Matrix-binding proteins of *Staphylococcus aureus*: functional analysis of mutant and hybrid molecules

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The fibrinogen-binding protein ClfA and the collagen-binding protein Cna are surface-associated adhesins of *Staphylococcus aureus*. ClfA has a dipeptide repeat region R composed mainly of serine and aspartate residues, more than 40 of which are required along with the 28-residue region W, the LPXTG motif and region M to display the ligand-binding region A on the cell surface in a functional form. Cna has a 61-residue region W and at least one 187-residue region B linking the collagen-binding region A to peptidoglycan. A *cna* mutant of *S. aureus* lacking region B was shown to bind collagen at the same level as wild-type Cna⁺ cells, indicating that region B is not necessary for ligand binding. Furthermore, altering the number of B repeats did not influence the level of collagen binding. In order to study the ability of C-terminal domains of Cna and ClfA to support functional ligand-binding activity of different adhesins, chimeric proteins were constructed and expressed in *S. aureus*. Surprisingly, the presence of a single Cna B domain and a nonapeptide linker located between ClfA region A and Cna region WM failed to support fibrinogen binding by *S. aureus* cells, despite the fact that ClfA region A was detected on the bacterial surface by immunoblotting. In contrast, the ClfA region A–Cna region B hybrid expressed as a recombinant protein in *Escherichia coli* did bind fibrinogen in Western ligand blots and in an ELISA-type assay. It is concluded that Cna region B cannot support functional display of ClfA region A on the bacterial cell surface. However, the ClfA dipeptide repeat region R and region WM did promote functional surface expression of the Cna collagen-binding domain in a hybrid Cna–ClfA protein.

Keywords: *Staphylococcus aureus*, clumping factor, collagen-binding protein, dipeptide repeat, fibrinogen-binding protein

INTRODUCTION

Staphylococcus aureus causes a variety of infections both in healthy individuals and in hospitalized patients, ranging from skin infections to infections associated with indwelling medical devices (Waldvogel, 1995). The ability of bacteria to adhere to biomaterial surfaces that become coated with host plasma and matrix proteins is a major determinant for initiating a foreign-body infection (Vaudaux *et al.*, 1995; Waldvogel, 1995). Furthermore, haematogenous spread of *S. aureus* from a

colonized device can lead to serious invasive infections such as endocarditis and septic shock (Waldvogel, 1995).

S. aureus strains express surface receptors designated MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) that interact with proteins in the extracellular matrix such as fibrinogen (McDevitt *et al.*, 1994; Ní Eidhin *et al.*, 1998), collagen (Switalski *et al.*, 1989), fibronectin (Signäs *et al.*, 1989), vitronectin (Liang *et al.*, 1995) and elastin (Park *et al.*, 1991). Some MSCRAMM proteins exhibit characteristics of a family of cell-wall-associated proteins with (i) a putative secretory signal sequence at the N-terminus and (ii) signals at the C-terminus that are required for anchoring the protein to the cell wall, including an LPXTG motif which is required for accurate sorting and

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correct orientation in the cell wall, a hydrophobic membrane-spanning region and a positively charged C-terminus that is thought to act as a signal to stop secretion. Following cleavage between the T and G of LPXTG by a novel transpeptidase (sortase), the carboxyl end of threonine is amide-linked to a free amino group of the peptidoglycan, covalently linking the protein to the cell wall (Schneewind *et al.*, 1995). Recently, three new proteins have been identified in *S. aureus* with features characteristic of cell-wall-associated proteins (Josefsson *et al.*, 1998). The Sdr proteins have an additional 110–113-residue sequence B repeated two to five times located between the putative ligand-binding A domain and the Ser-Asp repeat region R. A dimer of the SdrB domain is quite similar to the B repeat of Cna (Josefsson *et al.*, 1998).

