A Gene Cluster That Encodes Histone Deacetylase Inhibitors Contributes to Bacterial Persistence and Antibiotic Tolerance in *Burkholderia thailandensis*

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ABSTRACT Persister cells are genetically identical variants in a bacterial population that have phenotypically modified their physiology to survive environmental stress. In bacterial pathogens, persisters are able to survive antibiotic treatment and reinfect patients in a frustrating cycle of chronic infection. To better define core persistence mechanisms for therapeutics development, we performed transcriptomics analyses of *Burkholderia thailandensis* populations enriched for persisters via three methods: flow sorting for low proton motive force, meropenem treatment, and culture aging. Although the three persister-enriched populations generally displayed divergent gene expression profiles that reflect the multimechanistic nature of stress adaptations, there were several common gene pathways activated in two or all three populations. These include polyketide and nonribosomal peptide synthesis, Clp proteases, mobile elements, enzymes involved in lipid metabolism, and ATP-binding cassette (ABC) transporter systems. In particular, identification of genes that encode polyketide synthases (PKSs) and fatty acid catabolism factors indicates that generation of secondary metabolites, natural products, and complex lipids could be part of the metabolic program that governs the persistence state. We also found that loss-of-function mutations in the PKS-encoding gene locus BTH_I2366, which plays a role in biosynthesis of histone deacetylase (HDAC) inhibitors, resulted in increased sensitivity to antibiotics targeting DNA replication. Furthermore, treatment of multiple bacterial pathogens with a fatty acid synthesis inhibitor, CP-640186, potentiated the efficacy of meropenem against the persister populations. Altogether, our results suggest that bacterial persisters may exhibit an outwardly dormant physiology but maintain active metabolic processes that are required to maintain persistence.

IMPORTANCE The discovery of antibiotics such as penicillin and streptomycin marked a historic milestone in the 1940s and heralded a new era of antimicrobial therapy as the modern standard for medical treatment. Yet, even in those early days of discovery, it was noted that a small subset of cells (~1 in 10^9) survived antibiotic treatment and continued to persist, leading to recurrence of chronic infection. These persisters are phenotypic variants that have modified their physiology to survive environmental stress. In this study, we have performed three transcriptomic screens to identify persistence genes that are common between three different stressor conditions. In particular, we identified genes that function in the synthesis of secondary metabolites, small molecules, and complex lipids, which are likely required to maintain the persistence state. Targeting universal persistence genes can lead to the development of clinically relevant antipersistence therapeutics for infectious disease management.

KEYWORDS *Burkholderia*, antibiotic resistance, bacterial persistence, stress, transcriptomics

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Many microbes can undergo phenotypic changes and enter a seemingly dormant metabolic state, termed bacterial persistence, as a survival mechanism during periods of environmental stress. In bacteria, these persisters become recalcitrant to lethal doses of antibiotic and subsequently contribute to the onset of chronic infectious disease. Although persisters do not pass antibiotic tolerance to their progeny through genetic inheritance, persistence can exacerbate antibiotic resistance by decreasing antibiotic efficacy, prolonging treatment time, and generating a reservoir of surviving cells from which antibiotic-resistant mutations can evolve (1–3).

Both stochastic and deterministic mechanisms are thought to contribute to bacterial persistence. In the former case, random fluctuations in gene transcription, translation, or protein stability lead to phenotypic variability within bacterial populations (4). Alternatively, in the latter case, phenotypic divergence could be predetermined by epigenetic memory carried in a small subpopulation of cells that slowly recover from nutrient starvation, exposure to toxins, or harsh environmental conditions. The role of stress has been implicated in the establishment of persistence, including the stringent response to starvation, DNA damage, SOS signaling, and oxidative stress. Genes that encode stress response mechanisms were activated in persisters enriched by exposure to antibiotics (5–8). Furthermore, low acidity, temperature variations, and osmotic stress were also shown to significantly increase the number of persisters in bacteria (9–13).

Multiple studies have identified a variety of genes that play a role in persistence. Disrupted equilibrium of toxin-antitoxin (TA) systems, which regulate essential cellular activities, including protein translation, DNA stability, and cell membrane integrity, leads to metabolic shutdown similar to that of the stringent response to stress (14–18). Other studies have profiled persister transcriptomes using various enrichment methods, including antibiotic lysis of susceptible bacterial populations (5, 19, 20), flow cytometry based on dormancy markers (6), and analysis of stationary-phase bacteria (8, 11, 21). However, the already highly heterogeneous nature of persisters has been further confounded by the different stressor conditions used between the experimental groups, thus generating transcriptomic data sets that were biased to the particular phenotypic assay. Currently, there is no single essential persistence gene or mechanism that can be targeted for development of antipersistence drugs.

To identify core persistence genes that are ubiquitously activated in response to various stress conditions, we performed transcriptomics analyses of *Burkholderia thailandensis* persisters enriched by three experimental conditions, flow sorting for low proton motive force (PMF), meropenem (Mpm) treatment, and culture aging. *B. thailandensis* E246, a nonpathogenic saprophyte, shares close genetic similarity with two highly infectious pathogens, *Burkholderia mallei* and *Burkholderia pseudomallei*, classified as select agents due to their high mortality rates and ability to establish chronic infections with high percentage of relapse (22). Although lacking the virulence factors found in its two closely related pathogens, *B. thailandensis* E264 carries highly conserved genes that regulate antibiotic resistance and stress adaptation mechanisms (23) and is used as a surrogate for *B. pseudomallei* in infection studies *in vitro*. We had previously shown that *B. thailandensis* harbors a high level of persisters within logarithmically expanding populations (24). Furthermore, *Burkholderia* species encode various stress adaptation mechanisms that can drive the persistence phenotype, including efflux pumps, biofilm formation, quorum sensing systems, and various catabolite repression/activation systems known to induce the stringent response (25–28).

