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MOLECULAR PATHOGENESIS

The *Staphylococcus aureus* AirSR Two-Component System Mediates Reactive Oxygen Species Resistance via Transcriptional Regulation of Staphyloxanthin Production

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ABSTRACT *Staphylococcus aureus* is an important opportunistic pathogen and is the etiological agent of many hospital- and community-acquired infections. The golden pigment, staphyloxanthin, of *S. aureus* colonies distinguishes it from other staphylococci and related Gram-positive cocci. Staphyloxanthin is the product of a series of biosynthetic steps that produce a unique membrane-embedded C_{30} golden carotenoid and is an important antioxidant. We observed that a strain with an inducible *airR* overexpression cassette had noticeably increased staphyloxanthin production compared to the wild-type strain under aerobic culturing conditions. Further analysis revealed that depletion or overproduction of the AirR response regulator resulted in a corresponding decrease or increase in staphyloxanthin production and susceptibility to killing by hydrogen peroxide, respectively. Furthermore, the genetic elimination of staphyloxanthin during AirR overproduction abolished the protective phenotype of increased staphyloxanthin production in a whole-blood survival assay. Promoter reporter and gel shift assays determined that the AirR response regulator is a direct positive regulator of the staphyloxanthin-biosynthetic operon, *crtOPQMN*, but is epistatic to alternative sigma factor B. Taken together, these data indicate that AirSR positively regulates the staphyloxanthin-biosynthetic operon *crtOPQMN*, promoting survival of *S. aureus* in the presence of oxidants.

KEYWORDS: *S. aureus*, staphyloxanthin, transcriptional regulation, AirSR, two-component regulatory systems

Staphylococcus aureus is a leading bacterial agent of hospital- and community-acquired infections ranging from infective endocarditis and osteomyelitis to soft tissue infection (1). *S. aureus* possesses a vast array of adhesion and virulence factors that allow the organism to infect any part of the body and efficiently evade the immune system. *S. aureus* colonies are orange to golden (hence the species name, *aureus*), which was previously used as a distinguishing characteristic and is now recognized as the product of an important virulence factor (2).

The distinctive golden pigment of *S. aureus* is the biosynthetic product of *crtOPQMN* and *aldH* (3, 4). The final product of this six-enzyme-biosynthetic pathway is a membrane-embedded golden C_{30} triterpenoid carotenoid, α-D-glucopyranosyl-1-*O*-(4,4′-diaponeurosporen-4-oate)-6-*O*-(12-methyltetradecanoate), termed staphyloxanthin (STX) (5). STX is an antioxidant (6) and an important virulence factor of *S. aureus*, promoting intracellular phagocyte survival, and is linked to resistance to reactive oxygen species (ROS) produced by the NADPH oxidase system within the phagocyte phagosome (2).

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Currently, it is known that the stress response alternative sigma factor B (SigB; σ^B) regulates STX production, as a σ^B deletion mutant is white and lacks STX. This regulation is likely direct, as a consensus σ^B DNA binding motif was identified in the *crt* promoter, but direct interaction has not been confirmed (7). Additionally, cold shock protein A (CspA) is a positive regulator, likely in a σ^B-dependent manner (8). The metabolism of *S. aureus* is also known to influence pigment production, as mutants with a defective tricarboxylic acid cycle or inability to perform oxidative phosphorylation show increased pigmentation (9, 10). In addition to protein-mediated regulation, the small, stable RNA A (SsrA transfer-messenger RNA [tmRNA]) acts as an antisense RNA, base-pairing in the 5′ untranslated region (UTR) of *crtMN* to regulate STX production (11).

Two-component signal transduction systems (TCSs) are important regulators of staphylococcal metabolism (12–15) and coordinate expression of virulence factors. Many of the 16 known TCSs, including the quorum-sensing Agr system (16, 17), the regulator of staphylococcal accessory exoproteins SaeRS system (18–20), and autolysin-related locus ArlRS system (21), are well characterized and extensively studied. The oxygen-sensing and redox-signaling AirSR TCS is a global regulator; including regulation of pathways for nitrate respiration and lactose catabolism, as well as virulence factors (12, 14, 15, 22).

During characterization of the several AirSR mutant strains, it was observed that overexpression of the *airR* response regulator increased colony pigmentation. STX carotenoid is an important staphylococcal antioxidant. Further, it is known that the AirSR TCS phosphorylation relay is activated in the presence of oxygen or mild oxidants (12). Therefore, it was hypothesized that, in the presence of oxidants, the AirSR TCS transcriptionally regulates the STX-biosynthetic operon, *crtOPQMN*, thereby increasing the antioxidant capacity of the cell. In support of this hypothesis, data are presented demonstrating that the AirSR TCS directly transcriptionally activates the *crtOPQMN* operon and modulates STX carotenoid production. Moreover, AirSR's regulation of staphyloxanthin influences *S. aureus* susceptibility to hydrogen peroxide (H_2O_2) and survival in human whole blood.

RESULTS

Overproduction of AirR enhances the golden color of *S. aureus* colonies. The *airSR* (formerly *yhcSR*) TCS was initially characterized as an essential operon in the methicillin-resistant *S. aureus* (MRSA) strain WCUH29 (NCIMB 40771) (23), but it was subsequently determined that the essentiality of the TCS is strain dependent (22). Construction of isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible WCUH29 Δ*airS*::P*spac-airS* and WCUH29 Δ*airR*::P*spac-airR* strains was undertaken before the strain dependence of *airSR* essentiality was recognized (23, 24). Subsequently, in the absence of the P*spac* promoter inducer IPTG, *airS* expression was increased almost 3-fold over endogenous *airS* expression, as measured by semiquantitative reverse transcription (RT)-PCR, presumably due to "leaky" P*spac* promoter activity (data not shown) (25). The leaky expression prevented IPTG-dependent titration of the growth of each strain.

