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The Loading Module of Mycosubtilin: An Adenylation Domain with Fatty Acid Selectivity

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Mycosubtilin, a potent antifungal natural product of the iturin class of cyclic lipopeptides, was isolated from *Bacillus subtilis*.[1] The iturin family of lipopeptide antibiotics is characterized by a β-amino fatty acid containing octapeptide (Figure 1).[2] Cloning and sequencing of the gene cluster responsible for the biosynthesis of mycosubtilin reveals four open reading frames (ORFs): *fenF, mycA, mycB, mycC*. MycA, a single 459 kDa protein, contains elements of polyketide synthases (PKS), nonribosomal peptide synthetases (NRPS), fatty acid synthetases, and amino transferases (AMT). It is predicted that palmitic acid is loaded by an acyl ligase (AL) onto acyl carrier protein 1 (ACP_1) and malonyl-CoA is loaded onto ACP_2 in trans by FenF, then condensed via a ketosynthase (KS) domain to give a β-keto thioester that is reductively aminated by the AMT domain (Scheme 1).[3] The β-amino fatty thioester is then presumably passed on to the NRPS portion of MycA through a non-canonical condensation (C) and peptidyl carrier protein (PCP) didomain.

Hybrid PKS/NRPS synthetases are unique combinations of NRPS and PKS synthetase units. NRPS and PKS natural products have been a valuable source of many drugs, suggesting that hybrid PKS/NRPS natural products could provide another class of structurally diverse medicinal theraputics.[4] The lipopeptide natural products such as surfactin, calcium dependent antibiotic, and daptomycin are composed of nonribosomally synthesized cyclic peptides appended with an *N*-acyl fatty acid. Daptomycin, under the trade name Cubicin, is the first of this class of natural products to have been approved for human clinical use in treating vancomycin resistant infections.[5] A number of other lipopeptides are being investigated for clinical uses but have been limited by deleterious side-effects. Recently it has been shown that the activity of these natural products is significantly influenced by the length and structure of the fatty acid.[6] In the fermentation of these natural products there has been some flexibility in the length of fatty acid group incorporated. These two facts taken together, coupled with a complete biochemical understanding of this process, may allow for the utilization of this relaxed specificity in fatty acid side chains for novel hybrid PKS/NRPS-based natural products.

The fatty acid priming of ACP_1 for MycA is proposed to occur via the AL domain. Loading of ACP_1 can be envisioned to occur in one of two pathways, first being the fatty acid coenzyme A (CoA) ligase mechanism. The fatty acid is activated as an acyl−adenylate then reacts with CoA to form an acyl−CoA thioester intermediate that undergoes transthiolation with the HS−phosphopantetheine of ACP_1. Alternatively, there are a number of fatty acyl−AMP ligases (FAAL) recently described that activate the fatty acid as an acyl−adenylate which is directly transferred to an ACP domain.[7] Using

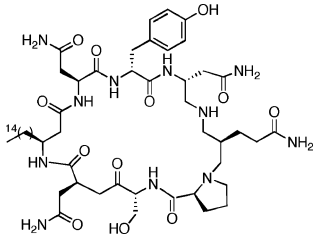
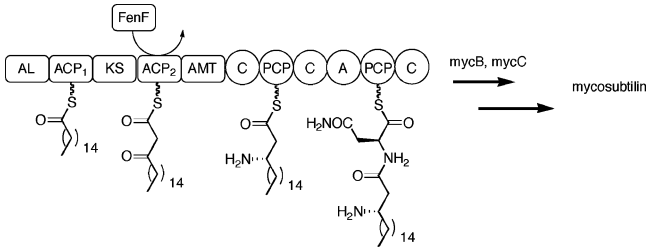


Figure 1. Mycosubtilin.

Scheme 1. Mycosubtilin Biosynthesis



a series of assays we report below how the fatty acid is incorporated in mycosubtilin.

To explore fatty acid incorporation we sought to reconstitute the activity of the AL−ACP_1 didomain in vitro. The AL−ACP_1 (MycA10) didomain was cloned and expressed in *E. coli* as a N-terminally His_6-tagged 77 kDa protein. The activity of the ACP domain to be primed was confirmed by incorporation of a radiolabel upon incubation with [^{14}C]-acetyl CoA and Sfp.[8] Loading of the fatty acid was examined with radioactive decanoic acid, which was a compromise between lipophilic character and aqueous solubility of potential substrates and was used with great success in the production of daptomycin. Incubation of holo-MycA10 with $MgCl_2$, ATP and [1-^{14}C]-decanoic acid was followed by SDS-polyacrylamide gel electrophoresis (PAGE) and the protein bound radioactivity was analyzed by autoradiography. A clear time dependent incorporation of the radiolabel was observed corresponding to covalent loading of decanoic acid. By contrast, incubation of holo-MycA10 with [1-^{14}C]-decanoyl-CoA followed by SDS-PAGE and autoradiography gave no significant incorporation of the radiolabel.

