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Morphologic Study of *Staphylococcus aureus* L-Form, Reverting, and Intermediate Colonies In Situ†

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Staphylococcus aureus **strains of bovine origin were induced to L-form by exposure to 100 U of penicillin in brain heart infusion broth supplemented with 5% NaCl, 5% sucrose, and 10% horse serum. L-forms were cultured on similarly supplemented brain heart infusion agar containing no antibiotic. Light and electron microscopic examination of plastic-embedded L-form colonies revealed a variety of morphologic types. The primary site of growth appeared to be the core area below the agar surface, consisting mainly of pleomorphic budding forms. At the surface, these forms gave rise to large spherules with a gradation from smaller to larger spherules toward the periphery of the colony. Some colonies progressed to reverting forms with the growth of bacterial cells containing cell wall. In addition to L-forms, intermediate colony forms were observed that lacked typical L-form morphology and progressed rapidly to the parent cell form on subculture to bovine blood agar. Description of these forms will be useful in the search for similar morphologic types in vivo during antibiotic treatment of chronic *S. aureus* bovine mastitis.**

Induction of *Staphylococcus aureus* to L-form during therapy of bovine mastitis has been postulated as one mechanism whereby the organism could withstand antibiotic therapy, reemerging with a remanifestation of symptoms (12, 15). The organism is thought to pass through a cycle of induction to L-form from parent cells and then reversion back to the parent, possibly via an intermediate or reverting form. Reversion of L-forms to the parent bacterial form is not unusual and has been reported often in the literature (3, 8–10, 14). Green et al. (8) postulated such a reproductive cycle for *Enterococcus* (*Streptococcus*) *faecalis*. Schonfeld and De Bruijn (14) reported on reversion of both *S. aureus* and *E. faecalis* and suggested an intermediate stage in the process. Reversion of *Bacillus subtilis* in which gelatin was required for reversion was described by Landman et al. (10). There appears to be considerable species differences in the requirements for reversion.

The L-form state in vivo may serve as a dormant phase in which the organism merely waits out the presence of antibiotics. Such a mechanism has been postulated to function in bovine mastitis (12). Alternatively, the L-form may be a more active state with multiplication and growth. Beaman and Scates (1) has described such a process in experimental *Nocardia* infections in mice.

There is some strain variation in the ease of induction of *S. aureus* of bovine origin to L-form and for other organisms (11, 13). The ultrastructural characteristics of selected strains of *S. aureus* in various stages of induction and reversion in vitro may offer some insight into what occurs in vivo. Morphologic studies of L-forms have revealed a variety of forms present in typical agar colonies and in broth cultures. These forms range from very small granular forms resembling the elementary bodies of mycoplasma to very large spherules many times the size of the bacterial cells. The different morphologic types appear in various areas of the L-form colony and vary with colony age and with its stability in the L-form state. Several researchers have suggested roles for these various morphologic types (8, 14). We have examined the ultrastructural characteristics of L-form, intermediate, and reverting colonies of *S. aureus* strains isolated from bovine mastitis at various stages of growth.

Staphylococcus aureus strains were obtained from the Mastitis Research Laboratory Culture Collection. All strains had been previously isolated from cases of bovine mastitis and were maintained frozen at −70°C in 20% glycerin and Trypticase soy broth (TSB) (BBL Microbiology Systems, Cockeysville, Md.). Organisms were identified as *S. aureus* by morphologic characteristics and by using the API Staph-Trac System (Analytab Products, Plainview, N.Y.). For induction of L-forms, strains shown previously to be inducible to L-form were subcultured from the frozen stock to Trypticase soy agar (BBL) with 5% bovine blood. After 24 h

