INFECTION AND IMMUNITY, Dec. 2010, p. 5223–5232 Vol. 78, No. 12
0019-9567/10/$12.00 doi:10.1128/IAI.00762-10


# Comparison of the Regulation, Metabolic Functions, and Roles in Virulence of the Glyceraldehyde-3-Phosphate Dehydrogenase Homologues *gapA* and *gapB* in *Staphylococcus aureus*▽

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Received 15 July 2010/Returned for modification 5 August 2010/Accepted 18 September 2010

**The Gram-positive bacterium *Staphylococcus aureus* contains two glyceraldehyde-3-phosphate dehydrogenase (GAPDH) homologues known as GapA and GapB. GapA has been characterized as a functional GAPDH protein, but currently there is no biological evidence for the role of GapB in metabolism in *S. aureus*. In this study we show through a number of complementary methods that *S. aureus* GapA is essential for glycolysis while GapB is essential in gluconeogenesis. These proteins are reciprocally regulated in response to glucose concentrations, and both are influenced by the glycolysis regulator protein GapR, which is the first demonstration of the role of this regulator in *S. aureus* and the first indication that GapR homologues control genes other than those within the glycolytic operon. Furthermore, we show that both GapA and GapB are important in the pathogenesis of *S. aureus* in a *Galleria mellonella* model of infection, showing for the first time in any bacteria that both glycolysis and gluconeogenesis have important roles in virulence.**

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key glycolytic enzyme, the primary function of which is the oxidative phosphorylation of glyceraldehyde-3-phosphate (G3P) to 1,3-diphosphoglycerate (1,3dPG) during glucose metabolism. However, over the past decade research has shown that GAPDH homologues from both eukaryotes and prokaryotes are in fact multifunctional and can be localized in unexpected cell fractions, such as the cell surface or the nucleus (6, 12, 23, 33). The additional roles identified for GAPDH include protein binding and cell signaling in many organisms, immune evasion in bacteria, and even maintenance of the cell in higher eukaryotes (2, 3, 17, 23, 25, 26, 31). Many of these surface-localized proteins have been shown to be enzymatically active and are usually transcribed from the same open reading frame (ORF) as their cytoplasmic counterpart (8, 12, 23, 24).

While many bacteria contain a single GAPDH gene, a number of species have multiple GAPDH homologues. There are three GAPDH homologues in *Escherichia coli*: *gapA* which encodes a GAPDH protein, *gapB* which encodes an erythrose-4-phosphate dehydrogenase (E4PDH), and *gapC*, the function of which is unknown (4, 14, 28, 32, 39). The Gram-positive bacterium *Bacillus subtilis* also has two *gap* homologues, but in this case they both encode GAPDH proteins, each with opposing roles in glucose metabolism; this differs from the more common case of one GAPDH performing both roles (10). The *B. subtilis gapA* GAPDH is functional only in glycolysis, converting G3P to 1,3dPG, and has specificity for $NAD^+$ as a cofactor. The *gapB* GAPDH is gluconeogenic and specifically uses the cofactor NADPH for conversion of 1,3dPG to G3P during gluconeogenesis (10). This is consistent with the normal role of the $NADP^+$/NADPH couple in biosynthetic pathways and $NAD^+$/NADH in catabolism. In gluconeogenesis, *B. subtilis gapA* expression is repressed by the Deo-like regulator CcgR due to the absence of the glycolytic precursor fructose-1,6-bisphosphate (FBP). In the absence of FBP, CcgR binds to an operator sequence between the *ccgR* promoter and open reading frame, blocking transcript elongation rather than initiation of transcription and thus repressing expression of the glycolytic operon (9, 21, 40). There is no evidence that CcgR is involved in the regulation of *gapB* (9, 10).

*Staphylococcus aureus* is an extremely adaptable and versatile organism and is notably a commensal and opportunistic pathogen capable of causing a diverse range of infections in many human and animal body sites. Therefore, *S. aureus* is a significant problem for public health care, veterinary medicine, and the food industry (20, 37). Hospital-acquired methicillin-resistant *S. aureus* (HA-MRSA) still continues to be a concern, and there is an increasing incidence of community-acquired MRSA (CA-MRSA) infections worldwide, which are severe infections in otherwise healthy individuals and so are very unusual for a normally opportunistic pathogen. Furthermore, there is an increasing incidence of MRSA in animals, which is of particular concern as these animals may act as reservoirs for transmission of MRSA to humans and are costly to the food animal production industry. Consequently, further investigation of the fundamental nature of *S. aureus* physiology is essential.

*S. aureus* has two GAPDH homologues which share approximately 40% sequence identity with one another; they have been termed *gapA* (also known as *gapC* in a bovine mastitis isolate [13]) and *gapB*. The *gapA* gene is located within the

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▽ Published ahead of print on 27 September 2010.

TABLE 1. Bacterial strains used in this study

| Strain | Description | Reference or source |
|---|---|---|
| *E. coli* | | |
| TOP10 | *E. coli* cloning strain | Invitrogen |
| BL21 DE3 | IPTG-inducible T7 expression strain | 34 |
| *S. aureus* | | |
| RN4220 | Restriction deficient 8325-4 | Laboratory stock |
| 8325-4 | Wild-type 8325 cured of prophages | 15 |
| 8325-4 *gapA* strain | 8325-4 Δ*gapA*::*tet* | This study |
| 8325-4 *gapB* strain | 8325-4 Δ*gapB*::*spc* | This study |
| 8325-4 *gapA gapB* strain | 8325-4 Δ*gapA*::*tet* Δ*gapB*::*spc* | This study |
| 8325-4 *gapR* strain | 8325-4 Δ*gapR*::*spc* | This study |

glycolytic operon alongside several other glycolytic enzymes and has previously been shown to encode a glycolytic GAPDH (35). A homologue of the *B. subtilis* glycolytic regulator CcgR is also found in the *S. aureus* glycolytic operon and is known as *gapR*. The *gapB* gene is located as a single open reading frame alongside genes involved in DNA replication and repair.

The roles of GapB and GapR in *S. aureus* have not been determined to date. GapB is assumed to be a gluconeogenic GAPDH in *S. aureus* due to homology to the Gram-positive model organism *B. subtilis*; however, no functional evidence has been published to confirm this (7, 30). Although both organisms have *gapA* and *gapB* homologues, aspects of carbon metabolism may differ between these species due to the different environments they inhabit, and therefore the assumption that these genes behave in the same way may not be accurate. In fact, there is evidence suggesting that GapB may not be a gluconeogenic GAPDH in *S. aureus*. GapB protein purified from *B. subtilis* shows a 50-fold higher catalytic efficiency with $NADP^+$ than with $NAD^+$ (10), whereas purified recombinant GapB protein from an *S. aureus* bovine mastitis isolate shows very little $NAD^+$-dependent GAPDH activity and no $NADP^+$-dependent GAPDH activity (13). Therefore, the purpose of this work is to investigate the metabolic function of GapB in *S. aureus* and identify its role in carbon metabolism and virulence.

