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Decreased susceptibility to antibiotic killing of a stable small colony variant of *Staphylococcus aureus* in fluid phase and on fibronectin-coated surfaces

Christian Chuard†, Pierre E. Vaudaux*, Richard A. Proctor and Daniel P. Lew

Division of Infectious Diseases, University Hospital, Geneva, Switzerland and Department of Medical Microbiology and Immunology, University of Wisconsin, Madison, WI, USA

The frequency of small colony variants of staphylococci associated with persistent, antibiotic resistant and relapsing infections is probably underestimated. These variants demonstrate decreased metabolism, leading to slow growth, increased resistance to cell-wall-active antibiotics, and decreased uptake of aminoglycoside antibiotics. This altered phenotype arises from defects in menadione and haemin biosynthesis resulting in impaired electron transport and decreased ATP concentrations. The recent acquisition of a stable small colony variant (SCV strain JB1), generated from strain 6850 of *Staphylococcus aureus*, allowed us to study the susceptibilities to antibiotic killing of parent and variant strains. Because differences in susceptibilities have been found between unattached and surface-adherent organisms, we tested both strains in solid and fluid-phase assays. Suspensions of SCV strain JB1 exposed to 8 x MIC of either oxacillin, vancomycin or fleroxacin, exhibited lower reductions in viable counts than the parent strain 6850, especially when high bacterial concentrations (1–2 x 10^7 cfu/mL) of either strain were tested. Susceptibility to antibiotic killing of bacteria attached to fibronectin-coated coverslips was markedly influenced by their growing or non-growing state on the surface. In the latter condition, surface-bound SCV organisms were highly resistant to the bactericidal action of oxacillin or vancomycin in contrast to the parental strain which was normally eliminated by each antimicrobial agent. In conclusion, the decreased susceptibility of the stable SCV strain of *S. aureus* to bactericidal concentrations of antibiotics may help to explain the persistence of such organisms in chronic infections.

Introduction

Staphylococcal small colony variants (SCVs) have been isolated from clinical specimens[1–10] and found in association with foreign body infections.[1,5,11–13] Recently, *Staphylococcus aureus* SCVs have been found to cause very persistent and antibiotic-resistant infections.[10,14] The ability to persist in patients is probably related to the ability of *S. aureus* SCVs to survive within mammalian cells without lysing these cells.[10,14–16] This intracellular locale is thought to shield the SCVs from host defences and from antibiotics that have limited ability to accumulate within mammalian cells.[14]

Two intrinsic metabolic characteristics of *S. aureus* SCVs which contribute to decrease their susceptibility to cell-wall-active antibiotics and aminoglycosides[10,12,14,15,17–21] are their slow mode of growth[10,14,15] and their reduced import of drugs due to the decreased electrochemical gradient,[17,19,20,22] respectively. The metabolic alterations that result in the *S. aureus* SCV phenotype can be explained on the basis of interruptions in electron transport which lead to decreased ATP production.[10,14–16] The reduced levels of ATP are believed to be responsible for the slow mode of growth, decreased production of α-toxin, and the ability of these organisms to persist within cultured endothelial cells.[10,14,15] The defects in electron transport, which are frequently caused by impaired biosynthesis of menaquinone or haemin and can be reversed by menadione or

†Present address: Service de Médecine, Hôpital Cantonal, 1700 Fribourg, Switzerland.

*Corresponding address: Division of Infectious Diseases, University Hospital, CH 1211 Geneva 14, Switzerland.
Tel: +41-22-3729826; Fax: +41-22-3729830.

haemin supplementation, respectively,[10,14,15] also lead to a significant reduction in the electrochemical gradient promoting aminoglycoside uptake and bacterial killing.[17,19,20,22] Overall, the phenotypic characteristics and frequently intracellular location of *S. aureus* SCVs allow them to counteract normal host defences and standard antimicrobial agents.[10,14]

Several metabolic characteristics of SCVs are shared by organisms recovered from foreign-body infections,[13,23–27] in particular a slow mode of growth on artificial surfaces and decreased in-vitro and in-vivo susceptibility to bactericidal antibiotics. A direct consequence of this is the necessity to evaluate the antibiotic activity not only against suspensions of SCVs or normally growing organisms, but also against bacteria attached to artificial surfaces. This study was made possible by the recent generation of a stable, menadione-auxotrophic SCV strain, derived from a clinical isolate of *S. aureus*, whose phenotypic and functional properties have been previously described in detail.[15] The phenotypic alterations of the menadione-auxotrophic SCVs have a profound impact on their susceptibility to antibiotic killing compared with that of the isogenic parental strain of *S. aureus*, especially when organisms were growing slowly on artificial surfaces.

