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Disruption of Quorum Sensing and Virulence in *Burkholderia cenocepacia* by a Structural Analogue of the *cis*-2-Dodecenoic Acid Signal

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ABSTRACT Quorum sensing (QS) signals are widely used by bacterial pathogens to control biological functions and virulence in response to changes in cell population densities. *Burkholderia cenocepacia* employs a molecular mechanism in which the *cis*-2-dodecenoic acid (named *Burkholderia* diffusible signal factor [BDSF]) QS system regulates *N*-acyl homoserine lactone (AHL) signal production and virulence by modulating intracellular levels of cyclic diguanosine monophosphate (c-di-GMP). Thus, inhibition of BDSF signaling may offer a non-antibiotic-based therapeutic strategy against BDSF-regulated bacterial infections. In this study, we report the synthesis of small-molecule mimics of the BDSF signal and evaluate their ability to inhibit BDSF QS signaling in *B. cenocepacia*. A novel structural analogue of BDSF, 14-Me-$C_{16:\Delta2}$ (*cis*-14-methylpentadec-2-enoic acid), was observed to inhibit BDSF production and impair BDSF-regulated phenotypes in *B. cenocepacia*, including motility, biofilm formation, and virulence, while it did not inhibit the growth rate of this pathogen. 14-Me-$C_{16:\Delta2}$ also reduced AHL signal production. Genetic and biochemical analyses showed that 14-Me-$C_{16:\Delta2}$ inhibited the production of the BDSF and AHL signals by decreasing the expression of their synthase-encoding genes. Notably, 14-Me-$C_{16:\Delta2}$ attenuated BDSF-regulated phenotypes in various *Burkholderia* species. These findings suggest that 14-Me-$C_{16:\Delta2}$ could potentially be developed as a new therapeutic agent against pathogenic *Burkholderia* species by interfering with their QS signaling.

IMPORTANCE *Burkholderia cenocepacia* is an important opportunistic pathogen which can cause life-threatening infections in susceptible individuals, particularly in cystic fibrosis and immunocompromised patients. It usually employs two types of quorum sensing (QS) systems, including the *cis*-2-dodecenoic acid (BDSF) system and *N*-acyl homoserine lactone (AHL) system, to regulate virulence. In this study, we have designed and identified an unsaturated fatty acid compound (*cis*-14-methylpentadec-2-enoic acid [14-Me-$C_{16:\Delta2}$]) that is capable of interfering with *B. cenocepacia* QS signaling and virulence. We demonstrate that 14-Me-$C_{16:\Delta2}$ reduced BDSF and AHL signal production in *B. cenocepacia*. It also impaired QS-regulated phenotypes in various *Burkholderia* species. These results suggest that 14-Me-$C_{16:\Delta2}$ could interfere with QS signaling in many *Burkholderia* species and might be developed as a new antibacterial agent.

KEYWORDS AHL, BDSF, *Burkholderia cenocepacia*, quorum sensing, virulence

Burkholderia cenocepacia is a Gram-negative opportunistic pathogen belonging to the *Burkholderia cepacia* complex (Bcc), which is a group of at least 20 closely related bacterial species (1, 2). This bacterium can cause life-threatening infections in

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susceptible individuals, particularly in cystic fibrosis and immunocompromised patients (3). *B. cenocepacia* mostly utilizes two types of quorum sensing (QS) systems, the *N*-acylhomoserine lactone (AHL) and *cis*-2-dodecenoic acid (BDSF) systems, to regulate biological functions and virulence (4–6). The major AHL system is the *cep* system, which consists of the AHL synthase CepI and the transcriptional regulator CepR. CepI predominantly synthesizes both *N*-octanoyl-homoserine lactone (C8-HSL) and smaller amounts of *N*-hexanoyl-homoserine lactone (C6-HSL). When the cell population density reaches a threshold level, these AHL signals will bind to CepR, cause a conformational change in the regulatory protein, and result in the modulation of the target gene expression levels (7, 8). In the BDSF system, the bifunctional crotonase $RpfF_{Bc}$ synthesizes the BDSF signal, which then binds to and stimulates the BDSF receptor protein RpfR to decrease intracellular cyclic diguanosine monophosphate (c-di-GMP) levels, resulting in the transcriptional modulation of target genes and controlling motility, biofilm formation, protease production, and virulence (9–11).

Antibiotics have been extensively used to control and prevent infectious diseases that are caused by bacterial and fungal pathogens. However, the extensive use of this live-or-die selection pressure has fostered the emergence of superbugs that are resistant to conventional antibiotics. Infections caused by antibiotic-resistant pathogens are becoming increasingly common (12, 13) and are now major health care and public concerns. The development of antibiotic resistance in *B. cenocepacia* and other Bcc species has also become a serious concern in the medical community (14), leading to an emergent need for new strategies and novel drugs to efficiently treat infections caused by these pathogens.

An attractive approach to avoid the emergence of superbugs is to target bacterial virulence systems rather than their essential cellular processes. This strategy can reduce selective survival pressures and slow the emergence of drug resistance (15–17). Many studies have indicated that QS inhibitors can impair QS-dependent functions in bacteria. Therefore, the design and development of novel QS inhibitors to treat infectious diseases caused by bacterial pathogens that employ QS to regulate virulence is a valuable approach to combat bacterial pathogens and prevent the emergence of drug resistance. This strategy has already been successfully utilized to develop novel drugs to target QS and other signaling systems in a number of bacterial pathogens (18–27), making anti-QS drugs attractive alternatives to antibiotics.

The BDSF system is a conserved QS system in *Burkholderia* species and in many other bacterial pathogens (28), regulating biological functions and playing a key role in pathogenesis. Therefore, the BDSF system is an excellent candidate target to treat diseases caused by these bacterial species. Here, we report the design of BDSF system inhibitors from unsaturated fatty acid derivatives and evaluate their activities to inhibit QS signaling and virulence. We identified an unsaturated fatty acid compound (*cis*-14-methylpentadec-2-enoic acid [14-Me-$C_{16:\Delta2}$]) that is capable of interfering with *B. cenocepacia* QS signaling and virulence. We demonstrate that 14-Me-$C_{16:\Delta2}$ reduced the QS signal production and the expression levels of their synthase-encoding genes. In other *Burkholderia* species, 14-Me-$C_{16:\Delta2}$ also inhibited motility and biofilm formation, which are BDSF-regulated phenotypes. In summary, the goal of our study was to determine the applicability of targeting the BDSF QS system to promote the development of novel anti-QS therapeutics against *B. cenocepacia*.