In this study we used a number of complementary approaches which demonstrate that *S. aureus* GapB is essential for gluconeogenesis. We also demonstrated that the glycolytic regulator GapR not only regulates *gapA* expression but also has a novel role in the regulation of *gapB*. Furthermore, both *gapA* and *gapB* showed attenuated virulence in the *Galleria mellonella* invertebrate model of infection, showing that both glycolysis and gluconeogenesis play important roles in virulence.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** All of the strains used in this study are listed in Table 1. *E. coli* strains were grown on Luria agar (LuA) or in broth (LuB), and *S. aureus* strains were grown in Trypticase soy broth (TSB) (BD Diagnostics) or Tris minimal (TM) medium, a modified minimal Tris succinate medium omitting succinate (29). Where necessary, ampicillin (100 μg/ml), kanamycin (50 μg/ml), tetracycline (10 μg/ml), erythromycin (10 μg/ml), spectinomycin (100 μg/ml), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg/ml) were added to the media. Bacterial cultures were grown at 37°C with aeration unless otherwise stated. Additional carbon sources were added to the medium to a final concentration of 1% (wt/vol) unless otherwise stated.

**Construction of mutant strains.** The *gapA* open reading frame and 1,282 bp of flanking sequence were PCR amplified using primers Gap30F and PGKSR, and a 402-bp internal deletion was created by digestion with ClaI. A terminatorless tetracycline cassette was inserted at this position, and the construct was cloned into the *E. coli-S. aureus* shuttle vector pMAD, which has a temperature-sensitive Gram-positive origin of replication (1). The 5′ and 3′ regions of the *gapB* open reading frame with flanking sequences were PCR amplified separately using the primer pair GapB5F and GapB5R and the pair GapB3F and GapB3R, to leave a 94-bp deletion within the *gapB* coding region. A PCR-amplified spectinomycin cassette (primers SpecF and SpecR) was inserted into this deletion site via an NheI site, and the construct was also cloned into pMAD. A similar approach was used for construction of the Δ*gapR* mutant, using the primer pair GapBF and GapRPR and the pair GapR3′F and GapAPR and a terminatorless spectinomycin cassette (primers SpecF and SpecRNT). All primer sequences are given in Table 2.

The mutagenesis plasmids were introduced into *S. aureus* strain RN4220, and mutagenesis was carried out as previously described (35), using TM medium for growth and selection of the Δ*gapA* mutant and Luria medium for growth and selection of the Δ*gapB* and Δ*gapR* mutants. Plates also contained 40 μg/ml X-Gal to allow blue/white selection of potential mutants (1). Mutagenesis of Δ*gapA* and Δ*gapR* was carried out directly in strain 8325-4, while mutagenesis of Δ*gapB* was performed in strain RN4220 followed by transduction by phage 11 into strain 8325-4. A Δ*gapA* Δ*gapB* double mutant was also produced by phage transduction of the Δ*gapB* mutation into the Δ*gapA* mutant background. All mutants were confirmed by PCR analysis (data not shown).

**Expression and purification of GAPDH proteins.** The open reading frames of *gapA*, *gapB*, and *gapR* were PCR amplified from 8325-4 chromosomal DNA (Table 2), cloned into the *E. coli* expression vector pLEICS03 (Protein Expression Laboratory [Protex], University of Leicester, United Kingdom), and transformed into *E. coli* Top10. The vector pLEIC03 allows inducible expression by isopropyl-β-D-thiogalactopyranoside (IPTG) and introduces an N-terminal $His_6$ tag which can be cleaved by tobacco etch virus (TEV) protease. Clones were sequenced before being transformed into the *E. coli* expression strain BL21(DE3). Cultures were grown in LuB until an optical density at 600 nm ($OD_{600}$) of 0.6 was reached, and expression was induced by the addition of IPTG to a final concentration of 100 μM. Cells were grown overnight at 20°C, and the proteins were purified using Ni-nitrilotriacetic acid (NTA) affinity chromatography. Where necessary, the hexahistidine tag was removed with TEV protease. Final protein concentrations were determined using a Bradford standard assay (5).

**Growth curves and 5-h growth assays.** Five milliliters of TSB medium (with antibiotics where necessary) was inoculated and grown overnight. The bacteria were then resuspended in fresh medium (TSB or TM medium) to an $OD_{600}$ of 0.05. Cultures were incubated at 37°C, and the $OD_{600}$ was taken either every hour for 7 h or after 5 h of growth against a blank reading of medium only. A final reading was then taken after 24 h of growth. Each assay was repeated a minimum of three times on separate days, and the results were averaged and are presented alongside the standard deviation of the data. *P* values were derived using a Student's *t* test at 4 h for growth in TSB and at 5 h for growth in TM medium.

**GAPDH assay.** For whole-cell GAPDH activity assays (23), cultures were grown in TSB medium overnight, and the $OD_{600}$ of the suspension was used to equalize the culture for cell growth. Cells were washed in distilled $H_2O$ ($dH_2O$) and then resuspended in 1 ml of GAPDH assay buffer (pH 7.5) containing 50 mM $Na_2HPO_4$, 5 mM EDTA, 40 mM triethanolamine, 2 mM DL-glyceraldehyde-3-phosphate (G3P), and either 2 mM $NAD^+$ or 2 mM $NADP^+$. Samples were incubated in a 37°C water bath for 30 min; the cells were then pelleted, and the supernatant was extracted. Production of NAD(P)H was detected by measuring the absorption of the suspension at 340 nm. For purified proteins, 200 ng of protein was mixed with GAPDH assay buffer (as above, but 4 mM G3P was used) to a final volume of 200 μl. This was carried out in triplicate for each sample in a 96-well plate, and samples were incubated at 37°C in a FLUOstar Omega plate reader. The absorption was measured at 340 nm after 30 min, and an average reading from the three wells was recorded for each sample. Each GAPDH assay (whole cell and protein) was repeated three times on separate days, and the results were averaged and are presented alongside the standard error of the data. *P* values were derived using a Student's *t* test.

**Northern blotting.** *S. aureus* cells were cultured in 10 ml of TM medium for 5 h with aeration, and RNA was extracted and analyzed by Northern blotting as previously described (18). DNA probes for *gapA*, *gapB*, *gapR*, and 16S rRNA were constructed by PCR using the primer pairs presented in Table 2. Each

