Engineered Biosynthesis of a Novel Amidated Polyketide, Using the Malonamyl-Specific Initiation Module from the Oxytetracycline Polyketide Synthase

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Tetracyclines are aromatic polyketides biosynthesized by bacterial type II polyketide syntheses (PKSs). Understanding the biochemistry of tetracycline PKSs is an important step toward the rational and combinatorial manipulation of tetracycline biosynthesis. To this end, we have sequenced the gene cluster of oxytetracycline (oxy and otc genes) PKS genes from Streptomyces rimosus. Sequence analysis revealed a total of 21 genes between the otrA and otrB resistance genes. We hypothesized that an amidotransferase, OxyD, synthesizes the malonamate starter unit that is a universal building block for tetracycline compounds. In vivo reconstitution using strain CH999 revealed that the minimal PKS and OxyD are necessary and sufficient for the biosynthesis of amidated polyketides. A novel alkaloid (WJ35, or compound 2) was synthesized as the major product when the otx-encoded minimal PKS, the C-9 ketoreductase (OxyJ), and OxyD were coexpressed in CH999. WJ35 is an isoquinolone compound derived from an amidated decaketide backbone and cyclized with a novel regioselectivity. The expression of OxyD with a heterologous minimal PKS did not afford similarly amidated polyketides, suggesting that the otx-encoded minimal PKS possesses novel starter unit specificity.

Tetracyclines are among the most important antibiotics known to mankind in the last half century (15). The broad-spectrum antimicrobial activities of tetracyclines have resulted in their widespread clinical use against infectious gram-positive and gram-negative bacteria (21). The emergence of microbial resistance to tetracyclines has severely limited their effectiveness and has prompted the search for analogs that can overcome the known modes of antibiotic resistance (14, 40, 55). The recently published, elegant total synthesis of 6-deoxytetracyclines by the ketosynthase (KS), the chain length factor (CLF, or KS), and the acyl carrier protein (ACP) (12). Dedicated tailoring enzymes then transform the carbon backbone into fused, richly substituted compounds. Shunt products of the oxytetracycline pathways have been characterized, which enabled deduction of the amidated backbone. Using a specific starter unit affords a novel isoquinolone compound derived from an amidated decaketide backbone.

MATERIALS AND METHODS

Bacterial strains and general techniques for DNA manipulation. Streptomyces coelicolor strain CH999 was used as a host for the transformation of shuttle vectors. Protoplast preparation and polyethylene glycol-assisted transformation were performed as described by Hopwood et al. (23). Escherichia coli XL1-Blue (Stratagene) was used for the manipulation of plasmid DNA. Streptomyces rimosus (ATCC 10970) was obtained from ATCC and was cultured for extraction of genomic DNA. Unmethylated DNA was obtained using the methylase-deficient strain GM2163 (New England Biolabs).
Sequencing of oxy (ote) cluster. The complete genomic DNA library of S. rimosus was constructed using a pWEB cosmid cloning kit (Epipcenter). The cosmids clone pYT264, which harbors the 21.2-kb oxy gene cluster flanked by ote1 (18) and otrB, was identified by PCR screening. A combination of shotgun and primer-walking techniques was used to obtain the sequence information. Open reading frames (ORFs) were detected and analyzed using Frameplot software (http://www.nih.go.jp/~H11011) and the putative roles of the proteins were assigned using protein-protein BLAST and Pfam analysis. To simplify naming and analysis of the cluster for subsequent studies, the genes were renamed oxyA to oxyT in a linear fashion. The five previously sequenced genes of the cluster are cross-referenced in Table 1.

Construction of shuttle plasmids for biosynthesis. The following primers were used to amplify the individual genes: for oxyA, 5'-GGTTAATTACGAGGGATCCAGCATGGCAATACGCCAGGACG-3' (PstI/BamHI) and 5'-GGTCTAGAGCACTCGTGCCGGCTGTCCTC-3'; for oxyB, 5'-GGCACTATGCGAGGGAGCCGGCAGTACCGCGCCGATCGTAAAGCCAAGCCGCGCCTCGCCCCC-3' (SpeI/BamHI) and 5'-GGGCTAGAGGCTCAGCGGCGCCTCGCCCCC-3'; for oxyC, 5'-GGGCTAGAGGCTCAGCGGCGCCTCGCCCCC-3'; for oxyD, 5'-AATTCTAGAGGGAGGCCGCTGACCA-3', 5'-AAAATCTAGAAGGGAGGCCGCTGACCA-3'; for oxyE, 5'-AATTCTAGAGGGAGGCCGCTGACCA-3', 5'-AAAATCTAGAAGGGAGGCCGCTGACCA-3'; for oxyF, 5'-AATTCTAGAGGGAGGCCGCTGACCA-3', 5'-AAAATCTAGAAGGGAGGCCGCTGACCA-3'.

