OVERPRODUCTION OF THE ACYL CARRIER PROTEIN COMPONENT OF A TYPE II POLYKETIDE SYNTHASE STIMULATES PRODUCTION OF TETRACENOMYCIN BIOSYNTHETIC INTERMEDIATES IN *Streptomyces glaucescens*

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The development of microorganisms with improved antibiotic production is an important goal in the commercialization of new pharmaceuticals or in lowering the cost of established drugs. We report a way to achieve this for biosynthetic intermediates of an antibiotic made by the polyketide pathway whose earliest steps involve a Type II multienzyme complex. Introduction of the *tcmKLM* β-ketoacyl : ACP synthase and acyl carrier protein (ACP) genes or just the *tcmM* ACP gene into the tetracenomycin (Tcm) C-producing *Streptomyces glaucescens* wild-type strain, or its *tcmN* or *tcmO* blocked mutants, on high copy vectors under the control of strong promoters caused a 2 to 30-fold overproduction of Tcm D3 and some other biosynthetic intermediates (or shunt products) and a 25 to 30% increase in Tcm C production relative to the control strains carrying the plasmid vector only. However, Tcm C production was not greater than that obtained with the vector-free wild-type strain. The unexpected effect of increased ACP on Tcm D3 production suggests that the level of this protein can influence either the activity or level of the three other components of the Tcm polyketide synthase.

There are now many commercial applications of recombinant organisms to produce proteins useful in human and veterinary medicine as well as enzymes used in microbial and chemical processes. In contrast, the use of genetically engineered organisms for the commercial production of specialty chemicals, including vitamin C,[1] indigo,[2] and benzoquinone,[3] and antibiotics,[4~10] is a comparatively under-developed field. For the antibiotic producing bacteria, CHATER has adumbrated the ways to construct overproducing strains.[11] Introduction of extra copies of positively-acting regulatory genes, which often are part of the cluster of antibiotic biosynthesis and self-resistance genes, into wild-type organisms (reports of their effect in commercial high-producers have not appeared) affords the simplest way to attain significant yield increases. This has become an increasingly attractive way to obtain high-producing strains since the number of cloned genes for the biosynthesis of antibiotics in *Streptomyces* spp. and related organisms has grown very fast in recent years.[11]

Comparison of the genes for the biosynthesis of aromatic polyketides such as actinorhodin,[12] granaticin,[13] and tetracenomycin C[14] (Tcm C) has revealed a high similarity in the organization of the polyketide synthase (PKS) genes.[15,16] The sequences of three of the proteins encoded by these genes, the predicted β-ketoacyl : ACP synthase, acyl carrier protein (ACP) and ketoacyl reductase, are quite similar to the corresponding enzymes of Type II fatty acid synthases from bacteria and plants.[15,16] In

view of their wide-spread occurrence among actinomycetes,[15,16] the questions naturally arise whether new, so-called hybrid antibiotics can be produced by interspecies exchange of PKS genes from different producers of aromatic polyketide antibiotics and whether these genes could be used in the overproduction of antibiotics. HOPWOOD and co-workers have reported the production of two new polyketide antibiotics by introduction of all or part of the cloned actinorhodin genes into strains that produce the related benzoisochromane quinones, granaticin and medermycin;[17] and, more recently, have described an unexpected increase in the formation of pigmented substances believed to be actinorhodin and apparent intermediates of its biosynthesis by the manipulation of several Type II ACP genes.[18]

We have cloned from *Streptomyces glaucescens*[19,20] all of the biosynthetic and resistance genes required for Tcm C biosynthesis on a 12.6 kb DNA fragment[14,21~26] whose introduction into *Streptomyces lividans* by transformation results in the heterologous production of Tcm C.[21] Since we have not found evidence for a dedicated positively-acting regulatory gene in this cluster, we have focused on the genes governing the earliest steps of Tcm C biosynthesis to see if we could develop a way to increase antibiotic production. Different plasmids were constructed to determine the minimal number of genes required for the production of Tcm F2, the first detectable intermediate of Tcm C biosynthesis (Fig. 1); these turned out to be the *tcmKLMN* genes.[26] Here we report the effect of plasmids containing different combinations of the β-ketoacyl: ACP synthase (*tcmK* and *tcmL*), the ACP (*tcmM*), a multifunctional cyclase-dehydratase-3-*O*-methyltransferase (*tcmN*), and the C-8 *O*-methyltransferase (*tcmO*) genes, on the production of Tcm C and its biosynthetic intermediates in *S. glaucescens*. In some cases, additional copies of these genes give rise to higher production of Tcm C or its biosynthetic intermediates and(or) shunt products of the Tcm pathway.

