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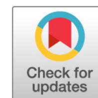

The Siderophore Product Ornibactin Is Required for the Bactericidal Activity of *Burkholderia contaminans* MS14

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ABSTRACT *Burkholderia contaminans* MS14 was isolated from soil in Mississippi. When it is cultivated on nutrient broth-yeast extract agar, the colonies exhibit bactericidal activity against a wide range of plant-pathogenic bacteria. A bacteriostatic compound with siderophore activity was successfully purified and was determined by nuclear magnetic resonance spectroscopy to be ornibactin. Isolation of the bactericidal compound has not yet been achieved; therefore, the exact nature of the bactericidal compound is still unknown. During an attempt to isolate the bactericidal compound, an interesting relationship between the production of ornibactin and the bactericidal activity of MS14 was characterized. Transposon mutagenesis resulted in two strains that lost bactericidal activity, with insertional mutations in a nonribosomal peptide synthetase (NRPS) gene for ornibactin biosynthesis and a *luxR* family transcriptional regulatory gene. Coculture of these two mutant strains resulted in restoration of the bactericidal activity. Furthermore, the addition of ornibactin to the NRPS mutant restored the bactericidal phenotype. It has been demonstrated that, in MS14, ornibactin has an alternative function, aside from iron sequestration. Comparison of the ornibactin biosynthesis genes in *Burkholderia* species shows diversity among the regulatory elements, while the gene products for ornibactin synthesis are conserved. This is an interesting observation, given that ornibactin is thought to have the same defined function within *Burkholderia* species. Ornibactin is produced by most *Burkholderia* species, and its role in regulating the production of secondary metabolites should be investigated.

IMPORTANCE Identification of the antibacterial product from strain MS14 is not the key feature of this study. We present a series of experiments that demonstrate that ornibactin is directly involved in the bactericidal phenotype of MS14. This observation provides evidence for an alternative function for ornibactin, aside from iron sequestration. Ornibactin should be further evaluated for its role in regulating the biosynthesis of secondary metabolites in other *Burkholderia* species.

KEYWORDS *Burkholderia contaminans* MS14, antibacterial activity, ornibactin

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The genus *Burkholderia* is composed of Gram-negative, rod-shaped, motile, environmental, versatile, non-spore-forming bacteria that have been identified in many diverse ecological niches (1). Currently, 88 species have been recognized in the genus *Burkholderia* (2). The bacteria have the ability to use a large array of carbon sources to synthesize secondary metabolites (3, 4). The *Burkholderia cepacia* complex is a group of *Burkholderia* species that includes soil isolates and opportunistic bacteria that cause lung disease in immunocompromised individuals (5). The *B. cepacia* complex group is

composed of 9 different genomovars and at least 18 different species (6). Conversely, some strains of *Burkholderia cepacia* are related to the promotion of plant growth and are considered to be plant-growth-promoting bacteria (PGPB). For example, *B. cepacia* strains could protect crops from the damping-off diseases caused by *Pythium* species and *Rhizoctonia solani* (3). Interest in the use of *Burkholderia* species or their secondary metabolites in agriculture has increased. In addition, multiple antimicrobials produced by *Burkholderia* species, such as occidiofungin (7), pyrrolnitrin (4), pyoluteorin (8), and AFC-BC11 (9), have been identified.

Siderophores are small-molecule, ferric-ion-specific, chelating agents secreted by bacteria and fungi growing under low-iron stress. They scavenge iron from the environment and make it available to the microbial cells (10). Siderophores are bacteriostatic agents that can inhibit the growth of pathogenic microorganisms by depleting iron in the soil (11). Many siderophores are synthesized by nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs). NRPSs and PKSs are large multimodular enzymes that are involved in natural product synthesis in many microorganisms (12). NRPSs, which are involved in the biosynthesis of oligopeptides, are grouped by active sites termed modules, with each module being required to catalyze one single cycle of product length elongation. The number and order of the modules of a NRPS protein mainly follow the "collinearity rule" (13).

Ornibactin is a product of nonribosomal peptide synthesis with siderophore activity. Ornibactin production in *Burkholderia cepacia* was shown to be critical for establishing an infection in a murine chronic respiratory infection model (14). In the same report, however, the authors noted that ornibactin appeared to be important for bacterial adherence or colonization. Furthermore, the report showed that the absence of ornibactin production led to a significant increase in the production of salicylic acid, suggesting that ornibactin production represses salicylic acid biosynthesis. These are interesting observations, but no direct link to an alternative function for ornibactin could be made. There is a long-term understanding that the role of ornibactin in virulence involves providing a source of iron in iron-restricted environments. To date, the best described function for ornibactin is its ability to sequester iron within the lung from iron-binding proteins, such as lactoferrin. This activity is crucial for survival within the respiratory mucus (15, 16).

In this study, we showed that *B. contaminans* MS14 produces a bactericidal compound that has a broad spectrum of activity toward Gram-negative bacterial plant pathogens. In addition, the bacteriostatic compound ornibactin was isolated and was shown to be an important component for the bactericidal phenotype of MS14. It was demonstrated that growing an ornibactin synthesis mutant in proximity to a *luxR* mutant restored MS14 bactericidal activity. We also showed that the addition of ornibactin to colonies of the ornibactin synthesis mutant restored the bactericidal phenotype, thus providing a direct observation of an alternative function for ornibactin. The genes involved in the initiation and regulation of biosynthesis, as well as regulatory elements, have significant diversity among *Burkholderia* species, suggesting that ornibactin has other functions within *Burkholderia*. The findings suggest that ornibactin is an important component for the production, or possibly the function, of the bactericidal secondary metabolite produced by *B. contaminans* MS14, and they support studies looking into its alternative functions within other bacterial species.

