Cloning, Expression in *Escherichia coli* and Nucleotide Sequence of a Tetracycline-resistance Gene from *Streptomyces rimosus*

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Determinants of tetracycline resistance have been cloned from two different tetracycline-producing industrial strains of *Streptomyces* into *Streptomyces lividans* using the plasmid vector pUT206. Three plasmids, pUT250 and pUT260 with a 9.5 and a 7.5 kb insert respectively of *Streptomyces rimosus* DNA, and pUT270 with a 14.0 kb insert of *Streptomyces aureofaciens* DNA, conferring resistance to tetracycline, have been isolated. By *in vitro* sub-cloning, a similar fragment of 2.45 kb containing the tetracycline resistance gene (*tet347*) was further localized on these plasmids. The *S. rimosus* gene has been cloned into *Escherichia coli* and expressed under the control of λ pL or Lpp promoters. Differential protein extraction of *E. coli* cells revealed the presence of an additional membrane-embedded protein in tetracycline-resistant cells. On the basis of available restriction endonuclease maps, the *tet347* gene is probably identical to the *tetB* gene from *S. rimosus* recently identified by T. Ohnuki and co-workers as responsible for the reduced accumulation of tetracycline. The nucleotide sequence of a 2052 bp DNA fragment containing the Tc^R structural gene from *S. rimosus* has been determined. The amino acid sequence of the *tet347* protein (*M_r* 35818) deduced from the nucleotide sequence shows a limited but significant homology to other characterized tetracycline transport acting determinants from pathogenic bacteria.

INTRODUCTION

Streptomyces spp. are Gram-positive multicellular bacteria of industrial importance. More than 60% of the naturally occurring antibiotics are produced by *Streptomyces* species, including many which are of great medical importance such as tetracyclines (Bérdy, 1974). The producing strains are resistant to their own antibiotic product during the idiophase stage of production (Demain, 1974). Mechanisms of self-resistance include inactivation of the antibiotic by antibiotic-modifying enzymes, alteration of target sites or reduced uptake of the antibiotic (Vining, 1979). In recent years, many genes encoding resistance to a large variety of antibiotics have been cloned and expressed in *Streptomyces lividans* from chromosomal DNA of producing actinomycetes. Many of these genes confer a resistance to common antibiotics such as aminoglycosides (Crameri & Davies, 1986; Distler & Piepersberg, 1985), macrolides (Stanzak *et al.*, 1986; Thompson *et al.*, 1982) and tetracyclines (Ohnuki *et al.*, 1985a) in similar ways to plasmid-borne resistance genes found in clinically important bacteria (Davies, 1986). Tetracycline resistance is the most frequently encountered in nature (Martin *et al.*, 1986). In Gram-negative bacteria, five genetically different classes of tetracycline-resistance determinants have been defined (Marshall *et al.*, 1986). In Gram-positive streptococci, three distinct tetracycline-resistance genes have been identified (Burdett *et al.*, 1982). The characterization of

Abbreviation: ORF, open reading frame.

these determinants revealed that the resistance is due mainly to an energy-dependent efflux of the antibiotic from the cell and also an action at the level of protein synthesis, which is the target of the antibiotic. Nucleotide sequences, models of regulation and analyses of gene product have been reported for many of these determinants, which are generally inducible by sub-inhibitory amounts of tetracycline. Two resistance genes from *Streptomyces rimosus* ATCC 10970 were cloned and characterized by Ohnuki *et al.* (1985a). One was shown to interfere with protein synthesis at the ribosome level (*tetA*) and the other with tetracycline uptake (*tetB*). That the resistance genes are directly or indirectly involved in antibiotic production can be inferred from two facts: washed mycelia taken at the production stage are more resistant to tetracycline than mycelia of the trophophase, and tetracycline-sensitive mutants of *Streptomyces aureofaciens* and *S. rimosus* no longer produce the antibiotic. The regulation of genes encoding enzymes of secondary metabolism in *Streptomyces* represents interesting but unsolved problems. The cloning and nucleotide sequence analysis of the Tc^R functional gene unit should contribute to a better understanding of expression and regulation mechanisms of secondary metabolite genes. In this respect, it would be valuable to compare the promoter nucleotide sequences of Tc^R genes cloned from a low tetracycline-producing *S. rimosus* strain such as ATCC 10970 with those of industrial strains which, as a consequence of mutation and selection steps, are highly resistant to tetracycline. A relationship between resistance and productivity appears to be a general rule among improved antibiotic-producing *Streptomyces*. Amplification by gene cloning of resistance determinants in *S. rimosus* or *S. aureofaciens* may result in high-yielding clones, as illustrated in *Streptomyces fradiae* (Crameri & Davies, 1986).

