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SdrC induces staphylococcal biofilm formation through a homophilic interaction

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Summary

The molecular pathogenesis of many *Staphylococcus aureus* infections involves growth of bacteria as biofilm. In addition to polysaccharide intercellular adhesin (PIA) and extracellular DNA, surface proteins appear to mediate the transition of bacteria from planktonic growth to sessile lifestyle as well as biofilm growth, and can enable these processes even in the absence of PIA expression. However, the molecular mechanisms by which surface proteins contribute to biofilm formation are incompletely understood. Here we demonstrate that self-association of the serine-aspartate repeat protein SdrC promotes both bacterial adherence to surfaces and biofilm formation. However, this homophilic interaction is not required for the attachment of bacteria to abiotic surfaces. We identified the subdomain that mediates SdrC dimerization and subsequent cell-cell interactions. In addition, we determined that two adjacently located amino acid sequences within this subdomain are required for the SdrC homophilic interaction. Comparative amino acid sequence analysis indicated that these binding sites are conserved. In summary, our study identifies SdrC as a novel molecular determinant in staphylococcal biofilm formation and describes the mechanism responsible for intercellular interactions. Furthermore, these findings contribute to a growing body of evidence suggesting that homophilic interactions between surface proteins present on neighbouring bacteria induce biofilm growth.

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Introduction

Bacterial biofilms are communities of microorganisms growing attached to biotic or abiotic surfaces. Within the biofilm, bacteria encase themselves in a self-secreted matrix with precise micro-architectural properties that allow free circulation of nutrients, water and metabolites (Costerton *et al.*, 1978). Besides acting as a scaffold, the matrix enables microbes to survive and persist as reservoirs, avoid desiccation or antimicrobials, and subvert host defences. In clinical settings, biofilms allow pathogens to establish chronic and/or infections that are refractory to treatment (Costerton *et al.*, 1999). Thus, it has long been recognized that their effective therapy management is challenging.

Widely known as an opportunistic pathogen, *Staphylococcus aureus* is a frequent cause of biofilm-related infections (Otto, 2008). The transition of staphylococci from planktonic organisms to sessile growth is triggered by environmental cues or receptor availability (Donlan, 2002). Overall bacterial surface hydrophobicity determines the degree of attachment to inert materials (Pascual *et al.*, 1986). In the case of *S. aureus*, colonization of abiotic components is attributed to teichoic acids and autolysin Atl (Gross *et al.*, 2001; Houston *et al.*, 2011). Attachment to host tissues and synthetic surfaces coated with plasma proteins is facilitated by members of the MSCRAMM subfamily of cell-wall-anchored (CWA) proteins (Ponnuraj *et al.*, 2003; Foster *et al.*, 2014). For example, fibrinogen-binding proteins, such as clumping factor A ClfA, ClfB, fibronectin (Fn)-binding protein FnBPA, FnBPB (McDevitt *et al.*, 1994; Ni Eidhin *et al.*, 1998; Schwarz-Linek *et al.*, 2003; Keane *et al.*, 2007), and collagen-binding protein (Cna) (Patti *et al.*, 1993) initiate adherence, and as a result, favour biofilm formation. Upon attachment, bacteria proliferate and synthesize a scaffolding matrix composed of intercellular polysaccharide adhesin (PIA) (Cramton *et al.*, 1999), CWA proteins (Cucarella *et al.*, 2001; Corrigan *et al.*, 2007; O'Neill *et al.*, 2008; Merino *et al.*, 2009; Schroeder *et al.*, 2009; Abraham and Jefferson, 2012) and

extracellular DNA (eDNA) (Izano *et al*., 2008). Accumulating evidence suggest that CWA proteins important for biofilm growth include biofilm associated protein (Bap) (Cucarella *et al*., 2001), ClfB (Abraham and Jefferson, 2012), FnBPA, FnBPB (O'Neill *et al*., 2008), *Staphylococcus aureus* surface protein SasC (Schroeder *et al*., 2009), SasG (Corrigan *et al*., 2007) and surface protein A (SpA) (Merino *et al*., 2009). However, Bap has been found only in bovine mastitis isolates (Cucarella *et al*., 2001). While ClfB, FnBPA, FnBPB, SpA and SasC are found in the majority of strains, SasG is present only in a subset of isolates (Corrigan *et al*., 2007; Schroeder *et al*., 2009). The molecular mechanism by which these proteins contribute to intercellular interaction is largely unknown, but recent studies suggest the involvement of metal-ion dependent interactions between CWA proteins present on adjacent cells (Conrady *et al*., 2008; 2013; Geoghegan *et al*., 2010; 2013; Abraham and Jefferson, 2012).

The initiation and development of biofilm appears to be chiefly regulated by the quorum sensing accessory gene regulator (Agr) system in response to cell density. Typically, at low density staphylococci express surface proteins involved in cell attachment and biofilm accumulation (Periasamy *et al*., 2012). When the population reaches high density, Agr activation triggers the upregulation of toxin, proteases and surfactants involved in biofilm dispersal and bacterial dissemination. Conversely, inactivation of Agr leads to an augmented biofilm phenotype, in part due to the deregulation of surface proteins involved in adherence and biofilm growth. In agreement, recent studies have indicated that these mutants form a thicker and more resistant biofilms presumably due to perpetual expression of CWA proteins that engage newborn cells within the structure. Notably, *agr* spontaneous mutants are frequently isolated from biofilms (Shopsin *et al*., 2010).

Despite their lack of sequence similarity, MSCRAMMs share common structural organization. An amino terminal signal sequence is followed by the ligand-binding A region subdivided into three subdomains (N1, N2 and N3), where N2 and N3 each adopt an IgG-like fold. In a subset of MSCRAMMs, the A-region is followed by a B-region containing repeated β-sandwich modules of unknown function (Fig 1A). In the case of the Sdr subfamily (serine-aspartate repeats) of MSCRAMMs, the B-region is accompanied by a repeat (R) domain composed of multiple Ser-Asp dipeptide repeats. The carboxyl terminal section of the proteins contains the LPXTG motif required for cell wall anchoring (reviewed in Foster *et al*., 2014; Fig. 1A). The ligand-binding activity of MSCRAMMs to host molecules has been shown to proceed via a 'dock, lock and latch' mechanism in which the ligand peptide 'docks' into the groove formed between the N2 and N3 subdomains. The ligand is 'locked' in place by interacting with amino acids residues in the C-terminal extension of the N3 domain. The complex is stabilized by the insertion of the subsequent 'latch' region into the N2 domain through a β-strand complementation (Ponnuraj *et al*., 2003).

Using phage display, we previously identified peptides targeting the putative ligand-binding region of SdrC. Further analysis of one peptide motif identified β-neurexin as a host ligand for SdrC (Barbu *et al*., 2010). In this study, we report that two other consensus sequences identified by our phage display screen are responsible for SdrC self-association and that this interaction is inhibited by Mn^{2+}. As a result, SdrC promotes bacterial intercellular interactions and contributes to biofilm formation. Exploiting heterologous expression in the non-pathogenic bacterium *Lactococcus lactis*, we show that SdrC also triggers bacterial adhesion to abiotic surfaces. However, bacterial attachment to uncoated inert surfaces is independent of self-association. Moreover, our results indicate that the SdrC contribution to biofilm formation is dependent on the strain background.

Results

The N2 subdomain mediates SdrC self-association

We demonstrated previously the feasibility of screening phage display libraries to identify binding partners for MSCRAMMs. We identified peptides binding to the orphan MSCRAMM SdrC after three consecutive rounds of selection with a 12-amino-acid random phage library. Although several consensus sequences were identified, only one motif matched a protein found in the human protein sequence database (β-neurexin) (Barbu *et al*., 2010). In an effort to identify ligands corresponding to the other consensus amino acids (RPGSV and VDQXT) (Fig. 1A), we repeated the similarity search using a staphylococcal protein sequence database. We found that these sequences are located adjacent to each other (positions 247–251 and 288–292) in the C-terminal region of the N2 subdomain of SdrC itself (Fig. 1A). Selected purified phage clones specifically bound recombinant $SdrC_{N2N3}$ ($rSdrC_{N2N3}$), whereas they did not bind to $rSdrG_{N2N3}$, a similar MSCRAMM from *S. epidermidis* (Fig. 1B).

