Chemistry & Biology
Article

Heterologous Expression and Genetic Engineering of the Tubulysin Biosynthetic Gene Cluster Using Red/ET Recombineering and Inactivation Mutagenesis

Yi Chai,[1,4] Shiping Shan,[1,3,4] Kira J. Weissman,[1,5] Shengbiao Hu,[1,3] Youming Zhang,[2] and Rolf Müller[1,*]
[1]Helmholtz Institute for Pharmaceutical Research Saarland, Helmholtz Centre for Infection Research, Department of Pharmaceutical Biotechnology
[2]Research & Development, Gene Bridges GmbH
Saarland University, Saarbrücken 66041, Germany
[3]College of Life Science, Key Laboratory of Microbial Molecular Biology of Hunan Province, Hunan Normal University, Changsha 410081, Hunan Province, People's Republic of China
[4]These authors contributed equally to this work
[5]Present address: Molecular and Structural Enzymology Group, UMR 7214 CNRS-UHP:AREMS, Henri Poincaré University, Nancy I, Faculté de Médecine, Batiment Biopôle, 9 Avenue de la Forêt de Haye, B.P. 184, 54506 Vandoeuvre-Lès-Nancy, France
*Correspondence: rom@mx.uni-saarland.de
DOI 10.1016/j.chembiol.2012.01.007

SUMMARY

Although the tubulysin (tub) biosynthetic gene cluster has been located in two myxobacterial strains, it appears in both cases to be incomplete as obvious candidates for acyl transfer and oxidation functions are lacking. Here, we report the engineering of a heterologous expression system for the tub biosynthetic pathway from strain *Cystobacter* sp. SBCb004. The entire tub core cluster was reconstituted from two cosmids using Red/ET recombineering and heterologous expression achieved in strains *Pseudomonas putida* and *Myxococcus xanthus*. Availability of the heterologous expression system and the natural producer strain SBCb004 provided a platform for the functional investigation of various biosynthetic genes by targeted inactivation. In addition, BLAST analysis of SBCb004 genome data was used to identify multiple candidate monooxygenases, whose involvement in tubulysin assembly was evaluated using a combination of knockout mutagenesis and heterologous expression.

INTRODUCTION

The tubulysins are a large family of highly cytotoxic natural products that have attracted considerable interest from both the academic and industrial sectors. These secondary metabolites are active in the low picomolar range against a wide range of cancer cell lines, including multi-drug-resistant tumors (Dömling and Richter, 2005), and they also exhibit antiangiogenic properties (Kaur et al., 2006). Furthermore, their growth inhibition potential exceeds that of the anticancer drugs epothilone, vinblastine, and taxol, by 20- to 1,000-fold (Steinmetz et al., 2004). Mode-of action studies have demonstrated that their anticancer effects derive from disruption of microtubules assembly (Khalil et al., 2006). Together, these characteristics make the tubulysins promising candidates for development as antineoplastic agents.

Recently, the tubulysin family was expanded to encompass 33 unique compounds (**1**–**33**; Figure S1 available online) from myxobacterial strains *Angiococcus disciformis* An d48, *Archangium gephyra* Ar 315, and *Cystobacter* sp. SBCb004 (Sasse et al., 2000; Steinmetz et al., 2004; Ullrich et al., 2009; Chai et al., 2010). The shared linear core of the metabolites (referred to as pretubulysin) comprises five amino acids (*N*-methyl pipecolic acid, isoleucine, valine, cysteine, and phenylalanine or tyrosine) and two units of acetate. The molecules differ in the extent of reductive processing and methylation that occurs during biosynthesis of the core structure by a hybrid polyketide synthase (PKS)-nonribosomal peptide synthetase (NRPS) "assembly line," as well as post-assembly line oxidation and acylation reactions. Interestingly, the pretubulysins retain good bioactivity (Ullrich et al., 2009). For example, the cytotoxicity of pretubulysin D (**15**) is only 3- and 5-fold lower, respectively, than that of the mature tubulysins A (**1**) and D (**4**). This result prompts interest in generating further simplified tubulysin derivatives by genetic engineering of the biosynthetic gene cluster.

A conserved, ca. 40 kb, tub biosynthetic gene cluster has been identified from both An d48 and SBCb004 (Sandmann et al., 2004; Chai et al., 2010). Analysis of the clusters reveals the anticipated genes for the hybrid NRPS-PKS megaenzymes, as well as six additional genes. Full deployment of all catalytic domains within the NRPS-PKS system yields, respectively, pretubulysins D (**15**) and A (**14**), while the pretubulysin variants arise from the skipping of specific domains (**18**–**33**) or even an entire PKS module (**16**, **17**) (Chai et al., 2010). The mature tubulysins (**1**–**9**) incorporate acyl groups at C11 and C39 (for the compound numbering, see Figure S1, structure 1) generated by post-assembly line oxidation and acylation, while the remaining metabolites (**10**–**13**) include a single acyl group at C39. Among the six accessory genes in the cluster, only the function of *tubZ* as a lysine cyclodeaminase for starter unit biosynthesis has been demonstrated (Khaw et al., 1998; Gatto et al., 2006; Ullrich et al., 2009; Chai et al., 2010). The gene product of *tubA* has been hypothesized to function as an acyl transferase based on its

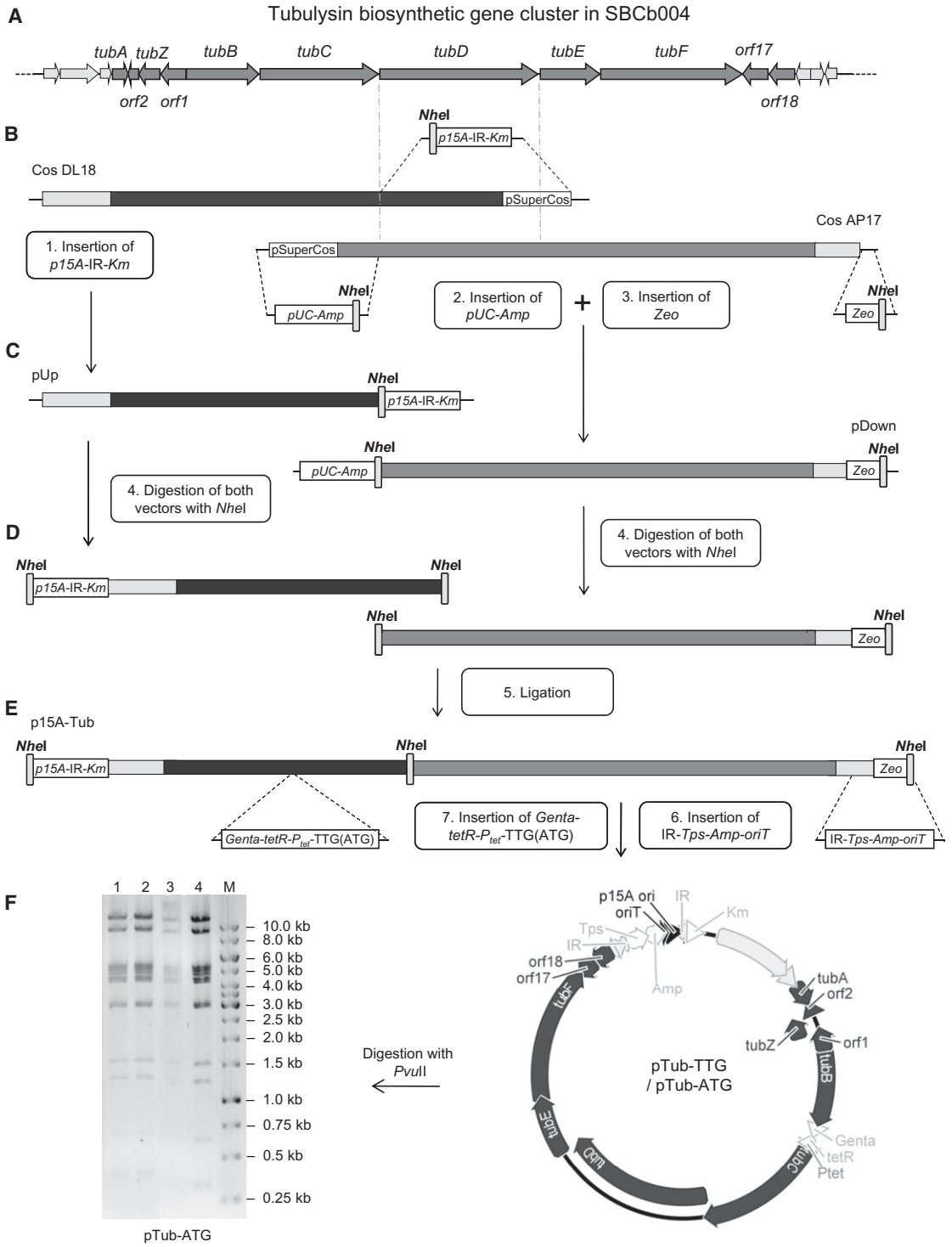

Figure 1. Reconstitution of the Tubulysin Biosynthetic Gene Cluster

(A) The identified tubulysin biosynthetic gene cluster from strain SBCb004.

