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Pseudomonas aeruginosa-Candida albicans Interactions: Localization and Fungal Toxicity of a Phenazine Derivative[▽]

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Phenazines are redox-active small molecules that play significant roles in the interactions between pseudomonads and diverse eukaryotes, including fungi. When *Pseudomonas aeruginosa* and *Candida albicans* were cocultured on solid medium, a red pigmentation developed that was dependent on *P. aeruginosa* phenazine biosynthetic genes. Through a genetic screen in combination with biochemical experiments, it was found that a *P. aeruginosa*-produced precursor to pyocyanin, proposed to be 5-methyl-phenazinium-1-carboxylate (5MPCA), was necessary for the formation of the red pigmentation. The 5MPCA-derived pigment was found to accumulate exclusively within fungal cells, where it retained the ability to be reversibly oxidized and reduced, and its detection correlated with decreased fungal viability. Pyocyanin was not required for pigment formation or fungal killing. Spectral analyses showed that the partially purified pigment from within the fungus differed from aeruginosins A and B, two red phenazine derivatives formed late in *P. aeruginosa* cultures. The red pigment isolated from *C. albicans* that had been cocultured with *P. aeruginosa* was heterogeneous and difficult to release from fungal cells, suggesting its modification within the fungus. These findings suggest that intracellular targeting of some phenazines may contribute to their toxicity and that this strategy could be useful in developing new antifungals.

Many diverse bacterial species secrete diffusible, redox-active phenazine compounds. Over 100 phenazine derivatives are produced by different bacterial species, with pseudomonads, streptomycetes, and *Burkholderia* spp. included among the best-known phenazine producers. Phenazines have antibiotic properties toward bacterial and eukaryotic species, and the side chain substituents on the phenazine backbone contribute to the biological activities of specific compounds. The production of phenazines has been shown to be important for antagonistic interactions among microbes. For example, phenazine-1-carboxylate (PCA) secreted by *Pseudomonas fluorescens* contributes to biocontrol activity against fungal phytopathogens such as *Gaeumannomyces graminis* (46, 47), and phenazine-1-carboxamide produced by *Pseudomonas chlororaphis* PCL1391 is essential for inhibition of the fungus *Fusarium oxysporum*, which causes tomato root rot (6). Many toxic effects have been reported for different phenazines, and much of their toxicity depends on their redox activity and their ability to generate reactive oxygen species (21, 22, 30, 42).

Pseudomonas aeruginosa, a common gram-negative soil bacterium and an opportunistic human pathogen, is well known for its ability to produce a blue phenazine, called pyocyanin, which is toxic to numerous bacteria and fungi and damages mammalian cells (21, 24, 35, 42, 52). *P. aeruginosa* culture supernatants also contain PCA, 1-hydroxyphenazine, and phenazine-1-carboxamide. In addition, *P. aeruginosa* can produce two red pigments, aeruginosins A and B (5-methyl-7-amino-1-carboxymethylphenazinium betaine and 5-methyl-7-amino-1-carboxy-3-sulfo-methylphenazinium betaine, respectively), after prolonged incubation. Unlike the other phenazines produced by *P. aeruginosa*, aeruginosins A and B are highly water soluble, and their biological activities are much less well characterized (15, 20).

Pseudomonads synthesize PCA from chorismate by the products of the genes within the *phzABCDEFG* operon (32, 33) (Fig. 1A). In the *P. aeruginosa* genome, there are two highly similar *phzABCDEFG* operons, *phzA1* to *phzG1* and *phzA2* to *phzG2* (45). The production of pyocyanin from PCA requires two additional enzymes, namely, PhzM, which catalyzes methylation at N-5, yielding the proposed intermediate 5-methyl-phenazine-1-carboxylate (5MPCA) (32), and PhzS, which catalyzes the conversion of the 1-carboxylate moiety to a hydroxyl group (32) (Fig. 1B). The *phzM* and *phzS* genes are adjacent to the *phzA1*-to-*phzG1* operon (Fig. 1A) (45). While both its precursor, PCA, and its derivative, pyocyanin, are detected at near millimolar concentrations in culture supernatants, the PhzM intermediate, proposed to be 5MPCA, has not been detected in supernatants and has been proposed to be unstable (4, 13, 39). In *P. aeruginosa*, phenazine production is controlled by a number of regulators, including those involved in cell density-dependent signaling, referred to as quorum sensing. Mutants that are unable to participate in signaling via C_4-acylhomoserine lactone, synthesized by RhlI (3, 25), or the *Pseudomonas* quinolone signal (PQS) are defective in pyocyanin production (12).

Numerous reports indicate that *P. aeruginosa* and *Candida albicans* can coexist in a variety of different opportunistic infections (1, 10, 16, 36), and a number of different molecular interactions between these two organisms have been described (18, 19, 23, 24). Here we report the formation of a red pigment in *P. aeruginosa-Candida albicans* cocultures grown on solid medium. Through a combination of genetic, biochemical, and microscopic experiments, it was determined that a pyocyanin

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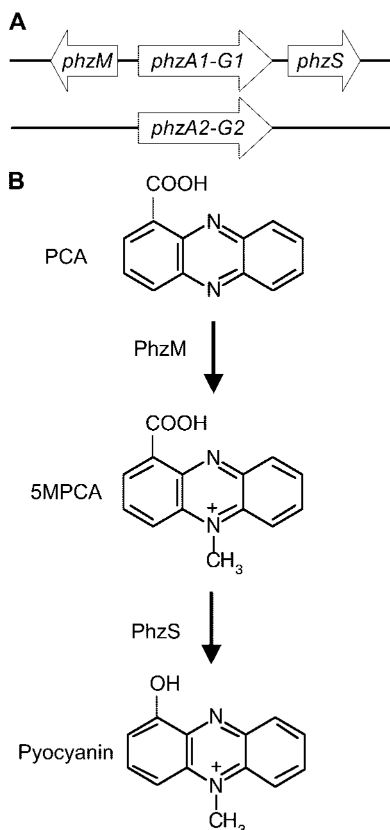

FIG. 1. *P. aeruginosa* phenazine biosynthetic genes and structures of pyocyanin and its immediate precursors. (A) *P. aeruginosa* has two redundant operons encoding the enzymes necessary for PCA production (*phzABCDEFG*). *phzM* and *phzS* are present as single copies. (B) Proposed biosynthetic pathway modified from reference 32. The 5MPCA intermediate has not been detected in *P. aeruginosa* cultures, while PCA and pyocyanin are readily detected in culture supernatants (4). Aeruginosin A has an amino substitution at position 7, and aeruginosin B has amino and sulfonate substitutions at positions 7 and 3, respectively.

precursor, 5MPCA, was necessary and likely sufficient for the formation of the red pigmentation. Further characterization showed that the red pigment accumulated within fungal cells, where it remained redox active, and that its formation correlated with decreased fungal viability. We propose that the intracellular accumulation of a 5MPCA-derived product within target cells may represent an important aspect of phenazine-mediated antagonism between *P. aeruginosa* and other species, including fungi.

