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Characterization of HmqF, a Protein Involved in the Biosynthesis of Unsaturated Quinolones Produced by *Burkholderia thailandensis*

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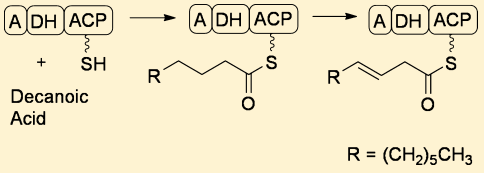
Supporting Information

ABSTRACT: The opportunistic pathogen *Burkholderia thailandensis* produces a number of structurally similar unsaturated quinolones involved in quorum sensing. However, little is known about the biosynthesis of these unsaturated quinolones. In this study, we have characterized the starting point of the biosynthesis of unsaturated quinolone molecules produced in *B. thailandensis*. We have shown by using in vitro enzymology, liquid chromatography, and mass spectrometry that protein HmqF is involved in the biosynthesis of unsaturated quinolones produced by *B. thailandensis*. HmqF consists of three domains: an adenylation domain (A domain), a dehydrogenase domain (DH domain), and an acyl carrier domain (ACP). The three domains (A, DH, and ACP) were cloned and expressed individually in *Escherichia coli*, and their reactivity was studied using high-performance liquid chromatography (HPLC) and mass spectrometry (MS) based assays. Our in vitro studies show that the A domain catalyzes ATP-dependent activation of medium chain (C6−C14) fatty acids without activation by coenzyme A (CoA). Results from competition assays are consistent with decanoic acid being the preferred substrate. Incubation of the ACP domain with 4′-phosphopantetheine transferase and CoA led to the formation of phosphopantetheinylated ACP (Ppant-ACP). In a Ppant ejection assay using tandem MS (MS/MS), a mass consistent with the mass of a cyclic variant of dephosphorylated Ppant was detected. We further demonstrated that Ppant-ACP could be loaded with medium chain fatty acids in the presence of ATP and the A domain. MS analysis was consistent with the formation of Ppant-ACP thiol esters of the fatty acids. MS/MS Ppant ejection experiments confirmed the loss of 2H in samples of fatty acid-loaded Ppant-ACP in the presence of the DH domain. HPLC analysis of benzyl amide ligation products allowed us to conclude that dehydrogenation produced *trans*-β,γ-unsaturation in the fatty acid chains. Our results are in good agreement with naturally observed quinolone molecules produced by *B. thailandensis*, which predominately produce nine-carbon *trans*-β,γ-unsaturated alkyl chain quinolone molecules.



The genus *Burkholderia* comprises more than 40 species that are ubiquitously distributed in nature as they have been isolated from soil, water, plant rhizosphere, insects, and infected humans.[1] Many *Burkholderia* species have developed beneficial interactions with their plant hosts and have been used for the biological control of plant pathogens and plant growth promotion. However, some members of this genus have emerged as serious opportunistic pathogens. For example, *Burkholderia cepacia* can colonize lungs of people suffering from cystic fibrosis.[2] Two other species, *Burkholderia mallei* and *Burkholderia pseudomallei*, are well-characterized animal and human pathogens. *B. pseudomallei* is the causative agent of melioidosis, an infectious disease of major public health importance in southeast Asia and northern Australia.[3]

In the past few decades, the frequency of opportunistic infections has increased in people with compromised immune systems resulting from HIV infections, cystic fibrosis, and advanced age. Opportunistic infections are caused by bacteria that do not normally cause disease in healthy individuals. As the rate of drug resistance grows in bacteria while the number of new antibiotics continues to decline, the need for novel approaches to the treatment of bacterial infections is clear. An improved understanding of how opportunistic populations of bacteria are controlled in the environment could provide novel therapeutic approaches to the treatment of opportunistic bacterial infections.[4]

In nature, many bacteria exhibit features similar to those of multicellular organisms and coordinate their behavior by means of chemical communication or quorum sensing (QS).[5,6] QS is a cell-to-cell communication system that allows bacteria to regulate their gene expression in response to cell density. This is primarily achieved by release of small diffusible signal molecules often termed autoinducers as they induce their own synthesis.[7] As the density of the bacterial population increases, the level of synthesis of QS signal molecules increase, thereby increasing their concentration in the external environment. Once a critical threshold concentration is reached, a response regulator is activated (or repressed) to control the expression of QS-regulated genes.

Burkholderia species release a large variety of extracellular products, including proteases, toxins, and siderophores, many of which are under QS control. Recent studies have documented that *Burkholderia* species produce secondary metabolites called 4-hydroxy-3-methyl-2-alkylquinolones

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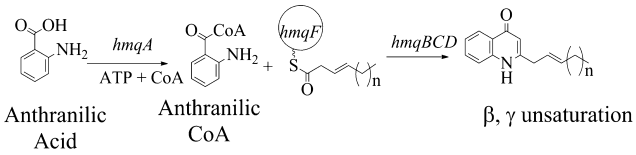
(HMAQs) involved in QS.[2,8] Vial et al. have recently identified 29 different structurally similar HMAQ derivatives from three *Burkholderia* species (*Burkholderia ambifaria*, *B. pseudomallei*, and *Burkholderia thailandensis*).[2] HMAQs are similar to the well-characterized QS molecules produced by *Pseudomonas aeruginosa* called 4-hydroxy-2-alkylquinolines (HAQs). *P. aeruginosa* produces more than 50 HAQs but produces a number of species specific molecules that are characterized by a hydroxyl group at position 3 of the quinolone ring and unsaturation of position 1′ in the alkyl side chain.[8] In contrast to *P. aeruginosa*, *Burkholderia* HMAQs have a methyl group at position 3, and their alkyl chain is predominantly unsaturated with the double bond at position 2′ in contrast to position 1′ in *P. aeruginosa*.[9]

In *Burkholderia* species, the gene cluster *hmqABCDEFG* has been shown to encode enzymes that mediate the biosynthesis of HMAQs.[9,10] The *hmqABCDEFG* operon is homologous to the *pqsABCDE* operon in *P. aeruginosa*, which is involved in the synthesis of HAQs with the addition of two additional genes further downstream of the *pqsE* homologue, named *hmqF* and *hmqG*. The methylation was shown to be a result of *hmqG*, the last gene in the operon that was shown to be a putative methyltransferase.[2,3] Currently, little is known about the biosynthesis of HMAQs produced by *Burkholderia*. It has been shown that anthranilic acid is the precursor of QQS molecules produced by *P. aeruginosa* and *Burkholderia* species.[9] Bredenbruch et al. have shown via feeding studies that HAQ molecules in *P. aeruginosa* are synthesized via a common biosynthetic pathway involving head-to-head condensation of anthranilic acid and β-keto fatty acids.[10] However, it is still unclear if *pqsBCD* genes in *P. aeruginosa* and the homologous cluster *hmqABCD* in *Burkholderia*, which all are homologous with β-keto fatty acid acyl carrier proteins, are responsible for the total synthesis of the β-keto fatty acid intermediate or if they divert intermediates from fatty acid biosynthesis into the pathway leading to QQS molecules. It is also unclear if *hmqBCD* genes are responsible for unsaturation in quinolones produced by *Burkholderia* or if this occurs because of the pool of fatty acid intermediates recruited by *Burkholderia*. Until the recent identification of the *hmqF* gene in *B. thailandensis*,[2] there was no clear source of unsaturated medium chain fatty acids found in quinolone molecules. Understanding the biosynthesis of these QQS molecules will shed light on how the subtle variations in the structures of QQS molecules are created and how this affects quorum sensing.

