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Basic Study

Mutations of the *brpR* and *brpS* genes affect biofilm formation in *Staphylococcus aureus*

Allison Zank, Lillian Schulte, Xavier Brandon, Lauren Carstensen, Amy Wescott, William R Schwan

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Abstract

BACKGROUND

In the United States, *Staphylococcus aureus* (*S. aureus*) kills tens of thousands of individuals each year and the formation of a biofilm contributes to lethality. Biofilm-associated infections are hard to treat once the biofilm has formed. A new stilbene drug, labeled SK-03-92, was shown to kill *S. aureus* and affected transcription of two genes tied to a putative two-component system (TCS) we have named *brpR* (biofilm regulating protein regulator) and *brpS* (biofilm regulating protein sensor).

AIM

To determine if BrpR and BrpS regulate biofilm formation, *brpR* and *brpS* mutants were assessed using biofilm assays compared to wild-type *S. aureus*.

METHODS

A combination of biofilm and quantitative real-time-polymerase chain reaction assays were used. In addition, bioinformatic software tools were also utilized.

RESULTS

Significantly more biofilm was created in the *brpR* and *brpS* mutants vs wild-type cells. Quantitative real-time polymerase chain reactions showed the *brpS* mutant had differences in transcription of biofilm associated genes that were eight-fold higher for *srtA*, two-fold lower for *lrgA*, and 1.6-fold higher for *cidA* compared to wild-type. Bioinformatic analysis demonstrated that the *S. aureus* *brpR/brpS* TCS had homology to streptococcal late-stage competence proteins involved in cell-death, increased biofilm production, and the development of persister cells.

CONCLUSION

Our study suggests that *brpR/brpS* is a TCS that may repress *S. aureus* biofilm production and be linked to late-stage competence in *S. aureus*.

Key Words: Biofilm; Two-component system; Stilbene; *Staphylococcus aureus*; Late-stage competence; SK-03-92

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Core Tip: *Staphylococcus aureus* is a primary cause of skin/soft tissue infections. In this study, we have shown that two previously uncharacterized genes, *brpR* and *brpS*, encode proteins that we believe comprise a two-component system that regulates biofilm formation in *S. aureus*.

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INTRODUCTION

Staphylococcus aureus (*S. aureus*) is a significant pathogen of humans, causing more than 700000 skin/soft tissue infections, nearly 120000 bloodstream infections, and close to 20000 deaths *per* year in the United States[1-3]. Because drug resistance within this species continues to increase, new drugs are needed to treat human infections. Our research group has developed a new stilbene drug labeled SK-03-92 with efficacy against all Gram-positive bacteria that were tested, including methicillin-resistant *S. aureus*[4]. An mRNA microarray was performed on SK-03-92 treated *vs* untreated *S. aureus* cells to try to elucidate the mechanism of action of the drug[5]. From this microarray, the genes for a putative two-component system (TCS) (annotated as MW2284/MW2285) were the most downregulated at the transcriptional level. Moreover, transcription of the *srtA* gene (encoding sortase A) was upregulated and the *lrgA* gene encoding an anti-holin was downregulated following SK-03-92 treatment. Additionally, SK-03-92 treatment led to a high degree of persister cells and greater biofilm formation. Because of the effect on biofilm formation, the MW2284 gene was labeled *brpR* (biofilm regulating protein regulator) and the MW2285 gene was labeled *brpS* (biofilm regulating protein sensor).

Transcriptional changes of the *srtA* and *lrgA* genes as well as high numbers of persister cells suggested that SK-03-92 treatment may induce late-stage competence in *S. aureus*. Although competence allows DNA uptake to occur in heavily stressed cells, transformation is only one effect of bacterial competence. During early competence, which occurs prior to transformation, a large proportion of the stressed bacterial population die *via* holin-induced autolysis[6]. It is this phenomenon that supplies environmental DNA (eDNA) to the remaining cells for DNA uptake. Additionally, the surplus eDNA provides scaffolding for the rapid formation of a biofilm[7]. The final stage of natural competence is metabolic dormancy[8]. Current estimates show that only the youngest 1% of the original population survive to become a dormant cell. Thus, when faced with resource competition, a thriving bacterial colony has the ability to rapidly transform itself into a small group of latent (*i.e.* persister) cells living within a biofilm. These surviving cells re-emerge once environmental resources again become plentiful. This is one strategy used by bacterial cells to survive antibiotic challenge and re-infect the host[9].

The initiation of competence has been shown to rely on a symphony of genetic switches that begin to harmonize when short-sequence amino acids, known as competence stimulating pheromones (CSPs) bind to certain specific membrane proteins. The initiation of the CSP alarmone response has been well characterized in streptococcal species[10]. These membrane proteins are autoinducers that comprise one half of a specific TCS[11]. In *Streptococcus pneumoniae* (*S. pneumoniae*) and *Streptococcus mutans* (*S. mutans*), this response is initiated following the interaction with self-produced autoinducing pheromones known as CSPs, which are short, 14 residue peptides. The CSP is then received by the membrane bound sensor kinase ComD (*S. pneumoniae*)[12] or BrsM (*S. mutans*)[13]. Next, ComD or BrsM phosphorylate the cytoplasmic response regulator ComE (*S. pneumoniae*)[12] or BrsR (*S. mutans*)[13], which ultimately controls programmed cell death and persistence. In *S. aureus*, neither the CSP nor the TCS by which CSPs are received have yet been identified.