In this study, we identified genes encoding polyketide synthases (PKSs) and fatty acid catabolism factors that are activated in persisters enriched via various stress conditions, suggesting that generation of secondary metabolites, natural products, and complex lipids can contribute to resiliency to antibiotics. These *B. thailandensis* PKS systems have been previously characterized as factories that generate a novel class of histone deacetylase (HDAC) inhibitors (29). Given the role of HDAC inhibitors as suppressors of the host immune response to microbial infections (30), our study has uncovered a link between persistence and the establishment of chronic infections. Identification of universal persistence genes or mechanisms that leads to the develop-
Characterization of persister phenotype. (A) Analysis of proton motive force (PMF) using fluorescence-activated cell sorting (FACS). Mid-log-growth-phase *B. thailandensis* cultures (OD_{600} of 0.2 to 0.3) were incubated in LB-Miller broth containing 50 μg/ml SHX, 20 μg/ml Ofl, or 20 μg/ml Mpm for 2 h at 37°C prior to addition of 1 μg/ml DiBAC_{4}(3). Stationary cells (STAT) were collected from 14-day-old cultures on LB-Miller nutrient agar plates and incubated for 30 min at 37°C in LB-Miller broth containing 1 μg/ml DiBAC_{4}(3). Flow cytometry analysis was performed on the BD FACSAria instrument using a fluorescein isothiocyanate (FITC) filter (Em: 530/30) after excitation with the 488-nm laser. Mid-log-phase cells not treated with DiBAC_{4}(3) were used to position the signal threshold gate. One representative experiment out of five independent replicates is shown. (B) Metabolic index of *B. thailandensis* sorted for DiBAC_{4}(3) retention. Mid-log-phase *B. thailandensis* cultures were treated with 1 μg/ml DiBAC_{4}(3) and sorted into subpopulations with low FI (FITC-A, 500 to 10^3 fluorescence units [FU]) and high FI (FITC-A, 10^3 to 10^6 FU). Ten thousand cells were collected by FACS and combined with BacTiter-Glo reagent to quantify cellular ATP. Presented are the average and standard deviation values of percent bacterial survival calculated from 10 CFU inocula originating from three independent bacterial populations. (C) Evaluation of bacterial tolerance to cytotoxic antibiotic concentrations. (Left) Mid-log-phase *B. thailandensis* cultures were treated with 1 μg/ml DiBAC_{4}(3) and sorted to subpopulations with low FI (FITC-A, 500 to 1,000 FU) and high FI (FITC-A, 10^3 to 10^6 FU). Ten thousand cells were collected by FACS and grown to early log phase (OD_{600} of ~0.2) prior to addition of 10 μg/ml Mpm. Persister populations surviving 24 h of antibiotic treatment were calculated as CFU/ml recovered on LB-Miller agar plates after antibiotic treatment relative to the CFU/ml prior to antibiotic exposure. Average and standard deviation values were derived from three independent experiments. (Middle) Mid-log-phase *B. thailandensis* cultures were subjected to 10 μg/ml Ofl or 10 μg/ml Mpm for 24 h. Percentage of surviving persisters was determined after 24 h of antibiotic exposure. Average and standard deviation values were derived from five independent experiments. (Right) A single CFU from 1-, 7-, 14-, and 21-day-old *B. thailandensis* cultures was inoculated into LB-Miller broth and cultured at 37°C until OD_{600} reached ~0.2, followed by addition of 10 μg/ml Ofl for 24 h. Presented are the average and standard deviation values of percent bacterial survival calculated from 10 CFU inocula originating from three independent bacterial populations.

RESULTS

Phenotypic screens for enrichment of *B. thailandensis* persister populations. To cast a wide net for discovery of genes and gene networks involved in bacterial persistence, we applied multiple methods to enrich for *B. thailandensis* persisters, including flow sorting for low proton motive force (PMF), cell lysis using the β-lactam antibiotic meropenem (Mpm), and culture aging on solid medium via nutrient starvation. First, we found that a subpopulation (~0.5%) of mid-log-phase *B. thailandensis* intracellularly retained the fluorescent dye DiBAC_{4}(3), signifying membrane depolarization (Fig. 1A). Bacteria normally maintain an electric potential gradient across their cytoplasmic membrane, which is essential for ATP synthesis. We further observed that the percentage of cells that retained DiBAC_{4}(3) increased upon treatment with the amino acid analog serine hydroxamate (SHX; 10%), an inhibitor of cellular metabolism.
through the stringent response (31), and antibiotics such as ofloxacin (Ofl; 6%) and Mpm (38%) (Fig. 1A).

We next isolated exponentially dividing cells that exhibited high and low fluorescence intensity (FI) upon incubation with DiBAC₄(3) and found that FI was inversely correlated with metabolic index, as measured by the total ATP pool (Fig. 1B). Upon expansion of sorted cells in nutrient-rich medium, we observed a >100-fold increase in the survival rate of cells that originated from populations sorted for high FI compared to low FI after 24-h treatment with twice the minimal bactericidal concentration (2× MBC) of OfI (Fig. 1C, left panel). All together, these results suggest that persisters exhibit low PMF compared to wild-type cells and that we can leverage this property to sort persisters that display enhanced DiBAC₄(3) uptake via increased fluorescence. A decrease in PMF has been previously linked to cell dormancy and multidrug tolerance phenotype in bacterial persistence (32–35).

Our second assay was based on enrichment for Burkholderia persisters through lysis of the actively dividing cells with Mpm, a broad-spectrum carbapenem antibiotic that inhibits cell wall synthesis. In a previous study, we had reported varied potencies of antibiotics against B. thailandensis persisters, suggesting that there are different mechanisms that lead to persister establishment (24). Treatment with Mpm has led to improved outcomes in melioidosis patients diagnosed with severe sepsis (36). We found that Mpm is less effective at killing Burkholderia persisters than the DNA gyrase inhibitor OfI (Fig. 1C, middle panel). Furthermore, treatment with the MIC of Mpm significantly increased the population of B. thailandensis cells with low PMF (and high FI) within the span of one replication cycle, 2 h of exposure (Fig. 1A). For our transcriptome studies, we collected RNA from bacterial populations that survived 2× MIC of Mpm after more than 2 cycles of replication, with ~80% enrichment of persister populations (see Fig. S1A in the supplemental material).

