UNIVERSITY OF WISCONSIN-LA CROSSE

Graduate Studies

THE BACTERICIDAL MECHANISM OF ACTION OF
THE SK-03-92 ANTIBIOTIC AGAINST *STAPHYLOCOCCUS AUREUS* AND THE
ERADICATION OF SK-03-92-INDUCED BACTERIAL PERSISTENCE

A Manuscript Style Thesis Submitted in Partial Fulfillment of The Requirements
for the Degree of Master of Science in Clinical Microbiology

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THE BACTERICIDAL MECHANISM OF ACTION OF
THE SK-03-92 ANTIBIOTIC AGAINST STAPHYLOCOCCUS AUREUS AND THE
ERADICATION OF SK-03-92-INDUCED BACTERIAL PERSISTENCE

By Allison Zank

We recommend acceptance of this thesis in partial fulfillment of the candidate’s requirements for the degree of Master of Science – Clinical Microbiology.

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Infections caused by *Staphylococcus aureus* kill approximately 52 Americans every day and are particularly lethal when a biofilm has formed. The development of persister cells, and thus a chronic infection, further increases the chances of developing a lethal biofilm-associated infection. The SK-03-92 antibiotic is a promising drug that kills *S. aureus*, but the killing mechanism has not yet been characterized. Bioinformatic analysis indicated that SK-03-92 kills *S. aureus* by inducing late-stage competence, a phenomenon characterized by cell-death, increased biofilm production, and the development of persister cells. To test this, the supernatants of *S. aureus* exposed to SK-03-92 were recaptured and diluted to reduce the residual amount of drug to non-bactericidal levels. This material was named 3HSN (three-hour supernatant). Testing of 3HSN showed results consistent with the late-stage competence model. Namely, 3HSN exposure killed 91% more *S. aureus* than SK-03-92 alone and increased the output of biofilm produced by *S. aureus* by 20.7-fold per cell. A scheme to kill persister cells was also developed. We hypothesized that adding concentrated malate (pH ~ 7) to persister-inducing levels of SK-03-92 would force persister cells out of dormancy and allow SK-03-92 to kill 100% of the population. This hypothesis was confirmed experimentally. In sum, *in silico* and *in vitro* analysis indicated that SK-03-92 kills *S. aureus* by causing the cells to release a suicide-inducing pheromone, and the addition of 125 mM of malate to bactericidal levels of SK-03-92 produces a fully effective antibiotic that causes 100% cellular death.
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INTRODUCTION

Staphylococcus aureus infection

*Staphylococcus aureus* is an opportunistic pathogen that is part of the normal flora of approximately 30% of humans (1). Although *S. aureus* does not normally cause infection on healthy skin, the bacteria often infects skin and soft tissue (SSTIs, 2). If SSTIs progress to internal tissues or the blood stream, these bacteria may cause a variety of potentially serious infections (1). In fact, *S. aureus* has now become the leading cause of bacteremia and endocarditis in the United States (3, 4), causing an estimated 119,247 bloodstream infections and 19,832 associated deaths in 2017 (5). Both skin/soft tissue infections as well as endocarditis are associated with biofilms created by the *S. aureus* bacteria (6, 7).

*Staphylococcus aureus* infection: biofilms

For the human host, the difference between a minor *S. aureus* infection and a life-threatening disease lies in the biofilm forming ability of the bacteria. Planktonic *S. aureus* cells are readily killed by antibiotics, but once the species progresses to the biofilm stage it is often chemotherapeutically untreatable (8). In fact, cells living in biofilms are estimated to be 1,000-1,500 times more resistant to antibiotic treatment than planktonic cells (9). This is due, in part, to the impermeability of staphylococcal biofilms by the drugs of choice for *S. aureus* infection (10). In such cases, invasive relapsing infection is often the norm, and is usually accompanied by a drastic increase in patient mortality (11).
A biofilm is defined as a sessile community of irreversibly attached cells embedded in a matrix, and chronicity is not only due to the impermeability of biofilms, but also to the lifestyle of the bacteria living within them (12). The lifestyle of bacteria in a biofilm is distinct from the lifestyle afforded to single-celled organisms (13). The biofilm lifestyle offers an evolutionary advantage that allows the maintenance of homeostasis under a wide array of environmental circumstances through shared nutrients, shelter, and intercellular communication. For this reason, cells living in a biofilm are often compared to multicellular organisms. Interestingly, most bacteria do not live in a unicellular, planktonic state, but instead live within biofilm matrices (14).

The formation of biofilms has four main stages: adhesion to a surface, microcolony formation, biofilm maturation, and dispersal (15). Attachment occurs through the display of surface proteins. This is mediated by \textit{S. aureus} surface proteins called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) encoded by the \textit{can}, \textit{clfA}, \textit{clfB}, \textit{ebps}, \textit{eno}, \textit{fib}, \textit{fnbA}, \textit{fnbB}, \textit{icaA}, \textit{icaB}, \textit{icaC}, \textit{icaD} genes (16). During microcolony formation and biofilm maturation, bacteria are cocooned in an extracellular polymeric substance, composed of both self-produced and scavenged material. This accounts for ~90% of the biomass (17, 18). Self-produced elements of the \textit{S. aureus} biofilm matrix include a polysaccharide capsule encoded by the \textit{cap5} gene, cell wall teichoic acids encoded by \textit{tagB}, \textit{tarF}, and \textit{tarJ2}, and MSCRAMM surface proteins (19-22). The scavenged material is mainly DNA, called extracellular DNA (eDNA), because it is collected from the environment after the lysis of nearby cells (23). eDNA acts as a stabilizing scaffold for the three-dimensional biofilm to build itself upon and is considered to be one of the most vital elements of the biofilm matrix. It
functions by adsorbing to and extending from the cell surface to promote adhesion to abiotic surfaces through acid-base interactions. The widespread importance of eDNA in biofilm formation is undisputed. Once a biofilm is mature, and new cells are actively forming, a process can occur in which the youngest bacteria become dormant. This is a function of environmental stress or stress caused by nutritional deprivation within the biofilm. These nascent, dormant cells are called persister cells or small colony variants (SCVs) and mature biofilms can be imagined as adherent communities of persister cells (24). To spread from one host system to the next, persister cells break free of a biofilm via a complex mechanism called dispersal. Dispersal is considered to be the final step in the biofilm lifecycle (24-26).

**Staphylococcus aureus infection: sortase A**

Once persister cells disperse, bacteria must again express the ability to attach to surfaces. This is how the process of biofilm formation continues. Both dispersion and attachment are primarily dependent on the expression of an enzyme called sortase A (SrtA; 27, 28; Fig. 1). SrtA is a membrane anchored transpeptidase that mediates the

![Figure 1. Visualization of the tertiary structure of the sortase A enzyme. Calculated by 35-hour processing through I-TASSER servers (Yang Zhang Lab, University of Michigan) and visualized with PyMOL software. I-TASSER predicted topology: cytoplasmic residues are shown in red, transmembrane residues in blue, and ligand binding residues are shown in yellow. The estimated global accuracy of the model is C score = 0.83, estimated TM score = 0.66±0.14; estimated RMSD = 4.2Å.](image-url)
attachment of secreted proteins with an LPXTG motif to the bacterial cell wall (25). At least ten proteins associated with S. aureus surface anchoring and early biofilm formation contain the LPXTG motif, including all MSCRAMMs (e.g. clumping factors A and B, fibrinogen binding protein, laminin binding protein, collagen binding protein, elastin binding protein, and bone sialo-protein binding protein; 29-32).

Sortase A functions by cleaving the peptide backbone between the C-terminal threonine (T) and the glycine (G) of the LPXTG motif to form an acyl-enzyme intermediate (33, 34). The acyl-enzyme is resolved by the nucleophilic attack of the amino-group found in the pentaglycine cross bridge in lipid II, the precursor for peptidoglycan synthesis (29, 32). The product of this reaction, surface protein linked to lipid II, is then incorporated into peptidoglycan via the transglycosylation and transpeptidation reactions of cell wall synthesis (35-37). S. aureus mutants lacking the srtA gene fail to anchor surface proteins and thus cannot attach, this results in an impaired ability to cause animal infections (22, 38, 39). Because srtA mutants are attenuated in animal models of infection, SrtA has become a target of several anti-infective drug studies (40-43).

**Sortase A is affected by bacterial phytoalexins**

The secret to a better understanding of the genetic control of srtA transcription may lie in the study of a certain class of plant-derived antimicrobials called phytoalexins. Many such compounds have been shown to specifically target SrtA, but to date, no information exists regarding how phytoalexins affect srtA transcription (44-46). As sessile beings, plants can only use chemical defenses against pests and pathogens, this involves the production of low-molecular-mass secondary metabolites. Collectively,
these compounds are known as phytoalexins (47). Phytoalexins include molecules with both stilbene and terpenoid moieties, and tens of thousands of potentially antibiotic stilbene/terpenoid lead compounds have been isolated from plants and microbial sources (48, 49). One class of chemical analogues, called terpenes, is pictured in figure 2.

Terpenes interfere with vital functions like quorum sensing, cellular surface adhesion, and biofilm formation (50-55). Terpenes shown to act as antibiotics include SK-03-92, resveratrol, pterostilbene, catechin, epicatechin, curcumin, and monoterpenes (56-60).

Stilbenes are a type of terpene. Although it is well established that many terpenoid drugs impede pathogenic infectivity, the mechanism of action of the stilbenoid/terpenoid class of drugs is poorly understood and no stilbenoid or terpenoid-based pharmacological drug has yet been brought to market as a viable class of antibiotic (61).

**Plant phytoalexins: SK-03-92**

In order to better understand phytoalexin-based antibiotic compounds, a modified terpene, labelled SK-03-92, has been investigated for its antibacterial activity (62). The compound (E)-3-(2-(benzo[b]thiophen-2-yl)vinyl)-5-methoxyphenol (SK-03-92) is an analog of the terpenoid (E)-3-hydroxy-(5)-methoxystilbene that was extracted from the leaves of the shrub *Comptonia peregrina* (L.) *peregrina*, also known as sweet fern. Parts of the sweet fern plant were used medicinally in the past by members of the Ho-Chunk Nation of indigenous Americans (63). The modified terpene antibiotic, SK-03-92, is a...
1.3 nm long lipophilic stilbenoid that contains a polar head and a reactive nucleophilic trans double-bonded carbon at its core. The SK-03-92 drug shows broad *in vitro* efficacy at low minimum inhibitory concentrations against a variety of Gram-positive bacterial pathogens, including *Bacillus cereus*, *Clostridioides difficile*, *Clostridium perfringens*, *Corynebacterium urealyticum*, *Listeria monocytogenes*, *Streptococcus agalactiae*, *S. bovis*, *S. mitis*, and a number of strains of *S. aureus* (64). Besides the activity against Gram-positive bacteria, SK-03-92 also exhibits activity against several Gram-negative species and many mycobacterial species (64).

