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The Major Cold Inducible Gene of *S. aureus*, *cspB*, modulates susceptibility to antimicrobials

By

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By

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B.S. Microbiology, University of Florida, 2003

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An abstract of a dissertation submitted to the Faculty of the Graduate School of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Graduate Division of Biological and Biomedical Science
Program in Microbiology and Molecular Genetics

2010

The Major Cold Inducible Gene of *S. aureus*, *cspB*, Modulates Susceptibility to Antimicrobials

By Brea Duval

Staphylococcus aureus is a major human pathogen causing a varied group of diseases ranging in severity from mild skin to invasive, often deadly infections that can impact the function of multiple organs and systems. While part of the normal human flora, problems with antibiotic resistant strains have been ever increasing and the arsenal of effective drugs available for treatment has been steadily dwindling. *S. aureus* is able to cause such diverse diseases because of its ability to survive a variety of environments, which would subject the bacteria to changes in temperature, pH, ionic strength and mediators of innate immunity. Previous work from our laboratory defined the role of the major cold shock protein of *S. aureus*, CspA, in the bacteria's response to cold stress as well as its role in resistance to antimicrobial peptides. Two other members of the *cspA* family cold shock protein-encoding genes, *cspB* and *cspC*, were found in the genome of strain COL, a methicillin resistant clinical isolate.

Previous microarray analysis identified *cspB* as the major cold-inducible gene in *S. aureus*. Therefore, the aim of this work was to define the role of *cspB* found in strain COL in antibiotic resistance and cold stress. In addition to having an important role under cold-shock conditions, CspB seems to have important functions under normal (37 °C) growth conditions. We found that insertional inactivation of *cspB* resulted in dramatic phenotypic changes that resembled those previously ascribed to staphylococcal small colony variants (SCVs). These changes include loss of pigment, slow growth, and an increase in resistance to several clinically relevant antimicrobials. Interestingly, while complementation of a *cspB* insertional mutant showed restoration of all of these phenotypic changes to wild type levels, the two notable exceptions were lack of complete complementation of biofilm forming capacity and loss of resistance to methicillin. This loss of methicillin resistance was shown to be due to loss of the type I SCC*mec* cassette. Taken together, this data suggests that *cspB* plays important role in many aspects of staphylococcal pathogenesis including suggesting a novel role for this stress response protein in involvement in the SCV phenotype.

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Dedication

This is for Louise and Nellie. Both of you put taking care of me before taking care of yourselves. Thanks for picking cotton before you even knew who I was so that I could get to where I am. I love you both. Nellie, I miss you so much. It still hurts to know you aren't around, but I know you're watching me all the time. I love you.

Acknowledgements

No one ever accomplishes a feat like this alone. You might think you're the one getting the degree, but for me there were lots and lots of people emotionally invested in my successful completion of this journey. I was lucky to have had some pretty good traveling partners.

I'd like to thank Bill for allowing me to conduct this work in his laboratory over the past few years. I have learned so much as a member of his lab and I know I'll take these lessons with me throughout the rest of my life. Thank you for all of your support and guidance over the years. To the rest of the Shafer lab, thank you for your support and for making my time in lab so memorable.

To my committee, thank you for your advice, support, and guidance. They are truly appreciated.

I want to thank my Mom and Dad. I could not have asked for better parents. They have been overwhelmingly supportive of me all of my life. I blame them for getting me my first chemistry set (and many more chemistry sets after that) and always encouraging me to set goals and pursue my dreams. Thank you for putting up with all of the really strange things I did when I was little. You obviously knew something I did not. I love you so very much.

To my brother Charles, thanks for being a great brother. I'm so thankful for the many conversations on the Marvel Universe. I've learned a lot from you in a bunch of ways, and I look forward to finally having the time to spend more time with you and Jasmyne, and go to Wizard World one year.

To my aunt, Anne Simpson, thank you for telling people I was working with Nobel Prize winners even before I got to graduate school. Just the fact that you believe that tells me how much you have always believed in me. I'm so blessed to be your niece. To Jordana, thank you for always being there to listen and for trying to show me how much more I am than what I thought. You and Tilda have made such a huge difference these past few years. Thanks.

To the rest of my family, I know I wasn't always around when I should have been, but that all is about to change. I'm so glad that God has blessed me with the family he's blessed me with. I've really lucked out.

To Kelly, everyone needs an archenemy and you have been a spectacular one. You're almost a better friend. Almost. Thank you for our many conversations on the major failures of the Universe, our favorite topic. I'm sure we'll have way more time to discuss that now. Super excited.

To Natassia, from our first conversation where I explained to you what lycanthropy was, I knew you were going to be a special and enduring presence in my life even if you did laugh in my face and look at me like I was crazy. You are always there to keep things real and Lord knows I need that. Remember me when you are living your fabulous, jetsetting life.

To Crystal, you have truly been a blessing. Thank you for all of your input, support, and friendship throughout all of this. I am so glad we found each other to lean on. You'll be writing this same thing soon.

Finally, for the most epic personality I have ever encountered. Nellie, I love you so much and I miss you every second of every day. You have no idea how much you mean to me. I'm so much of a better person for knowing you.

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I. *Staphylococcus aureus*

A. Background

The genus *Staphylococcus* contains over twenty species of Gram-positive bacteria. While two species in particular are adept at causing disease in humans and animals, it is *S. aureus* that has emerged in recent years as one of the most serious bacterial public health pathogens faced worldwide. With an increase in multidrug-resistant strains in both community and hospital settings, it is ironic to think that this bacteria was first described around the time that Joseph Lister began to transform surgery from a "hazardous lottery into a safe and soundly based science" by advocating the use of antiseptic surgical procedures.

Staphylococci were first described by Alexander Ogston in 1880. Ogston, a Scottish surgeon and admirer of Lister's work, was interested in finding the cause of suppuration in post-surgical wounds. Because of poor antiseptic techniques, death from post-operative wounds which we now understand to be due to sepsis, was common. Ogston, through a series of simple yet brilliant experiments was able to show that the "micrococci," as he described them, found in the pus from these wounds was able to cause similar disease and death in laboratory animals. Ogston described two types of micrococci from pus, ones that grew in long chains (which had been previously described as streptococci), and ones that grew in grape-like clusters (208). In 1882, Ogston named the clustered micrococci staphylococci, from the Greek *staphyle* meaning "bunch of grapes." Three years later in 1884, the German scientist, Anton J. Rosenbach, described two different types of these staphylococci that he was able to isolate in pure culture.

Based on their difference in appearance he named the two species *Staphylococcus albus* (now *Staphylococcus epidermidis*), from the Latin "albus" for white, and *Staphylococcus aureus*, from the Latin "aurum" for gold (243).

More than 100 years after its discovery, the significance of *S. aureus* in clinical infections has increased tremendously. Before the introduction and widespread clinical usage of ß-lactam antibiotics in 1940, invasive staphylococcal infections were often fatal (267). While the introduction of antibiotics greatly reduced these deaths, by the late 1940s, the numbers of penicillin-resistant strains outnumbered those that were penicillin-sensitive in hospitals (19). By 2005, there were ~94,360 invasive methicillin-resistant *S. aureus* (MRSA) infections annually with an incidence rate of 31.8 cases per 100,000 and a mortality rate of 6.3 per 100,000, and that figure is expected to increase (151). The problem of MRSA infections is no longer limited to hospital settings with the community-acquired (CA) MRSA strains causing sometimes fatal infections in otherwise healthy individuals. This is a dramatic shift from roughly 10 years ago when most staphylococcal infections in the community were caused by methicillin-susceptible *S. aureus* (MSSA) rather than MRSA strains (189).

S. aureus seems to be supremely suited to being a human pathogen. It is readily passed from individual to individual through hand-to-hand contact, which complicates treatment in health care settings where non-symptomatic health care workers, despite following proper protocols, can pass the bacteria to patients (311). MRSA can also survive for weeks at a time on surfaces such as fabrics and plastics, further aiding in its ability to spread (199). The diverse array of virulence factors it possesses as well as its

ability to survive inside and outside of its host ensure that *S. aureus* will remain a challenge for health care professionals for years to come.

B. *S. aureus* Methods of Immune Evasion

S. aureus is a Gram-positive, cluster-forming coccus capable of inhabiting most areas of the human body. From 1997 to 1999 *S. aureus* was the most reported cause of blood stream, skin and soft tissue, and lower respiratory tract infections in the United States (64). Its remarkable ability to adapt to and evade the host's immune response is coordinated by an astounding array of virulence factors, each targeting some aspect of the host's innate immune defenses. These methods include inhibition of opsonization and phagocytosis, inhibition of chemotaxis of immune cells to infection sites, factors that enhance resistance to host antimicrobial peptides, factors that cause an increase in resistance to reactive oxygen species (ROS), and lysis of immune cells (FIG. 1).

i. Inhibition of chemotaxis of immune cells.

Recruitment of immune cells to sites of infection is an essential first step in fighting infection in the host. The process of immune cell recruitment is controlled by chemokines, which are cytokines that are specifically involved in this chemotactic recruitment (178). Production of interleukin-8 (IL-8), a chemokine responsible for neutrophil recruitment, has been shown to be induced by *S. aureus* lipotechoic acid (LTA) and capsular polysaccharide in peripheral blood monocytes (276). Many *S. aureus* virulence factors act as superantigens (156) that promote the secretion of IL-8 in human monocytes, such as toxic shock syndrome toxin-1 (TSST1), enterotoxin A (SEA) and enterotoxin B (SEB) (157). Other important chemoattractants required for neutrophil

recruitment include platelet-activating factor, leukotriene B4, and members of the complement pathway C3a and C5a (62, 241).

Recently, de Haas et al. described a protein secreted by *S. aureus* that specifically targets the response of monocytes and neutrophils to C5a and formylated peptides (58). This group was able to demonstrate that the chemotaxis inhibitory protein of *S. aureus* (CHIPS), greatly reduced C5a mediated neutrophil recruitment in a mouse peritonitis model (FIG.1). The group also showed that this 14.1 kD protein is phage encoded and was present in more than 60% of clinical isolates.

ii. Inhibition of opsonization and phagocytosis

The complement pathway is an integral part of the host innate immune system and is composed of over 30 soluble and cell-membrane associated proteins (88). The three distinct pathways of the complement system are the classical pathway, the alternative pathway, and the lectin pathway (88). While the three pathways recognize different antagonists, the ultimate goal of each pathway is the formation of the membrane attack complex (MAC), also called the C5b-9 complex, which can lyse target cells by the formation of pores in their phospholipids bilayers (88, 190). The central component of the complement system is C3 and cleavage byC3 convertase results in the production of C3a and C3b, the latter of which is covalently attached to the microbial cell surface during a process called opsonization (88). Opsonization is important for phagocytes to efficiently recognize and engulf pathogens and this process is further aided by antibodies such as IgG (242). Recently, a gene encoded on *S. aureus* pathogenicity island 5 (SaPI5), the same pathogenicity island encoding CHIPS (242), was described as an inhibitor of this process. Of the four genes encoded on this pathogenicity island, three have been

shown to interact with the human immune system and Rooijakkers et al. have named the protein encoded by the *scn* locus staphylococcal inhibitor of complement (SCIN) (242). SCIN can inhibit phagocytosis as well as C3b deposition on bacterial membranes and while it is still unclear how SCIN inhibits all three complement pathways, it has been shown that this protein can inhibit dissociation of C3 convertases (242). Deposition of complement on the staphylococcal cell surface can be further impeded by the surface anchored clumping factor A (ClfA) which has been shown to inhibit the opsonophagocytic activity of both neutrophils and macrophages (113, 212). In addition to ClfA, *S. aureus* can secrete extracellular fibrinogen-binding protein C, which binds free C3, inhibiting its downstream opsonization functions (108).

a. *S. aureus* capsular polysaccharide

S. aureus can further evade the host innate immune response by inhibiting phagocytosis. Like most bacteria that cause invasive disease, *S. aureus* produces extracellular capsular polysaccharide. Encapsulation of *S. aureus* was first described over 80 years ago in studies using highly encapsulated strains that produced mucoid colonies. These strains resisted phagocytosis, and were more virulent in mice than non-mucoid strains (94, 152, 187, 307, 308). As capsular serotyping techniques improved, an important distinction was observed between the prototypical mucoid or heavily encapsulated strains (M and Smith diffuse), which produce type 1 and type 2 capsular polysaccharide, respectively (8), and the so called "microencapsulated" strains which produce non-mucoid colonies on solid agar surfaces (115, 166). Production of capsular polysaccharide type 1 is mediated by the *cap1* locus, which is located within a 50-kb region located on a staphylococcal cassette chromosome (SCC) element similar to that of

the SCC*mec* cassette responsible for methicillin resistance (see section II) (177) . It was shown that the microencapsulated strains seemed to predominate among clinical isolates, and produce either type 5 or type 8 capsular polysaccharide (115, 166). Serotype 5 and 8 capsular polysaccharide-producing *S. aureus* strains account for ~25% and 50%, respectively, of human clinical and commensal isolates, and have also been isolated from livestock and feed animals such as cows, poultry, pigs, and horses (8, 56, 107, 115, 166, 227, 271, 272, 285).

Serotype 1 and 2 strains have been shown to be more virulent in mice when the mice are challenged intraperitoneally than their isogenic capsular polysaccharide negative strains (165, 170) as well as spontaneous nonmucoid variants (50, 152, 187, 254, 268). This increased virulence has been attributed to the protection from effective opsonophagocytic killing by polymorphonuclear leukocytes (9, 165, 222, 223, 295, 308, 315) and the production of alpha toxin, which results in death within 24 to 48 h (50, 152). The virulence associated with capsular type 5 strains has been studied in mouse models of bacteremia (282) as well as in mouse models of renal and subcutaneous abscess formation (226). To study the role capsular polysaccharide plays in virulence, Thakker et al. used strain Reynolds, a well studied capsular polysaccharide type 5 strain and were able to demonstrate that the parent strain showed an LD_{50} that was ten-fold lower than a capsule negative mutant (282). Furthermore, the wild type capsule 5 strain was shown to be more virulent than the capsule negative mutant in models of both renal and subcutaneous abscess formation (226). It should be noted that the aforementioned experiments utilized bacteria grown under conditions used to maximally express capsule, and that bacteria grown in this manner have been shown *in vitro* to resist

opsonophagocytic killing in the presence of either complement or antibodies and strain Reynolds grown in this manner showed killing by human polymorphonuclear leukocytes only after exposure to both capsular specific antibodies and complement (282).

b. Protein A

Staphylococcal protein A is a 42 kDa protein anchored to the cell wall by covalent attachment and found in more than 90% of strains (222, 251). The *spa* locus, which encodes protein A, is widely used to type and distinguish among staphylococcal strains due to the polymorphism of region X (82). Protein A contains either four or five regions that can all bind the Fc fragment of immunoglobulin G (IgG) (77, 288). The binding of the Fc fragment of IgG presents the F(ab')2 fragments in an incorrect orientation on the bacterial cell surface, preventing the antibody from being recognized by Fc receptors on the neutrophil cell surface (78). It has been further shown that protein A- deficient mutants are phagocytosed more efficiently than their wild type counterparts *in vitro* (89) and that protein A mutants are less virulent in several different mouse infection models (211, 216).

iii. Resistance to host antimicrobials

Upon successful engulfment of *S. aureus* by human neutrophils, the bacteria has at its disposal a significant arsenal of virulence factors all aimed at preventing killing by host antimicrobials within the phagosome. Antimicrobial peptides (AMPs) are small, generally positively charged amphipathic molecules that disrupt bacterial membranes (233). AMPs can be found contained within the granules of neutrophils where they can be used to kill extracellular bacteria or released into phagosomes to kill phagocytosed

pathogens (233). They are also synthesized by epithelial cells and secreted to the extracellular fluid during inflammation.

The cathelicidins are a family of antimicrobial peptides highly conserved in all mammals (321). The only member of this family of AMPs found in humans is CAP-18 (or FALL-39) (33). CAP-18, like other cathelicidins, is processed by serine proteases which cleave the C-terminal domain into a 37 amino acid peptide called LL-37 (273). LL-37 has been shown to be a potent anti-staphylococcal AMP (284, 286) and mice lacking the murine homolog to LL-37, CRAMP, have been shown to be more susceptible to invasive Group A streptococcal infection (204). Aureolysin, an extracellular metalloprotease produced by *S. aureus* has been shown to cleave LL-37 inactivating it and this activity is thought to contribute to staphylococcal resistance to LL-37 *in vitro* (262).

Another strategy *S. aureus* uses to avoid killing by AMPs is by decreasing the net negative charge of the cell surface. The *dltABCD* operon has been identified in *S. aureus* and *S. xylosus* and inactivation of the operon in both strains has been linked to an increase in susceptibility to several different AMPs, including defensins and protegrins (221). This operon causes resistance to AMPs by adding D-alanines to cell wall teichoic acids, thereby contributing to an increase to the net positive surface charge and decreasing the affinity for AMPs for the bacterial cell surface (306). It has been also shown that enhanced expression of the *dlt* operon contributes to *S. aureus* resistance to daptomycin (318), an anionic lipopeptide whose activity depends on the presence of calcium ions (39, 48, 59) and is widely used as an antistaphylococcal antibiotic. Furthermore, mutations in *mprF*, which encodes an enzyme that adds lysines to

phosphatidylglycerols on the bacterial membrane, have also been shown to be associated with an increase in AMP and daptomycin resistance (319).

The chromosome of *S. aureus* encodes several other genes involved in modifying peptidoglycan to increase its resistance to host antimicrobial peptides. Lysozyme is an important protein involved innate immunity that is found in a variety of bodily secretions such as breast milk, tears, and saliva (128). Lysozyme is also found in immune cells such as neutrophils, monocytes, and macrophages (128) where it is used to lyse invading bacteria by cleaving the ß-1,4 glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in peptidoglycan (225, 250). Lysozyme is important in innate immunity for its broad spectrum activity against many invasive bacterial pathogens; however *S. aureus* is completely resistant to killing by lysozyme. A recent investigation has attributed this resistance to lysozyme mediated killing in staphylococci to *O*-acetylation of the C-6 in *N*-acetylmuramic acid, which is mediated by *O*-acetyltransferase A (OatA) (23). Interestingly, *oatA* is only found in pathogenic species of staphylococci such as *S. aureus* and *S. epidermidis,* which are generally resistant to lysozyme, but non-pathogenic species lacking *oatA* are sensitive to lysozyme mediated killing (23).

Staphylokinase is encoded on the same pathogenicity island, SaPI5, as SCIN and CHIPS, whose roles in the inhibition of phagocytosis and neutrophil chemotaxis respectively have been previously discussed (See B.i. and B.ii.). Staphylokinase is encoded by the *sak* gene and binds human α-defensins inhibiting their bactericidal properties (32, 127). In addition to aiding in resistance to antimicrobial peptides, staphylokinase has also been shown to activate plasmin, which causes local proteolysis and can aid the bacteria in entering into host cells (215, 246).

iv. Resistance to reactive oxygen species

S. aureus has a characteristic hue, which is used to distinguish it from other staphylococci. As previously mentioned, Anton J. Rosenbach named *S. aureus* after the Latin "aurum" meaning "gold" based on its appearance. The hallmark golden pigment of *S. aureus* is called staphyloxanthin, and is actually an important part of the organism's defense against oxygen-dependent killing by immune cells. Staphyloxanthin is a C_{30} triterpenoid carotenoid, which is synthesized in a complex and multi-step process by the *crtOPQMN* operon (10, 220). Carotenoid pigments in other organisms have been shown to be important antioxidants for their ability to react to and quench reactive oxygen species (160). Because of its structural similarity to other well studied carotenoids such as ß-carotene, Liu et al. hypothesized that staphyloxanthin could be used by *S. aureus* to evade killing by the oxygen-dependent arm of the host's immune system (173). They were able to demonstrate that mutants in *crtM*, which encodes an essential dehydrosqualene synthase responsible for the production of staphyloxanthin, were less able to survive in whole human or mouse blood and were also more susceptible to killing by purified human neutrophils (173). Furthermore, *S. aureus crtM* mutants were less virulent in a mouse subcutaneous abscess model of infection than their wild type counterparts (173). Perhaps most interesting from their study, when the *crtMNOPQ* operon is expressed *in trans* in nonpigmented *Streptococcus pyogenes*, the resistance of these cells to neutrophil-killing also increases (173). From these data, it is clear that staphyloxanthin plays an important role in *S. aureus*' ability to evade the host immune response.

Staphyloxanthin is not the only molecule that aids *S. aureus* in resisting oxygen-dependent killing by immune cells. *S. aureus* also produces extracellular catalase, encoded by *katA,* which has been shown to be a virulence factor responsible for increased protection from neutrophil killing (181). The presence of catalase in *S. aureus* has also been shown to give the bacteria a competitive advantage over other colonizers of the nasal mucosa against species that produce hydrogen peroxide such as *Streptococcus pneumoniae* (53).

