THE NATURE OF PROBLEM BACTERIA: IS RESISTANCE ENOUGH?

Staphylococcal Small Colony Variants Have Novel Mechanisms for Antibiotic Resistance

Richard A. Proctor, Barbara Kahl, Christof von Eiff, Pierre E. Vaudaux, Daniel P. Lew, and Georg Peters

From the Institute of Medical Microbiology, University of Muenster, Germany; and the Division of Infectious Diseases, Department of Medicine, Geneva University Hospital, Geneva, Switzerland; and the Departments of Medicine and Medical Microbiology/Immunology, University of Wisconsin, Madison, Wisconsin, USA

Over the past 4 years, a variant subpopulation of *Staphylococcus aureus* has been characterized that is defective in electron transport. These organisms grow slowly and are typical of the previously described small colony variants (SCVs). Indeed, many earlier papers included data that are consistent with defective respiratory activity in SCVs. We present a hypothesis that serves as biochemical basis for the development of SCVs. These variants are particularly interesting because they have been associated with very persistent infections, and they are more resistant to many antibiotics than normal *S. aureus*. Because of their slow growth, atypical colonial morphology, and unusual biochemical profile, they are easily missed or misidentified in the clinical laboratory. This is of some significance, as this subpopulation is more resistant to antibiotics than the parent population from which they arose. When an infection is particularly resistant to therapy, persists for a long period, or fails to respond to apparently adequate antimicrobial therapy, clinicians and clinical laboratory personnel should consider special efforts to search for SCVs.

Staphylococci are some of the most feared pathogens because of their ability to cause overwhelming sepsis and death. While many of these fears were alleviated with the advent of antibiotics, the emergence of staphylococci resistant to multiple antibiotics has renewed concern and led to the featuring of these organisms in both the lay [1, 2] and biomedical press [3–7] as a new threat in the postantibiotic era. Because vancomycin is the only drug with dependable activity against methicillin-resistant strains of *Staphylococcus aureus* [4, 6, 7], the emergence of some strains of *S. aureus* with intermediate resistance to vancomycin has heightened the fears of a pan antibiotic-resistant strain [3]. These concerns are reinforced by the existence of a number of species of gram-positive cocci that are vancomycin resistant, including some strains of coagulase-negative staphylococci [5]. A number of recent articles have addressed the problem of staphylococcal antibiotic resistance from the standpoint of classic forms of resistance, e.g., enzymatic degradation (production of β-lactamases and aminoglycoside modifying enzymes), altered penicillin-binding proteins (methicillin resistance), and enhanced export (quinolones) [4, 6–9]; however, staphylococci may have additional mechanisms for resisting therapy that extend beyond these classic mechanisms. In this review, we will present a hypothesis concerning a biochemical basis for the development of ''phenotypic resistance,'' wherein a subpopulation of organisms occurs as slow-growing variants that are more resistant than the original population to many commonly used antibiotics.

Prolonged Asymptomatic Persistence

Even when *S. aureus* is exquisitely susceptible to penicillin G, antibiotic therapy must be prolonged, and complete eradication of the organisms is not guaranteed [10]. For example, several years ago we saw a man who had a recurrence of a *S. aureus* infection that occurred following a shrapnel injury sustained in World War II. His infection was reactivated when he developed a hematoma following an ankle sprain. He had no other predisposing factors such as being immunocomprised because of vascular insufficiency, diabetes, rheumatoid arthritis, or prednisone therapy. A symptom-free interval of >40 years had passed since he was apparently cured by treatment with penicillin G, and the organism was susceptible to 0.01 μg of penicillin G/mL. His nose was colonized with *S. aureus* that

Financial support: This work was supported by funds from the Department of Medicine Research Committee, Medical School Research Committee, and the Graduate School at the University of Wisconsin; by the Wisconsin Affiliate of the American Heart Association; by the Center for Interdisciplinary Clinical Research of the Medical Faculty at the Münster University; and by the Swiss National Foundation for Scientific Research (Grant #3200-045810.95/1). R. A. P. was supported by an Alexander von Humboldt Distinguished Researcher Award through the Alexander von Humboldt Foundation, Bonn, Germany.

Reprints or correspondence: Dr. Richard A. Proctor, 407 SMI, Departments of Medicine and Medical Microbiology/Immunology, University of Wisconsin Medical School, Madison, Wisconsin 53705.

Clinical Infectious Diseases 1998;27(Suppl 1):S68–74

1058–4838/98/2702–0010$03.00

were β-lactamase producers. His ankle apparently was still infected with a staphylococcal strain from the 1940s.

This case raises several questions. Where did the bacteria persist? What mechanisms allowed them to resist antibiotic therapy? What were the signaling mechanisms for the bacteria to become reactivated? Why must staphylococcal infections be treated for so much longer than other gram-positive bacterial infections to achieve a cure? How can such a virulent organism persist without causing symptoms for so many years? Answers to some of these questions may relate to more recent data on variant forms of staphylococci that may resist antibiotic therapy by ''nonclassic'' resistance mechanisms.

