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A Peptidyl Transesterifying Type I Thioesterase in Salinamide Biosynthesis

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Abstract

Salinamide A belongs to a rare class of bicyclic depsipeptide antibiotics in which the installation of a (4-methylhexa-2,4-dienyl)glycine handle across a hexadepsipeptide core contributes to its chemical complexity and biological properties. Here we report the genetic and biochemical basis for salinamide construction in the marine bacterium *Streptomyces* sp. CNB-091 that involves a novel intermolecular transesterification reaction catalyzed by a type I thioesterase. Heterologous biosynthesis studies revealed the central role of the nonribosomal peptide synthetase Sln9 in constructing and installing salinamides distinctive acylglycine “basket” handle. Biochemical characterization of the Sln9 thioesterase domain established the transesterification of desmethylsalinamide E’s serine residue with acylated glycyl thioesters to yield desmethylsalinamide C.

Graphical Abstract

Uncovering the biosynthetic logic for salinamide A assembly has illuminated a type I thioesterase, encoded within a tetradomain non-ribosomal peptide synthetase (NRPS) module, as playing a pivotal role in the installation of an acyl-glycine handle across the relatively simple hexadepsipeptide core. Catalyzing an intermolecular peptidyl transesterification, this unprecedented reaction mediates the transfer of an acylated glycine from a carrier protein (CP) to a serine hydroxyl group within an offloaded non-ribosomal peptide product.

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Supporting information for this article is available on the WWW under <http://xxxxxxx>

Keywords

biosynthesis; coupling; intermolecular; peptides; thioesterase

Non-ribosomal peptides (NRPs) have long been recognized as a highly diverse and important class of natural products, both in terms of structure and function.^[1] Their construction by large, multifunctional assembly line proteins involves ATP-dependent activation and transfer of amino acid precursors to arrayed carrier protein (CP) domains. Once the mature peptide intermediate is assembled by the non-ribosomal peptide synthetase (NRPS), a myriad of protein off-loading reactions await to yield the released peptide product.^[2] The most common terminating reaction is catalyzed by thioesterase (TE) proteins that typically occupy the terminal domain of the NRPS and function to release the product as linear or macrocyclized peptides.^[3] Here, we report a new role for a terminating TE that involves an intermolecular transesterification reaction associated with the construction of the salinamides, a family of unusual bicyclic depsipeptides that exhibit anti-inflammatory and antibacterial properties.

The first in this class of depsipeptides to be isolated from the marine actinomycete *Streptomyces* sp. CNB-091 were salinamides A (**1**) and B (**2**).^[4] This series of NRPs has since been expanded through the isolation and characterization of various derivatives to include salinamides C (**3**), D (**4**), E (**5**) and F (**6**).^[5] All salinamides share a common depsipeptide core comprised of a beta-hydroxyacid moiety derived from isobutyrate and propionate, and six amino acid residues – L-serine, L-phenylalanine, *p*-hydroxy-L-phenylglycine (*p*-HPG), L-threonine, D-threonine and D-isoleucine (or D-valine in the case of **4**). The simplest of the salinamide derivatives, **5**, is methylated at the *p*-HPG position. The remaining salinamide derivatives are all decorated with a (4-methylhexa-2,4,-dienyl)glycine moiety tethered to the serine hydroxyl group (Ser-OH) via an ester bond. Within **1**, **2**, **4** and **6** this extended serine side chain is cyclized onto the phenoxy group of the *p*-HPG residue (*p*-HPG-OH) to create a “basket” handle across the cyclic core. Probing the metabolic origins of the carbon residues within the salinamides previously revealed that the Ser-modified acylglycine side chain is derived from the condensation of 2-methylbutyrate and malonate (Figure 1).^[6]

Identification of this series of salinamide derivatives and the isotope incorporation data suggests the involvement of a novel biosynthetic pathway, in which a mature cyclic depsipeptide is processed by the addition of a (4-methylhexa-2,4,-dienyl)glycine unit to the Ser-OH. Subsequent oxidative cyclization of this side chain onto *p*-HPG-OH would yield the bicyclic depsipeptide **1**. This biosynthetic scenario suggested the possibility of new peptide side chain modification enzymology involving a CP-bound substrate. Precedent examples are nonexistent. Thiotemplated assembly line synthesis of ester linkages are

known to involve NRPS condensation (C) domain chemistry, such as for the didemnins and hectochlorins, in which hydroxyacid rather than amino acid building blocks are incorporated into NRP products.^[7] That scenario however does not fit salinamide assembly in which the ester is derived in a very different manner involving a side chain hydroxyl. Standalone C-domains are rather known from polyketide biosynthesis to attach carboxylic acid substrates to ketide hydroxyls as in C-1027 and fumonisin biosynthesis.^[8] Although this mechanism is plausible for salinamide esterification, we show a new role for a terminal TE domain in catalyzing a transesterification reaction.

