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Characterization of the Tautomycetin Biosynthetic Gene Cluster from *Streptomyces griseochromogenes* Provides New Insight into Dialkylmaleic Anhydride Biosynthesis[#]

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Abstract

Tautomycetin (TTN) is a highly potent and specific protein phosphatase inhibitor isolated from *Streptomyces griseochromogenes*. The biological activity of TTN makes it an important lead for drug discovery, whereas its rare dialkylmaleic anhydride moiety and structural similarity to tautomycin (TTM), another potent phosphatase inhibitor with tremendous medicinal potential, draws attention to novel biosynthetic chemistries responsible for its production. To elucidate the biosynthetic machinery associated with TTN production, the *ttn* biosynthetic gene cluster from *S. griseochromogenes* was isolated and characterized, and its involvement in TTN biosynthesis confirmed by gene inactivation and complementation experiments. The *ttn* cluster was localized to a 79 kb DNA region, consisting of 19 open reading frames that encode two modular type I polyketide synthases (TtnAB), one type II thioesterase (TtnH), eight proteins for dialkylmaleic anhydride biosynthesis (TtnKLMNOPRS), four tailoring enzymes (TtnCDFI), two regulatory proteins (TtnGQ), and one resistance protein (TtnJ). A model for TTN biosynthesis is proposed on the basis of functional assignments from sequence analysis, which agrees well with previous feeding experiments, has been supported by in vivo gene inactivation experiments, and is supported by analogy to the recently reported *ttm* cluster. These findings set the stage to fully investigate TTN biosynthesis and to biosynthetically engineer new TTN analogues.

[#]Dedicated to Dr. David G. I. Kingston of Virginia Polytechnic Institute and State University for his pioneering work on bioactive natural products.

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Nucleotide Sequence Accession Number. The nucleotide sequence reported in this paper has been deposited in the GenBank database under accession number EU035755.

Supporting Information Available: Targeted gene inactivation (Table S1), mutant confirmation (Table S2 and Figures S1, S2, S3, S4), mutant complementation (Table S3), ¹H and ¹³C NMR and UV–vis spectra for TTN M-1 (Figures S5 and S6), and UV–vis (Figure S7) and mass spectra (Figure S8) of the TTN M-1 and three uncharacterized new compounds accumulated by the *Δttm* mutant strain SB13004. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Tautomycin (TTN), originally isolated from *Streptomyces griseochromogenes* in 1989, is structurally similar to tautomycin (TTM) (Figure 1A).^{1,2} Both polyketides were initially described as antifungal antibiotics capable of inducing morphological changes in leukemia cells. More importantly, both compounds were found to specifically inhibit the protein phosphatases (PPs) PP1 and PP2A.^{3,4} PP1 and PP2A are two of the four major serine/threonine PPs that regulate an array of cellular processes including, but not limited to, cell cycle progression, gene expression, calcium transport, muscle contraction, glycogen metabolism, phototransduction, and neuronal signaling.^{5,6} Many human diseases are characterized by an altered interplay between phosphatases and kinases, and thus the selective inhibition of PP1 and PP2A has been proposed to be an attractive goal for rational anticancer drug design.⁷ For instance, TTN has been suggested as a potential drug for colorectal cancer because of its regulation of Raf-1 activity through inhibition of PP1 and PP2A in a cell-type-specific manner.⁸ PP1 and PP2A inhibition by TTM and TTN heightens interest in the possible application of combinatorial biosynthesis methods as an integral tool for the discovery of new therapeutics based on the anhydride-capped polyketide scaffold of TTM and TTN.

In contrast to other naturally occurring PP1 and PP2A inhibitors, such as okadaic acid (OA),⁹ fostriecin,¹⁰ cantharidin,¹¹ microcystin-LR,¹² and calyculin-A¹³ (Figure 1B), TTM and TTN exhibit a high degree of PP1 selectivity. TTM inhibits PP1 and PP2A with IC₅₀ values of 22–32 nM while showing a slight preference for PP1.^{12,14–17} Conversely, TTN preferentially inhibits PP1 by a factor of ~40-fold relative to PP2A (IC₅₀ = 1.6 nM for PP1 versus 62 nM for PP2A).¹⁸ By virtue of its high selectivity for PP1 inhibition, TTN represents not only an interesting drug lead but also a powerful biochemical tool with which to elucidate the roles of PP1 in various biological pathways.

Despite their similarities of structure and activity, TTN, but not TTM, has been identified as a potent immunosuppressor of activated T cells in organ transplantation.¹⁹ Inhibition of T cell proliferation by TTN was observed at concentrations 100-fold lower than those needed to achieve maximal inhibition by cyclosporine A (CsA). CsA and FK506 exert their pharmacological effects by binding to the immunophilins; the resulting complex binds to and inhibits the Ser/Thr phosphatase calcineurin albeit with potentially deleterious effects due to the physiological ubiquity of calcineurin.^{20–22} TTN exerts immunosuppressive activity in a manner completely different from those of CsA and FK506 by blocking tyrosine phosphorylation of intracellular signal mediators downstream of the Src tyrosine kinases in activated T cells. This leads to cell-specific apoptosis due to cleavage of Bcl-2, caspase-9, caspase-3, and poly(ADP-ribose) polymerase, but not caspase-1.^{19,23} The activated T cell specificity of TTN thus suggests this unique polyketide as a significant lead in the search for immunosuppressive drugs superior to CsA and FK506.

The gross structure of TTN was deduced by chemical degradation and spectroscopic analysis,²⁴ and the relative and absolute stereochemistry was established by comparison of spectral data for degradation products of TTN with those of synthetic fragments (Figure 1).²⁵ Both TTM and TTN exist as a tautomeric mixture consisting of two interconverting anhydride and diacid forms in approximately a 5:4 ratio under neutral conditions (Figure 1A).^{2,24,26} Since the major structural differences between TTM and TTN reside in the region distal to the dialkylmaleic anhydride, it has been proposed that these differences might be responsible for variations in their PP1 selectivity.^{15,27–29}

The purpose of this study was to clone and characterize the *ttn* biosynthetic gene cluster. A long-term goal of this combinatorial biosynthesis program focused on TTN is to develop novel PP1- and PP2A-specific inhibitors and T cell-specific immunosuppressors, in a manner independent of and complementary to total chemical synthesis. We report here on (i) the cloning and sequencing of the *ttn* gene cluster; (ii) development of an expedient genetic system

for *S. griseochromogenes*; (iii) determination of the *ttn* gene cluster boundaries; (iv) bioinformatics analysis of the *ttn* cluster and a proposal for TTN biosynthesis; and (v) genetic characterization of the TTN pathway to support the proposed pathway.

Integral to this work has been the elucidation, enabled by accurate assignment of the *ttn* cluster boundaries, of all genes responsible for dialkylmaleic anhydride biosynthesis. Natural products containing a dialkylmaleic anhydride moiety are well-known (Figure 1C). This report, combined with previous work on the *ttn* cluster, now enables rapid access to their biosynthetic gene cluster as well as genome mining of microorganisms for new dialkylmaleic anhydride-containing natural products. Furthermore, to better understand the timing of dialkylmaleic anhydride introduction, a $\Delta ttnM$ mutant was prepared, which produced the C-3' deshydroxy analogue TTN M-1 (Figure 1A). This approach to new TTN analogues is made possible by the convenient genetic system described herein that enables exploitation of the complete TTN biosynthetic gene cluster.