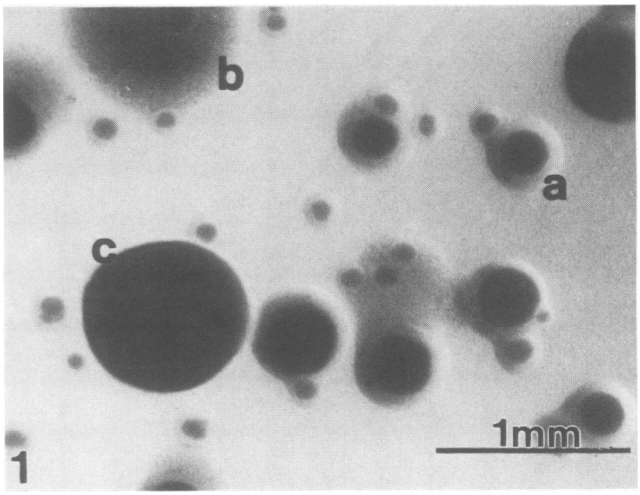

FIG. 1. A variety of colonial types observed at 4 to 5 days in culture include typical L-forms (a), reverting colonies (b), and intermediate colonies (c). Magnification, ×25.

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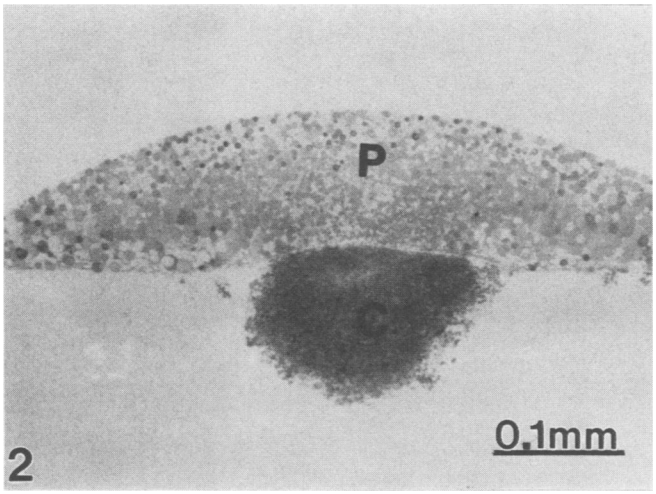

FIG. 2. Cross section through midportion of a 4-day-old L-form colony illustrating dense core area (C) embedded in the agar and the less dense, peripheral area (P) above the agar surface. Magnification, ×170.

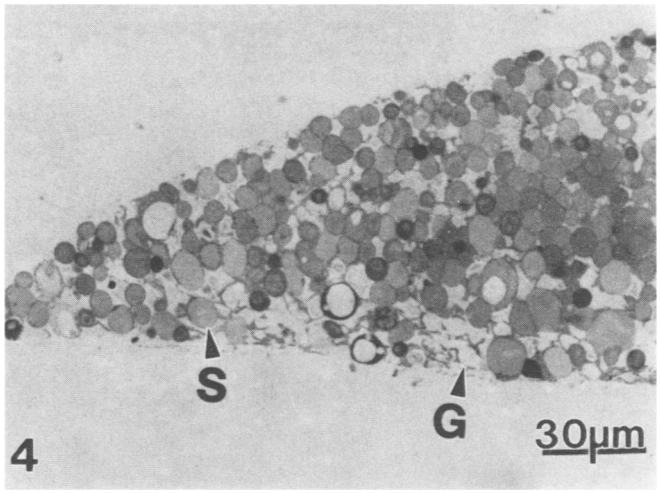

FIG. 4. Cross section of peripheral area of L-form colony composed of larger spherules (S) and ghost forms (G). Magnification, ×465.

on Trypticase soy agar with 5% bovine blood, two or three well-isolated colonies were subcultured to TSB and incubated for 2 to 4 h at 35°C after which the TSB suspension was adjusted to a turbidity approximating a 0.5 McFarland standard. For induction of L-forms, 0.1 ml of the TSB broth suspension was added to 9.9 ml of brain heart infusion (BHI) broth (BBL) supplemented with 5% NaCl, 5% sucrose, 0.5% yeast extract (Difco Laboratories, Detroit, Mich.), 10% horse serum (Sigma Chemical Co., St. Louis, Mo.), and 100 U of penicillin (Sigma). The osmolality of the supplemented broth and agar was 1,860 mosmol. The BHI broth was warmed to 35°C before inoculation. After 10 min of incubation, 0.1-ml samples were plated to BHI agar with the same supplements as the BHI broth. Plates were incubated at 35°C and examined daily for L-form, intermediate, or reverting colonies. Initially, several strains were examined; however, little or no morphologic difference from strain to strain was observed. Therefore, one strain, *S. aureus* Newbould 305 ATCC 29740, was selected as a representative organism. All subsequent work in this study was done on this strain.