Considering that the peptide sequences identified by phage display are found in the bait protein, we reasoned that SdrC interacts with itself. We compared the binding of SdrC subdomains to each other using a solid-phase binding assay. Recombinant polypeptides containing the N2 subdomain showed significant levels of binding to N2 subdomain-containing proteins relative to N1 and B segments (Fig. 2A; $P < 0.01$). To establish binding specificity, either N2 or N2N3 recombinant proteins were immobilized on microtitre plates and probed with increasing con-

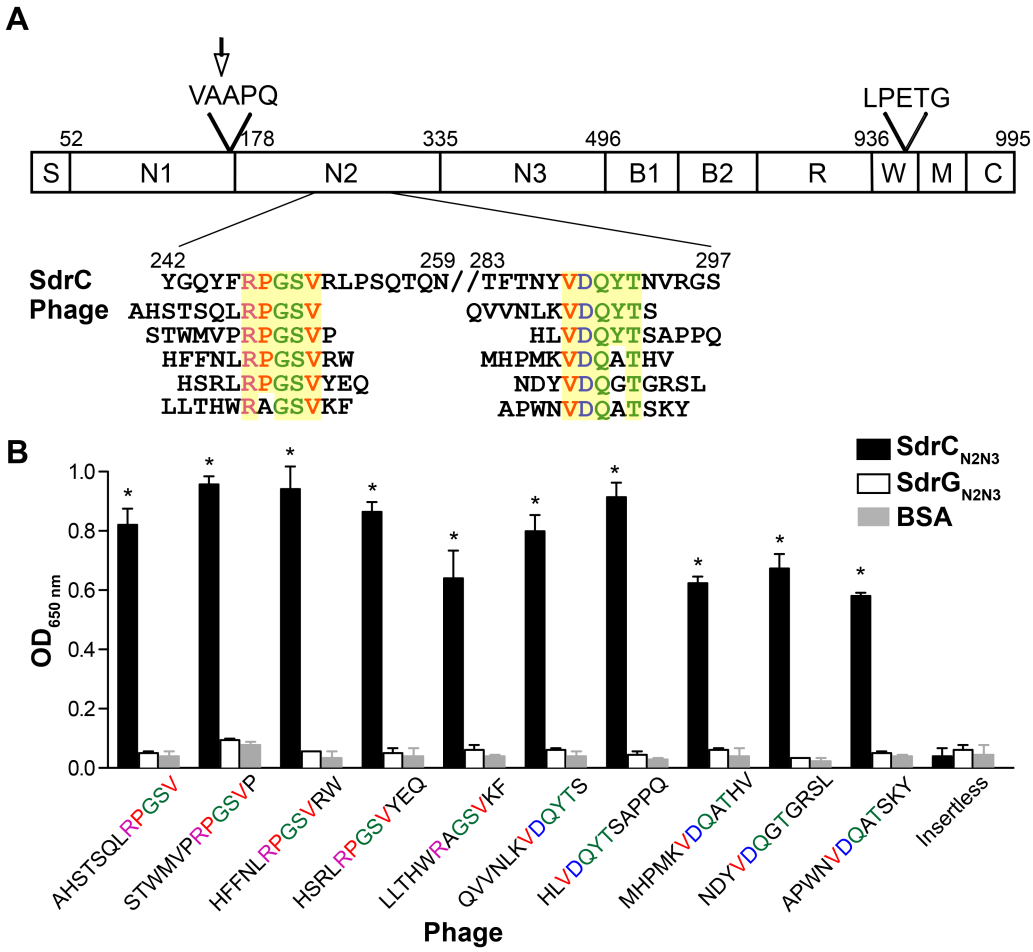


Fig. 1. Specificity of enriched peptide-displaying phage.
A. Schematic overall representation of SdrC domains. S, signal sequence; A region, composed of N1, N2 and N3; B repeats, B1 and B2; R, serine-aspartic acid repeat region; W, wall-spanning fragment; M, transmembrane domain; C, cytoplasmic tail; LPETG, cell wall anchoring motif; VAAPQ, cleavage site; the arrow indicates the enzymatic cleavage position. Also shown are the enriched phage-displayed peptides aligned with the relevant SdrC sequence. Consensus sequences are highlighted in yellow.
B. Purified phage clones binding to immobilized $SdrC_{N2N3}$, $SdrG_{N2N3}$ and BSA. Binding was detected with an anti-M13-HRP antibody. The data shown are representative of three individual experiments performed in triplicate. Bars represent mean ± standard error of the mean (SEM); $^{*}P < 0.001$.

centrations of biotin-labelled subdomains. We found that N2-containing proteins bound in a dose-dependent and saturable manner to both proteins. The apparent dissociation constant (corresponding to the concentration needed for half maximum binding) ranged between 0.2 and 0.3 μM (Fig. 2B and C). To further assess the binding specificity, we tested the ability of consensus peptides to inhibit N2-mediated SdrC self-association. Purified phage clones displaying each of the self-binding motifs inhibited the binding of biotin-labelled $rSdrC_{N2}$ to immobilized $rSdrC_{N2N3}$ by nearly 50% (Fig. 2D). Of importance, the binding was completely inhibited only when both phage clones displaying consensus sequences were combined, suggesting that the identified binding sites act cooperatively (Fig. 2D). An insertless phage had no effect on the N2 subdomain interaction with its partner. Similarly, antibodies against the N2N3 domain of SdrC inhibited the N2-containing domains binding to each other (Fig. 2E). In contrast, antibodies against $ClfB_{N2N3}$, a related staphylococcal MSCRAMM, had no effect on $rSdrC_{N2}$ binding to $rSdrCN_{N2N3}$ (Fig. 2E).

To determine the effects of N2 subdomain association, we compared the gel-permeation profiles of $rSdrC_{N2}$ (calculated theoretical mass 19 kDa), $rSdrC_{N3}$ (19.1 kDa) and $rSdrC_{N2N3}$ (36.8 kDa) subdomains. Both $rSdrC_{N2}$ and $rSdrC_{N2N3}$ eluted as two major distinct peaks (Fig. 2F) where the peaks corresponding to the larger isoforms contained most of the protein. The relative molecular masses calculated from the linear regression based on column calibration with globular proteins revealed a 40 kDa

