

NOTES

Thymidine-Dependent Small-Colony Variants of *Staphylococcus aureus* Exhibit Gross Morphological and Ultrastructural Changes Consistent with Impaired Cell Separation

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Thymidine-dependent small-colony variants (SCV) of *Staphylococcus aureus* exhibited unusual colony morphology with “fried-egg” or pinpoint white colonies on agar plates and pleomorphic cocci as determined by Gram staining. Electron microscopy revealed enlarged cocci with incomplete or multiple cross walls consistent with impaired cell separation. Fried-egg SCV phenotypes could be reversed by thymidine supplementation.

Staphylococcus aureus is a major human pathogen causing significant morbidity and mortality in both community- and hospital-acquired infections (5). Today, the prevalence of methicillin-resistant *S. aureus* (MRSA) is steadily increasing, and treatment of staphylococcal infections has become more difficult due to the emergence of multidrug-resistant strains (5, 8). Infections caused by *S. aureus* range from mild skin and wound infections to acute life-threatening diseases such as endocarditis, pneumonia, arthritis, and osteomyelitis. However, *S. aureus* can also cause a chronic type of disease with recurrent infections, as demonstrated for osteomyelitis (7, 10) and cystic fibrosis (CF) (3; Kahl et al., submitted). These infections have been associated with the isolation of subpopulations of *S. aureus* with the small-colony variant (SCV) phenotype. In contrast to normal *S. aureus* with typical colony size, pigmentation, and hemolysis on Columbia agar, SCVs are often altered in their electron transport activity (4) or in their ability to synthesize thymidine 1 (2). Thus, SCVs grow as small, nonpigmented, nonhemolytic colonies which produce greatly reduced amounts of α -hemolysin, thereby persisting intracellularly in vitro systems (1).

Recently, we demonstrated high prevalence and long-term persistence of normal and SCV *S. aureus* in the airways of CF patients during a 6-year prospective study (B. C. Kahl, A. Duebbers, G. Lubritz, J. Haerberle, H. G. Koch, B. Ritzerfeld, M. Reilly, E. Harms, R. A. Proctor, M. Herrmann, and G. Peters, submitted for publication). In many patients, normal and SCV *S. aureus* isolated at the same time were isogenic, as determined by pulsed-field gel electrophoresis (3; Kahl et al., submitted). Most SCVs (148 of 212; 69.8%) were thymidine

dependent, and nearly all of them emerged due to long-term treatment with trimethoprim-sulfamethoxazole (SXT), thus being SXT resistant, as was also described by Gilligan et al. (2). Importantly, treatment with SXT often represents a last choice in patients with infections caused by MRSA because more than 50% of multidrug resistant MRSA strains are still SXT susceptible (8). However, during long-term treatment with SXT, chances for selection or induction of thymidine-dependent SCVs arise. Accordingly, reports on thymidine-dependent MRSA SCVs causing fatal infections are increasing (9). However, due to their unusual morphology, the correct identification of these variants is extremely difficult, and therefore SCVs are easily missed or misdiagnosed as normal flora in the routine laboratory. This report illustrates this unusual morphology and correlates it with the underlying ultrastructural changes present in thymidine-dependent SCVs.

In contrast to the growth of normal *S. aureus* on Columbia sheep blood (Oxoid, Wesel, Germany) agar (Becton Dickinson, Heidelberg, Germany) (Fig. 1, N), thymidine-dependent SCVs grew either as “fried-egg” colonies with translucent edges surrounding a smaller, elevated pigmented center (Fig. 1A, SCV) or as pinpoint colonies approximately 10 times smaller (Fig. 1B, SCV) than normal *S. aureus*. In contrast to homogenous cocci of the isogenic normal *S. aureus* (Fig. 2A, panel III), Gram staining of thymidine-dependent SCVs revealed pleomorphic cocci, including colonies of sizes resembling fungi (Fig. 2A, panels I and II). Since growth on Columbia blood agar and Gram staining morphology were highly unusual for *S. aureus*, extended identification procedures such as PCR of the highly conserved *nuc* gene in addition to standard procedures were necessary (3) to confirm species identification.

