Carriage of both the *fnbA* and *fnbB* genes and growth at 37 °C promote FnBP-mediated biofilm development in methicillin-resistant *Staphylococcus aureus* clinical isolates

Eoghan O'Neill,^{1,2} Hilary Humphreys² and James P. O'Gara¹

Correspondence
James P. O'Gara
jim.ogara@ucd.ie

¹UCD School of Biomolecular and Biomedical Science, Ardmore House, University College Dublin, Belfield, Dublin 4, Ireland

²Department of Clinical Microbiology, Royal College of Surgeons in Ireland, Beaumont Hospital, Dublin 9, Ireland

The *Staphylococcus aureus* FnBPA and FnBPB proteins promote acid-induced biofilm accumulation. Methicillin-resistant *S. aureus* (MRSA) isolates from device-related infections with both *fnbA* and *fnbB* produced significantly more biofilm than isolates with either gene alone. Under mildly acidic growth conditions, FnBP-mediated biofilm and *fnbA* and *fnbB* transcript levels were substantially higher during growth at 37 °C than at 30 °C. Thus, in addition to a lowered pH, carriage of both *fnbA* and *fnbB* and growth at 37 °C promote MRSA biofilm development, further supporting a role for the FnBPA and FnBPB surface proteins in the pathogenesis of MRSA device-related infections.

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INTRODUCTION

Since the early 1950s, polymers have been used in the construction of medical devices such as central and peripheral intravascular lines, surgical drains and prosthetic joints (Montdargent & Letourneur, 2000). Bacteria such as staphylococci that originate from the patient's microflora or exogenously from healthcare personnel or the hospital environment can gain access to the medical device by migration externally from the skin or, as in the case of lines and drains, internally from the catheter hub or port. Occasionally, devices may become haematogenously seeded from another focus of infection. More uncommonly, infusate contamination leads to catheter-related bloodstream infection (O'Grady, 2002). Guidelines on the prevention of device-related infection therefore focus on (i) education and training of healthcare providers who insert and maintain intravascular lines and drains, (ii) maximal sterile barrier precautions during device insertion and (iii) appropriate skin antisepsis prior to device insertion (O'Grady, 2002).

Following insertion into the patient, medical devices are rapidly coated with a conditioning film composed primarily of host-derived extracellular matrix proteins. Some of these host proteins can act as receptors for bacterial attachment and can thus play an important role in the early stages of biofilm development (O'Gara, 2007).

Staphylococcus aureus can bind to a wide range of host matrix proteins, adherence to which is mediated by a group of *S. aureus* surface proteins termed MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (Foster & Hook, 1998). Following this initial attachment to an implanted device, biofilm formation involves a cellular accumulation process. In staphylococci, the exopolysaccharide termed polysaccharide intercellular adhesin or polymeric *N*-acetylglucosamine (PIA/PNAG) and the surface proteins accumulation-associated protein (Aap), biofilm-associated protein (Bap) and *S. aureus* surface protein G (SasG) have all been implicated in biofilm accumulation (reviewed by O'Gara, 2007). We recently described a new *S. aureus* biofilm accumulation phenotype mediated by the multi-functional fibrinogen- and fibronectin-binding proteins FnBPA and FnBPB. FnBP-mediated biofilm development appears to be more commonly associated with methicillin-resistant *S. aureus* (MRSA) isolates than with methicillin-sensitive *S. aureus* (MSSA) isolates (O'Neill *et al.*, 2008). Furthermore, FnBP-promoted biofilm development by MRSA isolates is triggered by mild acid stress, whereas PIA/PNAG-mediated biofilm development by MSSA isolates is generally induced by osmotic stress (Fitzpatrick *et al.*, 2005; O'Neill *et al.*, 2007, 2008). FnBP-mediated biofilm development is dependent on the *Staphylococcus* accessory regulator SarA but not at the level of *fnbA* and *fnbB* transcription (O'Neill *et al.*, 2008).

Here, we examined the relationship between carriage of the *fnbA* and/or *fnbB* genes and biofilm-forming capacity in a

Abbreviations: CC, clonal complex; MLST, multilocus sequence typing; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *S. aureus*.

collection of *S. aureus* clinical isolates clinically implicated in device-related infection. Furthermore, we examined temperature regulation of FnBP-mediated biofilm formation and *fnbA* and *fnbB* transcription in four MRSA isolates.