TABLE 2. Primers and probes used in this study

| Description or function | Primer or probe name | Sequence (5′-3′) |
|---|---|---|
| *gapA* mutagenesis | Gap30f | TTTCTAGATTCGTACCAGCCAGAGGT |
| | PGKSR | CAACTGGTTTATGTGGATC |
| Tetracycline cassette | tetNxF | AAGGCGCCATGCTAACATAGCATTACGG |
| | tetNxR | AAGGCGCCCGATTTAGAAATCCCTTTGAG |
| *gapB* mutagenesis | GapB5F | GGGGATCCCCTTGAGATTGTTAGAAGA |
| | GapB5R | GGGCTAGCCATTAACTATTCCAAACTG |
| | GapB3F | GGGCATATGGCATTATTCCTACTTCTAC |
| | GapB3R | GAAAACTGCTCTCTTGTG |
| *gapR* mutagenesis | GapBF | GGGGATCCGCTAATGATAAGTAGTATTTAG |
| *gapR* probe | GapRPR | GGGGCTAGCCCAGTTACAGCAACTATC |
| | GapR3′F | AAAACCCGGGCACAAGGTCAAATTGTCC |
| *gapA* probe | GapAPR | CACTCTAGAGCGGAGAAGCGTTTGTGC |
| Spectinomycin cassette | SpecF | GGGGCTAGCGATATAAAATAGGTACTAATC |
| | SpecR | GGGGCTAGGGCCATATGCAAGGGTTTATTG |
| | SpecRNT | GGGGCTAGCAAACCCGGGTGTTTCCACCATTTTTTC |
| Northern probe | GapAPF | CACAGATCTGGAAGGCCATTATAATGGCAG |
| | GapBPF | GAATGGTATTACGTATTGC |
| | GapBPR | GTGCTCCAATTTGCTCAG |
| | GapRPF | GTGAAAGACTTATTGCAAG |
| | 16SF | GATCCTGGGTCAGGATG |
| | 16SR | CTAGAGTTGTCAAAGGATG |
| GapA protein expression | GapA expF | TACTTCCAATCCATGGCAGTAAAAGTAGCAATTAAT |
| | GapA expR | TATCCACCTTTACTGTCATTATTTAGAAAGTTCAGCTAAGTA |
| GapB protein expression | GapB expF | TACTTCCAATCCATGTCAACGAATATTGCAATTAAT |
| | GapB expR | TATCCACCTTTACTGTCAGAAGTCAGAGTTAGGCTATAAATTA |
| GapR protein expression | GapR expF | TACTTCCAATCCGTGAAAGACTTATTGCAAGCACA |
| | GapR expR | TATCCACCTTTACTGTCATCTTATTCAAGTATTATCTTTGCT |

experiment was repeated at least twice, and in each case blots were stripped and reprobed with the 16S probe to demonstrate even loading of the RNA in each well and to quantify expression levels where necessary. Transcripts were evaluated using ImageJ, version 1.41, software (http://rsbweb.nih.gov/ij/).

***G. mellonella* infection model.** *G. mellonella* larvae were purchased from Vine House Farms, Ltd., Spalding, United Kingdom, and were stored at 4°C. Prior to use, larvae were maintained at room temperature overnight. Inocula for the larvae were grown overnight with shaking at 37°C in Falcon tubes in 5-ml volumes of CY broth (10 g/liter Casamino Acids, 10 g/liter yeast extract, 5.9 g/liter NaCl) supplemented with appropriate antibiotics. Bacteria were pelleted by centrifugation (13,000 × *g* for 2 min) and washed and resuspended in phosphate-buffered saline (PBS) to an optical density of 0.125 at 600 nm. Inocula (20 μl; containing approximately $5 \times 10^7$ bacteria or PBS alone as a control) were injected into the larval hemolymph adjacent to the fourth proleg using a syringe with a 29-gauge needle and a Tridak Stepper repetitive dispenser (Intertronics, Kidlington, United Kingdom). Groups of 10 infected or control larvae were maintained at 37°C, and viability was determined every 24 h over a 72-h period. The results of three independent experiments were combined, and survival curves were calculated using the Kaplan-Meier method. Significant differences between survival curves were calculated using a log rank test.

## RESULTS

**Purified recombinant GapB protein does not have GAPDH activity.** In order to investigate GapB enzymatic function in *S. aureus*, the *gapA* and *gapB* open reading frames from *S. aureus* strain 8325-4 were cloned into the *E. coli* protein expression vector pLEICS03 (Protex, University of Leicester, United Kingdom). The glycolytic operon regulator GapR was also cloned into this vector to act as a negative control as it is a regulatory protein and therefore should not have any glycolytic enzyme activity. The recombinant proteins were expressed in *E. coli*, and 200 ng of purified protein was used to assay for GAPDH activity in the presence of either $NAD^+$ or $NADP^+$ as cofactor.

The GapA protein had very high levels of activity with $NAD^+$ but showed much less activity with $NADP^+$ ($P < 0.001$) (Fig. 1A), showing that GapA has a reduced affinity for $NADP^+$ and therefore is likely to have a primary role as a glycolytic GAPDH rather than a role in biosynthesis. However, neither GapB nor the negative-control GapR demonstrated any detectable enzyme activity with either $NAD^+$ or $NADP^+$ (Fig. 1A). The possibility that GapB enzyme activity was affected by the presence of the His tag was eliminated by testing for activity after TEV protease cleavage to remove the tag (data not shown). Thus, these results suggest that either GapB is not a GAPDH or the recombinant protein is nonfunctional.

**GapB has $NADP^+$- but not $NAD^+$-dependent GAPDH activity *in vivo*.** Although the purified GapB lacks $NADP^+$-dependent GAPDH activity, the key amino acid residues that determine $NADP^+$ specificity ($Ala_{32}$ and $Asn_{187}$) and G3P binding ($Thr_{179}$ and $Thr_{208}$) are present, as determined from a number of GAPDH homologues (10), which would suggest a gluconeogenic GAPDH role for this protein. Therefore, to further investigate the function of GapB in *S. aureus*, deletion/insertion mutations were introduced into the *gapA* and the *gapB* loci in strain 8325-4. A terminatorless tetracycline cassette was introduced into the *gapA* locus to allow expression of the remainder of the glycolytic operon so that only the function of GapA would be impaired. Expression of the downstream genes was confirmed by Northern blotting using a phosphoglycerate kinase (PGK) probe (data not shown). The *gapB* locus is monocistronic, so a spectinomycin cassette with promoter and terminator was introduced in strain RN4220. The mutation was then transduced by phage into strain 8325-4, removing the possibility of secondary mutations in the genome. A double mutant was also produced by phage transduction of the *gapB* mutation into the 8325-4 Δ*gapA* strain.

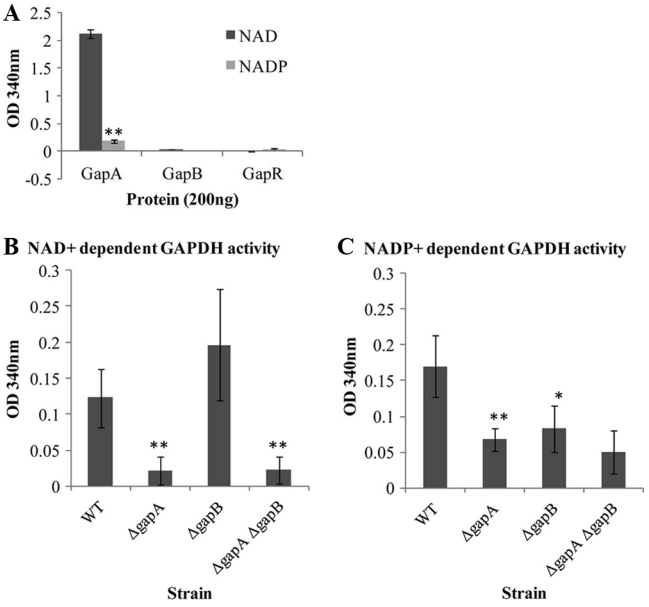


FIG. 1. Enzyme activity of *S. aureus* GAPDH proteins in the presence of either $NAD^+$ or $NADP^+$ as a cofactor. (A) GAPDH activity of N-terminal His-tagged proteins $His_6$-GapA, $His_6$-GapB, and $His_6$-GapR. Whole-cell $NAD^+$-dependent GAPDH activity (B) and whole-cell $NADP^+$-dependent GAPDH activity (C) were determined for the 8325-4, 8325-4 Δ*gapA*, 8325-4 Δ*gapB*, and 8325-4 Δ*gapA* Δ*gapB* strains. Cells were grown overnight in TSB medium and equalized for growth. The data show the average result of three repeats, and the error bars indicate the standard error. Significant differences relative to the wild-type (WT) strain are indicated as follows: ∗, $P < 0.01$; ∗∗, $P < 0.001$.

