Inhibition of co-colonizing cystic fibrosis-associated pathogens by *Pseudomonas aeruginosa* and *Burkholderia multivorans*

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INTRODUCTION

Cystic fibrosis (CF) is a recessive genetic disease characterized by chronic respiratory infections and inflammation causing permanent lung damage. Recurrent infections are caused by Gram-negative antibiotic-resistant bacterial pathogens such as *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex (Bcc) and the emerging pathogen genus *Pandoraea*. In this study, the interactions between co-colonizing CF pathogens were investigated. Both *Pandoraea* and Bcc elicited potent pro-inflammatory responses that were significantly greater than *Ps. aeruginosa*. The original aim was to examine whether combinations of pro-inflammatory pathogens would further exacerbate inflammation. In contrast, when these pathogens were colonized in the presence of *Ps. aeruginosa* the pro-inflammatory response was significantly decreased. Real-time PCR quantification of bacterial DNA from mixed cultures indicated that *Ps. aeruginosa* significantly inhibited the growth of *Burkholderia multivorans*, *Burkholderia cenocepacia*, *Pandoraea pulmonicola* and *Pandoraea apista*, which may be a factor in its dominance as a colonizer of CF patients. *Ps. aeruginosa* cell-free supernatant also suppressed growth of these pathogens, indicating that inhibition was innate rather than a response to the presence of a competitor. Screening of a *Ps. aeruginosa* mutant library highlighted a role for quorum sensing and pyoverdine biosynthesis genes in the inhibition of *B. cenocepacia*. Pyoverdine was confirmed to contribute to the inhibition of *B. cenocepacia* strain J2315. *B. multivorans* was the only species that could significantly inhibit *Ps. aeruginosa* growth. *B. multivorans* also inhibited *B. cenocepacia* and *Ps. apista*. In conclusion, both *Ps. aeruginosa* and *B. multivorans* are capable of suppressing growth and virulence of co-colonizing CF pathogens.

Abbreviations: Bcc, *Burkholderia cepacia* complex; CF, cystic fibrosis; CFS, cell-free supernatant; HHQ, 2-heptyl-4 quinolone; PQS, *Ps. aeruginosa* quinolone signal; QS, quorum sensing.
mechanisms of pathogenicity (Costello et al., 2011; Jørgensen et al., 2003; Stryjewski et al., 2003). Pandoraea colonization has been associated with CF and non-CF patients and often occurs when the patient has already succumbed to chronic colonization with another pathogen (Schneider et al., 2006). Nine species have been identified within the genus to date (Coenye et al., 2000; Daneshvar et al., 2001; Sahin et al., 2011). Pandoraea apista has been associated with epidemic spread and rapid patient decline (Atkinson et al., 2006; Jørgensen et al., 2003), while Pandoraea pulmonicola is the most commonly identified Pandoraea species among Irish CF patients (Costello et al., 2011).

It has recently been established that the CF microbiome is a complex, polymicrobial environment (Sibley et al., 2011). CF patients are rarely colonized by one single pathogen but by multiple pathogens with a range of possible virulence determinants that are regulated in response to changes in the bacterial environment (Duan et al., 2009). Investigation of the virulence of a particular bacterial species is usually carried out on single pathogens in isolation; co-colonizing bacteria are generally not taken into account. Competition between pathogens may result in the inhibition of one co-colonizing species by another in an environment where the bacterial species must compete for limited nutrients. It was recently shown that Ps. aeruginosa can inhibit planktonic B. cenocepacia; however, no other CF-associated pathogens were examined (Bragonzi et al., 2012). Alternatively, interactions between pathogens may have implications on the virulence associated with bacteria including repression or complementation of the virulence of another pathogen (Lavigne et al., 2008; Sibley et al., 2008). Co-colonizing pathogens can interact via their quorum sensing (QS) systems; for example, B. cenocepacia can utilize, and is regulated by, acylhomoserine lactones from Ps. aeruginosa and vice versa (Lewenza et al., 2002; Riedel et al., 2001). Furthermore, in the case of CF pathogens, the pro-inflammatory host responses elicited by many of these contribute to hyperinflammation in the CF airways leading to irreversible tissue damage (Bonfield et al., 1995; Machen, 2006). Given that B. multivorans, B. cenocepacia and members of the genus Pandoraea are all capable of eliciting potent pro-inflammatory responses (Bamford et al., 2007; Caraher et al., 2008), we wanted to investigate the pro-inflammatory response in the presence of more than one of these pathogens.

In this study the interactions between Ps. aeruginosa, Bcc and the emerging CF pathogen Pandoraea were examined. Our starting hypothesis was that these pathogens would result in a further elevation in inflammation when they are co-colonizing. To examine this, levels of IL-6 and IL-8 secreted from lung epithelial cells induced by Ps. aeruginosa, Bcc and Pandoraea species either singly or in combination were examined. The lack of an enhanced cytokine response when two pathogens colonized lung cells in combination led us to examine the competition between co-colonizing pathogens and the factors that may contribute to the ability of Ps. aeruginosa to inhibit co-colonizing pathogens.

