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Genetic and Environmental Investigation of a Novel Phenylamino Acetamide Inhibitor of the *Pseudomonas aeruginosa* Type III Secretion System

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ABSTRACT Traditional antibiotics target essential cellular components or metabolic pathways conserved in both pathogenic and nonpathogenic bacteria. Unfortunately, long-term antibiotic use often leads to antibiotic resistance and disruption of the overall microbiota. In this work, we identified a phenylamino acetamide compound, named 187R, that strongly inhibited the expression of the type III secretion system (T3SS) encoding genes and the secretion of the T3SS effector proteins in *Pseudomonas aeruginosa*. T3SS is an important virulence factor, as T3SS-deficient strains of *P. aeruginosa* are greatly attenuated in virulence. We further showed that 187R had no effect on bacterial growth, implying a reduced selective pressure for the development of resistance. 187R-mediated repression of T3SS was dependent on ExsA, the master regulator of T3SS in *P. aeruginosa*. The impact of 187R on the host-associated microbial community was also tested using the *Arabidopsis thaliana* phyllosphere as a model. Both culture-independent (Illumina sequencing) and culture-dependent (Biolog) methods showed that the application of 187R had little impact on the composition and function of microbial community compared to the antibiotic streptomycin. Together, these results suggested that compounds that target virulence factors could serve as an alternative strategy for disease management caused by bacterial pathogens.

IMPORTANCE New antimicrobial therapies are urgently needed, since antibiotic resistance in human pathogens has become one of the world's most urgent public health problems. Antivirulence therapy has been considered a promising alternative for the management of infectious diseases, as antivirulence compounds target only the virulence factors instead of the growth of bacteria, and they are therefore unlikely to affect commensal microorganisms. However, the impacts of antivirulence compounds on the host microbiota are not well understood. We report a potent synthetic inhibitor of the *P. aeruginosa* T3SS, 187R, and its effect on the host microbiota of *Arabidopsis*. Both culture-independent (Illumina sequencing) and culture-dependent (Biolog) methods showed that the impacts of the antivirulence compound on the composition and function of host microbiota were limited. These results suggest that antivirulence compounds can be a potential alternative method to antibiotics.

KEYWORDS *Pseudomonas aeruginosa*, T3SS, phyllosphere, antimicrobial, ExsA

Pseudomonas aeruginosa is an opportunistic human pathogen that causes acute and chronic infections in immunocompromised populations, such as cystic fibrosis (CF) patients (1). This pathogen is the major cause of lung infection and the leading cause of morbidity and mortality in CF patients (2). Although less known, *P. aeruginosa* can also infect plants, including *Arabidopsis* (3, 4). To date, antibiotics are the most

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widely used therapies for treating human infections caused by *P. aeruginosa* (5); however, as antibiotic therapies target cellular components and/or metabolic pathways essential for bacterial survival (i.e., cell wall synthesis and protein synthesis), the strong selective pressure caused by excessive antibiotic administration leads to the development and enrichment of multidrug-resistant (MDR) forms of *P. aeruginosa* (6, 7). In addition, the therapeutic targets of antibiotics are conserved in a wide range of nonpathogenic bacteria; as a result, antibiotic therapies alter host-associated microbiota, such as gut microbiota (8, 9), and can lead to serious health problems, such as *Clostridium difficile* infection and inflammatory bowel disease (10, 11). Recovery of microbiota after antibiotic treatment with antibiotics can take years (12), which could potentially shorten the life span of the host (9, 13). While resistance to newly discovered antibiotics is nearly inevitable (14), alternative anti-infection therapies that alleviate the occurrence of antibiotic resistance and preserve the microbiota are urgently needed.

Antivirulence therapy has emerged as a promising alternative therapeutic strategy for the management of bacterial infections. This therapy targets bacterial virulence instead of survival; therefore, it exerts little or no selective pressure over the bacteria and reduces the emergence of resistance (15, 16). Moreover, the narrow-spectrum nature of targeting virulence factors of the pathogen has the potential advantage of minimizing the damage to the host microbiota (17). One of the essential virulence factors for the pathogenicity of *P. aeruginosa*, particularly for acute infection, is the type III secretion system (T3SS) (18). T3SS is a needle-like structure that directly translocates type III effector proteins from the bacterial cytoplasm into the host cells (19), causing not only damage of the host tissue but also the death of immune cells, such as macrophages (20). It has been shown that a fully functional T3SS is critical for disease initiation and pathogen survival in the early stages of infection (21). Thus, T3SS is an excellent target for the development of a new antivirulence therapy.

In *P. aeruginosa*, the expression of genes encoding the T3SS secretion apparatus, effectors, and other T3SS-secreted proteins is under the control of the master regulator ExsA, an AraC family transcriptional activator (22). Mutation of *exsA* yielded a complete repression of T3SS gene expression and abolishment of the T3SS-mediated cytotoxicity in cultured mammalian cells (23). The activity and function of ExsA are regulated by a partner switch mechanism mediated by three proteins, ExsC, ExsD, and ExsE (24). Under a condition of low calcium or during host cell contact, ExsE, an antagonistic protein of ExsC, is secreted from bacterial cells. The absence of ExsE then frees ExsC to bind ExsD, an antagonistic protein of ExsA. The binding of ExsC to ExsD releases ExsA, and the free ExsA activates the expression of the T3SS-encoding genes (25). ExsA also autoregulates the transcription of itself by binding to the promoter of the *exsCEBA* operon (*exsC* promoter) (26) (Fig. 1). Besides the ExsCEBA regulatory cascade, the transcription of *exsA* is modulated by Vfr (virulence factor regulator)/cAMP complex and Crc (catabolite repression control protein). The posttranscriptional regulator RsmA (repressor of secondary metabolism) positively regulates the translation of *exsA* through an unknown mechanism (27–29).

Through a synthetic compound library screening, we identified a phenylamino acetamide compound named 187R that potently inhibited the expression of T3SS genes in *P. aeruginosa* without affecting bacterial growth. Further research demonstrated that 187R dramatically decreased the protein levels of ExsA, not via regulation of its transcription or translation, suggesting that 187R likely has a posttranslational effect on the master regulator ExsA. The impacts of 187R on the function and composition of host-associate microbial communities were also evaluated using the phyllosphere of *Arabidopsis*, a plant host of *P. aeruginosa*. Our data showed that 187R preserved the composition and function of the host-associated microbial community better than antibiotics. These results suggested that 187R is a potent antivirulence compound that inhibits the *P. aeruginosa* T3SS and has little impact on the host-associated microbial community.