Small Colony Variants

Small colony variant (SCV) subpopulations of staphylococci have been described for many decades [11–53]. Aminoglycosides and penicillins were recognized as having higher MICs for some of these organisms [15, 21–24, 26, 27, 29–41, 54–60], but the mechanism for their increased resistance to antibiotics was unclear, and they were described as a heterogeneous population. Recently, we have proposed that most clinical SCVs can be tied together by a common thread, which is alterations in electron transport [57, 58]. A decrease in electron transport activity may also account for their resistance to several antibiotics as well as provide a mechanism for persisting within host tissues [57, 58].

Many SCVs are menadione or hemin auxotrophs. Staphylococcal SCVs are defined by colony size 10 times smaller than the parent strain [15, 21, 43, 57]. This small size of many clinical and laboratory SCVs on tryptic soy or Mueller-Hinton agar is often due to auxotrophy for thiamine, menadione, or hemin [16–18, 23, 24, 29, 42, 46, 49–51, 53–57, 58–61]. When the medium is supplemented with 1 μg/mL of these compounds, SCVs grow as rapidly as the parent strains [54–60]. The large majority of clinical isolates from our studies and from studies in the literature are auxotrophic for one of these three substances.

Electron Transport and SCVs

Menadione, hemin, and thiamine are required for biosynthesis of electron transport chain components. Menadione is isoprenylated to form menaquinone, the acceptor of electrons from nicotinamide adenine dinucleotide (NADH)/flavin adenine dinucleotide ($FADH_2$) in the electron transport chain. Hemin is required for the biosynthesis of cytochromes, which accept electrons from menaquinone and complete the electron transport chain. Thiamine is required for menadione biosynthesis [61]; hence, thiamine auxotrophs are also menadione auxotrophs. Many previous reports also noted decreased respiratory or dye reducing activity in staphylococcal SCVs [16, 17, 23, 24, 29, 41, 49, 62], which is also consistent with reduced electron transport activity. Therefore, mutants in menadione or hemin biosynthesis will be auxotrophs and will be deficient in electron transport.

Reduced electron transport results in multiple phenotypic changes. Interruption of electron transport results in a decreased electrochemical gradient and reduced quantities of adenosine triphosphate (ATP). Because ATP is used in so many cellular reactions, the phenotype of menadione or hemin auxotrophic *S. aureus* SCVs differs markedly from that of the parent strain. At 18 hours, the colonies are very small, nonpigmented, and nonhemolytic [54–59]. SCVs have a weak coagulase reaction which may take 18–24 hours to become positive. Large amounts of ATP are required for cell wall biosynthesis; thus the slow growth leads to small colonies, and electron transport is directly linked to the biosynthesis of carotenoid pigments, rendering the colonies nonpigmented. The limited hemolysis and slow coagulase reaction is in part related to decreased amino acid biosynthesis and uptake (both energy-dependent processes); consequently, the biosynthesis of nonessential proteins for survival appears to be reduced. Biochemical reactions tested in the clinical microbiology laboratory show use of glucose and fructose but not mannitol or other sugars. Thus, SCVs are often difficult to recognize as staphylococci, and they may be misidentified by clinical laboratory personnel [54].

We have very recently reported a site-directed *hemB* mutant whose phenotype is that of a typical clinical hemin auxotrophic SCV [59]. The mutant organism grows slowly, is more resistant to antibiotics than the parent strain, shows reduced coagulase and hemolytic activity, and is able to persist within cultured endothelial cells; in addition, all of the phenotypic changes can be reversed by complementation with *hemB* or by the addition of hemin to the growth medium.

Staphylococcal SCVs are more resistant to antibiotics than the parent strain. An electrochemical gradient is required for the import of positively charged molecules, such as aminoglycosides and some lantibiotics, into the bacterium [55, 63]. Lantibiotics are positively charged, lathionine-containing, antibacterial peptides that are produced by a number of gram-positive bacterial species [64]. *S. aureus* SCVs are more resistant to lantibiotics and gentamicin [55, 63]. In addition, the slow growth of these organisms reduces the effectiveness of cell wall–active antibiotics such as β-lactams [21–23, 30, 34–36, 40]. With menadione or hemin auxotrophic SCVs, the resistance to antibiotics can be reversed by supplying the auxotrophic substance [54–59, 63].

SCVs and the Clinical Laboratory

SCVs present a challenge for susceptibility testing because the clinical isolates are often a mixed population of parent strains and SCVs [54, 65, 66]. Even a small percentage (e.g., 0.1%) of normally growing organisms will rapidly replace the SCVs in liquid medium in an overnight culture because the doubling time of normal *S. aureus* is ~20 minutes, whereas SCVs double in ~180 minutes; hence, the SCVs may be over-

grown to such an extent that they may not be included in the inoculum used for susceptibility testing. Furthermore, SCVs are not always stable once removed from the host, thus providing another possibility for not including them in susceptibility testing. On solid medium, the normal colonies are apparent within 18 hours, a time when SCVs are difficult to see. Consequently, these strains are again easily missed. Because SCVs and normal staphylococci have the same appearance on gram stain, there is no reason to suspect a mixed culture (normal and variant-growth types). With only the wild-type strain being tested, the more resistant SCV strain is not included in the testing, and a major reporting error will occur.

