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Biochemistry

Cloning and heterologous expression of a gene cluster for the biosynthesis of tetracenomycin C, the anthracycline antitumor antibiotic of *Streptomyces glaucescens*

(antibiotic resistance/pigment production genes)

HAIDEH MOTAMEDI AND C. RICHARD HUTCHINSON†

School of Pharmacy and Department of Bacteriology, University of Wisconsin, Madison, WI 53706

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ABSTRACT **Through complementation of mutations specifically blocking the biosynthesis of tetracenomycin C by *Streptomyces glaucescens* and selecting for resistance to tetracenomycin C in *Streptomyces lividans*, all of the genes for the production of tetracenomycin C were inserted in pIJ702, a high copy-number *Streptomyces* gene cloning vector. The *tcm* biosynthetic and resistance genes occur as a single cluster in the *S. glaucescens* genome and are expressed in heterologous streptomycete hosts like *S. lividans*, resulting in the overproduction of pigmented intermediates of the tetracenomycin C biosynthetic pathway.**

Anthracycline antibiotics like daunorubicin (**1**) and doxorubicin (**2**) are widely used in cancer chemotherapy but not without severe toxicological problems (1) and with a high production cost due to the complex metabolite mixtures that are characteristic of the producing strains (2). Solutions to such problems should be found through studies of the genetics of anthracycline antibiotic production and application of the knowledge gained for strain improvement and the creation of additional anthracycline metabolites with increased potency or lessened toxicity. As a first step in these directions, we initiated a study of the genetics of tetracenomycin C (TcmC) production. TcmC (**3**) is an anthracycline antibiotic with significant antitumor activity (3) that is produced by *Streptomyces glaucescens* GLA.0 (ETH 22794), a genetically well-characterized streptomycete (4). The biochemical parallels in the biosynthetic pathways of compounds **1**, **2**, and **3** (5–7) may reflect an underlying genetic similarity and should enable the production of hybrid anthracycline antibiotics by genetic engineering.

To set the stage for cloning the *tcm* genes, we isolated a set of TcmC-nonproducing mutants (6) and characterized the metabolites that accumulated in each mutant (7). From the latter information and the cosynthetic properties of these mutants, we constructed a hypothetical pathway for the biosynthesis of compound **3** and determined the positions of 10 *tcm* mutations that blocked this pathway at several points (6). Here we report the cloning of all of the genes required for TcmC biosynthesis through the complementation of the *tcm* mutations and the linkage between *tcm* biosynthetic and resistance genes. By examining the cloned *tcm* DNA, we show that the production and resistance genes form a single cluster in *S. glaucescens* and are expressed in heterologous streptomycete hosts.

MATERIALS AND METHODS

Bacteria, Plasmids, and Growth Media. The strains of *S. glaucescens* used in this study are described in ref. 6. *Streptomyces lividans* TK 24 (8) and plasmid pIJ702 (9) were obtained from David Hopwood (John Innes Institute, Norwich, U.K.). *S. glaucescens* strains were grown in the liquid R2YE plus $NaNO_3$ at 0.3 g/liter and monosodium L-glutamate at 3 g/liter (R2YENG) medium described by Hintermann *et al.* (10). Their protoplasts were regenerated on the same medium containing 2.2% (wt/vol) agar. Other conditions for the growth and sporulation of this species were as described in ref. 6. *S. lividans* TK 24 was grown in R5 medium (11), and protoplasts of this strain were regenerated on R2YE medium (11). Strains transformed with pIJ702 and its derivatives were grown under selection with thiostrepton (50 μg/ml).

DNA Isolation. Chromosomal DNA was isolated from *S. glaucescens* by the method of Hopwood *et al.* (11). Plasmid DNA was isolated by the minilysate method of Kieser (12), which was scaled-up 10-fold for the preparation of large amounts of DNA.