Scanning electron microscopy (SEM) of SCVs revealed heterogeneous bacteria of different sizes in addition to cells which

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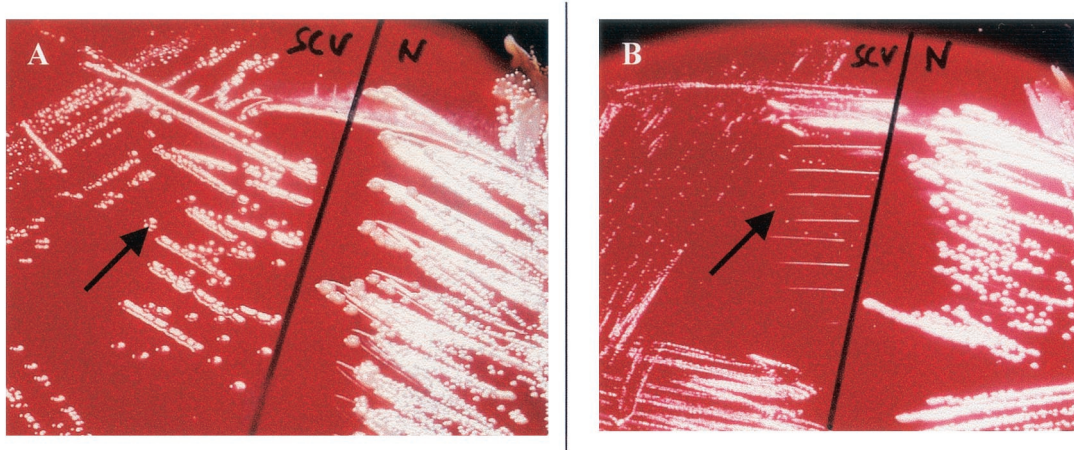


FIG. 1. Growth of thymidine-dependent SCVs with fried-egg appearance and with pinpoint colonies on Columbia blood agar. (A) Fried-egg thymidine-dependent SCVs (left, arrow) in comparison to the isogenic normal *S. aureus* (right, N). (B) Pinpoint SCVs (left, arrow) in comparison to the isogenic normal *S. aureus* (right, N). All strains were grown overnight at 37°C.