In this study, we have shown that mutations of the *brpR* and *brpS* genes in *S. aureus* strain Newman showed greater biofilm formation and transcriptional changes of the *srtA* and *lrgA* genes than wild-type *S. aureus*. Furthermore, we have used bioinformatic tools to show that the *brpR/brpS* TCS has homology to the BrsR/BrsM[13] and ComE/ComD[12] late-stage competence TCSs in *S. mutans* and *S. pneumoniae*, respectively. These findings suggest that the *brpR/brpS* TCS may be specific for the reception and resultant signal cascade of a molecule that induces late-stage competence in *S. aureus*.

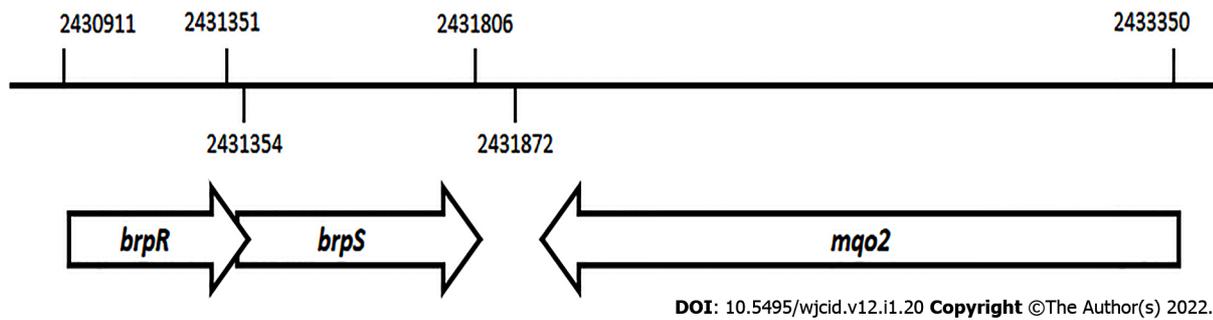


Figure 1 Schematic representation of the chromosomal position and organization of the *brpR*, *brpS*, and *mqo2* genes in the *Staphylococcus aureus* strain MW2 genome.

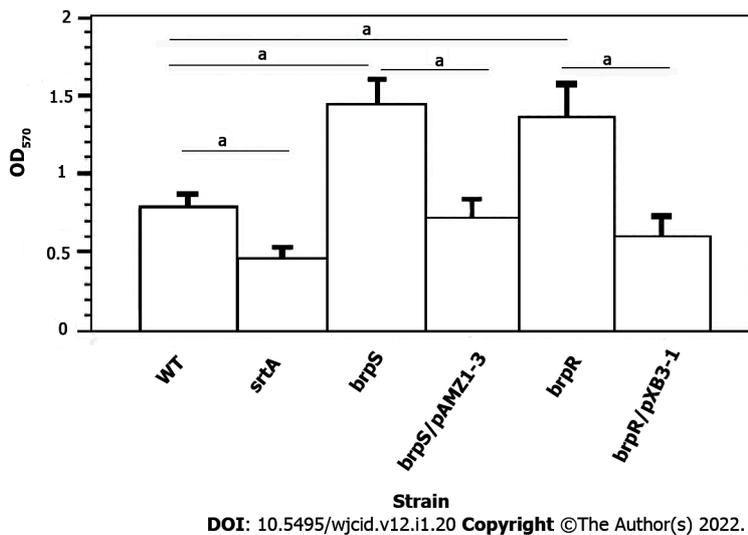


Figure 2 Effect of *brpR* and *brpS* mutations and complementation on *Staphylococcus aureus* biofilm formation. All experiments represent the mean \pm SD from five different runs done in triplicate for each strain. Biofilm formation was done on wild-type *Staphylococcus aureus* (*S. aureus*) strain Newman (WT, open column), *S. aureus* Newman *srtA* mutant (black column), *S. aureus* Newman *brpS* mutant (right striped column), *S. aureus* Newman *brpS* mutant/pAMZ1-3 (left striped column), *S. aureus* Newman *brpR* mutant (dark right striped column), and *S. aureus* Newman *brpR* mutant/pXB31 (dark left striped column). Differences were statistically compared by analysis of variance where $^aP < 0.001$.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions

All of the bacterial strains and plasmids used in this study are shown in Table 1. The *S. aureus* parent strain Newman was isolated from a human infection[14]. 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 [15]. Strains NE272 (*brpS* mutant), NE671 (*brpR* mutant), and NE1787 (*srtA* mutant) are erythromycin-resistant (Em^R) mutants representing part of the Nebraska Transposon Mutant Library created by the University of Nebraska Medical Center by *mariner* transposon mutagenesis[15] and obtained from the Network on Antimicrobial Resistance in *S. aureus* (NARSA) strain repository (Table 1). The *E. coli* strain DH5 α is a cloning strain with mutations that enable high-efficiency transformation[16]. *S. aureus* strain RN4220 is a transformation efficient strain of *S. aureus*[17].

To clone the *brpR* and *brpS* genes for complementation studies, plasmid pALC2073 was used. This plasmid carries ampicillin and chloramphenicol resistance genes, *E. coli* and Gram-positive origins of replication, and a *xyl/tetO* tetracycline inducible promoter[18].

All media was purchased from Thermo Fisher Scientific (Thermo Fisher Scientific, Pittsburgh, PA, United States). All antibiotics were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, United States). *E. coli* strains were grown in Luria (LB) broth shaken at 250 rpm at 37 °C or on Luria agar (LA) incubated at 37 °C. The *E. coli* strains carrying the pALC2073 plasmid were selected for in media containing 100 μ g/mL ampicillin.