**METHODS**

**Bacterial strains.** The strains used in this study are listed in Table 1. Bcc and Pandoraea strains were purchased from BCCM/LMG, University of Ghent, Belgium, while Ps. aeruginosa ATCC 27853 was obtained from the American type Culture Collection. PA14 mutant library (Liberati et al., 2006) and Pandoraea isolates were routinely grown on trypticase soy agar (TSA) or in TS broth at 37 °C. Bcc and Ps. aeruginosa strains were routinely grown on Luria–Bertani (LB) agar or in LB broth at 37 °C.

**Cytokine secretion from CF lung epithelial cells.** The CF bronchial epithelial cell line CFBE41o– is homozygous for the most common CF mutation (AF508 mutation). These cells were routinely cultured on coated flasks as recently described (Bevivino et al., 2012). IL-6 and IL-8 secretion from CFBE41o– cells was measured using ELISA as described by Caraher et al. (2008). CFBE41o– cells were seeded at 4 × 10^5 cells per well and incubated for 24 h prior to infection with bacterial strains individually for 24 h (m.o.i. 1:50) prior to analysis of cytokine levels. For double infections, the cells were exposed to the first strain (OD_{600} of 0.6; m.o.i. 1:25) 24 h after seeding and then the second strain (OD_{600} of 0.6; m.o.i. 25:1) a further 24 h later, to model an incoming additional infection to an already colonized epithelium. The infected cell cultures were then incubated for an additional 24 h before pro-inflammatory cytokine analysis of supernatants.

**Determination of growth inhibition by agar diffusion assay.** Agar well diffusion plates were prepared with 60 ml TSA containing 2% (v/v) Tween 80 and 5% (v/v) of stationary phase indicator cultures (Ps. aeruginosa ATCC 27853, Pa. pulmonicola LMG 18108, Pa. apista LMG 16407, B. multivorans LMG 13010 or B. cenocepacia J2315). Wells (18 × 6 mm) were inoculated with challenge culture (100 μl) and incubated for 24 h at 37 °C. After incubation, zones of inhibition and overgrowth were measured using digital callipers.

**Table 1. Species and strains used in this study and their sources**

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Source</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Ps. aeruginosa</td>
<td>ATCC 27853</td>
<td>Non-CF blood culture</td>
<td>Medeiros et al. (1971)</td>
</tr>
<tr>
<td>Ps. aeruginosa</td>
<td>Pa14</td>
<td>Burn isolate</td>
<td>Rahme et al. (1995)</td>
</tr>
<tr>
<td>B. cenocepacia</td>
<td>J2315</td>
<td>CF patient</td>
<td>Mahenthiralingam et al. (2000)</td>
</tr>
<tr>
<td>B. multivorans</td>
<td>LMG 13010</td>
<td>CF patient (Belgium)</td>
<td>Mahenthiralingam et al. (2000)</td>
</tr>
<tr>
<td>Pa. palmonicola</td>
<td>LMG 18108</td>
<td>CF patient (USA)</td>
<td>Coenye et al. (2000)</td>
</tr>
<tr>
<td>Pa. apista</td>
<td>LMG 16407</td>
<td>CF patient (Denmark)</td>
<td>Coenye et al. (2000)</td>
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Three independent experiments were performed for each pair of strains examined. The results are presented as the mean zone of clearance ± SD.

Quantification of bacterial growth by real-time PCR. Quantification of individual strains in co-cultures was determined by real-time PCR. Species-specific primers were designed for B. cenocepacia J2315 and B. multivorans LMG 13010 against the 16S ribosomal GidB methyl-transferase gene and partial 16S sequences were used to design primers for Pa. apista LMG 16407, Pa. pulmonicola LMG 18108 and Ps. aeruginosa ATCC 27853 to enable quantification of a single strain in a mixed culture. Primer 3 Software was used for primer generation and BLAST software for specificity. All primers (Table 2) were 18–20 bp long with a melting temperature of 58–60 °C and a DNA G+C content of 40–60 mol% generating products of 100–120 bp in length. The specificity of each set of primer pairs was confirmed using real-time PCR. Primer efficiency for each pair was calculated using standard curves ranging from 2 × 10^7 to 2 × 10^9 c.f.u. To ascertain whether one pathogen had an effect on the growth or survival of the other, each culture was grown singly in 1.5 ml as a control and mixed in 3 ml volumes.

Exponential-phase cultures (2 × 10^7 exponential-phase c.f.u.) of each strain were inoculated into separate and mixed growth tubes. DNA extraction, from standardized cultures (2 × 10^7 c.f.u.), was carried out using a Qiagen DNeasy blood and tissue DNA extraction kit. Real-time PCR analysis was carried out on 24 h co-cultured bacterial samples and single controls. Standard curves were run for each strain and time PCR analysis was carried out on 24 h co-cultured bacterial samples. Each analysis was run in triplicate on three separate occasions.