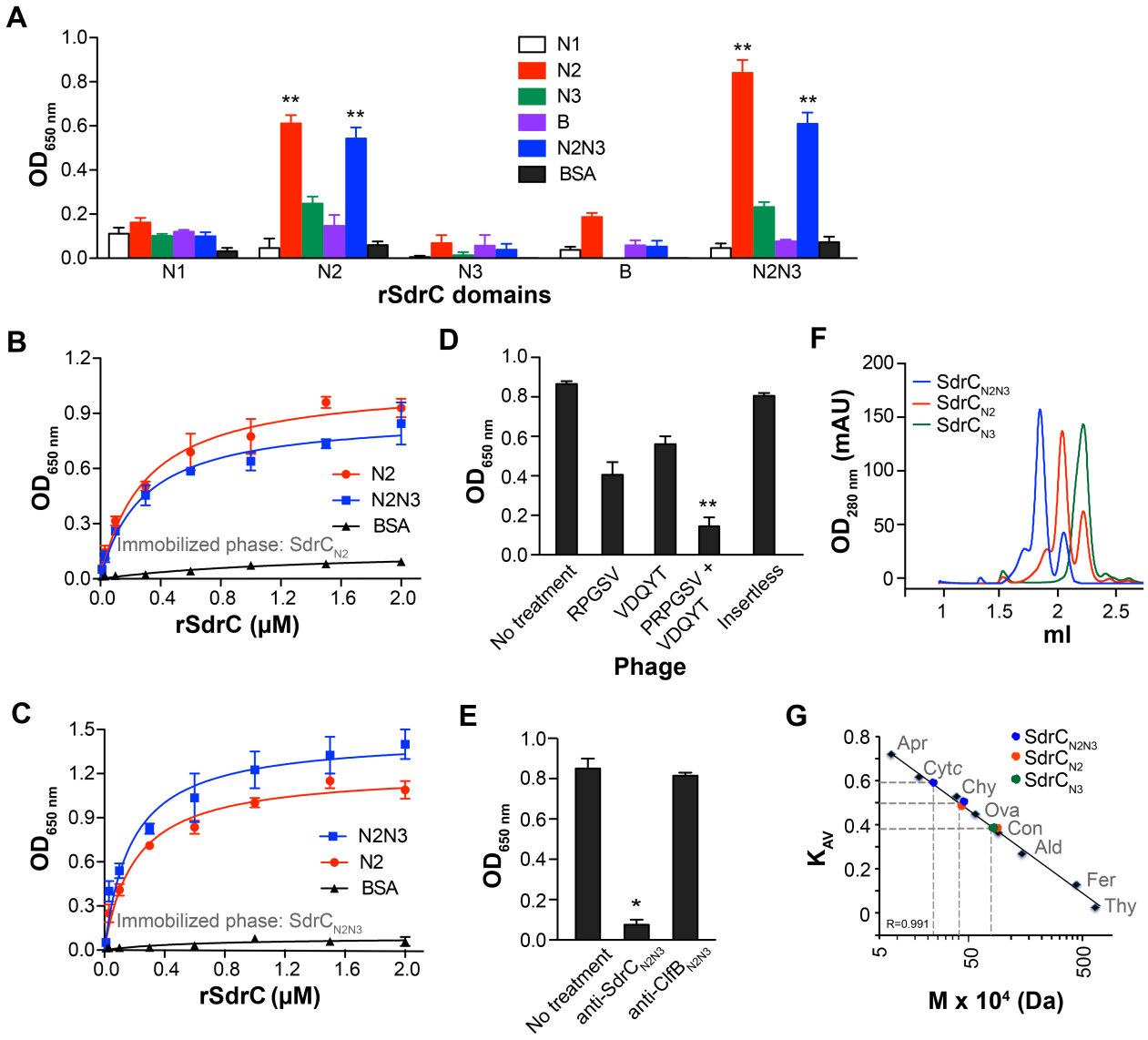


Fig. 2. The N2 subdomain mediates SdrC dimerization.
A. Biotin-labelled recombinant SdrC subdomains (1 μM) binding to unlabelled segments. The interaction was detected with avidin-HRP.
B and C. Dose-dependent and saturable binding of N2 (red) and N2N3 (blue) subdomains to one another. Increasing concentrations of biotin-labelled subdomains were incubated with either immobilized (B) unlabelled N2 or (C) N2N3. The apparent dissociation constant (K_D) calculated using the equation $\Delta P = \Delta P_{max}$ [protein]/(K_D + [protein]) ranged between 0.2 and 0.3 μM.
D and E. Inhibition of N2N3 subdomain dimerization by phage displaying consensus peptide sequences (D) or anti-$SdrC_{N2N3}$ antibodies (E). Immobilized recombinant $SdrC_{N2N3}$ protein was first allowed to interact with phage or antibodies and then incubated with biotin-labelled recombinant $SdrC_{N2}$ protein. Insertless phage and anti-ClfB antibodies were used as negative controls. Binding was detected with an anti-M13-HRP antibody or avidin-HRP. The data shown are representative of three individual experiments performed in triplicate. Bars represent mean ± SEM; *$P < 0.001$, **$P < 0.01$.
F. N2 subdomain-mediated dimer formation demonstrated by gel permeation chromatography. Pure recombinant SdrC subdomains were separated on a Sephadex 200 5/150 GL column at a flow rate of 0.3 ml min^{-1}.
G. Interpolated relative molecular masses of SdrC species from the linear regression based on column calibration with thyroglobulin (Thy, 659 kDa), ferritin (Fer, 440 kDa), aldolase (Ald, 158 kDa), conalbumin (Con, 75 kDa), ovalbumin (Ova, 44 kDa), chymotrypsin (Chy, 29 kDa), cytochrome *c* (Cyt*c*, 12.4 kDa), aprotinin (Fer, 6.5 kDa). Gel phase distribution coefficients (K_{AV}) were calculated from the respective elution volumes (Ve) and represented as a function of molecular mass.

dimeric species and an 18 kDa form indicative of a monomeric state for $rSdrC_{N2}$ (Fig. 2F and G). Similarly, $rSdrC_{N2N3}$ eluted as a 76 kDa dimer and a 36 kDa monomeric form (Fig. 2F and G). In contrast, $rSdrC_{N3}$ eluted from the column as a single peak with a relative molecular mass of 19 kDa, which is similar to the calculated theoretical mass of the monomer (Fig. 2F and G). These results indicate that the N2 subdomain mediates SdrC self-association.

