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CspA regulation of *Staphylococcus aureus* carotenoid levels and σ^B activity is controlled by YjbH and Spx.

Niles P. Donegan[a,#], **Adhar C. Manna**[a], **Ching Wen Tseng**[b,*], **George Y. Liu**[b], **Ambrose L. Cheung**[a,#]

[a]Department of Microbiology and Immunology, Geisel School of Medicine at Dartmouth, Hanover, New Hampshire, USA;

[b]Department of Pediatrics, Cedars-Sinai Medical Center, Los Angeles, California, USA

SUMMARY

Staphyloxanthin, a carotenoid in *S. aureus*, is a powerful antioxidant against oxidative stresses. The *crtOPQMN* operon driving pigment synthesis is under the control of σ^B. CspA, a cold-shock protein, is known to control σ^B activity. To ascertain genes that regulate *cspA*, we screened a transposon library that exhibited reduced *cspA* expression and pigmentation. We found that the adaptor protein YjbH activates *cspA* expression. Spx, the redox-sensitive transcriptional regulator and a proteolytic target for YjbH and ClpXP, complexes with αCTD of RNAP prior to binding the *cspA* promoter to repress *cspA* activity. Increased *cspA* expression *in trans* in the inactive *spx* C10A mutant of JE2 did not enhance pigment production while it did in JE2, suggesting that *cspA* is downstream to Spx in pigmentation control. As the staphyloxanthin pigment is critical to *S. aureus* survival in human hosts, we demonstrated that the *cspA* and *yjbH* mutants survived less well than the parent in whole blood killing assay. Collectively, our studies suggest a pathway wherein YjbH and ClpXP proteolytically cleave Spx, a repressor of *cspA* transcription, to affect σ^B-dependent carotenoid expression, thus providing a critical link between intracellular redox sensing by Spx and carotenoid production to improve *S. aureus* survival during infections.

GRAPHICAL ABSTRACT:

This study investigates the pathway whereby YjbH and ClpXP proteolytically cleave Spx, a repressor of *cspA* transcription, to affect σ^B-dependent carotenoid expression, thus providing a critical link between intracellular redox sensing by Spx and carotenoid production to improve *S. aureus* survival during infections.

ABBREVIATED SUMMARY:

[#]Address correspondence to 1) Ambrose Cheung at ambrose.cheung@dartmouth.edu., 2) Niles Donegan at niles.donegan.med@dartmouth.edu.

[*]Present address: Kite Pharma, Inc., Santa Monica, California.

AUTHOR CONTRIBUTIONS:

Niles Donegan performed most of the experiments and wrote the paper. Adhar Manna conducted specific experiments for EMSA and supplemental figure S4. Chin Wen Tseng helped design experiment with Niles Donegan. George Y. Liu did the whole blood killing assay and Ambrose L. Cheung helped design the experiment and edited the manuscript.

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The carotenoid pigment (also called staphyloxanthin) in *S. aureus* is a powerful antioxidant that protects the bacteria from oxidative stresses encountered during growth and infection. In this paper, we conducted experiments to ascertain genetic factors that govern the expression of this pigment in *S. aureus*.

Keywords

Carotenoid; oxidative stress; Spx; YjbH; CspA; *S. aureus*

INTRODUCTION

The golden pigmentation of the human pathogen *Staphylococcus aureus* results from accumulation of staphyloxanthin, a membrane-localized triterpenoid carotenoid (Katzif *et al.*, 2003; Duval *et al.*, 2010). This compound is thought to act as a free-radical scavenger and singlet oxygen quencher, enabling *S. aureus* to survive oxidative stresses stemming from ROS (Pelz *et al.*, 2005). The *crtOPQMN* operon encodes the proteins responsible for the biosynthesis of staphyloxanthin (Pelz *et al.*, 2005), and is regulated by a series of regulators (Lan *et al.*, 2010; Fey *et al.*, 2012) within the alternative sigma factor σ^B system (Kullik *et al.*, 1998). In unstressed conditions, σ^B (encoded by *sigB*) is bound in an inactive state by its anti-sigma factor RsbW, and is only freed to bind RNA polymerase to initiate transcription at σ^B-dependent promoters when its upstream regulator RsbU dephosphorylates RsbV to disrupt the σ^B-RsbW complex (Benson and Haldenwang, 1993; Senn *et al.*, 2005).

Other regulators of pigmentation in *S. aureus* include *cspA* [also called *msaB* (Elbarasi, 2014)] and *cspB* (Katzif *et al.*, 2005; Duval *et al.*, 2010) wherein σ^B activity appears to be positively dependent on *cspA* (Katzif *et al.*, 2005). Csps are small (~7 kD) proteins found in bacteria (Schindler *et al.*, 1999) and archaea (Giaquinto *et al.*, 2007) that have a compact β-barrel structure for binding single-stranded DNA (Zeeb, 2003) and RNA (Bae *et al.*, 2000), presumably to stabilize mRNA and promoters and to enhance translation (Phadtare and Severinov, 2010).

All Csps in *S. aureus* share considerable identity to those in *E. coli* (~60%) and *B. subtilis* (~76%), as well as amongst themselves (~80–90%). However, each Csp appears to impact different pathways in *S. aureus* (Duval *et al.*, 2010; Lioliou *et al.*, 2012), with *cspA* primarily affecting biofilm development (Elbarasi, 2014), protease production (Sahukhal and Elasri, 2014) and cold shock response (Katzif *et al.*, 2005; Anderson *et al.*, 2006). Regulation of these Csp homologs appears equally as diverse, with some *csp* promoters controlled by transcription factors (Constantinidou *et al.*, 2006; Uppal *et al.*, 2014), while other *csp* genes are modulated by regulatory RNAs at their 5' untranslated regions (Jiang *et al.*, 1996) that are then targeted post-transcriptionally for degradation by RNases (Lioliou *et al.*, 2012) and toxin-antitoxin modules (Younghoon Kim *et al.*, 2010; Samin Kim *et al.*, 2016). Post-translational regulation may also occur, as with the intracellular protease Lon which can affect CspD levels in *E. coli* (Langklotz and Narberhaus, 2011).

In this study, we sought to explore the role of *csp* homologs and other genes in controlling σ^B and the ensuing carotenoid pigmentation (i.e. staphyloxanthin) in *S. aureus*. We have

identified that YjbH, an adaptor of the ClpXP proteolytic system (Engman *et al.*, 2012), acts indirectly as an activator of *cspA*. In addition, Spx, a redox-sensing transcription factor targeted by ClpXP and YjbH for degradation, was found to repress *cspA* levels by directly interacting with the *cspA* promoter via the α-subunit of RNA polymerase (Michiko M Nakano *et al.*, 2010). Activation of CspA via Spx degradation by the YjbH-ClpXP proteasome complex would lead to increased pigmentation via enhanced σ^B activity. Together, these results reveal a novel pathway wherein staphyloxanthin, a *S. aureus* pigment that enhances bacterial survival upon oxidative stress, is controlled by a redox-sensitive regulator in *S. aureus*.

RESULTS

Carotenoid expression in *csp* mutants.

In prior studies, it has been reported that *cspA* (Katzif *et al.*, 2005) and *cspB* mutants (Duval *et al.*, 2010) harbor lower levels of carotenoid pigment. Given the role staphyloxanthin plays in *S. aureus* resistance to oxidative stress, we wanted to further evaluate this phenomenon since individual mutants have not been compared to each other on carotenoid levels in identical genetic background nor has the impact of *cspC* been examined. Accordingly, we created *cspA* and *cspC* mutants of *S. aureus* strain SH1000 using allelic replacement (Arnaud *et al.*, 2004). Due to the reported slower growth (Duval *et al.*, 2010) for the *cspB* mutant, a SH1000 strain disrupted for *cspB* was created via transduction of the kanamycin resistance cassette from strain BD1 to improve the frequency of homologous recombination. We observed that both *cspA* and *cspB* mutants revealed lower pigmentation than the wildtype SH1000, with the Δ*cspA* strain having the lower of the two. In contrast, a Δ*cspC* strain of SH1000 retained pigmentation comparable to the wildtype SH1000 (Fig. 1A), indicating *cspC* is not a major determinant of staphyloxanthin pigmentation in *S. aureus*.

σ^B activity is reduced in a strain lacking *cspA* but not *cspB*.

Given that the *ΔsigB*, Δ*cspA* and Δ*cspB* strains of *S. aureus* all exhibited reduced carotenoid levels, the contribution of each *csp* gene product to σ^B activity was examined. In contrast to other protein-based regulators, σ^B activity cannot be determined by Western blot because it is the free form of σ^B, not the σ^B-RsbW complex, that determines SigB activity. For this reason, transcription from the σ^B-dependent *asp23* promoter (Gertz *et al.*, 1999) is a more reliable measure of overall σ^B activity. Accordingly, *S. aureus* reporter strains with the plasmid containing a transcriptional fusion of the *asp23* promoter to gfp_{uvr} (pALC2201) were constructed (Palma *et al.*, 2006). Overnight cultures of these strains with pALC2201 (Cm resistant) were back-diluted to an OD_{650}=0.1 in fresh TSB with chloramphenicol followed by serial GFP fluorescence and OD_{650} measurements. While the *asp23*-dependent GFP levels were unaltered in Δ*cspB* and Δ*cspC* mutants compared to the wildtype strain SH1000 (Fig. 1B), the GFP level in the Δ*cspA* strain of SH1000 was significantly lower, but not completely absent. This result indicated that among the three *csp* genes in *S. aureus*, only *cspA* was involved in carotenoid production that is linked to the σ^B-dependent *asp23* promoter. We thus elected to concentrate our ensuing analysis on factors affecting *sigB*-dependent pigmentation attributable to *cspA*.

yjbH is an activator of *cspA* transcription.

The transcription of *cspA* is initiated from two promoters, one 514 bp upstream of the *cspA* translation start site that co-transcribes *msaA* and *cspA* (P_{msaA}) (Sahukhal and Elasri, 2014), and the other from a σ^A-dependent promoter 112 bp upstream of the *cspA* initiation codon (P_{cspA}) (Uppalapati *et al.*, 2017). While P_{cspA} is better characterized (Katzif *et al.*, 2003; Lioliou *et al.*, 2012; Uppalapati *et al.*, 2017) than P_{msaA}, there were conflicting data (Sahukhal and Elasri, 2014) regarding the relative activities of these two promoters. To address this, we created transcriptional fusions of each of these promoters to gfp_{uvr} and compared their fluorescence in wildtype SH1000. We observed that the promoter upstream of *cspA* (P_{cspA}) was consistently much stronger than that of *msaA* (P_{msaA}) (Fig. S1). Due to this significant difference, we limited further investigations to the transcriptional regulation of P_{cspA}.

To expand our understanding on how pigmentation is controlled through *sigB* and *cspA*, we screened the Nebraska JE2 transposon library for mutants with pigmentation defects (Fey *et al.*, 2012). These mutant strains were transformed with pALC8135 (P_{cspA}::gfp_{uvr}) and fluorescence compared to wild-type JE2 containing the identical plasmid. Using this technique to screen the entire mutant library, a mutant strain with diminished *cspA* promoter activity was found to have a transposition insertion into *yjbH* (NE896) (Fig. 2A). To validate this, a SH1000 strain with a clean deletion of *yjbH* was created using allelic exchange (Arnaud *et al.*, 2004), as well as its subsequent chromosomal replacement. These strains, together wild type SH1000, were transformed with pALC8135. Similar to our results in the JE2 transposon mutant, deletion of *yjbH* in SH1000 resulted in decreased *cspA* transcription (Fig. 2B) compared to wild type and chromosomal replacement strains, validating the results of our screen and suggesting that *yjbH* plays a role in activating *cspA* transcription.

Subsequent transcriptional analyses of *cspA* in *yjbH* mutants by Northern blots were unfortunately complicated by apparent cross-hybridization among *csp* homologs. For example, a *cspA* probe still hybridized with a transcript in a Δ*cspA* mutant, and similar result was seen for a *cspC* probe to RNA from the Δ*cspC* strain of SH1000 (Fig. S2A). This is likely due to the significant homology shared among the *csp* paralogs in *S. aureus* [e.g. *cspB* and *cspC* share 68% and 78% nucleotide identity to *cspA*, respectively (Fig. S2B)]. To verify that the decreased pigmentation in a Δ*yjbH* mutant occurred through changes in *crt* mRNA levels, analysis of the *crt* transcript was examined by Northern blotting. Overall, the *crt* transcript encoding *crtOPQMN* was lower in a Δ*yjbH* strain compared to the wildtype SH1000 (Fig. 2C), indicating that deletion of *yjbH* leads to reduced carotenoid production, likely due to decreased *crt* transcription by way of diminished *cspA* expression.