MATERIALS AND METHODS

Strains and growth conditions. All strains used in these studies are included in Table 1. Fungal strains were grown at 30°C on YPD (2% peptone, 1% yeast extract, 2% glucose) solidified, when required, with 2% agar. Strains of *Pseudomonas* spp. and *Escherichia coli* were grown on LB, also at 30°C. All clinical isolates were obtained in compliance with federal guidelines and institutional policies. Liquid cultures were aerated in a roller drum. For assessment of swimming motility, *P. aeruginosa* strains were inoculated into LB containing 0.3% agar from a freshly streaked LB-grown culture, followed by incubation at room temperature for 6 to 24 h. Pyocyanin production by *P. aeruginosa* transposon mutants was determined by growth in LB medium for 16 h at 37°C with vigorous aeration.

***P. aeruginosa-C. albicans* cocultures.** *P. aeruginosa* was inoculated onto preformed lawns of *C. albicans* SC5314 or a *tup1*/*tup1* mutant, either by using a sharp toothpick or spotting 10-μl drops of a 7×10^7-CFU/ml suspension onto the surface of the plate-grown fungal culture. The *C. albicans* lawns were prepared by spreading 350 μl of a YPD-grown overnight *C. albicans* culture onto the plate by use of glass beads, followed by incubation at 30°C for 48 h. After inoculation with *P. aeruginosa*, the cocultures were incubated at 30°C for an additional 24 to 96 h.

Complementation analysis was performed on filter-grown *C. albicans* cultures so that the *C. albicans* lawns could be transferred to fresh antibiotic-containing medium (150 μg/ml tetracycline) to maintain selection for the complementing plasmids over the course of the experiment. In these assays, a sterile Nylon 66 Plus Transphor transfer membrane was applied to the surface of a YPD plate, followed by inoculation with the *C. albicans tup1*/*tup1* mutant as described above. Prior to inoculation with *P. aeruginosa*, the nylon filter was transferred to a fresh YPD plate containing tetracycline. The *C. albicans* lawns were inoculated with 10-μl drops of *P. aeruginosa* cultures diluted to an absorbance (optical density at 600 nm [OD_{600}]) of 0.01. Plates were incubated at 30°C for 48 h prior to being photographed.

To achieve larger amounts of red-pigmented cocultures, full-plate cocultures were prepared by flooding established lawns of the *C. albicans tup1*/*tup1* mutant with a dilute bacterial suspension (OD_{600}, 0.05), followed by incubation at 30°C for 24 to 48 h. To visualize the redox activity of the red pigment, growth from two full-plate cocultures was scraped from the surface of the agar and resuspended in 50 mM potassium phosphate buffer, pH 7. Fungal cells were sedimented by centrifugation for 3 min at $3{,}000 \times g$ and then washed free of bacteria by repeating the procedure until the supernatant was no longer turbid. Bacteria were sedimented from the first wash by centrifugation at $4{,}500 \times g$ for 10 min. To completely reduce or oxidize the suspension of fungal cells from the coculture, a few crystals of dithionite or 10 μl of a 3% solution of H_2O_2 was added to a 1-ml cell suspension.

Genetic screen for mutants defective in red pigment formation. A *P. aeruginosa* strain PA14 Tn*5* mutant library containing ~9,000 random insertion mutants (29) was screened on 2-day lawns of the filamentous *C. albicans tup1*/*tup1* mutant (2) grown on YPD. Inocula from frozen stocks stored in 96-well plates were first grown on LB agar for 24 h at 30°C or 48 h at room temperature prior to transfer of the *P. aeruginosa* strains to the fungal lawns by use of a 48-prong inoculation device (Dan-Kar Corp.). After inoculation with *P. aeruginosa*, cocultures were incubated at 30°C. Mutants with altered zones of pigmentation were retrieved from the master plate and retested at least three times in triplicate. The identities of the mutants were determined by arbitrary PCR as described previously (38).

Pyocyanin production in single-species and mixed-species cultures. Pyocyanin levels in *C. albicans-P. aeruginosa* cocultures were assessed by extracting the blue pigment from plates. In these assays, lawns of the *C. albicans tup1*/*tup1* mutant were grown on YPD for 48 h at 30°C as described above, except that one-half of the plate was covered with sterile cellophane before spreading of the fungal inoculum. The cellophane, together with cells growing on it, was peeled off after growth of the fungus to provide an area of "conditioned" medium for measuring pyocyanin production in the absence of fungus. Three or four well-separated drops of a suspension of *P. aeruginosa* PA14 were added to both the lawn and the lawn-free areas as described above. After different periods of growth, sections of the agar plate that encompassed the entire region of bacterial growth surrounding the inoculation point (1.5 cm^2), including the underlying agar, were removed, transferred to tubes, and vortexed vigorously in 2 ml of sterile water. Numbers of viable *P. aeruginosa* cells were determined by plating serial dilutions of this suspension on LB agar. To recover the pyocyanin, the agar suspension was then mixed with 1 ml chloroform and incubated at 4°C in the dark until the agar fragments were no longer blue. The chloroform layer was recovered, and the aqueous layer was reextracted with 1 ml chloroform. Pyocyanin levels were determined spectrophotometrically at 690 nm (E_{mM} at 690 nm = 4.2) (43) after extracting the combined chloroform layers two times with 0.4 ml 50 mM HCl, combining the extracts, and neutralizing the suspension with 200 μl of 0.1 M ammonium acetate, pH 7.

Fungal viability assays. To assess the viability of *C. albicans* during coculture with *P. aeruginosa*, established lawns of yeast-form *C. albicans* SC5314 grown for 48 h were spotted with suspensions of the *P. aeruginosa* strains as described above and further incubated for various intervals. Agar cores (4-mm diameter) from the inoculated areas or control regions were taken in triplicate with inverted Pasteur pipettes after defined times of incubation. For a given strain at a given time point, the triplicate samples were taken from three distinct inoculation points. Each of the cores was vortexed vigorously in 1 ml sterile water, and the total number of fungal cells was determined microscopically using a hemocytometer. Numbers of viable *C. albicans* cells were determined by serial dilution followed by plate counts, using YPD plates containing 150 μg/ml tetracycline to

TABLE 1. Bacterial and fungal strains used in this study

Strain	Description	DH no.[a]	Source or reference
Pseudomonas aeruginosa strains			
PA14 WT	WT	123	41
PA14 *phzM*::Tn*M*	Tn*M* mutant, pyocyanin negative	693	28
PA14 *phzS*::Tn*M*	Tn*M* mutant, pyocyanin negative	698	28
PA14 WT/pUCP26	WT with empty plasmid from reference 51	942	51
PA14 *phzM*::Tn*M*/pUCP26	Mutant with empty plasmid from reference 51	944	This study
PA14 *phzM*::Tn*M*/pUCP-M	Mutant complemented with the *phzM* gene on a plasmid (32)	945	This study
PA14 *phzS*::Tn*M*/pUCP26	Mutant with empty plasmid from reference 51	946	This study
PA14 *phzS*::Tn*M*/pUCP-S	Mutant complemented with the *phzS* gene on a plasmid (32)	947	This study
PA14 Δ*phz*	In-frame deletion mutant of *phzA1* to *phzG1* and *phzA2* to *phzG2*	933	9
PA14 *flgK*::Tn*5*	Tn*5* mutant, nonmotile		37
PA14 *pqsA*::Tn*5*	Tn*5* mutant, lacks PQS		This study
PAO1 WT	WT	20	45
PAO1 Δ*phzM*	Mutant lacking the *phzM* gene, pyocyanin negative	296	32
PAO1 Δ*phzS*	Mutant lacking the *phzS* gene, pyocyanin negative	295	32
Clinical isolates	Isolates from respiratory sputum	211 to 228 and 74	This study
Other *Pseudomonas* strains			
Pseudomonas fluorescens SWB25		245	G. O'Toole lab
Pseudomonas putida KT2440		468	G. O'Toole lab
Pseudomonas chlororaphis PCL1391		469	G. O'Toole lab
Fungal strains			
C. albicans SC5314	WT	65	11
C. albicans tup1/tup1 mutant	BCa2-10; *tup1/tup1 URA3/ura3*	36	2
Saccharomyces cerevisiae Σ1278b	Σ1278b	347	F. Winston lab
S. cerevisiae BY4742	*MAT*α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	195	F. Winston lab
S. cerevisiae BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	196	F. Winston lab
Environmental yeast isolate		121	This study
Escherichia coli strains			
DH5α/pUCP-M			32
DH5α/pUCP26			32

