AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

RESEARCH ARTICLE

Studies of *Pseudomonas aeruginosa* Mutants Indicate Pyoverdine as the Central Factor in Inhibition of *Aspergillus fumigatus* Biofilm

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ABSTRACT *Pseudomonas aeruginosa* and *Aspergillus fumigatus* are common opportunistic bacterial and fungal pathogens, respectively. They often coexist in airways of immunocompromised patients and individuals with cystic fibrosis, where they form biofilms and cause acute and chronic illnesses. Hence, the interactions between them have long been of interest and it is known that *P. aeruginosa* can inhibit *A. fumigatus in vitro*. We have approached the definition of the inhibitory *P. aeruginosa* molecules by studying 24 *P. aeruginosa* mutants with various virulence genes deleted for the ability to inhibit *A. fumigatus* biofilms. The ability of *P. aeruginosa* cells or their extracellular products produced during planktonic or biofilm growth to affect *A. fumigatus* biofilm metabolism or planktonic *A. fumigatus* growth was studied in agar and liquid assays using conidia or hyphae. Four mutants, the *pvdD pchE*, *pvdD*, *lasR rhlR*, and *lasR* mutants, were shown to be defective in various assays. This suggested the *P. aeruginosa* siderophore pyoverdine as the key inhibitory molecule, although additional quorum sensing-regulated factors likely contribute to the deficiency of the latter two mutants. Studies of pure pyoverdine substantiated these conclusions and included the restoration of inhibition by the pyoverdine deletion mutants. A correlation between the concentration of pyoverdine produced and antifungal activity was also observed in clinical *P. aeruginosa* isolates derived from lungs of cystic fibrosis patients. The key inhibitory mechanism of pyoverdine was chelation of iron and denial of iron to *A. fumigatus*.

IMPORTANCE Interactions between human pathogens found in the same body locale are of vast interest. These interactions could result in exacerbation or amelioration of diseases. The bacterium *Pseudomonas aeruginosa* affects the growth of the fungus *Aspergillus fumigatus*. Both pathogens form biofilms that are resistant to therapeutic drugs and host immunity. *P. aeruginosa* and *A. fumigatus* biofilms are found *in vivo*, e.g., in the lungs of cystic fibrosis patients. Studying 24 *P. aeruginosa* mutants, we identified pyoverdine as the major anti-*A. fumigatus* compound produced by *P. aeruginosa*. Pyoverdine captures iron from the environment, thus depriving *A. fumigatus* of a nutrient essential for its growth and metabolism. We show how microbes of different kingdoms compete for essential resources. Iron deprivation could be a therapeutic approach to the control of pathogen growth.

KEYWORDS *Aspergillus fumigatus*, *Pseudomonas aeruginosa*, biofilms, intermicrobial interaction, iron, mutants, pyoverdine

Received 25 May 2017 **Accepted** 2 October 2017

Accepted manuscript posted online 16 October 2017

Citation Sass G, Nazik H, Penner J, Shah H, Ansari SR, Clemons KV, Groleau M-C, Dietl A-M, Visca P, Haas H, Déziel E, Stevens DA. 2018. Studies of *Pseudomonas aeruginosa* mutants indicate pyoverdine as the central factor in inhibition of *Aspergillus fumigatus* biofilm. J Bacteriol 200:e00345-17. https://doi.org/10.1128/JB.00345-17.

Editor George O'Toole, Geisel School of Medicine at Dartmouth

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Pseudomonas aeruginosa and *Aspergillus fumigatus* are the most prominent bacterium and fungus, respectively, in the airways of cystic fibrosis (CF) patients (1–4). They have been associated with deterioration of lung function (1, 5–14), and it has been suggested that their combined presence in those airways has an even worse potential (15, 16). Both of these microbes are also prominent opportunists in immunocompromised patients, particularly neutropenic patients (17, 18).

In studies of *P. aeruginosa-A. fumigatus* interactions, it has long been noted in *in vitro* studies that *P. aeruginosa* products can be toxic to *A. fumigatus*. These *P. aeruginosa* products include phenazines such as pyocyanin (5-*N*-methyl-1-hydroxyphenazine) (19–22), 1-hydroxyphenazine (19, 21, 22), phenazine-1-carboxamide, phenazine-1-carboxylic acid (22), and dirhamnolipids (23). Other *P. aeruginosa* products have been noted to be toxic to other fungi, though their effects on *A. fumigatus* have not been studied, including 3,4-dihydroxy-2-heptylquinoline (PQS) and phenazin-1-ol (24), 3,4-dihydroxy-*N*-methyl-4-(4-oxochroman-2-yl)butanamide (25), and lipopolysaccharide (26). However, studying individual molecules for toxicity does not give insight into their relative contributions in a mixture, how the molecules would behave in the presence of other *P. aeruginosa* and/or *A. fumigatus* metabolites, or whether the concentrations studied are relevant to those that occur in intermicrobial interactions (27, 28). An alternative approach to begin to understand the intermicrobial factors involved is to study *P. aeruginosa* mutants and examine the effects of removing molecules, groups of them, or pathways by mutational analysis. We studied a series of mutants (Table 1) defective in the expression of most known *P. aeruginosa* virulence functions, including the production of bioactive molecules and secondary metabolites previously reported to interact with eukaryotic cells, and compared their inhibitory activity against *A. fumigatus* biofilms, as well as planktonic *A. fumigatus* growth, to that of the parent *P. aeruginosa* strain.

RESULTS

PA14 *pvdD pchE*, *pvdD*, *lasR rhlR*, and *lasR* mutants are most impaired in their antifungal activities. Twenty-four PA14 mutants (Table 1) and wild-type PA14 were tested for the ability to interfere with *A. fumigatus* metabolism by using bacterial live cells (LC), *P. aeruginosa* planktonic culture filtrate (PCF), or *P. aeruginosa* biofilm culture filtrate (BCF) as the agents and *A. fumigatus* biofilm formation or preformed *A. fumigatus* biofilm as the target (resulting in six possible assay combinations for each assay method). The results will be considered in turn and then summarized at the end of Results.

Effects of LC, BCF, and PCF on *A. fumigatus* biofilm formation. As *A. fumigatus* biofilm forms from conidia in agar, *P. aeruginosa* LC inhibition of growth can be manifested by clear zones around a well in which the LC are placed. Figure 1A shows a summary of four inhibition zone experiments. In these assays, 12 mutants produced zones of inhibition that were significantly smaller than the inhibition zones of wild-type PA14 LC. These are ranked by zone size in Table 2. An example of *A. fumigatus* inhibition zones generated by *P. aeruginosa* LC is shown in Fig. S1 in the supplemental material.

When *P. aeruginosa* supernatants were used in the wells instead of live cells and *A. fumigatus* grown from conidia were used, no zones of inhibition were produced. However, there was inhibition of metabolism of *A. fumigatus* forming biofilm, and this could be measured by the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay (bioassay-conidium-agar-metabolic [BCAM] assays). Figure 1B shows the *A. fumigatus* inhibition results obtained with PCF of mutants 1 to 24 and wild-type PA14. In these assays, nine mutants demonstrated significantly impaired antifungal activity (ranked from most to least impaired Table 2). Figure 1C shows the results of this assay but with BCF. Four mutants showed significantly inhibited antifungal activity (ranked in Table 2).

In all three agar assays of *A. fumigatus* biofilm formation (Table 2), the *pvdD pchE* and *pvdD* mutants had impaired antifungal activity. The *pvdD pchE* mutant was the most impaired mutant in all three assays. The *lasR rhlR* and *lasR* mutants were also impaired

TABLE 1 PA14 mutants used in this study

Mutant no.	Mutation(s)	Result(s) of mutation(s)[a]	Reference(s)
1	*pvdD pchE*	Pyoverdine-pyochelin double-siderophore mutant	This study
2	*pqsE*	Gene mediates regulatory activity of MvfR system (see *mvfR*); HAQs similar to wild type but defective in pyocyanin, HCN	87
3	*mvfR*	Regulates transcription of *pqsABCDE* operon; underproduction of various QS-regulated factors related to loss of HAQs, phenazines, proteases, HCN, lectins, others (94)	88
4	*pqsA*	First step in HAQ synthesis; gene for anthranilate coenzyme A ligase lost; loss of extracellular quinolones, HAQ biosynthesis, including HHQ, PQS, DHQ; also decreased activity of MvfR (89)	87
5	*pqsH*	Product converts HHQ into PQS; loss of 2-heptyl-3-hydroxy-4(1H)-quinolone synthase; loss of PQS biosynthesis, thus decreased activity of MvfR; MvfR not as negatively affected as in *pqsA* mutant since *pqsH* mutant still produces HHQ and other HAQs that can act as ligands of MvfR	90
6	*lasR rhlR*	Double mutant defective for most QS-regulated metabolites, including phenazines, rhamnolipids, AHL, HAQs, proteases, HCN, chitinase, elastase, others	91
7	*lasR*	Lacks several QS-regulated factors, including proteases, oxo-C_{12}-HSL; delayed activation of *pqsABCDE* and of RhlR QS pathway	87
8	*rsmA*	Global posttranscriptional regulator mutant; various effects, including less rhamnolipids, more phenazines, and HCN (92)	93
9	*pqsA pqsH* not polar	Same predicted outcome as *pqsA* mutant; in both, *pqsA* mutation is nonpolar on downstream genes in operon, thus not preventing their transcription	90
10	*pvdD*	Loss of pyoverdine (siderophore)	93
11	*rhlR*	Lacks several QS-regulated factors; loss of rhamnolipids, phenazines, HCN, lectins, C_4-HSL	94
12	ΔHSI-I ΔHSI-II	Double-deletion mutant defective in 2 of 3 type VI secretion systems	95
13	*pvcA*	Loss of paerucumarin and pseudoverdin	93
14	*rhlA*	Loss of rhamnolipids (93)	96
15	*phzC1 phzC2*	Double-phenazine mutant (completely abrogated); no pyocyanin	Unpublished[b]
16	*pchE*	Loss of pyochelin (siderophore)	93
17	*exoU*	Loss of exotoxin U via type III secretion	93
18	*rsmY rsmZ*	Loss of genes for coding small regulatory RNAs; antagonistic to RsmA; decreased production of C_4-HSL, phenazines, chitinase (92)	This study
19	ΔHSI-II ΔHSI-III	Double-deletion mutant defective in 2 of 3 type VI secretion systems	95
20	ΔHSI-I ΔHSI-III	Double-deletion mutant defective in 2 of 3 type VI secretion systems	95
21	*pqsA pqsH* polar	*pqsA*::TnPhoA, *pqsH*::Gm; mutation in *pqsA* is polar (thus theoretically preventing transcription of downstream genes in operon); Kan^r Gm^r	Unpublished[b]
22	*chiC*	MrT7 transposon mutant; Gm^r	93
23	*lecA*	MrT7 transposon mutant; Gm^r	93
24	*hcnA*	MrT7 transposon mutant; Gm^r	93
25	None	None (parental strain UBCPP-PA14)	77, 78

[a]Abbreviations: HAQ, 4-hydroxy-2-alkylquinolones; HCN, hydrogen cyanide; AHL, acylhomoserine lactones; HSL, homoserine lactone; HHQ, 4-hydroxy-2-heptylquinoline; PQS, 3,4-dihydroxy-2-heptylquinoline (97); HQNO, 4-hydroxy-2-heptylquinoline-*N*-oxide. Kan, kanamycin; Gm, gentamicin.
[b]Unpublished laboratory strain (available from E. Déziel).

in all three assays and among the four highest ranking mutants in all three assays. The *rhlR*, *pvcA*, and polar *pqsA pqsH* mutants were impaired in two of the three assays.

Effects of PCF and BCF on preformed *A. fumigatus* strain 10AF biofilm in agar (BHAM) assays. The effects of supernatants on preformed *A. fumigatus* strain 10AF biofilm are summarized in Table 3. Figure 2A shows mutants 1 to 24, as well as wild-type PA14, tested in these bioassay-hypha-agar-metabolic (BHAM) assays with PCF. Five mutants showed significantly decreased antifungal activity, with again the two pyoverdine mutants, the *pvdD* and *pvdD pchE* mutants, and the two impaired *las* quorum-sensing (QS) system mutants, the *lasR* and *lasR rhlR* mutants, prominent (Table 3). With BCF, the picture was nearly identical to that obtained with PCF (Fig. 2B and Table 3). Thus, fewer mutants were impaired in the inhibition of preformed *A. fumigatus* biofilm than in the inhibition of *A. fumigatus* biofilm formation (compare Tables 2 and 3), although four mutants, the *pvdD pchE*, *pvdD*, *lasR rhlR*, and *lasR* mutants, appear prominently as impaired mutants on these lists against both forming and preformed *A. fumigatus* biofilms.

