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Brief Communication

N-Acylation during Glidobactin Biosynthesis by the Tridomain Nonribosomal Peptide Synthetase Module GlbF

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SUMMARY

Glidobactins are hybrid NRPS-PKS natural products that function as irreversible proteasome inhibitors. A variety of medium chain 2(*E*),4(*E*)-diene fatty acids *N*-acylate the peptidolactam core and contribute significantly to the potency of proteasome inhibition. We have expressed the initiation NRPS module GlbF (C-A-T) in *Escherichia coli* and observe soluble active protein only on coexpression with the 8 kDa MbtH-like protein, GlbE. Following adenylation and installation of Thr as a T-domain thioester, the starter condensation domain utilizes fatty acyl-CoA donors to acylate the Thr_1 amino group and generate the fatty acyl-Thr_1-S-pantetheinyl-GlbF intermediate to be used in subsequent chain elongation. Previously proposed to be mediated via acyl carrier protein fatty acid donors, direct utilization of fatty acyl-CoA donors for *N*-acylation of T-domain tethered amino acids is likely a common strategy for chain initiation in NRPS-mediated lipopeptide biosynthesis.

INTRODUCTION

Syrbactins comprise a family of bacterial *N*-acylated cyclic depsipeptides with a 12 membered macrolactam ring that covalently targets the active site of proteasomes (Groll et al., 2008). The electrophilic functionality is an α,β-unsatured amide moiety within the macrolactam scaffold that undergoes Michael addition by the N-terminal Thr_1–OH active site nucleophile within the proteasome (Figure 1). Two subfamilies within the syrbactins are the syringolins produced by the plant pathogen *Pseudomonas syringae* B301D-R (Waspi et al., 1998) and the glidobactins from *Burkholderia* K481-B101 (Oka et al., 1988a) which differ most dramatically in the substituents *N*-acylating the peptidolactam core.

The 12 membered macrolactam is assembled by a nonribosomal peptide synthase (NRPS) from a tripeptide X_1-Lys_2-X_3 framework where X_3 is Ala in glidobactins and Val in syringolins. In each case, X_3 is further elongated on a hybrid nonribosomal peptide synthetase (NRPS)-polyketide synthase (PKS) assembly line via a Δ^2-enoyl group (thereby introducing the electrophile that captures the proteasome) arising from partial processing of a malonyl unit by the PKS module (Amrein et al., 2004; Schellenberg et al., 2007) (see Figure S1 available online). X_1 is an *N*-acylated Val_1 in syringolins and an *N*-acylated Thr_1 in glidobactins. The *N*-acyl moieties are important determinants for potent inhibition of the proteasome, and they differ in syringolins and glidobactins. In syringolins, there is an additional Val_0 attached to Val_1 as an *N*-ureido-Val_0 while in glidobactin Thr_1 is *N*-acylated by medium chain fatty acid monomers. We recently described how the unusual ureido linkage is fashioned by a NRPS module in syringolin assembly (Imker et al., 2009) and now have turned to understanding the parallel step in glidobactin assembly.

Our attention in this work is on the *N*-acylation of Thr_1 in glidobactin scaffolds by dienoic acids, predominantly 2(*E*),4(*E*)-dodecadienoic acid. Bioinformatic analysis indicated that the tridomain NRPS GlbF harbors an N-terminal "starter" condensation (C) domain suggesting a direct function for this protein in initiation of glidobactin biosynthesis (Figure 2). Although starter C domains have been repeatedly proposed to catalyze *N*-acylation in peptide natural products (Baltz et al., 2005; Rausch et al., 2007), in vitro characterization has been lacking. We demonstrate that the starter C domain of the GlbF protein catalyzes chain-initiating *N*-acylation of the Thr_1-S-pantetheinyl-GlbF intermediate with fatty acyl-CoAs as cosubstrates without involvement of an in *trans* ACP.