The ligand-binding activities of the clumping factors ClfA and ClfB and the collagen-binding proteins (Cna) are located in their N-terminal A domains (McDevitt *et al.*, 1995; Patti *et al.*, 1995; Ní Eidhin *et al.*, 1998). In ClfA, a 308-residue dipeptide repeat composed mainly of serine and aspartate residues separates region A from the 31-residue region W. In Cna, a 187-residue B domain separates the ligand-binding A region from the 61-residue region W (Patti *et al.*, 1992). In the fibronectin-binding proteins (FnBPA and FnBPB) the ligand-binding domain is located in the C-terminal D repeats. In FnBP, the 92 C-terminal residue proline-rich region W is sufficient for efficient display of a lipase enzyme in a chimeric lipase–FnBP protein on the surface of *Staphylococcus carnosus* (Strauss & Götz, 1996) and presumably for fibronectin binding in the native protein. In ClfA, more than 72 residues are required to link region A and the LPDTG motif in order to display the fibrinogen-binding domain on the *S. aureus* cell surface (Hartford *et al.*, 1997).

All characterized Cna⁺ proteins carry one, two, three or four copies of the 187-residue region B (Gillaspy *et al.*, 1997; Switalski *et al.*, 1993), the function of which is unknown. Given the interest in using the anchoring mechanisms of surface proteins to display antigens on the surface of Gram-positive cocci (Ståhl & Uhlén, 1997) we constructed chimeric ClfA–Cna proteins to determine if the C-terminal sequences of Cna or ClfA could support functional surface expression of another ligand-binding activity. In addition, we constructed mutants of Cna in order to determine the role of the B region in surface expression of collagen-binding activity.

METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. Strain RN4220 was the recipient used for introducing plasmids into *S. aureus* by transformation. Plasmids were subsequently transduced to 8325-4 *clfA1 Δspa* (strain DU5941). This protein A-deficient mutant prevented non-specific interaction between protein A and rabbit IgG in Western immunoblotting.

Bacterial growth media and antibiotics. *Escherichia coli* strains harbouring plasmids were routinely grown in L-broth

or L-agar (Miller, 1972). For protein expression studies, *E. coli* was grown in Terrific broth (Sambrook *et al.*, 1989) supplemented with phosphate buffer. *S. aureus* cultures were grown in trypticase soy broth (TSB) or agar (TSA), brain-heart infusion (BHI) broth or Mueller–Hinton (MH) broth. Ampicillin (100 µg ml⁻¹) was used for the selection of plasmids in *E. coli* and chloramphenicol (10 µg ml⁻¹), erythromycin (3 µg ml⁻¹) or tetracycline (2 µg ml⁻¹) for selection of plasmids and/or chromosomal markers in *S. aureus*.

Transformation and transduction. *E. coli* XL-1 Blue cells were made competent by CaCl₂ treatment (Sambrook *et al.*, 1989). Recombinant plasmids were introduced into *S. aureus* by electroporation (Oskouian & Stewart, 1990). Chimeric shuttle plasmids were transduced from *S. aureus* RN4220 to DU5941 using phage 85 (Foster, 1998).

Manipulation of DNA. DNA manipulations were performed by standard procedures (Sambrook *et al.*, 1989). Chromosomal DNA of *S. aureus* strains was prepared by the method of Lindberg *et al.* (1972). Plasmid DNA for cloning and sequence analysis was purified by WizardPlus Minipreps (Promega) followed by phenol and chloroform extraction. DNA modification enzymes were purchased from New England BioLabs or Boehringer Mannheim.

PCR amplification of *clfA* and *cna* gene fragments. Oligonucleotide primers are listed in Table 2. Restriction sites were incorporated at the 5' end of each primer to facilitate cloning of PCR products. For amplification of *clfA* gene fragments, plasmid pCF3 (McDevitt *et al.*, 1994) was used as a template unless otherwise stated. For amplification of *cna* gene fragments, *S. aureus* strain FDA574 was used as template. The PCR reaction mixtures with Vent polymerase (New England BioLabs) were as previously described (Hartford *et al.*, 1997). All reactions were carried out with a 1 min denaturation step at 94 °C, a 1 min annealing step at 50–58 °C depending on the primer pair, and elongation for 1–2 min at 72 °C depending on the length of fragment to be amplified. This standard cycle was repeated 30 times and was followed by incubation at 72 °C for 10 min. Amplified DNA was purified with Wizard PCR Preps (Promega), cleaved with restriction enzymes and ligated with vector cut with the appropriate enzymes.