In *S. aureus* and *B. subtilis*, YjbH functions as an adaptor for the ClpXP proteolytic complex by recognizing and ushering specific proteins such as redox-sensitive Spx (Kommineni *et al.*, 2011; Engman *et al.*, 2012) to the proteolytic pocket of ClpP for processing. To determine this possibility for regulation of *cspA* by YjbH, we examined whether a *S. aureus clpP* mutant displayed a similar decrease in *cspA* transcription. Indeed, a SH1000 Δ*clpP* strain containing pALC8135 displayed decreased fluorescence comparable to a SH1000 Δ*yjbH* strain (Fig. 2D), suggesting that *yjbH*-mediated regulation of *cspA* may occur through ClpP-dependent proteolytic activity.

Given the linkage of *cspA* to proteolytic components of *S. aureus*, we examined whether direct proteolytic control of CspA exists in *S. aureus*. In *E. coli*, CspD is degraded by the Lon protease (Langklotz and Narberhaus, 2011); however, *S. aureus* lacks a Lon ortholog and, in its place, ClpP controls *S. aureus* various protein levels via ClpP-mediated degradation (Donegan *et al.*, 2010; Cohn *et al.*, 2011). Thus, a SH1000 strain containing a plasmid (pALC7342) with xylose-inducible expression of CspA (Forsyth *et al.*, 2002) was used to follow CspA protein levels over time. Aliquots of cells were taken pre- (t=0') and post-translational stalling (t=60') by adding 50 μg/ml erythromycin at various growth phases, as well as during both cold (16oC) and heat (44oC) shock. CspA levels were then examined by Western blots with a rabbit anti-CspA antibody (a kind gift of Sam Katzif, Midwestern University). Under no condition was CspA observed to be degraded (data not shown), suggesting that CspA is not post-translationally regulated.

cspA transcription is subject to negative autoregulation.

As the 5' UTR of *cspA* in both *E. coli* and *S. aureus* is controlled at both the transcriptional and post-transcriptional levels (Jiang *et al.*, 1996; Uppalapati *et al.*, 2017), we were interested in whether a *S. aureus* strain lacking *cspA* showed changes in *cspA* promoter activity. Fluorescence levels for a Δ*cspA* strain of SH1000 carrying pALC8135 were compared to wildtype with pALC8135. Cells lacking *cspA* showed a strong increase in P_{cspA}-dependent fluorescence compared to wildtype (Fig. 3), suggesting a negative feedback loop for the *cspA* locus.

Impact of *cspA* on transcription of the *yjbH* locus.

To ascertain if *cspA* can impact *yjbH*, we first examined the transcription of the *yjbH* locus (Fig. 4A). Northern blot analysis revealed two distinct transcripts of ~1.2 kb and 1.9 kb that hybridized with an *yjbH* probe (Fig. 4B), with the smaller of the two showing greater intensity and peaking at stationary phase (OD_{650}=1.7). The proximity of the *hbo* gene, also called *yjbI* (Austin *et al.* 2019) and located just 44 bp upstream of *yjbH*, coupled with a strong *rho*-independent terminator (*ΔG*=−16 kcal/mol) (de Hoon *et al.*, 2005) downstream of *yjbH* (Fig. 4A), implicates the 1.2 kb transcript to encompass *hbo-yjbH*. The larger 1.9 kb *yjbH*-containing transcript likely originates from a σ^A promoter upstream of *sa0862*, an uncharacterized gene immediately upstream of *hbo*. The exact function of the *hbo* (*yjbI*), coding for a putative truncated hemoglobin homolog with high oxygen affinity (Pathania, 2002), and its relationship to YjbH is unclear.

The linkage of *hbo* to *yjbH* was verified by probing for *hbo* transcript in wildtype and *ΔyjbH* strains (see Fig. 4A). The 1.2 kb and 1.9 kb bands in the SH1000 wildtype strain were replaced by ~360 and a ~1.0 kb bands in the isogenic Δ*yjbH* strain (Fig. 4C), indicating that *hbo* was part of both abbreviated transcripts that encompass *yjbH*. Therefore, the promoters of both *hbo* and *sa0862* are active, yielding co-transcription with *yjbH*, with the proximal (P_{hbo}) promoter stronger than the distal promoter (P_{sa0862}).

With the above information available, the effect of *cspA* on *yjbH* transcription was then evaluated by Northern blots of RNA from SH1000 wildtype, isogenic Δ*cspA* and Δ*yjbH* strains with a radiolabeled *yjbH* probe. As shown in Fig. 5A, the 1.2 kb *hbo-yjbH* transcript

at an OD_{650nm} of 1.1 was increased in a Δ*cspA* mutant compared to the wildtype, while the effect was not as prominent as the lesser transcribed 1.9 kb *sa0862-hbo-yjbH* transcript (Fig. 5A). These results indicate that *yjbH* is repressed by *cspA* and that most of the effects of *cspA* on *yjbH* transcription is through the 1.2 kb *hbo-yjbH* transcript. We then confirmed this finding of *cspA*-mediated repression of *yjbH* with the P_{hbo} promoter that controls *hbo-yjbH* transcription (Fig. 5B) as this promoter appeared to be the stronger of the two and also mostly affected by *cspA* (Fig. 5A). We constructed the plasmid (pALC8134) containing the 90 bp region upstream of *hbo* containing the putative *hbo* promoter (Pathania, 2002), and transcriptionally fused it to gfp_{uvr}. The recombinant plasmid was transformed into SH1000 wildtype, *ΔcspA* and Δ*yjbH* strains. Similar to the results of the Northern blot of *yjbH* in a Δ*cspA* strain, deletion of *cspA* resulted in increased P_{hbo} activity as detected by fluorescence (Fig. 5B). As we have shown (Fig. 3) that a regulatory loop exists for the regulation of the *cspA* promoter in a *cspA* mutant, we also assessed whether a similar type of regulation existed for *yjbH*. However, no variation of fluorescence was observed between wildtype and the Δ*yjbH* mutant harboring pALC8134, indicating a lack of feedback of YjbH on its own promoter (Fig. 5B).

Expression of *cspA* can complement the loss of *yjbH*.

As *yjbH* and *cspA* are necessary for full pigmentation in *S. aureus* (Engman *et al.*, 2012; Fey *et al.*, 2012) (Fig. 1A), we examined whether these two genes control carotenoid production along a similar pathway. Accordingly, we created Δ*yjbH* and Δ*cspA* strains with a multi-copy plasmid (pEPSA5) capable of inducible *cspA* (pALC7342) or *yjbH* (pALC7091) expression. The empty vector was used as a control. Carotenoid was then extracted from cells grown overnight at 37oC with 0.5% xylose induction and normalized to cell density. Notably, pigmentation was not restored in Δ*sigB* strains expressing either *yjbH* or *cspA* (Fig. 6), indicating that *sigB* is epistatic to both *yjbH* and *cspA*. However, pigment production was diminished in the Δ*yjbH* strain but was restored upon expressing *cspA* exogenously under a xylose-inducible promoter, while a Δ*cspA* strain carrying pEPSA5::*yjbH* was not able to restore pigmentation (Fig. 6). As expected, the Δ*yjbH* mutant strain expressing *yjbH* from the pEPSA5 plasmid, displayed high pigmentation. These data imply that *cspA* and *yjbH* share a common pathway in the synthesis of carotenoid, with *cspA* epistatic to *yjbH*.

yjbH regulates the *spx* promoter through Spx.

Spx is a redox-sensitive transcription factor that responds to redox and oxidative stresses (Zuber, 2004), and adjusts its transcriptome accordingly via changes in its interaction presumably with the α subunit of RNA polymerase (Nakano *et al.*, 2010). However, we have also encountered a scenario where Spx of *S. aureus* can bind directly to the target promoter (unpublished data). Importantly, intracellular Spx levels are controlled by YjbH, an adaptor protein that directs Spx for ClpXP-mediated proteolysis (Engman *et al.*, 2012). Given this involvement of YjbH in Spx and also in carotenoid regulation as delineated above, we surmise that *cspA* expression may be linked to Spx.

Transcriptional analyses have revealed *spx* to be transcribed from both a proximal (P1) and distal (P2) promoters (Jousselin *et al.*, 2013). Upon introducing plasmids containing *spx* P1 or P2 promoter fused to gfp_{uvr} into isogenic *yjbH* strains, it was observed that there was less

fluorescence from both *spx* P1 and P2 promoters in the *yjbH* mutant compared to wildtype (Fig. 7). This finding concurs with a previous report (Jousselin *et al.*, 2013), showing that *spx* transcription from its promoter is likely subject to increased auto-repression from elevated Spx levels, which accumulate in the absence of YjbH-mediated degradation by ClpXP.

The effect of Spx on *cspA* transcription.

As Spx and CspA are involved in redox stress and cold shock response, respectively, it is reasonable to surmise that Spx may somehow interact with *cspA*, given that Spx has been shown to be preferentially enriched on all three *csp* promoters in *B. subtilis* (Rochat *et al.*, 2012). Accordingly, a Δ*spx* mutant of *S. aureus* strain 8325–4 and the chromosomal replacement strain *spx-c* (by inserting a native copy of *spx* into Δ*spx* strain), both containing the *cspA* promoter::*gfp* construct (pALC8135), were grown and assessed for absorbance and GFP fluorescence. A potential issue for the Δ*spx* mutant in the 8325–4 background is that it may harbor suppressor mutations and possibly a mild fitness defect (Pamp *et al.*, 2006). Therefore, the Δ*spx* mutant of 8325–4 was complemented with a native copy of *spx* chromosomally for comparison, expecting whatever mutations in the Δ*spx* mutant would be carried over to the chromosomal replacement strain. Our results (Fig. 8A) showed that *cspA* promoter activity was higher in the 8325–4 Δ*spx* mutant than the chromosomal replacement strain at both time points, indicating that *spx* acts as a repressor for *cspA*. Consistent with this observation is that over-expression of *spx* from pEPSA5 (pALC7113) in wild type strain SH1000, predicted to have lower *cspA* expression due to the native and exogenous copy of *spx* (Fig. 8A), had a lower level of carotenoid pigment vs. the vector control (Fig. 8B), consistent with our hypothesis that *spx* is a repressor of *cspA*, a positive regulator of σ^B and carotenoid expression.

The *cspA* promoter is directly controlled by Spx.

We next examined the mechanistic control of Spx on the *cspA* promoter. While Spx has been shown to bind DNA when complexed with the α subunit of RNA polymerase, RpoA (Shunji Nakano *et al.*, 2004; Michiko M Nakano *et al.*, 2010), it does not eliminate the possibility that Spx can interact solely with the target promoter. With regard to the binding of RpoA, studies have shown that the C-terminal domain of RpoA, called the αCTD, recognizes UP elements in promoter DNA and interacts with transcription regulators like Spx in an association that can either enhance or occlude nearby DNA elements (Shunji Nakano *et al.*, 2004; Chan *et al.*, 2012). To evaluate this possibility for the *cspA* promoter, combinations of purified αCTD and Spx were evaluated for their ability to bind a 300 bp *cspA* promoter fragment in EMSA studies.

On its own, αCTD and Spx shifted radiolabeled *cspA* promoter DNA poorly (Fig. 9, lanes 2 and 10). However, when both proteins were pre-incubated together, more intense shifting of the *cspA* promoter fragment was observed (Fig. 9, lanes 3 and 4). The binding of αCTD and Spx together was also specific for the *cspA* promoter, as challenge with 100-fold excess of unlabeled *cspA* promoter was able to abolish this shift (Fig. 9, lane 5), while challenge with cold non-specific DNA did not (Fig. 9, lane 6).

Given Spx's role as a redox-sensitive transcription factor in both *S. aureus* (Pamp *et al.*, 2006) and *B. subtilis* (Michiko M Nakano *et al.*, 2010), we were curious whether Spx in *S. aureus* would show redox-dependent binding to αCTD and the *cspA* promoter. Indeed, the presence of 1 mM DTT which reduces Spx to an inactive state abolished a shift of the radiolabeled *cspA* promoter with αCTD and Spx (Fig. 9, Lane 9), suggesting that the oxidized form of Spx is important for this interaction.

Effect on Spx on pigment production.

Recognizing that Spx interacts with αCTD to bind the *cspA* promoter, we were curious as to the biologic effect of this interaction on pigmentation, an important virulence factor of *S. aureus*. We have shown earlier that increased expression of *spx* repressed carotenoid pigment production (Fig. 8B). To confirm this finding, we examined pigment production in the *spx* C10A mutant in the JE2 background. This mutant (gift of Vanai Thomas, Univ. of Nebraska Medical School), lacking the CXXC motif to fabricate the active (oxidized) form of Spx (Shunji Nakano *et al.*, 2004), has no growth defect and could elaborate strong carotenoid pigment (Fig 8C), consistent with high *cspA* expression (Fig. 8A) in association with elevated σ^B-dependent *asp23* activity (Fig. 10A). Over-expression of *cspA* from an exogenous promoter in the *spx* C10A mutant with plasmid pALC7342 (V+*cspA*) led to a modest but insignificant increase in carotenoid production vs. the vector control (V) (Fig. 8C), consistent with already elevated *cspA* expression in this mutant. In contrast to the *spx* C10A mutant, enhanced expression of *cspA* with pALC7342 in the wild type JE2 resulted in a significantly higher carotenoid level than its counterpart carrying the pEPSA5 vector alone (Fig. 8D). Together, these data support a model whereby Spx represses *cspA* expression to reduce carotenoid expression.