All *S. aureus* strains were grown in brain heart infusion (BHI) broth with 1% (wt/vol) glucose (BHI-G) or trypticase soy broth shaken at 250 rpm at 37 °C. Agar grown *S. aureus* cultures were passaged on BHI agar at 37 °C. The *mariner* transposon mutant strains were grown in media with 5 μ g/mL of

Table 1 Bacterial strains and plasmids used in this study

Bacterial strain	Description	Ref.
<i>E. coli</i>		
DH5	Transformation efficient <i>E. coli</i> strain	[16]
<i>S. aureus</i>		
Newman	<i>S. aureus</i> clinical isolate	[14]
JE2	<i>S. aureus</i> USA300 MRSA strain	[15]
NE272	<i>S. aureus</i> JE2 <i>brpS::mariner</i> mutant	[15]
NE671	<i>S. aureus</i> JE2 <i>brpR::mariner</i> mutant	[15]
NE1787	<i>S. aureus</i> JE2 <i>srtA::mariner</i> mutant	[15]
RN4220	Transformation-efficient <i>S. aureus</i> strain	[17]
Newman <i>brpR</i>	<i>S. aureus</i> Newman <i>brpR::mariner</i> mutant	This study
Newman <i>brpS</i>	<i>S. aureus</i> Newman <i>brpS::mariner</i> mutant	This study
Newman <i>srtA</i>	<i>S. aureus</i> Newman <i>srtA::mariner</i> mutant	This study
Plasmids		
pXB3-1	pALC2073 plasmid with the <i>brpR</i> gene inserted	This study
pALC2073	Cloning vector with Ap ^r , Cm ^r , and Tc ^r genes, and a Tc-inducible promoter	[18]
pAMZ1-3	pALC2073 plasmid with the <i>brpS</i> gene inserted	This study

erythromycin (Em^s). *S. aureus* strains carrying the pALC2073, pXB3-1, or pAMZ1-3 plasmid were selected for in media containing 10 µg/mL of chloramphenicol (Cm¹⁰). To induce the *xyl/tetO* promoter on the pXB3-1 and pAMZ1-3 plasmids, tetracycline at 0.25 µg/mL was added to the growth medium.

Transduction of *S. aureus*

The *brpR::mariner*, *brpS::mariner*, and *srtA::mariner* mutations were transduced into strain Newman using the φ80a bacteriophage [19]. The transductants were then selected for on BHI agar containing Em^s. All mutations were verified by polymerase chain reaction (PCR) and biofilm assays.

Construction of *brpS* and *brpR* complementing plasmids

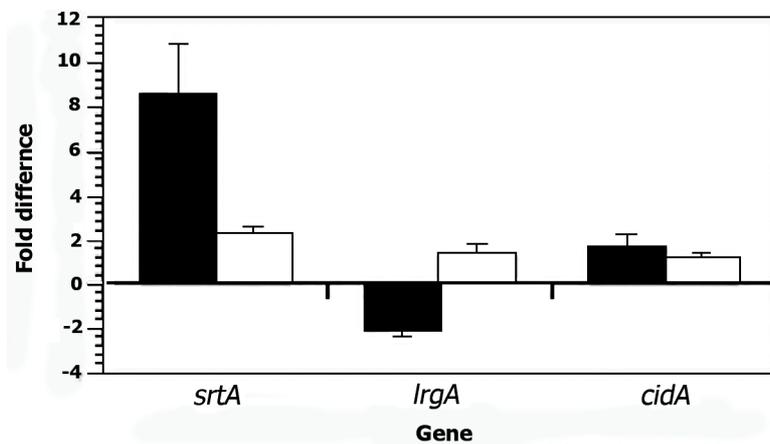
The *brpR* complementing plasmid was constructed using the pALC2073 backbone [18]. Isolation of pALC2073 plasmid DNA followed the manufacturer's instructions for the Qiagen QiaPrep plasmid isolation kit (Qiagen, Germantown, MD, United States). The full-length coding region of the *S. aureus* strain MW2 *brpR* gene was PCR amplified using the MW2284I/MW2284M primers (Integrated DNA Technologies, Coralville, IA, United States; Table 2) and the following PCR conditions: 35 cycles, 94 °C 1 min, 57 °C 1 min, 72 °C 1 min. *S. aureus* strain MW2 chromosomal DNA served as a template. The *brpR* DNA was amplified to have a *KpnI* site on the 5' end and an *EcoRI* site on the 3' end. PCR amplified *brpR* gene product was digested with *KpnI* and *EcoRI* (New England Biolabs, Ipswich, MA, United States), and then ligated with *KpnI/EcoRI* cut pALC2073 plasmid DNA using T₄ DNA ligase (New England Biolabs). Ligated DNA was transformed into *E. coli* strain DH5α cells [16]. Transformants were selected on LA containing 100 µg/mL ampicillin, and one resulting plasmid, plasmid pXB3-1, was used for further experiments.

A recombinant *brpS* complementing plasmid was also constructed. Isolation of pALC2073 plasmid DNA used the same plasmid isolation kit described above. The full-length coding region of the *S. aureus* strain MW2 *brpS* gene was PCR amplified using the GEX-5XB/GEX-5XC primers (Table 2) and the following PCR conditions: 35 cycles, 94 °C 1 min, 57 °C 1 min, 72 °C 1 min. *S. aureus* strain MW2 chromosomal DNA was used as the template. The *brpS* DNA was amplified to have a *KpnI* site on the 5' end and an *EcoRI* site on the 3' end. PCR amplified *brpS* gene product was digested with *KpnI* and *EcoRI* (New England Biolabs), and then ligated with *KpnI/EcoRI* cut pALC2073 plasmid DNA using T₄ DNA ligase (New England Biolabs) immediately downstream from the tetracycline-inducible *xyl/tetO* promoter on pALC2073. Ligated DNA was transformed into *E. coli* strain DH5α cells [16]. Transformants were selected on LA containing 100 mg/mL ampicillin, and one resulting plasmid, plasmid pAMZ1-3, was used for further experiments.