Construction of *cna* B repeat mutants. Fragments of the *cna* gene were amplified by PCR and cloned into the *S. aureus*–*E. coli* pCU1 shuttle plasmid. A 1751 bp fragment encoding the N-terminal signal peptide and region A and carrying 149 bp of non-coding sequence was amplified using primers F1 (incorporating an *Eco*RI site) and R1 (incorporating a *Bam*HI site). Fragments of the *cna* gene encoding the C-terminal domains were amplified by PCR using F2 (incorporating a *Bam*HI site) and R2 (incorporating a *Hind*III site). The forward primer for amplification of the C-terminal coding regions of the *cna* gene corresponded to the end of the B1 repeat of *cna* of FDA574. This sequence is also present at the end of the B2 and B3 repeats. Two mismatches are present between the F2 primer and the B3 coding sequence. Therefore, using this primer in a PCR reaction with the R2 primer generates three PCR products, of 1460 bp, 899 bp and 338 bp. The 1460 bp fragment encoded the last 7 residues of the B1 domain, regions B2 and B3, the wall-spanning region W and the membrane-spanning region M of Cna. The 899 bp fragment encoded the last 7 residues of the B2 domain, region B3 and the wall-anchoring region, while the 338 bp fragment encoded the last 7 residues of the B3 domain and the wall-anchoring region. It should be noted that the *Bam*HI site introduced between region A and region B (or region W) coding sequences encodes glycine and serine. The 1751 bp

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant genotype/markers	Relevant properties	Source/reference
<i>E. coli</i>			
XL-1 Blue	F'::Tn10 <i>proA</i> ⁺ <i>B</i> ⁺ <i>lacI</i> ^q $\Delta(lacZ)M15/recA1$ <i>endA1</i> <i>gyrA96</i> (Nal ^R) <i>thi</i> <i>hsdR17</i> (r _k ⁻ m _k ⁺) <i>supE44 relA1 lac</i>	Cloning host, with IPTG-inducible expression	New England Biolabs
<i>S. aureus</i>			
RN4220		Restriction-deficient 8325-4 derivative; stable maintenance of shuttle plasmids	Kreiswirth <i>et al.</i> (1983)
Newman		ClfA positive	Duthie & Lorenz (1952)
FDA574		Cna positive	
Phillips		Cna positive, osteomyelitis isolate	
DU5941	<i>clfA1</i> ::Tn917 Δspa ::Tc ^R	Clumping factor- and protein A-deficient strain; <i>cna</i> absent	Hartford <i>et al.</i> (1997)
Plasmids			
pQE30	Ap ^R	His-tag fusion protein expression vector	Qiagen
pCU1	Ap ^R Cm ^R	<i>S. aureus</i> – <i>E. coli</i> shuttle plasmid	Augustin & Götz (1990)
pCN4	Ap ^R Cm ^R	Expresses Cna region A fused to ClfA region RWM in pCU1	This study
pCF72	Ap ^R Cm ^R	Expresses ClfA region A fused to Cna region BWM in pCU1	This study
pCN1, pCN2, pCN3	Ap ^R Cm ^R	Express Cna variants with 2, 1 or 0 B repeats in pCU1	This study
pCF77	Ap ^R Cm ^R	<i>clfA</i> gene with <i>Bam</i> HI site separating the A and R encoding domains in pCU1	Hartford <i>et al.</i> (1997)
pClfA	Ap ^R	pQE30 expressing ClfA region A plus 72 residues from region R	This study
pClfA–Cna	Ap ^R	pQE30 expressing ClfA region A fused to Cna region B	This study

Table 2. Primers

Primer set	Primer sequence (5'–3')	Plasmid
F2	GCCGGATCCGTGACGAATAAATATACGCCA	pCF72
R2	GGCAAGCTTATAGAACTAAGAATAGCC	
F1	GCCGAATTCCTTTTCGAGAAGCGGTGAAGAAC	pCN1
R1	GGCGGATCCGATTGGTCTTTTCAGTATTAGT	
F2	GCCGGATCCGTGACGAATAAATATACGCCA	pCN3
R2	GGCAAGCTTATAGAACTAAGAATAGCC	
F3	GCCGGATCCGATTCTGACCCA	pCN4
R3	GCCAAGCTTGTCTAGTTTCAACGACTCA	
ClfAF1	TATGGATCCGTAGCTGCAGATGCACCG	pClfA
ClfAR1	GCCAAGCTTGTCTATCTGAATCCGAGTCATT	
ClfAF1	TATGGATCCGTAGCTGCAGATGCACCG	pClfA–Cna
CnaR1	GGCAAGCTTTGGCGTATATTTATTCGTCAC	

