Preventing *Staphylococcus aureus* biofilm formation using molecules that target fibronectin binding proteins

1. Department of Microbiology, Moyne Institute of Preventive Medicine, School of Microbiology and Genetics, Trinity College Dublin, Ireland.
2. Unit of Biochemistry, Department of Molecular Medicine, University of Pavia, Pavia, Italy.

Leanne M. C. Hays[1], Marta Zapotoczna[1], Giampiero Pietrocola[2], Pietro Speziale[2] and Joan A. Geoghegan[1].

1. Fibronectin binding proteins and biofilm accumulation

- The fibronectin binding proteins (FnBP) A and B are important factors mediating biofilm in clinically relevant lineages of *S. aureus*; CC8 and CC22 including CA- and HA-MRSA strains (Speziale *et al*., 2014 *Front Cell Infect Microbiol.* 10;4:171.). Thus, FnBPs are an attractive target for anti-biofilm agents.
- FnBPs expressed on the surface of adjacent cells form homophilic interactions allowing cells to aggregate and biofilm to accumulate (Speziale *et al*., 2014 *Front Cell Infect Microbiol.* 10;4:171.).
- FnBPs are members of the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) family of surface proteins which all contain two tandem IgG-like folded subdomains termed N2 and N3 (Foster et al., 2014 *Nat Rev Microbiol.* 12:49-62., Fig 1).
- This study set out to further investigate FnBP interactions in biofilm accumulation and, using this information, identify novel inhibitors of FnBP-mediated biofilm.

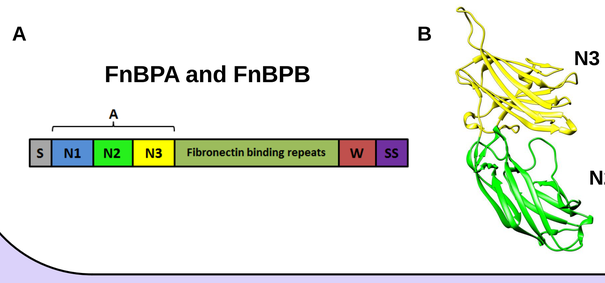

FIG 1. Domain organization of FnBPs. (A) Schematic representation of FnBPA and FnBPB. The N-terminal signal sequence (S) is followed by the A domain which consists of three subdomains; N1, N2 and N3. This is followed by a long unstructured repeat region composed of fibronectin binding repeats, a cell wall spanning domain (W) and a sorting signal (SS). (B) Crystal structure of FnBPA N2 and N3 subdomains. FnBPA N2N3 is shown in ribbon with subdomains N2 and N3 coloured green and yellow, respectively (PDB ID=4B5Z)

2. FnBPA-FnBPA interactions are mediated by subdomain N2.

- FnBPA homophilic interactions were previously localised to subdomains N2 and N3 (3).
- This study set out to further localise the regions involved:

The N2 and N3 subdomains were expressed individually with N-terminal hexahistidine tags (His-).

The ability of the N2 and N3 proteins to bind GST-tagged FnBPA$_{N2N3}$ was compared to His-FnBPA$_{N2N3}$, a recombinant polypeptide of both N2N3 subdomains (Fig. 2A).

- Subdomain N2 bound GST-FnBPA$_{N2N3}$ in a dose-dependent manner with a similar binding profile to the N2N3 subdomain protein.
- Subdomain N3 did not bind GST-FnBPA$_{N2N3}$ at any of the concentrations tested.

These data indicate that subdomain N2, and not N3, is important for FnBPA-FnBPA homophilic interactions.

