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In Vitro Spheroplast and L-Form Induction Within the Pathogenic Nocardiae

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Six strains of *Nocardia asteroides*, two strains of *N. caviae*, and two strains of *N. braziliensis* were grown in medium supplemented with glycine, lysozyme, D-cycloserine, glycine plus lysozyme, and glycine plus D-cycloserine. It was shown that three strains of *N. asteroides*, and two strains of *N. caviae*, readily formed spheroplasts and/or protoplasts when grown in the presence of glycine plus either lysozyme or D-cycloserine. This process was studied by both phase contrast microscopy and electron microscopy. The induced cultures were then plated on hypertonic medium for the isolation of L-forms. It was shown that the organisms differed greatly in their ability to produce spheroplasts and subsequently grew as L-forms or transitional-phase variants.

The recovery of stable nocardial L-forms from infected macrophages (7) has generally been of low frequency and required a great deal of time. Because of the high frequency of reversion to parental type, attempting to obtain nocardial L-forms by this method severely restricted our ability to evaluate the biological, ultrastructural, and biochemical characteristics of these organisms. Therefore, experiments were designed to induce large numbers of nocardial L-forms in vitro.

There are many reports of bacterial L-forms being induced by treatment with antibiotics, enzymes, and certain amino acids (13). The induction process with any of these chemical substances required the initial formation of cell wall-defective organisms, such as protoplasts or spheroplasts.

The conversion of *Nocardia* into spheroplasts or protoplasts has not been previously reported. With other bacterial cells, this conversion was generally achieved by the addition of exogenous substances to the growth medium, which then produced either a degradative effect on components in the microbial cell walls (i.e., degradative enzymes such as lysozyme) or which interfered with biosynthetic pathways in the cell wall (e.g., antibiotics such as penicillin). However, the *Nocardia*, like the *Mycobacterium*, have been observed to be resistant to the action of most of these inductive substances (2, 8).

The close structural similarity between the cell walls of the mycobacteria and the nocardiae suggested that procedures used to convert various mycobacterial species to spheroplasts might also work with *Nocardia*. Several investigators reported that *Mycobacterium smegmatis*, *M. phlei*, and other rapidly growing mycobacteria were converted to spheroplasts when they were grown in the presence of lysozyme and glycine (1, 12, 15). On the basis of these observations, we studied the effect of these compounds on the morphology and structure of several strains of *Nocardia asteroides*, *N. caviae*, and *N. braziliensis*.

MATERIALS AND METHODS

Organisms. *N. asteroides* 10905 was supplied by J. Rozanis, University of Western Ontario, London, Canada. *N. asteroides* 14759 and 19247 were obtained from the American Type Culture Collection (ATCC), Rockville, Md. *N. asteroides* GUH-2 was isolated at autopsy from a patient at Georgetown University Hospital, and *N. asteroides* GUH-5 was isolated from the sputum of a patient with fatal systemic nocardiosis at Georgetown University Hospital. *N. asteroides* 287 and *N. caviae* 112 were human isolates supplied by W. Causey, University of Chicago Hospitals and Clinics, Chicago, Ill. *N. braziliensis* 19296 and 19096 and *N. caviae* 14629 were obtained from ATCC, Rockville, Md.

Induction of spheroplasts. The nocardiae were grown in brain heart infusion broth (BHI-B) for 48 h. The resulting growth was centrifuged at low speed for 5 min to remove clumps of bacteria. Two milliliters of the supernatant was transferred to either BHI-B (Difco) or Sauton chemically defined medium. All media contained 0.35 M sucrose plus one of the following additives: (i) glycine, 0.5, 1.0 and 1.5% (wt/vol); (ii) lysozyme, 10, 20, 100, 200, and 400 μg/ml; (iii) glycine (1.2% [wt/vol]) and lysozyme (20 μg/ml); (iv) D-cycloserine, 50 and 100 μg/ml; and (v) glycine (1.2% [wt/vol]) and D-cycloserine (50 μg/ml). The cultures were incubated for 8 days at 34 C. The induction of spheroplasts was monitored by phase contrast and electron microscopy.

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Isolation of L-forms. Cultures producing spheroplasts were transferred to tubes and centrifuged at approximately 1,000 × *g* for 10 min to remove the majority of whole cells. The spheroplast suspensions were then cultured on Barile, Yarguchi, and Eveland medium, containing 10% heat-inactivated horse serum, 3.0% (wt/vol) NaCl, and 0.9% agar (BYEL medium) for the isolation of nocardial L-forms (3, 7). The plates were incubated at 34 C aerobically and at 37 C under 5% CO_2 in air. The developing colonies were observed using phase contrast microscopy. After 2 to 3 weeks, the L-phase colonies were inoculated onto fresh BYEL medium by agar-block transfers.

Indirect immunofluorescence. Nocardial L-forms and L-form revertants were shown to be derived from *N. asteroides* by indirect immunofluorescent staining (Table 1). Cover slip impressions of nocardial L-forms and smears of revertants were fixed in cold methanol and processed as described by Bourgeois and Beaman (7). Counterstaining with Evan blue was found not to be necessary.

The specificity of the antiserum raised in rabbits against the cytoplasmic extract of *N. asteroides* 10905 was tested by determining its ability to give specific fluorescence with a wide variety of mycobacteria and nocardiae and also with *Staphylococcus aureus, Bacillus subtilus, Escherichia coli,* and *Corynebacterium diphtheriae* (Table 1).

Photographs of immunofluorescent slides were made using a Nikon fluorescent microscope. High-speed Ektachrome film (Kodak) was exposed for 10 to 20 min, and the resultant slides were copied by using Kodak Plus-X film.

Acridine orange staining. To differentiate nocardial L-forms from potential "pseudocolonies" (13), cover slip impressions of these altered forms were stained with acridine orange. Cover slip impressions were fixed in cold methanol and stained with buffered acridine orange solution for 5 min (13). The slides were then rinsed with water and fixed on a slide with a drop of McIlvaine buffer (pH 3.8) (13). Slides were viewed with a Nikon fluorescent microscope, and pictures were taken with high-speed Ektachrome film (Kodak).