Three ferric uptake regulator homologues are found in the genome of *S. aureus*: *fur*, *perR*, and *zur*. In *S. pyogenes*, *perR* has been shown to be induced in response to peroxide stress (148). Horsburgh et al. recently determined several genes in the PerR regulon, and found genes that were involved in oxidative stress and iron storage (116). Furthermore, they were able to show that a *perR* mutant was less virulent in a murine abscess model than its wild type parent strain. Genes under the transcriptional control of *perR* include *katA*, alkyl hydroperoxide reductase (*ahpCF*), bacterioferritin comigratory protein (*bcp*), thioredoxin reductase (*trxB*), ferritin (*ftn*), and *mgrA*, a ferritin-like Dps homologue (116). Both iron storage and resistance to oxidative stress are important to pathogenicity. It has been suggested that the ability to both sense peroxide and also regulate iron storage contributes to the ability of *S. aureus* to survive intracellularly in granulocytes (71, 203, 323), which has been determined to contribute to infection (103).

v. Lysis of host cells

S. aureus produces several different cytotoxic molecules that act both on immune cells and non-immune cells. Lysis of host cells has been determined to be an important

contributor to staphylococcal virulence and the roles of the staphylococcal hemolysins (α-,β-,γ-, and δ-hemolysin) and the leukocidins will be discussed below.

a. Staphylococcal hemolysins

1. α-hemolysin

S. aureus α-hemolysin is perhaps the most closely studied staphylococcal cytotoxin and its role in disease has been well characterized. α-hemolysin (*hla*) was first cloned in 1984 (102) and has since been identified in most clinical *S. aureus* isolates. The mature protein is dermo- and neuronecrotic in addition to being hemolytic and is secreted as a 33.2 kDa water soluble monomer that is capable of self-assembly on the surface of target cells (28, 99, 169). Once the protein localizes to the membrane of a target cell, the monomers insert themselves into the cell membrane where they self-assemble into a heptamer (99, 289). These heptamers form mushroom shaped 1- to 2-nm pore-like channels in the lipid bilayer of the target cell causing cell lysis (99). Lysis of target cells occurs through the efflux of K^+ and the influx of Na^+, Ca^+, and small molecules with molecular weights of less than 1,000 Daltons, which results in rapid swelling and finally lysis (292). Interestingly, the specificity of the pores formed by α-hemolysin does not seem to be uniform across all cell types susceptible to lysis by α-hemolysin. For example, pores found in the membranes of keratinocytes and lymphocytes are much smaller, allowing only for the passage of monovalent ions (129, 303).

Sensitivity to α-hemolysin mediated lysis differs greatly based on cell type and species. Binding of α-hemolysin to human erythrocytes has been shown to not be mediated by any specific receptor (114). Accordingly, large amounts, approximately 1μM, are required for lysis of these cells (114). In contrast, rabbit erythrocytes are readily

lysed by α-hemolysin requiring 1,000 times less protein (1 nM) to cause lysis (26, 29). Although a receptor has yet to be identified, it is believed this increased sensitivity to α-hemolysin is due to the presence of a specific receptor for the toxin (114).

2. β-hemolysin

β-hemolysin or sphingomyelinase C, is encoded by *hlb* which was first cloned by Projan et al. (231) and Coleman et al. (51). *hlb* produces a secreted exotoxin with a molecular weight of 35 kDa (51, 231) that is produced in large quantities by a number of animal isolates (25). However, it is not dermonecrotic in guinea pigs and does cause death in mice (97). However, the high expression of β-hemolysin in animals could give strains producing the toxin a competitive advantage over toxin-negative strains. This hypothesis is supported by the decreased survival of an *hlb* negative strain compared to its wild type parent strain in murine mammary glands (67).

In 1935 Glenny and Stevens showed that β-hemolysin was significantly more hemolytic for sheep erythrocytes, but demonstrated little hemolytic activity against rabbit erythrocytes (97). Furthermore, it has been shown that this toxin has phospholipase *c*-like activity (68) and that this activity is specific for sphingomyelin and lyso-phosphatidylcholine (69). The difference in susceptibility to erythrocytes isolated from different species might be explained by the difference in content of sphingomyelin in each blood cells isolated from each species (69).

3. δ-hemolysin

The delineation between coagulase-negative and coagulase-positive staphylococci is an important factor as it relates to disease caused by these organisms. While coagulase-negative staphylococci (CoNS) (i.e. *S. epidermidis*, *S. saprophyticus*) lack many of the

virulence factors found in *S. aureus*, they are nevertheless capable of causing disease in humans and other animals. It has been shown, however, that between 50 and 70% of CoNS produce the exotoxins α-, β-, and δ-hemolysin (1). Kleck and Donahue were able to isolate δ-hemolysin from *S. epidermidis* (150) and it has been shown that over 97% of *S. aureus* strains produce this toxin (67).

In *S. aureus*, δ-toxin is part of the accessory gene regulator (*agr*) locus, one of the master regulatory networks in *S. aureus*, which controls the expression of many virulence factors such as α-hemolysin. It is encoded by *hld*, which is divergently transcribed from the other transcript of the locus, *agrBDCA* (296). *agrBDCA* encodes the RNA II transcript and *hld* encodes δ-hemolysin, or RNA III, which is the effector molecule for the *agr* system.

The peptide encoded by RNAIII, δ-hemolysin, is 26 amino acids long with a molecular weight of 2.9 kDa (140). δ-hemolysin can lyse erythrocytes from a variety of mammalian species such as humans, horses, rabbits, sheep and goat (158) and can also disrupt membrane-bound organelles, spheroplasts and protoplasts (81). Its role in disease, however, is less clear. Mellor et al. have shown that six individual proteins of δ-hemolysin can aggregate to form cation-selective channels in the membrane of target cells which leads to cell lysis (186). However, there is evidence that the action of δ-hemolysin on cell membranes is different depending on the concentration of the peptide in the medium. At low concentrations, below a threshold, it is hypothesized that the peptide adsorbs to the membrane, transiently crossing and weakening it by inducing strain caused by membrane curvature (187). However, when the peptide is working at a concentration above the threshold, δ-hemolysin acts similarly to detergents such as

Triton-X 100, solubilizing the membrane resulting in micelle formation and leading finally to cell lysis (22, 283). From these observations, it is clear that δ-hemolysin is extremely important in innate immune evasion and coordinating other aspects of staphylococcal virulence.

b. γ-hemolysin and the staphylococcal leukocidins

Of the six secreted cytotoxins produced by *S. aureus*, three are composed of two separate water soluble protein subunits named on the basis of the speed of their elution on an ion-exchange column. These components are S (for slow-eluting protein) and F (for fast-eluting protein) (312, 313). The cytotoxins discussed in this section are γ-hemolysin (Hlg), and Panton-Valentin leukocidin (PVL). While γ-hemolysin is considered to be a hemolysin, it will be discussed below with the staphylococcal leukodicin PVL due to its bicomponent structure.

1. γ-hemolysin

γ-hemolysin was first described in 1938 by Smith and Price (269). The genes for γ-hemolysin are transcribed from the *hlg* locus which consists of *hlgA*, *hlgC* and *hlgB* (52, 54, 139). Both *hlgC* and *hlgB* are transcribed as a single message and *hlgA* is transcribed separately with the mature proteins having molecular weights between 32 and 34 kDa (67) .Two separate toxins are formed from the combinations of the proteins encoded by these genes: HlgA+HlgB and HlgC+HlgB. HlgA and HlgC represent the respective F proteins of the toxins while HlgB encodes the S protein (154). The name γ-hemolysin is sometimes used to refer to the other bicomponent toxins of *S. aureus* as a group due to their high degree of identity (65%) (228), but it does refer to a distinct locus found in nearly every *S. aureus* strain. γ-hemolysin can lyse erythrocytes from humans and other

mammals and the activity of its subunits in combination with other staphylococcal leukocidins will be discussed below.

2. Panton-Valentine leukocidin

The Panton-Valentine leukocidin (PVL) was first identified in 1932 by Sir Philip Noel Panton and Francis Valentine (214). The researchers were able to demonstrate that there was a correlation between strains harboring the toxin and an increased severity of skin infections which were often fatal (214). The PVL locus was sequenced and cloned by Prevost et al. (228) and is encoded by *lukS-PV* and *lukF-P.* This is a phage encoded gene present in roughly 2% of *S. aureus* strains investigated (228). The role of PVL in the epidemiology of CA-MRSA will be discussed in a later section (see section II).

Interest in PVL has increased in recent years due to its prevalence in MRSA strains implicated in causing fatal necrotizing pneumonias in healthy individuals (95); however, PVL has a varied effect on immune cells. PVL shows a high specificity for granulocytes such as neutrophils; however, it can also lyse macrophages from human and non-human species (279). At lower concentrations, PVL can induce apoptosis in neutrophils, but high concentrations lead to rapid cell lysis (90). It has also been shown that upon challenge with PVL, neutrophils immediately degranulate releasing leukotriene B4 and IL-8 (155), which are important in the inflammatory response and it is believed that this increase in pro-inflammatory cytokines by the host actually aids in PVL pathogenesis by recruiting and activating neutrophils.

Recent work has demonstrated conflicting evidence for the role of PVL in staphylococcal virulence. Labandeira-Rey et al., while investigating the role of PVL in a mouse necrotizing pneumonia model, suggested that PVL may act as a global regulator in strains

USA300 and USA400 (163). The authors specifically investigated the role that PVL might play on *spa* expression by introducing PVL into mutants containing deletions in *sarS*, a master global regulator of many staphylococcal virulence factors, which also regulates the expression of *spa*. The authors demonstrated that production of Spa was abolished in the *sarS* deletion mutants expressing PVL when the presence of the protein was assayed using Western blot analysis. However, Diep et al. tried to confirm this observation by proteomic and microarray analysis and found that PVL had no impact on gene expression in USA300 or USA400 strains (66). Furthermore, Labandeira-Rey et al. suggested that PVL decreased survival of mice in pneumonia model of infection (163). They suggested that a *pvl* deletion mutant was less virulent than the isogenic wild type parent strain. This observation was also refuted and it has been shown that a *pvl* deletion mutant is no less virulent than its isogenic wild type parent in a mouse model of necrotizing pneumonia (37) and a recently developed rabbit model studying the role of PVL in causing necrotizing pneumonia may shed new light on the mechanisms by which this cytotoxin causes such damage (65). Taken together, this data suggests that PVL is an important virulence factor against cells of the innate immune system, however its overall role in staphylococcal pathogenesis should not be overestimated.

II. Methicillin resistant *Staphylococcus aureus* (MRSA)

Before the introduction of penicillin, the mortality rate due to infections caused by *S. aureus* was greater than 80% (275) and after its introduction, penicillin was touted as a

wonder drug in the treatment of staphylococcal infections. However, by the 1960s most staphylococcal strains were resistant to penicillin. This section outlines the evolution and current impact of MRSA in hospital and community settings and also details the intricacies of chromosomally mediated resistance to β-lactam antibiotics and problems with multi-drug resistant strains.

A. The evolution of MRSA in the antibiotic era

Sir Alexander Fleming discovered penicillin quite serendipitously in 1928. In a publication Fleming was asked to edit shortly after the start of WWII in 1946 when the drug was in short supply, Fleming warned that "…penicillin is not a 'cure-all'. There are many of our most common ailments on which it has no effect. When the supply of penicillin is plentiful many patients will demand it from their doctor for degenerative nerve conditions and the like for which there is not any possibility of its doing good" (75). While the drug was shown to be extremely effective against *S. aureus* and a number of other bacteria, the first penicillin-resistant *S. aureus* were reported in hospitals and the community in 1942 (234).

The mechanism of resistance to penicillin had been first described in 1940 when plasmid produced β-lactamases present in *E. coli* were shown to be capable of hydrolyzing the β-lactam ring present in penicillin (275). In the 1950s there were pandemic *S. aureus* infections and the use of penicillin in the hospital increased dramatically. In addition to the resistance seen to penicillin and penicillin derivatives in the 1940s, by this time the first *S. aureus* isolates, which showed resistance to tetracycline, erythromycin, streptomycin, and chloramphenicol, were also seen (219).

Methicillin, a semisynthetic penicillinase-resistant penicillin derivative, was introduced in Europe in 1959 and showed immediate success against these epidemic strains. However, in 1961, the very first methicillin-resistant *Staphylococcus aureus* isolated in the UK was described in the literature (125).

B. SCC*mec*

The chromosomally mediated resistance to β-lactam antibiotics that was first seen in 1961 was mediated by a different mechanism than the plasmid mediated resistance seen in the early 1940s. The staphylococcal cassette chromosome *mec* (SCC*mec*) is responsible for this alternative resistance to β-lactam antibiotics seen in *S. aureus*. SCC*mec* carries the *mecA* gene, which encodes an alternative penicillin binding protein, PBP2a. In the presence of β-lactams, PBP2a allows cell wall synthesis to continue (85). There is much debate about exactly how *S. aureus* acquired this genetic insert. One theory is that all MRSA share common ancestry with a single clonal isolate (159), but more recent evidence seems to support the theory that SCC*mec* found its way into multiple clones that share different genetic lineages (72, 74, 194, 232).

Enright et al. used multi-locus sequence typing (MLST) to trace the evolutionary history of *S. aureus* in order to support the theory that SCC*mec* was disseminated in multiple clones, giving rise to the diversity of clones seen today (72). The authors used MLST on 359 MRSA, epidemic MRSA (EMRSA), and glycopeptide intermediately susceptible *Staphylococcus aureus* (GISA) strains and compared the results to 553 MSSA strains to identify the first MRSA clone and its MSSA ancestor. They were able to demonstrate that GISA, MRSA, and EMRSA all arose from clones with different

sequence types and that only 38 of the over 250 sequence types were shown to contain MRSA. While the authors provided a thorough description of the evolutionary relationships among MRSA clones, and suggested that an MSSA from sequence type 8 was the likely common ancestor of many MRSA isolates, the exact origins of SCC*mec* and how it found its way into the staphylococcal chromosome remain unclear. It is believed, however, that an ancestor of PBP2a originated from a similar penicillin binding protein with 87% identity found in *S. scuiri* (314).

Expression of *mecA* is under the control of *mecI*, and a signal transduction protein encoded by *mecRI* (24). In the absence of methicillin or another related β-lactam antibiotic, transcription of *mecA* is repressed by MecI, but when the drug is present MecI is cleaved by MecRI, allowing MecI to bind at the operator region of *mecA* (24). However, in some SCC*mec* types both *mecI* and *mecRI* can be non-functional due to deletions caused by the presence of insertion sequences IS*431* or IS*1272* leading to derepression of *mecA* (122).

There are currently five major SCC*mec* types that have been described in the literature and they are categorized on the basis of the allotypes of their *ccr* complexes, which encode site specific recombinases believed to be responsible for movement of the cassette, as well as on the presence or absence of other genes (120, 142, 209, 310). The five major classes are further delineated (A-E) by their *mecI-mecRI-mecA*-IS*431* or *mec* complex (120, 122, 142) and these differences are the basis for several different methods for typing strains based on their *mec/ccr* complexes using multiplex PCR (80, 210, 322). The five cassettes differ in size and composition but all integrate at the same site near the origin of replication of the staphylococcal chromosome, bacterial chromosome

attachment site (*attBSCC*) (122). *attBSCC* is located downstream of *orfX*, which is an open reading frame well conserved in most clinical *S. aureus* isolates, but has no known function (121). The structure and contents of the five major SCC*mec* types will be discussed below.

i. SCC*mec* type I

Strain NCTC 10442, which was the first described MRSA strain in the literature, harbors the SCC*mec* type I cassette (61). The SCC*mec* type I cassette contains a Class B *mec* complex (IS*1272*–Δ*mecR1*–*mecA*– IS*431*) (FIG. 2A.) which has an insertion of IS*1272* at the penicillin binding domain of *mecRI* causing derepression of *mecA* transcription. The type I cassette also contains the *pls* gene, which encodes the methicillin-resistant surface protein and is also referred to as the plasmin sensitive surface protein, which promotes adherence of MRSA to nasal epithelial cells (238). The SCC*mec* type I cassette is a mid-sized cassette (34.3 kb) and while rarely seen among reported HA-MRSA strains, it has been classically associated with strains considered to be HA-MRSA (72, 121).

ii. SCC*mec* type II

The SCC*mec* type II (FIG. 2B) cassette is 53.0 kb and contains additional antibiotic resistance genes (121). This cassette has several integrated plasmids (pUB110, pI258 and pT181) as well as a transposon (Tn*554*). Strains harboring plasmid pUB110 show resistance to most aminoglycosides such as kanamycin, bleomycin, and tobramycin and this is specifically mediated by *ant(4')*. Resistance to penicillins and mercury is encoded on plasmid pI258 (122) and pT181, which also exists as a non-integrated plasmid in strain COL, which is a type I strain, and encodes resistance to tetracycline

(122). The Tn*554* transposon is responsible for inducible resistance to macrolides, lincosamides and streptogramins (191). The SCC*mec* type II cassette also contains a class A *mec* complex and a type 2 *ccrAB* complex.

The first type II strain was identified in Japan in 1982 (N315) and quickly spread worldwide. Recent work on MRSA isolated in Japan has identified a gene named *fudoh*, important in colony spreading as defined by motility on soft agar. *S. aureus* has always been defined as a non-motile bacterium, but recent work by Kaito et al. (138) has described the ability of certain MRSA strains to spread on soft agar. The authors investigated the differences in motility between type IV strains, normally found in the community with type II strains, often associated with hospital acquired infections. Using 10 MSSA strains and 40 MRSA strains, the authors found that strains lacking an SCC*mec* cassette spread nearly twice as far as those harboring one. Furthermore, when the SCC*mec* cassette was deleted in MRSA strains that were attenuated in their ability to spread on soft agar, the strains were able to spread just as well as the MSSA strains. The authors found a region they termed *fudoh* (meaning "non-motile" in Japanese). This gene encodes a 70 amino acid protein, which was shown to inhibit colony spreading. *fudoh* has been specifically identified as being harbored on the SCC*mec* type II and III cassettes. Expression of *fudoh* has been shown to inhibit the production of hemolysins and also attenuates virulence in a murine model of systemic infection (138). The authors hypothesize that the presence of *fudoh* may explain the difference in virulence seen in HA-type II strains and CA-type IV strains as the CA strains are typically more virulent, but lack multiple antibiotic resistance determinants seen in hospital acquired strains.

iii. SCC*mec* type III

The first SCC*mec* type III (FIG. 2C) was described in 1985 in strain 85/2082, which was isolated in New Zealand (291) and is 66.9 kb (121), making it the largest of the cassette types. Like SCC*mec* type II strains, type III strains often contain multidrug resistance determinants pUB110, pI258, pT181, and Tn*554* (164). Also, like the type II cassette, strains containing the type III cassette are often associated with hospital infections. SCC*mec* type II cassettes contain a class A *mec* complex and a type 3 *ccrAB* complex.

iv. SCC*mec* type IV

The SCC*mec* type IV (FIG. 2D) cassette is classically associated with highly virulent CA-MRSA strains. The type IV SCC*mec* is the smallest of the cassettes and ranges in size from 20.9 to 24.3 kb (179). Its role in the virulence of CA-MRSA will be covered in section C.

v. SCC*mec* type V

The SCC*mec* type V (FIG. 2E) is only slightly larger than the SCC*mec* type IV, with a size of approximately 27.6 kb (122). The first isolate identified as bearing this cassette type was strain WIS, a community-acquired isolate from Australia (122). SCC*mec* type V has a Class C *mec* complex (*IS431*-Δ*mecR1*-*mecA*-*IS431*) and along with SCC*mec* type IV has been documented as being responsible for as much as 87% of all CA-MRSA cases identified at some hospitals (290).

C. Community Acquired Methicillin Resistant *S. aureus* (CA-MRSA)

The first widespread infections caused by CA-MRSA were reported in the early 1990s in Western Australia (287), however, outbreaks among intravenous drug users in Michigan in 1980 are the first documented reports in the literature of what we know as CA-MRSA today (42). In 1999, an outbreak was reported in the Minnesota-North Dakota area that was associated with four deaths in young children that did not have risk factors (recent hospitalization, invasive or surgical procedures, intravenous drug use, prolonged exposure to antibiotics) that would have pre-disposed them to MRSA infection (43, 45, 260, 261). The lack of these risk factors in the children lead healthcare workers to assume the infections were caused by MSSA, and this delay in treatment could have contributed to the deaths in these pediatric cases. The genome of one of the isolates, MW2, was sequenced and it was discovered that it had a type IV cassette (13) and this strain has become the prototypical CA-MRSA strain. In the United States, the major pulsed-field gel electrophoresis type responsible for the first outbreaks was USA400 (3), which has now been overtaken in frequency as the cause of CA-MRSA infections by USA300 (149, 151, 189).

The definition of a CA-MRSA infection is still somewhat confusing and is varied in the literature (247). Time-based definitions for infections present upon admission to a hospital or diagnosed within 48-72 hours are considered to be community acquired (247). However, the lack of risk-factors in a patient that would pre-dispose them to an HA-MRSA infection is now often used to define a CA-MRSA infection, since evidence exists that suggests at least 47.5% of healthy individuals in the community had at least one risk factor for HA-MRSA infections, and that this might actually lead to an overestimation of

the number of CA-MRSA infections reported annually (247). It is generally accepted, however, that because of the dominance of the type IV SCC*mec* cassette in typed CA-MRSA strains, the most reliable method of defining a CA-MRSA isolate is on the basis of its SCC*mec* type (IV or V) (63). One of the key differences between HA- and CA-MRSA lies in the type of cassette present. When compared to HA-MRSA strains that carry type I, II, or III cassettes, which generally encode resistance to antibiotics in addition to β-lactams, type IV SCC*mec* cassettes are often much smaller, and strains harboring these cassettes have demonstrated an increased growth rate *in vitro* when compared to HA-MRSA isolates (168, 209). This increased growth rate and fitness might account for the predominance of CA-MRSA in the community.

CA-MRSA can manifest its pathogenicity in a variety of diseases. It is the most common cause of skin and soft tissue infections, causing ~77-95% of the cases of cellulitis and abscesses (84, 141). The cellulitis and abscesses attributed to CA-MRSA are usually seen in the form of furunculosis, or boils, which when damaged by scratching or other irritation can crust over and cause abscesses. Impetigo can also occur as a result of a CA-MRSA skin infection and in a study of 172 isolates implicated in these types of infections, 93% of isolates carried the PVL genes (171). The PVL toxin was also shown to be present in 85% of the isolates sequenced by Lina et al. (171) causing community-acquired necrotizing pneumonia and several groups have demonstrated a link between CA-MRSA with a type IV cassette and presence of the PVL toxin (261, 293).

D. Epidemiology

A recent study by Klevens et al. detailed the increasing healthcare burden of MRSA in this country. In 2005, it is estimated that over 94,360 invasive MRSA infections occurred in the United States and of those cases 18,650 were fatal (151). When comparing the incidence rate for MRSA infections in that same year (31.8 per 100,000) with the incidence rates of other important public health pathogens, the incidence rate of MRSA was more than double that of *S. pneumoniae*, which was a distant second with an incidence rate of 14.0 (151). Based on the results in this study, it is estimated that MRSA-related deaths outnumber those caused by AIDS (151) and for this reason the public health importance of invasive MRSA infections cannot be overlooked.