Results

Cloning and Sequencing of the *ttn* Biosynthetic Gene Cluster

The TTN gene cluster was cloned by using the dialkylmaleic anhydride biosynthesis gene *ttnP* and the crotonyl CoA reductase (*ccr*) gene for ethylmalonyl CoA biosynthesis as probes (Figure 2A). The cosmid library of the TTN producer *S. griseochromogenes* was constructed and screened by colony hybridization first using a 958-bp fragment of *ttnP* as a probe (probe 1). From 3000 colonies four positive cosmids (pBS13001–pBS13004) were identified and confirmed by PCR and Southern analyses (Figure 2A). A 568 kb fragment of the *ccr* gene was next amplified by PCR from *S. griseochromogenes* used as the second probe (probe 2). Similar screening of the cosmid library with probe 2 afforded four positive cosmids (pBS13009–pBS13012), PCR and Southern analyses of which confirmed that the two loci, identified with probes 1 and 2, respectively, overlap (Figure 2A). A total of 125 kb continuous DNA region was finally localized, 79 kb of which was ultimately sequenced on both strands. The overall G+C content for the sequenced region was 71.6%. The sequence was deposited in GenBank database under the accession number EU035755. Twenty-one complete open reading frames (orfs) were identified, among which 19 were designated as *ttn* genes (Figure 2B).

Corresponding homologues and the proposed function of each *ttn* gene product are summarized in Table 1. The deduced gene products include two large PKSs composed of a total of 10 modules, eight enzymes involved in dialkylmaleic anhydride biosynthesis, four tailoring enzymes, two regulatory proteins, and one resistance protein. While this work was in progress, a partial *ttn* cluster from *Streptomyces* sp. CK4412 was reported, which included 14 (i.e., spanning from *ttnG* to *orfI*) of the 21 orfs reported here; the cluster boundaries however were not determined.³⁰ While not identical, the two clusters are highly homologous with protein amino acid sequences ranging from 97% to 99% identity.

Determination of the *ttn* Gene Cluster Boundaries

The *ttn* gene cluster boundaries were defined by combining bioinformatics analysis and gene inactivation (Figure 2B). For the upstream boundary, *orf(-1)* encodes a putative transposase. Given the improbable role of a transposase during TTN biosynthesis, *orf(-1)* most likely lies beyond the *ttn* cluster. Immediately downstream of *orf(-1)* is a putative regulatory gene, *ttnQ*. Inactivation of *ttnQ*, affording the mutant strain SB13001, completely abolished TTN production, establishing its indispensability for TTN biosynthesis. For the downstream boundary, *orfI* encodes a putative polyprenyl phosphomannosyltransferase. Inactivation of *orfI*, affording mutant strain SB13002, had little impact on TTN production, excluding the involvement of *orfI* in TTN biosynthesis. Immediately upstream of *orfI* is *ttnL*, a homologue

of *ttnL* that has been confirmed to be essential for dialkylmaleic anhydride biosynthesis, hence essential for TTN biosynthesis.³¹

Genes Encoding Modular PKSs for Biosynthesis of the Polyketide Moiety of TTN

Two large orfs, *ttnA* and *ttnB*, that encode modular type I PKSs responsible were identified within the *ttn* cluster (Figures 2B and 3). The *ttnA* gene encodes the loading module and extension modules 1–5, whereas *ttnB* encodes extension modules 6–9 and has a C-terminal thioesterase domain for release of the full-length polyketide chain. Together, the TTN PKS of TtnA and TtnB consists of one loading module and nine extension modules and catalyzes nine rounds of decarboxylative condensation, using one malonyl CoA as a starter unit (loading module) and four malonyl CoA (modules 2, 4, 7, and 9), four methylmalonyl CoA (modules 1, 3, 5, and 6), and one ethylmalonyl CoA (module 8) as extender units, for initiation, elongation, and termination of the biosynthesis of the polyketide backbone of TTN (Figure 3).

Domain functions were deduced by sequence homology to known PKS domains.³² The loading module contains a mutated ketosynthase (KSq), an acyltransferase (AT), and an acyl carrier protein (ACP) domain, and each of the nine extension modules is minimally characterized by ketosynthase (KS), AT, and ACP domains. All KS domains contain the CHH catalytic triad required for the decarboxylative condensation reaction. All the ACP domains feature the highly conserved signature motif of DSL, in which the serine residue acts as the site for 4'-phosphopantetheinylation, a post-translational modification essential for polyketide biosynthesis by converting the apo-ACPs into the functional holo-ACPs. The choice of the loading module and the extender unit is dictated by the corresponding AT domains, for which the specificity is predicted on the basis of sequence comparison with ATs of known substrates (Figure 4).

The nine extension modules are also characterized with additional domains such as ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) domains, the presence of which accounts for the reductive modification of the β -keto group of the growing polyketide intermediate during each cycle of elongation. Functional KR domains, featuring the conserved consensus sequence GxGxxGxxA associated with NADP(H) binding, are found for all extension modules, except for KR in extender module 3, which contains a 16-amino acid deletion in the catalytic domain and, therefore, is inactive. Functional DH domains, containing the conserved consensus sequence HxxxGxxxxP, are identified for modules 5, 6, 7, and 8, excluding the DH domain in module 1, which contains a YxxxGxxxxP motif and, therefore, is inactive. In addition, intact DH domains are also present in extension modules 3 and 4, although their activities appear to be unnecessary in these modules. Finally, functional ER domains, having the conserved sequence GxGxAxxxxA, are predicted for modules 5, 6, and 7 (Figure 3).

The TE domain at the C-terminus of TtnB terminates polyketide biosynthesis by liberating the full-length polyketide intermediate from the TTN PKS biosynthetic machinery (Figure 3). Finally, in addition to the chain-terminating TE domain embedded within TtnB, a discrete type II TE (TEII), TtnH, remote from TtnA and TtnB within the *ttn* gene cluster, was also identified. TtnH may serve as an “editing” enzyme for mis-primed or stalled TtnA or TtnB PKS during polyketide chain elongation.

To support the predicted PKS function, *ttnA* was inactivated by using the PCR targeting strategies (Table S1, Supporting Information). Cosmid pBS13014, in which a 422 bp DNA region within the *ttnA* gene was replaced with the *aac(3)IV/oriT* cassette, was introduced into *S. griseochromogenes*. Apramycin-resistant and kanamycin-sensitive exconjugants were selected as double crossover recombinant mutants, named SB13003, for which the desired Δ *ttnA* genotype was confirmed by PCR and Southern blot analysis (Figure S1, Supporting

Information). Fermentation of SB13003, with the wild type strain as a positive control, followed by extraction and HPLC analysis revealed that inactivation of *ttnA* completely abolished TTN production, consistent with the indispensable role proposed for TtnA in TTN biosynthesis (Figure 6II).

Genes Encoding Enzymes for Biosynthesis of the Dialkylmaleic Anhydride Moiety of TTN

Comparison of the TTM and TTN biosynthetic gene clusters revealed eight conserved enzymes, TtnKLMNOPRS, strongly supporting the involvement of these genes in dialkylmaleic anhydride moiety biosynthesis (Figure 5A).³¹ These conserved orfs include (i) TtmO/TtnO, a putative citryl-CoA lyase; (ii) TtmP/TtnP, a putative CoA transferase; (iii) TtmR/TtnR, a putative dehydratase; (iv) TtmM/TtnM, a putative hydroxylase; (v) TtmK/TtnK, a putative esterase; (vi) TtmS/TtnS, a putative cyclase; (vii) TtmL/TtnL, a phosphatidylethanolamine-binding protein; and (viii) TtmN/TtnN, an apparently conserved hypothetical protein. The coordination of these enzymatic activities for biosynthesis of the dialkylmaleic anhydride moiety is postulated (Figure 5B).