(B) Two overlapping cosmids Cos DL18 and Cos AP17.

(C) An *NheI-p15A*-IR-*Km* cassette was used to modify Cos DL18 via Red/ET recombineering to replace the pSuperCos backbone as well as the *tubD* region, resulting in plasmid pUp. Two rounds of Red/ET recombination were carried out on Cos AP17, leading to the plasmid pDown. Cassette *pUC-Amp-NheI* was used to exchange the pSuperCos backbone and the *tubC* region, while the inserted zeocin restriction gene was used as the selection marker in the following stitching step.

(D) Digestion of both plasmids with *NheI*. Plasmid pUp was linearized while plasmid pDown was cut into two pieces: a 29 kb fragment (containing the 3′ end of the gene cluster) and a 4 kb fragment.

similarity to NRPS condensation (C) domains (Sandmann et al., 2004), but the cluster does not contain any other clear candidates for additional acyl transferases or oxidases. Furthermore, none of the other four conserved open reading frames (*orf*s) *1*, *2*, *17*, and *18* has an obvious role to play in tubulysin biosynthesis.

We aimed to deepen our understanding of the post-assembly line aspects of tubulysin biosynthesis, as well as to facilitate the engineering of further derivatives for evaluation as drug leads. For this, we endeavored to reconstitute the entire gene cluster using Red/ET recombineering technology. The heterologous expression of natural product biosynthetic pathways by Red/ET recombination is of increasing interest in biotechnology and drug discovery (Zhang et al., 2000; Zhang et al., 1998), as it allows in principle the transfer of large biosynthetic gene clusters from the natural producer strain into other more suitable and easily culturable heterologous hosts. With an expression system in hand, it then becomes possible not only to explore the biosynthesis in detail by genetic engineering in *Escherichia coli* but also to deliberately modify the pathway in order to generative novel compounds (Wenzel and Müller, 2005; Bode and Müller, 2006; Wenzel and Müller, 2007). Over the past decade, several gene clusters have been expressed heterologously by using Red/ET recombination to assemble the complete gene sets, including myxochromide and myxothiazol in *Pseudomonas putida* (*P. putida*) and the myxobacterium *Myxococcus xanthus* (*M. xanthus*) (Wenzel et al., 2005; Gross et al., 2006; Perlova et al., 2006a), phenalinolactone in *Streptomyces* strains (Binz et al., 2008), and epothilones in *Streptomyces coelicolor*, *M. xanthus*, and *E. coli* (Tang et al., 2000; Julien and Shah, 2002; Mutka et al., 2006; Fu et al., 2008).

Here we report the successful reconstitution of the tub biosynthetic gene cluster from two cosmids using Red/ET. Transfer of the cluster into two different hosts, *P. putida* and *M. xanthus*, was facilitated by the introduction of a gene cassette encoding a transposon. The obtained heterologous expression plasmid was then exploited to investigate the roles of genes located at the 5′ end of the cluster, and the results were compared to the equivalent mutations in the natural host *Cystobacter* SBCb004. The choice of this strain in preference to the second native producer, *A. disciformis*, was motivated by the availability of complete genome sequence data, as well as the relative amenability of SBCb004 to cultivation and genetic manipulation. Consistent with our earlier proposal (Sandmann et al., 2004), our results strongly support the role of TubA as one of the missing acyltransferases in the tubulysin pathway. We also attempted to boost tubulysin yields in strain SBCb004 by manipulating the promoter region. Finally, we used BLAST analysis of the sequenced SBCb004 genome to identify seven candidate monooxygenases; results of inactivation of gene *633P1* are consistent with its role as one of the missing tubulysin hydroxylases.

RESULTS AND DISCUSSION

Reconstitution of the Tubulysin Biosynthetic Gene Cluster of SBCb004

The tub biosynthetic gene cluster from strain SBCb004 was found to be located on two cosmids (Cos DL18 and AP17) within a cosmid genomic library (data not shown). Cos DL18 contained the 5′ end of the gene cluster (ending in the middle of *tubD*), while the 3′ end was located on Cos AP17 (starting in the middle of *tubC*) (Figures 1A and 1B); the region of overlap that encompassed the incomplete *tubC* and *tubD* genes was approximately 14 kb. As a first step in reconstituting the cluster, we replaced the pSuperCos cosmid backbone and the incomplete *tubD* gene on Cos DL18 with a cassette containing the p15A replication origin (*p15A*), an inverted repeat (IR), and a kanamycin selection marker (*Km*) flanked by an *Nhe*I site, resulting in plasmid pUp, which harbors the 5′ region of the tub gene cluster from *tubA* to *tubC* (Figure 1C). *p15A* is a low copy origin, which has been found to aid further Red/ET modification steps, the *Nhe*I site facilitated subsequent cluster assembly, and the inserted IR was part of the designed transposition system that enabled efficient integration of the cluster into the host genome. Cos AP17 was also modified by replacement of the pSuperCos backbone as well as the *tubC* fragment with a gene cassette containing the pUC origin of replication (*pUC*) and an ampicillin resistance gene (*Amp*) flanked by an *Nhe*I site (Figure 1C). To enable efficient selection in the subsequent ''stitching'' step, a zeocin resistance gene (*Zeo*) with a flanking *Nhe*I site was introduced at the opposite end of the cosmid to yield plasmid pDown, which harbors the 3′ end of the gene cluster encompassing *tubD* to *orf18* (Figure 1C). Both pUp and pDown were then digested with *Nhe*I (Figure 1D). The resulting 29 kb fragment from pDown was ligated with the linearized pUp, and the ligation mixture was transformed into *E. coli* GB05-red cells (Fu et al., 2008). Screening for zeocin resistance yielded the desired construct p15A-Tub, which harbors the 11 tub biosynthetic genes in the correct orientation (Figure 1E). The genetic organization of the reconstituted tub gene cluster on plasmid p15A-Tub is identical to the parent in SBCb004 with one only exception. The wild-type genes *tubC* and *tubD* overlap by 11 bp, whereas in construct p15A-Tub, they are separated by 29 bp (which includes the introduced *Nhe*I site) (Figure 1E).

A transposition cassette comprising the MycoMar transposase gene (*Tps*), two corresponding IRs, the origin of transfer *oriT*, and an ampicillin resistance gene was designed to enable transfer and integration of the gene cluster into the chromosome of heterologous host strains. The mariner transposon MycoMar is frequently used in Gram-negative hosts for genetic modification. In the heterologous expression of epothilone and myxochromide S (Fu et al., 2008), the MycoMar transposon-mediated transfer of large gene sets was more efficient than homologous

(E) Ligation of linearized pUp and 29 kb digested DNA fragment from pDown to reconstitute the entire tubulysin gene cluster in one plasmid, p15A-Tub.
(F) Insertion of the IR-*Tps*-*Amp*-*oriT* cassette into the construct to generate a transpositional system. Two modification cassettes *Genta*-*tetR*-P_{tet}-TTG *and* *Genta*-*tetR*-P_{tet}-ATG were then inserted in front of gene *tubC*, resulting in two heterologous expression constructs pTub-TTG and pTub-ATG, respectively. The identity of the resulting plasmids was confirmed by *Pvu*II restriction analysis and sequencing. In the DNA gel, M represents the DNA marker and lines 1–4 represent four distinct clones of plasmid pTub-ATG. The size of each band (bp) is as following [from largest to smallest]: 11,890, 9,172, 5,285, 5,025, 4,767, 4,447, 4,250, 3,144, 3,017, 1,547, 1,275, 663, and 372.
All primers used are listed in Table S1.

recombination using a smaller construct. Thus, as a powerful delivery tool for large transgenes, it was used in the tubulysin heterologous expression system to facilitate the transformation and integration of the gene cluster into the genome of host strains. The incorporation of *oriT* was previously shown to be necessary for transposon-mediated integration of plasmids into the chromosome of *P. putida* (Fu et al., 2008). Introduction of the IR-*Tps-Amp-oriT* cassette into construct p15A-Tub (the second IR is already on the plasmid) also enabled the deletion of the zeocin resistance gene and several *orfs* downstream of the cluster (*orf21–orf23*), resulting in plasmid pTps-Tub.