Sequencing of the oxy tet cluster. 2574 ZHANG ET AL. APPL. ENVIRON. MICROBIOL.

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The biosynthetic pathway was established previously by Hunter and coworkers based on an exhaustive analysis of shunt products. The assignments of the tailoring enzymes are based on their functions obtained through protein-protein BLAST analysis. We confirmed in this report the role of OxyD and the minimal PKS in the biosynthesis of an amidated decaketide. We also confirmed the role of OxyA as the C-9 ketoreductase.

FIG. 1. Proposed biosynthetic pathway of oxytetracycline (1) in S. rimosus. The biosynthetic pathway was established previously by Hunter and coworkers based on an exhaustive analysis of shunt products. The assignments of the tailoring enzymes are based on their functions obtained through protein-protein BLAST analysis. We confirmed in this report the role of OxyD and the minimal PKS in the biosynthesis of an amidated decaketide. We also confirmed the role of OxyA as the C-9 ketoreductase.

TABLE 1. Genes in the oxytetracycline biosynthetic cluster and deduced roles based on sequence homology

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<tr>
<th>Gene</th>
<th>Start position</th>
<th>Stop position</th>
<th>Predicted size (kDa)</th>
<th>Cts homolog (reference)a</th>
<th>Protein (reference)</th>
<th>Deduced role</th>
<th>% Identity</th>
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<td>MtmO1 (32)</td>
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<td>47</td>
</tr>
</tbody>
</table>

a We compared the partially annotated chlorotetacycline PKS cluster (43, 51) from Streptomyces aureofaciens with the oxy-c encoded PKS cluster.

b Gene names in parentheses are loci of the oxy (ote) PKS previously identified through mutant complementation studies. Sequence information for these genes is not available publicly, except for that shown in bold. The cluster was renamed in this study to facilitate sequence analysis. References for previously sequenced genes are listed in the text.

c No stop codon was detected. The putative protein size was based on alignment with closely related acyl-CoA ligases.

d A 60-bp deletion at the 3' end was found in the deposited otec1 sequence and has been corrected here.
GTACGGGAATCCGGGTACCCCT-3'. The introduced restriction sites are shown in italics, and the restriction enzymes are indicated in parentheses. The optimal ribosome binding site was introduced at the 5' end of each gene and is underlined. All oxy genes were amplified from pYT264, and multicistronic cassettes were constructed using the compatible XbaI/SpeI cohesive ends for most of the genes, except for oxyA and oxyB, which were cloned as a single PacI/XbaI cassette. Different combinations of genes were introduced into pYT315 (a pRMS-derived vector) to yield the constructs shown in Table 2.

**Culture conditions and purification of polyketides.** Strains were grown on solid R5 plates with 25 mg/liter thriostrepton at 30°C for 7 to 10 days. For analytical high-performance liquid chromatography (HPLC) analysis, a well-pigmented plate was chopped into fine pieces and extracted with 50 ml of ethyl acetate-methanol-acetic acid (89%-9.8%-1.2%). Extracts were dried over anhydrous Na2SO4. The solvent was removed in vacuo, and the residue was dissolved in 2 ml of acetone to be loaded onto two preparative thin-layer chromatography (TLC) plates (20-cm 

Fig. 2. Organization of oxy (ote) biosynthetic gene cluster. The biosynthetic enzymes are located between the two resistance genes otrA and otrB. Previously sequenced genes are boxed. For details of enzyme function assignments, see Table 1.

**RESULTS**

Sequencing of oxytetracycline gene cluster. To study the origin of the amide moiety observed among all tetracyclines and to gain additional insight into the biosynthesis of tetracyclines, we sequenced the oxytetracycline (oxy and ote) gene cluster using a combination of shotgun sequencing and cosmid walking techniques. The gene cluster was previously mapped to be between the two resistance genes otrA (18) and otrB (2, 10, 38). A total of 21 ORFs were clustered between otrA and otrB (Fig. 2 and Table 1), including the previously sequenced minimal PKS (oteY1-1, oteY1-2, and oteY1-3) (28), cyclase (oteD1) (46), and ATC oxygenase (oteC) (2, 45) genes. Sequencing information for the remainder of the cluster, which has been studied through mutant complementation experiments (24), was not available from GenBank prior to this study. To simplify designation of the gene cluster, we named the oxy genes oxyA to oxyT from end to end, as graphically represented in Fig. 2. The functions of the proteins were assigned based on sequence similarities to known aromatic PKS enzymes and are listed in Table 1.

