Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections

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Abstract | Small colony variants constitute a slow-growing subpopulation of bacteria with distinctive phenotypic and pathogenic traits. Phenotypically, small colony variants have a slow growth rate, atypical colony morphology and unusual biochemical characteristics, making them a challenge for clinical microbiologists to identify. Clinically, small colony variants are better able to persist in mammalian cells and are less susceptible to antibiotics than their wild-type counterparts, and can cause latent or recurrent infections on emergence from the protective environment of the host cell. This Review covers the phenotypic, genetic and clinical picture associated with small colony variants, with an emphasis on staphylococci, for which the greatest amount of information is available.

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Bacterial small colony variants (SCVs) were first described in 1910, as an aberrant form of *Eberthella typhosa* (now known as *Salmonella enterica* serovar Typhi, *S. typhi*)[1,2]. As the name implies, the most conspicuous feature of these SCVs was the formation of colonies that were nearly one-tenth the size of the colonies associated with wild-type bacteria (FIG. 1). Since their first description, SCVs have been described for a wide range of bacterial genera and species, including *Staphylococcus aureus*[3], meticillin (formerly methicillin)-resistant *S. aureus*[4], *Staphylococcus epidermidis*[5], *Staphylococcus capitis*[6], *Pseudomonas aeruginosa*[7], *Burkholderia cepacia*[8], *Salmonella* serovars[9], *Vibrio cholerae*[10], *Shigella* spp.[11], *Brucella melitensis*[12], *Escherichia coli*[13], *Lactobacillus acidophilus*[14], *Serratia marcescens*[15] and *Neisseria gonorrhoeae*[16] (TABLE 1). Furthermore, SCVs from many genera and species have been recovered from clinical specimens, including abscesses[17–25], blood[6,10,23,26–32], bones and joints[23,33–38], the respiratory tract[4,26–28,39–41] and soft tissues[4,21,24,25,27,36,42–46].

Initially, research focused on explaining the slow growth rate of SCVs, and strains were examined to identify a requirement for a specific substance that could restore wild-type growth rates. Compounds such as menadione, haemin, thiamine, unsaturated fatty acids and CO_2 were found to stimulate the growth of SCVs[47–49], with most isolates examined showing growth stimulated by the first three compounds[50]. These early studies also characterized SCVs as having a lower capacity for oxidative phosphorylation than wild-type strains[50]. In light of the modern understanding of bacterial metabolism, the auxotrophic data from SCVs point to defects in pathways that are involved in electron transport: menadione is used to form a quinone (menaquinone) and haemin, and thiamine is required for menadione biosynthesis. The clinical importance of SCVs that are defective in electron transport was first recognized when a group of patients who were infected with persistent, recurrent and antibiotic-resistant *S. aureus* SCVs was described[47].

This Review covers the clinical importance of SCVs, summarizes the information that links electron-transport deficiencies and thymidine biosynthetic deficiencies to the SCV phenotype, and provides a perspective on the future of research on SCVs. Although SCVs have been associated with many genera of bacteria, they have been most extensively studied for staphylococci, so *S. aureus* is used as an example organism in this Review. As appropriate, data obtained from studying other organisms are provided to augment or to contrast the biochemical and genetic data that are presented for *S. aureus*.

Auxotrophic phenotypes of SCVs

Although many alterations in bacterial metabolism cause slow growth, only a limited number of defects are found in SCVs from clinical specimens, with two groups of SCVs being consistently recovered: SCVs that are deficient in electron transport, and SCVs that are

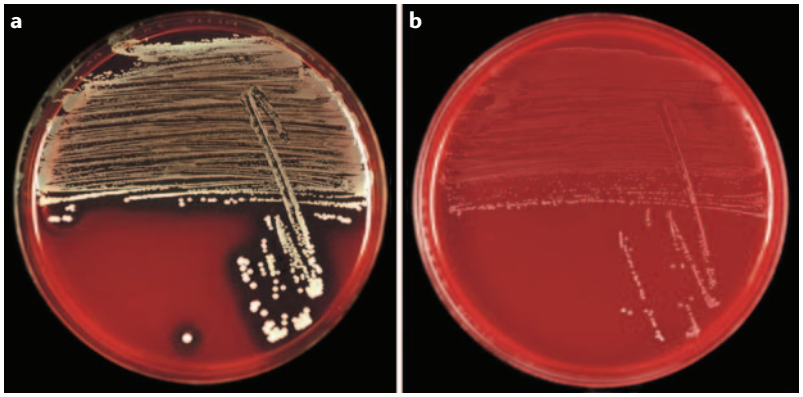

Figure 1 | **Small colony variants.** Columbia blood-agar plates that show the normal (**a**) and the small colony variant (**b**) phenotype of *Staphylococcus aureus* are shown.

deficient in thymidine biosynthesis (FIG. 2). The electron-transport-defective SCVs are defective in the biosynthesis of menadione or haemin, and this phenotype can be reversed by supplementation with menadione or haemin (discussed later), as is typical for auxotrophic defects. Additional features of the typical *S. aureus* SCV phenotype include decreased respiration, decreased pigmentation, decreased haemolytic activity, decreased coagulase activity, increased resistance to aminoglycosides, and an unstable colony phenotype[47–49]. As is shown in FIG. 2, growth rate, aminoglycoside susceptibility, pigmentation and toxin production can be linked to electron transport, thereby providing a unifying hypothesis for the phenotype (discussed in more detail in the next section). Because thiamine is required for the biosynthesis of menadione, SCVs that are auxotrophic for thiamine are a subtype of menadione auxotrophs.