Inhibition of bacterial growth by CFS. The effect of secreted factors on pathogen growth was examined using single or co-culture CFS. Exponential-phase cultures (2 × 10^7 c.f.u.) were inoculated individually into 5 ml broth. Co-cultures were prepared by inoculating 2 × 10^7 c.f.u. of each pathogen into 10 ml LB, for 24 h, at 37 °C. Bacterial cultures were centrifuged and the supernatants were removed and filter-sterilized (0.22 μm filters). CFS from either a single culture or a co-culture was added to LB at a ratio of 1:1. In total, 3 ml of broth was inoculated into a shake flask at 37 °C for 24 h. The c.f.u. for each sample was then calculated by serial dilution and plating (in duplicate). Each test was carried out at least three times.

A range of dilutions of Ps. aeruginosa or B. multivorans CFS were tested against B. cenocepacia J2315 growth to determine the potency of each pathogen CFS. Serial dilutions of CFS (10–100%) were prepared in sterile water and an aliquot of 3 ml was added to 3 ml of fresh media. B. cenocepacia J2315 (2 × 10^7 c.f.u.) was inoculated into each sample and incubated with shaking at 37 °C for 24 h. B. cenocepacia J2315 c.f.u. was determined as described above.

To investigate if inhibition was proteinaceous in nature, proteinase K (50 μg ml⁻¹) was incubated with individual CFS from each of the strains for 24 h followed by protease inhibitor prior to testing. Alternatively, CFS was heated for 15 min at 100 °C. Growth inhibition testing was then carried out as described above.

Functional screening of the non-redundant PA14 transposon mutant library. The genetic factors underpinning Ps. aeruginosa antagonism of CF-associated pathogens were investigated by both global and targeted approaches. The complete PA14 mutant library (Liberati et al., 2006) was transferred to bioassay agar plates and grown overnight at 37 °C. The following day, an overlay of B. cenocepacia J2315 in 0.3% (w/v) soft agar was added to the plates and incubated overnight at 37 °C. Zones of growth surrounding colonies were subsequently marked and the mutants involved were identified in the mutant library database. In tandem with this approach, mutants were selected for singular analysis on the basis of their role in virulence and pathogenesis in Ps. aeruginosa. Singular analysis of all candidate mutants was performed using CFS, which was extracted from overnight cultures by pelleting bacteria for 10 min before sterile filtering with 0.2 μm filters. B. cenocepacia J2315 overnight culture was diluted to an OD₆₀₀ of 0.1 with sterile broth and combined 1:1 with mutant CFS in a 100-well Bioscreen plate, to a total volume of 200 μl. The OD₆₀₀ of each plate was read every 15 min for 42 h on a Bioscreen analyser (Oy Growth Curves Ab). To investigate whether the antagonistic role of Ps. aeruginosa quinolone signal (PQS)/2-heptyl-4 quinolone (HHQ) was direct or indirect, the growth of B. cenocepacia J2315 was also monitored in the presence of synthetic HHQ and PQS (10 μM). Each analysis was performed in duplicate at least three times.

Effect of pyoverdine on growth of CF pathogens. The role of Ps. aeruginosa pyoverdine on growth of the other species was examined by inoculating exponential-phase cultures (2 × 10^7 c.f.u.) into wells of a 96-well plate, and incubating with a range of concentrations of desferripyoverdine (0.39–25 μg ml⁻¹, EMC Microcollections) in duplicate and measurement of OD₆₀₀ after 24 h. The effect of proteinase K or heat treatment on the inhibitory capacity of pyoverdine was assessed by incubation of 40 μM pyoverdine with 50 μg proteinase K ml⁻¹ for 24 h followed by addition of protease inhibitor or incubation at 100 °C for 15 min. Inhibition studies were carried out as above for untreated pyoverdine (MIC) at a final pyoverdine concentration of 20 μM. Each analysis was performed in duplicate at least three times. Pyoverdine was quantified from Ps. aeruginosa ATCC 27853 as previously described (Bayase et al., 2000) by spectrophotometric analysis of replicate CFS to obtain the Cₘₐₓ and then the absorption measured at 380 nm. Pyoverdine was quantified against a pyoverdine standard curve.

Statistical analysis. Statistical analysis of variance was carried out using Minitab software with 95% confidence interval.

<table>
<thead>
<tr>
<th>Species and strain</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>Ps. aeruginosa ATCC 7853</td>
<td>5′-GGACCGGCTGAGTATGCCTA-3′</td>
<td>5′-GAAGATCCCCCATTTCTCC-3′</td>
</tr>
<tr>
<td>Pa. pulmonicola LMG 18108</td>
<td>5′-TGCGTAGATGTTGAGGAA-3′</td>
<td>5′-TTTAGGGCCGTCAGACC-3′</td>
</tr>
<tr>
<td>Pa. apista LMG 16407</td>
<td>5′-TGCGTAGATGTTGAGGAA-3′</td>
<td>5′-TTTAGGGCCGTCAGACC-3′</td>
</tr>
<tr>
<td>B. multivorans LMG 13010</td>
<td>5′-AAGAAGTCGCGTTCACAG-3′</td>
<td>5′-GAATTTTTCGCCGACCT-3′</td>
</tr>
<tr>
<td>B. cenocepacia J2315</td>
<td>5′-GGCTGAAGGCGTGTGAAAAT-3′</td>
<td>5′-AAGGGCGGATACGAT-3′</td>
</tr>
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RESULTS