A putative oxytetracycline biosynthetic pathway was previously established, largely aided by the identification of metabolic shunt products (Fig. 1) (9, 24). The minimal PKS (OxyA, OxyB, and OxyC) accepts a malonamyl starter unit and condenses eight equivalents of malonate to afford an amided decaketide backbone. Two distinct nitrogen-inserting enzymes (OxyD and OxyQ) were found to be encoded in the oxy gene cluster. OxyD is an amidotransferase and participates in the biosynthesis of the amide starter unit (see below). OxyQ is homologous to aspartate/tyrosine/aromatic aminotransferases involved in amino acid metabolism (22) and is likely the enzyme that transaminates C-4 of 4-keto-ATC, using pyridoxal 5'-phosphate as a cofactor. OxyR, the putative pyridoxamine 5-phosphate oxidase, is translationally coupled to OxyQ with overlapping stop/start codons. A small set of proteins homologous to OxyR are found in the literature, with the most notable being ActVA-ORF2 (11), an enzyme of unknown function involved in actinorhodin biosynthesis.

Two ORFs (oxyJ and oxyM) encoding NADPH-dependent ketoreductases (KR) are present in the gene cluster. OxyJ catalyzes regiospecific C-9 reduction of the oxytetracycline backbone (see below). The specific role of OxyM in the oxy cluster is unresolved (24) and perhaps reflects redundancy in function. Only one KR gene is present in the recently sequenced chlorotetracycline gene cluster (51). The bifunctional cyclase/dehydratase OxyK (OtcD1) was previously identified by Petkovic and coworkers and was assigned to catalyze formation of the D ring (46). OxyN shows strong sequence similarity to second-ring cyclases (such as DpsY from the daunorubicin cluster [33]) and presumably catalyzes the aldol reaction between the C-5 carbonyl and the acidic C-14 methyl
TABLE 2. Plasmid constructs and resulting polyketide products

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genes</th>
<th>Major product</th>
<th>Approx. yield (mg/liter)</th>
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<tr>
<td>pYT319</td>
<td>oxyABC</td>
<td>SEK15</td>
<td>30</td>
</tr>
<tr>
<td>pYT318</td>
<td>oxyABCJ</td>
<td>RM20b</td>
<td>30</td>
</tr>
<tr>
<td>pWJ35</td>
<td>oxyABCDJ</td>
<td>Compound 2</td>
<td>20</td>
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<tr>
<td>pWJ35a</td>
<td>oxyABCDJactIII</td>
<td>Compound 2</td>
<td>20</td>
</tr>
<tr>
<td>pWJ40</td>
<td>actI ORFs I-II oxyCDJ</td>
<td>Mutacin</td>
<td>20</td>
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<tr>
<td>pWJ48</td>
<td>tcmlKL oxyCDJ</td>
<td>RM20b</td>
<td>&lt;5</td>
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</table>

a Streptomyces coelicolor strain CH999 was used as the host for polyketide biosynthesis. Each plasmid is derived from pRM5.

yleny to form the C ring. Subsequent formation of the B ring should be spontaneous, as seen for other aromatic polyketides such as aklanonic acid (33). Formation of the A ring is presumably catalyzed by OxyI, a small protein that is homologous to MtmX. MtmX has been implicated in catalyzing the formation of the final ring during mithramycin biosynthesis (32).

