

Ablation of the *otcC* Gene Encoding a Post-polyketide Hydroxylase from the Oxytetracycline Biosynthetic Pathway in *Streptomyces rimosus* Results in Novel Polyketides with Altered Chain Length*

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Oxytetracycline (OTC) is a 19-carbon polyketide antibiotic made by *Streptomyces rimosus*. The *otcC* gene encodes an anhydrotetracycline oxygenase that catalyzes a hydroxylation of the anthracycline structure at position C-6 after biosynthesis of the polyketide backbone is completed. A recombinant strain of *S. rimosus* that was disrupted in the genomic copy of *otcC* synthesized a novel C-17 polyketide. This result indicates that the absence of the *otcC* gene product significantly influences the ability of the OTC “minimal” polyketide synthase to make a polyketide product of the correct chain length. A mutant copy of *otcC* was made by site-directed mutagenesis of three essential glycine codons located within the putative NADPH-binding domain. The mutant gene was expressed in *Escherichia coli*, and biochemical analysis confirmed that the gene product was catalytically inactive. When the mutant gene replaced the ablated gene in the chromosome of *S. rimosus*, the ability to make a 19-carbon backbone was restored, indicating that *OtcC* is an essential partner in the quaternary structure of the synthase complex.

Oxytetracycline (OTC)⁴ and related tetracycline polyketide compounds are potent inhibitors of bacterial protein synthesis displaying broad-spectrum activity against both Gram-positive and Gram-negative pathogens. Although the clinical use of the tetracyclines has declined in recent years due to the emergence of resistance in these bacteria, OTC is still the first drug of choice for the treatment of intracellular infections caused by *Rickettsia*, *Chlamydia*, and mycoplasma in penicillin-sensitive patients incapable of tolerating macrolides (1). Gly-

cyclines are a new class of semi-synthetic tetracycline derivatives that exemplify how modification of a known antibiotic can be a successful strategy to obtain new compounds effective against pan-drug-resistant strains (2). To this end we have been elucidating the biosynthetic pathway leading to OTC in the producing actinomycete *Streptomyces rimosus*, as a potential route to clinically useful OTC products by combinatorial biosynthesis (3).

Polyketide synthases (PKSs) catalyzing the formation of aromatic products such as OTC are dissociable enzyme complexes of largely monofunctional proteins that follow a mechanistic pathway similar to fatty acid biosynthesis (4). The synthesis of aromatic Type II polyketides usually begins with the condensation of an acetate unit derived from the decarboxylation of a malonyl-S-acyl carrier protein (ACP) to a malonyl-S-ACP extender unit. This reaction is catalyzed by a heterodimeric ketosynthase chain length factor (KS α -KS β) (5, 6). Preference for non-acetate primers has also been recognized in a number of aromatic PKSs including OTC, although presumably decarboxylative chain initiation catalyzed by the heterodimer can still occur in the absence of these non-acetate starter units (7). Manipulating the mechanism of non-acetate priming has led to biosynthesis of novel polyketides (8, 9). The active site of the KS α also catalyzes iterative elongation of the polyketide chain by a decarboxylative condensation of additional malonyl moieties. The malonyl-S-ACP intermediates are recruited following acyl transfer between malonyl-CoA and holo-ACP catalyzed by a malonyl-CoA:ACP acyl transferase; self-malonylation of the ACP has been demonstrated also *in vitro* but is of limited physiological relevance *in vivo* (9–12). The crystal structure of a KS α -KS β polymerase has recently been resolved and suggests that elongation occurs inside an amphipathic tunnel at the heterodimer interface followed by the first cyclization of the acyl chain (13).

Based on limited *in vivo* experimental evidence, the minimal number of enzymes required to form a polyketide of the requisite chain length was assumed originally to be the KS α -KS β heterodimer and the ACP (14, 15). Further *in vitro* mutagenesis studies certainly implicate a level of control by the heterodimer over chain length (16). It is now recognized that disruption of genes encoding proteins upstream and downstream of the “minimal” PKS can also mediate chain length control. These proteins, including ketoreductases (KR), aromatases (ARO), and cyclases (CYC), play essential catalytic roles in modifying the growing poly- β -oxo-intermediate and *in vivo* and *in vitro* evidence suggests that these proteins must also exert considerable structural integrity on the dissociable complex during polyketide biosynthesis (16, 17). To date there is no three-dimensional structure for an aromatic PKS complex,

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY916128.