Thymidine-auxotrophic SCVs have a phenotype that is nearly identical to SCVs with a defect in electron transport, and the basis for this is not understood. It is suspected that thymidine auxotrophs are double mutants, because they can be recovered from pus, which is rich in thymidine released by the activity of *S. aureus* DNase. The model in FIG. 2 shows that the uptake of thymidine by *S. aureus* SCVs occurs through NupC, a ten-membrane-spanning protein that requires an electrochemical gradient to facilitate thymidine uptake[51,52]. In *Bacillus subtilis*, mutation of *nupC* causes a decrease in pyrimidine uptake to one-fifth the normal amount[51]. Similar to *B. subtilis*, only one *nup*-family gene is found in the *S. aureus* genome, whereas the *E. coli* genome has two: *nupG* and *nupC*[51]. Although the electrochemical gradient and the requirement for menaquinone in thymidine biosynthesis provide some connection between the two types of SCV, the exact link between them is an area of continuing investigation.

Other SCVs have occasionally been isolated (for example, CO_2 auxotrophs[19–21,25,28,53]), and SCVs for which the auxotrophism cannot be defined have also been found. CO_2 is a non-specific stimulant for *S. aureus* growth[54], and it is found in anaerobic chambers *in vitro*. We have not found CO_2 auxotrophs in our studies, and reports of such findings might stem from non-specific stimulation of growth, which could include stimulation of electron-transport variants. We suspect that SCVs might also arise from other defects (such as defects in F_0F_1-ATPase and cytochromes) that would not result in auxotrophy for menadione or haemin yet would result in a deficiency in electron transport.

Electron-transport-defective SCVs

Several genetic mutations can produce the electron-transport-defective SCV phenotype: mutations in *menD*, *hemB* and *ctaA*[55–57]. Mutations in *menD* and *hemB* block the biosynthesis of menadione, which is used in menaquinone biosynthesis, and haem, which is used in cytochrome biosynthesis (FIG. 3); both menaquinone and haem are components of the electron-transport system. The *ctaA* mutation blocks the biosynthesis of haem A[57], which is also involved in cytochrome biosynthesis, and *ctaA* mutants have features of a typical SCV, including reduced colony size, reduced expression of the regulatory RNA RNAIII, decreased production of α-toxin and toxic-shock-syndrome toxin 1, and increased aminoglycoside resistance. Interruption of *hemL* in *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) results in SCVs that produce persistent infections in an animal model[58]. In addition, an *E. coli* SCV with a mutation in *hemB* was recovered from the site of a chronic and recurrent hip infection[38].

Defining the precise genetic lesion in *S. aureus* SCVs from clinical isolates has proven difficult, but reversal of the phenotype by supplementation provides strong support for the defect being in the menadione and haemin biosynthetic pathways. The menadione-auxotrophic phenotype can be reversed by supplementation with

Table 1 | **Small colony variants of non-staphylococcal bacteria recovered from human specimens**

Microorganism	Type or site of infection and/or specimen	References
Brucella melitensis	Subacute bacterial endocarditis (blood culture)	12
Burkholderia cepacia	Lung and other airway specimens from patients with cystic fibrosis	8
Burkholderia pseudomallei	Experimental melioidosis	87
Escherichia coli	Chronic prosthetic-hip infection; urinary-tract infection; human faeces	13,38,120,121
Lactobacillus acidophilus	Human faeces	14
Neisseria gonorrhoeae	Gonorrhoea (urethra, cervix and vagina)	68
Pseudomonas aeruginosa	Lung and other airway specimens from patients with cystic fibrosis	86,88,89
Salmonella serovars	Typhoid fever	2,122

O-succinylbenzoate, but not with isochorismate, thereby indicating that the block in the menadione biosynthetic pathway occurs between these two compounds[59]. We expect that defining the specific genetic changes that are involved will help our understanding of the ability of SCVs to revert to the parental phenotype.

To characterize the phenotype of a genetically defined *S. aureus* SCV and to test the hypothesis that defects in electron transport promote the development of intracellular persistence, a stable electron-transport mutant was generated by interrupting *S. aureus hemB*[55]. This hypothesis arose from the observation that SCVs were found to cause recurrent infections many years after the initial infection[47]. *S. aureus* SCVs were also found to persist in mammalian cells *in vitro*, indicating that the recurrent infection might be a consequence of persistence in the protective environment of the host cell[59]. Haem is the prosthetic group of cytochromes, which have an essential role in electron transport, and *hemB* encodes one of the enzymes that are involved in biosynthesis of the porphyrin ring that is present in the haem prosthetic group. The *S. aureus hemB* mutant mimics the typical characteristics of clinical SCVs: tiny colonies on solid agar, and slow growth in liquid medium; decreased pigment formation; reduced haemolytic activity (0.25% versus 89% for wild-type *S. aureus*); decreased coagulase activity; increased resistance to aminoglycosides (minimum inhibitory concentration (MIC) for gentamicin is

