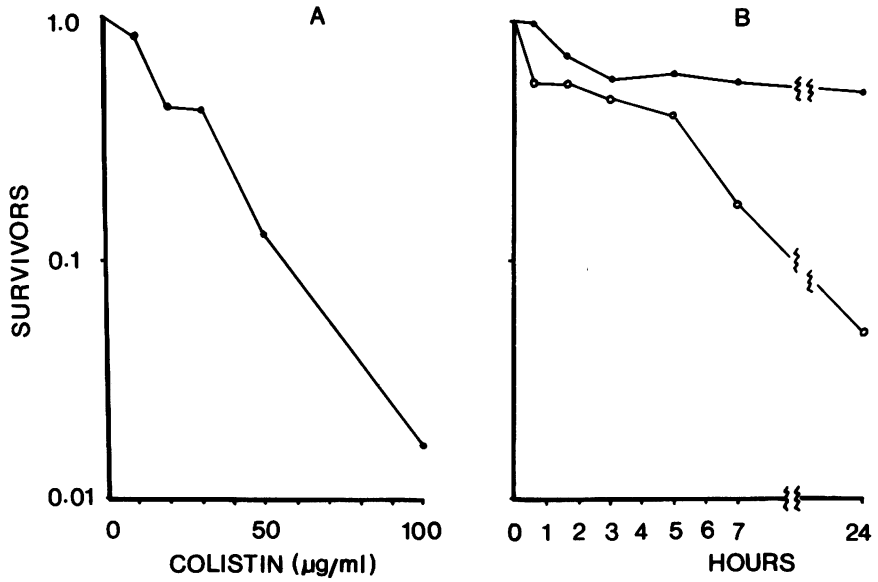

FIG. 1. Antibacterial action of colistin on *M. aurum*. (A) Survivors remaining after inoculation of media containing different concentrations of colistin. The tubes were inoculated with about 10^7 bacilli, and survivors in the presence of colistin were determined after 5 days of incubation at 37°C, when the control bacterial population reached about 10^8 bacilli per ml. (B) Survivors remaining after treatment of an actively growing culture of *M. aurum* with colistin for 1 to 24 h (at 37°C). Colistin was used at concentrations of 15 (●) and 100 (○) μg/ml. (Results with control and with 5 and 50 μg of colistin per ml not shown.)

The DNA content during synchronization experiments was followed by ^{32}P labeling of the bacilli. In this case, $^{32}P_i$ (in acid-free, carrier-free solution, 10 mCi/ml, code PBS 13; Amersham International, Buckinghamshire, United Kingdom) was added at a final concentration of 10 μCi/ml to the growth medium containing colistin. After 5 h of treatment at 37°C, the antibiotic was removed as described above and the bacteria were resuspended in new medium supplemented with 10 μCi of $^{32}P_i$ per ml. At regular intervals thereafter, sample portions were centrifuged and the bacteria were treated with 4 N NaOH at 40°C for 2 h. NaOH was then washed away by centrifugation, and the bacterial residue was washed three times with 10% cold trichloroacetic acid. Aliquots of the trichloroacetic acid-washed bacilli were then sampled for counting with a Beckman scintillation counter. The NaOH-resistant, trichloroacetic acid-insoluble material was considered to be mostly DNA.

Ultrastructural studies. Samples of the bacteria during the synchronization experiment (colistin posttreated at 0, 3, and 7 h), and bacteria treated for 5 h with lethal concentrations of colistin (50 and 100 μg/ml) were fixed for electron microscopy. In all cases, the bacteria were observed after standard lead citrate coloration and Thiéry cytochemical staining for polysaccharides with the periodic acid-thiocarbohydrazide-silver proteinate method (13). An untreated *M. aurum* culture served as a parallel control in all the experiments. The POL of *M. aurum* treated with 100 μg of colistin per ml was also observed by the RR staining procedure (15). All the procedures for fixation, dehydration, embedding, and specific stainings were carried out as described earlier for mycobacteria (12–16). Thin sections were cut on a LKB ultratome III, mounted on copper grids, and observed with a Siemens 101 electron microscope.