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⁴ The abbreviations used are: OTC, oxytetracycline; ACP, malonyl-S-acyl carrier protein; ARO, aromatase; ATC, anhydrotetracycline; CYC, cyclase; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond connectivity; HPLC, high-performance liquid chromatography; KR, ketoreductase; KS α -KS β , heterodimeric ketosynthase-chain length factor; PKS, polyketide synthase.

Ablation of Hydroxylation Effects Polyketide Chain Length

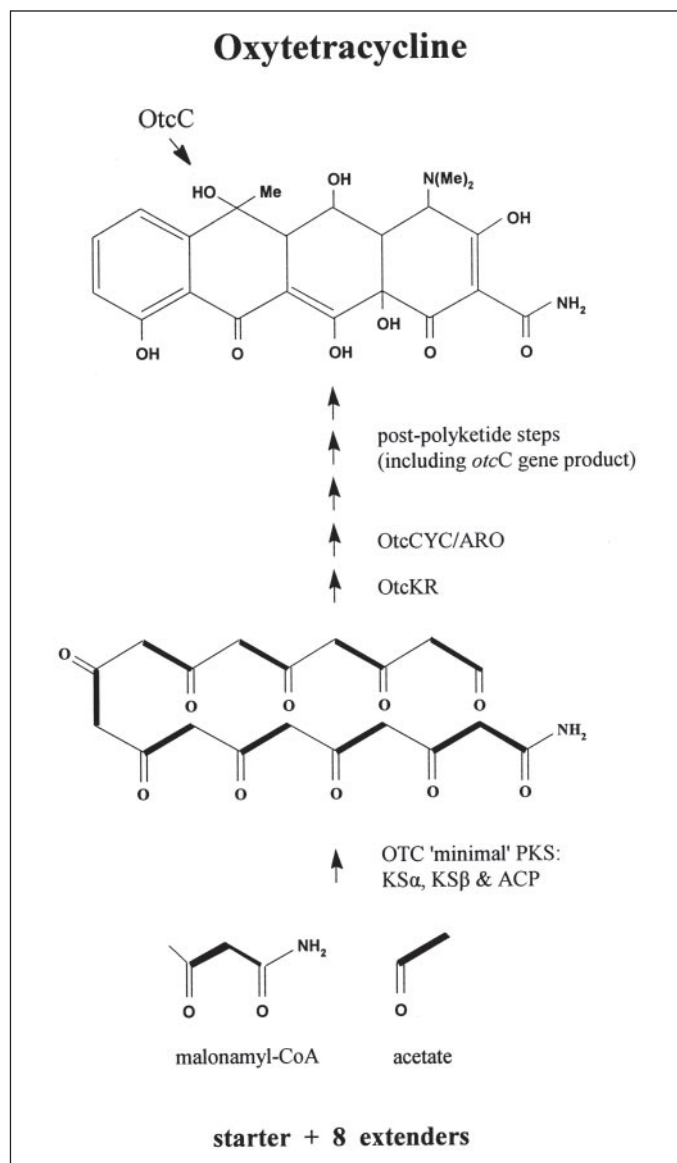


FIGURE 1. Biosynthesis of oxytetracycline from malonyl-CoA starter unit and malonyl-ACP by the PKS enzymes. Hydroxylation of the tetracyclic nucleus at position C-6 is indicated.

although yeast two-hybrid experiments have validated independent *in vitro* mutagenesis studies suggesting a head-to-tail heterodimeric structure for the daurubicin producing minimal PKS (18). Excluding the crystal structure of the actinorhodin producing KS α -KS β heterodimer (13), only five other aromatic PKS components have been resolved, indicating only limited protein-protein interactions pertaining to chain length control (19–23). Understanding these interactions will be crucial to controlling chain length and ultimate success of strategies for combinatorial biosynthesis.