RESULTS

Antibacterial activity of MS14. *Burkholderia contaminans* MS14 was shown previously to produce a potent antifungal named occidiofungin (17). In this study, zone-of-inhibition plate assays of strain MS14 grown on nutrient broth-yeast extract (NBY) demonstrated significant bactericidal activity against a broad array of plant bacterial pathogens (Fig. 1 and Table 1). Culture of agar plugs within the zones of inhibition from the indicator bacterial strain *Erwinia amylovora* did not yield any viable colonies, supporting the classification of the MS14 antibacterial product as a bactericidal compound. *Xanthomonas citri* pv. malvacearum, one of the most destructive pathogens on

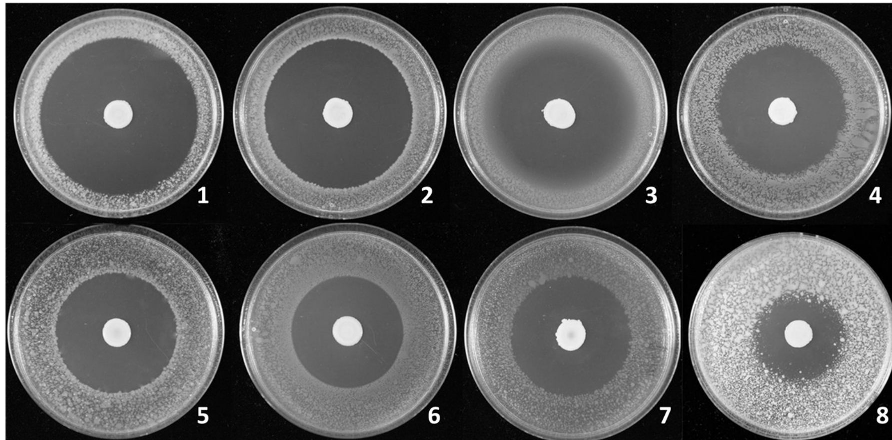

FIG 1 Antibacterial activity of *Burkholderia contaminans* MS14 against *Xanthomonas citri* pv. malvacearum MSCT1 (1), *Pectobacterium carotovorum* EC101 (2), *Ralstonia solanacearum* (3), *Pseudomonas syringae* pv. syringae B301D (4), *Erwinia amylovora* 2029 (5), *Burkholderia glumae* 291 (6), *Escherichia coli* (7), and *Clavibacter michiganensis* subsp. *michiganensis* Lu-01 (8). Aliquots (5 μl) of bacterial suspensions (optical density at 420 nm [OD_{420}] values of 0.3) were inoculated onto the centers of NBY plates. After the plates had been incubated for 3 days at 28°C, the NBY plates were oversprayed with a suspension of indicator pathogenic bacteria (OD_{420} values of 0.3). Inhibition zones were measured from the margins of bacterial colonies 24 h later. The results show that MS14 has broad-spectrum antibacterial activities against the tested pathogens.

cotton (18), was best inhibited by strain MS14, with a 36-mm-radius bactericidal zone on the plate. *Pectobacterium carotovorum* EC101, which is the pathogen of bacterial soft rot on potatoes and other vegetables (19), and *Ralstonia solanacearum*, which causes bacterial wilt of tomatoes and potatoes (20), were also significantly inhibited by strain MS14. The apple and pear fire blight pathogen *Erwinia amylovora* (21) and the bacterial panicle blight pathogen *Burkholderia glumae* (22) were also highly sensitive to MS14, with inhibition zone radii of 23 mm and 22 mm, respectively. Plate assays revealed that strain MS14 could significantly inhibit the Gram-positive bacterium *Clavibacter michiganensis* subsp. *michiganensis*, which is the pathogen of a major tomato disease, namely, tomato wilt and canker (23). However, another Gram-positive bacterium, *Bacillus megaterium*, was not very sensitive to the growth of strain MS14, compared to the other pathogenic bacteria tested. Overall, these data indicate that the cell metabolites of strain MS14 have possible applications as potent broad-spectrum antibacterial agents against plant pathogens. Mutagenesis analysis of MS14 generated the antibacterial-defective mutants MS14MT357 and MS14MT577, which retained similar antifungal patterns, compared to the wild-type strain (Fig. 2). These data indicate that

TABLE 1 Antibacterial activities of *Burkholderia contaminans* MS14

	Inhibition zone radius (mm)[a]		
Indicator pathogenic bacterium	**MS14**	**MT357**	**MT577**
Xanthomonas citri pv. malvacearum MSCT1	36 ± 1.66	0	0
Pectobacterium carotovorum subsp. *carotovora* EC101	33 ± 0.86	0	1 ± 0.5
Ralstonia solanacearum 102	31 ± 0.08	0	0
Pseudomonas syringae pv. syringae B301D	29 ± 1.00	0	0
Erwinia amylovora 2029	23 ± 0.85	0	0
Burkholderia glumae 291	22 ± 0.08	4 ± 1.55	5 ± 1.30
Escherichia coli JM109	22 ± 0.77	0	0
Clavibacter michiganensis subsp. *michiganensis* 1-07	17 ± 0.07	0	0
Bacillus megaterium KM	2.5 ± 0.04	0	0

[a]Mean ± standard deviation.

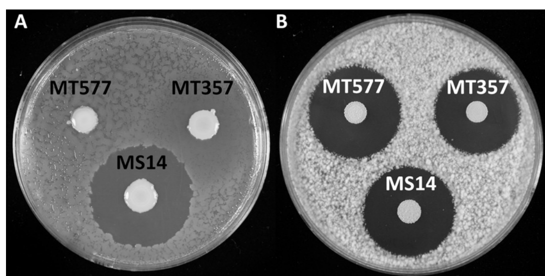

FIG 2 Plate bioassays of antimicrobial activities of *Burkholderia contaminans* strain MS14 and the mutants MS14MT357 (*luxR*::Tn*5*) and MS14MT577 (*orbI*::Tn*5*). (A) Loss of antibacterial activity of strain MS14 against the bacterial indicator *Erwinia amylovora* in MS14MT357 and MS14MT577. (B) Antifungal activities of strains MS14, MT357, and MT577 against the fungal indicator *Geotrichum candidum*. The results show that the genes disrupted in the mutants are not related to production of the antifungal activity of strain MS14.

the antibacterial mechanism is independent of production of the antifungal occidiofungin.

