Mini-review: Formation, antibiotic resistance and clinical outcome of infections associated with small colony variants of staphylococci

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Small colony variants (SCVs) comprise a slow-growing subpopulation of bacteria with distinctive phenotypic and pathogenic traits and reduced antibiotic susceptibility. *Staphylococcus aureus* and *S. epidermidis* can form a subpopulation of bacteria known as SCVs. Phenotypically these variants characterized by slow growth, reduced pigmentation, changed pattern of carbohydrate utilization, changes in virulence factor expression, and ability to revert to the normal phenotype. This multifarious phenotype can be explained by deficiencies in electron transport. In clinical staphylococci isolates, SCV phenotype is mostly due to mutations in hemin, menadione or thymidine genes. The recovery of SCVs from clinical materials was described early at the beginning of the last century. However, the relationship of these variants with chronic, persistent and recurring infections such as osteomyelitis, endocarditis and cystic fibrosis patients and soft-tissue infection was only described in the past three decades.

To genetically study SCVs that switching with high frequency to normal phenotype, a stable mutant in electron transport was generated by interrupting one of the hemin biosynthetic genes, *hemB*, in both *S. aureus* and *S. epidermidis*. The *S. aureus* mutant exhibited characteristics typical of clinical SCVs such as slow growth, colorless, low coagulase activity, reduced hemolytic activity, and resistance to aminoglycosides. Furthermore, the mutant was able to persist within cultured endothelial cells due to depressed α-cytotoxin activity. Similarly the *S. epidermidis* mutant showed these typical characteristics of clinical SCVs plus its ability to build a dense biofilm layers that make the eradication of this bacteria very difficult, especially in implant-associated infections. It was suggested that the intracellular location of this subpopulation might shield these variants from host defenses and antibiotics, thus providing one explanation for the difficulty in removing SCVs from host tissues. Hence, a defect in the electron-transport system allows SCVs to resist aminoglycosides and persist intracellularly in endothelial cells. Due to their slow growth characteristics, SCVs can be missed, misidentified or overgrowth by other bacteria in the clinical laboratory. So, any persistent infection that is difficult to treat should be carefully searched by clinicians and laboratory staff for this subpopulation of staphylococci.

Keywords Staphylococci; small colony variants; formation; clinical outcome; antibiotic resistance; biofilm formation

1. Introduction

Small colony variants (SCVs) constitute a slow-growing auxotrophic subpopulation of bacteria with distinctive phenotypic and pathogenic traits [1]. SCVs, also known as dwarf colony variants, were described first in *Salmonella Typhi* in 1910 by Jacobsen [2]. A part from *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS), SCVs were detected in a wide variety of bacterial specimens. In species such as *S. aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Burkholderia pseudomallei*, *Salmonella* spp. and *Neisseria gonorrhoeae* SCVs were isolated as naturally occurring subpopulations in clinical specimens or following in vitro exposure to antibiotics [3-7].

In the last three decades, a renewed interest in staphylococcal infections due to SCVs has emerged, since an association between the occurrence of *S. aureus* and *S. epidermidis* SCVs and persistent and relapsing infection was described. In patients whose acute infection initially responded to antimicrobial treatment and which recurred after long disease-free intervals or those with persisting infections despite appropriate antibiotic treatment, SCVs of *S. aureus* were recovered. In the past seven decades, a large number of patients have been described with various infections due to *S. aureus*, *S. epidermidis* and other CoNS staphylococci SCVs [8-28].

The first device-related infection due to *S. aureus* SCVs has been described illustrating the poor clinical and microbiological response to even prolonged antimicrobial therapy in patients infected with this *S. aureus* subpopulation [29]. SCVs of *S. aureus* have been recognized for many years, however, relatively little is known regarding infections caused by SCVs of CoNS. In the past years, several cases of pacemaker infections due to SCVs of CoNS (*S. epidermidis*, *Staphylococcus capitis*, and *Staphylococcus lugdunensis*) have been reported [7].

