Relaxed Specificity of the Oxytetracycline Polyketide Synthase for an Acetate Primer in the Absence of a Malonamyl Primer

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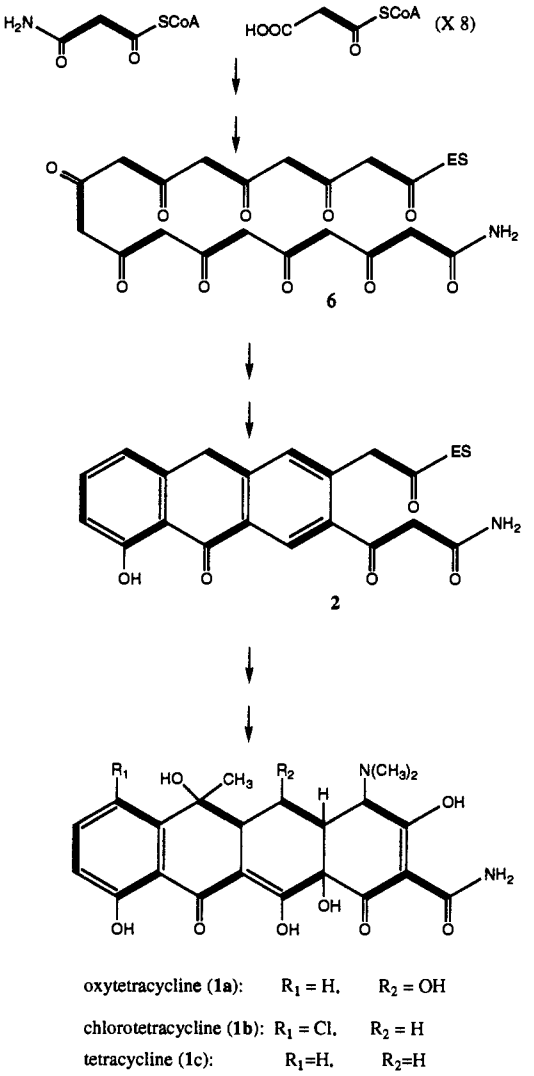
The tetracyclines (**1**) are commercially important broad-spectrum antibiotics produced by several actinomycetes. For more than three decades, the biosynthetic pathways of these polyketides have been the subject of chemical[1-8] and genetic[9-11] analysis. As shown in Figure 1, the overall pathway can be divided into two stages: (i) biosynthesis of a (presumably enzyme-bound) anthrone intermediate (**2**) from a malonamyl coenzyme A starter unit and eight malonyl coenzyme A extender units[5,7,8] and (ii) its subsequent conversion into various tetracyclines (**1a–c**). Most of our knowledge about the latter stage of the pathway comes from structural and isotopic labeling studies on the products of blocked mutants.[6] In contrast, however, mutants blocked in the first stage of the pathway do not produce detectable products. As a result, the only insights into the early steps in tetracycline biosynthesis have been derived from NMR analysis of ^{13}C-labeled tetracyclines.[7,8]

With the advent of genetic engineering methodologies in actinomycetes (reviewed in refs12 and 13) and their application to *Streptomyces rimosus*, it has been possible to clone an entire gene cluster for biosynthesis of a tetracycline, specifically oxytetracycline (**1a**).[11] More recently, the genes encoding the oxytetracycline (*otc*) polyketide synthase (PKS) have been localized and sequenced[14] and were found to be homologous to other aromatic PKS genes, such as those involved in the biosynthesis of actinorhodin, granaticin, and tetracenomycin; all of them are type II PKSs consisting of a series of separate polypeptides for catalysis of the various reactions in polyketide chain assembly, reduction, and cyclization.[15] Since the *otc* PKS is involved in the earliest catalytic steps in the pathway, these studies have opened the door for the application of genetic engineering methodologies to analyze the early stages of tetracycline biosynthesis.