Identification of genes involved in production of the antibacterial product. Mutants of strain MS14 were created by EZ-Tn5 transposon insertion and were tested for antibacterial activity against our indicator strain of *Erwinia amylovora*. Two mutants that lost activity in the overlay assay were named MS14MT357 and MS14MT577; the plasmid rescue method was used to obtain plasmids pDP357 and pDP577, respectively, from the genomes of the mutants. Plasmid details are shown in Table 2. BLAST analysis using the DNA sequence generated from plasmid pDP357 (rescued from the mutant MS14MT357) against the MS14 genome showed that the disrupted gene NL30_RS14390 is 672 bp in size and is a *luxR* family transcriptional regulator (see Fig. S1 in the supplemental material). Sequence analysis of pDP577 revealed that the disrupted gene in mutant MS14MT577 is at locus NL30_RS14890, which is 9,663 bp in size (Fig. S2) and encodes a 3,219-amino-acid peptide. The deduced peptide of NL30_RS14895 shares 93% identity with the product of the *orbI* gene in *Burkholderia cenocepacia* J2315, which is one of the two NRPS genes for siderophore ornibactin biosynthesis (24). Given the size of the gene product in the MS14MT577 mutant, complementation cannot be achieved. The downstream genes are also involved in ornibactin biosynthesis; therefore, any possible polar effects would be on genes involved in the synthesis of the same product.

TABLE 2 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source
Escherichia coli		
Ec100D	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK* λ$^-$ *rpsL* (Str^r) *nupG pir*$^+$(*DHFR*)	Epicentre Corp.
Burkholderia contaminans		
MS14	Wild-type strain	
MS14MT357	*luxR*::Tn*5* derivative of MS14; Km^r	This study
MS14MT577	NRPS gene::Tn*5* derivative of MS14; Km^r	This study
Plasmids		
pDP357	EZ-Tn5 carrying 1.2-kb genomic DNA of MS14MT357; Km^r	This study
pDP577	EZ-Tn5 carrying 0.9-kb genomic DNA of MS14MT577; Km^r	This study
pMLS7	Expression vector of *Burkholderia*; Tp^r	Lefebre and Valvano (25)
pDP357-2	pMLS7 carrying 828-bp BamHI and HindIII fragment containing intact *luxR* gene; Tp^r	This study

[a]Km^r, kanamycin resistant; Tp^r, trimethoprim resistant.

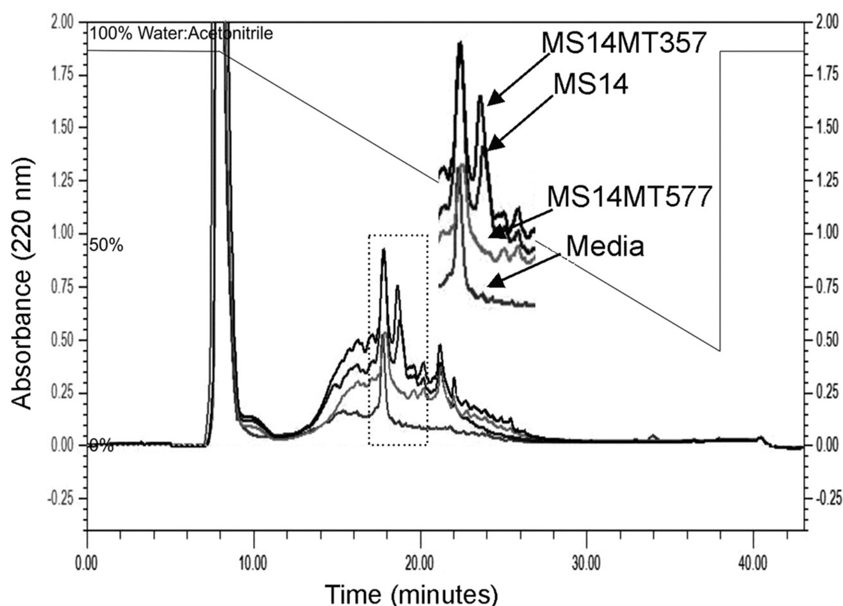

FIG 3 RP-HPLC chromatograms. An overlay of the chromatograms at 220 nm of the final purification step of the wild-type MS14 fraction, MS14MT357, and MS14MT577, using a C_{18} column (4.6 by 250 mm), is shown. Extracted medium was run as a negative control. The dotted box represents the region that was enlarged.

Complementation of the mutated *luxR*-type gene. The intact *luxR*-type transcriptional regulator gene was cloned into the *Burkholderia* expression vector pMLS7 by using the primer pair LuxRF (5′-CTGAGGATCCATTCAAACTAAACGAACGGGG-3′) and LuxRR (5′-GACGAAGCTTTGGCTCAGCGCGTTTC-3′), with the addition of BamHI and HindIII enzyme-digesting sequences, respectively. The cloned *luxR* genes were regulated by the S7 ribosomal protein promoter (25). The generated plasmid, pDP357-2, was transformed into the mutant MS14MT357 to be expressed constitutively. Plate bioassays revealed that the antibacterial activities of the mutants against *Erwinia amylovora* had fully been restored to the wild-type level, compared with the strain MS14 (Fig. S3). Considering that the EZ-Tn5 transposome was reported previously to have no polar effects leading to the inactivation of downstream genes (26) and that *luxR* gene complementation could fully restore MS14 antibacterial activities, we think that downstream genes were unlikely to have been affected by the insertional mutagenesis. The results demonstrated that the LuxR family transcriptional regulator is essential for the observed bactericidal activity in strain MS14.

Isolation and characterization of products from MS14MT357 and MS14MT577. The wild-type strain MS14 and the mutant strains MS14MT357 and MS14MT577 were cultured and antimicrobial compounds were extracted following an identical procedure. Extracts were analyzed on a reverse-phase high-performance liquid chromatography (RP-HPLC) column to determine differences among the isolated products. Wild-type MS14 and MS14MT357 had comparable peaks at the retention time of 18 min, eluting in 64:36 water/acetonitrile (Fig. 3). The mutant strain MS14MT577 did not produce a similar product at this retention time. In a MIC assay, the fraction at 18 min was the only product that exhibited any inhibitory activity against *E. amylovora*, but this activity was clearly not bactericidal. The initial Diaion HP-20 extracts had bactericidal activity in a zone-of-inhibition plate assay, but the bactericidal activity was not recovered from any RP-HPLC fraction. Isolation of the bactericidal compound has not yet been achieved.

