2014

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Published in *The Journal of Biological Chemistry* 289 (2014), pp. 29701-29711; doi: 10.1074/jbc.M114.584979

Submitted June 2, 2014. Revised August 13, 2014. Published online September 5, 2014

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

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Abstract

Carbon metabolism and virulence determinant production are often linked in pathogenic bacteria, and several regulatory elements have been reported to mediate this linkage in *Staphylococcus 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 *psm*α, 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 Δ*ccpE* derivative revealed increased disease severity in the Δ*ccpE* 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*.

Keywords: Bacterial Pathogenesis, *Staphylococcus aureus* (*S. aureus*), Transcription Regulation, Tricarboxylic Acid Cycle (TCA Cycle) (Krebs Cycle), Virulence Factor

Introduction

Carbon catabolite repression is a common mechanism utilized by bacteria to optimize their transcriptomes in response 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 *Staphylococcus 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 tricaboxylic 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 intermediary 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).

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristic(s)[a]	Ref. or source
S. aureus		
923	CA-MRSA, clinical isolate of pulsotype USA300, Oxa^r	26
HOM 354	923 Δ*ccpE*::*lox66-aphAIII-lox71*, Oxa^r, Kan^r	This study
HOM 355	SA564 Δ*ccpE*::*lox66-aphAIII-lox71*, Kan^r	
Newman	Laboratory strain (ATCC 25904); CP-5 producer	25
SA564	Low passage human isolate	27
THa	RN4220 Δ*ccpE*::*lox66-aphAIII-lox71*, Kan^r	6
TH01	Newman Δ*ccpE*::*lox72*	6
TH01c	TH01 harboring plasmid pTH2c *cis*-integrated at the NWMN_0640 locus, leading to a duplication of the NWMN_0640 gene, *ccpE*$^+$, Tc^r	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^r	32

a. The following abbreviations were used: CA-MRSA, community associated MRSA; Cm^r, chloramphenicol resistant; Kan^r, kanamycin resistant; MLST, multi-locus sequence type; Oxa^r, methicillin/oxacillin resistant; Tc^r, 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), *fnbA* (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 transcription of the aconitase-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-to-medium volume ratio of 10:1. The Δ*ccpE* 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.

Table 2. Primers used in this study

Primer		Sequence (5′–3′)
Northern probe primer		
hla-F	Forward	AGAAAATGGCATGCACAAAAA
hla-R	Reverse	TGTAGCGAAGTCTGGTGAAAA
RNAIII-F	Forward	GTGATGGAAAATAGTTGATGAG
RNAIII-R	Reverse	GTGAATTTGTTCACTGTGTCG
Real-time RT PCR primer		
capA	Forward	GAAAATACGCAACTTATCAACATCCA
capA	Reverse	TTTTTTCCGAATCTTGTTTATGACC
crtM	Forward	ACGCTTTTGACTTGTTACCAGAAGA
crtM	Reverse	AAATTGCCCAAACCGCTTT
Hla	Forward	GAACCCGGTATATGGCAATCAA
Hla	Reverse	GGAAGTTCTCTGCTGCTTTCATAG
RNAIII	Forward	AGGAGTGATTTCAATGGCACAAG
RNAIII	Reverse	TGTGTCGATAATCCATTTTACTAAGTCA
Psma	Forward	ATCAACAACTCATCACTATGTTAAATCAAC
Psma	Reverse	GCCATCGTTTTGTCCTCCTGT
gyrB	Forward	GACTGATGCCGATGTGGA
gyrB	Reverse	AACGGTGGCTGTGCAATA
EMSA primer		
agr P2/3	Forward	CCATCACATCTCTGTGATCTAG
agr P2/3	Reverse	CTCTCCTCACTGTCATTATACG
capA p	Forward	ATCATATGATTATAAGCAATAA
capA p	Reverse	GTAATACTTCTTTAATTTTTG
hla p	Forward	TAATTAATACCCTTTTTCTCTATTTC
hla p	Reverse	GTTACTGAGCTGACTATACGTGTTTTCAT
psma p	Forward	CAAATTCATGAGCTTAACCTC
psma p	Reverse	GAATTGTTCGATTAAGCTTTTG