In this study, we sought to characterize biosynthesis of 2′-unsaturated quinolone molecules (HMAQs) produced by *B. thailandensis*. HmqF is homologous to domains contained in bacterial polyketide and nonribosomal peptide synthases. On the basis of this homology to PKS and NRPS's, we propose that HmqF is responsible for the biosynthesis of unsaturated quinolones in *B. thailandensis* starting from anthranilic CoA. To test our hypothesis, we cloned the three domains of HmqF (A, ACP, and DH domains) from *B. thailandensis* and heterologously expressed these three domains individually in *Escherichia coli* (Figure S1 of the Supporting Information). We propose that HmqF has two main functions. First, via the activation of medium chain fatty acids via an adenylation domain and covalent attachment to the phosphopantetheine group of the ACP domain, the tethered fatty acid undergoes regio- and stereoselective dehydrogenation by the dehydrogenase domain, resulting in a *trans*-β,γ-unsaturated fatty acyl-*S*-ACP moiety. At this point, the unsaturated fatty acids could be passed on to the remaining biosynthetic proteins for the synthesis of unsaturated quinolones (Scheme 1).

Scheme 1. Proposed Biosynthesis of Unsaturated Quinolones Produced by *B. thailandensis* HmqF



MATERIALS AND METHODS

Materials. Chemically competent *E. coli* BL21(DE3) cells were purchased from Invitrogen. Plasmid pET28a was purchased from Novagen. All restriction endonucleases were obtained from New England Biolabs. Synthetic DNA oligonucleotides were purchased from Integrated DNA Technologies and used without further purification. Genomic DNA was purified with a DNeasay kit from Qiagen. All other DNA isolation and purification steps were performed using a QIAprep Spin Miniprep Kit from Qiagen. DNA sequencing was performed by Genewiz Inc.

Cloning of *hmqF*. The three domains of *hmqF* [*A domain* (residues 1−1833), *DH domain* (residues 2026−3108), and *ACP* (residues 3274−3972)] were cloned individually from genomic DNA of *B. thailandensis* E264. *A domain*, *DH domain*, and *ACP* genes were amplified via PCR from genomic DNA using primer pairs listed in Table 1. PCR was conducted using Phusion high-fidelity DNA polymerase (New England Biolabs). The amplicons were digested with the enzymes listed in Table 1 and cloned into appropriate restriction sites of a pET-28a plasmid with N-terminal hexahistidine tags. The resulting plasmids were checked by DNA sequencing and transformed into *E. coli* BL21(DE3) cells for protein production.

Protein Expression and Purification. The recombinant plasmids (*A-domain*-pET28a, *DH domain*-pET28a, and *ACP*-pET28a) were transformed in chemically competent *E. coli* BL21(DE3) cells and plated on LB-agar plates supplemented with kanamycin (50 μg/mL) and grown overnight. A single colony was picked and cultured in 100 mL of LB supplemented with kanamyin (50 μg/mL) and grown overnight at 37 °C with rapid agitation. Four 2.8 L Fernbach flasks containing 1 L of sterilized LB supplemented with kanamycin (50 μg/mL) were inoculated with 10 mL of the overnight culture each. Cultures were grown at 37 °C with rapid agitation for 1.5 h, after which the temperature was decreased to 15 °C. The OD_{600} was monitored regularly until it reached 0.4−0.6; isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 500 μM to induce gene expression, and then cells were further incubated at 15 °C overnight. The cell culture was centrifuged at 6000*g* and 5 °C for 10 min. The cell pellet was resuspended in 30 mL of buffer A [50 mM NaCl, 10% glycerol, 2 mM BME, 25 mM imidazole, and 50 mM Tris (pH 8.0)] and sonicated for a total of 2 min with alternating 5 s on and off pulses. Cell debris was removed by centrifugation at 32000*g* for 1 h at 4 °C. The clarified cell lysate was loaded onto a column filled with 5 mL of Ni-NTA resin pre-equilbrated with buffer A at a flow rate of 1 mL/min. The bound proteins were washed with lysis buffer for 30 min and eluted by applying a linear gradient of 0 to 100% buffer B [50 mM NaCl, 10% glycerol,

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Table 1. Oligonucleotide Pairs Used for PCR Amplification with Restriction Sites

oligonucleotide primer (5′−3′)	restriction site	expression vector	target gene
CGGCCGGGATCCGTGAGATCTTCGGCGC	*Bam*HI	pET28a	A
GCACGAGAGCTCCTACGCCTGCGACGCC	*Sac*I		
CCGGCCGGATCCGCCAAGGCAGCCGA	*Bam*HI	pET28a	DH
ACGTCCGAGCTCGGCGGCGGCTCGTCGG	*Sac*I		
GCCGTGGGATCCGACCCCGAGCTGAAG	*Bam*HI	pET28a	ACP
CCGCTTAAGCTTGCTAGTGCCGGTCCATTC	*Hind*III		

2 mM BME, 250 mM imidazole, and 50 mM Tris (pH 8.0)] with a flow rate of 1 mL/min over 30 min. Fractions containing the desired protein were monitored by SDS−PAGE, pooled, and dialyzed against buffer C [50 mM NaCl, 25 mM Tris (pH 8.0), 2 mM $MgCl_2$, 1 mM BME, and 10% glycerol]. The recombinant protein was then concentrated with Amicon concentrators with a 10 kDa cutoff membrane (Centriprep centrifugal filters, Millipore). The concentrated protein was then flash-frozen in liquid nitrogen as BB's and stored at −80 °C. The final concentration of protein was determined by a Bradford assay.

Adenylation Domain Assays. *Fatty Acid Activation.* To investigate the mechanism of the A domain and its selectivity toward activation of fatty acids, reaction mixtures (50 μL) were prepared using 5 μM A domain, 5 mM $MgCl_2$, and 2.5 mM ATP in 50 mM Tris buffer (pH 8.0), and the reaction was initiated by the addition of the fatty acid substrate to a final concentration of 1 mM. Fatty acid solutions (propionic, hexanoic, octanoic, decanoic, lauric, myristic, and palmitic acid) were prepared as 0.1 M solutions in ethanol. As a control, pure ethanol with no fatty acid was added in an equal volume to one of the reaction mixtures. To test the role of CoA in fatty acid activation by the A domain, identical reactions were performed with the addition of 1 mM CoA. The reaction mixtures were incubated for 4 h at room temperature and the reactions quenched with 50 μL of 50% acetonitrile and 0.1% formic acid. The samples were spun at 18000*g* for 5 min in a microcentrifuge (Beckman Coulter), and the supernatant was subjected to high-performance liquid chromatography (HPLC) and Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) analysis. The reactions were performed in triplicate.

Fatty Acid Mixture Competition Assays. Reaction mixtures (50 μL) containing 5 μM A domain, 5 mM $MgCl_2$, and 2.5 mM ATP in 50 mM Tris buffer (pH 8.0) were prepared. Reactions were initiated by the addition of the substrates to following final concentration: 1 mM hexanoic acid, 1 mM octanoic acid, 1 mM decanoic acid, and 1 mM lauric acid. The reaction mixtures were incubated at room temperature, and 50 μL aliquots of the reaction mixture were quenched with 50 μL of 50% acetonitrile and 0.1% formic acid at regular time points. The samples were spun at 18000*g* for 5 min in a microcentrifuge, and the supernatant was subjected to HPLC and FT-ICR-MS analysis. The reactions were performed in triplicate.

