Signal-mediated interactions between *Pseudomonas aeruginosa* and *Candida albicans*

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Pseudomonas aeruginosa causes infections in a wide variety of hosts and is the leading cause of mortality in cystic fibrosis (CF) patients. Although most clinical isolates of *P. aeruginosa* share common virulence determinants, it is known that strains evolve and change phenotypically during CF lung infections. These changes can include alterations in the levels of *N*-acyl homoserine lactones (HSLs), which are secreted signal molecules. In the CF lung, fungi, especially *Candida albicans* and *Aspergillus fumigatus*, may coexist with *P. aeruginosa* but the implications for disease are not known. Recent studies have established that signalling can occur between *P. aeruginosa* and *C. albicans*, with the bacterial molecule 3-oxo-C12HSL affecting *Candida* morphology, and the fungal metabolite farnesol reducing levels of the *Pseudomonas* quinolone signal and pyocyanin in *Pseudomonas*. Whether these interactions are common and typical in clinical strains of *P. aeruginosa* was addressed using CF isolates that produced varied levels of HSLs. It was found that, whereas some clinical *P. aeruginosa* strains affected *C. albicans* morphology, others did not. This correlated closely with the amounts of 3-oxo-C12HSL produced by the isolates. Furthermore, it was established that signalling is bidirectional and that the *C. albicans* molecule farnesol inhibits swarming motility in *P. aeruginosa* CF strains. This work demonstrates that clinical isolates of these opportunistic pathogens can interact in strain-specific ways via secreted signals and illustrates the importance of studying these interactions to fully understand the microbial contribution to disease in polymicrobial infections.

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INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium that is found in a myriad of environments. As well as being an opportunistic human pathogen, it is also capable of causing disease in eukaryotes such as plants, mammals, insects and nematodes (Diggle *et al.*, 2003; Giamarellou, 2000; Rahme *et al.*, 1995, 2000; Tan & Ausubel, 2000; Whitchurch *et al.*, 1995). It is also the principal cause of mortality in cystic fibrosis (CF) populations (Diggle *et al.*, 2003; Drenkard & Ausubel, 2002; Govan & Deretic, 1996; Smith *et al.*, 1996). *P. aeruginosa* communicates using chemical signals to sense cell density and alter gene expression and virulence factors in a process known as quorum sensing (QS) (Heurlier *et al.*, 2006; Schuster & Greenberg, 2006; Shiner *et al.*, 2005; Smith & Iglewski, 2003; Williams, 2002). The major cell-cell signalling molecules in *P. aeruginosa* are the *N*-acyl homoserine lactones (HSLs) 3-oxo-C12HSL and C4HSL, and the quinolone 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal; PQS). QS regulates many

virulence features including the formation of biofilms and production of factors such as hydrogen cyanide, pyocyanin, proteases and rhamnolipids (Calfee *et al.*, 2005; Cugini *et al.*, 2007; Diggle *et al.*, 2003, 2006, 2007; McGrath *et al.*, 2004; Wade *et al.*, 2005).

Together with *P. aeruginosa*, the dimorphic yeast *Candida albicans* is one of the most commonly isolated micro-organisms from CF patient sputum (Bakare *et al.*, 2003; Bauernfeind *et al.*, 1987; Hughes & Kim, 1973). Although *C. albicans* is a common non-pathogenic commensal of the skin and mucosal flora of healthy individuals, in compromised individuals it is able to initiate invasive growth that may result in serious disease and death (Hube, 2006; Naglik *et al.*, 2004; Pfaller & Diekema, 2007). *C. albicans* can exist as yeast or filamentous forms and, in response to certain cues, including temperature and host factors, it switches from yeast to hyphal (filamentous) growth. Interestingly, both morphological forms are important for virulence and thus the ability to morphologically transform is an important virulence trait (Calderone & Fonzi, 2001; Gow, 1997; Gow *et al.*, 2002; Liu, 2002; Mitchell, 1998; Whiteway & Oberholzer, 2004). Like *P. aeruginosa*, *C. albicans* employs secreted signals to regulate gene expression and virulence. Actively growing yeast cells

Abbreviations: CF, cystic fibrosis; HSL, *N*-acyl homoserine lactone; PQS, *Pseudomonas* quinolone signal; PVC, polyvinyl chloride; QS, quorum sensing.

secrete farnesol, a 12-carbon sesquiterpene, which acts as a virulence factor and a repressor of the switch from yeast to hyphal growth in *C. albicans* (Enjalbert & Whiteway, 2005; Hogan, 2006a, b; Hornby *et al.*, 2001; Navarathna *et al.*, 2007a, b; Nickerson *et al.*, 2006).

