



# Surface Dependent Inhibition of *Mycobacterium abscessus* by Diverse *Pseudomonas aeruginosa* Strains

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**ABSTRACT** Both *Pseudomonas aeruginosa* and *Mycobacterium abscessus* are bacteria that cause pulmonary infection in people with inflammatory lung disease, including individuals with cystic fibrosis (CF). These bacterial species inhabit the same environmental reservoirs (soil and water) and can be coisolated in the lungs of people with CF. We investigated the interaction of these bacteria and found an antagonistic interaction favoring *P. aeruginosa* that was observed in biofilms but not in planktonic cultures. This antagonism extended to multiple *P. aeruginosa* strains and against *Mycobacterium smegmatis*. We tested known *P. aeruginosa* mutants for genes that can play roles in interbacterial contact-dependent (type III and type VI secretion systems) and contact-independent (quorum sensing, type II secretion) antagonism pathways to interrogate the mechanism of action. Our results indicate that well-known mechanisms of interbacterial competition are not responsible for the antagonism of *P. aeruginosa* toward *M. abscessus*, suggesting a novel antibacterial strategy.

**IMPORTANCE** The biofilm lifestyle is favored by many organisms, and understanding interbacterial interactions that occur between coisolated bacterial species can provide new information regarding bacterial defense mechanisms and antibacterial targets. This may also provide insights into possible interbacterial interactions impacting host immunity during coinfection. Here, we investigate an antagonistic interaction favoring *P. aeruginosa* over *M. abscessus* exclusively in dual-species biofilms and not in liquid coculture.

**KEYWORDS** *Pseudomonas aeruginosa*, *Mycobacterium abscessus*, interbacterial interaction, antagonism, biofilms, bacteria-bacteria interactions, cell-cell interaction, cystic fibrosis pathogens, nontuberculous mycobacteria

Many bacterial species are found in diverse communities and can be coisolated from various infections. These various bacterial species can be found in biofilms, which can be defined as a community of microbial cells that are enclosed in a polymeric extracellular matrix. Different types of interactions can take place in these communities that are either beneficial or detrimental to the survival of the bacterial species of interest. This study focuses on the interaction between *Pseudomonas aeruginosa* and *Mycobacterium abscessus*, which are pathogens that can be found in the same environmental reservoirs, including water sources and soil (1, 2), and are coisolated from various pulmonary infections, including in people with cystic fibrosis (pwCF).

*P. aeruginosa*, a Gram-negative opportunistic pathogen, is known to cause nosocomial infections and is commonly isolated in pwCF (3). *M. abscessus*, a rapidly growing nontuberculous *Mycobacterium* (NTM), has also shown an increased prevalence in chronic lung infections in pwCF (4, 5). *M. abscessus* has two morphotypes, rough and smooth, which are mainly distinguished by the presence of glycopeptidolipids (GPL) in the cell wall of the smooth and absence of GPL in the rough. Both morphotypes can survive intracellularly in macrophages, and both can form biofilms (6).

CF is an autosomal recessive genetic disease that leads to progressive lung failure and chronic disorders in other organs of the body, including the pancreas and the liver

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(7). The reduced mucociliary clearance in the lung due to mutation of CFTR can sustain bacterial infections, which can develop into biofilms over time making them more challenging to treat (8, 9).

Notably, *P. aeruginosa* and *M. abscessus* are both found in the same environmental reservoirs (1, 2). Previous studies have shown that *M. abscessus* had specific genes common to *P. aeruginosa* that are thought to have been transferred through horizontal gene transfer from distantly related environmental bacteria (10). Both organisms have also been coisolated from pwCF and other respiratory infections, including studies showing that NTM-positive pwCF are more likely to be infected with *P. aeruginosa* (11–14). Case studies of severe pulmonary infections of *M. abscessus* and *P. aeruginosa* coinfection in patients with pneumonia have also been reported (15).