Materials and methods

Bacterial strains

The origin and phenotypes of *S. aureus* 6850, a clinical isolate from a patient with metastatic infection, and its menadione-auxotrophic SCV strain JB1 have recently been described in detail.[15] SCV strain JB1 was generated from strain 6850 by a single in-vitro passage in tryptic soy broth (Difco, Detroit, MI, USA) containing 1 mg/L of gentamicin (Sigma, St Louis, MO, USA). It was phenotypically stable upon multiple passages in broth, and reversion to the parental phenotype was not observed (frequency: $<10^{-9}$) as determined by dilution plating on tryptic soy agar (Difco). As shown previously,[15] the parental strain 6850 demonstrated a typical *S. aureus* phenotype and was highly haemolytic on rabbit blood agar plates, whereas SCV strain JB1 showed greatly reduced colony size and growth rate, diminished haemolytic activity, lack of pigmentation, and lower susceptibility to gentamicin (MIC = 8 mg/L) than strain 6850 (MIC = 1 mg/L).

Antimicrobial susceptibility by agar dilution testing

Since expression of the SCV phenotype by strain JB1 requires a menadione-deficient medium,[15] antibiotic susceptibility of the variant should be evaluated under slow growing auxotrophic conditions. Indeed, menadione supplementation not only transforms SCV into a fast growing haemolytic strain, but also restores its gentamicin susceptibility to the level expressed by the parental strain 6850 as previously described.[15] This implies that standard criteria used for estimating the MICs[28] and MBCs[29] of antibiotics against fast growing organisms need to be re-evaluated and adapted to the slow mode of growth of strain JB1. Mueller–Hinton agar (MHA, Difco) was selected for MIC determinations because of its low menadione content which fully maintained the SCV phenotype for several days. Antibiotic-containing MHA plates for agar dilution testing of the MICs of oxacillin (Sigma), vancomycin (Lilly, Giessen, Germany) and fleroxacin (Hoffmann–La Roche, Basel, Switzerland) were prepared as recommended by the National Committee for Clinical Laboratory Standards.[28] Overnight cultures of strains 6850 and JB1 were diluted 100-fold and 5-fold, respectively, to yield suspensions containing approximately 10^7 cfu/mL. Ten microlitre portions of either 6850 or JB1 were transferred to the surface of the antibiotic-counting MHA plates, yielding a final inoculum of 10^5 cfu/plate. The plates were incubated in air at 35°C and read after 24 and 48 h for strain 6850, and after 48, 72 and 96 h for strain JB1. The MIC of each antimicrobial agent was defined as the lowest concentration that completely inhibited growth, in conditions that allowed confluent growth of either 6850 or JB1 on antibiotic-free MHA plates.

Killing assays of fluid phase bacteria

Comparison of the bactericidal activity of each antimicrobial agent against standard ($1–2 \times 10^6$ cfu/mL) or high ($1–2 \times 10^7$ cfu/mL) concentrations of SCV and parental organisms in suspension was performed in Mueller–Hinton broth (MHB) whose low menadione content is appropriate for preserving the SCV phenotype in liquid phase.

Standard ($1–2 \times 10^6$ cfu/mL) concentrations of strains 6850 and JB1 were obtained by growing organisms for 3 h in MHB at 37°C without agitation, starting from an inoculum of 2×10^5 cfu/mL. High ($1–2 \times 10^7$ cfu/mL) concentrations of SCV and parental strains were derived from overnight cultures of strains 6850 and JB1 in MHB without agitation, followed by low-speed centrifugation and suspension of appropriate dilutions of either strain in fresh MHB medium.

Either standard or high concentrations of strains 6850 or JB1 were incubated for 24 h at 37°C with 8 × MIC of each agent, namely 2 mg/L of oxacillin, 8 mg/L of vancomycin or 8 mg/L of fleroxacin. The number of viable organisms was evaluated by subculturing 50 μL of 10-fold serially diluted portions of each culture on MHA after 0 and 24 h of incubation. Cfu counts were read at 48 h for strain 6850. For strain JB1, cfu counts were either read at 96 h on plain MHA, or at 48 h after being subcultured on MHA supplemented with 2 mg/L of menadione (Sigma) as previously described.[15] Control experiments verified that cfu counts of strain JB1 read at 48 h on menadione-supplemented MHA were equivalent to those scored after 96 h on unsupplemented agar.

