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Regulation of *Staphylococcus aureus* Immunodominant antigen B (IsaB)

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Abstract

Staphylococcus aureus is a leading cause of nosocomial and community-acquired infections and increased prevalence of antibiotic resistance has necessitated the search for novel therapeutic targets. The immunodominant surface antigen B induces an antibody response during septicemia and has therefore been proposed as a vaccine target. Because it appears to be expressed during infection but not during colonization, we sought to characterize the regulation of *isaB* expression by internal transcription factors and external stimuli. We found that expression of *isaB* was stimulated by glucose, human serum, and plasma. Furthermore *isaB* transcript levels increased in the absence of the global staphylococcal accessory regulator SarA and decreased in the absence of the carbon catabolite regulator CcpA. Interestingly, glucose and CcpA-mediated *isaB* expression appeared to be related to the decrease in pH subsequent to carbon metabolism, rather than a direct response to the carbon source and could be prevented by buffering the growth medium.

Keywords

Staphylococcus aureus; transcription; vaccine; virulence

INTRODUCTION

Staphylococcus aureus is an important opportunistic pathogen, causing significant morbidity and mortality on a global scale and is a leading cause of nosocomial infections. Antibiotic resistance is a mounting clinical problem and methicillin resistant *S. aureus* (MRSA) infections surpass AIDS as a cause of death in the United States (Gorwitz *et al.*, 2006). In order to find new therapeutic targets to treat or prevent infections with antibiotic-resistant *S. aureus* it is essential to identify virulence factors important in infection and to understand their regulation. The immunodominant antigen B (IsaB) was shown by Lorenz *et al.* to be immunogenic during septicemia suggesting that it may be a target for vaccine therapy (Lorenz *et al.*, 2000a). In an effort to characterize its function, we have demonstrated that IsaB is a secreted and partially surface-associated protein and that has the ability to bind extracellular nucleic acids (Mackey-Lawrence *et al.*, 2009).

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Little is known about the regulation of *isaB*. The finding that anti-IsaB antibodies are produced during septicemia but not during colonization suggests that expression of IsaB is controlled in such a way that it is only expressed during infection (Lorenz *et al.*, 2000b). Reports of global transcript analysis, suggest that *isaB* expression is induced in response to neutrophil exposure, in biofilms, under anaerobic conditions, and following internalization in human epithelial cells, again, suggesting a role in immune evasion and virulence (Fuchs *et al.*, 2007; Garzoni *et al.*, 2007; Resch *et al.*, 2005; Voyich *et al.*, 2005). Virulence factor regulation in *S. aureus* is carefully orchestrated by 3 sigma factors, 16 two-component systems, a quorum sensing system, and a large repository of transcriptional regulators (George and Muir, 2007; Pragman and Schlievert, 2004; Somerville and Proctor, 2009). In this study, we sought to characterize the regulation of *isaB* in *S. aureus* by mapping the promoter region and by determining which external stimuli and transcriptional regulators contribute to expression. We found that expression of *isaB* was induced in response to a simple carbon source such as glucose, however this response was absent in strains lacking the carbon catabolite regulator (CcpA). Furthermore, we found evidence that the CcpA-mediated control and response to glucose were due to acidification of the medium rather than in direct response to the carbon source. Finally it was observed that the staphylococcal accessory regulator A (SarA) is a repressor of *isaB* expression.