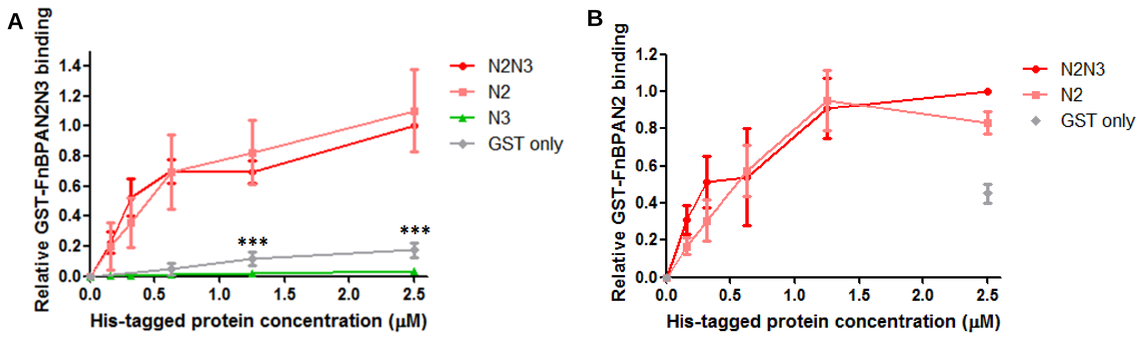

FIG 2. FnBPA homophilic interactions are mediated by subdomain N2. Binding of recombinant FnBPA subdomain proteins to immobilised GST-FnBPA$_{N2N3}$ (A) and GST-FnBPA$_{N2}$ (B). Values are expressed relative to the A_{450nm} reading measured for the highest concentration of His-N2N3 (2.5 μM = 1.0). *P*-values were calculated using an unpaired Student' t-test where ***, represents a *p*-value of ≤0.001.

- To investigate if subdomain N2 binds N2, His-FnBPA$_{N2}$ and His-FnBPA$_{N2N3}$ binding to GST-FnBPA$_{N2}$ was assessed (Fig. 2B).
- His-FnBPA$_{N2}$ and His-FnBPA$_{N2N3}$ bound GST-FnBPA$_{N2}$ in a similar dose-dependent manner.

These data indicate that FnBPA-FnBPA interactions consist of N2-N2 binding *in vitro*.

3. Sequence variation in the N2 subdomain does not affect FnBPA homophilic interactions

- Seven isotypes of FnBPA have been identified that share only 75-84 % identity in their N2 subdomains (Foster et al., 2014 *Nat Rev Microbiol.* 12:49-62.). Only isotype I of FnBPA has been shown to mediate biofilm (Geoghegan et al., 2013 *J Bacteriol.* 195(11):2675-83). Here, we assessed if other FnBPA isotypes could also mediate biofilm formation.
- DNA encoding the N123 subdomains of isotype I of FnBPA was replaced with sequence encoding N123 of isotypes III, IV, V and VI on a multicopy plasmid pFnBA4 and the plasmid introduced into *S. aureus*.
- Expression of FnBPA from all plasmids was confirmed by their ability to bind fibronectin (Fig. 3A).
- All proteins were capable of mediating biofilm accumulation (Fig. 3B) indicating that the sites of FnBPA homophilic interactions are likely to be conserved.

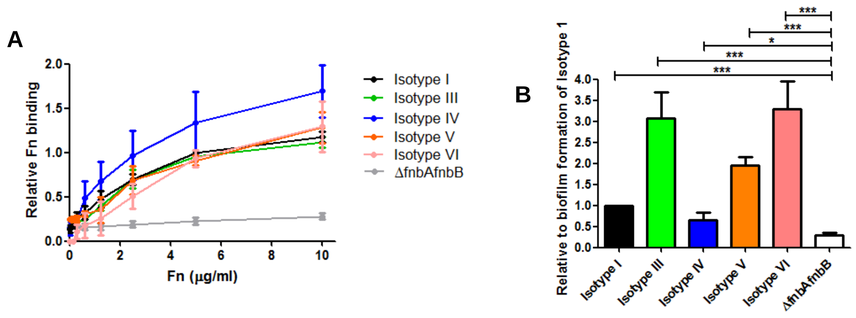

FIG 3. Assessment of the ability of BH1CCΔ*fnbAfnbB* pFnBA4 isotype I, III, IV, V and VI strains to adhere to fibronectin and mediate biofilm accumulation. A. All pFnBA4 plasmids express functional FnBPA proteins which bind fibronectin. Values are expressed relative to BH1CCΔ*fnbAfnbB* pFnBA4 isotype I binding the highest concentration of fibronectin (fn) (10 μg/ml = 1.0). B. All FnBPA chimeric proteins mediate biofilm. All values are expressed as % biofilm formation relative to BH1CCΔ*fnbAfnbB* pFnBA4 isotype I set as 100 %. *P*-values were calculated using an unpaired Student's t-test where * and *** represent *p*-values of ≤0.05 and ≤0.001, respectively.