The natural start codon of *tubC* is the rare TTG. As we worried that this would limit the efficiency of expression in the target heterologous hosts (Vervoort et al., 2000), we also aimed to alter the codon using Red/ET recombineering. First, we tried to insert a gentamicin resistance gene (*Genta*) in front of *tubC*, as well as to change the start codon to ATG, but no correct clones were obtained: all of the resulting recombinants incorporated a mutation at the 5′ end of *tubC*. This problem had been encountered previously during attempts to insert a constitutive promoter in front of the myxochromide gene cluster (Y.Z. and R.M., unpublished data). It may be the case that expression of the downstream tub gene cluster (*tubC-tubF*) when driven by the gentamicin constitutive promoter is toxic to the *E. coli* host. Therefore, to stabilize the tubulysin heterologous expression plasmid in *E. coli*, we aimed to insert an inducible promoter in 5′ of *tubC* to regulate the expression of the downstream genes. For this, we generated a gene cassette containing a gentamicin resistance gene and an inducible promoter *tetR*-P_{tet}. The *tetR*-P_{tet} is a tetracycline-based regulatory system that is generally used to selectively control downstream gene expression. It includes a tetracycline resistance gene (*tetR*) (encoding the tetracycline repressor protein TetR) and an 81 bp gene sequence, which harbors bidirectional promoters that overlap with two highly homologous control sequences. In the absence of the inducer tetracycline, TetR protein dimers inhibit the bidirectional promoters by binding to the two control sequences, shutting down the transcription of the following gene/operon under control of P_{tet}. When tetracycline is present, the TetR protein complexes with it in preference to the control region, resulting in expression of the target genes. The cassette was amplified by PCR using primers with short homology arms to enable integration between the *tubB* and *tubC* genes, as well as to modify the start codon; one version contained the original TTG start codon of *tubC*, and the second, a mutant ATG. The resulting fragments were then inserted into pTps-Tub. This strategy yielded the desired plasmids pTub-TTG and pTub-ATG (Figure 1F). In these plasmids, the gentamicin resistance gene and the tetracycline-inducible promoter *tetR*-P_{tet} are located in front of *tubC*, an organization different from that in the wild-type cluster. The integrity of all obtained recombination constructs was verified either by restriction analysis using a diagnostic set of enzymes or by sequencing.

Heterologous Expression of the Tubulysin Gene Cluster in *P. putida*

Both constructs pTub-TTG and pTub-ATG were introduced into the host organism *P. putida* by triparental conjugation. Positive clones were then cultivated in PMM medium in the presence of tetracycline to activate the *tetR*-P_{tet} promoter, and the culture extracts were analyzed by high pressure liquid chromatography-mass spectrometry (HPLC-MS). Two members of the pretubulysin subfamily, pretubululysin A (**14**) and tyrosine pretubulysin A (**16**) (Figure S1), were observed in the extracts of both *P. putida*::pTub-TTG and *P. putida*::pTub-ATG strains, although they were both produced at disappointingly low levels (the yield of pretubulysin A from *P. putida*::pTub-TTG was approximately 0.04 μg/l, and that from *P. putida*::pTub-ATG was 0.2 μg/l) (Figure 2). Nonetheless, this result demonstrates that, as predicted, the core gene cluster contains all genes required to produce the polyketide-nonribosomal peptide skeleton of the tubulysins (Chai et al., 2010; Sandmann et al., 2004). The fact that neither of the products incorporated the acyl groups of the mature tubulysins demonstrated that at least some of the post-assembly line genes are absent from the heterologous expression construct (i.e., one or more acyl transferases might have been present but unable to act due to the absence of a required oxidase). Alternatively, some genes may not be expressed because the promoters are not recognized in the heterologous host. Unexpectedly, we also detected trace amounts of tubulysin A in *P. putida*::pTub-TTG(ATG). A possible explanation for this finding is that another oxidase and/or acyltransferase encoded in the *P. putida* chromosome was able to complement the missing post-assembly line function(s) in tubulysin A biosynthesis. We have previously observed a similar complementation event in strains An d48 and SBCb004: inactivation of the cyclodeaminase gene *tubZ* did not abolish biosynthesis of the tubulysins; instead, the mutant strains generated substantial amounts of pretubulysins D and A as well as small quantities of tubulysin D/A (Ullrich et al., 2009; Chai et al., 2010). This result suggested that the genomes harbored additional cyclodeaminase-encoding genes, and indeed BLAST analysis of the SBCb004 genome revealed two genes with homology to ornithine cyclodeaminases (Altschul et al., 1997). We assume that the *P. putida* genome encodes a number of oxidases and acyltransferases, but that their efficiency toward the tubulysins as substrates is poor, explaining the low yield of tubulysin A from the heterologous expression strain.

L-pipecolic acid (Pip) serves as the starter unit for tubulysin biosynthesis. Reasoning that the Pip supply might limit the overall yields, we attempted to increase tubulysin production by feeding Pip to cultures of *P. putida*::pTub-TTG(ATG). Although racemic D,L-pipecolic acid was used in all the experiments, only the L-isomer is incorporated into the tubulysins (Figure S2) (Chai et al., 2010). As hoped, we obtained a substantial increase in tubulysin titers. For example, the production of pretubulysin A by *P. putida*::pTub-ATG increased from 0.2 μg/l to 1.76 μg/l in the presence of 0.1 mg/ml supplemental Pip. Thus, it appears that the activity of the heterologous TubZ in *P. putida* limits the overall yields from the biosynthetic pathway.

In order to explore the effect of the start codon on tubulysin biosynthesis in the heterologous expression strain, we compared the yields of pretubulysin A from *P. putida*::pTub-TTG and *P. putida*::pTub-ATG. Production by strain *P. putida*::pTub-ATG containing the modified ATG start codon was higher than that incorporating the wild-type TTG (0.2 μg/l vs. 0.04 μg/l). Similarly,

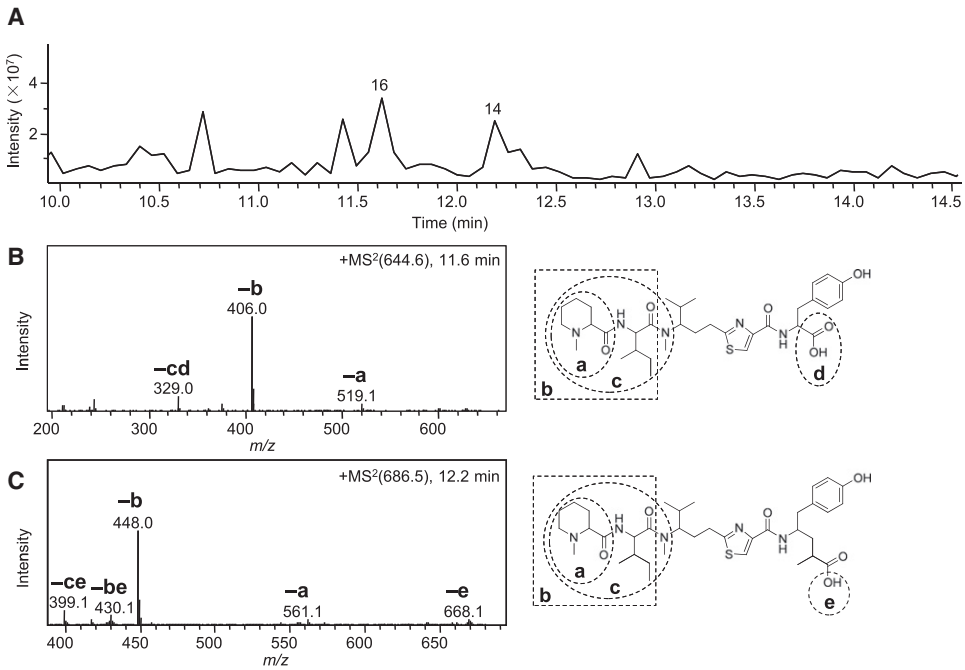

Figure 2. Analysis of Tubulysin Production by Strain *P. putida*::pTub-ATG Grown at 30°C and Supplemented with 0.1 mg/ml Pipecolic Acid

(A) HPLC-MS analysis (base peak chromatogram [BPC]) of *P. putida*::pTub-ATG.