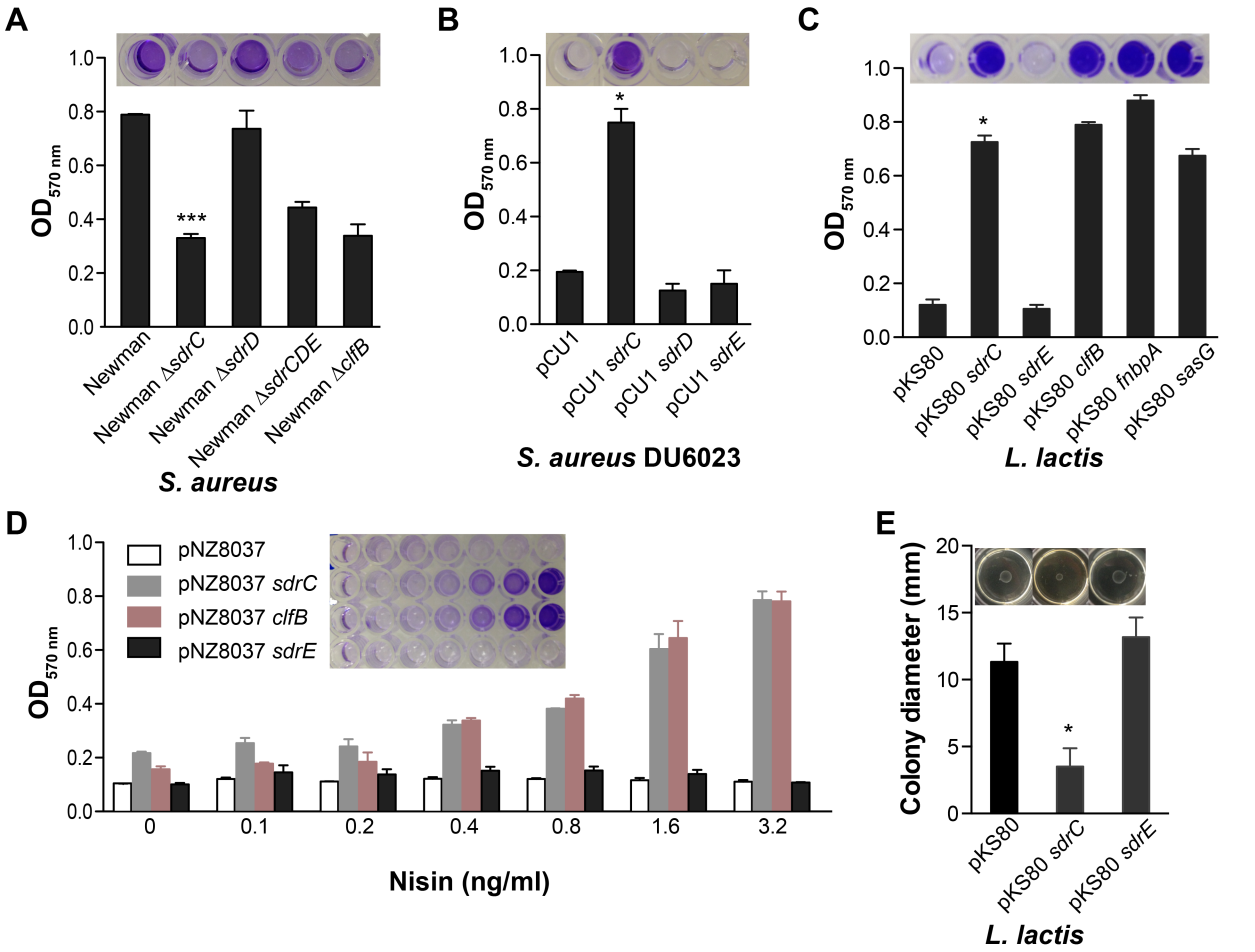


Fig. 3. SdrC contributes to staphylococcal biofilm formation.
A and B. Biofilm formation on plastic plates in TSB 1% glucose medium (TBSG) by (A) *S. aureus* Newman, Newman Δ*sdrC*, Newman Δ*sdrD*, Newman Δ*sdrCDE*, and Newman Δ*clfB* or (B) *S. aureus* Newman DU6023 pCU1, DU6023 pCU1 *sdrC*, DU6023 pCU1 *sdrD*, and DU6023 pCU1 *sdrE*. Bacteria were added to microtitre wells at $OD_{600} = 0.01$.
C and D. Biofilm formation in M17 medium containing 0.5% glucose (GM17) by (C) *L. lactis* pKS80 or *L. lactis* pKS80 constitutively expressing SdrC, SdrE, ClfB, FnBPA or SasG or (D) *L. lactis* pNZ8037, *L. lactis* pNZ8037 *sdrC*, and *L. lactis* pNZ8037 *clfB*. Bacteria were added to microtitre wells at $OD_{600} = 0.01$. Heterologous protein expression was induced with increasing concentrations of nisin. Static biofilm formation was measured by staining with 0.5% crystal violet (CV). The data shown are representative of three individual experiments performed in triplicate. Bars represent mean ± SEM; $^{*}P < 0.001$, $^{***}P < 0.05$. Representative images of wells containing the CV extracted from previously stained biofilm are shown above the graphs.
E. *L. lactis* pKS80, pKS80 *sdrC*, pKS80 *sdrE* colony spreading motility overnight incubation at 30°C. Bacteria from overnight cultures (2 μl) were spotted on soft agar GM17 plates. The data shown are representative of six individual experiments. Bars represent mean ± SEM; $^{*}P < 0.001$. Representative images of colony swarming motility are shown above the graphs.

SdrC contributes to staphylococcal biofilm formation

An earlier study investigating biofilm formation by *S. aureus* SA113, an Agr-defective isolate, revealed that *sdrC* is highly expressed in mature biofilm (Resch *et al.*, 2005). Since surface molecule self-association may lead to intercellular interactions, we hypothesized that SdrC may be involved in biofilm formation. We compared the biofilm growth of the parent strain *S. aureus* Newman (a strain which does not express SasG, and does not anchor FnBPA and FnBPB on the cell-wall), and *clfB*, *sdrC*, *sdrD*, *sdrCDE* mutants. A mutation in *sdrC* significantly inhibited biofilm formation, whereas an *sdrD* single mutant had no detectable effect (Fig. 3A). Biofilm formation by Newman *clfB* (Abraham and Jefferson, 2012) or Δ*sdrCDE* was reduced compared to the wild-type strain (Fig. 3A). Complete elimination of biofilm was not detected because these strains likely express additional factors involved in biofilm accumulation. To clarify this, we assessed the biofilm formed by *S. aureus* Newman mutant (DU6023) (Corrigan *et al.*, 2009), a strain defective in the CWA proteins ClfA, ClfB, IsdA,

IsdB, SdrC, SdrD and SdrE, complemented with SdrC on a multicopy plasmid. We found that SdrC expression restored biofilm formation (Fig. 3B). To determine whether additional staphylococcal factors are necessary for SdrC activity, we exploited the non-pathogenic bacterium *L. lactis*. Biofilm accumulation by lactococci constitutively expressing CWA proteins known to promote this type of growth was significant relative to the parent strain carrying the empty vector pKS80. SdrC conferred biofilm-forming activity similar to that of ClfB, FnBPA and SasG, whereas SdrE expression did not contribute to biofilm growth (Fig. 3C). Furthermore, a strong correlation between inducible surface protein expression and biofilm formation was observed for both SdrC and ClfB (Fig. 3D).

Previous work showed that MSCRAMMs, such as FnBPA and ClfB, involved in cell-cell aggregation and biofilm formation antagonize colony spreading motility on soft agar consistent with the hypothesis that these proteins promote intercellular adherence (Tsompanidou *et al.*, 2012). Similarly, SdrC also reduced the efficiency of spreading of lactococci on wet surfaces, whereas SdrE had no effect (Fig. 3E). These data demonstrate that SdrC contributes to staphylococcal intercellular interactions and subsequent biofilm formation *in vitro*.

Inhibition of SdrC self-association inhibits biofilm accumulation

To further investigate the mechanism of SdrC self-association, we determined the effect of anti-SdrC$_{N2N3}$ antibodies on biofilm formation. SasG-driven lactococcal biofilm was not affected by the antiserum, whereas biofilm formation by *L. lactis* pKS80 *sdrC* was completely eliminated (Fig. 4A). Next, we compared the abilities of different recombinant protein segments to inhibit biofilm formation. The rSdrC$_{N3}$ protein had no effect on *L. lactis* pKS80 *sdrC* biofilm (Fig. 4B). Both rSdrC$_{N2}$ and rSdrC$_{N2N3}$ inhibited biofilm accumulation in a dose-dependent manner (Fig. 4B). Despite repeated attempts, we were unable to determine a concentration at which biofilm was completely eradicated by the addition of recombinant SdrC fragments. We hypothesized that the residual biofilm is due to an SdrC-dependent bacterial adherence to plastic. Thus, we assessed the ability of *L. lactis* pKS80 and *L. lactis* pKS80 *sdrC* resuspended in PBS to adhere to plastic for 1 h in the presence of either rSdrC$_{N2N3}$ or anti-SdrC$_{N2N3}$ antibodies. We detected complete inhibition of bacterial attachment to plastic in the presence of antibodies, whereas rSdrC$_{N2N3}$ had no significant effect (Fig. 4C). These results suggest that SdrC promotes both bacterial adherence to plastic and biofilm growth. However, SdrC self-association is important only for biofilm accumulation.

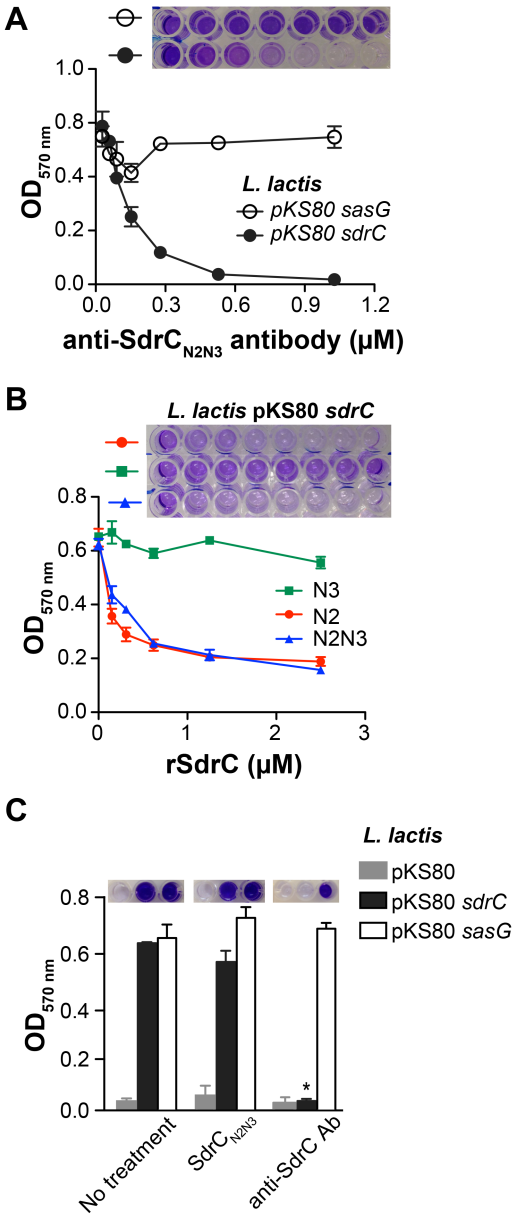


Fig. 4. Inhibition of SdrC dimerization disrupts biofilm formation.
A. *L. lactis* pKS80 *sdrC* biofilm inhibition by anti-SdrC$_{N2N3}$ serum (range, 0–1 μM). *L. lactis* pKS80 *sasG* was used as negative control.
B. *L. lactis* pKS80 *sdrC* biofilm inhibition by either recombinant SdrC$_{N2}$, SdrC$_{N3}$, SdrC$_{N2N3}$ proteins (range, 0–2.4 μM). Increasing concentrations of proteins were added to the plates at the same time as bacteria and incubated for 24 h at 30°C.
C. Inhibition of *L. lactis* pKS80 *sdrC* initial adherence to 96-well microtitre plates by anti-SdrC antibodies. Bacteria were grown overnight, washed in PBS, resuspended at OD_{600} = 1 and added to plastic plates in the presence of either recombinant SdrC$_{N2N3}$ (3 μM) or antibodies (1 μM). After 1 h, unbound bacteria were removed by washing with PBS. *L. lactis* pKS80 and *L. lactis* pKS80 *sasG* was used as negative control. Bound bacteria were stained with 0.5% CV. The data shown are representative of three individual experiments performed in triplicate. Bars represent mean ± SEM; *$P < 0.001$. Representative images of wells containing the CV extracted from previously stained biofilm are shown above the graphs.