The slow growth of SCVs makes testing by disk diffusion or by automated overnight methods invalid because the colonies may be too small to be seen on agar or detected by optical density measurements in automated systems. Testing must be performed by broth or agar dilution MIC methods.

SCVs, Biopolymers, and Resistance

S. aureus SCVs are not only more resistant when tested by classic methods, but they are also much more resistant to antibiotics when adherent to a biopolymer surface [67]. In one study, during logarithmic growth phase in broth or on solid phase (i.e., adherent to a polymer surface), both parent and SCV strains were susceptible to oxacillin, vancomycin, and fleroxacin; however, two orders of magnitude more of the SCVs than the parent strain remained alive (1–2 vs. 3–4 $\log_{10}$ reduction when antibiotics were at eight times the MIC) [67]. In contrast, stationary phase SCVs were much more resistant to killing with only a 10-fold reduction when in fluid phase and almost no reduction when SCVs were grown on a solid phase. Of the three antibiotics tested, fleroxacin reduced adherent, stationary phase SCVs almost one $\log_{10}$, whereas the other antibiotics had essentially no effect on colony counts [67]. Thus, while normal bacteria show a modest decrease in susceptibility when adherent, *S. aureus* SCVs demonstrate a dramatic reduction in susceptibility once they are attached to a surface and have reached stationary phase, such that these adherent organisms have shown nearly complete resistance to the antibiotics that have been tested.

Staphylococcal SCVs may persist and resist antibiotic therapy in patients because they can survive within cultured endothelial cells. The reduced production of α-toxin by *S. aureus* SCVs allows these bacteria to persist within mammalian cells [55, 59]. These variants are phagocytized by cultured endothelial cells, yet they do not damage the monolayer [55, 59]. This can be related to the reduced α-toxin production by *S. aureus* SCVs. Similarly, site-directed mutants for α-toxin also persist within cultured endothelial cells [68]. This location within host cells will shield these variants from host defenses and from antibiotics that have a limited ability to cross the plasma cell membrane.

Antibiotics are not particularly effective against SCVs within endothelial cells. The activity of antibiotics against an *S. aureus* menadione auxotrophic SCV strain, JB-1 [55], was tested by adding parent or SCV strains to cultured bovine endothelial cells [55]. After 3.5 hours, the monolayers were washed, and lysostaphin was added to kill the extracellular bacteria. Antibiotics were then added at 0–16 times the MIC, and the cells were then cultured for another 24 hours in a CO_2 incubator. At 24 hours, a trypsin-EDTA solution was added to loosen the cells, and the cells were washed in phosphate buffered saline. Bacteria were released by lysis of the endothelial cells with distilled water plus gentle sonication, and plate counts were determined on trypticase soy agar. We found that clarithromycin, clindamycin, ciprofloxacin, and novobiocin plus rifampin were ineffective at killing intracellular SCVs (R. A. Proctor et al., unpublished observations). Methicillin at 16 times the MIC was marginally effective against the parent strain in that it reduced the number of colonies about one order of magnitude, but it had little effect on the SCVs. Combining menadione at 2 μg/mL with methicillin increased the killing of *S. aureus* JB-1 so that it was killed as well as the parent strain.

Trimethoprim-sulfamethoxazole was the most effective antibiotic, as it usually lowered counts of JB-1 two orders of magnitude, but rifampin or ciprofloxacin did not further enhance the killing. None of the antibiotics were able to sterilize the infected monolayer. The finding that a number of antibiotics that penetrate the plasma membrane were ineffective against *S. aureus* SCVs was disappointing, but it is consistent with the difficulty in eradicating these organisms in patients [54].

Recovery of SCVs from Patients

Antibiotic therapy can produce SCVs. *S. aureus* SCVs can regularly be harvested when broth cultures are exposed to gentamicin or other aminoglycosides [27, 55]. These variants are like the clinical isolates in that they are predominantly menadione and hemin auxotrophs [16, 17, 23, 24, 29, 46, 50, 51, 54, 57]. Anecdotal reports have associated SCVs with the use of antibiotics, often for prolonged periods, with the recovery of *S. aureus* SCVs [15, 21–24, 26, 29–41, 54–60, 65, 66].

The frequency of *S. aureus* SCVs among clinical isolates has not been established by prospective studies, except for the one blood culture study where stable SCVs were recovered from 1% of patients [18]. Because SCVs can be selected by chronic antibiotic exposure, we looked for their presence in patients chronically exposed to aminoglycosides.

