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Cloning and Heterologous Expression in *Streptomyces lividans* of *Streptomyces rimosus* Genes Involved in Oxytetracycline Biosynthesis

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The anhydrotetracycline (ATC) oxygenase enzyme which carries out the conversion of ATC to dehydrotetracycline was purified and the N-terminal amino acid sequence was determined. The sequence displays a significant similarity to that of the *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens*. This is consistent with the activity of the oxygenase, i.e., addition of a hydroxyl moiety to an aromatic ring structure. Oligonucleotide probes were designed and used to clone the corresponding fragment of chromosomal DNA from *Streptomyces rimosus*. This DNA fragment was used to screen a cosmid library, allowing the isolation of flanking DNA sequences. Surprisingly, the gene was located within the previously cloned cluster of genes involved in the synthesis of the biosynthetic intermediate ATC and not as had been expected (P. M. Rhodes, N. Winskill, E. J. Friend, and M. Warren, J. Gen. Microbiol. 124:329–338, 1981) at a separate locus on the other side of the chromosome. Subcloning of an appropriate DNA fragment from one of the cosmid clones onto pIJ916 produced *Streptomyces lividans* transformants which synthesized oxytetracycline.

Oxytetracyline (OTC) is a commercially important broad-spectrum antibiotic produced by *Streptomyces rimosus*. OTC is a member of the polyketide class of antibiotics which includes, among others, avermectin, monensin, erythromycin, and other tetracyclines such as 7-chlorotetracycline.

The OTC biosynthetic pathway of *S. rimosus* has been the subject of both biochemical and genetic analysis (15). Figure 1 indicates the proposed biosynthetic pathway from malonyl coenzyme A to the final product. The isolation and characterization of mutants blocked at various points in the pathway indicated that the genes involved in OTC biosynthesis are clustered into two distinct positions mapping at diametrically opposite locations on the circular map of *S. rimosus*. The loci thought to be responsible for all pathway steps leading to the production of anhydrotetracycline (ATC) were assigned to the "4 o'clock" locus, whereas the genes encoding enzymes for the final part of the pathway, including those responsible for the production of a flavinlike cofactor (cosynthetic factor 1 [CSF1]) were assigned to a 10 o'clock location.

The isolation (3; M. J. Butler, E. J. Friend, I. S. Hunter, F. S. Kaczmarek, D. A. Sugden, and M. Warren, Mol. Gen. Genet., in press) of two genes, *otrA* and *otrB*, involved in host resistance to OTC and the analysis of flanking DNA indicated that the 4 o'clock cluster was organized as a single group of biosynthetic genes flanked by the two resistance loci (Fig. 2). However, the genes responsible for the conversion of ATC to OTC remained unidentified. This paper describes the isolation of the gene (*otcC*) encoding the ATC oxygenase, the enzyme catalyzing the hydroxylation of ATC to dehydrotetracycline. *otcC* unexpectedly mapped within the 4 o'clock cluster. This result led to experiments designed to test the hypothesis that all of the structural genes required for OTC biosynthesis were contained within this cluster. Confirmation of this hypothesis was achieved by the heterologous expression of OTC by *Streptomyces lividans* and *Streptomyces albus* transformants containing a 34-kilobase (kb) *Eco*RI fragment, including *otrA* and *otrB*, cloned in pIJ916.

MATERIALS AND METHODS

Bacteria and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Media and culture conditions. Growth and maintenance of *S. lividans* were essentially as described previously (7). *S. albus* G and *S. rimosus* strains were maintained on Emerson agar. For the preparation of chromosomal DNA from *S. rimosus*, strains were grown in Trypticase soy broth (BBL Microbiology Systems) for 2 days at 30°C. *Escherichia coli* strains were generally propagated on Luria medium. *E. coli* transformants were grown in either Luria broth or SOC broth (Bethesda Research Laboratories, Inc.) containing ampicillin at 100 $\mu g \cdot ml^{-1}$.

Transformations. High-efficiency transformation of *E. coli* DH5α was achieved using Library Grade (Bethesda Research Laboratories, Inc.) competent cells, according to the protocol of the supplier. Transformation of the *dam* hosts was by the standard calcium chloride method (as in reference 13). Regeneration and transformation of *S. lividans* protoplasts were essentially as described previously (7) except that *S. lividans* mycelium was grown for 3 days instead of 2 days in YEME medium containing glycine and $MgCl_2$ at 0.5% and 5 mM, respectively. *S. rimosus* protoplast formation, regeneration, and transformation were carried out as described by Butler et al. (in press).

S. albus G mycelium was grown at 34°C for 18 h in Trypticase soy broth, using spring flasks as described by Hopwood et al. (7). Protoplast formation, regeneration, and transformation conditions were the same as those used for *S. rimosus*.