Analysis of the pro-inflammatory cytokine response of CFBE41o- cells elicited by Ps. aeruginosa, Bcc and Pandoraea during single and co-infection

CF pathogens and, in particular, Bcc and Pandoraea strains have been shown to elicit considerable pro-inflammatory responses in lung epithelial cells (Caraher et al., 2008; Kaza et al., 2011). Combinations of pro-inflammatory co-colonizing pathogens were examined to investigate whether dual infections exacerbated the pro-inflammatory effects. As previously described, secretion of inflammatory cytokines IL-8 and IL-6 (Fig. 1) from CFBE41o- cells indicated a potent inflammatory response was elicited following infection with either Pandoraea or Bcc strains, and this was greater than that of Ps. aeruginosa (*P<0.05) when inoculated individually. Surprisingly, the secretion of IL-8 and IL-6 by CFBE41o- cells, elicited by Pa. pulmonicola LMG 18108, Pa. apista LMG 16407, B. multivorans LMG 13010 or B. cenocepacia J2315 was significantly reduced when co-cultured with Ps. aeruginosa ATCC 27853, in comparison with single cultures (*P<0.05). In all combinations, the inflammatory response of all co-cultures tested was reduced to the level of Ps. aeruginosa ATCC 27853 during single infection. This suggested that Ps. aeruginosa was capable of suppressing the inflammatory response induced by the presence of other pathogens during co-colonization.

Agar well diffusion analysis of growth inhibition

Agar well diffusion assays were carried out to determine if the growth of the strains was compromised in the presence of other strains. Each strain was infused in agar and all competing strain cultures were inoculated into wells throughout the agar. Ps. aeruginosa ATCC 27853 was capable of growth into B. multivorans LMG 13010-infused agar (Fig. 2a). The growth of B. cenocepacia J2315 or Pa. apista LMG 16407 was both overgrown and inhibited by Ps. aeruginosa ATCC 27853 culture, as evidenced by the zone of clearance extending beyond the Ps. aeruginosa growth around the wells (Fig. 2b, d). Both strains were also significantly inhibited by Ps. aeruginosa (P=0.002 and P=0, respectively). Ps. aeruginosa ATCC 27853 also overgrew Pa. pulmonicola LMG 18108 (Fig. 2c), inhibiting its growth (Fig. 2c, P=0.0133). The growth of Ps. aeruginosa ATCC 27853 in infused agar was unaffected by any of the pathogens in wells, (Fig. 2e, i, m, q), indicating Ps. aeruginosa ATCC 27853 may be a dominant pathogen in co-colonizing communities. Interestingly, no species had a reproducible observed effect on the growth of B. multivorans LMG 13010 (Fig. 2a, j, n, r)

B. multivorans LMG 13010 clearly inhibited both B. cenocepacia J2315 (P=0) and Pa. apista LMG 16407 (P=0) (Fig. 2f, h). In addition, agars infused with both B. cenocepacia J2315 and Pa. apista LMG 16407 were also overgrown by Pa. pulmonicola LMG 18108 (Fig. 2o, p). B. cenocepacia J2315 did not inhibit any of the strains examined. Interestingly, growth of both B. cenocepacia J2315 and Pa. apista LMG 16407 was either inhibited or overgrown by three of four challenging pathogens (Fig. 2b, f, o). These results, summarized in Fig. 2(u), indicate that growth of both of these pathogens would potentially be challenged by Ps. aeruginosa, B. multivorans and Pa. pulmonicola in a polymicrobial environment.

Quantification of Ps. aeruginosa, Bcc and Pandoraea species in co-culture using real-time PCR

Agar diffusion assays suggested that certain co-colonizing pathogens could out-compete others. To quantify this, the ability of Ps. aeruginosa ATCC 27853 to inhibit the growth of the other CF pathogens in co-culture was examined using real-time PCR with species-specific primers. Pa. pulmonicola LMG 18108 growth was significantly inhibited...
by *Ps. aeruginosa* ATCC 27853 when both strains were co-cultured together (*P*<0.05). This was shown by a reduction in growth of 67% compared with the control, and is consistent with the agar diffusion assay (Fig. 3a). In contrast, *Pa. pulmonicola* had no impact on *Ps. aeruginosa* growth. *Pa. apista* LMG 16407 growth was also inhibited over 10-fold.

**Fig. 2.** Effects of pathogens on the growth of a competing pathogen in agar well diffusion assays. The five pathogens which were each plated as indicator strains within the agar are shown at the top of each column of images and the test strains were inoculated in the wells as follows: *Ps. aeruginosa* (a–d); *B. multivorans* (e–h); *B. cenocepacia* (i–l); *Pa. pulmonicola* (m–p) and *Pa. apista* (q–t). White arrows indicate overgrowth (b) and zone of clearance (d). (u) Mean (± SD) zone of clearance from three independent experiments.
Ps. aeruginosa ATCC 27853 when both strains were co-cultured together (P<0.05), growing to only 8.7% of control (Fig. 3b), while Ps. aeruginosa growth was unaffected by Pa. apista LMG 16407. B. cenocepacia J2315 growth was almost completely inhibited by Ps. aeruginosa ATCC 27853 (P<0.05) and, again, Ps. aeruginosa ATCC 27853 growth was not significantly altered by the presence of B. cenocepacia (Fig. 3c).