Hexanoic versus Decanoic Competition Assays. Another set of assays were run to directly compare the selection of decanoic acid versus hexanoic acid by the A domain. In these experiments, two sets of assays were run, one at a low concentration of decanoic acid (50 μM) and another at a high concentration of decanoic acid (1 mM), while the concentration of hexanoic acid was increased in each case. In these assays, a typical reaction mixture (50 μL) containing 5 μM A domain, 5 mM $MgCl_2$, 2.5 mM ATP, and decanoic acid (50 μM or 1 mM) in 50 mM Tris buffer (pH 8.0) was incubated with increasing concentrations (50 μM to 4 mM) of hexanoic acid. The reactions were initiated by the addition of the fatty acid substrates and the mixtures incubated for 4 h at room temperature followed by quenching with 50 μL of 50% acetonitrile and 0.1% formic acid. The samples were spun at 18000*g* for 5 min, and the supernatant was subjected to HPLC and FT-ICR-MS analysis.

HPLC−MS Analysis. The separations were performed on a Kinetex C18 column [50.0 mm × 2.10 mm, pore diameter of 100 Å, particle size of 2.6 μm (Phenomenex, Torrance, CA)]. The column was equilibrated for 10 min with 5% buffer A and 95% buffer B and then with the following gradient: 5% A and 95% B to 95% A and 5% B over 12 min, followed by 95% buffer A and 5% B from 12 to 14 min, and then the gradient back to 5% A and 95% B from 14 to 16 min (buffer A being acetonitrile and 0.1% formic acid and buffer B being water and 0.1% formic acid). The separations were conducted at a flow rate of 0.2 mL/min, and product elution was monitored at 260 nm. The products were quantified by integration of HPLC peaks at 260 nm. HPLC analysis was performed using Hystar from Bruker Daltonics. The LC eluents were directly infused into the mass spectrometer. Mass spectrometry (MS) analyses were performed on a 7.0 T Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonics Inc., Billerica, MA). The instrument was externally calibrated with sodium trifluoroacetate clusters (0.1 mg/mL in acetonitrile) as they give evenly spaced, singly charged peaks in the mass range of 150−3000 Da. The following typical instrument settings were used for broadband FT-ICR-MS analysis: source accumulation set to 100 ms/scan and collision cell accumulation set to 500 ms/scan. The capillary voltage was set to 4.5 kV; the skimmer voltage was set to 30.0 V. All data sets were collected at 512 K. MS analysis was performed using Apex from Bruker Daltonics. The HPLC system (1200 series) was from Agilent Technologies Inc. (Santa Clara, CA).

Acyl Carrier Protein (ACP). *Generation of holo-ACP.* To the reaction mixture [50 mM Tris (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT] were added 50 μM apo-ACP, 0.5 mM CoA, and 2 μM 4′-phosphopantetheine transferase Sfp from *Bacillus subtilis* to a final volume of 100 μL, and the mixture was incubated for 1.5 h at 30 °C to generate holo-ACP. CoA was omitted as an apo-ACP control. The reaction was quenched with 50 μL of 50% acetonitrile and 0.1% formic acid. The intact apo- and holo-ACPs were characterized by HPLC−MS and ESI-FT-ICR-MS/MS analysis.

Generation of Fatty Acid-Loaded ACP. A typical reaction mixture (50 μL) for the generation of fatty acid-loaded holo-ACP was prepared using 50 μM holo-ACP, 5 μM A domain, 5 mM $MgCl_2$, and 2.5 mM ATP in 100 mM Tris buffer (pH 8.0). To the reaction mixtures were added 2 mM fatty acids (hexanoic, octanoic, decanoic, lauric, and myristic acid) individually followed by incubation for 1 h at 30 °C. The reaction was quenched with

50 μL of 50% acetonitrile and 0.1% formic acid. Following quenching, the samples were subjected to HPLC−MS and ESI-FT-ICR-MS/MS analysis.

HPLC−MS Analysis. The intact proteins were purified on a Jupiter C5 column [150.0 mm × 2.0 mm, pore diameter of 100 Å, particle size of 5 μm (Phenomenex)] using a gradient from 5% A and 95% B to 95% A and 5% B over 30 min, where buffer A was acetonitrile with 0.1% formic acid and buffer B was water with 0.1% formic acid. The separations were conducted at a flow rate of 0.2 mL/min, and absorbance was monitored at 215 nm. The LC eluents were directly infused into the mass spectrometer. The identities of intact apo-ACP, holo-ACP, and fatty acid-loaded ACPs were confirmed by FTMS using the instrument settings described above. The instrument was externally calibrated with sodium trifluoroacetate clusters (0.1 mg/mL in acetonitrile).

Phosphopantetheinyl Ejection Assay. Intact ACPs (holo-ACP and fatty acid-loaded ACP) were characterized by a phosphopantetheinyl (Ppant) ejection assay using tandem mass spectrometry (ESI-MS/MS). In this assay, intact ACP reaction mixtures were first desalted by solid phase extraction (SPE) on a 1 mL, 50 mg C4 column (Silicycle, Quebec City, QC) before MS/MS characterization. SPE was performed by first equilibrating C4 columns with 5% buffer A (acetonitrile with 0.1% formic acid), followed by activation of the columns with 0.1% formic acid. The samples were then loaded onto columns and washed with 0.1% formic acid followed by elution with 0.4 mL of 50% acetonitrile and 0.1% formic acid. Desalted intact ACP samples were directly infused into the mass spectrometer for ESI-MS/MS. MS/MS was achieved by collision-activated dissociation (CAD) by subjecting the ions to −15 to −25 V in the accumulation hexapole. The hexapole amplitude was set to 1500 $V_{p\text{-}p}$; the collision cell accumulation was set to 500 ms/scan, and 10−20 scans were acquired per spectrum. All data sets were collected at 512 K. The instrument was externally calibrated with sodium trifluoroacetate clusters (0.1 mg/mL in acetonitrile).

Dehydrogenase Domain Assays. *DH Domain Flavin Cofactor Analysis.* A 50 μL sample of the purified DH domain was boiled for 5 min, and the denatured protein was removed by centrifugation. Flavin present in the supernatant was analyzed by HPLC on a Kinetex C18 column (50.0 mm × 2.10 mm, pore diameter of 100 Å, particle size of 2.6 μm) with the following gradient: 5% A and 95% B to 95% A and 5% B over 25 min (buffer A being acetonitrile and 0.1% formic acid and buffer B being water and 0.1% formic acid). The separations were conducted at a flow rate of 0.2 mL/min, and absorbance was monitored at 445 nm. Additionally, the identity of the cofactor was confirmed by mass spectrometry.

DH Domain Unsaturation Assays. Typical reaction mixtures (100 μL) for detection of unsaturation in fatty acids by the DH domain containing 50 μM fatty acids (hexanoic, octanoic, decanoic, and lauric acid) loaded holo-ACPs were incubated with 5 μM DH domain and 250 μM FAD for 30 min at 30 °C. The reactions were quenched with 15 μL of formic acid. Following quenching, the samples were subjected to HPLC−MS and ESI-FT-ICR-MS/MS analysis.