The bacteriostatic product was isolated from wild-type MS14, and the structure was characterized by correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), nuclear Overhauser effect spectroscopy (NOESY), and heteronuclear single quantum co-

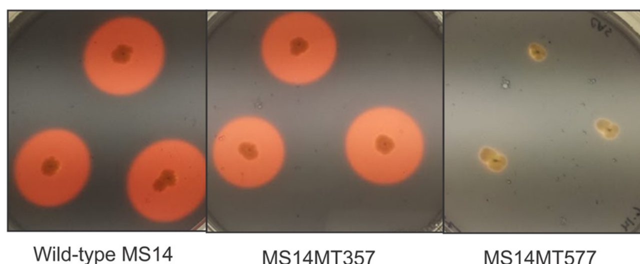

FIG 4 Chrome azurol S (CAS) plate assay. The wild-type MS14 and MS14MT357 strains have clear zones of siderophore activity, while the MS14MT577 strain has lost this activity due to the absence of ornibactin production.

herence (HSQC) nuclear magnetic resonance (NMR) (Fig. S4, S5, S6, and S7) and mass spectrometry. NMR analysis revealed that the purified product contained TOCSY spin systems for 3-hydroxyoctanoic acid (HOA), ornithine (Orn), aspartic acid (Asp), serine (Ser), and putrescine (Put) (Fig. S8 and Table S1). Furthermore, nuclear Overhauser effects (NOEs) were observed in the NOESY experiment, confirming the assigned position of each residue within the structure. NOEs were observed between Orn(Nδ-OH)1λ and Asp(β-OH)2NH, Orn(Nδ-OH)1α and Asp(β-OH)2NH, Asp(β-OH)2α and Ser3NH, Ser3α and Orn(Nδ-OH)4NH, Orn(Nδ-OH)4δ and formyl, Orn(Nδ-OH)4α and PutNH, and Ser3α and PutNH$_3^+$ (Fig. S9 and S10). The isolated product was structurally determined to be ornibactin-F, with a mass of 737 Da. In addition, a chrome azurol S (CAS) plate assay was used to demonstrate that the isolated product had siderophore activity (Fig. 4). The observed lack of the product in the MS14MT357 strain is to be expected, given that the mutation is within the biosynthesis pathway for ornibactin.

Restoration of bactericidal activity through coculture of MS14MT357 and MS14MT577. The isolated ornibactin-F product does not account for the bactericidal activity observed in the wild-type strain. This is supported by the lack of bactericidal activity in the MS14MT357 strain, which is capable of producing the same ornibactin product as wild-type MS14. Therefore, ornibactin is not directly responsible for the observed bactericidal activity. The relationship between ornibactin production and the LuxR family transcriptional regulator for synthesis of the bactericidal compound was further evaluated using plate overlay assays (Fig. 5). The mutant strains MS14MT357 and MS14MT577 were spotted on a plate in the shape of a V, with the colonies at the bottom being mixed cultures. Siderophore bacteriostatic activity was observed around

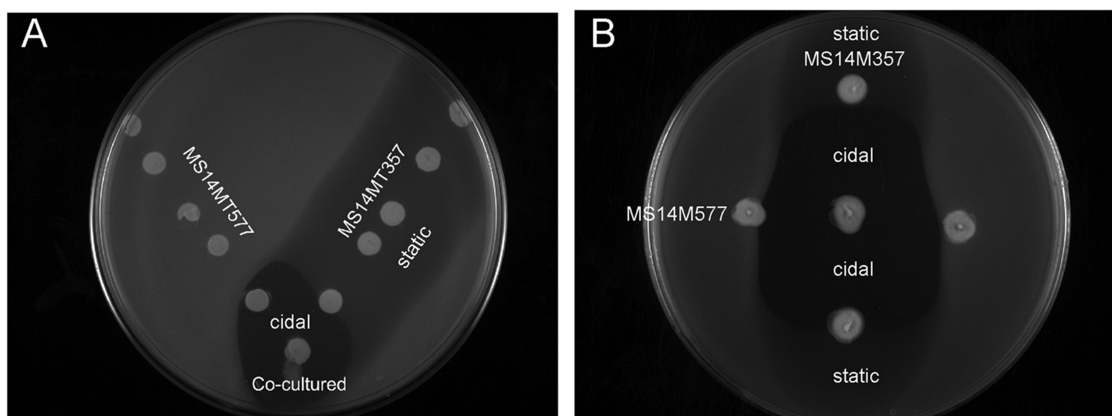

FIG 5 Bioassay for antibacterial activity. The mutant strains MS14MT357 and MS14MT577 were grown in proximity in a V assay (A) and a vertical assay (B). Mixed cultures are present at the bottom of the V in the V assay and in the center in the vertical assay. Both assays show that the mixed cultures produce bactericidal activity and that the bactericidal activity is also present in stabs of the two strains grown in close proximity ("cidal" indicates the bactericidal region on the plate). The bacteriostatic activity of ornibactin production in the MS14MT357 strain is visible in the observed growth reduction of the indicator strain ("static" indicates the bacteriostatic region on the plate).

