MsaB and CodY Interact To Regulate *Staphylococcus aureus* Capsule in a Nutrient-Dependent Manner

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ABSTRACT *Staphylococcus aureus* has a complex regulatory network for controlling the production of capsule polysaccharide. In *S. aureus*, capsule production is controlled by several regulators in response to various environmental stimuli. Previously, we described MsaB as a new regulator that specifically binds to the *cap* promoter in a growth phase- or nutrient-dependent manner. In addition to MsaB, several other regulators have also been shown to bind the same region. In this study, we examined the interactions between MsaB and other nutrient-sensing regulators (CodY and CcpE) with respect to binding to the *cap* promoter in a nutrient-dependent manner. We observed that *msaABCR* and *ccpE* interact in a complex fashion to regulate capsule production. However, we confirmed that *ccpE* does not bind *cap* directly. We also defined the regulatory relationship between *msaABCR* and CodY. When nutrients (branched-chain amino acids) are abundant, CodY binds to the promoter region of the *cap* operon and represses its transcription. However, when nutrient concentrations decrease, MsaB, rather than CodY, binds to the *cap* promoter. Binding of MsaB to the *cap* promoter activates transcription of the *cap* operon. We hypothesize that this same mechanism may be used by *S. aureus* to regulate other virulence factors.

IMPORTANCE Findings from this study define the mechanism of regulation of capsule production in *Staphylococcus aureus*. Specifically, we show that two key regulators, MsaB and CodY, coordinate their functions to control the expression of capsule in response to nutrients. *S. aureus* fine-tunes the production of capsule by coordinating the activity of several regulators and by sensing nutrient levels. This study demonstrates the importance of incorporating multiple inputs prior to the expression of costly virulence factors, such as capsule.

KEYWORDS CodY, *Staphylococcus aureus*, capsule, *cspA*, gene regulation, *msaB*

Staphylococcus aureus is a human commensal bacterium that often asymptotically colonizes the anterior nares and skin of healthy individuals. However, *S. aureus* is an opportunistic pathogen that can also cause life-threatening infections (1, 2). The process by which the bacterium adapts from a commensal lifestyle to a pathogenic one is linked to a plethora of regulatory loci of *S. aureus* (1–4). The fine-tuning of these regulatory loci is necessary for the organism to alter basic metabolic processes and to activate an arsenal of virulence factors, such as the production of capsular polysaccharides, which are required for successful colonization and infection (5–8). The *in vitro* expression of many of these virulence factors, including both extracellular and surface-associated proteins, is tightly regulated to specific phases of growth. For instance, many surface-bound proteins (i.e., fibronectin-binding protein, coagulase, and protein A) are expressed during the exponential phase of growth, whereas many secreted proteins, such as extracellular proteases and capsule polysaccharide, are predominantly expressed during the post-exponential phases of growth (6, 9–11). However, the environmental factors or host factors encountered *in vivo* that impact the regulation of

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expressed virulence factors remain poorly understood. Recently, several nutrient-sensing regulatory proteins have been identified as forming an important crossroad for the switch from a commensal to a pathogenic state, a step that is essential for the establishment of an infection (7, 12–14). This nutrient-dependent regulation is necessary for the organism to adapt to and survive during the different nutrient-limiting or stressful conditions encountered within the host during the colonization and infection processes. The production of the capsule polysaccharide has been shown to be an important factor in this switch from a commensal to a pathogenic form by aiding the ability of the pathogen to survive during infection (8, 15–18). *S. aureus* capsule polysaccharide has been shown to have a major role in bacterial virulence during infection by facilitating the survival of the pathogen inside the host, primarily via acting as an antiphagocytic factor to escape phagocytic uptake (19–21). As previously mentioned, *in vitro* capsule polysaccharide production is regulated in a growth phase-dependent manner and is suppressed during the early and mid-exponential growth phases and activated during the late and post-exponential growth phases (22–25). However, our previous data, as well as those from other groups, suggest that, in addition to growth phase, nutrient availability is also critical for the control of capsule production (8, 22, 25–27).

S. aureus produces four main serotypes of capsule, including the heavily encapsulated serotypes CP1 and CP2 and the microcapsulated serotypes CP5 and CP8. However, in the clinical context, serotypes CP5 and CP8 are considered the most significant and are present in 70 to 80% of clinical isolates (28–30). In *S. aureus*, capsule production is encoded by a single operon that contains 16 genes (31, 32). The genes that encode the CP5 and CP8 serotypes are very similar, including serotype-specific genes that are flanked by a nearly identical common region (33). Based on the similarities of the gene sequences of these two serotypes, it is thought that the regulation of the two serotypes is also similar (25, 33). The *cap* operons of these two serotypes have nearly identical promoters, which are located directly upstream from the *capA* open reading frame (ORF). Detailed analysis of the *cap* operon promoter region indicated that it is highly regulated (31). Generally, the activity of the *cap* promoter correlates with capsule polysaccharide synthesis, suggesting that regulation predominately occurs at the level of transcription (25, 34, 35). Several regulatory proteins have been shown to bind to the *cap* operon promoter region, increasing the regulatory complexity of this region. These proteins include the two-component systems AirSR and KdpDE and the nutrient- or stress-sensing regulatory proteins CcpE, CodY, RbsR, SpoVG, and MsaB (25, 36–41). The complexity of this region results in capsule polysaccharide being tightly regulated in a growth phase-dependent manner, where it is suppressed during the early and mid-exponential growth phases and activated during the late and post-exponential growth phases (22–25).

In our previous studies, we have shown that deletion of the *msaABCR* operon and/or *msaB* alone reduced *cap* transcription, as well as the production of capsule (25). We have also observed that when we delete *msaB*, it can only be complemented by the complete *msaABCR* operon, thus suggesting that the intact *msaABCR* operon is needed for the expression and/or function of *msaB* (25). Additionally, we have shown that the MsaB protein, a product of the *msaABCR* operon, binds a 10-bp regulatory repeat located directly upstream from the *cap* promoter and activates capsule production (25). We found that MsaB only binds to the *cap* promoter region during the late and post-exponential phases of growth under nutrient-rich growth conditions, even though MsaB is present throughout all growth phases. However, when the nutrient concentrations are altered, the binding ability of MsaB changes, which leads to observable changes in the expression of the *cap* transcript, as well as the production of capsule polysaccharide. This indicates that nutrients are important for the regulatory interaction of MsaB in binding to and activating the *cap* promoter (25). This led us to hypothesize that the binding ability of MsaB may be altered by changes in nutrient concentrations or that the MsaB binding site may be masked by other regulatory proteins under these conditions. Two nutrient-sensing regulatory proteins, CodY and CcpE (catabolite con-

TABLE 1 Expression of *cap*-binding genes in the *msaABCR* mutant relative to their expression in the wild-type strain

Gene	Mean fold change $\pm$ SE ^a
<i>airR</i>	1.25 $\pm$ 0.04
<i>airS</i>	2.25 $\pm$ 0.29
<i>ccpE</i>	>50
<i>codY</i>	−3.21 $\pm$ 0.26
<i>kdpD</i>	1.26 $\pm$ 0.08
<i>kdpE</i>	1.24 $\pm$ 0.14
<i>rbsR</i>	1.86 $\pm$ 0.13
<i>spoVG</i>	1.51 $\pm$ 0.11

^aGenes with a pronounced fold change value equal to or greater than ± 3.0 (shaded) were marked for further study. Results are representative of at least three independent experiments for each sample set.

trol protein E), have been shown to bind to the *cap* promoter region as repressors of *cap* production (37, 39). In this study, we show that complex regulatory interactions are present between MsaB and the other nutrient-dependent *cap* regulators, CodY and CcpE. We observed that nutrient concentrations influence the binding capability of these regulators within the *cap* promoter region. Interestingly, we observed that not only do these regulators control the *cap* promoter region, but the MsaB, CodY, and CcpE regulators appear to be responsible for the transcriptional regulatory control of each other's genes in complex and incoherent feed-forward loops. We found that mutation of any of these regulators differentially alters the transcription of genes of the other regulators, as well as the *cap* transcript, further demonstrating the tight regulatory control of *cap*. The interactions between these putative global regulatory proteins seem to lie at the crossroad between basic metabolic processes and the coordinated control of the production of virulence factors required for the bacterium to establish infection.

