RESEARCH LETTER

Fibronectin-binding proteins are required for biofilm formation by community-associated methicillin-resistant *Staphylococcus aureus* strain LAC

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Abstract

Community-associated methicillin-resistant *Staphylococcus aureus* of the USA300 lineage is emerging as an important cause of medical device-related infection. However, few factors required for biofilm accumulation by USA300 strains have been identified, and the processes involved are poorly understood. Here, we identify *S. aureus* proteins required for the USA300 isolate LAC to form biofilm. A mutant with a deletion of the *fnbA* and *fnbB* genes did not express the fibronectin-binding proteins FnBPA and FnBPB and lacked the ability to adhere to fibronectin or to form biofilm. Biofilm formation by the mutant LACΔ*fnbAfnbB* could be restored by expression of FnBPA or FnBPB from a plasmid demonstrating that both of these proteins can mediate biofilm formation when expressed by LAC. Expression of FnBPA and FnBPB increased bacterial aggregation suggesting that fibronectin-binding proteins can promote the accumulation phase of biofilm. Loss of fibronectin-binding proteins reduced the initial adherence of bacteria, indicating that these proteins are also involved in primary attachment. In summary, these findings improve our understanding of biofilm formation by the USA300 strain LAC by demonstrating that the fibronectin-binding proteins are required.

Introduction

Staphylococcus aureus is a commensal bacterium that is carried persistently in the anterior nares of about 20% of the human population. The organism is a leading cause of infections associated with indwelling medical devices such as central venous catheters, cardiovascular devices and artificial joints (O'Gara & Humphreys, 2001; Zimmerli *et al.*, 2004). The ability to form multicellular communities known as biofilm is crucial to success in device-related infection. Bacteria in the biofilm matrix are more resistant to neutrophils and macrophages and are difficult to inhibit with antibiotics (Stewart & Costerton, 2001; Vuong *et al.*, 2004; Otto, 2006).

Attachment to the surface of a biomedical device is the first stage of biofilm formation. Adhesion to surfaces that have been conditioned by host plasma proteins is promoted by microbial surface components recognising adhesive matrix molecules (MSCRAMMs) (Vaudaux *et al.*, 1995). The accumulation phase of biofilm formation can be mediated by the *icaADBC*-encoded polysaccharide intercellular adhesin (Heilmann *et al.*, 1996) or alternatively by *S. aureus* surface proteins such as Bap (Cucarella *et al.*, 2001), SasG (Geoghegan *et al.*, 2010), SasC (Schroeder *et al.*, 2009), fibronectin-binding proteins (O'Neill *et al.*, 2008) and protein A (Merino *et al.*, 2009).

For certain strains of healthcare-associated methicillin-resistant *S. aureus* (HA-MRSA), the accumulation phase of biofilm formation is mediated by the cell wall-associated fibronectin-binding proteins A and B (FnBPA and FnBPB (O'Neill *et al.*, 2008; Vergara-Irigaray *et al.*, 2009). FnBPs are multifunctional proteins which also promote bacterial attachment to fibrinogen, elastin and fibronectin (Fig. 1). The fibrinogen- and elastin-binding sites have been localised to subdomains N2 and N3 of the N-terminal A region which form independent immunoglobulin-like folded
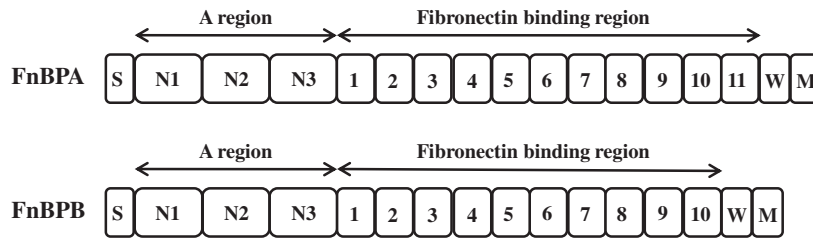
Fig. 1. Schematic representation of FnBPA and FnBPB. The position of the signal sequence (S) and the wall (W) and membrane (M)-spanning regions are indicated. The A region comprises subdomains N1, N2, N3. The fibronectin-binding region is composed of a series of tandemly repeated motifs which bind to fibronectin.

