Tricarboxylic Acid Cycle-Dependent Synthesis of *Staphylococcus aureus* Type 5 and 8 Capsular Polysaccharides[▽]

Marat R. Sadykov,[1] Theodoric A. Mattes,[1] Thanh T. Luong,[2] Yefei Zhu,[1] Shandra R. Day,[3] Costi D. Sifri,[3] Chia Y. Lee,[2] and Greg A. Somerville[1]*

Department of Veterinary and Biomedical Sciences, University of Nebraska, Lincoln, Nebraska[1]; Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, Arkansas[2]; and Division of Infectious Diseases and International Health, University of Virginia Health System, Charlottesville, Virginia[3]

Staphylococcus aureus capsule synthesis requires the precursor *N*-acetyl-glucosamine; however, capsule is synthesized during post-exponential growth when the availability of *N*-acetyl-glucosamine is limited. Capsule biosynthesis also requires aerobic respiration, leading us to hypothesize that capsule synthesis requires tricarboxylic acid cycle intermediates. Consistent with this hypothesis, *S. aureus* tricarboxylic acid cycle mutants fail to make capsule.

Staphylococcus aureus produces two major exopolysaccharides: poly-*N*-acetylglucosamine (PNAG) and capsule. PNAG is synthesized by enzymes encoded by genes within the intercellular adhesin (*ica*) operon (*icaADBC*) (5, 15) during the exponential growth phase when C_6 carbohydrates are abundant (5, 10, 12). In contrast, capsular polysaccharides are predominantly synthesized during the post-exponential growth phase when C_6 carbohydrates are in short supply (13). The most commonly encountered *S. aureus* capsule types, 5 and 8, are synthesized from the amino sugars *N*-acetyl-D-fucosamine, *N*-acetyl-L-fucosamine, and *N*-acetyl-D-mannosaminuronic acid (17). Interestingly, the biosynthetic precursor of the capsular sugars is UDP-*N*-acetylglucosamine (11), the same amino sugar used in synthesizing PNAG. *N*-acetylglucosamine is synthesized from the glycolytic intermediate fructose 6-phosphate, and in a rich medium containing glucose, abundant levels of fructose 6-phosphate will be generated by glycolysis. As stated, capsule is most abundantly synthesized in the post-exponential phase of growth when glucose is growth limiting (4, 13).

In the absence of glucose, fructose 6-phosphate can be synthesized by gluconeogenesis from the tricarboxylic acid (TCA) cycle intermediate oxaloacetate. To do this, oxaloacetate undergoes an ATP-dependent decarboxylation and phosphorylation by phosphoenolpyruvate carboxykinase (*pckA*) to generate phosphoenolpyruvate (PEP) (19). Gluconeogenesis can then generate fructose 6-phosphate from PEP, which can be used for UDP-activated *N*-acetylglucosamine biosynthesis. Support for the idea that post-exponential-phase capsule biosynthesis requires TCA cycle activity and PEP carboxykinase can be found in the observation that capsule is made during aerobic growth (6). In the Dassy and Fournier study, respiratory chain inhibitors were used to show that respiratory activity or a high proton motive force is required for post-exponential-growth-phase capsule biosynthesis (6). Although this was an

excellent study, it was unclear as to why inhibiting respiratory activity prevented capsule biosynthesis. One consequence of inhibiting respiratory activity is the accumulation of reducing potential in the form of NADH. As NADH accumulates, the intracellular concentration of NAD^+ decreases and the activity of NAD^+-requiring enzymes similarly decreases. During the post-exponential growth phase, the primary consumer of NAD^+ is the TCA cycle; therefore, inhibiting respiratory activity inhibits TCA cycle activity. Further support that post-exponential capsule biosynthesis requires TCA cycle activity can be seen in transposon mutagenesis studies that found inactivation of PEP carboxykinase, the TCA cycle (*citB*, *citC*, *citG*, or *odhA*), or *N*-acetylglucosamine biosynthesis (*glmM*) reduces killing in a *Caenorhabditis elegans* killing assay to the same extent as do mutations in capsule genes (1, 2). Despite these observations suggesting that TCA cycle activity is required for capsule biosynthesis, capsule accumulation has never been measured in TCA cycle-deficient *S. aureus* strains. The objective of this study was to determine if TCA cycle activity was required for the biosynthesis of capsule types 5 and 8.