RESULTS

MsaB, CodY, and CcpE coordinately regulate transcription of *cap* promoter activity and, therefore, capsule production. In this study, we further investigated the nutrient-dependent regulation of capsule production by MsaB. We examined interactions between these DNA-binding *cap* regulators and MsaB. We tested whether mutating the *msaABCR* operon had any regulatory effect on the known DNA-binding regulators of *cap*, including AirSR, CcpE, CodY, KdpDE, RbsR, and SpoVG, in the mid-exponential growth phase. Interestingly, we found that the *msaABCR* operon mutation resulted in a change in the transcriptional regulation of both of the nutrient-responsive regulators *ccpE* (upregulated) and *codY* (downregulated). However, no regulatory changes were observed for any of the other regulators of *cap*, suggesting that no direct regulatory interactions occur between *msaABCR* and these regulators (Table 1). Thus, for this study, we focused on the nutrient-dependent regulators CodY and CcpE to determine how they may interact with MsaB to coordinately control *cap* and, ultimately, capsule production in response to nutrient stimuli.

To examine the nutrient-dependent regulatory mechanism of the *cap* operon, we generated individual and double mutants with *codY*, *ccpE*, *codY msaABCR*, and *ccpE msaABCR* mutations. We compared the *cap* transcripts, as well as total capsule production, of these mutants to those of the *msaABCR* deletion mutant, the wild-type strain UAMS-1, and the *msaABCR* complementation strain. We found that individual deletion of *msaABCR*, *codY*, or *ccpE* results in differential levels of *cap* transcription in the post-exponential growth phase (by −23.4-fold, 5.4-fold, and −10.3-fold, respectively) (Table 2). This confirmed that all three regulators contribute to the transcription of the *cap* operon. Deletion of the *msaABCR* operon had the largest effect on *cap* transcription. This confirms our previous finding that deletion of the *msaABCR* operon leads to decreased *cap* gene expression, resulting in capsule production being abolished (25). Next, we measured the total capsule production in the *codY* individual mutant, as well as in the double mutant, and compared it to the capsule production level of the wild-type strain. In the *codY* mutant, we observed an increase in capsule production in

TABLE 2 Expression of *capE* in mutants relative to its expression in the wild-type strain

Mutation(s)	Fold change in <i>capE</i> expression of mutant relative to that of wild-type (mean $\pm$ SE) in indicated exponential growth stage ^a			
	Early	Mid-	Late	Post-
$\Delta msaABCR$	-1.645 ± 0.03	-3.925 ± 0.09	-12.53 ± 1.29	-23.41 ± 1.79
$\Delta msaABCR$ complement	1.08 ± 0.01	-1.12 ± 0.04	1.07 ± 0.02	1.18 ± 0.06
$\Delta codY$	-2.715 ± 0.81	-3.51 ± 0.94	-5.515 ± 1.24	-4.415 ± 1.11
$\Delta codY$ complement	-1.18 ± 0.03	-1.23 ± 0.09	1.24 ± 0.07	1.16 ± 0.12
$\Delta ccpE$	-2.925 ± 0.96	-3.92 ± 1.02	-3.08 ± 0.89	-10.33 ± 1.46
$\Delta ccpE$ complement	1.02 ± 0.08	1.03 ± 0.09	-1.01 ± 0.02	-1.13 ± 0.05

^aFold change values equal to or greater than ± 3.0 are considered to show pronounced differential gene expression. Results are representative of at least three independent experiments for each sample set.

the post-exponential growth phase, when capsule is maximally produced, thus confirming the role of CodY as a repressor of capsule production (Fig. 1A). These results suggest that CodY negatively regulates capsule production at the posttranscriptional level, possibly via CodY's regulatory effect on other global regulators, such as the Agr system (37, 42). With respect to the *msaABCR codY* double mutant, we observed that the double mutation resulted in capsule production being undetectable (Fig. 1A). This shows that MsaB is essential for the expression and production of capsule even in the absence of repression by CodY. As mentioned above, we found that mutation of *ccpE* resulted in downregulation of *cap* transcription, suggesting that CcpE possibly functions as an activator of *cap* (Table 2). The mutation of *ccpE* alone resulted in a decrease in total capsule production compared to the level in the wild-type strain, although there were still detectable levels of capsule (Fig. 1B). Additionally, we compared the capsule production phenotype of the double mutant to those of the single *ccpE* and *msaABCR* mutants to confirm their roles in capsule production. Interestingly, we observed that in the *ccpE msaABCR* double mutant, capsule production was detectable at a level similar to that in the *ccpE* individual mutant (Fig. 1B). These results suggested that *ccpE* may be epistatic to *msaB*. This is the first evidence of any detectable level of capsule production in any of the *msaABCR* individual or double mutants. These findings suggest that the double mutation of *ccpE msaABCR* likely affects other unknown *cap* regulators, resulting in the observed basal level of capsule production. However, further experiments are needed to investigate this possibility.

FIG 1 Mutation of *codY* results in more total capsule production, but mutation of *ccpE* results in less total capsule production. Capsule production was assessed in wild-type (WT) UAMS-1 and the respective individual and double mutants with *msaABCR* (A and B), *codY* (A), and *ccpE* (B) mutations. Samples were serially diluted as indicated and dot blotted directly onto the membrane. The blots were processed using capsule polysaccharide (CP)-specific antibodies. Results are representative of three independent experiments for each sample set.

FIG 2 *msaABC* regulates *codY* growth phase dependently. (A) Absolute quantification of *codY* in the four growth phases. Amplicons of *codY* were converted to copies per microliter and then serially diluted and used as templates for RT-qPCR. Bars represent standard errors. Asterisks indicate the *P* value cutoff as follows: ***, $P \leq 0.001$. (B) Chromatin immunoprecipitation (ChIP) using an anti-MsaB antibody was performed to determine whether the putative promoter regions of *codY* bind to MsaB from whole-cell extracts of strains in different growth phases (early, mid-, late, and post-exponential phases). Lanes are labeled as follows: WT, whole-cell extract from the wild-type strain and anti-MsaB antibody; $\Delta msaABC$, negative control, representing the whole-cell extract from the *msaABC* deletion mutant and anti-MsaB antibody; (+) Control, PCR product amplified from the genomic DNA of UAMS-1. These results are representative of three independently treated samples.

MsaB directly controls the transcription of both *codY* and *ccpE*. To acquire a more comprehensive understanding of the observed coordinated regulation between MsaB, CodY, and CcpE with respect to the regulation of *cap*, we tested whether the mutation of the *msaABC* operon had a regulatory effect on *codY* or *ccpE* transcription. First, we compared the absolute transcript level of *codY* in the *msaABC* deletion mutant to those of the wild-type UAMS-1 and the *msaABC* complementation strains. We found that the deletion of the *msaABC* operon resulted in a significant downregulation of *codY* transcripts in the late and post-exponential growth phases (Fig. 2A).

