A dual transacylation mechanism for polyketide synthase chain release in enacyloxin antibiotic biosynthesis

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SUMMARY

Polyketide synthases assemble diverse natural products with numerous important applications. The thioester intermediates in polyketide assembly are covalently tethered to acyl carrier protein domains of the synthase. Several mechanisms for polyketide chain release are known, contributing to natural product structural diversification. Here we report a dual transacylation mechanism for chain release from the enacyloxin polyketide synthase, which assembles an antibiotic with promising activity against *Acinetobacter baumannii*. A non-elongating ketosynthase domain transfers the polyketide chain from the final acyl carrier protein domain of the synthase to a separate carrier protein and a nonribosomal peptide synthetase condensation domain condenses it with (1S, 3R, 4S)-3, 4-dihydroxycyclohexane carboxylic acid. Molecular dissection of this process reveals that non-elongating ketosynthase domain-mediated transacylation circumvents the inability of the condensation domain to recognize the acyl carrier protein domain. Several 3, 4-dihydroxycyclohexane carboxylic acid analogues can be employed for chain release, suggesting a promising strategy for producing enacyloxin analogues.

INTRODUCTION

Polyketides are a large and structurally diverse family of specialized metabolites with a wealth of applications in medicine and agriculture, such as the treatment of infectious diseases and cancer, the protection of crops, and animal health.[1] Many industrially-important polyketides are assembled by type I modular polyketide synthase (PKS) biosynthetic assembly lines.[2] These remarkable molecular machines are capable of constructing a wide range of structurally complex carbon skeletons from acyl thioester building blocks via a series of condensation and modification reactions. With the exception of the loading module, each module in such PKSs is typically responsible for one round of chain elongation and minimally contains an acyl carrier protein (ACP) domain, which is post-translationally modified via attachment of a phosphopantetheine prosthetic "arm" to a conserved serine residue and a ketosynthase (KS) domain, which catalyzes chain elongation.[2] Modular PKSs fall into two distinct phylogenetic groups that are typically distinguished by the presence or absence of acyltransferase (AT) domains in their modules. In *cis*-AT PKSs, each module contains an AT domain that is responsible for loading an (alkyl)malonyl extender unit onto the adjacent ACP domain, whereas in *trans*-AT PKSs individual modules lack AT domains. Instead, a single AT catalyzes the transfer of malonyl extender units from coenzyme A onto the ACP domains in each module of the PKS.

Modular PKSs employ several strategies for generating structural diversity, such as the incorporation of a range of starter and extender units, various α- and β-carbon processing reactions after each round of chain extension and several distinct mechanisms for release of the fully-assembled polyketide chain.[1] The most frequently encountered and well-studied chain release mechanism involves macrolactonization catalyzed by a thioesterase (TE) domain appended to the C-terminus of the final PKS module.[3] Examples of TE domains that catalyze

chain release via thioester hydrolysis or macrolactamization are also known.[3,4] In addition, several other types of catalytic domains have been reported to catalyze chain release. Examples include thioester reductase domains, which catalyze reductive release to form an aldehyde or primary alcohol,[5,6] α-oxoamine synthase domains, which release the polyketide chain via decarboxylative condensation with the α-carbon of an amino acid,[7,8] and KS-like domains that catalyze condensation of the polyketide chain with a glyceryl-ACP to form a 4-hydroxymethyl-2-acyltetronic acid (**Supplementary Fig. 1**).[9]

Enacyloxin IIa (**1**) is a polyketide antibiotic[10,11] with clinically-relevant activity against *Acinetobacter baumannii* (MIC = 3 μg/ml),[12] a problematic multidrug-resistant Gram-negative pathogen. It selectively inhibits bacterial protein biosynthesis by binding to ribosomal elongation factor Tu.[13-15] We recently identified the enacyloxin biosynthetic gene cluster in *Burkholderia ambifaria* AMMD and proposed a pathway for enacyloxin biosynthesis, involving construction of the 25-carbon acyl chain by an unusual modular PKS containing a mixture of *cis* and *trans*-AT subunits (Bamb_5925-5919).[12] Release of the fully-assembled polyketide chain from the final (Bamb_5919) subunit is proposed to proceed via an unusual dual transacylation mechanism. A non-elongating ketosynthase (KS^0) domain appended to the C-terminus of Bamb_5919 is hypothesized to transfer the polyketide chain from the adjacent ACP domain to the C-terminal peptidyl carrier protein (PCP) domain of Bamb_5917 (**Fig. 1a**). Such KS^0 domains lack a conserved His residue required for chain elongation and are commonly found in *trans*-AT PKSs (**Supplementary Fig. 2**). They are proposed to transfer biosynthetic intermediates between ACP domains,[16, 17] but biochemical evidence for this is limited to a single partially characterized example,[18] and their functional significance remains unclear. Once the fully assembled enacyloxin polyketide chain has been transferred to the PCP domain of Bamb_5917 it is proposed to be released via condensation with the C-3 hydroxyl

