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Review

Exploring *Staphylococcus aureus* Virulence Factors; Special Emphasis on Staphyloxanthin

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Staphylococcus aureus **is a well-known pathogen that can cause diseases in humans. It can cause both mild superficial skin infections and serious deep tissue infections, including pneumonia, osteomyelitis, and infective endocarditis. To establish host infection, *S. aureus* manages a complex regulatory network to control virulence factor production in both temporal and host locations. Among these virulence factors, staphyloxanthin, a carotenoid pigment, has been shown to play a leading role in *S. aureus* pathogenesis. In addition, staphyloxanthin provides integrity to the bacterial cell membrane and limits host oxidative defense mechanisms. The overwhelming rise of *Staphylococcus* resistance to routinely used antibiotics has necessitated the development of novel anti-virulence agents to overcome this resistance. This review presents an overview of the chief virulence determinants in *S. aureus*. More attention will be paid to staphyloxanthin, which could be a possible target for anti-virulence agents.**

Keywords: ***Staphylococcus aureus*****, virulence, anti-virulence therapy, staphyloxanthin**

Introduction

Staphylococcus aureus is an aggressive bacterium that causes a variety of hospitals and community associated illnesses. This bacterium is able to induce a wide range of diseases, which could be categorized into three types; superficial lesions such like soft tissue infection, toxinoses like scalded skin syndrome, food poisoning and toxic shock syndrome in addition to systemic and life-threatening infections, like osteomyelitis, endocarditis, brain abscesses, pneumonia, meningitis and bacteraemia [2]. *S. aureus* is widely distributed in nature; however, direct contact, typically skin-to-skin contact with a colonized or infected individual, is the primary mode of *S. aureus* transmission [3–6]. Staphylococcal capacity to colonize healthy people asymptomatically is one of its most important biological features. *S. aureus* can colonize several body mucosal sites including throat, the nostrils (nares), dedicated parts of the skin as the groin, axilla, and perineum since these skin areas are often wet in addition to the rectum [7].

The relative high burden of *S. aureus* in community and health care settings possess a global challenge. Infections with *S. aureus*, particularly methicillin-resistant *S. aureus* (MRSA), can result in increased mortality, morbidity, and economic loss, exerting pressure on healthcare systems around the world [8]. The acquisition of *mec*A gene that confers resistance of *S. aureus* to methicillin, can further favour epidemic spread by encouraging the acquisition of additional virulence traits [9]. Several epidemic and pandemic MRSA clones are expanding in both hospitals and in the community, creating novel clinical syndromes in some instances [10].

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MRSA infections undergoing prolonged vancomycin therapy have resulted in the rise of vancomycin resistant *S. aureus* strains [11]. Additionally, small-colony variants of *S. aureus* allow for development of persistent, recurrent and antibiotic-resistant infections in the host [7, 12]. As a consequence, treatment of *S. aureus* has been very complicated owing to the development of strains with resistance to multiple antibiotics, which are called 'super bugs' [13].

S. aureus has developed a complex regulatory network to manage virulence factors production, allowing the pathogen to thrive in different environmental conditions. Since they're not required for growth, regulatory machinery and virulence factors are known as accessory genes. The pathogenesis of *S. aureus* is established via these accessory components. These factors involves both cell surface components and extracellular proteins that are directly secreted into environment [14].

Staphylococcal Virulence Factors

Based on their mechanisms, *S. aureus* virulence factors are divided into two groups. The first group comprises the invasion and inflammation associated virulence factors, which involve colonization, production of extracellular molecules that aid adhesion, as well as evasion of the host defensive mechanisms. The second one includes virulence factors as toxins, which are extracellularly released for harming host tissue and enhancing both bacterial dissemination and biofilm formation, which are vital in certain infections [15].

Surface Proteins (Adhesins)

S. aureus surface is enriched with various adhesin proteins, which are covalently attached to peptidoglycan layer, and involved in adhesion and colonization step. According to the presence of motifs identified through structure function analysis, cell wall anchored proteins (CWA) are divided into four groups [16]. The first and most predominant group is the microbial surface component recognizing adhesive matrix molecule (MSCRAMM) family, which includes collagen-binding protein, fibronectin-binding proteins A and B, clumping factor A and B proteins and Sdr proteins (from SD Repeat) [16, 17]. SdrC, SdrD, and SdrE are the three proteins encoded by the *sdr* locus, but not all *S. aureus* strains have all three genes [18]. MSCRAMMs help bacteria adhere to the plasma as well as various host extracellular matrices including collagen, fibrinogen and fibronectin. Apart from adhesion, MSCRAMMs additionally promote bacterial pathogenicity by invading host cells and tissues, evading immunological responses, and establishing biofilms [19].