The backbone of OTC, consisting of 19 carbon atoms, is probably derived from an aminated starter unit that forms the carboxamido group of the final structure (24, 25), to which eight acetyl extender units are added sequentially (Fig. 1). Molecular genetic analysis of OTC biosynthesis showed that the gene cluster is localized (26, 27) on the 8 Mb linear *S. rimosus* chromosome (28). The entire OTC cluster has been cloned and sequenced from M15883, a derivative of the strain M4018 (29). The entire OTC gene cluster was also cloned from the *S. rimosus* strain R6-500. Its restriction map is indistinguishable from that of the

strain M15883 (30). The *otcC* gene is a post-polyketide OTC biosynthetic gene whose product hydroxylates the tetracyclic nucleus at the C-6 position (anhydrotetracycline (ATC) oxygenase), many steps after the nascent polyketide chain has been synthesized and folded. The gene had been located by a "reverse genetics" approach, using a degenerate oligonucleotide designed against the amino-terminal peptide sequence of the purified enzyme protein (27) lying outside the transcription units of all genes involved in biosynthesis and folding of the OTC backbone, some 20 kb from the OTC "minimal" PKS (31). The combined results of transcriptional analysis (32) revealed co-transcription from the *otcCp1* promoter of *otcC*, *otcZ* encoding a biosynthetic methyltransferase (33), and the resistance gene *otrA*. During vegetative growth, *otrA* is also transcribed independently of *otcCp1* from its own promoter. In this paper, we present data on the disruption of the *otcC* gene and the influence of its post-polyketide gene product on the polyketide chain length.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Culture Conditions—The OTC producer *S. rimosus* R6-500 (34) was used for the disruption of the *otcC* gene. The strain was maintained and grown in fermentation medium as described previously (35, 36). *Streptomyces* plasmid pPFZ46 (26) was used as a source of the *otcC* gene and flanking sequences. *Escherichia coli* plasmid pIBI24 (37) was used to construct a disrupted *otcC* gene by the insertion of the *gmr* gene, which confers resistance to gentamicin (38). *Streptomyces* plasmid pIJ486 (39), carrying the *tsr* gene, that confers resistance to thiostrepton, was used to construct a bifunctional vector capable of replication in *E. coli* and *Streptomyces*. All DNA manipulations were in *E. coli* strain TG1 (40). Plasmids were passaged through *E. coli* GM31 (*dcm*) (41) to generate unmethylated DNA prior to transformation of *S. rimosus*.

General DNA Techniques—The general methods used for DNA manipulation in *Streptomyces* were described by Kieser *et al.* (42) and those in *E. coli* by Sambrook *et al.* (43). DNA was introduced into *S. rimosus* by electrotransformation, as described by Pigac and Schrepf (44).

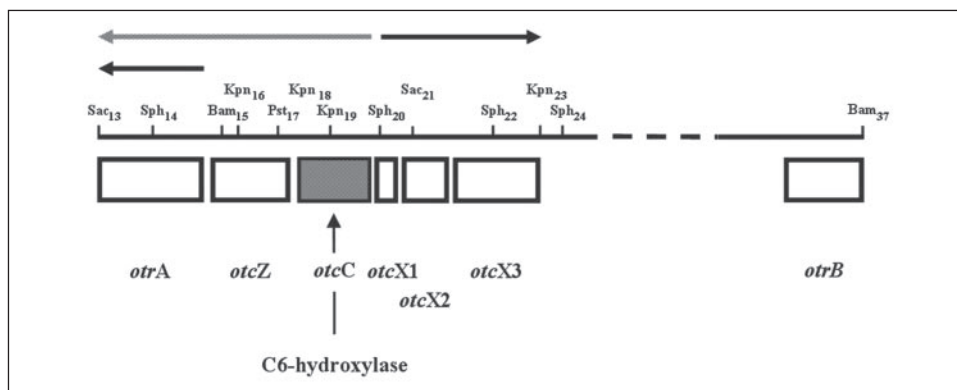
Site-directed PCR Mutagenesis—Mutations in the *otcC* gene were introduced by site-directed mutagenesis using PCR (45) with the primers mp1 (5'-GCC GCC GCG CCG ACC GCC CTG ATG CTC-3') and mp2 (5'-GQC GGT CGG CGC GGC GGC GGC GAT CA-3') were used. The underlined nucleotides denote point mutations: G \rightarrow C and C \rightarrow G.

