ISSN 1466-2523

Animal Health Research Reviews **12**(1); 33–45
doi:10.1017/S1466252311000065

Staphylococcus aureus small colony variants (SCVs) and their role in disease

Heba Atalla*, Carlton Gyles and Bonnie Mallard

Department of Pathobiology, Ontario Veterinary College, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Received 8 December 2010; Accepted 28 April 2011

Abstract

Persistent or difficult-to-treat *Staphylococcus aureus* infections in animals and humans may be related to small colony variants (SCVs) that can hide inside host cells and modulate host defenses. *S. aureus* SCVs have gained much attention in human medicine but have been underestimated and overlooked in veterinary medicine. Recently, an SCV isolated from a dairy cow with a history of chronic mastitis was shown to possess similar phenotypic and transcriptomic properties to those of human SCVs. SCVs form small, colorless, non-hemolytic colonies after 48 h, are only slowly coagulase positive, fail to ferment mannitol, and can revert to the parental phenotype. The phenotype of SCVs is mostly related to alterations in hemin and/or menadione biosynthesis or to thymidine deficiency. Transcriptomic analysis of SCVs shows up-regulation of genes involved in glycolytic and arginine–deiminase pathways, capsular biosynthesis; increased sigma B activity; and down-regulation of genes for α-hemolysin, coagulase and effector molecule RNA III of the global virulence regulator *Agr*. Similar results are reported at the protein level. SCVs are less virulent but successful persisters in infection models. SCVs persist longer and at higher numbers within non-phagocytes than do their parents. SCVs survive within spacious vacuoles up to 24 h within cultured bovine mammary epithelial cells, likely due to up-regulation of protective mechanisms that counteract the lethal acidic environment of the phagolysosome. Persistence of SCVs within host cells may explain failures in antimicrobial therapy and vaccinations.

Keywords: bovine mastitis, *Staphylococcus aureus*, small colony variant, SCV, MAC-T cells, internalization assay, transmission electron microscopy, phagosome maturation

Introduction

Staphylococcus aureus small colony variants (SCVs) are naturally occurring populations that were first described about 100 years ago, often as 'G' forms or 'dwarf' colonies of many bacterial species including *S. aureus* (Swingle, 1935; Wise and Spink, 1954; Goudie and Goudie, 1955; Proctor *et al.*, 2006; von Eiff *et al.*, 2006). In early reports SCVs were suggested as gonidial mutants or 'G' forms that developed within specialized mother cells under unfavorable conditions and had exceedingly small size, possibly representing a primitive phase of the bacterial life cycle (Swingle, 1935; Wise and Spink, 1954). Other studies reported the isolation of 'dwarf' colonies from animals and humans following antibiotic treatment (Goudie and Goudie, 1955; Sompolinsky *et al.*, 1974).

S. aureus SCVs have been extensively investigated in human medicine because of their association with persistent and relapsing infections (Proctor *et al.*, 2006), but have been mainly overlooked in veterinary medicine. However, in one study almost 40 years ago a dwarf-colony variant of *S. aureus* was linked to chronic bovine mastitis in an Israeli herd (Sompolinsky *et al.*, 1974). The authors suggested that the frequent use of antibiotics for the treatment of bovine mastitis may have selective advantages for staphylococci with metabolic disorders but their biological significance was not fully understood.

*Corresponding author. E-mail: hatalla@uoguelph.ca

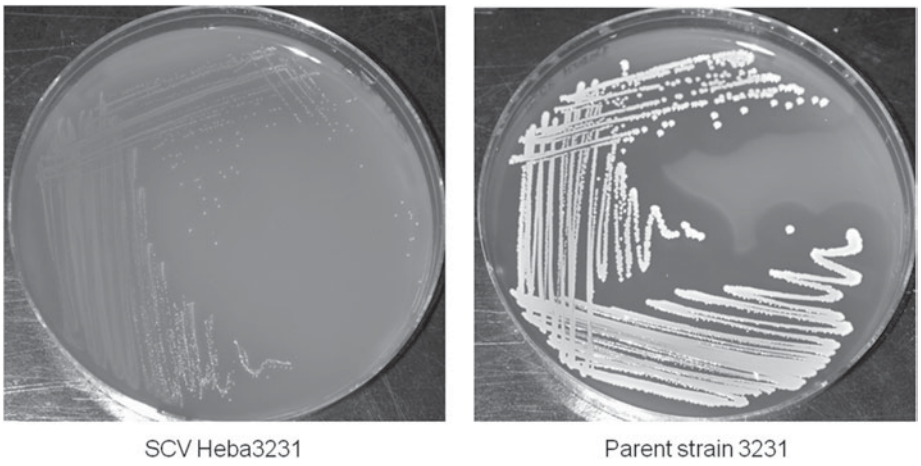

Fig. 1. Morphology of small-colony variant (SCV) Heba3231 on Columbia blood agar plate compared to its parent phenotype. The bovine SCV Heba3231 formed tiny non-hemolytic colonies that are typical for the SCV compared to its parent strain 3231.

In the mid-1990s, the clinical importance of *S. aureus* SCVs that are defective in electron transport gained considerable attention, and SCVs were linked to persistent and relapsing human infections (Proctor *et al.*, 1995). Subsequently, clinical SCVs were frequently isolated from humans with persistent and relapsing infection including septicemic arthritis, osteomyelitis, unmanageable wound infections or cystic fibrosis following antimicrobial therapy with gentamicin or trimethoprim–sulfamethoxazole (von Eiff *et al.*, 1997; Abele-Horn *et al.*, 2000; Sadowska *et al.*, 2002; Kahl *et al.*, 2003; Lattar *et al.*, 2009). Naturally occurring SCVs have been selected by the intracellular environment of cultured endothelial cells (Vesga *et al.*, 1996) as well as following *in vitro* exposure to certain concentrations of antibiotics, especially aminoglycosides, that eliminate or inhibit the growth of the parental phenotype and permit the SCV phenotype to become detectable (Wise and Spink, 1954; Musher *et al.*, 1977; Miller *et al.*, 1980; Balwit *et al.*, 1994; Massey *et al.*, 2001; Sadowska *et al.*, 2002; Schaaff *et al.*, 2003).

More recently, Atalla *et al.* (2008) reported the isolation of naturally occurring bovine SCVs from dairy cows with a history of chronic intramammary *S. aureus* infection and suggested possible association between persistent bovine mastitis and the formation of SCVs. Colonies with the appearance of SCV were detected in initial cultures of quarter milk samples of 1 of 11 cows, but these colonies reverted to the typical large-colony phenotype upon subculture. Following *in vitro* enrichment in the presence of sub-inhibitory concentrations of gentamicin (1 μg ml^{-1}), *S. aureus* of the SCV phenotype were recovered from six of the cows. These results are likely due to the fact that naturally occurring SCVs are often found in mixed population and are easily outgrown by bacteria of the parent phenotype; the findings are consistent with previous observations on human SCVs by Balwit *et al.* (1994) and Sadowska *et al.* (2002).

Phenotypic properties of *S. aureus* SCVs

S. aureus SCVs exhibit atypical morphological and biochemical properties relative to the parental phenotype that seem to be associated with disruption in the electron transport system or inability to synthesize thymidine (Gilligan *et al.*, 1987; Balwit *et al.*, 1994; McNamara and Proctor, 2000; Proctor *et al.*, 2006; von Eiff *et al.*, 2006; Atalla *et al.*, 2008). Typically, SCVs have a slow growth rate and require up to 48 h to form visible colonies on the solid agar medium supplemented with 5% rabbit or sheep blood (Fig. 1). They form tiny colonies that are about one-tenth the size of the parent strain, are non-pigmented, non-hemolytic or have a greatly reduced zone of hemolysis (Proctor and Peters, 1998; Kahl *et al.*, 2003; Atalla *et al.*, 2008). Reduced α-hemolysin production by *S. aureus* SCVs has been suggested to be responsible for reduced virulence in a nematode model of infection and their high capacity for intracellular persistence within non-professional phagocytes compared with their wild-type parental strains (Bates *et al.*, 2003; Jonsson *et al.*, 2003; Brouillette *et al.*, 2004; Proctor *et al.*, 2006; Sifri *et al.*, 2006). While SCVs utilize glucose and fructose, they fail to ferment mannitol or other sugars (Becker *et al.*, 2007; Atalla *et al.*, 2008).

SCVs have reduced coagulase production and require more than 18 h of incubation to be coagulase positive (Proctor *et al.*, 1995; Atalla *et al.*, 2008). Unlike other secreted proteins, the exoprotein coagulase is expressed during the exponential growth phase by almost all *S. aureus* strains including bovine isolates (Lebeau *et al.*, 1994). The expression of this protein is controlled by the accessory gene regulator (*agr*) locus and accordingly its expression is inhibited during the post-exponential phase. However, a previous study demonstrated that *S. aureus* strains that are *agr*-deficient continuously expressed an intermediate level of coagulase during the growth cycle

https://doi.org/10.1017/S1466252311000065 Published online by Cambridge University Press

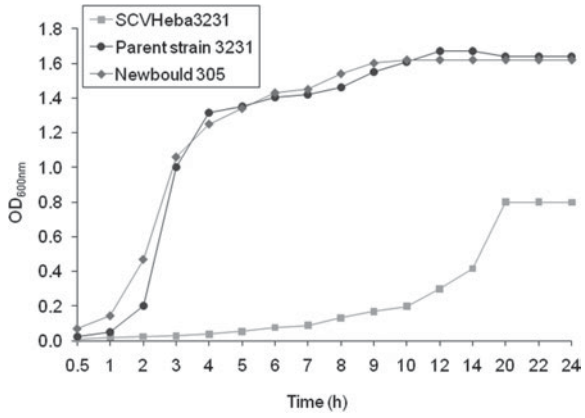

Fig. 2. Growth curve of SCV Heba3231 in brain heart infusion (BHI) broth with gentamicin in comparison with its parent strain and the prototypic strain Newbould 305.