Plasmid DNA from *E. coli* was purified with a Qiagen Plasmid Miniprep Kit (Qiagen) and electroporated into the *S. aureus* strain RN4220 [20] using a GenePulser, (Bio-Rad, Hercules, CA, United States) under the following conditions: 100 W capacitance, 25 mF resistance, 2.5 kV charge voltage, 4 s.

Table 2 Primers used in this study

Primer	Sequence
GEX-5XC	5'- CCTAGGAGATCCTTTTCIGTC -3'
GEX-5XB	5'- GTTAATTTTACTAAACTTAAG -3'
MW2284I	5'- GAGCAGGTACCATGATGAAACTCAATTTATTATCAATGCAAAAAG-3'
MW2284M	5'- CAGCGAATTCTCAATGGTGATGGTGATGATTGATAATCGCTCCTTTATAGATTTAAAA -3'
CidA1	5'- TGCAACGATACATGTTCTATG -3'
CidA2	5'- CTACAACCTAGGAATCATCATTTG -3'
LrgA1	5'- GCATCAAAAACAGCACACTTT -3'
LrgA2	5'- GACTTCGCCTAACTTAACAGC -3'
SaFtsZ1	5'- GGTGTAGGTGGTGGCGGTAA -3'
SaFtsZ2	5'- TCATTGGCGTAGATTGTC -3'
SrtA1	5'- TCGCTGGTGGTACTTATC -3'
SrtA2	5'- CAGGTGTGCTGGTCTGGA -3'



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Figure 3 Quantitative reverse transcribed-polymerase chain reaction results of *Staphylococcus aureus* Newman *cidA*, *lrgA*, and *srtA* transcription in wild-type bacteria (standardized to 0) compared to a *Staphylococcus aureus* Newman *brpS* mutant (black column) and the complemented *brpS* mutant (white column). The data represents the mean \pm SD from three separate runs.

Transformants were selected for on BHI agar containing Cm¹⁰ after one hour of expression in BHI broth. Finally, plasmid DNA was re-isolated from one *S. aureus* strain RN4200 transformant carrying either the pXB3-1 or pAMZ1-3 plasmid using the method noted above with one alteration. The *S. aureus* cells were incubated with 50 μ L of lysostaphin (10 mg/mL; Remel, San Diego, CA, United States) for 60 min at 37 °C prior to the first step to facilitate lysis of the staphylococcal cells. Each isolated plasmid DNA sample was then cut with the *KpnI* and *EcoRI* restriction endonucleases to verify the insertion. The *S. aureus* strain Newman was then transformed with 10 mL of pXB3-1 or pAMZ1-3 plasmid DNA using electro- poration as outlined above and transformants selected for on BHI agar containing Cm¹⁰.

Biofilm assays

To determine the effect of the *brpR* and *brpS* mutations on the ability of *S. aureus* Newman to form a biofilm, biofilm assays were performed[21]. Briefly, cultures of the *S. aureus* were grown at 37 °C with shaking (250 rpm) overnight in BHIG broth with the appropriate antibiotic(s). Each strain was then diluted 1:100 in BHI-G and 220 μ L of the solution was placed in microtiter wells in triplicate in a 96-well microtiter plate. The microtiter plates were statically incubated for 24 h at 37 °C to allow a biofilm to form. Each well was then rinsed three times with sterile water. The biofilms were then allowed to settle (10 min), stained with crystal violet dye (0.1% wt/vol) for 10 min, and then washed with sterile water. After allowing the well contents to dry fully in a sterile hood, the dried contents were incubated in 33% acetic acid at room temperature for 30 min. The contents of the well were vigorously curettaged. The optical densities were measured on a SpectraMax M3 96-well microtiter plate reader (Molecular

A query: BrsM (*S. mutans*); subject: BrpS (*S. aureus*)

Sequence ID: Query_163319 Length: 148 Number of Matches: 1

Range 1: 54 to 133		Graphics		▼ Next Match	▲ Pr
Score	Expect	Method	Identities	Positives	Gaps
26.6 bits(57)	6e-05	Compositional matrix adjust.	21/84(25%)	46/84(54%)	8/84(9%)
Query 56	ISIIILLWLLIGVVFFLGDFIFKYTDWSITKATIMHFITTYVGFPLAMLAG----	WFPLTV	111		
	+ + ++ L+GV+ +FK S+ +++H++ LPL +LAG WF +T				
Sbjct 54	VQLFIFALLGVLQGFASSLFFKNEKLSLLTSLIHYLFIV---	LPL-LLAGSYLHWFYMTR	109		
Query 112	HYLIIFTIIFIVVYVLIWIIQFFK	135			
	Y + ++ ++Y+LI++ +F+				
Sbjct 110	KYFVFSFLLVSIIYILIYLFICYFE	133			

B Query: BrsR (SMU2080) Subject: BrpR (mw2284)