To determine whether MsaB regulates *codY* transcription directly, we tested the binding of MsaB to the *codY* promoters using a chromatin immunoprecipitation (ChIP) assay. Briefly, *codY* has two putative promoter regions: one promotes the expression of the *xerC-clpQY-codY* operon, and the other promotes the expression of a 1.4-kb product that contains the *codY* ORF, the latter of which is responsible for producing the *codY* transcript alone (43). We tested for MsaB binding to both putative promoter regions. Interestingly, we found that MsaB binds to both putative promoters during late and post-exponential growth phases but not during early or mid-exponential growth phases (Fig. 2B). Taken together, these results show that MsaB directly regulates both the *xerC-clpQY-codY* operon and the 1.4-kb transcript containing the *codY* ORF in a growth phase-dependent manner. These results suggest that, in addition to binding to and activating *cap* in the late and post-exponential phases of growth, MsaB also regulates the *cap* repressor CodY in a growth-dependent manner. The results of this experiment show that MsaB has very tight and multidimensional regulation of the *cap* promoter.

To investigate the regulatory control exerted by the *msaABC* operon on CcpE, we compared the absolute transcript level of *ccpE* in the *msaABC* deletion mutant to the levels in the wild-type UAMS-1 and *msaABC* complementation strains. We found that the deletion of the *msaABC* operon resulted in a significant upregulation of *ccpE* transcripts during all growth phases of *S. aureus* strain UAMS-1 (Fig. 3A). CcpE has also been shown to regulate capsule production in *S. aureus*. However, there have been contradicting reports on the role or mechanism of this regulation. Ding and colleagues described *ccpE* as a direct binder of the *cap* operon, having a repressor role (39). Conversely, *ccpE* has also been described as promoting capsule formation in *S. aureus* (44). However, this regulation was found to be nondirect, as it was observed that *ccpE*

FIG 3 *msaABCR* regulates *ccpE* in all phases of growth. (A) Absolute quantification of *ccpE* in the four growth phases. Amplicons of *ccpE* were converted to copies per microliter and then serially diluted and used as templates for RT-qPCR. Bars represent standard errors. Student's unpaired *t* test was used to compare the results for the wild type and the respective mutants. Asterisks indicate the *P* value cutoff as follows: ***, *P* ≤ 0.001. (B) Chromatin immunoprecipitation (ChIP) using an anti-MsaB antibody was performed to determine whether the putative promoter region of *ccpE* binds to MsaB from whole-cell extracts of strains in different growth phases (early, mid-, late, and post-exponential phases). Lanes are labeled as follows: WT, whole-cell extract from the wild-type strain and anti-MsaB antibody; $\Delta msaABCR$, negative control, representing the whole-cell extract from the *msaABCR* deletion mutant and anti-MsaB antibody; (+) Control, PCR product amplified from the genomic DNA of UAMS-1. These results are representative of three independently treated samples.

did not bind *cap* (44). In our study, we found the *ccpE* mutant exhibited a decrease in *cap* transcripts, as well as total capsule production, suggesting that CcpE is an activator of *cap* transcription and capsule production.

To determine whether MsaB directly regulates *ccpE* to induce the strong regulatory effect observed by mutation of the *msaABCR* operon in UAMS-1, we tested whether MsaB could bind to a putative promoter region upstream from the *ccpE* ORF using ChIP. We observed that MsaB bound to the putative promoter region of *ccpE* during all phases of growth (Fig. 3B). These results suggest that MsaB directly regulates *ccpE* as a strong repressor of *ccpE* transcription. Taken together, these results suggest that MsaB has an important role in regulating CodY and CcpE, which in turn are involved in the overall nutrient-dependent regulation of *cap* transcription and capsule production in *S. aureus*.

CodY and CcpE both regulate *msaB* transcription and/or MsaB's DNA-binding ability. In an effort to gain a comprehensive understanding of the regulatory interactions between MsaB, CodY, and CcpE and determine how they regulate the *cap* transcript and, ultimately, capsule production, we also tested whether either the *codY* or the *ccpE* mutation had any regulatory impact on MsaB. First, the transcription of *msaB* in the *codY* or *ccpE* mutant was measured during all phases of growth using real-time quantitative PCR (RT-qPCR). Interestingly, we found that the mutation of either *codY* or *ccpE* decreased *msaB* expression (Table 3). This was particularly evident in the early, late, and post-exponential phases of growth, with little effect observed in the mid-exponential growth phase. We observed that the mutation of either gene (*codY*

TABLE 3 Expression of *msaB* in mutants relative to its expression in the wild-type strain

Mutation	Fold change in <i>msaB</i> expression of mutant relative to that of wild type (mean ± SE) in indicated exponential growth stage ^a			
	Early	Mid-	Late	Post-
$\Delta codY$	−2.38 ± 0.54	−1.525 ± 0.09	−3.31 ± 0.27	−3.31 ± 0.39
<i>codY</i> complement	1.12 ± 0.02	1.01 ± 0.07	−1.02 ± 0.04	−1.07 ± 0.09
$\Delta ccpE$	−3.265 ± 0.39	−1.505 ± 0.06	−2.76 ± 0.26	−5.955 ± 0.94
<i>ccpE</i> complement	−1.21 ± 0.04	1.08 ± 0.07	−1.11 ± 0.09	−1.23 ± 0.02

^aFold change values equal to or greater than ±3.0 were considered to show pronounced differential gene expression. Results are representative of at least three independent experiments for each sample set.

FIG 4 CodY binds to *cap* promoters in early, mid-, and late exponential growth phases, but CcpE does not bind to *cap* promoters in any phase of growth in UAMS-1 strain. Chromatin immunoprecipitation (ChIP) using an anti-His antibody was performed to determine whether the *cap* promoter region binds CodY (A) or CcpE (B) from whole-cell extracts of strains in different growth phases (early, mid-, late, and post-exponential phases). 6×His-*codY* and 6×His-*ccpE* complementation vectors were used to complement *codY* or *ccpE* in the respective individual mutant, as well as in the *codY msaABC* or *ccpE msaABC* double mutant strain. Lanes are labeled as follows: WT, negative control, representing whole-cell extract from the wild-type strain and anti-His antibody; $\Delta codY$ or $\Delta ccpE$, negative control, representing whole-cell extract from the *codY* or *ccpE* deletion mutant and anti-His antibody; $\Delta codY \Delta msaABC$ or $\Delta ccpE \Delta msaABC$, negative control, representing whole-cell extract from the *codY msaABC* or *ccpE msaABC* double mutant and anti-His antibody; $\Delta codY$ or $\Delta ccpE$ 6×His-complement, whole-cell extract from the *codY* or *ccpE* mutant complemented with 6×His-*codY* or 6×His-*ccpE* and anti-His antibody; $\Delta codY \Delta msaABC$ or $\Delta ccpE \Delta msaABC$ 6×His-complement, whole-cell extract from the *codY msaABC* or *ccpE msaABC* double mutant complemented with 6×His-*codY* or 6×His-*ccpE* and anti-His antibody; (+) Control, PCR product amplified from the genomic DNA of UAMS-1. These results are representative of triplicate independently treated samples.

or *ccpE*) had the greatest regulatory effect on the amount of *msaB* transcript produced during the late and post-exponential phases of growth. Importantly, this is when the regulatory activity of MsaB is the most evident, as it specifically activates *cap* transcription during these growth phases. These results suggest that complex regulatory interactions occur between MsaB, CodY, and CcpE and that these regulatory interactions are necessary for the tight control of capsule production.

