CcpA coordinates central metabolism and biofilm formation in *Staphylococcus epidermidis*

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Staphylococcus epidermidis is an opportunistic bacterium whose infections often involve the formation of a biofilm on implanted biomaterials. In *S. epidermidis*, the exopolysaccharide facilitating bacterial adherence in a biofilm is polysaccharide intercellular adhesin (PIA), whose synthesis requires the enzymes encoded within the intercellular adhesin operon (*icaADBC*). *In vitro*, the formation of *S. epidermidis* biofilms is enhanced by conditions that repress tricarboxylic acid (TCA) cycle activity, such as growth in a medium containing glucose. In many Gram-positive bacteria, repression of TCA cycle genes in response to glucose is accomplished by catabolite control protein A (CcpA). CcpA is a member of the GalR–LacI repressor family that mediates carbon catabolite repression, leading us to hypothesize that catabolite control of *S. epidermidis* biofilm formation is indirectly regulated by CcpA-dependent repression of the TCA cycle. To test this hypothesis, *ccpA* deletion mutants were constructed in strain 1457 and 1457-*acnA* and the effects on TCA cycle activity, biofilm formation and virulence were assessed. As anticipated, deletion of *ccpA* derepressed TCA cycle activity and inhibited biofilm formation; however, *ccpA* deletion had only a modest effect on *icaADBC* transcription. Surprisingly, deletion of *ccpA* in strain 1457-*acnA*, a strain whose TCA cycle is inactive and where *icaADBC* transcription is derepressed, strongly inhibited *icaADBC* transcription. These observations demonstrate that CcpA is a positive effector of biofilm formation and *icaADBC* transcription and a repressor of TCA cycle activity.

Received 12 May 2011

Revised 13 September 2011

Accepted 27 September 2011

INTRODUCTION

Staphylococcus epidermidis is an opportunistic pathogen that primarily infects immunocompromised individuals or those with implanted biomaterials (e.g. catheters). As life expectancies and the availability of new biomaterials have increased, the number of people living with implanted biomaterials has also increased. One consequence of implanting biomaterials is that it predisposes individuals to device-related infections (Barrau *et al.*, 2004; El-Ahdab *et al.*, 2005; Piper *et al.*, 2001). These infections are often difficult to treat due to the

formation of a bacterial biofilm in which the bacteria are encapsulated in an exopolysaccharide matrix (Donlan, 2001; von Eiff *et al.*, 2002). As with most virulence factors and mechanisms, the formation of biofilms and exopolysaccharide synthesis are strongly influenced by the availability of nutrients and environmental conditions (Deighton & Borland, 1993; Dobinsky *et al.*, 2003; Vuong *et al.*, 2005). In particular, *S. epidermidis* biofilm formation is enhanced during growth in media containing glucose (Dobinsky *et al.*, 2003), suggesting a carbon catabolite-responsive regulator activates genes necessary for biofilm formation and/or represses genes that inhibit biofilm formation.

In Gram-positive bacteria, carbon catabolite repression is primarily mediated by the catabolite control protein A

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Abbreviations: cre, catabolite responsive elements; PIA, polysaccharide intercellular adhesin; TCA, tricarboxylic acid.

(CcpA) (Henkin *et al.*, 1991; Seidl *et al.*, 2006, 2008b). CcpA largely functions as a repressor; however, it also activates transcription of genes involved in fermentation and overflow metabolism (Shivers *et al.*, 2006; Sonenshein, 2007). CcpA regulatory activity is controlled by interactions with the phosphorylated co-repressors, histidine-containing protein (HPr) or catabolite repression HPr (Crh) (Deutscher *et al.*, 1994, 1995; Galinier *et al.*, 1997; Wray *et al.*, 1994). In addition to HPr and Crh, the activity of CcpA is regulated by the glycolytic intermediates glucose-6-phosphate and fructose-1,6-bisphosphate (Lopez & Thoms, 1977). These intermediates increase the ATP-dependent phosphorylation of HPr and/or Crh by increasing HPr kinase activity (Deutscher & Saier, 1983; Galinier *et al.*, 1997). During growth in nutrient-rich media, CcpA is complexed with phosphorylated HPr or Crh and blocks transcription initiation by binding to catabolite responsive elements (cre) located in or near carbon catabolite repressed promoters (Kim *et al.*, 2005; Nicholson *et al.*, 1987). *S. epidermidis* has both CcpA and HPr homologues; however, it appears to lack a Crh homologue.

In *Staphylococcus aureus*, CcpA represses the synthesis of capsular polysaccharides and toxigenic exoproteins and promotes biofilm formation (Seidl *et al.*, 2006, 2008a, b). On this latter point, the biofilm-promoting function of CcpA is thought to occur via its activity as a repressor of tricarboxylic acid (TCA) cycle genes (Seidl *et al.*, 2009). Repression of TCA cycle activity derepresses synthesis of the biofilm-promoting exopolysaccharide polysaccharide intercellular adhesin (PIA) (Sadykov *et al.*, 2008; Vuong *et al.*, 2005). These observations and the fact that *S. epidermidis* biofilm formation appears to be controlled by a catabolite responsive regulator led us to inactivate the *ccpA* gene from *S. epidermidis* strain 1457 (Mack *et al.*, 1992) and assess the effects on growth, biofilm formation and virulence.