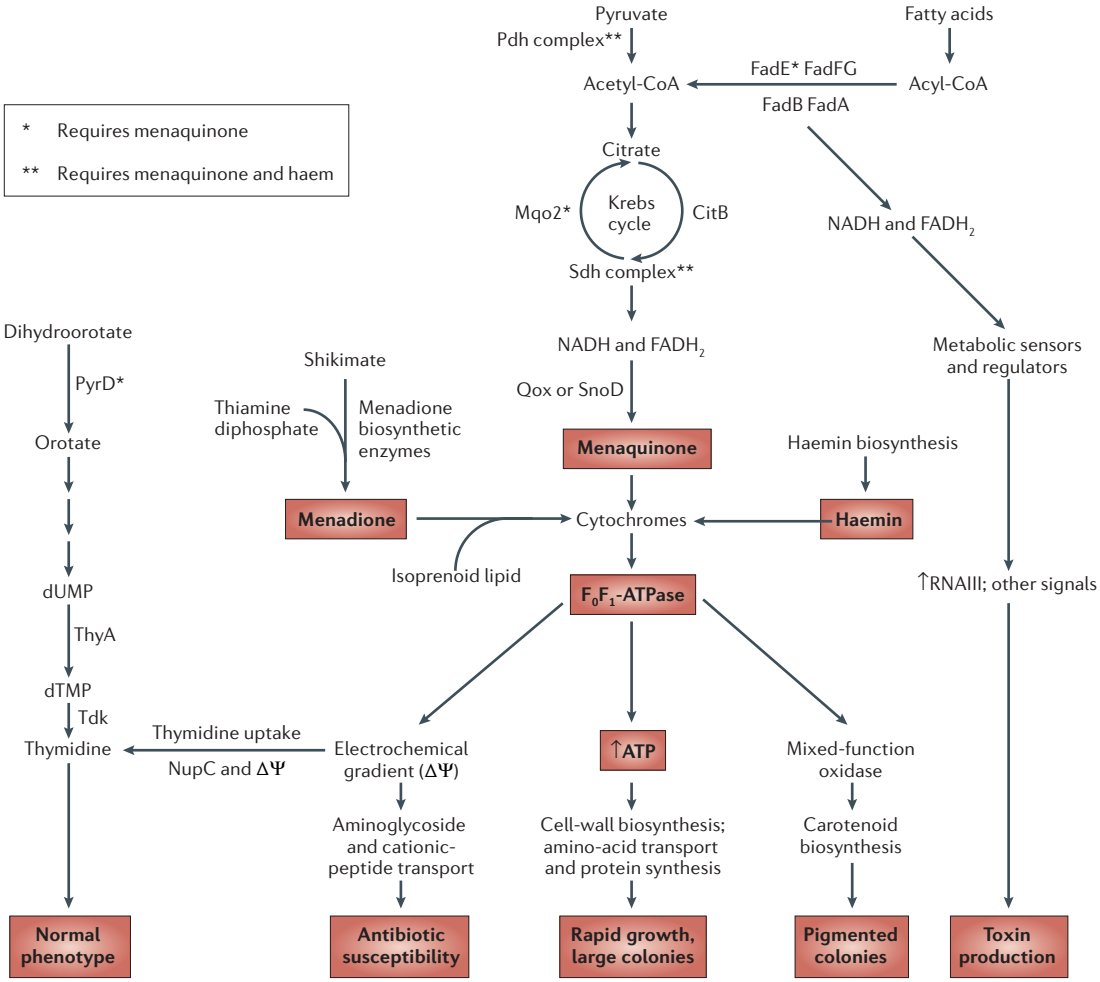

Figure 2 | **Hypothetical model of the metabolic and energetic pathways that are associated with the small colony variant (SCV) phenotype.** Defects in electron transport decrease the amount of ATP that is used for cell-wall biosynthesis (leading to a slower growth rate and, therefore, smaller colonies), reduce membrane potential (ΔΨ) (resulting in decreased uptake of cationic compounds) and decrease pigment formation. These associations are clear, but it should be emphasized that there are gaps in our knowledge and that this is a working model. Although *Staphylococcus aureus* SCVs show marked decreases in the regulatory RNA RNAIII, the metabolic sensors that link energetics to toxin production are unknown. Similarly, although thymidine auxotrophs show a phenotype that is almost identical to electron-transport variants, the only link that has been established between these phenotypes is that the uptake of thymidine requires a membrane potential, a feature that is also defective in electron-transport-deficient SCVs. CitB, aconitase; FadA, β-ketothiolase; FadB, 3-hydroxyacyl-CoA dehydrogenase; FadE, electron-transport flavoprotein; FadFG, acyl-CoA dehydrogenase; Mqo2, malate:quinone oxidoreductase; NupC, nucleoside-uptake protein; Pdh, pyruvate dehydrogenase; PyrD, dihydroorotate dehydrogenase; Qox, quinol oxidase (aerobic); Sdh, succinate dehydrogenase; SnoD, staphylococcal Nuo orthologue (microaerophilic NADH oxidase); Tdk, thymidylate kinase; ThyA, thymidylate synthase.

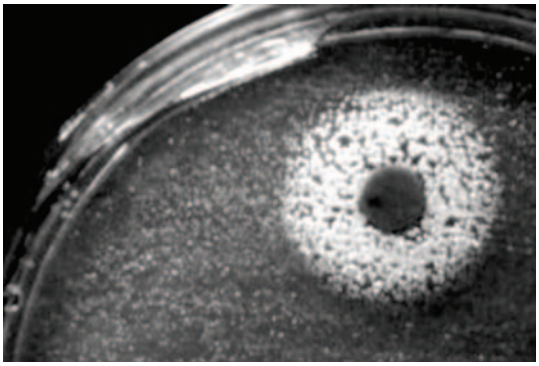

Figure 3 | **Haemin-auxotrophic small colony variants.** Shown is a chemically defined-medium agar plate that illustrates the auxotrophism of *Staphylococcus aureus* small colony variants growing as normal-sized colonies only within the diffusion zone of a disc loaded with haemin.

0.5 μg per ml compared with less than 0.031 μg per ml for wild-type *S. aureus*; and for kanamycin, 2.0 μg per ml compared with 0.25 μg per ml); and atypical biochemical reactions, such as reduced fermentation of lactose, turanose and mannitol, as well as no reduction of nitrate and reduced use of *N*-acetylglucosamine[55].

All of the characteristics of the SCV phenotype are reversed by growing the *hemB* mutant in the presence of haemin at a concentration of 1 μg per ml or by complementing the mutant with intact *hemB*. Western- and northern-blot analyses showed that *S. aureus* α-toxin protein and mRNA were produced in the parent strain but were not detectable in the *hemB* mutant[55]. Consequently, in a model of endovascular infection used to determine intracellular persistence[59], it was shown that, relative to the parent strain, over 200-fold more *hemB*-mutant bacteria persisted in cultured endothelial cells after 24 or 48 hours in the presence of lysostaphin[55]. The intracellular location might shield SCVs from host defences and from antimicrobial agents, thereby providing one explanation for the difficulty in clearing *S. aureus* SCVs from host tissue[40,47,55,59,60].