In addition to its chemical similarity to the phytoalexins shown in Fig. 3, the pharmacophore of SK-03-92 also chemically resembles two synthetic cancer drug compounds that affect body axis patterning in human cells (Reaxys; 65-67; Fig. 3). Analogously, the SK-03-92 drug was shown to cause morphological defects in *Saccharomyces cerevisiae* (yeast) cells and in the human monocytic cancer cell line THP-1 (68). These deformities were directly linked to punctate mitochondria observed with apoptotic death at low concentrations of SK-03-92 and diffuse mitochondria observed with necrotic death at high concentrations of SK-03-92. Mitochondria are bacterial endosymbionts that inhabit eukaryotic cells, but necrotic signaling has not yet been documented in any bacterial species (69).
Considering that SK-03-92 analogs often affect sortase A activity, it is unsurprising that a previous study showed increased formation of biofilm and an upregulation of \textit{srtA} transcription in \textit{S. aureus} treated with the SK-03-92 drug (62). However, that study also noted that an increased proportion of dormant (persister) cells formed in response to the drug. Prior to that study, terpenoid-induced bacterial persistence had not yet been studied, nor had it been linked to phytoalexin action.

**Persister cells**

Bacterial persister cells are cells that are still alive but have stopped growing. Unlike resistant cells that grow in the presence of antibiotics, persister cells stop growing in response to antibiotics but regain antibiotic susceptibility once they mature (70). These cells comprise a small fraction of exponentially growing cells and are considered to become a “significant fraction” of the stationary phase. This significant fraction, however, is typically 1% of the bacterial population (70). Surprisingly, in \textit{S. aureus} exposed to SK-03-92, 10% percent of the cells became persisters (62).

The formation of persister cells in response to antibiotics has been frequently documented. This dormant state can be observed by a typical biphasic killing pattern, with an initial rapid killing of the bulk of the population and an observed plateau where only the persister subpopulation remains alive (71). This biphasic pattern is observed both with increasing concentrations of antibiotics and with increasing treatment time (72, 73). When no death of a certain population occurs, persister cells cause infective relapse (74).

Persister cells are also multidrug tolerant. This is because antibiotics work by interfering with the physiology of mature or growing cells. The difference between
antibiotic resistant cells and persister cells must be emphasized, the tolerance of persisters to antibiotics is only a function of immaturity and not due to antibiotic resistance. Once growth resumes, persister cells again become susceptible to killing by antibiotic drugs and are indistinguishable from other cells (70, 75, 76). This transience makes persisters difficult to study, as it is nearly impossible to observe a cell that exists but simply does not grow.

Due to the difficulties in studying persister cells, the trigger signal that causes this dormancy is not understood. The percentage of persister levels that form, however, has been shown to depend on the nutritional environment (77). The resumption of normal growth from the persister phenotype is also nutrient dependent. For instance, *E. coli* has YehUT (BtsSR), that triggers persisters to resume normal growth in the presence of high levels of extracellular pyruvate and low levels of extracellular serine. Both conditions must be met to provoke growth (78). In addition to resumption of normal growth, the BtsSR system also triggers the formation of persister cells (79).

**Competence: early stage and late stage**

The formation of persister cells in response to environmental nutritional deficiency intimately links persister cell formation to bacterial competition. Like all other undomesticated life forms, microorganisms face a constant battle for resources within their environment. In the case of bacteria faced with competing organisms, cells become dormant with environmental nutrient depletion. This process is called competence. The hallmark of competence is the acquisition of DNA from the environment. However, not all of the cells in a competent community are capable of DNA uptake (80). Instead, these non-competent cells sacrifice themselves to produce the nutrients and eDNA needed to
rapidly form a biofilm (81). Although induced cell suicide in unicellular organisms is counteintuitive, for cells living in a biofilm, cell death is an altruistic behavior in which the death of a fraction of the cells benefits the rest of the population (82). All this improves the long-term survival of the species (83, 84). The death of this subpopulation also promotes the formation of channels that facilitate the transport of water and nutrients through existing or newly forming biofilm (85).

Dormancy only occurs as a last resort and during the latest stage of competence. Although the uptake of DNA originally gave the phenomenon of competence its name, competence is now understood to be a complex, multiphasic process. Other behavioral adaptations, besides DNA uptake, have now been documented to occur during this process. In general, the stages of the development of bacterial competence are broken down into two stages: early-competence or late-competence. During early-competence, the production and release of bacteriocins and pheromones occurs. These compounds act to either kill competitors in the environment (bacteriocins) or signal danger to cells located in the interior of the biofilm (pheromones, 86-88). Pheromones also control the expression of virulence factors and initiate biofilm formation, both within the species that is releasing them and also within other species that frequent the same ecological niche (89). Late competence is not well characterized, but sortase A upregulation in Gram-positive bacteria during this stage is suggested by the unanimous findings of increased biofilm formation during this stage (22, 90-92). Cell death, increased biofilm production, and persister cell formation have also been shown to rapidly and simultaneously occur in late-stage competence (Fig. 4). Fascinatingly, these same
processes also occur during the treatment of *S. aureus* with SK-03-92. This suggests that the SK-03-92 drug may act as an inducer of late-stage competence.

**Figure 4. Schematic model of competence-specific quorum sensing.** Two phases of competence development are controlled by signal transduction stimulated by pheromones through a transmembrane sensor for early genes, and by sigma factors transcribed during early-competence for late genes.

**Holin induced cell suicide**

Cell death during late competence occurs by an interesting mechanism in which certain members of the colony induce other cells to commit suicide. Cell-suicide is facilitated by cytoplasmic proteins known as holins and antiholins (93). Holins are filamentous proteins that form ulcerated lesions on the internal surface of living cells when stoichiometrically dominant, but prior to that, are held inactive by antiholins. The release of holins by antiholins ultimately causes cell death by causing the cell to burst. This causes the cytoplasmic contents to spill into the environment. It is in this elegant
way that large amounts of eDNA are released into the environment so that biofilms have a framework on which to build. (23)

Pores in the membrane formed by holins do not participate in the uptake of extracellular materials, but instead simply produce leaky cells. In *S. aureus*, holins are formed by CidA proteins, encoded by the *cidA* gene. Staphylococcal antiholin protein (LrgA) is encoded by the *lrgA* gene (84). The stoichiometric imbalance described above can occur between the holin and antiholin by LrgA degradation, the transcriptional upregulation of the *cidA* gene, or transcriptional downregulation of the *lrgA* gene (Fig. 5).

![Figure 5](image)

Figure 5. Model for the control of autolysis by holins and antiholins. Holin proteins are post transcriptionally regulated by antiholins. Antiholins inhibit holin activity and prevent cell lysis. When antiholins are stoichiometrically outnumbered by holins, holins pierce the cell wall. The cell then develops pores and dissipation of the membrane potential occurs. This causes spillage of the cytoplasmic components into the intercellular milieu and results in the death of the cell.

**Microarray showed antiholin downregulation in response to SK-03-92**

Holin/antiholin action was implicated by RNA microarray analysis as a potential cause of death in *S. aureus* exposed to SK-03-92. The same microarray also showed that transcript abundance of the *lrgA* antiholin gene fell without a concurrent transcriptional downregulation of the *cidA* holin gene (62). This suggests that when *S. aureus* is exposed to the SK-03-92 stilbene drug, the stoichiometry shifts so that LrgA antiholin levels go
down while CidA levels remain at wild-type levels. The observed rapid killing of 
*S. aureus* cells by the SK-03-92 drug could be due to an imbalance of holin and antiholin 
levels. When merged with the findings that the SK-03-92 also induces increased biofilm 
production and increased persister cell formation, these data suggest that SK-03-92 may 
act to stimulate late-stage competence in *S. aureus*.

**Two-component regulatory systems**

The systems used to induce bacterial competence are well understood. 
Gram-negative bacteria predominantly use N-acyl homoserine lactone molecules, and 
Gram-positive bacteria use small peptides. In both cases, the pheromones responsible for 
such signaling are classified as autoinducers (94). Autoinducing pheromones are received 
by transmembrane signal-transducing proteins called two-component systems (TCS; 95). 
In a TCS, signaling occurs when a sensor kinase protein (usually membrane bound) is 
stimulated by an environmental stimulus to modify the phosphorylated state of a cognate 
cytoplasmic response regulator protein (Fig. 6; 96). The phenomenon of TCS protein 

![Figure 6. Schematic representation of the elements of a prototypical two-component regulatory system (TCS). A TCS generally consists of a membrane-bound environmental sensor kinase (SK) protein and a cognate cytoplasmic response regulator (RR) protein. In response to specific signals, the SK modifies the phosphorylated state of the RR. The RR, in turn, can then positively or negatively regulate transcription.](image)

phosphorylation is a universal homeostatic-device employed by prokaryotes in response 
to stress, such as that experienced during resource competition (97).
One putative TCS was profoundly affected when *S. aureus* cells were treated with SK-03-92. This was confirmed by both microarray analysis and qRT-PCR (62). These tests, which compared the transcriptome of *S. aureus* exposed to SK-03-92 to that of wild-type *S. aureus*, showed that the drug caused *S. aureus* (MW2) to transcriptionally downregulate production of both the TCS putative response regulator MW2284, and the putative sensor kinase MW2285. Follow-up tests showed that mutating *S. aureus* MW2284 and MW2285 genes caused the cells to produce more biofilm than wild-type *S. aureus* when grown in BHI-G broth (brain heart infusion with 1% glucose; 62). For this reason, we now refer to MW2284 as biofilm regulating protein regulator (BrpR) and MW2285 as biofilm regulating protein sensor (BrpS).

**Competence initiation**

The initiation of competition-induced competence, also known as the competence stimulating pheromone (CSP) alarmone response, has been narrowed-down to a specific TCS in multiple bacterial species (81-84, 86, 98). However, due to its role as the key etiological agent in the infectious disease of dental caries, *S. mutans* is a well-studied model organism by oral microbiologists (99). Thus, the CSP alarmone response has been fairly well characterized in both *S. mutans* and *S. pneumoniae* (86). The general consensus is that the response functions the same way in both species. Initially, competence begins with the external reception of self-produced auto-inducing pheromones called CSPs. This only initiates early-competence if an internal protein called XIP (SigX inducing peptide) is also present within the cell (100). When this occurs, the CSP alarmone response is initiated by the secretion of a short, 14 residue peptide called CSP (ComC, SMU1915). CSP is then received by the membrane bound
sensor kinase ComD (SMU1916). This event phosphorylates the cytoplasmic response regulator ComE (SMU1917), which ultimately controls programmed cell death and persistence via the CipI protein (SMU1913c). CipI works in a similar manner to the holin/antiholin model described above. As shown in Figure 7, CipI silences CipB (SMU1914c) by antagonism, but if the concentration of CipB stoichiometrically dominates the CipI concentration, cell suicide is induced. Cell-suicide induction is caused by the expression of a self-acting autolysin called LytF_{sm} (SMU836). The gene that encodes LytF_{sm} is a late competence gene called lytF_{sm} whose transcription is controlled by sigma factor X (SigX/ComX1; SMU1997). SigX is the sigma factor...
responsible for expression of the general stress response in all Gram-positive bacteria. Additionally, SigX is specific for the transcription of all genes within the competence regulon, including the CSP-encoding gene (101). In some organisms, SigX is named SigH, but phylogenetic studies have shown that SigH and SigX are closely related and the SigH/X DNA binding sequence (CGAATA..15-17 nt..GTT) is the same for both sigma factors (102-106). Thus, the difference between SigX and SigH is in name only. In Clostridium, Mycobacterium, and Staphylococcus it is called SigH, whereas in Bacillus and Streptococcus it is called SigX.

