**Staphylococcus aureus** Small Colony Variants Are Induced by the Endothelial Cell Intracellular Milieu

Omar Vesga, Michael C. Groeschel, Marijke F. Otten, Dorothy W. Brar, James M. Vann, and Richard A. Proctor

Recent studies have reported that *Staphylococcus aureus* small colony variants (SCVs) can cause highly persistent infections in humans and in cultured endothelial cells. To understand the process by which SCVs of *S. aureus* appear in subjects who have not received antibiotic treatment, bovine endothelial cells were coincubated with a wild *S. aureus* strain for 72 h in the presence of lysostaphin. Intracellular bacteria were harvested and screened for stable SCVs. Intracellular bacteria developed the SCV phenotype at a greater rate than control bacteria not exposed to endothelial cells: The intracellular induction rate was $10^{-1}$ versus a spontaneous rate of $<10^{-7}$. This observation suggests that SCVs are induced by the intracellular milieu and suggests a possible mechanism for the intriguing pathophysiology of tissue persistence of staphylococci.

Small colony variants (SCVs) of *Staphylococcus aureus* are found in patients with infections that persist despite appropriate antibiotic treatment or that recur after long disease-free intervals [1, 2]. SCVs are usually isolated from patients who have been receiving antibiotics, and several in vitro and in vivo studies have demonstrated that aminoglycosides produce the SCV phenotype from *S. aureus* [3]. However, SCVs have also been recovered from humans with staphylococcal infections in the absence of antibiotic pressure [1, 2] and from animals with experimental endocarditis [4].

Here we used an in vitro model for endovascular persistence [5] to assess whether SCVs of *S. aureus* are generated inside endothelial cells (ECs) at a greater rate than the spontaneous rate observed under control conditions [6].

**Materials and Methods**

*Bacterial inoculum preparation.* *S. aureus* 6850 was the parent strain in all experiments. Bacterial inoculum ($10^7$/mL) was counted in a Petroff-Hausser chamber as described [3, 7], and viability was tested by plating in duplicate $10^{-5}$, $10^{-6}$, and $10^{-7}$ dilutions (100 µL) on tryptic soy agar (TSA; Difco, Detroit). After 24 h at 35°C, colony-forming units (cfu) were counted and screened for the spontaneous presence of SCVs.

*EC monolayer cultures.* Bovine aortic EC line Cad28 was derived as described [3, 8]. Briefly, EC monolayers were grown to confluency ($3.0 \times 10^5 \pm 0.5$ cells/mL) in plastic wells containing 900 µL of EC culture medium, infected to obtain a final bacterial concentration of $10^7$/mL, incubated with 5% CO$_2$ at 37°C for 3.5 h, and exposed to 1.0 mL of fresh medium containing 10 µg of lysostaphin (Applied Microbiology, New York). Lysostaphin does not penetrate the intact EC membrane but it lyases extracellular *S. aureus* [3, 7, 9]. Optimal inoculum ($10^4$ bacteria/mL) and final harvest point (72 h) were determined during 32 preliminary assays done under the same conditions. During the preliminary assays, we used $10^6$, $10^5$, $10^4$, $10^3$, and $5 \times 10^2$ bacteria/mL of ECs, infected the same number of ECs, and harvested cells at 0, 24, 48, 72, 96, and 120 h. Incubation in the presence of lysostaphin was continued until the final phase of each experiment (72 h later). Then, $100 \mu$L of the supernatant was cultured to confirm lysostaphin activity (the supernatant must remain sterile), and ECs were lysed to release intracellular bacteria. Dilutions of harvested bacteria were plated in duplicate on TSA. After incubation for 24 h, all cfu were screened by dissecting microscope for the presence of stable SCVs. To be considered stable, SCVs must remain $\geq 10$ times smaller than the parent strain after three consecutive passes on TSA. The SCV formation rate was calculated by dividing the number of intracellular stable SCVs by total intracellular cfu (small and normal-sized colonies) found after 34 experiments.