In a further study, it was shown that a greater number of *hemB* mutants bound to fibrinogen and fibronectin than was the case for the parent strain, and this was found to correlate with increased expression of adhesins at the bacterial surface, as assessed by flow cytometry[61]. Using real-time quantitative reverse-transcriptase PCR (RT-PCR), the *hemB* mutant was shown to have higher expression of the clumping factor A (*clfA*) and fibronectin-binding protein (*fnb*) genes than the wild-type parent. In addition, *hemB* mutants in different *S. aureus* backgrounds were also more efficiently internalized by human embryonic kidney cells than were their parent strains, presumably because of increased surface display of fibronectin-binding adhesins[61].

To study the reduction in aminoglycoside susceptibility, SCVs and their corresponding parent strains, including clinical isolates and defined mutants, were analysed for a decrease in membrane potential ($\Delta\Psi$), because $\Delta\Psi$ is known to facilitate aminoglycoside uptake[7,62,63]. SCVs that were grown in a chemically defined medium with glucose and enhanced buffering capacity generated an initial $\Delta\Psi$ that was comparable to that of their parent strains (−120 to −140 mV); however, after the glucose had been consumed, $\Delta\Psi$ fell as low as −60 mV. Although $\Delta\Psi$ also decreased for the parent strains, it recovered rapidly as Krebs-cycle metabolism became established. SCVs, however, cannot activate the Krebs cycle[64], and they failed to restore $\Delta\Psi$. Accordingly, the susceptibility of SCVs to aminoglycosides was one-tenth to one-thirtieth that of the parent strain. Similar results were found for cationic peptides that require a $\Delta\Psi$ for activity against staphylococci[65]. Cationic peptides in host cells might help SCVs to maintain their phenotype. *S. aureus* SCVs are also more resistant to cell-wall-active antibiotics: slow growth, and therefore reduced cell-wall division, reduces the efficacy of β-lactam antibiotics[66,67].

Most recently, in a proteomic approach, two-dimensional gel electrophoresis combined with matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometry was used to characterize the *S. aureus hemB* mutant[64]. Because the electron-transport chain is interrupted in the *hemB* mutant, it should be unable to use oxygen or nitrate as a terminal electron acceptor. The concentration of proteins involved in the glycolytic pathway (glyceraldehyde-3-phosphate dehydrogenase, enolase and phosphoglycerate kinase) and fermentation pathways (lactate dehydrogenase, alcohol dehydrogenase and pyruvate formate lyase) was increased in all phases of growth in the mutant. So, this study strongly indicates that the *hemB* mutant generates ATP from glucose or fructose by substrate-level phosphorylation. Analyses of the fermentation reactions showed that the main product was lactic acid. Transcriptional analysis of *citB*, which encodes aconitase, showed that its expression was downregulated in the *hemB* mutant. In addition, the arginine-deaminase pathway, an anaerobic pathway that generates ATP and releases ammonia, was induced, and this would help to neutralize the lactic acid that is released by SCVs. It is also of note that the *hemB* mutant expressed significantly reduced amounts of most of the extracellular virulence factors, except proteases and adhesins.

The SCV phenotype that has been described for other species and genera shows many of the same features. These include the following: auxotrophy for haemin, thiamine or menadione (*E. coli*, *N. gonorrhoeae* and *S. typhimurium*)[13,68,69]; reduction in formation of pigment (pyocyanin in *P. aeruginosa*)[7,70]; reduction in fermentation of sugars (*E. coli* and *Shigella* spp.)[7,13,71]; ability to revert to the parent phenotype (*Shigella* spp., *E. coli*, *Proteus* sp., *Klebsiella pneumoniae*, *Providencia stuartii*, *Enterobacter cloacae*, *S. marcescens*, *S. typhimurium* and *Citrobacter freundii*)[10–13,15,16,38,58,71–73]; and reduction in activity of the electron-transport chain, as assessed by decreased respiration, decreased reduction of the dye methylene blue or of tetrazolium dye, decreased oxidative metabolism of sugars and decreased production of ATP (*Shigella* spp., *E. coli*, *Salmonella* serovars and *P. aeruginosa*)[7,38,69,71,72].

In a recent study, NRK-49F fibroblasts were infected with *S. typhimurium*, and 103 SCVs were isolated[58]. Three SCVs that were able to persist intracellularly were found to

have defects in *hemL*, *aroD* and *ldh*. HemL and AroD are involved in haem biosynthesis and menadione biosynthesis, respectively, in *Salmonella* serovars. These SCVs were also found to induce less cell lysis than their wild-type counterparts. Additionally, although the SCVs were less virulent than their wild-type counterparts, they persisted better in BALB/c mice[58]. These data are reminiscent of the phenotype and clinical course of staphylococcal SCVs, and they indicate that the SCV phenotype might be a general mechanism for bacterial persistence in mammals.

Thymidine-biosynthetic-defective SCVs

Thymidine-dependent SCVs have been shown to emerge particularly after long-term trimethoprim sulphamethoxazole (SXT) treatment of patients with cystic fibrosis (CF)[39,41,74]. Analysis of SCVs isolated from the airways of patients with CF showed that 122 of 176 SCVs were thymidine dependent[39,41]. As a result of long-term SXT treatment of patients with CF to suppress infections, the thymidine-dependent SCVs that emerged were SXT resistant, in contrast to the parent strains from the same patient, which were SXT susceptible[39,41]. Although SXT interferes with the tetrahydrofolic-acid pathway, tetrahydrofolic acid is a co-enzyme for thymidylate synthase, catalysing the synthesis of dTMP from dUMP[75]. We have found that a clinical thymidine-auxotrophic strain that produces a typical SCV can be complemented with *thyA* (which encodes thymidylate synthase)[76].