To investigate the putatively nutrient-dependent binding of CodY or CcpE to the *cap* promoter, we used constructs labeled with histidine at the 5' end that are controlled by their native promoters to preserve complex regulation for complementation assays (5' 6×His-*codY* or 5' 6×His-*ccpE*, respectively). These constructs were transformed into the *codY* or *ccpE* mutant, respectively, for *trans* complementation and were used to measure the binding of CodY or CcpE via the ChIP assay. Using these constructs, we observed that under nutrient-rich conditions, CodY bound to the *cap* promoter during the early, mid-, and late exponential growth phases but not during the post-exponential growth phase (Fig. 4A), consistent with other studies that have examined CodY regulatory activity (13, 37, 45). We have previously observed that under nutrient-rich conditions (tryptic soy broth [TSB]), MsaB binds to and activates the *cap* promoter during late and post-exponential growth phases but not during early or mid-exponential phases. These observations suggest that when CodY is bound to the *cap* promoter under nutrient-rich conditions during early, mid-, and late exponential phases, the MsaB binding site may not be accessible for MsaB to bind to the *cap* promoter region. Additionally, we also observed that if we altered nutrient concentrations in the medium, the MsaB binding ability during these phases was altered, suggesting that a nutrient-dependent interaction is present (25).

To determine whether CodY binding alters MsaB binding to the *cap* promoter in a growth phase-dependent manner, we studied MsaB binding activity in the *codY* mutant. We used ChIP to determine whether the mutation of *codY* alters MsaB binding

FIG 5 CodY alters the binding ability of MsaB in early/mid-exponential growth phases and capsule is detectable in early and mid-exponential growth phases in *codY* mutants. (A) Chromatin immunoprecipitation (ChIP) using anti-MsaB antibody was performed to determine whether the mutation of *codY* or *ccpE* alters binding of the promoter region of *cap* to MsaB from whole-cell extracts of strains in different growth phases (early, mid-, late, and post-exponential phases) compared to the results for wild-type UAMS-1. Lanes are labeled as follows: WT, whole-cell extract from the wild-type strain and anti-MsaB antibody; $\Delta msaABC$, negative control, representing the whole-cell extract from the *msaABC* deletion mutant and anti-MsaB antibody; $\Delta codY$, whole-cell extract from the *codY* mutant and anti-MsaB antibody; $\Delta ccpE$, whole-cell extract from the *ccpE* mutant and anti-MsaB antibody; (+) Control, PCR product amplified from the genomic DNA of UAMS-1. (B and C) Total capsule polysaccharide (CP) production was assessed in wild-type UAMS-1 and the respective mutants with *msaABC*, *codY*, and *msaABC codY* mutations in early exponential (B) and mid-exponential (C) phases. These results are representative of triplicate independently treated samples.

under these nutrient-rich growing conditions. Interestingly, we found that when *codY* is mutated, MsaB binds to the *cap* promoter during all phases of growth, including the early and mid-exponential phases (Fig. 5A). Next, we tested whether this observed MsaB binding in the *codY* mutant led directly to capsule production. We observed detectable amounts of capsule in the *codY* mutant during both the early and mid-exponential growth phases (Fig. 5B and C). These results suggest that, through an undetermined mechanism, the binding of CodY to the *cap* promoter inhibits or blocks MsaB binding to *cap*. However, when CodY is not bound or is not present (mutation), MsaB can directly bind to and activate *cap*, leading to capsule production.

Interestingly, we found no evidence of CcpE binding to the *cap* promoter region during any phase of growth (Fig. 4B). This is consistent with the study by Hartmann et al., which found no evidence of CcpE binding to the *cap* promoter (44), but inconsistent with the study by Ding et al. (39). Additionally, mutation of *ccpE* did not appear to have any effect on the ability of MsaB to bind to the *cap* promoter (Fig. 5A). These results suggest that CcpE regulates *cap* indirectly through an undetermined mechanism and that CcpE does not have any effect on MsaB binding to the *cap* promoter.

MsaB and CodY interact with the *cap* promoter region in a nutrient-dependent manner. Capsule production has been shown to be largely regulated by environmental nutrient conditions. With respect to the regulatory function of CodY, when nutrient levels are high, capsule production is repressed by CodY binding to the *cap* promoter. However, when nutrient concentrations begin to decline, CodY undergoes a conformational change that results in CodY not binding to its DNA targets (46, 47). Moreover,

FIG 6 *msaB* and *capE* are both upregulated in strains grown in CDM without BCAAs versus CDM with branched-chain amino acids (BCAAs). RT-qPCR was used to measure the relative fold changes in the expression of *capE*, *msaB*, *codY*, and *ccpE* in mid-exponential growth phase in strains subjected to CDM with no BCAAs compared to their expression in the wild-type UAMS-1 strain grown in whole CDM. These results are representative of at least three independent experiments for each sample set.

CodY senses the availability of nutrients, specifically GTP and branched-chain amino acids (BCAAs), for its regulatory function (48–51). Previously, we used the prediction tool NsitePred to analyze the MsaB sequence and found that MsaB contains predicted nutrient-sensing domains (15 phenylalanine, 29 histidine, and 30 phenylalanine residues) for nucleotides, including GTP, ADP, and AMP, respectively (data not shown). To explore how the interactions between MsaB, CodY, and the *cap* promoter may be mediated by nutrients, we used a chemically defined medium (CDM) as a nutrient-defined medium to determine how *codY*, *msaB*, and ultimately, *cap* are affected by the presence or absence of BCAAs. We found that shifting cells from growth in CDM with BCAAs to growth in CDM without BCAAs resulted in the upregulation of *cap* in the wild-type strain (3.69-fold), resulting in more total capsule production. Under the same conditions in the wild-type strain, *msaB* transcripts were upregulated (3.47-fold) but *codY* and *ccpE* transcripts were both unaltered (Fig. 6). These results suggest that the bacterium senses this nutrient limitation and increases transcription of *msaB*, which in turn increases *cap* as a response to the nutrient-depleted conditions. However, it is also possible that in the absence of the BCAAs, capsule is depressed by CodY due to its reduction in activity. The mechanism for this process is still unknown.

Previous studies have shown that BCAA concentrations control CodY's binding affinity for its targets (13, 46, 47, 52). Using CDM, we tested how the presence or absence of BCAAs affects the *cap* promoter binding activity of CodY. We found that under chemically defined growth conditions in CDM with BCAAs, CodY was bound to the *cap* promoter during early and mid-exponential but not late or post-exponential growth phases. However, in cells shifted from CDM with BCAAs to CDM without BCAAs, CodY did not bind to the *cap* promoter during any phase of growth (Fig. 7). Given this nutrient-dependent binding of CodY, we hypothesized that the binding ability of MsaB may be altered by the binding of CodY under different nutrient conditions. Additionally, we used CDM with BCAAs and CDM without BCAAs to determine whether the *cap* promoter binding ability of MsaB during the mid-exponential phase of growth is dependent on CodY binding. Surprisingly, the binding activity of CodY in the presence or absence of BCAAs did not seem to have any effect on the ability of MsaB to bind to *cap* under these nutrient-defined conditions (Fig. 8A). This suggests that the ability of MsaB to bind to *cap* is not dependent on BCAAs or the CodY binding activity alone. Additionally, we also tested the ability of MsaB to bind to the *cap* promoter during all phases of growth under nutrient-defined conditions. We observed that under these conditions, MsaB bound to the *cap* promoter during all phases of growth (Fig. 8B).