Electron microscopy. Normal nocardial cells, colonies, spheroplasts, and colonies of altered forms were fixed for electron microscopy as previously described (7).

Scanning electron microscopy. Cover slip impressions of nocardial L-forms were fixed in 3.0% glutaraldehyde in Kellenberger buffer (pH 6.5). Cover slips were then dehydrated through a series of ethanols (50, 75, 95, and 100%) and critical-point-dried in a Sorvall critical-point dryer. Dried specimens were finally coated with gold-palladium in a Hummer (Technics, Inc.) and examined with an Etec Autoscan scanning electron microscope at 20 kV. In both procedures, photomicrographs were taken on Polaroid film type 55P/N.

RESULTS

Six strains of *N. asteroides,* two strains of *N. braziliensis,* and two strains of *N. caviae* were grown in BHI-B for 48 h. Clumps of bacteria were removed, and 2 ml of the supernatant was transferred into either BHI-B supplemented with 0.35 M sucrose or Sauton chemically defined medium containing 0.35 M sucrose. In addition, each of the media had specific additions as given in Materials and Methods and shown in Table 2. The cultures were incubated for 8 days at 34 C, and the changes in cell morphology were monitored daily by phase contrast and electron microscopy. It was found that the strains differed in their ability to form spheroplasts (Table 2).

Initially, the conversion of *N. asteroides* 10905 was followed as the model system, since it is this strain from which we isolated L-forms and transitional-phase variants from mouse peritoneal and rabbit alveolar macrophages (6, 7). When grown in BHI-B with sucrose for 48 h, the cells of this strain exhibited typical nocardial morphology (Fig. 1A). However, after 24 h in medium containing both lysozyme and glycine, nocardial filaments appeared swollen and pleomorphic (Fig. 1B). At 5 days postinoculation, the majority of filaments were extremely swollen and several spheroplasts were observed eminating from the ends of filaments (Fig. 1C). By 8 days, the *Nocardia* were totally converted to bacterial spheroplasts (Fig. 1D). The same morphological changes occurred when *N. asteroides* 10905 was inoculated into Sauton medium supplemented with sucrose and lysozyme plus glycine.

Using this procedure, other strains of *Nocardia* were found to differ greatly in their ability to produce spheroplasts. *N. asteroides* GUH-5, grown in medium containing lysozyme and glycine, formed spheroplasts indistinguishable from those of *N. asteroides* 10905, so that after 8 days of incubation only spheroplasts could be found (Fig. 1D). *N. caviae* 112 and *N. asteroides* 287 were intermediate in their response to lysozyme and glycine. After 8 days of incubation, the filaments of these two strains exhibited central swellings and terminal bulbs, in addition to numerous free spheroplasts. In 8-day cultures of *N. asteroides* 14759, only an occasional spheroplast was observed, and the remaining cells had no apparent central swelling. Bulbous cells were not observed. The other strains, *N. asteroides* GUH-2 and 19247 and *N. braziliensis,* were not altered by growth in media supplemented with lysozyme and glycine.

The conversion of the *Nocardia* to spheroplasts was studied by electron microscopy. Initially, *N. asteroides* 10905 and 287 exhibited typical nocardial morphology (Fig. 2). However, when these cells were transferred to media containing lysozyme (20 μg/ml) and glycine (1.2% [wt/vol]), the cell wall appeared to disassociate from the cell (Fig. 2). Prolonged growth

TABLE 1. *Specificity of norcardial antisera used for immunofluorescent microscopy*

Organisms	Source	Fluorescence[a]
Nocardia asteroides 14759 (grey)	ATCC	±
N. asteroides 14759 (white)	ATCC	−
N. asteroides 10905	Original stock culture (J. Rozanis, Univ. of West Ontario)	++
N. asteroides 10905	Mouse macrophage isolate	+++
N. asteroides 10905 L-form	Macrophage induced	+++
N. asteroides L-form	In vitro induced	+++
N. asteroides 10905	L-form R_1 revertant	+++
N. asteroides 10905	L-form R_2 revertant	+++
N. asteroides 10905	L-form R_3 revertant	+++
N. asteroides 10905	L-form R_4G revertant	+++
N. asteroides 10905	L-form animal revertant (Anirev)	+++
N. asteroides 10905	L-form R_3R revertant (pigmented)	+++
N. asteroides 19247	ATCC	++
N. asteroides GUH-1	Human isolate (G.U. Hosp.)[b]	+++
N. asteroides GUH-2	Human isolate (G.U. Hosp.)	++
N. asteroides GUH-2 L-form	Mouse spleen	+++
N. asteroides GUH-2	L-form revertant	++
N. asteroides GUH-3	Human isolate (G.U. Hosp.)	++
N. asteroides GUH-4	Human isolate (G.U. Hosp.)	++
N. asteroides GUH-5 L-form	C.S.F. Patterson (G.U. Hosp.)	+++
N. asteroides GUH-5 L-form	Mouse spleen	+++
N. asteroides GUH-5 (TPV)[c]	In vitro induced	+++
N. asteroides GUH-5	L-form revertant C.S.F. Patterson (G.U. Hosp.)	++
N. asteroides GUH-6	Human isolate (G.U. Hosp.)	++
N. asteroides 287BC	Bing Crosby isolate (CDC)[b]	+++
N. asteroides 287BC (TPV)[c]	In vitro induced	+++
N. braziliensis 19296	ATCC	−
N. braziliensis 19096	ATCC	−
N. caviae 112	Human isolate (CDC)	−
N. caviae 112	In vitro L-form	−
N. caviae 14629	ATCC	−
N. caviae 14629	TPV (in vitro)	−
N. caviae 112 L-form	Mouse spleen	−
N. caviae 260	Human isolate (CDC)	−
N. farcinica C	Stock culture (J. Rozanis, Univ. of West Ontario)	−
N. rubra 721-A (smooth)	Beaman isolate (Univ. of Kansas)	−
N. rubra 721-A (rough)	Beaman isolate (Univ. of Kansas)	−
N. pellegrino	Stock culture (J. Rozanis, Univ. of West Ontario)	−
N. serratia	Stock culture (J. Rozanis, Univ. of West Ontario)	−
Nocardia sp. MDEIR	Human isolate (Univ. of North Carolina)	−
Mycobacterium fortuitum 6841	ATCC	−
M. phlei 11758	ATCC	−
M. smegmatis 14468	ATCC	−
M. kansasii	G.U. stock culture	−
Corynebacterium diphtheriae	G.U. stock culture	−
Staphylococcus sp.	Lab isolate (G.U.)	−
Bacillus cereus	A.K. Saz strain (G.U.)	−
Escherichia coli	G.U. stock culture	−
Mouse peritoneal macrophages	Swiss-Webster mice	−