Killing assays of surface-bound S. aureus

Procedures for attachment and growth of *S. aureus* on fibronectin-coated surfaces have been previously described in detail.[23] In brief, polymethylmethacrylate coverslips (8 × 8 mm) were coated individually with a monolayer of fibronectin (300 ng/cm^2).[23] After being rinsed in PBS, each fibronectin-coated coverslip was incubated for 60 min in a shaking water bath at 37°C with a 1 mL suspension containing 2×10^7 cfu of washed overnight cultures of either *S. aureus* 6850 or JB1, prepared as described above. The medium for bacterial attachment was PBS with 1 mM Ca^{2+} and Mg^{2+}. At the end of the attachment period, the fluids containing unbound bacteria were drained and the coverslips rinsed in PBS.[23] Growth of surface-bound *S. aureus* 6850 or JB1, in MHB at 37°C without agitation, was followed as previously described[23] for various periods of time ranging from 2 to 48 h. Cfu counts of solid phase organisms were performed after their detachment from the coverslips by low-output sonication and trypsin.[23] This method was shown microscopically to detach surface-bound *S. aureus* efficiently and to be harmless for the bacteria.[23]

At selected time points during growth on coverslips, namely at 3 or 24 h, bactericidal assays on attached *S. aureus* 6850 and JB1 were performed in parallel. For each condition and bacterial strain, duplicate coverslips were gently rinsed and transferred to 1 mL of fresh MHB containing the same antimicrobial agents at the same concentrations as those used for fluid-phase organisms. At the end of the 24 h incubation period at 37°C, organisms were detached from coverslips as described above. Killing of surface-bound bacteria was estimated as the average decrease (expressed in $\log_{10}$ cfu per coverslip) in colony counts of antibiotic-exposed organisms compared with those measured on parallel coverslips before antibiotic exposure.[23]

Data analysis

Each experiment was performed at least three times and the results were expressed as mean ± standard deviation (S.D.). Differences between means were estimated by Student's *t*-test with two-tailed significance levels. *P* values of <0.05 were considered statistically significant.

Results

Antibiotic susceptibility testing by agar dilution

Similar MICs of oxacillin, vancomycin and fleroxacin were found by agar dilution against *S. aureus* 6850 and JB1, namely 0.25 mg/L, 1 mg/L and 1 mg/L, respectively.

Comparison of the growth characteristics of strains 6850 and JB1

As expected from its metabolic properties, the menadione auxotrophic strain JB1 showed altered growth characteristics in the menadione-deficient MHB medium compared with its isogenic parent. Not only was the generation time in MHB of strain JB1 significantly longer than that of strain 6850 (Figure 1a), but also the final concentration (5×10^7 cfu/mL) reached at the plateau phase by fluid-phase growing SCV was much lower than that of the parental strain (10^9 cfu/mL).

In contrast to fluid-phase conditions, the maximal number of surface bound bacteria scored at 24 h, at the end of the growing period on fibronectin-coated coverslips, was almost equivalent for strain 6850 (2.9×10^7 cfu/coverslip) and JB1 (2.3×10^7 cfu/coverslip), regardless of the lower growth rate of SCVs compared with parental organisms (Figure 1b).