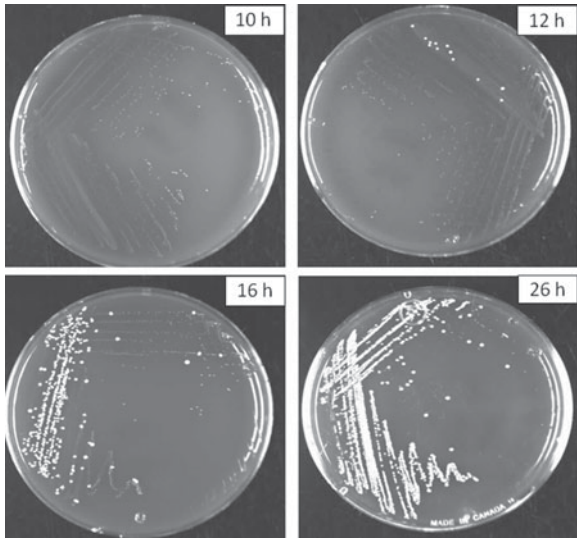

Fig. 3. Reversion of *S. aureus* SCV Heba3231 to the typical phenotype on Columbia blood agar plate when cultured in an antibiotic-free medium.

(Lebeau *et al.*, 1994). This observation could explain the delayed coagulase activity of *S. aureus* strains with the SCV phenotype. Coagulase-deficient mutants were not altered in their *in vitro* adherence to fibrinogen and in their infectivity in the rat model of endocarditis (Moreillon *et al.*, 1995). The role of coagulase in the pathogenesis of bovine mastitis has never been established (Phonimdaeng *et al.*, 1990; Baddour *et al.*, 1994; Sutra and Poutrel, 1994; Moreillon *et al.*, 1995; Kerro *et al.*, 2002).

Growth curve characteristics of *S. aureus* SCVs

Growth curve analysis of SCVs shows an extended lag phase (Kahl *et al.*, 2005; Atalla *et al.*, 2008) and a slower generation time compared with the parental strain (Wise and Spink, 1954; Proctor *et al.*, 1998). For example, the bovine SCV Heba3231 grew slowly in brain heart infusion (BHI) broth containing 1 μg ml^{-1} gentamicin and reached a plateau at a low cell density compared with its parent strain 3231 and the prototype strain Newbould 305 (Fig. 2). The rate of growth of the SCV was approximately one-ninth that of the parent (Atalla *et al.*, 2008).

A common feature of naturally occurring and *in vitro*-induced SCV *S. aureus* is the tendency to revert to the parental phenotype after cessation of antibiotic exposure (Swingle, 1935; Wise and Spink, 1954; Massey *et al.*, 2001; Atalla *et al.*, 2008). Reversion also occurs spontaneously on transfer from stock culture or after prolonged incubation in a culture medium, most often as 1–2 large colonies among numerous small colonies (Swingle, 1935). While the bovine SCV Heba3231 was stable on solid media, the colonies reverted to the parent phenotype in antibiotic-free BHI broth (Fig. 3) and the reversion was cell density dependent, occurring at >20×10^6 colony-forming unit (CFU) ml^{-1}.

Phenotypic switching between an SCV and a wild-type strain may be explained by a number of mechanisms, but the exact mechanism is not known. Regulatory mechanisms likely play a role in SCV selection (Proctor *et al.*, 1994; Hoffman *et al.*, 2006; Mitchell *et al.*, 2008), but mutational changes in genes with putative functions in hemin or menadione biosynthesis were reported, especially in gentamicin-induced SCVs (Schaaff *et al.*, 2003) or in SCVs from patients receiving systemic antibiotic therapy (Lannergård *et al.*, 2008). Because SCVs are unstable in most *in vitro* systems and the genetic changes in clinical isolates are undefined, constructed SCV mutants have been generated by disrupting the *hemB* and/or *menD* genes in *S. aureus* (von Eiff *et al.*, 1997; Bates *et al.*, 2003; Brouillette *et al.*, 2004). These genetically stable SCV mutants mimic the typical phenotypic features of clinical SCVs and have been successfully employed for phenotypic, transcriptomic and proteomic characterizations of SCVs and for the assessment of their pathogenic potential (Bates *et al.*, 2003; Jonsson *et al.*, 2003; Brouillette *et al.*, 2004; Senn *et al.*, 2005; Moisan *et al.*, 2006; Seggewiss *et al.*, 2006; Sifri *et al.*, 2006; von Eiff *et al.*, 2006; Besier *et al.*, 2007; Chatterjee *et al.*, 2008; Lannergård *et al.*, 2008).

Auxotrophism in *S. aureus* SCVs

The phenotypic characteristics of *S. aureus* SCVs are mostly related to alterations in hemin and/or menadione biosynthesis required for the electron transport system or to thymidine deficiency, but SCVs with other metabolic disorders have been reported (Acar *et al.*, 1978; Proctor *et al.*, 2006; Seggewiss *et al.*, 2006; Besier *et al.*, 2007; Kohler *et al.*, 2008). Hemin and menadione are required for the biosynthesis of cytochromes and menaquinone to complete the electron transport chain and generate large quantities of ATP. The production of ATP is required for biosynthesis of cell-wall teichoic acid, rapid growth and

https://doi.org/10.1017/S1466252311000065 Published online by Cambridge University Press

formation of large colonies, carotenoid biosynthesis, protein biosynthesis and increased electrochemical gradient to positively charged antibiotics including aminoglycosides and cationic peptides (Proctor *et al.*, 1998, 2006). Thymidine is required for the synthesis of electron transport chain components, yet the mechanisms involved in thymidine-dependent SCV are unlikely to be related to the electron transport defect (Kahl *et al.*, 2003). Thymidine-dependent SCVs emerged after long-term therapy of cystic fibrosis patients with trimethoprim sulphamethoxazole (STX) that interferes with a coenzyme required for downstream DNA synthesis (Proctor *et al.*, 2006). The survival of the STX-resistant SCV is dependent on exogenous thymidine that is abundant in airway secretions. In addition to the typical pinpoint colony morphology, thymidine-dependent SCVs often exhibit a 'fried-egg' appearance with translucent edges surrounding an elevated pigmented center (Kahl *et al.*, 2003).

Investigation of auxotrophism showed that supplementation of growth medium with hemin, menadione or thymidine restores the size of colonies (Wise and Spink, 1955; Balwit *et al.*, 1994; Kahl *et al.*, 1998; Brouillette *et al.*, 2004; Atalla *et al.*, 2008). The bovine SCV strain Heba3231 failed to grow on chemically defined medium supplemented with 1, 5 or 10 μg ml^{-1} hemin, menadione and/or thymidine suggesting that the bovine SCV may have unknown metabolic defects and require additional nutrients to support its growth (Atalla *et al.*, 2008). While the bovine SCV failed to grow on Muller Hinton (MH) agar plates, it formed minute colonies in 48 h on chocolate MH or when MH was supplemented with 5% sheep blood. Similarly, supplementation of MH with 1 μg ml^{-1} hemin or menadione enhanced SCV growth in 18 or 48 h. However, it failed to grow on MH supplemented with thymidine (Atalla *et al.*, 2008).

Antimicrobial susceptibility of *S. aureus* SCVs

In humans, *S. aureus* is problematic in healthcare and community settings and is featured as a 'superbug' for its increasing antimicrobial resistance (Foster, 2004; Lindsay and Holden, 2006). Antimicrobial therapy of clinical or subclinical *S. aureus* mastitis is generally not successful. Most antibiotic treatments result in short-term clinical cure and persistent or relapsing infection of the treated quarters (Barkema *et al.*, 2006). The inability of antimicrobial drugs to clear most *S. aureus* infections including bovine mastitis, coupled with the increasing rise of multidrug-resistant strains in humans, has led to extensive antimicrobial resistance studies involving bovine *S. aureus* isolates (Erskine *et al.*, 2004; Barkema *et al.*, 2006).