Selected genes (*ttnM*, *ttnP*, *ttnR*, and *ttnS*) were next inactivated to investigate their roles in dialkylmaleic anhydride, hence TTN biosynthesis. In each case, the target gene was replaced in vitro by the *aac(3)IV/oriT* cassette using the PCR targeting strategies, yielding a mutated cosmid (Table S1, Supporting Information). Upon introduction of the mutated cosmids into wild type *S. griseochromogenes*, apramycin-resistant and kanamycin-sensitive double crossover recombinant strains were selected, for which the desired mutant genotypes were finally confirmed by PCR and Southern blot analyses (Table S2, Supporting Information). Assigned names for each mutant strain are SB13004 ($\Delta ttnM$), SB13005 ($\Delta ttnP$), SB13006 ($\Delta ttnR$), and SB13007 ($\Delta ttnS$), respectively (Table S2, Supporting Information). Additionally, genetic complementation experiments were carried out to eliminate the possibility of polar effects. Plasmids pBS13017, pBS13018, and pBS13019, containing intact *ttnM*, *ttnP*, and *ttnR* genes under the control of *ErmE** promoter, were introduced into SB13004, SB13005, and SB13006, yielding SB13009, SB13010, and SB13011, respectively (Table S3, Supporting Information).

These recombinant strains were fermented alongside the wild type strain as a positive control, and TTN production was examined by HPLC analysis of the fermentation extracts. All four gene inactivation mutant strains failed to produce TTN, firmly establishing the essential roles these genes play in TTN biosynthesis (Figure 6). Moreover, under no circumstances were TTN intermediates detected in the SB13005 ($\Delta ttnP$), SB13006 ($\Delta ttnR$), or SB13007 ($\Delta ttnS$) mutant strain, consistent with the proposed critical functions of *ttnP*, *ttnR*, or *ttnS* in dialkylmaleic anhydride biosynthesis (Figures 5 and 6V, VII, IX). TTN production was partially restored upon expression of a functional copy of the targeted gene in trans position, as exemplified by *ttnP* (pBS13022) and *ttnR* (pBS13023) to SB13005 ($\Delta ttnP$) and SB13006 ($\Delta ttnR$), respectively, to approximately 60% (SB13010) and 80% (SB13011) of the levels observed for the wild type strain (Figure 6VI, VIII). [The *tmcD* gene, the homologue of *ttnP* from the recently reported partial *ttn* cluster from *S. sp.* CK4412, has also been inactivated. The resultant $\Delta tmcD$ mutant strain also abolished TTN production, although no in vivo complementation to $\Delta tmcD$ was reported.³⁰] In contrast, the SB13004 ($\Delta ttnM$) mutant strain accumulated four new compounds, with TTN M-1 being the predominant product (Figure 6III). Introduction of the *ttnM* expression construct (pBS13021) into SB13004 partially restored TTN production to approximately 30% (SB13009) of the level seen for the wild type strain with concomitant disappearance of the four new compounds (Figure 6IV). The latter result suggests that TtnM-mediated oxidation likely precedes convergence of the dialkylmaleic anhydride and polyketide halves of TTN. This is contrary to earlier postulates invoking TtnM-mediated oxidation as the last step in TTN biosynthesis (Figure 3).³⁰

The identity of TTN produced by the *S. griseochromogenes* wild type and recombinant strains was confirmed by MS and ^1H and ^{13}C NMR analysis; all spectra were identical to those of authentic TTN. The four new compounds produced by SB13004 were found to have UV-vis spectra identical to that of TTN, suggesting they all contain the dialkylmaleic anhydride moiety (Figure S7, Supporting Information). The dominant compound, TTN M-1, was isolated, and its structure established by MS, UV-vis, ^1H NMR, ^{13}C NMR, and other 2D NMR methods as that of C3' deshydroxy-TTN (Figure 1A). The three minor products of SB13004 fermentation were analyzed by HPLC-MS. Molecular weights for TTN M-2, TTN M-3, and TTN M-4 were found to be 576.4, 606.4, and 606.4 amu, respectively (Figure S8, Supporting Information), but detailed structural elucidation was not pursued in the current study due to their minute production titers.

Genes Encoding Tailoring Enzymes

Compared to the nascent polyketide chain released by the TtnB terminal TE domain, the mature polyketide moiety of TTN has the following two varying functionalities: (i) a carbonyl group at C-5 position and (ii) the terminal diene structure. While TtnI (a cytochrome P450 hydroxylase) serves as a candidate for C-5 oxidation, the terminal diene structure calls for the nascent polyketide chain to undergo decarboxylation and dehydration upon release from TtnB. The latter are probably catalyzed by TtnC (a putative flavoprotein decarboxylase) or TtnD (a putative UbiD family decarboxylases) and TtnF (a putative L-carnitine dehydratase), respectively. The exact timing of carbonyl group formation, decarboxylation, and dehydration, however, needs to be determined by further experiments.

Regulatory and Resistance Genes

Regulatory and resistance proteins have also been unveiled upon sequencing the complete *ttn* cluster. The two regulatory genes identified within the *ttn* cluster are *ttnG*, which codes for a protein with 33% identity to the regulatory protein ThcG (AAD28307) from *Rhodococcus erythropolis*, and *ttnQ*, which codes for a protein with 41% identity to SareDRAFT_1231 (ZP_01648842) from *Salinispora arenicola* CNS205. Both TtnG and TtnQ belong to the LuxR family of transcription factors with the classical LuxR helix-turn-helix (HTH) motif proximal to each protein's C-terminus. Typically activated for DNA binding through associations with autoinducers such as *N*-(3-oxohexanoyl)-L-homoserine lactone, the LuxR homologues TtnG and TtnQ are intriguing since both lack an N-terminal autoinducer binding domain.³³ Additionally, TtnG contains a TTA leucine codon suggesting a possible dependence on *bldA*, the structural gene of tRNA^{UUA}.³⁴

Identification of TtnG and TtnQ as regulatory protein candidates may have a bearing on metabolic engineering efforts to improve TTN titers. As described in the determination of the cluster boundary section, inactivation of *ttnQ*, affording mutant strain SB13001, completely abolished TTN production, a finding that agrees with TtnQ being a positive regulator (Figure 6X). TTN production was partially restored to approximately 70% (SB13008) of the level seen for the wild type strain upon introduction of the *ttnQ* expression construct (pBS13020) into SB13001 (Figure 6XI). Similarly, *tmcN*, the homologue of *ttnG* from the recently appearing partial *ttn* cluster from *S. sp.* CK4412, has also been inactivated. The resultant $\Delta tmcN$ mutant strain completely lost its ability to produce TTN, as would be expected for a pathway-specific positive regulator.³⁵

Common resistance mechanisms by which microorganisms protect themselves from the potentially deleterious effects of their own bioactive natural products include intracellular compound modifications or sequestration, modification of the normally sensitive target so as to render it impervious to the effects of the natural product, and extracellular export.³⁶ Within the *ttn* cluster one such transporter protein candidate coded for by *ttnJ* was found. This putative

resistance protein, TtnJ, was found to have 49% identity to the cytoplasmic membrane multidrug transporter RHA1_ro04399 (YP_704343) from *Rhodococcus* sp. RHA1. It thus appears that *S. griseochromogenes* may derive TTN resistance via an export mechanism although further studies are warranted to confirm this postulate.

Discussion

Cloning Strategy for the *ttn* Cluster Using Dialkylmaleic Anhydride Synthesis Genes as Probes

Inspired by the unusual dialkylmaleic anhydride moiety shared by TTM and TTN, we conceived of and developed a cloning strategy for dialkylmaleic anhydride-containing natural products, which proved highly effective in cloning the *ttn* gene cluster from *S. griseochromogenes*. Recent cloning and sequencing of the *ttn* cluster unveiled the importance of eight genes, *ttnKLMNOPRS*, in the biosynthesis of the dialkylmaleic anhydride moiety of TTM.³¹ Using this knowledge we utilized *ttnP*, which encodes a putative acyl-CoA transferase/carnitine dehydratase, as the heterologous probe, to localize and clone the corresponding locus associated with TTN biosynthesis by screening a cosmid library (with ~3000 clones) derived from the TTN producer *S. griseochromogenes* (Figure 2). Sequence analysis, however, revealed that all cosmids within the identified locus have PKS encoding sequences at one end, thus necessitating the need to exploit an additional probe, unrelated to *ttnP*, to rescreen the cosmid library for clones covering the entire *ttn* cluster.

