AMERICAN SOCIETY FOR MICROBIOLOGY | Clinical Microbiology Reviews

Clinical Significance and Pathogenesis of Staphylococcal Small Colony Variants in Persistent Infections

Barbara C. Kahl,[a] Karsten Becker,[a] Bettina Löffler[b]

Institute of Medical Microbiology, University Hospital Münster, Münster, Germany[a]; Institute of Medical Microbiology, Jena University Hospital, Jena, Germany[b]

(continued)

Published 9 March 2016

Citation Kahl BC, Becker K, Löffler B. 2016. Clinical significance and pathogenesis of staphylococcal small colony variants in persistent infections. Clin Microbiol Rev 29:401-427. doi:10.1128/CMR.00069-15.

Address correspondence to Barbara C. Kahl, kahl@uni-muenster.de.

SUMMARY

Small colony variants (SCVs) were first described more than 100 years ago for *Staphylococcus aureus* and various coagulase-negative staphylococci. Two decades ago, an association between chronic staphylococcal infections and the presence of SCVs was observed. Since then, many clinical studies and observations have been published which tie recurrent, persistent staphylococcal infections, including device-associated infections, bone and tissue infections, and airway infections of cystic fibrosis patients, to this special phenotype. By their intracellular lifestyle, SCVs exhibit so-called phenotypic (or functional) resistance beyond the classical resistance mechanisms, and they can often be retrieved from therapy-refractory courses of infection. In this review, the various clinical infections where SCVs can be expected and isolated, diagnostic procedures for optimized species confirmation, and the pathogenesis of SCVs, including defined underlying molecular mechanisms and the phenotype switch phenomenon, are presented. Moreover, relevant animal models and suggested treatment regimens, as well as the requirements for future research areas, are highlighted.

INTRODUCTION

The recognition that several pheno- or morphotypes exist for certain bacterial species is much older than our knowledge about the existence of the genotypic heterogeneity of bacterial species. However, the amount of current scientific knowledge on the genesis of phenotypic variants and the mechanisms of switches between different phenotypes is much smaller than the amount of data on genomic variations. This holds particularly true for the phenomenon of the small colony variant (SCV) phenotype, which is displayed by several staphylococcal and other species and has been known for more than 100 years.

Only investigations in the past 2 decades have led to a profound understanding of the importance of the SCV phenotype as a general strategy for bacterial survival and, furthermore, as a strategy for intracellular persistence of the otherwise extracellularly growing staphylococci. This lifestyle change, which is accompanied by a profound conversion of the microorganism's metabolism, drastically affects the host-pathogen interplay, with major clinical consequences leading to chronic and relapsing, often therapy-refractory courses as a main feature of SCV-caused infections. Moreover, with its reduced expression of virulence factors important for acute infection, the SCV lifestyle fits into a broader concept of persistence for long-term survival of pathogens within their hosts.

Early Reports and Concepts on the SCV Phenomenon

The first reports on the so-called "Zwergkolonieen" (old German spelling for "dwarf colonies") of staphylococci could already be found in microbiological textbooks published at the beginning of the 20th century (1). During the following decades, reports on SCVs designated them as a specific kind of staphylococcal "dissociants" or as "dwarf" and "G" ("gonidial") colonies (2–7). An overview of clinical reports on *S. aureus* SCVs between 1950 and 1980, prior to their definitive association with persistent and relapsing infections, is given by Proctor et al. (8). *S. aureus* SCVs are characterized by their small colony size, slow growth, and downregulated virulence genes, while genes important for biofilm formation and adhesion are mostly upregulated (9). Regarding their nature and medical impact, early speculations hypothesized SCVs to have a position in the life cycle of the microorganism or to represent a state of temporarily decreased metabolism; both hypotheses are surprisingly close to our present state of knowledge (10–13). Soon, it was discovered that SCVs may have specific growth requirements and may reveal CO_2 dependence to restore the normal phenotype (14–16).

However, the association of staphylococcal SCVs with chronic and relapsing courses of infection based on an intracellular lifestyle has been recognized only in more recent years. The first papers which described a distinct SCV-associated clinical syndrome were published by Proctor et al. in 1994 and 1995, and they focused on relapsing *Staphylococcus aureus* infections with a broad spectrum of different manifestations, including bacteremia, purulent sinusitis, osteomyelitis, and septic arthritis, and the culture of SCVs despite intensive treatment (8, 17).

TABLE 1 Examples of natural SCV-like phenotypes in nonstaphylococcal species

Microorganism[a]	Reference	Phenotypic changes[b]	Source	Infection or underlying disease	Comments[c]
Gram-positive organisms					
Bacillus licheniformis	217	Increased susceptibilities to cefuroxime and cefotaxime	Blood culture	Bacteremia due to pacemaker lead infection	Parallel isolation of isogenic NP organisms from pacemaker leads
Enterococcus faecalis	218	Decreased inhibition zone diameters with benzylpenicillin	Umbilical exudate	Recurrent omphalitis	Oleate dependent
Enterococcus faecium	110	Reduced alpha-hemolysis	Blood culture	Sepsis in patient with acute myeloid leukemia	Parallel isolation of isogenic NP organisms from blood
Streptococcus figurinus	219	Reduced alpha-hemolysis, increased resistance to induced autolysis	Biopsy samples from periprosthetic tissue	Prosthetic joint infection	Total knee arthroplasty with gentamicin-containing cement
Gram-negative organisms					
Burkholderia cepacia complex	220	Increased MICs of meropenem, cefotaxime, and trimethoprim-sulfamethoxazole	Respiratory tract specimens	Cystic fibrosis	Found in 1.7% of CF patients over a 22-month period
Enterobacter aerogenes	221	Increased MICs of aminoglycosides	Blood culture	Phlebitis, sepsis	Patient had received suboptimal gentamicin therapy
Escherichia coli	222	Transparent colonies	Urine	Urinary tract infections	Deficiencies in cysteine, thiamine, thymidine, and/or glutamine
Pseudomonas aeruginosa	223	Increased MICs of antipseudomonal agents	Respiratory tract specimens	Cystic fibrosis	Found in 38.4% of *P. aeruginosa*-positive CF patients over a 2-yr period

[a] Restricted to publications since 1970.
[b] Changes in comparison to the normal phenotype; restricted to those beyond reduced growth and formation of colonies with a drastically reduced size.
[c] NP, normal phenotype.

OCCURRENCE OF CLINICAL SCVs IN STAPHYLOCOCCAL SPECIES

Most reports and clinical studies on staphylococcal SCVs describe their occurrence in the human-adapted, coagulase-positive species *S. aureus* subsp. *aureus*, and details are given in the following sections. Concerning other coagulase-positive species, known to be primarily animal associated, a human-derived *mecA*-positive *S. pseudintermedius* SCV isolate was recently described (18). Unusually for the staphylococcal SCV phenotype, this stable SCV was strongly adherent to solid agar media, similar to sticky colonies formed by *Rothia mucilaginosa* (*Stomatococcus mucilaginosus*). Some SCV observations have been described for coagulase-negative staphylococcal (CoNS) species, particularly for *S. epidermidis*, *S. capitis*, and *S. warneri*, mostly in association with foreign body-related infections (3, 19–24). Also, for *S. lugdunensis*, a coagulase-negative species with a unique status almost resembling *S. aureus* in some aspects (25), SCVs have been reported for pacemaker-related and prosthetic joint infections (PJIs) (20, 26).

OCCURRENCE OF CLINICAL SCVs IN NONSTAPHYLOCOCCAL SPECIES

The phenomenon of the generation of a slower-growing phenotype resulting in smaller colonies on solid agar media is not restricted to members of the *Staphylococcus* genus. As described for staphylococcal SCVs, the first reports were published at the beginning of the 20th century, e.g., for *Salmonella enterica* serovar Typhi ("*Eberthella typhosa*") and other *Salmonella* serovars, *Serratia marcescens*, *Vibrio cholerae*, and *Shigella* species (for a summary of those early descriptions, see reference 12). Mostly, case reports are documented for certain Gram-positive and Gram-negative species (Table 1).

CLINICAL INFECTIONS

Staphylococcus aureus has long been known as a versatile human pathogen which causes a variety of community- and hospital-acquired infections, which range from local and harmless skin infections, such as furuncles and abscesses, to severe systemic infections, such as osteomyelitis, pneumonia, endocarditis, or sepsis (27). One special feature of *S. aureus* infections is their chronic and recurrent nature despite appropriate antibiotic treatment (17). Within the last 20 years, many reports have described the association of such recurrent infections with the occurrence of SCVs of *S. aureus*, a special phenotype with attenuated virulence, thereby facilitating intracellular survival and evasion of the immune system (9).

Although SCVs have been described to occur during the normal growth cycle in *in vitro* studies (28), their occurrence during acute *S. aureus* infections has not been described. However, SCVs have been reported to be cultured from patients with chronic recurrent infections, indicating their selection and optimized fitness compared to the normal *S. aureus* phenotype during persistent infections and antibiotic therapy (Table 2). Many SCVs have been characterized in terms of their underlying auxotrophism, meaning that specific substrates, such as hemin, menadione, or thymidine, support the growth of these SCVs or give enhanced growth (CO_2) (9).

Based on which antibiotics are used to treat patients, SCVs with particular genetic mutations can be expected. While aminoglycoside therapy is associated with the emergence of menadione- or hemin-dependent SCVs (29), trimethoprim-sulfamethoxazole

TABLE 2 Reports on staphylococcal SCVs recovered from human and animal clinical specimens since 2000

SCV-associated colonization, infection, or syndrome[a,b]	Species	Clinical details	Reference[c]
Circulatory system infections			
Pacemaker-related bloodstream infection	*S. aureus*	Recurrent (7 mo) bloodstream infection due to infected cardiac pacemaker electrode	34
Sepsis	*S. epidermidis*	Fatal case in a neutropenic patient suffering from acute myeloid leukemia despite catheter removal	19
Pacemaker-related bloodstream infection	*S. lugdunensis*	Recurrent (10 mo) bloodstream infection due to infected ventricular pacemaker lead	26
Bacteremia	*S. aureus*	Recurrent cardiac pacemaker-related bacteremia in a hemodialysis patient	201
Bacteremia, spinal process	*S. aureus*	MRSA-SCV with rifampin resistance and reduced linezolid susceptibility from a spinal aspirate; SCV phenotype as a result of permanent activation of the bacterial stringent response	149
Endocarditis	*S. aureus*	Isolation from blood from a child with subaortic ventricular septal defect	208
Bacteremia	*S. aureus*	No further clinical details	159
Prosthetic valve and pacemaker endocarditis with left ventricular assist device infection	*S. aureus*	Thymidine-dependent SCVs isolated from blood culture and left ventricular assist device infection	38
Integumentary system infections			
Recurring purulent skin infections	*S. aureus*	Isolation from skin specimens from a patient with Darier's disease (keratosis follicularis) over a period of 28 mo	89
Chronic fistulous wound infection with abscess	*S. aureus*	Isolation from wound swabs during the course of Lichtenstein repair of an inguinal hernia after explantation of a synthetic mesh	209
Nasal colonization	*S. aureus*	Isolation from nasal swabs of 3/125 AIDS patients	93
Nasal colonization	*S. aureus*	Hemodialysis patient with thymidine-dependent MRSA-SCVs who was treated with trimethoprim-sulfamethoxazole due to pulmonary infection with *Stenotrophomonas maltophilia*	202
Mastitis (bovine)	*S. aureus*	Isolation from foremilk samples from 1/11 chronically infected cows	210
Exit site infection	*S. aureus*	Peritoneal dialysis patient with persistent and recurrent exit site infection	211
Nasal colonization	*S. pseudintermedius*	Isolation from nasal swabs during pre-bone marrow transplantation screening in a leukemic patient with no rhinosinusitis or animal contact	18
Chronic rhinosinusitis	*S. aureus*	Isolation from sinonasal biopsy specimens	101
Diabetic foot ulcers	*S. aureus*	SCV detection in 4/47 *S. aureus*-positive samples; all SCV isolates were MRSA	187
Skeletal system infections			
Chronic osteomyelitis	*S. aureus*	Combination with osteopetrosis, femoral instability, and fracture of the femoral neck	103
Hip prosthesis-associated infections	*S. aureus*	Five cases with treatment failures prior to SCV isolation despite several surgical revisions and up to 22 mo of administration of antibiotics	39
Prosthetic joint infection	*S. epidermidis*	Isolation from explanted prosthetic joints; 9/11 isolates were *mecA* positive, and 5/11 showed aminoglycoside resistance; no auxotrophisms for hemin, menadione, or thymidine, but 3 CO_2-auxotrophic SCVs	42
	S. aureus	SCV recovery from sonicate fluid culture	105
	S. epidermidis, *S. aureus*, *S. lugdunensis*, *S. capitis*/*S. caprae*	SCV detection in fluid samples from 38/113 patients	20
Prosthetic joint infection and aseptic loosening	*S. epidermidis*, *S. warneri*	Isolation from synovial fluid and explanted prosthetic components from 6/31 culture-positive patients with revision of total hip prosthesis	24

(Continued on following page)

TABLE 2 (Continued)

SCV-associated colonization, infection, or syndrome[a,b]	Species	Clinical details	Reference[c]
Nervous system infections			
Brain abscess	*S. aureus*	Isolation from pus and tissue specimens from a patient with facial paresis and a 3-mo history of seizures 10 yr after neurosurgical operation for subarachnoid hemorrhage with febrile episode	212
Ventriculoperitoneal shunt-related meningitis	*S. aureus*	Isolation of MRSA-SCVs from cerebrospinal fluid and the shunt tip during the course of recurrent (>5 mo) episodes of meningitis	188
Multifocal and other infections, untargeted studies			
Multiorgan infection	*S. aureus*	Isolation from subcutaneous abscess in the left lower leg followed 3 mo later by multifocal pyomyositis and osteomyelitis with spinal epidural abscess and acute acalculous cholecystitis	213
Soft tissue infection, tympanitis, bronchitis, peritonitis, sepsis	*S. aureus*	Culture of thymidine-dependent SCVs from 5 patients with various underlying chronic infections	214
Catheter-related bacteremia, endocarditis, mediastinitis, spondylodiscitis, wound infections, respiratory infections	*S. aureus*	Isolation from clinical specimens from 14 patients during a 3-yr period, including two SCVs from nasal colonization	123
Osteomyelitis, abscess, bacteremia	*S. aureus*	No further clinical details	138
Irritable bowel syndrome	*Staphylococcus* sp.	Isolation from brush samples from the duodenum	215

[a] Not including SCVs recovered from airways of cystic fibrosis patients.
[b] Human infections, if not otherwise stated.
[c] Publications since 2000. For older reports, see the reviews of Proctor et al. (9) and von Eiff and Becker (104).

(SXT) therapy is strongly associated with the emergence of thymidine-dependent (TD) SCVs (30–32). However, for many SCVs, such an association of treatment and auxotrophism is still not clear. For example, it is not known if a special antibiotic regimen induces and selects for CO_2-dependent SCVs or as yet uncharacterized SCVs. Recent experimental research suggests that additional antimicrobial compounds, such as moxifloxacin and clindamycin, promote the formation of SCVs (33).

For this review, 46 clinical studies dealing with SCVs were retrieved from the literature, spanning the time frame from 1951 to 2014 (Table 2). These studies describe the occurrence of more than 700 staphylococcal SCVs, mostly identified in *S. aureus* and *S. epidermidis* but also in *S. capitis* or *S. lugdunensis*, in more than 350 patients. Most studies were performed on patients with device-related infections ($n = 15$), skin and soft tissue infections ($n = 14$), prosthetic joint infections ($n = 13$), osteomyelitis ($n = 9$), or cystic fibrosis (CF) ($n = 10$) (Tables 2 and 3).

Foreign Body-Related Infections

Pacemaker-related infections. Four studies describe SCVs recovered from pacemaker-related infections (21, 22, 26, 34). This type of infection seems to be especially associated with the culture of coagulase-negative staphylococci, since only one case with *S. aureus* (34), but two cases with *S. epidermidis* (21, 22) and one case each with *S. capitis* (21) and *S. lugdunensis* (26), have been published. All patients had a long history of having their pacemaker in place. Infection initiated after manipulation of the systems, with a change of the battery (26) or replacement of the pacemaker (22), or after tooth extraction (21). Although all patients initially responded to antibiotic treatment, all patients presented with

TABLE 3 Reports of *S. aureus* SCVs recovered from airway specimens from cystic fibrosis patients

Study authors, yr	No. of investigated patients	No. (%) of patients with SCVs	Study period (mo)	Auxotrophism (n)[a]	Reference
Sparham et al., 1978	14	7 (50)	7	Thy (7)	57
Gilligan et al., 1987	200	20 (10)	12	Thy (20)	58
Kahl et al., 1998	78	26 (33)	34	Thy (41), Hem (10), Men (2), Thy+Hem (25)	30
Vergison et al., 2007	627	25 (4)	7	ND	59
Besier et al., 2007	252	20 (8)	12	Thy (15), Thy+Men (1), NI (8)	60
Schneider et al., 2008	98	8 (8)	3	ND	61
Green et al., 2011	260	17 (6)	6	ND	62
Yagci et al., 2013	248	20 (8)	11	Thy (5), Thy+Hem (1), NI (42)	63
Wolter et al., 2013	100	24 (24)	24	Thy (57), Hem (4), Men (1), CO_2 (1)	31
Morelli et al., 2015	222	28 (13)	Not known	Thy (27)	216

[a] Numbers of SCVs with the specified auxotrophisms. Thy, thymidine dependent; Hem, hemin dependent; Men, menadione dependent; Thy+Hem, combined auxotrophisms for thymidine and hemin; ND, not determined; NI, auxotrophisms were tested but not identified; CO_2, CO_2 dependent.

recurrent high fever and chills and had to be treated for extended periods of up to months. Neither transesophageal echocardiography nor blood cultures revealed staphylococcal growth for all patients. Various combinations of antibiotic regimens including vancomycin, rifampin, ampicillin-sulbactam, and gentamicin were applied. However, infections caused by SCVs were resolved only once the device was totally removed, which in one case required open heart surgery (34). Such surgical interventions are not only necessary in order to eradicate SCV-related infections but also mostly needed to resolve normal *S. aureus*-initiated infections.

Baddour et al. showed that their SCVs were strong biofilm (slime) formers, indicating that biofilm formation is a special feature for the pathogenesis of SCVs involved in pacemaker infection (22). Interestingly, all authors reported the difficulty of species identification of bacteria grown by conventional methods and the phenotypic variation of the cultured bacteria. Identification was mostly performed by 16S rRNA gene sequencing. Today, bacterial identification would be much easier and faster by means of matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (35). The clonality of isolates with phenotypic variation was mostly proven by pulsed-field gel electrophoresis (PFGE).

Prosthetic valve infectious endocarditis. Surprisingly, there are no systematic clinical studies which investigate the prevalence of staphylococcal SCVs in patients with prosthetic valve infections. From the literature, we retrieved only one definite report, which reported the culture of *S. aureus* SCVs from one patient with an aortic valve prosthesis infection (36). The patient was treated with nafcillin because of *S. aureus* bacteremia and suspected aortic valve prosthesis infection. No further information on the patient's clinical condition and treatment was provided. The SCV reverted to normal growth after a few subcultures. Comparison to the normal phenotype revealed that the SCV lacked α-hemolysin and was significantly less virulent in an intraperitoneal mouse model (36). Unfortunately, no information about the prevalence of SCVs was reported in a recent prospective multicenter study which investigated the impact of early surgery in patients with *S. aureus* prosthetic valve infective endocarditis (37).

Heart assist device infection. Maduka-Ezeh et al. described the case of a 34-year-old woman who received a left ventricular assist device (LVAD) as a bridge until transplantation after an anterior wall myocardial infarction (38). Her tricuspid valve was replaced at the same time. One month after surgery, the patient developed fever and chills caused by methicillin-resistant *S. aureus* (MRSA), which was isolated from the blood culture and was susceptible to gentamicin, rifampin, linezolid, and SXT. In the following time, the patient experienced several episodes of fever and chills and was treated with different antibiotic regimens, one of which included SXT for several weeks. Despite antibiotic treatment and abscess drainage around the LVAD generator, the infection persisted, with intermittent swelling, redness, and swelling around the umbilical drive line exit site. The infection could be resolved only by removal of the device and the tricuspid valve. A thymidine-dependent SCV was cultured from around the LVAD during removal, which was attributed to the prolonged treatment with SXT. There are no further publications which report the culture of SCVs from patients carrying LVADs, although it can be assumed that in this clinical setting the emergence of SCVs can be expected if patients are treated with antibiotics for prolonged periods without device removal. Whenever possible, device removal is the optimal intervention, not only in order to eradicate SCV-related infections but also for normal *S. aureus*-related infections.

PJIs. Persistent and relapsing prosthetic joint infections (PJIs) caused by staphylococcal SCVs have been the focus of several studies (8, 39, 40) (Fig. 1). The first case was reported by Proctor et al., who described a patient with a history of hip prosthesis 5 years prior to an elective surgery, after which MRSA bacteremia occurred (8). Ten of 12 blood cultures within a 38-day period revealed *S. aureus* SCVs. At that time, no signs of endocarditis or hip prosthesis infection were diagnosed. The patient was treated with vancomycin for 8 weeks. Six months later, the patient was readmitted to the hospital with fever, pain, and swelling of the right hip. A right hip aspirate contained purulent fluid, from which MRSA and coagulase-negative staphylococci were cultured. The right hip prosthesis was removed surgically.

