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In Vitro Analysis of Carboxyacyl Substrate Tolerance in the Loading and First Extension Modules of Borrelidin Polyketide Synthase

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Supporting Information

ABSTRACT: The borrelidin polyketide synthase (PKS) begins with a carboxylated substrate and, unlike typical decarboxylative loading PKSs, retains the carboxy group in the final product. The specificity and tolerance of incorporation of carboxyacyl substrate into type I PKSs have not been explored. Here, we show that the first extension module is promiscuous in its ability to extend both carboxyacyl and non-carboxyacyl substrates. However, the loading module has a requirement for substrates containing a carboxy moiety, which are not decarboxylated *in situ*. Thus, the loading module is the basis for the observed specific incorporation of carboxylated starter units by the borelidin PKS.

Type I polyketide synthase (PKS) enzymes are modular "assembly lines" that give rise to large and complex molecules through successive condensations of 3-carboxyacyl-CoA substrates (recently reviewed by Khosla et al.).[1] The "textbook" understanding of polyketide biosynthesis initiation in type I PKS systems proceeds through two main mechanisms (reviewed by Hertweck).[2] In the first, as is present in the well-studied erythromycin PKS,[3] an AT-ACP didomain loading module selects an acyl starter unit that is then transferred to and extended by the downstream module. In the second mechanism, the AT domain loads a 3-carboxyacyl substrate (e.g., malonyl-CoA), which is subsequently decarboxylated by an accompanying KS^{Q} domain prior to chain translocation. Besides the presence of an upstream KS^{Q} domain, AT domains in such a tripartite system are identified by the presence of a conserved arginine residue thought to interact with the carboxylate moiety of the substrate.[4] Some loading modules (e.g., in lipomycin PKS) have been shown to promiscuously load a variety of acyl-CoA substrates;[5] however, there have been few such *in vitro* studies examining substrate tolerance in PKS loading modules.

The structure of borrelidin would suggest it uses a mechanism different from the two described above for polyketide biosynthesis initiation (Figure 1).[6] Borrelidin is to the best of our knowledge the only *cis*-AT-containing PKS proven to begin with a loaded carboxyacyl substrate [*trans*-1,2-cyclopentanedicarboxylic acid (*t*1,2-CPDA)] that is extended without *in situ* decarboxylation by a KS^{Q} domain.[7] Subsequent work demonstrated that the borrelidin PKS can process a variety of non-natural starter substrates by the production of several borrelidin analogues in a starter unit deficient strain fed with a panel of more than 40 acyl and carboxyacyl acids.[8] Only diacids with a particular geometric arrangement of carboxylates were found to serve as starter units, which the authors suggest is a result of selectivity imposed by the PKS loading module (BorA1). However, in the *in vivo* system, it was not possible to determine whether this selectivity resulted from the failure of the substrate to enter the cell, the inability of the endogenous CoA ligase to recognize the substrate, or the diversion of the substrate or its CoA thioester into other metabolic pathways, nor could the authors determine the stage in the polyketide biosynthesis at which this selectivity is imposed.

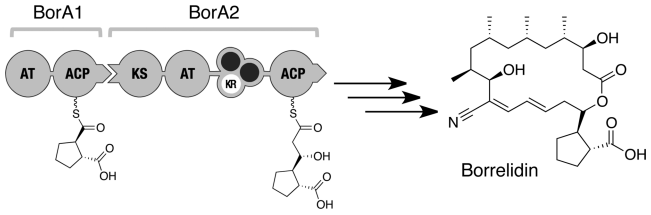

Figure 1. Partial schematic of borrelidin biosynthesis highlighting the loading and first extension modules.

To improve our understanding of substrate selectivity toward acyl and carboxyacyl substrates in the borrelidin PKS, we heterologously expressed and purified the loading and first extension modules and tested their activity *in vitro* with a panel of CoA substrates. Because pendant polyketide products are covalently attached to the phosphopantetheine arm of the acyl carrier proteins, tandem mass spectrometry (MS/MS) methods such as the "PPant ejection assay"[9] provide a convenient and sensitive way to qualitatively monitor enzyme activity. Briefly, the tryptic peptide containing the acylated PPant prosthetic group is selected by a quadrupole mass filter and subjected to

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collision-induced dissociation (CID) to eject the (acyl)PPant arm that is detected as a daughter ion via a quadrupole or time-of-flight mass analyzer.