There is no evidence to support a widespread increase in penicillin resistance of bovine *S. aureus* isolates over the past 35 years (Erskine *et al.*, 2004; Call *et al.*, 2008). In fact, recent studies show a decrease in penicillin resistance in several countries during the past 10 years (Werckenthin *et al.*, 2001; Erskine *et al.*, 2002; Vintov *et al.*, 2003; Bennedsgaard *et al.*, 2006; Hendriksen *et al.*, 2008). Penicillin-resistant *S. aureus* seem to be a herd problem often related to inappropriate use of penicillin (Bennedsgaard *et al.*, 2006). While methicillin-resistant *S. aureus* (MRSA) has been a stumbling block for human antimicrobial therapy in community and healthcare settings, methicillin resistance seems uncommon among bovine isolates (Moon *et al.*, 2007; Monecke *et al.*, 2007; Morgan, 2008). Transmission of MRSA between cows and attendants has recently been reported in one province in Canada (Patrick Boerlin, Department of Pathobiology, Ontario Veterinary College, personal communication), Belgium (Vanderhaeghen *et al.*, 2010), Hungary (Juhász-Kaszanyitzky *et al.*, 2007) and Korea (Lee, 2003). Therapeutic success of bovine *S. aureus* mastitis is dependent on pathogen, cow and treatment-related factors, and SCVs may be of considerable importance in internalization of bovine mastitis-associated *S. aureus* strains within cells of the mammary gland, making them inaccessible to β-lactam antibiotics (Barkema *et al.*, 2006). Furthermore, SCVs have novel mechanisms for antibiotic resistance and appear to be selected by exposure to certain antimicrobials (Proctor *et al.*, 1998).

Clinical SCVs are often more resistant to antimicrobial compounds especially to aminoglycosides compared to wild-type parent strains (Proctor *et al.*, 1998; Seifert *et al.*, 1999; von Eiff *et al.*, 2000; Brouillette *et al.*, 2004; Atalla *et al.*, 2008). An interruption of the electron transport chain affects the electrochemical gradient across the bacterial cell membrane to positively charged antibiotics including aminoglycosides and cationic peptides. The minimum inhibitory concentration (MIC) of gentamicin for the bovine SCV Heba3231 was at least 16 times that of its parent strain and the prototype strain Newbould 305, but there was no difference in MICs of other antimicrobials (Atalla *et al.*, 2008). Reduced susceptibility to gentamicin is typical of a respiratory deficient mutant due to alteration in the electromembrane potential. However, clinical isolates are often found in mixed populations of SCV and the parent, and even when SCVs constitute a high percentage of colonies, they are easily overgrown in overnight broth culture, presenting a challenge for antimicrobial testing (von Eiff *et al.*, 2006). Interestingly, while the bovine prototype strain Newbould 305 and its isogenic *hemB* mutant were susceptible to the beta-lactam antibiotic cephapirin in *in vitro* assays, the *hemB* mutant was over 100 times more persistent in the mouse mammary gland than was Newbould 305 during antimicrobial therapy with cephapirin (Brouillette *et al.*, 2004).

Pathogenicity of *S. aureus* SCVs

Pathogenicity of *S. aureus* SCVs of human or bovine origin has been evaluated in a number of infection

https://doi.org/10.1017/S1466252311000065 Published online by Cambridge University Press

models including a mouse model of arthritis (Jonsson *et al.*, 2003), a rabbit model of endocarditis (Bates *et al.*, 2003), a nematode *Caenorhabditis elegans* model of infection (Sifri *et al.*, 2006), a mouse model of mastitis (Brouillette *et al.*, 2004) and in dairy cows (Atalla *et al.*, 2009). Studies have reported that SCVs varied from being highly virulent, equally virulent or less virulent compared to their wild-type strains. In the mouse model of arthritis, the *hemB* mutant was more highly virulent than the wild-type strains and its severity was likely related to excessive production of destructive proteases (Jonsson *et al.*, 2003). In the rabbit model of endocarditis, a *hemB* mutant was as virulent as its wild-type parent strain as determined by bacterial dissemination, and as sensitive to oxacillin therapy, suggesting possible reversion of the *hemB* mutant to the parental phenotype due to the availability of hemin in the embolic infarcts; a *menD* mutant strain showed less colonization and more resistance to oxacillin (Bates *et al.*, 2003).

In an early study, rabbits injected intravenously with the parent strains most often became emaciated and died, whereas those receiving the SCV appeared healthy and lacked any macroscopic lesions, suggesting reduced virulence of the SCV strains (Swingle, 1935). In *C. elegans*, although the *hemB* mutant successfully colonized the nematode gut, it was less virulent than the wild-type strain (Sifri *et al.*, 2006), mostly associated with reduced production of α-toxin and other virulence determinants. Although the bovine Newbould *hemB* mutant has reduced ability to colonize the mouse mammary gland, it was greater than 100 times more persistent than the parent strain Newbould 305 (Brouillette *et al.*, 2004). Additionally, intramammary challenge of dairy cows with the SCV Heba3231 or the Newbould *hemB* strain resulted in mastitis that was mild compared with that induced by their wild-type parent strains, based on systemic and localized signs (Atalla *et al.*, 2009). The pathogenicity of SCVs appears to rely on their ability to survive intracellularly compared to toxin-producing wild-type strains (von Eiff *et al.*, 1997; Kahl *et al.*, 1998; von Eiff *et al.*, 2000; Vaudaux *et al.*, 2002; Moisan *et al.*, 2006; Sendi and Proctor, 2009). The intracellular environment provides protection from host defenses and antibiotics and allows for long-term persistence in the host.

Prevalence of *S. aureus* SCVs

In view of the aforementioned unusual phenotypic characteristics, *S. aureus* SCVs present a challenge to clinicians and clinical microbiologists and are easily overlooked by diagnostic laboratories (Proctor *et al.*, 2006; von Eiff, 2008). The estimated frequency of occurrence of human *S. aureus* SCVs varies between 1 and 30% of clinical samples (Proctor *et al.*, 2006). *S. aureus* SCV was found in ∼29% of patients with osteomyelitis (von Eiff *et al.*, 1997), 17–46% of patients with cystic fibrosis who were chronically colonized with *S. aureus* (Kahl *et al.*, 2003; Besier *et al.*, 2007), and in about 1% of isolates in a general microbiology laboratory (Acar *et al.*, 1978). Naturally occurring bovine SCV was detected in three out of six (50%) *S. aureus*- positive milk samples from dairy cows with a history of chronic mastitis (Atalla *et al.*, 2008). Considering that SCVs grow slowly and are easily overgrown by their parents and readily revert to the typical parental phenotype, there is pressing need for improved isolation techniques that enrich for the SCV population. This will allow for further studies to determine the prevalence of *S. aureus* SCV in dairy cows.

Transcriptomics, clonal characteristics of *S. aureus* SCVs

DNA microarrays were used to compare the transcriptome of *S. aureus* strains with normal phenotype to their artificially created SCV mutants (Seggewiss *et al.*, 2006; Moisan *et al.*, 2006; Kohler *et al.*, 2008) or naturally occurring SCVs (Moisan *et al.*, 2006; Atalla *et al.*, 2008). A *S. aureus*-specific DNA microarray was used to determine the heterogeneity of gene expression associated with the bovine SCV Heba3231 phenotype (Atalla *et al.*, 2008). The DNA array contained 460 genes including virulence, secretion, general stress response, regulatory systems, iron transport, antibiotic resistance and general biosynthesis genes (amplified from DNA of *S. aureus* strains N315, MRSA COL and Mu50). Genes encoding for the effector molecule of the *agr*, α-toxin and coagulase were down-regulated in SCV Heba3231, whereas genes encoding for fermentative and arginine–deiminase pathways, capsular biosynthesis and those associated with up-regulation of the alternative factor *sigB* were up-regulated (Table 1). Of note, genes involved in iron metabolism were down-regulated in SCV Heba3231.

While naturally occurring SCVs of bovine and human origins share common features with genetically defined SCVs that explain the SCV phenotypic characteristics, they had a unique transcriptional signature (Moisan *et al.*, 2006; Atalla *et al.*, 2008). For example, genes that are positively controlled by the alternative factor *sigB*, such as those encoding for capsular biosynthesis and shock proteins, were up-regulated in SCV Heba3231 (Atalla *et al.*, 2008) and clinical human SCVs isolated from cystic fibrosis patients (Moisan *et al.*, 2006).

Increased activity of the alternative factor *sigB* is known to up-regulate the transcription of several surface adhesins and is associated with adherence, invasion and persistence within host cells (Moisan *et al.*, 2006; Atalla *et al.*, 2008; Mitchell *et al.*, 2008). Naturally occurring SCVs with high *sigB* activity were successful persisters within host cells compared to Newbould *hemB* and a *sigB* mutant that was shown to be a poor persister (Moisan *et al.*, 2006) as well as compared to their wild-type parental strains (Atalla *et al.*, 2008, 2010a).

https://doi.org/10.1017/S1466252311000065 Published online by Cambridge University Press

Table 1. Expression of some marker genes in naturally occurring bovine small colony variant (SCV) Heba3231 compared to the genetically constructed Newbould hemB mutant

Gene	Protein encoded by marker gene	Fold change in SCV[a]	
		SCV Heba 3231[b]	Newbould *hemB*[c]
hla	α-toxin	−8.1	1.8
hld	α-hemolysin/RNAIII	−8.4	−5.3
coa	Coagulase	−2.6	−4.5
ldh	L-lactose dehydrogenase	1643	52.3
adh	Alcohol dehydrogenase	7.4	30.9
arcA	Arginine de-iminase	5.4	30.9
capA	Capsule biosynthesis	6.3	1.3
asp23	Alkaline shock protein – *sigB* activity marker	3.5	−4.4

A ⩾2-fold increase or decrease was considered significant.
[a]A decrease is indicated by minus sign.
[b]Atalla *et al.* (2008).
[c]Moisan *et al.* (2006).