METHODS

Bacterial strains, bacteriophage, plasmids and growth conditions. Bacterial strains, bacteriophage and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in 2 × YT broth or on 2 × YT agar (Sambrook *et al.*, 1989). *S. aureus* and *S. epidermidis* strains were grown in tryptic soy broth (TSB; BD Biosciences) or on TSB containing 1.5% (w/v) agar. *S. epidermidis* cultures were inoculated 1:200 from overnight cultures (normalized for growth) into TSB, incubated at 37 °C, and aerated at 225 r.p.m. with a flask-to-medium ratio of 7:1. Bacterial growth was assessed by measuring the OD₆₀₀. When used, antibiotics were used at the following concentrations: 100 µg ampicillin ml⁻¹ for *E. coli*, 10 µg chloramphenicol ml⁻¹, 10 µg erythromycin ml⁻¹ and 10 µg tetracycline ml⁻¹.

Construction of *ccpA* mutants in *S. epidermidis*. All primers (Table 2) used for PCR amplification of strain 1457 genomic DNA were designed using the *S. epidermidis* RP62A genome sequence. The *ccpA* mutant was constructed by replacing a 595 bp region, which included the *ccpA* gene, with an erythromycin resistance gene (*ermB*) using the gene splicing by overlap extension (gene SOEing) technique

(Horton *et al.*, 1990). *ermB* was amplified from pEC4 using primers *ccpA-ermB-f* and *ccpA-ermB-r*, which contain sequences homologous to the *ccpA* gene. Primers *BamHI-aroA-f* and *ermB-ccpA-r* were used for amplification of a 1.1 kb region upstream of the *ccpA* gene. Primers *ermB-ccpA-f* and *SacI-fhs-r* were used to PCR amplify a 1.2 kb region downstream of the *ccpA* gene. The resulting 3.3 kb PCR product consisted of the 1 kb *ermB* gene with DNA flanking the *ccpA* gene and contained *SacI* and *BamHI* sites that were used for ligation into pTS1-d (Sadykov *et al.*, 2008) digested with *SacI* and *BamHI* to generate plasmid pMRS15. Plasmid pMRS15 was used to construct strain 1457-Δ*ccpA* mutant (1457-Δ*ccpA*::*ermB*) using the temperature shift protocol of Foster (1998). The *ccpA* mutation of strain 1457-Δ*ccpA* was transferred into the methicillin-resistant *S. epidermidis* strain 1057 by phage transduction using *S. epidermidis* phage 71. Allelic replacement of the internal region of the *ccpA* genes by the *ermB* cassette was verified by PCR and Southern blot analysis.

Construction of the *S. epidermidis ccpA* complementation strain. For complementation of the *ccpA* mutation, a 1.9 kb PCR product containing the wild type *ccpA* allele and a portion of the upstream region was combined in a SOEing reaction with a 2.3 kb *tetM* gene and 1.4 kb of the downstream region of *ccpA*. The *tetM* gene was amplified from pWF105 using primers *ccpA-tetM-f* and *fhs-tetM-r*. Primers *BamHI-aroA-f* and *tetM-ccpA-r* were used to amplify a 1.9 kb fragment containing the intact *ccpA* gene. A 1.4 kb region of the *ccpA* downstream region was amplified using *tetM-fhs-f* and *KpnI-fhs-r* primers. The resulting 5.6 kb PCR product contained *KpnI* and *BamHI* sites that were used for ligation into pTS1-d (Sadykov *et al.*, 2008) to generate plasmid pMRS62. Plasmid pMRS62 was used to restore the wild type *ccpA* allele to strain 1457-Δ*ccpA*. Restoration of the *ccpA* gene was verified by PCR and Southern blot analysis.

Measurement of acetate and glucose concentrations in culture supernatants. Acetate and glucose concentrations were determined with kits purchased from R-Biopharm and used according to the manufacturer's protocol.

Enzymic activity assays. Aconitase activity assays were performed as described by Sadykov *et al.* (2010). Isocitrate dehydrogenase enzymic activity assays were performed as described previously (Somerville *et al.*, 2003). Protein concentrations were determined by using the modified Lowry assay (Pierce Chemical).

Transcript analysis. Northern blot analysis was performed as described by Sadykov *et al.* (2008). Four micrograms of total RNA was loaded per lane. Probes for Northern blotting were generated by PCR amplification of the internal DNA fragments of the *glmU*, *icaD*, *pfkA* and *glnA* (*femC*) genes and labelled using the North2South random prime labelling kit (Pierce). Detection was performed using the Chemiluminescent Nucleic Acid Detection Module (Pierce). PIA biosynthesis is encoded within the *icaADBC* four gene operon. To assess *icaADBC* transcription, the polycistronic *icaADBC* mRNA was probed for *icaD* and not each individual gene.

RNA isolation and real-time RT-PCR were carried out as described by Chatterjee *et al.* (2005), using the *icaD* primer pair (Arciola *et al.*, 2001). The level of *icaD* mRNA was normalized against the internal control *gyrB*. Transcripts amounts were expressed as the fold difference relative to the control gene ($2^{\Delta\Delta CT}$, where ΔCT represents the difference in threshold cycle between the target and control genes).

