The *Burkholderia pseudomallei* hmqA-G Locus Mediates Competitive Fitness against Environmental Gram-Positive Bacteria

Sherry Mou,a Conor C. Jenkins,b,c Udoka Okaro,a Elizabeth S. Dhummakupt,c Phillip M. Mach,c David DeShazer*a

aFoundational Sciences Directorate, Bacteriology Division, United States Army Medical Research Institute of Infectious Diseases, Frederick, Maryland, USA
bExcet Inc., Springfield, Virginia, USA
cDEVCOM Chemical Biological Center, Aberdeen Proving Ground, Maryland, USA

ABSTRACT  *Burkholderia pseudomallei* is an opportunistic pathogen that is responsible for the disease melioidosis in humans and animals. The microbe is a tier 1 select agent because it is highly infectious by the aerosol route, it is inherently resistant to multiple antibiotics, and no licensed vaccine currently exists. Naturally acquired infections result from contact with contaminated soil or water sources in regions of endemicity. There have been few reports investigating the molecular mechanism(s) utilized by *B. pseudomallei* to survive and persist in ecological niches harboring microbial competitors. Here, we report the isolation of Gram-positive bacteria from multiple environmental sources and show that ~45% of these isolates are inhibited by *B. pseudomallei* in head-to-head competition assays. Two competition-deficient *B. pseudomallei* transposon mutants were identified that contained insertion mutations in the *hmqA-G* operon. This large biosynthetic gene cluster encodes the enzymes that produce a family of secondary metabolites called 4-hydroxy-3-methyl-2-alkylquinolines (HMAQs). Liquid chromatography and mass spectrometry conducted on filter-sterilized culture supernatants revealed five HMAQs and N-oxide derivatives that were produced by the parental strain but were absent in an isogenic *hmqD* deletion mutant. The results demonstrate that *B. pseudomallei* inhibits the growth of environmental Gram-positive bacteria in a contact-independent manner via the production of HMAQs by the *hmqA-G* operon.

IMPORTANCE *Burkholderia pseudomallei* naturally resides in water, soil, and the rhizosphere and its success as an opportunistic pathogen is dependent on the ability to persist in these harsh habitats long enough to come into contact with a susceptible host. In addition to adapting to limiting nutrients and diverse chemical and physical challenges, *B. pseudomallei* also has to interact with a variety of microbial competitors. Our research shows that one of the ways in which *B. pseudomallei* competes with Gram-positive environmental bacteria is by exporting a diverse array of closely related antimicrobial secondary metabolites.

KEYWORDS HMAQ, melioidosis, microbial competition

*Burkholderia pseudomallei*, *Burkholderia mallei*, and *Burkholderia thailandensis* are closely related species that display notable differences in pathogenic potential, host specificity, and environmental persistence (1, 2). *B. pseudomallei* and *B. mallei* are the etiological agents of melioidosis and glanders, respectively, and are tier 1 select agents in the United States due to their potential for misuse as biological weapons (3). While numerous mammalian species are susceptible to melioidosis (4, 5), naturally occurring glanders is most commonly associated with equines, felines, and humans (6–8). *B. mallei* is a host-adapted pathogen that does not persist in the environment for prolonged periods outside its animal host. *B. thailandensis*, on the other
hand, is an environmental saprophyte with poor pathogenic potential for animals and humans (9, 10).

Environmental survival is critical for *B. pseudomallei* and *B. thailandensis* as both reside in soil, water, and/or rhizosphere sources in tropical and subtropical regions around the world (11, 12). These ecosystems are composed of complex mixtures of microbial species and abiotic materials. To survive and persist in these environments, bacteria have evolved contact-dependent and contact-independent strategies to compete for finite resources (13, 14). Many Gram-negative bacteria utilize type VI secretion systems (T6SSs) as contact-dependent contractile nanomachines to puncture competitor bacteria and deliver toxic effector proteins (15–17). *B. thailandensis* employees a T6SS, termed T6SS-1, to compete with other bacteria, including *Pseudomonas putida*, *Pseudomonas fluorescens*, and *Serratia proteamaculans* (18). T6SS-1, also referred to as T6SS-6 (19), is conserved in *B. pseudomallei* and presumably plays a similar role in contact-dependent inhibition of Gram-negative bacteria. Both species also possess ~15 large biosynthetic gene clusters that produce a variety of small-molecule secondary metabolites that serve as siderophores, cytotoxins, antibiotics, and virulence factors (20–23). Many of these molecules mediate contact-independent interactions with microbial competitors, and some of them have been structurally and functionally characterized, including malleipeptin, syrbaatin, malleilactone, bactobolin, acybolin, thailandamide, thailandenes, malleobactin, pyochelin, terphenyl, isonitrile, and 4-hydroxy-3-methyl-2-alkenylquinolines (HMAQs).

There has been great interest in the HMAQs produced by *Burkholderia* sp. recently (21, 24–27). The *Burkholderia hmqA-G* gene cluster encodes the enzymes responsible for generating HMAQs from anthranilic acid and $\beta$-keto fatty acids (28–30). The biosynthetic pathway generates multiple HMAQ congeners that differ from one another based on the length of the unsaturated aliphatic side chains. The most abundant HMAQ congeners in *B. thailandensis* and *B. pseudomallei* are those with C9 side chains (30). N-Oxide derivatives of HMAQs, HMAQ-NOs, are generated by an unlinked gene (*hmql*) encoding a monooxygenase (25). Both HMAQs and HMAQ-NOs act as antimicrobial agents, but HMAQ-NOs tend to have enhanced inhibitory activity (25–27, 31, 32).

The goal of this study was to investigate the competitive interaction between *B. pseudomallei* and environmental bacteria at the molecular level. The results show that the molecules produced by the *B. pseudomallei hmqA-G* locus, including HMAQs and HMAQ-NOs with C7 and C9 unsaturated alkyl side chains, are solely responsible for contact-independent inhibition of Gram-positive bacteria isolated from diverse environmental sources.

**RESULTS**

**Isolation and characterization of environmental bacteria for competition studies.**

*B. pseudomallei* is present in soil, water, and the rhizosphere in many tropical regions around the world (33, 34), and it encounters microbial competitors in all of these diverse habitats. In an attempt to better characterize *B. pseudomallei* interactions with bacterial competitors at the molecular level, we isolated bacteria from diverse environmental sources, including soil (S), river water (R), river sediment (RS), stream water (ST), and the rhizosphere (RZ). Bacteria forming colonies that could be easily distinguished from *B. pseudomallei* colonies on solid medium due to morphology, pigmentation, and/or $\beta$-galactosidase production were selected for further characterization. Fifty-five distinct bacterial colonies were selected, and PCR products were generated from purified genomic DNA using universal 16S rRNA primers 533F (35) and 1492R (36). The nucleotide sequences of the partial 16S rRNA genes were used to search against the non-redundant nucleotide collection database using BLASTN (37), and the top nucleotide hits indicated that we isolated 34 Gram-positive bacteria and 21 Gram-negative bacteria (data not shown). In this study, we focused on the competitive interaction of *B. pseudomallei* with environmental Gram-positive bacteria, and the Gram-negative bacteria were not further characterized. Fourteen distinct Gram-positive bacterial species...
were identified in our collection, and their evolutionary relationships are shown in Fig. 1. There were five Bacillus species isolated from S, R, ST, and RZ, two Exiguobacterium species isolated from R and RS, two Paenibacillus species isolated from S, one Neobacillus species isolated from S, one Priestia species isolated from RS, one Cytobacillus species isolated from R, one Cellulosimicrobium species isolated from RS, and one Microbacterium species isolated from RS (Fig. 1).