Sendi et al. conducted a very thoroughly performed 4-year prospective study, during which they identified 5 of 83 cases (6%) of PJI associated with *S. aureus* SCVs, which were cultured from joint aspirations or intraoperative tissues (39). The histories of the patients included the first infection period for all patients, the primary focus, the presence of bacteremia, the number of surgical revisions, and the antibiotic therapy in the months prior to the detection of SCVs. In all cases, staphylococcal infection persisted or relapse occurred until SCVs were isolated. Importantly, a transmission electron microscopy image of the periprosthetic tissue of one patient revealed intracellular bacteria residing within fibroblasts. Since SCVs from this patient were cultured only from synovial specimens, it is very likely that these intracellular bacteria represent SCVs. These intracellular bacteria retrieved from an *in vivo* tissue support the hypothesis that SCVs hide intracellularly, where they are protected against host defense and antibiotic therapy and from where they can escape and cause recurrent infection (9, 41). The authors concluded from their study that in case of treatment failure of staphylococcal PJI despite appropriate antibiotic therapy, SCVs should be actively sought. Moreover, they suggest removing all involved foreign material, with an implant-free interval of 6 to 8 weeks, combined with antimicrobial treatment based on MIC testing (39), which is especially required for resolving infections caused by SCVs but also for infections caused by bacteria with normal *S. aureus* phenotypes.

Maduka-Ezeh et al. determined the prevalence of CoNS SCVs in 31 patients with staphylococcal PJIs (42). They recovered 11 SCVs (35%), all belonging to *S. epidermidis*, none of which were auxotrophic for hemin, menadione, or thymidine and only five of which were resistant to aminoglycosides. Unexpectedly, only two *S. epidermidis* SCVs were proficient biofilm formers. The authors recovered organisms of several different phenotypes with clonal identity from the same specimen, as assessed by PFGE and different susceptibility patterns. They concluded that *S. epidermidis* SCVs can frequently be retrieved from PJIs and that these SCVs differ in characteristics typical for *S. aureus* SCVs, such as special auxotrophisms, resistance types, and biofilm formation.

During a 22-month prospective study, Bogut et al. studied cases of PJI according to the definition of Parvizi et al. (24), which includes clear infective signs, such as a sinus tract communicating with the prosthesis, a pathogen isolated from two separate tissue samples or fluids, and elevated C-reactive protein (CRP) and elevated leukocyte counts in the synovial fluid. Nineteen patients met the criteria for PJI, and 12 patients met the critera for the diagnosis

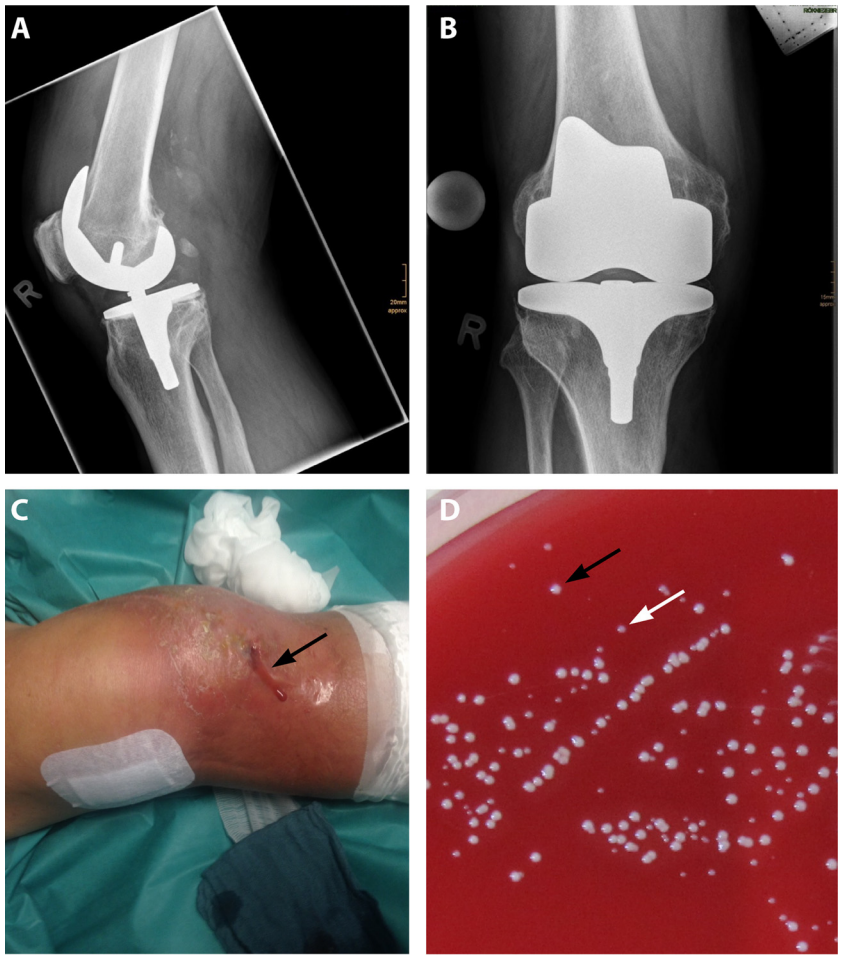

FIG 1 Periprosthetic knee joint infection of a patient with a history of clinical and infectious laboratory parameters for 2 weeks. A two-stage revision was performed. (A and B) X-rays without specific changes. (C) Clinical presentation 2 to 3 weeks after the first symptoms occurred. Inflamed skin with draining pus after incision of the skin (black arrow) can be seen. (D) *S. aureus* colonies grown from a specimen of chronically infected bone tissue. On the Columbia blood agar plate, a high phenotypic diversity of large (black arrow), small, and very small (white arrow) phenotypes, which represent dynamic SCVs, is visible. (Panels A, B, and C courtesy of W. Kluge, Hufeland-Klinikum Weimar, Germany; reprinted with permission.)

of aseptic loosening (AL). The authors identified 8 SCVs in 6 patients, half of them with the diagnosis of AL. All SCVs represented CoNS, including 7 *S. epidermidis* and 1 *S. warneri* isolate; all were hemin auxotrophs, and one was both hemin and menadione dependent. Since biofilm formation has been suggested to have an important impact in prosthetic joint infections and other persistent infections due to interference with the host response and antibiotic therapy, the authors also determined the presence of the *ica* operon, which confers biofilm formation, in the cultured staphylococci (25, 43). Surprisingly, only one *S. epidermidis* isolate carried the *ica* operon. Yet biofilm formation could occur by *ica*-independent mechanisms or could result from bacteria with normal phenotypes, as all SCVs except one were cultured in a mixture with normal-phenotype bacteria.

Unfortunately, both studies which described the occurrence of coagulase-negative SCVs in PJIs did not present data about the clinical history of the patients and prior antibiotic treatment.

In a very recent study, Tande et al. identified 38 patients (34%) among 113 patients with staphylococcal PJI in whom the infection was associated with SCVs (20). The authors used stored fluid containing bacteria, which was removed by vortexing and sonication from arthroplasty components of patients undergoing surgery for PJI. Comparison of patients with and without SCVs revealed that patients with SCVs had a longer history of implanted joints, a longer period since the last surgery, and a longer duration of PJI symptoms, that there were more of these patients with prior surgery for the PJI, and that more patients with SCVs had received antibiotics for at least 120 days in the prior 6 months. More than 50% of SCVs represented *S. epidermidis* (57.9%), followed by *S. aureus* (26.3%) and *S. capitis/caprae* (5.3%) and *S. lugdunensis* (7.9%). Remarkably, auxotrophism was identified in only a few isolates, with auxotrophism for CO_2 (19%) being the most frequent and with only one isolate each showing a combined auxotrophism for CO_2 and menadione or hemin. Although a larger number of patients with SCVs had an aminoglycoside in the cement, no decreased susceptibility toward aminoglycosides in SCVs was detected by applying categorization as susceptible or resistant according to CLSI guidelines. However, von Eiff et al. showed that in all patients with osteomyelitis who were treated with gentamicin beads, hemin- or menadione-auxotrophic *S. aureus* SCVs were cultured which showed higher MICs of gentamicin (up to 32-fold) for the SCVs than for the corresponding pa-

rental strain, but not necessarily leading to a switch of the categorization to resistant (44). In their analysis, the authors did not find differences in treatment failures between patients with and without SCVs, while they reported that patients with *S. aureus* were four times more likely to experience treatment failure. However, the authors did not report the bacterial phenotypes for patients with treatment failures, which may have been due to the emergence of SCVs, especially since antimicrobial-loaded cement spacers were used. Moreover, the data revealed that patients with SCVs had a history of prolonged and recurrent infection of their PJIs, which is the typical characteristic seen for patients with SCVs, thereby underlining the important the role of SCVs in PJI.

Subacute/chronic bone infections. Staphylococci, including *S. aureus* and coagulase-negative staphylococci, are by far the most frequent pathogens involved in bone and joint infections (40, 45). In general, osteoarticular infections are difficult to treat and in most cases require a combined treatment of surgical interventions and long-term antibiotic regimens (45, 46). Nevertheless, infections that have apparently been treated successfully have a high recurrence rate and can relapse even after several years (8, 44). In most cases, a high phenotypic diversity of infecting pathogens, including normal, small, and SCV-like phenotypes, is found on agar plates when tissue samples of chronic bone infections are plated. The tendency of staphylococci to cause chronic bone and joint infections is only marginally understood but might originate in the complex bacterial interaction with bone tissue (46).

Osteomyelitis. The impact of SCV formation in chronic bone infections was first investigated by Proctor et al., who analyzed five patients with chronic bone and/or joint infections and recovered SCVs on agar plates that represented phenotypic variants of a single bacterial strain which were not genotypically distinct *S. aureus* strains (8). Already for these five patients various percentages and degrees of stability of SCVs were described, and there were differences in the auxotrophisms to menadione, hemin, and CO_2, suggesting that SCV formation is not a uniform phenomenon. The clinical data of these patients revealed that they were poorly responsive to antibiotic treatment regimens, which might be explained by the slowly growing bacteria or the intracellular location (8). In the following years, there were some worldwide reports showing the development of SCVs in different staphylococcal species. In particular, the reduced susceptibility to aminoglycosides was mentioned early and later confirmed in other studies as well (42, 44, 47, 48). With respect to bone infections, for which local gentamicin treatment is frequently used, the recovery of SCVs following placement of gentamicin beads has been reported (44). These results are in line with laboratory data demonstrating that SCVs can be generated by *in vitro* gentamicin selection (see Pathogenesis).

The exact location of bacteria during chronic osteomyelitis is still not precisely known, and several options are discussed here.

(i) Adherence to bone matrix. *S. aureus* is known to express a multitude of surface proteins with adhesive functions, called adhesins (49). Different adhesins exhibit binding to components of the extracellular bone matrix, e.g., to collagen, and many different adhesins reveal redundant functions. Consequently, tight *S. aureus* adhesion to extracellular bone matrix was found to be a general characteristic of different *S. aureus* clinical isolates (50). Therefore, the bone matrix is a possible location that shelters bacteria, in particular in the acute stage of the infection, when bacteria arrive in the bone tissue and tightly adhere to initiate an infection.

(ii) Intracellular location. Although *S. aureus* was classically considered an extracellular pathogen, it became more and more clear that this bacterium can efficiently invade different types of host cells, including fibroblasts and osteoblasts. Hudson et al. and Ellington et al. demonstrated the uptake of *S. aureus* into cultured osteoblasts (51, 52). A comparative analysis with other cell types revealed that osteoblasts take up many fewer bacteria than, for example, endothelial cells (53); nevertheless, bacteria within osteoblasts are able to persist for several days, while increasingly forming SCVs (50). Although the bacterial intracellular location is very difficult to prove within the *in vivo* situation, cell culture experiments strongly suggest that intracellular persistence within osteoblasts may be a source for chronic osteomyelitis.

(iii) Dead bone fragments (sequestra). In chronic forms of osteomyelitis, infected bone fragments without a blood supply can develop. In this case, surgical debridement is urgently required to clear the infection focus because the bacteria cannot be reached by antibiotics or the immune system (45, 54). Dead bone fragments may be an optimal basis for bacterial biofilm formation that promotes SCV development as well (55, 56).

Cystic Fibrosis Airway Infection

During the last 40 years, nine prospective studies dealing with the culture of *S. aureus* SCVs from the airways of almost 2,000 cystic fibrosis (CF) patients (the numbers of patients in the different studies ranged from 14 to 594) during a period of 3 to 34 months (mean, 13 months) have been published from 6 countries, including the United Kingdom, United States, Germany, Belgium, Switzerland, and Turkey (30, 31, 57–63) (Table 3). This special interest in SCV occurrence and prevalence in CF is due to some special characteristics of CF airways, which are explained briefly in the following paragraph. For a deeper understanding, the reader is referred to recent reviews (64–68).

Diagnostic implications for the culture of *S. aureus* SCVs from CF airways. In almost all of the presented studies, it was pointed out that it is extremely important to use appropriate culture conditions which allow the detection of SCVs. Furthermore, the laboratory should actively search for SCVs. This is especially important in the case of MRSA-SCVs, which would have been missed in the study by Wolter et al. (31). MRSA airway infections in CF have implications not only for hygiene issues but also for treatment and the impact on the clinical course of the disease. It has been reported that CF patients with MRSA airway infection have worse lung function in the group of patients between 8 and 21 years of age and a higher risk of death (69, 70).

CF—a hereditary disease with chronic recurrent bacterial airway infections. CF is one of the most common hereditary diseases in the Caucasian population, with approximately 70,000 affected people worldwide (71). Although the knowledge about the pathogenesis of the disease and treatment options increased during the last decades, with life expectancy today reaching 40.7 years (71), more than 80% of patients still succumb to lung insufficiency as a consequence of chronic and recurrent bacterial airway infections (65). Due to changes caused by the mutated cystic fibrosis transmembrane regulator gene, which codes for an ion channel, the airways of CF patients are characterized by a highly viscous mucous layer which impairs mucociliary clearance, thereby facilitating colonization and infection by particular microorganisms (66, 67). In CF, there is an age-dependent bacterial infection pattern of the airways, with *S. aureus* being one of the first pathogens. Later, *Pseudomonas aeruginosa*, which is the most prevalent pathogen in

CF, replaces *S. aureus* and infects more than 80% of adult CF patients (72). Although CF patients are regularly treated with antibiotics directed against the respective pathogens, the same *S. aureus* clone persists in the airways for many years (73–75). Such persistence is even more pronounced for *P. aeruginosa*, which in many patients persists lifelong once chronic airway infection has been established (76). Thus, CF airways provide a unique niche where bacteria face a hostile environment, with neutrophil influx (77), hypoxia (78), competition with coinfecting species (79), and antibiotic selective pressure (80), which lead to various forms of bacterial adaptation, including the emergence of SCVs (30).

Clinical studies addressing special culture conditions for airway specimens. Sparham et al. studied the culture conditions most suitable for the detection of *S. aureus* from the complex CF sputum (57, 81). To enhance the culture of *S. aureus* from viscous CF sputum samples, the authors liquefied and sonicated sputa prior to culture on routine agar plates and selective agar, which was mannitol salt agar (BBL and Oxoid). Sonication and culture on BBL mannitol salt agar increased the number of isolates with a normal *S. aureus* phenotype and also allowed the culture of thymidine-dependent (TD) *S. aureus* (in 7/14 patients [50%]). TD *S. aureus* was later defined as a special subgroup of SCVs, because TD *S. aureus* differs in some characteristics from the typical features of previously described hemin- and menadione-dependent SCVs with tiny, nonpigmented, nonhemolytic colonies on Columbia blood agar plates (82). In addition to this phenotype, TD-SCVs can exhibit various phenotypes, including pinpoint colonies and a "fried egg" appearance with translucent colonies (83). Sparham et al. already stated that there was an association of TD *S. aureus* with patients with SXT treatment (57). Later, Gilligan et al. determined the prevalence of TD *S. aureus* in the airways of 200 CF patients during a 1-year period (58). TD-SCVs were cultured from 10% ($n = 20$) of their patients. Again, the authors determined an association of long-term SXT treatment (at least 30.9 months) with the emergence of TD *S. aureus* (58). All TD *S. aureus* strains were SXT resistant and failed to grow on Mueller-Hinton agar.

In 1998, our group published the first study which determined the prevalence of *S. aureus* SCVs in CF airways. During a 34-month period, 26 of 78 CF patients (33%) harbored SCVs, with or without an isogenic normal *S. aureus* strain, in their airways (30). In 19 of 26 patients, SCVs could be isolated for up to 31 months, indicating the persistence of SCVs (Fig. 2). Most SCVs were thymidine dependent ($n = 41$) or combined hemin-thymidine dependent ($n = 25$), while 10 SCVs were only hemin dependent and 2 were only menadione dependent. Again, there was an association of treatment with SXT and the emergence of TD-SCVs (30). Furthermore, we showed that SCVs were better able to persist intracellularly in eukaryotic cells than their isogenic normal *S. aureus* strain, supporting the hypothesis that *S. aureus* and especially SCVs can hide intracellularly, where they are protected against the host defense and antibiotic therapy. Yagci et al. reported a prevalence of 8.1% ($n = 20$) in their 248 CF patients during an 11-month study (63). Six of 48 SCVs (12.5%) were thymidine dependent and SXT resistant, and there was a history of SXT treatment in five patients. Patients with SCVs were older than patients with only normal *S. aureus*.

Green et al. conducted a 6-month study of 260 patients, in which the authors aimed to analyze the emergence of SCVs during long-term treatment with azithromycin (62). They reported the presence of SCVs in 4.1% of patients at the beginning, with no increase of SCV emergence during the study period. As can be expected, macrolide-resistant normal *S. aureus* increased significantly in the treatment group. However, 6 months of azithromycin therapy seems to be too short for the emergence of SCVs considering that prolonged therapy with SXT of more than a year induces and selects TD-SCVs. Furthermore, so far, no association of macrolide treatment and SCV emergence has been reported.

Vergison et al. (59) investigated the diagnostic performance of nine CF centers in Belgium, including 627 patients with a 4% prevalence of SCVs. The authors stated that obviously only two CF centers directly searched for SCVs. Therefore, the prevalence of SCVs in Belgium was underestimated in this study due to suboptimal culture procedures of laboratories serving CF centers (59).

Besier et al. related, for the first time, the isolation of SCVs from respiratory specimens to the clinical status of CF patients. In their 1-year prospective study, a prevalence of 8% of patients with *S. aureus* SCVs was determined (20 of 252 CF patients). Patients with SCVs were older, were more likely to be coinfected with *P. aeruginosa*, and had worse lung function (60). SCVs were more resistant to SXT, gentamicin, ciprofloxacin, and fosfomycin. Using a logistic regression model, the authors determined lower weight, advanced age, and prior SXT treatment to be independent risk factors for the culture of SCVs. Most SCVs were thymidine dependent (62.5%), while 6 SCVs were unstable and reverted back to the normal *S. aureus* phenotype.

Schneider et al. also showed an association between advanced pulmonary disease and prolonged antibiotic treatment in their short-term study (3 months) of analyzing data on 98 patients (61). The prevalence of *S. aureus* SCVs was approximately 8%. Patients with *S. aureus* SCVs were older and had been treated longer with SXT and aminoglycosides. Unfortunately, the authors did not determine the underlying auxotrophism of the cultured SCVs.

Recently, Wolter et al. published a study of SCV prevalence in 100 CF children during a 2-year study (31). In that study, the prevalence of SCVs was 24%, mostly consisting of TD-SCVs (95%), with 7% hemin and 1% (each) menadione and CO_2 dependencies. Patients with SCVs had a greater drop in lung function during the study period. A multivariable regression model suggested that TD-SCVs were most likely selected by SXT treatment.

Results and conclusions drawn from clinical studies of CF airway infections. So far, it is not clear if SCVs on their own are more virulent than organisms with the normal phenotype or if the culture of this adaptive phenotype is a marker of advanced airway disease. There are some important aspects involved in the estimation of the virulence of SCVs. (i) There could be a discrepancy between the growth of SCVs *in vitro* on agar plates and the growth of SCVs in infected airways, where sufficient amounts of thymidine, hemin, menadione, and CO_2 may be present, which would allow reversion of SCVs to the normal phenotype. (ii) Moreover, if SCVs with different mutations occur in the same place, it is possible that these SCVs exchange metabolites leading to wild-type virulence (84). (iii) Transcriptional analysis revealed that SCVs have a less virulent phenotype than that of normal *S. aureus* (85–87). However, SCVs are specialized for extracellular and also intracellular persistence due to decreased expression of alpha-toxin, which easily lyses eukaryotic cells, and increased expression of cell wall-associated genes, which facilitate colonization to extracellular matrix proteins and internalization into eukaryotic cells. (iv) In competition experiments, TD-SCVs had a survival advantage *in vitro* and in a chronic murine pneumonia model under SXT

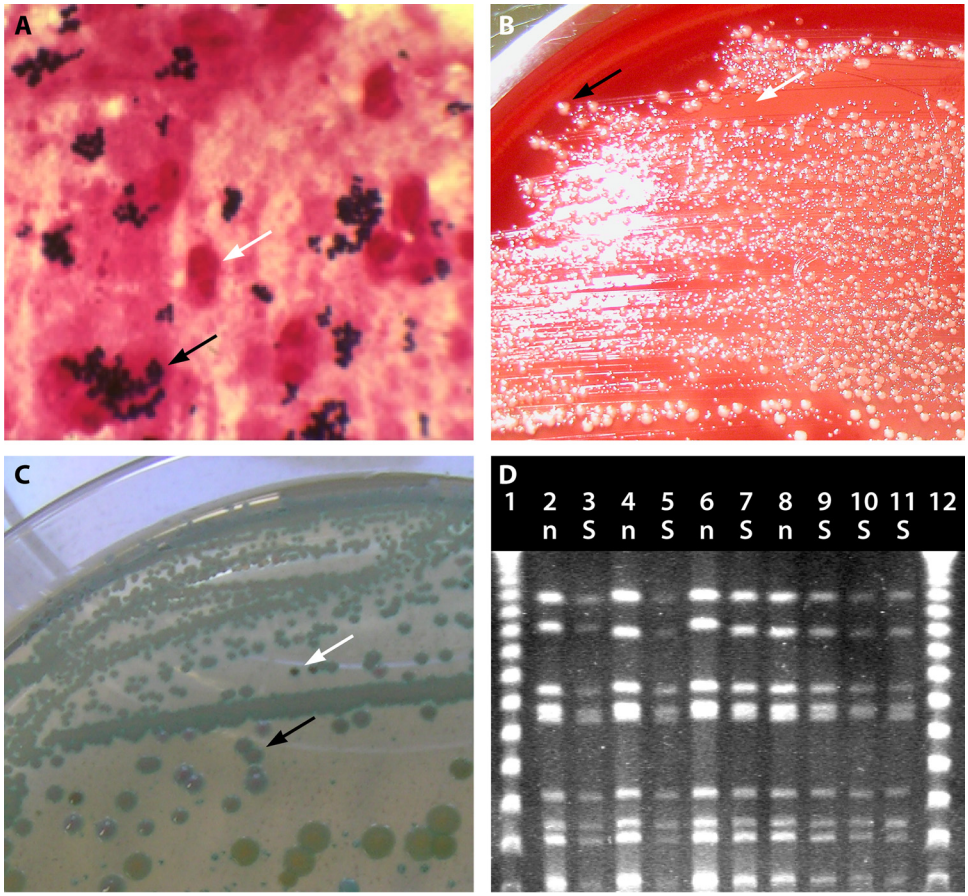

FIG 2 *S. aureus* recovered from the airways of a cystic fibrosis patient. (A) Gram staining of a sputum specimen from a CF patient. Gram-positive cocci (black arrow) representative of *S. aureus* and numerous neutrophils (white arrow) are visible. (B) Mixture of large (black arrow) and small (white arrow) colonies cultured from a sputum sample, indicative of a mixture of normal *S. aureus* and *S. aureus* SCVs, on Columbia blood agar. (C) Growth of large (black arrow) and small (white arrow) colonies typical for a mixture of normal *S. aureus* and *S. aureus* SCVs on SAID chromogenic agar, used as a selective agar for *S. aureus*, from a sputum sample from a CF patient. (D) Pulsed-field gel electrophoresis of normal *S. aureus* and *S. aureus* SCVs, which persisted in the airways of an individual CF patient from 1994 until 2000. n, normal *S. aureus*; S, SCV; lane 1, marker; lane 2, normal *S. aureus* (NS) 6/1994; lanes 3 and 4, SCV and NS isolated together in September 1995; lanes 5 and 6, SCV and NS isolated together in June 1995; lanes 7 and 8, SCV and NS isolated in January 1997; lane 9, SCV from May 1998; lane 10, SCV from October 1999; lane 11, SCV from January 2000; lane 12, marker.

selective pressure over normal *S. aureus*, which partly explains the high prevalence of TD-SCVs during SXT treatment (88).

