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RESEARCH ARTICLE

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

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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

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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, syrbactin, 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 β-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 C_9 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 C_7 and C_9 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 β-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 nonredundant 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

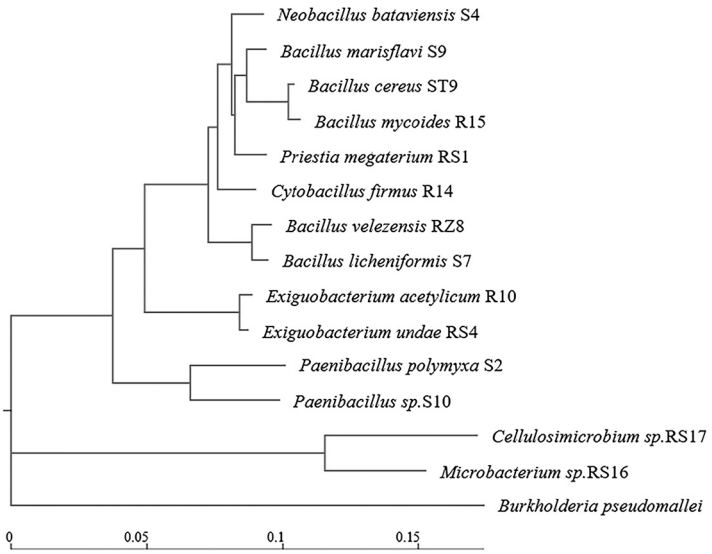


FIG 1 Phylogenetic tree based on partial 16S rRNA sequences of Gram-positive bacterial isolates from soil (S), river water (R), river sediment (RS), stream water (ST), and rhizosphere (RZ) samples. NGPhylogeny.fr was used to build the tree in "FastME/OneClick" mode, and it utilized MAFFT for multiple alignment, BMGE for automatic alignment curation, FastME for tree inference, and Newick Display for tree rendering (65). The sequence of the *B. pseudomallei* 16S rRNA gene was used as an outgroup. The scale bar indicates the number of substitutions per site.

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 $\sim 1 \times 10^7$ 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

