The Catabolite Control Protein E (CcpE) Affects Virulence Determinant Production and Pathogenesis of Staphylococcus aureus*

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Background: Carbon metabolism and virulence are often linked in pathogenic bacteria.

Results: Deletion of the catabolite control protein E (CcpE) affects the expression of virulence factors and pathogenicity of S. aureus.

Conclusion: Our data suggest that CcpE acts as an attenuator of virulence in S. aureus.

Significance: CcpE may serve to link S. aureus nutritional status to virulence determinant biosynthesis.

Carbon metabolism and virulence determinant production are often linked in pathogenic bacteria, and several regulatory elements have been reported to mediate this linkage in S. aureus. Previously, we described a novel protein, catabolite control protein E (CcpE) that functions as a regulator of the tricarboxylic acid cycle. Here we demonstrate that CcpE also regulates virulence determinant biosynthesis and pathogenesis. Specifically, deletion of ccpE in S. aureus strain Newman revealed that CcpE affects transcription of virulence factors such as capA, the first gene in the capsule biosynthetic operon; hla, encoding α-toxin; and psma, encoding the phenol-soluble modulin cluster α. Electrophoretic mobility shift assays demonstrated that CcpE binds to the hla promoter. Mice challenged with S. aureus strain Newman or its isogenic ΔccpE derivative revealed increased disease severity in the ΔccpE mutant using two animal models; an acute lung infection model and a skin infection model. Complementation of the mutant with the ccpE wild-type allele restored all phenotypes, demonstrating that CcpE is negative regulator of virulence in S. aureus.

to the availability of carbon sources (reviewed in Ref. 1). Similarly, many pathogenic bacteria use this same mechanism to link the nutritional status with the transcription of virulence factors (reviewed in Ref. 2). In S. aureus, carbon catabolite repression is mediated by several regulators such as the catabolite control protein A (CcpA),5 a glucose-responsive member of the LacI/GalR family of transcriptional regulators (3), CodY, a pleiotropic repressor that responds to GTP and branched-chain amino acids (4), and RpiRc, a putative ribose-responsive regulator that belongs to the RpiR family of transcriptional regulators (5). Recently, we identified CcpE as another potential carbon catabolite responsive element of S. aureus that controls transcription of tricarboxylic acid (TCA) cycle genes (6). In addition to regulating metabolism, CcpA, CodY, and RpiRc also regulate virulence factor expression (3–5).

CcpA regulates the expression of exotoxins, such as α-toxin (encoded by hla) and toxic shock syndrome toxin-1 (encoded by tst), and capsule formation in a glucose-responsive manner (3, 7, 8). In addition, CcpA promotes biofilm formation under in vitro conditions (9) and alters antibiotic susceptibility in methicillin-resistant S. aureus (MRSA) and glycopeptide intermediate-resistant S. aureus (3). More recently, CcpA was reported to mediate proline and arginine auxotrophies during in vitro growth (10, 11), and to contribute to infectivity of S. aureus in a murine model of staphylococcal abscess formation (10).

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5 The abbreviations used are: CcpE, catabolite control protein E; TCA, tricarboxylic acid; MRSA, methicillin-resistant S. aureus; qRT-PCR, real-time reverse transcription PCR; CP-5, Capsular Polysaccharide 5; BALF, bronchoalveolar lavage fluid; G-CSF, granulocyte-colony stimulating factor.
**Attenuation of Pathogenesis by CcpE of S. aureus**

**TABLE 1**

| Strain or plasmid | Relevant genotype or characteristic(s) | Ref. or source |
|-------------------|----------------------------------------|---------------|
| *S. aureus* 923   | CA-MRSA, clinical isolate of pulsortype USA300, Oxa' | 26            |
| HOM 354          | 923 ΔccpE::lox66-aphAIII-lox71, Oxa', Kan' | This study    |
| HOM 355          | SA564 ΔccpE::lox66-aphAIII-lox71, Kan' | 25            |
| Newman strain    | Laboratory strain (ATCC 25904); CP-5 producer | 27            |
| SA564            | Low passage human isolate                | 27            |
| THa              | RN4220 ΔccpE::lox66-aphAIII-lox71, Kan' | 6             |
| TH01             | Newman ΔccpE::lox72                       | 6             |
| TH01c            | TH01 harboring plasmid pTH2c cis-integrated at the NWMN_0640 locus, leading to a duplication of the NWMN_0640 gene, cepE', Tc' | 6             |

**Plasmids**

| pSB2035          | Escherichia coli-S. aureus shuttle plasmid, harboring the cat gene conferring chloramphenicol resistance, and a gfp-lux dual reporter system under the control of the agr P3 promoter; Cm' | 32            |

* a The following abbreviations were used: CA-MRSA, community associated MRSA; Cmr, chloramphenicol resistant; Kanr, kanamycin resistant; MLST, multi-locus sequence type; Oxa', methicillin/oxacillin resistant; Tc', tetracycline resistant.