Restriction enzyme sites are underlined.

fragment was ligated to the 1460 bp, the 899 bp or the 338 bp *cna* fragment and cloned into pCU1 cut with *Eco*RI and *Hind*III to generate pCN1, pCN2 and pCN3, respectively. Thus, the Cna protein encoded by pCN1 retained two B repeats, region W and region M of Cna, that of pCN2 expressed one B repeat, while that of pCN3 lacked region B altogether (see Fig. 1e). The in-frame fusions were verified by DNA sequence analysis.

Construction of a chimeric *clfA-cna* gene. Previously, we described expression of *clfA* variants using an *S. aureus*-*E. coli* shuttle plasmid (Hartford *et al.*, 1997). The construction of *clfA-cna* derivatives was facilitated by the presence of a unique *Bam*HI site created in the *clfA* gene of pCF77. Fragments of the *cna* gene were amplified by PCR using F2 (incorporating a *Bam*HI site) and R2 (incorporating a *Hind*III site) as described above. The 899 bp fragment encoding the last 7 residues of the B2 domain, region B3, the wall-anchoring region W and the membrane-spanning region M of Cna was cut with *Bam*HI and *Hind*III to replace the *Bam*HI-*Hind*III fragment of pCF77 encoding region RWM of ClfA to generate an in-frame fusion between region A of ClfA and region BWM of Cna (plasmid pCF72; Fig. 1d).

Construction of a chimeric *cna-clfA* gene. A *cna-clfA* derivative was generated by PCR amplifying the N-terminal coding regions of Cna (signal sequence and region A) using primers F1 and R1 and fusing to the PCR amplified C-terminal coding sequences of ClfA (region R, the LPDTG motif and transmembrane-spanning region of ClfA) using the primer pair F3 (incorporating a *Bam*HI site) and R3 (incorporating a *Hind*III site) to generate pCN4 (Fig. 1f).

SDS-PAGE, Western immunoblotting and Western affinity blotting. Preparation of cell-wall proteins from stabilized protoplasts by lysozyme treatment was performed as previously described (Hartford *et al.*, 1997). SDS-PAGE was performed by standard methods (Laemmli, 1970) through a 4.5% stacking gel and a 7.5% or 10% polyacrylamide separating gel. Proteins were transferred electrophoretically to PVDF (Boehringer Mannheim) by the semi-dry system (Bio-Rad) in Tris/HCl (48 mM), glycine (39 mM) and methanol (20%). Membranes were incubated for 15 h at 4 °C in blocking reagent (Boehringer Mannheim). Polyclonal rabbit anti-ClfA region A antiserum raised against the purified recombinant fibrinogen-binding ClfA truncate (residues 220–559) was used at a dilution of 1:500 and protein A conjugated to horseradish peroxidase (Sigma) was used to detect ClfA proteins using chemiluminescence (Boehringer Mannheim). Polyclonal rabbit anti-Cna region A antiserum was used at a dilution of 1:1000. For detection of fibrinogen-binding proteins by affinity blotting, purified recombinant ClfA and ClfA-Cna proteins were fractionated by SDS-PAGE, transferred to PVDF and incubated with fibrinogen conjugated to horseradish peroxidase and detected by chemiluminescence (Boehringer Mannheim).

Measurement of cell clumping. *S. aureus* strains to be tested for clumping were grown statically for 5 h in Mueller-Hinton broth, harvested by centrifugation at 3000 g for 10 min and washed in PBS. A suspension of 4×10^8 c.f.u. in a 20 µl volume was added to 50 µl of doubling dilutions of fibrinogen (starting at 1 mg ml⁻¹, Kabi) in a microtitre plate. The highest dilution of fibrinogen promoting visible clumping of the bacterial suspension after 5 min was defined as the titre.

Adherence of bacterial cells to immobilized fibrinogen and collagen. The assay for bacterial adherence to immobilized fibrinogen was performed as previously described (Hartford

et al., 1997). The same procedure was used for measuring adherence to collagen using type II collagen (Sigma). Strains to be tested for adherence to collagen were grown for 15 h at 37 °C in 100 ml TSB in a 250 ml flask at 200 r.p.m.