FIG 7 *codY* binds to *cap* promoter in strains grown in CDM with BCAAs but not in CDM without BCAAs. Chromatin immunoprecipitation (ChIP) using an anti-His antibody was performed to determine whether the *cap* promoter region binds CodY from whole-cell extracts grown under nutrient-defined conditions of CDM with BCAAs versus CDM without BCAAs in different growth phases (early, mid-, late, and post-exponential phases). The 6×His-*codY* complementation vector was used to complement *codY* in the *codY* mutant, as well as in the *codY msaABCR* double mutant strain. Lanes are labeled as follows: CDM (+BCAAs), chemically defined medium with BCAAs; CDM (–BCAAs), chemically defined medium without BCAAs; WT, negative control, representing whole-cell extract from the wild-type strain and anti-His antibody; $\Delta codY$, negative control, representing whole-cell extract from the *codY* deletion mutant and anti-His antibody; $\Delta codY \Delta msaABCR$, negative control, representing whole-cell extract from the *codY msaABCR* double mutant and anti-His antibody; $\Delta codY \Delta 6XHis$ -complement, whole-cell extract from the *codY* mutant complemented with 6×His-*codY* and anti-His antibody; $\Delta codY \Delta msaABCR \Delta 6XHis$ -complement, whole-cell extract from the *codY msaABCR* double mutant complemented with 6×His-*codY* and anti-His antibody; (+) Control, PCR product amplified from the genomic DNA of UAMS-1. These results are representative of triplicate independently treated samples.

These findings, in addition to those described above, suggest that the ability of MsaB to bind to the *cap* promoter is altered by the binding ability of CodY under nutrient-rich conditions. However, under nutrient-defined conditions, MsaB is able to bind *cap* independently of CodY's binding activity.

FIG 8 MsaB binds to the *cap* promoter under nutrient-defined conditions in all phases of growth. Chromatin immunoprecipitation (ChIP) using an anti-MsaB antibody was performed to determine whether the promoter region of the *cap* operon binds to MsaB from whole-cell extracts of strain grown under nutrient-defined conditions of CDM with BCAAs versus CDM without BCAAs in the mid-exponential phase of growth (A) or in CDM with BCAAs in different growth phases (early, mid-, late, and post-exponential phases) (B). Lanes are labeled as follows: CDM (+BCAAs), chemically defined medium with BCAAs; CDM (–BCAAs), chemically defined medium with no branched-chain amino acids; Early, Mid, Late, and Post, whole-cell extracts from strains in the respective growth phase when grown in whole CDM; WT, whole-cell extract from the wild-type strain and anti-MsaB antibody; $\Delta msaABCR$, negative control, representing the whole-cell extract from the *msaABCR* deletion mutant and anti-MsaB antibody; $\Delta codY$, whole-cell extract from the *codY* mutant strain and anti-MsaB antibody; $\Delta ccpE$, whole-cell extract from the *ccpE* mutant strain and anti-MsaB antibody; (+) Control, PCR product amplified from the genomic DNA of UAMS-1. These results are representative of triplicate independently treated samples.

FIG 9 Total capsule production is increased in WT grown in CDM without BCAAs versus CDM with BCAAs. (A) Total capsule polysaccharide (CP) production was assessed in wild-type UAMS-1 and the respective *msaABC* and *codY* mutants, along with the *msaABC codY* double mutant, to compare CP production in CDM with BCAAs versus CDM with no BCAAs in the mid-exponential growth phase. (B) Image analysis of total CP production in the wild-type UAMS-1 grown in CDM with BCAAs versus CDM with no BCAAs was performed using the ImageJ analysis software. Results are represented as the average percentage of total coverage area of the respective blots. Student's unpaired *t* test was used to compare the results for the wild type to the results for the respective mutants. ***, $P \leq 0.001$. (C) Total CP production was assessed in wild-type UAMS-1 and the respective *msaABC* and *codY* mutants, along with the *msaABC codY* double mutant, to compare the levels of CP production in CDM with BCAAs in the late exponential growth phase. These results are representative of triplicate independently treated samples.

Given the nutrient-dependent regulation of *cap* and *msaB* transcription, as well as the observed changes in the ability of MsaB to bind *cap* under these nutrient-defined conditions, we tested the total capsule production under nutrient-defined growth conditions. We compared the wild-type strain to the *msaABC*, *codY*, and *msaABC codY* mutants grown in CDM with BCAAs or in cells shifted to CDM without BCAAs. As mentioned above, we observed upregulation of the *cap* and *msaB* transcripts in the wild-type UAMS-1 strain in CDM without BCAAs relative to their levels in CDM with BCAAs (Fig. 6). This correlated with an increase in total capsule production (Fig. 9A). To confirm these findings, we quantified the intensities of the blots using ImageJ software analysis. We found a significant increase (approximately 50%) in the total intensity of the blots in cells shifted to CDM without BCAAs compared to that in cells grown in CDM with BCAAs (Fig. 9B). Interestingly, under the conditions tested, no capsule production was detected in the *codY* mutant, which was different from the observed result under the nutrient-rich conditions (TSB). As a result of these findings, we tested the total capsule production under these nutrient-depleted conditions during the late exponential phase of growth. We observed that capsule production was detectable in the *codY* mutant during the late exponential phase of growth under nutrient-depleted conditions (Fig. 9C). These results suggest that the observed binding of MsaB in the *codY* mutant may not be sufficient to lead to capsule being produced or that the mutation

of *codY* affects an unknown *cap* regulator under these nutrient-defined conditions. However, when the cells transition from mid-exponential growth to late exponential growth, MsaB binding becomes sufficient to directly activate the production of capsule via an unknown mechanism. Taken together, these results show that MsaB and CodY are both responsible for the complex temporal regulation and production of capsule in response to nutrients and/or growth phase. Further studies are needed to fully understand this complex nutrient-dependent and/or growth phase-dependent regulatory mechanism.

DISCUSSION

Capsule polysaccharide is a well-characterized virulence factor of *S. aureus* and is involved in the evasion of phagocytic uptake during certain types of infections (8). The regulation of capsule production in *S. aureus* is very complex and involves several global regulators that tightly control the expression of *cap*, predominately at the transcriptional level (25, 36–41). The complexity of this regulation enables the pathogen to fine-tune capsule expression based on environmental or host-specific signals, including nutrients and other stress-related factors (8, 45). We have previously identified MsaB, of the *msaABCR* operon, as a DNA-binding transcriptional activator of the *cap* operon promoter. Interestingly, the binding of MsaB seemed to be either growth phase dependent or mediated in a nutrient-dependent manner (25). In this study, we demonstrated that, in addition to directly regulating *cap*, the *msaABCR* operon (MsaB) is also involved in regulating two other major nutrient-dependent regulators of *cap*, CodY and CcpE. The findings of this study suggest that MsaB has dual regulatory roles as a transcriptional activator of both *cap* and *codY* and as a repressor of *ccpE*.

CodY has been described as a key nutrient-dependent global regulator (transcriptional repressor) that not only directly regulates *cap* but also directly controls many metabolic and virulence factors in *S. aureus* (7, 13, 37, 42, 43, 45, 53, 54). Pohl and colleagues described CodY's regulation of capsule in detail (43). CodY senses GTP and BCAA concentrations within the growth environment of the bacterium. The presence or absence of these nutrients results in a conformational change within the CodY protein structure (46, 47, 52). This conformational change of the protein results in a decrease in the DNA-binding affinity of CodY for its targets under conditions of low nutrient concentrations (43, 48, 49, 51). CodY is part of a four-gene operon that produces a large, 4.1-kb transcript (43). As described previously (43), this operon is composed of the genes *xerC*, *clpQ*, *clpY*, and *codY*. The *xerC* gene is thought to encode a tyrosine recombinase, and *clpQY* codes for the ATP-dependent heat shock protease HslVU (55). Within this operon, *codY* can be transcribed by itself as a shorter transcript that invades the 3' end of the *clpY* gene, producing a 1.4-kb transcript containing the *codY* ORF (43). Importantly, to the best of our knowledge, no other gene has been identified as directly regulating *codY* transcription in *S. aureus*. In addition to MsaB directly regulating *codY*, we also showed that MsaB and CodY putatively compete for binding sites within the *cap* promoter region in a growth phase- or nutrient-dependent manner. We observed that in the *codY* mutant, MsaB binding is altered, resulting in MsaB binding to the *cap* promoter during all phases of growth under nutrient-rich conditions. This binding of MsaB led directly to the activation of *cap* and resulted in capsule production during early and mid-exponential phases. However, when CodY was present in the wild-type strain, MsaB did not bind to the *cap* promoter under these same growth conditions, resulting in no detectable capsule production.