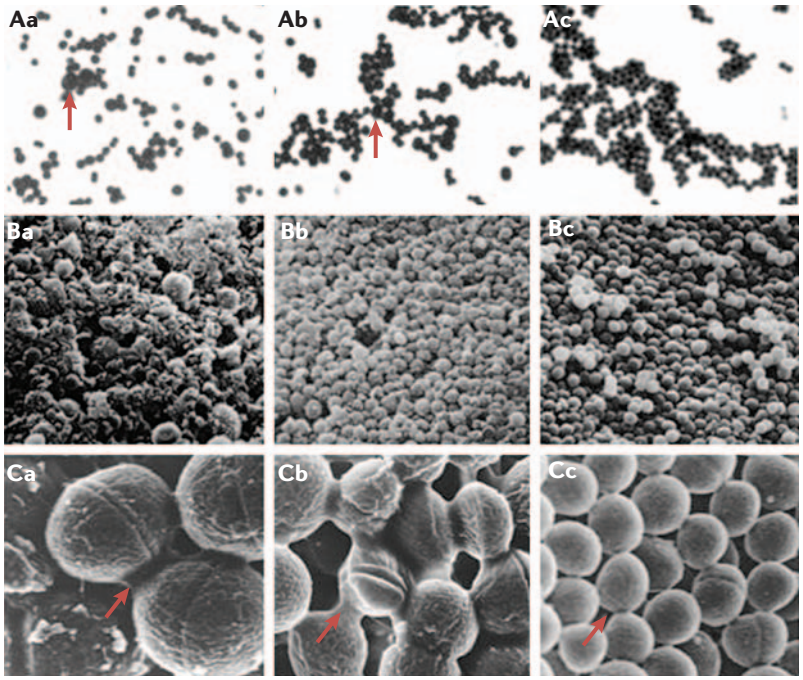

Figure 4 | **Thymidine-auxotrophic small colony variants (SCVs).** Gram stains (**A**) and scanning electron micrographs at low resolution (**B**) and high resolution (**C**) of thymidine-auxotrophic *Staphylococcus aureus* SCVs (**a**,**b**) and the normal *S. aureus* parent strain (**c**) are shown. There are two SCV phenotypes: 'fried egg' SCVs (**a**) and pin-point-colony SCVs (**b**). In **Aa** and **Ab**, arrows point to large cocci. In **B** and **C**, arrows point to the increase in intercellular substance that is present in SCVs compared with normal *S. aureus*. Original magnification of image is ×6,700 (**B**) and ×35,000 (**C**). This image is reproduced, with permission, from REF. 77 © (2003) American Society for Microbiology.

Because dTMP is essential for DNA synthesis, SXT-susceptible *S. aureus* strains are inhibited by therapy with SXT. To resist exposure to SXT, thymidine-dependent SCVs bypass the SXT-inhibited pathway by taking up extracellular thymidine. So, the survival of thymidine-dependent SCVs depends on exogenous thymidine, and thymidine is expected to be abundant in airway secretions of patients with CF, owing to the presence of necrotic cells (which release DNA, which in turn can be digested by *S. aureus* DNase). The phenotype of thymidine-dependent SCVs is related to the amount of thymidine that is present in the medium. We suspect that thymidine-dependent SCVs also have another mutation (in *nupC*) that slows the uptake of thymidine, as proposed in FIG. 2. (*B. subtilis nupC* mutants can take up thymidine, but the rate at which this occurs is one-fifth that of wild-type *B. subtilis*[51].) Moreover, when SCVs leave the pulmonary secretions and enter tissues or cells, the concentration of thymidine would be low, allowing them to maintain the SCV phenotype. If large amounts of thymidine are available, then SCVs grow similarly to wild-type *S. aureus*[39].

Thymidine-dependent SCVs have two colony types: 'fried egg' SCVs, with translucent edges surrounding a smaller elevated, pigmented centre; and pin-point colonies, which are nearly one-tenth the size of normal *S. aureus* colonies[77] (FIG. 4). Gram staining of thymidine-dependent SCVs showed pleomorphic cocci, and electron microscopy showed enlarged cocci with incomplete or multiple cross walls, indicating impaired cell separation[77]. Very similar cell-wall changes were also found in haemin-auxotrophic *S. aureus* SCVs (FIG. 5). All phenotypic changes can be reversed by supplementation with thymidine. Thymidine auxotrophs have also been recovered from non-pulmonary sites in patients who have received antibiotics (but not trimethoprim or sulphamethoxazole)[78]. Transcriptional analysis of the global virulence regulators *agr* and *sarA*, the alternative stress regulator *sigB*, and dependent virulence genes such as *hla* and *spa*, using northern-blot analysis or RT-PCR, showed that expression of these regulators was downregulated in thymidine-dependent SCVs compared with isogenic normal strains of *S. aureus*[79]. Thymidine-dependent SCVs had an agr^- phenotype with increased *spa*, and decreased *hla*, transcription. On supplementation with thymidine, transcription of *agr*, *spa* and *hla* was fully or partially restored in most SCVs. Furthermore, it was possible to activate the *agr* response of SCVs by adding autoinducing peptide isolated from the isogenic wild-type *S. aureus* strain to the SCVs in early log-phase growth. These data show that the *agr* system is inactive in SCVs. That *sigB* and *sarA* expression, which are independent of *agr*, were restored after thymidine supplementation indicates that regulatory mechanisms other than the autoinducing-peptide system were responsible for the various alterations in the expression of regulators. Multiple changes in regulator and virulence gene expression render thymidine-dependent SCVs less virulent but allow them to survive in the hostile environment that is present in the airways of patients with CF, thereby illustrating adaptation of the bacteria during long-term persistence.

