Chelating agents exert distinct effects on biofilm formation in *Staphylococcus aureus* depending on strain background: role for clumping factor B

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Staphylococcus aureus is a leading cause of catheter infections, and biofilm formation plays a key role in the pathogenesis. Metal ion chelators inhibit bacterial biofilm formation and viability, making them attractive candidates as components in catheter lock solutions. The goal of this study was to characterize further the effect of chelators on biofilm formation. The effect of the calcium chelators ethylene glycol tetraacetic acid (EGTA) and trisodium citrate (TSC) on biofilm formation by 30 *S. aureus* strains was tested. The response to subinhibitory doses of EGTA and TSC varied dramatically depending on strain variation. In some strains, the chelators prevented biofilm formation, in others they had no effect, and they actually enhanced biofilm formation in others. The molecular basis for this phenotypic variability was investigated using two related strains: Newman, in which biofilm formation was inhibited by chelators, and 10833, which formed strong biofilms in the presence of chelators. It was found that deletion of the gene encoding the surface adhesin clumping factor B (*clfB*) completely eliminated chelator-induced biofilm formation in strain 10833. The role of ClfB in biofilm formation activity in chelators was confirmed in additional strains. It was concluded that biofilm-forming ability varies strikingly depending on strain background, and that ClfB is involved in biofilm formation in the presence EGTA and citrate. These results suggest that subinhibitory doses of chelating agents in catheter lock solutions may actually augment biofilm formation in certain strains of *S. aureus*, and emphasize the importance of using these agents appropriately so that inhibitory doses are achieved consistently.

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INTRODUCTION

Staphylococcus aureus is a leading cause of nosocomial infections and of bloodstream infections (BSIs) associated with colonized intravenous catheters. Catheter-associated BSIs increase mortality rates, prolong patients' stay and increase healthcare costs (Centers for Disease Control and Prevention, 2011). An important virulence factor in such infections is the ability to adhere to and form a biofilm on the surface of the catheter.

Bacterial biofilms are communities in which intercellular bonds enforced by polymeric compounds and intercellular communication mediated by signalling molecules result in a bastion that is sheltered from sheer forces, antimicrobial agents and host immune components. Consequently,

biofilm-related infections are very difficult to treat therapeutically and, whilst antibiotics may eradicate the BSI and temporarily relieve the clinical signs of infection, the biofilms often persist and act as a nidus for relapsing infection (Hall-Stoodley *et al.*, 2004). Recent guidelines for prevention of catheter-related infections recommend the use of intraluminal antimicrobial lock solutions or catheter lock solutions (CLSs) (Mermel *et al.*, 2009; O'Grady *et al.*, 2011; Raad *et al.*, 2003). Heparin has traditionally been used to lock catheter lumens during interdialytic periods, but studies supporting the inhibitory effects of chelating agents on biofilm formation make the use of CLSs composed of trisodium citrate (TSC) appealing (Banin *et al.*, 2006; Percival *et al.*, 2005; Shanks *et al.*, 2006). Whilst TSC is not currently approved as a CLS in the USA (O'Grady *et al.*, 2011), use of these agents is becoming more widespread and CLSs including Citra-Lock and DuraLock-C are based on TSC. Metallic cations such as Ca²⁺ and Mg²⁺ play a role in microbial adherence, biofilm formation and bacterial growth. These divalent cations can

Abbreviations: BSI, bloodstream infection; CLS, catheter lock solution; EGTA, ethylene glycol tetraacetic acid; RACE, rapid amplification of cDNA ends; TSC, trisodium citrate.

A supplementary figure is available with the online version of this paper.

stimulate cell–cell adhesion and aggregation through their interactions with cell-wall teichoic acids (Dunne & Burd, 1992; Sarkisova *et al.*, 2005). Therefore, removal of free cations from the milieu reduces intercellular adhesion and biofilm formation. Furthermore, chelating agents can reduce biofilm formation by inhibiting the production of the staphylococcal polysaccharide intercellular adhesin poly-*N*-acetylglucosamine (Juda *et al.*, 2008; Ozerdem Akpolat *et al.*, 2003). However, *S. aureus* has many components in addition to poly-*N*-acetylglucosamine that can contribute to adhesion and biofilm formation.

The surface of *S. aureus* is coated with a variety of adhesins that are capable of binding different host proteins present in plasma and/or the extracellular matrix. Proteinaceous adhesins can also mediate bonds between bacterial cells (Corrigan *et al.*, 2007; Cucarella *et al.*, 2002). There are at least 28 different *S. aureus* proteins that promote binding to 18 different host proteins (Clarke & Foster, 2006). Adhesins that mediate such binding to host proteins are termed microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). *S. aureus* can express four distinct fibrinogen-binding MSCRAMMs: clumping factors A and B (ClfA and ClfB) (Ní Eidhin *et al.*, 1998) and the fibronectin-binding proteins FnbpA and FnbpB, which bind to fibronectin and fibrinogen (Wann *et al.*, 2000). Catheter surfaces are rapidly coated with blood components including fibrinogen and fibronectin and subsequently serve as efficient substrates for *S. aureus* binding.

