Short Communication

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Antibacterial activities of stationary phase culture filtrates of *Aspergillus fumigates*

Abstract

Our previous studies performed for the development of polymicrobial biofilm showed that simultaneous coculturing of *Aspergillus fumigatus* conidia or sporelings with *Pseudomonas aeruginosa* resulted in the killing of *A. fumigatus* cells whereas hyphae older than 12h were recalcitrant to the bacterial fungicidal activity. Since *A. fumigatus* produces an array of antimicrobial molecules, we examined the antibacterial activity of *A. fumigatus* culture filtrates on bacterial growth to examine the possible role of such small molecules minimizing the fungicidal activity of *P. aeruginosa* by spectrophotometric and colony forming unit assays. Culture filtrates collected from 48h stationary phase cultures inhibited the growth of *P. aeruginosa* and *S. aureus*, but not that of *Candida albicans*. Colony forming unit assay of culture filtrate-treated *P. aeruginosa* cells showed that the effect was bacteriostatic. Culture filtrate incubated in a boiling water bath for 15min retained its antibacterial activity compared to that of the unboiled culture filtrate. These results show that stationary phase liquid cultures of *A. fumigatus* produce a heat stable bacteriostatic compound(s) in liquid culture that is released into the growth medium inhibits the growth of *P. aeruginosa*.

Keywords: Culture filtrate, Stationary phase, Antibacterial activity, *Aspergillus fumigates, Pseudomonas aeruginosa*

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Introduction

Simultaneous static coculturing of *A. fumigatus* conidia or sporelings with *P. aeruginosa* cells *in vitro* results in the initial growth inhibition and eventual death of *A. fumigatus* cells,[1] perhaps elicited by *P. aeruginosa* produced small diffusible extracellular molecules released into the culture medium.[2,3] In contrast, *A. fumigatus* hyphae grown for 12h or longer from conidia at 35°C in a rich fungal growth medium such as Sabouraud's dextrose (SD) broth are relatively unaffected by the fungicidal effect of *P. aeruginosa* culture filtrate.[1] The lack of effect of *P. aeruginosa* culture filtrate on *A. fumigatus* hyphae could be due to either innate resistance of hyphae to the fungicidal factor(s) and/ or due to the production of an antibacterial factor(s) by the growing *A. fumigatus* hyphae that either inhibits bacterial growth or blocks the fungicidal activity. Since *A. fumigatus* is known to produce an array of small molecules[4] possessing antimicrobial properties to safeguard its survival, the latter scenario is a likely event in cocultures of *P. aeruginosa* and *A. fumigatus*. We therefore investigated the antibacterial activity of *A. fumigatus* culture filtrates obtained from 24h and 48h cultures of *P. aeruginosa*.

Materials and methods

A. fumigatus 53470 (a clinical isolate obtained from the Microbiology Laboratory of Henry Ford Hospital, Detroit, MI, USA) was used in this study. Cultures were grown for 24h and 48h in SD broth at 35°C in 6-well Costar cell culture plates. The culture filtrate (CF) was collected and filter-sterilized (0.22µm Millipore filters). For growth inhibition studies, one ml aliquots of the CF supplemented with fresh SD broth was inoculated with $1 x 10^7$ cells/ ml *P. aeruginosa* 56402 (a clinical isolate obtained from the Microbiology Laboratory of Henry Ford Hospital in Detroit) in 6-ml polystyrene culture tubes (6 replications) and incubated on a gyratory shaker (170 rpm) at 35°C for 24h. The growth inhibition of *P. aeruginosa* was determined spectrophotometrically by determining the optical density at 490nm and by colony forming unit (CFU) assay.

The heat stability of the antibacterial factor(s) of the CF was examined after incubating 1ml aliquots in a boiling water bath for 15min followed by the growth inhibition assays. Heat untreated CF and SD broth (1ml aliquots) were used as controls.

The bactericidal effect of the CF was examined by treating $1x10^7$ cells with 1ml CF at 35 °C for 24h. The viability of the treated cells was examined by plating 0.01ml aliquots of the diluted culture on SD agar and determining the CFUs after 24h growth.

Results and discussion

As shown in Figure 1, both spectrophotometric and CFU assays provided similar results. *A. fumigatus* CF collected from the 48 h stationary phase cultures almost completely inhibited the growth of *P. aeruginosa* (control: $3.867x10^{11}±2.161x10^{11}$ CFU/ml; CF: $9.333x10^6±1.146x10^7$ CFU/ml; p=0.0071) but that obtained from 24h old culture showed no significant growth inhibition (control: $7.392x10^{10}±1.312x10^{11}$CFU/ml; CF: $5.851x10^{10}±2.389x10^{10}$ CFU/ml; p=0.540). A CFU assay of *P. aeruginosa* cells treated with CF showed that the *A. fumigatus* produced factor was bacteriostatic and no significant reduction of CFUs was obtained for the CF-treated group compared to the initial inoculum whereas the control group showed a CFU increase of 3.5 to 4 logs after 24h incubation at 35°C (Figure 1 panel (c): SD vs. 48h CF and panel (d): SD vs. CF). The CF boiled for 15 min in a water bath retained its antibacterial activity ($7.450x10^6±3.882x10^6$ CFU/ml) compared to the heat untreated culture filtrate ($7.717x10^6±4.490x10^6$ CFU/ml) suggesting that the *A. fumigatus* produced inhibitory factor is a heat stable molecule(s) (p=0.774). In addition to *P. aeruginosa*, the stationary phase culture filtrate had similar inhibitory effect on the growth of *S. aureus* whereas the growth of *C. albicans* was unaffected.