One of the important features of the SCVs, particularly of *S. aureus* SCV, its ability to persist intracellularly. This intracellular location may provide a survival niche for these bacteria because the microorganisms are protected against antibiotic therapy and host defenses. This intracellular persistence is influenced by efficient invasion attributed to high expression of adhesion–fibronectin binding protein and down-regulation of α-toxin and proteinase which contribute to inflammation and tissue destruction [13, 14, 21, 30, 31]. However, this bacterial phenotype switching is an integral part of the infection process that enables the bacteria to hide inside host cells, which can be a reservoir for chronic and therapy-refractive infections [32].
2. Formation and biology of staphylococci SCVs

*Staphylococcus aureus* SCVs are a naturally occurring slow-growing subpopulation of the species *S. aureus* which are defined by tiny colonies about 10 times smaller than the parent strain as shown in Figure 1. The colonies are in contrast to the normal phenotype *S. aureus* - mostly nonpigmented and nonhemolytic. Most characteristics of the SCVs can be tied together by a common thread, which are alterations in electron transport. Specially, the following features are very likely linked to an interruption of electron transport: (i) slow growth of small colonies because cell wall synthesis requires large quantities of ATP, (ii) decreased pigment formation as carotenoid biosynthesis requires electron transport, (iii) resistance to aminoglycosides such as gentamicin because their uptake requires the large membrane potential generated by electron transport, and (iv) mannitol fermentation negative because utilization of a sugar alcohol as mannitol is decreased when electron transport is not used [33, 34]. The morphotype of clinically and experimentally-derived SCVs on solid agar is often due to auxotrophy for thymidine, thiamine, menadione, or hemin [9, 13, 14, 33-35]. When the medium is supplemented with these compounds, SCVs grow as rapidly as the parent strains. Thiamine, menadione, and hemin are required for biosynthesis of electron transport chain components, while the mechanism for thymidine auxotroph is not related to the electron transport.

Concerning the components of the electron transport chain as depicted in Figure 2, menadione is isoprenylated to form menaquinone, the acceptor of electrons from nicotinamide adenine dinucleotide (NADH)/flavin adenine dinucleotide (FADH$_2$). Hemin is required for the biosynthesis of cytochromes, which accept electrons from menaquinone and transport them to the ATP synthesis complex in the cell membrane. Thiamine is required for menadione biosynthesis; hence, thiamine auxotrophs are also menadione auxotrophs. Many previous and recent reports also noted decreased respiratory activity in staphylococcal SCVs, which is also consistent with reduced electron transport activity [9, 17, 18, 22, 33, 34, 36-38].

Thymidine-dependent SCVs are often associated with prior trimethoprim-sulfamethoxazole (TMP/SMX) therapy [13, 39, 40]. TMP/SMX interferes with the tetrahydrofolic acid (THF) pathway. THF acts as a co-enzyme for thymidylate synthetase, which catalyzes the synthesis of dTMP from dUMP [41]. Therefore, TMP/SMX therapy inhibits the synthesis of dTMP or thymidine. Since thymidine is essential for DNA synthesis, susceptible strains are affected by TMP/SMX treatment. Interestingly, if extracellular thymidine is provided, as likely present in bronchial secretions from cystic fibrosis (CF) patients with pus, neutrophils and dying cells, some bacteria bypasses the blocked pathway by using extracellular thymidine resources thereby surviving TMP/SMX therapy. However, in the absence of thymidine the bacteria are not able to grow. These mutations and/or changes in regulation of important *S. aureus* regulators apparently lead to the absolute dependency upon extracellular thymidine resources in the absence of
TMP/SMX in thymidine-dependent SCVs. Moreover, formation of SCV phenotype can also be influenced by differentially expressed non-coding RNAs in wild-type strains of *S. aureus* [42].

**Fig. 2** Metabolic diagram showing selected changes of the carbohydrate metabolism due to the electron transport defect of the SCV mutant leading to an increase of the NADH levels [14].

