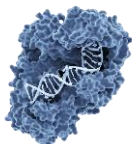

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Review Article

Role of SarA in *Staphylococcus aureus*: A Virulence Target For Therapeutic Strategies

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Abstract: Methicillin-resistant *Staphylococcus aureus* (MRSA) infection gives rise to significant morbidity and carries a grave prognosis, resulting in the demise of approximately 21.8% of afflicted individuals on a yearly basis *Staphylococcus aureus* has the capability to induce a myriad of diverse diseases, a phenomenon attributed to its extensive array of virulence factors and formation of biofilms. The regulation of key virulence determinants, crucial for pathogenicity, is intricately controlled by the staphylococcal accessory regulatory (*sarA*) system. SarA plays a crucial role in the pathogenic mechanisms of *S. aureus* and the

development of biofilms, while simultaneously modulating the synthesis of multiple virulence factors and influencing the expression of specific colonization determinants, and mutations in *sarA* partially limit the extent of *S. aureus* biofilms formation. In this review, we present an overview of the current understanding of the molecular mechanisms underlying the regulation of *sarA* gene expression, with a particular emphasis on its relevance in the development and sustenance of antimicrobial resistance, along with in the processes of biofilm formation and activation of virulence genes in MRSA. This review demonstrated that suppressing the expression of *sarA* gene exerts a notable impact on both biofilm development and the pathogenicity of MRSA strains, thereby offering a hopeful approach to the efficient management and treatment of MRSA infections.

Keywords: *Staphylococcus aureus*; MRSA; *sarA*; virulence factors; biofilms; *agr*; SDG 3 Good health and well-being

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1. Introduction

Staphylococcus aureus, a Gram-positive pathogen, demonstrates high versatility and adaptability. It can colonize the skin and mucous membranes as a non-pathogenic commensal[1]. Furthermore, *S. aureus* can proliferate within the bloodstream and diverse tissue compartments, giving rise to the onset of severe pathological disorders[2]. It is widely recognized as a predominant etiological factor of both hospital-acquired and community-acquired infections on a global scale[3,4]. The spectrum of *S. aureus*-related ailments encompasses a wide range of manifestations, ranging from minor cutaneous infections to critical conditions like pneumonia, osteomyelitis, and endocarditis. Recurrence of infection represents a notable facet of *S. aureus*-associated diseases, with a prevalence of 8-33% in cases of skin, soft tissue, and bloodstream infections, contributing significantly to human morbidity and mortality[5]. Through the administration of antibiotics on a global scale, bacterial infections are effectively managed and prevented. However, the widespread and improper utilization of antibiotics exerts selective pressure on the bacterial population, thereby fostering the emergence of antibiotic resistance[6,7]. Consequently, the emergence of MDR strains significantly diminishes the therapeutic effectiveness of antibiotics against the bacterial strain[8]. As stated by the World Health Organization (WHO), antimicrobial resistance presents a substantial peril to global public health[9]. Methicillin-resistant *Staphylococcus aureus* (MRSA) is considered one of the extensively concerning multidrug-resistant (MDR) pathogens and has been recognized as one of the high-priority ESKAPE pathogens, thereby new antimicrobial development is urgently needed by the WHO[10].

S. aureus exhibits a multitude of cell surface structures and releases virulence factors. The global regulatory systems, such as the accessory gene regulator (*agr*) and staphylococcal

accessory regulatory (*sarA*), primarily govern the secretion of these virulence factors and the formation of biofilms[11,12]. SarA is a DNA-binding protein consisting of 124 amino acid residues, which is encoded by the *sarA* locus. The *sarA* locus consists of three overlapping transcripts regulated by three distinct promoters, namely P1, P3, and P2[13]. From a structural perspective, SarA is composed of five α-helices, a β-hairpin turn, and a C-terminal region within each monomeric unit[14]. Based on the analysis of DNA binding, the SarA protein is postulated to exert its regulatory impact on target genes through direct interaction with their promoter regions, indirect modulation of regulators (e.g., *agr* promoter binding), or stabilization of mRNA during the logarithmic growth phase, thereby orchestrating the modulation of target gene expression[15,16]. The experimental results demonstrate that *sarA* mutants impede biofilm formation and restrict virulence in models of osteomyelitis, infective endocarditis, and septic arthritis, thereby emphasizing the pivotal role of *sarA* in promoting pathogenicity[17,18]. In this review, we aimed to discuss the regulatory role of *sarA* in *S. aurues* biofilm formation and virulence factor production, delineate the molecular mechanisms underlying *sarA* expression regulation, and explore the potential pharmacological approaches targeting *sarA* as a promising therapeutic strategy against *S. aureus* infections.

2. Overview of *Staphylococcus aureus* – an ESKAPE Pathogen

S. aureus is a significant human pathogen responsible for a broad scope of diseases, and it has escalated to a significant global concern owing to the appearance of resistant strains and their swift spread. This has led to high morbidity and mortality rates in affected individuals[19]. This versatile pathogen was traditionally believed to predominantly colonize the nasal cavity, but further investigations have revealed its ability to inhabit various skin sites and even the intestinal tract, resulting in a broader scope of dissemination[20,21]. *S. aureus*-mediated skin infections, including furuncles, abscesses, and wound infections, represent moderate to severe dermatological conditions with a substantial morbidity rate and considerable pain[22]. Notably, the fatality rate associated with bacteraemia infections caused by *S. aureus* exceeds that of numerous highly consequential infectious diseases. In the United States, it surpasses the combined mortality resulting from AIDS, tuberculosis, and viral hepatitis, emphasizing the severity of the problem posed by the pathogen[23]. Owing to the metabolic flexibility of *S. aureus*, it showcases adaptively and colonization capabilities across various environmental contexts, thus significantly impacting its pathogenic potential. The extensive array of virulence determinants in *S. aureus*, including extracellular toxins and surface-associated structural factors, are closely associated with the induction of pathogenicity and the establishment of persistent infections within the host. Due to their

elevated prevalence, these infections impose a significant public health burden, warranting the development of more efficacious interventions[24].

MRSA strains are known for their remarkable clonal architecture, with a limited number of specific lineages associated with pandemic clones[25,26]. Early reports of MRSA infection were confined to hospital settings and primarily affected immunocompromised individuals. It was not until the late 1980s and early 1990s that community-acquired (CA) MRSA infections were documented in the Oceania region, thereby initiating the dissemination of this significant pathogen across the globe. Based on observations, these strains have been observed to impact individuals who lack the typical risk factors associated with MRSA infection[25,27]. Furthermore, both CA-MRSA and hospital-acquired (HA)-MRSA possess the ability to elicit nasal cavity infections, thus presenting difficulties in distinguishing between CA- and HA-MRSA[19]. The globally prevalent clonal complexes (CC) of MRSA identified by multilocus sequence typing (MLST) encompass CC1, CC5, CC8, CC22, CC30, and CC45[25,27].