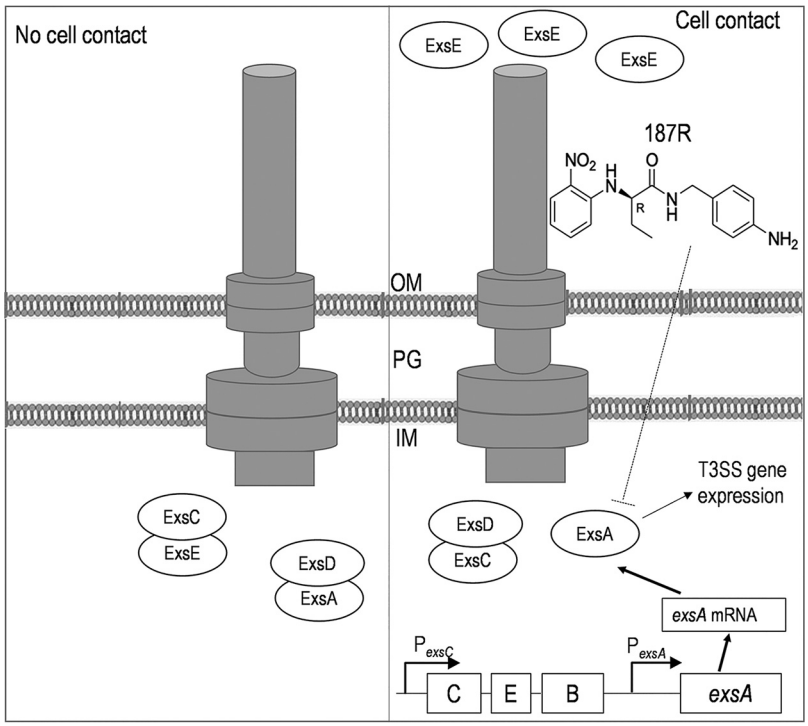

FIG 1 Regulatory cascade of T3SS gene expression in *P. aeruginosa* and the effect of compound 187R. The expression of T3SS is under the control of master regulator ExsA, an AraC family transcriptional activator. The activity and function of ExsA are regulated by a partner switch mechanism. When triggered by the low calcium level or host cell contact, ExsE is secreted; as a result, it frees the anti-anti-activator ExsC. ExsC then binds to the anti-activator ExsD, and the binding of ExsC to ExsD releases ExsA. The free ExsA activates the expression of the T3SS. 187R reduced ExsA protein abundance through a posttranscriptional mechanism.

RESULTS

Screen for compounds that inhibit T3SS in *P. aeruginosa*. To discover compounds that inhibited T3SS but not bacterial growth in *P. aeruginosa* PAO1, we screened a synthetic compound library consisting of five different categories of compounds: category 1, phenoxy and phenylamino acetamides (compounds 187, 187R, 187S, 373, and 412 to 416); category 2, cinchona alkaloids (382 to 387); category 3, inines (399 to 402, 405, 406, and 417 to 433); category 4, aminophenyl propanamides and acids (407 to 411); and category 5, phenyl acrylates (394 to 398) (see Table S1 in the supplemental material). The inhibitory effects on the T3SS upon adding a 250 μM concentration of these compounds was evaluated by measuring the promoter activity of *exoS*, encoding a T3SS effector protein, when PAO1 cells were cultured in a T3SS-inducing medium. The screening results for all the compounds are listed in Table S1. Among all the compounds screened, TS187, TS373, TS382, TS384, and TS405 displayed strong inhibition of *exoS* expression, in contrast to the DMSO control treatment. Further screening in a HeLa cell infection assay suggested that all the cells incubated with compounds at a concentration of 250 μM showed cell rounding and detachment, except for cells exposed to compound TS187. To determine the effects of different conformational isomers of 187, we synthesized two different 187 conformational isomers (187R and 187S) (Fig. 2) and compared their inhibitory effects on *exoS* expression. While 187R strongly inhibited the *exoS* promoter activity (Fig. 3A) without interfering with the growth of bacteria (Fig. 3B), 187S had no T3SS-inhibitory bioactivity (Fig. 3A). The dose-dependent effectiveness of 187R on *P. aeruginosa exoS* promoter activity was evaluated under the T3SS-inducing condition. The minimal concentration of 187R required to reach its maximum inhibition of *exoS* promoter activity was between 16.125 and 32.25 μM (Fig. 3C).

187R repressed T3SS expression in *P. aeruginosa* PAO1 and negatively regulated T3SS-mediated cytotoxicity toward mammalian cells. To determine whether 187R inhibited the expression of other T3SS genes besides *exoS* in *P. aeruginosa* PAO1, we further measured the expression of two other representative genes of T3SS, *exsD*

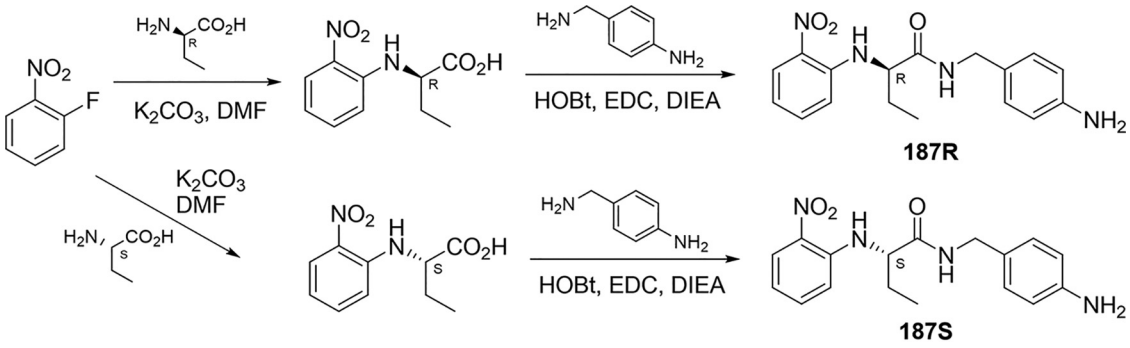

FIG 2 Synthetic routes for 187R and 187S.

and *exoT*, which encode an antiactivator and an effector protein, respectively. The results showed that the expression of both genes tested was significantly decreased when bacterial cells were exposed to 187R (Fig. 4A and B), suggesting that 187R not only inhibits the expression *exoS* but also of other T3SS regulon genes. As a confirmation of our promoter activity results, we also measured the synthesis and secretion of T3SS effector protein ExoS (48.3 kDa) in the presence or absence of 187R (250 μM). Consistent with the decreased *exoS* promoter activity, significant reductions of the intracellular and secreted ExoS protein levels were detected in the presence of 187R (Fig. 4C and Fig. S1). Additionally, we confirmed that 187R was able to inhibit T3SS in another *P. aeruginosa* strain, PAK, by probing its intracellular ExoS and ExoT protein levels (Fig. 4D). Together, these results demonstrated that 187R greatly reduces the T3SS gene expression and the secretion of the T3SS effector.

Next, we evaluated the activity of 187R in T3SS-mediated cytotoxicity toward mammalian cells. *In vitro*-cultured HeLa cells tightly attached to the culture plates, forming a monolayer of mammalian cells. When exposed to *P. aeruginosa*, the T3SS-mediated cytotoxicity resulted in HeLa cell rounding, lifting, and cell death (30). Our results showed that, compared to the DMSO-treated control, which displayed cell rounding and lifting, 187R-treated HeLa cells displayed better attachment to the plate surface at 3.5 h postinfection (Fig. 5A). The number of HeLa cells attached to the plate surface in the 187R-treated group showed no significant differences from those infected by a *pscJ* mutant (a T3SS mutant of *P. aeruginosa* that lacks the basal substructure of the secretion apparatus) or of HeLa cells without inoculating bacteria (Fig. 5A). In addition to the cell adhesion, we also observed a significant difference in cell morphology between the 187R-treated and DMSO-treated (control) HeLa cells after *P. aeruginosa* inoculation. Cell rounding, a typical phenotype observed in DMSO-treated control HeLa cells, was not observed in the presence of 187R (Fig. 5B, C, and D), indicating that 187R reduced the T3SS-mediated cytotoxicity. In summary, 187R is a potent *P. aeruginosa* T3SS inhibitor that reduces the *P. aeruginosa* T3SS under both low-calcium and host cell contact-inducing conditions.