Sequence ID: Query_98161 Length:147 Number of Matches 1

Range 1:2 to 147		Graphics		▼ Next Match	▲ Previc
Score	Expect	Method	Identities	Positives	Gaps
64.3 bits(155)	9e-19	Compositional matrix adjust.	44/146(30%)	76/146(52%)	2/146(1%)
Query 1	MKVKI IKDSNFKEPLLQIYTRHIDKQTQRVIDFIQQ--RPNIVYGYKNCLEFIPLEKVI	58			
	MK+ + ++ E + I+ ++ Q +I+ + + + + GY +K + I + VI				
Sbjct 2	MKLNLFINAKETESYIDIHAPKMNDHVQSIINAVNDLTKSHTLVGYIDKEIHIINVSVDVI	61			
Query 59	RIFTENQVLIQTLTDTYLAKQRLYFFEQELGTPFIRISQGEIINIKYIKQLNLTIRGSI	118			
	NK V T + K RLY E++L FIRIS+ EI+N YI++L L G I				
Sbjct 62	TFQVINKNVTAITSNQKFKLRLRYELEKQLPQHFIRISKSEIVNKYYIEKLLLEPNGLI	121			
Query 119	EVTFKNGTVSFVARRSLKRFKEQLNL	144			
	+ K+ ++ +RR LK KE+L++				
Sbjct 122	RMYLKDAHITYSSRRYLKSIKERLSI	147			

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Figure 4 Bioinformatic comparison of the BrsRM proteins of *Streptococcus mutans* with the BrpRS proteins of *Staphylococcus aureus*.

A: BrsM, a two-component system membrane protein responsible for activating competence in response to sensing competitor organisms within a niche, shares sequence similarity with BrpS; B: BrsR, which is the cognate response regulator to BrsM, shares sequence similarity with BrpR. 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.

Devices, San Jose, CA, United States) at an optical density of 570 nm. In addition to wild-type Newman cells, Newman *brpR* and Newman *brpS* mutant strains as well as *brpR* and *brpS* mutants containing the pXB3-1 or pAMZ1-3 plasmids were tested and compared with a Newman *srtA* transposon mutant strain that served as a negative control. To achieve statistical significance, the biofilm assays were performed a minimum of five times in triplicate for each strain.

Quantitative real-time PCR

Total RNA was isolated from *S. aureus* strains grown to early logarithmic phase in BHI broth with shaking (250 rpm) incubated at 37 °C using a High Pure RNA Isolation kit ΔΔ (Roche Diagnostics, Indianapolis, IN, United States) with an additional lysostaphin treatment step to help lyse the *S. aureus* cell walls and a DNase I digestion to digest contaminating DNA. To confirm RNA concentration and ensure the integrity of each RNA sample, an aliquot of each RNA sample was analyzed on a Nanodrop machine (Thermo Scientific, Waltham, MA, United States) and electrophoresed through 0.8% agarose gels. The cDNA for each strain was then synthesized from 2 μg of total RNA according to manufacturer's instruction using a First-Strand Synthesis kit (Life Technologies, Carlsbad, CA, United States). All quantitative real-time PCR (qRT-PCR) trials were performed according to manufacturer's instruction using the iTaq Universal SYBR Supermix kit (BioRad, Hercules, CA, United States). Oligonucleotide primers that targeted the *ftsZ*, *srtA*, *lrgA*, and *cidA* genes were synthesized (Table 2) by Integrated DNA Technologies. To perform qRT-PCR, the minimum information for publication of quantitative real-time PCR experiments guidelines were followed and the *ftsZ* housekeeping gene was used as a standardization control [22]. All replicates were performed at least three times on a CFX96 qPCR instrument (BioRad, Hercules, CA, United States) under the following conditions: 94 °C, 20 s; 55

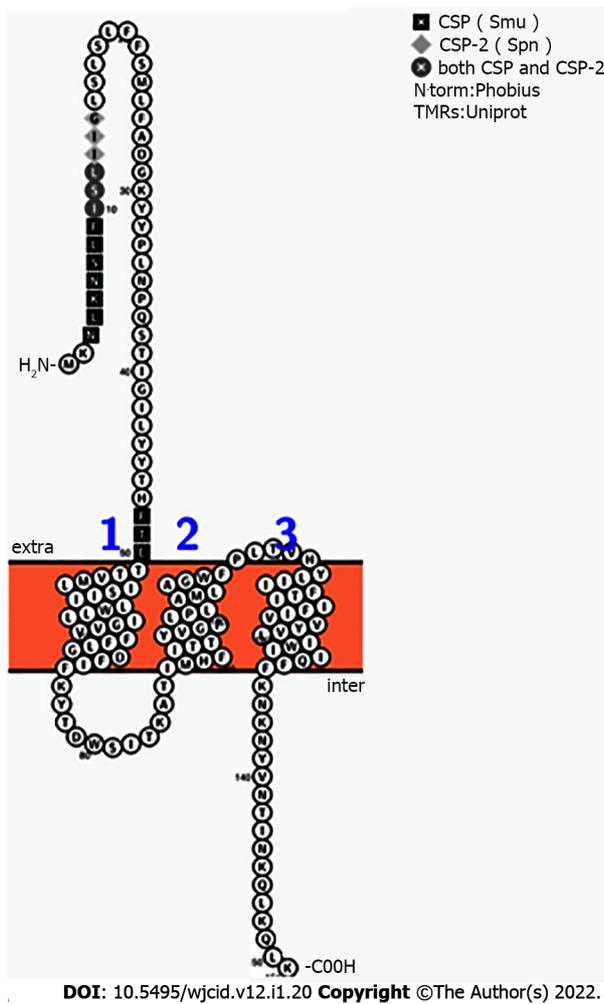


Figure 5 Highlight of amino acid residues shared by competence stimulating pheromone, competence stimulating pheromone-2, and BrpS. The predicted exterior segment of BrpS, which spans N'-1-MKNLKNLSLFIISLIIGLSLFLFSMLFADGKYYPNLPQSTIGILYYTHFT-50-C', was compared to competence stimulating pheromone (CSP) and CSP-2 by BLASTp. The competence stimulating peptides of *Streptococcus mutans* (1-SGSLSTFFRLFNRSFTA-18, CSP) and *Streptococcus pneumoniae* (1-EMRISRILDFLFLRKK-17, CSP-2) has 56% similarity to CSP and 30% similarity to CSP-2. CSP: Competence stimulating pheromone.