[a] −, No specific fluorescence; ±, some fluorescence (weak); ++, good fluorescence; +++, excellent, bright green fluorescence.

[b] G.U. Hosp., Georgetown University Hospital; CDC, Center for Disease Control.

[c] TPV, Transitional-phase variants.

TABLE 2. *Spheroplasts formed after 8 days of incubation*[a]

Organism	Glycine (1.2% [wt/vol]) + D-cycloserine (50 μg/ml)	D-Cycloserine (100 μg/ml)	Glycine (1.2% [wt/vol]) + lysozyme (20 μg/ml)	Lysozyme (400 μg/ml)	Glycine (1.5% [wt/vol])
Nocardia asteroides 10905	+++[b]	−	+++	−	−
N. asteroides 14759 (ATCC)	+	−	+	−	−
N. asteroides 19247 (ATCC)	+	−	−	−	−
N. asteroides GUH-2	−	−	−	−	−
N. asteroides GUH-5	+++	−	+++	−	−
N. asteroides 287 (CDC)[c]	++	−	++	−	−
N. caviae 112 (CDC)	++	−	++	−	−
N. caviae 14629 (ATCC)	++	−	++	−	−
N. braziliensis 19295	±	−	−	−	−
N. braziliensis 19096	+	−	+	−	−

[a] BHI-B + 0.35 M sucrose at 34 C.

[b] +++, Approximately 100% conversion to spheroplast; ++, >50% conversion to spheroplast; +, some spheroplasts detected; ±, some spheroplasts detected in some experiments but not in all; −, no spheroplasts detected.

[c] CDC, Center for Disease Control.

in the presence of these compounds led to partial degeneration or loss of the cell wall, which resulted in spheroplast formation (Fig. 2A to D). Ultrastructurally, spheroplasts of *N. asteroides* 10905 and 287 and *N. caviae* 112 were indistinguishable (Fig. 3A to C). They appeared to be bound only by a unit membrane, and

