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Analysis of the Tetronomycin Gene Cluster: Insights into the Biosynthesis of a Polyether Tetronate Antibiotic

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The biosynthetic gene cluster for tetronomycin (TMN), a polyether ionophoric antibiotic that contains four different types of ring, including the distinctive tetronic acid moiety, has been cloned from Streptomyces *sp. NRRL11266. The sequenced* tmn *locus (113 234 bp) contains six modular polyketide synthase (PKS) genes and a further 27 open-reading frames. Based on sequence comparison to related biosynthetic gene clusters, the majority of these can be assigned a plausible role in TMN biosynthesis. The identity of the cluster, and the requirement for a number of individual genes, especially those hypothesised to contribute a glycerate unit to the formation of the tetronate ring, were confirmed by specific gene disruption. However, two large genes that are predicted to encode together a multifunctional PKS of a highly unusual type seem not to be involved in this pathway since deletion of one of them did not alter tetronomycin production. Unlike previously characterised polyether PKS systems, oxidative cyclisation appears to take place on the modular PKS rather than after transfer to a separate carrier protein, while tetronate ring formation and concomitant chain release share common mechanistic features with spirotetronate biosynthesis.*

Introduction

Acyltetronic acids are abundant and diverse polyketide natural products that have been isolated from both terrestrial and marine micro-organisms that show an impressive range of antibiotic, antitumour and antiviral effects.[1] They include spirotetronic acids,[2–5] such as the antitumour compound chlorothricin (**1**;[2] Figure 1) and the protein phosphatase inhibitor RK-682 (**2**).[6] Particular interest attaches to the tetronate polyethers tetronomycin (TMN) **3**[7] and tetronasin (TSN) **4**[8] because, although they are chemically almost identical, they differ from one another in their configuration at every one of ten comparable asymmetric centres (Figure 1). This raises intriguing questions about the stereochemical course of polyketide chain formation on the respective polyketide synthase (PKS) multienzymes and about the evolution of the respective structural genes. In addition, as with the other acyltetronic acids, there is great interest in understanding how the extensive and unusual cyclisations that follow upon polyketide chain assembly are orchestrated. Future attempts to engineer such pathways, to produce potentially valuable analogues, will require a detailed knowledge of the nature and timing of these ring closures.

The biosynthetic origin of some of the carbon atoms of the tetronate (4-hydroxy-[5*H*]furan-2-one) ring system has not been established either for TMN or TSN.[9] In chlorothricin **1**[10] and versipelostatin,[11] feeding studies with isotopically labelled precursors have suggested that a 3-carbon glycerol-derived precursor is condensed with a polyketide chain that contains a 3-ketoacyl thioester moiety. Recently, the gene clusters for two spirotetronate biosynthetic pathways have been reported.[2,5] The precursor to chlorothricin, from *Streptomyces antibioticus* DSM 40725,[2] is assembled by a modular PKS,[12,13] and the full-length chain is transferred from the PKS by transesterification to an unusual enoylpyruvoyl-acyl carrier protein (ACP) unit. This adduct then undergoes tetronate ring closure and further reactions to generate the core of chlorothricin.[2] The chlorothricin gene cluster[2] contains a gene with significant similarity to the FkbH family of proteins, which catalyze the production of glyceryl-ACP from 1,3-bisphosphoglycerate.[14,15] This suggests that 1,3-bisphosphoglycerate is also the precursor of the nonpolyketide carbons of the tetronic acid ring in chlorothricin.[2] With minor modifications, a similar biosynthetic origin for the tetronate ring of the related spirotetronate kijanimicin has been proposed.[5] Despite the profound overall differences in the final structure between the spirotetronates and TMN, it is attractive to propose a common pathway that leads to the acyltetronic acid moiety.[16] In an alternative hypothesis for TMN biosynthesis, the full-length polyketide would be released from the PKS by transfer to a discrete ACP, which is catalysed by a modified ketosynthase (KS) as in other polyether biosynthetic pathways that have been studied,[17,18] before post-PKS processing is initiated. The detailed sequence and mutational analysis of the TMN gene cluster presented here together pro-

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Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author: details of the bacterial strains and plasmids used in this study, constructs for gene inactivation and complementation, and analysis of mutant strains.

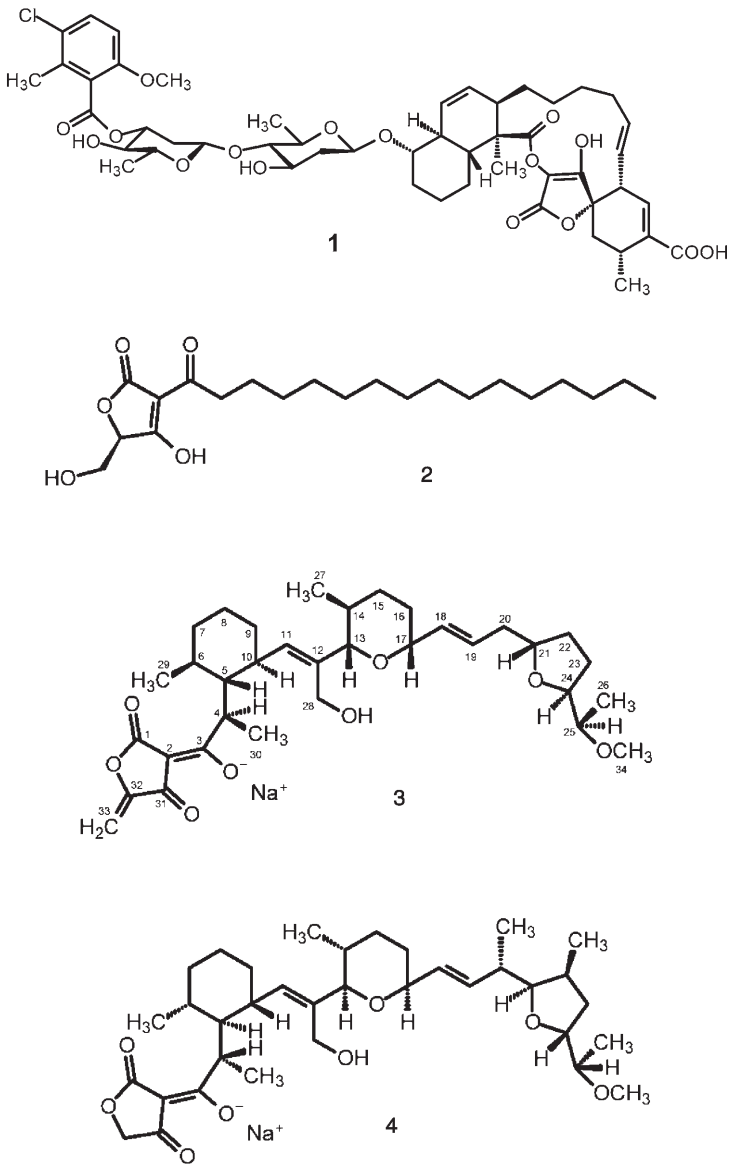


Figure 1. Structure of chlorothricin (**1**), RK-682 (**2**), tetronomycin (**3**) and tetronasin (**4**).