°C, 30 s; and 72 °C, 1 min for 35 cycles. The level of target gene transcript from each strain was estimated against the *ftsZ* gene standard curve. Additionally, crossover points for all genes were standardized to the crossover points for *ftsZ* in each sample using the $2^{-\Delta\Delta CT}$ formula[23].

Bioinformatic tools

The sequenced genomes of *S. aureus* strains MW2 and Newman used in this study are publicly available on GenBank (NCBI, genome assembly ASM1126v1)[24-26]. The protein annotations for all of the bacterial strains included in this study were found on BioCyc or GenBank[26,27]. BioCyc was also used to search for brpRS homologs downstream of the *mgo2* gene. The UniProt Consortium was used to obtain amino acid FASTA sequences[28]. Domain motifs were sought using NetPHOS, ExPASy, Prosite, and GenomeNet[28-30]. Protter was used to two-dimensionally visualize brpS and brsM[31]. I-TASSER and PyMOL were used together to three dimensionally visualize BrpR and BrpS[32,33]. I-TASSER and PyMol were also used to visually verify DNA binding in residues predicted by DP-Bind[34]. 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[35].

Statistical analysis

Calculation of the means, standard deviations, and paired Student's t-tests were performed using Microsoft Excel. $P < 0.05$ were considered significant.

RESULTS

Alignment of the *brpS*/*brpR* genes

An alignment of the *brpR* and *brpS* genes that encode the BrpR and BrpS proteins is seen on the *S. aureus* MW2 genome sequence (Figure 1)[24]. These genes overlap in a unidirectional in-tandem sequence. The overlapping *brpRS* genes lie just 66 base pairs upstream from the *mgo2* gene, encoding one of the two malate: Quinone oxidoreductases (MQO2) produced by *S. aureus*. The bi-functional MQO2 protein is able to generate oxaloacetate through an oxidation of malate as well as donate electrons to the electron transport chain[36].

Mutations in the *brpR* and *brpS* genes cause greater biofilm formation in *S. aureus*

To confirm biofilm formation was linked to BrpR and BrpS, individual *brpR* and *brpS* mutations were moved to the *S. aureus* strain JE2 background[15] to the *S. aureus* strain Newman via transduction. Biofilm production of both mutants was compared to the unmutated wild-type *S. aureus* Newman strain. Significantly more biofilm material was produced by the *brpS* and *brpR* mutants (1.8-fold and 1.73-fold higher, respectively, $P < 0.001$) compared to wild-type. Complementation of the *brpR* and *brpS* mutants caused biofilm expression to either return to wild-type levels or there was less biofilm material formed (Figure 2). The *srtA* mutant displayed a 1.73-fold decline in the biofilm forming ability compared to the wild-type strain ($P < 0.001$). This suggested that the putative BrpRS TCS may repress *S. aureus* biofilm production.

Transcription of *srtA* and *lrgA* are regulated by a *brpS* mutation

Previously, we showed *srtA* transcription was elevated and *lrgA* transcription was lower after SK-03-92 treatment of *S. aureus* cells compared to untreated cells[5]. The *srtA* gene encodes sortase A[37] and the *lrgA* gene encodes an anti-holin[38] that are important for the formation of biofilms[39,40]. Total RNA was collected during the mid-exponential growth phase from the *S. aureus* Newman *brpS* mutant, *S. aureus* Newman *brpS* mutant containing the pAMZ1-3 plasmid, and wild-type *S. aureus* Newman cells. Each RNA sample was converted to cDNA for qRT-PCRs analysis. The *brpS* mutant displayed 8.5-fold higher *srtA* transcription ($P < 0.008$), 2-fold lower *lrgA* transcription ($P < 0.016$), and 1.6-fold higher *cidA* transcription ($P < 0.43$) vs the wild-type strain (Figure 3). Complementation of the *brpS* mutation caused *srtA* transcription to drop to a 2.2-fold increase, a 1.3-fold increase in *lrgA* transcription, and a 1.2-fold increase in *cidA* transcription. These results demonstrated that transcription of some biofilm-associated genes was regulated by a mutation in the *brpS* gene.

BrpR/BrpS homology to other TCS proteins

BrpR and BrpS homologs were identified by BLAST analyses in multiple Gram-positive bacterial pathogens, including *Bacillus cereus*, *Clostridioides difficile*, *Enterococcus faecalis*, *Lactobacillus species*, *Staphylococcus haemolyticus*, *Streptococcus pneumoniae* ComD/ComE, and *S. mutans* BrsR/BrmM as well as three other bacterial species (*Escherichia coli* YehT/YehU, *Mycobacterium tuberculosis* YehT/YehU, and *Chlamydia trachomatis*)[26,35]. Of these, the *S. mutans* BrsR/BrmM TCS that senses CSP and then induces late-stage competence showed the highest homology (Figure 4)[12].

