Expanded Glucose Import Capability Affords *Staphylococcus aureus* Optimized Glycolytic Flux during Infection

Nicholas P. Vitko, Melinda R. Grosser, Dal Khatri, Thurlow R. Lance, Anthony R. Richardson

Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA

N.P.V. and M.R.G. contributed equally to this report.

ABSTRACT  Acquisition of numerous virulence determinants affords *Staphylococcus aureus* greater pathogenicity than other skin-colonizing staphylococci in humans. Additionally, the metabolic adaptation of *S. aureus* to nonrespiratory conditions encountered during infection (e.g., hypoxia, nitric oxide, iron chelation) has been implicated as contributing to *S. aureus* virulence. Specifically, *S. aureus* has been shown to ferment glycolytic substrates in nonrespiratory environments encountered within the host. Here, we show that *S. aureus* has acquired unique carbohydrate transporters that facilitate the maximal uptake of host sugars and serve to support nonrespiratory growth in inflamed tissue. The carbohydrate substrates of 11 *S. aureus* transporters were identified, and at least four of their genes encode glucose transporters (*glcA*, *glcB*, *glcC*, and *glcU*). Moreover, two transporter genes (*glcA* and *glcC*) are unique to *S. aureus* and contribute disproportionately to the nonrespiratory growth of *S. aureus* on glucose. Targeted inactivation of sugar transporters reduced glucose uptake and attenuated *S. aureus* in a murine model of skin and soft tissue infections. These data expand the evidence for metabolic adaptation of *S. aureus* to invasive infection and demonstrate the specific requirement for the fermentation of glucose over all other available carbohydrates. Ultimately, acquisition of foreign genes allows *S. aureus* to adopt a metabolic strategy resembling that of infiltrating host immune cells: high glycolytic flux coupled to lactate excretion.

IMPORTANCE  The bacterial pathogen *Staphylococcus aureus* causes a wide range of human infections that are costly and difficult to treat. *S. aureus* differs from closely related commensal staphylococci in its ability to flourish following the invasion of deeper tissue from the skin surface. There, *S. aureus* primarily uses glucose to grow under respiration-limiting conditions imposed by the immune system. It was previously unclear how *S. aureus* thrives in this environment when other *Staphylococcus* species cannot. Our results provide evidence that *S. aureus* has acquired an expanded repertoire of carbohydrate transporters. In particular, four glucose transporters contribute to efficient *S. aureus* growth during infection. Thus, *S. aureus* has evolved to maximize glucose uptake abilities for enhanced glycolytic flux during tissue invasion. This dependence on glucose acquisition for *S. aureus* virulence may also explain links between serious infectious complications associated with diabetic patients exhibiting elevated blood glucose levels.

*Staphylococcus aureus* is a Gram-positive coccus that asymptptomatically colonizes healthy human skin (1, 2). However, a compromised skin barrier or mucous membrane can lead to severe *S. aureus* infections, including: skin and soft tissue infections (SSTIs), bacteremia, osteomyelitis, pneumonia, and toxic shock syndrome (3–5). Many other species of staphylococci (e.g., *S. epidermidis*, *S. haemolyticus*, *S. saprophyticus*, etc.) also colonize human skin but cause disease far less frequently and with less severity than *S. aureus* (6). This difference has been extensively studied and is generally attributed to the combined presence of numerous unique virulence factors in the *S. aureus* genome, such as toxins, adhesins, antiphagocytic factors, and protein A (7–9). Absent from this explanation is the contribution of metabolic adaptation.

The *S. aureus* life cycle can plausibly be described as low-level growth on the skin surface with periodic penetration of deeper tissue environments marking a phase of enhanced growth and increased incidence of transmission. Major physiological differences between the skin surface and underlying tissue include oxygen concentrations, micronutrient availability, nitrogen sources, carbon sources, and pH (10–12). In general, the skin surface has lower levels of carbohydrates and peptides, relatively high levels of oxygen, and an acidic pH. Sterile tissue, on the other hand, contains an abundance of carbohydrates and peptides, lower levels of free oxygen, and a more neutral pH. However, invasion of sterile tissue by *S. aureus* leads to the activation of several innate immune responses that combine to limit bacterial respiration (e.g., iron chelation, nitric oxide [NO] production, and robust oxygen consumption by innate immune cells) (13–17). Thus, natural selection would dictate that *S. aureus* has adapted to take advantage of the unique metabolites present within sterile tissue (e.g., peptides and carbohydrates) in a manner compatible with increased resistance to host inflammation (i.e., respiration inhibition).
Recently, we demonstrated that *S. aureus* requires both glycolysis and lactate fermentation for SSTIs and bloodstream infections and that only carbohydrate support the growth of *S. aureus* under both high NO stress and anaerobiosis (i.e., nonrespiratory conditions) (13, 16). Additionally, the lack of abundant iron during infection limits respiration and necessitates high glycolytic flux coupled to lactate excretion (15, 17). This metabolic strategy, which is similar to that of activated immune cells, allows for the generation of ATP in a redox-balanced, respiration-independent manner. However, aside from the presence of a unique lactate dehydrogenase gene (*ldh1*) in the *S. aureus* genome that promotes enhanced redox balancing during respiration inhibition, there is a lack of molecular evidence supporting a contribution of metabolic adaptation to infection as a distinguishing characteristic of *S. aureus* (13). Given the high evolutionary conservation of glycolysis among the kingdoms of life, we postulated that metabolic adaptation to promote high glycolytic flux would most easily be achieved by the acquisition of additional carbohydrate importers (18).

