**Staphylococcus aureus** small colony variants are resistant to the antimicrobial peptide lactoferricin B

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**Objectives:** To determine whether **Staphylococcus aureus** small colony variants (SCVs) are resistant to the antimicrobial peptide lactoferricin B. To assess if deficiency in transmembrane potential, a common characteristic of SCVs that are haemin- or menadione-auxotrophs, affects the uptake of the peptide into the bacterial cytoplasm.

**Methods:** A broth microdilution technique was used for susceptibility testing to determine the MIC of lactoferricin B for SCVs with three different auxotrophisms (haemin, menadione or thymidine) and their isogenic parent strains. Both clinical isolates and genetically defined mutants were used. The internalization of lactoferricin B in a hemB mutant and the respective parent strain was studied using transmission electron microscopy and immunogold labelling.

**Results:** All SCVs showed reduced susceptibility to lactoferricin B irrespective of their auxotrophy compared with their isogenic parent strains. The MIC for all SCVs was >256 mg/L, whereas the MICs for the parent strains ranged from 16–256 mg/L. Surprisingly, the hemB mutant contained significantly more lactoferricin B intracellularly than the respective parent strain.

**Conclusions:** The resistance mechanism of SCVs towards the antimicrobial peptide lactoferricin B is presumably caused by the metabolic changes present in SCVs rather than by a changed transmembrane potential of SCVs or reduced uptake of the peptide.

**Keywords:** cellular uptake, electron microscopy, immunolabelling, transmembrane potential, metabolic resistance, antimicrobial resistance, resistance mechanism

**Introduction**

**Staphylococcus aureus** small colony variants (SCVs) are a subpopulation of bacteria, which are implicated in recurrent and antibiotic refractory infections, probably due to their ability to evade the host immune defence.¹ SCVs show different characteristics compared with normal **S. aureus**, including slow growth resulting in a small colony size, reduced carbohydrate metabolism, lack of pigment and altered virulence factor expression.²³ Two major types of SCVs are found in clinical isolates: (i) electron transport defective SCVs; and (ii) thymidine-auxotrophs. Electron transport defective SCVs are characterized by being haemin- or menadione-auxotrophs. Haemin and menadione are essential in the formation of cytochromes and menaquinone, respectively. Disruption of the electron transport chain causes reduced production of ATP, important for many metabolic processes in bacteria including cell wall biosynthesis, amino acid transport and protein synthesis. Loss of electron transport also reduces the transmembrane potential, which is required for the effect of many cationic antimicrobial compounds like aminoglycosides, lantibiotics and antimicrobial peptides, resulting in decreased susceptibility to these agents.¹⁴⁶ Thymidine-dependent SCVs emerge as a result of long-term trimethoprim/sulfamethoxazole therapy in cystic fibrosis patients.⁸ While trimethoprim/sulfamethoxazole interferes with the tetrahydrofolic acid (THF) pathway, THF acts as a coenzyme for thymidylate synthetase, which catalyses the synthesis of dTMP from dUMP.
Resistance to lactoferricin B in SCVs

Since dTMP is essential for DNA synthesis, susceptible *S. aureus* strains are affected by trimethoprim/sulfamethoxazole therapy. However, thymidine-dependent SCVs are resistant to trimethoprim/sulfamethoxazole and survive if extracellular thymidine is provided.

Antimicrobial peptides or host defence peptides are an integral part of the host defence against invading microorganisms. Since SCVs are able to survive and persist *in vivo*, resistance to these peptides might be a prerequisite. However, SCVs are not universally resistant to all antimicrobial peptides. Magainin and human neutrophil defensin-1 have similar activity against SCVs and normal *S. aureus*, whereas SCVs are resistant to protamine and thrombin-induced platelet microbicidal protein. Sadowska et al. have shown that under exposure to subMIC concentrations of the antimicrobial peptide protamine, both haemin- and menadione-dependent SCVs emerge.