RESULTS

14-Me-$C_{16:\Delta2}$ is a potential inhibitor of *B. cenocepacia* QS. To identify potential inhibitors of QS systems in *B. cenocepacia*, we first tested the effect of the candidate compounds (see Table S1 in the supplemental material) on the β-galactosidase (β-Gal) activity of a *B. cenocepacia* strain H111 carrying a lectin-encoding *bclACB* operon-*lacZ* promoter fusion plasmid, as this operon is positively controlled by both the BDSF and AHL systems (5, 9). The reporter strain, H111(P*bclACB-lacZ*), was inoculated and grown in LB medium supplemented with 22 candidate compounds at a final concentration of 100 μM. β-Gal activity was measured when the cell cultures reached an

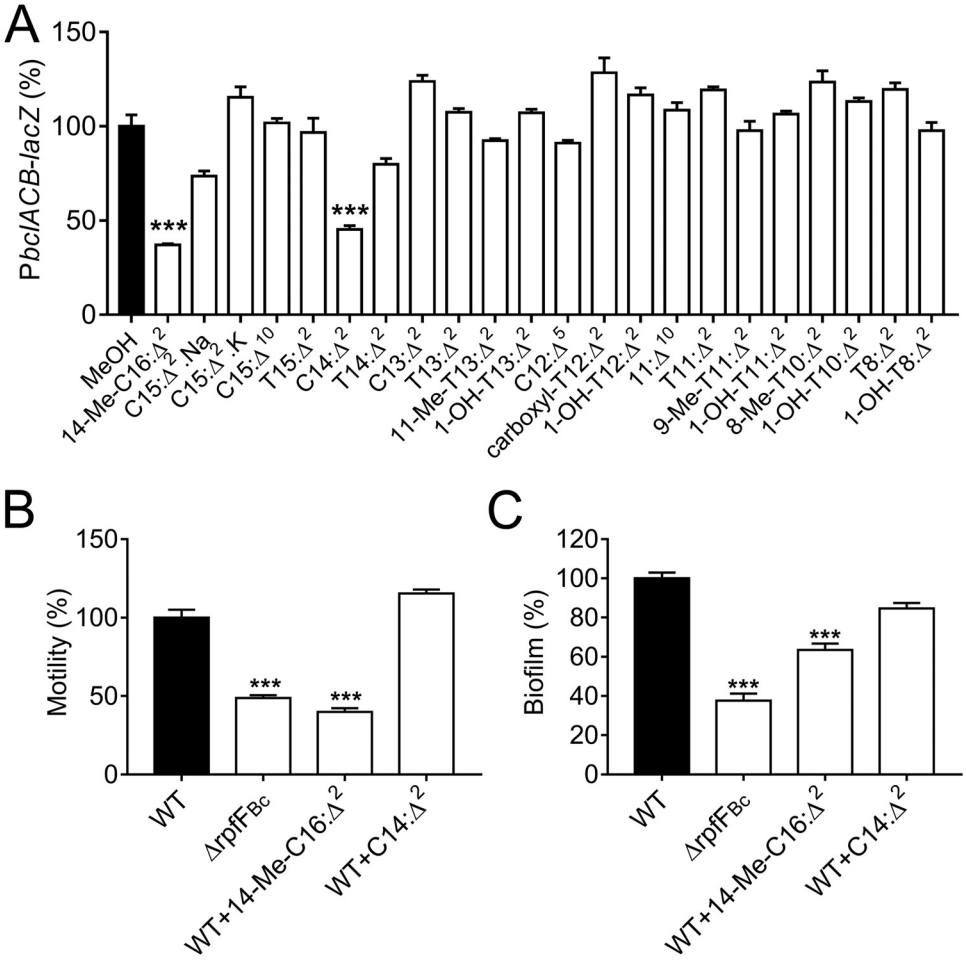

FIG 1 Evaluation of QS inhibition in *B. cenocepacia* H111 with the candidate compounds (100 μM). (A) Effect of exogenous addition of 100 μM candidate compounds on *bclACB* gene expression, as determined by using a P*bclACB-lacZ* fusion reporter strain. These compounds are listed in Table S1. (B and C) Quantitative analysis of swarming motility (B) and biofilm formation (C) of *B. cenocepacia* H111 with the addition of the leading compounds (100 μM).

optical density at 600 nm (OD_{600}) of ~1.5. Bioassay results showed that two candidate compounds (14-Me-$C_{16:\Delta2}$ and $C_{14:\Delta2}$) exerted significant inhibition on *bclACB* gene expression (Fig. 1A). We next tested the ability of 14-Me-$C_{16:\Delta2}$ and $C_{14:\Delta2}$ to interfere with the biological functions regulated by QS in *B. cenocepacia* H111. Data showed that swarming motility (Fig. 1B) and biofilm formation (Fig. 1C) were strongly reduced in the presence of 14-Me-$C_{16:\Delta2}$, but the compound $C_{14:\Delta2}$ did not show inhibitory activity in swarming motility and biofilm formation. Taken together, 14-Me-$C_{16:\Delta2}$ can act as a potential inhibitor of *B. cenocepacia* QS. In this study, 14-Me-$C_{16:\Delta2}$ was synthesized in large quantity from 11-methyl-dodecanol (Fig. S1A), and its chemical structure was confirmed via ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra (Fig. S1B and C and 2A).

14-Me-$C_{16:\Delta2}$ inhibits QS-regulated phenotypes in *B. cenocepacia*. Interestingly, exogenous addition of 14-Me-$C_{16:\Delta2}$ at final concentrations of 5 to 100 μM did not appear to inhibit the growth of *B. cenocepacia* (Fig. S1D). However, 14-Me-$C_{16:\Delta2}$ clearly inhibited the expression of *bclACB*, as the addition of 100 μM 14-Me-$C_{16:\Delta2}$ reduced *bclACB* expression in the wild-type strain at all growth stages (Fig. 1A and 2B). We next tested the different concentration of 14-Me-$C_{16:\Delta2}$ on phenotypes controlled by the *B. cenocepacia* QS systems. As expected, swarming motility was inhibited by 14-Me-$C_{16:\Delta2}$ in a dose-dependent manner, as the addition of 5, 20, 50, and 100 μM 14-Me-$C_{16:\Delta2}$