Preparation of plasmid DNA. Small- and large-scale plasmid preparations from *E. coli* transformants were by the alkaline lysis procedure (13). Small-scale preparations from *Streptomyces* transformants were essentially as in the alkaline lysis procedure of Kieser (10), without including bromocresol green in the lysis step. The large-scale preparation

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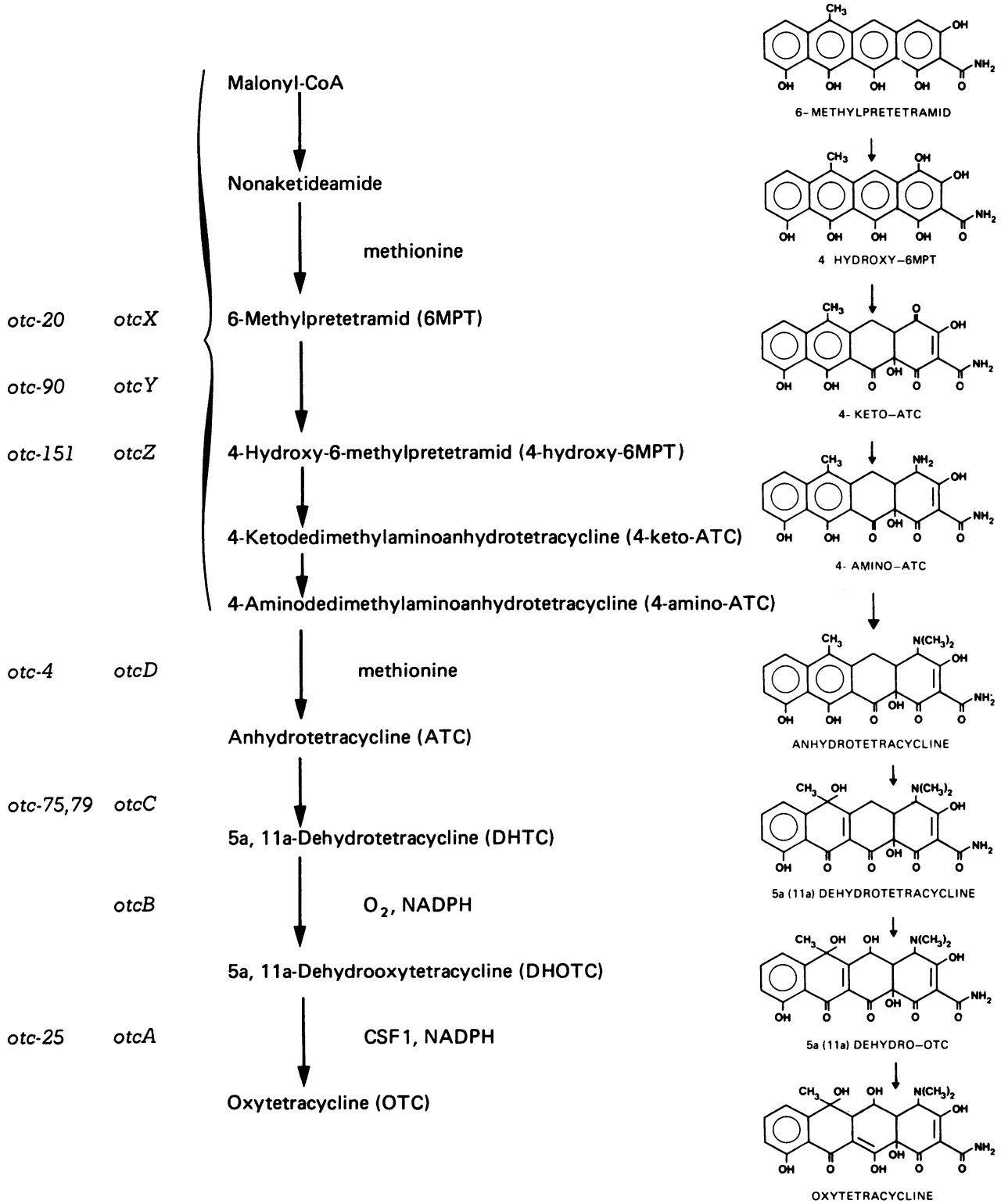

FIG. 1. Biosynthesis of OTC, indicating the steps at which various OTC-negative mutants are blocked. CoA, Coenzyme A.

of plasmid DNA was by the neutral lysis method described by Hunter (8).

Preparation of chromosomal DNA. High-molecular-weight chromosomal DNA from *S. rimosus* was purified as follows. Approximately 5 to 10 g (wet weight) of harvested mycelium was washed with 50 ml of 10.3% sucrose and centrifuged at 7,000 rpm for 10 min in a Sorvall GSA rotor, and the pellet was suspended in 40 ml of TE (10 mM Tris, 1 mM EDTA; pH 7.5). Lysozyme (egg white muramidase, grade 1, Sigma Chemical Co.) was added to a final concentration of 1