To determine if TCA cycle activity was required for *S. aureus* capsular polysaccharide biosynthesis, the amount of capsule produced by wild-type strains SA564 and UAMS-1 was compared to that produced by isogenic aconitase mutant strains SA564 *acnA*::*ermB* and UAMS-1 *acnA*::*tetM*. (Strains UAMS-1 and SA564 are low-passage, human isolates recovered from patients with osteomyelitis or toxic shock syndrome, respectively [8, 20].) In addition, to make certain that any differences in capsule accumulation were not specific to the aconitase gene, the genes coding for citrate synthase (*citZ*) and isocitrate dehydrogenase (*citC*) were inactivated in strain SA564, and the ability of these mutants to produce capsule was also determined. All TCA cycle mutants were constructed as previously described (18, 21, 25) by replacing *acnA-citB*, *citZ*, or *citC* in the capsule type 5-producing strain SA564 and *acnA-citB* in the capsule type 8-producing strain UAMS-1 with a *tetM* or *ermB* cassette. To minimize the possibility that any phenotypes were the result of random mutations occurring during temperature shifts, all mutations were backcrossed into the appropriate wild-type strain by using
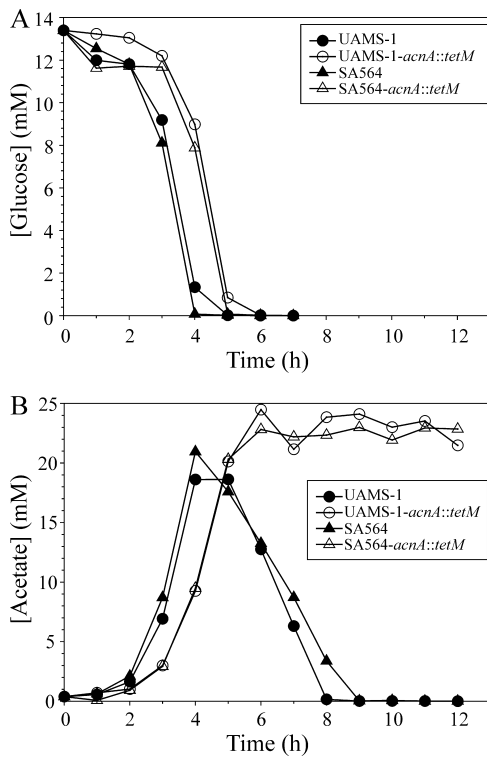
FIG. 1. Glucose depletion (A) and acetate accumulation and depletion (B) in the culture supernatants of wild-type strains SA564 and UAMS-1 and aconitase SA564 *acnA*::*tetM* and UAMS-1 *acnA*::*tetM* mutant strains, plotted as a function of time. Bacterial cultures were grown in tryptic soy broth (Difco) at 37°C, with a flask-to-medium ratio of 10:1, and aerated at 225 rpm. The results are representative of three independent experiments.

transducing phage 85 (16). Genetic inactivation of each gene was confirmed by PCR, Southern blotting (data not shown), and phenotypic assays. The phenotypic assays consisted of enzyme assays (i.e., aconitase, isocitrate dehydrogenase, and citrate synthase [22]), growth curves, and measuring the glucose and acetate concentrations present in the culture medium over time (Fig. 1 and data not shown). Acetate and glucose concen-
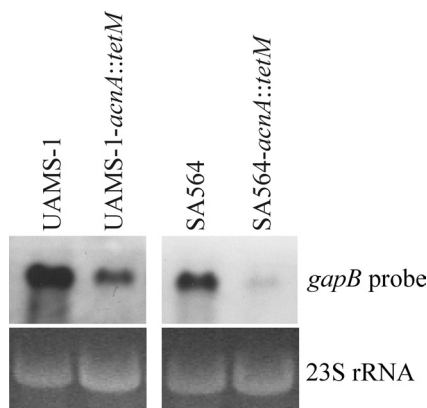

