Pseudomonas aeruginosa Inhibits the Growth of *Cryptococcus* Species

Antonella Rella · Mo Wei Yang · Jordon Gruber · Maria Teresa Montagna · Chiara Luberto · Yong-Mei Zhang · Maurizio Del Poeta

Abstract *Pseudomonas aeruginosa* is a ubiquitous and opportunistic bacterium that inhibits the growth of different microorganisms, including Gram-positive bacteria and fungi such as *Candida* spp. and *Aspergillus fumigatus*. In this study, we investigated the interaction between *P. aeruginosa* and *Cryptococcus* spp. We found that *P. aeruginosa* PA14 and, to a lesser extent, PAO1 significantly inhibited the growth of *Cryptococcus* spp. The inhibition of growth was observed on solid medium by the visualization of a zone of inhibition of yeast growth and in liquid culture by viable cell counting. Interestingly, such inhibition was only observed when *P. aeruginosa* and *Cryptococcus* were co-cultured. Minimal inhibition was observed when cell–cell contact was prevented using a separation membrane, suggesting that cell contact is required for inhibition. Using mutant strains of *Pseudomonas* quinoline signaling, we showed that *P. aeruginosa* inhibited the growth of *Cryptococcus* spp. by producing antifungal molecules pyocyanin, a redox-active phenazine, and 2-heptyl-3,4-dihydroxyquinoline (PQS), an extracellular quorum-sensing signal. Because both *P. aeruginosa* and *Cryptococcus neoformans* are commonly found in lung infections of immunocompromised patients, this study may have important implication for the interaction of these microbes in both an ecological and a clinical point of view.

Keywords *Cryptococcus* spp. · *Pseudomonas aeruginosa* · Quorum sensing · 2-heptyl-3,4-dihydroxyquinoline · Pyocyanin

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Introduction

Cryptococcus neoformans is an important, widely distributed, fungal human pathogen that causes a life-threatening meningoencephalitis in immunocompromised

hosts. In recent years, it has been shown to cause serious pulmonary infection also in individuals with competent immune system [1, 2]. The cryptococcal infection develops after exposure to fungal basidiospores or encapsulated yeast found ubiquitously in the environment, such as pigeon guano and eucalyptus trees. The port of entry of *Cryptococcus* species is the lung. When spores or yeasts are inhaled, the infection can be restricted to the lung or can disseminate to other tissues. Following inhalation, alveolar macrophages are the first line of defense against *Cryptococcus,* contributing to the clearance or containment of the fungal pathogen in the granuloma [3]. This occurs normally in immunocompetent subjects. In immunocompromised subjects, the yeast cells can disseminate from the lung to the brain within host cells (intracellularly) or in the bloodstream (extracellularly), leading to the development of life-threatening disease [4–6]. Thus, the lung is an important organ for the containment of cryptococcal infection.

The respiratory tract is also a common organ infected by *Pseudomonas aeruginosa* (*Pa*), a ubiquitous Gram-negative bacterium commonly found in soil and water, following aspiration of the organism from the upper respiratory tract, especially in patients on mechanical ventilation. Alternatively, pneumonia may occur as a result of bacteremia spread to the lungs. This is observed commonly in patients following chemotherapy-induced neutropenia or in patients with AIDS [7, 8]. In *Pa* and in other bacteria, modulation and coordination of gene expression are influenced by population density via the production of small molecules that impact the production of virulence factors. This mechanism based on cell–cell communication is known as quorum sensing (QS).

QS molecules are capable of not only controlling gene expression in *Pa* but also affecting host immune response and the growth of other microorganisms [9]. Several groups have demonstrated the capacity of *Pa* to inhibit yeast growth in vitro, namely *Candida* spp., *Aspergillus fumigatus* and *Saccharomyces cerevisiae* [10, 11]. Interest in the inhibitory effect of several strains of *Pa* against *C. neoformans* in vitro started in 1954, when Fisher et al. reported that strains of *Pa* produced a substance that inhibits the growth of *C. neoformans* in vitro, but the inhibitor has not been successfully separated [12]. Twenty years later, Teoh-Chan et al. reported an initial attempt to isolate and characterize the inhibitory substances involved in

1974 [13]. However, the literature about the inhibitory effect of *Pa* on *C. neoformans* is equivocal [12, 13]. In this study, we revealed the mechanism used by *Pa* to inhibit the growth of *Cryptococcus* spp. through the production of QS molecules. We found that the *Pa* PQS and, more profoundly, pyocyanin do significantly inhibit cryptococcal growth. Interestingly, this inhibition was mainly detected when *C. neoformans* was co-cultured with *Pa*, suggesting that physical contact triggers the production of antifungal molecules in *Pa*.

Materials and Methods

Experimental Strains

Cryptococcus neoformans var. *grubii* serotype A strain H99, *C. neoformans* var. *neoformans* serotype D strain JEC21, *C. gattii* serotype B (strain MMRL 1336) and serotype C (strain MMRL 1343) [the latter two strains were a kind gift from Wiley Schell, Duke University Medical Center, Durham, NC, USA), and *P. aeruginosa* wild-type strains PAO1 and PA14 were used in this study. PAO1 and PA14 are the two commonly used laboratory strains of *Pa*. Numerous reports showed that the clinical isolate PA14 is significantly more virulent than PAO1 in a wide range of hosts [14, 15]. *P. aeruginosa* PAO1 mutant strains *pqsAB*, *pqsB* and *pqsE* and *E. coli* strain DH5α were used in this study. *Cryptococcus* species were grown at 30°C in YPD broth (2% peptone, 1% yeast extract, 2% dextrose, BD) with shaking at 250 rpm. PAO1 and PA14 were cultured in two different media LB and YPD. *Pa* showed similar profile of growth in both media. PAO1 mutant strains were grown in LB (Luria–Bertani) at 37°C with shaking at 250 rpm.

