The *tcmVI* Region of the Tetracenomycin C Biosynthetic Gene Cluster of *Streptomyces glaucescens* Encodes the Tetracenomycin F1 Monooxygenase, Tetracenomycin F2 Cyclase, and, Most Likely, a Second Cyclase

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Certain mutations in the *tcmVI* region of the *Streptomyces glaucescens* chromosome affect formation of the D ring of the polyketide antibiotic tetracenomycin C (TCM C). This region lies immediately upstream from the TCM C polyketide synthase genes (*tcmKLM*), and the nucleotide sequence reveals the presence of three small genes, *tcmH*, *tcmI*, and *tcmJ*. On the basis of the phenotypes of mutants and the effects of these genes, when coupled on a plasmid with the *tcmKLMN*¹⁷⁷ genes (*tcmN*¹⁷⁷ is a 3'-truncated version of *tcmN*), on the production of TCM intermediates in a TCM[−] mutant, the *tcmH* gene encodes the C-5 monooxygenase that converts TCM F1 to TCM D3, the *tcmI* gene encodes the D-ring cyclase that converts TCM F2 to TCM F1 (mutations in this gene are responsible for the type VI phenotype), and the *tcmJ* gene most likely encodes the B-ring cyclase that acts in the biosynthesis of TCM F2. Furthermore, it appears that the N-terminal domain of the *tcmN* gene product (encoded by the *tcmN*¹⁷⁷ gene) acts later in the biosynthesis of TCM F2 than the product of *tcmJ*, suggesting that the N-terminal domain of the TcmN protein is the C-ring cyclase.

Tetracenomycin (TCM) C is an aromatic polyketide antibiotic produced by the filamentous, gram-positive soil bacterium *Streptomyces glaucescens* (32). We have been studying the biosynthesis of TCM C as a model for the entire family of aromatic polyketides, which includes such clinically important compounds as the tetracycline antibiotics and the chemotherapeutic agents daunorubicin and doxorubicin.

The initial steps in the mechanism of polyketide biosynthesis resemble the mechanism of long-chain fatty acid biosynthesis: small fatty acid units (acetate, propionate, butyrate, etc.) are sequentially condensed to yield extended linear precursors (3, 16, 18). Polyketide biosynthesis differs from fatty acid biosynthesis, however, because the condensation reactions of polyketide chain growth are not always followed by the cycles of reduction and dehydration that characterize the biosynthesis of fatty acids. As a result, the linear intermediate for the biosynthesis of aromatic polyketide metabolites like TCM C is a reactive poly- β -ketone that provides the framework for subsequent elaboration.

The tetracyclic backbone of TCM C is most likely formed by cyclization of a 20-carbon linear decaketide (Fig. 1). Four independent cyclizations and dehydrations are envisaged on the path from the putative linear precursor to the first tetracyclic intermediate, TCM F1. These biochemical analogs of the aldol and Claisen reactions of organic chemistry are characteristic of the biosynthesis of many polyketide metabolites (3, 16, 18) but have only recently been studied enzymatically. In fact, the TCM F2 cyclase, which catalyzes the fourth step in

Fig. 1, is the first example of an enzyme that catalyzes such reactions to be purified (25).

Previously, we cloned the *S. glaucescens* genes that are required for the biosynthesis of TCM C (19), and we have reported the DNA sequence of most of the gene cluster (7, 12, 30), including the polyketide synthase genes (1). Here we report the DNA sequence of the region that lies immediately upstream of the *tcmKLM* polyketide synthase genes. Three small genes are contained in this region, and we provide evidence that the first gene (*tcmH*) encodes the C-5 monooxygenase that oxidizes TCM F1 to TCM D3, that the second gene (*tcmI*) encodes the D-ring cyclase that converts tricyclic TCM F2 to tetracyclic TCM F1, and that the third gene (*tcmJ*) probably encodes the B-ring cyclase that is involved in the formation of TCM F2. The *tcmHIJ* genes are transcribed along with the *tcmGKLMNO* genes under control of a promoter upstream of *tcmG* (6); the three remaining genes of the cluster, *tcmA*, *-R*, and *-P*, produce monocistronic transcripts (6, 12).

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. The bacteriophages M13mp18 and M13mp19 (33) and the plasmids pIJ702 (17), pIJ486 (31), pGM160 (21), pELE37 (11), pHM57 (19), and pWHM721 (30) have been described previously. The *S. glaucescens* strains used are described in Table 1. Plasmids pGEM -3Zf(−), -5Zf(+), and -7Zf(+) were purchased from Promega (Madison, Wis.).

Media, enzymes, and chemicals. R2YENG liquid medium (pH 6) and agar plates (19) and HT plates (20) were prepared as described previously. Dry medium and Bacto-Agar were purchased from Difco Laboratories (Detroit, Mich.). The salts CaCl₂, KH₂PO₄, MgCl₂, NaCl, NaOH, and NaNO₃ were obtained from Mallinckrodt (Paris, Ky.), and K₂SO₄ was from Columbus Chemical Industries (Columbus, Wis.). TES [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer was

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FIG. 1. Scheme for the early-intermediate steps in the biosynthesis of TCM C. The putative linear decaketide, the presumed monocyclic and bicyclic precursors, and the known intermediates TCM F2, TCM F1, TCM D3, and TCM A2 are shown. Each arrow represents a single biochemical step.

purchased from Sigma (St. Louis, Mo.). Restriction endonucleases were obtained from either Amersham (Arlington Heights, Ill.), Bethesda Research Laboratories, New England Biolabs (Beverly, Mass.), Promega, or United States Biochemical. T4 DNA ligase was supplied by Bethesda Research Laboratories. Buffers for these enzymes were either used as provided or prepared as described by Sambrook et al. (23). The antibiotic thiostrepton was supplied by Sal Lucania, Squibb Institute for Medical Research (Princeton, N.J.).