Infections Involving Skin, Mucous Membranes, Soft Tissues, and Wounds

Little is known about the occurrence of staphylococcal SCVs as possible colonizers of the skin or mucous membranes. von Eiff et al. demonstrated the uptake and persistence of *S. aureus* SCVs in cultured human HaCaT keratinocytes (89). In line with this, Gläser et al. recently showed that skin-derived antimicrobial peptides exhibit less activity against SCVs (90). While the nares of approximately 10 to 35% of people are permanently colonized with *S. aureus*, and almost all are colonized with CoNS (91), SCVs are rarely isolated from the nasal cavities of healthy people. Since thymidine-dependent SCVs have been retrieved from the blood culture of a patient with AIDS (92), von Eiff et al. determined the presence of SCVs in the anterior nares of AIDS patients as a source of severe infections (93). However, in only 3 of 125 (2.4%) patients were the anterior nares positive for *S. aureus* SCVs, while the normal phenotype was identified in 29.6% of patients within that study (93).

A fatal case of a persistent deep-seated hip abscess in an AIDS patient (CD4 cell count, 20/μl) was caused by a methicillin-resistant *S. aureus* (MRSA) SCV (92). Nine months subsequent to a traffic accident with cerebral trauma resulting in spastic hemiparesis and 2 months after intramuscular injection to treat recurrent hip abscesses, SCVs were recovered from several blood cultures, the abscess, and a postmortem specimen.

An *S. aureus* SCV-caused chronic wound infection has been reported for a patient subsequent to herniotomy (94). Thirteen months after surgery, a subcutaneous, pus-generating abscess and chronic fistula with signs of chronic granulomatous inflammation were observed, and a menadione- and hemin-auxotrophic SCV isolate was cultivated.

S. aureus SCVs cooccurring with normal-phenotype isolates were recovered continuously from skin specimens from a Darier's (Darier-White) disease patient (89). This genodermatosis, also known as keratosis follicularis, is characterized by wide variations

in clinical severity and is often exacerbated by pyogenic infection (95, 96). Seven genotypically different strains, including those with methicillin resistance, were isolated subsequently or partly overlapping for a 28-month period, with times of persistence reaching 16 months (89).

S. aureus has been linked to the pathogenesis of chronic rhinosinusitis with nasal polyps (CRSwNP), and the pathogen has been demonstrated in the mucosa of CRSwNP patients by peptide nucleic acid-fluorescence *in situ* hybridization (PNA-FISH) (97, 98). However, the role of *S. aureus* SCVs in CRS is unclear. While some studies failed to detect SCVs in nasal lavage fluid and mucosal biopsy specimens from CRS patients (99, 100), other groups were able to cultivate intramucosal SCVs (*S. aureus* and other species) from respective sinonasal tissues of CRS patients (101, 102).

DIAGNOSIS

The uncommon physiological, metabolic, and morphological features of SCVs are challenging for the routine diagnostic laboratory. In fact, in general, they are difficult to recover, to identify, and to store. Even in the case of cooccurring staphylococcal cells displaying the normal phenotype, detection and also reporting of SCVs are of particular importance for the clinician because they (i) may be an indication of a chronic course of infection and (ii) may necessitate specific therapeutic considerations.

Choice of Specimens for Culture

Apart from CF diagnostics, there are no systematic studies which have analyzed the preanalytics and the choice of specimens for SCV recovery. From a theoretical viewpoint regarding SCV physiology and metabolism, the use of tissue specimens rather than swabs and rapid transport to the diagnostic laboratory might be vital to ensure the cultivation of SCVs and to avoid a phenotypic switch to the normal phenotype due to an early disintegration of the intracellular location of the SCV cells.

Non-CF-associated infections. So far, there have been no studies comparing the effectiveness of SCV culture from various human specimens in PJI and other infections. However, from personal experiences obtained from respective studies, it seems to be important to gain access to high-quality material, such as joint aspirates, intraoperative tissue samples, biopsy specimens, or fluids (44, 89, 103, 104). If devices or bones are being removed, sonication of samples improves the culture of SCVs, as reported by Piffaut et al. (105).

Cystic fibrosis. For CF patients, it is more likely to culture SCVs from sputum cultures, because the emergence of SCVs is associated with advanced age of patients, who are more likely to produce sputum (60, 61). However, SCVs can also be cultured from deep throat swabs, which allow growth of SCVs if the correct culture conditions are used (60).

Culture and Presumptive Identification

Since SCVs differ from the wild-type phenotype in their generation time, growth requirements, colony morphology, and many metabolic and other physiological characteristics, they are difficult to detect, often overlooked, or misidentified (Table 4). Due to an approximately 6-fold-reduced generation time, SCVs often need more than 24 h (mostly 48 to 72 h) to become visible on solid media. Their occurrence in mixed cultures with the parental strain and their instability and tendency to revert to the normal phenotype further hamper their recovery and identification. Table 4 shows key differences of the phenotypes.

TABLE 4 Key characteristics of SCVs compared to the normal phenotype

Characteristic	Description[a]	
	SCV	Normal phenotype (NP)
Colony size after 24 h of incubation	Invisible or pinpoint (1/10 the size of NP)	1–3 mm
Pigmentation	Not present or reduced	Unpigmented or colored gradation of yellow-orange
Hemolysis	Not present or reduced	Weak to strong zone of beta-hemolysis

[a] Characteristics are species and strain dependent. Those of the most prevalent staphylococcal species encountered in human specimens are given here; for divergent features of other staphylococcal species, refer to respective reviews and textbooks (25, 115).

Colony morphology. While the normal staphylococcal phenotype is characterized by medium-sized colonies, reaching 1 to 3 mm in diameter within 24 h, SCV colonies have a pinpoint size that is about 1/10 the size of the parental strain (9). In contrast to isogenic parental strains of a given species, SCVs are nonpigmented or show a strongly reduced pigmentation. In particular, thymidine-dependent SCVs often exhibit an unusual appearance of "fried egg"-like colonies, with translucent edges surrounding a smaller, elevated pigmented center (83). SCVs are also characterized by a missing or decreased beta-hemolysis zone. Thus, in the case of inexperienced examiners, their colonial appearance might easily be mistaken for slower-growing corynebacteria or nonhemolytic streptococci. In addition, primary misidentification as CoNS species has been reported several times (92, 94). Since they often occur together with a wild-type *S. aureus* strain, there might be the misimpression of a mixed culture.

Culture media and conditions. Apart from the reduced growth, staphylococcal SCVs can be cultivated on Columbia blood agar incubated in either air or CO_2. They also grow on other solid media and enrichment broths used for cultivation of Gram-positive cocci, such as chocolate agar (in 5% CO_2). On selective or chromogenic solid agar media developed for cultivation of staphylococci, most SCV strains do not exhibit a change of color (106). For example, most SCVs are mannitol salt agar negative. Use of selective or chromogenic plates should always be accompanied by the inoculation of blood agar plates.

Note that SCVs are very rapidly overgrown in mixed cultures, which particularly with enrichment broths. Careful treatment of the specimens and meticulous examination of the agar plates—ideally with the aid of a magnifying glass—are prerequisites for SCV diagnostics.

Species Confirmation

Biochemical procedures. Irrespective of the underlying auxotrophies, SCVs show a type of anaerobic metabolism and common metabolic features in central carbon metabolism characterized by a reduced carbon flux through the citric acid cycle (85, 107, 108). These metabolic changes and their reduced growth are the main reasons that SCVs typically show a lack or at least reduction of biochemical reactions, leading to non- or misidentification in the classical extensive scheme for fermentation, oxidation, hydrolysis, and degradation of several substrates originally published by Kloos and Schleifer (109). The same holds true for the application

of modern commercial identification systems. Moreover, catalase, clumping factor, and coagulase reactions or other assays used for presumptive identification are also delayed positive or even negative. Thus, all diagnostic approaches based on biochemical procedures are unreliable in the case of SCVs.

MALDI-TOF MS. So far, systematic studies evaluating the usability of MALDI-TOF MS analysis for staphylococcal SCVs are missing. However, if the mass range of 2,000 to 20,000 Da usually applied for diagnostic purposes is used, which represents predominantly ribosomal proteins, the underlying methodological principle should yield identification results mostly independent of the displayed phenotype of a given isolate. Indeed, observations showed that this approach is reliable for staphylococcal SCV identification if the amount of colonial material used is equal to the amount applied for staphylococci displaying the normal phenotype (K. Becker, unpublished observation). Interestingly, for *Enterococcus faecium*, it was published that an SCV phenotype was precisely identified by MALDI-TOF MS analysis (110).

PCR and sequencing. Molecular targets that have been proven for *S. aureus* identification (e.g., *nuc*, *clfA*, *eap*, *coa*, and *sodM*) (111–116) work well for the identification of *S. aureus* SCVs. The same holds true for respective genus-specific targets and those determined for the detection of other staphylococcal species (25, 117). Note that the Gram-positive nature of the cell wall may warrant special lysis conditions and that the use of lysostaphin is recommended if problems occur with the extraction of staphylococcal nucleic acids (118).

As for the normal staphylococcal phenotype, 16S rRNA and, in particular, *rpoB* gene sequencing is useful to ultimately determine the species and subspecies, respectively. However, for the identification of staphylococcal SCVs, the known limitations of public sequence databases have to be considered (119, 120).

In conclusion, species-specific PCR assays and sequencing of selected universal phylogenetic marker genes are the most specific approaches to identify and verify the species affiliation of SCV isolates.

Phenotypic Switch and Determination of the SCV Phenotype

Determination of SCV auxotrophies helps us to understand the underlying mechanisms leading to the generation of this distinct phenotype. However, phenotypic instability is a common phenomenon observed for clinical SCV isolates and was already described in very early reports as having "always been sudden and spontaneous" (121). Independently of the use of solid or broth media, this phenotype reversion may occur after only overnight incubation or after days or weeks of incubation (Fig. 3). Mostly, only some SCV cells switch back to the normal phenotype, but the whole culture may revert. This instability hinders diagnostics and analysis of this phenotype.

Determination of auxotrophies. Auxotrophies (also designated auxotrophisms) for hemin, menadione, and thymidine can be tested by the application of disks to the top of a solid agar plate inoculated with the test isolate, as done in principle for conventional agar disk diffusion assays (30). Auxotrophy can be categorized as positive if a zone of growth surrounds the impregnated disks after an incubation period of 18 h or longer. In this case, disks will be impregnated with solutions (15 μl) of hemin at 1 μg/ml, menadione at 10 μg/ml, and thymidine at 100 μg/liter (89). For some of these compounds, standard disks are also commercially available. Combined auxotrophies are detectable by impregnation of the disks with several compounds. Since conventional agar media may contain hemin, menadione, or thymidine, test isolates should be inoculated on chemically defined medium (CDM) agar (122). The exact composition of the CDM agar is a prerequisite for auxotrophy determination; even traces of auxotrophy-determining substances must be strictly avoided.

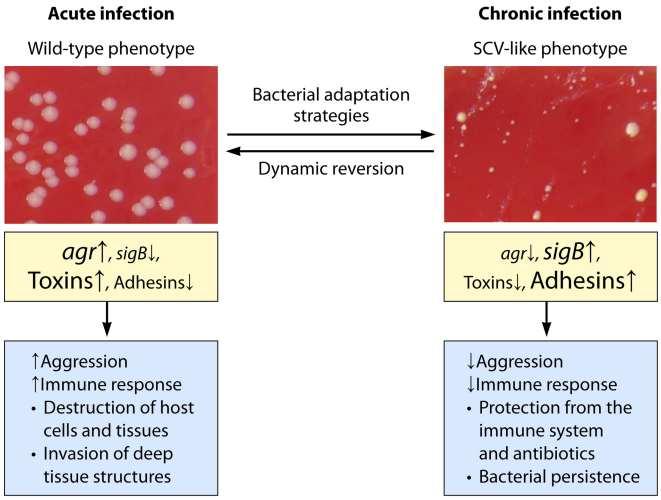

FIG 3 Scheme of dynamic SCV development in the course from acute to chronic infection. During acute infection, bacteria show high-level expression of *agr* and regulated toxins causing inflammatory and cytotoxic reactions. In the chronic course of the infection, bacteria apply adaptation mechanisms to resist cellular degradation that involve formation of dynamic SCV-like phenotypes, downregulation of *agr*, and upregulation of SigB. When leaving the intracellular shelter, the SCVs can rapidly revert back to the fully aggressive wild-type phenotype, which can cause a new episode of infection.

CO_2 dependence of SCVs can be detected by the use of biocarbonate solutions by applying 100 ml of a 1% or 1.5% aqueous solution of $NaHCO_3$ to the bottom of a jar with inoculated CDM agar plates, resulting in approximately 0.75% free CO_2 at 37°C (16). To generate an anaerobic atmosphere, other systems, e.g., commercially available systems, are also useful (123).

Growth curve analysis and spectroscopic approach. At some expense, growth curve analysis can be used to demonstrate and keep track of the low cell division rate of clinical and mutant SCVs compared to that of the normal phenotype, which is a prerequisite for experimental approaches to study the nature of SCVs (85, 108, 124–126). Typically, SCVs have been measured by determining the optical density (OD) at 578 nm over 12 h or longer. In nutrient-rich complex media, rapidly growing staphylococcal cells of the normal phenotype display—after a short lag phase—an exponential growth phase characterized by high cell division rates and high metabolic activity starting about 1 h after inoculation and lasting over a period of approximately 8 to 10 h, with a maximum OD of 14 to 15. The highest cell division rates can be observed 2 to 6 h after inoculation. This phase is followed by the stationary phase, which shows a conspicuously reduced or discontinued rate of staphylococcal cell division (OD of 9 to 10 after 24 h) (125). In contrast, SCV phenotypes reveal distinct growth characteristics. Strikingly, no clearly distinguishable growth phases are observable, because the SCVs grow constantly in a linear manner, with drastically reduced cell division rates not reaching the exponential phase. This was shown for defined mutants displaying the SCV

phenotype, for gentamicin-induced SCVs, and for naturally occurring SCVs (125, 126). Extended lag phases may occur. Often, SCVs are not able to reach ODs above 1 to 3, even after incubation for more than 24 h (125, 126).

Instability of clinical SCV isolates, i.e., reversion of a subpopulation back to the normal phenotype, is relatively easy to perceive by the naked eye on solid agar media. However, this switch is hard to trace in broth media and rapidly leads to overgrowth of the SCV population. Aberrant growth curve courses might be indicative of those events but are coupled with waste of time and experimental efforts. To overcome these drawbacks, Fourier transform infrared (FTIR) spectroscopy was shown to offer a real-time readout tool to study the nature of SCVs under liquid culture conditions (127). Analysis of FTIR-generated spectra of SCVs and their parental strains resulted in a clear discrimination between both phenotypes; thus, it is possible to ensure and monitor the SCV phenotype by a fast, direct, and noninvasive approach.

Susceptibility Testing

All time- and density-influenced methods of susceptibility testing are altered by the changed physiology and metabolism of the SCV phenotype (104, 128). Since these alterations are nonuniform, standardization of respective assays is difficult. For determination of the methicillin resistance of SCV isolates, the detection of the *mecA* (or *mecC*) gene by PCR or other molecular methods and the modified use of an anti-PBP2a slide latex agglutination assay with a drastically increased number of colonies should be applied (129).

Conventional testing. Due to the low growth rate and reduced metabolism of SCVs, all kinds of routine methods to test susceptibility against antibiotics are difficult or impossible to perform, as these methods were developed for rapidly growing bacteria. Consequently, MIC data should be evaluated with caution. Even though some reports for specific strains suggest that MICs are, in general, similar for SCVs and their wild-type phenotype counterparts, pharmacodynamic studies indicate that the effects of several antibiotics against SCVs are markedly reduced. Results on this subject were recently summarized in a review (130).

A conventional method that can provide indications on the susceptibility of SCVs to antibiotics is the agar diffusion method or the use of Etests that are based on the bacterial growth on agar plates. In applying these methods to test SCVs, the bacteria have to grow for several days on agar plates. Incubation with CO_2 can support the bacterial growth. Nevertheless, the readout of the plates is often difficult and sometimes does not provide clear results for all antimicrobial compounds tested.

Intracellular testing in monocytes and nonprofessional phagocytes. Several studies have demonstrated that SCVs are induced/selected within the intracellular environment and that they can persist intracellularly in larger bacterial numbers than those of bacteria with wild-type phenotypes (41, 89, 131, 132). Consequently, analysis of antibiotic activity against persisting SCVs within the intracellular milieu is of particular importance. Some *in vitro* models have been developed that investigate the intracellular antibiotic activity against stable SCVs (mainly *hemB* and *menD* mutants). A frequently used model was established in mouse macrophages that take up bacteria but also cell remnants from red blood cells (133, 134). Therefore, hemin-auxotrophic SCVs are supplemented within the host cells and exhibit normal growth and virulence, like those of wild-type strains, whereas thymidine- and menadione-dependent strains grow slowly or fail to grow intracellularly. In a systematic overview, the activities of various antibiotics of different classes were tested to compare the intracellular activity with the activity in culture medium. Although maximal intracellular activities remained lower than extracellular activities (irrespective of the level of drug accumulation), some compounds (oxacillin, levofloxacin, garenoxacin, moxifloxacin, and oritavancin) exhibited intracellular bactericidal effects (134). In the next step, antimicrobial activity was tested in this model when macrophages were infected with a stable SCV strain in comparison with the normal phenotype. Most antibiotics tested caused a reduction of bacterial counts, whereas all drugs showed higher intracellular activity against normal than against SCV strains (133). The authors also used their infection model to test antibiotic combinations with rifampin or oritavancin against intracellular SCVs, and they achieved a further decrease of the intracellular bacterial growth (135). Finally, concentration-dependent testing revealed that maximal efficacies of antibiotics were similar against wild-type and SCV strains, despite their different modes of infection (136).

Taken together, these complex cell culture infection models indicate that SCVs exhibit more tolerance against treatment while located within host cells. Yet some questions remain open, such as the influence of the different formation mechanism of SCVs and the development of resistance/tolerance during intracellular long-term persistence.

Animal Models for Studying the Formation and Persistence of SCVs

Data from *in vivo* studies. Different animal models have been established to test the ability of SCVs to infect the host, to spread an infection, and to persist within host tissues for long periods. The different published animal models are summarized in a recent review (130) and highlight the following aspects. (i) Hemin-dependent mutants often show reversion within the host organisms, as they can easily be supplemented with heme provided during the infection course, e.g., endocarditis. As a result, virulence and treatment did not differ from those for bacteria with wild-type phenotypes (137). Consequently, hemin-dependent mutants are most likely not representative of the SCVs that cause chronic tissue infections *in vivo*. In support of these findings, a menadione biosynthetic mutant persisted in the kidneys, whereas a hemin biosynthetic mutant did not persist (137). (ii) Gentamicin treatment as monotherapy easily selected for SCVs. Although these SCVs were less virulent than the parent strain, they caused persistent infection and were able to colonize different organs (48). (iii) Animal models show that different types of stable SCVs colonize host tissue and persist, although they cause less inflammation and fewer symptoms (138–140). These findings indicate that although SCVs represent a heterogeneous bacterial population, they are all characterized by an increased ability to persist. (iv) SCVs may account for bacterial persistence under antibiotic pressure. In a murine mastitis model, cephalosporin treatment was much less effective at reducing the amount of bacterial *hemB* mutant SCVs than reducing the number of bacteria with the wild-type phenotype, although both strains tested as being equally susceptible *in vitro* (141). (v) Chronic animal infection models revealed that SCVs develop in a dynamic manner during the infection course (131). When animals were infected with a fully virulent organism with a wild-type phenotype and developed a septic stage, the bac-

teria disseminated to various organs. In particular, in bone tissue, bacterial persistence was observed for up to 2 months and was accompanied by a high rate of SCV formation (142). Antibiotic treatment with different compounds was always less effective in the chronic than in the acute stage of the infection (33). (vi) In contrast to systemic treatment, local treatment with high levels of antibiotics over a prolonged time was successfully tested in a chronic osteomyelitis model in rabbits (143). These results suggest that high antibiotic doses are required to eliminate persisting bacteria, such as SCVs.