Recently, we developed a potentially general strategy for the biosynthesis of novel polyketides by the functional expression of recombinant PKSs in a specially constructed *S. coelicolor*



oxytetracycline (**1a**): R_1 = H, R_2 = OH

chlorotetracycline (**1b**): R_1 = Cl, R_2 = H

tetracycline (**1c**): R_1 = H, R_2 = H

Figure 1. Biosynthetic pathway for tetracyclines: **6** is a hypothetical carbon backbone formed by the minimal *otc* PKS as a nonaketide with a malonamyl CoA starter; it would be converted by a C-9 reduction and cyclization to a still enzyme-bound anthrone (**2**). This would be converted by the later-acting enzymes of the pathway to the tetracyclines (**1a–c**).

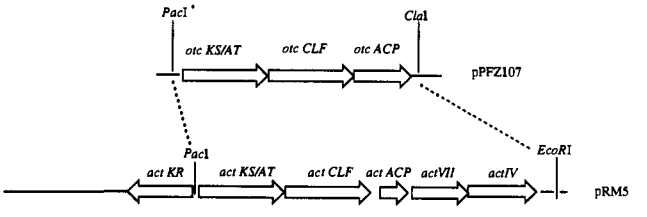


Figure 2. Construction of pSEK20: pSEK20 was constructed by replacing the *act* genes of pRM5[16] (as shown) with the genes encoding the *otc* KS/AT, CLF, and ACP. To facilitate this, a unique *Pac*I restriction site (indicated with an asterisk) was introduced upstream of the *otc* KS/AT.

expression system.[16] Analysis of the structures of the novel molecules revealed key features of the mechanisms by which the type II PKSs for bacterial aromatic compounds control their catalytic specificities.[16-20] On the basis of the results of our earlier

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studies on related aromatic PKSs, it could be anticipated that the essential components of the minimal *otc* PKS (consisting of a bifunctional ketosynthase/putative acyltransferase (KS/AT), a chain length determining factor (CLF), and an acyl carrier protein (ACP)) are responsible for (i) the biosynthesis of a polyketide backbone precursor of a defined length and (ii) a regiochemically precise initial cyclization of the nascent polyketide chain. Furthermore, the results allowed the prediction that coexpression of the actinorhodin ketoreductase (*act* KR) with the above three components of the *otc* PKS should lead to reduction of the C-9 carbonyl of the polyketide backbone. However, since the polyketide backbone of oxytetracycline is thought to start with an unusual malonamyl primer[10,11] instead of the more typical acetate primer used by *S. coelicolor* to make its native polyketide, actinorhodin, it was not a foregone conclusion that the minimal *otc* PKS would be able to make any product in the heterologous host. Even if it managed to do so, the structure of the polyketide product could not be predicted with confidence.

To address this problem, we cloned the genes encoding the *otc* KS/AT, CLF, and ACP into the *S. coelicolor* expression system,[21] along with the *act* KR gene (Figure 2). The resulting plasmid, pSEK20, was introduced by transformation into CH999, the genetically engineered expression host strain of *S. coelicolor* A3(2) that lacks the entire set of actinorhodin biosynthetic genes.[16]

Upon growth under conditions described earlier,[19] recombinant CH999/pSEK20 was found to produce two aromatic polyketides. ^{1}H and ^{13}C NMR spectroscopy revealed that these molecules were identical to RM20b (**3**) and RM20c (**4**) (Figure 3), which were earlier identified in a recombinant strain expressing the minimal tetracenomycin (*tcm*) PKS and the *act* KR, i.e., CH999/pRM37.[19] The amounts of RM20b and RM20c recovered from CH999/pSEK20 were comparable to those in CH999/pRM37 reported earlier. Sodium [1,2-$^{13}C_2$]acetate feeding experiments were carried out (as before[19]) to confirm that the polyketide backbones of RM20b and RM20c produced by CH999/pSEK20 were indeed derived from 10 acetate units (Figure 3).