The polyketide origin of TTN has been previously supported by feeding experiments, which also revealed the importance of an ethylmalonyl-CoA extender unit (Figure 3).³⁷ Therefore, we used a crotonyl-CoA reductase gene *ccr* as an alternative probe (probe 2) to screen the *S. griseochromogenes* genomic library.³⁸ The application of probe 2 allowed localization of the entire *ttn* biosynthetic gene cluster to a 125 kb continuous DNA region, consistent with the recently reported partial sequence of the *ttn* cluster from *Streptomyces* sp. CK4412 (Figure 2).³⁰ Summarized in Table 1, the *ttn* cluster was found to consist of 19 orfs encoding two modular type I polyketide synthases (TtnAB), one type II thioesterase (TtnH), four tailoring enzymes (TtnCDFI), two regulatory proteins (TtnGQ), one resistance protein (TtnJ), and eight proteins for dialkylmaleic anhydride biosynthesis (TtnKLMNOPRS); *ttnHIJQRS* represent the new TTN biosynthesis genes not previously reported.³⁰ The dialkylmaleic anhydride synthesis genes *ttnR* and *ttnS* bear particular significance, as they reside at a location remote from the rest of the anhydride synthesis subcluster and help define this subcluster as a potentially useful tool in future genome mining efforts to identify new dialkylmaleic anhydride-containing natural products and their respective producers.

Boundary Determination to Discover the Putative *ttn* Cluster

Critical to the functional assignment of all genes within the *ttn* cluster has been the determination of the cluster boundaries. An efficient genetic system of *S. griseochromogenes* was developed allowing the *ttn* gene cluster boundaries to be determined by combining bioinformatics analysis with gene inactivation. The upstream boundary is between *orf(-1)* and *ttnQ*. While *orf(-1)*, coding for a transposase, is not anticipated to be involved in TTN biosynthesis, inactivation of *ttnQ* completely abolished TTN production, defining the most upstream gene of the *ttn* cluster. The downstream boundary is between *ttnL* and *orf1*. Inactivation of *orf1* had a negligible effect upon TTN production, excluding it from the *ttn* cluster. The *ttnL* gene, a homologue of *ttnL* proposed to be involved in the dialkylmaleic anhydride moiety biosynthesis of TTM,³¹ therefore defines the most downstream boundary of the *ttn* cluster. As such, *ttnL* is within the *ttn* gene cluster. Taken together, these results allowed determination of the *ttn* cluster boundaries with a high degree

of confidence and precision, and the *ttn* cluster, spanning 76 kb DNA and bracketed by *orf* (–1) and *orf*1, consists of 19 orfs (Figure 2B and Table 1).

A Proposed Pathway for TTN Biosynthesis

A pathway featuring unique chemistry for dialkylmaleic anhydride biosynthesis and a type I PKS selecting and incorporating three different starter and extender units for TTN production has been proposed (Figure 3). This proposal is based on the findings from cloning and sequencing of the *ttn* gene cluster from *S. griseochromogenes*, precise determination of the *ttn* gene cluster boundaries, deduced function of the orfs within the *ttn* cluster, and in vivo analysis of selected genes from the *ttn* cluster by targeted gene inactivation and mutant complementation. Also pivotal in formulating the cogent proposal for TTN biosynthesis are our previous findings from the characterization of the *ttn* biosynthetic gene cluster.³¹ The proposed pathway agrees well with previous feeding experiments using isotopically labeled precursors and provides further insight into the recent report of the partial *ttn* cluster from *S. sp.* CK4412.³⁰

Biosynthesis of the TTN Polyketide Moiety

The polyketide moiety of TTN is biosynthesized by two large modular type I polyketide synthases (TtnA and TtnB), whose domain organization is colinear with the TTN core scaffold excluding predicted nonfunctional DH or KR domains, a common occurrence in modular PKSs.³² The TtnA and TtnB PKSs together catalyze nine rounds of decarboxylative condensation using one acetyl-CoA as the starter unit (loading module) and one ethylmalonyl ACP (module 8), four malonyl-CoA (modules 2, 4, 7, and 9), and four methyl malonyl-CoA (modules 1, 3, 5, and 6) as extender units. The TtnA and TtnB PKSs exhibit the presence of multiple ATs for various starter and extender units, a striking feature that could be exploited to engineer novel TTN analogues via combinatorial biosynthesis approaches (Figure 3).

As presently envisioned, the production of the TTN polyketide scaffold by the TtnA and TtnB PKSs precedes a minimum of three steps needed to complete the biosynthesis of TTN. These transformations include (i) esterification to converge the dialkylmaleic anhydride moiety with the polyketide; (ii) installation of the ketone oxidation state at C-5; and (iii) decarboxylative dehydration to install the terminal diene. Having cloned and sequenced the complete *ttn* cluster, we are now well positioned to experimentally investigate the timing of these events.

Biosynthesis of the Dialkylmaleic Anhydride Moiety

Comparative analysis, especially in the context of the recently sequenced *ttn* biosynthetic gene cluster,³¹ has revealed a set of eight enzymes that are likely involved in the formation of the dialkylmaleic anhydride moiety of TTM and TTN. The precise roles for TtmL/TtnL, TtmN/TtnN, and TtmS/TtnS could not be proposed on the basis of bioinformatics analysis alone. However, they are highly conserved between the *ttn* and *ttn* clusters (Figure 5A), inactivation of which, as exemplified by *ttnS*³¹ previously and *ttnS* in the current study, completely abolished TTM and TTN production, respectively, unambiguously establishing the indispensability of these genes for TTM and TTN biosynthesis. On the other hand, early feeding experiments have suggested propionate and α -ketoglutarate as biosynthetic precursors for the dialkylmaleic moiety.³ The unveiling of these conserved enzymes now allows us to propose a pathway for dialkylmaleic anhydride minimally involving (i) TtmO/TtnO to initiate the pathway by catalyzing aldol condensation with methylmalonyl-CoA as the nucleophile and the α -carbonyl of R-ketoglutarate as the site of attack; (ii) TtmP/TtnP to catalyze anhydride ring closure with concomitant release of CoA; (iii) TtmR/TtnR to catalyze the subsequent dehydration, thus installing the olefinic moiety; (iv) TtmM/TtnM to catalyze the hydroxylation at C-3', as supported by in vivo gene inactivation and genetic complementation experiments; and (v) finally TtmK/TtnK to catalyze convergence of the completed dialkylmaleic anhydride

and polyketide moieties (Figures 3 and 5B). Importantly, the $\Delta ttnM$ mutant strain SB13004 does not accumulate the C-3' deshydroxy TTN analogue TTN M-1 as the sole metabolite, as might be expected of a completely convergent biosynthesis in which the polyketide moiety is coupled to the dialkylmaleic anhydride unit with TtnM-catalyzed C-3' hydroxylation as the final step for TTN biosynthesis. Thus, already, a firm understanding of the *ttn* cluster in its entirety has permitted insight into the timing of TTN biosynthetic steps.

Dialkylmaleic Anhydride Biosynthesis Genes and Genome Mining

The genes coding for dialkylmaleic anhydride biosynthesis in both the *ttn* and *ttm* clusters are now known. The genes *ttnKLMNOPRS* are highly similar to *ttmKLMNOPRS*, although two crucial differences differentiate the two subclusters. First, it is evident that the relative positioning of the *ttn* genes with respect to each other is significantly different from that of the *ttm* system. Second, and perhaps most significant, is that the *ttn* genes almost all reside proximal to one another barring a ~5 kb gap isolating *ttnKL* from *ttnMNOPRS* (Figure 5A). Conversely, the *ttm* dialkylmaleic anhydride synthesis genes are characterized by clustering of *ttnKPONML* and the remote upstream positioning of *ttnR* and *ttnS* by approximately 62 kb (Figure 5A). That *ttnRS* are so remote from the rest of the dialkylmaleic anhydride biosynthetic genes stands in contrast to expectations based not only on previous findings with the *ttm* cluster but also on typical subcluster patterns commonly observed in biosynthetic gene clusters. Importantly, *ttnRS* represent previously unassigned genes during the partial sequencing of the *ttn* cluster from *S. sp.* CK4412.³⁰ Identification and functional assignments for *ttnRS* relating to dialkylmaleic anhydride synthesis were achieved here, in large part, by our ability to accurately define the *ttn* cluster boundaries. Natural products containing dialkylmaleic anhydride moiety are well known (Figure 1C). Having now characterized the subcluster responsible for dialkylmaleic anhydride biosynthesis in both the *ttm* and *ttn* clusters, we anticipate the usefulness of these genes in both cloning of biosynthetic gene clusters of other dialkylmaleic anhydride-containing natural products and genome mining of microorganisms for new ones.