Effects of BCF and PCF on forming and preformed *A. fumigatus* strain 10AF biofilms under hypoxic conditions. To more closely mimic some focal CF lung

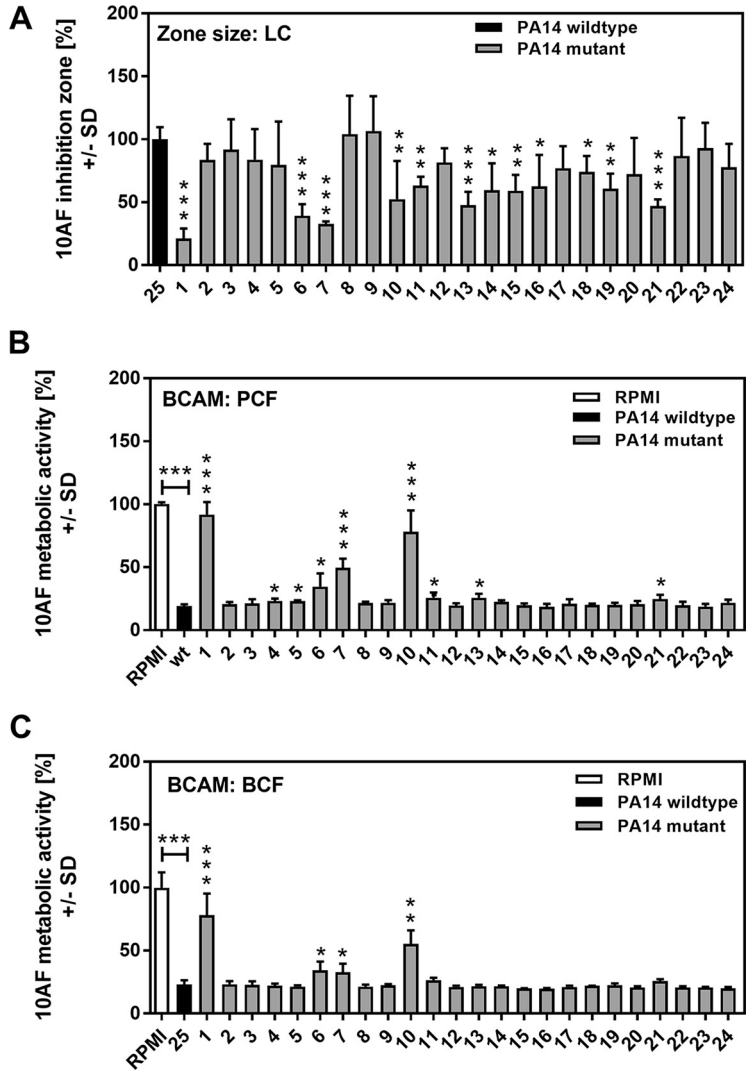

FIG 1 Effects of LC, PCF, and BCF on *A. fumigatus* strain 10AF biofilm formation and metabolism. (A) Wells in agar with *A. fumigatus* conidia were loaded with wild-type or mutant PA14 LC suspensions and incubated, and the area of the fungus-free (inhibition) zone around each well was measured. The inhibition zone created by wild-type PA14 LC was regarded as 100% inhibition, and inhibition zones created by mutants were compared to this. The results of four experiments are combined, with duplicates of each study article in each. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ (mutant [no. 1 to 24] versus wild type [no. 25]). (B and C) Agar and *A. fumigatus* strain 10AF conidia were distributed into 96-well cell culture plates that were loaded with wild-type or mutant PA14 PCF (B) or wild-type or mutant PA14 BCF (C) and incubated at 37°C for 24 h. *A. fumigatus* strain 10AF metabolism was evaluated by XTT assay. Metabolism in the presence of RPMI medium alone was regarded as 100% *A. fumigatus* strain 10AF metabolic activity. The data shown are the mean ± SD of four independent experiments (with duplicates of each group studied in each experiment). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. Vertical asterisks, mutants (no. 1 to 24) versus wild-type PA14 (no. 25); horizontal asterisks, RPMI medium versus wild-type PA14. Bars: 1, *pvdD pchE* mutant; 2, *pqsE* mutant; 3, *mvfR* mutant; 4, *pqsA* mutant; 5, *pqsH* mutant; 6, *lasR rhlR* mutant; 7, *lasR* mutant; 8, *rsmA* mutant; 9, *pqsA pqsH* not polar mutant; 10, *pvdD* mutant; 11, *rhlR* mutant; 12, ΔHSI-I ΔHSI-II mutant; 13, *pvcA* mutant; 14, *rhlA* mutant; 15, *phzC1 phzC2* mutant; 16, *pchE* mutant; 17, *exoU* mutant; 18, *rsmY rsmZ* mutant; 19, ΔHSI-II ΔHSI-III mutant; 20, ΔHSI-I ΔHSI-III mutant; 21, *pqsA pqsH* polar mutant; 22, *chiC* mutant; 23, *lecA* mutant; 24, *hcnA* mutant.

conditions, we repeated part of our experiments under hypoxic conditions. PCF of wild-type PA14, the *pvdD pchE* and *pvdD* mutants, which had shown the lowest overall antifungal activity, and the *pchE* and *phzC1 phzC2* mutants was tested in BCAM and BHAM assays under hypoxic conditions. Our results obtained under hypoxic conditions closely resemble those obtained under normoxic conditions (compare Fig. 3A to Fig. 1B and Fig. 3B to Fig. 2A), with the *pvdD* and *pvdD pchE* mutations affecting the antifungal activity of *P. aeruginosa* supernatants significantly.

TABLE 2 Ranking of PA14 mutant LC, PCF, and BCF activities against *A. fumigatus* strain 10AF forming biofilm[a]

	Mutation(s) ranked on basis of:		
Rank	Inhibition zone size, agar-LC	XTT conversion, agar-PCF	XTT conversion, agar-BCF
1	*pvdD pchE*	*pvdD pchE*	*pvdD pchE*
2	*lasR*	*pvdD*	*pvdD*
3	*lasR rhlR*	*lasR*	*lasR rhlR*
4	*pqsA pqsH* polar	*lasR rhlR*	*lasR*
5	*pvcA*	*rhlR*	
6	*phzC1 phzC2*	*pvcA*	
7	*rhlA*	*pqsA pqsH* polar	
8	ΔHSI-I ΔHSI-II	*pqsA*	
9	*pchE*	*pqsH*	
10	*rhlR*		
11	*rsmY rsmZ*		
12	*pvdD*		

[a]All assays started from *Aspergillus* conidia. Ranking from 1 to *n* represents highest to lowest loss of antifungal activity. Ranking was performed as described in Materials and Methods (Fig. 1). Only isolates statistically significantly different from the parent strain are listed.

Effects of BCF and PCF on planktonic *A. fumigatus* strain 10AF growth (MIC). Because there are demonstrated differences in *P. aeruginosa* activity against biofilm and planktonic *A. fumigatus* (29), we also investigated the activity of the mutants against planktonic *A. fumigatus*. Our results show that PCF and BCF derived from the *pvdD pchE*, *pvdD*, *lasR rhlR*, and *lasR* mutants interfered with planktonic fungal growth significantly less than wild-type supernatants did (Fig. 4). In addition, PCFs of the *pqsA*, *pqsH*, ΔHSI-I ΔHSI-II, and *pvcA* mutants, as well as BCF of the *rsmY rsmZ* mutant, showed significantly less antifungal activity. The reduction of antifungal activity by these mutants was much weaker than the reduction of antifungal activity by mutated inactivation of *pvdD pchE*, *pvdD*, *lasR rhlR*, or *lasR* (Table 4). Table S1 details the geometric mean values and ranges for all of the mutants shown in Table 4.

Overview of different assays of mutants as inhibitors of *A. fumigatus*. In summary, when the activities of all of the mutants tested in all seven normoxic assays were compared, the *pvdD pchE*, *pvdD*, *lasR rhlR*, and *lasR* mutants consistently showed the greatest magnitude of decreased antifungal activity (Table 5).

Pyoverdine is a crucial factor in *P. aeruginosa* antifungal activity. One common feature of mutants we found impaired in antifungal activity is the loss or lack of pyoverdine production. Figure 5A visualizes pyoverdine production in all 24 PA14 mutants, as well as the wild type. The *pvdD pchE* and *pvdD* mutants show complete loss of pyoverdine production, while the *lasR rhlR* and *lasR* mutants show substantial reductions of pyoverdine production. Some mutants actually show slightly increased pyoverdine production, i.e., the *pqsE*, *rsmA*, *rhlR*, *pchE*, *lecA*, and *hcnA* mutants. The most significant overproducer of pyoverdine is the *pchE* mutant; this was not unexpected, since it cannot produce the other siderophore, pyochelin, thus corroborating our

TABLE 3 Ranking of PA14 mutant PCF and BCF activities against *A. fumigatus* strain 10AF preformed biofilm[a]

	Mutation(s) tested on:	
Rank	Agar-PCF	Agar-BCF
1	*pvdD*	*pvdD*
2	*pvdD pchE*	*pvdD pchE*
3	*lasR*	*lasR rhlR*
4	*rsmA*	*lasR*
5	*lasR rhlR*	

[a]All assays started from *Aspergillus* hyphae. The readout in both assays was XTT conversion. Ranking from 1 to *n* represents the highest to lowest loss of antifungal activity. Ranking was performed as described in Materials and Methods. Only isolates statistically significantly different from the parent strain are listed.

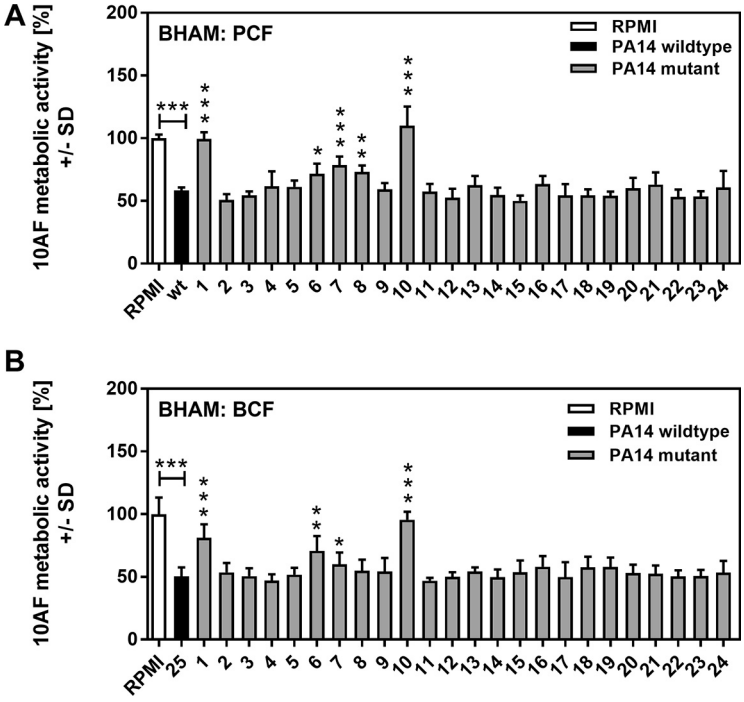

FIG 2 Effects of PCF or BCF on *A. fumigatus* strain 10AF preformed biofilm growth and metabolism. *A. fumigatus* strain 10AF conidia in agar were distributed into 96-well cell culture plates. Plates were incubated at 37°C for 24 h to allow hyphal growth and then loaded with wild-type or mutant PA14 PCF (A) or wild-type or mutant PA14 BCF (B) and incubated at 37°C for 24 h. *A. fumigatus* strain 10AF metabolism was evaluated by XTT assay. Metabolism in the presence of RPMI medium alone was regarded as 100% *A. fumigatus* strain 10AF metabolic activity. The data shown are the mean ± SD of four independent experiments (with duplicates of each group studied in each experiment). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ (mutant [no. 1 to 24] versus wild type [no. 25]). Horizontal asterisks, RPMI medium versus wild-type PA14. Bars: 1, *pvdD pchE* mutant; 2, *pqsE* mutant; 3, *mvfR* mutant; 4, *pqsA* mutant; 5, *pqsH* mutant; 6, *lasR rhlR* mutant; 7, *lasR* mutant; 8, *rsmA* mutant; 9, *pqsA pqsH* not polar mutant; 10, *pvdD* mutant; 11, *rhlR* mutant; 12, ΔHSI-I ΔHSI-II mutant; 13, *pvcA* mutant; 14, *rhlA* mutant; 15, *phzC1 phzC2* mutant; 16, *pchE* mutant; 17, *exoU* mutant; 18, *rsmY rsmZ* mutant; 19, ΔHSI-II ΔHSI-III mutant; 20, ΔHSI-I ΔHSI-III mutant; 21, *pqsA pqsH* polar mutant; 22, *chiC* mutant; 23, *lecA* mutant; 24, *hcnA* mutant.

assays. Figure 5B shows that the ability of wild-type or mutant PA14 to grow in RPMI medium and pyoverdine production do not correlate. Specifically, the lack of pyoverdine production in the *pvdD pchE*, *pvdD*, *lasR rhlR*, and *lasR* mutants is not related to their inability to grow in the medium.