Two primers were used to amplify and clone the mutated *otcC* gene: forward, *otcCp1* (NH₂ terminus): 5'-AGC GCA TAT GCG GTA CGA CGT GGT GAT CGC-3' and reverse, *otcCp2* (COOH terminus): 5'-TGG GAA GCT TCG GGC GCG GAA GGG CAG GGA-3'; thus the NH₂- and COOH-terminal regions were modified (in italics) by introducing NdeI and HindIII restriction sites (underlined).

A primer was designed to introduce an NdeI site at the beginning of the *otcC* gene: using Stratagene's ExSiteTM PCR site-directed mutagenesis system: mp3 (5'-CAT ATG GAT CCG AGG AGC GTG TAT GCG GTA-3', NdeI site underlined) and a second, mp4 (5'-ATC AGT GGG TTG CCG CCG AGC ATG-3'). *Pfu* DNA polymerase (Stratagene) was used to amplify the *otcC* gene.

Biochemical Methods—cell-free extracts of *E. coli* BL21(DE3) were prepared by sonic disruption of washed cell pellets. SDS-polyacrylamide gel electrophoresis was performed using the procedure of Laemmli (46). The molecular mass of the enzyme was estimated from the relative mobilities of standard proteins. Protein concentrations were determined by the Coomassie Blue binding assay (47), with bovine serum albumin as a standard. The ATC oxygenase activity was determined as described previously (48, 49).

FIGURE 2. Physical and restriction map of the oxytetracycline gene cluster indicating the position of *otcC* that encodes the C-6-specific hydroxylase downstream from the putative methylase encoded by *otcZ*.



Isolation and Purification of Metabolites—The fermentation broths (2.0 liters) from *S. rimosus* R6-500 and recombinants ZGL1 and ZGL5 were saturated with NaH_2PO_4 and extracted with ethyl acetate (3×2 liters). The evaporated solid was fractionated by silica gel chromatography. Compound C1-1 eluted on a methanol gradient (8–16%) in chloroform. It was purified further on a Sephadex LH-20 column (6% methanol:94% chloroform). Compound C1-2 eluted from the silica gel in ethyl acetate (65–80% in *n*-hexane), while compound C1-3 eluted in ethyl acetate (30–45%) in *n*-hexane. The extraction, concentration, and purification of polyketides were monitored by TLC on silica gel plates using chloroform/methanol/water (9:1:0.1) or acetonitrile/methanol/0.2 M oxalic acid (1:4:4) as mobile phases and by high performance liquid chromatography (HPLC) on an Eclipse XDB-C8 column with gradient elution using acetonitrile, 0.1% trifluoroacetic acid (pH 2.2) as the mobile phase. Commercially available OTC was used as a control in TLC and HPLC.

Structural Elucidation of Metabolites—The structures were resolved largely on the basis of two-dimensional homonuclear and heteronuclear NMR (400 MHz) studies using the COSY-45 technique for direct H-H coupling, the HMQC technique for 1-bond (1J), H-C interactions, and the HMBC technique for 2-bond (2J) and 3-bond (3J) H-C interactions, as described previously (3).

RESULTS

Construction of an *otcC* Disruption Mutant in *S. rimosus*—The *otcC* gene was subcloned from pPFZ46 onto *E. coli* plasmid pIBI24. The gene was sequenced initially using a universal primer and then by gene “walking” using the derived sequence to specify the next primer. Sequencing of both strands used “Big-Dye” terminators on an ABI 373 DNA sequencer at the University of Strathclyde, by standard protocols. The annotated sequence has been deposited at GenBankTM with accession number AY916128.

To mark the *otcC* gene in *S. rimosus* and to replace it with a mutated copy, the gene was inactivated by insertion of a *gmr* marker that confers resistance to gentamicin. A bifunctional vector containing the *otcC* gene, pUC for replication in *E. coli*, and a thiostrepton-resistance gene (*tsr*) for selection in streptomycetes was constructed in two steps. First, both the 4.5-kb SphI₁₄–SacI₂₁ *S. rimosus* genomic fragment carrying the *otcC* gene with a XbaI linker (between KpnI₁₈–KpnI₁₉ sites) (Fig. 2) and a 1.6-kb XbaI fragment carrying the *gmr* marker were cloned to create pGLW74. Second, pGLW74 was linearized and ligated to the *Streptomyces* vector pIJ486 to create shuttle vector pGLW75. This construct, which was expected to result in insertional inactivation of the *otcC* gene in the host genome upon recombination, was introduced into *S. rimosus* R6-500. Many primary transformants that were resistant to thiostrepton (the vector marker) were obtained. These were propagated under

