Clumping factor A of *Staphylococcus aureus* inhibits phagocytosis by human polymorphonuclear leucocytes

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Abstract

Staphylococcus aureus is a major cause of nosocomial and community-acquired infection. It expresses several factors that promote avoidance of phagocytosis by polymorphonuclear leucocytes. Clumping factor A (ClfA) is a fibrinogen-binding surface protein of *S. aureus* that is an important virulence factor in several infection models. This study investigated whether ClfA is an antiphagocytic factor, and whether its antiphagocytic properties were based on its ability to bind fibrinogen. In *S. aureus*, ClfA was shown to be of equal importance to protein A, the antiphagocytic properties of which are well established. ClfA expressed in a surrogate Gram-positive host was also found to be antiphagocytic. A ClfA mutant that was unable to bind fibrinogen had a similar antiphagocytic effect to native ClfA in the absence of fibrinogen. ClfA inhibited phagocytosis in the absence of fibrinogen, and showed enhanced inhibition in the presence of fibrinogen.

Introduction

Staphylococcus aureus causes a wide variety of diseases, including abscesses, septicaemia, septic arthritis and infective endocarditis. Immunity to staphylococcal disease involves the binding of complement and opsonizing antibodies to the bacterial surface, which leads to activation of phagocytosis by human polymorphonuclear leucocytes (PMNL) (Cunnion *et al.*, 2003; Rooijakkers *et al.*, 2005). Once *S. aureus* penetrates the barrier of the skin PMNL are the critical first line of defence (Verdrengh & Tarkowski, 1997).

Staphylococcus aureus expresses several factors that promote avoidance of phagocytosis by PMNL (Foster, 2005; Rooijakkers *et al.*, 2005). Protein A is a virulence factor in experimental infection models of subcutaneous infection, murine sepsis and septic arthritis and staphylococcal pneumonia (Patel *et al.*, 1987; Gomez *et al.*, 2004; Palmqvist *et al.*, 2002). It is believed to be antiphagocytic due to its ability to bind the Fc region of immunoglobulin G (IgG) (Gemmell *et al.*, 1991). Capsular polysaccharide is a virulence factor in a murine bacteraemia model (Thakker *et al.*, 1998). The antiphagocytic effect of capsule is due to its ability to impede binding by opsonizing antibodies that recognize antigens on the cell surface, as well as to inhibit complement fixation (Thakker *et al.*, 1998; Cunnion *et al.*, 2003). This contributes to the virulence of *S. aureus* by preventing bacterial clearance from the bloodstream, liver and spleen (Luong & Lee, 2002). Poly-*N*-acetylglucosamine also contributes to virulence in animal models of bacteraemia and renal abscess formation by inhibiting complement-mediated phagocytic uptake by PMNL (Kropec *et al.*, 2005).

Clumping factor A (ClfA) is a fibrinogen-binding surface protein of *S. aureus* (McDevitt *et al.*, 1997). It contains an N-terminal secretory signal peptide, followed by a 520-residue region A that contains the ligand-binding domain, region R consisting primarily of SD dipeptide repeats and a C-terminal domain that allows anchoring to the cell wall peptidoglycan (McDevitt *et al.*, 1995).

Clumping factor A is an important virulence factor in several infection models, including rat experimental endocarditis (Moreillon *et al.*, 1995), murine sepsis and septic arthritis (Josefsson *et al.*, 2001) and rabbit infective endocarditis (Vernachio *et al.*, 2003). By comparing wild-type *S. aureus* with ClfA-deficient mutants and by transfer of *clfA* to *Streptococcus gordonii* (Stutzmann Meier *et al.*, 2001) and to *Lactococcus lactis*, ClfA was identified as an important factor in mediating endovascular infection (Que *et al.*, 2001). ClfA-deficient strains are also attenuated in a murine model of sepsis and septic arthritis (Josefsson *et al.*, 2001; Vernachio *et al.*, 2003). In this model immunization with recombinant region A of ClfA was protective, as was passive immunization with human IgG containing a high titre against ClfA (Josefsson *et al.*, 2001).