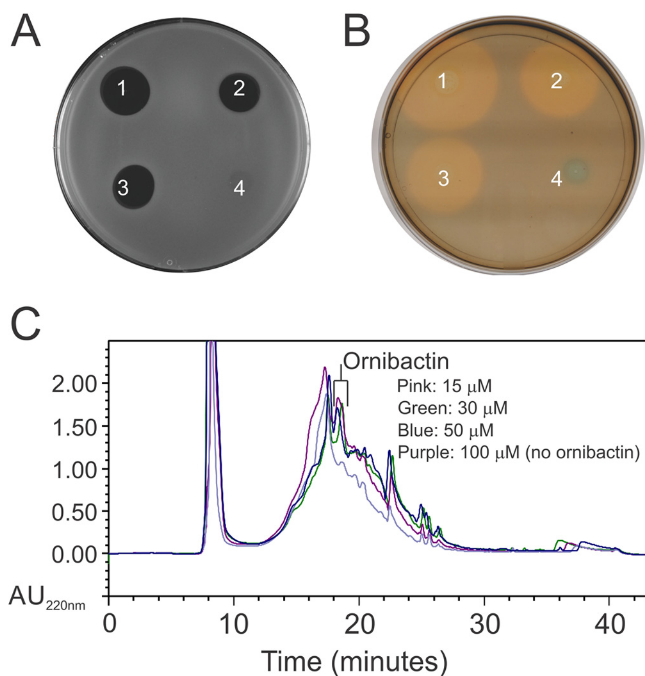

FIG 6 Bioactivity with supplemented ferric iron. (A) Antibacterial activity against *E. amylovora* of Diaion HP-20 extracts of cultures grown with ferric iron at 15 (1), 30 (2), 50 (3), and 100 (4) μM. (B) Chrome azurol S (CAS) plate assay of Diaion HP-20 extracts of cultures grown with ferric iron at 15 (1), 30 (2), 50 (3), and 100 (4) μM. (C) RP-HPLC chromatograms of Diaion HP-20 extracts of cultures grown with ferric iron at 15, 30, 50, and 100 μM. No siderophore or bactericidal activity is observed in cultures grown with 100 μM ferric iron.

each colony of the MS14MT357 strain along the right side of the V, while MS14MT577 did not inhibit the growth of the indicator strain along the left side of the V. The bactericidal activity, which was evident in the clear zone of inhibition at the bottom of the V, was present only when the MS14MT357 and MS14MT577 mutants were grown in close proximity. Similarly, when the strains were grown perpendicular to each other, a clear bactericidal zone of inhibition was present when cultures were mixed at the center of the plate (Fig. 5B). Synthesis of both the LuxR family transcriptional regulator and ornibactin is required for production of the bactericidal compound. To test this observation, the bactericidal and siderophore activities were tested with elevated concentrations of ferric iron. Increasing concentrations of ferric iron have been shown to regulate the synthesis of ornibactin (27). If ornibactin is directly involved in the regulation of the bactericidal product, then bactericidal activity should be absent at the same ferric iron concentrations that inhibit ornibactin biosynthesis. A concomitant loss of siderophore activity and bactericidal activity was observed for Diaion HP-20 extracts with increasing concentrations of available ferric iron (Fig. 6A and B). The loss of siderophore and bactericidal activities corresponded to loss of the ornibactin product observed by RP-HPLC (Fig. 6C). The data support the observation that ornibactin is directly required for the bactericidal activity of MS14. One possibility is that an ornibactin by-product is responsible for the observed bactericidal activity. This would suggest that the *luxR* gene NL30_RS14395 product is involved in the regulation of a product that modifies ornibactin. This scenario is unlikely, given that this product would presumably be isolated by the same extraction method as used to isolate ornibactin. It is more likely that ornibactin has a regulatory role in MS14 and promotes the synthesis of a bactericidal secondary product. This is supported by the observation that bactericidal activity was restored only in the mutant deficient in ornibactin production and not in the *luxR* regulatory mutant strain. When the ornibactin-deficient mutant MS14MT577 was grown on plates made from the culture broth of the MS14MT357 strain, the clear bactericidal zone of inhibition was restored (Fig. 7A). In a related assay,

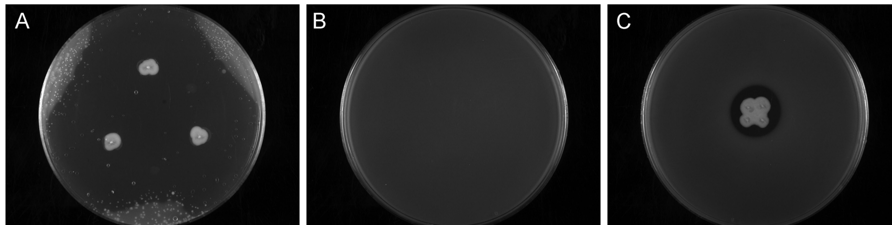

FIG 7 Bioassays of MS14MT577. (A) MS14MT577 strain grown on a plate made from a 3-day-old culture broth of the MS14MT357 strain and overlaid with the *E. amylovora* indicator strain after 3 days of growth. (B) Negative control in which the indicator strain was overlaid 3 days after 100 μg of ornibactin was spotted in the center of the plate. (C) Four inoculated colonies of MS14MT577 with ornibactin spotted in the center of the plate and overlaid with the indicator strain 3 days later. Bactericidal activity of the MS14MT577 strain was restored when MS14MT577 was grown on the culture broth of the MS14MT357 strain or when ornibactin was added directly.

100 μg of ornibactin was added to the center of a plate that had been previously stabbed with the MS14MT577 mutant (Fig. 7B and C). The addition of ornibactin restored the bactericidal activity of the MS14MT577 mutant strain, demonstrating that ornibactin has an activity aside from just sequestering iron in *B. contaminans* MS14.

Genetic architecture of ornibactin biosynthesis loci among *Burkholderia* species. The relationship between the production of ornibactin and *B. cepacia* complex virulence remains unclear (14). Given the demonstrated function of ornibactin in MS14 bactericidal activity, ornibactin biosynthesis loci were compared among *Burkholderia* species (Fig. 8). *B. cenocepacia* was selected as the reference because the ornibactin biosynthesis locus is best described in strain J2315 (24, 28). Using this reference, the ornibactin loci were identified for 13 *Burkholderia* species, including the pathogenic species *Burkholderia multivorans* ATCC 17616, *Burkholderia mallei* ATCC 23344, *Burkholderia thailandensis* E264, *Burkholderia oklahomensis* EO147, *Burkholderia pseudomallei* 1026b, and *B. pseudomallei* K96243 (29–32), the PGPB *Burkholderia lata* 383, *Burkholderia ambifaria* AMMD, and *Burkholderia phytofirmans* PsJN (33–35), and the soil isolates *B. cepacia* GG4, *Burkholderia vietnamiensis* G4, *Burkholderia phymatum* STM815, and *Burkholderia xenovorans* LB400 (36–38). We also analyzed the plant pathogens *B. glumae* BGR1 and *Burkholderia gladioli* BSR3 (39, 40); however, the ornibactin biosyn-

