A *Burkholderia cepacia* complex non-ribosomal peptide-synthesized toxin is hemolytic and required for full virulence

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Members of the *Burkholderia cepacia* complex (Bcc) have recently gained notoriety as significant bacterial pathogens due to their extreme levels of antibiotic resistance, their transmissibility in clinics, their persistence in bacteriostatic solutions, and their intracellular survival capabilities. As pathogens, the Bcc are known to elaborate a number of virulence factors including proteases, lipases and other exoproducts, as well as a number of secretion system associated effectors. Through random and directed mutagenesis studies, we have identified a Bcc gene cluster capable of expressing a toxin that is both hemolytic and required for full Bcc virulence. The Bcc toxin is synthesized via a non-ribosomal peptide synthetase mechanism, and appears to be related to the previously identified antifungal compound burkholdine or occidiofungin. Further testing shows mutations to this gene cluster cause a significant reduction in both hemolysis and *Galleria mellonella* mortality. Mutation to a glycosyltransferase gene putatively responsible for a structural-functional toxin variant causes only partial reduction in hemolysis. Molecular screening identifies the Bcc species containing this gene cluster, of which several strains produce hemolytic activity.

Introduction

Members of the *Burkholderia cepacia* complex (Bcc) are Gram-negative bacilli ubiquitous in soil and water sources. These bacteria embody a diverse array of metabolic capabilities, among them environmental contaminant degradation,[1,2] fungal phytopathogen suppression[3-7] and nitrogen fixation.[8] For these reasons, the Bcc has been identified as a powerful class of bioremediation and plant growth promoting agents.[9] However, the potential usefulness of the Bcc has been compromised by their opportunistic pathogenicity and concerns surrounding their extensive antibiotic and biocide resistance.[10-12] For immune-compromised patients, such as those with the heritable genetic disease cystic fibrosis (CF), the Bcc represent a singular threat because of their adept transmissibility in clinical settings,[13,14] their extremely high antibiotic resistance,[15] their persistence in disinfectant solutions[16-18] and their ability to survive in vivo.[19-22] Upon gaining access to the lung, members of the Bcc produce a number of virulence factors toxic to the host and instigate immune system stimulation that ultimately severely reduces lung function. As well, the Bcc occasionally create what has been termed "cepacia syndrome," a disease characterized by an acute and severe necrotizing pneumonia, sepsis and death.[9]

Toxic factors produced by the Bcc include proteases, lipases and type III secretion system effectors.[9] Early reports suggested that only approximately 4% of Bcc isolates exhibit β-hemolytic activity when sheep erythrocytes were tested.[23] However, later studies showed that up to 39% of clinical Bcc isolates possessed hemolytic activity when using sheep erythrocytes.[24] One source of this hemolytic activity may arise from a β-hemolysin,[24] although unlike *Pseudomonas aeruginosa*, Bcc phospholipase activity does not correlate with the presence of this hemolytic activity. In 1994, the isolation and purification of two related hemolysins with antifungal properties was published by Abe and Nakazawa.[3] The hemolytic activity of these "cepalysins" was inhibited by sterols, suggesting that they require an interaction with cholesterol in the erythrocyte membrane to produce a biological effect. Hutchison et al.[25] showed that a lipopeptide toxin produced by *Burkholderia cenocepacia* could cause hemolysis of erythrocytes, apoptosis of human neutrophils, and an increase in neutrophil degranulation. Fehlner-Gardiner et al.[26] identified components of the Bcc general secretory pathway that were involved in the secretion of hemolytic and phospholipase C activities, but that were not necessary for Bcc intracellular survival within *Acanthamoeba polyphaga.* More recently, Bevivino et al.,[27] using ram erythrocytes, found that almost all *Burkholderia ambifaria* isolates were hemolytic and that the percentage of hemolytic environmental *B. cenocepacia* isolates was markedly higher than the percentage of hemolytic clinical isolates. Similarly, Carvalho et al.[28] indicated that almost all of the 59 Bcc clinical isolates from a reference CF center in Rio de

Janeiro, Brazil produced several different exoproducts except for hemolysin (as tested against human erythrocytes). From these results, it is evident that some Bcc members produce hemolytic toxins, although their importance toward pathogenicity is unclear.

Several secondary metabolite compounds with biological effects are also produced by the Bcc, either through polyketide synthetase (PKS) or non-ribosomal peptide synthetase (NRPS) enzyme complexes. Known secondary metabolite toxins produced by the Bcc include the broad-spectrum antifungal agent "pyrrolnitrin,"[29] a group of antifungal and antitumor agents deemed "cepafungins,"[30] hemolytic peptides named "cepalysins,"[3] related antifungal compounds "cepacidine A1 and A2"[7,31] and the recently described antifungal cyclic peptide "occidiofungin" or "burkholdine,"[32-34] which may be similar to molecules previously isolated from Bcc strains as "xylocandins."[35,36] While these latter compounds show a broad range of activity against fungi, in this manuscript, we identify through random and directed mutagenesis a gene cluster in *Burkholderia vietnamiensis* strain DBO1[37] that is homologous to the gene cluster that synthesizes occidiofungin/burkholdine, and demonstrate that this gene product is also strikingly β-hemolytic. This study also examines the prevalence of this gene cluster within the Bcc and the lethality of this compound in an invertebrate infection model.

Results

Identification of an NRPS cluster through plasposon mutagenesis. The hemolytic patterns of the Bcc species *B. vietnamiensis* DBO1 and *B. ambifaria* AMMD, which form β-hemolysis (yellow clearing) on sheep blood agar, were investigated by randomly mutagenizing DBO1 with plasposon pTn*Mod*-OTp'.[38] Mutants were screened on sheep blood agar + 100 μg/mL Tp, and those deficient in hemolysis were carried forward for analysis, which revealed plasposon insertion sites in an uncharacterized gene cluster containing NRPS-encoding genes, as well as regulator and transporter-encoding genes. Further insertion mutation analysis identified genes essential to the hemolytic activity, and allowed delineation of the ends of the gene cluster. DNA sequence analysis of the *B. vietnamiensis* DBO1 gene cluster demonstrated 99.9% identity to a nearly identical gene cluster in the sequenced genome of *B. ambifaria* strain AMMD. As shown in **Figure 1**, and with functional activity for each gene shown in **Table 1**, the core Bcc NRPS gene cluster is approximately 50.5 kb in length and is comprised of 13 ORFs arranged identically in both strains. In *B. ambifaria* AMMD, the NRPS cluster of open reading frames (ORFs) is located on chromosome 3 and extends from basepair 1,101,558 to 1,152,073 for a total of 50,515 base pairs. BLASTN analysis[39] identified only one other closely related gene cluster in the GenBank database, a gene cluster of Bcc member *B. contaminans* strain MS14 encoding occidofungin biosynthesis proteins. Not only are these 13 genes in MS14 similarly arranged (syntenic) to the NRPS genes in AMMD/DBO1, each ORF in the AMMD/DBO1 gene cluster has as its most closely related database entry an ORF in the MS14 occidofungin biosynthetic cluster. For example, the *B. ambifaria* AMMD 3176 amino acid adenylation protein Bamb_4672 is 89% identical (2812/3176) and 93% similar (2944/3176) to the same protein in *Burkholderia contaminans* MS14, along the entire sequence (12/3176 gaps). For comparison, the next closest database relative is an uncharacterized protein identified in *Acidobacterium capsulatum* ATCC 51196, that is only 52% identical (1629/3138) and 68% similar (2119/3138) to Bamb_4672 across 3138/3176 amino acids, with 72/3138 gaps. This analysis indicates that the gene clusters identified to be responsible for the hemolytic activity observed in strain DBO1 (and by homology AMMD), are closely related to the occidofungin biosynthesis gene cluster identified in *B. contaminans* MS14, and to few other genes in the known bacterial genetic complement.
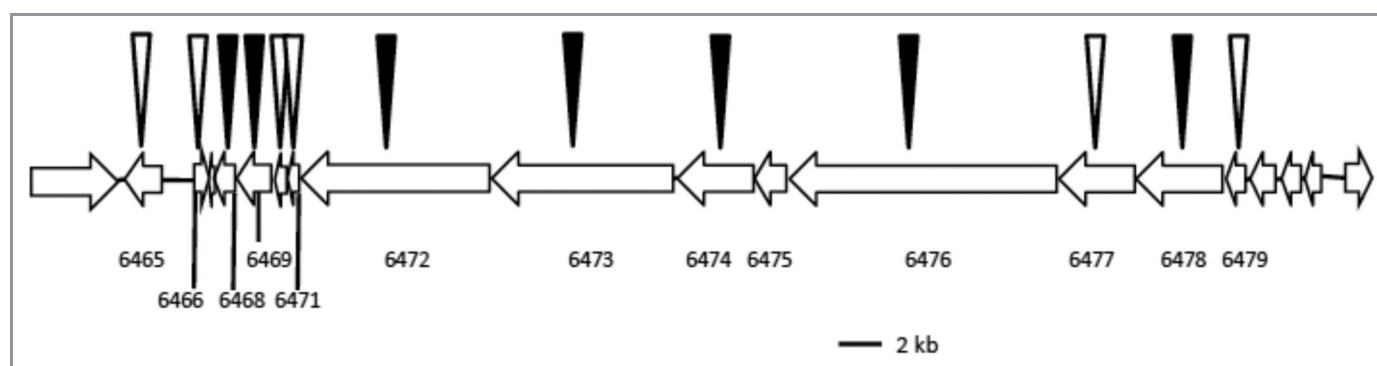