structures (Keane *et al.*, 2007; Geoghegan *et al.*, 2013). Subdomains N2N3 also mediate the accumulation phase of *S. aureus* biofilm formation in these HA-MRSA strains (Geoghegan *et al.*, 2013). The fibronectin-binding region is located C-terminally to the A region and comprises 11 (FnBPA) or 10 (FnBPB) tandemly repeated motifs which recognise type I modules of fibronectin (Sottile *et al.*, 1991; Schwarz-Linek *et al.*, 2003). The binding of FnBPs to fibronectin promotes the internalisation of bacteria into epithelial and endothelial cells through the formation of a fibronectin bridge to the α5β1 integrin (Peacock *et al.*, 1999; Sinha *et al.*, 1999).

Methicillin-resistant *S. aureus* (MRSA) causes significant morbidity and mortality. Healthcare-associated MRSA causes infection in those with a predisposing risk factor or illness, while community-associated (CA-)MRSA infection can occur in otherwise healthy individuals. CA-MRSA strains of the USA300 lineage (clonal complex 8, CC8) are the major cause of serious skin and soft tissue infections in the USA (David & Daum, 2010). USA300 strains are also emerging as an important cause of medical device-related infection and prosthetic-joint-related infection (Kourbatova *et al.*, 2005). CA-MRSA strains are generally much more virulent than HA-MRSA (Otto, 2010; Rudkin *et al.*, 2012). HA-MRSA strains often do not express *agr* RNAIII (Rudkin *et al.*, 2012), whereas RNAIII is very highly expressed in CA-MRSA strains of the USA300 lineage (Cheung *et al.*, 2011). As *agr* is the master regulator of gene expression in *S. aureus*, differences in RNAIII production are responsible for pleiotropic effects on gene expression (Novick, 2003; Thoendel *et al.*, 2011). Biofilm formation by HA-MRSA strains has been studied in detail (O'Neill *et al.*, 2007, 2008; Pozzi *et al.*, 2012; Geoghegan *et al.*, 2013), but much less is known about how other clinical strains (in particular CA-MRSA isolates) form biofilm. USA300 strain LAC forms biofilm *in vitro* and *in vivo* (Lauderdale *et al.*, 2009, 2010; Thurlow *et al.*, 2011; Zielinska *et al.*, 2012), and recent studies have focused on elucidating the composition of the biofilm matrix. Both extracellular DNA and proteins are important structural components of the

LAC biofilm matrix (Lauderdale *et al.*, 2009; Kiedrowski *et al.*, 2011; Mootz *et al.*, 2013). The *S. aureus* proteins that mediate biofilm formation by LAC have not been identified. This study set out to determine whether fibronectin-binding proteins are expressed by LAC and whether they play a role in biofilm formation.

Materials and methods

Culture conditions and reagents

Escherichia coli was grown in lysogeny broth at 37 °C. *Staphylococcus aureus* was grown in tryptic soy broth (TSB, Oxoid) or brain heart infusion (BHI, Oxoid) broth at 37 °C. Media were supplemented with glucose (1% w/v), ampicillin (100 μg mL^{-1}) or chloramphenicol (10 μg mL^{-1}) where appropriate. Stationary phase cultures were grown for *c.* 16 h. For exponential phase, bacteria were diluted 1 : 200, washed in BHI and allowed to grow to an OD$_{600 \text{ nm}}$ of 0.3 or 0.4. Unless otherwise stated, all reagents were obtained from Sigma.

Strain construction and plasmids

Strain BH1CC is a HA-MRSA isolate (O'Neill *et al.*, 2007) and BH1CCΔ*fnbAfnbB* an isogenic knockout mutant (Geoghegan *et al.*, 2013). LAC is a CA-MRSA isolate of the USA300 lineage (Diep *et al.*, 2006). Deletion of *fnbA* and *fnbB* to generate strain LAC Δ*fnbAfnbB* was achieved by allelic replacement using pIMAYΔ*fnbAfnbB* as previously described (Monk *et al.*, 2012; Geoghegan *et al.*, 2013). Plasmids pFnBA4 and pFnBB4 are multicopy plasmids expressing the entire *fnbA* and *fnbB* genes, respectively (Greene *et al.*, 1995). Plasmid pC221 (Projan *et al.*, 1985) is an *S. aureus* plasmid carrying the *cat* gene and served as a control to ensure that chloramphenicol acetyltransferase activity did not affect the ability of bacteria to adhere to fibronectin or to form biofilm. All shuttle plasmids were purified from *E. coli* DC10B (Monk *et al.*, 2012) and transformed into *S. aureus* made electrocompetent as previously described (Monk *et al.*, 2012).