B. multivorans LMG 13010 was the only strain that had any impact on Ps. aeruginosa ATCC 27853, although Ps. aeruginosa had a greater effect on the B. multivorans strain. In the presence of Ps. aeruginosa ATCC 27853, B. multivorans LMG 13010 was inhibited by 89% of the control (P<0.05), while Ps. aeruginosa ATCC 27853 growth was also inhibited by 28% in this co-culture (Fig. 3d). This suggests that when these two species co-infect, the competition between them may result in an overall reduction in growth of both species.

In summary, Ps. aeruginosa ATCC 27853 significantly decreased the growth of all co-cultured pathogens tested, while its own growth remained unchanged in the presence of all strains except B. multivorans LMG 13010.

**Inhibition of Pandoraea species and Bcc by Ps. aeruginosa is innate rather than responsive**

To examine whether the effect of Ps. aeruginosa on growth of the other species was innate or elicited in response to the presence of a potentially competing pathogen, the effect of Ps. aeruginosa CFS versus whole cells was examined. Individual strains (2 × 10^7 c.f.u.) were inoculated into the
CFS of a second strain, and growth was measured after 24 h. To examine whether the inhibitory factor was elicited in response to co-culture with a competing pathogen, CFS from co-cultured strains was inoculated with either one of the two strains (2 × 10^7 c.f.u.) and the growth at 24 h was compared with growth in monoculture CFS (which had not been exposed to a competing pathogen). Controls consisting of each pathogen grown in CFS from the same strain were used to determine growth in the absence of an inhibitory factor.

Monoculture *P. aeruginosa* ATCC 27853 CFS significantly reduced the growth of *P. pulmonicola* LMG 18108 (P<0.05), showing that the inhibition of this organism was innate (Fig. 4a). Interestingly, when *P. pulmonicola* LMG 18108 was inoculated back into CFS prepared from a co-culture of both species, its growth was not inhibited, indicating the potential for this pathogen to partially resist inhibition by *P. aeruginosa* after a period in co-culture. When the *P. aeruginosa* CFS was treated with proteinase K, its ability to inhibit the growth of *P. pulmonicola* was virtually eliminated, as evidenced by a return to the control growth level, indicating that the inhibitory factor is proteinaceous in nature. In contrast, heat and EDTA had little effect.

Growth of *P. apista* LMG 16407 was dramatically inhibited by *P. aeruginosa* ATCC 27853 CFS (Fig. 4b) and, unlike *P. pulmonicola*, prior co-culture did not allow

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**Fig. 4.** Inhibition of growth by *Ps. aeruginosa* ATCC 27853 (a–d), *B. multivorans* (e–g) or *Pa. pulmonicola* (h) CFS and the effect of treatment with proteinase K, heat or EDTA, where appropriate. Growth of test strains were monitored by plating following exposure to competing pathogen CFS or CFS from co-cultured pathogens and compared with growth in test strain CFS. Each experiment was carried out at least three times in duplicate. *Statistically significant difference compared with growth in test strain CFS, P<0.05.*
Pa. apista to overcome this effect. Proteinase K, heat or EDTA did not alter the inhibitory effect of Ps. aeruginosa CFS, indicating that in this case the inhibitory factor is neither proteinaceous nor heat-labile and does not require chelatable metals and is therefore different from that which affects Pa. pulmonicola.

Growth of B. multivorans was reduced by monoculture Ps. aeruginosa CFS but not by the CFS prepared from both strains cultured together, showing that B. multivorans can also resist inhibition by Ps. aeruginosa after a period in co-culture (Fig. 4c). The inhibitory effect of Ps aeruginosa CFS on B. multivorans was partially reduced following proteinase K treatment and was unaffected by heat treatment, comparable with that observed for Pa. pulmonicola. This indicates that a heat-resistant protein is at least partly responsible for inhibition of B. multivorans LMG 13010. However, non-proteinaceous virulence factors may also be involved in the inhibition of this pathogen. Growth of B. cenocepacia J2315 was not able to overcome the inhibition by Ps. aeruginosa ATCC 27853. As with inhibition of Pa. apista, the factor that inhibited B. cenocepacia was neither proteinaceous nor heat-labile.

In summary, Ps. aeruginosa secretes proteinaceous heat-resistant substance(s) that are capable of inhibiting Pa. pulmonicola and B. multivorans. Both of these latter organisms have the ability to resist this inhibition after 24 h in co-culture. In addition, the growth of both B. cenocepacia and Pa. apista is inhibited by secreted substances that are neither heat-labile nor proteinaceous. Furthermore, in contrast to B. multivorans and Pa. apista, neither B. cenocepacia nor Pa. apista showed any resistance to this inhibitory factor after 24 h in co-culture.

**Inhibition of CF pathogens by B. multivorans**

CFS from B. multivorans LMG 13010 and Pa. pulmonicola LMG 18108 had no effect on each other (Fig. 4g, h). In contrast, Pa. apista LMG 16407, B. cenocepacia J2315 was not able to overcome the inhibition by Ps. aeruginosa ATCC 27853. As with inhibition of Pa. apista, the factor that inhibited B. cenocepacia was neither proteinaceous nor heat-labile.