DNA sequence analysis. Pieces (≤ 800 bp) of the 1.2-kb *Bam*HI-*Bcl*I fragment that carries the *tcmVI* region were subcloned from a derivative of pHM57 into replicative-form M13mp18 and M13mp19 and were sequenced as outlined previously (30). Overlapping sets of subclones were generated, and all restriction endonuclease sites that were used for subcloning were sequenced across to confirm their place in the sequence. In this fashion, the complete nucleotide sequence of both strands of the 1.2-kb fragment was determined. Open reading frames (ORFs) in this sequence were identified by using the Genetics Computer Group (8) program CODON PREFERENCE. No significant inverted repeat structures occur in this region, according to the program STEMLOOP (8). The similarity between the *tcmHIJ* gene products and the

actVa-ORF6, *whiE*-ORFVII, and *whiE*-ORFII gene products, respectively, was discovered by using TFASTA (8) to search the GenBank (release 60.0) and EMBL (release 19.0) genetic sequence data banks. The percent identity between related amino acid sequences was determined by using GAP (8).

Complementation analysis. The DNA fragments depicted in Fig. 4 were subcloned in the coding orientation downstream of the melanin gene (*melC1*) promoter of pIJ702. Frequently, the fragments were subcloned first in pGEM-3Zf(-), -5Zf(+), or -7Zf(+) in order to obtain suitable flanking restriction sites for subsequent introduction into pIJ702. In addition, each of the constructs was designed so that at least one translational stop codon intervenes between the *melC* genes and the inserted fragment in the reading frame of interest. This is presumed to prevent translational readthrough that would complicate interpretation of the results.

Protoplasts of strains WMH1092, WMH1093, and WMH1500 (Table 1) were prepared and transformed with the constructed plasmids described above and pIJ702 (as a control) by the method of Hopwood et al. (15) with minor modifications. Cells were then grown and screened for the TCM C⁺ phenotype by bioassay against a thiostrepton-resistant (Th^r) derivative of *Streptomyces coelicolor* M111 (*hisA1 uraA1 strA1 SCP1 SCP2**), as described previously (20, 30). Colonies from each transformation were also grown in 5 ml of R2YENG containing 10 μ g of thiostrepton per ml at 30°C for 3 days and assayed for the presence of TCM C and its fluorescent biosynthetic intermediates by thin-layer chromatography (TLC) of culture broth extracts (30).

Construction of *S. glaucescens* WMH1500 ($\Delta tcmJ$). To delete codons 37 to 108 of the *tcmJ* gene, a plasmid-borne copy of the gene was digested with *Bgl*II and *Mro*I, and both restriction enzyme sites were filled in with the Klenow fragment of DNA polymerase I. The plasmid was then ligated closed to form the $\Delta tcmJ$ in-frame deletion derivative. After extension of the *tcm* DNA on both flanks of the $\Delta tcmJ$ construct by addition of the appropriate segments from the gene cluster (1, 7), a 2.7-kb

TABLE 1. *S. glaucescens* strains

Strain	Original designation (20)	Genotype	Reference(s)
WMH1061 ^a	GLA.2-36	<i>tcmK</i>	20, 29
WMH1065 ^a	GLA.5-10	<i>tcmM</i>	20, 29
WMH1068 ^a	GLA.11-47	$\Delta tcmL$	20, 29
WMH1077	GLA.5-1	<i>tcmGHIJKLMNO</i> ^b	7, 20, 30
WMH1092	GLA.8-21	<i>tcmHI</i>	This work, 20
WMH1093 ^a	GLA.10-48	<i>tcmI</i>	This work, 20
WMH1500 ^a		$\Delta tcmJ$	This work

^a Type strain.

^b These genes are not expressed (6).

*Bam*HI-*Bgl*II fragment that carries the *tcmHI*(Δ)*K* genes and a portion of the *tcmL* gene was transferred to the temperature-sensitive replicon pGM160, similarly digested. The resulting plasmid was transformed into wild-type *S. glaucescens* GLA.0, and the transformants were allowed to grow on R2YENG plates in the presence of thiostrepton (20 μ g/ml) for 3 days at 30°C. To select for chromosomal recombinants, the plates were transferred to 39°C (the nonpermissive temperature for plasmid replication), and after 5 days, new growth and/or sectors were streaked onto HT plates containing thiostrepton (50 μ g/ml). These were then grown at 39°C for 3 days. To cure the integrated plasmid, the Th^r colonies were first protoplasted and then regenerated at 30°C on HT plates without thiostrepton until sporulation. Spores were next transferred to additional HT plates and were again grown until sporulation at 30°C. Finally, spores from the second nonselective plating were replica plated onto HT plates with and without thiostrepton (50 μ g/ml), and thiostrepton-sensitive (Th^s) colonies were chosen for Southern analysis to determine which of the merodiploids had resolved to leave the Δ *tcmJ* gene in the chromosome. Strain WMH1500 carries Δ *tcmJ* in a background that is otherwise isogenic with the wild-type strain.

Construction and analysis of plasmids pWHM724 to pWHM731. Plasmids pWHM724 to pWHM730 (see Fig. 5A) are derivatives of pWHM721, which expresses the *tcmKLM* genes and a 3'-truncated version of the *tcmN* gene (*tcmN*¹⁷⁷) under the control of the *ermE** promoter (30). To construct these plasmids, segments of DNA from the *tcmVI* region of *S. glaucescens* were inserted into pWHM721 between the *ermE** promoter and the *tcmKLMN*¹⁷⁷ genes. In each case, the transcript start site and at least the first 3 nucleotides (nt) of the putative transcript should be identical.