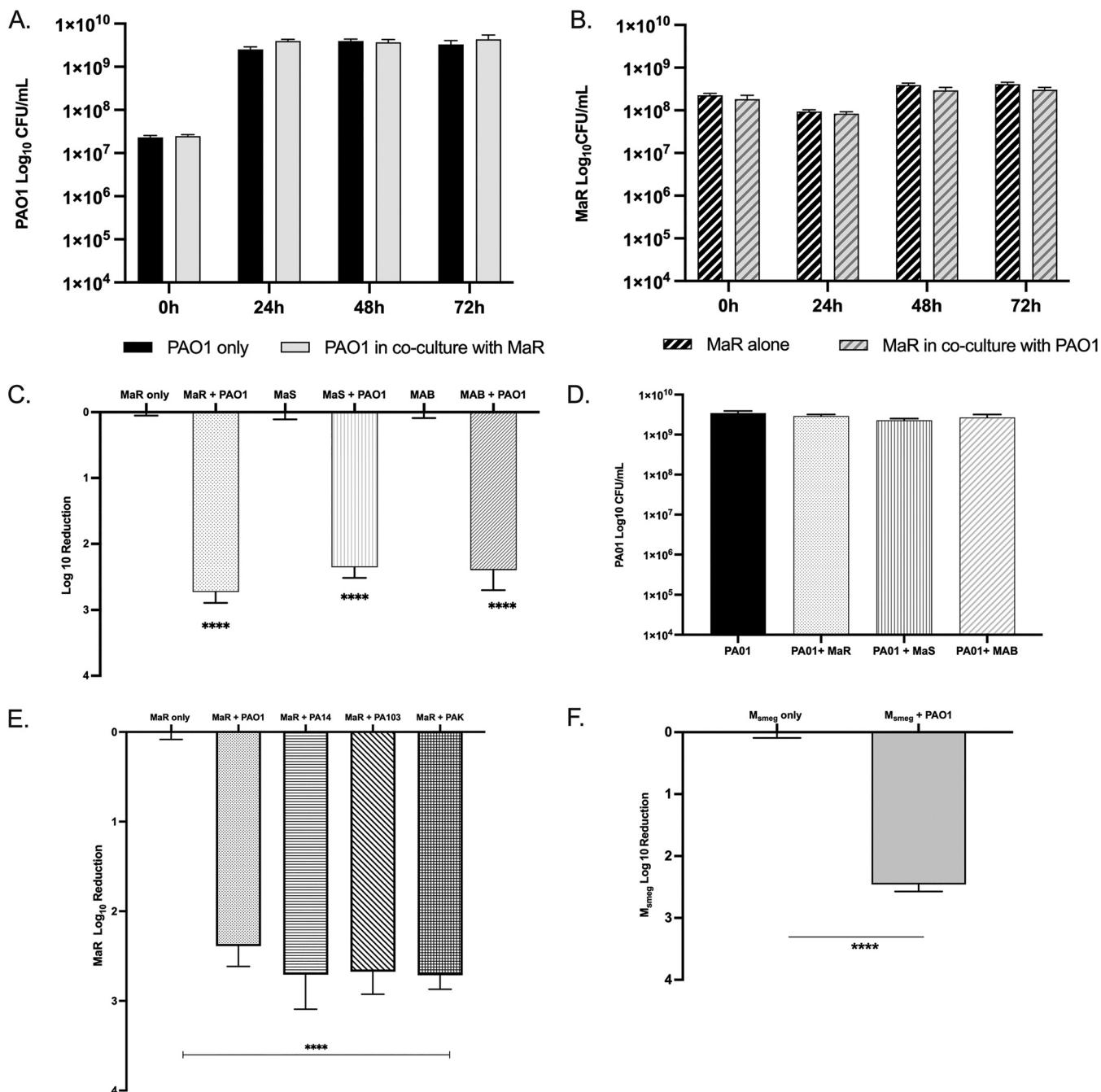
There is limited understanding, however, of the interaction between these two important opportunistic pathogens. One study suggested that *M. abscessus* can degrade *Pseudomonas* quinolone signal (PQS), which is a quorum-sensing (QS) molecule (16). This finding was compelling, as *P. aeruginosa* is equipped with multiple virulence factors, including various secretion systems and secondary metabolites, allowing it to have a survival advantage over other microbes in cocultures, including biofilms (17, 18). Here, we examine the interaction between these two bacterial species and report that *P. aeruginosa* can antagonize *M. abscessus* in colony biofilms (19) but not in liquid coculture. Furthermore, we report that this antagonism does not appear to be mediated by known mechanisms of *P. aeruginosa* interbacterial antagonism, suggesting a novel antibacterial strategy.

## RESULTS

***P. aeruginosa*-mediated antagonism of *M. abscessus* in biofilms.** To initially examine the interaction between *P. aeruginosa* and *M. abscessus*, we utilized agar plates and liquid coculture. Across all time points analyzed for the liquid coculture, there was no difference in planktonic growth yields of either *P. aeruginosa* (Fig. 1A) or *M. abscessus* (Fig. 1B). This was consistent for both the rough and smooth *M. abscessus* variants (see Fig. S1A in the supplemental material). This indicated that when grown in liquid coculture there is no antagonism between the two organisms. We hypothesized that the antagonism between the two organisms might be density and/or lifestyle dependent and further investigated this using a colony biofilm model (8, 19).