CodY in *S. aureus* regulates the expression of virulence factors such as the *cap* operon (encoding proteins required for capsule biosynthesis), *coa* (encoding coagulase), *fshA* (encoding fibronectin-binding protein A), *hla, icaADBC* (encoding factors required for synthesis of polysaccharide intercellular adhesin), and *spa* (encoding protein A) (4, 12, 13). Although inactivation of *codY* did not markedly affect infectivity of *S. aureus* strain Newman in a murine abscess model, it restored the virulence of a mutant lacking the major (p)ppGpp synthase/hydrolase enzyme RSH to wild-type levels, suggesting that RSH-dependent derepression of CodY-regulated genes is important for virulence of *S. aureus* (14). More recently, it was found that inactivation of *codY* decreased the infectivity of the community-associated MRSA (CA-MRSA) USA300 isolate 923 in two murine infection models (15).

The pentose phosphate pathway regulator RpiRc alters the synthesis of several virulence factors such as protein A, capsular polysaccharide, and hemolysins, to decrease the transcription of RNAIII, the regulatory RNA of the *agr* locus, and a major regulator of virulence factor production in *S. aureus*, and to promote biofilm formation under *in vitro* conditions (5). These observations on RpiRc suggest that this regulator might also affect virulence in *vivo*; however, this has not been tested.

CcpE directly affects expression of the aconitate-encoding gene *citB*, increases TCA cycle activity during *in vitro* growth (6), and decreases pigment production in *S. aureus* (16). Because both TCA cycle activity (17–22) and pigment production (16, 23, 24) affect virulence determinant synthesis and/or infectivity of *S. aureus*, it is likely that CcpE modulates the expression of virulence factors and pathogenicity in this medically important pathogen. To test this hypothesis, we assessed the effect of *ccpE* deletion in *S. aureus* strain Newman (25) on the transcription of select virulence factors and on its role in infectivity using two unrelated murine infection models. Our data demonstrate that CcpE affects the transcription of virulence determinants, and infectivity of *S. aureus* in both *in vivo* infection models.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions**—The bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown in Luria-Bertani Lennox (LB-L) medium (BD Biosciences) at 37 °C and aerated at 230 rpm with a flask-volume medium ratio of 10:1. The ΔccpE mutants HOM 354 and HOM 355 were obtained by phage transducing the *lox66-aphAIII-lox71*—tagged *ccpE* deletion of THa (6) into strains 923 (26) and SA564 (27), respectively.

**Transcriptional Analyses**—For Northern blot experiments, overnight cultures of *S. aureus* were diluted to an *A*<sub>600</sub> of 0.05 into fresh pre-warmed LB-L and grown at 37 °C with 230 rpm of aeration. Samples were removed from the cultures at the indicated times and centrifuged at 9,000 × g and 4 °C for 2 min, the culture supernatants were discarded, and the cell pellets were snap frozen in liquid nitrogen. Total RNAs were isolated according to Ref. 28, and blotting, hybridization, and labeling were performed as described (29). Primer pairs hla-F/hla-R and RNAII-F/RNAII-R (Table 2) were used to generate digoxigenin-labeled hla- and RNAII-specific probes by PCR labeling, respectively.