Cloning Procedure. pIJ702‡ was completely digested with *Bgl* II and treated with calf intestine alkaline phosphatase (Boehringer Mannheim). Chromosomal DNA from *S. glaucescens* GLA.0 was partially digested with *Bam*HI or *Mbo* I and size-fractionated on 10–40% sucrose gradients (11)

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Abbreviations: Tcm, tetracenomycin; Thio^R, thiostrepton resistant.
†To whom requests for reprints should be addressed.
‡Insertional inactivation of the *mel* gene in pIJ702 (9) could not be used as preliminary determination of the cloning efficiency because all of the TcmC$^-$ mutants produced melanin.

to an average size of 8–18 kilobase pairs (kb). Vector DNA (1 μg) was ligated with insert DNA (5 μg) in ligation buffer (200 μl) plus T4 DNA ligase (1 unit) at 12°C overnight.

Protoplasts of *S. glaucescens* TcmC$^-$ mutants and *S. lividans* TK 24 were prepared by minor modifications of the method of Hopwood *et al.* (11), then transformed with 100-μl aliquots of the ligation mixture and regenerated on R2YENG or R2YE plates following the protocols described for *S. lividans* (11). After overnight incubation at 30°C, thiostrepton-resistant (Thio^R) transformants were selected by overlaying the regeneration plates with soft nutrient agar containing sufficient thiostrepton for a final concentration of 50 μg/ml, followed by growth at 30°C for 3–4 more days.

The Thio^R colonies from the transformation of *S. glaucescens* strains were transferred onto master Hickey–Tresner sporulation plates (6) containing thiostrepton and R2YE plug plates (96-well microtiter plates). After 3–4 days incubation at 30°C, the plugs were screened for the TcmC$^+$ phenotype by bioassay with *Streptomyces coelicolor* M111 (*hisA1 uraA1 strA1* SCP1 SCP2*) as described (6). Colonies showing a zone of growth inhibition were streaked on Hickey–Tresner plates containing thiostrepton, then plasmid DNA was isolated from 5-ml cultures of single colonies and used to retransform the original host to confirm that the recombinant plasmid complemented the *tcm* mutation.

Confluent lawns of sporulated Thio^R *S. lividans* TK 24 transformants were replica plated onto R2YE containing crude TcmC at 70 μg/ml and thiostrepton. (Crude TcmC was prepared by extracting a 50-ml culture of the GLA.0 strain with 25 ml of ethyl acetate, evaporating the extract to dryness on a rotary evaporator, and dissolving the powdered residue in acetone.) Colonies growing under these conditions were streaked onto the same medium, then plasmid DNA was isolated from single colonies and used to retransform TK 24 to confirm that the recombinant plasmid carried the *tcm* resistance gene.

RESULTS

Cloning of *tcm* Biosynthetic and Resistance Genes. By inserting fragments of *Bam*HI-digested total DNA from *S. glaucescens* GLA.0 into the *Bgl* II site of the high copy-number *Streptomyces* vector pIJ702 (9), then transforming different types of TcmC$^-$ mutants (6) and screening for TcmC$^+$ colonies, we obtained 3 clones out of 7500 Thio^R transformants that complemented the *tcmII* mutation in the GLA.4-48 strain. No complementing clones were isolated when an identical number of transformants of the GLA.5-57 (*tcmIII*) strain or 3000 transformants of the GLA.5-1 (*tcmIc*) strain were screened. All the Thio^R transformants were found to be TcmC$^+$ upon retransformation of GLA.4-48 with plasmid DNA isolated from the 3 clones, thus confirming the presence of DNA complementing the *tcmII* mutation in the recombinant plasmids.

Restriction mapping of these plasmids established the presence of 6.9-, 7.9-, and 12.15-kb DNA inserts in plasmids pHM49, pHM10, and pHM57, respectively (Fig. 1 *A–C*). The insert in pHM10 overlapped that of pHM49 and was located in the same relative orientation in the vector. pHM57 contained the same insert as pHM10, but in the opposite orientation relative to vector DNA, and also had a 0.45-kb extension to the right and a 3.8-kb extension to the left of this insert.