The effects of TSC and other chelators on biofilm formation have not been defined fully and we therefore designed this study to examine their effects. Because TSC at low concentration has been shown to affect biofilm formation positively, not through its chelating activity but rather by acting as a tricarboxylic acid cycle intermediate, we also investigated the effect of ethylene glycol tetraacetic acid (EGTA) to confirm effects that might be due to metal ion chelation (Shanks *et al.*, 2006, 2008). Similar to TSC, EGTA has a high affinity for Ca^{2+} . We tested the effect of the chelating agents TSC and EGTA on a panel of *S. aureus* strains, comprising three laboratory strains and 27 cardiac device-associated infection isolates. Whilst TSC and EGTA effectively inhibited biofilm formation in some of the isolates, they failed to prevent biofilm formation in others and, surprisingly, they actually augmented biofilm formation significantly in some strains. Despite the fact that strains Newman and 10833 are related phylogenetically, they exhibited very different responses, in that the chelators eliminated biofilm formation in the Newman strain but actually augmented it in strain 10833. We therefore focused our investigation on the molecular basis of the differential effect on this strain pair and found that ClfB was required for biofilm formation in the presence of EGTA.

METHODS

Strains and growth conditions. *Escherichia coli* CH3-Blue (Bioline) and *S. aureus* RN4220, a restriction-deficient strain derived from the

laboratory strain NCTC 8325, were used for initial cloning. *S. aureus* strains tested for biofilm formation in the presence and absence of EGTA included strain SA113 (ATCC 35556), derived from laboratory strain NCTC 8325; strain Newman, a strongly coagulase-producing isolate from a case of osteomyelitis; strain 10833, a strong clumping factor-producing isolate; and a panel of 27 isolates from cardiac-associated infections. Strains 10833 and Newman appear to have originated from the same isolate. Strain Newman was deposited by E. S. Duthrie in 1950 and given the strain designation ATCC 13420 (NCTC 8178) and strain Newman D2c (NCTC 10833, referred to in this manuscript as 10833) was deposited by J. Hawiger in 1972 and given the strain designation ATCC 25904 (Grundmeier *et al.*, 2004). The concentration of free calcium in tryptic soy broth (TSB) and TSB supplemented with different concentrations of EGTA was determined using a QuantiChrom Calcium Assay kit (BioAssay Systems) according to the manufacturer's instructions. All strains used in this study were grown aerobically at 37 °C.

Static biofilm assay. Biofilm assays were performed essentially as described by Christensen *et al.* (1985). Briefly, overnight planktonic cultures of *S. aureus* were diluted to a final OD_{600} of 0.015 in fresh medium and 200 µl culture was aliquoted into individual wells of a 96-well Cell Bind plate (Corning). The cells were grown in TSB supplemented with 1 % glucose (TSBG). To determine the appropriate dose of EGTA and TSC for the experiments, we performed standard MIC assays and used 0.5 MIC in all subsequent studies. MICs were similar for all strains used in the study, and 0.5 MIC was equal to 12.5 mM EGTA or 12.5 mM TSC. To restore Ca^{2+} levels, 12.5 mM CaCl_2 was added. The plates were incubated at 37 °C for 18 h, spent medium was removed and the wells were washed once with 200 µl sterile water, dried and stained with safranin. The biofilms were inspected visually; digital images were obtained and the biofilms were quantified by dissolving the stain with 200 µl 33 % (v/v) acetic acid and determining A_{415} using a 96-well plate spectrophotometer (ELx800 Microplate Reader; BioTek). Replica plates were used to determine growth at OD_{595} by resuspending the biofilms by vigorous pipetting. The results for these experiments were compiled from three biological replicates, each of which contained five technical replicates.

Clumping assay. Overnight cultures of strains 10833 and Newman were diluted to a final OD_{600} of 0.015 in 2 ml TSBG or TSBG containing 12.5 mM EGTA. The cultures were incubated, without shaking, at 37 °C for 18 h. The tubes were vortexed lightly and inspected visually for clumping.

Quantitative real-time RT-PCR analysis. Overnight cultures of strains 10833 or Newman were grown in TSB, diluted 1:20 in fresh TSBG or TSBG supplemented with 12.5 mM EGTA or 12.5 mM TSC and incubated at 37 °C for 2 h with shaking at 200 r.p.m. To isolate RNA, approximately 5×10^8 bacteria were resuspended in 500 µl Buffer RLT (Qiagen) and added to 2 ml tubes containing 1 µm glass beads. Cold acid phenol (500 µl) was added and the cells were lysed using a FastPrep FP120 cell disrupter (Thermo Scientific). The beads were removed by centrifugation and the supernatant was purified using an RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Contaminating DNA was digested with Turbo DNase (Ambion) for 1 h at 37 °C and 4 µg RNA was converted to cDNA using a Tetro cDNA synthesis kit (Bioline) according to the manufacturer's instructions using 10 pmol gene-specific reverse primer (Table 1). Control reactions lacked reverse transcriptase (RT). Real-time RT-PCRs were carried out in a total volume of 25 µl, containing 2 µl cDNA or a no reverse transcriptase control (diluted 1:500 for 16S rRNA gene reactions and 1:5 for other genes), 1 pmol forward and reverse primers (Table 1), 8.5 µl nuclease-free deionized water and 12.5 µl SYBR Green Sensimix (Bioline). Real-time RT-PCR was performed using an iQ5 Multicolor Realtime PCR (Bio-Rad) cycloer under the following conditions: 94 °C for 2 min, followed by 40 cycles of 94 °C for 10 s,