The correlation between mutants lacking pyoverdine production and their decreased antifungal activity demonstrated in our assays indicates that pyoverdine is a major *P. aeruginosa* factor conferring antifungal activity. To test this hypothesis, we diluted pure pyoverdine in RPMI medium and investigated its impact on *A. fumigatus* strain 10AF forming and preformed biofilm metabolism. Figure 6A shows that pyoverdine concentrations as low as 3.125 μM significantly and dose dependently interfered with the metabolism of *A. fumigatus* strain 10AF forming biofilm, whereas preformed *A. fumigatus* strain 10AF biofilm metabolism was affected by pyoverdine at $\geq$1.56 μM (Fig. 6B). Comparison to a pyoverdine standard curve revealed that wild-type PA14 PCF contained about 30 μM pyoverdine (data not shown). Supplementation with only 10 μM pyoverdine was able to restore antifungal activities of the *pvdD pchE*, *pvdD*, *lasR rhlR*, and *lasR* mutants against forming *A. fumigatus* strain 10AF biofilm (Fig. 6C) and of the *pvdD pchE*, *pvdD*, and *lasR* mutants against preformed *A. fumigatus* strain 10AF biofilm (Fig. 6D).

Since pyoverdine is a siderophore produced by *Pseudomonas* that binds iron and facilitates iron uptake (30, 31), we investigated if complexing of iron during *Aspergillus* growth, thus decreasing iron bioavailability, might be the reason for its antifungal effects. We therefore compared the metabolism of *A. fumigatus* strain 10AF during

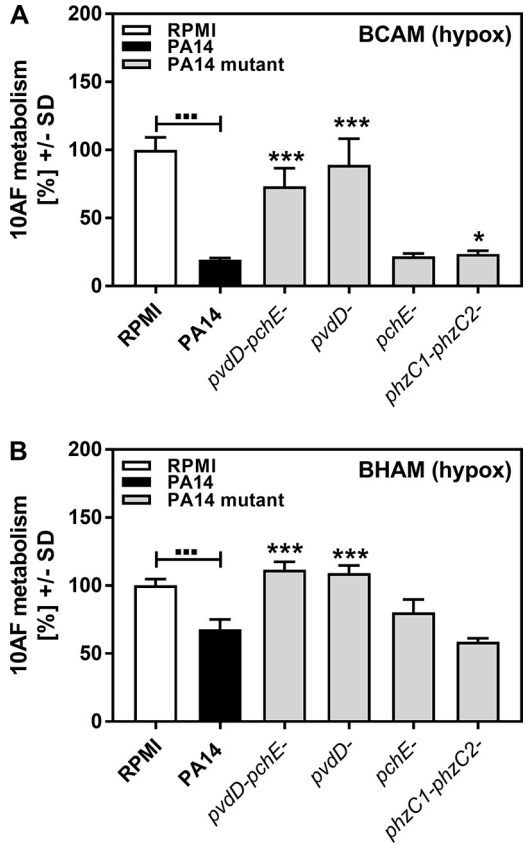

FIG 3 Effects of PCF on *A. fumigatus* strain 10AF forming and preformed biofilm metabolism under hypoxic conditions. *A. fumigatus* strain 10AF conidia in agar were distributed into 96-well cell culture plates. Plates were either loaded immediately after solidification (BCAM assay) (A) or incubated at 37°C for 24 h to allow hyphal growth (BHAM assay) (B). Plates were loaded with wild-type or mutant PA14 PCF and incubated under hypoxic conditions at 37°C for 24 h. *A. fumigatus* strain 10AF metabolism was evaluated by XTT assay. Metabolism in the presence of RPMI medium alone was regarded as 100% *A. fumigatus* strain 10AF metabolic activity. The data shown are the mean ± SD of four replicates. *, $P \leq 0.05$; ***, $P \leq 0.001$ (mutant versus wild-type PA14). Other comparisons are indicated by the ends of the brackets above the bars.

biofilm formation and in preformed biofilm in the presence of pyoverdine with or without an excess of iron ions. Our results show that detrimental effects of pyoverdine on both forming *A. fumigatus* strain 10AF biofilm (Fig. 6E) and preformed *A. fumigatus* strain 10AF biofilm (Fig. 6F) were abolished by iron supplementation.

***P. aeruginosa* predominantly affects *A. fumigatus* by iron starvation.** As shown in Fig. 6E and F, iron was able to interfere with the antifungal activity of PA14. To support the idea that iron starvation was the reason for the observed antifungal activity of PA14 supernatants, we performed Northern blot assays of *A. fumigatus* cultivated in the presence of wild-type or *pvdD pchE* mutant PA14 PCF. Our results show that wild-type PA14 PCF, in contrast to *pvdD pchE* mutant PCF, resulted in increased expression of *A. fumigatus* genes that are known to be responsive to iron starvation (*hapX*, *sidA*, and *mirB*), as opposed to the reduced expression of genes indicating iron availability (*sreA*, *cccA*, *cycA*, and *acoA*) (Fig. 7A). The hypothesis that iron denial affects *A. fumigatus* is further supported by the fact that pyoverdine increased *A. fumigatus* siderophore production by about 3-fold, as measured by chrome azurol S (CAS) assay (Fig. 7B). Induction of *A. fumigatus* siderophore production is a counteraction to lower iron levels in the fungal environment. Additionally, we found that iron concentrations as low as 60 μM were able to significantly interfere with the antifungal activity of PA14 PCF, supporting the relevance of iron for counteracting the effects of *P. aeruginosa* on *A. fumigatus* (Fig. 7C).

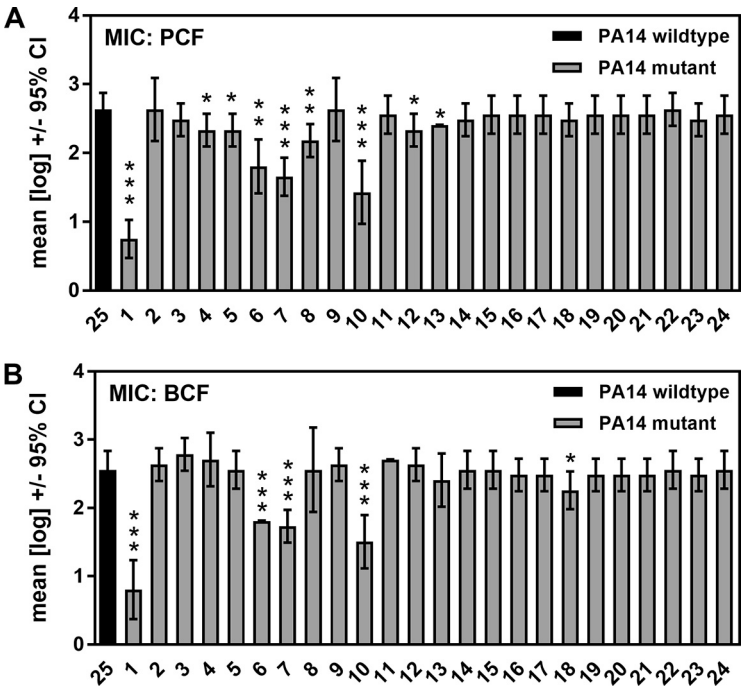

FIG 4 Effects of PCF or BCF on *A. fumigatus* strain 10AF planktonic growth (MIC). *A. fumigatus* strain 10AF conidia were incubated in the presence of RPMI medium and PCF dilutions (A) or RPMI medium and BCF dilutions (B) at 37°C for 48 h. Fungal growth was determined in 2-fold dilution steps for wild-type (no. 25) and mutant (no. 1 to 24) PA14 (dilutions, 1:2 to 1:1,024). For each supernatant, the last dilution showing fungal growth less than that in RPMI medium alone was determined. The means of four independent experiments were determined. The data shown are the mean ± SD. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ (mutant [no. 1 to 24] versus wild type [no. 25]). Bars: 1, *pvdD pchE* mutant; 2, *pqsE* mutant; 3, *mvfR* mutant; 4, *pqsA* mutant; 5, *pqsH* mutant; 6, *lasR rhlR* mutant; 7, *lasR* mutant; 8, *rsmA* mutant; 9, *pqsA pqsH* not polar mutant; 10, *pvdD* mutant; 11, *rhlR* mutant; 12, ΔHSI-I ΔHSI-II mutant; 13, *pvcA* mutant; 14, *rhlA* mutant; 15, *phzC1 phzC2* mutant; 16, *pchE* mutant; 17, *exoU* mutant; 18, *rsmY rsmZ* mutant; 19, ΔHSI-II ΔHSI-III mutant; 20, ΔHSI-I ΔHSI-III mutant; 21, *pqsA pqsH* polar mutant; 22, *chiC* mutant; 23, *lecA* mutant; 24, *hcnA* mutant. CI, confidence interval.

Iron concentrations and pyoverdine affect signaling pathways in *P. aeruginosa* leading to the expression of virulence factors, i.e., exotoxin A (ExoA), PrpL protease, and pyoverdine itself (32). Using a *P. aeruginosa* mutant that is unable to express pyoverdine but is still able to produce PvdS-dependent virulence factors (*pvdA fpvR* mutant) in comparison with a *P. aeruginosa* mutant that does not express pyoverdine but expresses small amounts of ExoA and PrpL protease (*pvdA* mutant) and a *P. aeruginosa* mutant that is unable to produce pyoverdine, as well as ExoA and PrpL protease (*pvdS*

TABLE 4 Ranking of PA14 mutant BCF and PCF activities against planktonic *A. fumigatus* strain 10AF growth[a]

	Mutation(s) tested for effect on antifungal activity of:	
Rank	**PCF**	**BCF**
1	*pvdD pchE*	*pvdD pchE*
2	*pvdD*	*pvdD*
3	*lasR*	*lasR*
4	*lasR rhlR*	*lasR rhlR*
5	*rsmA*	*rsmY rsmZ*
6	ΔHSI-I ΔHSI-II	
7	*pqsA*	
8	*pqsH*	
9	*pvcA*	

[a]BCF and PCF were evaluated by four MIC tests each. Only mutants with statistically significant differences from wild-type PA14 are shown. Ranking from 1 to *n* represents the highest to lowest loss of antifungal activity.

TABLE 5 PA14 mutations producing significant loss of antifungal activity in at least one of seven assays

Mutation(s)	No. of assays with significant loss/total
pvdD, *pvdD pchE*, *lasR*, *lasR rhlR*	7/7
pvcA	3/7
pqsA, *pqsH*, *pqsA pqsH* polar, *rhlR*, *rsmA*, ΔHSI-I ΔHSI-II	2/7
rhlA, *phzC1 phzC2*, *pchE*, *rsmY rsmZ*	1/7

mutant), we found no significant differences in the low antifungal activities of these mutants (Fig. 7D). Hence, PvdS-dependent expression of ExoA or PrpL protease does not contribute to the antifungal activity of *P. aeruginosa*. The predominant mode of action of pyoverdine appears to be deprivation of *A. fumigatus* of iron, resulting in fungal inhibition.

It has to be noted that although pyoverdine appears to have primacy over other potential *P. aeruginosa* microbial inhibitors (Table 5), other mutations are likely to affect the antifungal activity of *P. aeruginosa* to a lesser extent, since pyoverdine mutants were not constantly found completely impaired in their antifungal activity (Fig. 1 to 4 and 7D).