Representative colonies were selected at various times throughout the 10-day incubation period and processed for microscopy. Colonies for microscopic study were covered with a drop of agar containing 5% NaCl and 5% sucrose. After the covering agar solidified, an agar plug containing the colony was cut from the plate with a scalpel and placed in 5% glutaraldehyde in 0.1 M cacodylate buffer containing 5% sucrose for 2 h at room temperature. Glutaraldehyde-fixed agar blocks were postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 2 h, dehydrated in ethanol, and embedded in epoxy resins. Thick plastic sections (0.5 to 1 μm) for light microscopy were taken on a Porter Blum MT-5000 ultramicrotome, stained with toluidine blue, and examined by using a Zeiss standard 18 research microscope. Thin

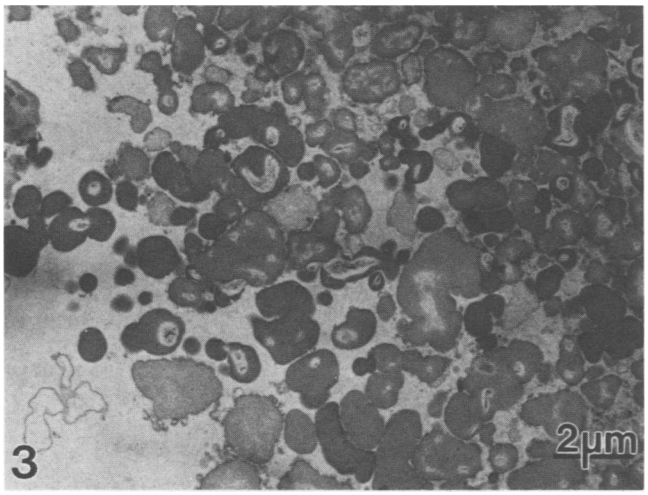

FIG. 3. Electron micrograph of the core area illustrating pleomorphic elemental forms with several at the budding stage. Magnification, ×5,430.

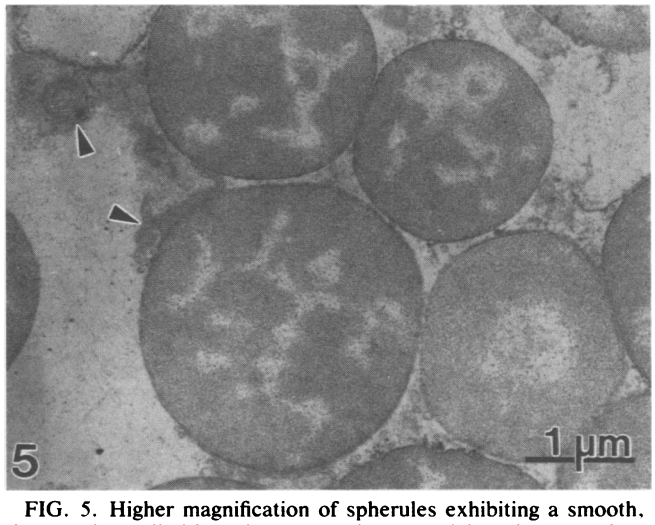

FIG. 5. Higher magnification of spherules exhibiting a smooth, electron-dense limiting plasma membrane and less-dense nuclear and cytoplasmic areas. Arrowheads indicate concentric, membranous material. Magnification, ×13,800.