Biofilm forming ability. Bacterial cultures were grown in TSB supplemented with 1% glucose for 24 h at 37 °C in glass culture tubes with aeration (150 r.p.m.) or under static conditions in 24-well plates. Growth medium was inoculated with 0.5 McFarland units. Following 24 h incubation, tubes and/or wells were washed three times with PBS, air-dried, stained with 0.1% safranin for 30 s and

Table 1. Bacterial strains, bacteriophage and plasmids used in this study

Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Tc, tetracycline; ts, temperature-sensitive origin of replication.

Bacterial strain, bacteriophage or plasmid	Relevant properties	Reference or source
Strains		
DH5 α	<i>E. coli</i> cloning host	Invitrogen
RN4220	<i>S. aureus</i> restriction-deficient mutant of strain 8325-4	Novick (1991)
1457	<i>S. epidermidis</i>	Mack <i>et al.</i> (1992)
1457- <i>acnA</i>	Strain 1457 <i>acnA</i> insertion mutant, Tc ^R	Sadykov <i>et al.</i> (2008)
1457- Δ <i>citZC</i>	Strain 1457 <i>citZC</i> deletion mutant, Em ^R	Sadykov <i>et al.</i> (2008)
1457- Δ <i>ccpA</i>	Strain 1457 <i>ccpA</i> deletion mutant, Em ^R	This study
1457- <i>acnA-ccpA</i>	Strain 1457 <i>acnA</i> and <i>ccpA</i> double mutant, Tc ^R Em ^R	This study
1457- Δ <i>ccpA</i> (comp)	Strain 1457 with restored wild-type <i>ccpA</i> allele, Tc ^R	This study
1057	Methicillin-resistant <i>S. epidermidis</i> (MRSE)	Nedelmann <i>et al.</i> (1998)
1057- Δ <i>ccpA</i>	Strain 1057 <i>ccpA</i> deletion mutant, Em ^R	This study
Bacteriophage		
ϕ 71	<i>S. epidermidis</i> 1457 transducing phage	Dean <i>et al.</i> (1973)
Plasmids		
pTS1-d	Shuttle vector, pE194ori ^{ts} , ColE1, Ap ^R Cm ^R	Sadykov <i>et al.</i> (2008)
pEC4	pBluescript II KS(+) with <i>ermB</i> inserted into <i>Clal</i> site, Ap ^R Em ^R	Brückner (1997)
pMRS15	Derivative of pTS1-d, with Δ <i>ccpA::ermB</i>	This study
pMRS62	Derivative of pTS1-d, with <i>ccpA_tetM</i>	This study

Table 2. Primers used in this study

Primer designation	Nucleotide sequence (5'–3')
<i>Bam</i> HI-aroA-f	CCAGGATCCGATGCTGTAGCACAAGATTTGCAGG
<i>ermB-ccpA-r</i>	GACGAATCCCTCCTTCGTCGTTGTACGTTTACTTGCAAGACC
<i>ccpA-ermB-f</i>	GCAAGTAAACGTACAACGACGAAGGAGGGATTTCGTCATGTTG
<i>ccpA-ermB-r</i>	CACTCCAGCATCTTGTGCACGCGACTCATAGAATTATTCCTCCC
<i>ermB-ccpA-f</i>	GGAAATAATTCTATGAGTCGCGTGCACAAGATGCTGGAGTGAAAG
<i>Sac</i> I-fhs-r	GAAGAGCTCCTAGGTAAGTCCGTCGAGTTGTACCAC
<i>tetM-ccpA-r</i>	CGTAAGAGCATATTTGTAAAGGAATCTCCTTACTGAGTTGTCCCACGGTATTC
<i>ccpA-tetM-f</i>	AGAATACCGTGGGACAACCTCAGTAAGGAGATTTCCTTTACAAATATGCTCCTTACG
<i>fhs-tetM-r</i>	GATTGATTGAGATACAATGAGTGAATATGACGATCTCCTCCTTTCCACTTTAATTC
<i>tetM-fhs-f</i>	GAATTAAAGTGAAAAGGAGGAGATCGTCATATTCATTCATTGTATCTGAATCAATC
<i>Kpn</i> I-fhs-r	GAAGGTACCGTGCATTTTCAAAGCACGAATC
<i>gcaD-f</i>	CGATTATTCTGGCAGCAGGTAAG
<i>gcaD-r</i>	CGATTGATACGTTGTTGCAAAGC
<i>ica</i> Probeforward	GACAGTCGCTACGAAAAG
<i>ica</i> Probereverse	CCGAATAATTTGTAAATTTCC
<i>pfkA-f</i>	GAAGAAGATTGCAGTTTAACTAGCGG
<i>pfkA-r</i>	GCTACATCCTTAATATCTGTATTGACTTCTGG
<i>femC-f</i>	GATGTTTGATGGTTCATCTATTGAAGGTTTCG
<i>femC-r</i>	GCAGTATCAGTCAATTGTAAATCACCTTCAG
<i>aroA-f</i>	GAATTACTATCGAAGCGAGGAGAATTAGC
<i>fhs-r</i>	CAACTGCTGATGGATCGAAACC
<i>fhs-r1</i>	CAAGATTAACAAGTCCCGCTTCAAC
<i>gyrB-RT-F1</i>	CTAATGCTGATTACGACGCGTAA
<i>gyrB-RT-D1</i>	TCTGTAGGACGCATTATTGTTGAAA
<i>icaD-RT-F</i>	ATGGTCAAGCCCAGACAGAG
<i>icaD-RT-D</i>	CGTGTTTTCAACATTTAATGCAA

washed three times with distilled water. For 24-well plate cultures, the adhering dye was dissolved with 30 % acetic acid, and the absorption was measured at 530 nm.