To prospectively study a group of patients who frequently have staphylococcal infections and receive large quantities of aminoglycosides, we looked at consecutive cultures for patients with cystic fibrosis over a 33-month period [65]. We chose patients with cystic fibrosis because *S. aureus* is the second most common pathogen isolated from these patients' sputum specimens [69], and they receive large quantities of antibiotics. *S. aureus* SCVs were recovered from >25% of patients with

cystic fibrosis [65]. A total of 78 SCV strains were recovered from these patients and found to be menadione, hemin, and/or thymidine auxotrophs. Thymidine auxotrophy was the most frequent (53% of strains), and double auxotrophy with thymidine plus hemin auxotrophy was also seen frequently (32%). Overall, menadione or hemin auxotrophy were present in 47% of SCVs. The thymidine auxotrophy probably relates to the frequent and prolonged use of trimethoprim-sulfamethoxazole as prophylaxis in these patients. The MICs of gentamicin for the SCVs were higher, and these organisms were resistant to trimethoprim-sulfamethoxazole.

As has been found in other clinical cases and animal models where SCVs are recovered, the SCVs were able to persist for a long period in patients with cystic fibrosis [65]. A clinical hemin auxotrophic SCV isolate from a patient with cystic fibrosis was able to persist within cultured endothelial cells [65]. When the SCVs were found in mixed cultures with normally growing *S. aureus,* either continuously or intermittently, the normal strain and SCVs proved to be clonal by pulsed-field gel electrophoretic analysis.

Some care must be exercised in the selection of media for susceptibility testing when thymidine auxotrophs are recovered because we found that some batches of Mueller-Hinton agar contained concentrations of thymidine that were high enough to cause reversion of an SCV to the parental phenotype, even though these batches were claimed to be ''low thymidine.'' Organisms with the normal phenotype are more susceptible to sulfonamide antibiotics [65]. In addition, the slow growth of SCVs might lead one to consider the use of a more enriched medium for susceptibility testing (e.g., brain-heart infusion medium; however, this medium contains relatively large amounts of menadione, which will relieve the SCV phenotype of menadione auxotrophs).

We monitored *S. aureus* isolates from patients with osteomyelitis [66]. This study included four patients with osteomyelitis who had recurrent disease following surgical placement of gentamicin beads in the infected bones. This represents another group of patients with chronic exposure to aminoglycosides. By comparison, no SCVs were recovered from the 10 patients with *S. aureus* osteomyelitis who did not receive gentamicin beads, and none of these 10 patients had recurrent disease. The SCVs were hemin or menadione auxotrophs, and they showed resistance to gentamicin [66]. This study does not necessarily indicate that gentamicin beads will not provide a net positive value in the treatment of osteomyelitis because more patients may be cured with gentamicin beads than without them. However, surgical placement of gentamicin beads essentially replicates the broth culture method for production of SCVs, where large numbers of *S. aureus* are exposed to gentamicin, and SCVs are produced with great regularity [27, 55]. Thus, if osteomyelitis recurs in patients after the use of gentamicin beads, clinicians should search especially hard for the presence of SCVs, as they are likely to be more resistant to antibiotic therapy.

The intracellular milieu of mammalian cells can also select for SCVs. When wild-type *S. aureus* strains were allowed to persist within cultured endothelial cells for 72 hours, SCVs were harvested at a surprisingly high rate [56]. The SCVs obtained from cocultivating endothelial cells with *S. aureus* were predominantly menadione auxotrophs, and they were more resistant to gentamicin than the normal strain, even though they had never been exposed to aminoglycosides. The rate of selection in host tissues exceeded the rate of selection that can be achieved by exposing *S. aureus* to positively charged antibiotics in vitro [56]. These observations help to explain the isolation of *S. aureus* SCVs from patients who have not received antibiotics [15, 17, 21, 33]. While the mechanism for SCV production within the intracellular milieu is unknown, we have found that a number of positively charged, bactericidal compounds (i.e., lantibiotics, gentamicin, and protamine) are able to select for SCVs (unpublished observations and [63]). Thus, SCVs can emerge and be more resistant than the parent strain to antibiotics, even in the absence of antibiotic pressure.

Potential Therapeutic Advantages to Controlling SCV Formation

While the association of electron transport defects in clinical SCV isolates occurs quite frequently, the factors that allow organisms to switch from one phenotype to another are unknown. Formation of SCVs at a relatively high rate may offer a survival advantage because they can persist intracellularly and resist antibiotics. If clinicians were able to control when the bacteria grow rapidly and when they form SCVs, this might be therapeutically valuable. For example, reversing the SCV phenotype by adding menadione enhances susceptibility to antibiotics and decreases the ability of the organisms to reside within cultured cells. Perhaps adding vitamin K (the isoprenylated form of menadione) to standard antibiotic regimens might reduce the rate of the development of SCVs or prevent the establishment of SCVs as chronic intracellular pathogens. This might be particularly valuable in the treatment of endovascular and bone infections, for which therapy currently must be very prolonged to assure eradication [10].