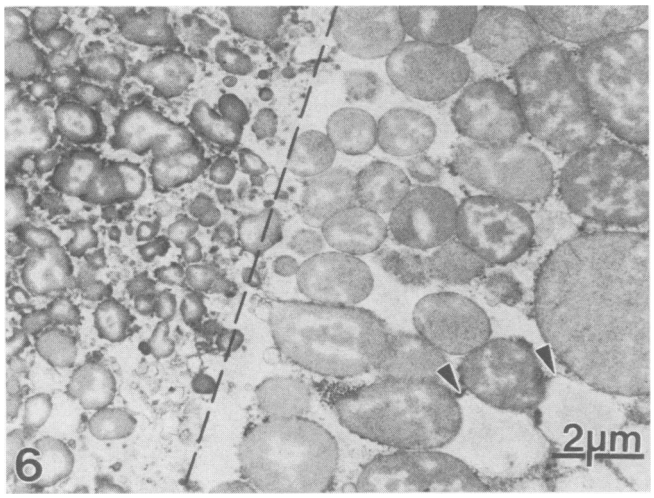

FIG. 6. Interface of sub- and supra-agar colonial area (----) illustrating the dramatic transformation from pleomorphic elemental forms to large spherules. Ghost forms (indicated by arrowheads) adjacent to spherules appeared empty, having irregular, limiting membranes. Magnification, ×6,100.

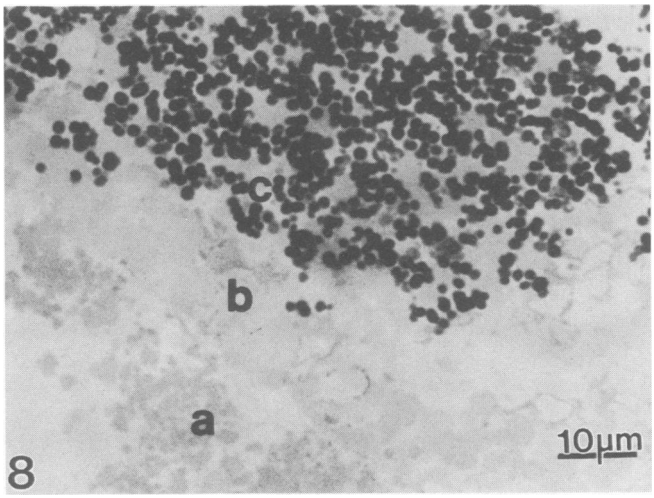

FIG. 8. Higher magnification of reverting colony illustrating the progression from elemental core forms (a) through spherules (b) to parent forms (c). Magnification, ×1,000.

sections (60 to 80 nm) for transmission electron microscopy were sectioned as above, stained in uranyl acetate and lead citrate, and examined by using a Philips EM 300 electron microscope operating at 60 kV.

Exposure to 100 U of penicillin G per ml induced typical L-form colonies from the selected strains of *S. aureus* (Fig. 1). Cross-sectional views of in situ glutaraldehyde-fixed L-form colonies revealed considerable morphologic differences between the portion of the colony above the agar and that below and within the agar. However, there were no morphologic differences between bacterial strains. Light microscopic examination of toluidine blue-stained cross-sectional views through the midportion of 3- to 4-day-old colonies revealed a dense core area embedded in the agar with a large, less dense area covering the core and extending out around the core on the agar surface (Fig. 2). The subagar core area was composed mainly of pleomorphic forms, ranging from 0.2 to 2 μm, typical of those described by others as elemental or granular forms (4, 7), and appeared to be the more actively growing portion of the colony. At the ultrastructural level, pleomorphic elemental forms displayed an electron-dense limiting unit membrane and less-dense nuclear and cytoplasmic areas (Fig. 3). Blebs of variable size protruding from elemental bodies suggested multiplication by budding in many instances (Fig. 3). The periphery of the colony was above the surface of the agar and was composed of large spherules of up to 9 μm in diameter and empty ghost forms (Fig. 4). Spherules exhibited an electron-dense limiting unit membrane and less-dense nuclear and cytoplasmic areas (Fig. 5). Concentric, filamentous debris was often found adhering to spherules as well as ghost forms.