*B. pseudomallei* outcompetes environmental Gram-positive bacteria in a contact-mediated competition assay. Competition between bacterial species is multifaceted and can be mediated by contact-independent mechanisms, such as the export of antibiotics and siderophores, or by contact-dependent interactions such as T6SS and contact-dependent growth inhibition (CDI) (14). A qualitative and quantitative contact-mediated bacterial competition assay (38) was employed to assess the ability of *B. pseudomallei* to compete with the 14 environmental Gram-positive bacteria. Briefly, *B. pseudomallei* and environmental competitors were grown in liquid broth and diluted to ~1 × 10⁷ CFU/ml, and 10-μl aliquots of each were spotted onto the surface of LB agar or LB agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Fig. 2A). Twenty-microliter aliquots of 1:1 mixtures of *B. pseudomallei* and environmental competitors were also spotted onto the solid medium and incubated for 48 h at room temperature (RT). *B. pseudomallei* is not pigmented and does not produce β-galactosidase, and the colonies formed are off-white in color, whereas Gram-positive bacteria that are pigmented or β-galactosidase positive form colonies that are orange, yellow, or blue (Fig. 2). A qualitative observation of bacterial competition can be assessed by looking at the relative color of the *B. pseudomallei* plus competitor mixtures compared to that of the competitors grown solitarily (Fig. 2A). A competition mixture that is off-white in color indicates that *B. pseudomallei* inhibited the competitor, and a competition mixture that is pigmented or blue in color indicates that the competitor was not inhibited, or only partially inhibited, by *B. pseudomallei*.

The competition assay can also be quantitated by resuspending the bacterial spots in phosphate-buffered saline (PBS), performing serial dilutions, and spreading aliquots onto agar plates. The quantity of bacterial competitors present in the competition mixtures can be assessed by enumerating the number of *B. pseudomallei* off-white colonies and comparing it to the number of Gram-positive pigmented or blue colonies.
The results of quantitative contact-mediated competition assays between *B. pseudomallei* Bp82 and the 14 environmental strains are shown in Table 1. The Bp82/environmental strain solitary growth ratio was compared to the Bp82/environmental strain mixed growth ratio and used to assess the fold difference between the two growth indices. *B. pseudomallei* Bp82 outcompeted *Neobacillus bataviensis* S4, *Bacillus velezensis* RZ8, *Bacillus licheniformis* S7, *Bacillus mycoides* R15, *Cellulosimicrobium* sp. strain RS17, and *Microbacterium* sp. strain RS16 but was outcompeted by *Cytobacillus firma* R14, *Bacillus marisflavi* S9, *Priestia megaterium* RS1, and *Paenibacillus polymyxa* S2. There was no significant competitive difference between *B. pseudomallei* Bp82 and *Bacillus cereus* ST9, *Exiguobacterium acetylicum* R10, *Exiguobacterium undae* RS4, and *Paenibacillus* sp. strain S10 (Table 1). The results show that 43% of the Gram-positive bacteria isolated from a variety of environmental sources are inhibited by *B. pseudomallei* in a contact-mediated competition assay. *N. bataviensis* S4 was used in further assays as a representative of the Gram-positive environmental species that were outcompeted by *B. pseudomallei*.

**N. bataviensis growth is inhibited by *B. pseudomallei* filter-sterilized supernatant.** While a contact-mediated competition assay was employed as described above to assess the ability of *B. pseudomallei* to compete with Gram-positive environmental bacteria, it seemed plausible that contact-independent antimicrobial mechanisms might also be mediating the competitive fitness observed in this assay. To assess the importance of contact-independent growth inhibition, we compared the ability of *B. pseudomallei* Bp82 to compete with *N. bataviensis* S4 on solid medium (contact mediated) and in liquid medium (contact independent). The Bp82/S4 mixed growth ratio was

![Fig 2](image_url)
100-fold higher than the Bp82/S4 solitary growth ratio on agar medium (Fig. 3A) and 10-fold higher in broth medium (Fig. 3B). This finding suggests that B. pseudomallei inhibits N. bataviensis in a contact-independent manner. We next grew Bp82 to saturation in LB broth, filter sterilized the supernatant, and applied 5-ml and 10-ml aliquots to a lawn of S4 on LB agar with X-Gal. Figure 3C shows that after incubation, the growth of an S4 lawn was inhibited by the filter-sterilized supernatant. For comparison, a Bp82 colony was inoculated on the S4 lawn, and it produced a similar zone of antimicrobial activity around the colony (Fig. 3C). Three virulent B. pseudomallei strains, 1026b, K96243, and MSHR346, also produced zones of clearing around their colonies when grown on an agar lawn of S4 supplemented with X-Gal (see Fig. S1 in the supplemental material), suggesting that this is an inherent B. pseudomallei phenotype. Further support for contact-independent growth inhibition by a Bp82 filterable exported product(s) was obtained by coculturing Bp82 and S4 in broth cultures separated by a 0.22-mm filter (see Fig. S2A). The Bp82/S4 mixed growth ratio was 5-fold higher than the Bp82/S4 solitary growth ratio when the broth cultures were separated by a cell-impermeable filter (Fig. S2B). Taken together, the results suggest that B. pseudomallei exports a filterable antimicrobial compound(s) that mediates competition with N. bataviensis. It is important to emphasize that any growth inhibition observed when cells are in close proximity could be due to contact-dependent inhibition or contact-independent growth inhibition. The B. pseudomallei exported antimicrobial product(s) is likely more concentrated when the cells are grown in close contact on agar medium than when grown in broth medium, which may explain why there is enhanced antimicrobial activity in the contact-mediated competition assay (Fig. 3).

A transposon screen identifies the B. pseudomallei hmqA-G locus as the source of the exported antimicrobial compound(s) that inhibits environmental Gram-positive bacteria. B. pseudomallei Bp82 colonies export an antimicrobial product(s) into the extracellular milieu that eliminates the growth of N. bataviensis S4 (Fig. 3C) and this phenotype was exploited to develop a transposon screen to identify Bp82 mutants that cannot inhibit the growth of S4. The transposon used in the screen was TnMod-OKm′, a self-cloning mini-Tn5 derivative (39). Preliminary studies indicated that Bp82 transposon mutants incubated on a lawn of S4 for 24 h at RT produced zones of inhibition that were too large to allow efficient screening (see Fig. S3), and so incubation for 24 h at 37°C was used instead. Approximately 8,000 Bp82 transposon mutants were screened, and two, termed SMM1 and SMM2, did not exhibit antimicrobial activity.

### Table 1: Results of contact-mediated competition assays between B. pseudomallei Bp82 and Gram-positive environmental isolates

| Environmental isolate       | Bp82 outcomes in mixed culture | Outcompetes Bp82 in mixed culture | Fold differencea |
|-----------------------------|-------------------------------|----------------------------------|------------------|
| Neobacillus bataviensis S4  | X                             | 117 ± 3                          |                  |
| Bacillus velezensis R28     | X                             | 60 ± 18                          |                  |
| Bacillus licheniformis S7   | X                             | 111 ± 14                         |                  |
| Cytopacillus firmus R14     |                               | 14 ± 4                           |                  |
| Bacillus marisflavi S9      | X                             | 2 ± 0.4                          |                  |
| Bacillus cereus ST9         |                               | ND                               |                  |
| Bacillus mycoides R15       | X                             | 124 ± 43                         |                  |
| Priestia megaterium R51     |                               | 149 ± 25                         |                  |
| Exiguobacterium acetylicum R10 |                         | ND                               |                  |
| Exiguobacterium undae RS4   |                               | ND                               |                  |
| Paenibacillus polymyxa S2   |                               | 42 ± 2                           |                  |
| Paenibacillus sp. S10       |                               | ND                               |                  |
| Cellulosimicrobium sp. RS17 | X                             | 12 ± 3                           |                  |
| Microbacterium sp. RS16     | X                             | 51 ± 12                          |                  |

aFold difference between the Bp82/environmental isolate ratio when the bacteria are grown in mixed culture compared to when the bacteria are grown alone. Three independent pairs of cultures were performed for each Bp82-environmental isolate competition assay, and the results were recorded as the mean ± the standard deviation.