In summary, Ps. aeruginosa secretes proteinaceous heat-resistant substance(s) that are capable of inhibiting Pa. pulmonicola and B. multivorans. Both of these latter organisms have the ability to resist this inhibition after 24 h in co-culture. In addition, the growth of both B. cenocepacia and Pa. apista is inhibited by secreted substances that are neither heat-labile nor proteinaceous. Furthermore, in contrast to B. multivorans and Pa. apista, neither B. cenocepacia nor Pa. apista showed any resistance to this inhibitory factor after 24 h in co-culture.
not heat or EDTA treatment, indicating the role of a heat-resistant inhibitory protein in this inhibition. Co-culture CFS did not significantly inhibit growth of *Pa. apista* LMG 16407, indicating *Pa. apista* LMG 16407 is capable of resisting this to some degree when these two pathogens are grown together (Fig. 4e). *B. cenocepacia* J2315 was strongly inhibited not only by *B. multivorans* LMG 13010 CFS but also co-culture CFS (Fig. 4f). The measured c.f.u. did not increase past the starting inoculum, indicating that *B. cenocepacia* J2315 could not grow in the presence of *B. multivorans* LMG 13010 CFS. The inhibitory factor was not affected by EDTA, proteinase K or heat treatment. In summary, *B. multivorans* secretes proteinaceous factor(s) that inhibit *Pa. apista* and non-proteinaceous factor(s) that inhibit *B. cenocepacia*.

**Ps. aeruginosa CFS and B. multivorans CFS are equally capable of inhibiting *B. cenocepacia* growth**

As both *Ps. aeruginosa* ATCC 27853 and *B. multivorans* LMG 13010 were capable of significantly inhibiting the growth of co-colonizing pathogens, a comparison of a series of dilutions of each CFS was tested against *B. cenocepacia* J2315 growth to determine which CFS was more inhibitory. There was no significant difference in CFS potency between these species. Both species have comparable ability to inhibit *B. cenocepacia*, with the CFS from both pathogens showing a clear inhibition above a threshold 50% dilution (Fig. 5).

**Ps. aeruginosa inhibition of *B. cenocepacia* growth is QS-regulated**

To elucidate the genetic factors underpinning antagonism of *B. cenocepacia* growth by *Ps. aeruginosa*, the non-redundant PA14 transposon mutant library (Liberati et al., 2006) was screened both by overlay of the complete library and by targeted analysis of CFS from mutants selected on the basis of their role in virulence regulation. PA14 CFS was first tested to ensure that it inhibited *B. cenocepacia* J2315 and was comparable to *Ps. aeruginosa* ATCC 27853 (data not shown). The global screen implicated a role for QS regulation in J2315 inhibition, which was further
inhibition is not due to iron limitation. This indicates that the B. cenocepacia J2315 (Fig. 6c). Given that PQS has been neither PQS nor HHQ had any direct effect on growth of none of the folX cis of cellular redox potential in higher organisms; four katA genes involved in the oxidative stress response (e.g. katA, katB, katE, ohr); xdhAB, an important modulator of cellular redox potential in higher organisms; four peptidyl-prolyl cis-trans isomerases (ppiA, ppiB, ppiC1, ppiD); and genes involved in the synthesis of the pyoverdine siderophore (pvdQA, pvdE, pvdH, pvdGL). A large region involved in phosphonate metabolism, phnPNMLK–phnD was also unable to suppress growth of the overlay, while mutants of the phoBR–phoU–pst phosphate responsive virulence system were still antagonistic to growth.

Ps. aeruginosa ATCC 27853 produces a green QS-regulated fluorescent metabolite, pyoverdine, to scavenge iron when nutrients are limited or in stressed growth conditions (Becerra et al., 2003; Visca et al., 2007). PQS-regulated pyoverdine was confirmed to be a significant contributor to B. cenocepacia J2315 inhibition as this pathogen was able to grow at a significantly greater rate in pvdS, pvdE, pvdF and pvdL mutant CFS than in wt CFS (P<0.05) (Fig. 6b). As none of the pvd mutants was capable of producing an active pyoverdine siderophore it is apparent that pyoverdine has a profound effect on B. cenocepacia J2315 growth. Neither PQS nor HHQ had any direct effect on growth of B. cenocepacia J2315 (Fig. 6c). Given that PQS has been shown to operate as an iron trap, this indicates that the inhibition is not due to iron limitation.

Pyoverdine is a Ps. aeruginosa ATCC 27853 virulence factor associated with competition and inhibition of co-colonizing pathogens

The functional genomic analysis implicated pyoverdine in antagonism of B. cenocepacia J2315 growth. To confirm the antagonistic activity of pyoverdine, and to determine its spectrum of activity, all four pathogens (B. multivorans LMG 13010, B. cenocepacia J2315, Pa. pulmonicola LMG 18108 and Ps. apista LMG 16407) were grown in the presence of purified pyoverdine. Each of the four pathogens tested was maximally inhibited at 12.5 µM pyoverdine (Fig. 7).