(B) MS^2 fragmentation pattern of tyrosine pretubulysin A (**16**).

(C) MS^2 fragmentation pattern of pretubulysin A (**14**).

In (B) and (C) the mass spectra are labeled to indicate the fragments lost to generate each daughter peak.

in the presence of 0.1 mg/ml supplemental Pip, production of pretubulysin A by the ATG-modified strain was 1.76 μg/l, approximately 3-fold higher than that by the TTG strain (0.66 μg/l). This result supports the idea that the rare TTG codon can inhibit translation of TubC and, ultimately, lower the overall yields of the tubulysins. Thus, optimization of start codons may be a useful general strategy for improving titers from heterologous expression constructs in the future.

During heterologous expression of myxochromide S in *P. putida*, it was noted that the cultivation temperature dramatically affected the product yield: cultivation at 16°C resulted in 1,000-fold more myxochromide S than incubation at 30°C (Wenzel et al., 2005). In order to test the effect of temperature on tubulysin heterologous expression in *P. putida*, we cultivated *P. putida*::pTub-ATG at both 30°C and 16°C. Surprisingly, we obtained the opposite result: under supplementation with 0.1 mg/ml Pip, the yield of pretubulysin A at 30°C (1.76 μg/l) was 2-fold higher than that at 16°C (0.84 μg/l). In the case of myxochromide S, a P_m promoter was introduced in front of the gene cluster in order to boost metabolite production by upregulating expression of the biosynthetic genes. Thus, high expression at 30°C may have resulted in improper folding and/or aggregation of the large PKS and NRPS proteins, so that the overall yield of myxochromides was reduced. In the case of tubulysin, however, expression is under the presumably weak, native promoter(s) from the SBCb004 genome. Thus, the influence of temperature on the *P. putida*::pTub-ATG strain is relatively small and consistent with the general preference of *P. putida* for growth at 30°C.

Heterologous Expression of the Tubulysin Gene Cluster in *M. xanthus*

Given the closer relationship between *M. xanthus* and the native tubulysin producer SBCb004, we anticipated that *M. xanthus* might, despite its inferior growth characteristics, be a better heterologous expression host strain for tub biosynthesis. To test this hypothesis, construct pTub-ATG was transformed into *M. xanthus* DK1622 by electroporation, correct *M. xanthus*::pTub-ATG clones were fermented in CTT medium under tetracycline induction, and the culture extracts were analyzed by HPLC-MS. Expression of the gene cluster in DK1622 produced a similar metabolite profile as *P. putida*::pTub-ATG, but the tubulysins were obtained at higher yield. For example, in the presence of 0.1 mg/ml supplemental Pip, the yield of pretubulysin A from *M. xanthus*::pTub-ATG (0.19 mg/l) was 100-fold higher than that from *P. putida*::pTub-ATG (1.76 μg/l). In addition, because of the higher titers, we were able to detect more tubulysin variants from the *M. xanthus*::pTub-ATG strain via their MS^2 fragmentation pattern during HPLC-MS analysis. These variants included tyrosine pretubulysin A (**16**), *N*-desmethyl 12-keto pretubulysin A (**18**), *N*-desmethyl 12-hydroxy pretubulysin A (**20**), *N*-desmethyl pretubulysin A (**22**), and also trace amounts of tubulysin A (**1**). Again, biosynthesis of tubulysin A is likely due to spontaneous action of oxidase and/or acyltransferase enzymes encoded in the *M. xanthus* DK1622 genome. It is worth mentioning that, although *M. xanthus* yielded more tubulysin, *P. putida* retains several advantages as a heterologous expression host strain, including ease of cultivation and transformation efficiency

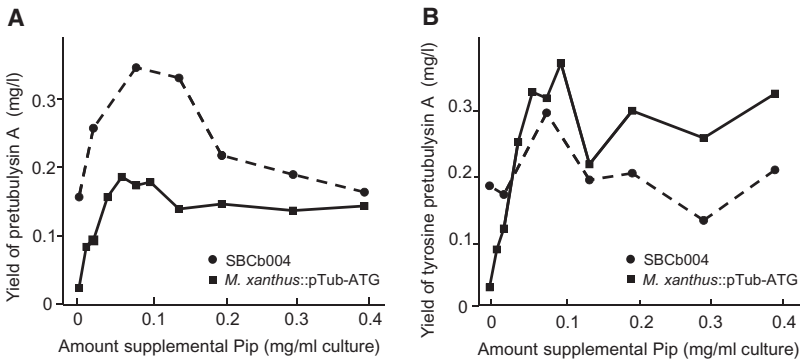

Figure 3. Feeding Experiments with Strain SBCb004, Indicated by a Dashed Line, and Strain *M. xanthus*::pTub-ATG, Indicated by a Solid Line
(A) Yield of pretubulysin A.
(B) Yield of tyrosine pretubulysin A.
See also Figure S2.

(>50-fold higher than *M. xanthus* on average). Thus, it was desirable to try to optimize production titers from this strain.

Supplementation with Pip also increased production of *M. xanthus*::pTub-ATG. In order to quantify the relationship between the Pip supply and pretubulysin production, we fed various amounts of Pip to *M. xanthus*::pTub-ATG. In order to estimate the production yield, we generated a reference curve using synthetic pretubulysin D (**15**) (a kind gift of Professor U. Kazmaier) (Ullrich et al., 2009). Assuming that the ionization efficiency of the derivatives was the same as that for pretubulysin D, the yields of all tubulysins were judged based on their relative peak areas in the HPLC-MS chromatogram in comparison with the standard curve. Four individual clones of *M. xanthus*::pTub-ATG were grown in parallel, and the quantities of the two major products pretubulysin A and tyrosine pretubulysin A were determined from the average of the four values. Yields from the SBCb004 wild-type strain were used as a benchmark (Figure 3). As the integration of the cluster into the *M. xanthus* genome was transposon mediated, the site of integration is likely to vary between randomly selected clones. In the case of *Streptomyces*, it has been observed that integration into different loci can affect the protein expression and production yield (Kuhstoss et al., 1991). However, the four randomly selected clones, which likely represent different integration sites (although this was not directly determined), produced similar amounts of the tubulysins. For example, in the presence of 0.08 mg/ml supplementary Pip, the yields of pretubulysin A from the four clones were 0.176, 0.154, 0.165, and 0.173 mg/l, respectively (average yield, 0.167 ± 0.008 mg/l). A similar result was also observed when this mariner transposon strategy was used to integrate epothilone and myxochromide gene clusters into *M. xanthus* (Fu et al., 2008).

In the case of SBCb004, production of both compounds initially increased during supplementation with Pip (to approximately 0.08 mg/ml) but declined thereafter, suggesting an inhibitory effect on the cells. The yields of both metabolites from *M. xanthus*::pTub-ATG also initially increased with the concentration of Pip, but no further augmentation was seen at concentrations above 1 mg/ml (Figure 3). In addition, in the case of pretubulysin A, the overall yields remained approximately 50% lower than that of supplemented SBCb004, although they were comparable to the nonsupplemented wild-type. These results confirm that the supply of L-pipecolate is limiting to tubulysin biosynthesis in both strains, but equally that supplementation can only boost metabolite quantities to a modest extent. Indeed, a possible reason for the relatively low titers from the heterologous system is that the 29 bp inserted fragment separating the *tubC* and *tubD* genes, which interrupted their coupled translation, may have negatively affected the initiation of *tubD* translation and, therefore, the overall multienzyme stoichiometry. It is worth mentioning that the yields of the tubulysins currently limit further development of the compounds, as neither synthetic nor fermentative approaches have led to sufficient production titers. Only the less potent pretubulysin offers access in reasonable yield via chemical synthesis (Ullrich et al., 2009).

