Bacterial proteases and haemostasis dysregulation in the CF lung


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BACTERIAL PROTEASES AND HAEMOSTASIS DYSREGULATION IN THE CF LUNG

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Running title: Bacterial proteases and haemostasis dysregulation in CF
Abstract

Background: Pathogenic bacteria which chronically colonise the cystic fibrosis (CF) lung produce a number of virulence determinants, including distinct proteolytic activities. The potential role bacterial proteases play on haemostatic dysregulation within the CF lung is however poorly defined, despite haemoptysis being a common complication in CF.

Methods: The potential impact of known CF pathogens (Pseudomonas aeruginosa and Burkholderia cepacia complex spp.) on haemostasis was examined for their ability to degrade fibrinogen and dysregulate fibrin clot formation and platelet aggregation.

Results: Results demonstrate that key CF pathogens growing as a biofilm on mucin exhibit considerable fibrinogenolytic activity, resulting in fibrinogen breakdown, impaired clot formation, and modulation of platelet aggregation. Human neutrophil elastase may also contribute to fibrinogen breakdown and dysregulated clot formation at high concentration.

Conclusion: Bacterial-derived proteases may play an important role in the dysregulation of airway haemostasis, and potentially contribute to episodes of haemoptysis within the CF lung.

Keywords: cystic fibrosis; haemostasis; haemoptysis; coagulation; platelet aggregation; Pseudomonas aeruginosa, Burkholderia cenocepacia; Burkholderia multivorans.

Abbreviations: cystic fibrosis (CF), biofilm-conditioned cell-free broth (BC-CFB)
Introduction

As a major interface between the body and outside world, the lung is continually under threat from both internal and external insults which have the potential to damage the extensive pulmonary vasculature. When damage does occur a cascade of well-defined coagulation proteases enable vascular repair and haemostasis through the aggregation of platelets, which are stabilised by the formation of a fibrin mesh that forms a temporary plug (stable clot).

Shortly after birth, CF lungs become colonised by a number of bacterial species; however, chronic infection with Pseudomonas aeruginosa is particularly problematic as the bacterium employs several strategies to evade the host immune system leading to progressive pulmonary decline and increased morbidity (1). Furthermore, the most common Burkholderia cepacia complex (BCC) species to infect CF airways in the UK and USA, B. cenocepacia and B. multivorans (2), are also strongly associated with a rapid clinical deterioration (3). These pathogenic bacteria secrete a range of virulence factors, including proteases that are known to interfere with critical host processes (4;5). Haemoptysis is a common complication of acute pulmonary exacerbation which typically resolves following antibiotic therapy. In some cases, however, the condition can be life-threatening due to airway obstruction and haemodynamic instability, with the rate of bleeding the most important factor determining mortality (6). Although P. aeruginosa-derived proteases have been shown previously to degrade and subsequently affect the activity of fibrinogen (7;8), their clinical relevance, particularly in regard to their impact on CF lung haemostasis, is unclear.

The aim of the present investigation was to examine the effect of CF-relevant bacterial proteases (from P. aeruginosa, B. multivorans and B. cenocepacia) on fibrinogen degradation and the downstream impact on in vitro fibrin clot formation. We also sought to determine whether bacterial filtrates modulate platelet aggregation. We hypothesized bacterial proteases would interfere with these haemostatic processes.
2. Materials and Methods

2.1. Bacterial strains and culture conditions: The bacterial isolates used in the following studies typically cause lung infections in the CF respiratory tract including \textit{P. aeruginosa} (PA0219, BCH10-3, PA0025 and PA0049), and the BCC spp. \textit{B. multivorans} (C1962, C1576, C5393) and \textit{B. cenocepacia} (ET-12 lineage) (J2315, K56-2, BC7, C5424). The isolates were obtained from clinical settings (including a selection of both non-epidemic and epidemic strains) and the environment (Table 1). BCC organisms were grown on MAST \textit{cepacia} selective agar, supplemented with \textit{B. cepacia} Selectatab (Ticarcillin 100 mg/L, Polymyxin B 300,000 units/L) (Mast Diagnostics, Merseyside, UK). \textit{P. aeruginosa} isolates were grown on Müller-Hinton agar (Oxoid Ltd., Basingstoke, Hampshire, UK). Agar plates were incubated aerobically at 37°C until sufficient growth was obtained.