The level of *asp23* transcription is temporally different in *cspA* and *yjbH* mutants.

Given that *cspA* regulates pigment through *sigB* (Katzif *et al.*, 2005), and *yjbH* is necessary for *cspA* transcription via reduced Spx expression, we measured the impact of each on σ^B activity over time by following the fluorescence in strains with an *asp23* promoter fusion with GFP. As a control, a Δ*sigB* strain was provided as a baseline for the impacts of *cspA* and *yjbH* on σ^B activity (Katzif *et al.*, 2005). As displayed in Fig. 10A, σ^B-dependent *asp23* promoter activity in a Δ*cspA* mutant of SH1000 was lower compared to the wild-type, but still higher than that found in a Δ*sigB* strain despite a lack of differences in growth among strains (Fig. 10B), thus mirroring the significant, but not total, decrease in pigmentation in a Δ*cspA* mutant (Fig. 1A). In contrast, the *asp23* activity of the Δ*yjbH* strain of SH1000 was equivalent to that seen in wild type up through the end of post-exponential phase (Fig. 10A). However, following entry into late or post stationary phase, *asp23* promoter activity diverged, with levels increasing in a wildtype strain, while the level of *asp23* promoter activity of the Δ*yjbH* strain had a modest increase but significantly lower than that of the wild type, consistent with a lower σ^B activity in the mutant. We also observed that chromosomal replacement in the respective *cspA* and *yjbH* mutants (*csp+* and *yjbH+*) yielded slightly higher *asp23* promoter activity than the wild type. The reason for this moderate increase is not clear at this point.

Both *cspA* and *yjbH* are necessary for survival in whole blood.

cspA has been previously shown to be important for survival of *S. aureus* phagocytosed by neutrophils (Samanta *et al.*, 2016), purportedly due to alteration of capsular activity via binding of CspA to the *cap* promoter. We were interested as to whether the increased susceptibility of a *S. aureus cspA* mutant to neutrophils extended to strains lacking *ΔyjbH*, given the genetic linkage between *yjbH* and *cspA* delineated above. Furthermore, as *sigB* has also been shown to be involved in capsule regulation via the *arlRS* and *yabJ-spoVG* operons (Meier *et al.*, 2007), a *S. aureus ΔsigB* strain was included as a control in this experiment. Accordingly, we exposed *S. aureus* wild type, Δ*cspA, ΔyjbH* and Δ*sigB* strains to human phagocytic cells (mostly PMNs) in a whole blood killing assay. Both *ΔsigB* and Δ*cspA* strains were much less able to survive compared to the wildtype (Fig. 11). Importantly, a SH1000 Δ*yjbH* strain survived even less well than the *sigB* and *cspA* mutants, suggesting a greater involvement of *yjbH* in resistance to oxidative stress and oxidative killing by PMN, possibly through post-translational control of a *cspA*-independent but *yjbH*-dependent pathway in *S. aureus*.

DISCUSSION

Staphyloxanthin, the carotenoid pigment accounting for the golden yellow color in *S. aureus*, is a major virulence factor of *S. aureus* (Song *et al.*, 2009). During infections, staphyloxanthin, as a powerful antioxidant, has been shown to impair neutrophil killing by evading death due to reactive oxygen species produced by the neutrophils (Liu *et al.*, 2005). In previous studies, the cold shock protein CspA has been found to regulate *sigB*-dependent carotenoid production in *S. aureus* (Katzif *et al.*, 2005). However, the pathways that control CspA expression have not been well defined. In addition, there are two additional Csp paralogs (e.g. CspB and CspC) the function of which is poorly defined. Our study here has clarified the roles of these Csp paralogs in carotenoid pigment production in *S. aureus*. In previous reports (Katzif *et al.*, 2005; Duval *et al.*, 2010), attempts at determining the *csp*-dependent phenotypes were ill-defined as strains were created with polar disruptions of *csp* paralogs in divergent backgrounds or large deletion of chromosomal region surrounding *csp* genes (Sahukhal and Elasri, 2014). In this study, only the impact of clean deletions and/or non-polar insertions in *csp* genes in a single genetic background (i.e. SH1000) on carotenoid production were examined and compared to a Δ*sigB* strain. We established that *cspA* plays a larger role in carotenoid production than *cspB* and that *cspC* has no role in *S. aureus* pigmentation (Fig. 1A). In contrast to CspA, the part played by CspB in pigmentation is independent of σ^B. The significance of this is yet unclear, but a screen of carotenoid-deficient mutants in the JE2 transposon library (Fey *et al.*, 2012) with unaltered *asp23* activity also revealed σ^B-independent expression of staphyloxanthin in diverse strains (data not shown). This is in agreement with a previous report, showing that both σ^B-dependent and -independent pathways are capable of modulating carotenoid production in *S. aureus* (Lan *et al.*, 2010).

A recent study (Sahukhal and Elasri, 2014) suggested that the vast majority of *cspA* mRNA in *S. aureus* existed as a single-gene transcript generated by post-transcriptional processing of a larger *msaA-cspA* transcript (Sahukhal and Elasri, 2014). However, the results from our

studies (Fig. 2B) and other labs (Uppalapati *et al.*, 2017) support the notion that the *cspA* promoter is considerably more active than the *msaA* promoter (Fig. S1). One possibility for this difference could be the use of luminescent construct in other studies (Sahukhal and Elasri, 2014) vs. the fluorescent reporter in our studies, as luminescence in *S. aureus* is affected by intracellular redox state and growth phase (e.g. less activity during stationary phase) (Galluzzi and Karp, 2007).

In elucidating the mechanism that controls carotenoid production in *S. aureus*, we have discovered a novel pathway wherein the stress-response pathway of CspA-σ^B is linked to the redox sensitive transcriptional regulator Spx (Fig. 12). The discovery of Spx in the repression of *cspA* (Fig. 8) has prompted us to examine factors that mediate post-transcriptional control of Spx. In conducting a *de novo* screen of the Nebraska JE2 transposon library for mutants that exhibited reduced pigmentation, we discovered one of the mutants to be an *yjbH* mutant. YjbH is an adaptor protein that ushers specific protein(s) to the ClpXP proteolytic core for degradation (Fig. 12). Our observation that the Δ*yjbH* and the Δ*clpP* mutants displayed reduced *cspA* promoter activity vs. the parent SH1000 (Fig. 2D) is consistent with increased Spx level due to reduced Spx degradation in the mutants which, in turn, represses *cspA* more than the wildtype.

Our model (Fig. 12) also predicts reduced carotenoid production in the Δ*yjbH* mutant (Fig. 6). As both *cspA* and *yjbH* positively regulate pigment production, we also verified that *cspA* is epistatic to *yjbH* as supported by findings that complementation of *cspA* in a Δ*yjbH* mutant could restore carotenoid production (while the reverse was not possible) (Fig. 6). YjbH regulates Spx post-translationally by recognizing a C-terminal signal on Spx and guiding it to the chaperone ClpX, which channels it for degradation by ClpP (Chan *et al.*, 2012). Together, these data suggest that *yjbH* lies upstream of *spx* and *cspA* in a pathway controlling *sigB*-dependent carotenoid production.

Interestingly, a recent paper by Austin *et al* also reported the contribution of YjbH to pigmentation and host colonization in *S. aureus* (Austin *et. Al.* 2019). While some of our findings are complementary (e.g. YjbH controls σB and pigmentation by altering Spx level), other findings in this paper are additive (e.g. *cspA* is the intermediary between Spx and σB. We have also provided a more comprehensive model to explain the genetic pathway that controls pigmentation. It is surprising that Austin *et al* described increased colonization of the *yjbH* mutant in kidneys and spleens vs. the wild type in a murine eye infection model, in contrast to our human whole blood killing assay wherein the *yjbH* mutant was more susceptible than the wild type. As mouse survival with these mutants was not measured in the Austin paper, it is unclear if these mutants would impact survival in this particular murine model. It should be mentioned that infection of an eye where it is immunologically inert is an unusual model for disseminated infection for *S. aureus*. Based on our whole blood killing assay, we would predict decreased survival of the *yjbH* mutant vs. the parental strain in the murine dissemination model of infection due to reduced pigment production.

Spx contains an internal disulfide redox sensing motif 10-CXXC-14 that affects its interaction with RNA polymerase (Shunji Nakano *et al.*, 2004). The function of Spx as an activator or repressor appears to requires a small G/C rich element upstream of RpoA αCTD

DNA binding site (Rochat *et al.*, 2012). As our EMSA data show that Spx interacts with the *cspA* promoter through the αCTD of RpoA, we conclude that Spx is a direct repressor of the *cspA* promoter. Such regulation is consistent with ChIP-chip data from *B. subtilis*, showing that Spx is enriched at promoters of all three *csp* homologs (Rochat *et al.*, 2012). Notably, failure of a previous two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) to detect CspA (~7 kD) in the proteome of Spx (Pamp *et al.*, 2006) was likely due to its inability to detect proteins below 20 kD. In addition, we also showed that the repressive effect of Spx on carotenoid production can be bypassed by expressing *cspA* from xylose-inducible promoter whereas this induction effect is muted in a *spx* C10A mutant where *cspA* expression is already high.

It is still possible that Spx governs *cspA* expression through other mechanisms of regulation. Maturation of the *cspA* transcript has been shown to be modulated by the binding of *msaR*, an antisense small RNA to the 5' UTR of *cspA*. Generation of this small RNA and the subsequent maturation of the *cspA* transcript is controlled by ribonucleases (Galluzzi and Karp, 2007; Lioliou *et al.*, 2012) which themselves could be targets for Spx-mediated regulation.

We have demonstrated auto-repression of *cspA*, based on up-regulation of the *cspA* promoter in a *cspA* mutant. Predicated on the up-regulation of *yjbH* in a *cspA* mutant (Fig. 5A), we propose that the pathway for auto-repression of *cspA* may occur via YjbH and Spx which, in turn, binds αCTD of RNAP and then onto the *cspA* promoter to repress *cspA* transcription (Fig. 3). According to this scheme, transcription of *hbo-yjbH* is up-regulated in a *cspA* mutant (Fig. 5A and 5B), increasing YjbH levels which contribute to increased Spx proteolysis. Depletion of Spx would lead to de-repression of the *cspA* promoter (Fig. 8A), resulting in up-regulation of the *cspA* promoter in a *cspA* mutant (Fig. 3). Alternatively, we recognize that Csp homologs can alter gene expression through their ability to bind mRNA and DNA promoter and terminator elements (Phadtare and Severinov, 2010), thus CspA may bind the *cspA* promoter directly for the repressive effect.

The significance of the transcription of *hbo* and *yjbH* (or *yjbIH*) as an operon and its relevance to the larger *cspA/sigB* regulatory network is still unclear. The putative truncated hemoglobin protein produced from the *hbo* gene was recently shown to have a high oxygen affinity and provide an advantage for growth under low oxygen conditions when expressed exogenously in *E. coli* (Pathania, 2002). Any relationship linking these characteristics of Hbo to oxidative stress or carotenoid production is not clear at this point. Interestingly, we have also found via *cspA* promoter-GFP fusion studies with pALC8135 that the *cspA* promoter activity in strain SH1000 was significantly elevated upon exposure to 10 mM of hydrogen peroxide, suggesting that the *cspA* gene is also responsive to oxidative stress (Fig. S4). Given the role of Spx, CspA and carotenoid in response and resistance to oxidative stress, the linkage seems plausible.

Carotenoid pigment is a powerful antioxidant that has been found to impair neutrophil killing mediated by the superoxide anion that is generated as a result of the oxidative burst. As we have uncovered the role of *yjbH-spx-cspA-sigB* axis (Fig. 12) in the control of pigment production, we wanted to verify the survival of the *yjbH* and *cspA* mutant in whole

blood killing assay where PMNs are the predominant phagocytic cells capable of generating superoxide anion and hydrogen peroxide inside the phagolysosomes. We observed that both the *yjbH* and *cspA* mutants survived less well than the parent SH1000. Remarkably, the *yjbH* mutant has the lower survival rate than *sigB and cspA* mutants, suggesting that YjbH may harbor *cspA*-independent pathway for resistance to oxidative killing by PMNs. Accordingly, a transcriptomic analysis of *cspA* and *yjbH* mutant may provide clues for the differential survival rates between these two mutants.

EXPERIMENTAL PROCEDURES:

Bacterial strains and culture conditions.

Table 1 contains a list of bacterial strains used in these studies. *E. coli* strains were grown in Lysogeny Broth (LB), and *S. aureus* grown in Trypticase Soy Broth (TSB), both shaking at 250 rpm at 37°C. Cell density was measured via optical densities (OD) at 650 nm using either an 18-mm borosilicate glass tube in a Spectronic 20D+ spectrophotometer or an Infinite M1000 Pro reader (Tecan).