Bacterial cell immunoblots. Late-exponential-phase cells (OD₆₀₀ 0.7–0.8) were harvested by centrifugation and washed twice in PBS buffer. The suspensions (10 µl) and twofold dilutions were placed onto nitrocellulose membranes and allowed to dry for 15 min. Membranes were placed in blocking buffer (5% skim milk in PBS) and developed by standard immunoblotting procedures described above.

Expression of ClfA and ClfA-Cna in *E. coli*. Fragments of the *clfA* gene were amplified by PCR and cloned into plasmid pQE30. A 1233 bp fragment encoding the C-terminal part of region A of ClfA plus 72 residues from region R (residues 220–631) was amplified using primers ClfAF1 (incorporating a *Bam*HI site) and ClfAR1 (incorporating a *Hind*III site) to generate pClfA, which expressed a fusion protein with a short N-terminal segment containing six histidine residues (which allow purification by Ni²⁺-chelating chromatography). Fragments of the *clfA-cna* fusion gene were amplified from pCF72 using primers ClfAF1 (incorporating a *Bam*HI site) and CnaR1 (incorporating a *Hind*III site) and cloned into pQE30 to generate pClfA-Cna, expressing a hybrid ClfA-Cna protein with a single B domain. Expression and purification of proteins with N-terminal His-tags was performed as described by O'Connell *et al.* (1998).

ELISA-type assay for fibrinogen-binding activity. Purified recombinant ClfA and ClfA-Cna proteins were analysed by ELISA to detect fibrinogen-binding activity. Briefly, wells in microtitre plates (Sarstedt) were coated with fibrinogen (5 µg ml⁻¹, Kabi Pharmacia/Chromogenix) in sodium carbonate buffer [15 mM Na₂HCO₃, 35 mM NaH(CO₃)₂, 3.2 mM NaN₃, pH 9.6] for 15 h at 4 °C. The plates were washed with PBS and 0.05% Tween-20 (PBS-T) and blocked with 2.5% bovine serum albumin supplemented with 0.05% Tween-20 in PBS, at 37 °C for 1 h. Following washing, purified recombinant protein in PBS was added and plates were incubated for 2 h at 37 °C. The wells were washed with PBS-T and monoclonal antibody raised against Clf41 (221–559) was added for 1 h at 37 °C. Following further washing with PBS-T, rabbit anti-mouse IgG conjugated to horseradish peroxidase (1:1000) was added for 1 h at 37 °C and developed with 100 µl of chromogenic substrate (580 µg tetramethylbenzidine ml⁻¹, 0.0001% H₂O₂ in 0.1 M sodium acetate buffer, pH 5.0) for 10 min. The reaction was stopped by the addition of 50 µl 2 M H₂SO₄ and the A₄₅₀ was measured in an ELISA plate reader (Lab Systems Multiskan Plus).

RESULTS

Construction and expression of Cna and a chimeric Cna-ClfA variant

To analyse the role of the B region of Cna in functional surface expression of the collagen-binding A domain, three mutants of the *cna* gene from strain FDA574 were constructed (Fig. 1e). Plasmid pCN1 expressed Cna with two B repeats linked to the C-terminal region W, the LPKTG motif and the membrane-spanning domain. Plasmid pCN2 expressed Cna with one B repeat while pCN3 lacked the B region altogether, leaving the N-terminal region A domain fused directly to the C-terminal regions W and M. Expression of variant *cna*

Fig. 1. (a) Schematic representation of the ClfA protein. S, signal sequence; A, ligand-binding region; R, dipeptide repeats; W, wall-spanning region; M, membrane-spanning region; ++, positively charged C-terminus. The position of the LPDGTG motif is indicated. (b) Schematic representation of the Cna protein. Abbreviations as in (a); B refers to the 187-residue B repeat. Native Cna proteins can have one, two, three or four B repeats. (c) Structure of the chimeric protein expressed by pCF77. Note that the *Bam*HI site between region A and region R coding sequences introduces two additional amino acids. (d) Structure of the ClfA-Cna hybrid MSCRAMM. The thin line linking regions A and B indicates the position of the fusion and is drawn to allow alignment of the 3' ends of the genes. (e) Structure of Cna mutants with different B domains. (f) Structure of the Cna-ClfA hybrid MSCRAMM. Restriction sites in (c-f) are indicated as follows: E, *Eco*RI; B, *Bam*HI; H, *Hind*III. (g) Structure of recombinant ClfA and ClfA-Cna proteins expressed in *E. coli*.