Experimental

Strains and Plasmids

The *S. glaucescens* GLA.0 wild-type strain and the *S. glaucescens* WMH1082 (formerly GLA. 4~48) and WMH1090 (formerly GLA. 12~41) strains were from MOTAMEDI *et al.*[27] The plasmids pIJ702,[28] pIJ486,[29] pELE42 and pELE37,[30] pWHM722 and pWHM723,[26] and pWHM701[31] are described elsewhere. A 1.6 kb *Mro*I-*Bgl*II DNA fragment carrying the *tcmN* gene[26] was cloned into pIJ702 downstream of the *melC1* promoter as pWHM700.

Culture Conditions

R2YENG liquid medium[27] was used for Tcm C production and R2YENG agar for transformation and regeneration of protoplastes of *S. glaucescens*. Strains were maintained on HT sporulation medium.[27] Thiostrepton (obtained from Sal Lucania, Squibb Institute for Medical Research, Princeton, N.J.) concentrations of 50 μg/ml on solid medium and 10 μg/ml in liquid medium were used for selective growth of *S. glaucescens* transformants. Samples of cultures were taken after 72, 96 and 120 hours, and Tcm C and its biosynthetic intermediates were extracted from 1 ml aliquots with 0.5 ml ethyl acetate containing glacial acetic acid (20 μl/ml). Authentic samples of Tcm D3 and Tcm B3 were kindly provided by A. ZEECK (Universität Göttingen, Germany).