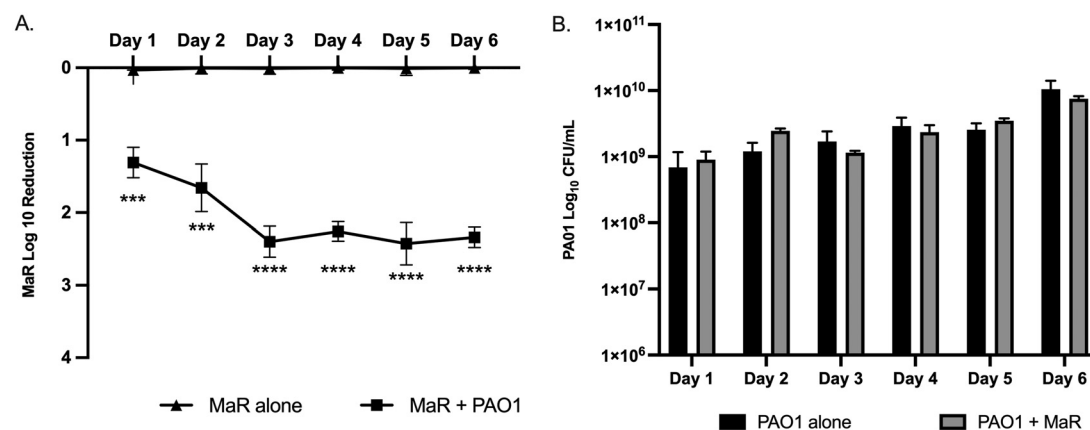
To account for the aggregative nature of *M. abscessus*, liquid cocultures of MaR and PAO1 were grown in either 7H9 + Tween or 7H9 medium (Fig. S1B and C). No antagonism was observed in either medium. The change in the growth of MaR compared to that of PAO1 in liquid monocultures shows a visible optical density (OD) change over 72 h (see Fig. S4B in the supplemental material). We further considered the difference in growth rates between the organisms. Liquid cocultures with various initial suspension of *P. aeruginosa* PAO1 (optical density at 600 nm [OD<sub>600</sub>], 0.05, 0.150, and 0.5) were cocultured with *M. abscessus* MaR at an OD<sub>600</sub> of 0.150 and showed that there is no significant difference in growth of MaR when in coculture with PAO1 at different starting ODs (Fig. S1D and E). Similarly, when bacteria were suspended in different volumes of media, no statistical difference was observed in *M. abscessus* growth (Fig. S1F and G).

To interrogate growth in biofilms, *M. abscessus* and *P. aeruginosa* dual-species biofilms were grown for 4 days on membrane filters placed on 7H10 medium at a multiplicity of infection (MOI) of 10:1 (19). Biofilm biomass and survival of each organism in dual-species biofilms were quantified by CFU and compared to growth in single-species colony biofilms. Both variants of *M. abscessus* were used, as they readily form biofilms (6). Interestingly, there was a significant decrease in *M. abscessus* abundance when grown in dual-species biofilms with *P. aeruginosa* compared to that of as *M. abscessus* single-species biofilm (Fig. 1C; note log scale). Antagonism was observed with each *M. abscessus* morphotype and the ATCC 19977 reference strain (MAB), which is a mixture of ~30% rough (MaR) and 70% smooth (MaS) morphotypes and the source of our isolated morphotypes (Fig. 1C). In contrast, there was no statistical significance in *P. aeruginosa* growth in dual-species biofilms compared to single-species biofilms (Fig. 1D), suggesting a unidirectional antagonism in biofilms.



**FIG 1** (A) Log<sub>10</sub> CFU graph of PAO1 grown alone versus in planktonic coculture with MaR for 72 h. There was no statistical significance between the two conditions. (B) Log<sub>10</sub> CFU/mL of MaR grown alone versus in planktonic coculture with PAO1 for 72 h (MaS data is found in Fig. S1A in the supplemental material). There was no statistical significance between the two conditions. (C) Log<sub>10</sub> reduction CFU graph of MaR, MaS, and MAB (ATCC strain) grown in a single species biofilm compared to growth in a dual-species biofilm with PAO1. Unpaired *t* test; \*\*\*\*, *P* < 0.0001; *n* = 3. (D) Log<sub>10</sub> CFU graph of PAO1 grown in a single-species biofilm compared to growth in dual-species biofilm with MaR, MaS, or MAB. (E) Log<sub>10</sub> reduction graph of MaR grown alone compared to growth with PAO1, PA14, PA103, and PAK. Unpaired *t* test; \*\*\*\*, *P* < 0.0001; *n* = 3. (F) Log<sub>10</sub> reduction graph of *Mycobacterium smegmatis* in a single-species biofilm compared to growth in dual-species biofilm with PAO1. Unpaired *t* test; \*\*\*\*, *P* < 0.0001; *n* = 3. Three biological replicates with two technical replicates were completed for each CFU study, except for that in panel F with two biological and technical replicates. All biofilm data represent day 4 CFU.

*P. aeruginosa* must be viable for antagonism in dual-species biofilms since heat-killed *P. aeruginosa* and *M. abscessus* MaR do not show an antagonistic interaction (see Fig. S2A in the supplemental material). We also evaluated the role of the membrane filters in antagonism by spotting mono- and dual-species bacterial suspensions of *M. abscessus* and *P. aeruginosa* onto 7H10 agar, which also resulted in an antagonism of *M. abscessus* by *P. aeruginosa* (Fig. S2B). To further account for the difference in



**FIG 2** (A) Log<sub>10</sub> reduction graph of kinetic biofilm over 6 days comparing MaR growth in single-species biofilm to dual-species biofilm with PAO1. Unpaired *t* test; \*\*\*, *P* < 0.001; \*\*\*\*, *P* < 0.0001; *n* = 3. Two filters were placed on each plate with three biological replicates. A similar log reduction was observed with the smooth morphotype (see Fig. S2E in the supplemental material). (B) Log reduction CFU graph of kinetic biofilm over 6 days comparing PAO1 growth in single-species biofilm to dual-species biofilm with MaR. Two filters were placed on each plate with three biological replicates.

