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The Agr Quorum-Sensing System Regulates Fibronectin Binding but Not Hemolysis in the Absence of a Functional Electron Transport Chain

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Staphylococcus aureus **is responsible for numerous chronic and recurrent infections, which are frequently associated with the emergence of small-colony variants (SCVs) that lack a functional electron transport chain. SCVs exhibit enhanced expression of fibronectin-binding protein (FnBP) and greatly reduced hemolysin production, although the basis for this is unclear. One hypothesis is that these phenotypes are a consequence of the reduced Agr activity of SCVs, while an alternative is that the lack of a functional electron transport chain and the resulting reduction in ATP production are responsible. Disruption of the electron transport chain of *S. aureus* genetically (*hemB* and *menD*) or chemically, using 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO), inhibited both growth and Agr activity and conferred an SCV phenotype. Supplementation of the culture medium with synthetic autoinducing peptide (sAIP) significantly increased Agr expression in both *hemB* mutant strains and *S. aureus* grown with HQNO and significantly reduced staphylococcal adhesion to fibronectin. However, sAIP did not promote hemolysin expression in *hemB* mutant strains or *S. aureus* grown with HQNO. Therefore, while Agr regulates fibronectin binding in SCVs, it cannot promote hemolysin production in the absence of a functional electron transport chain.**

Staphylococcus aureus is responsible for a broad range of infections, including furunculitis, abscesses, bacteremia, infective endocarditis, osteomyelitis, septic arthritis, pneumonia, and meningitis, and for colonization of the airways of people with cystic fibrosis (CF) (1–3).

The ability of *S. aureus* to cause such diverse infections is underpinned by a huge array of different virulence factors and immune evasion molecules, which are tightly regulated by a number of global regulators, including the quorum-sensing master virulence regulator Agr (accessory gene regulator) (2, 4–7). The Agr locus encodes two divergent promoters: P2, which transcribes RNAII, and P3, which transcribes RNAIII. P2 controls the expression of a four-gene operon, *agrBDCA*, which encodes the quorum-sensing *agr* autoactivation circuit. Specifically, *agrD* encodes the quorum-sensing molecule autoinducing peptide (AIP), which is processed and secreted by AgrB. AgrC and AgrA constitute a two-component signaling system, which detects AIP and completes the quorum-sensing part of the Agr system. The secreted AIP binds the transmembrane-bound histidine kinase signal receptor AgrC and activates it; it subsequently undergoes transautophosphorylation and transfers the phosphate to AgrA, the response regulator. The phosphorylated AgrA activates the P2 promoter, thereby completing the autoinduced activation circuit (6). Therefore, Agr is activated as the *S. aureus* population density increases, up to a maximum level in stationary phase. In addition to quorum sensing, there is also evidence that Agr acts as a diffusion-sensing system to signal bacterial uptake into host cell vacuoles (8). AgrA, in addition to regulating the Agr quorum-sensing system from P2, also drives transcription from the P3 promoter that transcribes the RNAIII transcript, the major effector molecule of the Agr system (6). RNAIII inhibits expression of surface protein (e.g., protein A and fibronectin binding proteins) genes and promotes expression of hemolytic exotoxin genes (e.g., *hld* and *hla*) (6, 9, 10).

While a combination of antibiotic therapy and a potent immune response are usually sufficient to resolve most *S. aureus* infections, a significant number develop a chronic or relapsing course (1, 11–13). Several studies of *S. aureus* isolated from the sites of chronic and recurrent infections have revealed the presence of slow-growing, weakly pigmented small-colony variants (SCVs) (14–24). As their name suggests, SCVs form small colonies on agar plates, have significantly reduced hemolytic activity, and are often resistant to aminoglycoside or sulfonamide antibiotics (14, 15, 20, 24).

SCVs typically arise from wild-type bacteria via mutations in genes associated with biosynthetic pathways, including those of menaquinone, heme, and thymidine, and are a normal part of the *S. aureus* life cycle (25–29). The loss of menaquinone or heme production results in loss of the electron transport chain, leading to decreased membrane potential and reduced ATP production and growth rate (20, 30, 31). Additionally, the loss of heme biosynthesis may impact other aspects of *S. aureus* biology, for example, the production of catalase (20).

The ability of SCVs to survive in host tissues for extended periods is believed to be due, at least in part, to their ability to persist within host cells without triggering apoptosis, which shields them from immune surveillance and therapeutic antibiotics (23, 32, 33). Invasion of, and persistence within, host cells is enabled by the high level of fibronectin-binding protein (FnBP) expression and

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TABLE 1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics	Source or reference
S. aureus		
SH1000	Functional *rsbU*$^+$ strain repaired for *rsbU* from parental laboratory strain NCTC8325-4	42
SH1001 (*agr* mutant)	SH1000 *agr*::*tet*	42
SH1000 *hemB*::Tn	SH1000 transduced with DNA from a USA300 *hemB*::Tn mutant; Erm^r; heme auxotroph	This study
SH1001 *hemB*::Tn	SH1001 transduced with DNA from a USA300 *hemB*::Tn mutant; Erm^r; heme auxotroph	This study
SH1000-P3	pCL55$_{\text{agr IR P3-GFP}}$ integrated into the *geh* locus of SH1000; reports P3 activity	44
SH1000-P2	pCL55$_{\text{agr IR P2-GFP}}$ integrated into the *geh* locus of SH1000; reports P2 activity	44
SH1001-P3	pCL55$_{\text{agr IR P3-GFP}}$ integrated into the *geh* locus of SH1001; reports P3 activity	44
SH1001-P2	pCL55$_{\text{agr IR P2-GFP}}$ integrated into the *geh* locus of SH1001; reports P2 activity	44
SH1000 *hemB*::Tn-P3	SH1000-P3 transduced with DNA from a USA300 *hemB*::Tn mutant; Erm^r; heme auxotroph; reports P3 activity	This study
SH1000 *hemB*::Tn-P2	SH1000-P2 transduced with DNA from a USA300 *hemB*::Tn mutant; Erm^r; heme auxotroph; reports P2 activity	This study
SH1001 *hemB*::Tn-P3	SH1001-P3 transduced with DNA from a USA300 *hemB*::Tn mutant; Erm^r; heme auxotroph; reports P3 activity	This study
SH1001 *hemB*::Tn-P2	SH1001-P2 transduced with DNA from a USA300 *hemB*::Tn mutant; Erm^r; heme auxotroph; reports P3 activity	This study
USA300 LAC	CA-MRSA strain of the USA300 lineage	43
USA300 *hemB*	USA300 in which *hemB* has been deleted	This study
USA300 *menD*	USA300 in which *menD* has been deleted	This study
USA300 *hemB*-P3	USA300 *hemB;* reports P3 activity	This study
USA300 *menD*-P3	USA300 *menD*; reports P3 activity	This study
E. coli		
DC10B	Δ*dcm* in DH10B (Δ*hsdRMS endA1 recA1*) *dam* methylation only; high-efficiency cloning strain that bypasses the restriction modification system of *S. aureus*	46
Plasmids		
pCL55$_{\text{agr IR P3-GFP}}$	pCL55 A^r Cm^r containing the *agr* P2-P3 intergenic region with *gfp* expression under the control of P3	44
pCL55$_{\text{agr IR P2-GFP}}$	pCL55 A^r Cm^r containing the agr P2-P3 intergenic region with *gfp* expression under the control of P2	44

decreased cytolysin (hemolysin) expression exhibited by SCVs (33–36). However, the regulation of these two key virulence factors in SCVs is unclear (20). Both of these phenotypic properties might conceivably be explained by the significantly reduced Agr activity in SCVs (6, 37). Alternatively, there is overwhelming evidence that the metabolic state of bacteria, which is dramatically altered in SCVs relative to the wild type, can have dramatic effects on virulence factor production (38–40). Therefore, it is possible that the loss of the electron transport chain and associated shift in the metabolic profile, rather than reduced Agr activity, are responsible for the altered virulence factor profile of SCVs. Indeed, Vaudaux et al. provided evidence that the enhanced expression of fibronectin-binding protein in SCVs was *agr* independent (36), while Proctor et al. showed that chemical inhibition of the electron transport chain abrogated hemolysin production (41).