bND, no significant difference in competitive index in mixed growth relative to solitary growth.
activity against S4. The sites of the Tn\text{Mod-OKm}\textsuperscript{9} insertions in SMM1 and SMM2 were mapped to \textit{hmqA} and \textit{hmqD}, respectively (Fig. 4A). The \textit{hmqA-G} locus encodes biosynthetic enzymes involved in the production of 4-hydroxy-3-methyl-2-alkenylquinolines (HMAQs) and their N-oxide counterparts (HMAQ-NOs) in \textit{B. pseudomallei}, \textit{B. thailandensis}, and some members of the \textit{Burkholderia cepacia} complex (21, 23–25, 28, 30). Figure 4B shows that Bp82 produces a zone of inhibition on an S4 lawn, but SMM1 and SMM2 do not. Klaus et al. (25) recently reported that \textit{B. thailandensis} \textit{hmq} transposon mutants similarly exhibited diminished antimicrobial activity against \textit{Bacillus subtilis} 168, a bacterial strain utilized as a laboratory workhorse for decades (40).

In an attempt to further characterize role of the \textit{B. pseudomallei} \textit{hmqA-G} locus in competitive interactions with Gram-positive environmental bacteria, we constructed in-frame deletion mutations in \textit{hmqA}, \textit{hmqD}, and \textit{hmqF} in Bp82. The resulting mutants, termed Bp82 \textit{ΔhmqA}, Bp82 \textit{ΔhmqD}, and Bp82 \textit{ΔhmqF} (Table 2), did not produce zones of inhibition on an S4 lawn (Fig. 4C). The full-length \textit{hmqD} gene, which encodes a 3-oxoacyl-(acyl carrier protein) synthase, was cloned into the broad-host-range vector pBHR2 and conjugated to Bp82 \textit{ΔhmqD} for complementation studies. The Bp82 \textit{ΔhmqD} (pBHR2-hmqD)/S4 mixed growth ratio on agar medium was >1,000-fold higher than the Bp82 \textit{ΔhmqD} (pBHR2)/S4 mixed growth ratio, indicating that the \textit{ΔhmqD} mutation was complemented by \textit{hmqD} in trans and does not have a polar effect on downstream genes (Fig. 5). Importantly, the \textit{hmqA} and \textit{hmqF} mutations were also complemented by providing the corresponding wild type genes in trans on pBHR2 (see Fig. S4), indicating that these deletion mutations did not have polar effects on downstream genes. The results suggest that the only \textit{B. pseudomallei}-exported antimicrobial product(s) with activity against \textit{N. bataviensis} under these growth conditions is produced by the \textit{hmqA-G} locus. As demonstrated above, Bp82 was able to outcompete six environmental Gram-positive species in a contact-mediated competition assay (Table 1). We next conducted contact-mediated competition assays with Bp82 \textit{ΔhmqD} to examine the importance of the \textit{B. pseudomallei} \textit{hmqA-G} locus in providing a competitive advantage against the environmental isolates. Figure 6 shows that the Bp82/environmental isolate and Bp82 \textit{ΔhmqD/environmental isolate mixed growth ratios were dramatically different. The data clearly
demonstrate that Bp82 ΔhmqD cannot compete in a contact-mediated competition assay with N. bataviensis, B. velezensis, B. licheniformis, B. mycoides, Microbacterium sp., or Cellulosimicrobium sp. (Fig. 6A to F). Taken together, the results indicate that the B. pseudomallei hmqA-G locus produces an antimicrobial product(s) that facilitates competition with Gram-positive bacterial isolates obtained from soil, river water, river sediment, and the rhizosphere.

**B. pseudomallei Bp82 supernatant contains HMAQ and HMAQ N-oxide derivatives that are absent in Bp82 ΔhmqD supernatant.** We next performed liquid chromatography-mass spectrometry on Bp82 and Bp82 ΔhmqD saturated broth culture supernatants to identify compounds associated with the antimicrobial activity of the former. Three 4-hydroxyl-3-methyl-2-alkenylquinolines (HMAQs) and two HMAQ N-oxides (HMAQ-NOs) were identified in the supernatant extract of Bp82, but were absent in Bp82 ΔhmqD (Fig. 7). The HMAQs differed from one another in the length of the unsaturated alkyl chains, including congeners with C7, C8, and C9 side chains (Fig. 7B, D, and E). The HMAQ-NOs identified were congeners with C7 and C9 unsaturated alkyl side chains (Fig. 7C and F). The production of these HMAQ congeners (family A) and HMAQ-NO congeners (family C) by B. pseudomallei 1026b was initially described in 2008 by Vial et al. (30). In a recent study, HMAQ and HMAQ-NO congeners with C7 and C8 alkyl side chains were chemically synthesized, and their antimicrobial activities against Gram-negative and Gram-positive bacteria were assessed (27). While both displayed antimicrobial activity against Gram-positive bacteria, the HMAQ-NO congeners displayed superior antimicrobial activity relative to that of the HMAQ congeners. The liquid chromatography-mass spectrometry (LC-MS) results demonstrate that the B. pseudomallei Bp82 hmqA-G locus produces at least five HMAQ and HMAQ-NO molecules that provide a competitive advantage against a variety of environmental Gram-positive bacteria in coculture growth assays.
**DISCUSSION**

Microorganisms inherently engage in exploitative competition (passive) and interference competition (active) in an attempt to survive and persist in diverse environmental niches (13, 14). Exploitative competition occurs when a microbe depletes the limiting nutrients in a shared niche, and interference competition involves a microbe damaging another microbe.
via contact-dependent or contact-independent mechanisms. Microbial competition assays are often employed to study these behaviors, and they commonly involve mixing two strains or species together and examining their relative survival under defined growth conditions (13, 14, 38, 41). Such experiments cannot replicate the natural environment of the competitors but can provide information about the bacterial factors that facilitate competition under specific laboratory conditions. There are relatively few published studies examining the ability of B. pseudomallei to compete with environmental bacteria. Ngamdee et al. reported that multiple B. pseudomallei strains inhibited the growth of B. thailandensis when cocultured on LB agar for 24 h at 37°C, but the inhibition only occurred when the B. pseudomallei/B. thailandensis ratio was $\geq 100:1$ (42). The mechanism of growth inhibition was not investigated but could involve CDI. B. pseudomallei strains contain an assortment of modular CDI systems that serve to inject protein toxins, often tRNases or DNases, directly into target bacteria, resulting in death in the absence of specific immunity proteins (43). Recent studies suggest that CDI systems are highly specific and probably only result in growth inhibition of closely related strains or species (44). While B. pseudomallei can potentially utilize CDI to target bacteria within the genus Burkholderia, it probably cannot be used to target more distantly related Gram-negative or Gram-positive bacteria. Lin et al. isolated Burkholderia cenocepacia and Burkholderia multivorans strains from soil in Taiwan that exported a soluble substance that produced zones of inhibition on a B. pseudomallei agar lawn (45). The antimicrobial substance was filterable but was not further characterized. Interestingly, the presence of B. cenocepacia and B. multivorans in agricultural crop soil was inversely correlated with the presence of B. pseudomallei, suggesting that these Burkholderia species might be antagonistic to B. pseudomallei in the soil (45).