Recent work on antibiotic-producing *Streptomyces* has revealed that some genes which encode resistance mechanisms may be linked to the antibiotic biosynthesis genes on the chromosome (Chater & Bruton, 1985; Distler & Piepersberg, 1985; Murakami *et al.*, 1986; Ohnuki *et al.*, 1985b; Rhodes *et al.*, 1984; Stanzak *et al.*, 1986). Therefore, another advantage of the cloning of Tc^R genes is their use as specific DNA probes to identify the gene clusters encoding the associated antibiotic biosynthesis pathways. Also of interest would be a computer-assisted comparison between nucleotide sequences of Tc^R genes from producing *Streptomyces* and plasmid-borne Tc^R genes widely spread among Gram-negative and Gram-positive bacteria, to determine a possible evolutionary relationship among them. For these reasons, we decided to clone genes conferring resistance to tetracycline from industrial strains of *S. aureofaciens* and *S. rimosus*.

METHODS

Bacterial strains, plasmids and phages. Donor DNAs of tetracycline-resistance genes were extracted from PG2, a tetracycline-hyperproducing strain of *S. aureofaciens*, and PG3, an oxytetracycline-hyperproducing strain of *S. rimosus*. These strains, used commercially for production of tetracyclines, were obtained from Pointet-Girard (Rhône-Poulenc); their derivation is unclear. *S. lividans* 1326, used as a host throughout, was provided by D. A. Hopwood, John Innes Institute, Norwich, UK. The plasmid pUT206, derived from pIJ702 (Katz *et al.*, 1983), was used as a vector for initial cloning and sub-cloning experiments (Marcel *et al.*, 1987).

Escherichia coli strains HB101 and DH5 (Hanahan, 1985) were used as host organisms for transformation. The *E. coli* plasmids pINI-A1 (Masui *et al.*, 1983), pUT19 and pUT40 (unpublished data) served as intermediate vectors for construction of hybrid plasmids. *E. coli* strains JM101 and JM107 and M13 phages mp18, mp19 (Norrand *et al.*, 1983), um20 and um21 (IBI Co., cat. no. 33700 and 33720) were used for the determination of the nucleotide sequence.

Culture conditions. GAPY agar medium (Drocourt & Tiraby, 1983) was used for maintenance and sporulation of *Streptomyces* strains. YEME and R2YE media were used for DNA preparation and transformation of *S. lividans* as described by Chater *et al.* (1982). Antibiotic agar medium no. 2 (Biomerieux), pH 6.0, containing 2 g glucose l⁻¹ was used for tetracycline selection (by replica plating of transformants) and determinations of minimal inhibitory concentration (MIC).

Streptomyces cultures for DNA preparations were made in trypticase soya broth (Biomerieux) supplemented with 5 g yeast extract (Difco) l⁻¹; protoplast regeneration media for *S. rimosus* and *S. aureofaciens* were R2YE (Chater *et al.*, 1982) and M4 respectively. M4 consisted of 130 g sucrose, 4 g glucose, 4 g yeast extract, 10 g malt extract, 0.25 g K₂SO₄, trace elements (Okanishi *et al.*, 1974), 0.01 g K₂HPO₄, 0.59 g CaCl₂·7H₂O, 2 g MgCl₂·6H₂O, 20 g agar, and distilled water to 1 litre total volume, pH adjusted to 7.2. All cultures were grown using standard *Streptomyces* culture conditions (Hopwood *et al.*, 1985). *E. coli* strains were grown at 37 °C in Luria broth or on Luria agar.

Biochemicals and enzymes. Restriction enzymes, T4 DNA ligase and DNA polymerase I (Klenow fragment) were obtained through commercial suppliers and used according to the suppliers' instructions. Lysozyme and proteinase K were obtained from Merck and type IV agarose from Sigma. IPTG and X-gal were obtained from Genofit.

Thiostrepton was a gift from the Sofca company (Courbevoie, France) and tetracycline was from Pointet-Girard (Villeneuve la Garenne, France).

DNA isolation. Plasmid and chromosomal DNAs from *Streptomyces* were prepared by the methods described by Marcel *et al.* (1987). Both plasmid and chromosomal DNAs were further purified on a DEAE-cellulose (Whatman DE52) mini-column prepared in a pipette tip as described by Marcel *et al.* (1987). Plasmids from *E. coli* HB101 and M13 replicative-form DNA from infected *E. coli* JM101 were prepared by the methods described by Maniatis *et al.* (1982) and by Sanger (1977).