It is possible that ClfA acts as a virulence factor in certain infections by inhibiting phagocytosis, as well as promoting adhesion to fibrin and fibrinogen. This study investigated whether ClfA is antiphagocytic and if this effect is due to its ability to bind fibrinogen.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains are listed in Table 1. *Staphylococcus aureus* strains were cultured on trypticase soy agar or broth (Oxoid, Basingstoke, UK) at 37 °C with shaking at 200 r.p.m. for liquid cultures. *Lactococcus lactis* strains were cultured in M17 medium (Difco, Detroit, MI) containing 0.5% glucose without shaking at 30 °C. Antibiotics were incorporated where appropriate at the following concentrations: 10 μg mL^{-1} chloramphenicol, 5 μg mL^{-1} erythromycin and 10 μg mL^{-1} kanamycin.

Transduction

A null mutation in protein A was transduced from *S. aureus* 8325-4 *spa*::Kan^{R} (Patel *et al*., 1987) by generalized phage transduction using phage 85 (Foster, 1998) to strains Newman (Duthie & Lorenz, 1952) and Newman *clfA* (McDevitt *et al*., 1994) with selection for kanamycin resistance. The fidelity of transductants was verified by Southern blotting and by Western immunoblotting of solublized cell wall proteins with anti-ClfA polyclonal antisera and with horseradish peroxidase-conjugated goat IgG.

PMNL isolation

Fresh whole blood was obtained from healthy volunteers, heparinized and mixed with an equal volume of phosphate-buffered saline (PBS). This was centrifuged through step gradients of Histopaque (ρ = 1.077, Sigma, St Louis, MO) and Ficoll-paque (ρ = 1.119, Amersham, Chalfont St Giles, UK) and PMNL were aspirated from the buffy coat between the Ficoll and Histopaque layers. Cells were washed in RPMI 1640 medium [containing 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 25 mM glutamine and 0.05% (v/v) human serum albumin] and resuspended in water. After a 30 s osmotic shock to lyse contaminating red blood cells, 10 × PBS was added and the cells were again washed in RPMI. PMNL were counted in a haemocytometer (bright line, Neubauer) and adjusted to 5×10^6 cells per mL in RPMI. This procedure typically yielded > 97% PMNL with > 95% viability, as determined by trypan blue exclusion. All reagents used in PMNL isolation were certified endotoxin-free.

Phagocytosis of bacteria by human PMNL

Whole bacterial cells grown to stationary phase were washed twice in PBS and labelled with 30 μg mL^{-1} fluorescein isothiocyanate (FITC) in PBS for 1 h at 37 °C with shaking. Cells were washed three times in PBS and enumerated in a Neubauer haemocytometer (Brand GmbH, Wertheim, Germany), adjusted to 1×10^9 CFU mL^{-1} in RPMI and stored frozen at − 20 °C. FITC-labelling of bacteria did not affect their ability to bind fibrinogen (data not shown). Bacteria were thawed on ice and diluted to 5×10^7 CFU mL^{-1} in RPMI. Pooled human serum was diluted in RPMI and fibrinogen (Calbiochem, Schwalbach, Germany) was added to serum where necessary. The final concentration of fibrinogen, where present, was the same percentage as the serum concentration, calculated as 3 mg mL^{-1} = 100%. Bacteria (50 μL) were opsonized in 10 μL diluted serum (with or

Table 1. Strains and plasmids used in this study

	Properties	References
Strain		
Staphylococcus aureus		
Newman	NCTC 8178	Duthie & Lorenz (1952)
Newman *clfA*	*clfA*::Erm^{R*}	McDevitt *et al*. (1994)
Newman *spa*	*spa*::Kan^{R}	This study
Newman *spa clfA*	*spa*::Kan^{R} *clfA*::Erm^{R}	This study
Lactococcus lactis		
MG1363	Plasmid-free derivative of NCD 0712	Gasson (1983)
NZ9800	MG1363 bearing nisin resistance plasmid [Cam^{R}]	Kuipers *et al*. (1993)
Plasmid		
pKS80	*L. lactis* expression vector [Erm^{R}]	Wells *et al*. (1993)
pKS80 *clfA*	pKS80 containing *clfA* coding sequence [Erm^{R}]	O'Brien *et al*. (2002)
pKS80 *spa*	pKS80 containing *spa* coding sequence [Erm^{R}]	O'Brien *et al*. (2002)
pNZ8037	Nisin-inducible *L. lactis* expression vector [Cam^{R}]	de Ruyter *et al*. (1996)
pNZ8037 *clfA*	pNZ8037 containing *clfA* coding sequence [Cam^{R}]	Loughman *et al*. (2005)
pNZ8037 *clfA*PY	pNZ8037 containing *clfA* coding sequence, incorporating mutations [Cam^{R}]	Loughman *et al*. (2005)