YNB Plate Assay

C. neoformans var. *grubii* serotype A, *C. neoformans* var. *neoformans* serotype D and *C. gattii* serotype B and serotype C were grown overnight, and the cells were washed twice with sterile water and resuspended in YNB broth (Yeast Nitrogen Broth, Sigma-Aldrich). 300 μl of 1×10^8 cells/ml were spread on YNB agar plates using a glass spreader. Then, sterile filter paper disks were placed on the plates. Both PAO1 or PA14 were grown overnight at 37°C in LB media, and 10-μl

drops of an 8×10^9 CFU/ml *Pa* cultures were spotted on the filter disks, following by incubation at 30°C for up to 72 h. The same procedure was used for PAO1 mutant strains: *pqsAB, pqsB* and *pqsE*.

Survival Assay of *C. neoformans* H99 Co-cultured with *Pa* Strains

C. neoformans and *Pa* strains PAO1 or PA14 were grown at 30°C in YPD broth overnight. Different *C. neoformans* dilutions were performed (10^7, 10^6, 10^5, 10^4 and 10^3 cells/ml) and inoculated with 3×10^9 CFU/ml *Pa* strains onto 96-well plate. The plate was incubated at 30°C for 24 h. *C. neoformans* and *Pa* cells viability was determined by CFU counting on YNB and LB plates, respectively. YNB and LB plates were used to count *C. neoformans* and *Pa* cells in mixed culture conditions, respectively. On YNB plates, *C. neoformans* was growing faster than *Pa,* allowing us to count *C. neoformans*, whereas LB plates do not support the growth of *C. neoformans* and were chosen to count *Pa.*

C. neoformans H99-PAO1 or PA14 Interaction

Both PAO1 and PA14 were grown in 100 ml of YPD at 30°C for 24 h. *C. neoformans* was grown in 50 ml of YPD at 30°C for 24 h. *Pa* and *C. neoformans* cultures were washed twice with sterile water and harvested at $8,000 \times g$ for 20 min and at 3,000 rpm for 10 min, respectively. *Pa* cultures were split into two flasks: *Pa* was cultured alone in one flask, and *Pa* was co-cultured with *C. neoformans* in the other flask, for 48 h at 30°C. *Pa* alone and the *Pa–C. neoformans* co-culture were pelleted by centrifugation at $8,000 \times g$ for 20 min. The supernatants were filtered with vacuum-driven disposable filtration system with 0.22-µm pore size (Millipore), frozen and lyophilized using a bench top freeze dryer. 500 mg of dried co-culture supernatants (1.26×10^{15} CFU *Pa* strains and 1×10^9 CFU *C. neoformans*) and *Pa* supernatants (1×10^{15} CFU *Pa* strains) was resuspended in 1 ml of YPD broth. *Cryptococcus neoformans* was grown overnight and 300 µL of 1×10^8 cells/ml culture was spread on YPD agar plates using a glass spreader. Different amounts of dried co-culture supernatants (e.g., 12.5, 25, 50, 75 and 100 mg) and dried *Pa* supernatants (100 mg) resuspended in 200 µl of YPD were added into 8-mm wells made on the YPD plates.

The plates were incubated at 30°C for up to 72 h. We used 100 mg of dried YPD broth resuspended in 200 µl of YPD as a control.

U-Tube Assay

A sterile membrane with 0.22-µm pore size was used to separate the two sides of the U-tube. In one side of the tube, 25 ml of YPD with 1.5×10^9 CFU/ml of either *Pa* strains was cultured, whereas in the other side, 25 ml of YPD with 1×10^5 cells/ml of *C. neoformans* was inoculated. Separately, Erlenmeyer flasks were used for controls, including co-cultures of *C. neoformans* with PAO1 or PA14, *C. neoformans* with *E. coli* and cultures of *C. neoformans* alone and PAO1 or PA14 alone, maintaining the same number of *C. neoformans* and *Pa* used in the U-tube. The U-tube and the flasks were incubated for 48 h at 30°C. *C. neoformans* and *Pa* cell viability was determined by CFU counting on YNB and LB plates, respectively.

Fungal Viability Assay in Physiological Condition

Overnight cultures of *C. neoformans* in YPD broth at 30°C and *Pa* strains in LB broth at 37°C were pelleted, washed with sterile water and resuspended in DMEM buffered with 50 mM HEPES (pH 7.2). 1.5×10^9 CFU/ml of PAO1 or PA14 and 2×10^4 cells/ml of *C. neoformans* were co-cultured at 37°C with 5% CO_2 for 24 and 48 h. *C. neoformans* and *Pa* cells viability was determined by CFU counting.

Mutant Generation

The *pqs* mutant strains were created in strain PAO1 by gene replacement described previously [16]. Briefly, a DNA fragment, which contained a gentamicin resistance cassette flanked by the 5′ and 3′ ends of the target gene, was cloned into pEX18ApGW [16]. The resultant plasmid was conjugated from *E. coli* strain SM10 into the *Pa* wild-type strain PAO1 with the selection of gentamicin (30 µg/ml) on *Pseudomonas* isolation agar plate (BD, Becton, Dickinson and Company). Merodiploids formed via a single crossover event was resolved through 5% sucrose selection in the presence of gentamicin. The gentamicin resistance cassette was subsequently removed by Flp recombinase, resulting in an unmarked deletion mutant of the target gene. The

presence of the deletion in the correct region was verified by PCR and sequencing.