Lactoferricin B is a multifunctional cationic antimicrobial peptide derived from bovine lactoferrin, with antibacterial, antiviral, antiparasitic, antitumour and immunomodulating activities. The antibacterial mode of action of lactoferricin B is likely to be multitargeted involving the outer surface, cytoplasmic membrane and intracellular targets. Resistance to lactoferricin B has been possible to induce in *S. aureus*, and proteases have previously been described to be involved in susceptibility to lactoferricin B. So far, the SCV phenotype was not observed in the lactoferricin B-resistant *S. aureus*. However, since SCVs are frequently unstable and media were not appropriate to culture SCVs, they might have been missed without actively searching for SCVs.

To determine whether SCVs are resistant to lactoferricin B, the susceptibility of SCVs with different auxotrophies was determined. Furthermore, the internalization of lactoferricin B was compared between a *hemB* mutant mimicking the SCV phenotype and the isogenic parent strain using transmission electron microscopy and immunogold labelling.

**Materials and methods**

**Growth conditions and bacterial strains**

Bacterial strains used in this study are listed in Table 1. Mueller–Hinton (MH) broth (Difco, Detroit, USA) was used as growth and assay medium for all strains. Erythromycin (5 mg/L) (Astra, Södertälje, Sweden) was used in agar plates and overnight broth cultures for the genetically defined mutants (*hemB::ermB; menD::ermC*) to maintain pure cultures of the mutants. The *menD* mutant was constructed as described in Bates et al. except that the COL strain was used as the parent strain.

**Determination of MIC**

The MIC was determined using a standard broth microdilution technique. Briefly, 2-fold dilutions (2–256 mg/L) of lactoferricin B in double distilled water (125 μL) were added to microtitre plates (Nunc, Roskilde, Denmark) and exponentially growing bacteria (125 μL) were added resulting in a final bacterial concentration of ~10⁶ cfu/mL. The MIC was determined as the lowest concentration that inhibited growth after 24 and 48 h for the normal strains and after 48 h of incubation for the SCVs. Lactoferricin B was prepared by pepsin digestion of bovine lactoferrin and purified by reverse phase HPLC (Centre for Food Technology, Queensland, Australia).

**Immunolabelling, transmission electron microscopy and determination of intracellular content of lactoferricin B**

The intracellular localization and amount of lactoferricin B in *S. aureus* 8325–4 and the isogenic *hemB* mutant I-10 exposed to 30 mg/L lactoferricin B for 30 min were determined as previously described. The Mann–Whitney non-parametric test was used for comparison of intracellular peptide content.

**Results and discussion**

The MIC of the antimicrobial peptide lactoferricin B was determined for SCVs with different auxotrophies and their isogenic parent strains (Table 1). The MICs after 48 h for all SCV phenotypes (haemin-, menadione- or thymidine-auxotrophs) were higher than 256 mg/L. Among the strains with normal phenotype, the MIC after 24 h varied from 16–256 mg/L, with the COL strain being the most resistant (MIC = 256 mg/L) and the A22616/5 strain the most susceptible (MIC = 16–32 mg/L). No significant differences in MICs were observed after 48 h compared with 24 h studying the normal strains. Further detailed determination of the MIC for the SCVs was not possible as lactoferricin B aggregates at higher

### Table 1. Bacterial strains used in the study and MIC values of lactoferricin B [MIC determination was done in parallel and on two separate occasions (n = 4)]