2.2. Cell-Free Broth Preparation: A single colony was inoculated into 50 ml Tryptone Soya Broth (TSB; Oxoid Ltd, UK) supplemented with 0.2% (w/v) sucrose before overnight culture at 37°C under mild agitation (100 RPM). The culture was then adjusted \textasciitilde 5x10^7 CFU/ml and an aliquot (200 µl) transferred to wells of a polystyrene 96-well microtitre plate, which had previously been coated with 1 mg/ml porcine stomach mucin, Type III (Sigma Aldrich, UK). The plates were incubated for 24 hours at 37°C under mild agitation (100 RPM) to allow attachment of the cells to the surface of the well. Following incubation, non-adherent cells were removed and the wells washed three times with sterile PBS. TSB containing 0.2% (w/v) sucrose (200 µl) was added to each well and the plates incubated at 37°C under mild agitation for a further 48 hours. Following incubation, culture supernatants were harvested, centrifuged for 20 minutes at 12,100 x g before filter sterilisation (0.20 µm pore size; VWR International). Bacterial biofilm formation on mucin was validated by a crystal violet binding assay (Supplementary Fig. 1) and Congo red staining (Supplementary Fig. 2). The resultant biofilm-conditioned cell-free broth (BC-CFB) samples were aliquoted and stored at -20°C for further experiments.
2.3. Fibrinogen zymography: BC-CFBs (25 µl) were analysed under non-reducing conditions using 10% SDS-PAGE gels containing 0.1% (w/v) fibrinogen from human plasma (Sigma Aldrich, UK). Following electrophoresis, gels were immersed in a renaturation/incubation buffer, 50 mM Tris/HCl, pH 7.5 containing 1 mM CaCl₂·H₂O, 150 mM NaCl and 2.5% (v/v) Triton X-100 (Sigma-Aldrich, UK) for 48 hours at 37°C. Clear bands of lysis indicative of protease activity were visualized by staining the gels overnight with 0.1% (w/v) Coomassie Brilliant Blue R-250 (Sigma-Aldrich, UK) in acetic acid, isopropanol and double distilled water (DDW) (10:25:65).

2.4. Fibrinogen degradation (SDS-PAGE): Fibrinogen from human plasma (Sigma Aldrich, USA) was added to BC-CFB samples at a final concentration of 1 mg/ml. Samples were incubated at 37°C for 48 hours, resolved by SDS-PAGE (NUPAGE® Novex 4-12% Bis-Tris gels, Invitrogen, USA) and then stained with Coomassie Brilliant Blue R-250.

2.5. Protease inhibitor studies: BC-CFB samples were pre-incubated for 45 minutes at 37°C in the presence of each individual inhibitor (as outlined in the results section), prior to incubation with fibrinogen (1 mg/ml). Samples were incubated at 37°C for 48 hours then subjected to SDS-PAGE.