The near iron transporter (NEAT) motif protein family is the second group of CWA proteins. The iron-regulated surface determinant (Isd) proteins contain one (for IsdA), two (for IsdB) or three (for IsdH) NEAT motifs, which can attach to haem or haemoglobin. IsdA possess a hydrophilic stretch at its C-terminus, that decreases cell surface hydrophobicity and contributes antimicrobial peptide and bacterial lipid resistance [20, 21]. The third group is the three-helical bundle family consisting of protein A, only identified in *S. aureus*. Protein A binding to the Fc region of immunoglobulin activates TNFR1, induces TNF-α-like responses and interferes with opsonization [22]. The last group is the G5-E repeat family, which includes *S. aureus* surface protein G, which participates in adhesion to desquamated epithelial cell and biofilm formation [23].

Polysaccharide Capsule

S. aureus capsular polysaccharides (CP) are classified according to their immunological specificity [24]. CP is believed to enhance *S. aureus* virulence by allowing bacteria to resist phagocytosis and killing by polymorphonuclear phagocytes [25]. There have been 13 distinctive serotypes of *S. aureus,* with type 5 (CP5) and type 8 (CP8) being the most prevalent in clinical isolates [26]. CP5 and CP8 expression has been proved to be crucial for *S. aureus* to evade killing by opsonophagocytosis. The genes specifying CP in *S. aureus* are encoded by a 17.5-kb region with 16 highly conserved genes, *capABCDEFGHIJKLMNOP* (97–99% identity between serotypes), and four genes, *capHIJK*, specifying chemical diversity among serotypes. The three sugar residues in CP5 and CP8 have the same repeat unit, however their glycosidic linkage and acetylation are different [27, 28].

Biofilm Formation

A biofilm is a sessile microbial population where cells are adhered to a surface or to each other and are encased in an extracellular polymeric matrix that protects them

[29, 30]. Biofilms from different bacterial species have different compositions. The biofilm matrix consists mainly of extracellular polysaccharide, proteins, DNA in addition to lipids [31]. In biofilms, bacterial cells have different phenotypes in terms of proliferation, gene expression and protein production [32]. Throughout infection, biofilm growth is significant because it provides a barrier against various clearance systems. The biofilm matrix can obstruct and hence prevents certain types of immune defenses including the macrophages from entry into the biofilm matrix [33]. Moreover, biofilm cells display increased tolerance to metal toxicity, UV damage, anaerobic conditions, salinity, acid exposure, pH gradients, bacteriophages and antibiotics [34, 35].

S. aureus can attach and develop biofilm on both biotic and abiotic surfaces causing a high burden of biofilm related infections that lead to billions of healthcare costs each year all over the world [36]. Staphylococci are amongst the most common pathogens to infect indwelling medical devices, such as implanted artificial heart valves, catheters, and joint prosthesis, because they are commensal on human skin and mucus surfaces [37]. The biofilm structures vary from a monolayer of scattered single cells to a thick mucus multi-layered structure with channels that allow both liquid and gas passage in addition to the transport of nutrients and waste components [38].

Immunomodulatory Proteins

S. aureus secretes proteins that have been demonstrated to have a significant impact on the host innate and adaptive immune systems. These proteins include staphylokinase (SAK), extracellular fibrinogen binding protein (Efb), chemotaxis inhibitory protein of *S. aureus* (CHIPS), the staphylococcal complement inhibitor (SCIN), formyl peptide receptor-like-1 inhibitory protein, and extracellular adherence protein (Eap) [39].

SAK, known as staphylococcal fibrinolysin, is a 16 kDa plasminogen (PLG) activator protein that is secreted by many *S. aureus* strains. PLG is converted to plasmin (PL) by SAK, which cleaves human IgG, in addition to human C3b and C3bi, from the bacterial cell wall, resulting in disrupting human neutrophil phagocytosis. Furthermore, SAK suppresses the bactericidal activity of α-defensins by binding to α-defensins in a different site than its plasminogen-binding site [40]. Efb is a 15.6 kDa extracellular protein that binds to fibrinogen and inhibits the complement cascade by binding to complement C3b. Efb hinders the opsonization (classical pathway) and subsequently phagocytosis. Apart from fibrinogen binding site, C3b bind to Efb at a different site. Notably, Efb has the ability to bind both fibrinogen and C3b at the same time [41].