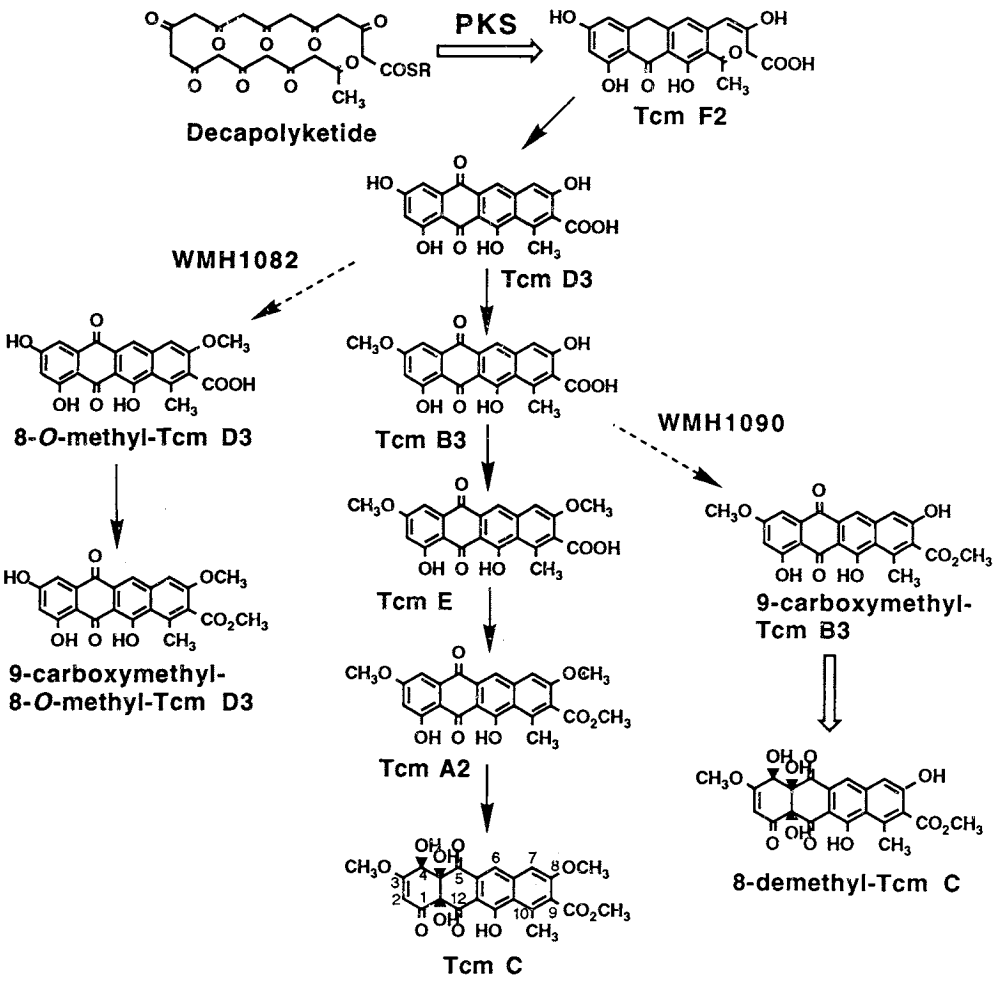
Analytical Techniques

Quantification of Tcm C and its biosynthetic intermediates was performed by reversed phase high performance liquid chromatography (HPLC) on a Waters C_{18} Novo-Pak column, 10 cm × 5 cm i.d. (Millipore, Milford, MA). A 20 μl portion of the ethyl acetate extract was injected, and the compounds were eluted with a linear 0.1% acetic acid - acetonitrile gradient (20 to 100% acetonitrile in 10 minutes, flow rate 2 ml/minute) and their UV absorbance was detected at 280 nm.

Isolation of 8-*O*-Methyl Tcm D3

A 250 ml portion of a culture of *S. glaucescens* WMH1082 (pELE37) grown for 96 hours in R2YENG was extracted twice with the same volume of ethyl acetate and glacial acetic acid (20 μl/ml culture). The extract was concentrated and evaporated to dryness, and the resulting residue was washed with petroleum ether and further purified by flash chromatography on silica gel (Merck, 200~400 mesh). The silica gel column was washed with $CHCl_3$, then $CHCl_3$-methanol (9:1) and finally $CHCl_3$-methanol-acetic acid (9:1:0.1). Fractions containing 8-*O*-methyl Tcm D3 were pooled and concentrated, and after addition of 0.1% acetic acid, 8-*O*-methyl Tcm D3 was extracted from the concentrated solution with ethyl acetate. The ethyl acetate extract was concentrated to dryness and the resulting residue was dissolved in methanol and diluted with 0.1% acetic acid to give a final methanol concentration of approx. 10%. The 8-*O*-methyl Tcm D3 in this solution was concentrated with a reversed phase Sep-Pac C_{18} cartridge (Millipore), and the resin was washed with 0.1% acetic acid and double distilled H_2O then eluted with methanol to yield the pure compound. Thin layer chromatography (TLC) was performed on

Fig. 1. Tcm C biosynthetic pathway.



The pathway begins with formation of a putative decapolyketide from acetate and malonate by the Type II polyketide synthase (PKS). Solid and open arrows indicate single or multiple step transformations, respectively. Strains WMH1082 and WMH1090 have mutations that block the pathway at the dotted arrows and cause the formation of the shunt products shown on the left and right sides of the scheme.

silica gel plates (Merck) with cyclohexane-ethyl acetate-acetic acid (55:45:5) and the separated compounds were visualized under UV-light. Tcm D3, 8-*O*-methyl Tcm D3 and Tcm B3 exhibited different retention times and diagnostic UV spectra by HPLC analysis. Authentic samples of Tcm D3 and Tcm B3 co-eluted with the metabolites produced by *S. glaucescens* GLA.0 and the recombinant strains on TLC and HPLC.

Structure Elucidation of 8-*O*-Methyl Tcm D3

EI-MS were determined at 40 eV on a Finnegan 4000 mass spectrometer. ^{1}H NMR spectra (300 MHz) were take on a Bruker Aspect 3000 spectrometer in dimethyl-sulfoxide-d_6. 8-*O*-Methyl Tcm D3 was methylated with diazomethane and the reaction mixture purified on silica gel plates (2.0 mm, Merck) with $CHCl_3$-methanol (9:1). UV spectra were recorded with a Pharmacia Ultrospec III spectrophotometer in methanol and a Hewlett-Packard HP 1090 M diode-array HPLC detector. 8-*O*-Methyl Tcm D3 λ_{max} in MeOH: 484, 312, and 270 nm; EI-MS *m/z* (% of base peak): 394 (72%), 379 (40%, $M-CH_3$), 361 (14%), 347 (100%), 333 (24%), and 319 (55%). Tcm B3 EI-MS *m/z* (% of base peak): 394 (30), 376 (79%, $M-H_2O$), 350 (100%, $M-CO_2$), and 320 (69%). Tcm A2 (obtained by diazomethane treatment of 8-*O*-methyl Tcm D3) EI-MS *m/z* (% of base peak): 422 (100%), 407 (65%, $M-CH_3$), 391 (40%), 361 (74%), and 333 (52%). The ^{1}H NMR data for Tcm B3, 8-*O*-methyl Tcm D3 and Tcm A2 are listed in Table 1.