2.6. In vitro clotting turbidimetric assay: Fibrin clots were formed in 96-well polystyrene microtitre plates by first pre-incubating a mixture of purified human fibrinogen (Sigma Aldrich, USA) in Tris-buffered saline (TBS; 50 mM Tris/HCl, pH 7.4, containing 150 mM NaCl) and 2.5 mM CaCl₂ for 10 minutes. Following addition of the relevant BC-CFB or unconditioned broth as a mock control, samples were incubated for 48 hours at 37°C. Thrombin from human plasma (Sigma Aldrich, USA) was then added to trigger clot formation. Clot turbidity (A₄₀₅) was measured over a period of 1 hour using an EL808 plate reader (Biotek Instruments, Winooski, VT, USA). Final reactant concentrations were fibrinogen (2.6 mg/ml), 2.5 mM CaCl₂ and 1 nM thrombin (from human plasma) (Sigma-Aldrich) in a total volume of 200 µl.
2.7. Platelet aggregation assay: Blood was collected from consenting healthy human donors (ethical approval was granted by the School of Pharmacy Research Ethics Committee, Queen’s University, Belfast), and centrifuged at 240 x g for 15 minutes. The platelet-rich plasma (PRP)-containing supernatant was carefully removed and centrifuged again at 150 x g for 10 minutes to remove red blood cells (RBC). To obtain washed platelets, prostaglandin E1 (final concentration 1 µM) was added to stabilise the RBC-free PRP before further centrifugation at 640 x g for 15 minutes. The supernatant containing platelet-poor plasma was removed, and the pellet re-suspended in 10 ml of the following modified Tyrode’s solution: 137 mM NaCl; 11.9 mM NaHCO₃; 0.4 mM NaH₂PO₄; 2.7 mM KCl; 1.1 mM MgCl₂; 5.6 mM glucose; pH 7.4. The platelet count was adjusted to 200 x 10⁶/ml, and the platelets allowed to rest at room temperature for at least 1 hour. Prior to use, 1 mM CaCl₂ was added to the platelet-containing suspension. Fibrinogen (at a working concentration of 10 mg/ml) was added to BC-CFB and incubated for 48 hours at 37°C before adding to washed platelets to achieve a final fibrinogen concentration of 0.2 mg/ml. Aggregation induced by 6 µM ADP was monitored using two BioData PAP-4 aggregometers (Alpha Laboratories, Eastleigh, UK). The assay was performed at 37°C with a sample stir speed of 1000 RPM. The aggregometer automatically generated values for the final extent of aggregation, measured as a percentage of a theoretical maximum represented by platelet poor plasma.

2.8. Statistical analysis: Differences between groups (different species or strains of bacteria) were determined by non-parametric Mann-Whitney U-test. A p value of < 0.05 was considered significant. Data analysis was performed using Prism 5 for Windows Software version 5.00 (GraphPad software Inc, CA, USA).
3. Results

3.1. Bacterial filtrates display fibrinogenolytic activity

The ability of the bacterial filtrates to degrade fibrinogen (1 mg/ml) was assessed by fibrinogen zymography. All four *P. aeruginosa* strains (PA0219, BCH10-3, PA0025 and PA0049) produced a number of fibrinogenolytic activities (Fig. 1A). PA0219 showed a band of lysis corresponding to ~55 kDa, in addition to some lesser lower molecular weight activities, whereas BCH10-3 had a dominant band at ~100 kDa with additional active species ranging from 50-100 kDa. PA0025 and PA0049 also showed several bands of lysis between ~40 and 100 kDa, with a major species of ~70 kDa present in both. Of the four *B. cenocepacia* strains (J2315, K56-2, C5424 and BC7), only J2315 displayed substantial fibrinogenolytic activity (Fig.1A). Multiple bands of degradation were produced by J2315 at the following MWs; ~100 kDa, ~70 kDa, ~55 kDa, ~40 kDa and ~20 kDa. BC7 in contrast displayed only a single discernible band with a molecular weight of 100 kDa and some other minor, more diffuse activities. *B. multivorans* strains (C1962, C1576 and C5393) exhibited fibrinogenolytic activity to a lesser degree, though faint bands were visible at ~70 kDa, ~55 kDa, ~40 kDa and ~20 kDa (Fig. 1A).