MATERIALS AND METHODS

Strains and growth conditions

Strain 10833 is positive for clumping factor (ATCC 25904), is positive for capsular polysaccharide CP5, and is closely related to the sequenced strain Newman. Strain ALC1342, a derivative of strain RN6390 in which the *sarA* gene was replaced with an erythromycin resistance cassette by homologous recombination, was kindly provided by Ambrose Cheung, Dartmouth Medical School (Cheung and Manna, 2005). 10833 Δ *sarA*::erm was produced by transducing the mutation from ALC1342 using phage 80 α (Kasatiya and Baldwin, 1967). SA113 is closely related to NCTC 8325 and is capsular polysaccharide negative. RN4220 is a restriction deficient laboratory strain from Dr. Richard Novick (Skirball Institute of Molecular Medicine, New York University, NY). The strains were grown at 37 °C on tryptic soy agar plates and liquid cultures were either in Luria Bertani broth (LB) or LB+1% glucose (LBG) overnight with shaking at 200 rpm. For some experiments, overnight *S. aureus* cultures were diluted 1:100 in human serum or human plasma (MP Biomedicals, Solon, OH). In others, the cultures were diluted 1:100 in LB containing 50mM HEPES pH 7.0. To assess the effect of pH, cultures were diluted 1:100 in 100 mL LB, grown for 2 hr, collected, suspended in 10mL LB pH-adjusted using HCl to pH 4, 5, 6, 7, or 8, and incubated for 30 min prior to RNA extraction or 2 hr prior to Western analysis. *E. coli* strain CH3-Blue (Bioline, Taunton, MA) was used for cloning and was grown in LB supplemented with 100 μ g ampicillin / ml at 37 °C overnight.

RNA Extraction

RNA was purified using the Qiagen RNeasy® mini kit (Qiagen, Valencia, CA) with modifications. Approximately 1×10^9 bacteria were collected and resuspended in 500 μ l RLT® lysis buffer (Qiagen) and added to Lysing Matrix B® tubes (MP Biomedicals, Solon, OH). 500 μ l Acid Phenol:Choloroform (Ambion, Austin, TX) was added and the samples were processed in a FastPrep-FP120 (MP Biomedicals) two times at a speed setting of 6.0 for 45 seconds (sec), with a 5 minutes (min) incubation on ice in between. Samples were centrifuged at 14,000 rpm for 7 min at 4 °C. The aqueous phase was collected, combined with 500 μ l 100% ETOH (Invitrogen, Carlsbad, CA), and the samples were loaded onto RNeasy columns (Qiagen). RNA was then purified according to manufacturer's instructions and eluted with 100 μ l nuclease-free H₂O. Contaminating DNA was removed

using the TurboDNA-free kit (Ambion) and RNA concentration and purity were determined spectrophotometrically.

Northern Blot Analysis

Northern analysis was performed using the NorthernMax®–Gly Kit (Ambion, Austin, Texas) according to the manufacturer's instructions. RNA (20 µg) from cultures grown for 6 hr in LBG was added to ULTRAhyb®–Oligo Hybridization Buffer (Ambion). The ULTRAhyb®–Oligo Hybridization Buffer protocol was followed per the manufacturer's instructions from pre-hybridization until the final washes. IsabNorthern (5'CGTTTTAGCTAATTTAATACCATTGAATTTACATTATCATACTTAATCGCG3') was used as the probe. BrightStar®–BioDetect Kit (Ambion) was used following the manufacturer's instructions for detection and the blots were exposed to autoradiographic film.

Real-Time Reverse Transcriptase PCR

For RT-PCR, 1 µg of RNA was converted into cDNA using Superscript II Reverse Transcriptase kit (Invitrogen, Carlsbad, Ca) in the presence of 1 pM *isaB*QPCRRev (5' AACGACAAACCAGATGCCTAA3') and 1 pM 16sQPCRRev (5' TATGCATCGTTGCCCTTGGA3') following the manufacturer's instructions. The cDNA and controls lacking reverse transcriptase were diluted 1:10 in ddH₂O to detect *isaB* transcript and 1:1000 to detect *16s* transcript. These samples were subjected to real-time RT-PCR using the SensiMix SYBR & Fluorescein kit (Bioline, Randolph, Ma), 1 pM *isaB*QPCRRev+*isaB*QPCRfwd (5' GCAAAAGGAAACGAAGCAAG3') or 16sQPCRRev+16sQPCRfwd (5' GAACCGCATGGTTCAAAGT3'), and the following cycle conditions: 1 cycle 95 °C for 3 min.; 40 cycles of 10 sec 95 °C, 30 sec 58 °C and 15 sec 72 °C (real-time data collection) followed by a melt-curve. Cycle thresholds from the *isaB* samples and 16S rRNA samples were used to determine normalized expression (*E*) of *isaB*, using the equation $E = 1,000 * \{2^{-(16s\ ct - isaB\ ct)}\}$. Samples lacking the reverse transcriptase enzyme were assembled as controls to detect contamination of the RNA samples with genomic DNA, and each PCR run included a no template control.