*Control cultures.* To determine the spontaneous rate of SCV formation from *S. aureus* 6850, parallel experiments were done under the culture conditions described above, but without ECs or lysostaphin. After incubation for 72 h, $100 \mu$L of bacterial culture was diluted ($10^{-5}$, $10^{-6}$, and $10^{-7}$) and plated in duplicate on TSA. The dilution containing the highest number of SCVs ($30-300$ cfu/streak) was counted, although SCVs were screened through all three dilutions. The spontaneous SCV formation rate was calculated as in the intracellular experiments. To determine whether lysostaphin enhanced SCV production, 13 culture tubes containing a range of enzyme concentrations (1:10 successive dilutions from $100 \mu$g/mL to 1.0 fg/mL and a control without lysostaphin) in 10 mL of tryptic soy broth (Difco) and $10^7$ bacteria/mL were incubated for 24 h in air at 35°C. Four $100-\mu$L samples from each of three dilutions were plated per tube, and cfu were counted and SCVs screened as detailed above. This experiment was repeated three times.
Results

three times.

bacteria/mL) was plated to evaluate bacterial viability (31% present in the original inoculum. This experiment was repeated counted per streak at the 10⁻⁵ dilution, giving a total of Scv aureus 740 Concise Communications JID 1996; 173 (March)

taining 2.0 µg of menadione or 20 µg of hemin and by growing tested for auxotrophism by using diffusion disks on TSA con­

not detected in 34 control experiments in which we screened

ysis was done with control cultures at 72 h: 198 ~

formation rates in 34 experiments.

Before ECs were infected, 100 µL from the inoculum (10⁸ bacteria/mL) was plated to evaluate bacterial viability (31% ± 7% of 10⁷ bacteria/mL of ECs = 3.1 ± 0.7 × 10⁶ viable bacteria/mL) and to screen for the presence of spontaneous SCVs at time zero (table 1). On average, 310 ± 70 cfu were counted per streak at the 10⁻³ dilution, giving a total of ~25,000 cfu from 34 experiments. Stable SCVs were not found in this or the two higher dilutions (2500 additional cfu screened). Based on the absolute number of cfu screened, we found <3.64 × 10⁻³ spontaneous stable SCVs. The same analysis was done with control cultures at 72 h: 198 ± 29 cfu per streak were counted at the 10⁻⁶ dilution. Stable SCVs were not detected in 34 control experiments in which we screened ~15,000 cfu in this dilution plus an additional 1500 cfu in the 10⁻⁷ dilution. The 10⁻³ dilution could not be screened because

the large number of cfu per plate prevented evaluation. From the absolute number of cfu visually screened, the spontaneous rate from control cultures at 72 h was ~6.06 × 10⁻⁵ (table 1).

Stable SCVs were not found at 0, 24, and 48 h after infection of ECs during 32 preliminary experiments during which ~16,400 intracellular colonies were screened. Because a significant number of stable SCVs started to appear 72 h after infection, all 34 subsequent experiments were harvested at this point. A total of 2.70 × 10⁵ intracellular cfu was obtained from the last 34 experiments. We visually screened ~14,000 intracellular colonies and found >100 intracellular SCV strains; from these, we selected 14 stable strains for further testing. Not all of the 14 stable SCV strains were equally represented intracellularly. We assessed a total of 660 colonies (table 2): E3-5 was the most abundant strain (500 cfu); each of the remaining 13 strains was represented by 5 or 10 cfu. From the number of cfu visually screened, we determined that the intracellular SCV formation rate was 10⁻³ (14/14,000). When the intracellular rate was compared with the spontaneous rates calculated from starting inoculum cultures at time zero or from parallel control experiments at 72 h, P ~< .001 (table 1).

The rate of SCVs spontaneously present in S. aureus 6850 was detected by selection of the variants with gentamicin, an antibiotic to which SCVs are resistant. The MICs for the wild type and the stable variant strains were 1.0 and 8.0 µg of gentamicin/mL, respectively. After three experiments in which 3.0 × 10⁷ bacteria were plated on TSA containing 4.0 µg of gentamicin/mL, only 1 stable SCV was isolated. Wild type and nonstable colonies did not grow on the plates with this concentration of gentamicin. The viability of starting inocula was 30%, leaving 9.0 × 10⁶ viable bacteria to be screened. Considering that only one stable SCV cfu was selected by gentamicin, the spontaneous rate of SCVs was 1.11 × 10⁻⁷ (1/9.0 × 10⁶) (table 1).