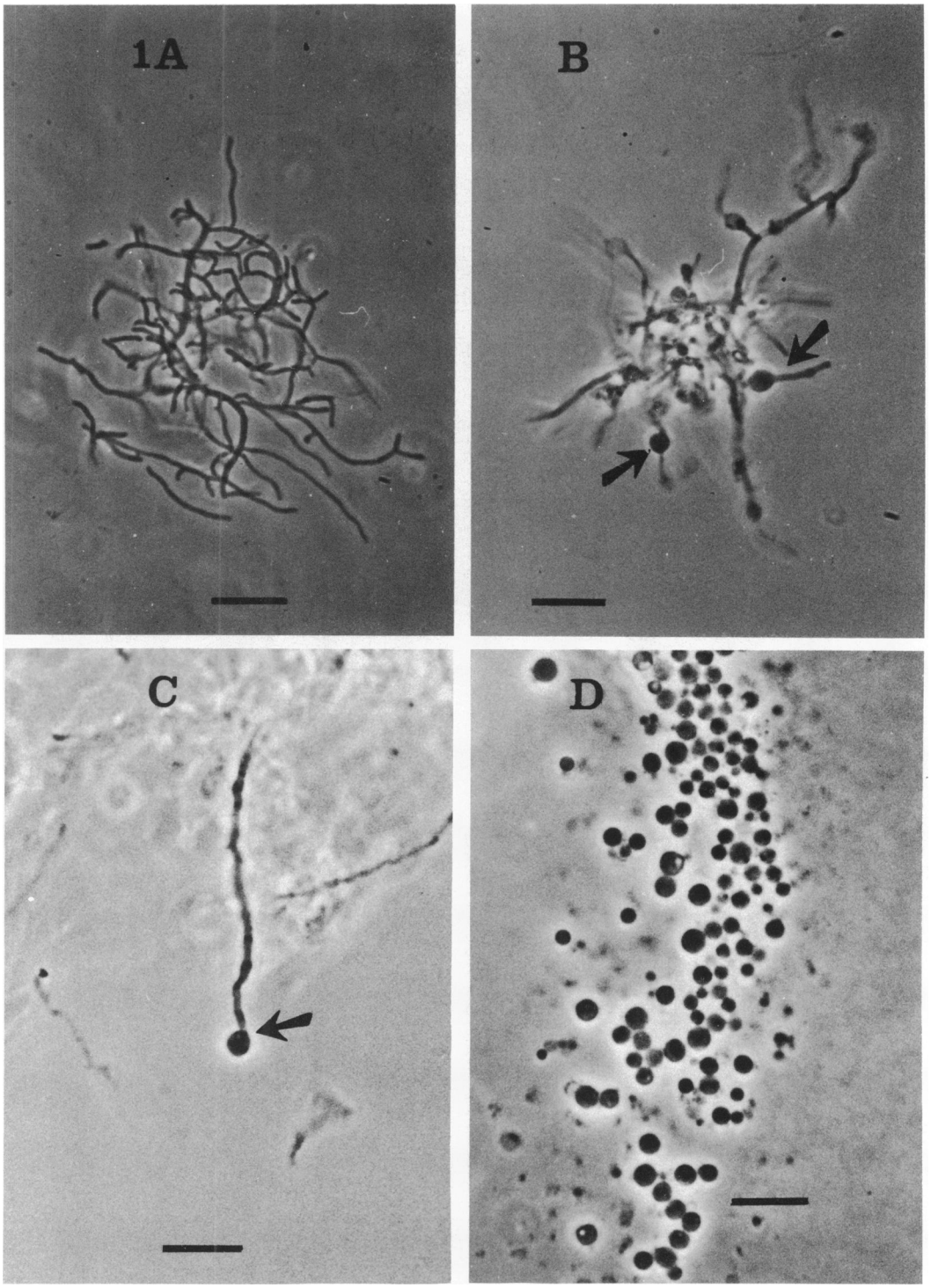
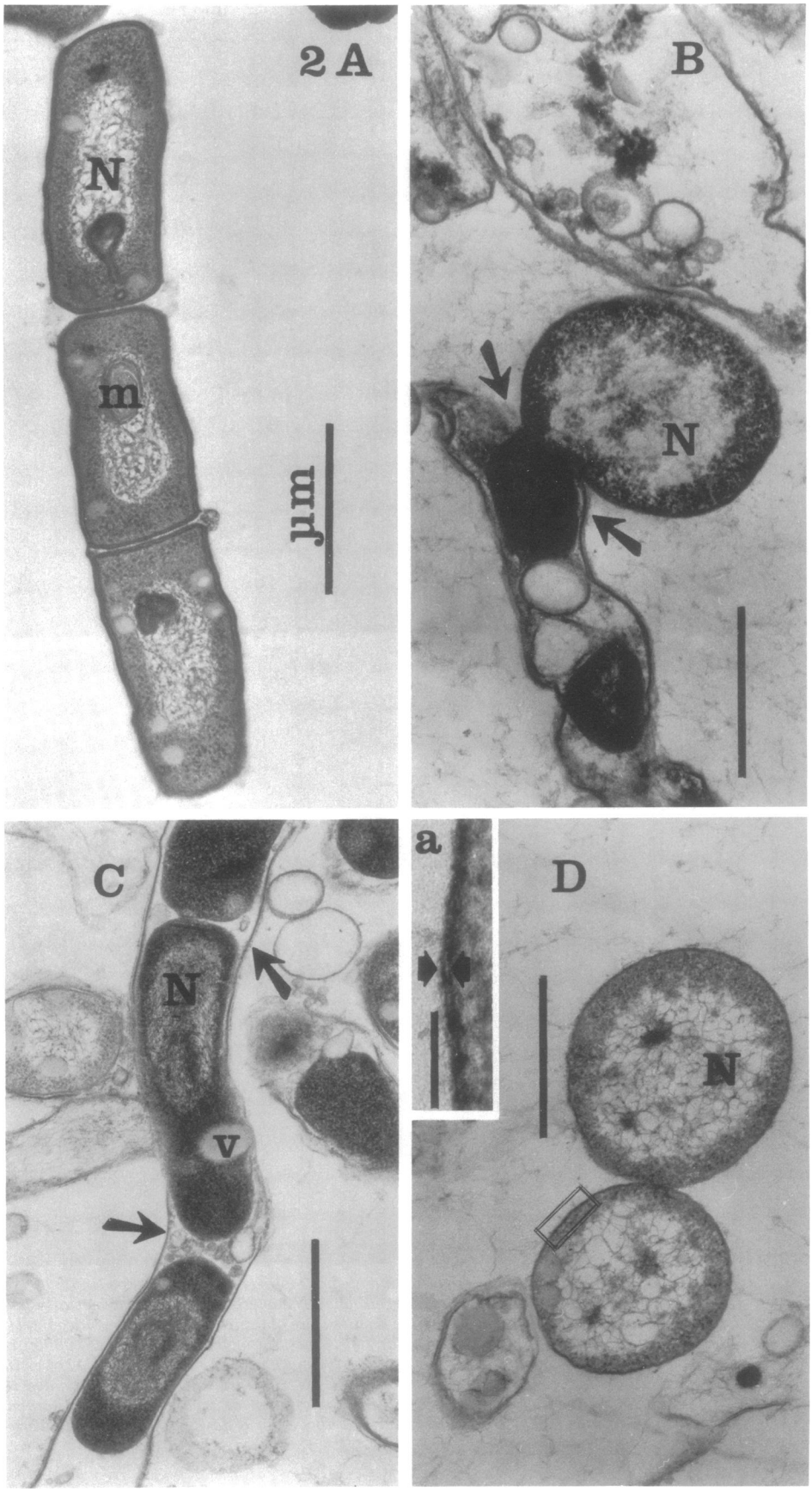