### 3. Persistent infections due to SCVs of staphylococci

The ability to interrupt electron transport and to form a variant subpopulation gives *S. aureus* a number of survival advantages that extend beyond simply increased resistance to antibiotics. Although SCVs have been recognized for many decades following the first description as dwarf-colony or G variants [12, 17, 43], connecting this phenotype to persistent and recurrent infections has only in recent times been appreciated [14, 33, 44-46]. Proctor and colleagues first reported a model that related the multiple changes in phenotypic characteristics of *S. aureus* SCVs, alterations in electron transport, and the clinical pattern of persistent and relapsing infection [14]. They described five patients with unusually persistent and/or antibiotic-resistant infections due to SCVs of *S. aureus*. All SCV strains were nonhemolytic and nonpigmented and grew very slowly on routine culture media in an ambient atmosphere. Strains available for further studies were shown to be auxotrophs that reverted to normal colony forms in the presence of menadione, hemin, and/or CO₂ supplement [14]. The incidence of SCVs in clinical specimens has been found to range from 1% to more than 30% in different studies. *S. aureus* SCVs were found in about 1% of 1,110 isolates in a general microbiology laboratory [9]. Analysis of sputa from 72 patients with CF has shown that more than 70% (52 of 72) are chronically colonized with *S. aureus*, and of these samples, 46% (24 of 52) contained SCVs [47]. In a study of *S. aureus* recovered from bone specimens or deep-tissue aspirates of patients with osteomyelitis, *S. aureus* SCVs were found in ~29% of patients (4 of 14). So, SCVs are not rare, but they can be difficult to recover [48]. Moreover, an outbreak with methicillin-resistant SCVs of *S. aureus* in an intensive care unit (ICU) was described [49]. The investigators conducted a retrospective study analyzing nine previously identified cases of MRSA SCV-related sepsis occurring in 11-months period. Clinical and epidemiological data were collected and compared to patients with MRSA (non-SCV) infection. MRSA SCVs were typed by ribotyping and random amplification of polymorphic DNA. Comparing SCV and non-SCV infection, the investigators reported a higher hospital mortality (100% vs. 50%, p<0.05) and ICU-mortality (71.5% vs. 12.5%, p<0.05) respectively, in patients infected with SCVs. Compared to the control group, SCV-infected patients received more antimicrobial agents (85.7% vs. 62.5%); other data did not achieve statistically significant differences. While the authors in this study described the first known outbreak with MRSA SCVs, the investigation confirmed previous features of infections due to *S. aureus* SCVs [49].

Most recently, two studies from Turkey and USA revealed high prevalence of *S. aureus* SCVs isolated from cystic fibrosis patients [27, 50] and for the first time a case of infective endocarditis caused by *S. aureus* SCVs in India [28].
3.1. Osteomyelitis infections.

Several investigators have shown that SCVs can often be recovered from cultures of normal *S. aureus* strains that have been exposed to gentamicin or other aminoglycosides [14, 36, 51-57]. Beads containing gentamicin are used as an adjunct to systemic antibiotic therapy and debridement to treat patients with osteomyelitis. The beads release the aminoglycoside slowly (over weeks to months), providing a sustained local concentration of the antimicrobial agent. To examine whether the slow release of gentamicin into the local environment is an efficient way to select for SCVs in vivo, a case–control study was initiated [30]. Bone specimens or deep-tissue aspirates from patients who were suspected to have osteomyelitis were screened for SCVs, and only those patients for whom *S. aureus* was recovered from at least one specimen were included in the study. Fourteen patients who carried *S. aureus* and had clinical signs of chronic osteomyelitis were found in an 18-month period. Menadione- or hemin-auxotrophic *S. aureus* SCVs were recovered only from those patients who had previously been treated with gentamicin-containing beads. Large and small colony types were recovered from three of these patients, and therapy failed despite using antimicrobials with *in vitro* activity against these isolates. By contrast, ten other patients with normal *S. aureus* (and no previous placement of gentamicin-containing beads) had no relapses of osteomyelitis within more than 1 year of primary diagnosis after active antibiotics were given. No other important differences between the two groups were detected in a clinical evaluation. Small digests of the whole bacterial DNA of all isolates from each patient were typed by pulsed-field gel electrophoresis (PFGE), and despite large phenotypic differences, the isolates were found to be clonal. The MICs for gentamicin were up to 32-fold higher for the SCVs (1 µg/ml) than for the parent strain (less than 0.031 µg/ml), whereas no differences were found in susceptibilities to other antimicrobial agents [1].

3.2. Cystic fibrosis infections.

CF patients—especially children and adolescents—are often colonized with *S. aureus* [47]. The results of a 6-year prospective study that analyzed the prevalence and persistence of *S. aureus* in CF patients demonstrated that the airways of 52/72 (72.2%) patients were persistently colonized/infected by normal and SCV *S. aureus* with a median persistence of 37 months (range 6–70 months) [47]. Twenty-eight patients harbored only the normal *S. aureus* phenotype, 22 were infected by isogenic normal and/or *S. aureus* SCVs, and two patients harbored SCVs only. The investigators were able to observe the emergence of SCVs from normal *S. aureus* in two patients. Furthermore, six patients, who initially harbored both normal and *S. aureus* SCVs, subsequently lost the normal strain, while SCVs persisted for extended periods. The longer persistence of the SCV phenotype indicates a survival advantage of SCVs as compared to the normal phenotype in the hostile milieu of the airways, possibly due to optimized adaptation of the SCVs [48, 58]. SCVs were also detected to occur in 42% of patients (8 of 19) with CF who were colonized or infected with *Burkholderia cepacia* [59]. Two of these patients developed fatal systemic infections caused by SCVs after receiving a lung transplant [1, 59]. Most recently, Wolter et al. performed a two-year study of 100 children with CF using culture techniques sensitive for *S. aureus* SCVs, and evaluated associations with clinical characteristics. *S. aureus* SCVs infection was detected among 24% of participants and was significantly associated with a greater drop in lung function during the study (p=0.007) [50]. Also in another CF study conducted in Turkey, the investigators recovered *S. aureus* SCV from 8.1% of CF patients examined during a period of 11 months[27].