Figure 1. The NRPS cluster giving rise to toxic activity in *B. vietnamiensis* DBO1. The cluster appears similar to that described in *B. contaminans* MS14 by Gu et al.[32] except that an additional two genes, homologs of *6477* and *6478* in *B. ambifaria* AMMD, are present upstream of the described MS14 cluster that are required for virulence in DBO1. Filled vertical arrows indicate genes disrupted by plasposon mutagenesis; unfilled vertical arrows indicate genes disrupted through targeted mutagenesis. Genes are named according to the sequenced *B. ambifaria* AMMD genome: *6465*, encoding an FAD-linked dioxygenase homolog; *6466*, encoding a LuxR homolog; *6467*, encoding a hypothetical protein with a DNA binding motif; *6468*, encoding a LuxR homolog; *6469*, encoding a cyclic peptide transporter; *6470*, encoding a hypothetical protein; *6471*, encoding a glycosyltransferase; *6472*, encoding an adenylation domain-containing protein (ADCP); *6473*, encoding an ADCP; *6474*, encoding an ADCP; *6475*, encoding a β-lactamase; *6476*, encoding an ADCP-polyketide synthetase (PKS) hybrid; *6477*, encoding a PKS; *6478*, encoding a PKS; *6479*, encoding a taurine dioxygenase. Only genes *6466* through *6478* appear to be involved in toxin production.

Table 1. Bacterial strains and plasmids constructed for this study

Bacterial strains	Genotype or relevant phenotype	Hemolytic activity	Source
B. vietnamiensis			
DBO1	Parent strain	+	37
6465::Tp^R	DBO1 with targeted oriTp' insertion in gene 6465, encoding an FAD-linked dioxygenase	+	This study
6466::Tp^R	DBO1 with targeted oriTp' insertion in gene 6466, encoding a putative LuxR regulator	−	This study
6468::Tp^R	DBO1 with oriTp' plasposon insertion in gene 6468, encoding a putative LuxR regulator	−	This study
6469::Tp^R	DBO1 with oriTp' plasposon insertion in gene 6469, encoding a putative peptide transporter	−	This study
6470::Tp^R	DBO1 with targeted oriTp' insertion in gene 6470, encoding a hypothetical protein	+	This study
6471::Tp^R	DBO1 with targeted oriTp' insertion in gene 6471, encoding a putative glycosyltransferase within the NRPS gene cluster	+/−	This study
6472::Tp^R	DBO1 with oriTp' plasposon insertion in gene 6472, encoding a putative adenylation domain-containing protein	−	This study
6473::Tp^R	DBO1 with oriTp' plasposon insertion in gene 6473, encoding a putative adenylation domain-containing protein	−	This study
6474::Tp^R	DBO1 with oriTp' plasposon insertion in gene 6474, encoding a putative adenylation domain-containing protein	−	This study
6476::Tp^R	DBO1 with oriTp' plasposon insertion in gene 6476, encoding a putative adenylation domain-containing protein and polyketide synethetase	−	This study
6477::Tp^R	DBO1 with targeted oriTp' insertion in gene 6477, encoding a putative polyketide synthetase	−	This study
6478::Tp^R	DBO1 with oriTp' plasposon insertion in gene 6478, encoding a putative polyketide synthetase within the NRPS gene cluster	−	This study
6479::Tp^R	DBO1 with targeted oriTp' insertion in gene 6479, encoding a putative taurine metabolism protein	+	This study
DBO1/pSCRhaTc	DBO1 carrying pSCRhaTc	+	
6466::Tp^R/pSCRhaTc	DBO1 mutant 6466::Tp^R carrying pSCRhaTc	−	
6466::Tp^R/p6466	6466::Tp^R carrying pSCRha-6466^{His}	+	This study
E. coli			
DH5α	Cloning host strain	NA	Invitrogen
Plasmids			
pJET1.2/blunt	Cloning vector, Amp^R	NA	Fermentas
pTnMod-OTp'	Plasposon used for random mutagenesis, Tp^R	NA	38
pSCRha-6466^{His}	Rhamnose-inducible plasmid pSCRhaB2 modified with Tc resistance cassette and 6x histidine-tagged gene 6466 for genetic complementation	NA	This study
pSCRhaTc	pSCRha-6466^{His} carrying no 6466^{His} insert	NA	43

+, full clearing; +/−, partial clearing; −, no clearing; NA, not applicable.

Uniquely structured toxin is a broad-specificity hemolysin. Bcc strains DBO1 and AMMD were grown on blood agar with hemocytes from various sources, including bovine, rabbit, sheep, horse and human, to determine whether hemolysis was limited to certain mammals. Both strains exhibited hemolytic activity on all types of blood agar (data not shown). Clearing on sheep blood agar produced large yellow zones of lysis, indicative of a β-hemolysis (**Fig. 2**), while cleared zones on other blood types appeared colorless. Mutants in DBO1 defective in producing zones of clearing on one type of blood agar were defective in producing zones of clearing on the other types of blood agar. In order to determine whether the diffusible hemolytic activity observed was active against bacterial cells, Bcc strains producing hemolytic activity were streaked on sheep blood plates and cross-streaked (either concurrently or after one day's growth) with either a panel of Gram-negative and Gram-positive bacterial species including various pseudomonads, *Escherichia coli*, *Staphylococcus* and *Bacillus* species, or 25 different Bcc strains. In both conditions, the Bcc hemolytic activity was not inhibitory to the growth of any other bacteria tested, even though hemolytic activity was observable in the underlying blood agar.