For the quantification of transcripts by real-time reverse transcription PCR (qRT-PCR), RNA isolations and qRT-PCRs were carried out essentially as described (30). The cDNA (20 ng/reaction) was used for real-time amplification using the

**TABLE 2**

| Primer | Sequence (5′–3′) |
|--------|-----------------|
| *Northern probe primer* |                 |
| hla-F  | Forward AGAAAAATTCATATATACGTCATCA             |
| hla-R  | Reverse TGTAGCGAAGTCTGGTGAAAA                 |
| RNAII-F| Forward GTATGGAAATTATTGATAGCG                |
| RNAII-R| Reverse GTGATTTTTCATCCTTCCTT               |
| *Real-time RT PCR primer* |               |
| capA   | Forward GAAACCATACAAAGTATTTACATTAC          |
| capA   | Reverse TTTTTCTGGAATTGCTTTTGTAGGC          |
| capM   | Forward ACCGGTTTTATTTGTTCTATCA              |
| capM   | Reverse AAACCGGTATTACGCTTATCA               |
| Hla    | Forward GGAAGTTCTCTGTGCTTTTC                 |
| Hla    | Reverse CGAAATCTTCCTTGTGCTTTTAG             |
| RNAII  | Forward AGGACCTTTTAACGCTTACGACAA            |
| RNAII  | Reverse TGTGATTTTTCATCCTTCCTTCCTT          |
| Poma   | Forward ATCAAATGCTACTACAAATTTAATCAAC         |
| Poma   | Reverse GGGACATCTTTTTCCTTCCTTCCTT          |
| gryB   | Forward GATCCTGATGACGTGTTGA                 |
| gryB   | Reverse AACCTGTCACATTGCAATA                 |
| *EMSA primer* |                        |
| agr P2/3| Forward CCTCCCTCCAACTCTAGTTA               |
| agr P2/3| Reverse AGATCGGATATTTTACTA                 |
| capA p | Forward GTAAGCTCTCCAACTCTAGTTA             |
| capA p | Reverse CTCCCTCCAACTCTAGTTA               |
| hla p  | Forward TTAATAACGCTTTCCTTCCTTCCTT          |
| hla p  | Reverse GAGCACTGCTTTCCTTCCTTCCTT          |
| pona p | Forward GAAATTTCTTAACACTCT                     |
| pona p | Reverse GAAATTTCTTAACACTCT                     

For the quantification of transcripts by real-time reverse transcription PCR (qRT-PCR), RNA isolations and qRT-PCRs were carried out essentially as described (30). The cDNA (20 ng/reaction) was used for real-time amplification using the
primes listed in Table 2. mRNA levels were normalized against the mRNA level of gyrB, which is constitutively expressed under the conditions analyzed (31). The amounts of transcripts were expressed as the n-fold difference relative to the control gene (2^ΔΔCt, where ∆ΔCt represents the difference in threshold cycle between the target and control genes).

Electrophoretic Mobility Shift Assays—DNA probes for electrophoretic mobility shift assays (EMSAs) were generated by PCR using S. aureus strain Newman chromosomal DNA as a template, and primer pairs (Table 2) that amplified the DNA regions preceding the capA, icaA, icaD, purA, and psma ORFs. The 5′-ends of the double-stranded PCR products were labeled using [γ-32P]ATP and T4 polynucleotide kinase. A typical assay mixture contained (in 20 μl) 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 5 mM MgCl2, 0.1 μg of nonspecific competitor (poly(dI-dC)), 2.5% (v/v) glycerol, 0.05% (v/v) Igepal, radioactive DNA probe (2000 cpm ml⁻¹) and various amounts (0, 15, 65, 130, and 200 nM) of purified CcpE. After 20 min of incubation at room temperature, 20 μl of this mixture was loaded into a native 5% (w/v) polyacrylamide Tris borate-EDTA Ready Gel (Bio-Rad) and electrophoresed in 1% Tris borate-EDTA (v/v) buffer for 1 h at 100 V cm⁻¹. Radioactive species were detected by autoradiography using direct exposure to films. Radioactivity labeled promoter probes shifting with CcpE were additionally coincubated with increasing amounts of a nonspecific promoter probe and cold competitor, respectively, to demonstrate specificity of the shifting reaction.

Luciferase Assay—For assaying luciferase activities of S. aureus cells harboring plasmid pSB20235 (32), bacteria were cultured in LB supplemented with 10 μg ml⁻¹ chloramphenicol. Luciferase measurements were carried out essentially as described (33). 200-μl samples of the cell suspensions were removed at the time points indicated, and transferred to the wells of a 96-well clear-bottomed black plate (Greiner). The plate was placed in a Wallac Victor2 1420 Multilabel Counter (PerkinElmer Life Sciences), and luminescence readings were taken for 5 s at 37 °C.