Table 1. Oligonucleotides used in this study

Primer description	Sequence (5'→3')
16S RT Fwd	TCCGGAATTATTGGGCGTAA
16S RT Rev	CCACTTTCCTCTTCTGCACTCA
clfA RT Fwd	CTGGGCTTCAGTGCTTGTAG
clfA RT Rev	TCCTGTTGTGCTGGATTTTG
clfB RT Fwd	TCGAACGATACAACGCAATC
clfB RT Rev	TGTTGAAGCTGGCTCTGTTG
clfB Soe1	AGATCTGCACAAGGTAAGTTTGTGGA
clfB Soe2	CTCGAGCGTCTAATCGAATACTTATT
clfB Soe3	CAGAATAAGTATTTCGATTAGACGCTC
clfB Soe4	AGATCTCCTTGTTC AATT CAGCAATGA
clfB-pCL15 Fwd	GGGAAAGGGCTCCAGTTGAAAAAAG
clfB-pCL15 Rev	GGGAAAGGGGAATTCTTACGCTTTTT
clfBPROFWD	TGTTGTAAAGATCATAAAATTGGTT
clfBREV	TTACGCTTTTTCTTTATGATCTTGCTTG
ClfBRACE	GTACCTACTGTAAACGTCTAATCG
ClfBRACEnest	TCGAATACTTATTCTGCTTATTTCGAC

54 °C for 15 s and 72 °C for 15 s. The normalized amount of transcript for each gene was expressed as the fold difference relative to the control gene ($2^{\Delta C_t}$, where ΔC_t represents the difference in threshold cycle between the target gene and the 16S rRNA gene). Samples were performed in triplicate and each experiment was repeated to ensure reproducibility. The data were combined and the results are represented as the relative fold difference when comparing cells grown in 12.5 mM EGTA or 12.5 mM TSC with cells grown in control TSBG.

Construction of *clfB* deletion and complementation mutants.

The *clfB* gene was replaced with an erythromycin resistance cassette in *S. aureus* strain RN4220 by homologous recombination using the pMAD vector (kindly provided by Michel Débarbouillé and Maryvonne Arnaud, Pasteur Institute, Paris, France) as described previously by Arnaud *et al.* (2004). Genomic DNA from strain SA113 was used as a template for PCR using primers *clfB* Soe1 and *clfB* Soe4 and the PCR product was cloned into pCR4-TOPO (Invitrogen). The resulting construct was amplified using primers *clfB* Soe2 and *clfB* Soe3 (Table 1), digested with *XhoI* and ligated to an erythromycin resistance cassette excised from plasmid pSC57 with *XhoI*. The resulting plasmid was digested with *BamHI* and the region surrounding the *clfB* gene and the intervening erythromycin cassette were ligated to pMAD. This construct was electroporated into RN4220 (Lee, 1993). Once gene replacements in RN4220 were confirmed, the mutations were then transduced into strains 10833 and Newman using phage 80 α (Kasatiya & Baldwin, 1967) and transductants were selected on tryptic soy agar (TSA) plates containing 10 μ g erythromycin ml⁻¹.

For complementation of the deletion, *clfB* was cloned into the IPTG-inducible vector pCL15, which was kindly provided by Dr Chia Lee (University of Arkansas, USA) (Luong & Lee, 2006). Primers *clfb*-pCL15 Fwd and *clfb*-pCL15 Rev (Table 1) were used to amplify the *clfb* gene from SA113 genomic DNA. The PCR product was digested with *BamHI* and *EcoRI* and ligated to pCL15 to produce *clfb*-pCL15. Plasmids were purified and transformed into RN4220 and selected on tryptic soy agar (TSA) containing chloramphenicol before the constructs were transduced into strains 10833 and Newman by phage 80 α . Strains harbouring *clfb*-pCL15 and pCL15 were cultured in the presence of 10 μ g chloramphenicol ml⁻¹ and 1 mM IPTG.

Analysis of the *clfB* promoter. Genomic DNA was isolated from strains 10833 and Newman using a DNeasy tissue kit (Qiagen). The *clfb* gene and 0.4 kb 5'-proximal to the gene were amplified using

primers *clfb*PROFWD and *clfb*BREV and sequenced (Eurofins MWG Operon). The *clfb* transcriptional start site was determined using a FirstChoice RLM-RACE kit (Ambion). RNA (10 μ g) was incubated in 1 $\times$ reaction buffer in a 20 μ l total volume with 1 U terminator enzyme (Epicentre Biotechnologies) to degrade rRNA, tRNA and partially degraded mRNA for 1 h at 37 °C. The reaction was terminated by phenol extraction and ethanol precipitation and the RNA was subjected to 5' rapid amplification of cDNA ends (RACE) analysis according to the manufacturer's protocol. Primer ClfBRACE was used for the initial PCR and ClfBRACEnest for the nested PCR (Table 1). The nested PCR product was cloned into pCR4TOPO (Invitrogen) and sequenced (Eurofins MWG Operon).