To understand the mechanism responsible for the installation of the salinamide “handle”, we obtained a draft genome sequence of *Streptomyces* sp. CNB-091. The high quality of this draft sequence resulted in the 8.2 Mbp genome being split across 50 scaffolds, with a GC content of 71.6%. Subsequent bioinformatic analysis of this draft genome unveiled the full biosynthetic potential of *Streptomyces* sp. CNB-091.^[9] A vast array of putative secondary metabolite gene clusters were illuminated, with a total of 38 clusters encoding the synthesis of polyketides, NRPs, terpenes and ribosomally synthesized and post-translationally modified peptide natural products. Intriguingly the salinamides are, to date, the only class of molecules reported from *Streptomyces* sp. CNB-091, thus indicating that *Streptomyces* sp. CNB-091 is a relatively untapped resource with regards to its potential to produce diverse natural products.

From the 38 biosynthetic gene clusters, we identified a single candidate cluster for salinamide biosynthesis. This 47.6 kbp, 14 coding sequence NRPS-polyketide synthase (PKS) hybrid gene cluster was designated the *sln* locus (Figure 1a). The *sln* cluster contains four genes encoding NRPS proteins and one encoding a PKS/NRPS hybrid, and genes encoding enzymes responsible for precursor biosynthesis and post-NRPS tailoring enzymes. Significantly, the domain structure and module organization of the encoded NRPS-PKS megasynthetase was consistent with the structures of the salinamides in which two amino acid residues are D configured (Val/Ile-2 and Thr-5) and another (Phe-4) is methylated.^[10] Moreover genes supporting the biosynthesis of the non-proteinogenic amino acid residue *p*-HPG were subclustered in a gene cassette (*slnI-3*).^[11] One salient feature of the *sln* cluster is the presence of two genes, *sln6* and *sln9*, both of which encode tetradomain NRPS modules containing a C-terminal TE domain. This led to the proposal that one of these type I TE domains play a central role in the addition of the (4-methylhexa-2,4,-dienoyl)-glycine moiety onto the Ser-OH, rather than this being the product of C domain chemistry (Figure 1B).

To validate that this putative biosynthetic gene cluster is responsible for the production of salinamides, we directly captured the *sln* locus from gDNA via yeast-based transformation associated recombination (TAR) in the multipurpose expression plasmid pCAP01.^[12] Following capture, the *sln* cluster was integrated into the genome of the model actinomycete *Streptomyces coelicolor* M1146 for heterologous expression.^[13] Fermentation of this genome minimized strain revealed the production of the full suite of salinamides, confirming the *sln* locus is responsible for salinamide biosynthesis (Figure 2).

Successful capture and heterologous expression of the salinamide gene cluster provided a mechanism by which to interrogate the *in vivo* biosynthesis of salinamides. We deleted several *sln* genes via λ -Red mediated recombination and analyzed chemical extracts of the mutants by HPLC-MS. Firstly we focused upon the biosynthesis of the salinamide depsipeptide core via the canonical NRPS/PKS hybrid. This was probed through the deletion of *sln6*, which encodes the putative final module in this assembly line terminating with the cyclizing TE. As anticipated, this mutation completely abolished salinamide production (Figure S1-C). A loss of salinamide production was also observed upon deletion of the LuxR regulatory protein (Figure S1-B). We then turned our attention to the tailoring genes within the pathway. Deletion of the *O*-methyltransferase *sln4* resulted in the loss in production of **3** and **5** with concomitant production of **1** and a new desmethylsalinamide, the postulated intermediate **7**, which was subsequently isolated and characterized via HR-MS and NMR spectroscopy (Figure S1-F). The production of **1** was uniquely abolished with the deletion of the cytochrome P450 (CYP450)-encoding *sln10* gene, as all other salinamides including **3** and **5**, were maintained in the mutant (Figure S1-E). This observation is highly suggestive that the CYP450 is responsible for catalyzing the closure of the salinamide A basket handle between the *p*-HPG and the side chain diene. Furthermore these results suggest that **3** and **5** are end of pathway products rather than intermediates towards the more chemically elaborate **1** (Figure 1B).

