CidR and CcpA Synergistically Regulate *Staphylococcus aureus* *cidABC* Expression

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ABSTRACT The death and lysis of a subpopulation of *Staphylococcus aureus* cells during biofilm development benefit the whole bacterial population through the release of an important component of the biofilm matrix, extracellular DNA. Previously, we have demonstrated that these processes are affected by the gene products of the *cidABC* operon, the expression of which is controlled by the LysR-type transcriptional regulator, CidR. In this study, we characterized *cis*- and *trans*-acting elements essential for the induction of the *cidABC* operon. In addition to a CidR-binding site located within the *cidABC* promoter region, sequence analysis revealed the presence of a putative catabolite responsive element (*cre* box), suggestive of the involvement of the catabolite control protein A (CcpA) in the regulation of *cidABC* expression. This was confirmed using electrophoretic mobility shift assays and real-time reverse transcriptase PCR analysis demonstrating the direct positive control of *cidABC* transcription by the master regulator of carbon metabolism. Furthermore, the importance of CcpA and the identified *cre* site for the induction of the *cidABC* operon was demonstrated by examining the expression of P*cidABC*-*lacZ* reporter fusions in various mutant strains in which the genes involved in carbon metabolism and carbon catabolite repression were disrupted. Together the results of this study demonstrate the necessity of both transcriptional regulators, CidR and CcpA, for the induction of the *cidABC* operon and reveal the complexity of molecular interactions controlling its expression.

IMPORTANCE This work focuses on the characterization of *cis*- and *trans*-acting elements essential for the induction of the *cidABC* operon in *S. aureus*. The results of this study are the first to demonstrate the synergistic control of *cidABC* expression by transcriptional regulators CidR and CcpA during carbohydrate metabolism. We established that the full induction of *cidABC* expression depends on the metabolic state of bacteria and requires both CidR and CcpA. Together, these findings delineate regulatory control of *cidABC* expression under different metabolic conditions and provide important new insights into our understanding of cell death mechanisms during biofilm development in *S. aureus*.

KEYWORDS regulation of gene expression, glycolysis, carbon catabolite repression, *Staphylococcus aureus*

Staphylococcus aureus infections often involve the formation of a biofilm, a structured community of bacteria held together by a self-produced matrix that consists of extracellular polymeric substances (EPS) (1). The EPS matrix plays an important role in the adherence between cells and surface materials, retains water within the biofilm, and serves as a barrier protecting bacteria from antimicrobial agents and the host immune response (2). The composition of the EPS matrix is diverse in nature and varies between bacterial species and strains. It is typically comprised of different molecules,

including polysaccharides, lipids, proteins, and extracellular DNA (eDNA) (3, 4). In *S. aureus*, eDNA is an important structural component of the biofilm matrix that is released by the lysis of a subpopulation of cells via a process termed programmed cell death (PCD) (4–7).

Although the understanding of bacterial PCD in *S. aureus* envisions a holin-antiholin action of the membrane-associated *cidA* and *lrgA* gene products (8–10), several studies have revealed that activities of metabolic enzymes of the CidR regulon, consisting of the *cidABC* and *alsSD* operons, also have a direct impact on bacterial cell death during the stationary phase of growth (5, 11, 12). It has been demonstrated that the *cidC*-encoded pyruvate:menaquinone oxidoreductase (13, 14) contributes to the cell death through acetate-mediated acidification of the growth medium leading to protonation of bacterial cytoplasm (12). In contrast, the gene products of the *alsSD* operon, involved in acetoin biosynthesis, promote cell survival under these conditions by consuming protons and, thus, increasing cytoplasmic pH (11, 12). These findings are consistent with a previously proposed hypothesis that the balance between the prolife and prodeath functions of these enzymes determines cell fate during growth (8). In accordance with this, our recent work demonstrated alternative functions of the CidA and CidB proteins in modulating CidC activity and revealed the involvement of the CidR regulon in limiting acetate-mediated cell death in staphylococcal populations (15). Specifically, it has been shown that under conditions of excess glucose, both CidB and CidC promote the generation of acetate and cell death, whereas the activation of *cidA* and *alsSD* expression by CidR opposes the effects of CidB and CidC by directing carbon flux toward the production of acetoin (15).

The *cidABC* and *alsSD* operons represent the only genes directly controlled by the transcriptional regulator CidR (11, 12, 16). CidR is a member of the LysR-type transcriptional regulator (LTTR) family of proteins, which are typically activated through the binding of a specific small molecule to a conserved ligand-binding domain (16, 17). It has been suggested that pyruvate might serve as an inducer molecule for activation of CidR, as its intracellular concentrations under acidic stress conditions, in the presence of excess glucose, will modulate the metabolic status of the cells (10). While CidR is a major transcriptional regulator of the *cidABC* and *alsSD* operons, the regulatory network controlling expression of these operons is more complex. Recently, we have shown that expression of *cidABC* genes is also controlled by the SrrAB two-component system and affected by cellular respiration (18). This regulation appears to be superimposed on the regulation mediated by CidR, providing an exquisite level of transcriptional control of the *cidABC* operon. Furthermore, in agreement with the necessity of glucose for the induction of the *cidABC* operon (16), earlier work has suggested involvement of the master regulator of carbon metabolism, CcpA, in *cidABC* expression (19). In particular, using Northern blot analysis, Seidl et al. (19) demonstrated that inactivation of the *ccpA* gene in *S. aureus* leads to a significant decrease in the accumulation of *cidABC* transcripts during the exponential phase of growth. Interestingly, in addition to the transcriptional control of *cidABC* expression, recent work by Hussein et al. (20) revealed the presence of a functional thermosensor within the 5′ untranslated region (UTR) of the *cidA* gene, which posttranscriptionally facilitates increased *cidABC* expression at lower temperatures.