RESULTS

Antibacterial action of colistin. Determined by the broth dilution method, the MIC of colistin for *M. aurum* was 5 μg/ml in RVB_{10} liquid medium. Continued incubation of the cultures, however, showed that the drug merely delayed growth (bacteriostatic action). This was verified by performing surface plate counts from cultures containing increasing concentrations of the antibiotic and incubated until the control had reached a bacterial concentration of about 10^8 CFU/ml (Fig. 1A). When colistin was added to actively growing cultures (containing about 10^8 CFU/ml) at 5, 15, 50, and 100 μg/ml and the survivors were determined by surface plate counts at regular intervals, it was found that colistin did not cause cell death at 5 μg/ml, caused a slight initial drop in viability at 15 μg/ml, and a lethal effect was apparent at concentrations above 50 μg/ml (Fig. 1B). As a result of these data, we studied the antibacterial effect of colistin on *M. aurum* treated with 50 and 100 μg of the antibiotic per ml.

Synchronized cell division. The average division time of *M. aurum* in the RVB_{10} medium as about 4 h, 24 min (Fig. 2A). *M. aurum* was treated with 15 μg of colistin per ml for 5 h at 37°C, the antibiotic was washed off, and the bacilli were resuspended in new medium; surface plate counts made thereafter at regular intervals showed that the bacterial concentration remained constant for 3 h and then doubled at 4 h. The synchronization experiments were repeated six times (Fig. 2B).

When $^{32}P_i$-prelabeled bacteria were treated in the same manner and resuspended in a new medium supplemented with $^{32}P_i$, the relative ^{32}P content of the DNA more than doubled after an initial lag of 2 h (Fig. 2C). These data show that colistin induced a synchronized cell division in *M. aurum* and that DNA replication started in 120 min, preceding by 60 min the initiation of cell division.

For electron microscopy, we sampled bacteria at 0, 3, and 7 h during the synchronization experiment (Fig. 3). The examination of bacteria did not show any ultrastructural lesions, and the cytoplasmic membrane retained its usual asymmetrical profile after both lead citrate staining (Fig. 3D, F, and H) and Thiéry staining (Fig. 3E, G, and I).

As only the cytoplasmic membrane, but not any other cell components, were stained by the Thiéry method, it was

possible to examine large numbers of bacteria at a lower magnification (×5,000); this led to the observation of some striking new morphological features. These observations are represented ultrastructurally at a higher magnification in Fig. 3A (0 h), 3B (3 h), and 3C (7 h) and are summarized in Table 1 and show that, immediately after the removal of colistin (0 h), the bacterial cytoplasm contained considerable amounts of Thiéry-positive material (possibly polysaccharides) which gradually disappeared.

The number of cells with mesosome-like structures was the highest in the 3-h sample; the number of cells with finished division septa, however, remained about the same in all the samples studied (Table 1).

Ultrastructural modifications caused by lethal concentrations of colistin. When *M. aurum* bacilli treated with lethal concentrations of colistin (50 and 100 μg/ml) were studied by electron microscopy, the following observations were made. Normal untreated control *M. aurum* (Fig. 4A) had a triple-layered cell wall (outer electron-dense layer, middle electron-transparent layer, and inner electron-dense peptidoglycan layer), a double-layered, asymmetrical cytoplasmic membrane, and a dense cytoplasm. RR staining revealed a uniform, 7- to 9-nm-thick POL in the control bacteria (Fig. 4B).

Colistin at 50 μg/ml produced serious cytoplasmic membrane damage; the four major types of membrane injuries are represented in Fig. 4C, D, F, and G, which varied from peripheral deformations (Fig. 4D) to polar accumulation of membrane-like structures (Fig. 4G). Such membrane damage was never observed in control bacteria. All these intracytoplasmic membrane structures had symmetrical profiles.

When *M. aurum* cells were treated with 100 μg of colistin per ml, many bacilli had unusual intracytoplasmic membrane structures (Fig. 4J and K), bacteria showed overall cytoplasmic membrane invaginations (Fig. 4E), and the outer electron-dense layer of the cell wall was often less uniformly visible than in untreated cells after lead citrate coloration (Fig. 4E).