RESULTS

GlbF as a Stand-Alone Tridomain C-A-T Module Predicted to Carry Out Glidobactin Chain Initiation: Heterologous Protein Production

To evaluate the 118 kDa GlbF as the putative glidobactin biosynthesis chain initiation module, the 3252 bp *Burkholderia* K481-B101 *glbF* gene was subcloned into various vectors for expression in *E. coli* as His_6-tagged constructs. No protein expression was detected despite variations in tag location, IPTG-induced expression, or growth temperature. We noted the presence of the predicted 8 kDa orf *glbE*, encoded just upstream of *glbF* (Figure 2). GlbE is a member of the MbtH family, with the founding member MbtH encoded in the mycobactin siderophore biosynthetic pathway. We recently reported that, during characterization of vicibactin biosynthesis, it was optimal to coexpress the MbtH homolog VbsG with the NRPS VbsS, and the VbsGS complex coeluted during purification (Heemstra et al., 2009).

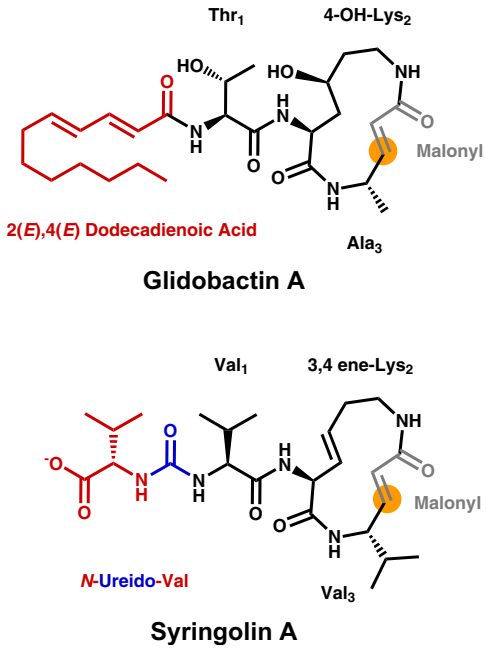


Figure 1. Chemical Structures of Glidobactin A and Syringolin A

To that end, tandem coexpression of GlbE and GlbF led to production of soluble GlbF (Figure S2). Notably, nickel affinity purification of C-His_6-tagged GlbF (120 kDa) resulted in copurification of the untagged GlbE (8 kDa). Likewise, nickel affinity purification of N-His_6-tagged GlbE resulted in copurification of the untagged GlbF. Washing of the resin-bound C-His_6 GlbF for 16 hr with no salt, 2 M NaCl, 1% Triton X-100, 1 mM DTT, or 1 mM ATP/1 mM Thr containing buffer was not sufficient to disrupt the GlbEF interaction (data not shown). Consequently, the complex was further purified by gel filtration and the proteins separated on 4%–15% gradient SDS-PAGE gel. The two bands were excised and amino acid content was analyzed by ion exchange following in-gel acid hydrolysis. This analysis showed that the complex consisted of a 1.71:1 GlbE:GlbF stoichiometry when GlbE was His_6-tagged and 1.65:1 stoichoimetry when GlbF was His_6-tagged. With pure, soluble GlbEF complex obtainable at yields of ~10 mg/liter we could proceed to evaluation of GlbF NRPS activity.

The Adenylation Domain of GlbF Activates and Loads L-Thr as a Thioester on the Holo Form of the T Domain

Based on the domain organization of the two NRPS megasynthetases in the glidobactin gene cluster, GlbF was the most likely candidate for activation and incorporation of the first amino acid, thereby initiating glidobactin biosynthesis. Additionally, the 10 amino acid Stachelhaus motif (DFWNIGMVHK) predicts Thr is the substrate for the GlbF A domain (Stachelhaus et al., 1999; Rausch et al., 2005). Reversible generation of amino acid adenylates allows for quantification of activation by the classical ATP-$^{32}PP_i$ exchange assay. When this assay was carried out with a panel of representative amino acids, GlbF showed strong selectivity toward Thr and Ser substrates (Figure S3). Full kinetic analysis further refined the specificity; while activation of Thr showed prototypical adenylation parameters (k_{cat} = 0.8 s^{-1}, K_M = 0.7 mM, k_{cat}/K_M = 1.3 × 10^3 M^{-1} s^{-1}), saturation kinetics were not achievable for Ser at concentrations less than 10 mM (k_{cat}/K_M ~1 × 10^1 M^{-1} s^{-1}) (Figure S3).