HPLC−MS Analysis. The DH domain reaction products were purified on a Jupiter C5 column [150.0 mm × 2.0 mm, pore diameter of 100 Å, particle size of 5 μm (Phenomenex)] using a gradient from 5% A and 95% B to 95% A and 5% B over 30 min (buffer A being acetonitrile and 0.1% formic acid and buffer B being water and 0.1% formic acid). The separations were conducted at a flow rate of 0.2 mL/min, and absorbance was monitored at 215 nm. The LC eluents were directly infused into the mass spectrometer. The identities of intact DH domain reaction products were confirmed by FTMS using the instrument settings described above. The instrument was externally calibrated with sodium trifluoroacetate clusters (0.1 mg/mL in acetonitrile).

Phosphopantetheinyl Ejection Assay. The DH domain reaction products were characterized by a phosphopantetheinyl (Ppant) ejection assay using tandem mass spectrometry (ESI-MS/MS). In this assay, intact DH domain reaction mixtures were first desalted by solid phase extraction (SPE) on a 1 mL, 50 mg C4 column. C4 columns were equilibrated with 5% buffer A (acetonitrile and 0.1% formic acid), followed by activation of column with 0.1% formic acid. Reaction samples were then loaded onto C4 columns, washed with 0.1% formic acid, eluted with 0.4 mL of 50% acetonitrile and 0.1% formic acid, and directly infused into the mass spectrometer for ESI-FT-ICR-MS/MS analysis.

Time Course DH Domain Unsaturation Assay. Typical reaction mixtures (100 μL) for determining the time dependence of unsaturation with the DH domain containing 50 μM decanoic acid-loaded holo-ACPs were incubated with 5 μM DH domain and 250 μM FAD for 5, 10, 20, and 30 min at 30 °C. A control reaction was also run without the DH domain. The reactions were quenched with 15 μL of formic acid. Following quenching, the reaction mixtures were first desalted by solid phase extraction (SPE) on a 1 mL, 50 mg C4 column. C4 columns were equilibrated with 5% buffer A (acetonitrile and 0.1% formic acid), followed by activation of the column with 0.1% formic acid. Reaction samples were then loaded onto C4 columns, washed with 0.1% formic acid, eluted with 0.4 mL of 50% acetonitrile and 0.1% formic acid, and directly infused into the mass spectrometer for the phosphopantetheinyl ejection assay using tandem mass spectrometry (ESI-MS/MS) as described above.

Amide Ligation Assay of the Location of the Double Bond. To characterize the location of the double bond in the unsaturated fatty acids, complete reaction mixtures (200 μL) of DH domain assays in 0.1 M HEPES buffer (pH 7.5) were incubated with excess (50 mM) benzylamine and 100 μL of acetonitrile at 50 °C for 2 h to form amide ligation products. Precipitated proteins were removed by centrifugation at 18000*g* for 5 min. Protein pellets were washed twice with 25 μL of HEPES buffer and combined with the supernatant, and the combined fractions were concentrated with a speed-vac and subjected to HPLC−MS analysis.

HPLC−MS Analysis. The unsaturated amide ligation products along with their synthetic standards were run on a Kinetex C18 column (50.0 mm × 2.10 mm, pore diameter of 100 Å, particle size of 2.6 μm). The column was equilibrated with 5% buffer A followed by a gradient from 5% A and 95% B to 95% A and 5% B over 25 min (buffer A being acetonitrile and 0.1% formic acid and buffer B being water and 0.1% formic acid). Separations were conducted at a flow rate of 0.2 mL/min, and absorbance was monitored at 260 nm. The LC eluents were directly infused into the mass spectrometer for mass identification using the FTMS instrument settings described above.

RESULTS AND DISCUSSION

Adenylation Domain (A Domain). A domains are responsible for the selection and activation of fatty acids in

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the presence of ATP to form acyl-adenylates.[11] We performed a series of assays to study the scope of the A domain's substrates for comparison with naturally observed quinolone molecules produced by *B. thailandensis*. The N-terminally His_6-tagged A domain was overexpressed in *E. coli*, resulting in a soluble protein product with an apparent molecular mass of 74 kDa, in agreement with the calculated mass based on the amino acid sequence (Figure S1 of the Supporting Information).

Fatty Acid Activation. Fatty acid activation can occur in one of two pathways, the first being the fatty acid CoA ligase mechanism. In this mechanism, fatty acid is first transiently activated as an acyl-adenylate, which reacts with CoA to form an acyl-CoA thioester intermediate. The second pathway is via fatty acyl-AMP ligases where fatty acid activation stops at the acyl-adenylate.[12] The A domain was incubated with fatty acids and ATP in the presence and absence of CoA to determine the mechanism of fatty acid activation. No acyl-CoA thioesters were observed by LC−MS, arguing that fatty acids are activated as acyl-adenylates (Figure S2 of the Supporting Information).

To assess substrate specificity, the A domain was incubated in the presence of ATP with fatty acids starting from propionic acid to palmitic acid. Mass spectrometry confirmed the formation and identity of acyl-adenylates with a high degree of accuracy (<5.0 ppm error) (Table S1 of the Supporting Information). Acyl-adenylate formation was quantified by HPLC via integration of peaks at 260 nm (Figure 1). Figure 1 shows that

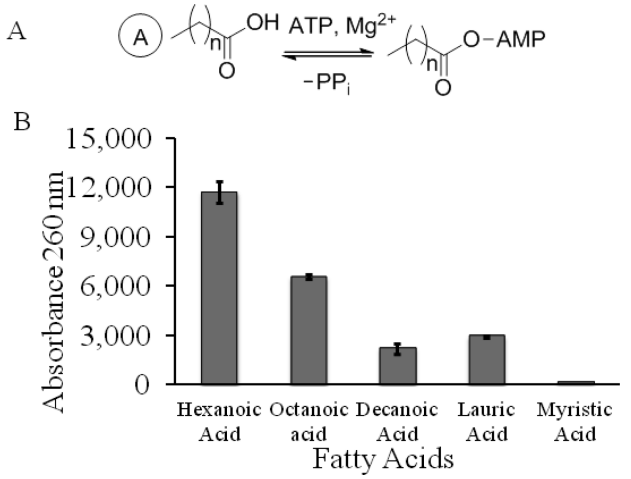
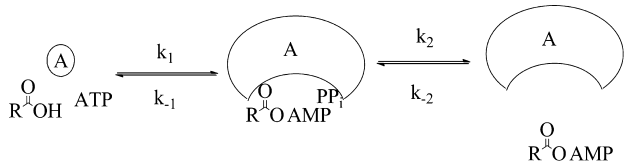


Figure 1. Adenylation domain assays. (A) Schematic representation of the mechanism of fatty acid activation by the A domain in the presence of ATP. (B) Quantitation of acyl-adenylates by integration of HPLC peaks at 260 nm. Assays were performed by incubating the A domain with fatty acids (1 mM) in the presence of ATP.