Chronic osteomyelitis model. To analyze the development of SCVs within the whole host organism, a chronic infection model in which the bacteria persist in tissue for long periods (e.g., several weeks) is required. One type of infection that has a high tendency to develop to chronicity is osteomyelitis (45). There are several osteomyelitis models published, but rarely has the development of SCVs been documented or investigated; in some models, stable SCVs have been used (143). Only recently was a hematogenous osteomyelitis model in mice established that develops from an acute septic infection into a chronic stage in which the bacteria persist within the bone tissue for long periods (142). During a time course of 2 months, the mice did not resolve the inflammation within bones, which increased in volume up to 5-fold. The histopathological findings closely resembled the findings for patients suffering from chronic osteomyelitis, e.g., osteoclast activity and formation of bone sequestra. The analysis of the persisting bacteria revealed that a high percentage of bacteria with SCV-like phenotypes that still had the capacity to revert back to the fully virulent phenotype had formed in the bone tissue. Nevertheless, the systemic inflammatory parameters in the mice returned to control levels, although there were still persisting bacteria and inflammation in bone tissues (142). This model is suited for the study of dynamic bacterial adaptation processes and alterations in the immune system that fail to completely clear the infection.

Chronic lung infection model. To study the impact of SCVs on the pathogenesis of lung infections in CF, it would be necessary to use a chronic lung infection model which mimics CF airway disease. Recently, a model was initiated which uses bacteria implemented into agar beads, which are placed directly via incision into the trachea (75). The authors of that study followed airway infection for 2 weeks. In their model, *S. aureus* efficiently colonized the lungs of the mice and caused chronic pneumonia with high bacterial loads in the lungs without killing too many mice. Such a model would be suitable for studying the emergence of SCVs, but it should go on for at least some months.

Three-dimensional tissue model of osteoblasts for orthopedic infections. Lee et al. recently established a three-dimensional tissue model to study orthopedic implant infections. They seeded osteoblasts in a microfluidic device and infected the cells with different morphotypes of *S. epidermidis* to study the impact on tissue structure development by infecting strains. *S. epidermidis* isolates were cultured under different conditions in the microfluidic device prior to infection of osteoblasts to yield different morphotypes, such as SCVs, which were induced by rifampin, and sessile bacteria in biofilms (144). Biofilm bacteria and dead cells did not significantly interfere with tissue-like structures of osteoblasts, while SCVs did not affect adherence and spreading of cells but killed osteoblasts within 2 days. Unfortunately, the authors did not use a normal *S. epidermidis* isolate to differentiate effects caused by SCV or normal *S. epidermidis*. Furthermore, such a model would also be suitable for testing *S. aureus* SCVs or SCVs of other species.

PATHOGENESIS

Defined Underlying Mechanisms and Mutations

While there are some common features of SCVs regarding colony morphology and reduced growth, analyses of the underlying mechanisms leading to the formation of SCVs suggest that there is no common metabolic change typical for SCVs, but rather an array of several more or less understood possibilities of altered metabolism to reach this phenotype.

Underlying mutations have been identified for hemin-, menadione-, and thymidine-dependent SCVs but not for CO_2-dependent SCVs or SCVs with as yet unidentified auxotrophisms (32, 145–148). However, by using whole-genome sequencing, Gao et al. identified and confirmed a further mechanism for SCVs due to mutations in *relA*, with a permanent activation of the stringent stress response (148, 149). Just recently, Bui et al. studied a stable SCV induced by host-generated chemical stresses by full genomic characterization (150, 151). These studies revealed a new type of stable SCVs, which are characterized by mutations in *mgrA* and *rsbU* (150).

An aggravating circumstance for studying the metabolic changes is the instability of the vast majority of clinically derived or *in vitro* (without genetic engineering)-produced SCVs. Some stable SCVs have been gained by the use of concentrations of gentamicin that exceed the MIC and the minimal bactericidal concentration (MBC) (152). Serial subcultures of colonies displaying the SCV morphotype for up to 10 generations enabled selection of stable variant forms (152). Also, other stable or partially stable SCVs were generated by passages in media containing aminoglycosides, gentamicin, kanamycin, streptomycin, or amikacin (29, 125, 153–156).

To enable targeted analyses of the metabolic changes leading to the SCV phenotype, several genetically defined mutants displaying the SCV phenotype have been constructed in the past. This was done mainly by interrupting the hemin biosynthetic gene *hemB*, encoding the aminolevulinic acid dehydratase (125, 126), or by disrupting a menaquinone biosynthesis bifunctional protein encoded by *menD* (137) (Fig. 4).

So far, the most advanced model for studying the SCV phenotype consists of a clonal strain sextet comprising a clinical wild-type isolate and its corresponding clinical SCV isolate plus a spontaneous *in vitro* revertant strain with a normal phenotype as well as a genetically defined *hemB* mutant plus the complemented *hemB* mutant and a gentamicin-induced SCV (125). Applying this sextet for proteome analysis, it was shown that the physiological variations between both phenotypes were indeed more complex than reflected by defined mutants and their complemented counterparts. Interestingly, the natural revertant also retained dominant protein features characteristic of the clinical SCV phenotype (125).

Alterations in Electron Transport and Their Impact on Metabolism

For the transfer of electrons, menaquinone, the first electron acceptor, and heme, which receives electrons from menaquinone and is used in cytochrome biosynthesis, are required (157). Functional or genetic defects in the biosynthesis of these compounds

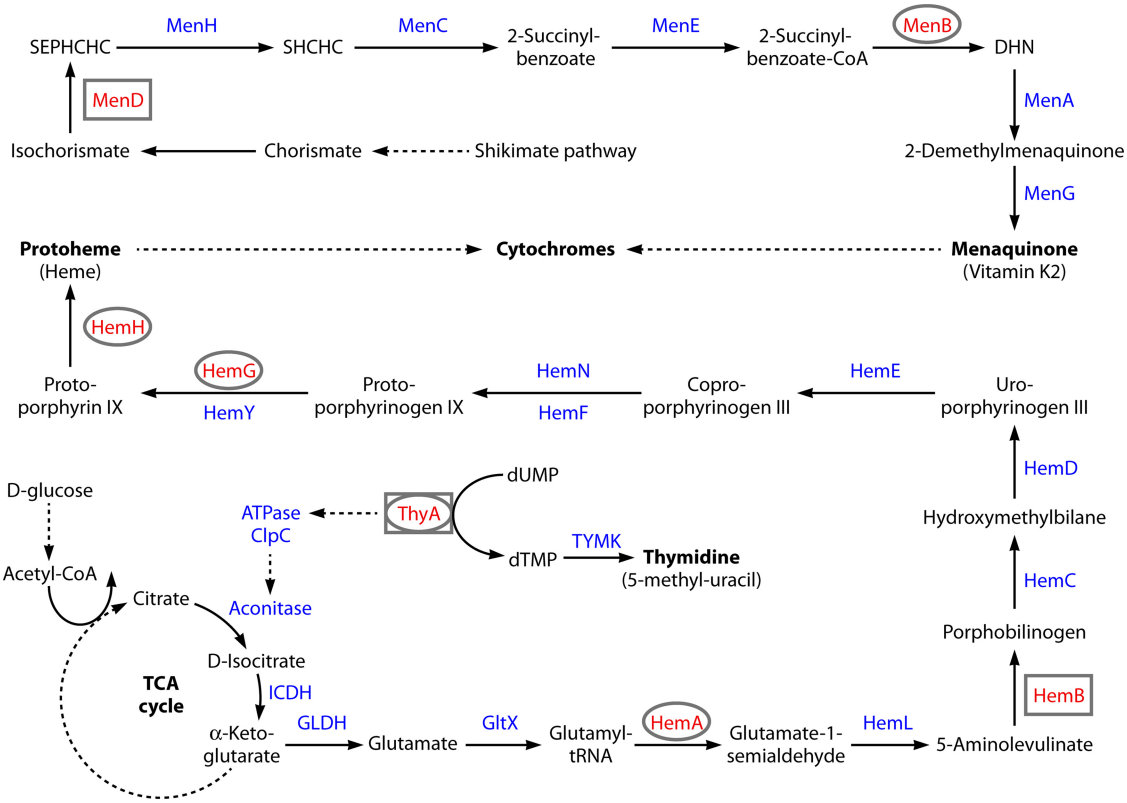

FIG 4 Overview of the menaquinone heme and thymidine pathways. Enzymes encoded by genes known to lead to the SCV phenotype if disabled are shown in red (knockout-generated SCVs are indicated with rectangular boxes, and naturally occurring or gentamicin-induced SCVs are indicated with ovals). DHN, 1,4-dihydroxy-2-naphthoate; GLDH, glutamate dehydrogenase; GltX, glutamate-tRNA ligase; HemA, glutamyl-tRNA reductase; HemB, 5-aminolevulinic acid dehydratase (porphobilinogen synthase); HemC, porphobilinogen deaminase; HemD, uroporphyrinogen III synthase; HemE, uroporphyrinogen decarboxylase; HemF, coproporphyrinogen III oxidase; HemG, protoporphyrinogen oxidase; HemH, protoheme ferrolyase (ferrochelatase); HemL, glutamate-1-semialdehyde aminotransferase; HemN, oxygen-independent coproporphyrinogen III oxidase; HemY, oxygen-dependent protoporphyrinogen oxidase; HSDC, (1*R*,6*R*)-6-hydroxy-2-succinylcyclohexa-2,4-diene-1-carboxylate; ICDH, isocitrate dehydrogenase; MenA, 1,4-dihydroxy-2-naphthoate octaprenyltransferase; MenB, 1,4-dihydroxy-2-naphthoyl-CoA synthase; MenC, *o*-succinylbenzoate synthase; MenD, SEPHCHC synthase; MenE, 2-succinylbenzoate-CoA ligase; MenF, isochorismate synthase; MenG, demethylmenaquinone methyltransferase; MenH, SHCHC synthase (SHCHC synthase activity was previously attributed to MenD); SEPHCHC, 2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate; SHCHC, (1*R*,6*R*)-2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate; TYMK, deoxythymidylate kinase; ThyA (TYMS), thymidylate synthase. Pathways are according to the Kyoto Encyclopedia of Genes and Genomes (KEGG), release 74.0 (http://www.genome.jp/kegg/).

lead to defects in electron transport, and thus the amount of ATP that is available for cell wall biosynthesis is drastically reduced (158). Consequently, this electron transport-defective SCV phenotype reveals a lower growth rate, decreased pigment formation, and a reduced membrane potential ($\Delta\Psi$) (9). Supplementation with these compounds, as described above, results in a switch back to the normal phenotype.

The first report on SCVs auxotrophic for hemin (an iron-containing porphyrin named protoporphyrin IX [protoheme IX, heme B]) was given by Jensen and Thofern in 1953 (154). The first evidence substantiating this observation on a molecular level was given by knocking out the *hemB* gene, encoding a porphobilinogen synthase (Fig. 4). von Eiff and colleagues generated an SCV in the *S. aureus* 8325-4 strain background that displayed typical features of clinical SCVs, including a low growth rate, changed biochemical characteristics, decreased pigment formation, reduced hemolytic activity, and decreased susceptibility to aminoglycosides (126). Later, it was shown that defects in *hemH*, encoding the terminal enzyme of the heme biosynthetic pathway, and in *hemA* were responsible for the phenotype change in gentamicin-induced hemin-auxotrophic SCVs (146). More recently, a *hemG* frameshift mutation resulting in a truncated protoporphyrin oxidase (HemG) was detected within the course of a whole-genome sequencing project for the investigation of a neonatal MRSA outbreak (159).

Similar to heme, a structure called menadione (2-methyl-1,4-naphthoquinone; provitamin "vitamin K_3"), which is used in menaquinone biosynthesis, is mandatory for aerobic as well as anaerobic electron transport phosphorylation (Fig. 4). Bacteria harboring the vitamin K synthesis machinery produce a multitude of menaquinones (so-called "vitamins K_2"). Menadione-auxotrophic SCVs recovered from clinical specimens were found to harbor mutations in the *menB* gene, encoding the naphthoate synthase (145). The mutations comprised a 9-bp deletion, a frameshift mutation that resulted in a premature stop codon, and a point mutation that caused an amino acid substitution. In the *S. aureus* 8325-4 background, a mutant of *menD* was constructed and displayed the typical features of staphylococcal SCVs; however, a higher level of expression of the *arc* operon than that in the *hemB* mutant of the same strain background was found (137, 160). The

arc operon contains the *arcABDC* genes, involved in ATP production from arginine catabolism under anaerobic conditions (161). In the presence of arginine, the arginine deiminase pathway allows staphylococci and other bacteria to grow anaerobically. The electron transport-defective SCV mutants compensate for the loss of citric acid cycle utilization and electron transport by stimulation of this pathway (162).

Also, *ctaA* mutants exhibit typical characteristics of SCVs due to blocked biosynthesis of HemA, which is involved in cytochrome biosynthesis (163).

Since heme and menaquinone are vital for the electron transport to both oxygen and nitrate, the respective auxotrophic SCV phenotypes show a "reduced" metabolic status (108). Compared to isolates with a normal *S. aureus* phenotype, the main common feature of electron transport-deficient SCVs, mutants, and clinically derived isolates, as well as thymidine-dependent SCVs and their respective mutants, was the downregulation or absence of the tricarboxylic acid (TCA) cycle (86, 108, 125). By transcriptional, proteomic, and reporter metabolite analyses as well as isotopologue profiling, it was shown that the expression of the *acnA* gene, encoding the aconitase catalyzing the second and third TCA cycle steps via *cis*-aconitic acid for the synthesis of isocitric acid, was reduced, thus resulting in a reduced carbon flux via this cycle (85, 108, 125, 162). However, in contrast to a *hemB* mutant and gentamicin-induced SCVs, clinical SCVs showed no prominent upregulation of glycolytic proteins (125).

Since RNAIII, which acts as the effector molecule of the *agr* (accessory gene regulator) locus, encoding the *S. aureus* quorum sensing apparatus, is produced in *S. aureus* during all growth phases, at least on a basal level (see "Role of the *agr* quorum sensing system") (164, 165), it was an astounding observation that there was no RNAIII production in electron transport-deficient SCVs (86, 125, 162, 166). This phenomenon is not necessarily the result of a reduced concentration of the quorum-sensing peptide due to the reduced growth rate of the SCV (148). We (86) and Pader et al. (167) showed that exogenous autoinducing peptide (AIP) is able to trigger RNAIII expresion in TD-SCVs (86) and in menadione- and hemin-dependent SCVs (167), thereby demonstrating that synthetic AIP is able to activate Agr in at least some SCVs. Based on cDNA libraries built from total RNA, which was collected from different growth phases of an *S. aureus* strain pair displaying both the normal and the SCV phenotype, phenotype-specific expression of non-protein-encoding RNAs (npcRNAs) was shown (168, 169). This may indicate a probable impact of npcRNAs for the generation of SCVs and the expression of their variant features.

Alterations in Thymidine Metabolism and Thymidine Dependency

TD-SCVs emerge if patients are treated with trimethoprim-sulfamethoxazole for extended periods (30, 32, 58–60, 63, 92). These antibiotics inhibit the synthesis of tetrahydrofolic acid, which serves as a cofactor for thymidylate synthase (*thyA*), an essential protein required for the conversion of thymidine from uracil (170) (Fig. 4). TD-SCVs depend on external thymidine for their growth, which is provided in infected tissues via cell degradation and pus (32). In addition to the small colonies described for all other SCVs, TD-SCVs can also grow with a "fried egg" colony appearance on Columbia blood agar plates (83). By light microscopy, TD-SCVs are characterized by a heterogeneous appearance, with small and large cells (83). Furthermore, TD-SCVs are impaired in cell division, as demonstrated by transmission electron microscopy, with several or even not intact division planes (83). TD-SCVs carry mutations in *thyA*, which can occur throughout the *thyA* gene (32, 147). A constructed Δ*thyA* deletion mutant displayed the same features as those described for clinical TD-SCVs, with attenuated infection in an acute mouse pneumonia model compared to infection with the wild type (140). In comparison to the gene expression in wild-type *S. aureus*, microarray analyses revealed downregulation of important regulator (*agr*, *sarA*, and *arlRS*) and virulence (*hla*, *hlb*, *sspAB*, and *geh*) genes and upregulation of virulence genes important for colonization (*fnbA*, *fnbB*, *spa*, *clfB*, *sdrC*, and *sdrD*) (140), all of which support the chronic infection type of TD-SCVs.

CO_2-Dependent SCVs

Several reports of *S. aureus* and CoNS SCVs with CO_2 dependency have been published (4, 14–16, 20, 42, 123, 171, 172). While all CO_2-dependent SCVs from earlier times were isolated from patients with skin and soft tissue infections (4, 14–16, 171, 172), in one of the latest studies, 14 CO_2-dependent SCVs were isolated from 14 individual patients with various infections, including surgical wound infection, respiratory infections, device-related infections, spondylodiscitis, and skin and soft tissue infection, in a 3-year prospective study conducted in a university hospital in Spain (123). These isolated SCVs were identified to be CO_2 dependent by exhibiting typical small colonies without pigmentation and hemolysis on Columbia blood agar but reversion to normally sized colonies by growth under 5% CO_2 (123). In further studies, CO_2-dependent *S. epidermidis* SCVs were found to be associated with PJI (20, 42). However, so far, there have been no attempts to characterize the metabolic changes or the underlying mechanism of CO_2 dependence in more detail. It is interesting that for this kind of SCVs, an irregular cell wall layer and tri- and tetraploid cells, along with many dividing forms, were also described (172).

Dynamic Phenotype Switching Phenomenon

In the past decades, research on SCVs has largely focused on SCV mutants, such as *hemB* or *menD* mutants, that exhibit a stable SCV phenotype and can easily be used in infection models (107, 126, 137). These experiments provide results on the interaction of SCVs with their host cells and on tolerance to antibiotics but leave the question open regarding the mechanism of formation of SCVs during infection. However, differences between SCV and normal phenotypes are more complex than those mirrored by defined electron transport chain-interrupting mutants (125). In particular, it remained largely unclear whether SCVs are only a marginal phenomenon or are an integral part of chronic infections. To investigate this question, various *in vitro* and *in vivo* long-term infection models were established. Cell culture systems or mice were infected with fully aggressive bacteria with wild-type phenotypes, and the infecting bacteria were analyzed during the whole infection course (131, 142). In different types of cell culture models, the first SCVs appeared after 2 to 3 days of infection, with the percentage of SCVs continuously increasing over longer infection times, reaching almost 90% after 4 weeks. It was very remarkable that the recovered SCVs were not stable but rapidly reverted back to the fully aggressive wild-type phenotype after a few subculture steps in rich medium. From these results, it was concluded that SCVs always form in a very dynamic manner during host cell infection but

still keep the ability to rapidly revert to the fully aggressive wild-type phenotype when leaving their host cells (Fig. 3). This dynamic switching phenomenon suggests either that regulatory mechanisms are involved in the early formation of dynamic SCVs or that reversible phase variation occurs by insertion or deletion of tandem repeats, which has been described to occur for mucoid *S. aureus* isolates or for *Escherichia coli*, but so far not for *S. aureus* SCVs (173, 174).

Role of the *agr* Quorum Sensing System

agr is a well-known quorum sensing system that upregulates the expression of secreted toxins and exoenzymes (165). These factors contribute to inflammation and cell/tissue destruction (175). In the acute stage of infection, the *agr* system is highly upregulated in infecting bacteria (131, 176, 177). Upregulation of *agr* and secreted virulence factors apparently helps bacteria to defend against invading immune cells and to destroy and invade tissue to establish infection in deep tissue structures. After host cell invasion, *agr* is downregulated, which is characteristic of persisting SCV-like bacteria. Testing of different mutants for regulatory factors revealed that *agr*-related activity needs to be downregulated for long-term persistence. Yet because *agr* interacts closely with other regulatory factors, such as the transcription factor SarA, that can partly compensate for the lack of *agr* expression, these factors need to be silenced as well. Taken together, these results demonstrate that proinflammatory and cytotoxic activities mediated via the *agr* system need to be silenced to enable for bacterial long-term persistence and SCV formation (53) (Fig. 3). Although the expression of *agr*-related virulence genes has been studied in various SCVs and by different groups, so far there is no information about the role of superantigen expression in SCVs, which may be an interesting area for future studies.

Role of SigB during Long-Term Infection

SigB is a stress-related transcription factor that helps bacteria to cope with different types of stress conditions (178). In addition, SigB has been described as an important regulator of virulence in SCVs because it promotes biofilm formation and the expression of adhesins (179, 180). The use of *sigB* mutants in cell culture infection models revealed that a defective *sigB* system resulted in a lack of persistence and in the bacterial disability to form SCV-like cells. Furthermore, the expression of SigB and adhesins was upregulated during chronic infection courses (176). Consequently, *sigB* can be described as an important regulatory factor that is required for bacterial long-term persistence within host cells and for the dynamic formation of SCVs (Fig. 3). Until now, it has been understood only marginally which intracellular signals are involved in activating *sigB* and how these signals are transferred to enhance SCV formation.

Other Factors That Induce the Formation of SCVs during *In Vivo* Infection

Stringent stress response. A new mechanism for the formation of SCVs was described for SCVs which were isolated from a patient with chronic MRSA bacteremia, who was treated with various antibiotics, including vancomycin, linezolid, rifampin, and ciprofloxacin, leading to antibiotic resistance and the SCV phenotype (149). In addition to a few other mutations, the authors identified a single nucleotide substitution in *relA* by whole-genome sequencing. This point mutation resulted in reduced RelA hydrolase activity, with a permanent activation of the stringent stress response as determined by levels of ppGpp (149). At present, such a mechanism has been reported only once, since testing of this mechanism is rather laborious and difficult to perform.