To determine genome relatedness between SCV Heba3231 and its parent strain 3231, genomic compositions were studied by comparative genomic hybridization using a DNA microarray containing 460 genes representing sequences of *S. aureus* strains N315, MRSA COL and Mu50 (Atalla *et al.*, 2008). Results were also compared to those for the Newbould *hemB* mutant and its parent strain, the prototype Newbould 305 (Moisan *et al.*, 2006) to identify genetic differences between bovine isolates that may influence host–pathogen interaction. Clonal characterization confirmed that wild-type strain 3231 and the SCV Heba3231 are of the same clone but were different from the prototype strain Newbould 305 and its isogenic *hemB* mutant (Table 2).

Important differences included the presence of a set of pyrogenic superantigens such as staphylococcal enterotoxin D (SED) in strains 3231 and SCV Heba3231 that appear to play a role in immune response modulation. In our recent study (Atalla *et al.*, 2010a, b), differences in antibody-mediated immune response (AMIR) and cell-mediated immune response (CMIR) were clearly evident in the response to SCV Heba3231 and its parent strain 3231 compared with the *hemB* mutant and its parent strain Newbould 305, following intramammary infection (IMI) of cattle. Predominance of IgG_2 antibody in sera and induction of CMIR to the SCV Heba3231 were noted and are typical of a type 1-biased immune response. These findings correlate with the ability of the bovine SCV to adapt to an intracellular lifestyle for better adaptation to the bovine mammary gland and for long-term persistence. Similar to the SCV Heba3231, the parent strain 3231 stimulated both AMIR and CMIR. However, polarization of the immune response toward type 1 or type 2 was not evident. In fact, involvement of both types 1 and 2 in domestic species including cattle is the usual outcome in response to most pathogens (Estes and Brown, 2002; Crawley *et al.*, 2005).

On the other hand, both the *hemB* strain and its parent Newbould 305 stimulated production of specific antibody responses predominated by IgG_1 and failed to induce DTH, which is typical for a type 2-biased immune response. The differences in immune response seem to be related to differences in virulence attributes of the *S. aureus* strains involving mostly exoproteins such as SED and staphylococcal enterotoxin-like gene *sen* that are expressed by SCV Heba3231 and 3231 strains but not the other strains. Specifically, SED has been reported to mediate a type 1 biased immune response characterized by predominance of IgG2 antibody in secreted milk during experimental IMI of dairy cattle (Tollersrud *et al.*, 2006).

Table 2. Genome relatedness between naturally occurring bovine SCV (Heba3231) and its parent strain 3231 compared to the genetically constructed Newbould *hemB* mutant and its parent strain the prototype Newbould 305 as determined by comparative genomic hybridization

Gene	Strains 3231 and SCV3231	Strains Newbould 305 and its *hemB* mutant
Virulence regulator		
agr type 1	−	+
TRAP type 2	−	+
agr type 2	+	−
TRAP type 2	+	−
Surface component		
cap type 5	−	+
cap type 8	+	−
Can	+	−
Pyrogenic superantigen		
Sed	+	−
She	+	−
Sei	+	−
Sem	+	−
Sen	+	−
Seo	+	−
luk SF	+	−

https://doi.org/10.1017/S1466252311000065 Published online by Cambridge University Press

Intracellular survival of *S. aureus* SCVs

Traditionally, *S. aureus* has been viewed as an extracellular pathogen. However, there are several reports of its ability to invade and survive intracellularly within professional phagocytes including neutrophils and monocyte-derived macrophages (Gresham *et al.*, 2000; Voyich *et al.*, 2005), as well as non-professional phagocytic cells such as cultured bovine aortic endothelial cells (BAEC) (Vann and Proctor, 1987; Atalla *et al.*, 2008) and a bovine mammary epithelial cell line (MAC-T) (Almeida *et al.*, 1996; Bayles *et al.*, 1998; Atalla *et al.*, 2010a, b). Unlike facultative intracellular pathogens that survive inside infected phagocytes and mammalian cells for long periods of times, internalized *S. aureus* often reside for a short time, escape into the cytosol and induce apoptosis in *in vitro* infection models (Bayles *et al.*, 1998; Menzies and Kourteva, 1998; Murai *et al.*, 1999; Kahl *et al.*, 2000; Wesson *et al.*, 2000; Smagur *et al.*, 2009).

Compared to wild-type *S. aureus* strains, clinical SCVs of human and bovine origin were shown to invade and persist longer inside non-phagocytic cells in culture with minimal deleterious effects compared to wild-type strains (Moisan *et al.*, 2006; Schröder *et al.*, 2006; Atalla *et al.*, 2008, 2010a, b). Monolayer MAC-T cells with internalized SCV Heba3231 following exposure at multiplicity of infection (MOI) 100 appeared healthy with minimal loss of integrity of the cell monolayer; viable CFU were detected in cell lysates 96 h after infection. By contrast and in line with previous observations (Bayles *et al.*, 1998; Menzies and Kourteva, 1998) cells infected with either the parent strain 3231 or Newbould 305 underwent progressive loss of viability at 3.8 h to complete detachment of cell monolayers by 24 h after infection. There was a marked reduction in viable bacteria in cell lysates at 3.8 h and complete absence at 24 h. The minimal tissue damage caused by the SCV Heba3231 is likely related to its diminished α-toxin production as determined by phenotypic and transcriptional analyses (Atalla *et al.*, 2008), whereas the considerable tissue damage caused by the wild-type strains with the continuous loss of viable bacteria was likely due to expression of the membrane damaging α-toxin and subsequent elimination by lysostaphin of bacteria released into the extracellular fluid.

Role of *S. aureus* SCVs in pathogenesis

The pathogenesis of *S. aureus* infections involves several stages including entry into the host, rapid replication, avoidance or subversion of innate defenses, attachment and colonization, tissue invasion and tropism and post-invasion events (Frost *et al.*, 1977; Sutra and Poutrel, 1994; Alexander and Hudson, 2001; Kerro *et al.*, 2002; Garzoni and Kelley, 2009; Sinha and Fraunholz, 2010). The outcome of the interaction between the host and a microbe is influenced by virulence that is a function of certain factors produced by the microbe (Casadevall, 2008). *S. aureus* is equipped with several virulence factors that promote colonization, infection (caused by attachment, growth of invading bacteria and subversion of host defenses) and overt disease (often as a result of damaging toxins or induced host response).

In the case of bovine mastitis, access of *S. aureus* into the mammary gland often occurs as a consequence of disruption of the teat or mucosal barriers. Breaching of the protective physical barriers allows *S. aureus* to gain access to the sterile environment of the mammary gland either through progressive colonization of the teat canal and/or transfer with milk into the teat sinus at the end of milking (Sutra and Poutrel, 1994). While little is known about the clinical importance of *S. aureus* SCVs in spontaneous bovine mastitis, intramammary challenge of dairy cows through the teat canal with ∼5000 CFU of SCV Heba3231 per gland-induced localized signs of mastitis in 3/5 cows and virtual absence of systemic response during the 5-days post-challenge (Atalla *et al.*, 2009). Bacterial shedding in milk from SCV Heba3231 challenged quarters was generally low and first detected at day 4 post-challenge (Atalla *et al.*, 2009). While bacteria were not detected in all challenged quarters at any of the sampling points, SCVs were recovered from the milk of three cows during the first 5 days, and at days 21 and 36 post-challenge, and of all five cows at day 14 post-challenge. Somatic cell scores were significantly higher (P<0.5) during the first week and up to day 36 post-challenge compared to day 0 before challenge (Atalla *et al.*, 2009).

Upon breaching the compromised anatomical barriers, *S. aureus* invades the bovine MAC-T by receptor-mediated endocytosis involving adhesion to host cells, signal transduction and cytoskeletal rearrangement and subsequent bacterial uptake (Almeida *et al.*, 1996; Bayles *et al.*, 1998; Dziewanowska *et al.*, 1999; Lammers *et al.*, 1999; Sinha *et al.*, 1999, 2000; Alexander and Hudson, 2001; Menzies, 2003; Garzoni and Kelley, 2009). The intimate adherence of *S. aureus* to the MAC-T is the first and most crucial event for teat colonization and development of IMI (Frost, 1975; Frost *et al.*, 1977; Wanasinghe, 1981; Gudding *et al.*, 1984; Opdebeeck *et al.*, 1988; Sordillo *et al.*, 1989; Cifrian *et al.*, 1994; Hensen *et al.*, 2000; Kerro *et al.*, 2002). The high capacity for adhesion of *S. aureus* to epithelial cells was linked to the high prevalence of chronic mastitis in dairy herds with relapsing acute episodes compared to the sporadic infections associated with coliforms (Frost, 1975; Frost *et al.*, 1977).