Many studies have described nutrients as an important factor that facilitates capsule production within different environmental niches of *S. aureus* (8, 22, 26, 27). Based on the findings of this study, we suggest that the described nutrient-dependent binding activities of MsaB and CodY lead directly to capsule production. When nutrient concentrations are altered, specifically in CDM in the presence or absence of BCAAs, the transcription of *msaB* and *cap* is upregulated and the ability of MsaB to bind to the *cap* promoter is altered, resulting in MsaB binding during all phases of growth. This binding of MsaB under nutrient-defined conditions results in capsule being produced abun-

dantly during mid-exponential growth phase, suggesting that the binding of MsaB under nutrient-defined conditions leads directly to capsule production. It is unclear why MsaB binds to the *cap* promoter during all growth phases under nutrient-defined conditions, whereas under nutrient-rich conditions, it only binds the *cap* promoter during late and post-exponential phases. This suggests that factors other than nutrients may be involved in the binding behavior of MsaB. Additionally, under these same nutrient-defined conditions, there was no capsule production detected in the *codY* mutant (no *cap* repression) in CDM with BCAAs or CDM without BCAAs. This suggests that, even though MsaB binds under these conditions, the level of binding may not be sufficient to completely activate *cap* transcription or the mutation of *codY* alters another *cap* regulator under these nutrient-depleted conditions that leads to the inhibition of *cap* production. Taken together, these results suggest that MsaB may be responding directly to the concentration of nutrients, resulting in an increase in its ability to bind to the *cap* promoter, and that under chemically modified growth conditions, the binding ability of MsaB is independent of CodY activity. Indeed, based on amino acid sequence analysis, MsaB appears to contain nutrient (nucleotide) binding regions, including GTP, ADP, and AMP, suggesting that it may directly bind and respond to nucleotide or nutrient molecules.

Previously, there have been conflicting reports on the regulatory role of CcpE with respect to *cap* regulation. As mentioned above, Ding et al. described *ccpE* as a direct repressor of the *cap* operon (39). However, Hartmann et al. described *ccpE* as promoting capsule formation in *S. aureus* but described CcpE as not binding directly to the *cap* promoter (39, 44). In our study, we observed that *ccpE* promotes capsule formation. However, under the conditions tested, we did not find any evidence of CcpE binding directly to the *cap* promoter, suggesting that the regulatory effect of CcpE with respect to *cap* is not direct and may be dependent upon another *cap* regulator. Importantly, in the *ccpE* mutant during the post-exponential growth phase *msaB* (*cap* activator) was also downregulated (−6.0-fold). These results suggest that the observed effect on capsule production resulting from the mutation of *ccpE* may be dependent on the regulatory effect that the mutation has on the *msaB* transcript under these conditions. Additionally, another interesting finding observed in this work, with respect to MsaB and CcpE, is that in the *msaABCR ccpE* double mutant, there was still detectable capsule production, even though it was decreased, similar to what was observed in the *ccpE* mutant alone. These findings suggest that *ccpE* may be epistatic to *msaB*. However, this seems to contradict our findings that MsaB represses the transcription of *ccpE* (Fig. 3A). This indicates that the regulatory relationship between *msaABCR*, *ccpE*, and *cap* is complex and may involve other factors. Further studies are necessary to better understand these interactions.

This work focused on the nutrient-dependent regulation of capsule production in *S. aureus* with respect to the nutrient-dependent regulators MsaB, CodY, and CcpE. The findings from this work suggest that *cap* regulators CodY (repressor) and MsaB (activator) both directly respond to changes in nutrients within the environment of the bacterium and, in turn, tightly control *cap* expression and, ultimately, the production of capsule polysaccharide. However, many other regulators and factors are responsible for the control of capsule production that do not have a known direct link to nutrients. Based on previous studies and the results of this work, we propose a working model that describes how MsaB and CodY control capsule production (Fig. 10). In this model, we propose that under high-nutrient conditions, represented by growth in TSB broth, CodY is bound to the *cap* promoter region, repressing the *cap* promoter (Fig. 10A). However, as nutrients become limited, the binding affinity of CodY decreases as a result of a conformational change in the CodY protein structure, resulting in the loss of transcriptional repression. Under conditions in which CodY is not bound to the *cap* promoter, the MsaB binding site is available, allowing MsaB to bind to and activate the *cap* promoter (Fig. 10B). This proposed mechanism suggests that regulatory interactions occur between CodY and MsaB as a direct response to nutrient availability. The regulatory control of capsule production by both MsaB and CodY described in this work

FIG 10 Working model for *cap* operon regulation by MsaB and CodY. (A) When nutrients are abundant, CodY binds to the CodY binding site (CBS) in the promoter region of the *cap* operon and represses *cap* transcription. (B) When nutrient levels decrease, a conformational change in the CodY structure occurs, resulting in a decrease in the affinity of CodY for the promoter. Detachment of CodY from the promoter allows MsaB to access the MsaB binding site (MBS). In addition, MsaB appears to have a greater binding ability under low nutrient levels. Binding of MsaB activates the transcription of the *cap* operon.

is representative of a mixture of incoherent feed-forward loops or (FFLs). This type of regulatory mechanism is defined as consisting of three genes: a regulatory gene X that regulates gene Y, and gene Z (Fig. 11A). The gene Z promoter is regulated by both X and Y, as well as X regulating gene Z independent of Y (56, 57). In our case, MsaB directly regulates both the *cap* operon (activation) and *codY*, which in turn regulates capsule production. This is representative of an incoherent type 1 FFL (Fig. 11B). Alternatively, CodY directly regulates both capsule production (repression) and *msaB*, which subsequently regulates capsule production. This is representative of an incoherent type 3 FFL (Fig. 11C). These regulatory interactions allow both regulators to coordinately control the *cap* promoter and, ultimately, capsule production. Furthermore, the findings from this work suggest that this regulatory control of capsule production by MsaB and CodY is dependent on the growth phase and/or nutrient stimuli in *S. aureus*. Additionally, both CodY and MsaB have also been described to have roles in the regulation of virulence, as well as MsaB possibly acting as an RNA chaperone interacting with RNA to regulate *cap* and other virulence determinants in *S. aureus* (7, 13, 25, 37, 42, 43, 53, 54, 58–60). Findings from this work may have implications for the interaction of *S. aureus* with the host during the transition from a

FIG 11 Incoherent feed-forward loops (FFLs) representing regulation of capsule production by MsaB and CodY. (A) Examples of the four different types of incoherent FFLs are shown that describe two regulators, “X” and “Y” (activators or repressors), controlling one gene, “Z.” (B) Highlights the incoherent type 1 FFL with respect to MsaB regulation of capsule production, as well as the *codY* transcript. (C) Highlights the incoherent type 3 FFL with respect to CodY regulation of capsule production, as well as the *msaB* transcript. (B and C) Both schematics are representative of the findings described in this work with respect to the regulation of capsule production by MsaB and CodY (*cap* activator and repressor, respectively).

