Golden Pigment Production and Virulence Gene Expression Are AFFECTED BY METABOLISMS IN Staphylococcus aureus

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The pathogenesis of staphylococcal infections is multifactorial. Golden pigment is an eponymous feature of the human pathogen Staphylococcus aureus that shields the microbe from oxidation-based clearance, an innate host immune response to infection. Here, we screened a collection of S. aureus transposon mutants for pigment production variants. A total of 15 previously unidentified genes were discovered. Notably, disrupting metabolic pathways such as the tricarboxylic acid cycle, purine biosynthesis, and oxidative phosphorylation yields mutants with enhanced pigmentation. The dramatic effect on pigment production seems to correlate with altered expression of virulence determinants. Microarray analysis further indicates that purine biosynthesis impacts the expression of ~400 genes involved in a broad spectrum of functions including virulence. The purine biosynthesis mutant and oxidative phosphorylation mutant strains exhibit significantly attenuated virulence in a murine abscess model of infection. Inhibition of purine biosynthesis with a known small-molecule inhibitor results in altered virulence gene expression and virulence attenuation during infection. Taken together, these results suggest an intimate link between metabolic processes and virulence gene expression in S. aureus. This study also establishes the importance of purine biosynthesis and oxidative phosphorylation for in vivo survival.

Staphylococcus aureus causes a variety of infections in humans, ranging from minor skin and wound infections to life-threatening diseases (31). The pathogenesis of staphylococcal infections is a multifactorial process that depends on the expression of different virulence factors controlled by multiple regulatory systems in conjunction with environmental and nutritional signals (46). The high degree of variability in the expression of virulence genes is modulated by a complex network regulated by factors such as the agr locus (RNAIII), SarA, and SigB (5, 9), which allows the bacterium to adapt to changing environmental conditions for survival and developing infection.

The species epithet of S. aureus reflects its characteristic surface pigmentation (aureus, meaning “golden” in Latin) (43). The yellowish-orange (golden) pigment produced by S. aureus has been linked to virulence, owing to its antioxidant property (29, 30). The golden pigmentation of S. aureus is the product of a C30 triterpenoid carotenoid biosynthesis pathway, and the carotenoid pigment biosynthesis genes are organized in an operon crtOPQMN controlled by the alternative sigma factor SigB (3, 39). Since many virulence genes are coordinately regulated in S. aureus (5, 9, 31), we hypothesized that genes affecting pigmentation may also influence the production of virulence determinants and have an impact on the pathogenesis of S. aureus.

Herein, we present an analysis of S. aureus golden pigment biosynthesis and regulatory pathways at the genomic level by screening the Phoenix (ΦN3E) library, a collection of defined transposon insertions into 1,812 open reading frames of S. aureus strain Newman (1). This study indicates an intimate link between metabolic processes and virulence gene expression. It demonstrates the importance of purine biosynthesis and oxidative phosphorylation for in vivo survival and pathogenesis of S. aureus. Our results show that targeting purine biosynthesis is a promising strategy to develop anti-S. aureus therapies.

MATERIALS AND METHODS

Bacterial strains and chemical reagents. S. aureus strain Newman is a human clinical isolate (13) that stably maintains an agr phenotype, i.e., quorum-controlled toxin secretion and the ability to cause animal disease (1, 37). S. aureus was propagated in tryptic soy broth (TSB) (Difco) with shaking at 250 rpm or on tryptic soy agar (TSA) (Difco). The transposon mutant collection, the Phoenix library (ΦN3E), used in this study was described previously (1). Adenine (Acros Organics) was dissolved in 0.05 M HCl, and the same amount of HCl was used as a control. 6-Thioguanine (6-TG) (Sigma-Aldrich) and mycophenolic acid (MPA) (AK scientific, Inc.) were dissolved in dimethyl sulfoxide (DMSO) or 0.1 M NaOH, followed by neutralization with HCl. Amino acid requirements for growth were determined by growth with chemically defined medium (HHW, for Hussain, Hasting, and White) medium (18).