3.2 Assessment of protease inhibitors on fibrinogenolytic activity

Each of the bacterial isolates (J2315, PA0025, BCH10-3, PA0049 and PA0219) that displayed high levels of fibrinogenolytic activity (Fig. 1A), were further investigated by fibrinogen zymography in the presence or absence of protease inhibitors (Fig. 1B-1F). These included serine protease inhibitors developed in-house (9) that irreversibly inactivate trypsin- (lanes 3,6), elastase- (lane 4), and chymotrypsin-like (lane 5) proteases. The commercially available inhibitors EDTA, phosphoramidon and 1,10 phenanthroline (lanes 7-9, respectively) were also used. EDTA is an effective chelator of divalent cations whereas 1,10 phenanthroline complexes with most metal ions and therefore inhibits metalloproteinases that require divalent cations, such as zinc, for activity. Phosphoramidon has been shown to
be a potent inhibitor of thermolysin and several other metalloproteinases, but only weakly inhibits collagenase and possesses no inhibitory activity against trypsin, papain, chymotrypsin or pepsin. In these studies, EDTA attenuated degradation of fibrinogen elicited by BC-CFB (J2315, PA0025, BCH10-3 and PA0049 although not PA0219) (Fig. 1B-F) indicating that either a protease reliant on divalent metal ions for activity is responsible for the degradation of fibrinogen observed with these strains, or that EDTA disrupts the quaternary structure of fibrinogen making it a less effective substrate for the enzymes. The inhibitor 1,10 phenanthroline reduced fibrinogen degradation caused by PA0025 (Fig. 1C) and PA0049 (Fig. 1E). The serine protease inhibitors partially inhibited fibrinogenolytic activity from strains PA0025, BCH10-3 and PA0049. For J2315, all of the inhibitors (except EDTA) increased the ~40 kDa activity. This suggests that multiple proteases are responsible for fibrinogen degradation, possibly as a cascade, and that the inhibitors (except EDTA) act selectively.

3.3 Degradation of the fibrinogen molecule by BC-CFB

Fibrinogen integrity was investigated by SDS-PAGE analysis after incubation of the molecule with BC-CFB samples (Fig 2A-C). Degradation of fibrinogen was evident in the presence of each bacterial sample (PA0219, C5393 and J2315), however the profiles varied from that elicited by the endogenous regulator thrombin (Fig. 2A). Proteolytic processing of fibrinogen by PA0219 was almost completely prevented in the presence of EDTA and 1,10 phenanthroline (Fig. 2A), with some partial protection offered by the individual serine protease inhibitors. In contrast, EDTA provided only limited protection to the fibrinogen molecule against degradation by B. multivorans proteases (C5393) (Fig. 2B); in this case the \( \gamma \) sub-unit of fibrinogen appeared to be more resistant. The most significant degradation of fibrinogen was observed in the presence of BC-CFB from the B. cenocepacia isolate J2315. These activities were resistant to inhibition by all of the compounds tested, although some partial protection was provided by one of the in-house trypsin-like inhibitors and EDTA (Fig. 2C).
3.4. Effect of BC-CFB on *in vitro* fibrin clot formation

As BC-CFB degraded fibrinogen we proceeded to determine the impact of bacterial proteases on fibrin clot formation using an *in vitro* turbidimetric clotting assay. The basis of this assay involves monitoring the thrombin-mediated conversion of soluble fibrinogen to insoluble fibrin which gives rise to a measurable increase in sample turbidity. When fibrinogen was pre-treated with each of the BC-CFB samples we found that the ability to form clots was markedly reduced in all instances (p<0.05) (Fig. 3A-C). Typical values of clot formation for BC-CFB treated samples were less than 20% of the thrombin-native fibrinogen control (Fig. 3A-C), except for the *B. cenocepacia* strain BC7 which was slightly higher (~30% of control) (Fig. 3B). We observed no difference in the rate of clotting between *P. aeruginosa*, *B. cenocepacia* and *B. multivorans* when we performed a sub-analysis (Fig. 3D).

3.4 Evaluation of neutrophil elastase on *in vitro* fibrin clot formation

Neutrophil elastase (NE) is a key host-derived protease found at significant levels in CF airways. Co-incubation experiments demonstrated fibrinogen degradation at NE concentrations of 1 μg/ml, but not lower levels (0.1-0.5 μg/ml) (Fig. 4A). Consistent with this observation, significantly impaired thrombin-induced *in vitro* clotting was observed after fibrinogen was incubated with NE at 1 μg/ml (p<0.05), but remained unaffected at the lower range of 0.1-0.5 μg/ml (Fig. 4B).