In the current study, we investigated in greater detail the *cis*- and *trans*-acting regulatory elements critical for the control of *cidABC* expression. We identified the nucleotide sequences of the CidR-binding site and c̲atabolite r̲esponsive e̲lement (*cre* box) within the *cidABC* promoter region and demonstrated the importance of these *cis*-acting elements in the control of *cidABC* expression. These findings were supported by the demonstration of specific interactions of CidR and CcpA with these *cis*-acting elements. In agreement with this, a transposon mutagenesis approach to identify additional factors involved in the induction of *cidABC* expression revealed genes involved in carbon metabolism and carbon catabolite repression (CCR). Overall, the results of this study suggest a model in which CcpA and CidR work synergistically to induce *cidABC* expression under conditions of carbon overflow.
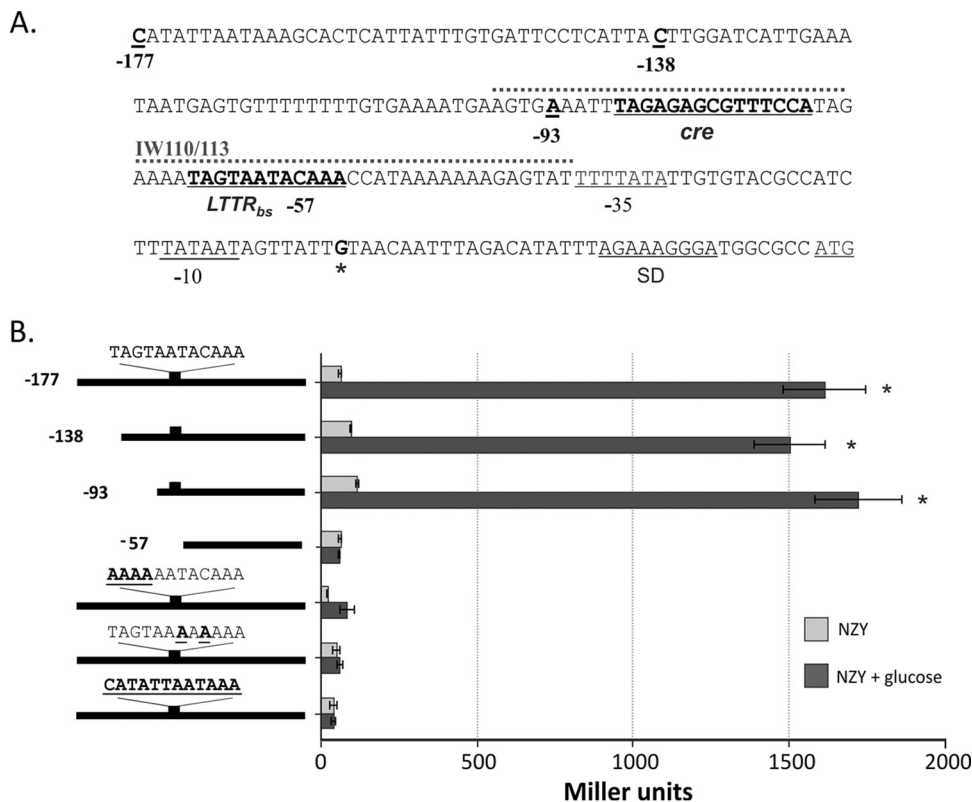
FIG 1 Identification of *cis*-acting elements in the *cidA* promoter region. (A) Nucleotide sequence of the *cidA* promoter. The −35 and −10 elements, Shine-Dalgarno (SD) sequence and translational start codon are underlined. The transcriptional start point is marked by an asterisk and presented in bold. The positions of truncated DNA fragments used for the deletion analysis of *cidA* promoter activity, the putative CidR binding site, and identified catabolite responsive elements (*cre*) are underlined and presented in bold. A 60-bp biotin-labeled DNA fragment used in EMSAs (IW110/113) is marked by a dotted line. (B) Deletion and mutational analysis of *cidA* promoter activity. Wild-type *S. aureus* cells (strain UAMS-1) containing *lacZ* reporter fusions with truncated *cidA* promoter fragments or the full-length *cidA* promoter with mutations in the putative CidR-binding site were grown under inducing conditions for 6 h and subsequently assayed for β-galactosidase activity. The length of the *cidA* promoter fragments used for the deletion analysis and the wild-type sequence of the CidR-binding site with generated mutations are shown in bold and underlined on the left. The results are representative of at least three independent experiments. Statistical significance in β-galactosidase activity between the wild-type strain containing *cidA* promoter fragments in the presence or absence of glucose (*) was determined by Student's *t* test ($P \leq 0.01$).

RESULTS

Identification of a *cis*-acting element required for CidR-mediated induction of *cidABC* expression. To characterize the molecular interactions associated with CidR-mediated regulatory control, we first sought to identify potential *cis*-acting DNA elements important for the binding of CidR to the promoter region of the *cidABC* operon. Although LTTR binding sites typically encompass the consensus sequence, T-N_{11}-A motif (17), the identification of the putative CidR-binding site within the *cidABC* promoter region is difficult because of the AT-rich nature of the *S. aureus* DNA. We, therefore, constructed reporter plasmids with a nested set of truncations of the sequences spanning the *cidABC* promoter region (Fig. 1A) that were fused to the *lacZ* reporter gene and then introduced these constructs into the *S. aureus* wild-type (wt) strain, UAMS-1. Because the CidR-mediated induction of *cidABC* transcription exhibits glucose-inducible expression under conditions of acidic stress (11, 21), we grew each reporter strain in the presence or absence of 35 mM glucose and measured the β-galactosidase activity associated with the cells after 6 h of growth. Consistent with previous results (16), *cidABC* promoter activity was induced by the full-length construct (designated −177) after 6 h of growth in the presence of glucose (Fig. 1B) in a CidR-dependent manner, suggesting that the *cis*-acting element important for the
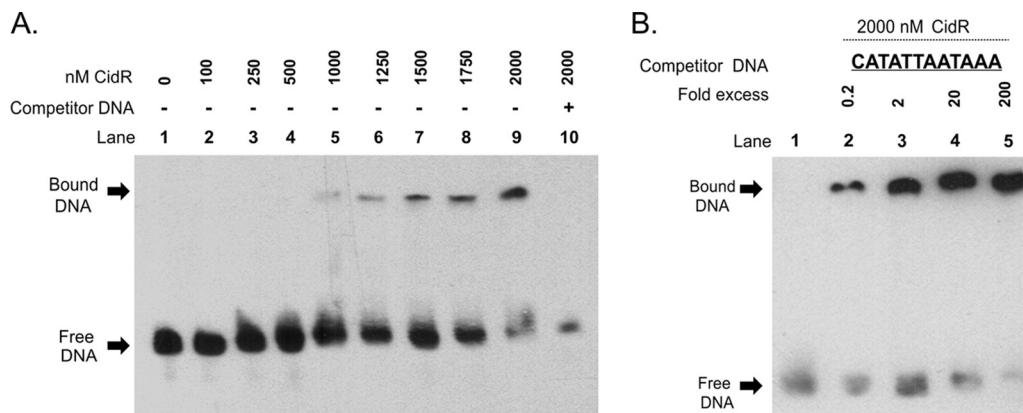
A.

B.

FIG 2 CidR binds to the 12-bp *cis*-acting element within the *cidA* promoter. (A) Electrophoretic mobility-shift assay (EMSA) was performed using increasing amounts of purified CidR protein and biotin-labeled *cidA* promoter DNA as a target. Lanes 1 and 10 contain a no-protein control and a 200-fold excess of unlabeled competitor DNA control, respectively. (B) EMSA performed with *cidA* promoter using increasing concentrations of mutated competitor DNA and a constant amount of purified CidR protein. Lane 1 contains wild-type biotin-labeled *cidA* promoter fragment only. Reaction mixtures were incubated for 30 min at room temperature and separated in a 6% TBE polyacrylamide gel.