Recent reports have suggested that the epidemiology associated with MRSA infections is changing, especially among those strains associated with infections in the community. CA-MRSA infections have been shown to be increasing in prevalence. For example, in a study by Carleton et al. it has been shown that there is an expanding community reservoir for CA-MRSA infections caused by SCC*mec* type IV bearing isolates and these have overtaken SCC*mec* type II isolates in the community coinciding with a >4-fold increase in methicillin resistance over a seven-year period (40). Furthermore, the types of populations affected by MRSA differ among infections caused by HA- and CA-MRSA. CA-MRSA is a major cause of concern among several different populations that either share close contact or common equipment. These populations include “closed populations” such as Native Americans (105), inmates (213), military recruits (324), and competitive athletes (145).

Comparison of HA- and CA-MRSA infections yields further insights on the types of populations generally affected by each. To further understand the differences in HA- and CA-MRSA infections based on populations, Naimi et al. (195) conducted a study using a surveillance network comprised of twelve laboratory facilities in Minnesota. Of the 4612 unique patients that showed a positive result for an *S. aureus* infection in a clinical culture, 25% of the infections were caused by MRSA. When the MRSA infections were classified on the basis of whether they were HA- or CA-MRSA, 12% of the isolates were CA-MRSA and 85% were HA-MRSA. The median age among the HA-MRSA patients was 68 as compared to 23 for the CA-MRSA patients. Naimi et al. also found that CA-MRSA infections were more often found among non-whites (195). Fridkin et al. (84) and Klevens et al. (151) also had similar findings to those reported by Naimi et al. in regards to MRSA infections among different racial groups. Both groups found that incidence rates among blacks were higher than other racial groups (196). While there are limited studies that address the exact reasons blacks might be more likely than whites to suffer from MRSA infections, it has been hypothesized that risk factors such as increased underlying illness within the black community (diabetes, HIV etc.), differences in immune response, and socioeconomic factors such as decreased access to healthcare could all contribute to this disparity in infection rates (176).

Clinical manifestations seem to differ among HA- and CA-MRSA infections as well. CA-MRSA tends to manifest as skin and soft tissue infections in the forms of abscesses, folliculitis, and impetigo (195). Interestingly, they found that only 2% of the CA-MRSA cases were pneumonia. The study also demonstrated that bloodstream and urinary tract infections tended to be caused primarily by HA-MRSA (195).

Outside of the United States, the statistics associated with MRSA infections vary greatly depending on the part of the world. Northern Europe and Scandinavia have had tremendous success in controlling the spread of MRSA in both the community and in hospitals, but the degree of success of southern European countries is much different. In Sweden, the government run initiative Strama (The Swedish Strategic Programme Against Antibiotic Resistance) aggressively uses local county run multidisciplinary groups that provide doctors with feedback as to occurrences of resistance and also antibiotic usage among patients (188). The aggressive surveillance and monitoring seen in Sweden has lead to it having one of the lowest rates of MRSA infections (<1%) in the entire world (188). As a result, Strama's activities have lead to an overall decrease in antibiotic usage in Sweden (188). In contrast to Sweden, in a study comparing the prevalence of MRSA among *S. aureus* isolates found in hospitals in Italy and Portugal with isolates taken from hospitals in Switzerland and the Netherlands, up to 58% of the isolates from Italy and 54% of the isolates from Portugal were MRSA, while only 2% of the isolates found in the Netherlands and Switzerland were positive for *mecA* (76). Also in contrast to the picture of MRSA infections in the United States where the incidence is increasing, the prevalence of MRSA in Europe as a whole has remained fairly stable over the past few years (277).

III. Antibiotic resistance mechanisms of *S. aureus*

The role of the SCC*mec* cassette in antibiotic resistance has been well studied and characterized (60, 61). With the nearly universal resistance of staphylococci to methicillin and other β-lactam antibiotics, however, there have been many attempts at

developing with alternative antibiotic therapies for these types of infections. There has been success with many drugs, tempered almost immediately by reports of resistant bacteria. For example, daptomycin, a cyclic lipopeptide antibiotic displays a novel mechanism in its staphylococcalcidal activity, where it acts by inserting itself into the bacterial cell membrane causing membrane depolarization, potassium ion efflux, and cessation of DNA, RNA, and protein synthesis (59). However, since its introduction in 2003, there have been an increasing number of reports of daptomycin resistant clones, although the mechanism of this resistance remains unknown (132, 265). *S. aureus* uses a wide array of tactics aimed at causing resistance to the antibiotics used in the treatment of its infections. An understanding of these mechanisms of resistance is key to the development of new therapy regimens for drug-resistant staphylococcal infections and examples of major themes seen in staphylococcal resistance to antibiotics will be discussed below.

A. Target Modification

Perhaps the best example of target modification as a strategy for resistance to a selected antibiotic in *S. aureus* is in the case of vancomycin. Vancomycin was first isolated from a soil sample isolated in Borneo and sent to a chemist at Eli Lilly by a friend on a mission there in 1952 (7). The drug was first dubbed "compound 05865" and was shown to have activity against many Gram-positive bacteria including penicillin-resistant staphylococci, but was also active against *Neisseria gonorrhoeae* (91). The drug was approved by the USDA in 1958, after being used to successfully treat several staphylococcal endocarditis patients who had experienced previous treatment failure

while on other antibiotics (7, 91). The clinical usage of vancomycin exploded in the 1980s with the dramatic increases seen in MRSA infections and widespread oral usage of vancomycin is thought to be responsible for the emergence of vancomycin-resistant enterococci (VRE) (92). Horizontal gene transfer events between VRE and staphylococci are believed to be responsible for the appearance of vancomycin-resistant *Staphylococcus aureus* (VRSA) and the first VRSA isolate was described in 2002 taken from a wound in the foot of a diabetic patient (44, 264).

Vancomycin is perhaps the best studied member of the glycopeptide family of antibiotics. Vancomycin acts by targeting a key step in peptidoglycan synthesis. Two molecules of D-alanine are joined together by a ligase in the cytoplasm and the resulting D-Ala-D-Ala is then added to a uracil diphosphate–*N*-acetylmuramyl-tripeptide to form a pentapeptide. The pentapeptide, uracil diphosphate–*N*-acetylmuramyl-pentapeptide, is bound to a lipid carrier after the addition of *N*-acetylglucosamine and the molecule is translocated to the outer leaflet of the cytoplasmic membrane where it is incorporated into the nascent peptidoglycan by a transglycolase. There it is used in the final transpeptidation step forming a cross-bridge responsible for the integrity of the peptidoglycan (237). Vancomycin binds to the C-terminus of the D-Ala-D-Ala residue of the pentapeptide blocking cross-linking by transpeptidation (237).

Vancomycin resistance is encoded by operons that generally encode genes for the addition of alternative residues of the pentapeptide (D-lactate or D-serine instead of D-alanine) crucial in the final transpeptidation step (12). High level resistance to vancomycin in *S. aureus* is mediated exclusively by a transposon, Tn*1546*, which carries an operon encoding nine polypeptides of varying function (12). Gene expression from

this VanA-type resistance element is mediated by the *vanR/vanS* two component regulatory system (11). The *vanA* and *vanH* genes on this element encode genes responsible for addition of alternative terminal dipeptides (D-Ala-A-Lac), for which vancomycin has a lowered affinity (11). In VanA-type resistant strains, inducible resistance is also seen to teicoplanin (11).

There have been several reports of the incompatibility of high level vancomycin resistance with methicillin resistance. While they both work on inhibiting cell wall synthesis, methicillin acts on an earlier stage of the process, preventing the synthesis of peptidoglycan. The alternative penicillin binding protein responsible for β-lactam resistance in MRSA, PBP2a, is not able to crosslink peptidoglycan with *vanA* modified terminal peptide residues (79, 259). There have been reports of MRSA isolates passaged in the presence of sublethal concentrations of vancomycin showing a decrease in their resistance to oxacillin (4, 236, 263). Further evidence of incompatibility of these two methods of antibiotic resistance was recently described by Noto et al. (207) who demonstrated site-specific excision of the entire SCC*mec* cassette in response to prolonged exposure to vancomycin. The authors suggest that this loss of the SCC*mec* cassette might help the bacteria compensate for the fitness cost associated with acquiring high level vancomycin resistance. It should be noted that excision of the SCC*mec* cassette in the strains used by Noto et al. was not mediated by *vanA*. Cell wall thickening is often seen in vancomycin intermediately susceptible *Staphylococcus aureus* (VISA) isolates and isolates exposed to vancomycin over long periods of time (83, 270). The thicker cell wall seen in some VISA isolates has also been linked to daptomycin resistance in some

cases (34, 55), although a definitive mechanism for this type of resistance has yet to be elucidated.

B. Drug efflux

Exporting antimicrobials is a common strategy used among bacteria in their attempt to achieve resistance to antimicrobials. *S. aureus* utilizes both plasmid and chromosomally-mediated efflux systems that recognize a wide variety of antimicrobials to accomplish this. Active drug transport systems are categorized into five families. These families are: ABC superfamily drug transporters, MFS drug transporter family, the MATE family of drug transporters, the SMR family transporters, and the SepA transporter.

i. ABC superfamily

Transporters in the ABC superfamily are seen not only in bacteria, but are also found in cancer cells (111). The functional unit of the characterized staphylococcal ABC drug transporters are composed of several transmembrane domains linked covalently to two nucleotide-binding domains and it is believed that hydrolysis of ATP induces conformational changes in the transmembrane domain that helps move bound antimicrobials outside of the cell (57). The staphylococcal ABC transporters remain poorly characterized, but recently the crystal structure of the transporter Sav1866 and it was shown to share ~30% identity with the human P-glycoprotein transporter and the LmrA multidrug transporter found in lactococcal species (57).

Staphylococcal ABC family transporters recognize diverse antimicrobials. The Vga(A) protein is a plasmid encoded 522 amino-acid protein which shares ~35% amino-acid identity with the *msrA* determinant that is responsible for efflux of macrolides and

type B streptogramins in *S. epidermidis* (5). Vga(A) has been shown to recognize and export several different antibiotics such as clindamycin, type A streptogramins, lincomycin and pristinamycin with inducing varying degrees of resistance (47).

ii. Major facilitator superfamily drug transporters

Members belonging to the major facilitator superfamily (MFS) of drug transporters are found in all classes of living organisms and most drug transporters present in *S. aureus* belong to this family (245) including the NorA and QacA multidrug efflux pumps, which are among the best characterized bacterial multidrug transporters. QacA was the first bacterial multidrug transporter described and can be carried on staphylococcal multidrug resistance plasmids (281). Expression of QacA is regulated by the repressor QacR, which is divergently transcribed from the *qacA/qacB* locus (104). QacR has been shown to bind substrates recognized by the QacA/QacB pump and QacR complexed with any of these molecules has been shown to be unable to bind DNA upstream of *qacA/qacB* leading to derepression of the expression of those genes (104). Both the QacA and QacB transporters give *S. aureus* resistance to a variety of antimicrobials and they take their name from the resistance they give to monovalent cationic antimicrobials such as quaternary ammonium compounds. The pump can also recognize disinfectants such as chlorhexidine and dyes such as ethidium bromide (172). Both *qacA* and *qacB* are prevalent among MRSA isolates and in a recent survey of MRSA isolates in Europe, either *qacA* or *qacB* was found in 63% of those isolates (184).

Unlike the plasmid encoded QacA/QacB multidrug transporters, the NorA transporter is chromosomally encoded. The NorA efflux pump was first identified in a screen of plasmid cloned resistance determinants from a fluoroquinolone resistant *S.*

aureus isolates that would transfer that resistance to *E. coli* (320). Sequence comparisons of the 388 amino-acid sequence of NorA with the multidrug transporter Bmr in *B. subtilis* gave insight on the other types of antimicrobials that the pump might recognize (202). Like Bmr, NorA recognizes dyes such as acridine orange, ethidium bromide and rhodamine 6G, and antibiotics such as chloramphenicol, puromycin, and norfloxacin (202). NorA also demonstrates similarity with tetracycline efflux pumps seen in Gram-negative bacteria and has been shown to play an important role in chromosomally-mediated resistance to tetracycline (132, 320).

The TetA(K) transporter is also responsible for tetracycline resistance in *S. aureus* and is among the best characterized staphylococcal multidrug transporters. The *tetA(K)* determinant was first identified on *S. aureus* plasmid pT181 (147). pT181 has been found in both MRSA and MSSA clinical isolates as well as among coagulase-negative staphylococci and some isolates taken from domestic animals (252, 253). TetA(K) and the related resistance determinant TetA(L) show about 60% sequence identity (96, 218) and both recognize and export tetracycline complexed with divalent metal ions in exchange for protons (316).

iii. MATE Family Transporters

The multidrug and toxin extrusion (MATE) family of transporters rely on the protein motive force and sodium motive force to pump drugs from the bacterial cell and homologs have also been found in all classes of living organisms (119). To date however, only one staphylococcal MATE family transporter has been identified. MepA was first identified in two *S. aureus* strains that did not express levels of *norA* significant enough to confer resistance and that had been exposed to increasing concentrations of

moxifloxacin and ethidium, and norfloxacin respectively (130). One operon containing three genes, *mepR*, *mepA* and *mepB* showed a significant increase in transcription in both strains (130). *mepR* encodes a 139 amino-acid MarR family transcriptional regulator, *mepA* encodes a MATE family transporter with twelve transmembrane domains, and *mepB* encodes a protein with no similarity to any proteins of known function (130). Expression of the *mep* operon has been shown to be induced by exposure to tigecycline and has been shown to be present in all sequenced staphylococcal strains (185). MepR negatively regulates the expression of *mepA* as well as *mepR* and possibly *mepB* (161). *mepA* recognizes a variety of mono- and bivalent cationic antimicrobial peptides as well as fluoroquinolones and glycyclines (131, 186) and has also been shown to play a role in virulence as *mepA* mutants have been shown to produce increased levels of protein A (87).

iv. SMR family transporters and SepA

Members of the small multidrug resistance (SMR) family of transporters, such as QacG, QacH, and QacJ have been identified as being carried on several staphylococcal plasmids (31, 112, 146), although QacC is the best characterized staphylococcal SMR family transporter. *qacC* has been found in MRSA isolates from Europe and Asia and like QacA, has been implicated in resistance to quaternary ammonium complexes and dyes such as ethidium (184, 206). QacC has also been shown to confer resistance to β-lactam antibiotics (86).

A novel staphylococcal transporter has been recently described by a Japanese group. *sepA* was found in a screen of chromosomal fragments from *S. aureus* that would confer resistance to benzalkonium and chlorhexidine to *E. coli* (198). SepA is a

chromosomally encoded efflux pump that confers resistance to quaternary ammonium compounds, dyes and mediates the specific efflux of ethidium (198). It is thought that SepA might have evolved from an SMR family transporter due to its pattern of conserved amino-acid residues in its transmembrane domains, but its predicted topology is distinct from SMR family members (198).

C. Phenotypic shift

S. aureus is one of many bacteria that use an alternative colony phenotype as a strategy to evade antimicrobial therapy. The small colony variant (SCV) phenotype was first described 100 years ago in *Salmonella typhi* (123) and since then has been described in many Gram-negative and Gram-positive bacteria including MRSA (38), MSSA (124), *Vibrio cholerae* (278), and *Neisseria gonorrhoeae* (235). Because of the growing problem with antibiotic resistance in *S. aureus*, this alternative phenotype often implicated in persistent and drug resistant infections, has been the subject of aggressive studies as of late. *S. aureus* small colony variants demonstrate the following characteristics: pinpoint colonies on agar, decreased pigment production, reduced hemolytic activity, increased resistance to aminoglycosides, decreased coagulase activity, and altered metabolism (300). While no genetic mechanism has been suggested for this phenotypic shift, there is evidence of a genetic component and the role of SCVs in antibiotic treatment failure and possible genetic causes will be discussed below.

i. The SCV phenotype

S. aureus SCVs are a slow growing subpopulation of bacteria that have been isolated from diverse sites of infections including abscesses (2, 256), bloodstream infections (302), bone and joint infections (240, 241, 300, 301), respiratory tract (38, 134), and soft tissues (229, 257, 298). Besides a difference in colony size, small colony variants differ significantly in other ways from their respective parent strains and clinical identification of SCVs are often difficult because they can be overgrown by normal sized colonies and easily missed on plates. Clinical SCV isolates are difficult to grow in the laboratory because they have fastidious growth characteristics and clinical SCVs have a tendency to revert to their large colony phenotype upon repeated passages further complicating the ability to study the genetic mechanisms involved in this process (230). There are two major groups of SCVs reported in clinical laboratories: SCVs with defects in electron transport and SCVs with thymidine biosynthesis deficiencies (230).

Clinical SCV isolates with defects in electron transports generally have one of two major auxotrophies, either for menadione or haemin (36, 135, 239, 299, 301, 302, 303). This auxotrophy complicates identification in clinical laboratories, but reversal of this phenotype can occur if the SCV is grown on media supplemented with either compound depending on the specific auxotrophy. This lack of proper electron transport leads to pleiotropic changes in phenotype seen in SCVs including decreased respiration, lack of pigmentation, decrease in toxin production, increased resistance to aminoglycoside antibiotics, and an unstable colony phenotype seen in the high reversion rates *S. aureus* SCVs demonstrate after repeated passage on media (229) and the proposed genetic mechanisms seen in this phenotype will be discussed in detail in a later

section. Clinical *S. aureus* isolates demonstrating thymidine auxotrophies display a similar phenotype to those with growth requirements for haemin or menadione. These isolates are often recovered from pus (46) and from the lungs of patients with cystic fibrosis (27). Electron microscopy analysis of thymidine-dependent SCV isolates reveals an irregular cell morphology as well as improper separation (136). Clinical thymidine-dependent *S. aureus* SCV isolates also demonstrate important changes in cell metabolism that will be discussed in detail in another section.

ii. Clinical manifestations and role in disease of *S. aureus* SCV isolates

It is becoming clear that the role of SCVs in clinical infections is an important aspect of staphylococcal pathogenesis that deserves intense scrutiny. The first indication that *S. aureus* SCVs might play a role in persistent and drug-resistant infections came from five patients who displayed unusually hard to treat and severely antibiotic-resistant infections (229). Three of the five patients had recurrent osteomyelitis, one presented recurrent sinusitis, and the final patient had recurrent septic arthritis and muscle abscesses. Initial isolation of all bacteria cultured from each patient demonstrated that SCVs made up between 20 and 100% of the phenotype of the culture and four out of five were resistant to gentamicin (229). The isolates were all non-pigmented, non-hemolytic, and required either haemin or menadione for growth. Comparison of the PFGE SmaI restriction pattern of one of the isolates that showed a moderate reversion rate to the large colony phenotype from day 0 to day 14 demonstrated that while the colony phenotype changed, the SmaI pattern remained the same (229). Analysis such as this confirms the elusive nature of the exact genetics mechanisms responsible for this colony switching phenotype.

Since the initial reports of *S. aureus* SCVs and their role in persistent and drug-resistant infections, these isolates have been associated with a myriad of clinical diseases and complications. In a recent study of MRSA isolates displaying the SCV phenotype and implicated in sepsis during an outbreak at a long term care facility, patients infected with SCV-MRSA demonstrated a mortality rate of 100% when compared to those patients with non-SCV-MRSA infections (248). Furthermore, the SCV-infected patients received more antibiotic treatment than the non-SCV infected patients highlighting once again the problems of treatment burdens of SCV-infected patients.

Apart from their role in bacteremia, SCVs are often implicated in osteomyelitis infections. Treatment of *S. aureus* osteomyelitis often requires systemic antibiotic therapy, debridement of the bone, and implantation of gentamicin-containing beads at the site of infection (302). Because of their improper membrane potential, *S. aureus* SCVs often demonstrate increased resistance to aminoglycoside antibiotics, and it has been shown that the slow release of gentamicin from beads used in osteomyelitis treatment can result in SCVs being isolated from the sites of bead placement where the initial isolates demonstrated a large colony phenotype (18, 229, 300). Further studies investigating the role of gentamicin bead placement in selecting for *S. aureus* SCVs in osteomyelitis have indicated that this form of treatment is indeed an efficient way to select for menadione and haemin auxotrophic isolates (300). This data indicates the need for clinicians to alter their treatment protocols in cases of relapsing osteomyelitis and further illustrate the clinical impact of SCVs in persistent and drug-resistant infections.

Post-surgical complications from infections associated with medical devices such as pacemakers, are most commonly caused by staphylococci. There have been many cases

reported of persistent pacemaker-related bloodstream infections caused by staphylococcal small colony variant isolates (14, 257). These types of infections respond poorly to antibiotic therapy and are frequently misdiagnosed (274, 302) and can often be resolved only by removal or replacement of the device.

Small colony variants are commonly found in infections associated with the lungs of cystic fibrosis (CF) patients. In a recent study of adolescents with CF, wild type *S. aureus* and *S. aureus* SCVs were found to persistently colonize the lungs of 52 of the 72 patients involved with the study (134). In six of the patients who initially showed both wild-type *S. aureus* and SCV *S. aureus* to be present in their lungs, after a period of 36 months only SCVs were able to be isolated from their sputum, suggesting that the persistence of SCVs may give them a competitive advantage over wild-type *S. aureus* for survival over extended periods of time (134).

Polymicrobial infections are common in the lungs of CF patients and one organism that is commonly co-isolated from the sputum of these patients with staphylococcal SCVs is *Pseudomonas aeruginosa*. Recent investigations into the role that polymicrobial communities might play in the persistent infections seen in the lungs of CF patients have revealed that *P. aeruginosa* can induce the phenotypic shift of *S. aureus* from wild-type to SCV. *P. aeruginosa* secretes several exotoxins that act as respiratory inhibitors to surrounding bacteria. These include pyocyanin (110), hydrogen cyanide (41), and quinoline *N*-oxides (180), and it has been shown that *S. aureus* is particularly sensitive to all of these toxins and that they act by specifically targeting the electron transport chain (297). Biswas et al. (30) have recently suggested that the small colony phenotype exhibited by *S. aureus* commonly infecting the lungs of CF patients may be

used as a survival strategy when growing in the presence of *P. aeruginosa*. The defects seen in the electron transport chain of SCV isolates found in the lungs of CF patients can actually be selected for in the presence of these extracellularly secreted toxins (30).