Unexpectedly, it was observed that the golden color of WCUH29 Δ*airR*::P*spac-airR* colonies was remarkably enhanced relative to the wild-type (WT) *S. aureus* strain WCUH29 and WCUH29 Δ*airS*::P*spac-airS* colonies. This phenotype was observed on both Trypticase soy agar (TSA) plates (Fig. 1A) and sheep's blood agar plates (Fig. 1B). The enhanced golden pigment of the WCUH29 Δ*airR*::P*spac-airR* strain was observed only under aerobic growth conditions; when the bacteria were grown anaerobically, the golden pigment was absent in all the strains (Fig. 1C). The golden pigment of *S. aureus* is a potent antioxidant (2). Further, oxidants, such as oxygen, are known to activate the AirSR TCS (12), and therefore, it was hypothesized that AirSR regulates the expression of golden pigment in response to the presence of environmental oxidants.

The amount of staphyloxanthin produced parallels the amount of AirR produced by *S. aureus* WCUH29. The predominant pigment produced by *S. aureus* is an alcohol-soluble membrane-embedded golden C_{30} triterpenoid carotenoid, α-D-gluco-

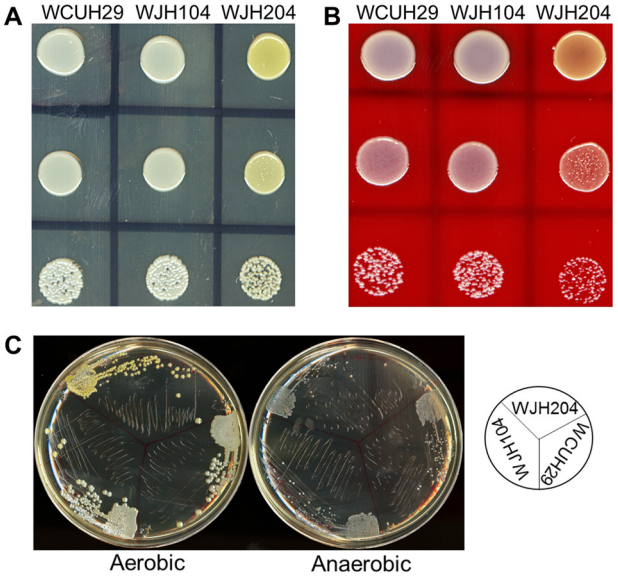

FIG 1 Overproduction of AirR enhances the golden color of *S. aureus*. (A and B) Overnight cultures of the strains were serially diluted, and 10 μl of the 10^{-3}, 10^{-5}, and 10^{-7} dilutions was plated on TSA (A) and 5% sheep's blood agar (B) plates and incubated for 24 h at 37°C. (C) Each strain was streaked on TSA plates and incubated aerobically or anaerobically. The diagram on the right shows the placement of the strains on the plates. WJH104, WCUH29 Δ*airS*::P*spac-airS*; WJH204, WCUH29 Δ*airR*::P*spac-airR*.

pyranosyl-1-*O*-(4,4′-diaponeurosporen-4-oate)-6-*O*-(12-methyltetradecanoate), termed staphyloxanthin (STX), that is the product of the biosynthetic pathway encoded by *crtOPQMN* and *aldH* (3, 4). The initial observational data suggested golden-pigment production was linked to AirSR TCS expression. The amount of golden pigment produced during overproduction of AirR or RNA interference (RNAi) knockdown of the *airSR* TCS was quantified to support the hypothesis that golden-pigment production was linked to AirR production. The WCUH29 Δ*airR*::P*spac-airR* strain produced 35% more golden pigment than wild-type WCUH29 (Fig. 2A). Furthermore, the JSAS909 strain, expressing an *airSR*-specific antisense RNA, produced 50% less golden pigment than control WCUH29/pYH3 (Fig. 2B) (23), further suggesting that the AirSR TCS regulates golden-pigment production.

To confirm that the golden pigment was indeed staphyloxanthin, a *crtM* markerless deletion in *S. aureus* WCUH29 was constructed. As expected, the colonies were devoid of their trademark golden pigment (2). Subsequently, the TetR-regulated empty-control (pYH4) and AirR overproduction (pAirR) plasmids were electroplated into the Δ*crtM* strain, resulting in strains WCUH29 Δ*crtM*/pYH4 and WCUH29 Δ*crtM*/pAirR, respectively. The WCUH29 wild-type empty-control (WCUH29/pYH4) and AirR-overproducing

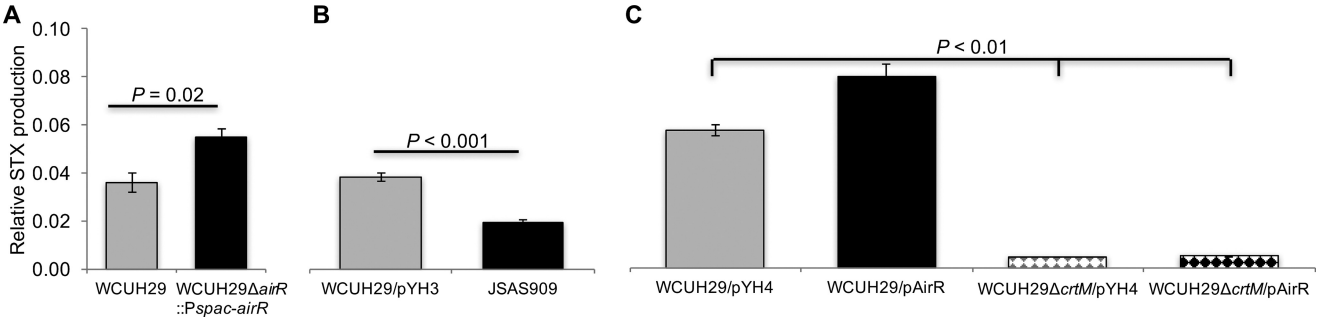

FIG 2 The AirSR TCS regulates the production of STX. All the strains were cultured for 24 h at 37° with shaking. *S. aureus* strains were cultured with 200 μM IPTG (A), 500 ng/ml ATc (B), and 250 ng/ml ATc (C). The OD_{600} was measured after washing. STX was extracted in methanol, and the OD_{450} was measured. Relative STX values were calculated as follows: OD_{450}/OD_{600}. The data represent the means ± standard errors of the mean (SEM) of the results of at least three independent experiments. JSAS909 is WCUH29 with an *airS* antisense RNA-expressing plasmid.