Effects of pharmacologically decreased pyoverdine levels on the antifungal activities of *P. aeruginosa* supernatants. It has been shown previously that 5-fluorocytosine (5FC) is able to decrease pyoverdine production by *P. aeruginosa* strain PAO1 (33). Using 10 μM 5FC, we found a similar effect on PA14 (Fig. 8A). PCF produced

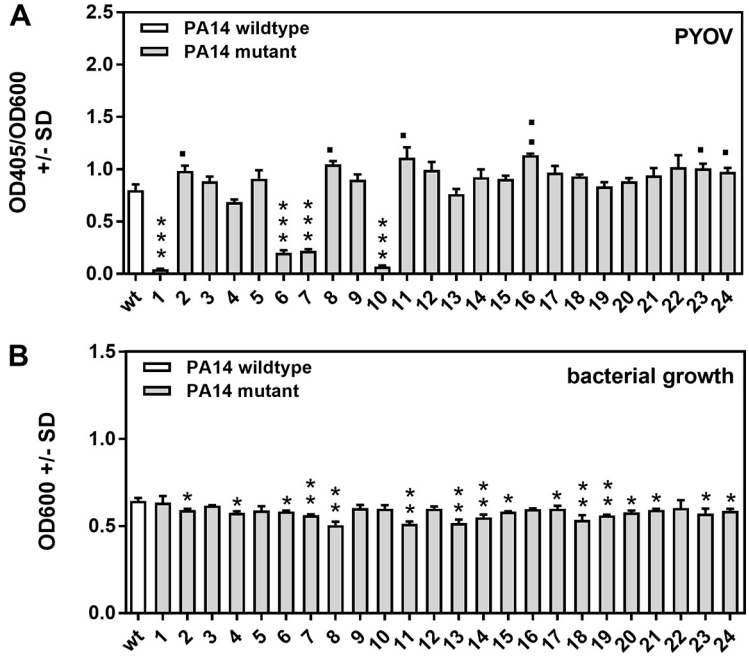

FIG 5 Pyoverdine production by wild-type and mutant PA14. Wild-type or mutant PA14 bacteria (5×10^7/ml) were incubated in RPMI medium overnight. Bacterial growth was measured spectrophotometrically at 600 nm. PCF was harvested, and pyoverdine (PYOV) production was measured at 405 nm (A). Measurements were normalized to bacterial growth (B) with the formula relative pyoverdine expression = OD_{405}/OD_{600}. Experiments in both panels were performed three times (four replicates of each group in each experiment), and the results were combined. ***, $P \leq 0.001$ (decrease in pyoverdine production or bacterial growth in mutants compared to wild-type PA14); ■, $P \leq 0.05$; ■■, $P \leq 0.01$ (significant increase in pyoverdine production compared to wild-type PA14). Bars: 1, *pvdD pchE* mutant; 2, *pqsE* mutant; 3, *mvfR* mutant; 4, *pqsA* mutant; 5, *pqsH* mutant; 6, *lasR rhlR* mutant; 7, *lasR* mutant; 8, *rsmA* mutant; 9, *pqsA pqsH* not polar mutant; 10, *pvdD* mutant; 11, *rhlR* mutant; 12, ΔHSI-I ΔHSI-II mutant; 13, *pvcA* mutant; 14, *rhlA* mutant; 15, *phzC1 phzC2* mutant; 16, *pchE* mutant; 17, *exoU* mutant; 18, *rsmY rsmZ* mutant; 19, ΔHSI-II ΔHSI-III mutant; 20, ΔHSI-I ΔHSI-III mutant; 21, *pqsA pqsH* polar mutant; 22, *chiC* mutant; 23, *lecA* mutant; 24, *hcnA* mutant.

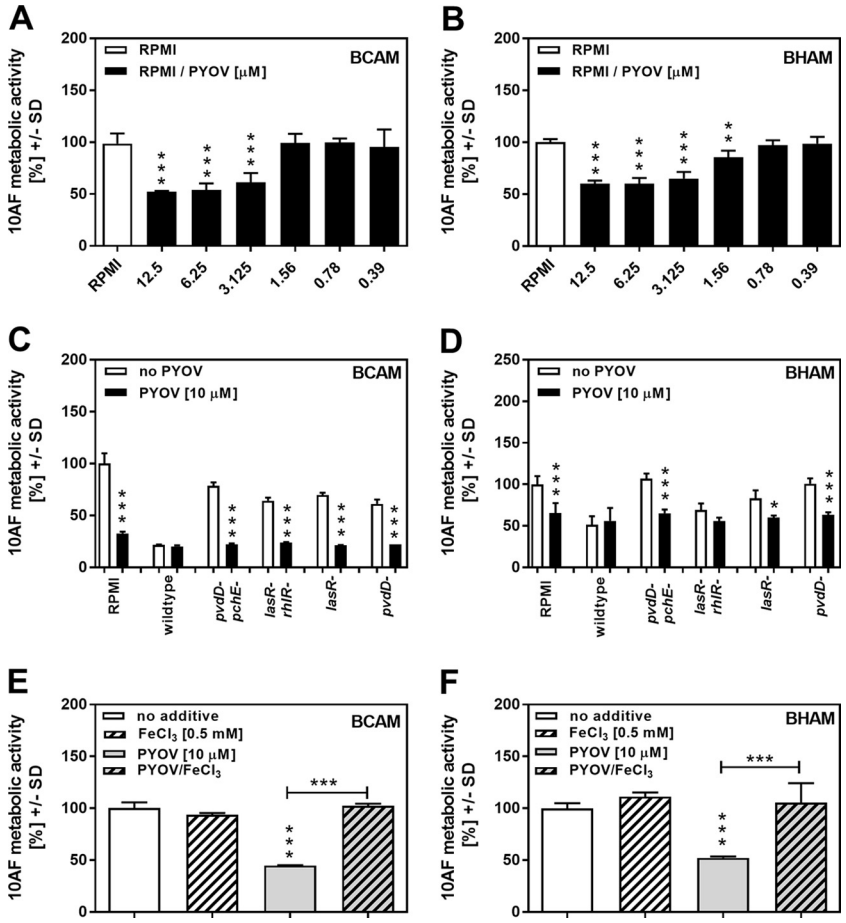

FIG 6 Effects of pyoverdine on *A. fumigatus* strain 10AF biofilm metabolism and antifungal activities of PA14 mutants. (A and B) Increasing concentrations of pyoverdine (PYOV) in RPMI 1640 (0.39 to 12.5 μM) were assayed for activity against *A. fumigatus* strain 10AF biofilm formation (A) or preformed *A. fumigatus* strain 10AF biofilm (B). Fungal metabolism was measured by XTT assay. Statistical analysis was performed by one-way ANOVA of the RPMI 1640 control versus pyoverdine concentrations: **, $P \leq 0.01$; ***, $P \leq 0.001$. (C and D) Antifungal activities of wild-type PA14 and pyoverdine-lacking mutants with or without supplementation with 10 μM pyoverdine were measured against forming *A. fumigatus* strain 10AF biofilm (C) or preformed *A. fumigatus* strain 10AF biofilm (D). No pyoverdine supplementation versus pyoverdine supplementation: *, $P \leq 0.05$; ***, $P \leq 0.001$. (E and F) *A. fumigatus* strain 10AF conidia were incubated in the presence of RPMI 1640 (unstriped bars) or RPMI 1640 supplemented with 0.5 μM $FeCl_3$ (striped bars) with (gray bars) or without (white bars) 10 μM pyoverdine. *A. fumigatus* strain 10AF metabolism was evaluated by XTT assay. Metabolism in the presence of RPMI 1640 without $FeCl_3$ or pyoverdine was regarded as 100% *A. fumigatus* strain 10AF metabolic activity. Vertical asterisks, comparison with RPMI 1640; horizontal asterisks, RPMI 1640 containing pyoverdine versus RPMI 1640 containing pyoverdine and $FeCl_3$. ***, $P \leq 0.001$. Experiments in panel A were performed twice, those in panel B were performed three times, those in panel D were performed six times, and representative results are shown. Every experiment had four replicates per group studied.

in the presence of 10 μM 5FC, i.e., containing less pyoverdine, was found to be significantly less active against *A. fumigatus* biofilm formation than supernatant produced without 5FC (Fig. 8B, right side). It has to be noted that 10 μM 5FC by itself showed significant antifungal effects (Fig. 8B, left side) (34), which might partially mask the loss of antifungal activity in 5FC-containing *P. aeruginosa* supernatants.

Because of the perfusion of lungs with blood, exacerbated by the presence of blood components in airways as a result of chronic inflammation in the lungs of individuals with CF, both *P. aeruginosa* and *A. fumigatus* are exposed to blood components in CF lungs. Blood, owing to its heme component, can be a vast source of iron for organisms expressing heme oxygenases (35, 36). *P. aeruginosa* possesses two heme uptake systems, encoded by *has* and *phu* (37), and expresses the heme oxygenase HemO (38) and thus is able to use hemin as an iron source. We investigated whether addition of

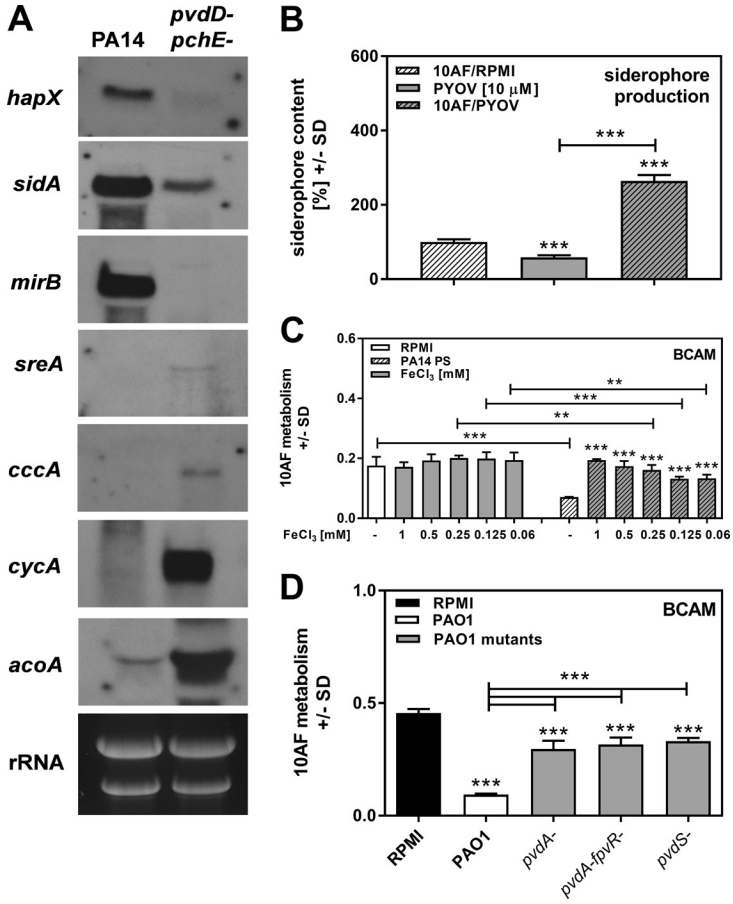

FIG 7 Effects of *P. aeruginosa* siderophores on *A. fumigatus* iron metabolism. (A) *A. fumigatus* strain AfS77 conidia were grown at 37°C in 15 ml of liquid 2YT medium for 15 h. Subsequently, 10 ml of the culture supernatant was replaced with 10 ml of wild-type or *pvdA pvdE* mutant PA14 PCF and incubated for 3 h. A Northern blot analysis was performed for genes inducible by iron starvation (*hapX*, *sidA*, and *mirB*) and genes repressed by iron starvation (*sreA*, *cccA*, *cycA*, and *acoA*). Measurement of rRNA served as a loading control. (B) *A. fumigatus* strain 10AF conidia were incubated in the presence of RPMI 1640 (striped bar) or RPMI 1640 supplemented with 10 μM pyoverdine (gray striped bar) for 24 h. Supernatants were sterile filtered. As a control, 10 μM pyoverdine (PYOV) solution in RPMI 1640 was prepared (solid gray bar). A CAS assay measuring siderophore content in *A. fumigatus* supernatants was performed, and the results were normalized to those of an XTT assay prepared in parallel with the CAS assay measuring fungal metabolism. Siderophore production by *A. fumigatus* was regarded as 100% and compared to all other bars. Other comparisons are indicated by the ends of the brackets above the bars. ***, $P \leq 0.001$. Pyoverdine itself as a siderophore was measurable by CAS assay. (C) RPMI 1640 or PA14 PCF with or without increasing amounts of $FeCl_3$ was subjected to a BCAM assay measuring effects on the metabolism of *A. fumigatus* strain 10AF forming biofilm. *A. fumigatus* strain 10AF metabolism was evaluated by XTT assay. Metabolism in the presence of RPMI 1640 alone was regarded as 100% *A. fumigatus* strain 10AF metabolic activity. The data shown are the mean $\pm$ SD from four replicates. **, $P \leq 0.01$; ***, $P \leq 0.001$ (RPMI 1640 versus RPMI 1640 containing $FeCl_3$ [left side] or PCF versus PCF containing $FeCl_3$ [right side]). Other comparisons are indicated by the ends of the brackets above the bars. (D) Agar and *A. fumigatus* strain 10AF conidia were distributed into 96-well cell culture plates that were loaded with wild-type or mutant PAO1 PCF and incubated at 37°C for 24 h. *A. fumigatus* strain 10AF metabolism was evaluated by XTT assay. Metabolism in the presence of RPMI medium alone was compared to metabolism in the presence of PCF. Other comparisons are indicated by the ends of the brackets above the bars. ***, $P \leq 0.001$.