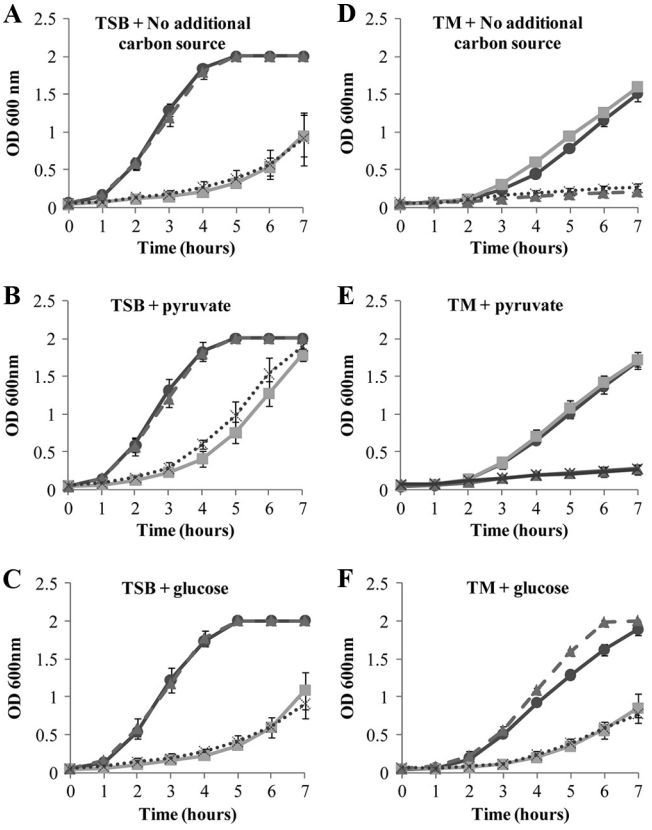


FIG. 2. Growth of the 8325-4 (black line; ●), 8325-4 Δ*gapA* (gray line; ■), 8325-4 Δ*gapB* (dashed line; ▲), and 8325-4 Δ*gapA* Δ*gapB* (dotted line; X) strains in TSB without the addition of any additional carbon source (A) or with the addition of 0.056% pyruvate (B) or 0.75% glucose (C), added at time zero. Growth of the same strains, indicated as above, was measured in TM medium without the addition of an additional carbon source (D) and with the addition of 0.056% pyruvate (E) or 1% glucose (F), added at time zero. Cultures were grown at 37°C with aeration, and growth was measured as the optical density of the culture at 600 nm. Experiments were repeated a minimum of three times on different days, and the data presented are the average of three repeats, with error bars indicating the standard deviations.

Whole-cell GAPDH activity was tested for each of the strains in the presence of both $NAD^+$ and $NADP^+$. In both the 8325-4 Δ*gapA* and 8325-4 Δ*gapA* Δ*gapB* strains, the $NAD^+$-dependent GAPDH activity was approximately 5-fold lower than that in the wild-type strain ($P < 0.001$) (Fig. 1C). There was no significant difference in activity between either the wild type and the Δ*gapB* mutant or between the Δ*gapA* mutant and the double mutant, which indicates that *in vivo* GapB does not show $NAD^+$-dependent GAPDH activity (Fig. 1C). This is consistent with GapA being solely responsible for $NAD^+$-dependent GAPDH activity in *S. aureus.*

Surprisingly, $NADP^+$-dependent GAPDH activity was found to be significantly lower in both the Δ*gapA* ($P < 0.001$) and the Δ*gapB* mutants ($P < 0.01$) than in the wild type (Fig. 1D) (by 2.5-fold and 2-fold, respectively). Therefore, both *S. aureus* GapA and GapB proteins demonstrate $NADP^+$-dependent activity *in vivo* in contrast to the purified proteins, which show no GapB $NADP^+$-dependent GAPDH activity. However, $NADP^+$-dependent GAPDH activity is not significantly lower in the double mutant than in either of the single mutants, indicating that another enzyme maybe responsible for this residual activity, which may also play a role in this process *in vivo*.

**GapA is not responsible for both glycolytic and gluconeogenic GAPDH activity in *S. aureus*.** To investigate the role of each GAPDH homologue in the growth of *S. aureus*, 24-h growth curves were carried out on the 8325-4, 8325-4 Δ*gapA*, 8325-4 Δ*gapB*, and 8325-4 Δ*gapA* Δ*gapB* strains in the complex medium TSB in the presence of either glucose or pyruvate as alternative carbon sources. TSB contains a mix of Casamino Acids and nitrogenous substances from the digest of casein and soybean meal, with 0.25% glucose (wt/vol) as the primary carbon source. Additional carbon sources were added to the medium to assess the effect of the mutations in response to changes in carbon flow. Glucose and pyruvate were chosen as they both increase carbon flow into the tricarboxylic acid cycle (TCA) cycle and increase the growth rate, but the addition of pyruvate should bypass the need for a functional glycolytic pathway.

Primary carbon metabolism was seen to be unaffected in the Δ*gapB* mutant as there was no significant difference in growth compared to the wild type under any of the growth conditions tested (Fig. 2). However, both the Δ*gapA* mutant and the Δ*gapA* Δ*gapB* double mutant showed a decrease in growth in both TSB and TSB with glucose compared to growth of the wild-type strain (Fig. 2A and C). This growth defect was significantly alleviated by the addition of pyruvate ($P < 0.01$) (Fig. 2B). Therefore, glycolysis is disrupted in the Δ*gapA* strains, but

the processes downstream such as the TCA cycle are still functional.

To further investigate the roles of GapA and GapB in carbon metabolism, growth assays were conducted in a defined medium where the carbon source could be tightly controlled. Tris minimal (TM) medium was chosen as it contains no glucose; in this medium Casamino Acids (1%, vol/vol) act as both the carbon and nitrogen sources. In TM medium in the absence of additional carbon, the Δ*gapA* mutant grew at a rate comparable to that of the wild type, indicating that this strain is able to utilize secondary carbon sources and that gluconeogenesis must be functional.

In contrast, the growth of both the Δ*gapB* mutant and the Δ*gapA* Δ*gapB* double mutant was severely limited in the absence of glucose (Fig. 2D). Furthermore, the addition of pyruvate enhanced the growth rate of the wild type and the Δ*gapA* mutant but not that of either Δ*gapB* mutants, suggesting that a loss of *gapB* results in a loss of ability to synthesize glucose (Fig. 2E). Addition of glucose significantly increased the growth of the Δ*gapB* mutant ($P < 0.001$) to a level comparable to that of the wild-type strain, whereas growth of the Δ*gapA* mutant was significantly lower in the presence of glucose ($P < 0.001$) (Fig. 2F). Glucose also enhanced the growth of the Δ*gapA* Δ*gapB* double mutant but only to a level similar to that of the Δ*gapA* mutant under these conditions.