Phage transduction and determination of transposon insertion sites. To determine whether unrelated mutations gave rise to the observed pigment production phenotype, all the mutant S. aureus strains used in this study were lysed with phage 845, and transposon insertions were transduced into wild-type strain Newman. The transduced bursa aurealis insertion sites were again mapped by PCR and DNA sequencing. All experiments were performed as described previously (1).

Measurement of carotenoid pigment and thin-layer chromatography (TLC) analysis. Colonies of S. aureus Newman and its isogenic mutants were washed by methanol-acetomitrile (1:3, vol/vol).
RNA isolation and Northern blotting. Bacterial strains were grown in TSB or on TSA plates (a 20-μl overnight culture was plated) before being harvested for RNA extraction. The harvest and cell lysis follow the enzymatic lysis procedure described in the RNAprotect Bacteria Reagent Handbook (40) and with mechanical disruption (Fast Prep FP120 instrument; Qiogene, Heidelberg, Germany). An RNeasy Mini Kit (Qiagen) was used for subsequent RNA purification. To isolate the RNA for microarray analysis, all S. aureus strains were grown at 37°C overnight in TSB, diluted 200-fold in 10 ml of fresh TSB in a 50-ml conical tube (BD Biosciences), and incubated at 37°C with shaking at 250 rpm for 48 h (OD_{600} of 8.5 to 10). Two volumes of the RNAprotect Bacteria Reagent (Qiagen) were added to 1 volume of cell culture and treated at room temperature for 30 min. Total RNA was isolated by using an RNaseasy Mini Kit (Qiagen) as described above, except that cells were lysed with 0.5 mg of lysozyme (Sigma) per ml for 1 h at 37°C. RNA concentration and purity were determined by absorbency at 260 and 280 nm. Northern blotting was performed following previously reported procedures (6). Primers used for amplification of DNA fragments in Northern blotting are listed in Table S1 in the supplemental material. Briefly, the primers were crtM-F/NF/crtM-F/NR for the crtM gene, sigB-F/NF/sigB-F/NR for the sigB gene, sarA-F/NF/sarA-F/NR for the sarA gene, RNAAII-H/RNAII-NF for RNAAII, qoxB-chF/qoxB-chR for the qoxB gene, Cit-chF/Cit-chR for the citF gene, Sav1073FNF/Sav1073FNR for the purH gene, clpX-FNF/clpX-FNR for the clpX gene, saxFNF/saxAFNR for the saxF gene, codY-FNF/codY-FNR for the codY gene, and norA_1/norA_2 for the norA gene.

Construction of plasmids for complementation of pigment mutants. The previously constructed shuttle plasmids pYJ335 (20) and pKOR1 (2) were used in this study. To construct pYJ335::qoxB, a 3,143 bp DNA fragment containing qoxB was amplified from strain Newman genomic DNA using primer pairs tetRFor399/SAV1117R and tetRFor399/PurAioR were used to prime the PCR. To construct pYJ335::qoxB, a 2,121 bp DNA fragment containing Sav1115 was amplified by using the primers Sav1115FNR and Sav1115FNF. To construct pYJ335::SAV1108, the LysRFCC22/LysRFCC22 primer pair was used. DNA fragments were cloned into pYJ335 as described previously (8). To construct pYJ335::SAV1117 and pYJ335::purA, primer pairs Sav1117F/ Sav1117R and PurAio/PurAioR were used, respectively. In this study, the primers used to PCR amplify from strain Newman genomic DNA were used to select the plasmid clones in which SAV1117 and purA are located downstream of the xyl-I promoter as described previously (7, 20). To construct pKOR1::qoxB, qoxB-ACD was amplified from Newman’s genomic DNA by using the primers FC-qoxBF and FC-qoxBR and cloned to pKOR1 as described previously (2). The selected plasmid clones were sequenced to confirm that no additional mutations were introduced by PCR. The correct plasmids were transformed into RN4220 and then into the mutant strain by electroporation. The pYJ335 vector or pKOR1 alone was transformed into RN4220 and then into the mutant strain and wild-type Newman as the control.