Transcriptional Analyses — For Northern blot experiments, overnight cultures of *S. aureus* were diluted to an A_{600} 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 RNAIII-F/RNAIII-R (Table 2) were used to generate digoxigenin-labeled *hla*- and *RNAIII*-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 primers 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 ($2^{-\Delta CT}$, where ΔC_T 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*, *hla*, *hld*, *purA*, and *psm*α ORFs. The 5′-ends of the double-stranded PCR products were labeled using [γ-^{32}P]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 $MgCl_2$, 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 cultivated in LB-L 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-phenylindole (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 5×10^7 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 1×10^7 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) (Figure 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 (Figure 1*B*), luciferase activity assays revealed a growth phase-dependent transcription of *RNAIII* (Figure 1*C*). In addition, we observed that deletion

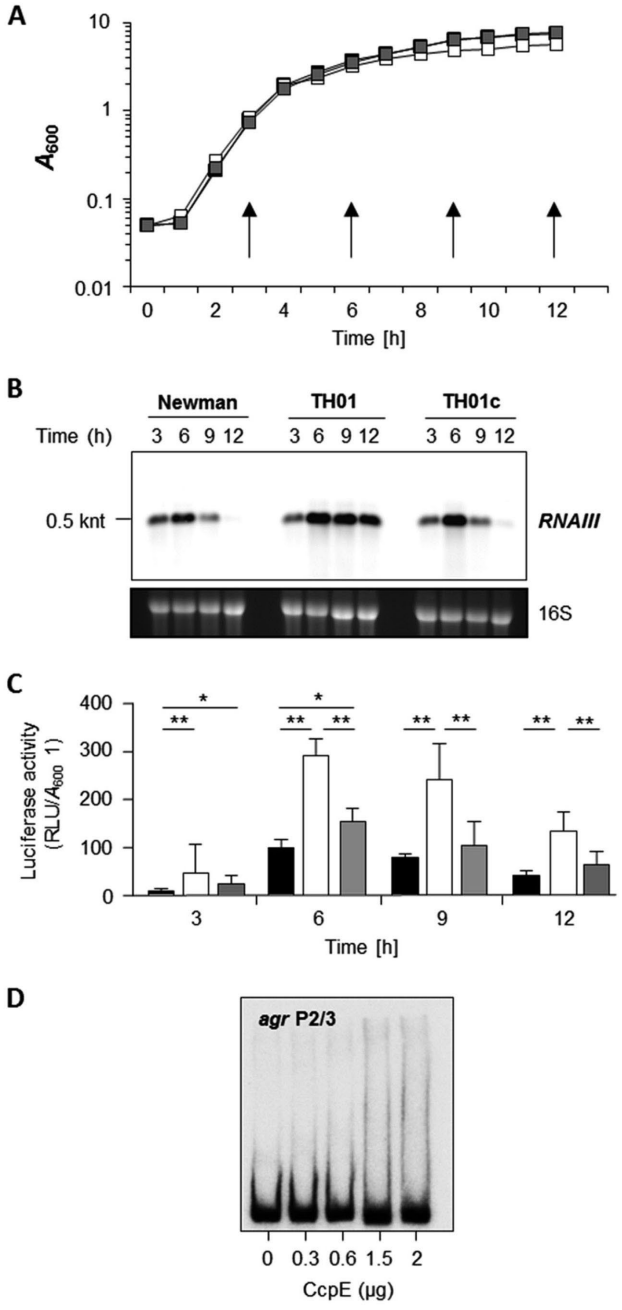

Figure 1. Effect of the *ccpE* deletion on *RNAIII* transcription in *S. aureus* Newman. *A*, growth characteristics of *S. aureus* strains Newman (*black symbols*), TH01 (*white symbols*), and TH01c (*gray symbols*) cultured in LB-L at 37 °C and 230 rpm. Time points of sampling for downstream applications (reporter assays, qRT-PCRs) are indicated by *arrows*. *B*, Northern blot of *RNAIII* transcription in strains Newman, TH01 (Δ*ccpE*), 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 RNA loading. *C*, *agr* P3 promoter-driven luciferase activities of plasmid pSB2035 harboring derivatives of strains Newman (*black bars*), TH01 (*white bars*), and TH01c (*gray bars*) during growth in LB-L. Luciferase activities were 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*, binding activity of CcpE to the *agr* P2/3 promoter region. The PCR-amplified DNA fragments were radioactively labeled and incubated with the amount of purified CcpE indicated. The results are representative of at least two independent experiments. *RLU*, relative light units.