Pyocyanin Assay

For pyocyanin quantification, cultures of PAO1 or *pqs* mutants were grown overnight in LB at 37°C with shaking (250 rpm) to reach OD_{600} of 2.0. Pyocyanin was extracted with chloroform from cell-free supernatants, acidified by 0.2 N HCl and assayed spectrophotometrically at 520 nm as previously described [17]. Concentrations, expressed in mg/l, were determined by multiplying the optical density at 520 nm by 17.072 [17].

Microtiter Assay

C. neoformans was grown overnight, washed and resuspended in YNB buffered with 25 mM HEPES (pH 7.2). 2×10^4 cells/ml of *C. neoformans* were incubated in a 96-well plate containing 4-hydroxy-2-heptylquinoline (HHQ) [*Qingdao Vochem*], 3,4-dihydroxy-2-heptylquinoline (PQS) [*Sigma-Aldrich*], 2,4-dihydroxyquinoline (DHQ) [*Fluka*] and pyocyanin [*Cayman Chemical*] at different concentrations (1.5, 3.5, 7, 14, 28, 56, 112 and 225 µg/ml). The compounds were dissolved in DMSO. The final concentration of DMSO was less than 1–1.5%. The plate was incubated for 48 h at 30°C. The growth of yeast cells was assessed by monitoring optical density at 495 nm (OD_{495}), and the surviving viable yeast cells were determined by CFU counting.

Statistics

Data from each experimental group were subjected to an analysis of normality and variance. Statistical significance between the means of different experimental data sets composed of normally distributed values was analyzed using two-tailed Student's *t* test. For all statistical tests, standard deviation with *P*-values less than 0.05 was considered significant. All experiments were done twice, with similar results each time, or three times when statistics was applied.

Results

Pa Inhibited Growth of *Cryptococcus* spp.

Previous reports reveal that *Pa* inhibits the growth of different microbes [10–13]. However, the mechanism that underlines the inhibitory effects of *Pa* on *Cryptococcus* species has not been investigated. Our results showed that when PAO1 or PA14 was inoculated on paper disks on YNB plate, which contained a lawn of *Cryptococcus* species, a zone of inhibition developed around the disks within 24 h. The diameters of the zone of inhibition of PA14 against serotypes, A, B, C and D of *Cryptococcus* were 23, 25, 29 and 30 mm, respectively. In contrast, reduced diameters of the zone of inhibition of PAO1 against *Cryptococcus* species were observed (16, 12, 23 and 25 mm) (Fig. 1).
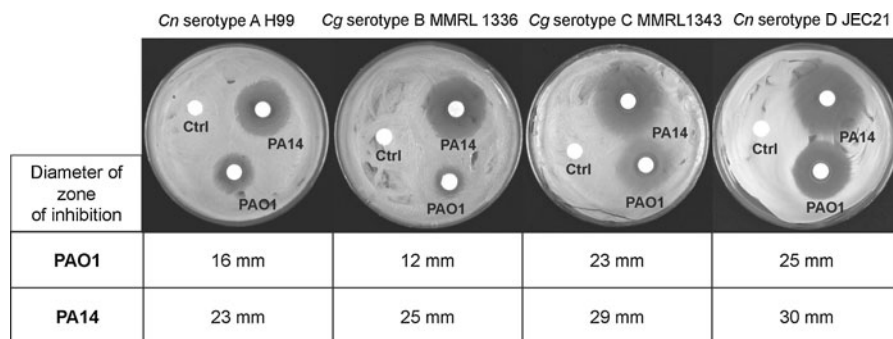

Diameter of zone of inhibition	*Cn* serotype A H99	*Cg* serotype B MMRL 1336	*Cg* serotype C MMRL1343	*Cn* serotype D JEC21
PAO1	16 mm	12 mm	23 mm	25 mm
PA14	23 mm	25 mm	29 mm	30 mm

Fig. 1 *Pa* inhibited the growth of *Cryptococcus* spp. PAO1 and PA14 were inoculated on paper disks, on YNB plates with *C. neoformans* var. *grubii* serotype A (*Cn* serotype A H99), *C. gattii* serotypes B (*Cg* serotype B MMRL 1336), *C. gattii* serotypes C (*Cg* serotype C MMRL1343) and *C. neoformans* var. *neoformans* serotype D (*Cn* serotype D JEC21). Control disks containing LB medium were placed on the plates (indicated with *ctrl* on the plates). The diameters of the zone of inhibition were determined after 72 h at 30°C

### *Pa* Fungicidal Effect Against *C. neoformans* in Co-culture

To determine whether the inhibitory effects of *Pa* on *C. neoformans* growth were fungistatic or fungicidal, we performed a viability assay of *C. neoformans* in co-cultures with PAO1 or PA14. Different *C. neoformans* dilutions were mixed with *Pa* strains (Fig. 2). After co-incubation of PAO1 and *C. neoformans*, we observed, in the first 4 dilutions, 1 log of decrease in *C. neoformans* cell number compared with the start culture. The last dilution showed no remaining viable cells of *C. neoformans* (Fig. 2a). In contrast, in the first 3 dilutions of co-cultures of PA14 and *C. neoformans,* we observed 1 log of decrease in *C. neoformans* cells number, but there was no remaining viable cells of *C. neoformans* in the last two dilutions (Fig. 2c). These results showed that the inhibitory effect of *Pa* on *C. neoformans* is fungicidal and the killing effect is dependent on the relative cell density. To evaluate whether *Pa* growth is affected in co-culture with *C. neoformans*, the number of *Pa* cells was counted. The results showed that *C. neoformans* does not affect *Pa* growth (Fig. 2b, d). Both PAO1 and PA14 grew in the condition tested, and therefore, the different inhibitory effect is not the result of different growth rate of PAO1 and PA14.