Therefore, the aim of this study was to determine whether the virulence factor profile of electron transport chain-defective *S. aureus* is due to reduced Agr activity or the loss of a functional electron transport chain.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* SH1000 was chosen for the study because it has intact *agr* and *sigB* regulatory elements (42). USA300 was also employed to demonstrate that key phenotypic effects of loss of a functional electron transport chain occur in a clinically relevant strain (43). *S. aureus* was cultured either in 3 ml tryptic soy broth (TSB) in 30-ml universal tubes at 37°C with orbital shaking (180 rpm) for phenotypic analyses or in 200 μl TSB in microtiter plates for growth and Agr activity analyses (see below) (44). In some assays, *S. aureus* was grown in the presence of 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) (10 μg ml^{-1}; Santa-Cruz Biotechnology) (45); synthetic type 1 AIP, as described previously (46) (1 to 10 μM; Peptide Synthetics); hemin (1 μg ml^{-1}; Sigma-Aldrich); or menadione (1 μg ml^{-1}; Sigma-Aldrich). The concentration of synthetic AIP (sAIP) used was based on previous measurements of the concentration of active (unoxidized) AIP (~10 μM) in stationary-phase *S. aureus* culture supernatants (46). Where appropriate, the following antibiotics were included in the medium: chloramphenicol (10 μg ml^{-1}) and erythromycin (10 μg ml^{-1}).

Construction of strains. SH1000 *hemB*::Tn and SH1001 (Δ*agr* mutant) *hemB*::Tn were produced via transduction of DNA from USA300 *hemB*::Tn using Φ11 and selection for erythromycin resistance. Transductants had a classic SCV phenotype, including slow growth, small colonies on TS agar, lack of pigmentation, and resistance to gentamicin (see Fig. S1 in the supplemental material) (45). A wild-type phenotype was restored upon supplementation of the culture medium with hemin (see Fig. S1 in the supplemental material) (reference 45 and data not shown). USA300 *hemB* and *menD* mutations were generated using pIMAY (47), resulting in deletion of nucleotides 159 to 901 from *hemB* and 72 to 1554 from *menD*. Mutations were confirmed by DNA sequencing. Both the *hemB* and *menD* mutants displayed a typical SCV appearance, as described previously (27, 29, 48, 49) (see Fig. S1 in the supplemental material).

Reporter constructs, consisting of the *agr* P2-P3 intergenic region fused with *gfp* under the control of either the P2 or P3 promoter, in pCL55 were passaged through *Escherichia coli* DC10B and transformed directly into *S. aureus* strains by electroporation (44). Transformants were selected for by using chloramphenicol, and insertion of the construct into the *geh* locus was confirmed by PCR (44).

Growth and Agr expression reporter assays. Growth and Agr expression reporter assays were performed as described previously (44). Briefly, stationary-phase cultures were diluted 1:50 in a final volume of 200 μl TSB (with or without HQNO, hemin, or sAIP, as described above) and added to the wells of black microtiter plates with clear bottoms (Corning). The plates were incubated at 37°C with shaking (500 rpm) in a Polarstar Omega multiwell plate reader. Bacterial growth was measured via optical

density at 600 nm (OD_{600}) readings, and green fluorescent protein (GFP) expression from the P2 or P3 promoter was measured as relative fluorescence units (excitation filter, 485 nm; emission filter, 520 nm) simultaneously every 30 min for a total of 17 h (44).

Real-time PCR analysis of RNAII and RNAIII transcripts. RNA extraction and real-time (RT) PCR were performed as described previously (44). Briefly, *S. aureus* SH1000 wild type or *hemB*::Tn was grown to either mid-logarithmic or stationary phase, samples were taken, and bacterial cells were lysed using lysostaphin and SDS. RNA was stabilized using TRIzol (Life Technologies), purified using a Qiagen RNeasy kit, and used to generate cDNA with a Qiagen Omniscript reverse transcription kit and random hexanucleotides (Promega). The reaction conditions and details of primers for the amplification of *hld*, *agrA*, and *gyrA* have been described previously (44).

Fibronectin binding. Bacterial attachment to immobilized human plasma fibronectin was determined as described previously (50, 51). Briefly, fibronectin (1 μg $well^{-1}$) was immobilized onto plastic Nunc Maxisorp Immuno modules in PBS at 4°C for 16 h. The remaining binding sites were blocked with 300 μl 3% (wt/vol) bovine serum albumin. Bacteria were cultured in 3 ml TSB to stationary phase, washed twice in PBS, and adjusted to equivalent densities in PBS (~10^8 ml^{-1}) before 100 μl of each suspension was added to wells and incubated statically for 1 h at 37°C. The wells were then washed thrice with PBS before adherent bacteria were fixed with 0.25% (wt/vol) paraformaldehyde and stained with crystal violet (0.5% [wt/vol]) for 1 min. Excess dye was removed by 3 rounds of PBS washing, and the bound dye was solubilized with 7% (vol/vol) acetic acid (100 μl). Solubilized crystal violet was quantified by absorbance (A_{595}) measurement using a microplate reader, and the values were converted to bacterial numbers by the use of standard plots (52).

Determination of hemolytic activity. Hemolysis of sheep erythrocytes was employed as a measure of hemolytic activity, as these cells can be lysed by at least two major *S. aureus* hemolysins (alpha and delta toxins) (53). The hemolytic activity of culture supernatants was determined essentially as described previously (54). Bacteria were removed from spent culture medium by centrifugation (13,000 × *g*; 10 min), and the supernatant was diluted in 2-fold steps using fresh TSB. Each dilution (100 μl) was mixed with a 2% sheep blood suspension in PBS and incubated statically at 37°C for 1 h. Unlysed blood cells were removed by centrifugation, and the supernatant was transferred to the wells of a new microtiter plate. Erythrocyte lysis was determined by measuring the A_{450} of the supernatant (54). Erythrocytes incubated with fresh TSB or TSB containing 1% TX-100 served as negative and positive controls, respectively. All values were related to the A_{450} reading of undiluted supernatant from wild-type cultures (i.e., represented as relative percent hemolysis). For experiments using sAIP, TSB (1 ml) was inoculated with ~2×10^9 CFU *S. aureus* and incubated at 37°C with shaking in the absence or presence of HQNO and/or sAIP (10 μM). This ensured that all cultures had sufficient numbers of bacterial cells to reasonably expect detection of hemolytic activity.