Finally, we subjected cells to nutrient starvation by long-term incubation on LB-Miller agar plates at 4°C, as a model for population aging and biofilm-induced resistance to antibiotics that could naturally occur in nutrient-limited soil microcosms. In this case, population aging is comparable to long-term stationary phase, bearing the complexity of respiratory and metabolic adaptations that are quite different from aging resulting from asymmetrical division. We observed a gradual increase in the survival rates of B. thailandensis persisters isolated from 7-, 14-, and 21-day-old cultures on agar plates (Fig. 1C, right panel). As a measure of tolerance, we used two doses of MBC of OfI that normally killed ≥99.9% of the bacterial populations isolated from a 1-day culture on LB agar. At least ~60% of the cells collected from 14-day-old cultures had low PMF (Fig. 1A, B.th. STAT). In summary, our transcriptome screens were based on (i) physical separation of persisters via cell sorting (low PMF), (ii) ~80% persister enrichment following Mpm treatment (Mpm), and (iii) aged bacterial populations harboring ~60% of cells with the persister phenotype (aged).

**Genome-wide transcriptome analysis of persisters.** We performed RNA Mi-Seq transcription profiling of B. thailandensis gene expression patterns (i) in DiBAC₄(3)-treated mid-log-phase cultures sorted by high FI (low PMF), (ii) in cells lysed with 2× MBC of meropenem for 4 h (Mpm treated), and (iii) following 21 days of aging on nutrient agar at 8°C (aged). We analyzed relative changes in gene expression between persister-enriched and the following controls processed in parallel: (i) DiBAC₄(3)-treated cells sorted for low FI, (ii) untreated exponential cultures, and (iii) cells following 1 day of growth on nutrient agar.

Hierarchical clustering analysis of the global transcriptomes revealed good data reproducibility between experimental replicates, where control samples from all tested conditions clustered together and showed distinct gene expression patterns from the persister-enriched samples (Fig. S1B). Applying principal-component analysis (PCA), we observed that the transcriptome of the aged population showed the most distinct differential gene expression (Fig. 2A). The persisters enriched via cell sorting for low PMF or exposure to Mpm displayed relatively convergent global gene expression patterns compared to the other...
profiles to their respective control groups. These results suggest that the mechanism governing resilience in the aged populations differs significantly from that of bacterial membrane depolarization and Mpm tolerance. We also note that the low-PMF controls (red dots in Fig. 2) clustered with the controls from the other phenotypic assays on the left side of the plot, signifying that DiBAC4(3) dye itself did not contribute to the persister phenotype through interference with bacterial membrane function.

DESeq statistical analysis revealed that persisters with low PMF had the highest number (300) of differentially expressed genes (log2-fold) in persister populations enriched via three methods (low PMF, Mpm, and aged). The P value cutoffs applied for differentially regulated genes were ≤0.001 for the populations with low PMF, and P values of ≤0.01 were used for Mpm-treated and aged populations featuring higher variability. Colored ovals in the legend show five functional groups of interest with gene representatives differentially expressed in persister-enriched populations.

**FIG 2** Comparative analysis of genome-wide transcriptomics data from three persistence phenotypic screens. (A) Principal-component analysis of RNA-seq data was performed using DESeq2 as part of PiReT analyses on the EDGE bioinformatics platform. The control and persister populations for the three experimental methods are indicated in the legend. (B) Comparison of the number of differentially expressed genes (log2-fold) in persister populations enriched via three methods (low PMF, Mpm, and aged). The P value cutoffs applied for differentially regulated genes were ≤0.001 for the populations with low PMF, and P values of ≤0.01 were used for Mpm-treated and aged populations featuring higher variability. Colored ovals in the legend show five functional groups of interest with gene representatives differentially expressed in persister-enriched populations.

Genes encoding polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs) were upregulated in persisters from all three screens (Fig. 2B and Fig. 3A). PKSs polymerize simple fatty acids into a large variety of secondary metabolite products
called polyketides. Due to the complexity of polyketide synthesis, PKS genes are organized into operon systems (Fig. S2A). There are 11 gene clusters with predicted PKS and NRPS functions in the *Burkholderia* genome (29). We found PKS genes from two operons, including BTH_I2366 and BTH_I1677, to be upregulated in low-PMF and Mpm-treated persisters, whereas aged persisters showed upregulated PKS genes located only on chromosome I (Fig. 3A). PKS operons often include NRPS genes that work in cooperation with PKSs to synthesize polyketide-peptide hybrid macromolecules. An NRPS gene, BTH_I1956, located in the PKS operon on chromosome I was upregulated in both low-PMF and aged persisters (Fig. 3A).

Four genes that encode various classes of Clp proteases (BTH_I1046, BTH_I1895, BTH_I0140, and BTH_I1476) were primarily upregulated in persisters with low PMF (Fig. 3B). The ATP-binding subunit of type B Clp protease (BTH_I1895) was expressed at higher levels in all three screens. Clp proteases have been previously linked to persistence through proteolytic regulation of toxin-antitoxin systems (5, 37) and participate in bacterial stress response mechanisms via the degradation of misfolded proteins (38, 39) and activation of outer membrane biosynthesis enzymes (40).

The three screens yielded other upregulated genes that likely play a role in regulating bacterial persistence. We observed overexpression of mobile elements in bacteriophage operon genes from primarily the low-PMF screen (Fig. 3C and Table S1).
Bacteriophage genes have previously been associated with biofilm formation and antibiotic tolerance in *Pseudomonas aeruginosa* (41, 42). Genes regulating lipid biosynthesis were identified in all three screens (Fig. 2B, Fig. 3D, and Tables S1 to S3). We detected upregulation of multiple gene loci involved in fatty acid catabolism in persisters, including acyl coenzyme A (acyl-CoA) dehydrogenase (ACAD), which catalyzes fatty acid β-oxidation (BTH_I0060 and BTH_I3298), and long-chain acyl-CoA ligase, which catalyzes the breakdown of complex fatty acids (BTH_I2096 and BTH_I2529) (43–45). ATP-binding cassette (ABC) transporter systems for amino acids, such as the arginine/ornithine transporter (BTH_I2383), were upregulated in persisters from the low-PMF and aged screens (Fig. 2B, Fig. 3E, and Tables S1 and S2).