CidR-mediated control was intact in this construct. As can be seen in Fig. 1B, removal of the regions 5′ to the nucleotides at positions −177, −138, and −93 upstream of the transcription start site had a minimal effect on *cidABC* expression, indicating the absence of *cis*-acting regulatory elements involved in *cidABC* induction upstream of the −93 position. In contrast, the −57 construct does not demonstrate induction of *cidABC* expression, suggesting that a required *cis*-acting element is present in the region between nucleotides −93 and −57. Analysis of the sequence within this region revealed the presence of a 12-bp element (TAGTAATACAAA) (Fig. 1A) that is one base pair shorter than typical LTTR binding sites. Interestingly, this sequence is located in a region that contains what was previously predicted to be the *cidABC* promoter (22).

To test the importance of the identified *cis*-acting element for *cidABC* expression, we altered the nucleotide sequence of this element by introducing different mutations into the −177 P*cidABC*-*lacZ* reporter construct and then examined the impact of these mutations on induction of *cidABC* promoter activity by measuring β-galactosidase activity in the wild-type cells as performed above. As shown in Fig. 1B, all generated mutations in the CidR-binding site abolished expression of the reporter gene, confirming the critical role of the identified *cis*-acting element for the induction of *cidABC* expression.

CidR binds to the identified specific *cis*-acting element within the *cidABC* promoter region. Although the above mutagenesis experiments demonstrated that the TAGTAATACAAA element is important for the induction of *cidABC* promoter activity, these results alone are not sufficient to conclude that this sequence represents the CidR-binding site. To address this, we performed electrophoretic mobility shift assays (EMSAs) using a C-terminal His tag-labeled CidR protein and a 60-bp biotin-labeled DNA fragment spanning the region containing the putative CidR-binding site (Fig. 1A). As shown in Fig. 2A, incubation of purified CidR protein with this DNA fragment resulted in a dose-dependent shift in the migration of the target DNA. Furthermore, addition of 200-fold excess of unlabeled specific competitor DNA effectively blocked formation of the CidR-DNA complexes, suggesting that CidR binding to the target DNA was specific (Fig. 2A). In agreement with this, CidR binding was not observed in the EMSAs with DNA fragments originating from sequences located upstream and downstream of the 60-bp target region and not containing the putative CidR-binding site (see Fig. S1 in the supplemental material). To further demonstrate the specificity of the protein-DNA interactions between CidR and its *cis*-acting element, we performed EMSAs with a *cidABC* promoter fragment containing a mutated CidR-binding site. As seen in Fig. 2B, competitor DNA in which the entire CidR-binding element in the *cidABC* promoter

region was replaced with a random nucleotide sequence failed to block binding of CidR to the labeled target DNA. Combined, these data demonstrate that CidR directly regulates *cidABC* expression by specifically binding to the identified 12-bp TAGTAATA CAAA nucleotide sequence.

CcpA is a positive regulator of *cidABC* transcription. Previously, the inactivation of the *ccpA* gene in *S. aureus* was shown to cause a drastic decrease in *cidABC* transcript accumulation under conditions of excess glucose, suggesting the involvement of the master regulator of carbon metabolism, CcpA, in the control of *cidABC* expression (19). Consistent with this, the *cidABC* promoter region was found to contain a putative *cre* box upstream of the CidR-binding site (Fig. 1A), indicative of the direct positive regulation of this operon by CcpA. To demonstrate that CcpA is involved in the direct transcriptional control of *cidABC* expression, we performed a quantitative real-time reverse transcriptase PCR (RT-PCR) analysis using primers specific to the *cidA* and *cidR* genes. Using this approach, we found that the lack of CcpA during growth under conditions of acetate-mediated acidic stress in the presence of glucose (inducing conditions) abolishes *cidABC* expression, even though it leads to a slight increase in the *cidR* transcript accumulation (Fig. 3A).

Due to the fact that inactivation of the *ccpA* gene in staphylococci diminishes production of acetate by directing carbon flux into the tricarboxylic acid (TCA) cycle (23, 24), we asked whether the observed decrease in *cidABC* transcription in the *ccpA* mutant was simply a result of insufficient acidification of the medium or a direct effect of this transcription factor on the *cidABC* promoter. Circumstantial evidence against the former model was previously generated by demonstrating that disruption of the major acetate-generating pathway, the Pta-AckA pathway, in *S. aureus* causes a significant increase in *cidABC* transcription while simultaneously reducing the ability of the bacteria to acidify the culture medium in the presence of excess glucose (25, 26). To more directly examine the role of culture acidification in the CcpA-mediated control of *cidABC* expression, we combined the *ackA* and *ccpA* mutations in a single strain and performed a quantitative RT-PCR analysis of *cidABC* expression during exponential growth. Of note, similar to the single mutants, the *ackA ccpA* double mutant exhibited a severe impairment of acetate generation, resulting in a reduced acidification of the culture medium (Fig. 4). As shown in Fig. 3B, the previously observed effect of the *ackA* mutation on *cidABC* expression was completely eliminated by the presence of the *ccpA* mutation, demonstrating that CcpA-mediated induction of *cidABC* expression does not require acidification of the culture medium. Furthermore, in agreement with the results of Northern blot analysis (19), the absence of CcpA during exponential growth significantly decreases the levels of *cidABC* transcription compared to those of the wild-type strain, underlying the necessity for glucose in the induction of *cidABC* expression (Fig. 3B).