Analysis of Tcm C resistance

Tcm C resistance was tested using gradient plates[32] with 0 to 500 μg/ml Tcm C in HA medium (4 g/liter yeast extract, 4 g/liter glucose, 10 g/liter malt extract, pH 7.2) at 27 °C.

Results

Production of Metabolites in *S. glaucescens* GLA.0

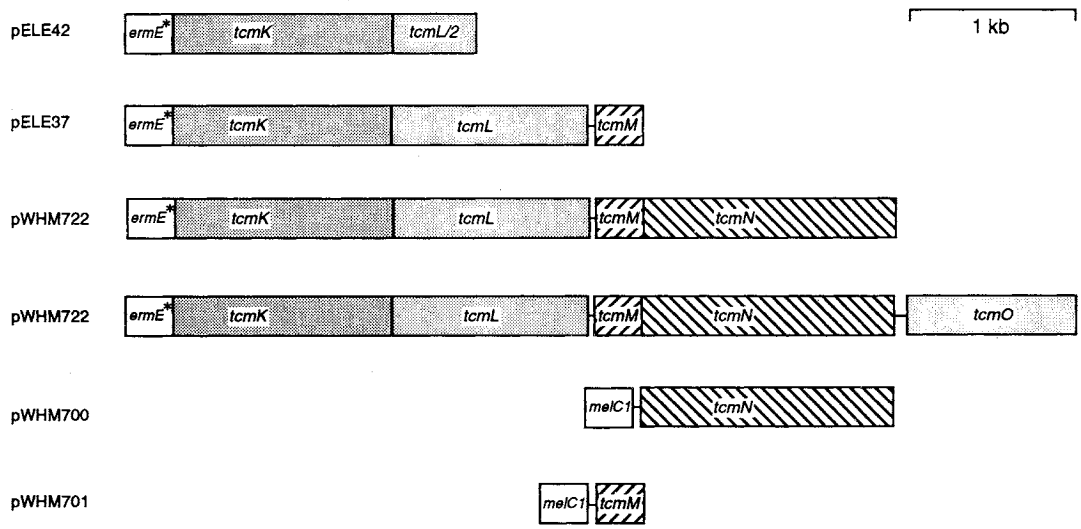
Upon tranformation of the *S. glaucescens* GLA.0 wild-type strain with a vector carrying the *tcmKLM* genes (pELE37,[30] Fig. 2), the cultures acquire an intense redness (most of the Tcm C intermediates have an orange or red color). Several known intermediates of Tcm C biosynthesis like Tcm D3, Tcm B3 and Tcm A2 (Fig. 1) were identified by HPLC and TLC of ethyl acetate extracts of acidified (pH 4) cultures. Tcm D3 is the most abundant intermediate and its production is increased by a factor of 20 to 30 (Fig. 3). Tcm D3 and Tcm B3 are usually not detected in *S. glaucescens* wild-type cultures or blocked mutants, although their existence was proposed at the time of the initial work on Tcm C biosynthesis in *S. glaucescens* mutants.[27,33] (Both of these compounds were isolated from a blocked mutant of the elloramycin producer,

Table 1. ^{1}H NMR data[a].

Position	Tcm B3	8-*O*-Methyl Tcm D3	Tcm A2[b]	Tcm A2[b]
10-CH_3	3.00	2.8	2.86	2.85
O-CH_3	3.9	3.96	3.93	3.92
O-CH_3			3.96	3.95
O-CH_3			3.97	3.96
2-H	6.72 d[c] (2.5)	6.55 d (2.3)	6.68	6.67
4-H	7.08 d (2.5)	7.09 d (2.3)	7.36	7.35
6-H	7.78 s	8.07 s	8.03	8.05
7-H	7.12	7.6	7.09	7.1
1-OH	12.3	12.2	12.44	N.D.[d]
11-OH	14.95	14.8	14.74	N.D.