P. aeruginosa and *C. albicans* are frequently co-isolated from CF patient sputum and a variety of *in vitro* and *in vivo* studies have established that these two species can interact. *In vitro* and *in vivo*, there is evidence to suggest that *P. aeruginosa* may inhibit *C. albicans* growth in the host (Bauernfeind *et al.*, 1987; Burns *et al.*, 1999; Gupta *et al.*, 2005; Kaleli *et al.*, 2007; Kerr, 1994a, b). Furthermore, several *in vitro* studies using well-characterized laboratory strains have identified more complex interactions between these human pathogens. Certain strains of *P. aeruginosa* are cytotoxic to the filamentous form of *C. albicans* but are unable to attach to or kill *C. albicans* yeast cells (Hogan & Kolter, 2002). In addition, it has been reported that 3-oxo-C12HSL and structurally related molecules can inhibit and even reverse the switch from yeast to hyphal growth in *C. albicans* (Hogan *et al.*, 2004). That study also showed that a mutant defective in producing 3-oxo-C12HSL lost the ability to influence *C. albicans* morphology (Hogan *et al.*, 2004). Interestingly, recent research has shown that these signalling interactions between *P. aeruginosa* and *C. albicans* may in fact be bidirectional. It was found that addition of farnesol leads to decreased PQS production in *P. aeruginosa* and that this decrease leads to a reduction in the PQS-regulated virulence factor pyocyanin (Cugini *et al.*, 2007). Those studies open up an intriguing scenario whereby secreted factors from *P. aeruginosa* and *C. albicans* have mutually antagonistic effects on important virulence traits.

It is known, however, that strains of *P. aeruginosa* isolated from CF patients show significant phenotypic and genotypic variation (Finnan *et al.*, 2004; Martin *et al.*, 1995; Oliver *et al.*, 2000; Wolfgang *et al.*, 2003). These variations in

P. aeruginosa CF strains are likely to alter how *P. aeruginosa* interacts with the host and other organisms including *C. albicans*. In this study, we took advantage of a set of seven characterized *P. aeruginosa* CF clinical isolates to investigate possible interaction between bacterial isolates and the yeast *C. albicans*. We addressed specifically the question of whether secreted factors from clinical isolates would affect *C. albicans* growth or morphology. Using cell-free supernatants we were able to focus on the role of HSL molecules in mediating these effects. In addition, we examined the effect of the yeast signal, farnesol, on these bacterial isolates.

METHODS

Strains, culture conditions and compounds. All strains used in this study (apart from the HSL⁻ mutant) were clinical isolates and are listed in Table 1. All strains were characterized genetically previously and were shown to be genotypically distinct (Adams *et al.*, 1998; Finnan *et al.*, 2004). *P. aeruginosa* PAO1 and CF strains were routinely cultured at 37 °C in Luria-Bertani (LB) broth or on LB plates. Antibiotics for selection of the *P. aeruginosa* mutant strain were used at the following concentrations: 100 µg gentamicin ml⁻¹ and 200 µg tetracycline ml⁻¹. *C. albicans* was grown in non-filament-inducing medium (YNB) and filament-inducing medium (YNBNP) as described by Hogan *et al.* (2004). Farnesol (*trans, trans*-farnesol) was purchased from Fluka and diluted in methanol as required. Purified C12HSL was purchased from Sigma and diluted in acetonitrile as required.

Swarming motility assay. Swarming was analysed on agar plates containing 0.5 % Eiken agar and 8 g l⁻¹ Eiken nutrient broth (Eiken Chemical Co.) supplemented with 5 g glucose l⁻¹. Bacteria from an overnight culture were inoculated onto the centre of plates using a sterile pipette tip and the plates were incubated overnight at 37 °C. A positive result was recorded as growth over the agar surface away from the inoculation point. To determine the effect of farnesol, swarming media were supplemented with 100 µM farnesol.