FIG. 2. Northern blot analysis of *gapB* (glyceraldehyde-3-phosphate dehydrogenase) at 6 h postinoculation. The results presented are representative of two independent experiments.
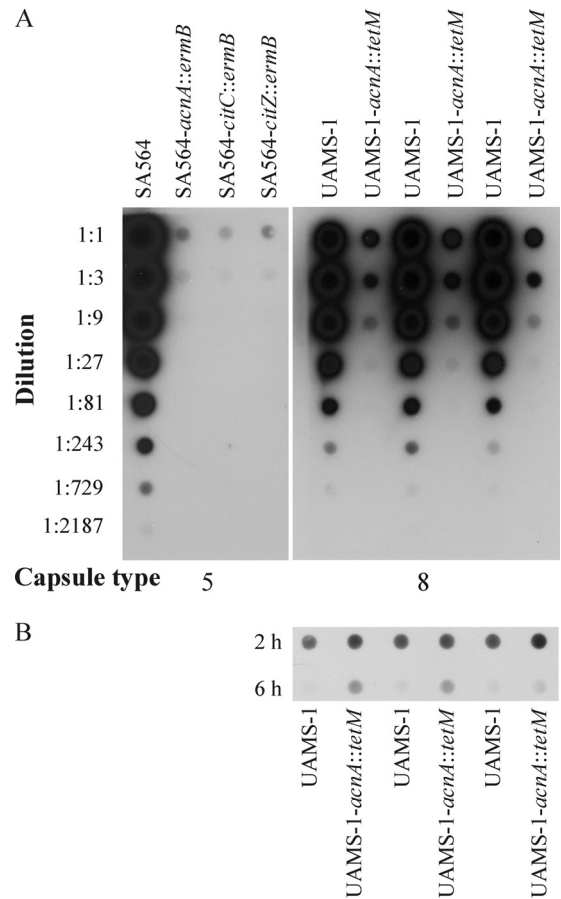

FIG. 3. TCA cycle activity is required for capsule biosynthesis but not for PNAG synthesis. (A) For the capsule blots, bacteria were harvested after overnight growth in tryptic soy broth at 37°C, with a flask-to-medium ratio of ~20:1, and aerated at 225 rpm. Left panel, type 5 capsule immunoblot of wild-type strain SA564 and three TCA cycle mutants (*citZ*, *citC*, and *acnA-citB* mutants). The results are representative of experiments performed at least three times. Right panel, type 8 capsule immunoblot of wild-type strain UAMS-1 and the isogenic aconitase mutant strain UAMS-1 *acnA*::*tetM*. The results are from three independent experiments. (B) PNAG immunoblot of strains UAMS-1 and UAMS-1 *acnA*::*tetM*. PNAG immunoblot analyses were performed as described previously (26) on samples collected after 2 and 6 h of growth. The results are from three independent experiments.