group of (1*S*, 3*R*, 4*S*)-3, 4-dihydroxycyclohexane carboxylic acid (DHCCA). Bamb_5915, which shows sequence similarity to condensation (C) domains, typically responsible for peptide bond formation between PCP-bound amino acyl thioesters in nonribosomal peptide synthetase (NRPS) multienzymes, is hypothesized to catalyze this reaction (**Fig. 1a**).[12]

Here, we report an extensive set of genetic and biochemical experiments that establish the role played by Bamb_5915, Bamb_5917 and the KS^0 domain of Bamb_5919 in the unusual chain release mechanism employed by the enacyloxin PKS. These experiments demonstrate that the Bamb_5919 KS^0 domain overcomes the inability of Bamb_5915 to recognize the Bamb_5919 ACP domain by transferring the fully assembled polyketide chain to the PCP domain of Bamb_5917. We also show that Bamb_5915 possesses relaxed substrate specificity, indicating that it has the potential to be exploited for the production of novel enacyloxin analogues.

RESULTS

Bamb_5915 and Bamb_5917 are required for enacyloxin biosynthesis

To establish whether Bamb_5915 and Bamb_5917 are required for enacyloxin IIa biosynthesis, we created in-frame deletions in the corresponding genes. Because our originally identified enacyloxin producer, *B. ambifaria* AMMD, proved refractory to genetic manipulation using a homing endonuclease-based mutagenesis system[19], these deletions were created in *B. ambifaria* BCC0203 (also known as *B. ambifaria* BC-F[20]). The enacyloxin biosynthetic gene cluster in *B. ambifaria* BCC0203 has the same organization as that in the AMMD strain and shows a very high degree of sequence similarity (**Supplementary Fig. 3**). UHPLC-ESI-Q-TOF-MS analyses of extracts from agar cultures showed that enacyloxin production is abolished in the *bamb_5915* and *bamb_5917* mutants. Complementation of these mutants via in *trans*

expression of deleted genes restored enacyloxin production (**Fig. 1b**). These results show that Bamb_5915 and Bamb_5917 play an essential role in enacyloxin biosynthesis.

The Bamb_5919 KS^0 domain is a carrier protein transacylase

To investigate the function of the Bamb_5919 KS^0 domain, we overproduced Bamb_5917, its C-terminal PCP domain (lacking the N-terminal domain of unknown function), and the ACP and KS^0 domains of Bamb_5919 (both individually and as an ACP-KS^0 di-domain) in *E. coli* as N-terminal His_6-fusion proteins, and purified them to homogeneity using nickel affinity chromatography (**Supplementary Fig. 4**). The identity of all purified proteins was confirmed by UHPLC-ESI-Q-TOF-MS (**Supplementary Fig. 4**; note that Bamb_5917, and the ACP/PCP domains of Bamb_5919 and Bamb_5917 are produced in their *apo*-forms, presumably because the *E. coli* phosphopantetheinyl transferase is unable to recognize them).

We first sought to establish the function of the KS^0 domain, which has been hypothesized to catalyze the transfer of the fully assembled polyketide chain from the Bamb_5919 ACP domain to the Bamb_5917 PCP domain. To investigate this hypothesis, we converted the Bamb_5917 PCP domain to its *holo*-form by incubating it with CoA and the phosphopantetheinyl transferase Sfp (**Supplementary Fig. 5**). Similarly, an *S*-acetyl derivative of the *holo*-ACP domain from Bamb_5919 was created by incubating the *apo*-protein with Sfp and acetyl-CoA (**Supplementary Fig. 6**). The acetylated Bamb_5919 ACP domain was incubated with the Bamb_5919 KS^0 domain and the Bamb5917 *holo*-ACP domain to examine whether the KS^0 domain can transfer the acetyl group (which serves as a simple mimic of the fully assembled enacyloxin polyketide chain) from the ACP domain to the PCP domain. UHPLC-ESI-Q-TOF-MS analyses of the resulting mixture showed that 40.2 ± 1.4 % of the PCP domain underwent