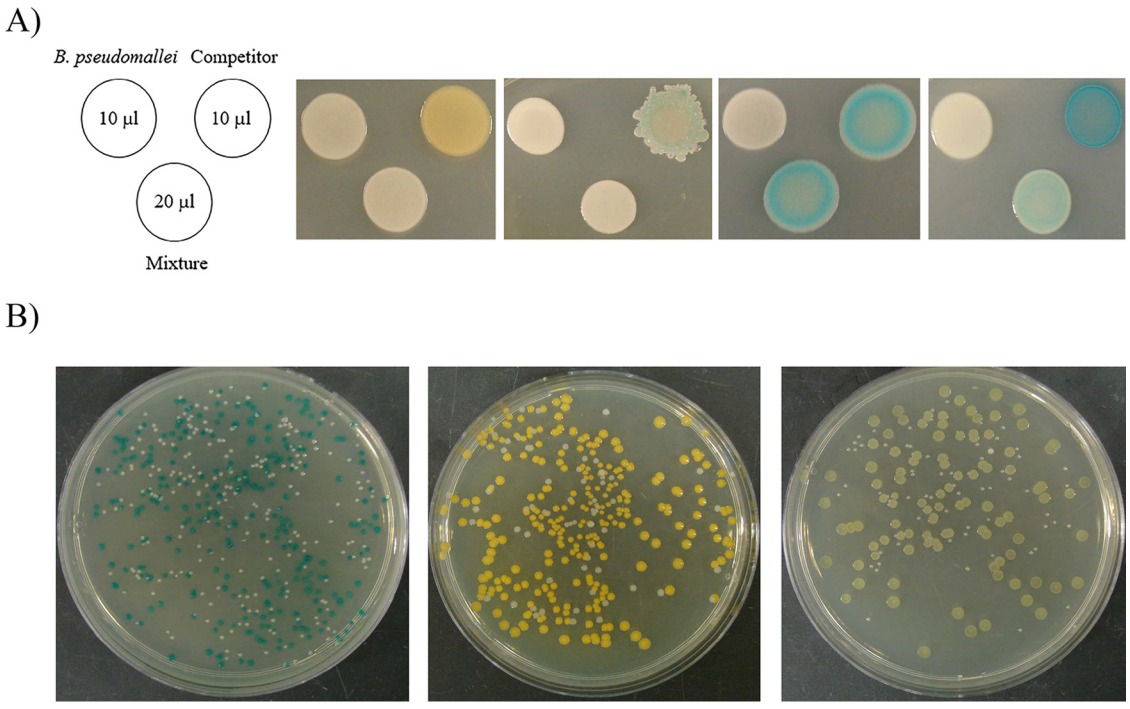


FIG 2 Contact-mediated competition assay used in this study. (A) Qualitative observation of bacterial competition between *B. pseudomallei* and Gram-positive environmental bacteria. Bacteria were grown to stationary phase in liquid broth and diluted to $\sim 1 \times 10^7$ CFU/ml, and aliquots of *B. pseudomallei* (10 μl), environmental competitor (10 μl), and a 1:1 mixture of *B. pseudomallei* plus competitor (20 μl) were spotted onto LB agar with or without X-Gal and incubated for 48 h at RT (left). The appearance of competition assays conducted with four different environmental competitors is shown. The competition mixtures that remain an off-white color indicate that *B. pseudomallei* is able to inhibit the competitor, and the mixtures that are pigmented or blue indicate that *B. pseudomallei* is unable to eliminate the competitor. (B) Quantitative assessment of bacterial competition between *B. pseudomallei* and Gram-positive environmental bacteria. The bacterial spots were resuspended in PBS, serially diluted, and spread onto agar plates to determine the number of viable bacteria present. The appearance of agar plates from three different *B. pseudomallei* plus competitor mixtures are shown. *B. pseudomallei* colonies are off-white in color and can be easily distinguished from competitors that produce β-galactosidase (left) or competitors that are pigmented (center and right).

(Fig. 2B). 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 firmus* 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

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 difference[a]
Neobacillus bataviensis S4	X		117 ± 3
Bacillus velezensis RZ8	X		60 ± 18
Bacillus licheniformis S7	X		111 ± 14
Cytobacillus firmus R14		X	14 ± 4
Bacillus marisflavi S9		X	2 ± 0.4
Bacillus cereus ST9			ND[b]
Bacillus mycoides R15	X		124 ± 43
Priestia megaterium RS1		X	149 ± 25
Exiguobacterium acetylicum R10			ND
Exiguobacterium undae RS4			ND
Paenibacillus polymyxa S2		X	42 ± 2
Paenibacillus sp. S10			ND
Cellulosimicrobium sp. RS17	X		12 ± 3
Microbacterium sp. RS16	X		51 ± 12

[a]Fold 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.

[b]ND, no significant difference in competitive index in mixed growth relative to solitary growth.

>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-μl and 10-μl 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 Tn*Mod*-OKm′, a self-cloning mini-Tn*5* 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