A TcmC sensitive strain, *S. lividans* TK 24 (8), was used as the host to clone the *tcm* resistance gene by using pIJ702 with *Mbo* I-digested GLA.0 DNA inserted into its *Bgl* II site. Three different plasmids were obtained from five TcmC-resistant clones and designated as pHM151 (one isolate), pHM152 (two isolates), and pHM153 (two isolates). After confirming by retransformation that these three plasmids carried the *tcm* resistance gene, restriction mapping showed (Fig. 1*F*) that pHM153 contained a 5-kb DNA insert that was also present in the opposite orientation in pHM151. Plasmid pHM151 had an 8.05-kb insert representing a 2.05-kb extension to the left and a 1-kb extension to the right of the insert in pHM153 (Fig. 1*E*). pHM152 carried the entire insert of pHM151 in the same orientation plus a 4-kb extension to its right (Fig. 1*D*).

Complementation Patterns of the *tcm* Clones. To learn if the plasmids isolated from the 6 clones carried other *tcm* genes, representatives of the 10 TcmC$^-$ mutants identified (6) were transformed with each of the 6 plasmids, and the transformants were scored for the TcmC$^+$ phenotype. pHM49 strongly complemented the *tcmIa, -Id, -II, -IV,* and *-V* mutations but only weakly complemented the *tcmVI* mutation (Table 1) as judged by the size of the zone of growth inhibition. pHM10 showed a similar complementation profile indicating that its 1 kb of extra DNA does not encode any detectable functions. Both plasmids complemented the *tcmIb* mutation in only 5–10% of the transformants tested. This implies that each of these plasmids contained only a part of the *tcmIb* gene, the less-than-complete complementation possibly resulting from the need for recombination with the genomic DNA to observe the TcmC$^+$ phenotype (14). pHM57, which contains the largest insert of GLA.0 DNA, complemented all seven of these *tcm* mutations (Table 1) to the same extent.

All three of the plasmids carrying the *tcm* resistance gene complemented the *tcmVII* mutation. pHM151 in addition complemented the *tcmIc* and *tcmIII* mutations in 30–50% of the cases. This plasmid was very unstable in all of the TcmC$^-$ mutants except for GLA.4-16 (*tcmVII*). Plasmid pHM152 could not be maintained in any of the TcmC$^-$ strains. Despite our inability to verify this directly because of its instability, pHM152 should have a complementation profile similar to pHM151 since it carries the same insert plus some additional DNA. All three of these plasmids were relatively stable in the TK 24 strain.

Clustering of *tcm* Genes. The possibility that the 0.45-kb *Bam*HI fragment on the right end of the pHM57 insert (Fig. 1*C*) was the same as the *Bam*HI fragment on the left end of the pHM152 (Fig. 1*D*) and pHM151 (Fig. 1*E*) inserts was tested by Southern hybridization. A 2-kb *Sst* I–*Kpn* I fragment (sites 24 to 29 of Fig. 1), which contains ≈300 base pairs of the presumptive overlapping sequence, was labeled with biotinylated dUTP and used to probe different restriction digests of pIJ702, pHM57, and pHM152 (Fig. 2*A*). Vector DNA did not hybridize with this probe; *Sst* I–*Kpn* I-digested pHM57 DNA hybridized only to a band of identical size as the probe, as expected; *Sst* I–*Pst* I-digested pHM152 DNA yielded a 1.9-kb fragment from a *Pst* I site in the vector through sites 28–32 (Fig. 1*D*) that hybridized to the probe; and *Bam*HI-digested pHM152 DNA gave a 1.8-kb fragment from a *Bam*HI site in the vector through sites 28–30 (Fig. 1*D*) that hybridized to the probe. Since these latter two fragments each contain the entire 0.45-kb fragment covering sites 28–30 in Fig. 1, the results establish that the inserts of plasmids pHM57 and pHM152 share a 0.45-kb sequence of overlapping DNA as shown in Fig. 1 *C* and *D*.