acetylation (**Fig. 2a**). Due to the similar bond enthalpies for the linkages broken/formed in this reaction, an approximately 1:1 mixture of the ACP and PCP thioesters is produced. The level of acetylation of the Bamb_5917 PCP domain significantly decreased when the Bamb_5919 KS^0 domain was omitted from the reaction (8.4 ± 0.9 %), or when it was replaced with a C1988A mutant (in which the thiol group has been removed from the active site Cys residue; 7.9 ± 0.8 %). These data are consistent with the hypothesis that the KS^0 domain catalyses translocation of the fully assembled polyketide chain in enacyloxin biosynthesis from the Bamb_5919 ACP domain to the Bamb_5917 PCP domain. Substitution of the Bamb_5919 ACP and KS^0 domains with the ACP-KS^0 di-domain, or the Bamb_5917 PCP domain with full-length Bamb_5917 in these experiments gave analogous results (**Supplementary Fig. 7 and 8**). This shows that neither the covalent linkage between the ACP and KS^0 domains, nor the N-terminal appendage of the PCP domain present in the wild type system are critical for catalysis of the transacylation reaction.

To directly probe the acylation of the active site Cys residue in the Bamb_5919 KS^0 domain during the transfer of the acetyl group from the Bamb_5919 ACP domain to the Bamb_5917 PCP domain, the KS^0 domain was incubated with a 10-fold molar excess of the acetylated Bamb_5919 ACP domain. Analysis of the protein mixture by UHPLC-ESI-Q-TOF-MS showed that the KS^0 domain undergoes acetylation (35.1 ± 2.3 %) (**Fig. 2b**). In contrast, no acetylation was observed in such analyses when the acetylated Bamb_5919 ACP domain was incubated with the C1988A mutant of the KS^0 domain.

Bamb_5915 catalyzes chain release from Bamb_5917

The role played by Bamb_5915 (a homologue of NRPS C domains) in enacyloxin biosynthesis was similarly investigated via overproduction in *E. coli* as an N-terminal His_6 fusion protein,

purification using nickel affinity chromatography and confirmation of identity by UHPLC-ESI-Q-TOF-MS (**Supplementary Fig. 4**). The *S*-acetyl derivative of the Bamb_5917 *holo*-PCP domain was created by incubating the *apo*-protein with acetyl-CoA and Sfp (**Supplementary Fig. 6**). Incubation of this simple mimic of the Bamb_5917-bound enacyloxin polyketide chain with Bamb_5915 and chemically synthesized racemic DHCCA resulted in the production of a monoacetylated DHCCA derivative, as evidenced by UHPLC-ESI-Q-TOF-MS analyses (**Supplementary Fig. 9**). The greater thermodynamic stability of the ester bond formed in this reaction than the thioester bond in the acetylated PCP drives product formation. No acetylation of DHCCA was observed when Bamb_5915 was omitted from the reaction. We were unable to confirm the structure of the monoacetylated DHCCA derivative produced in the Bamb_5915-catalysed reaction, due to its propensity to undergo rearrangement during purification and the difficulty of preparing authentic standards. To circumvent this problem, we synthesized racemic (1*S*, 3*R*, 4*S*)-3-amino-4-hydroxycyclohexane carboxylic acid (AHCCA), an analogue of DHCCA containing an amino group in place of the C-3 hydroxyl group. Incubation of AHCCA with the acetylated Bamb_5917 PCP domain and Bamb_5915 resulted in a monoacetylated product (**Fig. 3a**). The identity of this product was confirmed as *N*-acetyl-AHCCA by comparison with a chemically-synthesised standard (**Fig. 3a**). None of this product was formed when Bamb_5915 was omitted from the reaction (**Fig. 3a**), or when an H205A mutant of Bamb_5915 (in which the active site His residue known to play an important role in catalysis in NRPS C domains has been altered) was employed (**Supplementary Fig. 10**).