commensal to a pathogenic form. Studies are under way to investigate whether the described regulatory mechanism between CodY and MsaB is responsible for the regulatory control of other virulence mechanisms possessed by *S. aureus*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. For this study, we used *S. aureus* strain UAMS-1 as a representative strain for the clinically significant capsule serotype CP8 (Table 4), as was done in our previous study (25), and the strain USA300_LAC as a control for select transcriptional experiments (see

TABLE 4 Strains and plasmids used in this study

Strain or plasmid	Relevant feature(s)	Reference or source
Strains		
<i>E. coli</i> TOP10	F [−] ϕ 80 <i>lacZ</i> Δ <i>M15</i> <i>recA1</i>	Life Technologies
<i>S. aureus</i> strains		
UAMS-1	CP8-producing strain	
UAMS-1Δ <i>msaABC</i> R	<i>msaABC</i> R operon knockout	25
UAMS-1Δ <i>msaABC</i> R comp.	UAMS-1Δ <i>msaABC</i> R(pCN34- <i>msaABC</i> R operon)	25
USA300 JE2	<i>codY</i> ::Tn transposon mutant	NARSA
USA300 JE2	<i>ccpE</i> ::Tn transposon mutant	This study
UAMS-1Δ <i>codY</i>	<i>codY</i> ::Tn transposon mutant	This study
UAMS-1Δ <i>msaABC</i> R/Δ <i>codY</i>	Δ <i>msaABC</i> R Δ <i>codY</i> double mutant	This study
UAMS-1Δ <i>codY</i> 6×His complement	UAMS-1Δ <i>codY</i> 6×His(pCN34-6×His- <i>codY</i>)	This study
UAMS-1Δ <i>msaABC</i> R/Δ <i>ccpE</i> 6×His complement	UAMS-1Δ <i>ccpE</i> 6×His(pCN34-6×His- <i>ccpE</i>)	This study
UAMS-1Δ <i>ccpE</i>	<i>ccpE</i> ::Tn transposon mutant	This study
UAMS-1Δ <i>msaABC</i> R/Δ <i>ccpE</i>	Δ <i>msaABC</i> R Δ <i>codY</i> double mutant	This study
Plasmids		
pCN34	Amp ^r Erm ^r ; shuttle vector, low copy no.	25; NARSA
pCN34(Cm ^r)- <i>msaABC</i> R operon	1.7-kb PCR fragment containing <i>msaABC</i> R operon cloned into pCN34(Cm ^r)	25, 62
pCN34(Cm ^r)-6×His- <i>codY</i>	<i>codY</i> 5′ end labeled with 6×His cloned into pCN34(Cm ^r)	This study
pCN34(Cm ^r)-6×His- <i>ccpE</i>	<i>ccpE</i> 5′ end labeled with 6×His cloned into pCN34(Cm ^r)	This study
pD861- <i>msaB</i>	6×His- <i>msaB</i> protein expression vector	This study

Fig. S1 in the supplemental material). The restriction-deficient laboratory strain *S. aureus* RN4220 and *Escherichia coli* strain DH5 α were used to move plasmid constructs into the strains of choice through transformation and phage transduction as described previously (58, 61, 62). *S. aureus* strains were routinely grown at 37°C with shaking (250 rpm) with a flask-to-medium volume ratio of 10:1 in tryptic soy agar (TSA) or tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI) as appropriate, or for the experiments with altered nutrient conditions, strains were grown at 37°C with shaking (250 rpm) with a flask-to-medium volume ratio of 10:1 in chemically defined medium (CDM) as a nutrient-defined medium, as described previously, with specific nutrients added (43). When required, either erythromycin (10 μ g ml⁻¹) or chloramphenicol (10 μ g ml⁻¹) was added to TSB or TSA for selection. The *E. coli* strain was grown at 37°C with shaking (250 rpm) in Luria-Bertani (LB) medium, with ampicillin added (100 μ g ml⁻¹) when required for selection.

Generation of transposon mutants and complementation. The *codY* and *ccpE* mutants were generated by insertion of a transposon in the *codY* or *ccpE* ORF. Briefly, strains NE1555 (SAUSA300_1148) (*CodY*) and NE1560 (SAUSA300_0658) (*CcpE*) were obtained from the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) collection (BEI Resources, Manassas VA). These strains contain the *bursa aurealis* mariner-based erythromycin resistance expression transposon within the *codY* or *ccpE* region, respectively. The mutations were mobilized by generalized transduction, using bacteriophage ϕ 11 (25, 60, 62), into wild-type UAMS-1. The introduction of *codY*::Tn and *ccpE*::Tn mutations was verified by PCR, followed by sequencing and phenotypic assays (37, 39, 42–44). For *trans* complementation, the *codY* ORF (1.4-kb *codY* transcript with its native promoter) or *ccpE* ORF, each having 6 histidine residues at the 5' end (6 $\times$ His-*codY* or 6 $\times$ His-*ccpE*), was cloned into the pCN34 low-copy-number vector modified by changing the kanamycin-selectable marker to a chloramphenicol resistance marker as described previously (25, 62, 63). These complementation vectors were used in the RT-qPCR experiments and phenotypic assays. In addition, we used these complementation vectors with the 5'-end histidine residues (6 $\times$ His-*codY* or 6 $\times$ His-*ccpE*) to perform the chromatin immunoprecipitation (ChIP) assay.

RNA extraction, reverse transcription, and RT-qPCR. The expression of *capE* and *msaB* was measured by RT-qPCR in the wild type, the corresponding mutants, and complemented strains. Briefly, an aliquot of an overnight culture was normalized to an optical density of 600 nm (OD₆₀₀) of 0.05 and then grown at 37°C with shaking (250 rpm) to the appropriate growth phase. After the cells had grown to the appropriate growth phase (early, mid-, late, or post-exponential phase), cells were pelleted by centrifugation at 10,000 $\times$ *g*, treated with the RNeasy Protect Bacterial reagent (Qiagen, Valencia CA), and stored at -80°C until analysis. For the analysis, the samples were thawed on ice and total RNA was extracted as previously described (25, 58, 62). RNA concentration and quality were analyzed by measuring absorbance and interpreting the absorbance ratio at 260 nm/280 nm using a Nanodrop spectrophotometer (Thermo Scientific). Reverse transcription was performed with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA), using 1 μ g of the total RNA isolated according to the manufacturer's protocol. Next, RT-qPCR was performed using the appropriate primers (Table 5) and the relative fold change in gene expression was calculated using *gyrA*, the 16S rRNA gene, and *rpoB* as endogenous control genes. Fold change data represented in the main text were calculated with *gyrA* (Tables 1 and 3). Fold change data calculated using the 16S rRNA gene and *rpoB* as endogenous controls are shown in Tables S1, S2, S5, and S6, respectively (10, 25, 58, 62). The results were consistent for all the control genes used, and the experiments were repeated in triplicate in independent assays.

Absolute quantification of the *codY* and *ccpE* transcripts. Absolute quantification of the *codY* and *ccpE* transcripts was performed by the method previously described by others and our previous work (25, 64). The *codY* and *ccpE* genes were amplified from chromosomal DNA using primers external to the primers used for RT-qPCR. The PCR amplicons were purified and their concentrations were measured using a Nanodrop spectrophotometer (Thermo Scientific). The corresponding concentrations were converted to copies per microliter using a previously described method (65). Tenfold serial dilutions (10⁻¹ to 10⁻⁸) of these amplicons were used as templates for RT-qPCR. Standard curves were generated by plotting threshold cycle (*C_t*) values against the log of the copy numbers (log starting quantity [SQ]). Starting quantities of "unknown" wild-type samples (early, mid-, late, and post-exponential cDNA of UAMS-1) were calculated by plotting the respective *C_t* values on the standard curve. Copy numbers were measured by 10^{5Q}. The respective copy numbers of *codY* or *ccpE* were normalized to those of *gyrA* (Table 2), 16S rRNA (Table S3), or *rpoB* (Table S4) as endogenous control genes and plotted against the standard curve to obtain the absolute transcript copy numbers of *codY* or *ccpE*. The results were consistent for all control genes used. The experiment was repeated in triplicate in independent assays.