Determination of fibronectin-binding protein expression. Expression of fibronectin-binding proteins was determined by blot overlay of total protein extracts using human plasma fibronectin (Sigma). Wild-type *S. aureus* SH1000 bacteria grown in the absence or presence of HQNO, *hemB*::Tn, or SH1001 were grown in the absence or presence of 10 μM sAIP for 16 h. The bacteria were washed three times by alternate rounds of centrifugation and resuspension in PBS before adjustment to equivalent cell numbers via OD_{600} measurements. Approximately 10^9 CFU of each strain from each set of growth conditions was lysed with lysostaphin. Insoluble material was removed by centrifugation, and soluble protein was separated by SDS-PAGE. The separated proteins were blotted onto nitrocellulose membranes, and the remaining protein binding sites were blocked with 5% skim milk powder in Tris-buffered saline (TBS) as described previously (44). Fibronectin-binding proteins A and B were detected by overlaying blots with human plasma fibronectin (5 μg ml^{-1} in TBS). The blots were then washed three times with TBS-Tween (0.1%) before detection of bound fibronectin using an anti-human fibronectin polyclonal antiserum raised in rabbit (Sigma), followed by horseradish peroxidase-conjugated antibodies against rabbit IgG. Bound antibody was detected using enhanced chemiluminescence (ECL) detection as described previously (44).

RESULTS

Disruption of the electron transport chain inhibits growth and reduces Agr activity. Previous work has shown that the *Pseudomonas* exoproduct HQNO blocks the electron transport chain of wild-type *S. aureus* and confers an SCV-like phenotype, including slow growth and aminoglycoside resistance (45). Therefore, this provided an ideal tool to delineate the relative contributions of electron transport chain deficiency and lack of Agr activity to the virulence phenotype of SCVs.

Growth of wild-type *S. aureus* SH1000 in the presence of HQNO significantly reduced both the growth rate and final density relative to the wild type grown in the absence of HQNO. In comparison, the SH1000 *hemB*::Tn mutant had an even lower rate of growth, but the final density was identical to that of the wild type grown with HQNO (Fig. 1A). Similarly, USA300 mutants lacking *hemB* or *menD* had significantly reduced growth rates and final densities in stationary phase (Fig. 1D).

We then employed a fluorescent reporter construct to measure expression from the P3 promoter of the Agr operon during bacterial growth (Fig. 1B and E) (44). Because the strength of the fluorescent signal is affected by the total number of bacteria present, we accounted for differences in culture densities between strains and culture conditions by plotting fluorescence over the OD_{600} readings (Fig. 1C and F) (44). As reported previously, P3 expression in both wild-type *S. aureus* strains increased rapidly as the culture entered stationary phase (Fig. 1B and E) (44). In contrast, P3 activity in the *hemB*::Tn (SH1000) and Δ*hemB* (USA300) mutants was barely detectable (Fig. 1B and E). Similar results were seen for the Δ*menD* mutant (USA300) (Fig. 1E). P3 activity in wild-type SH1000 cultures exposed to HQNO were also very low, although slightly greater than that seen in *hemB*::Tn mutant cultures (Fig. 1B and C). The very low level of P3 activity in mutants and bacteria grown with HQNO was not simply an artifact of the reduced cell density, as shown in Fig. 1C and F. Therefore, even allowing for differences in bacterial cell density (Fig. 1C and F), Agr activity is reduced in response to loss of a functional electron transport chain, regardless of whether this occurs chemically (HQNO) or genetically (*hemB* and *menD*).

To ensure that reduced fluorescence was not simply a consequence of the impaired metabolic status associated with loss of the electron transport chain, we measured RNAIII (*hld*) transcript by RT-PCR. This demonstrated significantly lower levels of RNAIII transcript in the SH1000 *hemB*::Tn mutant than in the wild type (see Fig. S2 in the supplemental material). In addition, and in keeping with our previous findings (44), compared with *agr*-defective strains that harbored reporter constructs but did not express GFP, we could not detect a GFP-induced growth defect in our reporter strains (see Fig. S3 in the supplemental material).

To determine whether the Agr activity data in Fig. 1 correlated with the virulence factor phenotype of each strain, we measured both fibronectin binding and hemolytic activity. In SH1000, as expected from the P3 expression data, the *hemB*::Tn mutant and the wild-type *S. aureus* bacteria grown in the presence of HQNO bound strongly to fibronectin-coated plastic, attaching at levels similar to those of an *agr*-defective mutant (Fig. 2A). In contrast,

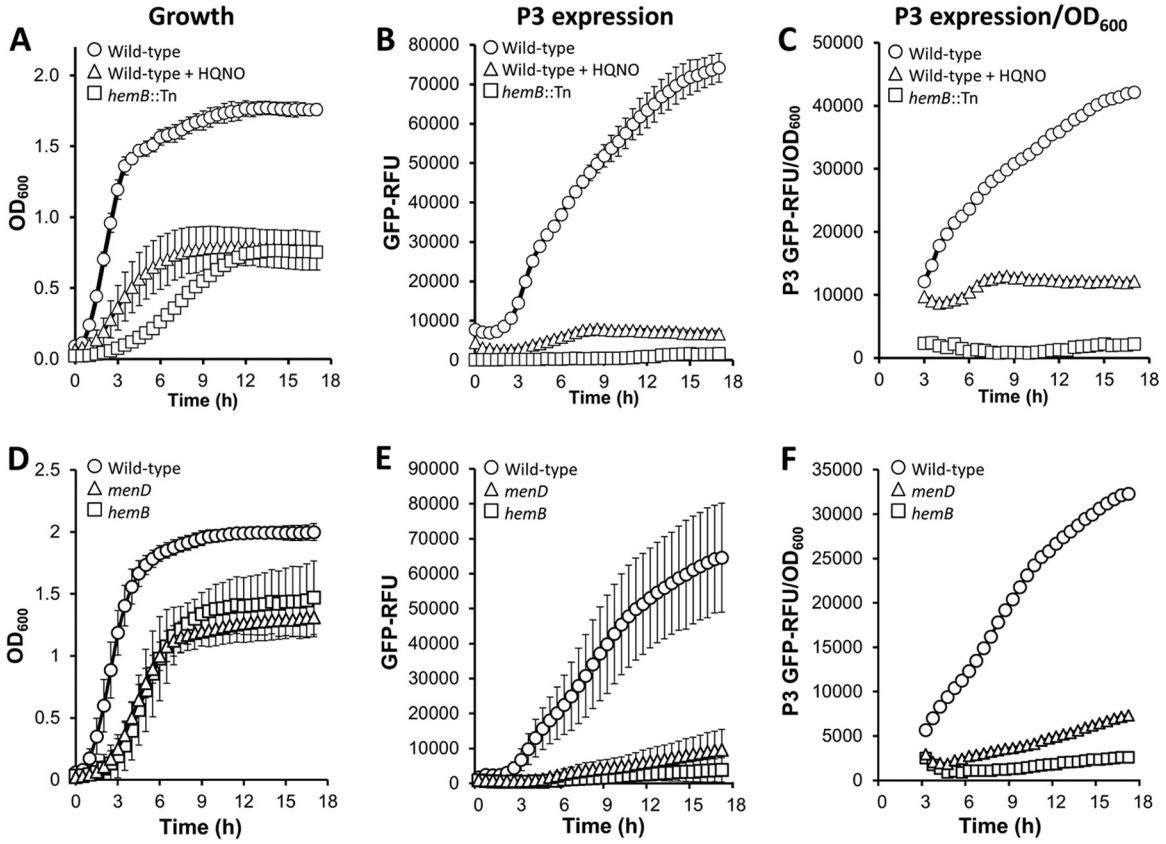

FIG 1 Loss of the electron transport chain reduces growth and Agr expression. Shown are growth (A and D), P3 expression (B and E), and P3 expression corrected for growth (C and F) of *S. aureus* SH1000 (A to C) and USA300 (D to F) and derived strains. (A to C) SH1000 grown in the absence or presence of HQNO and a *hemB*::Tn mutant in TSB as determined by the OD_{600} (growth) and A_{520} (GFP fluorescence) measurements. (D to F) USA300 wild type, Δ*hemB*, and Δ*menD* grown in TSB. The measurements are as described for panels A to C. The data represent the means of at least 4 independent experiments, each in triplicate. The error bars represent the standard deviations of the mean. RFU, relative fluorescence units.