of *ccpE* increased transcription of *RNAIII*, confirming that the increased amount of *RNAIII* in the TH01 mutant is due to increased *RNAIII* transcription and not due to decreased *RNAIII* degradation. To test whether 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 (Figure 1*D*). 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 (Figure 2*A*), 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 (Figure 2*B*). 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 (Figure 2*C*). 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 (Figure 2*D*). 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 invertable 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 (Figure 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 (Figure 3*A*). 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 poitive cells

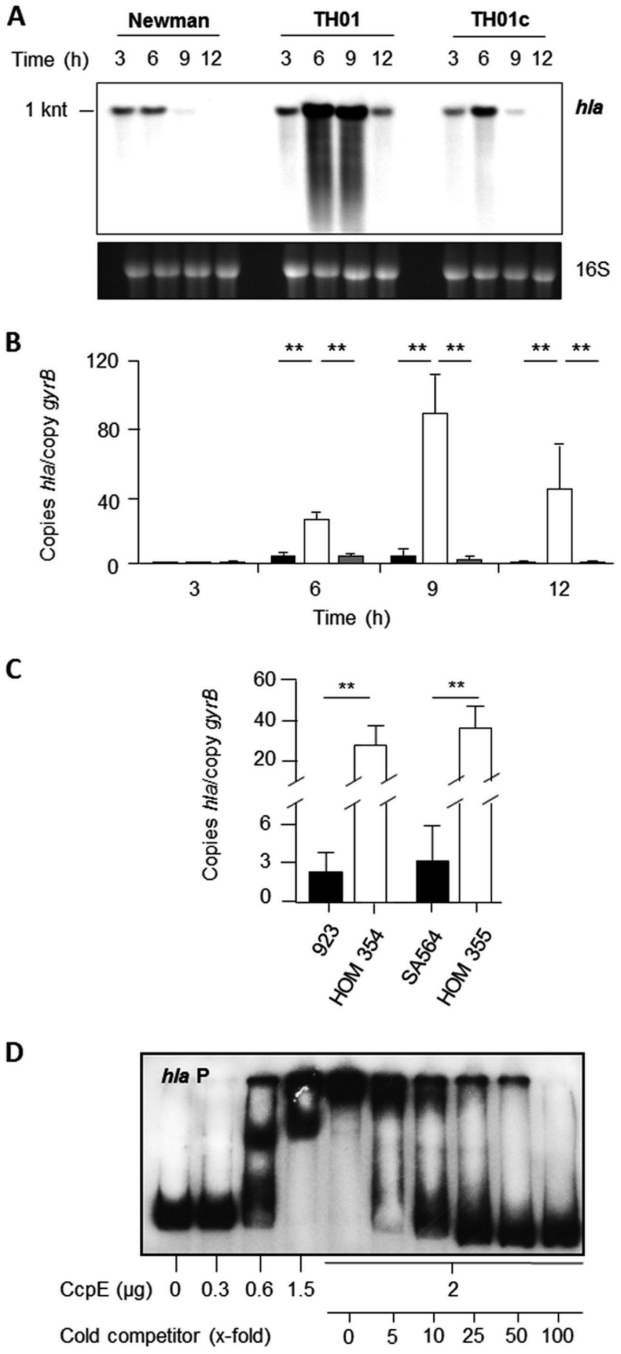

Figure 2. Effect of the *ccpE* deletion on *hla* transcription in *S. aureus*. *A,* Northern blot of *hla* transcription in strains Newman, TH01 (Δ*ccpE*), and the complemented TH01c during growth in LB-L. Approximate transcript sizes are indicated on the *left*. Ethidium bromide-stained 16 S rRNA are presented to indicate equivalent RNA loading. *B*, quantitative transcript analysis of *hla* by qRT-PCR of strains Newman (*black bars*), TH01 (*white bars*), and TH01c (*gray bars*) during growth in LB-L. *C,* quantitative transcript analysis of *hla* by qRT-PCR of strains 923, HOM 354 (923 Δ*ccpE*), SA564, and HOM 355 (SA564 Δ*ccpE*) after 9 h of growth in LB-L. mRNA levels are expressed relative to gyrase B (in numbers of copies per copy of *gyrB*). The data presented in *B* and *C* are the mean ± S.D. of three independent experiments each determined in duplicate. Mann-Whitney *U* test: *, $p < 0.05$; **, $p < 0.01$. *D*, binding activity of CcpE to the *hla* promoter of strain Newman. The PCR-amplified DNA fragments (100 ng/lane) were radioactively labeled and incubated with the amount of purified CcpE in the absence and presence of cold competitor as indicated. The results are representative of at least two independent experiments.