To quantify the effect of different NRPS mutations on hemolytic activity, we developed a high-throughput liquid human blood hemolysis assay using hemoglobin release (absorbance at wavelength 570 nm) as a marker. As shown in **Figure 3**, we first tested this assay against wild-type strain DBO1, and two constructed mutants including 6466::Tp^R, a putative *luxR* homolog mutant and 6477::Tp^R, a putative polyketide synthetase mutant, neither of which produce zones of clearing on sheep blood agar. Although hemocytes were inoculated with similar numbers of
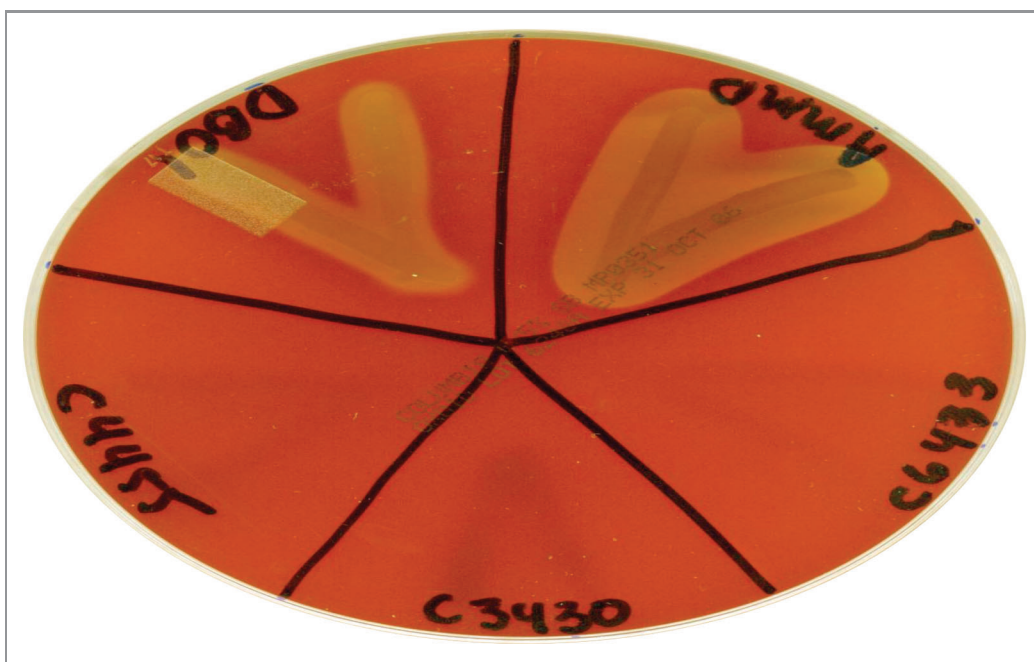
Figure 2. Bcc hemolytic activity on TSA 5% sheep blood agar. *B. vietnamiensis* strain DBO1 and *B. ambifaria* strain AMMD ("V" streaked) produce large transparent yellow hemolytic zones of clearing on an inverted sheep blood plate following overnight growth at 30°C. *B. cenocepacia* strains C4455 and C6433 and *B. multivorans* strain C3430 produce no such hemolytic zones of clearing and are barely visible through the opaque growth media.

bacterial cells (**Fig. 3B**), only wild-type strain DBO1 was able to release substantial amounts of hemoglobin (**Fig. 3A**) or reduce the counted number of red blood cells (**Fig. 3C**).

As described previously,[33,34] the NRPS product occidiofungin/burkholdine is a cyclic lipopeptide comprising eight amino acids. At least two forms of the peptide exist. One form contains a xylose side chain (occidiofungin/burkholdine 1229), which is presumably added to the peptide by the predicted glycosyltransferase encoded within the NRPS gene cluster. The other form contains no xylose side chain (burkholdine 1097). While the xylose-containing peptide was shown to have antifungal activity, the peptide lacking xylose displayed little activity.[32,33] To determine whether we would observe differences in hemolytic activity produced by the different *B. vietnamiensis* DBO1 NRPS cluster mutants, including the glycosyltransferase mutant *6471*::Tp^R, DBO1 mutants were incubated with human erythrocytes for a period of two days, and the absorbance at 570 nm of the supernatants was taken each day. As shown in **Figure 4**, there are three potential hemolytic phenotypes demonstrated by the strains. First, there is a fully hemolytic phenotype characteristic of both wild-type DBO1 and strains containing mutations to genes outside of the NRPS gene cluster (*6465* and *6479*). In addition, genes *6469* and *6470,* although by location included as a part of the NRPS gene cluster, are not essential to the production of hemolytic activity, as their mutation does not significantly alter wild-type hemolytic activity. Second, there is an intermediate hemolytic phenotype exhibited by mutant *6471*::Tp^R, likely due to the lack of a xylose addition to the final product, as described for occidofungin/burkholdine.[30,33] Whereas xylose addition to occidiofungin/burkholdine appears to be essential for antifungal

activity, the absence of NRPS product glycosylation in DBO1 reduces but does not eliminate the hemolytic activity of the NRPS-derived compound in *B. vietnamiensis* DBO1. Finally, there is a complete abrogation of hemolysis in strains containing mutations to genes *6466* (encoding a LuxR regulator), *6469* (encoding a cyclic peptide transporter), *6472* [encoding an adenylation domain-containing protein (ADCP)], *6473* (encoding an ADCP), *6474* (encoding an ADCP), *6476* [encoding an ADCP-polyketide synthetase (PKS)], *6477* (encoding a PKS), and *6478* (encoding a PKS). To prove that the DBO1 genes identified by mutagenesis were indeed responsible for the defect in hemolytic activity observed, genetic complementation was achieved for cluster gene *6466* (**Fig. 4**). When gene 6466 is cloned behind a rhamnose inducible promoter in plasmid pSCRhaTc-*6466*^{His} (forming plasmid p66), and introduced into DBO1 mutant *6466*::Tp^R, the addition of 0.2% rhamnose results in full restoration of hemolytic activity as measured by hemoglobin release, whereas *6466*::Tp^R/p66 without added rhamnose remains non-hemolytic.