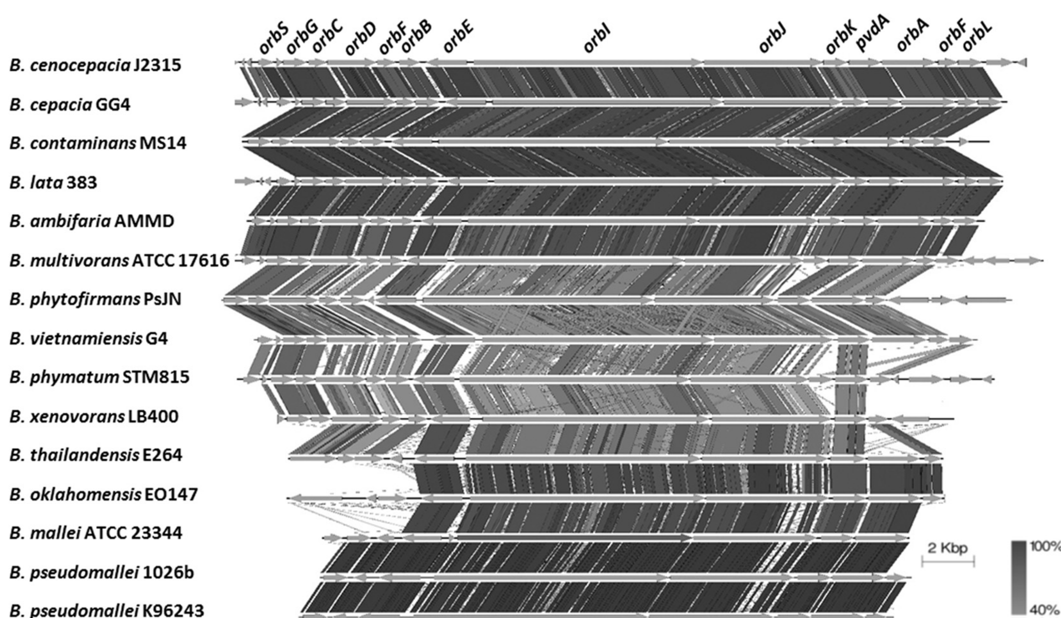

FIG 8 Ornibactin biosynthesis locus genetics of *Burkholderia* species. The ornibactin loci showed a high degree of conservation of the *orbL*, *orbJ*, *orbE*, and *pvdA* genes among the *Burkholderia* species compared; those genes are responsible for the biosynthesis and secretion of ornibactin. The bar at the right bottom indicates the degrees of similarity (40 to 100%).

thesis loci were not identified for the two species. The comparison of ornibactin loci demonstrated a high degree of conservation of the *orbI*, *orbJ*, *orbE*, and *pvdA* genes, which are responsible for ornibactin biosynthesis and ornibactin export across cytoplasmic membranes. The MS14 NRPS genes NL30_RS14890 and NL30_RS14895 share 90% nucleotide identity with the *orbJ* and *orbI* genes, respectively, in *B. cenocepacia* J2315, and the deduced peptides share 93% identity with those in J2315. In contrast, the genes from *orbS* to *orbB* and from *orbA* to *orbL*, which are involved in ornibactin biosynthesis initiation, regulation, transportation, and modification, show significant diversity among the studied *Burkholderia* genomes. In this analysis, the ornibactin products appear to be fairly consistent across *Burkholderia* species. The main question is why the regulatory elements for ornibactin production are so diverse, for a product that is thought to have a limited function in sequestering iron. The genomic comparison further illustrates the need to evaluate the possible functions ornibactin may have within *Burkholderia*.

DISCUSSION

The findings from this study demonstrate that MS14 has antibacterial activities against a wide range of plant-pathogenic bacteria, including *Erwinia amylovora*, *Xanthomonas citri* pv. malvacearum, and *Clavibacter michiganensis* subsp. *michiganensis*. Random mutagenesis studies resulted in the identification of two MS14 mutants with losses in antibacterial production. Both of the mutations occurred within regions that are not directly involved in the biosynthesis of the bactericidal product. Bactericidal activity could be restored by growing the ornibactin NRPS mutant and the LuxR family transcriptional regulatory mutant in proximity, suggesting that ornibactin production is essential for production of the antibacterial compound. We also showed a significant amount of diversity among *Burkholderia* species for the initiation and regulation of ornibactin biosynthesis. Given the genomic diversity of these regions, ornibactin presumably has evolved to have functional roles in addition to iron sequestration and iron uptake.

A LuxR family transcriptional regulator and the synthesis of ornibactin were essential for the production of a bactericidal compound in *B. contaminans* MS14 under the culture conditions tested. Mutations in each of the *luxR* and *orbI* genes resulted in a loss of production of the bactericidal activity. The LuxR bacterial protein family is one of the largest groups involved in the regulation of cell functions to control a variety of phenotypes (41). The results of this research suggest that production of the bactericidal activity in MS14 is regulated by the *luxR* gene (NL30_RS14390) via quorum-sensing communication. Currently, there are multiple mechanisms for the LuxR proteins to regulate cell functions in nearly all known Gram-negative quorum-sensing systems (42). Further studies are needed to understand the details of the regulation of bacterial activity. As shown previously, the genetic locus NL30_RS14890 is a homolog of the *orbI* gene of *B. cenocepacia* J2315, and the deduced peptide of NL30_RS14890 shares 93% identity with the OrbI protein, which is one of the two synthetases for production of the siderophore ornibactin (24). Mutation of the *orbI* gene in *B. contaminans* MS14 led to the ornibactin-negative phenotype, which is consistent with the result obtained in *B. cenocepacia* J2315 (24). Therefore, current data support the idea that *luxR* gene transcriptional regulation of the biosynthesis genes for the unknown bactericidal compound(s) and the *orbI* gene is involved in the biosynthesis of the siderophore ornibactin, which is associated with the production of bactericidal activity.