METHODS

***S. aureus* strains and growth conditions.** The 217 *S. aureus* isolates (118 MRSA and 99 MSSA) used in this study were from clinically diagnosed, device-related infections collected retrospectively from January 2002 to June 2005. These isolates, which were obtained from a 720-bed tertiary referral centre in Ireland, were collected from blood cultures and line tips, as described previously (O'Neill *et al.*, 2007, 2008), and were deemed clinically significant following review by a clinical microbiologist. The genetic background of these isolates was determined previously by sequencing the X-repeat region of the *spa* gene and performing multilocus sequence typing (MLST) on selected isolates (O'Neill *et al.*, 2007). The MRSA isolates represented 16 *spa* types, whereas the MSSA isolates were more genetically diverse and represented 67 *spa* types. Furthermore, three clones identified by *spa* types 46, 382 and 781 accounted for 90 % of the MRSA isolates, and all but one of our MRSA isolates for which a clonal complex (CC) could be inferred belonged to CC8 or CC22 (O'Neill *et al.*, 2007). The previously characterized MRSA isolates (O'Neill *et al.*, 2007, 2008) BH1CC (SCCmec type II, MLST type 8, CC8), BH3 (SCCmec type II, MLST type 8, CC8), BH4 (SCCmec type II, MLST type 8, CC8) and BH10 (SCCmec type IV, MLST type 22, CC22), which were found to be amenable to genetic manipulation and representative of both CCs in our isolate collection, were used to examine temperature regulation of FnBP-mediated biofilm formation and *fnbA* and *fnbB* transcription in this study. *S. aureus* strains were grown at 30 or 37 °C in brain-heart infusion (BHI; Oxoid) medium. BHI broth was supplemented where indicated with 1 % glucose or 4 % NaCl.

Biofilm assays. Semi-quantitative measurements of biofilm formation were determined using tissue culture-treated, 96-well polystyrene plates (Nunc) (Nunc), based on the methods of Christensen *et al.* (1985) and Ziebuhr *et al.* (1997) as described previously (O'Neill *et al.*, 2007, 2008). Bacteria were grown in individual wells of 96-well plates at 37 °C in BHI medium or BHI medium supplemented with 4 % NaCl or 1 % glucose. After 24 h growth, the plates were washed vigorously. This involved three rounds of plunging the plates into a large volume of distilled water and decanting to remove unattached bacteria. The plates were subsequently dried for 1 h at 60 °C prior to staining with a 0.4 % crystal violet solution. The A_{492} of the adhered, stained biofilms was measured using a microtitre plate reader. The laboratory strain RN4220 and its isogenic Δica mutant were used as positive and negative controls, respectively, in all experiments. Biofilm formation by each strain was measured in a minimum of three independent experiments and the mean results are presented. A biofilm-positive phenotype was defined as having an A_{492} value ≥ 0.17 .

Genetic techniques. Genomic DNA was prepared as described previously (O'Neill *et al.*, 2008). PCR with previously described primers (O'Neill *et al.*, 2008) was used to determine the presence or absence of the *fnbA* and *fnbB* genes in our 217 clinical isolates. PCRs were carried out using Taq DNA polymerase (Invitrogen) and 3 mM MgCl₂ under the following conditions: 95 °C for 30 s, 54 °C for 30 s and 72 °C for 3 min for 36 cycles. All oligonucleotide primers used for PCR were supplied by MWG-Biotech.

RNA purification, RT-PCR and analysis of RT-PCR data were performed as described previously (O'Neill *et al.*, 2008) on the four

well-characterized MRSA isolates. Briefly, following overnight growth in BHI broth, 50 µl bacterial cells was grown in 5 ml BHI broth (supplemented with 1 % glucose) and incubated with shaking (200 r.p.m.) at 37 or 30 °C until the cells reached an OD₆₀₀ of 2.0 (exponential phase). The constitutively expressed *gyrB* gene was used as an internal standard in these experiments using the primers described by Goerke *et al.* (2000). The following primer sets were used: for *fnbA*, SAFnbAFOR (5'-CGCGGATCCGGTACAGATGT-AACAAGTAAAG-3') and SAFnbAREV (5'-GACGCGTCGACTT-AATTCGGACCATTCTTCTCATT-3'); for *fnbB*, SAFnbBFOR (5'-CGCGGATCCGTGGAAGTAGAAGAAGGTAG-3') and SAFnbAREV (5'-GACGCGTCGACTTAAATTAGGTTCCCTTAGTTTATC-3'); and for *gyrB*, SAgyrBFOR (5'-TTATGGTGCTGGGCAAATACA-3') and SAgyrBREV (5'-CACCATGTAAACCACCAGATA-3'). Reverse transcription was performed at 50 °C for 30 min, terminated at 94 °C for 15 min and followed by 20 cycles of PCR for *gyrB* and up to 30 cycles of PCR for *fnbA* and *fnbB* amplification.