Immediately above the agar surface, the pleomorphic core forms became spherical and larger, and upon electron microscopic examination, a gradation from small to large spherules was observed toward peripheral areas in less-

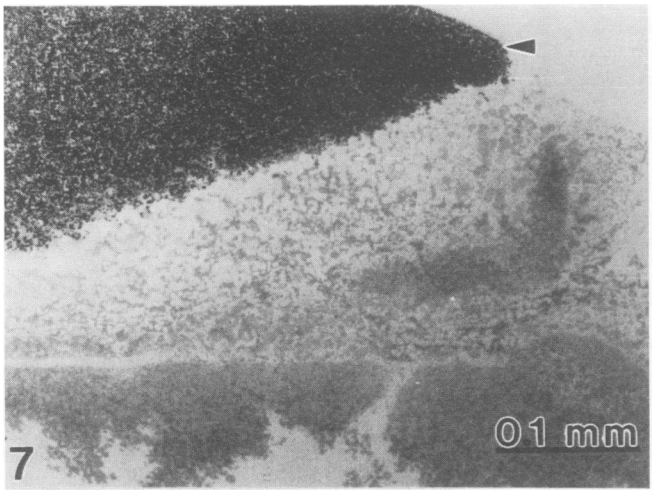

FIG. 7. Portion of reverting colony exhibiting dense core area and peripheral area with parent forms (arrowhead) in close association with the supra-agar layer composed of spherules and ghost forms. Magnification, ×166.

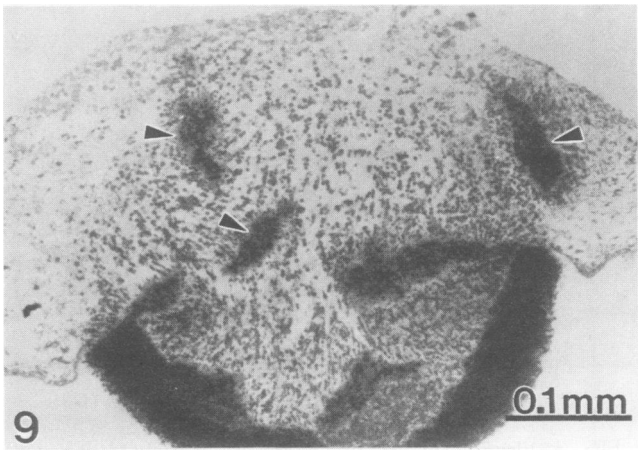

FIG. 9. Cross section through secondary dense core areas (arrowheads) of an L-form colony. Magnification, ×175.

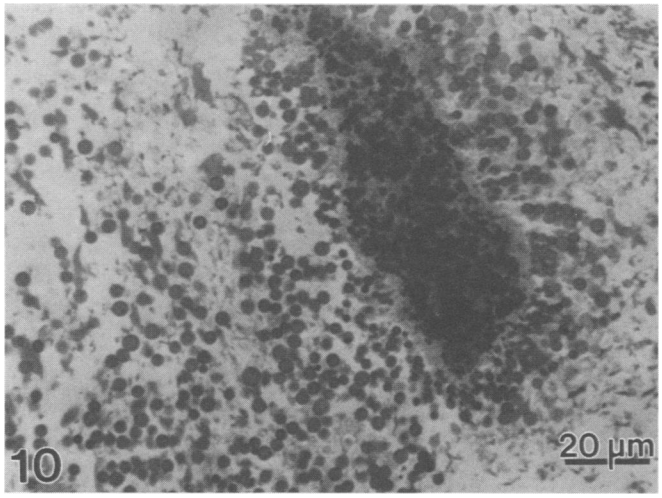

FIG. 10. Higher magnification of a secondary dense core areas in Fig. 9 illustrating large spherules emanating toward peripheral areas. Magnification, ×525.

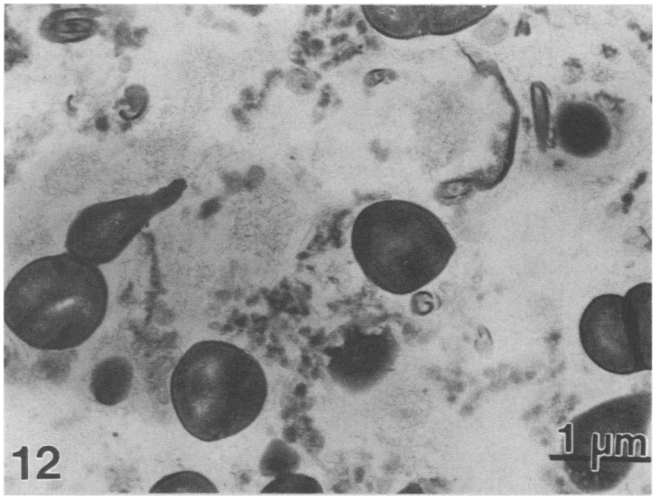

FIG. 12. Higher magnification of an intermediate colony illustrating coccal forms among fibrillar debris and cell wall material. Magnification, ×13,700.