Bacterial carbohydrate transporters can be divided into those that modify the sugar during transport (i.e., phosphotransferase system [PTS] transporters) and those that do not (i.e., primary and secondary active transporters) (19–21). PTS transport proceeds via a phosphorelay system that transfers the phosphoryl group of phospho(enol)pyruvate (PEP) through a series of carrier proteins (EI and HPr) to a transporter (EII) and then on to the sugar as it is imported. PTS sugar transporters are composed of at least three subunits: EIIA, EIIB, and EIIC. The EIIA and EIIB subunits transfer the phosphoryl group from HPr to the sugar, while the EIIC subunit acts as a sugar-specific transmembrane receptor. Interestingly, the EI subunits may be encoded as individual polypeptides or fused into multisubunit proteins. PTS-dependent carbohydrate transport is unique to bacteria and is the predominant form of sugar uptake. PTS-dependent transport is also functionally linked to the transcriptional regulation of cellular metabolism via carbon catabolite repression (mediated by CcpA in Gram-positive bacteria), which further contributes to overall metabolic efficiency.

In this report, we show that *S. aureus* exhibits better nonrespiratory growth than other skin-dwelling staphylococci and partially attribute this phenomenon to an increased capacity for carbohydrate import. More specifically, we identify the carbohydrate transporters: SAUSA300_0191 (see Table S1). Importantly, the 10 unique *S. aureus* alleles are predicted to encode glucose transporters: SAUSA300_0191 (glcA), SAUSA300_0194, SAUSA300_0236 (glcC), and SAUSA300_0259 (see Table S1). Importantly, glucose is (i) largely absent from the skin surface, (ii) the most abundant free carbohydrate in human serum, and (iii) used by activated innate immune cells to both produce and resist inflammatory radicals.

**Carbohydrate uptake in *S. aureus* is mostly PTS dependent and contributes disproportionately to nonrespiratory growth.** The majority (21/29) of putative *S. aureus* carbohydrate transport proteins are predicted to be PTS proteins. Thus, we decided to test the contribution of PTS-dependent carbohydrate transport to the nonrespiratory growth of *S. aureus* by using a PTS-deficient strain of *S. aureus* (ptsH-H15A). The H15A substitution in PtsH prevents the transfer of the phosphoryl group from EI to PtsH, thereby inhibiting PTS-dependent sugar uptake but not directly affecting interactions with CcpA, the master regulator of carbon catabolite repression (21). To confirm the efficacy of this mutation and identify PTS-dependent substrates, we compared the aerobic growth of wild-type (WT) and ptsH-H15A mutant *S. aureus* on 16 different carbohydrates. Previous studies demonstrated PTS-dependent utilization of at least seven carbohydrates (lactose, fructose, galactose, maltose, sucrose, glucose, and mannitol) by *S. aureus* (19). In line with these observations, we found that loss of PTS-dependent sugar uptake prevented *S. aureus* growth on 10 carbohydrates (mannose, fructose, galactose, mannitol, N-acetylgluosamine, N-acetylmannosamine, maltose, sucrose, trehalose, lactose, and turanose) and reduced its growth on two carbohydrates (glucose and maltotriose) but did not affect its growth on ribose (Table 1). These data show that *S. aureus* carbohydrate utilization is largely PTS dependent.