With confirmation that the GlbF A domain activates Thr to generate Thr-AMP in the first reversible half reaction, we then validated the expected second half reaction, transfer of the Thr moiety to the HS-pantetheinyl arm of the T domain of GlbF.

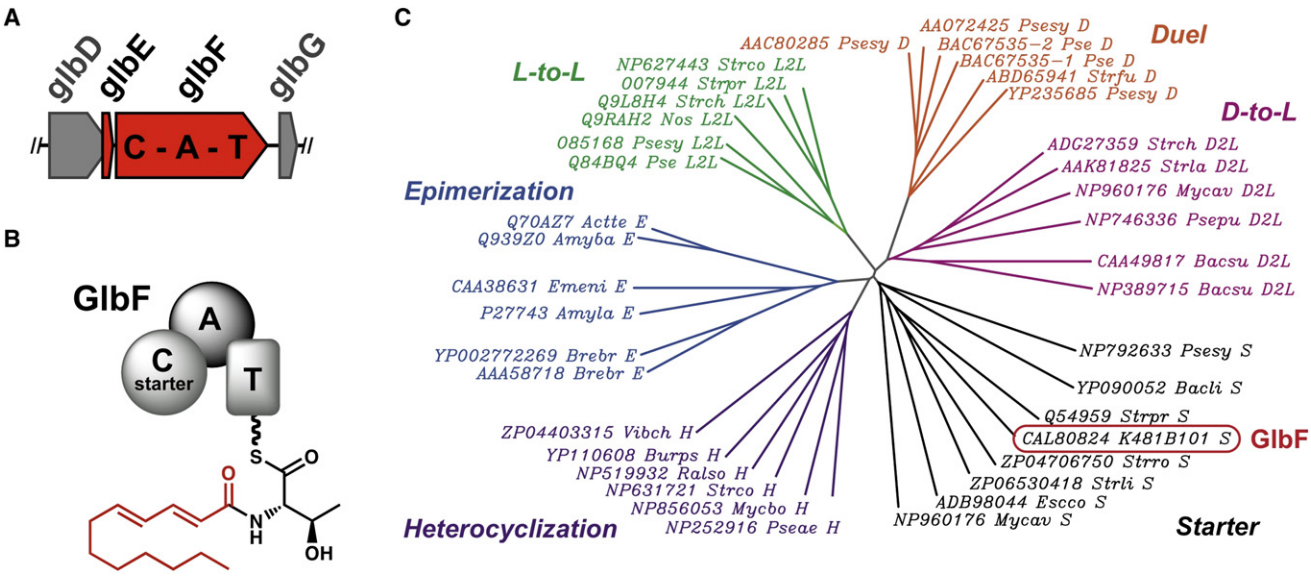


Figure 2. Characterization of the Glidobactin Initiation Module, GlbF

(A) Partial glidobactin cluster.

(B) GlbF module organization and putative thioester product.

(C) Phylogenetic analysis of C domains including the GlbF starter C domain.

See also Figures S1 and S2.