Organisms competing for resources induce SigX production

As previously mentioned, competence can be either auto-induced or caused by the release of competence stimulating peptides from species frequenting the same bacterial ecological niche. In one study, a S. mutans brsRM deletion mutant (SMU2080/SMU2081) was grown in co-culture with Aggregatibacter actinomycetemcomitans. The brsRM mutant had a stronger quorum sensing response that was responsible for coordinating streptococcal competence development than the wild-type strain. Interestingly, sigX transcripts were more abundant in the mutant compared to wild-type. This showed that BrsRM is a quorum sensing regulon responsible for the coordination of the development of streptococcal competence (107). As we shall see, the BrsRM late-competence-stimulating TCS of S. mutans appears to be the S. aureus analog to BrpRS.

Quorum sensing promoter

While sigma factor H/X binding is an internal trigger of competence, a unique DNA binding sequence has also been identified as an internal trigger of competence and
bacteriocin production (104). This sequence is an upstream element of the \textit{qsrAB} gene, which contains a hybrid motif that accommodates separate transcriptional regulators. The discovery of this hybrid binding pattern facilitated the finding that the secretion of bacteriocins is more closely linked to competence than previously thought. Moreover, it provides a searchable motif for the bioinformatic discovery of competence genes.

**HYPOTHESIS AND OBJECTIVES**

As bacteria become more resistant to current antibiotic treatment, the human race desperately needs new antibiotic drugs. The ability to incorporate phytoalexin drugs into the antibiotic armamentarium could provide many leads. Unfortunately, the mechanism of phytoalexin antibiotic action is unknown. SK-03-92 is a promising phytoalexin-based antibiotic that has been shown to be highly non-toxic to eukaryotic cells (108). Unfortunately, the finding that SK-03-92 stimulates increased persister cell formation poses a major obstacle to the development of both SK-03-92 and possibly other phytoalexin drugs. Based on the information presented above, we hypothesized that the mechanism of action of the SK-03-92 drug is to incite the release of a compound that induces late-stage competence in \textit{S. aureus}. Additionally, based on the \textit{in silico} research presented below, we hypothesized that high levels of environmental malate can reverse persistence induced by SK-03-92.

Thus, the specific aims of this research were to:

1. Determine the mechanism by which SK-03-92 kills \textit{S. aureus}.
2. Expand the effect of SK-03-92 by preventing drug-induced dormancy.
MATERIALS AND METHODS: IN SILICO

Bioinformatic references

The sequenced genomes of *S. aureus* strains MW2 and Newman used in this study are publicly available on GenBank (109, 110, 111; NCBI, genome assembly ASM1126v1). The protein annotations for all of the bacterial strains included in this study were found on BioCyc or GenBank (111, 112). BioCyc was also used to search for BrpRS homologs downstream of the *mqo2* gene. UniProt was used to obtain amino acid FASTA sequences (113). Domain motifs were sought using NetPHOS, ExPASy, Prosite, and GenomeNet (114-117). Protter was used to two-dimensionally visualize BrpS and BrsM (118). I-TASSER and PyMOL were used together to three dimensionally visualize BrpR and BrpS (119, 120). I-TASSER and PyMol were also used to visually verify DNA binding in residues predicted by DP-Bind (121). Finally, protein sequence homology analyses were performed by BLASTp (NCBI) with the following parameters: max target sequences=100, automatically adjusted parameters for short input sequences, expect threshold=10, word size=3, max matches in a query range=0, matrix=BLOSUM62, gap costs=11 existence and 1 extension, and a conditional compositional score matrix adjustment (122).
MATERIALS AND METHODS: IN VITRO

Bacterial strains, plasmids, and growth media

The bacterial strains and plasmids used in this study are listed in Table 1. The

*S. aureus* parent strain, Newman (ATCC 13420), was isolated from a human infection (123). The JE2 strain, created by the University of Nebraska Medical Center, is the

*S. aureus* parent strain USA300 LAC CA-MRSA cured of its plasmids (124).

Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DH5α</td>
<td>Transformation efficient <em>E. coli</em> strain</td>
<td>124</td>
</tr>
<tr>
<td>S. aureus Newman</td>
<td><em>S. aureus</em> clinical isolate</td>
<td>122</td>
</tr>
<tr>
<td>S. aureus JE2</td>
<td><em>S. aureus</em> USA300 MRSA strain</td>
<td>123</td>
</tr>
<tr>
<td>S. aureus NE272</td>
<td><em>S. aureus</em> JE2 <em>brpS</em> mariner mutant</td>
<td>123</td>
</tr>
<tr>
<td>S. aureus NE671</td>
<td><em>S. aureus</em> JE2 <em>brpR</em> mariner mutant</td>
<td>123</td>
</tr>
<tr>
<td>S. aureus NE1787</td>
<td><em>S. aureus</em> JE2 <em>srtA</em> mariner mutant</td>
<td>123</td>
</tr>
<tr>
<td>S. aureus RN4220</td>
<td>Transformation-efficient <em>S. aureus</em> strain</td>
<td>125</td>
</tr>
<tr>
<td>Newman <em>brpR</em></td>
<td><em>S. aureus</em> Newman <em>brpR</em> deletion mutant</td>
<td>This study</td>
</tr>
<tr>
<td>Newman <em>brpS</em></td>
<td><em>S. aureus</em> Newman <em>brpS</em> deletion mutant</td>
<td>This study</td>
</tr>
<tr>
<td>Newman <em>srtA</em></td>
<td><em>S. aureus</em> Newman <em>srtA</em> mariner mutant</td>
<td>This study</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAD123</td>
<td>Cloning vector with Ap' and Cm' genes</td>
<td>127</td>
</tr>
<tr>
<td>pAB1-5</td>
<td>pAD123 plasmid with <em>brpR</em> gene inserted (Ap', Cm')</td>
<td>126</td>
</tr>
<tr>
<td>pALC2073</td>
<td>Cloning vector with Ap', Cm', and Tc' genes, and a Tc-inducible promoter</td>
<td>131</td>
</tr>
<tr>
<td>pAMZ1-3</td>
<td>pALC2073 plasmid with the <em>brpS</em> gene inserted</td>
<td>This study</td>
</tr>
</tbody>
</table>

*S. aureus* strains NE671 (*brpR*), NE272 (*brpS*), and NE1787 (*srtA*) are erythromycin resistant mutants created by the University of Nebraska Medical Center by mariner transposon mutagenesis (124). Strains JE2, NE671, NE272, and NE1787 were obtained
from the NARSA Strain Repository (Network on Antimicrobial Resistance in *Staphylococcus aureus*) that served as a repository for the Nebraska Transposon Mutant Library (124). The *E. coli* strain DH5α is a cloning strain with mutations that enable high-efficiency transformation (125). *S. aureus* strain RN4220 is a transformation efficient strain engineered to lack endonucleases (126).

The pAB1-5 plasmid was created by a graduate student at the University of Wisconsin – La Crosse using the pAD123 plasmid as the backbone to clone the *brpR* gene into the EcoRI/BamHI restriction endonuclease sites (127). Plasmid pAB1-5 contains ampicillin and chloramphenicol resistance genes, as well as *E. coli* and Gram-positive origins of replication (128). Like plasmid pAB1-5, plasmid pALC2073 also carries ampicillin and chloramphenicol resistance genes and *E. coli* and Gram-positive origins of replication; however, plasmid pALC2073 also carries tetracycline resistance genes and a tetracycline inducible promoter (128/129).

All media and all citric acid cycle metabolites were purchased from Thermo Fisher Scientific (Thermo Fisher Scientific, Pittsburgh, PA, USA). All antibiotics were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). *S. aureus* strains were grown in brain heart infusion (BHI) broth or trypticase soy broth (TSB) shaken at 250 rpm at 37 °C; or statically, on brain heart infusion agar at 37 °C. The transposon mutant strains were grown in media with 5 μg/mL of erythromycin (Em5). *S. aureus* strains carrying the pAB1-5 plasmid were selected for in 10 μg/mL of chloramphenicol (Cm10), whereas strains carrying the pALC2073 plasmid were grown in media with Cm10 and 0.25 μg/mL of tetracycline (Tc0.25) to induce the xyl/tet promoter. *E. coli* strains were grown in Luria-Bertani (LB) broth shaken at 250 rpm at 37 °C or LB agar at 37 °C; and
E. coli strains carrying the pALC2073 plasmid were selected for in media containing 100 μg/mL ampicillin (Ap^{100}).

**Creation of brpR, brpS, and srtA deletion strains**

As previously discussed, an mRNA microarray showed a reduction in the transcriptional abundance of brpR and brpS mRNA, and an increase in the transcriptional abundance of srtA mRNA when S. aureus was exposed to the SK-03-92 drug. To verify this finding, brpR, brpS, and srtA mutants were created S. aureus strain Newman by transduction (130). Briefly, a φ80α bacteriophage was propagated (100:1) on soft agar containing CaCl (5 mM) in the JE2 mutants NE671 (brpR mutation), NE272 (brpS mutation), or NE1787 (srtA mutation) for 6 h. The phage was then titered and harvested by centrifugation (6,000 xg, 4 °C, 10 min). Bacteriophage from the filter isolates were then filter sterilized and used to transduce the gene of interest into the S. aureus recipient strain RN4220 (10^{10} pfu/mL; Acrodisk 0.2 µm; Pall Corporation, Port Washington NY; 129). The transductants were then selected for on BHI agar containing Em^{5}. To transfer the mutation to S. aureus Newman, this generalized transduction procedure was repeated, using S. aureus Newman as the recipient strain. All mutations were verified by biofilm assay and selected for on BHI agar containing Em^{5}.

**Creation of a brpS complementing plasmid**

To satisfy Koch’s molecular postulates, a recombinant brpS complementing plasmid was also constructed. First, the full-length coding region of the S. aureus (MW2) brpS gene was polymerase chain reaction (PCR) amplified using the following primers and conditions: GEX-5XC 5’- CCT AGG AGA TCT CTT TCT GTC -3’ and GEX-5XB 5’- GTT AAT TTT ACT AAA CTT AAG -3’; 35 cycles, 94°C 1 min, 57°C 1 min, 72°C
1 min. The *brpS* DNA was amplified to have a *Kpn*I site on the 5’ end and an *EcoR*I site on the 3’ end. Then, this *brpS* gene PCR product was digested with *Kpn*I and *EcoR*I, ligated with T₄ DNA ligase immediately downstream from the tetracycline-inducible *xyl/tet* promoter on *Kpn*I/*EcoR*I cut pALC2073 plasmid DNA, and transformed into *E. coli* strain DH5α cells (131). Transformants were selected on LA containing 100 µg/mL ampicillin. From one transformant, plasmid pAMZ1-3 was the end result.