S. aureus adhesion is mediated by the expression of several surface adhesion molecules that bind directly to host cells or through bridging ligands such as fibronectin (Fn), fibrinogen and collagen (Clarke and Foster, 2006). Binding of *S. aureus* to epithelial cells often occurs through Fn that acts as a bridging molecule between Fn-binding protein (FnBP) and host cells α5β1-integrins

https://doi.org/10.1017/S1466252311000065 Published online by Cambridge University Press

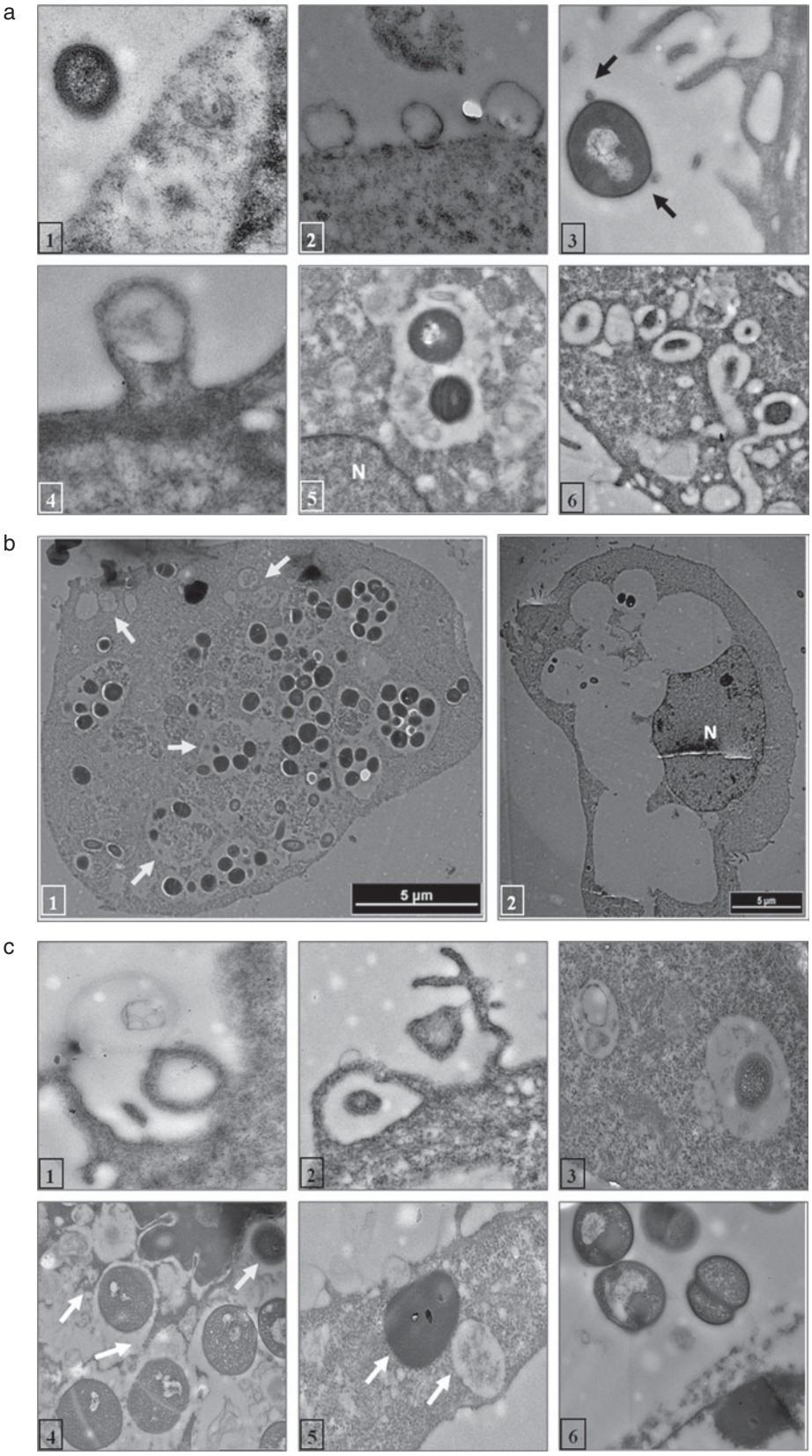

Fig. 4. (a**)** Transmission electron micrographs (TEM) of bovine mammary epithelial cells (MAC-T) infected with SCV Heba3231 (MOI 100), demonstrating the proposed sequence of events of SCV uptake: (1) SCV in close proximity with epithelial cell surface, (2) adherence of SCV to the epithelial cell surface, (3) elongation of epithelial cell surface around adherent SCV, (4) formation of pseudopod-like structure surrounding SCV and (5 and 6) one and two SCVs enclosed within a vacuole at 1 and

https://doi.org/10.1017/S1466252311000065 Published online by Cambridge University Press

(Lammers *et al.*, 1999; Dziewanowska *et al.*, 2000). In addition, FnBPs can bind to both heat shock protein 60 (HSP60) expressed on the cell surface and Fn linked to α5β1-integrins, or may bind directly to HSP60 independent of β1-integrins (Dziewanowska *et al.*, 2000). The interaction between *S. aureus* and α5β1-integrins leads to integrin clustering that triggers a signaling cascade across the cell membrane and activation of several protein tyrosine kinases (PTK). The signal transduction leads to cytoskeletal rearrangement through polymerization of actin microfilaments and bacterial uptake in a membrane-bound vacuole via a zipper-like mechanism (Bayles *et al.*, 1998; Dziewanowska *et al.*, 1999; Sinha and Herrmann, 2005). *S. aureus* internalization occurs in a time-dose-dependent manner (Sinha and Herrmann, 2005).

As with the wild-type parents SCV adhesion and internalization by host cells appear to be mediated by Fn–FnBP interaction (Vaudaux *et al.*, 2002; Moisan *et al.*, 2006; Sendi and Proctor, 2009). In SCVs, surface adhesins such as FnBP and fibrinogen-binding proteins are over-expressed under the influence of increased *sigB* activity and down-regulation of the *agr* locus compared to parent strains; the enhanced expression promotes efficient adhesion and internalization by host cells (Vaudaux *et al.*, 2002; Moisan *et al.*, 2006). Noteworthy, Fn is not expressed on the apical surface of epithelial cells in the bovine mammary gland, but on the surface of myoepithelial cells located beneath the luminal epithelial cells (Lammers *et al.*, 1999). Accordingly, Fn may not be accessible for early tissue adhesion and establishment of IMI, instead it may play a role in the spread of infection. In addition to the role of FnBPs in the pathogenesis of *S. aureus* infections, the involvement of other surface proteins such as Eap that compensate for the loss of FnBPs and wall teichoic acid have been reported in successful *S. aureus* internalization within human eukaryotic cells (Hussain *et al.*, 2008; Weidenmaier and Peschel, 2008).

Unlike entry into cells which can be a microbe or host-active process, the intracellular survival strategy is determined largely by the microbe (Casadevall, 2008). Once inside the host cell, microbial pathogens use different strategies to avoid the lethal phagolysosomal environment through preventing lysosomal fusion or acidification (e.g. *Salmonella* spp. and *Mycobacterium tuberculosis*), surviving within the harsh lysosome environment (e.g. *Coxiella burnetii*) or by escaping from their endocytic vesicles into the cytoplasm (e.g. *Listeria monocytogenes*) (Gruenheid and Finlay, 2003; Luzio *et al.*, 2007). Despite being recognized to survive well within non-phagocytic cells (Garzoni and Kelley, 2009; Sinha and Fraunholz, 2010), trafficking pathway studies present conflicting results regarding the fate of *S. aureus* enclosed within vacuoles. The different bacterial fates are likely related to strain variation and variations in their gene expression, the type of *in vitro* infection model, MOI and the time points at which the cells are examined (Lowy *et al.*, 1988; Almeida *et al.*, 1996; Bayles *et al.*, 1998; Kahl *et al.*, 2000; Atalla *et al.*, 2010a, b).

Recent ultrastructural analysis of MAC-T cells infected with the SCV Heba3231 and its parent strain (MOI 100) provided an insight into intracellular aspects of pathogenesis (Atalla *et al.*, 2010a, b). Both SCV Heba3231 and its parent strain adhered, and were internalized within MAC-T cells following the same sequence of events including adherence of bacteria to the epithelial cell surface, formation of pseudopod-like structures around the adherent bacteria, and engulfment of bacteria within endocytic vesicles (Fig. 4a and c). Nevertheless, differences were seen in their intracellular fates. While SCV Heba3231 remained localized within the endocytic membrane at 24 h of incubation (Fig. 4b), the wild-type strain 3231 escaped from the vacuoles into the host cytosol at 3.5 h and induced complete destruction of the epithelial cells by 24 h (Fig. 4C4 and 6), similar to previous observations (Bayles *et al.*, 1998; Schröder *et al.*, 2006). In agreement with other studies (Bayles *et al.*, 1998; Schröder *et al.*, 2006), the highly toxigenic parent strain 3231 likely escaped from the endocytic vacuole into the cytoplasm due to the release of α-toxin and proteases; in the cytoplasm, the organisms replicate rapidly causing cell death, contributing to spread of infection and development of acute mastitis.

Survival of *S. aureus* SCV within non-phagocytic cells is a successful strategy for persistence within the host as the intracellular compartment shields the SCV from immune defenses and antimicrobial therapy. In view of the SCV Heba3231 transcriptome and up-regulation of genes involved in alternative respiration (Atalla *et al.*, 2008), it is conceivable that internalized SCV Heba3231 use the arginine–deiminase pathway to produce ATP and the released ammonia to counteract the lethal acidic environment of the phagolysosome, contributing to SCV survival and persistence (Atalla *et al.*, 2010a, b).