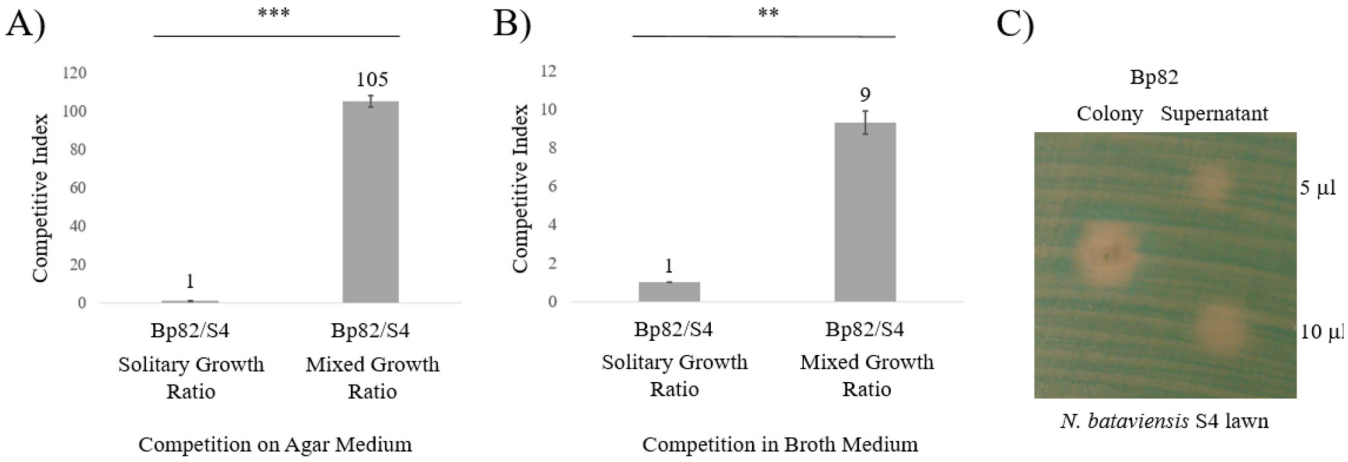


FIG 3 *B. pseudomallei* Bp82 inhibits *N. bataviensis* S4 in a contact-independent manner. (A) Bp82 competition assay with S4 on solid medium (contact mediated). (B) Bp82 competition assay with S4 in broth medium (contact independent). Three independent pairs of cultures were performed for each competition assay, and the results were recorded as the mean ± the standard deviation. (C) Bp82 culture supernatant contains a filterable antimicrobial product(s) that inhibits S4 growth. A Bp82 colony was inoculated on an S4 lawn on LB agar with X-Gal, and it produced a zone of inhibition around the colony (left); the growth of the S4 lawn was also inhibited by 5-μl and 10-μl aliquots of filter-sterilized Bp82 culture supernatant (right). **, $P < 0.01$; ***, $P < 0.001$.

activity against S4. The sites of the Tn*Mod*-OKm′ insertions in SMM1 and SMM2 were mapped to *hmqA* and *hmqD*, respectively (Fig. 4A). The *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 *B. pseudomallei*, *B. thailandensis*, and some members of the *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 *B. thailandensis hmq* transposon mutants similarly exhibited diminished antimicrobial activity against *Bacillus subtilis* 168, a bacterial strain utilized as a laboratory workhorse for decades (40).

In an attempt to further characterize role of the *B. pseudomallei hmqA-G* locus in competitive interactions with Gram-positive environmental bacteria, we constructed in-frame deletion mutations in *hmqA*, *hmqD*, and *hmqF* in Bp82. The resulting mutants, termed Bp82 Δ*hmqA*, Bp82 Δ*hmqD*, and Bp82 Δ*hmqF* (Table 2), did not produce zones of inhibition on an S4 lawn (Fig. 4C). The full-length *hmqD* gene, which encodes a 3-oxoacyl-(acyl carrier protein) synthase, was cloned into the broad-host-range vector pBHR2 and conjugated to Bp82 Δ*hmqD* for complementation studies. The Bp82 Δ*hmqD* (pBHR2-*hmqD*)/S4 mixed growth ratio on agar medium was >1,000-fold higher than the Bp82 Δ*hmqD* (pBHR2)/S4 mixed growth ratio, indicating that the Δ*hmqD* mutation was complemented by *hmqD* in *trans* and does not have a polar effect on downstream genes (Fig. 5). Importantly, the *hmqA* and *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 *B. pseudomallei*-exported antimicrobial product(s) with activity against *N. bataviensis* under these growth conditions is produced by the *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 Δ*hmqD* to examine the importance of the *B. pseudomallei hmqA-G* locus in providing a competitive advantage against the environmental isolates. Figure 6 shows that the Bp82/environmental isolate and Bp82 Δ*hmqD*/environmental isolate solitary growth ratios were not significantly different, indicating that there were no growth differences between the *B. pseudomallei* strains and the environmental isolates when grown apart (Fig. 6). In comparison, the Bp82/environmental isolate and Bp82 Δ*hmqD*/environmental isolate mixed growth ratios were dramatically different. The data clearly