Western analysis

Cells were collected, washed with PBS, resuspended in 100 µL 40mM Tris-Cl containing 100 mM NaCl and 50 µg lysostaphin, and incubated for 1 hr at 37°C. The lysed bacteria were sonicated to shear DNA, and equal amounts of protein from LB pH 5-8 were analyzed by denaturing PAGE using NuPAGE® 4-12% Bis-Tris gels (Invitrogen) according to manufacturer's instructions. The proteins were transferred onto a PVDF membrane and the membrane was blocked 1 hr in PBS containing 5% skim milk. The blot was probed with a 1:5,000 dilution of *IsaB*-specific rabbit antisera in PBS containing 0.05% tween (PBST) and 0.5% skim milk followed by a 1:10,000 fold dilution of horseradish peroxidase conjugated protein A (Sigma-Aldrich, St. Louis, Mo) in PBST. Proteins were detected using the ECL Plus detection system (Amersham).

Deletion of *ccpA*

We replaced the *ccpA* gene with an erythromycin resistance cassette (*erm*) in *S. aureus* strain RN4220 using the pMAD vector (kindly provided by Michel Débarbouillé and Maryvonne Arnaud Pasteur Institute, Paris, France). The *ccpA* gene and surrounding sequence were amplified from total DNA from strain 10833 using primers CcpADELFWD (5' GGATCCCCATATGATTTCCAAGGCCTAGG3') and CcpADELREV (5' GGATCCGAATGGTCAATTTAAGCATATGCACCG3') and the PCR product was cloned into the pCR4-TOPO vector and transformed into CH3 Blue *E. coli*. The plasmid

was amplified with primers CcpAXhoFWD (5'GGGCTCGAGCCTCACAGAATTGAATACCGAGG3') and CcpAXhoREV (5'GGGCTCGAGGAGACACGCGCTTCTCTTGC3'). The PCR product was treated with *DpnI* to digest the original methylated plasmid; it was then digested with *XhoI* and ligated to the *erm* cassette excised from plasmid pSC57 (Cramton *et al.*, 1999) with *XhoI*. The region surrounding the *ccpA* gene and the intervening *erm* cassette were excised with *BamHI* and ligated to pMAD. This construct was purified from *E. coli* and electroporated into strain RN4220 as described by Lee (Lee, 1993). Mutants in which a double-crossover event had replaced the chromosomal copy of *ccpA* with the *erm* cassette were selected as previously described (Mackey-Lawrence *et al.*, 2009). The mutation was transduced to strains 10833 and SA113 using phage 80 α (Kasatiya & Baldwin, 1967) to produce strains 10833 Δ *ccpA::erm* and SA113 Δ *ccpA::erm*.

RESULTS

The *isaB* transcript is monocistronic

As shown in Fig. 1A, *aur*, the gene encoding the metalloprotease aureolysin, is located immediately downstream from *isaB*. We used Northern blot analysis to determine whether or not the two genes were co-transcribed. As shown in Fig. 1B, a probe specific for *isaB*, detected a transcript of approximately 500 nucleotides (nt), which is consistent with the predicted 528 bp size of the monocistronic *isaB* transcript. If *aur* and *isaB* were cotranscribed, they would yield a transcript of approximately 2,481 nt but no transcripts over ~500 nt were detected. These results suggest that the *isaB* transcript is monocistronic.