Biofilm formation was also monitored under flow conditions using the BioFlux 200 system (Fluxion Biosciences) and a 48-well microfluidic device. Experiments were performed essentially as described by Benoit *et al.* (2010) with minor modifications. Microfluidic channels of the BioFlux flowthrough device were covered with TSB supplemented with 1 % glucose for 5 min at a flow rate of 5 $\mu\text{N cm}^2$, and subsequently inoculated with bacteria (0.5 McFarland) at a flow rate of 2 $\mu\text{N cm}^2$ from the 'outlet' well until the bacterial solution flooded the viewing channel of the device. Bacteria were allowed to attach for 15 min at 37 °C. Afterwards, fresh medium was pumped from the 'inlet' to the 'outlet' well through the microfluidic channels at a constant flow rate of 5 $\mu\text{N cm}^2$ and 37 °C. Biofilm formation was monitored after 18 h by light microscopy using an inverted microscope (Leica DMI 4000 B; Leica Mikrosysteme). Phase-contrast images were obtained using a digital camera (Leica DFC 420 C) and the Leica application suite software V3.6.

Mouse virulence. Non-obese diabetic female mice (NOD/LtJ; Charles River Laboratories) were kept under specific pathogen-free conditions in the Animal House of the Department of Biomedicine, University Hospital Basel, according to the regulations of the Swiss veterinary law. Female NOD mice spontaneously develop type 1 diabetes mellitus usually between 15 and 30 weeks of age (Leiter, 1993). Animals of 12 weeks of age were tested weekly for increased urinary glucose levels, and subsequently analysed for blood glucose levels. Mice with blood glucose $\geq 15 \text{ mmol l}^{-1}$ were included as diabetic animals.

Mice were anaesthetized and sterile catheter segments were inserted subcutaneously as described by Kristian *et al.* (2008) and Rupp *et al.* (1999). Catheters were infected with 20 μl pyrogen-free saline containing 10^4 c.f.u. *S. epidermidis* strain 1457 and its isogenic 1457- ΔccpA mutant, respectively, before the incisions were closed with wound clips. The diameter of swelling/oedema was measured daily using a caliper. Ten days after infection, mice were euthanized by CO_2 or intraperitoneal injection of 500 mg thiopental kg^{-1} , and the catheters and surrounding tissues were aseptically removed and separated. Bacteria adherent to the catheters were detached by vortexing in 0.9 % saline, 0.15 % EDTA, followed by sonication for 2 min at 250 W. Tissue samples were homogenized in 1 ml 0.9 % NaCl. Serial dilutions were plated on Mueller–Hinton agar to enumerate bacterial load in catheters and tissues.

Statistical analysis. The significance of changes was assessed using Student's *t*-test or Mann–Whitney Rank Sum Test. *P*-values < 0.05 were considered significant.

RESULTS

Growth characteristics of *S. epidermidis* strain *ccpA* deletion mutant

CcpA is a TCA cycle repressor and an activator of genes involved in fermentation and overflow metabolism (Shivers *et al.*, 2006; Sonenshein, 2007). These observations suggest that deletion of *ccpA* would increase carbon flow into the TCA cycle and away from overflow metabolism. Because staphylococci have evolved to utilize nutrients rapidly but inefficiently when they are in abundance, redirecting carbon into the more efficient TCA cycle would likely result in a slower growth rate. Consistent with this suggestion, deletion of *ccpA* in strain 1457 resulted in a slightly decreased growth

rate relative to strain 1457; nevertheless, the growth yields of both strains were similar (Fig. 1a). In *Bacillus subtilis*, deletion of *ccpA* impairs growth on minimal media (Ludwig *et al.*, 2002). This growth impairment is the result of CcpA regulating glutamate and branched chain amino acid biosynthesis; hence, growth can be restored by the addition of amino acids to the minimal medium. While it is possible that amino acid auxotrophies in strain 1457- ΔccpA contribute to its slower growth rate relative to strain 1457, growth studies were conducted in the amino acid- and peptide-rich medium TSB, minimizing this possibility. Importantly, complementation of the *ccpA* deletion restored the growth rate to that of strain 1457. If the decreased growth rate was due to carbon being directed away from overflow metabolism and into the TCA cycle, then the accumulation of organic acids in the culture medium of strain 1457- ΔccpA will be less than in the culture medium of the wild type strain 1457. To assess the accumulation of organic acids in the culture medium, the pH and acetic acid concentrations were measured during growth (Fig. 1b, c). In the exponential growth phase, the pH of the culture medium of strain 1457- ΔccpA remained more basic than did the pH of the culture medium of the wild type strain 1457. Acidification of the culture medium was restored to wild type levels by complementing the *ccpA* deletion with a wild type *ccpA* gene. Similar to the culture medium pH profiles, strain 1457- ΔccpA accumulated less acetate in the medium than did the wild type strain or strain 1457- ΔccpA (comp). These data suggest that CcpA regulates overflow and TCA cycle metabolism in *S. epidermidis*.