Adherence to catheter tubing. Overnight cultures of strains 10833 and Newman were diluted to an OD₆₀₀ of 0.015, inoculated into test tubes with TSBG or TSBG plus 12.5 mM EGTA and a piece of intravenous catheter tubing of ~2 cm (BD), and incubated at 37 °C for 18 h without shaking. After overnight incubation, the catheter tubes were removed using an aseptic technique, washed once with deionized water, sonicated in 1 ml PBS and plated on TSA to determine the number of c.f.u. The assay was performed in triplicate and the results are represented as the mean c.f.u.

Statistical analysis. Statistical significance was determined using an unpaired Student's *t*-test, and *P*<0.05 was considered significant.

RESULTS

Chelators exert different effects on biofilm formation in different strains

Biofilm assays were performed to determine the effect of EGTA and TSC on biofilm formation in *S. aureus* strain SA113. Because we wanted to determine the effect of these compounds on biofilm formation and not growth, we determined the MICs for EGTA and TSC. We found that 12.5 mM EGTA and 12.5 mM TSC were subinhibitory (0.5 MIC), so these concentrations were used for all subsequent experiments. We quantified the free calcium levels in TSBG and TSBG containing the chelators and found that TSBG contained 4.2 mM Ca²⁺, whilst free Ca²⁺ was not detectable in TSBG containing 12.5 mM EGTA or TSC. As shown in Fig. 1(a), 12.5 mM EGTA and 12.5 mM TSC allowed bacterial growth to occur but completely eliminated biofilm formation (*P*<0.0001). The addition of Ca²⁺ individually or in concert with either EGTA or TSC restored biofilm formation. To extend our findings, we analysed biofilm formation under similar chelating conditions with strains 10833 and Newman (Fig. 1b). The biofilm phenotype was significantly inhibited by EGTA and TSC in strain Newman, similar to what was observed with SA113. However, in strain 10833, EGTA actually increased biofilm formation relative to the diminished or eliminated biofilms observed with strain Newman (Fig. 1b, *P*<0.0001), and inhibition by TSC was significantly greater in strain Newman than it was in strain 10833.

We next tested a panel of clinical isolates from the bloodstream of patients with cardiac device-associated infections (Table 2). Out of 27 isolates, biofilm formation was inhibited (by at least 25%) by both chelators in five

Fig. 1. TSC and EGTA exerted different effects on biofilm formation depending on strain. (a) Strain SA113 was grown in TSBG alone or supplemented with 12.5 mM $CaCl_2$, 12.5 mM EGTA, 12.5 mM TSC, 12.5 mM $CaCl_2$ + 12.5 mM EGTA, or 12.5 mM $CaCl_2$ + 12.5 mM TSC in 96-well microtitre plates. The wells were either resuspended to determine overall growth (OD_{595}) or washed to remove non-adherent bacteria and stained with safranin to gauge biofilm formation. Plates were scanned using a flatbed scanner (right panel) and shown here to represent technical duplicates from one representative plate. The safranin stain was dissolved in acetic acid and quantified at A_{415} . Quantification of growth (dark grey bars) versus biofilm formation (light grey bars) under the respective conditions with the respective media conditions is shown. (b) Strains 10833 and Newman were grown in TSBG alone or supplemented with 12.5 mM EGTA or 12.5 mM TSC and static biofilm assays were performed. ** $P < 0.0001$.

isolates, was not significantly affected by the chelators in three isolates and was augmented by both chelators in eight isolates. The rest of the strains exhibited variable responses to TSC versus EGTA. These results demonstrated that, whilst subinhibitory concentrations of EGTA and TSC inhibited the biofilm phenotype in certain strains of *S. aureus*, they actually stimulated the biofilm phenotype in other strains.

ClfB transcript levels are stimulated by chelators

Because strains 10833 and Newman are closely related, we hypothesized that the diametric phenotype in the presence of chelators would have a relatively straightforward molecular basis and chose to focus our study on this

strain pair (Maira-Litrán *et al.*, 2005). Strong clumping factor activity of strain 10833 has been documented previously and the fibronectin-binding activity of ClfA and ClfB is inhibited by Ca^{2+} (Duthie & Lorenz, 1952). We therefore hypothesized that clumping factor activity might play a role in biofilm formation under chelating conditions. To test this hypothesis, we compared the clumping activity of the two strains in the absence and presence of 12.5 mM EGTA (Fig. 2a). EGTA induced clumping in strain 10833, whereas strain Newman did not exhibit any significant clumping.

ClfA- and ClfB-mediated clumping is usually induced in the presence of fibrinogen. However, we hypothesized that, in the presence of a chelating agent, these surface adhesins could induce clumping in the absence of fibrinogen. We therefore investigated the relative expression of the clumping factor genes *clfA* and *clfB* when bacterial cultures were incubated in TSBG containing 12.5 mM EGTA or 12.5 mM NaCl relative to expression in TSBG alone. The levels of *clfA* transcript were unchanged in EGTA in the two strains (data not shown). However, in strain Newman, *clfB* transcript levels were lower in the presence of EGTA but were increased threefold in the presence of EGTA in strain 10833 (Fig. 2b). Additionally, we observed a 1.5-fold increase in *clfB* transcript levels in strain 10833 in 12.5 mM TSC, whereas the *clfB* transcript was repressed by TSC in strain Newman. In summary, the expression of *clfB* was negatively regulated in the presence of EGTA or TSC in strain Newman but positively regulated by the chelators in strain 10833.