Besides giving the bacteria a competitive advantage over wild-type cells during prolonged infection, there is accumulating evidence that staphylococcal SCVs allow the bacteria to persist for long periods of time within non-professional phagocytes. There have been several studies demonstrating that *S. aureus* can be successfully phagocytosed and can survive for extended periods of time within many different kinds of cells including endothelial (18), epithelial (21, 137), and osteoblasts (118, 196). The staphylococcal cell surface expresses a large number of proteins termed microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) that mediate binding to various host cell surface receptors to facilitate bacterial attachment and adherence (217). Among these MSCRAMMs, fibronectin-binding proteins (FnBPs) present on the staphylococcal cell surface, specifically mediate attachment to the host cell through forming bridges with $\alpha_5\beta_1$-integrin (70, 266). Vaudaux et al. demonstrated that in a model *hemB* insertional mutant displaying the small colony phenotype internalization in a human embryonic kidney cell line were internalized more than their wild-type parent strain (294). There is also evidence that small cationic peptides can select for cells with mutations in *hemB* and *menD* (244). This evidence indicates that once internalized, host cell cationic proteins might select for small colony variants. Furthermore, these cells may be able to persist intracellularly for years once internalized and this might play a role in recurrent infections. Clement et al. described three patients with chronic reoccurring rhinosinusitis over a three year period (49). Biopsy and staining of patients'

nasopharyngeal cells with a specific *S. aureus* monoclonal antibody raised against staphylococcal peptidoglycan when imaged with confocal microscopy revealed large numbers of bacteria during asymptomatic periods. Furthermore, when the cells isolated during symptomatic and asymptomatic time periods demonstrated a clonal relationship, suggesting that persistence of *S. aureus* can be caused by intracellular reservoirs of the bacteria within non professional phagocytes (49). von Eiff et al. also demonstrated that a model staphylococcal SCV strain could persist within cultured epithelial cells (299). Taken together, the SCV phenotype seems to enhance the bacteria's ability to cause persistent and recurrent infections by increasing staphylococcal uptake by non-professional phagocytes.

iii. Causes and consequences of the SCV phenotype

The specific genetic causes responsible for the small colony variant phenotype are not yet fully understood. However, evidence suggests that there are many genes involved in this phenomenon. Because of their high reversion rate to the large colony phenotype, it is often difficult to study clinical SCV isolates in the laboratory. To circumvent this problem, model SCV strains have been created that exhibit the hallmarks of the small colony variants seen in clinical laboratories. In *S. aureus*, *hemB* codes for porphobilinogen synthase (also known as aminolevulinic acid dehydratase [133, 299]), which is responsible for converting delta-aminolevulinic acid to porphobilinogen, which is essential for heme biosynthesis. Heme is a prosthetic group required for cytochromes, which are molecules responsible for proper electron transport (100). von Eiff et al. made a strain with a stable SCV phenotype by insertionally inactivating *hemB* in *S. aureus* 8325-4 (299). Their observations were based on previous work that had shown electron

transport deficient mutants of *S. aureus* were found to be phagocytized by and not lyse cultured epithelial cells (18). Like many clinical staphylococcal SCVs, the phenotypic changes associated with this insertional *hemB* mutant could be reversed when grown on media supplemented with haemin (299).

Proteomic analysis of an insertional *hemB* mutant has revealed many proteins involved in fermentation and glycolysis as being up-regulated. Kohler et al. used 2D gel electrophoresis to compare the patterns between a model SCV *hemB* insertional mutant and its parent strain (153). MALDI-TOF analysis revealed that these proteins included lactate dehydrogenase, enolase, alcohol dehydrogenase, and arginine deiminase (153). In order to confirm their proteomic analysis, the authors harvested total mRNA from the *hemB* and wild-type strains at different phases of growth for use in Northern blot analysis. The authors were able to show that genes involved in glycolytic processes as well as genes involved in fermentation were strongly up-regulated in the *hemB* mutant compared to the parent strain.

In order to gain a more complete picture of the transcriptional changes that occur in a model SCV mutant, Seggewiss et al. performed a detailed microarray analysis comparing the transcriptional profile of an insertional *hemB* mutant with that of its isogenic parent strain (255). The authors found that there were 170 genes differentially regulated between the *hemB* mutant and the parent strain, with 122 of those being significantly up-regulated and 48 of those being significantly down-regulated. Similar to the findings of Kohler et al., the authors found that there were many genes involved in fermentation and glycolysis that were up-regulated in the mutant. The authors were able to further demonstrate that because the *hemB* insertional mutant was unable to make as

much ATP as the parent strain because of defects in electron transport, many genes involved in the TCA cycle were significantly down-regulated. In order to compensate for this lack of ATP, genes involved in the arginine deiminase (AD) pathway, which has been shown to be important to other bacteria such as *Streptococcus suis* when grown under microaerophilic or anaerobic conditions (106), were significantly up-regulated. Other genes indicated in staphylococcal stress responses that were found to be significantly up-regulated from this study include genes involved in capsular polysaccharide biosynthesis (*cap5* and *cap8* operons), heat shock (*htrA*), and SA2494 which encodes a cold shock protein, *cspB*. While the roles of capsular polysaccharide and heat shock proteins have been well defined in *S. aureus*, work with cold shock proteins has been limited. Their roles outside of allowing the bacteria to survive suboptimal temperatures is not well understood; however, there is growing evidence that they provide important cellular functions under normal conditions, and besides the work of Seggewiss et al. linking this stress response protein to a change in colony phenotype, further work is needed to clearly define the role of *cspB* in *S. aureus* pathogenesis.

IV. Cold shock proteins

Bacteria have adapted a number of ways to cope with changes in their local environment. In order to survive, they must be able to successfully respond to changes in pH, osmolarity, and temperature. For bacteria living in soil, in particular, an efficient system of responding to cold stress is essential to their survival. The cold shock response is a physiological response to a downshift in temperature characterized by the inhibition of general protein synthesis and the increased synthesis of cold shock proteins.

Cold shock proteins belong to the oligonucleotide binding domain (OB) family of proteins (192). These proteins have all been shown to bind ssRNA or ssDNA with high affinity (193), which may shed some light on their specific functions in bacteria. Interestingly, the OB domain characteristically found in these proteins has been found in initiation factor 1 (IF1) from *E. coli* (258), archael initiation factor-1a from *Methanococcus jannaschii*, ribosomal protein L2 from *Bacillus stearothermophilus*, ribosomal protein S12 from *Thermus thermophilus*, and eIF1a in humans (193). All of these proteins recognize "Y-box" motifs (ATTGG) which are *cis*-acting DNA regulatory elements (183). The members of this family bind RNA or ssDNA but do not bind dsDNA (126, 174, 175), with the sole exception of this in bacteria so far being the binding of CspA from *E. coli* to a double-stranded 110 bp fragment of the promoter region of the H-NS protein operon in crude cell extract (162). The general role of cold shock proteins in Gram-negative (*E. coli*) and Gram-positive (*B. subtilis*) bacteria as well as the specific findings on how cold shock proteins might influence antibiotic susceptibilities in *S. aureus* will be discussed below.

A. Cold shock proteins in *E. coli* and *B. subtilis*

The specific roles of cold shock proteins in bacteria are still an area of active research with many of their functions still remaining unclear. However, work done in *E. coli* and *B. subtilis* has given some insight into how these proteins might function *in vivo*. CspA was the first cold-shock protein to be described in detail from *E. coli* and since its discovery several CspA family members have been described in Gram-negative and Gram-positive bacteria (98, 280) and there is some evidence that these Csps might have

redundant functions (16, 126). In *E. coli*, there are nine members of this family of proteins (CspA-CspI) and they can either be constitutively produced or induced upon cold stress (98, 167, 197, 304). Among the constitutively produced proteins from *E. coli*, CspC and CspE have been suggested to act as regulators of *rpoS* and *uspA* (224). Further work on CspE from *E. coli* has shown that it plays a role in camphor resistance in *E. coli* by maintaining nucleoid integrity when the bacteria are exposed to the terpenoid (109, 117). Another constitutively produced Csp, CspD has also been shown to be associated with the nucleoid in *E. coli* during the late exponential phase of growth (93) where it can bind to single stranded regions of the replication fork, and when overexpressed can lead to cell death by blocking DNA replication (317).

E. coli contains three cold inducible proteins belonging to the CspA family, CspA (98), CspB (167), and CspG (197). Among the cold inducible Csps in *E. coli*, CspA is the major cold inducible protein (73, 98) with more than 10^6 copies of its mRNA being present under cold shock conditions (35). Analysis of the three-dimensional structure of CspA demonstrates that it has a β-barrel structure with five β-strands containing RNP1 (β2 strand) and RNP2 (β3 strand) RNA binding domains (201, 249). CspA has been shown to nonspecifically bind RNA (126) and CspA has also been show to negatively regulate its own expression (15). A further insight into how CspA from *E. coli* might function *in vivo* was shown by Bae et al. (17). The authors were interested in further defining the roles of CspA, CspC and CspE. To accomplish this, the authors overexpressed each of those three Csps independently in an *E. coli* strain and then looked at their influence on the expression of several other non-CspA family cold shock genes. The authors were able to demonstrate that overexpression of either CspA, CspC or CspE

was able to increase the expression of those cold induced transcripts when the bacteria were grown at 37° C. The authors also demonstrate that this mechanism was due to the CspA family of cold shock proteins acting as transcriptional antiterminators, preventing the formation hairpins that lead to pausing of transcription (17).

Besides the work done in *E. coli* demonstrating the ability of CspA family cold shock proteins to bind to mRNAs stabilizing them, work in *Bacillus subtilis* has shown that the roles of these Csps in Gram-positive organisms might be slightly different. *Bacillus subtilis* contains a homolog to the *E. coli* CspA that has been named CspB and the two proteins share 61% identity (309). Three cold-inducible Csps, CspB, CspC, CspD, have been identified in *B. subtilis* and analysis of individual deletion mutants has revealed that at least one copy of a CspA family of cold shock proteins is required for proper cellular functions at normal temperatures and that CspB is the major cold inducible CspA family member in *B. subtilis* (101). Apart from being induced by cold stress, CspB and CspC are both induced during stationary phase and cells with a *cspB*/*cspC* double deletion in *Bacillus subtilis* showed pleiotropic changes in protein synthesis, as well as an inability of the cells to form endospores and lysis upon entry into stationary phase (101, 305). Clues to the role of CspB in *B. subtilis* might lay in its crystal structure. Comparison of the crystal structure of *B. subtilis* CspB with IF-1 from *E. coli* shows a high degree of similarity upon superimposition (305). Furthermore, *B. subtilis* CspB has been shown to co-localize with ribosomes *in vivo* (182, 306) and it has been suggested that these proteins might act like ribosomal chaperones or alternative transition initiation factors based on these findings, although it is still unclear whether or not this is the case.

B. Cold shock proteins in *S. aureus*

Information on the role of cold shock proteins outside of *B. subtilis* and *E. coli* is limited. While cold shock proteins have been identified and described in bacteria such as *Listeria monocytogenes* (20) and *Yersinia enterocolitica* (200), there are still many questions as to their specific functions. In *S. aureus*, there have been three cold shock genes (*cspA, cspB, cspC*) identified as belonging to the CspA family of cold shock proteins. Previous work with CspA from *S. aureus* has demonstrated that it is the major Csp present at 37°C (143). Interestingly, an *S. aureus* strain (COL) with a Tn*551* insertion in the 5′ UTR of *cspA* demonstrated an increased resistance to a host derived antimicrobial peptide, CG 117-136 (143). This was the first indication that *S. aureus*, in addition to using its cold shock proteins to adapt to growth under cold stress, might also have recruited them to be a part of its arsenal against host defenses during infection. Work from Katzif et al. (144) also demonstrated that a *cspA* insertional mutant, in addition to having a cold-sensitive phenotype, was also less pigmented than its isogenic parent and that pigment production in *S. aureus* strain COL is regulated by *cspA* through a mechanism that involves the alternative sigma factor, σ^B. Investigations into the functions of the other two cold shock genes present in *S. aureus*, however, are limited. Recently, though, Anderson et al. (6) reported that the major cold inducible gene in *S. aureus* strain UAMS-1 is *cspB.* Outside of these findings, the role of *cspB* in *S. aureus* is unknown. To that end, it was the goal of this dissertation to address the following specific aims:

1. To define the role of the major cold inducible gene, *cspB* from *S. aureus* strain COL in response to cold stress.

2. To define the role of *cspB* in *S. aureus* in resistance to antimicrobials.

3. To understand the significance of *cspB* in the SCV phenotype.

Figure Legends

FIG. 1. *S. aureus* evasion of host innate immune defenses. This diagram illustrates the various methods *S. aureus* uses to evade the host innate immune system. Figure from Nizet 2007 (205).

FIG. 2. Comparison of the various SCC*mec* types. Diagrams of the various SCC*mec* types are shown. The cassettes are as follows: (A) type I, (B) type II, (C) type III, (D) type IV, (E) type V. The major elements important to each cassette are shown. The diagrams are not drawn to scale.

Figures

FIG. 1. *S. aureus* evasion of host innate defenses.

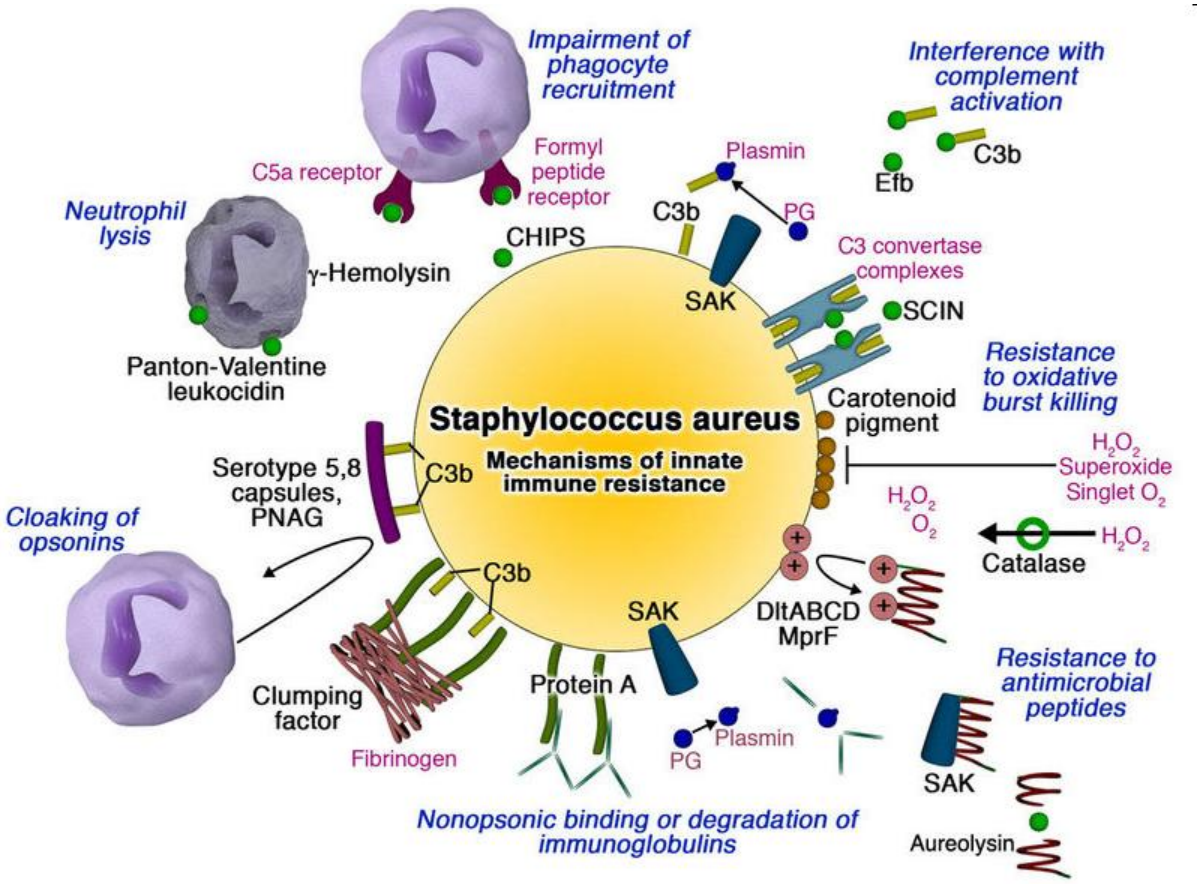
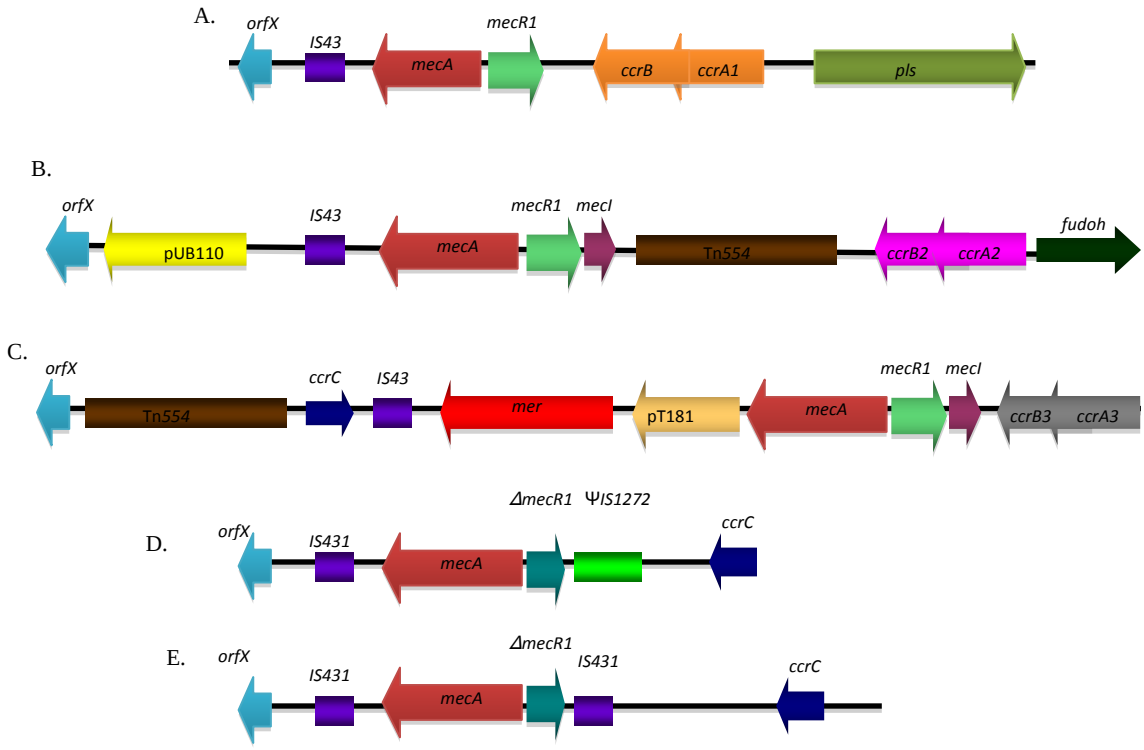


FIG. 2. Comparison of the various SCC*mec* types.



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Chapter 2: Altered Growth, Pigmentation and Antimicrobial Susceptibility Properties of *Staphylococcus aureus* Due to Loss of the Major Cold Shock Gene *cspB*

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Published in

Antimicrobial Agents and Chemotherapy, 2010

Volume 54 pgs. 2283-90

This manuscript was jointly written by Brea Duval and William Shafer with editorial input from Sarah Satola.

Abstract

An insertional mutation made in the major cold shock gene *cspB* in *Staphylococcus aureus* strain COL, a methicillin-resistant clinical isolate, yielded a mutant that displayed a reduced capacity to respond to cold shock and many phenotypic characteristics of *S. aureus* small-colony variants: a growth defect at 37 °C, a reduction in pigmentation and altered levels of susceptibility to many antimicrobials. In particular, a *cspB* null mutant displayed increased resistance to aminoglycosides, trimethoprim-sulfamethoxazole and paraquat and increased susceptibility to daptomycin, teicoplanin and methicillin. With the exception of increased susceptibility to methicillin, which was due to a complete loss of the type I SCC*mec* cassette, these properties were restored to wild type levels by complementation when *cspB* was expressed in *trans*. Taken together, our results link a stress response protein (CspB) of *S. aureus* to important phenotypic properties that include resistance to certain antimicrobials.

Introduction

Staphylococcus aureus is a major global public health problem causing serious, often life- threatening infections in the community and hospital settings that are becoming more difficult to manage with current antibiotic therapy regimens (7). The emergence of methicillin-resistant *S. aureus* (MRSA) in the hospital and community settings, coupled with the increasing number of persistent MRSA infections (25), and multi-drug resistant strains, is a growing problem not just for immunocompromised patients, but also for otherwise healthy individuals. The virulence of *S. aureus* strains is multi-factorial and involves the production of extracellular toxins, surface structures that mediate interaction with host cells and resistance to host defenses, transcriptional regulatory processes that control virulence gene expression and metabolic schemes that allow for adaptation to stresses imposed by the local environment within and outside of the human or animal host.

The capacity of *S. aureus* to respond to environmental stress conditions has been the subject of recent investigations (1,33), and is imperative for its survival in hostile environments such as extreme temperature. *S. aureus* can effectively respond and adapt to a decrease in temperature by the expression of cold shock proteins (Csps). This cold shock response likely plays a significant role in the ability of *S. aureus* to survive refrigeration and subsequently cause food-borne illnesses. Constitutive and inducible expression of cold shock proteins (CSPs) is linked to a bacterial response to lower temperatures (16). CSPs have been extensively studied in both *E. coli* and *B. subtilis*, and their roles in DNA- and RNA-binding have been investigated (15,16). These proteins belong to several diverse classes, some of which are constitutively produced, while others

are induced upon cold stress (16). While the role of each of these proteins is unclear, the major Csp in *E. coli* acts as an RNA chaperone that prevents the formation of undesired secondary structures during cold shock and actively promotes transcription (4). In previous communications, we (20,21) reported that mutations within or upstream of the cold shock gene *cspA* decreased pigment production by *S. aureus* strain COL through a SigB-dependent mechanism and increased bacterial resistance to a cationic antimicrobial peptide of human lysosomal cathepsin G. The decrease in production of the carotenoid pigment, staphyloxanthin, by the *cspA* mutant was of interest as it acts as an antioxidant, protecting staphylococci from neutrophil killing (24).