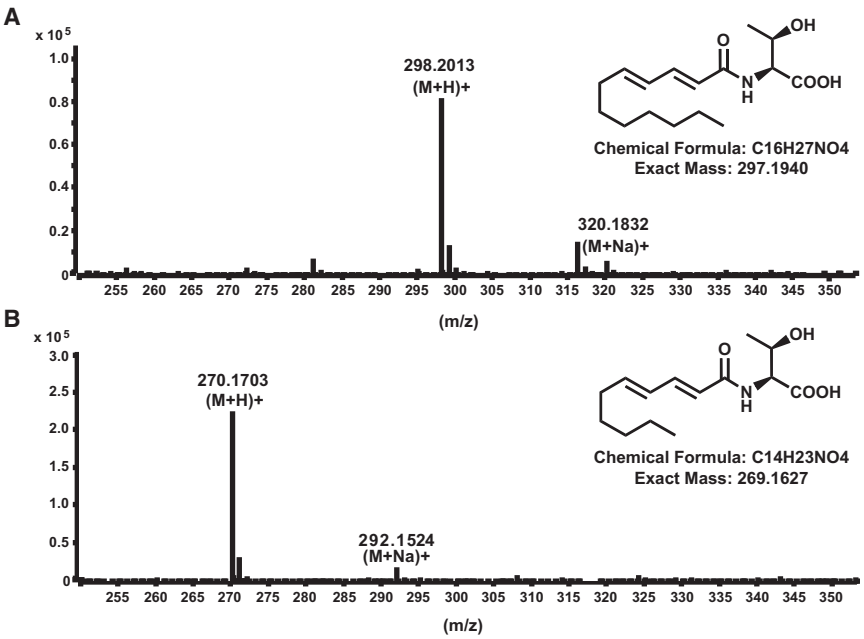


Figure 3. HR LC-MS Spectra of Condensation Products Hydrolyzed from the GlbF T Domain

(A) 2(*E*),4(*E*) dodecadienoyl-Thr Product.
(B) 2(*E*),4(*E*) decadienoyl-Thr Product.
See also Figures S3, S4, and S5.

For this to occur, the apo form of GlbF must be posttranslationally modified such that Ser1018 becomes phosphopantetheinylated in the holo form. This was accomplished with purified GlbF by use of the broad specificity PPTase Sfp using coenzyme A as the cosubstrate (Quadri et al., 1998). Covalent loading of ^{14}C-Thr onto the holo T domain of GlbF occurs to 70% calculated stoichiometry (Figure S4). ^{14}C-Ser is also loaded to approximately the same level as Thr, albeit more slowly which is likely a reflection of suboptimal adenylation.

The Condensation Domain of GlbF Catalyzes Auto *N*-Acylation of the Thr$_1$-S-T Domain Using Fatty Acyl-CoA Donors

The putative *N*-acylation activity of the GlbF starter C domain requires access to activated fatty acid donors. Although ACPs had been proposed to be the activating unit used in the biosynthesis of other *N*-acylated peptidyl natural products, no ACP was found in the glidobactin gene cluster (Schellenberg et al., 2007). Furthermore, early feeding studies indicated that the unusual 2(*E*),4(*E*) diene fatty acids were intermediates shunted from β-oxidation of long-chain fatty acids (Numata et al., 1988). This observation suggested that the usual diene fatty acids were biosynthesized as CoA thioesters without involvement of dedicated ACPs. Therefore, we synthesized the CoA thioesters of the C10 and C12 2(*E*),4(*E*) diene fatty acids as these acids were accessible by commercial or synthetic methods and represented both glidobactin A as the most prominent variant (C12 diene) and glidobactin F as a minimally produced variant (C10 diene). The CoA ester of 2(*E*),4(*E*) decadienoic acid was synthesized using standard PyBOP coupling using commercial 2(*E*),4(*E*) decadienoic acid (Kopp et al., 2008). In contrast, the CoA ester of synthetically prepared 2(*E*),4(*E*) dodecadienoic acid was prepared after activation with ethyl choroformate.