Chain transfer is necessitated by Bamb_5915 carrier protein specificity

Having established that Bamb_5915 is able to catalyse chain release from Bamb_5917, we next investigated whether this enzyme can also offload acyl groups from the Bamb_5919 ACP

domain. Thus, the acetylated Bamb_5919 ACP domain was incubated with Bamb_5915 and AHCCA. No products with an *m/z* corresponding to mono-acetylated AHCCA could be detected by UHPLC-ESI-Q-TOF-MS analysis of the reaction mixture, indicating that Bamb_5915 is unable to recognise acyl groups bound to the Bamb_5919 ACP domain (**Fig. 3b**). These data are consistent with the ability of the Bamb_5919 KS^0 domain to transfer acyl groups from the Bamb_5919 ACP domain to the Bamb_5917 PCP domain and strongly indicate that such transacylation is required to circumvent the intrinsic carrier protein specificity of Bamb_5915. To directly test this hypothesis, we incubated the acetylated Bamb_5919 ACP domain with the Bamb_5919 KS^0 and Bamb_5917 *holo*-PCP domains, Bamb_5915 and AHCCA. UHPLC-ESI-Q-TOF-MS analyses confirmed that *N*-acetyl-AHCCA is produced in this reaction (**Fig. 4**). When the Bamb_5919 KS^0 domain was omitted from the reaction or replaced by the C1988A mutant, small amounts of *N*-acetyl-AHCCA were still produced (**Fig. 4**). This likely results from uncatalyzed transfer of the acetyl group from the Bamb_5919 ACP domain to the Bamb_5917 PCP domain via direct transthioesterifcation, which has been observed in related systems.[21] It is not the result of the spontaneous transfer of the acetyl group from the acetylated Bamb_5919 ACP domain to AHCCA, because no *N*-acetyl-AHCCA was detected when Bamb_5915 was omitted from the reaction (**Fig. 4**). Analogous results were obtained when the acetylated full length Bamb_5917 protein was used in place of the acetylated Bamb_5917 PCP domain (**Supplementary Fig. 11**). This indicates that the cryptic N-terminal domain of Bamb_5917 plays no specific role in the chain release process and is likely an evolutionary remnant. However, other roles for this domain (e.g. in mediating protein-protein interactions with other components of the biosynthetic machinery) cannot be excluded. To provide further evidence for the relevance of these findings to chain release from the enacyloxin PKS, we repeated the experiments with acyl groups that more closely mimic the fully assembled

enacyloxin polyketide chain. Thus, dodecanoylated and (2*E*, 4*E*)-2, 4-hexadienoylated-Bamb_5917_ACP domain were incubated separately with the Bamb_5919 KS^0 and Bamb_5917 *holo*-PCP domains and Bamb_5915. In both cases, a monoacylated AHCCA derivative was produced (**Supplementary Figs 12 and 13**). No product could be detected when Bamb_5915 or the Bamb_5919 KS^0 domain were omitted from reaction, or when the C1988A mutant of the KS^0 domain was used (**Supplementary Figs 12 and 13**). Taken together, our data show that Bamb_5915 is unable to interact productively with the Bamb_5919 ACP domain. This necessitates transfer of the fully assembled enacyloxin polyketide chain to the Bamb_5917 PCP domain (catalysed by the Bamb_5919 KS^0 domain) where it is released by Bamb_5915 via condensation with DHCCA.

Bamb_5915 tolerates various acyl acceptor and donor analogues

The observation that Bamb_5915 can tolerate an acetyl thioester and AHCCA in place of the natural acyl donor and acceptor, respectively, prompted us to further investigate the substrate tolerance of this enzyme. To probe acyl acceptor specificity, we incubated Bamb_5915 with the acetylated Bamb_5917 PCP domain and several chemically-synthesized and commercially available DHCCA analogues. Analysis of the reaction mixtures by UHPLC-ESI-Q-TOF-MS showed that Bamb_5915 is able to accept a variety of carbocyclic DHCCA analogues with variations in ring size, degree of unsaturation, substitution pattern and stereochemistry (**Fig. 5a and Supplementary Fig. 9**). Moreover, the enzyme was also able to utilise acyclic DHCCA analogues, such as γ-aminobutyric acid (**Fig. 5a**). Indeed, the minimal determinant of substrate acceptance by Bamb_5915 appears to be the 1, 3-juxtaposition of a nucleophile and a carboxyl group (**Fig. 5a and Fig. 5b**). The acyl donor specificity of Bamb_5915 was investigated by assessing its ability to catalyse acylation of AHCCA with several N-acetyl

cysteamine (NAC) thioester analogues of the native substrate (**Supplementary Fig. 14**). While various straight chain thioesters (e.g. acetyl, propionyl, and (2E, 4E)-2, 4-hexadienoyl) were well tolerated by Bamb_5915, little or no acylation of AHCCA was observed when the enzyme was incubated with α-branched substrates, such as isobutyryl, pivaloyl and serinyl-NAC thioesters.