vide a detailed model of the TMN pathway and indicate that the mechanism of polyketide chain release from the PKS is tetronic acid ring formation, as for the spirotetronates. Further support for a common mechanism for tetronate formation comes from our recent identification of a specific glyceryl-acyl-carrier protein as a potential intermediate in supplying additional carbon atoms for the tetronate ring.[19]

Results and Discussion

Cloning, sequence analysis and organisation of the TMN gene cluster from *Streptomyces* sp. NRRL11266

The strategy used to clone the TMN cluster was to screen a cosmid library of genomic DNA from the tetronomycin-producing strain with a hybridisation probe that consisted of a 1.2 kb DNA fragment that encodes part of the KS domain of module 2 of the erythromycin-producing PKS. Since KS domains are the most highly conserved of domains in canonical modular PKSs, this probe is expected to detect all polyketide biosynthetic gene clusters that contain such multienzymes. Positive colonies bearing PKS-related sequences at their ends were subjected to restriction mapping. Initial inspection of the deduced sequence from one region, to which several overlapping cosmids were mapped (Figure S1), suggested that it might contain the TMN gene cluster. Further cosmids were identified by chromosome walking. In total, a region of about 126 kb of contiguous DNA was identified in this way. The overlapping cosmids cySH7, cy119 and cy1XSE5 were shotgun sequenced, together with extensive portions of flanking plasmids, to give a total of 113 234 kb of DNA sequence. Computer-assisted analysis and comparison with genes in public databases revealed 33 open-reading frames (orfs) the positions of which are shown in Figure 2, and the putative functions of which are summarised in Table 1. The sequence data have been deposited in the EMBL/GenBank databases under the accession number AB193609.

The TMN gene cluster is bounded by regulatory genes

Several individual orfs from the putative TMN gene cluster were inactivated, as described in the Experimental Section and in the Supporting Information, and the effects of each inactivation on the production of TMN were studied by LC-MS analysis of crude extracts from the mutant strains (see the Experimental Section, and the Supporting Information). Inactivation of *tmn5*, which encodes a putative LuxR-type transcriptional regulator[20] on the left-hand side of the cluster as shown in Figure 2, completely abolished TMN production. A downstream orf, *tmn6*, would encode a discrete thioesterase (TEII) that is highly typical of modular PKS-containing gene clusters, and might play an activating (but not essential) role by hydrolyzing mis-acylated PKS active sites.[12,13] Consistent with this, specific disruption of *tmn6* lowered the production of TMN to 2–3% of wild-type levels. This result incidentally confirms that the results of disruption of the adjacent *tmn5* were not due to consequent loss of *tmn6* function.

The left-hand flanking sequence (some 10–11 kb; Figure 2) contained additional orfs the products of which appeared not to be related to TMN biosynthesis, these included a secreted endoglucanase, squalene synthetase, pyridoxamine phosphate oxidase and a putative regulatory protein (Y.D., unpublished data). Therefore, for the present, *tmn5* is taken to represent the left-hand boundary of the cluster. On the right-hand side of the cluster, disruption of the actII-orf4-like gene *tmn18*, which is predicted to encode a pathway-specific (SARP) regulatory protein[21] like that found in the CHL cluster (ChlF2), also