Due to the widespread prevalence of antibiotic resistance in most isolates of *S. aureus*, treating *S. aureus* infections faces a formidable challenge. Especially noteworthy is the fact that all known β-lactam antibiotics are ineffective against MRSA due to resistance, thereby rendering MRSA a major clinical menace[28]. According to the 2018 report by the WHO, patients infected with MRSA are at a 64% higher risk of mortality compared to those without MRSA infection, underscoring the seriousness of this matter[29]. In response to the looming threat of bacterial resistance, the Chinese government has launched the "National Action Program to Contain Bacterial Resistance (2016-2020)." However, despite these efforts, the prevalence of MRSA remains alarmingly high, with a rate of 34.4% reported in 2016[30]. In 2017, the G20 initiated an international ten-year research and development (R&D) program on antibiotic resistance, underscoring the pressing need to address this global concern[31]. MRSA strains produce an array of toxins and virulence factors that can circumvent human defence systems, resulting in severe infections. In addition, diseases caused by MRSA in animals, such as poultry-associated lameness and bovine mastitis, can result in substantial financial losses[32]. Considering the elevated occurrence of MRSA infections and their profound repercussions on humans and animals alike, the development of innovative strategies to ameliorate this ubiquitous public health menace is of paramount importance.

Bacterial biofilm formation and the production of virulence factor genes are crucial aspects contributing to the development of bacterial drug resistance. The clinical severity of MRSA is primarily determined by the production of various toxins and adhesion proteins, which are crucial for *S. aureus* to evade the immune system and persist in the host[33]. The

virulence of MRSA is heightened by its ability to adhere, synthesize, and develop biofilms, which are facilitated by the expression of several toxins that confer protection against host defences[34].

3. Virulence Factors, Biofilm Formation and Quorum Sensing of *S. aureus*

3.1. Virulence Factors and Their Associated Genes

The profound adaptability of *S. aureus* to resist host defences and induce a wide spectrum of infections is attributable to its capacity to manifest an extensive array of virulence determinants[35]. *S. aureus* virulence factors encompass surface proteins that mediate adherence and infiltration of host cells, exoproteins that facilitate circumvention of the immune system, and an array of pore-forming and hemolytic toxins[36]. Coordinated regulation of these virulence determinants is pivotal for effective infection[37]. Among the many virulence factors encoded by *S. aureus*, *staphylococcal* enterotoxin A (SEA) is a major contributor to *S. aureus*-mediated gastroenteritis and T-cell activation through its immunomodulatory properties as a superantigen[38]. The α-toxin, encoded by the *hla* gene, is a critical virulence factor responsible for the development of pneumonia, sepsis, septic arthritis, brain abscesses, and corneal infections in *S. aureus*. In human strains of *S. aureus*, the modulation and control of virulence factors are commonly regulated by global virulence regulators, such as *sarA*, *agr*ABCD system, and *KdpDE* two-component system (*KdpDE*)[39]. Furthermore, the regulation of virulence and resistance determinants located on mobile genetic elements (MGEs), including staphylococcal cassette chromosomes, pathogenic islands, plasmids, phages, transposons, and insertion sequences, is governed by the global gene regulators in *S. aureus*[40].

MRSA strains generate organic acids through carbohydrate metabolic pathways, with acetic acid being the predominant organic acid produced by these bacteria[41]. The production of organic acids contributes to a decrease in the pH of the infection site, promoting microbial biofilm formation[42]. Biofilms exert a pivotal role in the pathogenic mechanisms of infections in both human and animal hosts. They enhance bacterial adherence to epithelial cells, facilitate the transmission of diverse toxins, and contribute to the clinical presentations of infections[43–45].

3.2. Biofilm Formation and Biofilm-Related Genes

The biofilm formation by *S. aureus* on the surfaces of implanted medical devices promotes bacterial persistence and enhances the severity of infections caused by this pathogen[46,47]. Biofilms exert a dampening effect on metabolic activity, thereby endowing

inherent resistance to antibiotics. Moreover, the biofilm matrix impedes antibiotic permeation by reducing the rate of diffusion, thereby manifesting an obstructive effect that augments antibiotic resistance[48–50]. The development of physiologically dormant persister cells during biofilm growth further facilitated the emergence of antibiotic tolerance[15,51]. Biofilms formed on surfaces within the food industry have acquired resistance to disinfectants[52].

Biofilm is a complex community of sessile microorganisms embedded within substances (EPS) or a mucosal layer[53]. It is a sophisticated architecture comprising proteins, DNA, and polysaccharides, commonly known as polysaccharide intercellular adhesin (PIA) or poly-N-acetylglucosamine (PNAG) material[54]. PIA, comprising β-1,6 N-acetylglucosamine residues, is encoded by the intercellular adhesion (*ica*) locus, encompassing the structural genes *ica*ADBC and the regulatory gene *icaR*. Initially, PIA was identified as a critical component involved in biofilm formation. In addition, the PIA is considered to be the hallmark component in the development of mature biofilms, which result in notorious multi-layered clustering matrices of cells and are responsible for cell-to-cell adhesion[55]. However, studies demonstrated that bacterial strains devoid of the *ica* locus, essential for PIA synthesis, are still capable of biofilm formation[56]. Moving forward, fibronectin-binding proteins (FnBPs) were demonstrated to substitute PIA in PIA-independent biofilms, and these FnBP-mediated biofilms are particularly prevalent in highly virulent MRSA isolates[57].

Biofilm formation represents a multifaceted process wherein substantial genetic and physiological alterations occur within bacterial cells during their intricate progression. Extracellular DNA (eDNA) derived from lysed bacterial cells constitutes a vital constituent of the mature biofilm matrix and serves as a pivotal factor in biofilm formation[58]. In *S. aureus*, the regulation of extracellular DNA (eDNA) release predominantly involves the autolysin Atl and the *cidA*/*lrgA*-mediated holin/antiholin system[59–61].