DISCUSSION

The dual transacylation mechanism elucidated here for chain release from the enacyloxin modular polyketide synthase has several unusual features. First, it involves a rare intermolecular esterification reaction catalyzed by a standalone C domain,[22] which is a novel mechanism for modular PKS chain release. Second, the standalone C domain appears to possess uncommonly broad substrate tolerance towards both acyl acceptors and acyl donors, and third, the polyketide chain must be translocated by a KS^0 domain from a carrier protein domain that is not recognized by the C domain, to one that is, for chain release to occur.

KS^0 domains are a common feature of *trans*-AT PKSs,[16] but their functional significance has remained unclear. Our data show that the KS^0 domain appended to the C-terminus of Bamb_5919 functions as a transacylase, consistent with the hypothesis that it shuttles the fully assembled polyketide chain from the upstream ACP domain to the PCP domain of Bamb_5917. This is necessary because Bamb_5915, an NRPS-like C domain that catalyzes polyketide chain release via intermolecular esterification with DHCCA, can recognize acyl donors when they are attached to the Bamb-5917 PCP domain, but not the Bamb_5919 ACP domain. Indeed, KS^0 domains are commonly found at the interface between PKS and NRPS subunits in hybrid *trans*-AT PKS/NRPS assembly lines (**Fig. 6**),[16] where they have been shown to play an essential role,[17] and are proposed to translocate an acyl thioester intermediate from

an upstream ACP/PCP domain to a downstream PCP/ACP domain.[16] Thus, the inability of C (and other NRPS catalytic) domains to recognize acyl donors when they are attached to *trans*-AT PKS ACP domains appears to be a general phenomenon. Our results suggest that KS^0 domains have been recruited during the evolution of many such hybrid systems as "adapters" to overcome this obstacle. In the accompanying manuscript, we show that protein-protein interactions mediated by mutually-compatible docking domains are primarily responsible for the specific recognition of the Bamb_5917 PCP domain by Bamb_5915.[23] While it seems very likely that such interactions also underlie similar carrier protein incompatibilities in other systems, further experiments will be required to establish their precise nature.

In the broader context, KS^0 domains are frequently found juxtaposed between up- and downstream ACP/PCP domains in several other types of *trans-AT* PKS architecture.[16] Common examples include: ACP-KS^0-PCP-Cy; PCP-KS^0-ACP-KS; ACP-KS^0-ACP-TE; ACP-KS^0-OMT-ACP; ACP-KS^0-DH-ACP; and ACP-KS^0-ER-ACP (domain abbreviations not defined previously are as follows: Cy, heterocyclisation; KR, ketoreductase; DH, dehydratase and related domains, including enoyl isomerases and pyran synthases; OMT, O-methyltransferase; ER, enoyl reductase) (**Fig. 6**). In the all of these cases, it seems likely that the catalytic domains downstream of the KS^0 domains cannot interact productively with the ACP/PCP domains upstream of them. The KS^0 domains therefore transfer the acyl chains attached to these ACP/PCP domains to the downstream ACP/PCP domains, which can be recognized by the adjacent catalytic domains, allowing the reactions they catalyze to proceed. Modules that recruit *trans*-acting oxygenases, which modify the growing polyketide chain during its assembly, also employ KS^0 domains.[16] These appear to transfer the requisite intermediate onto an ACP domain that can be recognized by the oxygenase. Further experiments will be required to test these hypotheses, but one obvious consequence is that in each case the

ACP/PCP domain downstream of the KS^0 domain must avoid being loaded with a malonyl extender unit by the *trans*-acting AT. Whether this is controlled solely by protein-protein interactions, as demonstrated for selective ACP recognition by the *trans*-AT KirCII in kirromycin biosynthesis,[24] or more complex phenomena remains to be established. There is no selective pressure to maintain the HGTGT motif in a KS domain when it is positioned upstream of an ACP or PCP domain that cannot be malonylated. Thus KS^0 domains probably evolved from elongating KS domains via the random accumulation of mutations in the HGTGT motif (**Supplementary Fig. 2**).