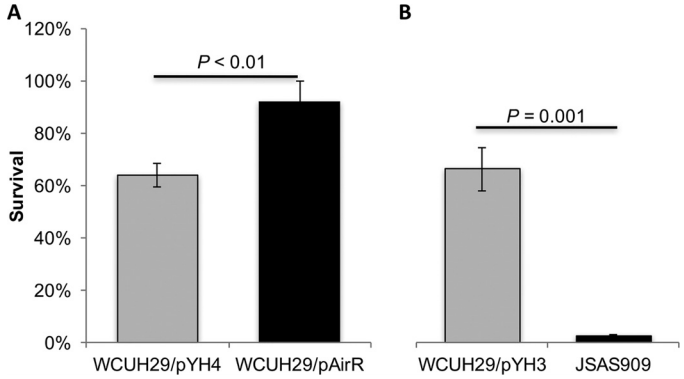

FIG 3 The AirSR TCS is important for H_2O_2 resistance. All the strains were cultured for 24 h at 37°C with shaking. The *S. aureus* strains were cultured with 250 ng/ml ATc (A) and 500 ng/ml ATc (B). Approximately 5×10^8 CFU was incubated in sterile PBS with 1.5% hydrogen peroxide at 37°C for 1 h. JSAS909 is WCUH29 with an *airS* antisense RNA-expressing plasmid. The percent survival was calculated as follows: (number CFU_{final}/number CFU_{input}) $\times$ 100. The data represent the means $\pm$ SEM of the results of at least three independent experiments.

(WCUH29/pAirR) strains (22), along with the new Δ*crtM* mutant strains, were cultured with the TetR inducer anhydrotetracycline (ATc) and assayed for golden-pigment production. Consistent with the previous AirR overproduction results, the WCUH29/pAirR strain produced 30% more golden pigment than WCUH29/pYH4 (Fig. 2C). Regardless of a strain's ability to overproduce AirR, golden pigment was absent in the Δ*crtM* strains (Fig. 2C). These data indicate that the golden pigment is indeed STX and the AirSR TCS regulates STX production in *S. aureus* WCUH29.

The AirSR TCS mediates resistance to hydrogen peroxide killing. The membrane-bound STX carotenoid protects *S. aureus* from ROS, such as hydrogen peroxide (2, 6). Therefore, AirSR would be expected to be important for ROS resistance as a potential redox sensor and regulator of STX production. To test this hypothesis and to determine if the AirSR TCS is important for *S. aureus* resistance to killing by H_2O_2, the ATc-regulated AirR overproduction strain and *airS* antisense RNA *S. aureus* strains were utilized for an H_2O_2 susceptibility assay. The ATc-induced overproduction of AirR significantly enhanced the survival of WCUH29/pAirR compared to the WCUH29/pYH4 control strain (92% versus 64%) (Fig. 3A). Conversely, depletion of AirSR by RNAi dramatically reduced the ability of JSAS909 to resist H_2O_2-mediated killing relative to the WCUH29/pYH3 control strain (2% versus 66%) (Fig. 3B). These data indicate that the AirSR TCS is an important mediator of H_2O_2 resistance and that it contributes to protection from ROS, likely through regulation of STX biosynthesis (2).

The AirSR TCS is a positive transcriptional regulator of *crtOPQMN* but is epistatic to alternative sigma factor B. The precursors of staphyloxanthin are the product of the biosynthetic *crtOPQMN* operon; therefore, a *crt* promoter-*lux* (*luxABCDE*) reporter plasmid was constructed in order to determine the impact of altered AirR levels on *crt* transcription. In both of the AirR overproduction strains, WCUH29 Δ*airR*::P*spac-airR* and WCUH29/pAirR, the maximum relative luminescence units (RLU) of the *crt-lux* reporter were 5- and 2-fold higher, respectively, than those of the respective controls (Fig. 4A and B). The AirSR TCS appears to be a positive transcriptional regulator of the *crtOPQMN* operon.

During the course of these investigations, a community-acquired MRSA (CA-MRSA) USA300 Δ*airSR* mutant was reported (26). The parental wild-type strain and Δ*airSR* mutant, AH1263 and AH2084, respectively, were kindly provided to our laboratory by Alex Horswill. To further determine if AirSR regulates *crt* transcription, the *crt-lux* reporter was introduced into AH1263 and AH2084. The maximum RLU level of the *crt-lux* reporter was reduced more than 4.5-fold in the AH2084 *airSR* mutant relative to the AH1263 parental strain (Fig. 4C). These data confirm that the AirSR TCS is a positive transcriptional regulator of the STX-biosynthetic operon, *crtOPQMN*.

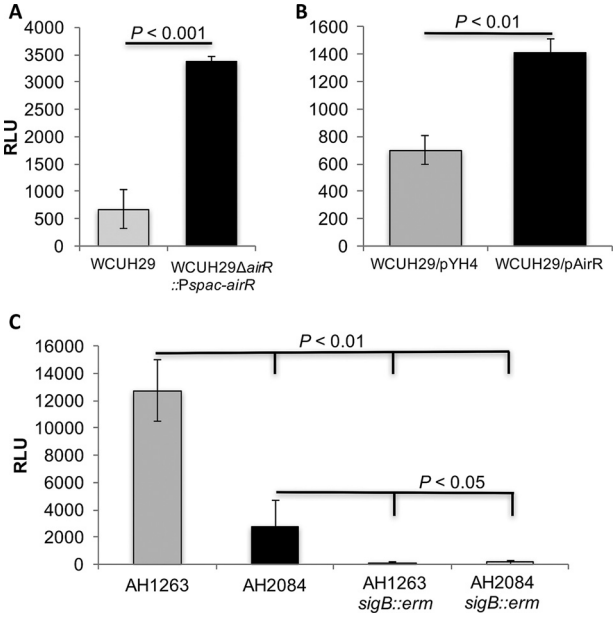

FIG 4 The AirSR TCS transcriptionally regulates *crtOPQMN* and is epistatic to alternative sigma factor B. (A and B) All the strains were cultured overnight without IPTG or ATc. Following overnight growth, the strains were diluted, and 250 μM IPTG (A) and 25 ng/ml ATc (B) were added to the respective cultures. (C) No inducer was added to CA-MRSA USA300 strains AH1263 WT and AH2084 (AH1263 Δ*airSR*). The data represent the maximum RLU after 2 h of growth. All the strains carry the P*crt-lux* reporter. The RLU were calculated by dividing the luminescence reading by the OD_{600} reading. The data represent the means ± SEM of the results of at least three independent experiments.