To see if the genomic organization of *tcm* DNA is identical to that of the cloned *tcm* DNA, the same probe was hybridized with *Bam*HI and *Xho* I–*Eco*RI-digested GLA.0 DNA. Hybridizing fragments of ≈7 kb were seen in the *Bam*HI digest (Fig. 2*B*), which represents sites 15–28 in Fig. 1, and of ≈8.3 kb in the *Xho* I–*Eco*RI double digest (Fig. 2*B*), representing sites 17–36 in Fig. 1. Since pHM152 contains the unique *Eco*RI site and no *Xho* I sites (Fig. 1*D*), these results confirm the lack of DNA rearrangement during the cloning experiments.

Expression of *tcm* Genes in Homologous and Heterologous Streptomycete Hosts: Evidence for Cloning of the Entire *tcm*

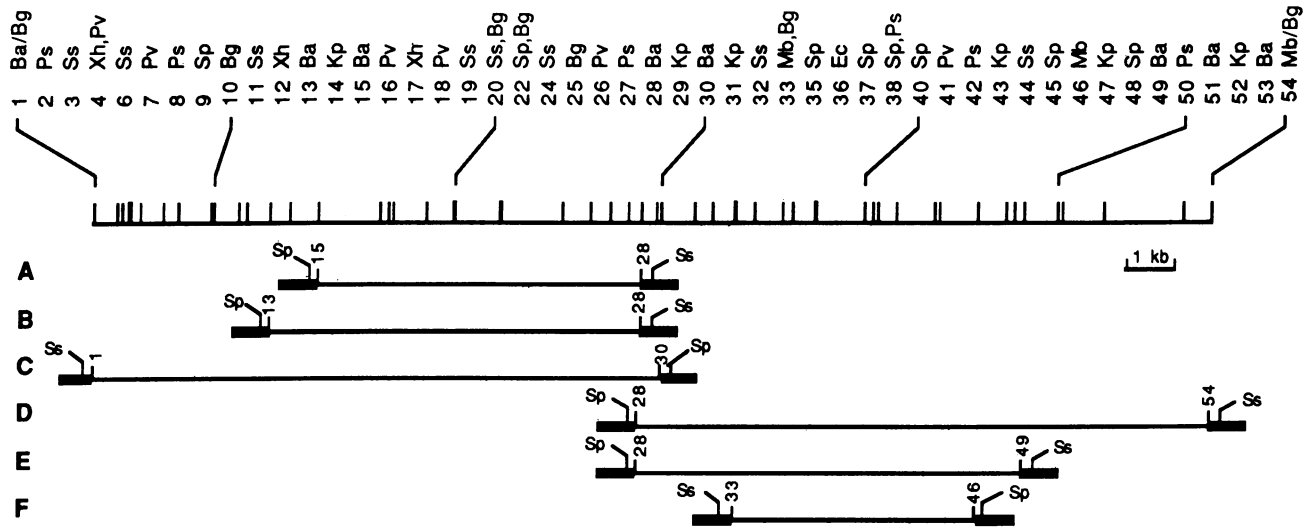

FIG. 1. Restriction map of the *tcm* gene cluster as deduced from the restriction maps of the following six plasmids isolated from TcmC$^+$ clones. (*A*) pHM49. (*B*) pHM10. (*C*) pHM57. (*D*) pHM152. (*E*) pHM151. (*F*) pHM153. Thin line, DNA cloned from *S. glaucescens* GLA.0; thick line, a portion of the vector DNA. Only the first restriction site flanking the *Bgl* II cloning site in the vector DNA is shown, and the *Mbo* I sites (numbers 33 and 46) are the two *Mbo* I/*Bgl* II junction sites of pHM153. Restriction site abbreviations: *Bam*HI (Ba), *Bgl* II (Bg), *Eco*RI (Ec), *Kpn* I (Kp), *Mbo* I (Mb), *Pst* I (Ps), *Pvu* II (Pv), *Sph* I (Sp), *Sst* I (Ss), and *Xho* I (Xh). Restriction maps were constructed from the results of single and double restriction enzyme digestions. The sizes of DNA fragments fractionated in agarose gels were measured from the gel photographs with the aid of microcomputer programs [ref. 13; K & H Biosoft, Frederick, MD].