Transcriptional start site of *isaB*

The transcriptional start site (TSS) of *isaB* was determined by 5' RACE analysis. As Fig. 2 indicates, the TSS matched the TSS predicted by BPROM (Softberry software). The TSS was 39 bp upstream from the start codon, indicating that the *isaB* transcript contains a 39 nt 5'-untranslated region. Upon analysis of the promoter region, we found a putative SarA consensus binding sequence of ATTTTAT, which overlaps the predicted -35 site within the *isaB* promoter (Chien *et al.*, 1999; Sterba *et al.*, 2003). We therefore investigated the role of SarA as a regulator of *isaB*.

SarA represses *isaB* expression

SarA, is a well characterized transcription factor considered to be a global regulator of many virulence genes, and a previously published report using microarray analysis suggests that it represses *isaB* (Fujimoto *et al.*, 2009). We compared levels of *isaB* transcript in 10833 versus its isogenic *sarA* deletion mutant using Northern analysis and realtime RT-PCR. As shown in Fig. 3A, *isaB* transcript levels were elevated in the 10833 Δ *sarA::erm* deletion mutant as observed by northern blot analysis.

In order to further quantify the level of SarA-mediated repression, realtime RT-PCR was used to measure *isaB* transcript levels over time. Overnight cultures of the wild-type and Δ *sarA* strains were diluted 1:100 in fresh media, and samples were collected at 2, 4, 6, and 24 hours. At all time-points, strain 10833 Δ *sarA::erm* contained more *isaB* transcript than 10833 (Fig. 3B). Interestingly, *isaB* transcript continued to accumulate in strain 10833 Δ *sarA::erm* over the time-course, whereas transcript levels began to decrease after 4 hr in the wild-type strain resulting in an 88-fold lower level of *isaB* transcript in 10833 at 24 hr. These data support the conclusion that SarA is a negative regulator of *isaB* expression.

***isaB* is induced during growth in plasma and serum**

IsaB induces an immune response during septicemia suggesting that it is expressed in blood (Lorenz *et al.*, 2000a). We assessed blood components for their ability to induce *isaB* expression. RNA was purified from *S. aureus* cultures grown in LB, human plasma, or serum. After two hours of incubation in plasma or serum *isaB* transcript levels were increased by more than 5-fold relative to LB (Fig. 4), suggesting that *isaB* expression is induced by blood components.

Simple sugars induce expression of *isaB*

We tested the effect of carbohydrate availability on *isaB* expression. As shown in Fig. 5A, upon addition of 1% glucose, *isaB* transcript increased approximately 14-fold relative to media without glucose. Fructose and sucrose, both simple sugars that can be used in glycolysis were also tested for their ability to induce *isaB*, again utilizing realtime RT-PCR (Jankovic and Brückner, 2007). Both fructose and sucrose were capable of increasing detectable *isaB* transcript by approximately 13- and 8-fold, respectively.

A time course of glucose induction was performed to determine when the maximal induction of *isaB* expression occurred, and its duration. Time points were collected at 2, 4, 6, 8, 24 hrs post-dilution into fresh LBG. Interestingly, there was a significant delay between the time glucose was added and the time when elevated levels of *isaB* transcript were observed. By 6 hr following glucose addition, there was an 8-fold increase in transcript levels and by 24 hr there was an approximately 20-fold increase (Fig. 5B). Similar experiments were performed on the unrelated *S. aureus* strain Sa113 and the same trend was observed with *isaB* expression peaking between 8-24 hours (data not shown).