3.5 Effect of BC-CFB on platelet aggregation

Platelet aggregation, a key component of haemostasis, requires the binding of fibrinogen with activated integrin αIIbβ3 to provide critical cross-links that serve to stabilise the clot. Platelet aggregation was assayed using washed platelets collected from human donors to determine if BC-CFB samples had any effect on this process. The majority of bacterial strains examined reduced the extent of platelet aggregation in comparison to the control. In particular, the *P. aeruginosa* strain PA0219, *B. multivorans* strain C5393 and *B. cenocepacia*
strains J2315 and BC7 resulted in complete impairment of this process (p<0.05) (Fig 5A-C). A number of strains (PA0025, BCH10-3, and K56-2) elicited a modest inhibitory effect on platelet aggregation, whereas C1962 and PA0049 had no effect (Fig. 5A-C). The *B. multivorans* strains produced a variable response (Fig. 5D) with C1576 causing an increase in platelet aggregation (p=0.076) (Fig. 5B).
4. Discussion

Using a mucin-based biofilm assay to better mimic the environment of the CF lung, we have demonstrated in vitro that CF-relevant bacterial proteases have the potential to dysregulate haemostasis via the breakdown of fibrinogen, thus contributing to significant impairment of fibrin clot formation and platelet aggregation. Our study indicates that multiple bacterial proteases, the precise identification of which remains unknown, are involved in the observed degradation of fibrinogen.

Fibrinogen is a soluble, rod-like 340 kDa plasma glycoprotein composed of three pairs of polypeptides (two $\alpha$, two $\beta$ and two $\gamma$) linked together by a number of disulphide bridges. During the process of coagulation the N-terminal ends of the $\alpha$ and $\beta$ peptides are cleaved by the catalytic action of thrombin to produce insoluble fibrin monomers which then polymerise to form a three dimensional network of fibres, the fibrin clot. Plasma fibrinogen concentrations range from 2-4 g/L under physiological conditions however it is often elevated (by several fold) in diseases with an inflammatory component, including CF (10-13).

Extracellular proteases from $P$. aeruginosa, the major CF lung pathogen, have previously been shown to degrade fibrinogen (7). Here we confirm proteases secreted from CF-relevant bacteria ($P$. aeruginosa, $B$. cenocepacia and $B$. multivorans) possess considerable fibrinogenolytic activity, causing degradation of the fibrinogen molecule and significantly reduced in vitro fibrin clot formation. For some isolates, e.g. the $B$. cenocepacia isolate J2315 (14;15), very high levels of fibrinogenolytic activity were observed which correlated with complete fibrin degradation and abrogation of in vitro clot formation. J2315 is a strain of the ET12 lineage, and the index strain for transmission of this lineage among CF patients in the UK and Canada in the 1990s. Interestingly, ‘cepacia syndrome’, a progressive pneumonic illness often associated with ET-12 infection, as well as other BCC species, results in a rapid decline in CF lung function that is often fatal (3). While the development of
haemoptysis is a common feature of ‘cepacia syndrome’, no direct link has yet been established.

In addition to dysregulation of haemostasis, degradation of fibrinogen may also contribute to the chronic inflammatory cycle within the CF lung since fibrinogen products are known to act as a potent chemotactic agent for leukocytes, including neutrophils (16;17), and can induce fibroblast proliferation (18). Interestingly, leucocytosis is a clinical characteristic of ‘cepacia syndrome’ (19). Furthermore, the CF lung itself may represent a potential source of fibrinogen as alveolar cells have been shown to secrete the molecule when stimulated with IL-6, a pro-inflammatory cytokine elevated in CF airways (20). Further work is warranted to better understand the wider role of fibrinogen degradation products and their role in the CF lung.

Leukocyte proteases including neutrophil elastase, a key enzyme associated with CF pulmonary pathophysiology (21), have been long documented as able to degrade fibrinogen (22;23). Interestingly, high levels of neutrophil elastase (1 μg/ml) were necessary to degrade fibrinogen and impair clotting, whereas lower concentrations (<0.5 μg/ml) had no effect (Fig. 4). Whilst it is possible to achieve elastase levels (μg) in the CF lung which could start to impact on the coagulation process, these studies have shown that considerably lower concentrations of bacterial proteases (as present in our BC-CFB preparations) can impair clotting. Our data therefore indicates that host and/or bacterial proteases may feasibly contribute to a haemoptytic event. Additional work is, however, necessary to better understand their individual contributions.