At the transcriptional level, *cspB* is the major cold shock gene in *S. aureus* (1) and its expression was impacted in *S. aureus* strain A22223I, a clinical osteomyelitis isolate, by a mutation in *hemB,* required for hemin biosynthesis (40). Mutations in *hemB* have been linked to the small colony variant (SCV) phenotype often displayed by *S. aureus* strains isolated from sites of persistent or antibiotic-resistant infections (36). These naturally occurring SCV subpopulations have been isolated from patients with a wide variety of infections such as device-related infections, skin and soft tissue infections, osteomyelitis and persistent airway infections in cystic fibrosis patients (19,35,44). SCVs frequently require exogenous hemin or menadione for growth, which has been implicated in their reduced membrane potential (26). Other hallmark features of SCVs include their reduced level of pigmentation, resistance to aminoglycosides, and reduced production of virulence factors (19,35,41,44,46). We created a non-polar insertional mutation in the coding sequence of *cspB* and introduced this mutation into *S. aureus* COL. A *cspB* null mutant was found to exhibit many properties previously observed with SCVs and most of these

properties were reversed by complementation. The sole exception was that of susceptibility to methicillin, which was due to excision of the type I SCC*mec* cassette.

Materials and Methods

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* COL is a clinical MRSA isolate containing the type I SCC*mec* cassette (13,38); it is the parent strain for BD1 and BD2. Strain BD1 contains an *aphA-3* insertion in *cspB* and was created by electroporation using pBD1 as previously described by Katzif *et al.* (20). Strain BD2 is a complemented version of BD1 and contains a wild type copy of *cspB* expressed in *trans* from pBD2; the construction of pBD1 and pBD2 are described below. All *S. aureus* strains were grown on tryptic soy agar (TSA) or in trypticase soy broth (TSB) (BD Pharmaceuticals, Wilson, NC) with or without antibiotic selection. *E. coli* TOP10® cells (Invitrogen, Carlsbad, CA) were grown in Luria-Bertani broth (LB) or on LB agar at 37 °C.

Cold shock and gene expression analysis. *S. aureus* strains were grown in 50 ml of TSB at 37 °C with shaking (200 RPM) and growth was measured at an optical density of 600 nm. At mid-logarithmic phase the culture was split into two 10 ml samples and these were incubated at 15 °C (cold-shock) or 37 °C (control) for one hour. Growth at both temperatures was monitored by optical density measurement and viability was determined by dilution plating onto TSA. In order to determine expression of *csp* genes, RNA from control and cold-shocked cultures of strain COL was prepared as described by Katzif *et al*. (21). For RT-PCR analysis, SuperScript® II Reverse Transcriptase (Invitrogen, Inc.) was used with 500 ng of RNA from each sample according to the

manufacturer's instructions for cDNA synthesis. Amplitaq (Applied Biosystems, Foster City, CA) was used to generate transcriptional PCR products from the cDNA templates. The primers used to generate all transcriptional products are summarized in Table 2. The control transcripts in each were *sigB,* the transcript for an alternative sigma factor in *S. aureus* and *asp23*, the transcript for the alkaline shock protein, which is a SigB-regulated transcript (14,21). PCR products from all transcripts were separated on a 1.5% agarose gel, stained with ethidium bromide and imaged using the ChemiDoc XRS (Bio-Rad, Quantity One Quantitation software, Hercules, CA) and scanning densitometry was carried out.

Isolation of Chromosomal and Plasmid DNA. Chromosomal and plasmid DNA were both isolated as previously described by Katzif *et al*. (20). For isolation of chromosomal DNA, the DNeasy Tissue Kit (Qiagen, Valencia, CA) was used with the following modifications: lysostaphin was used at a concentration of 50 µg/ml instead of lysozyme, a one hr incubation at 37°C was used, and 4 µl of RNase A (final concentration of 0.4µg/ml) was added to each sample after lysis and incubated at room temperature for 10 minutes. Chromosomal DNA was eluted in 100µl of AE buffer (Qiagen, Inc.) and the eluate was then applied to a spin column and eluted after a 5 minute incubation. High-copy plasmids were isolated from 5 ml overnight cultures of TOP10® *E. coli* cells (Invitrogen) using the Qiagen mini-preparation technique. Low-copy plasmids propagated in *E. coli* were also isolated using the Qiagen mini-preparation technique as described by Katzif *et al*. (21).

Plasmid construction and genetic exchange procedures. Plasmid pBD1 was used to introduce a nonpolar insertion using the *aphA-3* cassette encoding kanamycin resistance

into the open reading frame of *cspB*. Plasmid pBD2 was constructed to re-introduce *cspB* on a low copy vector *in trans*. Plasmids were constructed essentially as previously described (2,5,17,20). To construct pBD1, a 1.4Kb region containing *cspB* was PCR-amplified from genomic DNA from strain COL. Primers cspB5241 and cspB3743S were used to amplify a fragment containing *cspB* with a 3′ SmaI restriction site. All restriction enzymes were obtained from Promega (Madison, WI). Primers csp5723S and cspB31617 were used to amplify a 3′ region containing *cspB*, with an internal SmaI site. Both fragments were ligated together and the entire fragment was cloned into the pCR 2.1 vector (Invitrogen). This plasmid was transformed into *E. coli*, isolated, and digested with KpnI and XbaI. The fragment containing *cspB* was gel purified and ligated onto plasmid pUC19 digested with KpnI and XbaI. The *aphA-3* cassette was released from plasmid pUC18K by SmaI and then ligated into the SmaI site of the *cspB* coding region on plasmid pUC19. The entire *cspB* fragment containing the kanamycin-resistance cassette was isolated, purified, and then ligated onto a temperature sensitive *E. coli – S. aureus* expression vector, pBT2. The resulting plasmid, pBD1, was maintained in *S. aureus* strain RN4220 and moved into strain COL for allelic exchange. Following the method of Brückner for allelic exchange in *S. aureus* (5), strains containing plasmid pBD1 were grown on TSA at either 30°C for maintenance of the plasmid or at 42°C to select for the double crossover with the appropriate antibiotic. Liquid cultures of *S. aureus* were prepared using TSB and were grown at the appropriate temperature for either maintenance or loss of the plasmid. A similar procedure was used to create plasmid pBD2, which was used for complementation of strain BD1, but the *aphA-3* cassette was not inserted into the SmaI site. Staphylococcal phage Φ11, was used for transduction of

pBD2 into strain BD1 as previously outlined by Shafer and Iandolo (42). Phage Φ11 was induced using mitomycin C (1µg/ml), and used to infect RN4220 (pBD2) to obtain a transducing lysate. Transductants of strain pBD1 were selected on TSA plates containing 25 µg/ml of chloramphenicol per ml. The presence of pBD2 in representative transductants was confirmed by digesting isolated plasmid DNA with KpnI and XbaI followed by agarose gel electrophoresis.

Pulse field gel electrophoresis (PFGE) and Southern hybridization. PFGE was performed as outlined by PulseNet, The National Molecular Subtyping Network for Foodbourne Disease Surveillance of the CDC (Atlanta, GA) (43). Agarose plugs containing cells were prepared from 175 µl of overnight cultures of each strain grown in brain-heart infusion broth (Becton Dickinson Microbiology Systems, Cockeysville, MD) and digested with SmaI (New England Biolabs, Ipswich, MA). Plugs were placed into the well of a 1% (w/v) SeaKem® Gold (Cambrex Biosciences Rockland Inc., Rockland, ME) agarose gel and run with the following parameters: 200 v (6 v/cm), 14°C, 5 s initial switch, 40 s final switch for 21 hours. The gel was stained using ethidium bromide, de-stained, and then visualized using UV light. For Southern hybridization, a 391 bp fragment of *mecA* was PCR- amplified from *S. aureus* COL genomic DNA using primers mecA5F and mecA5R and used as a probe in Southern blot analysis. This *mecA* probe was prepared using the DIG DNA Labeling Kit (Roche Diagnostics Corporation, Indianapolis, IN) following the manufacturer's instructions. The digoxigenin (DIG)-labeled *mecA* fragment was confirmed by agarose gel electrophoresis. Southern blot analysis was performed as described by Satola *et al*. (39).

Antimicrobial susceptibility and pigment determinations. To determine the MICs of aminoglycosides (amikacin and tobramycin), methicillin, and paraquat, a modified disc-diffusion protocol from Chen and Morse (9) was used. Briefly, *S. aureus* strains were grown in TSB overnight and then diluted 10-fold in TSB. 200 µl samples were then plated onto TSA and incubated at 37 °C for 1 hr. Whatman filter paper discs (1.0 cm in diameter, Biometra, Goettingen, Germany) were soaked in a solution of the antimicrobial at various concentrations and then placed on the surface of the TSA plate and incubation was continued at 37 °C for 24 hr. Zones of growth inhibition were determined by measuring the diameter of the growth inhibition. All disc-diffusion assays were performed in triplicate. To determine the MIC values of daptomycin, gentamicin, teicoplanin and trimethoprim-sulfamethoxazole, Etest® strips (bioMérieux, Inc., Durham, NC) were used according to the manufacturer's instructions with the following changes: cells were grown in TSB at 37 °C for at least 20 h. For determination of MIC values after cold-shock, cells were grown at 37 °C until mid-log phase, the culture was then split into equal volumes and half was allowed to continue to grow at 37 °C or 15 °C for one hour. Etest® strips for daptomycin, gentamicin, or trimethoprim-sulfamethoxazole were then applied as per the manufacturer's instructions with plates from each temperature being allowed to grow at 37 °C or 15 °C.

Pigment produced by *S. aureus* strains was visualized by inspection of colonies grown on TSA and was quantitated by the methanol extraction protocol previously described (29). Briefly, overnight cultures of each *S. aureus* strain were grown in 5 ml of TSB at 37 °C for 24 h growth with the appropriate antibiotic. Cells were harvested from 850µl of the culture after it was diluted to 10^8 CFU/ml, centrifuged and washed once with an equal

volume of phosphate-buffered saline (PBS). The cells were re-suspended in 200 µl methanol and heated at 55 °C for 3 min. The supernatant was removed from the cell debris after spinning for 1 min at 13,000 rpm. The extraction was repeated once, the supernatants from each extraction were pooled into one tube, methanol was added to yield a final volume of 1 ml and the absorbance was measured at 465 nm.

Results and Discussion

***cspB* is the major cold inducible gene in *S. aureus* strain COL.** Previous work by Anderson *et. al*. (1) indicated that *cspB* is the major cold-inducible gene in strain UAMS-1. Although CSPs have been implicated in maintaining the fidelity of bacterial gene expression during exposure to low temperature (4,18), certain *csps* are expressed at 37°C and may have functions during normal growth. Earlier work by Katzif *et al*. (20) showed that production of CspA at 37 °C can influence levels of staphylococcal susceptibility to cationic antimicrobial peptides (CAP) and pigment production. Interestingly, a *hemB* mutant of *S. aureus* strain A22223I that displayed an SCV phenotype had altered levels of *cspB* expression compared to its *hemB* $^{+}$ parent (40). Accordingly, we sought to define the functions of CspB in this pathogen. As our previous studies (20, 21) on CAP resistance and pigment production in *S. aureus* were performed with strain COL, we first examined the levels of *csp* transcripts in this strain when a logarithmically growing culture was shifted from 37 °C to 15 °C. Using RNA extracted from control and cold-shocked cultures of strain COL and RT-PCR reactions to detect transcripts from the three main *csp* genes (*cspA*, *cspB* and *cspC*) and two control genes (*sigB* and *asp23*) not differentially expressed during cold shock, we determined that *cspB* is the major cold shock gene expressed by *S. aureus* COL (Figure 1). We found that the *cspA* transcript

was more abundant than either the *cspB* or *cspC* transcript in the culture maintained at 37 °C, but the *cspB* transcript predominated in the 15 °C culture; the ratios obtained when comparing the peak values at 15 °C to 37 °C were: 1.34 for *cspA*; 2.79 for *cspB* and 1.58 for *cspC.*

Loss of *cspB* in *S. aureus* COL leads to a severe growth defect and reduced pigmentation. In order to study the function of *cspB* in *S. aureus* COL, we created a null mutant (strain BD1) that contained a non-polar *aphA-3* insertion in the *cspB* coding sequence. To verify that phenotypic differences (see below) were linked to this mutation, we also created a complemented strain (BD2) that had *cspB* expressed in *trans* from pBD2. Strain BD1 demonstrated many phenotypic differences compared to parent strain COL and complemented strain BD2. The most obvious differences were the smaller, pinpoint colonies formed by BD1 that were less pigmented than the larger colonies from either parental strain COL or complemented strain BD2 (data not presented). The reduced level of pigmentation seen with colonies or cell pellets (data not presented) of BD1 compared to those of COL and BD2 was confirmed by measuring the level of methanol-extractable carotenoids (Figure 2). When growth of these strains in TSB at 37 °C was monitored, we observed that the parent and complemented strains grew similarly, but BD1 had a severe growth defect (Fig. 3A). Strain BD1 was also less proficient in responding to cold shock than parent strain COL (Figure 3B), which was reversed by complementation (data not presented). The growth defect and reduced pigment properties of BD1 were stable as spontaneous revertants could not be isolated (data not presented). We also found that additional *cspB::aphA-3* mutants of COL expressed growth and pigment phenotypes resembling BD1 (data not presented).

Although complementation with the wild type *cspB* gene expressed from pBD1 was able to restore both normal growth and levels of pigment production, we were concerned that second site mutations in *hemB* and/or *menD*, which have been associated with the SCV property of *S. aureus* (45), might exist in BD1 and contribute to some of its SCV-like phenotypes. However, we found that the coding sequences of *hemB* and *menD* were identical in all three strains (data not presented). Since BD1 exhibited growth and pigment properties resembling previously reported SCVs (26,35,46) yet had wild type *hemB* and *menD* sequences, we concluded that multiple mechanisms contribute to the appearance of SCVs in *S. aureus*, including expression of *cspB*.

Loss of *cspB* impacts levels of antimicrobial resistance. Since SCVs have been reported to display increased resistance to certain antimicrobials (19,26,35), notably aminoglycosides, and BD1 exhibited SCV-like characteristics, we examined antimicrobial resistance levels of BD1 and compared them to parental strain COL and complemented strain BD2. BD1 was more resistant than COL or BD2 to a panel of aminoglycosides (gentamicin, amikacin, and tobramycin), trimethoprim-sulfomethoxazole (TMS) and paraquat (Table 3 and Figure 4). In contrast, BD1 was more sensitive than COL and BD2 to daptomycin, which is an anionic antimicrobial lipopeptide whose activity depends on the presence of calcium ions (6,10,12), and teicoplanin (Table 3). Interestingly, however, when compared to COL, both BD1 and BD2 were very sensitive to methicillin, which we subsequently found to be due to loss of the type I SCC*mec* cassette (see below).

Since loss of *cspB* influenced the antimicrobial susceptibility profile of staphylococci, we tested if exposure of *S. aureus* COL to cold shock would change its level of

susceptibility to antibiotics. For this purpose, a mid-logarithmic culture was shifted from 37 °C to 15 °C and the MICs of daptomycin, gentamicin and trimethoprim-sulfamethoxazole were determined by Etest® incubated at 15 °C and 37 °C for both the control (37 °C) and cold-shocked cultures (15 °C) (Table 4). For the control culture grown and maintained at 37 °C, there was a nearly four-fold increase in susceptibility to both daptomycin and trimethoprim-sulfamethoxazole when the Etest® assay was incubated at 15 °C compared to the Etest® assay incubated at 37 °C (p = 0.0021 and 0.002, respectively). For the cold-shocked culture, an increase in susceptibility to daptomycin (2.4 fold, p = 0.0021) and trimethoprim-sulfamethoxazole (1.8 fold, p = 0.013) when the Etest® assay was incubated at 15 °C. Additionally, the cold-shocked culture was more susceptible to trimethoprim-sulfamethoxazole (3.2 fold, p = 0.006) when the Etest® strip assay was incubated at 37 °C. A similar decrease in daptomycin resistance after exposure to cold temperature was observed in strains BD1 and BD2 (data not presented). Hence, we propose that membrane changes independent of *cspB* likely account for the increased susceptibility of staphylococci to daptomycin when the bacteria are exposed to 15 °C.

Loss of the type I SCC*mec* cassette in BD1. Recent work by Noto *et al.* documented spontaneous excision of *mecA* from clinical *S. aureus* strains in response to their prolonged exposure to vancomycin (31). Our observation that strains BD1 and BD2 were, in contrast to their MRSA parental strain COL, highly susceptible to methicillin, lead us to investigate the presence of the *mecA* gene and the full type I SCC*mec* cassette in these strains. Using PCR primers to amplify the *mecA* gene, a product corresponding to the *mecA* gene (~1.6 Kb) was obtained from genomic DNA from parent strain COL but not

BD1 or BD2, indicating that *mecA* was absent in the mutant and complement. To examine the extent of the deletion in this region, PCR was used to amplify several genes (e.g., *orfX*, *mecA*, *ccrA*, and *pls*) that spanned the entire SCC*mec* cassette in COL (Fig. 5B). While these genes (*orfX*, *mecA*, *ccrA* and *pls*) were readily amplified from COL chromosomal DNA, PCR products were not obtained when BD1 and BD2 DNA preparations were used as templates (Figure 5A). To further examine the extent of this deletion, PFGE and Southern blot hybridization analyses were employed. The PFGE analysis demonstrated a unique SmaI pattern of strain COL versus strains BD1 and BD2 in that the latter strains lacked an approximate 200 kB fragment that was present in strain COL (see arrow in Fig. 5C). As analysis of the COL genome sequence (cmr.jcvi.org) showed that the type I SCC*mec* cassette is harbored on a 204 kB SmaI fragment, we hypothesized that this roughly 200 kB fragment in COL contained the *mecA* cassette. Indeed, Southern blot hybridization analysis on the gel shown in Fig.5C that used a 391 bp *mecA* gene fragment probe showed this to be the case and that no bands in the SmaI digests of BD1 and BD2 hybridized to the probe. Taken together with PCR analysis (Figure 5A), we conclude that the entire type I SCC*mec* cassette was deleted in BD1 and BD2, indicating that these strains have at least a 30 kB deletion compared to parental strain COL. As BD1 and BD2 have a unique band corresponding to approximately 170 kB (see asterisk in Fig. 5C); we tentatively conclude that it was generated due to deletion of the complete type I SCC*mec* cassette.

We have confirmed that *cspB* is the major cold shock gene in *S. aureus* COL during growth at low temperature (Fig.1). Not unexpectedly, a *cspB* null mutant (BD1) of this MRSA strain had a reduced capacity to grow at 15 °C. Unexpectedly, however, it

displayed several properties previously reported by others (26,35,46) for SCVs of clinical and laboratory strains of *S. aureus*. SCVs are phenotypically distinct subpopulations of *S. aureus* that have been implicated in persistent and drug resistant infections (36). While it has been previously shown that mutations in certain genes (e.g., *hemB* and *menD*) involved in components of the electron transport chain (7,22,30,40,45,46) can lead to the phenotypes seen in SCVs, the exact genetic mechanisms that allow *S. aureus* to accomplish this phenotypic shift remain unclear. Although BD1 shares many properties previously described in SCVs (e.g., slow growth rate, decreased pigmentation and resistance to aminoglycosides), there are important differences, notably the absence of *hemB* or *menD* mutations in BD1. Given the many similarities between our *cspB* insertional mutant and SCVs, we propose that *cspB* could play a part in this phenotype shift. In this respect, Seggewiss *et al*. (40) noted that expression of *cspB* (SA2494) in *S. aureus* A22223I was up-regulated in a model SCV *hemB* insertional mutant of this strain when compared to its isogenic parent. Although our results seem at variance with those of Seggewiss *et al.* (40), it is important to note that our groups worked with different strains (COL vs. A22223I). Moreover, our work was performed in a *hemB*$^{+}$ background, while that of Seggewiss *et al.* was done with a *hemB* mutant and it might respond differently at the transcriptional level to cold shock. The altered antimicrobial susceptibility profile of BD1 compared to parental strain COL was of interest due to its increased resistance to aminoglycosides and paraquat and susceptibility to daptomycin and methicillin. While the aminoglycoside/paraquat resistance and daptomycin susceptibility properties of BD1 were reversed by complementation with the wild type *cspB* expressed in *trans*, complementation failed to re-establish methicillin resistance. For

reasons that remain unclear, BD1 (and other *cspB* null mutants of strain COL [data not presented]) had an apparent deletion of the type I SCC*mec* cassette (Figure 4). We do not yet know if CspB contributes to maintenance of this cassette, or if the deletion of the cassette was a secondary event that helps to reduce a fitness cost associated with loss of CspB production. It has been hypothesized (31) that deletion of SCC*mec* reflects an attempt by staphylococci to gain a competitive advantage over those that still harbor an intact SCC*mec*.

Resistance to aminoglycosides in staphylococcal SCVs has been previously attributed to a diminished or inadequate membrane potential (34,36). It is unknown if this is the reason for the resistance phenotype seen in BD1 (Table 3 and Figure 4). However, the extreme resistance to paraquat (Table 3) seen in BD1 is suggestive of this mechanism. Paraquat is a redox cycling agent that exerts its toxicity by producing superoxide anions in the presence of oxygen, which can then form other reactive oxygen species such as hydrogen peroxide and hydroxyl radicals (3). In bacteria, it has been shown that protection against paraquat toxicity can be imparted by increasing the cellular levels of superoxide dismutase (28) and that the reduced form of paraquat can cross the bacterial membrane (37). These observations suggest that the positively-charged reduced form of paraquat is either unable to cross the bacterial membrane and exert its normal activity in the cytoplasm or that the protection seen in BD1 is due to increased levels of superoxide dismutase. If paraquat is unable to cross the bacterial membrane due to improper membrane potential, this likely explains the increased resistance to aminoglycosides seen in BD1.