RESULTS AND DISCUSSION

Relationship between carriage of the *fnbA* and *fnbB* genes and biofilm-forming capacity in *S. aureus* clinical isolates

Mutation of the *S. aureus* *fnbA* and *fnbB* genes is accompanied by loss of glucose-induced biofilm development in MRSA isolates (O'Neill *et al.*, 2008). Growing *S. aureus* in BHI medium with 1 % glucose lowers the final culture pH to 5 compared with approximately 8.5 in BHI medium without glucose, and this mildly acidic pH correlates with induction of FnBP-promoted biofilm (O'Neill *et al.*, 2008). In addition, the expression of either the *fnbA* or *fnbB* gene on multicopy plasmids can independently promote biofilm development in both MRSA and MSSA clinical isolates (O'Neill *et al.*, 2008), suggesting that the FnBPA and FnBPB proteins can substitute functionally for each other and that expression of both proteins may enhance biofilm-forming capacity. To investigate this possibility, we examined the correlation between the rate of *fnbA* and *fnbB* carriage and the mean biofilm-forming capacity in BHI medium supplemented with 1 % glucose in our previously described collection of 217 clinical MRSA ($n=118$) and MSSA ($n=99$) isolates (O'Neill *et al.*, 2007). PCR demonstrated that 40/118 (34 %) of the MRSA isolates possessed both *fnbA* and *fnbB* compared with 68/99 (69 %) of the MSSA isolates (Table 1). Of the MRSA isolates, 77/118 (65 %) were positive for the *fnbA* gene alone compared with 19/99 (19 %) of the MSSA isolates, while only 1/118 (0.9 %) of the MRSA isolates and 8/99 (8 %) of the MSSA isolates were positive for *fnbB* alone. None of our MRSA clinical isolates and only 4/99 (4 %) of our MSSA isolates lacked both the *fnbA* and *fnbB* genes.

Carriage of both the *fnbA* and *fnbB* genes together was associated with a significantly higher level of biofilm production in MRSA isolates than carriage of either gene alone ($P < 0.0001$, Student's *t*-test) (Fig. 1). Similarly, MSSA isolates possessing either the *fnbA* or *fnbB* gene alone were capable of significantly higher levels of acid-induced biofilm production than MSSA isolates lacking

Table 1. Carriage of the *fnbA* and *fnbB* genes in a collection of 217 *S. aureus* isolates

Genotype	MRSA (n=118)	MSSA (n=99)	Total (n=217)
<i>fnbA</i> ⁺ / <i>fnbB</i> ⁺	40 (33 %)	68 (69 %)	108 (50 %)
<i>fnbA</i> ⁺ / <i>fnbB</i> ⁻	77 (65 %)	19 (19 %)	96 (44 %)
<i>fnbA</i> ⁻ / <i>fnbB</i> ⁺	1 (0.9 %)	8 (8 %)	9 (4 %)
<i>fnbA</i> ⁻ / <i>fnbB</i> ⁻	0	4 (4 %)	4 (2 %)

both genes ($P=0.01$, Student's *t*-test) (Fig. 1). However, unlike MRSA, MSSA isolates with both the *fnbA* and *fnbB* genes did not form significantly more acid-induced biofilm than isolates with either gene alone ($P=0.78$, Student's *t*-test) (Fig. 1), suggesting a less important role for the FnBPs in MSSA biofilm development. However, as observed in MRSA isolates (O'Neill *et al.*, 2008), expression of *fnbA* or *fnbB* from multicopy plasmids dramatically increased acid-induced biofilm development in a number of MSSA isolates (O'Neill *et al.*, 2008). These findings indicate that acid-induced, FnBP-mediated biofilm development is more commonly associated with MRSA isolates and that carriage of both the *fnbA* and *fnbB* genes, as opposed to either gene alone, is associated with significantly higher levels of biofilm production. In this context, it is interesting to note that, similar to their ligand-binding activity, the *fnbA* and *fnbB* genes could functionally substitute for each other as biofilm adhesins and that expression of both proteins may enhance biofilm-forming capacity.