These results show that gluconeogenesis must be functional in the Δ*gapA* mutant as it is able to grow on Casamino Acids as the sole carbon source. Consequently, the GapA GAPDH is not responsible for both glycolytic and gluconeogenic GAPDH activity in *S. aureus*. Indeed, our data suggest that GapB is involved in the gluconeogenic pathway as the Δ*gapB* mutant is unable to grow in the absence of glucose, and pyruvate did not improve the growth, which indicates a loss of gluconeogenesis and an inability to produce *de novo* glucose for use in other essential processes.

**A Δ*gapB* mutant is unable to utilize secondary carbon sources.** To determine the functional role of GapB in secondary carbon metabolism, the mutant strains were tested for their ability to utilize a wider range of secondary carbon sources. Figure 3A demonstrates where a variety of different carbon sources can enter either glycolysis or gluconeogenesis. The secondary carbon sources tested included succinate, a TCA cycle intermediate, and glutamate, an amino acid (both of which are substrates for gluconeogenesis), and glycerol as it can enter both the glycolytic and gluconeogenic pathways through conversion to G3P. Strains were grown in TM medium with each additional carbon source, and growth was measured after 5 h (Fig. 3B). The Δ*gapA* mutant was able to grow in the presence of pyruvate, succinate, and glutamate and in the absence of additional carbon at the same rate as the wild type. However, growth was significantly inhibited in the Δ*gapA* mutant compared to growth of the wild type by the presence of both glucose ($P < 0.001$) and glycerol ($P < 0.01$). Conversely, Δ*gapB* mutant growth was severely inhibited in TM medium unless glucose or glycerol was present. Glycerol enters the gluconeogenic pathway after conversion to G3P (Fig. 3A). Therefore, GapB must function before this point in the pathway for glycerol to recover the growth defect in the Δ*gapB* mutant. Therefore, the most likely function of GapB in *S. aureus* is as a gluconeogenic GAPDH.

**Transcriptional control of *gapA* and *gapB* is also reciprocal with respect to glucose, and both genes are repressed by GapR.** If the GAPDH proteins are active in opposing pathways, then they should be expressed only in response to their individual stimuli and not at the same time. Northern blot analysis was used to determine the expression of *gapA* and *gapB* after 5 h of growth in TM medium in the presence and absence of 1% glucose. When glucose is added to the medium at the start of growth, it leads to an increased growth rate, which affects cell density and the pH of the medium, two stimuli that could potentially alter gene expression. To confirm that any transcriptional differences were due to the presence or absence of glucose and not changes in cell density, growth rate, or pH, glucose was added to cultures at either time zero or at 1 h (glucose pulse) before the cells were harvested. The *gapA* probe hybridizes to multiple transcripts of various sizes due to surrounding genes being cotranscribed with *gapA* (Fig. 4A). The *gapB* probe appears to hybridize to three distinct transcripts although the sizes of these suggest that they are all transcribed from within the *gapB* reading frame and are not due to cotranscription with surrounding genes. Figure 4B and C show that, in the presence of glucose, *gapA* is induced while *gapB* is repressed. This occurs under both glucose and glucose pulse conditions, indicating that the effect is due to the presence of glucose and not the changes associated with an increase in growth rate. In contrast, in the absence of glucose and in the presence of Casamino Acids only, *gapA* transcription is repressed while *gapB* transcription is induced (Fig. 4B and C and Table 3). Consequently, these data indicate that transcription of *gapA* and *gapB* is reciprocally controlled by glucose levels, which is further evidence that they act in opposing pathways.

In *B. subtilis* the glycolytic operon is repressed by CcgR under gluconeogenic conditions (8, 35). Sequence analysis shows that there is no evidence of a *ccgR*-like consensus binding sequence in the putative *S. aureus gapR* promoter region. However, to determine whether the *ccgR* homologue *gapR* plays a similar regulatory role in *S. aureus*, a mutation was introduced into the *gapR* locus in strain 8325-4. A deletion/insertion method was used to incorporate a terminatorless spectinomycin resistance cassette into *gapR* to allow constitutive expression of the downstream genes of the glycolytic operon. To assess transcription of the glycolytic operon by Northern blotting, a *gapR* probe which hybridized upstream of the inserted spectinomycin cassette was used in place of the *gapA* probe so that expression of the spectinomycin promoter was not detected. Overall, *gapR* appears to be expressed at a lower level than *gapA* in wild-type *S. aureus* although the response to glucose is consistent (Fig. 4D). This is interesting as it demonstrates differential levels of expression between the operon regulator and the glycolytic genes even though they form a polycistronic transcript. Due to an apparent lack of a promoter upstream of *gapA*, this variable expression may be a product of posttranscriptional processing of the polycistronic gene transcripts, as seen in *B. subtilis* (17).

Expression of *gapR* and the glycolytic operon increased under both glycolytic and gluconeogenic conditions in the Δ*gapR* mutant compared to the wild type (Fig. 4D). This indicates that GapR represses the glycolytic operon under gluconeogenic conditions and limits the level of expression in the presence of