Although enacyloxin IIa has promising activity against *A. baumannii*,[12] a multi-drug resistant pathogen for which new antibiotics are urgently required, it is unlikely to find direct clinical application. Enacyloxin analogues with enhanced potency, reduced toxicity and greater chemical stability are therefore needed. The broad acyl acceptor tolerance of Bamb_5915 demonstrated by our work indicates that it may be possible to produce enacyloxin analogues with modifications to the DHCCA moiety via biosynthetic engineering. One attractive strategy for doing this involves feeding of DHCCA analogues to mutants of *B. ambifaria* blocked in DHCCA biosynthesis. Bamb_5915 is also able to accept a range of thioesters as acyl donors, suggesting that it may be possible to make analogues of enacyloxin with modifications to the polyketide chain via the manipulation of tailoring genes and other biosynthetic engineering strategies. Together, such approaches offer the potential to deliver a library of enacyloxin analogues that illuminate the structure-activity relationship of the natural product, which would be an important milestone in its development towards therapeutic application.

In conclusion, our results provide important insights into the mechanism and specificity of chain release from the enacyloxin polyketide synthase. They not only suggest a general role for KS^0 domains in overcoming an intrinsic incompatibility between several types of catalytic

domains and certain types of carrier protein domain in *trans*-AT PKSs, but also define plausible approaches for the production of enacyloxin analogues via biosynthetic engineering.

METHODS

See the Supplementary Information for a description of the methods employed.

Data availability

The genome sequence of *B. ambifaria* BCC0203 was deposited in the European Nucleotide Archive (Accession No. ERS782625). All other data are available from the authors upon request**.**

REFERENCES

1. Hertweck, C. The biosynthetic logic of polyketide diversity. *Angew. Chem. Int. Ed. Engl.* **48**, 4688–4716 (2009).

2. Fischbach, M. A. & Walsh, C. T. Assembly-line enzymology for polyketide and nonribosomal peptide antibiotics: logic, machinery, and mechanisms. *Chem. Rev.* **106**, 3468–3496 (2006).

3. Du, L. & Lou, L. PKS and NRPS release mechanisms. *Nat. Prod. Rep.* **27**, 255–278 (2010).

4. Tang, G.-L., Cheng, Y.-Q. & Shen, B. Leinamycin biosynthesis revealing unprecedented architectural complexity for a hybrid polyketide synthase and nonribosomal peptide synthetase. *Chem. Biol.* **11**, 33–45 (2004).

5. Awodi, U. R., Ronan, J. L., Masschelein, J., de Los Santos, E. L. C. & Challis, G. L. Thioester reduction and aldehyde transamination are universal steps in actinobacterial polyketide alkaloid biosynthesis. *Chem. Sci.* **8**, 411–415 (2017).

6. Mullowney, M. W., McClure R. A., Robey M. T., Kelleher N. L., Thomson R. J. Natural products from thioester reductase containing biosynthetic pathways. *Nat. Prod. Rep.* **35**, 847-878 (2018).

7. Mo, S. J. *et al.* Elucidation of the *Streptomyces coelicolor* pathway to 2-undecylpyrrole, a key intermediate in undecylprodiginine and streptorubin B biosynthesis. *Chem. Biol.* **15**, 137-148 (2008).

8. Hu, D. X., Withall, D. M., Challis, G. L. & Thomson, R. J. Structure, chemical synthesis, and biosynthesis of prodiginine natural products. *Chem. Rev.* **116**, 7818–7853 (2016).

9. Sun, Y. *et al. In vitro* reconstruction of tetronate RK-682 biosynthesis. *Nat. Chem. Biol.* **6**, 99–101 (2010).

10. Watanabe, T., Izaki, K. & Takahashi, H. New polyenic antibiotics active against gram-positive and-negative bacteria. I. Isolation and purification of antibiotics produced by *Gluconobacter* sp. W-315. *J. Antibiot.* (Tokyo). **35**, 1141-1147 (1982).

11. Watanabe, T. *et al.* New polyenic antibiotics active against Gram-positive and Gram-negative bacteria. IV. Structural elucidation of enacyloxin IIa. *J. Antibiot.* (Tokyo). **45**, 470–475 (1992).

12. Mahenthiralingam, E. *et al*. Enacyloxins are products of an unusual hybrid modular polyketide synthase encoded by a cryptic *Burkholderia ambifaria* Genomic Island. *Chem. Biol.* **18**, 665–677 (2011).

13. Cetin, R. *et al.* Enacyloxin IIa, an inhibitor of protein biosynthesis that acts on elongation factor Tu and the ribosome. *EMBO J.* **15**, 2604–2611 (1996).

14. Parmeggiani, A. *et al.* Enacyloxin IIa pinpoints a binding pocket of elongation factor Tu for development of novel antibiotics. *J. Biol. Chem.* **281**, 2893–2900 (2006).