Galleria mellonella **infection model.** *B. vietnamiensis* DBO1 strains (wild-type, *6466*::Tp^R and *6466*::Tp^R/p66) were grown overnight and inoculated at 5×10^6 cfu into *G. mellonella* (greater wax moth) larvae. Larval death counts were taken every 24 h. The results indicate that the DBO1 NRPS product is toxic to the *G. mellonella* moth larvae, whereas a gene *6466* LuxR knockout mutant lacking the ability to produce the hemolysin has greatly reduced virulence. Genetic complementation of this gene knockout, as described above for hemoglobin release, showed similar restoration of the toxic effects toward *G. mellonella* upon the addition of rhamnose. Since moth larvae do not contain red blood
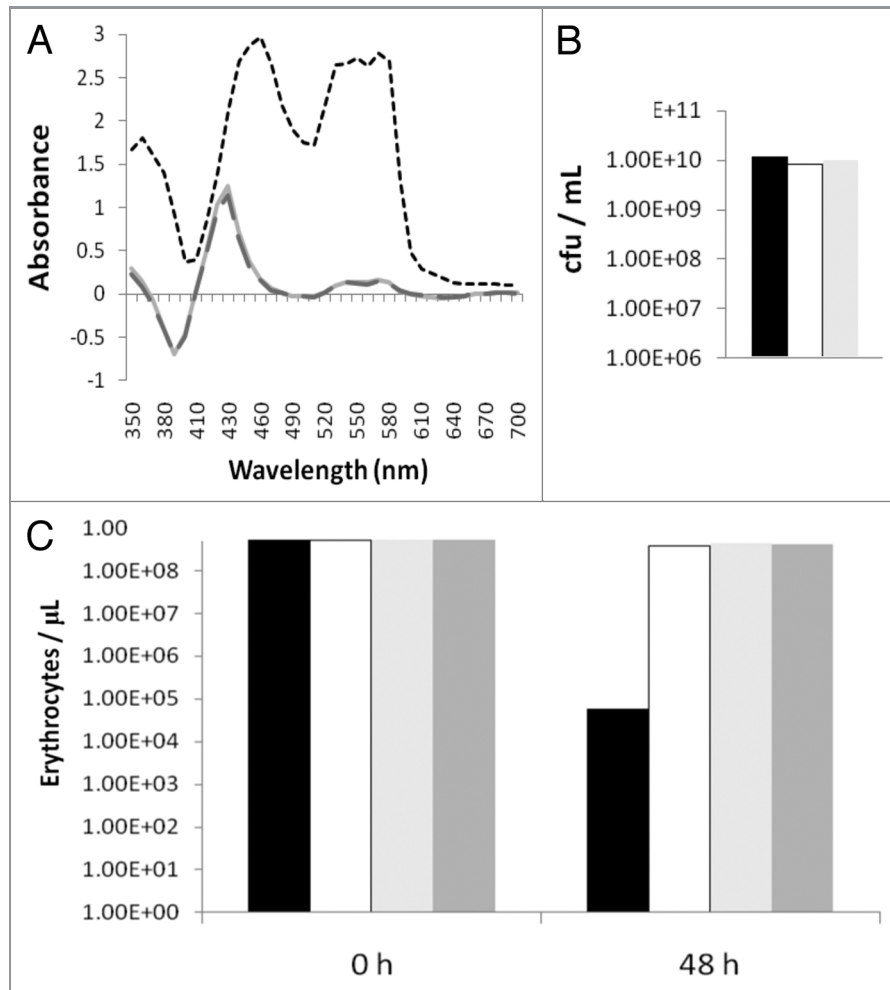
Figure 3. (A) Absorbance of blood broth supernatants across a range of wavelengths after 24 h incubation with wt DBO1 and two NRPS cluster mutants. Dotted black line, wild-type DBO1; solid gray line, *6466*::oriTp^R (*luxR* mutant); dotted gray line, *6477*::oriTp^R (polyketide synthetase mutant). (B) Viable plate counts after 24 h. Black, wild-type DBO1; white, *6466*::oriTp^R; gray, *6477*::oriTp^R. (C) Erythrocyte counts. Black, wild-type DBO1; white, *6466*::oriTp^R; light gray, *6477*::oriTp^R; dark gray, blank control (no bacteria added).

cells, the results illustrated in **Figure 5** suggest that the hemolytic NRPS product is not simply a hemolysin sensu stricto, but rather a cytotoxin, possibly active against several cell types including fungi (as demonstrated for *B. contaminans* MS14 occidofungin/burkholdine), insects, amoebae and higher organisms. To further this hypothesis, Bcc strains exhibiting NRPS hemolytic activity, as well as isogenic mutants defective in hemolysin production, were tested in a *Dictyostelium discoideum* feeding infection model.[40] We were unable to detect differences in virulence toward grazing *D. discoideum* in Bcc strains either producing or not producing the NRPS toxin/hemolysin. We also observed that constructed mutant *6471*::*Tp^R*, which is unable to produce the NRPS toxin glycosyltransferase, and that produced intermediate levels of hemoglobin release in the liquid hemolysis assay, did not produce intermediate levels of death in the *G. mellonella* infection model as compared with wild-type DBO1 and NRPS structural mutants such as *6477*::*Tp^R* (data not shown). Instead, *6471*::*Tp^R* behaved similarly to wild-type NRPS toxin-producing strains and produced wild-type levels of *G. mellonella* death.

Prevalence of the NRPS gene cluster in the Bcc species. In order to determine the prevalence of occidiofungin/burkholdine-like compounds across members of the Bcc, we screened all available Bcc isolates in our library using PCR primers designed from homologous regions between known sequences of *B. contaminans* MS14 and *B. ambifaria* AMMD. The primers were also designed to amplify the regions within these genes showing no homology to other sequenced genes. Of 54 isolates screened, 13 yielded PCR products for at least two of the primer sets, and of those 13, ten yielded products in all three primer sets (**Table 2**). Using the same assay as described above, hemolysis of human erythrocytes by each of the 13 strains was investigated. Of the ten Bcc strains positive for all three PCR products, seven of them demonstrated high levels of hemolytic activity against human erythrocytes. Of the three Bcc strains positive for only two of the three PCR products, only one of these exhibited any hemolytic activity, and this was low level of activity (**Fig. 6**). In addition, analysis of other *Burkholderia* genomes currently available in the GenBank database indicate that no additional
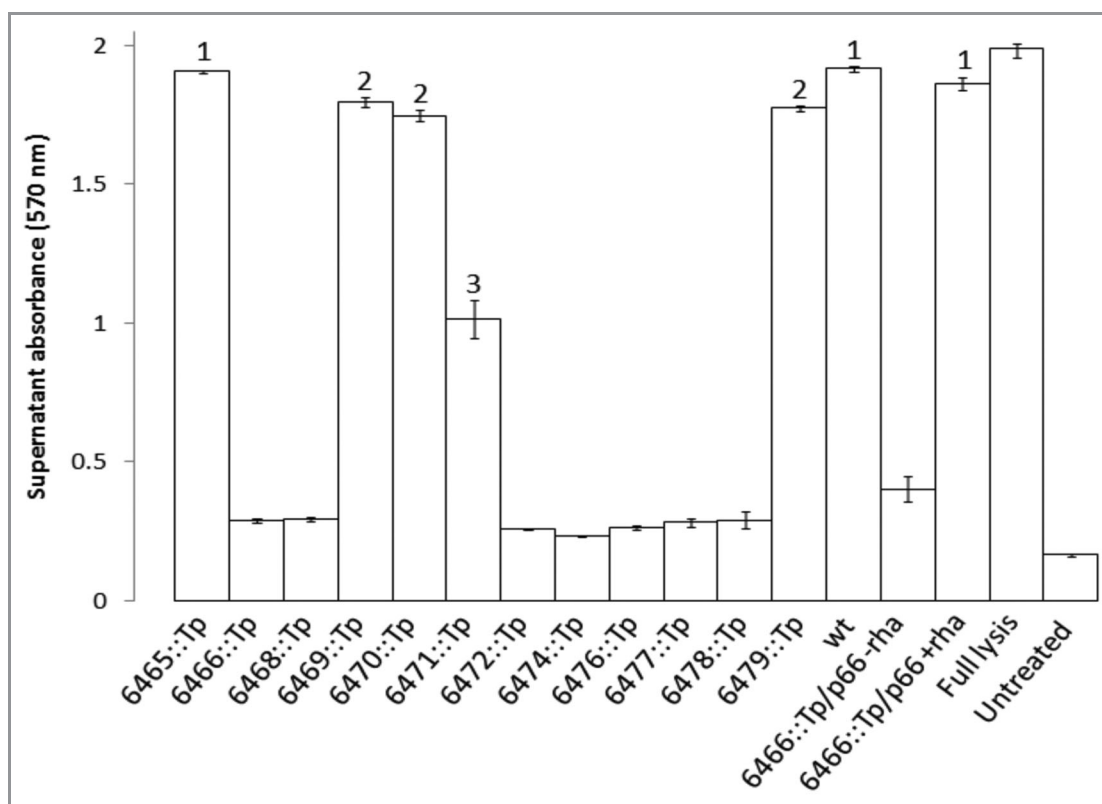
Figure 4. Lysis of human erythrocytes by *B. vietnamiensis* DBO1 requires the intact NRPS cluster, including biosynthetic genes, Lux regulators and transporter. Supernatant absorbance (570 nm) was taken of each strain incubated in TSB + 5% human erythrocytes after shaking at 30°C for 48 h. Wt = wild type; *6466*::Tp^R/p66 −rha and *6466*::Tp^R/p66 +rha = complemented mutant *6466*::Tp^R/pSCRhaTc-*6466*^{His} incubated with and without 0.2% rhamnose, respectively. Error bars denote standard deviation around the mean from three independent trials performed in triplicate. Means labeled with different digits (1–3) are statistically different (p < 0.05, n = 3).