*Erm^{R}, Kan^{R} and Cam^{R}; resistance to erythromycin, kanamycin and chloramphenicol, respectively.

without fibrinogen) for 10 min at 37 °C, followed by addition of 50 μL prewarmed PMNL and incubation at 37 °C with vigorous shaking. The final bacteria : PMNL ratio was 10 : 1. Reactions were stopped after 5–15 min by addition of 100 μL ice-cold 1% (w/v) paraformaldehyde in PBS. The percentage of PMNL bearing FITC-labelled bacteria (% phagocytosis) was determined by flow cytometric analysis of 5000 cells with manual gating using a FACScan flow cytometer (Becton Dickinson, Oxford, UK). The percentage of internalized bacteria was determined by trypan blue quenching of extracellular fluorescence (Benne *et al.*, 1997; Su *et al.*, 2001). Addition of 20 μg mL^{-1} (final concentration) trypan blue (Merck, Schwalbach, Germany) demonstrated that $>$ 85% bacteria associated with PMNL were internalized (data not shown). The absence of bacterial clumping during the assay was verified microscopically. Samples were prepared in triplicate and all experiments were repeated a minimum of three times using the blood of different donors. Statistical analyses were performed using the Student's *t*-test for paired data in Kaleidagraph (Synergy software). *P* values $<$ 0.01 were considered significant.

Whole cell dot immunoblotting

Quantitation of the relative amounts of protein expressed by *L. lactis* strains was carried out by whole cell dot immunoblotting, as described previously (Loughman *et al.*, 2005).

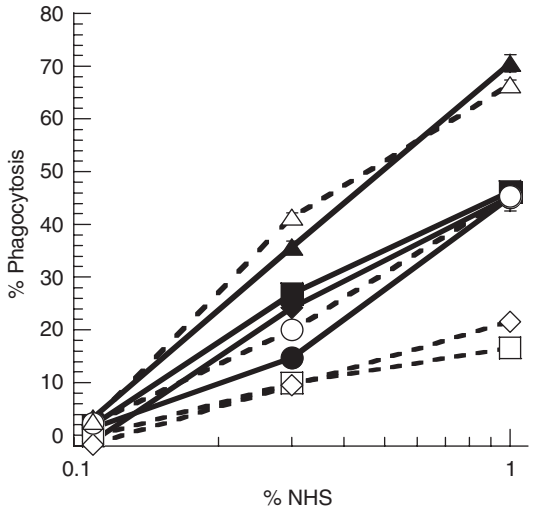

Fig. 1. Phagocytosis of Newman strains by human polymorphonuclear leucocytes (PMNL). Freshly isolated human PMNL were incubated with *Staphylococcus aureus* Newman strains grown to stationary phase and opsonized with varying concentrations of normal human serum in the absence (solid lines) or presence (dashed lines) of physiologically relevant concentrations of fibrinogen. Wild-type Newman (squares) expresses both clumping factor A (ClfA) and protein A, Newman *spa* (diamonds) is protein A-deficient, Newman *clfA* (circles) is ClfA-deficient and Newman *spa clfA* (triangles) is deficient in both protein A and ClfA. The percentage of PMNL containing bacteria is expressed as % phagocytosis. All samples were prepared in triplicate. Data are representative of at least three independent experiments using three different donors.

Results

In order to investigate the antiphagocytic effects of protein A and ClfA, strains Newman, Newman *clfA*, Newman *spa* and Newman *spa clfA* were tested for their uptake by fresh human PMNL in the presence of human serum opsonins. Increasing concentrations of serum resulted in increased phagocytic uptake of all strains, indicating that the process was dependent on serum opsonins. At 1% normal human serum (NHS) strain Newman was phagocytosed by approximately 46% of PMNL (Fig. 1). Newman *spa* and Newman *clfA* were taken up by approximately 45% of PMNL at this serum concentration, whereas the Newman *spa clfA* double mutant was taken up by 72% of PMNL ($P < 0.001$). This demonstrated that both protein A and ClfA can contribute to the inhibition of phagocytosis of *Staphylococcus aureus* Newman, and that the absence of one factor was compensated for by the presence of the other. The absence of both factors led to a significant increase in phagocytic uptake.