15. Zuurmond, A. M., Olsthoorn-Tieleman, L. N., Martien de Graaf, J., Parmeggiani, A. & Kraal, B. Mutant EF-Tu species reveal novel features of the enacyloxin IIa inhibition mechanism on the ribosome. *J. Mol. Biol.* **294**, 627–637 (1999).

16. Helfrich, E. J. N. & Piel, J. Biosynthesis of polyketides by *trans*-AT polyketide synthases. *Nat. Prod. Rep.* **33**, 231–316 (2016).

17. Huang, Y., Tang, G.-L., Pan, G., Chang, C.-Y. & Shen, B. Characterization of the ketosynthase and acyl carrier protein domains at the LnmI nonribosomal peptide synthetase–polyketide synthase interface for leinamycin biosynthesis. *Org. Lett.* **18**, 4288-4291 (2016).

18. He, H.-Y., Tang, M.-C., Zhang, F. & Tang, G.-L. *Cis*-Double Bond Formation by Thioesterase and Transfer by Ketosynthase in FR901464 Biosynthesis. *J. Am. Chem. Soc.* **136**, 4488–4491 (2014).

19. Flannagan, R. S., Linn, T. & Valvano, M. A. A system for the construction of targeted unmarked gene deletions in the genus *Burkholderia*. *Environ. Microbiol.* **10**, 1652–1660 (2008).

20. Li, W. *et al.* Broad spectrum antibiotic activity and disease suppression by the potential biocontrol agent *Burkholderia ambifaria* BC-F. *Crop Prot.* **21**, 129–135 (2002).

21. Thomas, I. Martin, C. J., Wilkinson, C. J., Staunton, J. & Leadlay P.F. Skipping in a hybrid polyketide synthase: evidence for ACP-to-ACP chain transfer. *Chem. Biol.* **9**, 781-787 (2002).

22. Lin, S., Van Lanen, S. G. & Shen, B. A free-standing condensation enzyme catalyzing ester bond formation in C-1027 biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 4183-4188 (2009).

23. Kosol, S. *et al.* Molecular basis for condensation domain-mediated chain release from the enacyloxin polyketide synthase. *Nat. Chem.* Submitted for publication.

24. Ye, Z., Musiol, E. M., Weber T. & Williams, G. J. Reprogramming acyl carrier protein interactions of an acyl-CoA promiscuous *trans*-acyltransferase. *Cell Chem. Biol.* **21**, 636-646 (2014).

SUPPLEMENTARY INFORMATION is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.M. and P.K.S. contributed equally to this work. J.M., P.K.S. and G.L.C. designed the experiments. P.K.S. cloned the *B. ambifaria* genes into pET151 and created the site-directed mutants. J.M., P.K.S. and C.H. carried out the *in vitro* biochemical experiments. C.J., Z.L.Y. and E.M. identified strain BCC0203 as amenable to genetic manipulation, established selection

conditions, and constructed and complemented the *B. ambifaria* gene deletion mutants. C.J. analysed the genome sequence of strain BCC0203 and made the initial comparisons to the genome of the AMMD strain. C.H., R.H., D.M.R., P.K.S. and J.M. synthesized substrates. J.M. and G.L.C. wrote the manuscript with input from the other authors.

COMPETING INTERESTS

The broad substrate specificity of Bamb_5915 has been exploited for the production of enacyloxin analogues leading to the following patent application. Applicant: the University of Warwick; Names of Inventors: G.L. Challis, J. Masschelein, C. Hobson, X. Jian; Application Number: International (PCT) Patent Application No. PCT/GB2018/051058; Status of Application: filed 23 April 2018.

FIGURE LEGENDS

Fig 1: Proposed mechanism for chain release from the enacyloxin PKS and confirmation that Bamb_5915 and Bamb_5917 are involved in enacyloxin biosynthesis. (**a**) Proposed dual transacylation mechanism for chain release from the type I modular PKS responsible for enacyloxin IIa (**1**) biosynthesis. The KS^0 domain at the C-terminus of Bamb_5919 (the final PKS module) is proposed to transfer the fully-assembled polyketide chain from the upstream ACP domain to the Bamb_5917 PCP domain. Bamb_5915, which shows sequence similarity to NRPS C domains, catalyzes condensation of the resulting thioester with (1*S*, 3*R*, 4*S*)-3,4-dihydroxycyclohexane carboxylic acid. (**b**) Deletion of the genes encoding Bamb_5915 and Bamb_5917 abolishes enacyloxin production in *B. ambifaria* BCC0203. Extracted ion chromatograms at *m/z* = 724.2267 ± 0.005 (corresponding to the $[M+Na]^+$ ion for enacyloxin IIa) from UHPLC-ESI-Q-TOF-MS analyses of extracts from agar-grown cultures of wild type *B. ambifaria* BCC0203 (top), the *bamb_5917* (second from top) and *bamb_5915* (second from bottom) mutants. The chromatograms for extracts from the *bamb_5915* and *bamb_5917* mutants complemented by *in trans* expression of the deleted genes are also shown (bottom and third from bottom, respectively). The peak corresponding to enacyloxin IIa is indicated with an asterisk. The other peaks are isomers resulting from light / acid-promoted isomerization during isolation / analysis.