Development of the SCV phenotype may not always be a clinically disadvantageous situation. SCVs cause less tissue damage than do normally growing staphylococci because of their low production of exotoxins, and the presence of SCVs would be much preferable to that of a highly virulent pathogen in patients with acute bacteremia. The use of drugs that interfered with electron transport might be particularly valuable as a short-term measure if such drugs could rapidly turn off toxin production. Drugs have been developed that interfere with shikimate metabolism in *Escherichia coli,* and shikimate is a biosynthetic precursor of menadione [70]. We have recently examined the effects of an electron transport inhibitor on *S. aureus* and found that α-toxin production and damage to cultured endothelial cells was reduced (R. A. Proctor, unpublished ob-

servations). Drugs such as these would not have to be bactericidal and would represent a new class of anti–virulence factor drug. Allowing clinicians to control when *S. aureus* isolates grow rapidly and when they are SCVs might reduce the mortality associated with acute staphylococcal sepsis, reduce the length of therapy, and increase the rate of clearance of bacteria from persistently infected patients.

SCVs in Other Species

The emphasis of this review has been on *S. aureus;* however, coagulase-negative staphylococci have been reported to form SCVs, and these strains have been particularly difficult to clear from infected heart valves [35, 71]. Furthermore, a wide variety of other species are known to form SCVs (e.g., *Pseudomonas aeruginosa, Salmonella typhimurium, Shigella* species, *Brucella abortus, E. coli, Lactobacillus acidophilus, Serratia marcescens,* and *Neisseria gonorrhoeae*) [72–80]. Many of the SCVs from these species are described as respiratory deficient and/or have been characterized as deficient in electron transport [73–76, 79, 80]. These SCVs also display increased resistance to aminoglycosides.

SCVs and Spores

In many regards, SCVs can be thought of as spore equivalents of nonsporulating bacteria. Their reduced rate of growth and uptake of substances from the environment is very slow; hence, SCVs show some similarity to spores. One of the factors that favors both sporulation and SCV formation is limited nutrients in the medium, such as aged cultures, which favors both processes [33, 47, 52, 74, 80]. When *B. subtilis* sporulates, one of the early steps is a burst of menadione biosynthesis, which is followed by a complete shutdown of menadione production as sporulation proceeds [81, 82]. If exogenous menadione is added to the medium, then *Bacillus cereus* will not sporulate even when all of the conditions favor sporulation because menadione stimulates respiration and activation of spores [83]. Consequently, the biochemical strategy used during sporulation by *Bacillus* species is quite similar to the formation of menadione-auxotrophic *S. aureus* SCVs. Perhaps the ability to form SCVs is an evolutionary strategy for survival during times of limited resources and has now been adapted by pathogenic bacteria to survive within the host when the conditions are unfavorable, e.g., when antibacterial peptides and antibiotics are present. Clearly, the ability to form subpopulations of bacteria is of value to the microorganism as it offers additional possibilities for survival. The observation that this behavior is exhibited by many species suggests that it is important for bacterial survival.

Summary

Subpopulations of bacteria present several challenges to clinicians and clinical laboratory personnel. With *S. aureus* SCVs, the first challenge is finding and correctly identifying them as *S. aureus*. Next, susceptibility testing must be done by agar dilution or by broth MIC methods because the slow growth of the organisms will not allow accurate determinations by the disk diffusion method or automated methods. Susceptibility testing must be conducted with medium that has low levels of thymidine and menadione. The laboratory should be particularly alert for *S. aureus* SCVs when samples come from patients who have received long-term therapy or when an infectious disease has been unusually persistent, antibiotic resistant, or recurrent. However, the host milieu can select for *S. aureus* SCVs, which means that patients may have SCVs even when they have not received antibiotic therapy. The presence of subpopulations of bacteria that are able to persist within host cells also reemphasizes that the determination of MICs has major limitations for predicting efficacy of a drug. Finally, when these variant bacteria are established in stationary phase and adherent to surfaces, they are exceptionally resistant to antibiotics.