CHIPS is a 14.1 kDa protein that specifically disrupt neutrophils and monocytes response to formylated peptides and C5a. In vitro, CHIPS effectively suppresses neutrophil recruitment, however in vivo, high CHIPS concentrations are necessary. Furthermore, CHIPS completely blocks C5a cellular activation [42]. SCIN is a 10 kDa extracellular protein that blocks efficiently all complement pathways, including the lectin, classical and alternative pathway. SCIN successfully prevents opsonization and phagocytosis in *S. aureus*. SCIN hinders activation of the human complement cascade through binding to human C3 convertases and blocking C3b deposition. In addition, SCIN inhibits C5a-induced neutrophil responses [43].

Toxins

Hemolysins. Hemolysins are class of proteins defined by their ability to cause holes or pores in the target cell membrane resulting in cell lysis. Red blood cells (RBCs) lysis is mediated by hemolysins as alpha (α), beta (β), gamma (γ), and delta (δ) toxins. The *hla* gene encodes the α-hemolysin (α-toxin), which is the most well-studied virulence component of *S. aureus*. Most of *S. aureus* clinical isolates produce α-hemolysin, which is capable of lysing different human and animal cells, such as leukocytes, erythrocytes, platelets, epithelial cells and fibroblasts [44, 45]. On the other hand, specific *S. aureus* strains produce β-hemolysin, a 35-kDa protein encoded by the *hlb* gene. As a result of its characteristic action on sheep blood agar plates, β-toxin is formally recognized as the hot-cold toxin. β-toxin reacts with sheep RBCs at 37℃ but does not lyse them. However, if these RBCs are then subjected to cold (4℃), they would lyse. Furthermore, β-toxin is cytotoxic for lymphocytes especially proliferating T cells, erythrocytes and neutrophils [46, 47].

S. aureus γ-hemolysins are bicomponent toxins which are encoded by three genes *hlg*A, *hlg*B, and *hlg*C. Staphylococcal γ-toxins have been suggested to have a role in progression of the toxic shock syndrome (TSS)

together with TSS toxin 1 (TSST-1), as this hemolysin is more detected within TSS isolates [48]. In addition to the previously mentioned hemolysins, another one is known as delta-hemolysin, which is encoded by the *hld* gene. Most of *S. aureus* isolates (97%) produce δ-hemolysin that lyses neutrophils, human erythrocytes in addition to various mammalian cells. The haemolytic activity of δ-hemolysin has been explained by three possible mechanisms. For instance, δ-hemolysin could adhere to cell surface forming transmembrane pores, thereby destabilizing the plasma membrane. δ-hemolysin acts as a detergent at high concentrations that solubilizes the membrane [46, 47].

Panton-Valentine Leukocidin. Panton-Valentine Leukocidin (PVL) is found within community-associated MRSA [49]. PVL is considered as a type of membrane pores forming proteins. It is made up of two protein subunits (LukS-PV and LukF-PV) that work collectively to generate pores on the host cell membrane, causing leakage of cell contents and finally cell death. PVL shows a high affinity toward leukocytes [50].

Phenol-Soluble Modulins (PSMs). PSMs are amphipathic peptides found in staphylococci They have been recently correlated with highly pathogenic *S. aureus* strains [51]. *S. aureus* secretes four PSMα peptides which are encoded in the *psmα* locus, two PSMβ peptides, encoded in the *psmβ* locus, while the δ-toxin, encoded within RNAIII [52]. PSMs have surfactant-like characteristics which facilitate bacterial spreading and growth in environments with oil/water interfaces, such as on the skin, facilitating stapylococcus epithelial colonization [53]. Moreover, PSMs contribute to biofilm development, a phenotype believed to be essential for staphylococcal colonization and biofilm structuring [54, 55].

Staphylococcal Exfoliative Toxins. Staphylococcal exfoliative toxins (ETs) induce a syndrome called staphylococcal scalded skin syndrome. This syndrome primarily affects newborns, infants in addition to adults with renal dysfunction and immunological disorders [56]. Infected people will experience skin blistering, as well as the damage of superficial skin layers associated with dehydration and secondary infections. ETs break the desmoglein protein, which causes the skin epidermis to detach by disrupting the desmosomal cell binding [57]. Subsequently, this disruption of epidermal layer of the skin leads to more progression of infection. Furthermore, ETs are superantigens, however they are weaker than others like TSST-1 [58].