Genetic Modification of the Tubulysin Heterologous Expression System

As noted earlier, the conserved tubulysin biosynthetic gene cluster in both An d48 and SBCb004 strains contains several *orfs* whose functions have been unclear to date. These include the genes *tubA*, *orf2*, and *orf1* at the 5′ end of the gene set. The observation that they share a high degree of mutual sequence homology (56%–78% identity/73%–90% similarity) (Chai et al., 2010) suggests that they play a role in the biosynthesis. The product of gene *tubA* shows similarity to NRPS C domains. Of particular note, a sequence (DHxxxDx) within core motif C3 is closely similar to a proposed signature sequence (HHxxxDG) for enzymes that effect acyl transfer, such as chloramphenicol acyltransferase, and dihyrolipoamide acyltransferase (De Crécy-Lagard et al., 1995). Thus, TubA was identified early on as a candidate to serve as one of the missing acyl transferases in the tubulysin pathway (Sandmann et al., 2004). However, neither *orf1* (predicted to encode an anion transporting ATPase) nor *orf2* (similar to hypothetical proteins) has an obvious role to play in tub biosynthesis. Therefore, to obtain further insights into the potential functions of these genes in the tubulysin pathway, we aimed to inactivate these genes on the pTub-ATG expression construct.

For this, we replaced the target genes by a chloramphenicol resistance gene (*Cm*), using Red/ET recombineering. As genes *orf1*, *tubZ*, and *orf2* are likely to form an operon, deletion of any gene would likely affect the function of all downstream genes due to polar effects. Therefore, in order to obtain the clearest picture from the results, in each case, we deleted the target gene together with any downstream gene(s) (Figure 4). The modified deletion constructs pTubA, pOrf2, pTubZ (deletion of both *tubZ* and *orf2*), and pOrf1 (deletion of the entire *orf1-tubZ-orf2* operon) were screened using low-salt Luria broth (LB) plates containing ampicillin and chloramphenicol and then verified by restriction analysis (Figure S3). The modified constructs were transformed into *M. xanthus* DK1622, and the production profile of positive clones was analyzed by HPLC–MS.

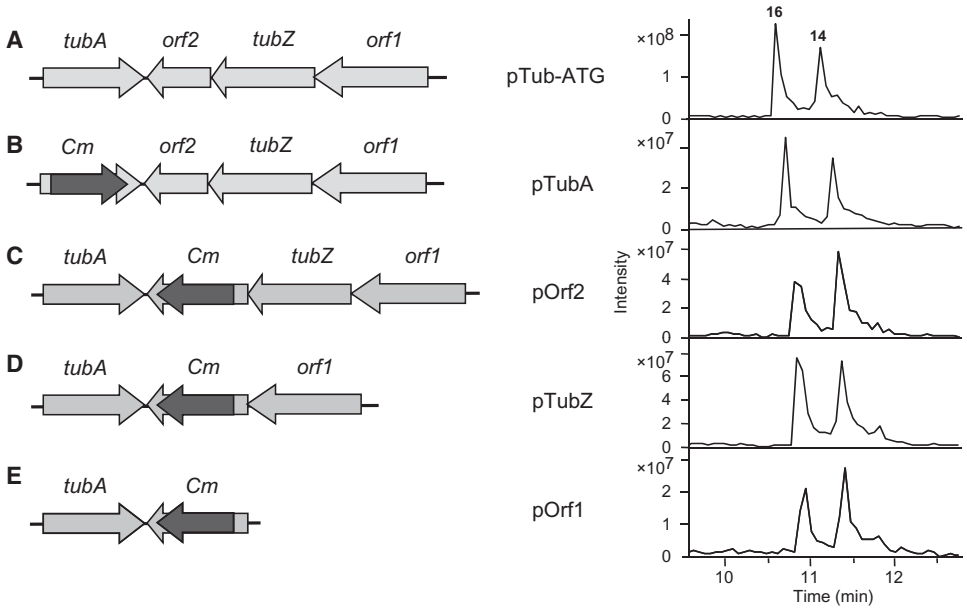

Figure 4. Genetic Modification of the Tubulysin Heterologous Expression System

(Left) Map of the modified region within the deletion constructs.

(Right) The corresponding HPLC-MS analysis of the *M. xanthus* deletion strains in the presence of 0.08 mg/ml pipecolic acid. The extracted ion chromatogram [EIC] at *m/z* 686.5 $[M+H]^+$ (pretubulysin A [**14**]) and *m/z* 644.4 $[M+H]^+$ (tyrosine pretubulysin A [**16**]) is shown.

(A) *M. xanthus*::pTub-ATG strain.

(B) *M. xanthus*::pTubA strain.

(C) *M. xanthus*::pOrf2 strain.

(D) *M. xanthus*::pTubZ strain.

(E) *M. xanthus*::pOrf1 strain.

Construct restriction analysis is shown in Figure S3.

The resulting strains still produced both pretubulysin A and tyrosine pretubulysin A, albeit in reduced yields. For example, when fed with saturating Pip (0.08 mg/ml), the titers of pretubulysin A from all mutants were lower than that from *M. xanthus*::pTub-ATG strain (pTubA 24% relative yield, pOrf2 45%, pTubZ 51%, and pOrf1 18%) (Figure 4). In addition, even with Pip supplementation (0.08 mg/ml), no tubulysin A was detected from these strains. The production of the pretubulysin subfamily compounds in the absence of *orfs 1*, *2*, and *tubA* indicates that these genes are not essential for assembling the tubulysin core structure. However, the reduction in yield relative to that from the pTub-ATG strain shows that they nonetheless contribute to pretubulysin biosynthesis in some manner. It remains formally possible, however, that the significant modification to the cluster architecture caused by the gene inactivations may have had some effect on the expression of the biosynthetic enzymes.

Mutagenesis of the Tubulysin Gene Cluster in Strain SBCb004

In order to confirm these results, we aimed to repeat the gene inactivation experiments in the SBCb004 wild-type strain, as the higher metabolite titers would permit the identification of metabolites that are produced at low yields. For this, we generated insertion knockout mutants of genes *tubA* and *orf1* in strain SBCb004 (Figure S4), as described previously (Chai et al., 2010). Unfortunately, we were unable to obtain an *orf2* knockout mutant due to the small size of the gene (660 bp). By HPLC-MS analysis, mutant SBCb004-*tubA*$^-$ was found to produce a similar amount of pretubulysin A as the wild-type and small amounts of several additional pretubulysin derivatives based on their MS^2 fragmentation patterns, including tyrosine pretubulysin A (**16**), *N*-desmethyl 12-keto pretubulysin A (**18**), *N*-desmethyl 12-hydroxy pretubulysin A (**20**), and the *N*-hydroxymethyl 12-keto pretubulysin A (**32**). However, in no case was acylated tubulysin present in culture extracts (Figure 5). The same result was found for a *tubA* knockout in strain An d48 (data not shown). The fact that no mature tubulysins were observed from any of the *tubA* inactivation mutants in both the heterologous expression strain and the natural producers strongly suggests that TubA serves as an acyltransferase in tubulysin assembly. Nonetheless, the proposed function of TubA remains to be directly demonstrated, for example, in vitro with recombinant protein.