To assess whether pyoverdine secreted by Ps. aeruginosa ATCC 27853 was comparable to inhibitory levels, it was quantified from replicate Ps. aeruginosa ATCC 27853 stationary-phase cultures. Pyoverdine was present in Ps. aeruginosa ATCC 27853 CFS at a mean concentration of 49±10.24 µM. This indicates this virulence factor was present in Ps. aeruginosa CFS at a sufficient concentration to inhibit the growth of all four pathogens.

DISCUSSION

A key factor in the pathogenesis of CF pathogens is the pro-inflammatory response that is elicited in response to infection. Both Pandoraea and Bcc in particular elicit a potent IL-6 and IL-8 response in epithelial cells (Bamford et al., 2007; Caraher et al., 2008). At the outset we wanted to examine whether co-infection with two pathogens would enhance the overall pro-inflammatory effect, exacerbating the potential for lung damage to the patient. Unexpectedly the inflammatory response was suppressed rather than enhanced by the presence of Ps. aeruginosa.

Both the agar diffusion and real-time PCR experiments demonstrated that this suppressive effect was most likely to be as a result of inhibition of growth of the other pathogens rather than a specific effect on their overall pro-inflammatory abilities. A dominance of Ps. aeruginosa ATCC 27853 over the other bacterial species examined was apparent. As summarized in Fig. 8, Ps. apista, Pa. pulmonicola and B. cenocepacia did not inhibit the growth of Ps. aeruginosa and could not grow in the presence of Ps. aeruginosa. Ps. apista was also inhibited by B. multivorans, suggesting that this Pandoraea species may not establish infection easily in the presence of either B. multivorans or Ps. aeruginosa. Surprisingly, B. cenocepacia was challenged by the presence of three of four pathogens, suggesting that this epidemic strain may thrive best in the absence of other organisms. Although Ps. aeruginosa has been shown previously to inhibit B. cenocepacia (Bragonzi et al., 2012), the ability of B. multivorans or other CF-associated pathogens to inhibit this bacterium has not been demonstrated previously. Interestingly, the only species that could compete to any degree with Ps. aeruginosa was B. multivorans, which may be a factor in Ps. aeruginosa colonizing 80% of adults with CF.
The dominance of *Ps. aeruginosa* parallels with findings in the clinic, in particular with non-CF patients who acquire Bcc or *Pandoraea* infections. Non-CF patients are typically not previously colonized with *Ps. aeruginosa* and may be more susceptible to infections with Bcc or with *Pandoraea* species, in the absence of the supressing effects of *Ps. aeruginosa*. Jones et al. (2001) concluded that when a non-CF patient is colonized by Bcc alone it can cause serious lung disease, in the absence of *Ps. aeruginosa*. Furthermore, *Pandoraea* may be a more virulent pathogen when not in co-colonization with *Ps. aeruginosa* as the former has been indicated as the cause of death in a bilateral lung transplant patient who did not have CF and was not co-colonized with *Ps. aeruginosa* (Stryjewski et al., 2003).

It is becoming more apparent that the polymicrobial nature of CF lower lung infection is an important aspect of lung disease in these patients (Sibley et al., 2011). Pathogen diversity in the CF lung correlated with a lower incidence of permanent lung damage (Cox et al., 2010). While younger patients had microbial communities that were richly diverse, older patients with significantly poorer lung function had microbial communities that had significantly lower diversity. In addition, the communities in older patients were phylogenetically related species which occurred as a result of species replacement.

*B. multivorans* was the only pathogen that showed an ability to antagonize *Ps. aeruginosa*. Growth inhibition of both of these pathogens by each other may indicate an overall reduction in bio-burden, when these pathogens are co-infecting. The bacteriostatic activity of *B. multivorans* CFS indicates that it is an inherent characteristic. This contrasts with recent work describing a screen of 268 Bcc isolates across 19 species and subgroups. No antagonistic activity towards Bcc, *S. aureus* or *Candida albicans* was observed in any *B. multivorans* isolate tested (Mahenthiralingam et al., 2011). In addition, none of the isolates were antagonistic towards *Ps. aeruginosa*. The lack of *B. multivorans* antagonistic activity may be due to the fact that the latter studies were carried out under conditions which maximized secondary metabolite production and biofilm formation. We did not focus on the biofilm form of growth in this study, as neither *Pandoraea* species examined has the ability to form biofilms (Caraher et al., 2008).

Masyahit et al. (2009) showed that *B. multivorans* displayed the greatest capacity for fungal inhibition in comparison to *B. cenocepacia* and *Ps. aeruginosa*. Although *B. cenocepacia* is considered the more virulent of the two Bcc species, these findings indicate *B. multivorans* may have a more competitive advantage in polymicrobial environments such as the CF lung. This is significant in the context of this pathogen being the most commonly isolated Bcc species in recently colonized CF patients in North America and Europe (LiPuma, 2010). *B. multivorans* and *Pa. pulmonicola* strains were not inhibited by one another, indicating that these two pathogens could coexist independently in the lung.