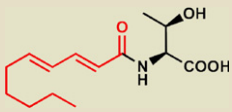
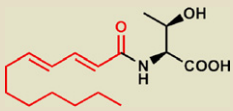
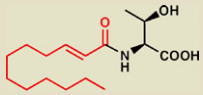
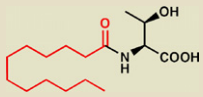
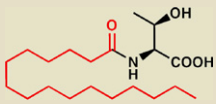
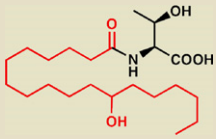
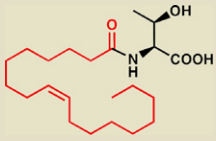
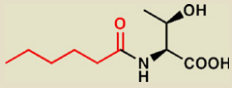
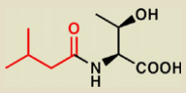
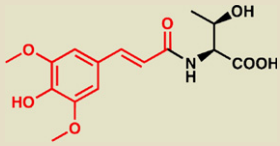
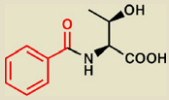
Condensation between fatty acyl-CoA donors and Thr$_1$-S-pantetheinyl-GlbF was tested by in situ generation of the holo GlbF protein followed by addition of the fatty acyl-CoA and ATP. After the 2 hr, the protein was precipitated in methanol and the protein pellet subjected to base hydrolysis to cleave the thioester-bound product. After workup to remove protein and concentrate the sample, the liberated product was analyzed by HR LC-MS for formation of the condensation product. Both 2(*E*),4(*E*) dodecadienoyl-Thr and 2(*E*),4(*E*) decadienoyl-Thr were readily detected with (M+H)+ ions of 270.1703 (expected *m/z* 270.1706) and 298.2013 (expected *m/z* 298.2019), respectively (Figure 3). To confirm that the GlbF starter condensation domain was responsible for acylation of the T-domain-bound Thr, the conserved catalytic His common to all condensation domains in the H**H**xxxDG motif (Stachelhaus et al., 1998) was replaced with Ala by site-directed mutagenesis. When assayed as above for condensation activity, no product was detected from reaction with the H141A mutant protein despite being active in adenylation and loading assays.

In the glidobactin family, six of the seven structurally characterized natural products vary only in the constitution of the *N*-acyl fatty acid. This variability suggests a propensity toward C domain promiscuity and prompted us to test condensation with structurally diverse acyl-CoA donors (Table 1; Figures S5 and S6). While donors that deviated from the fatty acid family were not utilized (e.g., benzoyl and isovaleryl CoAs), surprisingly, long-chain fatty acid donors were accepted, including palmitoyl- and oleoyl-CoA. However, while the fully saturated dodecanoyl-CoA was not a donor, the singly desaturated 2(*E*) dodecenoyl-CoA sufficed. Short-chain fatty acyl-CoAs, either saturated or desaturated, were not utilized. These results are consistent with semisynthetic SAR studies conducted shortly after the discovery of glidobactins (Oka et al., 1988b). Despite the ability of GlbF to activate and load Ser (*vide supra*), 2(*E*),4(*E*) dodecadienoyl-Ser and 2(*E*),4(*E*) decadienoyl-Ser were not observed. However, none of the glidobactin variants isolated contain Ser at X$_1$ suggesting specificity at this position is strict.

DISCUSSION

While *N*-acylation has been shown to be a critical feature for efficacy in several cases, including the clinically relevant antibiotic daptomycin (Huber et al., 1988; Baltz et al., 2005), little understanding has been acquired as to the timing and mechanism of acylation. Several groups have noted the conspicuous presence of an N-terminal condensation domain in the initiating module of NRPS assembly lines of *N*-acylated natural product biosynthetic

Table 1. Acyl-CoA Donors Tested for Condensation to Thr_1-S-Pantetheinyl-GlbF

CoA Donor	Hydrolyzed Condensation Product	Expected m/z (M+H)+	Found m/z (M+H)+	Δ ppm[a]
2(*E*),4(*E*) decadienoyl-CoA		270.1706	270.1703	1.27
2(*E*),4(*E*) dodecadienoyl-CoA		298.2019	298.2013	0.81
2(*E*) dodecenoyl-CoA		300.2176	300.2168	−0.44
dodecanoyl-CoA		302.2332	None	–
palmitoyl-CoA		358.2958	358.2962	−1.18
12-hydroxy-stearioyl-CoA		402.3220	None	–
oleoyl-CoA		384.3115	384.3109	0.26
hexanoyl-CoA		218.1393	None	–
isovaleryl-CoA		204.1237	None	–
sinapioyl-CoA		326.1241	None	–
benzoyl-CoA		224.0924	None	–

See also Figures S5 and S6.