The *agr* and *sarA* genes, as well as microbial surface molecules, including laminin-binding protein (Eno), clumping factors A and B (ClfA and ClfB), *fnbA*, elastin binding protein (EbpS), and fibrinogen binding protein (*Fib*) genes, are the predominant genes involved in the formation and maintenance of biofilms. They exhibit a high affinity for extracellular matrix proteins of host cells, facilitating the process of biofilm formation[62]. The presence of *sarA*, *agr*, *icaA*, *icaD*, and eight MSCRAMM genes was found to be mutually associated in 11.7% of MRSA strains, playing a crucial role in the production and maintenance of biofilms[63]. Within these genes, the genes *icaA* and *icaD* play a predominant role in the biosynthesis of PIA, which constitutes the principal component of the extracellular

polysaccharide matrix enveloping bacterial cells within the biofilm. N-acetylglucosamine serves as a key constituent within this polysaccharide matrix[63,64]. Furthermore, penicillin-binding protein 2a (PBP2a) is an essential component during the biofilm formation process[65,66]. MRSA strains carrying the *mecA* gene, which is associated with genetic elements, express the PBP2a protein. This protein exhibits low affinity for all β-lactam antibiotics, leading to resistance to penicillin in MRSA strains. Additionally, MRSA strains carried with the *mecA* gene promote the formation of protein-based biofilms, impeding the development of PIA-dependent biofilms[60,65].

The development of biofilms is a multifaceted process that involves various stages and encompasses three pivotal phases: initial adhesion, biofilm maturation, and bacterial cell dispersion[34]. *S. aureus* exhibits the ability to generate two distinct types of biofilm matrix: *ica*-dependent biofilms and *ica*-independent biofilms. Of these, the most crucial biofilm generated by MRSA isolates is the *ica*-independent biofilm[67]. The primary constituents of the biofilm matrix are proteins, particularly fibronectin-binding proteins[56]. These proteins play crucial roles in the development and robustness of biofilms, promoting both the initial attachment phase and the maturation stage of biofilm formation[57,68]. Meanwhile, the biofilm matrix exhibits a high level of antibiotic resistance, thereby augmenting the bacterium's pathogenic potential.

3.3. Quorum Sensing

The quorum sensing (QS) mechanism allows bacteria to perceive and respond to various environmental stimuli by regulating the bacterial community. During the transition from exponential growth to the stationary phase, QS reduces the expression of multiple cell surface proteins while increasing the expression of numerous virulence factors, enabling intercellular communication and promoting biofilm formation[22,69]. QS pathway operates through the exchange of diffusible autoinducer molecules produced by bacteria in the extracellular environment. Upon reaching a threshold concentration of accumulated autoinducers in the surroundings, diffusion back into the cells occurs, triggering gene expression[70]. Interfering with QS through various approaches, such as inhibiting signal molecule synthesis, degradation of signal molecules, or disruption of signal molecule binding to response regulators, can effectively reduce virulence and facilitate bacterial eradication[71].

The regulation of bacterial virulence is a complex and multifaceted process involving the interplay of various regulatory systems. Among these systems, the accessory gene regulator (*agr*) and *sar* regulators have been identified as critical regulators of staphylococcal virulence. In addition to their notable roles in controlling the expression of QS and biofilm-related genes, these regulators also play a pivotal role in the regulation of peptidoglycan

hydrolases, which are enzymes responsible for cell wall remodelling during bacterial growth and division[72]. Furthermore, sigma factor B (σ^B) has also been implicated in the regulation of *staphylococcal* virulence factors.

The functional *agr* regulatory system plays a crucial role in the pathogenicity of *S. aureus* by tightly regulating the expression of an extensive repertoire of virulence factors[73]. The *agr* operon is organized around two distinct promoters, P2 and P3, which give rise to two primary transcripts, RNAII and RNAIII, respectively. RNAII encodes AgrB, AgrD, AgrC, and AgrA, while RNAIII serves as a post-transcriptional regulatory factor for multiple virulence genes[74]. Additionally, the *sar* locus has been identified as a second regulatory locus that is responsible for the *agr*-dependent regulation of staphylococcal virulence[75].

The signal sensed by the *agr* system is an autoinducing peptide (AIP). Once the extracellular concentration of the AIP, generated from the AgrD precursor, reaches a threshold level, it triggers the activation of the *agr* system. AgrC binds to AIP and phosphorylates AgrA, leading to the activation of the P2 and P3 promoters and subsequent transcription of *agr* system targets. The membrane-localized enzyme AgrB is involved in the maturation and export of AIP. RNAIII encodes the delta-toxin-encoding gene *hld*, and these structural regions play a regulatory role in the expression of various virulence factors. Additionally, other regulators, including SarA, SrrAB, SarR, and SarX, can either modulate the activity of the *agr* system by either enhancing or inhibiting its function (Figure 1)[76].