Although the role of the cold shock proteins in *S. aureus* remains unclear, previous work on these proteins in *E. coli* suggest they play roles as RNA chaperones (4,18), transcriptional anti-terminators (4) and as an alternative initiation factor during translation. We are currently determining the mechanism(s) by which CspB functions in *S. aureus* under normal growth conditions and during cold shock so as to understand how it regulates production of virulence factors and levels of bacterial susceptibility to antimicrobials.

Acknowledgements

We thank Christal Hembree for help with the PFGE analyses, P. Johnson for help with figures and Lane Pucko for help with manuscript preparation. This work was supported by NIH grant AI043316 and funds from a VA Merit Award from the Medical Research Service of the Department of Veterans Affairs, both to W.M.S. W.M.S. is the recipient of a Senior Research Career Scientist Award from the Veterans Affairs Research Service. S.S. is supported by the Centers for Disease Control and Prevention's Emerging Infections Program.

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Figure Legends

FIG. 1. Densitometry analysis of selected *S. aureus* transcripts before and after cold stress. RNA was prepared as previously described and a Bio-Rad® Chemi-doc Xl was used to measure density of electrophoresed cDNA. Intensity is reported in proprietary units as a function of location on the gel. The peak intensity was reported for each transcript at 37 °C (A) and after cold shock for 1 hour at 15 °C (B).

FIG 2. Pigment production by strains COL, BD1, and BD2. Levels of pigment in the test strains were quantified using a methanol extraction protocol adapted from Morikawa *et al.* (29) and the result for each strain is the average of three different samples done in triplicate. The differences in pigment between COL vs. BD1 and BD1 vs. BD2 were significant (*p* value = 0.001).

FIG. 3. Growth differences observed between staphylococcal strains. (A) Growth experiments were carried out in TSB at 37 °C. Growth was monitored each hour by reading the optical density at 600nm from cultures started at an identical optical density of 0.05. (B) For cold shock experiments, strains were initially both grown at 37 °C until their respective mid-log phases. The cultures were then split (see arrow) into equal volumes and either grown at 37 °C or shifted to 15 °C. Growth of each culture was monitored hourly at OD_{600}.

FIG 4. Disk diffusion analysis of selected antimicrobials. Disk diffusion assays were performed as previously described (9). Concentrations of amikacin (A), tobramycin (B), and methicillin (C), are given in µg/ml, while the concentration of paraquat (D) is given in µmol. The zone of inhibition is defined as the diameter of the zone of clearance

surrounding the impregnated filter disk as measured in centimeters. Zones of inhibition were measured after plates were incubated overnight at 37 °C.

FIG. 5. Loss of type I SCC*mec* genes in strains BD1 and BD2. Selected genes associated with the type I SCC*mec* cassette were amplified using PCR. As a control, 16S rRNA was amplified in each reaction (A). The primers are listed in Table 2 and each strain used as template is indicated above. (B) The genetic organization of selected genes on the type I SCC*mec* cassette. This diagram illustrates the approximate size and orientation of select genes on the cassette. This diagram is not drawn to scale. (C) The PFGE SmaI restriction patterns of strains COL, BD1, and BD2 were compared; from left to right and the lane order is the SmaI fragments of the *Salmonella* serotype Branderup strain H9812 genome (STD), COL, BD1 and BD2. The size of SmaI fragments near the 200 kB fragment from COL are indicated. Southern blot analysis was used to confirm deletion of the type I SCC*mec* cassette. A DIG-labeled *mecA* PCR product was used to probe for the fragment containing the SCC*mec* cassette in the staphylococcal genomes and this probe hybridized only with the 200 kB SmaI fragment (see arrow) from strain COL (data not shown; see arrow in figure). The asterisk next to the nearly 170 kB band shows the unique SmaI fragment in strain BD1 and BD2 that likely resulted from loss of the type I SCC*mec* cassette.

Table 1. Bacterial strains and plasmids

Strain or Plasmid	Genotype	Source
E. coli strains		
TOP10	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ* ΔM15 Δ*lacX*74 *deoR recA*1 *araD139* Δ(*ara-leu*) 7697 *galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
S. aureus strains		
RN4220	*rsbU res* mutant strain used for electroporation with *E. coli*-replicated plasmids	J. Iandolo (23)
8325-4 (Φ11)	8325-4 harboring phage Φ11	J. Iandolo (32)
BD1	Nonpolar *aphA-3* Km^r cassette insertion into the 5 prime end of the *cspB* coding region	This study
BD2	Complement of strain BD1 using plasmid pBD1	This study
Plasmids		
pBT2	Low-copy-number *E. coli-S. aureus* shuttle vector with	(5)

	Amp^r in *E. coli*, and Cm^r in *S. aureus* with temperature sensitive origin of replication	
pBD1	pBT2 construct containing 2.4Kb region with *aphA-3* cassette inserted into the 5 prime end of the *cspB* coding region	This study
pBD2	pBT2 construct containing 1.4kb region with wild type *cspB*	This study
pUC19	High-copy-number *E. coli* host Amp^r	(47)
pUC18K	pUC18 with *aphA*-3 nonpolar Km cassette Amp^r	(27)
pCR2.1	High-copy-number PCR cloning vector; Amp^r and Km^r in *E. coli*	Invitogen

Table 2. Oligonucleotide primers used in this investigation

Primer	Sequence (5′-3′)	Reference
cspB3743S	CATTTTACTGTCCCGGGATTC	This study
cspB5723S	GAATCCCGGGACAGTAAAATG	This study
cspB5241	GCGTAGTTACAACACCAATTATAG	This study
cspB31617	CGTATGTAATTGAATCTGAGTAAAC	This study
sigB52673	ATGGCGAAAGAGTCGAAATC	This study
sigB33421	CTATTGATGTGCTGCTTCTT	This study
asp2351	GAAAACTTCTGGTCCACGAG	This study
asp233512	GCAGCGATACCAGCAATTTT	This study
rjmec	TATGATATGCTTCTCC	(10)
ORFX1r	AACGTTTAGGCCCATACACCA	(10)
ccrA5	CGACAGAGCACTACAAAGCA	This study
ccrA3	GCTGCTCGTGATTGAGTGTA	This study
mecA5F	CATATGACGTCTATCCATTT	This study
mecA5R	TCACTTGGTATATCTTCACC	This study
mecA5	GTTGTAGTTGTCGGGTTTGG	This study
mecA3	CCGTTCTCATATAGCTCATC	This study

Table 3. MIC Data Summary

Strain	MIC values			
	Dp	Gn	Tc	Tm
COL	1.5	0.3	3.0	2.0
BD1	0.064	32	0.75	>32
BD2	2.0	0.3	2.0	1.0

[a] Values were determined by Etest® strips according to the manufacturer's instructions for daptomycin (Dp), gentamicin (Gn), teicoplanin (Tc), and trimethoprim-sulfamethoxazole (Tm).

Table 4. Influence of growth and assay temperature on the antibiotic susceptibilities of *S. aureus*

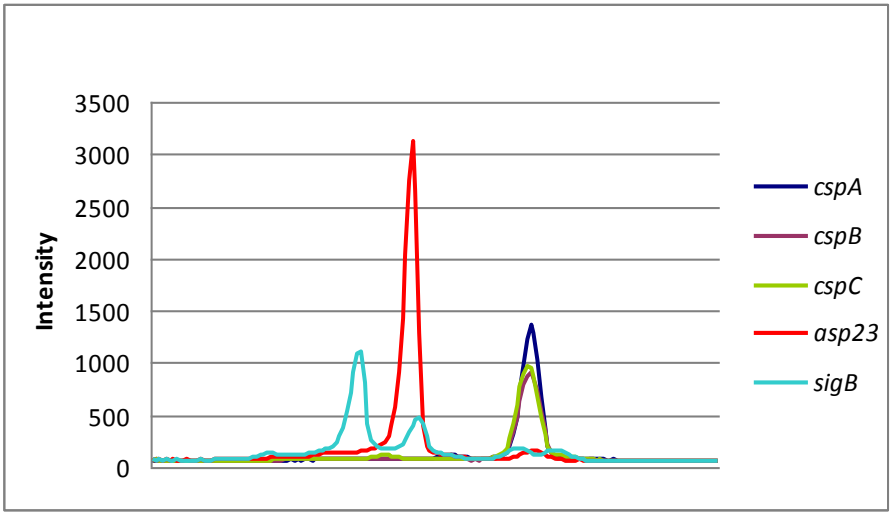
Antibiotic	Growth Temperature	Assay Temperature		
	37° C	15° C	37° C	*p* [a]
		Average MIC (μg/ml)		
Daptomycin		0.46 ± 0.07	1.5 ± 0.0	0.0021
Gentamicin		0.21 ± 0.04	0.34 ± 0.07	0.057
Trimethoprim-Sulfamethoxazole		0.25 ± 0.0	1.33 ± 0.29 [b]	0.002
	15° C			
Daptomycin		0.63 ± 0.21	1.5 ± 0.0	0.0021
Gentamicin		0.29 ± 0.08	0.38 ± 0.0	0.12
Trimethoprim-Sulfamethoxazole		0.23 ± 0.03	0.42 ± 0.07 [b]	0.013

[a] *p* values shown in the table are from a comparison of results from assays performed at 15° vs. 37° C

[b] MIC comparison of *S. aureus* strain COL grown at 37° C or 15° C and tested for antibiotic susceptibility to trimethoprim-sulfamethoxazole at 37° C, *p* value = 0.00

FIG. 1.

A.


3500
3000
2500
2000
1500
1000
500
0
Intensity
cspA
cspB
cspC
asp23
sigB

B.

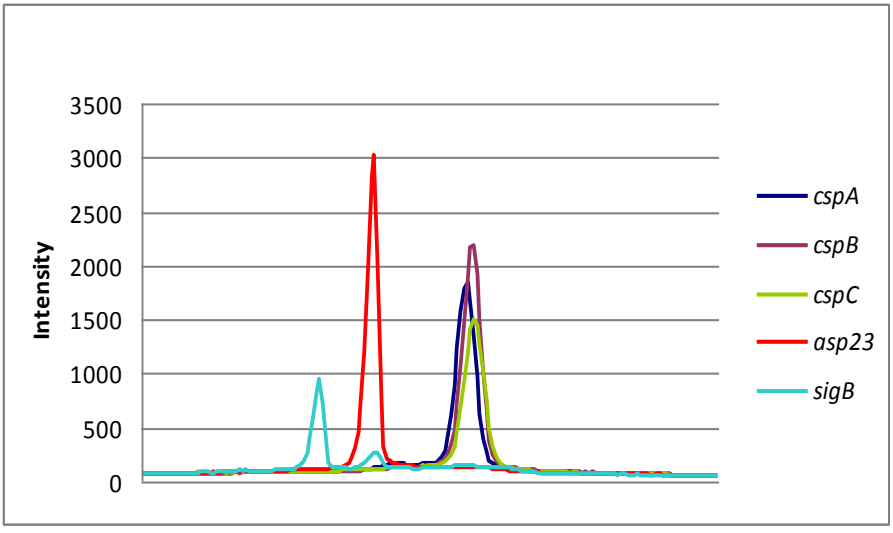

3500
3000
2500
2000
1500
1000
500
0
Intensity
cspA
cspB
cspC
asp23
sigB

FIG. 2.

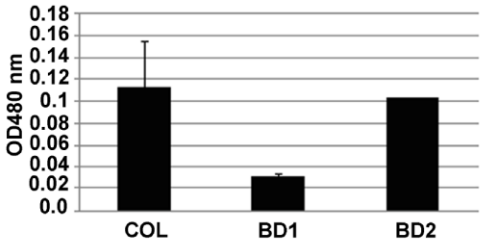
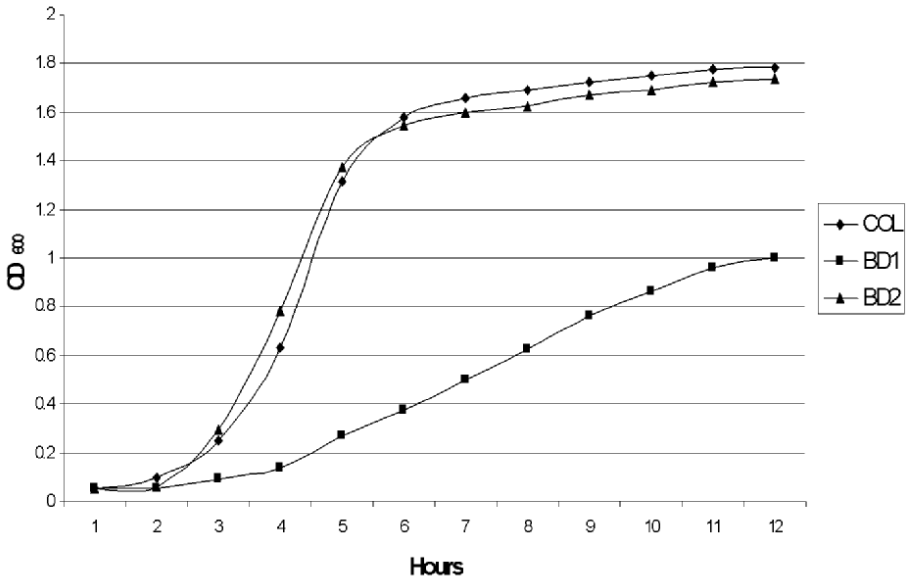


FIG. 3.

A.


2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD 600
1 2 3 4 5 6 7 8 9 10 11 12
Hours
CCL
BD1
BD2

B.

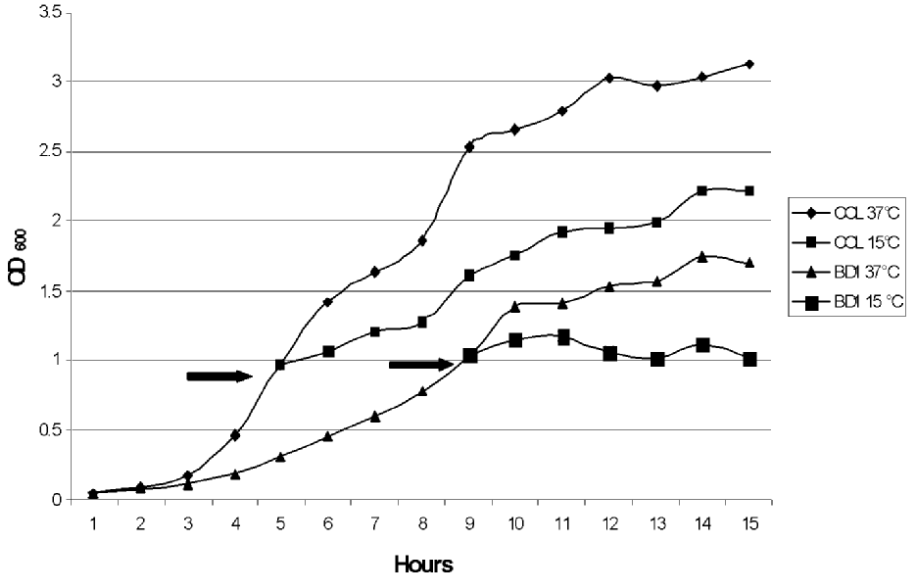

3.5
3
2.5
2
1.5
1
0.5
0
OD 600
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Hours
CCL 37°C
CCL 15°C
BDI 37°C
BDI 15 °C

FIG. 4.

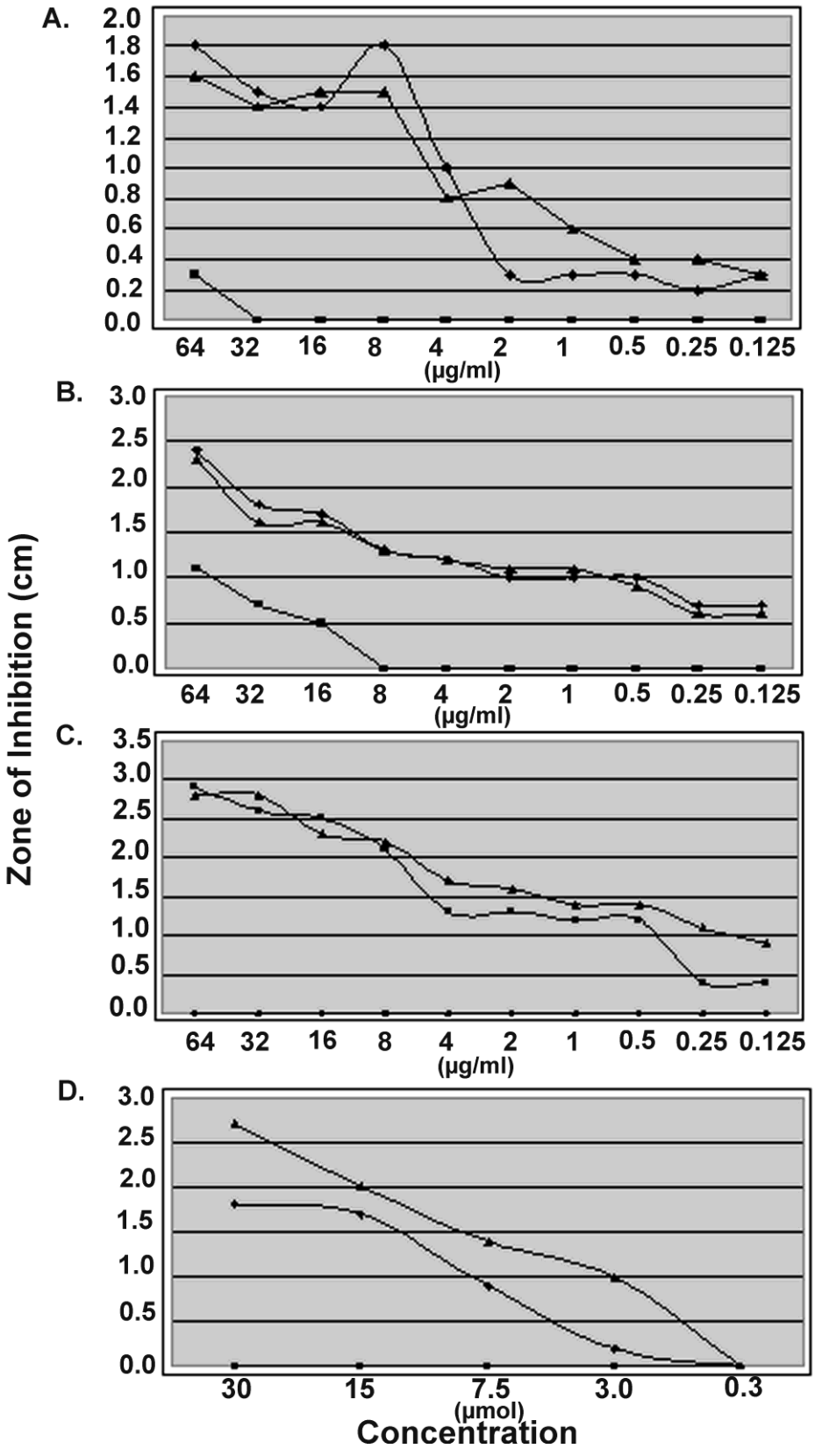
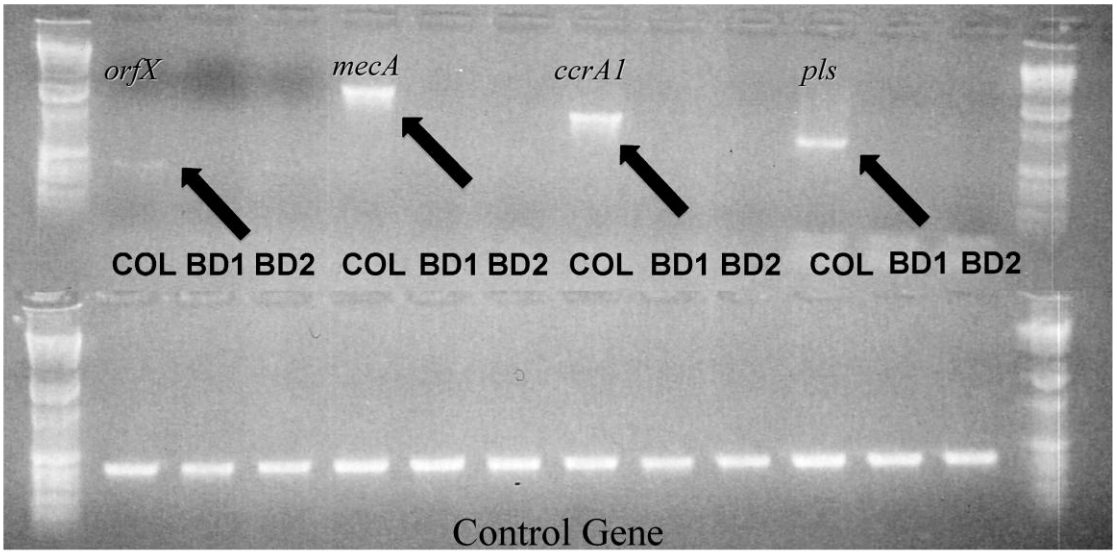

A.
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
64 32 16 8 4 2 1 0.5 0.25 0.125
(µg/ml)
B.
3.0
2.5
2.0
1.5
1.0
0.5
0.0
64 32 16 8 4 2 1 0.5 0.25 0.125
(µg/ml)
C.
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
64 32 16 8 4 2 1 0.5 0.25 0.125
(µg/ml)
D.
3.0
2.5
2.0
1.5
1.0
0.5
0.0
30 15 7.5 3.0 0.3
(µmol)
Zone of Inhibition (cm)
Concentration

FIG. 5.