3.5 h. Scale bars: 1 μm. (b) TEM of MAC-T cells infected with SCV Heba3231 (MOI 100) at 24 h showing: (1) epithelial cell packed with large vacuoles each containing 5–10 bacteria, and (2) epithelial cell occupied by large spacious vacuole that incorporates small vacuoles and contains few bacteria. Bars=5 μm. (c) TEM of MAC-T cells infected with 3231 strain (MOI 100) demonstrating the proposed sequence of events of 3231 uptake: (1) adherence of 3231 to epithelial cell surface and formation of elongated appendages, (2) formation of pseudopod-like structure surrounding 3231, (3) single coccus of 3231 strain within large vacuole, (4) cocci of 3231 strain partially surrounded by a degraded vacuolar membrane – some appear to be dividing and are entirely free in the cytoplasm at 3.5 h, (5) membrane-bound vacuole (phagosome) in contact with electron dense lysosome, (6) cocci of 3231 strain released from damaged MAC-T cells at 24 h. Scale bars=1 μm.

https://doi.org/10.1017/S1466252311000065 Published online by Cambridge University Press

Conclusion

Persistent *S. aureus* infection has been related to the extraordinary versatility of this pathogen and the development of diverse strategies to overcome the host immune system and antimicrobial therapy. The ability of strains of *S. aureus* to form slow growing subpopulations called SCVs is a unique feature that may sustain intracellular persistence and modulate innate and adaptive defense mechanisms. While *S. aureus* SCVs have been extensively studied in human medicine and often linked to persistent and recurrent human infections, they are neglected and often overlooked in veterinary medicine.

Among the common features of *S. aureus* SCV are the up-regulation of genes for surface molecules that allow for intimate adherence to and internalization by host cells, and down-regulation of genes for secreted toxins and enzymes to facilitate persistence for the lifetime of the infected host cells. Internalization and persistence of the bovine SCV within MAC-T cells has major implications. First, it highlights the potential role of bovine MAC-T in persistent infection. Second, it explains the inability of antimicrobial therapy to clear IMI and of currently available vaccines to provide protection against *S. aureus* mastitis. Third, it indicates the need for effective CMIR and AMIR to control persistent strains, especially those that generate SCVs. Lastly, it explains the frequent failure to detect *S. aureus* in milk from cows with subclinical mastitis.

References

Abele-Horn M, Schupfner B, Emmerling P, Waldner H and Goring H (2000). Persistent wound infection after herniotomy associated with small-colony variants of *Staphylococcus aureus*. *Infection* **28**: 53–54.

Acar JF, Goldstein FW and Lagrange P (1978). Human infections caused by thiamine- or menadione-requiring *Staphylococcus aureus*. *Journal of Clinical Microbiology* **8**: 142–147.

Alexander EH and Hudson MC (2001). Factors influencing the internalization of *Staphylococcus aureus* and impacts on the course of infections in humans. *Applied Microbiology and Biotechnology* **56**: 361–366.

Almeida RA, Matthews KR, Cifrian E, Guidry AJ and Oliver SP (1996). *Staphylococcus aureus* invasion of bovine mammary epithelial cells. *Journal of Dairy Science* **79**: 1021–1026.

Atalla H, Gyles C, Jacob CL, Moisan H, Malouin F and Mallard B (2008). Characterization of a *Staphylococcus aureus* small colony variant (SCV) associated with persistent bovine mastitis. *Foodborne Pathogens and Disease* **5**: 785–799.

Atalla H, Gyles C, Wilkie B, Leslie K and Mallard B (2009). Somatic cell scores and clinical signs following experimental intramammary infection of dairy cows with a *Staphylococcus aureus* small colony variant (*S. aureus* SCV) in comparison to other bovine strains. *Veterinary Microbiology* **137**: 326–334.

Atalla H, Gyles C and Mallard B (2010a). Persistence of a *Staphylococcus aureus* small colony variants (*S. aureus* SCV) within bovine mammary epithelial cells. *Veterinary Microbiology* **143**: 319–328.

Atalla H, Wilkie B, Gyles C, Leslie K, Mutharia L and Mallard B (2010b). Antibody and cell-mediated immune responses to *Staphylococcus aureus* small colony variants and their parental strains associated with bovine mastitis. *Developmental and Comparative Immunology* **34**: 1283–1290.

Baddour LM, Tayidi MM, Walker E, McDevitt D and Foster TJ (1994). Virulence of coagulase-deficient mutants of *Staphylococcus aureus* in experimental endocarditis. *Journal of Medical Microbiology* **41**: 259–263.

Balwit JM, van LP, Vann JM and Proctor RA (1994). Gentamicin-resistant menadione and hemin auxotrophic *Staphylococcus aureus* persist within cultured endothelial cells. *Journal of Infectious Diseases* **170**: 1033–1037.

Barkema HW, Schukken YH and Zadoks RN (2006). Invited Review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine *Staphylococcus aureus* mastitis. *Journal of Dairy Science* **89**: 1877–1895.

Bates DM, von EC, McNamara PJ, Peters G, Yeaman MR, Bayer AS and 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. *Journal of Infectious Diseases* **187**: 1654–1661.

Bayles KW, Wesson CA, Liou LE, Fox LK, Bohach GA and Trumble WR (1998). Intracellular *Staphylococcus aureus* escapes the endosome and induces apoptosis in epithelial cells. *Infection and Immunity* **66**: 336–342.

Becker K, Bierbaum G, von EC, Engelmann S, Gotz F, Hacker J, Hecker M, Peters G, Rosenstein R and Ziebuhr W (2007). Understanding the physiology and adaptation of staphylococci: a post-genomic approach. *Int. Journal of Medical Microbiology* **297**: 483–501.

Bennedsgaard TW, Thamsborg SM, Aarestrup FM, Enevoldsen C, Vaarst M and Christoffersen AB (2006). Resistance to penicillin of *Staphylococcus aureus* isolates from cows with high somatic cell counts in organic and conventional dairy herds in Denmark. *Acta Veterinaria Scandinavia* **48**: 24.

Besier S, Ludwig A, Ohlsen K, Brade V and Wichelhaus TA (2007). Molecular analysis of the thymidine-auxotrophic small colony variant phenotype of *Staphylococcus aureus*. *International Journal of Medical Microbiology* **297**: 217–225.

Brouillette E, Martinez A, Boyll BJ, Allen NE and Malouin F (2004). Persistence of a *Staphylococcus aureus* small-colony variant under antibiotic pressure *in vivo*. *FEMS Immunology and Medical Microbiology* **41**: 35–41.

Call DR, Davis MA and Sawant AA (2008). Antimicrobial resistance in beef and dairy cattle production. *Animal Health Research Reviews* **9**: 159–167.

Casadevall A (2008). Evolution of intracellular pathogens. *Annual Review of Microbiology* **62**: 19–33.

Chatterjee I, Kriegeskorte A, Fischer A, Deiwick S, Theimann N, Proctor RA, Peters G, Herrmann M and 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*. *Journal of Bacteriology* **190**: 834–842.

Cifrian E, Guidry AJ, O'Brien CN, Nickerson SC and Marquardt WW (1994). Adherence of *Staphylococcus aureus* to cultured bovine mammary epithelial cells. *Journal of Dairy Science* **77**: 970–983.

Clarke SR and Foster SJ (2006). Surface adhesins of *Staphylococcus aureus*. *Advances in Microbial Physiology* **51**: 187–224.

Crawley AM, Mallard B and Wilkie BN (2005). Genetic selection for high and low immune response in pigs: effects on

immunoglobulin isotype expression. *Veterinary Immunology and Immunopathology* **108**: 71–76.

Dziewanowska K, Carson AR, Patti JM, Deobald CF, Bayles KW and Bohach GA (2000). Staphylococcal fibronectin binding protein interacts with heat shock protein 60 and integrins: role in internalization by epithelial cells. *Infection and Immunity* **68**: 6321–6328.

Dziewanowska K, Patti JM, Deobald CF, Bayles KW, Trumble WR and Bohach GA (1999). Fibronectin binding protein and host cell tyrosine kinase are required for internalization of *Staphylococcus aureus* by epithelial cells. *Infection and Immunity* **67**: 4673–4678.

Erskine R, Cullor J, Schaellibaum M, Yancey B and Zecconi A (2004). *Bovine Mastitis Pathogens and Trends in Resistance to Antimicrobial Drugs*. Verona, WI: National Mastitis Council, pp. 400–414.

Erskine RJ, Walker RD, Bolin CA, Bartlett PC and White DG (2002). Trends in antibacterial susceptibility of mastitis pathogens during a seven-year period. *Journal of Dairy Science* **85**: 1111–1118.

Estes DM and Brown WC (2002). Type 1 and type 2 responses in regulation of Ig isotype expression in cattle. *Veterinary Immunology and Immunopathology* **90**: 1–10.

Foster TJ (2004). The *Staphylococcus aureus* "superbug". *Journal of Clinical Investigation* **114**: 1693–1696.

Frost AJ (1975). Selective adhesion of microorganisms to the ductular epithelium of the bovine mammary gland. *Infection and Immunity* **12**: 1154–1156.

Frost AJ, Wanasinghe DD and Woolcock JB (1977). Some factors affecting selective adherence of microorganisms in the bovine mammary gland. *Infection and Immunity* **15**: 245–253.

Garzoni C and Kelley WL (2009). *Staphylococcus aureus*: new evidence for intracellular persistence. *Trends in Microbiology* **17**: 59–65.

Gilligan PH, Gage PA, Welch DF, Muszynski MJ and Wait KR (1987). Prevalence of thymidine-dependent *Staphylococcus aureus* in patients with cystic fibrosis. *Journal of Clinical Microbiology* **25**: 1258–1261.