Deletion of *clfB* eliminates EGTA-induced biofilm formation in strain 10833

To investigate further the role of clumping factor B in the differential effect of chelators on biofilm formation in strains Newman and 10833, isogenic *clfB* deletion mutants were produced and biofilm assays were performed (Fig. 3). Deletion of *clfB* in strain 10833 eliminated biofilm formation significantly ($P < 0.0001$) when the strains were grown in TSB + 12.5 mM EGTA but did not significantly affect biofilm formation in the absence of EGTA. Real-time RT-PCR demonstrated that, in strains complemented *in trans* with *clfB*-pCL15, 1 mM IPTG restored *clfB* transcript levels to those of the parental strains (data not shown). Complementation of gene expression restored biofilm formation. In strain Newman, biofilm formation in TSBG was unaffected by the deletion, but the weak biofilm formed in the presence of EGTA was eliminated by the *clfB* mutation (Figs 1b and 3b). Complementation of the gene restored biofilm levels to that observed with the wild-type strain in the presence of EGTA. The effect of the *clfB* deletion on biofilm formation by both strains was similar in TSBG + 12.5 mM TSC (data not shown). To determine whether ClfB was an important biofilm mediator in other strains or whether the phenomenon was restricted to strain 10833, we deleted *clfB* in additional isolates. Cardiac device

Table 2. Effect of 12.5 mM EGTA or 12.5 mM TSC on biofilm formation in 27 cardiac device isolates

^{UP}, Strains that were observed to show a significant increase in biofilm formation with EGTA and TSC relative to the TSBG control ($P < 0.0001$) (shown in bold); ^{DOWN}, strains that had significantly decreased biofilm formation with EGTA and TSC ($P < 0.0001$) (shown in italic).

Strain no.	Biofilm (%) in 12.5 mM EGTA relative to TSBG	Biofilm (%) in 12.5 mM TSC relative to TSBG
Strain 1 ^{DOWN}	29.78 ± 0.07	31.75 ± 0.04
Strain 2 ^{DOWN}	21.06 ± 0.17	74.33 ± 0.04
Strain 3 ^{UP}	125.23 ± 0.06	163.48 ± 0.04
Strain 4	59.25 ± 0.23	173.68 ± 0.07
Strain 5 ^{UP}	161.67 ± 0.05	174.34 ± 0.06
Strain 6	101.70 ± 0.03	82.23 ± 0.04
Strain 7	103.96 ± 0.04	79.01 ± 0.03
Strain 8	132.61 ± 0.05	63.27 ± 0.06
Strain 9 ^{UP}	130.58 ± 0.03	140.28 ± 0.06
Strain 10 ^{UP}	126.37 ± 0.04	145.30 ± 0.04
Strain 11	134.86 ± 0.06	80.62 ± 0.06
Strain 12 ^{DOWN}	6.95 ± 0.01	32.27 ± 0.02
Strain 13	54.26 ± 0.14	92.15 ± 0.10
Strain 14	117.36 ± 0.04	91.35 ± 0.06
Strain 15	209.29 ± 0.10	105.01 ± 0.05
Strain 16	125.52 ± 0.03	90.60 ± 0.04
Strain 17	127.56 ± 0.06	97.27 ± 0.07
Strain 18	113.92 ± 0.02	60.38 ± 0.04
Strain 19 ^{UP}	126.88 ± 0.06	136.82 ± 0.07
Strain 20	203.33 ± 0.07	106.29 ± 0.03
Strain 21 ^{DOWN}	16.49 ± 0.08	45.24 ± 0.03
Strain 22 ^{DOWN}	7.92 ± 0.00	30.65 ± 0.02
Strain 23 ^{UP}	432.11 ± 0.16	243.17 ± 0.06
Strain 24 ^{UP}	314.24 ± 0.08	140.09 ± 0.05
Strain 25 ^{UP}	757.19 ± 0.06	162.41 ± 0.02
Strain 26	73.16 ± 0.01	180.31 ± 0.01
Strain 27	115.06 ± 0.07	126.01 ± 0.07

isolates 19 and 24 both exhibited augmented biofilm formation in the presence of TSC and EGTA. Deletion of the *clfB* gene in these strains severely reduced biofilm formation (Fig. S1, available in JMM Online). Together these results suggested that biofilms formed in the presence of EGTA are *ClfB* dependent. In certain strains, such as 10833, 19 and 24, *ClfB* appeared to be expressed more highly under these conditions, resulting in the production of a thicker biofilm.

Gene sequence and transcriptional start site are conserved in strains 10833 and Newman

To determine whether the difference in *clfB* expression in strains 10833 and Newman was due to a mutation in the gene or promoter, we sequenced the *clfB* gene and the upstream region. The *clfB* gene from strain 10833 was identical to that of strain Newman except for the presence of additional serine-aspartate (SD) repeats, and the upstream promoter regions were 100% identical to the previously published strain Newman sequence (Baba *et al.*, 2008). We also determined the transcriptional start site using 5'-RACE and found that transcription started at the same nucleotide in both strains (Fig. 4).

Increased adherence of strain 10833 to catheters in the presence of EGTA

To test further the hypothesis that chelators such as EGTA may not effectively inhibit biofilm formation on catheters by all *S. aureus* strains, we quantified adherence to catheter tubing. Adherence of strain Newman to catheter tubing was inhibited by EGTA (Fig. 5). However, a sixfold increase in adherence of strain 10833 was observed in the presence of 12.5 mM EGTA relative to that for the TSBG control.