Plasmid pWHM730 carries the entire *tcmVI* region (starting at nt 1; see Fig. 3) in its natural position upstream from the *tcmK* gene. The other plasmids are similar, except that they lack various pieces of the *tcmVI* region. Plasmid pWHM724 is missing nt 402 to 1097, leaving the intact *tcmH* gene. For pWHM725, nt 1 to 294 and 882 to 1097 have been removed, yielding the *tcmI* gene. In pWHM726, only *tcmJ* is present, starting at nt 707. Plasmid pWHM727 expresses both *tcmH* and *tcmI* but lacks nt 882 to 1097. Similarly, pWHM728 expresses two genes, *tcmI* and *tcmJ*; in this case nt 1 to 294 have been removed. Finally, pWHM729 encodes the *tcmH* and *tcmJ* genes, with the intervening nucleotides (nt 402 to 656) deleted. For each construct, the deletion endpoints coincide with convenient restriction enzyme sites in the *tcmVI* region, and these sites are shown above their position in the nucleotide sequence in Fig. 3. The 5' or 3' overhangs that were generated during restriction enzyme digestion were either filled in with the Klenow fragment of DNA polymerase I or removed with nuclease S1, as appropriate. The deletions of intervening DNA in plasmids pWHM725, pWHM727, and pWHM729 are in frame, creating small remnant ORFs that should continue to be expressed by using the natural start and stop codons. The deletion in pWHM724 is out of frame, however, frameshifting the last 30 codons of the *tcmJ* gene. This frameshifted ORF ends 5 nt downstream of the original *tcmJ* translation stop codon and now overlaps the ATG start codon of the *tcmK* gene. This does not seem to interfere with expression of *tcmK* or any of the other downstream genes, though, since pWHM724 still complements the *tcmK*, *tcmL*, and *tcmM* type strains.

Construct pWHM731 was formed by replacing the 1.7-kb *Eco*RI-*Bsp*HI fragment of pELE37 with its homolog from pWHM726 that carries the *ermE** promoter and the *tcmJK*

FIG. 2. Phenotype of the *S. glaucescens tcmVI* mutants WMH1092, WMH1093, and WMH1500 determined by TLC of culture extracts. Strains WMH1092 and WMH1093 accumulate the tricyclic intermediate TCM F2. Strain WMH1500 also accumulates TCM F2, but this mutation (Δ *tcmJ*) is leaky and does not block production of TCM C. Designations on the left indicate the bands that correspond to the known intermediates of TCM C biosynthesis. "B", band consists of TCM B1, TCM B2, and/or TCM B3 (32, 34).

genes. The resulting plasmid is identical to pWHM726 except that it lacks the *tcmN*¹⁷⁷ gene.

Correct construction of plasmids pWHM724 to pWHM731 was verified by restriction analysis, DNA sequence analysis of unnatural fusion junctions, and appropriate complementation behavior in *tcmI*, *tcmJ*, *tcmK*, *tcmL*, and *tcmM* type strains.

To analyze the effects of the plasmids on the biosynthesis of TCM intermediates in a TCM⁻ background, protoplasts of *S. glaucescens* WMH1077 (7, 20) were prepared and transformed to Th^r with the above-described plasmids and pIJ486 (the ultimate parent of this series of plasmids), as described previously (30). Several colonies from each transformation were then picked and grown in 5 ml of R2YENG (containing 20 μ g of thiostrepton per ml) at 30°C for 3 to 4 days. Prior to extraction with 1 ml of ethyl acetate, the pH of each culture was lowered by addition of 1 M KH₂PO₄ (pH 4.8) (500 μ l), and a portion (50 μ l) of the extract was then analyzed for the presence of TCM C and its fluorescent intermediates by TLC on silica gel plates in chloroform-methanol-acetic acid (8:2:0.25, vol/vol).

Nucleotide sequence accession number. The DNA sequence of the entire *tcm* gene cluster has been deposited at EMBL and GenBank with the accession number M80674.

RESULTS

Sequence analysis of the *tcmVI* region. The *tcmVI* region of the TCM C biosynthetic gene cluster of *S. glaucescens* lies immediately upstream from the TCM C polyketide synthase genes (*tcmKLM*). Two mutants from a group of 33 independent TCM⁻ mutants carry lesions in this region (strains WMH1092 and WMH1093 [Table 1]), and both mutants accumulated the early tricyclic intermediate TCM F2 (26) (Fig. 2). This suggests that the *tcmVI* region encodes the cyclase responsible for the formation of the D ring of TCM C (Fig. 1). Since enzymes that catalyze intramolecular aldol condensations such as that required for the formation of the D ring had not been previously described, we determined the nucleotide sequence of the *tcmVI* region (Fig. 3).

FIG. 3. Nucleotide sequence of the *tcmVI* region. The putative start sites and potential ribosome binding sites of the *tcmH*, *tcmI*, *tcmJ*, and *tcmK* genes are underlined. Amino acid translations are provided for the *tcmH*, *tcmI*, and *tcmJ* gene products and the N terminus of the *tcmK* gene product. The restriction sites used in this study are shown above their positions in the nucleotide sequence. All of the sites shown are unique. *, translational stop codons.

Surprisingly, the 1.2-kb *Bam*HI-*Bcl*II fragment that encompasses the *tcmVI* region (Fig. 4) carries three short ORFs. The first ORF (*tcmH*) extends from position 58 to position 396 and would encode a protein of 112 residues (calculated M_r , 12,700). The second ORF (*tcmI*) begins 2 nt downstream from the end of the first ORF and continues to position 728. The product of this ORF would comprise 109 amino acids with a calculated M_r of 12,900. Finally, the third ORF (*tcmJ*) most likely begins with a GTG codon at position 775 and ends at position 1191, 1 nt upstream from the predicted start of the *tcmK* gene. The third ORF should encode a protein of 138 residues (calculated M_r , 14,700). There are other possible in-frame start sites for the third ORF, a GTG codon at position 742 or an ATG codon at position 799, but a comparison of the putative product of this ORF with its homolog in the *whiE* spore pigment gene cluster of *S. coelicolor* (5) gives the best match, assuming that translation initiates at position 775. Furthermore, only the GTG codon at position 775 is preceded by a likely ribosome binding site on the basis of complementarity to a sequence near the 3' end of *Streptomyces lividans* 16S rRNA (2), a feature that we have found to characterize the beginnings of all genes (including the three described here) in the *tcm* gene cluster (1, 7, 12, 30).