We performed loading/extension assays in which the loading and extension modules (BorA1 and BorA2) were co-incubated with different starter CoA substrates along with malonyl-CoA and NADPH (the extender unit and reducing substrate for BorA2). We tested *t*1,2-CPDA CoA (CPDA-CoA), borrelidin's natural starter, along with an aliphatic analogue, cyclopentanemonocarboxylic acid CoA (CPMA-CoA) (Figure 2A;

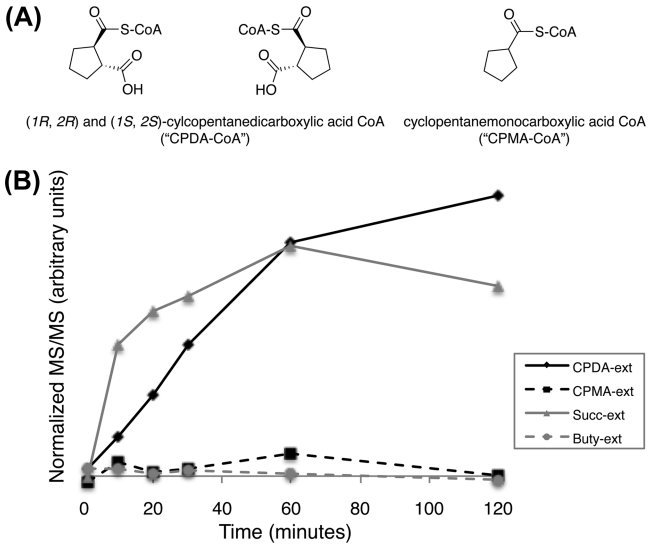

Figure 2. (A) Synthetic substrates used in this study. (B) Loading/extension assay with BorA1 and BorA2 showing normalized MS/MS transitions for extension products.

see the Supporting Information for details of synthesis). As expected, when the CPDA-CoA substrate is provided, its extended/reduced product, the 3-(2-carboxycyclopentyl)-3-hydroxypropionyl moiety (CPDA-ext), accumulated on the BorA2 ACP (Figure 2B) in a time-dependent manner. In contrast, the CPMA-CoA substrate was not accepted by the loading AT as neither CPMA-ACP nor its extension product (CPMA-ext) was detected (see Figure 3 of the Supporting Information for loading ACP data). Performing the loading/extension assay with two additional substrates—succinyl-CoA and another aliphatic analogue, butyryl-CoA—surprisingly revealed that while succinyl-ACP was not detected on the loading ACP [which we attribute to instability (Figure 4 of the Supporting Information)], we infer its loading and extension by the detection of its extension product, 3-hydroxyhexanedioic-ACP (Succ-ext). As with the CPMA-CoA substrate, when butyryl-CoA was tested, neither butyryl-ACP nor its extension product (Buty-ext) was detected.

We next focused on the substrate specificity of BorA2 by performing chain extension assays using acylated loading ACP (ACP0) as a starting substrate. In these experiments, the loading ACP was expressed in its apo form as a monodomain and then phosphopantetheinylated *in vitro* with the same panel of four CoA substrates via Sfp.[10] These (carboxy)acyl-ACP substrates were incubated in the presence of (holo) BorA2, and product formation was monitored using the PPant ejection assay. Because both CPDA-CoA enantiomers generated during the synthesis were presumably loaded onto the apo ACP by Sfp, we investigated if BorA2 preferentially extends one enantiomer over the other by refining the liquid chromatography method with a shallower gradient in an attempt to resolve the different possible diastereomer products (see the Supporting Information for details).

Interestingly, examination of CPDA-ACP0 extension revealed two clear peaks repeatedly observed in an approximately 2:3 ratio (Figure 3A, top panel), and analysis via high-