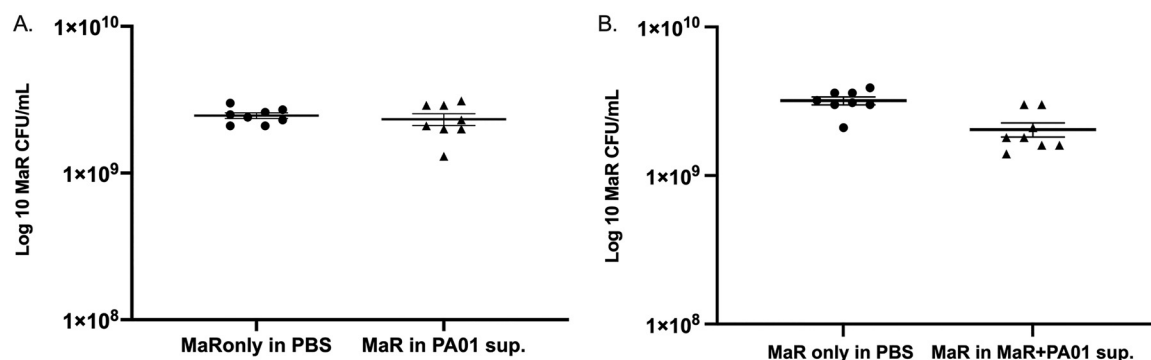
growth between the two bacteria, in addition to the MOI of 10:1 ratio of *M. abscessus* to PAO1, biofilms grown at MOIs of 1:1 and 100:1 resulted in the antagonism of *M. abscessus* (Fig. S1C and D). Remarkably, antagonism was also observed in experiments with three other *P. aeruginosa* strains (PA14, PA103, PAK) (Fig. 1E) and with another NTM bacterium, *Mycobacterium smegmatis* (Fig. 1F), showing that the antagonistic interaction is evident in multiple *P. aeruginosa* strains and against another NTM isolate.

**Temporal analysis of *M. abscessus* and *P. aeruginosa* interaction in biofilm.** To investigate the viability of *M. abscessus* in coculture with *P. aeruginosa*, dual-species biofilms were evaluated over 6 days. MaR CFU decreased significantly at days 1 and 2 in the dual-species biofilm, compared to a single-species biofilm and remained stable through days 3 to 6 (Fig. 2A), while *P. aeruginosa*, unlike MaR, showed unchanged growth over the 6 days (Fig. 2B). The same results were obtained with the smooth morphotype (MaS) (see Fig.S2E in supplemental material).

Kinetic biofilm growth studies suggested that *M. abscessus* might be killed or its growth inhibited by *P. aeruginosa* when in biofilm cocultures. We hypothesized that *M. abscessus* viability, measured by CFU, would decrease steadily over 6 days when in a dual-species biofilm with *P. aeruginosa*. However, *M. abscessus* viability remained constant from days 3 to 6 (Fig. 2A).

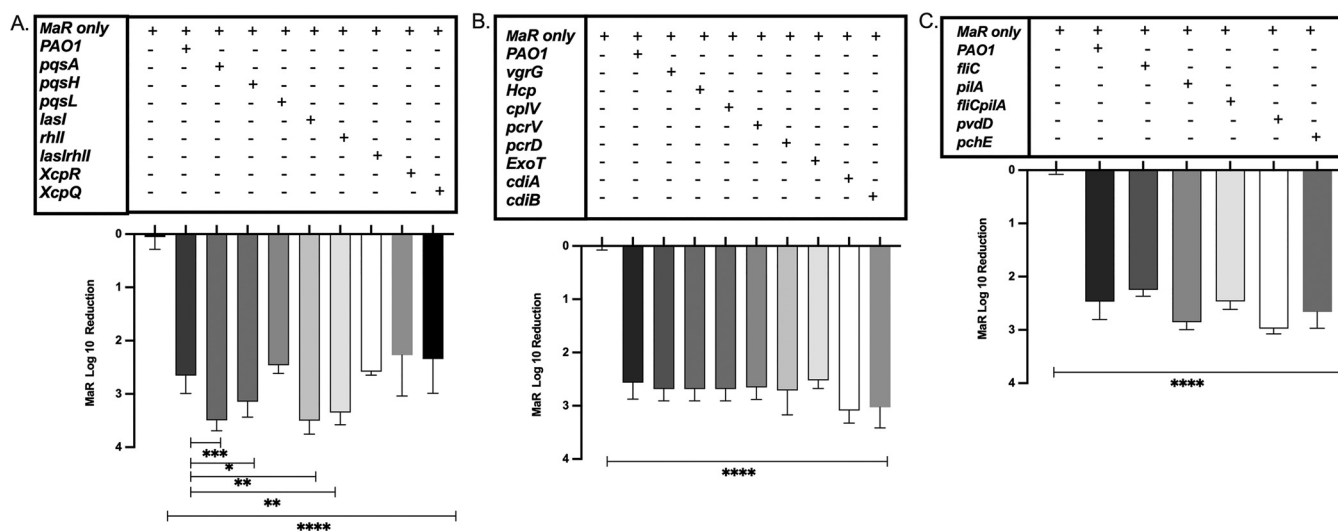
**Antagonism against *M. abscessus* is not maintained in the supernatant recovered from single *P. aeruginosa* only or from mixed biofilms.** Since *P. aeruginosa* can secrete various factors to antagonize the growth of other microorganisms, we evaluated if the antagonism toward *M. abscessus* resulted from *P. aeruginosa*-secreted factors. *M. abscessus* biofilms were grown using an inoculum of *M. abscessus* resuspended in supernatants harvested from *P. aeruginosa* single-species biofilm or dual-species *P. aeruginosa* and *M. abscessus* biofilms. There was a slight decrease in growth of MaR when resuspended in supernatant harvested from *P. aeruginosa*-only biofilm (Fig. S2F), but there was no significant difference in growth when resuspended in dual-species biofilm supernatant (Fig. S2G). In the case of MaS, there was no significant difference in growth when resuspended in MaS only and dual-species biofilm supernatant (Fig. S2H and I).