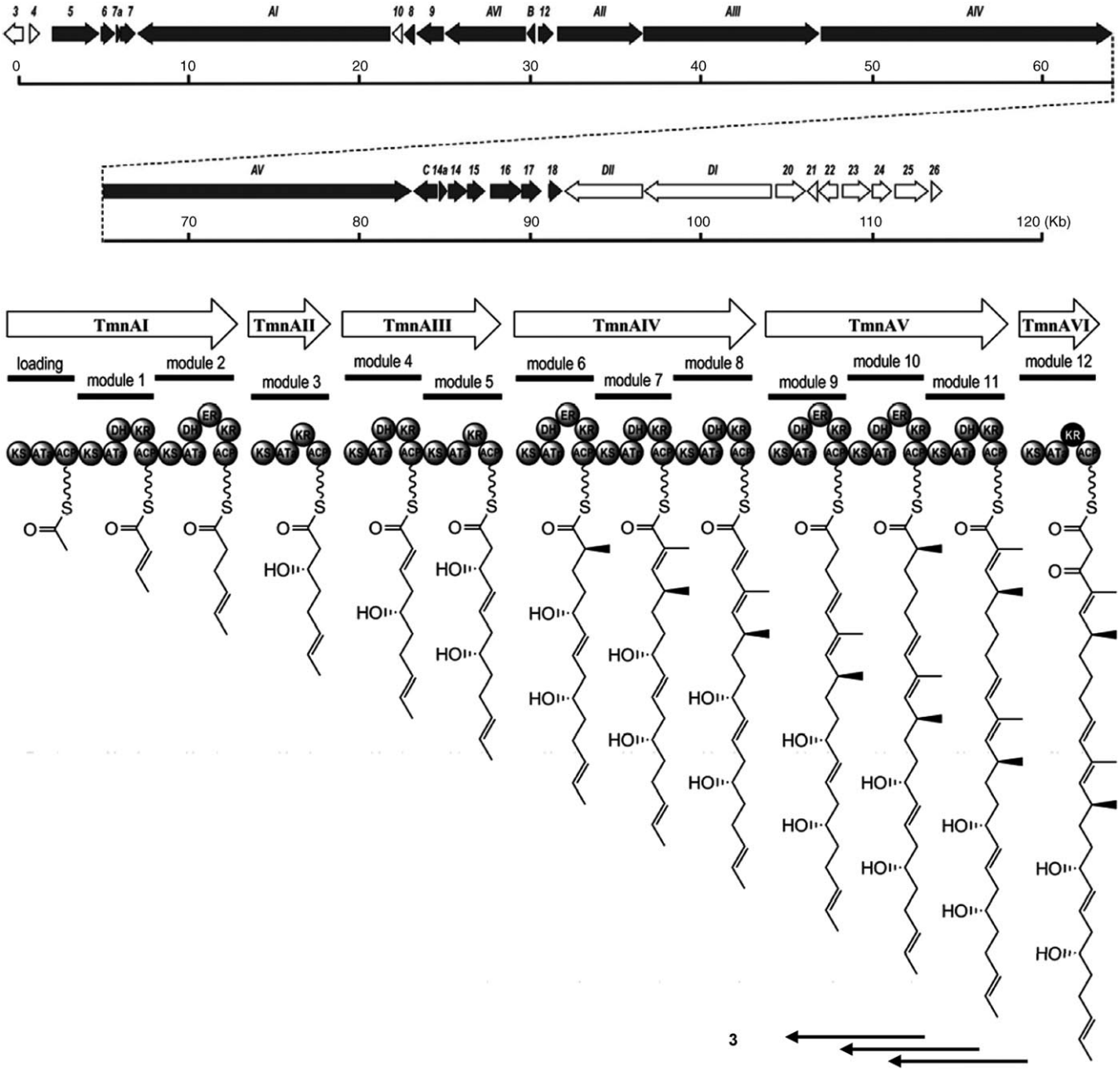


Figure 2. Genetic organization of the tetronomycin biosynthetic gene cluster and polyketide chain extension on the tetronomycin PKS. Those orfs proposed to be involved in TMN biosynthesis are shaded. Their proposed functions are summarized in Table 1; KS, ketosynthase; AT, acyltransferase; DH, dehydratase; ER, enoyl reductase; KR, ketoreductase; ACP, acyl carrier protein. The inactive KR domain of module 12 is shaded.

abolished TMN production. In contrast, the adjacent large genes, which are labelled *tmnDI* and *tmnDII* (Figure 2), are very unlikely to be essential for TMN production, even though they encode PKS-related functions (Table 1) because disruption of *tmnD1* (Supporting Information) had no effect on the production of TMN. These genes, together with the flanking genes *tmn20–25*, appear to be involved in the production of another, as yet unidentified, polyketide secondary metabolite. The PKS-related activities housed by *tmnDI* and *tmnDII* are an unusual mixture: *tmnDI* would encode a conventional loading module of a modular PKS (acyltransferase (AT) and ACP domains), together with domains KS-ACP-KS-ACP-KR that comprise two extension modules typical of a so-called trans-AT modular PKS,[22,23] except that the ketoreductase (KR) domain would normally be found on the N-terminal side of the adjacent ACP domain. Meanwhile, *tmnDII* would encode a more conventional extension module (KS, AT, dehydratase (DH) and ACP domains) and a thioesterase/cyclase chain-termination activity (TE).

Table 1. Deduced function of orfs in the TMN biosynthetic gene cluster.

orf	Size[a]	Homologue and origin	Identity/ similarity [%]	Proposed function
tmn3	300	YP_906523, *Mycobacterium ulcerans* Agy99	48/61	LuxR-family transcriptional regulator
tmn4	155	YP_645372, *Rubrobacter xylanophilus* DSM 9941	74/81	pyridoxamine 5′-phosphate oxidase related, FMN-binding protein
tmn5	910	NP_824078, *Streptomyces avermitilis* MA-4680	32/48	LuxR-family transcriptional regulator
tmn6	263	NP_821582, *Streptomyces avermitilis* MA-4680	56/68	type II thioesterase
tmn7a	75	AAZ77704(ChlD2), *Streptomyces antibioticus*	42/53	acyl-carrier protein
tmn7	292	AAZ77705(ChlD3), *Streptomyces antibioticus*	60/74	pyruvate/2-oxoglutarate dehydrogenase
tmnA1	4872	CAD55506, *Streptomyces coelicolor* A3(2)	52/63	PKS: loading module (KS-Q, ATa, ACP); module 1 (KS, ATa, DH, KR, ACP); module 2 (KS, ATa, DH, ER, KR, ACP)
tmn10	223	ZP_01191507, *Mycobacterium flavescens* PYR-GCK	44/66	DedA-family membrane-associated protein
tmn8	190	AAZ77701(ChlL), *Streptomyces antibioticus*	23/41	putative ribosomal protein L15P
tmn9	500	AAZ77700(ChlE3), *Streptomyces antibioticus*	39/52	FAD-dependent oxygenase
tmnAVI	1574	ABC84471, *Streptomyces violaceusniger*	47/58	PKS: module 12 (KS, ATa, ACP)
tmnB	141	ABC84467, *Streptomyces violaceusniger*	54/82	epoxide hydrolase
tmn12	300	CAA42929, *Saccharopolyspora erythraea* NRRL 2338	42/60	methyltransferase
tmnAII	1658	AAQ94246, *Saccharopolyspora erythraea*	52/63	PKS: module 3 (KS, ATa, KR, ACP)
tmnAIII	3408	AAG23263, *Saccharopolyspora spinosa*	49/60	PKS: module 4 (KS, ATa, DH, KR, ACP); module 5 (KS, ATa, KR, ACP)
tmnAIV	5657	AAZ77696(ChlA3) *Streptomyces antibioticus*	49/60	PKS: module 6 (KS, ATp, DH, ER, KR, ACP); module 7 (KS, ATp, DH, KR, ACP); module 8 (KS, ATa, DH, KR, ACP)
tmnAV	5963	ABB86408, *Streptomyces hygroscopicus* subsp. *duamyceticus*	49/62	PKS: module 9 (KS, ATa, DH, ER, KR, ACP); module 10 (KS, ATp, DH, ER, KR, ACP); module 11 (KS, ATp, DH, KR, ACP)
tmnC	473	ABC84466, *Streptomyces violaceusniger*	50/65	epoxidase
tmn14a	80	CAA82742, *Rhodococcus fascians*	45/57	ferredoxin
tmn14	400	AAT45294, *Streptomyces tubercidicus*	51/65	P450 monooxygenase
tmn15	346	AAZ77702(ChlM), *Streptomyces antibioticus*	63/76	3-oxoacyl-ACP synthase III
tmn16	633	AAZ77703(ChlD1), *Streptomyces antibioticus*	58/70	phosphatase and glyceryl transferase
tmn17	345	AAZ77706(ChlD4), *Streptomyces antibioticus*	48/61	putative hydrolase or acyltransferase
tmn18	256	AAZ77687(ChlF2), *Streptomyces antibioticus*	58/71	putative pathway-specific activator
tmnDII	1508	CAD60099, *Anabaena circinalis* 90	31/47	PKS: (KS, AT, DH, ACP, TE)
tmnD1	2488	YP_632115, *Myxococcus xanthus* DK 1622	37/52	PKS: (ATa, ACP, KS, ACP, KS, ACP, KR)
tmn20	584	NP_820905, *Coxiella burnetii* RSA493	36/53	acyltransferase-family protein
tmn21	232	CAE51169, *Streptomyces resistomycificus*	38/49	TetR-family transcriptional regulator
tmn22	367	YP_250094, *Corynebacterium jeikeium* K411	32/46	hypothetical membrane protein
tmn23	530	AAZ94406, *Streptomyces neyagawaensis*	69/79	putative phosphoesterase
tmn24	415	CAE13512, *Photorhabdus luminescens* subsp. *laumondii* TTO1	35/53	putative ATP-dependent carboxylate-amine ligase domain protein
tmn25	663			unknown protein
tmn26	235			unknown protein