Gene Cluster. Recombinant plasmid pHM57, when introduced into wild-type and TcmC$^-$ strains of *S. glaucescens*, caused the overproduction of an orange-red pigment that was identified by TLC and structural analysis (NMR, IR, and UV spectroscopy) as a mixture of the known metabolites TcmD1 and TcmE (7). This finding suggested that pHM57 carried all of the genetic information for the production of TcmE, a relatively late intermediate of the TcmC biosynthetic pathway (6, 7). We tested this possibility by transforming *Streptomyces ambofaciens* ATCC 15154, *Streptomyces lasaliensis* NRRL 3382R, and *S. lividans* TK 24 with pHM57. The transformants all produced an orange-red pigment very early in their growth that was proven to be a mixture of TcmD1 and TcmE (data not shown).

Since the overlapping inserts in pHM57 and pHM152 represent ≈24 kb of contiguous DNA from *S. glaucescens*, we wondered if this would be enough to encode the entire TcmC biosynthetic pathway. To examine this possibility, *S. lividans* TK 24(pHM57) and TK 24(pHM152) transformants were grown in a mixed liquid culture, and, at 3 days, the culture was extracted with ethyl acetate to recover TcmC and similar metabolites (7). TLC (Fig. 3) plus bioautography (data not shown) of this extract showed that TcmC, verified by chemical [conversion to the 3-ethoxy-3-demethoxy derivative of TcmC (7)] and spectral [IR, NMR, and UV (7)] analyses, and several related metabolites (TcmA2, -B, -D, and -E) were produced by the mixed culture and the wild-type GLA.0 strain, whereas such compounds were not found in extracts of TK 24 and TK 24(pHM152) cultures. As noted above, TK 24(pHM57) produced TcmD1 and TcmE, the two metabolites that also are accumulated by the GLA.4-16 (*tcmVII*) mutant (6, 7).

The observation that all of the TcmC production genes reside in pHM57 and pHM152 was confirmed by a double transformation experiment. *S. lividans* TK 24(pHM57) was

Table 1. Complementation patterns of plasmids containing *tcm* DNA

	TcmC$^-$ mutant phenotype									
Plasmids	Ia	Ib	Ic	Id	II	III	IV	V	VI	VII
pHM10	+*	±†	−	+	+	−	+	+	±‡	−
pHM49	+	±†	−	+	+	−	+	+	±‡	−
pHM57	+	+	−	+	+	−	+	+	+	−
pHM151§	−	−	+	−	−	+	−	−	−	+
pHM152§	−	−	+	−	−	+	−	−	−	+
pHM153§	−	−	−	−	−	−	−	−	−	+

*+, the TcmC$^+$ phenotype; −, the TcmC$^-$ phenotype.
†≈5% of the transformants were TcmC$^+$.
‡Weak complementation was observed.
§These plasmids also conferred the TcmC-resistant phenotype to *S. lividans* TK 24.