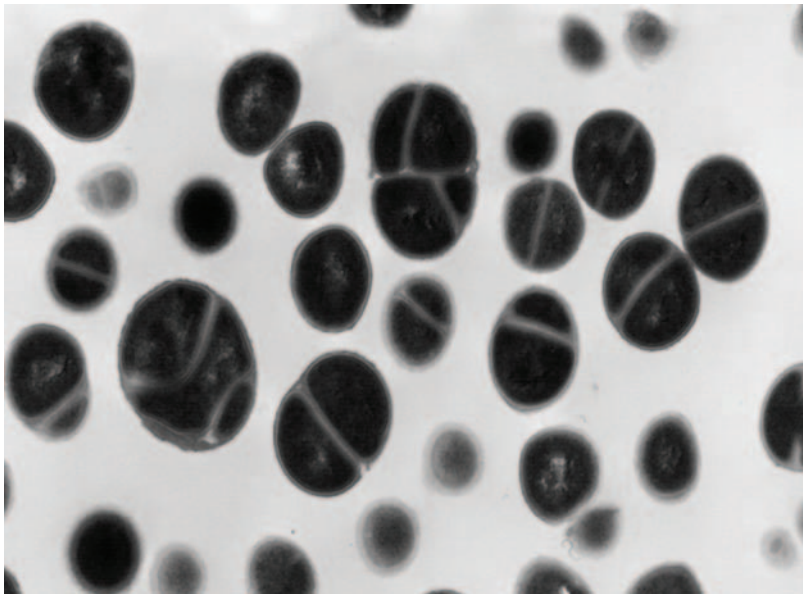

Figure 5 | **Altered cellular morphology of haemin-auxotrophic small colony variants.** Shown is a transmission electron micrograph depicting haemin-auxotrophic *Staphylococcus aureus* small colony variants with incomplete, branched and multiple cross walls, without regular cell separation.

The clinical presentation of SCV infections

That there is a connection between SCVs and persistent, recurrent infections has only been appreciated since the mid-1990s[17,33,39,47,80]. A model that relates the multiple changes in the phenotypic characteristics of *S. aureus* SCVs and the alterations in electron transport to the clinical pattern of persistent and relapsing infection was initially reported in 1995 (REF. 47). Unusually persistent and/or antibiotic-resistant infections due to *S. aureus* SCVs were described for five patients. In further analysis, SCVs were shown to be auxotrophs that reverted to normal colony forms with the addition of menadione, haemin and/or CO_2 (REF. 47). Following this study, interest in *S. aureus* SCVs and coagulase-negative staphylococci blossomed, and many cases and prospective studies were reported[6,18,34,37,40–43,81–85] (see Supplementary information S1 (table)), as were clinical cases of Gram-negative SCVs[8,38,86–90].

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 ~1% of 1,110 isolates in a general microbiology laboratory[23]. 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[41]. 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)[33]. Finally, three *N. gonorrhoeae* SCVs were found in 135 cervical specimens (~0.02%)[68]. So, SCVs are not rare, but they can be difficult to recover (see BOX 1 for details on how SCVs can be identified in the clinical microbiology laboratory).

Disease states associated with SCVs

Cystic fibrosis. Patients with CF, especially children and adolescents, are often colonized with *S. aureus*[39]. The results of a 72-month prospective study that analysed the prevalence and persistence of *S. aureus* in patients with CF showed that the airways of 72% of patients (52 of 72) were persistently infected with wild-type *S. aureus* and *S. aureus* SCVs, with a median persistence of 37 months (ranging from 6 to 70 months)[41]. Twenty-eight patients harboured only wild-type *S. aureus*; 22 patients were infected with isogenic wild-type *S. aureus* and *S. aureus* SCVs; and two patients harboured only *S. aureus* SCVs. Researchers observed the emergence of SCVs from wild-type *S. aureus* in two patients after 36 and 48 months of observation. Furthermore, six patients who initially harboured both wild-type *S. aureus* and *S. aureus* SCVs subsequently lost the wild-type strain, whereas the SCVs persisted for extended periods. The longer persistence of the SCV phenotype indicates a survival advantage for SCVs over the normal phenotype in the hostile milieu of the airways, possibly as a result of optimized adaptation of SCVs.

P. aeruginosa SCVs also have a role in patients with CF. During a 24-month period, *P. aeruginosa* SCVs were isolated from 38% of patients (33 of 86) who carried *P. aeruginosa*[90]. Compared with patients with CF who did not have SCVs, the presence of *P. aeruginosa* SCVs was associated with chronic lung infection, as well as more severely compromised lung function and daily inhalation of tobramycin or colistin. Compared with wild-type *P. aeruginosa*, *P. aeruginosa* SCVs showed increased fitness under stationary growth conditions, increased piliation, increased twitching motility, increased formation of biofilms and greater adhesion to a respiratory cell line[89]. Furthermore, *P. aeruginosa* SCVs showed a marked upregulation of the type III secretion system (as assessed in microarray studies), as well as increased cytotoxicity against macrophages and increased virulence in a mouse model of respiratory-tract infection[88]. Increased expression of the type III secretion system has been associated with increased uptake of *P. aeruginosa* by host cells[88,89,91]. In addition, *P. aeruginosa* SCVs have been shown to be more resistant to aminoglycosides and more damaging to host cells[92]. So, these SCVs present a complex situation in which there is increased resistance to antibiotics and increased host-cell uptake, possibly increasing persistence, but also increased host-cell damage, which would result in increased release from host cells. *P. aeruginosa* SCVs have also been found in a mouse model of corneal infection[92]. When challenged with *P. aeruginosa* and treated with gentamicin, SCVs that can persist in corneal epithelial cells emerge. These SCVs are a focus for reactivation of infection, a feature that is highly reminiscent of *S. aureus* SCVs.