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Characteristics</th>
<th>MIC (mg/L)</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>8325-4</td>
<td>NCTC 8234 cured of prophages, plasmid-free</td>
<td>64 64</td>
<td>NCTC</td>
</tr>
<tr>
<td>I-10</td>
<td>SCV, <em>hemB</em> mutant; 8325-4 <em>hemB::ermB</em>, Ery&lt;sup&gt;R&lt;/sup&gt;, haemin-auxotroph</td>
<td>&gt;256</td>
<td>von Eiff et al.¹¹</td>
</tr>
<tr>
<td>COL</td>
<td>clinical isolate, methicillin resistant (<em>mecA</em> positive)</td>
<td>256 256</td>
<td>Kohler et al.³</td>
</tr>
<tr>
<td>COL <em>hemB</em></td>
<td>SCV, <em>hemB</em> mutant; COL <em>hemB::ermB</em>, Ery&lt;sup&gt;R&lt;/sup&gt;, haemin-auxotroph</td>
<td>&gt;256</td>
<td>Baumert et al.⁷</td>
</tr>
<tr>
<td>COL <em>menD</em></td>
<td>SCV, <em>menD</em> mutant; COL <em>menD::ermC</em>, Ery&lt;sup&gt;R&lt;/sup&gt;, menadione-auxotroph</td>
<td>&gt;256</td>
<td>this study</td>
</tr>
<tr>
<td>A22616/5</td>
<td>clinical isolate, wild-type strain of A22616/3</td>
<td>16–32 32</td>
<td>von Eiff et al.¹⁵</td>
</tr>
<tr>
<td>A22616/3</td>
<td>SCV; clinical isolate; menadione-auxotroph</td>
<td>&gt;256</td>
<td>von Eiff et al.¹⁵</td>
</tr>
<tr>
<td>F3829-I</td>
<td>clinical isolate, wild-type strain of F3829-I</td>
<td>64–128 128</td>
<td>Kahl et al.⁸</td>
</tr>
<tr>
<td>F3829-I</td>
<td>SCV; clinical isolate; thymidine-dependent; pin-point colonies</td>
<td>&gt;256</td>
<td>Kahl et al.⁸</td>
</tr>
</tbody>
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NCTC, National Collection of Type Cultures.
concentrations in MH broth. These results indicate that SCVs are resistant to lactoferricin B and that the resistance is a result of a mechanism or property that is common between all SCV phenotypes independent of the underlying auxotrophy. For other antimicrobial peptides, differences in resistance between haem- and menadione-auxotrophic SCVs have been observed. One common property for the haem- and menadione-auxotrophic SCVs is a defective electron transport chain resulting in a decreased transmembrane potential. The transmembrane potential is required for import or interaction of cationic compounds with the cytoplasmic membrane, and several antimicrobial peptides are shown to require a threshold transmembrane potential to be active.

It has previously been shown that the transmembrane potential of the hemB mutant I-10 dropped immediately (to values below -100 mV) when glucose expired and other nutrients such as acetate and lactate did not allow further growth. To examine if an intact electron transport chain and transmembrane potential is involved in internalization of lactoferricin B, the hemB mutant I-10 and the parent strain 8325-4 were exposed to 30 mg/L lactoferricin B for 30 min. Electron microscopy and immunogold labelling showed a significantly higher amount of intracellular labelling of lactoferricin B in the hemB mutant than in the parent strain (P < 0.0001) (Figure 1). This suggests that lactoferricin B does not require an intact transmembrane potential to interact with and cross the cytoplasmic membrane. Thus, the resistance mechanism in SCVs is not due to reduced uptake of the peptide as observed for aminoglycosides for haemin- and menadione-auxotroph SCVs.

The resistance mechanism might therefore be dependent on other metabolic factors caused by or independent of a defective electron transport chain. Lactoferricin B is more potent against metabolically active bacteria and it could be speculated that the resistance observed in SCVs is due to the reduced metabolic activity of SCVs, which might alter the target for lactoferricin B. Lactoferricin B has been shown to inhibit bacterial macromolecular synthesis in other Gram-positive and Gram-negative bacteria. Therefore, reduced activity or alteration of a target involved in either DNA, RNA or protein synthesis might be responsible for the resistance occurring in SCVs.

These findings show that SCVs (haemin-, menadione- or thymidine-auxotrophs) are resistant to the antimicrobial peptide lactoferricin B, and that the resistance mechanism may be a form of a metabolic resistance and not due to reduced uptake. These results also suggest that lactoferricin B crosses the membrane through passive diffusion or promotes the uptake itself. Further studies are warranted to determine the exact mechanism of resistance.

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Transparency declarations

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References

Resistance to lactoferricin B in SCVs