To move the pAMZ1-3 plasmid into *S. aureus* Newman, plasmid DNA from *E. coli* was purified with a Qiagen Plasmid Miniprep Kit (Qiagen, Germantown MD; 132). Then, the purified DNA was electroporated into the *S. aureus* strain RN4220, according to an established protocol under the following conditions: 100 Ω capacitance, 25 µF resistance, 2.5 kV charge voltage, 4 s (133, 129; GenePulser, Bio-Rad, Hercules CA). After one hour of expression in SMMP broth (sucrose, maleic acid/MgCl₂/Penassy, see Appendix A), each strain was selected for on BHI agar containing Cm₁⁰. Finally, plasmid DNA was reisolated from one of the strain RN4200 transformants using the method noted above with 50 µL of lysostaphin (10 mg/mL, 60 min, 37 °C) added prior to the first step to facilitate lysis of the staphylococcal cells. This isolated plasmid DNA was then cut with the *EcoR*I and *Kpn*I restriction endonucleases to verify insertion. The *S. aureus* Newman target strain was then transformed by electroporation using the method and conditions outlined above. Prior to experimentation, the mutant was grown in BHI-G broth containing its respective antibiotic media (250 rpm, 37 °C) and stored at -80 °C.
Biofilm assays of brpR and brpS deletion mutants and complements

To determine the effect of the brpR and brpS mutations on the ability of S. aureus Newman to form a biofilm, a biofilm assay was performed (134). Briefly, cultures of the S. aureus were grown overnight in BHI-G broth with the appropriate antibiotic(s) shaken at 250 rpm, 37 °C. Each strain was then diluted 1:100 and 220 μL placed in microtiter wells in triplicate, and statically incubated for 24 h at 37 °C to allow a biofilm to form. Each well was then rinsed three times with sterile water. To preserve the integrity of the biofilm, one-half of the supernatant was left in each well prior to rinsing. After a final evacuation of all well contents, the biofilms were allowed to settle (10 min) and then treated with crystal violet dye (0.1%; see Appendix B) for 10 min. The wells were then allowed to fully dry. The dried contents were incubated in 33% acetic acid at room temperature for 30 min and vigorously curettaged. Extracted biofilm was then diluted 1:5 in 33% acetic acid so that the optical densities could be measured on a SpectraMax M3 96-well microtiter plate reader (Molecular Devices, San Jose CA) at an optical density of 570 nm. The percent biofilm formation was calculated by dividing the OD_{570} of each treatment by the OD_{570} of S. aureus biofilm formation in BHI-G broth. In addition to wild-type Newman cells, Newman brpR and Newman brpS mutant strains as well as brpR and brpS mutants containing the pAB1-5 or pAMZ1-3 plasmids were tested. A Newman srtA transposon mutant was used as a negative control. Calculation of the means, standard errors of the mean (SEM) and analysis of variance (ANOVA) were performed using Microsoft Excel. The biofilm assay was performed a minimum of 10 times in triplicate for each strain to achieve statistical significance. A p-value <0.05 was considered significant.
**3HSN\(^1\) production**

To determine whether a suicide inducing signal-compound was produced by *S. aureus* exposed to SK-03-92, the secretions of drug-exposed cells were isolated and evaluated for antibiotic activity. First, *S. aureus* was exposed to SK-03-92 (4 \(\mu\)g/ml or SK-03-92\(^4\) with the superscript denoting the drug concentration in \(\mu\)g/mL). This concentration was achieved by statically incubating 1 mL SK-03-92\(^8\) in BHI-G broth mixed with 1 mL of *S. aureus* (1 x 10\(^7\) CFU/mL in phosphate buffered saline) at 37 °C for three hours and filter sterilizing the resulting supernatant (0.2 \(\mu\)M). We named this compound three-hour supernatant (4 \(\mu\)g/ml or 3HSN\(^4\) with the superscript denoting the concentration of residual SK-03-92 in the diluent). Because the residual concentration of the SK-03-92 drug was still at bactericidal levels, the 3HSN was further diluted to 1 \(\mu\)g/mL in BHI-G during experimentation. Loss of 3HSN efficacy was prevented by performing all experiments within 4 days of 3HSN production.

**S. aureus viability in 3HSN\(^1\) and SK-03-92\(^1\)**

The ability of populations of *S. aureus* Newman to survive in 3HSN\(^1\) for 24 h was assessed. To accomplish this, 3HSN\(^4\) was diluted in BHI-G broth (1:1) and then further diluted (1:1) in phosphate buffered saline (PBS – see Appendix C) within 96-well microtiter plates containing 1 x 10\(^6\) CFU/mL *S. aureus* cells. Each mixture was then statically incubated for 24 h at 37 °C. After incubation, the remaining viable cells were serially diluted 10-fold in PBS and each dilution plated onto BHI agar. Quantitation was made by counting the colony forming units after 18-24 h of growth and calculating the CFU/mL (135). For best practice, all cells used in the original bacterial dilutions were freshly grown on TSA for less than 24 h prior to suspension and evaporation of the media.
from the original growth mixture was mitigated by sealing the seams of the microtiter plates with tape prior incubation. Untreated cells were defined as *S. aureus* Newman grown in BHI-G, and the percent-viability was calculated by dividing the number of cells treated with 3HSN¹ by the number of untreated cells. These assays were performed a minimum of four times. The mean, standard deviation, percent-viability, and analysis of variance (ANOVA) were calculated using Microsoft Excel.

**Growth curves**

In addition to colony counts, the impact of 3HSN¹ on *S. aureus* growth was also assessed. This was done by comparing the turbidity of *S. aureus* Newman grown in 3HSN¹ to the turbidity of *S. aureus* Newman grown in SK-03-92¹ or BHI-G at OD₆₀₀. For this portion of the study, 1 × 10⁶ CFU/mL of *S. aureus* Newman was diluted (1:1) in either BHI-G, 3HSN² in BHI-G, or SK-03-92² in BHI-G, and incubated at 37 °C (250 rpm). Growth measurements were taken every 2 h for 10 h, and then again at 24 h at OD₆₀₀. Means, standard deviations, and analysis of variance were calculated using Microsoft Excel. The growth curves were performed three times using strains grown on varying media (BHI agar or TSA) on three different days.

**S. aureus** biofilm production in 3HSN¹ and SK-03-92¹

To compare how the amount of biofilm formed by *S. aureus* Newman was affected by 3HSN¹, SK-03-92¹, and BHI-G, the same biofilm assay outlined above was performed (134). Briefly, overnight cultures of *S. aureus* Newman were added to microtiter wells containing 3HSN or SK-03-92 in concentrations that would produce a final SK-03-92 concentration of 1 µg/µL. After 24 h of static incubation at 37 °C, the wells were processed as described above. The Newman *srtA* mutant was used as a
negative control. Means, standard error of the mean, and ANOVA analyses were performed using Microsoft Excel. These biofilm assays were performed a minimum of 10 times for each strain to achieve statistical significance.

**S. aureus viability in SK-03-92 supplemented with malate**

In an effort to eradicate persister cell formation in SK-03-92, cell viability in media containing the drug spiked with high concentrations (125 mM) of malate was measured. Briefly, a 2X strength mixture of malate in SK-03-92 drug was diluted in microtiter wells (1:1) with *S. aureus* Newman in PBS (1 x 10^6 CFU/mL). After 24 h of static growth at 37 °C, the well contents were serially diluted 10-fold and 100 µl aliquots spread onto BHI plates. After 24 hours of incubation, the surviving colony forming units were counted (CFU/mL) and compared among treatments. Control wells contained 125 mM of fumarate, oxaloacetate; or human-cytoplasmic concentrations of the same metabolites (2.5 mM fumarate, 3.2 mM malate, 0.24 mM oxaloacetate). Evidence of persister formation was also sought using control wells containing SK-03-92. All compounds were diluted in BHI-G and untreated cells grown in BHI-G were considered to be baseline growth. Prior to testing, the pH of all metabolites was adjusted to 7.2 (± 0.3). For best practice, all cells used in the original bacterial dilutions were freshly grown on TSA for less than 24 h prior to suspension and the seams of the microtiter plates were sealed with tape to mitigate evaporation. The percent-viability was calculated by dividing the viability of treated cells by the baseline. The viability assays were performed a minimum of 4 times. Means, standard deviations, and statistical analysis (ANOVA) were calculated using Microsoft Excel.
RESULTS: IN SILICO

The relation of the two-component system operon brpRS to mqo2

From the *S. aureus* MW2 genome sequence (109), the genes that encode the BrpR and BrpS proteins overlap in a unidirectional in-tandem sequence, indicating a probable co-translational overlap. This suggests that BrpRS is a two-protein system. Further, the *brpRS* genes are located just 66 base pairs upstream from the *mqo2* gene, so named because it encodes one of two malate:quinone oxidoreductases (MQO2) in *S. aureus* (Fig. 8). A MQO2 protein is bi-functional, both generating oxaloacetate by irreversibly oxidating malate and also donating electrons to the electron transport chain (136). The close proximity of the *mqo2* gene to the genes that encode BrpRS suggests that malate may play a role in BrpRS function, and thus, the SK-03-92-mediated response.

The amino acid sequences of the BrpRS proteins are homologous to the amino acid sequences of the BrsRM proteins

The SK-03-92 antibiotic has been shown to affect the BrpR and BrpS proteins, but the function of those proteins is unknown. The BrpR protein is predicted to contain a LytTR motif and bind DNA, whereas the BrpS protein is predicted to be a three-pass transmembrane protein (Figs 9 and 10; 113, 119). To gain insight into the possible function of these proteins, the amino acid sequences of both the BrpR and BrpS proteins

![Figure 8. Schematic representation of the chromosomal position and organization of the brpR, brpS, and mqo2 genes in the S. aureus strain MW2 genome.](image-url)
Figure 9. Predicted structures of the A) BrpR protein and B) BrpS protein. Calculated by Protter.

Figure 10. Visualization of the tertiary structure of the BrpR protein bound to DNA (left) and of BprS (right). Calculated by 35-hour processing through 1-TASSER servers (Yang Zhang Lab, University of Michigan) and visualized with PyMOL software.

were aligned to other bacterial proteins using an amino acid Alignment Search (BLASTp). This comparison showed that BrpR shares amino acid homology with the streptococcal TCS response regulator BrsR, and BrpS shares amino acid homology with
the streptococcal TCS sensor protein BrsM (Fig. 11). These streptococcal proteins belong to a two-component system responsible for sensing CSP and inducing late-stage competence in *S. mutans* (BrsRM). This affirmed BrpRS as a potential competence stimulating system. The detailed results of that alignment follow: the expect value (E), which signifies the number of times that an unrelated database sequence would obtain a match by chance, is $6 \times 10^{-5}$ for BrsR/BrpR and $9 \times 10^{-19}$ for BrsM/BrpS. BLAST identities (ID) are defined as the percentage of identical residues between two sequences. The percent identity for BrsR/BrpR is 30% and 25% for BrsM/BrpS. Positive scores
(POS) indicate residues that are homologous but not identical. The positive match values are 52% for BrsR/BrpR and 54% for BrsM/BrpS. Gaps are used by BLAST software to force the best match and typically indicate phylogenetic distance. Finally, there are two gaps between the BrsR/BrpR BLAST and eight between BrsM/BrpS.