Fig 2: Acyl transfer and active site acylation assays reveal that the KS^0 domain of Bamb_5919 functions as a transacylase. (a) Deconvoluted mass spectra from incubation of the *holo*-Bamb_5917 PCP domain with the acetylated Bamb_5919 ACP domain in the presence of the Bamb_5919 KS^0 domain (top) and the C1988A mutant of the KS^0 domain (middle), and in the absence of the KS^0 domain (bottom). The data demonstrate that the KS^0 domain is able to transfer an acetyl group from the ACP domain to the PCP domain and that the active site Cys residue of the KS^0 domain is required for this reaction. Due to the similar bond enthalpies for the thioester linkages broken/formed in the reaction catalyzed by the KS^0 domain an approximately 1:1 mixture of starting material and product is formed. (**b**) Deconvoluted mass spectra of the Bamb_5919 ACP domain (left), the Bamb_5919 KS^0 domain (top right) and the C1988A mutant of the Bamb_5919 KS^0 domain (bottom right), following incubation of the wild type and mutant KS^0 domains with a 10-fold excess of acetyl-Bamb_5919 ACP. Transfer of the acetyl group from the ACP domain to the KS^0 domain is observed for the wild type enzyme (top), but not for the mutant in which the active site Cys residue has been mutated to Ala (bottom). The data shown are from a single measurement and are representative of three independent experiments.

Fig. 3: Functional characterization of Bamb_5915. Extracted ion chromatograms at *m/z* = 224.089 ± 0.005 (corresponding to the $[M+Na]^+$ ion for N-acetyl-AHCCA) from UHPLC-ESI-Q-TOF-MS analyses of Bamb_5915-calaysed reactions. (**a**) Incubation of the acetylated Bamb_5917 PCP domain with AHCCA and Bamb_5915 results in a monoacetylated product with the same retention time as a chemically synthesized authentic standard of *N*-acetyl-AHCCA. (**b**) No acetylated products are observed when Bamb_5915 and AHCCA are incubated with the acetylated Bamb_5919 ACP domain.

Fig. 4: *In vitro* reconstitution of chain release from the enacyloxin PKS. Extracted ion chromatograms at *m/z* = 224.089 ± 0.005 (corresponding to the $[M+Na]^+$ ion for N-acetyl-AHCCA) from LC-ESI-Q-TOF-MS analyses of synthetic *N*-acetyl-AHCCA (top), and the product of the reaction of the acetylated Bamb_5919 ACP domain with the Bamb_5915 KS^0 domain, the Bamb_5917 holo-PCP domain, Bamb_5915 and AHCCA (second from top). The bottom three chromatograms are from control reactions in which Bamb_5915 has been omitted (third from bottom), the Bamb_5919 KS^0 domain has been omitted (second from bottom), and the C1988A mutant of the Bamb_5919 KS^0 domain has been used in place of the wild type enzyme (bottom).

Fig. 5: Bamb_5915 tolerates a wide range of acyl acceptors. Overview of DHCCA analogues tested as substrates for Bamb_5915 using acetylated Bamb_5917 as an acyl donor. (**a**) Structures of DHCCA analogues converted to monoacetylated products by Bamb_5915. (**b**) DHCCA analogues not accepted as substrates by Bamb_5915.

Fig. 6: Examples of *trans*-AT PKS architectures containing KS^0 domains. In each case, we hypothesize that the catalytic domain downstream of the KS^0 domain is unable to interact with the upstream ACP/PCP domain. The KS^0 domain thus transfers the substrate from the upstream to the downstream carrier protein domain, which the neighbouring catalytic domain is able to interact with, allowing the reaction it catalyses to proceed. Domains (other than the KS^0 domain) we propose are able to engage in productive protein-protein interactions are shown in the same colour.