DISCUSSION

Catheter-related BSIs are a significant iatrogenic complication associated with the use of implanted intravascular devices, and incidence increases proportionally with the length of time that the catheter is left in place. Use of chelating agents such as TSC in CLS has been shown previously to disrupt the process of bacterial adherence and biofilm formation on the synthetic surface, and can help prevent catheter colonization (Shanks *et al.*, 2008; Weijmer *et al.*, 2002). However, catheter-related infections still occur, suggesting that bacteria are able to form biofilms even in the presence of antibiofilm CLS. This study

Fig. 2. Expression of *clfB* in the presence of TSC and EGTA. (a) Strains 10833 and Newman were incubated without shaking in TSBG alone or supplemented with 12.5 mM EGTA at 37 °C for 18 h. The tubes were briefly vortexed and then imaged. (b) Total RNA was isolated from strains 10833 and Newman induced in TSBG, TSBG + 12.5 mM EGTA and TSBG + 12.5 mM TSC during the mid-exponential phase of growth. Transcript levels of the clumping factor gene *clfB* were analysed by real-time RT-PCR. Individual genes were normalized to the 16S rRNA gene and the relative fold difference is shown with respect to expression in TSBG. Black bars represent relative expression of the gene expressed by strain 10833, whilst grey bars are representative of strain Newman. ** $P < 0.0001$.

indicated that certain strains of *S. aureus* are resistant to the antibiofilm effects of chelating agents, and the results suggest that, in certain cases, subinhibitory concentrations of the chelators could enhance biofilm formation.

We found that TSC, which was expected to inhibit biofilm formation, was effective in two out of three laboratory strains but did not inhibit biofilm formation to the same extent in the third strain, 10833. TSC has been shown to augment biofilm formation by serving as a TCA cycle intermediate; however, the concentration used in this study (12.5 mM) was considerably higher than the concentration that induces biofilm formation (Shanks *et al.*, 2006, 2008). Furthermore, this concentration was high enough to remove all detectable free Ca^{2+} from the medium. We

Fig. 3. Deletion of *clfB* eliminates biofilm formation in the presence of EGTA. *S. aureus* strains 10833 (a) and Newman (b) and their isogenic *clfB* mutant and complementation derivatives were grown in 96-well polystyrene plates in TSBG (dark grey bars) or TSBG + 12.5 mM EGTA (light grey bars) and assessed for biofilm formation. Biofilms were stained for visualization (right panel) and quantified as A_{415} (left graph). The complementation mutants were induced (I) with 1 mM IPTG or uninduced (U). ** $P < 0.0001$.

therefore hypothesized that the effect was due to Ca^{2+} chelation, rather than to effects on the TCA cycle. To test this, we used EGTA in addition to TSC. Whilst EGTA inhibited biofilm formation in strains SA113 and Newman, it actually augmented it in strain 10833, suggesting that the difference in biofilm-forming capacity of strain 10833 was due to a differential response to Ca^{2+} chelation. When we tested a panel of cardiac device isolates, we found that eight out of 27 of the isolates exhibited increased biofilm formation in the presence of both EGTA and TSC. However, some strains were affected differently by the two compounds, in that some strains exhibited significantly increased biofilm formation in EGTA but less in TSC, or a decrease in biofilm formation in EGTA and an increase in TSC. Despite this, the resistance of strains to the antibiofilm effect of the two chelating agents displayed a trend in the same direction, in that all strains that exhibited increased biofilm formation in EGTA either exhibited an

Fig. 4. Schematic representation of the *clfB* gene displaying the transcriptional start site (+1, underlined) for strains 10833 and Newman, which was identical for the two strains, as determined by 5'-RACE analysis.

increase in TSC or only a slight decrease, and all strains that displayed a decrease in biofilm formation in EGTA displayed either a decrease in TSC or only a slight increase. The differential effects of TSC and EGTA may be attributable to a number of side-effects of TSC. TSC chelates not only Ca^{2+} ions but other divalent cations as well, it is a TCA cycle intermediate, and it is a weak acid and can alter the pH of the growth medium. EGTA can bind Mg^{2+} in addition to Ca^{2+} but with much lower affinity. Therefore, EGTA is considerably more specific as a Ca^{2+} -chelating agent, whereas TSC may exert additional effects on the bacteria and on their ability to form a biofilm.

The concentrations of the chelators used in this study were subinhibitory, suggesting that they specifically affected the biofilm phenotype. The concentration of TSC used in this

study was lower than the concentrations used in catheter lock solutions. However, subinhibitory doses would be expected during dialysis, as the CLS is removed from the catheter lumen.

Although it is likely that the molecular basis of the capacity for biofilm formation under chelating conditions varies from strain to strain, we focused our studies on strains 10833 and Newman. Due to their genetic similarity, we hypothesized that the contrasting effect of chelating agents on biofilm formation in these strains was based on the differential expression of just one or a few genes. Indeed, we found that biofilm formation by strain 10833 in the presence of EGTA or TSC required expression of the *clfB* gene. Thus, whilst there may be other genetic differences between strains 10833 and Newman that contribute to the differences in their capacity to establish biofilms in the presence of chelating agents, differential *clfB* expression appears to play a key role. Further supporting the role for ClfB, deletion of *clfB* from selected cardiac device-associated isolates (strains 19 and 24) that exhibited increased biofilm formation in the presence of EGTA and TSC lost this phenotype when *clfB* was deleted within these strains. This was a novel finding, as ClfB has not been implicated previously in biofilm formation. However, other surface-associated adhesin proteins are known to play a role in biofilm formation and, in particular, the biofilm-associated protein (Bap), similar to the clumping factor proteins, has an EF-hand domain and is regulated by Ca^{2+} .