Growth and adhesion assays. *P. aeruginosa* growth curves were performed in LB medium at 37 °C using a Bioscreen C microtiterplate reader. To determine the effect of farnesol, LB media were

Table 1. Strains used in this study

Strain	Country of isolation	Year of isolation	Reference
<i>Pseudomonas aeruginosa</i>			
Clinical isolates			
CF139	Ireland	1994	Adams <i>et al.</i> (1998)
CF198	Ireland	1994	Adams <i>et al.</i> (1998)
CF95	Ireland	1993	Adams <i>et al.</i> (1998)
CF93	Ireland	1993	Adams <i>et al.</i> (1998)
CF144	Ireland	1994	Adams <i>et al.</i> (1998)
CF177	Ireland	1994	Adams <i>et al.</i> (1998)
CF242	Ireland	1994	Adams <i>et al.</i> (1998)
Controls			
PAO1 (WT)			Holloway & Morgan (1986)
PAO1Δ <i>lasRI</i> ::Gm Δ <i>rhlRI</i> ::Tc			Beatson <i>et al.</i> (2002)
<i>Candida albicans</i>			
SC5314			Gillum <i>et al.</i> (1984)

supplemented with 30, 100 or 300 μM farnesol. Adhesion on polyvinyl chloride (PVC) was measured after 12 h static incubation using a crystal violet assay essentially as described previously (Burrowes *et al.*, 2006). In experiments to determine the effects of farnesol, 300 μM farnesol was added at each starting inoculation.

Detection of HSLs by TLC. HSL extraction with dichloromethane and detection by TLC was carried out as described previously (Baysse *et al.*, 2005) and by Shaw *et al.* (1997) using the reporter strains *Chromobacterium violaceum* CV026, capable of sensing short-chain HSL molecules (C4HSL and C6HSL) through the production of a violet pigment (Ravn *et al.*, 2001), and *Agrobacterium tumefaciens* NTI, which carries a *lacZ* transcriptional fusion under the control of an HSL-inducible promoter for long-chain HSLs (3-oxo-C12HSL) (Shaw *et al.*, 1997).

Supernatant preparation and morphology studies. *P. aeruginosa* strains were grown overnight for 16 h in LB liquid media until an approximate OD_{600} 1.2 was reached. Cells were harvested by centrifugation and the supernatant was passed through a Nalgene vacuum filter unit with a 0.2 μM polyethersulfone membrane (Sigma). The sterile supernatant was immediately lyophilized and resuspended at a concentration of $\times 20$ in sterile purified water. The sterile, concentrated supernatant was used immediately or stored for short periods of time at -80°C before use. Analysis of *C. albicans* morphology in liquid medium was carried out as described by Hogan *et al.* (2004) with minor adjustments. Briefly, *C. albicans* was grown as yeast cultures overnight at 30°C in YNB medium. Yeast cells were diluted in YNBNP filament-inducing medium to OD_{600} 0.05 and incubated at 37°C . Upon inoculation from YNB to YNBNP, sterile concentrated supernatant from the *P. aeruginosa* strains was added at a final concentration of $\times 2$ from a $\times 20$ stock. After 6 h growth, cells were examined microscopically at $\times 400$ magnification and the images were digitally captured.

RESULTS AND DISCUSSION

Production of HSLs is strain dependent

In a previous study, we surveyed phenotypic characteristics including motility and production of specific virulence