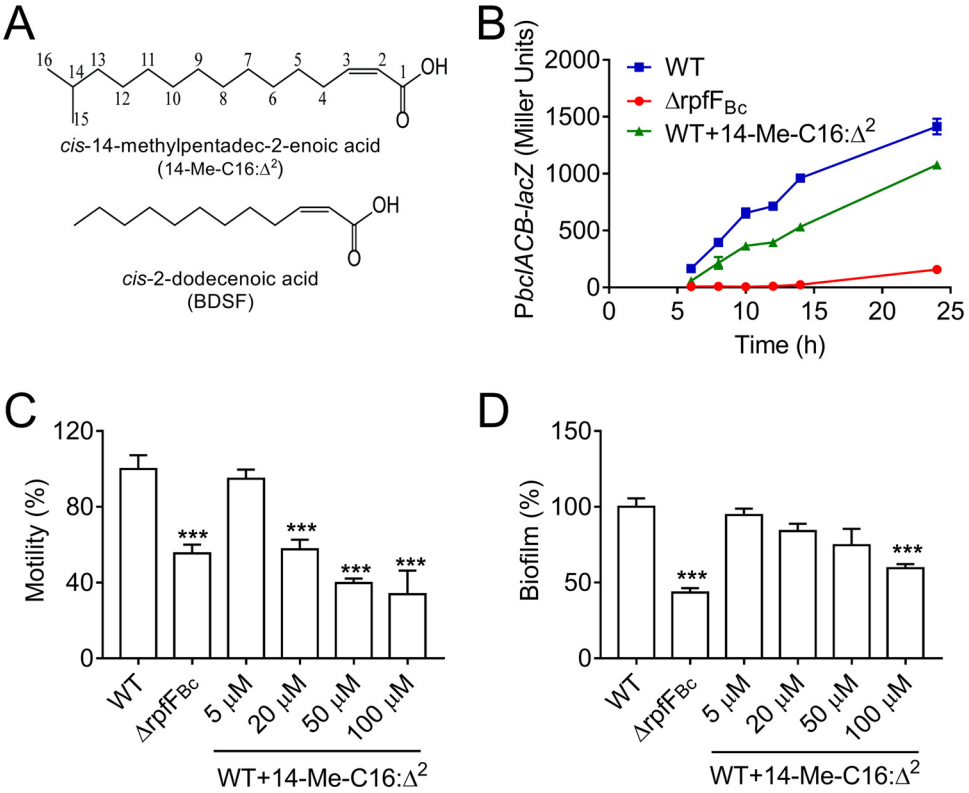

FIG 2 Effect of 14-Me-$C_{16:\Delta2}$ on QS-regulated functions of *B. cenocepacia* H111. (A) The chemical structure of 14-Me-$C_{16:\Delta2}$ compared to BDSF. (B) Effects of 14-Me-$C_{16:\Delta2}$ on *bclACB* gene expression were measured by assessing β-galactosidase activity. (C and D) Quantitative analysis of swarming motility (C) and biofilm formation (D) of *B. cenocepacia* H111 with the addition of 14-Me-$C_{16:\Delta2}$. Cells were treated with various concentrations of 14-Me-$C_{16:\Delta2}$ and incubated statically at 37°C, and the results are expressed as the relative values compared to those of the untreated controls. The results are based on three independent experiments. Error bars represent the means ± standard deviations (SD). Significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) between the tested compound and its control ($n = 3$) are shown.

reduced *B. cenocepacia* swarming motility by 5.2, 37.3, 42.4, and 60.2%, respectively (Fig. 2C).

Biofilm formation is a virulence trait of *Burkholderia* species that has been associated with the persistence of infections and the increased antibiotic tolerance of biofilm-associated cells compared with those of planktonic cells. In *B. cenocepacia*, QS systems regulate the expression of surface proteins, lectins, and extracellular DNA, which are all important components for biofilm matrix structures (29, 30). Therefore, we were interested to further investigate the inhibitory effect of 14-Me-$C_{16:\Delta2}$ on the ability of *B. cenocepacia* to form biofilms. After growing *B. cenocepacia* in microtiter dish wells in the presence of 0, 5, 20, 50, or 100 μM 14-Me-$C_{16:\Delta2}$, the addition of 100 μM 14-Me-$C_{16:\Delta2}$ was observed to cause a 37.3% reduction in biofilm formation (Fig. 2D). We also found that the addition of 14-Me-$C_{16:\Delta2}$ could significantly increase the antibiotic susceptibility of *B. cenocepacia* cells to chloramphenicol (Table S2), suggesting that 14-Me-$C_{16:\Delta2}$ showed an obviously synergistic effect with this antibiotic against *B. cenocepacia*.

14-Me-C16:Δ² decreases BDSF signal production. Viable QS inhibition strategies consist of the inhibition of signal sensing or signal synthesis and of signal degradation. In *B. cenocepacia*, the BDSF system positively regulates AHL signal production and *cepI* expression at the transcriptional level (9). To determine the mechanism by which 14-Me-$C_{16:\Delta2}$ acts upon QS signaling in *B. cenocepacia*, we first analyzed whether 14-Me-$C_{16:\Delta2}$ is involved in the competitive inhibition of the BDSF signal binding to the receptor protein RpfR using isothermal titration calorimetry (ITC). The results showed

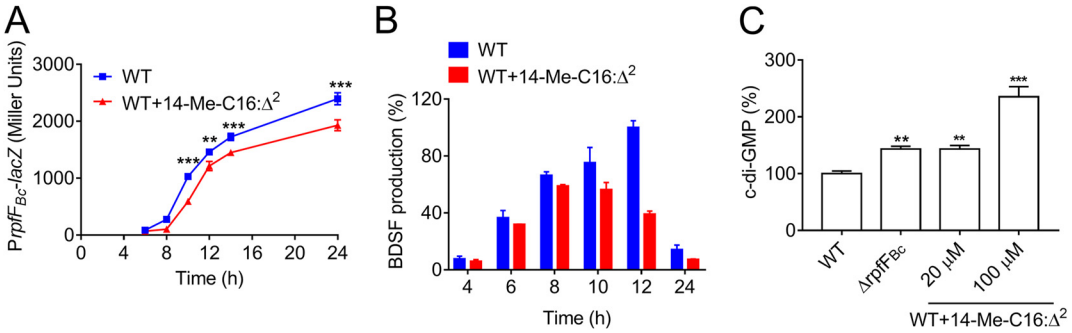

FIG 3 Effect of 14-Me-$C_{16:\Delta2}$ on the BDSF QS system in *B. cenocepacia* H111. (A) Effects of 14-Me-$C_{16:\Delta2}$ on *rpfF*$_{Bc}$ gene expression were measured by assessing β-galactosidase activity of the *rpfF*$_{Bc}$-*lacZ* transcriptional fusions in the H111 wild-type (WT) strain in the presence or absence of 100 μM 14-Me-$C_{16:\Delta2}$. (B) Quantitative analysis of BDSF production in *B. cenocepacia* H111 with the addition of 14-Me-$C_{16:\Delta2}$ at different time points. The relative amounts of signal molecules were calculated on the basis of their peak areas. For convenient comparison, the peak BDSF of the WT at 12 h was arbitrarily defined as 100% and used to normalize the signal ratios of the different time points. (C) Quantitative analysis of c-di-GMP levels in *B. cenocepacia* H111 in the presence or absence of 14-Me-$C_{16:\Delta2}$ (100 μM). The relative amounts of signal molecules were calculated on the basis of their peak areas. For convenient comparison, the peak c-di-GMP of the WT was arbitrarily defined as 100% and used to normalize the signal ratios of the different treatments. The results are based on three independent experiments. Error bars represent the means $\pm$ standard deviations (SD). Significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) between the tested compound and its control ($n = 3$) are shown.