In this study, we sought to examine how B. pseudomallei competes with environmental bacteria at the molecular level. We isolated Gram-positive bacteria from soil, river water, river sediment, stream water, and rhizosphere sources in the Middle Atlantic region of the United States. It is important to emphasize that these bacteria are not specific to this geographical region and have been isolated from diverse environmental sources worldwide (46–51). We employed a modified contact-mediated competition assay (38) to explore the relative competitive fitness of B. pseudomallei with 14 environmental Gram-positive bacteria. B. pseudomallei outcompeted 6/14 environmental bacteria in this assay, including N. bataviensis, B. velezensis, B. licheniformis, B. mycoides, Cellulosimicrobium sp., and Microbacterium sp. (Table 1 and Fig. 6). B. velezensis is a member of the “operational group of Bacillus amyloliquefaciens” due to the close relatedness of these species (52). In recent studies conducted in Thailand, six B. amyloliquefaciens isolates were obtained from soil samples that were devoid of B. pseudomallei, and it was hypothesized that the lack of B. pseudomallei might
correspond to the presence of antimicrobial compounds in this niche (53, 54). In further studies, the researchers found that *B. amyloliquefaciens* inhibited *B. pseudomallei* growth in a liquid coculture assay and that *B. amyloliquefaciens* supernatant contained an antimicrobial peptide that produced zones of inhibition on *B. pseudomallei* agar lawns (53, 54). These studies suggest that *B. amyloliquefaciens* exports secondary metabolites with antimicrobial properties into the surrounding environment that may prevent niche colonization by *B. pseudomallei*. In comparison, we found that *B. pseudomallei* outcompeted *B. velezensis* by 60-fold in an *hmqD*-dependent manner (Table 1 and Fig. 6B). Bacterial exported secondary metabolites, namely, *Bacillus* antimicrobial peptides or *Burkholderia* HMAQs and HMAQ-NOs, may be responsible for the different competitive fitness results reported in these studies. Variability in the secondary metabolites produced by the *B. pseudomallei* strains utilized seems unlikely given that both studies utilized 1026b and its isogenic derivatives (Table 2 and see Fig. S1 in the supplemental material).

**FIG 6** *B. pseudomallei* outcompetes environmental Gram-positive bacteria in an *hmqD*-dependent manner. Contact-mediated competition assays were performed with *B. pseudomallei* Bp82, Bp82 ΔhmqD, and *N. bataviensis* S4 (A), *B. velezensis* RZ8 (B), *B. licheniformis* S7 (C), *B. mycoides* R15 (D), *Microbacterium* sp. RS16 (E), and *Cellulosimicrobium* sp. RS17 (F). The fold difference between the *B. pseudomallei/environmental isolate ratio when the bacteria were grown alone (solitary growth ratio) or in mixed culture (mixed culture ratio) was used to establish the competitive indexes. The bacteria were incubated at RT for 48 h on solid medium, and the surviving competitors were quantitated. Three independent pairs of cultures were performed for each competition assay, and the results were recorded as the mean ± the standard deviation. NS, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
**B. pseudomallei** HMAQs Inhibit Gram-Positive Bacteria

Vol. 9 Issue 1 e00102-21 MicrobiolSpectrum.asm.org

FIG 7  *B. pseudomallei* Bp82 supernatant contains 4-hydroxy-3-methyl-2-alkenylquinolines (HMAQs) and their N-oxide counterparts (HMAQ-NOs) that are absent in Bp82 ΔhmqD supernatant. (A) Chromatograms of Bp82 and Bp82 ΔhmqD supernatant extracts. Chromatographic retention times for the

(Continued on next page)
material) (54). On the other hand, the results reported here may differ from those of the Sermswan laboratory (54) due to differences in the secondary metabolites produced by the *B. velezensis* and *B. amyloliquefaciens* strains used or in the competition assays employed. Future studies are warranted to address these possibilities.

The results presented here demonstrate that the secondary metabolites produced by the *hmqA-G* locus are critical for *B. pseudomallei* competition with environmental bacteria. There has been limited research on HMAQ and its derivatives in *B. pseudomallei* (29, 30), but much work has been conducted on these molecules in *B. thailandensis* (23, 25, 28, 31, 32). The *B. thailandensis hmq* genes were recently shown to be important for inhibiting the growth of *B. subtilis* 168, a legacy strain used to study *Bacillus* biology for decades (25, 40). *B. pseudomallei* and *B. thailandensis* produce a similar array of HMAQ and HMAQ-NO congeners, but species-specific differences also exist (30). The species variability is largely due to the relative amount of each congener produced, the length of the alkyl chains, and the presence or absence of unsaturation in the alkyl chains (30). The most common HMAQ and HMAQ-NO congeners produced by *B. pseudomallei* and *B. thailandensis* are those with unsaturated C₉ alkyl chains. Piochon et al. (27) chemically synthesized HMAQs and their N-oxide counterparts with unsaturated C₉, C₁₀, and C₁₁ alkyl side chains and evaluated their relative antimicrobial activity against nine bacterial species. HMAQs were not as effective as HMAQ-NOs, which exhibited superior activity against Gram-positive bacteria relative to that against Gram-negative bacteria (27). There is growing evidence that the diverse compounds produced by the *B. thailandensis hmq* genes may act synergistically to inhibit bacterial growth by acting on different targets (25, 32). Wu and Seyedsayamdost demonstrated that two of these molecules, 4-hydroxy-3-methyl-2-(2-nonenyl)-quinoline (HMNQ) and 2-heptyl-4(1'H)-quinoline N-oxide (HQNO), both inhibit pyrimidine biosynthesis by acting on a common target but interrupt the proton motive force by acting on different targets (32). They further speculated that antimicrobial resistance to the products of the *hmq* gene cluster may be difficult to attain due to the diversity of target mutations that would be required. In support of this notion, we never identified *N. bataviensis* resistant colonies emerge in areas of growth inhibition around any of the *B. pseudomallei* agar lawns examined during this research study (data not shown). These results suggest that the five *B. pseudomallei* HMAQ and HMAQ-NO congeners identified here (Fig. 7), and the closely related derivatives identified by others (30), may act synergistically to target environmental Gram-positive bacteria. It is currently unclear if all of the congeners, or only a few, are responsible for the growth inhibition we observed in the current study.

Klaus et al. found that *B. thailandensis* HMAQ and HMAQ-NO congeners were largely cell associated and that the molecules present in culture supernatants could not be passed through a 0.2-μm membrane filter (25). The *B. pseudomallei* HMAQ and HMAQ-NO congeners, on the other hand, were present in culture supernatants and were filterable (Fig. 3C and S2). In fact, the *B. pseudomallei* HMAQ and HMAQ-NO molecules prepared for LC-MS analysis were directly extracted from filter-sterilized culture supernatants. It is not known how HMAQ and HMAQ-NO are exported from bacteria, but it is possible that *B. pseudomallei* possesses a novel transporter that facilitates passage of these molecules out of the cell that is absent in *B. thailandensis*. This might result in fewer cell-associated HMAQs and HMAQ-NOs in *B. pseudomallei* and a higher proportion present in the supernatant. The transposon screen utilized in this study was low throughput, and a saturating transposon mutagenesis screen might be necessary to identify a putative *B. pseudomallei*-specific HMAQ transporter. It was recently shown that *B. thailandensis* outer

FIG 7 Legend (Continued)

compounds of interest are indicated by vertical arrows. Associated mass spectra for these retention times are shown in panels B to F. Fragmentation spectra and precursor mass structure of HMAQ-C₇:₁ (B), HMAQ-C₇:₁ N-oxide (C), HMAQ-C₈:₁ (D), HMAQ-C₉:₁ (E), and HMAQ-C₁₁:₁ N-oxide (F). The HMAQ derivatives shown in B to F all possess alkyl chains that are unsaturated with the double bond at position 1'. (G) Tabulated data identifying the molecular formula, weight, and chromatographic area counts associated with the mass of interest.
membrane vesicles (OMVs) have antimicrobial activity and contain HMAQ-C₉ (31). The purified OMVs ranged in size from 0.02 to 0.1 μm and should theoretically pass through a 0.2-μm membrane filter. B. pseudomallei also secretes OMVs of a similar size, but it is currently unclear if they package HMAQs (55). Further work will be necessary to determine why the molecules produced by the B. thailandensis hmq-encoded biosynthetic enzymes are not filterable while those produced by B. pseudomallei are.