Bcc genomes (n = 8) contain NRPS genes or gene clusters similar to those identified in AMMD, DBO1 or MS14 (**Table 2**). It is important to note that the apparent presence of these NRPS genes is required for hemolytic activity but does not guarantee its production, possibly due to small, acquired mutations in the NRPS gene cluster in some Bcc strains. Overall, hemolytic or toxic activity produced by an NRPS-derived occidiofungin/burkholdine-like compound appears to be limited to the Bcc species *B. ambifaria*, *B. contaminans*,[32,33] *Burkholderia pyrrocinia* and *B. vietnamiensis*.

Discussion

Bcc species *B. contaminans* MS14 produces cellular toxins that are synthesized using a non-ribosomal peptide synthase pathway, which is characteristic of complex secondary metabolite compounds. These compounds, known as occidiofungins or burkholdines, have been previously shown to have antifungal activity,[32,33] but herein we show that these compounds, or compounds closely related to occidiofungins/burkholdines (based partially on biosynthetic gene cluster similarities), also possess high levels of hemolytic activity. Besides individual ORF homology and syntenic organization between the gene clusters in MS14 and AMMD/DBO1, the hemolysis results from the glycosyltransferase

mutant in DBO1 suggest that the NRPS-derived compounds from the three Bcc strains are similar. Prior structural analysis of the MS14 NRPS compound suggests that the ring peptide can be glycosylated by xylose, and that without this glycosylation, the antifungal properties of occidiofungin/burkholdine are lost. However, genetic inactivation of *B. vietnamiensis* DBO1 NRPS gene cluster homolog *6471*, encoding the putative glycosyltransferase, does not eliminate hemolytic activity completely, but does reduce hemolytic activity by approximately half, whereas in *G. mellonella*, the unglycosylated compound still exhibits wild-type bacterial toxicity. This suggests that the unglycosylated form of the occidiofungin/burkholdine-like compound is fully toxic towards some organisms such as in insects, but only partially active against other cell types, including erythrocytes and fungal cells. Because constructed mutant *6471*::Tp^R does not produce intermediate levels of killing in the *G. mellonella* infection model as compared with wild-type DBO1 and NRPS structural mutants, this suggests that the organismal assays used to test toxicity are less sensitive than the hemoglobin release cellular lysis assay.

To examine whether other Bcc strains possess the NRPS gene cluster, PCR primer sets localized to unique sequences were used to probe for the presence of two NRPS genes. This analysis identified 13 of 55 Bcc strains potentially carrying the NRPS gene cluster. Further analysis with another unique PCR primer pair
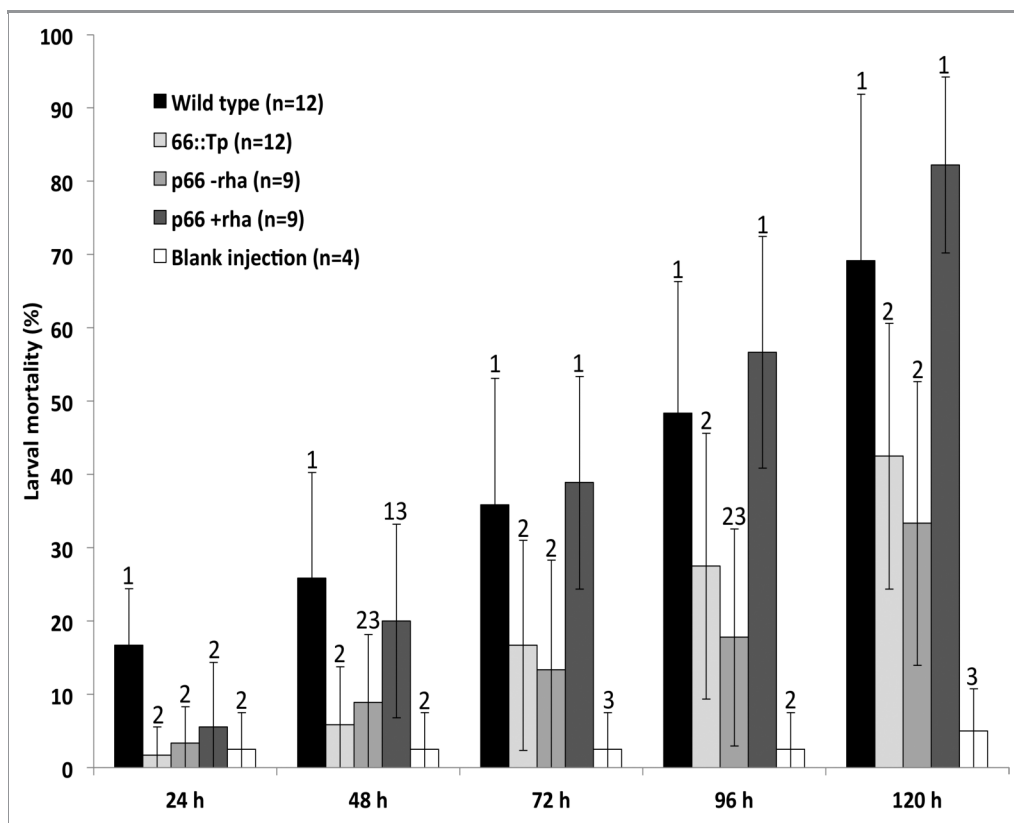
Figure 5. DBO1 requires a LuxR homolog regulator for virulence against *Galleria mellonella* larvae. Five microliters of bacterial suspension, corresponding to 5×10^6 colony forming units, was inoculated into 10 larvae in n independent trials. The infected larvae were incubated at 30°C and viability counts were taken at 24 h intervals. [66TpR = strain *6466*::TpR; p66 = *6466*::TpR carrying complementation vector pSCRha-*6466His*]. All results are means shown +/− SD. Means labeled with different digits (1–3) are statistically different (p < 0.01).

reduced this number to ten strains that carried three NRPS genes. Upon functional testing, only seven of these ten strains exhibited hemolysis of human erythrocytes, suggesting that two strains (*B. pyrrocinia* LMG 14294 and *B. vietnamiensis* LMG 10929) possessed undefined mutations to the hemolytic gene cluster. In addition, only two PCR products were amplified from *B. ambifaria* CEP0996; however, this strain exhibited hemolytic activity in this assay. This suggests that either one set of primer pairs was degenerate from the CEP0996 genomic sequence to allow amplification of the NRPS ortholog from this strain even though a functional protein was expressed, or that this strain produces a hemolytic toxin different from occidiofungin/burkholdine. In terms of prevalence, it is interesting to note that this NRPS gene cluster has not been identified in the two most clinically important Bcc species, *Burkholderia multivorans* and *B. cenocepacia*. Although these two species are found both as environmental and clinical isolates, and clinical Bcc strains can exhibit relatively little difference from strains found in the environment,[27] the identification of this NRPS gene cluster in Bcc species better adapted to soil environments than in association with humans suggests that this gene cluster evolved to protect the Bcc from ecological niche predators such as fungi and amoeba rather than as a virulence factor to assist invasion of human tissue.