The addition of a physiologically relevant concentration of fibrinogen had no significant impact on the level of uptake of the ClfA-deficient strains Newman *clfA* and Newman *spa clfA* (Fig. 1). However, the levels of uptake of both the wild-type strain and of Newman *spa* in 1% NHS were significantly reduced in the presence of fibrinogen ($P < 0.005$ and $P < 0.01$, respectively). This indicated that strains bearing ClfA on their cell surface had enhanced antiphagocytic properties in the presence of soluble fibrinogen. The presence of fibrinogen had no effect on the phagocytic uptake of cells lacking ClfA.

Bacterial cells used in this assay were grown to stationary phase to minimize any possible contribution of the fibrinogen-binding protein clumping factor B, which is absent in stationary phase due to proteolysis and cessation of *clfB* transcription (McAleese *et al.*, 2001). The fibronectin-binding proteins are also predominantly expressed during the exponential phase of growth and can also bind fibrinogen. However, the *fnbA* and *fnbB* genes of strain Newman each have a frameshift mutation that results in secretion of truncated forms of the proteins (Grundmeier *et al.*, 2004). Thus, ClfA was the only known fibrinogen-binding surface protein expressed by the cells in this assay.

In order to investigate further the antiphagocytic properties of ClfA, the protein was expressed on the surface of the Gram-positive surrogate host *Lactococcus lactis* (O'Brien *et al.*, 2002). Increasing concentrations of serum led to increased uptake of *L. lactis* cells (Fig. 2), indicating that efficient opsonization of the bacteria was required to facilitate phagocytosis. *Lactococcus lactis* cells bearing an empty plasmid vector (pKS80) were taken up by 50% of PMNL at

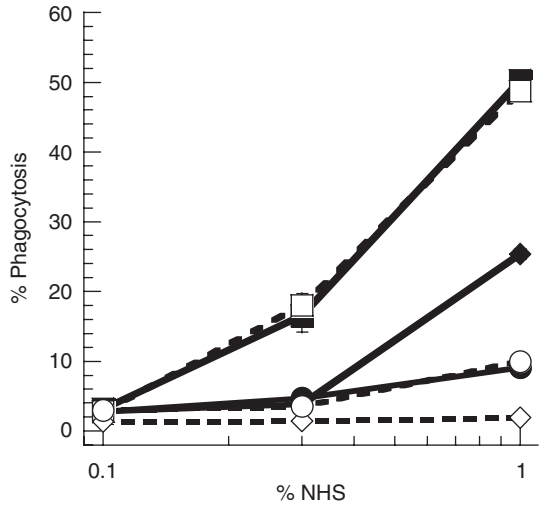

Fig. 2. Phagocytosis of *Lactococcus lactis* strains expressing clumping factor A (ClfA) or protein A. Freshly isolated human polymorphonuclear leucocytes (PMNL) were incubated with *L. lactis* MG1363 strains expressing no protein (pKS80, squares), ClfA (pKS80 *clfA*, diamonds) or protein A (pKS80 *spa*, circles) grown to stationary phase and opsonized with varying concentrations of normal human serum in the absence (solid lines) or presence (dashed lines) of physiologically relevant concentrations of fibrinogen. The percentage of PMNL containing bacteria is expressed as % phagocytosis. All samples were prepared in triplicate. Data are representative of at least three independent experiments using three different donors.

