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Minireview

MINIREVIEW

Staphylococcus aureus pathogenesis in diverse host environments

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One sentence summary: *Staphylococcus aureus* uses a complex regulatory network to adapt to different environments to support its different lifestyles.
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ABSTRACT

Staphylococcus aureus is an eminent human pathogen that can colonize the human host and cause severe life-threatening illnesses. This bacterium can reside in and infect a wide range of host tissues, ranging from superficial surfaces like the skin to deeper tissues such as in the gastrointestinal tract, heart and bones. Due to its multifaceted lifestyle, *S. aureus* uses complex regulatory networks to sense diverse signals that enable it to adapt to different environments and modulate virulence. In this minireview, we explore well-characterized environmental and host cues that *S. aureus* responds to and describe how this pathogen modulates virulence in response to these signals. Lastly, we highlight therapeutic approaches undertaken by several groups to inhibit both signaling and the cognate regulators that sense and transmit these signals downstream.

Keywords: *Staphylococcus aureus*; MRSA; pathogenesis; gene regulation

INTRODUCTION

Staphylococcus aureus, aptly called a 'Janus-faced' bacterium (Broker, Holtfreter and Bekeredjian-Ding 2014), is a commensal organism and a debilitating pathogen. In the USA, ∼20% of the adult population carry *S. aureus* in their nares persistently, whereas ∼30% of the population is intermittently colonized by *S. aureus* (Wertheim *et al.* 2005). Nasal carriage of *S. aureus* in children is substantially higher, ranging from 45% to 70% (Wertheim *et al.* 2005). While colonization is typically not harmful to the host, *S. aureus* may breach innate host defenses and gain access to deeper tissues, causing a variety of superficial and invasive infections (Wertheim *et al.* 2005; Tong *et al.* 2015). For example, in healthy individuals in the community, *S. aureus* frequently causes minor skin and soft tissue infections such as impetigo, folliculitis and cutaneous abscesses. More rare but severe infections in the community include pyomyositis (Tong *et al.* 2015), necrotizing fasciitis (Foster 1996; Tong *et al.* 2015) and necrotizing pneumonia (Sader *et al.* 2016; Kale and Dhawan 2016). In nosocomial settings, *S. aureus* can initiate infections at surgical sites or from implanted medical devices including artificial heart valves, catheters, prosthetic joints and orthopedic implants (Richards *et al.* 1999; Brooks and Jefferson 2012; Hogan *et al.* 2015; Tong *et al.* 2015). During bacteremia, *S. aureus* circulates in blood and can seed vital organs (Archer *et al.* 2011), resulting in disseminated infections such as endocarditis, osteomyelitis and descending urinary tract infections (Foster 1996; Wertheim *et al.* 2005). The ability of this pathogen to persist in a wide variety of host niches ranging from skin (von Eiff *et al.* 2001; Montgomery, David and Daum 2015) to abiotic devices (Scherr *et al.* 2014) and deep-seated tissues makes it difficult to eradicate, resulting in recurrent infections.

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Staphylococcus aureus has caused havoc in both the community and healthcare setting, resulting in a high socioeconomic burden in both developed and developing nations. For example, a large-scale study evaluating skin and soft tissue infections between 2001 and 2009 estimated treatment costs of hospitalized patients in the USA to vary between ~$12 000 and $23 000 depending on the year and the patients' age group (Suaya *et al.* 2014). Management of *S. aureus* is complicated by the emergence of 'super bugs' that have become resistant to multiple antibiotics, as in the case of methicillin-resistant and vancomycin-resistant *S. aureus* (MRSA and VRSA, respectively). The average number of MRSA infections in the USA has been estimated to be ~80 000 cases with a mortality rate of ~11 000 individuals per year (Klevens *et al.* 2007; Malani 2014). Studies suggest that total treatment costs for MRSA infections are on the order of double that of MSSA infections (Filice *et al.* 2010). Thus, there is a critical need for new treatment strategies to manage *S. aureus* infections, especially infections with methicillin-resistant strains.

Importantly, *S. aureus* infections are most often derived from colonizing flora present on mucosal membranes or the skin of the infected host (Wertheim *et al.* 2005). Inasmuch as the commensal and invasive lifestyles are radically different, it is likely that the bacterium undergoes extensive adaptation while transitioning between the two states. Thus, understanding how *S. aureus* regulates its virulence in response to host environments is crucial to devising effective treatment strategies.

Staphylococcal virulence regulation involves a complex web of global regulatory circuits that sense environmental signals and influence the activation of master regulators, which act alone and in concert to modulate gene expression. In addition to external stimuli, *S. aureus* responds to cell density by means of an autoinduced, quorum-sensing signal. In the following section, we provide a brief overview of staphylococcal autoinduced and environmental signaling systems. We will also introduce additional regulators that play into these networks, and discuss the specific host signals that they respond to.

First identified in 1986, the accessory gene regulatory (Agr) quorum-sensing, two-component system (TCS) is still the most characterized master regulator of virulence in *S. aureus* (Recsei *et al.* 1986). Comprehensive reviews of this quorum-sensing system have been published (Lyon and Novick 2004; Novick and Geisinger 2008; Painter *et al.* 2014; Singh and Ray 2014; Wang and Muir 2016). Briefly, *S. aureus* produces basal levels of a peptide signaling molecule called the auto-inducing peptide (AIP). Accumulation of AIP triggers a series of signal transduction events that in turn activate expression of the *agr* locus. The *agr* locus consists of two divergent promoters, P2 and P3, that encode AgrBDCA and the major regulatory RNA effector RNAIII, respectively. When bacterial cell density surpasses a certain threshold (quorum), accumulated AIP binds to the histidine kinase, AgrC, which in turn phosphorylates the response regulator AgrA. Activated AgrA can directly regulate virulence genes (Queck *et al.* 2008), induce its own P2 promoter to increase the transcription of *agrBDCA* in a positive feedback loop and activate the adjacent P3 promoter to drive the transcription of RNAIII (Novick *et al.* 1993). The remaining two genes in the *agrP2* operon, *agrD* and *agrB*, respectively encode the AIP propeptide, and a transmembrane endopeptidase involved in the processing and export of the mature protein product.