References

1. Revenge of the killer microbes. Time **1994**;Nov 12:58–65.
2. Gorman C. Germ warfare. A drug-resistant staph strain has doctors on edge. Time **1997**;Sept 1:65.
3. Hiramatsu K, Hanaki H, Ino T, et al. Methicillin resistant *Staphylococcus aureus* clinical strain with reduced susceptibility to vancomycin. J Antimicrob Chemother **1997** (in press).
4. Tomasz A. Multiple-resistant-pathogenic bacteria. A report on the Rockefeller University workshop. N Engl J Med **1994**;330:1247–51.
5. Schwalbe RS, Stapleton JT, Gilligan PH. Emergence of vancomycin-resistant coagulase-negative staphylococci. N Engl J Med **1987**;316:927–31.
6. Peters G, Becker K. Epidemiology, control, and treatment of methicillin-resistant *Staphylococcus aureus.* Drugs **1996**;52 (suppl 2):50–4.
7. Mulligan M, Murray-Leisure KA, Ribner BS, et al. *Staphylococcus aureus:* a consensus review on the microbiology, pathogenesis and epidemiology with implications for prevention and management. Am J Med **1993**;94:313–28.
8. Kaatz GW, Seo SM, Ruble CA. Mechanisms of fluoroquinolone resistance in *Staphylococcus aureus.* J Infect Dis **1991**;163:1080–6.
9. Archer GL, Climo MW. Antimicrobial susceptibility of coagulase-negative staphylococci. Antimicrob Agents Chemother **1994**;38:2231–7.
10. Waldvogel FA. *Staphylococcus aureus* (including toxic shock syndrome). In: Mandell GL, Bennett JE, Dolan R, eds. Mandell, Douglas and Bennett's principles and practice of infectious diseases. New York: Churchill Livingstone, **1995**:1754–77.
11. Hale JH. Studies on *Staphylococcus* mutation: a naturally occurring ''G'' gonidial variant and its carbon dioxide requirements. Br J Exp Pathol **1951**;32:307–13.
12. Sherris JC. Two small colony variants of *Staphylococcus aureus* isolated in pure culture from closed infected lesions and their carbon dioxide requirements. J Clin Pathol **1952**;5:354–5.
13. Goudie JG, Goudie RB. Recurrent infection by a stable dwarf-colony variant of *Staphylococcus aureus.* J Clin Pathol **1955**;8:284–7.
14. Thomas MEM, Cowlard JH. Studies on a CO_2-dependent *Staphylococcus.* J Clin Pathol **1955**;8:288–91.
15. Quie PG. Microcolonies (G variants) of *Staphylococcus aureus.* Yale J Biol Med **1969**;41:394–403.
16. Kaplan M, Dye WE. Growth requirements of some small-colony-forming variants of *Staphylococcus aureus.* J Clin Microbiol **1976**;4:343–8.