4. Recombinant FnBPA and FnBPB can form heterophilic interactions *in vitro*

- We investigated if recombinant FnBPA and FnBPB proteins can form a heterophilic interaction.
- His-FnBPB$_{N2N3}$ was observed to bind GST-FnBPA$_{N2N3}$ in a dose-dependent manner with a similar binding profile to His-FnBPA$_{N2N3.}$ (Fig. 4A).
- Furthermore, the N2 subdomain of FnBPB bound GST-FnBPA$_{N2N3}$ and GST-FnBPA$_{N2}$ in a similar manner to His-FnBPA$_{N2}$ (Fig. 4B, C), despite the N2 subdomains sharing only 45 % amino acid identity.

These data indicate that recombinant FnBPA and FnBPB can form a heterophilic interaction *in vitro* and that their N2 subdomains are involved.

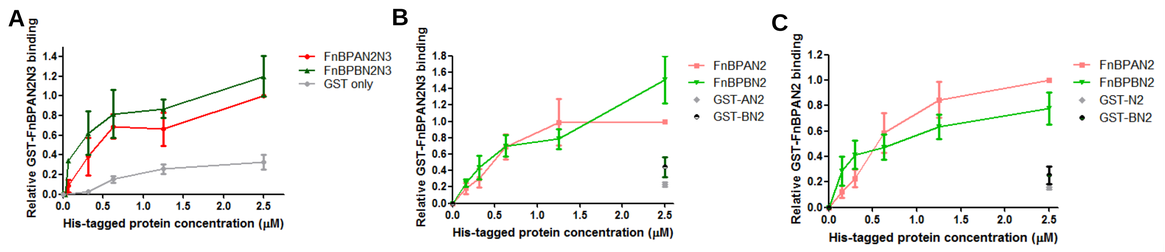

FIG 4. Recombinant FnBPA and FnBPB form heterophilic interactions. A. Binding of His-FnBPB$_{N2N3}$ and His-FnBPA$_{N2N3}$ to GST-FnBPA$_{N2N3.}$ B and C. Binding of His-FnBPB$_{N2}$ and His-FnBPA$_{N2}$ to GST-FnBPA$_{N2N3}$ (B) and GST-FnBPA$_{N2}$ (C). Values are expressed relative to the A_{450nm} reading measured for the highest concentration of His-FnBPA$_{N2N3}$ (A) or His-FnBPA$_{N2}$ (B and C) (2.5 μM = 1.0).

5. Identification of novel inhibitors of FnBP-mediated biofilm

- Using information from the interaction studies presented here, small molecules from the Zinc library were docked onto the crystal structure of FnBPA N2N3.
- Five small molecules, termed LH1-5, were selected and assessed for their ability to inhibit FnBP-mediated biofilm of clinical isolates and recombinant FnBPA interactions *in vitro*.
- LH1, LH2, LH3 and LH5 significantly inhibited FnBP-mediated biofilm of two HA-MRSA strains from different genetic lineages, BH1CC; a CC22 isolate and DAR70; a CC45 isolate (Fig. 5A and B).
- Small molecules LH1, LH3 and LH5 inhibited recombinant FnBPA-FnBPA interactions *in vitro* (Fig. 5C).

These data indicate that LH1, LH3 and LH5 are inhibitors of FnBP-mediated biofilm accumulation.