It has been well established that the alternative sigma factor B (SigB) plays an important role in the regulation of *crt* operon expression (7, 27, 28). To exclude the possibility that downregulation of transcription from the *crt* promoter is due to an unidentified deficiency of *sigB* in WCUH29, *sigB* allelic-replacement mutants were created. The hemolytic activity of the new WCUH29 *sigB*::*erm* mutant on sheep's red blood cell agar was dramatically increased compared to the WCUH29 WT control (data not shown). These data are consistent with previous reports and further demonstrate the integrity of the SigB regulatory circuit in WCUH29 (29, 30). To further decipher the transcriptional regulation of *crt*, *sigB* allelic mutations were constructed in AH1263 and the *airSR* mutant, AH2084. The AH1263 *sigB*::*erm* strain had dramatically increased hemolytic activity relative to the parental AH1263 (data not shown). The hemolytic activity of AH2084 was slightly reduced compared to that of the AH1263 strain. Consistent with functional SigB regulation, the AH2084 *sigB*::*erm* had noticeably increased hemolytic activity relative to the parental AH2084, but it still appeared to be slightly reduced relative to that of the AH1263 *sigB*::*erm* mutant (data not shown). These data indicate that the SigB regulatory circuit is intact and functional in AH1263 and derivative strains. Furthermore, *airSR* and *sigB* may regulate hemolytic activity independently.

To determine the relationship between AirSR and SigB on transcription from the *crt* promoter, the *crt* promoter-*lux* reporter system was electroporated into AH1263 *sigB*::*erm* and AH2084 *sigB*::*erm*. The AH1263 *sigB*::*erm* strain had a maximum RLU 100-fold less than that of the AH1263 WT, while the maximum RLU was reduced more than 15-fold in the AH2084 *sigB*::*erm* relative to the parental AH2084 (Fig. 4C). No difference in maximum RLU was observed between an AH1263 *sigB* single mutant and an AH2084 *airSR sigB* double mutant (Fig. 4C). These results indicate that *airSR* is epistatic to *sigB*, as AirSR regulation of *crt* is dependent on the presence of SigB.

AirR directly binds the promoter region of *crtOPQMN*. To determine if AirR directly regulates the *crtOPQMN* operon, an electrophoretic mobility shift assay (EMSA) was performed with purified AirR-His and a biotinylated P*crt* probe. The electrophoretic

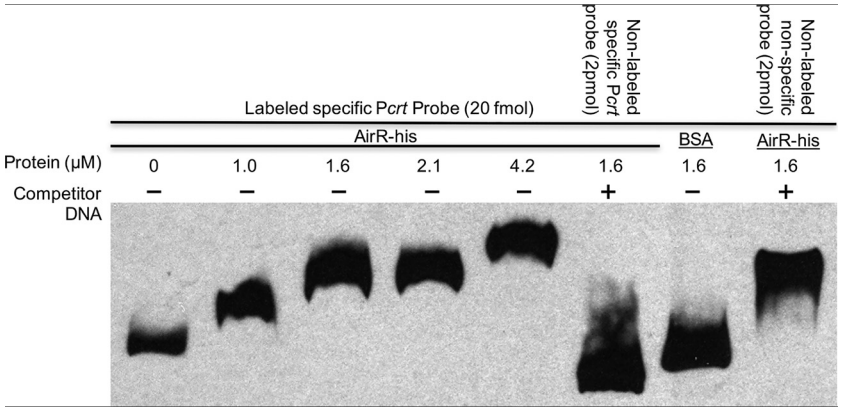

FIG 5 AirR specifically binds the *crt* promoter region. The electrophoretic mobility of the labeled promoter fragment alone is shown in the first lane. Increasing amounts of AirR (1.0, 1.6, 2.1, and 4.2 μM) were incubated with labeled *crt* promoter probe. −, absence of competitor DNA; +, addition of 100-fold excess competitor DNA. BSA (1.6 μM) and a nonspecific unlabeled probe (an internal fragment of *sspA*) were used as nonspecific binding controls. For each reaction, 20 fmol of biotin-labeled *crt* promoter probe was included. All the reaction mixtures totaled 20 μl.

mobility of the P*crt* probe during native PAGE was increasingly retarded as the concentration of AirR-His increased (Fig. 5, lanes 1 to 5), indicating that AirR binds the *crt* promoter probe. To determine the specificity of this binding, 100-fold excess of nonlabeled specific P*crt* probe was added to the reaction mixture, resulting in almost complete elimination of labeled specific P*crt* retardation, indicating that the nonlabeled specific probe competed with the labeled probe for the limited amount of AirR-His (Fig. 5, compare lanes 4 and 6). To further confirm the specificity of the binding, a nonspecific bovine serum albumin (BSA) protein control was added to the reaction mixture (Fig. 5, lane 7), and no apparent probe shift was observed, indicating that the mere presence of protein did not inhibit the mobility of the P*crt* probe. The inclusion of 100-fold excess of a nonlabeled internal fragment of the *sspA* gene (22) in the reaction mixture did not interfere with the AirR-His–P*crt* probe interaction. These data indicate that the AirR response regulator binds the *crtOPQMN* promoter and that recognition of the promoter is specific.

Loss of staphyloxanthin eliminates AirR-mediated enhanced survival in human blood. Previously, the AirSR TCS was recognized as an important factor for survival in human blood (22). The apparent global regulatory role of the TCS makes the assessment of AirSR-specific regulation of any virulence factor's contribution to overall *S. aureus* survival and pathogenesis cumbersome (12, 14, 15, 22). To facilitate the evaluation of a specific virulence factor's contribution to AirSR-mediated survival in blood, the virulence factor of interest is removed from the system by targeted gene deletion and the AirR response regulator is overproduced; in this case, *crtM* was deleted to prevent STX biosynthesis (reference 22 and data not shown). Using this principle, the significance of AirR-mediated STX production for *S. aureus* survival in human blood was evaluated. As seen previously, the ATc-induced WCUH29/pAirR strain had significantly enhanced survival in human blood throughout the experiment (Fig. 6). Overproduction of AirR in the STX-less WCUH29 Δ*crtM*/pAirR strain was significantly decreased relative the WCUH29/pAirR strain throughout the experiment. Interestingly, there was not a significant statistical difference between the WCUH29/pYH4 control strain and the STX-less vector control strain, WCUH29 Δ*crtM*/pYH4, at any time in the experiment, contrary to a previous report (2). Excitingly, there was not a statistical difference in survival between WCUH29 Δ*crtM*/pYH4 and WCUH29 Δ*crtM*/pAirR at any time in the experiment. Taken together, these data indicate that the enhanced production of STX facilitated by overproduction of AirR is a significant factor contributing to AirR-mediated enhanced survival of *S. aureus* in human blood (22).