acyl-adenylates were formed reproducibly for hexanoic, octanoic, decanoic, lauric, and myristic acid. No acyl-adenylates were observed for fatty acids with chains shorter than six carbons and longer than 14 carbons. Myristoyl adenylate was formed in very small amounts. This is because the A domain is selective only toward medium chain fatty acids (C6−C12), and as myristic acid is a long chain fatty acid, it is not the preferred substrate. The observed substrate selectivity of the A domain toward the activation of medium chain fatty acids corresponds well with the naturally observed HMAQ produced by *B. thailandensis*.[2,3] However, contrary to our expectations, Figure 1 also shows that maximal acyl-adenylate formation was observed for hexanoyl-adenylate. The most naturally abundant quinolone molecule produced by *B. thailandensis* is 4-hydroxy-3-methyl-2-nonenylquinole (HMNQ);[2] we had expected that decanoic acid would be the preferred substrate of the A domain on the basis of our proposed scheme of biosynthesis of unsaturated quinolones by HmqF (Scheme 1). To explain this discrepancy, we hypothesize that in the first half of the reaction, the A domain catalyzes the reaction between ATP and carboxylic acid of the acyl group to form the acyl-adenylate and pyrophosphate (Scheme 2). It has been demonstrated that in a similar protein

Scheme 2. Proposed Rates of Fatty Acid Selection and Formation of Acyl-Adenylate by a Domain in the Presence of ATP (k_1) Followed by the Slow Release of Acyl-Adenylate from the A Domain in the Absence of Holo-ACP (k_2)



EntF, which has an adjacent A and PCP domain, if A domain is subjected to a similar assay in the absence of the PCP, the extent of formation of seryl-AMP is extremely low. Catalytic turnover requires the release of the thermodynamically activated aminoacyl-AMP from the EntF active site.[13] Thus, this assay measures the rate of release of acyl-adenylates by the A domain in the absence of the ACP acceptor. Our experiments were performed in absence of the holo-ACP acceptor. Therefore, we were measuring the artificial release or leak rate of the acyl-adenylate from the A domain. Furthermore, release of acyl-adenylate from the A domain would be predicted to be the slowest for the substrate that has the highest affinity for the A domain and conversely higher for nonoptimized substrates. As myristoyl adenylate is not the preferred substrate for the A domain, Figure 1 shows the formation of the adenylate in small amounts and not the release of myristoyl adenylate from the A domain. Therefore, the formation of decanoyl-adenylate was slowest of all the substrates tested, leading us to believe that decanoic acid is the preferred substrate for the A domain. Hence, we propose that in absence of holo-ACP, release of acyl-adenylate from the A domain is unfavorable and characterized by an extremely slow leak rate (k_2) (Scheme 2).

Fatty Acid Mixture Competition Assays. To improve our understanding of the substrate preference of the A domain, a series of competition experiments was designed to probe the initial association of the fatty acid with the A domain. In an assay where multiple substrates are present, the A domain should preferentially select from a mixture of substrates the preferred length fatty acids. Once the fatty acid is bound by the A domain and reacts to form the acyl-adenylate, it prevents catalytic turnover until the acyl-adenylate is released. HPLC analysis of the resulting mixture of acyl-adenylates should be indicative of which substrate is preferred. To this end, the A domain was incubated with equal concentrations C6, C8, C10, and C12 carbon fatty acids and quenched at regular time points followed by HPLC analysis to quantify the individual acyl-adenylate. Figure 2A is a plot of formation of the individual acyl-adenylates when fatty acids were added together to the reaction mixture with respect to time. The plot shows that there is no hexanoyl-adenylate formed at any time point;

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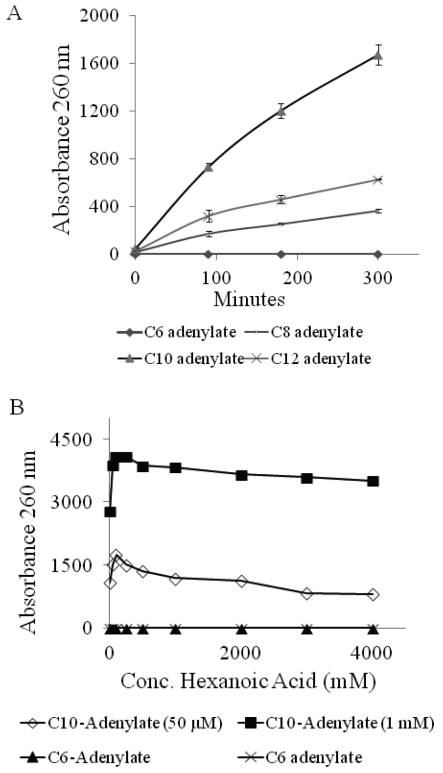

Figure 2. Fatty acid mixture competition assays. (A) Formation of acyl-adenylates with respect to time with a fatty acid mixture (hexanoic, octanoic, decanoic, and lauric acid) incubated with the A domain and ATP. (B) Formation of decanoyl-adenylate at a low concentration of decanoic acid (50 μM) and a high concentration of decanoic acid (1 mM) with respect to increasing concentrations of hexanoic acid (50 μM to 4 mM). No hexanoyl-adenylate formation occurred in the presence of decanoic acid.

similarly, less octanoyl-adenylate and lauryl-adenylate is formed over time with decanoyl-adenylate showing considerably more product formation over time.

Hexanoic versus Decanoic Competition Assays. To further probe substrate scope, we designed a direct competition assay to measure the ability of the enzyme to select for decanoic acid at increasing concentrations of hexanoic acid (0.05−4.0 mM). Figure 2B shows formation of decanoyl-adenylate at low (0.05 mM) and high (1 mM) concentrations of decanoic acid with respect to increasing concentrations of hexanoic acid added to the reaction mixture. The plot also shows that there is no formation of hexanoyl-adenylate (in the presence of decanoic acid in the assay mixture) even when hexanoic acid was present at 80-fold greater concentrations (4 mM vs 0.05 mM).

The results of both competition assays show that the substrate preferred by the A domain is decanoic acid. The single-substrate assay now when considered as a measure of the leak rate of the thermodynamically activated acyl-adenylates would be expected to exhibit lower turnover rates or acyl-adenylate release for the preferred substrates and the high rates of release of hexanoyl- and octanoyl-adenylates that reflect the fact that these substrates have lower affinities and higher leak rates. These results are consistent with the fact that HMAQs produced by *B. thailandensis* produce minor amounts of C7 and C11 alkyl chains, the majority being the C9 alkyl chain that would presumably arise from the activation of decanoic acid. No HMAQs were isolated with C5 alkyl chains that would correspond to activation of hexanoic acid, and this is in good agreement with our substrate competition assays.[2]

Analysis of the control reaction in which no fatty acids were added to the reaction mixture shows by extracted ion chromatogram (EIC) a mixture of acyl-adenylates (Figure S3 of the Supporting Information). The acyl-adenylates could not be quantified as they were not formed in appreciable amounts for detection by UV. Attempts to further characterize the kinetics of acyl-adenylate formation have been hampered by fatty acid contamination from enzyme preparations, resulting in high background rates of acyl-adenylate formation preventing to date the use of an enzyme assay such as the ATP−PP_i exchange assays that would measure the ability to form enzyme-bound acyl-adenylates reversibly and release the unlabeled PP_i product into solution to exchange with $[^{32}P]PP_i$.[13]

Acyl Carrier Protein (ACP). ACPs are a part of the carrier protein (CP) superfamily, which comprises a number of different members. These members are named according to the substrates they carry. The ACPs of fatty acids and polyketide synthases carry acetate, malonate, and growing acyl units, and the peptidyl carrier protein (PCP) carries amino acids or peptidyl units. These CPs react with activated substrates to form thioesters on the terminal thiol of the phosphopantetheinyl group, thereby covalently attaching the substrate to the CP.[11,14] We performed a variety of well-documented assays to characterize loading of acyl-adenylates formed by the A domain in the presence of ATP onto the phosphopathetheinyl arm of the ACP domain of HmqF in trans.