Shotgun cloning procedure. *S. aureofaciens* and *S. rimosus* chromosomal DNAs were partially cleaved with *Mbo*I into fragments of 2–15 kb and ligated separately to pUT206 cleaved by *Bgl*II and dephosphorylated. For each type of ligation, vector (0.5 µg) and genomic (2.5 µg) fragments were mixed and heated to 70 °C for 10 min, precipitated in 1 vol. 2-propanol, and suspended at a DNA concentration of 50 µg ml⁻¹ in ligation buffer (Maniatis *et al.*, 1982) with T4 DNA ligase. The ligation reaction was maintained at 16 °C for 24 h. Half of each ligation mixture was used for transformation of *S. lividans* 1326 protoplasts as described by Hopwood *et al.* (1985). After 16–18 h incubation at 27 °C, each of the six total regeneration plates for any transformation experiment was overlaid with 2.5 ml soft agar supplemented with thiostrepton (200 µg ml⁻¹). After 1 week incubation, the soft layer containing transformed colonies of each plate was scraped, vortexed and equally spread onto two large square plates (NUNC Inter-Med 243 × 243 mm) containing 150 ml antibiotic no. 2 medium supplemented with thiostrepton (20 µg ml⁻¹). One plate was supplemented with tetracycline at 50 µg ml⁻¹ and the other at 150 µg ml⁻¹. From each of the few plates containing colonies after 3–4 d incubation at 27 °C, two colonies were picked.

Restriction enzyme mapping. Plasmid DNAs were digested with various restriction enzymes according to the conditions described by Maniatis *et al.* (1982). Plasmids and restriction fragments were analysed by 0.6% slab agarose gel electrophoresis using TAE buffer (Maniatis *et al.*, 1982). DNA molecular size standards were *Hind*III fragments of bacteriophage λ.

Sub-cloning experiments. After partial digestion with *Mbo*I, DNA fragments from the initial plasmids (pUT250, pUT260, pUT270) were separated by the electro-elution method on preparative agarose gels and then purified with DEAE-cellulose mini-columns. These fragments (for each initial plasmid) were ligated with *Bgl*II-linearized and dephosphorylated pUT206 and used to transform *S. lividans* 1326. For sub-cloning experiments of the *Tc^R* genes, direct tetracycline selection was obtained on regeneration plates using modified R2YE (containing one-tenth the usual MgCl₂), modified soft agar R2YE (containing no salts) and final concentrations of 5 µg thiostrepton ml⁻¹ and 150 µg tetracycline ml⁻¹.

Determination of the MIC for tetracycline. MIC determinations were done on antibiotic no. 2 agar medium, pH 6.0, containing 2 g glucose l⁻¹. Thiostrepton (20 µg ml⁻¹) or ampicillin (10 µg ml⁻¹) were added to this medium for *Streptomyces* and *E. coli* clones respectively.

Nucleotide sequence analysis. According to the sequence analysis strategy (Fig. 5), all the sub-fragments of the *Tc^R* region contained on pUT1954 were recovered after digestion (and Klenow treatment when necessary) by electro-elution from preparative agarose gel. Fragments were sub-cloned into M13 mp18, mp19, um20 or um21 to obtain the insert in both orientations whenever possible. Ligation mixtures were transfected into competent *E. coli* JM107 cells and white plaques were screened for the presence of DNA inserts. Single-stranded DNAs were isolated and sequenced by the dideoxy method of Sanger *et al.* (1977) using [³⁵S]dATP (Amersham) and M13 17-mer sequencing primer (–20) (Biolabs). Reaction mixtures were separated by electrophoresis (at a constant temperature of the external glass plate of 49–50 °C) on 6% sequencing gels which were then exposed to Trimax type XM (Kodak) autoradiography films. All computations of DNA or protein sequences were carried out using the PC GENE program (Genofit) on a Persona 1600 Logabax computer.

Probe preparation and blot hybridization. A restriction fragment containing the *Tc^R* gene from *S. rimosus* was obtained by linearization of pUT1953 by *Kpn*I and labelled using a multiprime DNA-labelling system purchased from Amersham. The [³⁵S]dCTP-labelled probe was hybridized to a sheet of Biotyne membrane (Pall) in which *Bgl*II-digested chromosomal DNAs or *Kpn*I-digested pUT1953 DNA had been blotted according to the Pall protocol.