RNAIII is the key effector molecule linking the Agr TCS and virulence. It is an RNA molecule that binds to the 5′ region of target mRNAs and post-transcriptionally represses or activates virulence factors such as various toxins and immune modulatory proteins, either acting directly or by influencing their upstream regulators. One of the principal targets of RNAIII is another critical virulence regulator, the **r**epressor **o**f **t**oxins (Rot). Rot positively and negatively modulates the activity of target promoters by directly binding to promoter elements (Said-Salim *et al.* 2003; Geisinger *et al.* 2006; Killikelly *et al.* 2015). During the onset of infection, the *agr* locus is thought to be inactive due to the presence of few bacteria and low levels of AIP, resulting in high levels of Rot. Rot in turn upregulates the expression of immune evasion proteins and adhesins that help dodge first-line, innate immune defenses (Said-Salim *et al.* 2003; Benson *et al.* 2011, 2012; Xue *et al.* 2012; Montgomery, David and Daum 2015; Mootz *et al.* 2015). These virulence proteins are critical for the initial stages of the infection. Later, after infection is established and quorum is reached, RNAIII levels increase, Rot translation is inhibited, and toxins and exo-enzymes responsible for lysis of immune cells and tissue destruction are expressed (Said-Salim *et al.* 2003; Mootz *et al.* 2015).

The SarA protein family members are an additional set of global regulators with broad consequences on transcription of staphylococcal virulence genes (Cheung and Zhang 2002). SarA can directly bind the *agr* P2 and P3 promoters, albeit with different affinities, causing increased transcription of *agrBDCA* and higher abundance of RNAIII (Cheung *et al.* 1992; Chien *et al.* 1999). Evidence also exists that in binding to the *agr* P2 promoter, SarA bends DNA and enhances the ability of AgrA to activate the P2 and P3 promoters (Morfeldt, Tegmark and Arvidson 1996; Cheung, Eberhardt and Heinrichs 1997; Chien and Cheung 1998; Chien *et al.* 1999). Additionally, SarA affects virulence independently of *agr* by binding directly to promoters of genes encoding for many virulence factors (Cheung and Ying 1994; Cheung, Eberhardt and Heinrichs 1997; Chan and Foster 1998; Sterba *et al.* 2003).

Another critical regulator of *S. aureus* virulence is encoded by the *saeRS* locus (Giraudo *et al.* 1994). Similar to *agr*, the *sae* locus encodes a TCS, SaeRS (Giraudo *et al.* 1999). However, unlike Agr, which is a 'self'-sensing system, SaeRS senses external stimuli and modulates virulence genes by binding to consensus sequences in promoter regions, directly influencing their transcription (Nygaard *et al.* 2010; Sun *et al.* 2010). SaeS serves as a sensor of environmental cues, and SaeR directly upregulates virulence in response to these signals (Montgomery, Boyle-Vavra and Daum 2010; Benson *et al.* 2012; Olson *et al.* 2013). While the *sae* locus is downstream of *agr* and is regulated by RNAIII via Rot (Li and Cheung 2008), it also has select functions that are epistastic to Agr (Novick and Jiang 2003). The transcription pattern of *sae* is complex; environmental signals such as changes in pH, high concentrations of sodium chloride and subinhibitory levels of certain antibiotics regulate its expression (Novick and Jiang 2003; Kuroda *et al.* 2007). Additionally, *sae* promoter activity is affected by exposure to phagocytosis-related signals such as hydrogen peroxide and antimicrobial peptides produced by neutrophils such as alpha defensins (Geiger *et al.* 2008; Flack *et al.* 2014) and calprotectin (Cho *et al.* 2015).

Despite our extensive knowledge of staphylococcal virulence factors and their regulation, subsequent treatments and vaccines based on this information have not been successful. The development of effective therapeutics is hampered by our limited understanding of *in vivo* signals that enhance or inhibit virulence. Results derived from *in vitro* studies or animal models of infection may not apply to the *in vivo* situation in humans. For instance, therapeutics under development that seek to inhibit *in vitro* expressed virulence effectors may not be effective for treatment of clinical infections in which they are not expressed or produced (Fowler and Proctor 2014). Thus, there is a critical

need to understand signals in the human host that *S. aureus* encounters and adapts to, which results in its ability to modulate virulence. Below, we summarize several well-characterized host signals that are critical for *S. aureus* fitness, and address how the host modulates levels of such signals during an infection to inhibit *S. aureus* growth. We discuss how, in turn, *S. aureus* uses host signals as cues to modulate virulence and tolerate host stresses. Lastly, we highlight how knowledge of host signals and regulators critical for fitness of this pathogen has informed the development of therapeutics aimed at modifying and preventing *S. aureus* disease.

HOST SIGNALS AND *STAPHYLOCOCCUS AUREUS* RESPONSES

Molecular oxygen

Oxygen levels in host

Molecular oxygen (O_2) is critical for *S. aureus* growth both *in vitro* and in host tissues. *In vivo*, O_2 vary by tissue sites (Carreau *et al.* 2011). For example, the arterial blood O_2 content is 68–95 mmHg, while venous blood contains ∼40 mmHg O_2 (Park, Myers and Marzella 1992). The skin possesses a wide range of O_2 concentrations depending on the depth from the surface (8–35 mmHg). The intestinal lumen is completely anaerobic and contains <2 mmHg O_2 (Zeitouni *et al.* 2016), whereas critical organs such as the kidneys and liver contain relatively high levels of O_2 (∼50–72 mmHg and ∼30—40 mmHg, respectively) (Brezis and Rosen 1995; Brooks *et al.* 2004; Carreau *et al.* 2011). Thus, tissues contain wide range of O_2 levels, from being essentially anaerobic (intestines) to comparatively O_2 replete (blood rich tissues). During an infection, rapid recruitment of energy-consuming immune cells such as activated neutrophils can increase O_2 demands more than 50-fold (Gabig, Bearman and Babior 1979; Colgan and Taylor 2010), triggering oxygen deficiency (hypoxia) at sites of infection (Schaffer and Taylor 2015; Zeitouni *et al.* 2016). Additionally, tissue-resident macrophages, dendritic cells and T cells induce inflammation, in turn altering vascular structures, leading to restricted blood flow to tissues and reducing O_2 levels dramatically (Colgan and Taylor 2010).