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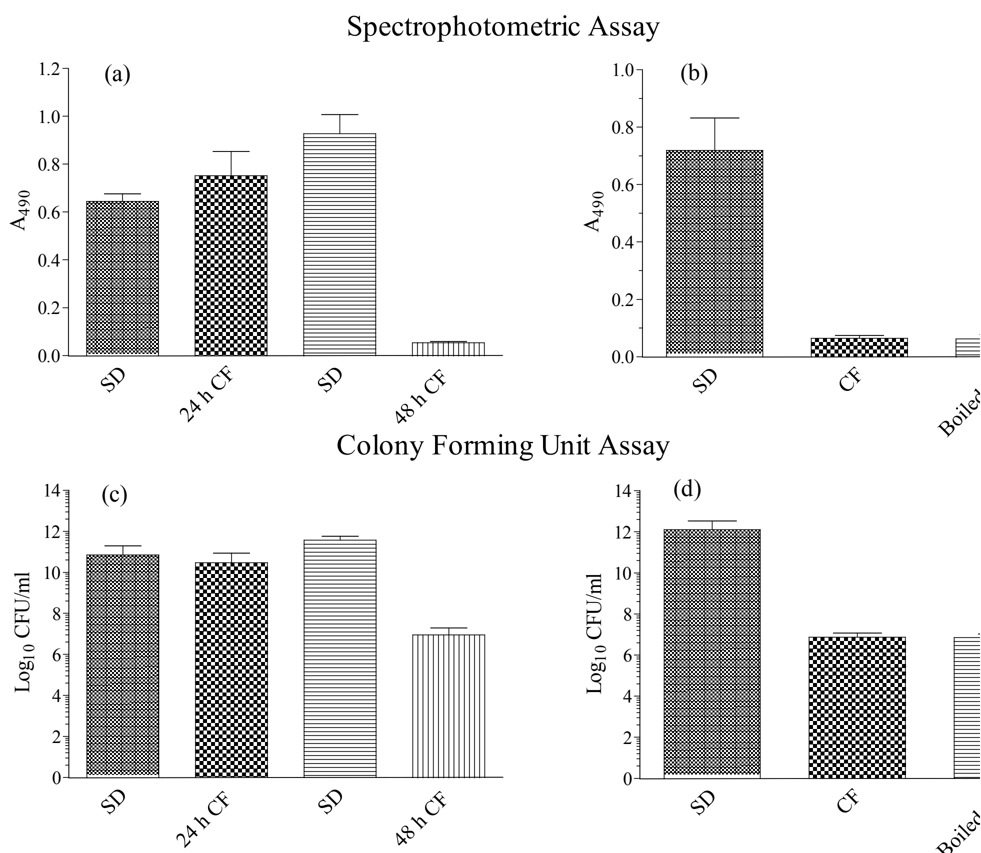

Figure 1 Effects of *A. fumigatus* culture filtrates obtained from 24 h and 48 h cultures on the growth of *P. aeruginosa*. We evaluated the effects of culture filtrate on the viability of *P. aeruginosa* cells by spectrophotometric (Panels (a) and (b)) and CFU assays (Panels (c) and (d)), and both methods provided similar results. *P. aeruginosa* cells (1×10^{7} cells/ ml) were incubated with 24h or 48h *A. fumigatus* culture filtrates for 24h at 35°C and the bacterial growth was determined by optical density at 490 nm and CFU assays. Panels (b) and (d) show the effect of boiling on the antibacterial activity of culture filtrate. Legends: SD, Sabouraud's dextrose broth (growth control); CF, culture filtrate. The experiment was repeated two times and the vertical bar on each histogram represents the standard deviation of two independent experiments. The data were analyzed by Student's *t*-test using GraphPad Prism Version 5.0 for Windows (GraphPad Software, Inc., La Jolla, CA, USA) and a *p* value ≤0.05 was considered significant.

Mycotoxins, including those produced by *A. fumigatus*, are low molecular weight organic molecules produced by the fungus to protect itself from predators and competitors in its ecological niche. Among the many toxins produced by *A. fumigatus,* gliotoxin[4] is the most potent and well studied, and it possesses a variety of cytotoxic effects, including triggering apoptosis. We postulated that the CF mediated inhibition of *P. aeruginosa* is due to gliotoxin or an *A. fumigatus* produced antibacterial factor(s) perhaps similar to that produced by *C. albicans* against *P. aeruginosa* in mixed cultures.[5] Bruns et al.,[6] and Sekonyela et al.,[7] have recently investigated gene expression profiles of young (24h) and mature (48h) *A. fumigatus* biofilms by Transcriptomics and Proteomics. Not surprisingly, in matured biofilms and liquid shake cultures overall metabolic activity was significantly reduced but energy is channeled to the enhanced expression of genes coding for the biosynthesis of secondary metabolites. In particular, proteins of the gliotoxin secondary metabolite gene cluster were induced in mature biofilm and shake cultures as determined by real-time PCR and HPLC analysis. Our experiments showed that *A. fumigatus* stationary phase CF inhibited *P. aeruginosa* growth due to the production a heat stable bacteriostatic compound(s) secreted into the culture medium, and this bacterial growth inhibitory factor(s) may be at least in part responsible for the poor fungicidal effect of *P. aeruginosa* CF on *A. fumigatus* hyphae.

Conclusion

Mature biofilms and shake cultures of *A. fumigatus* is capable of producing an antibacterial factor perhaps similar to gliotoxin to inhibit *P. aeruginosa*, and this factor may be at least partly responsible for rending protection to *A. fumigatus* from the fungicidal activity of *P.* aeruginosa in polymicrobial biofilm.

Acknowledgments

None.

Conflicts of interest

Authors declare that there is no conflict of interest.

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