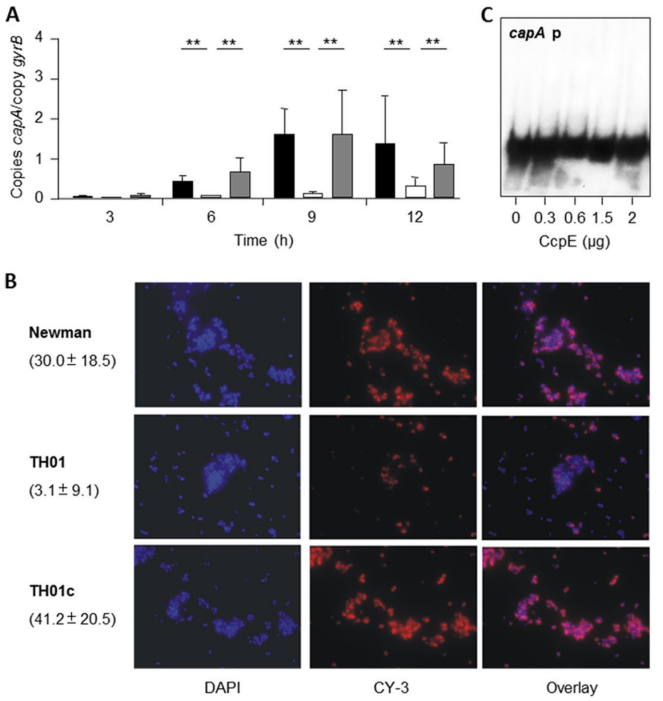

Figure 3. Effect of the *ccpE* deletion on capsule formation in *S. aureus* Newman. *A*, quantitative transcript analysis of *capA* by qRT-PCR of strains Newman (*black bars*), TH01 (Δ*ccpE*, *white bars*), and TH01c (complemented derivative, *gray bars*) during growth in LB-L. mRNA levels are expressed relative to gyrase B (in numbers of copies per copy of *gyrB*). The data presented are the mean ± S.D. of three independent experiments each determined in duplicate. Mann-Whitney *U* test: *, $p < 0.05$; **, $p < 0.01$. *B*, CP-5 expression of strains Newman, TH01, and TH01c during growth in LB-L. Bacteria were grown to an A_{600} of 0.5, stained with DAPI, marked with CP-5-specific monoclonal antibodies, and stained with Cy-3-conjugated anti-mouse antibodies (*CY-3*). *Numbers in parentheses* indicate the mean fluorescence intensities ± S.D. per cell ($n = 80$). *C,* binding activity of CcpE to the *cap* promoter. The PCR-amplified DNA fragments were radioactively labeled and incubated with the amount of purified CcpE indicated. The results in *B* and *C* are representative of at least two independent experiments.

was observed with the Δ*ccpE* mutant (Figure 3*B*). Although about 69 ± 6% of the Newman cells and 75 ± 8% 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 ± 7% of the cells emitted detectable amounts of fluorescence. Similarly, a ~10-fold decrease in the mean fluorescence intensity per cell was observed with the Δ*ccpE* mutant (Figure 3*B*) 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 (Figure 3*C*), indicating that CcpE indirectly influences *cap* operon transcription and capsule formation.

CcpE Alters Transcription of the Phenol-soluble Modulin a (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, *psm*α and *psm*β, which are transcriptionally affected by regulators such as SarA and AgrA (40, 47). To determine whether CcpE

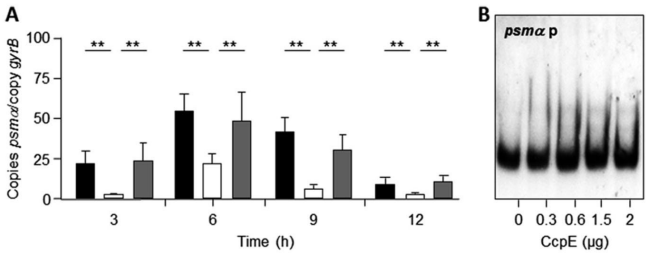

Figure 4. Effect of the *ccpE* deletion on *psm*α transcription in *S. aureus* Newman. *A*, quantitative transcript analysis of *psm*α by qRT-PCR of strains Newman (*black bars*), TH01 (*white bars*), and TH01c (*gray bars*) during growth in LB-L. mRNA levels are expressed relative to gyrase B (in numbers of copies per copy of *gyrB*). The data presented are mean ± S.D. of three independent experiments each determined in duplicate. Mann-Whitney *U* test: *, $p < 0.05$; **, $p < 0.01$. *B*, binding activity of CcpE to the *psm*α promoter. The PCR-amplified DNA fragments were radioactively labeled and incubated with the amount of purified CcpE indicated. The results are representative of at least two independent experiments.