non-selective conditions to eliminate the plasmid. After three rounds of subculture, the transformants were plated onto media containing gentamicin; more than 50% of the colonies were resistant to gentamicin. Chromosomal DNA was isolated from a representative number of thiostrepton-sensitive, gentamicin-resistant colonies and compared with DNA from the parental R6 strain. Southern blot hybridization confirmed that the *gmr* marker had been inserted into the target *otcC* gene through the expected double crossover (data not shown). A transformant designated ZGL1 was fermented to determine whether insertion of the *gmr* marker had led to inactivation of the *otcC* gene.

Characterization of Metabolites Produced by *S. rimosus* Mutant ZGL1—Preliminary TLC profiles of extracts from the fermentation broths of the *otcC*-disrupted strain *S. rimosus* ZGL1 did not contain OTC, but two products that were not detected in extracts from wild-type *S. rimosus* R6-500 could clearly be visualized. Further HPLC analysis revealed five potentially novel compounds (data not shown). Three of these compounds designated C1-1, C1-2, and C1-3 were major metabolites that could be purified in sufficient quantities for structural determination. However, full structural determination of compounds C1-2 and C1-3 were not possible because of conflicting NMR assignments that warrant closer investigation beyond the scope of this present investigation. Moreover, the empirical formula of C1-3 was $\text{C}_{19}\text{H}_{17}\text{NO}_7$ with a mass calculated to be 371.1005 (error 0.8 ppm) indicating that it might be a methyl derivative of C1-1 formed during the isolation procedure. Although the remaining compounds were also purified, structural determination was not possible because of conflicting NMR assignments that warrant closer investigation beyond the scope of this present investigation.

The molecular formula of compound C1-1 was established as $\text{C}_{18}\text{H}_{15}\text{NO}_7$ by fast atom bombardment mass spectroscopy data (observed monoisotopic mass m/z 358.0927 ($M + H$)⁺, calculated m/z 357.3228, error 3.9 ppm]. The structure of this compound was resolved by the analysis of various NMR experiments (^1H , ^{13}C , and two-dimensional homonuclear and heteronuclear NMR studies using the COSY-45 technique for direct H-H coupling, the HMQC technique for 1-bond (1J) H-C interactions, and the HMBC technique for 2-bond (2J) and 3-bond (3J) H-C interactions) (Fig. 3, A and B). The NMR assignments are given in TABLE ONE.

For compound C1-1 the quaternary aliphatic carbon (δ 69.4) was coupled with two protons, H-1 and H-8, that belong to separate aromatic rings and with methyl protons (δ 1.51) suggesting that a methyl group is attached directly to it. Since the resonance was still low for a sp^3 hybrid, it was concluded that it must also carry a hydroxyl group (δ 6.29). There were no correlations for a carbonyl carbon (δ 191.5) with any proton; this was assigned to the opposite site of the quaternary aliphatic carbon in the same ring of the anthraquinone. The signal of the