Characterization of ACPs. The N-terminally His_6-tagged apo-ACP was overexpressed in *E. coli* and purified as described in detail in Materials and Methods. The apo-ACP was characterized by SDS−PAGE, resulting in a soluble protein product with an apparent molecular mass of 14 kDa in agreement with the calculated mass based on the amino acid sequence (Figure S1 of the Supporting Information). LC−MS analysis of the intact protein in Figure 3A shows that the intact apo-ACP eluted at 20.7 min with an intact average molecular mass of 14213.4 Da, which was in close agreement with the theoretical mass of 14213.8 Da. Figure 3A shows two additional peaks observed with less intensity than the major peak with average molecular masses of 14391.4 and 14472.4 Da. These masses are 178.0 and 259.0 Da higher than the major peak, respectively. These two excess masses (+178 and +259 Da) are a result of phosphogluconoylation of α-amino groups of the N-terminal His tag in *E. coli*. The +259 Da modifications are from addition of the 6-phosphogluconyl group to the α-amino group of the N-terminal His tag. This is due to acylation by 6-phosphoglucono-1,5-lactone, produced from glucose 6-phosphate by glucose-6-phosphate dehydrogenase in *E. coli*. A subsequent removal of the phospho group by host cell phosphatases, such as alkaline phosphatase, results in the modification of 178.0 Da.[15]

Apo-ACP was converted to its holo form by incubation with 4′-phosphopantetheine transferase Sfp from *Bacillus subtilis* and CoA followed by its characterization by LC−MS. Figure 3B shows that intact holo-ACP eluted at 19.4 min with an intact average molecular mass of 14553.6 Da, 340.2 Da higher than the mass of apo-ACP and within the 0.12 Da mass shift expected for phosphopantetheinylation. Additionally, gluconoylation peaks were observed also with masses of 14731.6 Da (178.0 Da higher) and 14812.6 Da (259.0 Da higher).

Phosphopantetheinyl Ejection Assay. Further characterization of the holo-ACP was performed by the phosphopantetheinyl

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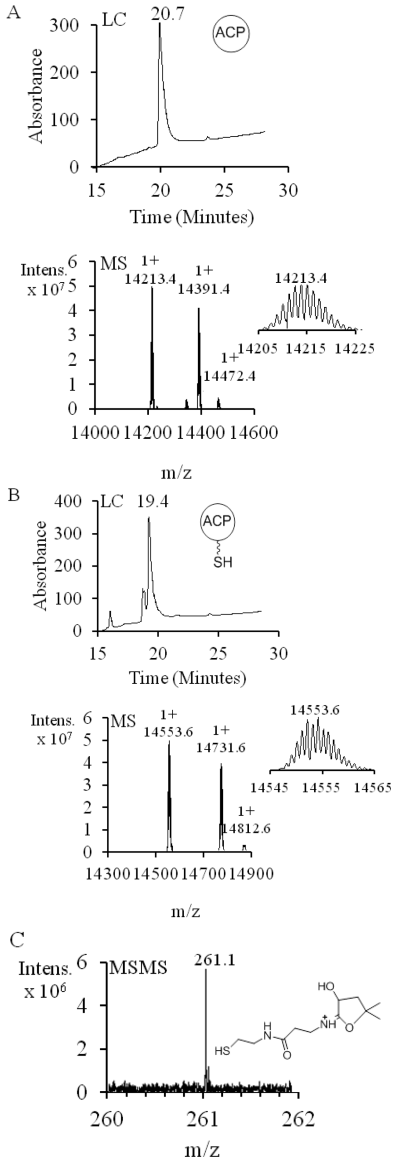

Figure 3. Characterization of intact ACPs. (A) LC–MS characterization of apo-ACP. The inset shows the isotopic distribution of the +12 charge state of apo-ACP. (B) LC–MS characterization of holo-ACP. The inset shows the isotopic distribution of the +12 charge state of holo-ACP. (C) MS/MS data of the Ppant ejection fragment from holo-ACP.

(Ppant) ejection assay using tandem mass spectrometric method CAD. The Ppant ejection assay is a fragmentation event observed by either CAD or IRMPD that results in elimination of the phosphopantetheinyl moiety from carrier domains found on NRPSs and PKSs. It has been used to characterize substrates, intermediates, and products loaded on the phosphopantetheinyl arm of carrier domains.[11,14,16] The Ppant ejection assay from the holo-ACP resulted in observation of the 261.1 Da fragment shown in Figure 3C. This fragment is within the 5.0 ppm error of the expected value and consistent with the empirical formula of the rearranged Ppant ejection fragment. The Ppant fragment (Figure 3C) that we observe is the one in which following the elimination reaction, phosphate anion is left behind on the protein and the carbonyl of an amide displaces the pantetheinylate moiety, forming a five-membered ring with a protonated imine that is positively charged. The mechanism is shown in Figure S4 of the Supporting Information.[16]

Characterization of Fatty Acid-Loaded ACP. Next, fatty acids loaded on holo-ACP were characterized by LC–MS. Holo-ACP was incubated with fatty acids starting from hexanoic acid to myristic acid, ATP, and the A domain. Figure 4B shows results of decanoic acid loaded on holo-ACP. Decanoic

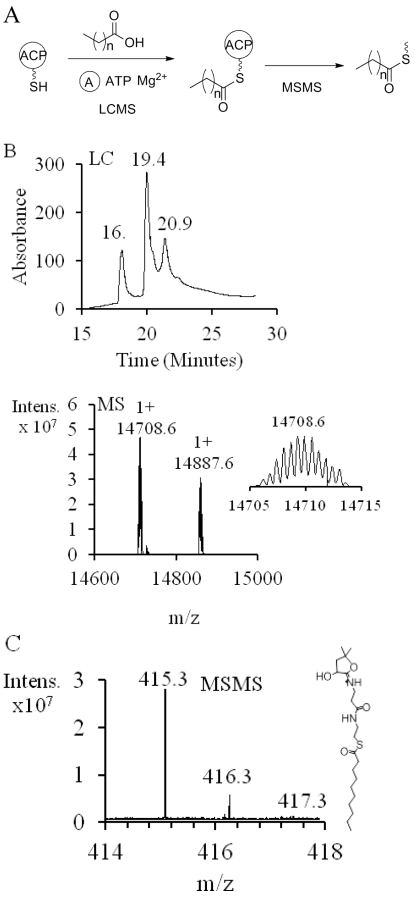

Figure 4. Loading of fatty acids onto holo-ACP. (A) Schematic representation of the experimental setup. LC–MS characterization of holo-ACP loaded with fatty acids followed by MS/MS characterization of Ppant ejection fragments. (B) LC–MS data of decanoyl-*S*-ACP. Decanoyl-*S*-ACP eluted at 20.9 min. LC data also show the formation of decanoyl-adenylate eluting at 16.9 min and holo-ACP eluting at 19.4 min. (C) MS/MS data of the decanoyl-*S*-Ppant ejection fragment from decanoic acid-loaded holo-ACP.

acid-loaded ACP eluted at 20.9 min with an intact average molecular mass of 14708.6 Da, which was shifted by +155.0 Da from that of holo-ACP. This +155.0 Da shift in mass was consistent with the formation of decanoyl-*S*-ACP. The LC trace shows two additional peaks eluting at 16.9 and 19.4 min. MS analysis showed that the peak eluting at 16.9 min corresponds to decanoyl-adenylate and the peak eluting at 19.4 min corresponds to holo-ACP. Further, the Ppant ejection assay resulted in the fragment with a mass of 415.2 Da (Figure 4C), consistent with the empirical formula of the decanoyl-*S*-Ppant ejection product and within the 5.0 ppm error of the expected value. Table S2 of the Supporting Information lists masses of all fatty acid-loaded holo-ACPs. Additionally, each reaction was characterized by Ppant ejection assays, and each of the observed fragments is listed in Figure S5 of the Supporting Information.