3.3. Implant-associated infections.

Staphylococci are the most common causes of medical device-associated infections. Cases of persistent pacemaker-related bloodstream and ventriculoperitoneal-shunt infections caused by *S. aureus* SCVs have been reported [7, 29, 60]. These cases illustrate the poor clinical and microbiological response to prolonged antimicrobial therapy in patients who are infected with these variants. Also, *S. epidermidis* and *S. capitis* SCVs have been linked to implanted medical devices [7, 61, 62]. In patients with prosthetic heart-valve endocarditis and infection of pacemaker electrodes, these SCVs were identified using sequence analysis of a portion of the 16S-rRNA gene [7]. Device-related infections with SCVs respond poorly to antibiotic therapy and can be misidentified by automated diagnostic systems [7, 60]. These device-related infections emphasize that SCVs might also have a role in intravascular-device-related infections. The reported cases also illustrate that complete removal of any foreign-body material is essential for the complete cure of prosthetic intra vascular-device-related infections caused by staphylococcal SCVs, and this idea is consistent with previous *in vitro* models in which *S. aureus* SCVs were found to be almost completely resistant to antibiotics [1, 63]. Most recently, *in vitro* studies demonstrated the ability of *hemB* *S. epidermidis* SCVs mutant to persistently colonize catheter and form abscesses in a mouse model [64].

3.4. Further infection or colonization by SCVs.

The first known case of persistent and antibiotic-resistant skin infection due to different phenotypes and genotypes of *S. aureus*, including clonally different *S. aureus* SCVs was described in 1999 [51]. *S. aureus* strains were derived from a 39-year-old patient with Darier’s disease, who was hospitalized several times due to his continuously worsening skin condition. Over a period of 28 months, 119 *S. aureus* isolates were derived from 53 different clinical specimens,
predominantly obtained from different areas of the affected skin and from the anterior nares. Phenotypic characterization of the isolates showed that hemin-auxotrophic SCVs, as well as different S. aureus strains with normal phenotype, were associated with the skin infection. In contrast to S. aureus that showed typical colony size, pigmentation, and hemolysis on Columbia agar, S. aureus SCVs grew as nonhemolytic, nonpigmented, tiny colonies. In addition to these strains, S. aureus that exhibited typical colony size (but not pigmentation) and hemolysis were recovered as well as SCVs that grew as pinpoint colonies but exhibited pigmentation and hemolysis. Moreover, methicillin resistance was found in isolates of both the normal and the SCV phenotype. SCVs had up to 32 times higher gentamicin MICs than clonally identical strains of the normal phenotype. Molecular typing revealed seven genotypes involved over this period, including four different SCV genotypes. One clone, growing on one occasion as a pinpoint colony and another as S. aureus with the normal growth phenotype, persisted over 18 months; the other clones were isolated during periods of 1 week and 5, 7, 13, and 16 months. One of the two SCVs selected for an intracellular persistence assay within keratinoctyes belonged to the clone that persisted in the infected patient for 18 months. This SCV was internalized as assessed by transmission electron microscopy, and it persisted intracellularly in contrast to the normally growing strain [51]. More recently, a menadione auxotrophic S. aureus SCV was isolated from a 3 1/2 year old female patient that was admitted in the pediatric ward in Bankura, West Bengal, India with symptoms of respiratory distress, swelling of the legs and fever. Chest auscultation showed pansystolic precordial murmur. Echocardiography was carried out which revealed a subaortic ventricular septal defect (VSD). Antibiotic susceptibility test showed sensitivity to tetracycline, amikacin, erythromycin, cotrimoxazole and ofloxacin and resistance to oxacillin. The SCV morphotype was selected since the patient was on aminoglycoside antibiotic treatment. This is the first report of S. aureus SCV from blood of a patient with VSD having infective endocarditis [28]. In conclusion, the intracellular persistence of S. aureus SCVs and its phenotype switching considered as an effective bacterial strategy to escape host immune response and establish a chronic infection [32].