The above results demonstrate that, even in the absence of additional proteins encoded by the *otc* gene cluster (or elsewhere in the *S. rimosus* genome), the minimal *otc* PKS is able to turn over with the same efficiency as other minimal PKSs.[20] Furthermore, the *otc* PKS is intrinsically capable of synthesizing an acetate-derived polyketide backbone (**5**) that is isosteric with the predicted polyketide backbone precursor of oxytetracycline (**6**),[8] even though it requires one additional condensation cycle. (Alternatively, an acetoacetate primer could be used to synthesize the RM20b/RM20c backbone via eight condensation cycles.) On the basis of their results of isotopic labeling studies, Thomas and Williams concluded that, while nitrogen insertion into **6** may indeed occur via a malonamate primer unit, an equally acceptable scheme would involve the biosynthesis of a nonaketide backbone starting with a malonate primer unit, followed by subsequent formation of the amide moiety.[8] The results reported here favor the former hypothesis, since neither **3** nor **4** starts with a malonate

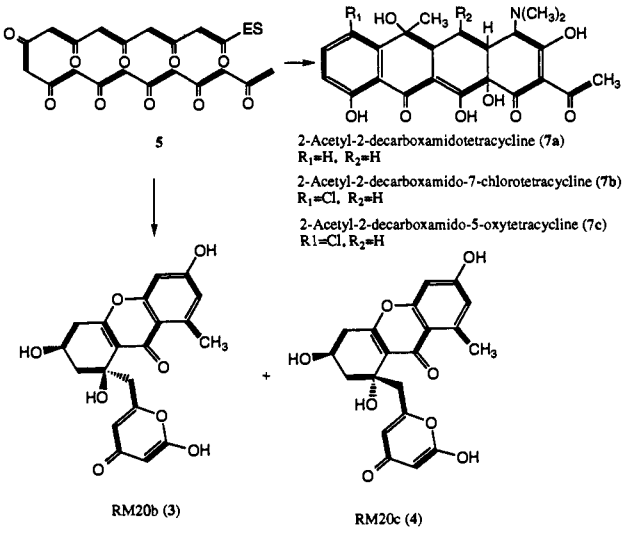

Figure 3. Polyketide products of the *otc* PKS in CH999/pSEK20 and mutants of *S. rimosus* and *S. aurofaciens*: RM20b and RM20c are produced by CH999/pSEK20, whereas the 2-acetyl-2-decarboxamidotetracyclines are synthesized by genetically uncharacterized mutants of *S. rimosus* and *S. aureofaciens*.

primer, even though both malonyl CoA and a malonyl CoA transferase must be available during polyketide biosynthesis. However, since the biosynthesis of **6** in *S. rimosus* could theoretically require the presence of two separate malonyl CoA transferases for starter and extender unit transfer to the PKS, the latter hypothesis cannot be altogether ruled out. Nevertheless, our results are consistent with earlier reports of the biosynthesis of 2-acetyl-2-decarboxamidotetracyclines (**7a–c**) by genetically uncharacterized mutants from *Streptomyces aureofaciens*[22] and *S. rimosus*.[4] They also confirm earlier predictions that the PKS dictates the regiospecificity of the first aldol condensation of the nascent polyketide backbone,[18] and that the *act* KR is able to reduce the nascent polyketide backbone product of any homologous aromatic PKS at C-9.[16,17] Finally, our results provide a conceptually novel starting point for studying the early steps in oxytetracycline biosynthesis by incrementally expressing additional *otc* enzymes in CH999/pSEK20. Structural analysis of the polyketide products of such a nested series of recombinant strains could lead to new insights into the pathway and mechanisms for oxytetracycline biosynthesis. At the same time, as illustrated above, such an approach could give rise to engineered polyketides that might otherwise not be accessible via conventional mutagenesis methodologies starting from naturally occurring producer strains.

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(21) In order to achieve this, the 5′ end of the gene encoding the *otc* KS/AT [14] was resequenced, and a unique *Pac*I restriction site, together with a ribosome binding site, was engineered (as before[16]) immediately upstream of this gene in pPFZ107 (an *Sst*I subclone of pPFZ518[11] containing the *otc* genes of interest). The 2.9-kb *Pac*I–*Cla*I region containing the genes encoding the KS/AT, CLF, and ACP was then cloned into the *Pac*I–*Eco*RI sites of pRM5.[16]

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