Table 2 shows the biochemical characterization of the SCV strains. There were 9 (64%) menadione auxotrophs and 3 (22%)

### Table 1. Presence of stable small colony variants (SCVs) of S. aureus 6850 after 34 experiments under different conditions.

<table>
<thead>
<tr>
<th>Bacterial source</th>
<th>No. of stable scv visually detected</th>
<th>Total cfu visually screened</th>
<th>Scv formation rate (scv/total cfu)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>starting inoculum¹</td>
<td>0</td>
<td>2.75 × 10⁴</td>
<td>&lt;3.64 × 10⁻¹</td>
</tr>
<tr>
<td>control cultures²</td>
<td>0</td>
<td>1.65 × 10⁴</td>
<td>&lt;6.06 × 10⁻¹</td>
</tr>
<tr>
<td>endothelial cells⁵</td>
<td>14</td>
<td>1.40 × 10⁴</td>
<td>1.00 × 10⁻¹</td>
</tr>
<tr>
<td>gentamicin plates⁴</td>
<td>1</td>
<td>9.00 × 10⁴</td>
<td>1.11 × 10⁻¹</td>
</tr>
</tbody>
</table>

* Total cfu = wild type size + scv size, 0, replaced by 1 to enable proportions calculation (result is preceded by <).

¹ Cultures done at time point zero (rate calculation: 1/2.75 × 10⁴).
² Cultures done at 72 h (rate calculation: 1/1.65 × 10⁴).
³ Intracellular cultures harvested at 72 h (rate calculation: 14/1.40 × 10⁴).
⁴ P = .001 when intracellular culture rates are compared with starting inoculum and control culture rates.
⁵ Spontaneous rate of SCVs in any S. aureus 6850 inoculum as calculated by gentamicin selected on solid medium (1/9.00 × 10⁶).

### Results

In addition, to find the number of stable SCVs in any starting S. aureus 6850 culture, 1 mL of bacteria (10⁷ log phase growing cells) not previously exposed to gentamicin was plated on TSA containing four times the MIC for the wild type strain. Growth of stable SCVs at the concentration at which wild type bacteria do not survive represents the spontaneous number of variants initially present in the original inoculum. This experiment was repeated three times.

**Characterization of SCV strains.** All stable SCV isolates were tested for auxotrophy by using diffusion disks on TSA containing 2.0 µg of menadione or 20 µg of hemin and by growing in the presence of 5% CO₂ [3]. Gram’s stain characteristics, hemolytic activity (sheep blood agar; Difco), coagulase production (tube coagulase test; GIBCO BRL, Gaithersburg, MD), and susceptibility tests for lysostaphin and gentamicin were evaluated by standard procedures.

**Statistical analysis.** We used the z-test with Yates’s correction to compare proportions between intracellular and control SCV formation rates in 34 experiments.

### **Table 1.** Presence of stable small colony variants (SCVs) of *S. aureus* 6850 after 34 experiments under different conditions.
CO₂ auxotrophs, and 2 (14%; E3-2 and E4-10) showed no auxotrophy for hemin, menadione, or CO₂. Growth was enhanced by 5% CO₂ incubation in all of the menadione-auxotrophic strains, and growth of the CO₂ auxotrophs was enhanced by menadione in 1 (E3-8). Ten (71%) were nonhemolytic; the other 4 were weakly hemolytic. When the auxotrophy was relieved with menadione or CO₂ (12 strains), 8 strains (66.7%) showed the same hemolytic activity as that of S. aureus 6850, 1 (8.3%) showed significant but weaker hemolysis than the parent strain, and 3 (25%) remained weakly hemolytic even when they were supplemented. Hemolytic activity correlated with the degree of reversion to the parent strain phenotype that each SCV strain achieved once the auxotrophy was relieved, with stronger hemolysis in the strains that regained full growth rate and pigmentation.