17. Borderon E, Horodniceanu T. Mutants déficients à colonies naines de *Staphylococcus:* étude de trois souches isolées chez des malades porteurs d'ostéo synthèses. Annals Microbiology Institute Pasteur **1976**; 127A:503–14.
18. Acar JF, Goldstein FN, Lagrange P. Human infections caused by thiamine or menadione-requiring *Staphylococcus aureus.* J Clin Microbiol **1978**; 8:142–7.
19. Spagna VA, Fass RJ, Prior RB, Slama TG. Report of a case of bacterial sepsis caused by a naturally occurring variant form of *Staphylococcus aureus* [letter]. J Infect Dis **1978**;138:277–8.
20. Wise RI. Small colonies (G variants) of staphylococci: isolation from cultures and infections. Ann NY Acad Sci **1956**;65:169–74.
21. Wise RI, Spink WW. The influence of antibiotics on the origin of small colony (G variants) of *Micrococcus pyogenes* var. *aureus.* J Clin Invest **1954**;33:1611–22.
22. Nydahl BC, Hall WL. The treatment of staphylococcal infection with nafcillin with a discussion of staphylococcal nephritis. Ann Intern Med **1965**;63:27–43.
23. Devriese LA. Hemin-dependent mutants isolated from methicillin-resistant *Staphylococcus aureus* strains. Antonie Van Leeuwenhoek Journal of Microbiology and Serology **1973**;39:33–40.
24. Yegian D, Gallo G, Toll MW. Kanamycin resistant staphylococcus mutants requiring hemin for growth. J Bacteriol **1959**;78:10–2.
25. Browning CH, Adamson HS. Stable dwarf-colony forms produced by *Staphylococcus pyogenes.* J Pathol Bacteriol **1950**;62:499–500.
26. Pelletier LL Jr, Richardson M, Feist M. Virulent gentamicin-induced small colony variants of *Staphylococcus aureus.* J Lab Clin Med **1979**;94: 324–34.
27. Musher DM, Baughn RE, Templeton GB, Minuth JN. Emergence of variant forms of *Staphylococcus aureus* after exposure to gentamicin and infectivity of the variants in experimental animals. J Infect Dis **1977**; 136:360–9.
28. Hale JH. Studies on staphylococcal mutation: characterization of the ''G'' (gonidial) variant and factors concerned in its production. Br J Exp Pathol **1947**;28:202–10.
29. Sasarman A, Surdeanu M, Portelance V, Dobardzic R, Sorrea S. Classification of vitamin K deficient mutants of *Staphylococcus aureus.* J Gen Microbiol **1971**;65:125–30.
30. Schnitzer RJ, Canagni LJ, Back M. Resistance of small colony variants (G forms) of a *Staphylococcus* toward the bacteriostatic activity of penicillin. Proc Soc Exp Biol Med **1943**;53:75–8.
31. Lacy RW, Mitchell AAB. Gentamicin-resistant *Staphylococcus aureus.* Lancet **1969**;2:1425–6.
32. Lacy RW. Dwarf-colony variants of *Staphylococcus aureus* resistant to aminoglycoside antibiotics and to a fatty acid. J Med Microbiol **1969**; 2:187–97.
33. Hoffstadt RE, Youmans GP. *Staphylococcus aureus:* dissociation and its relation to infection and to immunity. J Infect Dis **1932**;51:216–42.
34. Bulger RJ. A methicillin-resistant strain of *Staphylococcus aureus.* Clinical and laboratory experience. Ann Intern Med **1967**;67:81–9.
35. Baddour LM, Christensen GD, Lowrance JH, Simpson WA. Pathogenesis of experimental endocarditis. Rev Infect Dis **1989**;11:452–63.
36. Youmans GP, Williston EH, Simon M. Production of small colony variants of *Staphylococcus aureus* by the action of penicillin. Proc Soc Exp Biol Med **1945**;58:56–7.
37. Wilson SG, Sanders CC. Selection and characterization of strains of *Staphylococcus aureus* displaying unusual resistance to aminoglycosides. Antimicrob Agents Chemother **1976**;10:519–25.
38. Barbour RGH. Small colony variants (''G'' forms) produced by *Staphylococcus pyogenes* during the development of resistance to streptomycin. Aust J Exp Biol Med Sci **1950**;28:411–20.
39. Miller MH, Wexler MA, Stiegbigel NH. Single and combination antibiotic therapy of *Staphylococcus aureus* experimental endocarditis: emergence of gentamicin mutants. Antimicrob Agents Chemother **1978**;14: 336–43.
40. Chambers HF, Miller MM. Emergence of resistance to cephalothin and gentamicin during combination therapy for methicillin-resistant *Staphylococcus aureus* endocarditis in rabbits. J Infect Dis **1987**;155:581–5.
41. Chin YM, Harmon SA. Genetic studies of kanamycin resistance in *Staphylococcus aureus.* Jpn J Microbiol **1971**;15:417–23.
42. Sompolinsky D, Cohen M, Ziv G. Epidemiological studies on thiamine-less dwarf-colony variants of *Staphylococcus aureus* as etiologic agents of bovine mastitis. Infect Immun **1974**;9:217–28.
43. Kolle W, Hetsch H. Die experimentelle bacteriologie und die infectionskrankheiten mit besonderer berueckichtigung der immunitaetslehre. In: Urban, Schwarzenberg, eds. Medizinioche mikrobiologie Vol. 1. 3rd edition. Berlin: Springer Verlag, **1913**:21–8.
44. Burke V, Swartz H, Klise KS. Morphological life cycle of a staphylococcus-like organism and modification of the cycle. J Bacteriol **1943**;45: 415–30.
45. Youmans GP, Delves E. The effect of inorganic salts on the production of small colony variants by *Staphylococcus aureus.* J Bacteriol **1942**; 44:127–36.
46. Sasarman A, Surdeanu M, Sabados J, Greceanu V, Horodniceanu T. Menaphthone requiring mutants of *Staphylococcus aureus.* Rev Can Biol **1968**;23:333–40.
47. Swingle EL. Studies on a small variant of *Staphylococcus aureus.* J Bacteriol **1935**;29:467–90.
48. Slifkin M, Merkow LP, Kreuzberger SA, Engwall C, Pardo M. Characterization of CO_2 dependent microcolony variants of *Staphylococcus aureus.* Am J Clin Pathol **1971**;56:584–92.
49. Sompolinsky D, Geller ZE, Segal S. Metabolic disorders in thiamine-less dwarf strains of *Staphylococcus aureus.* J Gen Microbiol **1967**;48: 205–13.
50. Weinberg ED. Vitamin requirements of dwarf colony variants of bacteria. J Infect Dis **1950**;87:299–306.
51. Jensen J. Biosynthesis of hematin compounds in a hemin requiring strain of *Micrococcus pyogenes* var. *aureus.* J Bacteriol **1957**;73:324–33.
52. Swingle EL. Studies on small colony variants of *Staphylococcus aureus.* Proc Soc Exp Biol Med **1934**;31:891–3.
53. Youmans GP. Production of small colony variants of *Staphylococcus aureus.* Proc Soc Exp Biol Med **1937**;36:94–8.
54. Proctor RA, van Langevelde P, Kristjansson M, Maslow JN, Arbeit RD. Persistent and relapsing infections associated with small colony variants of *Staphylococcus aureus.* Clin Infect Dis **1995**;20:95–102.
55. Balwit JM, van Langevelde P, Vann JM, Proctor RA. Gentamicin-resistant menadione and hemin auxotrophic *Staphylococcus aureus* persist within cultured endothelial cells. J Infect Dis **1994**;170:1033–7.
56. Vesga O, Groeschel MC, Otten MF, Brar DW, Vann JM, Proctor RA. *Staphylococcus aureus* small colony variants are induced by the endothelial cell intracellular milieu. J Infect Dis **1996**;173:739–42.
57. Proctor A. Microbial pathogenic factors: small colony variants. In: Bisno AL, Waldvogel FA, eds. Infections associated with indwelling medical devices. 2nd ed. Washington, DC: American Society for Microbiology, **1994**:77–90.
58. Proctor RA, Balwit JM, Vesga O. Variant subpopulations of *Staphylococcus aureus* can cause persistent and recurrent infections. Infect Agents Dis **1994**;3:302–12.
59. von Eiff C, Heilmann C, Proctor RA, Woltz C, Peters G, Götz F. A site directed *Staphylococcus aureus hemB* mutant is a small clony variant which persists intracellularly. J Bacteriol **1997**;179:4706–12.
60. Lewis LA, Li K, Bharosay M, et al. Characterization of gentamicin-resistant respiratory-deficient (Res^-) variant strains of *Staphylococcus aureus.* Microbiol Immunol **1990**;34:587–605.
61. Bentley R, Meganathan R. Biosynthesis of vitamin K (menaquinone) in bacteria. Microbiol Rev **1982**;46:241–80.
62. Sasarman A, Sanderson KE, Surdeanu M, Sonea S. Hemin-deficient mutants of *Salmonella typhimurium.* J Bacteriol **1970**;102:531–6.
63. Koo SP, Bayer AS, Sahl H-G, Proctor RA, Yeaman MR. Staphylocidal action of thrombin-induced platelet microbicidal protein (tPMP) is not