that 14-Me-$C_{16:\Delta2}$ did not bind to RpfR (Fig. S2A). In addition, 14-Me-$C_{16:\Delta2}$ did not inhibit the binding of the BDSF signal to its receptor protein RpfR (Fig. S2B and C), suggesting that 14-Me-$C_{16:\Delta2}$ does not interfere with the ability of RpfR to sense the BDSF signal. We next investigated whether 14-Me-$C_{16:\Delta2}$ exerts its inhibitory effect on BDSF signal synthesis. The data showed that treatment of cells with 14-Me-$C_{16:\Delta2}$ at a final concentration of 100 μM resulted in a significant decrease in the expression of *rpfF*$_{Bc}$ at all growth stages, especially in the earlier growth stage (Fig. 3A).

The inhibitory activity of 14-Me-$C_{16:\Delta2}$ on BDSF signal synthesis was also assessed by liquid chromatography-mass spectrometry (LC-MS) analysis. In agreement with the above-mentioned results, 14-Me-$C_{16:\Delta2}$ reduced BDSF production at all growth stages (Fig. 3B). The production of BDSF in the wild-type strain supplemented with 100 μM 14-Me-$C_{16:\Delta2}$ was reduced to 39.2% of that observed in the untreated wild-type strain at 12 h postinoculation (Fig. 3B).

Our recent studies demonstrated that the disruption of *rpfF*$_{Bc}$ or *rpfR* results in an increase in intracellular c-di-GMP levels (9). In addition, modified intracellular c-di-GMP levels can cause changes in the production of virulence factors and AHL signal molecules, as well as alter the biosynthesis of different biofilm matrix components (9, 31). The observed inhibition in BDSF signal synthesis by 14-Me-$C_{16:\Delta2}$ motivated us to investigate the effect of 14-Me-$C_{16:\Delta2}$ on intracellular c-di-GMP levels in *B. cenocepacia* H111. The LC-MS analysis showed that treatment of the wild-type strain with 14-Me-$C_{16:\Delta2}$ at final concentrations of 20 μM and 100 μM caused approximately a 1.4- and 2.4-fold increase in the intracellular c-di-GMP levels, respectively (Fig. 3C). Taken together, the results showed that the disruption of *rpfF*$_{Bc}$ gene expression by 14-Me-$C_{16:\Delta2}$ resulted in a notable increase in intracellular c-di-GMP levels, consequently affecting target gene expression.

14-Me-$C_{16:\Delta2}$ decreases AHL signal production. Recent studies showed that the BDSF system controls AHL signal production by regulating the expression of the AHL synthase CepI at the transcriptional level (9, 11). We next sought to measure AHL signal production in the wild-type H111 strain in the presence or absence of 14-Me-$C_{16:\Delta2}$. Our results revealed a notable reduction in AHL signal production in the wild-type strain when it was treated with 14-Me-$C_{16:\Delta2}$ (Fig. 4A). To test whether 14-Me-$C_{16:\Delta2}$ affected *cepI* (AHL synthase-encoding gene) transcription, we used *cepI* promoter-*lacZ* fusions and measured their activities in *B. cenocepacia* H111 strains. In agreement with the above-mentioned results, when the wild-type strain was grown in the presence of

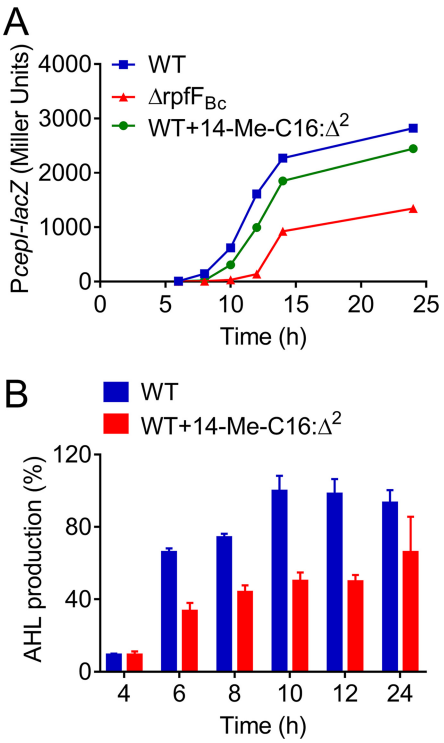

FIG 4 Effect of 14-Me-$C_{16:\Delta2}$ on the AHL QS system in *B. cenocepacia* H111. (A) Effects of 14-Me-$C_{16:\Delta2}$ on *cepI* gene expression were measured by assessing β-galactosidase activity of the *cepI-lacZ* transcriptional fusions. *B. cenocepacia* H111 was grown in the presence or absence of 14-Me-$C_{16:\Delta2}$ (100 μM). Samples were taken from each culture at the OD_{600} values for the indicated time intervals. (B) Quantitative analysis of AHL production in *B. cenocepacia* H111 in the presence or absence of 100 μM 14-Me-$C_{16:\Delta2}$ at different time points. For convenient comparison, AHL production of the WT at 10 h was arbitrarily defined as 100% and used to normalize the signal ratios of the different time points. The results are based on three independent experiments. Error bars represent the means $\pm$ SD. Significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) between the tested compound and its control ($n = 3$) are shown.

14-Me-$C_{16:\Delta2}$ at a final concentration of 100 μM, a decrease in *cepI* expression was observed at various growth stages (Fig. 4B).

14-Me-$C_{16:\Delta2}$ attenuates *B. cenocepacia* virulence. Previous studies showed that both the BDSF and AHL QS systems play vital roles in the pathogenesis of *B. cenocepacia*. Because the addition of 14-Me-$C_{16:\Delta2}$ inhibited the production of BDSF and AHL signals, we evaluated the efficacy of 14-Me-$C_{16:\Delta2}$ on *B. cenocepacia* H111 virulence using an A549 cell line infection model. Cytotoxicity was measured by quantifying the release of lactate dehydrogenase (LDH) into the supernatants of cultured cells. As expected, treatment with 14-Me-$C_{16:\Delta2}$ led to a reduction in bacterial virulence against this cell line (Fig. 5). When the wild-type H111 strain was incubated with A549 cells in the presence of 20, 50, and 100 μM 14-Me-$C_{16:\Delta2}$, the observed cytotoxicity levels were reduced to 74, 59, and 46% of that of the untreated group at 8 h postinoculation, respectively (Fig. 5).