The literature on NRPS and PKS systems indicates that associated dedicated glycosyltransferases are not uncommon. Although the putative xylose moiety appears to enhance hemolytic activity of the Bcc NRPS-derived compound, the exact mechanism through which this is achieved awaits further investigation. Walsh et al.[41] describes a possible function of such glycosyltransferases as having a role in imparting polarity or solubility to hydrophobic compounds, thus improving their access into target cell surfaces. The results presented in this and previous publications on occidiofungin/burkholdine[32,33] suggest that glycosylation is important for full toxicity of the compound.

Several key questions remain with respect to the function of some ORFs found within the NRPS cluster. For example, gene *6470* downstream of the glycosyltransferase *6471* encodes a hypothetical protein that upon mutagenesis, appears to have no effect on hemolytic or toxic activity. Gene *6467*, overlapping the two *luxR* homolog ORFs, is a small gene encoding a potential product of only 110 amino acids, with no homology to any known protein. This may be a truncated ORF that was introduced along with the upstream *luxR* family transcriptional regulator gene *6468* during acquisition and assembly of the NRPS cluster. Finally, gene *6475*, situated between two large genes encoding proteins with amino acid adenylation domains, encodes a 538 amino acid protein that contains a β-lactamase

Table 2. Bacterial strains analyzed in this study

Species	Strain	PCR products	Hemolysis	Source/info
B. cepacia	ATCC25416^T	ND	NT	Onion rot
B. cepacia	LMG18821	ND	None	CF isolate
B. cepacia	ATCC17759	ND	None	Soil
B. cepacia	CEP521	ND	None	CF isolate
B. multivorans	LMG13010^T	ND	None	CF isolate
B. multivorans	ATCC17616	ND	None	Anthranilate enrichment
B. multivorans	PC249–2	ND	None	ATCC17616 mutant
B. multivorans	C5393	ND	None	CF isolate
B. multivorans	C3430	ND	None	CF isolate
B. multivorans	C5274	ND	None	CF isolate
B. multivorans	C5568	ND	NT	CF isolate
B. multivorans	CGD1	ND*	NT	CGD isolate
B. multivorans	CGD2	ND*	NT	CGD isolate
B. cenocepacia	J2315^T	ND$^{(*)}$	None	CF isolate
B. cenocepacia	K56–2	ND	None	CF isolate
B. cenocepacia	PC184	ND	NT	CF isolate
B. cenocepacia	715j	*6472*	None	CF isolate
B. cenocepacia	K63–3	*6472*	NT	CF isolate
B. cenocepacia	C1257	ND	None	CF isolate
B. cenocepacia	C4455	ND	None	CF isolate
B. cenocepacia	C5424	ND	NT	CF isolate
B. cenocepacia	C6433	ND	None	CF isolate
B. cenocepacia	BC7	ND	None	CF isolate
B. cenocepacia	CEP511	ND	None	CF isolate
B. cenocepacia	AU1045	ND*	NT	CF isolate
B. cenocepacia	HI2424	ND*	NT	Soil rhizosphere
B. cenocepacia	MC0–3	ND*	NT	Soil rhizosphere
B. cenocepacia	D1	ND	NT	Environmental isolate
B. stabilis	LMG14294^T	*6472, 6474, 6476*	None	CF isolate
B. stabilis	LMG18870	ND	None	CF isolate
B. vietnamiensis	LMG10929^T	*6472, 6474, 6476*	None	Soil rhizosphere
B. vietnamiensis	DB01	*6472, 6474, 6476*	High	Phthalate enrichment
B. vietnamiensis	LMG18835	*6472, 6474, 6476*	High	CF isolate
B. vietnamiensis	G4	ND$^{(*)}$	NT	Trichloroethene enrichment
B. dolosa	LMG18943^T	ND	None	CF isolate
B. dolosa	L06	ND	NT	CF isolate
B. dolosa	AU0645	ND	NT	CF isolate
B. dolosa	STM1441/LMG21443	ND	None	Soil rhizosphere
B. dolosa	CEP021	ND	NT	CF isolate
B. dolosa	E12	ND	NT	CF isolate
B. ambifaria	AMMD^T	*6472, 6474, 6476*	High	Soil rhizosphere
B. ambifaria	CEP0996	*6472, 6474, 6476*	High	CF isolate
B. ambifaria	LMG17828	*6472, 6474, 6476*	None	Soil
B. ambifaria	M53	*6472, 6474, 6476*	High	Soil
B. pyrrocinia	LMG14191^T	*6472, 6474*	Low	Soil rhizosphere

Table 2. Bacterial strains analyzed in this study (continued)

Species	Strain	PCR products	Hemolysis	Source/info
B. pyrrocinia	LMG21822	*6472, 6474, 6476*	Moderate	Soil
B. pyrrocinia	LMG21823	*6472, 6474, 6476*	None	Water
B. pyrrocinia	LMG21824	*6472, 6474, 6476*	High	CF isolate
B. anthina	W92[T]	ND	NT	Soil rhizosphere
B. anthina	J2552	ND	NT	Soil rhizosphere
B. anthina	C1765	ND	NT	CF isolate
B. anthina	AU1293	*6472*	NT	CF isolate
B. ubonensis	Bu	ND*	NT	Soil
B. contaminans	MS14	*6472, 6474, 6476**	NT	Soil
B. lata	383	ND*	NT	Soil
Burkholderia sp	JS150	ND	NT	*p*-dichlorobenzene enrichment
R. pickettii	ATCC27511[T]	ND	NT	Patient isolate
R. pickettii	YH105	*6472, 6474*	None	*p*-nitrobenzoate enrichment

Superscript "T" following the strain name denotes the Type strain of each species. ND, not detected. NT, not tested. Asterisks indicate the result was obtained using genomic analysis. CF, cystic fibrosis; CGD, chronic granulomatous disease.

domain. Rather than providing cellular resistance to antibiotics, it is likely that this protein is somehow involved in modifying the cyclic peptide structure, as all homologs to this particular protein are associated with NRPS and PKS gene clusters. Because we were unable to mutagenize gene *6475*, a definitive function for the associated protein remains to be assigned. All other genes in the strain DBO1 NRPS gene cluster are essential for hemolytic activity.