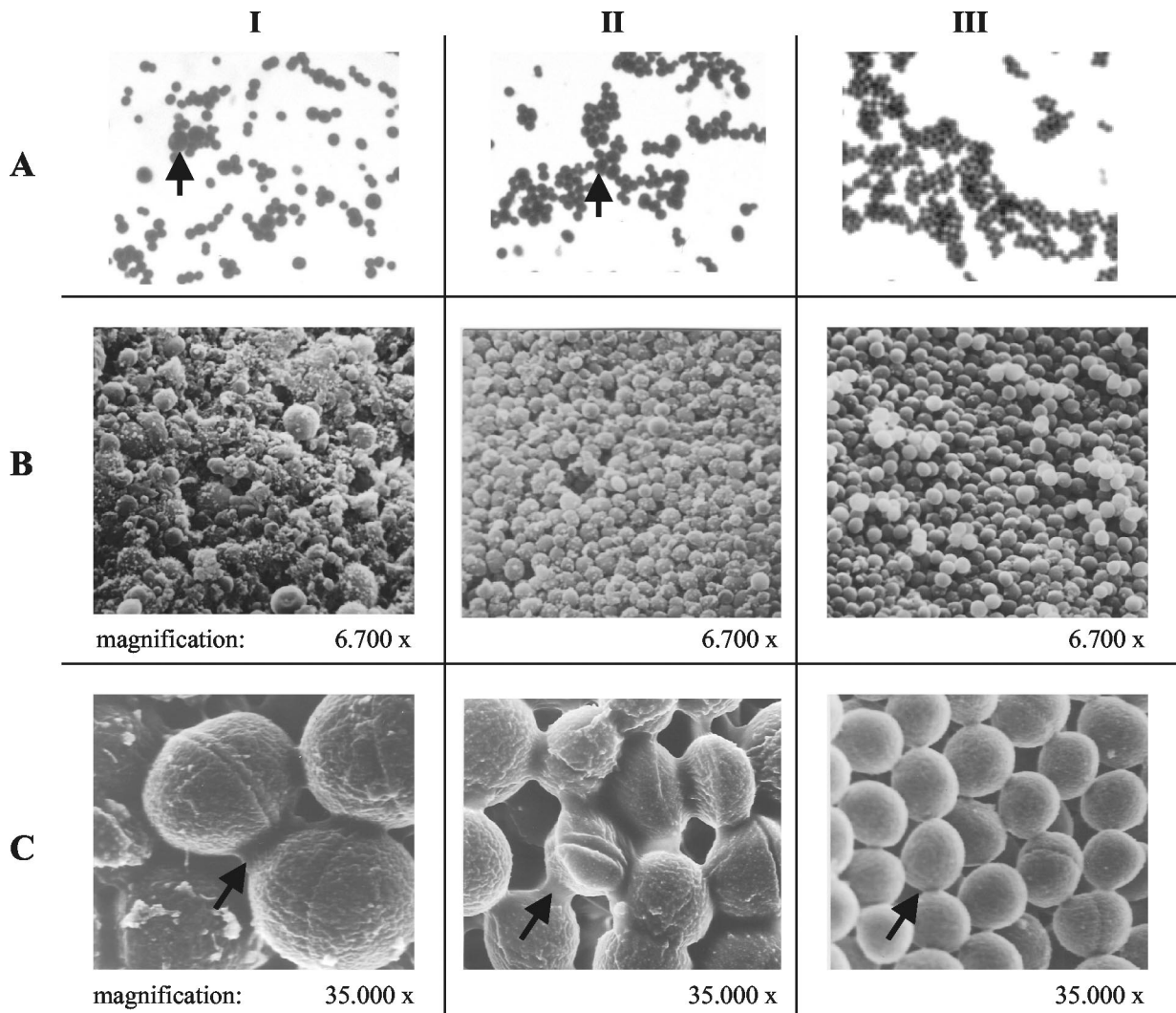


FIG. 2. Gram staining and SEM of fried-egg SCVs (panels I), pinpoint SCVs (panels II), and normal *S. aureus* (panels III). (A) Gram staining. Arrows indicate large cocci. (B and C) Low- and high-magnification SEM, respectively. Arrows indicate the enhanced intercellular substance present in SCVs compared to normal *S. aureus*.