187R inhibited T3SS by antagonizing the master regulator ExsA without inhibiting its transcription or translation. In *P. aeruginosa*, the expression of genes encoding the secretion apparatus and T3SS effector proteins are under the control of the master regulator ExsA (18). To elucidate the mechanism of how 187R inhibits T3SS in *P. aeruginosa*, we first measured the protein levels of ExsA in the presence or absence of 187R. ExsA protein was detected in the DMSO control, and its level was reduced by approximately 2.5-fold in the 187R-treated samples (Fig. 6A). The transcription of *exsA* could be activated through the *exsC* or *exsA* promoter region, generating *exsCEBA* polycistronic mRNA or *exsA* mRNA, respectively. Therefore, two reporter plasmids measuring *exsC* or *exsA* promoter activity were generated (Table 1). To determine if the observed repression on ExsA by 187R was the result of transcriptional regulation, we measured the promoter activity of genes *exsC* and *exsA* and found that *exsC* promoter activity significantly increased (Fig. 6B). Although *exsA* promoter activity was

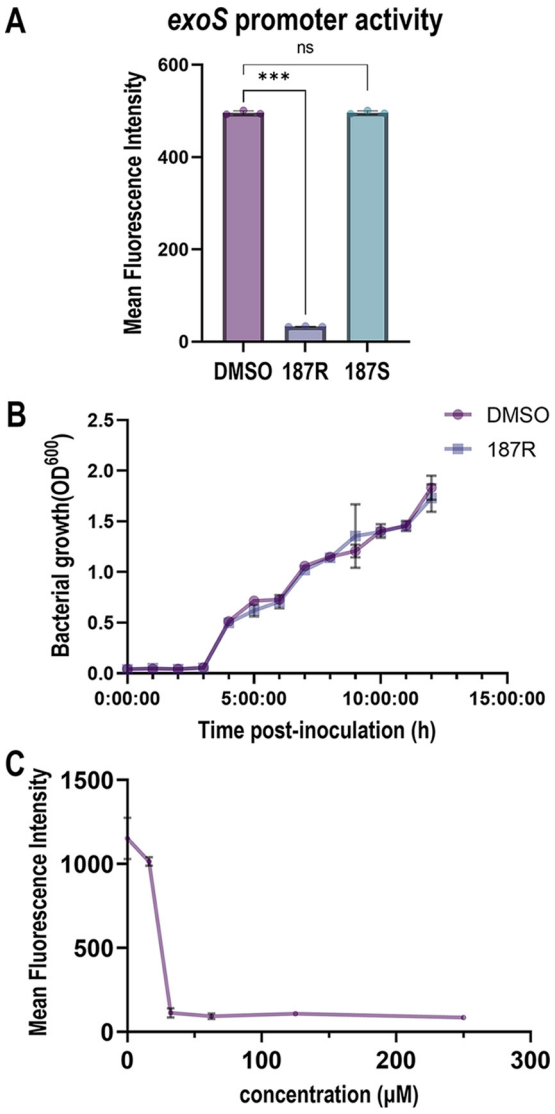

FIG 3 Effects of 187R on *P. aeruginosa exoS* expression and growth. (A) Promoter activity of *exoS* was measured in the presence of 187R (250 μM), 187S (250 μM), or DMSO in *P. aeruginosa* PAO1. Bacterial cells harboring the plasmid pProbe-AT-*exoS* were incubated with 187R, 187S, or DMSO for 6.5 h in T3SS-inducing medium, and the mean fluorescence intensity was measured. Data represent the means of three biological replicates. (B) 187R did not affect the growth of *P. aeruginosa*. At 250 μM, 187R was added to T3SS-inducing medium. Cell density was measured every hour for 12 h. Data represent the means of three biological replicates. (C) Evaluation of the dose-dependent effect of 187R on the promoter activity of *exoS*. Cells of PAO1 were cultured in T3SS-inducing media containing various concentrations of 187R for 6.5 h, followed by the measurement of *exoS* promoter activity. Student's *t* test was performed to compared the 187R-treated and DMSO-treated groups. ***, $P < 0.001$.

statistically different in the presence of 187R, the decrease was marginal (Fig. 6C). Reverse transcription-quantitative PCR (qRT-PCR) analysis revealed a comparable *exsA* mRNA level in the presence of 187R or DMSO (Fig. 6D), suggesting that 187R does not inhibit the transcription of *exsA*.

An alternative hypothesis to account for the decreased ExsA protein level in the presence of 187R could be that 187R hampers the translation of *exsA*. Since ExsA can be synthesized from either *exsCEBA* polycistronic mRNA or *exsA* mRNA, we generated two translational fusion reporters, including an *exsCEBA*′-′*lacZ* translation fusion and an *exsA*′-′*lacZ* translational fusion (Fig. 7A and Table 1). We compared the translation of *exsCEBA* and *exsA* in the presence or absence of 187R. The results showed that the

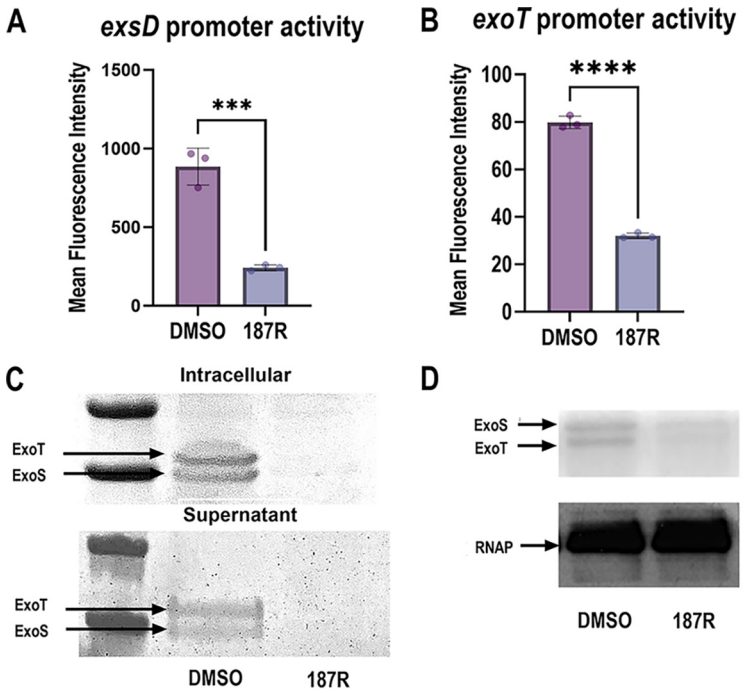

FIG 4 Expression of other T3SS genes upon 187R treatment. (A) *exsD* promoter activity in PAO1 treated with 187R (250 μM) or DMSO for 6.5 h. Data represent the means of three biological replicates. (B) *exoT* promoter activity in *P. aeruginosa* cells treated with 187R or DMSO. *, $P < 0.05$; ***, $P < 0.001$. Data represent the means of three biological replicates. (C and D) Western blots of the protein levels of ExoS in PAO1 (C) and PAK (D) treated with 187R and DMSO, respectively, for 6.5 h. Cross-reacting ExoT bands are also shown. Representative data are from three independent experiments.