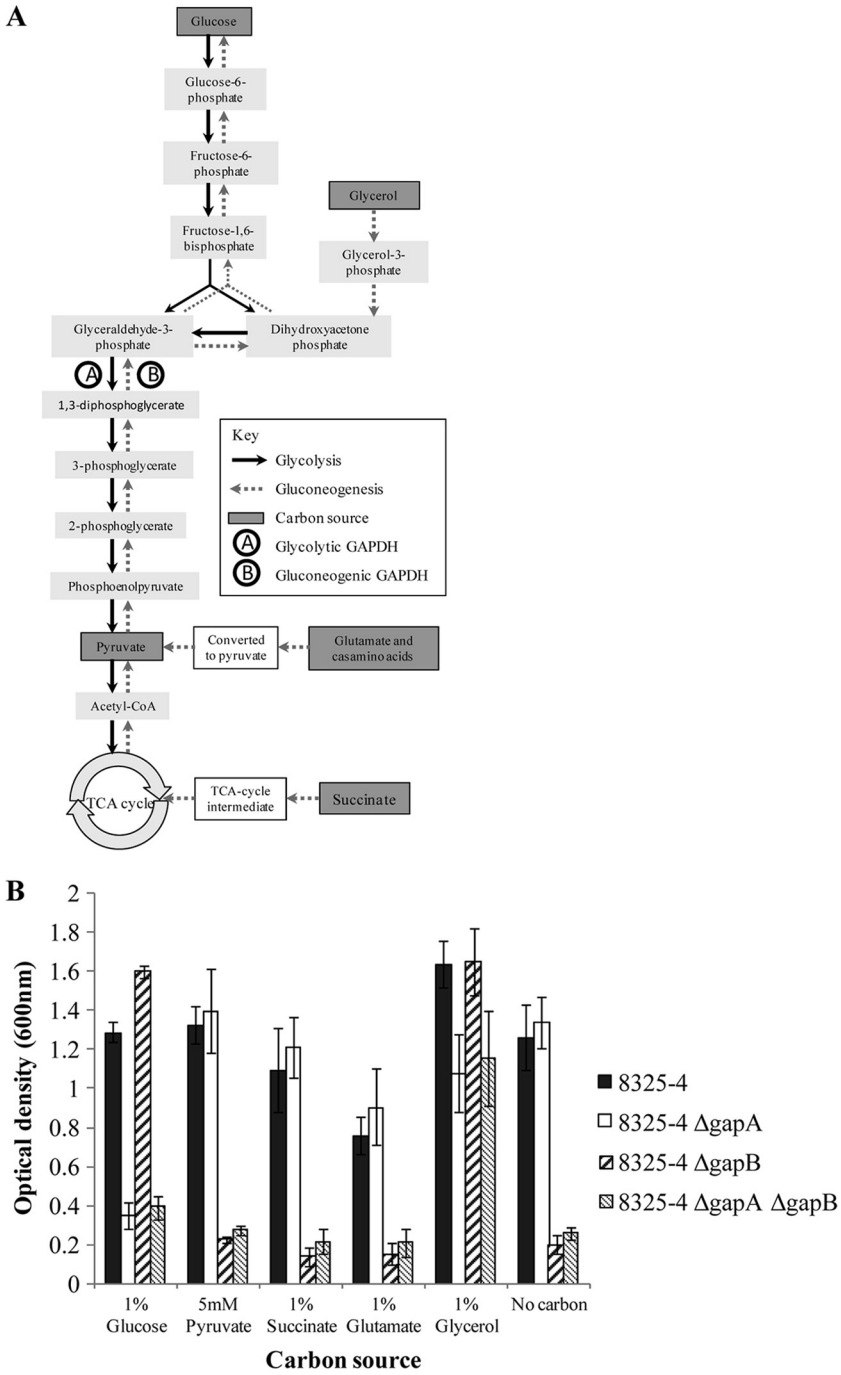


FIG. 3. (A) Flow chart indicating where the various carbon sources can enter glycolysis and gluconeogenesis during carbon metabolism. (B) Graph showing the level of growth of the 8325-4, 8325-4 Δ*gapA*, 8325-4 Δ*gapB*, and 8325-4 Δ*gapA* Δ*gapB* strains after a 5-h incubation in TM medium supplemented with an additional carbon source. Cultures were grown at 37°C with aeration, and growth was measured as the optical density of the culture at 600 nm. Each experiment was repeated three times on different days, and the data presented are averages, with error bars indicating the standard deviations. CoA, coenzyme A.

glucose. Interestingly, the expression of *gapB* compared to that in the wild type was also increased under gluconeogenic and glycolytic conditions in the Δ*gapR* mutant (Fig. 4E). These data are supported by densitometry analysis which is presented as a ratio of mRNA/16S transcript levels (Table 3).

Therefore, *S. aureus* GapR also represses the transcription of *gapB* and limits its expression under gluconeogenic conditions. This indicates that the function of GapR is not limited to control of the glycolytic operon but is also involved in regulating gluconeogenic metabolism in *S. aureus*. To our knowledge this is the first evidence in any bacteria that a CcgR-like homologue acts to regulate genes other than those of the glycolytic operon.

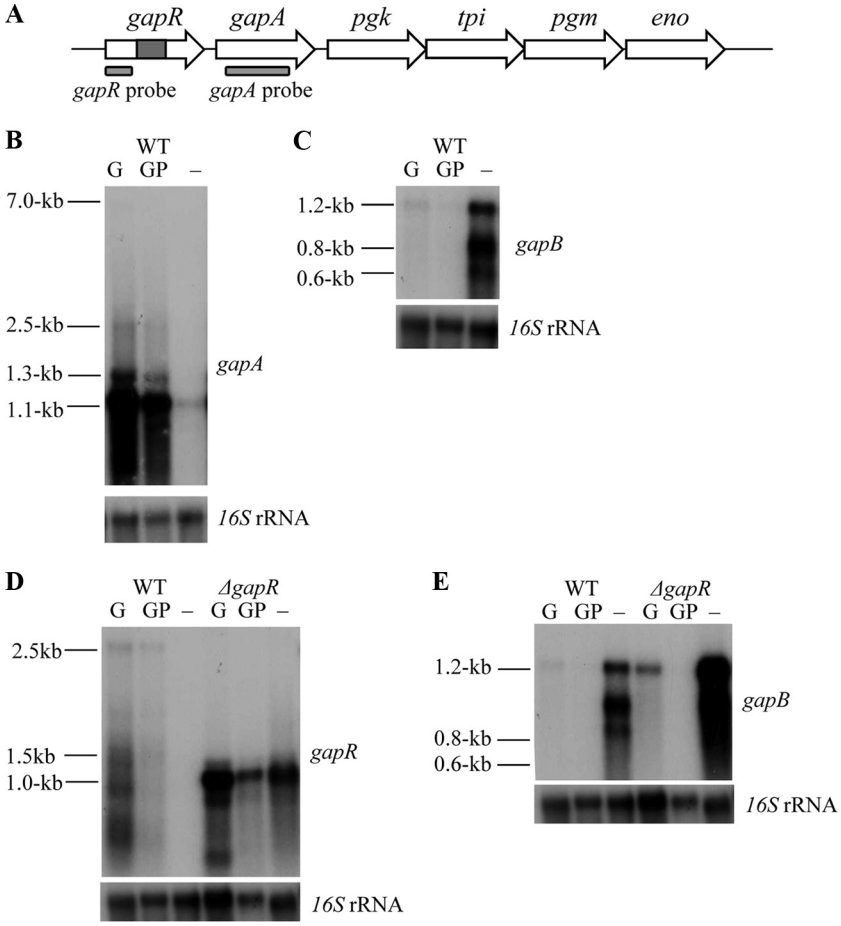


FIG. 4. (A) Schematic representation of the glycolytic operon with a Δ*gapR* mutation, indicating the position of the *gapR* and *gapA* probes used for Northern blot analysis. Genes are putative operon regulator (*gapR*), glyceraldehyde-3-phosphate dehydrogenase (*gapA*), phosphoglycerate kinase (*pgk*), triphosphate isomerase (*tpi*), phosphoglycerate mutase (*pgm*), and enolase (*eno*). Northern blot analysis is shown of *gapA* transcript (B) and *gapB* transcript (C) expression in response to glucose induction in strain 8325-4 and *gapR* transcript (D) and *gapB* transcript (E) expression in response to glucose induction in both wild-type (WT) 8325-4 and 8325-4 Δ*gapR* strains. Total RNA was extracted from cells grown for 5 h in TM broth with 1% glucose added at time zero (G), 1% glucose added 1 h before cells were harvested (GP), and without glucose (−). Gels presented are representative of experiments that were repeated two times using RNA extracts from cultures grown on different days, with similar results observed each time. Blots were stripped and rehybridized with a control probe (16S) to ensure equal loading of RNA in each case.