[a] From our lab collection.

inhibit bacterial growth. Viable bacterial cell counts were obtained by similar plating of cells on LB agar medium lacking antibiotics. For methylene blue staining, similar suspensions of cocultures (2.5 μl) were mixed with 0.05% methylene blue (2.5 μl) and incubated for 10 min prior to examination by bright-field or differential interference contrast microscopy.

Epifluorescence microscopy of red-pigmented fungal cells. Cocultures of *P. aeruginosa* and *C. albicans* yeast cells were grown as described above for the viability assays. A small amount of the coculture growth was removed with a micropipette tip and resuspended in 500 μl of 50 mM phosphate buffer. Suspensions were incubated with 0.03% H_2O_2 for at least 20 min to ensure that pigments were oxidized before being viewed with a Zeiss filter set 20 (excitation, 550 nm; emission, 580 to 640 nm). Fixation of the yeast cells with 0.4% formaldehyde did not interfere with fluorescence and stabilized the fluorescence for more than 3 days. All images were captured using a fixed capture time and light intensity.

Preparation and spectral analysis of purified phenazines and fungus-associated red pigment. PCA was prepared from spent culture supernatants of *P. aeruginosa* strains by published methods (32). The product of PhzM activity on PCA was prepared as described previously (32), by incubating PCA with *E. coli* DH5α/pUCP-M, which contains a plasmid carrying the *phzM* gene. Cells from 50 ml of an overnight culture of this *E. coli* strain in LB with tetracycline (15 μg/ml) at 37°C were harvested by centrifugation, washed once in M63 medium (34) containing 0.2% glucose, and then resuspended in 5 ml of the same medium amended with 1 mM methionine and 0.4 mM PCA. The suspension was shaken vigorously at 37°C for 6 to 7 h. As controls, *E. coli* cells containing the pUCP26 vector and incubated with PCA and *E. coli*/pUCP-M cells incubated without PCA were treated identically. *E. coli* cells were removed by centrifugation, and the supernatant was acidified to pH 5 with 2 M HCl before being extracted three times with 3 ml chloroform to remove unreacted PCA. Recovery and quantitation of PCA in the chloroform fraction indicated that approximately 5 to 15% of PCA had been consumed in *E. coli*/pUCP-M compared to that in control cultures. The aqueous fraction of the extracted medium was neutralized with 2 M NH_4OH and concentrated under reduced pressure to a final volume of 200 μl. The solution containing the PhzM product was deep red, while the control solution was pale yellow.

Pyocyanin was extracted from 24-h LB-grown cultures of *P. aeruginosa* PA14 by cycling three times between chloroform at neutral pH and 20 mM HCl (7). Aeruginosins A and B were recovered as water-soluble red pigments from 6-day supernatants from *P. aeruginosa* PA14 cultures grown in Holliman's medium (20). Approximately 200 ml of the culture supernatant was applied to chromatographic columns. Aeruginosin A bound weakly to a 2.5- by 15-cm column of the C_{18} hydrophobic interaction medium LRP-2 (Whatman) equilibrated with 20 mM NH_4HCO_3, from which it was recovered by elution with 10% acetonitrile in 20 mM NH_4HCO_3 in a volume of about 50 ml. Aeruginosin B, which passed through the hydrophobic interaction column, bound to a column (1.5 by 12 cm) of DEAE Sephadex in 20 mM NH_4HCO_3, from which it was eluted with a gradient of NH_4HCO_3 (20 to 200 mM) in a volume of about 25 ml. The aeruginosin-containing eluates were concentrated under reduced pressure. The UV-visible spectra of both compounds corresponded to those previously reported (15, 20).

The fungus-associated red pigment was partially purified from the fungal fraction from full-plate cocultures (described above). The purification of the pigment is described in more detail in Results. The approximate concentration of the red pigment was determined using the extinction coefficient for aeruginosins A and B (15, 20). The absorbance and fluorescence spectra were obtained using

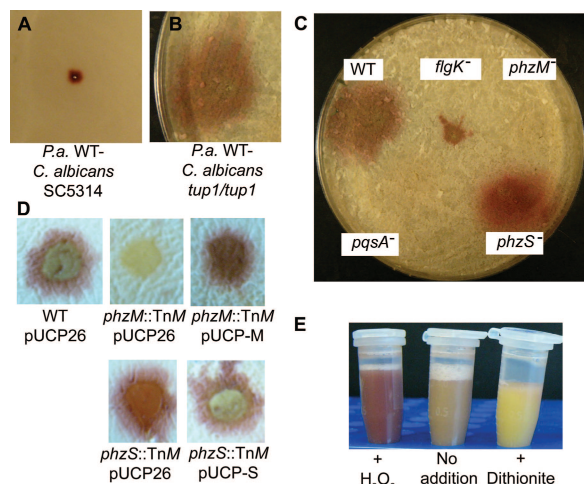

FIG. 2. Plate cocultures of *C. albicans* with *P. aeruginosa* strains. (A to C) *C. albicans* lawns were grown for 48 h at 30°C on YPD plates before point inoculation from LB agar-grown *P. aeruginosa* strains. Plates were photographed after coculture for 48 h. (A) *C. albicans* SC5314 with *P. aeruginosa* (*P.a.*) PA14 WT. (B) *C. albicans tup1*/*tup1* mutant with *P. aeruginosa* PA14 WT. (C) *C. albicans tup1*/*tup1* mutant with PA14 WT, *flgK*::Tn*5*, *pqsA*::Tn*M*, *phzS*::Tn*M*, and *phzM*::Tn*M* strains. (D) Complementation of mutations in *phzM*::Tn*M* and *phzS*::Tn*M* strains in the coculture assay. *P. aeruginosa* strains were inoculated onto the *C. albicans tup1*/*tup1* lawns as 10-μl drops and incubated at 30°C. Vector controls for WT, *phzM*::Tn*M*, and *phzS*::Tn*M*/(pUCP26) strains were also included. (E) Redox activity of fungal-associated pigment. *C. albicans tup1*/*tup1* cells from two plate-grown *P. aeruginosa* PA14 WT cocultures were separated from the bacteria by centrifugation, resuspended in 5 ml 50 mM phosphate buffer, pH 7, and divided into three tubes. The suspensions were photographed several minutes after aeration (center), after the addition of 20 μl 3% hydrogen peroxide (left), and after the addition of dithionite (right).

a 1-cm-path-length quartz cuvette in a SpectraMax M5 (Molecular Devices) spectrophotometer.