Tailoring Steps

After, or during, the assembly line construction of the polyketide core, a minimum of three steps are needed to finish the biosynthesis of TTN: (i) hydroxylation and oxidation to install the C-5 ketone, which is probably catalyzed by the cytochrome P450 hydroxylase candidate TtnI; (ii) decarboxylation and dehydration to afford the terminal diene, which are most likely catalyzed by decarboxylases TtnC and/or TtnD, and the L-carnitine dehydratase TtnF, respectively; and (iii) TtnK-catalyzed esterification to couple the dialkylmaleic anhydride moiety with the polyketide chain (Figure 3). The availability of these candidate genes and the establishment of an expedient genetic system for *S. griseochromogenes* now set the stage to investigate these events by in vivo and in vitro experiments.

Timing of Dialkylmaleic Anhydride Coupling to Polyketide

As with TTM biosynthesis, bioinformatics analysis of the *ttn* gene cluster alone fell short of differentiating the timing for the coupling between the dialkylmaleic anhydride and polyketide moieties. The dialkylmaleic anhydride unit could be coupled to the full-length (convergent model) or a growing (linear model) polyketide intermediate, after which time additional processing may proceed. The convergent model would suggest that inactivation of genes encoding dialkylmaleic anhydride synthesis would abolish TTN production but allow for the accumulation of the polyketide moiety or metabolites thereof. The *ttnKPRS* genes are outstanding candidates to test this postulate since they are all likely to play a role in dialkylmaleic anhydride biosynthesis and installation in TTN. Inactivation of any one of the four genes would be anticipated to lead to accumulation of a biosynthetic intermediate or

metabolites devoid of the dialkylmaleic anhydride moiety. For *ttnPRS*, this was not found to be the case; inactivation of *ttnP*, *ttnR*, or *ttnS* completely abolished production of TTN while at the same time failing to afford any polyketide or related metabolites, as was the case for *ttnM* reported previously.³¹ These data suggest that these gene products perform early biosynthetic functions preceding release of the full-length TTN polyketide intermediate from the TtnA and TtnB PKSs. Also possible is that inactivation of *ttnPRS* could prohibit the ensuing enzyme-dependent events related to polyketide elongation and/or release from the TtnA and TtnB PKSs. Fundamentally, these results are not consistent with a convergent biosynthesis of TTN but rather support a linear pathway in which coupling of the dialkylmaleic anhydride to the TTN polyketide precedes completion of the full-length polyketide backbone and its liberation from the PKS (Figure 3).

Conversely, TtnM, an Fe(II)- α -ketoglutarate-dependent oxygenase, is an excellent candidate to catalyze hydroxylation at C-3' and thus constitutes a key enzyme involved in dialkylmaleic anhydride biosynthesis. In vivo gene inactivation of *ttnM* afforded the mutant strain BS13004, unable to produce TTN but yielding, instead, four new TTN congeners, all devoid of the 3' hydroxyl moiety. This is in stark contrast to the results of *ttnPRS* inactivation in which no intermediates were detected under all conditions examined. That TTN M-1 was the major congener produced by BS13004 supports the following assertions about TTN biosynthesis: (i) TtnM is directly responsible for C-3' hydroxylation; (ii) the timing of C-3' hydroxylation in relation to TTN construction is such that its abolishment does not significantly impact the activity of enzymes involved in late stage TTN synthesis; and/or (iii) enzymes downstream of the C-3' hydroxylation event are sufficiently promiscuous so as to not be significantly impaired by the lack of substrate C-3' hydroxylation. More importantly, the production of multiple TTN congeners following *ttnM* inactivation supports further a linear biosynthetic logic for TTN. Attachment of an incomplete dialkylmaleic anhydride unit, such as the C-3'-deshydroxyl dialkylmaleic anhydride in the Δ *ttnM* mutant strain, midway during polyketide elongation can be envisioned to interfere with TtnA and TtnB PKS-catalyzed chain elongation, leading ultimately to multiple polyketide products. Supporting the notion that convergence of the dialkylmaleic anhydride and polyketide units occurs early during polyketide synthesis thus affording a linear synthesis is that all TTN analogues found display UV-vis spectra similar to TTN, supporting the presence of the anhydride moiety. It is also unlikely that inactivation of TtnM would lead to multiple products due to changes in tailoring enzyme (TtnICDF) efficiencies. Finally, it is significant that TTN production in the mutant strains SB13004 (Δ *ttnM*), SB13005 (Δ *ttnP*), and SB13006 (Δ *ttnR*) could be partially restored by expressing functional copies of each inactivated enzyme in trans position, thereby excluding the possibility of polar effects.

Taken together, our ability to correlate specific gene inactivation events, as exemplified here with *ttnM*, with specific structural modifications to the natural product, supports the significance of the genetic system described in ultimately dissecting the biosynthesis of TTN and in allowing for the production and development of new TTN analogues. This is particularly significant because of TTN's unique niche as a PP1-specific inhibitor and an activated T cell-specific immunosuppressor mechanistically different from CsA and FK506. Moreover, the structural similarities and implied biosynthetic parallels between TTN and TTM attract great interest and amplify the possible significance of studies in TTN biosynthesis. The results reported here set an excellent stage for future investigations of TTN biosynthesis and engineering in addition to providing the valuable tool of a bona fide dialkylmaleic anhydride biosynthesis cluster for use in genome mining studies to discover new anhydride-containing natural products and their producers.

Experimental Section

Bacterial Strains and Plasmids

Escherichia coli DH5 α was used as the host for general subcloning.³⁹ *E. coli* XL 1-Blue MR (Stratagene, La Jolla, CA) was used as the transduction host for cosmid library construction. *E. coli* ET12567/pUZ8002⁴⁰ was used as the cosmid donor host for *E. coli*–*Streptomyces* conjugation. *E. coli* BW25113/pIJ790 and *E. coli* DH5 α /pIJ773 were provided by John Innes Center (Norwich, UK) as a part of the REDIRECT Technology kit.⁴¹ The *S. griseochromogenes* wild type strain has been described previously.^{2,26} SuperCos1 (Stratagene) was used to construct the *S. griseochromogenes* genomic library.

Biochemicals, Chemicals, and Media

Common biochemicals and chemicals were acquired from standard commercial sources. *E. coli* strains carrying plasmids were grown in Luria–Bertani (LB) medium with appropriate antibiotics selection.³⁹ All media for *Streptomyces* growth were prepared according to standard protocols.⁴² ISP-4 and tryptic soy broth (TSB) were from Difco Laboratories (Detroit, MI), and modified ISP-4 is ISP-4 supplemented with 0.05% yeast extract and 0.1% tryptone.⁴³

DNA Isolation and Manipulation

Plasmid extractions and DNA purifications were carried out using standardized commercial kits (Qiagen, Santa Clarita, CA). Genomic DNAs were isolated according to the literature protocol.⁴² The digoxigenin-11-dUTP labeling and detection kit (Roche Diagnostics Corp, Indianapolis, IN) was used for preparation of DNA probes, and detection by colony and Southern hybridization was carried out according to the protocols provided by the manufacturer.