In Gram-positive bacteria, the DNA-binding activity of CcpA is triggered or enhanced by interactions with its coregulator, histidine-containing protein (HPr), which in the presence of the glycolytic intermediate, fructose-1,6-bisphosphate, is phosphorylated on residue Ser46 by its cognate HPr kinase (27). To demonstrate the essentiality of the glycolytic flux (i.e., fructose-1,6-bisphosphate) for the CcpA-mediated regulation of *cidABC* expression, we compared the impact of alternative carbon sources on *cidABC* induction using a P*cidABC*-*lacZ* reporter plasmid. In this approach, we grew wild-type cells in medium containing either glucose (35 mM), fructose (42 mM), or glycerol (70 mM) as a carbon source and then measured β-galactosidase activity after 6 h of growth to assess the activity of the *cidABC* promoter. As anticipated, the addition of fructose to the culture medium induced *cidABC* promoter activity in a similar manner to that of glucose since catabolism of both of these sugars yields fructose-1,6-bisphosphate (Fig. 3C). In contrast, glycerol, which catabolism bypasses the formation of fructose-1,6-bisphosphate, did not induce *cidABC* promoter activity (Fig. 3C). Combined, the results of these experiments demonstrate that CcpA-mediated regulation of
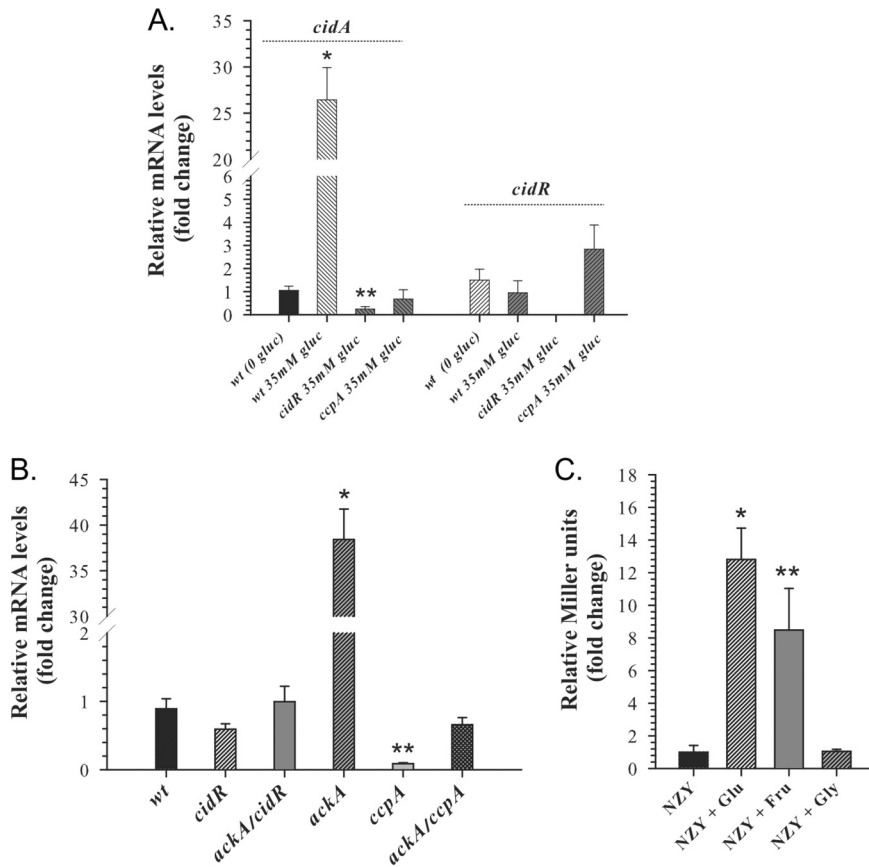
FIG 3 CcpA is required for induction of *cidABC* expression. (A) Relative transcript levels of the *cidA* and *cidR* genes determined by quantitative RT-PCR after 6 h of growth. Transcript levels in the wild-type strain, *cidR*, and *ccpA* mutants grown in TSB containing 35 mM glucose (inducing conditions) are presented as a fold difference compared to those for the wild-type strain grown in TSB without glucose. Statistical significance between the wild-type strain grown with or without glucose (*) as well as between the *cidR* mutant grown under inducing conditions and wild-type strain grown in the absence of glucose (**) was determined by Student's *t* test ($P \leq 0.01$). (B) Relative *cidA* transcript levels determined by quantitative RT-PCR after 3 h of growth in TSB containing 14 mM glucose. Transcript levels in the *cidR*, *ackA*, *ccpA*, *ackA cidR*, and *ackA ccpA* mutants are presented as fold differences compared to those in the wild-type strain. Statistical significance between the wild-type strain and *ackA* mutant (*) and between the wild-type strain and *ccpA* mutant (**) was determined by Student's *t* test ($P \leq 0.005$). (C) Effect of alternative carbon sources on *cidABC* expression. Wild-type strain cultures containing the P$_{cidABC}$-*lacZ* reporter plasmid were grown either in plain NZY medium or NZY medium supplemented with 35 mM glucose, 42 mM fructose, or 70 mM glycerol. After 6 h of growth, samples were collected and assayed for β-galactosidase activity. Statistical significance between the wild-type strain grown in plain NZY and NZY supplemented with glucose (*) or fructose (**) was determined by Student's *t* test ($P \leq 0.01$).

cidABC expression requires carbon flux through the glycolytic machinery and occurs independently of acidification of the culture medium during growth.

CcpA directly regulates *cidABC* expression by binding to the *cidABC* promoter. The above experiments and the presence of a putative *cre* site within the *cidABC* promoter region strongly suggest direct positive regulation of *cidABC* expression by CcpA. To provide experimental evidence that CcpA directly binds within the *cidABC* promoter region, we performed EMSA experiments similar to those described above for CidR. In this approach, a C-terminal His tag-labeled CcpA protein was affinity purified and incubated with the same 60-bp biotin-labeled *cidABC* promoter fragment (Fig. 1A) that was previously used in the experiments to assess DNA binding by CidR. The results presented in Fig. 5A demonstrate that CcpA binds to the target DNA in a dose-dependent manner. Furthermore, the addition of a 200-fold excess of unlabeled specific competitor DNA effectively blocked the formation of higher-order CcpA-DNA complexes, demonstrating the specificity of the CcpA interaction with the target DNA.
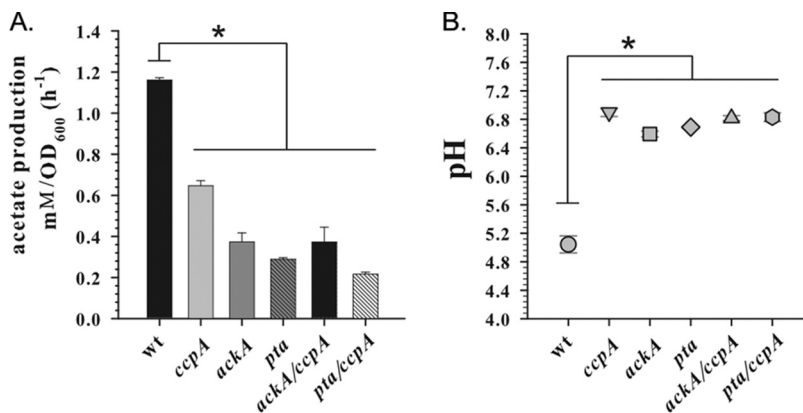
FIG 4 Inactivation of *ccpA* and Pta-AckA pathway decreases production of acetate and consequent acidification of the culture medium. (A) Acetate production rates of the UAMS-1 wild-type strain and *ccpA*, *ackA*, *pta*, *ackA ccpA*, and *pta ccpA* mutants determined after 6 h of growth on TSB supplemented with 35 mM glucose. (B) pH values of the culture medium of the UAMS-1 wild-type strain and *ccpA*, *ackA*, *pta*, *ackA ccpA*, and *pta ccpA* mutants determined after 6 h of growth on TSB supplemented with 35 mM glucose. Statistical significance between the wild-type strain and the mutants (*) was determined by Student's *t* test ($P \leq 0.01$).