The latter observation suggested that high concentrations of colistin probably also deformed the outer surface of *M. aurum*, and for this reason we decided to examine the POL of bacteria treated with 100 μg of colistin per ml by RR coloration. Figures 4H and I show RR-stained *M. aurum* cells. In the first case, the bacilli were double stained with both RR and lead citrate, and both the POL and other cell components are apparent; in the second case, the bacteria are stained only with RR, and only the POL selectively stains. As shown in Fig. 4H and I, cells treated with 100 μg of colistin per ml contained a patchy and diffused POL, with patches measuring 14 to 30 nm, compare with 7 to 9 nm in the case of a uniformly distributed POL from control cells (Fig. 4B). Lysing cells, on the other hand, showed a loss of major polysaccharide components from POL and a symmetrical cytoplasmic membrane (Fig. 4L).

The action of both 50 and 100 μg of colistin per ml on the cytoplasmic membrane of *M. aurum* was studied after selective staining by the Thiéry cytochemical method for polysaccharides. Colistin at concentrations of both 50 μg/ml (Fig. 5A) and 100 μg/ml (Fig. 5C) caused membrane deformities. The evidence for lesions in the cytoplasmic membrane was its fragmentation and the staining of both its outer and inner layers after staining by the Thiéry procedure, showing a transition from its normal asymmetrical to a symmetrical profile.

A patchy appearance of POL and the accumulation of Thiéry-positive material (possibly polysaccharides) in the cell cytoplasm could be revealed by staining with both RR and the Thiéry cytochemical method (Fig. 5B). Thiéry staining also confirmed a polar accumulation of membrane-like structures, along with peripheral membrane deformities (Fig. 5D). In the case of lysing cells, the cytoplasmic membrane stained symmetrically and the inner wall layer, which is normally Thiéry negative, turned weakly Thiéry positive (Fig. 5E), as observed earlier during *M. smegmatis* cell lysis (16).

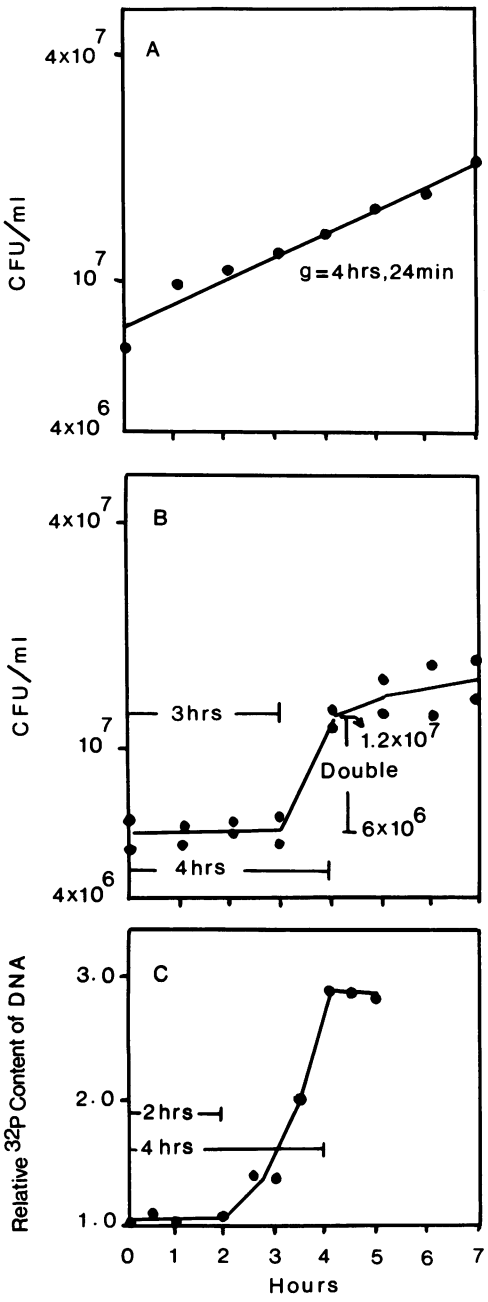

FIG. 2. Synchronization of cell division in *M. aurum*. (A) Control, untreated *M. aurum*; (B) actively growing cells treated with 15 μg of colistin per ml for 5 h at 37°C, washed free of drug, and incubated in drug-free medium; (C) Incorporation of $^{32}P_i$ into cells treated as described for panel B. The detailed procedure is described in the text.