Direct observation of the activated intermediates may provide a more accurate picture as to the mechanism of fatty acid loading. Accumulation of the reactive acyl−adenylate or acyl−CoA intermediate should occur with apo-MycA10 because of the absence of the thiol nucleophile of the phosphopantetheine cofactor of the apo-ACP domain. Incubation of apo-MycA10 with [1-^{14}C]-decanoic acid, $MgCl_2$ and ATP both in the presence and absence of CoA followed by TLC and autoradiography showed new spots of identical retention factor (R_f) distinct from both decanoic acid and

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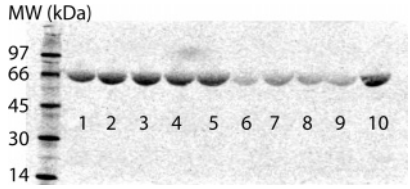

Figure 2. Substrate screen for MycA10 loading. Autoradiography of a SDS-PAGE gel contains mycA10 chased with [1-^{14}C] decanoic acid after incubation with no substrate (lane 1); acetic acid (lane 2); propionic acid (lane 3); butyric acid (lane 4); hexanoic acid (lane 5); trans-2-decenoic acid (C10, lane 6); decanoic acid (C10, lane 7); myristic acid (C14, lane 8); palmitic acid (C16, lane 9); no substrate (lane 10).

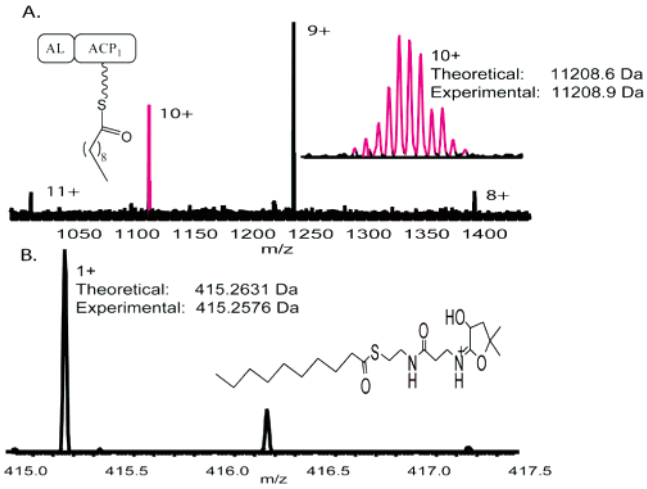

Figure 3. (a) FTMS broadband spectrum of decanoic acid loaded MycA10 active site peptide; (inset) enlargement of the 10+ charge state. (b) Partial FT mass spectrum of the PPant ejection product from loaded MycA10 with the chemical structure of ejection product (see inset).

an authentic [1-^{14}C]-decanoic-CoA standard. This result is consistent with the previous result that acyl−CoAs are not involved in the loading of ACP_1. To further corroborate the acyl-adenylate mechanism two reactions were compared, incubating [1-^{14}C]-decanoic acid and ATP, or [8-^{14}C]-ATP and decanoic acid with MycA10. Analysis by TLC gave spots of identical R_f consistent with the decanoyl−adenylate intermediate.

To examine the substrate specificity of the loading domain of MycA a simple chase experiment was used.[9,10] Holo-MycA10 was incubated with the putative unlabeled acid substrate and ATP under standard condition for 30 min; the small molecules were removed and chased with additional ATP and [1-^{14}C]-decanoic acid. Any unlabeled substrate that was activated and subsequently loaded would block the [1-^{14}C]-decanoic acid chase loading leading to little or reduced bound radioactivity when examined by SDS-PAGE and autoradiography (Figure 2).

High-resolution Fourier-transform mass spectrometry (FTMS) was used to further characterize the loading of a fatty acid on MycA. Holo-MycA10 was incubated with decanoic acid in the presence or absence of ATP for 1.25 h and then proteolytically digested with trypsin for 15 min. The peptide mixture was separated by reverse phase liquid chromatography connected directly to a 7 T LTQ-FT mass spectrometer (ThermoFisher, San Jose, CA). All samples were subjected to two FTMS experiments, the first being data-dependent tandem mass spectrometry of intact peptides for thiolation domain active site mapping and the second being a phophopantetheinyl (PPant) ejection assay for further characterization of the loaded substrates.[11]

Analysis of a tryptic digest of holo-MycA10 revealed a 95 amino acid peptide with a mass of 11053.4 Da, 340.0 Da higher than the theoretical mass of the apo-peptide and within 0.1 Da of the mass shift expected for phosphopantetheinylation of this peptide. The tandem MS data using collision induced dissociation (CID) confirmed that it indeed contained the active site serine of the MycA10 thiolation domain and localized the modification to one of two serine residues on the peptide (one of which is predicted to be the active site serine). Upon incubation of holo-MycA10 with decanoic acid and ATP, the mass of this peptide (11208.9 Da) was shifted by +155.5 Da, consistent with formation of decanoyl-*S*-enzyme (Figure 3a). Further, the PPant ejection assay resulted in observation of the 415.2576 Da ion shown in Figure 3b. This ion is within 5 mDa of the expected value and consistent with the empirical formula of the deaonoyl-*S*-PPant ejection product (Figure 3b, inset).

The data presented clearly characterizes the function and mechanism by which fatty acids are loaded in biosynthesis of mycosubtilin. The loading domain functions in an analogous fashion to A-T didomains of NRPS systems where the substrate is activated as (amino)acyl−adenlyate and then directly loaded on the thiolate of the PPant arm of the carrier protein. The loading domain of MycA presents an entry into the understanding the initial steps in the biosynthesis of lipopeptides of hybrid PKS/NRPS origin. In conjunction with an understanding of fatty acid incorporation the observed tolerance for loading a variety of fatty acids may provide for the combinatorial biosynthesis of lipopeptides of hybrid PKS/NRPS origin.

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Supporting Information Available: Experimental details of protein preparation and analysis of loading, including detailed descriptions of FTMS studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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