The effect of CcpA and pH on *isaB* expression

Since glucose induced the expression of *isaB*, we hypothesized that CcpA could play a role in its regulation. An isogenic *ccpA* deletion mutant was constructed and realtime RT-PCR was used to analyze *isaB* transcript levels in 10833 and 10833 Δ *ccpA::erm*. Samples were taken at time-points 2, 4, 6, 8, and 24 hrs following dilution in LB or LBG. Deletion of *ccpA* resulted in a dramatic decrease in the amount of *isaB* transcript in the presence of glucose (Fig. 6A). These findings suggested that *isaB* expression is activated through the carbon catabolite control system, mediated by CcpA.

Because we consistently noted a delay between glucose addition and *isaB* induction, we considered the possibility that the effect was actually due to a decrease in the pH of the growth medium (Gaupp *et al.*, 2010; Seidl *et al.*, 2009). To determine whether the *ccpA* deletion affected the pH of the growth medium, the pH of the media was monitored over time. We found that the media in which 10833 Δ *ccpA::erm* was cultured exhibited a delay in acidification compared to the wild-type strain 10833 (Fig. 6B).

Glucose-mediated *isaB* induction is dependent upon acidification

To address the possibility that a drop in pH was involved in the elevated *isaB* expression in the presence of glucose, a time-course was performed, however in addition to the normal LBG culture, 50 mM HEPES was added to LBG to buffer the media to pH 7.0 and prevent or delay the acidification of the media without affecting growth (Seidl *et al.*, 2006). When the pH of the media was buffered to maintain a neutral pH longer we did not observe the typical levels of *isaB* induction by glucose (Fig. 7a). Furthermore, when 10833 and 10833 Δ *ccpA::erm* were added to LB adjusted to pH 5, 6, 7, or 8, *isaB* transcript levels were elevated at low pH within 30 minutes (Fig. 7b). To ensure that the increased transcript levels translated into increased surface expression of IsaB protein, we performed Western analysis and found that IsaB was expressed at pH 5 and 6 but not at pH 7 or 8 (Fig 7c). Therefore, it

is likely that the decrease in pH, a consequence of carbon metabolism, is in fact turning on *isaB* expression. The delayed drop in pH is likely responsible for the observed effect that deletion of *ccpA* had on *isaB* levels, and therefore from these studies it appears that CcpA may not have a direct role in the regulation of *isaB*.

DISCUSSION

These studies show that blood components and simple carbon sources or their acidic metabolic byproducts lead to increased transcription of *isaB*. As aerobic bacteria in culture grow, CO₂ and acetic acid accumulate in the media, which leads to a decrease in the pH from ~7.5 to ~4.5 over only a few hours. Despite incubation of cultures in the presence of oxygen, the culture to vessel volume ratio used in our studies was likely high enough to result in the development of anaerobic conditions. This would lead to the accumulation the production of additional acidic byproducts and a further reduction in pH. From our time-course studies we observed that *isaB* expression does not reach peak levels until a time-point at which all of the glucose in the culture has likely been depleted, and the pH has dropped to approximately 5 (Seidl *et al.*, 2008; Seidl *et al.*, 2009). Therefore we speculated that acidic conditions were actually behind the induction of *isaB*. We buffered the medium to significantly delay acidification following carbohydrate metabolism. We found that once the media was buffered and the drop in pH delayed, induction of *isaB* by glucose was severely attenuated. Another interesting finding was that the pattern of *isaB* induction of cultures grown in the buffered media was strikingly similar to what was observed for the CcpA mutant. By repeating the CcpA time-course and monitoring the change in pH we found that the CcpA mutation was associated with a significant delay in the acidification of the media. This is likely due to impairment of glucose utilization, which would prevent the accumulation of acetic acid in the media. Therefore, we were able to conclude that pH, not glucose or CcpA was responsible for the majority of the *isaB* induction. This data also raises the possibility that changes in the expression of other genes in response to the deletion of *ccpA* could be an indirect effect of pH.