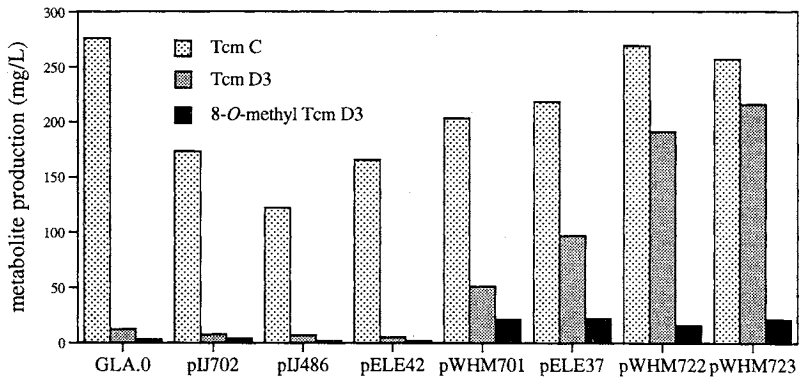
[a] In DMSO-d_6: ppm, (*J*, Hz).
[b] In $CDCl_3$.
[c] Multiplicity.
[d] N.D., not determined.

Fig. 2. Plasmid constructions used in this study.



pELE42, pELE37, pELE722 and pELE723 were constructed from pIJ486 and the cloned *tcm* genes are transcribed under the control of the *ermE** promoter.[28] pWHM700 and pWHM701 were constructed from pIJ702 and the cloned *tcm* genes are transcribed under the control of the *melC1* promoter.

Fig. 3. Production of Tcm metabolites in *S. glaucescens* GLA.0 upon transformation with different plasmids.



Streptomyces olivaceus, and a similar pathway was postulated for Tcm C and elloramycin biosynthesis.[34] We also identified a metabolite that has not been previously described in *S. glaucescens* cultures: 8-*O*-methyl Tcm D3 (Fig. 1). Subsequent examination revealed, however, that this metabolite is present in cultures of the GLA.0 strain but at a 10-fold lower concentration.

Transformants with additional copies of the *tcmKLM* genes (pELE37) also produce 25 to 30% more Tcm C than cultures containing just the plasmid vector alone (pIJ486), although the presence of either of the high copy plasmid vectors pIJ486[29] or pIJ702[28] has a negative effect on the production of Tcm C by the GLA.0 strain (Fig. 3). A smaller increase in the production of Tcm C and its biosynthetic intermediates relative to the control (pIJ702) is also seen with just additional copies of the *tcmM* ACP gene (pWHM701[31]), but this effect is less pronounced than with *tcmKLM*. Extension of pELE37 by the

Fig. 4. Production of Tcm metabolites in the *tcmN* mutant *S. glaucescens* WMH1082 upon transformation with different plasmids.

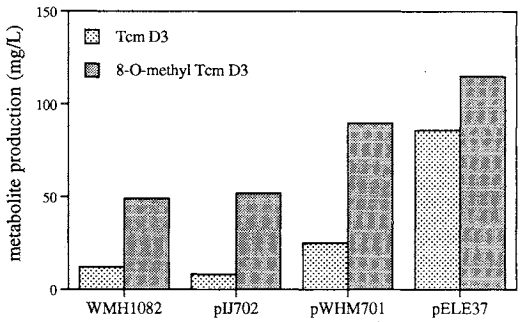
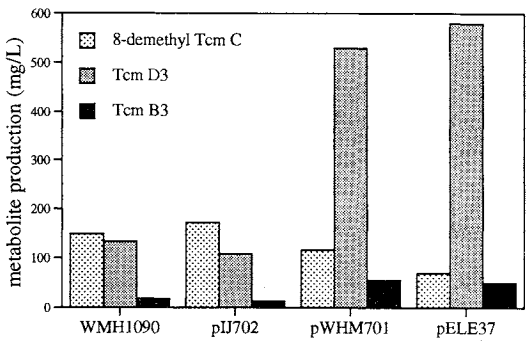


Fig. 5. Production of Tcm metabolites in the *tcmO* mutant *S. glaucescens* WMH1090 upon transformation with different plasmids.