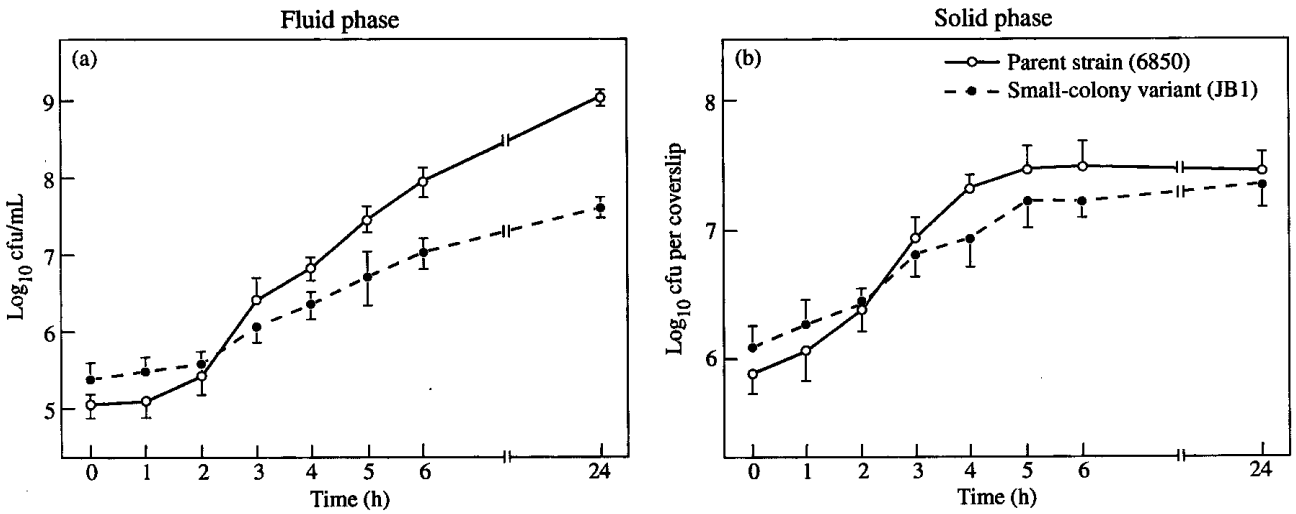

Figure 1. Growth curves of parental strain 6850 (○) and SCV strain JB1 (●) of *S. aureus* in fluid phase (a) and on fibronectin-coated coverslips (b). Results are means ± S.D.

Susceptibilities of fluid-phase organisms to bactericidal antibiotics

The susceptibilities of parental and SCV strains to bactericidal concentrations of either oxacillin (2 mg/L), vancomycin (8 mg/L) or fleroxacin (8 mg/L) were tested against standard or high concentrations of bacteria. SCV strain JB1 exposed to each antibiotic at standard ($1–2 \times 10^6$ cfu/mL) concentrations was eliminated to a slightly lower extent than the parental strain 6850, whose reduction in viable counts was >3 $\log_{10}$ cfu/mL with each antibiotic (Figure 2). In these conditions, differences in the reduction of strain JB1 versus 6850 were significant ($P < 0.05$) for oxacillin and vancomycin but not fleroxacin. Additional observations showed that when strains JB1 and 6850 were incubated for 3 h at 37°C with the same antibiotics at identical concentrations, the SCV showed virtually no decrease (<0.3 $\log_{10}$ cfu/mL) in viable counts compared with the parent which showed an average decrease of 1.5 $\log_{10}$ cfu/mL (data not shown).

Figure 2 also shows the differences in elimination of high concentrations ($1–2 \times 10^7$ cfu/mL) of SCV or parental organisms exposed to each antimicrobial agent in identical conditions. Whereas cfu counts of strain 6850 decreased by >3 $\log_{10}$ cfu/mL with each antibiotic, elimination of strain JB1 by the same agents hardly exceeded 1 $\log_{10}$ cfu/mL ($P < 0.01$).

Susceptibilities of solid-phase organisms to bactericidal antibiotics

SCV strain JB1 growing on fibronectin-coated surfaces exposed to the bactericidal antibiotics showed a somewhat lower elimination than the parental strain 6850 which reached significance ($P < 0.05$) with all antibiotics (Figure 3). Major quantitative differences were observed with nongrowing organisms on the solid phase. In contrast to the parental strain which was still significantly reduced by each antimicrobial agent, surface-bound SCV organisms were fully resistant to the bactericidal action of oxacillin or vancomycin. The only antimicrobial agent capable of showing a bactericidal effect on surface-bound SCVs was fleroxacin.

Discussion

Chronic staphylococci infections, particularly those associated with the presence of foreign bodies, present a major therapeutic dilemma because of the poor clearance of adherent bacteria from the implanted devices by host defences and antibiotics.[23,–26,30] In view of the dramatic decrease in antimicrobial susceptibility expressed by surface-bound *S. aureus* SCVs, these data yield further support to the notion that any SCV subpopulation emerging during intensive therapy of foreign-body staphylococcal infections might be particularly difficult to eradicate.

While *S. aureus* SCVs can regularly be produced by exposure of normal *S. aureus* strains to certain categories of antibiotics, especially the aminoglycosides,[17,19,20,22] they can also arise without antibiotic pressure.[10,15] This is supported by recent studies which demonstrated that passage of *S. aureus* in cultured mammalian cells[15,16] can induce SCV production. Consequently, SCVs might be found in patients following antibiotic treatment or *de novo*.