**Glucose inhibits growth in a Δ*gapA* mutant and promotes growth in a Δ*gapB* mutant in a reciprocal manner.** *In vivo* normal blood glucose levels are within the range of 3.6 to 5.8 mM, and even under trauma conditions the glucose levels increase to only 12 mM (19). Consequently, the 1% glucose concentration used here is much higher than physiological levels. Therefore, to determine the level of glucose required to switch from *gapA* expression to *gapB* expression and thus determine whether glucose will have a similar effect on *S. aureus* growth *in vivo*, the GAPDH mutant strains were grown in TM medium for 5 h in the presence of varied concentrations of glucose, ranging from 0.001% to 0.2% (Fig. 5A). At 0.001% glucose the Δ*gapA* mutant and wild-type strains are able to grow, but both the Δ*gapB* and the Δ*gapA* Δ*gapB* mutants have severe growth defects. As glucose concentrations are increased, the growth of the wild type and Δ*gapB* mutant also increased while the growth of the Δ*gapA* mutant is decreased. Above 0.05% glucose the Δ*gapB* mutant grows at a comparable level to that of the wild-type strain while little or no growth can be detected in either the Δ*gapA* or the Δ*gapA* Δ*gapB* double mutant strains. The point at which the switch from gluconeogenic to glycolytic metabolism occurs is approximately 0.01% glucose. The process by which this switch occurs is likely to be carbon catabolite repression (CCR). In the presence of a readily metabolized carbon source such as glucose, the genes

TABLE 3. Densitometry analysis of Northern blots

| Probe (figure no.) | Transcript levels in the WT with:[a] | | | Transcript levels in the Δ*gapR* strain with:[a] | | |
|---|---|---|---|---|---|---|
| | Glucose | Glucose pulse | No carbon | Glucose | Glucose pulse | No carbon |
| *gapA* (4B) | 1.08 | 0.97 | 0.15 | NA[b] | NA | NA |
| *gapB* (4C) | 0.08 | 0.07 | 1.09 | NA | NA | NA |
| *gapR* (4D) | 0.90 | 0.26 | 0.04 | 0.94 | 0.56 | 0.77 |
| *gapB* (4E) | 0.08 | 0.07 | 1.09 | 0.33 | 0.15 | 1.42 |

[a] Values represent the ratio of mRNA/16S transcript levels.
[b] NA, not applicable.

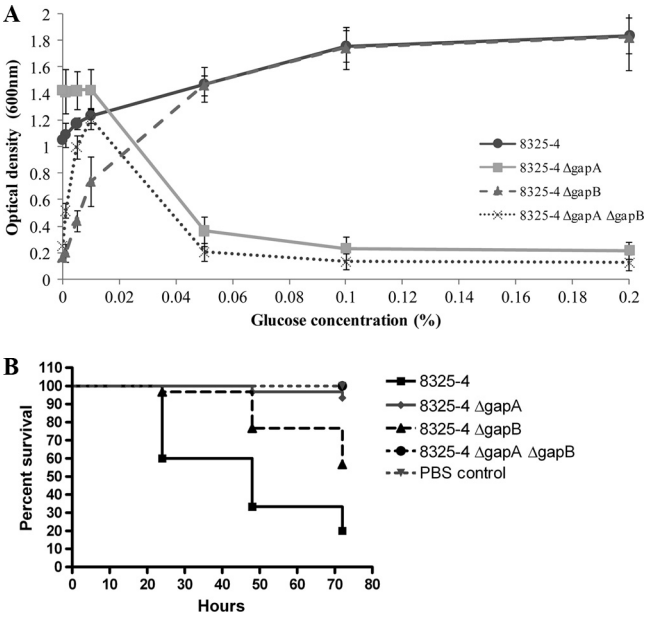


FIG. 5. (A) Growth of 8325-4 and isogenic Δ*gapA*, Δ*gapB*, and Δ*gapA* Δ*gapB* mutants after 5 h of incubation in TM medium with varied concentrations of glucose. Cultures were grown at 37°C with aeration, and growth was measured as the optical density of the culture at 600 nm. Each experiment was repeated three times on different days, and results are presented as averages with error bars indicating the standard deviations. (B) Effect of the GAPDH mutations on the pathogenesis of *S. aureus* infection in *G. mellonella*. The graph indicates the percent viability of infected *G. mellonella* larvae at 24, 48, and 72 h postinfection with *S. aureus* 8325-4 wild-type strain and the isogenic Δ*gapA*, Δ*gapB*, and Δ*gapA* Δ*gapB* mutants. With the PBS negative control, larvae showed 100% survival at all time points. The results from three independent experiments were combined and used to produce survival curves using the Kaplan-Meier method.

responsible for secondary carbon metabolism are repressed, ensuring sequential utilization of available carbon sources based on how favorable they are to the bacteria. When glucose reaches 0.01%, growth of the Δ*gapA* mutant begins to decrease while growth of the Δ*gapB* mutant begins to increase, indicating repression of secondary carbon metabolism genes and derepression/induction of glucose metabolism. Therefore, Fig. 5A clearly demonstrates that at 0.01% glucose the bacteria switch from utilizing the highly abundant Casamino Acids as their carbon source to utilizing the favorable but limited carbon source of glucose.

As both pathways are disrupted in the double mutant, it could be assumed that the strain would be unable to grow in any of the concentrations of glucose, but unexpectedly the growth of the double mutant increases with glucose concentration until 0.01%, and then growth levels rapidly decrease as glucose concentrations further rise. This suggests that 0.01% glucose is sufficient for growth without the need for *de novo* glucose from gluconeogenesis. The reason that we fail to see this level of growth in the Δ*gapB* single mutant may be because the functional glycolytic pathway will utilize this glucose, limiting its availability for other metabolic functions in *S. aureus*. Therefore, the regulation of carbon metabolism is tightly controlled in *S. aureus* to ensure that the bacteria utilize environmental carbon sources in the most efficient way possible.

**Both *gapA* and *gapB* are required for pathogenesis in an invertebrate model of infection.** To determine the importance of both GapA and GapB in the wider role of host infection, larvae of *Galleria mellonella* were infected with wild-type 8325-4 and each of the isogenic GAPDH mutant strains. In this infection model, pathogenesis of the bacterial strain is measured by the percent viability of the infected larvae at 24, 48, and 72 h postinfection. The results show that both *gapA* and *gapB* are required for a full pathogenic phenotype of *S. aureus* in this model (Fig. 5B). Survival of the Δ*gapA* mutant ($P < 0.0001$), the Δ*gapB* mutant ($P = 0.0003$), and the double mutant ($P < 0.0001$) is significantly reduced compared to that of the wild-type strain. At 72 h postinfection the percent viability of the *Galleria* was lower in the larvae infected with the wild-type strain (20%) than in larvae infected with either the Δ*gapA* (93.3%) or the Δ*gapB* (56.7%) strain, showing that virulence was reduced in each of the mutants. Furthermore, pathogenesis of *S. aureus* was attenuated entirely in the double mutant strain, with 100% survival of the *Galleria* seen at all time points. This indicates that the loss of either *gapA* or *gapB* results in a reduction in virulence in *S. aureus*, which shows that at least during infection in this model, *S. aureus* relies on both primary and secondary carbon metabolism to utilize any available carbon source in the host environment.

## DISCUSSION

In this study we have shown that in the pathogenic organism *S. aureus*, the GAPDH homologue GapB is an essential component in anabolic carbon metabolism. We also showed for the first time that the regulator *gapR* not only controls expression of *gapA* and the glycolytic operon but also regulates the level of *gapB* expression during gluconeogenesis. In addition, we have demonstrated that GapA and GapB are important for virulence in an invertebrate model of infection, showing that both glycolysis and secondary carbon metabolism play important roles during *S. aureus* infection.