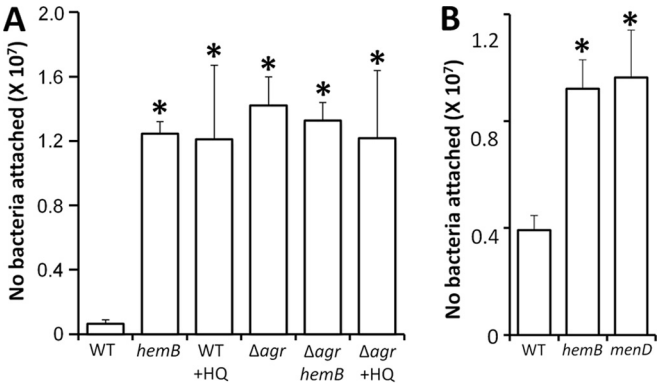

FIG 2 Loss of the electron transport chain results in enhanced fibronectin binding. (A) Attachment of wild-type (WT) *S. aureus* SH1000 grown in the absence or presence (+HQ) of HQNO and a *hemB*::Tn mutant (*hemB*) to immobilized human fibronectin. Also shown are values relating to the attachment of *agr*-defective SH1001 (Δ*agr*) grown in the absence or presence of HQNO and of a *hemB* Δ*agr* strain. (B) Attachment of WT USA300 and *hemB* and *menD* mutants. The values represent the mean averages of at least 4 experiments done in triplicate, and the error bars represent the standard deviations of the mean. Values that are significantly greater ($P = 0.05$ by Student's *t* test) than that of the wild type are indicated by asterisks.

wild-type SH1000 grown in the absence of HQNO bound fibronectin weakly (Fig. 2A). Similar results were seen with USA300 strains, with the *hemB* and *menD* mutants attaching to fibronectin-coated wells at significantly higher levels than the wild type (Fig. 2B).

In contrast to the adhesion assay, strong hemolytic activity was detected in culture supernatant from wild-type *S. aureus* SH1000 grown in the absence of HQNO but was barely detectable in spent medium from the *hemB*::Tn mutant or the wild type grown with HQNO (Fig. 3A). Similarly, wild-type USA300 had high hemolytic activity while very weak activity was seen in the culture supernatants of *hemB* and *menD* mutants (Fig. 3B). Therefore, both the strong fibronectin binding and weak hemolytic activity of bacteria without functional electron transport chains were consistent with the Agr activity data (6, 9, 10, 14, 20, 36).

sAIP activates Agr expression in the absence of the electron transport chain. If Agr alone controls virulence factor expression in the absence of the electron transport chain, then modulation of Agr activity should influence both fibronectin binding and hemolytic activity. To determine whether electron transport-deficient *S. aureus* could respond to AIP, we added sAIP to the culture medium of *S. aureus* SH1000 *hemB*::Tn or the wild type in the absence or presence of HQNO and measured both growth and expression from the P3 promoter of Agr. In the wild-type grown without

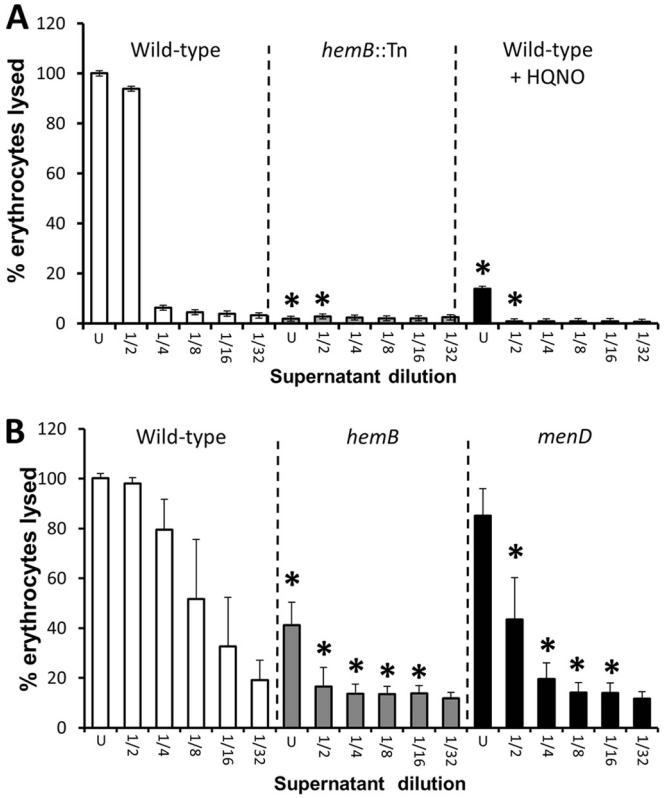

FIG 3 Loss of the electron transport chain results in significantly reduced hemolytic activity. Shown are hemolytic activities of culture supernatants of wild-type *S. aureus* SH1000 and derived strains (A) and USA300 and derived mutants (B). Culture supernatants were used undiluted (U) or after serial 2-fold dilutions, as indicated. The data represent the means of at least 4 independent experiments, each in triplicate. The error bars represent the standard deviations of the mean. Values that are significantly different ($P < 0.05$ by Student's *t* test) from those of the wild type at equivalent dilutions are indicated by asterisks.

HQNO, AIP caused a slight, dose-dependent reduction in bacterial density (but not the growth rate) in stationary phase and, as a consequence, also a slight decrease in P3 expression levels (Fig. 4A and B). However, when fluorescence was related to the OD_{600}, readings confirmed that the overall fluorescence per unit of biomass was unchanged in the wild type by the addition of sAIP (Fig. 4B and C). This suggests that expression from P3 is maximal in wild-type *S. aureus* and that exogenous sAIP cannot enhance it without adversely affecting growth. Synthetic AIP caused a much more significant decrease in the growth of the *hemB*::Tn mutant. Notably, the 1 μM and 10 μM doses caused identical inhibition of the *hemB*::Tn growth rate and final density in stationary phase (Fig. 4A). Exogenous sAIP triggered Agr expression in the *hemB*::Tn mutant, and when corrected for differences in bacterial density (OD_{600}), Agr expression in the presence of sAIP was similar to that seen in the wild type (Fig. 4B and C).