FIG. 1. *Phase contrast micrographs of in vitro induction of spheroplasts of N. asteroides 10905 by lysozyme and glycine. Similar morphological changes were observed when N. asteroides 10905 was serially passed in medium containing* D*-cycloserine and glycine. The same results were obtained with N. asteroides GUH-5. (A) N. asteroides 10905 grown in BHI-B for 48 h at 34 C. Bar represents 5 μm. (B) N. asteroides 10905 grown in BHI-B containing 0.35 M sucrose, lysozyme (20 μg/ml), and glycine (1.2%) at 34 C for 24 h. Arrows indicate central swelling in nocardial filaments. Bar represents 5 μm. (C) N. asteroides 10905 grown, as described in (B) above, for 5 days at 34 C. Arrows indicate nocardial spheroplast at terminal end of filament. Bar represents 5 μm. (D) N. asteroides 10905 grown, as described above, for 8 days. Nocardial cells were totally converted to spheroplasts. Bar represents 5 μm.*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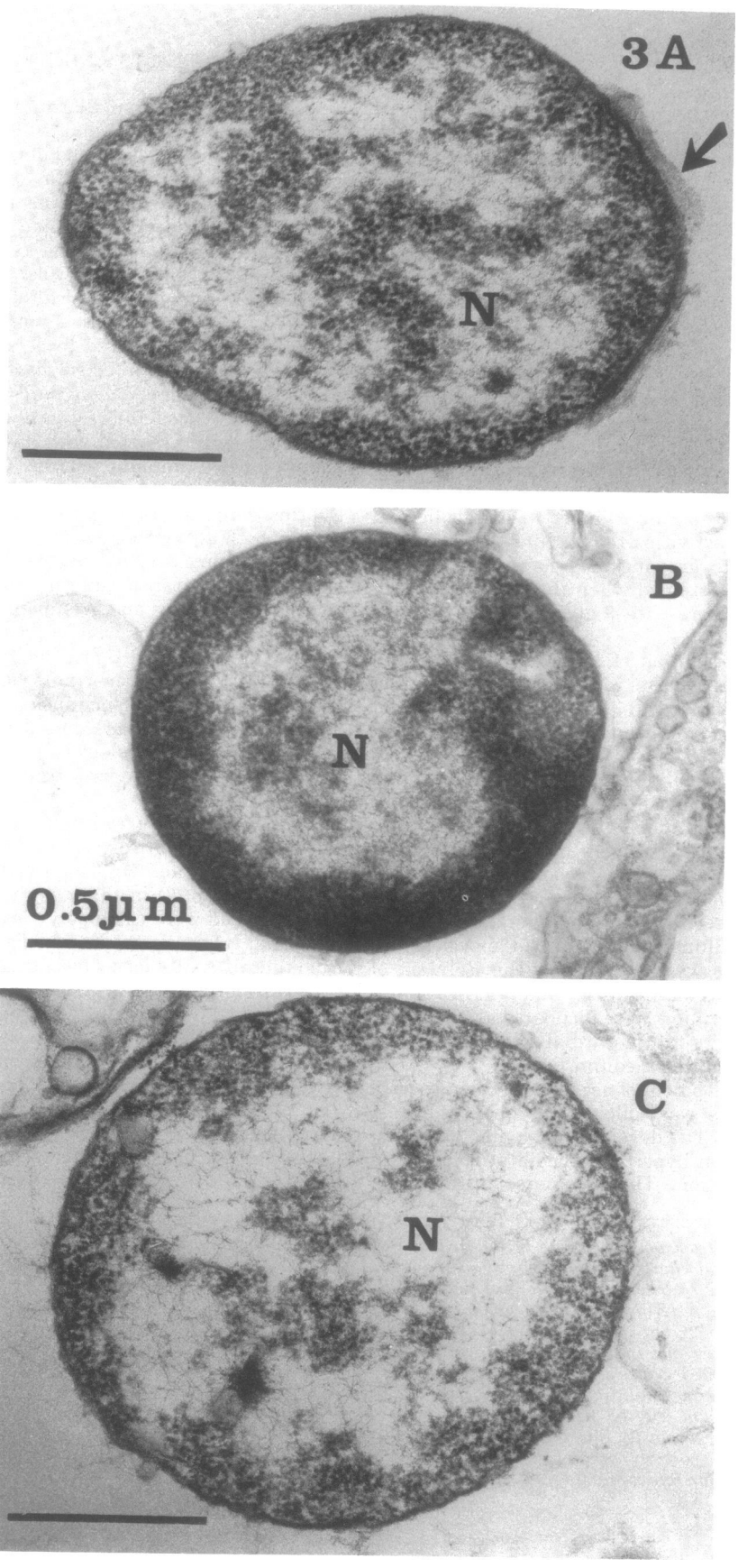
therefore resembled protoplasts. However, we refrain from using this terminology since we have no chemical evidence that they totally lacked cell wall material. The nuclear material within the in vitro induced spheroplasts changed as compared with the parental strains (compare Fig. 2A and 3B). The nuclear region changed from a relatively compact, coarse fibrilar makeup (Fig. 2A) to a more diffuse, fine fibrilar structure (Fig. 2D and 3). It is interesting to note that many of the spheroplasts growing as L-forms (Fig. 5A and B) did not possess this type of nuclear structure. In fact, the nuclear region of many (but not all) of these cells was so diffuse as to be difficult to clearly distinguish (Fig. 5A and B). We do not believe these changes to be artifacts of preparation; instead, they reflect specific changes in metabolic and structural activities that occur as the spheroplasts begin growing as L-forms. Similar results were obtained when the cells were incubated in the presence of glycine plus D-cycloserine (Table 2).

For the isolation of nocardial L-forms, cultures of spheroplasts were plated on BYEL medium as described. Both nocardial L-forms and typical *Nocardia* were isolated aerobically at 37 C under 5% CO_2 in air. L-form colonies developed primarily on plates that lacked large numbers of typical *Nocardia;* therefore, dilutions of spheroplast suspensions frequently yielded greater numbers of L-colonies. Many investigators have observed that plating high concentrations of L-forms frequently stimulated the reversion of these organisms (13). BYEL medium that contained D-cycloserine (250 μg/ml) was selective for the isolation of transitional-phase variants and L-form colonies when these plates were incubated at 37 C in 5% CO_2 in air. Typical nocardial colonies were not recovered on this medium.

The development of nocardial L-colonies from spheroplasts was followed by phase contrast microscopy. The developing colonies were first detectable on hypertonic media at 2 to 4 days postinoculation. These colonies initially appeared as a cluster of small granules and large refractile bodies (Fig. 4A and B). By 9 days, the majority of these colonies had developed a central core that extended into the agar (Fig. 4C). The refractile granules at the periphery of these colonies appeared to remain on the surface (Fig. 4C). After 14 days, typical L-form colonies had formed. These colonies exhibited "fried egg" morphology, typical of other bacterial L-forms (Fig. 4D). The nocardial L-forms induced in vitro appeared to be similar to L-forms isolated from infected macrophages (7). Those derived from *N. asteroides* (10905, 287, GUH-5) gave good fluorescence with anti-*N. asteroides* serum reacted with antirabbit fluorescein-labeled immunoglobulin G (Table 1); however, *N. caviae* L-forms did not. All of the L-forms were catalase negative; they were positive for both deoxyribonucleic acid and ribonucleic acid using acridine orange fluorescence, and they were transferable by the agar-block technique of Dienes and Weinberger (9). Many of these altered forms were successfully transferred nine times on BYEL medium without antibiotics. Very few revertants were observed; therefore these organisms appear to be stable L-forms. However, those that did revert to typical nocardial morphology after one or two transfers were considered transitional-phase variants (Table 3).