Platelet aggregation is the process by which platelets adhere to each other at the site of injury and form a primary haemostatic plug, which is further stabilised by the conversion of fibrinogen to fibrin. A functional platelet assay was used to assess whether bacterial samples affect platelet aggregation. Fibrinogen degradation products have been shown previously to modulate (both inhibit and accelerate) platelet aggregation (24). We found 5/11 bacterial
samples abrogated aggregation (PA0219, C5393, J2315, BC7 and C5424), 4/11 elicited a more modest inhibitory effect (PA0025, BCH10-3, and K56-2) whereas 2/11 (C1962 and PA0049) did not modulate platelet aggregation. Interestingly, one bacterial isolate C1576 actually enhanced platelet aggregation. At present it remains unclear as to the underlying reasons for this variable impact of BC-CFB on platelet aggregation, but factors other than protease activity may be responsible. As well as playing a key role in haemostasis, platelets play a major role in inflammation and asthma (25) and platelet abnormalities are apparent in CF patients who display thrombocytosis (26), increased platelet reactivity and increased secretion of mediators that may negatively impact the lung (27;28). An inverse relationship between platelet aggregation and pulmonary function has also been reported (29;30). It would be of particular interest to examine the impact of BC-CFB on platelets isolated from CF patients and to further determine the downstream impact on platelet-derived inflammatory mediators.

Haemoptysis is a common complication in CF. Although often minor in nature, with slight streaking of mucus, massive haemoptysis (defined as acute bleeding >240 ml/ 24h, or recurrent bleeding >100 ml/d over several days) (31) occurs in 4.1% of patients and is associated with a significant deterioration in lung function over the following year and a higher rate of mortality (32). Massive haemoptysis is more prevalent in older patients with more advanced disease and represents a growing problem particularly given the continued improvement in life expectancy (33;34). The pathogenesis of haemoptysis is not very well understood, but is attributed to infection and inflammation-driven changes in the pulmonary vasculature, including weakened bronchial arteries and abnormal new vessel formation (angiogenesis) which are susceptible to bleeds into the airway lumen during acute infection (33;35). Our results showing that CF-relevant bacterial proteases negatively impact key haemostatic processes suggest these enzymes may play a role in the excessive haemorrhagic tissue damage seen in the later stages of CF lung disease.
Overall we have demonstrated that CF-related pathogens have the ability when grown as biofilms to secrete a number of proteases that collectively degrade fibrinogen, and prevent normal clot formation upon the addition of thrombin. We further demonstrate that the majority of BC-CFBs investigated resulted in impaired platelet aggregation. These findings are important as platelet aggregation and fibrin clot formation are critical components of haemostasis, and may therefore be of significance in the context of haemoptytic events observed in CF lung disease.

Acknowledgements

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5. References


Table 1: General information on the different bacterial isolates used in this study.

<table>
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<th>Isolate designation</th>
<th>Isolate Source</th>
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NK = Not known
Fig. 1. Fibrinogen zymography of BC-CFB. Fibrinogen degradation was investigated by incorporating fibrinogen (1 mg/ml) into the gel matrix prior to polymerisation of the gel. The sample profiling was carried out by SDS-PAGE, followed by Coomassie Blue staining. (A) BC-CFB (10 μl) was added to each lane. Lanes were as follows: molecular weight marker; TSB control (lane 1), PA0219 (lane 2), BCH10-3 (lane 3), PA0025 (lane 4), PA0049 (lane 5), J2315 (lane 6), K56-2 (lane 7), C5424 (lane 8), BC7 (lane 9), C1962 (lane 10), C1576 (lane 11) and C5393 (lane 12). (B-E) BC-CFB samples that displayed considerable fibrinogenolytic activity (B: J2315, C: PA0025, D: BCH10-3, E: PA0049, and F: PA0219) were then analysed in the presence of protease inhibitors. Lanes were
as follows: pre-stained protein markers; negative broth control (lane 1); positive bacteria-only control (lane 2); bacteria/inhibitor samples: trypsin inhibitor (lane 3); elastase inhibitor (lane 4) chymotrypsin inhibitor (lane 5); trypsin inhibitor (lane 6); EDTA (lane 7); phosphoramidon (lane 8) and 1,10-phenanthroline (lane 9). DDW control (lane 10).