Treatment of yeast with the PhzM product. In order to determine if the *E. coli*-synthesized product of PhzM activity on PCA was sufficient to lead to red pigmentation and intracellular fluorescence of *C. albicans* SC5314 yeast cells, 50 μl of aqueous extract (described above) was added to 0.5 ml of overnight *C. albicans* culture grown in liquid YPD (OD_{600} of 15). As controls, *C. albicans* cultures received equivalent volumes of aqueous extracts from *E. coli*/pUCP26 incubated with PCA and *E. coli*/pUCP-M incubated without the PCA precursor. After 24 h at 30°C, the *C. albicans* suspensions were collected by centrifugation, washed two times with 50 mM phosphate buffer, pH 7, and resuspended in 100 μl of 50 mM phosphate buffer for analysis by visual inspection, epifluorescence microscopy, and enumeration of the number of CFU.

RESULTS

Red pigment formation in *P. aeruginosa*-fungal cocultures. When *P. aeruginosa* strain PA14 was point inoculated onto established lawns of *C. albicans* strain SC5314 yeast and further incubated at 30°C, a red-pigmented zone developed around the inoculation point within 12 h and continued to develop over 48 h (Fig. 2A). Red pigmentation also developed when *P. aeruginosa* was inoculated onto established lawns of filamentous *C. albicans* cells, as demonstrated using the constitutively filamentous *C. albicans tup1*/*tup1* mutant (Fig. 2B) (2). Inoculation of *P. aeruginosa* onto a lawn of the filamentous *C. albicans* strain led to a larger zone of pigmentation than that formed on *C. albicans* yeast (Fig. 2A and B). Neither *P. aeruginosa* nor *C. albicans* produced any red pigmentation when grown alone under similar conditions. While the red pigmentation was observed regardless of whether the cocultures were grown on rich or minimal agar plates, i.e., YPD or M63 medium with 0.2% glucose, respectively (34), no obvious red pigmentation was observed when *P. aeruginosa* and *C. albicans* were grown together in liquid cultures of the same medium composition.

To determine if the development of red pigmentation was specific to *P. aeruginosa* and *C. albicans*, other strains of *P. aeruginosa*, other *Pseudomonas* species, and other fungal strains were analyzed in coculture assays. A similar, but paler, red pigmentation was observed when *P. aeruginosa* strain PA14 was cultured on lawns of *Saccharomyces cerevisiae* BY4742, BY4741, and Σ1278b and three unidentified environmental yeast-like fungi isolated from soil samples and plant material (data not shown). Upon coculture with the *C. albicans tup1*/*tup1* mutant, *P. aeruginosa* strain PAO1 and 17 of 19 clinical *P. aeruginosa* isolates gave rise to red pigmentation to various degrees. No red pigmentation was observed upon incubation of *Pseudomonas putida*, *Pseudomonas fluorescens*, or *Pseudomonas chlororaphis* on lawns of *C. albicans* (data not shown).

Identification of genes involved in coculture pigmentation. To identify *P. aeruginosa* genes involved in the formation of red pigmentation, strains from a collection of *P. aeruginosa* strain PA14 Tn*5* transposon mutants (29) were screened, with 48 mutants per plate, in the coculture plate assay described above. An initial screen of approximately 9,000 random Tn*5* insertions found mutants with an altered pattern of pigmentation (more than 75 mutants), 5 mutants that lacked pigmentation, and 2 mutants with increased red pigmentation.

Role of swimming motility in coculture interactions. All of the *P. aeruginosa* mutant strains categorized as having an altered pattern of red pigmentation gave rise to a compact red ring around the point of inoculation which was much smaller than that formed by the wild-type (WT) strain on lawns of the *C. albicans tup1*/*tup1* mutant. Mutants in this class included *flgK*::Tn*5* (Fig. 2C), *fleN*::Tn*5*, *flgF*::Tn*M*, *flgM*::Tn*M*, *fliF*::Tn*M*, and *fliD*::Tn*M* mutants (data not shown). All of these transposon mutants are predicted to lack a functional flagellum, and they did not swim in a 0.3% agar swim assay (data not shown). When nonmotile *P. aeruginosa* mutants were tested in cocultures with *C. albicans* SC5314 yeast, there were no obvious differences between the mutants and the WT; all remained at the point of inoculation (Fig. 2A and data not shown). These data suggest that flagellar motility is necessary for *P. aeruginosa* movement across *C. albicans* filaments and that *P. aeruginosa* is incapable of this flagellum-dependent motility across lawns of *C. albicans* in the yeast form. When *P. aeruginosa* mutants defective in type IV pilus-mediated twitching motility (*pilB*::Tn*5* or *pilC*::Tn*5* mutant) or in the production of rhamnolipids (*rhlA*::Gm mutant), which are necessary for swarming motility on an agar surface, were assayed on lawns of the filamentous *C. albicans tup1*/*tup1* mutant, the zones of red pigmentation were indistinguishable from those formed by WT *P. aeruginosa* strain PA14 (Fig. 2B and data not shown) (37, 40).

To determine the relationship between the area of the *P. aeruginosa* colony on the *C. albicans* filaments and the size of the zone of the red pigment, samples from plate-grown fungal cocultures with either a WT or nonmotile (*flgK*) strain were

taken at 2-mm intervals from the point of inoculation and plated onto medium selective for *P. aeruginosa*. These assays showed that bacteria were present throughout the red-pigmented zone and that no bacteria were detected outside the red-pigmented area, indicating that the red pigmentation developed only in regions where the bacterial and fungal cells were in close proximity and that the red pigment did not diffuse away from the coculture region.

Involvement of phenazine-related genes in red pigment formation. Those mutants that lacked pigment production were altered in quorum sensing (three independent hits in *pqsA*, one in its regulator, *mvfR*, and one in *rhlR*). The two mutants with increased pigment production had insertions in *phzF1* and *phzS*, which both encode enzymes involved in pyocyanin biosynthesis. To further characterize the role of quorum-sensing regulation in phenazine production, mutants involved in quorum sensing and phenazine biosynthesis from the PA14 nonredundant mutant collection (28) were analyzed. Again, several mutants defective in signaling by PQS (*pqsA*::Tn*M* and *mvfR*::Tn*M* mutants) or C_4-homoserine lactone (*rhlR*::Tn*M* mutant), two quorum-sensing molecules, completely lacked red pigmentation upon coculture with *C. albicans* (Fig. 2C and data not shown). A mutant with a disrupted *rhlI* gene had a more variable phenotype, and *lasI* and *lasR* mutants produced pigment on fungal lawns. None of these mutants produced pyocyanin in single-species *P. aeruginosa* cultures.