Nonrespiratory fermentation of carbohydrates is inherently less energy efficient than respiration. Consequently, *S. aureus* must consume three times the amount of glucose under fermentative growth as under respiratory conditions in order to produce equivalent biomass (dry weight) (Fig. 3A). Maintaining this elevated level of sugar catabolism necessitates more efficient uptake of carbohydrates, particularly under nonrespiratory conditions. Accordingly, despite the large reduction in carbohydrate utilization in the PTS-deficient strain, aerobic growth of *S. aureus* Lac in complex medium is largely unaffected (Fig. 3B). In contrast, the PTS-deficient mutant exhibited a significant reduction in growth under anaerobic conditions (Fig. 3C), under NO stress (Fig. 3D),
and under metal-limited conditions (Fig. 3E). Similar results were obtained with *S. aureus* COL (data not shown). Thus, the increased reliance of *S. aureus* on carbohydrate transport during nonrespiratory growth implicates carbohydrate transporter acquisition as a possible mechanism of metabolic adaption of *S. aureus* to infection.

**Substrate identification for individual *S. aureus* PTS proteins.** To identify the sugar specificity of individual *S. aureus* PTS proteins, we used a modified version of the microplate version of the “biological filter” method (12). Substrate utilization was assessed for 24 h using the cell growth and metabolism array microplate system (Bacterial Signatures, BD Biosciences). This system has been described previously (5). Microplate arrays were inoculated with 100 μL of an overnight culture in TSB with 0.5% glucose and incubated at 37°C for 24 h. The absorbance was measured at 650 nm, and the average growth rates were calculated for each strain and substrate. Statistical significance was calculated with a Student two-sided *t* test (***, *P* ≤ 0.001). Abs, absorbance.

**FIG 1.** *S. aureus* displays better anaerobic growth than CoNS. Anaerobic growth of *S. aureus* (SA) COL and LAC, *S. epidermidis* (SE) RP62A, *S. haemolyticus* (SH) ATCC 29970, and *S. saprophyticus* (SS) ATCC 15305 in TSB (A) and CDM plus 25 mM glucose (C) (*n* = 3). Corresponding average growth rates for TSB and CDM plus glucose are displayed in panels B and D, respectively (*n* = 3; error bars show the pooled standard error of the mean). Growth rates were calculated from 2 to 4 h (*S. aureus* LAC) and 3 to 5 h (*S. aureus* COL, *S. epidermidis*, *S. haemolyticus*, and *S. saprophyticus*) in TSB and from 2 to 8 h in CDM plus 25 mM glucose. Statistical significance was calculated with a Student two-sided *t* test (***, *P* ≤ 0.001). Abs, absorbance.

**FIG 2.** *S. aureus* Encodes enhanced carbohydrate transport capability. Shown is a Venn diagram depicting the presence and conservation of putative carbohydrate transport proteins in the genomes of *S. aureus* COL, *S. epidermidis* RP62A, *S. haemolyticus* JCSC1435, and *S. saprophyticus* ATCC 15305. *S. aureus* encodes more overall transporters (*n* = 22) and the highest number of unique transporters not found in any of the other species (*n* = 10).
Lastly, we found that attenuation of specific to glucose (see Table S2 in the supplemental material) transporter was able to independently complement the aerobic growth of the S. aureus (24, 25). In support of these observations, we found that the mtlFA as encoding fructose and mannitol importers, respectively, selected carbohydrates. Previous studies identified predicted PTS protein-encoding genes for aerobic growth defects on maltotriose, GlcU more than that of the PEP-dependent PTS transporters GlcA, GlcB, GlcC, or GlcU increased its glucose uptake to 98, 110, 105, or 56% of the WT level, respectively. The lack of significant uptake complementation by GlcU could be explained by the fact that GlcU encodes a member of the glucose/ribose porter family, a family of secondary active transporters that rely on proton motive force (PMF) for energy (26, 27). The dense cell pellet conditions required to perform these uptake assays likely have a negative impact on PMF. This would specifically decrease the activity of PMF-dependent GlcU more than that of the PEP-dependent PTS transporters GlcA, GlcB, and GlcC. Importantly, the S. aureus ΔG4 mutant is still capable of importing glucose and exhibits residual aerobic growth on glucose. These data indicate the presence of an additional glucose transporter(s).

To rule out the contribution of other PTS-dependent transporters to S. aureus glucose uptake, we compared the aerobic growth of WT and ΔG4, ptsH-H15A ΔglcU, and ptsH-H15A Δglk mutant S. aureus COL. Glucose kinase, encoded by glk, is responsible for phosphorylating intracellular glucose taken up by non-PTS transporters. Thus, without glk, intracellular glucose cannot be catabolized unless transported via PTS proteins. The growth of the S. aureus ΔG4 and ptsH-H15A ΔglcU mutants was indistinguishable, whereas the S. aureus ptsH-H15A Δglk mutant exhibited no residual growth in CDM with glucose as the primary carbon source (see Fig. S1 in the supplemental material). These data indicate that one or several unidentified non-PTS-dependent transporters are responsible for the remaining S. aureus ΔG4 glucose uptake observed.