Oxidative stress. Another new mechanism of SCV formation was reported very recently. In that study, the authors investigated the impact of reactive oxygen species on SCV formation and persistence by challenging SH1000 with sublethal concentrations of hydrogen peroxide, which led to the emergence of gentamicin-resistant SCVs (181). Using whole-genome sequencing, the authors identified mutations in the DNA repair system RexAB, recombinase A, and polymerase V. These SCVs were rather stable and more resistant to H_2O_2, which was accomplished by increased expression of catalase. While it is surprising that SCVs emerged with increased catalase activity, which has been shown to be decreased in many other SCVs with other underlying mutations, the use of the particular wild-type strain (SH1000) and the special inducing conditions might explain such diverse results (181).

Coculture with other pathogens. *S. aureus* can often be coisolated with *P. aeruginosa* from various sites and infections, such as catheters, endotracheal tubes, skin, eyes, and especially the respiratory tract of CF patients. Several studies which determined the prevalence of *S. aureus* SCVs in CF patients revealed that *S. aureus* SCVs were associated with increased age, worse lung function, and coculture with *P. aeruginosa* (30, 31, 60, 61). Such an association of *S. aureus* SCVs and *P. aeruginosa* is most likely not just an accident but at least partly due to a survival strategy that *S. aureus* uses to overcome the toxic effects of 4-hydroxy-2-heptylquinoline *N*-oxide (HQNO) or pyocyanin, which drive the emergence of respiratory-deficient SCVs (182, 183). Such SCVs are known to be hemin or menadione dependent and are resistant to aminoglycosides, which are often used to treat *P. aeruginosa* airway infection either systemically or by inhalation therapy. Thus, by inducing the SCV phenotype, *P. aeruginosa* facilitates survival of these SCVs during antibiotic therapy.

The intracellular environment and phagosomal escape. The intracellular environment was already described by the group of Proctor to induce the formation of SCVs (132). In further work, the intracellular milieu was increasingly found to be a shelter for SCVs, since SCVs can persist within host cells in larger numbers than those of bacteria with wild-type phenotypes (30, 41, 89). During persistence, SCVs largely avoid activation of the host immune response or cytotoxic effects, but they persist within morphologically intact host cells for long periods. This "silent" persistence can explain why chronic infections often lack clear inflammatory symptoms. Yet the persisting SCVs can escape from their intracellular shelter, revert back to the fully aggressive wild-type phenotype, and be the source for a new acute episode of an infection (8, 9, 82, 131).

The localization of the intracellularly persisting bacteria is not precisely known. Recent work showed that staphylococci can escape from their initial phagosomes to the cytoplasm by expressing delta-toxin and/or the PSM-alpha protein, whereas some strain-specific differences are possible (184, 185). The location where bacteria form SCVs and persist for long periods might be the cytoplasm, but this is not precisely known. Cell culture experiments after infection with defined regulatory mutants demonstrated that an absence of the *agr* and/or *sarA* system allowed the mutants to avoid phagosomal escape and enabled bacteria to persist within their initial phagosomes in large numbers for long periods (53).

From these results, it can be concluded that phagosomal escape to the cytoplasm is a characteristic feature of virulent strains that express *agr*- and/or *sarA*-regulated virulence factors but that wild-type bacteria can change to SCVs after phagosomal escape within their host cells.

CURRENT AND FUTURE TREATMENT REGIMENS

Staphylococcal SCVs are challenging in many ways, in terms of (i) treatment by antibiotic agents and (ii) susceptibility testing. Basically, a given SCV isolate mirrors the genetically encoded resistance pattern of its parental strain. Thus, β-lactamase-producing SCVs and *mecA*-based MRSA-SCVs have been described repeatedly (31, 63, 92, 186–188). However, in addition to and beyond these classical mechanisms of antibiotic resistance described for staphylococci, the SCV phenotype is viewed as a prime example of the expression of phenotypic resistance, often also called functional resistance. This means that despite an isolate testing as susceptible *in vitro*, a treatment regimen administering respective antibiotic agents might fail clinically. Moreover, in addition to the SCV phenotype-generated alterations in antibiotic susceptibilities, the implications of the intracellular lifestyle as well as biofilm-caused functional resistance due to foreign body-related infections should be considered. For example, SCVs in biofilms can become completely resistant to clinically achievable concentrations of antibiotics (128).

In the context of antibiotic treatment of staphylococci in general, one should keep in mind that the administration of antibiotics itself may lead to the formation of SCVs (33). Respective phenotypic effects of antibiotic agents have been described since the earliest times of their use (7).

A detailed overview of current *in vitro* studies as well as animal and clinical data on the resistance patterns of SCVs was recently given by Garcia et al. (130).

Effect of SCV Phenotype on Susceptibility Testing

Susceptibility testing is challenging in the case of SCVs because routinely applied methods have been developed for testing of rapidly growing microorganisms but not for slow-growing pathogens, such as SCVs. In addition, use of both a disk or gradient diffusion assay and MIC determination by agar or broth dilution assays, as well as (semi)automated methods, could lead to invalid results due to difficulties in detection of growth either by visual inspection or measured by optical density measurements. Also, the standard agar media used according to the guidelines are often suboptimal or unusable for the cultivation of SCVs. Thus, in general, the results obtained by testing SCVs by applying disk diffusion assays or methods to determine MICs should be interpreted cautiously. Mostly, longer incubation times have to be applied before readable results are gained.

Apart from growth rate-related limitations, agar or broth dilution assays show the best practicability and validity for SCV susceptibility testing. If one is available, testing of a parallel isolated parental strain exhibiting the normal phenotype may help to determine antibiotic susceptibilities not depending on the phenotype. For that purpose, the clonality of both recovered SCV and wild-type strains should be substantiated, e.g., by PFGE, multilocus sequence typing (MLST), or—in the case of *S. aureus*—*spa* typing.

Influence of SCV Phenotype on Antibiotic Susceptibilities

For most antibiotics, based on MIC studies, SCVs and their normal phenotype counterparts show similar categorizations in terms of resistance; however, in comparing isogenic pairs consisting of the normal and the SCV phenotype, some SCVs may show reduced susceptibilities (130, 186). Aside from this, the altered metabolism of SCVs because of the phenotype switch leads to changes in susceptibility to distinct antibiotics. The regular loss of activity of SCVs toward antifolate agents and aminoglycosides is explicable by the metabolic mechanisms leading to the auxotrophism of respective SCV types (189). Negative effects on the bactericidal activity of antibiotics due to SCV phenotype characteristics have been reported for sessile and planktonic as well as extra- and intracellularly occurring SCV cells (128, 136, 190).

Keep in mind that the intracellular location of SCVs protects them from the action of all antibiotics that cannot penetrate the host cell's membrane. Thus, only compounds reaching the intracellular milieu have, in principle, the capacity to act toward intracellularly persisting SCV cells. Note that the possibility of the cooccurrence of parental cells with a normal phenotype should be considered.

The decreased growth rate of SCVs may generally affect the efficacy of antibiotics, particularly those that are active against dividing microorganisms. Nevertheless, there was no general direct correlation shown between the growth rates of intracellular SCV mutants and their susceptibilities to antimicrobial agents (136).

As known since the first years of the discovery of penicillin as the first β-lactam, and possibly due to the low growth rate and therefore reduced cell division of SCVs, the activity of cell wall-active antibiotics may be reduced in the case of SCVs (7, 191–193). However, intracellular menadione-dependent SCVs were shown to be hypersusceptible to β-lactams in a cell culture model based on interoperation of vacuolar acidic pH and oxidant species (194). For a compound of the novel MRSA-active class of cephalosporins, ceftobiprole, MRSA-SCVs showed equal or slightly increased MIC values (186). Testing of the *in vitro* activities of vancomycin, a compound of another antibiotic class with cell wall activity, against a hemin-auxotrophic clinical isolate and a genetically defined (Δ*hemB*) *S. aureus* SCV revealed markedly attenuated killing activities compared to those for the parental isolates (195).

The reduced antibiotic susceptibility of SCVs toward aminoglycosides due to a reduction in transmembrane potential, which impairs aminoglycoside uptake in *S. aureus* (189, 196), is well studied. However, despite the increased MICs, gentamicin may remain highly bactericidal *in vitro* against SCV mutants (136). Note that aminoglycosides may induce the formation of the SCV phenotype, as shown *in vitro* and *in vivo* (see Pathogenesis).

Thymidine-dependent SCVs have been shown to be resistant to trimethoprim-sulfamethoxazole (39, 86). They are not susceptible to those antifolate agents because they are not capable of converting dUMP to dTMP as a result of mutations in the *thyA* gene, encoding the thymidylate synthase (147).

Independent of the SCV auxotrophism, fluoroquinolones in general appeared to be highly effective against SCVs, although some SCVs may exhibit increased MIC values (186). For ciprofloxacin, MIC values were shown to be higher for SCV strains than for isogenic wild-type isolates under neutral (pH 7.2) and acidic (pH 5.8) conditions (197). For two other fluoroquinolones included in that study, i.e., moxifloxacin and levofloxacin, there was no marked difference in activity against SCVs compared with their parental strains under both

pH conditions. This also held true for finafloxacin, which exhibits optimal activity under slightly acidic conditions (197).

Testing of linezolid against isogenic SCV-normal phenotype pairs, categorized as methicillin-susceptible *S. aureus* (MSSA) and MRSA, respectively, the MSSA- and MRSA-SCVs showed predominantly increased MIC values (186).

While no higher activity of tigecycline was noted against SCV strains than against strains with normal phenotypes in a model of human THP-1 macrophages, significantly increased MICs of tigecycline compared to those for simultaneously isolated wild-type strains were reported for a collection of SCVs from patients with cystic fibrosis (63, 133).

For the cyclic lipopeptide daptomycin, sustained bactericidal activity against *hemB* mutants displaying the SCV phenotype was observed, although early killing activity was attenuated (190). Extracellularly, daptomycin demonstrated similar levels of effectiveness alone or in combination with gentamicin and/or rifampin against both parental and SCV strains (47).

Prolonged sublethal exposure to triclosan resulted in survivors resembling SCVs and exhibiting decreased susceptibility to triclosan (198, 199). Triclosan induction of SCVs resulted in significant increases of susceptibilities to antibiotics in the majority of cases (200).

Contemporary Therapeutic Strategies

Currently, the optimal treatment for infections caused by staphylococcal SCVs is not known. However, in consideration of the consequences in the context of the SCV phenotype switch, a chosen therapeutic regimen should address (i) the general resistance characteristics of SCVs according to their auxotrophies, (ii) their location within host cells, and (iii) the results of susceptibility tests applied to the parental strain, if available, and/or the results of testing the SCV isolate itself. Moreover, the pharmacokinetic aspects resulting from the respective infected tissue have to be considered. Thus, the armamentarium applicable for a given SCV infection is generally much more limited than that for the parental strain. However, since the cooccurrence of the parental cells and/or a reversion to the normal phenotype cannot be excluded, the use of agents active against the normal phenotype should not be neglected in the treatment of SCV infections, even if normal *S. aureus* isolates have not been cultivated. Consequently, an "ideal" anti-SCV treatment regimen should include antibiotics that are able to penetrate the SCV-containing host cells and to maintain their bactericidal activity against this slowly growing phenotype.

For a foreign body-related infection where the removal of the colonized device is associated with serious clinical risks or is even impossible, continuous suppressive treatment by antibiotics may be considered not only for infections caused by the normal *S. aureus* phenotype but also in the case of SCVs. In a chronic hemodialysis patient with several episodes of bacteremia due to an *S. aureus* SCV-infected pacemaker, which could not be removed, administration of cefuroxime axetil was able to keep the patient free of bacteremia relapse for at least 2 years (201).

The only agent with antistaphylococcal activity that fulfills these requirements is rifampin; however, the rapid emergence of high-level resistance prohibits its application in monotherapy. Thus, if an isolate tests as susceptible, a combination with rifampin is important to include an antistaphylococcal agent with proven intracellular activity. In intracellular models, antibiotic combinations including rifampin were shown to be particularly effective at eradicating intracellular SCVs (47, 133). Independent of phenotype, a clinical SCV isolate with high-level rifampin resistance due to an *rpoB* mutation has been reported (149). Also, other rifampin-resistant SCVs have been observed (39). In a case series of five patients with hip prosthetic joint infections due to SCVs, administration of a fluoroquinolone (levofloxacin)—if the SCVs tested susceptible—mostly in combination with rifampin, was associated with successful outcomes (39). In these cases, antimicrobial therapy was given for an implant-free interval of 6 to 8 weeks within a 2-stage exchange, without implantation of a spacer. Note that therapy became successful only after removal of the implant.

In the case of penicillin-resistant MSSA SCVs, the administration of staphylococcus-active β-lactam antibiotics, such as isoxazolyl penicillins or cephalosporins of the first and second generations, has been recommended. For penicillin-susceptible isolates, the use of penicillin should be considered. In case of doubt of penicillin susceptibility, penicillin therapy can be supplemented with a β-lactamase inhibitor; alternatively, a penicillinase-stable penicillin or a first-generation cephalosporin should be used for therapy.

In several cases, methicillin-resistant SCVs have been isolated from infection or colonization, sometimes without response to the given antibiotics, even if they tested susceptible *in vitro* (89, 92, 187, 188, 202). If an MRSA-SCV infection has to be treated, the administration of glycopeptides represents a therapeutic option. However, vancomycin is not bactericidal against SCVs (136). Moreover, Lenhard et al. (203) showed recently in mixed-population experiments that vancomycin favorably selects for the growth of the SCV subpopulation. Thus, in cases of persistent severe MRSA infections with proven or possible SCV occurrence, a glycopeptide combination regimen or alternative MRSA-active antibiotics should be considered.

PREVENTIVE STRATEGIES

In general, there are no clear preventive strategies available to avoid the formation of SCVs and chronic infection development. Because bacterial persistence mechanisms start early during infection, the most important strategy is to clear an infection in the acute stage, before bacterial adaptation with the development of SCVs starts to emerge. Therefore, the strategy to "hit hard and early" is particularly true for the prevention of SCVs and chronic infections.

To deliver high levels of antibiotics to the infection focus at an early stage, local antibiotic applications are available. Particularly in orthopedic surgery, various antibiotic delivery system vehicles, such as antibiotic-loaded bone cement, bioabsorbable or biodegradable material, and coating of prosthetic material, are available. Although the number of clinical studies is small and consensus guidelines are lacking, local antibiotic administration for prophylaxis of bacterial infections is used during many surgical procedures. By far the most frequently used antibiotic compound for local treatment is gentamicin (204, 205). Various methods of local applications and studies on local antibiotic use for surgical site prophylaxis have been summarized in published reviews (206, 207). Based upon available data, these reviews draw the conclusion that although some methods appear to be safe, the efficacy of local antibiotic administration (especially gentamicin) for the prophylaxis of surgical site infections remains uncertain, as subinhibitory concentrations of gentamicin induce the formation of SCVs. Nevertheless, the rationale behind the local antibiotic application is to provide high antibiotic doses at the site of surgery or infection without systemic exposure of the patient. In this way, bacterial pathogens can be killed efficiently before they establish an infection. This might even be applicable for persisting

SCVs that are found to be susceptible to high antibiotic concentrations (143). Yet if the infecting pathogens are not readily reached by the high antibiotic concentrations, e.g., in bone sequestra or due to malperfusion of inflamed or necrotic tissue areas, the bacteria are exposed only to low or subinhibitory concentrations of antibiotics that might even favor SCV formation. At least for low doses of gentamicin, an SCV-promoting activity has been reported that could even promote the development of a chronic infection (29, 44). Due to these inconsistent possible actions, more systematic clinical studies are required to work out clear consensus guidelines for the local use of antibiotics.

CONCLUSIONS

In this review, in juxtaposition to the normal staphylococcal phenotype, we summarize the various clinical infection types with emergence of SCVs, different mechanisms of SCV formation, and their characteristics, virulence, and resistance/tolerance mechanisms against antibiotics. SCVs represent a very heterogeneous bacterial population found in different staphylococcal species, with different formation mechanisms and virulence levels. Nevertheless, a general characteristic of SCVs is the fact that they are often isolated from chronic and therapy-refractory infections, most likely due to selection of these phenotypes during antibiotic therapy. During the course of infection, different mechanisms of formation of SCVs might take place in a time-dependent manner. Early after infection, the first SCVs rapidly form via regulatory mechanisms that enable the bacteria to dynamically react to changing environmental conditions. In this way, the infecting pathogens can hide within host cells and escape from the immune response and from antimicrobial treatments. Within their intracellular shelter, the bacteria are most likely well protected. Later, if the host defense mechanism and antibiotic treatment do not successfully clear the infection focus, chronically infecting bacteria might apply further strategies to form permanent SCVs, such as defined mutations in the electron transport system or in thymidine synthesis, depending on the infection type and on the selective antibiotic pressure. For successful management of infections suspected or confirmed to include staphylococcal SCVs, the specific requirements of sample collection, laboratory diagnostics, and treatment should be considered.

ACKNOWLEDGMENTS

This work was partly funded by grants from Transregio34/C12 to B.L., from BMBF:CSCC/StaphBone to B.L., from the Deutsche Forschungsgemeinschaft (DFG) within the SPP1316 program to K.B. (grant BE 2546/1-2), and from Transregio34/C7 to B.C.K.

REFERENCES

1. **Kolle W, Hetsch H.** 1906. Die experimentelle Bakteriologie und die Infektionskrankheiten mit besonderer Berücksichtigung der Immunitätslehre. Ein Lehrbuch für Studierende, Ärzte und Medizinalbeamte. Urban & Schwarzenberg, Berlin, Germany.
2. **Hoffstadt RE, Youmans GP.** 1934. The genetic significance of the dissociants of *Staphylococcus aureus*. J Bacteriol **27:**551–560.
3. **Borderon E, Horodniceanu T.** 1976. Mutants déficients a colonies naines de *Staphylococcus*: étude de trois souches isolées chez des malades porteurs d'ostéosynthèses. Ann Microbiol (Paris) **127:**503–514.
4. **Goudie JB, Goudie RB.** 1955. Recurrent infection by a stable-dwarf-colony variant of *Staphylococcus aureus*. J Clin Pathol **8:**284–287. http://dx.doi.org/10.1136/jcp.8.4.284.
5. **Spink WW, Osterberg K, Finstad J.** 1962. Human endocarditis due to a strain of CO_2-dependent penicillin-resistant staphylococcus producing dwarf colonies. J Lab Clin Med **59:**613–619.
6. **Hale JH.** 1947. Studies on *Staphylococcus* mutation: characteristics of the "G" (gonidial) variant and factors concerned in its production. Br J Exp Pathol **28:**202–210.
7. **Schnitzer RJ, Camagni LJ, Buck M.** 1943. Resistance of small colony variants (G-forms) of a *Staphylococcus* toward the bacteriostatic activity of penicillin. Proc Soc Exp Biol Med **53:**75–78. http://dx.doi.org/10.3181/00379727-53-14192.
8. **Proctor RA, van Langevelde P, Kristjansson M, Maslow JN, Arbeit RD.** 1995. Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*. Clin Infect Dis **20:**95–102. http://dx.doi.org/10.1093/clinids/20.1.95.
9. **Proctor RA, von Eiff C, Kahl BC, Becker K, McNamara P, Herrmann M, Peters G.** 2006. Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. Nat Rev Microbiol **4:**295–305. http://dx.doi.org/10.1038/nrmicro1384.
10. **Hadley P, Delves E, Klimek EJ.** 1931. The filterable forms of bacteria. 1. A filterable stage in the life history of the Shiga dysentery bacillus. J Infect Dis **48:**1–159.
11. **Hoffstadt RE, Youmans GP.** 1932. *Staphylococcus aureus*. Dissociation and its relation to infection and to immunity. J Infect Dis **51:**216–242.
12. **Swingle EL.** 1935. Studies on small colony variants of *Staphylococcus aureus*. J Bacteriol **29:**467–489.
13. **Chinn BD.** 1936. Characteristics of small colony variants with special reference to *Shigella paradysenteriae* Sonne. J Infect Dis **59:**137–151. http://dx.doi.org/10.1093/infdis/59.2.137.
14. **Sherris JC.** 1952. Two small colony variants of *Staphylococcus aureus* isolated in pure culture from closed infected lesions and their carbon dioxide requirements. J Clin Pathol **5:**354–355. http://dx.doi.org/10.1136/jcp.5.4.354.
15. **Hale JH.** 1951. Studies on *Staphylococcus* mutation: a naturally occuring "G" gonidial variant and its carbon dioxide requirements. Br J Exp Pathol **32:**307–313.
16. **Thomas MEM, Cowlard JH.** 1955. Studies on a CO_2-dependent *Staphylococcus*. J Clin Pathol **8:**288–291. http://dx.doi.org/10.1136/jcp.8.4.288.
17. **Proctor RA, Balwit JM, Vesga O.** 1994. Variant subpopulations of *Staphylococcus aureus* as cause of persistent and recurrent infections. Infect Agents Dis **3:**302–312.
18. **Savini V, Carretto E, Polilli E, Marrollo R, Santarone S, Fazii P, D'Antonio D, Rossano A, Perreten V.** 2014. Small colony variant of methicillin-resistant *Staphylococcus pseudintermedius* ST71 presenting as a sticky phenotype. J Clin Microbiol **52:**1225–1227. http://dx.doi.org/10.1128/JCM.02861-13.
19. **Adler H, Widmer A, Frei R.** 2003. Emergence of a teicoplanin-resistant small colony variant of *Staphylococcus epidermidis* during vancomycin therapy. Eur J Clin Microbiol Infect Dis **22:**746–748. http://dx.doi.org/10.1007/s10096-003-1029-9.
20. **Tande AJ, Osmon DR, Greenwood-Quaintance KE, Mabry TM, Hanssen AD, Patel R.** 2014. Clinical characteristics and outcomes of prosthetic joint infection caused by small colony variant staphylococci. mBio **5:**e01910-14. http://dx.doi.org/10.1128/mBio.01910-14.
21. **von Eiff C, Vaudaux P, Kahl BC, Lew D, Emler S, Schmidt A, Peters G, Proctor RA.** 1999. Bloodstream infections caused by small-colony variants of coagulase-negative staphylococci following pacemaker implantation. Clin Infect Dis **29:**932–934. http://dx.doi.org/10.1086/520462.
22. **Baddour LM, Barker LP, Christensen GD, Parisi JT, Simpson WA.** 1990. Phenotypic variation of *Staphylococcus epidermidis* in infection of transvenous endocardial pacemaker electrodes. J Clin Microbiol **28:**676–679.
23. **Baddour LM, Christensen GD.** 1987. Prosthetic valve endocarditis due to small-colony staphylococcal variants. Rev Infect Dis **9:**1168–1174. http://dx.doi.org/10.1093/clinids/9.6.1168.
24. **Bogut A, Niedzwiadek J, Koziol-Montewka M, Strzelec-Nowak D, Blacha J, Mazurkiewicz T, Marczynski W, Plewik D.** 2014. Characterization of *Staphylococcus epidermidis* and *Staphyloccocus warneri* small-colony variants associated with prosthetic-joint infections. J Med Microbiol **63:**176–185. http://dx.doi.org/10.1099/jmm.0.066068-0.
25. **Becker K, Heilmann C, Peters G.** 2014. Coagulase-negative staphylococci. Clin Microbiol Rev **27:**870–926. http://dx.doi.org/10.1128/CMR.00109-13.
26. **Seifert H, Oltmanns D, Becker K, Wisplinghoff H, von Eiff C.** 2005. *Staphylococcus lugdunensis* pacemaker-related infection. Emerg Infect Dis **11:**1283–1286. http://dx.doi.org/10.3201/eid1108.041177.