Mn^{2+} inhibits SdrC-mediated biofilm formation

Metal ions often influence CWA-mediated staphylococcal biofilm formation. While Zn^{2+} is required for FnBPA and SasG promoted biofilm (Conrady *et al.*, 2008; 2013; Geoghegan *et al.*, 2010; 2013), ClfB-dependent biofilm formation is inhibited by Ca^{2+} (Abraham and Jefferson, 2012). To determine whether SdrC self-association is metal ion dependent, we conducted an initial screen where biofilm accumulation was assessed in the presence of selected metal ions (1 mM). In this assay, Mn^{2+} abolished biofilm formation by *L. lactis* pKS80 *sdrC,* whereas other metal ions tested had no effect (Fig. 5A). As expected, Ca^{2+} eliminated lactococcal ClfB-promoted biofilm (Abraham and Jefferson, 2012). Chelation with EDTA (1 mM) restored Mn^{2+} inhibited SdrC-driven biofilm formation (Fig. 5B). Furthermore, Mn^{2+} (1 μM) inhibited the binding of biotin-labelled $rSdrC_{N2}$ to immobilized $rSdrC_{N2N3}$ (Fig. 5C). These results indicate that the self-association of SdrC and its contribution to biofilm is inhibited by Mn^{2+}.

The contribution of SdrC to biofilm formation is strain-dependent

We compared the biofilm formation on plasma-coated and uncoated plastic by common laboratory strains and clinical isolates in the presence of $rSdrC_{N2}$ subdomain (3 μM). When the biofilm was grown on bare plastic, we detected a reduction of nearly 50% in biofilm accumulation by strains Newman, Cowan, MRSA252, RN4220, RN6390 and RN691. Biofilm formation *in vitro* by strains USA300, MW2 and Phillips was not affected by the incubation with $rSdrC_{N2}$ (Fig. 6A). In addition, we investigated SdrC-mediated biofilm formation by 20 clinical isolates. Similar to the strains commonly used in the laboratory, SdrC contributed to biofilm formation in approximately 50% of the clinical isolates, suggesting that the contribution of SdrC to biofilm formation is dependent on the strain background (Fig. 6A). To mimic the conditions that arise *in vivo* when implanted devices are rapidly covered with plasma proteins, a low amount of plasma (20 ng total protein ml^{-1}) was added to microtitre plates, and biofilm was allowed to accumulate on the surfaces of the coated wells. Overall, we found that biofilm development was more robust on coated plastic (Fig. 6B). Interestingly, $rSdrC_{N2}$ was able to reduce biofilm formation on plasma-coated plastic even by strains such as USA300 and MW2. A similar trend was observed for biofilm accumulation by the clinical isolates tested (Fig. 6B). Notably, half of the clinical isolates used in our study were δ haemolysin negative consistent with an Agr-deficient phenotype. Indeed, biofilm formation by these strains was enhanced and only three isolates were affected by the exogenous addition of $SdrC_{N2}$. In contrast, inhibition of SdrC self-association reduced biofilm formation in seven of the δ haemolysin positive strains.

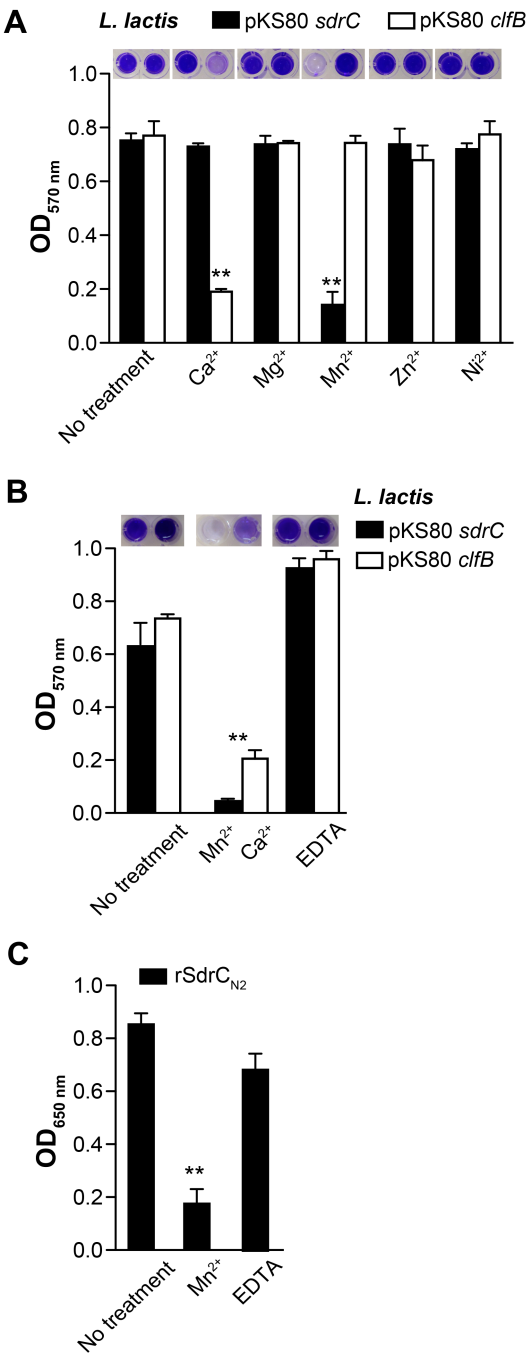
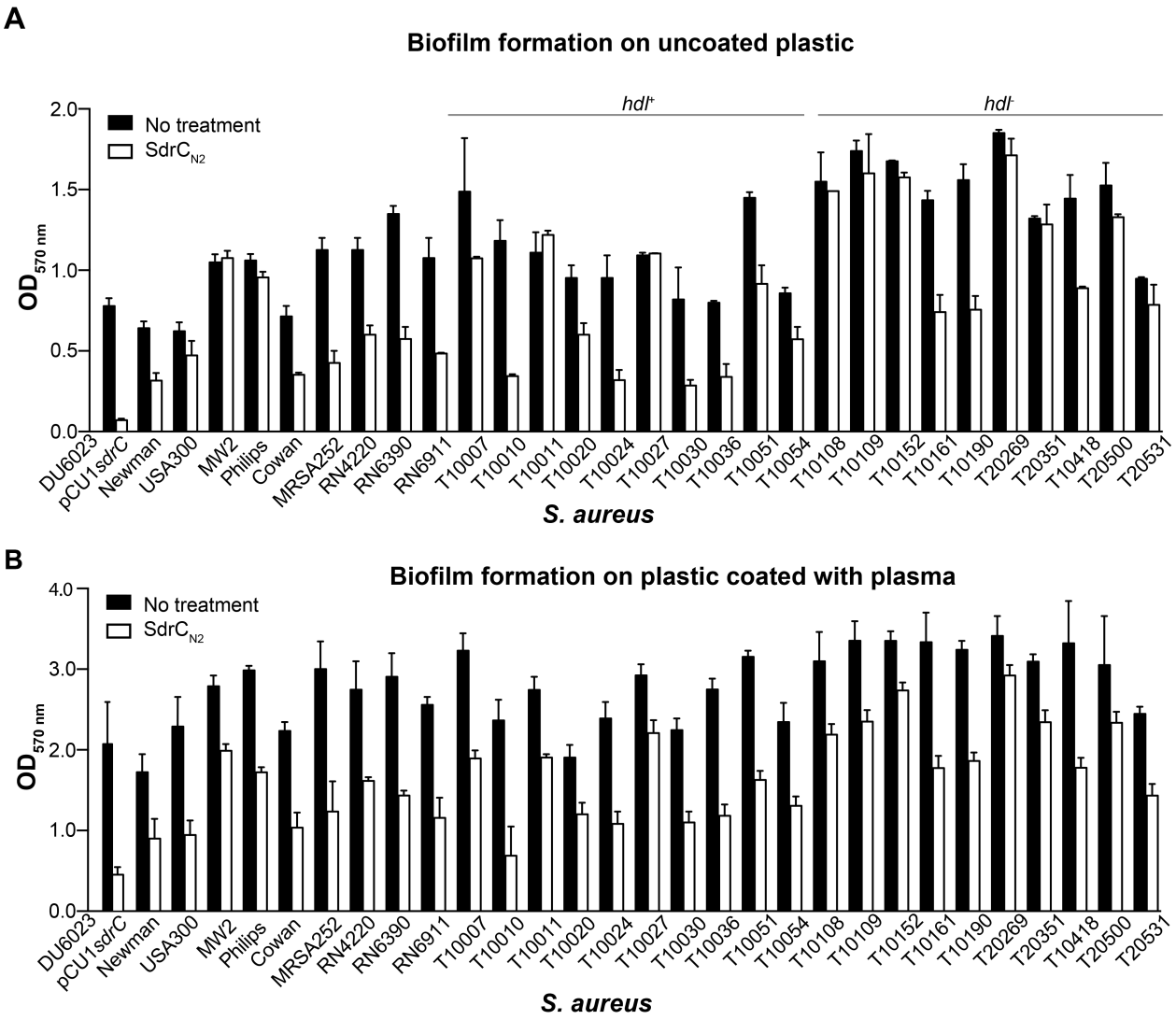