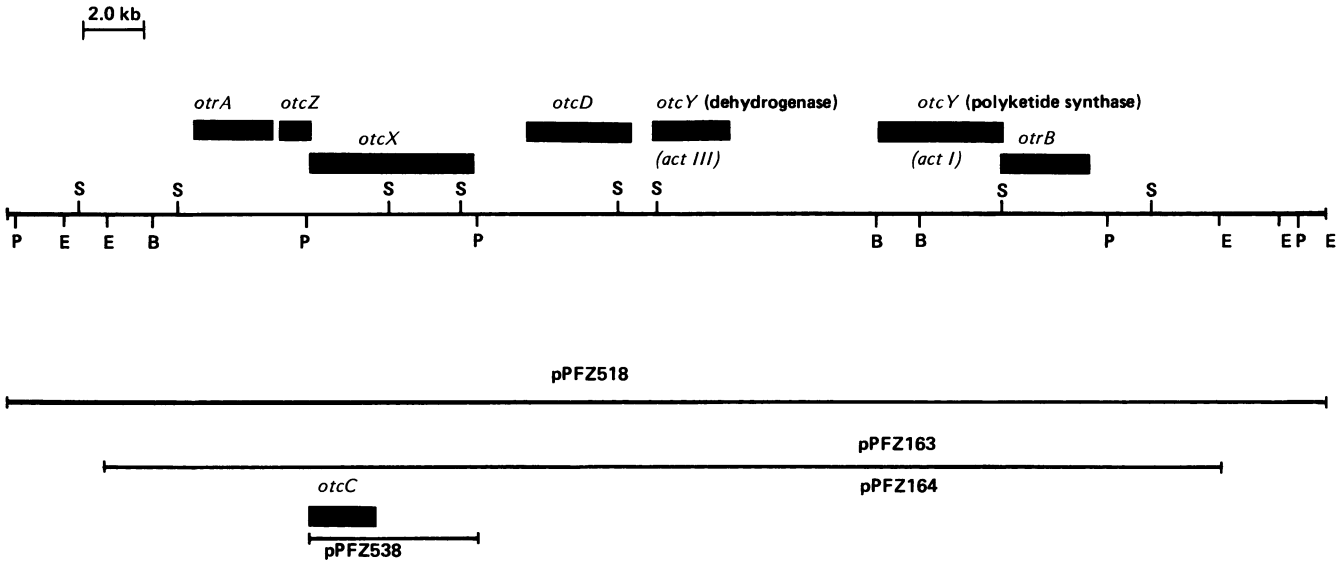

FIG. 2. Organization of the genes of the *otrA-otrB* biosynthetic cluster. The relative positions of DNA fragments exhibiting homology with the actinorhodin genes *actI* and *actIII* (12) are indicated. Both are contained within an *Sst*I fragment which complements the *otcY* mutation (Butler et al., in press). Abbreviations: E, *Eco*RI; P, *Pst*I, S, *Sst*I; B, *Bgl*II.

mg · ml^{-1}, and the mixture was incubated for 45 to 60 min at 37°C. Sarcosyl (*N*-lauroylsarcosine, sodium salt, Sigma Chemical Co.) was added with gentle inversion to a 1% final concentration, with complete lysis normally occurring after a few minutes. CsCl (Ultrapure, Bethesda Research Laboratories, Inc.) was added at 1.05 g · g of lysate^{-1}. CsCl was added slowly to avoid aggregation of nucleic acids and proteins. Ethidium bromide (Sigma) was added to a final concentration of 10 μg · ml^{-1}, and the lysate was centrifuged at 40,000 rpm for 20 h in a Sorvall TV865B vertical rotor. Chromosomal DNA, generally visible as a creamy-colored viscous band around the middle of the tube, was carefully removed by a wide-mouthed pipette. Four volumes of TE was gently mixed in with DNA in a conical flask, and 2 volumes of ice-cold ethanol (99% BDH Analar) was added carefully down the side of the flask. DNA was spooled off by a thick glass rod, washed in 70% ethanol, and dissolved by standing overnight at 4°C in 10 to 20 ml of TE. In some preparations a standard neutral phenol-chloroform extraction stage at this point, followed by respooling in ethanol, served to efficiently remove all residual protein. Long-term storage of aliquoted DNA was under ethanol at −20°C. The purity and concentration of chromosomal DNA were assessed by UV absorbance and electrophoresis in a 0.4% agarose gel.

Preparation of a cosmid library of *S. rimosus* chromosomal DNA in *E. coli*. Chromosomal DNA extracted from *S. rimosus* by the above method was partially digested with *Sau*3A1, using standard methodology (13). Material from four separate digestions was further fractionated by NaCl gradient centrifugation (1.5 to 5 M NaCl in TE), and the fractions were analyzed by 0.4% agarose gel electrophoresis. Fractions enriched for 35- to 45-kb fragments were pooled and concentrated by ethanol precipitation. A 0.5-μg amount of this material was ligated in a volume of 10 μl with 0.1 μg of pPFZ514 (previously digested with *Bgl*II and dephosphorylated using calf intestinal alkaline phosphatase [Boehringer Corp. (London), Ltd.]). Ligations were carried out overnight at 15°C in the presence of 1 μl (10 Weiss units) of T4 DNA ligase (Bethesda Research Laboratories, Inc.).

Ligated DNA was packaged in vitro, and host cells were transformed according to the Promega Biotech Packagene protocol. A total of 4,000 transformants were picked, grown in 150 μl of SOC medium (Bethesda Research Laboratories, Inc.) containing ampicillin (100 μg · ml^{-1}) in microtiter dishes (Nunc) for 2 days at 30°C, and stored as glycerol stocks at −70°C. Twelve representative transformants all carried plasmids in the appropriate size range, as judged by restriction enzyme analysis of alkaline lysis "miniprep" plasmid DNA. Optimum yield and integrity of cosmid clones were obtained by growing cells in SOC medium (containing ampicillin at 100 μg · ml^{-1}) for 24 h at 30°C.

The library was replicated onto sterilized squares of nylon membranes (1.2 μm, Pall Biodyne), grown in triplicate overnight at 37°C in Nunc bioassay dishes on Luria agar plus ampicillin (100 μg · ml^{-1}). Colonies were lysed by standard methods (13).

Purification and labeling of oligonucleotides. Two 42-base oligonucleotides (see Results) were prepared by the Biochemistry Department, Glasgow University, using an Applied Biosystems DNA synthesizer. Final purification was achieved by elution in 0.5 M ammonium acetate of material loaded onto a preparative 7 M urea–10% polyacrylamide gel, using UV shadowing on a thin-layer chromatography plate to visualize full-length oligonucleotides.