Analysis of the nucleotide sequence spanning the *cidABC* promoter region revealed the presence of a potential *cre* box sequence, TAGAGAGCGTTTCCA, upstream of the CidR-binding site (Fig. 1A), which has only one mismatched nucleotide (underlined) (see Fig. S2 in the supplemental material) from the *cre* site consensus in *Bacillus subtilis*, WTGNAANCGNWNNCW (28). To demonstrate the ability of CcpA to recognize and bind this putative *cre* site, we constructed a P*cidABC*-*lacZ* transcriptional reporter fusion in which this element was substituted with random sequence GCAGTGTATCCAGGC and then analyzed its ability to generate β-galactosidase activity in *S. aureus*. As shown in Fig. 5B, the elimination of the putative *cre* site from the *cidABC* promoter region
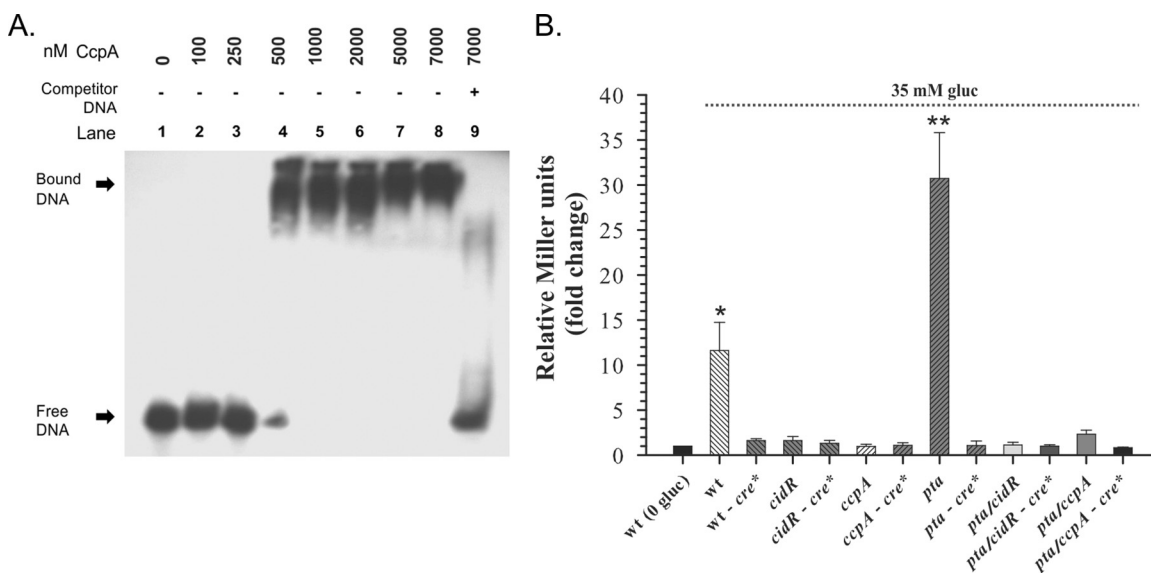

FIG 5 CcpA directly regulates *cidABC* expression by binding to the *cre* site within the *cidA* promoter. (A) EMSA was performed using increasing concentrations of purified CcpA protein (100 to 7,000 nM) and biotin-labeled wild-type *cidA* promoter DNA as a target. Reaction mixtures were incubated for 30 min at room temperature and separated on a 6% TBE polyacrylamide gel. Lanes 1 and 9 contain a no-protein control and a 200-fold excess of unlabeled competitor DNA control, respectively. (B) Effect of *cre* site presence on *cidABC* expression. *S. aureus* cultures with *lacZ* reporter plasmids containing *cidA* promoter DNA fragment with native or mutated (*cre**) *cre* site were grown in plain TSB or TSB medium supplemented with 35 mM glucose. After 6 h of growth, samples were collected and assayed for β-galactosidase activity. Statistical significance between the wild-type strain grown in plain TSB and wild-type strain (*) or *pta* mutant (**) grown under inducing conditions was determined by Student's *t* test ($P \leq 0.001$).
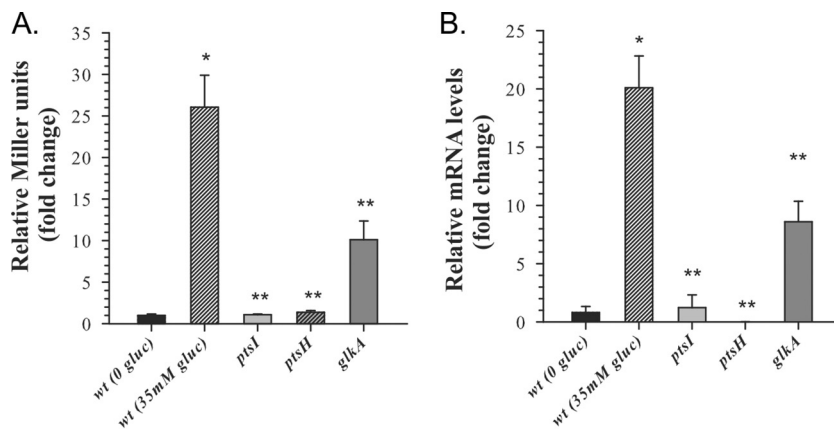
FIG 6 Relative induction of *cidABC* expression in isolated transposon mutants. (A) Mutants from the transposon mutagenesis screen were tested for *cidABC* expression under inducing conditions (NZY medium supplemented with 35 mM glucose). After 6 h of growth, samples were collected and assayed for β-galactosidase activity. Statistical significance between the wild-type strain grown under inducing conditions and wild-type strain grown in plain NZY (*), as well as between wild-type strain and *ptsI*, *ptsH*, and *glkA* mutants grown under inducing conditions (**), was determined by Student's *t* test ($P \leq 0.01$). (B) Relative *cidA* transcript levels determined by quantitative RT-PCR after 6 h of growth in TSB containing 35 mM glucose. Statistical significance between the UAMS-1 wild-type strain grown with or without glucose (*), as well as between the UAMS-1 wild-type strain and *ptsI*, *ptsH*, and *glkA* mutants grown under inducing conditions (**), was determined by Student's *t* test ($P \leq 0.01$).

drastically decreased β-galactosidase production in wild-type cells under inducing conditions. Furthermore, the absence of this site eliminates the positive impact of the *pta* mutation on *cidABC* promoter activity similarly to the lack of CcpA (Fig. 5B). Combined, the results of these experiments demonstrate that the CcpA-mediated control of *cidABC* expression occurs through the direct binding of CcpA to the *cidABC* promoter region, which requires the presence of a functional *cre* site.

Identification of potential effectors of *cidABC* expression. The results of RT-PCR analysis (Fig. 3B) and β-galactosidase assays (Fig. 5B) revealed that induction of *cidABC* promoter activity by CcpA and CidR requires both acidification of the culture medium and the presence of glucose, suggesting that expression of the *cidABC* operon under conditions of carbon overflow is modulated by the metabolic status of the cells. Therefore, to demonstrate the direct linkage of *cidABC* expression to carbohydrate metabolism and to identify metabolic genes that affect expression of this operon, we used an unbiased transposon mutagenesis approach. For this purpose, we generated a reporter strain, a derivative of *S. aureus* JE2 (29), that contains a P$_{cidABC}$-*lacZ* fusion construct inserted into the *geh* gene within the *S. aureus* chromosome (strain KB6001). To confirm the suitability of this reporter strain for screening, we measured β-galactosidase activity after 6 h of growth in the presence of glucose (inducing conditions) and demonstrated that the reporter strain reflects the anticipated *cidR*-dependent control of the *cidABC* promoter activity (Fig. 6A). Next, we performed transposon mutagenesis of the KB6001 strain as previously described (29) and screened mutants on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) indicator plates for the colonies that displayed a decrease in the β-galactosidase activity. To verify screening results, we measured β-galactosidase activity in mutant cultures grown for 6 h in tryptic soy broth (TSB) supplemented with 35 mM glucose as described above. Using this approach, we identified 23 genes where transposon insertions led to decreased *cidABC* promoter activity (see Table S3 in the supplemental material). Of particular interest, the results of this screen together with subsequent β-galactosidase activity assay (Fig. 6A) revealed three genes involved in carbohydrate metabolism that affect *cidABC* promoter activity. Two of these genes, *ptsI* and *ptsH*, encode the components of a phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) (i.e., phosphoenolpyruvate protein phosphotransferase and phosphocarrier protein HPr