DNA manipulations.

E. coli plasmid purification was performed per the manufacturer's instructions (Omega), while plasmid isolation from *S. aureus* was performed as described previously (Schenk and Laddaga, 1992). Transformation of *S. aureus* was achieved via electroporation with a MicroPulser (Biorad), using plasmid DNA directly isolated from either *E. coli* ALC7884 or ALC7885 (Jones *et al.*, 2015), for p15A and pUC-based vectors, respectively. Oligonucleotides were synthetized by IDT Inc.

Protein expression and purification.

Expression of Spx or αCTD (residues 245 to 314 of the RNA polymerase α subunit RpoA) was performed by PCR amplification of each gene with 5' *Nde*I and 3' *Eco*RI sites, digesting with the same enzymes, and subcloning into the equivalently digested pET28a. *E. coli* BL21(DE3)pLysS strains harboring each resulting plasmid (pALC8681 or pALC8682) were induced with 1 mM IPTG at 37oC for 4 hours (Studier *et al.*, 1990). Harvested cells were lysed ultrasonically and purified with HisPur Cobalt Resin (ThermoFisher). His_6-αCTD was digested with thrombin to remove the hexahistidine tag using a Thrombin Cleavage/Capture Kit (Millipore). Protein was then aliquoted, stored in 10 mM Tris-Cl, 1 mM EDTA, 100 mM NaCl, 10% glycerol, pH 8.0 at −80oC, verified for purity with AcquaStain (Bulldog Bio) and authenticated by mass spectrometry at the University of Vermont Spectrometry Facility.

in trans expression in *S. aureus*.

Inducible systems in pEPSA5 for *cspA*, *yjbH* and *spx* expression were created by PCR amplifying each gene with a *sarA* ribosome binding site and 5' *Eco*RI and 3' *Bam*HI sites, digesting the PCR product with these enzymes, and ligating into similarly digested pEPSA5 (Forsyth *et al.*, 2002).

Cell lysate preparation and Western blot analyses.

Protein lysates were prepared as described before (Donegan *et al.*, 2010) except that translation was stalled with 50 μg/ml erythromycin upon harvest.

Transcriptional fusions to gfp_{uvr}.

Constructs containing the promoter regions of *cspA*, *msaA* (the gene upstream of *cspA*) and *hbo-yjbH* (also called *yjbIH*) were created to control gfp_{uvr} by PCR amplification of each region with flanking 5' *Eco*RI and 3' *Xba*I restriction sites. These regions corresponded to: P_{hbo}, 90bp upstream of the *hbo* start codon; P_{msaA}, 500bp upstream of the *msa* start codon; P_{cspA}, 500bp upstream of the *cspA* start codon. The correspondingly digested PCR fragments were then ligated into the *Eco*RI and *Xba*I sites upstream of promoterless gfp_{uvr} in pALC1484.

Construction of deletion and mutant strains.

Strains deleted for *cspA* (*sa1234*), *cspC* (*sa0747*) or *yjbH* (*sa0860*) were constructed using the temperature-sensitive allelic replacement plasmid pMAD as described (Arnaud *et al.*, 2004). In brief, chromosomal regions 1000 bp up- and downstream of each gene of interest were amplified by PCR with primers proximal to the deletion site, joined by gene sewing, ligated into pMAD, transformed into ALC7885 (a derivative of *E. coli* DC10B) and then electroporated into *S. aureus* strain SH1000. The mutant was then generated by a temperature shift strategy to yield the mutant (Arnaud *et al.*, 2004). The pMAD constructs used to replace deletion of either *cspA* or *yjbH* were created by amplifying the appropriate gene and 1000 bp flanking regions from the wild type SH1000 chromosome, and then utilizing allelic replacement as described above. As the genes replaced above are either transcribed monocistronically (e.g. *cspC* or *cspA* where the major transcript is monocistronic) or the last gene in a two-gene transcript (e.g. *yjbH*), it is unlikely that the chromosomal gene replacement would have significant polar effect downstream. Successful allelic exchanges were verified by colony PCR and chromosomal sequencing. Transduction of the kanamycin-resistance cassette *aphA* immediately upstream of *cspB* from *S. aureus* strain COL to SH1000 was performed using phage 80α (Schwesinger and Novick, 1975).

A chromosomal C10A mutation of *spx* in MRSA strain JE2 was a gift from Vanai Thomas at the University of Nebraska. The *spx* C10A mutant cannot be oxidized due to the disruption of the 10-CXXC-14 motif.

Isolation of RNA and Northern blot hybridization.

RNA isolation and purification and subsequent Northern blot analysis were performed as previously reported (Cheung *et al.*, 1994).

Electrophoretic mobility shift assays (EMSA).

To decipher interactions between proteins and the *cspA* promoter, a PCR product consisting of −300 to +1 of the *cspA* promoter was first end-labeled with γ−32P-ATP (Perkin Elmer) using T4 polynucleotide kinase (NEB) and purified by MicroSpin G-50 columns (GE). His_6-Spx and/or αCTD were pre-incubated for 10 min at 25oC in 18 μL of binding buffer (20

mM Tris-HCl pH 7.8, 50 mM NaCl, 5 mM MgCl2, 10% glycerol) containing 0.5 μg of calf thymus DNA. 2 μL of radiolabeled DNA (20,000 cpm) was then added and incubated at 25oC for 15'. Non-radioactive competitor DNA was either the *cspA* promoter (specific) or a 285 bp *sarX* fragment (non-specific). Samples were then resolved on 6% polyacrylamide gels in TGE buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA) at 150 V and visualized with a Typhoon Phosphorimager (GE).

Carotenoid assay.

Strains were grown overnight at 37oC on Tryptic Soy agar (TSA) with antibiotics and, as needed, 0.2% xylose. Cells were scraped into 500 μL of phosphate buffered saline (PBS), vortexed 5' for homogenization and measured for OD_{650} in a microplate reader to determine cell density. The culture was pelleted and resuspended in 1 ml methanol and pigment extracted by vortexing for 5' and incubation at 55oC for 10'. After brief centrifugation, the supernatant was measured at OD_{450} in a microplate reader and normalized for cell density at OD_{650}.

Fluorescence analysis.

For static assays, GFP fluorescence was measured at 485 nm and 516 nm for excitation and emission, respectively, and cell density recorded by measuring absorbance at 650 or 600 nm. For kinetic assays, 150 μL of cells were grown at 37oC with shaking (250 rpm) in a sterile 96 well plate with a transparent bottom (Costar plate 3632). Auto-fluorescence from sterile media was subtracted from each time-point and divided by OD_{650} to normalize for cell density.

Whole blood killing assay.

A whole blood killing assay with *S. aureus* strains was performed as previously described (Kyme *et al.*, 2012).

Software analysis.

Scanned images from non-saturated exposed film were analyzed densitometrically using ImageJ 1.51g (NIH). Statistical significance was determined with the unpaired two tailed t-test using Prism 7.0a (Graphpad) where needed.

STUDY APPROVAL

Peripheral blood was obtained from healthy adult donors by the General Clinical Research Center at Cedars-Sinai Medical Center. The study was approved by the Cedars-Sinai Medical Center Institutional Review Board and Office of Research Compliance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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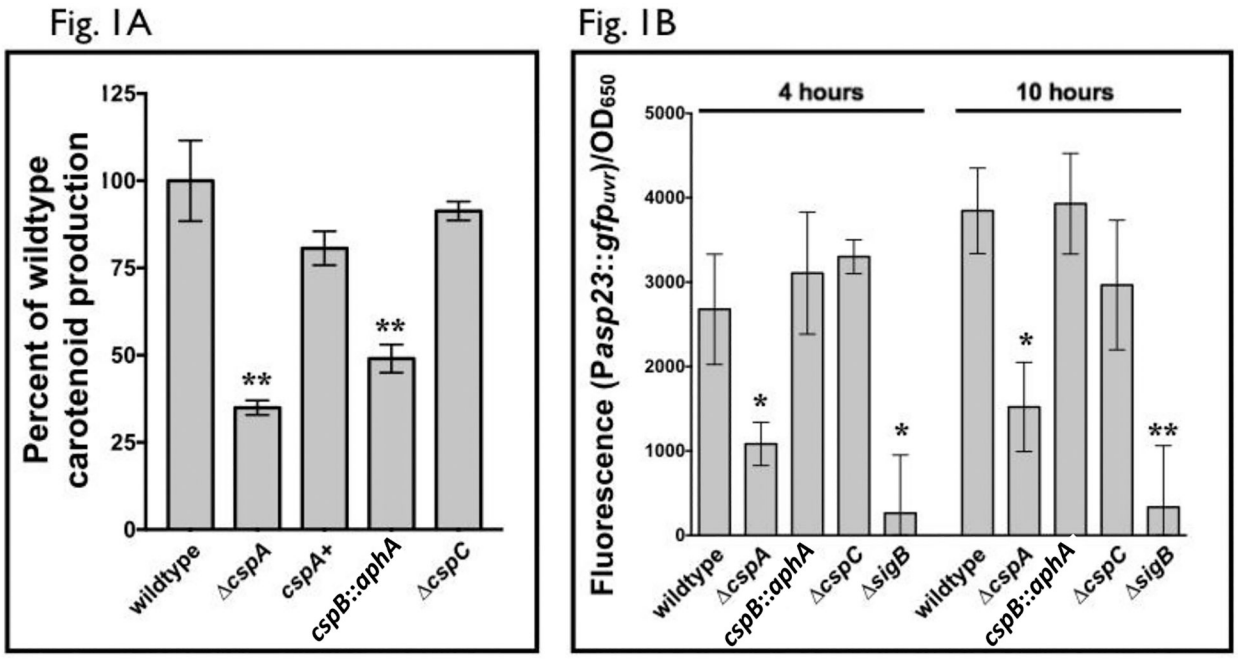

Figure 1: The impact of *S. aureus csp* homologs on carotenoid production and σ^B activity.

(A) Carotenoid was extracted from each of three biological replicates of *S. aureus* SH1000 wildtype and *csp* mutant strains that were grown overnight on tryptic soy agar. These levels were normalized to cell density and compared to one another as a percentage of wildtype. The experiments were performed thrice at different days, with consistent data within each experiment, but showing variation between experiment. Accordingly, percentages from one representative experiment were displayed. The *cspA+* strain denotes the *cspA* complement mutant strain ALC8212, and *cspB*::*aphA* indicates the *cspB* mutant strain ALC8581.

(B) GFP fluorescence generated from σ^B-dependent *asp23* promoter was measured for *S. aureus* SH1000 wildtype, *ΔsigB* and *csp* mutant strains transformed with the plasmid pALC2201 carrying the *asp* promoter driving *gfp*, at 4 and 10 hours of growth in TSB. Cell densities were simultaneously measured at OD_{650}, and fluorescence was normalized to this value. The experiments were performed using three biological replicates and repeated three times. A representative experiment is displayed.

The asterisks in Fig. 1A and 1B indicate statistical significance between wildtype and mutant strains, determined using Student t-test (*, $P < 0.05$; **, $P < 0.005$).

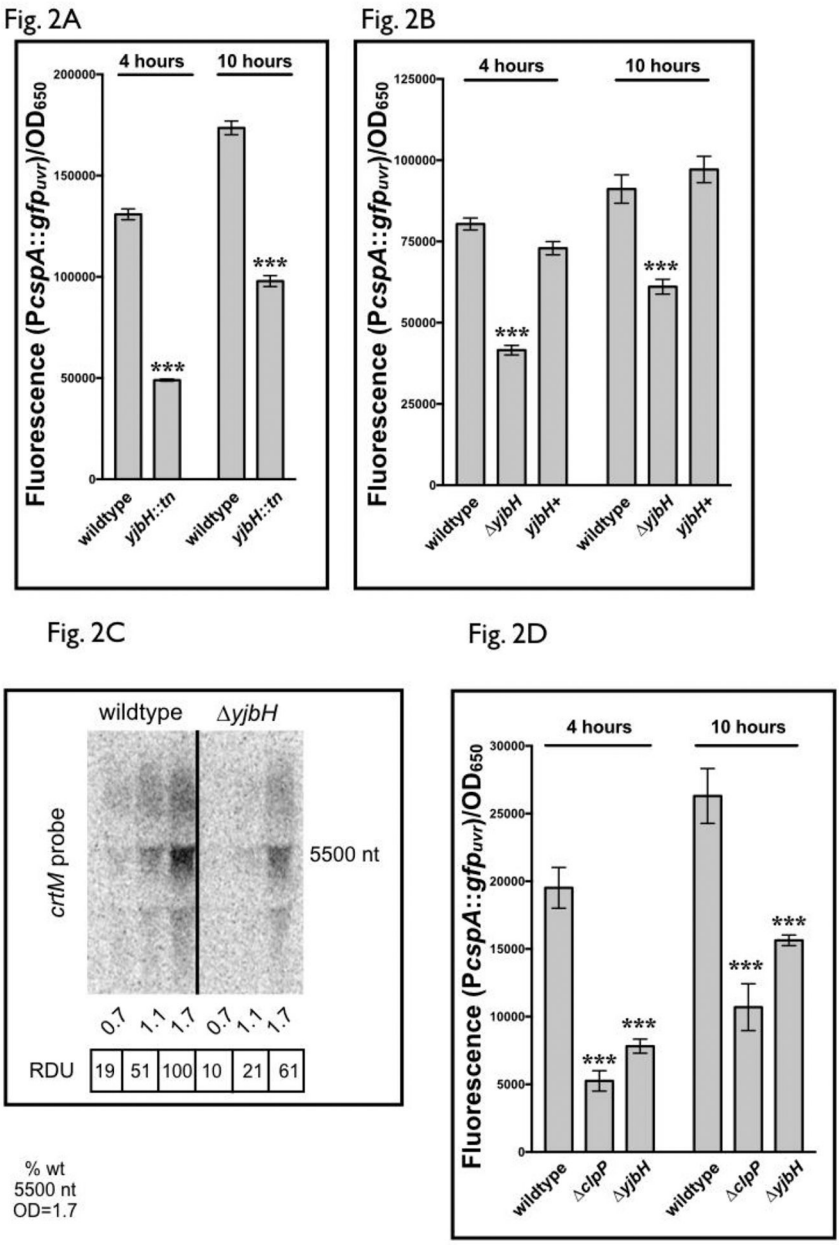

Figure 2: *yjbH* regulates *cspA* promoter activity in *S. aureus*.