In contrast, exogenous sAIP did not significantly affect the growth of *S. aureus* in the presence of HQNO. This is explained by P3 reporter data, which show that while sAIP promoted Agr activity, it was not to levels seen in the wild type or the *hemB*::Tn mutant when adjusted for OD_{600} measurements (Fig. 4C). Therefore, while the *hemB*::Tn mutant is extremely sensitive to the presence of sAIP, *S. aureus* grown in the presence of HQNO does not respond as strongly.

The effect of sAIP on wild-type USA300 was similar to that seen with SH1000, with the quorum-sensing molecule causing a slight growth defect but no significant alteration in P3 activity (Fig. 4D, E, and F). Growth of USA300 *hemB* was not significantly affected by sAIP at 10 μM despite P3 activity increasing significantly, although not to the same levels as seen in SH1000 *hemB*::Tn (Fig. 4D, E, and F).

To investigate the apparently high degree of sensitivity of the SH1000 *hemB*::Tn mutant to sAIP, we determined the expression of the RNAII transcript, which encodes the quorum-sensing circuit. Analysis of expression from P2 in the *hemB*::Tn mutant using a *gfp* reporter construct revealed very low levels of activity (see Fig. S4 in the supplemental material). These findings were confirmed by RT-PCR analysis, which showed that *agrA* expression in the *hemB*::Tn strain was significantly lower than that of the wild type in exponential phase (see Fig. S4 in the supplemental material). Therefore, the potent response of the *hemB*::Tn mutant to sAIP is not due to elevated RNAII and thus to expression of the AgrC sensor kinase that detects AIP.

Finally, to ensure that sAIP-mediated growth inhibition was solely due to activation of Agr expression, rather than any off-target effects of sAIP, we replicated this experiment with strains lacking *agr*. No inhibition of growth was seen for any of the *agr*-defective strains when grown with sAIP (see Fig. S5 in the supplemental material).

Exogenous sAIP results in reduced binding of electron transport chain-defective *S. aureus* to fibronectin. To determine whether Agr was responsible for the regulation of fibronectin binding in the absence of the electron transport chain, wild-type *S. aureus* grown with or without HQNO and the *hemB*::Tn mutant were cultured with or without sAIP, and their abilities to bind to fibronectin were determined. As described above, attachment of wild-type *S. aureus* to fibronectin was weak in the absence of sAIP, while bacteria grown in the presence of sAIP exhibited even weaker binding (Fig. 5A). The addition of sAIP to the culture media of both the *hemB*::Tn mutant and the wild type grown with HQNO led to a significant, dose-dependent decrease in fibronectin binding to levels similar to those seen for the wild type (Fig. 5A). To ensure that sAIP itself did not directly inhibit adhesion to fibronectin, these experiments were repeated with *agr*-negative mutants. All Agr-defective strains bound fibronectin strongly, and this was not affected by the presence of sAIP, ruling out a direct inhibitory effect (Fig. 5A). In addition, we examined the expression levels of fibronectin-binding proteins A and B in bacteria grown in the absence or presence of sAIP using a blot overlay approach. In keeping with the adhesion data, wild-type *S. aureus* SH1000 grown in either the absence or presence of sAIP had very weak expression of FnBPs (see Fig. S6 in the supplemental material). Both the *hemB*::Tn mutant and the wild type grown in the presence of HQNO had very high levels of FnBP expression, which was dramatically reduced in bacteria grown with sAIP (see Fig. S6 in the supplemental material). Finally, an *agr* mutant had moderately high levels of fibronectin-binding protein, which was unaffected by the presence of sAIP in the culture medium. Therefore, FnBP expression in the absence of the electron transport chain is regulated by Agr. However, additional regulators may enhance FnBP expression in the absence of an electron transport chain beyond that seen in an *agr* mutant with a competent electron

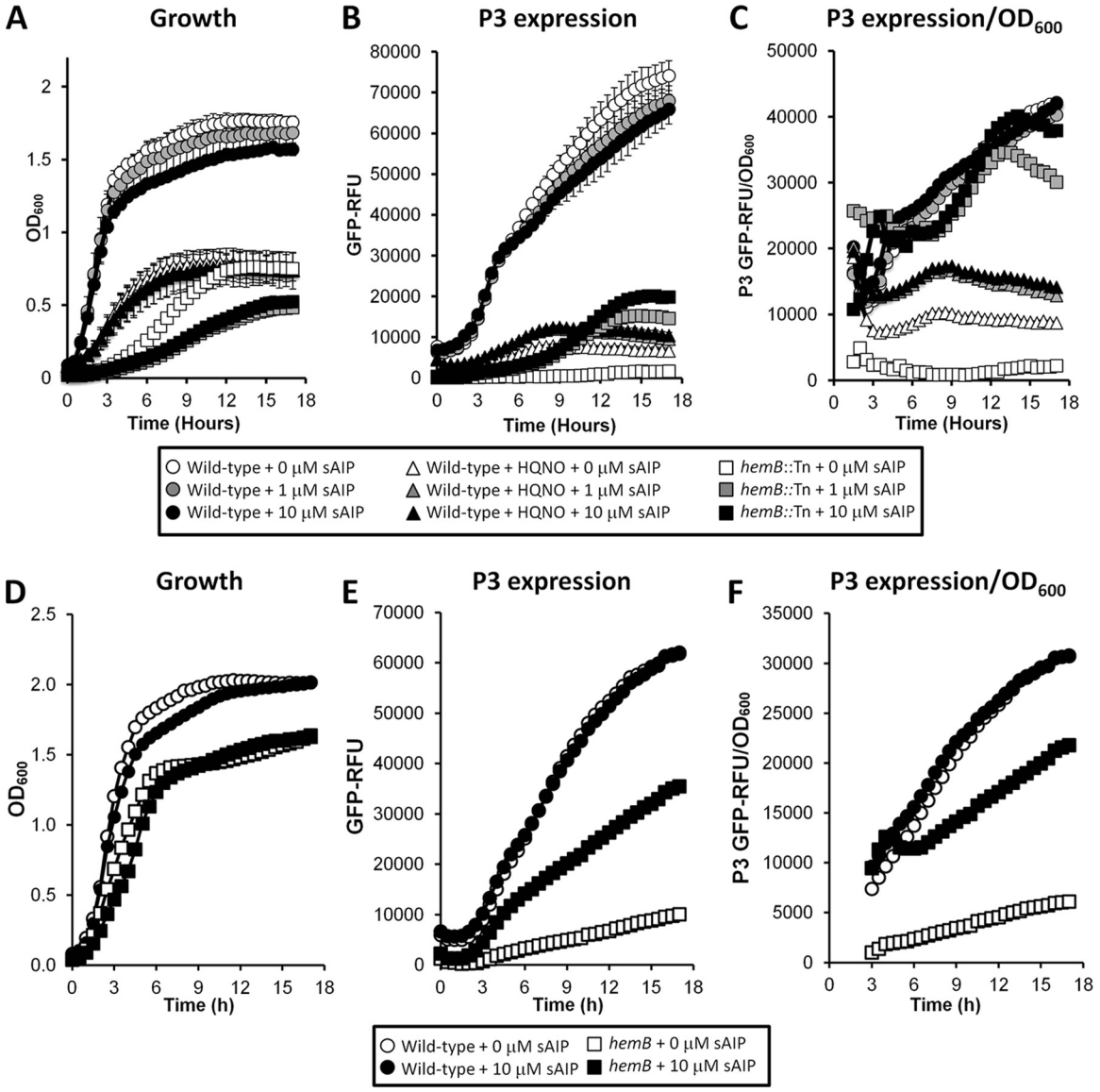

FIG 4 Synthetic AIP enhances *agr* expression in the absence of a functional electron transport chain. Shown are growth (A and D), P3 expression (B and E), and P3 expression corrected for growth (C and F) of SH1000 and derived strains (A to C) and USA300 and derived strains (D to F). (A to C) Wild-type *S. aureus* grown in the absence or presence of HQNO and that of a *hemB*::Tn mutant in TSB only or TSB supplemented with 1 μM or 10 μM sAIP. (D to F) Wild-type *S. aureus* and a *hemB* mutant grown in the absence or presence of 10 μM sAIP. The data represent the means of at least 4 independent experiments, each in triplicate. The error bars were omitted to enhance clarity but were all within 5% of the mean.

transport chain. In addition, protein A expression varied identically to FnBP expression, demonstrating that this immune evasin is also subject to regulation by Agr in the absence of a functional electron transport chain (see Fig. S6 in the supplemental material).