[a] Numbers refer to amino acid residues.

Biosynthesis of the polyketide backbone of TMN

Six genes in the TMN cluster, *tmnAI–tmnAVI*, encode the modular PKS that assembles the polyketide chain of TMN (Figure 2). Detailed analysis of the constituent enzymatic domains (Table 1) confirmed the presence of a loading module and of twelve extension modules, but with no evidence for a C-terminal polyketide chain-releasing enzyme. In all other polyether clusters that have been sequenced, genes (*acpX*, *ksX*) have been found whose products assure an alternative mechanism of chain release onto a discrete ACP, where oxidative cyclisation then takes place.[17] However, no equivalent genes are present in the TMN cluster; this suggests a different mode of chain release from the TMN PKS.

The linear pentaene polyketide structure of the product predicted by examination of the individual modules in the PKS ac-

cords exactly with that expected to give rise to TMN, assuming the normal co-linear functioning of a canonical modular PKS.[24] In particular, the two carbons added by the action of the last module, module 12, appear to provide two of the ring carbons of the tetronic acid ring. This is consistent with precursor feeding experiments previously conducted on TSN, when these carbons were found to be labelled with ^{13}C-acetate.[25] More exotic mechanisms, in which a highly unusual extender unit is added by PKS module 12, are excluded, because inspection of the specificity motifs of the acyltransferase (AT) domain of this module[26–28] (Figure 3) shows clearly that malonyl-CoA is the preferred substrate to provide the extender unit at this position. Curiously, the AT domain of extension module 3 differs significantly in its sequence from all the other AT domains (Figure 3), and might at first glance have been taken to accept methylmalonyl-CoA even though it is, in fact, specific for malonyl-CoA. Any misincorporation of propionate instead of acetate at this position would have given rise to a homologue of tetronomycin, and LC-MS of crude extracts showed no evidence of the presence of such a compound (Supporting Information).

Analysis of the ketoreductase (KR) domains revealed that KR domain of module 12 lacks several essential active site residues[29] (Figure 3); this is consistent with the production of a β-ketoacyl thioester as the full-length product of the PKS. Certain sequence motifs at KR active sites[29,31] of modular PKSs have been shown to correlate with the stereochemical course of the reduction, and the KR domains of TMN modules 3 and 5 differ in these sequence motifs from those at the active sites of the other nine active KR domains (Figure 3); this is in full agreement with the alcohol configurations at C-17 and C-21 of the product being as shown in Figure 2.