We also noted other interesting gene expression patterns among the three screens. *N*-acytymuramoyl-L-alanine amidases (NAMLAAs), which function in bacterial cell wall remodeling, were upregulated in cells with low PMF (BTH_I0722) and Mpm treatment (BTH_I10537) but remained silenced in aged cells (Tables S1 to S3). Finally, the gene encoding MarR, multiple antibiotic resistance transcription factor (BTH_I2391), was upregulated in aged cultures but not in cells exposed to Mpm (Tables S2 and S3).

**Validation of RNA-seq differential gene expression using RT-qPCR.** To validate the RNA sequencing (RNA-seq) results, we performed targeted RT-qPCR using culture aging (for 7, 14, and 21 days) and antibiotic treatment (Mpm, Ofl, and ceftazidime (Cef) at 1× MBC) as stress factors for the enrichment of persister cells (Table 1). We observed consistent upregulation of the PKS gene BTH_I2366 in response to all tested aging and antibiotic stress conditions. NRPS (BTH_I1956) expression was upregulated in response to the three antibiotics but not in the aged cultures. Upregulation of PKS and NRPS transcripts was the only common gene expression pattern seen by RT-qPCR across bacteria treated with each of the three antibiotics. Genes encoding ClpP (BTH_I1046) and ClpA/B (BTH_I1040) were upregulated in persisters from both aging and antibiotic stress cultures. However, we noted that Ofl treatment inhibited *clp* expression compared to the β-lactam antibiotics Mpm and Cef. We observed a similar divergent effect of Ofl on the cell wall-remodeling enzyme NAMLA (BTH_I10537), which was downregulated in *Burkholderia* treated with Ofl but upregulated upon Mpm and Cef treatment. The gene encoding HK97 phage major capsid protein (BTH_I1047) was upregulated in the aged cultures but not in response to antibiotics. The RT-qPCR results also confirmed the RNA-seq data showing that MarR (BTH_I2391) was upregulated in the aged (14- and 21-day) cultures. Additionally, MarR transcript levels were increased in response to Cef but remained the same with Mpm and Ofl treatment, indicating divergent cell responses to different antibiotics and mechanisms of action.

**Polyketide synthase gene function augments bacterial resilience to antibiotics targeting DNA replication.** Upon identification of the PKS gene BTH_I2366 as a general persistence biomarker in *B. thailandensis* (Fig. 3A), we further investigated its role in bacterial survival. We tested the antibiotic sensitivity of three transposon mutants (BT02489, BT06658, and BT08684), generated by random insertion of Tn23 transposon sequences within the BTH_I2366 gene locus (46). Treatment with quinolone
antibiotics Ofl and ciprofloxacin (Cipro), which inhibit DNA replication, led to a significant decrease in the number of persisters in the mutants compared to the wild type (WT), indicating that transposon mutagenesis of BTH_I2366 increased the susceptibility of persister cells to antibiotic killing (Fig. 4A). Among the mutant strains, BT08684 exhibited the highest variability in persister cell survival upon antibiotic treatment. This observation prompted the study of possible reverse BT08684 mutations, resulting from spontaneous Tn23 excisions. PCR analysis of genomic DNA from BT08684 cell populations that exhibit wild-type sensitivity to Ofl displayed loss of the transposon insertion in the BTH_I2366 gene (Fig. S2B), thus validating the role of PKS gene function in antibiotic tolerance.

Complementation of the BTH_I2366 mutant background is challenging due to the broad-spectrum antibiotic resistance of the mutant strains, both natural (resulting from

![Graph showing persister population (%)](image)

**FIG 4** PKS gene activity is a determinant of *B. thailandensis* persistence. (A) PKS BTH_I2366 loss of function increased *B. thailandensis* sensitivity to antibiotics that target DNA replication. Mid-log-growth bacterial cultures (OD$_{600}$ of ~0.2 to 0.3) were treated with 1× MBC of Mpm (10 μg/ml), Ofl (10 μg/ml), and Cipro (5 μg/ml) for 24 h. The percentages of persister populations were determined as the ratio of antibiotic-surviving CFU/ml versus the cell number (CFU/ml) prior to antibiotic exposure. Presented are data from four independent antibiotic treatment experiments. The significance of the registered sensitivity to antibiotic treatment was evaluated using one-way ANOVA, where # indicates a P value of <0.002. (B) The PKS-encoding BTH_I2366 gene is responsible for membrane depolarization upon Mpm treatment. Wild-type *B. thailandensis* and mutant strains were grown to mid-log phase (OD$_{600}$ of 0.2 to 0.3) and were treated with 10 μg/ml Mpm for 3 h. Bacterial cultures were incubated in 1 μg/ml DiBAC$_{4}(3)$ for 30 min prior to flow cytometry analysis on the BD FACS Aria using an FITC filter (Em: 530/30). Gate P2 (top panel) represents the population of cells with low PMF that retain the fluorescent dye, shown in green. The total analyzed bacterial population (P1) is shown in red. Data are representative of five independent experiments.

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Complementation of the BTH_I2366 mutant background is challenging due to the broad-spectrum antibiotic resistance of the mutant strains, both natural (resulting from
β-lactamase activity or efflux pump-mediated exclusion of aminoglycosides) and acquired (Tn23-associated trimethoprim resistance [Tpr]), that limited our ability to use selective pressure for transgene delivery. Thus, we generated a *B. thailandensis* strain that expresses green fluorescent protein (GFP) with a TpR selection marker incorporated 25 bp downstream of the glucosamine-6-phosphate synthetase (GlmS) stop codon using the mini-Tn7 system. This *B. thailandensis* strain, designated *B. thailandensis* TpR-Tn7, served as a persistence control to evaluate the impact of transposon-acquired antibiotic resistance from a mobile element. We did not observe a significant difference in the survival rates of *B. thailandensis* wild type (WT) and the *B. thailandensis* TpR-Tn7 strain (Tn7-G) when treated with quinolone antibiotics (Fig. 4A).