multifunctional *tcmN* gene (pWHM722[26]) and the *tcmO* 8-*O*-methyltransferase gene (pWHM723[26]) does not change the type of metabolite produced, but the production of Tcm C and Tcm D3 is significantly higher. An incomplete set of PKS genes (pELE42[30] which contains *tcmK* and about one-half of *tcmL*: Fig. 2) or the *tcmN* gene alone (pWHM700, data not shown) do not affect the production of Tcm C, its intermediates, or shunt products of the biosynthetic pathway. The highest production of Tcm C, Tcm D3, Tcm B3 (data not shown) and 8-*O*-methyl Tcm D3 is obtained when *S. glaucescens* carries pWHM722 or pWHM723 with all 5 of the *tcm* genes (Fig. 3). However, Tcm C production is not higher in any of the recombinant strains compared to Tcm C production by the untransformed GLA.0 strain. This may be due to the inhibitory effects of the plasmid vectors since THOMAS *et al.*[35] have described similar depressive effects on antibiotic production due to the introduction of a plasmid cloning vector into a streptomycete that produces an anthelmintic macrolide. In the latter case, the effects were shown to be independent of the type of plasmid vector or antibiotic used for selection.

To substantiate our findings concerning the structure of 8-*O*-methyl Tcm D3, we analysed cultures of *S. glaucescens* blocked mutants that should product Tcm D3 and 8-*O*-methyl Tcm D3 (WMH1082) or Tcm D3 and Tcm B3 (WMH1090). Strain WMH1082 has a mutation in the *tcmN* gene that blocks methylation of the hydroxyl-group at position 3 (Fig. 1). As expected, Tcm D3 and 8-*O*-methyl Tcm D3 are the main compounds produced by this mutant. Introduction of the *tcmKLM* genes (pELE37) or the *tcmM* gene (pWHM701) into this strain causes increased production of Tcm D3 (3 to 10-fold) and 8-*O*-methyl D3 (2-fold) (Fig. 4). Strain WMH1090, on the other hand, has a mutation in the *tcmO* 8-*O*-methyltransferase gene and thus should not produce 8-*O*-methyl Tcm D3 (Fig. 1). Indeed, Tcm D3 is the predominant compound produced by this mutant. We analyzed culture extracts of the WMH1090, WHM1090 (pELE37) and WMH1090 (pWHM701) strains, respectively, and found Tcm D3, Tcm B3 and 8-demethyl Tcm C,[33] but as expected no 8-*O*-methyl Tcm D3 (Fig. 1). The production of Tcm D3 and Tcm B3 in the WMH1090 (pELE37) or WMH1090 (pWHM701) strains is elevated by factors of 4 and 3, respectively (Fig. 5).

Elucidation of the Structure of 8-*O*-Methyl Tcm D3

8-*O*-Methyl Tcm D3 exhibits a molecular ion at *m/z* 394 by low resolution electron impact mass spectrometry (EI-MS). Tcm B3 also gives this molecular ion, but the fragmentation patterns of Tcm B3 and 8-*O*-methyl Tcm D3 are quite different (see Experimental). For instance, 8-*O*-methyl Tcm D3 has a characteristic fragment ion at $M^+ - 15$.[33] Treatment of 8-*O*-methyl Tcm D3 with diazomethane gives Tcm

A2, a known intermediate of Tcm C biosynthesis, whose structure was confirmed by EI-MS and ^{1}H NMR analysis (see Experimental).

Discussion

The economic production of an antibiotic often necessitates raising the yield from the initial microbial isolate considerably. Traditionally, this has been done by optimization of the fermentation process parameters, and(or) by mutation and screening for variants with increased antibiotic production. Impressive yield increases have been accomplished in this manner but can take many years to achieve (a 41,000-fold increase in penicillin production has been achieved over a 50 year period, for example.[36]) Moreover, this route frequently results in strains with special growth requirements or genetic instabilities. Now, however, identification of the regulatory and structural genes for antibiotic production opens a non-empirical way to achieve this goal.[11] For example, introduction of several copies of the *actII-orf4* regulatory gene into the actinorhodin producer *S. coelicolor* increases antibiotic production 30 to 40-fold.[37,38] Similarly, greater than a 100-fold increase is seen in the production of ε-rhodomycinone and up to a 10-fold increase in daunorubicin, key intermediates of doxorubicin (adriamycin) biosynthesis, when the wild-type *Streptomyces peucetius* ATCC 29050 strain is transformed with the *dnrI* and *dnrR*$_2$ regulatory genes.[39] Additional copies of structural genes that govern a limiting biosynthetic step may also improve antibiotic production; a notable achievement is the enhanced conversion of isopenicillin N to cephalosporin C by introduction of extra copies of the *cefEF* genes into a high-producing strain of *Cephalosporium acremonium*.[4]