factors in a small collection of *P. aeruginosa* CF isolates (Finnan *et al.*, 2004). No defining virulence traits were identified but, as HSLs have since been shown to exert effects on *C. albicans* (Hogan *et al.*, 2004), we tested each of the strains for their ability to produce both long- and short-chain HSLs (Fig. 1). HSL production is known to be growth-phase dependent; therefore, we tested each strain at three stages of growth in liquid medium using a well-established bioassay. Although not strictly quantitative, this bioassay facilitates comparisons of relative HSL levels among strains. All strains produced the long-chain molecule 3-oxo-C12HSL (Fig. 1, top panel), with clear variation between strains observed. Only three strains, PAO1, CF144 and CF242, produced significant amounts of the short-chain molecules C4HSL and C6HSL, although trace levels could be detected in all strains. Another minor spot, perhaps C5HSL or 3-oxo-C4HSL, was also visible in these strains (Fig. 1, bottom panel, circled spot). HSL production was growth-phase dependent in all cases, with the highest levels being typically associated with stationary phase cultures. It is clear from this analysis that significant variation in HSL production exists within this small group of clinical isolates, with two strains, CF144 and CF242, resembling the laboratory strain PAO1 and categorized as 'high producers' of long-chain HSLs, short-chain HSLs, or both. These data are consistent with how *P. aeruginosa* is believed to behave in the CF lung. In this condition, the same lineage of *P. aeruginosa* can persist in the lung for years, even decades, undergoing many evolutionary changes due to selective and adaptive pressures within the host (Nguyen & Singh, 2006; Smith *et al.*, 2006). As an infection progresses from the acute to chronic phase, virulence factors, including production of HSLs, appear to be counter-selected by the host (Nguyen & Singh, 2006; Smith *et al.*, 2006). Interestingly, our set of CF isolates showed a large degree of variation in their production of

Fig. 1. Production of HSLs is strain dependent. Strains were grown to mid-exponential phase (A_{600} 0.6), late-exponential phase (A_{600} 1.0) and stationary phase (A_{600} 1.6) and HSLs were extracted twice and detected using TLC as described in Methods. For each image, three lanes represent three growth phases (A_{600} 0.6, 1.0, 1.6) in that order, as shown for strain CF139. The top panel shows long-chain HSLs and the bottom panel short-chain HSLs. The arrow and circle indicate an extra uncharacterized HSL.

both long- and short-chain HSLs (Fig. 1) and this is consistent with adaptation of these strains to the host.

Cell-free supernatant from *P. aeruginosa* can influence *C. albicans* morphology in an HSL-dependent manner

Pure HSLs and structurally similar molecules can prevent the yeast to hyphal switch in *C. albicans* but it was not known whether this effect could also occur with complex bacterial secretions from clinical strains. The variation in levels of HSLs is also likely to impact on how different strains of *P. aeruginosa* interact with both the host and with other micro-organisms such as *C. albicans* that may be present in the lung. In support of this, we found that supernatants from some strains, when added to actively growing cultures of *C. albicans*, inhibited the yeast to hyphal switch in an HSL-dependent manner (Fig. 2). Supernatants from the high HSL producer, CF144, and the low HSL producer, CF177, were added to *Candida* cultures as they were induced to switch from the yeast to the hyphal form. We also included supernatant from the characterized strain *P. aeruginosa* PAO1, which produces high levels of HSLs (Fig. 1). We found that strains that produced high amounts of HSLs (PAO1 and CF144) inhibited the yeast to hyphal switch (Fig. 2c, d), whereas the low/non-producing strain (CF177) was unable to influence the transition (Fig. 2a, b). These data suggest that this is an

Fig. 2. Cell-free supernatant from *P. aeruginosa* influences *C. albicans* morphology. Supernatants from *P. aeruginosa* strains were sterilized by filtration and concentrated $\times 20$ by lyophilization and resuspension in purified water. Sterile, concentrated supernatants, at a final concentration of $\times 2$, were then added to *C. albicans* yeast cells that were induced to grow in filament form. Supernatants from strains PAO1 and CF144 inhibited the yeast to hyphal switch (c, d), whereas supernatant from strain CF177 (b) did not. (a) *C. albicans* hyphal control, (b) *C. albicans*+CF177 supernatant, (c) *C. albicans*+PAO1 supernatant and (d) *C. albicans*+CF144 supernatant. Magnification $\times 400$.