Hybridization conditions. For hybridizations with labeled oligonucleotides, between 20 and 30 μg of completely digested *S. rimosus* chromosomal DNA was loaded per lane of an 0.8% agarose gel and transferred by capillary action onto a nylon membrane (Hybond, Amersham International plc) in the presence of 0.4 M NaOH (14). The same method was followed for the transfer of DNA for hybridizations with nick-translated plasmid inserts, except that only 2 μg of digested material was loaded per lane.

Routinely, 100 ng of each oligonucleotide was end labeled by T4 polynucleotide kinase (Bethesda Research Laboratories, Inc.) in the presence of 100 μCi of [γ-^{32}P]ATP (New England Nuclear Corp.). Prehybridization and hybridization were carried out in 6× SCP (1× SCP is 0.1 M NaCl–0.03 M Na_2HPO_4–1 mM EDTA, pH 6.2) with 1% sarcosyl and 200

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Bacterial strains		
S. rimosus		
M24167	OTC^r	This work
M4018	OTC^r	15
M15883	OTC^s	This work
M23283	OTC^r restriction negative	9
otc-79	OTC$^-$	15
otc-75	OTC$^-$	15
otc-19	OTC$^-$ CSF1$^-$	15
otc-56	OTC$^-$ CSF1$^-$	15
S. lividans		
1326	SLP2$^+$ SLP3$^+$	7
TK64	Str^r Pro$^-$, plasmid-free	7
S. albus G R42	Restriction negative	4
E. coli		
ED8767	*hsdR hsdM recA56 mcrA mcrB supE sup*F Met$^-$	N. Murray
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1*φ80 *dlacZ*ΔM15 (*lacZYA argF*)	6
CB51	F$^-$ Ara$^-$ Lac$^-$ Pro$^-$ Thi$^-$ Dam$^-$ Str^r	I. S. Hunter
GM2929	F$^-$ *dam-13*::Tn*9 dcm-6 hsdR2 recF143 galK2 galT22 ara-14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL136 hisG4 tsx-78 mtl-1 glnV44*	T. Kieser (John Innes Institute)
Plasmids		
pUC18	Amp^r	19
pUC19	Amp^r	19
pIJ916	Thiostrepton resistant, low-copy-number *Streptomyces* plasmid	11
pIJ922	Thiostrepton resistant, low-copy-number *Streptomyces* plasmid	11
pTBE	Amp^r cosmid	F. Grosveld (5)
pPFZ514	pTBE, modified (see text)	This work
pPFZ518	Ca. 45 kb, partial *Sau*3A fragment from M24167 cloned in pPFZ514	This work
pPFZ538	5.2-kb *Pst*I fragment (ATC oxygenase clone) in pUC19	This work
pPFZ542	0.3-kb *Sau*3A fragment from pPFZ538 cloned in pUC19	This work
pPFZ105	*Pst*I fragment of pPFZ538 in high-copy-number plasmid pPFZ12	Chambers and Hunter (3)
pPFZ161	pIJ922 with *Pst*I fragment from pPFZ538	This work
pPFZ162	As pPFZ161, with insert in opposite orientation	This work
pPFZ163	pIJ916 + 34-kb *Eco*RI fragment from pPFZ518	This work
pPFZ164	As pPFZ163, with insert in opposite orientation	This work
pPFZ46	Cloned *S. rimosus* DNA containing the OTC resistance gene *otrA* in pPFZ12	Butler et al. (in press)
pPFZ57	Cloned *S. rimosus* DNA containing the OTC resistance gene *otrB* in pPFZ12	Butler et al. (in press)

μg of heparin · ml^{-1}. Boiled probe was added to fresh, prewarmed solution, and hybridization was carried out overnight at 55°C. Filters were washed four times at 55°C in 250 ml of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate). For higher-stringency hybridizations with nick-translated DNA, 100 ng of purified plasmid insert DNA was generally labeled by nick translation according to the kit method (Bethesda Research Laboratories, Inc.), using both labeled dGTP and dCTP.

Prehybridization and hybridization conditions were as above, but at 68 instead of 55°C. Filters were washed at 68°C in 250 ml of 5× SSC–0.1% sodium dodecyl sulfate and 5× SSC, followed by two successive washes in 0.1× SSC. Autoradiography was generally carried out at −70°C, using Amersham Hyperfilm and a Kodak X-Omat cassette.

DNA sequencing. The direct plasmid sequencing protocol outlined in the kit methods of Boehringer Corp. (using Klenow enzyme) and U.S. Biochemical Corp. (Sequenase enzyme) was followed, except that the concentrations of dGTP and dCTP were four times that recommended due to the high G+C content of *Streptomyces* DNA. In general, 2 μg of total denatured plasmid was used per reaction.

Purification and analysis of OTC from culture medium. OTC was eluted from agar plates in an equal volume of pH 1.7 HCl. The retention index on high-pressure liquid chromatography columns was assessed by S. V. Hammond, using criteria of strong anionic exchange (a proprietary resin) and reverse-phase mobility (Millipore μBondapack C_{18}). A wavelength scan of the purified material by a diode array detector was carried out by S. V. Hammond and M. Pacey.