[30]), where seryl-phosphorylated HPr under conditions of excess glucose also acts as a corepressor in CcpA-mediated carbon catabolite repression (CCR) (31). The third gene, *glkA*, encodes glucokinase, which together with glucose permease (encoded by *glkU*) comprises a PTS-independent system that transports glucose and catalyzes the formation of the very first glycolytic intermediate, glucose-6-phosphate (32). Interestingly, the β-galactosidase activity assays and RT-PCR analysis of two different *S. aureus* strains revealed a range of effects of these genes on *cidABC* expression. As can be seen in Fig. 6A and B, inactivation of either *ptsI* or *ptsH* genes completely abolished induction of *cidABC* promoter activity, while the lack of glucokinase during growth decreases its activity approximately 2.5 fold, suggestive of the different contributions of products of these genes to glucose transport and subsequent carbon flux through the glycolytic machinery (33). Together, the results of these experiments reveal a strong correlation of *cidABC* expression with glucose uptake and glycolytic activity, which are required for the induction of *cidABC* expression by CcpA, demonstrating the critical role of carbohydrate metabolism in the regulation of this operon.

DISCUSSION

In *S. aureus*, cell death and lysis during biofilm development are known to be affected by the gene products of the *cidABC* operon (5, 22). Based on growth conditions, this operon can be expressed in a form of two overlapping transcripts designated *cidABC* and *cidBC* (34). It has been shown that expression of the smaller *cidBC* transcript depends on sigma factor B (21), while induction of the full-length *cidABC* transcript is controlled by the LTTR family transcriptional regulator, CidR, and requires both acidification of the culture medium and excess glucose (16, 34). Microarray analysis revealed two operons, *alsSD* and *cidABC*, that are directly controlled by the transcriptional regulator, CidR (11), and whose gene products are involved in catabolism of pyruvate formed under excess glucose conditions. The products of the *alsSD* operon are acetolactate synthase and acetolactate decarboxylase of the 2,3-butanediol pathway, converting pyruvate to the neutral by-product, acetoin, whereas the *cidC*-encoded pyruvate:menaquinone oxidoreductase decarboxylates pyruvate to acetate, leading to acidification of the culture medium that causes acidic stress and subsequent cell death (11, 12, 14). It has been hypothesized that an intricate balance in the metabolic activities of the *alsSD* and *cidC* gene products, directing carbon flux at the pyruvate node, determines cell fate during aerobic growth with an excess of glucose (8). In support of this, the results of our recent study provided a new mechanistic insight into the role of the CidR regulon during overflow metabolism and have extended the understanding of the contribution of individual Cid proteins to carbon flux at the pyruvate node (15). Specifically, we have shown that the *cidB* gene product enhances the generation of acetate by CidC, while, in contrast, CidR-mediated induction of the *cidA* and *alsSD* expression has a negative effect on CidC activity and directs carbon away from acetate and toward the production of acetoin (15). Therefore, by revealing opposing functions of the CidA and CidB proteins in modulating CidC activity, we established that the major function of the CidR regulon is to limit acetate-mediated cell death in the bacterial population during growth under conditions of carbon overflow (15).

In the present study, we further characterized the regulatory network controlling *cidABC* expression in *S. aureus* and clarified the metabolic requirements essential for the induction of this operon. Using computational analyses, mutagenesis approaches, and subsequent β-galactosidase activity assays, we identified *cis*-acting elements within the *cidABC* promoter region (Fig. 1A) and then confirmed their necessity for the binding of the transcriptional regulators, CidR and CcpA (Fig. 2A and Fig. 5A). Importantly, β-galactosidase activity assays and RT-PCR analyses revealed that CcpA and CidR are involved in the direct positive control of *cidABC* expression (Fig. 3A and B), where the complete induction of the *cidABC* promoter depends on acidification of the culture medium in the presence of glucose and requires the synergistic contribution of both transcriptional regulators (Fig. 3A and Fig. 5B). Interestingly, in contrast to the CidR-
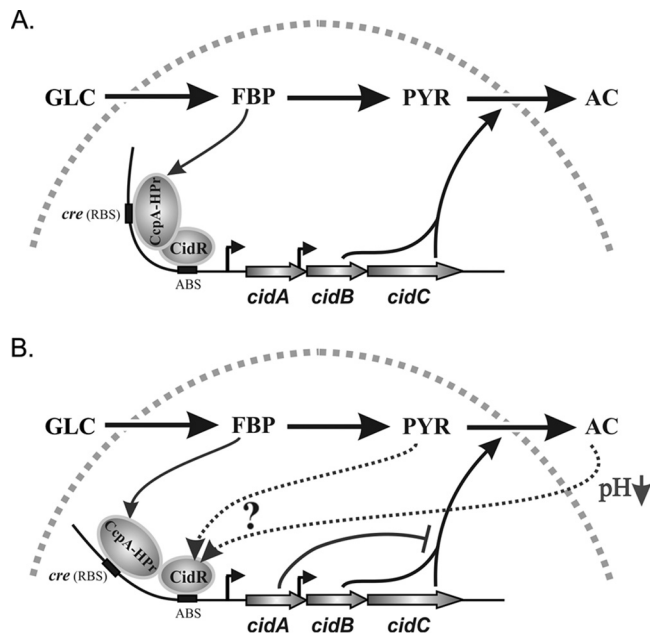
FIG 7 A conceptual model demonstrating synergistic regulation of *cidABC* expression by CidR and CcpA-HPr (Ser-P) complex under conditions of carbon overflow without (A) and with (B) acidic stress. Metabolites presented in the scheme include the following: GLC, glucose; FBP, fructose 1,6-bisphosphate; PYR, pyruvate; AC, acetate.

mediated induction of *cidABC* expression during acidic stress, the positive regulation of the *cidABC* promoter by CcpA is independent of acidification of the culture medium (Fig. 3A and B; Fig. 5B) but requires the presence of glycolytic intermediate fructose-1,6-bisphosphate (Fig. 3C). In agreement with this, the transposon mutagenesis approach revealed genes involved in glucose transport and CCR, the inactivation of which has a negative impact on *cidABC* expression as it affects glycolytic flux and the regulatory function of CcpA (Fig. 6).