We considered the possibility that *P. aeruginosa*-secreted factors might be diluted, as the biofilms were harvested into 5 mL of phosphate-buffered saline (PBS). Hence, we concentrated the supernatants 10-fold; however, this did not recapitulate the antagonism, as no significant difference in the survival of the *M. abscessus* rough morphotype resulted when grown in either *P. aeruginosa*-only supernatant (Fig. 3A) or supernatants harvested from *P. aeruginosa* and *M. abscessus* dual-species biofilms (Fig. 3B). These results suggested that the antagonism is surface dependent and requires cell-cell contact between *P. aeruginosa* and *M. abscessus*.



**FIG 3** (A) Log<sub>10</sub> CFU graph of MaR grown in 10-fold concentrated supernatant harvested from PAO1 biofilm. There is no statistical significance; mean ± standard error of the mean (SEM) (n = 2); one technical replicate and two biological replicates. (B) Log<sub>10</sub> CFU graph of MaR grown in concentrated supernatant harvested from PAO1 and MaR biofilm. There is no statistical significance; mean ± SEM (n = 1); one technical replicate and two biological replicates.

**Known *P. aeruginosa* contact-independent antagonism pathways are not responsible for *M. abscessus* antagonism.** We next investigated the mechanism responsible for the *P. aeruginosa*-mediated antagonism of *M. abscessus* by testing well-known *P. aeruginosa* contact-independent mechanisms. We hypothesized that PQS might play a role in the observed antagonism, as it has been shown in other systems (20). We evaluated dual-species biofilms of *M. abscessus* and *P. aeruginosa* *pqsA*, *pqsH*, and *pqsL* mutants. Antagonism of *M. abscessus* when grown with each of the PQS mutants was similar to that of wild-type PAO1 (Fig. 4A). Since PQS production is regulated by other *P. aeruginosa* QS systems, we also tested *P. aeruginosa* *lasI* and *rhII* QS mutants. The antagonism was maintained in dual-species biofilms of *M. abscessus* and the *P. aeruginosa* QS mutants (*lasI*, *rhII*, and *lasI rhII* mutants) (Fig. 4A). These results were consistent across both morphotypes of *M. abscessus* (see Fig. S3A in the supplemental material). These data indicate that QS does not play a role in the observed antagonism against *M. abscessus*. We further examined biofilm cocultures with MaR and *P. aeruginosa* type II secretion system (T2SS) mutants, *xcpQ* and *xcpR* mutants, to test for other secreted molecules (Fig. 4A). Antagonism was also maintained in coculture with T2SS mutants.



**FIG 4** (A) Log<sub>10</sub> reduction graph of MaR growth compared to that of *P. aeruginosa* QS (*lasI*, *rhII*, and *lasI rhII* mutants), PQS biosynthesis mutants (*pqsA*, *pqsL*, and *pqsH* mutants), and T2SS mutants (*xcpR* and *xcpQ* mutants) (n = 3). Unpaired t test; \*\*\*\*, P < 0.0001. Unpaired t test comparing PAO1 to *pqsA*, *pqsH*, *lasI*, and *rhII* mutants; \*\*\*, P = 0.0001; \*, P = 0.0121; \*\*, P = 0.0028; and \*\*, P = 0.0084, respectively. (B) Log<sub>10</sub> reduction graph of MaR growth compared to that of *P. aeruginosa* T6SS (*vgrG*, *Hcp*, and *cplV* mutants), T2SS (*pcrV*, *pcrD*, and *exoT* mutants), and CDI mutants ( $\Delta cdiA_{PA0041}$  and  $\Delta cdiB_{PA2463}$  mutants) (n = 3). Unpaired t test; \*\*\*\*, P < 0.0001. (C) Log<sub>10</sub> reduction of MaR growth compared to that of *P. aeruginosa* motility mutants (*fliC*, *pilA*, and *fliC pilA* mutants) and iron sequestering mutants (*pvdD* and *pchE* mutants) (n = 3). Unpaired t test; \*\*\*\*, P < 0.0001. All experiments evaluated two technical replicates and three biological replicates. See Table S1 in the supplemental material for a list of mutants used in this study. MaS results can be found in the supplemental material (see Fig. S3A to C).