In this study, we demonstrate that B. pseudomallei HMAQ derivatives are exported antimicrobials that are critical for competitive interactions with a variety of environmental Gram-positive bacteria. B. pseudomallei is an opportunistic pathogen that inherently resides in soil and/or water and can be transmitted to animals and humans via these contaminated sources. Bacterial survival and persistence in such niches require competition with complex microbial communities (13, 14). The B. pseudomallei results presented here, and those presented in a recent B. thailandensis study (25), indicate that both species rely on the hmqA-G and hmqL genes for competitive fitness against Gram-positive bacteria. Interestingly, the host-adapted pathogen B. mallei has lost the hmq genes and cannot persist for prolonged periods in the environment (30, 56). Taken together, the data support the notion that HMAQ and HMAQ-NO derivatives are important for survival and persistence in environmental sources containing complex microbial communities (21). There has been little information published about the importance of these molecules in B. pseudomallei virulence, but Price et al. (57) found that the hmqA-G genes were upregulated in chronically adapted cystic fibrosis (CF) patient isolates following growth in artificial CF sputum medium. Further studies will be necessary to determine if the products encoded by the hmqA-G locus are important for B. pseudomallei virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 2. Escherichia coli and B. pseudomallei K96243 (58), 1026b (59), and MSHR346 were grown at room temperature (RT) or 37°C on Luria-Bertani (LB) agar (Lennox formulation; Sigma-Aldrich) or in LB broth. One hundred micrograms per milliliter adenine HCl and 5 μg/ml thiamine HCl were added to solid and liquid media for growth of the purM select agent strain B. pseudomallei Bp82 (60). Broth cultures were grown in 14-ml Falcon round-bottom polypropylene test tubes with snap caps (Fisher Scientific) containing 3 ml of LB and shaking at 250 rpm unless indicated otherwise. When appropriate, antibiotics were added at the following concentrations: 25 μg/ml kanamycin (Km) and streptomycin (Sm) for E. coli, and 25 μg/ml polymyxin B (Pm) and 500 to 1,000 μg/ml Km for B. pseudomallei. For induction studies, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. A 20-mg/ml stock solution of the chromogenic indicator 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was prepared in PBS and added to solid and liquid plates for blue/white screening in E. coli TOP10 or E. coli 10G chemically competent cells. All manipulations with B. pseudomallei select agent strains were carried out in a class II microbiological safety cabinet located in a designated biosafety level 3 (BSL3) laboratory. Other strains were handled in a class II microbiological safety cabinet located in a designated BSL2 laboratory.

Environmental bacteria were isolated from several sources in Frederick, MD, USA (latitude and longitude coordinates, 39.396509 and −77.368223, respectively) during 2017 to 2020. Bacterial river (R) isolates were obtained by spreading aliquots of Monocacy River water serially diluted in sterile phosphate-buffered saline (PBS) onto the surfaces of sheep blood agar plates, LB agar plates, LB agar plates supplemented with 60 μg/ml X-Gal, and 1× Difco M9 minimal Salts (Becton, Dickinson and Company) agar plates containing 0.4% glucose. The plates were incubated at 37°C for 2 days, and colonies that could be easily distinguished from B. pseudomallei colonies on solid medium due to morphology, pigmentation, and/or β-galactosidase production were selected for further characterization. A similar strategy was employed for water obtained from a stream (ST) that feeds directly into the Monocacy River. River sediment (RS) was obtained using sterile 50-ml conical tubes, resuspended in PBS, serially diluted in PBS, and spread onto agar plates as described above. Bacterial soil (S) isolates were obtained by using a hand trowel and placing soil 3 to 4 in. below the surface into 50-ml conical tubes. Approximately 2 g of soil was resuspended in PBS, vigorously vortexed, and serially diluted in PBS, and aliquots were spread onto agar plates as described above. Bacterial rhizosphere (RZ) isolates associated with the root microbiome of lawn weeds were placed into sterile 50-ml conical tubes and processed like the soil samples.

DNA manipulation. Restriction enzymes (Roche Molecular Biochemicals and New England BioLabs), Antarctic phosphatase (New England BioLabs), and T4 DNA ligase (Roche Molecular Biochemicals) were used according to the manufacturer’s instructions. When necessary, the End-It DNA end repair kit (Epicentre) was used to convert 5’ or 3’ protruding ends to blunt-ended DNA. The DNA fragments used in the cloning procedures were excised from agarose gels and purified with a PureLink Quick gel extraction kit (Invitrogen). Bacterial genomic DNA was prepared from overnight LB broth cultures with the
GenElute bacterial genomic DNA kit (Sigma-Aldrich). Plasmids were purified from overnight LB broth cultures by using the Wizard Plus SV miniprep DNA purification system (Promega).

PCR amplifications. The PCR primers used in this study are shown in Table 2. The PCR products were sized and isolated by using agarose gel electrophoresis, cloned using the pCR2.1-TOPO TA cloning system (Promega), and environmental Gram-positive bacteria. Briefly, centrifugation, washed with sterile PBS, and diluted to 1× FailSafe PCR PreMix D (Epicentre), 1.25 U FailSafe PCR enzyme mix (Epicentre), 1 μM PCR primers, and approximately 200 ng of genomic DNA. Genomic DNA was isolated from all environmental bacterial isolates, and their 16S rRNA genes were PCR amplified using the primers 533F and 1492R (Table 2) and cloned into pCR2.1-TOPO. The Bacillus mycoides R15 and Bacillus cereus ST9 16S rRNA genes were also PCR amplified using Rd1 and Fd1 (Table 2). Colony PCR was utilized to screen for B. pseudomallei deletion mutants. Briefly, sucrose-resistant and Km-sensitive colonies were resuspended in 50 μl of water, and 5 μl was added to the PCR mixture rather than purified genomic DNA. PCR cycling was performed using a Mastercycler pro S (Eppendorf) and heated to 97°C for 5 min. This was followed by 30 cycles of a three-temperature cycling protocol (97°C for 30 s, 55°C for 30 s, and 72°C for 1 min) and 1 cycle at 72°C for 10 min. For PCR products larger than 1 kb, an additional 1 min per kb was added to the extension time.

DNA sequencing. DNA inserts cloned into pCR2.1-TOPO were PCR amplified with M13 forward and M13 reverse primers (Table 2), and unincorporated deoxynucleoside triphosphates (dNTPs) and primers were removed using the DyeEx 2.0 spin kit (Qiagen). The PCR products were then sequenced using the ABI BigDye Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific) and an Applied Biosystems SeqStudio genetic analyzer (Thermo Fisher Scientific) according to the manufacturer’s instructions. The nucleotide sequences were analyzed with DNAStar Lasergene 17 software.