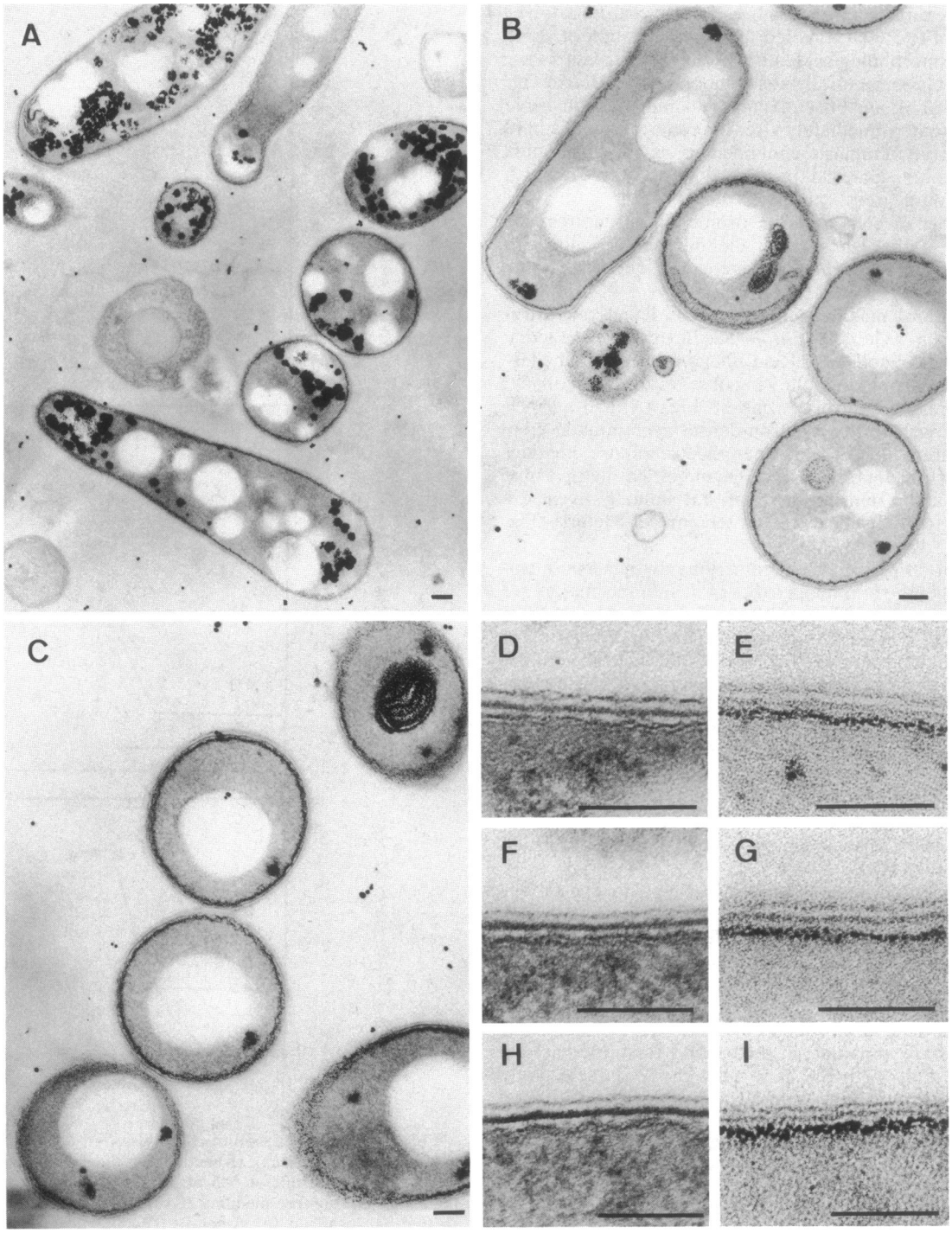

FIG. 3. Ultrastructure of *M. aurum* during a synchronization experiment (for details, see the text, Fig. 2B, and Table 1). Bacteria at lower magnification are shown at 0 h (A), 3 h (B), and 7 h (C) after Thiéry cytochemical staining. The ultrastructural details of the cell envelope are shown in the insets after both lead citrate coloration at 0 h (D), 3 h (F), and 7 h (H) and Thiéry cytochemical staining at 0 h (E), 3 h (G), and 7 h (I). Bar, 100 nm.