RESULTS

For the isolation of the gene encoding ATC oxygenase, the strategy followed was to use oligonucleotide hybridization probes based on the known amino acid sequence of the purified protein.

Purification of ATC oxygenase. ATC oxygenase catalyses the formation of 5a,11a-dehydrotetracycline by hydroxyla-

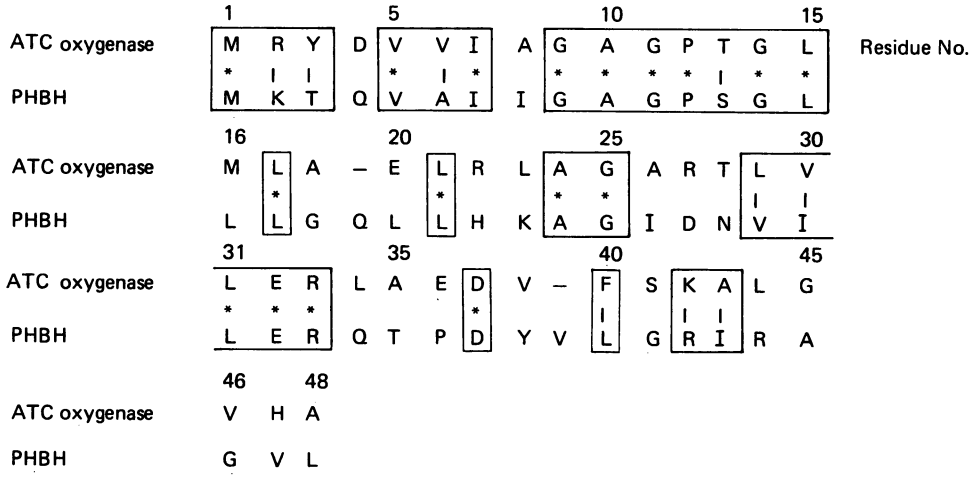

FIG. 3. Comparison of amino acid sequences at ATC oxygenase and *p*-hydroxybenzoate hydroxylase (PHBH; from *P. fluorescens*). Glycine residues at positions 9, 11, and 14 are involved in providing the flexibility of a peptide backbone required to create a flavin-binding domain. Symbols: *, identical; | , chemically similar; —, unknown amino acid.

tion of ATC and requires NADPH. By using purified ATC substrate, cell-free lysates of a commercial strain of *S. rimosus* M24167 were assayed for ATC oxygenase activity by spectrophotometric methods as described previously (2). In addition, the formation of OTC during the assay was confirmed by high-pressure liquid chromatography analysis.

By using a novel separation technique (M. J. Butler and B. N. Gedge, submitted for publication), cell-free lysates were purified by successive fast protein liquid chromatography with Q-Sepharose and Mono-Q matrices. ATC oxygenase activity correlated with the appearance of a major polypeptide species of 52 kilodaltons, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (result not shown). The molecular mass of ATC oxygenase from the chlorotetracycline producer *Streptomyces aureofaciens* was reported to be 100,000 daltons (1), suggesting that the active species in *S. rimosus* may be a dimer.

N-terminal amino acid sequence analysis of electroeluted material was performed by J. B. S. Findlay and J. Keen, Department of Biochemistry, University of Leeds. The first 48 amino acids, apart from residues 19 and 39, were unequivocally assigned and displayed a strong homology with the flavin-binding domain of the enzyme *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* (Fig. 3). The 0.25-nm-resolution X-ray structure of the *P. fluorescens* enzyme has been published previously (18).

Isolation of the ATC oxygenase gene. From the amino-terminal sequence of the purified protein, two single 42-base oligonucleotides (Fig. 4) were designed by reverse translation of two nonoverlapping regions of sequence, utilizing the inherently biased codon usage observed for cloned *Streptomyces* genes to assign bases at ambiguous positions.

The two oligonucleotides were end labeled and hybridized separately at low stringency against Southern blots of digested chromosomal DNA from *S. rimosus* M24167 (Fig. 5). Specific signals were observed only with sequence 1 oligonucleotide, presumably due to a lower degree of mismatch relative to that of sequence 2 (see below). Sequence 1 oligonucleotide specifically hybridized to an ca. 5-kb *Pst*I fragment, a 6.5-kb *Sst*I fragment, and large (>20-kb) *Bam*HI and *Bgl*II fragments. The 5-kb *Pst*I fragment specifically hybridizing to sequence 1 was isolated after colony hybridization of a pool of *E. coli* DH5α transformants containing M24167 chromosomal DNA enriched for *Pst*I fragments in the 4- to 6-kb size range cloned in pUC19.

The *Pst*I insert from one representative plasmid, pPFZ538, showed a specific hybridization pattern identical to that of the oligonucleotide (Fig. 5), although two additional *Sst*I bands appeared which could be explained by overlap of the 5-kb *Pst*I fragment with three *Sst*I fragments.