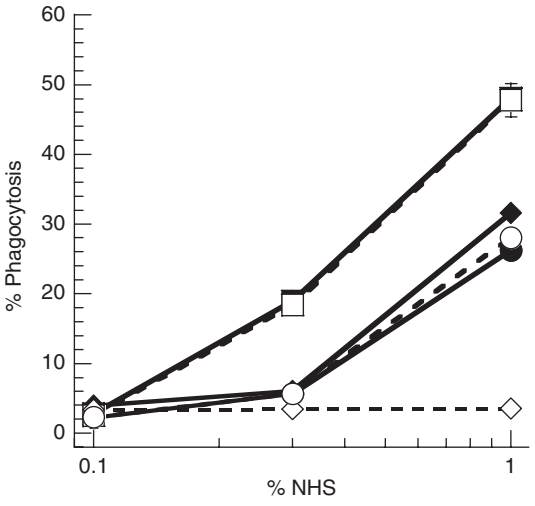

Fig. 3. Phagocytosis of *Lactococcus lactis* strains expressing clumping factor A (ClfA) or ClfAPY. Freshly isolated human PMNL were incubated with *L. lactis* NZ9800 strains expressing no protein (pNZ8037, squares), ClfA (pNZ8037 *clfA*, diamonds) or ClfAPY (pNZ8037 *clf*APY, circles) grown to stationary phase and opsonized with varying concentrations of normal human serum in the absence (solid lines) or presence (dashed lines) of physiologically relevant concentrations of fibrinogen. The percentage of polymorphonuclear leucocytes (PMNL) containing bacteria is expressed as % phagocytosis. All samples were prepared in triplicate. Data are representative of at least three independent experiments using three different donors.

1% NHS. The addition of fibrinogen had no significant impact on the level of uptake of these bacteria. *L. lactis* expressing ClfA (pKS80 *clfA*) were taken up by significantly fewer PMNL (25% at 1% NHS, $P = 0.002$ compared with *L. lactis* pKS80). The addition of fibrinogen further reduced this level to 2% ($P = 0.001$). *Lactococcus lactis* expressing protein A were taken up by only 9% of PMNL at 1% NHS ($P = 0.003$ compared with *L. lactis* pKS80), however the addition of fibrinogen did not affect the level of uptake of this strain. Protein A showed very potent inhibition of phagocytosis, but showed no fibrinogen-dependent reduction of uptake. ClfA was capable of inhibition of phagocytosis in the absence of fibrinogen, and showed increased inhibition in the presence of fibrinogen.

In order to investigate whether the effect of fibrinogen was due to direct binding to ClfA, a derivative of ClfA with substitutions in residues essential for fibrinogen binding (ClfAPY) was used (Loughman *et al.*, 2005). *Lactococcus lactis* NZ9800 allows inducible expression of genes cloned into the vector pNZ8037. The relative levels of expression of ClfA and ClfAPY were monitored by whole cell dot immunoblotting and shown to be equal before cell labelling (data not shown). *Lactococcus lactis* NZ9800 bearing the empty vector (pNZ8037) was taken up by approximately 48% of PMNL at 1% NHS both in the presence and absence of fibrinogen (Fig. 3). *Lactococcus lactis* NZ9800 expressing ClfA (pNZ8037 *clfA*) was taken up by approximately 32% of PMNL at the same serum concentration in the absence of fibrinogen, and the level of uptake was further reduced to approximately 4% in the presence of fibrinogen ($P < 0.001$ compared with *L. lactis* pNZ8037). *Lactococcus lactis* expressing ClfAPY (pNZ8037 *clf*APY), which does not bind fibrinogen (Loughman *et al.*, 2005) was taken up by approximately 27% of PMNL at 1% NHS. However, the addition of soluble fibrinogen did not influence the level of uptake of this strain (Fig. 3). This indicated that direct binding of fibrinogen to ClfA on the surface of the bacteria caused increased inhibition of phagocytosis by human PMNL.

Discussion

The importance of ClfA in the virulence of *Staphylococcus aureus* in a variety of animal models suggests that the protein might have an antiphagocytic effect. This study investigated a possible role for ClfA in the resistance of *S. aureus* to phagocytosis by human PMNL. The experiments described here show clearly that the well known antiphagocytic action of protein A (Rooijakkers *et al.*, 2005) can be clearly demonstrated in our *in vitro* phagocytosis assay. Null mutation of the gene responsible in virulent staphylococci

(Newman *spa*) resulted in increased sensitivity to phagocytosis. Moreover, introduction of the gene into the nonvirulent *Lactococcus lactis* (pKS80 *spa*) showed clear inhibition of phagocytosis. In the same system, it was also shown that ClfA is an important antiphagocytic factor for *S. aureus*, with both fibrinogen-dependent and fibrinogen-independent mechanisms. In *S. aureus* Newman, ClfA was shown to be of equal importance to protein A. Using ClfA expressed in a surrogate Gram-positive host we could also show that it possesses strong antiphagocytic properties. A ClfA mutant that was unable to bind fibrinogen had a similar antiphagocytic effect as native ClfA in the absence of fibrinogen, further establishing the existence of a fibrinogen-independent mechanism.