Finally, fibronectin-binding results similar to those of SH1000 were seen with USA300, with attachment of both the wild-type and *hemB* strains to fibronectin being significantly reduced after growth in the presence of sAIP (Fig. 5B).

Taken together, these data demonstrate that fibronectin binding is under the control of Agr in *S. aureus* in both the presence and absence of a functional electron transport chain.

Exogenous sAIP does not trigger hemolysin production in *S. aureus* in the absence of the electron transport chain. Next, we sought to determine whether reduced Agr expression was also directly responsible for the lack of hemolysin production in the absence of the electron transport chain. Initially we examined the hemolytic activity of culture supernatants from bacteria grown in the presence or absence of sAIP at 10 μM, which triggered maximal P3 activity in all strains (Fig. 4). In contrast to the fibronectin-binding assays shown in Fig. 5, the addition of sAIP to cultures of wild-type SH1000 in either the absence or presence of HQNO had no significant effect on hemolytic activity (Fig. 6A). Similarly, sAIP had no effect on the hemolytic activity of SH1000 *hemB*::Tn (Fig. 6A). In keeping with these findings, the hemolytic activity of both wild-type USA300 and the *hemB* mutant was unaffected by growth in the presence of sAIP at 10 μM (Fig. 6B).

Because the level of hemolytic activity in culture supernatants can be affected by the density of cells in the culture, this was ad-

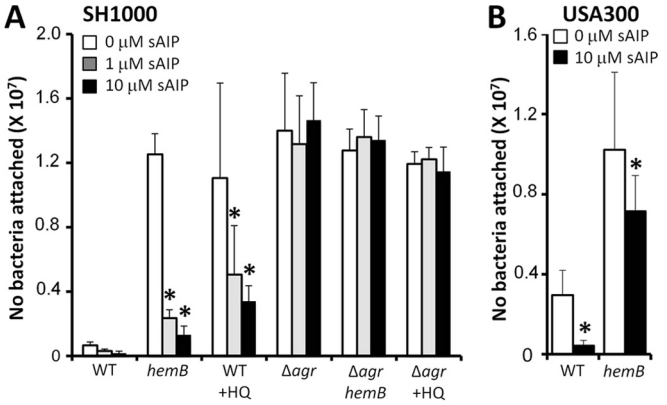

FIG 5 Agr regulates fibronectin binding in both the presence and absence of the electron transport chain. (A) Fibronectin binding of wild-type *S. aureus* SH1000 grown in the absence (WT) or presence (WT + HQ) of HQNO and a *hemB*::Tn mutant (*hemB*). Also shown are the fibronectin-binding levels of *agr*-deficient mutants grown in the absence (Δ*agr*) or presence (Δ*agr* + HQ) of HQNO or in the *hemB*::Tn mutant background (Δ*agr hemB*). The culture medium consisted of TSB only or TSB supplemented with 1 μM sAIP or 10 μM sAIP. (B) Fibronectin binding of wild-type *S. aureus* USA300 and a *hemB* mutant grown in the absence or presence of 10 μM sAIP. Experiments were repeated 4 times in triplicate. Values that are significantly different ($P < 0.05$ by Student's *t* test) from those seen in the absence of sAIP are indicated by asterisks.

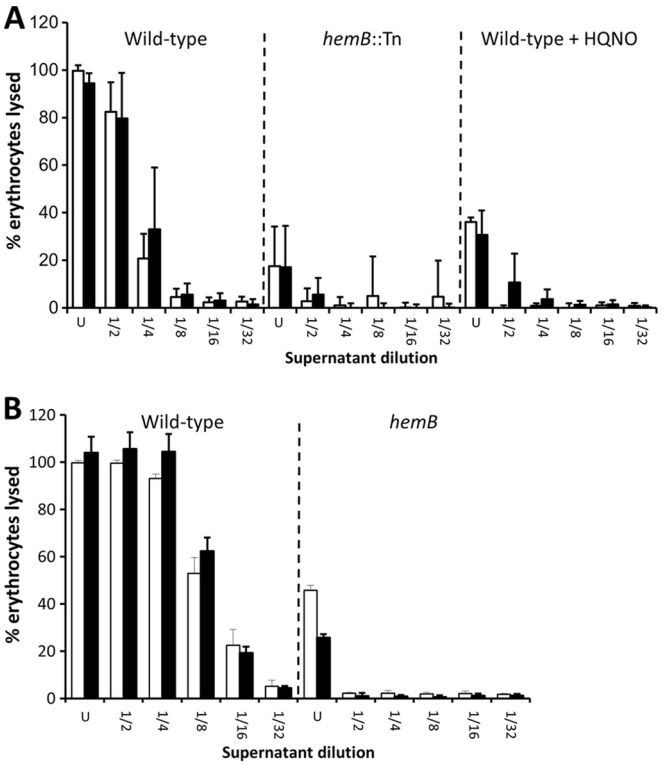

FIG 6 Agr activation by sAIP does not promote hemolytic activity in the absence of a functional electron transport chain. (A) Hemolytic activities of culture supernatants of wild-type SH1000 grown in the absence or presence of HQNO and of a *hemB*::Tn mutant in the absence (open bars) or presence (filled bars) of 10 μM sAIP. (B) Hemolytic activities of culture supernatants of wild-type USA300 and a *hemB* mutant in the absence (open bars) or presence (filled bars) of 10 μM sAIP. The data represent the mean averages of 4 experiments performed in triplicate, and the error bars represent the standard deviations of the mean. None of the values obtained from cultures containing sAIP were significantly different from those without sAIP at identical dilutions.

dressed by adjustment of all strains to ~2×10^9 CFU ml^{-1} in TSB, with or without HQNO or sAIP, and incubation for 16 h at 37°C. The addition of sAIP to cultures of wild-type *S. aureus* had no effect on the already strong hemolytic activity, suggesting that native AIP levels trigger maximal hemolysin production (Fig. 7). In contrast, neither the *hemB*::Tn mutant nor wild-type *S. aureus* cultured with HQNO produced significantly higher levels of hemolytic activity in the presence of sAIP, despite increased Agr expression and high bacterial densities (Fig. 4B and 7).