The structure of the isolated siderophore determined from our analysis is the same as that reported previously for ornibactin-F (43). The mass for this product also is similar to the reported mass for ornibactin-F, i.e., 737 Da (43). Ornibactin (27) is a tetrapeptide siderophore that was reported to be produced first by *Pseudomonas* (44) and then by several *B. cenocepacia* strains (27, 45). The ornibactin gene cluster contains two core NRPS genes (24), each of which is composed of an amino acid adenylation domain and a condensation domain. The two domains are core components of the NRPS mechanism (46). These domains for ornibactin biosynthesis were conserved among the

Burkholderia species that were compared (Fig. 8), suggesting that there is little deviation from the core structure of the ornibactin product.

Bactericidal activity is associated with the presence of ornibactin and is absent when ornibactin production is inhibited with higher iron concentrations. Concentrations of ≥15 μM ferric iron were enough to suppress ornibactin biosynthesis by members of the *Burkholderia cepacia* complex. Ornibactin production in MS14 was shown to be suppressed with the addition of 100 μM ferric iron. At that iron concentration, there was a concomitant loss of bactericidal activity (Fig. 6). Comparison of the ornibactin gene cluster with previously sequenced genomes of 14 *Burkholderia* species, including plant-growth-promoting strains and mammal-pathogenic strains, indicated that the ornibactin biosynthesis gene cluster commonly exists among other *Burkholderia* species. The diversity in the regulatory and transport regions within the gene cluster for ornibactin biosynthesis supports additional regulatory roles for the compound in *Burkholderia* species. In the case of MS14, ornibactin does have a clear role in promoting the bactericidal activity of the strain. Given the requirement of ornibactin for production of the bactericidal compound in MS14, ornibactin may also be crucial for the synthesis of other secondary metabolites in other bacterial systems. Ornibactin possibly has a broader function for virulence, aside from just sequestering iron. It is clear that ornibactin has an alternative function aside from iron sequestration in MS14, and a better understanding of this alternative activity could possibly promote the isolation of novel secondary metabolites or yield a more complete picture of the role of ornibactin in bacterial virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture medium. Bacterial strains and plasmids used in this study are listed in Table 2. *Escherichia coli* strain TransforMax EC100DTM pir+ (Epicentre Biotechnologies, Madison, WI) was used for plasmid rescue cloning and was cultured in Luria-Bertani (LB) medium at 37°C. Nutrient broth-yeast extract (NBY) agar (47) was used for cultures of *Burkholderia* strains and for plate bioassays of antimicrobial activities. Potato dextrose agar (PDA) (Difco, Detroit, MI) was used for plate bioassays to evaluate antifungal activities. Antibiotics (Sigma Chemical Co., St. Louis, MO), if applicable, were added to media at the following concentrations: trimethoprim, 100 mg/ml; kanamycin, 100 mg/ml for *Escherichia coli* and 300 mg/ml for *Burkholderia* strains.

Bioassay for antimicrobial activities. *B. contaminans* MS14 and its mutants used in this study were evaluated for antibacterial activities against *Erwinia amylovora* 2029 and other pathogenic indicators by using NBY plate bioassays. The bioassay was similar to that described by Scholz-Schroeder and colleagues (48). Briefly, MS14 and mutants were grown overnight in 5 ml of NBY liquid medium at 28°C. The bacterial cells were then collected by centrifugation and suspended in sterile distilled water to an optical density (OD) of 0.3 (approximately 2×10^8 CFU/ml). Aliquots (5 μl) of bacterial suspensions were inoculated onto the centers of NBY plates. After the plates had been incubated for 2 days at 28°C, NBY plates were oversprayed with a suspension of the indicator bacterial strains (OD at 420 nm [OD_{420}] of 0.3) and PDA plates were oversprayed with the indicator fungus *Geotrichum candidum* F-260 (OD_{420} of 0.3). Inhibition zones were measured from the margins of bacterial colonies 12 to 24 h later, and the sizes of the zones were compared between MS14 and the mutants. MS14MT577 was grown on plates made from the culture supernatants of extracted media from soft agar stabs. Supernatants were collected as described below and were used to make 1.5% agar plates. The MS14MT577 strain was stabbed into the culture supernatant agar plates and grown for 3 days at 28°C. The plates were placed in an oven at 60°C for 1 h, to kill the MS14MT577 strain, before being overlaid with *E. amylovora* 2029. *E. amylovora* was grown to an OD_{600} of 0.2 and was diluted 20-fold in NBY agar (0.75%). Five milliliters of the soft agar suspension was poured over the plates, and the plates were placed at 28°C for 1 day. In another assay, four colonies were stabbed into the center of NBY agar plates before 100 μg of ornibactin (10 μg/μl stock solution in 35% acetonitrile) was added to the center of the colonies. The plates were incubated for 3 days before being overlaid with the indicator bacterial strain embedded in the top agar, as described above. Three replicates of the plate bioassays were performed independently, and standard deviations of the means were calculated. To differentiate bactericidal activity from bacteriostatic activity, agar plugs (1 by 1 cm each) within the zones of inhibition of the indicator bacterial strain *Erwinia amylovora* were picked up and suspended in 1 ml of NBY medium; 100 μl of the suspension was plated. Bactericidal activity was indicated by no growth of the indicator bacterial strain from the agar plugs cut from the clear zones of inhibition. The initial CFU (time zero) from an agar plug (1 by 1 cm) suspended in 1 ml of medium was $\sim 8 \times 10^4$ CFU.

Random mutagenesis. The EZ-Tn5 <R6Kγori/KAN-2>Tnp transposome kit was used, as recommended by the manufacturer (Epicentre Biotechnologies, Madison, WI), to characterize the genes dedicated to the antibacterial activity of MS14. MS14 acquired kanamycin resistance on NBY plates with the EZ-Tn5 transposon insertion into the genome, and mutants could be selected on NBY plates supplemented with 300 μg/ml kanamycin. The mutants that exhibited reduced or no antibacterial activity against *Erwinia amylovora* were isolated; 16S rRNA and *recA* genes were cloned and sequenced to confirm that the resulting mutants were derivatives of strain MS14. Plasmid rescue cloning was

performed according to the transposome kit instructions, to generate the plasmids pPD357 and pPD577 (Table 2). To confirm that the rescue plasmid contained the transposon sequence, a portion of the Tn*5* transposon sequence was amplified by PCR with the primers R6kF1 (5′-GGGTAGCCAGCAGCATCCT-3′) and R6kR1 (5′-CATGATCGTGCTCCTGTCGTT-3′). The positive rescue clones were sequenced for further analysis. Sequence analysis was accomplished using the Lasergene Cloning Suite (version 12; DNASTAR, Inc., Madison, WI). Genes were searched against the *B. contaminans* MS14 reference genome (49). The BLASTn comparison of genomes was visualized with the BLAST Ring Image Generator (BRIG) (50).