Given the operon structure of genes *orf1*, *tubZ*, and *orf2*, we anticipated polar effects on *tubZ* and *orf2* from disruption of *orf1*, and thus inactivation of *orf1* was assumed to knockout the entire operon. We have shown previously that insertion mutants of *tubZ* in both the An d48 and SBCb004 produce more pretubulysin D and A and substantially less tubulysin D and A than the respective wild-type strains (Ullrich et al., 2009; Chai et al., 2010). The production profile from the *orf1* mutant (SBCb004-*orf1*$^-$) closely resembled that of the *tubZ* knockout strain (Figure 5). This result confirms that strain SBCb004 must contain an additional cyclodeaminase function capable of

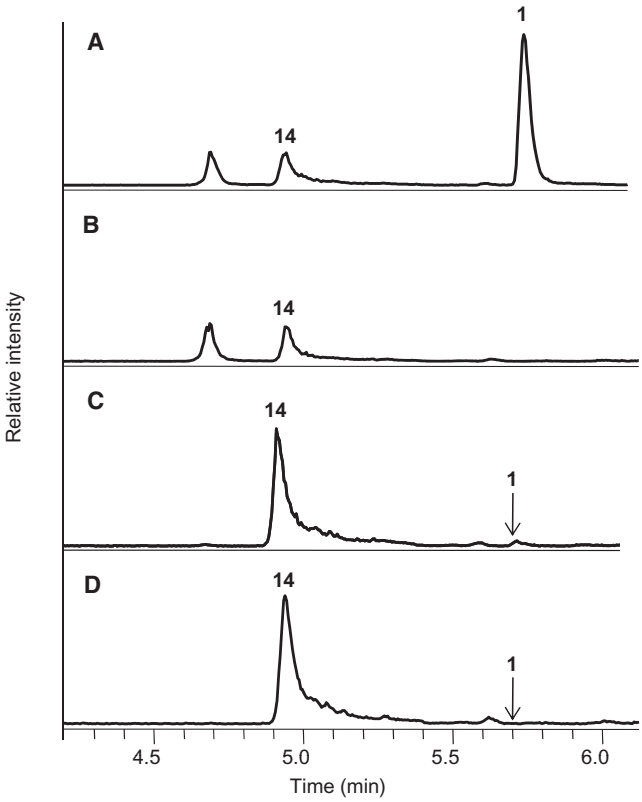

Figure 5. High-Resolution MS Analysis of the Inactivation Mutants in SBCb004

The EIC at *m/z* 686.5 $[M+H]^+$ (pretubulysin A [**14**]) and *m/z* 844.4 $[M+H]^+$ (tubulysin A [**1**]) is shown.

(A) SBCb004 wild-type.

(B) Mutant SBCb004-*tubA*$^-$.

(C) Mutant SBCb004-*tubZ*$^-$ (Chai et al., 2010).

(D) Mutant SBCb004-*orf1*$^-$.

Primers used are listed in Table S2, and detailed results of inactivation mutants are also shown in Figure S4.

supplying L-pipecolate to the assembly line in the absence of a functional TubZ. In contrast to mutants *M. xanthus*::pOrf1 *and M. xanthus*::ptubZ, however, the SBCb004-*orf1*$^-$ and SBCb004-*tubZ*$^-$ inactivation strains were also found to produce small amounts of tubulysin A. The most probable explanation for this result is that tubulysin A was also present in the *M. xanthus* strains but at yields too low to be detected by our HPLC-MS method. The fact that inactivation of these genes affected the extent of post-assembly line modification resulting in the accumulation of the pretubulysins suggests that *orfs 1* and *2* serve to recruit the oxidases and/or acyl transferases to the pathway via specific protein-protein interactions. In summary, although the results of gene inactivation in both *M. xanthus* and SBCb004 strains did not definitively identify the functions of genes *orf1* and *orf2*, the results clearly indicate that the genes are involved in tub biosynthesis.

Promoter Insertion into Strain SBCb004

Developing natural products as drug leads depends critically on obtaining sufficient amounts of the target compounds. The mature tubulysins are produced at relatively low yields by the natural producer strains An d48 (0.48 mg/ml tubulysin D) and SBCb004 (0.35 mg/ml tubulysin A), as well as by both heterologous expression hosts *P. putida* and *M. xanthus*, even in the presence of supplemental start unit Pip. Furthermore, the titers of the tubulysin variants are even lower. These observations prompted us to look for molecular methods for increasing the yields of tubulysin. We have previously demonstrated that overexpression of single genes or multigene transcriptional units by promoter exchange in other myxobacterial strains can lead to increased secondary metabolite formation (Richter et al., 2008; Meiser and Müller, 2008; Fu et al., 2008; Buntin et al., 2010). Thus, we aimed to insert a strong constitutive Tn5-derived *npt* promoter directly in front of the tubulysin core gene cluster *tubB-tubF* in strain SBCb004. This strategy had been used successfully for myxochromide and ajudazol overproduction in *M. xanthus* and *Chondromyces crocatus*, respectively (Buntin et al., 2010). The required modification was also achieved by homologous recombination (Figure S4). Unfortunately, the inserted Tn5 promoter did not upregulate the production but instead decreased the titers of all the major metabolites (by one third relative to the wild-type strain). A possible explanation for this finding is that the promoter insertion method somehow affected normal expression of the tubulysin assembly line, but determining the exact cause of the reduction in yields will require further investigation.

Identification of a Candidate for the Missing Monooxygenase in the SBCb004 Genome

All evidence suggests that the tubulysin oxidase(s) and, possibly, a second acyl transferase are absent from both clusters in strains An d48 and SBCb004 and so are presumed to be located elsewhere in the genomes. Such split cluster PKS/NRPS architecture is unusual for *Streptomyces* but has increasing precedent in the myxobacteria (Carvalho et al., 2005; Kopp et al., 2005; Perlova et al., 2006b). In order to locate the missing oxygenase(s), we used a P450-encoding gene *ajuJ* from the ajudazol biosynthetic gene cluster of the myxobacterium *Chondromyces crocatus* Cm c5 (Buntin et al., 2008) as a probe in a BLAST analysis (Altschul et al., 1997) of whole genome sequencing data from strain SBCb004, obtained by shotgun sequencing using 454 technology (Margulies et al., 2005; Rothberg and Leamon, 2008). This analysis identified 49 candidate oxygenases. Annotation of the flanking regions in each case identified six P450 genes and one monooxygenase gene that were co-located with at least one acyl transferase, indicating their possible involvement in the tubulysin pathway (Figure S5). Therefore, these seven genes were targeted by knockout mutagenesis in SBCb004, using the method described earlier. The tubulysin production profiles of six mutants (SBCb004-*24P*$^-$, SBCb004-*45M*$^-$, SBCb004-*302P*$^-$, SBCb004-*333P*$^-$, SBCb004-*399P*$^-$, and SBCb004-*1042P*$^-$) were essentially identical with that of the SBCb004 wild-type, indicating that the mutated enzymes are not involved in tubulysin assembly. However, culture extracts of a strain containing a mutated P450 gene from contig 633 (*633P1*) produced only several pretubulysin subfamily compounds **14**, **18**, **20**, and **28** (Figure S1), while tubulysin variants incorporating post-PKS hydroxylation were completely absent. In addition, the production of the pretubulysins decreased dramatically (Figure 6).

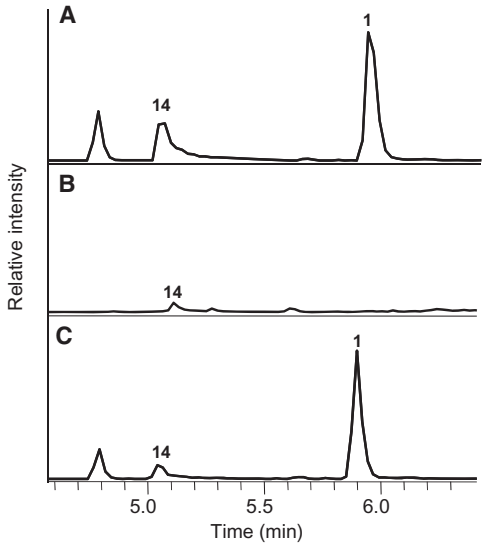

Figure 6. High-Resolution MS Analysis of P450 Gene Mutants in SBCb004

The EIC at *m/z* 686.5 $[M+H]^+$ (pretubulysin A [**14**]) and *m/z* 844.4 $[M+H]^+$ (tubulysin A [**1**]) is shown.

(A) SBCb004 wild-type.

(B) SBCb004-*633P1*$^-$ mutant.

(C) SBCb004-*633P2*$^-$ mutant.

Primers used are listed in Table S2, and the detailed analysis of the P450 genes is presented in Figure S5.

This result identified gene *633P1* as a promising candidate for one of the missing oxygenases. However, it is unclear why the yields of the pretubulysin should have been affected by its inactivation.

It is interesting that gene *633P1* is located in close proximity to a second P450 gene *633P2* as well as an acyltransferase *633AT* (Figure S5), identifying these additional genes as candidates for participation in tubulysin biosynthesis. However, inactivation of gene *633P2* in SBCb004 strain yielded a metabolite profile identical to that of the wild-type strain, likely excluding its involvement in the pathway (Figure 6). Thus, the P450 *633P1* may carry out both of the post-assembly line oxidations in tubulysin assembly.