Analysis of protein extracts in *E. coli*. Cultures of DH5 transformed by pUT19 grown in LB medium (25 ml) were harvested at early stationary phase. The periplasmic fraction was obtained according to the osmotic shock procedure of Neu & Heppel (1965). The resulting pellet was further fractionated into membrane and cytoplasmic proteins as follows. The pellet was resuspended in 3 ml 10 mM-Tris/HCl (pH 8.0), 1 mM-EDTA containing 1 mg lysozyme ml⁻¹ and incubated for 15 min at 37 °C. This preparation was then freeze-thawed three times before addition of 3 ml water, 20 mM-MgSO₄ and 100 µg DNAase I (Boehringer) ml⁻¹. After 15 min at 37 °C, the mixture was centrifuged (30 min, 48000 g). The supernatant corresponded to the cytoplasmic protein extract, and the pellet, resuspended in 3 ml 0.01 M-Tris/HCl (pH 7.5), represented the membrane protein extract.

RESULTS

Isolation of Tc^R transformants

After ligation of *S. aureofaciens* or *S. rimosus* *Mbo*I partially digested DNAs with *Bg*/II-cleaved pUT206, about 2000 Thio^R *S. lividans* transformants were obtained on each Petri dish. The selection of Tc^R clones cannot be directly realized on R2YE regeneration medium because of its high content of interfering MgCl₂. The semi-confluent lawn of transformed colonies of each Petri dish was resuspended in broth and spread onto antibiotic no. 2 agar medium supplemented with tetracycline at final concentrations of 50 or 150 µg ml⁻¹. Using *S. aureofaciens* DNA as donor, only two Tc^R colonies arose, on the same selective plate, with 50 µg tetracycline ml⁻¹. These transformants were named *S. lividans* 206-SA1 clones. Using *S. rimosus* DNA as donor, cells scraped from two master plates gave rise to numerous Tc^R colonies at both concentrations of tetracycline. Resistant clones obtained from colonies on the same Petri dish were presumed identical and named *S. lividans* 206-SR1 and *S. lividans* 206-SR2.

Analysis by agarose gel electrophoresis of covalently closed circular DNA from mini-preparations indicated the presence of plasmid DNA larger than pUT206 in all of the three types of Tc^R clones. *S. lividans* 206-SA1 showed a plasmid (pUT270) with a 14.0 kb insert of *S. aureofaciens* DNA. *S. lividans* 206-SR1 and 206-SR2 showed plasmids (pUT250 and pUT260) with a 9.5 and a 7.5 kb insert respectively of *S. rimosus* DNA.

Plasmids pUT250, pUT260 and pUT270 were separately retransformed into *S. lividans* 1326. In each case, approximately 80% of the Thio^R transformants were also Tc^R, indicating that a Tc^R gene was carried by all three plasmids.

Subcloning of the DNA fragment determining tetracycline resistance

In order to localize the Tc^R genes carried by pUT250, pUT260 and pUT270, each plasmid was partially digested by *Mbo*I using conditions that gave DNA fragments ranging from 0.5 to 5 kb in length. The DNA fragments created by *Mbo*I digestion were ligated with plasmid pUT206 linearized with *Bg*/II. The products of the ligation reactions were introduced into *S. lividans* 1326 and direct selection of Tc^R and Thio^R transformants was performed on R2YE modified medium overlaid, after 18 h at 27 °C, with soft nutrient agar containing thiostrepton and tetracycline at final concentrations of 5 µg ml⁻¹ and 150 µg ml⁻¹, respectively. Mycelia and spores from colonies that appeared on the regeneration medium after 5–7 d at 27 °C were streaked on a medium with increasing concentrations of tetracycline to evaluate their level of resistance to this antibiotic. Three highly resistant clones harbouring hybrid plasmids with the smallest inserts, 3.45 kb in pUT253, 2.45 kb in pUT266 and 3.85 kb in pUT273, derived from pUT250, pUT260 and pUT270 respectively, were retained for subsequent analysis.

The MICs for tetracycline of *S. lividans* harbouring these various plasmids were determined. The resistance level of *S. lividans* transformants was increased 10-fold with plasmids carrying *S. aureofaciens* inserts (300 µg ml⁻¹ versus 30 µg ml⁻¹) and 30-fold with plasmids carrying *S. rimosus* DNA (1000 µg ml⁻¹ versus 30 µg ml⁻¹) compared to the sensitive host. In the latter case *S. lividans* became as resistant to tetracycline as the two industrial producing strains (*S. aureofaciens* PG2 and *S. rimosus* PG3) used as donors.

Restriction endonuclease digestion of these plasmids revealed the presence of a similar region of 2.45 kb (see hatched regions in Fig. 1), suggesting that the Tc^R gene was contained within this sequence of DNA. The restriction endonuclease cleavage map of this region appears to be quite comparable to the *tetB* gene cloned from the relatively low tetracycline-producing *S. rimosus* ATCC 10970 by Ohnuki *et al.* (1985a).