Putative structures of the BrpS protein

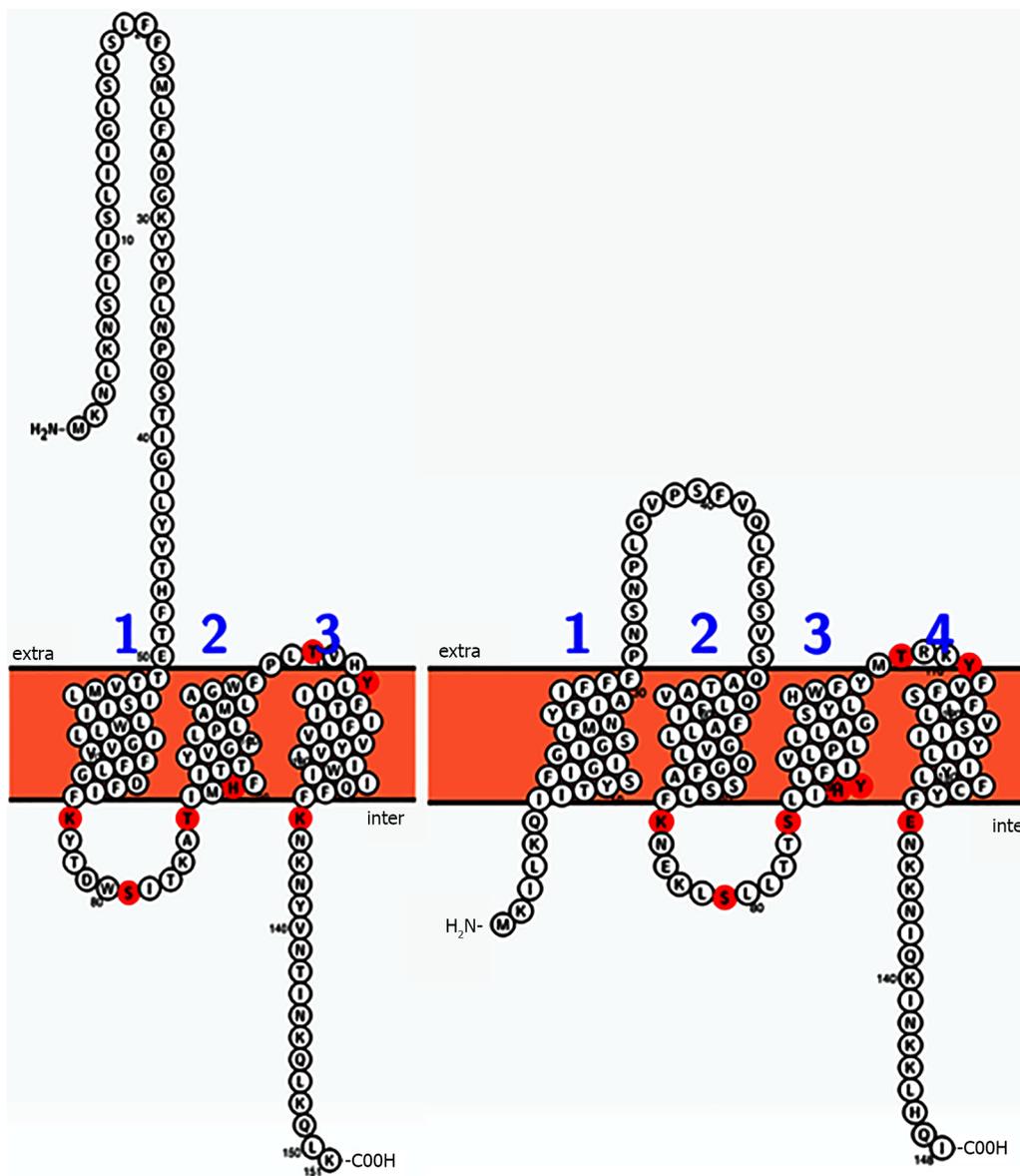
S. aureus BrpS and *S. mutans* BrmM have a similar arrangement of reactive residues. Lysine, serine, threonine, histidine, tyrosine, and glutamic acid residues were illuminated on a 2-dimensional Protter-generated image of each protein (Figure 5)[29]. This mapping suggested that BrpS and BrmM are partitioned into distinct functional domains separated by the membrane. Functionality appears to occur at the intercellular loop (staphylococcal N'-76-KYTDWSITKAT-86-C'), at the extracellular loop (staphylococcal N'-108-PLTVHY-113-C'), and within a single reactive residue near the membrane at K135 (staphylococcal) within the C'-terminal tail region.

Additionally, the region at the N'-terminus of *brpS* displays sequence homology with the secreted *S. mutans* and *S. pneumoniae* CSPs (Figure 6). The segment of *brpS* spanning the regions from N'-1-MKNLKNLSLFIIGLSLFFSMLFADGKYYPNPQSTIGILYYTHFT-50-C' showed 56% similarity with CSP (*S. mutans*, 1-SGSLSTFFRLFNRSFTQ A-18) and 30% similarity to CSP-2 (*S. pneumoniae*, 1-EMRISRILDFLFRKK-17).

DISCUSSION

S. aureus causes 65% of biofilm-associated infections per year[40,41]. Biofilms provide a defense against host immune defenses as well as most antibiotics. An understanding of what regulates *S. aureus* biofilm formation could lead to treatment options that target this process in *S. aureus*.

Both sortase A (SrtA) and antiholin (LrgA) are important *S. aureus* proteins needed for creation and maintenance of biofilms. Sortase A promotes the covalent anchoring of surface proteins to the cell wall



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Figure 6 Comparison of the sequences and predicted topologies of the putative two-component system membrane sensor BrpS (left, *Staphylococcus aureus*) and BrsM (right, *Streptococcus 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.

of *S. aureus*[42] that are important in the first stage of biofilm formation. Cell death releases eDNA that is tied to holin/antiholin action. An integral part of mature *S. aureus* biofilms is eDNA[7]. The function of the antiholin LrgA is to prevent cell autolysis by complexing with CidA holins[38,43].