Biofilms have also been shown to induce hypoxia (Lone *et al.* 2015). Biofilms are complex microbial communities attached to surfaces or other cells that have a protective extracellular matrix, and can thus promote *S. aureus* colonization (Lister and Horswill 2014). Formation of biofilms by *S. aureus* on medical implants and host tissues makes this pathogen a leading cause of device-related infections, and results in dangerous, chronic and recurrent infections (Lister and Horswill 2014). *In vitro* experiments demonstrate that anaerobic conditions induce expression of 'biofilm' genes, as evidenced by induction of *icaADBC* (Cramton *et al.* 2001), whose gene products lead to the production and transport of extracellular polysaccharide adhesins that help in attachment of bacterial cells to each other, to host cells and to surfaces (Vuong *et al.* 2004; O'Gara 2007). Thus, depletion of O_2 may either be a by-product of bacterial growth or a strategy employed by the bacterium to induce biofilm.

Osteomyelitis or infection of the bones is a low oxygen, biofilm-associated infection. *Staphylococcus aureus* is the major cause of osteomyelitis in adults and children, accounting for 70%–90% of infections in the latter (Bocchini *et al.* 2006; Hatzenbuehler and Pulling 2011; Pendleton and Kocher 2015). Bone and bone marrow are considered hypoxic, due to low blood flow to these tissues (Mader *et al.* 1980; Spencer *et al.* 2014). Upon infection with *S. aureus*, O_2 levels plummet further (Wilde *et al.* 2015), similar to what happens with O_2 levels in device-related *S. aureus* biofilms (described above). Importantly, *in vitro* studies indicate that hypoxic conditions increase *S. aureus* cytotoxin production, suggesting that reduced O_2 states promote *S. aureus* pathogenesis (Wilde *et al.* 2015). Moreover, *S. aureus* can induce hypoxia even in tissues that have relatively higher levels of O_2, like the kidneys (Vitko, Spahich and Richardson 2015), leading to formation of O_2-restricted microenvironments, such as abscesses. *Staphylococcus aureus* can then disseminate from these abscesses, become bacteremic and seed a variety of vital organs (Rubinstein 2008; Cheng *et al.* 2009; Sheen *et al.* 2010; Dahl, Hansen and Bruun 2013; Lister and Horswill 2014). Collectively, these observations suggest that *S. aureus* promotes hypoxia in tissues, which is a key signal for *S. aureus* biofilm formation and enhanced staphylococcal virulence (summarized in Fig. 1).

Staphylococcus aureus responses to hypoxia

Under conditions of decreased oxygen, *S. aureus* readily uses nitrate and nitrite as its final oxygen acceptors. In the absence of these two terminal O_2 acceptors, the bacterium will switch to fermentative metabolism (Burke and Lascelles 1975; Pagels *et al.* 2010). Hypoxic or anaerobic conditions result in two major challenges: inability to replenish the NADH/NAD+ pools and inefficient ATP synthesis (Green and Paget 2004). *Staphylococcus aureus* is less versatile in comparison to other facultative aerobes such as *Escherichia coli* because it has less complex fermentative pathways and lacks cytochrome oxidases present in the latter (Burke and Lascelles 1975). However, it has several sensors by which it can quickly recognize hypoxia/anaerobiosis and turn on nitrate respiration and fermentation. Under anaerobic conditions, *S. aureus* upregulates genes in glycolysis, fermentation and anaerobic respiration and represses genes in the Krebs cycle—the main pathway responsible for NADH generation (Fuchs *et al.* 2007). In addition, genes involved in nitrate and nitrite reduction pathways are upregulated (Fuchs *et al.* 2007). Thus, concomitant with its ability to promote hypoxia, *S. aureus* has multiple regulatory pathways to respire in low oxygen conditions.

The staphylococcal respiratory response AB (SrrAB) TCS is critical for anaerobic growth of *S. aureus in vitro* (Throup *et al.* 2001; Yarwood, McCormick and Schlievert 2001; Kinkel *et al.* 2013). SrrAB was found bioinformatically due to its homology to the O_2-responsive TCS in *Bacillus subtilis* called ResDE (Yarwood, McCormick and Schlievert 2001). The ligand responsible for SrrAB activation is currently unknown, although Kinkel *et al.* (2013) offer menaquinone as the most likely candidate based on various inducers of SrrAB. This hypothesis has been supported by Schlievert *et al.* (2013), who demonstrate that menaquinone analogs affect both *S. aureus* growth and alter toxin production in a SrrAB-dependent manner. SrrAB is induced under nitric oxide stress, detoxifies nitric oxide (Kinkel *et al.* 2013; Grosser *et al.* 2016) and is required for efficient biofilm formation (Ulrich *et al.* 2007; Kinkel *et al.* 2013). Several studies have demonstrated the contribution of SrrAB to *S. aureus* metabolism and pathogenesis. However, studies on the relationship between SrrAB and virulence produced seemingly conflicting results. Deletion of *srrAB* was shown to decrease bacterial recovery from infected kidneys in mice (Throup *et al.* 2001), and an *srrAB* mutant was attenuated in osteomyelitis. These results suggest that SrrAB enhances virulence (Wilde *et al.* 2015). In contrast, *in vitro* studies indicate that SrrAB represses virulence by negatively influencing *agr* P2/P3 and presumably virulence (Throup *et al.* 2001; Pragman *et al.* 2004). However, this apparent paradox was recently resolved by the demonstration that the *in vivo* attenuation of the mutant during osteomyelitis is independent of RNAIII (Wilde *et al.* 2015).