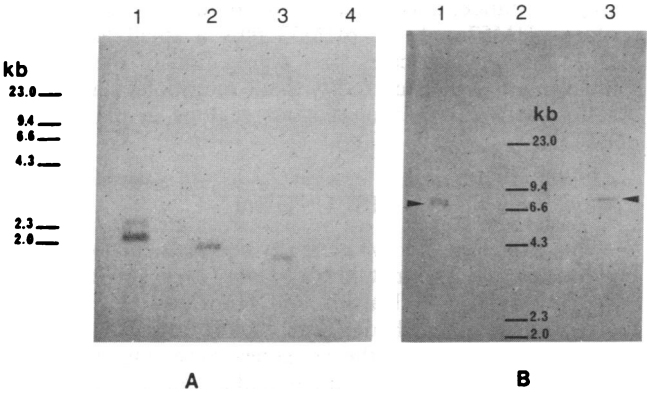

FIG. 2. Southern hybridizations using the *Sst* I–*Kpn* I fragment from pHM57 as the probe. (*A*) The positions of *Hin*dIII-digested λ DNA fragments are indicated. Lanes: 1, *Kpn* I/*Sst* I-digested pHM57 DNA (the upper band is due to a nick-translated contaminant coming from the left end of pHM57, Fig. 1); 2, *Sst* I/*Pst* I-digested pHM152 DNA; 3, *Bam*HI-digested pHM152 DNA; 4, *Bgl* II-digested pIJ702 DNA. (*B*) Total DNA from *S. glaucescens* GLA.0 digested with *Bam*HI (lane 1) or *Xho* I and *Eco*RI (lane 3); *Hin*dIII λ DNA fragments (lane 2). The 2-kb *Sst* I–*Kpn* I fragment on the right end of the pHM57 insert DNA, Fig. 1, was isolated by electroelution onto a NA-45 DEAE membrane (Schleicher & Schuell), as suggested by the manufacturer, and labeled with biotinylated-11-dUTP (Bethesda Research Laboratories) by nick-translation (15). DNA fragments were transferred to nitrocellulose (Schleicher & Schuell BA85) according to Maniatis *et al.* (16). Southern hybridization and detection of hybridized bands were according to the supplier with slight modifications.

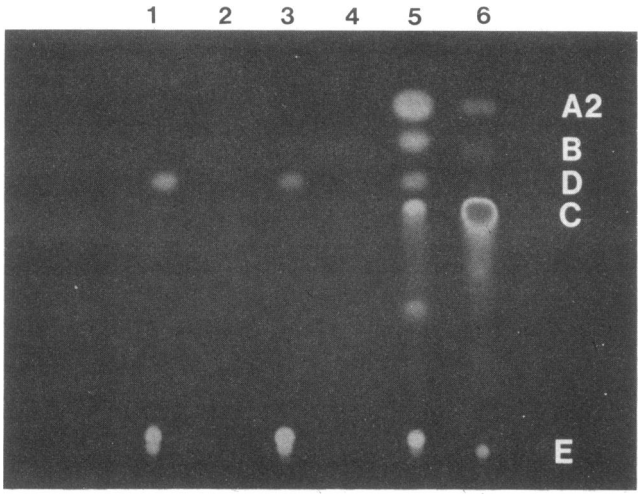

FIG. 3. Thin layer chromatogram of the fluorescent compounds produced by cultures of the following *Streptomyces* spp. Lanes: 1, *S. glaucescens* GLA.4-16 (*tcmVII*); 2, *S. lividans* TK 24; 3, TK 24(pHM57); 4, TK 24(pHM152); 5, mixed culture of TK 24(pHM57) and TK 24(pHM152); and 6, *S. glaucescens* GLA.0. The white letters indicate the positions of the following Tcm metabolites: TcmA2 (A2), TcmB1 (B), TcmD1 (D), TcmC (C), and TcmE (E). Mixed culture of *S. lividans* TK 24(pHM57) and TK 24(pHM152), and TLC and bioautography of the ethyl acetate extract of this culture were carried out as described (6). Tcm metabolites were identified by comparison with authentic reference standards (7).

transformed with plasmid pHM152, the transformed colonies were regenerated with selection for Thio^R, and the primary transformants were screened for antibiotic production. TcmC was produced by 2–5% of these colonies (verified by the yellow color of young colonies plus TLC and bioautography of colony extracts), which was possible because cotransformation of TK 24(pHM57) with pHM152 provided the necessary resistance to TcmC. This property was lost upon serial transfer with selection for Thio^R or for Thio^R plus TcmC resistance, and the resulting colonies were shown to contain pHM57 only or pHM57 plus a small amount of pHM152, which indicates that segregation of the primary transformants had occurred without intermolecular recombination between the homologous regions in pHM57 and pHM152.

DISCUSSION

We chose to clone the *tcm* genes by complementation of *tcm* mutations in blocked mutants of *S. glaucescens* following the precedent set by Malpartida and Hopwood (17) with the cloning of the actinorhodin gene cluster from *S. coelicolor*. Since they showed that the *act* genes were clustered in one segment of DNA (17), we anticipated a similar result for the *tcm* genes. But it was not at all certain that the biosynthetic and resistance genes would be located in the same DNA region, even though at the outset of our work there were two indications that this could be the case (17–19). That the *tcm* production and resistance genes are indeed clustered [and chromosomally located since the *tcmVII* mutation mapped between the *S. glaucescens his-2* and *met-2* loci (20) (E. Wendt-Pienkowski and C.R.H., unpublished data)] suggests that this is a common trait among the *Streptomyces* for their antibiotic production genes. Other discoveries (21) have confirmed this hypothesis with only one exception, the genes for the production of oxytetracycline that exist as two clusters in the *Streptomyces rimosus* genome (22).