**BrpRS protein homology in diverse bacterial species**

The ubiquity of BrpRS and BrsRM homologs was also explored. The reason for this was multifactorial. First, a previous study showed a wide range of SK-03-92 efficacy against multiple bacterial species (64). Thus, it was thought that SK-03-92 may induce species other than *S. aureus* to produce late-competence stimulating pheromones. Also, because the potential scope of this research may define the mechanism for novel, plant-based antibiotics, it was prudent to explore the plausibility of the effect of SK-03-92 on homologs to these specific proteins. Finally, although the downregulation of BrpRS and BrsRM seem to have a strong effect on bacterial viability, the functions of these proteins is not yet well defined. If BrpRS homologs are ubiquitous among multiple species, it is plausible that an advancement in the understanding of bacterial physiology may be achieved by understanding the function of these proteins. Thus, BLAST was performed to determine the ubiquity of BrpRS and BrsRM homologs among the full catalog of organisms in the NCBI database (Tables 2 and 3). This resulted in the finding that BrsRM homologs are produced by a vast diversity of genera. Of note, the well-studied metabolite-sensing YehUT TCS was among the proteins showing similarity to BrpRS. YehUT is a TCS found in a number of significant pathogens (e.g. *Salmonella enterica*, *E. coli*, and *M. tuberculosis*) that has not been fully
characterized but has been shown to be a carbon starvation protein that triggers persister cells to mature (78).

DNA sequences similar to *brpRS* are found in a diverse variety of bacterial species, but the diversity of species that bear *mqo2* genes upstream from *brpRS* gene homologs remained in question. This portion of the study found that the location of the system next to an *mqo2* gene is somewhat unique (Table 4). Aside from *S. aureus*, the genes that encode BrpRS homologs were found immediately downstream from the *mqo2* gene in *Lactobacillus sp.* (wkB8), *Staphylococcus haemolyticus* (51-48), and *Streptomyces sp.* (WAC00263). In other species, [e.g. *Chlamydia trachomatis* serovar L2b (H11MS), *E. coli* (K-12), *M. tuberculosis* (2926STDY5723586), *S. mutans* (UA159), and *S. pneumoniae* (R6)] it was not. These data also suggest that malate may be a key metabolite for the reversal of persistence in the pathogens listed in Table 4.

**Stress-induced, intergenic, sigma factor binding site patterns**

Bioinformatic analysis of the staphylococcal DNA also supported the idea that BrpRS is a stress response TCS. This is consistent with the idea that SK-03-92 causes pheromones to be produced in response to resource competition. A highly similar pattern of sigma factor binding sites can be found when the intergenic regions of *brpRS* and *srtA* are compared. Interestingly, all of the sigma factors found within this pattern (RpoD, SigB, RpoD, NFY, RpoD, SigB, SigB) are specific to the prokaryotic stress-response (Fig. 12). Although RpoD (SigA) is not technically considered to be a stress-induced sigma factor, it is nearly identical to the central regulator of the general stress response (SigS). In fact, these proteins are so similar that studies measuring SigA must block SigS expression lest false positive values arise. This pattern can be found spanning the entire
intergenic region upstream from brpRS. With respect to srtA, though, the pattern is only located within the enhancer region. Enhancer regions traditionally function by being constitutively blocked by repressors during times of inactivity. When these repressors are released, the likelihood that transcription will occur vastly increases. These data support the previously published suggestion that BrpR may block the enhancement of SrtA (62).

Table 2. BrpR amino acid sequence homologs are found among diverse species.

<table>
<thead>
<tr>
<th>Species and strain</th>
<th>Gene name</th>
<th>Expect</th>
<th>Identities</th>
<th>Positives</th>
<th>Gaps</th>
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<td><em>Bacillus cereus</em> (AF007599)</td>
<td>CN450 14065</td>
<td>3x10^{-9}</td>
<td>32%</td>
<td>59%</td>
<td>0/91</td>
</tr>
<tr>
<td><em>Clostridoides difficile</em> (P59)</td>
<td>QUI 0705</td>
<td>5x10^{-16}</td>
<td>31%</td>
<td>55%</td>
<td>9/143</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em> (CVM N59689F)</td>
<td>CUM91 15205</td>
<td>7x10^{-10}</td>
<td>32%</td>
<td>58%</td>
<td>1/94</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (K-12)</td>
<td>YehT/b2125/BtsR</td>
<td>4x10^{-9}</td>
<td>29%</td>
<td>55%</td>
<td>5/98</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em> (2926STDY5723586)</td>
<td>YehT</td>
<td>7x10^{-8}</td>
<td>27%</td>
<td>48%</td>
<td>9/100</td>
</tr>
<tr>
<td>Lactobacillus sp. (wk88)</td>
<td>LACWK88_RS02825</td>
<td>5x10^{-21}</td>
<td>31%</td>
<td>51%</td>
<td>10/150</td>
</tr>
<tr>
<td><em>Chlamydia trachomatis</em> (H11MS)</td>
<td>ERS133246_02042</td>
<td>2x10^{-23}</td>
<td>33%</td>
<td>57%</td>
<td>2/147</td>
</tr>
<tr>
<td><em>Staphylococcus haemolyticus</em> (51-48)</td>
<td>AK212_RS00835</td>
<td>1x10^{-25}</td>
<td>54%</td>
<td>72%</td>
<td>0/146</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em> (R6)</td>
<td>SPR0159</td>
<td>6x10^{-29}</td>
<td>40%</td>
<td>64%</td>
<td>0/147</td>
</tr>
<tr>
<td><em>Streptococcus mutans</em> (UA159)</td>
<td>SMU2080</td>
<td>9x10^{-9}</td>
<td>30%</td>
<td>52%</td>
<td>2/146</td>
</tr>
</tbody>
</table>

Table 3. BrpS amino acid sequence homologs are found among diverse species.

<table>
<thead>
<tr>
<th>Species and strain</th>
<th>Gene name</th>
<th>Expect</th>
<th>Identities</th>
<th>Positives</th>
<th>Gaps</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus cereus</em> (AF007599)</td>
<td>CN450 14060</td>
<td>3x10^{-2}</td>
<td>42%</td>
<td>62%</td>
<td>0/24</td>
</tr>
<tr>
<td><em>Clostridoides difficile</em> (P59)</td>
<td>QUI 0704</td>
<td>2x10^{-1}</td>
<td>26%</td>
<td>47%</td>
<td>6/82</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em> (CVM N59689F)</td>
<td>CUM91 15200</td>
<td>1x10^{-3}</td>
<td>24%</td>
<td>40%</td>
<td>18/109</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (K-12)</td>
<td>YehU/b2126/BtsS</td>
<td>5x10^{-4}</td>
<td>30%</td>
<td>48%</td>
<td>0/27</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em> (2926STDY5723586)</td>
<td>YehU</td>
<td>2.1x10^{-4}</td>
<td>36%</td>
<td>50%</td>
<td>0/22</td>
</tr>
<tr>
<td>Lactobacillus sp. (wk88)</td>
<td>LACWK88_RS02820</td>
<td>2x10^{-25}</td>
<td>40%</td>
<td>59%</td>
<td>5/135</td>
</tr>
<tr>
<td><em>Chlamydia trachomatis</em> (H11MS)</td>
<td>ERS133246_02041</td>
<td>3x10^{-21}</td>
<td>28%</td>
<td>52%</td>
<td>5/141</td>
</tr>
<tr>
<td><em>Staphylococcus haemolyticus</em> (51-48)</td>
<td>AK212_RS00840</td>
<td>1x10^{-32}</td>
<td>57%</td>
<td>76%</td>
<td>0/151</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em> (R6)</td>
<td>SPR0158</td>
<td>6x10^{-32}</td>
<td>37%</td>
<td>56%</td>
<td>2/151</td>
</tr>
<tr>
<td><em>Streptococcus mutans</em> (UA159)</td>
<td>SMU2081</td>
<td>6x10^{-5}</td>
<td>25%</td>
<td>54%</td>
<td>8/84</td>
</tr>
</tbody>
</table>

Table 4. The presence of mgo2 genes immediately downstream from BrpRS homologs. The expression of a malate:quinone oxidoreductase downstream from two-protein systems similar to BrpRS is not found in organisms sharing a niche with *S. aureus*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cytoplasmic regulator protein</th>
<th>Membrane sensor protein</th>
<th>MGO2</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus sp.</em></td>
<td>LACWK88_RS02825</td>
<td>LACWK88_RS02820</td>
<td>yes</td>
</tr>
<tr>
<td><em>Staphylococcus haemolyticus</em></td>
<td>AK212_RS00835</td>
<td>AK212_RS00840</td>
<td>yes</td>
</tr>
<tr>
<td><em>Streptomyces sp.</em></td>
<td>BOG92 07930</td>
<td>BOG92 07935</td>
<td>yes</td>
</tr>
<tr>
<td><em>Chlamydia trachomatis</em></td>
<td>ERS133246_02042</td>
<td>ERS133246_02041</td>
<td>no</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>b2125, JW5352, BtsR, YehT</td>
<td>b2126, JW5352, BtsS, YehU</td>
<td>no</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>YehT</td>
<td>YehU</td>
<td>yes</td>
</tr>
<tr>
<td><em>S. mutans</em></td>
<td>SMU2080</td>
<td>SMU2081</td>
<td>no</td>
</tr>
<tr>
<td><em>S. pneumoniae</em></td>
<td>Spr0159</td>
<td>Spr0158</td>
<td>no</td>
</tr>
</tbody>
</table>
Figure 12. Comparison of DNA binding site motifs in the intergenic region preceding \textit{brpRS} and the enhancer region of \textit{srtA}. A highly similar pattern of DNA binding sites specific for stress-response sigma factors is located in the intergenic region upstream of \textit{brpR} and \textit{srtA}. SigA/S = dark gray, SigB = black, NFY = light gray.

The alignment of BrpR to stress-induced sigma factors

Evidence was sought to support the proposition that the putative BrpR response regulator can antagonize the sigma factor binding site pattern discussed above. To do this, sequence homology between the cytoplasmic BrpR and the sigma factors was explored using BLASTp. This showed that homology between BrpR and the identified sigma factors was highly likely. Those results, found in table 5, follow: SigA': expect (E)=2x10^{-1}, percent identity (ID)= 60\%, positives (POS)=80\%, gaps (G)=0/10; SigA'': E=4 \times 10^{-1}, ID=23\%, POS=50\%, G=4/40; SigA'''': E=8.6, ID=27\%, POS=53\%, G=6/30; SigB: E=1.6, ID=26\%, POS=47\%, G=0/19; and SigS: E=4x10^{-1}, ID=55\%, POS=81\%, G=0/11. Sigma factor B and sigma factor S each contain short single sequences homologous to sequences at the N-terminus and central region of BrpR, respectively. Sigma factor A, on the other hand, contains three sequences homologous to BrpR of varying length. These are found throughout the protein at the N-terminus, central region, and C-terminus of BrpR. In sum, these alignments support the idea that BrpR can repress the enhancement of \textit{srtA}, by antagonizing the sigma factor binding sites listed above, under non-stress conditions.
Table 5. Alignment of BrpR to sigma factors A, B, and S. A’, A’’, and A ‘’’ represent three different alignments between BrpR and sigma factor A. Found at the N’-terminus, central region, and C’-terminus of SigA. The BLASTp (NCBI) algorithm parameters follow: max target sequences=100, automatically adjusted parameters for short input sequences, expect threshold=10, word size=3, max matches in a query range=0, matrix=BLOSUM62, gap costs=11 existence and 1 extension, and a conditional compositional score matrix adjustment.