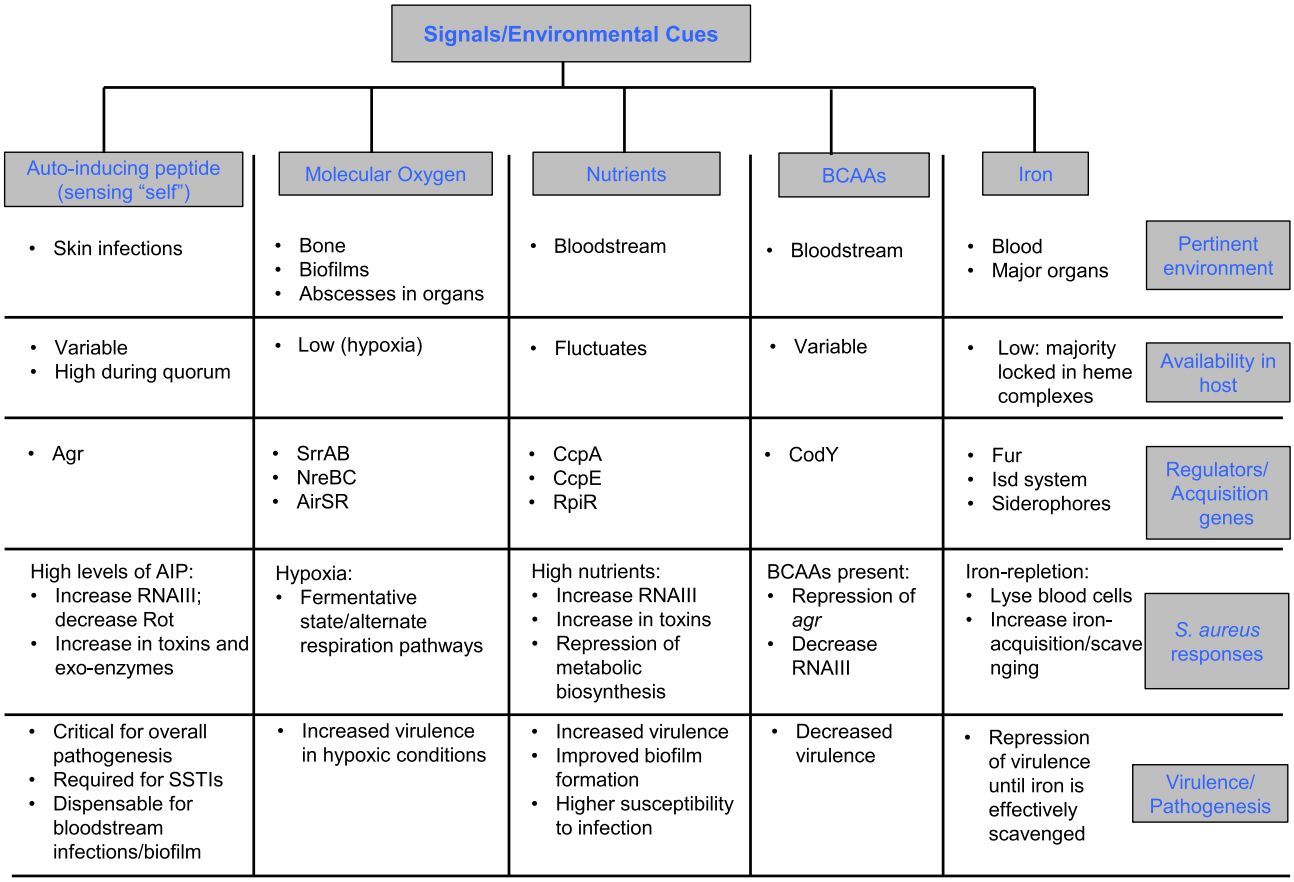

Figure 1. Summary of host signals encountered by *S. aureus* and the response to these cues. *Staphylococcus aureus* senses 'self' or external environmental cues via various sensors and regulators that transmit these signals to alter metabolism and virulence.

Both hypoxia and the *srrA* deletion resulted in enhanced expression of phenol-soluble modulins (PSMs) in an AgrA-dependent, but RNAIII-independent manner (Queck *et al.* 2008). Thus, although SrrAB represses RNAIII *in vitro*, it is an activator of virulence during osteomyelitis.

NreBC is another TCS involved in O_2 sensing and nitrogen regulation that was first identified in *S. carnosus* (Fedtke *et al.* 2002). NreBC is encoded in an operon with NreA. The exact function of NreA is unclear although there are hints that it is a nitrate sensor (Hall and Ji 2013). The sensor histidine kinase NreB is a fumerate and nitrate reductase-type, cytoplasmic protein containing four conserved cysteine residues that together comprise an Fe-S cluster (Kamps *et al.* 2004). The presence of O_2 renders the NreBC TCS inactive due to oxidation of the Fe-S cluster, while the absence of O_2 leads to reduction of the Fe-S cluster, causing dimerization and activation of the NreC response regulator, and ultimately induction of the nitrate reductase system (Kamps *et al.* 2004; Hall and Ji 2013). Inactivation of NreBC abrogates the ability of *S. aureus* to reduce nitrate, forcing the bacterium to upregulate fermentative pathways for survival (Fedtke *et al.* 2002; Schlag *et al.* 2008; Yan *et al.* 2011). Under anaerobic and nitrate respiration conditions, the NreABC locus was shown to induce nitrite and nitrate reductase genes (Schlag *et al.* 2008). However, no phenotypes have been described for this system *in vivo*.

The third sensor of O_2 in *S. aureus* is the AirSR/YhcSR TCS, a pleiotropic regulator that is essential for *S. aureus* survival (Sun *et al.* 2005). It is involved in the positive regulation of the NreBC TCS during anaerobic growth of bacteria (Yan *et al.* 2011). Expression of *airSR* increases by the addition of exogenous nitrate, but not nitrite, suggesting its exclusive role in nitrate respiration (Yan *et al.* 2011). Consistent with this hypothesis, downregulation of *airSR* leads to poor growth of *S. aureus* under anaerobic growth in media containing nitrate (Yan *et al.* 2011). Similar to NreBC, AirR binds to the promoters of the nitrate reductase gene, *narG* (Yan *et al.* 2012). Likewise, the activity state of this TCS is determined by oxidation of an Fe-S cluster present in AirS (Sun *et al.* 2012b). Depletion of AirSR using antisense RNA interference results in decreased *S. aureus* survival in human blood, presumably owing to decreased *S. aureus* protease production. Additionally, proteases that are important for *S. aureus* pathogenesis are regulated by AirSR at the promoter level (Hall *et al.* 2015).

In addition to directly sensing oxygen, *S. aureus* also produces Rex, a protein that senses NAD+/NADH pools, allowing the bacterium to monitor its metabolic state independently of O_2 (Somerville and Proctor 2009). Rex activation leads to increased levels of enzymes involved in fermentative pathways, nitrate/nitrite reductases and *srrAB* (Pagels *et al.* 2010). Lastly, the response regulator of the AgrAC TCS, AgrA, has been shown to modulate virulence factor production in response to oxygen levels in the cell. It is thought that under oxidative stress, intramolecular disulfide bonds between two cysteine residues within the AgrA active site impede AgrA DNA-binding activity, thus affecting transcription of various virulence factors (Sun *et al.* 2012a,b). Taken together, *S. aureus* has multiple regulatory proteins that interact in a complex manner to counteract low oxygen states in the host. These genetic elements perform dual functions by activating genes required to handle hypoxic stress

and enable virulence by increasing expression of toxins and proteases.