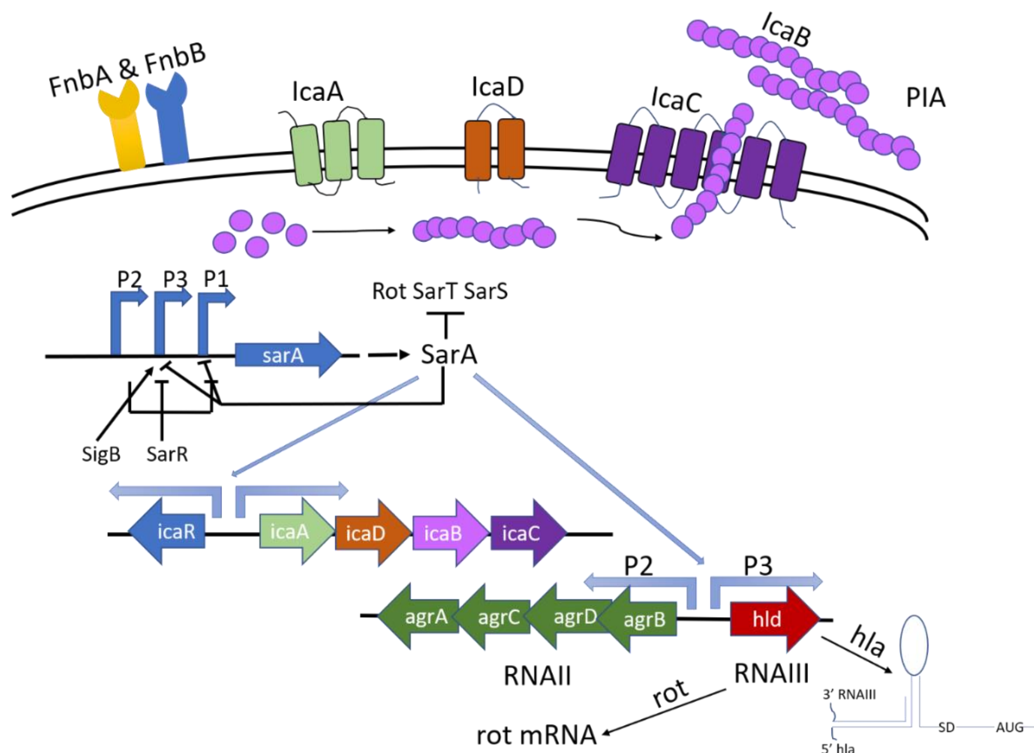

Figure 1. The *Staphylococcal* quorum-sensing system. The expression of the *sarA* gene is governed by three distinct promoters (P1, P2, and P3), with alternative sigma factor (SigB) facilitating the expression of *sarA* through its binding to the P3 promoter. SarR acts as a modulator by binding to all three promoters, interfering with the auto-regulatory function of SarA. Additionally, SarA serves as a negative regulator for three SarA-like proteins, namely SarH1, SarT, and Rot. Furthermore, SarA plays a pivotal role in activating the *agr* system, whereby the extracellular concentration of the AgrD precursor reaches a threshold level, triggering the activation of the Agr system. This activation occurs through the binding of AgrC to AIP and subsequent phosphorylation of AgrA, ultimately leading to the activation of the P2 and P3 promoters and transcription of *agr* system targets. The RNAIII transcript encodes the delta-toxin gene (*hld*)[76].

4. An Overview of *SarA* in *S. aureus*

SarA is unequivocally recognized as the central regulator of biofilm formation in *S. aureus*, exerting a critical role in the modulation of virulence factors through its involvement in transcriptional regulation and modulation of mRNA stability, has been found to positively regulate both the *agr* system and biofilm accumulation, making it a key factor in *S. aureus* pathogenesis[11, 12]. SarA locus plays a crucial role in the pathogenesis of *S. aureus*, and *SarA*, the principal virulence regulator, also serves a vital post-translational function by limiting protease-mediated degradation and thus enhancing the accumulation of various virulence factors. In the early 2000s, analysis of *S. aureus* genomic sequencing data indicates the presence of a minimum of nine major homologs of the SarA protein in the majority of genomes[77,78].

4.1. Molecular Basis of SarA

SarA is a DNA-binding protein with a molecular weight of 14.7 kDa, and it is involved in regulating a total of 120 genes, with 72 genes being upregulated and the remaining genes being downregulated. Each monomer of SarA consists of five α-helices, a β-hairpin turn, and a C-terminal region[14]. The *sarA* locus consists of a 1.2 kb DNA region that encompasses three overlapping transcripts driven by the sarAP1, sarAP3, and sarAP2 promoters, each promoter encodes a primary 372 bp *sarA* open reading frame (ORF), and all three transcripts terminate at the same site, resulting in the production of the SarA protein with a molecular weight of 14.7 kDa. These DNA-binding proteins engage in specific interactions with AT-rich inverted repeat or palindromic sequences located within target promoters, thereby governing the expression of multiple genes[79]. The monomeric structure of SarA primarily consists of five α-helices and three β-strands. The interaction between α1-helices from each monomer plays a significant role in dimer formation. The dimerization interface exhibits a hydrophobic nature and is conserved across related proteins. The characteristic helix-turn-helix (HTH) motif, formed by α3 and α4, is responsible for DNA binding and is commonly found in transcription factors. Within the three antiparallel β-strands (β1, β2, and β3), the β2 and β3 strands form a β-hairpin structure known as the "wing,"

which interacts with the minor groove of the target DNA[80]. Furthermore, *sarA* mutants exhibited reduced virulence in various infection models, such as osteomyelitis, infective endocarditis, and arthritis[17,18], suggesting the crucial role of *sarA* in regulating virulence (Figure 1).

4.1.1. Other SarA Homologs

Based on structural data, it has been observed that members belonging to the SarA protein family exhibit a comparable mode of DNA binding[80]. Nevertheless, owing to differences in their activation domains, distinct SarA homologs display diverse functional properties.

4.1.2. SarR

SarR, a homologue of the 13.6 kDa SarA regulatory protein, acts as a negative regulator of *sarA* gene expression by binding to the promoters of the *sarA* locus, including P1, P3, and P2. Importantly, SarR specifically binding to the P2 promoter inhibits the expression of the *agr* genes[81]. Notably, SarR exhibits a higher affinity for the *agr* promoter compared to SarA. Thus, SarR can effectively substitute SarA to maintain negative regulation of *agr* activity[82].

4.1.3. SarT

The expression of the *sarT* gene is controlled by the regulatory factors *agr* and *sarA*, both of which exert inhibitory effects on *sarT* transcription. Although SarT exerts minimal influence on the expression of SarA and SarR, the absence of SarT in a mutant strain leads to elevated mRNA levels of the *agr* effector RNAIII. Hence, there appears to be a reciprocal regulatory relationship between SarT and the *agr* system, potentially forming a negative feedback loop. In addition to this regulatory mechanism, SarT also exerts repression on the expression of *hla* gene[83]. The synthesis of SarT is controlled by ArtR, a small noncoding RNA. The ArtR molecule interacts with the 5' untranslated region of the *sarT* mRNA, leading to its degradation and subsequent reduction in SarT production[84]. Remarkably, the expression of ArtR is under positive regulation by *agr*, indicating its potential role as a mediator in the repression of *sarT* by *agr*[76].

4.1.4. SarS

SarS, alternatively referred to as SarH1 is a 29 kDa protein belonging to the SarA protein family[85]. Similar to other members of the SarA family, the regulatory network of SarA is intricately connected with other SarA family proteins and the *agr* system. The

expression of SarS is significantly inhibited by SarA and *agr*, as evidenced by previous studies[86]. Conversely, SarT has been found to induce the upregulation of SarS expression[87]. SarS functions as a dual regulator, acting as a transcriptional repressor for *hla* and a positive regulator for surface *spa*. Interestingly, the *spa* gene is positioned immediately downstream of the *sarS* locus. The *agr* system controls the expression of SarT, which subsequently enhances the expression of SarS. In turn, SarS positively regulates the production of the *spa*[87]. SarS potentially contribute to the dissemination of antibiotic resistance through SCCmec[88].