expression of *exsCEBA′-′lacZ* significantly increased (Fig. 7B) while the expression of *exsA′-′lacZ* was not changed in the presence of 187R (Fig. 7C), suggesting that 187R does not inhibit the translation of *exsA* or *exsCEBA*. A recent study showed that RpII, a ribosomal large subunit protein L9, directly interacts with the 5′ untranslated region (UTR) of *exsA* and represses its translation (31). We confirmed that overexpression of

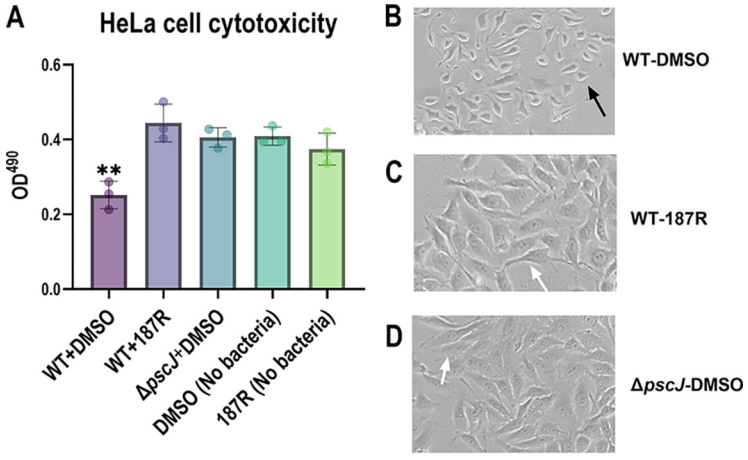

FIG 5 Cytotoxicity of *P. aeruginosa* on HeLa cells. (A) HeLa cell lifting assay. HeLa cells were incubated with *P. aeruginosa* for 3.5 h in the presence of 187R or DMSO. Three replicates were monitored per condition. Wild type, *P. aeruginosa* PAK; *pscJ*-, PAK Δ*pscJ*; no bacteria, no bacteria and only DMSO or 187R (250 μM) added into the HeLa cell culture. Significant differences were found between the DMSO-treated wild-type infection group and all other groups (**, $P < 0.01$). No significant difference was found among all other groups. (B to D) HeLa cell morphology postinfection in the presence of DMSO (B), 187R (C), or the *pscJ* mutant (D). Black arrow indicates cell rounding induced by T3SS effectors, and white arrows show normal cell morphology.

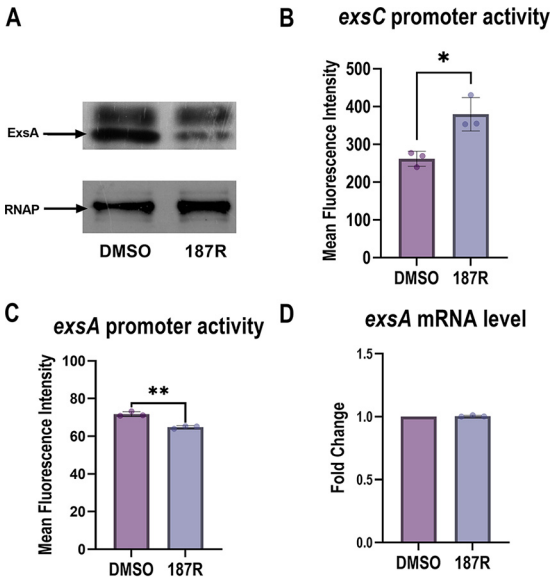

FIG 6 Impact of 187R on *P. aeruginosa* T3SS master regulator *exsA*. (A) PAO1 ExsA protein levels in *P. aeruginosa* cells treated with DMSO or 187R (250 μM). RNA polymerase (RNAP) was used as a loading control. (B and C) Promoter activities of PAO1 *exsC* (B) and PAO1 *exsA* (C) in *P. aeruginosa* cells treated with 187R or DMSO for 6.5 h. *, $P < 0.05$; **, $P < 0.01$. Data represent the means of three biological replicates. (D) mRNA level of *exsA* was measured by qRT-PCR after incubating with 187R or DMSO for 6.5 h. The *rpsL* gene served as an internal control. Three independent experiments with three technical replicates were performed. Data show one of the representative experiments.

rplI significantly repressed the expression of our *exsA*′-′*lacZ* translational fusion but had a negligible impact on the empty vector (Fig. S2). In summary, our results indicated that 187R reduces the protein levels of ExsA but does not affect the transcription or translation of *exsA*. It is likely that 187R antagonizes ExsA protein through an unknown mechanism at the posttranslational level.

Impact of 187R on T3SS in various *Enterobacteriaceae* pathogens. T3SS is a conserved virulence factor in many *Enterobacteriaceae* pathogens of animals and plants. Unlike *P. aeruginosa*, which processes a Ysc family T3SS, *Enterobacteriaceae* pathogens such as *Dickeya dadantii* and *Erwinia amylovora* have Hrc1 family T3SSs that do not have an ExsA ortholog (32–34). Thus, it is unlikely that 187R could inhibit the T3SS of *D. dadantii* or *E. amylovora*. To evaluate the above hypothesis, we tested the T3SS gene expression in *D. dadantii* and *E. amylovora* in the presence or absence of 187R. Unlike what was observed in *P. aeruginosa*, expression of *hrpA*, which encodes the major subunit of the T3SS Hrp pilus, was not affected when exposed to 187R in either *D. dadantii* or *E. amylovora* under the T3SS-inducing condition (Fig. S3). On the other hand, 187R indeed inhibited the expression of T3SS effector proteins ExoS and ExoT in *P. aeruginosa* PAK (Fig. 4D), like those observed in PAO1. These results indicated that it is likely that bacteria that process the Hrc1 T3SS family are not sensitive to 187R.

187R has the potential to not harm the host microbiome. Although it has been proposed that antivirulence therapies can potentially minimize the damage to the hosts' microbiota (17), there is a lack of experimental validation of such. To address this question and evaluate if 187R has the potential to not harm the host microbiome, we compared the impact of 187R with that of the antibiotic streptomycin on the phyllosphere microbiota of *Arabidopsis*, a plant host of *P. aeruginosa*, via spraying compounds on leaves. A total of 19 phyla, with *Proteobacteria* and *Bacteroidetes* as the two most abundant ones, were observed in each of the treatment and control groups. Compared to control groups, an altered *Proteobacteria* and *Bacteroidetes* ratio has been observed in the microbiota of streptomycin-treated leaves. In contrast, such a substantial change in microbes' relative abundances was not shown in the 187R-treated leaves (Fig. 8A). Principal-coordinates analysis (PCoA) of the microbial communities with