4. Identification of SCVs in clinical laboratory

For S. aureus with normal phenotype normally no requirements for more sophisticated genotypic methods or molecular phenotypic procedures are needed in order to achieve accurate species identification. However, recovery and identification of SCVs may be difficult because of their fastidious growth characteristics. For SCVs extended conventional culture and identification techniques are needed. SCVs are rapidly overgrown and are easily missed when the normal S. aureus is present since SCVs grow about nine times slower than S. aureus with normal phenotype [33, 36].

The variants may be identified by their atypical colonial morphology with tiny, mostly nonpigmented and nonhemolytic colonies slowly growing following 24-72 hours incubation on rabbit blood agar. Unusual biochemical reactions (mannitol salt agar negative), and reduced coagulase activity (incubation for > 18 hours is needed) are also typical features of the variants as compared to the S. aureus isolate with normal phenotype. Because some SCVs grow more rapidly in the presence of CO₂ and on rich medium such as Schaedler’s agar which contains hemin, SCVs can be mistakenly identified as anaerobic organisms [7, 17, 18, 22]. Due to the characteristics of SCVs described above, isolates suspicious for S. aureus SCVs should be confirmed as S. aureus molecularly by testing the species-specific nuc and coa genes [65, 66]. A technique for in situ detection and identification of S. aureus SCVs was reported based on an in situ hybridization method with fluorescence-labeled oligonucleotide probes specific for staphylococcal 16S rDNA [67, 68].

Fourier-Transform Infrared Spectroscopy (FTIR) allowed a rapid and reproducible tool for the examination of different subpopulations of S. aureus on solid and in broth media. Phenotypically, the SCVs of S. aureus gave an FTIR fingerprint that was easily recognizable and was different from their parent strains. This technique could be used as a noninvasive approach to investigate dynamic processes of reversion of SCVs to the normal phenotype and vice versa [69].

Auxotrophy for hemin may be tested by using standard disks, and auxotrophy for thymidine or menadione by impregnating disks with 15 μL of thymidine at 100 mg/L or menadione at 10 mg/L. Test isolates should be inoculated on chemically defined medium and/or on Mueller-Hinton agar as described previously [23].

Concerning susceptibility testing, SCVs also present a challenge. Due to the different doubling times of normal and SCV phenotype, even a small percentage of normally growing organisms will rapidly replace the SCVs in liquid medium in an overnight culture. Hence, the SCVs may be overgrown to such an extent that they may not be included in the inoculum used for susceptibility testing [63]. In addition, because of the slow growth of the SCVs, phenotypical tests for susceptibility testing such as disc diffusion, Etest, microdilution test, determination of MICs by automated susceptibility testing systems (e.g. by VITEK®2) as well as slide latex agglutination tests (e.g. for determination of MRSA) may fail to detect SCVs as resistant to defined antimicrobial agents [33, 36, 63]. Therefore, the detection of antibiotic resistance genes such as the mecA gene [70] combined with the detection of genes specific for S. aureus such as the nuc gene [65] by molecular methods should be used for reliable diagnosis and susceptibility testing of S. aureus SCVs. Kipp et al. showed that only detection of the mecA gene by PCR and the MRSA-Screen latex agglutination test...
using a high inoculum were shown to be reliable methods to rapidly detect methicillin resistance in these variants [71, 72].

For molecularly typing S. aureus with SCVs phenotype, same typing techniques such as PFGE, spa typing and multilocus sequence typing that used for typing of S. aureus with normal phenotype can be used. Most of the previous phenotypic and genotypic methods and techniques can be used in the identification and diagnosis of CoNS SCVs.

5. Antibiotic resistance and therapy of staphylococci SCVs

Interruption of electron transport reduces the electrochemical gradient across the bacterial membrane, resulting in a decreased uptake of antimicrobial agents that require a charge differential to be active, such as aminoglycosides. Therefore, these substances should not be used, although single strains with SCV phenotype might be susceptible to gentamicin or other aminoglycosides. To investigate the reduced aminoglycoside susceptibility, the membrane potential (ΔΨ) of clinical SCVs was analyzed since ΔΨ is involved in aminoglycoside uptake [52]. SCVs growing in a chemically defined medium with glucose and enhanced buffering capacity generated an initial ΔΨ of ~120 to ~140 mV, which is comparable to the parent strains. However, once glucose was consumed, the membrane potential dropped below ~100 mV. Accordingly, the susceptibility of SCVs to aminoglycosides dropped 10–30-fold when compared to the parent strain. S. aureus SCVs are often also more resistant to cell wall active antibiotics [33]. Slow growth, and hence reduced cell-wall division, reduces the efficacy of β-lactam antibiotics [33, 36]. To test whether SCVs acquire chromosomally encoded resistance phenotypes differently from parent strains with normal phenotype, the mutation rates and the accumulation of mutations in the target genes of isolates exposed to ciprofloxacin, rifampicin, and mupirocin were investigated. The in vitro activities of these compounds were measured in SCVs and their corresponding parent strains before and after ten serial passages in antibiotic-containing medium, followed by sequencing of the target genes [73]. All isolates tested became resistant to ciprofloxacin, rifampicin, and mupirocin.