4.1.5. Rot

The "repressor of toxins" (Rot) regulatory protein belongs to the SarA-like family and has a molecular weight of 15.6 kDa. Mutation of the rot locus in an *agr*-deficient background leads to the reestablishment of toxin production and protease activity, thereby restoring the virulence of the bacterium in a rabbit model of endocarditis[89,90]. In the context of toxin regulation, Rot functions as a transcriptional repressor for enterotoxin B (*seb*), *hla*, proteases encoded by the *spl* and *ssp* operons, and lipase (*geh*). It exerts control over the expression of these virulence factors, inhibiting their transcription and subsequent production. Rot directly binds to the *seb* promoter, resulting in the direct repression of Enterotoxin B[89]. In contrast, the repression of *hla* is mediated indirectly through the SaeRS Two-Component System (TCS). Rot represses sae transcription from the P3 promoter, leading to a subsequent decrease in *hla* expression[91].

Additionally, Rot serves as a facilitator of numerous virulence factors, exerting a positive regulatory influence on their expression. Rot directly upregulates the *spa* and the SarA-family protein SarS[92]. By binding to the promoter region of these genes, Rot acts as a positive regulator for the superantigen-like proteins (Ssl)[93]. RNAIII, the primary mediator of the *agr* system, inhibits the translation of the *rot* gene[94]. In addition to RNAIII, Rot expression is also suppressed by SarA through direct interaction with the Rot promoter[95], and by the σ^B during the stationary growth phase[96].

4.1.6. MgrA

MgrA belongs to the SarA protein family and holds significant importance. According to the microarray study, MgrA exerts positive regulatory control over a set of 175 genes and negative regulatory control over another set of 180 genes in the strain Newman of *S. aureus*[97], including *hla*, coagulase, *spa*, nuclease, extracellular serine protease, and capsule biosynthesis genes encoding key virulence determinants[98], MgrA is involved in the

regulation of SarZ expression, which in turn modulates exoprotein production[99]. Additionally, surface proteins are positively regulated by MgrA, while extracellular proteins are negatively regulated by MgrA[99]. The transcription of the *mgrA* gene occurs through two distinct promoters, namely P1 and P2. Notably, the mRNA transcript originating from the P2 promoter is subject to stabilization by RNAIII[100].

In addition to the regulation of MgrA production by RNAIII, there is an alternative mechanism mediated by small RNAs. Subsequent investigations into MgrA have revealed its capacity to modulate the *lytRS*, *lrgAB*, and *arlRS* two-component systems (TCS), all of which play crucial roles in *S. aureus* autolysis[101]. MgrA plays a significant and dualistic role in the formation of biofilms in *S. aureus*. It has been reported that mutations in *mgrA* can both promote[102] and inhibit[103] biofilm formation. This discrepancy is likely attributed to the use of different strains of *S. aureus*. A study revealed that MgrA inhibits eight major surface proteins, including Ebh, SraP, and SasG, which are involved in preventing fibrinogen clotting. Fibrinogen-mediated clotting increases the production of *agr*-mediated virulence factors and decouples quorum sensing from actual cell density[104].

4.2. The biological role of SarA System-Mediated Regulation:

4.2.1. SarA Regulation of Virulence:

The analysis of DNA microarrays and proteomics has demonstrated that *sarA* governs the regulation of over 100 genes[105,106]. The *sarA* locus plays a pivotal role in the modulation of virulence factors. SarA exerts its regulatory functions on numerous virulence factors either through *agr*-dependent or -independent pathways[107]. In the *agr*-dependent pathway, SarA enhances the expression of the *agr* locus. In the *agr*-independent pathway, it interacts with AT-rich inverted repeat or multiple sequences located on the promoters of various target virulence genes to regulate the expression of multiple genes[105]. The expression of *SarA* was significantly reduced in the *agr*-dysfunctional MRSA compared to the *agr*-functional MRSA, suggesting that the defect was upstream of *agr*[108]. Notably, SarA represses the production of cell-surface proteins, such as fibronectin-binding protein and *spa*, and positively regulates the production of extracellular factors, including β-haemolysin, lipase, and autolysin[106]. SarA also modulates the accumulation of virulence factors at the intracellular level and extracellular protease-mediated degradation[109]. SarA orchestrates the regulation of multiple virulence factors, spanning gene transcription, post-transcriptional regulation, intracellular accumulation, and extracellular degradation. Furthermore, the presence of *sarA* has been correlated with isolates harbouring the *ica*ADBC genes, specifically implicating its

association with the *ica*ACD locus[34,110], indicating a potential linkage between these genes in contributing to *S. aureus* pathogenesis.

Additionally, SarA also exerts regulatory control over the expression of diverse virulence factors through pathways that are dependent-*mecA* on or independent[18], *mecA* gene represses lipase production, which is considered a hallmark of the *sarA* regulon[111,112]. Studies have shown that the absence of sarA and sigB inhibits the expression of *mecA*, and that increases in lipase activity are associated with a decrease in *sarA* gene expression[18,112]. Apart from *SarA*[76] and other homologues SarR[82,113], repressor of toxins(Rot)[90,114], SarS[85,114], SarT[83,91], and SarU[115], also have significant roles in regulating virulence factor expression.

4.2.2. SarA-Mediated Biofilm Formation

SarA is also involved in the expression of genes that encode surface-associated binding proteins, which contribute to biofilm formation[116,117]. The biofilm formation process in *S. aureus* is regulated by two genetic loci, the *sarA* and *agr* quorum-sensing systems, which play crucial roles in controlling virulence and the mechanisms underlying the development of infection[110,118], and it is controlled by *sarA* through the *agr*-independent pathway[119]. SarA serves as a pivotal determinant in biofilm formation, as the *sarA* gene has been identified in 84% of biofilms derived from MRSA isolates[34,120]. SarA, through an *agr*-independent mechanism, also plays a pivotal role in mediating biofilm formation by facilitating the interactions between cells and surfaces, as well as cell-to-cell interactions mediated by *fnbPA*, *fnbPB*, and other virulence factors[121]. Additionally, *sarA* negatively regulates the expression of autolysin (*atl*)[105]. SarA exerts regulatory control over multiple virulence genes implicated in the formation of the biofilm matrix, encompassing extracellular proteases, nucleases, and *fnBP*. Notably, *sarA* positively regulates biofilm formation by enhancing the expression of *fnb* genes and suppressing the protease activity that typically involves in degradation and remodelling of surface adhesins[121]. Moreover, SarA collaboratively interacts with the two-component saeRS system to inhibit the expression of extracellular proteases, thereby mitigating the depletion of critical proteins essential for the biofilm matrix assembly[116]. Notably, the *sarA* gene has been found to be strongly associated with the polysaccharide poly-N-acetylglucosamine (PNAG)-dependent biofilm formation and development in *S. aureus*[122].