TABLE 1 Bacteria strains, plasmids, and primers used in this study

Strain	Description	Reference or source
P. aeruginosa strains		
PAO1	Wild-type strain	33
PAK	Wild-type strain	34
PAK Δ*pscJ*	*pscJ* mutant of PAK	34
PAO1 Δ*rpoS*	PAO1 *rpoS101*::*aacCI*	67
D. dadantii 3937	Wild-type strain	Lab stock
E. amylovora 273	Wild-type strain	68
E. coli DH5α	ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*	69
Plasmids		
pPROBE-AT	Cloning vector for transcriptional *gfp* fusions; Cb^r	50
exoS-gfp	pPROBE-AT containing *exoS* promoter; Cb^r	6
exoT-gfp	pPROBE-AT containing *exoT* promoter; Cb^r	This study
exoD-gfp	pPROBE-AT containing *exoD* promoter; Cb^r	This study
exsC-gfp	pPROBE-AT containing *exsC* promoter; Cb^r	This study
exsA-gfp	pPROBE-AT containing *exsA* promoter; Cb^r	This study
cdrA-gfp	pPROBE-AT containing *cdrA* promoter; Cb^r	This study
pSW205	*lacZ* translational fusion vector carrying *P. aeruginosa* 1.8-kb stability fragment; Cb^r	51
exsA '-' lacZ	pSW205 carrying *exsA* transcriptional start site to +52 codon of *exsA*, driven by LacUV5 promoter	This study
exsCEBA '-' lacZ	pSW205 carrying *exsC* transcriptional start site to +52 codon of *exsA*, driven by LacUV5 promoter	This study
pBBR1-MCS-5	Expression plasmid with *lac* promoter	70
pBBR1-MCS-5-*rplI*	pBBR-MCS-5 containing *rplI*	This study
pAT-*hrpA*	pPROBE-AT containing *D. dadantii hrpA* promoter; Ap^r	71
pAT-*hrpA*	pPROBE-AT containing *E. amylovora hrpA* promoter; Ap^r	72
Primers[a]		
exoD-gfp		
P*exoD*-F	ATGGATCCATCGTCGACATCGCCATGGA	
P*exoD*-R	ATGAATTCGCTTCTCGGGAGTACTGCTT	
exoT-gfp		
P*exoT*-F	TAGGATCCCACCAAGAGCCCGTCGCTGC	
P*exoT*-R	ATGAATTCCCAGGCGCCCGGCCACGGC	
exsC-gfp		
P*exsC*-F	ATTGTCGACGCAGAAGGTCGAGGACCAGATG	
P*exsC*-R	ATTGAATTCGATACGGCCTGCGAACTCGGC	
exsA-gfp		
P*exsA*-F	ATTGTCGACTACATTGCCTGCTGTTTCGG	
P*exsA*-R	ATTGAATTCGGCCAAGAGATTTGGCTCC	
cdrA-gfp		
P*cdrA*-F	ATTGTCGACGCAGTTGCAGCTCGTCGAA	
P*cdrA*-R	ATTGAATTCCGGACGGACCATGAAAATCT	
exsA'-'lacZ and *exsCEBA'-'lacZ* translational fusion		
Post *exsA* F	AAAGAATTCAGGCTTTACACTTTATGCTTCCGGCTCGTATAATGTGTGG CGTGCTCATGGCTTTGAAAATC	
Post *exsA* R	AAAGGATCC CGCCAGGCAAAAAGTGGAAT	
Post *exsCEBA* F	AAAGAATTCAGGCTTTACACTTTATGCTTCCGGCTCGTATAATGTGTGG AGCTTTAGGAGGCGCCCCCA	
rplI overexpression vector		
pBBR-rplI-F	TAGAACTAGTGGATCCTTACTCAGCGACGATGATCAGCTTCA	
pBBR-rplI-R	CGGTATCGATAAGCTTATGGAAGTCATCCTGCTGGAAAAAGT	
qRT-PCR primer		
rpsL F	TGAAGGTCACAACCTGCAAGAGCA	
rpsL R	AACGACCCTGCTTACGGTCTTTGA	
exsA F	CAAGGGAAAGGACAGCCGAA	
exsA R	ACGCTCGACTTCACTCAACA	

[a]For primers, the description column reports the sequence (5′ to 3′), and underlining indicates restriction enzyme sites.

different treatments based on Unifrac distance showed that the streptomycin-treated phyllosphere was well separated from the 187R- or DMSO-treated phyllosphere along PC1 (Fig. 8B), indicating that streptomycin, but not 187R, altered the phyllosphere to a greater extent. Overall, these results suggested that the antivirulence compound 187R has a negligible impact on the microbial community composition compared to antibiotics.

The changes in microbial metabolic function, such as carbon metabolism, reflect the viability and metabolism of the microbial community. To test whether the

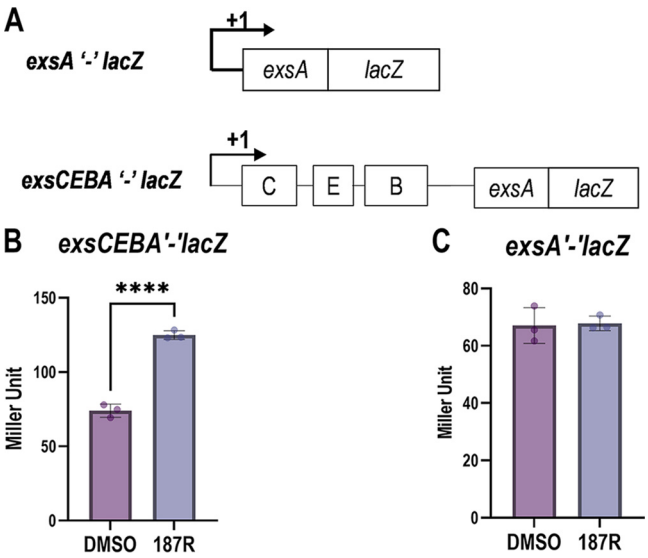

FIG 7 Impact of 187R on *P. aeruginosa* T3SS master regulator *exsA* at the translational level. (A) Illustration of the *exsA* translational fusion reporter plasmid construction. (B and C) *exsA'-'lacZ* (B) and *exsCEBA'-'lacZ* (C) expression in the presence of 187R (250 μM) or DMSO for 6.5 h. ****, $P < 0.0001$. Data represent the means of three biological replicates.

composition of a phyllosphere in 187R- and streptomycin-treated leaves could comprehensively represent the corresponding functional profiles, we used an EcoPlate assay to test the carbon metabolism (an important function related to microbe survival and nutrient cycling) of 187R-, DMSO-, and streptomycin-treated phyllospheres. The PCA (principal-component analysis) for carbon profiles of all nine samples suggested that the carbon profiles of all three streptomycin-treated samples were well separated from the DMSO- and 187R-treated samples along dimension 1 (Dim1), which explained 49.69% of the variation (Fig. 8C). Further analysis showed that in the streptomycin-treated phyllosphere, the metabolism of nine carbon sources (2-hydroxy benzoic acid cannot be used by the phyllosphere) was significantly decreased compared to those of the DMSO-treated samples. However, metabolism of only one carbon source (phenylethylamine) showed a significant reduction in the 187R-treated phyllosphere compared to the DMSO-treated phyllosphere (Fig. S4). These results confirmed that there are drastic changes in streptomycin-treated phyllosphere microbiota but not in the 187R-treated ones, suggesting that compared to the antibiotic streptomycin, the antivirulence compound 187R exhibited less impact on a microbial community's carbon metabolism.