DISCUSSION

The polymyxins are cyclic, polycationic peptides with a fatty acid chain attached through an amide linkage. Because these antibiotics contain a polar head in addition to their nonpolar fatty acid chain, they are amphipathic in nature and tend to form micelles in their aqueous state. Mainly active against the gram-negative microorganisms, they are less active against gram-positive bacteria (20). They appear to act by disrupting the structure and functions of the cytoplasmic membrane (5, 11) and the outer membrane (1a, 21, 23) of gram-negative bacteria, owing to their interaction with both the lipopolysaccharides and phospholipids (4, 22, 24), which is sufficient to alter all other major biochemical processes of the cell. They also have an electrostatic interaction, as they competitively replace divalent cations like mg^{2+} and Ca^{2+}

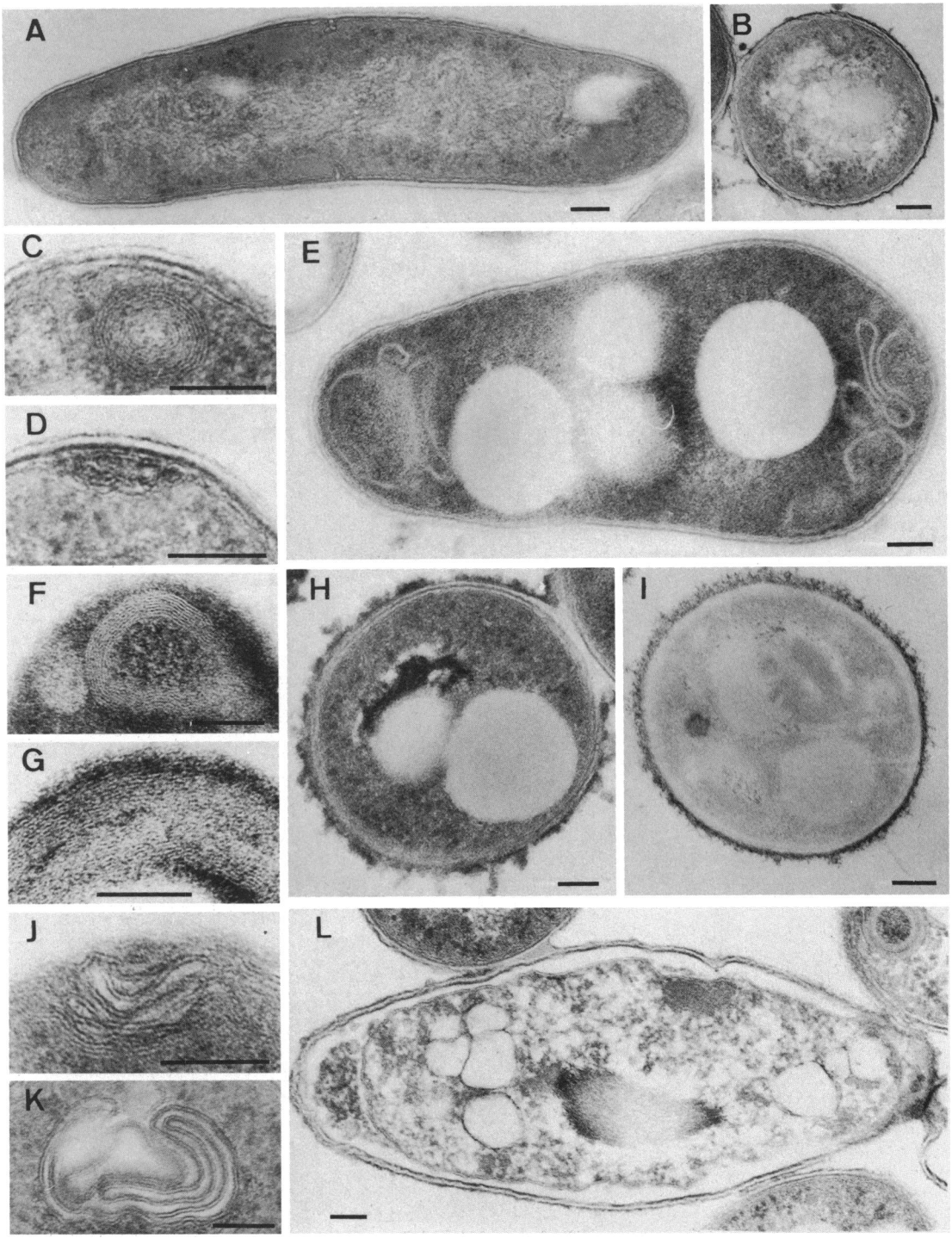

FIG. 4. Effect of lethal concentrations of colistin on *M. aurum* ultrastructure as revealed by lead citrate and RR colorations. (A) Normal, untreated control bacteria after lead citrate staining; (B) control bacilli stained with RR; (C, D, F, G) ultrastructural details of bacilli treated with 50 μg of colistin per ml, observed after lead citrate staining; (J, K) bacilli treated with 100 μg of colistin per ml after lead citrate staining; (E) overall cytoplasmic membrane invaginations caused in a bacterium after treatment with 100 μg of colistin per ml (lead citrate coloration); (H, I) patchy appearance of POL in bacterium treated with 100 μg of colistin per ml and stained with both RR and lead citrate (H) or with RR alone (I); (L) bacterium lysing after treatment with 100 μg of colistin per ml and stained with both RR and lead citrate. Bar, 100 nm.

from negatively charged phosphate groups on the membrane lipids (10). Despite these earlier observations, direct evidence for interaction between colistin (polymyxin E) and the cytoplasmic membrane is still lacking for both gram-negative and -positive bacteria (20).

The present investigation shows two distinct observations: (i) colistin caused a synchronized cell division of *M. aurum* when used in sublethal concentrations, and (ii) bacteriostatic concentrations of the drug did not produce detectable modifications of the *M. aurum* cell envelope, whereas