Of clinical relevance, these studies have found that *isaB* expression increases significantly following culture in either serum or plasma. Again supporting the hypothesis that as a virulence factor, IsaB is expressed in vivo during septicemia. However as these fluids are composed of a great number of factors, it is not clear from our study which components are responsible for stimulating increased *isaB* transcription. These results further increase the complexity of the regulation of *isaB* as the same signals or proteins that sense acidic conditions are not likely to be stimulated in serum/plasma as these biological fluids are inherently buffered to maintain a tight homeostasis at neutral pH. However, *isaB* is induced following phagocytosis, where *S. aureus* would be exposed to low pH inside the phagolysosome of neutrophils. Important future experiments would be to elucidate which mechanisms, such as one of the 16 two-component systems in *S. aureus*, are responsible for each stimulus (Somerville & Proctor, 2009).

Finally these studies have demonstrated that SarA is a negative regulator of *isaB*, although it is currently unknown if this is due to direct or indirect regulation. The predicted promoter of *isaB* contains a consensus 7 base pair SarA binding consensus sequence, which overlaps the -35 region, suggesting that *isaB* could be directly regulated by SarA (Chien *et al.*, 1999; Sterba *et al.*, 2003). It is interesting to note that a recent study by Fujimoto *et al.*, has suggested that SarA is affected by pH, which may explain our findings that *isaB* expression was regulated by pH and SarA (Fujimoto *et al.*, 2009).

It is interesting to speculate that *isaB* expression in vivo could be induced by factors such as high local concentrations of nucleic acids found in neutrophil extracellular traps (NETs), or

within the phagolysosome of viable neutrophils. We have found that IsaB binds nucleic acids to the bacterial cell surface (Mackey-Lawrence *et al.*, 2009). Therefore, in these compartments, IsaB could bind to extracellular nucleic acids and protect *S. aureus* from killing. Through these studies we have begun to define the regulatory network of *isaB* and have provided additional evidence suggesting that IsaB would be expressed during bacteremia and infection, further supporting its role as an important virulence factor, and potential therapeutic target of *S. aureus*.

Acknowledgments

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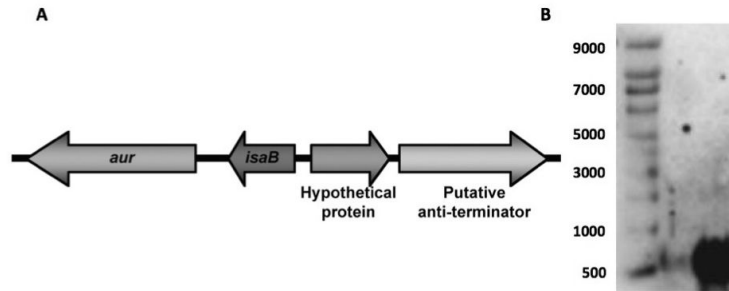


Fig 1. The *isaB* transcript is monocistronic

A. Illustration of *isaB* and surrounding genes showing the gene for aureolysin (*aur*) immediately downstream. **B.** Northern analysis revealed a single ~500nt transcript detected with an *isaB*-specific probe. Left lane, RNA size marker, right lane, *isaB* transcript.

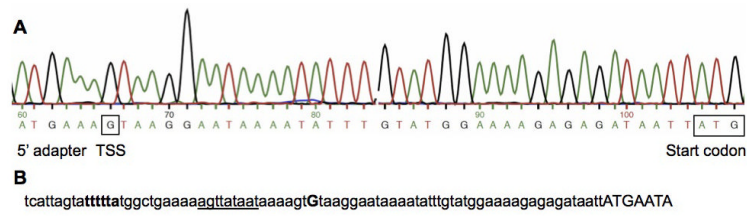


Fig 2. Identification of the *isaB* transcriptional start site

A. Sequence data from 5'-RACE analysis showing the fusion between the adapter and the 5'-end of the transcript (boxed "G"), and the start codon of the *isaB* gene. **B.** BPRM predicted promoter indicating the predicted -35 region in bold characters, the predicted -10 region underlined, the predicted transcriptional start site (uppercase "G"), and the start of the *isaB* gene in uppercase.