Enhanced Inhibition of *C. neoformans* by Co-culture Supernatants

To understand the mechanism by which *Pa* inhibits fungal growth, we screened the effect of culture supernatants of PAO1 or PA14 and the effect of *Pa*–*C. neoformans* co-culture supernatants on *C. neoformans* growth. Supernatants were prepared as outlined in the Materials and Methods. We inoculated different amounts of dried co-cultured supernatants (e.g., 12.5, 25, 50, 75 and 100 mg) and dried *Pa* supernatants (e.g., 100 mg), on YPD plate, which contained a lawn of *C. neoformans*. As a control, we used dried YPD, to exclude any effects from the high concentration of salts. A diameter of zone of inhibition of 15, 16, 17 and 20 mm was observed with 25, 50, 75 and 100 mg of PAO1-*C. neoformans* supernatant, respectively (Fig. 3a), whereas we observed a diameter of zone of inhibition of 15, 18, 21 and 22 mm with PA14-*C. neoformans* supernatant (Fig. 3c). The dried PAO1 supernatant did not result in a clean zone of inhibition, whereas with dried PA14 supernatant, we estimated a

Fig. 2 *Pa* has fungicidal effect against *C. neoformans* in co-culture. Different *C. neoformans* (*Cn*) dilutions were mixed with *Pa* strains and incubated in 96-well plates. The viability of *Cn* in co-culture with PAO1 (**a**) and PA14 (**c**) was determined by CFU counting on YNB plates. The viability of PAO1 (**b**) and PA14 (**d**) in co-culture with *Cn* was determined by CFU counting on LB plates. The inhibitory effect of *Pa* on *Cn* is fungicidal and is dependent on the relative cell density
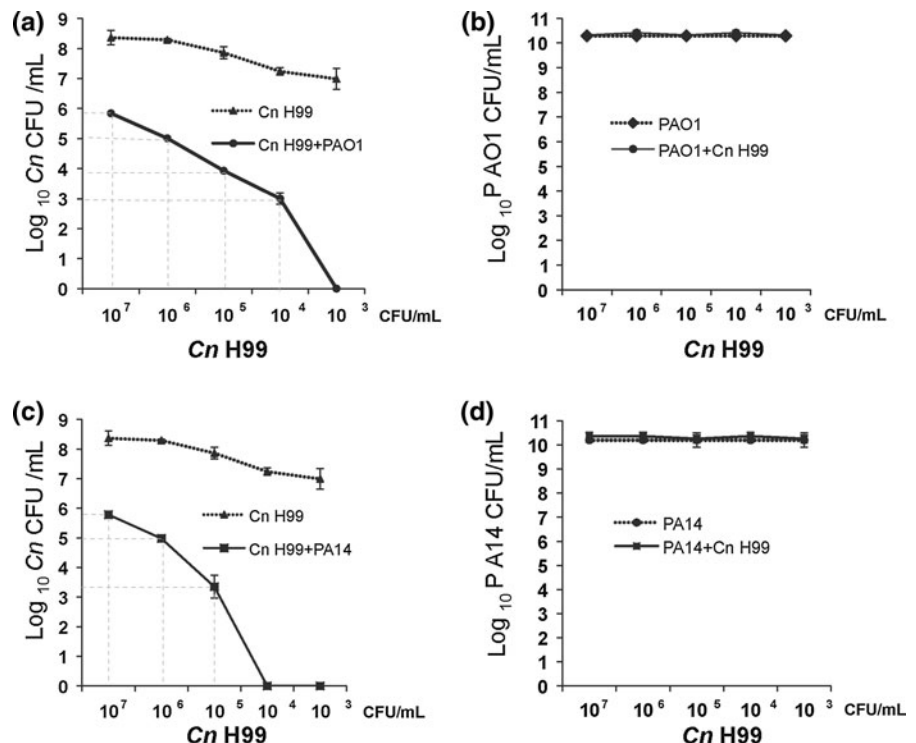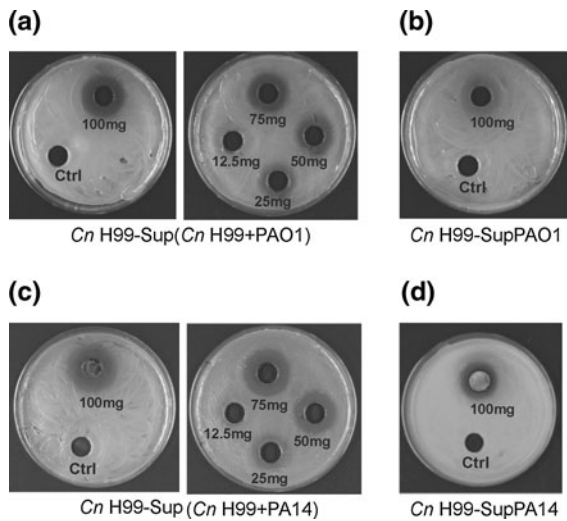
Fig. 3 Enhanced inhibition of *C. neoformans* by *Pa* in co-culture supernatants. Dried co-culture supernatants [Sup(*Cn* H99 + PAO1), Sup(*Cn* H99 + PA14)] (**a**, **c**) and dried *Pa* supernatants (SupPAO1, SupPA14) (**b**, **d**), resuspended in YPD broth, were inoculated into 8-mm wells on YPD plates, which contained a lawn of *C. neoformans* (*Cn* H99). The diameters of the zone of inhibition were determined

zone of inhibition of 17 mm (Fig. 3b, d). These results suggest that the physical contact between *Pa* and *C. neoformans* triggers the production of antifungal molecules by *Pa* that inhibit *C. neoformans* growth. *Pa* supernatant alone was not able to inhibit *C. neoformans* growth as much as *C. neoformans–Pa* supernatant.