Since we have not identified a regulatory gene in the *tcm* cluster,[21] we decided to introduce additional copies of the *tcmKLM* PKS genes to see whether this would affect production of Tcm C and its biosynthetic intermediates or cause the production of shunt products. Introduction of additional copies of *tcmKLM* and, interestingly, *tcmM* alone gave rise to higher Tcm C yields compared to control experiments with the plasmid vector itself. These cultures did not produce more Tcm C than untransformed wild-type cultures, however, suggesting the plasmid vector or thiostrepton antibiotic has a repressive effect on Tcm C production. In addition to the increased yield of Tcm C compared to the controls, the recombinant cultures also accumulate Tcm C biosynthetic intermediates indicating that, when flux through the early part of the pathway is increased, a biosynthetic step subsequent to the production of Tcm D3 becomes limiting. Several reasons for this are possible. First, a particular enzyme may become over-saturated, at its normal titer, causing more of its substrate to accumulate or be diverted into a shunt product. Alternatively, the methylation steps that require *S*-adenosyl-L-methionine[22,26] might become limiting due to the intracellular levels of this cofactor. Tcm C may also inhibit the growth of *S. glaucescens*. WEBER *et al.*[19] reported the MIC to be 100 μg/ml using an agar plate diffusion assay; we obtained a MIC of 150 μg/ml (after 72 hours) using gradient plates[32] that were inoculated with a spore suspension of *S. glaucescens*. Tcm C resistance is confered by the *tcmA* gene whose product is though to act as a metabolite export pump powered by the transmembrane electrochemical gradient.[23] Therefore, high Tcm C concentrations might be suicidal for the cell (Tcm C interacts with DNA presumably by intercalation[20]) if the antibiotic cannot be removed efficiently from the cytoplasm. Finally, Tcm C or any of its pathway intermediates might retard one or more of the biosynthetic steps by feedback inhibition or repression.

The detection of Tcm C biosynthetic intermediates Tcm D3 and 8-*O*-methyl Tcm D3 that had not been isolated from *S. glaucescens* cultures previously was a consequence of the introduction of pELE37, pWHM701, pWHM722 and pWHM723. This discovery was also made easier because the cultures were extracted at pH 4 instead of at neutral pH as in earlier work[33] where we had identified only the methyl esters of 8-*O*-methyl Tcm D3 and Tcm B3 (Fig. 1). We cannot explain why 8-*O*-methyl Tcm D3 accumulates to a higher extent than Tcm B3 in many of the recombinant strains. Before this can be understood, enzymatic studies will have to be done to determine the *Km*, V_{max} and relative titers of each of the *O*-methyltransferases involved in Tcm C biosynthesis and to see whether any of these steps are regulated by feedback inhibition or repression.

The ability of extra copies of the *tcmM* gene to increase metabolite yields nearly as much as all three of the PKS genes (*tcmKLM*) or these genes plus the *tcmN* cyclase-dehydratase-3-*O*-methyltransferase

or *tcmO* 8-*O*-methyltransferase genes (Figs. 3 to 5) is surprising. *S. glaucescens* (pWHM701) strains definitely overproduce the *tcmM* ACP[31] (the percentage increase has not been quantitated), but we had not originally assumed that the amount of this one protein would limit Tcm C biosynthesis in the wild-type cells. It appears, however, that excess ACP either increases the substrate concentration (malonyl*S*ACP in this case[31]) in an unsaturated system, or somehow increases the level of the *tcmK* and *tcmL* proteins or the overall activity of the PKS complex, which is believed to consist minimally of the *tcmKLMN* gene products.[40] The results of KHOSLA *et al.*[18] can be explained in the same way, although they did not explictly demonstrate overexpression of the various ACP genes studied. Overproduction of bacterial and plant fatty acid synthase ACP's, in contrast, appears to be deleterious to growth in *Escherichia coli*[41,42] or has no observable effect on fatty acid biosynthesis in tobacco plants.[43]

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