A *P. aeruginosa phzM*::Tn*M* mutant, which lacks the phenazine biosynthetic enzyme necessary for methylation of PCA (Fig. 1B), formed no color upon coculture with *C. albicans* (Fig. 2C). In contrast, the *phzS*::Tn*M* mutant, which is defective in reduction of the 1-carboxylate group of PCA to an alcohol (Fig. 1B), produced more red pigmentation than the WT strain (Fig. 2C and D). Complementation analysis indicated that red pigmentation was largely restored to the *phzM* mutant by providing the *phzM* gene in *trans* and that the hyperpigmentation phenotype of the *P. aeruginosa phzS*::Tn*M* mutant was corrected by providing the *phzS* gene on a plasmid (Fig. 2D). Complementation of the *phzS* mutant also restored the production of pyocyanin in cocultures (Fig. 2D). The *phzH* mutant, which converts PCA to phenazine-1-carboxamide, produced slightly more red pigment than the WT strains did (data not shown) (32). While mutants defective in the *phzABCDEFG* biosynthetic genes involved in PCA biosynthesis were not identified in our assays as being defective in pigment production, likely due to the fact that there are redundant *phzABCDEFG* operons (Fig. 1A), the *P. aeruginosa* PA14 Δ*phzA1-G1* Δ*phzA2-G2* mutant (Δ*phz* mutant) (9) did not give rise to red pigmentation upon coculture with *C. albicans* (data not shown). Experiments with *P. aeruginosa* strain PAO1 similarly showed that the Δ*phzM* strain completely lacked production of the red pigment and that the PAO1 Δ*phzS* strain produced more red pigment than the WT strain did (data not shown). Because the fungus-associated red pigment required functional *phzABCDEFG* genes and *phzM*, but not *phzS* or *phzH*, we hypothesized that this pigment was derived from the PhzM product, proposed to be 5MPCA.

Our genetic experiments indicated that red pigmentation was dependent on a precursor to pyocyanin but not on pyocyanin itself. To determine if pyocyanin formation was negatively impacted by growth with *C. albicans*, its levels were determined in single-species and mixed-species cultures. Quantitative analysis of pyocyanin levels in agar core samples from *P. aeruginosa-C. albicans* cocultures grown on plates for 24 h detected 9.6 ± 1 nmol pyocyanin per core. The amount of pyocyanin was similar (9.7 ± 1.2 nmol pyocyanin per core) for single-species *P. aeruginosa* cultures grown on medium conditioned by *C. albicans* but in the absence of fungal cells. The numbers of *P. aeruginosa* CFU per unit area based on the core diameter were similar for both cultures ($1.56 \times 10^6 \pm 0.45 \times 10^6$/mm^2 and $1.2 \times 10^6 \pm 0.36 \times 10^6$/mm^2, respectively) at the 24-h time point. After 48 h, the levels of pyocyanin were higher in cocultures (106.8 ± 8.5 nmol per core) than the amounts in *P. aeruginosa* single-species cultures (39.3 ± 6.3 nmol per core), even though the numbers of *P. aeruginosa* CFU per unit area were similar ($1.65 \times 10^6 \pm 1.6 \times 10^6$/mm^2 and $1.64 \times 10^6 \pm 0.66 \times 10^6$/mm^2 in cocultures and single-species cultures, respectively). No red coloration was observed when *P. aeruginosa* was grown on *C. albicans*-conditioned medium without fungal cells. Because pyocyanin formation was similar or greater in *P. aeruginosa-C. albicans* cocultures than in single-species *P. aeruginosa* cultures, we propose that production of a 5MPCA product other than pyocyanin in cocultures is not likely due solely to inhibition of the final step involved in pyocyanin production.

Redox activity of the red pigment. When *P. aeruginosa-C. albicans* cocultures were harvested from the surface of the agar medium and resuspended in phosphate buffer, the cell suspension was similarly red pigmented (Fig. 2E). The color of the cell suspension changed from red to buff upon standing at room temperature for several minutes, and the red pigment was restored upon vigorous aeration. Similarly, the redness was intensified upon the addition of dilute H_2O_2, and the cell suspension converted from red to buff upon addition of a few crystals of dithionite (Fig. 2E). Color changes upon addition of reducing and oxidizing agents could be observed repeatedly using the same coculture suspension, suggesting that the pigment could be reversibly oxidized and reduced.

Correlation between pigment production and fungal viability in coculture. To determine if the formation of red pigmentation correlated with an altered viability of *C. albicans* SC5314, the survival of *C. albicans* was assessed by both methylene blue staining, which is indicative of a cell being metabolically inactive or membrane compromised, and comparing the total number of yeast cells determined by microscopic direct counts to the numbers of CFU. In *C. albicans* control cultures and *C. albicans* SC5314 cocultures with the Δ*phz* or *phzM*::Tn*M* strain, $<1\%$ of the cells were stained by methylene blue even after 48 h of coincubation (Fig. 3A). Yeast cells incubated with WT *P. aeruginosa* exhibited 14 and 26% methylene blue staining at 24 and 48 h, respectively. Cocultures with the *phzS* mutant, which led to increased red pigmentation, contained approximately twice as many methylene blue-stained cells (32 and 50% at 24 and 48 h, respectively).

Viability determined by combining direct counts with determination of CFU strongly supported the results from the methylene blue staining experiments. In the absence of bacteria, *C. albicans* yeast viability was 86% at the 72-h time point, as measured by these means (Fig. 3B). When *C. albicans* was cocultured with the *P. aeruginosa* PA14 strains, the viability in

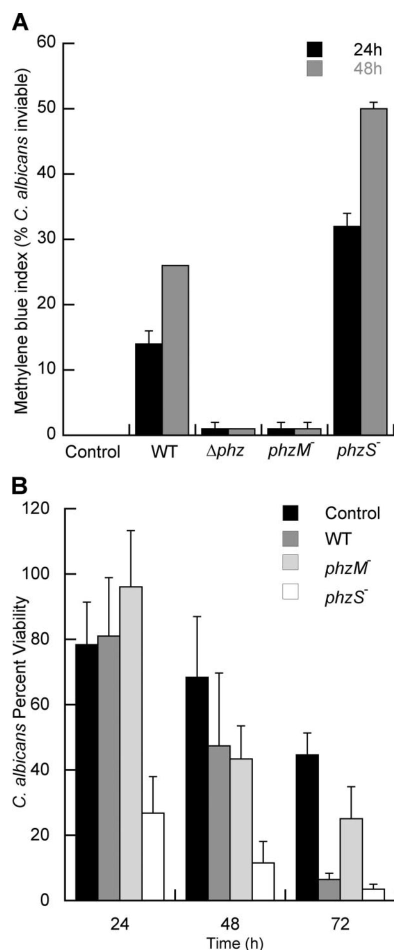

FIG. 3. Survival of *C. albicans* in coculture with strains of *P. aeruginosa* PA14. Established lawns of *C. albicans* SC5314 yeast were inoculated with *P. aeruginosa* suspensions. Core samples from plates were taken for counts of total cells by microscopy and of viable cells (CFU) after incubation for various times. (A) *C. albicans* methylene blue staining after incubation alone or with *P. aeruginosa* PA14 WT, Δ*phz* (which lacks the genes necessary to synthesize PCA), *phzM*::Tn*M*, and *phzS*::Tn*M* strains. (B) Survival of *C. albicans* after 72 h alone (control) or in coculture with the *P. aeruginosa* PA14 WT, *phzM*::Tn*M*, or *phzS*::Tn*M* strain. Percent viability represents the fraction of the total cells that gave rise to visually detectable colonies within 24 h. These experiments were repeated as three completely separate experiments, with similar results each time.

the presence of *P. aeruginosa* was reduced to 12% for WT *P. aeruginosa* and 3% for the *phzS* mutant. In contrast, *C. albicans* cocultured with the *phzM* strain had 76% viability. Analysis of variance found the differences between the WT and mutant strains to be statistically significant ($P < 0.05$). The differences in fungal killing were not due to differences in *P. aeruginosa* growth, as the numbers of *P. aeruginosa* CFU were equivalent (9.6×10^7 to 10.4×10^7 CFU per core) in all of the cocultures included in this experiment. These data indicate that the presence of the *phzM* gene, which leads to the formation of 5MPCA, is required for much of the killing of *C. albicans* by *P. aeruginosa* in this assay.