(A) *cspA* promoter activity in *S. aureus* JE2 wildtype and a strain disrupted for *yjbH* by the mariner transposon. GFP fluorescence generated from the *cspA* promoter was measured at 4 and 10 hours for *S. aureus* JE2 wildtype and NE896 (*yjbH* transposon mutant), carrying the plasmid pALC8135 with *cspA* promoter driving *gfp*. Cell densities were simultaneously measured at OD_{650} and fluorescence normalized to OD_{650}. The value for each strain represents the mean of two biological replicates that were read in triplicate, and the experiments were repeated three times. A representative experiment is displayed.

(B) *cspA* promoter activity in *S. aureus* SH1000 wildtype, *de-novo* constructed Δ*yjbH* strains and *yjbH* mutant with chromosomal replacement. Fluorescence of *S. aureus* SH1000 wildtype, Δ*yjbH* and *yjbH+*, its chromosomally complemented strain, carrying the plasmid

pALC8135 was measured at 4 and 10 hours of growth in TSB. Cell densities were simultaneously measured at OD_{650} and fluorescence normalized by it. The value for each strain represents the mean read in triplicate and the experiments were repeated three times. A representative experiment is displayed. The *yjbH+* strain indicates the *yjbH* complement strain ALC8121.

(C) A representative Northern blot of the *crtOPQMN* transcript in SH1000 wildtype and Δ*yjbH* strains. RNA obtained from SH1000 wildtype and Δ*yjbH* cells at various optical densities (15 μg of cellular RNA per lane) was resolved on a denaturing agarose gel, blotted to Hybond XL membrane and hybridized with a 300-bp 32P-radiolabeled *crtM* DNA probe. The relative densitometric units (RDU) of each ~5.5k nt band were calculated relative to wildtype at OD_{650}=1.7. The experiment was repeated at least two times with consistent results.

(D) *cspA* promoter activity in *S. aureus* SH1000 wildtype and strains deleted for Δ*clpP* and Δ*yjbH*. Fluorescence of *S. aureus* SH1000 wildtype, Δ*clpP* and Δ*yjbH* carrying the plasmid pALC8135 was measured at 4 and 10 hours of growth in TSB. Cell densities were simultaneously measured at OD_{650} and fluorescence normalized by it. The value for each strain represents the mean of two biological replicates that were read in triplicate, and the experiments were repeated three times. A representative experiment is displayed.

The asterisks in Fig. 2A, 2B and 2D indicate statistical significance between wildtype and mutant strains, determined using Student t-test (***, P <0.0005).

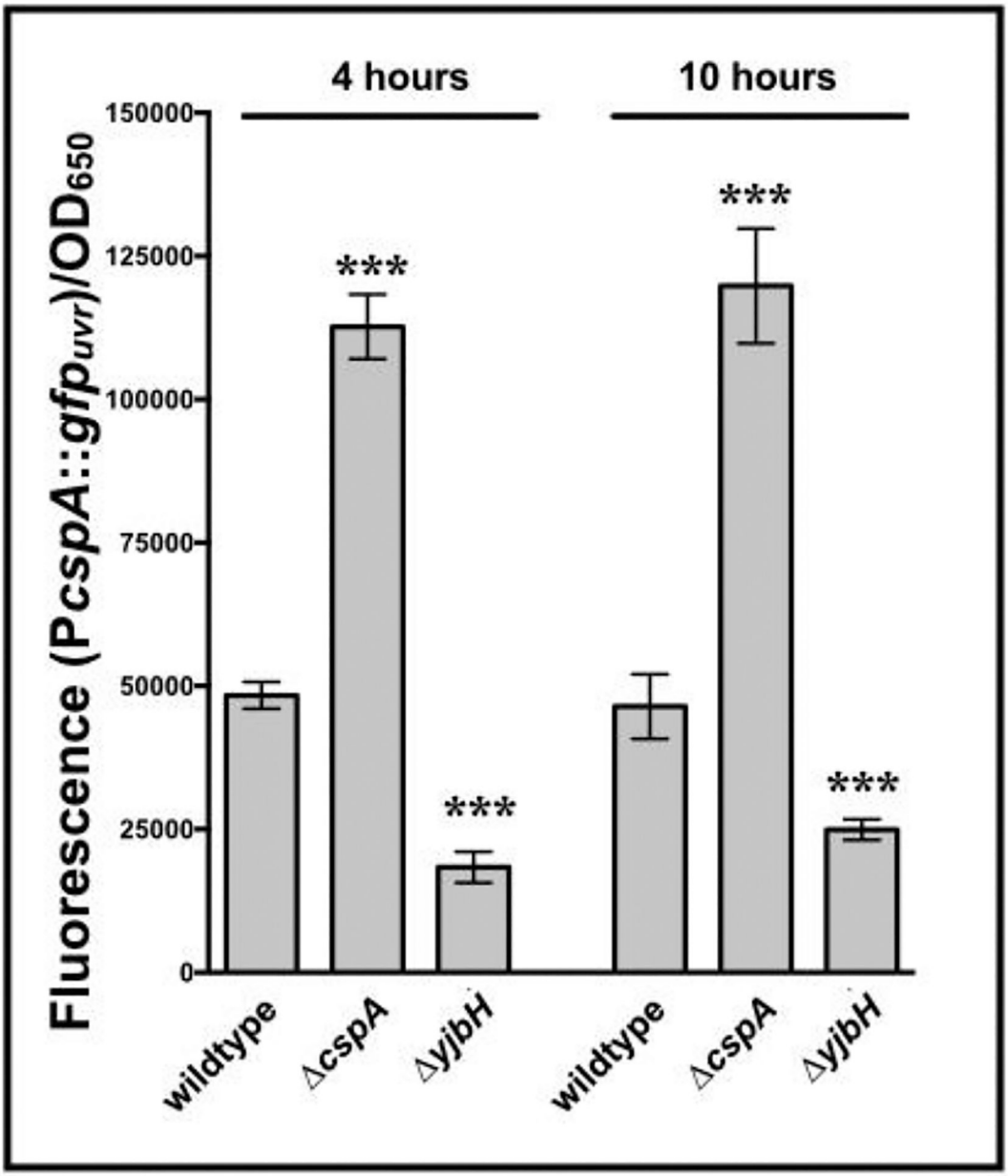

Figure 3: *cspA* represses the activity of its own promoter and is regulated by the *hbo-yjbH* locus.

cspA promoter activity in *S. aureus* SH1000 strains deleted for *cspA* or *yjbH*. Fluorescence of *S. aureus* SH1000 wildtype, *ΔcspA* and Δ*yjbH* strains carrying the plasmid pALC8135 at 4 and 10 hours of growth in TSB. Cell densities were simultaneously measured at OD_{650} and fluorescence normalized by it. The value for each strain represents the mean of two biological replicates that were read in triplicate, and the experiments were repeated three times. A representative experiment is displayed. The asterisks indicate statistical significance between wildtype and mutant strains, determined using Student t-test (***, P <0.0005).

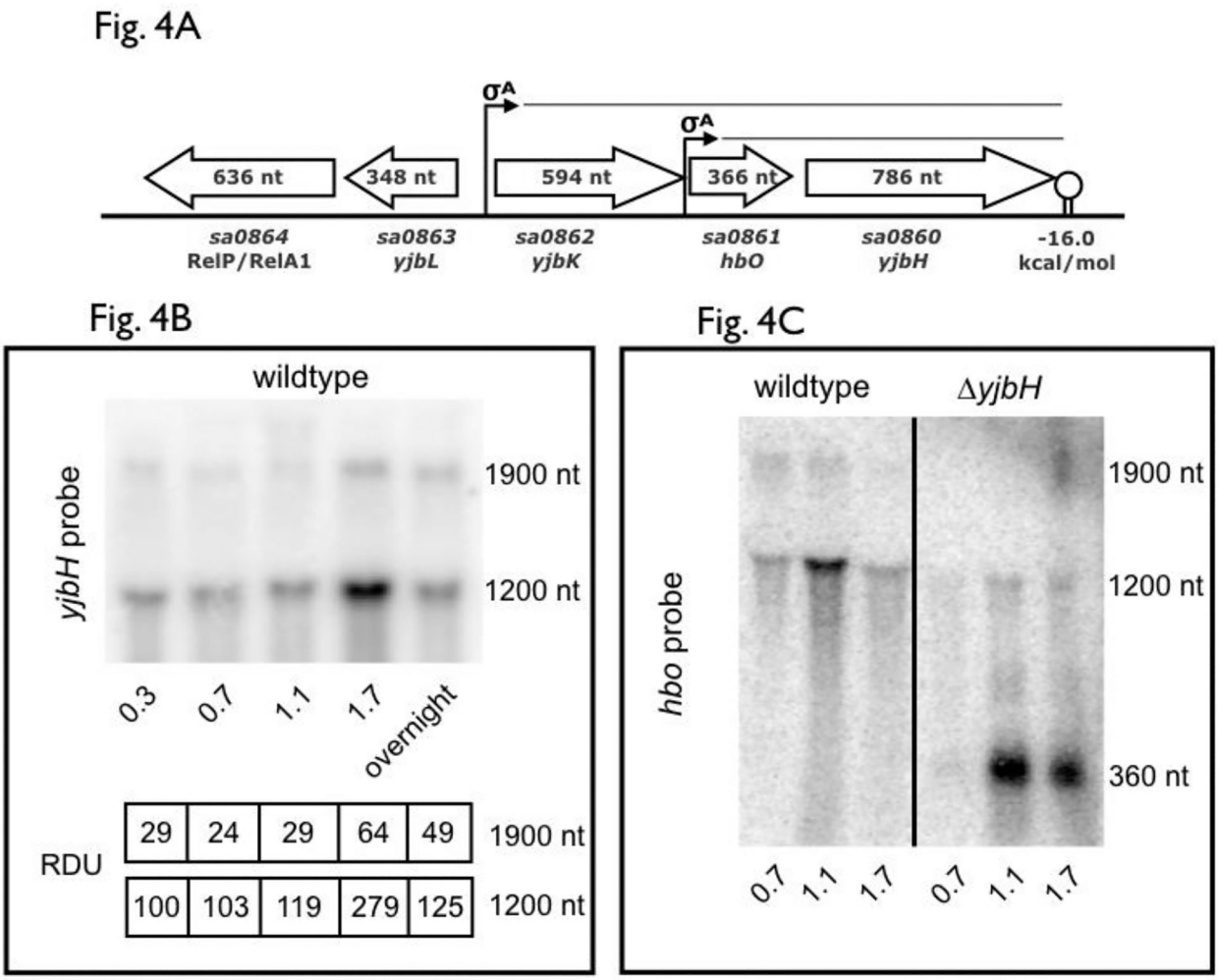

Figure 4: *yjbH* operon structure and transcriptional analysis.

(A) Operon and promoters of genetic elements neighboring *yjbH*. The location for the σ^A-dependent promoters directing *hbo* (also called *yjbI*) and *yjbH* transcription as well as that for *sa0862-hbo-yjbH* and the coding sequences for *yjbH* and its surrounding genes are shown in relation to one another. Predicted *yjbH* transcripts from transcriptional analyses and *in vitro* terminator data are also shown.