Ablation of Hydroxylation Effects Polyketide Chain Length

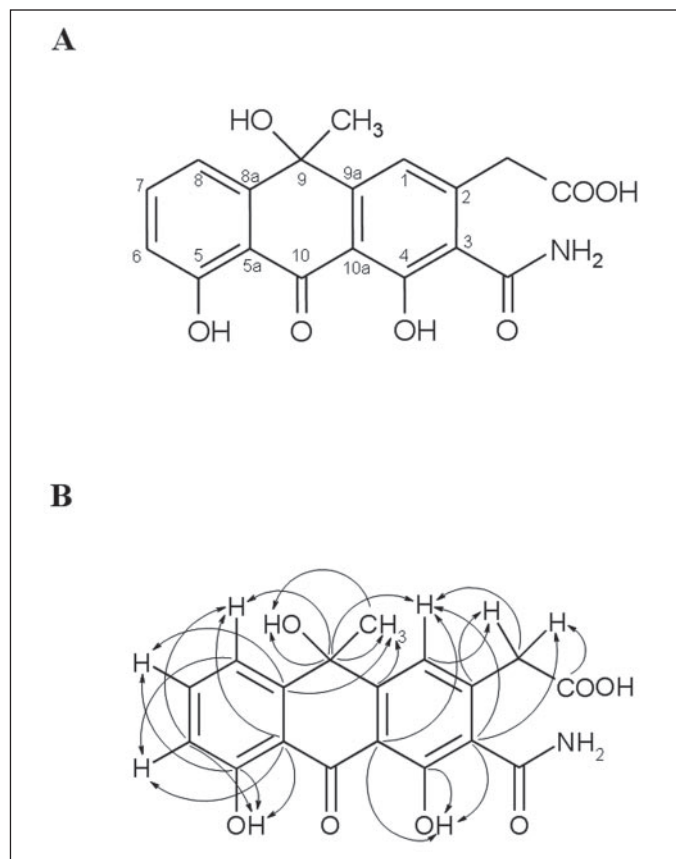


FIGURE 3. The structure of the isolated polyketide compound C1-1 (A) and its long range ¹H-¹³C coupling observed in the NMR spectra (B).

carbonyl carbon in the ¹³C NMR spectrum at 171.1 ppm was assigned as carboxylic rather than as a carboxamido carbonyl because of the more electrophilic environment (δ 166.9). Thus, compound C1-1 was identified as (3-carbamoyl-4,5,9-trihydroxy-9-methyl-10-oxo-9,10-dihydroan-tracen-2-yl)-acetic acid.

Construction and Analysis of an *otcC* Gene Encoding a Functionally Inactive ATC Oxygenase in *S. rimosus*—A 2.6-kb fragment of the *otcC* gene (from PstI₁₇-SacI₂₁) (Fig. 2), containing the NADPH binding site, was chosen for PCR mutagenesis. The mutated gene was generated using mp1 and mp2 primers containing the single-base changes (see “Experimental Procedures”) that altered the three glycine residues of the GXGXXG motif, involved in binding of NADPH, to alanines. This strategy ensured that the structure of OtcC was changed minimally and that mutations were non-polar on downstream sequences. All of the sequence alterations were confirmed by nucleotide sequence analysis, while checking that no other base had been mutated. The entire mutated *otcC* gene was assembled by a three-piece ligation of the 150 bp SalI-SphI₂₀ fragment of the PCR product and fragments containing wild-type *otc* DNA from SphI₁₄-SalI and SphI₂₀-SacI₂₁ to give pGLW96.

The NH₂-terminal region of the mutant *otcC* gene was re-sequenced to confirm that no other sequence alteration was introduced. The mutated region was then exchanged into the chromosome of *S. rimosus* R6-ZGL1 from the replacement shuttle vector pGLW97 (pGLW96::pIJ486), by transformation. Numerous recombinants were recovered and analyzed. After the vector was segregated, several independent recombinants were identified as having lost the vector DNA by a Tsr^S phenotype and simultaneous loss of the *gmr* marker. To confirm that the Gm^S Tsr^S phenotype of the mutants was linked to the mutation of the *otcC* gene, the *otcC* gene was amplified from genomic DNA using

primer *otcCp1* introducing a NdeI site at the NH₂ terminus and primer *otcCp2* containing a HindIII site at the COOH terminus. Amplification was achieved with 25 cycles of denaturation at 94 °C for 30 s followed by annealing at 55 °C for 30 s and by extension at 72 °C for 2 min. The resulting 1.6-kb PstI₁₇-SphI₂₀ PCR product from each putative mutant was purified and analyzed using restriction enzyme NarI to identify mutant fragments. NarI digestion showed five fragments in the wild-type cells and four in the mutant cells, since one NarI site within the *otcC* coding region was lost as a result of replacement with the *otcC* mutant gene. This result confirmed that the *otcC::gmr* of strain ZGL1 had been replaced. Three positive PCR fragments (from recombinants named ZGL5.1, ZGL5.2, and ZGL5.3) were digested with NdeI and HindIII. The fragment was cloned directly and sequenced to reconfirm the desired alteration of the *otcC* sequence (data not shown).