Fig. 5. Manganese inhibits SdrC-mediated biofilm formation.
A. *L. lactis* pKS80 *sdrC* biofilm formation in the presence of metal ions. Bacteria (OD_{600} = 0.01) and metal ions (1 mM) were added to microtitre wells at the same time and incubated for 24 h at 30°C. *L. lactis* pKS80 *clfB* was used a positive control.
B. EDTA metal chelation restores the ability of both SdrC and ClfB to promote biofilm growth of heterologous host *L. lactis*. The data shown are representative of two individual experiments performed in triplicate.
C. Biotin-labelled $rSdrC_{N2}$ binding to immobilized $rSdrC_{N2N3}$ in the presence of Mn^{2+} (1 μM) or Mn^{2+} (1 μM) chelated with EDTA (10 μM). Bars represent mean ± SEM; $^{**}P < 0.01$. Representative images of wells containing the CV extracted from previously stained biofilm are shown above the graphs.



C

	R P G S V		V D Q Y T
Newman	TTCCGTCCAGGATCAGTAAGATTACCT	//	AACTATGTAGATCAATATACAAAT
StaAur208671	TTCCGTCCGGGTTCTGTAAGATTACCT	//	AACTATGTAGATCAATATACAAAT
StaAur202941	TTCCGTCCGGGTTCTGTAAGATTACCT	//	AACTATGTGGATCAATATACAAAT
StaAur224518	TTCCGTCCGGGTTCTGTAAGATTACCA	//	AATTACGTTGATCAATATACAAAT
StaAur72157	TTCCGTCCAGGTTCTGTAAGATTACCT	//	AATTATGTAGATCAATACACAAAT

Fig. 6. SdrC contribution to biofilm formation is strain-dependent.
A and B. Biofilm formation by staphylococcal laboratory and clinical strains in the presence of recombinant SdrC$_{N2}$ protein (3 μM), (A) plastic and (B) immobilized plasma (20 ng protein ml^{-1}). The data shown are representative of four individual experiments performed in triplicate. Bars represent mean ± SEM.
C. SdrC nucleic acid sequences from 134 staphylococcal genomes deposited in the PATRIC database were analysed for mutations in the dimerization site identified by phage display. The consensus sequence present in *S. aureus* Newman is highlighted in yellow; examples of polymorphisms are highlighted in green.

To determine whether polymorphisms in the primary amino acid sequence are related to the differential involvement of SdrC in biofilm formation, we analysed the corresponding nucleotide sequences of all strains described above. We detected no differences in the sequence of the identified SdrC dimerization sites (Fig. 6C). We also compared the nucleic acids and the amino acids sequences of the SdrC dimerization sites from 134 sequenced *S. aureus*

strains deposited in the PathoSystems Resource Integration Center (PATRIC) database (http://patric.vbi.vt.edu). DNA alignments revealed several single nucleotide polymorphisms (SNPs) corresponding to amino acids residues P248, G249, S250, V288 and Y291 (Fig. 6C). Multiple polymorphisms occurred very often in the same *S. aureus* isolate (89%), while single SNPs were very rare (3%). Importantly, all SNPs resulted in synonymous codons suggesting that the site of SdrC dimerization is conserved.

Discussion

Biofilms are important for human health because they protect microorganisms from both antimicrobials and host immune defences (Costerton *et al*., 1999). *S. aureus* is a common cause of biofilm formation on indwelling medical devices or implants (Otto, 2008). Although significant progress has been made in understanding how staphylococci adhere to inert surfaces and develop sessile communities, the identification of molecular factors involved in this process is still underway. In addition to exopolysaccharides (Cramton *et al*., 1999) and eDNA (Izano *et al*., 2008), several CWA proteins have been shown to participate in biofilm accumulation. Interestingly, biofilm formation by staphylococcal clinical isolates could be completely eradicated after treatment with either carbohydrate-degrading enzymes or proteases, indicating that surface proteins and polysaccharides are equally important (Rohde *et al*., 2007).

In this study, we demonstrate that the surface protein SdrC contributes to staphylococcal biofilm formation *in vitro*. Using a combinatorial peptide screening approach, we identified two amino acid sequences located adjacent to each other within the N2 subdomain, which appear to act cooperatively to promote SdrC dimerization and, as a result, biofilm expansion. Interestingly, both peptides were necessary to completely inhibit SdrC self-association. We speculate that rather than binding to each other, these sequences either have an as yet unidentified binding site located within the N2 subdomain or bind to the same residues from another SdrC molecule. Although the mechanistic details are poorly understood, the N2N3 subdomains of three other CWA proteins (FnBPA, FnBPB and SasC) have been implicated in biofilm formation (Schroeder *et al*., 2009; Geoghegan *et al*., 2013). In addition, SasG mediates biofilm accumulation via Zn^{2+}-dependent B repeat dimerization (Conrady *et al*., 2008; 2013; Geoghegan *et al*., 2010). Thus, homophilic interactions between either N2N3 or B repeat subdomains appear to be a common mechanism by which CWA proteins promote biofilm growth. Intriguingly, the N2N3 domain is also the ligand-binding domain of FnBPs for Fg and of SdrC for β-neurexin (Patti *et al*., 1993; Schwarz-Linek *et al*., 2003; Barbu *et al*., 2010). In the case of FnBPA, the 'dock, lock and latch' mechanism of Fg-binding is not essential for biofilm formation (Geoghegan *et al*., 2013). Thus, it is possible that SdrC follows the same trend. Nonetheless, it is still unknown if the presence of the host ligand in solution interferes with biofilm growth.

Precisely why staphylococci require several CWA proteins with presumably similar mechanisms of action to maintain intercellular interactions is still unclear. It is possible that these molecules are expressed at different stages during infection. *In vitro*, FnBPs, ClfB and SasG are displayed during early exponential phase regardless of the growth medium (Novick and Jiang, 2003; Ythier *et al*., 2012), while SdrC expression is more robust during late exponential to stationary phase in RPMI or glucose-containing TSB (Ythier *et al*., 2012; and our data). Thus, temporal regulation of CWA protein expression may be beneficial for bacteria depending on the environmental conditions (e.g. the type of infection or stage of growth). Conflicting with previously reported data, recent studies have shown that FnBPs are expressed at high levels throughout the growth cycle in methicillin-resistant clinical isolates and this correlates with their contribution to biofilm formation (Geoghegan *et al*., 2013). Consistent with these observations, our attempts to eliminate biofilm accumulation by commonly used laboratory strains and clinical isolates by interfering with SdrC dimerization was also strain-dependent and not caused by polymorphisms in the self-association site. We speculate that the extent of SdrC contribution to biofilm formation depends on strain-specific genetic and molecular factors.

It is also possible that that co-ordinate expression of MSCRAMMs involves a spatial component. A recent study investigating the role of surface adhesins in *Vibrio cholerae* biofilm formation has demonstrated that two molecules, Bap1 and RbmA, have distinct roles in agreement with their localization (Absalon *et al*., 2011). While RbmA reinforces interactions within the biofilm, Bap1 is expressed at the surface interphase where it stabilizes adhesion and recruits cells that have not yet made the transition from planktonic living to sessile lifestyle. We conclude that further investigation is necessary to understand the genetic and spatially segregated factors involved in SdrC regulation *in vivo* and *in vitro*.