(B) *yjbH* transcription in SH1000 during logarithmic and stationary growth phases. RNA taken from SH1000 wildtype cells (15 μg per lane) at time points was resolved on a denaturing agarose gel, blotted to Hybond XL membrane and hybridized with a 300-bp 32P-radiolabeled *yjbH* DNA fragment. The relative densitometric units (RDU) of the 1.9k and 1.2k nt bands were calculated relative to 1.2k nt levels in the wildtype at OD_{650}=0.7 (set at 100). This is a representative blot of an experiment that had been repeated at least two times, with consistent results across different experiments

(C) *hbo* transcription in SH1000 wildtype and Δ*yjbH* strains. RNA obtained from SH1000 wildtype and Δ*yjbH* cells at various optical densities (15 μg per lane) was resolved on a denaturing agarose gel, blotted to Hybond XL membrane and hybridized with a 200-bp 32P-radiolabeled *hbo* DNA fragment.

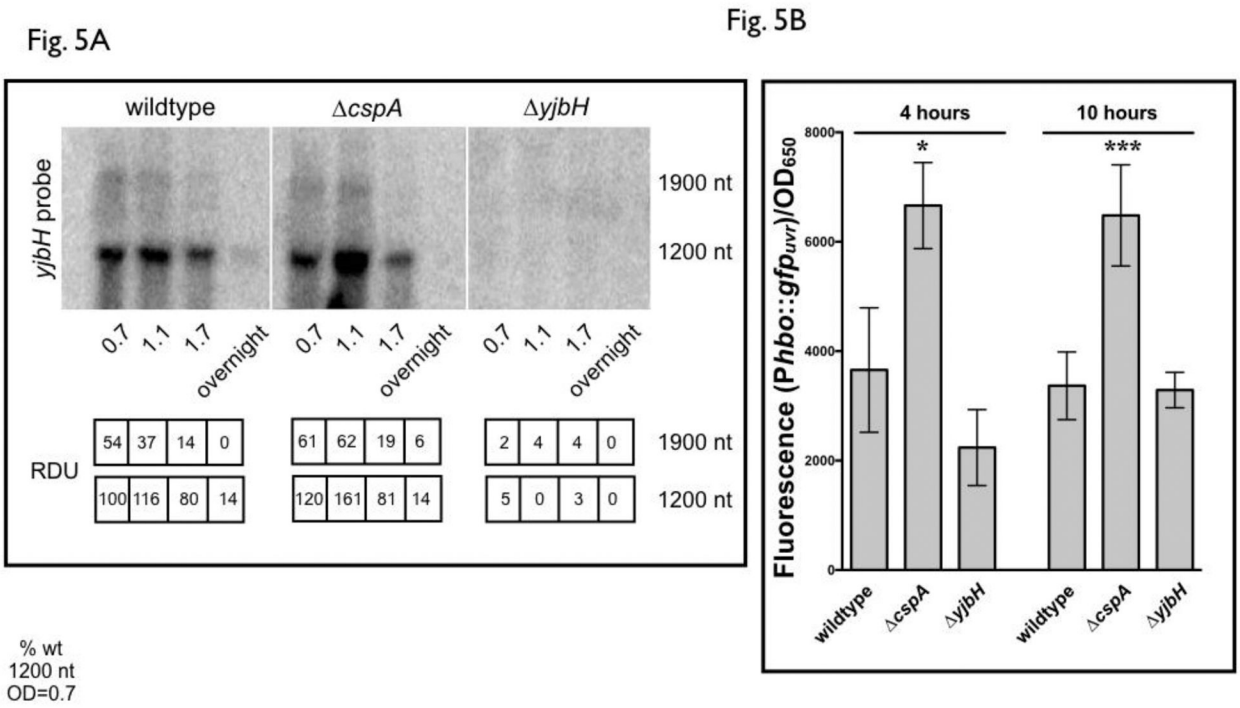

Figure 5: Transcriptional activity of the *hbo-yjbH* promoter is affected by *cspA*.

(A) *yjbH* transcription in SH1000 wildtype, Δ*cspA* and Δ*yjbH* strains. RNA obtained from SH1000 wildtype, Δ*cspA* and Δ*yjbH* cells at various optical densities (15 μg per lane) was resolved on a denaturing agarose gel, blotted to Hybond XL membrane and hybridized with a 200-bp 32P-radiolabeled *yjbH* DNA fragment. The relative densitometric units (RDU) of the 1.9k and 1.2k nt bands were calculated relative to 1.2k nt levels in the wildtype at OD_{650}=0.7 (set at 100). The hybridization analysis was repeated twice with similar results. A representative blot was shown.

(B) *hbo-yjbH* promoter activity in *S. aureus* SH1000 strains deleted for *cspA* or *yjbH*. Cell density-normalized GFP fluorescence of *S. aureus* SH1000 wildtype, *ΔcspA* and Δ*yjbH* strains carrying the *hbo-yjbH* promoter reporter plasmid (pALC8134) at 4 and 10 hours of growth in TSB was shown. Cell densities were simultaneously measured at OD_{650} and fluorescence normalized by it. The value for each strain represents the mean of two biological replicates that were read in triplicate, and the experiments were repeated three times. A representative experiment is displayed. The asterisks indicate statistical significance between wildtype and mutant strains, determined using Student t-test (*, $P < 0.05$; ***, $P < 0.0005$).

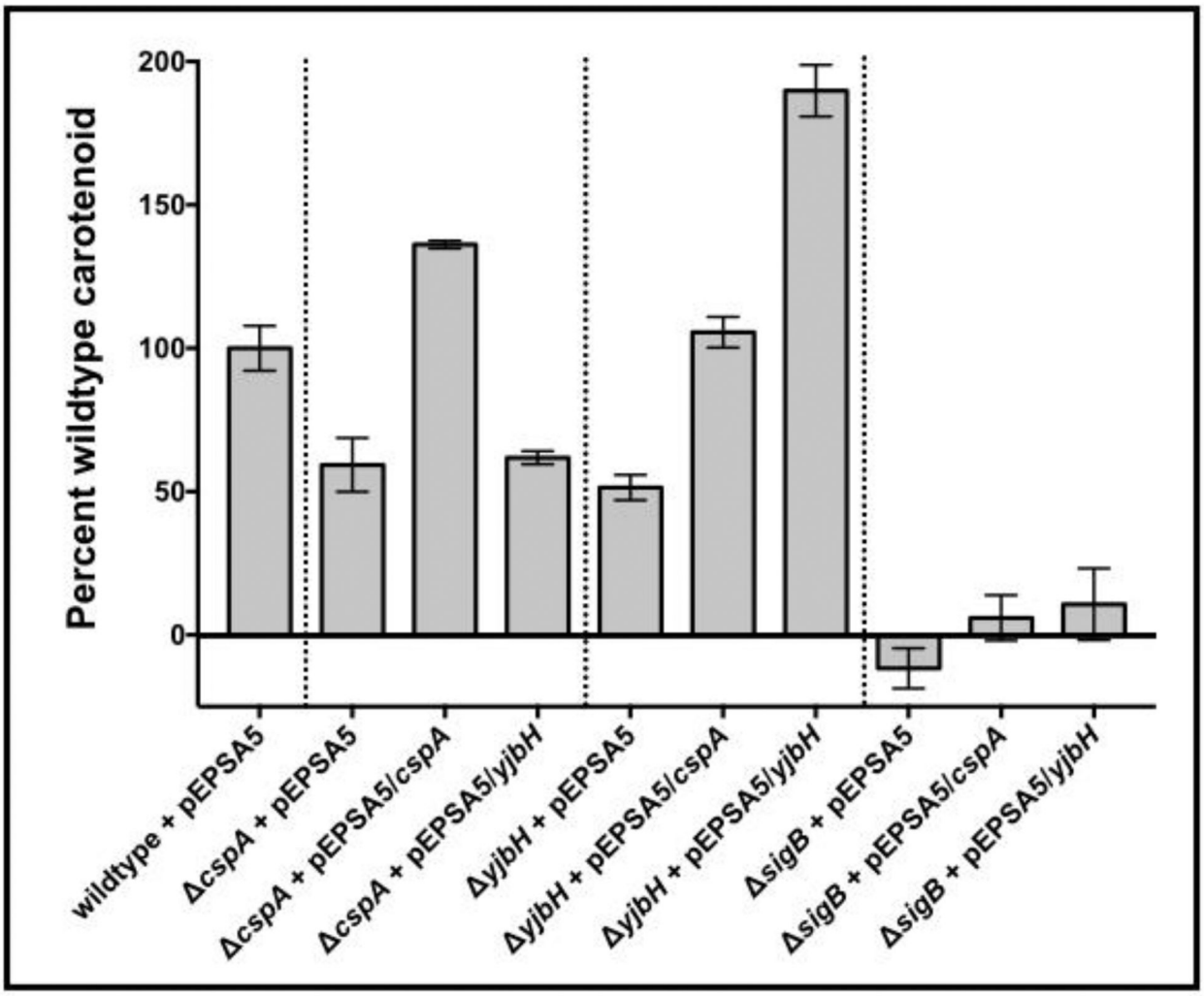

Figure 6: Expression of *cspA in trans* cross-complements carotenoid production in a SH1000 Δ*yjbH* strain.

Carotenoid was extracted from two biological replicates each of *S. aureus* SH1000 wildtype, Δ*cspA, ΔyjbH* and *ΔsigB* mutant strains with either pEPSA5, pALC7091 (pEPSA5::*yjbH*) or pALC7342 (pEPSA5::*cspA*) that were grown overnight on solid Tryptic Soy agar. The carotenoid levels were normalized to cell density and compared as a percentage of wildtype set at 100%. The experiments were repeated three times, with representative percentages from one experiment displayed. The asterisks indicate statistical significance between wildtype and mutant strains, determined using Student t-test (**, $P < 0.005$; ***, $P < 0.0005$).

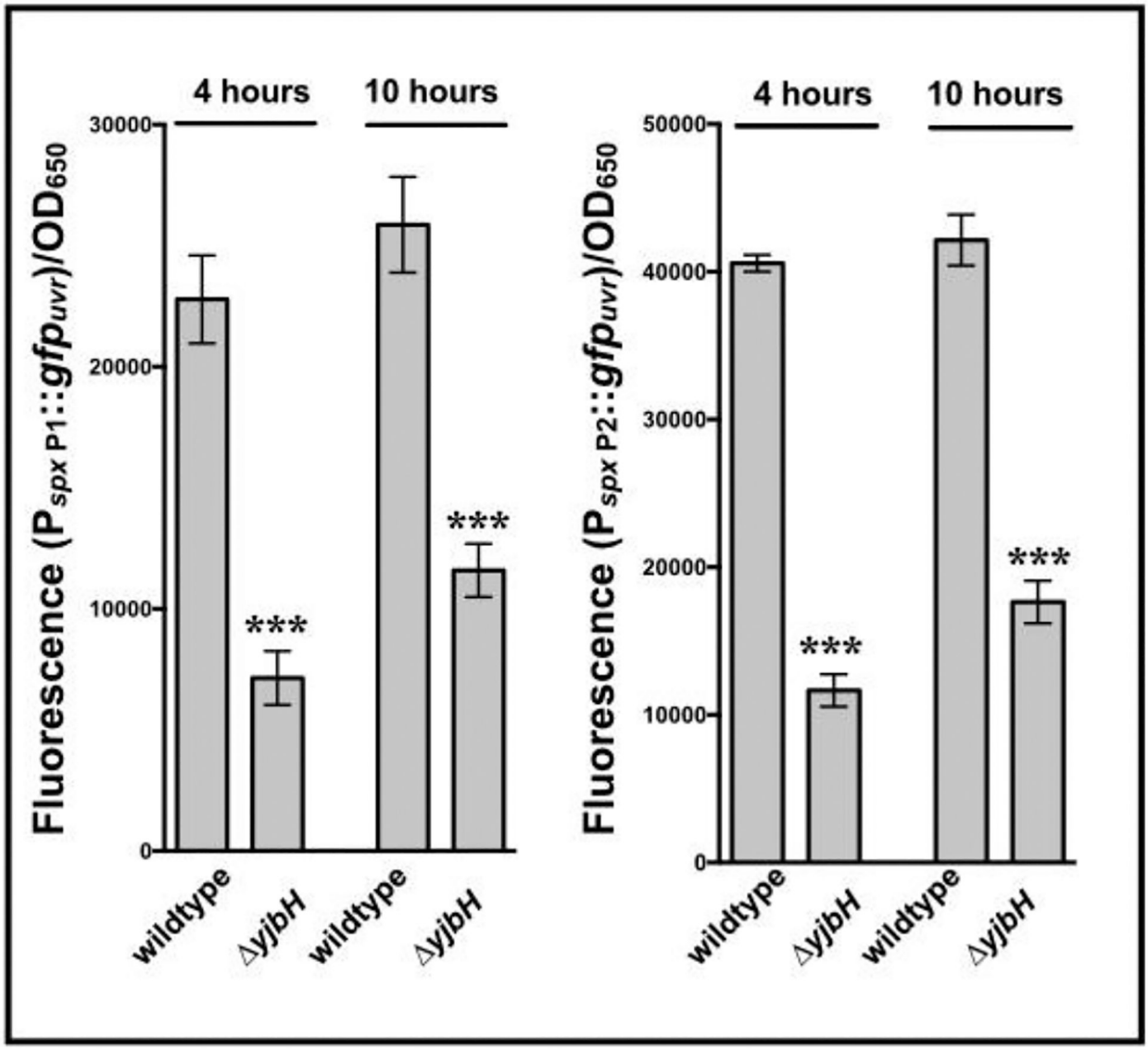

Figure 7: Activation of *spx* promoter in a Δ*yjbH* strain vs. the parent.