SCVs were also detected to occur in 42% of patients (8 of 19) with CF who were colonized or infected with *B. cepacia*[8]. Two of these patients developed fatal systemic infections caused by SCVs after receiving a lung transplant.

Box 1 | **Isolating and identifying small colony variants in the clinical microbiology laboratory**

In contrast to the normal staphylococcal phenotype, small colony variants (SCVs) have fastidious growth requirements. SCVs can be identified as non-pigmented, non-haemolytic, pin-point colonies after 24–72 hours of incubation on rabbit-blood agar. In addition to their atypical colony morphology, they also typically have a deficiency or a reduction in biochemical reactions (for example, mannitol-salt-agar-negative). Most *Staphylococcus aureus* SCVs are coagulase-positive by the tube test only after incubation for more than 18 hours. So, these uncommon morphological and physiological features of SCVs present a challenge to clinical microbiologists in terms of recovery and identification[115,116].

To avoid misidentification, a prerequisite for the recovery and isolation of SCVs is the application of extended conventional culture and identification techniques[117]. Recently, we showed that the most accurate and rapid method for detection of both the species *S. aureus* and the SCV phenotype of *S. aureus* is to inoculate specimens on both Columbia blood agar and the chromogenic agar *S. aureus* ID agar (bioMérieux SA)[117]. Of note, SCVs are rapidly overgrown and are easily missed when normal *S. aureus* is present, because SCVs divide at about one-ninth the rate of *S. aureus* that have a normal phenotype. Isolates that are suspected to be *S. aureus* SCVs, which might give a false-negative coagulase test, should be confirmed as *S. aureus* by testing for the species-specific genes *nuc* and *coa* or by diagnostic sequencing of 16S rDNA sequences of coagulase-negative staphylococcal SCVs[118]. Another diagnostic approach was shown in a study of a patient with a brain abscess caused by a meticillin-resistant *S. aureus* (MRSA) SCV, in which brain tissue was examined using a 16S-rRNA-directed *in situ* hybridization technique[18].

For several reasons, SCVs also present a challenge with regard to susceptibility testing. First, SCVs are often in a mixed population with normal *S. aureus*. Even when present as a small proportion of the total number of bacteria, normally growing organisms rapidly replace SCVs in liquid medium in an overnight culture, thereby rendering susceptibility testing (as well as recovery) of SCVs difficult[117]. Second, the slow growth rate of SCVs makes standardization of testing difficult, because a slow growth rate alters diffusion tests and times for measuring susceptibility. Last, irrespective of their auxotrophism, errors can occur when these variants are resistant to oxacillin and are tested by disc-diffusion tests, Etests (AB Biodisk), microdilution tests, automated susceptibility-testing systems, as well as anti-penicillin-binding-protein-2a (PBP2a) latex agglutination tests (MRSA-Screen, Denka Seiken)[117,119]. As a consequence, detection of *mecA* by molecular methods, or use of an anti-PBP2a latex agglutination test and a markedly increased inoculum of bacteria (approximately a loopful, with 100–200 SCV colonies), should be applied for reliable diagnosis or validation of MRSA SCVs[117,119].

Osteomyelitis. 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[24,47,59,62,63,65,66,93–95]. 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[33]. 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 haemin-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. *Sma*I digests of the whole bacterial DNA of all isolates from each patient were typed by pulsed-field gel electrophoresis, 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 per ml) than for the parent strain (less than 0.031 μg per ml), whereas no differences were found in susceptibilities to other antimicrobial agents.

In a follow-up study, *S. aureus* SCVs were again often recovered following previous placement of gentamicin-containing beads (C.v.E., R.A.P. and G.P., unpublished observations). However, SCVs were also cultivated from specimens taken from patients who had no previous placement of gentamicin-containing beads, and *S. aureus* with a normal phenotype were also cultivated following local prophylaxis with gentamicin-containing beads, without the simultaneous isolation of SCVs. Nevertheless, although the patient milieu is clearly complex, the results provide evidence that gentamicin-containing bead placement might be an efficient way to select *in vivo* for *S. aureus* SCVs[33].

Device-related infections. Staphylococci are the most common cause of infections associated with medical devices. Cases of persistent pacemaker-related bloodstream and ventriculoperitoneal-shunt infections caused by *S. aureus* SCVs have been reported[6,43,85]. 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[5,6,30]. 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[6]. Device-related infections with SCVs respond poorly to antibiotic therapy and can be misidentified by automated diagnostic systems[6,85]. 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 intravascular-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[96].

The pathogenesis of SCV infections

Uptake by host cells. An intracellular location provides a survival niche for bacteria, because the microorganisms are protected against antibiotic therapy and host defences. In the past decade, several studies have shown that *S. aureus* is not only an extracellular pathogen but also an intracellular pathogen, owing to effective uptake of these bacteria by non-professional phagocytes, such as endothelial and epithelial cells, fibroblasts, osteoblasts and keratinocytes[42,59,61,97–101].