Coexpression of the Tubulysin Gene Cluster and Gene *633P1* in *P. putida*

To attempt to confirm the involvement of gene *633P1* in tubulysin biosynthesis, we introduced the gene into construct pTub-ATG as a cassette along with a zeocin resistance gene and a P_{tet} promoter sequence via Red/ET recombination (Figure S5). The resulting pTub-633P1 construct was conjugated into *P. putida*, and expression induced with tetracycline. However, analysis by HPLC-MS showed that strain *P. putida*::pTub-633P1 generated pretubulysin A (**14**) and tyrosine pretubulysin A (**16**) but that no metabolites incorporating post-assembly line hydroxylation and/or acylation were present. These data were surprising, given the clear results of inactivation of *633P1* in the wild-type strain, but may indicate that the P450 requires additional gene products to be present in order to be active, which are currently absent from the heterologous expression construct (e.g., ferredoxins and/or ferredoxin reductases). In any case, further work will be required to confirm the function of *633P1* as the tubulysin monooxygenase.

SIGNIFICANCE

The tubulysins are a 33-membered family of hybrid polyketide (PK)-nonribosomal peptide (NRP) natural products with promising cytotoxic activity against multi-drug-resistant tumors. They are active in the low picomolar range against a range of cancer cell lines and are, therefore, promising candidates for development as antineoplastic agents. We have recently identified a shared gene cluster for tubulysin biosynthesis in two myxobacterial strains, *Angiococcus disciformis* An d48 and *Cystobactor sp.* SBCb004 (which is responsible for assembly of the PK-NRP backbone of the metabolites). Here, we have applied Red/ET recombineering to reconstitute the 11-gene (ca. 40 kb) cluster from two cosmids, with the addition of a transposon to facilitate heterologous expression. The engineered gene cluster was then transferred into two heterologous hosts, *Pseudomonas putida* and the myxobacterium *Myxococcus xanthus*, resulting in the production of pretubulysin A and tyrosine pretubulysin A, predicted products of the cluster. This result sets the stage for manipulation of the gene set toward the generation of bioactive analogs. As proof of principle, we exploited the expression system to investigate the function of several genes whose role in the biosynthesis had previously been unclear. Confirmation of the results was obtained by engineering the equivalent mutations in the wild-type strain SBCb004. Although the core tubulysin cluster gives rise to bioactive pathway intermediates, it lacks the oxidase and acyl transferase functions responsible for generating the mature tubulysins. By BLAST analysis coupled with insertion mutagenesis, we have identified a candidate for one of the missing oxygenases in the SBCb004 genome, setting the stage for the complete reconstitution of tubulysin biosynthesis.

EXPERIMENTAL PROCEDURES

Reconstitution of the Tubulysin Biosynthetic Gene Cluster

Through Red/ET recombineering, the 5′ end of the tub gene cluster on Cos DL18 was cloned into a minimal linear cloning vector p15A-IR-Km, flanked by an *Nhe*I site (the cassette was amplified by PCR using primers 15A-Km-tub5 and 15A-Km-tub3), and Red/ET recombineering with Cos DL18 resulted in plasmid pUp. Two rounds of recombineering were used to engineer the tubulysin genes on Cos AP17. First, a minimal cloning vector pUC-Amp incorporating an *Nhe*I site (primers ask-tub5 and ask-tub3) was designed to replace both the pSuperCos backbone and the *tubC* fragment on Cos AP17. A cassette containing a zeocin resistance gene and an *Nhe*I site (primers Zeo-tub5 and Zeo-tub3) was then subcloned into the above mentioned plasmid, resulting in plasmid pDown. The two modified vectors were then digested with *Nhe*I, and the 29 kb fragment resulting from vector pDown was ligated to linearized pUp using T4 DNA ligase. The ligation mixture was introduced into GB05-red competent cells by electroporation, and plasmids harboring the full-length tub core gene cluster in the correct orientation (p15A-Tub) were verified by restriction analysis. To enable expression in the heterologous expression host strains, a conjugation/transposition cassette IR-*Tps-Amp-oriT* was generated (oligos Tps-amp5 and Tps-amp3) and inserted into plasmid p15A-Tub. In order to modify the start codon of gene *tubC*, two gene cassettes (*Genta-tetR*-P_{tet}-TTG and *Genta-tetR*-P_{tet}-ATG) were

generated using primer pairs Genta-tub5 and TetR-tub-TTG as well as Genta-tub5 and TetR-tub-ATG, respectively. The cassettes were then inserted directly 5′ of *tubC* by Red/ET recombineering, and clones containing correct plasmids pTub-TTG or pTub-ATG were selected for both gentamicin and ampicillin resistance. Primers are listed in Table S1, and all generated constructs were verified either by restriction analysis or by sequencing.

Genetic Modification of the Tubulysin Heterologous Expression System in *E. coli*

A series of inactivation cassettes containing a chloramphenicol resistance gene was generated by varying the homology arms through different primer pairs (Table S1). Primers orf2-for and orf2-rev were used to inactivate gene *orf2*, primers tubZ-for and orf2-rev were used to delete both *tubZ-orf2* genes, and primers orf1-for and orf2-rev were used to replace the whole *orf1-tubZ-orf2* operon. Primers tubA-for and tubA-rev were used to delete gene *tubA*. The cassettes were introduced into plasmid pTub-ATG, and the resulting deletion vectors were screened using both chloramphenicol and ampicillin.

Coexpression of the Tubulysin Gene Cluster and Gene *633P1*

Cassette *Zeo*-P_{tet}-*633P1* was designed to introduce gene *633P1* at the 5′ end of the tub gene cluster on the heterologous expression construct pTub-ATG. Gene *633P1* was obtained by PCR from the SBCb004 genome using primers 633P1-1 and 633P1-2 (Table S1) and subsequently cloned into pCR2.1-TOPO. The resulting pTOPO-633P1 plasmid was linearized by digestion with *Eco*RI. DNA fragment *Zeo*-P_{tet} (zeocin resistance gene and P_{tet} promoter sequence) was amplified using primers P450-zeo5 and P450-zeo3 (Table S1). Due to the presence of *tetR* in the plasmid pTub-ATG, it was only necessary to introduce the P_{tet} promoter sequence in order to regulate expression of *633P1*. The two linear fragments *pTOPO-633P1* and *Zeo*-P_{tet} were then joined by linear-to-linear recombination in *E. coli* strain YZ2005 (Fu et al., 2010; Zhang et al., 2000), and correct plasmids (pTOPO-Zeo-P_{tet}-633P1) were selected by zeocin resistance. DNA fragment *Zeo*-P_{tet}-*633P1* was then released using *Eco*RI, and then one-step Red/ET recombination was carried out to insert this cassette into the vector pTub-ATG. The resulting positive clones were selected on low-salt LB agar plates containing ampicillin and zeocin.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, two tables and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2012.01.007.

ACKNOWLEDGMENTS

The authors thank Professor Uli Kazmaier and his colleagues from the Institute for Organic Chemistry for providing the synthesized pretubulysin D and the deuterium labeled L-/D-pipecolic acid and Eva Luxenburger and Daniel Krug from the Department of Pharmaceutical Biotechnology for their skillful help with analytical technologies. Work in R.M.'s laboratory was funded by the Bundesministerium für Bildung und Forschung and the Deutsche Forshungsgemeinschaft.

Received: July 5, 2011
Revised: December 22, 2011
Accepted: January 2, 2012
Published: March 22, 2012

REFERENCES

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J.H., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. *25*, 3389–3402.

Binz, T.M., Wenzel, S.C., Schnell, H.J., Bechthold, A., and Müller, R. (2008). Heterologous expression and genetic engineering of the phenalinolactone biosynthetic gene cluster by using red/ET recombineering. Chem. Bio. Chem. *9*, 447–454.

Bode, H.B., and Müller, R. (2006). Analysis of myxobacterial secondary metabolism goes molecular. J. Ind. Microbiol. Biotechnol. *33*, 577–588.

Buntin, K., Rachid, S., Scharfe, M., Blöcker, H., Weissman, K.J., and Müller, R. (2008). Production of the antifungal isochromanone ajudazols A and B in *Chondromyces crocatus* Cm c5: biosynthetic machinery and cytochrome P450 modifications. Angew. Chem. Int. Ed. Engl. *47*, 4595–4599.