Given the interest in developing the Bcc NRPS-derived compound known as occidiofungin or burkholdine as an antifungal agent for agricultural (crop preservation and protection) or pharmaceutical use, it is important to note its toxic and hemolytic activities. Clearly, the development of biological-based fungicides should include extensive analysis of their toxic properties. A previous study[33] has noted that occidiofungin/burkholdine produces aberrant cell membrane morphology in fungi similar to what has
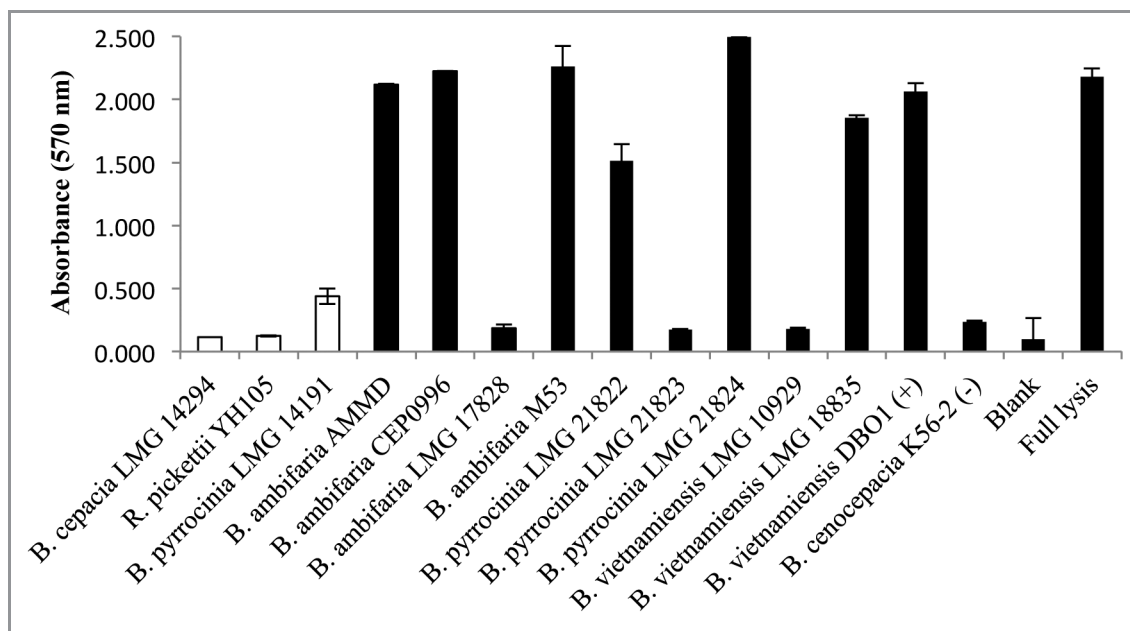

Figure 6. Human erythrocyte lysis generally requires the presence of the NRPS cluster in *B. cepacia* complex isolates. Forty-seven Bcc strains, one untyped *Burkholderia* species, and two *R. pickettii* strains and were screened using PCR primers for three of the non-ribosomal peptide biosynthesis genes within the cluster. Of the 50 strains, 13 were positive for at least two of the genes, and 10 were positive for all three genes tested. White columns = strains positive for two of the genes, black columns = strains positive for all three genes tested. A_{570} of the supernatants were taken after 48 h incubation of the strains in TSB + 5% human erythrocytes in triplicate. Error bars = +/− SD of triplicates.

been described for the echinocandin class of antifungal compounds, which disrupt β-glucan polymerization. A recent study[42] describes occidiofungin's in vitro potency against *Candida* species, as well as its chemical stability in the presence of human serum. This finding suggests that if occidiofungin/burkholdine displays pathogenic traits against humans, the compound may resist degradation within the body. Furthermore, a toxicological evaluation of occidiofungin is described where B6C3F1 mice were given a single dose of occidiofungin up to 20 mg/kg body weight or a daily dose for 5 d at 2 mg/kg body weight. Histological examination of treated mice did not identify organ toxicity; however, overall effects were a reduction in body and organ weights, and anemia or neutropenia were not tested. Our results suggest that the Bcc occidiofungin/burkholdine-like cyclic peptide targets and disrupts components of the membranes of eukaryotic cells (but not prokaryotic cells), especially erythrocytes, potentially binding to cholesterol or another cell scaffolding carbohydrate component. Future experiments will be aimed at further investigation of Bcc NRPS-derived hemolysins and their mechanism of action against erythrocytes.

Materials and Methods

Bacterial strains, plasmids, antibiotics and culture conditions. Luria-Bertani (LB) broth was prepared in distilled water to half concentration for Bcc strains and full concentration for *Escherichia coli* strains. Sheep, horse, bovine and rabbit blood broth (Dalynn Biologicals) and human blood broth (Canadian Blood Services) were prepared to 5% v/v blood in trypticase soy broth (TSB). Agar plates were prepared with 1.5% w/v agar. Antibiotics were included where necessary to the following concentrations: ampicillin (amp), 100 μg/mL; trimethoprim (Tp), 100 μg/mL; tetracycline (Tc), 100 μg/mL. All antibiotics were purchased from Difco through BD Canada. *E. coli* DH5α was used for plasmid manipulation. *E. coli* strains were grown at 37°C, while DBO1 was grown at 30°C. All liquid cultures were shaken at 225 rpm. High-copy number plasmid pJET (Amp^R) was employed in cloning experiments. For random plasposon mutagenesis, pTn*Mod*-OTp' (Tp^R) was used.[38] To complement gene *6466*, pSCRhaB2 was used.[43] A list of bacterial strains and plasmids used or constructed is shown in **Table 1**.

Random plasposon mutagenesis. A random plasposon insertion mutant library was created in *B. vietnamiensis* DBO1 using plasposon pTn*Mod*-OTp' as previously described.[38] Cells containing integrated plasposons were selected on 1/2 LB + 100 μg/mL trimethoprim. Twenty thousand mutants were patched onto 5% sheep blood TSA agar, incubated at 30°C and observed for hemolysis after 48 h. Mutants exhibiting loss of hemolysis were carried forward and their plasposon insertion sites were isolated as previously described.[38] To ensure that the random plasposon mutants obtained carried authentic mutations responsible for the observed phenotype, plasposon site of insertion plasmid clones were reintroduced into wild-type DBO1, and following recombination and selection on 1/2 LB + 100 μg/mL trimethoprim, and PCR analysis to ensure proper integration, the reconstructed mutants were again tested for hemolytic activity on 5% sheep blood TSA agar.

Targeted mutagenesis. Once the NRPS cluster had been identified through random plasposon mutagenesis, mutations in the remaining genes within the cluster were created through a targeted insertion mutagenesis strategy using homologous recombination. Primers were designed to amplify open reading frames (ORFs) and XbaI sites at either end. A full list of primers is shown in **Table 3**. PCR amplification was performed using TopTaq (Qiagen Inc.). In many cases, because of imperfect local homology between the AMMD and DBO1 sequences, techniques were used to enhance primer binding in order to obtain PCR products. DNA bands were extracted from agarose gel using GeneClean (Fermentas), ligated to pJET1.2 (Fermentas) and cloned in *E. coli* DH5α (Invitrogen Corp.), and DNA sequencing of the cloned fragments was performed to confirm the identity of the product. To generate plasmids containing insertions, the OriTp^R region was extracted from pTn*Mod*-OTp' by digestion with BglII and XbaI, ligated to the PCR products using T4 ligase (Promega Corp.) and the three-way ligation product was

Table 3. Oligonucleotides used in this study

Oligonucleotides	Sequence
Mutagenesis*	
m6465–1	GGTTCGACATTCTGACGTT
m6465–2	CCTCTATGTGCCGAACAG
m6466–1	AGAGTCAGATGTTCGCGAAG
m6466–2	GTCTCGACCGTGCTTTCC
m6470–1	AAGCGGCGTTCGTCAAGTC
m6470–2	AGGTGGCTGAGTTCGACATTG
m6471–1	AAGGTCTGCATCAATCTGG
m6471–2	AGGGAATAGGTCAGCGGC
m6477–1	GCCGTTCTGCAACTACATCC
m6477–2	AGGCGGTCGGTCAGTTCG
m6479–1	AT**GGTACC**GGCGTCCTTCGAATC
m6479–2	AT**AAGCTT**GGCAGACGTCGGGTT
Complementation*	
c6466–1	ATAATA**CATATG**<u>CATCATCACCATCACCAC</u>GTT-CGCGAAGCTTG
c6466–2	AT**TCTAGA**CTACGCCGCCGACGCGCAC
Sequencing	
pJET-F	CGACTCACTATAGGGAGAGCGGC
pJET-R	AAGAACATCGATTTTCCATGGCAG
pBBR-F	AACAGCTATGACCATG
pBBR-R	AATACGACTCACTATAG
Bcc cluster screening	
s6472–1	TACATGCTCGACGACGCGCT
s6472–2	ATGTTGTAGGTGGCCGACGGG
s6474–1	TCTCGACCAGCGGGCAATACC
s6474–2	TCCTCGATCATGAAGCGCAG
s6476–1	AAGGTCACGTGGTTCGGCTCG
s6476–2	ATTTCGCGGACCAGTTCGGC