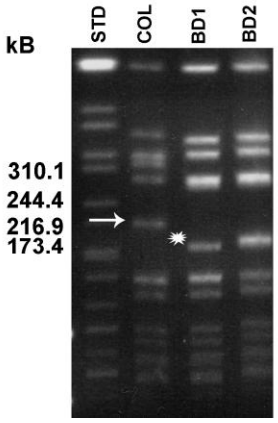
A.


orfX
mecA
ccrA1
pls
COL BD1 BD2
COL BD1 BD2
COL BD1 BD2
COL BD1 BD2
Control Gene

B.


orfX
IS431mec
mecA
mecR1
ccrB
ccrA
pls

C.


kB
STD
COL
BD1
BD2
310.1
244.4
216.9
173.4

Chapter 3: Unpublished Observations

1. Loss of *cspB* impacts the cell surface of *S. aureus*
2. Microarray analysis
3. Biofilm analysis

1. Loss of *cspB* impacts the cell surface of *S. aureus*

Previous work has shown that SCVs often have significant changes in colony morphology when evaluated by electron microscopy (12). Electron microscopy of thymidine-auxotrophic SCVs has shown that these bacteria often have an altered appearance from their wild type parent strains (12) with incomplete or multiple cross walls. We wanted to find out whether or not the small colony size of strain BD1 was due to the individual cells having a smaller size and whether or not loss of *cspB* leads to changes in the cell surface properties of *S. aureus*. To answer these questions, scanning electron microscopy (SEM) was performed with the help of Dr. Hong Yi at the Robert P. Apkarian Integrated Electron Microscopy Core at Emory University. Strains COL, BD1, and BD2 were all subjected to SEM analysis at various magnifications. Figure 1 demonstrates the major phenotypic differences seen by the SEM analysis that was performed. Strains COL (Fig. 1A) and BD2 (Fig. 1C) showed abundant amounts of an unknown extracellular material on their respective cell surfaces. Interestingly, this extracellular material was nearly absent on the cell surface of BD1 (Fig. 1B) in all electron micrographs taken at the same magnification (10,000x). Previous work by Kahl *et al*. demonstrated that in their clinical thymidine-dependent SCVs, there was actually more of a similar extracellular material than observed on the wild type strains when the strains were subjected to SEM analysis; however the authors did not further investigate the biofilm forming capacity of these mutants (12). In their communication, the authors

also did not hypothesize what this material might be. We hypothesize that this material could be polysaccharide intracellular adhesin (PIA), which is responsible for facilitating cell-cell adhesion of *S. aureus* and is also important in biofilm formation (6). In our microarray analysis (to be discussed below), we found that genes belonging to the *ica* locus, which is responsible for producing the extracellular polysaccharide required by *S. aureus* for biofilm formation (6), to be significantly down-regulated (2.4-2.7 fold) due to loss of CspB. More detailed studies involving the use to monoclonal antibodies against PIA to probe the surface of our strains for differences in this surface antigen will need to be done to test this hypothesis.

2. Microarray analysis

Based on the drastic phenotypic differences between strains COL and BD1 seen on plates, as well as the dramatic cell surface differences seen from our SEM analysis, we were interested in understanding the global transcriptional changes that might have occurred due to loss of *cspB* in strain BD1. To ascertain the changes in transcription between our strains, we employed microarray analysis with the help of Dr. Allison Gillaspie, Ms. Mandi Gipson, and Dr. David Dyer at the Oklahoma University Health Sciences Center. Previously published microarray analysis performed on genetically defined model SCV strains (15,17), has shown that SCVs have changes in many genes compared to their isogenic parent strains. These genes include those involved primarily in pathogenesis and metabolism (15).

We wanted to use microarray analysis to determine whether or not the types of transcriptional changes seen in other genetically defined SCV strains would be seen in

strain BD1, which demonstrated many aspects of the SCV phenotype. To accomplish this, strains COL and BD1 were sent to Dr. Gillaspie and Ms. Gipson for microarray analysis. Both strains were grown in TSB to 10 hours, at which time cells were harvested for RNA extraction. Cells were harvested in triplicate and after RNA was extracted, the samples were pooled. cDNA was then synthesized and biotin labeled and the labeled cDNA was sent to the University of Iowa for analysis using an Affymetrix GeneChip *S. aureus* Genome Array. This genomic array contains over 3300 open reading frames (*orfs*) from four different strains of *S. aureus*. The *orfs* included on this chip are from strains COL (TIGR), NCTC 8325 (University of Oklahoma), N315 (National Institute of Technology and Evaluation, Japan), and Mu50 (National Institute of Technology and Evaluation, Japan).

In our analysis, only genes that showed a statistically significant ($p < 0.05$) fold change of greater than 1.5 in strain BD1 when compared to the wild type were considered. In all, 870 genes were shown to be differentially regulated due to loss of CspB, which represents 31% of the total genes found in strain COL (tigr.org). This total consists of 457 genes that were significantly down-regulated, and 413 that were significantly up-regulated (Fig. 2). It has been previously shown that staphylococcal SCVs can show significant down-regulation in transcripts involved in pathogenesis and virulence (14). Of the 457 genes that were differentially down-regulated in strain BD1 as compared to strain COL, 93 of those genes encode proteins that are involved in virulence and pathogenesis. Of particular interest to us was the change in transcription of genes in the *icaADBC* operon, which is responsible for production of polysaccharide intracellular adhesion (PIA), which is required for biofilm formation in staphylococci (6). Based on

our SEM analysis, which showed a decrease in an extracellular material on the cell surface of BD1 as compared with strain COL, we wanted to ascertain whether or not it was reasonable to assume that loss of *cspB* could lead to a decrease in transcription of genes responsible for PIA production. Divergently transcribed from *icaADBC* locus is *icaR*, which specifically represses transcription of the *icaADBC* operon (9). Previous work defining the changes in transcription of the *ica* operon in *Staphylococcus epidermidis* has shown that these SCVs show an increase in transcription of genes found at this locus (1). In our microarray analysis, three of the four genes found in the *ica* operon (*icaA*, *icaB*, and *icaD*), met our requirements and showed significant down-regulation. These genes were shown to be down-regulated between 2.4 (*icaA*, *icaB*) and 2.7 fold (*icaD*). Of note, *icaR* did not appear to be subject to CspB regulation and possible reasons to explain the down-regulation of this operon will be discussed below.

The *icaADBC* locus is also regulated by another repressor, TcaR. TcaR, a MarR family transcriptional regulator, is a part of the teicoplanin resistance locus (*tcaRAB*), which is responsible for modulating resistance to teicoplanin in *S. aureus* (4). *tcaR* has also been shown to be a secondary repressor of the *ica* locus (10). In our microarray analysis, we found that *tcaR* was up-regulated ~2.0 fold. We hypothesize that CspB is somehow indirectly negatively regulating *tcaR*, and therefore is contributing to the decrease of expression of genes belonging to the *ica* locus (Figure 3). It has been previously been shown that IcaR (6) and TcaR (10) can both regulate expression at the *ica* locus. From our microarray analysis, we saw an approximate 2.0 fold increase in expression of the *tcaR* transcript in our *cspB* insertional mutant versus the wild type parent strain. We used this information to form a model that provides a mechanism for

how CspB negatively regulates the expression of *tcaR* through binding of CspB to *tcaR* mRNA (Figure 3). Cold shock proteins have been shown to bind mRNAs stabilizing them and preventing the formation of secondary structures that would inhibit translation during cold shock conditions (2). It is possible that under normal growth temperatures CspB binds to the *tcaR* mRNA and destabilizes it thereby inhibiting its translation. To test this hypothesis, RNA binding assays would need to be undertaken to assess the ability of CspB to bind to *tcaR* mRNA. While it would seem most likely that CspB would bind to the mRNA of the *tcaR* transcript, it is also possible that CspB might somehow act at the transcriptional machinery, repressing the expression of the mRNAs (Figure 3). We believe that this increased expression of *tcaR* could partially explain the lack of extracellular material on the cell surface of strain BD1 seen in our SEM analysis if this material is PIA.

3. Biofilm analysis

Biofilms are an important aspect of staphylococcal pathogenesis that have been the subject of several recent investigations (7,8). Biofilms are one strategy that *S. aureus* uses to evade the host immune response and antibiotic therapies (13). Previous work has illustrated that menadione-dependent staphylococcal SCVs form much more robust and ordered biofilms with more PIA than their isogenic parent strains (16).

Based on our microarray analysis where genes found at the *ica* locus were significantly down-regulated in strain BD1 when compared to strain COL, we wanted to investigate the biofilm-forming phenotype of all three strains of interest. To do this, we used a biofilm assay protocol previously reported by Beenken *et al*. (3). Briefly,

overnight cultures of all three strains were grown in TSB supplemented with 0.5% glucose and 3.0% sodium chloride, which has been shown to enhance the biofilm forming capacity of *S. aureus* (Beenken *et al*. 2003). The following morning, 200 µl of 10^5 cfu/ml of each strain were inoculated into a 96-well, non-treated, polystyrene plate (Corning) that had been pre-coated with 20% human plasma. The plates were allowed to incubate for 48 hrs at 37° C without shaking and the cells were fixed with 100% ethanol. The fixed cells were stained with 0.41% (w/v) crystal violet, washed twice with PBS, and the wells were allowed to dry. After the wells containing the cells were dry, the stained cells were resuspended in 100 µl of 100% ethanol and the absorbance was read at OD_{595}.

Figure 4 demonstrates the difference in absorbance as read at 595 nm for all three strains. Strain COL made significantly more biofilm than strain BD1 ($p = 0.010$). Interestingly, however, while COL made more biofilm than the complemented strain BD2, the difference in biofilm formation between these two strains was not statistically significant ($p = .064$). When comparing strain BD1 with strain BD2, however, the difference in biofilm formed between these two strains was statistically significant ($p = .010$). Taken together with our microarray data, as well as previously published information on staphylococcal biofilms, we propose that PIA is not sufficient in *S. aureus* strain COL for biofilm formation and that Pls, the plasmin-sensitive surface protein, is an important cell surface component required for this process.

While previous studies have shown that SCVs can make a more robust biofilm than their isogenic parent strains (16), our *cspB* insertional mutant seems to make significantly less biofilm than parent strain COL. We think this is an important distinction between our *cspB* insertional mutant and other previously described SCV strains. We

believe that strain BD1 represents an important alternative SCV, with many of the phenotypic characteristics previously ascribed to SCVs, but with novel differences that might be important in clinical identification of these difficult to treat staphylococcal strains. Interestingly, we also see this reduced capacity to form a biofilm in the complemented strain BD2, although the difference between parent strain COL and complemented strain BD2 did not achieve statistical significance. We hypothesize that complementation of strain BD1 with *cspB* is sufficient to restore wild type levels PIA, but it is not sufficient to bring the biofilm forming ability of strain BD2 back to that of strain COL. Both BD1 and BD2 lack the type I SCC*mec* (Chapter 2). Present on the SCC*mec* of strain COL is *pls*, which encodes the plasmin-sensitive surface protein that has been previously implicated in being an important virulence factor in the establishment of septic arthritis in a mouse model of infection (11). It has also been further suggested that *pls* is important for adhesion of the bacteria at various stages of growth (11). Based on these results and what has been previously discussed in the literature, we propose that presence of PIA in *S. aureus* is not sufficient to establish a biofilm and that *pls* plays an important role in biofilm formation (Figure 5). In the complemented strain BD2, there is an intermediate biofilmin phenotype when compared to strain COL. It is possible that while the *ica* locus might be sufficient to restore some of the biofilm-forming ability of strain BD2, *pls* is required to bring back wild type levels of biofilm formation. *In vivo*, while Pls has been shown to have anti-adhesive properties during some phases of growth (5), it has also been shown to be important in the establishment of septic arthritis and it is also thought that the role of Pls having both

adhesive and anti-adhesive properties changes across different growth phases of the bacteria (11).

In order to test whether or not Pls is responsible for increasing the capacity of strains BD1 and BD2 to form a biofilm, first the type I SCC*mec* would have to be moved back into these strains via transduction. These strains complemented with the SCC*mec* containing *pls* would then be subjected to biofilm assays to determine their ability form a biofilm. If it is shown that restoring the SCC*mec* restores biofilm formation, plasmids containing *pls* would then be moved into strains BD1 and BD2 and these *pls* complemented strains would be used in biofilm assays. If these assays confirm that addition of *pls* is the only gene from the SCC*mec* that is required to re-establish the biofilm-forming ability of these strains that would confirm our hypothesis that Pls indeed plays a role in the biofilm forming ability of type I SCC*mec* containing *S. aureus*. If, however, addition of *pls* is not sufficient to re-establish the biofilm forming capacity of our type I SCC*mec* strains, a library of the cassettes generated through digesting it with a specific restriction enzyme could be constructed and these fragments could be added to plasmids and re-introduced into strains BD1 and BD2. These newly constructed strains containing plasmids carrying different fragments of the cassette could then be tested for their biofilm forming capacity. The genes present on each plasmid found in strains that were once again able to form a biofilm could be identified and then placed on individual plasmids and re-introduced into BD1 and BD2 where biofilm assays would again be carried out. This could identify the gene, or genes responsible for aiding in biofilm formation found on the type I SCC*mec* cassette.

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Figure Legends

FIG. 1. SEM analysis of strains COL, BD1 and BD2. Strains COL (A), BD1 (B) and BD2 (C) were subjected to scanning electron microscopy. The red arrow in each image represents the presence of extracellular material. All scans were taken at a maginification of 10,000x.

FIG. 2. Microarray analysis of strain COL vs. BD1. A total of 870 genes were shown to be differentially regulated between the targets strains. The criteria genes in this study needed to meet in order to be considered for analysis were to show a statistically significant fold change of greater than 2.0.

FIG. 3. Proposed model for CspB dependent regulation of the *ica* locus by TcaR. TcaR acts as a secondary repressor of the *ica* locus. Based on microarray analysis, as well as previously published work on the *tca* and *ica* loci, we propose that CspB can act on TcaR in several different way: (1) CspB binds TcaR preventing its binding at either the *ica* or *tca* locus, (2) CspB binds *tcaR* mRNA negatively regulating TcaR translation, or (3) CspB binds at the *tca* locus, blocking transcription of *tcaR*.

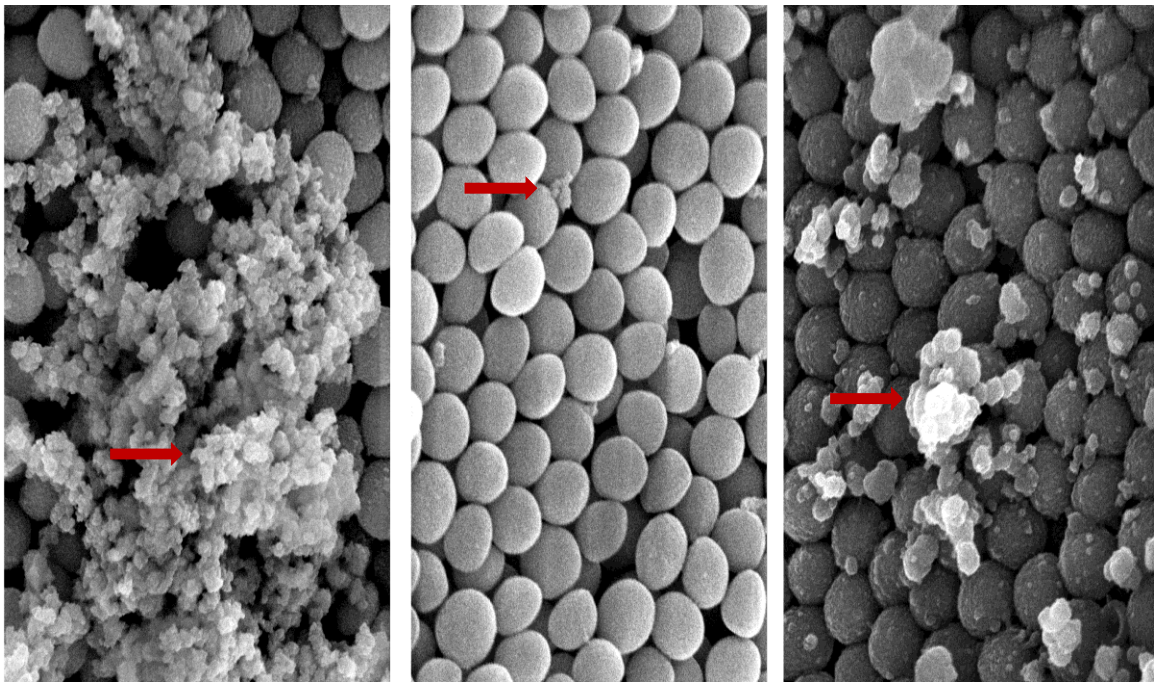
FIG. 4. Biofilm formation assays of strain COL, BD1, and BD2. Biofilm assays were performed as previously described (3). The difference in total biofilm between COL and

BD1 was statistically significant (p = .010), while the difference between COL and BD2 was not statistically significant (p = .064). The difference between BD1 and BD2 however was also statistically significant (p = .011).

FIG. 5. Proposed model for Pls dependent biofilm formation in *S. aureus* strain COL. The proposed model for Pls dependent biofilm formation in strain COL is depicted. It is proposed that this cell surface protein plays a crucial role in this process by acting to facilitate adhesion between cells.

Figures

FIG. 1



(A) (B) (C)

FIG. 2.

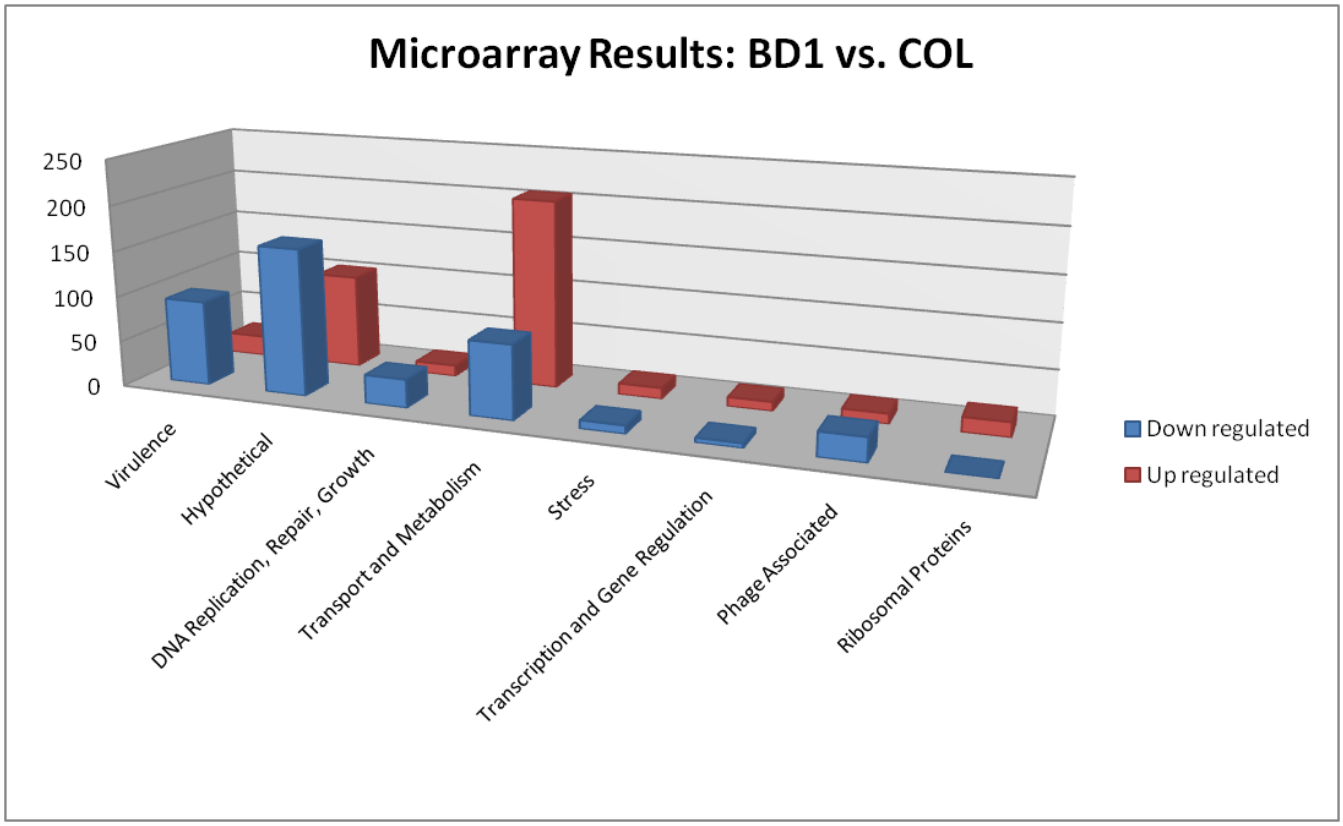

Microarray Results: BD1 vs. COL
250
200
150
100
50
0
Virulence
Hypothetical
DNA Replication, Repair, Growth
Transport and Metabolism
Stress
Transcription and Gene Regulation
Phage Associated
Ribosomal Proteins
Down regulated
Up regulated

FIG. 3.