Genomic Library Construction and Screening

S. griseochromogenes genomic DNA was partially digested with *Sau*3AI to yield a smear of about 45–55 kb, dephosphorylated with shrimp alkaline phosphatase, and ligated into SuperCos1 pretreated with *Xba*I, dephosphorylated, and digested with *Bam*HI. The resulting ligation mixture was packaged with the Gigapack III Gold (Stratagene) and transduced into *E. coli* XL 1-Blue MR to generate the genomic library. The transduced cells were spread onto LB plates containing ampicillin (200 μ g/mL), and the plates were incubated at 37 °C overnight. The titer of the primary library was approximately 10⁵ cfu per μ g of DNA. The average size of the inserts for the cosmid library was determined to be 40 to 45 kb by restriction enzyme analysis of 10 randomly selected cosmids.

The genomic library was first screened by colony hybridization, and then the positive clones were rescreened by PCR and confirmed by Southern hybridization. Probe 1 was the 958 bp fragment of *ttnP*, which was amplified from pBS6004 using *ttnPFP* (5'-GCGGACCGGCCAGTCGATC-3') and *ttnPRP* (5'-TTCGGCCATGCGCACGAC-3').³⁷ Probe 2 was a 568 kb fragment of the *ccr* gene, which was amplified from *S. griseochromogenes* genomic DNA using the following pair of primers: P2F: 5'-GCACGACCTGCCCTATCAC-3'/P2R: 5'-GAAGCGACGCCACTCCTT-3'. As shown in Figure 2, a 125 kb DNA region containing the whole *ttn* biosynthetic gene cluster was represented by the eight selected overlapping cosmids pBS13001, pBS13002, pBS13003, pBS13004, pBS13009, pBS13010, pBS13011, and pBS13012.

DNA Sequencing and Analysis

The 79 kb region was sequenced on both chains by the dideoxynucleotide chain termination method. Sequencing reactions were run using Big Dye Terminator mix (Applied Biosystems,

Foster City, CA), purified using CleanSeq magnetic beads (Agencourt Biosciences, Beverly, MA), and sequenced by the University of Wisconsin Biotechnology Center (Madison, WI). Sequence assembly and contig alignments were carried out using the Seqman program in the Lasergene software package (DNASTAR, Inc., Madison, WI). Orf assignments and their function predictions were accomplished with the FramePlot 2.3.2 program and Blast programs, respectively.

Gene Inactivation

Target genes were inactivated by the REDIRECT Technology according to the literature protocol.⁴¹ Briefly, the apramycin resistance gene *aac(3)IV/oriT* cassette was used to replace an internal region of the target gene. Mutant cosmids pBS13013 ($\Delta orfI$), pBS13014 ($\Delta ttnQ$), pBS13015 ($\Delta ttnA$), pBS13016 ($\Delta ttnM$), pBS13017 ($\Delta ttnP$), pBS13018 ($\Delta ttnR$), and pBS13019 ($\Delta ttnS$) were constructed (Table S1, Supporting Information) and introduced into *S. griseochromogenes* by conjugation from *E. coli* ET12567/pUZ8002 according to the literature procedure with the following modifications.⁴² *S. griseochromogenes* spores were suspended in TSB medium and heat-shocked at 45 °C for 15 min, followed by incubation at 30 °C for 6 h. Germinated spores were mixed with *E. coli* ET12567/pUZ8002 harboring the mutant cosmid and spread onto modified ISP-4 plates freshly supplemented with 20 mM MgCl₂. After incubation at 28 °C for 16 to 22 h, each plate was overlaid with 1 mL of sterile H₂O containing apramycin at a final concentration of 10 µg/mL and nalidixic acid at a final concentration of 50 µg/mL. Incubation continued at 28 °C until exconjugants appeared. The desired double crossover mutants, selected by the apramycin-resistant and kanamycin-sensitive phenotype, were isolated as SB13001 ($\Delta orfI$), SB13002 ($\Delta ttnQ$), SB13003 ($\Delta ttnA$), SB13004 ($\Delta ttnM$), SB13005 ($\Delta ttnP$), SB13006 ($\Delta ttnR$), and SB13007 ($\Delta ttnS$), the genotypes of which were verified by PCR and selectively confirmed by Southern analysis (Table S2 and Figures S1, S2, S3, and S4, Supporting Information). To construct the expression plasmids for mutant complementation, the *ttnQ*, *ttnM*, *ttnP*, and *ttnR* genes were amplified, digested with *NsiI* and *XbaI*, and cloned into the same sites of pBS6027 to yield pBS13020 (for *ttnQ* expression), pBS13021 (for *ttnM* expression), pBS13022 (for *ttnP* expression), and pBS13023 (for *ttnR* expression). Introduction of the expression constructs into the corresponding mutants by conjugation afforded strains SB13008, SB13009, SB13010, and SB13011, in which the $\Delta ttnQ$, $\Delta ttnM$, $\Delta ttnP$, and $\Delta ttnR$ mutations were complemented by the constitutive expression of functional copies of *ttnQ*, *ttnM*, *ttnP*, and *ttnR*, respectively, under the control of the *ErmE** promoter (Table S3, Supporting Information).

Production and HPLC Analysis of TTN

For TTN production, both seed and production media consisted of 2% glucose, 0.5% soluble starch, 0.05% beef extract, 0.3% yeast extract, 1% soybean flour, 0.2% NaCl, and 0.0025% K₂HPO₄, pH 7.0. The wild type and recombinant strains were fermented under literature conditions with minor modifications, and a two-stage fermentation process was adopted.^{2,26} Briefly, a spore suspension (5 µL) of the wild type or recombinant strains was first inoculated into 50 mL of seed medium in a 250 mL flask and incubated at 28 °C and 250 rpm for 2 days. The resulting seed culture was used to inoculate the production medium (5 mL into 50 mL of production medium in a 250 mL flask for production analysis or 50 mL into 500 mL of production medium in a 2 L flask for isolation) and incubated at 28 °C and 250 rpm for an additional 4 days. The fermentation broth was adjusted to pH 4.0 with 1 N HCl and centrifuged and filtered to remove the mycelia. The resulting supernatant was extracted twice with an equal volume of EtOAc. The combined EtOAc extracts were concentrated in vacuo to afford an oily residue. The latter was dissolved in CH₃CN, filtered through a 0.2 µm filter, and subjected to HPLC analysis. The HPLC chromatography system consisted of Varian ProStar 210 pumps and a ProStar 330 photodiode array detector (Varian, Walnut Creek, CA), developed with a

linear gradient from 15% to 80% CH₃CN/H₂O in 20 min followed by an additional 10 min at 80% CH₃CN/H₂O at a flow rate of 1 mL/min and UV detection at 272 nm.

Production and Purification of TTN M-1 from SB13004

S. griseochromogenes SB13004 recombinant strain was fermented similarly to that above in large scale for isolation. After fermentation, the fermentation broth (15 L) was adjusted to pH 4.0 with 1 N HCl and centrifuged, and the resulting supernatant filtered to remove the mycelia. The resulting supernatant was loaded and passed through 3 L of XAD-16 resin. The resin was washed with deionized water and then eluted with acetone (6 L). The acetone eluates were combined and solvent was then evaporated to dryness, yielding a residue. The residue was dissolved in 0.5 L of H₂O, adjusted to pH 4.0 with 1 N HCl, and then extracted twice with an equal volume of EtOAc. The combined EtOAc extracts were concentrated in vacuo to afford an oily residue. The latter was dissolved in a small amount of CHCl₃/Me₂CO (1:1) and mixed with 3 g of RP-C₁₈ silica gel. The dried RP-C₁₈ silica gel containing sample was loaded onto a RP-C₁₈ silica gel flash column developed by stepwise elution with 20–80% CH₃CN in H₂O, yielding 12 fractions, each of which was analyzed by analytical HPLC. Fractions eluted with 50% CH₃CN were combined and concentrated under vacuum to provide an oily residue, which was further purified by RP-C₁₈ silica gel flash column chromatography developed by stepwise elution with 10–80% CH₃CN in H₂O to afford 3'-deshydroxy-TTN (31 mg).