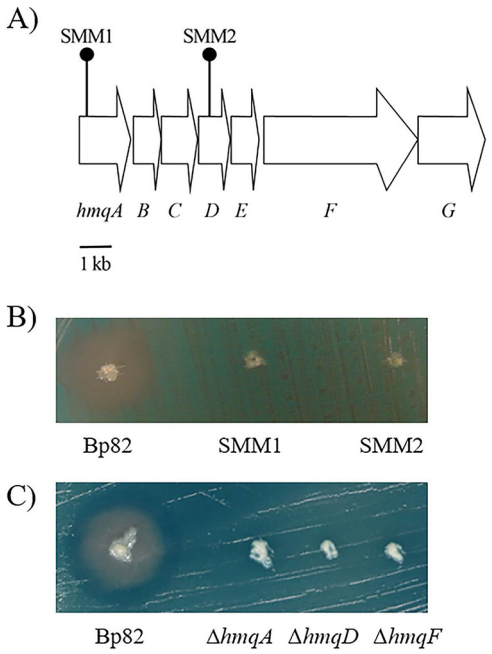


FIG 4 The *B. pseudomallei hmqA-G* locus mediates antimicrobial activity against *N. bataviensis* and other Gram-positive environmental species. (A) Genetic map of the *hmqA-G* gene cluster, locus tags *BP1026B_II0535-BP1026B_II0541* in *B. pseudomallei* 1026b and *BPSS0481-BPSS0487* in *B. pseudomallei* K96243. The location and direction of transcription of genes are represented by arrows. The location of Tn*Mod*-OKm′ insertions in SMM1 and SMM2 are shown schematically by round-top push pins. The unlinked *hmqL* gene, locus tag *BP1026B_II2272* in 1026b and *BPSS2111* in K96243, is not shown. A 1-kb scale is shown at the bottom. (B) Bp82 produces a zone of inhibition on a lawn of S4, but SMM1 and SMM2 are unable to produce such zones. (C) The deletion mutations in *B. pseudomallei* Bp82 Δ*hmqA*, Bp82 Δ*hmqD*, and Bp82 Δ*hmqF* do not produce zones of inhibition on a lawn of S4. The inoculated LB agar plates containing X-Gal were incubated for 48 h at RT.

demonstrate that Bp82 Δ*hmqD* 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 Δ*hmqD* supernatant.** We next performed liquid chromatography-mass spectrometry on Bp82 and Bp82 Δ*hmqD* 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 Δ*hmqD* (Fig. 7). The HMAQs differed from one another in the length of the unsaturated alkyl chains, including congeners with C_7, C_8, and C_9 side chains (Fig. 7B, D, and E). The HMAQ-NOs identified were congeners with C_7 and C_9 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 C_7 and C_9 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.

TABLE 2 Strains, plasmids, and primers used in this study

Strain or plasmid	Relevant characteristics or sequence (5′→3′)[a]	Source or Reference
Strains		
E. coli		
TOP10	General cloning and blue/white screening	Life Technologies
E. cloni 10G	General cloning and blue/white screening	Lucigen
S17-1	Mobilizing strain with transfer genes of RP4 integrated on chromosome, Sm^r, Tp^r	66
INV110	*dam* and *dcm* deficiencies to allow restriction digestion with Dam- and Dcm-sensitive restriction enzymes	Invitrogen
B. pseudomallei		
1026b	Isolated in Thailand from a human case of septicemic melioidosis with skin, soft tissue, and spleen involvement, Pm^r	59
Bp82	1026b Δ*purM* derivative; adenine and thiamine auxotroph	60
SMM1	Bp82 derivative, *hmqA*::Tn*Mod*-OKm′	This study
SMM2	Bp82 derivative, *hmqD*::Tn*Mod*-OKm′	This study
Bp82 Δ*hmqA*	Bp82 derivative harboring a 438-bp in-frame deletion mutation in *hmqA* (Δ*hmqA*)	This study
Bp82 Δ*hmqD*	Bp82 derivative harboring a 318-bp in-frame deletion mutation in *hmqD* (Δ*hmqD*)	This study
Bp82 Δ*hmqF*	Bp82 derivative harboring a 948-bp in-frame deletion mutation in *hmqF* (Δ*hmqF*)	This study
K96243	Isolated in Thailand from a diabetic patient with a clinical history of short incubation, septicemic infection, and rapid progression to death	58
MSHR346	Isolated in 1995 from the sputum of a melioidosis patient admitted to Royal Darwin Hospital, Northern Territory, Australia	P. Keim
Plasmids		
pCR2.1-TOPO	3,931-bp TA vector, pMB1 *oriR*, Km^r, Ap^r	Life Technologies
pCR2.1-*hmqA*	pCR2.1-TOPO containing 1,639-bp PCR product generated with hmqA-up and hmqA-dn	This study
pCR2.1-Δ*hmqA*	pCR2.1-*hmqA* digested with MluI and religated resulting in a 438-bp in-frame deletion in *hmqA*	This study
pCR2.1-*hmqD*	pCR2.1-TOPO containing 1,142-bp PCR product generated with hmqD-up and hmqD-dn	This study
pCR2.1-Δ*hmqD*	pCR2.1-*hmqD* digested with ClaI and religated resulting in a 318-bp in-frame deletion in *hmqD*	This study
pCR2.1-*hmqF*	pCR2.1-TOPO containing 1,995-bp PCR product generated with hmqF-up and hmqF-dn	This study
pTn*Mod*-OKm′	Minitransposon vector, pMB1 *oriR*, RP4 *oriT*, Tn*5 tnp*, Km^r	39
pSMM1No	Plasmid obtained from SMM1 by *in vitro* cloning with NotI	This study
pSMM2No	Plasmid obtained from SMM2 by *in vitro* cloning with NotI	This study
pMo130	Suicide vector for allelic exchange in *Burkholderia*, ColE1 *ori*, RK2 *oriT xylE sacB*, Km^r	62
pMo130-Δ*hmqA*	pMo130 containing the NheI insert from pCR2.1-Δ*hmqA*	This study
pMo130-Δ*hmqD*	pMo130 containing the SpeI-XbaI insert from pCR2.1-Δ*hmqD* cloned into the NheI site	This study
pMo130-*hmqF*	pMo130 containing the SpeI-XbaI insert from pCR2.1-*hmqF* cloned into the NheI site	This study
pMo130-Δ*hmqF*	pMo130-*hmqF* digested with BclI and religated resulting in a 948-bp in-frame deletion in *hmqF*	This study
pBHR2	Broad-host-range plasmid, Km^r	19
pBHR2-*hmqA*	pBHR2 containing EcoRI insert from pCR2.1-*hmqA*, constitutively expresses *hmqA*	This study
pBHR2-*hmqD*	pBHR2 containing EcoRI insert from pCR2.1-*hmqD*, constitutively expresses *hmqD*	This study
pBHR2-*hmqF*	pBHR2 containing EcoRI insert from pCR2.1-*hmqF*, constitutively expresses *hmqF*	This study
Primers		
553F	GTGCCAGCAGCCGCGGTAA	35
1492R	ACCTTGTTACGACTT	36
Rd1	AAGGAGGTGATCCAGCC	67
Fd1	AGTTTGATCCTGGCTCAG	67
M13 Forward	GTAAAACGACGGCCAG	Life Technologies
M13 Reverse	CAGGAAACAGCTATGAC	Life Technologies
TnMod-LT2	TTCCTGGTACCGTCGACATG	This study
hmqA-up	GCTAGCGTGAGCACTGACGATGGAC	This study
hmqA-dn	GCTAGCTCAGGCCGCCTGCACGTCTTC	This study
hmqD-up	TCGCCGCGGCGTCGTGAAGC	This study
hmqD-dn	TCAGCTTCGATAGAGCGCGC	This study
hmqF-up	ACGAACAACTCGCGACGCTC	This study
hmqF-dn	ACTCCGAGCCGAGGATCTTC	This study