Nutrients and metabolic signals

Availability in the host

Carbohydrates (carbon sources) are critical for cellular growth and metabolism. They serve as the precursors and metabolic intermediates in pathways such as glycolysis, the pentose phosphate pathway and the tricarboxylic acid (Krebs) cycle. Glucose is the preferred carbon source of most organisms (Monod 1942). In humans, glucose is produced and stored in the liver until it is transported into the bloodstream for distribution throughout the body. Glucose serves as the major energy source for many cell types and as a result, its homeostasis is carefully regulated (Nordlie, Foster and Lange 1999). This is not surprising given that glucose is the most abundant free carbohydrate in human serum (Psychogios *et al.* 2011). In humans, blood glucose levels in the 80–130 mg/dL range are considered normal, while <70 and >200 mg/dL are indicative of hypoglycemia and hyperglycemia, respectively (Association AD 2016). To thrive under these diverse nutritional conditions, *S. aureus* tightly controls and modulates gene expression in a coordinated fashion based on particular environmental cues (Somerville and Proctor 2009). For example, under hypoxic states during an infection, *S. aureus* increases its glycolytic flux to balance the inefficient fermentation of carbohydrates. Likewise, to accommodate increased glucose consumption, *S. aureus* has adaptive mechanisms to increase its glucose uptake during infection (Vitko *et al.* 2016).

Staphylococcus aureus pathogenesis seems to be closely linked to glucose availability *in vitro* and in humans. For instance, biofilm formation by *S. aureus* is enhanced by the addition of glucose to media (Waldrop *et al.* 2014). *In vivo* studies have demonstrated that diabetic mice are more susceptible to *S. aureus* infections and are significantly deficient in clearing *S. aureus* compared to their non-diabetic counterparts (Rich and Lee 2005). Likewise, diabetic patients are at a higher risk for *S. aureus* pneumonia (Equils *et al.* 2016) and are more susceptible to *S. aureus*-mediated foot infections (Dunyach-Remy *et al.* 2016). Importantly, hospitalized patients who are hyperglycemic seem to be at a higher risk of *S. aureus* infection (Pomposelli *et al.* 1998).

Staphylococcus aureus *responses to carbohydrate availability and metabolism*

In low glucose conditions, *S. aureus* assumes a low-energy 'starvation' state (Watson, Clements and Foster 1998). Watson *et al.* found that although over 99% of *S. aureus* cells lose viability in response to glucose starvation within the first few days of culture, the surviving population can remain viable for months. Cells in this long-term starvation state are smaller and denser than cells grown in the presence of glucose. Additionally, marked changes in RNA and protein synthesis profiles are observed during the early stages of nutrient starvation (Watson, Clements and Foster 1998). When starved cells are given complex medium containing glucose, they recover from their starvation state, rapidly increasing RNA synthesis and protein production to support growth (Clements and Foster 1998).

Staphylococcus aureus adapts to nutritionally diverse environments by prioritizing utility of primary versus secondary carbon sources. This process, best characterized in *Bacillus subtilis*, is known as carbon catabolite repression (CCR) (Titgemeyer and Hillen 2002; Warner and Lolkema 2003; Gorke and Stulke 2008). CcpA is a highly conserved transcription factor that plays important roles in CCR (Henkin *et al.* 1991; Saier *et al.* 1996). In response to the presence of rapidly metabolized carbon sources such as glucose or other glycolytic intermediates, HPr kinase phosphorylates the signaling intermediate HPr (Deutscher and Saier 1983). Phosphorylation allows HPr to complex with CcpA and together, this phospo-HPr-CcpA complex binds to catabolite responsive elements to modulate the expression of target genes (Deutscher *et al.* 1995; Miwa *et al.* 2000). Starvation-induced genes are among these target genes that have been shown to be regulated by CcpA in Gram-positive bacteria (Leboeuf *et al.* 2000). Of note, serine phosphorylated Crh, an HPr homolog, has also been shown to complex with CcpA in CCR but this interaction is up to 10-fold weaker and results in a less robust phenotype (Galinier *et al.* 1997; Martin-Verstraete, Deutscher and Galinier 1999). Notably, in *S. aureus*, the expression of RNAIII is significantly increased in the presence of glucose under constant pH, but not in a Δ*ccpA* mutant, where the effect of glucose on RNAIII expression is markedly decreased (Seidl *et al.* 2006). Collectively, these observations suggest that high glucose triggers a signal cascade through CcpA that upregulates RNAIII expression and ultimately modulates virulence gene expression.

Additionally, CcpA modulates the expression of genes involved in the glycolytic pathway through CCR. In response to high levels of glucose, CcpA represses the TCA cycle by downregulating the expression of critical TCA cycle enzymes (Strasters and Winkler 1963; Seidl *et al.* 2008, 2009). Thus, as glucose is depleted from the media or is otherwise limited during nutrient starvation, the TCA cycle is progressively derepressed. This process is under the control of a second carbon catabolite protein, CcpE (Hartmann *et al.* 2013). CcpE binds to citrate, the first intermediate of the TCA cycle, and adopts a predominantly tetrameric (active) state. Active CcpE binds to and regulates target promoters, including those of TCA cycle enzymes (Hartmann *et al.* 2013; Ding *et al.* 2014). Metabolomic, microarray and transcriptional analyses show that not only is CcpE involved in modulating the carbon flow through the TCA cycle, it is also a major regulator of virulence genes such as those involved in the synthesis of virulence factor capsular polysaccharides and superantigen-like proteins (Ding *et al.* 2014). Whether this global regulation observed in the metabolomics analyses is due to its direct action on virulence gene promoters or indirectly due to its effects on metabolism is unknown but is likely influenced by both (Hartmann *et al.* 2013; Ding *et al.* 2014).