trations were determined with kits purchased from R-Biopharm and used according to the manufacturer's protocol. As expected, inactivation of the TCA cycle did not impair glucose catabolism; however, it completely inhibited the catabolism of acetate in all TCA cycle mutant strains. (In staphylococci, acetate catabolism requires a fully functioning TCA cycle [9, 21, 23].) The inhibition of acetate catabolism will decrease the availability of oxaloacetate and PEP, preventing gluconeogenesis. To assess the effect of TCA cycle inactivation on gluconeogenesis, Northern blot analysis was performed using a probe for the glyceraldehyde-3-phosphate dehydrogenase gene *gapB*. Although several enzymes involved in glycolysis and gluconeogenesis can function in the catabolic and anabolic directions, many bacteria, including staphylococci, use two distinct glyceraldehyde-3-phosphate dehydrogenase enzymes encoded by
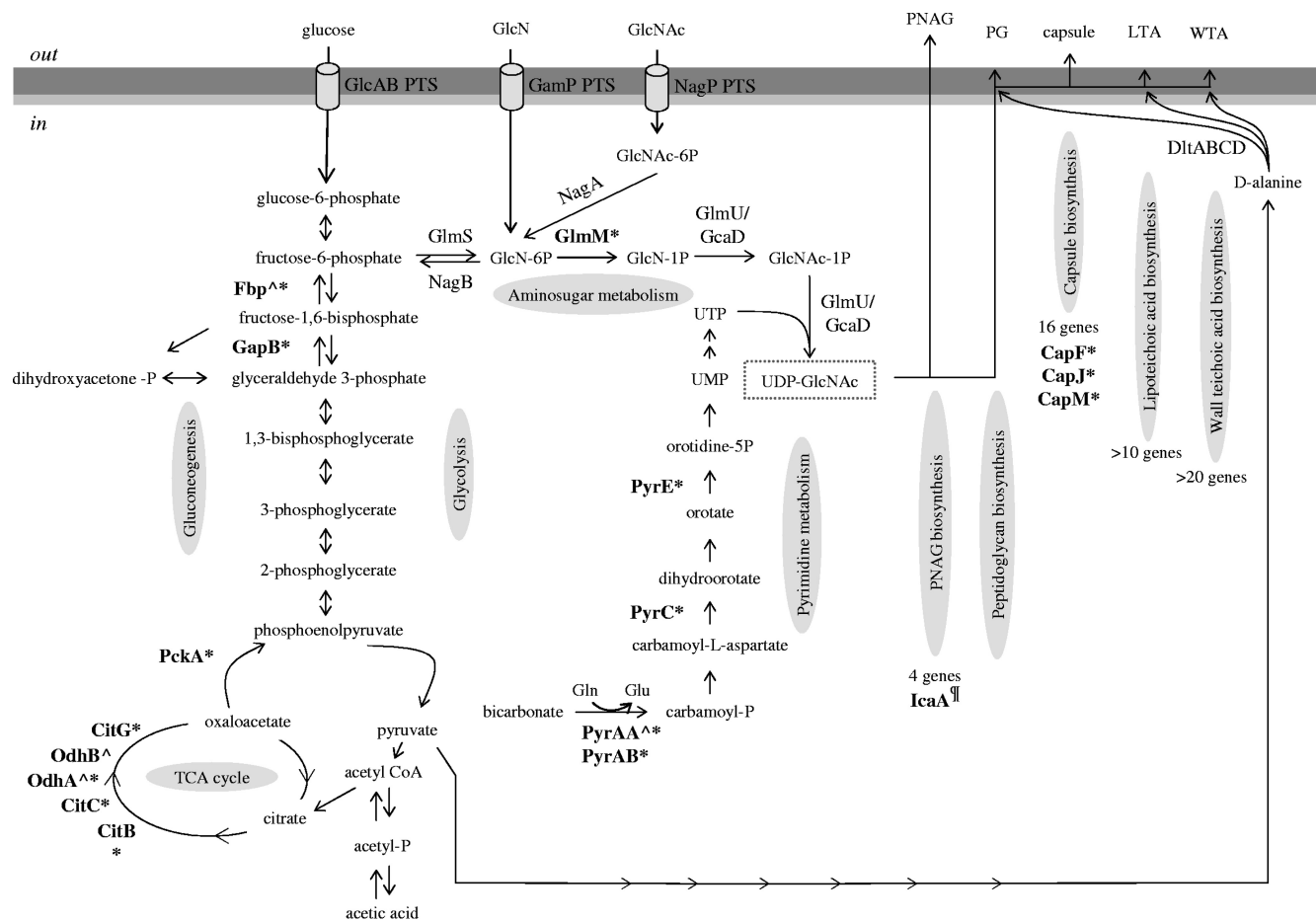
FIG. 4. Schematic representation of metabolic genes and pathways important for UDP-*N*-acetylglucosamine, capsule, and PNAG biosynthesis. For comparison with previous work, boldface type indicates products of virulence-associated genes identified in *C. elegans* killing assays. ^, Begun-Sifri screen (2); *, Bae et al. screen (1); ¶, PNAG biosynthetic locus important for *Staphylococcus epidermidis* and *S. aureus* virulence (3). Mutants in committed steps of gluconeogenesis (PckA, GapB, Fbp), *N*-acetylglucosamine metabolism (GlmM/FemD), and UTP biosynthesis (PyrAA, PyrAB, PyrC, PyrE) suggest that UDP-*N*-acetylglucosamine is a pivotal metabolic intermediate. UDP-*N*-acetylglucosamine serves as a biosynthetic precursor for peptidoglycan, biofilm, capsule, and teichoic acids. Biofilm and capsule have been shown to be important in both nematode and mammalian infections by staphylococci.