Heterologous expression in *L. lactis* indicated that SdrC is involved in both initial bacterial adherence to plastic as well as biofilm expansion. However, initiation of biofilm is independent of dimerization and is likely related to the overall hydrophobicity of the CWA proteins. The ability of *S. epidermidis* AtlE and *S. aureus* SasC to promote surface attachment has been attributed to their overall hydrophobicity (Heilmann *et al*., 1996; Gross *et al*., 2001; Houston *et al*., 2011). Our analysis revealed that the percent of hydrophobic amino acids in SdrC (21.2%) is similar to that of that of AtlE (26%) (Heilmann *et al*., 1996)

or SasC (25%) (Schroeder *et al*., 2009). Furthermore, the ability of SdrC to promote lactococcal biofilms demonstrates that the contribution of SdrC to this process is independent of both autolysin and PIA activity.

Previous studies have shown that chelation of Zn^{2+} inhibits biofilm formation by FnBPs and SasG expressing strains (Geoghegan *et al*., 2010; 2013). Thus, the use of metal ion-depleting agents, such as trisodium citrate, as catheter lock solutions has become more widespread (Shanks *et al*., 2006; O'Grady *et al*., 2011). More recent work has demonstrated that only a subset of strains is susceptible to chelators, whereas biofilm formation by a significant number of clinical isolates is enhanced by metal ion-depletion (Abraham *et al*., 2012). Our data revealed that, similar to ClfB (Abraham and Jefferson, 2012), SdrC-mediated biofilm formation is inhibited by Mn^{2+} and restored by chelating agents. The selective advantage, if any, of differential susceptibility to metal ions remains to be determined. It is possible that the presence of these cofactors may vary depending on the environmental conditions at the site of infection. On one hand, Ca^{2+} efflux and persistence in the skin is essential for rapid and effective wound healing (Xu and Chisholm, 2011). On the other hand, Mn^{2+} and Zn^{2+} chelation by the host innate immune protein calprotectin limits *S. aureus* growth in the abscess (Corbin *et al*., 2008). Moreover, within the biofilm eDNA acts as a metal chelator (Mulcahy *et al*., 2008). Thus, it is tempting to speculate that staphylococci have evolved their surface proteins to exploit and modulate environmental conditions, such as the availability of divalent metal ions.

In summary, we have demonstrated that SdrC promotes biofilm formation through homophilic interactions between the N2 subdomains likely occurring on neighbouring bacteria. We have also shown that additional genetic or molecular factors, and environmental conditions affect the biofilm-forming activity of SdrC. How our *in vitro* studies will correlate with biofilm formation *in vivo* remains to be determined, but a recent study investigating differential gene expression in biofilm-forming clinical isolates from skin indicated that *ica* operon and *sdrC* are highly expressed suggesting that SdrC may be important for *in vivo* biofilm (Shin *et al*., 2013). Nevertheless, our study identified and characterized a novel factor involved in staphylococcal biofilm formation. In addition, the mechanistic aspects described in this work contribute to the accumulating evidence that the self-association MSCRAMMs mediates biofilm formation.

Experimental procedures

Media and growth conditions

S. aureus (Table 1) was cultured in tryptic soy broth (TSB) at 37°C with shaking at 250 rpm. *L. lactis* pKS80 (Table 1) was cultured in M17 containing 0.5% glucose (GM17, Oxoid) and erythromycin (10 μg ml^{-1}) at 30°C without shaking. *L. lactis* pNZ8037 (Table 1) was cultured in GM17 supplemented with chloramphenicol (10 μg ml^{-1}). Overnight cultures were diluted 1:100, grown for another 3 h and surface protein expression was induced with nisin (0–3.2 ng ml^{-1}), unless otherwise mentioned (Corrigan *et al*., 2009).

For recombinant protein expression, plasmids pQE30-SdrC$_{52–178}$ (SdrC$_{N1}$), pQE30-SdrC$_{178–335}$ (SdrC$_{N2}$), SdrC$_{335–496}$ (SdrC$_{N3}$), SdrC$_{178–496}$ (SdrC$_{N2N3}$) were transformed into *E. coli* TOPP3 (Stratagene). Overnight starter cultures were diluted 1:50 in Luria–Bertani medium (LB) containing ampicillin (100 μg ml^{-1}) and incubated with shaking until mid-exponential phase was reached (OD$_{600}$ 0.6–0.8). Protein expression was induced by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside (final concentration) and growth was continuing for 4 h, after which bacteria were harvested by centrifugation, resuspended in PBS and frozen at –80°C.

Plasmid construction

Fragments encoding different domains of SdrC were amplified by PCR from *S. aureus* Newman genomic DNA, and oligonucleotide primers listed in Table 1. The PCR products were analysed by agarose gel electrophoresis and purified using a QIAquick gel extraction kit (Qiagen). To construct SdrC expression plasmids, a BamHI-HindIII fragment containing the appropriate gene segment was cloned into pQE30 (Qiagen). All plasmid constructs were sequenced to ensure the integrity of the amplified fragments (Baylor College of Medicine DNA Sequencing Core Facility).

Protein expression and purification

The His-tagged recombinant SdrC subdomains were purified by affinity chromatography with a 5 ml nickel-charged HiTrap column (GE Healthcare) and 5 ml anion or cation exchange Sepharose column (GE Healthcare) as described by Barbu *et al*. (2010). Fractions containing > 95% pure recombinant were dialysed against Tris-buffered saline (TBS), 10 mM EDTA, pH 7.4.

Phage display

The phage display screen was described earlier (Barbu *et al*., 2010).

Phage binding assays

Binding of phage displaying enriched peptides to immobilized rSdrC$_{N2N3}$ was determined as described (Barbu *et al*., 2010). Briefly, microtitre wells were coated with recombinant proteins overnight (1 μg per well in carbonate buffer pH 9.3). After blocking with PBS containing 3% BSA for 1 h, wells were incubated with 10^9 phage. Unbound phage were removed by washing with PBS six times and subsequently probed with an anti-M13-HRP antibody. Colour development was performed using TMB (Calbiochem) and the binding was