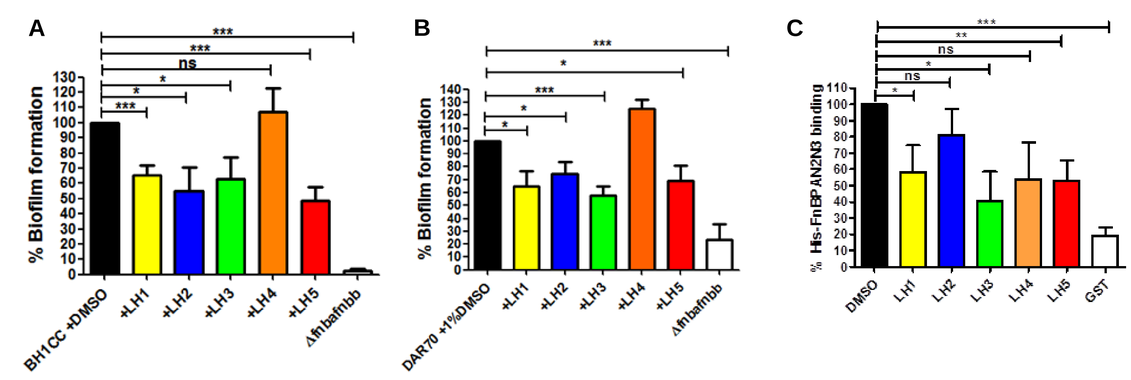

FIG 5. Inhibition of FnBP-mediated biofilm and homophilic interactions by small molecules. Small molecules LH1, LH2, LH3 and LH5 (100 μM) significantly reduced biofilm formation of HA-MRSA strains BH1CC (A) and DAR70 (B). Values were normalised as % biofilm formation relative to the DMSO control as 100%. B. LH1, LH3 and LH5 inhibited recombinant FnBPA-FnBPA homophilic interactions. Values were normalised as % His-FnBPA$_{N2N3}$ binding GST-FnBPA$_{N2N3}$ relative to the DMSO control as 100%. *P*-values were calculated using an unpaired Student's t-test where *, ** and *** represent *p*-values of ≤0.05, ≤0.01 and ≤0.001, respectively. *P*-values >0.05 are considered not significant (ns).

- To assess the specificity of the small molecules as anti-biofilm agents targeting FnBPs, small molecules were assessed for affects on bacterial growth (Fig. 6A) and for inhibition of biofilm formed by *S. aureus* strains RF122 and MSSA476 (Fig. 6B and C). RF122 and MSSA476 form protein-dependent biofilms which are not dependent on FnBPs.
- LH1-5 did not affect bacterial growth and did not significantly inhibit RF122 and MSSA476 biofilms.

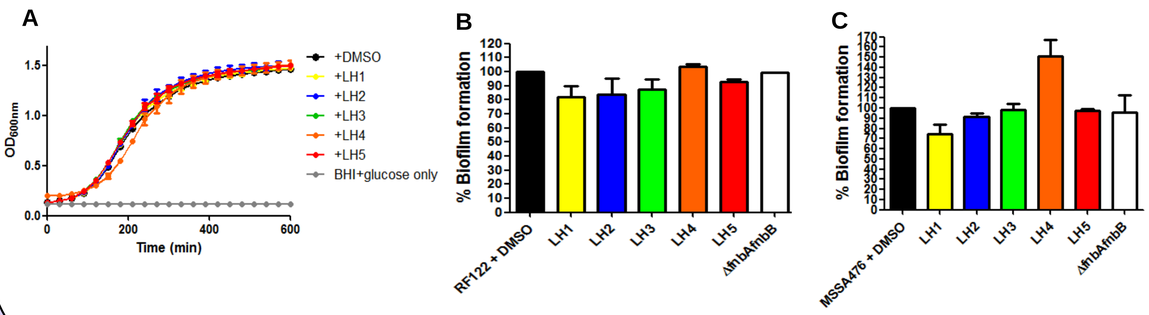

FIG 6. FnBP biofilm inhibitors do not affect bacterial growth or biofilm formed by RF122 and MSSA476 strains. A. Growth curves of BH1CC with small molecules LH1-5 or DMSO control. B and C. FnBP inhibitors did not significantly reduce RF122 and MSSA476 biofilms. Values were normalised as % biofilm formation relative to the DMSO control as 100%.

6. Conclusions and Future Direction

- FnBPA homophilic interactions are mediated by subdomain N2 and are conserved across FnBPA isotypes I, III, IV, V and VI despite considerable N2 sequence variation.
- Recombinant FnBPA and FnBPB can form heterophilic interactions *in vitro*.
- Using this information, several small molecules were identified which inhibit FnBP-mediated biofilm.
- These data highlight FnBPs as a novel, attractive target for the further development of anti-biofilm agents and the small molecules identified here may serve as scaffolds in further drug design.

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