Accumulation of red pigment within fungal cells. Several pieces of evidence suggested that the red pigment that formed in *P. aeruginosa*-*C. albicans* cocultures was present only in association with the fungal cells. First, while pyocyanin diffused into the agar below the *P. aeruginosa*-*C. albicans* coculture, the red pigmentation was observed only in the layer of cells atop the agar medium in cocultures of up to 7 days old and did not diffuse into the surrounding agar (data not shown). Second, centrifugal separation of bacteria from the fungal cells showed that the red pigmentation was associated with the fungal pellet and was not present in the bacterium-containing fractions. No red coloration was observed in water or phosphate buffer washes of the fungal pellet, suggesting that the pigment was not capable of readily diffusing away from fungal cells (data not shown). The red pigmentation of the fungal cell fraction was redox sensitive (Fig. 2E). Third, when an established lawn of the *C. albicans tup1*/*tup1* mutant was covered with cellophane prior to inoculation with *P. aeruginosa*, removal of the bacterial colony after a 24-h growth period revealed red pigmentation only of the fungal cells (data not shown). Culturing experiments confirmed that *P. aeruginosa* and *C. albicans* remained separate over the course of the cellophane separation experiment. Experiments describing the release and purification of the red pigment from fungal cells are described below.

Visualization of cells grown in coculture with *P. aeruginosa* by epifluorescence microscopy provided additional information regarding the location of the red pigmentation that formed in *P. aeruginosa*-*C. albicans* cocultures. After incubation of WT *P. aeruginosa* on lawns of *C. albicans* yeast, a small amount of the coculture was resuspended in buffer and then observed by epifluorescence microscopy. *C. albicans* that had been incubated with WT *P. aeruginosa* exhibited bright intracellular fluorescence (Fig. 4A). The intracellular fluorescence was evident at 24 h, and the percentage of fluorescing cells increased with time. *C. albicans* cells from cocultures with the *P. aeruginosa phzS*::Tn*M* strain, which led to increased production of the red pigment, were also brightly fluorescent (Fig. 4A). In contrast, only a faint background fluorescence was observed with *C. albicans* grown either in the absence of *P. aeruginosa* or in the presence of the *P. aeruginosa phzM*::Tn*M* strain, which does not support red pigmentation (Fig. 4A). In many *C. albicans* cells from cocultures with WT *P. aeruginosa*, the red fluorescence seemed brightest in the cytoplasm (Fig. 4A), but in very brightly fluorescing cells, such as those detected in cocultures with the *phzS*::Tn*M* strain, the fluorescence appeared throughout the cell (Fig. 4A). The level of fluorescence per cell was not even across the population. Incubation of the constitutively filamentous *tup1*/*tup1* mutant with WT *P. aeruginosa*, but not the Δ*phz* or *phzM* mutant, also yielded brightly fluorescent cells (Fig. 4B). The addition of dithionite, the reducing agent that changed the visual pigmentation of the culture from red to colorless (Fig. 2E), also suppressed fluorescence (data not shown).

Effects of PhzM product on red pigmentation, intracellular fluorescence, and viability of fungal cells. Because cocultures with the *P. aeruginosa phzM*::Tn*M* mutant formed no red pigment, while those with the *phzS* mutant overproduced the red pigment, we hypothesized that the PhzM product, proposed to be 5MPCA, is responsible for the red pigmentation. To test this hypothesis directly, the PhzM product was synthesized by feeding PCA to resting cells of an *E. coli* strain carrying the *phzM* gene on a high-copy-number plasmid, using a previously

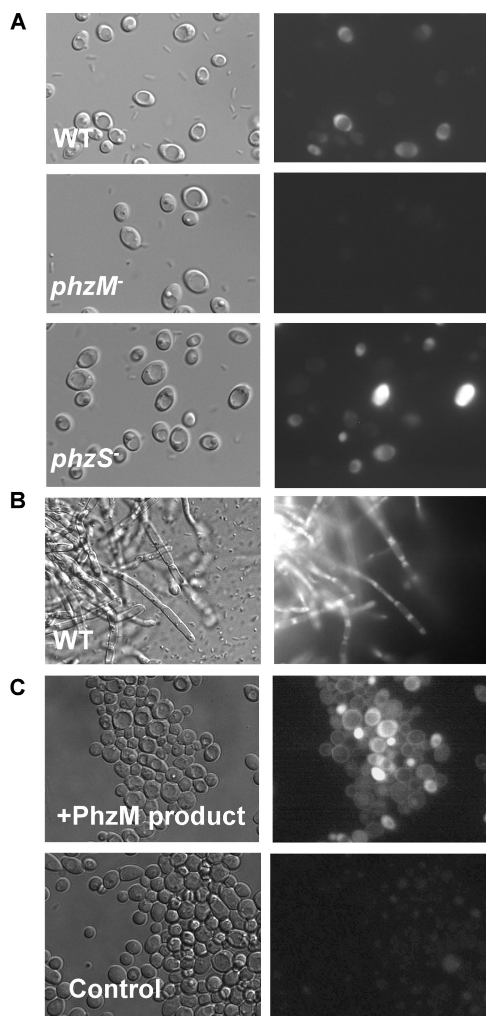

FIG. 4. Epifluorescence microscopy of *C. albicans* grown in coculture with *P. aeruginosa* or in medium containing the *E. coli*-synthesized PhzM product. For each pair of images, identical fields were photographed using differential interference contrast and Zeiss set 20 fluorescence optics for rhodamine. (A) *C. albicans* SC5314 from 72-h cocultures with *P. aeruginosa* PA14 WT, *P. aeruginosa phzM*::Tn*M*, or *P. aeruginosa phzS*::Tn*M* strain. (B) *C. albicans tup1*/*tup1* mutant from 72-h cocultures with *P. aeruginosa* PA14 WT. No fluorescence was observed in similar cocultures with a *P. aeruginosa* Δ*phzM* strain (not shown). (C) *C. albicans* SC5314 after 24 h of incubation with either ~200 μM 5MPCA prepared by incubating *E. coli*/pUCP-M (expressing the *phzM* gene) with PCA (+PhzM product) or extracts from a control preparation from *E. coli*/pUCP26 incubated with PCA (control).