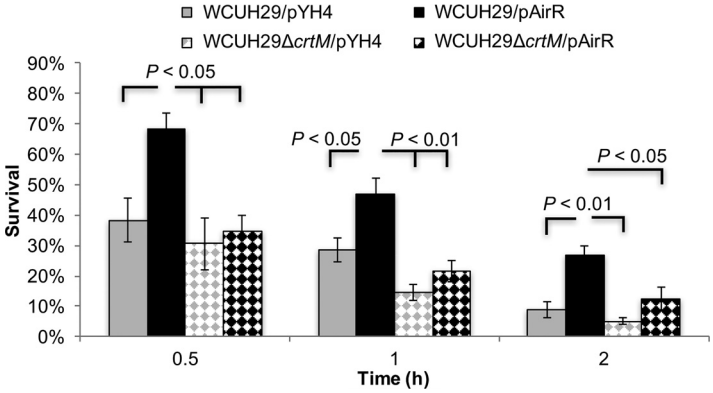

FIG 6 Absence of STX eliminates the enhanced survival of *S. aureus* resulting from AirR overproduction. Shown is the percent survival of *S. aureus* strains in freshly collected heparinized human blood with ATc. Bacteria were cultured overnight with ATc (250 ng/ml), and the following day were diluted, inoculated into blood with ATc (250 ng/ml), and incubated at 37°C in a rotisserie incubator. The percent survival was calculated as follows: (number CFU_{final}/number CFU_{input}) × 100. The data represent the means ± SEM of the results of four independent experiments.

DISCUSSION

The AirS sensor kinase uses a [2Fe-2S]-containing GAF domain as a redox sensor, and oxidation of AirS stimulates phosphorylation of the AirR transcriptional-response regulator (12). The STX carotenoid promotes virulence as an antioxidant against exogenous ROS and the neutrophil-mediated oxidative burst that occurs during phagocytosis (2, 31). In this study, we established for the first time that the AirSR TCS is important for the production of STX and resistance to H_2O_2 under normal atmospheric conditions. Further, we demonstrated that AirSR positively regulates the STX-biosynthetic operon, *crtOPQMN*, in a direct transcriptional-regulation manner, linking a redox sensor to the production of a protective carotenoid antioxidant. Our data further indicate that the AirSR regulatory system is epistatic to the alternative sigma factor B for transcriptional regulation of the *crt* operon, as transcription from the *crt* promoter was essentially the same in a *sigB* mutant regardless of the presence or absence of *airSR*.

Regulation of STX production is multifactorial, influenced by alternative virulence factor regulators, antisense RNA, and metabolic processes, as well as oxygen (7–11). Our quest to construct an IPTG-regulated *airS* and *airR S. aureus* strain ultimately proved unsuccessful due to high basal P*spac* activity, but we were able to observe that a consequence of increased AirR production was increased colony pigmentation. We confirmed that the pigment was indeed STX and that the levels of STX were directly correlated with the amount of AirSR TCS produced by *S. aureus*. Furthermore, it was determined that the AirSR TCS was important for resisting killing by H_2O_2. The AirR response regulator is a DNA binding transcription factor, and thus, it was conceivable the AirSR TCS regulated STX production.

Our observations suggested that the AirSR TCS regulated STX production, likely through regulation of the biosynthetic operon, *crtOPQMN*. In support of AirSR-mediated regulation of STX, reevaluation of our unpublished microarray data found two STX-biosynthetic genes, *crtM* (dehydrosqualene synthase) and *crtN* (dehydrosqualene desaturase), to be downregulated 2- to 3-fold at the mid-exponential phase of growth during *airS* antisense RNA induction when cultured aerobically (Y. Ji, unpublished data). Moreover, transposon disruption of *airR* in *S. aureus* Newman reportedly did not alter *crt* expression at the mid-exponential growth phase when cultured anaerobically (12), consistent with AirSR activation during aerobic growth and its acting as a likely positive regulator of *crtOPQMN*. Using a P*crt-lux* reporter, we confirmed that the AirSR TCS is a positive regulator of the *crtOPQMN* biosynthetic operon, as overproduction of AirR in *S. aureus* WCUH29 increased P*crt-lux* reporter activity and deletion of *airSR* in *S. aureus* USA300 decreased reporter activity.

Using a gel shift assay, it was determined that regulation of the biosynthetic operon is direct, as AirR bound the upstream promoter region of *crtOPQMN* specifically. The AirR DNA recognition motif is posited to be AAATNNAAAATNNNNTT based on the analysis of four AirR-bound promoters (32). Using the upstream promoter regions of 11 genes known to be directly bound by AirR and the motif discovery program MEME (12, 14, 22, 32, 33), we identified the motif (G/T)AA(C/A)ATNNA(C/A)AAAT in all 11 promoters. This updated potential recognition motif maintains the core AAATNNAAAAT sequence previously identified and is consistent with the sequence structure of other NarL-like response regulator binding sequences (34, 35). AirR belongs to the LuxR/NarL family of response regulators.

We previously determined that overproduction of AirR enhanced the survival of *S. aureus* in human blood and that AirR regulated the *sspAB* protease genes (22). Deletion of the *sspAB* gene only minimally decreased survival of the AirR overproduction strain in blood, however, indicating other virulence factors were contributing to the enhanced survival of the AirR overproduction strain, as well. In this study, genetic elimination of STX production by deleting *crtM* had a significant impact on survival for AirR overproduction, suggesting regulatory mechanisms or mutations that increase the production of AirSR or STX benefit *S. aureus* survival in blood.