Interestingly, treatment with Mpm led to a different outcome from that with the antibiotics targeting DNA replication (OfI and Cipro). First, we observed no difference between the survival rates of persisters in wild-type *B. thailandensis* and in BTH_I2366 mutants treated with Mpm (Fig. 4A). Second, there was a marked decrease in the number of DiBAC₄(3)-stained cells (~1 to 2%) for BTH_I2366 mutants BT02489 and BT06658 compared to wild type (20%) in response to Mpm, indicating that BTH_I2366 mutagenesis counteracted the membrane depolarization induced by an antibiotic that targets cell wall synthesis (Fig. 4B). Treatment with OfI slightly increased the population of persisters with low PMF, at ~6 to 8% compared to 40% with Mpm exposure (data not shown). However, we did not observe any significant difference between DiBAC₄(3) staining in the wild type and in the PKS mutants exposed to quinolone antibiotics. Collectively, these data indicate that PKS function contributes broadly to the establishment of a persistence phenotype in *B. thailandensis* exposed to antibiotics.

Natural products with HDAC inhibitor properties regulate DNA synthesis in *B. thailandensis*. A previous study had identified that BTH_I2366 is part of a megaenzyme PKS/NRPS gene cluster complex that produces natural products in the form of acyldepsipeptides with histone deacetylase (HDAC) inhibitor properties, termed burkholdacs (29). In that study, a galactose-regulated promoter (Ptac) was engineered to drive PKS/NRPS overexpression and the resultant natural products were synthetically modified via dithiothreitol reduction to increase their potency as HDAC inhibitors. Here, we observed that the conditioned medium of wild-type cells treated with Mpm for at least 12 h exhibited ~70% HDAC inhibition, indicating release of secondary metabolites with HDAC-inhibitory properties via Mpm-induced cell lysis (Fig. 5A). HDAC inhibition in conditioned medium was reduced to ~30 to 40% in Mpm-treated BTH_I2366 mutants, indicating that BTH_I2366 function is required for efficient production of metabolites with HDAC-inhibitory properties in response to antibiotic stress.

Given the key role of HDAC inhibitors in stimulating DNA replication in both eukaryotes (47) and bacteria (48), we measured DNA content in both the wild type and BTH_I2366 mutants by flow cytometry. DNA content was decreased by ~50% in the BTH_I2366 mutants compared to wild-type cells in the mid-log phase, consistent with reduced expression of HDAC inhibitors in the mutants (Fig. 5B). Interestingly, DNA content was comparatively increased in the BTH_I2366 mutants in stationary phase, suggesting that secondary metabolites with HDAC-inhibitory properties may negatively regulate DNA accumulation in stationary-phase bacteria. We found no difference in the rate of cell growth between the wild-type and mutant strains in nutrient-rich medium (data not shown).

The fatty acid synthesis inhibitor CP-640186 enhanced antibiotic activity against persister populations of various Gram-negative bacteria. Polyketide synthase function is closely linked to fatty acid synthesis, and the two pathways share common components, including acetyl-CoA and malonyl-CoA (49). We tested the effect of CP-640186, an inhibitor of mammalian acetyl-CoA carboxylase (mACC1/2), which catalyzes the synthesis of malonyl-CoA, on the survival of *B. thailandensis* persisters. Since the compound exhibited no antibacterial activity on its own (data not shown), we combined CP-640186 with Mpm treatment and observed an ~10-fold decline in persister survival in wild-type *B. thailandensis* (Fig. 6A). Interestingly, we registered no additive effect of CP-640186 on OfI potency against the persister population.
We further tested the effect of CP-640186 on Mpm potency against persisters from other Gram-negative pathogens, including *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium, and an attenuated strain of *Yersinia pestis* A1122. Upon addition of CP-640186 to Mpm treatment, we observed -3-, 4-, and 8-fold decreases in the percentage of *P. aeruginosa*, *S. Typhimurium*, and *Y. pestis* persister cells, respectively, compared to antibiotic monotherapy (Fig. 6B). Altogether, our results indicate that fatty acid metabolism is a viable target for development of antimicrobials to combat bacterial persistence.

**DISCUSSION**

Bacterial persistence is a transient survival mechanism that mediates noninherited antibiotic resistance and contributes to extended latency in chronic disease (2). Persisters comprise a small fraction (0.0001% to 1%) of isogenic bacterial populations and exhibit highly dynamic phenotypic changes in response to a variety of environmental stress conditions (52–54). Our functional genomics studies indicate that nutrient starvation and antibiotic toxicity stimulate gene clusters that drive production of secondary metabolites with HDAC-inhibitory properties. These natural products contribute to the establishment of a persistence phenotype manifested in bacterial membrane depolarization, low metabolic index, and bacterial resilience toward antibiotics targeting DNA and protein synthesis. A statistical comparison of the global transcriptome profiles
showed that persisters enriched by the three methods generated very different gene expression patterns, underscoring the multimechanistic and complex nature of bacterial persistence and antibiotic tolerance that can shape cellular phenotypic behaviors. However, there were also pathways activated in common.

We found upregulation of multiple gene loci that encode PKSs, in particular BTH_I2366, in B. thailandensis persisters enriched by all three phenotypic screens. PKSs form large modules of megaenzymes that synthesize a variety of natural products and complex lipids needed for cell growth and metabolism (55). Previous studies have characterized B. thailandensis natural products produced by the gene cluster that encompasses BTH_I2366 as histone deacetylase inhibitors (termed burkholdacs) (29). One possible role for the burkholdacs in bacterial cells is regulation of posttranscriptional modification (PTM) of proteins upon onset of stressful conditions to allow for quick adaptations. PTM through lysine acetylation is an evolutionarily conserved strategy for regulation of enzyme activity, DNA-protein interactions, and protein stability (48, 56, 57). Although the role of burkholdacs in global bacterial metabolism remains to be determined, our study underscores their contribution to bacterial resilience to antibiotics.