The Cell–Cell Contact was Important for *Pa* to Inhibit *C. neoformans*

To investigate whether *Pa–C. neoformans* cells contact was important for inhibition, we performed an assay in YPD media using a U-tube. We cultured *Pa* in one side and *C. neoformans* in the other side of the tube; the two sides were separated with a sterile membrane, which prevented mixing of *Pa* and *C. neoformans* but allowed free exchange of nutrients and extracellular molecules. The *C. neoformans* growth exhibited half a log reduction when cultured in the U-tube with PAO1 (Fig. 4a), whereas 1 log reduction was observed when *C. neoformans* was cultured with PA14 (Fig. 4c). A 4 log inhibition was observed when *C. neoformans* was co-cultured with *Pa* strains in Erlenmeyer flasks (Fig. 4a, c). These results suggest that the cell contact is important to

elicit a strong inhibition of *C. neoformans* by *Pa*. When *E. coli* was co-cultured with *C. neoformans,* no inhibitory effects were observed (Fig. 4a, c). Under these conditions, *Pa* growth was not affected by *C. neoformans* like observed before (Fig. 4b, d).

Pa Inhibited *C. neoformans* in Physiological Condition

To determine whether *Pa* inhibits the growth of *C. neoformans* in physiological condition, we co-cultured PAO1 or PA14 with *C. neoformans* in DMEM (pH 7.2) at 37°C with 5% CO_2 for 24 and 48 h. Both PAO1 and PA14 inhibited the growth of *C. neoformans* in DMEM; however, the degree of inhibition in DMEM (Fig. 5a, c) was significantly reduced compared to co-cultures in YPD (Fig. 4a, c). In DMEM, the growth of *C. neoformans* exhibited a 2 log reduction when co-cultured with PAO1 for 48 h (Fig. 5a), whereas a 4 log reduction was observed when *C. neoformans* was co-cultured with PAO1 in YPD (Fig. 4a). Similar results were observed with the PA14 strain (Figs. 4c, 5c). The reduced inhibition of *C. neoformans* growth by *Pa* could be the result of reduced replication of *Pa* in DMEM. *Pa* cell number counting showed that there was no significant increase in the number of *Pa* when co-cultured with *C. neoformans* in DMEM (Fig. 5b, d), suggesting that DMEM does not support the growth of *Pa* as well as YPD, in which *Pa* cell number increased by 1 log when co-cultured with *C. neoformans* (Fig. 4b, d). These results suggest that the inhibition of *C. neoformans* growth depends on the cell density of *Pa* and relative ratio of *Pa* and *C. neoformans*.

Effects of PQS Mutants of PAO1 on the Growth of *C. neoformans*

It has been shown that extracellular compounds secreted by *Pa*, including quorum-sensing molecules and phenazines (such as pyocyanin), affect the growth of different microbes [9, 11]. *Pseudomonas* quinolone signaling plays an essential role in activating the production of pyocyanin [18]. Proteins encoded by the *pqsABCDE* operon are required for both PQS synthesis and activation of pyocyanin production. Specifically, PqsA, PqsB, PqsC and PqsD are required for the formation of extracellular quinolones including
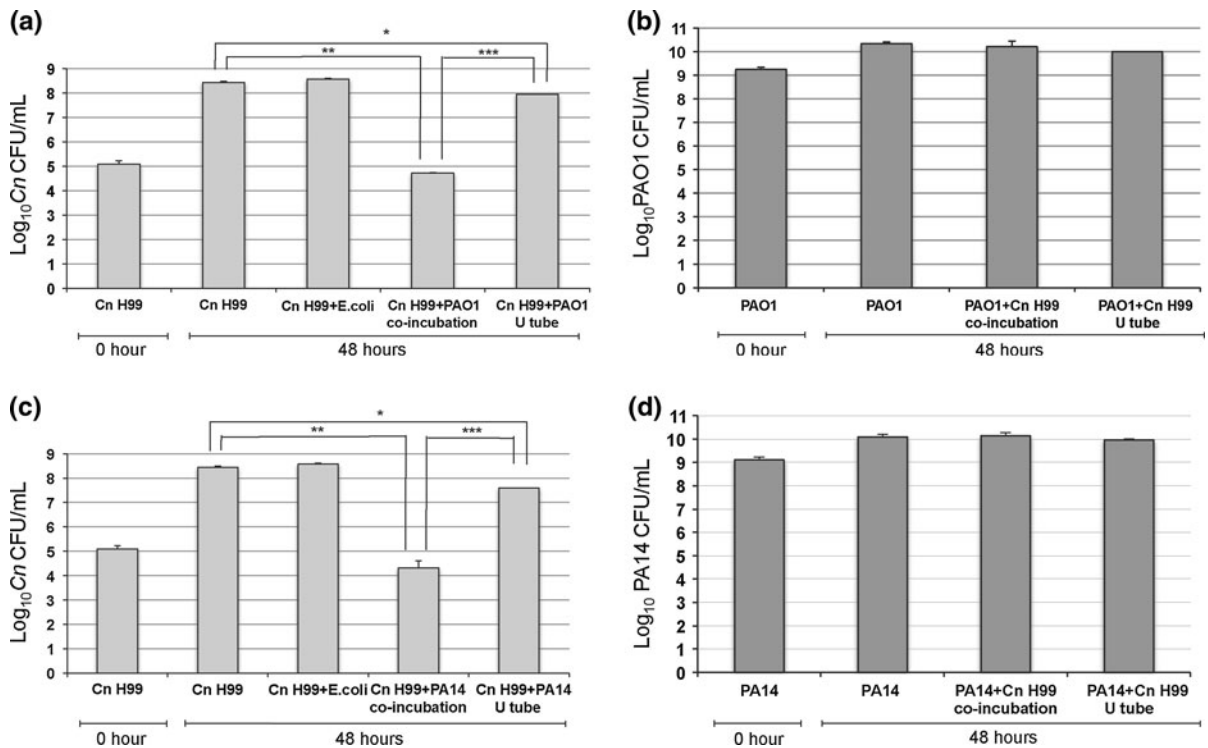
Fig. 4 Cell–cell contact is important for *Pa* to inhibit *C. neoformans*. An assay in YPD media using a U-tube was performed. A sterile membrane was used to separate the two sides of the U-tube, in which *Pa* strains and *C. neoformans* (*Cn*) were cultured. Separately, Erlenmeyer flasks were used for controls, including co-cultures of *Cn* with PAO1 or PA14, *Cn* with *E. coli* and cultures of *Cn* alone and PAO1 or PA14 alone.