ClfB regulation may occur on multiple levels. ClfB has a partial EF-hand domain, suggesting that it can coordinate divalent cations, and its autoaggregative activity is inhibited by Ca^{2+} and Mn^{2+} (Ní Eidhin *et al.*, 1998). Therefore, regulation at the level of activity would be suspected. However, we also found evidence that both EGTA and TSC regulated *clfB* expression at the transcript level and that the gene was regulated differentially in these two strains.

Isogenic deletion of *clfB* in strain 10833 strongly repressed biofilm formation and further weakened the phenotype in strain Newman in the presence of EGTA but did not affect biofilm formation in the absence of EGTA. This suggests that, despite the decreased *clfB* expression in the presence of EGTA in strain Newman, whatever ClfB is present on the surface of *S. aureus* contributes to biofilm formation under these conditions. The finding that biofilm formation was unaffected by the *clfB* deletion in TSBG suggested that other biofilm-related factors contribute to biofilm formation in the absence of EGTA but that ClfB is the major determinant of biofilm-forming potential in the presence

Fig. 5. Strain 10833 exhibited greater adherence to catheter tubing in the presence of EGTA than strain Newman. Bacteria were diluted in test tubes with TSBG (dark grey bars) or TSBG + 12.5 mM EGTA (light grey bars) along with a piece of Vialon-based catheter tubing of ~2 cm. The tubes were incubated for 18 h at 37 °C without shaking. The catheters were removed in a sterile fashion, washed to remove non-adherent bacteria, sonicated and plated to determine c.f.u. counts on TSA plates. ** $P < 0.0001$.

of EGTA. Biofilms produced by *S. aureus* are held together by a mixture of proteinaceous adhesins along with polysaccharides and extracellular DNA. Because so many factors can contribute to the formation of a biofilm, the phenotype may be difficult to target pharmacologically, and agents with antibiofilm activity against certain strains may not inhibit biofilm formation in other strains.

In summary, the capacity to form a biofilm under chelating conditions varies significantly among different strains of *S. aureus*. When Ca^{2+} is depleted by chelating agents, biofilm formation is eliminated in some strains, whereas it is retained or even augmented in others. ClfB has not been implicated in biofilm formation previously, but this study demonstrates a role for this surface protein in mediating biofilm formation under Ca^{2+} -depleted conditions. Whilst we found that there was an increase in *clfB* transcript levels in strain 10833 under chelating conditions, we do not yet know the full molecular basis for the differential ClfB-mediated biofilm formation. Studies to resolve this phenomenon are currently under way.