It remains an open question whether pathogenic Burkholderia species synthesize HDAC inhibitors in response to stress, including antibiotic exposure, to counteract the immune defenses of the eukaryotic host. Treatment of B. thailandensis with the antibiotic trimethoprim has been previously shown to induce synthesis of a variety of secondary metabolites (many of them PKS and NRPS products) that act as quorum-sensing molecules or antibiotics (58). Burkholderia species, including the highly pathogenic select agent B. pseudomallei, are known for their large genomes that contain multiple pathogenic islands and large numbers of natural product biosynthetic gene clusters (59). For example, gene cluster BTH_II0204–207/BPSS0130–133, which is highly conserved between B. thailandensis E264 and B. pseudomallei K96243, was found to produce chemical analogs of betulinan and terferol, which are potent inhibitors of eukaryotic phosphodiesterase (PDE) (60). As a catalyst for cAMP and cGMP hydrolysis, PDE has been implicated in host inflammatory responses and represents a therapeutic

FIG 6 Acetyl-CoA carboxylase inhibits persister formation in Gram-negative bacteria. (A) Combination of Mpm with acetyl-CoA carboxylase inhibitor CP-640186 reduced persisters in B. thailandensis. Mid-log-phase cultures (OD_{600} of 0.2 to 0.3) of B. thailandensis were treated with 10 μg/ml Mpm, 10 μg/ml Ofi, or a combination of antibiotic and 50 μg/ml CP-640186 for 24 h. The percentage of persisters surviving antibiotic treatment was calculated as previously described. Shown are average and standard deviation values derived from four independent experiments. (B) CP-640186 potentiated Mpm activity against persisters in three Gram-negative bacterial pathogens. Mpm at 2× MBC alone or in combination with 50 μg/ml CP-640186 was added to each bacterial species (OD_{600} of ~0.2). Persister populations were evaluated after 24 h of antibiotic exposure as described above. Shown are average and standard deviation values derived from four independent experiments.
target for inflammatory disease (63, 64). PKS-encoding gene islands are not specific to the *Burkholderia* genus and have been previously associated with pathogenicity in the gut microflora. A genotoxin encoded by the *pks* pathogenicity island in *Escherichia coli* was found to induce DNA double-strand breaks in host mammalian cells, resulting in higher mutation rates (61, 62).

In addition to PKSs, we also identified genes that regulate lipid catabolism in persisters. PKSs are analogous in architecture to, and share sequence homology with, type I fatty acid synthases, such as FAS-I, responsible for *de novo* fatty acid synthesis (49, 65). The two enzyme assembly lines share homologous domains (acyl carrier protein [ACP]) that carry the growing polyketide or fatty acid chains and utilize acyl-CoA and malonyl-CoA as precursors (49). We observed that Mpm treatment in combination with the inhibitor of malonyl-CoA synthesis (CP-640186) resulted in a decrease of persisters in multiple pathogens, including *B. thailandensis*, *P. aeruginosa*, *S. Typhimurium*, and attenuated *Y. pestis*. Our findings suggest that targeting acetyl-CoA carboxylase enzymatic function could be a viable therapeutic strategy for the elimination of persister populations in Gram-negative bacteria.

Fatty acids are a major component of the bacterial cell membrane and an important source of metabolic energy. Intermediates of the saturated fatty acid biosynthetic pathway provide short-chain 3-hydroxy fatty acids that are needed for synthesis of biotin, the lipid A component of Gram-negative bacteria outer membranes, and acyl-homoserine lactones (AHLs) of quorum-sensing molecules (66–68). The by-products of fatty acid degradation can fuel pathways for amino acid synthesis and energy production or serve as building blocks for a variety of secondary metabolites. We detected upregulation of multiple gene loci involved in fatty acid catabolism in persisters enriched by at least two of the three phenotypic screens, including acyl-CoA dehydrogenase (ACAD), which catalyzes fatty acid β-oxidation (BTH_I0060 and BTH_I3298), and long-chain acyl-CoA ligase, which catalyzes the breakdown of complex fatty acids (BTH_I12096 and BTH_I2529) (43–45). Other groups have reported activation of bifunctional enoyl-CoA hydratase (BTH_I10417) upon exposure of *B. thailandensis DW503* to 10× MIC of the fluoroquinolone antibiotic ciprofloxacin and the DNA-damaging chemical mitomycin C (8, 69). Enoyl-CoA hydratase is an essential enzyme in fatty acid β-oxidation, which catalyzes the complete oxidation of long-chain fatty acids to provide an energy source and building blocks for primary and secondary metabolites.

Our transcriptomics studies found that ~10% of genes differentially upregulated in persister populations encoded ABC transporters (Fig. 3E; see also Tables S1 to S3 in the supplemental material). ABC transporter systems are essential for the import and export of peptides, proteins, polysaccharides, and many other molecules to maintain metabolic homeostasis in the cell (70). All ABC transporter systems share a highly conserved ATP-hydrolyzing domain that couples ATP binding/ hydrolysis to the translocation of essential nutrients or expulsion of antibiotics from the periplasm, thus contributing to drug resistance (76). Various gene loci encoding branched-chain amino acid ABC transporters were upregulated in both the aged and low-PMF persister populations (Fig. 3E and Tables S1 and 2). Persisters with low PMF had 38 upregulated ABC transporter genes, including sugar and amino acid transporters (Table S1). Branched-chain amino acid ABC transporters and peptide ABC transporters have previously been reported to be significantly upregulated in stationary-phase *Listeria monocytogenes* and *B. thailandensis DW503* treated with 10× MIC of ciprofloxacin (8, 77). Additionally, other genes encoding ABC transporters were upregulated in *B. thailandensis* with low PMF (BTH_I11288 and BTH_I0880, Table S1) and in aged populations of *L. monocytogenes* (77).

Several independent transcriptomics studies have previously implicated bacteriophage and mobile element activation as universal stress response mechanisms that may contribute to antibiotic tolerance. For example, ciprofloxacin treatment of *B. thailandensis* at 10× MIC was found to stimulate bacteriophage encoding genomic islands and SOS response genes, and sublethal concentrations of β-lactam antibiotics...
activated phage development in various bacterial species, including *Burkholderia cepacia* (8, 78). Interestingly, we observed upregulation of phage operons in *B. thailandensis* persisters enriched by low PMF and aging but not in response to Mpm (Fig. 3C and Tables S1 to S3). These results demonstrate that species-specific gene regulatory mechanisms might drive divergent cellular responses to various antibiotic classes. Activation of bacteriophage operons in response to environmental stress can shape microbial evolution and accelerate genetic adaptations leading to antibiotic resistance (79).