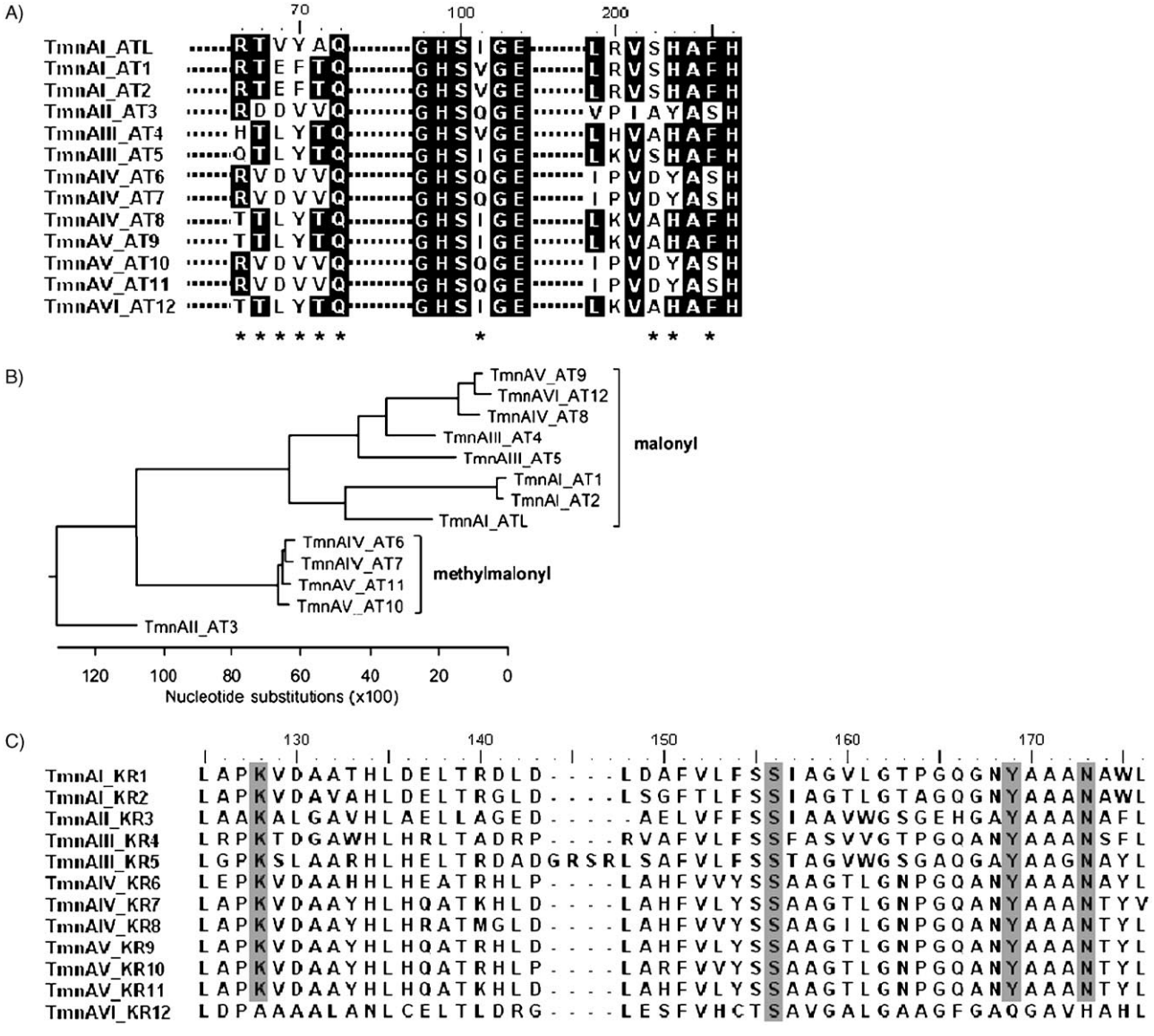


Figure 3. Multiple sequence alignment of the AT and KR domains from the tetronomycin PKS. A) Alignment of conserved motifs of AT domains from the TMN PKS modules. Asterisks indicate the conserved amino acid residues that determine the substrate specificity. B) Phylogenetic analysis of AT domains. The number of amino acid substitutions is proportional to the length of the horizontal lines. C) Alignment of the KR domains from the TMN PKS modules.

Origin of the three-carbon unit required for tetronic acid ring formation

Given that they each contain an acyltetronic acid moiety in their structures, it is significant that seven non-PKS orfs in the TMN cluster (*tmn7*, *7a*, *8* and *9*; *tmn15–17*) show high sequence similarity to genes in the chlorothricin (CHL) biosynthetic gene cluster[2] (Table 1). Most of these orfs also have counterparts in the recently reported kijanimicin gene cluster (for which the sequence is not yet publicly available).[5] In particular, *tmn15–17* resemble a set of adjacent orfs in the CHL gene cluster (Figure 4). For example, both *chlD1* and *tmn16* (albeit much more similar to each other) are predicted to encode a protein that is similar to authentic FkbH-family proteins. Members of this protein family have recently been shown to catalyze the conversion of 1,3-bisphosphoglycerate into glyceryl-ACP, as the first step in production of methoxymalonate and hydroxymalonate extender units for polyketide biosynthesis.[31] The orf *tmn7a* encodes an ACP, similar to ChlD2, which could serve as the substrate for this reaction to provide glyceryl-ACP for TMN biosynthesis. We have separately obtained experimental evidence that Tmn16 catalyses the production of glyceryl-Tmn7 protein from 1,3-bisphosphoglycerate in vitro, which strengthens this hypothesis.[19] To confirm a potential role for Tmn16 in provision of the three-carbon unit of the tetronate ring of TMN, in-frame deletion of *tmn16* was undertaken (Experimental Section). In the resulting mutant, TMN biosynthesis was found to be completely abolished. Production of TMN could be restored to normal levels by transformation of the mutant strain with a plasmid that bears a copy of the *tmn16* gene, which is expressed from its own promoter (Figure 5).

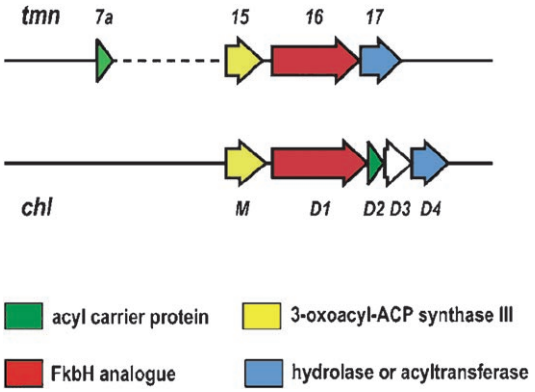


Figure 4. Comparison between *chl* and *tmn* gene cassettes implicated in tetronic acid ring formation.

A minimal mechanism for the formation of the tetronic acid ring in TMN, starting from glyceryl-ACP and the polyketide β-ketoacylthioester **5**, requires formation of both a C–C and a C–