Goudie JG and Goudie RB (1955). Recurrent infections by a stable dwarf-colony variant of *Staphylococcus aureus*. *Journal of Clinical Pathology* **8**: 284–287.

Gresham HD, Lowrance JH, TCaver TE, Wilson BS, Cheung AL and Lindberg FP (2000). Survival of *Staphylococcus aureus* inside neutrophils contributes to infection. *Journal of Immunology* **164**: 3713–3722.

Gruenheid S and Finlay BB (2003). Microbial pathogenesis and cytoskeletal function. *Nature* **422**: 775–781.

Gudding R, McDonald JS and Cheville NF (1984). Pathogenesis of *Staphylococcus aureus* mastitis: bacteriologic, histologic, and ultrastructural pathologic findings. *American Journal of Veterinary Research* **45**: 2525–2531.

Hendriksen RS, Mevius DJ, Schroeter A, Teale C, Meunier D, Butaye P, Franco A, Utinane A, Amado A, Moreno M, Greko C, Stark K, Berghold C, Myllyniemi AL, Wasyl D, Sunde M and Aarestrup FM (2008). Prevalence of antimicrobial resistance among bacterial pathogens isolated from cattle in different European countries: 2002–2004. *Acta Veterinaria Scandinavia* **50**: 28.

Hensen SM, Pavicic MJ, Lohuis JA and Poutrel B (2000). Use of bovine primary mammary epithelial cells for the comparison of adherence and invasion ability of *Staphylococcus aureus* strains. *Journal of Dairy Science* **83**: 418–429.

Hoffman LR, Deziel E, D'Argenio DA, Lepine F, Emerson J, McNamara S, Gibson RL, Ramsey BW and Miller SI (2006). Selection for *Staphylococcus aureus* small-colony variants due to growth in the presence of Pseudomonas aeruginosa. *Proceedings of the National Academy of Sciences USA* **103**: 19890–19895.

Hussain M, Haggar A, Peters G, Chhatwal GS, Herrmann M, Flock JI and Sinha B (2008). More than one tandem repeat domain of the extracellular adherence protein of *Staphylococcus aureus* is required for aggregation, adherence, and host cell invasion but not for leukocyte activation. *Infection and Immunity* **76**: 5615–5623.

Jonsson IM, von EC, Proctor RA, Peters G, Ryden C and 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. *Microbial Pathogenesis* **34**: 73–79.

Juhász-Kaszanyitzky E, Janosi S, Somogyi P, Dan A, van der Graaf-van Bloois L, van Duijkeren E and Wagenaar JA (2007). MRSA transmission between cows and humans. *Emerging Infectious Diseases* **13**: 630–632.

Kahl B, Herrmann M, Everding AS, Koch HG, Becker K, Harms E, Proctor RA and Peters G (1998). Persistent infection with small colony variant strains of *Staphylococcus aureus* in patients with cystic fibrosis. *Journal of Infectious Diseases* **177**: 1023–1029.

Kahl BC, Belling G, Becker P, Chatterjee I, Wardecki K, Hilgert K, Cheung AL, Peters G and Herrmann M (2005). Thymidine-dependent *Staphylococcus aureus* small-colony variants are associated with extensive alterations in regulator and virulence gene expression profiles. *Infection and Immunity* **73**: 4119–4126.

Kahl BC, Duebbers A, Lubritz G, Haeberle J, Koch HG, Ritzerfeld B, Reilly M, Harms E, Proctor RA, Herrmann M and Peters G (2003). Population dynamics of persistent *Staphylococcus aureus* isolated from the airways of cystic fibrosis patients during a 6-year prospective study. *Journal of Clinical Microbiology* **41**: 4424–4427.

Kahl BC, Goulian M, van WW, Herrmann M, Simon SM, Kaplan G, Peter G and Cheung AL (2000). *Staphylococcus aureus* RN6390 replicates and induces apoptosis in a pulmonary epithelial cell line. *Infection and Immunity* **68**: 5385–5392.

Kerro DO, van Dijk JE and Nederbragt H (2002). Factors involved in the early pathogenesis of bovine *Staphylococcus aureus* mastitis with emphasis on bacterial adhesion and invasion: a review. *Veterinary Quarterly* **24**: 181–198.

Kohler C, von EC, Liebeke M, McNamara PJ, Lalk M, Proctor RA, Hecker M and Engelmann S (2008). A defect in menadione biosynthesis induces global changes in gene expression in *Staphylococcus aureus*. *Journal of Bacteriology* **190**: 6351–6364.

Lammers A, Nuijten PJ, Kruijt E, Stockhofe-Zurwieden N, Vecht U, Smith HE and van Zijderveld FG (1999). Cell tropism of *Staphylococcus aureus* in bovine mammary gland cell cultures. *Veterinary Microbiology* **67**: 77–89.

Lannergård J, von EC, Sander G, Cordes T, Seggewiss J, Peters G, Proctor RA, Becker K and Hughes D (2008). Identification of the genetic basis for clinical menadione-auxotrophic small-colony variant isolates of *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **52**: 4017–4022.

Lattar SM, Tuchscherr LP, Caccuri RL, Centron D, Becker K, Alonso CA, Barberis C, Miranda G, Buzzola FR, von EC and Sordelli DO (2009). Capsule expression and genotypic differences among *Staphylococcus aureus* isolates from patients with chronic or acute osteomyelitis. *Infection and Immunity* **77**: 1968–1975.

Lebeau C, Vandenesch F, Greenland T, Novick RP and Etienne J (1994). Coagulase expression in *Staphylococcus aureus* is positively and negatively modulated by an *agr*-dependent mechanism. *Journal of Bacteriology* **176**: 5534–5536.

Lee JH (2003). Methicillin (Oxacillin)-resistant *Staphylococcus aureus* strains isolated from major food animals and their potential transmission to humans. *Applied and Environmental Microbiology* **69**: 6489–6494.

Lindsay JA and Holden MA (2006). Understanding the rise of the superbug: investigation of the evolution and genomic variation of *Staphylococcus aureus*. *Functional and Integrative Genomics* **6**: 186–201.

Lowy FD, Fant J, Higgins LL, Ogawa SK and Hatcher VB (1988). *Staphylococcus aureus*–human endothelial cell interactions. *Journal of Ultrastructure and Molecular Structure Research* **98**: 137–146.

Luzio JP, Pryor PR and Bright NA (2007). Lysosomes: fusion and function. *Nature Reviews Molecular Cell Biology* **8**: 622–632.

Massey RC, Buckling A and Peacock SJ (2001). Phenotypic switching of antibiotic resistance circumvents permanent costs in *Staphylococcus aureus*. *Current Biology* **11**: 1810–1814.

McNamara PJ and Proctor RA (2000). *Staphylococcus aureus* small colony variants, electron transport and persistent infections. *International Journal of Antimicrobial Agents* **14**: 117–122.

Menzies BE (2003). The role of fibronectin binding proteins in the pathogenesis of *Staphylococcus aureus* infections. *Current Opinion in Infectious Diseases* **16**: 225–229.

Menzies BE and Kourteva I (1998). Internalization of *Staphylococcus aureus* by endothelial cells induces apoptosis. *Infection and Immunity* **66**: 5994–5998.

Miller MH, Edberg SC, Mandel LJ, Behar CF and Steigbigel NH (1980). Gentamicin uptake in wild-type and aminoglycoside-resistant small-colony mutants of *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **18**: 722–729.

Mitchell G, Lamontagne CA, Brouillette E, Grondin G, Talbot BG, Grandbois M and 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. *Molecular Microbiology* **70**: 1540–1555.

Moisan H, Brouillette E, Jacob CL, Langlois-Begin P, Michaud S and Malouin F (2006). Transcription of virulence factors in *Staphylococcus aureus* small-colony variants isolated from cystic fibrosis patients is influenced by SigB. *Journal of Bacteriology* **188**: 64–76.

Monecke S, Kuhnert P, Hotzel H, Slickers P and Ehricht R (2007). Microarray based study on virulence-associated genes and resistance determinants of *Staphylococcus aureus* isolates from cattle. *Veterinary Microbiology* **125**: 128–140.

Moon JS, Lee AR, Kang HM, Lee ES, Joo YS, Park YH, Kim MN and Koo HC (2007). Antibiogram and coagulase diversity in staphylococcal enterotoxin-producing *Staphylococcus aureus* from bovine mastitis. *Journal of Dairy Science* **90**: 1716–1724.

Moreillon P, Entenza JM, Francioli P, McDevitt D, Foster TJ, Francois P and Vaudaux P (1995). Role of *Staphylococcus aureus* coagulase and clumping factor in pathogenesis of experimental endocarditis. *Infection and Immunity* **63**: 4738–4743.

Morgan M (2008). Methicillin-resistant *Staphylococcus aureus* and animals: zoonosis or humanosis? *Journal of Antimicrobial Chemotherapy* **62**: 1181–1187.

Murai M, Sakurada J, Seki K, Shinji H, Hirota Y and Masuda S (1999). Apoptosis observed in BALB/3T3 cells having ingested *Staphylococcus aureus*. *Microbiology and Immunology* **43**: 653–661.