Semiquantitative plate assays for detection of hemolysins and proteases. Briefly, hemolytic activity was analyzed on 5% sheep blood agar plates. Protease secretion was measured on 2% skim milk-TSA plates. Positive reactions, as indicated by the clearing halo surrounding the bacterial colonies, were determined to 24 to 48 h following inoculation at 37°C. Murine infection and abscess formation. All S. aureus strains were grown at 37°C overnight in TSB, diluted 100-fold in fresh medium, and incubated at 37°C for 2.5 h until the cultures reached OD_{600} of 1.0. Bacteria were collected by centrifugation, washed, and suspended in phosphate-buffered saline (PBS) buffer. Viability of staphylococci was enumerated by colony formation on TSA and produced increased levels of carotenoid pigment (SAV0672) and one gene encoding a hypothetical protein (Table 1 and Fig. 1). In this case, transposon insertion occurred in the third of the Newman genome. A total of 1,812 Phoenix iso-
To support the involvement of the TCA cycle, oxidative phosphorylation, and purine biosynthesis in pigment production, we performed complementation experiments with the citZ, ctaA, qoxB, and purA mutants. In addition, we also performed complementation experiments with SAV0672 and SAV1117 mutants. In all cases complementation of the pigment mutant with complementing plasmid restored pigment production to normal levels (Fig. 1C).

To test whether the enhanced yellow-orange pigmentation is a consequence of compromised growth, we used an approach with chemically defined medium (HHW) (18) in which a single amino acid was omitted to reveal growth-limiting amino acids. We found that the limitation of a single amino acid generally had little or no effect on the growth of the Newman strain. The main exceptions to this are proline, alanine, valine, leucine, and arginine, the limitation of which resulted in very poor growth. However, we did not observe any enhanced pigmentation when the wild-type Newman strain was grown in HHW medium with the omission of a growth-limiting amino acid.

Analysis of the expression of *crtM* in representative *S. aureus* pigment mutants. To test whether the expression level of the carotenoid biosynthesis *crtOPQMN* operon was altered in some of the mutants, we performed Northern blot analysis on total RNA isolated 24 h after bacterial growth on TSA plates and examined *crtM* transcript levels. Seven mutants (citZ, ctaA, purH, SAV0672, sigB, ispA, and *crtM* strains), representing groups listed in Table 1, were analyzed. The result showed that the transcript levels of *crtM* correlate with changes in pigment production in four mutants (ctaA, purH, SAV0672, and sigB strains) (see Fig. S2A in the supplemental material). The *crtM* mutant strain exhibits the expected nonpigmented phenotype as described previously (30, 39, 49), and the truncated *crtM* mRNA was detected (see Fig. S2A). Mutation of *ispA*, a putative geranyltranstransferase gene, generated a nonpigmented mutant but showed an elevated level of *crtM* compared to the wild-type strain (see Fig. S2A).

Inactivation of *citZ*, a gene encoding the first enzyme in the citric acid cycle, resulted in increased pigment production when cells were grown on TSA plates (Fig. 1A and Table 1). However, Northern blot analysis did not reveal any significant increase in the *crtM* mRNA level in the *citZ* mutant (see Fig. S2A in the supplemental material). We further examined the *crtM* mRNA level in *citG* and SAV2365 mutant strains by Northern blot analysis. Again, there was no significant increase in the *crtM* mRNA level in the *citG* or SAV2365 mutant strain compared to that in the wild-type strain when bacteria were grown on a TSA plate, despite the increased pigment production observed in these mutants (see Fig. S2B in the supplemental material). In addition, Northern blot analysis indicates that there was no significant change in the *crtM* level in a *qoxB* mutant compared to the wild-type level when cells were grown on TSA plates (24 h), despite the increased pigment production (see Fig. S2B).