Tn9 modKm mutagenesis and plasmid conj-OXm. TrNmod-OKm9 (39) was delivered to Bp82 via conjugation with E. coli S17-1 (pTnMod-OKm9) by using a membrane filter mating technique. Briefly, S17-1 (pTnMod-OKm9) was inoculated into 3 ml of LB broth containing Km and Sm and grown at 37°C for 18 to 20 h with shaking (250 rpm). B. pseudomallei was also grown under these conditions but without antibiotic selection. One hundred microliters of each saturated culture was added to 3 ml of sterile 10 mM MgSO4 mixed, and filtered through a 0.45-μm-pore-size nitrocellulose filter using a 25-mm Swinnex filter apparatus (Millipore). Filters were placed on LB plates supplemented with 10 mM MgSO4 and incubated for 8 h in a 37°C incubator. The filters were washed with 2 ml of sterile phosphate-buffered saline (PBS), and 100-μl aliquots were spread onto LB agar plates containing Km and Pm. Km’ and Pm’ colonies were identified after 48 h of incubation at 37°C. TrNmod-OKm’ contains a Km’ gene and a pbM1 conditional origin of replication that does not function in B. pseudomallei, allowing the rapid cloning of DNA transposon insertions into E. coli. The in vitro cloning of DNA flanking the Tn9mod-OKm9 insertion sites in B. pseudomallei SMM1 and SMM2 was performed by digesting total genomic DNA with the restriction endonuclease NotI, self-ligating, and transforming into an E. coli host (Table 2). The resulting plasmids were then sequenced with an outward facing primer (TrnMod-LT2) that binds to the left end of TrNmod-OKm9. The resulting sequence reactions revealed the junction of the transposon and B. pseudomallei genomic DNA. Plasmids pMo130 and pBHR2 and their derivatives were likewise conjugated to B. pseudomallei by using E. coli S17-1 as the donor strain (Table 2).

Screening for B. pseudomallei Bp82 transposon mutants that do not produce a zone of inhibition on lawns of N. bataviensis S4. Individual TrnMod-OKm’ mutants were picked from 150-by-15 mm polystyrene petri plates containing LB agar and X-Gal using sterile toothpicks. Prior to transfer, the agar medium was inoculated with N. bataviensis S4 by submerging a sterile swab into a saturated LB broth culture and spreading it across the entire surface of the agar in back-and-forth motions. The agar plate was rotated 90° three times, and this process was repeated and the surface of the agar was allowed to dry in a class II microbiological safety cabinet prior to inoculation with B. pseudomallei Bp82 transposon mutants. The plates were incubated at 37°C for 1 to 2 days and screened for mutants that did not produce zones of inhibition (clearing) around the colonies. Approximately 8,000 transposon mutants were screened by this method.

Construction of B. pseudomallei mutants. Gene replacement experiments with B. pseudomallei were performed using the sacB-based vector pMo130, as previously described (61–63). Recombinant derivatives of pMo130 (Table 2) were electroporated into E. coli S17-1 (12.25 kV/cm) and conjugated with B. pseudomallei Bp82 for 8 h. Pm was used to counterselect E. coli S17-1. The optimal conditions for the resolution of the sacB constructs were found to be LB agar lacking NaCl and containing 10% (wt/vol) sucrose with incubation at 37°C for 2 days. B. pseudomallei deletion mutants were identified by colony PCR using the primers flanking the deleted regions of the targeted genes (Table 2). As expected, the PCR products generated from the mutant strains were smaller than those obtained from the wild-type strain.

Bacterial competition assays. A modified qualitative and quantitative contact-mediated bacterial competition assay (38) was employed to assess the ability of B. pseudomallei to compete with the 14 environmental Gram-positive bacteria. Briefly, B. pseudomallei and environmental competitors were grown in LB broth at 37°C for 18 h, and three independent cultures of each strain were used for each competition assay performed. Two hundred microliters of each of the saturated cultures was pelleted by centrifugation, washed with sterile PBS, and diluted to 1×107 CFU/ml, and 10-μl aliquots of each competitor were spotted onto the surface of LB agar or LB agar containing X-Gal (Fig. 2A). One hundred microliters of B. pseudomallei and of the environmental competitor were combined and mixed, and a 20-μl aliquot of the 1:1 mixture was spotted onto the solid medium (Fig. 2A) and incubated for 48 h at RT. The remaining competition mixture was serially diluted in PBS, and 100-μl aliquots were spread onto LB agar or LB agar containing X-Gal to determine the input concentration of B. pseudomallei and environmental species present in the mixture. Following incubation, the bacteria present in each spot were resuspended in 1 ml of PBS using sterile swabs and serially diluted in PBS, and 100-μl aliquots were
spread onto LB agar or LB agar containing X-Gal and incubated for 1 to 2 days at 37°C to determine the number of CFU present. The quantity of each competitor present in the competition mixture was assessed by enumerating the number of B. pseudomallei off-white colonies compared to the number of Gram-positive pigmented or blue colonies. The fold difference between the B. pseudomallei/environmental isolate ratio when the bacteria were grown alone relative to the B. pseudomallei/environmental isolate ratio when the bacteria were grown in mixed culture was used to establish the overall competitive index. Three independent pairs of cultures were performed for each B. pseudomallei/environmental isolate competition assay, and the results were recorded as the mean ± the standard deviation.

Contact-independent liquid competition assays were performed as described above except that B. pseudomallei, the environmental competitor, and the B. pseudomallei plus environmental competitor mixtures were each added to 3 ml of LB broth and grown at RT for 48 h. For liquid competition experiments in which B. pseudomallei and N. bataviensis cocultures were separated by a cell-impermeable filter, Steriflip vacuum-driven sterile filter devices (Millipore Sigma) were utilized. Briefly, B. pseudomallei and N. bataviensis overnight cultures were diluted to an optical density at 600 nm (OD600) of 0.1, and 10-μl aliquots were added to 30 ml LB broth on each side of the Steriflip apparatus as depicted in Fig. S2A in the supplemental material. Parafilm was employed to firmly seal the 50-ml conical tubes to the filter apparatus, and the coculture chamber was incubated at RT for 48 h with mild agitation (120 rpm). The concentration of bacteria in each chamber after growth was determined by spreading 100-μl aliquots of serial dilutions onto LB agar and incubating for 1 to 2 days at 37°C. Three independent pairs of cultures were performed for each B. pseudomallei competition assay, and the results were recorded as the mean ± the standard deviation. For solitary growth cultures, Bp82 cultures (or S4 cultures) were grown on both sides of the apparatus, and the means were calculated and used to determine the Bp82/S4 solitary growth ratio.

**Extraction and preparation of 4-hydroxy-3-methyl-2-alkenylquinolines from B. pseudomallei cultures.** HMAQ and HMAQ-NO derivatives were isolated from 75-ml stationary-phase cultures of B. pseudomallei Bp82 and Bp82.ΔhmqD. Cells were removed from the culture by centrifugation, and the culture supernatant was sterilized by using a 0.2-μm filter (Millipore, Billerica, MA). The sterilized culture supernatants were extracted with equal volumes of acidified ethyl acetate and dried to completion under a constant stream of nitrogen gas. The samples were subjected to a metabolomics sample preparation procedure described by Dhummakupt et al. (64). Briefly, 100 μl of sample was mixed with an 8:1:1 acetonitrile-methanol-acetone solvent (Optima LC/MS grade; Fisher Scientific) containing isotopically labeled internal standards. Samples were stored at 4°C for 30 min until proteins precipitated and then centrifuged at 10,000 × g for 4 min at 4°C. The supernatant was removed, dried down, and resuspended in 100 μl of water with 0.1% formic acid (Optima LC/MS grade) and placed in a liquid chromatography (LC) vial.

**Liquid chromatography and mass spectrometry.** Reverse-phase high-performance liquid chromatography was utilized with solvent A being water with 0.1% formic acid (Optima LC/MS grade) and solvent B being acetonitrile (Optima LC/MS grade) containing 1% formic acid with a 2-μl injection volume. Conditions were 0% B from 0 to 8 min, 5% B at 8 to 12 min, 35% B at 12 to 15 min, 95% B at 15 to 18 min, and 0% B at 18 to 20 min with a linear gradient. A Cörtecs T3 column (Waters Corp., Milford, MA; 120 Å, 2.7 μm, 2.1 mm by 150 mm) with a Phenomen (Torrance, CA) SecurityGuard ultrahigh-performance liquid chromatography (UHPLC) phenyl 2.1-mm-inside-diameter (μL) guard column was utilized for separations.