14-Me-$C_{16:\Delta2}$ affects the expression of a wide range of genes controlled by the BDSF system. As the addition of 14-Me-$C_{16:\Delta2}$ resulted in reduced BDSF signal production, we examined whether 14-Me-$C_{16:\Delta2}$ affects the expression of BDSF-regulated genes. Quantitative reverse transcription-PCR (qRT-PCR) analyses revealed that cells treated with 14-Me-$C_{16:\Delta2}$ exhibited altered expression of many genes in the BDSF regulon (Fig. 6 and Table S3), indicating that 14-Me-$C_{16:\Delta2}$ inhibited BDSF signal production and affected the expression of the target genes of the BDSF system.

14-Me-$C_{16:\Delta2}$ inhibits QS-regulated phenotypes in many *Burkholderia* species. The results of our previous studies indicated that many *Burkholderia* species may

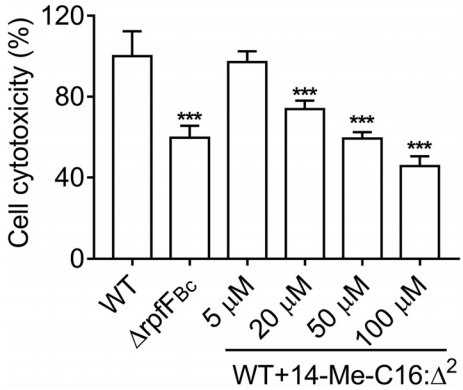

FIG 5 Effects of 14-Me-$C_{16:\Delta2}$ on the pathogenicity of *B. cenocepacia* were determined using an A549 cell line model system. The cell cytotoxicity of the WT was arbitrarily defined as 100% and used to normalize the signal ratios of the different treatments. The results are based on three independent experiments. Error bars represent the means ± SD. Significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) between the tested compound and its control ($n = 3$) are shown.

employ the BDSF system to regulate biological functions (11, 28, 32, 33). Therefore, the effect of 14-Me-$C_{16:\Delta2}$ on the QS-regulated phenotypes of different *Burkholderia* species was investigated. Swarming motility and biofilm formation were significantly inhibited in cells treated with 14-Me-$C_{16:\Delta2}$ for all the tested *Burkholderia* species, while their growth rates were unaffected (Fig. 7 and S3).

DISCUSSION

In this study, we identified *cis*-14-methylpentadec-2-enoic acid (14-Me-$C_{16:\Delta2}$) as an unsaturated fatty acid compound that is capable of interfering with the AHL and BDSF QS systems in *B. cenocepacia* (Fig. 3 and 4). 14-Me-$C_{16:\Delta2}$ reduced the production of QS signals and the expression of their synthase-encoding genes, resulting in the down-regulation of factors associated with QS system-regulated biological functions in *B. cenocepacia* H111 (Fig. 2). As expected, because the QS system targeted by 14-Me-$C_{16:\Delta2}$ is a nonessential cell pathway, this molecule did not exhibit antimicrobial activity against *B. cenocepacia* (Fig. S1). However, promising results were obtained using the A549 cell line model, where the effect of 14-Me-$C_{16:\Delta2}$ was clearly demonstrated *in vitro* (Fig. 5). Thus, these results demonstrate that 14-Me-$C_{16:\Delta2}$ has efficacy against *B. cenocepacia* virulence.

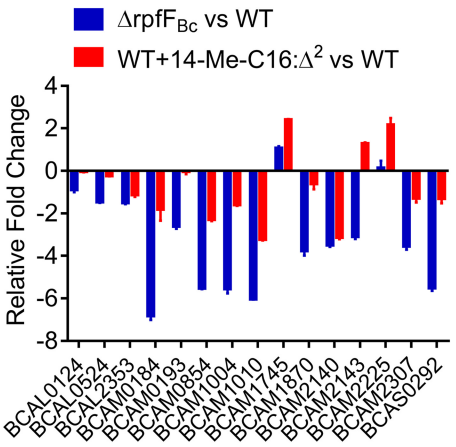

FIG 6 qPCR analysis of the genes that showed differential expression between the *rpfF$_{Bc}$* mutant strain and the wild-type stain, and between the wild-type strain in the presence and absence of 14-Me-$C_{16:\Delta2}$ (100 μM). The results are based on three independent experiments. Error bars represent the means ± SD.

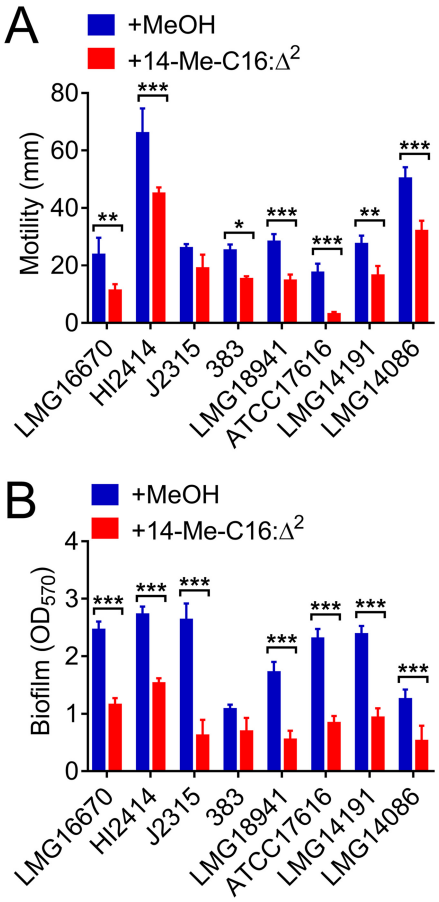

FIG 7 (A and B) Quantitative analysis of swarming motility (A) and biofilm formation (B) of different *Burkholderia* species in the absence or presence of 14-Me-$C_{16:\Delta 2}$ (100 μM). LMG16670, HI2414, J2315, 383, LMG18941, ATCC 17616, LMG14191 and LMG14086 represent *B. anthina* LMG16670, *B. cenocepacia* HI2414, *B. cenocepacia* J2315, *B. cepacia* 383, *B. dolosa* LMG18941, *B. multivorans* ATCC 17616, *B. pyrrocinia* LMG14191, and *B. stabilis* LMG14086, respectively. Cells were treated with various concentrations of 14-Me-$C_{16:\Delta 2}$ and incubated statically at 37°C, and the results are expressed as the relative values compared to that of the untreated controls. The results are based on three independent experiments. Error bars represent the means ± SD. Significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) between the tested compound and its control ($n = 3$) are shown.