the heme oxygenase inducer hemin, which also serves as a heme oxygenase substrate, would affect pyoverdine production by PA14, as well as the antifungal activity of PA14 supernatant produced in the presence of hemin. Our results show that 10 μM hemin abolished pyoverdine production by PA14 (Fig. 8C), whereas the bacteria grew better in the presence of hemin than in its absence, showing the dissociation between growth and pyoverdine production, as well as lack of hemin toxicity for *P. aeruginosa*. Hemin alone had no significant effect on *A. fumigatus* metabolism (Fig. 8D, left side). Plank-

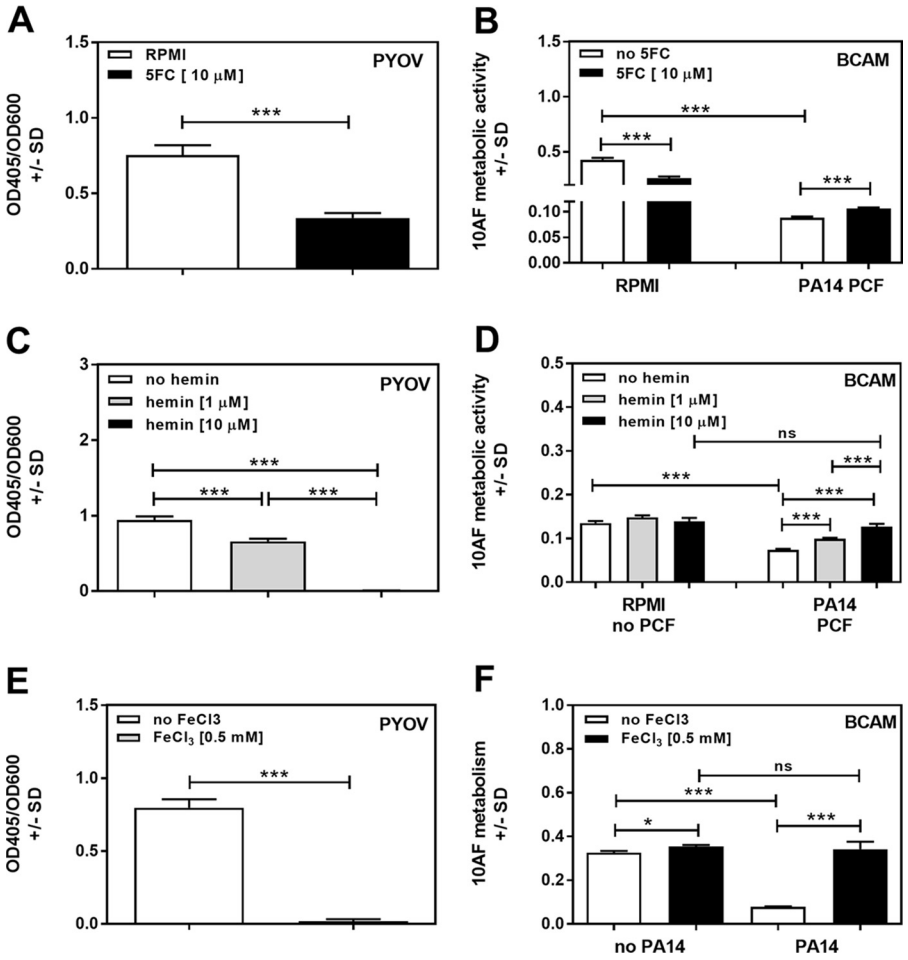

FIG 8 Effects of pyoverdine reduction on *A. fumigatus* strain 10AF biofilm metabolism. RPMI 1640 with or without 10 μM 5FC (A, B), 1 or 10 μM hemin (C, D), or 0.5 mM $FeCl_3$ (E, F) was inoculated with 5×10^7 PA14 bacteria/ml. Pyoverdine (PYOV) production was measured after 24 h of incubation (A, C, E). PCFs derived from panels A, C, and E were subjected to BCAM assays measuring effects on the metabolism of *A. fumigatus* strain 10AF forming biofilm (B, D, F). Groups being compared are indicated by the ends of the brackets above the bars. The experiments shown were performed three times (four replicates of each group in each experiment), and the results were combined. *, $P \leq 0.05$; ***, $P \leq 0.001$.

tonic PA14 supernatants produced in the presence of hemin showed significantly less antifungal activity than control supernatants produced without hemin (Fig. 8D, right side). Supernatants produced in the presence of 10 μM hemin had no significant antifungal effects on *A. fumigatus* strain 10AF forming biofilm (Fig. 8D), similar to the effects of PA14 mutants lacking pyoverdine (Fig. 1B and 4A). Thus, iron from blood in patients' lungs might suppress pyoverdine production by *P. aeruginosa* and aggravate the detrimental effects of *A. fumigatus* in, e.g., CF. These data are supported by Fig. 8E and F, showing that the presence of iron indeed interferes with the ability of *P. aeruginosa* to produce pyoverdine (Fig. 6E). Iron did not exert toxic effects on *P. aeruginosa* and significantly increased bacterial growth. Similar to our observations regarding hemin, iron abolished the antifungal properties of PA14 PCF (Fig. 8F). In the experiments presented in Fig. 8E and F, we used 0.5 mM iron but observed similar effects over a range of iron concentrations (1 to 0.1 mM; data not shown). The results support our finding that the ability to produce pyoverdine via iron denial is a major factor determining the antifungal activity of *P. aeruginosa*.

Corroboration of the studies with PA14. In addition to wild-type PA14, we also studied *P. aeruginosa* isolates PAO1 (producer of a type I pyoverdine, as is PA14) and PAK. PCFs of PAO1 and PAK also inhibited *A. fumigatus* strain 10AF biofilm formation

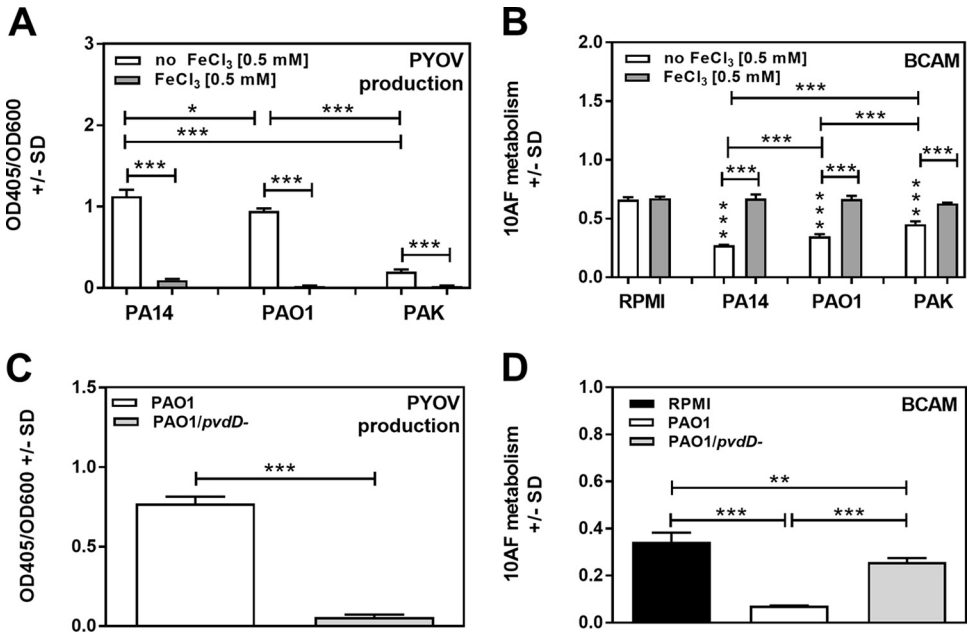

FIG 9 Pyoverdine production by *P. aeruginosa* PAO1, PAK, and PA14 and effects on *A. fumigatus* strain 10AF forming biofilm. (A) RPMI 1640 with or without 0.5 mM $FeCl_3$ was inoculated with *P. aeruginosa* strains PAO1, PAK and PA14 at 5×10^7 bacteria/ml. Pyoverdine (PYOV) production was measured after 24 h of incubation. (B) PCFs derived from panel A were studied in assays measuring effects on the metabolism of *A. fumigatus* strain 10AF forming biofilm (BCAM assay). Vertical asterisks, comparison with the negative control (RPMI 1640 alone). (C) Pyoverdine production by wild-type or *pvdD* mutant PAO1 bacteria (5×10^7/ml) was measured after 24 h of incubation. (D) PCFs derived from panel C were studied in assays measuring effects on the metabolism of *A. fumigatus* strain 10AF forming biofilm (BCAM). Groups being compared are indicated by the ends of the brackets above the bars. Representative results are shown. Each group in each experiment contained at least four replicates. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

($P \leq 0.01$ to 0.001 in four experiments) (Fig. 9B). Pyoverdine production (Fig. 9A) and inhibition of *A. fumigatus* biofilm formation by their PCFs were also abolished by iron ($P \leq 0.01$ to 0.001) (Fig. 9B). PAK produced less pyoverdine than PA14 or PAO1 ($P \leq 0.001$) (Fig. 9A), and also in three experiments, its PCF produced the least inhibition of *A. fumigatus* biofilm formation (Fig. 9B). We also studied a *pvdD* mutant in the PAO1 background; compared to the parent, its ability to inhibit *A. fumigatus* forming biofilm was reduced ($P \leq 0.01$ to 0.001 in four experiments) (Fig. 9D), its pyoverdine production was negligible (Fig. 9C), and added iron did not affect either inhibition of *A. fumigatus* or pyoverdine production. Representative experiments for each of these findings are shown in Fig. 9.

Compared to the effects of PCF from the PAO1 parent strain on preformed *A. fumigatus* biofilm, those of *pvdD* mutant-derived PCF were also reduced ($P \leq 0.05$ to 0.001 in three experiments). PAK-derived PCF showed less inhibition of *A. fumigatus* strain 10AF preformed biofilm than PA14- or PAO1-derived PCF ($P \leq 0.05$ to 0.001 in four experiments).

Study of pyoverdine types. *Pseudomonas* species generate three, possibly four, classes of pyoverdines, and *P. aeruginosa* strains produce one representative of each principal class (30, 31). For *P. aeruginosa*, these representatives are termed types, with every *P. aeruginosa* strain producing only one type. We measured the activity of each pyoverdine type to assess the global applicability of our observations on antifungal activity. Dose titrations of 2-fold dilutions of purified pyoverdine types ranging from 20 to 2.5 μM were tested against *A. fumigatus* strain 10AF biofilm formation. Significant inhibition of *A. fumigatus* biofilm formation by types I and III was observed at concentrations as low as 5 μM. However, 5 μM type II was not significantly inhibitory. At 2.5 μM, no type was different from the control (Fig. 10A). These results suggest that all three pyoverdine types are similarly active against *A. fumigatus* biofilm formation and type II is slightly less active.