<table>
<thead>
<tr>
<th>Sigma Factor</th>
<th>Expect</th>
<th>Percent-ID</th>
<th>Positives (%)</th>
<th>Gaps</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2x10^{-1}</td>
<td>60</td>
<td>80</td>
<td>0/10</td>
</tr>
<tr>
<td>A’’</td>
<td>4x10^{-1}</td>
<td>23</td>
<td>50</td>
<td>4/40</td>
</tr>
<tr>
<td>A ’’’</td>
<td>8.6</td>
<td>27</td>
<td>53</td>
<td>6/30</td>
</tr>
<tr>
<td>B</td>
<td>1.6</td>
<td>26</td>
<td>47</td>
<td>0/19</td>
</tr>
<tr>
<td>S</td>
<td>4x10^{-1}</td>
<td>55</td>
<td>81</td>
<td>0/11</td>
</tr>
</tbody>
</table>

It would be an error to assume that all residues homologous to the outlined sigma factors are capable of binding to DNA simply because they align. Thus, the sigma factor residue sequences with homology to BrpR were verified as DNA binding sequences. This showed that all regions estimated to share sequence homology with the SigA, SigB, and SigS were also DNA-binding motifs (Fig. 13).

![Figure 13. Visualization of the portions of BrpR that align to the stress-specific sigma factors SigA, SigB, and/or SigS. A pattern of DNA binding sites specific to those factors is found in the intergenic region preceding brpRS and the transcriptional enhancer region of srtA. Residues shown in bold were predicted to bind DNA by DP-Bind servers (Kutnetzov Lab, New York State University).](image)

**A QsrA/SigH binding pattern links competence to srtA transcription**

In addition to the likelihood that BrpR may act as a repressor of srtA enhancement, genetic bioinformatics also supported the proposition that srtA transcription is affected by competence. This portion of the study found that a QsrA/SigH/QsrA/SigH/SigH binding pattern, specific for bacteriocin production and competence, is located just 28 base pairs downstream from the ATG start codon of srtA in *S. aureus*. This pattern extends for ~200 bp (Fig. 14). Since SigH (MW0489) is the...
Figure 14. Highlight of SigH/QsrA binding motifs immediately preceding the ATG start codon of *srtA*. A distal SigH binding pattern sandwiched between two qsrA binding motifs and two proximal SigH motifs, all separated from the *srtA* start codon by a mere 28 nucleotides provides a direct link between competence and *srtA* transcription.
transcription factor that binds to this region to induce competence, a competitive SigH antagonist was sought within the *S. aureus* proteome. Only one BLASTp match was identified. Intriguingly, that match was the MQO2 protein located just downstream from BrpRS (Fig. 15). This surprising finding linked MQO2 production to competence-induced *srtA* repression. This finding also underscored the idea that the function of the BrpRS TCS and the upregulation of *srtA* transcription are somehow connected to malate conversion in *S. aureus*.

Figure 15. Sequence similarity between SigH and MQO2. BLASTp (NCBI) algorithm parameters: max target sequences=100, automatically adjusted parameters for short input sequences, expect threshold=10, word size=3, max matches in a query range=0, matrix=BLOSUM62, gap costs=11 existence and 1 extension, and a conditional compositional score matrix adjustment.
Reactive BrpS residues align dimensionally to BrsM

The shared functionality of BrpS and BrsM was further explored. By mapping and color coding the homologous and potentially reactive amino acid residues (e.g. lysine, serine, threonine, histidine, tyrosine, and glutamic acid), residues similar in topology and function were illuminated (118; Fig. 16). This also showed that BrpS is likely to be partitioned into four, distinct functional domains, separated by the membrane. These domains were designated follows: a leader peptide sequence spanning N’-1-MKNLKNSLFISLIIGLSSLFFSMLFADGKYYPLNPQSTIGILYYTHFT-50-C’; an extracellular loop from N’-108-PLTVHY-113-C’; and a cytoplasmic C-terminal tail covering the N’-135-KNKNYVNTINKQLQLK-151-C’ region. The next sections will individually analyze each of these segments and attempt to extrapolate meaning based on the similarities between the uncharacterized staphylococcal BrpS protein, the partially characterized streptococcal BrsM protein, and any bacterial component known to interact with BrsM protein.

Comparison of the leader sequence of BrpS to CSP and CSP-2

The leader sequence of BrpS shares sequence homology with the competence stimulating peptides secreted by *S. mutans* and *S. pneumoniae*. This segment of the protein, which spans N’-1-MKNLKNSLFISLIIGLSSLFFSMLFADGKYYPLNPQS TIGILYYTHFT-50-C’, was compared to CSP and CSP-2 by BLASTp. From the BLASTp analysis, we concluded that the competence stimulating peptides of *S. mutans* (1-SGSLSTFFRLFNRSFTQA-18, CSP) and *S. pneumoniae* (1-EMRISRIIILDFLFLRKK-17, CSP-2) has 56% similarity to CSP and 30% similarity to CSP-2 (Figs. 17, 18). This
Figure 16. Comparison of the sequences and predicted topologies of the putative TCS membrane sensor BrpS (left, *S. aureus*) and BrsM (right, *S. mutans*). According to this prediction, residues likely to be reactive (red) are topologically arranged in similar loci among both proteins. Intra is proposed to correspond with the cytoplasmic space, and extra is proposed to correspond with the extracellular milieu of the cell. Figures generated by Protter.

suggests that the leader peptide of BrpS antagonizes the residues responsible for sensing the competence stimulating pheromones of competitive species. Taking into account that BrpS is a putative membrane protein it is realistic to propose that portions of this protein may encounter CSP produced by these other species within this environment. At first glance, the BrpS leader sequence and the CSP-2 protein appear to share only a short residue similarity; but CSP-2 is only 17-residues long in its entirety, hence, the case can be made that sequence similarity is not likely due to chance. An E value of $9.8 \times 10^{-2}$ supports this. Unfortunately, it was not feasible to experimentally determine whether or not BrpS can sense CSP-2.
Figure 17. Comparison of the N’-terminal 50-peptide leader sequence of BrpS with the competence stimulating proteins CSP (S. mutans) and CSP-2 (S. pneumoniae). Processed by BLASTp (NCBI):
(A) Fifty six percent of CSP-2 is homologous with the leader sequence of BrpS. Residues 3..12: E=2.0x10^-3, ID=70%, POS=70%, G=1. Residues 48..50: E=5.7x10^-1, ID=67%, POS=100%, G=0.
(B) Thirty percent of CSP-2 is homologous to the leader sequence of BrpS. E=9.8x10^-2, ID=80%, POS 80%, G=0. Algorithm parameters: max target sequences=100, automatically adjusted parameters for short input sequences, expect threshold=10, word size=3, max matches in a query range=0, matrix=BLOSUM62, gap costs=11 existence and 1 extension, and a conditional compositional score matrix adjustment.

Figure 18. Visual representation of BrpS. Residues homologous to those of competence stimulating peptides CSP are shown in black and those homologous to CSP-2 are shown in light gray. Residues shared by both CSP and CSP-2 are dark gray. Generated by the Protter server.
Analysis of the cytoplasmic loop of BrpS and comparison to BrsM

*In silico* analysis of the cytoplasmic-loop portion of BrpS suggests that kinase activity is focused in this region. Unfortunately, the specific type of kinase located in this region appears to be indeterminable by bioinformatic means. Domain motifs within the N’-76-KYTDWSITKAT-86-C’ portion of the protein were sought using the online servers NetPHOS, ExPASy, Prosite, and GenomeNet.

Analysis of the extracellular loop of BrpS

We speculate that the extracellular loop of the protein, designated as N’-108-PLTVHY-113-C’, may be responsible for environmental sensing. This proposition is primarily due to the exterior location of this portion of the protein. The residues within this region most likely to be reactive, T110 and Y113, are homologous between BrpS and BrsM (Fig. 12). Both of these regions are only five residues in length but still share active residue homology. Again, residue homology within a very short sequence is not likely due to chance.

Analysis of the C-terminal tail of BrpS

The cytoplasmic C’-terminal tail of BrpS shares sequence similarity with the late-competence inhibiting protein CipI. The finding that the section N’-135-KNKNYVNTINKQLKQLK-151-C’, which comprises the C-terminal tail of BrpS, shares identity with the autolysis inhibitor CipI strengthens the proposition that BrpRS functions as an effector of late-competence in *S. aureus* (E=0.004, ID=38%, POS=61%, G=0/24; Fig. 19).

This cytoplasmic tail section also contains an uncharacterized phage holin motif. Using the same methods for a domain-motif search described in the section analyzing the
Figure 19. Comparison and visual representation of BrsM homology to CipI. (A) Primary sequence alignment of the C’-terminal 20-peptide tail sequence of BrpS with the CipI protein of *S. mutans* processed by BLASTp (NCBI). (B) Visualization of BrsM residues homologous to those of CipI (black) generated by the Protter server. Algorithm parameters: max target sequences=100, automatically adjusted parameters for short input sequences, expect threshold=10, word size=3, max matches in a query range=0, matrix=BLOSUM62, gap costs=11 existence and 1 extension, and a conditional compositional score matrix adjustment.

cytoplasmic loop of BrpS, a match was found between phage holin SPP1 family and the C-terminal tail region of BrpS (E=0.12). Although annotated as holins, members of the SPP1 family are not yet functionally characterized and do not necessarily function as holins. However, SPP1 is a double-stranded DNA phage that infects Gram-positive bacteria and is unequivocally linked to late-stage competence-induced autolysis. The finding of an SPP1 motif within this region supports the proposal that SK-03-92 induces bacterial suicide by late-stage competence.
RESULTS: *IN VITRO*

Increased biofilm production was observed when BrpR and BrpS were mutated

BrpRS activity has been indirectly linked to SrtA activity. Prior analysis by microarray and follow-up qRT-PCR demonstrated that the transcriptional abundance of srtA mRNA increased in brpR and brpS mutant strains when compared to wild-type cells (62). To verify this link between biofilm formation and BrpR and BrpS mutation, individual brpR and brpS mutations were transduced into *S. aureus* strain Newman. The relative biofilm production of each mutant was compared to biofilm production by wild-type *S. aureus* Newman. These experiments showed that the brpS and brpR mutants produced significantly more biofilm (200 and 300% respectively, *p*=0.03) than wild-type cells. When the brpR and brpS mutants were complemented, biofilm production returned to wild-type or sub-wild-type-levels in the complemented strains (Fig. 20). This suggested direct repression of biofilm production by the putative BrpRS TCS.

**Figure 20.** Effect of brpR and brpS mutations and complementation on *S. aureus* biofilm formation. All experiments represent the mean ± the standard error of the mean (SEM). Biofilm formation by wild-type *S. aureus* (WT) was considered to be 100% biofilm formation with the -srtA mutation representing a negative control. Ten biofilm assays done in duplicate were performed per strain. Differences were statistically compared by analysis of variance where * = *p*=0.05 and ** = *p* <0.005.