Microtitre plate biofilm assay

Biofilm assays were carried out as described by Geoghegan *et al.* (2010). Briefly, *S. aureus* was grown for 16 h in TSB and diluted 1 : 200 in BHI supplemented with glucose (1% w/v). Diluted bacteria (200 μL) were added to the wells of untreated flat-bottomed polystyrene plates (Sarstedt). Plates were incubated statically at 37 °C for 24 h. Wells were washed three times with PBS and dried by inversion for 30 min. Adherent cells were stained with crystal violet (0.5% w/v), and the $A_{570 \text{ nm}}$ was measured. Each experiment was repeated three times. Statistical significance was determined with the Student's *t*-test, using GRAPHPAD software.

Aggregation assay

Aggregation assays were carried out as described by Geoghegan *et al.* (2010). Bacteria were grown overnight in TSB and diluted to an $OD_{600 \text{ nm}}$ of 1.0 in BHI broth (5 mL) supplemented with 1% (w/v) glucose. Tubes were incubated statically at 37 °C for 24 h. One millilitre of broth was removed from the top of the tube, and the $OD_{600 \text{ nm}}$ was measured. The remaining culture was vortexed to resuspend the cells, and the $OD_{600 \text{ nm}}$ was measured again. The per cent aggregation was calculated using the following formula: $100 \times [(OD_{600 \text{ nm}} \text{ of vortexed sample} - OD_{600 \text{ nm}}$ of broth removed before vortexing)/($OD_{600 \text{ nm}}$ of vortexed sample)]. The experiment was repeated three times, and statistical significance was determined with the Student's *t*-test, using GRAPHPAD software.

Biofilm flow cell experiments

The BioFlux 1000z microfluidic system (Fluxion Biosciences Inc., San Francisco, CA) was used to assess biofilm formation under shear flow conditions. The growth medium for flow cell biofilms was BHI supplemented with glucose (1% w/v). The system was initiated by adding 200 μL of media to the output wells of a 48-well plate and priming the channels for 5 min at 5.0 dynes cm^{-2}. After priming, the media were aspirated from the output wells and replaced with a 50 μL of bacteria grown to early exponential growth phase and adjusted to an $OD_{600 \text{ nm}} = 0.8$. A further 50 μL of medium was added to the input wells, and the channels were seeded by pumping from the output wells to the input wells for 3–5 s at a speed of 3 dynes cm^{-2}. Bacteria were allowed to attach to the surface of the plate for 1 h at 37 °C. Excess inoculum solution was aspirated from the output wells, and a further 1 mL of medium was added to the input wells. The flow rate was set at 0.4 dyne cm^{-2} for 20 h (equivalent to 42 μL h^{-1}), and brightfield images were captured every 5 min at 10×

magnification. Initial attachment of the bacteria was assessed by capturing images after allowing 20 s of flow to remove unattached bacteria (0 h). A total of 289 images were captured, and the gain and exposure settings remained constant over the 20-h period for all images.

Fibronectin affinity blotting

To extract cell wall-associated proteins, cultures of *S. aureus* were harvested, washed in PBS and resuspended to give an $OD_{600 \text{ nm}}$ of 40 in lysis buffer (50 mM Tris-HCl, 20 mM $MgCl_2$, pH 7.5) supplemented with raffinose (30% w/v) and complete protease inhibitors (40 μL mL^{-1}, Roche). Cell wall proteins were solubilised by incubation with lysostaphin (100 μg mL^{-1}; AMBI, New York, NY) for 8 min at 37 °C. Protoplasts were removed by centrifugation at 12 000 *g* for 5 min, and the supernatant containing solubilised cell wall proteins was aspirated and boiled for 10 min in Laemmli sample buffer.