In contrast to the *act* genes (17), we were able to clone the *tcm* genes with the high copy-number vector pIJ702 in a homologous background. Nonetheless, we did encounter a severe instability of plasmids pHM151 and pHM152, which is the reason that only 30–50% of the pHM151 transformants exhibited complementation of the *tcmIc* and *-III* mutations. Our failure to obtain TcmC$^+$ clones in the primary cloning experiment with the GLA.5-1 and 5-57 strains also must have been due to the instability of recombinant plasmids with DNA from the same region as in the inserts of pHM151 and pHM152. The relative stability of pHM153 and the isolation of a stable, deleted form of pHM151 that lacked the entire insert in this plasmid except for sites 28–31 (this deleted plasmid was isolated through complementation of the *tcmIII* mutation in GLA.5-47 with pHM151), suggests that the observed instability is due to a region of ≈1 kb around *Sst* I site 32 in Fig. 1. DNA subcloned from this region in a low copy-number *Streptomyces* plasmid in *S. lividans* was also quite unstable, causing extensive deletions of vector DNA along with complete loss of insert DNA (unpublished data).

Expression of the production genes for two other antibiotics—actinorhodin (17) and erythromycin A (23)—has also been observed in heterologous hosts. While the production of the TcmC pathway metabolites TcmA2 and TcmE in *S. lividans* was clearly increased due to the gene-dosage effect resulting from the use of a high copy-number vector, such an effect was not seen with the *ery* genes (23), even though the cosmid shuttle vector used in this work contained an origin of replication from a high copy-number *Streptomyces* plasmid. Moreover, the presence of pHM57 in *S. lividans* TK 24 and in *Streptomyces* spp. that produce compounds **1** and **2** inhibited formation of the pigmented metabolites normally produced by these strains (H.M., E. Wendt-Pienkowski, and C.R.H., unpublished data). The reason for this effect is unknown (and a similar effect on oxytetracycline production has also been noted[§]), but it might facilitate the detection of new metabolites produced by the introduction of *tcm* genes into different *Streptomyces* spp., if "hybrid anthracycline antibiotics" can be produced in the way demonstrated by Hopwood *et al.* (24) with the *act* genes.

The results of subcloning experiments have confirmed the implication, from the complementation data (Table 1), that pHM57 carries the genes encoding early steps and that pHM152 carries genes for the late steps (plus the TcmC resistance gene) in the TcmC biosynthetic pathway[¶]. Of particular interest is that the 1.8-kb *Bgl* II fragment bounded by sites 23 and 25 (Fig. 1) complements the *tcmIa* mutation[¶], which presumably blocks the first step in the TcmC pathway, formation of the polyketide precursor of TcmF (6, 7). The *Bgl* II fragment is homologous to the *actI* gene (25), which corresponds to a mutation having the same effect on actinorhodin biosynthesis (26). This result has been used along with other data to support a proposition for extensive sequence homology among "polyketide synthase" genes and to show the value of this homology in expediting the cloning of genes for the production of other polyketide metabolites (25). Availability of such polyketide synthase genes will enable studies of the genetic organization and enzymology of the complex polyketide biochemical systems found in bacteria, fungi, and plants.

[§]Hunter, I. S., Sugden, D. A., Warren, M. & Butler, M. J. (1986) Fifth International Symposium on the Genetics of Industrial Microorganisms, Sept. 14–20, 1986, Split, Yugoslavia, p. 162.

[¶]Motamedi, H. & Hutchinson, C. R. (1986) Fifth International Symposium on the Genetics of Industrial Microorganisms, Sept. 14–20, 1986, Split, Yugoslavia, p. 159.

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