Capsular Polysaccharide 5 (CP-5) Production—CP-5 production was determined by indirect immunofluorescence as described (3), using mouse immunoglobulin M monoclonal antibodies to CP-5 (34). Quantification of CP-5-positive cells was done by determining the numbers of 4′,6-diamidino-2-phenylindol (DAPI) and CY-3-positive cells using the software program CellC (35) (Institute of Signal Processing, Tampere University of Technology, Finland). Immune fluorescence intensities were analyzed using the MetaVue™ Research Imaging System (Molecular Devices). Briefly, from each image 80 bacteria detected by DAPI were randomly selected and the regions were transferred to the corresponding CY-3-stained image. Intensities of the single bacteria were measured and the distribution of intensities analyzed by the software program GraphPad Prism (GraphPad Software, Inc.).

Pigment Measurements—Bacteria were harvested after 24 h of growth on tryptic soy agar and carotenoids were extracted as described (36). The optical densities at 465 nm of the methanol extracts were measured and normalized in reference to the values obtained with the wild-type extracts, which were set at 100.

Animal Models—Eight-week-old female C57BL/6N mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and kept under specific pathogen-free conditions according to the regulations of German veterinary law. All animal studies were performed with the approval of the local State Review Boards.

The murine lung infection model was done essentially as described (37). Eight-week-old C57BL/6N mice were slightly anesthetized by intraperitoneal injection of 2.6 mg of ketamin hydrochloride (Pfizer, Berlin, Germany) and 0.18 mg of xylazin hydrochloride (Bayer, Leverkusen, Germany) per mouse and infected intranasally with 5 × 10⁶ colony forming units (cfu) of S. aureus. Twenty-four hours post-infection, the animals were euthanized, the tracheae were cannulated, and a bronchoalveolar lavage was performed (three times with 1 ml of phosphate-buffered saline). The bronchoalveolar lavage fluid (BALF) was centrifuged at 300 × g for 10 min at 4 °C to obtain alveolar cells, which were suspended in 1 ml of PBS. Total cell numbers in BALF were determined using a Neubauer hemocytometer. To identify the bacterial load of the lungs 24 h post-infection, whole lungs were homogenized in 1 ml of PBS, and serial dilutions were plated onto sheep blood agar. CFU were counted after incubation overnight at 37 °C.

The footpad swelling model was carried out as described (38). Age-matched mice were inoculated subcutaneously with 1 × 10⁷ cfu of S. aureus into the left hind footpad, and footpad swelling was measured daily with a micrometric caliper in reference to the uninfected footpad.

Cytokine Determinations—Levels of murine interleukin-1β (IL-1β), keratinocyte-derived chemokine (KC), and granulocyte-colony stimulating factor (G-CSF) in cell-free BALFs and lung homogenates were determined by commercially available sandwich-type ELISAs, according to the manufacturer’s instructions (R&D Systems, Wiesbaden-Nordenstadt, Germany).

Statistical Analyses—Statistical significance was assessed using the Mann-Whitney U test. p values <0.05 were considered significant.

RESULTS

Influence of a ccpE Deletion on RNAIII Transcription—Given the importance of the agr locus for virulence determinant production in S. aureus (reviewed in Refs. 39 and 40), we tested whether CcpE affects transcription of this regulatory system. Northern blot analysis revealed that all three strains, Newman, TH01, and TH01c, produced RNAIII transcripts in a growth phase-dependent manner, with a peak transcription rate at the transition from the exponential growth phase to post-exponential growth phase (i.e. 6 h) (Fig. 1). However, deletion of ccpE increased the post-exponential growth phase accumulation of RNAIII transcripts in TH01, suggesting that CcpE negatively affects RNAIII transcription. To test this suggestion, we transformed an RNAIII transcriptional reporter plasmid, pSB2035, (32) into strains Newman, TH01, and TH01c. This plasmid harbors a gfp-luxABCDE dual reporter system under control of the RNAIII transcription-driving agr P3 promoter. Similar to the Northern blot data (Fig. 1B), luciferase activity assays revealed a growth phase-dependent transcription of RNAIII (Fig. 1C). In addition, we observed that deletion of ccpE increased transcrip-
Attenuation of Pathogenesis by CcpE of *S. aureus*

**FIGURE 1.** Attenuation of Pathogenesis by CcpE of *S. aureus* and not due to decreased RNAIII transcription.

**A**

- Growth characteristics of *S. aureus* strains Newman (black symbols), TH01 (white symbols), and TH01c (gray symbols) cultured in LB-L at 37 °C and 230 rpm. Time points of sampling for downstream applications (reporter assays, qRT-PCRs) are indicated by arrows. B, Northern blot of RNAIII transcription in strains Newman, TH01 (ΔccpE), and the complemented TH01c during growth in LB-L. Approximate transcript sizes are indicated on the left. Ethidium bromide-stained 16 S rRNA are presented to indicate equivalent growth in LB-L. Approximate transcript sizes are indicated on the left.