[a]r, resistant; s, susceptible; Sm, streptomycin; Tp, trimethoprim; Pm, polymyxin B; Km, kanamycin; Ap, ampicillin.

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

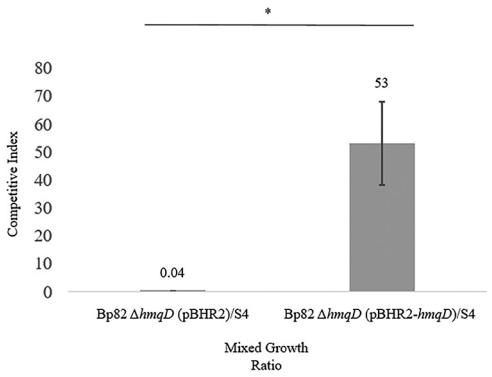


FIG 5 The in-frame Δ*hmqD* mutation can be complemented by *hmqD* when supplied in *trans* on the broad-host-plasmid pBHR2. Bp82 Δ*hmqD* (pBHR2) and Bp82 Δ*hmqD* (pBHR2-*hmqD*) were mixed with S4, and competitive indexes were assessed in contact-mediated competition assays (38). S4 outcompeted Bp82 Δ*hmqD* (pBHR2) by 25-fold, but Bp82 Δ*hmqD* (pBHR2-*hmqD*) outcompeted S4 by 53-fold. The mixtures 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. *, $P < 0.05$.

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 ≥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