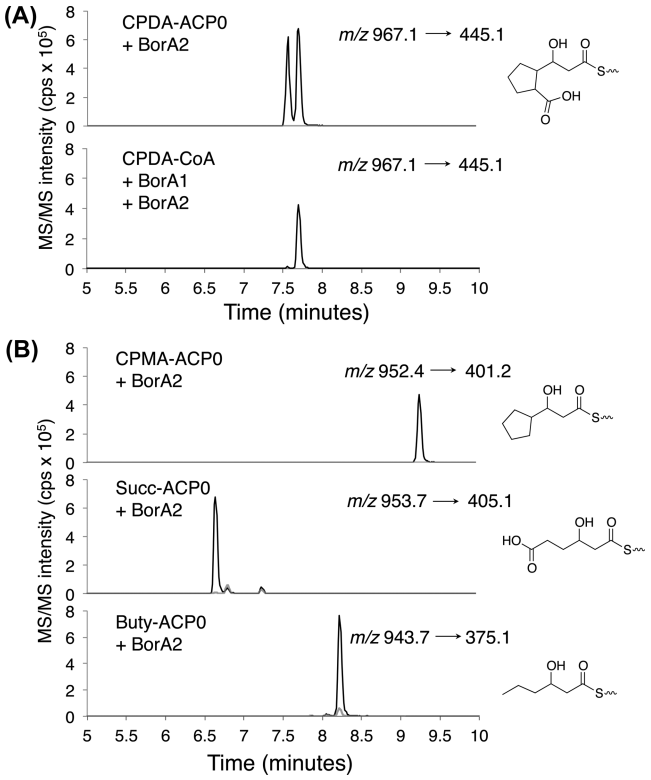

Figure 3. MS/MS chromatograms and structures of extension products (stereochemistry not shown for the sake of simplicity). (A) Comparison of CPDA-ext products upon generation by extension of CPDA-ACP0 synthetically loaded by Sfp (top) or AT domain-mediated loading (bottom). (B) Chromatograms of respective products for different acyl-ACP0 extension reactions. Negative controls (where appropriate) are shown as gray lines (see the Supporting Information for more details).

resolution time-of-flight mass spectrometry confirmed the two peaks have the same *m*/*z* value and isotopic distribution [expected *m*/*z* of 967.1270; *z* = 3 (see the Supporting Information)]. In contrast, upon examination of the CPDA samples from the loading/extension experiment using this method, only one peak was detected (Figure 3A, bottom panel), suggesting only one enantiomer (presumably 1*R*,2*R*) was loaded by the AT. Surprisingly, extension products for the CPMA- and butyryl-ACP substrates were also detected at levels comparable to those of the native CPDA-ACP extension product (Figure 3B). Taken together, these data show that while the loading AT domain of BorA1 accepts only dicarboxylates with a stereospecific arrangement as proposed by Moss et al.,[8] BorA2 is comparatively permissive in its ability to extend substrates with and without carboxy groups or ring structures and with little apparent stereoselectivity. No β-keto-ACP intermediates were detected for any substrates, suggesting the ketoreductase activity is rapid and substrate agnostic (data not shown).

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Demonstration of succinyl-CoA loading and extension in these *in vitro* studies is seemingly at odds with the precursor-directed biosynthesis findings of Moss and co-workers, who never detected the the succinate analogue of borrelidin. One possibility is that borrelidin PKS expression takes place only when succinyl-CoA levels are low enough not to compete with CPDA-CoA. Alternatively, it is possible that succinyl-CoA is occasionally loaded but is subjected to editing, perhaps by the type II thioesterase in the borrelidin cluster whose function is, as yet, unassigned.

As the majority of PKS starter units are achiral, stereospecificity in loading ATs has not been extensively investigated, though it is well-documented in extension ATs.[11] It is unclear if the loading AT in borrelidin PKS evolved to discriminate against one of two stereoisomers that may be present, or if only one species is biosynthesized and the stereoselectivity is incidental. Nevertheless, this *in vitro* study has demonstrated that a loading AT domain can discriminate between different stereoisomers and therefore has the capacity to impose another layer of specificity in starter unit selection.

Interestingly, the first approximately 300 residues of BorA1 align with other ketosynthases (Figure 6 of the Supporting Information), and some PKS domain analysis programs (MAPSI[12]) but not all (DoBiscuit[13]) annotate this region as a KS domain; however, its small size and lack of a recognizable active site motif suggest it is likely vestigial and performs no catalytic function. Taken together along with the observation that BorA1's AT contains the previously mentioned conserved arginine, these features suggest that BorA1 may have originally functioned as a typical 3-carboxyacyl loading/decarboxylating module whose substrate specificity shifted to a 4-carboxy substrate such as CPDA-CoA. Because canonical KS^Q-mediated decarboxylation is not possible on such substrates, selective pressure on the BorA1's putative KS^Q domain may have been relaxed, leading to its degeneration. Finally, this study has demonstrated broad substrate tolerance in BorA2, though it is currently unknown whether BorA2 is unique in its ability to extend carboxyacyl substrates.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis, experimental procedures, and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): J.D.K. has a financial interest in Amyris and Lygos. L.K. has a financial interest in Lygos.

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