B. cenocepacia infections are often associated with poor clinical outcomes and high mortality due to declines in lung function that lead to fatal pneumonia (1, 3, 34). These infections are often refractory to treatment with common antibiotics because of the emergence of multidrug-resistant (MDR) *B. cenocepacia* strains (14). This serious fact suggests that there is an urgent need to develop alternative strategies. Targeting bacterial virulence rather than cell growth is one attractive strategy. The significant advances of such "antivirulence" therapies include the inhibition of biofilm formation, motility, virulence, and QS signaling (12, 15–17, 35). In particular, the development of QS inhibitors as novel drugs to treat bacterial infections has attracted significant attention over the past 20 years (18–21, 23–27, 36). For example, a number of compounds have been screened and identified or synthesized as antagonists of QS systems, including diketopiperazines (37) and baicalein (38). The compounds with activities that interfere with the BDSF QS significantly inhibit *B. cenocepacia* virulence, suggesting that interference in QS signaling can be used as favorable therapeutic method to treat *B. cenocepacia* infections.

Diverse strategies have been explored to control QS signaling, including inhibition of signal synthesis and signal sensing and the promotion of signal degradation (39). However, specific *Burkholderia* species produce multiple types of QS signals and sense

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Phenotypes and/or characteristics[a]	Reference or source[b]
Strains		
Burkholderia spp.		
B. cenocepacia H111	Wild-type strain, genomovar III of the *B. cepacia* complex	42
H111(P*cepI-lacZ*)	H111 harboring reporter construct P*cepI-lacZ*	9
H111(P*bclACB-lacZ*)	H111 harboring reporter construct P*bclACB-lacZ*	11
H111(P*rpfF$_{Bc}$-lacZ*)	H111 harboring reporter construct P*rpfF$_{Bc}$-lacZ*	11
Δ*rpfF$_{Bc}$* mutant	BDSF-deficient mutant derived from H111 with *rpfF$_{Bc}$* being deleted	32
Δ*rpfF$_{Bc}$*(P*cepI-lacZ*) mutant	Δ*rpfF$_{Bc}$* mutant harboring reporter construct P*cepI-lacZ*	9
Δ*rpfF$_{Bc}$*(P*bclACB-lacZ*) mutant	Δ*rpfF$_{Bc}$* mutant harboring reporter construct P*bclACB-lacZ*	11
B. multivorans ATCC 17616	Soil, USA	E. Mahenthiralingam laboratory
B. cepacia 383	Soil, Trinidad	E. Mahenthiralingam laboratory
B. cenocepacia J2315	Cystic fibrosis isolate, UK	ATCC
B. stabilis LMG 14086	Respirator, UK	BccMtm
B. cenocepacia HI2414	Isolated from agricultural soil, USA	BccMtm
B. dolosa LMG 18941	Cystic fibrosis isolate, USA	BccMtm
B. anthina LMG 16670	Rhizosphere, UK	BccMtm
B. pyrrocinia LMG 14191	Soil, Japan	BccMtm
E. coli		
DH5α	*supE44 lacU169(80lacZ*ΔM15*) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 pir*	Laboratory collection
BL21	F$^-$ *ompT hsdS* (r_B^- m_B^-) *dcm*$^+$ Tet^r *gal* (DE3) *endA*	Stratagene
Plasmids		
pET-28a	Expression vector, Kan^r	Novagen
pET-*rpfR*	pET-28a containing *rpfR*	7

[a]Tet^r tetracycline resistance; Kan^r, kanamycin resistance.
[b]BccMtm, Belgian Coordinated Collections of Microorganisms.

them using different cognate receptors. Thus, simultaneous inhibition of all signal-specific QS pathways in *Burkholderia* spp. requires targeting the upstream region of the QS signals. Among these factors, RpfFR homologues function as the master QS regulators within *Burkholderia* QS pathways (11, 32, 40). Moreover, these homologs display high sequence similarity and structural similarity (28), making them attractive targets for *Burkholderia* QS inhibition. In this study, 14-Me-$C_{16:\Delta2}$ inhibited $RpfF_{Bc}$ activity and markedly affected the QS-regulated phenotypes in all the *Burkholderia* species examined (Fig. 7). The potent and broad activity of this molecule would be especially important in practical settings, since multiple *Burkholderia* species can cause pulmonary infections in hospital environments. In fact, multiple *Burkholderia* species, rather than a single species, are responsible for *Burkholderia* pathogenesis in naturally infected CF patients.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *Escherichia coli* strain and *Burkholderia* strains used in this work are listed in Table 1 and were grown at 28 or 37°C, as indicated previously (4, 9), with shaking at 250 rpm in Luria-Bertani (LB) broth. *B. cenocepacia* H111 and its *rpfF$_{Bc}$* deletion mutant were described previously (32). The following antibiotics were used to supplement media when necessary: ampicillin, 100 μg ml^{-1}; gentamicin, 50 μg ml^{-1}; kanamycin, 100 μg ml^{-1}; and tetracycline, 10 μg ml^{-1}. A549 human lung carcinoma cells (American Type Culture Collection #CCL-185) were grown in Dulbecco's modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum and 1× penicillin-streptomycin (Pen-Strep; Sigma) at 37°C.

Synthesis of 14-Me-$C_{16:\Delta2}$. 14-Me-$C_{16:\Delta2}$ was synthesized using 11-methyl-dodecanol as a starting material. 11-Methyl-dodecanol was used to synthesize 1-bromo-11-methyl-dodecane by bromination, which together with propiolic acid was subsequently used to synthesize 14-methylpentadec-2-ynoic acid under the catalytic action of hexamethylphosphoric triamide (HMPT) and *n*-butyllithium (*n*-BuLi). Finally, *cis*-14-methylpentane-2-enoic acid was synthesized by a one-step Pd-$BaSO_4$ catalytic hydrogenation reaction.

Construction of reporter strains and measurement of β-galactosidase activity. The *bclACB*, *cepI*, and *rpfF$_{Bc}$* reporters were introduced into the *B. cenocepacia* H111 wild-type and *rpfF$_{Bc}$* mutant strains by electroporation. Transconjugants were then selected on LB agar plates containing tetracycline. Measurements of β-galactosidase activity were made according to previously described methods (41). Bacteria were cultured at 37°C, and the cells were harvested to measure β-galactosidase activity.

Swarming motility assay. *Burkholderia* strains treated with candidate compounds were used for the swarming motility assay. Swarming motility was determined on semisolid agar (0.3%). Bacteria

were inoculated into the center of plates containing 0.8% tryptone, 0.5% glucose, and 0.3% agarose. The plates were incubated at 30°C for 18 h before the diameter of the colony was measured.