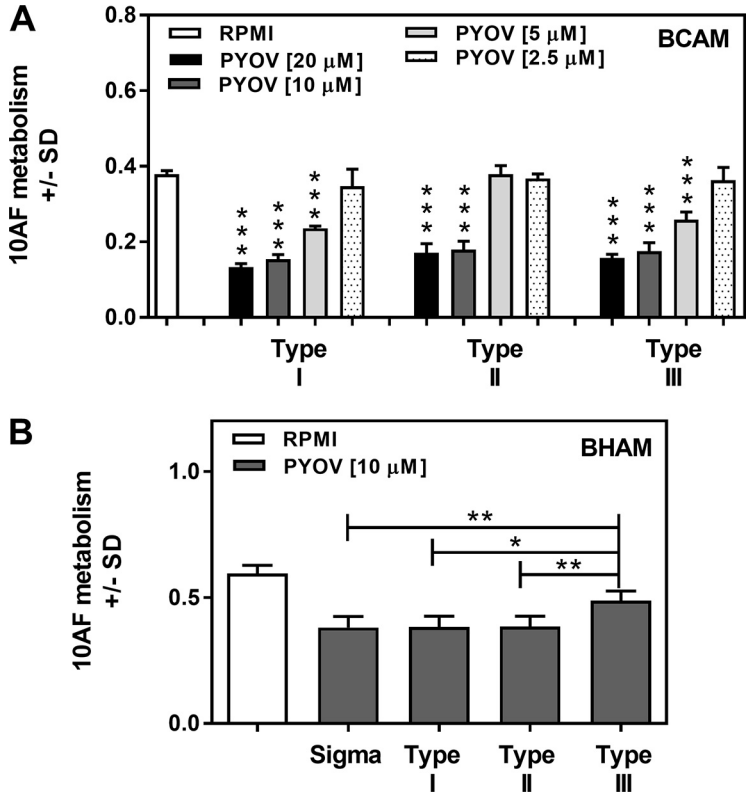

FIG 10 Titration of purified pyoverdines. (A) Pyoverdine types were titrated 2-fold from 20 to 2.5 μM against *A. fumigatus* strain 10AF biofilm formation. Mean results of four replicates of each group are shown. ***, $P \leq 0.001$ (versus RPMI 1640 control). (B) Part of a titration series against preformed *A. fumigatus* strain 10AF biofilm (10 μM comparison) is shown. Sigma is the commercial pyoverdine (PYOV; type I) used in the experiments whose results are shown in the preceding figures. Mean results of four replicates of each group are shown. Groups being compared are indicated by the ends of the brackets above the bars. *, $P \leq 0.05$; **, $P \leq 0.01$. All three types of pyoverdine inhibited preformed biofilm metabolism compared to the RPMI 1640 control. Type III, $P \leq 0.01$; type I or II, $P \leq 0.001$.

The dose titration against preformed *A. fumigatus* biofilm gave similar results. At 5 to 20 μM, inhibition by all three types was different from that of the control ($P \leq 0.01$), as was the case for 2.5 μM type I, whereas at that apparently limiting dilution, the other two types showed nonsignificant inhibition (data not shown). At 10 μM, pyoverdine types I and II produced significantly more inhibition of preformed *A. fumigatus* biofilm ($P < 0.001$) than the control, similar to the commercial pyoverdine (class I) used in all of the preceding parts of this study, as did *P. aeruginosa* type III ($P < 0.01$) (Fig. 10B). However, type III was significantly ($P \leq 0.05$) less active than the other three pyoverdine comparators. These results suggest that type I may be slightly more active against preformed *A. fumigatus* biofilm and type III may be slightly less active. These data were confirmed in a second experiment.

Pyoverdine production by clinical *P. aeruginosa* isolates correlates with their antifungal activity. To verify the clinical relevance of our observation that *P. aeruginosa* antifungal activity is related primarily to pyoverdine production, we compared the pyoverdine production (Fig. 11A) and antifungal activity (Fig. 11B) levels of 10 *P. aeruginosa* isolates derived from the lungs of CF patients. Our results showed a correlation between pyoverdine production and antifungal activity (Fig. 11C). Isolates producing small amounts of pyoverdine displayed low antifungal activity (isolates a, c, d, e, g, and k), while isolates with higher pyoverdine production showed increased antifungal activity (isolates b and i).

Pyoverdine does not act as a xenosiderophore for *A. fumigatus*. *P. aeruginosa* has been shown to be able to use several xenosiderophores of bacterial origin, as well

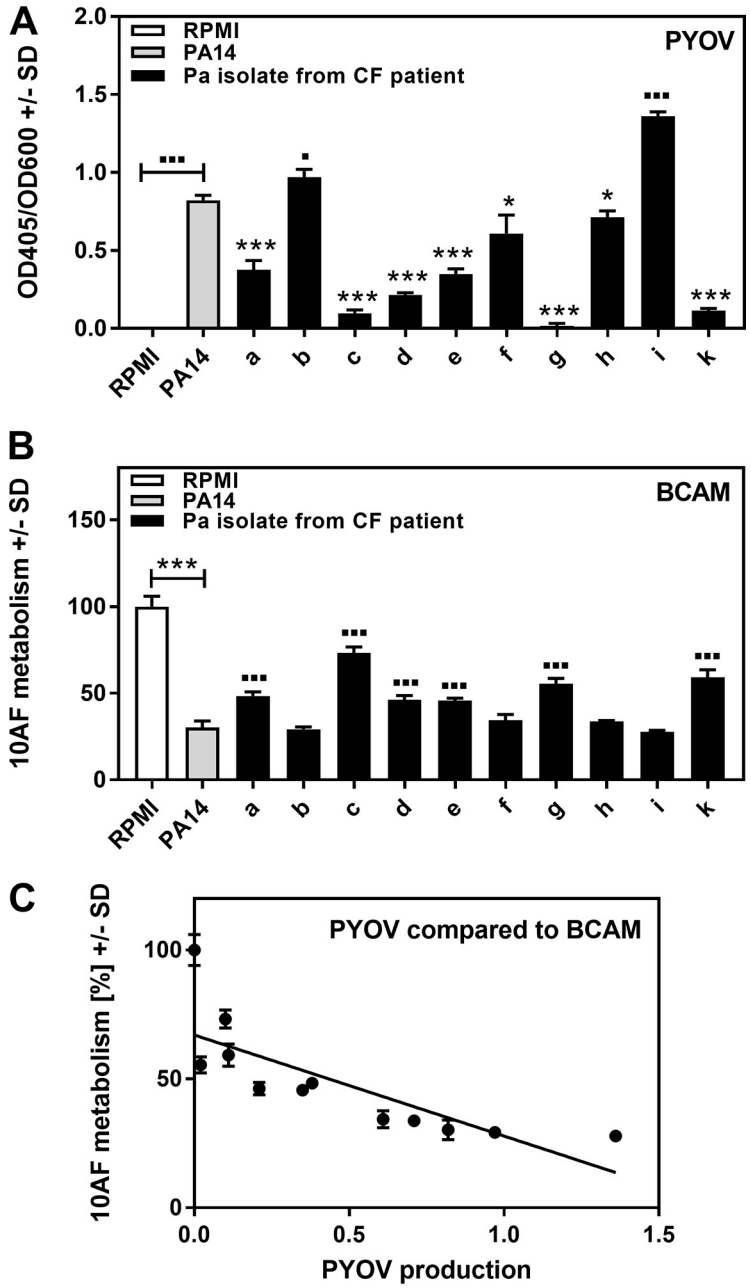

FIG 11 Correlation between pyoverdine production and antifungal activity of clinical *P. aeruginosa* (Pa) isolates. Ten CF isolates designated a to i and k, as well as PA14, were tested for pyoverdine (PYOV) production (A), as well as for activity against the metabolism of *A. fumigatus* strain 10AF forming biofilm (BCAM assay) (B). RPMI medium served as a control. Results of panels A and B were compared by linear regression curve analysis (panel C). Symbols: ■ or *, $P \leq 0.01$; ■■■ or ***, $P \leq 0.001$. Each comparison without brackets in panels A and B is PA14 versus 1 of the 10 CF isolates. Comparisons with brackets are as indicated. *, significant decreases; ■, significant increases. $n = 3$ for pyoverdine production. $n = 5$ for *A. fumigatus* strain 10AF metabolic activity.

as the fungal siderophore ferrichrome (reviewed in reference 39). To investigate whether pyoverdine or other *P. aeruginosa* siderophores could act as xenosiderophores for *A. fumigatus*, we performed a growth bioassay using the *A. fumigatus sidA ftrA* mutant (40) lacking both high-affinity iron uptake systems (siderophore biosynthesis and reductive iron assimilation). We incubated the mutant fungus in the presence of a large amount of iron (5 mM $FeSO_4$), the *A. fumigatus* endogenous siderophore ferricrocin (FC), or the fusarinine-type siderophore triacetylfusarinine C (TAFC) (40), enabling

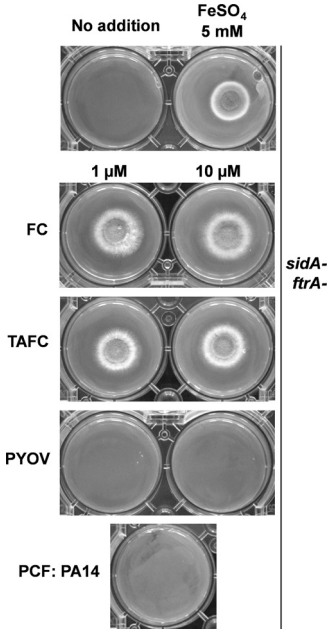

FIG 12 Measurement of xenosiderophores in PCF. *A. fumigatus sidA ftrA* mutant conidia (10^4) were point inoculated onto solid minimal medium agar with or without supplementation with $FeSO_4$ to a final concentration of 5 mM, pyoverdine (PYOV) to final concentrations of 1 and 10 μM, TAFC or FC to a final concentration of 1 or 10 μM, or 600 μl of wild-type PA14 PCF. The plates were incubated for 48 h at 37°C.

growth of the *sidA ftrA* mutant (Fig. 12, rows 1 to 3). Incubation of the *A. fumigatus sidA ftrA* mutant with pyoverdine (Fig. 12, row 4) or PA14 PCF containing all PA14 siderophores or chelators (Fig. 12, row 5) did not enable fungal growth. Our results show that PA14 PCF does not contain xenosiderophores usable by *A. fumigatus* at concentrations necessary to overcome the antifungal effects of pyoverdine.

DISCUSSION

P. aeruginosa has a relatively large genome compared to those of other bacteria and produces numerous extracellular products that are able to interfere with bacteria and fungi that might compete with *P. aeruginosa* in its ecological niches. Those niches include water and soil, as well as the human microbiome. Elaboration of these products explains the ubiquitous nature of *P. aeruginosa* and its success as a colonizer and pathogen. These products include toxins, as well as molecules that enable *P. aeruginosa* to compete with other microbes, such as molecules that can seize the crucial element iron from the environment or from the host and deny iron to these competitors (22, 39, 41, 42).

For decades (19), it has been known that *P. aeruginosa* can inhibit fungi, particularly *A. fumigatus*, a fungus that *P. aeruginosa* would encounter in human sinuses and airways, as well as in soil and water. A number of *P. aeruginosa*-produced molecules have been studied for the ability to inhibit *A. fumigatus*, as cited in the introduction, and the strengths and shortcomings of that approach have been discussed (such as the difficulty in knowing the relevance of the concentrations of materials studied *in vitro* to physiologic conditions). We have taken a different approach to investigate these questions, namely, to look at the effects of deletion mutations, to assess the importance of the subtraction of a gene on the intermicrobial activity against *A. fumigatus*. Because *P. aeruginosa* can modulate its metabolism and offensive weapons depending on the environment and the competitors (39, 43–46), we have studied the *P. aeruginosa-A. fumigatus* interaction in several different ways. We have studied the mutants in direct cellular interactions, as well the effects of extracellular *P. aeruginosa* products, and studied products of *P. aeruginosa* produced during planktonic growth, as well as from growth as *P. aeruginosa* biofilms. Initially, this was pursued in liquid in wells (47) and

here in agar too. We have studied the effects on planktonic *A. fumigatus* and on *A. fumigatus* trying to form biofilm, as well as on preformed *A. fumigatus* biofilm. Biofilm formation by *A. fumigatus* leads to morphological and physiologic changes that produce a protected environment for *A. fumigatus*, with special importance for diseases such as CF (47–56).