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REFERENCES

- Arnaud, M., Chastanet, A. & Débarbouillé, M. (2004). New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, Gram-positive bacteria. *Appl Environ Microbiol* **70**, 6887–6891.
- Baba, T., Bae, T., Schneewind, O., Takeuchi, F. & Hiramatsu, K. (2008). Genome sequence of *Staphylococcus aureus* strain Newman and comparative analysis of staphylococcal genomes: polymorphism and evolution of two major pathogenicity islands. *J Bacteriol* **190**, 300–310.
- Banin, E., Brady, K. M. & Greenberg, E. P. (2006). Chelator-induced dispersal and killing of *Pseudomonas aeruginosa* cells in a biofilm. *Appl Environ Microbiol* **72**, 2064–2069.
- Centers for Disease Control and Prevention (2011). Vital signs: central line-associated blood stream infections – United States, 2001, 2008, and 2009. *MMWR Morb Mortal Wkly Rep* **60**, 243–248.
- Christensen, G. D., Simpson, W. A., Younger, J. J., Baddour, L. M., Barrett, F. F., Melton, D. M. & Beachey, E. H. (1985). Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *J Clin Microbiol* **22**, 996–1006.
- Clarke, S. R. & Foster, S. J. (2006). Surface adhesins of *Staphylococcus aureus*. *Adv Microb Physiol* **51**, 187–224.
- Corrigan, R. M., Rigby, D., Handley, P. & Foster, T. J. (2007). The role of *Staphylococcus aureus* surface protein SasG in adherence and biofilm formation. *Microbiology* **153**, 2435–2446.
- Cucarella, C., Tormo, M. A., Knecht, E., Amorena, B., Lasa, I., Foster, T. J. & Penadés, J. R. (2002). Expression of the biofilm-associated protein interferes with host protein receptors of *Staphylococcus aureus* and alters the infective process. *Infect Immun* **70**, 3180–3186.
- Dunne, W. M., Jr & Burd, E. M. (1992). The effects of magnesium, calcium, EDTA, and pH on the *in vitro* adhesion of *Staphylococcus epidermidis* to plastic. *Microbiol Immunol* **36**, 1019–1027.
- Duthie, E. S. & Lorenz, L. L. (1952). Staphylococcal coagulase: mode of action and antigenicity. *J Gen Microbiol* **6**, 95–107.
- Grundmeier, M., Hussain, M., Becker, P., Heilmann, C., Peters, G. & Sinha, B. (2004). Truncation of fibronectin-binding proteins in *Staphylococcus aureus* strain Newman leads to deficient adherence and host cell invasion due to loss of the cell wall anchor function. *Infect Immun* **72**, 7155–7163.
- Hall-Stoodley, L., Costerton, J. W. & Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* **2**, 95–108.
- Juda, M., Paprota, K., Jaloza, D., Malm, A., Rybojad, P. & Goździuk, K. (2008). EDTA as a potential agent preventing formation of *Staphylococcus epidermidis* biofilm on polychloride vinyl biomaterials. *Ann Agric Environ Med* **15**, 237–241.
- Kasatiya, S. S. & Baldwin, J. N. (1967). Nature of the determinant of tetracycline resistance in *Staphylococcus aureus*. *Can J Microbiol* **13**, 1079–1086.
- Lee, J. C. (1993). *Electrotransformation of Staphylococci*. Totowa, NJ: Humana Press.
- Luong, T. T. & Lee, C. Y. (2006). The *arl* locus positively regulates *Staphylococcus aureus* type 5 capsule via an *mgrA*-dependent pathway. *Microbiology* **152**, 3123–3131.
- Maira-Litrán, T., Kropec, A., Goldmann, D. A. & Pier, G. B. (2005). Comparative opsonic and protective activities of *Staphylococcus aureus* conjugate vaccines containing native or deacetylated staphylococcal poly-N-acetyl- β -(1-6)-glucosamine. *Infect Immun* **73**, 6752–6762.
- Mermel, L. A., Allon, M., Bouza, E., Craven, D. E., Flynn, P., O'Grady, N. P., Raad, I. I., Rijnders, B. J., Sherertz, R. J. & Warren, D. K. (2009). Clinical practice guidelines for the diagnosis and management of intravascular catheter-related infection: 2009 update by the Infectious Diseases Society of America. *Clin Infect Dis* **49**, 1–45. [Erratum: *Clin Infect Dis* **50**, 457, 2010; and *Clin Infect Dis* **50**, 1079, 2010].
- Ní Eidhin, D., Perkins, S., Francois, P., Vaudaux, P., Höök, M. & Foster, T. J. (1998). Clumping factor B (ClfB), a new surface-located fibrinogen-binding adhesin of *Staphylococcus aureus*. *Mol Microbiol* **30**, 245–257.
- O'Grady, N. P., Alexander, M., Burns, L. A., Dellinger, E. P., Garland, J., Heard, S. O., Lipsett, P. A., Masur, H., Mermel, L. A. & other authors (2011). Guidelines for the prevention of intravascular catheter-related infections. *Am J Infect Control* **39** (Suppl. 1), S1–S34.
- Ozerdem Akpolat, N., Elçi, S., Atmaca, S., Akbayin, H. & Gül, K. (2003). The effects of magnesium, calcium and EDTA on slime production by *Staphylococcus epidermidis* strains. *Folia Microbiol (Praha)* **48**, 649–653.
- Percival, S. L., Kite, P., Eastwood, K., Murga, R., Carr, J., Arduino, M. J. & Donlan, R. M. (2005). Tetrasodium EDTA as a novel central venous catheter lock solution against biofilm. *Infect Control Hosp Epidemiol* **26**, 515–519.
- Raad, I., Chatzinikolaou, I., Chaiban, G., Hanna, H., Hachem, R., Dvorak, T., Cook, G. & Costerton, W. (2003). In vitro and ex vivo activities of minocycline and EDTA against microorganisms embedded in biofilm on catheter surfaces. *Antimicrob Agents Chemother* **47**, 3580–3585.
- Sarkisova, S., Patrauchan, M. A., Berglund, D., Nivens, D. E. & Franklin, M. J. (2005). Calcium-induced virulence factors associated with the extracellular matrix of mucoid *Pseudomonas aeruginosa* biofilms. *J Bacteriol* **187**, 4327–4337.

Shanks, R. M., Sargent, J. L., Martinez, R. M., Graber, M. L. & O'Toole, G. A. (2006). Catheter lock solutions influence staphylococcal biofilm formation on abiotic surfaces. *Nephrol Dial Transplant* **21**, 2247–2255.

Shanks, R. M., Meehl, M. A., Brothers, K. M., Martinez, R. M., Donegan, N. P., Graber, M. L., Cheung, A. L. & O'Toole, G. A. (2008). Genetic evidence for an alternative citrate-dependent biofilm formation pathway in *Staphylococcus aureus* that is dependent on

fibronectin binding proteins and the GraRS two-component regulatory system. *Infect Immun* **76**, 2469–2477.

Wann, E. R., Gurusiddappa, S. & Hook, M. (2000). The fibronectin-binding MSCRAMM FnbpA of *Staphylococcus aureus* is a bifunctional protein that also binds to fibrinogen. *J Biol Chem* **275**, 13863–13871.

Weijmer, M. C., Debets-Ossenkopp, Y. J., Van De Vondervoort, F. J. & ter Wee, P. M. (2002). Superior antimicrobial activity of trisodium citrate over heparin for catheter locking. *Nephrol Dial Transplant* **17**, 2189–2195.