In the absence of oxidants, the AirS $[2Fe\text{-}2S]^{+}$ oxygen sensor is maintained in a reduced form; thus, kinase activity of AirS is inactive and AirR is not phosphorylated (12), and subsequently, AirR does not promote transcription. Conversely, oxygen oxidizes the AirS $[2Fe\text{-}2S]^{2+}$ cluster. The oxidation of AirS results in phosphorylation and effector function activation of AirR, namely, transcriptional activation. As an AirSR-regulated operon, *crtOPQMN* transcription is increased, ultimately resulting in more STX production and protection from environmental oxidants.

The AirS kinase function is inactivated by strong oxidation, such as in the presence of millimolar concentrations of H_2O_2 *in vitro* (12), but modeling of the neutrophil phagosome suggests that the H_2O_2 concentration is only in the low micromolar range (36). It is therefore conceivable that mild oxidation of the AirS [2Fe-2S] cluster may occur in the phagosomes of neutrophils within an anaerobic wound site and promote AirSR activation and production of STX (37). Oxidation of AirS likely upregulates *crt* transcription and promotes STX biosynthesis as a way to counteract oxidative killing by the neutrophil. If oxygen were present in the phagosome, it, too, would promote AirSR-mediated upregulation of STX and antioxidant protection. In support of this suggestion, STX has been shown to be important for bacterial survival when *S. aureus* is cultured with neutrophils (2), and AirSR is important for survival in blood, where neutrophils constitute 50 to 60% of all leukocytes (22, 38). Furthermore, genetic mutations or pharmacological inhibition of the NADPH oxidase pathway in neutrophils eliminated the requirement for STX by *S. aureus* in both assays (2). Likewise, the data presented here show that the genetic disruption of STX biosynthesis completely eliminated the enhanced survival of *S. aureus* due to AirR-mediated STX overproduction.

The AirSR TCS is not essential in every *S. aureus* strain, as previously believed (26), but the importance of this undercharacterized system for regulation of virulence factors and resistance to phagocytosis is becoming more apparent. Investigations of the role of AirSR in responding to and resisting phagocytosis and the subsequent phagocytic oxidative burst are ongoing. It is now apparent that the AirSR TCS is an important modulator of *S. aureus* antioxidants, virulence factors, and, potentially, survival within phagosomes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DC10B (a gift from T. J. Foster) served as the host for all *in vitro* recombinant DNA (39). *E. coli* transformants were selected on brain heart infusion (BHI) (Difco) agar containing erythromycin (100 μg/ml) or Luria-Bertani agar containing ampicillin (100 μg/ml). *S. aureus* was cultured in Trypticase soy broth (TSB) (Difco) or on TSA at 37°C with appropriate antibiotics. All the bacterial cell cultures were incubated with shaking at 220 rpm. *S. aureus* transformants were selected on

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Description	Source
Strains		
DC10B	Dam$^-$ *E. coli* that allows direct electroporation of purified plasmid DNA into wild-type *S. aureus*	39
WCUH29	Human clinical MRSA isolate; *sigB*$^+$ *rsbU*$^+$	NCIMB40771
SASJ104	RN4220 *geh*::pFF71-*airS*; *airS*::*tet* Tet^r Cm^r	23
SASJ204	RN4220 *geh*::pFF71-*airR*; *airR*::*tet* Tet^r Cm^r	23
WCUH29 Δ*airS*::P*spac-airS*	WCUH29 *geh*::pFF71-*airS*; Δ*airS* Cm^r	This study
WCUH29 Δ*airR*::P*spac-airR*	WCUH29 *geh*::pFF71-*airR*; Δ*airR* Cm^r	This study
WCUH29 Δ*airR*::P*spac-airR*/P*crt-lux*	Carries P*crt-lux* reporter; Cm^r Kan^r	This study
WCUH29/pYH3	WCUH29 antisense control strain with empty pYH3; Erm^r	23
JSAS909	WCUH29 with pSAS909; Erm^r	23
WCUH29/pYH4	WCUH29 protein overproduction control with empty pYH4; Erm^r	22
WCUH29/pAirR	WCUH29 with pYH4-*airR*; Erm^r	22
WCUH29/pYH4/P*crt-lux*	WCUH29/pYH4 with with P*crt-lux* reporter; Erm^r Cm^r	This study
WCUH29/pAirR/P*crt-lux*	WCUH29/pAirR with P*crt-lux* reporter; Cm^r Cm^r	This study
WCUH29 Δ*crtM*	WCUH29 with in-frame deletion of *crtM*	This study
WCUH29 Δ*crtM*/pYH4	WCUH29 Δ*crtM* with pYH4; Erm^r	This study
WCUH29 Δ*crtM*/pAirR	WCUH29 Δ*crtM* with pAirR; Erm^r	This study
AH1263	USA300CA-MRSA; Erm^s (LAC*)	26
AH2084/JMB1241	AH1263 Δ*airSR*	26
AH1263 *sigB*::*erm*	AH1263 with deletion of *sigB*; Erm^r	This study
AH2084 *sigB*::*erm*	AH2084 with deletion of *sigB*; Erm^r	
AH1263/P*crt-lux*	AH1263 with P*crt-lux* reporter; Cm^r	This study
AH2084/P*crt-lux*	AH2084 with P*crt-lux* reporter; Cm^r	This study
AH1263 *sigB*::*erm*/P*crt-lux*	AH1263 *sigB*::*erm* with P*crt-lux* reporter; Cm^r Erm^r	This study
AH2084 *sigB*::*erm*/Pcrt-*lux*	AH2084 *sigB*::*erm* with P*crt-lux* reporter; Cm^r Erm^r	This study
Plasmids		
pYH3	Shuttle vector with a TetR-regulated inducible promoter; Erm^r	23
pSAS909	pYH3 with *airS* antisense downstream of TetR promoter; Amp^r Erm^r	23
pYH4	pYH3 with Amp^r removed; Erm^r	46
pAirR	*airR* cloned downstream of pYH4 TetR promoter; Erm^r	
pCY1006	*agr*-promoter-*luxABCDE* reporter system; Cm^r	44
P*crt-lux*	pCY1006 with *crt* promoter cloned upstream of *luxABCDE* in place of *agr* promoter; Cm^r	This study
pKOR1	Temperature-sensitive inducible allelic exchange plasmid for *S. aureus*; Cm^r	42
pKOR1-*crtM*	pKOR1 with in-frame *crtM* upstream/downstream deletion region; Cm^r	This study

TSA containing chloramphenicol (10 μg/ml) or erythromycin (5 μg/ml). Sheep's blood agar plates were obtained from BD. For anaerobic growth, TSA plates were placed in an oxygen-free nitrogen-hydrogen gas mixture COY chamber (Coy Laboratory Products) and incubated at room temperature.