HSL-dependent phenomenon. To rule out the possibility that other molecules in the supernatant may prevent CF177 from mediating the effect, we performed an add-back experiment in which pure C12HSL was added to CF177 supernatant. This supplemented supernatant now inhibited the yeast to hyphal transition (data not shown). Further support for the role for long-chain HSLs came from experiments that showed that a HSL⁻ mutant of PAO1, PAO1 Δ lasRI::Gm Δ rhIRI::Tc, also failed to inhibit the morphological switch (data not shown). Similar effects were reported previously with pure HSLs but the mechanism by which this effect is mediated is not known (Hogan & Kolter, 2002; Hogan *et al.*, 2004). The morphological switch in *C. albicans* involves the activity of two distinct signal transduction systems, a RAS-cAMP-protein kinase A pathway and a mitogen-activated protein kinase pathway, and it is believed that farnesol modulates one or both of these pathways in some way (Biswas *et al.*, 2007; Enjalbert & Whiteway, 2005; Hornby *et al.*, 2001; Liu, 2001; Sato *et al.*, 2004; Whiteway, 2000). As farnesol is also a 12-carbon molecule, it is tempting to speculate that 3-oxo-C12HSL recognizes the same target as the *C. albicans* auto-signal, but this remains to be determined.

Farnesol reduces swarming motility while having no effect on adhesion or growth in *P. aeruginosa*

We were also interested in the effects of the *Candida* secreted signal, the 12-carbon molecule farnesol, on the clinical strains of *P. aeruginosa*. We therefore examined the effects that addition of farnesol had on three *P. aeruginosa* traits that have been reported as being important for virulence: swarming motility, growth and biofilm formation. As biofilm formation is influenced by HSLs and PQS, and the addition of farnesol reduces PQS levels, we investigated the ability of each strain to adhere to polyvinyl chloride (PVC) plates in the presence and absence of farnesol. Despite the phenotypic differences in the strains (growth rates, HSL production and colony morphology), no significant difference in the ability of the bacteria to adhere to PVC was detected among the strains and addition of 300 μ M farnesol had no effect on this adherence (data not shown). We also observed no effect on the growth of *P. aeruginosa* in the presence of up to 300 μ M farnesol (data not shown). Differences between strains and effects of farnesol were observed, however, when swarming motility was assessed (Fig. 3). The high HSL-producing strains (PAO1, CF144 and CF242) were strong swimmers, the low producer CF95 a moderate swimmer, and the low producers CF139, CF198, CF93 and CF177 were unable to swarm (Fig. 3a, arrows mark the edge of the swarming colony). The ability of strains PAO1, CF144, CF242 and CF95 to swarm was almost completely abolished by the addition of 100 μ M farnesol. The effect of farnesol may be mediated via rhamnolipids as rhamnolipids are known to be required for swarming and are part-regulated by PQS, which is known to be downregulated by farnesol. The reduction in PQS, pyocyanin production and

Fig. 3. Farnesol inhibits swarming in *P. aeruginosa*. (a) Swarming motility of *P. aeruginosa* on 0.5% Eiken agar plates. (b) Media were supplemented with 100 µM farnesol and swarming was almost abolished. Swarm edges are indicated by arrows.

swarming motility may also have implications for the interaction between *P. aeruginosa* and the host.

Importance of interspecies signalling in disease

From the data above and in the literature, it is clear that the *in vivo* interaction between *P. aeruginosa* and *C. albicans* is likely to be complex and that it is possible to predict some consequences of these interactions. During the acute phase of infection when a strain of *P. aeruginosa* is secreting high levels of HSLs, any *Candida* cells in that niche will remain in the yeast form. In contrast, during a chronic infection when HSL concentrations are likely to drop, *C. albicans* may be able to trigger a switch to filamentous growth, possibly leading to formation of a fungal biofilm with the potential for invasive growth. Relatively little is known about the importance of fungal biofilms in disease, but this is an emerging topic of research. The possible cytotoxic effects of some strains of *P. aeruginosa* are an additional consideration as it has been shown that some strains of *P. aeruginosa* destroy *Candida* hyphae (but not yeast cells) (Hogan & Kolter, 2002). In contrast, fungal signals can affect *Pseudomonas* traits important for gene regulation and motility, and are likely to impact on the ultrastructure of the bacterial biofilm.

In conclusion, it is emerging that the structure of the microbial community in the lung may have significant consequences for the nature of disease in CF patients. The key challenges now are to determine mechanistically precise details of how *Pseudomonas* and *Candida* interact, and to devise methods to study this interaction *in vivo*. The ultimate aim will be to use this knowledge to manipulate, modify, or disrupt microbial ecology in the CF lung to the benefit of the patient. These data also have implications for interactions between bacteria and fungi that may occur in other clinical settings, for example the burn wound.

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