Restoration of the electron transport chain in the *hemB* or *menD* mutant via supplementation of the growth medium with hemin or menadione almost completely restored the growth phenotype and partially restored Agr activity (Fig. 8). However, despite the incomplete restoration of Agr activity, hemolytic activity was completely restored (Fig. 9A and B). Therefore, hemolysin production does not require maximal Agr activity but does require a functional electron transport chain.

DISCUSSION

During the course of infection, *S. aureus* frequently acquires mutations that affect the expression of virulence factors (55–59). However, the mechanisms by which these mutations influence the expression of specific virulence factors are not always clear. This is particularly true for small-colony variants, where mutations in biosynthetic-pathway genes result in disruption of the electron transport chain and loss of Agr activity (25–29).

In this report, we dissected the relative contributions of electron transport chain deficiency and reduced Agr expression to both fibronectin binding and hemolysis. The elevated fibronectin binding of electron transport chain-deficient bacteria was directly due to reduced Agr expression. In contrast, reduced hemolysin production was not simply a consequence of low Agr activity, and we therefore conclude that hemolysin production requires a functional electron transport chain, in addition to Agr expression.

The data in Fig. 4 indicate that Agr expression and growth are finely balanced in wild-type bacteria to maximize both growth and hemolysin production; exogenous sAIP caused a growth defect without increasing hemolytic activity. The sAIP-induced growth defect was even more pronounced in the *hemB*::Tn mutant, indicating that *S. aureus* lacking a functional electron transport chain can sense and respond to AIP despite low levels of RNAII transcript. Although sAIP enhanced Agr activation in *S. aureus* grown in the presence of HQNO, the effect on growth was minor compared to that in the *hemB*::Tn mutant. This may conceivably be due to interference between HQNO and sAIP, but we were unable to test this.

The mechanism(s) by which *S. aureus* detects and allocates resources to growth versus virulence factor production is unclear. However, in recent years, a number of gene products have been described which respond to metabolic signals and regulate virulence factor production accordingly (e.g., Rsh, CodY, CcpA, and SrrAB) (39, 40, 59–65). Of particular relevance, SrrAB has been shown to directly monitor electron transport chain activity and is capable of modulating both growth and the expression of several virulence factors 59, 60, 65). In addition, previous work has shown that the lack of Agr expression in electron transport chain-defi-

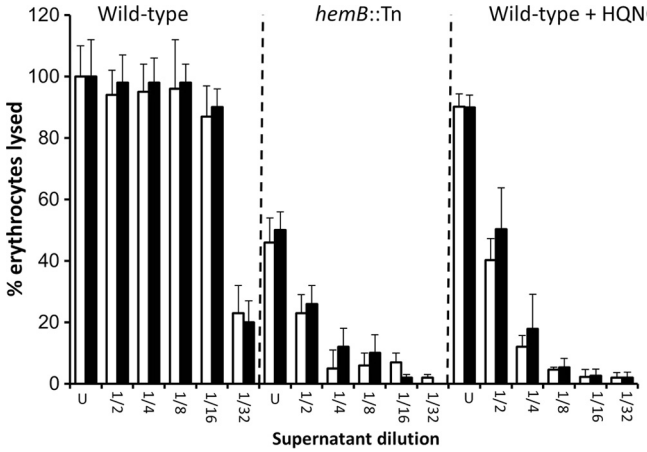

FIG 7 Agr activation by sAIP does not promote hemolytic activity in concentrated cultures in the absence of a functional electron transport chain. Wild-type SH1000 and a *hemB*::Tn mutant were concentrated to equivalent bacterial densities and cultured in TSB in the absence or presence of HQNO and the absence (open bars) or presence (filled bars) of 10 μM sAIP. The data represent the mean averages of 4 experiments performed in triplicate, and the error bars represent the standard deviations of the mean. None of the values obtained from cultures containing sAIP were significantly different from those without sAIP at identical dilutions.

cient SCVs is, at least in part, due to suppression by the alternative sigma factor SigB, which exhibits constitutive activity during all growth phases (66, 67). In a *sigB* mutant defective in menaquinone biosynthesis, Agr activity was significantly elevated, providing convincing evidence that regulation by SigB, rather than low growth density, is responsible for decreased activity of Agr in bacteria lacking the electron transport chain (67). Furthermore, the elevated SigB activity in SCVs may also explain the particularly high level of fibronectin-binding proteins seen in *S. aureus* lacking functional electron transport chains, above that seen in an *agr*-deficient mutant (see Fig. S6 in the supplemental material) (67).

However, our data demonstrate that this SigB-mediated suppression of Agr expression in SCVs can be overcome by exogenous sAIP, and this may have important implications for virulence factor expression *in vivo*. During infection, electron transport chain-deficient SCVs emerge from wild-type populations, and SCVs and wild-type bacteria are often coisolated from the same infection site (14–16, 18, 20–22, 24). This makes it likely that, *in vivo*, SCVs are exposed to AIP generated by wild-type *S. aureus*. Therefore, the phenotype of electron transport chain-deficient SCVs in the presence of wild-type bacteria will be very different from those observed in SCV monocultures, at least with respect to surface protein expression.