27. **Lowy FD.** 1998. *Staphylococcus aureus* infections. N Engl J Med **339:**520–532. http://dx.doi.org/10.1056/NEJM199808203390806.
28. **Edwards AM.** 2012. Phenotype switching is a natural consequence of *Staphylococcus aureus* replication. J Bacteriol **194:**5404–5412. http://dx.doi.org/10.1128/JB.00948-12.
29. **Balwit JM, van Langevelde P, Vann JM, Proctor RA.** 1994. Gentamicin-resistant menadione and hemin auxotrophic *Staphylococcus aureus* persist within cultured endothelial cells. J Infect Dis **170:**1033–1037. http://dx.doi.org/10.1093/infdis/170.4.1033.
30. **Kahl B, Herrmann M, Everding AS, Koch HG, Becker K, Harms E, Proctor RA, Peters G.** 1998. Persistent infection with small colony variant strains of *Staphylococcus aureus* in patients with cystic fibrosis. J Infect Dis **177:**1023–1029. http://dx.doi.org/10.1086/515238.
31. **Wolter DJ, Emerson JC, McNamara S, Buccat AM, Qin X, Cochrane E, Houston LS, Rogers GB, Marsh P, Prehar K, Pope CE, Blackledge M, Deziel E, Bruce KD, Ramsey BW, Gibson RL, Burns JL, Hoffman LR.** 2013. *Staphylococcus aureus* small-colony variants are independently associated with worse lung disease in children with cystic fibrosis. Clin Infect Dis **57:**384–391. http://dx.doi.org/10.1093/cid/cit270.
32. **Besier S, Ludwig A, Ohlsen K, Brade V, Wichelhaus TA.** 2007. Molecular analysis of the thymidine-auxotrophic small colony variant phenotype of *Staphylococcus aureus*. Int J Med Microbiol **297:**217–225. http://dx.doi.org/10.1016/j.ijmm.2007.02.003.
33. **Tuchscherr L, Kreis CA, Hoerr V, Flint L, Hachmeister M, Geraci J, Bremer-Streck S, Kiehntopf M, Medina E, Kribus M, Raschke M, Pletz M, Peters G, Löffler B.** 2016. *Staphylococcus aureus* develops increased resistance to antibiotics by forming dynamic small colony variants during chronic osteomyelitis. J Antimicrob Chemother **71:**438–448. http://dx.doi.org/10.1093/jac/dkv371.
34. **Seifert H, Wisplinghoff H, Schnabel P, von Eiff C.** 2003. Small colony variants of *Staphylococcus aureus* and pacemaker-related infection. Emerg Infect Dis **9:**1316–1318. http://dx.doi.org/10.3201/eid0910.0302000.
35. **Idelevich EA, Schüle I, Grünastel B, Wüllenweber J, Peters G, Becker K.** 2014. Rapid identification of microorganisms from positive blood cultures by MALDI-TOF mass spectrometry subsequent to very short-term incubation on solid medium. Clin Microbiol Infect **20:**1001–1006. http://dx.doi.org/10.1111/1469-0691.12640.
36. **Quie PG.** 1969. Microcolonies (G-variants) of *Staphylococcus aureus*. Yale J Biol Med **41:**394–403.
37. **Chirouze C, Alla F, Fowler VG, Jr, Sexton DJ, Corey GR, Chu VH, Wang A, Erpelding ML, Durante-Mangoni E, Fernández-Hidalgo N, Giannitsioti E, Hannan MM, Lejko-Zupanc T, Miró JM, Munoz P, Murdoch DR, Tattevin P, Tribouilloy C, Hoen B.** 2015. Impact of early valve surgery on outcome of *Staphylococcus aureus* prosthetic valve infective endocarditis: analysis in the International Collaboration of Endocarditis-Prospective Cohort Study. Clin Infect Dis **60:**741–749. http://dx.doi.org/10.1093/cid/ciu871.
38. **Maduka-Ezeh A, Seville MT, Kusne S, Vikram HR, Blair JE, Greenwood-Quaintance K, Arabia F, Patel R.** 2012. Thymidine auxotrophic *Staphylococcus aureus* small-colony variant endocarditis and left ventricular assist device infection. J Clin Microbiol **50:**1102–1105. http://dx.doi.org/10.1128/JCM.01170-11.
39. **Sendi P, Rohrbach M, Graber P, Frei R, Ochsner PE, Zimmerli W.** 2006. *Staphylococcus aureus* small colony variants in prosthetic joint infection. Clin Infect Dis **43:**961–967. http://dx.doi.org/10.1086/507633.
40. **Geipel U.** 2009. Pathogenic organisms in hip joint infections. Int J Med Sci **6:**234–240.
41. **Tuchscherr L, Heitmann V, Hussain M, Viemann D, Roth J, von Eiff C, Peters G, Becker K, Löffler B.** 2010. *Staphylococcus aureus* small-colony variants are adapted phenotypes for intracellular persistence. J Infect Dis **202:**1031–1040. http://dx.doi.org/10.1086/656047.
42. **Maduka-Ezeh AN, Greenwood-Quaintance KE, Karau MJ, Berbari EF, Osmon DR, Hanssen AD, Steckelberg JM, Patel R.** 2012. Antimicrobial susceptibility and biofilm formation of *Staphylococcus epidermidis* small colony variants associated with prosthetic joint infection. Diagn Microbiol Infect Dis **74:**224–229. http://dx.doi.org/10.1016/j.diagmicrobio.2012.06.029.
43. **Heilmann C, Schweitzer O, Gerke C, Vanittanakom N, Mack D, Götz F.** 1996. Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. Mol Microbiol **20:**1083–1091. http://dx.doi.org/10.1111/j.1365-2958.1996.tb02548.x.
44. **von Eiff C, Bettin D, Proctor RA, Rolauffs B, Lindner N, Winkelmann W, Peters G.** 1997. Recovery of small colony variants of *Staphylococcus aureus* following gentamicin bead placement for osteomyelitis. Clin Infect Dis **25:**1250–1251. http://dx.doi.org/10.1086/516962.
45. **Lew DP, Waldvogel FA.** 2004. Osteomyelitis. Lancet **364:**369–379. http://dx.doi.org/10.1016/S0140-6736(04)16727-5.
46. **Wright JA, Nair SP.** 2010. Interaction of staphylococci with bone. Int J Med Microbiol **300:**193–204. http://dx.doi.org/10.1016/j.ijmm.2009.10.003.
47. **Baltch AL, Ritz WJ, Bopp LH, Michelsen P, Smith RP.** 2008. Activities of daptomycin and comparative antimicrobials, singly and in combination, against extracellular and intracellular *Staphylococcus aureus* and its stable small-colony variant in human monocyte-derived macrophages and in broth. Antimicrob Agents Chemother **52:**1829–1833. http://dx.doi.org/10.1128/AAC.01480-07.
48. **Miller MH, Wexler MA, Steigbigel NH.** 1978. Single and combination antibiotic therapy of *Staphylococcus aureus* experimental endocarditis: emergence of gentamicin-resistant mutants. Antimicrob Agents Chemother **14:**336–343. http://dx.doi.org/10.1128/AAC.14.3.336.
49. **Clarke SR, Foster SJ.** 2006. Surface adhesins of *Staphylococcus aureus*. Adv Microb Physiol **51:**187–224. http://dx.doi.org/10.1016/S0065-2911(06)51004-5.
50. **Kalinka J, Hachmeister M, Geraci J, Sordelli D, Hansen U, Niemann S, Oetermann S, Peters G, Löffler B, Tuchscherr L.** 2014. *Staphylococcus aureus* isolates from chronic osteomyelitis are characterized by high host cell invasion and intracellular adaptation, but still induce inflammation. Int J Med Microbiol **304:**1038–1049. http://dx.doi.org/10.1016/j.ijmm.2014.07.013.
51. **Hudson MC, Ramp WK, Nicholson NC, Williams AS, Nousiainen MT.** 1995. Internalization of *Staphylococcus aureus* by cultured osteoblasts. Microb Pathog **19:**409–419. http://dx.doi.org/10.1006/mpat.1995.0075.
52. **Ellington JK, Reilly SS, Ramp WK, Smeltzer MS, Kellam JF, Hudson MC.** 1999. Mechanisms of *Staphylococcus aureus* invasion of cultured osteoblasts. Microb Pathog **26:**317–323. http://dx.doi.org/10.1006/mpat.1999.0272.
53. **Tuchscherr L, Bischoff M, Lattar SM, Noto Llana M, Pförtner H, Niemann S, Geraci J, Van de Vyver H, Fraunholz MJ, Cheung AL, Herrmann M, Völker U, Sordelli DO, Peters G, Löffler B.** 2015. Sigma factor SigB is crucial to mediate *Staphylococcus aureus* adaptation during chronic infections. PLoS Pathog **11:**e1004870. http://dx.doi.org/10.1371/journal.ppat.1004870.
54. **Cunha BA.** 2002. Osteomyelitis in elderly patients. Clin Infect Dis **35:**287–293. http://dx.doi.org/10.1086/341417.
55. **Archer NK, Mazaitis MJ, Costerton JW, Leid JG, Powers ME, Shirtliff ME.** 2011. *Staphylococcus aureus* biofilms: properties, regulation, and roles in human disease. Virulence **2:**445–459. http://dx.doi.org/10.4161/viru.2.5.17724.
56. **Mitchell G, Fugère A, Pépin Gaudreau K, Brouillette E, Frost EH, Cantin AM, Malouin F.** 2013. SigB is a dominant regulator of virulence in *Staphylococcus aureus* small-colony variants. PLoS One **8:**e65018. http://dx.doi.org/10.1371/journal.pone.0065018.
57. **Sparham PD, Lobban DI, Speller DC.** 1978. Isolation of *Staphylococcus aureus* from sputum in cystic fibrosis. J Clin Pathol **31:**913–918. http://dx.doi.org/10.1136/jcp.31.10.913.
58. **Gilligan PH, Gage PA, Welch DF, Muszynski MJ, Wait KR.** 1987. Prevalence of thymidine-dependent *Staphylococcus aureus* in patients with cystic fibrosis. J Clin Microbiol **25:**1258–1261.
59. **Vergison A, Denis O, Deplano A, Casimir G, Claeys G, DeBaets F, DeBoeck K, Douat N, Franckx H, Gigi J, Ieven M, Knoop C, Lebeque P, Lebrun F, Malfroot A, Paucquay F, Pierard D, Van Eldere J, Struelens MJ.** 2007. National survey of molecular epidemiology of *Staphylococcus aureus* colonization in Belgian cystic fibrosis patients. J Antimicrob Chemother **59:**893–899. http://dx.doi.org/10.1093/jac/dkm037.
60. **Besier S, Smaczny C, Von Mallinckrodt C, Krahl A, Ackermann H, Brade V, Wichelhaus TA.** 2007. Prevalence and clinical significance of *Staphylococcus aureus* small-colony variants in cystic fibrosis lung disease. J Clin Microbiol **45:**168–172. http://dx.doi.org/10.1128/JCM.01510-06.
61. **Schneider M, Mühlemann K, Droz S, Couzinet S, Casaulta C, Zimmerli S.** 2008. Clinical characteristics associated with isolation of small-colony variants of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from respiratory secretions of patients with cystic fibrosis. J Clin Microbiol **46:**1832–1834. http://dx.doi.org/10.1128/JCM.00361-08.
62. **Green N, Burns JL, Mayer-Hamblett N, Kloster M, Lands LC, Anstead**

M, Ratjen F, Saiman L. 2011. Lack of association of small-colony-variant *Staphylococcus aureus* strains with long-term use of azithromycin in patients with cystic fibrosis. J Clin Microbiol **49:**2772–2773. http://dx.doi.org/10.1128/JCM.00835-11.
63. **Yagci S, Hascelik G, Dogru D, Ozcelik U, Sener B.** 2013. Prevalence and genetic diversity of *Staphylococcus aureus* small-colony variants in cystic fibrosis patients. Clin Microbiol Infect **19:**77–84. http://dx.doi.org/10.1111/j.1469-0691.2011.03742.x.
64. **Yonker LM, Cigana C, Hurley BP, Bragonzi A.** 2015. Host-pathogen interplay in the respiratory environment of cystic fibrosis. J Cyst Fibros **14:**431–439. http://dx.doi.org/10.1016/j.jcf.2015.02.008.
65. **O'Sullivan BP, Freedman SD.** 2009. Cystic fibrosis. Lancet **373:**1891–1904. http://dx.doi.org/10.1016/S0140-6736(09)60327-5.
66. **Döring G, Gulbins E.** 2009. Cystic fibrosis and innate immunity: how chloride channel mutations provoke lung disease. Cell Microbiol **11:**208–216. http://dx.doi.org/10.1111/j.1462-5822.2008.01271.x.
67. **Boucher RC.** 2007. Cystic fibrosis: a disease of vulnerability to airway surface dehydration. Trends Mol Med **13:**231–240. http://dx.doi.org/10.1016/j.molmed.2007.05.001.
68. **Ratjen F, Doring G.** 2003. Cystic fibrosis. Lancet **361:**681–689. http://dx.doi.org/10.1016/S0140-6736(03)12567-6.
69. **Dasenbrook EC, Merlo CA, Diener-West M, Lechtzin N, Boyle MP.** 2008. Persistent methicillin-resistant *Staphylococcus aureus* and rate of FEV1 decline in cystic fibrosis. Am J Respir Crit Care Med **178:**814–821. http://dx.doi.org/10.1164/rccm.200802-327OC.
70. **Dasenbrook EC, Checkley W, Merlo CA, Konstan MW, Lechtzin N, Boyle MP.** 2010. Association between respiratory tract methicillin-resistant *Staphylococcus aureus* and survival in cystic fibrosis. JAMA **303:**2386–2392. http://dx.doi.org/10.1001/jama.2010.791.
71. **Cystic Fibrosis Foundation.** 2013. Cystic Fibrosis Foundation annual report 2013. Cystic Fibrosis Foundation, Bethesda, MD.
72. **Zolin A, van Rens J, Fox A, Iansa P, Preftitsi A, Pypops U, Gulmans V, Jung A, Mehta A, Viviani L, Vebert Olesen H, McKone E.** 2014. ECFS Patient Registry, annual report 2010. European Cystic Fibrosis Society, Karup, Denmark.
73. **Kahl BC, Duebbers A, Lubritz G, Haeberle J, Koch HG, Ritzerfeld B, Reilly M, Harms E, Proctor RA, Herrmann M, Peters G.** 2003. Population dynamics of persistent *Staphylococcus aureus* isolated from the airways of cystic fibrosis patients during a 6-year prospective study. J Clin Microbiol **41:**4424–4427. http://dx.doi.org/10.1128/JCM.41.9.4424-4427.2003.
74. **Branger C, Gardye C, Lambert-Zechovsky N.** 1996. Persistence of *Staphylococcus aureus* strains among cystic fibrosis patients over extended periods of time. J Med Microbiol **45:**294–301. http://dx.doi.org/10.1099/00222615-45-4-294.
75. **Hirschhausen N, Block D, Bianconi I, Bragonzi A, Birtel J, Lee JC, Dübbers A, Küster P, Kahl J, Peters G, Kahl BC.** 2013. Extended *Staphylococcus aureus* persistence in cystic fibrosis is associated with bacterial adaptation. Int J Med Microbiol **303:**685–692. http://dx.doi.org/10.1016/j.ijmm.2013.09.012.
76. **Cramer N, Wiehlmann L, Ciofu O, Tamm S, Høiby N, Tümmler B.** 2012. Molecular epidemiology of chronic *Pseudomonas aeruginosa* airway infections in cystic fibrosis. PLoS One **7:**e50731. http://dx.doi.org/10.1371/journal.pone.0050731.
77. **Cohen TS, Prince A.** 2012. Cystic fibrosis: a mucosal immunodeficiency syndrome. Nat Med **18:**509–519. http://dx.doi.org/10.1038/nm.2715.
78. **Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, Birrer P, Bellon G, Berger J, Weiss T, Botzenhart K, Yankaskas JR, Randell S, Boucher RC, Döring G.** 2002. Effects of reduced mucus oxygen concentration in airway *Pseudomonas* infections of cystic fibrosis patients. J Clin Invest **109:**317–325. http://dx.doi.org/10.1172/JCI0213870.
79. **Hogardt M, Heesemann J.** 2013. Microevolution of *Pseudomonas aeruginosa* to a chronic pathogen of the cystic fibrosis lung. Curr Top Microbiol Immunol **358:**91–118. http://dx.doi.org/10.1007/82_2011_199.
80. **Prunier AL, Malbruny B, Laurans M, Brouard J, Duhamel JF, Leclercq R.** 2003. High rate of macrolide resistance in *Staphylococcus aureus* strains from patients with cystic fibrosis reveals high proportions of hypermutable strains. J Infect Dis **187:**1709–1716. http://dx.doi.org/10.1086/374937.
81. **Sparham PD, Lobban DI, Speller DC.** 1978. Thymidine-requiring *Staphylococcus aureus*. Lancet **i:**104–105.
82. **von Eiff C, Peters G, Becker K.** 2006. The small colony variant (SCV) concept—the role of staphylococcal SCVs in persistent infections. Injury **37**(Suppl 2)**:**S26–S33. http://dx.doi.org/10.1016/j.injury.2006.04.006.
83. **Kahl BC, Belling G, Reichelt R, Herrmann M, Proctor RA, Peters G.** 2003. Thymidine-dependent small-colony variants of *Staphylococcus aureus* exhibit gross morphological and ultrastructural changes consistent with impaired cell separation. J Clin Microbiol **41:**410–413. http://dx.doi.org/10.1128/JCM.41.1.410-413.2003.
84. **Hammer ND, Cassat JE, Noto MJ, Lojek LJ, Chadha AD, Schmitz JE, Creech CB, Skaar EP.** 2014. Inter- and intraspecies metabolite exchange promotes virulence of antibiotic-resistant *Staphylococcus aureus*. Cell Host Microbe **16:**531–537. http://dx.doi.org/10.1016/j.chom.2014.09.002.
85. **Seggewiss J, Becker K, Kotte O, Eisenacher M, Khoschkhoi Yazdi MR, Fischer A, McNamara P, Al Laham N, Proctor R, Peters G, Heinemann M, von Eiff C.** 2006. Reporter metabolite analysis of transcriptional profiles of a *Staphylococcus aureus* strain with normal phenotype and its isogenic *hemB* mutant displaying the small-colony-variant phenotype. J Bacteriol **188:**7765–7777. http://dx.doi.org/10.1128/JB.00774-06.
86. **Kahl BC, Belling G, Becker P, Chatterjee I, Wardecki K, Hilgert K, Cheung AL, Peters G, Herrmann M.** 2005. Thymidine-dependent *Staphylococcus aureus* small-colony variants are associated with extensive alterations in regulator and virulence gene expression profiles. Infect Immun **73:**4119–4126. http://dx.doi.org/10.1128/IAI.73.7.4119-4126.2005.
87. **Moisan H, Brouillette E, Jacob CL, Langlois-Bégin P, Michaud S, Malouin F.** 2006. Transcription of virulence factors in *Staphylococcus aureus* small-colony variants isolated from cystic fibrosis patients is influenced by SigB. J Bacteriol **188:**64–76. http://dx.doi.org/10.1128/JB.188.1.64-76.2006.
88. **Kriegeskorte A, Lore NI, Bragonzi A, Riva C, Kelkenberg M, Becker K, Proctor RA, Peters G, Kahl BC.** 2015. Thymidine-dependent Staphylococcus aureus small-colony variants are induced by trimethoprim-sulfamethoxazole (SXT) and have increased fitness during SXT challenge. Antimicrob Agents Chemother **59:**7265–7272. http://dx.doi.org/10.1128/AAC.00742-15.
89. **von Eiff C, Becker K, Metze D, Lubritz G, Hockmann J, Schwarz T, Peters G.** 2001. Intracellular persistence of *Staphylococcus aureus* small-colony variants within keratinocytes: a cause for antibiotic treatment failure in a patient with Darier's disease. Clin Infect Dis **32:**1643–1647. http://dx.doi.org/10.1086/320519.
90. **Gläser R, Becker K, von Eiff C, Meyer-Hoffert U, Harder J.** 2014. Decreased susceptibility of *Staphylococcus aureus* small-colony variants toward human antimicrobial peptides. J Invest Dermatol **134:**2347–2350. http://dx.doi.org/10.1038/jid.2014.176.
91. **Kaspar U, Kriegeskorte A, Schubert T, Peters G, Rudack C, Pieper DH, Wos-Oxley M, Becker K.** 28 April 2015. The culturome of the human nose habitats reveals individual bacterial fingerprint patterns. Environ Microbiol http://dx.doi.org/10.1111/1462-2920.12891.
92. **Seifert H, von Eiff C, Fätkenheuer G.** 1999. Fatal case due to methicillin-resistant *Staphylococcus aureus* small colony variants in an AIDS patient. Emerg Infect Dis **5:**450–453. http://dx.doi.org/10.3201/eid0503.990319.
93. **von Eiff C, Lubritz G, Heese C, Peters G, Becker K.** 2004. Effect of trimethoprim-sulfamethoxazole prophylaxis in AIDS patients on the formation of the small colony variant phenotype of *Staphylococcus aureus*. Diagn Microbiol Infect Dis **48:**191–194. http://dx.doi.org/10.1016/j.diagmicrobio.2003.10.010.
94. **Abele-Horn M, Schupfner B, Emmerling P, Waldner H, Göring H.** 2000. Persistent wound infection after herniotomy associated with small-colony variants of *Staphylococcus aureus*. Infection **28:**53–54. http://dx.doi.org/10.1007/s150100050014.
95. **Burge SM, Wilkinson JD.** 1992. Darier-White disease: a review of the clinical features in 163 patients. J Am Acad Dermatol **27:**40–50. http://dx.doi.org/10.1016/0190-9622(92)70154-8.
96. **Sehgal VN, Srivastava G.** 2005. Darier's (Darier-White) disease/keratosis follicularis. Int J Dermatol **44:**184–192. http://dx.doi.org/10.1111/j.1365-4632.2004.02408.x.
97. **Sachse F, Becker K, von Eiff C, Metze D, Rudack C.** 2010. *Staphylococcus aureus* invades the epithelium in nasal polyposis and induces IL-6 in nasal epithelial cells in vitro. Allergy **65:**1430–1437. http://dx.doi.org/10.1111/j.1398-9995.2010.02381.x.
98. **Corriveau MN, Zhang N, Holtappels G, Van Roy N, Bachert C.** 2009. Detection of *Staphylococcus aureus* in nasal tissue with peptide nucleic

acid-fluorescence in situ hybridization. Am J Rhinol Allergy **23:**461–465. http://dx.doi.org/10.2500/ajra.2009.23.3367.