Thin sections of nocardial L-form colonies were composed entirely of membrane-bound bodies (Fig. 5A and B). These cells resembled type A L-forms (7) in that they totally lacked cell walls and were extremely variable in size. Nocardial L-forms were clearly distinguishable from their parental forms by both scanning and transmission electron microscopy (compare Fig. 2, 5, and 6). Scanning electron microscopy revealed that the L-colonies consist of clusters of small spherical forms that appeared to originate from an enlarged central body (Fig. 6A and B). Fibrilar debris was frequently associated with the L-form colonies. Similar material was observed in association with cell wall-defective forms of *Straphylococcus* (10). Fass et

FIG. 2. *Ultrastructural events in the in vitro formation of spheroplasts of N. asteroides 10905. Similar ultrastructural changes were observed in N. asteroides GUH-5 and 287 and N. caviae 112. (A) Electron micrograph of N. asteroides 10905 grown in BHI-B for 48 h at 34 C. N, Nuclear region; M, mesosomes. Bar represents 1 μm. (B) N. asteroides 10905 grown in BHI-B containing 0.35 M sucrose, lysozyme (20 mg/ml), and glycine (1.2%) at 34 C. A nocardial spheroplast is emerging through its degenerating cell wall (as shown in Fig. 1C). Arrows indicate area of nocardial cell that has been solubilized. Bar represents 1 μm. (C) N. asteroides 10905 grown as described in (B). This nocardial filament is in the initial stage of spheroplast formation. Arrows indicate areas where cell wall appears to be disassociating from the cell. V, Vacuole. Bar represents 1 μm. (D) N. asteroides 10905 grown as above and shown in Fig. 1D. These cells represent nocardial spheroplasts. Bar represents 1 μm. (Insert a) High magnification of the cell membrane. These spheroplasts (possibly protoplasts) appear to totally lack cell wall material and are bound by a unit membrane. Bar represents 0.1 μm.*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B
N
0.5µm
C
N