As indicated above, the CidR protein belongs to the LTTR family of transcriptional regulators. The canonical LTTR binding region contains two distinct binding sites separated by a few nucleotides, termed the r̲ecognition b̲inding s̲ite (RBS) and the a̲ctivation b̲inding s̲ite (ABS) (17). It has been suggested that the LTTR initially binds each site as a dimer, then dimers interact to form a tetramer, and finally the tetramer acts to bend the DNA, where the high angle of the bent DNA impedes the formation of the transcription bubble (17). In the presence of a specific ligand, the LTTR undergoes a conformational change, causing the tetramer to shift on the DNA and relax the angle. The new angle then allows for the formation of the transcription bubble, thus facilitating the initiation of transcription. This model of LTTR regulation has been termed the "sliding dimer" model (35).

Although the proposed LTTR-mediated regulation of gene expression requires two separate binding sites, to our surprise, the results of the current work revealed the presence of only one *cis*-acting element within the *cidABC* promoter region that is critical for the binding of CidR, suggesting that regulation of *cidABC* expression by this LTTR might vary from the classical model. Interestingly, in a close proximity (7 bp upstream) to the *cidR* binding site, we identified another *cis*-acting element critical for the induction of the *cidABC* promoter (Fig. 1A and Fig. 5B). This element has only a single mismatch from the canonical *cre* box sequence (28), and it is required for the binding of the master regulator of carbon metabolism, CcpA (27). Therefore, considering the mechanisms of LTTR-mediated regulation of gene expression (17) and based upon the results of current work, we propose a model that predicts the induction of *cidABC* expression through a synergistic interplay between CidR and CcpA and links its expression to the metabolic status during glucose catabolism (Fig. 7). In this model, we

speculate that the complete induction of *cidABC* expression will occur in two steps. First, during growth in medium with excess glucose, carbon flux through the glycolytic machinery, yielding fructose-1,6-bisphosphate, would lead to the formation of the CcpA-HPr (Ser-P) complex. Then, the CcpA-HPr (Ser-P) dimer will bind to the *cre* site within the *cidABC* promoter region, which will act as a functional analog of RBS (Fig. 7A). Simultaneously, the CidR dimer will bind to the ABS sequence. Subsequent protein-protein interactions between CidR and CcpA-HPr (Ser-P) dimers will bend the DNA leading to a basal level of expression of the *cidABC* operon (Fig. 7A). At the second stage, over time, the excretion of acetate into the culture medium during glucose catabolism will decrease external pH values close to the pK$_a$ of acetate (4.74), leading to formation of acetic acid (Fig. 7B). The generated weak acid will translocate across the membrane and dissociate within the bacterial cytoplasm resulting in its protonation. Then, the decrease in intracellular pH (pHi) will stimulate binding of a yet to be identified inducer molecule (acetate or pyruvate) to the conserved ligand-binding domains within the CidR dimer. The conformational changes caused by interaction of CidR with its inducer will affect protein-protein interactions with the CcpA-HPr (Ser-P) dimer, leading to relaxation of the DNA-bending angle and facilitating transcription (Fig. 7B).

Overall, the results of this study provide important insight into the signals required for the induction of *cidABC* expression during carbohydrate metabolism. Previously, we have considered the presence of glucose during growth mainly as a prerequisite for the acetate-mediated acidification of the culture medium that causes induction of the *cidABC* promoter activity by CidR (16, 34). The results of this work, however, reveal that glucose, *per se*, is an important component of this regulation, as its catabolism through the glycolytic machinery leads to the accumulation of fructose-1,6-bisphosphate that is required for CcpA-mediated control of *cidABC* expression. Furthermore, we established that the full induction of *cidABC* expression requires the synergistic contribution of CidR and CcpA, and proposed a conceptually novel model where the interaction of a LTTR with CcpA-HPr (Ser-P) complex under different metabolic conditions modulates the activity of the *cidABC* promoter. To support this model, we are currently planning *in vitro* experiments that will reveal protein-protein interactions between the CcpA-HPr (Ser-P) complex and CidR within the *cidABC* promoter region under conditions mimicking those observed in the current study.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. The UAMS-1-*ccpA* mutant as well as the UAMS-1-*pta ccpA* and UAMS-1-*ackA ccpA* double mutants were generated by bacteriophage Φ11-mediated transduction (36) of the *ccpA::tetL* allele from the JE2 *ccpA::tetL* strain (37) into UAMS-1, UAMS-1-*pta*, and UAMS-1-*ackA*, correspondingly. The replacement of the *ccpA* gene by the *ccpA::tetL* allele was verified by PCR using primers SAV1737-f and acuC-f. The UAMS-1-*pta cidR* and UAMS-1-*ackA cidR* double mutants were generated by bacteriophage Φ11-mediated transduction (36) of the *cidR::tet* allele from the KB1090 strain into UAMS-1-*pta* and UAMS-1-*ackA*, correspondingly. The UAMS-1 background in all mutants was confirmed by PCR using primers cna-f and cna-r (25).

S. aureus strains were grown in tryptic soy broth (TSB) medium (BD Biosciences) at 37°C with aeration at 250 rpm and a flask-to-medium ratio of 10:1. *Escherichia coli* DH5α was grown in Luria-Bertani medium (Fisher Scientific). Antibiotics were purchased from Sigma-Aldrich and Fisher Scientific and were used at the following concentrations: kanamycin, 50 μg/ml; chloramphenicol, 10 μg/ml; ampicillin, 100 μg/ml; and erythromycin, 5 μg/ml.

DNA manipulations. Oligonucleotides used in this study are listed in Table S2 in the supplemental material. Genomic DNA was isolated from *S. aureus* using Wizard Plus kits from Promega, Inc. Restriction endonucleases, DNA polymerases, and T4 DNA ligase used in this study were purchased from New England Biolabs and Invitrogen Life Technologies. Recombinant plasmids were isolated using the Wizard Plus SV Minipreps DNA purification system (Promega, Inc.). PCR was performed using the KOD polymerase kit (Novagen) in the Applied Biosystems GeneAmp PCR system 9700 (Life Technologies Corporation) with primers purchased from Integrated DNA Technologies and Eurofins Operon. DNA fragments were purified using the DNA Clean and Concentrator-5 kit (Zymo Research), and recombinant DNA plasmid products were sequenced at the University of Nebraska Medical Center DNA Sequencing Core facility and analyzed using Vector NTI (Invitrogen).

The *cidABC* promoter was amplified using the primers IW11 and IW15 and UAMS-1 genomic DNA as a template following digestion by BamHI and NheI and ligation into pIHW7 (18) to yield the *cidABC* reporter plasmid, pIHW10-*lac*.

A deletion analysis was performed by creating nested sets of DNA fragments spanning the *cidABC* promoter region, fusing them to the *lacZ* reporter gene, and then measuring β-galactosidase activity (see below) under inducing and noninducing conditions. To create 5′ end shortened DNA fragments of the *cidABC* promoter region, the reverse primer IW11 was paired with the forward primers, IW10, IW16, IW31, and IW22. Each generated PCR promoter fragment was digested with BamHI and NheI and then ligated into pIHW7 (18), yielding the plasmids, pIHW9, pIHW11-*lac*, pIHW22-*lac*, and pIHW17-*lac*. Specific point mutations in the *cidABC* promoter were made as previously described (38) using pIHW25 plasmid as a template.