GlcA and GlcC contribute disproportionately to the nonrespiratory growth of S. aureus on glucose. Next, we compared the aerobic, anaerobic, and NO-exposed growth of the various S. aureus glucose transporter mutants in CDM with glucose as the primary carbon source. The two triple mutants lacking both unique glucose transporters (Glca and GlcC), and thus only expressing GlcB or GlcU, grew significantly more poorly than the other triple mutants, in which either GlcA or GlcC remained functional (Fig. 4D and E). This suggests that the unique glucose transporters GlcA and GlcC contribute disproportionately to S. aureus growth on glucose under nonrespiratory conditions. The ability of either GlcA or GlcC alone to individually maintain WT growth under nonrespiratory conditions cannot be explained by its expression level. The glcA transcript levels were commensurate with those of glcB and glcU (see Fig. S2 in the supplemental material). Moreover, glcC transcription was less robust under all of the conditions tested. Furthermore, none of the glucose transporter genes responded to the presence or absence of glucose or to respiratory inhibition, with the exception of glcC, which showed modest induction under anaerobiosis. Thus, other factors must explain the ability of GlcA or GlcC to fully restore growth by itself, such as translation efficiency, protein stability, and/or affinity for glucose.

### TABLE 1 Identification of PTS-dependent carbohydrates that support growth of S. aureus

| Sugar   | COL WT | ptsH-H15A mutant | LAC WT | ptsH-H15A mutant |
|---------|--------|------------------|--------|------------------|
| Glucose | ++ +   | ++ +            | ++ +   | ++ +            |
| Mannose | ++ +   | ++ +            | ++ +   | ++ +            |
| Fructose| +      | +               | ++ +   | +               |
| Galactose| +    | +               | +      | +               |
| Ascorbate| +    | +               | +      | +               |
| Mannitol| + +   | + +             | + +    | + +             |
| Sorbitol| +     | +               | +      | +               |
| GlcNAc  | +      | +               | +      | +               |
| ManNAc  | +      | +               | +      | +               |
| Ribose  | ++ +   | + +             | ++ +   | + +             |
| Succrose| + +    | +               | ++ +   | +               |
| Trehalose| +     | +               | +      | +               |
| Lactose | +      | +               | +      | +               |
| Turanose| +      | +               | +      | +               |
| Maltotriose| + +  | + +             | + +    | + +             |

*+++, grows as well as when cultured with glucose; ++, grows to same terminal OD as when cultured on glucose, but delayed ≥10 h; +, does not grow to maximal terminal OD.*

proteins, we screened mutants with insertions in all of the predicted PTS protein-encoding genes for aerobic growth defects on selected carbohydrates. Previous studies identified fruA and mtlFA as encoding fructose and mannitol importers, respectively (24, 25). In support of these observations, we found that the mtlF and mtlA mutants were unable to grow on mannitol, while S. aureus COL, a natural fruA mutant, exhibited poorer growth than S. aureus JE2 on fructose. Additionally, we identified PTS transport proteins contributing to the uptake of nine other carbohydrates. Overall, our results link individual PTS transport proteins to the uptake of 11 of the 12 PTS-dependent sugars (Table 2). All phenotypes were confirmed in a second S. aureus strain (COL) background following transduction (data not shown). Consistent with the growth phenotypes of the ptsH-deficient mutant, not a single S. aureus PTS transposon mutant displayed a growth defect on glucose, suggesting that glucose uptake (i) is genetically redundant and (ii) likely requires both PTS and non-PTS-dependent transporters.

**S. aureus glucose transport is highly redundant.** To identify the S. aureus glucose transporters, four different candidate genes were mutated via allelic replacement (three PTS transporters [glcA, glcB, and SAUSA300_0236] and one non-PTS transporter [glcU]) and then combined into all possible double, triple, and quadruple mutants. The deleted genes were chosen on the basis of a combination of sequence similarity to known glucose transporters and high expression levels during aerobic growth on glucose (data not shown). We found that only the S. aureus quadruple mutant (ΔglcA ΔglcB ΔglcU ΔSAUSA300_0236) exhibited a substantial aerobic growth defect on glucose (the quadruple mutant is referred to here as S. aureus ΔG4, and the SAUSA300_0236 gene is referred to as glcC) (Fig. 4A). Additionally, each putative glucose transporter was able to independently complement the aerobic growth defect of the S. aureus ΔG4 mutant on glucose (Fig. 4B). Lastly, we found that attenuation of ΔG4 mutant growth was fairly specific to glucose (see Table S2 in the supplemental material) (ManNAc and GlcNAc were not tested, as GlcA and GlcC were previously implicated in their transport; see Table S1 in the supplemental material).