A complexity of our approach is that some genes of *P. aeruginosa* regulate downstream pathways, where the elaboration of a number of products could be affected at once. Despite this complexity and despite mutations that affect many downstream pathways, a clear pattern emerged, where a limited number of mutants consistently appeared deficient in *A. fumigatus* inhibition in multiple settings. These were the *pvdD*, *pvdD pchE*, *lasR*, and *lasR rhlR* mutants (Table 5). The first two of these mutants pointed to the importance of *P. aeruginosa* siderophores, and especially a single such molecule, pyoverdine, in the interplay with *A. fumigatus*. The three classes of pyoverdines appear to have similar iron binding properties and appear to have similar levels of activity. Type II pyoverdine is the one prevalent in CF strains (57).

Some studies have indicated that pyoverdine may have antifungal activity independent of its activity as a *P. aeruginosa* siderophore (58), but our studies, demonstrating reversal of pyoverdine inhibition in the presence of small amounts of iron and *A. fumigatus* siderophore induction in the presence of pyoverdine, indicate that iron denial is the main mechanism by which pyoverdine inhibits *A. fumigatus*. Although pyoverdine regulates the production of virulence factors (32), our studies indicate the importance of iron chelation by pyoverdine itself as key. Residual antifungal activity of pyoverdine mutants was independent of the mutant's ability to produce PvdS-dependent virulence factors. We could also demonstrate that wild-type PA14 PCF, but not *P. aeruginosa* siderophore-deficient PCF, affected *A. fumigatus* iron metabolism at a molecular level, inducing genes that are known to be upregulated during iron denial, e.g., *hapX* (59, 60), and suppressing the expression of genes that are expressed under high-iron conditions, e.g., *sreA* (61).

The prominent appearance of the other two mutants, the *lasR rhlR* and *lasR* mutants, suggests that QS-regulated metabolites also play an important role in *A. fumigatus* inhibition. Many of these products have potential as intermicrobial inhibitors, but the advantage of studying downstream mutants as we did enables us to suggest that it is possible that the effect of the *lasR rhlR* and *lasR* mutations is a result of the loss of the combined activity of many of the downstream products, since few of the single downstream mutants appear as consistently impaired (Table 5). However, we note that both the *lasR rhlR* and *lasR* mutants had diminished pyoverdine production as well, which may help explain their reduced inhibitory activity. This was rather unexpected, since the initial observation (62) of reduced production of pyoverdine in a *lasR* mutant has never since been confirmed at the regulatory level, likely because the QS transcriptome of *P. aeruginosa* is also dependent on culture conditions (63).

For completeness, inhibition assays in 12-well plates had first shown the dramatic impairment of the *pvdD pchE* mutant (47). In the more extensive studies using the 96-well format, this mutant also had impaired activity against biofilm formation in all three assays (LC, PCF, and BF) (47). The *lasR rhlR*, *pvdD*, and *rhlR* mutants also appear to be impaired in some 96-well biofilm formation assays, as also described for the agar assays here. Also, for comparison, only in wells was assay of inhibition of preformed biofilm possible with LC, and those assays and the well assays with PCF and BCF revealed several impaired mutants, including the *lasR* and *pvdD pchE* mutants (47). The only other study of the interaction of *P. aeruginosa* mutants with *A. fumigatus* that we are aware of is a study of only the *lasR* and *rhlR* mutants (64), which were deficient in inhibition compared to the wild type. Our results are consistent with that report.

Perhaps the most striking absence of effects on antifungal activity was observed regarding the double phenazine mutant. This mutant was similar to the wild type in activity both initially in wells (47) and in the present studies. This observation is striking, because several *in vitro* studies had emphasized the inhibitory power of phenazines, including pyocyanin, on *A. fumigatus* (19–22), whereas complete subtraction of these

molecules via mutation appeared to make little difference. This may suggest that the concentrations that have been previously studied *in vitro* are irrelevant, although we cannot exclude the upregulation of other factors in the mutant that could compensate for the loss of inhibiting activity. However, a recent study with another CF fungal pathogen has indicated that neither pyocyanin nor phenazine has a fungus inhibitory effect (65), and others have found pyocyanin to only be fungistatic (66).

In the present assays, we noted that there was some residual inhibition left for pyoverdine mutants. This suggests the presence of other inhibitors, which may contribute to the total fungal inhibition by wild-type *P. aeruginosa*. Some of these residuals in the *pvdD pchE* and *pvdD* mutants may correspond to the roster of absent molecules resulting from loss of QS-regulated metabolites in the *lasR rhlR* and *lasR* mutants, such as rhamnolipids (23). *P. aeruginosa* also produces iron chelators other than pyoverdine, such as pyochelin, PQS, and paerucumarin, but their absence appears to have a much lesser effect (Tables 1 and 5).

In lungs of CF patients, the microenvironment frequently contains *A. fumigatus*, as well as *P. aeruginosa* (15, 16). In our experiments, we used the well-studied *A. fumigatus* reference strain 10AF (29, 67–69). However, a recent survey found no differences among non-CF and CF *A. fumigatus* strains and included *A. fumigatus* strain 10AF, with respect to inhibition by *P. aeruginosa* products (70), indicating that the present findings are broadly applicable to *A. fumigatus*.

Using *P. aeruginosa* isolates from the lungs of 10 CF patients, we were able to demonstrate that the correlation between pyoverdine production and antifungal activity observed in PA14, PAO1, and PAK was also present in clinical samples. A prospective study would likely show that the ability to produce pyoverdine is correlated with clinical events and changes in the microbiome, contributing to a long-term prognosis for *P. aeruginosa-A. fumigatus* lung infections.

Although we were able to show in many assay settings that PA14 PCF has antifungal activity, there was the possibility that *P. aeruginosa* could also produce some profungal compounds, thus blunting the antifungal activity measured. A candidate class of such compounds is *P. aeruginosa* siderophores, which potentially could be used as xenosiderophores by *A. fumigatus*. Using bioassays measuring *A. fumigatus* growth, we did not find evidence of the use of *P. aeruginosa* siderophores by *A. fumigatus*.

The assays we used are a model system for a much more complex interplay in the *in vivo* setting. *P. aeruginosa* and *A. fumigatus* are competitors for iron in their microenvironment, and high pyoverdine expression antagonizes *A. fumigatus* metabolism and growth, which might support antifungal treatment. Most of the work presented here was performed under normoxic conditions. In CF progression, however, hypoxia may exist in focal lung sites (71–73). When experiments were repeated under hypoxic conditions, we observed the same outcomes, with pyoverdine being the principal mediator of antifungal activity on both forming and preformed *A. fumigatus* biofilms. This is consistent with our previous observations with nonmutant *P. aeruginosa* cells and filtrates under hypoxic conditions (74). As CF progresses, *P. aeruginosa* mutants frequently appear, and commonly these mutants are hypoproducers of pyoverdine (39). In the areas of hypoxia, the ratio of Fe^{3+} to Fe^{2+} decreases and *P. aeruginosa* depends more on the less avid Fe^{2+} transport into the bacterium for iron uptake via production of phenazines and a permease (39, 41, 75). The majority of *P. aeruginosa* isolates from CF patients we tested showed low pyoverdine expression. The resulting low antifungal activity of *P. aeruginosa* represents an unwanted advantage for *A. fumigatus*. Also, iron derived from blood, e.g., present in inflamed lungs, would decrease pyoverdine production and provide beneficial conditions for coexisting *A. fumigatus*. These conditions would be more favorable to *A. fumigatus* in the *P. aeruginosa-A. fumigatus* struggles and may help explain why *A. fumigatus* appears in CF airways later in the course of the disease (5, 6, 9, 10, 13, 14, 16). Evolution of *P. aeruginosa* may act in concert with other factors favoring *A. fumigatus* colonization, including repeated antibacterial courses and inhaled corticosteroids (76).

The present studies have focused on the effects of *P. aeruginosa* on *A. fumigatus*. As

shown above, iron sequestering by pyoverdine leads to an increase in *A. fumigatus* siderophore excretion, providing further evidence that *A. fumigatus* is iron starved by pyoverdine. In subsequent studies, we are examining the countering effects of *A. fumigatus*. Adding *A. fumigatus* supernatants, presumably containing *A. fumigatus* siderophores, at the time of a pyoverdine challenge of *A. fumigatus* blunts the inhibitory effect of pyoverdine. An *A. fumigatus sidA* mutant, which fails to produce *A. fumigatus* siderophores, was found to be hypersusceptible to *P. aeruginosa* products and to pyoverdine. This is consistent with the findings of the importance of siderophores and pyoverdine in the *P. aeruginosa* mutants in the present study, as a siderophoreless *A. fumigatus* mutant that has a decreased ability to defend itself in the contest for iron would be expected to be hypersusceptible to *P. aeruginosa*'s weapons. Our observations suggest that the competition between *A. fumigatus* and *P. aeruginosa* seems to rely on the relative amounts of siderophores produced by them, the speed at which they are produced, and their relative affinity for Fe.

MATERIALS AND METHODS

Materials. Pyoverdine, 5FC, $FeCl_3$, hemin, XTT, *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (DDAPS), and menadione were purchased from Sigma-Aldrich (St. Louis, MO). CAS was purchased from MP Biomedicals (Solon, OH). The type I (derived from *P. aeruginosa* ATCC 15692), type II (derived from *P. aeruginosa* ATCC 27853), and type III (derived from the *P. aeruginosa* strain Pa6) pyoverdines were purchased from EMC Microcollections GmbH, Tuebingen, Germany. The Sigma-Aldrich pyoverdine is of type I and was used throughout, except in experiments comparing the three types (obtained from EMC Microcollections GmbH).

Isolates. The *A. fumigatus* strain used in these studies, 10AF, is a virulent patient isolate (67, 68) that can be obtained from the ATCC (ATCC 90240). The ATCC 46645 *sidA ftrA* mutant (40) lacks the high-affinity iron permease (FtrA) essential for reductive iron assimilation, as well as L-ornithine-*N*5-mono-oxygenase (SidA), which is essential for siderophore production. The use of all microbes in our lab was approved by the CIMR Biological Use Committee (approval no. 001-03Yr.11). *P. aeruginosa* isolates from CF patient respiratory cultures were obtained after written informed consent for biobanking and subsequent use of the patients' specimens, approved by the Stanford Institutional Review Board, was obtained (29). No patient names are associated with any of the organisms. PA14, a widely studied *P. aeruginosa* strain (77, 78), is the parental strain of all of the PA14 mutants studied here. The PA14 mutants investigated here, their descriptions and phenotypes, and references for production are shown in Table 1. *P. aeruginosa* strains PAO1 (ATCC 15692) and PAK (ATCC 25102) and the PAO1 *pvdD* mutant were kindly provided by P. R. Secor, Departments of Microbiology and Medicine, University of Washington, Seattle, WA. The PAO1 *pvdA*, *pvdA fpvR*, and *pvdS* mutants were generated and described previously (79). None of these PAO1 mutants is able to produce pyoverdine, whereas they differ in the production of ExoA and PrpL protease virulence factors. Briefly, the *pvdA* mutant shows reduced production of PvdS-dependent ExoA and PrpL protease virulence factors, the *pvdA fpvR* mutant is fully able to direct PvdS-dependent virulence factor production, and the *pvdS* mutant does not produce ExoA and PrpL protease.

PA14 mutants. (i) Construction of the markerless PA14 Δ*rsmY* Δ*rsmZ* (*rsmY rsmZ*) mutant. Overnight cultures of the single PA14 Δ*rsmY* (receiver strain) mutant and the donor strain SM10 containing the pEXG2-*rsmZ* suicide plasmid (80) grown at 37°C were diluted to an optical density at 600 nm (OD_{600}) of 0.05 and subcultured until the mid-exponential phase was reached (an OD_{600} of 0.5). Once the desired OD was achieved, 1 ml of each culture was washed twice with 1 ml of phosphate-buffered saline (PBS). A 50-μl volume of both the donor and receiver strains was plated on lysis broth (LB) agar and incubated overnight at 37°C. On the following day, the biomass was scraped off the plate and resuspended in 1 ml of PBS. Serial dilutions (10^0 to 10^{-6}) were made, plated on LB agar supplemented with 100 μg/ml gentamicin and 25 μg/ml triclosan, and incubated overnight at 37°C. The colonies obtained were inoculated and incubated overnight at 37°C in LB supplemented with 100 μg/ml gentamicin for first recombinant screening. On the next day, a colony PCR assay of the gentamicin resistance cassette was performed to confirm the integration of the suicide plasmid. A second recombination was initiated by inoculating the first recombinants into LB with no salt (LBNS) and incubating them at 37°C overnight. On the following day, the cultures were serially diluted (10^0 to 10^{-7}) and a 100-μl volume was plated on LBNS agar containing 10% sucrose. The plates were incubated overnight at 30°C. Once colonies were visible, they were replated on both LBNS supplemented with 10% sucrose and LBNS supplemented with 100 μg/ml gentamicin before overnight incubation at 37°C to screen for second recombinants. Colonies that grew only on LBNS containing 10% sucrose and not on LBNS plus 100 μg/ml gentamicin were selected and verified by colony PCR for the presence of a truncated gene and loss of the *sacB* gene.