Biofilm formation assays. Biofilm formation assays were performed as described previously, with minor modifications (42). Overnight cultures of *Burkholderia* strains were inoculated into 200 μl of LB broth in 96-well microtiter plates at an OD_{600} of 0.05. Candidate compounds were added as indicated below, and the planktonic cells and medium were removed after culturing at 24 h. The crystal violet staining method was used to quantify biofilm mass, as described by Huber et al. (42). The crystal violet from stained biofilms was dissolved in 200 μl of 95% (vol/vol) ethyl alcohol, and the absorbance at 570 nm was measured.

Detection of MIC values. The MICs of 12 antibiotics (ampicillin, kanamycin, gentamicin, tetracycline, chloramphenicol, penicillin, trimethoprim, streptomycin, rifampin, tobramycin, apramycin sulfate, and cefotaxime) and the combinations of antibiotics with 14-Me-$C_{16:\Delta2}$ (100 μM) against *B. cenocepacia* were determined using broth microdilution according to the CLSI 2015 guidelines (43). Overnight culture of *B. cenocepacia* was diluted 1,000-fold for the subsequent tests. The cultures were placed in a 96-well plate and incubated at 37°C supplemented with different concentrations of antibiotics for 24 h. The MIC was defined as the lowest concentration of antibiotic in which bacterial growth in the well was not measurable by determination of the turbidity at 600 nm.

Quantification of BDSF and AHL signals. Bacteria were cultured overnight in LB broth with agitation at 37°C, inoculated into 1 liter of fresh LB medium, and incubated for 20 h. Cells were removed by centrifugation, and the supernatants were mixed with an equal volume of ethyl acetate. The ethyl acetate was collected and evaporated to dryness and dissolved in 1 ml of methanol. BDSF signals were measured by liquid chromatography-mass spectrometry (LC-MS) (33).

Quantification of AHL signals was performed using the β-galactosidase assay with the aid of the AHL reporter strain CF11, as described previously (44). Briefly, the reporter strain CF11 was grown in minimal medium at 28°C with shaking at 220 rpm for 12 h. The cultures were inoculated into the same medium supplemented with extracts containing AHL signals. After bacterial cells were harvested, β-galactosidase activity was assayed as described previously (41).

Quantification of c-di-GMP. *B. cenocepacia* H111 and its derivatives were grown in 400 ml of LB medium at 37°C to an OD_{600} of 2.5 to ~3.0 with shaking at 200 rpm. Formaldehyde (final concentration, 0.18%) was added to block the degradation of c-di-GMP. Cells were collected by centrifugation at 8,000 $\times$ *g* for 10 min at 4°C. The cell pellets were washed with 40 ml of phosphate-buffered saline (pH 7.0) containing 0.18% formaldehyde and were centrifuged at 8,000 $\times$ *g* for 10 min at 4°C. Next, the cell pellets were dissolved in water, boiled for 10 min, and cooled on ice for 10 min. Nucleotides were extracted using 65% ethanol. The supernatants were retained, and the extractions were repeated. The supernatants were concentrated and lyophilized, and the pellets were dissolved in 1 ml H_2O and filtered using polyvinylidene difluoride (PVDF) filters (0.22-μm pore size). c-di-GMP levels were measured by LC-MS (32).

Protein expression and purification. *rpfR* was fused to the expression vector pET-28a. The fusion gene construct was transformed into *E. coli* strain BL21. Affinity purification of the HIS-RpfR fusion protein was performed following the method described previously (32). Fusion protein was cleaved with PreScission protease (GE Healthcare; 2 units/100 μl of bound proteins) at 4°C overnight. The cleaved fusion proteins were eluted and analyzed by SDS-PAGE.

ITC analysis. The isothermal titration calorimetry (ITC) measurements were performed using an ITC-200 microcalorimeter following the manufacturer's protocol (MicroCal, Northampton, MA). In brief, titrations began with one injection of 1.5 μl of 14-Me-$C_{16:\Delta2}$ (500 μM) solution into the sample cell containing 350 μl of the RpfR solution (25 μM) in the ITC-200 microcalorimeter. The volume of 14-Me-$C_{16:\Delta2}$ injection was changed to 2 μl in the subsequent 18 injections. The heat changes accompanying injections were recorded. The titration experiment was repeated at least three times, and the data were calibrated with the final injections and fitted with the one-site model to determine the binding constant (K_d) using the MicroCal ORIGIN version 7 software.

Quantitative real-time PCR assays. *B. cenocepacia* H111 was cultured at 37°C in LB broth in the presence or absence of 14-Me-$C_{16:\Delta2}$ at a final concentration of 100 μM to the logarithmic-growth phase. *B. cenocepacia* H111 cultured without 14-Me-$C_{16:\Delta2}$ was used as the control. Total RNA was extracted with TRIzol reagent (Invitrogen), according to the manufacturer's instructions. RNA quality was determined by measuring the A_{260}/A_{280} and A_{260}/A_{230} values and by gel electrophoresis. Reverse transcription-PCR was performed using a cDNA synthesis kit (Promega), according to the manufacturer's instructions. Specific qRT-PCR primers (listed in Table 2) were used to amplify central fragments of approximately 200 bp in length from different genes. qRT-PCR was performed using SYBR green qPCR mastermixes (Thermo Scientific) and a 7300Plus real-time PCR system (Applied Biosystems). The *recA* gene was used as an internal reference (33). The relative expression levels of the target genes were calculated using the quantitation-comparative C_T ($\Delta\Delta C_T$) method according to Livak and Schmittgen (45).

Cytotoxicity assays. Cytotoxicity was assessed by measuring the release of LDH from A549 cells, which were routinely grown in DMEM with the addition of 10% fetal bovine serum (FBS) in 96-well tissue culture plates with 1 $\times$ 10^4 cells per well. Confluent A549 cells were washed and incubated in DMEM supplemented with 1% FBS before infection. The *B. cenocepacia* H111 wild-type strain and its derivatives were grown in LB medium at 37°C, centrifuged, and resuspended in culture medium to an OD_{600} of 1.0. A549 cells were infected with bacterial cells at 10^9 CFU/ml for 8 h, and then the culture supernatants were collected by centrifugation for 4 min at 250 $\times$ *g*. The LDH in the