[a] Delta parts per million calculated as [(observed m/z − expected m/z)/expected m/z] × 10^6.

gene clusters (Baltz et al., 2005; Farnet et al., 2007; Rausch et al., 2007). The presence of these domains in the initiation module indicates that amide bond formation occurs between the activated fatty acyl donor and the aminoacyl$_1$-S-pantetheinyl intermediate before chain elongation and involvement of the next downstream module. Accordingly, these domains were termed ''starter'' C domains by Rausch et al. during their careful phylogenetic analysis of condensation domain sub-types and include the N-terminal condensation domains of daptomycin, enterobactin, and surfactin (Rausch et al., 2007). We carried out a similar phylogenetic analysis including the GlbF sequence and found that this condensation domain also clearly grouped with other starter C domains (Figure 2).

Along with the proposed role of starter condensation domains as the catalyst for *N*-acylation, the nature of the activated fatty acid species has been the subject of consideration as well. The presence of acyl-CoA ligases and ACPs in several lipopeptide natural product gene clusters, including CDA, daptomycin, and A54145, led Baltz et al. to propose ACP-mediation as a general strategy in lipopeptide biosynthesis (Baltz et al., 2005). Indeed, the 2,3-epoxyhexanoyl moiety *N*-acylating the CDA peptide core was shown to be synthesized on a dedicated ACP encoded within the CDA gene cluster (Kopp et al., 2008). Although no in vitro biochemical analysis has been published directly showing condensation between the X_1 amino acid and ACP-activated fatty acids, this is likely the mechanism in those systems. Notably, *N*-acylation of Ser-S-pantetheinyl-EntF with 2,3-dihydroxybenzoate in enterobactin biosynthesis occurs via the ArCP (aryl-carrier protein) containing EntB (Gehring et al., 1998), giving further credence to starter C domain interaction with various carrier proteins. Steller et al. showed direct utilization of fatty acyl-CoA donors in *N*-acylation of Glu$_1$ during surfactin biosynthesis; however, this is thought to be largely mediated by the dual-function thioesterase/acyltransferase SrfD instead of the SrfA starter C domain alone (Steller et al., 2004). This conclusion was supported by the observed binding of myristoyl-CoA to SrfD during structural characterization of SrfD by NMR spectroscopy (Koglin et al., 2008).

All glidobactins are *N*-acylated by 2(*E*),4(*E*)-dienoic fatty acids which vary in chain length and extent of oxidation. Long-chain fatty acids are broken down by sequential removal of two carbon units during β-oxidation, and examination of these intermediates correspond to the novel fatty acids found in the glidobactin natural products. Given that degradation by β-oxidation occurs on CoA thioesters and no ACP was found in the glidobactin gene cluster, we hypothesized that the fatty acids used in glidobactin biosynthesis were activated as CoA thioesters. Alternatively, the CoA intermediate could be transferred to an in *trans* ACP domain for interaction with the GlbF starter C domain; however, our ability to locate remote ACPs was restricted by the fact that *Burkholderia* K481-B101 has not been sequenced. Therefore, we synthesized the CoA thioesters of 2(*E*),4(*E*)-dodecadienoic acid (glidobactin A, major variant) and 2(*E*),4(*E*)-decadienoic acid (glidobactin F, minor variant) to test for direct condensation to Thr$_1$-S-pantetheinyl-GlbF in an end point assay. Reaction products were chemically hydrolyzed from the GlbF T domain and analyzed by HR LC-MS. While no product was detected in the enzyme-free control reactions or with the GlbF H141A mutant, the wild-type enzyme produced both 2(*E*),4(*E*) dodecadienoyl-Thr and 2(*E*),4(*E*) decadienoyl-Thr (Figure 3). These results confirmed our hypothesis that the starter C domain of GlbF utilizes CoA thioesters as activated fatty acid donors to carry out stoichiometric self-modification of Thr$_1$ without requirement for an in *trans* ACP.