The pentose phosphate pathway (PPP) has also been implicated in linking metabolism to virulence, through the RpiR family of transcriptional repressors (Zhu *et al.* 2011). The RpiR family was first identified as regulators of ribose metabolism in *E. coli* (Sorensen and Hove-Jensen 1996) but members of this family have since been linked to a number of other catabolism pathways, including the PPP, in both Gram-negative and Gram-positive bacteria (Jaeger and Mayer 2008; Daddaoua, Krell and Ramos 2009; Kohler, Choong and Rossbach 2011). Although RpiR family members have a C-terminal sugar isomerase-binding domain, the actual ligand is unknown. Of the three RpiR homologs present in *S. aureus*, only RpiRb and RpiRc appear to modulate PPP gene regulation. RpiRc is an important regulator of virulence (Zhu *et al.* 2011; Balasubramanian *et al.* 2016; Gaupp *et al.* 2016). Recent work indicates that RpiRc senses metabolic shifts and represses virulence by modulating the expression of the *agr* locus. This results in the repression of RNAIII expression and thus increased translation of the repressor Rot (Balasubramanian *et al.* 2016). Additional work suggests that the effect of RpiRc on *agr* and virulence gene expression occurs via repression of *sarA*, a positive regulator of *agr* and virulence (Gaupp *et al.* 2016). Future

work is required to elucidate the metabolic signal(s) responsible for activating RpiRc and to understand the molecular mechanism that governs the intersection between the PPP and RpiRc's contribution to pathogenesis.

In addition to central metabolism, amino acid availability plays a critical role in *S. aureus* pathogenesis. The branched chain amino acids (BCAAs) valine, leucine and isoleucine, along with GTP, initiate the repressive activity of CodY, a global metabolic regulator in *S. aureus* and many Gram-positive bacteria (Guedon *et al.* 2001; Ratnayake-Lecamwasam *et al.* 2001; Shivers and Sonenshein 2004; Tojo *et al.* 2005; Sonenshein 2007). Upon sensing and binding intracellular GTP or BCAAs, the affinity of CodY toward consensus sequences (CodY binding boxes) increases. A dimerized CodY binds these *cis*-regulatory elements to control target gene expression (Shivers and Sonenshein 2004; den Hengst *et al.* 2005; Levdikov *et al.* 2006; Majerczyk *et al.* 2008). As expected, CodY activity is at its highest in exponential growth phase where nutrients are in excess (Majerczyk *et al.* 2008). As a result, metabolic pathways that are unnecessary in nutrient-replete environments are repressed. In *S. aureus*, the CodY regulon consists of over 200 genes, including biosynthesis genes of metabolic intermediates as well as those involved in virulence (Majerczyk *et al.* 2008, 2010; Pohl *et al.* 2009). Interestingly, CodY acts by direct binding to virulence gene promoters, and also indirectly through Agr. Although deletion of *codY* results in increased expression of *agrBDCA* and *rnaIII*, its low affinity to *agr* promoters suggests that direct transcriptional regulation is unlikely (Majerczyk *et al.* 2008). Instead, it appears that CodY prevents premature activation of *agr* during exponential growth phase, despite the presence of phosphorylated AgrA (Roux *et al.* 2014). Taken together, these data suggest that as GTP and/or BCAAs are depleted, CodY senses this change in nutritional state and progressively derepresses its target genes to increase the metabolic biosynthesis pathways and also regulate the expression virulence factors.

In summary, *S. aureus* must confront and adapt to diverse host environments, where levels of carbon-based nutrients naturally vary greatly. For example, when the nutrients are low, a subset of bacteria enters a low-energy long-term starvation state, which it encounters the nutrients that it needs. Factors such as CcpA, CcpE, RpiRc and CodY sense the changes in carbon state of the host and accordingly adjust the utilization of pathways involved in metabolism. Either in the process of or as a result of changes to the metabolic state, *S. aureus* differentially regulates virulence factor expression, thereby modifying its pathogenesis (Fig. 1).

Iron

Availability in the host

Iron is a vital nutrient across all domains of life. Although iron limitation inhibits cellular processes, iron abundance is toxic due to its highly reactive properties. As a result, iron metabolism in mammalian cells and in bacteria is tightly regulated to maintain homeostasis. Iron in vertebrates exists in four major forms: (i) as heme in hemoglobin, a tetrapyrrole molecule with high affinity for molecular oxygen; (ii) as iron–sulfur clusters in several critical enzymes; (iii) as extracellular storage molecules, such as transferrins found in serum and lactoferrins found in the lymphoid system (Hammer and Skaar 2011; Cassat and Skaar 2013); and (iv) intracellularly bound to ferritin (MacKenzie, Iwasaki and Tsuji 2008). Greater than 90% of iron in the host is intracellular, trapped in heme. As a result, free extracellular iron in human tissues is estimated to be around 10^{-18} M (Bullen, Rogers and Griffiths 1978), well below the concentration required for microbial life. Additionally, infection-induced inflammation leads to rapid decline in iron levels in blood serum (Cartwright *et al.* 1946; Darton *et al.* 2015). Finally, extracellular iron is often scavenged by host glycoproteins, further restricting iron availability for microbes during infection (Cassat and Skaar 2013). For example, NrampI, a phagosomal iron efflux pump that is important for bacterial clearance, is upregulated during certain infections (Loomis *et al.* 2014). The process of depriving microbes of iron has been cleverly coined as 'nutritional immunity' (Hammer and Skaar 2011; Cassat and Skaar 2013).

Staphylococcus aureus *responses to iron limitation*

Staphylococcus aureus has evolved intricate mechanisms to counter iron deficiency. Here, we focus on two well-studied mechanisms of iron acquisition: siderophore-mediated acquisition and heme-iron acquisition. Similar to many other pathogens, *S. aureus* produces several low molecular weight scavenging proteins called siderophores, out of which staphyloferrins A and B are the best characterized (Konetschny-Rapp *et al.* 1990; Hammer and Skaar 2011). These secreted factors capture extracellular iron bound to host glycoproteins by removing iron from loaded transferrins (Park *et al.* 2005). Siderophores are essential for bacterial growth in media where transferrin is the sole source of iron (Park *et al.* 2005). Once iron is removed from transferrins, the siderophore-bound iron is actively transported into the cell via ABC transporters (Skaar *et al.* 2004).

Although *S. aureus* culture filtrates have been long known to possess siderophore activity, Beasley *et al.* (2009) were the first to identify the genetic locus responsible for staphyloferrin A biosynthesis, called *sfa*. While this locus was important for *S. aureus* growth in iron-deplete media, it was dispensable for growth of the bacterium in serum, which is naturally iron deficient. This result was puzzling until the discovery that deletion of both *sfa* and a second poorly characterized siderophore operon (*sbn*) was required to abrogate *S. aureus* growth in serum. The *sbn* (siderophore biosynthesis gene cluster) operon contains nine genes encoding proteins required for biosynthesis of staphyloferrin B (Dale *et al.* 2004). Inactivation of at least one of the genes in this operon, *sbnE*, abolishes siderophore activity in culture filtrates and leads to moderate reduction in *S. aureus* colonization of murine kidneys (Dale *et al.* 2004).