Complementation analysis of the *tcmVI* region. To determine which of the three ORFs in the *tcmVI* region encodes the D-ring cyclase, we subcloned a series of DNA fragments from this region downstream of the *melC1* promoter of pJ702 and

examined the abilities of these fragments to complement the type VI mutations in strains WMH1092 and WMH1093 (Fig. 4 and Table 2). As expected, the 1.75-kb *Bam*HI-*Sst*I fragment that carries the entire *tcmVI* region restored production of TCM C to both of the type VI mutants (Fig. 4, fragment A). When this fragment was subdivided into segments representing each of the individual ORFs, the 399-bp *Bam*HI-*Nco*I fragment that carries the first ORF (*tcmH*) enabled low-level production of TCM C in strain WMH1092 but did not restore antibiotic synthesis to its wild-type level. Strain WMH1093, on the other hand, was unaffected (Fig. 4, fragment B). Interestingly, the 428-bp *Pst*I-*Nae*I fragment that encodes the second ORF (*tcmI*) also imparted low-level TCM C biosynthesis to strain WMH1092. More importantly, the second ORF fully complemented the lesion in strain WMH1093 (Fig. 4, fragment C). Finally, the 502-bp *Nae*I-*Bcl*II fragment that encodes the third ORF (*tcmJ*) had no effect on either strain (Fig. 4, fragment D).

From this analysis it is apparent that WMH1093 is a *tcmI* mutant. Since WMH1092 is weakly complemented by both the first and second ORFs, it is possible that this strain might be a *tcmHI* mutant. Indeed, the 733-bp *Bam*HI-*Nae*I fragment that carries both the first and second ORFs together restored wild-type TCM C production to strain WMH1092 (Fig. 4, fragment E). Thus, WMH1092 either carries mutations in both *tcmH* and *tcmI* or contains a polar mutation in *tcmH*. Since both WMH1092 and WMH1093 have the same phenotype

FIG. 4. Schematic illustration of the restriction fragments used to complement the type VI mutants (see Table 2). Roman numerals above the top bar indicate the relative locations of the eight phenotypic classes of TCM⁻ mutations (20) and the *tcmAR* TCM C resistance genes (12). Restriction sites are abbreviated as follows: Ba, *Bam*HI; Bc, *Bcl*I; Na, *Nae*I; Nc, *Nco*I; Ps, *Pst*I; and Ss, *Sst*I. All of these sites are unique.

(they accumulate the tricyclic intermediate TCM F2 [Fig. 2]) and since *tcmH* does not complement the *tcmI* mutation, it appears that the product of *tcmI* acts earlier in TCM C biosynthesis than the product of *tcmH*. Moreover, since the *tcmI* mutation seems to block formation of the D ring of TCM C, it is likely that *tcmI* encodes at least a component of the D-ring cyclase. Consistent with these results, recent biochemical evidence shows that *tcmI* is the gene for the D-ring cyclase (25), which produces the substrate for the product of *tcmH* (24).

We established the role of the *tcmH* gene by characterizing *tcmI* transformants of strain WMH1092, since the identity of the intermediate(s) now formed in the partially complemented double mutant should reveal the function of *tcmH*. Interestingly, whereas the principal compound observed in culture broth extracts of untransformed strain WMH1092 was TCM

F2 (in our TLC solvent system, a slowly migrating, aquamarine fluorescent substance), the *tcmI* transformant produced at least four fast-migrating yellow to yellow-orange fluorescent species (data not shown). The high mobility and fluorescent color of these compounds is characteristic of late intermediates in the TCM pathway, but the compounds produced by transformation of WMH1092 with the *tcmI* gene are distinct from the known late intermediates. Moreover, these compounds fluoresce with an unusual greenish tint compared with the known species. Since all of the anticipated late intermediates of TCM C biosynthesis have been characterized (32, 34), we presume that the compounds produced when WMH1092 is transformed with the *tcmI* gene are variants of these known species, most likely the C-5 naphthacenones. Some support for this notion comes from the finding that one of the fast-migrating intermediates was quantitatively converted to TCM A2 (on the basis of chromatographic evidence) when applied to silica gel and allowed to sit overnight in air (data not shown). Of the four steps on the path from TCM F1 to TCM A2, an oxidation and three O methylations (Fig. 1), only the C-5 oxidation would be expected to occur under these conditions. It therefore appears that the WMH1092 (*tcmI*) transformant, which is effectively a *tcmH* mutant, is unable to oxidize the C-5 position of the TCM C backbone. Thus, the *tcmH* gene must encode the C-5 monooxygenase. We have confirmed this by isolation and amino-terminal sequence analysis of this enzyme from *S. glaucescens* (24). Furthermore, the implication that the WMH1092 (*tcmI*) transformant produces the C-5 naphthacenone analog of TCM A2 indicates that oxidation of the C-5 position is not required before methylation can occur at the C-3 and C-8 hydroxyl and C-9 carboxy positions.

The C-5 monooxygenase encoded by the *tcmH* gene resembles the predicted ActVa-ORF6 protein, while the deduced proteins of the *tcmI* and *tcmJ* genes have analogs in the *whiE*

TABLE 2. Complementation behavior determined by TLC analysis of culture extracts of the DNA fragments from the *tcmVI* region in plasmid pIJ702 (Fig. 4)

DNA fragment	Description	Complementation with type VI mutant ^a :		
		WMH1092	WMH1093	WMH1500 ^b
None	pIJ702	—	—	—
A	<i>tcmHIJ</i>	++	++	++
B	<i>tcmH</i>	+	—	—
C	<i>tcmI</i>	+	++	—
D	<i>tcmJ</i>	—	—	++
E	<i>tcmHI</i>	++	++	—

^a ++, wild-type level of TCM C; +, increased level of TCM C but less than the wild-type level; —, no TCM C produced.