When challenged with variant acyl-CoA donors, GlbF specificity was restricted to use of the singly desaturated 2(*E*) dodecenoyl-CoA and the long-chain palmitoyl- and oleoyl-CoAs (Table 1; Figures S5 and S6). Nothing is known about the unusual tailoring that gives rise to the 2(*E*),4(*E*) configuration, and it is possible that 2(*E*) fatty acids undergo subsequent oxidation on the assembly line to generate the diene natural products. Likewise, the significance of the palmitoyl and oleoyl donors is unclear. As the two most common lipids in *Burkholderia* species (Yabuuchi et al., 1992), palmitoyl- and oleoyl-CoA pools may be substantial enough to generate novel acyl-Thr$_1$-S-pantetheinyl-GlbF intermediates. A detailed kinetic analysis would provide insight into the true selectivity; however, self-acylation of GlbF demands fast-reaction/single-turnover kinetics currently beyond the scope of our investigation. We suspect the absence of long-chain glidobactin variants is due to downstream editing of off-pathway species. Despite this, feeding of methyl linolenate to *Burkholderia* K481-B101 cultures resulted in isolation of a new glidobactin variant (Numata and Oka, 1992). Efforts to explore the promiscuity observed here ultimately requires probing condensation between noncanonical acyl-Thr$_1$-S-pantetheinyl-GlbF intermediates with Lys$_2$-S-pantetheinyl-GlbC in the next downstream module.

One particularly intriguing aspect of this work is the necessity for coexpression of the MbtH-like protein GlbE in order to obtain overexpressed and soluble GlbF (Figure S2). Only ~70 amino acids in length, MbtH-like proteins are found in dozens, but not all, NRPS gene clusters and have remained enigmatic from first observation. Knockout studies indicate they are essential for production of vicibactin, coelichelin, CDA, clorobiocin, and pyoverdine natural products, yet no catalytic function can be surmised (Carter et al., 2002; Drake et al., 2007; Lautru et al., 2007; Wolpert et al., 2007). The crystal structure of the MbtH-like protein from pyoverdine biosynthesis revealed a novel fold consisting of a three-stranded antiparallel β sheet and two α helices (Drake et al., 2007). That study also highlighted strictly conserved hydrophobic residues, prompting a chaperone function to be proposed.

A recent study in our group on vicibactin biosynthesis found that the MbtH-like protein VbsG was required for adenylation activity of the NRPS VbsS (Heemstra et al., 2009). In that work, it was optimal to coexpress VbsG and VbsS which then purified as a complex. When initial efforts to obtain heterologous expression of the NRPS GlbF failed, coexpression of GlbE and GlbF was pursued. Remarkably, direct cloning of the *glbE-glbF* construct from *Burkholderia* K481-B101 resulted in overexpression of soluble GlbE and GlbF, without modification of the RBS for *glbF* or intergenic spacing (Δ 5 bp). In this case, presumably GlbE serves a chaperone function giving rise to the stable formation of the 118 kDa GlbF. We have not yet been able to determine if GlbE is also required for optimal activity, as with the VbsG/VbsS pair, because we were unable to separate the complex in the absence of denaturing detergents. These results prompt continued examination of MbtH-like proteins as activators,

chaperones, or both in NRPS assembly lines. Regardless, we find that coexpression of MbtH-like proteins is of practical relevance and should be considered during heterologous expression of NRPS megasynthases.