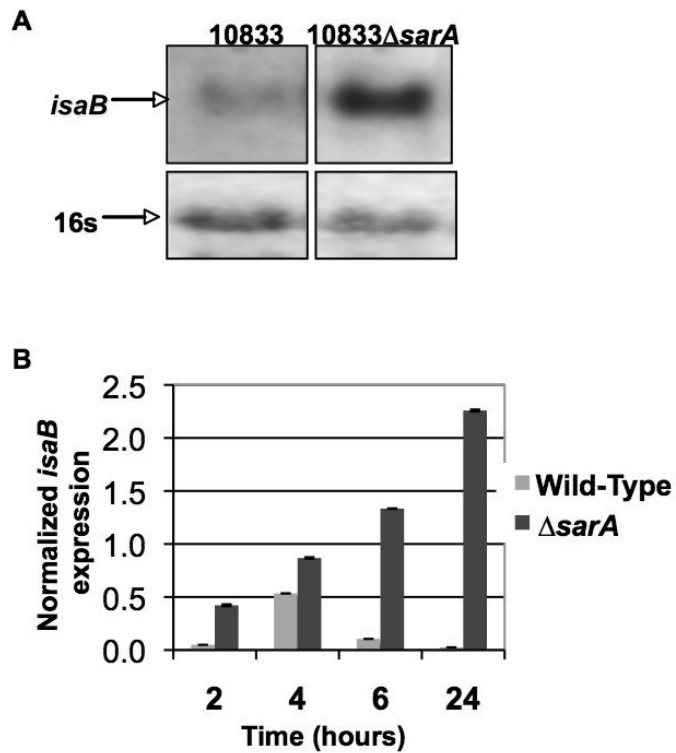


Fig 3. Deletion of *sarA* leads to increased levels of *isaB* transcript

RNA was isolated from *S. aureus* strain 10833 and its isogenic *sarA* deletion mutant and subjected to **A**, Northern analysis using an *isaB*-specific probe and **B**, realtime RT-PCR. For RT-PCR, RNA was purified from cultures at different time-points to quantify the effect of SarA on *isaB* expression during different stages of growth and threshold values were normalized to 16S. Error bars represent standard deviation within 3 replicates.

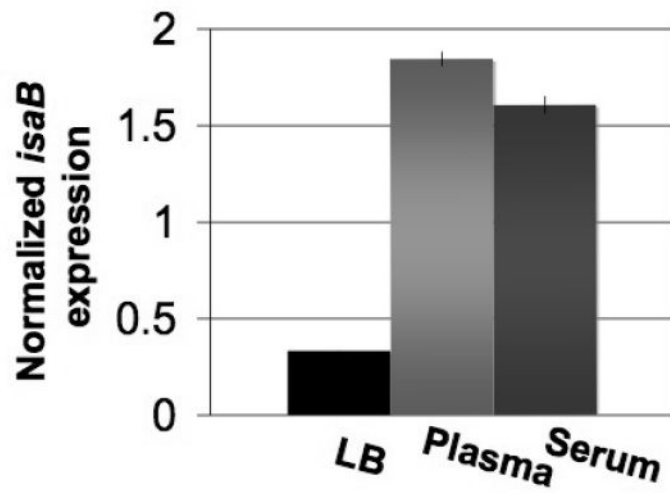


Fig 4. Levels of *isaB* transcript are elevated in human plasma and serum

S. aureus strain 10833 was cultured in LB, human plasma, or human serum for hours, RNA was purified and analyzed by realtime RT-PCR using *isaB*-specific primers. The threshold values were normalized to 16S. Error bars represent standard deviation within 3 replicates.