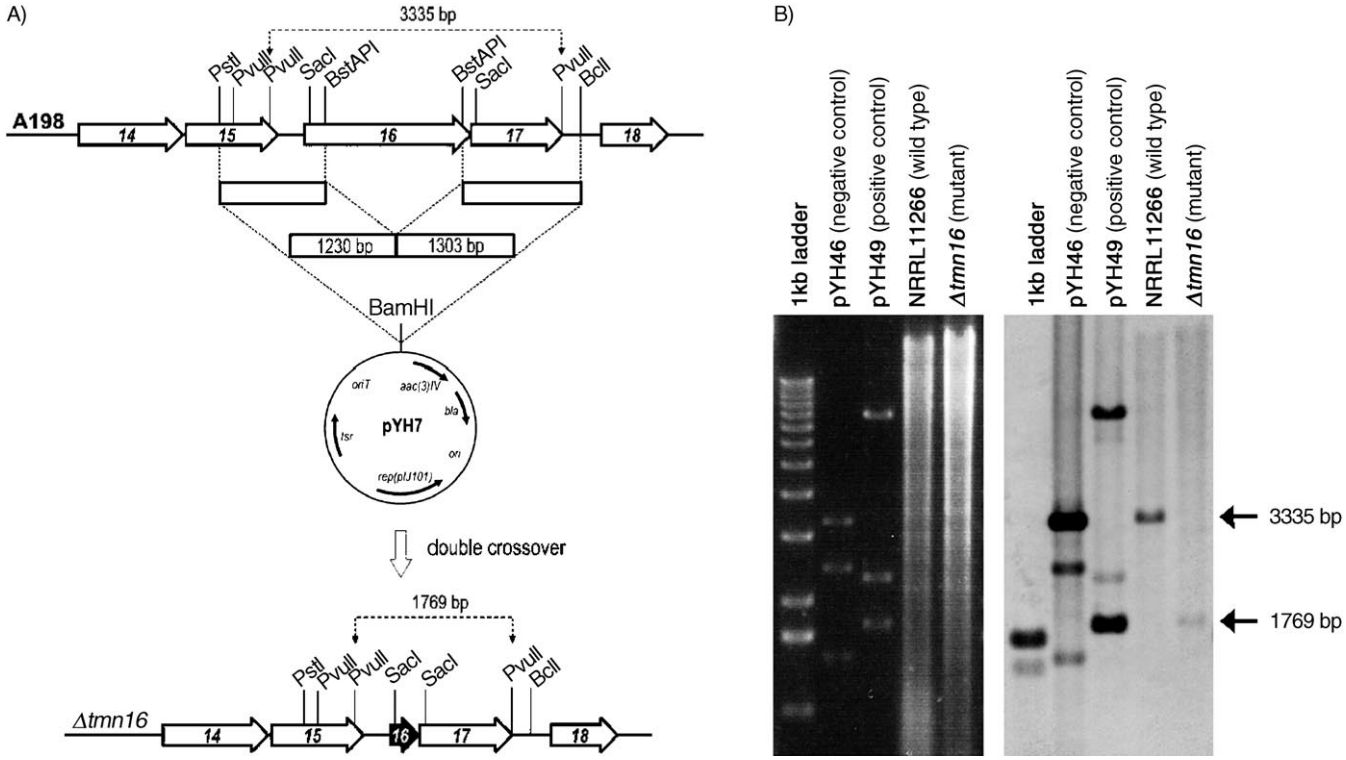
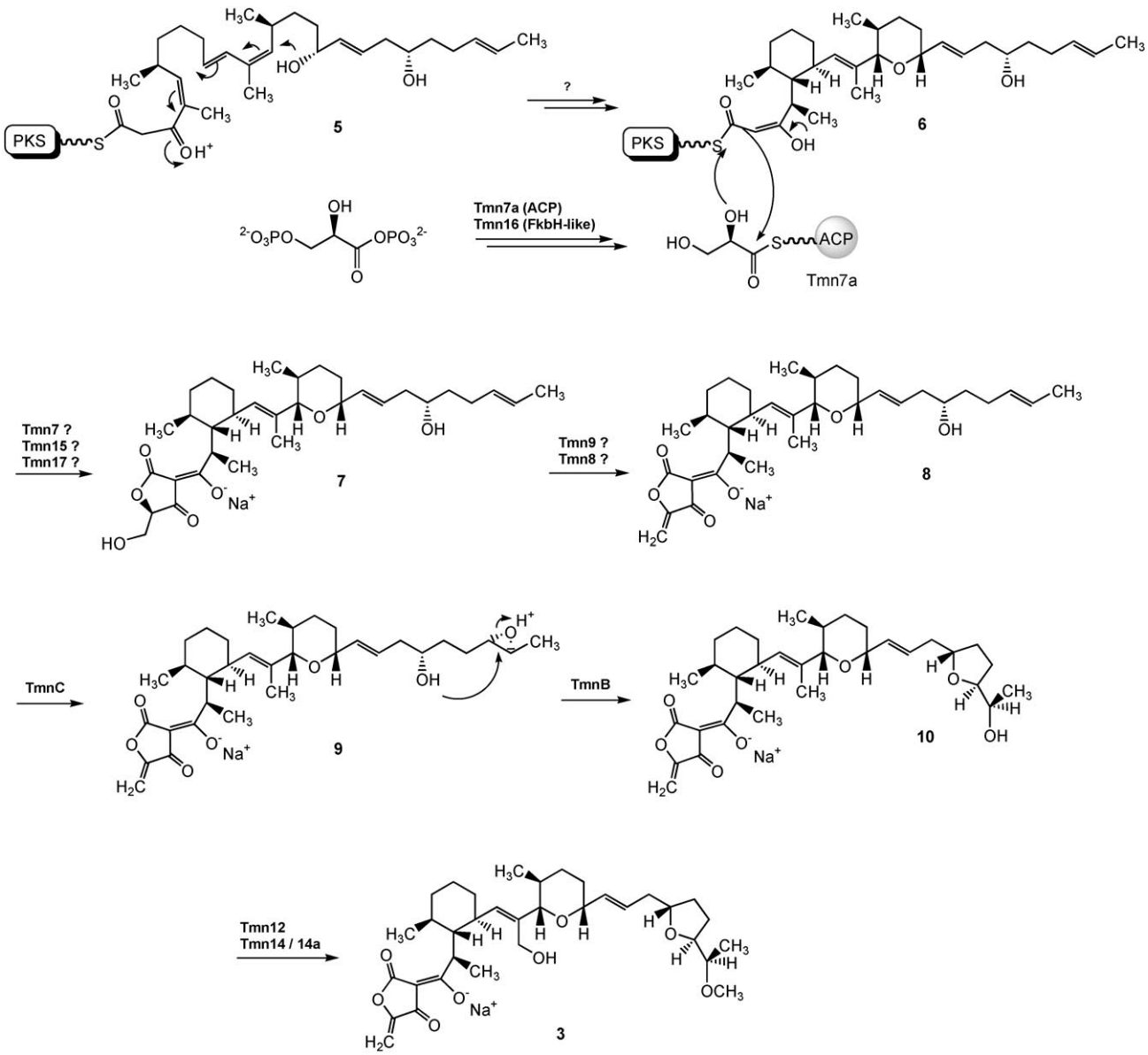


Figure 5. In-frame deletion of *tmn16*. A) Schematic representation of in-frame deletion in *tmn16* by using shuttle vector pYH7. The numbers between two PvuII sites represent the expected size of the fragments in *tmn* wild-type and *Δtmn16* mutant chromosomal DNA after digestion with PvuII and hybridisation to the probe (a 1805 bp SacI fragment that was obtained by digestion of plasmid pYH46—an intermediate construct that contained a complete *tmn16* gene). The sizes of the arms used for homologous recombination between construct and chromosome are indicated. B) Confirmation of *Δtmn16* by Southern blot analysis. All DNA samples were digested with PvuII. Plasmid pYH49 and pYH46 were used as positive and negative controls for the corresponding fragment in wild type and mutant, respectively.