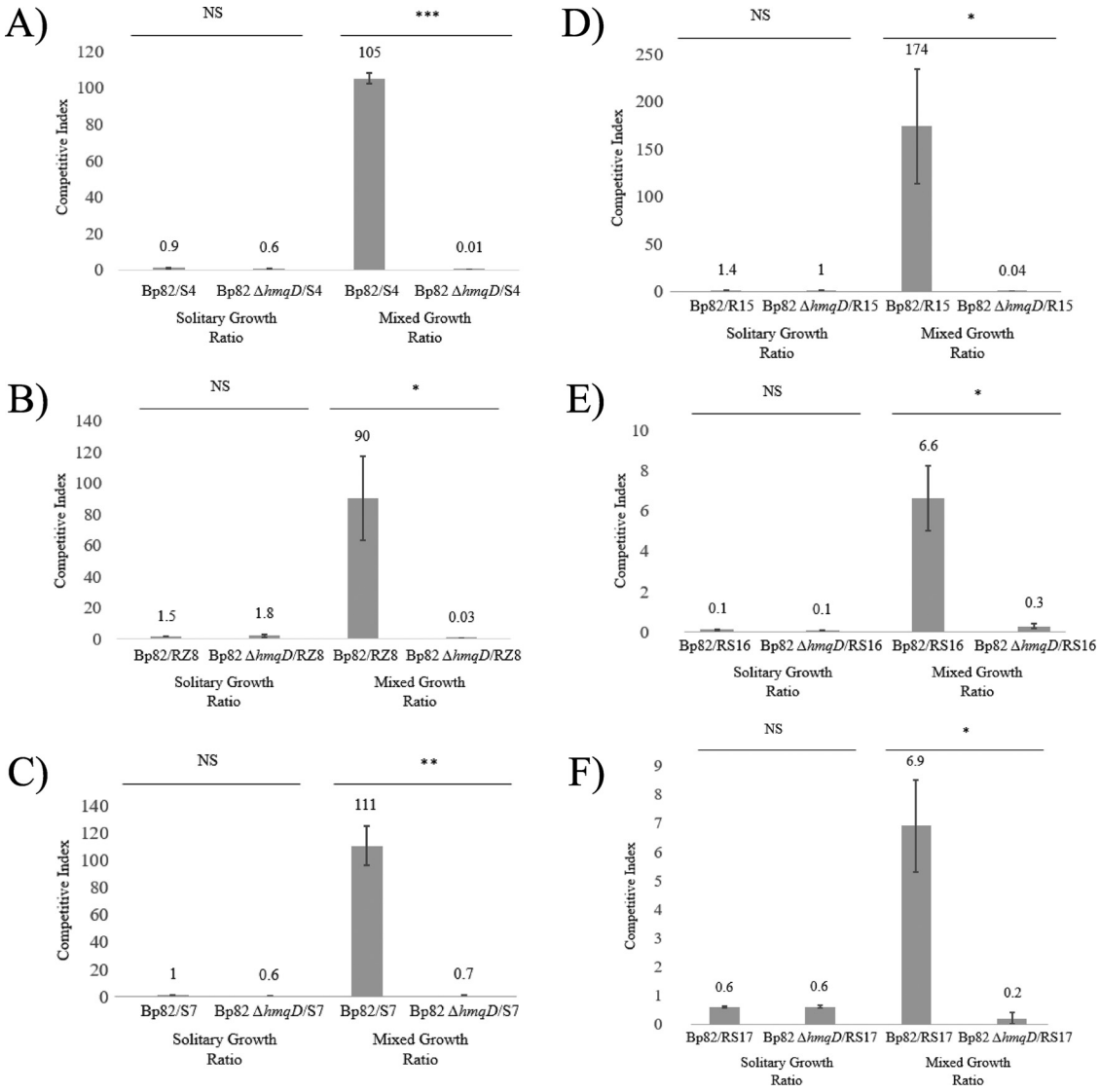
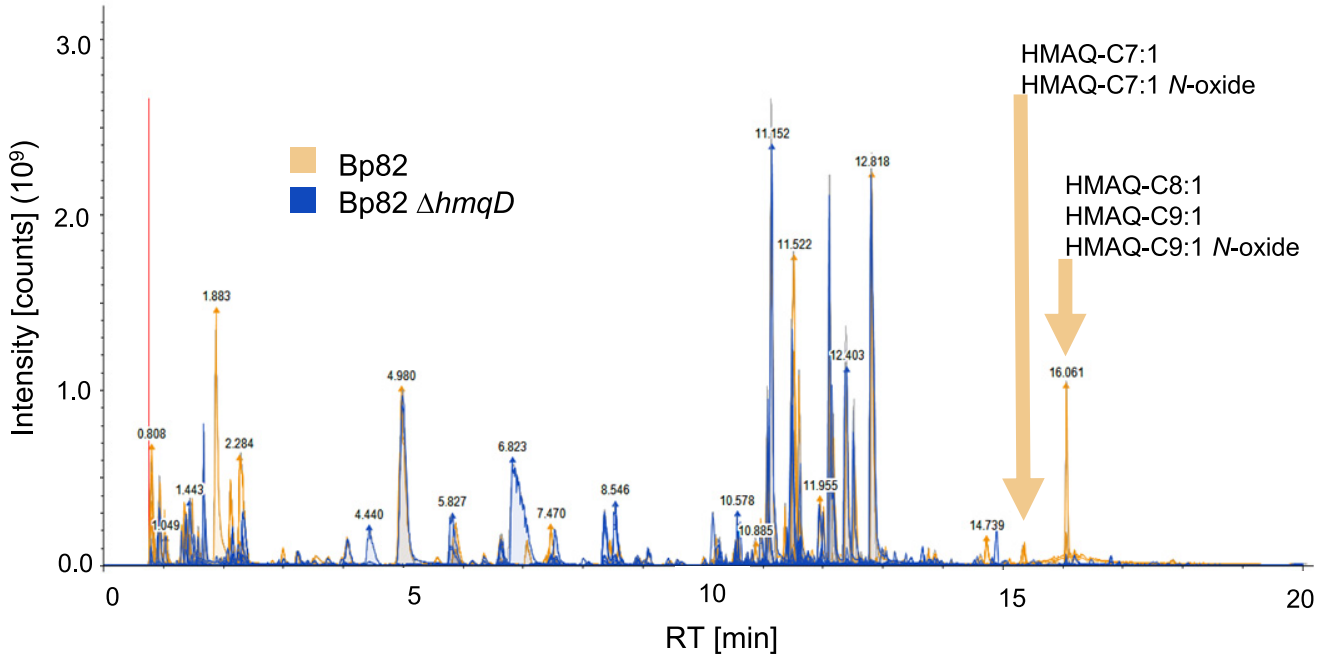
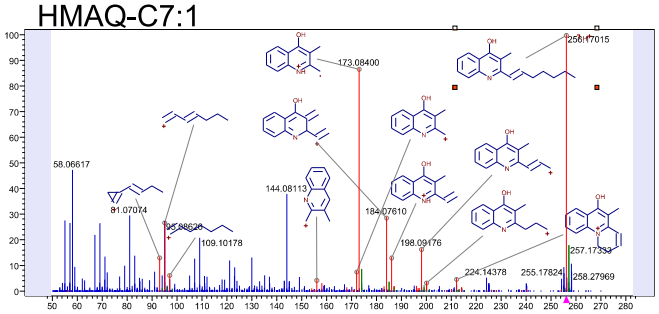
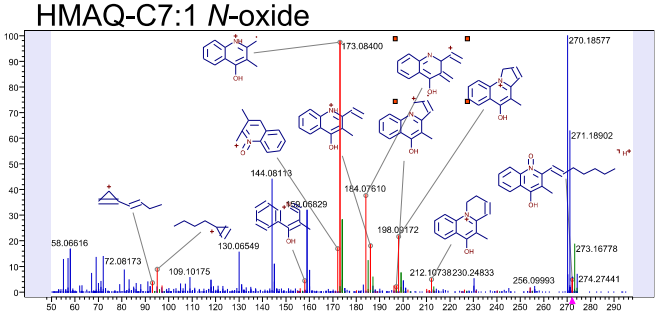