soley dependent on transmembrane potential ($\Delta\Psi$). Infect Immun **1996**; 64:1070–4.

64. Sahl H-G, Jack RW, Bierbaum G. Biosynthesis and biological activities of lantibiotics with unique post-translational modifications. Eur J Biochem **1995**;230:827–53.
65. Kahl B, Herrmann M, Schulze-Everding A, et al. Persistent infection with small colony variants of *Staphylococcus aureus* in patients with cystic fibrosis. J Infect Dis **1997**;177:1023–9.
66. von Eiff C, Bettin D, Proctor RA, et al. Recovery of small colony variants of *Staphylococcus aureus* following gentamicin bead placement for osteomyelitis. Clin Infect Dis **1997**;25:1250–1.
67. Chuard C, Vaudaux PE, Proctor RA, Lew DP. Decreased susceptibility to antibiotic killing of small colony variants of *Staphylococcus aureus* in fluid phase and on fibronectin-coated surfaces. J Antimicrob Chemother **1997**;39:603–8.
68. Vann JM, Proctor RA. Cytotoxic effects of ingested *Staphylococcus aureus* on bovine endothelial cells: role of *S. aureus* α-hemolysin. Microb Pathog **1988**;4:443–53.
69. Gilligan PH. Microbiology of airway disease in patients with cystic fibrosis. Clin Microbial Rev **1991**;4:35–51.
70. Ewart CDC, Jude DA, Thain JL, Nichols WW. Frequency and mechanism of resistance to antibacterial action of ZM 240401, (6S)-6-fluoro-shikimic acid. Antimicrob Agents Chemother **1995**;39:87–93.
71. Baddour LM, Simpson WA, Weems JJ Jr, Hill MM, Christensen GD. Phenotypic selection of small-colony variant forms of *Staphylococcus epidermidis* in the rat model of endocarditis. J Infect Dis **1988**;157: 757–63.
72. Goetz MB, Proctor RA, Gerber AU, Craig WA. Complement and neutrophil mediated killing of gentamicin-resistant small colony variants of *Pseudomonas aeruginosa* [abstract]. Clin Res **1981**;29:728A.
73. Bayer AS, Norman DC, Kim KS. Characterization of *Pseudomonas aeruginosa* isolated during unsuccessful therapy of experimental endocarditis. Antimicrob Agents Chemother **1987**;31:70–5.
74. Chinn BD. Characteristics of small colony variants with special reference to *Shigella paradysenteriae sonne.* J Infect Dis **1936**;59:137–51.
75. Colwell CA. Small colony variants of *Escherichia coli.* J Bacteriol **1946**; 52:417–22.
76. Huddleson IF, Baltzer B. The characteristics and dissociation pattern of type G (micro-colony type) of *Brucella abortus.* In: Studies in brucellosis, III. A series of five papers. East Lansing, Michigan: Michigan State College, **1952**:64–83.
77. Kopeleff N. Dissociation and filtration of *Lactobacillus acidopohilus.* J Infect Dis **1934**;55:368–89.
78. Morton HE, Shoemaker J. The identification of *Neisseria gonorrhoeae* by means of bacterial variation and the detection of small colony forms in clinical material. J Bacteriol **1945**;50:585–90.
79. Sasarman A, Horodniceanu T. Locus determining normal colony formation on the chromosome of *Escherichia coli* K12. J Bacteriol **1967**;94: 1268–9.
80. Sasarman A, Sanderson KE, Surdeanu M, Sonea S. Hemin-deficient mutants of *Salmonella typhimurium.* J Bacteriol **1970**;102:531–6.
81. Farrand SK, Taber HW. Changes in menaquinone concentration during growth and early sporulation in *Bacillus subtilis.* J Bacteriol **1974**;117: 324–6.
82. Hederstedt L. The Krebs citric acid cycle. In: Sonenshein AL, Hoch JA, Losick R, eds. *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. Washington, DC: American Society for Microbiology, **1993**:181–97.
83. Escamilla JE, Barquera B, Ramírez R, García-Horsman A, Arenal PD. Role of menaquinone in inactivation and activation of the *Bacillus cereus* forespore respiratory system. J Bacteriol **1998**;170:5908–12.