gapA (catabolic) and *gapB* (anabolic) (7); hence, *gapB* is an indicator of gluconeogenesis. Northern blots were preformed as previously described (18) using a PCR-generated *gapB* probe. During the post-exponential growth phase (6 h) when glucose is depleted and there is abundant acetate in the culture medium (Fig. 1B), the wild-type strains SA564 and UAMS-1 produced abundant transcript levels of *gapB* (Fig. 2). In contrast, the SA564 *acnA*::*tetM* and UAMS-1 *acnA*::*tetM* strains had dramatically decreased *gapB* mRNA levels relative to those of the wild-type strains (Fig. 2). As is normally the situation, there was strain-to-strain variation in the absolute amount of *gapB* mRNA present in these genetically distinct *S. aureus* strains (Fig. 2); however, the decreased level of *gapB* mRNA in the aconitase mutants relative to the wild-type strains was consistent. These data demonstrate that the normal post-exponential flow of carbon through the TCA cycle and gluconeogenesis is inhibited in TCA cycle mutants.

To determine if TCA cycle inactivation altered capsule biosynthesis, capsule accumulation was quantified from equivalent numbers of bacteria (1.25 optical density at 660 nm

[OD$_{660}$] units) grown overnight in tryptic soy broth, using capsule immunoblots essentially as described previously (14), except that immunoblots were developed using a chemiluminescent horseradish peroxidase (HRP) substrate (Millipore). Consistent with the hypothesis that post-exponential-growth-phase capsule biosynthesis requires TCA cycle activity, inactivation of any of the first three genes of the TCA cycle (*citZ*, *acnA-citB*, or *citC*) dramatically reduced capsule type 5 accumulation in strain SA564 (Fig. 3). Similarly, inactivation of the aconitase gene (*acnA-citB*) in strain UAMS-1 strongly inhibited capsule type 8 accumulation (Fig. 3A). To determine if TCA cycle inactivation impaired PNAG synthesis, PNAG immunoblot analyses were preformed as described previously (26). As expected, TCA cycle inactivation did not impair PNAG synthesis by strain UAMS-1 (Fig. 3B). (Strain SA564 does not produce PNAG under any growth conditions [C. Vuong and G. A. Somerville, unpublished observations]; thus, its ability to synthesize PNAG was not assessed.) These data demonstrate that the post-exponential-growth-phase biosynthesis of capsule requires TCA cycle activity.

During aerobic growth of *S. aureus* in a carbohydrate-containing medium, acetate accumulates in the medium until the carbohydrate concentration decreases to a level at which it can no longer sustain rapid growth (Fig. 1). The exit from the exponential phase of growth corresponds with the TCA cycle-dependent catabolism of acetate (21, 23). TCA cycle inactivation inhibits the post-exponential-growth-phase catabolism of acetate (Fig. 1), severely limiting the ability of *S. aureus* to generate oxaloacetate. Oxaloacetate is a dicarboxylic acid intermediate of the TCA cycle, and it is the substrate for PEP carboxykinase, which catalyzes the first committed step in gluconeogenesis to generate PEP. In the absence of PEP, *S. aureus* cannot drive hexose synthesis via gluconeogenesis (Fig. 2); hence, very little fructose 6-phosphate is made. When fructose 6-phosphate is in limiting supply, the ability of *S. aureus* to make UDP-*N*-acetylglucosamine, the precursor of capsular polysaccharides, is inhibited; thus, TCA cycle inactivation prevents capsule biosynthesis (Fig. 3A). In contrast to capsule biosynthesis, PNAG is synthesized during the exponential growth phase (Fig. 3B) when there is abundant glucose flowing through glycolysis and TCA cycle activity is repressed (18, 23, 24). In summary, the UDP-*N*-acetylglucosamine used in PNAG biosynthesis is derived from glycolysis, while the UDP-*N*-acetylglucosamine used in capsule biosynthesis is derived from gluconeogenesis (Fig. 4).

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