Proteins were separated on 7.5% (w/v) polyacrylamide gels, transferred onto PVDF membranes (Roche) and blocked in skimmed milk proteins (10% w/v). Human fibronectin (0.5 mg mL^{-1}, Calbiochem) was incubated with biotin (2 mg mL^{-1}) for 20 min at room temperature. The reaction was stopped by addition of 10 mM NH_4Cl. Excess biotin was removed by dialysis against PBS overnight at 4 °C. Blots were probed with biotin-labelled human fibronectin (15 μg mL^{-1}) and peroxidase-conjugated streptavidin (0.5 μg mL^{-1}, Genscript). Reactive bands were visualised using the LumiGLO reagent and peroxide detection system (Cell Signalling Technology).

Bacterial adherence to fibronectin

Microtitre plates (Sarstedt) were coated with doubling dilutions of a solution of human fibronectin (Calbiochem) in PBS overnight at 4 °C. Wells were blocked with 5% (w/v) bovine serum albumin (BSA) for 2 h at 37 °C. Washed bacteria were adjusted to an $OD_{600 \text{ nm}}$ of 1.0 in PBS, and 100 μL was added to each well and incubated for 1.5 h at 37 °C. Wells were washed with PBS, and adherent cells fixed with formaldehyde (25% v/v), stained with crystal violet and the $A_{570 \text{ nm}}$ measured. Each experiment was performed three times.

Results

LAC adhesion to fibronectin depends on FnBPs

Recently, conflicting evidence was published concerning the expression of FnBPs in the surface proteome of CA-MRSA strain LAC (Ventura *et al.*, 2010; Zielinska *et al.*, 2012; Kolar *et al.*, 2013). One aim of this study was to

determine conclusively whether FnBPs are expressed by LAC and whether they promote bacterial adherence to fibronectin. Solid phase assays were used to measure the adherence of bacteria to immobilised fibronectin. Strain BH1CC, a HA-MRSA isolate which expresses FnBPs at high levels throughout the growth cycle (Geoghegan *et al.*, 2013), was included as a control. LAC grown to either the exponential or the stationary phase of growth adhered to fibronectin in a manner dependent on the ligand concentration (Fig. 2a). The levels of adherence were similar to those seen for BH1CC. A mutant of BH1CC with a deletion of the closely linked *fnbA* and *fnbB* genes (BH1CCΔ*fnbAfnbB*) lacks the ability to adhere to fibronectin and served as a negative control (Fig. 2a).

To determine whether FnBPs were the only proteins promoting LAC adherence to fibronectin, an *fnbA fnbB* deletion mutant of LAC was constructed by allelic exchange. *Staphylococcus aureus* LACΔ*fnbAfnbB* did not adhere detectably to fibronectin unless a very high concentration of the ligand (5 μg mL^{-1}) was used (Fig. 2b). This low-level adherence was considered to be nonspecific because adherence of all other strains has reached saturation at the concentration of fibronectin. This demonstrates that adherence of LAC to fibronectin can be attributed to the expression of FnBPs. Complementation with plasmid pFnBA4, expressing FnBPA, or pFnBB4, expressing FnBPB restored the ability of the bacteria to adhere to fibronectin at levels higher than the wild-type (Fig. 2b). LACΔ*fnbAfnbB* carrying an empty control plasmid pC221 did not adhere to fibronectin (Fig. 2b). Together, these data show

that FnBPs are expressed by LAC and required for the bacteria to adhere to fibronectin.