99. **Niederfuhr A, Kirsche H, Deutschle T, Poppert S, Riechelmann H, Wellinghausen N.** 2008. *Staphylococcus aureus* in nasal lavage and biopsy of patients with chronic rhinosinusitis. Allergy **63:**1359–1367. http://dx.doi.org/10.1111/j.1398-9995.2008.01798.x.
100. **Gitomer SA, Ramakrishnan VR, Malcolm KC, Kofonow JM, Ir D, Frank DN.** 2015. Initial investigation of small colony variants of *Staphylococcus aureus* in chronic rhinosinusitis. Am J Rhinol Allergy **29:**29–34. http://dx.doi.org/10.2500/ajra.2015.29.4133.
101. **Tan NC, Cooksley CM, Roscioli E, Drilling AJ, Douglas R, Wormald PJ, Vreugde S.** 2014. Small-colony variants and phenotype switching of intracellular *Staphylococcus aureus* in chronic rhinosinusitis. Allergy **69:**1364–1371. http://dx.doi.org/10.1111/all.12457.
102. **Kim R, Freeman J, Waldvogel-Thurlow S, Roberts S, Douglas R.** 2013. The characteristics of intramucosal bacteria in chronic rhinosinusitis: a prospective cross-sectional analysis. Int Forum Allergy Rhinol **3:**349–354. http://dx.doi.org/10.1002/alr.21117.
103. **Rolauffs B, Bernhardt TM, von Eiff C, Hart ML, Bettin D.** 2002. Osteopetrosis, femoral fracture, and chronic osteomyelitis caused by *Staphylococcus aureus* small colony variants (SCV) treated by girdlestone resection—6-year follow-up. Arch Orthop Trauma Surg **122:**547–550.
104. **von Eiff C, Becker K.** 2007. Small-colony variants (SCVs) of staphylococci: a role in foreign body-associated infections. Int J Artif Organs **30:**778–785.
105. **Piffaut C, Lustig S, Laurent F, Chidiac C, Ferry T.** March 2013. Small colony variant-producing *S. aureus* prosthesis, joint infection highlighted by sonication and treated with prolonged high doses of daptomycin. BMJ Case Rep http://dx.doi.org/10.1136/bcr-2013-008637.
106. **Kipp F, Kahl BC, Becker K, Baron EJ, Proctor RA, Peters G, von Eiff C.** 2005. Evaluation of two chromogenic agar media for recovery and identification of *Staphylococcus aureus* small-colony variants. J Clin Microbiol **43:**1956–1959. http://dx.doi.org/10.1128/JCM.43.4.1956-1959.2005.
107. **von Eiff C, McNamara P, Becker K, Bates D, Lei XH, Ziman M, Bochner BR, Peters G, Proctor RA.** 2006. Phenotype microarray profiling of *Staphylococcus aureus menD* and *hemB* mutants with the small-colony-variant phenotype. J Bacteriol **188:**687–693. http://dx.doi.org/10.1128/JB.188.2.687-693.2006.
108. **Kriegeskorte A, Grubmüller S, Huber C, Kahl BC, von Eiff C, Proctor RA, Peters G, Eisenreich W, Becker K.** 2014. *Staphylococcus aureus* small colony variants show common metabolic features in central metabolism irrespective of the underlying auxotrophism. Front Cell Infect Microbiol **4:**141. http://dx.doi.org/10.3389/fcimb.2014.00141.
109. **Kloos WE, Schleifer KH.** 1975. Simplified scheme for routine identification of human *Staphylococcus* species. J Clin Microbiol **1:**82–88.
110. **Gröbner S, Beck J, Schaller M, Autenrieth IB, Schulte B.** 2012. Characterization of an *Enterococcus faecium* small-colony variant isolated from blood culture. Int J Med Microbiol **302:**40–44. http://dx.doi.org/10.1016/j.ijmm.2011.07.001.
111. **Vannuffel P, Gigi J, Ezzedine H, Vandercam B, Delmee M, Wauters G, Gala JL.** 1995. Specific detection of methicillin-resistant *Staphylococcus species* by multiplex PCR. J Clin Microbiol **33:**2864–2867.
112. **Zambardi G, Reverdy ME, Bland S, Bes M, Freney J, Fleurette J.** 1994. Laboratory diagnosis of oxacillin resistance in *Staphylococcus aureus* by a multiplex-polymerase chain reaction assay. Diagn Microbiol Infect Dis **19:**25–31. http://dx.doi.org/10.1016/0732-8893(94)90047-7.
113. **Valderas MW, Gatson JW, Wreyford N, Hart ME.** 2002. The superoxide dismutase gene *sodM* is unique to *Staphylococcus aureus*: absence of *sodM* in coagulase-negative staphylococci. J Bacteriol **184:**2465–2472. http://dx.doi.org/10.1128/JB.184.9.2465-2472.2002.
114. **Mason WJ, Blevins JS, Beenken K, Wibowo N, Ojha N, Smeltzer MS.** 2001. Multiplex PCR protocol for the diagnosis of staphylococcal infection. J Clin Microbiol **39:**3332–3338. http://dx.doi.org/10.1128/JCM.39.9.3332-3338.2001.
115. **Brakstad OG, Aasbakk K, Maeland JA.** 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. J Clin Microbiol **30:**1654–1660.
116. **Hussain M, von Eiff C, Sinha B, Joost I, Herrmann M, Peters G, Becker K.** 2008. *eap* gene as novel target for specific identification of *Staphylococcus aureus*. J Clin Microbiol **46:**470–476. http://dx.doi.org/10.1128/JCM.01425-07.
117. **Becker K, Skov RL, von Eiff C.** 2015. *Staphylococcus*, *Micrococcus*, and other catalase-positive cocci, p 354–382. *In* Jorgensen JH, Pfaller MA (ed), Manual of clinical microbiology, 11th ed. ASM Press, Washington, DC.
118. **von Eiff C, Kokai-Kun JF, Becker K, Peters G.** 2003. In vitro activity of recombinant lysostaphin against *Staphylococcus aureus* isolates from anterior nares and blood. Antimicrob Agents Chemother **47:**3613–3615. http://dx.doi.org/10.1128/AAC.47.11.3613-3615.2003.
119. **Mellmann A, Becker K, von Eiff C, Keckevoet U, Schumann P, Harmsen D.** 2006. Sequencing and staphylococci identification. Emerg Infect Dis **12:**333–336. http://dx.doi.org/10.3201/eid1202.050962.
120. **Becker K, Harmsen D, Mellmann A, Meier C, Schumann P, Peters G, von Eiff C.** 2004. Development and evaluation of a quality-controlled ribosomal sequence database for 16S ribosomal DNA-based identification of *Staphylococcus* species. J Clin Microbiol **42:**4988–4995. http://dx.doi.org/10.1128/JCM.42.11.4988-4995.2004.
121. **Youmans GP, Delves E.** 1942. The effect of inorganic salts on the production of small colony variants by *Staphylococcus aureus*. J Bacteriol **44:**127–136.
122. **van de Rijn I, Kessler RE.** 1980. Growth characteristics of group A streptococci in a new chemically defined medium. Infect Immun **27:**444–448.
123. **Gómez-González C, Acosta J, Villa J, Barrado L, Sanz F, Orellana MÁ, Otero JR, Chaves F.** 2010. Clinical and molecular characteristics of infections with CO_2-dependent small-colony variants of Staphylococcus aureus. J Clin Microbiol **48:**2878–2884. http://dx.doi.org/10.1128/JCM.00520-10.
124. **Sander G, Börner T, Kriegeskorte A, von Eiff C, Becker K, Mahabir E.** 2012. Catheter colonization and abscess formation due to *Staphylococcus epidermidis* with normal and small-colony-variant phenotype is mouse strain dependent. PLoS One **7:**e36602. http://dx.doi.org/10.1371/journal.pone.0036602.
125. **Kriegeskorte A, König S, Sander G, Pirkl A, Mahabir E, Proctor RA, von Eiff C, Peters G, Becker K.** 2011. Small colony variants of *Staphylococcus aureus* reveal distinct protein profiles. Proteomics **11:**2476–2490. http://dx.doi.org/10.1002/pmic.201000796.
126. **von Eiff C, Heilmann C, Proctor RA, Woltz C, Peters G, Götz F.** 1997. A site-directed *Staphylococcus aureus hemB* mutant is a small-colony variant which persists intracellularly. J Bacteriol **179:**4706–4712.
127. **Becker K, Laham NA, Fegeler W, Proctor RA, Peters G, von Eiff C.** 2006. Fourier-transform infrared spectroscopic analysis is a powerful tool for studying the dynamic changes in *Staphylococcus aureus* small-colony variants. J Clin Microbiol **44:**3274–3278. http://dx.doi.org/10.1128/JCM.00847-06.
128. **Chuard C, Vaudaux PE, Proctor RA, Lew DP.** 1997. Decreased susceptibility to antibiotic killing of a stable small colony variant of *Staphylococcus aureus* in fluid phase and on fibronectin-coated surfaces. J Antimicrob Chemother **39:**603–608. http://dx.doi.org/10.1093/jac/39.5.603.
129. **Kipp F, Becker K, Peters G, von Eiff C.** 2004. Evaluation of different methods to detect methicillin resistance in small-colony variants of *Staphylococcus aureus*. J Clin Microbiol **42:**1277–1279. http://dx.doi.org/10.1128/JCM.42.3.1277-1279.2004.
130. **Garcia LG, Lemaire S, Kahl BC, Becker K, Proctor RA, Denis O, Tulkens PM, Van Bambeke F.** 2013. Antibiotic activity against small-colony variants of *Staphylococcus aureus*: review of in vitro, animal and clinical data. J Antimicrob Chemother **68:**1455–1464. http://dx.doi.org/10.1093/jac/dkt072.
131. **Tuchscherr L, Medina E, Hussain M, Völker W, Heitmann V, Niemann S, Holzinger D, Roth J, Proctor RA, Becker K, Peters G, Löffler B.** 2011. *Staphylococcus aureus* phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. EMBO Mol Med **3:**129–141. http://dx.doi.org/10.1002/emmm.201000115.
132. **Vesga O, Groeschel MC, Otten MF, Brar DW, Vann JM, Proctor RA.** 1996. *Staphylococcus aureus* small colony variants are induced by the endothelial cell intracellular milieu. J Infect Dis **173:**739–742. http://dx.doi.org/10.1093/infdis/173.3.739.
133. **Nguyen HA, Denis O, Vergison A, Theunis A, Tulkens PM, Struelens MJ, Van Bambeke F.** 2009. Intracellular activity of antibiotics in a model of human THP-1 macrophages infected by a *Staphylococcus aureus* small-colony variant strain isolated from a cystic fibrosis patient: pharmacodynamic evaluation and comparison with isogenic normal-phenotype and revertant strains. Antimicrob Agents Chemother **53:**1434–1442. http://dx.doi.org/10.1128/AAC.01145-08.
134. **Barcia-Macay M, Seral C, Mingeot-Leclercq MP, Tulkens PM, Van**

Bambeke F. 2006. Pharmacodynamic evaluation of the intracellular activities of antibiotics against *Staphylococcus aureus* in a model of THP-1 macrophages. Antimicrob Agents Chemother **50:**841–851. http://dx.doi.org/10.1128/AAC.50.3.841-851.2006.

135. **Nguyen HA, Denis O, Vergison A, Tulkens PM, Struelens MJ, Van Bambeke F.** 2009. Intracellular activity of antibiotics in a model of human THP-1 macrophages infected by a *Staphylococcus aureus* small-colony variant strain isolated from a cystic fibrosis patient: study of antibiotic combinations. Antimicrob Agents Chemother **53:**1443–1449. http://dx.doi.org/10.1128/AAC.01146-08.
136. **Garcia LG, Lemaire S, Kahl BC, Becker K, Proctor RA, Denis O, Tulkens PM, Van Bambeke F.** 2012. Pharmacodynamic evaluation of the activity of antibiotics against hemin- and menadione-dependent small-colony variants of *Staphylococcus aureus* in models of extracellular (broth) and intracellular (THP-1 monocytes) infections. Antimicrob Agents Chemother **56:**3700–3711. http://dx.doi.org/10.1128/AAC.00285-12.
137. **Bates DM, von Eiff C, McNamara PJ, Peters G, Yeaman MR, Bayer AS, Proctor RA.** 2003. *Staphylococcus aureus menD* and *hemB* mutants are as infective as the parent strains, but the menadione biosynthetic mutant persists within the kidney. J Infect Dis **187:**1654–1661. http://dx.doi.org/10.1086/374642.
138. **Dean MA, Olsen RJ, Long SW, Rosato AE, Musser JM.** 2014. Identification of point mutations in clinical *Staphylococcus aureus* strains that produce small-colony variants auxotrophic for menadione. Infect Immun **82:**1600–1605. http://dx.doi.org/10.1128/IAI.01487-13.
139. **Jonsson IM, von Eiff C, Proctor RA, Peters G, Rydén C, Tarkowski A.** 2003. Virulence of a *hemB* mutant displaying the phenotype of a *Staphylococcus aureus* small colony variant in a murine model of septic arthritis. Microb Pathog **34:**73–79. http://dx.doi.org/10.1016/S0882-4010(02)00208-5.
140. **Kriegeskorte A, Block D, Drescher M, Windmüller N, Mellmann A, Baum C, Neumann C, Lorè NI, Bragonzi A, Liebau E, Hertel P, Seggewiss J, Becker K, Proctor RA, Peters G, Kahl BC.** 2014. Inactivation of *thyA* in *Staphylococcus aureus* attenuates virulence and has a strong impact on metabolism and virulence gene expression. mBio **5:**e01447-14. http://dx.doi.org/10.1128/mBio.01447-14.
141. **Brouillette E, Martinez A, Boyll BJ, Allen NE, Malouin F.** 2004. Persistence of a *Staphylococcus aureus* small-colony variant under antibiotic pressure in vivo. FEMS Immunol Med Microbiol **41:**35–41. http://dx.doi.org/10.1016/j.femsim.2003.12.007.
142. **Horst SA, Hoerr V, Beineke A, Kreis C, Tuchscherr L, Kalinka J, Lehne S, Schleicher I, Köhler G, Fuchs T, Raschke MJ, Rohde M, Peters G, Faber C, Löffler B, Medina E.** 2012. A novel mouse model of *Staphylococcus aureus* chronic osteomyelitis that closely mimics the human infection: an integrated view of disease pathogenesis. Am J Pathol **181:**1206–1214. http://dx.doi.org/10.1016/j.ajpath.2012.07.005.
143. **Joosten U, Joist A, Gosheger G, Liljenqvist U, Brandt B, von Eiff C.** 2005. Effectiveness of hydroxyapatite-vancomycin bone cement in the treatment of *Staphylococcus aureus* induced chronic osteomyelitis. Biomaterials **26:**5251–5258. http://dx.doi.org/10.1016/j.biomaterials.2005.01.001.
144. **Lee JH, Wang H, Kaplan JB, Lee WY.** 2011. Microfluidic approach to create three-dimensional tissue models for biofilm-related infection of orthopaedic implants. Tissue Eng Part C Methods **17:**39–48. http://dx.doi.org/10.1089/ten.tec.2010.0285.
145. **Lannergård J, von Eiff C, Sander G, Cordes T, Seggewiss J, Peters G, Proctor RA, Becker K, Hughes D.** 2008. Identification of the genetic basis for clinical menadione-auxotrophic small-colony variant isolates of *Staphylococcus aureus*. Antimicrob Agents Chemother **52:**4017–4022. http://dx.doi.org/10.1128/AAC.00668-08.
146. **Schaaff F, Bierbaum G, Baumert N, Bartmann P, Sahl HG.** 2003. Mutations are involved in emergence of aminoglycoside-induced small colony variants of *Staphylococcus aureus*. Int J Med Microbiol **293:**427–435. http://dx.doi.org/10.1078/1438-4221-00282.
147. **Chatterjee I, Kriegeskorte A, Fischer A, Deiwick S, Theimann N, Proctor RA, Peters G, Herrmann M, Kahl BC.** 2008. In vivo mutations of thymidylate synthase (encoded by *thyA*) are responsible for thymidine dependency in clinical small-colony variants of *Staphylococcus aureus*. J Bacteriol **190:**834–842. http://dx.doi.org/10.1128/JB.00912-07.
148. **Proctor RA, Kriegeskorte A, Kahl BC, Becker K, Löffler B, Peters G.** 2014. *Staphylococcus aureus* small colony variants (SCVs): a road map for the metabolic pathways involved in persistent infections. Front Cell Infect Microbiol **4:**99. http://dx.doi.org/10.3389/fcimb.2014.00099.
149. **Gao W, Chua K, Davies JK, Newton HJ, Seemann T, Harrison PF, Holmes NE, Rhee HW, Hong JI, Hartland EL, Stinear TP, Howden BP.** 2010. Two novel point mutations in clinical *Staphylococcus aureus* reduce linezolid susceptibility and switch on the stringent response to promote persistent infection. PLoS Pathog **6:**e1000944. http://dx.doi.org/10.1371/journal.ppat.1000944.
150. **Bui LM, Kidd SP.** 2015. A full genomic characterization of the development of a stable small colony variant cell-type by a clinical *Staphylococcus aureus* strain. Infect Genet Evol **36:**345–355. http://dx.doi.org/10.1016/j.meegid.2015.10.011.
151. **Bui LM, Turnidge JD, Kidd SP.** 2015. The induction of *Staphylococcus aureus* biofilm formation or small colony variants is a strain-specific response to host-generated chemical stresses. Microbes Infect **17:**77–82. http://dx.doi.org/10.1016/j.micinf.2014.09.009.
152. **Musher DM, Baughn RE, Templeton GB, Minuth JN.** 1977. Emergence of variant forms of *Staphylococcus aureus* after exposure to gentamicin and infectivity of the variants in experimental animals. J Infect Dis **136:**360–369. http://dx.doi.org/10.1093/infdis/136.3.360.
153. **Singh R, Ray P, Das A, Sharma M.** 2009. Role of persisters and small-colony variants in antibiotic resistance of planktonic and biofilm-associated Staphylococcus aureus: an in vitro study. J Med Microbiol **58:**1067–1073. http://dx.doi.org/10.1099/jmm.0.009720-0.
154. **Jensen J, Thofern E.** 1953. Chlorhämin (Ferriporphyrinchlorid) als Bakterienwuchsstoff. I. Zur Synthese der Hämatinfermente. Z Naturforsch B **8b:**599–603.
155. **Miller MH, Edberg SC, Mandel LJ, Behar CF, Steigbigel NH.** 1980. Gentamicin uptake in wild-type and aminoglycoside-resistant small-colony mutants of *Staphylococcus aureus*. Antimicrob Agents Chemother **18:**722–729. http://dx.doi.org/10.1128/AAC.18.5.722.
156. **Kafala B, Sasarman A.** 1994. Cloning and sequence analysis of the *hemB* gene of *Staphylococcus aureus*. Can J Microbiol **40:**651–657. http://dx.doi.org/10.1139/m94-103.
157. **Collins MD, Jones D.** 1981. Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implication. Microbiol Rev **45:**316–354.
158. **McNamara PJ, Proctor RA.** 2000. *Staphylococcus aureus* small colony variants, electron transport and persistent infections. Int J Antimicrob Agents **14:**117–122. http://dx.doi.org/10.1016/S0924-8579(99)00170-3.
159. **Köser CU, Holden MT, Ellington MJ, Cartwright EJ, Brown NM, Ogilvy-Stuart AL, Hsu LY, Chewapreecha C, Croucher NJ, Harris SR, Sanders M, Enright MC, Dougan G, Bentley SD, Parkhill J, Fraser LJ, Betley JR, Schulz-Trieglaff OB, Smith GP, Peacock SJ.** 2012. Rapid whole-genome sequencing for investigation of a neonatal MRSA outbreak. N Engl J Med **366:**2267–2275. http://dx.doi.org/10.1056/NEJMoa1109910.
160. **Kohler C, von Eiff C, Liebeke M, McNamara PJ, Lalk M, Proctor RA, Hecker M, Engelmann S.** 2008. A defect in menadione biosynthesis induces global changes in gene expression in *Staphylococcus aureus*. J Bacteriol **190:**6351–6364. http://dx.doi.org/10.1128/JB.00505-08.
161. **Maghnouj A, de Sousa Cabral TF, Stalon V, Vander WC.** 1998. The *arcABDC* gene cluster, encoding the arginine deiminase pathway of *Bacillus licheniformis*, and its activation by the arginine repressor ArgR. J Bacteriol **180:**6468–6475.
162. **Kohler C, von Eiff C, Peters G, Proctor RA, Hecker M, Engelmann S.** 2003. Physiological characterization of a heme-deficient mutant of *Staphylococcus aureus* by a proteomic approach. J Bacteriol **185:**6928–6937. http://dx.doi.org/10.1128/JB.185.23.6928-6937.2003.
163. **Clements MO, Watson SP, Poole RK, Foster SJ.** 1999. CtaA of *Staphylococcus aureus* is required for starvation survival, recovery, and cytochrome biosynthesis. J Bacteriol **181:**501–507.
164. **Novick RP, Ross HF, Projan SJ, Kornblum J, Kreiswirth B, Moghazeh S.** 1993. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J **12:**3967–3975.
165. **Novick RP, Geisinger E.** 2008. Quorum sensing in staphylococci. Annu Rev Genet **42:**541–564. http://dx.doi.org/10.1146/annurev.genet.42.110807.091640.
166. **Vaudaux P, Francois P, Bisognano C, Kelley WL, Lew DP, Schrenzel J, Proctor RA, McNamara PJ, Peters G, von Eiff C.** 2002. Increased expression of clumping factor and fibronectin-binding proteins by *hemB* mutants of *Staphylococcus aureus* expressing small colony variant phenotypes. Infect Immun **70:**5428–5437. http://dx.doi.org/10.1128/IAI.70.10.5428-5437.2002.
167. **Pader V, James EH, Painter KL, Wigneshweraraj S, Edwards AM.**

2014. The Agr quorum-sensing system regulates fibronectin binding but not hemolysis in the absence of a functional electron transport chain. Infect Immun **82:**4337–4347. http://dx.doi.org/10.1128/IAI.02254-14.