Construction of *S. aureus* WCUH29 Δ*airS*::P*spac-airS*, WCUH29 Δ*airR*::P*spac-airR*, WCUH29 Δ*sigB*, AH1263 Δ*sigB*, and AH2084 Δ*sigB* strains. *S. aureus* WCUH29 P*spac-airS* and -*airR* were constructed by ϕ11 phage transduction and selection of the pFF71-*airS* or -*airR* chromosomal fragment from SASJ104 and SASJ204, respectively (23, 40). Briefly, SASJ104 and SASJ204 were cultured and used to make separate ϕ11 lysates. The lysates were then used to infect wild-type *S. aureus* WCUH29, and transductants were selected on TSA with chloramphenicol. The pFF71 plasmid site-specifically integrates into the *S. aureus* chromosome at the ϕL54α *attB* site located in the lipase (*geh*) gene (41). Transductants that were Cm^r and lipase negative on Spirit Blue agar plates with lipase reagent (BD) were screened by diagnostic PCR using the PspacFor/AirROE-Rev primer set (listed in Table 2) to specifically identify colonies that had properly recombined the pFF71-*airS* or -*airR* chromosomal fragment into the chromosome. Subsequently, *airS* and *airR* were deleted from their native loci in the respective strains using pKOR1-*airS* and pKOR1-*airR* plasmids (42).

To determine whether *sigB* is involved in the regulation of the *crt* operon by *airSR*, we created *sigB* allelic-replacement mutants in wild-type WCUH29 and AH1263 strains, as well as AH2084, an *airSR* deletion knockout (KO) mutant, using phage transduction, as described previously (23, 40). The *sigB* mutants were selected with erythromycin and further confirmed by diagnostic PCR and DNA sequencing. The donor *sigB* allelic-replacement mutant was kindly provided by Knut Ohlsen (43).

Extraction of staphyloxanthin from *S. aureus* strains. To determine if AirSR contributed to the production of staphyloxanthin, *S. aureus* strains were cultured in 5 ml of TSB in a 50-ml conical vial with or without the respective inducer, as indicated: 200 μM IPTG or 500 ng/ml ATc. After 24 h, each culture was pelleted, washed twice in PBS, and resuspended in 5 ml of PBS. The optical density at 600 nm (OD_{600}) of the culture was read in 200-μl duplicates using a 96-well flat-bottom microtiter plate (Sarstedt) and a BioTek Synergy II spectrophotometer. One milliliter of culture was pelleted and resuspended in 1 ml of 100% methanol, with pipetting and vortexing until all large clumps of cells were dispersed. The methanol extractions were placed in a rotisserie incubator and rotated end over end at room temper-

TABLE 2 Oligonucleotide sequences

Primer	Sequence
Pspacfor	5′-GCTTGAATTCATTCAGAACGCTCGGTTGCC-3′
AirROE-rev	5′-TTGGCGCGCCCTATTTTATAGGAATTGTGAATTG-3′
crtM-pKOR1-L-For	5′-GGGG ACAAGTTTGTACAAAAAAGCAGGCT CAGCAAGTTCCAGTAGATGTCATTG-3′
crtM-pKOR1-L-Rev	5′-CATACTAGTCCTCCTATATTGAAATGCC-3′
crtM-pKOR1-R-For	5′-Phos-CATAGAATATAGGTGGTTGAATAATGAAGATTG-3′
crtM-pKOR1-R-Rev	5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTG ATCTGTCACATCAATATCTATACCG-3′
luxA Rev	5′-GCTCCAGTAACCATACGG TATC-3′
Pcrt For-lux	5′-TAGCGAATTCTAATGGTTATGCATCAGGAAGTAAC-3′
Pcrt Rev-lux	5′-TTCCCGGGCTAAATTGAATCACTCTCAATCATACTGAC-3′
Pcrt For-EMSA	5′-biotin-CTAATGGTTATGCATCAGGAAGTAAC-3′
Pcrt Rev-EMSA	5′-biotin-CTAAATTGAATCACTCTCAATCATACTGAC-3′
sigBFor seq	5′-ATGGCGAAAGAGTCGAAATCAG-3′
sigBRev seq	5′-AATTGCCGTTCTCTGAAGTCG-3′

ature for 2 h. The cellular debris was pelleted, and the OD_{450} (2) for each methanol extraction was measured in 200-μl duplicates. The relative STX levels were calculated as the OD_{450} divided by the respective OD_{600} for each sample. The experiment was repeated at least three times.

Hydrogen peroxide survival assay. To determine the contribution of *airSR* to the survival of *S. aureus* when challenged with oxidative stress, overnight cultures were washed with PBS and $\sim 2 \times 10^8$ CFU was incubated at 37°C in a 1.5% H_2O_2–phosphate-buffered saline (PBS) solution for 60 min (2). Serial dilutions were plated on TSA for enumeration of surviving CFU. Percent survival was calculated as the number of surviving CFU divided by the number of input CFU multiplied by 100 [(number CFU_{final}/number CFU_{input}) $\times$ 100].

Construction of a promoter-*luxABCDE* reporter fusion system. To investigate if the *airSR* TCS transcriptionally regulates the promoter of the *crtOPQMN* operon, we constructed a *crt* promoter-*lux* (*luxABCDE*) reporter using the pCY1006 vector (44), as previously described (45). The upstream *crt* promoter region was PCR amplified with primers Pcrt For-lux/Pcrt Rev-lux (listed in Table 2), purified, and digested with EcoRI and SmaI (NEB). The pCY1006 plasmid was digested with the same enzymes, and the *crt* promoter PCR fragment was ligated into the gel-purified vector backbone with T4 DNA ligase (Promega). The reconstructed P*crt-lux* promoter reporter (P*crt-lux*) was confirmed by diagnostic PCR using the promoter-specific forward primer and a plasmid-specific luxA rev reverse primer (listed Table 2). The plasmid was purified from *E. coli* DC10B (39) and electroporated into the *S. aureus* strains, as indicated in Table 1.