influences *psm* transcription, we assessed *psm*α and *psm*β transcription using qRT-PCR. Deletion of *ccpE* had a negligible effect on *psm*β transcription (data not shown); however, we observed a significant reduction of *psm*α transcripts in strain TH01 compared with the wild-type strain (Figure 4*A*). Complementation of TH01 with a *ccpE* wild-type allele restored *psm*α mRNA levels to that seen in the wild-type strain. EMSAs performed using CcpE and the *psm*α promoter as a probe failed to shift the radiolabeled probe with any of the protein concentrations tested (Figure 4*B*), suggesting an indirect effect of CcpE on *psm*α transcription.

CcpE Decreases Pigment Production – Most *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 *ccpE* into this mutant (Figure 5). The synthesis of staphyloxanthin is encoded within the *crtOPQMN* operon (48); hence, to determine whether CcpE affects transcription of *crtOPQMN*, we assessed *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 *crtOPQMN* appears to be independent of CcpE (Figure 5*C*). Similarly, inactivation of *ccpE* in strains 923 and SA564 significantly increased the pigment contents of mutant cells compared with wild-type, without affecting *crtM* transcription, suggesting that this phenomenon is not strain-dependent (Figure 5).

CcpE Attenuates Virulence in Two Murine Infection Models – Deletion of *ccpE* in *S. aureus* strain Newman augmented transcription of the global virulence regulator *RNAIII* (Figure 1) and increased α-toxin (*hla*) mRNA (Figure 2). Given the effect of CcpE on virulence factor transcription *in vitro*, we hypothesized that CcpE might alter infectivity of *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 (Figure 6). Strain TH01 significantly increased the bacterial load

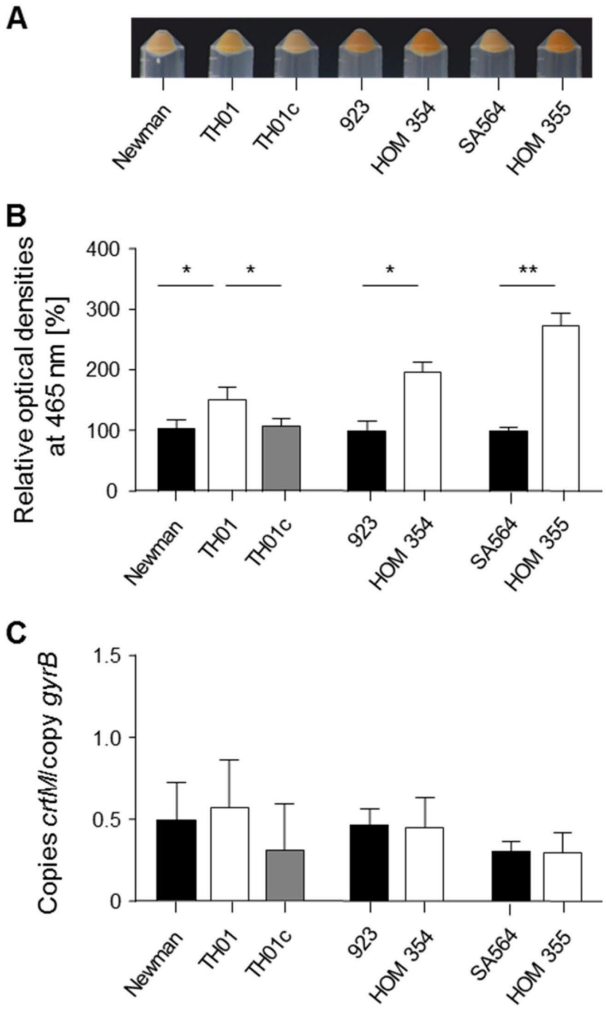

Figure 5. Effect of the *ccpE* deletion on pigment production in *S. aureus*. *A*, pigmentation displays of *S. aureus* strains grown for 24 h at 37 °C on tryptic soy agar plates. *B*, measurement of carotenoid pigment contents in *S. aureus* cells grown for 24 h at 37 °C on tryptic soy agar. The relative optical density units at 465 nm were normalized to those of wild-type, which were set at 100. The data presented are mean ± S.D. of five independent experiments. Mann-Whitney *U* test: *, $p < 0.05$; **, $p < 0.01$. *C*, quantitative transcript analysis of *crtM* by qRT-PCR of *S. aureus* cells grown for 24 h at 37 °C on tryptic soy agar. mRNA levels are expressed relative to gyrase B (in numbers of copies per copy of *gyrB*). The data presented are mean ± S.D. of three independent experiments each determined in duplicate.