Buntin, K., Irschik, H., Weissman, K.J., Luxenburger, E., Blöcker, H., and Müller, R. (2010). Biosynthesis of thuggacins in myxobacteria: comparative cluster analysis reveals basis for natural product structural diversity. Chem. Biol. *17*, 342–356.

Carvalho, R., Reid, R., Viswanathan, N., Gramajo, H., and Julien, B. (2005). The biosynthetic genes for disorazoles, potent cytotoxic compounds that disrupt microtubule formation. Gene *359*, 91–98.

Chai, Y., Pistorius, D., Ullrich, A., Weissman, K.J., Kazmaier, U., and Müller, R. (2010). Discovery of 23 natural tubulysins from *Angiococcus disciformis* An d48 and *Cystobacter* SBCb004. Chem. Biol. *17*, 296–309.

De Crécy-Lagard, V., Marlière, P., and Saurin, W. (1995). Multienzymatic non ribosomal peptide biosynthesis: identification of the functional domains catalysing peptide elongation and epimerisation. C. R. Acad. Sci. III *318*, 927–936.

Dömling, A., and Richter, W. (2005). Myxobacterial epothilones and tubulysins as promising anticancer agents. Mol. Divers. *9*, 141–147.

Fu, J., Wenzel, S.C., Perlova, O., Wang, J., Gross, F., Tang, Z., Yin, Y., Stewart, A.F., Müller, R., and Zhang, Y. (2008). Efficient transfer of two large secondary metabolite pathway gene clusters into heterologous hosts by transposition. Nucleic Acids Res. *36*, e113.

Fu, J., Teucher, M., Anastassiadis, K., Skarnes, W., and Stewart, A.F. (2010). A recombineering pipeline to make conditional targeting constructs. Methods Enzymol. *477*, 125–144.

Gatto, G.J., Jr., Boyne, M.T., 2nd, Kelleher, N.L., and Walsh, C.T. (2006). Biosynthesis of pipecolic acid by RapL, a lysine cyclodeaminase encoded in the rapamycin gene cluster. J. Am. Chem. Soc. *128*, 3838–3847.

Gross, F., Ring, M.W., Perlova, O., Fu, J., Schneider, S., Gerth, K., Kuhlmann, S., Stewart, A.F., Zhang, Y., and Müller, R. (2006). Metabolic engineering of *Pseudomonas putida* for methylmalonyl-CoA biosynthesis to enable complex heterologous secondary metabolite formation. Chem. Biol. *13*, 1253–1264.

Julien, B., and Shah, S. (2002). Heterologous expression of epothilone biosynthetic genes in *Myxococcus xanthus*. Antimicrob. Agents Chemother. *46*, 2772–2778.

Kaur, G., Hollingshead, M., Holbeck, S., Schauer-Vukasinović, V., Camalier, R.F., Dömling, A., and Agarwal, S. (2006). Biological evaluation of tubulysin A: a potential anticancer and antiangiogenic natural product. Biochem. J. *396*, 235–242.

Khalil, M.W., Sasse, F., Lünsdorf, H., Elnakady, Y.A., and Reichenbach, H. (2006). Mechanism of action of tubulysin, an antimitotic peptide from myxobacteria. Chem. Bio. Chem. *7*, 678–683.

Khaw, L.E., Böhm, G.A., Metcalfe, S., Staunton, J., and Leadlay, P.F. (1998). Mutational biosynthesis of novel rapamycins by a strain of *Streptomyces hygroscopicus* NRRL 5491 disrupted in *rapL*, encoding a putative lysine cyclodeaminase. J. Bacteriol. *180*, 809–814.

Kopp, M., Irschik, H., Pradella, S., and Müller, R. (2005). Production of the tubulin destabilizer disorazol in *Sorangium cellulosum*: biosynthetic machinery and regulatory genes. Chem. Bio. Chem. *6*, 1277–1286.

Kuhstoss, S., Richardson, M.A., and Rao, R.N. (1991). Plasmid cloning vectors that integrate site-specifically in Streptomyces spp. Gene *97*, 143–146.

Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A., Berka, J., Braverman, M.S., Chen, Y.J., Chen, Z., et al. (2005). Genome sequencing in microfabricated high-density picolitre reactors. Nature *437*, 376–380.

Meiser, P., and Müller, R. (2008). Two functionally redundant Sfp-type 4′-phosphopantetheinyl transferases differentially activate biosynthetic pathways in *Myxococcus xanthus*. Chem. Bio. Chem *9*, 1549–1553.

Mutka, S.C., Carney, J.R., Liu, Y., and Kennedy, J. (2006). Heterologous production of epothilone C and D in *Escherichia coli*. Biochemistry *45*, 1321–1330.

Perlova, O., Fu, J., Kuhlmann, S., Krug, D., Stewart, A.F., Zhang, Y., and Müller, R. (2006a). Reconstitution of the myxothiazol biosynthetic gene cluster by Red/ET recombination and heterologous expression in Myxococcus xanthus. Appl. Environ. Microbiol. *72*, 7485–7494.

Perlova, O., Gerth, K., Kaiser, O., Hans, A., and Müller, R. (2006b). Identification and analysis of the chivosazol biosynthetic gene cluster from the myxobacterial model strain *Sorangium cellulosum* So ce56. J. Biotechnol. *121*, 174–191.

Richter, C.D., Nietlispach, D., Broadhurst, R.W., and Weissman, K.J. (2008). Multienzyme docking in hybrid megasynthetases. Nat. Chem. Biol. *4*, 75–81.

Rothberg, J.M., and Leamon, J.H. (2008). The development and impact of 454 sequencing. Nat. Biotechnol. *26*, 1117–1124.

Sandmann, A., Sasse, F., and Müller, R. (2004). Identification and analysis of the core biosynthetic machinery of tubulysin, a potent cytotoxin with potential anticancer activity. Chem. Biol. *11*, 1071–1079.

Sasse, F., Steinmetz, H., Heil, J., Höfle, G., and Reichenbach, H. (2000). Tubulysins, new cytostatic peptides from myxobacteria acting on microtubuli. Production, isolation, physico-chemical and biological properties. J. Antibiot. (Tokyo) *53*, 879–885.

Steinmetz, H., Glaser, N., Herdtweck, E., Sasse, F., Reichenbach, H., and Höfle, G. (2004). Isolation, crystal and solution structure determination, and biosynthesis of tubulysins—powerful inhibitors of tubulin polymerization from myxobacteria. Angew. Chem. Int. Ed. Engl. *43*, 4888–4892.

Tang, L., Shah, S., Chung, L., Carney, J., Katz, L., Khosla, C., and Julien, B. (2000). Cloning and heterologous expression of the epothilone gene cluster. Science *287*, 640–642.

Ullrich, A., Chai, Y., Pistorius, D., Elnakady, Y.A., Herrmann, J.E., Weissman, K.J., Kazmaier, U., and Müller, R. (2009). Pretubulysin, a potent and chemically accessible tubulysin precursor from *Angiococcus disciformis*. Angew. Chem. Int. Ed. Engl. *48*, 4422–4425.

Vervoort, E.B., van Ravestein, A., van Peij, N.N., Heikoop, J.C., van Haastert, P.J., Verheijden, G.F., and Linskens, M.H. (2000). Optimizing heterologous expression in dictyostelium: importance of 5′ codon adaptation. Nucleic Acids Res. *28*, 2069–2074.

Wenzel, S.C., and Müller, R. (2005). Recent developments towards the heterologous expression of complex bacterial natural product biosynthetic pathways. Curr. Opin. Biotechnol. *16*, 594–606.

Wenzel, S.C., Gross, F., Zhang, Y., Fu, J., Stewart, A.F., and Müller, R. (2005). Heterologous expression of a myxobacterial natural products assembly line in pseudomonads via red/ET recombineering. Chem. Biol. *12*, 349–356.

Wenzel, S.C., and Müller, R. (2007). Myxobacterial natural product assembly lines: fascinating examples of curious biochemistry. Nat. Prod. Rep. *24*, 1211–1224.

Zhang, Y., Buchholz, F., Muyrers, J.P., and Stewart, A.F. (1998). A new logic for DNA engineering using recombination in *Escherichia coli*. Nat. Genet. *20*, 123–128.

Zhang, Y., Muyrers, J.P.P., Testa, G., and Stewart, A.F. (2000). DNA cloning by homologous recombination in *Escherichia coli*. Nat. Biotechnol. *18*, 1314–1317.