During infection, *Ps. aeruginosa* produces an extensive repertoire of extracellular virulence factors, including...
heat-labile and heat-stable haemolysins, exotoxins, phospholipases and proteases such as elastase, alkaline protease and staphyloytic protease (Banu et al., 2011), as well as pyocyanin (Lau et al., 2004) and phenazines (Laursen & Nielsen, 2004). Any of these virulence factors may contribute to inhibition of other pathogens during co-colonization, while the phenazines are instrumental in its ability to compete in ecological niches by suppression of the growth of competitors (Mazzola et al., 1992). Ps. aeruginosa has previously been shown to inhibit the growth of Candida during co-colonization. P. aeruginosa repressed C. albicans biofilm formation in a QS-dependent (Reen et al., 2011) and QS-independent (Holcombe et al., 2010) manner. In addition, while Ps. aeruginosa inhibited the growth of all Candida species tested, some of the yeast species were in turn inhibited by Ps. aeruginosa (Bandara et al., 2010; Treat et al., 2007). Therefore, the factors contributing to the inhibition by Ps. aeruginosa and B. multivorans are likely to be complex. In all cases, the factor(s) involved were heat stable. Studies on Ps. aeruginosa ATCC 27853 CFS indicated that at least one inhibitory metabolite is a heat-resistant peptide or protein, while the inhibitory effect of Ps. aeruginosa CFS on Pa. apista or B. cenocepacia was not significantly affected by proteolytic digestion, suggesting that non-peptide molecules are associated with inhibition of these pathogens, distinct from those affecting Pa. pulmoni-cola or B. multivorans. The significant reduction in inhibition of B. cenocepacia J2315 by PA14 mutants that were deficient in the production of active pyoverdine (pvdS, pvdE, pvdF and pvdL) demonstrates that the iron chelating siderophore pyoverdine is a major inhibitory factor for the epidemic strain B. cenocepacia J2315. The role of pyoverdine was confirmed by clear inhibition of both Bcc species and both Pandoraea species by pure pyoverdine at a concentration that was less than that produced by Ps. aeruginosa ATCC 27853. However, it is unlikely to be the only inhibitory agent towards B. multivorans or Pa. pulmoni-cola and future studies will elucidate this further.

QS regulation of the antagonistic factor is consistent with the emerging role played by QS in inter-species and inter-kingdom cell–cell communication. In particular, the Ps. aeruginosa quinolone signal PQS and its biological precursor HHQ have been shown to directly antagonize a broad spectrum of CF-associated bacterial and fungal pathogens (Reen et al., 2011, 2012); as well as targeting host-specific factors involved in infection (Kim et al., 2010a; b; Legendre et al., 2012). PQS has also been shown to control the expression of genes associated with iron scavenging and can itself act as an iron trap (Bredenbruch et al., 2006; Diggle et al., 2007). While it would be consistent with the iron-chelating activity of pyoverdine, purified PQS (and HHQ) did not antagonize the growth of B. cenocepacia J2315, indicating that it is the virulence activity of pyoverdine, and not iron scavenging per se, that underlies its inhibitory role during co-culture.

B. multivorans LMG 13010 CFS was also inhibitory to the growth of both Pa. apista LMG 16407 and B. cenocepacia J2315, and distinct inhibitory factors such as proteinase K had different effects on the inhibition. Interestingly, the CFS from Ps. aeruginosa and B. multivorans were equally capable of suppressing the more virulent species, B. cenocepacia. Future studies will address the nature of inhibition by B. multivorans.

It is not surprising that bacteria produce antagonistic factors to enhance their ability to compete with other micro-organisms in hostile environments. In particular, the genera Pseudomonas, Burkholderia and Pandoraea inhabit the natural environment including soil and rhizosphere. This has probably resulted in the evolution of multiple inhibitory systems to enable them to successfully compete in this complex and harsh environment. The overriding ability of Ps. aeruginosa to routinely secrete inhibitory factors to antagonize growth of four other late-colonizing CF pathogens must contribute to its dominance in the CF lung. Challenging pathogens may have difficulty adhering and colonizing an area which Ps. aeruginosa or, indeed, B. multivorans is already populating, mainly due to growth inhibition of the challenging pathogen(s). This may be a fundamental mechanism by which Ps. aeruginosa and B. multivorans compete with other pathogens during chronic infection. Indeed, the general nature of this response can be further investigated across a wide range of Ps. aeruginosa strains collated recently in an international reference panel (De Soyza et al., 2013).

Overall, pyoverdine clearly has a role in the inhibition of other late-colonizing pathogens by Ps. aeruginosa, with other factors likely to contribute to this antagonism. B. multivorans is also a robust pathogen in the presence of competing organisms. The interactions between CF pathogens are complex and require extensive further investigation to fully understand the virulence contributions of organisms in a polymicrobial environment. It will be important to elucidate the factor(s) involved in the inhibition of Burkholderia and Pandoraea species by Ps. aeruginosa, in addition to elucidation of the factors utilized by B. multivorans to inhibit Pa. apista, B. cenocepacia and Ps. aeruginosa. In particular, identification of any factor that inhibits Ps. aeruginosa would have a significant impact on future treatment of Ps. aeruginosa infection, as this pathogen colonizes up to 80% of adult CF patients.

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Inhibition among co-colonizing CF pathogens


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