Collectively, our results support a model in which bacterial persisters exhibit an outwardly dormant physiology but sustain active metabolic processes that are required to maintain persistence. We found that antibiotic treatment activated a *Burkholderia* gene cluster (BTH_12363–12369) that produces natural products with known HDAC-inhibitory effects on host immune responses. Our study also sheds light on the role of fatty acid catabolism in antibiotic tolerance, identifying PKS systems as biomarkers of persistence. The modular architecture of the PKS megaenzymes provides a highly adaptable platform for the generation of polyketides with diverse structures and biological activities. Further studies will be needed to understand the mechanistic link between the antibiotic-induced production of natural products with HDAC-inhibitory properties and the establishment of persistent infections through suppression of host immune defenses. We expect that a more comprehensive understanding of the persistence state will enhance our ability to develop broad-spectrum, high-efficiency inhibitors to target persister populations.

**MATERIALS AND METHODS**

**Bacterial strains, growth conditions, and reagents.** *B. thailandensis* E264, *P. aeruginosa* Migula (ATCC 25347), *S. Typhimurium* (Loeffler) Chi4064 (ATCC 53648), and attenuated *Y. pestis* A1122 bacterial cultures were maintained on LB-Miller agar plates at room temperature. To test antibiotic sensitivity and metabolic activity, cultures initiated from single colonies were grown overnight in 5 ml LB-Miller broth (ThermoFisher Scientific) at 37°C with agitation at 120 rpm. To make mid-log cultures, overnight stationary cultures were diluted 500-fold in fresh LB-Miller broth and incubated at 37°C with agitation at 120 rpm for 4 to 5 h until culture density reached an optical density of 600 nm (OD$_{600}$) of 0.2 to 0.3. Antibiotics were acquired from Sigma-Aldrich (St. Louis, MO): M2574 (Mpm), C0690500 (Cef), 1478108 (Ofl), 17850 (Cipro), and P20362 (CP-640186). The minimal bactericidal concentration (MBC) of antibiotics was determined for each new batch prior to performing persister assays according to standard CLSI protocols for MIC. MIC was determined as the lowest antibiotic concentration at which the OD$_{600}$ remained <0.5 during 24 h of bacterial culture with agitation. MBC was calculated as the antibiotic concentration at which ~90% loss of population viability was achieved.

**Metabolic index determination.** We measured the total ATP pool in bacterial populations using the BacTiter-Glo microbial cell viability assay (Promega, Madison, WI). We added equal volumes (100 μl) of BacTiter-Glo reagent and bacterial culture (100 μl) each and measured luminescence in white 96-well plates (Corning) using the BioTek Synergy HTX microplate reader. The cell number for the corresponding bacterial populations was derived from the OD$_{600}$ measurements correlated with CFU/ml. Cell number was derived from a standard curve reflecting the linear correlation between OD$_{600}$ (ranging from 0.1 to 0.5) and the CFU/ml. Metabolic index was calculated as the ratio between the ATP pool enumerated as relative light units (RLU) and cell number.

**Enumeration of persister populations.** We measured persister populations as a percentage of surviving cells after 24 h of antibiotic treatment (at 1× MBC or higher dose) relative to the number of cells (CFU/ml) prior to antibiotic exposure. Limiting dilutions (log$_{10}$) of bacterial cultures were plated before and after antibiotic treatment on antibiotic-free LB-Miller agar plates and incubated at 37°C for 24 h (*P. aeruginosa* and *S. Typhimurium*) or 48 h (*B. thailandensis*) or up to 5 days (*Y. pestis* A1122). In parallel, bacterial cultures were plated on LB-Miller agar plates containing antibiotic at 1× MIC to evaluate the occurrence of genetic antibiotic-resistant mutants.

**Enrichment of persister populations and RNA extraction.** We applied three different methods to enrich for *B. thailandensis* persisters for RNA-seq transcriptomic analysis.

1. **Low PMF.** We treated mid-log-phase *B. thailandensis* cultures (OD$_{600}$ of 0.2 to 0.3) with 1 μg/ml DiBAC$_4$(3) for 30 min, washed bacteria twice in dye-free LB-Miller broth, and resuspended bacteria in fresh LB broth to a density of OD$_{600}$ of ~0.1. Persisters were sorted using a blue laser (excitation (Ex)/emission (Em), 488/530(30) nm, respectively) with the BD FACSAria instrument (Becton Dickinson, NJ) (see Fig. S1A in the supplemental material). Total RNA was isolated from 10⁵ cells sorted for either high or low Fl. Collection of 10⁵ cells with high Fl was a lengthy process (about 4 h) given the low frequency of this population (0.5 to 1%). To restrict changes in the global transcriptome during sorting, cells were stained, sorted, washed, and resuspended in QiAzo lysis reagent (Qiagen, Germantown, MD) in batches within 15-min intervals.
(iii) Mpm-treated. We exposed *B. thailandensis* to 2× MBC of Mpm at the mid-log phase (OD_{600} of ~0.3) for 4 h based on our previous observation that the metabolic index increased rapidly within 1 to 3 h of antibiotic exposure followed by a sudden drop (24). Furthermore, we had detected ~80% absorption of the fluorescent dye DiBAC$_{4}(3)$ at this time point, which was indicative of high enrichment for persisters (Fig. S1A). Untreated *B. thailandensis* populations were used for the control samples. RNA was isolated from three independent bacterial cultures per experiment.

(iii) Aged. RNA was isolated from *B. thailandensis* cultured on LB-Miller agar plates for 21 days at 8 to 10°C. In addition to temperature stress and nutrient limitation, the low temperature allowed for the retention of moisture within the agar medium, which was essential for cell viability. Approximately 50 CFU was scraped from the agar cultures, resuspended in 1 ml phosphate-buffered saline (PBS), and incubated with 1,000 U lysozyme for 15 min at 37°C prior to lysing the cells in QiAprep reagent. Total RNA was isolated using the miRNeasy minikit (Qiagen) (24). Total RNA for the control samples was harvested from 1-day-old cultures and processed in parallel to the aged populations. Three independent starter cultures were used for sample preparation.