Fig. 2. Degradation of fibrinogen by BC-CFB with and without protease inhibitors. Fibrinogen (1 mg/ml) degradation was investigated by co-incubation with BC-CFB at 37°C for 48 hours with or without a pre-incubation step with protease inhibitors (45 minutes, 37°C). Samples were subjected to SDS-PAGE followed by Coomassie blue staining. Representative gels for (A) PA0219; (B) C5393 and (C) J2315 are shown. Each lane was as follows: 5 μg fibrinogen (lane 1), fibrinogen and thrombin (5 μg and 10 U, respectively) (lane 2), fibrinogen pre-incubated with BC-CFB (lane 3), fibrinogen/BC-CFB samples in the presence of in-house (QUB) protease inhibitors (lane 4: trypsin inhibitor; lane 5: chymotrypsin inhibitor; lane 6: elastase inhibitor; lane 7: trypsin inhibitor); fibrinogen/BC-CFB samples in the presence of commercially available protease inhibitors: EDTA (lane 8); phosphoramidon (lane 9); and 1,10-phenanthroline (lane 10). (Representative figure of n=3 experiments).
Fig. 3. BC-CFB impairs *in vitro* fibrin clot formation. Clot formation initiated by the addition of 1 nM thrombin determined in reactions containing fibrinogen that had undergone previous proteolytic processing by bacterial filtrates. Data shown in (A-C) are kinetic traces representative of the values obtained from three independent experiments for clot formation in the presence of (A) *P. aeruginosa* (B) *B. cenocepacia* and (C) *B. multivorans* isolates. The rate of clot formation was calculated for each individual isolate and expressed as a percentage of the thrombin/native fibrinogen control and the mean value plotted according to species group (D).
Fig. 4. The impact of neutrophil elastase (NE) on fibrinogen status and in vitro fibrin clot formation. (A) Fibrinogen (2.6 mg/ml) degradation was investigated after incubation with various doses of recombinant neutrophil elastase at 37°C for 48 hours. Samples were then subjected to SDS-PAGE followed by Coomassie blue staining. (B) Clot formation measured as a % thrombin control (no NE) when NE-fibrinogen samples (200 μl) were spiked with 1 nM thrombin to induce clotting (n=4).
Fig. 5. Assessment of the ability of washed platelets to undergo aggregation in the presence of bacterial-processed fibrinogen. Fibrinogen was incubated with BC-CFB from isolates of (A) *P. aeruginosa*, (B) *B. multivorans* and (C) *B. cenocepacia* for 48 hours at 37°C then added to washed platelets at a final fibrinogen concentration of 0.2 mg/ml. Platelet aggregation was measured upon the addition of 6 µM ADP. Results displayed are representative of the values obtained from three independent experiments. Platelet aggregation (%) after 360 seconds was plotted according to species and shown in (D).
Supplementary Methods

**Mucin coating of microtitre plate surfaces.** 96-well microtitre plates were pre-coated with porcine stomach mucin (Type-III, Sigma-Aldrich, USA) dissolved in sterile phosphate buffered saline (PBS, Sigma-Aldrich, USA). Optimization of mucin concentrations for bacterial adherence was investigated by preparation of doubling dilutions ranging from 5 mg·ml⁻¹ to 2.5 µg·ml⁻¹ in sterile PBS. The mucin solution (200 µl) was transferred to each well for coating and incubated at 4°C overnight. Excess mucin solution was decanted off and plates briefly washed with sterile PBS.