Plasmid pPFZ542 (Fig. 6) was obtained by subcloning a 0.3-kb *Sau*3A fragment from the pPFZ538 insert which hybridized (weakly) with the oligonucleotide (data not shown). An analysis of the available DNA sequence information (Fig. 7) indicated the presence of a *Sau*3A recognition site within the fragment hybridizing to oligonucleotide

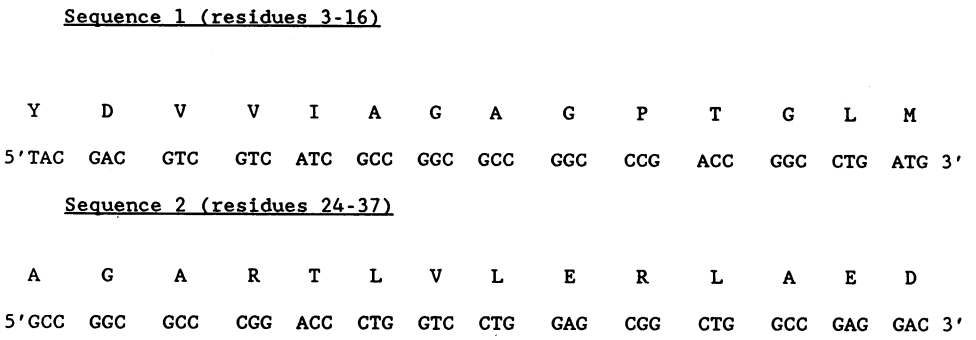

FIG. 4. Sequences of two 42-base oligonucleotides based on nonoverlapping regions of the amino acid sequence at the N terminus of ATC oxygenase (Fig. 3).

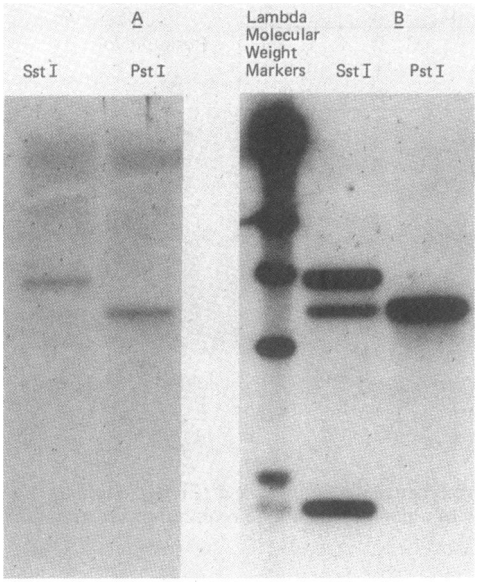

FIG. 5. Duplicate Southern blots of *S. rimosus* M24167 chromosomal DNA probed with oligonucleotide no. 1 (low stringency) (A) and pPFZ538 insert (high stringency) (B).

no. 1, so that no sequence was available in pPFZ542 for hybridization to 11 bases at the 5′ end of the oligonucleotide. Within the 31 remaining bases only two mismatches occurred, whereas oligonucleotide no. 2 contained at least six mismatches within 42 base pairs, presumably accounting for the original observed difference between the two probes in the specificity of hybridization against chromosomal DNA (Fig. 5). The weak hybridization observed with pPFZ542 and oligonucleotide no. 1 relative to that obtained against pPFZ538 (data not shown) highlights the potential danger of using *Sau*3A to cleave larger inserts for sequence analysis.

It is clear from Fig. 7, however, that in general the amino acid sequence predicted from the limited DNA sequence available corresponds with that obtained by N-terminal analysis (Fig. 3). There is a discrepancy, however, around residues 36 to 39 which may have been a result of a DNA sequencing artifact, but beyond this point there is good agreement between predicted and chemically derived amino acid sequences.

Location of *otcC*, the gene encoding ATC oxygenase. From the size of the purified protein and the position of the 5′ end of the ATC oxygenase gene, it appeared possible that its coding sequence would be contained entirely within the 5-kb *Pst*I fragment in pPFZ538. For the isolation of sequences flanking the putative ATC oxygenase gene (*otcC*), a genomic library was first constructed in *E. coli* ED8767. A library of M24167 chromosomal DNA partially digested with *Sau*3A was constructed, using the cosmid vector pTBE (5) modified by the addition of a linker at the *Bam*HI site. The linker contains a *Bgl*II cloning site flanked by two *Xba*I recognition sequences. It was hoped, by using this vector (pPFZ514), to subsequently remove intact cosmid inserts by *Xba*I excision, due to the general paucity of these sites in *Streptomyces* DNA. Excision of the *S. rimosus* 4 o'clock cluster as a single entity would also be possible since it lacks *Xba*I recognition sites (Fig. 2).

The integrity of the bank (4,000 colonies) was confirmed by first isolating 10 overlapping clones carrying all or part of the 4 o'clock cluster, using the *otrA* resistance gene from plasmid pPFZ46 as a hybridization probe. The *otrB* resistance gene from plasmid pPFZ57 was subsequently used to isolate a clone from this subset containing the whole cluster. Hybridization and restriction enzyme analysis (not shown) of the pPFZ518 insert (Fig. 2) confirmed its integrity.

The pPFZ538 insert was used to isolate from the bank DNA sequences flanking *otcC*. Surprisingly, the same 10 colonies which hybridized to the *otrA* fragment also hybridized to the pPFZ538 insert.

A comparison of the restriction map with that of pPFZ518 and flanking DNA, coupled with hybridization analysis (data not shown), indicated that *otcC* was situated adjacent to *otcZ151* at the *otrA* end of the 4 o'clock cluster, but separate from the *otcX20* locus (Fig. 2). However, no ATC oxygenase activity was detected by spectrophotometric assay (2) of *S. lividans* transformants containing the pPFZ538 insert in high-copy (pPFZ105) or low-copy (pPFZ161) *Streptomyces* vectors. These plasmids also failed to complement the OTC-nonproducing phenotype of putative ATC oxygenase-deficient mutants *otcC75* and *otcC79* (see Discussion). Functional evidence for the presence of the ATC oxygenase gene was obtained when pPFZ105 conferred ATC oxygenase activity onto the OTC-sensitive nonproducing *S. rimosus* strain M15883S, normally deficient in this enzyme due to deletion of the entire 4 o'clock cluster.