(ii) Construction of the *pvdD pchE* double mutant. A mutant fragment of *pvdD* containing a kanamycin resistance cassette flanked by flippase recognition sites (FRT) from pUC18-miniTn7T-kan-FRT was created by PCR overlap extension as described previously (81). The primers used to create the *pvdD*::*kan*-FRT fragment contained HindIII restriction sites, which allowed it to be digested and ligated into HindIII-digested pEX18Ap (82). The ligation product was then transformed into *Escherichia coli* DH5α, and positive clones were selected on 35 μg/ml kanamycin and 100 μg/ml carbenicillin. Confirmation was done by plasmid extraction (miniprep) and digestions with HindIII visualized by agarose gel electrophoresis.

The confirmed pEX18Ap-*pvdD*::*kan*-FRT construct was then transformed into *E. coli* SM10 λ*pir* with 100 μg/ml carbenicillin for selection. Replacement of the *pvdD* gene in the *pchE*::MrT7 mutant (83) was performed by a *sacB*-mediated strategy as described previously (82). Merodiploid selection was performed with 400 μg/ml kanamycin and 250 μg/ml carbenicillin. Ten percent sucrose was then used for counterselection of double recombinants on LBNS agar, and resistant colonies were screened for kanamycin resistance and carbenicillin sensitivity. Carbenicillin-sensitive clones were confirmed as *pvdD pchE* mutants by colony PCR. Absence of pyoverdine production was verified by cultivating selected clones in King's B medium and measuring fluorescence at 398 and 455 nm with a Cytation plate reader (BioTek, Winooski, VT).

***Pseudomonas* culture filtrate and cell production.** PA14 wild-type and mutant LC, PCF, and BCF were prepared as detailed previously (29). In brief, RPMI 1640 medium (Lonza, Walkersville, MD) was used and quantitated *P. aeruginosa* suspensions were grown for 24 h at 37°C. For BCF, a 2-h adhesion step and wash preceded 22 h of culture. PCF and BCF were filtered (0.22 μm) after the growth period.

Assays used to determine effects of *Pseudomonas* on *Aspergillus*. In our initial studies, we studied *A. fumigatus* biofilm formation and preformed *A. fumigatus* biofilm and the mutants in plastic wells, initially 12-well plates, as previously described (29). This platform has advantages and disadvantages; the advantages include large volumes that enable more studies of the supernatants, and the disadvantages include greater consumption of materials per study and fewer replicates possible for each variable studied. In further developments, a 96-well plate assay was used as described previously (69), which had the advantage of an increased number of possible replicates and decreased consumption of reagents but required more precision in the delivery of smaller quantities of materials per well. Results of studies of the mutants in wells were presented in a previously published abstract and poster (47). However, even the 96-well format does not allow tests that would include a study of all of the mutants on the same plate, allowing the same controls for all; thus, the well studies are based on eight replicates per test condition studied but studied one to three times for each condition and not all on the same plate with the same controls. Because of these limitations, the assays on agar plates described below were developed, which overcome the above-described limitations and also are less cumbersome to perform. For that reason, although the well assay results are briefly summarized in the Discussion, for comparative purposes and completeness, the focus of the present paper is on the newer assays, which we regard as more robust for the concurrent screening of multiple isolates.

Forming (BCAM) and preformed (BHAM) *A. fumigatus* strain 10AF biofilm plate assays on agar. A 1.25-g sample of Bacto agar (final experimental concentration, 1.25%; BD Biosciences, Durham, NC) was added to 25 ml of distilled water and autoclaved. After cooling to 56°C, 75 ml of RPMI 1640 medium and 2.5×10^4 *A. fumigatus* (strain 10AF) conidia/ml of agar were added. The conidium-containing agar was distributed into the inner 60 wells of sterile flat-bottom 96-well cell culture plates (Costar, Corning Inc., Corning, NY) at 100 μl/well. Upon agar solidification, plates were either loaded immediately with test articles (BCAM assays) or incubated at 37°C for 24 h before loading (BHAM assays). Immediately before loading, all of the empty peripheral wells of each 96-well plate were filled with 200 μl of sterile water to limit evaporation from the test wells. Test wells were loaded with 100 μl of test substances, e.g., *P. aeruginosa* wild-type or mutant planktonic or biofilm supernatant. On each supernatant test plate, all PA14 mutant and wild-type supernatants were present in duplicate. Control wells on each test plate contained 100 μl of RPMI 1640 medium, allowing the conversion of test results from each plate to percentages of the individual RPMI medium control (as 100%) for better comparison of results between plates. Loaded plates were incubated at 37°C for 24 h. On the agar, hyphal mats with biofilm form (48), as verified by optical microscopy, showing the same arrangement as has been studied in liquid wells, studied by optical, confocal, and electron microscopy (29, 83). Studies were also conducted in a hypoxic environment, as detailed previously (74). In brief, the assay plates were incubated in the Gas-Pak EZ Campy pouch system (Becton Dickinson, Franklin Lakes, NJ), generating an atmosphere of 10% oxygen (74). Plates were evaluated by XTT metabolic assay (29). Briefly, 100 μl of an XTT-menadione mixture (150 μg/ml XTT, 30 μM menadione) was added to each test well and incubated at 37°C for 30 min (BHAM assays) to 1 h (BCAM assays). Supernatant from each well (100 μl) was assayed with a plate reader (Opsys MR; DYNEX Technologies, Chantilly, VA).

Agar-based bioassay to measure effects of PA14 LC on *A. fumigatus* strain 10AF growth. Bacto agar (3 g) was dissolved in 50 ml of distilled water and autoclaved. After cooling to 56°C, 150 ml of RPMI 1640 medium and 2.5×10^4 *A. fumigatus* strain 10AF conidia/ml of agar were added. The conidium-containing agar was distributed into a plastic bioassay dish (Thermo Scientific, Roskilde, Denmark). The agar-conidium mixture described above was sufficient for one plate. Upon agar solidification, wells were cut into the agar with a vacuum pump attached to a metal suction tool. Each plate was loaded with 40 μl (OD_{610} of 0.4) of wild-type or mutant PA14 LC suspension per well in duplicate. Loaded plates were incubated at 37°C for 24 h. Plates were evaluated by measuring the area of the fungus-free zone around each well; the area of the well was subtracted. A mean was calculated for each duplicate value on the same plate. Each plate contained all of the mutants, wild-type PA14, and RPMI medium negative controls. The experiment was performed four times, resulting in four inhibition zone area values per wild type/mutant. The mean of the wild-type inhibition zones was regarded as 100% inhibition, and the inhibition zones of the mutants were scored into relation to that.

For visualization of inhibition zones (Fig. S1), we used 60- by 15-mm petri dishes (Corning Inc., Corning, NY) containing 2 ml of RPMI 1640 agar and 2.5×10^4 *A. fumigatus* (strain 10AF) conidia/ml of agar. Upon solidification, a single well was punched into the middle of each plate and filled with a wild-type or mutant PA14 LC suspension. After 24 h of incubation at 37°C, inhibition zones were measured, plates were washed with PBS, and photographs were taken.

MIC assay. MIC testing for wild-type and mutant PA14 PCF and BCF against planktonic *A. fumigatus* growth was performed by using the CLSI M38-A2 protocol for broth macrodilution (84). Briefly, an inoculum of 2×10^3 conidia/ml was added to 500 μl of fresh RPMI 1640 medium and combined with 500 μl of wild-type or mutant PA14 supernatant dilutions (1:2 to 1:2,048) in 5-ml polystyrene round-bottom tubes (BD Biosciences). Controls containing dilutions of 500 μl of RPMI 1640 instead of supernatants were added. A positive control contained 10^3 conidia in 1 ml of fresh RPMI 1640. The tubes were incubated at 37°C with shaking (100 rpm) for 48 h, and *A. fumigatus* strain 10AF growth was determined visually. The highest dilution with fungal growth diminished from the growth in the RPMI 1640 control was determined for wild-type and mutant supernatants. The experiment was performed four times.

Ranking of PA14 mutants. For each of four experiments per assay, wild-type and mutant PA14 effects on *A. fumigatus* strain 10AF were expressed as percentages of the RPMI 1640 control (in BCAM and BHAM assays, the RPMI 1640 control = 100% = undisturbed metabolism or growth) or percentages of wild-type PA14 activity (for zone sizes; the MIC is wild-type PA14 activity = 100% = maximal antifungal effect). The results of the four experiments per assay were combined and statistically evaluated with Student's *t* test. Mutants with antifungal effects significantly different from that of wild-type PA14 were ranked by their mean values, with a rank of 1 assigned to the mutant with the lowest antifungal activity.

Pyoverdine measurement. RPMI 1640 medium was incubated with PA14 (5×10^7 bacteria/ml) at 37°C and 100 rpm overnight. Bacterial growth was measured at 600 nm in a spectrophotometer (Genesys 20; Thermo Fisher Scientific Inc., Waltham, MA). All cultures were centrifuged at 200 $\times$ *g* for 30 min at room temperature. Supernatants were filtered (0.22 μm) to obtain sterile PCFs. For all PCFs, pyoverdine production was measured at 405 nm. Measurements were normalized to bacterial growth with the formula Relative PYOV production = OD_{405}/OD_{600}.

Northern blotting. An *A. fumigatus* wild-type strain (AfS77; 10^6 conidia/ml) was grown in 15 ml of liquid 2YT medium at 37°C for 15 h (22). Subsequently, 10 ml of the culture supernatant was removed and replaced with 10 ml of culture supernatant of wild type or *pvdA pvdE* mutant *P. aeruginosa* PA14, respectively, and culturing was continued for another 3 h. For Northern blot analysis, RNA was isolated with TRI Reagent (Sigma); 10 μg of total RNA was separated in formaldehyde-containing agarose gels, blotted onto Hybond-N+ membranes (Amersham Biosciences), and hybridized with digoxigenin-labeled probes. The primers used for generation of the hybridization probes were described previously (22).

Liquid CAS assay. We prepared 10× CAS assay reagent as described previously (85). One part 10× CAS reagent was combined with 9 parts siderophore-containing liquid, and the mixture was incubated at 37°C for 6 h. Mixtures were measured with a plate reader (Opsys MR; DYNEX Technologies) and compared to RPMI medium –1× CAS reagent as reference values.

***Aspergillus* growth assays.** The *A. fumigatus sidA ftrA* mutant strain (10^4 conidia) was point inoculated onto 2 ml of solid minimal medium (86) with or without supplementation with $FeSO_4$ to a final concentration of 5 mM, pyoverdine to final concentrations of 1 and 10 μM, TAFC to final concentrations of 1 and 10 μM, or 600 μl of wild-type PA14 PCF. The plates were incubated for 48 h at 37°C.

Statistical analysis. Results were analyzed with Student's *t* test if two groups were compared and one-way analysis of variance (ANOVA) combined with Tukey's posttest for multiple comparisons. All data in this study are expressed as the mean ± the standard deviation (SD). Data reported as percentages of the control value were compared with Student's *t* test after arcsin transformation of the proportions; these data are presented as percentages.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00345-17.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

ACKNOWLEDGMENTS

We thank Marife Martinez for excellent technical support.

These studies were partially supported by a gift from John Flatley (CIMR no. 3770) and by a grant from the Child Health Research Institute, Stanford Transdisciplinary Initiatives Program (CIMR no. 3777). This work was partially supported by the Austrian Science Fund/Infect-ERA program (FWF grant I1616/Infect-ERA project AspMetNet to H.H.). A.-M.D. is an associate student of the HOROS doctoral program (W1253).

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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