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

Strain	Description	Source or RE
E.coli		
XL1Blue	*recA1 endA1 gyrA96 thi-1 hsdR17* (r_k^- m_k^+) *supE44 relA1 lac [F' proAB*$^+$ *lacI*^{q}*ZΔM15* Tn*10* (Tet^r)]	Stratagene
TOPP3	Rif^r, [F' *proAB*$^+$ *lacI*^{q}*ZΔM15* Tn*10* (Tet^r) (Kan^r)]	Stratagene
S. aureus		
Newman	ST8, human clinical MSSA isolate	Duthie and Lorenz (1952)
Newman DU5943	*clfB*::Tet^r	McDevitt *et al.* (1994)
Newman DU5988	*sdrC*::pG$^+$Host	O'Brien *et al.* (2002)
Newman DU5989	*sdrD*::pG$^+$Host	O'Brien *et al.* (2002)
Newman DU5973	Δ*sdrCDE*::Tet^r	O'Brien *et al.* (2002)
Newman DU6023	*clfA*5 isdA *clfB*::Erm^r Δ*sdrCDE*::Tet^r	Corrigan *et al.* (2009)
LAC	ST8, human clinical MRSA isolate, USA300	Voyich *et al.* (2005)
MW2	ST1, human clinical MRSA isolate	Baba *et al.* (2002)
Philips	Human clinical isolate from patient with osteomyelitis; *cna*$^+$	Patti *et al.* (1993)
Cowan	ST30, MSSA, ATCC12598	Cowan *et al.* (1954)
MRSA252	ST36, MRSA	Holden *et al.* (2004)
RN4220	ST8, chemically mutagenized derivative of 8325-4, transformable with *E. coli* DNA	Kreiswirth *et al.* (1983)
RN6390	8325-4, *rsbU*	Peng *et al.* (1988)
RN6911	RN6390 Δ*agr*::tetM	Novick *et al.* (1993)
T10007, T10010, T10011, T10020, T10024, T10027, T10030, T10036, T10051, T10054	Clinical isolate; *hld* positive	
T10108, T10109, T10152, T10161, T10190, T20269, T20351, T10418, T20500, T20531	Clinical isolate; *hld* negative	
L. lactis		
MG1363	Plasmid-free derivative of NCD 0712; host for constitutive expression vector pKS80 and its derivatives	Gasson (1983)
NZ9000	MG1363 *nisA;* host strain for nisin-inducible expression vector pNZ8037 and its derivatives	Kuipers *et al.* (1997)
Plasmids		
pQE30	*E. coli* plasmid for protein expression with an N-terminal His-tag; Amp^r	Qiagen
pQE30 $SdrC_{N1}$	$SdrC_{N1}$ with an N-terminal His-tag; Amp^r	This study
pQE30 $SdrC_{N2}$	$SdrC_{N2}$ with an N-terminal His-tag; Amp^r	This study
pQE30 $SdrC_{N3}$	$SdrC_{N3}$ with an N-terminal His-tag; Amp^r	This study
pQE30 $SdrC_{N23}$	$SdrC_{N23}$ with an N-terminal His-tag; Amp^r	Barbu *et al.* (2010)
pQE30 $SdrC_B$	$SdrC_B$ with an N-terminal His-tag; Amp^r	This study
pCU1	*E. coli – S. aureus* shuttle vector; Amp^r, Cam^r	Augustin *et al.* (1992)
pCU1 *sdrC*	Derivative of pCU1 encoding *sdrC*; Amp^r, Cam^r	Barbu *et al.* (2010)
pCU1 *sdrD*	Derivative of pCU1 encoding *sdrD*; Amp^r, Cam^r	Corrigan *et al.* (2009)
pCU1 *sdrE*	Derivative of pCU1 encoding *sdrE*; Amp^r, Cam^r	Corrigan *et al.* (2009)
pKS80	*L. lactis* constitutive expression vector expression; Erm^r	Wells *et al.* (1996)
pKS80 *clfB*	Derivative of pKS80 encoding *clfB*; Erm^r	O'Brien *et al.* (2002)
pKS80 *sdrC*	Derivative of pKS80 encoding *sdrC;* Erm^r	O'Brien *et al.* (2002)
pKS80 *sdrE*	Derivative of pKS80 encoding *SdrE*; Erm^r	O'Brien *et al.* (2002)
pKS80 *fnbpA*	Derivative of pKS80 encoding *fnbpA*; Erm^r	Massey *et al.* (2001)
pKS80 *sasG*	Derivative of pKS80 encoding *sasG*; Erm^r	Roche *et al.* (2003)
pNZ8037	*L. lactis* nisin-inducible expression vector; Cam^r	de Ruyter *et al.* (1996)
pNZ8037 *clfB*	Derivative of pNZ8037encoding *clfB*; Cam^r	Miajlovic *et al.* (2007)
pNZ8037 *sdrC*	Derivative of pNZ8037encoding *sdrC*; Cam^r	Barbu *et al.* (2010)
pNZ8037 *sdrE*	Derivative of pNZ8037 encoding *sdrE*; Cam^r	This study

Oligonucleotides		Restriction site
$SdrC_{N1}$ forward	GCACGGATCCGGTGACTATGTATGGGAAGATACAA	BamHI
$SdrC_{N1}$ reverse	GCACAAGCTTTTATACATCAACTTCGCCACCCAT	HindIII
$SdrC_{N2}$ forward	GCACGGATCCCATACGAATGGAGAATTAAATCAA	BamHI
$SdrC_{N2}$ reverse	GCACAAGCTTTTAAGCTGCAACAGTATTCACTGC	HindIII
$SdrC_{N3}$ forward	GCACGGATCCCACAACAAGAACAAATGTTAATG	BamHI
$SdrC_{N3}$ reverse	GCACAAGCTTTTAATAATCGACAATGATTTCTTCGCTAT	HindIII
SdrCN2N3 forward	CCCGGATCCGGAACAAATGTTAATGATAAAGTACAT	BamHI
$SdrC_{N2N3}$ reverse	CCCAAGCTTTTATTTCTTTTGGTCGCCATTAG	HindIII
$SdrC_B$ forward	GCACGGATCCAAAGCACAACCGCTTATTTCA	BamHI
$SdrC_B$ reverse	GCACAAGCTTTTATTTCTTTTGGTCGCCATTAGC	HindIII

RE, restriction site; MSSA, methicillin sensitive *S. aureus*; MRSA, methicillin resistant *S. aureus*.

measured using a microtitre plate reader (Molecular Devices) at 650 nm.

Solid phase binding assays

To detect self-association, SdrC recombinant subdomains were labelled with biotin according to the manufacturer's instruction (EZ-Link-NHS-Biotin, Pierce). Microtitre wells were coated with unlabelled SdrC subdomains overnight at 4°C (1 μg per well in carbonate buffer pH 9.3). Coated wells were blocked for 1 h at room temperature with 2% BSA in TBS buffer. Increasing concentrations of biotin-labelled SdrC subdomains were added to the wells and incubated for 1 h at room temperature. Wells were then probed with avidin-HRP. Colour development was performed using TMB (Calbiochem) and the binding was measured using a microtitre plate reader (Molecular Devices) at 650 nm. For inhibition assays, either phage (10^9 TU) or 1 μM anti-$SdrC_{N2N3}$ antibodies were added to the wells before incubating with 1 μM biotin labelled $SdrC_{N2N3}$. Data presented represent the mean ± SD of three independent experiments performed in triplicate. The apparent dissociation constant (K_D) calculated using the equation $\Delta P = \Delta P_{max}[protein]/(K_D + [protein])$.

Gel permeation chromatography

To assess SdrC oligomerization, pure SdrC subdomains (1 mg ml^{-1}) were analysed on a gel filtration column (Superdex 200 5/150 GL, GE Healthcare) attached to an AKTA FPLC at a flow rate of 0.3 ml min^{-1} in TBS pH 7.4. The column was calibrated with the following proteins: thyroglobulin (660 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (4 kDa), chymotrypsin (29 kDa), cytochrome *c* (12.4 kDa) and aprotinin (6.5 kDa) (Gel Filtration Calibration Kits HMW & LMW, GE Healthcare). Gel phase distribution coefficients (K_{AV}) were calculated from the respective elution volumes (Ve), represented as a function of molecular mass and analysed by linear regression ($R = 0.9905$). The relative molecular mass of SdrC species eluted from the column was calculated by interpolation.

Biofilm formation

S. aureus was grown overnight in TSB and diluted 1:100 in TSB supplemented with 1% glucose and dispensed into sterile non-treated microtitre plates (100 μl per well). Overnight cultures of *L. lactis* were diluted in GM17 and added to sterile non-treated microtitre plates (275 μl per well). After 24 h incubation, plates were washed three times with PBS, dried and stained with 0.5% crystal violet (CV). Subsequently, the plates were rinsed with water and dried. The dye was dissolved in 200 μl of 95% ethanol and the absorbance was measured at OD_{570} (Corrigan *et al.*, 2007). For specific accumulation assays, microtitre wells were coated with plasma (20 ng protein ml^{-1} in PBS) at 4°C for 18 h and washed twice with sterile PBS. For competition assays, rSdrC subdomains, anti-SdrC antibodies or metal ions (1 mM for biofilm and 1 μM for recombinant proteins) were added to the plates at the same time as bacteria. For inhibition of biofilm by clinical isolates, 3 μM $SdrC_{N2}$ was added to the well at the same time as bacterial cells.

Adherence of bacteria to plastic

L. lactis was grown overnight in GM17 supplemented with the appropriate antibiotic, washed and resuspended in PBS at $OD_{600} = 1$. Bacteria were added to the plates (100 μl) and incubated at room temperature for 1 h. Unattached cells were removed by washing three times with PBS. Bacterial adherence was measured as described above. For inhibition assays, either 1 μM anti-$SdrC_{N2N3}$ serum or 3 μM $SdrC_{N2N3}$ were added to the wells before bacteria were allowed to adhere to plates. Data presented represent the mean ± SD of three independent experiments performed in triplicate.

Colony spreading

The colony spreading assay was performed as described (Kaito and Sekimizu, 2007). Briefly, a 2 μl aliquot of bacteria from overnight cultures was spotted on soft agar GM17 (0.24% agar) after plates were dried for approximately 10 minutes in a laminar flow cabinet. Dishes were then dried for an additional 5 minutes and incubated overnight at 37°C. Colony spreading assays were repeated six times.

Statistical analysis

For all assays, three independent experiments were carried out in triplicate. Comparisons of multiple treatment groups were performed by using two-way analysis of variance with post-hoc paired comparisons by Dunnett's test.

Calculations were made with InStat (GraphPad Software). Two-tailed *P*-values of less than 0.05 were considered statistically significant.

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