CcpA represses TCA cycle activity

The decreased accumulation of acetate in the culture medium of strain 1457- ΔccpA relative to strain 1457 (Fig. 1c) indicated that less carbon was entering into overflow metabolism. The more likely explanation for this observation was increased carbon flow into the TCA cycle. To determine if deletion of *ccpA* increased TCA cycle enzymic activity, the activities of aconitase and isocitrate dehydrogenase were measured in the exponential and post-exponential phases of growth (Fig. 2a, b). As expected, isocitrate dehydrogenase and aconitase activities were significantly increased in strain 1457- ΔccpA relative to strain 1457 ($P < 0.005$). Complementation of the *ccpA* deletion restored enzymic activity to the level of the wild type. Although *ccpA* deletion increased enzymic activity, it was less than a twofold difference, raising the possibility that *ccpA* deletion had an indirect effect on TCA cycle activity. In *B. subtilis*, CcpA regulates TCA cycle activity at the transcriptional level (Kim *et al.*, 2002). To determine if *ccpA* deletion affected transcription of *citZ* (citrate synthase) and *acnA/citB* (aconitase), quantitative RT-PCR was used to determine the relative levels of *citZ* and *acnA/citB* mRNA. In addition, we assessed the effect of *ccpA* deletion on *ilvD* (dihydroxy-acid dehydratase, an indicator of branched chain amino acid biosynthesis) and *glbB* (the large subunit of glutamate synthase) to see if changes in

Fig. 1. Growth characteristics of strains 1457 (○), 1457-Δ*ccpA* (▽) and 1457-Δ*ccpA*(comp) (□). Growth curves (a), culture medium pH profiles (b) and acetate accumulation and depletion from the culture medium (c) of these strains are shown. The data are representative of two independent experiments.

transcription could account for the slower growth rate of strain 1457-Δ*ccpA*. Similar to in *B. subtilis*, deletion of *ccpA* derepressed transcription of both *citZ* and *acnA/citB*

Fig. 2. CcpA deletion increases aconitase and isocitrate dehydrogenase activity and de-represses transcription during the exponential growth phase. Isocitrate dehydrogenase (a) and aconitase (b) activity was assessed during the exponential (3 h) and post-exponential (6 h) growth phases. The data are presented as the mean ± SEM of two independent experiments each determined in triplicate. (c) Quantitative RT-PCR analysis of the effects of *ccpA* inactivation on the quantity of *acnA/citB*, *citZ*, *ilvD* and *gltB*.

relative to the parental strain during the exponential growth phase (Fig. 2c). During the exponential growth phase, the mRNA levels of *ilvD* and *gltB* were low, suggesting that they do not account for the difference in

the growth rates between strains 1457 and 1457- Δ ccpA. In addition, these data demonstrate the normal temporal induction of transcription that coincides with the depletion of glucose and the release of catabolite repression. These data confirm that CcpA functions as a regulator of the TCA cycle in *S. epidermidis*.

CcpA regulates transcription of genes involved in PIA biosynthesis

TCA cycle activity regulates synthesis of the biofilm-promoting exopolysaccharide PIA both at the transcriptional level and by altering the availability of PIA biosynthetic precursors (Sadykov *et al.*, 2008; Vuong *et al.*, 2005). Specifically, deletion of any of the first three genes of the TCA cycle derepresses transcription of PIA biosynthetic genes (i.e. *icaADBC*) and PIA biosynthesis. Although it is possible that TCA cycle enzymes directly regulate *icaADBC* transcription, the more likely possibility is that a regulatory protein(s) responding to TCA cycle-induced changes in the metabolic status alters transcription of *icaADBC*. Notably, one of the metabolic changes caused by TCA cycle stress (i.e. any external stressor that is capable of altering TCA cycle activity) is an increase in the intracellular concentration of glucose-6-phosphate (Sadykov *et al.*, 2010). Because the regulatory activity of CcpA is enhanced by the glycolytic intermediate glucose-6-phosphate, this raised the possibility that CcpA both regulates TCA cycle activity (Fig. 2a, b) and responds to metabolic signals transduced by the TCA cycle (Brückner & Titgemeyer, 2002; Deutscher & Saier, 1983; Sonenshein, 2007; Warner & Lolkema, 2003). These observations led us to assess the effect of *ccpA* deletion on the transcription of a subset of genes involved in PIA biosynthesis; specifically, *icaD* [IcaD forms a heterodimeric UDP-*N*-acetylglucosamine transferase with IcaA (Gerke *et al.*, 1998)], *N*-acetylglucosamine biosynthesis (*glmU*; glucosamine-1-phosphate *N*-acetyltransferase), *glnA* (glutamine synthetase) and *pfkA* (6-phosphofructokinase) (Fig. 3a). In addition, to determine whether metabolic signals transduced by the TCA cycle require CcpA, the effect of *ccpA* deletion on transcription of these same genes in an aconitase (*acnA/citB*) mutant background was investigated (Fig. 3b). Of the genes involved in PIA biosynthesis that were examined, a putative cre site was identified [using the *B. subtilis* cre consensus site (TGWNANCGNTNWCA) as a template (Weickert & Chambliss, 1990)] in the promoter region of *pfkA* (TTTAAACGTTTACATC) and another putative cre site was found in the 3' portion of *glmU* (TGACGGCGCTAACAAA). During the exponential phase of growth when glucose was present in the medium, *ccpA* deletion decreased the transcription or stability of *pfkA* mRNA relative to strain 1457 (Fig. 3b). These data and the presence of a cre site in the promoter region of *pfkA* indicate that CcpA regulates *pfkA* transcription; however, as with *B. subtilis*, the effect of CcpA on glycolytic genes may be indirect (Tobisch *et al.*, 1999). Interestingly, aconitase inactivation caused a remarkable increase in *pfkA* mRNA relative to strain 1457 and CcpA was required for this