Staphylococcal Enterotoxins. Staphylococcal enterotoxins (SEs) are most frequent etiology of food-borne diseases with symptoms including vomiting and diarrhea. Enterotoxigenic *S. aureus* strains produce these toxins in food. SEs are thermostable and therefore they are not affected by cooking procedures. Over 20 SEs have been identified based on their antigenic structure [59]. The SEs are superantigens that induce activation and proliferation of T-cells, release of cytokine and cell death via apoptosis and potentially lethal toxic shock syndrome [60, 61].

Staphyloxanthin

The species epithet of *S. aureus* reveals its distinctive pigmentation (aureus, meaning "golden" in Latin) [62]. The golden pigmentation staphyloxanthin (STX) of *S. aureus* has a C_{30}-polyene backbone with alternating single and double bonds, which is the product of triterpenoid carotenoid biosynthesis pathway [63]. Carotenoids are structurally distinctive natural compounds, which typically have a lengthy carbon chain in the center with two terminal rings (Fig. 1). Carotenoids usually have 40 carbons, while others synthesized via different intermediates can have 30 or 50 carbons [64]. This carbon chain have a series of conjugated double bonds that confer STX its antioxidant capacity [65].

Staphyloxanthin Biosynthesis. Staphyloxanthin was distinguished as β-D-glucopyranosyl-1-O-(4,4'-diaponeurosporen-4-oate)-6-O-(12-methyltetradecanoate) by NMR spectroscopy, in which the triterpenoid carotenoid carboxylic acid at the C_1-position and a C_{15} fatty acid at C_6 position were esterified with glucose moiety [1]. The STX biosynthesis is primarily controlled by an operon

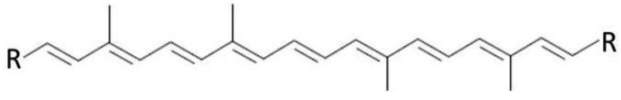

Fig. 1. Basic structure of carotenoid adopted from Fernandes *et al.* [66].

crtOPQMN, which encodes five different enzymes. Based on sequence similarities to known enzymes and product analyses of gene deletion mutants, the function of these enzymes was proposed [67].

As shown in Fig. 2, the head-to-head condensation of two C_{15} isoprenoid molecules of farnesyl diphosphate to generate dehydrosqualene is the initial step in STX biosynthesis, which is catalysed by dehydrosqualene synthase (CrtM). Consequently, the colorless dehydrosqualene (4,4' diapophytoene) is converted into the yellow intermediate 4,4'-diaponeurosporene by dehydrosqualene desaturase (CrtN) which is then oxidized by CrtP to form 4,4'-diaponeurosporenic acid. 4,4'-diaponeurosporenic acid is then esterified by a glycosyltransferase; CrtQ to give glycosyl 4,4'-diaponeurosporenoate. Lastly, the acyltransferase CrtO esterifies glucose at the C_6 position with the carboxyl moiety of 12-methyltetradecanoic acid to give orange end-product staphyloxanthin [1, 68].

Staphyloxanthin Function. STX has two proposed functions in the *S. aureus* cell; protection against oxidative stress and stabilization of the bacterial cell membrane [69]. Importantly, it has been shown that pigmented MRSA strains have a wide distribution in healthcare facilities and have the capacity to survive for a long period of time than those strains that are less pigmented [70]. Thus, pigmented *S. aureus* strains have more

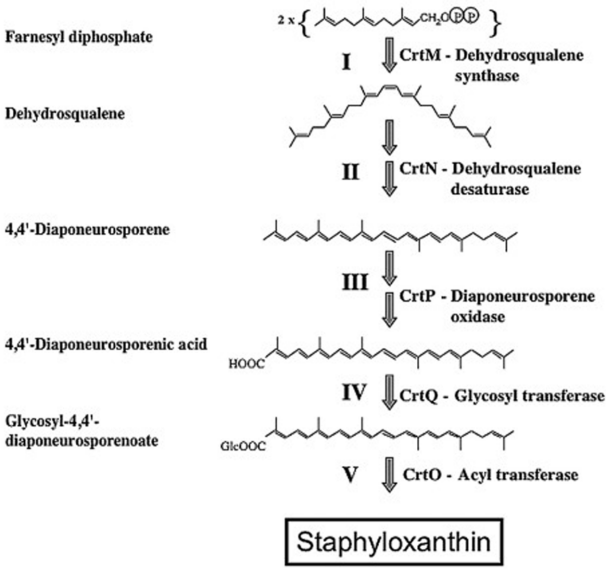

Fig. 2. Biosynthesis of staphyloxanthin adopted from Pelz *et al.* [1].