Further phenotypic characterization of the SCVs revealed that all strains formed grape-like clusters and were gram- and coagulase-positive when the auxotrophy was relieved. However, SCVs showed a greater tendency toward being gram-variable, exhibited smaller elliptical cocci and less microclustering, and were coagulase-negative in the absence of menadione or CO₂. Exceptions were strains E3-2 and E4-10, which did not show auxotrophy for the compounds tested but were coagulase-positive as SCVs (table 2).

All SCV strains, like the parent strains, were fully susceptible to 2.0 μg of lysostaphin/mL. Lysostaphin does not induce the SCV phenotype at sub-MICs between 1.0 fg/mL and 100 ng/mL, and the number of cfu after 24 h of incubation was the same as control cultures without lysostaphin. A total of ~12,000 cfu were screened in three experiments.

Discussion

The intracellular rate of stable SCV formation is four orders of magnitude greater than the rate obtained by gentamicin selection. Because gentamicin on agar kills wild type organisms while allowing SCVs already present on the inoculum to survive, it selects for the rare spontaneous event (~1 SCV in 10⁷ organisms). In contrast, finding 1 SCV in every 1000 cfu recovered from cultured EC (14/14,000) suggests that induction, rather than selection, has occurred. The abrupt appearance of SCVs between 48 and 72 h, with the lack of stable SCVs before 48 h, also supports the concept of intracellular induction of variants. If selection were occurring, a steady increase in cfu would have been found.

There may be a connection between SCV intracellular induction, intracellular persistence, and the pathophysiology of some S. aureus infections. In vitro experimentation [10, 11] suggests that the EC intracellular milieu protects slow-growing organisms from cell-mediated immunity. In addition, the intracellular space is reached only to a limited extent by β-lactams and aminoglycosides [12]. Even antibiotics with excellent intracellular concentrations and high in vitro antistaphylococcal activity, such as erythromycin and clindamycin, are not effective against intracellular S. aureus [13]. Furthermore, a recent evaluation of the bactericidal activity of antistaphylococcal antibiotics inside ECs [14] found surprising differences between conventional and intracellular MBCs: In the intracellular milieu for vancomycin, rifampin, and ciprofloxacin, respectively, these were 1024, 128, and 8 times higher [12, 13]. Independent of their localization with respect to the host cell, SCVs are more difficult to treat because of their slow growth [2, 15]. Antibiotics that require a metabolically active target are not as effective against SCVs (aminoglycosides [1, 3, 10] and β-lactams [1, 10, 15]). These advantages may permit S. aureus SCVs to survive much longer intracellularly, to revert to the parent strain, and to invade new tissues, thus perpetuating infection and producing late relapses [1, 2].

### Table 2. Biochemical characterization of small colony variants of S. aureus 6850 obtained after infection of endothelial cells.

<table>
<thead>
<tr>
<th>Strain</th>
<th>cfu*</th>
<th>Auxotrophy</th>
<th>Coagulase</th>
<th>Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 14)</td>
<td>(n = 660)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E3-2</td>
<td>5</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E3-5</td>
<td>500</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E3-8</td>
<td>50</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>E3-10</td>
<td>5</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E4-10</td>
<td>10</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E4-19A</td>
<td>10</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E4-19B</td>
<td>10</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E4-19C</td>
<td>10</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E4-19D</td>
<td>10</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E4-19E</td>
<td>10</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E4-19F</td>
<td>10</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E4-24B</td>
<td>10</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>E4-24D</td>
<td>10</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>E4-24H</td>
<td>10</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

NOTE. M = menadione, H = hemin, NS = nonsupplemented, S = supplemented with menadione or CO₂, NT = not tested because of nonauxotrophism.

* No. of cfu for each strain.
We believe our study is the first to demonstrate that the intracellular environment of the EC may induce formation of \textit{S. aureus} SCVs. These findings may provide an explanation for staphylococcal persistence in host tissues that causes chronic and relapsing infections over months to years\cite{2}. Genetic studies should help to clarify the molecular mechanisms that control conversion between these two populations.

References