Promoter activity from both the proximal (P1) and distal (P2) *spx* promoters were measured in *S. aureus* SH1000 strains deleted for *yjbH*. Cell density-normalized GFP fluorescence of *S. aureus* SH1000 wildtype and Δ*yjbH* strains carrying the P1 (pALC7803) and P2 (pALC7804) transcriptional fusion plasmids were taken at 4 and 10 hours of growth in TSB. Cell densities were simultaneously measured at OD_{650} and fluorescence normalized by it. The value for each strain represents the mean of three technical triplicates of two biological replicates, and the experiments were repeated three times. A representative experiment is displayed. The asterisks indicate statistical significance between wildtype and mutant strains, determined using Student t-test (***, P <0.0005).

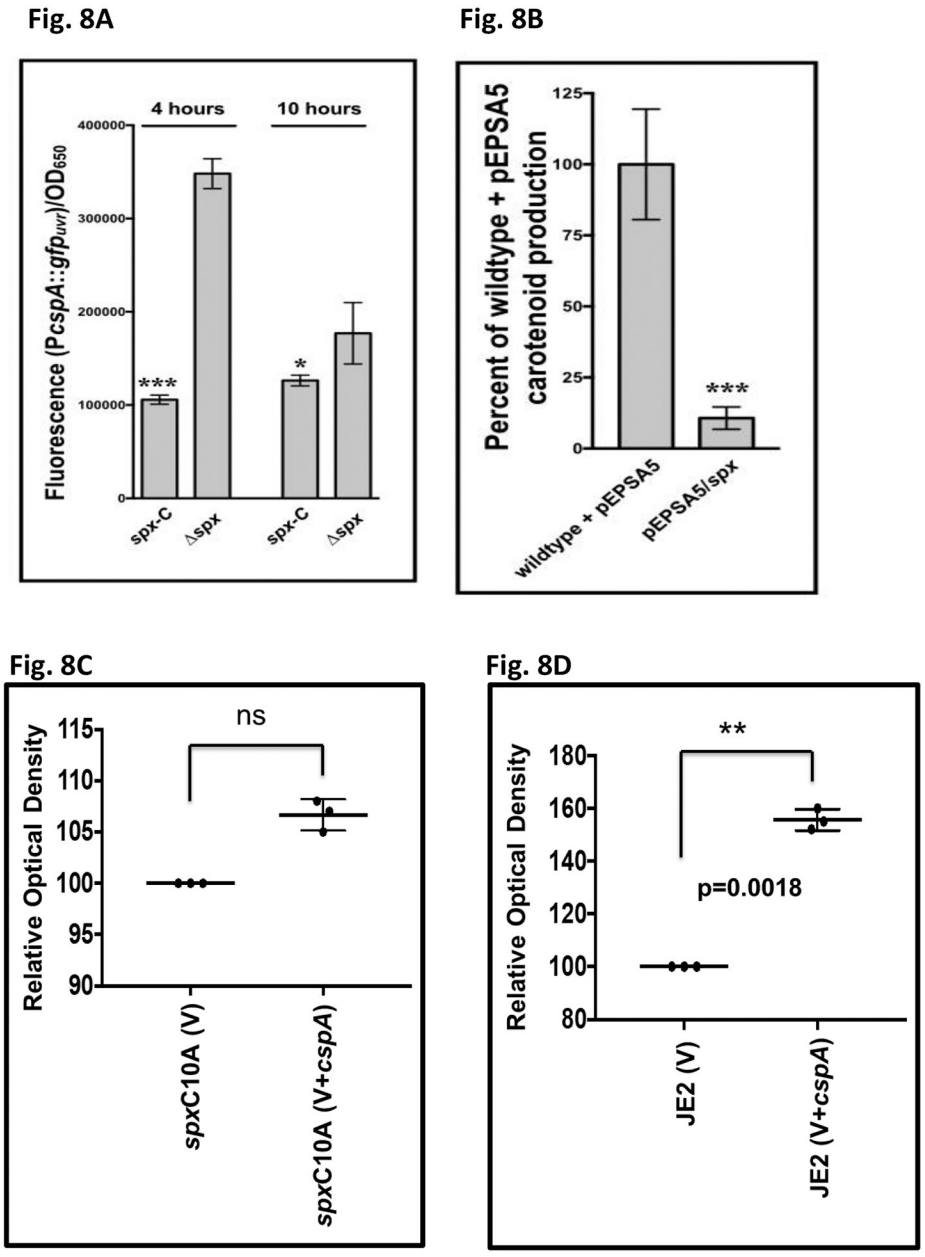

Fig. 8: Repression of the *cspA* promoter in a *spx* strain and effect of *spx* on pigment production.

(A) *S. aureus* 8325–4 Δ*spx* and its restored complement *spx-C* transformed with pALC8135 (P_{cspA}::*gfp*) were examined for fluorescence during growth in TSB at 4 and 10 hours. Cell densities at OD_{650} were measured simultaneously, and fluorescence normalized by it. The value for each strain represents the mean of three technical triplicates of two biological replicates, and the experiments were repeated three times. A representative experiment is displayed.

The asterisks indicate statistical significance between wildtype and mutant strains, determined using Student t-test (*, $P < 0.05$; **, $P < 0.005$).

(B) *S. aureus* SH1000 wildtype with either empty vector (pEPSA5) or vector over-expressing *spx* (pEPSA5::*spx*, pALC7133) were grown at 37oC overnight on solid agar with

0.2% xylose. Cells were scraped and resuspended in PBS, whereupon the cell density was measured, and carotenoid pigment extracted. Carotenoid levels were then normalized to cell density and compared to one another as a percentage of wildtype set at 100%. The experiments were repeated three times, with representative percentages from one experiment displayed. The asterisks indicate statistical significance between wildtype and mutant strains, determined using Student t-test (***, P <0.0005).

(C) Relative carotenoid pigment expression in *spx* C10A mutant carrying either pEPSA5 (V) or pEPSA5::*cspA* (V+*cspA*). Pigment from cells was extracted in triplicate as described in Fig. 8B. The *spx* C10A mutant with vector (V) was set at 100%. There was no significant difference between vector alone and vector expressing *cspA*.

(D) Relative carotenoid pigment expression in wild type JE2 cells carrying either pEPSA5 (V) or pEPSA5::*cspA* (V+*cspA*). Pigment from cells was extracted in triplicate as described above. The JE2 cells with vector (V) was set at 100%. Student t test was used to assess significance, with P value shown.

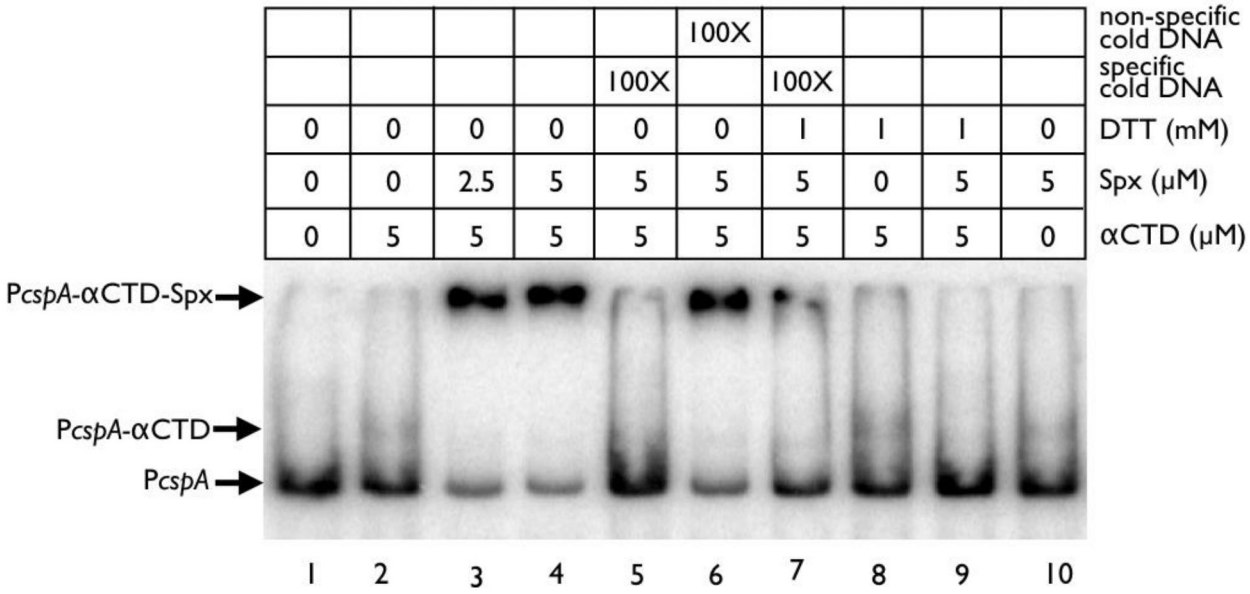

Figure 9: Spx and αCTD interact with the *cspA* promoter.

EMSA analysis of αCTD and Spx binding to the *cspA* promoter probe in reactions containing Spx or mixtures of Spx with αCTD under various conditions. Bands corresponding to the *cspA* promoter/αCTD and *cspA* promoter/Spx/αCTD complexes are marked with arrows. The 32P *cspA* promoter probe was generated by labeling a PCR product of the *cspA* promoter (−300 to +1) with γ−32P dATP using T4 polynucleotide kinase. An internal fragment of *sarX* was used as the non-specific cold challenge DNA, while unlabeled *cspA* promoter (−300 to +1) was used as the specific cold challenge DNA.

Fig. 10A

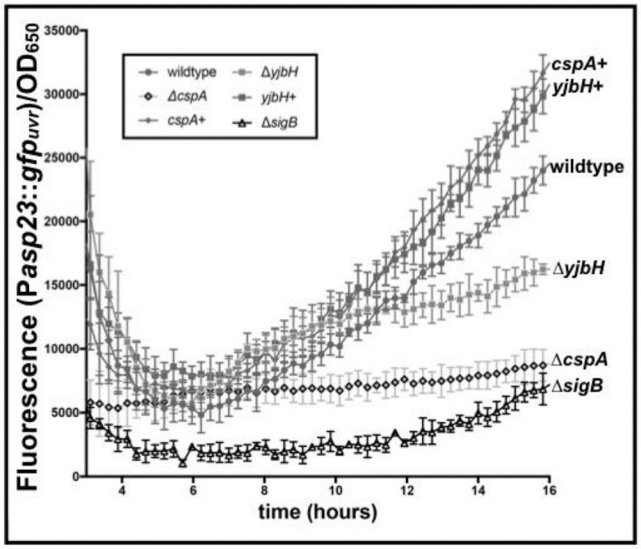

Fig. 10B

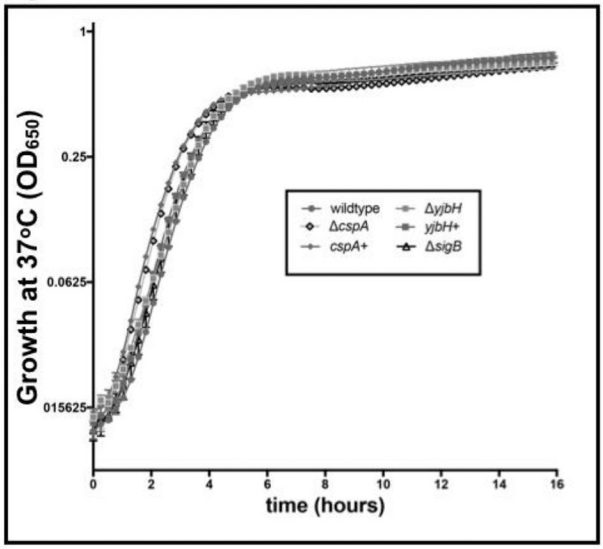

Figure 10: Strains lacking Δ*cspA or* Δ*yjbH* have differing profiles of the σ^B-dependent *asp23* promoter activity.

(A) Fluorescence and cell density (OD_{650}) were simultaneously measured in *S. aureus* wildtype (closed circle), Δ*cspA* (open diamond), *ΔyjbH* (lighter closed square) and Δ*sigB* mutant (open triangle) strains and their chromosomally complemented equivalents (*cspA+*, closed diamond, *yjbH+*, darker closed square) carrying the plasmid pALC2201 (*asp23* promoter driving *gfp*) during 16 hours of growth in TSB. GFP fluorescence was then normalized to OD_{650}, with each value representing the mean of two biological replicates that were read in triplicate.