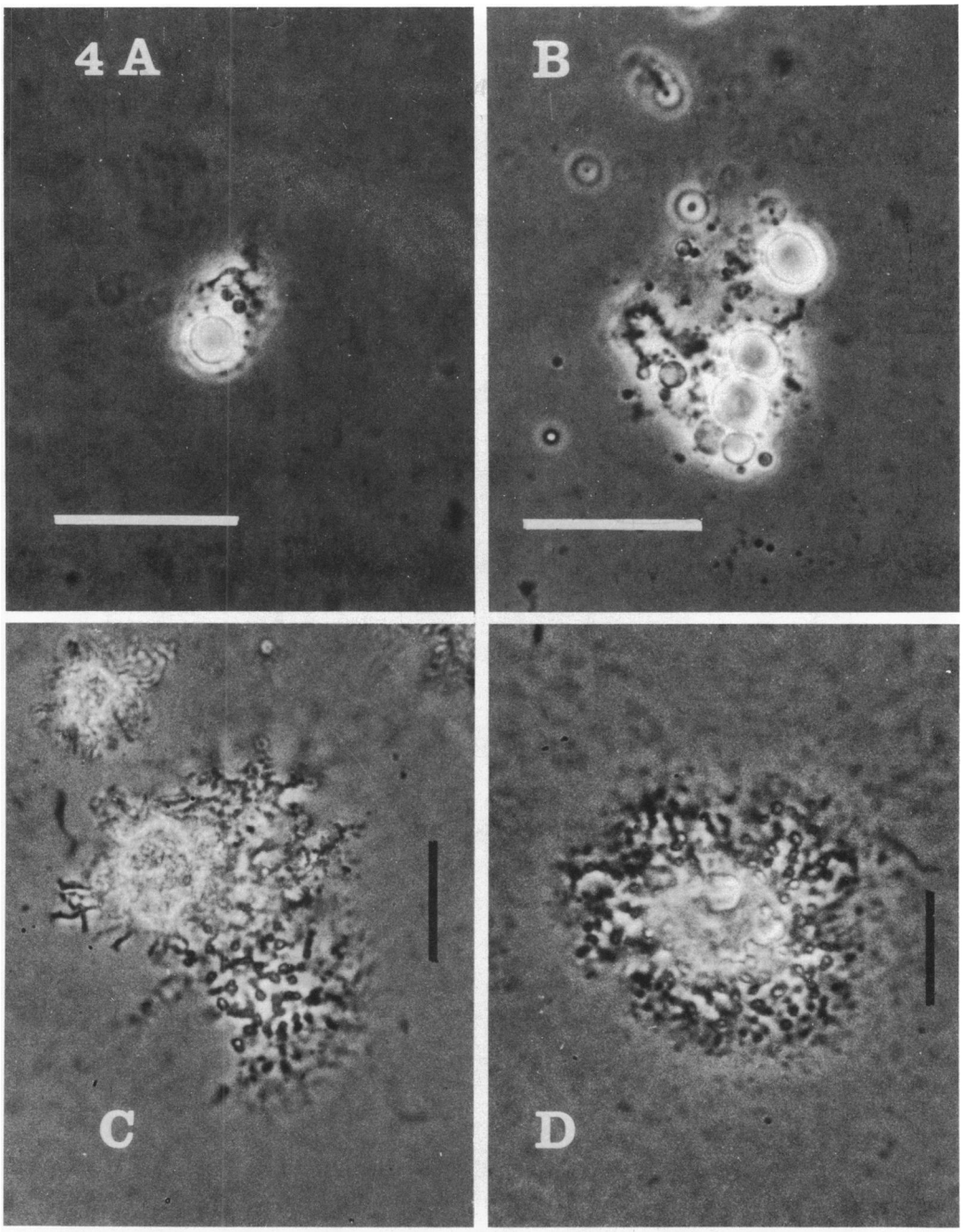

FIG. 4. *Phase contrast micrographs of development of L-form colonies of N. asteroides 10905 from spheroplasts on BYE medium containing 10% heat-inactivated horse serum, 3% NaCl, and 0.9% agar (BYEL). The spheroplasts were induced by growth of the organism in medium containing lysozyme and glycine. A similar developmental sequence was observed for 10905 L-forms derived from D-cycloserine- and glycine-induced spheroplasts as well as from spheroplasts of N. asteroides 287, GUH-5, and N. caviae 112. (A) Developing L-colony of N. asteroides 10905 on BYEL (2 days postinoculation). Bar represents 50 μm. (B) Developing L-colony of N. asteroides 10905 on BYEL (5 days postinoculation). Bar represents 50 μm. (C) Granular nocardial L-form growth on surface of agar (9 days postinoculation). Bar represents 50 μm. (D) Fully developed L-colony of N. asteroides 10905 on BYEL (14 days postinoculation). This L-colony shows "fried egg" morphology typical of other bacterial L-forms. Bar represents 100 μm.*

FIG. 3. *Ultrastructural comparison (electron micrographs) of spheroplasts of N. asteroides 10905, N. asteroides 287, and N. caviae 112. (A) Spheroplast of N. caviae 112. Arrow indicates small amount of cell wall remaining on the surface of this cell. Bar represents 1 μm. (B) Spheroplast of N. asteroides 287. This spheroplast appears ultrastructurally similar to spheroplasts of N. asteroides 10905. Bar represents 1 μm. (C) Spheroplast of N. asteroides 10905. This spheroplast appears to lack all remnants of cell wall material. Bar represents 1 μm.*

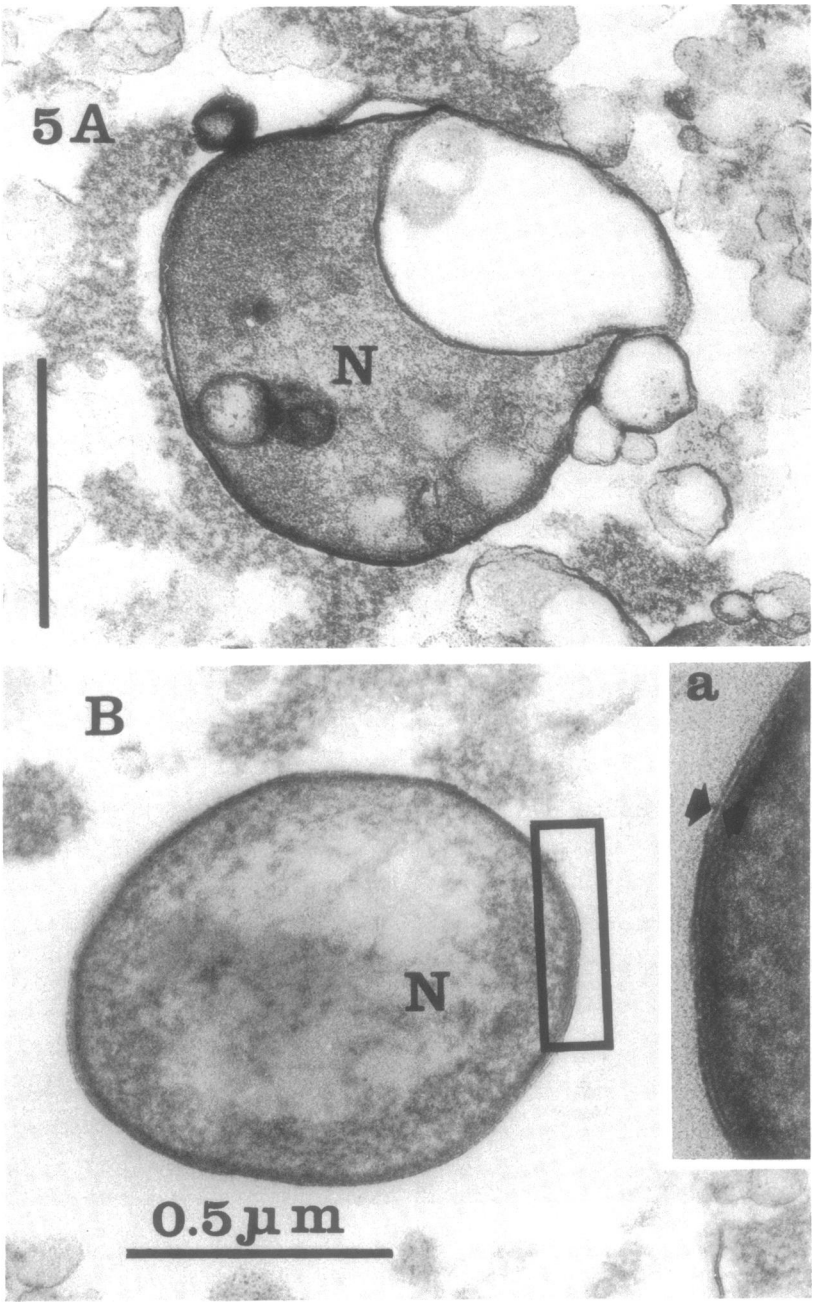

FIG. 5. *Ultrastructure of L-colonies of N. asteroides 10905 induced in vitro. (A) and (B) Electron micrographs of thin sections through L-colonies of N. asteroides 10905 grown on BYE medium containing 10% heat-inactivated horse serum, 3% NaCl, and 0.9% agar (BYEL) for 3 weeks. These L-forms resemble type A L-forms in that they were bound only by a unit membrane (Insert a). Bar represents 1 µm.*

al. (10) suggested that this material represented residual cell wall and membrane.