### Table 1. *S. aureus* mutants with reduced or enhanced pigmentation.

<table>
<thead>
<tr>
<th>Group, function, and strain</th>
<th>Pigmentation</th>
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<tr>
<td>Reduced pigmentation mutants</td>
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<td>SAV1117 strain</td>
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* - reduced pigmentation observed on TSA plates compared to the wild-type Newman strain; +, enhanced pigmentation observed on TSA plates compared to the wild-type Newman strain. Additional plus signs indicate greater relative enhancement of pigmentation.
Characterization of pur mutants. As described above, disrupting purine biosynthesis leads to a severe reduction of bacterial in vivo survival, and this result is consistent with the recent findings that adenosine is critical for S. aureus to escape host immune responses (47). Thus, we examined the possibility that purine biosynthesis may influence pigment production as well as the expression of other virulence determinants. purN, purH, purD, and purA mutants display increased pigmentation compared to the wild-type Newman strain; further enhanced pigmentation was observed in a purA mutant when bacteria were grown to late stationary phase (48 h) (see Fig. S2D in the supplemental material). Moreover, all four pur mutant strains

![Figure 1: Pigmentation of representative S. aureus pigment mutants and complementation experiments.](image1)

![Figure 2: Virulence of wild-type strain Newman and ctaA, qoxB, purH, and purA mutants.](image2)
exhibited decreased proteolytic activity compared to the wild type, as shown by the width of the cleared zone around colonies grown on a TSA plate containing 2% skim milk. Notably, disrupting purA resulted in a protease-defective phenotype (see Fig. S2D). These results suggest that purN, purH, and purD are functionally upstream of purA. While there was no significant change in the hemolytic activity of purN, purH, and purD mutants compared to the wild-type Newman strain, the purA mutant showed a growth defect on the TSA plate containing 5% sheep blood (see Fig. S2D).

To investigate whether disrupting purine biosynthesis affects bacterial growth in TSB medium, overnight cultures of the mutant strains were diluted in TSB medium and incubated at 37°C with shaking. The OD600 was measured as a function of time. As shown in Fig. S3 in the supplemental material, growth of the purN, purH, or purD mutant was slightly slower than that of the wild type in log phase, and all of these mutants reached stationary phase with total growth similar to that of the wild type. In contrast, the purA mutant showed the same growth rate as the wild type in the early log phase but reached the stationary phase with a lower total growth than the wild type.

**purA affects virulence gene expression.** Since purA is located downstream of the other three pur mutants (purN, purH, and purD) and exhibits the most dramatic phenotypes (see Fig. S2D in the supplemental material), we chose a purA mutant for further analysis. Complementation of the purA mutant with the pYJ335::purA plasmid fully restored proteolytic activity; the level of bacterial growth on skim milk or blood agar plates was comparable to that of the wild-type Newman strain (Fig. 3A).

Northern blot analysis revealed that the purA mutant (24 h after growth on a TSA plate) had significantly increased levels of sigB, RNAIII, and cna compared to the wild type (Fig. 3B). The upper transcript likely originated from P3, a sigB-dependent promoter (12, 33). It is well known that sigB and sarA are negative regulators of the production of protease in **S. aureus** (5, 9, 25, 26). Thus, the elevated levels of sarA or sigB mRNAs may contribute to the protease-defective phenotype of the purA mutant. Although the RNAIII level is dramatically enhanced in the purA mutant, we did not observe a significantly increased production of alpha toxin in this mutant. Since PurA is an enzyme used by the bacterium to produces adenine, we examined the complementation of a purA mutant with adenine. As shown in Fig. 3C, adenine was able to complement the expression of RNAIII and sigB associated with mutation of the purA gene. To determine whether adenine could reverse the attenuation of virulence of the purA mutant (Fig. 2), infected mice were repeatedly treated with subtoxic amounts (19) of adenine (total of 1 mg in 4 days with 4 injections). Unexpectedly, the infections of adenine were not sufficient for the purA mutant to multiply in vivo (see Fig. S4 in the supplemental material).

We next evaluated the expression of selected genes after bacteria were grown to exponential (3 h), postexponential (6 h), early stationary (12 h), stationary (24 h), and late stationary (48 h) phases. Both sigB and RNAIII were included in the study, as well as crmM, a gene essential for pigment biosynthesis in **S. aureus**. Mutation of purA had no significant effect on the expression of crmM, sigB, or RNAIII when bacteria were grown to exponential phase (Fig. 4). However, mutation of purA led to decreased crmM expression when bacteria were grown to
postexponential (6 h) or late stationary phase (48 h) but an elevated level of *crtM* mRNA when bacteria were grown to stationary phase (24 h). A similar expression pattern was also observed for *sigB*, with the exception that a decreased level of *crtM* transcript was observed in early-stationary-phase cells (12 h) while increased *sigB* expression was seen in the *purA* mutant compared to the wild type. Enhanced expression of RNAIII also occurred in the *purA* mutant compared to the wild type when bacteria were grown to stationary phase (12 h) (Fig. 4). These results show that the *purA* mutation affects the expression of global regulators such as *sigB* and RNAIII and that the effects are growth phase dependent.