Preliminary TLC profiles of extracts from the fermentation broths of *S. rimosus* strains ZGL5.1, ZGL5.2, and ZGL5.3 contained OTC as the major metabolite. Further HPLC analysis confirmed this by comparison of retention time and UV spectra against an OTC standard. There was no evidence for the production of compound C1-1 or any of the other major metabolites (data not shown).

Anhydrotetracycline Oxygenase Activity in *E. coli*—The activity of the wild-type *otcC* gene in cell-free preparations of *E. coli* BL21 DE3, quantified by conversion of anhydrotetracycline into 6-deoxycycline (49), was 11.2 nmol·min⁻¹·(mg protein)⁻¹ ± 0.16 (five determinations). Background activity (reduction in A₄₄₀ in the absence of NADPH) was 4% of this value. *E. coli* recombinants of the mutant *otcC* gene, which had the essential glycine residues in the NADPH binding site mutated, had no net enzyme activity in this assay (limit of detection <0.016 nmol·min⁻¹·(mg protein)⁻¹), confirming that the mutations had the desired effect.

DISCUSSION

ZGL1, a Strain Disrupted in *otcC*, Makes Novel Polyketides of Shorter Chain Length—The *otcC* gene encodes an anhydrotetracycline oxygenase that catalyzes hydroxylation at position C-6 during the biosynthesis of OTC in *S. rimosus*. Modification of existing antibiotics by combinatorial biosynthesis is a strategy that we wanted to evaluate as a route to new compounds with novel biological activity. To this end, the absence of *otcC* from the OTC cluster was expected to provide a biological route for production of doxycycline, which is currently made by a semi-synthetic route. A disrupted copy of *otcC* was constructed by introducing a gentamicin-resistance cassette into a restriction site within the coding region of *otcC*. The disrupted copy was exchanged for the wild-type copy located on the chromosome of the OTC production strain *S. rimosus* R6-500 by a double crossover event. Selection of stable integrants proved difficult, presumably because of the location of the OTC gene cluster within an amplifiable unit of DNA located close to one end of the linear chromosome predisposing this region to either DNA amplifications or deletions (34). One stable integrant, designated ZGL1, failed to produce OTC but instead synthesized at least five compounds absent from the wild-type strain. One of the compounds characterized was an anthrocycline polyketide but was of shorter chain length than OTC. This implies that the absence of the *otcC* gene product has a very strong effect on the ability of the minimal OTC producing PKS to determine chain length. This is consistent with earlier predictions that auxiliary PKS subunits such as KRs, AROs, and CYCs can significantly modulate the intrinsic specificity of the minimal PKS with respect to both the folding pattern and the chain length of the final product. For example, analysis of a set of actinorhodin (*act*) and griseolin (*gris*) ARO/CYC-derived hybrid proteins expressed together with

TABLE ONE

¹H NMR and ¹³C NMR chemical shift data for compound C1-1

All spectra were run in Me₂SO-*d*₆; resonance multiplicities and coupling constants (*J* in Hz) are given in parentheses. s, singlet; d, doublet; t, triplet; ABq, AB quartet; br. S, broad singlet.

H/C	δ_{H} <i>ppm</i>	δ_{C} <i>ppm</i>
1	7.40 (1H, s)	119.1
2		141.8
3		126.2
4 (OH)	12.41 (2H, br. s)	158.3
5 (OH)	11.97 (1H, s)	161.5
5a		113.0
6	6.95 (1H, d, <i>J</i> = 8.4 Hz)	116.0
7	7.69 (1H, t, <i>J</i> = 8.0 Hz)	137.4
8	7.42 (1H, d, <i>J</i> = 7.6 Hz)	117.1
8a		152.0
9 (OH)	6.29 (1H, s)	69.4
9a		151.5
10		191.5
10a		111.6
9-CH ₃	1.51 (3H, s)	38.7
2-CH ₂ -	3.72 (2H, ABq, <i>J</i> = 16 Hz)	39.1
COOH	12.41 (2H, br. s)	171.1
CONH ₂	7.74; 7.64 (2×H, br. s)	166.9
Errors (Hz)	0.19	0.60