TABLE 2 PCR primers used in this study

Primer	Sequence (5′–3′)[a]
For protein expression	
rpfR-His-F	CGCGGATCCATGGATGACGAAAACGATAGCGCGG
rpfR-His-R	CCCAAGCTTTCAGGCGATCAGCCTGAGCTTTCTC
For qPCR assay	
BCAL0017-F	AATGAAGACCCTCGCACAGG
BCAL0017-R	TCGATATCCGCGTAAAGCCC
BCAL0111-F	TCAGCGTAGTGGTTGAACCC
BCAL0111-R	TCGGTAATGACGGCGATCAG
BCAL0113-F	TTGATCGTGTTCAGCGTCGA
BCAL0113-R	AACTTCGCGACGCTCTACAA
flhD-F	CTACCAGCGAAATGCTCAGT
flhD-R	CATACCCATCGCCTTGTCTT
flhC-F	GAAGGAAATCACCCTCGCCA
flhC-R	AACAGCGACGAGTGGATGTT
flhF-F	ACATTCGCAAATTCACCGGC
flhF-R	CGAGTGCAACGATTTCGACG
BCAL0248-F	CAATCGCGCAAGTCCTGAAG
BCAL0248-R	ATGACCTGCGGAATGTCGTT
BCAL0251-F	GTGAAGGGTGGCCGTATTCT
BCAL0251-R	TTCTTGAGCGGCACCTTGAA
secY-F	CTAACAGCCCGAGTCTTGCA
secY-R	AGTTGATCCGGATCGATGCC
fill-F	CTCGATCACCGCGTTCTACA
fill-R	GATGCGTTTCGTCGATCAGC
BCAL0524-F	CAGATGGTGCTCAAGGAAGT
BCAL0524-R	GACATGTTCGCGAGGAACT
BCAL0566-F	CGGATCCTGGTTCTGCAACT
BCAL0566-R	TGACTTCCTCCAACACGACG
BCAL0571-F	TGTTGATCGAGATGCCGAGG
BCAL0571-R	CTGAAGGATCTCGCGCAGAT
BCAL0831-F	TTGCAGGTTGAGTTCGACGA
BCAL0831-R	TCCGTATTTGCCCCCGAAAA
BCAL0990-F	GCAAAACAAGAAGTCGCCGT
BCAL0990-R	ACGACTTTCTTGCCGCGATA
BCAL1059-F	CACGCTTCGGCATCTTCAAG
BCAL1059-R	CAGGTCGAACGGAATCACCA
BCAL1060-F	ATGATGTACATCGCCGCGAA
BCAL1060-R	GCATCACTTCCGCGATGAAC
leuE-F	TGAGCCTGCTGAATCCGAAG
leuE-R	GAAGATCAGCGTGCTCAGGT
gtrR-F	GTGAAGGAGGGACTGTTCCG
gtrR-R	GACGAACGACTTGAACAGCG
zmpB-F	GGAAGGCTTGTCGGAAGG
zmpB-R	CCAGTTGTAGACCCAGTGATAC
BCAM2374-F	CAATCTGCTCTCCGTCTCCG
BCAM2374-R	AATCGGTTCGTAGTGCGTGT
BCAM2627-F	CTATCGTCACTGGGTGTCGG
BCAM2627-R	CCTTGTGGATCGCATGACCT
BCAS0263-F	CGGACGAATTGCTGCACAAG
BCAS0263-R	TCGTACTGGTGCAACACGAT
BCAS0292-F	GCCGATCGAAGCGGAAAT
BCAS0292-R	CAAAGAGCCGGTTGTCGTTG
BCAS0638-F	TTGCCGAAGAGAACCTGGTC
BCAS0638-R	CCGAGCAGGGTGAAGTGATT
BCAL2337-F	TCCACGACAGGAAGTTCACG
BCAL2337-R	AAATCTCGATGGGCTTCGCA
BCAL2353-F	GTCGTTTCTGGGCAAGGTA
BCAL2353-R	CACGTCGTGATCGATGTAGTC
BCAL2946-F	GAGCGCACGATCTACATGGA
BCAL2946-R	AGGAAGTGATAGCCGATGCG
BCAL2978-F	GAACGTGTCGATGTGCATGG
BCAL2978-R	TCGGCTACGACATGCACTAC
BCAL3041-F	CGATGGTCATCAACGGCAAG
BCAL3041-R	GTTCTTCTCGCCCTTCTGCT
BCAL3405-F	TACAACATCGGCAAGCAGGT

(Continued on next page)

TABLE 2 (Continued)

Primer	Sequence (5′–3′)[a]
BCAL3405-R	GGCAACTGCTCGTAGGTGAT
bclB-F	CTTTACCCACGACGACCTTTAC
bclB-R	TCGTATTGCGGCAGTTTCTC
BCAM0193-F	TATCGGGAAGGCAGCTACT
BCAM0193-R	CCGTAGTAATGGATTTCGAGCA
BCAM0196-F	GGAGCCTGATCCATACGTCG
BCAM0196-R	CATCGCCATGTCGAGAAAGC
BCAM0854-F	GGGACGATGGCGATTTCTT
BCAM0854-R	GGTTCCATCACCGCATAGTC
BCAM0859-F	TCGCTACAACGATCTCGACG
BCAM0859-R	CTTGCCTTCGAGCGAGATCT
BCAM0862-F	GCAACAAGTTCTACGCGACC
BCAM0862-R	GTCGAAGCTGCCGTATTTGC
BCAM0948-F	GCATGATGATGAGCAAGCCG
BCAM0948-R	CCCAGTAGTCCGGATAACGC
BCAM1004-F	TGAACTACCGTGAATCGTATGG
BCAM1004-R	CAACGGTGTCCGTGATCTT
BCAM1005-F	GCGTCAGGTAGTAGATCGGC
BCAM1005-R	CGGTCGAGGAGCAGTTCTAC
BCAM1010-F	GTGGGAAGAGGACATCATCAAG
BCAM1010-R	CGAAGATCGTCGGCATGAATA
BCAM1745-F	GCGATGTCGATCAGGTTCTT
BCAM1745-R	TGGCCGTCATGTTCAGGTAC
BCAM1870-F	AGTTCGATCGCGACGATACC
BCAM1870-R	AGCGACTTCAGCAGATACGG
BCAM1871-F	CTCGAACGACAGGTTGACGA
BCAM1871-R	GTATTTGCTGCGCATCTCCG
BCAM2044-F	CGGTCACGTAAAGCTCGGTA
BCAM2044-R	ACGTTTACGATCGGCTTCCA
BCAM2060-F	GTTGAGCAGGAACAGGTCGA
BCAM2060-R	GTGCTGTACGTGAACCAGGA
BCAM2140-F	GCGACATCGCATTCATTCATC
BCAM2140-R	ATCGTGTCGGGCGAAATC
bapA-F	CTGTTGTTGGTGCGATCATTT
bapA-R	CAACGTCGTGCCGTCATA
BCAM2215-F	AATCGGTCGTTGACGGGAAA
BCAM2215-R	CTTCAGCGTGAACGTGTAGC
BCAM2225-F	TCGCTCAAACGTTTGCAGTG
BCAM2225-R	GAACCATGCGCGGATCAAC
aidA-F	GACGTTGTCCTGGTTGGTCA
aidA-R	CGCGTTACCGATGTACTCGT

[a]Restriction enzyme sites are underlined.

supernatant was measured, and the cytotoxicity was calculated relative to that of an uninfected control.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00105-19.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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