Two S-adenosylmethionine (SAM)-dependent methyltransferases (OxyF and OxyT) are encoded in the gene cluster. OxyT shows high sequence similarity to O-methyltransferases and is cotranscribed with OxyQ, OxyR, and OxyS (OtcC) (37), and it may therefore be associated with these downstream tailoring steps. We putatively assigned OxyT to dimethylation of the free amine present in 4-amino-ATC to yield ATC. OxyF is putatively assigned to methylate C-6 of pretetramid that yields 6-methyl-pretetramid. Four oxygenase genes are present in the oxy gene cluster, including those for the previously identified ATC oxygenase OxyS (OtcC) (2, 45). Interestingly, all four oxygenases are homologous to enzymes encoded in the mithramycin gene cluster in Streptomyces argillaceus (32). OxyE, OxyL, OxyG, and OxyS (OtcC) show strong sequence similarities to MtmOI, MtmOII, MtmOIII, and MtmOIV, respectively, indicating that the oxy and mtm gene clusters are evolutionarily closely related. Prado and coworkers showed that MtmOII is involved in an early hydroxylation step to yield the mithramycin precursor 4-demethylpremithramycinone (47). Thus, OxyL is assigned to catalyze an analogous reaction in the biosynthesis of compound 1, using 6-methyl-pretetramid as a substrate. OxyG is a small (11-kDa) quinone-forming oxygenase and is also homologous to ElmH from the tetracenomycin gene cluster (48). OxyG is therefore possibly involved in the quinone formation of ring A in 4-keto-ATC. OxyE is a flavin adenine dinucleotide-dependent monooxygenase and is putatively assigned to catalyze the C-5 oxidation of 5a,11a-dehydrotetracycline to yield 5a,11a-dehydroxysteroytetracycline.

The last step in the enzymatic cascade is the reduction of 5a,11a-dehydroxysteroytetracycline to compound 1. The gene encoding the reductase (TchA) responsible for the same step in chlorotetracycline biosynthesis was mapped outside the chlorotetracycline gene cluster (42). We have used degenerate primers and isolated a homologous ORF in the S. rimosus genome (result not shown).

Enzymes putatively involved in malonamyl starter unit biosynthesis. We previously showed that nonacetate-primed aromatic PKSs contain discrete initiation modules that typically consist of (i) a second ACP (ACP_p) that primes the minimal PKS with the nonacetate unit (34), (ii) a ketosynthase III that synthesizes the ACP_p-bound primer unit (39), and (iii) a potent acetyl-ACP thiolase that hydrolyzes the competing acetyl-ACP species that may otherwise initiate polyketide assembly with acetate (56). An ORF (oxyP) 15 kb downstream of the minimal PKS genes encodes an enzyme of high sequence homology to acetyl-ACP thiolases, such as ZhuC from the R1128 PKS (34) (49% identity) and FrmK from the frenolicin PKS (1) (43% identity). Based on sequence homology, OxyP may play an analogous role in the biosynthesis of compound 1 to ensure correct chain initiation by the malonamyl starter unit.

Surprisingly, no homologs of ketosynthase III and ACP_p are present in the oxy cluster, suggesting that the oxy-encoded PKS employs a distinct set of enzymes to initiate polyketide biosynthesis. OxyD, encoded immediately downstream of the minimal PKS genes (oxyABC), is a 613-amino-acid protein that shows high sequence identity (60%) to the type II (also known as the Ntm family) aspartate synthases (31). Aspartate synthase converts aspartic acid to asparagine in an ATP-dependent two-step reaction, using glutamine or ammonia as the amine donor (3–5). OxyD contains the conserved N-terminal nucleophilic cysteine (Cys2) which is involved in the hydrolysis of glutamine (64) as well as the conserved adenylation domain that activates the acid moiety of the amine acceptor (6). We hypothesize that OxyD may therefore amidate either malonyl-coenzyme A (malonyl-CoA) or malonyl-ACP to yield malonamyl-CoA or malonamyl-ACP (see Fig. 4), which then primes the oxy-encoded KS-CLF for chain elongation.

Biosynthesis of an amidated polyketide by extended minimal oxy-encoded PKS. To examine the roles of OxyD in the biosynthesis of an amidated polyketide in vivo, a series of Streptomyces coelicolor shuttle vectors derived from pRM5 were constructed (35). The plasmids were transformed into S. coelicolor strain CH999 (35) and were analyzed for polyketide biosynthesis (Table 2).

The minimal oxy-encoded PKS (oxyABC, in pYT319) produced ample amounts of SEK15 (see Fig. 4) (53) (30 mg/liter), while the addition of OxyJ (pYT318) yielded reduced deca- ketide RM20b as the major product (30 mg/liter), consistent with the data reported by Fu et al. (19). OxyJ is therefore the regiospecific C-9 ketoreductase in the oxy-encoded PKS. Therefore, to reconstitute the steps of oxytetracycline biosynthesis, OxyJ was coexpressed in all subsequent studies.