Because a large proportion of clinical *S. aureus* SCVs are menadione or haemin auxotrophs,[10,14] care must be exercised when studying these variants. Rich media such as brain heart infusion or Schaedler's broth have a high

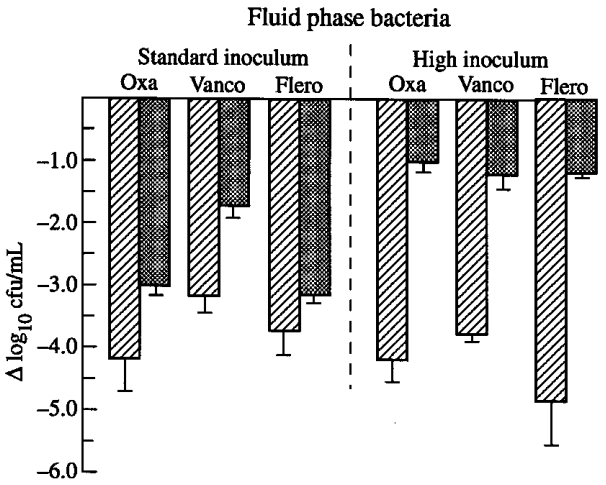

Figure 2. Killing of standard or high inocula of parental strain 6850 (▨) and SCV strain JB1 (■) of *S. aureus* in suspension by oxacillin (Oxa) 2 mg/L, vancomycin (Vanco) 8 mg/L or fleroxacin (Flero) 8 mg/L.

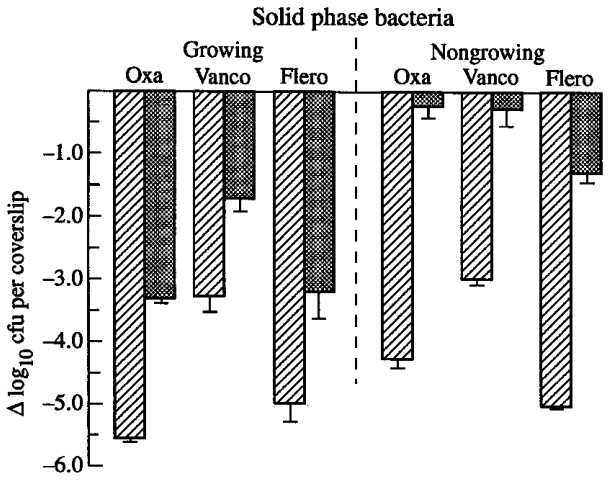

Figure 3. Killing of growing or nongrowing solid phase organisms of the parental strain 6850 (▨) and SCV strain JB1 (■) of *S. aureus* on fibronectin-coated coverslips by oxacillin (Oxa) 2 mg/L, vancomycin (Vanco) 8 mg/L or fleroxacin (Flero) 8 mg/L.

menadione content which will mask the SCV phenotype by supplementing the auxotrophy.[10,14] An additional problem related to the slow growth of SCVs is the difficulty encountered in using routine NCCLS guidelines for testing their susceptibility to antibiotics.[28,29] Because of the slow growth of *S. aureus* SCVs it is necessary to change standard time intervals for establishing the MICs by agar dilution in MHA.[28] Furthermore, MIC testing in menadione- and haemin-deficient liquid media such as MHB is not feasible because the growth of auxotrophic SCVs is not sufficient for turbidimetric comparison of controls and antibiotic-exposed cells.[28] The absence of a simple reliable assay for MIC determination of antimicrobials against SCVs grown in a liquid medium preserving the auxotrophic phenotypes also prevents evaluation of antibiotic MBCs against such variants. Also comparisons of the respective elimination rates of the SCV versus the parent by the bactericidal antibiotics in the fluid phase are difficult because of their quite different growth rates and plateau cell concentrations.

The characteristics of the in-vitro assay used to compare the susceptibilities to antimicrobial killing of *S. aureus* growing on fibronectin-coated surfaces have been previously described in detail.[23] This previous study documented a decrease in the susceptibility to antibiotic killing of *S. aureus* strain 120, a clinical isolate from an intravenous device-associated infection, at the end of exponential growth on fibronectin-coated surfaces. The susceptibility changes were not expressed to the same extent for all antibiotics, being moderate for fleroxacin and more important for oxacillin and vancomycin.[23] Whereas the parental strain 6850 of *S. aureus* used for this study was less influenced by growth conditions than the previously evaluated strain 120, the greater stability of strain 6850 to varying growth conditions allowed comparison in optimal conditions of its susceptibility to antimicrobial killing with that of its SCV derivative strain JB1.

The slow growth and atypical microbiological profile of *S. aureus* SCVs hinder their identification by clinical microbiology laboratories. They also present some unique problems when performing various types of antibiotic susceptibility testing which require significant changes from routine protocols. Finally, these organisms may present a major therapeutic challenge when they are adherent to foreign surfaces because of their conversion to a highly resistant state. Release of the SCVs from the adherent state leads to their reversion to a less resistant subpopulation. Hence, even carefully performed laboratory testing may greatly underestimate the magnitude of the resistance problem found in adherent *S. aureus* SCVs.

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