Structure Elucidation of 3'-Deshydroxy-TTN (TTN M-1)

TTN M-1 was obtained as a colorless gum. It has a molecular formula of C₃₃H₅₀O₉ as determined by HRMALDIMS, 16 mass units less than TTN, and consistent with a structure having one less hydroxyl group than TTN. The ¹H NMR spectrum of TTN did not show the 3'-hydroxyl group proton signal that can be easily discerned in TTN at δ 5.19, indicating that TTN M-1 lacks the hydroxyl group at the 3' position. Detailed analyses of its 2D NMR spectra confirmed this proposal. Compared with the spectroscopic data of TTN, the C-3' of TTN M-1 was shifted upfield by 32.2 ppm. In the HMBC spectrum of TTN M-1, H-2' and H-3' showed correlations with C-1' at δ 172.0, proving that TTN M-1 differed structurally from TTN only at the C-3' position, which lacks a hydroxyl group. NMR data of TTN M-1: ¹H NMR (CDCl₃, 500 MHz) δ 6.22 (1H, dd, *J* = 17.5, 10.5 Hz, 3-vinyl-CH), 6.04 (1H, s, H-4), 5.70 (1H, d, *J* = 17.5 Hz, 3-vinyl-CH₂), 5.42 (1H, d, *J* = 10.5 Hz, 3-vinyl-CH₂), 4.15 (1H, m, H-18), 3.71 (1H, m, H-16), 2.77 (2H, m, H-3'), 2.74 (2H, m, H-2'), 2.12 (3H, s, CH₃-5'), 1.29 (3H, d, *J* = 6.0 Hz, CH₃-18), 1.00–1.10 (6H, m, CH₃-13 and CH₃-17), 0.80–1.00 (9H, m, 1-CH₃, 7-CH₃, CH₃-9); ¹³C NMR (CDCl₃, 125 MHz) δ 217.8 (C-14), 200.8 (C-5), 172.0 (C-1'), 166.1 (C-6'), 165.9 (C-7'), 157.3 (C-3), 142.8 (C-5'), 142.1 (C-4'), 139.5 (3-vinyl-CH), 126.4 (C-4), 120.4 (3-vinyl-CH₂), 73.8 (C-12), 73.2 (C-18), 66.6 (C-16), 53.0 (C-13), 52.7 (C-6), 46.8 (C-15), 45.0 (C-8), 43.0 (C-17), 33.0 (C-10), 32.0 (C-11), 31.8 (C-3'), 30.0 (C-9), 27.0 (C-7), 20.8 (C-2), 20.1 (CH₃-7), 20.0 (C-2'), 19.5 (CH₃-9), 18.1 (CH₃-18), 14.1 (CH₃-13), 13.9 (CH₃-13), 10.0 (CH₃-5'), 9.8 (CH₃-17); (+)ESIMS *m/z* 591.1 [M + H]⁺; (+)HRMALDIMS *m/z* 613.3364 [M + Na]⁺ (calcd for C₃₃H₅₀O₉Na⁺, 613.3347).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

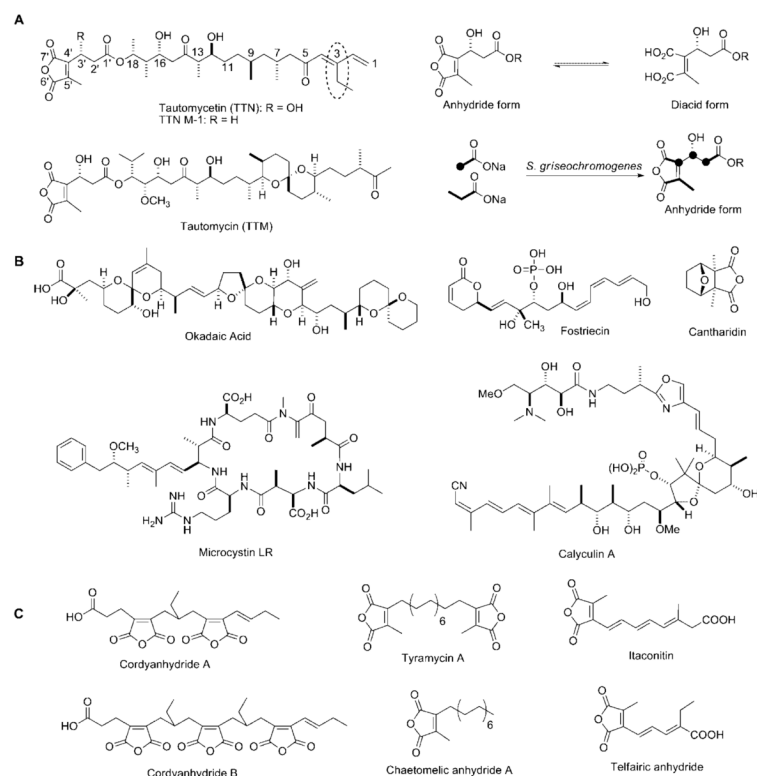
Acknowledgments

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**Figure 1.**

(A) Structures of tautomycin (TTM) and tautomycetin (TTN) in anhydride or diacid forms, structure of TTN M-1 produced by the $\Delta ttmM$ mutant strain SB13004 described herein, and biosynthetic origin of the dialkylmaleic anhydride by feeding experiments using ^{13}C -labeled acetate and propionate. (B) Selected natural product inhibitors of PP-1 and PP-2A. (C) Selected natural products containing a dialkylmaleic anhydride moiety.

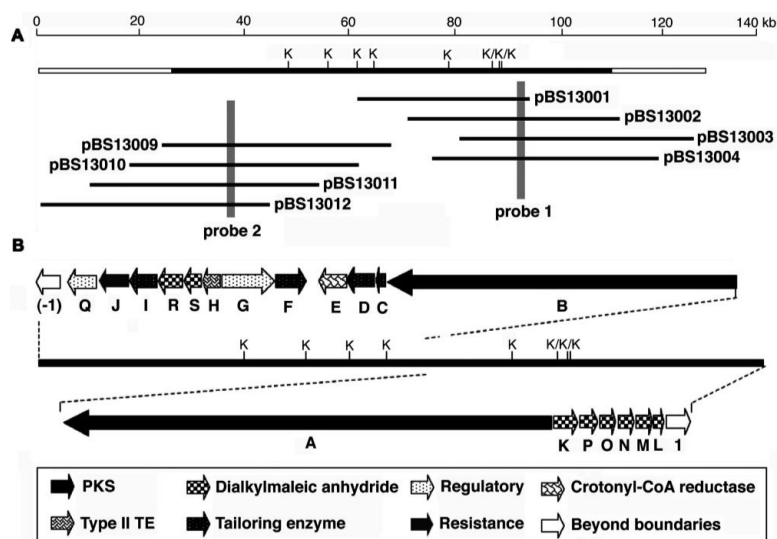


Figure 2.

(A) Restriction map of the 140 kb DNA region from *S. griseochromogenes* harboring the entire *ttn* gene cluster as represented by eight overlapping cosmids. Solid black bar indicates sequenced DNA region. (B) Genetic organization of the *ttn* gene cluster. Proposed functions for individual orfs are coded with various patterns and summarized in Table 1. K, *KpnI*.