**Inhibition of purine biosynthesis with small molecules.** To confirm the critical role of purine biosynthesis in regulating pigment production and virulence determinants, two known inhibitors of purine biosynthesis, 6-thioguanine (6-TG) and mycophenolic acid (MPA), were employed to test the inhibition of purine biosynthesis in *S. aureus* Newman. Enhanced pigmentation was observed when Newman was plated on TSA plates in the presence of 6-TG (Fig. 5A). Quantification of pigment production by extracting carotenoid products confirmed that both inhibitors afforded enhanced pigmentation in strain Newman (Fig. 5B). The *sigB* mutant displayed a colorless phenotype, and both 6-TG and MPA failed to enhance pigmentation of this mutant strain (data not shown), indicating that *sigB* is required for pigment production. Inhibition of purine biosynthesis in strain Newman also led to increased *sigB*, *sarA*, and RNAIII transcripts, as also observed with the *purA* mutant (Fig. 3B). While the P3 promoter of *sarA* is controlled by SigB, the increase in RNAIII is likely mediated in a SigB-independent manner since the same change was observed in Newman and *sigB* mutant strains after treatment with 6-TG or MPA (Fig. 5C). In addition, *S. aureus* Newman showed a 1.5-fold longer doubling time of exponential growth when TSB medium was supplied with 20 µg/ml 6-TG, and enhanced pigmentation was also observed (data not shown). Finally, we tested the effect of 6-TG on the *in vivo* survival of *S. aureus* in the murine abscess model. Mice treated with 6-TG exhibited a 1.5-log (31.6-fold) reduction (*P* < 0.008, t test) of bacterial loads in both liver and kidney (Fig. 5D and E).

**Microarray analysis of *purA* and *purH* mutants in late-stationary-phase cells.** Enhanced pigmentation was observed for *purA* mutant strains when bacteria were grown to stationary phase (Fig. 3; see also Fig. S2 in the supplemental material). To further study the roles of purine biosynthesis in starvation survival, we performed microarray analysis of transcriptional profiling of both the *purA* mutant and *purH* mutant in late-stationary-phase cells and compared the results with those in the wild-type Newman strain. From the microarray data we identified 399 genes with increased transcript levels (≥2.5-fold) and 8 genes with decreased transcript levels in the wild type versus the *purA* mutant (see Table S2 in the supplemental material). These 407 genes represent ~16% of the total of annotated genes in the *S. aureus* Newman genome. Grouping these genes according to their annotated function shows that they belong to several functional categories, primarily energy production and conversion, posttranslational modification, and transcription regulation (see Table S2). Interestingly, *citZ*, *citG*, *SAY2365*, *goxB*, *hemL*, and *SAV1117* exhibit decreased expression in the *purA* mutant compared to expression in the wild-type Newman strain in our microarray analysis. Considering that disrupting these genes led to enhanced pigment production (Fig. 1 and Table 1), we suspect that the decreased expression of these genes might contribute to the enhanced pigmentation observed in the *purA* mutant when it is grown to late stationary phase (Fig. 3D; see also Fig. S2).

We also compared gene expression in the wild-type Newman strain with expression in the *purH* mutant strain in late-stationary-phase cells (48 h). We identified 353 genes with increased transcript levels (≥2.5-fold) (see Table S2 in the supplemental material) and 12 genes with decreased transcript levels in the wild-type strain versus the *purH* mutant (see Table S2). About 195 genes out of 399 that are downregulated in the *purA*
mutant are also downregulated in the purH mutant (see Table S2). In addition, cluster analysis indicates that about 400 (98%) genes that displayed expression differences between the wild-type Newman and purA mutant strains also showed similar expression profiles in the purH mutant and Newman strain (Fig. 6A).