^b Strain WMH1500 normally produces low levels of TCM C and accumulates TCM F2. Successful complementation of this strain was judged by production of wild-type levels of TCM C and failure to accumulate TCM F2.

spore pigment cluster of *S. coelicolor*. A computer search of the GenBank and EMBL sequence libraries revealed that the deduced primary structure of the TcmH monooxygenase resembles that of the predicted product of *actVa*-ORF6 (4) from the actinorhodin (ACT) biosynthetic pathway of *S. coelicolor* (39% identity). This finding is consistent with the need in ACT biosynthesis for an oxidation equivalent to the C-5 oxidation of TCM C. The deduced enzymes of the *tcmI* and *tcmJ* genes also have analogs in *S. coelicolor*, although not in the ACT gene cluster. The TcmI cyclase is similar to the putative product of *whiE*-ORFVII of the *whiE* spore pigment gene cluster (40% identity), while the predicted product of the *tcmJ* gene is 43% identical to the putative WhiEII protein of the same gene cluster (5). The *tcmJ* and *whiE*-ORFII genes also occupy related locations in their respective gene clusters, immediately upstream from the polyketide synthase genes.

The metabolite produced by the *whiE* pathway is unknown, but in light of the extensive similarity between the *tcm* and *whiE* gene clusters (in addition to the TcmI and TcmJ proteins, the *whiE* gene cluster also encodes close analogs of the TcmK, TcmL, and TcmM proteins and the TcmN cyclase domain [1, 5, 16, 30]), it seems likely that the *whiE* gene cluster is responsible for the biosynthesis of an naphthacenequinone-like pigment.

The *tcmJ* gene influences the amount of TCM C produced but is not required for TCM C biosynthesis. Of the 33 original TCM C⁻ mutants (20), only 2 (strains WMH1092 and WMH1093) carry lesions in the *tcmVI* locus, and, as our complementation analysis shows, neither of these mutations maps to the *tcmJ* gene. Consequently, to determine the phenotype of a mutation in the *tcmJ* gene, we introduced an in-frame deletion of the *tcmJ* gene into the chromosome of wild-type *S. glaucescens*. For this purpose, a derivative of the temperature-sensitive replicon pGM160 (21) that carries the *tcmHIJK* genes, excluding codons 37 to 108 of the *tcmJ* gene, which had been excised in frame, was prepared. This plasmid was integrated into the chromosome of *S. glaucescens* via a single homologous recombination by selection for Th^r growth at 39°C, the nonpermissive temperature for plasmid replication. The integrants were then cured of the plasmid by protoplasting followed by two rounds of nonselective growth at 30°C. Th^s strains were recovered at high frequency (60%) and screened by Southern analysis to differentiate wild-type revertants from the desired $\Delta tcmJ$ variant. Strain WMH1500, which carries the $\Delta tcmJ$ lesion in an otherwise wild-type background, was selected for further study.

Removal of more than 50% of the *tcmJ* gene did not abolish biosynthesis of TCM C. Instead, as determined by bioassay and TLC culture extracts, strain WMH1500 still produced TCM C, but at a reduced level (Fig. 2). Indeed, chromatographic analysis of young cultures indicated that the initial output of TCM C was lowered more than 10-fold. Furthermore, the reduced yield of TCM C in strain WMH1500 was accompanied by the accumulation of TCM F2. In this regard, the $\Delta tcmJ$ mutant resembles the other two type VI strains. This finding is puzzling, however, and it suggests that mutation of *tcmJ* impairs the D-ring cyclization ascribed to the product of the *tcmI* gene. In essence, the $\Delta tcmJ$ mutant looks like a leaky *tcmI* mutant.

To confirm that the lesion in strain WMH1500 is confined to the *tcmJ* locus, we attempted to complement this mutant with the 502-bp *NaeI*-*BclI* fragment that carries the *tcmJ* gene (fragment D; Fig. 4 and Table 2). As anticipated, this fragment restored wild-type levels of TCM C production to the strain, and TCM F2 no longer accumulated (data not shown). Since the *NaeI*-*BclI* fragment does not encode any *tcmI* sequences, we conclude that strain WMH1500 owes its phenotype exclu-

FIG. 5. Schematic illustration of the plasmid-based partial *tcm* gene clusters used to transform *S. glaucescens* WMH1077. (A) Plasmid pWHM721 and its *tcmHIJ* derivatives (see Table 3). (B) Plasmid pELE37 and its *tcmJ/N*¹⁷⁷ derivatives (see Fig. 6).

sively to the absence of the *tcmJ* gene product. It is also apparent from this experiment that the $\Delta tcmJ$ mutation is not *cis* acting.

Unfortunately, the phenotype of strain WMH1500 does little to clarify the function of the *tcmJ* gene product. Moreover, the similarity between the *tcmJ* and *tcmI* phenotypes caused us to question whether *tcmI* alone encodes the D-ring cyclase or whether *tcmIJ* together are required for this activity. To address this and to reexamine the functions of the *tcmVI* locus, we constructed a set of partial *tcm* gene clusters based on plasmid pWHM721. In a previous study (30), we had shown that pWHM721, which expresses the *tcmKLM* polyketide synthase genes plus the N-terminal 177 codons of the *tcmN* gene (*tcmN*¹⁷⁷), is sufficient to cause biosynthesis of TCM F2 in the *S. glaucescens* TCM C⁻ strain WMH1077. This strain carries a mutation that essentially abolishes *tcmG* promoter activity for the putative *tcmGHJKLMNO* operon, but the *tcmP* O-methyltransferase and *tcmAR* resistance genes are still expressed (7). Since the *tcmHIJ* genes seem to be involved in the series of reactions leading from TCM F2 to later intermediates, the stepwise addition of these genes to the genes already expressed by pWHM721 should result in distinct changes in the pattern of TCM C intermediates produced by strain WMH1077 transformants. All possible combinations of the *tcmHIJ* genes were therefore added to pWHM721 to give plasmids pWHM724 to pWHM730 (Fig. 5A), and the TCM intermediates produced in strain WMH1077 were determined by TLC of culture broth extracts (Table 3).