Expression of fibronectin-binding proteins by *S. aureus* LAC

To determine whether both FnBPA and FnBPB are expressed by LAC, we solubilised proteins from the cell wall of bacteria grown to exponential phase and performed ligand affinity blotting with biotin-labelled fibronectin. As FnBPB contains fewer fibronectin-binding repeats (10) than FnBPA (11), these proteins can be separated by SDS-PAGE. Two reactive bands could be detected in the cell wall fraction of LAC, but not in the FnBP-deficient mutant LACΔ*fnbAfnbB* (Fig. 3a). The complemented strains LACΔ*fnbAfnbB* pFnBA4 and LACΔ*fnbAfnbB* pFnBB4 expressed the A or B proteins, respectively, confirming that the smaller protein is FnBPB (Fig. 3a). In the wild-type strain, a stronger band was detected for FnBPB, suggesting that this protein is expressed at a higher level by LAC than FnBPA (Fig. 3a). *Staphylococcus aureus* LACΔ*fnbAfnbB* pFnBA4 and LACΔ*fnbAfnbB* pFnBB4 expressed the A or B protein, respectively, at higher levels than the LAC wild-type strain (Fig. 3a). This is likely to be because *fnbA* and *fnbB* have been expressed from a multicopy plasmid and explains why the complemented strains adhered more strongly to fibronectin (Fig. 2b). To determine whether FnBPA and FnBPB were present on the surface of bacteria in the stationary phase of growth, cell wall extracts from cultures
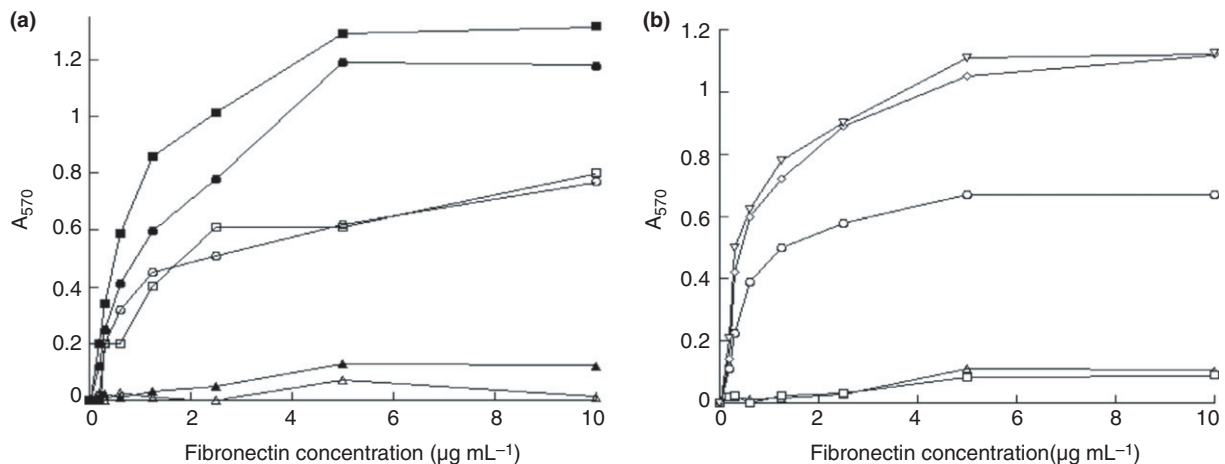

Fig. 2. Bacterial adherence to fibronectin. (a) LAC from exponential (●) or stationary phase (○), BH1CC from exponential (■) or stationary phase (□) and BH1CCΔ*fnbAfnbB* from exponential (△) or stationary phase (▲) were added to wells coated with fibronectin. (b) LAC (○), LACΔ*fnbAfnbB* (△), LACΔ*fnbAfnbB* pC221 (□), LACΔ*fnbAfnbB* pFnBA4 (◇) and LACΔ*fnbAfnbB* pFnBB4 (▽) were grown to stationary phase and added to wells coated with fibronectin. Adherent cells were stained with crystal violet, and the absorbance was read at 570 nm. Graphs shown are representative of three independent experiments.
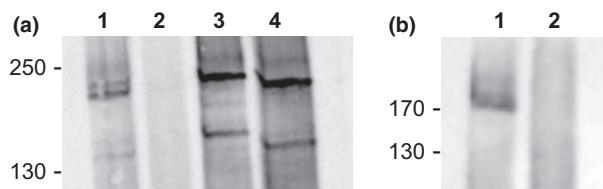
Fig. 3. Expression of FnBPA and FnBPB by LAC. Cell wall extracts were separated on 7.5% acrylamide gels, blotted onto PVDF membranes and probed with biotin-labelled fibronectin. Size markers (kDa) are indicated. (a) Cell wall extracts from LAC (1), LACΔ*fnbAfnbB* (2), LACΔ*fnbAfnbB* (pFnBA4) (3) and LACΔ*fnbAfnbB* (pFnBB4) (4) grown to exponential phase (OD$_{600\ nm}$ = 0.3). (b) Cell wall extracts from LAC (1) and LACΔ*fnbAfnbB* (2) grown to stationary phase.

were analysed by ligand affinity blotting with biotin-labelled fibronectin. Two reactive bands corresponding to FnBPA and FnBPB could be detected in the cell wall fraction of LAC, but not in LACΔ*fnbAfnbB* (Fig. 3b), indicating that FnBPs are intact on the surface of the bacterium in the stationary phase of growth. This correlates with the ability of the bacteria to adhere to fibronectin in stationary phase (Fig. 2a).