*Restriction sites shown in bold. 6x histidine tag underlined.

cloned into *E. coli* DH5α, with selection of transformants on LB + 100 μg/ml Tp. OriTp-insert plasmids were subsequently extracted from their DH5α hosts, electroporated into *B. vietnamiensis* DBO1, and transformants were selected on ½ LB + 100 μg/ml Tp. Mutations were confirmed by PCR and sequencing using previously described methods.[38]

Genetic complementation. To restore the mutated hemolysis phenotype to DBO1 mutant *6466::Tp^R*, we generated a PCR product of *B. ambifaria* AMMD gene *6466*, a putative LuxR regulator of the NRPS gene cluster, and cloned this into the rhamnose-inducible plasmid pSCRhaB2[43] modified with a tetra-cycline cassette from p34S-Tc[38] with SmaI and ligated into the unique EcoRV site of pSCRhaB2. The *6466^His* PCR construct was amplified with an N-terminal 6x histidine tag and NdeI and XbaI sites at the 5' and 3' termini (**Table 3**), respectively, and inserted into the NdeI and XbaI sites of pSCRhaB2 following its NdeI + XbaI digestion and purification from a 0.8% agarose gel using GeneClean. Triparental mating was performed to transform mutant *6466::Tp^R* with the plasmid; meanwhile, wild type DBO1 and *6466::Tp^R* were similarly transformed with a blank version of the same Tc^R-carrying plasmid, produced by digestion with NdeI + XbaI, purification with GeneClean, digestion with Mung Bean exonuclease (Promega Corp.) and self-ligation, to control for the physiological effects of the vector. Fifty pSCRhaTc-*6466^His* transformants were screened on blood agar with and without 0.2% rhamnose to identify transformants expressing hemolysin; of these, approximately half showed rhamnose-dependent hemolytic activity. Two complemented mutants were carried forward, and one of these was used in virulence experiments.

Liquid hemolysis assay. To quantitatively compare hemolytic activity produced by *B. vietnamiensis* DBO1 with that produced by mutants of the NRPS cluster constructed in DBO1 (and subsequently, other strains exhibiting positive PCR results for genes *6472*, *6474* and *6476*), a liquid hemolysis assay was developed. DBO1 and mutants were grown in 2 mL sheep blood broth for 48 h in duplicate. One milliliter of supernatant was assayed at 24 h and 48 h and absorbance was measured using an Ultrospec 3000 (Pharmacia Biotech) at a range of wavelengths from 350 nm to 700 nm (**Fig. 3**). Viable bacterial plate counts were obtained by serial dilution and plating on ½ LB and red blood cell counts were taken using a Bright-Line Hemacytometer (Hausser Scientific). This method measured released heme at the optimal wavelengths of 540 nm and 570 nm. A higher-throughput assay was then developed based on these results. The strains were grown for 24 h in four separate 200 μL TSB with appropriate antibiotics in a Costar® 3599 96 Well Culture Cluster (Corning Inc.). Complemented mutant *6466::Tp^R*/pSCRhaTc-*6466^His* was grown with and without 0.2% rhamnose. OD$_{600}$ was taken of all cultures, and three cultures falling within 10% of OD$_{600}$ = 1.1 were selected for each strain; cultures of mutant *6473::Tp^R* did not grow within this cut-off, so this strain was not used in the experiment. Five microliters of each cell suspension was added to quadruplicates of 200 μL 5% sheep blood in TSB in 96-well plates and incubated with shaking at 30°C; to reduce evaporation from wells, plates were plastic-wrapped. After 48 h, 180–200 μL was removed from each well to a microfuge tube and centrifuged at 1,000x g for 15 min. Carefully, avoiding any cell pellet, 140 μL of each supernatant was removed into a 96-well plate and OD$_{570}$ was measured. Averages of quadruplicates for each trial were taken and each quadruplicate average statistically represented 1 *n*. Student's t-tests were performed to determine statistical significance; to determine t-test type, an F-test was performed for each comparison.

Wax moth larva killing assay. To compare in vivo virulence of wild-type *B. vietnamiensis* DBO1 with NRPS cluster gene insertion mutants, *G. mellonella* (greater wax moth) larvae were infected. The standard protocol from Seed and Dennis[44] was followed with several adjustments. Bacterial inoculums used were 5×10^6 c.f.u. and were OD$_{600}$ standardized prior to injection. Ampicillin was omitted from the buffer due to its inhibitory activity against DBO1; to compensate for the lack of antibiotic, larvae were dipped in ethanol and dried on paper towel prior to injection. DBO1/pSCRhaTc, *6466::Tp^R*/pSCRhaTc and *6466::Tp^R*/pSCRhaTc-*6466^His* were grown at 37°C for 24 h on LB agar + Tp + Tc + 0.2% rhamnose, and an additional *6466::Tp^R*/pSCRhaTc-*6466^His* culture was grown without rhamnose for reduced *6466* expression. All strains except *6466::Tp^R*/pSCRhaTc-*6466^His* grown without rhamnose were injected with 0.4% rhamnose added to the 10 mM MgSO$_4$ suspension. The control injections included 10 mM MgSO$_4$, Tp, Tc, and 0.4% rhamnose. Injections were spread over a period of 4 d. Separate cultures were grown, suspended in buffer, and diluted to OD$_{595}$ = 0.170 (200 μL in a 96-well plate) for each set of 10 larvae to be injected, with each set of 10 larvae statistically representing 1 *n*. Infected larvae were then incubated at 30°C for 120 h. Mortality data were analyzed statistically by a Student's t-test; to determine t-test type, an F-test was performed for each comparison.

Prevalence of the hemolysin NRPS cluster in the Bcc. To determine the prevalence of the NRPS cluster among clinical and environmental Bcc isolates, 47 Bcc strains, one untyped *Burkholderia* species and two *Ralstonia picketii* strains were screened using primers (**Table 3**) based on regions of homology between the known sequences of *B. contaminans* and *B. ambifaria* AMMD but displaying no homology beyond those known sequences. Genes corresponding to *B. ambifaria* strain AMMD genes *6472*, *6474* and *6476* were chosen for this screen because these appear to be three of the most significant NRPS bio-synthetic genes of the cluster, and their larger size offered more options for primers fitting the above criteria. Colony PCR was performed on each isolate and the PCR products were separated on a 0.8% agarose gel. Strains exhibiting correctly sized products for at least two of the three primer pairs were analyzed further using the liquid hemolysis assay. An additional 10 strains were examined through bioinformatic comparison, although two of these strains had also been tested using PCR. Paired BLASTN[39] sequence analysis was performed using each of AMMD genes *6472*, *6474* and *6476* along with the GenBank deposited genome files, using default algorithmic settings. Overall, a total of 58 bacterial strains or isolates were tested for the presence of these NRPS genes, including 55 Bcc strains.

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