Addition of just the *tcmI* gene to the *tcmKLMN*¹⁷⁷ genes of pWHM721 (to give pWHM725) was sufficient to cause cyclization of TCM F2 to TCM F1 (Table 3). As expected from the

TABLE 3. Principal TCM C intermediates produced by transformants of *S. glaucescens* WMH1077 as determined by TLC of culture extracts

Transforming plasmid ^a	Description	TCM C intermediate
pIJ486	Control	None
pWHM721	<i>tcmKLMN</i> ¹⁷⁷	TCM F2
pWHM724	<i>tcmHKLMN</i> ¹⁷⁷	None
pWHM725	<i>tcmIKLMN</i> ¹⁷⁷	TCM F1
pWHM726	<i>tcmJKLMN</i> ¹⁷⁷	TCM F2 (10×) ^b
pWHM727	<i>tcmHIKLMN</i> ¹⁷⁷	None
pWHM728	<i>tcmIJKLMN</i> ¹⁷⁷	TCM F1 (5×) ^c
pWHM729	<i>tcmHIJKLMN</i> ¹⁷⁷	None
pWHM730	<i>tcmHIJKLMN</i> ¹⁷⁷	TCM D3

^a See Fig. 5A.

^b These transformants produce approximately 10-fold-increased amounts of TCM F2 relative to that produced by pWHM721 transformants.

^c These transformants produce roughly fivefold-increased amounts of TCM F1 relative to that produced by pWHM725 transformants.

results of the earlier complementation analysis, *tcmI* thus encodes the D-ring cyclase; the *tcmJ* gene product is not required for this reaction.

On the other hand, when just *tcmJ* was added to the genes of pWHM721 (as in pWHM726), production of TCM F2 was increased roughly 10-fold (Table 3). This finding suggests that the *tcmJ* gene product functions in one of the early steps that leads to the biosynthesis of TCM F2. Presumably, in the absence of *tcmJ* this step can still occur but at a lower, uncatalyzed rate. Some of the cyclizations and attendant dehydrations involved in the biosynthesis of TCM C might reasonably be supposed to behave in this fashion. Indeed, chemical evidence shows that linear polyketide chains can cyclize spontaneously in vitro (14).

When both *tcmI* and *tcmJ* were coupled (as in construct pWHM728 [Fig. 5A]), tetracyclic TCM F1 was again the principal product as it had been when *tcmI* was present alone (Table 3). Yet, the yield of TCM F1 was higher in this case, possibly reflecting the increased TCM F2 made available by the *tcmJ* gene product.

Interestingly, when the *tcmH* gene was added to the *tcmKLMN*¹⁷⁷ genes (to give construct pWHM724 [Fig. 5A]), chromatographic evidence indicated that the biosynthesis of all TCM intermediates was abolished (Table 3). This was true even if *tcmH* was coupled with *tcmI* (construct pWHM727) or *tcmJ* (construct pWHM729). Only when the *tcmHIJ* genes were present together (as in construct pWHM730) was the biosynthesis of TCM intermediates restored, resulting in the low-level accumulation of TCM D3 (Table 3).

The failure of the *tcmH*-containing constructs pWHM724, pWHM727, and pWHM729 to cause production of TCM intermediates in strain WMH1077 is unusual, and it might arise because these plasmids fail to express any of the encoded *tcm* genes. Yet, each of these plasmids complements the *tcmK*, *tcmL*, and *tcmM* type strains (and, where appropriate, the *tcmI* and *tcmJ* type strains), indicating that the downstream genes are expressed at least somewhat. A second possibility is that these plasmids somehow "poison" antibiotic production, but TCM C production was unimpaired when wild-type *S. glaucescens* was transformed with pWHM724, pWHM727, and pWHM729. It is unclear, therefore, why the three *tcmH*-containing constructs fail to direct the synthesis of any known TCM intermediates. (It seems unlikely that these three constructs produce a toxic compound in the WMH1077 background since all three constructs transform strain WMH1077 at high frequency; on the other hand, it is possible that the

FIG. 6. Phenotype determined by TLC of culture extracts of *S. glaucescens* WMH1077 transformed with plasmids pIJ486, pELE37, pWHM731, pWHM721, and pWHM726. The pIJ486 vector has no effect (the faintly visible material has a fluorescence very different from that of TCM F2). Plasmid pELE37 enables the production of small amounts of TCM F2 and several other unidentified compounds. Plasmid pWHM731 causes a roughly 10-fold increase in the levels of all of the compounds synthesized by the WMH1077(pELE37) transformant, whereas pWHM721 drives an increase in TCM F2 production alone. Plasmid pWHM726 boosts TCM F2 production approximately 100-fold in comparison to pELE37, and the levels of the other metabolites are increased also, but not as greatly as observed for the WMH1077(pWHM731) transformants. We presume that the unidentified metabolites are early TCM intermediates or their shunt products.

product of the *tcmP* gene, which is expressed in WMH1077, converts the compound(s) produced by these constructs into something not recognizable as a TCM metabolite.)

The *tcmJ* gene product acts earlier in TCM F2 biosynthesis than the N-terminal domain of the TcmN protein. Tricyclic TCM F2 is the earliest known intermediate in TCM C biosynthesis (26). Yet, even this compound is fairly advanced along the path from acetate and malonate to TCM C, and its synthesis should require the activities of several enzymes. Previously, we found that a 5' portion of the *tcmN* gene (*tcmN*¹⁷⁷) enables increased synthesis of TCM F2 when coupled with the *tcmKLM* polyketide synthase genes, suggesting that the N-terminal domain of the TcmN protein provides some of the activities required for TCM F2 biosynthesis (30). From the findings described here, the product of the *tcmJ* gene appears to function similarly. Therefore, to determine the order of the steps catalyzed by the *tcmJ* and *tcmN*¹⁷⁷ gene products, we again employed the partial *tcm* gene clusters and compared the effects of several genetic combinations of *tcmJ* and *tcmN*¹⁷⁷ on the synthesis of TCM intermediates (Fig. 5B and 6).