advantages than non-pigmented strains. Invading pathogens are attacked mainly by host phagocytes (neutrophils and macrophages) through release of reactive oxygen species (ROS), such as O_2^-, HOCl and H_2O_2, which are released by nicotinamide adenine dinucleotide phosphate oxidase [71]. STX- induced protection against host immune cells oxidative stress is attributed to the numerous double bonds in the pigment that are capable of quenching oxidation by ROS, and subsequently allows the bacteria to persist longer within the host [69, 72]. STX has been proven to be crucial for *S. aureus* infectivity. Bacteria that lack staphyloxanthin are non-pigmented and are more susceptible to hydrogen peroxide, superoxide radical, hypochlorite, hydroxyl radical and singlet oxygen. Furthermore, in mouse skin and systemic infection models, these non-pigmented cells are unable to cause illness [69, 72, 73].

In addition to being an antioxidant, staphyloxanthin can help to stabilise the structure of the cell membrane (CM) in the same manner as cholesterol does in the human cell membrane. Staphyloxanthin decreases CM fluidity and allows for membrane adaptation to various environmental conditions. Increased CM stiffness could enhance *S. aureus* survival to cationic antimicrobial peptides-mediated non-oxidative host defense [74, 75]. In the of work of Mishra *et al.* [75], the susceptibility of *crtM* mutant strain was assessed with its supplemented counterpart to a variety of antimicrobial agents including platelet microbicidal proteins, human alpha-defensin 1 and polymyxin B. The supplemented strain was less sensitive to AMPs, which was accounted to increased cell membrane rigidity. In contrast, Bayer *et al.* [76] found that membrane fluidity and AMP resistance were positively correlated. Therefore, *S. aureus* susceptibility in the face of non-oxidative host defenses is influenced by the net carotenoid homeostasis.

Genes Involved in Staphyloxanthin Production. STX biosynthesis genes in *S. aureus* are arranged in an operon *crtOPQMN* with a sigma B (σ^B) dependent promoter located in upstream of *crtO* and a termination region downstream of *crtN* [67]. SigB has an essential role not only in regulating staphyloxanthin biosynthesis but also in *S. aureus* biofilm formation as well as virulence expression [1, 63, 77–79]. Moreover, the *crtOPQMN* operon is positively regulated by *rsbUVW*-σ^B system [63,

80–82] and negatively regulated by the small RNA; SsrA RNA [83]. The biosynthesis of STX varies between strains and largely depends on environmental conditions [84]. As *sigB* is a positive regulator of *crtOPQMN* operon expression, direct and indirect impacts on *sigB* expression and activity affect pigment biosynthesis [85]. Additionally, a set of Rsb proteins encoded by *rsb* genes (*rsbUVWsigB*) regulate the activity of *sigB*. Both *rsbUVWsigB* system and *crtOPQMN* operon are found to be crucial for the *S. aureus* pigmentation [80, 82, 86].

CspA, a cold-shock protein, and AirR, an aeration-sensing response regulator, have been reported to have a positive effect on both *sigB* and pigmentation. Loss of CspA leads to a decrease in the expression of *crtMN* and *sigB* [87, 88]. Additionally, mutations and/or altered activities of some regulators including staphylococcal accessory regulator (*sarA*), accessory gene regulator (*agr*), arginine regulator (*argR*), and ClpP protease may impact expression of *sigB* and alter bacterial cell pigmentation [89, 90].

DnaK proteins are molecular chaperones of the Heat-shock proteins family, which present in all organisms [91–94]. Staphylococcal DnaK plays a fundamental role in preventing *S. aureus* from heat, oxidation and antibiotic stress. Furthermore, DnaK has a significant impact on pigmentation, autolysis as well as *in vivo* animal survival. *S. aureus* dnaK mutants can synthesize pigment, although they produce fewer carotenoids than the wild-type strain, resulting in colonies with pale yellow-orange color. Furthermore, the mutants were also found to be more susceptible to oxidative stress and to have a lower survival rate *in vivo* [92].