Rates of appearance of colonies with higher MICs were in the range 10^{-5} to 10^{-6} for ciprofloxacin, 10^{-6} to 10^{-7} for rifampicin, and 10^{-7} for mupirocin. Differences in mutation rates or MICs were not detected between SCVs exhibiting different aminoglycosides and their clonally identical parent strains with normal phenotype, indicating that this phenotype does not affect the development of ciprofloxacin, rifampicin or (low-level) mupirocin resistance in S. aureus [73]. Optimal therapy for infections due to S. aureus SCVs has not yet been defined. Reversion to the normal colony form may influence these microorganisms to be more susceptible to antibiotics. In the case of menadione auxotrophs, this reversal might be accomplished by administering vitamin K to patients. However, whether this will prove to be beneficial remains to be determined by clinical trials [48].

Recently, investigators evaluated the pharmacodynamics of daptomycin against defined S. aureus mutants displaying the SCVs phenotype and their parental strains with normal phenotype. They found that the bactericidal activity was achieved rapidly, within 2 h at concentrations > or =16 times the MIC against strains with normal phenotype, while against SCVs, bactericidal activity was achieved within 6 h at concentrations > or =16 times the MIC. They concluded that daptomycin represents a potential therapeutic option for infections caused by S. aureus strains displaying the SCV phenotype [74]. More recently, Garcia et al. have studied the intracellular fate of menD and hemB mutants of the COL methicillin-resistant S. aureus strain and the antibiotic pharmacodynamics profile against extracellular (broth) and intracellular (human THP-1 monocytes) bacteria. Compared to the parental strain, SCVs showed slower extracellular growth, reduced phagocytosis, and, for the menD SCV, lower intracellular counts at 24 h post-infection. Against extracellular bacteria, daptomycin, gentamicin, rifampin, moxifloxacin, and oritavancin showed similar profiles of activity against all strains, with a static effect obtained at concentrations close to their MICs and complete eradication as maximal effect. In contrast, vancomycin was not bactericidal against SCVs [75].

However, daptomycin and vancomycin pharmacodynamics were evaluated against a site-directed hemB mutant of S. epidermidis displaying the SCV phenotype and compared to that of the parental strain. The MICs of vancomycin were 2.0 and 4.0 mg/liter and of daptomycin were 0.25 and 0.25 mg/liter against the parental strain and the SCV mutant, respectively. The maximal killing effect decreased by 7.7-fold for vancomycin and 1.5-fold for daptomycin against the SCV mutant [76].

6. Characterization of a site-directed S. aureus hemB mutant with SCV phenotype

To characterize the phenotype of a genetically defined SCV of S. aureus and to test the hypothesis that defects in electron transport promote the development of intracellular persistence, von Eiff et al. generated a stable mutant in electron transport by interrupting hemB in S. aureus [34]. Heme is the prosthetic group of cytochromes, which plays an essential role in electron transport and the hemB gene is a member of the family of genes encoding enzymes of the porphyrin biosynthetic pathway.