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Fatty acid loading on holo-ACP was observed for hexanoic, octanoic, and decanoic acid; a low level of conversion was observed for lauric acid, and no conversion could be detected for myristic acid. Again, our results are consistent with the fact that medium alkyl chain fatty acids are incorporated in the biosynthesis of HMAQs in *B. thailandensis*.

Dehydrogenase Domain (DH Domain). Dehydrogenases belong to a class of enzymes that catalyze the dehydrogenation of fatty acids. To understand the substrate scope and regio- and stereochemistry of the reaction, we performed a variety of well-documented assays to characterize the unsaturation on fatty acid substrates loaded on the phosphopantetheinyl arm of holo-ACP by the DH domain.

The N-terminally His_6-tagged DH domain was overexpressed in *E. coli* BL21(DE3) cells and purified as described in Materials and Methods. The DH domain was characterized by SDS−PAGE, resulting in a soluble protein product with an apparent molecular mass of 55 kDa, in agreement with the calculated mass based on the amino acid sequence (Figure S1 of the Supporting Information). Dehydrogenases typically use an electron-accepting cofactor like FAD or FMN to oxidize their substrates via a reduction reaction. To determine the identity of the cofactor, the DH domain was heat denatured to release the cofactor, which was analyzed by LC−MS against FAD and FMN standards. HPLC analysis revealed the presence of FAD as an electron-accepting cofactor as both the FAD standard and the DH domain supernatant eluted at 9.5 min (Figure S6 of the Supporting Information). Further, the identity of the FAD cofactor was confirmed by mass spectrometry (Figure S6 of the Supporting Information).

Characterization of Unsaturated Fatty Acids. To detect unsaturation in fatty acids, fatty acid-loaded holo-ACPs were incubated with the DH domain and FAD; samples were subjected to the Ppant ejection assay (Figure 5A), and LC−MS analysis of the intact holo-ACP loaded with fatty acids was conducted (Figure S7 of the Supporting Information). A loss of 2 Da was observed for decanoyl acid-loaded holo-ACP (Figure 5B). This corresponds to the fragment with a mass of 413.2 Da, consistent with the calculated mass of the decenoyl-S-Ppant ejection product (within the 5.0 ppm error). A loss of 2 Da was also observed by MS analysis of the intact holo-ACP loaded with decanoic acid (Figure S7B of the Supporting Information). Figure S7A of the Supporting Information shows that the intact average molecular mass of decanoic acid-loaded holo-ACP in the absence of the DH domain is 14708.6 Da. Figure S7B of the Supporting Information shows that in the presence of the DH domain, dehydrogenation takes place, which results in loss of 2 Da leading to the formation of decenoyl acid-loaded holo-ACP with an intact molecular mass of 14706.6 Da. A loss of 2 Da from octanoyl acid-loaded holo-ACP was observed in trace amounts. Hexanoyl acid- and lauryl acid-loaded holo-ACPs did not show the loss of 2 Da in their Ppant ejection products. Our results are consistent with the fact that in *B. thailandensis* cultures, the most abundant unsaturated HMAQ congener has a nine-carbon unsaturated side chain.

Further, a time course DH domain assay was performed with decanoic acid-loaded holo-ACP followed by a Ppant ejection assay (Figure S8 of the Supporting Information) to determine the time dependence of the unsaturation reaction by the DH domain. Figure S8A of the Supporting Information shows that in the control assay where no DH domain was added, only the fragment at 415.3 Da was observed, corresponding to the decanoyl-S-Ppant ejection product. This confirms that there is

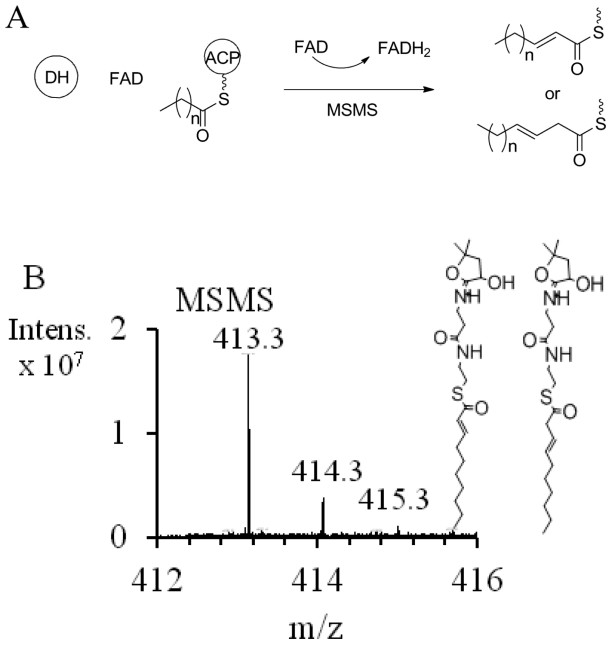

Figure 5. DH domain assay. (A) Schematic representation of the experimental setup and MS/MS characterization of Ppant ejection fragments of DH domain assay products. (B) MS/MS data of the decenoyl-*S*-Ppant ejection fragment. The Ppant fragment at 413.2 Da could correspond to either *trans*-α,β- or *trans*-β,γ-unsaturated decenoyl-*S*-Ppant fragment.

no unsaturated fatty acid present in the absence of the DH domain. Figure S8B−S8D of the Supporting Information shows the conversion of decanoyl-*S*-Ppant to decenoyl-*S*-Ppant as observed by the increase in the amount of the fragment at 413.2 Da corresponding to decenoyl-*S*-Ppant and the decrease in the amount of the fragment at 415.2 Da corresponding to decanoyl-*S*-Ppant with time. Further, Figure S8E of the Supporting Information shows the complete conversion of decanoyl-*S*-Ppant to decenoyl-*S*-Ppant over 30 min as only the fragment with a mass of 413.2 Da was observed.