A Thermo Fisher Scientific (Waltham, MA) Q Exactive hybrid quadrupole-Orbitrap mass spectrometer was utilized for mass analysis. A heated electrospray ionization (HESI-II; Thermo Fisher Scientific) probe ionization source was utilized in positive mode at 3.5 kV, the capillary temperature was set to 269°C, S-Lens radio frequency (RF) was set to 50 V, sheath gas was set at 53 arbitrary units (AU), auxiliary gas at 14 AU with heater temperature at 300°C, and sweep gas at 3 AU. A top-20 data-dependent acquisition (DDA) method with an inclusion list was employed, utilizing 70,000 R at the MS1 level and 15,000 R at the MS2 level with stepped collision energies of 30, 50, and 70. Inclusive masses for targeted MS2 fragmentation were those precursor masses of the mutant knockout pathway of interest and variants.

**Data processing.** Data processing was performed by Thermo Fisher Scientific’s Compound Discoverer 3.1. Searches were performed according to the default Targeted E&L workflow with FISh Scoring built-in workflow. Features were identified and aligned with a max retention time tolerance of 2 min and delta 10 ppm tolerance. Features were then filtered by a signal-to-noise ratio of 1.5. The aligned and filtered features were searched against all the 4-hydroxy-2-alkenylquinoline (HAQ) families listed by Vial et al. (30) with the expected compounds node and all possible ion adducts. Results were then filtered by identifications with MS2 DDA support.

**Data availability.** The GenBank accession numbers for the Gram-positive 16S rRNA sequences described in this study are Prieta megaterium RS1, MW756952; Exiguobacterium undae RS4, MW756953; Cytopbactillus firmus R14, MW756954; Bacillus mycoides R15, MW756955; Bacillus cereus ST9, MW756956; Neobacillus bataviensis S4, MW756957; Exiguobacterium acetylicum R10, MW756958; Bacillus marisflavi S9, MW756959; Paenbacillus sp. S10, MW756960; Paenbacillus polymyxa S2, MW756961; Bacillus licheniformis S7, MW756962; Bacillus velezensis RZ8, MW756963; Microbacterium sp. RS16, MW756964, and Cellulosimicrobium sp. RS17, MW756965.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 0.4 MB.
ACKNOWLEDGMENTS
This work was supported by DTRA/JSTO-CBD grant CB10207 (to D.D.) and a DTRA Chemical Biological Defense Research Associateship Award to U.O. via the National Academy of Sciences NRC Research Associateship Program.

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

REFERENCES
1. Galyov EE, Brett PJ, DeShazer D. 2010. Molecular insights into Burkholderia pseudomallei and Burkholderia mallei pathogenesis. Annu Rev Microbiol 64:495–517. doi: 10.1146/annurev.micro.112408.134030.
2. Kim HS, Schell MA, Yu Y, Ulrich RL, Sarria SH, Nierman WC, DeShazer D. 2005. Bacterial genome adaptation to niches: divergence of the potential virulence genes in three Burkholderia species of different survival strategies. BMC Genomics 6:174. doi: 10.1186/1471-2164-6-174.
3. Centers for Disease Control and Prevention, Department of Health and Human Services. 2012. Possession, use, and transfer of select agents and toxins: biennial review. Final rule. Fed Regist 77:61083–61111.
4. Gasspie I, Armstrong M, Norton R. 2020. Human melioidosis. Clin Microbiol Rev 33:e00006-20. doi: 10.1128/CMR.00006-19.
5. Sprague LD, Neubauer H. 2004. Melioidosis in animals: a review on epizootiology, diagnosis and clinical presentation. J Vet Med B Infect Dis Vet Public Health 51:305–320. doi: 10.1111/j.1439-4504.2004.00797.x.
6. Robins GD. 1906. A study of chronic glanders in man. Studies from the Royal Victoria Hospital, vol 2. Guertin Printing Company, Montreal, Canada.
7. Srinivasan A, Kraus CN, DeShazer D, Becker PM, Dick JD, Spacle L, Bartlett JG, Byrne WR, Thomas JL. 2001. GenBank in a microbiologist workshop. N Engl J Med 345:256–258. doi: 10.1056/NEJM200107263450404.
8. Steele JH. 1979. Glanders. p 339–362. In Steele JH (ed), CRC handbook series in zoonoses. CRC Press, Boca Raton, FL.
9. Brett PJ, DeShazer D, Woods DE. 1998. Burkholderia thailandensis sp. nov., description of a Burkholderia pseudomallei-like species. Int J Syst Bacteriol 48:317–320. doi: 10.1099/00207713-48-1-317.
10. Gee JE, Elrod MG, Gulvik CA, Haselow DT, Waters C, Liu L, Hoffmaster AR. 2018. Virulence genes in three Burkholderia pseudomallei isolated from infected wound, Arkansas, USA. Emerg Infect Dis 24:2091–2094. doi: 10.3202/eid2411.180082.
11. Janesboon J, Suangsombut V, Srinon V, Meethai T, Thairinjaroen CS, Amornchai P, Withatpanon T, Chanthrai N, Miyamoto M, Wuthiekanun V, Currie BJ, Stevens JM, Karbsrisate S. 2021. Detection and differentiation of Burkholderia species with pathogenic potential in environmental soil samples. PLoS One 16:e0245175. doi: 10.1371/journal.pone.0245175.
12. Triaknonboon S, Vuokdal V, Tharavichitkul P, Na-Gnam N, Suputtamongkol Y, Thairinjaroen CS, Karbsrisate S. 2019. Epimutations of aminoglycoside resistance in Burkholderia pseudomallei isolated from patients and soil in Thailand. Southeast Asian J Trop Med Public Health 30:756–759.
13. Ghoul M, Mitri S. 2016. The ecology and evolution of microbial competition. Trends Microbiol 24:833–845. doi: 10.1016/j.tim.2016.06.011.
14. Stubbsendieck RM, Straight PD. 2016. Multifaceted interfaces of bacterial competition. J Bacteriol 198:2145–2155. doi: 10.1128/JB.00275-16.
15. Hood RD, Singh P, Hsu F, Gütenier T, Carl MA, Trinidad RR, Silverman JM, Ohlson BB, Hicks KG, Plemel RL, Li M, Schwarz S, Wang WV, Merz AJ, Goodlett DR, Mougdj JG. 2010. A type VI secretion system of Dromomonas aeruginosa targets a toxin to bacteria. Cell Host Microbe 7:25–37. doi: 10.1016/j.chom.2009.12.007.
16. Russell AB, Hood RD, Bui NK, LeRoux M, Vollmer W, Mougdj JD. 2011. Type VI secretion delivers bactericidal effectors to target cells. Nature 475:343–347. doi: 10.1038/nature10244.
17. Yu KW, Xue F, Yu Y, Yang L. 2021. T6SS mediated stress responses for bacterial environmental survival and host adaptation. Int J Mol Sci 22:478. doi: 10.3390/ijms22020478.
18. Schwarz S, West TE, Boyer F, Chiang WC, Carl MA, Hood RD, Rohmer L, Tolker-Nielsen T, Skerritt SJ, Mougdj JG. 2010. Burkholderia type VI secretion systems have distinct roles in eukaryotic and bacterial cell interactions. PLoS Pathog 6:e1000106. doi: 10.1371/journal.ppat.1000106.
19. Schell MA, Ulrich RL, Ribot WJ, Brungsammer EE, Hines HB, Chen D, Lipcomb L, Kim HS, Mrazek J, Nierman WC, DeShazer D. 2007. Type VI secretion is a major virulence determinant in Burkholderia mallei. Mol Microbiol 64:1466–1485. doi: 10.1111/j.1365-2958.2007.05734.x.
36. Lane DJ. 1991. 16S/23S rRNA sequencing. p 115–175. In Stackebrandt E, Goodfellow M (ed), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, New York, NY.