Significantly, a new polyketide product was identified when OxyD was coexpressed with the minimal oxy-encoded PKS and OxyJ. The oxyD gene encoding the amidotransferase was cloned into pYT318 to yield pWJ35. CH999 transformed with pWJ35 produced an intense yellow pigmentation on solid R5 medium that was not observed before. HPLC analysis of the CH999/pWJ35 extract with positive ion extraction for m/z 388 showed that no trace of compound 2 was produced in the absence of OxyD, confirming the essential role of OxyD in the biosynthesis of compound 2. A sufficient amount of compound 2 was extracted from 2 liters of CH999/pWJ35 fermentation and was purified to homogeneity.
using reverse-phase preparative HPLC and normal-phase thin-layer chromatography. HRMS indicated a molecular formula of C_{19}H_{17}NO_{8} (m/z = 388.1027 [M + H]^+; difference of 0.0016), consistent with the molecular composition of an amidated decaketide that has been subjected to one ketoreduction at C-9 and two cyclization/dehydration events. When OxyJ was replaced by the heterologous ActIII KR (pWJ35; Table 2), no change in yield and selectivity of compound 2 was observed.

The structure of compound 2 was elucidated using a combination of 1D and 2D NMR experiments (Table 3) and is shown in Fig. 4. Data obtained from $^{13}$C, distortionless enhancement by polarization transfer (90, 135), and HMQC experiments identified 19 carbon signals, supporting the number of carbon atoms predicted by HRMS. No methyl carbon atom is present, consistent with the lack of an acetate starter unit. The presence of the α-pyrone moiety as a result of O-1/C-5 cyclization is clearly evident from both $^1$H (δ_{H12} = 5.3 and δ_{H14} = 6.1) and $^{13}$C (δ_{C3}) data. The C-6 methylene was assigned using $^1$H-$^{13}$C HMBC (Table 3). The linear connectivities among C-6 to C-9 were readily established using correlation spectroscopy and $^1$H-$^{13}$C HMBC. The $^1$H multiplet at δ_{H4} 4.5 is assigned to H-9 and is nearly identical to the H-9 protons present in mutactin (66) and YT46 (57), with each containing a 9-OH as a result of C-9 ketoreduction (Fig. 4). The assignments of the C-8/C-10 methylenes were readily achieved using correlation spectroscopy, HMBC, and HMQC experiments. The key assignment of the C-7 aliphatic ketone enabled us to eliminate the common C-7/C-12 intramolecular cyclization observed among aromatic polyketides (36). The remaining carbon backbone cyclizes and dehydrates through a C-13/C-18 heterocycle. The sharp proton singlet at δ 12.9, which shows long-range coupling to C-16, C-17, and C-18, is assigned to OH-17. The broad singlet at δ 10.2 is assigned to the NH proton. These assignments are consistent with the isquinolone protons observed in the natural product fredericamycin (7). The presence of a lactam ring was unambiguously supported by the $^1$H-$^{15}$N HMBC experiment, in which both H-10 and H-12 showed long-range coupling to the nitrogen (Table 3). Therefore, WJ35 is a shunt product of the oxy-encoded PKS in the absence of the first-ring cyclase.

### OxyD does not interact with heterologous minimal PKSs.

We assayed whether heterologous minimal PKSs that are normally primed by acetate can interact productively with OxyD and OxyC to yield amidate polyketides. The act-encoded KS-CLF (65) and the tcm-encoded KS-CLF (54) were each coexpressed in the presence of Act KR, OxyD, and OxyC in CH999. Heterologous KS-CLFs have been shown to function with the OxyC ACP to produce acetate-primed compounds (26). To our surprise, no amidated polyketides were recovered from these strains (Table 2). We were able to detect only acetate-primed mutactin and RM20b from CH999/pWJ40 and CH999/pWJ48, respectively. This is in sharp contrast to the broad compatibility between the R1128 initiation module and heterologous minimal PKSs (57). Hence, fundamental differences exist between oxy-encoded and other minimal PKSs that allow the former to accept both amide and acetate starter units.

### DISCUSSION

The tetracyclines are universally primed with a unique malonamate starter unit not found in any other polyketides. Past work has cloned five oxy (otc) genes and shed light on the minimal PKS. However, the initiation mechanism that leads to the production of the unique malonamate starter unit is not well understood. The lack of this information has hampered efforts to engineer the initiation of tetracycline biosynthesis. Understanding the biosynthesis and incorporation of the novel amide functionality is therefore a top priority in studying tetracycline biosynthesis.