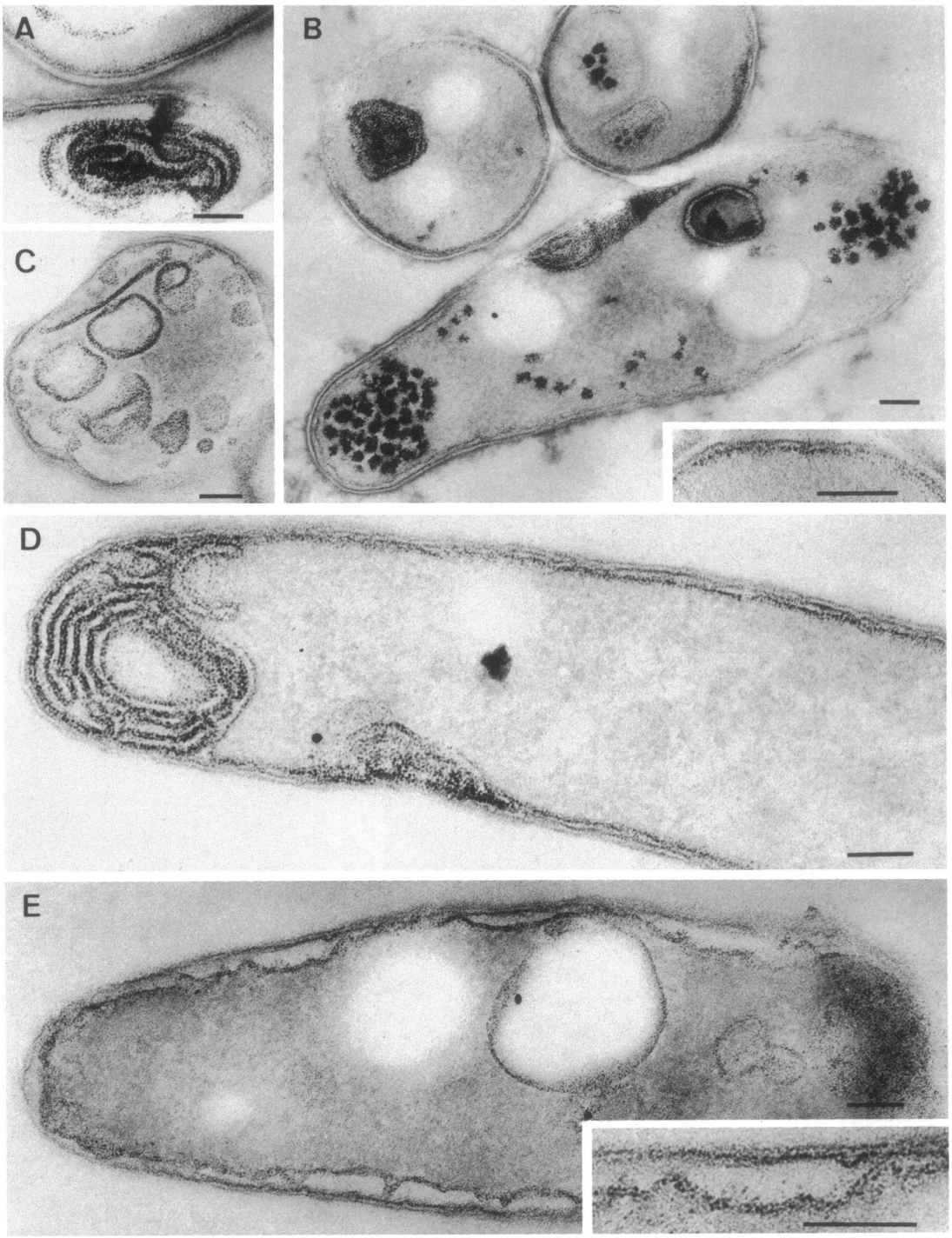

FIG. 5. Effect of lethal concentrations of colistin on *M. aurum* ultrastructure as revealed by Thiéry cytochemical staining. (A) Bacteria treated with 50 μg of colistin per ml; (B) a double staining of bacteria treated with 100 μg of colistin per ml by both RR and Thiéry cytochemical procedures; (C, D) cytoplasmic membrane damage (C) and polar accumulation of membrane (D) caused by 100 μg of colistin per ml; (E) bacterium lysing after treatment with 100 μg of colistin per ml. Bar, 100 nm.

the bactericidal concentrations resulted in gross alteration of the cell envelope at the level of the cell surface and the cytoplasmic membrane.

With regard to the first observation, this study shows that colistin at 15 μg/ml induced synchronized cell division in *M. aurum* (Fig. 2B). It also shows that DNA replication started at about 2 h after the antibiotic removal, whereas the cell division started at about 3 h after the antibiotic removal (Fig. 2C). Although there was no electron microscopic evidence for a reversible detachment of the bacterial genome under the effect of sublethal concentrations of colistin, the data shown indicate that colistin caused reversible biochemical lesions in the cell, probably reflected by the accumulation of Thiéry-positive material (possibly polysaccharides) in the

TABLE 1. Summary of ultrastructural observations after Thiéry cytochemical staining of *M. aurum* during a synchronization experiment[a]

Time (h)	No. (%) of cells with:			Total no. (%) of bacteria observed
	Intracellular polysaccharide	Division septa	Mesosomes	
0	214 (53.63)	16 (4.01)	18 (4.51)	399 (100)
3	153 (39.74)	18 (4.68)	63 (16.36)	385 (100)
7	105 (27.63)	8 (2.11)	34 (8.95)	380 (100)

[a] For details, see the text and Fig. 2B and 3.