In contrast, MSSA isolates with both genes did not form more acid-induced biofilm than isolates with either gene alone, although FnBPA and FnBPB may contribute to the MSSA biofilm phenotype under appropriate environmental conditions.

Fig. 1. Relationship between carriage of the *fnbA* and/or *fnbB* genes and biofilm-forming capacity in 217 *S. aureus* clinical isolates. Mean biofilm-forming capacity of 118 MRSA and 99 MSSA isolates grown for 24 h at 37 °C in BHI medium supplemented with 1 % glucose. Biofilm formation in tissue culture-treated 96-well plates was measured three times for each isolate. *, There were no MRSA isolates lacking both the *fnbA* and *fnbB* genes.

Regulation of FnBP-mediated biofilm development in *S. aureus*

The staphylococcal biofilm phenotype is tightly regulated and highly responsive to environmental conditions. As described above, FnBP-mediated biofilm development is induced by mild acid stress and, unlike PIA/PNAG-promoted biofilm, is unaffected by osmotic stress in medium supplemented with NaCl (O'Neill *et al.*, 2008). To investigate further the conditions that influence FnBP-mediated biofilm formation, we examined the impact of temperature on this phenotype in four previously characterized MRSA isolates, BH1CC, BH3, BH4 and BH10 (O'Neill *et al.*, 2007, 2008). BH1CC, BH3, BH4 and BH10 possess both *fnbA* and *fnbB* and produce FnBP-mediated biofilm (O'Neill *et al.*, 2007, 2008). These experiments revealed that mild acid-induced biofilm formation was significantly reduced at 30 °C compared with 37 °C ($P < 0.001$, Student's *t*-test) (Fig. 2a). Transcript levels of *fnbA* and *fnbB* in all four isolates were then measured using RT-PCR. Transcript levels of *fnbA* and *fnbB* were very low, necessitating up to 30 cycles of PCR compared with 20 amplification cycles for the *gyrB* housekeeping gene, possibly suggesting that transcriptional regulation of *fnbA* and *fnbB* does not play an important role in controlling FnBP-mediated biofilm. Nevertheless, expression of *fnbB* was measurably higher at 37 °C than at 30 °C in all four strains (Fig. 2b). Similarly, transcript levels of *fnbA* were also higher at 37 °C in three of the four MRSA strains tested (Fig. 2b).

The data presented in this study indicate that carriage of both *fnbA* and *fnbB* and growth at 37 °C correlate with enhanced acid-induced biofilm development in MRSA clinical isolates. However, it remains unclear whether temperature significantly influences FnBP-mediated biofilm formation at the level of *fnb* gene expression. We reported previously that FnBP-mediated MRSA biofilm was SarA-dependent, even though mutation of *sarA* was not accompanied by reduced *fnbA* or *fnbB* transcription (O'Neill *et al.*, 2008). SarA controls expression of the four major extracellular proteases in *S. aureus* and we also demonstrated that BH1CC, BH3, BH4 and BH10 biofilms were dispersed following treatment with exogenous V8 protease (O'Neill *et al.*, 2008). It therefore also seems possible that altered MRSA cell-surface characteristics at different growth temperatures may indirectly influence the ability of the cell-wall-anchored FnBPs to promote biofilm formation.

Fig. 2. Environmental regulation of biofilm development and *fnbA* and *fnbB* transcription in MRSA isolates. (a) Biofilm development by the MRSA isolates BH1CC, BH4, BH3 and BH10 grown at 37 or 30 °C in BHI medium supplemented with 1% glucose. Biofilm formation was measured at least three times and the standard deviation, which was less than 20%, is indicated. (b) Comparative measurement of *fnbA*, *fnbB* and *gyrB* (control) transcription in the MRSA isolates BH1CC, BH4, BH3 and BH10. RT-PCR analysis was performed on total RNA extracted from cultures grown at 37 or 30 °C to an OD₆₀₀ value of 2.0 in BHI medium supplemented with 1% glucose.

In any event, under conditions predisposing to infection *in vivo*, the temperature is likely to be at least 37 °C, whereas a number of the human colonization habitats of *S. aureus* such as the skin or nares are generally cooler. Our data indicate that changes in temperature and pH during transit of the pathogen from the skin to an infection milieu may correlate with activation of FnBP-mediated biofilm-forming capacity, further suggesting a potentially important role for this phenotype in *S. aureus* virulence.

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