DISCUSSION

Generally, the *Nocardia* are resistant to the action of many substances used for the induction of spheroplasts and L-forms in other bacteria (2, 8). For example, nocardial cells are resistant to the action of lysozyme (8), and cell wall inhibitors such as penicillin and D-cycloserine have little effect on most species of *Nocardia* (2). It has been suggested that the

complex nature of the nocardial cell wall is the primary cause of resistance in these organisms. The results presented above indicate that glycine potentiates the effects of both lysozyme and D-cycloserine on the integrity of the cell walls of certain nocardial strains. We found that neither lysozyme nor D-cycloserine alone had any apparent effect on nocardial morphology (Table 2). However, when these compounds were used in combination with glycine, they both converted cells of *N. asteroides* and *N. caviae* to spheroplasts which then grew as L-phase variants (Table 2 and Fig. 4). Glycine was shown to play a similar role in the induction of L-forms of group A, beta-hemolytic *Streptococcus* (14). In addition, it was shown that glycine and lysozyme converted the cells of several strains of *Mycobacterium* to spheroplasts (1, 15).

TABLE 3. *Isolation and growth of altered forms of Nocardia from in vitro induced spheroplasts*

Organism	Inducing agent		No. of transfers before reversion to parental type
	Glycine + D-cycloserine	Glycine + lysozyme	
Nocardia asteriodes 10905	L-form	L-form	>9
N. asteroides GUH-5	TPV[a]	TPV	3
N. asteroides 287	TPV	TPV	2
N. caviae 112	TPV	TPV	2

[a] TPV, Transitional-phase variants.

The mechanisms by which glycine affected bacterial morphology were studied by Hammes et al. (11). Using different species of bacteria, they demonstrated that glycine interfered with several steps in peptidoglycan biosynthesis (11). Glycine was incorporated into both the nucleotide-activated peptidoglycan precursors and the peptidoglycans of the organisms studied. Analysis of the primary structure of these precursors and their corresponding peptidoglycans indicated that glycine could replace L-alanine in position 1 and D-alanine in positions 4 and 5 of the peptidoglycan side chains. Hammes et al. (11) suggested that the replacement of D-alanine residues with glycine was reponsible for the altered morphology of organisms grown in the presence of excess glycine since glycine appeared to inhibit the cross-linking within the peptidoglycan. A reduction in the amount of peptidoglycan cross-linking might explain how glycine potentiates the effect of lysozyme on *Nocardia*.

Glycine might also have altered the composition and structural integrity of the outer layer

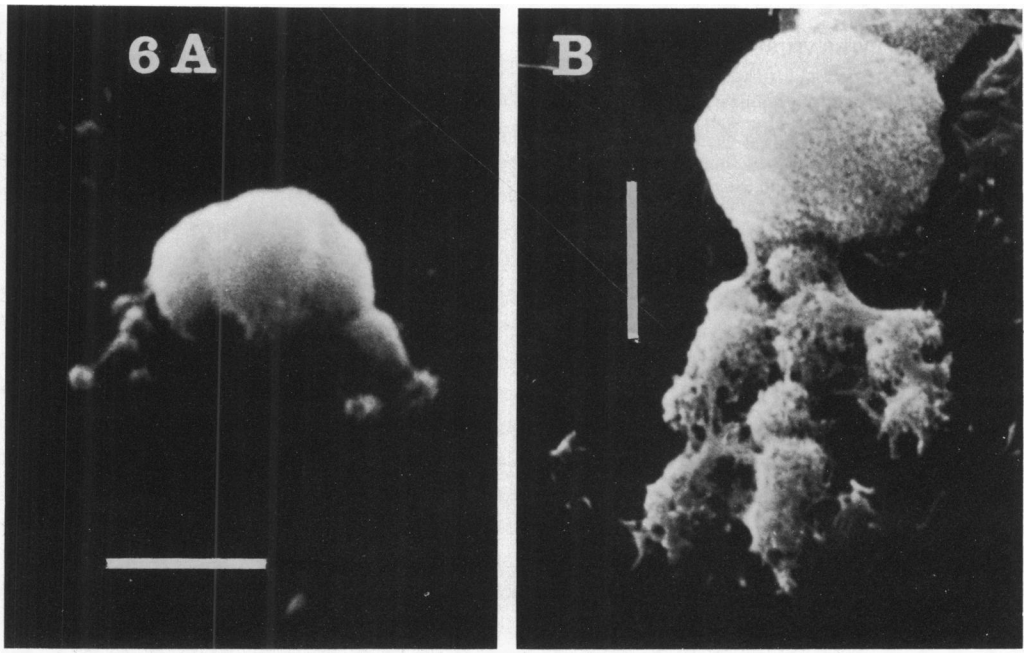

FIG. 6. *Scanning electron micrographs of surface morphology of N. asteroides 10905 L-forms. (A) Developing L-colony of N. asteroides 10905 grown on BYEL at 34 C. These L-colonies appeared to develop from an enlarged central body. Bar represents 10 μm. (B) Mature L-colony of N. asteroides 10905 grown on BYEL for 3 weeks at 34 C. The fibrilar debris associated with these organisms is typical of bacterial L-form colonies and may be residual cell wall and membrane. Bar represents 10 μm.*

of the cell wall of *Nocardia*. This layer was shown to be composed of peptides or proteins in close association with lipids (4, 5). We found that alanine was a major amino acid in these lipid-associated peptides (4, 5). Therefore, it seems reasonable to postulate that glycine might be substituted for the alanine in the outer layer of the nocardial cell wall. Substantial substitution of alanine by glycine in the outer layer, as in the peptidoglycan, might render the cell more susceptible to the action of lysozyme or D-cycloserine.

The observation that different strains of *N. asteroides* responded differently to glycine and lysozyme or glycine and D-cycloserine suggested that the cell wall structures of these strains were different. Preliminary data confirmed that there were major chemical differences between the cell walls of *N. asteroides* 10905 and *N. asteroides* 14759 (5). Further, cells of strain 10905 were readily converted into L-phase variants by alveolar and peritoneal macrophages (6, 7). In contrast, *N. asteroides* 14759 cells were not converted to variants, but instead rapidly grew out of the macrophages as gram-positive, beaded filaments (6).

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