In *S. aureus* it has been assumed that GapA is a glycolytic GAPDH protein and that GapB is a gluconeogenic GAPDH protein although there has been no published biological evidence (7, 30). In fact, recombinant $His_6$-tagged GapB protein cloned from a bovine mastitis isolate of *S. aureus* was shown to have little or no GAPDH activity with either $NAD^+$ or $NADP^+$ as a cofactor (13). We confirmed these findings with recombinant GapB from a human *S. aureus* isolate and were also able to demonstrate that this lack of activity is not due to the presence of the $His_6$ N-terminal tag. In *E. coli*, the second GAPDH homologue, GapB, functions as an $NAD^+$-dependent E4PDH in the pyridoxine (vitamin $B_6$) biosynthesis pathway (32, 38, 39). We have shown that this is not the case in *S. aureus* as GapB has no E4PDH activity with either $NAD^+$ or $NADP^+$ (data not shown).

However, our data suggest that although the recombinant GapB protein lacks specific GAPDH activity *in vitro*, *S. aureus* GapB is in fact a gluconeogenic enzyme. GapA cannot be solely responsible for gluconeogenic GAPDH activity in *S. aureus* as the Δ*gapA* mutant grows as well as the wild-type strain in medium lacking glucose, indicating that glycolysis is disrupted but that secondary carbon metabolism is still functional. In addition the Δ*gapB* mutant strain shows a reduction

in $NADP^+$-dependent GAPDH activity, is unable to grow in medium lacking glucose, and is unable to utilize secondary carbon sources. Thus, these data indicate a loss of anabolic carbon metabolism, most likely due to disruption of the gluconeogenesis pathway. Interestingly, the Δ*gapB* mutant is able to grow with glycerol as the primary carbon source. Glycerol enters the gluconeogenic pathway as G3P, the product of gluconeogenic GAPDH activity. Therefore, GapB must function before this stage in the gluconeogenic pathway in order for glycerol to recover the growth defect seen in the Δ*gapB* mutant.

The reciprocal growth response of the Δ*gapA* and Δ*gapB* mutants in response to glucose is mirrored in the reciprocal regulation of the transcription of *gapA* and *gapB*. Transcriptional analysis confirms that in wild-type *S. aureus gapA* expression is high in the presence of glucose but repressed under gluconeogenic conditions while *gapB* is expressed only in the absence of glucose. This is consistent with the GAPDH homologues in *B. subtilis* although transcription of these genes was determined by using integrated *lacZ* reporter systems in mutant strains rather than by analysis of wild-type RNA levels (10). By measuring the growth of Δ*gapA* and Δ*gapB* strains in variable concentrations of glucose, we have also been able to determine that as little as 0.01% glucose in the medium is sufficient for *S. aureus* to switch carbon metabolism from gluconeogenesis to glycolysis. Taken together, these data strongly suggest that in *S. aureus* GapA functions only in glycolysis and GapB functions only in gluconeogenesis.

Based on homology to *B. subtilis cggR*, the Deo-like regulator *gapR* has been proposed as the regulator of the glycolytic operon in *S. aureus* although no data on this gene have been published to date. Our study shows that in *S. aureus* GapR not only functions to regulate the glycolytic operon but also plays a role in the regulation of *gapB* transcription. In a Δ*gapR* knockout strain the expression of the glycolytic operon is derepressed under both glycolytic and gluconeogenic conditions. Interestingly, the expression of *gapB* is also higher in a *gapR* mutant under gluconeogenic conditions. This indicates a role of *gapR* in limiting the expression of *gapB* when glucose concentrations are low, which could indicate a very complex regulatory relationship between glycolysis and gluconeogenesis in *S. aureus*. In *B. subtilis* CggR represses the glycolytic operon in the absence of fructose-1,6-bisphosphate (FBP), an early product of glycolysis (9, 10, 22), by binding to a consensus sequence 32 bp downstream of the transcriptional start site of the operon and blocking elongation of the transcript (9). The transcription of the operon is also indirectly induced by carbon catabolite protein A (CcpA) under glycolytic conditions, providing multiple levels of control (22). Genome-wide sequence homology searches and transcriptome comparison between the *B. subtilis* wild type and a Δ*cggR* mutant strongly suggest that the function of CcgR is limited to the glycolytic operon in *B. subtilis*, whereas CcpA is a key regulator of carbon metabolism and controls many other genes (9, 36). Sequence analysis of the putative promoter regions of *S. aureus gapR* and *gapB* does not reveal a similar GapR target sequence in *S. aureus*, and therefore further work is required to determine the mechanism by which GapR represses transcription of genes in *S. aureus*. Interestingly, a recent microarray study has shown that in *S. aureus* CcpA is involved in carbon catabolite repression of *gapB* in the presence of glucose, but the effect on the induction of *gapA* expression was less clear (30). Therefore, further study of the regulation of both GAPDH homologues would need to investigate the interplay of GapR and CcpA on controlling gene expression.

The roles of glycolysis and gluconeogenesis in virulence have not been investigated in any bacteria to our knowledge. Therefore, we investigated the impact that each of the GAPDH homologues has on host virulence in a *Galleria mellonella* model of infection. Larvae of the greater wax moth *G. mellonella* have been shown to provide a useful insight into the pathogenesis of a wide range of bacterial and eukaryotic microbial infections including *S. aureus* (27). The *Galleria* model is particularly useful as these insects share many common aspects with mammalian innate immunity. Furthermore, *Galleria* infection model results consistently correlate with those of similar mammalian studies as bacterial strains that are attenuated in mammalian models demonstrate lower virulence in *Galleria*, regardless of the species of bacterium studied (11, 16, 27). Our data showed that both GapA and GapB are required during *S. aureus* infection. The loss of either of the proteins individually resulted in increased survival of the infected larvae while a loss of both proteins left the bacteria severely attenuated, and all of the infected larvae survived. Primary carbon utilization appears to be more important than secondary carbon utilization during infection as the *Galleria* showed 93.3% viability at 72 h postinfection with the Δ*gapA* mutant compared to 56.7% with the Δ*gapB* mutant, but it is still clear that both pathways are required for *S. aureus* to make use of all available carbon sources and infect the host to its full virulence potential. The main sugar present in the hemolymph is trehalose, but published literature does suggest that some glucose is also present. *S. aureus* is able to utilize trehalose which is broken down to glucose-6-phosphate by TreC. This feeds directly into glycolysis before the action of GapA. Therefore, the variation in the abilities of wild-type *S. aureus* and the Gap mutants to kill *Galleria* is in part due to the inability of the mutants to use the available carbon sources in the hemolymph, in agreement with the observations made *in vitro*. It is also possible that each of the proteins may have other "moonlighting" roles in infection, such as seen in *Streptococcus pyogenes*, where surface-associated GAPDH has been shown to have antiphagocytic properties and is involved in host cell adherence (3), but further study needs to be undertaken to determine any such roles in *S. aureus*.

In conclusion, the data presented in this study identify GapB as an essential component in secondary carbon use in *S. aureus*, and the evidence strongly suggests that it is essential for gluconeogenesis. Furthermore, both GapA and GapB are important during *S. aureus* infection and are required for pathogenesis. Thus, the data presented here may lead to a clearer understanding of the complex control of carbon metabolism in this important pathogen and the role that carbon metabolism plays in virulence during *S. aureus* infection.

## ACKNOWLEDGMENTS

This study was partially supported by a Doctoral Training Grant from the BBSRC to Joanne Purves.

We thank Xiaowen Yang at Protex (University of Leicester) for the cloning of pLEIC03 protein expression constructs and the Protein

Nucleic Acid Chemistry Laboratory (University of Leicester) for the processing of all sequencing data.

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*Editor:* A. J. Bäumler