*S. aureus* exposed to SK-03-92 secretes a suicide signal
Cell death arises during competence through a mechanism of suicide. Thus, experiments were designed to determine whether or not a suicide inducing compound is secreted by \textit{S. aureus} exposed to the SK-03-92 drug. \textit{S. aureus} cells were mixed with the recaptured bacterial secretions of \textit{S. aureus} treated with SK-03-92 for 3 h. These were named three-hour supernatant (3HSN$^X$, with the superscript denoting the concentration of residual SK-03-92 in the diluent). After first diluting the recaptured secretions ($\frac{1}{2}$ MIC in BHI-G) to diminish the potentially confounding effects produced by bactericidal levels of residual drug, we determined whether this solution killed more cells than the same concentration of SK-03-92 in media alone. To do this, \textit{S. aureus} cells were grown in both environments, and then the number of CFU/mL were compared to a baseline count of bacteria grown in BHI-G alone. This showed us that, compared to the untreated population, an average population reduction of 91% occurred in 3HSN$^1$-treated cells and an increased cell population formed in SK-03-92$^1$ exposed cells (Fig. 21).

![Figure 21. Effect of 3HSN$^1$ on \textit{S. aureus} viability. All experiments represent the mean CFU/ml of \textit{S. aureus} grown in either SK-03-92$^1$ or 3HSN$^1$ ± standard deviation from at least four runs. Cell growth in BHI-G was considered to be 100% survival. Differences were statistically compared by ANOVA, where ** = p<0.005.](image-url)
These data experimentally supported the hypothesis that cellular suicide is triggered when \textit{S. aureus} is exposed to SK-03-92 above the MIC.

**3HSN\textsuperscript{1} treatment caused a delay in \textit{S. aureus} growth**

The growth of \textit{S. aureus} in 3HSN\textsuperscript{1} was measured and compared to growth in SK-03-92\textsuperscript{1}. We observed a prolonged lag time in both 3HSN\textsuperscript{1} and SK-03-92\textsuperscript{1} as compared to growth in BHI-G. When compared to growth in BHI-G, a spike in growth was observed in media containing SK-03-92\textsuperscript{1} after 24 h, whereas a growth-reduction was observed in media containing 3HSN\textsuperscript{1} (Fig. 22). This mirrored the observation that fewer cells survived treatment with 3HSN\textsuperscript{1} than SK-03-92\textsuperscript{1} alone (Fig. 21).

![Figure 22. The effect of 3HSN\textsuperscript{1} on \textit{S. aureus} growth over 24 h. The growth conditions tested were BHI-G broth (light gray dashes), 3HSN\textsuperscript{1} in BHI-G (solid black line), and SK-03-92\textsuperscript{1} in BHI-G (light gray line). Aliquots of each culture were sampled every 2 h up to 10 h measuring the optical density (O.D.) at 600 nm and then again at 24 h. Means ± standard deviations are indicated from at least three separate runs. Differences were statistically compared by ANOVA, ** = p<0.005 (top=3HSN\textsuperscript{1}, bottom=SK-03-92\textsuperscript{1}).](image-url)
**3HSN¹ treatment caused an increase in S. aureus biofilm production**

If *S. aureus* cells treated by SK-03-92 are stimulated to undergo late-stage competence, an increase in biofilm production should occur. To test this, cells were grown in microtiter wells containing either 3HSN¹ or SK-03-92¹ and the amount of cellular biofilm production was measured. When the results were normalized to account for the amount of biofilm produced per cell, cells exposed to 3HSN¹ produced 20-fold more biofilm than those exposed to SK-03-92¹ (Fig. 23). This supported the hypothesis that SK-03-92 induces *S. aureus* to enter a state of late-stage competence.

![Figure 23. Effect of 3HSN on S. aureus biofilm formation.](image)

**Formation of persister cells at SK-03-92 concentrations of 8 µg/mL**

Previously published MIC data showed that SK-03-92 killed *S. aureus* at concentrations higher than 2 µg/mL, viability assays showed that persister cells form at 8 µg/mL. Here, we note that a key difference between MIC growth and growth during
viability assays lies in the amount of incubation time. Traditionally, *S. aureus* MIC assays incubate statically for approximately 18 hours, whereas viability assays provide an additional 6 hours of growth in media and 24 hours of incubation with no antibiotic pressure. Prior to metabolite exposure, tests were run to ensure that *S. aureus* formed persister cells when exposed to SK-03-92 at concentrations of 8 µg/mL (Fig 25). This test showed a 7% increase in cell viability at 8 µg/mL concentrations of SK-03-92 (SK-03-92<sup>8</sup>), which is less than the previously published 10% formation. This difference does, however, fall within the measured range standard deviations and probably accounts for the deviation.

Figure 24. Effect of varying SK-03-92 concentrations on *S. aureus* persister cell formation. All experiments represent the mean CFU/ml of *S. aureus* grown in either SK-03-92<sup>1</sup> or SK-03-92<sup>8</sup> ± standard deviation from at least three runs. Cell growth in BHI-G was considered to be 100% survival. Differences were statistically compared by ANOVA, where ** = p<0.005.
A high concentration of malate was lethal to all cells exposed to SK-03-92

An unfortunate side-effect of SK-03-92 treatment was the drastic increase of bacterial persister cell formation. As previously discussed, persister cells cause chronic infection to occur (70). For this reason, we aimed to expand the effect of SK-03-92 by reversing drug-induced dormancy. We rationalized that if a unique signal compound is required to cause species competing within a niche to emerge from persistence, such a compound must be common but yet not normally found in large quantities in the environment. Thus, we determined that since the brpRS operon is in close proximity to mgo2, high levels of malate added to media containing the drug may reverse persistence and allow SK-03-92 to kill more cells. To test this, excess malate was added to media containing SK-03-928 and viability was measured. No S. aureus cells survived exposure to SK-03-92 mixed with excess malate. The cellular population grew to 3.6 x 108 CFU/mL in BHI-G broth containing only SK-03-928, and 1.4 x 108 CFU/mL formed in unsupplemented BHI-G broth (Fig. 24). This supported our second hypothesis. As controls, we also grew S. aureus in SK-03-92 spiked with high levels of oxaloacetate and fumarate, and physiological levels of all metabolites. S. aureus cells exposed to SK-03-928 and physiological levels of malate showed no difference in viability, and an inverse killing pattern was observed when comparing the lethality of SK-03-928 supplemented with high versus physiological levels of the aforementioned compounds (Fig. 25). Supplementing SK-03-928 with physiological levels of fumarate or oxaloacetate proved to be lethal to all S. aureus cells, whereas supplementation with high levels of fumarate or oxaloacetate did not increase the lethality of the drug.
Figure 24. Effect of high levels of malate on SK-03-92 killing. Figures represent the percent of viable *S. aureus* grown in persister-cell inducing levels of SK-03-92 supplemented with high (125 mM) levels of fumarate, malate, or oxaloacetate compared to the percent of viable *S. aureus* grown in BHI-G broth, which was the negative control and is represented by the bar at 100% average viability. Error bars represent ± standard deviation. Single factor ANOVA analysis (\(\alpha=0.05\)) provided a p-value of 0.00 for the percent-viability in SK-03-92 with malate, the oxaloacetate control, and SK-03-92 with.

n = 11 (BHI-G, n = 7 (SK-03-92), n = 5 (fumarate), n = 4 (SK-03-92 and fumarate), n = 5 (malate), n = 4 (SK-03-92 with malate), n = 5 (oxaloacetate), n = 5 (SK-03-92 with oxaloacetate).
Figure 25. Effect of human cytoplasmic levels of malate on SK-03-92 killing. Figures represent the percent of viable of *S. aureus* grown in persister-cell inducing levels of SK-03-92 supplemented with physiological levels of fumarate (2.5 mM), malate (3.2 mM), or oxaloacetate (0.24 mM) compared to the percent of viable *S. aureus* grown in BHI-G broth, which was the negative control and is represented by the bar at 100% average viability. Error bars represent ± standard deviation. Single factor ANOVA analysis (α=0.05) provided a p-value of 0.00 for the percent-viability in SK-03-92 with malate, the oxaloacetate control and SK-03-92 with. n = 16 (BHI-G broth), n = 11 (SK-03-92), n = 6 (fumarate), n = 6 (SK-03-92 and fumarate), n = 5 (malate), n = 5 (SK-03-92 with malate), n = 4 (oxaloacetate), n = 4 (SK-03-92 with oxaloacetate).
DISCUSSION

As we enter the dawn of a post-antibiotic era, the development of new drugs to treat antibiotic-resistant infections is of vital importance. Plant-derived antibiotics are an obvious and untapped reservoir of potential compounds, but unfortunately, an unknown mechanism of action prevents the therapeutic development of these products. This study addresses two major questions: the lack of a current proposed antibacterial mechanism of action for phytoalexin drugs and induction of bacterial persistence by SK-03-92 antibiotic treatment. We hypothesized that the mechanism of action of the SK-03-92 drug is to incite the release of a compound that induces late-stage competence in \textit{S. aureus} and that high levels of malate could reverse cellular persistence and allow for total SK-03-92 efficacy.

A decrease in bacterial cell viability coupled with an increase in biofilm formation is a hallmark of late-stage competence and our results demonstrated that the SK-03-92 drug is likely causing the onset of this phenomenon. This was evidenced in three ways. Firstly, \textit{in silico} data strongly supported this proposition. Secondly, cell death by SK-03-92 exposure appeared to be caused by a compound produced by drug-exposed cells. This was consistent with the late-stage competence model and was made evident by the finding that \textit{S. aureus} viability fell 91% when exposed to secretions recaptured from SK-03-92-exposed \textit{S. aureus} cells. Finally, a 20.7-fold increase in staphylococcal biofilm production was observed in response to SK-03-92-exposed bacterial secretions, further supporting the late-stage competence model.

Consistent with the late-competence model, cell suicide and increased biofilm formation in the staphylococcal response to SK-03-92-exposure may be tied to
holin/antiholin action. LrgA is an antiholin that functions to prevent cell auto-lysis by binding CidA holins (84). A prior study showed a 3.2-fold reduction of \( lrgA \) transcription in cells exposed to SK-03-92 when compared to non-exposed cells, which showed no change in the transcriptional abundance of \( cidA \) transcripts (62). In addition to cell death, a non-functional \( lrgA \) gene has also been correlated to the increased production of bacterial biofilms (137). This is hardly a surprise, considering that cell lysis has been shown to be the mechanism by which eDNA is rapidly produced to act as a scaffolding for newly forming biofilms (23). However, the finding that \( lrgA \) transcriptional abundance is reduced in staphylococcal cells exposed to SK-03-92 does tie the mechanism of action of SK-03-92 to the dysregulation of \( lrgA, srtA, brpR, \) and \( brpS \) transcription in a novel way. That this combination of dysregulated genes can tie phytoalexin action to late-stage bacterial competence is particularly surprising. Not only do these data fill in some gaps in the understanding of this important phase of cell survival but they also illuminate which stage of the bacterial life-cycle may lead to be leading to chronic infection and antibiotic insufficiency.