The S. aureus hemB mutant mimicked the typical characteristics of clinical SCVs: (i) tiny colonies on solid agar (>10 fold smaller than the parent strain) and slow growth in liquid medium such as TSB or CDM; (ii) decreased pigment formation (whitish colonies versus golden yellow colored colonies of the parent strain); (iii) reduced hemolytic activities of these compounds were measured in SCVs and their
activity (> 90-fold reduction in percentage of lysis of RBCs compared with the parent strain 8325-4 [0.25% vs. 89%]); (iv) decreased coagulase activity (delayed coagulase reaction in the tube coagulase test, being positive after 22 h incubation at 37°C); (v) resistance to aminoglycosides (MIC for gentamicin was 16-fold higher for the mutant [0.5µg/ml] compared to the wild-type strain [<0.031 µg/ml]) and MIC for kanamycin was 8-fold higher for the mutant [MIC = 2.0 µg/ml] compared to the wild-type strain [MIC = 0.25 µg/ml]); (vi) changed biochemical characteristics such as reduced lactose-, turanose- and mannitol-fermentation, no nitrate reduction, and reduced N-acetyl-glucosamine utilization (analyzed in the API-systems and with conventional biochemicals). All features of the SCV phenotype were essentially reversed by growing the hemB mutant with hemin at a concentration of 1 µg/ml or by complementation of the mutant with intact hemB gene. In addition, Western blot analysis showed that α-toxin is produced in the parent strain, but was not detectable in the hemB mutant. Northern blot analysis, performed to determine whether reduced protein levels correlated with reduced transcription, showed that transcription of hla was high in the parent strain, however, not detectable in the non-complemented mutant. Finally, in a model of endovascular infection to determine the intracellular persistence, it was demonstrated that > 200-fold more hemB-mutant cells persisted intracellularly after 24 or 48 h incubation relative to the parent strain [34]. More recently, microarray analysis of SCV hemB mutants and their parental strains in endothelial cells in vitro showed that wild-type phenotypes up-regulated a large number of endothelial genes (including genes involved in innate immunity), whereas the SCVs did not cause these dramatic changes. The inflammatory response and cytotoxicity were strongest shortly after infection and largely decreased within the following days, which was accompanied by a fast elimination of intracellular wild-type bacteria. By contrast, SCVs survived within endothelial cells at high numbers which could be a reservoir for chronic infections [34].

The adhesions factors of the hemB mutant was studied [77]. Both adhesion to fibrinogen and fibronectin was shown to be significantly higher for the hemB mutant compared to its isogenic normally growing parent and correlated with the increased surface display of these adhesions assessed by flow cytometry. Real-time quantitative RT-PCR demonstrated increased expression of clfA and fnb genes by the hemB mutant compared to its isogenic parent. In addition, hemB mutants tested were also more efficiently internalized by human embryonic kidney cells as compared to their isogenic controls, presumably because of increased surface display of their adhesions [77]. While the studies with clinical isolates of SCVs suggested a link between electron transport defective strains and persistent infections, investigations with the defined hemB mutant displaying the SCV phenotype provided definitive evidence for these connections. The hemB mutant was phagocytized by cultured endothelial cells, but did not lyse these cells, because the mutant produced very little α-toxin (as shown on protein and transcription level). The intracellular location may shield the SCVs from host defenses and the effectiveness of antimicrobial agents, thus providing one explanation for the difficulty in clearing S. aureus SCVs from host tissues [30-32, 35, 78].

To assess the virulence of the hemB mutant of S. aureus, a murine septic arthritis model was performed [79]. For this purpose, mice were inoculated intravenously with either the wild type strain or with its hemB mutant mimicking the SCV phenotype. Mice inoculated with the hemB mutant displayed significantly lower severity (but not frequency) of arthritis on day three (p< 0.05), compared to mice inoculated with the wild type strain. Seventeen days after the inoculation, the SCV inoculated mice displayed also significantly lower bacterial burden in their kidneys and joints compared to the parent strain. However, there were no significant differences in mortality rate or weight decrease between the mutant and the parental strain inoculated mice. It was concluded that the finding that the mutant was almost as virulent as its isogenic parent strain might be due to increased amounts of protease production by SCVs, which would attack the joint cartilage [79]. In another animal model, investigators used a hemB mutant for their study which was essentially identical to that described above. The mutant was found to cause a persistent and antibiotic-resistant mastitis in mice [80]. The mutant invaded the epithelial cells as the parent strain, but it produced a more persistent and antibiotic refractory-infection. Finally, in a rabbit endocarditis model, the hemB mutant was compared to a menD mutant displaying the SCV phenotype, too [81]. While the infectivity of both mutants for heart valves was comparable to the parent strains, the menD mutant showed increased persistence in the kidneys and spleens in the presence of antibiotic therapy. The difference in persistence between the hemB and menD mutants probably arose from the repletion with hemin derived from lysed erythrocytes in emboli, which circumvents the hemB knockout-induced defect in the cytochrome system [81]. Regarding biofilm production in a menadione-auxotrophic S. aureus SCVs, the biofilms formed were highly structured, consisting of large micro-colonies separated by channels, and contained more biomass as well as significantly more polysaccharide intercellular adhesion (PIA) than its wild-type biofilms. The autoaggregation and increased biofilm-forming capacity of menadione-auxotrophic S. aureus SCVs was related to the enhanced production of PIA in these variants. This is the first report indicating the augmented expression of PIA in menadione-auxotrophic S. aureus SCVs [82].