37. McGinnis S, Madden TL. 2004. BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucleic Acids Res 32:W20–W25. https://doi.org/10.1093/nar/gkh435.

38. Hachani A, Lossi NS, Filloux A. 2013. A visual assay to monitor T6SS-mediated bacterial competition. J Vis Exp 2013:e51013. https://doi.org/10.3791/51013.

39. Dennis JJ, Zylstra GJ. 1998. Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of Gram-negative bacterial genomes. Appl Environ Microbiol 64:2710–2715. https://doi.org/10.1128/AEM.64.7.2710-2715.1998.

40. Zeigler DR, Prágai Z, Rodriguez S, Chevreux B, Muffler L, Albert T, Bai R, Wyss M, Perkins JB. 2008. The origins of 168, W23, and other Bacillus subtilis’ legacies strains. J Bacteriol 190:6983–6995. https://doi.org/10.1128/JB.00722-08.

41. Chandler JR, Heilmann S, Mittler JE, Greenberg EP. 2012. Acyl-homoserine lactone-dependent eavesdropping promotes competition in a laboratory co-culture model. ISME J 6:2219–2228. https://doi.org/10.1038/ismej.2012.69.

42. Ngamdee T, Tandhavanant S, Wikaiphat C, Reampong O, Wuthiekanun V, Salje J, Low DA, Peacock SJ, Chantratita N. 2015. Competition between Burkholderia pseudomallei and B. thailandensis. BMC Microbiol 15:56–56. https://doi.org/10.1186/s12866-015-0395-7.

43. Nikolakakis K, Amber S, Wilbur JS, Diner EJ, Aoki SK, Sooie PL, Tuanyok A, Keim PS, Peacock S, Hayes CS, Low DA. 2012. The toxin/immunity network of Burkholderia pseudomallei contact-dependent growth inhibition (CDI) systems. Mol Microbiol 84:516–529. https://doi.org/10.1111/j.1365-2958.2012.06839.x.

44. Dankar ES, Garcia EC, Cotter PA. 2017. Are CDI systems multicolored, facultative, helping greenbeards? Trends Microbiol 25:391–401. https://doi.org/10.1016/j.tim.2017.02.008.

45. Lin HH, Chen YS, Li YC, Tseng IL, Hisheh TH, Buu LM, Chen YL. 2011. Burkholderia multivorans acts as an antagonist against the growth of Burkholderia pseudomallei in soil. Microbiol Immunol 55:616–624. https://doi.org/10.1111/j.1348-0421.2011.00365.x.

46. Bertelé-Sellás A, Cervantes-Ceballos I, Tirado-Ballestras I, Maldonado-Rojas W, Alzate-Restrepo J, Olivero-Verbel J. 2020. Biodegradation of biodiesel-oil by Paenibacillus vireti. Int J Biol Macromol 175:2228. https://doi.org/10.1016/j.ijbiomac.2019.11.082.

47. Hernández-González IL, Moreno-Hagelsieb G, Olmedo-Álvarez G. 2018. Functional plasticity of the causative agent of melioidosis, Burkholderia pseudomallei. Proc Natl Acad Sci U S A 115:2440–2445. https://doi.org/10.1073/pnas.1703021106.

48. DeShazer D, Brett PJ, Carlyon R, Woods DE. 1997. Mutagenesis of Burkholderia pseudomallei with Tn5-OT182: isolation of motility mutants and molecular characterization of the flagellin structural gene. J Bacteriol 179:2116–2125. https://doi.org/10.1128/JB.179.7.2116-2125.1997.

49. Propst KL, Mima T, Choi KH, Dow SW, Schweizer HP. 2010. A Burkholderia pseudomallei AtpA mutant is avirulent in immunocompetent and immunodeficient animals: candidate strain for exclusion from select-agent lists. Infect Immun 78:3136–3143. https://doi.org/10.1128/IAI.01313-09.

50. Burtinck MN, Brett PJ, Harding SV, Ngugi SA, Rifat BW, Chantratita N, Scapin A, Milne TS, Dean RE, Fritz DL, Peacock SJ, Prior JL, Atkins TP, DeShazer D. 2011. The cluster 1 type VI secretion system is a major virulence determinant in Burkholderia pseudomallei. Infect Immun 79:1512–1525. https://doi.org/10.1128/IAI.01218-10.

51. Hamad MA, Zajdowicz SL, Holmes RK, Voskuil MI. 2009. An allelic exchange molecular characterization of the Burkholderia pseudomallei and Burkholderia mallei contact-dependent growth inhibition (CDI) operon. BMC Microbiol 9:257. https://doi.org/10.1186/1471-2180-9-257.

52. Lefèvre A, Lebrun S, Bidaud B, Delsad M, Landry JD, Skerjanec D, Bricogne G, Bonnet M, Pichon R, Beckstrom-Sternberg SM, Sañez M, Schutzer SE, Keim P, Nierman WC. 2010. Continuing evolution of Burkholderia mallei through genome reduction and large-scale rearrangements. Genome Biol Evol 2:112–116. https://doi.org/10.1093/gbe/evq043.

53. Price EP, Viberg LT, Krid TJ, Bell SC, Currie BJ, Sarovich DS. 2018. Transcriptomic analysis of longitudinal Burkholderia pseudomallei infecting the cystic fibrosis lung. Microb Genom 4:e000194. https://doi.org/10.1099/mgen.0.000194.

54. Holden MT, Tribble RW, Peacock SJ, Cerdeno-Tarraga AM, Atkins T, Crossman LC, Pitt T, Churchner C, Mungall K, Bentley SD, Sebaihia M, Thomson NR, Bason N, Beacham IR, Brooks K, Brown KA, Brown NF, Challs GL, Cherevach I, Chillingworth T, Cronin A, Cossert B, Davis P, DeShazer D, Feltwell T, Fraser A, Hance Z, Hauser H, Holroyd S, Jagels K, Keith KE, Maddison M, Moule S, Price C, Quail MA, Rabinowitch E, Rutherford K, Sanders M, Simmonds M, Songvilsai S, Stevens K, Tumapa S, Vesaratchavitch M, Whitehead S, Yeats C, Barrell BG, Oyston PC, Parkhill J. 2004. Genomic plasticity of the causative agent of melioidosis, Burkholderia pseudomallei. Proc Natl Acad Sci U S A 101:14240–14245. https://doi.org/10.1073/pnas.0403320101.

55. Potisap C, Khan MAW, Boonmee A, Rodrigues JLM, Wongratanacheewin S, Sermswan RW. 2018. Burkholderia pseudomallei-absent soil bacterial community results in secondary metabolites that kill this pathogen. AMB Express 8:136–136. https://doi.org/10.1186/s13568-018-0663-7.

56. Nieves W, Asakara S, Qazi O, Brown KA, Kurtz J, Aucoin DP, McLachlan JB, Roy CJ, Morici LA. 2011. A naturally derived outer-membrane vesicle vaccine protects against lethal pulmonary Burkholderia pseudomallei infection. Vaccine 29:8381–8389. https://doi.org/10.1016/j.vaccine.2011.08.058.

57. Losada L, Ronning CM, DeShazer D, Woods D, Fodarena N, Kim HS, Shabalina SA, PerezTur J, Brinkla L, Tan P, Nandi T, Crabtree J, Badger J, Beckstrom-Sternberg S, Sañez M, Schutzer SE, Keim P, Niemier WN. 2010. Continuing evolution of Burkholderia mallei through genome reduction and large-scale rearrangements. Genome Biol Evol 2:112–116. https://doi.org/10.1093/gbe/evq043.