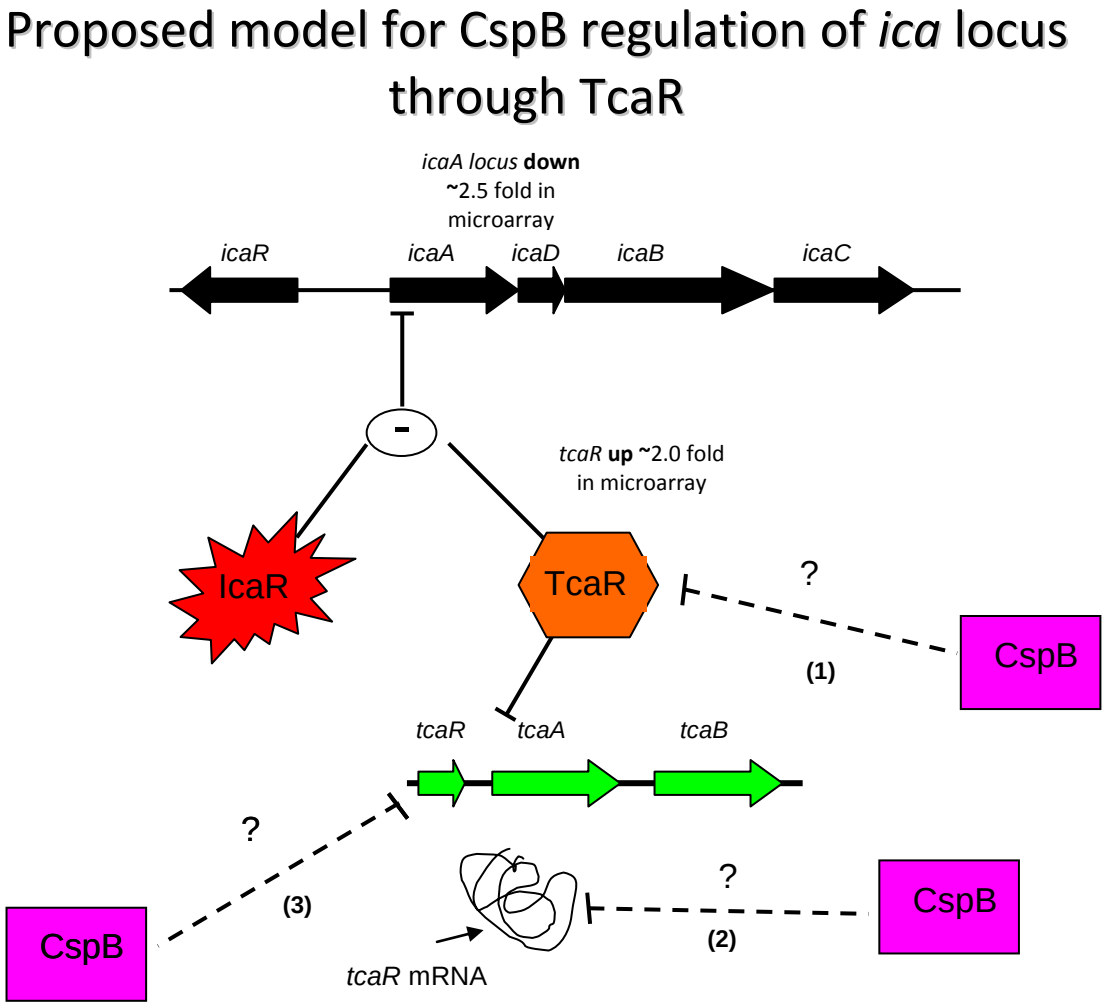
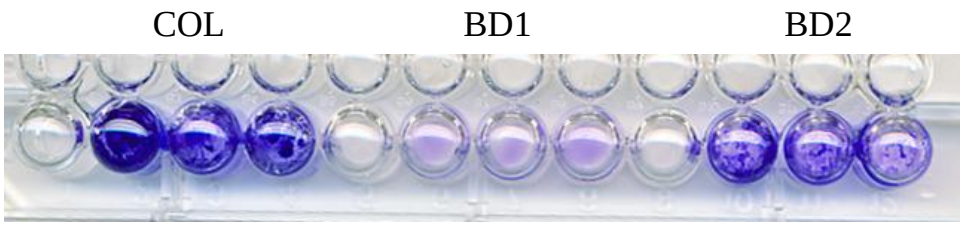

Proposed model for CspB regulation of *ica* locus through TcaR
icaA locus **down** ~2.5 fold in microarray
icaR
icaA
icaD
icaB
icaC
-
tcaR **up** ~2.0 fold in microarray
IcaR
TcaR
?
(1)
CspB
tcaR
tcaA
tcaB
?
(3)
CspB
tcaR mRNA
?
(2)
CspB

FIG. 4.

A.


COL
BD1
BD2

B.

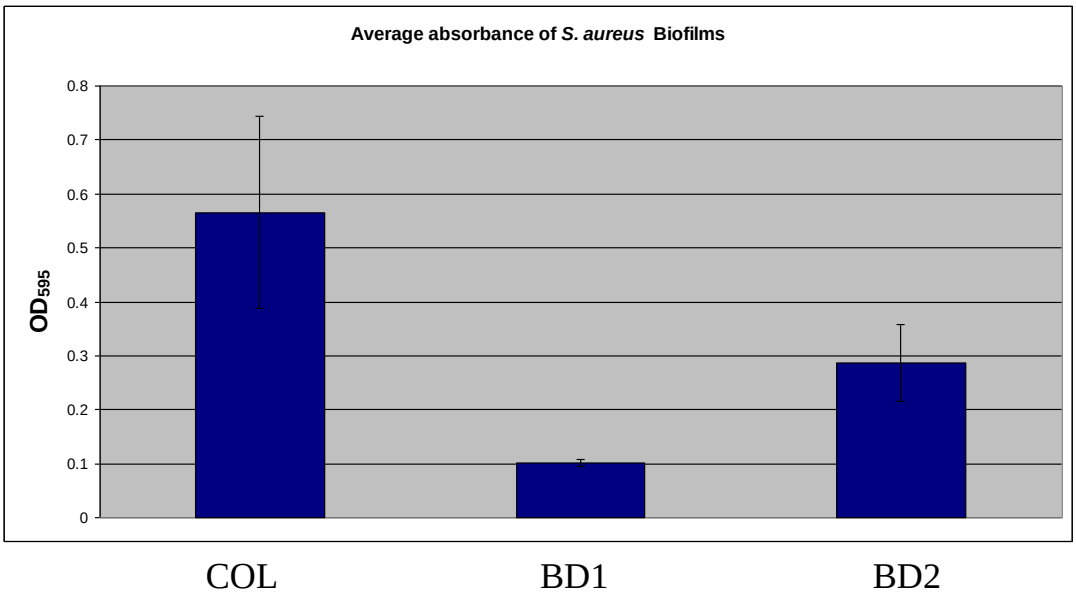
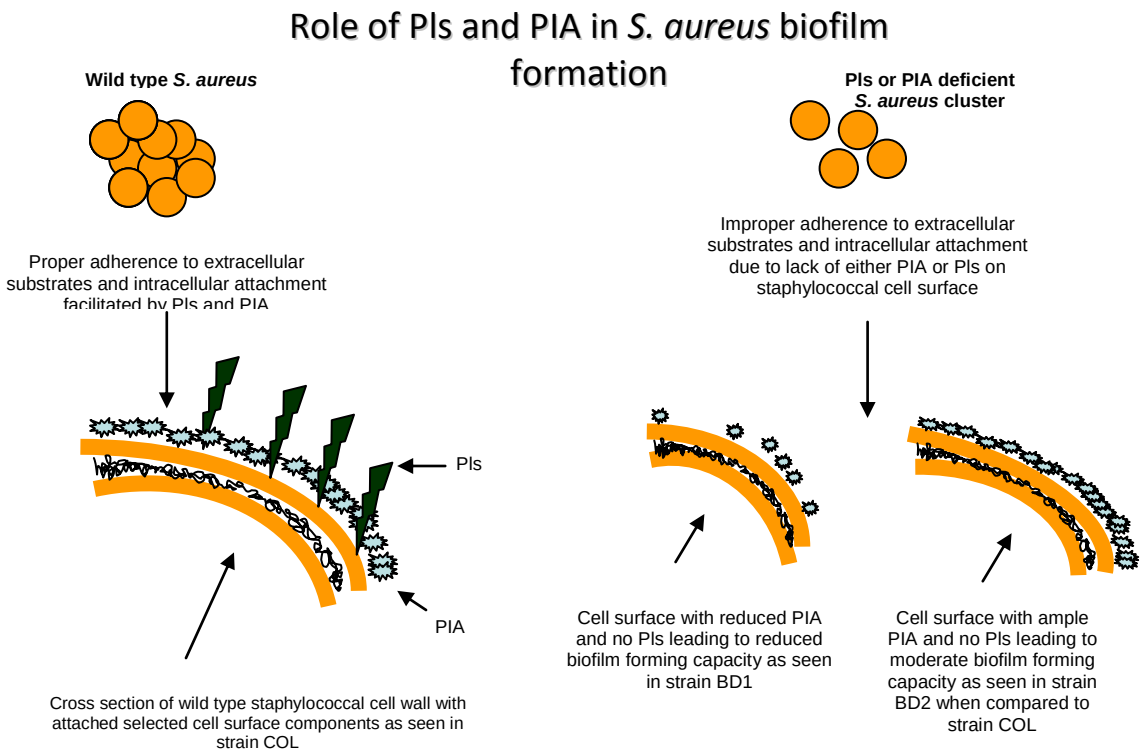

Average absorbance of S. aureus Biofilms
OD595
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
COL
BD1
BD2

FIG. 5.


Role of Pls and PIA in S. aureus biofilm formation
Wild type S. aureus
Proper adherence to extracellular substrates and intracellular attachment facilitated by Pls and PIA
Pls
PIA
Cross section of wild type staphylococcal cell wall with attached selected cell surface components as seen in strain COL
Pls or PIA deficient S. aureus cluster
Improper adherence to extracellular substrates and intracellular attachment due to lack of either PIA or Pls on staphylococcal cell surface
Cell surface with reduced PIA and no Pls leading to reduced biofilm forming capacity as seen in strain BD1
Cell surface with ample PIA and no Pls leading to moderate biofilm forming capacity as seen in strain BD2 when compared to strain COL

Chapter 4: Final Discussion

Staphylococcus aureus continues to be a serious and growing public health issue. By 2007, *S. aureus* was responsible for more deaths in this country than those attributed to AIDS (19). As has been previously discussed (Chapter 1, Section A), *S. aureus* contains an astounding array of virulence factors that allow it to evade the host immune response as well as treatment regimens. Many areas of staphylococcal pathogenesis have been well studied, but how *S. aureus* is able to distinctly regulate certain aspects of its pathogenesis still remain unclear. Part of *S. aureus*' success as a pathogen lies in its ability to survive various environmental stresses, particularly cold stress, which is problematic in the food service industry where *S. aureus* is the second most commonly reported cause of food poisoning in the United States (14). Based on previous work from our laboratory where the role of the cold shock protein *cspA* in antimicrobial resistance was investigated, (17, 18), we sought to define the role that the major cold inducible gene, *cspB*, plays in resistance to antimicrobials in a MRSA strain, COL. Interestingly, our *cspB* insertional mutant demonstrated many phenotypic differences from a previously characterized *cspA* insertional mutant in the same strain. In particular, the *cspB* mutant studied herein displayed many properties previously observed with small colony variants (SCV), including loss of pigment, slow growth, and change in antimicrobial susceptibilities.

SCVs present an important aspect of staphylococcal pathogenesis that deserves further study. These slow growing sub-populations of bacteria are often found at the sites of persistent or drug-resistant infections (28). While it is clear that SCVs have a clinically significant role, the genetic components responsible for this phenotypic switch remain

unknown. However, while clinical SCVs are difficult to maintain once isolated from patients, clinical SCVs generally fit into two categories: those deficient in electron transport and those deficient in thymidine-biosynthesis (30). Model SCV strains have been constructed by making mutations in *hemB* (30), *menD* (4), or *ctaA* (7) (see FIG. 1.). Much less is known about the specific genes involved in the SCV phenotype in other organisms. For example, a recently described SCV isolate of *Enterococcus faecalis* isolated from a patient with chronic valve endocarditis was not only stable after isolation, it also demonstrated many of the hallmarks of previously described SCVs (slow growth, change in antimicrobial susceptibility), but no detectable auxotrophy for thymidine (34). While it is clear that *cspB* plays a significant role for staphylococci under cold shock conditions (2, 11), the dramatic change in phenotype of strain BD1 at normal growth temperature seems to indicate an important role for *cspB* at 37°C as well. Cold shock proteins belong to the OB domain family of proteins (23), which have been shown to bind RNA and ssDNA (23). In *B. subtilis*, which has three *cspA* family cold shock genes, a *cspB/cspC* double mutant showed dramatic changes in protein synthesis as well as an inability to form endospores or enter into stationary phase (33). The authors were able to complement this double mutant by expression of IF1 from *E. coli in trans*. Analysis of crystal structures and protein sequences from both CspB from *B. subtilis* and IF1 from *E. coli* demonstrate a high degree of similarity (33). It is further suggested in this communication that CspB in *B. subtilis*, might function as an accessory initiation factor. Many Gram-positive bacteria lack any IF1 homolog and cold shock proteins therefore might serve as a functional replacement for the initiation of translation. While the lack of an IF1 homolog is common among Gram-positive bacteria, *S. aureus* does in fact contain

infA, which encodes IF1 (tigr.org). The question then arises as to why there is such a profound change in phenotype in a *cspB* insertional mutant? Protein alignments between *S. aureus* IF1 and CspB (Duval unpublished results) demonstrate a high degree of similarity, which might also suggest an overlap of function. It is possible that under normal conditions CspB acts in concert with IF1 to aid in the initiation of translation. Cold shock proteins have been shown to co-purify with ribosomes (22), suggesting that they might have some function at or near the translational machinery. Under cold shock conditions, the function of CspB in *S. aureus* may change and it may then take on a more traditional role for cold shock proteins where it binds RNA preventing the formation of secondary structures that would inhibit proper translation.

Strain BD1 has undergone a significant genetic rearrangement by loss of the type I SCC*mec*. Part of the success of MRSA as a pathogen is due to the stability of this methicillin resistance cassette (9). Previously, loss of the SCC*mec* has been seen only under very specific conditions including prolonged vancomycin stress or the acquisition of glycopeptide resistance (1, 24, 29), nutrient starvation (15), and exposure to UV light (3, 15). Strain BD1 demonstrates many aspects of the SCV phenotype (which will be discussed in detail later), including a decrease in growth rate. Previous work has demonstrated that clinical MRSA isolates harboring the much shorter type IV SCC*mec* grow much faster and are more fit than HA-MRSA isolates harboring larger cassettes (20, 25). It is possible that loss of the type I SCC*mec* in strain BD1 is an attempt by the cell to achieve greater fitness. Maintenance of the cassette could require a greater expenditure of cellular resources that are already compromised in the face of its significant change in colony morphology. *cspB*'s involvement in loss of the type I SCC*mec* is novel because it

links a gene outside of the SCC*mec* cassette with its specific excision. Site-specific excision of types I, II, III and IV SCC*mec* is mediated by the recombinases encoded by *ccrA* and *ccrB,* which form an operon carried on the cassette itself (32). While *cspB* does not encode a recombinase, the *cspA* mRNA has been shown to act as a thermosensor in *E. coli* (13) and this may be a clue to the link between loss of *cspB* and loss of the cassette. Perhaps part of *cspB*'s role at normal temperatures is to act as a general stress sensor for certain internal or external cellular stresses and the result of this process is loss of DNA that codes for non-essential processes. It should be noted, however, that in our observations there was only a single major deletion observed corresponding to the size of the type I SCC*mec* (11), which may mean that this process specifically targets the SCC*mec*. Genetic triggers that cause loss of this major determinant in *S. aureus* merit further investigation because an understanding of factors responsible for the maintenance of the cassette could lead to novel therapeutics for treatment of MRSA infections or more conservatively, adding methicillin back to treatment regimens for *S. aureus* infections under certain circumstances.

As indicated above, strain BD1 demonstrates many aspects of the small colony variant phenotype. Among these phenotypic similarities to previously described SCVs was a dramatic change in the antimicrobial resistances of strain BD1 when compared to the parent strain COL. One of the hallmarks of the SCV phenotype is an increase in resistance to aminoglycoside antibiotics, which is thought to be due to a diminished membrane potential (26). It is this reduced membrane potential that is thought to lead to the increased resistance of SCVs to aminoglycoside antibiotics such as gentamicin (5). Strain BD1 demonstrates this increased aminoglycoside resistance when compared to the

parent or complemented strain. It is reasonable, therefore, to conclude that loss of *cspB* leads to a decrease in membrane potential in strain COL. Interestingly, strain BD1 showed a decrease in susceptibility to trimethoprim-sulfamethoxazole (TMS). TMS is most often used in combination with rifampicin to treat chronic infections caused by SCVs (26, 27). With the observation that our *cspB* insertional mutant mimics other previously described SCVs and that it differs in its resistance to TMS, which is often used to treat clinical SCV infections, an understanding that SCVs might be varied not only in their clinical manifestations but also in their antimicrobial resistance profiles becomes important. Interestingly, an *Enterococcus faecalis* SCV isolated from a patient with aortic valve endocarditis also demonstrated a similar leave of resistance to TMS as determined by the Etest® method (34). The MIC of TMS in our case was >32 μg/ml. In contrast to the *Enterococcus faecalis* isolate previously described, there was demonstrated auxotrophy for hemin (34). *hemB* in strain BD1 was sequenced and compared to both the wild type strain COL and BD2 and both BD1 and BD2 were found to have wild type *hemB* sequences (10). Our *cspB* insertional mutant demonstrates the clinical importance of understanding the multiple causes and differences that might be found among SCVs *in vivo*.

The effect that loss of proper membrane potential has on virulence factor expression in staphylococcal SCVs has been well studied (30). Production of many staphylococcal virulence factors requires large amounts of available ATP, which SCVs do not produce. Most notably, many SCVs, both clinical isolates and laboratory strains, are non-pigmented (28, 30). Staphyloxanthin, the golden-hued pigment produce by *S. aureus*, has been shown to be a powerful antioxidant and is important in *S. aureus*'s

defense against oxidative killing by the innate immune system (21). While the exact metabolic sensors that link membrane potential with pigment production are unknown, the dramatic change in phenotype seen in BD1 gives further insights on the genes possibly involved in this phenotypic switch.

Previous studies have demonstrated that hemin-auxotrophic SCVs often have altered cellular morphology. Electron microscopy has revealed that these types of SCVs demonstrate incomplete or multiple cross cell walls (16). Our investigations into whether or not strain BD1 showed the same sort of cellular morphology demonstrated that BD1 showed normal cellular morphology as compared to both COL and BD1 (see unpublished observations). Our SEMs did, however, show that BD1 lacked extracellular material seen on the surfaces of COL and BD2. We believe this extracellular material to be poly-N-acetyl glucosamine (PNAG), which is a product of the *ica* operon responsible for cellular adhesion and biofilm formation (8). *S. aureus* uses PNAG to facilitate attachment to host cells and also to form biofilms (8, 12). It is important to note that microarray analysis of BD1 compared to strain COL revealed a reduction in expression of genes in the *ica* operon (unpublished observations), including *icaB,* which encodes a deacetylase responsible for modification of the polysaccharide, allowing it to properly associate with the bacterial cell surface (31). Accordingly, it is possible that BD1 lacks sufficient levels of IcaB, resulting in improper modification of the polysaccharide and a reduction in its accumulation on the cell surface. However, further studies are required to test this hypothesis.

There are many questions yet to be answered concerning the SCV phenotype of *S. aureus*. Most importantly, how do the bacteria make the decision *in vivo* to switch to this

alternative phenotype? The difference in how wild type *S. aureus* evades the host immune response, versus how SCVs accomplish this (Fig. 1), seems to be one of active process versus a more passive one. Wild type *S. aureus*, for example, actively secretes many different virulence factors, while SCVs seem to evade detection by the immune system by turning off expression of certain genes and even going as far as to reside within non-professional phagocytes (30). It is clear that SCVs have a competitive advantage over their wild type counterparts during persistence in long term staphylococcal infections (30). It is reasonable to think that such a drastic change in phenotype that is also accompanied by changes in metabolism, virulence factor expression, and antibiotic susceptibility might be governed by complex genetic mechanisms involving changes in the expression of multiple genes. The *in vivo* environment of an active or persistent staphylococcal infection would subject the cell to many different inputs: from cells that are going to maintain a wild type phenotype, from other SCV cells in its environment, and also from host immune cells. The cell then must be able to somehow process and make sense of all of these different inputs in order to make the switch to the SCV phenotype. The question remains: how does *S. aureus* coordinate this dramatic shift in phenotype? The more genes identified as playing some role in the process broadens our understanding of how this might be accomplished. Furthermore, knowledge of the phenotypic properties of genetically defined SCV mutants demonstrates that there are indeed multiple ways to achieve this phenotype. Whether or not this change can be defined by only a few genes remains unclear and this matter deserves extensive further study

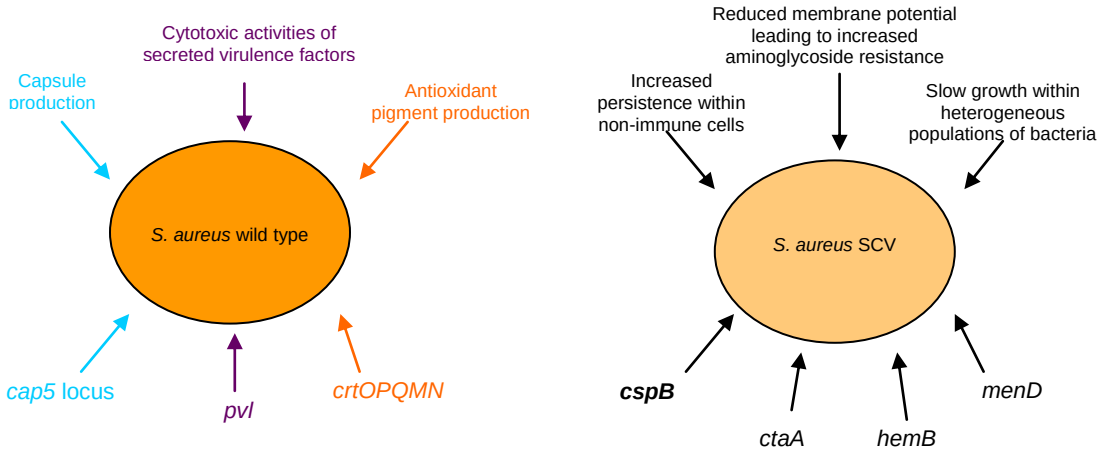
Figure Legend

FIG. 1. **Comparison of strategies used by wild type *S. aureus* versus *S. aureus* small colony variants (SCV) to evade innate immune defenses.** Genes involved in wild type *S. aureus* evasion strategies, and what those genes control are in the same color (right). Genes implicated in the SCV phenotype are summarized (left), along with survival strategies used by SCVs to cause persistent and drug resistant infections.

Figures

FIG. 1.

Strategies used by wild type *S. aureus* and SCVs to evade host innate immune defenses



Genes involved in staphylococcal innate immune evasion

Genes believed to be involved in the SCV

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