Fibronectin-binding proteins are required for biofilm formation by *S. aureus* LAC

Previous studies have shown that formation of biofilm by certain *S. aureus* strains is dependent on the expression of FnBPs (O'Neill *et al.*, 2008; Vergara-Irigaray *et al.*, 2009). The matrix of a LAC biofilm comprises protein and DNA (Lauderdale *et al.*, 2009; Kiedrowski *et al.*, 2011). An aim of this study was to determine whether FnBPs are

required for biofilm formation by LAC. The level of biofilm measured under static conditions in a microtitre plate assay was significantly reduced for LACΔ*fnbAfnbB* compared to the wild-type strain, indicating that FnBPs are required for LAC biofilm to form (Fig. 4a). Biofilm formation by LACΔ*fnbAfnbB* could be restored by expression of FnBPA or FnBPB from a plasmid demonstrating that both of these proteins can mediate biofilm formation when expressed by LAC (Fig. 4a). The density of biofilm was considerably thicker in the complemented strains than the wild-type presumably due to the gene dosage effect. Growth curve experiments were carried out as a control to ensure that all strains had a similar doubling time and that the final density of bacteria was similar for all strains (data not shown). To examine the role of FnBPs in cell accumulation, bacterial suspensions were allowed to settle for 24 h and the percentage aggregation was determined. LACΔ*fnbAfnbB* showed significantly reduced aggregation compared to the wild-type LAC strain (Fig. 4b). This suggests that FnBPs are crucial for the accumulation phase of LAC biofilm formation. The defect in biofilm accumulation could be restored by expression of either FnBPA or FnBPB from a plasmid (Fig. 4b). LACΔ*fnbAfnbB* carrying an empty control plasmid (pC221) did not aggregate or form biofilm.

We next examined biofilm formation under shear flow conditions. LAC formed robust biofilm in the flow cell, while LACΔ*fnbAfnbB* was defective in biofilm formation (Fig. 5). This confirmed a role for FnBPs in LAC biofilm formation and demonstrated that these proteins also participate in biofilm formed under shear flow. The biofilm phenotype of the LACΔ*fnbAfnbB* mutant was restored by expression of FnBPA or FnBPB from a plasmid
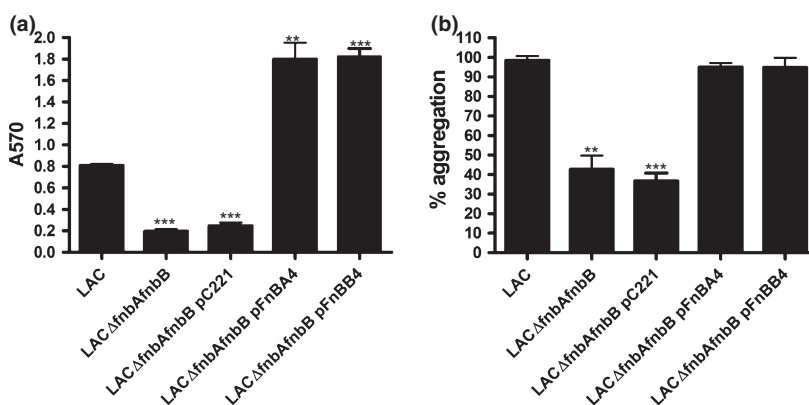