**B**

- Northern blot of RNAIII transcription in *S. aureus* strains Newman, TH01 (ΔccpE), and TH01c throughout a complete growth cycle (Fig. 2B). Consistent with our Northern blot data, we observed a growth phase-dependent transcription of *hla*, with a peak in the post-exponential growth phase (9 h). Deletion of *ccpE* resulted in a massive up-regulation (30 to 60-fold) of *hla* transcription in strain TH01. Complementation of TH01 with a *ccpE* wild-type allele restored *hla* mRNA levels to those seen in the wild-type strain.

**C**

- Northern blot of hla transcription in strains Newman, TH01 (ΔccpE), and TH01c during growth in LB-L. Luciferase activities determined at the time points indicated. Data shown are the mean ± S.D. of six independent experiments. Mann-Whitney U test: *p* < 0.05; **p** < 0.01.

**D**

- Luciferase activity (RLU/Amp) in *S. aureus* strains Newman, TH01, and TH01c throughout a complete growth cycle (Fig. 2B). Consistent with our Northern blot data, we observed a growth phase-dependent transcription of *hla*, with a peak in the post-exponential growth phase (9 h). Deletion of *ccpE* resulted in a massive up-regulation (30 to 60-fold) of *hla* transcription in strain TH01. Complementation of TH01 with a *ccpE* wild-type allele restored *hla* mRNA levels to those seen in the wild-type strain.

To conclude that this effect of CcpE was specific for *S. aureus* strain Newman, we deleted *ccpE* in two genetically unrelated *S. aureus* strains, CA-MRSA USA300 isolate 923 (26) and the low passage human isolate SA564 (27), and assessed *hla* transcription of these strain pairs (Fig. 2C). Deletion of *ccpE* again strongly increased the transcription of *hla* in both strains, suggesting that the repressive effect of CcpE on *hla* transcription is independent of the genetic background. To assess whether CcpE directly regulates transcription of *hla*, we performed EMSAs with the *hla* promoter as probe (Fig. 2D). A clear and dose-dependent shift of CcpE with the radioactively labeled *hla* promoter probe was observed, which was not affected by the addition of a nonspecific promoter probe (data not shown) but was invariable by adding excessive amounts of cold competitor, suggesting that CcpE directly controls transcription of *hla*.

**CcpE Promotes Capsule Formation**—Capsular polysaccharide is another important virulence factor of *S. aureus*, whose synthesis is intimately linked to the nutritional status of the bacterium (3, 5, 12). Our results (Fig. 3) demonstrate that in addition to CcpA, CodY, and RpiRc, CcpE also modulates transcription of the *cap* operon and the elaboration of a capsule. As expected, when *S. aureus* was cultivated in LB-L, the first gene of the *cap* operon (*capA*) was predominantly transcribed during the later stages of growth (Fig. 3A). Deletion of *ccpE* in TH01 strongly decreased accumulation of *capA* mRNA throughout the growth cycle. *cis*-Complementation of TH01 with the wild-type *ccpE* allele restored *capA* mRNA levels to those found in the isogenic wild-type strain Newman. Consistent with the transcriptional data, a reduced number of capsular polysaccharide positive cells was observed with the Δ*ccpE* mutant (Fig. 3B).

CcpE might exert this effect via direct binding to the *agr* P3 promoter, EMSAs were performed with purified CcpE and a radioactively labeled PCR probe covering the *agr* P2/3 promoter (Fig. 1D). No mobility shifts were observed over a range of protein concentrations, suggesting that CcpE does not directly interact with the *agr* P3 promoter to modulate RNAIII transcription.