While siderophores are adept at scavenging extracellular iron, the majority of iron in vertebrates is locked in complex with heme inside erythrocytes (Deiss 1983). Heme iron is obtained from lysis of erythrocytes by hemolysins and cytotoxins (Torres *et al.* 2006, 2010; Spaan *et al.* 2014, 2015). Following lysis, heme is captured and taken up by the iron-regulated surface determinant (Isd) system (Mazmanian *et al.* 2003). This specialized system consists of the cell wall anchored surface proteins IsdABCH, the transporters IsdDEF and the cytoplasmic degradation enzymes IsdIG (Muryoi *et al.* 2008). Briefly, the cell surface proteins IsdBH are important for binding hemoglobin to the surface of *S. aureus* (Torres *et al.* 2006), and work together with IsdAC to extract heme. Extracted heme is transported across the membrane via two ABC transporter clusters: IsdDEF (Mazmanian *et al.* 2003; Liu *et al.* 2008) and HtsABC (Skaar *et al.* 2004). IsdIG then degrades heme, releasing iron Skaar, Gaspar and Schneewind (2004). *In vivo, hts* mutants are severely attenuated in their ability to colonize liver and kidneys of mice (Skaar *et al.* 2004). Likewise, *isdB* mutants demonstrate reduced ability to infect murine kidneys and spleen (Torres *et al.* 2006).

The ferric uptake regulator (Fur) regulates iron metabolism in many Gram-negative and Gram-positive bacteria. By

amplifying *fur* from *S. aureus* and expressing it recombinantly, Xiong *et al.* (2000) showed that Fur is involved in regulating genes in ferrichrome uptake and has an iron-binding site, similar to that of Fur found in other organisms. There are a series of excellent reviews summarizing decades of work on Fur-mediated regulation of iron metabolism (Hantke 2001; Troxell and Hassan 2013; Fillat 2014). Briefly, in the presence of iron, Fur directly binds Fe^{2+} and in its holoform, acts as a repressor of iron acquisition genes. In *E. coli*, Fur acts by repressing the small regulatory RNA, RyhB (Masse and Gottesman 2002). In iron-deplete conditions, RyhB is derepressed due to the inactivation of apo-Fur. Using an antisense base-pairing mechanism, RyhB rapidly upregulates expression of iron acquisition genes and shuts down production of non-essential proteins that use or store iron (Masse and Gottesman 2002).

While there are no reports of *ryhB* in *S. aureus*, iron homeostasis in *S. aureus* is clearly Fur dependent. Both the siderophore biosynthesis operons, *sfa* and *sbn* (Dale *et al.* 2004; Cheung *et al.* 2009), and the *isd* locus involved in heme acquisition are under Fur control (Torres *et al.* 2010). Fur also connects iron metabolism and virulence gene expression in *S. aureus*: it positively impacts expression of immunomodulatory proteins such as coagulase, superantigen-like proteins and negatively regulates genes involved in virulence such as lipases and cytotoxins (Torres *et al.* 2010). Importantly, *fur* mutants are attenuated for virulence in a murine pneumonia model of infection. Additionally, *S. aureus* lacking *fur* is more susceptible to neutrophil-mediated killing (Torres *et al.* 2010).

In summary, *S. aureus* most likely encounters a gradient of iron concentrations when it traverses through different tissues. Under iron-starved conditions, *S. aureus* senses iron via Fur, upregulates siderophore and heme acquisition pathways, and represses virulence. When iron is abundant, either due to the natural reservoir of iron in the tissue or due to efficient acquisition of iron, *S. aureus* switches to a more pathogenic lifestyle characterized by enhanced virulence factor production (Fig. 1). While this review focuses solely on iron as a key element affecting *S. aureus* virulence, other metals such as manganese and zinc also alter *S. aureus* pathogenesis. Similar to *S. aureus*–iron interactions, specific regulatory proteins sense these metals and affect virulence, the host actively sequesters manganese and zinc, and *S. aureus* has evolved complicated transport mechanisms to acquire them (Cassat and Skaar 2012).

INHIBITION OF *STAPHYLOCOCCUS AUREUS* ENVIRONMENTAL SENSING: THERAPEUTIC POTENTIAL

Staphylococcus aureus relies on environmental cues derived from the host as it transitions between colonizing and invasive states. Accordingly, these cues are being targeted for the development of anti-*S. aureus* therapeutics involving inhibitory compounds, including natural and chemical inhibitors as well as antibodies that block environmental sensing. Both these regulatory mechanisms can be inhibited, albeit by different mechanisms. Here, we highlight the potential use of environmental sensing and signaling pathway inhibitors as novel anti-staphylococcal therapeutics.

By far, the largest category of therapeutics against *S. aureus*-sensing systems targets the Agr-mediated quorum-sensing system. As discussed above, Agr induces rapid and massive accumulation of harmful, tissue-degrading toxins and exo-enzymes that are critical for *S. aureus* pathogenesis (reviewed in Khan *et al.* 2015). A small molecular inhibitor of Agr, called savarin (*Staphylococcus aureus* virulence inhibitor), was identified in a screen for compounds that attenuated *agr* P3 promoter activity. Extensive analysis of savarin revealed that it alters binding of AgrA to DNA, and attenuates skin ulcers and abscesses in murine models of infection. Resistance to savarin was not observed after *in vitro* and *in vivo* passage, enhancing its attractiveness as a therapeutic (Sully *et al.* 2014). Others have undertaken an alternative approach by designing analogs or dominant negative AIP that competitively inhibit signal sensing. Tal-Gan *et al.* used an alanine-scanning approach to find mutations in AIP that disrupt binding and signaling of wild-type AIP via AgrC. These AIP mimetics were able to significantly reduce *S. aureus* hemolytic activity *in vitro*, suggesting efficient blockage of Agr-mediated signaling (Tal-Gan *et al.* 2013). Likewise, monoclonal antibodies such as AP4-24H11 have been designed to 'quench' quorum sensing by binding and neutralizing AIP. AP4-24H11 has demonstrated protection in intradermal infections of mice and reduced *S. aureus*-mediated lethality in systemic infection models (Park *et al.* 2007; Kirchdoerfer *et al.* 2011). Lastly, an US Food and Drug Administration-approved, non-steroidal anti-inflammatory compound called diflunisal significantly attenuates *S. aureus* toxin production (Khodaverdian *et al.* 2013), without altering bacterial growth (Hendrix *et al.* 2016). This compound is thought to inhibit phosphorylation of AgrA by the sensor kinase AgrC, thus abrogating quorum-sensing and toxin levels (Khodaverdian *et al.* 2013). Importantly, diflunisal has recently been shown to impede *S. aureus* cytotoxicity toward osteoblasts *in vitro*, due to reduced production of PSMs (Hendrix *et al.* 2016). Moreover, this compound has promising efficacy *in vivo*, in that it can moderately attenuate *S. aureus*-mediated cortical bone destruction in a murine model of osteomyelitis (Hendrix *et al.* 2016). Taken together, a variety of approaches exist to inhibit Agr that target different portions of the quorum-sensing cascade.