To confirm that glcA, glcB, glcC, and glcU encode glucose transporters, we performed radiolabeled-glucose uptake assays with WT S. aureus, the ΔG4 mutant, and the ΔG4 mutant containing plasmids constitutively expressing each of the four glucose transporters. The ΔG4 mutant exhibited significantly less glucose uptake than WT S. aureus (45% of the WT level) (Fig. 4C). Complementation of the ΔG4 mutant with glcA, glcB, glcC, or glcU increased its glucose uptake to 98, 110, 105, or 56% of the WT level, respectively. The lack of significant uptake complementation by glcU could be explained by the fact that glcU encodes a member of the glucose/ribose porter family, a family of secondary active transporters that rely on proton motive force (PMF) for energy (26, 27). The dense cell pellet conditions required to perform these uptake assays likely have a negative impact on PMF. This would specifically decrease the activity of PMF-dependent GlcU more than that of the PEP-dependent PTS transporters GlcA, GlcB, and GlcC. Importantly, the S. aureus ΔG4 mutant is still capable of importing glucose and exhibits residual aerobic growth on glucose. These data indicate the presence of an additional glucose transporter(s).
Rich medium provides alternative carbohydrates to support nonrespiratory growth of S. aureus. We hypothesized that the acquisition of additional glucose transporters might partially explain the enhanced nonrespiratory growth phenotypes exhibited by S. aureus in both CDM and rich medium. Thus, we compared the growth of WT S. aureus (normal transport) with that of the ΔH9004G4 (significantly reduced glucose uptake) and ΔptsH-H15AΔglcU ΔH9004 S. aureus (COL and LAC) mutants in Bacto tryptic soy broth (TSB; BD; catalog no. 211825) under respiratory and nonrespiratory conditions. We observed almost no growth defect in the ΔH9004G4 mutants under aerobic or nonrespiratory conditions (including NO stress, anaerobiosis, and metal chelation) (see Fig. S3 in the supplemental material). This suggests that glucose transport is nonessential for growth under nutrient-rich conditions, perhaps because of the presence or uptake of other carbohydrates. In line with this hypothesis, we observed an additive effect of the ΔglcU and ΔptsH-H15A mutations under aerobic conditions (see Fig. S3). However, the ΔptsH-H15AΔglcU mutant exhibited drastic growth rate reductions under nonrespiratory conditions, including anaerobiosis, NO-exposure, and metal chelation (see Fig. S3). These data indicate that carbon is not a limiting factor for S. aureus glucose uptake even when high glycolytic flux is required upon respiration inhibition.

Glucose uptake contributes to S. aureus virulence in a murine SSTI model. To investigate the relative contributions of glucose and other carbohydrates to S. aureus growth or survival during infection, C57BL/6 mice were subcutaneously infected with 1 × 10^7 CFU of WT or ΔG4, ΔptsH-H15A, ΔptsH-H15AΔglcU, or ΔglcU mutant S. aureus LAC. At 5 days postinfection, the S. aureus ΔG4 mutant was significantly attenuated by the abscess burden.
The robust import of glucose by the bacterium.

In order to accommodate an elevated level of glycolytic flux, *S. aureus* must efficiently acquire host carbohydrates. Importantly, glucose is the most abundant free carbohydrate in the human body, and elevated host glucose levels are associated with greater *S. aureus* disease (11, 22, 28–30). However, efficient glucose uptake is likely difficult in inflamed tissue spaces, given that infiltrating phagocytes rapidly consume tissue glucose by running a metabolic scheme not unlike Warburg metabolism (i.e., robust glucose uptake capabilities, as well as a highly active lactate dehydrogenase, *S. aureus* has distinguished itself from other skin-dwelling staphylococci and evolved to "mimic" the metabolic state of the host at sites of inflammation.

It should be noted that glucose is not the only substrate for the *S. aureus*-specific GlCA and GlcC transporters. We found that GlCA and GlcC are solely responsible for the uptake of GlcNAc and ManNAc, respectively, which may be indicative of a role in peptidoglycan homeostasis. However, the selective pressure for these specific GlCAs may be due to the rapid consumption of oxygen by active immune cells. *S. aureus* has evolved to thrive under all of these stresses provided it has a rich source of carbohydrates, particularly glucose. Under aerobic conditions, respiration contributes directly to PMF, which in turn is used to generate ATP. In the absence of respiration, the only source of ATP is substrate level phosphorylation. Moreover, PMF has to be adequately maintained by consumption of ATP and reversal of the F$_{1}$F$_{0}$-ATPase. Therefore, under nonrespiratory conditions, *S. aureus* requires enhanced glycolytic flux, as demonstrated by a $>$3-fold increase in glucose consumption (Fig. 3A).