The role of SigB in shaping the phenotype of *S. aureus* lacking

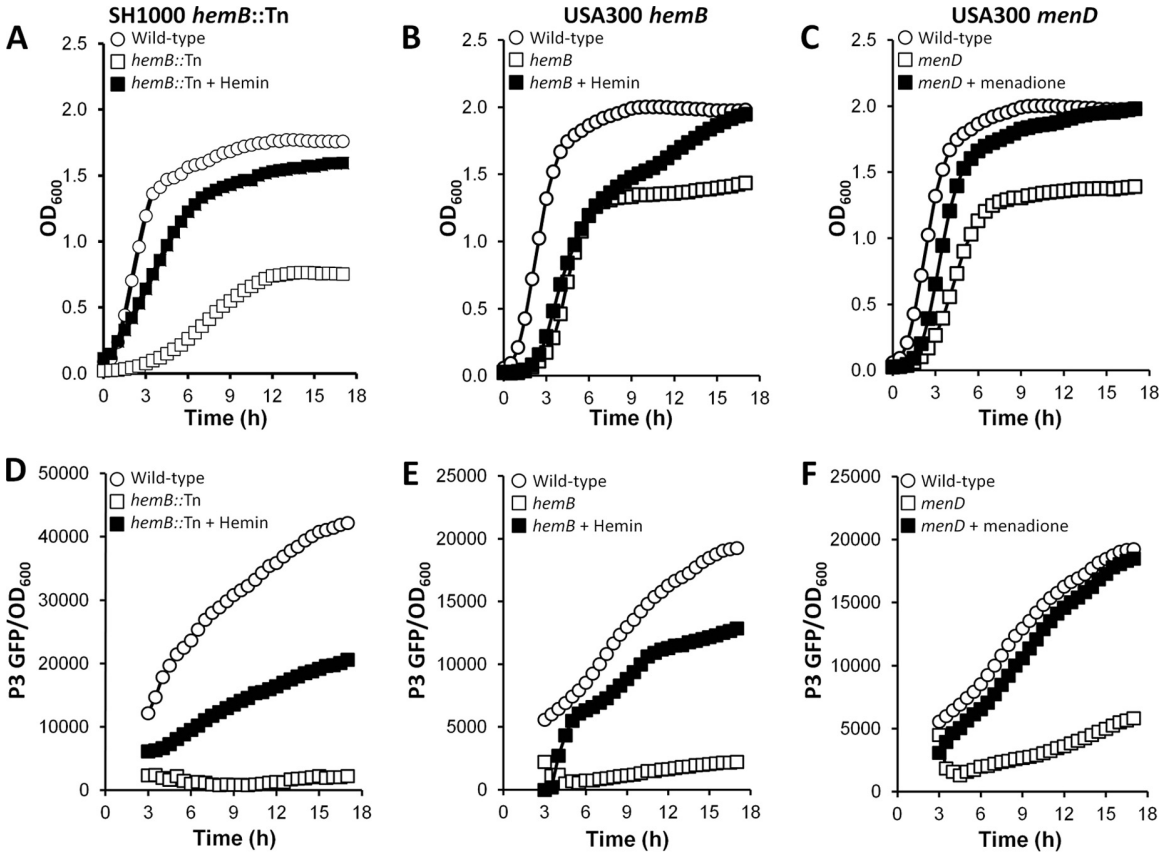

FIG 8 Hemin and menadione restore growth and Agr activity in *hemB* and *menD* mutants, respectively. (A, B, D, and E) SH1000 *hemB*::Tn (A and D) and USA300 *hemB* (B and E) were grown in the absence or presence of 1 μg ml^{-1} hemin, and growth (A and B) was measured via OD_{600} readings, while P3 activity (corrected for OD_{600} values) was measured to determine Agr activity (D and E). (C and F) USA300 *menD* was grown in the absence or presence of 1 μg ml^{-1} menadione, and growth (C) and P3 activity (F) were measured as described for the *hemB* mutants. The data represent the means of at least 4 independent experiments, each in triplicate. The error bars were omitted to enhance clarity but were all within 5% of the mean.

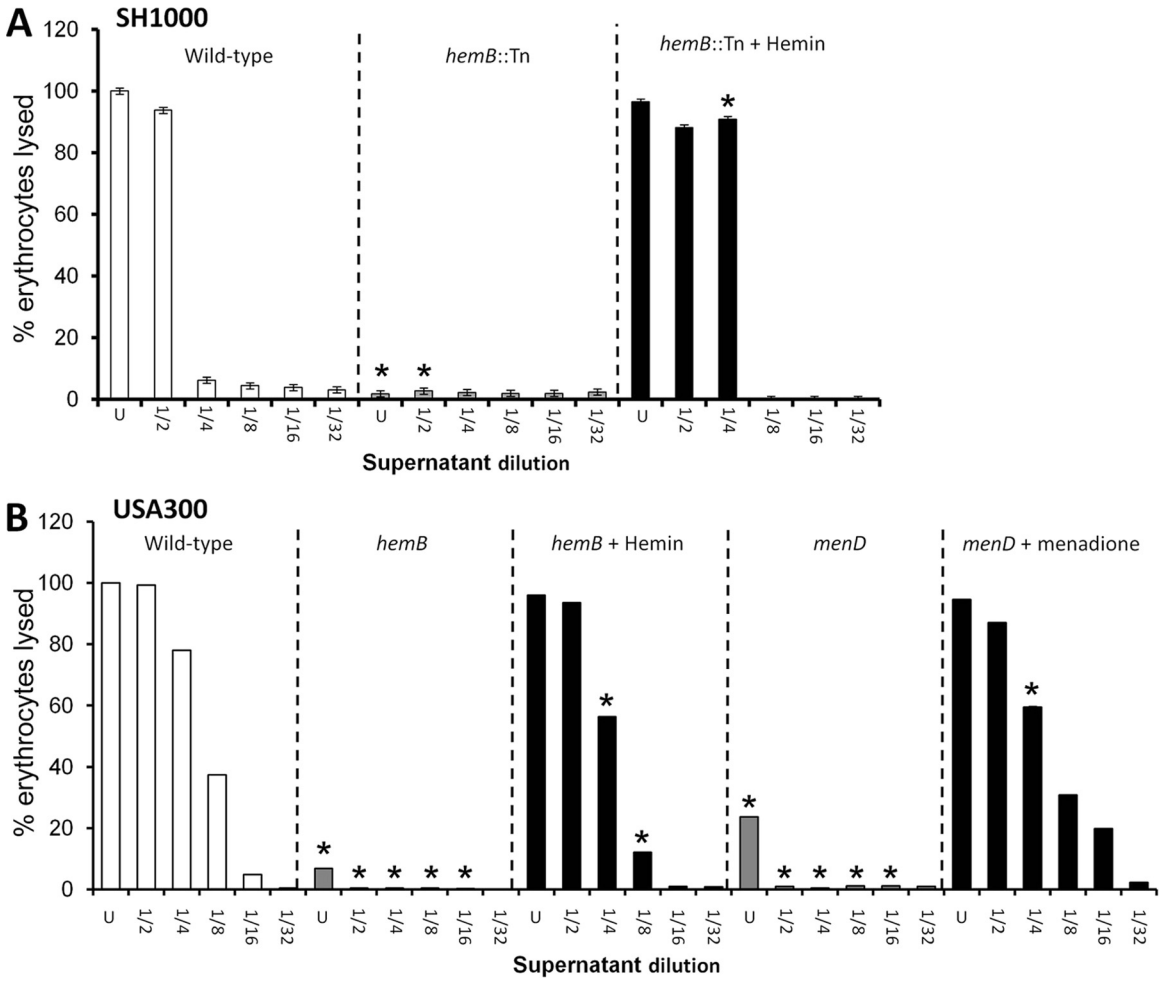

FIG 9 Hemin and menadione restore hemolytic activity in *hemB* and *menD* mutants, respectively. (A) SH1000 *hemB*::Tn was grown in the absence or presence of 1 μg ml^{-1} hemin, and the hemolytic activity of the culture supernatant was determined. Culture supernatant from wild-type SH1000 is included for comparison. (B) USA300 *hemB* and *menD* were grown in the absence or presence of 1 μg ml^{-1} hemin or menadione, and the hemolytic activity of the culture supernatant was determined. Culture supernatant from wild-type USA300 is included for comparison. The data represent the means of 4 experiments done in triplicate, and the error bars represent the standard deviations of the mean. Values that are significantly different ($P < 0.05$ by Student's *t* test) from those of the wild type at the same dilution are indicated by asterisks.

a functional electron transport chain may explain the apparent discrepancy between our data and those of Vaudaux et al. (36). The strain used by Vaudaux and coworkers, 8325, is defective in SigB production due to a mutation in *rsbU* (42). One of the strains used in this report, SH1000, is derived from 8325 but has a repaired copy of *rsbU*, restoring SigB activity (42). Furthermore, the undefined spontaneous mutant strain used by Vaudaux et al., which lacked production of RNAIII, was unusual in that it did not display enhanced *fnb* transcript or fibronectin-binding relative to the parent strain (36). This is at odds with studies using defined *agr*-deficient mutants, including in the 8325 background, which show significantly enhanced fibronectin binding, and suggests the possibility of additional mutations or pleiotropic effects (68–70). Therefore, the data presented in this report contribute to our growing understanding of the link between metabolism and the regulation of specific virulence factors by providing compelling evidence that decreased Agr expression is directly responsible for elevated fibronectin-binding protein expression in both electron transport chain-defective and -competent *S. aureus*. In contrast, the Agr-mediated regulation of hemolysin production is dependent upon a functional electron transport chain.

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