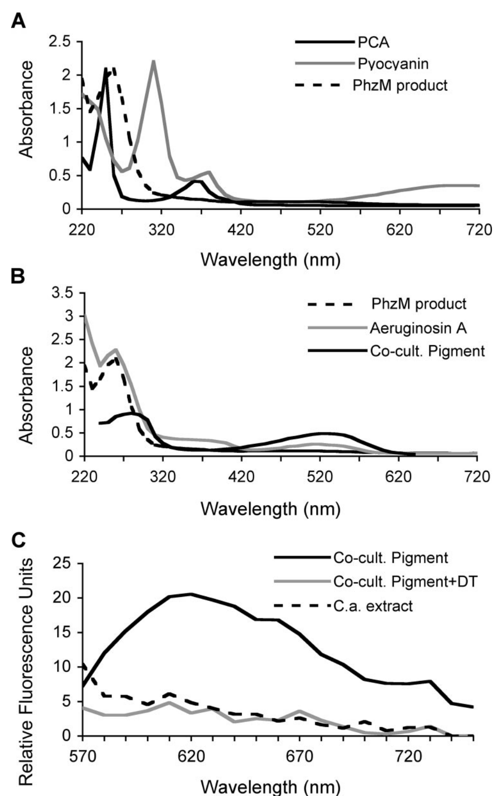

FIG. 5. Spectra of late intermediates in pyocyanin biosynthesis and the coculture pigment. All solutions were in 0.1 M NH_4HCO_3 and were normalized at the UV maximum. (A) Absorption spectra of PCA, the *E. coli*-synthesized PhzM product, and pyocyanin. (B) Absorption spectra of 5MPCA, aeruginosin A, and the partially purified coculture pigment. (C) Fluorescence emission spectra of partially purified red pigment from *C. albicans* cells grown in coculture with *P. aeruginosa* (solid lines). Excitation was done at 550 nm. The pigment was analyzed before (black line) and after (gray line) the addition of a few crystals of sodium dithionite (DT). A comparable fraction from *C. albicans* (C.a.) cells grown in the absence of *P. aeruginosa* was also analyzed (dashed line).

published protocol (32). The supernatants from *E. coli*/pUCP-M incubated with PCA were yellow-orange after 4 h and developed a reddish color after 8 h of incubation. The colored product(s) remained in the aqueous phases during chloroform extraction to remove the unreacted PCA. Quantification of the PCA extracted from the suspensions at the end of the incubation period suggested that ~10% of the PCA was acted upon by PhzM. The spectrum for the aqueous extracts from PCA incubation with PhzM was distinct from the spectrum for PCA (Fig. 5A).

When *C. albicans* SC5314 yeast cells were incubated in YPD medium with the PCA-free aqueous extracts containing the PhzM product for 24 h at 30°C, the *C. albicans* cells developed a reddish color that was visible by eye in the cell pellet after centrifugation, even after repeated washing with buffer. No pigmentation was observed in the *C. albicans* cell pellets after incubation with aqueous extracts from *E. coli*/pUCP-M cultures that did not receive PCA or from the vector control strain, *E. coli*/pUCP26, incubated with PCA. Epifluorescence microscopy of yeast cells incubated with extracts containing the PhzM product found that 8.5% ± 2.3% of cells fluoresced brightly and the remaining cells had increased fluorescence (Fig. 4C). The lack of fluorescence in yeast cells from the above-described control cultures confirmed that the 5MPCA product was necessary for development of the intracellular fluorescence (Fig. 4C). After 48 h, *C. albicans* yeast cells incubated with the PhzM product had reduced viability (36.7% ± 3.2%) in comparison to *C. albicans* yeast cells incubated with the control extract from *E. coli*/pUCP26 with PCA (52.7% ± 11.6% viable).

Comparison of 5MPCA-derived pigment within *C. albicans* cells to known *P. aeruginosa* phenazines. To determine the nature of the red-pigmented product observed within fungal cells, suspensions of *C. albicans* from *P. aeruginosa*-*C. albicans* cocultures were lysed and the red-pigmented products were analyzed. While the red product 5MPCA and derivatives synthesized from PCA by *E. coli*/pUCP-M are highly soluble in water, the red pigment within fungal cells was poorly soluble in either aqueous solutions (water or acidic or basic buffers) or organic solvents (chloroform, ethyl acetate, or ethanol). Mechanical or enzymatic and chemical disruption of cells, followed by centrifugation of the cell lysate at $10,000 \times g$, led to sedimentation of the majority of the pigment, yielding only a pale pink supernatant. Conditions that promote yeast autolysis, including incubation of dense cell suspensions at 37°C for 24 to 48 h, led to further, but far from complete, release of pigment in a time-dependent manner. Heat inactivation of the cell suspension prior to incubation under autolysis conditions prevented release of red-pigmented products into the aqueous supernatant. The observations suggest that pigment is aggregated or polymerized inside the fungus but can be released slowly by the action of endogenous fungal enzymes.

Partial purification of red pigment from autolyzed suspensions of the *C. albicans tup1*/*tup1* mutant from *P. aeruginosa* cocultures could be achieved by size-exclusion chromatography on Bio Gel P-2 (molecular weight, 200 to 2,000; Bio-Rad). The red fractions emerged close to the void volume, whereas pyocyanin and vitamin B_{12} eluted in later fractions that corresponded to their predicted molecular weights of 210 and 1,355, respectively (49). These data suggest that the red pigment molecule(s) is of a substantially larger size than that of other phenazines known to be produced by *P. aeruginosa*. For spectrophotometric analysis, the red fractions were further purified using a C_{18} hydrophobic interaction column (LRP-2; Whatman) in 1 M NH_4CO_3, followed by elution with 10% acetonitrile. The red fraction had a single broad absorbance maximum at ~530 nm in the visible region (Fig. 5B) but lacked the maximum at ~390 nm, which is characteristic of red *P. aeruginosa* phenazines aeruginosins A (Fig. 5B) and B (not shown), as reported previously by Holliman et al. (15, 20). The spectrum of the red pigment isolated from fungal cells also differed from those of other known *P. aeruginosa* phenazines, including PCA, pyocyanin, and the products of PhzM activity on PCA produced in *E. coli* (Fig. 5).

To determine if the fluorescence observed within *C. albicans* yeast cells from cocultures by epifluorescence microscopy could be attributed to the red pigment that was partially purified from *C. albicans*, the fluorescence spectrum of the partially purified material was measured with an excitation wavelength of 550 nm (Fig. 5C). Red fluorescence was detected with a maximum at 620 nm when the sample was aerated. Consistent with what was observed in the *C. albicans* whole-cell suspensions, the fluorescence was not observed in the same sample after reduction with dithionite (Fig. 5C). A comparable preparation from *C. albicans* cultures grown in the absence of *P. aeruginosa* yielded fractions with no detectable fluorescence (Fig. 5C).

While we were not able to determine the identity of the fungally associated pigment that formed upon incubation of *C. albicans* with the products of PhzM activity on PCA- or 5MPCA-producing strains, largely due to its insolubility, it is clear that it has properties that are distinct from those of the phenazines present in single-species *P. aeruginosa* cultures. We could detect this red pigment within *C. albicans* cells only after coculture with *P. aeruginosa* or upon incubation with the PhzM product, leading us to hypothesize that the pigment results from an activity on 5MPCA that occurs within the fungal cell. Incubation of fungal cells with the partially purified red pigment, aeruginosins A or B, PCA, or pyocyanin at concentrations of up to 1 mM did not give rise to red-pigmented fungal cells with intracellular fluorescence. It is important, however, that while treatment of the partially purified red pigment with 1 mg/ml proteinase K, DNase, RNase, boiling, acid (1 M HCl), base (1 M NaOH), or sodium dodecyl sulfate (1%) did not liberate a homogenous low-molecular-weight species, we cannot rule out the alternative hypothesis which states that 5MPCA or a derivative thereof is unaltered but aggregated in some way.