The inhibitory effect of *Pa* on *Cn*, in the U-tube and in co-culture, was determined by CFU counting (**a**, **c**). When *E. coli* and *Cn* were co-cultured, no inhibitory effects of *Cn* were observed (**a**, **c**). The growth of PAO1 and PA14 was not affected by *Cn* (**b**, **d**). *P* values were calculated by Student's *t* test, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

4-hydroxy-2-heptylquinoline (HHQ), 3,4-dihydroxy-2-heptylquinoline (PQS) and 2,4-dihydroxyquinoline (DHQ). PqsE is not required for the biosynthesis of quinolones but is essential for the activation of pyocyanin production. To determine whether the extracellular quinolones and pyocyanin are involved in the inhibition of *C. neoformans*, we tested the effects of *pqs* mutants of PAO1 on the growth of *C. neoformans*. Three mutant strains of PAO1 (*pqsAB*, *pqsB* and *pqsE*) were chosen to distinguish the effects of alkylquinolones (including HHQ and PQS), DHQ and pyocyanin. *pqsAB* mutant is defective in both PqsA and PqsB and does not produce any extracellular quinolones. *pqsB* mutant lacks alkylquinolones [19] but produces normal level of DHQ. *pqsE* mutant produces extracellular quinolones at the similar levels as the wild-type PAO1. In terms of pyocyanin production, *pqsAB* and *pqsE* mutants are defective, whereas *pqsB* secreted pyocyanin at a level that was about 50% of the

wild-type PAO1 (Fig. 6a). No inhibition on the growth of *C. neoformans* by the *pqsAB* mutant was observed (Fig. 6b). *pqsB* exhibited inhibition on *C. neoformans* growth on YNB plates (14 mm, diameter of the zone of inhibition), although the zone of inhibition of *pqsB* mutant was smaller than that of the wild type (17 mm) (Fig. 6b). *pqsE* mutant marginally inhibited *C. neoformans* growth with a small zone of inhibition (9 mm) outside the paper disk (Fig. 6b). These results, combined with the different compositions of extracellular compounds secreted by the mutants (Fig. 6), suggest that the inhibition of *C. neoformans* growth by *Pa* is mainly dependent on pyocyanin and, to a lesser degree, alkylquinolones such as HHQ and PQS.