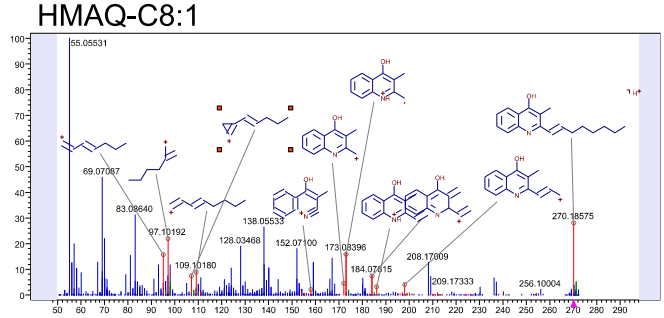
FIG 6 *B. pseudomallei* outcompetes environmental Gram-positive bacteria in an *hmqD*-dependent manner. Contact-mediated competition assays were performed with *B. pseudomallei* Bp82, Bp82 Δ*hmqD*, and *N. bataviensis* S4 (A), *B. velezensis* RZ8 (B), *B. licheniformis* S7 (C), *B. mycoides* R15 (D), *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; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

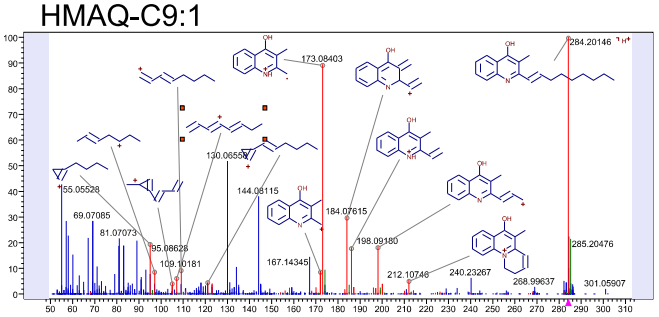
correspond to the presence of antimicrobial compounds in this niche (53, 54). In further studies, the researchers found that *B. amyloliquefaciens* inhibited *B. pseudomallei* growth in a liquid coculture assay and that *B. amyloliquefaciens* supernatant contained an antimicrobial peptide that produced 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