With that, SarA potentially modulates the long-term persistence of MRSA infections through its involvement in biofilm formation. The intricate interplay between eDNA, autolysins, and *sarA*, and their roles in the complex biofilm formation process, highlight the

complex and multifaceted nature of *S. aureus* biofilm formation. The broad-spectrum transcription factor SarA is crucial in regulating biofilm formation by modulating the activity of *atl* and *fnbps*, thereby enabling the bacterium to adapt to various environmental stressors including acid pressure or DNA damage. Furthermore, *sarA* exerts a significant influence on the expression of extracellular proteases, resulting in a negative effect on *atl* and *fnbps* activity[59]. The dynamic interplay between *sarA* and these key biofilm components underscores the intricate regulatory mechanisms that govern *S. aureus* virulence and pathogenicity.

4.2.3. Mutation studies of the sarA

Mutations in the *sarA* gene exhibit a significantly more prominent effect on biofilm formation compared to mutations in any other regulatory loci. Mutations in the *sarA* gene have been demonstrated to significantly reduce the ability of *S. aureus* to form biofilms, whereas mutations in *agr* have minimal impact on biofilm formation in most strains, consequently affecting the susceptibility to β-lactam antibiotics. Furthermore, in both in *vitro* and in *vivo* conditions, *sarA* mutants of USA300 strains LAC exhibited reduced biofilm formation and decreased expression of virulence factors, as well as diminished virulence was observed in animal models[123,124]. Recent studies demonstrated that *SarA* also plays a significant role in regulating extracellular protease production, which is directly associated with reduced biofilm formation and accumulation of *FnbA*/*FnbB*-associated virulence factors in an *atl*/*sarA* mutant[125].

Mutation of the *sarA* gene in *S. aureus* present exerts substantial inhibitory effects on the expression of multiple virulence factors, including ten widely recognized extracellular proteases such as aureolysin, *SspA*, *SspB*, *ScpA*, and *Aur*[63]. Proteases have the ability to restore high virulence in *sarA* mutants[109,124,126]. Additionally, in *vitro*, studies demonstrated that all *sarA* mutants display reduced biofilm formation in the presence or absence of antibiotics[127]. The functional interplay of *sarA* with other regulatory loci is critical for the virulence of *S. aureus*. The reversal of the increased biofilm formation in the *mgrA* mutant by *sarA* mutation, independent of aureolysin or *SspA* production[128]. However, concurrent mutations in both *sarA* and *agr* give rise to phenotypes resembling those observed in *sarA* single mutations, leading to the complete abrogation of virulence[18,129,130].

SarA is additionally involved in bacterial adaptation to host and environmental settings, as it modulates the expression of *sodA*, *budA*, and *budB* genes in response to fluctuations in redox potential and pH levels[131]. The presence of mecRI in *S. aureus* strains is linked to the increased level of RNAIII expression, which is regulated by SarA-mediated

transcription. In addition, the *sarA* gene is known to affect the transcription of hemolysins, which play an important role in the pathogenesis of *S. aureus* infections[132]. These findings underscore the importance of understanding the regulation and expression of virulence factors in *S. aureus* in order to develop effective strategies for the prevention and treatment of *S. aureus* infections. With that, these findings highlight that *SarA* plays a crucial role in modulating the pathogenic potential of *S. aureus*, and that targeting *SarA* and its downstream effectors could be a promising strategy for developing novel antimicrobial therapies.

5. Targeted Strategies for MRSA Mitigation

Achieving successful eradication of MRSA requires the administration of antibiotics at elevated concentrations and prolonged treatment durations, presenting formidable challenges in the management of MRSA infections. Unfortunately, persistent infections resulting from healthcare-associated infections related to medical devices often pose significant challenges to clinical treatment and frequently lead to treatment failures. This challenge has spurred researchers globally to investigate innovative solutions aimed at controlling biofilm formation and development, as well as the expression of virulence factors[133–135]. Additionally, MRSA demonstrates intrinsic resistance to aminoglycosides, macrolides, tetracyclines, chloramphenicol, and lincosamides[136]. Despite the ongoing development and utilization of novel antibiotics, bacteria strains can acquire increasing resistance to conventional antibiotics through continuous genetic mutations and transfer mechanisms. The rapid evolution of antibiotic resistance leads to the gradual obsolescence of once-effective conventional antibiotics each year. The emergence of antibiotic-resistant bacteria poses significant challenges in the treatment of MRSA infections. For the treatment of CA-MRSA infections, some clinicians also recommend avoiding the use of doxycycline, clindamycin, and trimethoprim/sulfamethoxazole, as they may exacerbate clinical outcomes[137]. However, our understanding of the antibiotic-mediated regulation of virulence-associated protein expression and its impact on the regulatory network controlling *S. aureus* virulence remains limited.

5.1. Modulate the Expression of Virulence Factors in S. aureus

Based on the aforementioned scenario, targeting virulence factors may present a promising therapeutic approach for treating infections. This strategy allows the disarming of bacteria without compromising their viability, thereby avoiding selective pressure on bacterial growth and reducing the likelihood of resistance emergence[117,138].

Several studies have focused on the impact of antibiotics on regulating the expression of virulence factors in *S. aureus*. For instance, subinhibitory concentrations of β-lactam antibiotics have been shown to induce the upregulation of Panton-Valentine leukocidin (PVL) production, an important virulence factor of *S. aureus* responsible for leukocyte destruction. PVL contributes to poor antibiotic diffusion at the site of infection. Antibiotics such as clindamycin and linezolid, which inhibit protein synthesis, can decrease the release of toxins, including PVL. In addition, SarA has been identified as a major regulator of PVL production[139], SarA positively regulates the transcription of PVL, and its absence leads to the inhibition of PVL production[140,141]. During the exponential growth phase, SarA activation facilitates the interaction with target gene promoter regions, resulting in the repression of toxin expression and subsequent upregulation of PVL expression[95].