The effects of quinolones and pyocyanin on *C. neoformans* were further investigated using a microtiter dilution method in YNB with different concentrations of HHQ, PQS, DHQ and pyocyanin (Fig. 7a). The viable *C. neoformans* cells were
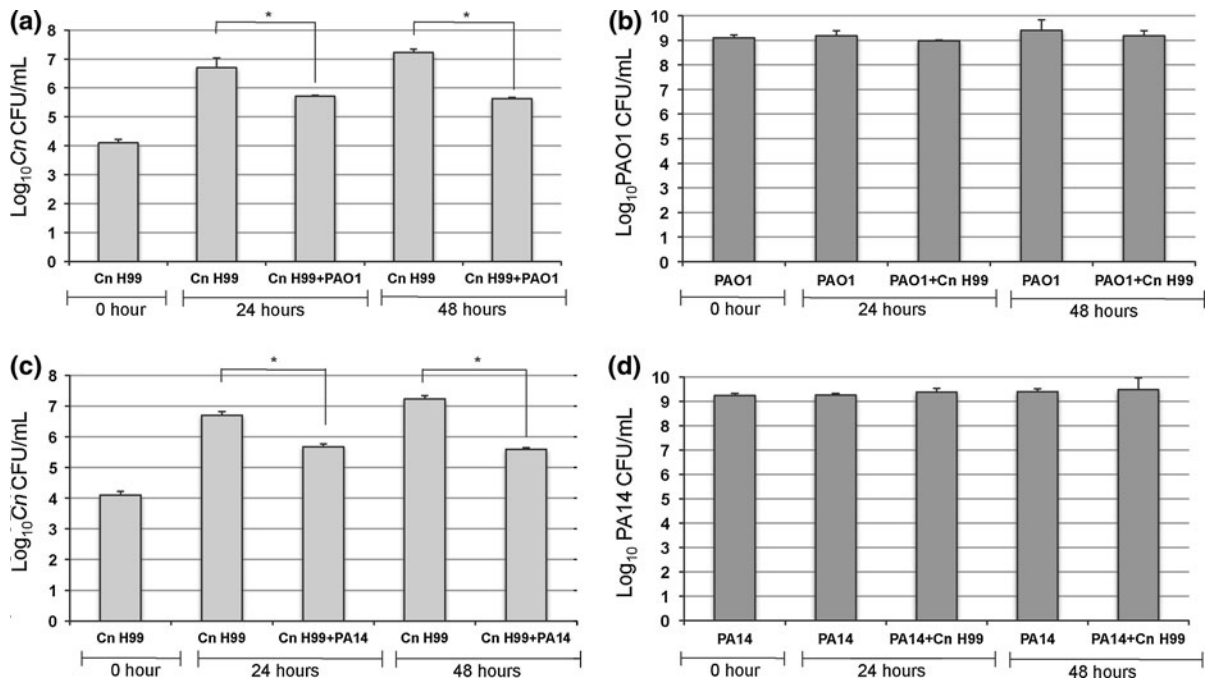
Fig. 5 *Pa* inhibited *C. neoformans* in physiological condition. PAO1 and PA14 were co-cultured with *C. neoformans* (*Cn*) in DMEM for 24 and 48 h. Viable *Cn* and *Pa* were measured by CFU on YNB and LB plates, respectively. *Cn* inhibition was observed with both *Pa* strains (**a**, **c**). *Pa* cells number counting showed no significant increase in the number of cells in DMEM (**b**, **d**). *P*-values were calculated by Student's *t* test, *$P < 0.05$

assessed by monitoring optical density at 495 nm and CFU. Both pyocyanin and PQS completely inhibited *C. neoformans* growth at 28 μg/ml. However, pyocyanin exhibited more pronounced inhibition than PQS at lower concentrations (less than 14 μg/ml), demonstrating that pyocyanin is a more potent antifungal agent than PQS (Fig. 7b). HHQ partially inhibited the growth of *C. neoformans* only at the highest concentration tested. No inhibition on *C. neoformans* by DHQ was observed. These results corroborated the data obtained with the *pqs* mutants in that pyocyanin is a major contributing factor in the inhibition of *C. neoformans*. We also determined the number of viable *C. neoformans* cells by withdrawing aliquots from the microtiter plates. Viable cells were present in wells with pyocyanin or PQS (data not shown), demonstrating that the inhibitory effects on *C. neoformans* growth are static but not fungicidal.

Discussion

In the present study, we investigated the ability of *Pa* strains PAO1 and PA14 to inhibit the growth of *Cryptococcus* species. Our results demonstrated that, in mixed culture conditions, *Pa* exhibited a fungicidal effect on *C. neoformans,* dependent on the relative cell density. Physical contact between *Pa* and *C. neoformans* was important for inhibition as demonstrated by experiments using U-tubes (Fig. 4), indicating that cell–cell contacts could activate the production of antifungal molecules by *Pa* that impeded *C. neoformans* growth. Our results also showed that the major antifungal molecule produced by *Pa* was the redox-active metabolite, pyocyanin. In addition, alkylquinolones, such as HHQ and PQS, exhibited antifungal proprieties against *C. neoformans.* These findings are consistent with earlier reports that showed *Pa* inhibited the growth of Gram-positive bacteria and yeasts by heat-stable molecules involving quorum-sensing mechanism [20, 21].

Our results showed that PA14 exhibited more potent inhibition of *Cryptococcus* growth than PAO1. The *p*-value of paired *t* test is 0.02, demonstrating a significant difference in the diameters of the zone of inhibition of PA14 and PAO1 against *Cryptococcus* species (Fig. 1). PAO1 and PA14 are two commonly used laboratory strains of *Pa*. Strain PAO1 was first
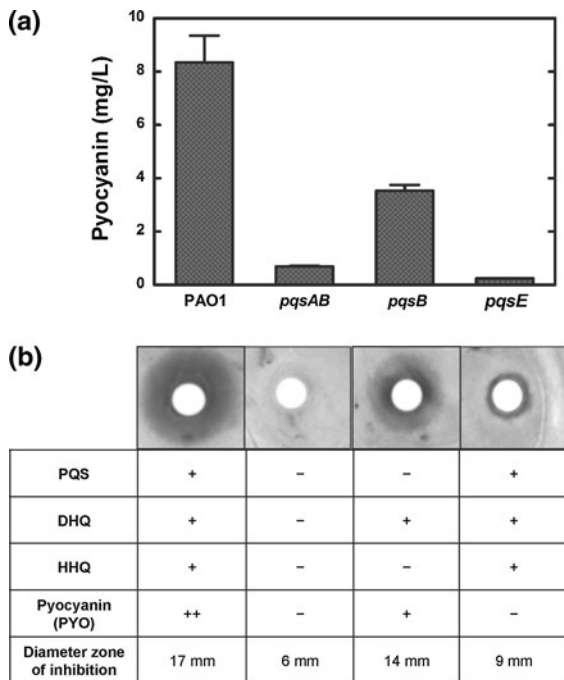
Fig. 6 Effects of *pqs* mutants of PAO1 on the growth of *C. neoformans*. The pyocyanin production was measured for all mutants (**a**). *pqsB* mutant secreted pyocyanin at a level that was about 50% of the wild-type PAO1 (**a**). PAO1, *pqsAB*, *pqsB* and *pqsE* were inoculated on paper disks, on YNB plates, which contained a lawn of *C. neoformans* (*Cn*). The diameters of the zone of inhibition were determined after 72 h at 30°C (**b**)

isolated in 1954 from a wound [22]. Numerous reports showed that the clinical isolate PA14 is significantly more virulent than PAO1 in multiple hosts, including mice, the nematode *Caenorhabditis elegans*, the insect