Agr inhibition is expected to be most effective as a therapeutic in clinical situations where this regulator is critical for pathogenesis (Fig. 1). CA-MRSA strains cause disease—primarily skin and soft tissue infections—in otherwise healthy community subjects. CA-MRSA strains have a 'hyperactive' *agr* locus and produce copious levels of toxins and proteases *in vitro*, in animal models of infection and in humans (Nastaly, Grinholc and Bielawski 2010; Date *et al.* 2014). In contrast, *S. aureus* strains isolated from hospitalized patients frequently have mutations that inactivate or severely impair the activity of the Agr-TCS (Shopsin *et al.* 2008; Traber *et al.* 2008). Presumably, disruption of barrier functions by disease and clinical intervention in the hospital environment permit *S. aureus* strains that lack full virulence to cause infection. Furthermore, Agr dysfunction has been associated with persistent rather than resolving bacteremia, and mortality (Fowler *et al.* 2004; Schweizer *et al.* 2011), perhaps because killing by host and synthetic antimicrobials is reduced in *agr*-dysfunctional isolates (reviewed in Painter *et al.* 2014). These observations suggest that there are situations *in vivo* where Agr activation is dispensable, or even deleterious for *S. aureus*. Thus, the clinical consequences of disabling Agr activity are not obvious; depending on the patient, efforts to use Agr and virulence as targets for new antimicrobials may be ill advised.

Recently, Arya *et al.* used a novel bioinformatics-based structural approach to design and synthesize a small molecule inhibitor of SarA (SarABI). As discussed above, SarA is a cytoplasmic transcription factor that activates genes critical for biofilm formation in an Agr-independent manner (Trotonda *et al.* 2005). SarABI acts by binding to the DNA-binding domain of the

transcription factor, forming a stable complex such that SarA's downstream regulatory events are blocked. Since SarA is a potent regulator of toxins and exo-enzymes and can act independently of Agr, SarABI may be useful to treat infections that are associated with low *agr* activity states (Arya *et al*. 2015). One such scenario may be biofilm infections; activation of *agr* is thought to cause dispersal of the biofilm (Boles and Horswill 2008); and *agr*-defective cells are frequently recovered from biofilms on prosthetic devices in humans (Kiedrowski and Horswill 2011). *Staphylococcus aureus* biofilms are difficult to treat and are often the cause of recurring infections in humans (Parsek and Singh 2003; Harris and Richards 2006). Promisingly, SarABI is a potent inhibitor of biofilm development both *in vitro* (on abiotic surfaces) and *in vivo* (on rat vascular graft infections). Similar to *agr* inhibitors, SarABI does not restrict bacterial growth, suggesting that its use is likely to not elicit bacterial resistance *in vivo* (Arya *et al*. 2015). Additional studies are urgently needed to determine the safety and efficacy of anti-SarA strategies such as SarABI.

Small molecules inhibitors have also been developed that antagonize critical metabolic pathways by targeting cytoplasmic rather than surface or secreted proteins. Tripathi *et al*. identified chemical inhibitors of the iron-scavenging siderophores called baulamycins. These compounds are natural antimicrobials that attenuate the function of the cytosolic synthetase involved in siderophore biosynthesis (Tripathi *et al*. 2014). In addition, compound screening has identified a chemical inhibitor of SaeRS, apparently at the transcriptional level (Long *et al*. 2013). Mechanisms by which these compounds inhibit their cognate receptors and downstream signaling are currently unknown.

In contrast to the numerous efforts undertaken to counteract *S. aureus* secreted proteins (reviewed in Missiakas and Schneewind 2016; Karauzum and Datta 2016; Giersing *et al*. 2016; Lacey, Geoghegan and McLoughlin 2016), far fewer therapeutics target intracellular regulators of virulence or their signaling molecules. While regulators that control multiple virulence effector proteins make attractive target candidates, designing such counteractive therapeutics has been challenging. First, antibody-based neutralization approaches—the current golden standard for treating several infectious diseases—are ineffective against cytoplasmic regulatory proteins, as these are inaccessible to antibodies. Second, finding chemical inhibitors that traverse into the bacterial cytoplasm but leave host cells unharmed can be a challenging task. Third, *S. aureus* has an array of regulators that interact with each other in a complex manner and perform redundant functions (such as various TCSs responding to oxygen or the intricate network of proteins involved in toxin production). Lastly, the ligands of many regulators are unknown. As such, designing competitors or quenchers is an underdeveloped area of study that could hold great promise.

CONCLUSIONS

In this review, we highlight key features of *Staphylococcus aureus* adaptation to the host environment during infection (summarized in Fig. 1). We delved into tissue specific environments and metabolic stresses that the bacterium may encounter during infection. Understanding the signals and regulatory elements that alter *S. aureus* pathogenesis in response to environmental signals is crucial to developing novel therapeutics. Thus, basic and clinical research studies should account for differential production of *S. aureus* virulence factors under various environmental conditions and disease states. The results may inform the design of *S. aureus* vaccines and therapeutic trials. Additionally, a better understanding of factors specific to an individual's condition, such as site of infection, immune competency and the virulence potential of the infecting strain under these conditions, may pave the way for 'personalized' management of *S. aureus* infections.

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