Fig. 3. Northern blot analysis of *icaD*, *glmU*, *pfkA* and *glnA* mRNA levels in the exponential (2 h) and post-exponential (6 h) phases of growth. (a) The diagram shows the relationship of the genes chosen for Northern analysis to the synthesis of PIA. The presence of a cre site in the promoter region of *pfkA* is indicated by a dagger ($\dagger$). -6-P indicates a 6-phosphate chain; -1,6-P indicates 1,6-bisphosphate; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; UOP, uniline diphosphate. (b) Representative Northern blots probed with PCR products for *icaD*, *glmU*, *pfkA* and *glnA*. 23S and 16S rRNA were visualized by ethidium bromide staining and used as loading controls. The results are representative of at least two independent experiments. (c) Quantitative transcript analysis of *icaD* in strains 1457, 1457- Δ ccpA, and 1457- Δ ccpA(comp) after 2 and 6 h of growth. The data are presented as the mean $\pm$ SD of four independent experiments each determined in duplicate. Statistical significance between the wild type and *ccpA* mutant was determined by using the Mann-Whitney test. * P <0.05; ** P <0.01.

increased level of *pfkA* mRNA. Similarly, CcpA was required for the transduction of TCA cycle metabolic signals into an increased level of *icaD* mRNA (Fig. 3b). This observation was interesting because Northern blot analysis revealed that inactivation of *ccpA* had a small effect on *icaD* transcription and no cre sites were identified in *icaADBC* or its promoter region (Fig. 3b and data not shown). Since it was possible that changes in *icaD* transcript levels of the wild-type and Δ *ccpA* mutant strains were below the detection limit of Northern blots, *icaD* transcription was assessed by real-time PCR (Fig. 3c). Using RT-PCR, we observed that inactivation of *ccpA* had a small but significant effect on *icaD* transcription (Fig. 3c). Specifically, deletion of *ccpA* decreased the exponential (2 h) and post-exponential growth phase (6 h) level of *icaD* mRNA by a factor of two to three. Complementation of the mutant with CcpA reversed this effect, suggesting this effect was CcpA-dependent. Neither *glmU* nor *glnA* transcription was affected by *ccpA* deletion during the exponential phase of growth; however, *ccpA* deletion derepressed *glnA* transcription during the post-exponential growth phase (Fig. 3b). Taken together, these data suggest that CcpA has a modest effect on some genes involved in PIA biosynthesis (i.e. *icaD* and *pfkA*). In addition, this suggestion is consistent with PIA immunoblot data demonstrating that *ccpA* inactivation had only a minor effect on PIA accumulation (Sadykov *et al.*, 2010).

CcpA is required for biofilm formation

The CcpA-dependent regulation of *pfkA* (Fig. 3b) and the modest effect of CcpA on *icaD* transcription (Fig. 3c) suggested that deletion of *ccpA* might hinder the ability of strain 1457 to form a biofilm. To test if *ccpA* deletion altered the ability of *S. epidermidis* to form a biofilm, strains 1457, 1057, 1457- Δ *ccpA*, 1057- Δ *ccpA* and 1457- Δ *ccpA*(comp) were grown in static biofilms and the biofilms were quantified using a dye retention assay (data not shown). Deletion of *ccpA* decreased the ability of *S. epidermidis* to form a biofilm, while complementation of the *ccpA* deletion restored the biofilm forming capacity (data not shown). Similarly, strains 1457- Δ *ccpA* and 1057- Δ *ccpA* grown in glass culture tubes (1:5 medium-to-flask ratio) with 150 r.p.m. aeration displayed a greatly diminished ability to form biofilms relative to strains 1457, 1057 and 1457- Δ *ccpA*(comp) (Fig. 4a). The effect of *ccpA* deletion on biofilm formation is most apparent in a microfluidic shear flow assay (Fig. 4b), where strain 1457- Δ *ccpA* does not form a biofilm. These data demonstrate that CcpA is critical for the ability of *S. epidermidis* to form a biofilm in a glucose-containing medium.