SIGNIFICANCE

_N_-acylation is an important avenue for diversification of peptide scaffolds and can have profound effects on SAR profiles. Daptomycin, a potent antibiotic in clinical use (as Cubicin) against Gram-positive pathogens including MRSA, was discovered through exploration of fatty acid variations in the constituents of the A21978C complex (Baltz et al., 2005). Likewise, _N_-acylation of the glidobactin macrolactam core was shown very early on to be critical for antimicrobial and antitumor function (Oka et al., 1988b). More recently, a chimeric syrbactin was produced synthetically by _N_-acylating the syringolin macrolactam core with a saturated fatty acid resulting in one of the strongest proteasome inhibitors reported to date (Clerc et al., 2009). Despite these examples, biochemical characterization of this step in natural product biosynthesis has remained largely unexplored. The work reported in this communication reveals the necessary components required for _N_-acylation in the glidobactin family of natural products. The single NRPS module GlbF uses fatty acyl-CoA donors as cosubstrates to initiate glidobactin biosynthesis. Furthermore, the importance of the MbtH-like protein GlbE in this work encourages inclusion of MbtH-like proteins during future in vitro characterization of other NRPS systems. Continued investigation into _N_-acylation tailoring will undoubtedly yield more insight for strategies to bioengineer natural product pathways.

EXPERIMENTAL PROCEDURES

Cloning and Purification of GlbEF

The bicistronic *glbE-glbF* insert was cloned from *Burkholderia* K481-B101 genomic DNA and ligated into His_6-pET vectors for heterologous expression in *E. coli* BL21 CodonPlus (Stratagene) using standard molecular biology techniques and is detailed in the Supplemental Experimental Procedures. Large-scale (2 L) production was carried out at 37°C with IPTG induction (0.5 mM) at an OD_{600} of 0.4 followed by continued growth at 37°C for an additional 5 hr. Protein was isolated by nickel affinity chromatography as previously described (Imker et al., 2009). Protein was visualized on a 4%–15% SDS-PAGE gel, and fractions containing the GlbEF complex in ≥95% purity were dialyzed against 250 mM NaCl buffered with 25 mM Tris-HCl (pH 7.9). The protein was concentrated to 7–10 mg/ml as determined by Bradford Assay using BSA as a standard and stored at −80°C until use.

Assay of Condensation Activity

Apo-GlbF (20 μM) was converted to holo-GlbF by incubation with 300 nM *Bacillus subtilis* Sfp and 200 μM CoA for ~30 min in a reaction that contained 5 mM $MgCl_2$, 0.5 mM TCEP, 5 mM Thr, and 40 mM NaCl buffered with 50 mM HEPES-HCl (pH 7.5). The reaction was initiated by addition of ATP and the acyl-CoA to 5 and 1.25 mM final concentrations, respectively. The final reaction volume was 250 μl. After 2 hr, 750 μl of cold methanol was added, and the protein precipitate was pelleted at 10,000 × g for 8 min. The supernatant was removed, and the pellet washed twice with 250 μl cold methanol. The pellet was dried under an air stream and then dissolved in 125 μl of 0.1 M KOH followed by heating at 70°C for 10 min. After cooling to room temperature and neutralization with HCl, the protein was precipitated overnight at −20°C by addition of 1 ml methanol. The hydrolysate was centrifuged at 10,000 × g for 15 min to remove precipitated protein, and the supernatant concentrated by SpeedVac. The residue was taken up in 100 μl acetonitrile and analyzed by Dual-ESI on an Agilent 6520 QTOF-LCMS in positive ion mode.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at doi: 10.1016/j.chembiol.2010.08.007

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Note Added in Proof

Kraas et al. have recently published the mechanism of *N*-acylation in surfactin biosynthesis. Their in vivo and in vitro results allow the same conclusions to be drawn as the work present here (Chem. Biol. *17*, 872-880).