in the lungs of mice relative to the wild-type and complemented strains (Figure 6*A*). Similarly, we observed a significant increase in total cells in BALFs of the TH01 challenged mice (Figure 6*B*), indicating a more severe infection. This increase in total cell numbers correlated with an increased number of neutrophils in BALFs of TH01 challenged mice (Figure 6*C*), and this also correlated with increased concentrations of the neutrophil mobilization stimulating factor G-CSF (49) (Figure 6*D*). Complementation of the Δ*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 Δ*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

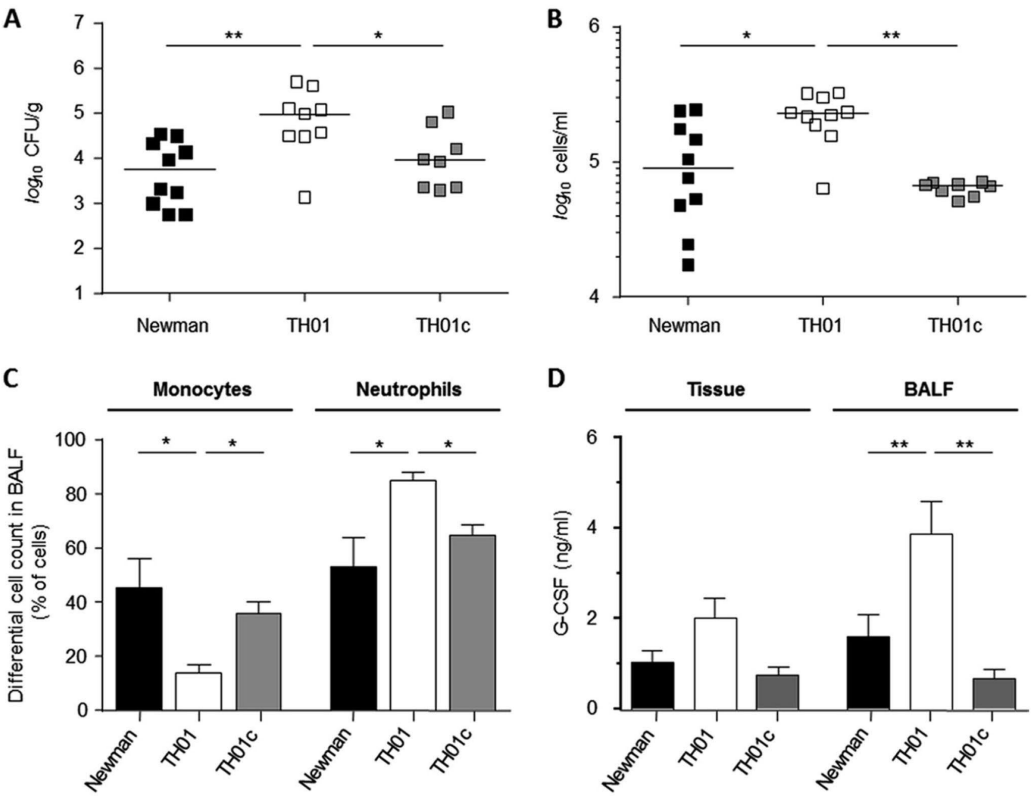

Figure 6. Effect of the *ccpE* deletion on infectivity of *S. aureus* Newman in an acute murine lung infection model. C57BL/6N mice were infected intranasally with 5×10^7 cells of *S. aureus* strain Newman (*black symbols*), TH01 (Δ*ccpE*, *white symbols*), and TH01c (complemented mutant, *gray symbols*), respectively (n = 8–10 per group). Mice were euthanized 24 h post-infection, BALFs were collected, and lungs were homogenized in PBS to determine the bacterial loads and cytokine concentrations in this tissue. *A,* bacterial loads in the lungs of infected mice. *B,* total eukaryotic cell contents in BALFs. Each *symbol* represents an individual mouse. *Horizontal bars* indicate the median of all observations. *C*, ratios of monocytes and neutrophils in BALFs of infected mice. *D,* G-CSF concentrations in lungs and cell-free BALFs of infected mice. Data are presented as mean ± S.E. (n = 8–10). Mann-Whitney *U* test: *, $p < 0.05$; **, $p < 0.01$.

with the SA564 *ccpE* mutant (Figure 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 (Figure 8). Consistent with our observations using a lung infection model, we observed enhanced footpad swelling in mice challenged with the Newman Δ*ccpE* mutant relative to the isogenic wild-type and complemented strains (Figure 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.