**Well-studied contact-dependent interbacterial antagonistic mechanisms do not mediate the antagonism of *P. aeruginosa* on *M. abscessus*.** As known contact-independent mechanisms did not appear to play a role in the antagonistic interaction between the two bacterial species, we next investigated contact-dependent bacterial systems. We tested mutants to evaluate whether the *P. aeruginosa* type VI secretion system (T6SS) and type III secretion system (T3SS), as well as the contact-dependent inhibition (CDI) system played a role in the observed antagonism. All *P. aeruginosa* T3SS, T6SS, and CDI mutants tested in dual-species biofilm cocultures with *M. abscessus* failed to reverse the antagonism against *M. abscessus* (Fig. 4B). These results were consistent for both morphotypes of *M. abscessus* (Fig. S3B).

**Neither motility nor iron sequestration are responsible for the antagonism of *M. abscessus*.** Since known contact-independent and -dependent antagonistic mechanisms of interbacterial interaction were not responsible for the observed antagonism of *M. abscessus*, we next evaluated other key mechanisms of interbacterial antagonism that have been reported for *P. aeruginosa*, including motility and iron sequestration (18). We tested dual-species biofilms with *M. abscessus* and the *P. aeruginosa* *fliC* and *pilA* motility mutants, which are responsible for swarming and twitching motility, respectively, as well as the *fliC pilA* double mutant. Interestingly, antagonism was still observed in the presence of the motility mutant (Fig. 4C) and was the same for both variants of *M. abscessus* (Fig. S3C). This finding suggested that the motility of *P. aeruginosa* is not required for antagonism against *M. abscessus*.

Finally, we further tested the two main iron sequestering mutants of *P. aeruginosa*, including pyoverdine (*pvdD*) and pyochelin (*pchE*) to examine if *P. aeruginosa* iron sequestration leads to delayed growth of *M. abscessus* and aids in the growth of *P. aeruginosa*. Antagonism of *M. abscessus* was maintained, however, with both *P. aeruginosa* iron-sequestering mutants (Fig. 4C).

To determine the interbacterial mechanism responsible for the observed antagonism between *M. abscessus* and *P. aeruginosa*, we tested various contact-independent and contact-dependent mechanisms of *P. aeruginosa*. Our results indicated that all of these tested systems along with motility and iron sequestration mutants do not appear to mediate the observed antagonism, suggesting a novel mechanism of interbacterial antagonism.

## DISCUSSION

Despite the increased prevalence of *M. abscessus* in CF and in other pulmonary infections and its common isolation with *P. aeruginosa*, there is little understanding of the interaction between these two opportunistic pathogens. Here, we examined the interaction between these organisms in liquid planktonic cultures and biofilm cocultures. To determine the mechanisms responsible for the observed antagonistic interactions between *M. abscessus* and *P. aeruginosa*, mutants of known contact-dependent and -independent antagonistic mechanisms of *P. aeruginosa* were examined. However, all *P. aeruginosa* mutant strains that we examined were unable to abrogate the antagonism against *M. abscessus*, suggesting that a novel or a more complex mechanism may play a role in the observed antagonism. One of the few reports in the literature that examines the interaction between *P. aeruginosa* and *M. abscessus* showed that *M. abscessus* has enzymes that can degrade PQS produced by *P. aeruginosa* (16). This study mainly focused on purified and isolated *M. abscessus* enzymes, while we tested the direct interaction between these organisms. Although no antagonism between *M. abscessus* and *P. aeruginosa* was observed in liquid cocultures, including in those tested at different starting bacterial concentrations to account for growth difference between *M. abscessus* and *P. aeruginosa*, significant antagonism was observed in dual-species biofilm cocultures, suggesting that the pathway responsible for the observed antagonism was surface associated and concentration dependent. Interestingly *P. aeruginosa* antagonism was also observed with another rapidly growing NTM, *Mycobacterium smegmatis*. *M. smegmatis* is considered a model organism for molecular genetics and biochemical studies for several *Mycobacterium* species as many of the genes encoded by *M. smegmatis* are conserved in other mycobacterial species.

We therefore tested multiple different contact-independent mechanisms of *P. aeruginosa* antagonism, including PQS and QS mutants. PQS is a product of one of the four QS pathways

in *P. aeruginosa* and plays a role in virulence factor regulation, iron sequestration, cytotoxicity, and biofilm formation (20, 22). PQS and other QS pathways lead to the production of different virulence factors that benefit *P. aeruginosa* when in association with other microbes, such as *Staphylococcus aureus* (21). Therefore, in addition to PQS, we also tested 2-heptyl-hydroxyquinoline-*N*-oxide (HQNO), a metabolite that acts in respiratory electron transfer (20, 22–24). PQS, HQNO (*pqsL*), and all other QS mutants tested, however, did not play a role in the *P. aeruginosa* interaction with *M. abscessus* (Fig. 4A). We also tested another contact-independent system of *P. aeruginosa*, T2SS, through which various toxins are released to the target. We evaluated T2SS-secreted factors using *xcpQ* and *xcpR* mutants of *P. aeruginosa* (25), but the antagonism was retained.