In this work, we sequenced the previously mapped oxy (otc) gene cluster from S. rimosus, which allowed us to propose that OxyD is involved in the formation of the malonamate starter unit. We reconstituted the minimal oxy-encoded PKS in CH999,
as well as an extended minimal PKS including OxyD. Signifi-
cantly, the coexpression of minimal PKS with the C-9 KR and
OxyD produced a unique, amidated polyketide 2 at a high yield
(Table 2). Establishing the origin of the amide unit and con-
structing the complete backbone required for tetracycline bio-
synthesis are significant steps towards rational bioengineering
of this family of compounds. Isolation of this novel isoquin-
olone 2 revealed several important biochemical properties of
the *oxy*-encoded PKS, as follows.

(i) OxyD is the only enzyme required to biosynthesize and
insert an amide starter unit into the polyketide backbone in the
heterologous host. CH999/pWJ35 produced predominantly the
amidated polyketide, indicating preferential incorporation of a
malonamyl unit over acetyl-OxyC by the *oxy*-encoded minimal
PKS, even in the absence of an acetyl-ACP editing enzyme. A
homologous enzyme (TcsG) from the chlorotetracycline PKS
(43) likely performs the same catalytic function as OxyD during
chlorotetracycline biosynthesis.

(ii) The amidated compound 2 is derived from an intact
decaketide (10-carbonyl) backbone, indicating that in the het-
erologous host CH999, the *oxy*-encoded minimal PKS is able to
maintain correct chain length control. We did not find any
truncated polyketide products in the extract of CH999/pWJ35,
preumably because these truncated products are present at
much lower levels in this strain and may have escaped our
purification and detection protocols. We did detect a truncated
polyketide in a pWJ35-derived construct coexpressing OxyK
(OtcD1) (unpublished data), consistent with the conclusion by
Petkovic and coworkers that the *oxy*-encoded PKS also synthe-
sizes truncated polyketides (46).

(iii) The most surprising structural feature of compound 2 is
perhaps the lack of C-7/C-12 cyclization. The decaketide back-
bone must undergo two unique cyclization steps (C-13/C-18 and
N-19/C-11) to yield the isoquinolone backbone. This is appar-
ently a dominant mode of cyclization, since no alternatively cyclized
amidated polyketides were detected in the fermentation extract.
The cyclization regioselectivity of compound 2 was completely
unanticipated, considering that C-9-reduced, acetate-primed de-
cketides cyclize solely between C-7 and C-12 (as observed in
RM20b/c) (49). From the crystal structures of *act*-encoded KR
(29) and *act*-encoded KS-CLF (25), it has been tempting to
propose that the C-7/C-12 connectivity is formed within the
active site of KS, prior to C-9 reduction by the KR. It is evident
from the novel structural features of compound 2 that C-9 keto-
reduction must take place independently of C-7/C-12 cyclization.
OxyJ must therefore recognize an uncyclized polyketide back-
bone to yield compound 2.

(iv) Furthermore, formation of the isoquinolone must take
place immediately after the complete assembly and release of
the polyketide product. Premature C-13/C-18 cyclization will
prevent the correct C-7/C-12 cyclization catalyzed by OxyK
during the tailoring steps of tetracycline biosynthesis (Fig. 1).
Malonate is a very rare metabolite under normal physiolog-
ical conditions and is a toxic compound due to its potent
inhibition of succinate dehydrogenase in the tricarboxylic acid
cycle (30), and hence it is unlikely to serve as a substrate for

**FIG. 4. Biosynthesis of compound 2 by the extended *oxy*-encoded minimal PKS.**
OxyD. Therefore, we hypothesize that biologically plausible malonyl substrates for OxyD are malonyl-CoA and malonyl-OxyC. Malonyl-OxyC may be preferred over malonyl-CoA for the following reasons. (i) Malonyl-CoA is involved in other essential cellular processes, including fatty acid biosynthesis. Amidation of a significant amount of the intracellular malonyl-CoA pool may therefore be detrimental to host strain viability (we did not observe any difference in the growth characteristics of CH999/pWJ35). Malonyl-OxyC, on the other hand, is dedicated to the oxy-encoded PKS and is not involved in the primary metabolism of the host. The robust growth of CH999/pWJ35 supports the above argument that the malonyl-CoA pool may therefore be detrimental to host strain viability. The robust growth of CH999/pWJ35 supports the above argument that the malonyl-CoA pool may therefore be detrimental to host strain viability.

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