Scheme 1. Proposed pathway for tetronomycin biosynthesis.

O bond, and dehydration to form the exocyclic methylene group. In the mechanism shown in Scheme 1, the relative timing of C–C and C–O bond formation is left undefined. Formation of the C–O bond, by attack of the primary hydroxyl group of the glyceryl moiety on the polyketide acyl thioester, is an acyltransferase/thioesterase-catalysed reaction that would release the polyketide chain from the PKS multienzyme. One candidate for the enzyme that could catalyse this reaction is Tmn7. When the *tmn7* gene was specifically disrupted, TMN production was abolished. It has been proposed for CHL biosynthesis[2] that the counterpart to Tmn7 (ChlD3) is a dehydratase enzyme that acts on glyceryl-ACP to form an enoylpyruvoyl–ACP intermediate. However, such an intermediate would rapidly tautomerize to pyruvoyl–ACP, as in the formation of the lactoyl moiety in the biosynthesis of bryostatin[32] and in lankacidinol[33] biosynthesis. Re-examination of the sequence similarity of ChlD3 and Tmn7 (61% identical, 73% similar to each other) to known enzymes suggests that these gene products might indeed function as an acyltransferase, with an acyl-CoA (or similar thioester substrate) as the acyl donor. Both bear a significant sequence resemblance to the C-terminal acyltransferase domain of the E2 core enzyme of authentic 2-oxoacid dehydrogenases (Table 1), including the conserved acyltransferase active site residues threonine (serine) and histidine.[34] The KijE protein in kijanimicin biosynthesis, which shows homology in its N-terminal domain to Tmn7[5] has also been canvassed as an acyltransferase, but is proposed instead to convert glyceryl–ACP to glyceryl–CoA, which is then the substrate for tetronate ring formation. Further work will be required to test these ideas. An alternative candidate for the acyltransferase that catalyses tetronic acid C–O bond formation (Scheme 1) is Tmn17, which, like its counterparts ChlD4

from the CHL gene cluster and the C-terminal domain of KijE from the kijanimicin cluster, shows significant sequence similarity to α/β-hydrolase proteins. Specific gene disruption of *tmn17* was also found to lead to complete loss of polyketide production, as was previously found for disruption of *chlD4* in CHL biosynthesis.[2] In the extensive 3′-untranslated region downstream of *tmn17* there is a predicted terminator structure, so it is unlikely that this effect is a polar one on the adjacent activator gene *tmn18*.

The product of each of the genes Tmn15, ChlM and KijM from the respective gene clusters is predicted to resemble ketoacyl-ACP synthase III (KSIII) or FabH-like enzymes, which catalyze the first condensation step in fatty acid biosynthesis in bacteria. Apparently *chlM* is not essential for CHL biosynthesis, because disruption of *chlM* did not ablate CHL biosynthesis.[2] In contrast, in the present study, when *tmn15* was inactivated by gene disruption, the resulting mutant did not produce TMN. This gene therefore remains a candidate to catalyse the formation of the C–C bond in the tetronic acid moiety of TMN (Scheme 1). It might be argued that the effect of *tmn15* inactivation actually reflects loss of function of a downstream gene, but this can be ruled out because in the above-described complementation experiment with *tmn16* this gene was well expressed from its own promoter, and was not dependent on expression from the *tmn15* promoter. The CHL and TMN clusters contain a conserved orf (*tmn9* and *chlE3*, respectively) that is predicted to encode a flavin-linked oxygenase, which is adjacent to a second conserved orf (*tmn8* and *chlL*, respectively) of unknown function. Disruption of *tmn9* was also found to abolish TMN production, but further work will be needed to elucidate its precise role in the biosynthetic pathway. The counterpart *kijA* in kijanimicin biosynthesis has been proposed to catalyse a dehydration step by arguing from the ability of the related flavin-dependent oxygenase JadH to catalyse in vitro a formally similar dehydration step in the formation of the aromatic polyketide, jadomycin.[35]

The post-PKS steps of TMN biosynthesis

The conversion of the hypothetical linear pentaene intermediate **5** into TMN requires the formation of a total of four rings: the tetronic acid ring; a carbocyclic and a tetrahydropyran ring; and a tetrahydrofuran ring; together with specific *O*-methylation and hydroxylation steps to generate TMN. The exact order of these processes remains to be unravelled, and there are no genes in the cluster whose products can as yet be specifically identified as participating in the closure of the tetrahydropyran and carbocyclic rings, but for illustration the mechanism of Scheme 1 shows initial concerted closure of the cyclohexane and pyran rings, in a possibly metal-assisted process,[36] to give **6**. Formation of the tetronate ring follows, with participation of glyceryl-Tmn7A (ACP; catalysed by the FabH-like Tmn15 and either Tmn7 or Tmn17) to give **7**, followed by dehydration to give **8**. The genes *tmnC* and *tmnB* closely resemble genes in other polyketide biosynthetic gene clusters whose products are, respectively, epoxidases and ring-opening epoxide hydrolases,[17,18,37,38] and TmnC and TmnB are therefore likely to govern the regiospecific and stereospecific oxidative cyclisation of **8** into **10** that gives rise to the tetrahydrofuran ring of TMN. The products of the genes *tmn14*/*tmn14a* (which strongly resemble authentic cytochrome P450 hydroxylases and their cognate ferredoxins) and *tmn12* (which strongly resembles *S*-adenosylmethionine-dependent methyltransferases) are predicted to catalyse, respectively, the specific hydroxylation at C-28 and the methyl transfer to the C-25 hydroxyl group, probably as the final steps in the pathway.