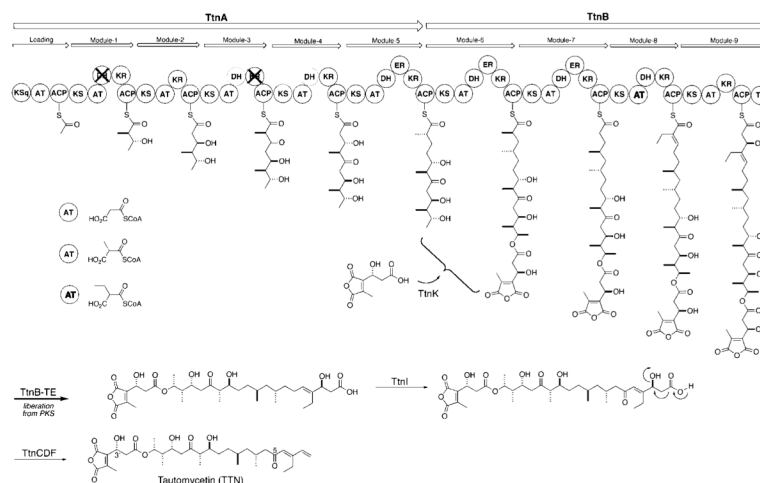


Figure 3.

Deduced module and domain organization of TtnA and TtnB PKSs and a linear model for TTN biosynthesis featuring the TTN PKS templated assembly of the TTN polyketide backbone featuring various starter and extender units, coupling of the dialkylmaleic anhydride to the elongating polyketide intermediate prior its reaching to full length, and other key tailoring steps. The AT domains are coded with various patterns to highlight their substrate specificity, “X” marks domains predicted to be inactive, and dotted circles indicate intact domains whose activities appear to be unnecessary. AT, acyl transferase; ACP, acyl carrier protein; KS, ketosynthase; DH, dehydratase; KR, ketoreductase; ER, enoylreductase; TE, thioesterase.

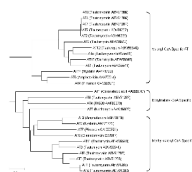


Figure 4. Phylogenetic analysis of TtnAB AT domains and their homologues from type I PKSs that specify malonyl-CoA, methylmalonyl-CoA, and ethylmalonyl-CoAs. Following each AT domain is the NCBI accession number. The scale bar represents 0.1 amino acid substitution per position.

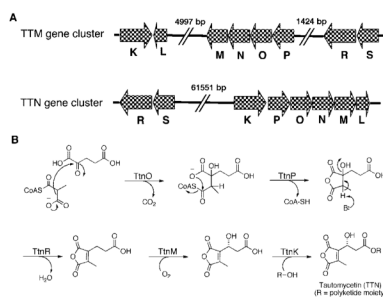


Figure 5.

(A) Comparative chromosomal spacing and orientation of dialkylmaleic anhydride synthesis genes *ttmKLMNOPRS* and *ttnKLMNOPRS*. Differentiating features of the two subclusters include both the relative positioning of orfs with relation to each other and the very remote location of *ttnRS* from the rest of the subcluster. Though both are putative transcriptional activators, *ttnQ* and *ttmQ*³¹ are not homologous proteins. (B) Proposed pathway for dialkylmaleic anhydride biosynthesis minimally involving TtnO, TtnP, TtnR, and TtnM and its coupling with the polyketide moiety catalyzed by TtnK.

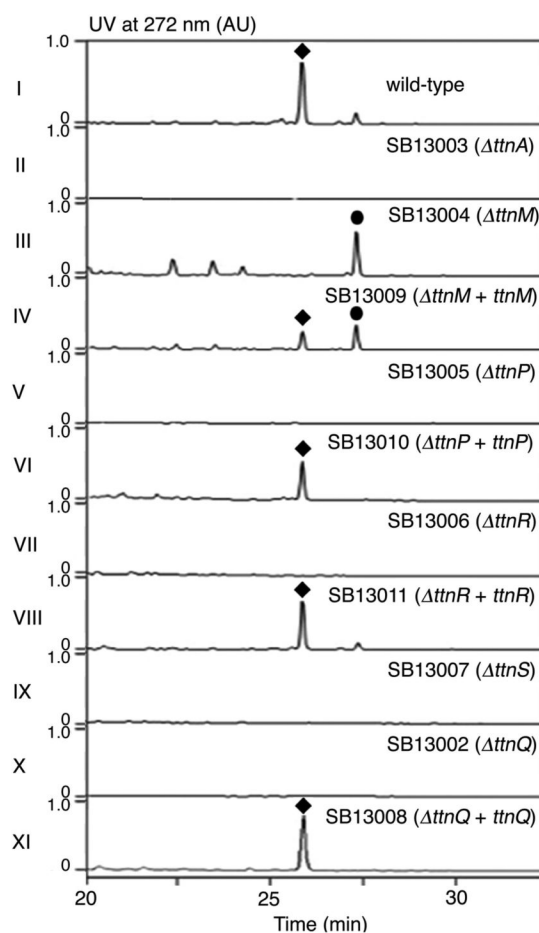


Figure 6.

HPLC analysis of *ttn* gene inactivation mutant strains and selected complementation strains. (I) Wild type *S. griseochromogenes*; (II) SB13003, $\Delta ttnA$ mutant; (III) SB13004, $\Delta ttnM$ mutant; (IV) SB13009, $\Delta ttnM$ complemented, (V) SB13005, $\Delta ttnP$ mutant, (VI) SB13010, $\Delta ttnP$ complemented, (VII) SB13006, $\Delta ttnR$ mutant; (VIII) SB13011, $\Delta ttnR$ complemented, (IX) SB13007, $\Delta ttnS$ mutant, (X) SB13002, $\Delta ttnQ$ mutant, (XI) SB13008, $\Delta ttnQ$ complemented. ◆ TTN, ● TTN M-1 (C-3' deshydroxy-TTN, Figure 1A).

Table 1**Deduced Functions of Open Reading Frames in the Tautomycin Biosynthetic Gene Cluster**

gene	size ^a	proposed function	homologue ^b	identity%/ similarity%
<i>orf(-1)</i>	262	transposase	MUL_2441 (YP_906264)	32/42
Upstream boundary of the <i>ttn</i> cluster				
<i>ttnQ</i>	472	transcriptional activator	StaR (BAC55205)	13/19
<i>ttnJ</i>	560	multidrug transporter	RHA1_ro04399 (YP_704343)	49/53
<i>ttnI</i>	449	cytochrome P450	EryF (1Z8Q_A)	30/43
<i>ttnR</i>	470	dehydratase	PrpD (2HP3_A)	24/38
<i>ttnS</i>	272	unknown	PFL_4035 (YP_261132)	27/39
<i>ttnH</i>	259	thioesterase	PikAV (AAC69333)	42/53
<i>ttnG</i>	926	regulatory protein	ThcG (AAD28307)	33/46
<i>ttnF</i>	505	L-carnitine dehydratase	CaiB (1XK7_B)	12/24
<i>ttnE</i>	444	crotonyl-CoA reductase	Ccr (AAA92890)	75/84
<i>ttnD</i>	485	UbiD family decarboxylases	UbiD (2IDB_A)	24/36
<i>ttnC</i>	209	flavoprotein decarboxylase	VdcB (AAD28781)	57/71
<i>ttnB</i>	7576	PKS modules 6–9		
<i>ttnA</i>	9528	PKS loading module and modules 1–5		
<i>ttnK</i>	465	esterase	PnbA (1QE3_A)	29/44
<i>ttnP</i>	383	CoA transferase	CaiB (1XVV_A)	24/41
<i>ttnO</i>	309	citryl CoA lyase	Mtb CitE (1Z6K_A)	24/37
<i>ttnN</i>	363	unknown	EhpF (AAN40895)	37/52
<i>ttnM</i>	339	hydroxylase	Plav_0577 (YP_001411857)	29/42
<i>ttnL</i>	185	unknown	Ybhb (1FUX_A)	25/33
Downstream boundary of the <i>ttn</i> cluster				
<i>orfI</i>	507	polyprenyl phospho-mannosyltransferase	MppI (AAU34200)	32/48

^aNumbers are in amino acids.

^bGiven in parentheses are NCBI accession numbers.