To confirm our microarray results, we performed Northern blot analysis of eight genes (qoxB, sigB, codY, citZ, lexA, clpX, norA, and saeS). The expression profiles of these eight genes as revealed by Northern blot analysis are consistent with the results derived from the microarray analysis (Fig. 6B; see also Table S2 in the supplemental material). Interestingly, the transcripts of these eight genes are upregulated in a guaB mutant compared to expression in the wild-type Newman strain (Fig. 6B). This result raises the possibility that the main phenotypic effect of the purine biosynthetic pathway in S. aureus is mediated primarily through purA or adenine biosynthesis. Further studies are required to elucidate the relative contributions from each pathway.

Mutation of either purA or purH led to mostly downregulation of genes in the S. aureus genome, perhaps suggesting a signal for nutrient limitation. Specifically, the expression levels of 54 genes were severely reduced (>6-fold) in both the purA mutant and purH mutant when bacteria were in late stationary phase (see Table S2 in the supplemental material). These genes encode proteins with diverse biological functions, which demonstrated the profound effects of purine biosynthesis on the physiology of S. aureus. Notably, genes involved in energy metabolism are severely repressed in a purA mutant. This includes genes involved in oxidative phosphorylation (qoxB, sigB, and tcaR), the TCA cycle (sucA and sucB), and β-oxidation (SA0211-SA0215). Expression levels of genes encoding functional components in protein turnover (SA2563, clpX, and SA1270) are also dramatically repressed in a purA or purH mutant. In addition, many regulatory elements are affected in purine biosynthesis mutants. Expression levels of 49 genes encoding proteins with potential regulatory functions (transcriptional regulators and kinases) are affected by purA or purH mutation (see Table S2). Significantly, 14 out of these 49 genes, such as rsbUVW-sigB, sarA, sarR, sarZ, rot, sacS-sacR, codY, tcaR, srrA, and ccpA, are the well-known genes involved in virulence gene regulation in S. aureus.

Disrupting of the TCA cycle and oxidative phosphorylation affect the expression of virulence determinants. As described above, purine biosynthesis affects virulence gene regulation in S. aureus. To further examine the possibility that the TCA cycle and oxidative phosphorylation may influence pigment production as well as the regulation of other virulence genes, the mRNA levels of global virulence regulators sigB, sarA, and RNAIII were measured in citZ, citG, and SA2365 mutants. Northern blot analysis revealed that significantly decreased levels of sigB and sarA mRNAs were detected in a citG mutant (Fig. 7A). In addition, inactivation of citZ, citG, or SA2365 gave rise to overproduction of RNAIII (Fig. 7A). Transforma-
tion of a ctaA mutant with the complementing plasmid pYJ335: citZ restored the wild type levels of RNAIII and sigB (Fig. 7B). We further employed semiquantitative plate assays to examine the production and secretion of hemolysin in these strains. As expected, citZ, citG, and SAV2365 mutants exhibit higher hemolytic activity than the wild-type strain (Fig. 7C). In addition, disrupting the sigB gene also led to enhanced hemolytic activity (Fig. 7C).

Both CtaA and QoxB are functional components of oxidative phosphorylation. We performed hemolysin activity assays on both mutants using TSA agar plates containing 5% sheep blood. Disrupting ctaA generated a strain with reduced hemolytic activity, consistent with a previous report (10). Inactivation of qoxB also resulted in reduced hemolytic activity (Fig. 7D). Complementation of the qoxB mutant with the pKOR1::qoxB plasmid partially restored hemolytic activity to the normal level (Fig. 7D). Northern blot analysis indicates that there is no significant change in sigB, sarA, and RNAIII mRNA levels in a qoxB mutant compared to levels in wild-type strain when bacteria are grown on TSA plates (24 h) despite the increased pigment production and decreased hemolytic activity observed in the qoxB mutant (see Fig. S2C in the supplemental material).