Plasmid pELE37 expresses just the *tcmKLM* polyketide synthase genes, and in the TCM⁻ background of *S. glaucescens* WMH1077, this plasmid directed the synthesis of trace quantities of TCM F2 and several other unidentified metabolites (Fig. 6). With just these genes we presume that the small amount of TCM F2 arises by the uncatalyzed cyclization and dehydration of the poly-β-ketoacyl chain produced from the polyketide synthase. Interestingly, when the *tcmJ* gene was

added to pELE37 (as in construct pWHM731 [Fig. 5B and 6]), the levels of TCM F2 and the other metabolites were increased more than 10-fold. Evidently, *tcmJ* plays a role in the biosynthesis of several compounds. In contrast, addition of *tcmN*¹⁷⁷ to the polyketide synthase genes of pELE37 (to give plasmid pWHM721) caused an increase only in TCM F2. The levels of the unidentified metabolites were not affected. Thus, although both *tcmJ* and *tcmN*¹⁷⁷ stimulate TCM F2 production, the *tcmJ* gene has a broader effect. When both *tcmJ* and *tcmN*¹⁷⁷ were coupled with the polyketide synthase genes (as in plasmid pWHM726), TCM F2 biosynthesis was increased nearly 100-fold over the amount produced by the *tcmKLM* genes alone. In this case, the levels of the other metabolites remained relatively low.

The *tcmJ* and *tcmN*¹⁷⁷ genes act additively in the biosynthesis of TCM F2, indicating that the products of these genes act independently, probably catalyzing separate steps in the formation of TCM F2. The slightly different effects of the two genes on the pattern of extractable metabolites in strain WMH1077 also support this notion. More importantly, the stimulatory effect of *tcmJ* on the production of several compounds, versus the specific effect of *tcmN*¹⁷⁷ on TCM F2 levels, suggests that the product of *tcmJ* acts earlier in the biosynthesis of TCM F2 than the product of *tcmN*¹⁷⁷: the metabolites that accumulate in the *tcmJ*KLM background are most likely early intermediates (but not their shunt products) that build up due to a bottleneck created by the absence of *tcmN*¹⁷⁷. This presumption is supported by the observation that all of these compounds disappeared and only TCM C and its known intermediates were formed when ethyl acetate extracts of the WMH1077(pWHM731) transformant culture were fed to a *tcmL* mutant (data not shown). Since it affects only TCM F2 production, the *tcmN*¹⁷⁷ gene product probably catalyzes the last step(s) in TCM F2 formation, namely, closure of the C ring and its subsequent dehydration. The *tcmJ* gene product could conceivably catalyze any of the earlier steps.

DISCUSSION

The nucleotide sequence of the 1.2-kb DNA fragment that lies immediately upstream from the TCM C polyketide synthase genes of *S. glaucescens* revealed three small genes (*tcmHII*) that are involved in the early-to-intermediate steps in the biosynthesis of TCM C. The *tcmH* gene encodes the C-5 monooxygenase that oxidizes TCM F1 to TCM D3 (Fig. 1), the *tcmI* gene encodes the D-ring cyclase that converts tricyclic TCM F2 to tetracyclic TCM F1, and the product of the *tcmJ* gene appears to act at an early step on the route to TCM F2. Both the TcmH monooxygenase and TcmI cyclase have been recently isolated, and their activities have been confirmed in vitro (24, 25).

The *tcmJ* gene, when coupled with the *tcmKLM* polyketide synthase genes on a high-copy-number plasmid, causes a roughly 10-fold increase in TCM F2 production in a TCM⁻ mutant (strain WMH1077). In this regard, the effect of *tcmJ* parallels the earlier findings with *tcmN*¹⁷⁷, which, when coupled with the *tcmKLM* genes, also directs increased biosynthesis of TCM F2 in strain WMH1077 (30). The *tcmN*¹⁷⁷ gene, however, acts only on TCM F2 production; no other compounds are observed to accumulate in the strain WMH1077 transformants. In contrast, the *tcmJ* gene boosts production of TCM F2 and several other unidentified metabolites.

Because of its specific effect on TCM F2 production, the product of *tcmN*¹⁷⁷ most likely catalyzes the last step(s) in TCM F2 biosynthesis, namely, closure (and perhaps the attendant dehydration) of the C ring (the third step in Fig. 1).

FIG. 7. TCM F2 and its hypothetical ACT analog. The product of the *tcmJ* gene is most likely responsible for the additional cyclization that distinguishes TCM F2 from its ACT counterpart.

Consistent with this assignment, the deduced product of *tcmN*¹⁷⁷ resembles the predicted N-terminal portion of the *actVII* gene product of *S. coelicolor* (22, 30), which has been implicated in the formation of the penultimate ring in the ACT monomer (27, 35).