DISCUSSION

A number of virulence inhibitors that target the *P. aeruginosa* T3SS have been discovered over the past 2 decades (35–38). For example, Moir and colleagues reported that phenoxy acetamides, including several 2,4-dichlorophenyloxy acetamides, as T3SS inhibitors that reduced the secretion of *P. aeruginosa* T3SS effectors (39, 40). An attractive target for developing T3SS inhibitors is the master regulator ExsA. As all the *P. aeruginosa* T3SS genes are ExsA dependent, inhibiting the synthesis or activity of ExsA would lead to a decrease, or even complete elimination, of all T3SS gene expression levels. In our work, a series of 2-nitrophenylamino acetamides were synthesized and screened. We found that compound 187R reduced ExsA protein levels in *P. aeruginosa* and dramatically decreased the T3SS-mediated cytotoxicity in HeLa cell assays. We further showed that the R enantiomer of compound 187 (187R) was potent, but the S enantiomer (187S) was not. Culture-independent Illumina sequencing and culture-dependent EcoPlate methods showed that 187R preserved the host-associated microbial

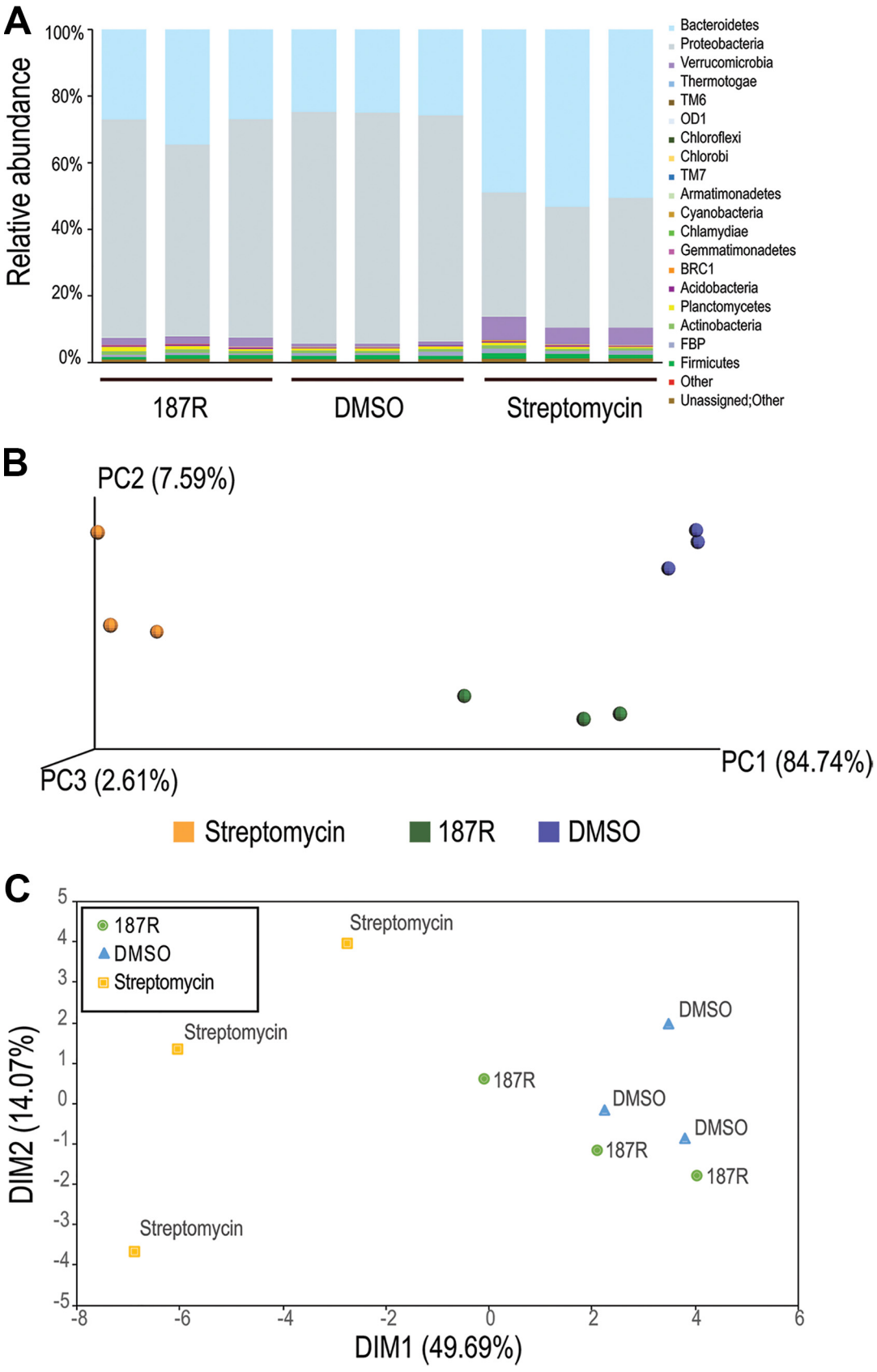

FIG 8 Culture-independent and -dependent analyses of the microbial community. (A) Relative abundances of phyllosphere microbiota treated with 187R, DMSO, and streptomycin overnight at the phylum level. (B) PCoA of 187R-, DMSO-, and

(Continued on next page)

community better than streptomycin in terms of microbial community composition and function.

The transcription and translation of T3SS master regulator *exsA* are regulated by three major regulatory components, including RsmA, Crc, and Vfr/cAMP. However, we found that 187R did not inhibit T3SS through these major regulatory components, because either the transcription or translation of *exsA* was inhibited (Fig. 6B, C, and D and Fig. 7B and C). To test whether 187R repressed T3SS via other known T3SS regulatory components in *P. aeruginosa*, we determined the impact of 187R on c-di-GMP signaling, sigma factor RpoS, and nitrite reductase NirS. The results suggested that 187R did not inhibit T3SS through these regulatory components (Fig. S5 to S7). Therefore, we hypothesized that 187R could directly interact with the ExsA protein and promote its degradation or modulate an unknown regulator that posttranslationally controls ExsA. Interestingly, we observed an increased expression of *exsC* (Fig. 6B) and a decreased expression of *exsD* (Fig. 4A), suggesting that more ExsA proteins should be available to bind its target promoter regions to initiate T3SS gene expression; however, 187R reduced the overall ExsA protein levels (Fig. 6A), resulting in a repression of the *P. aeruginosa* T3SS. In *P. aeruginosa*, it has been reported that the ~200-amino-acid amino-terminal domain (NTD) of ExsA can interact with ExsA protein and cause protein degradation (41). The binding of two ExsA monomers through NTD is crucial for the activation of *exsC* transcription via cooperating binding (an ExsA monomer binds to the high-affinity binding site and recruits another ExsA monomer to the low-affinity binding site) (42). Together with our observations of increased *exsC* promoter activity and decreased ExsA protein levels in the presence of 187R, we propose that it is likely that 187R interacts with the NTD of ExsA, which in turn enhances the ExsA-ExsA protein binding, causing increased *exsC* promoter activity, and promoting ExsA protein degradation via an unknown mechanism. Future studies will be conducted to evaluate this potential interaction between ExsA and 187R and how this interaction affects ExsA protein stability, ExsA-ExsA self-assembly, and/or interactions with target DNA. The regulatory mechanism of the other ExsA-dependent promoters, such as the *exsD* promoter, is not completely understood, and previous research suggested that the transcription of these genes might not be dependent on the cooperative binding of ExsA (41).