The inactivation of tricarboxylic acid cycle genes (*citZ, citG,* and *SAV2365*) was shown to enhance farnesyl diphosphate production through the flux of acetyl-CoA to the mevalonate pathway, resulting in more pigmentation. Moreover, acetyl-CoA shift to the mevalonate pathway did not show any elevation in *sigB* or *crtM* expression. Similarly, inhibiting oxidative phosphorylation genes (*qoxB* and *ctaA*) enhances bacterial pigmentation. *S. aureus* Δ*ctaA* mutant exhibited an elevation in *crtM* mRNA level. However, the *qoxB* mutant did not show any significant change in *crtM* and *sigB* mRNA levels. Furthermore, bacterial pigmentation was found to be more pronounced in purine biosynthetic (*purN*, *purH*, *purD*, or *purA*) mutants which was explained by augmentation of *sigB* expression [89].

Mutation in pyruvate dehydrogenase (*pdh*) leads to interruption in production of acetyl-CoA from pyruvate, which is a substrate for both fatty acids and staphyloxanthin biosynthesis and subsequently a drop in pigments biosynthesis. On the other hand, the branched-chain α-keto acid dehydrogenase (*bkd*) mutant revealed induced pigments biosynthesis. This was accounted by the availability of acetyl-CoA for STX biosynthesis with no consumption in branched chain fatty acid (BCFA) biosynthesis [89, 95].

Disrupting Staphyloxanthin Biosynthesis: A Novel Anti-virulent Therapy. The rise of antibiotic-resistant strains of *S. aureus* has rendered the existing bactericidal agents ineffective [13]. Hence, the newest strategy is to disarm bacteria rather than exerting selective pressure on it. Lack of STX has no effect on bacterial growth but would lead to high susceptibility of bacteria to ROS produced by host neutrophils. Thus, enzymes responsible for STX biosynthesis could be ideal targets for inhibiting pigment production and subsequently abolishing bacterial virulence [72].

• STX Inhibition by Targeting CrtM

Pigment inhibitors were first developed in 1967, near the same time that MRSA strains were discovered. Diphenylamine (DPA), a fatty acid synthetic inhibitor, was also reported at that time to partially inhibit carotenoid pigment production in several microorganisms without harming bacterial growth [96]. Next, many DPA derivatives were synthesized to characterize their inhibitory effect on STX formation, like 2-(2,3-dichloro-6-phenylphenoxy) ethylamine, and 2-(2,3-dichloro-6-phenylphenoxy)-N,N-diethylamine [97]. Following the characterization of STX biosynthetic pathway, blocking CrtM enzyme is considered as a possible mechanism for pigment inhibition. Drugs that have bisphosphonate functional groups, as zoledronate, were found to have a potent inhibitory activity against farnesyl diphosphate synthase (FPPs) while their antibacterial and CrtM inhibitory activites were minimal [98].

Further studies showed that CrtM structure resembles that of human squalene synthase (SQS), which indicates that SQS inhibitors reported as potential cholesterol biosynthesis inhibitors might also gain activity against

bacterial CrtM [73]. Three SQS inhibitors of phosphonosulfonates were shown to inhibit CrtM leading to loss of bacterial pigment with a decreased survival in human blood killing assay and mouse innate immune system. Furthermore, these inhibitors showed no toxicity on three human cell lines; NCI-H460, MCF-7 and SF-268 [99]. These results represent a possible anti-virulence therapy against *S. aureus* by targeting CrtM.