On the basis of its stimulatory effect on the levels of several metabolites (most likely early TCM intermediates or their shunt products), the *tcmJ* gene product appears to act earlier in the biosynthesis of TCM F2 than the product of *tcmN*¹⁷⁷. A possible role for the product of *tcmJ* is suggested from a comparison of the biochemistry and the genetics of TCM C production with those of ACT production (4, 9, 10, 22, 27, 35). As shown in Fig. 7, immediately prior to the final ring closure in the biosynthesis of TCM C or the ACT monomer, the corresponding intermediates differ primarily in size and the number of aromatic rings. The putative ACT intermediate is two acetyl units smaller and has one less ring. (By this time in its biosynthesis, it is likely that the ACT monomer has also undergone keto-reduction at C-3 [as shown] catalyzed by the product of the *actIII* gene [13] and therefore has lost one of the phenolic hydroxyl groups. The exact timing of this step, however, is unknown [16].) To account for the differences between the TCM and ACT intermediates shown in Fig. 7, the genetic data suggest that the *tcmJ* gene is at least partly responsible. While the evident similarities between the two intermediates are mirrored in the organizations of the *act* and *tcm* gene clusters (thus, *actI* ORFs 1, 2, and 3 and the N-terminal codons of *actVII*, which are responsible for the early steps in ACT biosynthesis, are analogous to the *tcmKLMN*¹⁷⁷ genes, respectively), there is no *tcmJ* analog in the *act* gene cluster. Since the additional cyclization that distinguishes TCM F2 from the early ACT intermediate should require a corresponding enzymatic activity, it seems most reasonable that the *tcmJ* gene encodes this cyclase. The stimulatory effect of the *tcmJ* gene on the biosynthesis of several compounds in addition to TCM F2 (Fig. 6) supports this idea, as it seems that the product of *tcmJ* acts relatively early in the biosynthesis of TCM F2. Of course, it is also conceivable that *tcmJ* encodes the enzyme responsible for the difference in chain length between the ACT and TCM intermediates. This seems less probable, though, because the determination of chain length, while slightly different for ACT and TCM, should require similar enzymes, and analogous genes for this activity would be expected in both gene clusters.

A *tcmJ* deletion mutant (strain WMH1500) still produces some TCM C, indicating that the step catalyzed by the putative TcmJ enzyme is chemically facile, as expected for an intramolecular aldol condensation such as the B-ring cyclization. Paradoxically, however, the deletion mutant also accumulates TCM F2. Since the TcmI cyclase is responsible for the conversion of TCM F2 to TCM F1, the accumulation of TCM F2 by strain WMH1500 suggests that the *tcmJ* gene product may somehow contribute to this activity. Yet, it is clear that *tcmJ* is not required for the activity of the TcmI cyclase, since

a plasmid that expresses just the *tcmIKLMN*¹⁷⁷ genes is sufficient to direct formation of TCM F1 in the TCM C⁻ mutant WMH1077. Moreover, purified TcmI protein has recently been shown to catalyze specifically the efficient conversion of TCM F2 to TCM F1 in vitro (25). Another possibility is that the remnant polypeptide (Δ TcmJ) in strain WMH1500 interferes with the activity of the TcmI cyclase. This might suggest that TcmI and TcmJ interact naturally in the wild-type strain, even though such an interaction is not required for the activity of either protein. We are currently examining this possibility.

Each of the *tcmHIJ* genes has an analog in *S. coelicolor*. The TcmH monooxygenase is 39% identical to the predicted product of *actVa*-ORF6 from the ACT gene cluster, the TcmI cyclase resembles the putative product of *whiE*-ORFVII from the *whiE* spore pigment gene cluster (40% identity), and the deduced TcmJ protein is similar to the expected product of *whiE*-ORFII (43% identity). The similarity between TcmH and the product of *actVa*-ORF6 was anticipated from the need in ACT biosynthesis for a monooxygenation analogous to the C-5 monooxygenation of TCM C (4). Unfortunately, the structure of the *whiE* metabolite and the functions of the putative *whiE*-ORFII and *whiE*-ORFVII proteins are unknown. There is, however, considerable similarity between the *tcm* and *whiE* gene clusters, suggesting that the *whiE* metabolite may be a naphthacenequinone-like pigment.

Finally, in spite of the similar proposed cyclase functions for the TcmI and TcmJ proteins and the N-terminal domain of the TcmN protein, there is very little resemblance among their derived primary structures. A comparison using the program GAP reveals that TcmI and TcmJ are the most similar (21% identity with several introduced gaps), while neither protein has much in common with the N-terminal domain of the TcmN protein (13% identity with TcmI and 11% identity with TcmJ). All three proteins or domains are, however, relatively small (<20 kDa).

At this point, our view of the early events in the biosynthesis of TCM C is as follows: (i) the *tcmKLM* genes encode the polyketide synthase that builds the linear decaketide chain and possibly also catalyzes the formation of the A ring, which more or less dictates the final structure of the TCM C backbone; (ii) the *tcmJ* gene product closes the B ring and perhaps participates in the attendant dehydration; (iii) the N-terminal domain of the TcmN protein then cyclizes (and dehydrates) the C ring to give TCM F2; (iv) the product of *tcmI* forms the D ring, giving TCM F1; and finally, (v) the TcmH monooxygenase oxidizes TCM F1 to the quinone TCM D3. The relationship between each cyclization and the attendant dehydration is unclear. It appears that separate enzymes for each dehydration do not exist, since DNA sequence analysis of the *tcm* cluster has failed to reveal any genes that might encode such dedicated activities. Consequently, either the dehydrations occur without catalysis immediately following ring closure or the cyclase enzymes may provide the required activities, as in the case of TcmI (25).

Only a few enzymatic functions in the proposed pathway for TCM C biosynthesis remain to be accounted for. These include (i) the malonyl coenzyme A:acyl carrier protein transferase that transfers malonate to the TcmM acyl carrier protein for use in polyketide chain growth, (ii) a thioesterase-like activity that, in part, may determine the polyketide chain length, and (iii) the cyclase that forms the A ring of the tetracyclic backbone. The last two activities are almost certainly encoded by the *tcmKLMN*¹⁷⁷ genes, since these alone are sufficient to drive the synthesis of TCM F2 (30) and analogs of these genes are common to all aromatic polyketide biosynthetic gene

clusters (16, 18). The malonyl coenzyme A:acyl carrier protein transacylase, on the other hand, may be scavenged from the fatty acid biosynthetic machinery of primary metabolism, since no gene for this activity has been found in any of the aromatic polyketide gene clusters (16, 18). We have recently cloned a gene from *S. glaucescens* that encodes this activity and are studying its role in both fatty acid and TCM C biosynthesis (28).

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