The impact of 187R on the *P. aeruginosa* host microbial community (*Arabidopsis* phyllosphere) was explored by culture-independent and culture-dependent methods. Our Illumina sequencing results suggested that compared to streptomycin, 187R did not significantly influence the *Arabidopsis* phyllosphere community in terms of composition. Streptomycin has been widely used for the treatment of bacterial diseases in public health and agriculture. It is also one of the broad-spectrum antibiotics used to treat *P. aeruginosa* infection, which has led to resistance in *P. aeruginosa* (43). A major limitation of culture-independent methods to examine the microbial community is that they cannot differentiate the viable from dead microbes and they reflect the absolute number of microbes in the ecosystem; therefore, the effects of treatments on the phyllosphere may be underestimated (44). Meanwhile, microbial communities are not only characterized by their species composition but also feature different functional genes (for example, genes related to carbon and nitrogen metabolism) that are responsible for the microbes' bioactivities, such as nutrient cycling and immunomodulation (45, 46). It has frequently been discussed that changing the microbial composition would not necessarily result in the alternation of a microbial community's function due to its high functional redundancy (47, 48). Our EcoPlate assay highlighted the effect of 187R on preserving the *Arabidopsis* phyllosphere metabolic functions, suggesting that

FIG 8 Legend (Continued)
streptomycin-treated phyllosphere microbiota. An Unifrac Monte Carlo significance test confirmed that all the streptomycin-treated phyllosphere were significantly different from the DMSO-treated phyllosphere ($P < 0.05$); no significant difference was observed between the 187R-and DMSO-treated phylospheres. (C) PCA of 187R-, DMSO-, and streptomycin-treated phyllosphere carbon profiles. The average OD_{590} values from three repeats were used for generating the figure. *, $P < 0.05$ between streptomycin- and DMSO-treated groups; #, $P < 0.05$ between 187R- and DMSO-treated groups.

the antivirulence compound 187R could potentially reduce the threat to host microbiota compared to streptomycin. In the streptomycin-treated phyllosphere, we observed that the carbon utilization profile was greatly altered in the microbial community (Fig. 8C and Fig. S4). Since streptomycin interferes with protein synthesis by disturbing the stability of the mRNA-ribosomal complex and induces misreading of the genetic code (49), it is likely that the accumulation of mutated proteins hampers bacterial activity and causes a decreased carbon metabolic rate. We found that 187R did not inhibit the T3SS of two plant pathogenic bacteria, *E. amylovora* and *D. dadantii*, which agrees with our results related to microbial composition and function. We are currently studying the impact of 187R on other pathogenic and nonpathogenic bacteria harboring the T3SS to further understand the specificity of this antivirulence compound.

In conclusion, in this study, a potent T3SS inhibitor, 187R, targeting the T3SS master regulator ExsA has been discovered. Using Illumina sequencing and a culture-dependent EcoPlate assay, we showed the impact of 187R on preserving *Arabidopsis* phyllosphere communities and metabolic functions in comparison with the antibiotic streptomycin. To our knowledge, this is the first report showing whether and how T3SS inhibitors influence host microbiota. To fully evaluate the potential of 187R, further studies are needed to understand both short- and long-term effects of 187R on microbial composition and function in various host plants and animals, different host tissues, and how hosts respond to this antivirulence compound targeting bacteria. In summary, this work indicates that targeting virulence factors is a potential alternative strategy for developing new antimicrobial therapies with the potential advantage of not affecting the host-associated microbiota.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and growth conditions. Bacterial strains, plasmids, and primers used in this study are listed in Table 1. *Escherichia coli* and *P. aeruginosa* were cultured in lysogeny broth (LB) medium (tryptone at 10 g/liter, yeast extract at 5 g/liter, and NaCl at 10 g/liter) at 37°C unless otherwise stated. LB with 200 mM NaCl and 10 mM nitrilotriacetic acid was used as the T3SS-inducing medium for *P. aeruginosa*. *D. dadantii* and *E. amylovora* were grown in M9 minimal medium (32) at 28°C for the induction of T3SS. When necessary, carbenicillin (Cb) was supplemented at a concentration of 100 μg/mL.

Reporter plasmid construction. Green fluorescent protein (GFP) reporter plasmids were constructed by cloning the promoter regions of *exsC*, *exsA*, *exsD*, and *exoT* into pProbe-AT, a broad-host-range vector with a promoterless *gfp* gene (50). To construct the *exsA*′-′*lacZ* translational fusion reporter plasmid, the 5′ UTR and the first 52 codons of *exsA* were cloned in frame into the translational *lacZ* fusion vector pSW205 (51). The *exsCEBA*′-′*lacZ* translational fusion reporter plasmid was constructed by in-frame cloning the region between the *exsC* transcriptional start site to 52 codons of *exsA* into the pSW205. The forward oligonucleotide primers for constructing these two translational fusion reporters were engineered to include an EcoRI restriction site and a *lacUV5* promoter.

Screen for *P. aeruginosa* T3SS inhibitors. *P. aeruginosa* strain PAO1 harboring the *exoS-gfp* transcriptional fusion reporter plasmid was cultured in LB medium overnight. The overnight culture was inoculated into T3SS-inducing medium in a 1:1,000 (inoculum:medium) ratio. The inducing medium was supplemented with a 250 μM concentration of a candidate compound from library of 60 synthesized compounds. The compound library was purchased from Changzhou Nimrod Biotech, Jiangsu, China. The purity of the purchased compounds was >95%. The synthetic compound library consisted of five different categories of compounds: category 1, phenoxy and phenylamino acetamides (compounds 187, 187R, 187S, 373, and 412 to 416); category 2, cinchona alkaloids (compounds 382 to 387); category 3, inines (compounds 399 to 402,405, 406, and 417 to 433); category 4, aminophenyl propanamides and acids (compounds 407 to 411); category 5, phenyl acrylates (compounds 394 to 398). Candidate compounds were dissolved in DMSO. *P. aeruginosa* treated with an equal amount of DMSO was used as a negative control. Bacteria were cultured in a test tube containing 5 mL of T3SS-inducing medium at 37°C for 7 h with shaking (200 rpm) prior to harvest. The harvested bacterial cells were diluted in phosphate-buffered saline (PBS). The GFP intensities of 10,000 cells were measured using a fluorescence-activated cell sorter (FACS; BD Biosciences, CA), and the mean fluorescence intensity was calculated using CellQuest Pro software (BD Biosciences, CA).

Synthesis of 187R and 187S. The synthesis procedures for 187R and its enantiomers are described in Fig. 2. (*R*)-2-Aminobutyric acid was reacted with 1-fluoro-2-nitrobenzene in the presence of potassium carbonate, and the resulting (*R*)-2-(2-nitrophenylamino) butyric acid was then coupled with 4-aminobenzyl amine to obtain 187R, a yellow solid. By employing the same procedure, 187S was obtained by replacing (*R*)-2-aminobutyric acid with (*S*)-2-aminobutyric acid (Fig. 2). Based on the high-performance liquid chromatography and nuclear magnetic resonance spectra, the purity of 187R and 187S were 98% and 95%, respectively. The detailed synthetic procedure is described in the supplemental material.