Galleria mellonella and the plant *Arabidopsis thaliana* [14, 15, 23], suggesting that the greater antifungal activity of PA14 may be due to its increased virulence. Although the genomes of the two *Pa* strains are very similar (92% of the PA14 genome is present in PAO1, and 96% of PAO1 genome is present in PA14 [24]), unique genes of PA14 have been extensively studied for their roles in virulence. However, Lee et al. showed that the presence of these genes was not correlated with PA14 virulence, and the virulence of *Pa* is both combinatorial and multifactorial [24]. Therefore, future studies to identify PA14 genes that are essential for the increased inhibition of *C. neoformans* growth may help decipher the roles of different virulence factor on the ability of *Pa* to cause diseases.

Previous reports have shown an interspecies competition between *Pa* and yeasts, and in this study, we have clearly demonstrated that *Pa* inhibits the growth of *C. neoformans*, whereas *Pa* growth is not affected by *Cryptococcus* strains. Hogan et al. [25] demonstrated that *Pa* formed a biofilm on *Candida albicans* filaments and killed the fungus; where as *C. albicans* produced farnesol, a major fungal QS molecule, which reduced the production of PQS and pyocyanin by *Pa* [18, 26]. Our data showed that the interaction of *Pa* and *C. neoformans* is not reciprocal; production of extracellular virulence molecules by *Pa* inhibited the growth of *Cryptococcus* strains, while *Pa* was not affected by the presence of *C. neoformans*. Our results also demonstrated that *Pa* exhibited a fungicidal effect when co-cultured with *C. neoformans* (Fig. 2), whereas the effects of pyocyanin and PQS were static, suggesting
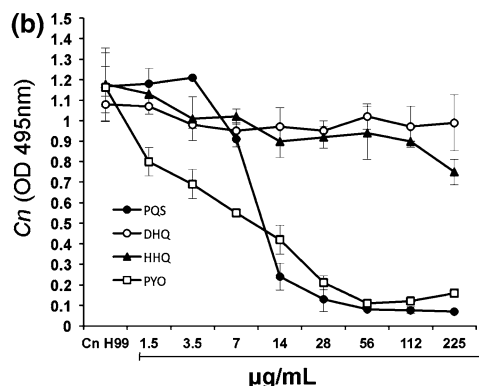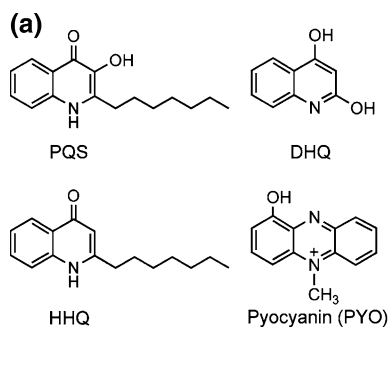

Fig. 7 Antifungal activity of pyocyanin and PQS against *C. neoformans*. Chemical structures of 3,4-dihydroxy-2-heptyl-quinoline (PQS), 2,4-dihydroxyquinoline (DHQ), 4-hydroxy-2-heptylquinoline (HHQ) and pyocyanin (**a**). *C. neoformans* (*Cn*)

was incubated in microtiter plates with different concentration of PQS, DHQ, HHQ and PYO. The growth of yeast cells was assessed by monitoring optical density at 495 nm (**b**)

that other unidentified exoproducts, such as proteases, hemolysin and rhamnolipids, may be involved in fungicidal activity of *Pa*. Moreover, cell–cell contact with *C. neoformans* may be important in mounting a fungicidal response of *Pa* on *C. neoformans*.

The lung of patients with cystic fibrosis is infected with a large spectrum of microbial pathogens. Over time, both the types of bacterial and their individual characteristics change [27, 28]. For instance, *Staphylococcus aureus* and *Haemophilus influenzae* are common bacteria found in CF patients at an early age. Eventually, more than 80% of CF patients harbor *Pa*. Filamentous fungal pathogens such as *A. fumigatus* and *C. albicans* are also frequently isolated from CF patients [29]. Fungal colonization could be the result of prolonged therapy with antibiotics and steroid, in addition to the defective mucus clearance. Hughes et al. [30] showed that in CF patients with *Pa* infection, only 10% had positive *C. albicans* skin tests, compared with 30% positivity in those free of *Pa*, suggesting the antifungal substance produced by *Pa* could prevent *Candida* infections. To date, there has been no report on the isolation of *Cryptococcus* species from the CF patients. Considering that both *Cryptococcus* and *Pa* are common lung pathogens, the lack of co-colonization could be the result of the antifungal effect of *Pa* on the growth of *C. neoformans*. Both pyocyanin and PQS accumulate intensively in the lung mucus of CF patients [31, 32], and these antifungal molecules may be important in the prevention of pulmonary cryptococcosis in CF patients.

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