When *S. aureus* is grown in plasma it becomes coated with soluble plasma components that interfere with bacterial adherence to immobilized ligands (Massey *et al.*, 2002). In this study, inactivation of both *clfA* and *spa* in a microencapsulated strain of *S. aureus* caused a significant increase in the level of phagocytosis. Although Newman can express a type 5 capsular polysaccharide, the growth conditions used in this study would not have promoted its expression at high levels. The presence of either ClfA or protein A alone seemed to be sufficient to inhibit phagocytosis. It appears that being coated with either immunoglobulin or fibrinogen, or a mixture of both, is sufficient to impede phagocytosis. This indicates that protein A and ClfA are two major antiphagocytic factors for *S. aureus*.

Anti-ClfA antibodies are present both in normal and convalescent sera (Colque-Navarro *et al.*, 2000; Dryla *et al.*, 2005). This implies that normal exposure to *S. aureus* as a commensal of the skin causes the formation of anti-ClfA antibodies, but that the antibodies in normal sera are not protective against invasive *S. aureus* disease. These antibodies have opsonizing activity (Josefsson *et al.*, 2001; Vernachio *et al.*, 2003; Patti, 2004). Immunization of mice with recombinant ClfA induced anti-ClfA antibody production, whereas infection of naïve mice with *S. aureus* Newman did not (Josefsson *et al.*, 2001). Perhaps the ability of ClfA on the surface of *S. aureus* cells to bind fibrinogen in the bloodstream prevents the development of high levels of specific antibodies directed against it. In addition, the recent observation that low levels of anti-ClfA antibodies stimulate platelet aggregation may go some way to explaining why the low levels of antibodies in normal serum are not protective against staphylococcal infection (Loughman *et al.*, 2005).

It is likely that the pooled human serum used in this study contained low levels of antibodies against ClfA (Dryla *et al.*, 2005; Loughman *et al.*, 2005). They might interact with ClfA in one of two ways. They may compete with fibrinogen to bind ClfA and promote phagocytosis, either alone via Fc receptors on the PMNL surface or by stimulating complement activation. Alternatively they may promote phagocytosis by binding to ClfA along with bound fibrinogen.

However, ClfA is antiphagocytic despite the presence of low levels of anti-ClfA antibodies in normal human sera. Perhaps opsonins bound to bacteria are prevented from interacting productively with Fc receptors and complement receptors on the PMNL surface, possibly through steric hindrance by bound fibrinogen, immunoglobulins bound to protein A, or other serum or bacterial factors. These may form a protective layer of host proteins around the bacterium, preventing access by antibodies to antigens on the cell wall surface such as peptidoglycan, teichoic acids and other proteins, or preventing interactions between antibodies bound to these antigens and Fc receptors on the PMNL surface. Such a protective layer might also interfere with complement deposition.

Attempts to measure the survival of bacteria following uptake gave paradoxical results. After 30 min *c.* 50% of wild-type bacteria were killed, however the ClfA and protein A mutants were killed less than wild type during this time (unpublished data), despite being taken up more efficiently (Fig. 1). This may have been caused by alteration of the fidelity of the phagosome due to changes in major surface proteins on the bacterial surface, as was observed by Gresham *et al.* (2000). The fluorescence-based assay used in this study allows analysis of bacterial uptake independently from the complex interactions involved in bacterial killing.

The presence of two mechanisms of resistance to phagocytic uptake, one fibrinogen-dependent and the other fibrinogen-independent may reflect the presence of different niches for the bacteria within the host during disease progression, in which fibrinogen is available or unavailable. For example, it appears likely that the ability of ClfA to inhibit phagocytosis independently of fibrinogen may be important in the murine sepsis and septic arthritis model, as depletion of free fibrinogen in the bloodstream led to aggravation of septic arthritis caused by ClfA-producing *S. aureus* strains (Palmqvist *et al.*, 2004). This fibrinogen-independent inhibition may also be important *in vivo* in environments with an elevated Ca^{2+} concentration, as Ca^{2+} inhibits fibrinogen binding by ClfA with an IC_{50} of 2.5 mM (O'Connell *et al.*, 1998).

The ability of ClfA to inhibit phagocytosis by human PMNL may explain its importance in *S. aureus* virulence in a variety of animal models of infection. It is likely also to be the reason that high-titre, but not low-titre anti-ClfA antibodies are protective.

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