To facilitate biofilm formation in the laboratory, glucose is often added to the culture medium in abundance (Dobinsky *et al.*, 2003; Lim *et al.*, 2004). One outcome of the addition of excess glucose is the induction of overflow metabolism and a decrease in the extracellular pH. This decreased pH is one factor that can contribute to biofilm formation. Because *ccpA* deletion alters the carbon flow,

Fig. 4. Deletion of *ccpA* reduces *S. epidermidis* biofilm forming capacity. (a) The ability of strains 1457, 1057, 1457- Δ *ccpA*, 1057- Δ *ccpA* and 1457- Δ *ccpA*(comp) to form biofilms under aerobic conditions was assessed in glass culture tubes that were stained with safranin (figure is representative of three independent experiments). (b) Biofilm formation monitored under shear flow conditions using the BioFlux 200 system and a 48-well microfluidic device.

the pH of the culture medium is more alkaline than it is with the wild type strain (Fig. 1), raising the possibility that the more alkaline pH is responsible for the decreased ability of strain 1457- Δ *ccpA* to form a biofilm. To test this possibility, static and aerated cultures of strains 1457, 1057, 1457- Δ *ccpA* and 1057- Δ *ccpA* were grown in TSB at pH 7.0

and 6.0, and the ability of the bacteria to form a biofilm was assessed (Fig. 4a and data not shown). Surprisingly, the decreased ability of *ccpA* deletion strains to form a biofilm was independent of changes in the extracellular pH.

CcpA is not required for virulence in a mouse catheter/biofilm infection model

CcpA is required for biofilm formation (Fig. 4); hence, it was reasonable to hypothesize that CcpA is required for virulence in a biofilm-promoting infection model. To test this hypothesis, we assessed the effect of *ccpA* deletion in a mouse catheter/biofilm infection model (Fig. 5). Because the regulatory activity of CcpA is affected by glycolytic intermediates, diabetic mice (blood glucose levels ≥ 15 mmol l⁻¹) were used to exacerbate any differences in virulence associated with *ccpA* inactivation. In contrast with our hypothesis, deletion of *ccpA* did not significantly alter the infectious outcomes in diabetic or non-diabetic mice in the catheter/biofilm infection model. Previously, we observed that the antiphagocytic properties of PIA were important for *S. epidermidis* virulence in our mouse catheter/biofilm infection model (Kristian, *et al.*, 2008). Therefore, the absence of any significant difference between the wild type and *ccpA* mutant strains in our mouse catheter infection model (Fig. 4) is consistent with the modest effect CcpA has on *icaADBC* transcription (Fig. 3b, c) and PIA accumulation (Sadykov, *et al.*, 2010). Alternatively, these data may indicate that biofilm formation is not regulated by the availability of carbohydrates *in vivo*.

CcpA decreases oxacillin susceptibility

Inactivation of *ccpA* in *S. aureus* increases methicillin and oxacillin susceptibility (De Lencastre *et al.*, 1999; Seidl *et al.*, 2006), raising the possibility that deletion of *ccpA* in *S. epidermidis* would increase oxacillin susceptibility. To determine if *ccpA* deletion altered the susceptibility of the methicillin-resistant strain 1057 to oxacillin, strains 1057 and 1057- Δ *ccpA* were grown for 48 h in Mueller–Hinton broth, or Mueller–Hinton broth supplemented with 2 % NaCl, with increasing concentrations of oxacillin, and c.f.u. ml⁻¹ was determined (Fig. 6). Similar to *S. aureus*, deletion of *ccpA* in strain 1057 caused an increase in the susceptibility to oxacillin, demonstrating that oxacillin resistance in *S. epidermidis* requires CcpA and suggesting that CcpA-mediated oxacillin resistance may be a common resistance mechanism.

DISCUSSION

Most *S. epidermidis* virulence determinants are involved in attachment, adherence, and the elaboration of a biofilm; hence, the primary mode of infection for *S. epidermidis* is its ability to colonize implanted biomaterials (von Eiff *et al.*, 2002). Extensive data have accumulated demonstrating that formation of *S. epidermidis* biofilms is dependent upon the bacterial metabolic state (Cramton *et al.*, 2001; Sadykov *et al.*, 2008, 2010; Schlag *et al.*, 2007; Vuong *et al.*, 2005). Specifically, *S. epidermidis* biofilms form under nutrient-rich conditions when TCA cycle activity is

Fig. 5. The virulence of *S. epidermidis* strain 1457 in a catheter-related infection model is independent of CcpA. Sterile catheter segments were inserted subcutaneously into non-diabetic (non-diab.; blood glucose levels <10 mM) and diabetic (blood glucose levels >15 mM) NOD mice and subsequently infected with 10^4 bacteria before the incisions were closed with wound clips. On day 10 after infection, oedema end points (a) at the insertion site were determined, and the catheters (b) and the surrounding tissues (c) were aseptically removed and separated. Bacteria adherent to the catheters were detached by vortexing in saline, and tissue samples were homogenized in 1 ml 0.9 % NaCl. For c.f.u. determinations, serial dilutions were plated on Mueller–Hinton agar. Statistical significance was determined by comparing mice from the same age group using the Mann–Whitney test. * $P < 0.05$; ** $P < 0.01$; ns, not significant. Data are presented as “box and whisker plots” showing the interquartile range (25–75 %; box), median (horizontal line), whiskers (bars) and min/max outliers (○).