Western blotting. A PAO1 overnight culture was inoculated in a 1:1,000 (inoculum:medium) ratio in T3SS-inducing medium supplemented with 250 μM 187R or DMSO (negative control). Bacterial cell pellets and supernatant were separated by centrifugation at 7 h postinoculation. For measuring the intracellular ExoS and ExsA proteins, 1 mL of bacterial culture (optical density at 600 nm [OD_{600}], ~0.7) was collected by centrifugation, and the cell pellets were resuspended in PBS. A 2× SDS-PAGE buffer was added into each sample, followed by boiling the mixture for 10 min. For measuring the secreted ExoS protein in the supernatant, the supernatant collected from the previous step was centrifuged again to remove any remaining cells. Trichloroacetic acid was added to the supernatant to reach a concentration of 10%. Secreted protein was pelleted by a 30-min centrifugation at 13,500 rpm. The protein pellets were resuspended in 2× SDS-PAGE buffer. The same samples were loaded on an SDS-PAGE gel as a loading control. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane and probed with a rabbit polyclonal antibody against ExsA (1:2,000 dilution; a gift from Chao Wang [25, 52]) or a chicken polyclonal antibody against ExoS (1:4,000 dilution; catalog number ab20031, Abcam, United Kingdom). A mouse antibody against RNA polymerase (neoclone, USA) was used as a control for the total protein quantity. The Western blotting band intensities were quantified by ImageJ (53).

HeLa cell cytotoxicity assay. T3SS-mediated cytotoxicity was evaluated using a cell lifting assay based on methods described previously (54). HeLa cells (1×10^5) were seeded in each well of a 12-well plate and cultured for 18 h at 37°C with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL). Before the infection assay, the HeLa cell culture medium was removed from the well and the cells were washed twice with PBS. HeLa cell culture medium without antibiotic and supplemented with 250 μM compound or DMSO was added into each well. In this assay, the PAK strain of *P. aeruginosa* was used, because it has higher and earlier T3SS expression than the PAO1 strain (55) and its T3SS is also repressed by 187R. Wild-type PAK strain and its T3SS mutant, Δ*pscJ* strain, were cultured overnight in LB medium at 37°C. The bacteria were collected by centrifugation, and the cell pellets were washed with PBS and resuspended in DMEM. HeLa cells were then infected with the bacteria at a multiplicity of infection (MOI) of 30. At 3.5 h postinfection, the medium was removed, and the HeLa cells were stained with crystal violet after rinsing twice with PBS. The plate was washed twice with PBS, and then 200 μL of 95% ethanol was added to the wells to dissolve the crystal violet. The OD_{490} of the ethanol solution with dissolved crystal violet was measured to determine the number of cells that attached to the surface.

β-Galactosidase assays. β-Galactosidase activity was determined as described previously (56). The samples were collected by centrifugation at 8,000 rpm for 3 min. Cell pellets were rinsed and resuspended in PBS. The expression levels of the reporter plasmids were determined by normalization of β-galactosidase activity (OD_{420}) to the bacterial growth (OD_{600}). Three biological replicates were included in each treatment group.

qRT-PCR analysis. The mRNA levels of *exsA* were measured by qRT-PCR. Bacterial cells were cultured in T3SS-inducing medium supplemented with 250 μM 187R or DMSO (negative control) for 7 h before harvest. An RNeasy minikit (Qiagen, Hilden, Germany) was used to isolate the total RNA. cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). The cDNA level of the *exsA* gene was quantified by qRT-PCR using SYBR green master mix (Applied Biosystems, CA). The expression level of *rpsL* was used as an endogenous control for data analysis (57).

Statistical analysis. Two-tailed Student's *t* test was used to assess the inhibition of 187R on the T3SS gene expression compared to the DMSO treatment. One-way analysis of variance combined with Tukey's honestly significant difference test was applied for multiple comparisons. The test was performed in R (version 3.5.0) (58).

Phyllosphere collection. To test the effect of 187R on the phyllosphere microbiota, 8-week-old *Arabidopsis thaliana* Col-1 plants maintained in potting soil in a growth chamber were transferred to 2-mL microcentrifuge tubes with the roots immersed in water. Leaves were sprayed twice (8 h between each spray) with a water solution containing 35 μM 187R in DMSO, DMSO alone (solvent of 187R), or streptomycin (200 ppm). Eighteen hours postinoculation, leaves were collected for analysis of phyllosphere microbiota profiling. The collected leaves were gently washed in PBS to remove the remaining compound and antibiotics. The washed leaves were placed in sterile tubes containing 30 mL PBS. The phyllosphere microbiota was collected by sonicating the test tubes in a water bath sonicator for 15 min. Samples were collected in triplicate for each treatment. Twenty to 24 plants were used for each treatment.

16S rRNA gene Illumina sequencing and data analysis. Cell pellets were collected from the phyllosphere microbiota. Genomic DNA was then extracted using the Qiagen Dneasy PowerSoil kit (Qiagen, Germany) following the manufacturer's instructions, and concentration was measured by Nanodrop spectrophotometer. Approximately 10 ng of DNA was added to each PCR mixture. The V4 region of the 16S rRNA gene was amplified using the 16S_515F and 16S_806R primers with Illumina sequencing adaptors (59). PCR amplification consisted of 95°C for 45 s, followed by 38 cycles of 95°C for 15 s, 78°C for 10 s, 60°C for 30 s, and 72°C for 30 s (60). Peptide nucleic acid (PNA) clamps mPNA and pPNA clamps (mPNA, GGC AAG TGT TCT TCG GA; pPNA, GGC TCA ACC CTG GAC AG) (61), which bind to and block the amplification of mitochondria and chloroplast DNA, respectively, were added to the PCR mixture at a final concentration of 0.75 μM. The PCR products were purified with a QIAquick gel extraction kit (Qiagen, Germany).

Microbial community Illumina sequencing data was analyzed using Qiime version 1.9.1 (62). The open-reference operational taxonomy unit (OTU) picking method using the GreenGenes reference database version 13.8 (63) was performed for OTU clustering and taxonomy assignments. Principal-coordinates analysis (PCoA) of microbial communities was performed based on the Unifrac distance matrix (64).

Biolog EcoPlate assay. In the EcoPlate assay, the ability of phyllosphere microbial communities treated with 187R (35 μM), DMSO, or streptomycin (200 ppm), utilizing the carbon sources were assessed. Each EcoPlate had 96 wells containing 31 carbon sources and one blank control in triplicates. Tetrazolium violet redox dye was used to evaluate the substrate (carbon source) metabolization. For the EcoPlate assay, 2 mL of PBS containing the suspended phyllosphere was diluted 1:20 (vol:vol) in sterile PBS. PBS containing the diluted phyllosphere suspension was aliquoted into each well (120 μL) of the Biolog EcoPlate. The carbon utilization was measured as the OD_{590} after 48 h of incubation using a plate reader. The results were analyzed by principal-component analysis (PCA) in R (version 3.5.0) (58) using the package FactoMineR (65). The heatmap with clustering analysis was generated based on the average OD_{590} value of each carbon source within each treatment group in R using the package gplots (66).

Data availability. The raw Illumina sequencing data were deposited in the Sequence Read Archive of the National Center for Biotechnology Information under the accession numbers SAMN13938370 to SAMN13938378.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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