In order to accommodate an elevated level of glycolytic flux, *S. aureus* must efficiently acquire host carbohydrates. Importantly, glucose is the most abundant free carbohydrate in the human body, and elevated host glucose levels are associated with greater *S. aureus* disease (11, 22, 28–30). However, efficient glucose uptake is likely difficult in inflamed tissue spaces, given that infiltrating phagocytes rapidly consume tissue glucose by running a metabolic scheme not unlike Warburg metabolism (i.e., robust glucose uptake capabilities, as well as a highly active lactate dehydrogenase, *S. aureus* has distinguished itself from other skin-dwelling staphylococci and evolved to "mimic" the metabolic state of the host at sites of inflammation.

### DISCUSSION

Many variables within host tissue necessitate nonrespiratory growth, including the production of immune radicals, the seques-
Transporters during infection is likely their affinity for glucose. This conclusion is drawn from the fact that the \textit{Δ\textit{g}lcA \textit{g}lcC} mutant, which is completely devoid of GlcNAc or ManNAc import, is fully virulent (data not shown). Thus, a role in cell wall homeostasis cannot explain the maintenance of these two genes. Rather, attenuation in the animal model of skin infection requires loss of either all carbohydrate transport (\textit{ptsH}-H15A) or specific loss of glucose transport (\textit{g}lc\textit{U}) (Fig. 5A). The fact that the \textit{ptsH}-H15A mutant alone (unable to utilize almost all carbohydrates, with the exception of glucose) is fully virulent implies that all other carbohydrates found within the host environment are incapable of sustaining \textit{S. aureus} in vivo.

In addition to meeting the energy needs of the cell, the effect of glucose on \textit{S. aureus} virulence factor regulation in the context of infection should not be ignored. Specifically, \textit{in vitro} glucose induces the expression of \textit{S. aureus} biofilm-related genes (\textit{cidA} and \textit{icaA}) and modulates the expression of the genes for a master virulence regulator (\textit{agr}/RNAIII), toxins (\textit{bla}, \textit{sec}, and \textit{tst}), and protein A (\textit{spa}) (32–36). This may explain the complete loss of lesion formation in mice infected with the \textit{Δ\textit{g}lc\textit{U}} mutant despite only a modest reduction in the viable CFU count (Fig. 5). Although we did not observe a loss of hemolytic activity in \textit{S. aureus} grown \textit{in vitro} on blood agar plates (see Fig. S4 in the supplemental material), this experiment is not quantitative and does not rule out a difference in the kinetics or cumulative levels of toxin production. Similarly, we found that the \textit{S. aureus} \textit{ptsH}-H15A \textit{Δ\textit{g}lc\textit{U}} mutant exhibited normal hemolysis but displayed reduced pigment formation. This defect may be explained as follows: (i) slow growth of

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig4}
\caption{Contributions of the identified glucose transporters to the nonrespiratory growth of \textit{S. aureus}. (A) Aerobic growth of WT and selected double, triple, and quadruple \textit{S. aureus} \textit{COL} glucose transporter mutants in CDM plus 25 mM glucose (\(n = 3\)). (B) Representative aerobic growth curve demonstrating complementation of \textit{S. aureus} \textit{COL} \textit{ΔG4} mutant growth in CDM plus 25 mM glucose by each individual glucose transporter (\(n = 3\)). (C) Percent \([U^{-14}C]\)glucose uptake by \textit{S. aureus} \textit{COL} \textit{ΔG4} relative to that of the WT, as well as \textit{ΔG4} complemented with each individual glucose transporter gene. Uptake by each strain was measured following 12 min of incubation with radiolabeled substrate and then normalized to that of the WT (\(n = 4\)). Statistical significance was calculated with a Student two-sided \(t\) test (**, \(P < 0.01\); ***,

\[P < 0.001\)). (D and E) Nonrespiratory growth rate of \textit{S. aureus} \textit{COL} \textit{ΔG4}, relative to that of the WT, compared to that of mutants expressing individual transporter genes from their native promoters. Strains were cultured anaerobically (D) or under NO stress (10 mM NOC-12–1 mM DEA-NO) (E) (\(n = 3\)). Abs, absorbance.
\end{figure}
the mutant in TSB may delay sigB activation of the crrOPQMN operon, and/or (ii) reduced carbohydrate uptake may limit the intracellular availability of glucose, a required substrate for staphyloxanthin production (37, 38). Regardless, our data indicate that carbohydrate uptake may also contribute to S. aureus infection via regulation of virulence factor production.