Fig. 6. Deletion of *ccpA* increased the susceptibility of methicillin-resistant *S. epidermidis* strain 1057 to oxacillin. Population analysis profiles were established by plating appropriate dilutions of overnight cultures of 1057 (■) and 1057-Δ*ccpA* (△) on Mueller–Hinton agar (a) and cation-adjusted Mueller–Hinton broth supplemented with 2% NaCl (b) containing increasing concentrations of oxacillin. The c.f.u. was determined after 48 h of incubation at 35 °C.

repressed (Cramton *et al.*, 2001; Dobinsky *et al.*, 2003; Mack *et al.*, 1992; Sadykov *et al.*, 2008; Vuong *et al.*, 2005).

In *S. epidermidis*, repression of the TCA cycle de-represses *ica* transcription and PIA biosynthesis, which facilitates biofilm formation (Fig. 3b; Sadykov *et al.*, 2008; Vuong *et al.*, 2005). As stated, the more likely hypothesis to explain how TCA cycle activity regulates *icaADBC* transcription and PIA biosynthesis is that changes in activity are transduced into metabolic signals that can be 'sensed' by regulators of *icaADBC* (Sadykov *et al.*, 2010; Somerville & Proctor, 2009). Regulation of the *icaADBC* operon is complex, involving at least two DNA-binding proteins (IcaR and SarA) and the alternative sigma factor σ^B (Conlon *et al.*, 2002; Handke *et al.*, 2007). If changes in TCA cycle activity produce metabolic signals that can be sensed by regulators of *icaADBC*, then regulatory mutants (i.e. *icaR*, *sarA* and *sigB*) whose TCA cycle activity is blocked should be unable to de-repress *icaADBC* transcription and/or PIA biosynthesis. We previously observed that addition of the aconitase-specific inhibitor, fluorocitric acid, to the culture medium of *S. epidermidis* strains 1457-*sigB* and 1457-*icaR* increased the accumulation of PIA relative to untreated control cultures (Sadykov *et al.*, 2008). These data suggest that TCA cycle-mediated de-repression of *icaADBC* transcription and PIA synthesis occur independently of IcaR and σ^B (Sadykov *et al.*, 2008). In a 1457 *sarA* mutant, the accumulation of PIA was similar irrespective of the presence of fluorocitric acid in the culture medium ($P > 0.05$). These data confirmed that PIA synthesis requires SarA but that there must be at least one additional regulator that is responsive to TCA cycle-associated metabolic signals.

Environmental stressors that repress TCA cycle activity increase the intracellular concentration of the glycolytic intermediate glucose-6-phosphate (Sadykov *et al.*, 2010). Glucose-6-phosphate increases phosphorylation of the

histidine-containing protein (HPr) by enhancing the activity of the HPr kinase (Deutscher & Saier, 1983), which increases its interaction with CcpA, thus activating CcpA (Brückner & Titgemeyer, 2002; Sonenshein, 2007; Warner & Lolkema, 2003). In *S. aureus*, CcpA is required for *ica* transcription, PIA accumulation and biofilm formation (Seidl *et al.*, 2008b). These observations raised the possibility that CcpA is the additional TCA cycle-responsive regulator required for *ica* transcription in *S. epidermidis*. Surprisingly, deletion of *ccpA* in *S. epidermidis* has only a minor effect on *ica* transcription (Fig. 3c) and PIA accumulation (Sadykov *et al.*, 2010); however, it inhibits biofilm formation (Fig. 4). In *S. aureus*, de-repression of the TCA cycle decreases PIA accumulation and delays biofilm formation (Zhu *et al.*, 2009); hence, the simplest explanation for CcpA-dependent biofilm formation in *S. epidermidis* is that CcpA represses TCA cycle activity, increasing *ica* transcription and PIA synthesis. If this explanation is correct, then inactivation of the TCA cycle in strain 1457-Δ*ccpA* should de-repress *ica* transcription and PIA accumulation. In contrast with this expectation, inactivation of aconitase in strain 1457-Δ*ccpA* failed to increase *ica* transcription (Fig. 3b) or PIA accumulation (Sadykov *et al.*, 2010). Although the mechanism by which CcpA facilitates biofilm formation has yet to be elucidated, it is clear that CcpA is required to transduce metabolic changes associated with TCA cycle stress into alterations in *icaADBC* transcription and PIA accumulation. Taken together, these observations demonstrate that CcpA is an essential positive effector of biofilm formation.

ACKNOWLEDGEMENTS

G. A. S. was supported by funds provided through the Hatch Act and the Institute of Agriculture and Natural Resources and by funds provided through the National Institutes of Health (AI087668). T. A. M. and M. H. were supported in part by funds provided through

the University of Nebraska's Undergraduate Creative Activities and Research Experiences. T.H., M.H. and M.B. were supported by the Deutsche Forschungsgemeinschaft (grant BI 1350/1-1). We are grateful to S. Balkow and IUL Instruments for providing the BioFlux 200 system.

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Edited by: T. Msadek