CcpE Directly Influences *hla* Transcription—α-Toxin is a major virulence factor of *S. aureus*, and its synthesis is regulated at multiple levels, including transcriptional and post-transcriptional mechanisms (3, 41–44). Regulation of *hla* transcription is also influenced by the carbon catabolite responsive elements CcpA and CodY (3, 4, 15, 45); hence, we hypothesized that *hla* transcription might be regulated by CcpE as well. Support for this hypothesis can be seen in the Northern blot analysis of *hla* transcription (Fig. 2A), where *hla* mRNA levels are much greater in the Δ*ccpE* mutant strain TH01 in all growth phases relative to the wild-type and *cis*-complemented derivative strain TH01c. To quantify the effect of *ccpE* deletion on *hla* transcription, we performed qRT-PCRs on strains Newman, TH01, and TH01c throughout a complete growth cycle (Fig. 2B). Consistent with our Northern blot data, we observed a growth phase-dependent transcription of *hla*, with a peak in the post-exponential growth phase (9 h). Deletion of *ccpE* resulted in a massive up-regulation (30 to 60-fold) of *hla* transcription in strain TH01. Complementation of TH01 with a *ccpE* wild-type allele restored *hla* mRNA levels to those seen in the wild-type strain. To exclude that this effect of CcpE was specific for *S. aureus* strain Newman, we deleted *ccpE* in two genetically unrelated *S. aureus* strains, CA-MRSA USA300 isolate 923 (26) and the low passage human isolate SA564 (27), and assessed *hla* transcription of these strain pairs (Fig. 2C). Deletion of *ccpE* again strongly increased the transcription of *hla* in both strains, suggesting that the repressive effect of CcpE on *hla* transcription is independent of the genetic background. To assess whether CcpE directly regulates transcription of *hla*, we performed EMSAs with the *hla* promoter as probe (Fig. 2D). A clear and dose-dependent shift of CcpE with the radioactively labeled *hla* promoter probe was observed, which was not affected by the addition of a nonspecific promoter probe (data not shown) but was invariable by adding excessive amounts of cold competitor, suggesting that CcpE directly controls transcription of *hla*.

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Although about 69% of the Newman cells and 75% of the TH01c cells incubated with the CP-5 antibodies produced clear fluorescence signals after 24 h of growth in LB-L, in the TH01 cell pool only 35% of the cells emitted detectable amounts of fluorescence. Similarly, a 10-fold decrease in the mean fluorescence intensity per cell was observed with the ccpE mutant (Fig. 3B) when compared with cells of the wild-type and complemented derivative TH01c. To determine whether the CcpE-dependent regulation of capA was due to an interaction with the capA promoter, EMSAs were performed with CcpE and a radioactively labeled probe of the capA promoter. In contrast to the hla promoter, CcpE did not shift the capA promoter probe at any of the CcpE concentrations tested (Fig. 3C), indicating that CcpE indirectly influences cap operon transcription and capsule formation.

CcpE Alters Transcription of the Phenol-soluble Modulin α (psmA) Cluster—Phenol-soluble modulins are a small group of cytolytic and immunomodulating peptides that are important virulence determinants of S. aureus, especially in CA-MRSA USA300 isolates (reviewed in Ref. 46). The S. aureus Newman genome harbors two psm operons, psmα and psmβ, which are transcriptionally affected by regulators such as SarA and AgrA (40, 47). To determine whether CcpE influences psm transcription,
tion, we assessed \( \text{psmA} \) and \( \text{psmb} \) transcription using qRT-PCR. Deletion of \( \text{ccpE} \) had a negligible effect on \( \text{psmb} \) transcription (data not shown); however, we observed a significant reduction of \( \text{psmA} \) transcripts in strain TH01 compared with the wild-type strain (Fig. 4A). Complementation of TH01 with a \( \text{ccpE} \) wild-type allele restored \( \text{psmA} \) mRNA levels to that seen in the wild-type strain. EMSAs performed using CcpE and the \( \text{psma} \) promoter as a probe failed to shift the radiolabeled probe with any of the protein concentrations tested (Fig. 4B), suggesting an indirect effect of CcpE on \( \text{psma} \) transcription.

**CcpE Decreases Pigment Production**—Most \( \text{S. aureus} \) strains produce the carotenoid pigment staphyloxanthin, which is responsible for the yellowish-orange appearance of this bacterium (48). In line with a previous publication (16), we noticed an increase in pigment production after 24 h of growth on tryptic soy agar, and this phenotype was reverted by introducing a functional \( \text{ccpE} \) into this mutant (Fig. 5). The synthesis of staphyloxanthin is encoded within the \( \text{crtOPQMN} \) operon (48); hence, to determine whether CcpE affects transcription of \( \text{crtOPQMN} \), we assessed \( \text{crtM} \) mRNA levels in strains Newman, TH01, and TH01c using qRT-PCR. Contrary to the findings reported by Lan and colleagues (16), our results suggest that transcription of \( \text{crtOPQMN} \) appears to be independent of CcpE (Fig. 5C). Similarly, inactivation of \( \text{ccpE} \) in strains 923 and SA564 significantly increased the pigment contents of mutant cells compared with wild-type, without affecting \( \text{crtM} \) transcription, suggesting that this phenomenon is not strain-dependent (Fig. 5).