the tetracenomycin (*tcm*) producing minimal PKS showed that the ARO could modulate the chain length specificity of the *tcm* minimal PKS (50). The frenolicin (*fren*) producing minimal PKS has also been shown to exhibit relaxed chain length specificity. When expressed alone this minimal PKS produces only octaketides. When the minimal PKS is co-expressed with *tcmN* (ARO/CYC) from the *tcm*-producing pathway, nonaketides are synthesized, and when the KR from the *act* producing PKS is introduced, a mixture of octaketides and nonaketides are synthesized (51). Polyketides of varying chain lengths have also been described for the *whiE* producing PKS that is dependent on the presence of the CYC protein (16, 52). These findings, together with the results presented here and from our previous work disrupting the *otcD1* (CYC/ARO) gene from the OTC producing gene cluster (3), indicate that the number of extender units are determined not only by the minimal PKS but also by the auxiliary PKS components. This implies that aromatic type II polyketide biosynthesis involves multienzyme complexes consisting not only of the enzymes involved in the formation of the polyketide chain but also protein-protein interactions within the complex influence the nature of products.

The novel compound C1-1 produced by the *otcC* mutant contained an amino group at one terminus of the polyketide chain supporting the hypothesis that starter precursor of OTC is malonamide. Similarly, when the *otcD1* (CYC/ARO) gene was previously disrupted (3), all four novel polyketides produced were derived from this starter unit. These results imply that the carboxamido group is present at the start of the biosynthetic process.

Products from Strain ZGL1 Suggest a Role for the Gene Product of otcZ—There are three methylation steps during OTC biosynthesis: the addition of two methyl groups to 4-amino-ATC had been assigned to the *otcD* gene (53), while the *otcZ* gene had been deduced to encode the methylase that would be involved in 6-methylation of the pretetramid structure (26). However, a sequence data search showed that *OtcZ* and the C-4 *N*-methylase in *Streptomyces aureofaciens* (54, 55), which has

been associated with in dimethylation of 4-amino-anhydrotetracycline, are close orthologs. Therefore, it is conceivable that the *otcZ* gene product could be involved in C-4 methylation. As insertional inactivation of *otcC* also likely affected the *otcZ* gene (which is normally co-transcribed with *otcC* as part of a polycistronic message; data not shown), and the ZGL1 mutant produces polyketides with a methyl group in the C-6 equivalent position, we now suggest a role of *OtcZ* in C-4 *N*-methylation. Careful disruption of the *otcZ* gene, without destroying the complex transcriptional pattern around this locus, is in progress to establish whether 6-demethyl-OTC will be made in the recombinant.

Restoration of a Structurally Intact but Catalytically Inactive OtcC Restores the Fidelity of the Polyketide Synthase—*OtcC* is a redox enzyme, with three essential glycines (GxGxxG) at the binding pocket. From this we postulated that *OtcC* is likely to have an active site located near the NH₂-terminal region and within the highly conserved flavin-nucleotide binding site. Since we predicted that glycine residues would play a role in hydroxylation activity of *OtcC*, these were altered by *in vitro* site-directed mutagenesis without altering the protein-reading frame. Expression of the mutant gene in *E. coli* showed that the protein was structurally intact but catalytically inactive. The mutant gene was used to replace the null mutation in ZGL1, creating ZGL5. The synthase, now in a recombinant that makes the *OtcC* structural protein, had regained its programming. Moreover the “6-hydroxyl” derivative was being made, but such adventitious hydroxylation of polyketide backbones by other hydroxylases in the cell is not unprecedented. The presence of the hydroxyl group at C-6 indicates that, in the absence of *otcC*, this reaction may be undertaken to some extent by other hydroxylases present in *S. rimosus*. In the initial cloning of the OTC gene cluster, the function of the *otcC* gene had to be validated by “reverse genetics” (27), as it was not possible to use a strategy that involved complementation. In hindsight, this is because of redundancy of functional specificity among some hydroxylases.

We have used cell-free extracts to investigate the activity of ATC oxygenase. Mutagenesis has shown that Gly⁹, Gly¹¹, and Gly¹⁴ residues play an essential role in the function of ATC oxygenase.

Taken together, these results provide compelling evidence, for the first time, that proteins with post-PKS modifying activities can also interact with the minimal PKS in a processive as well as a post-assembly manner.

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Ablation of Hydroxylation Effects Polyketide Chain Length

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