Fig. 4. (a) Biofilm formation by LAC. Biofilm was allowed to form for 24 h at 37 °C under static conditions in microtitre dishes. Biofilm was stained with crystal violet, and the absorbance was measured at 570 nm. Error bars represent the standard error of the mean values obtained from three independent experiments. **$P < 0.005$, ***$P < 0.0005$, indicating significant difference from values obtained for LAC. (b) Bacterial aggregation. Bacteria were diluted to OD$_{600\ nm}$ = 1.0 and incubated statically at 37 °C for 24 h. Bars represent the mean percentage aggregation values from three independent experiments, and error bars indicate the standard error of the mean. **$P < 0.005$, ***$P < 0.0005$, significant difference from values obtained for LAC.
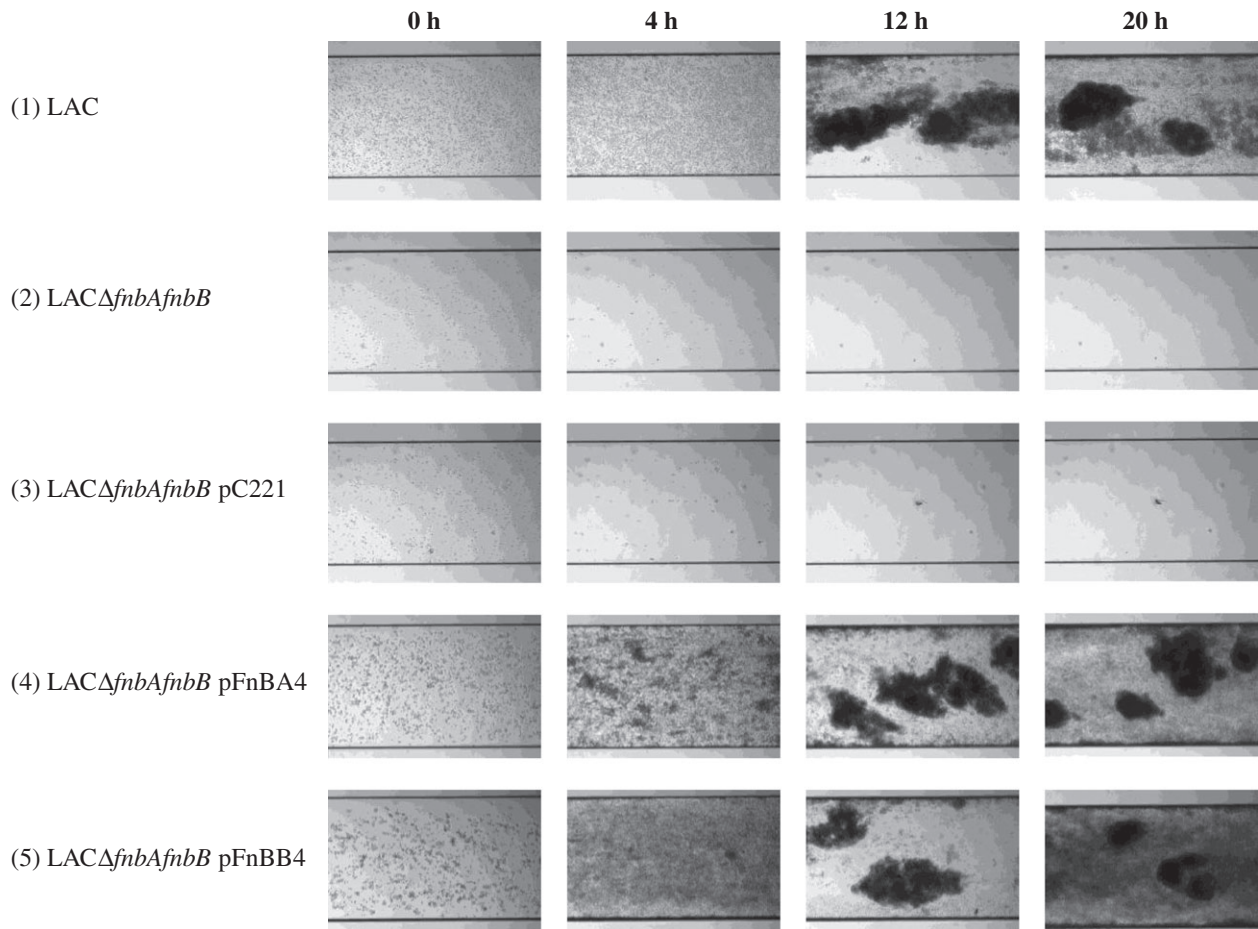
Fig. 5. Attachment and biofilm accumulation under flow conditions. Biofilms of LAC (1), LACΔ*fnbAfnbB* (2), LACΔ*fnbAfnbB* pC221 (3), LACΔ*fnbAfnbB* pFnBA4 (4) and LACΔ*fnbAfnbB* pFnBB4 (5) were grown in BHI supplemented with 1% glucose in the BioFlux 1000z instrument under a shear flow rate of 0.4 dyne cm^{-2}. Brightfield images were captured at 10× magnification at the timepoints indicated and are representative of two independent experiments.