Recently, Hilmi et al. studied and compared biofilm formation and immune stimulatory capacity in consecutive SCV isolates originating from a single patient. Despite the relatedness of the isolates, their results revealed significant differences in biofilm formation and immune stimulation determined by Toll-like receptor-2 activity between normal and SCV phenotype of clinical S. aureus isolate. The investigators stated that these variations in the extent of biofilm production could be attributed to differences in the expression of protein A (spa) and accessory gene regulator A (agrA) [83].
7. Characterization of a site-directed \textit{S. epidermidis} hemB mutant which mimics the SCV phenotype

While coagulase-negative staphylococci with their ability to form a thick, multilayered biofilm on foreign bodies have been identified as the major cause of implant-associated infections, no data are available for biofilm formation of staphylococcal small-colony variants. In the past years, a number of device-associated infections due to staphylococcal SCVs were described, among them several pacemaker infections due to SCVs of CoNS auxotrophic to hemin. To test the characteristics of SCVs of CoNS and in particular to study the ability of SCVs to form a biofilm on foreign bodies, Al Laham \textit{et al.} generated a stable mutant in electron transport by interrupting one of the hemin biosynthetic genes, \textit{hemB}, in \textit{S. epidermidis}. In fact, this mutant displayed a stable SCV phenotype with tiny colonies that are approximately 10- to 15-fold smaller than those of the parent strain. SCVs colonies showed strong adhesion to the agar surface and were difficult to detach with a loop, in contrast to the parental strain and the plasmid-complemented mutant. Interestingly, the SCV phenotype of the mutant was nearly restored to the wild-type phenotype (parent strain) by growth on Columbia blood agar, most probably due to uptake of hemin from red blood cells [84].

The \textit{hemB} mutant with SCV phenotype revealed considerable changes in biochemical characteristics, such as negative glucose, maltose, lactose, turanose, and mannitol fermentation; reduced fructose and sucrose fermentation was also noted. Moreover, no nitrate reduction or \textit{N}-acetylglucosamine utilization was observed, even after incubation for 48 h or 72 h. When tested for enzymatic activity by the API ZYM gallery, the SCV mutant showed increased activity of several enzymes; e.g., esterase and esterase lipase activities were higher than those of the parent strain with normal phenotype.

Testing for antibiotic resistance with Etest strips and the microdilution method, the MIC of gentamicin was $>20$-fold higher for the mutant with SCV phenotype (1.5 µg/ml) than for the parental strain with normal phenotype (0.064 µg/ml). The MICs of kanamycin, amikacin, and tobramycin were up to 32-fold higher for the SCV mutant (32 µg/ml) than for the parental strain (1 µg/ml) [84].

Regarding biofilm production, the SCV mutant formed weak biofilm in comparison to the parental strain. However, extending the incubation time up to 48 h or using a higher inoculum, the mutant produced significantly more amounts of biofilm on polystyrene in comparison to the parental and plasmid-complemented mutant strains. When grown under planktonic conditions, the mutant formed markedly larger cell clusters than the parental strain which were completely disintegrated by the specific beta-1,6-hexosaminidase dispersin B, but were resistant to trypsin treatment. In a dot blot assay, the mutant expressed larger amounts of PIA than the parent strain as shown in Figure 3.

![Fig. 3](image.png)

\textbf{Fig. 3} Semiquantitative detection of cell wall-associated PIA in \textit{S. epidermidis} O-47, the \textit{hemB} mutant, and the complemented mutant. Whereas PIA is detected in \textit{S. epidermidis} O-47 and the complemented mutant at dilutions of up to 1:8 and 1:4, respectively, a much stronger signal was found in the \textit{hemB} mutant at dilutions of up to 1:16. PIA-positive reference strain \textit{S. epidermidis} 1457 served as a control [84].

So, interrupting a hemin biosynthetic gene of \textit{S. epidermidis} resulted in a SCV phenotype which was comparable to that of the hemin-auxotrophic SCVs recovered from patients with persistent infections. Markedly larger cell clusters and the ability of the \textit{hemB} mutant to form biofilm are obviously related to the augmented expression of PIA. Thus, both SCV phenotype and biofilm formation might be regarded as different strategies of the bacteria to enable the effective survival in the host [84]. In recent study, Gunnar \textit{et al.} studied the catheter colonization and abscess formation due to \textit{S. epidermidis} SCV in a mouse model [64]. They investigated the impact of \textit{S. epidermidis} SCV on colonization of implanted PVC catheters and abscess formation in three different mouse strains and found that SCV colonization was
highest in CD-1 mouse strain. Further, this mutant showed higher resistant rate to vancomycin and daptomycin and higher implant-associated infections in comparison to its parental strain [64, 76].

References


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