**CcpE Attenuates Virulence in Two Murine Infection Models**—Deletion of \( \text{ccpE} \) in \( \text{S. aureus} \) strain Newman augmented transcription of the global virulence regulator \( \text{RNAIII} \) (Fig. 1) and increased \( \alpha \)-toxin (\( \text{hla} \)) mRNA (Fig. 2). Given the effect of CcpE on virulence factor transcription in vitro, we hypothesized that CcpE might alter infectivity of \( \text{S. aureus} \) in vivo. To address this hypothesis, we assessed the ability of Newman, TH01, and TH01c strains to cause disease in two different murine infection models. In a murine pneumonia model, C57BL/6N mice were infected intranasally with strains Newman, TH01, or TH01c, and the bacterial load in the lungs and the total amount of eukaryotic cells in BALFs at 24 h post-infection were determined (Fig. 6). Strain TH01 significantly increased the bacterial load in the lungs of mice relative to the wild-type and complemented strains (Fig. 6A). Similarly, we observed a significant increase in total cells in BALFs of the TH01 challenged mice (Fig. 6B), indicating a more severe infection. This increase in total cell numbers correlated with an increased number of neutrophils in BALFs of TH01 challenged mice (Fig. 6C), and this also correlated with increased concentrations of the neutrophil mobilization stimulating factor G-CSF (49) (Fig. 6D). Complementation of the \( \Delta \text{ccpE} \) mutant restored all virulence traits back to wild-type levels, confirming that all observed alterations were caused by CcpE. To exclude that this CcpE effect is specific for strain Newman, we additionally infected mice intranasally with strain SA564 and its \( \Delta \text{ccpE} \) derivative HOM 355, respectively. In line with our observations made with the strain triplet Newman/TH01/TH01c, we observed significantly increased cfu numbers in the lung tissues of mice that have been...
infected with the SA564 ccpE mutant (Fig. 7), demonstrating that this virulence diminishing effect of CcpE is not specific for strain Newman.

To substantiate these findings in another in vivo model, we utilized a murine footpad infection model (38). In this model, bacteria are inoculated into the left hind footpad of mice and footpad swelling ratios are determined on a daily basis for up to 12 days (Fig. 8). Consistent with our observations using a lung infection model, we observed enhanced footpad swelling in mice challenged with the Newman/H9004 ccpE mutant relative to the isogenic wild-type and complemented strains (Fig. 8). Swelling was most significantly increased early in the infection process (days 1 to 4) and in the later stages of the infection (days 8 to 12) when compared with the values obtained with the wild-type and TH01c challenged mice groups.

DISCUSSION

The nosocomial pathogen S. aureus is known to link its virulence factor production with central metabolic pathways (3–5, 8, 9, 13, 22). This linkage is mediated via at least three metabolite responsive regulators; namely, CcpA (3, 8), CodY (13), and RpiRc (5). Data presented here demonstrate that CcpE represents a fourth regulatory protein that connects virulence factor synthesis with the central metabolism, specifically the TCA cycle (Fig. 9) (6).

Although most effects of CcpE on virulence factor synthesis were indirect, possibly via regulation of TCA cycle activity (22, 27, 50), a direct link between CcpE and hla transcription was established. In a murine pneumonia model, α-toxin is a key virulence determinant involved in the pathogenesis of S. aureus (51, 52); specifically, the level of α-toxin correlated with disease severity in this animal model (53). Mechanistically, α-toxin increases cytokine synthesis, enhances neutrophil recruitment, and stimulates the NLRP3 (NOD-like receptor family, pyrin domain containing 3) inflammasome in lungs, leading to massive inflammatory response and tissue destruction (54, 55). Consistent with these observations, deletion of ccpE increased
In addition to directly interacting with hla, ccpE deletion increased RNAIII levels, which likely contributed to the altered pathogenesis of the ΔccpE mutant in both animal models. RNAIII is the RNA regulator of the agr locus encoded quorum sensing system (reviewed in Refs. 39 and 40) and it codes for a small lytic peptide called δ-toxin, which is a chemoattractant for neutrophils (50). RNAIII is predominantly transcribed when a threshold level of bacteria is achieved (56, 57). In its regulatory function, RNAIII promotes the expression of many exoproteins including α-toxin, either directly or via control of a repressor protein known as Rot (43, 58). Mutations in agr have been shown to attenuate virulence in several animal models (59–63) including murine models of pneumonia (52, 64) and skin infections (65–67). When the peptide δ-toxin is translated from RNAIII, it is produced in two forms; one without an N-terminal formyl group on the methionine, and one containing a formylated methionine (50). Formylated δ-toxin is a potent neutrophil chemoattractant, suggesting that increased neutrophils in the lungs on TH01-infected mice may be due to an increase in δ-toxin synthesis.