DISCUSSION

The studies reported here show that the *P. aeruginosa* product of PhzM activity on PCA, proposed to be 5MPCA, leads to the accumulation of a red-pigmented, redox-active compound within fungal cells. Like the *C. albicans* cells from cocultures, the partially purified pigment exhibited a red coloration that was colorless upon reduction and could readily be oxidized by aeration or the addition of hydrogen peroxide (Fig. 2E). Both the pigmentation in association with fungal cells and the partially purified pigment exhibited a red fluorescence only in an oxidized state. *P. aeruginosa* PA14 and PAO1 mutants that were defective in *phzM*, the gene responsible for the methylation of PCA to form 5MPCA, were incapable of red pigment formation. Furthermore, pseudomonads such as *Pseudomonas putida* and *Pseudomonas fluorescens*, whose genomes do not contain *phzM* (53), did not induce the accumulation of pigment on *C. albicans* lawns. In contrast, deletion of the *phzS* gene, which encodes the enzyme that acts upon 5MPCA to form pyocyanin, led to hyperpigmentation (Fig. 2), suggesting that the formation of pyocyanin competed for the 5MPCA pool. Disruption of the *phzH* gene, which encodes an enzyme that acts on PCA (32), also led to increased red pigment formation, suggesting that PhzH competes with PhzM for the PCA pool within cocultures.

The putative intermediate 5MPCA, which is responsible for the coculture pigment, has not been studied extensively. While its immediate precursor, PCA, and its immediate derivative, pyocyanin, accumulate in *P. aeruginosa* cultures, 5MPCA has not been detected in supernatants (4, 14). Because pyocyanin levels were higher in *P. aeruginosa*-*C. albicans* cocultures than in *P. aeruginosa* cultures grown in the absence of *C. albicans* but on *C. albicans*-conditioned medium, it does not appear that the release of the PhzM product was simply due to inhibition of the last PhzS-dependent step in pyocyanin production by *C. albicans*. In vitro assays with purified PhzM and PhzS indicate that PhzM activity is greatly enhanced or dependent on PhzS (39), suggesting that PhzM reaction kinetics are favorable only when the product, 5MPCA, is consumed. Furthermore, 5MPCA synthesized as a phenazinium chloride was found to be stable in acid but not tractable at neutral pH (14). The fact

that the fungally associated red pigment formed only when *P. aeruginosa* and *C. albicans* were grown in close contact with one another leads us to speculate that *C. albicans* uptake and subsequent modification of a small extracellular pool of 5MPCA may increase PCA conversion to 5MPCA and the formation of 5MPCA-derived products. In a medium that supports growth of *P. aeruginosa* on the surfaces of *C. albicans* hyphae (8), the fungal pellet develops a distinct pink coloration, and red fluorescent *C. albicans* cells were observed upon coculture with WT *P. aeruginosa* and *phzS* mutant strains (data not shown). The role that fungal cells play in promoting the formation and release of the 5MPCA intermediate is a subject of our future research.

The exact chemical nature of this coculture red pigment extracted from fungi remains elusive. Its UV-visible spectrum and solubility characteristics are distinct from those of phenazines known to be produced by monocultures of *P. aeruginosa*, including PCA, pyocyanin, and aeruginosins A and B, and from the product in extracts from PCA-fed cultures of *E. coli* carrying the *phzM* gene (15, 20) (Fig. 5A and B). 5MPCA is converted to a red phenazine, aeruginosin A, upon incubation with late *P. aeruginosa* culture supernatants or through a chemical reaction with concentrated ammonia (14). A similar, soluble red phenazine accumulates in supernatants of *phzS* mutant cultures, as shown previously (32) and confirmed by our laboratory (data not shown). While aeruginosin A and the red pigment in *phzS* culture medium are very soluble in liquid and agar media, the red phenazine derivative formed in yeast was located exclusively in yeast cells and did not diffuse into the surrounding medium. Incubation of *C. albicans* with purified aeruginosins did not give rise to red-pigmented yeast (data not shown). Nevertheless, the absorption spectrum of the coculture pigment does resemble that of aeruginosin in that it has an absorption maximum in the 520-nm range.

The concentration of the coculture pigment in the fungal cell fraction may indicate that fungal enzymes or other factors in yeast participate in the formation of the red pigment. At this time, however, we know little about the factor or factors that lead to the formation of the red product with poor solubility and an apparent molecular weight that is significantly larger than that of the 5MPCA precursor. Because formation of the red pigment is observed upon coculture with numerous fungi, including *S. cerevisiae*, any modifications are not unique to *C. albicans*. The red pigmentation that develops in *ade2* mutants of both *S. cerevisiae* and *C. albicans* grown in media with limiting concentrations of adenine, due to the conjugation of the toxic adenine biosynthetic precursors with glutathione (5, 44), is distinct from the pigment we observe in *P. aeruginosa*-*C. albicans* cocultures. The red pigment in *ade2* mutants is not redox sensitive (50). The addition of adenine to the agar medium does not alter pigmentation of *C. albicans* in our experiments (data not shown).

Previous reports have demonstrated the toxicities of pyocyanin and 1-hydroxyphenazine on *C. albicans* and *S. cerevisiae*, with MICs in the 100- to 500-μM range (24, 42). Our studies indicate that the product of PhzM activity on PCA also leads to decreased fungal viability. Because the precise nature of the 5MPCA product has not been described and purified preparations are reported to be unstable at neutral pH (14), direct quantitative comparison of its lethal effect on yeast to that of other phenazines has not been performed. The *phzS* mutant, which leads to increased *C. albicans* red pigmentation and fluorescence compared to those of *C. albicans* cocultured with WT *P. aeruginosa*, is also more lethal for yeast than the WT strain (Fig. 4). These data indicate that the putative product of the PhzM enzyme, 5MPCA, is more important for yeast killing than pyocyanin when the two species are grown in close proximity to one another. While pyocyanin has previously been demonstrated to play an important role in *P. aeruginosa* virulence (24, 42), previous studies by Lau et al. found that both the *phzM* and *phzS* mutants were attenuated in lung infections in vivo. It is interesting, however, that the *phzM* mutant exhibited a greater defect, as determined by competitive index, than the *phzS* mutant in these in vivo assays (26). Experiments with both partially purified pigment and whole *C. albicans* cells showed that the fungally associated red pigment retained redox activity within the fungus despite any modifications that may occur within the fungal cell (Fig. 2E and 5C). Reaction with oxygen is important for the toxicity of many different phenazines toward a variety of species (31), and we hypothesize that the redox activity of the fungally associated pigment contributes to the decreased *C. albicans* viability that we observe in its presence.

While many phenazines have been shown to have toxic effects toward a variety of species, including fungi, it is not yet known if previously characterized phenazine antibiotics are modified or sequestered by other organisms (22, 27, 31, 48). One report describes the production of an extracellular red pigment, with different properties from the pigment described here, that forms in cocultures of *Aspergillus sclerotiorum* and *Pseudomonas chlororaphis*, suggesting that other fungi may be able to modify certain bacterially produced phenazines (17); the biological activity of the modified *P. chlororaphis* phenazine is not known. One can envision many ways in which modification or aggregation of phenazines after secretion by the producing microbe could enhance or reduce the toxicity of the antibiotic, and these processes may be important factors to consider in the design of phenazine-producing biocontrol strains.

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