To contextualize our in vivo findings, one must also consider that respiration, iron acquisition, and the TCA cycle have all been shown to contribute to S. aureus virulence (39–42). Thus, we cannot accurately state that inflamed tissue spaces are strictly non-respiratory. However, it is clear from our work that S. aureus displays enhanced nonrespiratory growth phenotypes and that glycolysis-based fermentation is equally required for infection. These seemingly paradoxical findings can be reconciled by considering the temporal and spatial aspects of infection. For instance, NO production and oxygen availability are temporally regulated during S. aureus abscess development (14). Skin abscess-inducible NO synthase activity is highest 1 to 7 days after S. aureus injection and then wanes as the infection clears. However, it is clear from our work that S. aureus displays enhanced nonrespiratory growth phenotypes and that glycolysis-based fermentation is equally required for infection. These data suggest that S. aureus uses carbohydrate-based fermentative metabolism to overcome instances of high NO exposure encountered early during infection and instances of hypoxia later in infection. Moreover, bacteria within murine renal abscess have been shown to be relatively starved of iron (43). Thus, until bacterial numbers are reached such that efficient hemolysis releases hemoglobin into the tissue, allowing S. aureus sufficient iron to respire, S. aureus may rely on nonrespiratory metabolism to thrive, necessitating rapid import of glucose.

Regardless of when or why S. aureus glycolytic and glucose transporter-deficient mutants are attenuated during infection, the fact that they exhibit any attenuation at all emphasizes the importance of glucose to S. aureus disease. In particular, this observation may partially explain the unique susceptibility of uncontrolled diabetics to S. aureus infections (30). Diabetes is an important risk factor for S. aureus disease, with diabetic individuals exhibiting an increased incidence and severity of S. aureus STIs, bloodstream infections, and endocarditis (22, 28, 29, 44–46). If the susceptibility of diabetics to S. aureus infection is, in fact, augmented by the enhanced propensity of S. aureus to acquire and ferment glucose, then the development of novel S. aureus glycolysis inhibitors by Kumar et al. may constitute a particularly effective treatment for diabetics with S. aureus infections, one that both limits S. aureus growth and reduces its destructive capacity during infection (47).

MATERIALS AND METHODS

Bacterial strains and medium. All staphylococci were cultivated in TSB or CDM, wherein the primary carbon source could be modified (48). Individual carbohydrates added to CDM were carbon balanced to 25 mM glucose for all experiments, except the NO growth assay (see explanation below). Casamino Acids were added to the CDM at 0.5%. Chloramphenicol was added to TSB (10 μg/ml) and CDM (2.5 μg/ml) during the growth of plasmid-containing strains. All of the strains utilized in this study are listed in Table S3 in the supplemental material. All mutant strains, except the PTS insertion mutants, were generated via allelic replacement with Escherichia coli-S. aureus shuttle vectors pBT2ts, pBTK, pBTE, and pBTS and the new vector pBT2 as previously described (49). pBT2 was constructed by amplifying the tetK allele from S. aureus COL plasmid pT181 (tet.3A and tet.3B) and then cloning it into the XmaI site of
pB2Ts. The PTS insertion mutants were ordered from the Nebraska Mu-
tant Transposon Library (Network on Antimicrobial Resistance in Staph-
ylococcus aureus) and verified by PCR upon arrival (24). For the plasmids and
primers used for mutant construction, verification, and comple-
mentation, see Table S3. Importantly, all of the mutants used for virulence
studies were fully transduced, with the exception of the ptsH-H15A and
ptsH-H15A ΔglcU mutants. Since the ptsH-H15A mutation is markerless,
we constructed and verified three independent ptsH-H15A mutants in the
LAC background. We then separately transduced the ΔglcU mutation into
each of these three mutants and verified that each mutant grew identically
under aerobic conditions in glucose, Casamino acids, and TSB.

**Growth curves.** *Staphylococcus* cultures were grown overnight in TSB
at 37°C with shaking at 250 rpm. For aerobic, metal-restricted, and
NTreated bacterial growth curves, overnight cultures of *S. aureus* were
washed twice with phosphate-buffered saline (PBS) and diluted into TSB
with or without 2,2-dipyridyl (1 mM) or into CDM with or without car-
bon to an initial OD at 600 nm (OD660) of 0.04. Diluted cultures were then
washed twice with phosphate-buffered saline (PBS) and diluted into TSB
under aerobic conditions in glucose, Casamino acids, and TSB.