**RNA-seq procedure and analysis.** We utilized the RNA 6000 Pico kit (Agilent, catalog no. 5067-1513) to evaluate quality of RNA isolated from the bacterial cultures. Total RNA was depleted of rRNA using the Ribo-Zero Gram-negative bacterium kit (Illumina, catalog no. MR2GN126), and the concentration was determined with the Qubit RNA high-sensitivity (HS) assay (ThermoFisher Scientific, catalog no. Q32855). cDNA libraries were generated with the ScriptSeq v2 library preparation kit (Illumina, catalog no. SSV21106), titrated with the Qubit double-stranded DNA (dsDNA) HS assay (ThermoFisher Scientific, catalog no. Q32854), and sequenced on the Illumina Mi-Seq platform, generating paired-end 151-bp reads (25 to 40 million reads per sample). We used the high-sensitivity DNA kit (Agilent, catalog no. 5067-4626) to determine the average size of the library and normalized the input of sequencing libraries to 10 ng. RNA-seq data were applied to perform qPCR analysis of bacterial transcripts on the ABI 7500 Fast System platform (Applied Biosystems). Total RNA was isolated from bacterial cultures, RNA concentration was determined using an ND1000 NanoDrop spectrophotometer (Thermo Scientific), and RNA quality was determined using an ND1000 NanoDrop spectrophotometer (Thermo Scientific), and RNA quality was evaluated on a denaturing 1% agarose gel. Each RT-qPCR mixture (25 µl) was run in duplicate with transcript-specific forward and reverse primers (Table S4) at a final concentration of 600 pmol. Temperature melting curves of the PCR products showed a single amplicon for each primer pair. Purified PCR products were sequence verified for specificity. MinE transcripts served as an internal sample control since their relative abundance was comparable to the average transcript levels of the genes investigated in this study. RNA (mRNA) content in live bacterial cells.

**Histone deacetylase assay.** We applied the histone deacetylase (HDAC) activity assay (fluorometric) kit (Abcam, Cambridge, MA). The enzymatic reaction mixtures (50 µl) were prepared according to the manufacturer’s two-step protocol for quantification of HDAC activity. Trichostatin A (TSA; 1 µM) was used as the inhibitor control in the assay. Mid-log-phase *B. thailandensis* cultures were treated with 10 µg/mL Mmp and incubated for 12 to 24 h at 37°C in a 96-well plate (150 µl per well). Crude conditioned medium (30 µl) was then assayed for HDAC-inhibitory properties. The enzymatic reactions were conducted in duplicate at room temperature (RT) for 30 min prior to the addition of stop solution and developer. Fluorescence intensity was measured using a microplate reader (BioTek) at Ex/Em 350/30 nm. One hundred percent HDAC activity was defined as fluorescence intensity (F$_{100}$) in the absence of drug or *B. thailandensis* crude conditioned medium. Zero percent activity was defined as fluorescence intensity for each data set without HDAC nuclear extract (F$_{0}$). Percent HDAC inhibition was determined according to the equation % inhibition = [(1 − (F$_{0}$ − F$_{0}$))/F$_{100}$ − F$_{0}$)], where F$_{0}$ is the fluorescence intensity in the presence of TSA or *B. thailandensis* conditioned medium.

**Analysis of in vivo DNA content in bacterial cells.** Vybrant DyeCycle green stain (ThermoFisher Scientific) was used to analyze DNA content in logarithmically expanding (LOG) and stationary-phase (STAT) *B. thailandensis* cultures. DyeCycle green stain was added at a final concentration of 10 µM to 10^8 cells resuspended in 1 ml LB-Miller broth. Bacterial cells were incubated in the stain-containing medium for 30 min at 37°C and were analyzed by flow cytometry (BD FACsAnAria II) using an argon laser. Unstained cells were used as negative controls to position the gates for analysis of fluorescence intensity. The cell distribution plots of log scale of side scatter (SSC-A) versus forward scatter (FSC) were used to gate the total population of analyzed cells, followed by gating of log-scale fluorescence intensity at the 530/30 emission filter versus FSC cell distribution. Mean fluorescence intensity (MFI) and standard deviation data were obtained through BD FACSDiva software (Becton, Dickinson, NJ). MFI was used as a measure of DNA content in live bacterial cells.
**Generation of *B. thailandensis* TpR-Tn7 strain.** Insertion of the mini-Tn/7-Tp-GFP sequence into *B. thailandensis* E264 was facilitated via triparental mating of *B. thailandensis* E264 with *E. coli* BW29427 strains harboring pUC18T-mini-Tn7-Tp-gfpmut3 and pTNS2. Addgene plasmids 65033 and 64968, respectively (50). *E. coli* BW29427 is a dianigmoidalamic acid (DAP) auxotrophic mutant that provides convenient and efficient conjugation-mediated plasmid transfer followed by counterselection of the donor strain on DAP-deficient medium. The pTNS2 and pUC18T-mini-Tn7-Tp-gfpmut3 plasmids were independently transfected in *E. coli* BW29427 cells by heat shock and were maintained on ampicillin (Amp) or Tp medium, respectively, supplemented with 0.3 mM DAP. The pUC18T-mini-Tn7-Tp-gfpmut3 plasmid was mobilized into *B. thailandensis* E264 cells via triparental mating with the helper pTNS2 (carrying the transposable gene) at a 1:1:1 ratio of bacterial cells (10^8 in 100 μl each), collected on a 20-mm cellulose filter, and incubated on LB-Amp-DAP overnight at 37°C. Single clones of transformants were isolated on LB agar supplemented with 100 μg/ml Tp, and the insertion of Tn7-Tp-GFP sequences was confirmed via colony PCR with the primer pair glmS1-DN (5'-TGGGATCA) and Tn7 (5'-ATTAGCTTACGACGCTACACCC) as previously described (51).

**Statistical analysis.** Student’s t test and one-way analysis of variance (ANOVA) (under R version 3.4.2) were applied to determine the statistical significance between target and control samples. The Wilcoxon rank test was used to confirm the normal distributions for multiple-sample comparisons with a family error of <0.05.

**Data availability.** All transcriptomics data have been submitted to GEO under accession number GSE127838 and NCBI tracking system no. 19795101.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

FIG S1, TIF file, 0.2 MB.

FIG S2, TIF file, 1.7 MB.

TABLE S1, XLSX file, 0.04 MB.

TABLE S2, XLSX file, 0.02 MB.

TABLE S3, XLSX file, 0.01 MB.

TABLE S4, DOCX file, 0.01 MB.

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