demonstrating that both of these proteins can mediate biofilm formation when expressed by LAC (Fig. 5). The presence of a control plasmid pC221 did not affect the ability of LACΔ*fnbAfnbB* to form biofilm. Initial attachment of LACΔ*fnbAfnbB* (0 h) was reduced compared to LAC (Fig. 5). The defect in primary attachment could be restored by expression of either FnBPA or FnBPB from a plasmid, but not by the introduction of an empty control plasmid pC221 (Fig. 5). These data indicate that FnBPs are required for primary attachment by LAC.

Discussion

Community-associated MRSA of the USA300 lineage is an important emerging cause of device-related infection (Kourbatova *et al.*, 2005). The ability of LAC to form biofilm *in vitro* and in mouse infection models has been described (Lauderdale *et al.*, 2009; Thurlow *et al.*, 2011).

Previous studies identified eDNA and protein as important components of the LAC biofilm matrix, but the *S. aureus* proteins involved were not identified (Lauderdale *et al.*, 2009; Kiedrowski *et al.*, 2011). Here, we show that FnBPA and FnBPB are displayed on the surface of LAC and that the ability of LAC to adhere to fibronectin is dependent on FnBP expression. We identify a role for fibronectin-binding proteins during biofilm formation by the USA300 isolate LAC under shear flow and static conditions. Fibronectin-binding proteins promote both the accumulation phase and the primary attachment phase of biofilm formation. This is in agreement with a recent study demonstrating that primary attachment and biofilm accumulation by the *S. aureus* CC30 strain MW2 are mediated by FnBPs (Lei *et al.*, 2011). It is possible that factors in addition to eDNA and FnBPs are required for biofilm formation by LAC and dissection of the mechanisms involved warrants further investigation.

FnBPs have previously been shown to be required for certain HA-MRSA strains to form biofilm (O'Neill *et al.*, 2008). CA-MRSA is much more virulent than HA-MRSA. An important difference is that BH1CC and related HA-MRSA strains often do not express *agr* RNAIII (Rudkin *et al.*, 2012), whereas RNAIII is very highly expressed in CA-MRSA strains of the USA300 lineage (Cheung *et al.*, 2011). The *agr* system is the master regulator of gene expression in *S. aureus,* and differences in RNAIII production are responsible for pleiotropic effects on gene expression and bacterial virulence (Novick, 2003; Thoendel *et al.*, 2011). Studies with laboratory strains of *S. aureus* have shown that transcription of *fnbA* and *fnbB* is down-regulated in the postexponential phase of growth due to negative regulation by *agr* (Saravia-Otten *et al.*, 1997; Xiong *et al.*, 2004), but it is apparent that LAC does not follow this trend. Cheung *et al.* (2011) demonstrated that expression of *fnbA* was not affected and *fnbB* was only moderately down-regulated by *agr* activity in the LAC strain. Surface-associated FnBPs are degraded by proteases in the stationary phase of growth (McGavin *et al.*, 1997), and LAC produces high levels of extracellular proteases (Zielinska *et al.*, 2012; Kolar *et al.*, 2013). Therefore, it was unexpected that FnBPs would promote bacterial adherence to fibronectin in the stationary phase of growth. Despite this, FnBPs were present and intact in cell wall extracts isolated from LAC grown to stationary phase (Fig. 3b) and promoted bacterial adherence to fibronectin (Fig. 2a). Thus, it has been assumed incorrectly that FnBPs are absent and do not promote bacterial adhesion to fibronectin in the stationary phase of growth. It is clear that findings with laboratory strains do not apply to all clinical isolates of *S. aureus*. This study highlights the importance of studying surface protein function and contribution to biofilm formation and virulence in clinically relevant strains.

Acknowledgements

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