In this study, we have shown that mutations in either the *brpR* or *brpS* gene cause an increase in biofilm formation as well as transcriptional changes of the *srtA* and *lrgA* genes, which are linked events. Previous studies with transposon mutants of what was an uncharacterized gene, that we have named *brpS*, displayed better biofilm formation than the wild-type strain[44,45]. Strains with a mutated *lrgA* gene have also been shown to produce increased levels of biofilms[46]. Another study has shown that cell lysis caused eDNA to be rapidly produced that could act as a scaffolding for newly forming biofilms [10].

The *in silico* data; biofilm results with the *brpR* and *brpS* mutants; and the data from the transcript abundance changes of the *lrgA*, *srtA*, *brpR*, and *brpS* genes suggest that BrpR/BrpS comprise a TCS that may be involved in late-stage competence. From the *in silico* analysis, we speculate that the BrpR protein (that possesses an apparent LytTR DNA binding-motif[47]) may repress *srtA* transcription. Other proteins that have LytTR motifs, such as BrsR and ComE, have been shown to have multifunctional activities tied to activation and repression[47-49]. Further analysis is required to show that BrpR is capable of binding to this region.

From the data presented, we speculate that BrpS is a receptor for a CSP-like pheromone secreted by *S. aureus* as a response to competition for resources. The leader peptide of BrpS may function to competitively antagonize the extracellular receptor portion of BrpS from the CSPs of competitive species, such as *S. mutans* and *S. pneumoniae*, that inhabit the human upper respiratory tract.

A number of previously completed studies focused on biofilm production and bacterial cell viability due to interactions with CSP-like pheromones. A study by Zhang *et al*[49] demonstrated that within *S. mutans* there was a 76.3% decline in cell viability and biofilm mass increased by 89.3% following the addition of CSP to bacterial growth media[49]. In addition, supernatant collected from *S. mutans* that was co-cultured with *Aggregatibacter actinomycetemcomitans* caused a 1.3-fold rise in biofilm production within *S. mutans*[50]. Ample evidence of cell death after CSP exposure has been documented by several studies, however, biofilm production has not been normalized to the viable bacterial cells that remain [51-53]. Nevertheless, a number of Gram-positive bacterial species show cell viability and subsequent biofilm production correlate with the level of CSP added to the media. Further studies should be done to assess the actual increase in biofilm formation by taking into account the findings that competence is accompanied by massive cellular death.

We also believe that there may be a connection between metabolic dormancy and the BrpR/BrpS TCS. If malate production is interrupted after BrpR binds to the sigma factor binding sites, malate conversion to oxalacetate would be halted. As a consequence of this interruption, any acetyl groups generated by acetyl-CoA would not interact with citrate within the citric-acid cycle. Thus, too much acetyl-CoA would arise within the cell. Because these functional groups would be liberated, it is possible that there would be an epigenetic modification and BrpR would be rapidly released from the *srtA* gene enhancer region. By freeing BrpR from the *srtA* gene enhancer region, additional BrpR molecules would be available to interact with sigma factors, blocking transcription of *brpRS* that would lead to even less transcription of the *mgo2* gene. The work by Zhang *et al*[54] used profiling of lysine acetylomes in *S. aureus* and *E. coli* to identify a sequence motif, which supports our idea that BrpR may epigenetically block DNA-binding[54]. As part of that study, 412 proteins and 1361 lysine sites were cross-referenced against each other, which led to a conserved motif, N'-RLYELEQLxxxFIRISKxxEIVN-C', being identified. BrpR has this conserved motif, which is very well conserved among a number of bacterial species. By shutting down malate expression, persister cells could form suddenly as a response to late-stage competence or treatment with the SK-03-92 drug. Thus, BrpR repression of malate production could be connected to formation of persister cells that is a feature of late-stage competence.

CONCLUSION

Our study suggests that BrpR/BrpS is a TCS that may repress *S. aureus* biofilm production and be linked to late-stage competence in *S. aureus*.

ARTICLE HIGHLIGHTS

Research background

Staphylococcus aureus (*S. aureus*) is a primary cause of skin/soft tissue infections. Biofilm formation is a key component of *S. aureus* pathogenesis. Thus, an understanding of what regulates biofilm formation in *S. aureus* is important.

Research motivation

We were interested in characterizing two open reading frames that we thought were tied to biofilm formation in *S. aureus*.

Research objectives

Determine if mutations in the *brpR* and *brpS* genes affected biofilm formation and what the respective proteins had homologies with.

Research methods

We used biofilm assays and quantitative real-time-polymerase chain reaction (qRT-PCR) analysis to test *brpR* and *brpS* mutants compared to the parent strain of *S. aureus*. Bioinformatic tools were used to determine what roles the BrpR and BrpS proteins may play in *S. aureus* cells.

Research results

The biofilm and qRT-PCR analyses demonstrated that mutations in the *brpR* and *brpS* genes affected biofilm formation in *S. aureus* and led to transcriptional differences in key biofilm-related genes as compared to the parent strain. Further, the BrpR and BrpS proteins share homologies with proteins

involved in late-stage competence in streptococcal species.

Research conclusions

BrpR/BrpS are likely a new two-component system which regulates biofilm formation in *S. aureus*.

Research perspectives

A better understanding of a new regulator of *S. aureus* biofilm formation has been identified.

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FOOTNOTES

Author contributions: Zank A, Wescott A, and Schwan WR designed the research study; Zank A, Schulte L, Brandon X, Carstensen L, Wescott A, and Schwan WR performed the research; Zank A, Brandon X, and Schwan WR contributed new plasmids; Zank A, and Schwan WR analyzed the data and wrote the manuscript; and all authors have read and approved the final manuscript.

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Conflict-of-interest statement: Schwan WR holds a composition of matter and use patent covering the SK-03-92 Lead compound.

Data sharing statement: The authors will share their data with whomever asks.

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