FIG. 7. Analysis of representative S. aureus pigment mutants. (A) The transcripts of sigB, sarA, and RNAIII were measured for S. aureus strains grown on TSA plates at 37°C for 24 h. (B) Northern blot analysis of the transcripts of sigB and RNAIII. S. aureus strains were grown on TSA plates for 24 h before total RNA was harvested. (C and D) Hemolytic activity assay. Bacterial strains were inoculated onto plates with a toothpick and incubated at 37°C for 36 h.

DISCUSSION

Previous studies have revealed that the rsbUVW-sigB system and crtOPQMN operon are required for the production of S. aureus pigment (3, 17, 26, 38, 39, 49). We show here that golden pigment production of S. aureus is also affected by metabolic pathways such as purine biosynthesis, the TCA cycle, and oxidative phosphorylation (Table 1; Fig. 1). Mutations in these pathways lead to enhanced pigmentation of the pathogen, a result that has not been reported in the past. We have also demonstrated the importance of purine biosynthesis and oxidative phosphorylation for in vivo survival of S. aureus and pathogenesis of infection (Fig. 2).

Inactivation of purine biosynthetic genes (purN, purH, purD, or purA) or inhibition of purine biosynthesis led to enhanced pigmentation; this is most likely mediated by enhanced expression of sigB (Fig. 3, 4, and 5). In addition, the decreased expression of citZ, citG, SAV2365, qoxB, hemL, and SAV1117 (Fig. 6; see also Table S2 in the supplemental material) might contribute to the enhanced pigmentation observed in pur mutants when bacteria are grown to late stationary phase since disruption of these genes led to enhanced pigment production (Table 1).

While negatively modulating the expression of sigB when bacteria are grown on TSA plates (Fig. 3), purA seems to positively impact the expression of sigB when bacteria are grown to late stationary phase in TSB medium (Fig. 4 and 6). Indeed, the effect of purA on the expression of sigB is pleiotropic and is likely growth phase dependent (Fig. 4). These results are consistent with the notion that the interplay between metabolism and production of virulence factors depends on a large array of factors (46). It is well known that medium constituents (41), growth conditions (16, 48, 50), and stage of growth (Fig. 4) (5, 9, 31) all influence the expression of regulators in S. aureus. Additional work is needed to elucidate underlying mechanisms; however, our data do show a profound effect of purine biosynthesis on the expression of global regulators such as sigB, sarA, and RNAIII in S. aureus.

Disrupting either oxidative phosphorylation or purine biosynthesis leads to a reduction in bacterial in vivo survival, with the largest survival defect observed for the strains impaired for purine biosynthesis (Fig. 2). The increased pigmentation benefits bacteria for survival in certain environments (29, 30). However, disrupting purine biosynthesis may starve the bacteria of essential replicative precursors that also play an important role in S. aureus to escape host immune responses (47). In addition, intact purine biosynthesis is required for full proteolytic activity of S. aureus (Fig. 3). Moreover, gene expression profile analysis indicates that purA or purH is required for full expression of genes that are involved in a wide variety of cellular functions, including energy metabolism, regulation, stress response, and virulence, when S. aureus is grown to late stationary phase (see Table S2 in the supplemental material). Inactivation of some of these genes, for instance, qoxB (Fig. 5C), SAV0862 (1), clpX (15), clp (15), sodA (24), srtA (22), fshH (28), sarA (42), saeR-saeS (27), sarZ (7), sarA (4), sigB (21), rsbU (21), sucB (35), and dnuD (11), has been shown to result in attenuated virulence of S. aureus in vivo. Thus, the dramatic virulence attenuation of purA and purH mutant strains is most likely due to a combination of several effects, including nutrient limitation, energy metabolism, stress response, and altered...
global expression of bacterial virulence factors. It has been shown that reactive oxygen species (ROS)-mediated DNA damage is responsible for the attenuated virulence phenotype of *Salmonella enterica* serovar Typhimurium purine auxotrophs in mice (34). In the current study, the expression of 23 genes involved in DNA repair is also affected by *parA* or *parH* mutation (see Table S1).