Our data suggests that BrpS is a receptor for a CSP-like pheromone released by \( S. aureus \) in response to resource competition. The SK-03-92 lead drug is an analog of a compound that is most likely to have developed by convergent evolution to protect the sweet fern plant from bacterial colonization (64). This study suggests that (E)-3-hydroxy-(5)-methoxystilbene acts to chemically camouflage the plant as a competing bacterial cell and in turn causes colonizing bacteria to enter late stage competence through the production of CSP-like pheromones. However, the isolation of a putative staphylococcal CSP-like compound was not the goal of this study. Instead, we
aimed to infer whether a staphylococcal CSP-like compound was indeed present. To do this, we diluted recaptured supernatant to reduce residual SK-03-92 to ½-MIC concentrations, which also diluted any recaptured CSP-like compound. Our growth curve matched a previously published study which measured the growth of *S. mutans* exposed to 2 µM of purified CSP for 14 h (138). Although we did not use purified CSP, the growth trend indicates that a similar concentration of a staphylococcal CSP-like compound may be present in the sterilized supernatant used in this study. A more exact estimation could be made by isolating the putative staphylococcal CSP-like compound and rerunning the *in vitro* experiments. Of course, isolating and identifying such a compound would also strongly support the proposed competence-induced mechanism of SK-03-92 action.

Several prior studies have examined the effect of CSP-like pheromones on bacterial cell viability and production of biofilms. Zhang et al. observed that the addition of CSP to growth media induced an average 76.3% reduction in cell survival and an 89.3% increase in biofilm biomass in *S. mutans* (139). Another study used recaptured supernatant from *S. mutans* co-cultured with *Aggregatibacter actinomycetemcomitans* and found a 1.3-fold increase in *S. mutans* biofilm formation (107). Yet another study showed a 3-fold increase in biofilm production in *S. pneumoniae* exposed to 10 ng/mL of exogenous CSP-2 and a 10-fold increase in biofilm production when CSP-2 concentrations were raised to 100 ng/mL (140). Unfortunately, despite ample evidence that cell death occurs in response to CSP exposure, none of those studies normalized biofilm production to account for a reduced number of cells (80, 138, 140, 141). For this reason, we assert that our estimated 20.7-fold increase in biofilm formation is more
accurate than the previously published estimations. Nevertheless, the cited studies all show that adding CSP to media correlated to cell death and biofilm formation in several Gram-positive bacterial species. Thus, our findings are consistent with the bioinformatic suggestion that SK-03-92 induces late-stage competence in *S. aureus*.

We also appear to have discovered a method to eradicate persisters formed in response to SK-03-92 treatment. As already discussed, bacterial persistence is a major clinical problem. For this reason, any drug or treatment that produces persister cells is likely to be considered clinically irrelevant. An earlier study showed that ~10% of *S. aureus* cells treated with the SK-03-92 drug survived as persisters (62). This study showed that within 24 hours the remaining 90% of cells are eradicated by SK-03-92-antibiotic treatment. Together, this accounts for the entire staphylococcal population. We saw that the addition of high concentrations of malate to media containing bactericidal levels of the SK-03-92 drug synergistically eradicated all of the *S. aureus* cells. We attribute this observation to the likely possibility that malate drives small colony variants out of quiescence and allows SK-03-92 to kill the persister cells as they mature. Together, these numbers account for the entire staphylococcal population.

Antibiotic use can lead to a microbiome shift and serious side-effects can occur in patients taking antibiotic drugs. No antibiotic currently exists that can specifically target a single pathogen. Of the species traditionally found within the respiratory microbiome, the bioinformatic data showed *S. aureus* is the only such species with a *brpRS/mqo2* pairing. Contextually, this makes sense. If a unique signal is required to resuscitate dormant bacterial cells within a certain niche, dormant bacteria that colonize the same niche would not have evolved to re-emerge to the same signal. Hence, it can be postulated that while
persistence in *S. aureus* may be reversed by high levels of extracellular malate, persistence in other species must require a different metabolite to begin to mature. The discovery of an antibiotic that can be supplemented with a metabolite to eradicate a specific bacterial species is a major breakthrough. Our research suggests that we now have access to a synergistic mixture that can target an entire *S. aureus* population, and while other species may be affected by SK-03-92 treatment, they too can be expected to leave behind a population of persister cells that can colonize their host. Clearly, the potential therapeutic value of such an option opens new possibilities in the chemotherapeutic fight against bacterial pathogenicity.

The resurrection and killing of persister cells as an antibiotic mechanism appears to be unique to this study. Hence, there is no exact precedence in the literature that parallels this angle of research. However, one study showed that the addition of glucose to media containing daptomycin increased antibiotic killing (142). Conversely, glucose and multiple-nutrient starvation have been correlated with the loss of viability of all but 1% of a *S. aureus* population (143). The remaining cells are likely to be persisters. These studies support the idea that SK-03-92 persister-induction can be reversed to increase the efficacy of SK-03-92. Fortunately, malate is a very safe for human use and consumption. As a compound regularly used in cosmetic chemistry as an emollient, malate has been extensively tested on human subjects (144). The addition of malate to SK-03-92 for therapeutic purposes is not predicted to produce toxic side-effects in humans.

We believe that there is a metabolic rationale that underlies the SK-03-92 mechanism of action. A connection has been clearly established between SK-03-92, BrpR, BrpS, and SrtA by this work and another study (62). By taking a metabolic
perspective, a cyclical picture begins to form. Figures 11 and 12 suggest that the production of malate may be interrupted by the binding of BrpR to the sigma factor binding sites downstream from *brpRS*. This binding may stop the conversion of malate to oxaloacetate and prevent the acetyl groups provided by acetyl-CoA from being picked up by citrate during the citric-acid cycle. In this way, an abundance of acetyl-CoA becomes available within the cell. The liberation of these functional groups would allow the epigenetic modification, and rapid release, of BrpR from the enhancer region of the *srtA* gene. The release of BrpR from *srtA* could provide more BrpR to bind to and block sigma factors from transcribing *brpRS* that would further repress *mqo2* transcription. The idea that BrpR is epigenetically blocked from DNA-binding is supported by the work of Zhang et al, which comprehensively profiled the lysine acetylomes of *S. aureus* and *E. coli* to identify a sequence motif (145). In that study, a total of 1,361 lysine sites in 412 proteins were cross-referenced to produce a conserved motif, N’-RLYELEQLxxxxFI RISKxxxxIVN-C’, that can be found in BrpR and is extremely well conserved among diverse bacteria. The sudden formation of persister cells in response to the SK-03-92 drug or by late-stage competence could be due to bacterial cells naturally lacking the energy needed to develop. Thus, the very foundation of the SK-03-92 mechanism of action and late-stage competence persister cell formation may be tied to BrpR repressing the production of malate. Future work that includes DNA fingerprinting of these regions in response to various CSP- or drug-treatments could solidify this argument.

The addition of human cytoplasmic levels of fumarate (2.5 mM) to persister-inducing levels of SK-03-92 killed all *S. aureus* cells (Fig. 25), while persister cells survived about half of the time when high levels of fumarate (125 mM) were present with
the drug. Thus, it appears that low levels of exogenous fumarate added to SK-03-92 may be sufficient to eradicate surviving persister cells. Unfortunately, fumarate has been shown to cause a regulatory cascade within the nuclei of human cells that inhibits the nuclear factor kappa B system, modulates the production of certain cytokines, and induces apoptosis in certain T-cell subsets. This suggests that fumarate is an unsatisfactory candidate to use as a potential additive to SK-03-92 (146, 147, 148).

In silico analysis also supports the previously published proposition that BrpR, which contains a LytTR DNA binding-motif, represses srtA expression (62). It has been suggested that LytTR proteins cannot act as repressors, but two proteins directly connected with this work contain LytTR motifs (149, 150, 151). One of these is BrsR, the analog of BrpR found in S. mutans upon which much of this work is dependent, and the other is the previously discussed S. pneumoniae BrpR homolog ComE (151). Both BrsR and ComE are multifunctional and responsible for both transcriptional repression and activation. In silico analysis suggested by this study shows a stress-induced sigma factor binding site pattern in the intergenic regions upstream from both the srtA and brpRS genes. The finding that this pattern can be found in the enhancer region upstream from the srtA gene suggests that BrpR represses sortase A transcriptional abundance under non-stress conditions. Future studies can use the discovery of this pattern to determine whether or not BrpR does, in fact, bind to this region.

In this study, our original hypotheses appear to be correct. We asserted that the mechanism of action of the SK-03-92 drug is to incite the release of a compound that induces late-stage competence in S. aureus. This was heavily supported by the in silico comparison of BrpRS to BrsRM, the detection of a suicide-inducing compound in
secretions of *S. aureus* exposed to SK-03-92 drug, and the finding that the secretions of
*S. aureus* exposed to SK-03-92 drug cause an intensive increase in staphylococcal
biofilm production. Our other hypothesis that persister cells can be eradicated by the
addition of excessive malate to media containing SK-03-92 also appears to be correct.
Based on the growth curves and viability assays, a novel mechanism of SK-03-92 action
and a way to eradicate all *S. aureus* cells exposed to the SK-03-92 drug may have been
uncovered. Through this study, the possibility of using plant-based phytoalexins as
antibiotic drugs moved a step forward. Although this research strongly infers the
presence of a staphylococcal CSP-like molecule released by *S. aureus* exposed to
SK-03-92, further work is required to determine whether that compound is a small
molecule, a protein, or a combination of both.
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APPENDIX A

REAGENTS FOR ELECTROPORATION
APPENDIX A. Reagents for electroporation

1. 0.5 M sucrose

68.40 g sucrose
400.00 ml distilled water
-filter sterilized

2. 2X SMM

85.50 g sucrose
1.15 g maleic acid
2.02 g MgCl₂ x 6 H₂O
250.00 ml distilled water
-adjusted pH to 6.5
-filter sterilized

3. 4X PAB

17.50 g antibiotic medium #3 (Difco)
250.00 mL distilled water
-autoclaved to sterilize

4. SMMMP

-mixed equal volumes of 2X SMM and 4X PAB

5. 10X TBE

890 mM Tris Base
890 mM Borate
20 mM EDTA (pH 8.0)
APPENDIX B. Reagents for biofilm assay

1. 1% crystal violet

   0.01 g crystal violet dye
   200.00 mL distilled water

2. 33% acetic acid

   33.00 mL 100% acetic acid
   67.00 mL distilled water
APPENDIX C. Multiuse reagents

1. Phosphate buffered saline

1.44 g NaH$_2$PO$_4$
0.24 g KH$_2$PO$_4$
8.00 g NaCl
0.20 g KCl
1.00 L distilled water
pH adjusted to 7.2 with HCl or NaOH
APPENDIX D

REAGENTS FOR PCR
APPENDIX D. Reagents for PCR

1. Complete Wigler’s buffer

   20 mM Tris-HCl (pH 8.8)
   500 mM NH₄SO₄
   150 mM bovine serum albumin
   25 mM MgCl₂
   5% β-mercaptoethanol
   pH adjusted to 8.8 with HCl or NaOH

2. Additional reagents

   50 mM of each dNTP
   2 mM of each primer (50 pmol)
   5 units of Taq polymerase