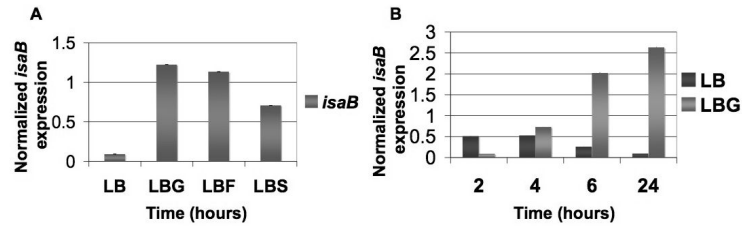


Fig 5. Levels of *isaB* transcript are elevated in the presence of a simple carbon source *S. aureus* 10833 was cultured in LB alone or supplemented with 1% glucose (LBG), 1% fructose (LBF), or 1% sucrose (LBS) and RNA was purified for analysis of *isaB* expression by realtime RT-PCR. **A.** *isaB* transcript levels were elevated by the addition of glucose, fructose, and sucrose. **B.** The increase in *isaB* transcript was not apparent until 4 hours following the addition of glucose but was sustained over a 24 hour period.

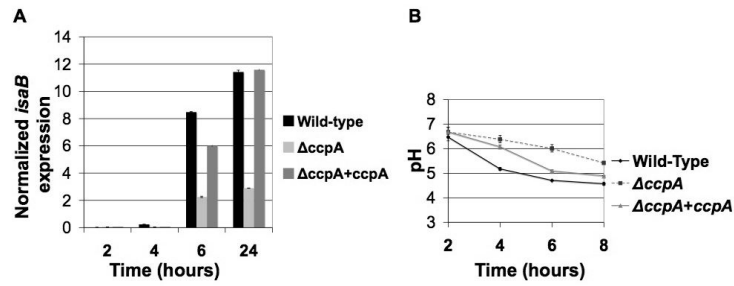


Fig 6. Levels of *isaB* transcript decrease in the absence of CcpA

RNA was purified from *S. aureus* 10833 and an isogenic *ccpA* deletion mutant and analyzed by realtime RT-PCR using *isaB*-specific primers. The threshold values were normalized to 16S. **A.** The *ccpA* deletion mutant expressed lower levels of *isaB* transcript at every time-point. **B.** The pH of the media in which the 10833 $\Delta ccpA::erm$ was grown did not decrease as rapidly as 10833. Error bars represent standard deviation within 3 replicates.

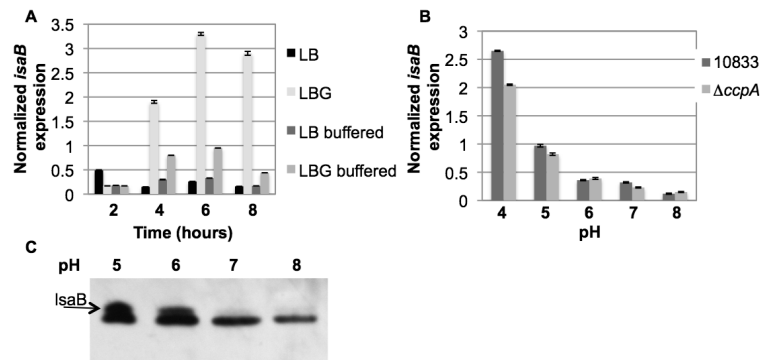


Fig 7. Glucose-induced *isaB* expression is mediated by decreasing pH

RNA was purified from **A.** 10833 or **B.** 10833 $\Delta ccpA::erm$, and analyzed by realtime RT-PCR using *isaB*-specific primers. The threshold values were normalized to 16S. **A.** cultures were in LB alone, LB supplemented with 1% glucose (LBG), LB buffered to pH 7.0, and LBG buffered to pH 7.0. **B.** cultures were in LB pH 5, 6, 7, or 8. **C.** Western analysis showing expression of IsaB in 10833 at different pH levels. The IsaB antibody cross-reacts with a similar sized antigen. IsaB is the upper band and is marked with an arrow.