We, therefore, considered that it was possible that secretory molecules not regulated by the known QS systems of *P. aeruginosa* might be mediating the antagonism. This entailed a closer evaluation of contact-dependent mechanisms of interbacterial interaction. Specifically, we evaluated T6SS and T3SS mutants. In both systems, a translocation apparatus delivers toxins into target cells (26–30). For example, *P. aeruginosa* can kill heterologous bacterial species, including *Vibrio cholerae* and *Acinetobacter* via T6SS (31), while the T3SS is known to promote virulence against host cells (32). We further examined the role of contact-dependent growth inhibition, which is a two-partner secretion pathway through which Gram-negative bacteria can deliver a toxic C terminus domain of the CdiA protein into neighboring cells when in contact (33). This phenomenon, first described in *Escherichia coli*, can provide an advantage in competitive coculture conditions (34). However, none of the tested mutants reversed the observed antagonism (Fig. 4B). One potential explanation for this result is that the complex cell wall structure of *M. abscessus*, composed of multiple peptidoglycans and mycolic acids (35), may reduce the effectiveness of contact-dependent mechanisms preventing delivery of these toxins.

Both motility and the ability to take up nutrients are important for bacteria to survive and compete in multispecies environments (18). *P. aeruginosa* can compete against nonmotile *Agrobacterium tumefaciens* by blanketing the bacterium in dual-species biofilms. In this study, the formation of smaller microcolonies and reduced blanketing was observed with a *P. aeruginosa pilA* mutant (17). We, therefore, examined the role of *P. aeruginosa* motility, hypothesizing that the lack of motility by *M. abscessus* would provide an advantage for *P. aeruginosa* against *M. abscessus*. However, in this case, motility did not appear to play a role in the *P. aeruginosa* antagonism against *M. abscessus*.

Finally, iron sequestration can also be advantageous in interbacterial interactions, as studies have shown that *P. aeruginosa* can use lysed *S. aureus* as an iron source (18). This again was not the case in our study, as the two major iron sequestration mutants tested, pyoverdine (*pvdD* mutant) and pyochelin (*pchE* mutant), also failed to reverse the antagonism against *M. abscessus* (Fig. 4C). Mycobacteria also have molecules with a high affinity for iron (mycobactins and exochelins) (36), but since the antagonism is not reversed in the presence of *P. aeruginosa* iron sequestration mutants, the antagonistic mechanism(s) at hand may be overriding the contribution of iron sequestration.

In summary, we report a novel antagonistic interaction between *P. aeruginosa* and *M. abscessus* that is observed only in biofilms and not in planktonic coculture, suggesting that the biofilm lifestyle and likely direct contact between *P. aeruginosa* and *M. abscessus* contribute to antagonism. Surprisingly, the well-known *P. aeruginosa* contact-dependent and -independent interbacterial mechanisms, as well as motility and iron sequestration, do not appear to play a role in this antagonism, suggesting a novel interbacterial strategy.

As most of the study is focused on investigating what pathways might be causing the antagonism by *P. aeruginosa*, future studies using transcriptome sequencing (RNA-Seq) and sequencing approaches will investigate *M. abscessus* in the interaction. The novel antagonistic pathway responsible for the observed antagonism is important to study the interaction between these two pathogens since they can be coisolated from the same environmental reservoirs and from individuals with respiratory disease including CF. A better understanding of the interaction, as well as providing new information regarding bacterial defense mechanisms

and antibacterial targets, will help to assess potential outcomes of this microbial interaction and the effects it can have on the host.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** All bacterial strains and 21 transposon mutants of *P. aeruginosa* that were used are listed in Table S1 in the supplemental material. The *P. aeruginosa* PAO1 strain and *M. abscessus* ATCC 19977 strain were used as wild-type strains. *M. abscessus* was obtained from American Type Culture Collection (ATCC) and grown as directed. The ATCC strain is a combination of about 30% of the rough morphotype (MaR) and 70% of the smooth morphotype (MaS). To select for *M. abscessus* when in coculture with *P. aeruginosa*, we used mCherry *M. abscessus*. For mCherry transformation, each morphotype was grown, and an mCherry cassette with kanamycin resistance (provided by Sarah Fortune, Harvard University) was electroporated into the *M. abscessus* strain (6). We combined mCherry reporter morphotype strains in the same ratio as the *M. abscessus* ATCC 19977 type strain.

A 7H9 (Thermo Fisher) broth medium containing 10% oleic acid-albumin-dextrose-catalase (OADC) (Hardy Diagnostics) and glycerol as well as a 7H10 (Thermo Fisher) agar medium containing 10% OADC and glycerol were prepared as directed. The 7H10 agar and 7H9 and 7H9 + Tween liquid media were used for biofilm and liquid cocultures, respectively, as both *M. abscessus* and *P. aeruginosa* can grow in both forms of media. To isolate for *P. aeruginosa*, we used *Pseudomonas* isolation agar (PIA) plates (*M. abscessus* does not grow) and 7H10 + kanamycin plates to isolate for *M. abscessus* (PAO1 does not grow) (see Fig. S4A in the supplemental material).