Alterations in the synthesis of virulence factors and RNAIII will likely alter the immune response to the infection. The BALF cytokine profiles of mice infected with strains Newman, TH01, and TH01c were similar with respect to keratinocyte...
derived chemokine and IL-1β, however, G-CSF was higher in BALFs and lung homogenates from TH01 challenged mice relative to mice infected with the wild-type strain. G-CSF was originally characterized in hematopoietic cells to stimulate the proliferation and differentiation of neutrophil granulocyte precursors. In addition, G-CSF functions to recruit polymorphonuclear leukocytes to the lung (68), and its expression in lung tissue is stimulated by microbial infections (69–72). Recently, Hua et al. (73) observed in a mouse pneumonia model that preimmunization with an anti-α-toxin antibody significantly decreased the G-CSF contents in BALFs of mice infected with S. aureus. Based on this observation, it is reasonable to speculate that an increase in α-toxin synthesis (Fig. 2) would increase G-CSF production (Fig. 6D), resulting in an increase in neutrophil recruitment (Fig. 6C).

Transcription of RNAIII is primarily promoted by AgrA, the response regulator of the two-component system encoded by the agr locus (74). In addition, AgrA also promotes transcription of the psm operons (40). Because we observed divergent effects of CcpE on RNAIII and psma transcription (Figs. 1 and 4), we can largely exclude that CcpE modulates RNAIII production via activation of AgrA. Similarly, the agr system promotes capsule synthesis (75–77); however, capA mRNA levels were decreased in the ΔccpE mutant despite an increase in RNAIII transcript levels (Figs. 1 and 3). Interestingly, Somerville and colleagues (20, 27) observed increased RNAIII levels and an impaired capsule biosynthesis in TCA cycle mutants in which inactivation on capsule synthesis is tied to a lack of oxaloacetate for gluconeogenesis (20), and it is still unclear how TCA cycle mutants despite an increase in RNAIII levels and an impaired capsule biosynthesis in TCA cycle mutants in which the aconitase-encoding gene citB (syn. acnA) was inactivated, demonstrating a link between TCA cycle activity, capsule formation, and RNAIII production. It is possible that CcpE modulates RNAIII transcription and capsule biosynthesis via regulation of TCA cycle activity. However, the effect of TCA cycle inactivation on capsule synthesis is tied to a lack of oxaloacetate for gluconeogenesis (20), and it is still unclear how TCA cycle activity affects transcription of RNAIII. A potential factor might be aconitase itself. This key enzyme of the TCA cycle is reported in Bacillus subtilis to act as a bifunctional protein that possesses enzymatic activity and functions as a RNA-binding regulatory protein (78–80). Similar to B. subtilis, apo-aconitase binds to ion-responsive elements in mRNA, raising the possibility of a direct interaction between aconitase and the highly structured RNAIII. Additionally, CcpE might affect RNAIII synthesis and capsule formation via pH alterations. We have recently shown that in vitro cultivation of the ccpE deletion mutant in LB-L led to a significantly reduced alkalinization of the culture medium during later stages of growth (6–12 h) compared with the wild-type culture (6). Alkaline growth conditions were previously reported to repress RNAIII production (81), and to augment capsule formation (82, 83), consistent with our findings of increased RNAIII transcription and decreased capA transcription in TH01 during the later growth stages in LB-L (Figs. 1 and 3).

In conclusion, CcpE modulates the expression of several major virulence factors of S. aureus, which affects its pathogenesis. Given its mostly repressive effect on virulence determinant production, it can be assumed that CcpE serves as an attenuator of virulence in this clinically important pathogen.

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