Analysis and isolation of the siderophore product. The wild-type strain MS14 was grown overnight at 28°C on modified NBY (487 ml distilled water, 2.5 g peptone, 1.5 g Todd-Hewitt broth, 1.0 g yeast extract, 1.0 g anhydrous K_2HPO_4, 0.25 g KH_2PO_4, and 1.5% agar, with 12.5 ml of 20% glucose and 0.5 ml of 1 M $MgSO_4$ added after autoclaving) agar plates. Colonies from the overnight NBY agar plates were stabbed into 500 ml of modified NBY soft agar (NBY medium with only 0.75% agar). The inoculum in soft agar was placed at 28°C for 3 days and then frozen at −80°C. The medium was thawed in a 65°C water bath for 1 h. The inoculum was then placed in 250-ml centrifuge bottles and centrifuged at 20,000 × *g* for 30 min. The collected supernatant was pooled, mixed with 1 g of the polyaromatic absorbance resin Diaion HP-20, and shaken for 1 h. The resin was allowed to settle before the supernatant was decanted, and the resin was suspended following the decantation of the medium in 10 ml of 50% acetonitrile in water. The extract was dried by lyophilization and suspended in 1 ml of 35% acetonitrile in water. The extracts were tested for siderophore and antibacterial activities by spotting 10 μl of the extract on a chrome azurol S (CAS) plate or on an NBY plate overlaid with *Erwinia amylovora* 2029. RP-HPLC was performed using a C_{18} column (4.6 by 250 mm; Grace-Vydac catalog no. 201TP54) on a Bio-Rad BioLogic DuoFlow F10 system with a QuadTec UV-visible detector. Fractions were separated by using a 30-min gradient from 90:10 to 20:80 (water with 0.1% trifluoroacetic acid/acetonitrile with 0.1% trifluoroacetic acid).

Structural determination of the siderophore product by NMR. A 2 mM sample of the purified bacteriostatic compound was prepared in acetonitrile-D_3 (Cambridge Isotopes)/H_2O (50:50). The NMR data were collected with a Bruker Avance III HD 600-MHz spectrometer and a Bruker Avance III HD 850-MHz spectrometer, using TCI CryoProbes for each spectrometer. The ^{1}H resonances were assigned according to standard methods (51), using COSY, TOCSY, NOESY, and ^{13}C-HSQC NMR experiments. NMR data were collected at 10°C. The carrier frequency was centered on the water resonance, which was suppressed minimally by using standard presaturation methods. A 2.0-s relaxation delay between scans was used. The TOCSY experiment was performed with a 60-ms mixing time, using the Bruker DIPSI-2 spinlock sequence. The NOESY experiment was performed with a 400-ms mixing time. The parameters for collecting the HSQC spectrum were optimized for the observation of aliphatic and aromatic CH groups. The spectral sweep widths for the TOCSY and NOESY experiments were 11.35 ppm in both dimensions. The spectral sweep widths for the HSQC experiment were 11.35 ppm in the proton dimension and 0 to 85 ppm in the carbon dimension. All two-dimensional data were collected with 2,048 complex points for the acquisition dimension and 256 complex points for the indirect dimensions, except for the HSQC data, which were collected with 2,048 and 128 complex points for the direct and indirect dimensions, respectively. Phase-sensitive indirect detection for the NOESY, TOCSY, and COSY experiments was achieved using standard Bruker pulse sequences. ^{1}H chemical shifts were referenced to the residual water peak (3.33 ppm). Data were processed with NMRPipe (52) by removing the residual water signal by deconvolution, multiplying the data in both dimensions by a squared sine-bell function with 45° or 60° shifts (for the ^{1}H dimension of HSQC), zero-filling once, and performing Fourier transformation and baseline correction. Data were analyzed with the interactive computer program NMRView (53).

Mass spectrometry of the siderophore product. The mass of the purified bacteriostatic product was confirmed by matrix-assisted laser desorption ionization (MALDI) using a Shimadzu/Kratos MALDI-TOF mass spectrometer in both the linear and reflectron modes. The isolated compound was further analyzed by an electrospray mass spectrometer using a Thermo Fisher Deca XP ion trap mass spectrometer. The compound was dissolved in acetonitrile/water (50:50 [vol/vol]) with 0.1% formic acid and injected into a 1-μl/min flow of the same solvent by using a Harvard syringe pump. The flow was sprayed using the nano-liquid chromatography interface. Tandem mass spectrometry was performed with singly charged ions using standard collision energy (34 V) and higher collision energy (50 V).

Plasmid construction for *luxR* gene complementation. The intact *luxR* gene was amplified using the primer pair LuxRF (5′-CTGAGGATCCATTCAAACTAAACGAACGGGG-3′) and LuxRR (5′-GACGAAGCTTTGGCTCAGCGCGTTTC-3′), in which restriction endonuclease cutting sites (underlined; for BamHI and HindIII, respectively) were added. The resulting PCR product, containing the intact wild-type *luxR* gene, was digested with BamHI and HindIII and then cloned into the expression vector pMLS7 to generate the plasmid pDP357-2, as described previously (54). The plasmid pDP357-2 was electroporated into competent MT357 cells to recover the wild-type characteristics. Empty vector was used as a negative control. Single colonies were picked from NBY plates supplemented with trimethoprim (100 μg/ml) and kanamycin (300 μg/ml). Plasmid was extracted from the colonies, and sequencing confirmed the existence of the resultant plasmid pDP357-2. Plate bioassays were used to evaluate the antibacterial activity of the resulting cells.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00051-17.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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We have no conflicts of interest to declare.

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