OD<sub>600</sub> values and their equivalent CFU values used in this study are as follows: for *M. abscessus*, an OD<sub>600</sub> of 0.150 is equivalent to  $2 \times 10^8$ . OD<sub>600</sub> values for PAO1 at 0.05, 0.150, and 0.5 are equivalent to  $2 \times 10^7$ ,  $5 \times 10^7$ , and  $2 \times 10^8$ , respectively.

**Liquid coculture.** Overnight cultures of *P. aeruginosa* and *M. abscessus* were diluted to OD<sub>600</sub> values of 0.05 and 0.150, respectively (16). *M. abscessus* colonies were scraped, suspended, and diluted in 7H9 from a 7H10 plate supplemented with kanamycin (100 μg/mL). As seen in Fig. S2J in the supplemental material, growing overnight cultures of *M. abscessus* in 7H9 + Tween versus scraping a loop full of an agar and diluting to the desired OD results in antagonism. *M. abscessus* and *P. aeruginosa* were pelleted and resuspended in 2 mL of 7H9 and incubated at 37°C and 200 rpm for 72 h. At every 24-h time point, aliquots were serially diluted and enumerated for CFU. Both 7H9 without Tween and 7H9 + Tween media were each tested, and no statistical difference was observed between the media (see Fig. S1B and C in the supplemental material). One milliliter of medium was used to replenish the cultures every 24 h. Replenishing the cultures with 2 mL of fresh medium versus 1 mL also resulted in antagonism (Fig. S1F and G).

**Colony biofilm.** Overnight cultures of *P. aeruginosa* were diluted to an OD<sub>600</sub> of 0.05. *M. abscessus* colonies from 7H10 supplemented with kanamycin (100 μg/mL) were scraped, suspended, and diluted in 7H9 to an OD<sub>600</sub> of 0.150 at an MOI of 1:10, respectively. Mixed cellulose esters membrane filters (25 mm, 0.45-μm pore size; Millipore) were UV sterilized on both sides for 5 min and placed on 7H10 solidified medium. One hundred microliters of bacteria cultures, either *M. abscessus* alone, *P. aeruginosa* alone, or mixed at a 1:1 ratio, was pipetted onto the filters to cover the entire membrane and incubated at 37°C with 5% CO<sub>2</sub> for 4 days. Filters were transferred to new 7H10 plates at day 2 of incubation. At day 4, each filter was harvested by scraping biomass, with a cell scraper, into 5 mL of PBS. Harvested biomass was serially diluted and enumerated for CFU on 7H10 agar supplemented with kanamycin (100 μg/mL) or PIA to quantify *M. abscessus* and *P. aeruginosa*, respectively. To prevent clumping, which would affect CFU values, harvested biofilm bacteria were vortexed with glass beads and diluted in 7H9 + Tween (6). There were two filters on every plate with two technical replicates and three biological replicates performed for both mCherry morphotypes of *M. abscessus*.

For the biofilm kinetic analysis, colony biofilms of *M. abscessus* and *P. aeruginosa*, either alone or mixed, were set up as above. Biofilms were grown for 6 days, with the filters transferred to new 7H10 plate every 2 days. Biofilm biomass was harvested and quantified for CFU as above for each day.

Heat killed biofilms were tested by heat killing *P. aeruginosa* at 80°C for 30 min and inoculating a colony biofilm in coculture with *M. abscessus*.

Biofilms were also set up in six-well plates without membrane filters. Three milliliters of 7H10 agar was poured into six-well plates and left at room temperature overnight. The following day, 50 μL of *M. abscessus* and *P. aeruginosa* monoculture and dual cultures were prepared using an OD<sub>600</sub> of 0.150 and 0.05, respectively, and were spotted onto the agar. At day 4, the biofilm was scraped with 3 mL of PBS and cell scraper and enumerated for CFU.

**Colony biofilm supernatant isolation.** Mixed *M. abscessus* and *P. aeruginosa* as well as single *P. aeruginosa* biofilms were grown for 4 days. For each replicate, six filters were harvested into 20 mL of PBS as mentioned above and centrifuged at  $2,100 \times g$  for 10 min to pellet cells and obtain supernatant. The supernatant was collected and filter sterilized twice using a 0.22-μm syringe filter. The sterile supernatant was collected into 50-mL conical tubes, frozen at -80°C overnight and lyophilized for 2 days. The resulting powder is resuspended in 2 mL of PBS for a 10-fold concentration.

**M. abscessus growth in biofilm supernatant.** *M. abscessus* (OD<sub>600</sub> of 0.150) was pelleted by centrifugation at  $2,100 \times g$  for 10 min, washed in 1 mL PBS, and resuspended in 1 mL of the lyophilized supernatant. As a control, *M. abscessus* pellet was resuspended in 1 mL of PBS. The *M. abscessus* biofilms were grown for 4 days. Biofilm biomass was harvested, serially diluted, and enumerated for CFU. The *M. abscessus* biofilms were also set up with unconcentrated supernatant (supernatant obtained from the initial harvest in 5 mL PBS).

**Data availability.** All data generated or analyzed during this study are included in this published article (and its supplemental information files).



## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.7 MB.

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We have no conflict of interest to declare.

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