In vitro capsule production assay. Total capsule production was determined using a dot blotting method described previously (66), with the following modifications described in our previous work (25). In brief, 2 ml of an 18-h culture, adjusted to an OD₆₀₀ of 5.0, was pelleted and resuspended in 100 μ l of phosphate-buffered saline (PBS). The suspension was sequentially treated with the following enzymes at 37°C: lysozyme (100 μ g ml⁻¹) for 15 min, DNase I (300 U ml⁻¹) for 15 min, and proteinase K (100 μ g ml⁻¹) for 1 h. The proteinase K was subsequently inactivated by heating at 75°C for 10 min. The crude capsule preparations were serially diluted and assayed by immunoblotting on a membrane using a CP8-specific antibody as described previously (25, 66).

ChIP assays. The chromatin immunoprecipitation (ChIP) assays were performed as previously described (67), with minor modifications as outlined in our previous work (25). Briefly, *S. aureus* cells were grown to the growth phase required for a given experiment and were treated with 1% formaldehyde and 10 mM sodium phosphate to facilitate the cross-linking of the MsaB, 6 $\times$ His-*CodY*, or 6 $\times$ His-*CcpE* proteins to their target binding sites. After 20 min, the cross-linking reaction was quenched by the addition of 0.1 volume of 3 M glycine. The cultures were subsequently washed with an equal volume of 100 mM

TABLE 5 Primers used in this study

Purpose and primer	Sequence (5'→3')
Real-time PCR	
RT <i>gyrA</i> F	GCCGTCAGTCTTACCTGCTC
RT <i>gyrA</i> R	AATAACGACACGCACACCAG
RT <i>capE</i> F	ACATTGGTGATGTGCGTGAT
RT <i>capE</i> R	TCACATGACGGCACTTGTTT
RT <i>airR</i> F	TGCTGATGGTTATGAAATGA
RT <i>airR</i> R	CATCTTGTCCTTAGGATGT
RT <i>airS</i> F	TTCCTAGCCAAAATGACAATA
RT <i>airS</i> R	TTCAGTATTTGGAGACGCTAC
RT <i>ccpE</i> F	GGGTGTTCTTTTGATTGG
RT <i>ccpE</i> R	TTGAACCAACTTGCACTTGT
RT <i>codY</i> F	ATCGCATCAAAAGTTGCAGA
RT <i>codY</i> R	CGTGATTCAATTACACCAGCA
RT <i>kdpD</i> F	TACCACACCATTCAAGTTAGA
RT <i>kdpD</i> R	GTAAACGAGAGGATTTTGAG
RT <i>kdpE</i> F	AATTCAAAGTCGTTTCACAAA
RT <i>kdpE</i> R	GAATTCATTGGTGTTAGATG
RT <i>rbsR</i> F	TATCGACAATACATATCATCC
RT <i>rbsR</i> R	GTATAGCCTTGATGGTCATTTT
RT <i>spoVG</i> F	AGCACTCGTTTCCATTACAT
RT <i>spoVG</i> R	TGTACGTTTACTTGGCATTG
ChIP and EMSA ^a	
<i>cap</i> -ChIP F	CTACTTTAGAGTATAATTATTTTAAATTC
<i>cap</i> -ChIP R	CCCTTAAAAATTTTCATTAAAAATTG
<i>codY</i> -ChIP F	TTTCCATGTATCTAAGCCGAG
<i>codY</i> -ChIP R	CATCAACATATTGTGGGGTAAT
<i>codY</i> operon F	TAAATAACACGCAATAAGTTGATTG
<i>codY</i> operon R	CTTGAATATGATTCAATACATTAC
<i>ccpE</i> -ChIP F	GTAATTCAAGCTGCAGCCATG
<i>ccpE</i> -ChIP R	TCGCTCTCTTTTCAACATGTCAC
6×His-labeled complement constructs	
6×His- <i>codY</i> comp F	ATCAGGGATCCATGCATCATCACCATCACCACCTCAAGAAAGCGCTAACCAAG
6×His- <i>codY</i> comp R	ATGTGAATTCTTATTTACTTTTTCTAATTCATCTAAG
6×His- <i>ccpE</i> comp F	ATCAGGGATCCATGCATCATCACCATCACCACATGATTATTGAGCATGCCCGTGA
6×His- <i>ccpE</i> comp R	ATGTGAATTCCTACGCTTTGGTTGTTCAACAAA

^aChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay.

phosphate buffer and pelleted by centrifugation to remove the excess formaldehyde. The cells were then resuspended in 750 μ l of IP buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5% glycerol, and 1% Triton X-100) and lysed by bead beating. The cell debris was removed by centrifugation, and the lysate was moved into a new tube. The cell lysates were then diluted with an additional 750 μ l of IP buffer, and the cellular DNA was sheared to a size of approximately 500 bp by further bead beating. After centrifugation at $10,000 \times g$, the supernatant was diluted with an additional 1 ml of IP buffer. To 500 μ l of the cleared lysate, anti-MsaB antibody or ChIP grade anti-His antibody (diluted 1:1,000; Abcam, Cambridge, UK) was added, and the mixture incubated with continuous mixing at room temperature for 2 h. The antigen-antibody mixture was then added to prewashed protein G-coupled magnetic beads (Thermo Scientific Pierce) and incubated under ambient conditions with continuous mixing for 1 h. The antigen-antibody-bead complexes were collected with a magnetic stand and washed, after which the antigen-antibody complexes were eluted, followed by decoupling of the antigen-antibody complexes. After the decoupling step, the DNA was extracted using the phenol-chloroform extraction method, followed by ethanol precipitation as previously described (25). The DNA was used as the template to detect the MsaB-, CodY-, or CcpE-bound promoter sequences by PCR amplification using promoter-specific primers. The *msaABCR* operon mutant or the *codY* and *ccpE* mutants without the 6×His-labeled complementation constructs were used as internal negative controls to demonstrate that the antibodies specifically enriched the tested promoter region.

Altered-nutrient experiments. To observe the effect of nutrient limitation on the production of capsule, as well as the regulatory effects of MsaB and CodY, we used a chemically defined medium (CDM) as previously described (43). Briefly, overnight cultures grown at 37°C with shaking (250 rpm) in TSB were diluted 1:10 in fresh, prewarmed CDM and then were incubated for an additional 2 h. Subsequently, the cells were normalized to a OD_{600} of 0.05 in fresh, prewarmed CDM for use as the starter culture. These cells were compared to cells grown under nutrient-rich growth conditions (TSB) in the early, mid-, late, and post-exponential phases of growth. Additionally, we compared cells grown in CDM with BCAAs or cells grown in CDM with BCAAs and then introduced into CDM without BCAAs (valine, leucine, and isoleucine; Sigma-Aldrich, USA). Briefly, exponential cultures in the appropriate growth phase, growing

in CDM with BCAAs, were collected by centrifugation at $10,000 \times g$ and resuspended in CDM without BCAAs. These cells were further incubated at 37°C with shaking (250 rpm) for an additional 30 min, after which they were collected by centrifugation. These cells grown in CDM without BCAAs were compared to cells grown similarly in CDM with BCAAs. Using these different nutrient conditions, we tested the capsule phenotype, as well as the binding profile of MsaB and CodY, under different nutrient conditions. Additionally, we observed how the change in nutrients plays a role in the regulatory interactions between these two regulators with respect to *cap*.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00294-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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