Musher DM, Baughn RE, Templeton GB and Minuth JN (1977). Emergence of variant forms of *Staphylococcus aureus* after exposure to gentamicin and infectivity of the variants in experimental animals. *Journal of Infectious Diseases* **136**: 360–369.

Opdebeeck JP, Frost AJ and O'Boyle D (1988). Adhesion of *Staphylococcus aureus* and *Escherichia coli* to bovine udder epithelial cells. *Veterinary Microbiology* **16**: 77–86.

Phonimdaeng P, O'Reilly M, Nowlan P, Bramley AJ and Foster TJ (1990). The coagulase of *Staphylococcus aureus* 8325–4. Sequence analysis and virulence of site-specific coagulase-deficient mutants. *Molecular Microbiology* **4**: 393–404.

Proctor RA, Balwit JM and Vesga O (1994). Variant subpopulations of *Staphylococcus aureus* as cause of persistent and recurrent infections. *Infectious Agents and Disease* **3**: 302–312.

Proctor RA and Peters G (1998). Small colony variants in staphylococcal infections: diagnostic and therapeutic implications. *Clinical Infectious Diseases* **27**: 419–422.

Proctor RA, van LP, Kristjansson M, Maslow JN and Arbeit RD (1995). Persistent and relapsing infections associated with small-colony variants of *Staphylococcus aureus*. *Clinical Infectious Diseases* **20**: 95–102.

Proctor RA, Kahl B, von EC, Vaudaux PE, Lew DP and Peters G (1998). Staphylococcal small colony variants have novel mechanisms for antibiotic resistance. *Clinical Infectious Diseases* **27** (suppl. 1): S68–S74.

Proctor RA, von Eiff C, Kahl BC, Becker K, McNamara P, Herrmann M and Peters G (2006). Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nature Reviews Microbiology* **4**: 295–305.

Sadowska B, Bonar A, von Eiff C, Proctor RA, Chmiela M, Rudnicka W and Rozalska B (2002). Characteristics of *Staphylococcus aureus*, isolated from airways of cystic fibrosis patients, and their small colony variants. *FEMS Immunology and Medical Microbiology* **32**: 191–197.

Schaaff F, Bierbaum G, Baumert N, Bartmann N and Sahl HG (2003). Mutations are involved in emergence of aminoglycoside-induced small colony variants of *Staphylococcus aureus*. *International Journal of Medical Microbiology* **293**: 427–435.

Schröder A, Kland R, Peschel A, von Eiff C and Aepfelbacher M (2006). Live cell imaging of phagosome maturation in *Staphylococcus aureus* infected human endothelial cells: small colony variants are able to survive in lysosomes. *Medical Microbiology and Immunology* **195**: 185–194.

Seggewiss J, Becker K, Kotte O, Eisenacher M, Yazdi MR, Fischer A, McNamara P, Al LN, Proctor R, Peters G, Heinemann M and 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. *Journal of Bacteriology* **188**: 7765–7777.

Seifert H, von Eiff C and Fatkenheuer G (1999). Fatal case due to methicillin-resistant *Staphylococcus aureus* small colony variants in an AIDS patient. *Emerging Infectious Diseases* **5**: 450–453.

Sendi P and Proctor RA (2009). *Staphylococcus aureus* as an intracellular pathogen: the role of small colony variants. *Trends in Microbiology* **17**: 54–58.

Senn MM, Bischoff M, von Eiff C and Berger-Bachi B (2005). sigmaB activity in a *Staphylococcus aureus hemB* mutant. *Journal of Bacteriology* **187**: 7397–7406.

Sifri CD, Baresch-Bernal A, Calderwood SB and von Eiff C (2006). Virulence of *Staphylococcus aureus* small colony variants in the *Caenorhabditis elegans* infection model. *Infection and Immunity* **74**: 1091–1096.

Sinha B, Francois PP, Nusse O, Foti M, Hartford OM, Vaudaux P, Foster TJ, Lew DP, Herrmann M and Krause KH (1999).

Fibronectin-binding protein acts as *Staphylococcus aureus* invasion via fibronectin bridging to integrin alpha5beta1. *Cellular Microbiology* **1**: 101–117.

Sinha B and Fraunholz M (2010). *Staphylococcus aureus* host cell invasion and post-invasion events. *International Journal of Medical Microbiology* **300**: 170–175.

Sinha B and Herrmann M (2005). Mechanism and consequences of invasion of endothelial cells by *Staphylococcus aureus*. *Thrombosis and Haemostasis* **94**: 266–277.

Smagur J, Guzik K, Magiera L, Bzowska M, Gruca M, Thogersen IB, Enghild JJ and Potempa J (2009). A new pathway of staphylococcal pathogenesis: apoptosis-like death induced by Staphopain B in human neutrophils and monocytes. *Journal of Innate Immunity* **1**: 98–108.

Sompolinsky D, Cohen M and Ziv G (1974). Epidemiological and biochemical studies on thiamine-less dwarf-colony variants of *Staphylococcus aureus* as etiological agents of bovine mastitis. *Infection and Immunity* **9**: 217–228.

Sordillo LM, Doymaz MZ and Oliver SP (1989). Morphological study of chronic *Staphylococcus aureus* mastitis in the lactating bovine mammary gland. *Research in Veterinary Science* **47**: 247–252.

Sutra L and Poutrel B (1994). Virulence factors involved in the pathogenesis of bovine intramammary infections due to *Staphylococcus aureus*. *Journal of Medical Microbiology* **40**: 79–89.

Swingle EL (1935). Studies on small colony variants of *Staphylococcus aureus*. *Journal of Bacteriology* **29**: 467–489.

Tollersrud T, Kampen AH and Kenny K (2006). *Staphylococcus aureus* enterotoxin D is secreted in milk and stimulates specific antibody responses in cows in the course of experimental intramammary infection. *Infection and Immunity* **74**: 3507–3512.

Vanderhaeghen W, Cerpentier T, Adriaensen C, Vicca J, Hermans K and Butaye P (2010). Methicillin-resistant *Staphylococcus aureus* (MRSA) ST398 associated with clinical and subclinical mastitis in Belgian cows. *Veterinary Microbiology* **144**: 166–171.

Vann JM and Proctor RA (1987). Ingestion of *Staphylococcus aureus* by bovine endothelial cells results in time- and inoculum-dependent damage to endothelial cell monolayers. *Infection and Immunity* **55**: 2155–2163.

Vaudaux P, Francois P, Bisognano C, Kelley WL, Lew DP, Schrenzel J, Proctor RA, McNamara PJ, Peters G and von Eiff C (2002). Increased expression of clumping factor and fibronectin-binding proteins by *hemB* mutants of *Staphylococcus aureus* expressing small colony variant phenotypes. *Infection and Immunity* **70**: 5428–5437.

Vesga O, Groeschel MC, Otten MF, Brar DW, Vann JM and Proctor RA (1996). *Staphylococcus aureus* small colony variants are induced by the endothelial cell intracellular milieu. *Journal of Infectious Diseases* **173**: 739–742.

Vintov J, Aarestrup FM, Zinn CE and Olsen JE (2003). Association between phage types and antimicrobial resistance among bovine *Staphylococcus aureus* from 10 countries. *Veterinary Microbiology* **95**: 133–147.

von Eiff C (2008). *Staphylococcus aureus* small colony variants: a challenge to microbiologists and clinicians. *International Journal of Antimicrobial Agents* **31**: 507–510.

von Eiff C, Heilmann C, Proctor RA, Woltz C, Peters G and Gotz F (1997). A site-directed *Staphylococcus aureus hemB* mutant is a small-colony variant which persists intracellularly. *Journal of Bacteriology* **179**: 4706–4712.

von Eiff C, Proctor RA and Peters G (2000). Small colony variants of Staphylococci: a link to persistent infections. *Berliner und Munchener Tierarztliche Wochenschrift* **113**: 321–325.

von Eiff C, Peters G and Becker K (2006). The small colony variant (SCV) concept – the role of staphylococcal SCVs in persistent infections. *Injury* **37** (suppl. 2): S26–S33.

Voyich JM, Braughton KR, Sturdevant DE, Whitney AR, Said-Salim B, Porcella SF, Long RD, Dorward DW, Gardner DJ, Kreiswirth BN, Musser JM and DeLeo FR (2005). Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. *Journal of Immunology* **175**: 3907–3919.

Wanasinghe DD (1981). Adherence as a prerequisite for infection of the bovine mammary gland by bacteria. *Acta Veterinaria Scandinavia* **22**: 109–117.

Weidenmaier C and Peschel A (2008). Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. *Nature Reviews Microbiology* **6**: 276–287.

Werckenthin C, Cardoso M, Martel JL and Schwarz S (2001). Antimicrobial resistance in staphylococci from animals with particular reference to bovine *Staphylococcus aureus*, porcine *Staphylococcus hyicus*, and canine *Staphylococcus intermedius*. *Veterinary Research* **32**: 341–362.

Wesson CA, Deringer J, Liou LE, Bayles KW, Bohach GA and Trumble WR (2000). Apoptosis induced by *Staphylococcus aureus* in epithelial cells utilizes a mechanism involving caspases 8 and 3. *Infection and Immunity* **68**: 2998–3001.

Wise RI and Spink WW (1954). The influence of antibiotics on the origin of small colonies (G variants) of *Micrococcus pyogenes* var. *aureus*. *Journal of Clinical Investigation* **33**: 1611–1622.