genes was studied in *S. aureus* DU5941, which lacks *cna*. Cell-wall-associated proteins expressed by strain DU5941 carrying plasmids pCN1, pCN2 and pCN3 were released by lysostaphin treatment and tested for Cna by Western immunoblotting using anti-Cna region A serum. Plasmids pCN1 and pCN2 expressed single immunoreactive proteins of 110 kDa or 87 kDa (Fig. 2, lanes 2 and 3), while plasmid pCN3 expressed two immunoreactive proteins, of 76 kDa and 73 kDa (Fig. 2, lane 4).

We then investigated whether region R of ClfA could substitute for the B domain of Cna. The dipeptide repeat occurs in four other surface protein of *S. aureus* (Josefsson *et al.*, 1998) and in two related proteins from *Staphylococcus epidermidis* (Nilsson *et al.*, 1998; K. McCrea, O. Hartford, T. J. Foster & M. Höök, unpublished) and seems capable of surface display of many different A domains. A hybrid protein (expressed by pCN4) was generated between Cna and ClfA which consisted of the N-terminal regions of Cna (signal sequence and region A) fused to the C-terminal region R, the LPDGTG sorting motif and transmembrane-spanning region of ClfA. Plasmid pCN4 expressed a

single hybrid immunoreactive protein of 130 kDa (Fig. 2, lane 1). The amount of immunoreactive Cna protein was compared by densitometric analysis. There was threefold less hybrid Cna-ClfA protein expressed by pCN4 than observed for the pCN1 protein (data not shown). The Cna-ClfA protein (846 residues) migrates more slowly than the Cna variant with two B repeats (949 residues). This is probably due to the negatively charged Ser-Asp dipeptide repeats which migrate aberrantly in SDS-PAGE.

Adherence of *S. aureus* *cna* mutants to immobilized collagen

S. aureus strains carrying plasmids with *cna* mutations were tested for their ability to adhere to immobilized collagen (Fig. 3). DU5941(pCN1) and DU5941(pCN2), expressing Cna with two or one B repeats respectively, adhered to immobilized collagen at levels similar to the control strain Phillips, as did cells expressing Cna with no B repeat (pCN3). Adherence of bacteria expressing the Cna-ClfA fusion protein (pCN4) was approximately 1.6-fold lower than that of the Cna derivatives. Thus the B repeats of Cna are not required for expression of

Fig. 2. Identification of Cna proteins by Western immunoblotting. Proteins released from the cell wall of *S. aureus* strains, grown for 15 h in TSB with aeration, were fractionated by SDS-PAGE (7.5% acrylamide), transferred to PVDF membranes and probed with anti-Cna region A antibody. Lanes: 1, DU5941(pCN4); 2, DU5941(pCN1); 3, DU5941(pCN2); 4, DU5941(pCN3).

Fig. 3. Bacterial adherence to immobilized collagen. Bacterial suspensions of 10^8 cells were added to microtitre plates containing immobilized collagen. Bound cells were fixed with formaldehyde and stained with crystal violet. The absorbance was measured at 570 nm. Column 1, strain Phillips; columns 2–6, strain DU5941 carrying the plasmids indicated. The mean value $\pm$ SE of five samples was determined; $n = 2$.

collagen-binding activity and the DS repeats of region R of ClfA can display the collagen-binding A domain of Cna in a functional form on the cell surface.

Construction and expression of a ClfA–Cna derivative

In order to determine if region B of Cna could substitute for region R of ClfA, we constructed a chimeric ClfA–Cna protein where region R plus the C-terminal region W, the LPDTG motif and the membrane-

Fig. 4. Identification of ClfA proteins by Western immunoblotting. Proteins released from the cell wall of *S. aureus* strains grown for 15 h in TSB with aeration were fractionated by SDS-PAGE (7.5% acrylamide), transferred to PVDF membranes and probed with anti-ClfA region A antibody. Lanes: 1, DU5941(pCF77); 2, DU5941(pCF72).