The disruption of *parA* can be complemented by adenine *in vitro* (Fig. 3) but not *in vivo* (see Fig. S4 in the supplemental material). A similar phenomenon has also been observed for adenine auxotrophs of *Bacillus anthracis*, and it was proposed that adenine production might be important for host invasion of the bacterium (19). Further experiments are required to test the hypothesis. We further showed that targeting purine bio-synthesis with 6-TG resulted in enhanced bacterial killing *in vivo*, as evidenced by lower bacterial counts in the livers and kidneys of tested mice (Fig. 5). Since significant differences exist between purine biosynthetic pathways in Gram-positive bacteria such as *S. aureus* and humans (23), optimized inhibitors that shut down purine biosynthesis in bacteria may offer an alternative therapeutic paradigm for treating *S. aureus* infections.

Inactivation of TCA cycle genes (*citZ, citG*, and *SAV2365*) led to enhanced pigmentation (Table 1) while no elevated level of *sigB* or *crtM* was observed (Fig. 7A; also see Fig. S2 in the supplemental material). Disrupting these genes yields mutant strains with enhanced hemolysin activity, a phenotype also observed in a *sigB* mutant (Fig. 7C). In addition, enhanced expression of *sigB* resulted in decreased hemolysin activity (25). Thus, the enhanced pigmentation of the TCA cycle mutant strains is less likely a result of enhanced activity of SigB. The golden pigment of *S. aureus* is synthesized from acetyl coenzyme A (acetyl-CoA) and acetoacetyl-CoA via the mevalonate pathway. It is tempting to propose that inactivation of the TCA cycle may direct acetyl-CoA flux to the mevalonate pathway, which could result in increased pigment biosynthesis. In *Staphylococcus epidermidis*, inactivation of the TCA cycle alters metabolic status, resulting in a redirection of carbon flow from growth into polysaccharide intercellular adhesin biosynthesis (44).

Disrupting *ctaA* and *qoxB* gives elevated levels of pigmentation (Fig. 1) and reduced hemolyc activity (Fig. 7). An elevated *crtM* mRNA level (see Fig. S2A in the supplemental material) was observed in the *ctaA* mutant. However, Northern blot analysis indicates that there is no significant change in *crtM, sigB*, and *RNAIII* mRNA levels in a *qoxB* mutant compared to levels in the wild-type strain when bacteria were grown on TSA plates (24 h) (see Fig. S2C). In *Bacillus subtilis*, the *qoxB* knockout causes a severe reduction of TCA cycle fluxes and an increased overflow metabolism (51). The function of *qoxB* in *S. aureus* awaits further investigation. In addition, disrupting either *ctaA* or *qoxB* leads to reduction of bacterial survival *in vivo* although enhanced pigmentation was observed for a *ctaA* mutant or *qoxB* mutant. These results further support the notions that the pathogenesis of staphylococcal infections is multifactorial (46) and that the reduced hemolysin activity observed for a *ctaA* mutant or *qoxB* mutant may contribute to the attenuated virulence.

Results from this work also suggest a link between metabolic processes and virulence gene expression in *S. aureus*. This network may play a pivotal role in the extraordinary flexibility of staphylococci to adapt to and cope with changing host environments. The TCA cycle has been proposed to act as a signal transduction pathway, which translates external environmental cues into intracellular metabolic signals in order to modulate the activity of transcriptional regulators in *S. epidermidis* (44). It has also been reported that the course of staphylococcal infections in mice is likewise dependent upon the nutritional state of the animals at the time of exposure to the infective dose (45). On the other hand, *S. aureus* cells grown in milk or milk whey *in vitro* show increased virulence in a mouse model (32). Thus, metabolic processes in both host and *S. aureus* may be crucial determinants of disease outcome. The findings reported here further emphasize the need for the study of metabolite requirements in the context of infection. We do not yet fully understand the impact of the altered expression levels of virulence regulators and virulence factors in the mutant strains. We speculate that the dramatic virulence reduction of these mutants is due to a combination of multiple factors, including nutrient limitation, energy metabolism, stress response, and altered global expression of bacterial virulence factors. Potent and bacterium-selective small molecules that inhibit purine biosynthesis may lead to the development of a new generation of anti-*S. aureus* agents.

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