Transformation of *S. lividans*, using 4 o'clock DNA. As the complementation result contradicted the previous report (15) which had suggested a non-4 o'clock location for *otcC*, it was necessary to determine whether all of the structural genes required for OTC biosynthesis were located on the pPFZ518 insert. Unfortunately, the entire insert could not be excised intact by *Xba*I digestion due to the (inadvertent) creation of a (*Xba*I-resistant) *dam* methylation site on the modified linker in cosmid pPFZ514. Attempts to transform intact pPFZ518 into various restriction-deficient and -profi-

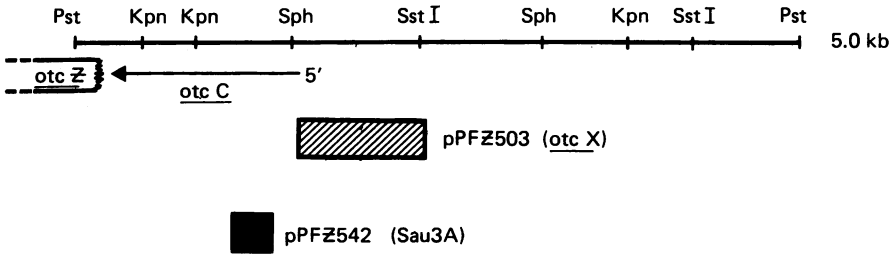

FIG. 6. Restriction map of pPFZ538 and position of subclone pPFZ542. The map indicates the location of *otcC* relative to the inferred location of *otcX* and *otcZ* (Fig. 1) biosynthetic genes. The direction of transcription of *otcC* is inferred from the sequences of the N-terminal coding region.

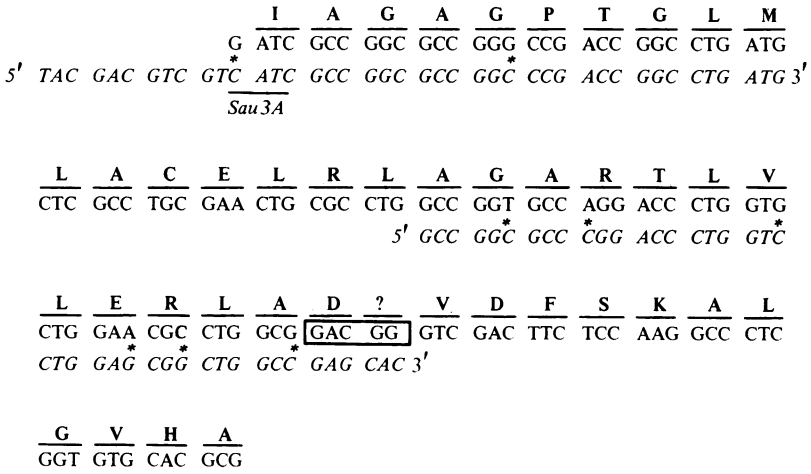

FIG. 7. Partial DNA sequence of pPFZ542. The predicted amino acid sequence is shown, in addition to the DNA sequence of two oligonucleotides based on the amino acid sequence by Edman degradation of purified protein. The sites of known mismatches are indicated (*). The boxed area represents an area including a sequencing artifact.

cient *dam E. coli* strains were unsuccessful (a similar vector, pPFZ543, has since been constructed in which the *Xba*I and *Bgl*II sites are separated by "stuffer" sequences, allowing complete *Xba*I digestion of the DNA made from *E. coli dam*$^+$ hosts). However, the entire cluster could be subcloned on a 34-kb *Eco*RI fragment (Fig. 2) in *S. lividans* 1326, using the low-copy-number vector pIJ916. The fragment was inserted in both orientations, in pPFZ163 and pPFZ164, and in both cases enabled the production of OTC by *S. lividans* 1326 grown on solid or in liquid medium. Transformants grown in liquid YEME medium (see Materials and Methods) made no more than 20 μg of OTC · ml^{-1}, and no attempt was made to optimize production. These transformants were resistant to a high level (250 to 500 μg · ml^{-1}) of OTC when grown on agar, whereas *S. lividans* (with or without pIJ916) is only resistant at levels of below 50 μg · ml^{-1}. The formation of OTC was detected by both anionic exchange and reverse-phase high-pressure liquid chromatography analysis (result not shown), coupled with UV absorption spectra (Fig. 8) in addition to normal bioassay (15). Also, the OTC production and resistance phenotypes were transferred at a high frequency by retransformation of *S. lividans* 1326, plasmid-free *S. lividans* TK64, and *S. albus* (r$^-$ m$^-$). Transformation of pPFZ163 and pPFZ164 into an OTC-sensitive nonproducing *S. rimosus* strain (deleted for the entire 4 o'clock cluster) gave rise to OTC-resistant transformants producing significant levels of OTC (result not shown).