**Comparison of bacterial adherence on mucin and non-treated surfaces.** An overnight bacterial culture suspension, adjusted to ~5×10⁷ CFU·ml⁻¹, was seeded to mucin-coated and uncoated wells and incubated at 37°C under mild agitation (100 RPM) for 24 hours. Following initial incubation, culture supernatants were aspirated and each well washed three times with sterile PBS for the removal of non-adherent bacteria. Biofilm formation was observed by crystal violet staining as previously described (1) with minor modifications as detailed below. The optimal concentration of mucin was 1 mg·well⁻¹, which was used in all subsequent culture experiments.

**Congo red binding assay for visualisation of adherent bacteria.** Congo red staining of biofilm was performed as previously described (2), with minor modifications. The congo red solution (50 µl of 1% (w/v) solution; Sigma-Aldrich, St. Louis, USA), in 50% (v/v) ethanol (EtOH) (VWR International, France), was added to the microtitre plate, shaken briefly and incubated for 15 minutes at room temperature (R/T). The staining solution was then decanted and each well washed with sterile PBS. Counter staining was achieved by adding 50 µl of 1% (w/v) ziehl carbol fuchsin, containing 0.1% (w/v) carbol fuchsin (Sigma-Aldrich, St. Louis, USA), 1% (v/v) EtOH, 5% (w/v) phenol (Sigma-Aldrich, St. Louis, USA), and allowed to stain bacteria for 6 minutes at R/T. The excess stain was removed and wells rinsed as before. Stained biofilms were visualised by phase-contrast microscopy (Nikon
Eclipse TE300, Nikon Instruments Inc, Surrey, UK) at x400 magnification and photographs taken using Nikon DXM1200 digital camera. Analysis was carried out using Lucia GF 4.60 digital imaging suite software (Laboratory Imaging, Prague, Czech Republic). Congo red solution stains EPS component pink or orange, while ziehl carbol fuchsin stains bacteria red or purple.

Supplementary Results

Comparison of bacterial biofilm formation between non-mucin and mucin-conditioned abiotic surfaces. The ability of bacteria to adhere and form biofilm on a mucin-conditioned surface compared to a non-mucin-conditioned surface was found to be statistically different (p < 0.0001) (Supplementary Fig. 1). Mucin-coating of polystyrene surfaces was used for all subsequent bacterial culture experiments.

Biofilm visualisation with Congo red binding assay. Biofilm formation between mucin- and non-mucin-conditioned surfaces, as shown by Congo red staining, was microscopically visualised using a single strain representative of each species (B. multivorans, C1576; B. cenocepacia, J2315; P. aeruginosa, PA0219). When cultured in the presence of mucin, C1576, J2315, and PA0219 demonstrated clear, compact aggregates indicative of biofilm-like structures attached to the mucin-conditioned surface of the microtitre plate and surrounded by a congo red-stained EPS matrix (Fig. 2.; E, F, and G respectively). However, when grown in the absence of mucin each of the bacterial strains appeared to demonstrate more dispersed formations of thin layers or aggregates of bacterial cells (Fig. 2; A, B, and C respectively). The negative control (no bacteria) demonstrated no observable bound dye eliminating the possibility of false negative results within the assay (Fig. 2; D and H respectively).
Supplementary Fig. 1. Bacterial adhesion at 24 hours without and with porcine stomach mucin (Type III; 1 mg·ml⁻¹) coating of an abiotic surface of a 96-well microtitre plate. Each absorbance value is the mean of measurements for isolates representing *B. multivorans* (*n* = 3), *B. cenocepacia* (*n* = 3), and *P. aeruginosa* (*n* = 3). Error bars denote ± SEM. The means were compared using the Mann-Whitney non-parametric test (*p* < 0.0001).
Supplementary Fig. 2. Biofilm formation at 48 hrs in the absence (A, B, C) or presence (E, F, G) of mucin coating of microtitre plate surfaces. Biofilm formation by *B. multivorans* strain (C1576, A and E respectively), *B. cenocepacia* strain (J2315, B and F respectively), *P. aeruginosa* strain (PA0219, C and G respectively), and non-bacterial control (D and H respectively). In the presence of mucin the bacteria formed defined structures. Bacteria were stained with congo red and then counter stained with ziehl carbol fuchsin (all images x400 magnification, Bars = 100 μm).
References