Targeting the virulence regulator SarA could be highly advantageous, as it can disrupt various virulence factors rather than individually targeting specific ones[142]. Moreover, SarA plays a crucial role in biofilm formation by regulating the expression of extracellular proteases, nucleases, and *fnbps* associated with biofilm formation, impacting the tissue persistence of pathogenic targets[127]. Most bacterial isolates obtained from wounds and pus samples exhibit positive *sarA* gene presence. Alterations in the *sarA* gene lead to methicillin resistance, as methicillin significantly influences the metabolic processes involved in biofilm formation, ultimately promoting robust biofilm development[143]. Approximately 46.9% of strains carrying the *sarA* gene display multidrug resistance, particularly towards erythromycin, ciprofloxacin, clindamycin, and gentamicin[116,124]. Furthermore, single nucleotide polymorphisms (SNPs) in *sarA* have been shown to affect the sensitivity to vancomycin (VAN)[144]. Inhibition of sarA expression could serve as an effective approach in controlling *S. aureus* infections, making it a noteworthy avenue for further investigation.

5.2. Natural Products, Synthetic Compounds, Synergistic Treatment Strategies and Drug Repurposing

Natural products derived from plants[145–148], microbes[149–151], and animals[152], present promising avenues for combating MRSA infections by targeting key regulatory genes and virulence factors through various mechanisms, including antibiofilm and antivirulence activities[153–157]. Among the different sources of natural products, plant extracts have been demonstrated for their ability to exert anti-MRSA activity through diverse mechanisms including the inhibition of *sarA* gene expression and downregulation of downstream virulence genes regulated by SarA, either via direct or indirect interactions with SarA.

Different plant extracts were shown to exhibit anti-MRSA activity via modulating the *sarA* gene. For instance, ethanolic extract of *Myrtus communis* L. exhibited significant

inhibition of biofilm formation and development. It was found to markedly suppress the expression of *sarA*, *icaA*, *icaD*, and *bap* genes, while showing no significant impact on *agr* gene[136], suggesting the involvement of regulation of biofilm formation targeting *sarA* in an *agr*-independent pathway. Similarly, ethanol extract from *Torilis japonica* (TJE) inhibited biofilm formation, suppressed hemolysis, and downregulated the expression of virulence factors such as *sarA*, indicating that TJE may reduce the production of virulence factors by downregulating the AgrA-RNAIII system in the *S. aureus* QS system[158]. Meanwhile, another study revealed a non-direct pathway of anti-MRSA activity via regulation of *sarA* exhibited by differential concentrations of essential oil extracted from *Chamaecyparis obtuse* leaves. When the concentration of essential oil exceeded 0.1 mg/mL, the growth of MRSA and the production of acid from glucose metabolism were inhibited. Furthermore, at concentrations greater than 0.1 mg/mL, the formation of MRSA biofilms was suppressed. The expression of *agrA* was inhibited at concentrations exceeding 0.2 mg/mL, while the inhibition of *sarA* was observed at a concentration of 0.3 mg/mL[159]. The ethanolic extract obtained from the root bark of *Ulmus pumila* exhibited potential antibacterial activity against MRSA. At sub-minimum inhibitory concentrations (63-125 μg/mL), it induced a significant downregulation in the expression of *mecA*, *sea*, *agrA*, and *sarA* genes[160], thereby postulating its mechanism of action involving the inhibition of agrA or sarA expression, resulting in reduced transcription of exotoxin-encoding genes. The ethanolic extract of *R. javanica* leaves inhibits biofilm formation at concentrations higher than 0.05 mg/mL, and significantly suppresses the gene expression of key virulence factors such as *mecA*, *sea*, *agrA*, and *sarA* at concentrations of 0.4-1.6 mg/mL[160,161]. *Ginkgo biloba* exocarp extract (GBEE) exhibits dose-dependent inhibition of biofilm formation, with the expression of relevant factors *icaA* and *sarA* downregulated after 6 hours of treatment. Furthermore, the expression of *hld* is inhibited through the downregulation of *sarA*[162].

Moreover, plant-derived secondary metabolites like dihydrocelastrol, dihydrocelastryl diacetate[163], quebrachitol[164] and eugenol[165] exert inhibitory effects on MRSA virulence factors by targeting *sarA*, *agr* and *ica* genes. Dihydrocelastrol and dihydrocelastryl were shown to inhibit biofilm formation and suppress hemolytic activity in MRSA[163]. In addition, quebrachitol inhibited the production of staphyloxanthin in MRSA. Eugenol significantly inhibited the formation of biofilms in both MRSA and MSSA clinical strains by markedly reducing the expression of biofilm- and enterotoxin- related genes, including *icaD*, *sarA*, and *sea* genes[165].

Several plant-derived monoterpenes were reported to exhibit antivirulence and antibiofilm activities against MRSA by targeting the SarA regulatory pathways. Myrtenol, a

bicyclic alcohol monoterpene, was shown to inhibit the synthesis of virulence factors of MRSA, including slime, lipase, α-hemolysin, staphyloxanthin and autolysin. Mechanistically, myrtenol was unveiled to down-regulate various virulence genes significantly, including *sarA*, *agrA, crtM, hld, geh*, *fnbA*, *fnbB*, *icaA* and *icaD*[166]. Myrtenol is one of the essential oil constituents of several aromatic plants, such as the genus *Myrtus*[167]. Meanwhile, another widely studied essential oil constituent, thymol is derived from the genus *Thymus* and is well known for its antibacterial and antibiofilm activities[168]. Thymol demonstrated potent inhibitory effects on biofilm formation in *Δagr* strains, while exhibiting negligible impact on biofilm formation in *ΔsarA* strains, highlighting the *sarA*-dependent antibiofilm activity of thymol[169]. As an isomer of thymol, carvacrol was also shown to exhibit anti-MRSA and antibiofilm activities by inhibiting *sarA* expression and interfering with SarA-*mecA* promoter binding[170]. Selvaraj *et al.*[171] reported that carcacrol can form anionic bonding and hydrogen bonding with SarA and CrtM, respectively, via molecular docking analysis.

Sapindus mukorossi methanolic extract (SMME) exhibited concentration-dependent antibiofilm activity, and molecular docking studies indicated close interactions between bioactive compounds identified in SMEE and the SarA protein, namely the oleic acid with the strongest interaction with SarA was suggested as the major constituent responsible for the antibiofilm potential of SMME[172]. Another study also identified a potent interaction between hesperidin and the SarA protein in molecular docking analyses, highlighting the ability of hesperidin to effectively inhibit the expression of key virulence factors, including *sarA*, *icaA*, *icaD*, and *crtM* [173].