For the substitution of the *cre* site in the pIHW10-*lac* plasmid with the sequence GCAGTGTATCCA GGC, we used outward primers cre*-cidABC-f and cre*-cidABC-r followed by self-ligation and transformation of the PCR fragment into *E. coli* DH5α to generate the plasmid pSW1. The elimination of the *cre* site in the plasmid pSW1 was confirmed by DNA sequencing using primers pCN51-S2-f and B-gal-S-r.

Construction of the *lacZ* reporter strain for transposon mutagenesis. For screening and identification of transposon mutants with altered *cidABC* expression, we created *S. aureus* strain KB6001 containing the P*cidABC*-*lacZ* reporter fusion integrated into the *geh* gene within the bacterial chromosome. The 5′ region upstream of *geh* from LAC-13c was amplified (using the primers IW27 and IW28) and ligated into the EcoRI and SacI sites of pCL52.2 (39) to generate the plasmid, pIHW20. The 3′ region downstream of *geh* was amplified using the primers IW29 and IW30 and ligated into the PstI and NheI sites of pIHW20 to generate the plasmid pIHW21. The P*cidABC*-*lacZ* reporter fusion construct was generated by PCR using the primers IW10 and IW44 and the pIHW9 plasmid as a template. Then, the PCR product was ligated into the PstI and BamHI sites of pIHW21 to produce the temperature-sensitive integration plasmid pIHW25. The JE2 *cidABC* reporter strain with the pIHW25 plasmid integrated into the chromosome was generated using allelic replacement methodology as described previously (40), selecting for blue colonies in the presence of 35 mM glucose and 50 μg/ml X-Gal. The reporter strain was confirmed by PCR and used as a host for further transposon mutagenesis as described previously (29).

β-Galactosidase assays. β-Galactosidase assays were performed as previously described (41). Briefly, 1.0 ml of cell culture collected by centrifugation was suspended in 1 ml of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0) and lysed using a FastPrep FP120 instrument (MP Biomedicals). The lysates were then centrifuged, and 700 μl of the supernatants was transferred to a 1.5-ml microcentrifuge tube. After the addition of 140 μl of ONPG (*o*-nitrophenyl-β-D-galactopyranoside; 4 mg ml^{-1}), the samples were incubated at 37°C until they turned slightly yellow (under an optical density at 420 nm [OD_{420}] of 1.0). To stop the reactions, 500 μl of 1 M sodium carbonate was added and the OD_{420} was measured. Protein concentrations were determined by performing Bradford assays using the protein assay dye solution from Bio-Rad. Miller units were calculated using protein concentration instead of OD_{600} (42).

Expression and purification of the CidR and CcpA proteins. To generate a plasmid for expression and purification of the CidR protein, the *S. aureus cidR* gene was amplified using the primers IW128cc and IW129cc and UAMS-1 genomic DNA as a template. Then, the PCR product was cloned into the NheI and XhoI sites of pET24b, and its integrity was confirmed by DNA sequencing. The resulting plasmid (pET24b-*cidR*) was transformed into the rare-codon enhanced *E. coli* strain BL21 derivative Rosetta 2 (Invitrogen) for protein expression. Similarly, to construct a plasmid for expression and purification of the CcpA protein, the *ccpA* gene was amplified from the UAMS-1 genomic DNA via PCR using the primers IW116 and IW117 and cloned into pET24b as described above. The resulting plasmid (pET24b-*ccpA*) was transformed into the *E. coli* strain BL21 (Invitrogen) for protein expression.

To produce large quantities of the CidR protein, the Rosetta 2 (pET24b-*cidR*) strain was grown in 1.0 liter of LB medium containing kanamycin and chloramphenicol at 37°C until the culture reached mid-exponential phase. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a final concentration of 0.4 mM to induce expression of CidR. The culture was transferred to a 20°C incubator and expressed overnight. To produce the CcpA protein, a similar approach was utilized, with the exception that the overnight cultures were grown at 30°C. Purification of the CidR and CcpA proteins was achieved by collecting the expressed cells by centrifugation and resuspending them in 50 ml of lysis buffer (100 mM phosphate and 300 mM NaCl). Phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 1.0 mM to inhibit protease activity. The cells were lysed by serial passage through an EmulsiFlex C3 system. The released recombinant proteins were purified using a HisPur Cobalt purification kit (Pierce Biotechnology) and desalted by ultrafiltration using an exchange buffer (100 mM Tris-HCl [pH 8.0], 150 mM KCl, 1 mM EDTA, and 0.1 mM dithiothreitol [DTT]). The purified proteins were stored at −20°C in an exchange buffer containing 40% glycerol.

Protein-DNA interactions. The binding of purified proteins to target DNA was demonstrated by electrophoretic mobility shift assays (EMSAs) using a LightShift chemiluminescent kit (Pierce Biotechnology) according to the manufacturer's instructions. The target DNA used for the CidR and CcpA EMSAs was made by annealing biotin-labeled 60-bp primers IW110 and IW113 (spanning the CidR and CcpA binding sites in the *cidABC* promoter region). Specific competitor DNA (annealed oligonucleotides IW110 and IW113) was added in 200-fold excess as per the manufacturer's instructions. Mutant-specific competitor DNA was made by annealing the primers IW176 and IW177 for the complete substitution of the CidR-binding site. The binding reaction mixture for each sample contained 10 mM Tris (pH 7.5), 50 mM KCl, 1 mM DTT, 2.5% glycerol, 1 μg of salmon sperm DNA, 5 mM $MgCl_2$, 0.05% NP-40, 10 mM EDTA, and 5.0 fmol of labeled DNA in a total volume of 20 μl. After 30 min of incubation at room

temperature, the samples were separated in a 6% polyacrylamide gel in 0.5× Tris-borate-EDTA (TBE) buffer at 85 V. The DNA was then transferred and cross-linked to the nylon membrane using a UV Stratalinker 1800 (Stratagene) cross-linker instrument. The labeled DNA fragments were visualized using an SRX-101a Imager (Konica Minolta).

Quantification of the mRNA transcripts. RNA isolation was carried out as described previously (43). Quantitative real-time PCR was performed using the *cidA*-, *cidR*-, and *sigA*-specific primers listed in Table S2. Total RNA (500 ng) was converted to cDNA using the QuantiTect reverse transcription kit (Qiagen). The samples were then diluted 1:50, and the cDNA products were amplified using the LightCycler DNA master SYBR green I kit (Roche Applied Science) following the manufacturer's instructions. The relative transcript levels were calculated using the comparative threshold cycle (C_T) method (44) with normalization to the amount of *sigA* transcripts present in the RNA samples. The results were recorded in duplicate and are representative of three independent experiments.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB .00371-19.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

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