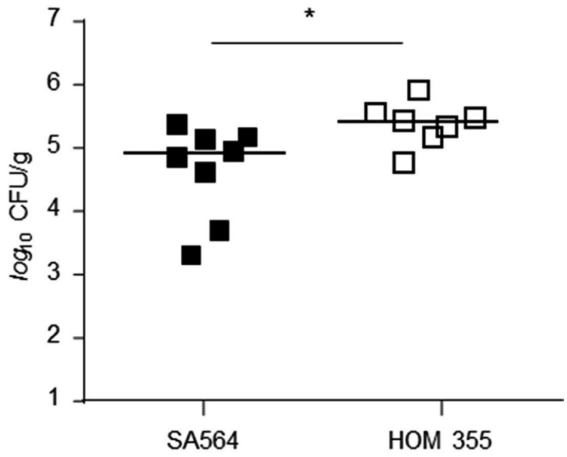

Figure 7. Effect of the *ccpE* deletion on infectivity of *S. aureus* SA564 in an acute murine lung infection model. C57BL/6N mice were infected intranasally with 5×10^7 cells of *S. aureus* strain SA564 (*black symbols*) and HOM 355 (Δ*ccpE*, *white symbols*), respectively (n = 7–8 per group). Mice were euthanized 24 h post-infection, and lungs were homogenized in PBS to determine the bacterial loads in this tissue. Each *symbol* represents an individual mouse. *Horizontal bars* indicate the median of all observations. Mann-Whitney *U* test: *, $p < 0.05$.

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 (Figure 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 *hla* transcription (Figure 2) and increased the bacterial loads and neutrophil

contents in the lungs of mice (Figs. 6 and 7), suggesting that CcpE might influence the virulence of *S. aureus* during lung infections via transcriptional regulation of *hla*.

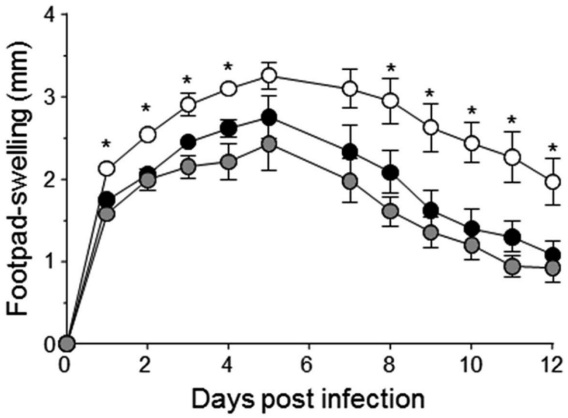

Figure 8. Effect of the *ccpE* deletion on infectivity of *S. aureus* Newman in a systemic murine footpad infection model. 1×10^7 cells of *S. aureus* strain Newman (*black symbols*) and its derivatives TH01 (Δ*ccpE*, *white symbols*), and TH01c (complemented mutant, *gray symbols*) were injected subcutaneously into the footpads of C57BL/6N mice, and swelling of the footpads were measured in reference to the uninfected footpads at the time points indicated. Data shown represent the mean ± S.D. of 8 mice per group. Mann-Whitney *U* test: *, $p < 0.05$.

In addition to directly interacting with *hla*, *ccpE* deletion increased *RNAIII* levels, which likely contributed to the altered pathogenesis of the Δ*ccpE* 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