(B) Growth curve of SH1000 wildtype and mutant strains. Cell densities of *S. aureus* wildtype (closed circle), Δ*cspA* (open diamond), *ΔyjbH* (lighter square) and Δ*sigB* mutant (open triangle) strains and their chromosomally complemented equivalents (*cspA+*, closed diamond, *yjbH+*, darker square) carrying the plasmid pALC2201 were measured at OD_{650}, with each value representing the mean of two biological replicates that were read in triplicate.

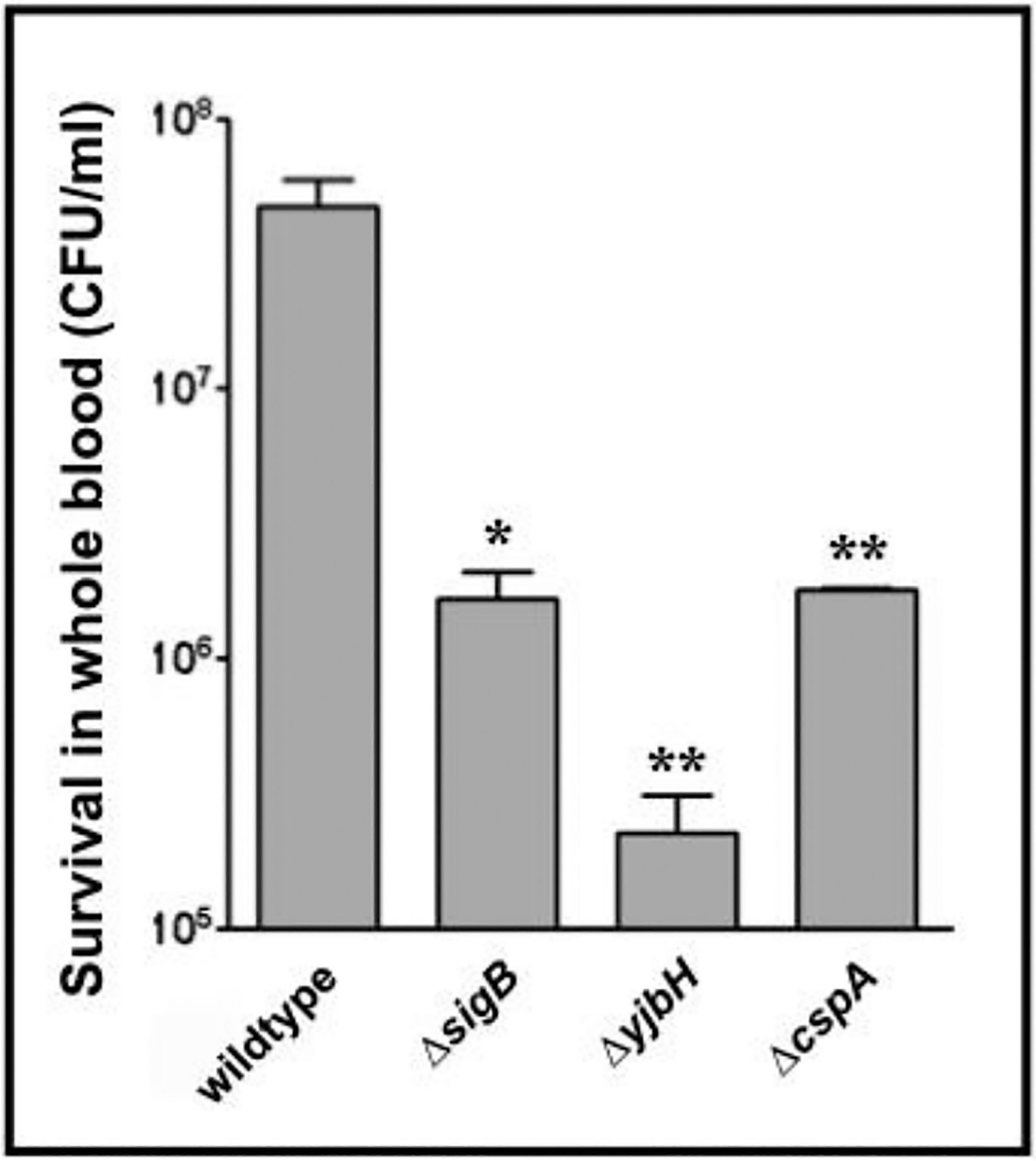

Figure 11: Human whole blood killing assay of SH1000 and mutant strains.

Blood from human volunteers was mixed with wildtype or mutant *S. aureus* strains (1 × 105 CFU/ml). Shown are surviving CFU at 3h. The experiment was performed three times and a representative experiment is displayed. The asterisks indicate statistical significance between wildtype and mutant strains, determined using Student t-test (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$).

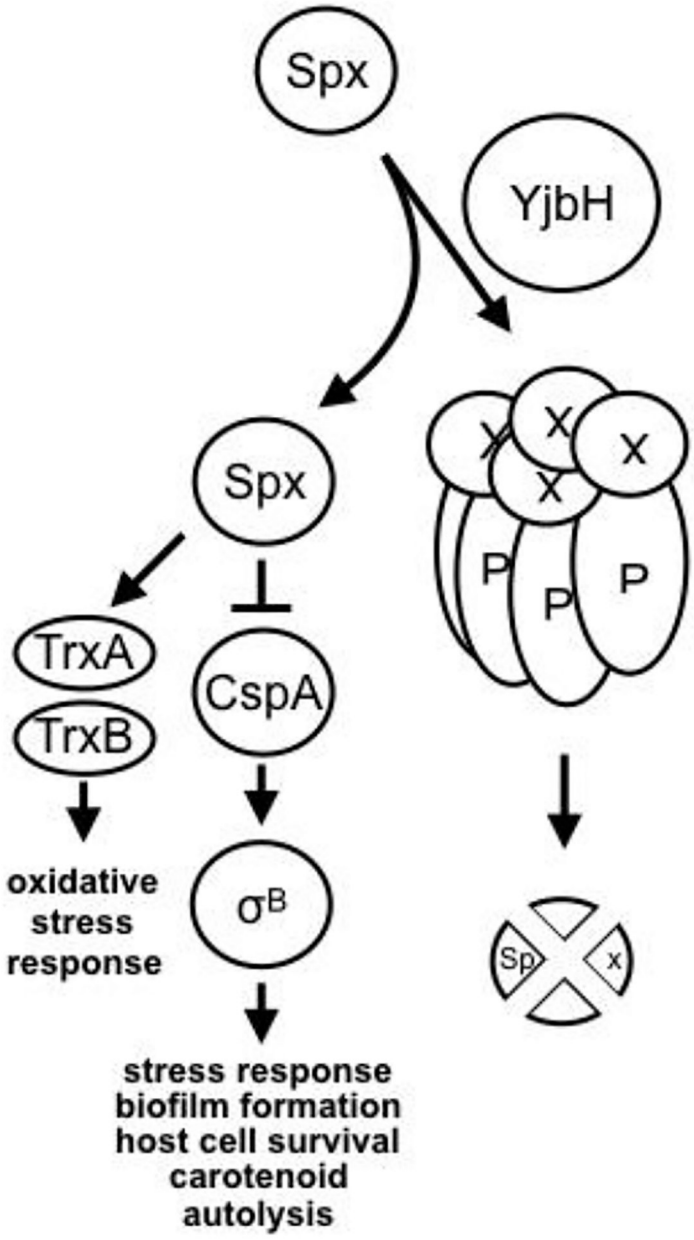

Figure 12: Proposed model for *sigB*-mediated carotenoid regulation in *S. aureus*.

The membrane pigmentation, staphyloxanthin, is a triterpenoid carotenoid. Staphyloxanthin encoded by the *crtOPQMN* operon is transcribed from a σ^B-dependent promoter. CspA acts as an activator of σ^B and its transcription is directly repressed by the redox-responsive transcriptional regulator Spx. Besides transcriptional control of *cspA*, there is also post-translational control because Spx is subjected to proteolytic degradation by the ClpXP protease as assisted by the adaptor YjbH. Spx also represses its own transcription. As part of the feedback, CspA can repress *yjbH* transcription.

TABLE 1:

Strain	Genotype	Source or reference
S. aureus		
BD1	COL with *aphA* inserted upstream of *cspB*	(Duval *et al.*, 2010)
JE2	USA300 LAC derivative cured of its plasmids	(Fey *et al.*, 2012)
NE896	JE2 *yjbH*::*tn*	(Fey *et al.*, 2012)
SH1000	8325–4 strain with repaired *rsbU* gene	(Horsburgh *et al.*, 2002)
SK12	SH1000 with Tn*551* insertion upstream of *cspA*	(Katzif *et al.*, 2003)
Δ*spx*	AR738, 8325–4 Δ*spx*	(Pamp *et al.*, 2006)
spx-c	8325–4 *spx* mutant with a native chromosomal copy of *spx*	(Pamp *et al.*, 2006)
ALC3085	SH1000 *sigB*::*erm*	(Schmidt *et al.*, 2004)
ALC5105	SH1000 Δ*clpP*	(Donegan *et al.*, 2010)
ALC7137	SH1000 Δ*yjbH*	(Donegan *et al.*, 2014)
ALC8112	SH1000 *ΔcspA*	This work
ALC8121	SH1000 *yjbH* mutant with a native chromosomal copy of *yjbH*	This work
ALC8212	SH1000 *cspA* mutant with a native chromosomal copy of *cspA*	This work
ALC8249	SH1000 Δ*cspC*	This work
ALC8581	SH1000 *cspB::aphA*	This work
ALC8702	MRSA JE2	(Fey *et al.*, 2012)
ALC8704	JE2 with *spx* C10A mutation	Vanai Thomas, Univ. of Nebraska Medical School
ALC8706	JE2 with *spx* C10A mutation chromosomally repaired	Vanai Thomas, Univ. of Nebraska Medical School
ALC8971	ALC8702 with pALC8135 (pALC1484 with *cspA* promoter driving gfp_{uvr})	This work
ALC8972	ALC8704 with pALC8135	This work
ALC8973	ALC8706 with pALC8135	This work
ALC8974	ALC8702 with pEPSA5 containing a xylose-inducible promoter	This work
ALC8975	ALC8702 with pALC7342 (pEPSA5::*cspA*)	This work
ALC8976	ALC8704 with pEPSA5	This work
ALC8977	ALC8704 with pALC7342	This work
E. coli		
XL-1 Blue	General cloning strain	Agilent
BL21 (DE3) pLysS	General expression strain	(Studier *et al.*, 1990)
ALC7884	DC10B with pCR2.1-AmpS::*hsdMS*-2CC8	(Jones *et al.*, 2015)
ALC7885	DC10B with pACYC184::*hsdMS*-2CC8	(Jones *et al.*, 2015)
Plasmid	Characteristics	Source or reference
pMAD	*E. coli*/*S. aureus* shuttle plasmid with the ori pE194ts; *bgaB* Ampr Ermr	(Arnaud *et al.*, 2004)
pET28a	*E. coli* expression vector	EMD Millipore
pEPSA5	Xylose-inducible shuttle vector; Ampr Cmr	(Forsyth *et al.*, 2002)
pSK236	*S. aureus*/*E. coli* shuttle vector with pUC19 cloned into the *Hin*dIII site of pC194	(Gaskill and Khan, 1988)

Strain	Genotype	Source or reference
pALC1484	pSK236 with a promoterless gfp_{uvr}, a cycle-3 *gfp* allele.	(Kahl *et al.*, 2000)
pALC2201	pALC1484 with *asp23* promoter fragment	(Palma *et al.*, 2006)
pALC7091	pEPSA5 with *yjbH* in *Eco*RI and *Bam*HI sites and *sarA* ribosome binding site	This work
pALC7113	pEPSA5 with *spx* in *Eco*RI and *Bam*HI sites and *sarA* ribosome binding site	This work
pALC7342	pEPSA5 with *cspA* in *Eco*RI and *Bam*HI sites and *sarA* ribosome binding site	This work
pALC7803	pALC1484 with *spx* P1 promoter in *Eco*RI and *Xba*I sites preceding the gfp_{uvr} gene	(Donegan *et al.*, 2014)
pALC7804	pALC1484 with *spx* P2 promoter in *Eco*RI and *Xba*I sites preceding the gfp_{uvr} gene	(Donegan *et al.*, 2014)
pALC8134	pALC1484 with *hbo-yjbH* promoter in *Eco*RI and *Xba*I sites preceding the gfp_{uvr} gene	This work
pALC8135	pALC1484 with *cspA* promoter in *Eco*RI and *Xba*I sites preceding the gfp_{uvr} gene	This work
pALC8640	pALC1484 with *msaA* promoter in *Eco*RI and *Xba*I sites preceding the gfp_{uvr} gene	This work
pALC8681	pET28a with *spx* and N-terminal hexahistidine fusion.	This work
pALC8682	pET28a with *αCTD* of *S. aureus* and N-terminal hexahistidine fusion.	This work