The biosynthesis of polyketide acyltetronates such as the antibiotic tetronomycin involves a particularly complex set of stereospecific transformations. Chain assembly and subsequent cyclisation steps appear to be tightly coupled, because the disruption of specific genes that are likely to be involved in tetronate ring formation did not lead to accumulation of detectable intermediates. Nevertheless, the insights afforded by our characterisation of the tetronomycin gene cluster open the way to the future deconvolution of the nature and timing of each step in the pathway, and of the determinants of stereochemical control. In particular, they provide evidence for polyketide chain release via formation of a tetronic acid ring. The ring is created by annealing a glyceryl-thioester unit, which is derived from 1,3-bisphosphoglycerate, onto a full-length linear polyketide,[19] as previously suggested for spirotetronate biosynthesis.[2,5] In principle, a very similar reaction sequence could also account for the production of the tetronate ring of tetronasin (**4**). In this case, additional enzyme-catalysed steps that lead to the loss of the side-chain carbon atom from the tetronate moiety would be required. Alternatively, the tetronate ring of tetronasin might be formed directly by annealing an unusual glycolyl-thioester (C-2) intermediate onto the full-length polyketide. Experiments to resolve this question are now in progress.

Experimental Section

Bacterial strains, plasmids and DNA manipulation: Bacterial strains and plasmids used during this study are summarised in Table S1. DNA manipulations were performed by using standard procedures for *E. coli*[39] and *Streptomyces*.[40]

Construction and screening of genomic cosmid library: A genomic library of *Streptomyces* sp. NRRL11266 was constructed in the cosmid vector SuperCos 1 (Stratagene). The genomic DNA was partially digested with BamHI, dephosphorylated by alkaline phosphatase SAP and ligated to the prepared SuperCos 1 vector without fractionation. *E. coli* XL1-Blue MR strain and Gigapack III XL packaging extract (Stratagene) were used for library construction according to the manufacturer's instructions. About 2000 colonies were screened by hybridization with a DIG-labelled PKS probe, which is a 1.2 kb conserved fragment of the erythromycin KS2 domain. Thirty positive colonies were obtained and analysed by restriction enzyme digestion.

Sequencing and annotation of the tetronomycin biosynthetic gene cluster: The overlapping cosmids cySH7, cy119, cy1XSE5, and plasmids pSTL, pKKP1, pMR from a pUC18-based plasmid library were identified by chromosome walking. Each cosmid or plasmid was sequenced by shotgun sequencing of a subclone library that consisted of 1.5–2.0 kb fragments (obtained through partial diges-

tion with Sau3AI) that were cloned in pHSG397 (Supporting Information). DNA sequencing was carried out with an ABI Prism 3700 DNA Analyzer automated sequencer (Applied Biosystems). The raw sequence data were processed and assembled with the Phred/Phrap/Consed software package (http://www.phrap.org). The annotation analysis of the sequence data was performed through database comparison with the BLAST search tools on the server of the National Center for Biotechnology Information, Bethesda, Maryland (http://www.ncbi.nlm.nih.gov).

Culture conditions: *Streptomyces* sp. NRRL11266 wild-type and mutants were grown in TSBY liquid medium (3% tryptone soy broth, 10.3% sucrose, 0.5% yeast extract) for isolating chromosomal DNA, and SFM solid medium (2% mannitol, 2% soya flour, 2% agar) for tetronomycin production. For liquid culture, the strains were grown at 28 °C and 200 rpm on a rotary incubator and harvested after 2–3 days, and for solid culture, the strains were grown at 28 °C for 7–10 days. *E. coli* strains were cultured in 2TY (1.6% tryptone, 1% yeast extract, 0.5% NaCl) medium at 37 °C with the antibiotic selection at a final concentration of 100 $\mu g\,mL^{-1}$ carbenicillin, 100 $\mu g\,mL^{-1}$ hygromycin B, 50 $\mu g\,mL^{-1}$ apramycin, 25 $\mu g\,mL^{-1}$ chloramphenicol or 25 $\mu g\,mL^{-1}$ nalidixic acid as appropriate.

Gene disruption and complementation: The constructs for gene disruption and complementation used in this study are summarised in the Supporting Information.

The constructs were introduced into *Streptomyces* sp. NRRL11266 (wild type) by conjugation by using donor strain ET12657/pUZ8002 on SFM plates. After incubating at 30 °C for 18 h, exconjugants were selected with water (1 mL) that contained apramycin (50 μg) and nalidixic acid (25 mg). Single colonies from this plate were transferred to an SFM plate that contained apramycin (50 $\mu g\,mL^{-1}$) to double check for antibiotic resistance. To screen the double-cross-over mutants, single colonies from the double-check plate were patched onto SFM plates that contained apramycin (50 $\mu g\,mL^{-1}$) and hygromycin B (100 $\mu g\,mL^{-1}$), respectively. Candidate clones with the correct phenotype ($Apr^R Hyg^S$) were used for further identification by PCR, sequencing or Southern blot analysis (Supporting Information).

For complementation of the in-frame mutant strain *Δtmn16*, a pSET152-derived construct, pYH93, was used, which carries the complete *tmn16* gene with its natural promoter (Supporting Information). The complementation plasmids were introduced into *Δtmn16* by conjugation, and exconjugants were selected with apramycin. Resistant colonies were confirmed by PCR and sequencing.

Production, isolation and analysis of tetronomycin: To assess tetronomycin production by *Streptomyces* sp. NRRL11266 (wild type) and its mutants, a plate that contained SFM solid medium (20 mL) was inoculated and grown as described above. After 7–10 days, a whole plate culture was extracted with ethyl acetate (50 mL). The organic phase was evaporated to dryness under reduced pressure by using a Speed-Vac. The residue was redissolved in methanol (800 μL) and clarified by centrifugation, then subjected to liquid chromatography–mass spectrometry (LC–MS) analysis. On-line LC–MS, LC–MS/MS and LC–MS^3 analysis were carried out with an LCQ mass spectrometer (Thermo Finnigan) by using positive-mode electrospray ionization. The LCQ was coupled to an HP 1100 LC (Agilent) that was fitted with a Prodigy C18 column (5 μm, 4.6× 250 mm; Phenomenex, Macclesfield, UK) equilibrated with ammonium acetate (20 mM) and methanol. Samples were eluted by using a gradient of 5–75% methanol over 5 min, then 75–95% methanol over 25 min. The mass spectrometer was set to full-scan (from *m/z* 150 to 1500), MS/MS and MS^3 modes with normalized collision energy of 35%.

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