For NO growth curves, 10 mM NOC-12 (Santa Cruz Biotechnology; cat-
alog no. 202246) and 1 mM DEA NONaOate (A. G. Scientific; catalog no.
D-1013) were added to the cultures at an OD660 of 0.15. To extend the
fermentative phase of *S. aureus* NO-resistant growth, an additional, iden-
tical, dose of NO donors was added to each well 1.5 h later. To ensure
continued substrate availability during such prolonged NO exposure
(*S. aureus* utilizes carbon inefficiently during NO-induced fermentation),
we used 50 mM glucose for these experiments. For anaerobic growth curves,
the overnight cultures were washed twice with PBS and diluted into
5 ml of prewarmed (37°C) TSB or CDM with or without carbon with
or without 50 mM potassium nitrate to an OD660 of 0.08. Cultures were
prepared in duplicate in 16- by 150-mm glass tubes containing 1-mm stir
bars. Following dilution, cultures were immediately transferred into a Coy
anaerobic chamber and grown at 37°C with stirring. Growth was moni-
tored hourly by reading absorbance at 650 nm.

**Growth rate and lag analysis.** Growth rates were calculated with the
formula \( \mu = \Delta \ln (A) / \Delta \text{time} \) (hours). The time intervals used for growth
rate analysis are experiment specific and thus are provided in the figure
legends. Lag time was calculated as the time (hours) until cultures reached
an OD660 of 0.2.

**Glucose yield calculation.** Glucose yield was measured in milligrams
of glucose consumed per milligram (dry weight) of biomass for *S. aureus* COL,
our primary laboratory strain. Glucose consumption was moni-
tored by enzymatically (R-Biopharm) determining glucose in 200-
l of CDM (20 mM glucose). The resus-
tended cells were added to a scintillation vial containing 4 ml of EcoScint
A scintillation fluid (National Diagnostics). To determine the level of
radioactivity in each sample, a Beckman LS6500 Multi-Purpose Scintilla-
tion Counter was used to measure counts per minute.

**Hemolysis assays.** To detect hemolysis activity, *S. aureus* LAC strains
(WT, ΔG4, ΔglcH15A [isolates 1 to 3], and ΔtsH-H15A ΔglcU [isolates 1 to 3])
were streaked onto blood agar (Remel; tryptic soy agar [TSA] with
sheep blood; catalog no. R01200) from freezer stocks and incubated at
37°C for 36 h. Plates were subsequently incubated at 4°C for 12 h and
then imaged with a digital microscope.

**Virulence assays.** For virulence assessment, 6- to 8-week-old female
C57BL/6 mice from The Jackson Laboratory (Bar Harbor, ME) were anes-
thetized with tribromoethanol (Avertin, 0.08 mg/kg; Acros Organics; cat-
alog no. 421430100) shaved (on the flank), and injected subcutaneously
(on the flank) with 1 \( \times 10^{7} \) CFU of WT or ΔG4, ΔglcU, ΔtsH-H15A, or
ΔtsH-H15A ΔglcH15A mutant *S. aureus* LAC in 20 μl of sterile PBS. Impor-
tantly, two separate isolates of the ΔtsH-H15A and ΔtsH-H15A ΔglcH15A mu-
tants were used for infection of at least five mice apiece. On day 5, mice
were euthanized and the abscesses were removed, homogenized in 500 μl
of PBS, and diluted plated on TSA to enumerate CFU. To control for
reversion of the ΔtsH-H15A mutation during infection, WT (positive con-
trol) and ΔtsH-H15A and ΔtsH-H15A ΔglcH15A mutant abscesses were plated
on CDM agar plus sucrose (25 mM), incubated at 37°C for 48 h, and then
inspected for colonies.
Fluorescence immunohistochemistry. The Hypoxyprobe-1 Omni kit (Hypoxyprobe Inc., Burlington, MA) was used for immunohistochemical detection of tissue hypoxia. Briefly, mice were injected intraperitoneally with 60 mg/kg pimonidazole HCl 30 min prior to euthanasia. Following euthanasia, infected tissues were fixed in 10% formalin, paraffin embedded, and sectioned (5 μm). Unstained sections were deparaffinized with a series of xylene and ethanol washes, followed by antigen retrieval in boiling 10 mM sodium citrate buffer (pH 6). Tissues were blocked with 10% donkey serum (Jackson Immunoresearch, West Grove, PA) and subsequently incubated with anti-Hypoxyprobe PAb2627AP (Hypoxyprobe Inc.). The primary antibody was detected by incubation with a biotinylated donkey anti-rabbit antibody, followed by incubation with streptavidin-conjugated Dylight 594 (Jackson Immunoresearch). Tissues were mounted with ProLong antifade gold containing 4’,6-diamidino-2-phenyindole (Invitrogen, Grand Island, NY) and imaged on an Olympus BX60 fluorescence microscope with iVision software v.4.0.0 (BioVision Technologies, New Minas, Nova Scotia, Canada).

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00296-16/-/DCSupplemental.

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