mature colonies (Fig. 6). However, this progression was not apparent in more-mature colonies. Many of the larger spherules appeared to disintegrate, leaving a filamentous matrix in which intact forms resided. Lack of osmotic protection and structural support provided by the agar may be responsible for this transformation. Often the large spherules above the surface contained inclusions similar to those described by Green et al. (8). However, a clear life cycle progression similar to that proposed by Green et al. (8) for *S. faecalis* could not be established. Some of the mature colonies began to exhibit significant morphologic changes, with apparent reversion of the colony originating near the edge of the central dense core and spreading throughout the colony (compare Fig. 1 with Fig. 7 and 8). A similar reversion process has been described by Kato et al. (9), using a defined reversion medium.

After 4 to 5 days of incubation, a variety of colony types were present ranging from very young typical L-forms to older mature L-forms (Fig. 1). Some L-forms developed secondary dense core areas and exhibited very dense bands of material within the primary core or above the original plane of the agar surface (Fig. 9). These secondary areas resembled the initial core morphologically with larger spherules appearing to originate from the denser areas (Fig. 10).

In addition to typical L-form colonies, exposure to penicillin often induced an intermediate-type colony. These were seen with typical L-form colonies on the same agar plate (Fig. 1). Intermediate colonies were typically larger than L forms, lacked a dense core area, and had a cut glass texture (Fig. 1). Cross-sections of plastic-embedded colonies confirmed the absence of core areas and illustrated the variable-sized coccal forms making up the colony (Fig. 11). Subculture of these forms to bovine blood agar invariably resulted in growth of fully reverted parent bacterial colonies. Electron microscopic evaluation revealed a variety of irregularly dividing coccal forms with true cell walls intermixed with empty ghost forms and portions of cell wall material (Fig. 12).

Exposure of *S. aureus* strains to penicillin under osmotic protection has long been recognized to result in loss of the cell wall without membrane lysis (2, 3, 5, 6, 14). The resulting membrane-bound forms are capable of growth on suitable media resulting in the L-form colony. In this study, a wide variety of morphologic forms were observed within L-forms at various stages of maturity. The progression of L-form colonies through reversion and toward the bacterial parent suggests that some of these observed forms are involved in this progression. The role of L-forms in therapy failure of bovine mastitis would require involvement of similar forms in vivo. The descriptions of morphologic types in this study should aid a search for similar forms in mammary tissue and milk samples to fully establish the link between L-form induction and recurrent *S. aureus* bovine mastitis.

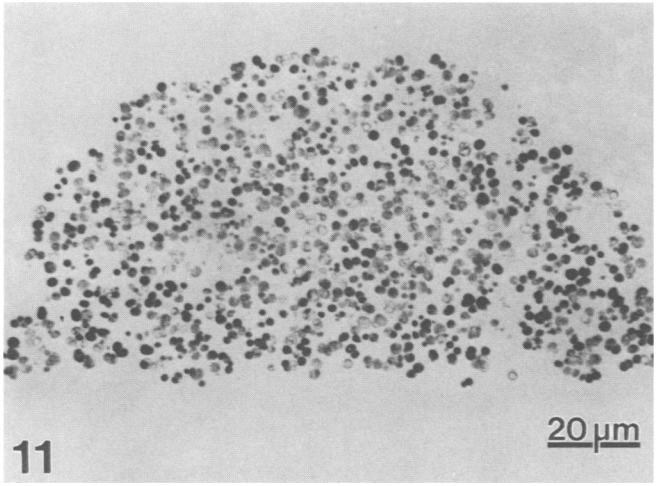

FIG. 11. Intermediate colony illustrating variable-sized coccal forms. Magnification, ×620.

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