• STX Inhibition by Targeting CrtN

Similar to CrtM, CrtN is another crucial enzyme in STX biosynthesis. CrtN has been shown to be a potential target to control *S. aureus* virulence and therefore, many compounds have been found to inhibit STX production through targeting CrtN. Diphenylamine, for example, has been proven to impede carotenoid pigments synthesis, owing to its moderate inhibitory action on CrtN [100]. A library of commercially available known drugs was screened for activity against STX production. It was found that the biosynthesis of STX pigment in *S. aureus* Newman was potently blocked by the FDA-approved allylamine antifungal drug naftifine [101]. Next, MRSA strains (USA400 MW2, USA300 LAC and Mu50) were additionally used to examine whether naftifine could additionally act on *S. aureus* mutant strains. The results showed that naftifine efficiently inhibited pigment biosynthesis in both the Newman strain as well as multidrug resistant *S. aureus*. Moreover, the incorporation of naftifine to staphylococcal cultures has no effect on *S. aureus* Newman growth indicating that it could inhibit STX biosynthesis selectively. Importantly, naftifine-treated cells were found to be more susceptible to both oxidative stress and whole blood killing as compared to untreated cells. Naftifine also has been reported to reduce staphylococcal virulence *in vivo* without altering *crtOPQMN* expression. However, naftifine inhibits pigment biosynthesis by competing with CrtN [101]. This was evidence that CrtN was a possible drug target against pigmented *S. aureus* virulence. Further structure activity relationship (SAR) analysis of the derivatives of naftifine elucidated the structural requirements for efficient pigment inhibition. SAR analysis indicated that the naphthyl moiety of naftifine is not essential for pigment inhibition. Also, it is acceptable to replace the naphthyl ring with other bulky aromatic rings such as benzofuran or quinoline. In addition, the N-methyl group is essential for high potency leading to a loss of pigment inhibitory activity, while functional groups that are too small or too large are unvaluable. Finally, regarding allyl linker that bind naphthylmethylamine with phenyl moiety, the potency decreases in the following order; 1,3-pentadienyl > propargyl > allyl > 2-methyl allyl = propyl = methenecyclopropanyl [102].

• STX Inhibition from Natural Product Library

Some natural compounds have been found to inhibit STX biosynthesis. Flavonoids are abundant throughout the plant kingdom with a variety of biological activities including antibacterial, antifungal, antiviral, antioxidant, and anticarcinogenic activities [103]. Flavone markedly inhibits both STX biosynthesis and α-hemolysin production with no effect on *S. aureus* planktonic growth. Importantly, the flavones-treated *S. aureus* cells were 100 times more liable to hydrogen peroxide which could be related to reduction in STX production [104]. Although the specific mechanism of flavone anti-virulence activity seems to be undistinguishable, previous report suggest that the screening of more flavonoids will generate additional therapeutic alternatives for combating *S. aureus*. Rhodomyrtone is acylphloroglucinol isolated from *Rhodomyrtus tomentosa* (Aiton) Hassk and that has been reported to exhibit a prominent bactericidal activity against many Gram-positive bacteria [105, 106]. Rhodomyrtone-treated *S. aureus* showed less pigment production as well as increased susceptibility to both hydrogen peroxide and singlet oxygen killing. Rhodomyrtone could probably function through induction of the CrtM enzyme activity and inhibition of the CrtN enzyme [107].

In a previous study, Sakai *et al.* [108] screened a microbial metabolite library by a paper-disk assay method. In addition, the activity of compounds from the natural product library (300 compounds) and actinomycete culture broths (1000 compounds) was assesed for their pigment inhibitory potential. The results indicated that four lipid metabolism inhibitors (dihydrobisvertinol, xanthohumol, cerulenin, and zaragozic acid) and two anthraquinones (tetrangomycin and 6-deoxy-8-O-methylrabelomycin) were capable of inhibiting STX production without affecting *S. aureus* viability. Since STX incorporates polyprenyl, sugar and acyl moieties in its

structure, lipid inhibitors have the capacity to hinder the formation of both the polyprenyl and acyl residues in staphyloxanthin. However, the precise mechanism of action of the two anthraquinones was uncertain. Similarly, a comprehensive screen of natural compound library containing 45,000 cultures was performed. Three compounds showed inhibitory action on MRSA pigment production without affecting bacterial viability. These compounds include citridone A that was first isolated from the culture broth of *Penicillium* spp. to potentiate miconazole activity against *Candida albicans*, FKI-1938; a series of thiodiketopiperazines graphiumins, that were collected from the culture of the marine derived fungus *Graphium* spp. and the diphenolic racemates (±)-tylopilusin A, (±)-tylopilusin B, and tylopilusin C isolated from the fruiting bodies of *Tylopilus eximius* [109–111].

In conclusion, the widespread expansion of bacterial multidrug resistance to antibiotics in addition to their adverse influence on human microbiota has led to an urgent need to develop new approaches to combat bacterial pathogens. The anti-virulence approaches have been recently introduced as an alternative strategy to fight various bacterial infections. *S. aureus* has an arsenal of virulence factors that works co-ordinately together in order to establish host pathogenesis. Among *S. aureus* virulence factors, staphyloxanthin has been reported as one of the striking targets for anti-virulent therapy. Therefore, staphyloxanthin inhibitors could be a promising approach for dealing with *S. aureus* infections.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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