A newly isolated naphthoquinone-derived carbon skeletons eleucanainones, from the bulbs of *Eleutherine Americana,* exhibits antimicrobial activity against MRSA by inhibiting the expression of *agrA*, *cidA*, *icaA*, and *sar* genes, as observed in in *vitro* studies[174]. Andrographolide sulfonate (AS), a compound extracted from *Andrographis paniculata*, effectively inhibits biofilm formation and improves biofilm permeability by downregulating the expression of key bacterial adhesion-related genes, including *agrD*, *sarA*, *clfA*, *fnbB*, *icaA*, and *cidA*[175].

In addition to phytochemicals, synthetic chemical compounds also offer promising avenues for the management of MRSA. A study evaluated the antibiofilm activities of synthetic *N,O*-acetals derived from 2-amino-1,4-napthoquinone and found that among all the derivatives, 2-(ethoxymethyl)-amino-1,4-naphthoquinone exhibited strongest inhibitory effects on biofilm accumulation by suppressing the *sarA*-*agr* regulatory system and downregulating the expression of *fnbA*, a gene that is positively regulated by *sarA*[176]. A

novel piperazine-based copper (II) complex, namely cPAmPiCaTc, was synthesized and shown to exert anti-MRSA activity by regulation of *sarA* and dihydrofolate reductase (*DFHR*) genes[177]. Interestingly, the study also showed that cPAmPiCaTc exhibits excellent blood compatibility, opening a new avenue for metallodrug therapeutics against MRSA infection. Another study designed and synthesised a novel SarA based inhibitor (SarABI), 4-[(2,4-difluorobenzyl)amino] cyclohexanol, via a *de novo* computer-aided discovery approach. The antibiofilm activity of SarABI was correlated with its effects on downregulating the expression of major virulence genes, such as *RNAIII*, *hld*, and *fnbA* of *S. aureus*[178].

With the growing understanding of the SarA regulatory mechanisms in the pathogenicity and antibiotic resistance of MRSA, numerous studies also evaluated the potential of these natural products as an adjuvant to enhance the efficacy of beta-lactam antibiotics to combat MRSA by targeting sarA regulatory pathways. Valliammai *et al.*[169] showed that thymol potentiated the antibacterial activity of rifampicin on both planktonic, biofilm and persister cells of MRSA. On the other hand, Li *et al.*[170] showed carvacrol improved the efficacy of cefotaxime and oxacillin in both murine models of MRSA bacteremia and subcutaneous catheter-related biofilm infection.

Repurposing of clinically available drugs is another promising strategy to search for anti-MRSA drugs which have been well studied for their toxicity and side effects. A study revealed that an anti-hypertensive drug, candesartan, exhibits antivirulence activities against *S. aureus* along with domperidone (anti-emetic drug) and miconazole (anti-fungal drug). They were shown to exhibit antibiofilm, antihaemolytic and anti-staphyloxanthin production activities. Particularly, candesartan, an angiotensin receptor blocker, exhibited the strongest inhibitory activities against expressions of virulence-related genes including *sarA*, *CrtM*, *SigB*, *AgrA*, *hla*, *FnbA*, and *icaA*[179].

6. Conclusions

The rise of MRSA as a virulent pathogen, characterized by its multidrug resistance and biofilm-forming capability, has underscored the urgency of discovering novel therapeutics for combating MRSA infections. The exploration of alternative antimicrobial agents has directed scientific attention toward natural products, representing a burgeoning field of investigation. However, the *sarA* locus has received significant attention due to its crucial role in the formation of *S. aureus* biofilm. Mutations in the *sarA* locus have been shown to hinder biofilm development, leading to heightened susceptibility to antibiotics and enhanced efficacy of therapeutic interventions in animal models. The virulence of *S. aureus*

is attributed to its capacity for adhesion and invasion, which are key factors in its pathogenicity. Adhesive capacity is intricately linked to the formation of biofilms, leading to enhanced resistance against antibiotics. QS is crucial for facilitating biofilm formation. This intricate system facilitates biofilm detachment through the upregulation of autoinducing peptides AIPs production or the downregulation of glucose levels. SarA protein with DNA-binding properties that promote *agr* expression and RNAIII initiation. Additionally, the double mutation of *agr* and *sarA* leads to the loss of virulence.

SarA regulates the production and development of biofilms and directly modulates the expression of multiple virulence factors, making it a key regulator of *S. aureus* pathogenicity. Conversely, *agr* is a binary regulatory system that governs the synthesis of toxins and adhesion based on the quorum-sensing population of bacteria prevalent in specific ecological niches. SarA exhibits a high affinity for the intergenic region between the P2 and P3 promoters within the agr system. Additionally, SarA promotes hemolysin expression through direct interaction with the RNAIII transcript, highlighting its ability to coordinate gene expression through intricate molecular mechanisms, thus underscoring its significance in bacterial pathogenicity. Furthermore, SarA demonstrates a strong affinity for genes encoding *FnbA* and *FnbB*, *Spa*, enterotoxin C, and PIA synthesis proteins. This agr-dependent and agr-independent regulatory mechanism highlights the potential of SarA as a therapeutic target for addressing MRSA infections.

The choice of bacterial strains for studying the anti-MRSA activity targeting SarA is currently unrestricted. However, ATCC 33591 is more commonly selected by researchers, and clinical MRSA strains isolated from other clinical sites samples, including blood samples from patients, are also frequently chosen. Studies investigated the effects of two chemical synthetic compounds, SarA inhibitors and 6-TG/MMF, as well as a natural compound, eugenol, on MRSA, using animal models in *vivo* studies. While the other listed compounds were only studied in *vitro*. The lack of efficacy testing in *vivo* raises concerns about the effectiveness of these compounds. Firstly, their efficacy rates are a cause for alarm, and secondly, toxicity testing of the extracts is lacking. The exploration of natural compounds with inhibitory activity against bacterial toxins and other virulence factors is an ongoing endeavour in the field of antimicrobials. Despite some drawbacks associated with natural compounds, their wide range of biological properties exhibited by both the compounds themselves and their derivatives remains an intriguing area worth exploring. Moreover, it is crucial to determine how natural products can demonstrate their efficacy in the face of chemical instability. Based on the structure-activity relationship, natural products can be

considered as a potential direction, even if their initial apparent activity is weak, as they can serve as lead compounds for further structural modifications to achieve higher efficacy.

Due to the intricate involvement of SarA in regulating the virulence circuitry of MRSA, two important questions arise when considering SarA as a therapeutic target: firstly, whether there are regulatory sites similar to SarA or even more directly targeted; secondly, whether the therapeutic strategies targeting SarA are influenced by the functional status of other regulatory sites. The answers to these questions may provide new insights for the development of novel therapeutic approaches against MRSA infections.

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