We next focused on the biosynthesis of the *N*-acylated glycine moiety and the mode of its transfer to the Ser-OH in **1** and **3**. Previously we showed that the acyl diene fragment originates from the condensation of isoleucine-derived tiglic acid and acetate,^[6] which is consistent with the activity of FabH-based enzymology. We thus deleted the FabH homologue *sln11*. Whilst the level of production of **5** was maintained, the production of all other salinamide derivatives was significantly reduced, suggesting genetic complementation by a host FabH gene (Figure S1-D). Inactivation of the Sln9 NRPS gene resulted in the elimination of **1** and **3** while maintaining the production of **5** and **7** (Figure S1-G). Based on these outcomes, we surmise that the tetradomain Sln9 activates glycine, condenses it with the Sln11 product 4-methyl-2,4-hexadienoyl-S-Sln12, and, involving unprecedented TE domain chemistry, transfers the acylglycine substrate to the Ser-OH residue in **7** to yield **8**. This proposed activity would signal a departure from the NRPS assembly line paradigm in which TEs normally catalyze offloading hydrolytic or intramolecular reactions, much like that observed with the C-terminal TE domain of Sln6.^[14]

To explore this proposal, we biochemically characterized the Sln-TE. We isolated the presumed substrate **7** from fermentation broths of the Sln4 MT mutant and synthesized two acylglycine-CP substrate mimics as *N*-acetylcysteamine and *N*-pantatheinylcysteamine thioesters (**9** and **10**, Figure 3a).^[15] We also cloned, expressed and purified the recombinant hexahistadyl-tagged Sln9-TE domain (Figure S17). Indeed, as hypothesized, upon incubation of His₆-Sln9-TE with **7** and the acylglycine-CP substrate mimics, **9** or **10**, we were able to observe the transfer of the acylglycine moiety onto the Ser-OH. This confirms that Sln9-TE represents the first example of an intermolecular-acting TE domain within a bacterial NRPS (Figure 3). This observation increases the scope of TE domain chemistry to

include the modification of NRP side chains through the transesterification of peptidyl substrates.

In conclusion, we have successfully characterized the biosynthetic logic of salinamide assembly. Direct capture of the salinamide biosynthetic gene cluster via TAR cloning facilitated heterologous expression studies which allowed for the genetic interrogation of the *sln* gene locus. Significantly, this work uncovered the first example of an intermolecular-acting TE domain within a bacterial NRPS to mediate the transesterification of a CP-bound substrate to a cyclic depsipeptide. Investigations to probe the substrate specificity of this TE are currently ongoing along with efforts to understand the structural features that enable the controlled acylation of peptide substrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Gene organization of the salinamide (*sln*) biosynthetic gene cluster from *Streptomyces* sp. CNB-091 (see Supporting Information for the proposed function assignments of the coding sequences). (B) Proposed biosynthesis of salinamides. The A domain of Sln8 is able to accept isoleucine and valine; consequently two different salinamide series are biosynthesized. All 'Ile' derivatives, **1–3** and **5–8**, are illustrated within this figure, however **4** is the only 'Val' derivative represented as it is a significant pathway product (see Figure 2). Abbreviations: A, adenylation; AT, acyltransferase; C, condensation; CP, carrier protein; E, epimerization; FAS, fatty acid synthase; *p*-HPG, hydroxyphenylglycine; KR, ketoreductase; KS, ketosynthase; MT, methyltransferase; TE, thioesterase.

Figure 2.
HPLC analysis of salinamide production in the native *S. sp.* CNB-091 (A) and the heterologous host *S. coelicolor* M1146 into which the pCAP*sln* vector has been integrated (B). UV absorption at 210 nm was monitored and the peaks corresponding to salinamides A–F (1–6) are highlighted.

Figure 3.

Biochemical characterization of the Sln9 type I TE domain with **7** as a substrate, **5** was also accepted as a substrate for Sln9-TE; the results of which are shown in Figure S19. (A) Intermolecular transesterification reaction catalyzed by the Sln9-TE domain. Synthesis of substrates **9** and **10** and the preparation of recombinant hexahistidine-tagged Sln9-TE are described in the Supporting Information; (B) HR-LCMS analysis of the Sln9 TE reaction, all traces are extracted ion chromatograms (EICs) for m/z 841.43 ($[M+H]^+$ ion for **7**) and 1006.51 ($[M+H]^+$ ion for **8**) (a) Control assay with heat-inactivated His₆-Sln9-TE domain using **9** as a substrate; (b) His₆-Sln9-TE domain enzymatic assay showing the conversion of **7** to **8** using substrate **9**; (c) Control assay with heat-inactivated His₆-Sln9-TE domain using **10** as a substrate; and (d) His₆-Sln9-TE domain enzymatic assay showing conversion of **7** to **8** using substrate **10**.