spanning regions of ClfA were replaced by one B repeat plus the C-terminal anchoring signals of Cna (pCF72; Fig. 1d). ClfA–Cna expression was monitored by analysing cell-wall proteins of *S. aureus* carrying the recombinant *clfA–cna* plasmid by Western immunoblotting with anti-ClfA-region A serum (Fig. 4). Plasmid pCF77 expressed three immunoreactive proteins, of >200 kDa, 185 kDa and 150 kDa (as described by Hartford *et al.*, 1997; Fig. 4, lane 1). The highest immunoreactive band probably corresponds to the native form of ClfA with the protein running at twice its molecular mass and the two lower bands are presumably degradation products (Hartford *et al.*, 1997). A similar pattern was observed with the ClfA–Cna variant. Plasmid pCF72 expressed a major immunoreactive protein of 155 kDa presumably corresponding to the native form (Fig. 4, lane 2) of the protein. In addition, a major breakdown product of 130 kDa was detected.

Binding of the *S. aureus* ClfA–Cna derivative to soluble fibrinogen

S. aureus cells carrying plasmids with the *clfA–cna* fusion were tested for cell clumping with soluble fibrinogen. Strain Newman, which carries a single copy of the *clfA* gene, gave a clumping titre of 1024. DU5941(pCF77), expressing the cloned wild-type gene on a multicopy plasmid, gave a clumping titre of 512. In contrast, DU5941(pCF72) was devoid of clumping activity. Thus, when the B domain and the C-terminal regions W and M of Cna are fused to region A of ClfA, the hybrid protein cannot participate in fibrinogen binding.

Binding of ClfA–Cna derivatives to immobilized fibrinogen

Bacteria expressing the hybrid ClfA–Cna protein were tested for binding to immobilized fibrinogen. As previously observed, pCF77, which expresses an engineered copy of the *clfA* gene, promoted adherence to immobilized fibrinogen (Hartford *et al.*, 1997). In contrast, no adherence to fibrinogen was observed with bacteria expressing ClfA fused to the B domain of Cna (pCF72) (data not shown).

To measure the level of surface expression of the ClfA–Cna chimeric proteins relative to ClfA, whole-cell dot immunoblotting was performed with exponential-phase bacterial cells and anti-ClfA serum. ClfA derivatives carrying pCF77 bound the antibody at a twofold dilution higher than strains carrying pCF72. This slightly reduced antibody binding is not sufficient to explain the complete lack of fibrinogen binding by pCF72-carrying cells. Taken together, these results indicate that region A of ClfA is located on the cell surface in the ClfA–Cna hybrid but region B of Cna cannot fulfil the role of region R of ClfA and results in a non-functional protein.

Fibrinogen binding of recombinant ClfA–Cna hybrid proteins

To investigate if the B region of Cna alters the conformation of region A of ClfA so that ligand binding cannot occur, a recombinant ClfA–Cna fusion protein was expressed in *E. coli* and tested for its ability to bind fibrinogen by Western affinity blotting and by an ELISA-type assay. A recombinant protein expressed by pClfA comprising residues 220–631 of ClfA (the ligand-binding region A residues 220–559 linked to 72 residues from region R) was included as a control. Initially, Western immunoblotting of *E. coli* lysates was performed with anti-ClfA antibodies to confirm that each construct expressed an immunoreactive protein. Lysates of *E. coli*(pClfA–Cna) induced with IPTG contained an immunoreactive protein of 58 kDa that reacted with anti-ClfA serum and bound fibrinogen in a Western affinity blot (data not shown). Plasmid pClfA expressed a fibrinogen-binding and immunoreactive protein of 46 kDa. When these recombinant proteins were analysed by ELISA, the ClfA–Cna recombinant protein bound immobilized fibrinogen as well as the control protein, ClfA (data not shown). Thus the presence of region B of Cna does not prevent region A of ClfA from binding fibrinogen when the protein was expressed in soluble form in the cytoplasm of *E. coli*.