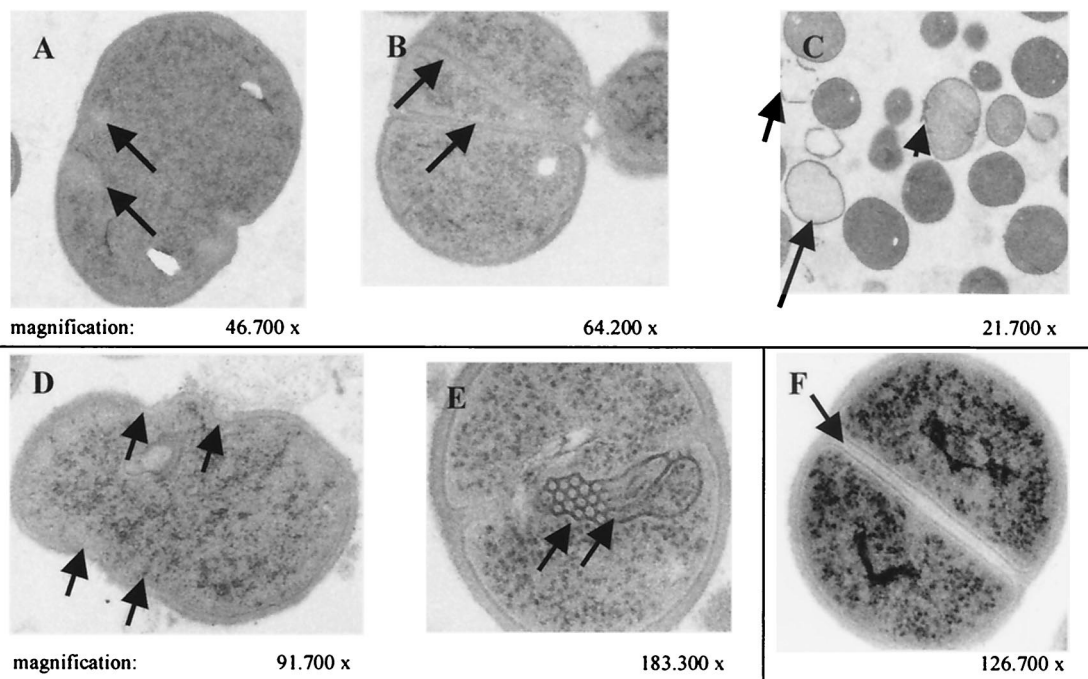


FIG. 3. TEM of fried-egg SCVs (A, B, and C), pinpoint SCVs (D and E), and normal *S. aureus* (F). (A and B) Arrows indicate incomplete or multiple cross walls. (C) Incomplete cross wall (small arrows), cellular debris (intermediate arrow), and empty cells (large arrows) are seen. (D and E) Pinpoint SCVs with atypical unrounded cells and mesosome-like structures (arrow). (F) Normal *S. aureus* cells displaying regular cell separation by a cross wall (arrow) surrounding a highly contrasting splitting system.

seemed to be empty or dented and were covered with debris (Fig. 2B, panels I and II), which was more pronounced in fried-egg SCVs than in pinpoint SCVs. This was in stark contrast to homogenous cocci of the isogenic normal *S. aureus* (Fig. 2B, panel III, and C, panel III), on which no debris was present. Some SCVs were nearly eight times larger than normal *S. aureus*, presumably due to impaired cell separation. Moreover, an intercellular substance was more abundant in SCVs than in the normal *S. aureus* (Fig. 2C). Transmission electron microscopy (TEM) disclosed large “swollen” cells with several uncompleted cross walls and a reduced or undetectable splitting system in both SCV phenotypes (Fig. 3A, C, and D), whereas only in pinpoint SCVs were mesosome-like structures at the cross wall site detectable (Fig. 3E). In addition, some fried-egg SCVs possessed branched and multiple cross walls without obvious cell separation (Fig. 3B), while other cells lacked the cytoplasm (Fig. 3C), and debris of cell walls of dead cells was visible (Fig. 3C). For comparison, the morphology of the isogenic normal *S. aureus* is shown in Fig. 3F, demonstrating regular cell separation by cross walls surrounding a highly contrasting rectangular splitting system. The same images were seen with *S. aureus* ATCC 29213 (data not shown). All structural alterations present in fried-egg SCVs could be reversed to normal *S. aureus* morphology by supplementation of the culture medium with 100 μg of thymidine per ml (data not shown). While fried-egg SCVs reverted with a frequency of 10^{-3} in Mueller-Hinton (MH) broth without thymidine, pinpoint SCVs did not grow without additional thymidine, being absolutely dependent on thymidine, and thus did not revert.

Interestingly, the morphological changes described in this

report were similar to those induced by in vitro treatment of *S. aureus* with trimethoprim (6). However, thymidine-dependent SCVs collected in our study were isolated not only from patients currently undergoing SXT therapy but also from patients who had received SXT therapy in the past but had not received it for months or years (3). To test if it is possible to induce SCVs in vitro by application of SXT to MH broth, we cultured *S. aureus* Newman in MH broth with 16/304 μg of trimethoprim-sulfamethoxazole per ml, which is above the MIC. While no SCVs were found in Newman cultured in MH broth alone, SCVs were isolated from Newman grown in MH broth with SXT. Although most SCVs reverted upon the first subculture, we were able to isolate several stable SCVs which grew as pinpoint SCVs, indicating the possibility of in vitro induction of SCVs by SXT application.

Although low concentrations of thymidine present in Columbia blood agar allow culture of thymidine-dependent SCVs (which fail to grow on most MH and chemically defined medium agars [3]), the atypical colony morphology together with the unusual Gram stain appearance may pose major identification problems to the clinical microbiologist. Therefore, thymidine-dependent SCVs might be easily missed in the routine laboratory without appreciation of these colony characteristics, resulting in erroneous species identification or even discard of these pathogens as contaminants. In view of the increasing prevalence of multiresistant MRSA, for which SXT may represent one of the few active drugs, it is important to be aware of the emergence of thymidine-dependent SCVs, especially as a result of long-term therapy necessary for chronic disease such as osteomyelitis, wound infections, or CF (3, 10). In conclusion, our data demonstrate that thymidine-dependent *S. aureus*

SCVs exhibiting unusual colony morphology and atypical Gram stain characteristics were associated with gross ultra structural changes consistent with impaired cell separation.

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