cytoplasm which progressively disappeared after the antibiotic was removed (Fig. 3A, B, and C; Table 1). Taking advanage of the specific staining of the mycobacterial cytoplasmic membranes by the Thiéry method (13), this study also showed a significant increase in mesosome-like structures in bacteria at the time of cell division (Table 1).

Because sublethal concentrations of colistin used for the synchronization studies did not cause apparent cell envelope damage (Fig. 3), we examined bacteria treated with lethal concentrations of colistin (50 and 100 μg/ml). Electron microscopic observations after the specific cytoplasmic membrane staining by the Thiéry method (Fig. 5) showed serious membrane damage and a transition from its usual asymmetrical to a symmetrical profile. These findings were interpreted to indicate a direct interaction of colistin with the cytoplasmic membrane. The lethal concentrations of colistin also caused significant alterations of the *M. aurum* cell surface, which could be followed by specific RR staining of the POL, as observed earlier for other mycobacteria (12–15). Cells treated with 100 μg of colistin per ml had a patchy and diffused POL, with patches measuring 14 to 30 nm (Fig. 4H and I), compared with a uniformly distributed POL in control bacteria (Fig. 4B), which measured only 7 to 9 nm.

Presuming that the patchy appearance of POL was a direct result of colistin interaction, a recent model (9) of the mycobacterial cell envelope gets preliminary support, according to which covalently bound mycolic acids on the mycobacterial cell surface must form a coherent outer monolayer, and free complex lipids (possibly lipopolysaccharides or lipoproteins) might insert in it to give a functional bilayer.

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