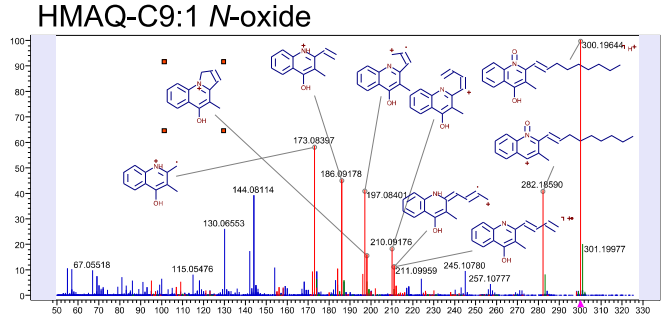












Molecule	Formula	Molecular Weight (Da)	Mutant Area Counts	Wild Type Area Counts
HMAQ-C7:1	C17 H21 N O	255.162	0	6.2241E+08
HMAQ-C7:1 N-Oxide	C17 H21 N O2	271.157	0	7.8290E+06
HMAQ-C8:1	C18 H23 N O	269.178	0	1.0004E+08
HMAQ-C9:1 N-Oxide	C19 H25 N O2	299.189	0	6.2839E+07
HMAQ-C9:1	C19 H25 N O	283.194	0	6.8775E+08

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 Δ*hmqD* supernatant. (A) Chromatograms of Bp82 and Bp82 Δ*hmqD* 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 C_9 alkyl chains. Piochon et al. (27) chemically synthesized HMAQs and their *N*-oxide counterparts with unsaturated C_7, C_8, and C_9 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-$C_{7:1}$ (B), HMAQ-$C_{7:1}$ *N*-oxide (C), HMAQ-$C_{8:1}$ (D), HMAQ-$C_{9:1}$ (E), and HMAQ-$C_{9:1}$ *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-C_9 (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 exempt 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 *N*,*N*-dimethylformamide, and 40 μl was spread onto the surface of plate medium for blue/white screening in *E. coli* TOP10 or *E. cloni* 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 kit (Life Technologies), and transformed into chemically competent *E. coli* 10G (Lucigen). The PCR amplifications were performed in a final reaction volume of 50 or 100 μl containing 1$\times$ 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 with the M13 forward and M13 reverse primers 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.

Tn*Mod*-OKm′ mutagenesis and plasmid conjugations. Tn*Mod*-OKm′ (39) was delivered to Bp82 via conjugation with *E. coli* S17-1 (pTn*Mod*-OKm′) by using a membrane filter mating technique. Briefly, S17-1 (pTn*Mod*-OKm′) 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 $MgSO_4$, 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 $MgSO_4$ 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^r and Pm^r colonies were identified after 48 h of incubation at 37°C. Tn*Mod*-OKm′ contains a Km^r gene and a pMB1 conditional origin of replication that does not function in *B. pseudomallei*, allowing the rapid cloning of DNA adjacent to the transposon's site of insertion in *E. coli*. The *in vitro* cloning of DNA flanking the Tn*Mod*-OKm′ 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 (TnMod-LT2) that binds to the left end of Tn*Mod*-OKm′. 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 Tn*Mod*-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 submersing 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 coinoculation 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 $\sim 1 \times 10^7$ 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 also 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 (OD_{600}) 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-N. bataviensis* 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 Δ*hmqD*. 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) with 0.1% 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 CORTECS T3 column (Waters Corp., Milford, MA; 120 Å, 2.7 μm, 2.1 mm by 150 mm) with a Phenomex (Torrance, CA) SecurityGuard ultrahigh-performance liquid chromatography (UHPLC) phenyl 2.1-mm-inside-diameter (i.d.) 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-alkylquinoline (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 *Priestia megaterium* RS1, MW756952; *Exiguobacterium undae* RS4, MW756953; *Cytobacillus firmus* R14, MW756954; *Bacillus mycoides* R15, MW756955; *Bacillus cereus* ST9, MW756956; *Neobacillus bataviensis* S4, MW756957; *Exiguobacterium acetylicum* R10, MW756958; *Bacillus marisflavi* S9, MW756959; *Paenibacillus* sp. S10, MW756960; *Paenibacillus 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.

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