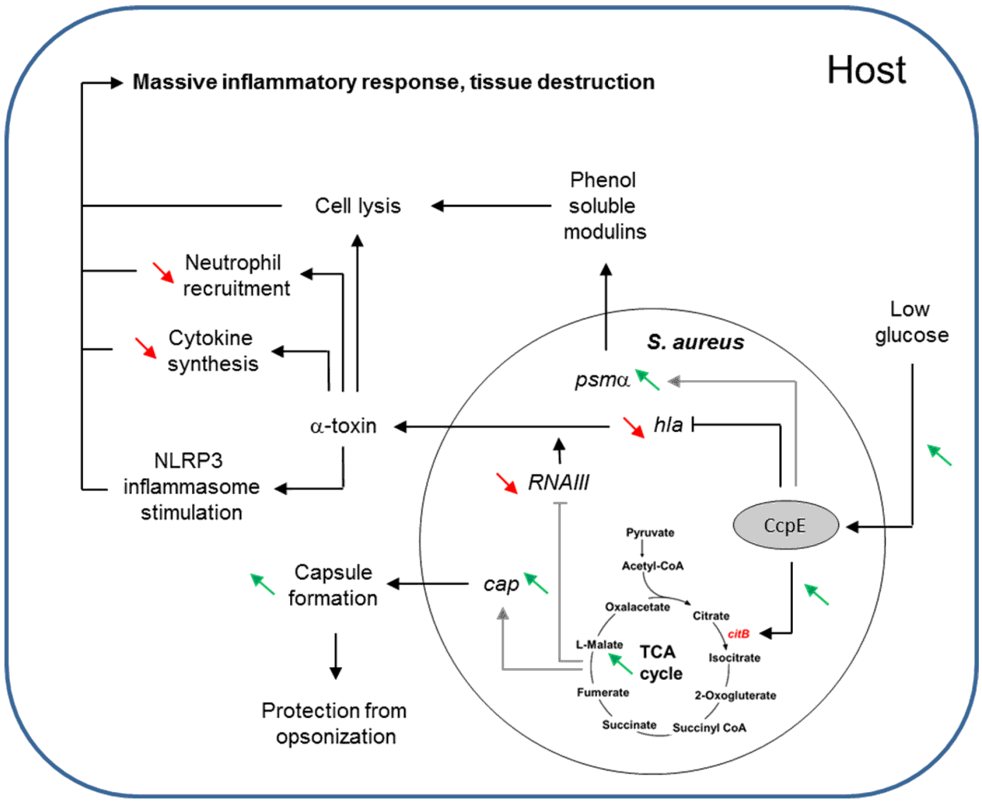

Figure 9. Proposed regulatory role of CcpE on virulence factor production and pathogenicity of *S. aureus*. Under glucose-rich conditions, TCA cycle activity is repressed in *S. aureus* via CcpA. When glucose concentrations become growth limiting, the transcription of TCA cycle genes is de-repressed in a CcpE-dependent manner, which directly promotes TCA cycle activity via direct transcriptional control of the aconitase encoding gene *citB*. An active TCA cycle augments capsule formation via an increased transcription of the *cap* operon. Additionally, it decreases pigment production and transcription of *RNAIII*, the master virulence regulator of the *agr* locus. CcpE also directly interferes with *hla* transcription, leading to reduced α-toxin synthesis, thereby decreasing the synthesis of cytokines, reducing the attraction of neutrophils, and impairing the pathogen-driven stimulation of the NLRP3 inflammasome in lungs. CcpE also enhances transcription of the *psmα* cluster by a yet unidentified mechanism, thereby increasing the lysis of white blood cells and stimulating an inflammatory response. Experimentally proven positive effects of CcpE are depicted by *green diagonal arrows* and negative effects by *red diagonal arrows*. Direct regulation of CcpE is displayed by *black connecting lines*, and indirect regulation by *gray lines*. *Arrows* indicate a stimulatory effect, and *perpendicular lines* a repressive effect.

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 (Figure 2) would increase G-CSF production (Figure 6*D*), resulting in an increase in neutrophil recruitment (Figure 6*C*).

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 *psm*α 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 Δ*ccpE* 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 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 an RNA-binding regulatory protein (78–80). Similar to *B. subtilis*, apoaconitase binds to iron-responsive elements in mRNA (G. A. Somerville, unpublished data.), 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.

Acknowledgments - We thank A. Honecker for excellent technical assistance. This work was supported by National Institutes of Health Grant AI087668 (to Greg A. Somerville) and Grants BI 1350/1-1 and BI 1350/1-2 from the Deutsche Forschungsgemeinschaft (DFG). Grégory Baronian and Virginie Molle were supported by a region Languedoc-Roussillon-Chercheur d'Avenir grant. Bettina Schulthess was supported by Swiss National Science Foundation Grant 31-117707. Christiane Wolz was supported by Deutsche Forschungsgemeinschaft Grant SFB766 project A7.

Abbreviations: CcpE, catabolite control protein E; TCA, tricaboxylic 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

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