SCV internalization is mediated by fibronectin bridging between the bacterial fibronectin-binding proteins (FnBPs) and the receptor $\alpha_5\beta_1$-integrin, which is present at the surface of eukaryotic cells[97,102,103]. A *hemB* mutant of *S. aureus* 8325-4 displayed more ClfA-mediated fibrinogen-binding sites and FnBP-mediated fibronectin-binding sites than the parent strain[61]. As a consequence, the rates of internalization of the *hemB* mutant by human embryonic kidney cells were higher than those of the wild-type strain or a haemin-supplemented mutant. By contrast, the intracellular location itself can trigger the emergence of SCVs[58,104], and this might be a mechanism for persistence even when there is no exposure to antibiotics. We have been able to select for haemin- and menadione-auxotrophic SCVs by exposing *S. aureus* to a cationic protein[40]. So, intracellular cationic proteins might be able to select for SCVs. Internalized normal *S. aureus* strains readily lyse endothelial cells[60], owing to expression of α-toxin, a pore-forming toxin. Recently, it has been shown that α-toxin is necessary and sufficient for the induction of apoptosis of Jurkat T cells and primary mononuclear cells[105,106]. Clinical haemin-auxotrophic, menadione-auxotrophic and thymidine-dependent SCVs persist longer in eukaryotic cells than in the corresponding wild-type strains[39,42,55,59,104]. This could be explained by the expression of less α-toxin by SCVs than by the normal phenotype, as determined by transcriptional analysis[55,61,64,79]. Interestingly, keratinocytes with internalized SCVs had a healthy appearance 48 hours after infection, whereas cells infected by the corresponding normal *S. aureus* underwent apoptotic or necrotic cell death[6]. A similar observation was made in another study in which the parent strain was highly destructive to cultured endothelial cells, whereas SCVs did not damage these cells[59]. Taken together, these data indicate that the SCV phenotype might be one of the survival strategies that *S. aureus* (and perhaps also *S. typhimurium*) uses for optimal internalization and survival in the host.

Virulence in animal models. In animal studies, *S. aureus* SCVs cause disease and can be re-isolated in pure culture[26,93,94,107–110]. Nevertheless, several studies that have been carried out using animal models indicate that *S. aureus* SCVs might be less virulent than normal strains[10,63,94,107,108,110], as determined by measuring lethal doses and fatality rates. This could be, in part, due to decreased resistance to serum[26] and/or a decreased rate of growth[47–49]. Despite this apparent decrease in virulence, *S. aureus* SCVs can persist as well as the parent strains in these animal models and human infections[4,21,26,27,30,32,36,56,94,107,109,110].

Many of the early studies used genetically undefined strains, but more recent studies using defined mutants led to similar results. To assess the virulence of SCVs in an animal model, the *hemB* mutant and its parent strain were tested in a mouse model of septic arthritis[111]. Mice inoculated intravenously with the *hemB* mutant had a higher frequency and a markedly greater severity of arthritis than mice exposed to the wild-type parent strain. Despite this, mice inoculated with the mutant strain had a markedly lower bacterial burden in the kidneys and joints than mice challenged with the parent strain. Notably, the *hemB* mutant produced almost 20-fold more protease *in vitro* than the parent strain. So, it was concluded that the *hemB* mutant is more virulent on a per-organism basis than its parent strain[111]. A *hemB* mutation was introduced into *S. aureus* Newbould, a strain that is associated with bovine mastitis[110]. Although the mutant showed less efficient colonization, it was over 100-fold more persistent than the parent strain under antibiotic (cephapirin) pressure.

To investigate relative infectivity and antibiotic-response profiles, a *hemB* mutant, a *menD* mutant and the parent strain (*S. aureus* 8325-4) were compared in a rabbit model of endocarditis[56]. With regard to seeding heart valves (with ~10^6 colony-forming units) or other target organs (the kidneys and spleen), there were no differences in the 95% infectious dose between strains. Furthermore, no differences were documented between the response of the *hemB* mutant to antimicrobial therapy with oxacillin and that of the parent strain in any target tissues. Compared with untreated control animals, considerable reductions in bacterial densities were observed in all tissues. By contrast, therapy with oxacillin markedly reduced bacterial densities of the *menD* mutant in cardiac vegetations but not in the kidneys or spleen. Reversal of the *menD* mutation restored the responsiveness of this strain to oxacillin (in the kidneys and spleen) to a level similar to that of the parent strain. So, SCVs can colonize multiple tissues *in vivo*, and the *menD* mutant in the spleen and kidneys resisted antibiotic therapy. The observed differences between the mutants tested are presumably a consequence of each organ probably being replete with haemin derived from the embolic infarctions that occur in these organs during the course of experimental endocarditis, thereby circumventing the *hemB*-knockout-induced defect in the cytochrome system.

Summary and future perspectives

SCVs have been found for many genera of bacteria, but they have been most extensively studied for staphylococci. The initial hypothesis from the clinical presentation of SCV infections was that SCVs produce subacute infections that are persistent, antibiotic resistant and recurrent[47]. Although this has been shown in many subsequent studies, we now know that *S. aureus* SCVs can also cause more aggressive infections in both humans and animal models. The high rate of selection by aminoglycosides[112,113] and in tissue culture[104] indicates that SCVs are part of the normal life cycle of staphylococci. This raises the possibility that intracellular SCVs might account for the difficulty in clearing *S. aureus* from the host even when stable SCVs are not found. Interestingly, *Salmonella*, a classic intracellular pathogen, seems to use the same strategies when it persists: that is, it also forms electron-transport-deficient SCVs at exceptionally high rates[58]. The genetic mechanism(s) for reversion to a rapidly growing form and the specific mutations in clinical isolates need to be defined by future investigations. Current areas of research are focused on defining the genetic basis of clinical SCVs, discovering the basis for the similarities between thymidine-auxotrophic SCVs and electron-transport-defective SCVs, and understanding the basis for the ability of SCVs to revert to a phenotype of rapid growth. Recent work by Heinemann *et al.*[114] used an *in silico* genome-scale reconstruction of the metabolic network of *S. aureus*, and the information learned from studying the metabolism of *S. aureus* SCVs proved to be highly instructive for constructing metabolic pathways. We suspect that these natural variants will continue to teach us about metabolism, signalling and virulence.

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Competing interests statement
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