168. **Abu-Qatouseh LF, Chinni SV, Seggewiss J, Proctor RA, Brosius J, Rozhdestvensky TS, Peters G, von Eiff C, Becker K.** 2010. Identification of differentially expressed small non-protein-coding RNAs in *Staphylococcus aureus* displaying both the normal and the small-colony variant phenotype. J Mol Med **88:**565–575. http://dx.doi.org/10.1007/s00109-010-0597-2.
169. **Khayrullina GA, Raabe CA, Hoe CH, Becker K, Reinhardt R, Tang TH, Rozhdestvensky TS, Kopylov AM.** 2012. Transcription analysis and small non-protein coding RNAs associated with bacterial ribosomal protein operons. Curr Med Chem **19:**5187–5198. http://dx.doi.org/10.2174/092986712803530485.
170. **Stryer L.** 1995. Biosynthesis of nucleotides, p 736–762. *In* Stryer L (ed), Biochemistry, 4th ed. W H Freeman, New York, NY.
171. **Wise RI.** 1956. Small colonies (G variants) of staphylococci: isolation from cultures and infections. Ann N Y Acad Sci **65:**169–174. http://dx.doi.org/10.1111/j.1749-6632.1956.tb36636.x.
172. **Slifkin M, Merkow LP, Kreuzberger SA, Engwall C, Pardo M.** 1971. Characterization of CO_2 dependent microcolony variants of *Staphylococcus aureus*. Am J Clin Pathol **56:**584–592.
173. **Brooks JL, Jefferson KK.** 2014. Phase variation of poly-N-acetylglucosamine expression in *Staphylococcus aureus*. PLoS Pathog **10:**e1004292. http://dx.doi.org/10.1371/journal.ppat.1004292.
174. **Iqbal S, Parker G, Davidson H, Moslehi-Rahmani E, Robson RL.** 2004. Reversible phase variation in the *phnE* gene, which is required for phosphonate metabolism in *Escherichia coli* K-12. J Bacteriol **186:**6118–6123. http://dx.doi.org/10.1128/JB.186.18.6118-6123.2004.
175. **Otto M.** 2014. Staphylococcus aureus toxins. Curr Opin Microbiol **17:**32–37. http://dx.doi.org/10.1016/j.mib.2013.11.004.
176. **Tuchscherr L, Löffler B.** June 2015. *Staphylococcus aureus* dynamically adapts global regulators and virulence factor expression in the course from acute to chronic infection. Curr Genet http://dx.doi.org/10.1007/s00294-015-0503-0.
177. **Grundmeier M, Tuchscherr L, Brück M, Viemann D, Roth J, Willscher E, Becker K, Peters G, Löffler B.** 2010. Staphylococcal strains vary greatly in their ability to induce an inflammatory response in endothelial cells. J Infect Dis **201:**871–880. http://dx.doi.org/10.1086/651023.
178. **Horsburgh MJ, Aish JL, White IJ, Shaw L, Lithgow JK, Foster SJ.** 2002. σB modulates virulence determinant expression and stress resistance: characterization of a functional *rsbU* strain derived from *Staphylococcus aureus* 8325-4. J Bacteriol **184:**5457–5467. http://dx.doi.org/10.1128/JB.184.19.5457-5467.2002.
179. **Mitchell G, Lamontagne CA, Brouillette E, Grondin G, Talbot BG, Grandbois M, Malouin F.** 2008. *Staphylococcus aureus* SigB activity promotes a strong fibronectin-bacterium interaction which may sustain host tissue colonization by small-colony variants isolated from cystic fibrosis patients. Mol Microbiol **70:**1540–1555. http://dx.doi.org/10.1111/j.1365-2958.2008.06511.x.
180. **Mitchell G, Brouillette E, Séguin DL, Asselin AE, Jacob CL, Malouin F.** 2010. A role for sigma factor B in the emergence of *Staphylococcus aureus* small-colony variants and elevated biofilm production resulting from an exposure to aminoglycosides. Microb Pathog **48:**18–27. http://dx.doi.org/10.1016/j.micpath.2009.10.003.
181. **Painter KL, Strange E, Parkhill J, Bamford KB, Armstrong-James D, Edwards AM.** 2015. *Staphylococcus aureus* adapts to oxidative stress by producing H_2O_2-resistant small-colony variants via the SOS response. Infect Immun **83:**1830–1844. http://dx.doi.org/10.1128/IAI.03016-14.
182. **Hoffman LR, Deziel E, D'Argenio DA, Lepine F, Emerson J, McNamara S, Gibson RL, Ramsey BW, Miller SI.** 2006. Selection for *Staphylococcus aureus* small-colony variants due to growth in the presence of *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A **103:**19890–19895. http://dx.doi.org/10.1073/pnas.0606756104.
183. **Biswas L, Biswas R, Schlag M, Bertram R, Götz F.** 2009. Small-colony variant selection as a survival strategy for Staphylococcus aureus in the presence of Pseudomonas aeruginosa. Appl Environ Microbiol **75:**6910–6912. http://dx.doi.org/10.1128/AEM.01211-09.
184. **Giese B, Glowinski F, Paprotka K, Dittmann S, Steiner T, Sinha B, Fraunholz MJ.** 2011. Expression of δ-toxin by *Staphylococcus aureus* mediates escape from phago-endosomes of human epithelial and endothelial cells in the presence of β-toxin. Cell Microbiol **13:**316–329. http://dx.doi.org/10.1111/j.1462-5822.2010.01538.x.
185. **Grosz M, Kolter J, Paprotka K, Winkler AC, Schafer D, Chatterjee SS, Geiger T, Wolz C, Ohlsen K, Otto M, Rudel T, Sinha B, Fraunholz M.** 2014. Cytoplasmic replication of *Staphylococcus aureus* upon phagosomal escape triggered by phenol-soluble modulin α. Cell Microbiol **16:**451–465. http://dx.doi.org/10.1111/cmi.12233.
186. **von Eiff C, Friedrich AW, Becker K, Peters G.** 2005. Comparative in vitro activity of ceftobiprole against staphylococci displaying normal and small-colony variant phenotypes. Antimicrob Agents Chemother **49:**4372–4374. http://dx.doi.org/10.1128/AAC.49.10.4372-4374.2005.
187. **Cervantes-García E, García-Gonzalez R, Reyes-Torres A, Resendiz-Albor AA, Salazar-Schettino PM.** 2015. *Staphylococcus aureus* small colony variants in diabetic foot infections. Diabet Foot Ankle **6:**26431. http://dx.doi.org/10.3402/dfa.v6.26431.
188. **Spanu T, Romano L, D'Inzeo T, Masucci L, Albanese A, Papacci F, Marchese E, Sanguinetti M, Fadda G.** 2005. Recurrent ventriculoperitoneal shunt infection caused by small-colony variants of *Staphylococcus aureus*. Clin Infect Dis **41:**e48–e52. http://dx.doi.org/10.1086/432577.
189. **Baumert N, von Eiff C, Schaaff F, Peters G, Proctor RA, Sahl HG.** 2002. Physiology and antibiotic susceptibility of *Staphylococcus aureus* small colony variants. Microb Drug Resist **8:**253–260. http://dx.doi.org/10.1089/10766290260469507.
190. **Begic D, von Eiff C, Tsuji BT.** 2009. Daptomycin pharmacodynamics against *Staphylococcus aureus hemB* mutants displaying the small colony variant phenotype. J Antimicrob Chemother **63:**977–981. http://dx.doi.org/10.1093/jac/dkp069.
191. **Proctor RA, Kahl B, von Eiff C, Vaudaux PE, Lew DP, Peters G.** 1998. Staphylococcal small colony variants have novel mechanisms for antibiotic resistance. Clin Infect Dis **27**(Suppl 1)**:**S68–S74. http://dx.doi.org/10.1086/514906.
192. **Bulger RJ.** 1969. In vitro studies on highly resistant small colony variants of Staphylococcus aureus resistant to methicillin. J Infect Dis **120:**491–494. http://dx.doi.org/10.1093/infdis/120.4.491.
193. **Devriese LA.** 1973. Hemin-dependent mutants isolated from methicillin-resistant *Staphylococcus aureus* strains. Antonie Van Leeuwenhoek **39:**33–40. http://dx.doi.org/10.1007/BF02578839.
194. **Garcia LG, Lemaire S, Kahl BC, Becker K, Proctor RA, Tulkens PM, Van Bambeke F.** 2012. Intracellular forms of menadione-dependent small-colony variants of methicillin-resistant *Staphylococcus aureus* are hypersusceptible to beta-lactams in a THP-1 cell model due to cooperation between vacuolar acidic pH and oxidant species. J Antimicrob Chemother **67:**2873–2881. http://dx.doi.org/10.1093/jac/dks325.
195. **Tsuji BT, von Eiff C, Kelchlin PA, Forrest A, Smith PF.** 2008. Attenuated vancomycin bactericidal activity against *Staphylococcus aureus hemB* mutants expressing the small-colony-variant phenotype. Antimicrob Agents Chemother **52:**1533–1537. http://dx.doi.org/10.1128/AAC.01254-07.
196. **Mates SM, Eisenberg ES, Mandel LJ, Patel L, Kaback HR, Miller MH.** 1982. Membrane potential and gentamicin uptake in *Staphylococcus aureus*. Proc Natl Acad Sci U S A **79:**6693–6697. http://dx.doi.org/10.1073/pnas.79.21.6693.
197. **Idelevich EA, Kriegeskorte A, Stubbings W, Kahl BC, Peters G, Becker K.** 2011. Comparative in vitro activity of finafloxacin against staphylococci displaying normal and small colony variant phenotypes. J Antimicrob Chemother **66:**2809–2813. http://dx.doi.org/10.1093/jac/dkr393.
198. **Bayston R, Ashraf W, Smith T.** 2007. Triclosan resistance in methicillin-resistant *Staphylococcus aureus* expressed as small colony variants: a novel mode of evasion of susceptibility to antiseptics. J Antimicrob Chemother **59:**848–853. http://dx.doi.org/10.1093/jac/dkm031.
199. **Latimer J, Forbes S, McBain AJ.** 2012. Attenuated virulence and biofilm formation in *Staphylococcus aureus* following sublethal exposure to triclosan. Antimicrob Agents Chemother **56:**3092–3100. http://dx.doi.org/10.1128/AAC.05904-11.
200. **Forbes S, Latimer J, Bazaid A, McBain AJ.** 2015. Altered competitive fitness, antimicrobial susceptibility, and cellular morphology in a triclosan-induced small-colony variant of *Staphylococcus aureus*. Antimicrob Agents Chemother **59:**4809–4816. http://dx.doi.org/10.1128/AAC.00352-15.
201. **Nielsen XC, Nielsen FT, Kurtzhals JA, Moser C, Boye K, Christensen JJ, Johansen UR, Westh H.** 2009. Management of recurrent pacemaker-related bacteraemia with small colony variant Staphylococcus aureus in a haemodialysis patient. BMJ Case Rep **2009:**bcr05.2009.1910. http://dx.doi.org/10.1136/bcr.05.2009.1910.
202. **Cleeve VJ, Perry JD, Cresswell G, Orr KE.** 2006. Thymidine-dependent

meticillin-resistant *Staphylococcus aureus*: a potentially unrecognized reservoir of MRSA in hospital patients? J Hosp Infect **63:**228–229. http://dx.doi.org/10.1016/j.jhin.2005.11.009.
203. **Lenhard JR, von Eiff C, Hong IS, Holden PN, Bear MD, Suen A, Bulman ZP, Tsuji BT.** 2015. Evolution of *Staphylococcus aureus* under vancomycin selective pressure: the role of the small-colony variant phenotype. Antimicrob Agents Chemother **59:**1347–1351. http://dx.doi.org/10.1128/AAC.04508-14.
204. **Diefenbeck M, Mückley T, Hofmann GO.** 2006. Prophylaxis and treatment of implant-related infections by local application of antibiotics. Injury **37**(Suppl 2)**:**S95–S104. http://dx.doi.org/10.1016/j.injury.2006.04.015.
205. **Knaepler H.** 2012. Local application of gentamicin-containing collagen implant in the prophylaxis and treatment of surgical site infection in orthopaedic surgery. Int J Surg **10**(Suppl 1)**:**S15–S20. http://dx.doi.org/10.1016/j.ijsu.2012.05.020.
206. **Huiras P, Logan JK, Papadopoulos S, Whitney D.** 2012. Local antimicrobial administration for prophylaxis of surgical site infections. Pharmacotherapy **32:**1006–1019. http://dx.doi.org/10.1002/phar.1135.
207. **Lalidou F, Kolios G, Drosos GI.** 2014. Bone infections and bone graft substitutes for local antibiotic therapy. Surg Technol Int **24:**353–362.
208. **Bhattacharyya S, Roy S, Mukhopadhyay P, Rit K, Dey J, Ganguly U, Ray R.** 2012. Small colony variants of *Staphylococcus aureus* isolated from a patient with infective endocarditis: a case report and review of the literature. Iran J Microbiol **4:**98–99.
209. **Göring H, Waldner H, Emmerling P, Abele-Horn M.** 2001. Chronisch fistelnde Wundinfektion nach Lichtensteinreparation einer Leistenhernie, verursacht durch eine Small-colony-Variante von *Staphylococcus aureus*. Chirurg **72:**441–443.
210. **Atalla H, Gyles C, Jacob CL, Moisan H, Malouin F, Mallard B.** 2008. Characterization of a *Staphylococcus aureus* small colony variant (SCV) associated with persistent bovine mastitis. Foodborne Pathog Dis **5:**785–799. http://dx.doi.org/10.1089/fpd.2008.0110.
211. **Coman G, Mardare N, Copacianu B, Covic M.** 2008. Infectie cutanata persistenta si recurenta cu *Staphyloccocus aureus* meticilino-rezistent, varianta cu colonii mici. Rev Med Chir Soc Med Nat Iasi **112:**104–107.
212. **Kipp F, Ziebuhr W, Becker K, Krimmer V, Höß N, Peters G, von Eiff C.** 2003. Detection of *Staphylococcus aureus* by 16S rRNA directed in situ hybridisation in a patient with a brain abscess caused by small colony variants. J Neurol Neurosurg Psychiatry **74:**1000–1002.
213. **Agarwal H, Verrall R, Singh SP, Tang YW, Wilson G.** 2007. Small colony variant *Staphylococcus aureus* multiorgan infection. Pediatr Infect Dis J **26:**269–271.
214. **Besier S, Zander J, Siegel E, Saum SH, Hunfeld K-P, Ehrhart A, Brade V, Wichelhaus TA.** 2008. Thymidine-dependent *Staphylococcus aureus* small-colony variants: human pathogens that are relevant not only in cases of cystic fibrosis lung disease. J Clin Microbiol **46:**3829–3832. http://dx.doi.org/10.1128/JCM.01440-08.
215. **Berstad A, Hauso O, Valeur J.** 2014. Intestinal staphylococcal small colony variants: a cause of medically unexplained physical symptoms? Microb Ecol Health Dis **25:**25817. http://dx.doi.org/10.3402/mehd.v25.25817.
216. **Morelli P, De Alessandri A, Manno G, Marchese A, Bassi M, Lobello R, Minicucci L, Bandettini R.** 2015. Characterization of *Staphylococcus aureus* small colony variant strains isolated from Italian patients attending a regional cystic fibrosis care centre. New Microbiol **38:**235–243.
217. **Idelevich EA, Pogoda CA, Ballhausen B, Wüllenweber J, Eckardt L, Baumgartner H, Waltenberger J, Peters G, Becker K.** 2013. Pacemaker lead infection and related bacteraemia caused by normal and small colony variant phenotypes of *Bacillus licheniformis*. J Med Microbiol **62:**940–944. http://dx.doi.org/10.1099/jmm.0.051987-0.
218. **Kubota N, Kuzumoto K, Hidaka E, Yoshizawa K, Yumoto K, Oana K, Ogiso Y, Nakamura T, Kawakami Y.** 2013. First isolation of oleate-dependent *Enterococcus faecalis* small-colony variants from the umbilical exudate of a paediatric patient with omphalitis. J Med Microbiol **62:**1883–1890. http://dx.doi.org/10.1099/jmm.0.062752-0.
219. **Zbinden A, Quiblier C, Hernandez D, Herzog K, Bodler P, Senn MM, Gizard Y, Schrenzel J, François P.** 2014. Characterization of *Streptococcus tigurinus* small-colony variants causing prosthetic joint infection by comparative whole-genome analyses. J Clin Microbiol **52:**467–474. http://dx.doi.org/10.1128/JCM.02801-13.
220. **Häußler S, Lehmann C, Breselge C, Rohde M, Classen M, Tümmler B, Vandamme P, Steinmetz I.** 2003. Fatal outcome of lung transplantation in cystic fibrosis patients due to small-colony variants of the *Burkholderia cepacia* complex. Eur J Clin Microbiol Infect Dis **22:**249–253.
221. **Rusthoven JJ, Davies TA, Lerner SA.** 1979. Clinical isolation and characterization of aminoglycoside-resistant small colony variants of *Enterobacter aerogenes*. Am J Med **67:**702–706.
222. **Borderon E, Horodniceanu T.** 1978. Metabolically deficient dwarf-colony mutants of *Escherichia coli*: deficiency and resistance to antibiotics of strains isolated from urine culture. J Clin Microbiol **8:**629–634.
223. **Häußler S, Tümmler B, Weissbrodt H, Rohde M, Steinmetz I.** 1999. Small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis. Clin Infect Dis **29:**621–625.

Barbara C. Kahl, M.D., is Associate Professor in the Department of Medical Microbiology at the Westfalian-Wilhelms University (WWU), Münster, Germany. She received her M.D. from the WWU and her Board Certification for Medical Microbiology and Epidemiology. She worked as a visiting researcher at the Rockefeller University, New York City, NY, from 1997 to 2000. Since the 1990s, Dr. Kahl's scientific interests have included the epidemiology and pathogenesis of *Staphylococcus aureus* infections, especially in the background of the hereditary disease cystic fibrosis (CF), with a focus on staphylococcal small colony variants. She has published more than 40 papers in peer-reviewed journals on staphylococci and CF.

Karsten Becker, M.D., is Professor of Medical Microbiology, assistant medical director, and group leader at the Institute of Medical Microbiology at the Westfalian Wilhelms University, Münster, Germany. He is chairman of the Diagnostic Procedures Standing Working Group of the German Society for Hygiene and Microbiology (DGHM) and received the Clinical Infectiology Research Award from the German Society for Infectiology. He is an author of several medical textbooks, encyclopedias, and guidelines and of more than 200 scientific publications. Since the 1990s, Dr. Becker's interests have included the epidemiology, pathogenesis, diagnosis, prevention, and therapy of staphylococcal and micrococcal infections. In particular, he has done extensive research on the intracellular lifestyle of the staphylococcal small colony variant (SCV) phenotype. Also, he has contributed to the detection, treatment, and clinical significance of SCVs. A further focus is on the epidemiology and characterization of methicillin-resistant *Staphylococcus aureus*. He is an academic editor and a member of the editorial boards of medical and bioscience journals.

Bettina Löffler, born in 1972, studied medicine at the Ludwig-Maximilians Universität in Munich, Germany. From 2000 to 2002 she had a DFG scholarship at the TNO, Vascular and Connective Tissue Research Division, in Leiden, the Netherlands. From 2002, she continued her carrier at the Institute of Medical Microbiology at the University of Münster. She established a Cellular Microbiology research group that developed a focus on host pathogen interactions during *Staphylococcus aureus* infections. The research group established different *in vivo* and *in vitro* long-term infection models to analyze *S. aureus* dynamic adaptation and persistence strategies during the whole infection process. In 2013, Dr. Löffler served as a professor and director at the Institute of Medical Microbiology at Jena University Hospital. In 2014, she moved with her research group to Jena and continued her research work on S. aureus virulence and persistence there.