DISCUSSION

A minimum number of residues are needed to link the biologically active N-terminal domains of the cell-wall-associated proteins ClfA and FnBPA in a functional form to the bacterial cell surface. More than 72 residues are required between region A of ClfA and the anchoring motif LPDTG for maximal binding of *S. aureus* to

fibrinogen (Hartford *et al.*, 1997). The 92-residue region W of *S. aureus* FnBPB must be sufficient for surface display of the immediately N-terminal fibronectin-binding D repeats and can support functional expression of a lipase on the surface of *S. carnosus* (Strauss & Götz, 1996). The collagen-binding protein (Cna) has a proline-rich wall-spanning region of 61 residues. All *S. aureus* Cna⁺ strains have at least one B domain of 187 residues between region W and the ligand-binding region A. We investigated whether the B domain of Cna is required as a surface display mechanism to project the ligand-binding region A domain on the bacterial cell surface. A mutant expressing one or two B domains adhered to immobilized collagen to the same extent as a deletion lacking the entire region B-encoding region of the *cna* gene. Thus the B domain of Cna does not contribute to collagen-binding activity and is not required for surface display of the ligand-binding A domain on the bacterial cell surface. However, the B domain of Cna may be necessary for surface expression of Cna in strains with capsular polysaccharide, especially as it is expressed at higher levels under *in vivo* conditions (Watson, 1989). The 8325-4 strain used in these studies is capsule negative (Wann *et al.*, 1999). A previous study suggested that masking of the collagen adhesin occurs in type 1 macroencapsulated strains (Gillaspay *et al.*, 1998). Presently it is unclear if the masking effect of the adhesin occurs in the clinically important type 5 and type 8 microencapsulated strains and if the B domain of Cna is required for surface display of the collagen-binding A domain.

This study shows that the 61-residue region W of Cna (plus nine additional residues) is sufficient for functional surface expression of the collagen-binding A domain in *S. aureus*. In ClfA, more than 72 residues (40 residues contributed by region R and 32 by region W) are required for functional expression of the fibrinogen-binding region A (Hartford *et al.*, 1997). As *S. aureus* surface proteins are modular in composition and most likely have evolved by *in vivo* gene fusion events, and because of the interest in expressing antigens on the surface of Gram-positive cocci using anchoring mechanisms of surface proteins, we investigated the possibility that C-terminal sequences of Cna and ClfA could support the functional expression of heterologous ligand-binding domains. Incorporating one single 187-residue B domain plus the C-terminal anchoring signals of Cna to the ligand-binding A domain of ClfA did not promote fibrinogen binding by *S. aureus* cells, suggesting that the B repeat prevents correct folding of the ligand-binding domain. However, a soluble ClfA–Cna fusion protein expressed in *E. coli* bound fibrinogen as well as a ClfA region A control. Thus, the B domain of Cna appears to alter the structure of the A domain of ClfA only when the hybrid protein is expressed on the bacterial cell surface.

Western immunoblotting analysis of ClfA released from *S. aureus* by lysostaphin treatment revealed three immunoreactive proteins, the native ClfA protein and two proteolytic breakdown products (Hartford *et al.*,

1997). This implies that residues at the extreme N-terminus of ClfA are cleaved from the protein, the bulk of which, including the functional part of region A (220–559), remains attached to the cell wall. A similar banding profile was observed with the ClfA–Cna variant, confirming that ClfA region A is susceptible to proteolytic degradation. In contrast, the Cna protein migrates at its predicted molecular mass by SDS-PAGE and is not subject to proteolysis. Interestingly, a lower amount of Cna–ClfA protein was detected relative to the Cna B domain variants. This might explain the slightly lower collagen binding of Cna–ClfA-expressing bacteria compared to pCN1.

A previous study showed that when the C-terminal end of Cna is fused to protein A, the hybrid protein is targeted to the membrane and is not covalently linked to peptidoglycan by sortase (Schneewind *et al.*, 1993). However, only newly synthesized proteins were analysed in these pulse-chase experiments. Proteins were considered to be membrane anchored if they were released into hot SDS after muramidase or lysostaphin treatment, whereas proteins that were covalently linked to peptidoglycan were released by lysostaphin. Here we found that much of the Cna protein is released by lysostaphin treatment, but a portion remains in the insoluble (membrane) fraction, despite repeated lysostaphin treatment (unpublished data). This suggests that sortase acts less efficiently on Cna than on other surface proteins.

In conclusion, this paper shows that the dipeptide repeat region R of ClfA is capable of supporting functional surface expression of a heterologous ligand-binding function whereas the B repeats of Cna are not. In addition, the B repeats of Cna have been shown not to be required for surface expression of collagen-binding activity by Cna.

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