The P*crt-lux* reporter plasmid was electroporated into several strains to measure the effect of the presence or absence of the AirR response regulator (Table 1). For analysis using strains overproducing AirR, strains WAirR and WJH204, and the respective controls, cultures were grown overnight in TSB at 37°C with shaking overnight. The overnight cultures were diluted 1:300 in TSB with the appropriate inducer, as indicated in the legend for Fig. 4, in a sterile 96-well flat-bottom white microtiter plate (CoStar), sealed with a Breathe-Easy sealing membrane (Electron Microscopy Sciences), and incubated at 37°C with shaking every 15 min in a BioTek Synergy II spectrophotometer. The bioluminescence intensities and optical densities of duplicate cultures were measured after 2 h of growth. The RLU were calculated by dividing the average bioluminescence reading by the average OD_{600} reading for each strain. The experiment was repeated three times with separate colonies of each strain.

For analysis of P*crt-lux* reporter activity in AH1263 (USA300 CA-MRSA), AH2084 (AH1263 Δ*airSR*), AH1263 *sigB*::*erm*, and AH2084 *sigB*::*erm*, cultures were grown overnight in TSB at 37°C with shaking overnight. The overnight cultures were diluted 1:100 in TSB and incubated at 37°C with shaking. The bioluminescence intensity and optical density at 600 nm of each culture were measured in duplicate in a 96-well flat-bottom white microtiter plate (CoStar) using a BioTek Synergy II spectrophotometer. The RLU were calculated by dividing the average bioluminescence reading by the average OD_{600} reading for each strain. The experiment was repeated three times with separate colonies of each strain.

Electrophoretic mobility shift assay. DNA for EMSA was amplified from the region upstream of the *crtOPQMN* operon. The P*crt*-EMSA For/Rev promoter primers listed in Table 2 were purchased with biotin conjugated to their 5′ termini. The dual 5′-biotin-labeled promoter fragment was obtained by high-fidelity PCR. A total of 300 μl of PCR was produced and loaded into a 2% agarose gel. The reaction mixture was electrophoresed and stained with ethidium bromide. The promoter fragment was cut from the gel and purified using the NucleoSpin gel cleanup kit (Macherey-Nagel). The probe was further purified by 5% native Tris-borate EDTA (TBE) PAGE, and the gel was stained with ethidium bromide. The DNA band corresponding to the calculated P*crt* fragment size was cut from the gel and repurified according to the NucleoSpin gel cleanup kit protocol. Lastly, a sample of the purified probe was electrophoresed on a 1.2% agarose gel to verify the size and purity of the biotin-labeled P*crt* probe.

The EMSA was performed using the LightShift Chemiluminescent EMSA kit (Thermo Scientific) essentially as described in the manufacturer's protocol. All the samples contained 1$\times$ LightShift binding buffer, 50 ng/μl poly(dI · dC), 2.5% glycerol, and 25 mM acetyl phosphate (Sigma). The labeled probe, AirR-His (22), and, where indicated, nonlabeled specific probe, BSA, and nonspecific nonlabeled probe

were all added to the concentrations outlined in the figures, and ultrapure water was added so that all the reaction volumes totaled 20 μl. The reaction mixtures were incubated at room temperature for 20 min, followed by addition of 5 μl of 5× loading buffer to each reaction mixture. Twenty microliters of each reaction mixture was loaded into a prerun 8% TBE native polyacrylamide gel and electrophoresed at 75 V for 3 h at 4°C. The samples were transferred to a nylon membrane, cross-linked, and processed as outlined in the manufacturer's protocol. BioMax light film (Kodak) was used to detect the chemiluminescent reaction.

Blood survival assay. A human blood survival assay was used to determine the contribution of staphyloxanthin to AirSR-mediated survival in humans. Bacteria were cultured in TSB with appropriate antibiotics overnight, and the inducer ATc was added where indicated. The overnight cultures were washed twice in sterile PBS and resuspended to an OD of 0.14 using a Behring photometer in PBS. Fresh venous human whole blood was collected, using heparin-containing Vacutainer tubes (BD), from outwardly healthy adult donors and then immediately used in the assay. The University of Minnesota Institutional Review Board approved the blood collection. Approximately 5×10^6 CFU in 50 μl of PBS was added to 450 μl of blood per microcentrifuge tube with appropriate antibiotics and ATc where indicated. The microcentrifuge tubes were capped, placed in a rotisserie incubator, and incubated at 37°C with end-over-end mixing. A 20-μl sample was removed from each sample at the indicated time points, serially diluted, and plated on TSA to determine the surviving CFU count for each sample. The percentage of surviving bacteria was calculated as follows: $CFU_{time\ point}/CFU_{initial\ input} \times 100$.

Gene deletion protocol. Deletion of *crtM* was carried out following the pKOR1 allelic-exchange protocol as described previously (42) and primer sets crtM-pKOR1-L-For/Rev and CrtM-pKOR1-R-For/Rev (listed in Table 2). The R-For primer was synthesized with a 5′ phosphate group. Each PCR fragment was purified, and the two fragments were ligated together with T4 DNA ligase (Promega). The ligation product was mixed with BP Clonase according to the manufacturer's instructions, and plasmid pKOR1, incubated at 25°C overnight, was then transformed into *E. coli* DC10B. The pKOR1-*crtM* KO plasmid was subsequently transformed into *S. aureus* WCUH29. White colonies were restreaked to fresh TSA plates, and deletion of *crtM* was confirmed by diagnostic PCR and absence of colony pigment.

Data analysis. Independent samples were statistically analyzed using a Student *t* test, with an alpha level of ≤0.05 considered significant. For data with more than two independent samples, a one-way analysis of variance (ANOVA) with a *post hoc* Tukey honestly significant difference (HSD) test was used to determine if there was statistical significance between samples, with an alpha level of ≤0.05 considered significant.

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We declare no conflicts of interest.

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