Characterization of the Location of Double Bond. To further characterize the location of the double bond (α,β or β,γ) in unsaturated fatty acids, HPLC-based comparison assays were performed using synthetic standards. Ppant ejection assays with DH domain products characterized the formation of unsaturated fatty acids; however, these assays could not ascertain the location of the double bond to be α,β or β,γ with respect to the carbonyl carbon. To characterize the location of the double bond, unsaturated fatty acid holo-ACP derivatives were incubated with benzylamine to form amide ligation products, and the reaction mixture was centrifuged, concentrated, and subjected to LC−MS analysis (Figure 6A) along with their α,β and β,γ synthetic standards.[17] Figure 6B shows extracted ion chromatograms at 260.2 Da along with retention times of two synthetic standards, *trans*-benzyldec-2-enamide and *trans*-benzyldec-3-enamide. *trans*-Benzyldec-2-enamide eluted at 14.3 min, whereas *trans*-benzyldec-3-enamide eluted at 13.7 min under conditions given in Materials and Methods. Figure 6C shows the EIC at 260.2 Da of benzyldecenamide from our reaction assay, which shows that benzyldecenamide eluted at 13.7 min. The retention time of the benzyldecenamide ligation product coincides with that of *trans*-benzyldec-3-enamide. This assay confirms that the DH domain forms the *trans*-β,γ-unsaturated fatty acid-loaded ACP. LC−MS

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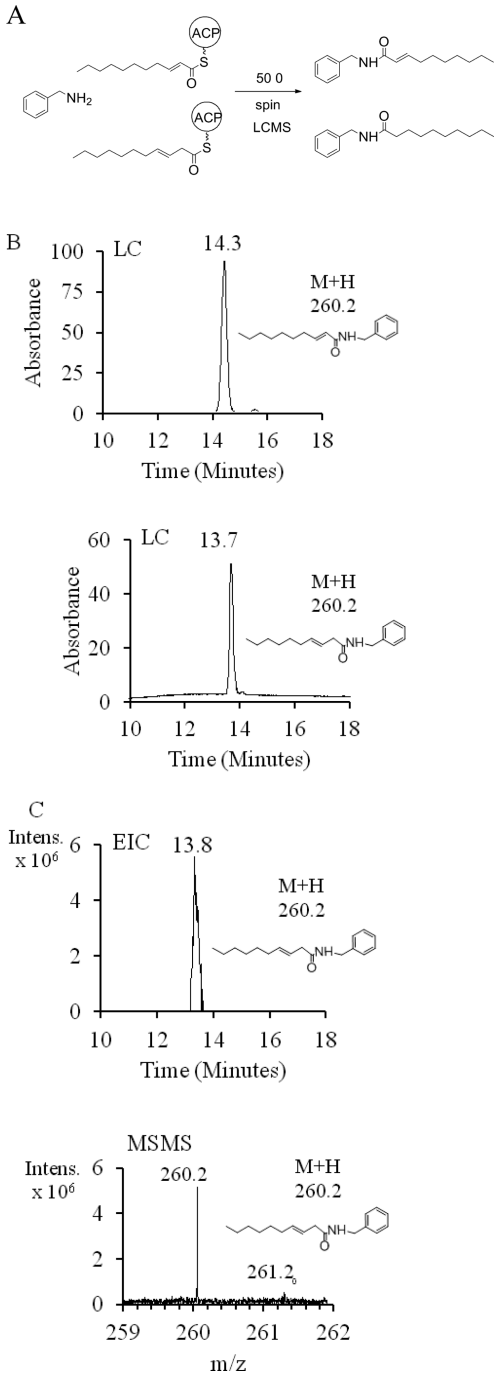

Figure 6. Characterization of the location of the double bond in unsaturated products formed by the DH domain. (A) LC–MS characterization of amide ligation products formed by the reaction of unsaturated DH domain assay products with benzylamine. (B) Extracted ion chromatograms at 260.2 Da with retention times of synthetic standards: *trans*-benzyldec-2-enamide and *trans*-benzyldec-3-enamide. (C) Extracted ion chromatogram with the retention time of the amide ligation product of the DH domain shows that it coelutes with *trans*-benzyldec-3-enamide. MS spectra show the mass of *trans*-benzyldec-3-enamide.

analysis was also performed on benzyloctenamide from the reaction assay, and it was compared with *trans*-benzyloctyl-2-enamide and *trans*-benzyloctyl-3-enamide. However, in this case, we did not observe any LC peak or extracted ion chromatogram showing that the benzyloctenamide product was not formed. Dehydrogenases mostly catalyze the desaturation at positions α and β of various CoA-conjugated fatty acids[18] because the α-hydrogen to the carbonyl carbon is more acidic and therefore more easily abstracted by a base than the β- and γ-hydrogens. To our surprise, the DH domain forms the less favorable β,γ-unsaturated fatty acid in our experiments. Mechanistically, the α-proton could be abstracted first to form the energetically favorable α,β-unsaturated fatty acid, followed by the isomerization to form the β,γ-unsaturated fatty acid. However, there is no literature to support the fact that isomerization of the α,β-unsaturated fatty acid to the β,γ-unsaturated fatty acid occurs. There is evidence of isomerization of 3-butenoylpantetheine to 2-butenoylpantetheine catalyzed by short chain acyl-CoA dehydrogenases.[19] In this case, the α-proton is abstracted from C2 of 3-butenoylpantetheine and reprotonated at C4 to form 2-butenoylpantetheine. However, there is no evidence of isomerization from the β,γ-unsaturated isomer to the α,β-unsaturated isomer.

Our results are consistent with the fact that in *B. thailandensis*, HMAQs identified in the highest concentration are *trans*-β,γ-unsaturated congeners with a C9 side chain. Vial et al. observed trace quantities of *trans*-β,γ-unsaturated congeners with a C7 side chain.[2] Although we saw trace quantities of the octenoyl-*S*-Ppant product in our DH domain assay via MS/MS, we did not observe α,β- or β,γ-unsaturated octanoic acid in our amide ligation assay via LC–MS.

CONCLUSION

In this work, we have biochemically characterized the protein HmqF and shown its involvement in the biosynthesis of unsaturated quinolones produced by *B. thailandensis*. Our results reveal that the three domains of HmqF (A, DH, and ACP) are involved in the biosynthesis of unsaturated HMAQs produced by *B. thailandensis*. Until now, there was no clear source for unsaturated medium chain fatty acids present in quinolone molecules. Our results reveal that HmqF is responsible for the recruitment of medium chain fatty acids via the A domain, which are then loaded onto the phosphopantetheinyl arm of holo-ACP, followed by dehydrogenation by the DH domain to give *trans*-β,γ-unsaturated fatty acyl-*S*-ACP products. Additionally, we have also shown that decanoic acid is the preferred substrate of HmqF, which is in good agreement with the observation that the most abundant HMAQ in *B. thailandensis* is the nine-carbon unsaturated alkyl chain that would arise from decanoic acid. The unsaturated fatty acids could then be passed on to remaining biosynthetic proteins (hmqBCD) for the biosynthesis of unsaturated quinolones.

The wide variety of quinolone molecules produced by *Burkholderia* species is surprising given the high fidelity of bacterial secondary metabolite biosynthetic pathways. Elucidating the biosynthesis of these QQS molecules will shed light on how the heterogeneity of QQS molecules can be produced by an individual bacterial species. Understanding importance of the subtle structural variations in the structures of the QQS molecule will be crucial in understanding the complex signaling pathways in QQS and provide the possibilities of novel approaches in the treatment of bacterial infection based on quorum sensing.

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■ ASSOCIATED CONTENT

Supporting Information

SDS−PAGE gels of A, ACP, and DH domains, extracted ion chromatograms of A domain assays, mechanism of Ppant fragmentation, Ppant fragments, Ppant fragment masses, acyl-adenylate masses, LC−MS of DH domain cofactor identification, DH domain unsaturation assay of intact holo-ACP loaded with decanoic acid, and time course DH domain unsaturaton assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CoA, coenzyme A; HIV, human immunodeficiency virus; LB, Luria broth; PCR, polymerase chain reaction; OD, optical density; ESI, electrospray ionization; FAD, flavin adenine dinucleotide; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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