DISCUSSION

The heterologous expression of OTC by both *S. lividans* and *S. albus* confirmed that all of the structural genes required for OTC biosynthesis had been isolated intact. There also exists a class of *S. rimosus* mutants deficient in OTC production whose mutations do not map at 4 o'clock on the *S. rimosus* chromosome. Some of these mutants (e.g., *otc-19* and *otc-56*) lack the ability to synthesize CSF1 required for the dehydrooxytetracycline dehydrogenase reaction, the final step in the OTC pathway. These mutants cosynthesize OTC when cultured with OTC-sensitive CSF1$^+$ strains. It would be expected that transformation of CSF1$^-$ strains with pPFZ163 and pPFZ164 would not re-

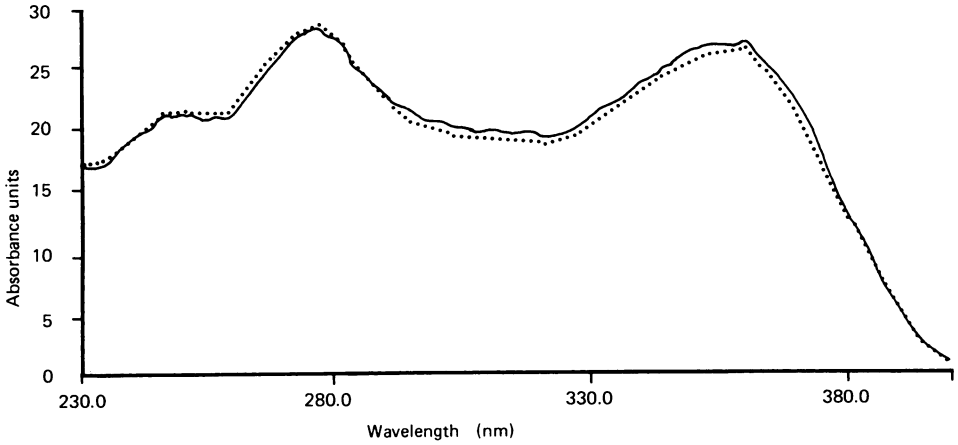

FIG. 8. UV absorption spectral analysis of OTC produced by *S. lividans* transformant LT156 (· · · · ·). The spectrum of purified OTC from *S. rimosus* is indicated (——).

store OTC production, but this still has to be tested. *S. lividans* and *S. albus* produce enough CSF1 (as detected by an agar strip bioassay test [15]) to allow some expression of the OTC pathway, although the function of CSF1 in these strains may not be limited to antibiotic biosynthesis, as illustrated by its involvement in enzymatic repair of UV-damaged DNA (17).

The failure of the ATC oxygenase high- and low-copy-number clones (pPFZ105 and pPFZ161) to complement the putative *otcC* mutants suggested that these mutants might harbor an additional mutation(s) in a locus required for ATC oxygenase activity. Coupled with the sequence homology with a flavin-binding domain at the N-terminus of the ATC oxygenase protein, this suggested the possibility of a non-CSF1 flavin cofactor pathway requirement for the enzyme.

Rhodes et al. (15) presented data indicating that CSF1$^-$ mutations and the *otc-75* mutation mapped at a 10 o'clock location, separate from the 4 o'clock cluster encoding the biosynthetic steps leading to the production of ATC. However, it is clear from the results of the cloning work presented in this paper that the structural gene for ATC oxygenase, *otcC*, in fact maps within the 4 o'clock cluster.

Although the *otc-75* mutant is clearly blocked in the conversion of ATC to dehydrotetracycline, it appears likely that the mutation mapping at 10 o'clock is within a gene involved in the synthesis of a flavin cofactor required for ATC oxygenase activity and not in *otcC* itself. In a preliminary cosynthesis experiment designed to test this hypothesis, OTC was produced when an *otc-75* CSF1$^+$ strain was cocultured with an OTC-sensitive (entire 4 o'clock cluster deleted) CSF1$^-$ strain. This result implies that a mutation in the *otc-75* strain maps outside the segment deleted in OTC-sensitive strains, i.e., outside the 4 o'clock cluster. Another non-CSF1 gene, *otcA*, was assigned to a 10 o'clock location by Rhodes et al. (15). The results of cosynthesis tests and precursor feeding experiments (15) indicated that the *otcA* gene product is responsible for the conversion of dehydrooxytetracycline to OTC, the final step in the biosynthetic pathway. However, there are no mapping data available to support the published map position of *otcA*, although again it is clear from this work that the structural gene must map within the 34-kb *Eco*RI fragment flanked by the two resistance loci.

Failure to detect ATC oxygenase activity in *S. lividans* harboring pPFZ105 and pPFZ161 may have been a consequence of several factors. One possibility is that the assay itself may not be sensitive enough when extracts of low-producing strains are used, as it did not successfully detect activity in extracts of *S. lividans* harboring the OTC pathway at a low copy number but nevertheless producing OTC. It may be that higher concentrations of specific cofactor or NADPH or both than are utilized in vivo are required for efficient in vitro activity. A second possibility is that the supply of metabolic precursors and NADPH required for high OTC output and ATC oxygenase activities may be limiting the production by *S. lividans* transformants and may influence the expression of the cloned *otcC* gene. The influence of these factors in *S. rimosus* is under investigation, using both high- and low-producing strains and their OTC-sensitive deletion derivatives.

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ADDENDUM

After submission of this paper, a protocol was independently published for the purification of ATC oxygenase from *S. aureofaciens*, and direct biochemical evidence was obtained for the existence of an ATC oxygenase-specific flavin cofactor (16).

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