Expression of the Tc^R determinant in E. coli

Considering the apparent homology of the inserted fragments in pUT253, pUT266 and pUT273, further subcloning experiments were done with pUT253 to define the position and the direction of transcription of the Tc^R determinant within the cloned insert. DNA fragments of pUT253 were inserted into *E. coli* plasmids as illustrated in Fig. 2. pUT253 DNA was partially cleaved with *Nco*I (pUT253 contains five *Nco*I sites, including three sites in the inserted

Fig. 1. Restriction endonuclease cleavage maps of the cloned DNA and localization of the *Tc^R* gene. Open bars represent the DNA fragments inserted into the *Bgl*II site of pUT206 and hatched bars represent the common DNA region shared by the three plasmids. Single lines indicate pUT206 sequences. Ba, *Bam*HI; Bg, *Bgl*II; Bs, *Bst*EII; C, *Cla*I; K, *Kpn*I; Mb, *Mbo*I; Ml, *Mlu*I; N, *Nco*I; P, *Pvu*II; S, *Sma*I.

fragment) and the digestion products were ligated to the pUT19 vector digested by *Nco*I. The pUT19 vector (our unpublished results), derived from pINI-A1 (Masui *et al.*, 1983), contains a unique *Nco*I site including the ATG codon of a phleomycine-resistance gene, and a unique *Bst*EII site in the ribosome-binding site region of this gene (unpublished). When *E. coli* HB101 was transformed by this ligation, all Ap^R *Tc^R* transformants harboured only one type of plasmid recombinant (pUT1953), corresponding to an *Nco*I–*Nco*I insert of 2.65 kb in the same orientation, suggesting that the expressed *Tc^R* activity was dependent on the Lpp promoter. The removal of the small *Bam*HI fragment from pUT1953, resulting in pUT1954, did not affect the expression of tetracycline resistance in *E. coli*.

In a further experiment, the *Tc^R* region of pUT1953 was sub-cloned by ligating a partial *Bst*EII digestion of pUT1953 into pUT40 cleaved by *Bst*EII. The pUT40 vector differs from pUT19 by the replacement of the Lpp promoter with the λ pL promoter preceding the unique *Bst*EII and *Nco*I sites. As with pUT1953, only one type of hybrid construction, named pUT4053, was characterized in the Ap^R *Tc^R* transformants, with an inserted fragment (2.25 kb) in the same orientation as in pUT1953. Deletion of the small *Bam*HI fragment gave pUT4054, which still expressed tetracycline resistance. A detailed construction map analysis of pUT1954 and pUT4054 showed that an identical *Streptomyces* fragment was inserted in both plasmids (see pUT1954 restriction map in Fig. 3).

Cells of *E. coli* HB101 or DH5 transformed by either pUT1954 or pUT4054 gave colonies of mucoid appearance on LM agar plates. The MIC of these strains for tetracycline on this medium was increased 10-fold compared to the sensitive parent strains (30 μ g ml⁻¹ versus 3 μ g ml⁻¹).

Cells of *E. coli* DH5 carrying either pUT1954 or pUT19 grown in LB medium were subjected to differential extraction for periplasmic, membrane and cytoplasmic proteins by the procedure described in Methods. Fig. 4 represents a SDS–PAGE gel of the three fractions from the two cultures. No differences were detectable in the mobility of periplasmic and cytoplasmic proteins of the two strains. In contrast, a band corresponding to an *M_r* of approximately 30 000 appeared more intense in the membrane fraction of the cells expressing the *Tc^R* gene in comparison to cells sensitive to tetracycline.

Hybridization of the cloned *Tc^R* gene with chromosomal DNA of *S. rimosus*

In order to confirm the origin of the cloned *Tc^R* gene in pUT1953, Southern hybridization of the inserted fragment was done with *Bgl*II-cleaved chromosomal DNA of *S. rimosus*. In this experiment, the hybrid plasmid pUT1954 was used as a radioactive probe. Radioactively

Fig. 2. Subcloning of the *S. rimosus* Tc^R gene from pUT253 in *E. coli* plasmids. The arrows indicate the direction of transcription of the relevant genes. Restriction endonuclease sites: Ba, *Bam*HI; Bs, *Bsr*EII; C, *Cla*I; K, *Kpn*I (Asp 718); N, *Nco*I.

labelled pUT1954 hybridized with a single 8 kb *Bgl*II fragment of *S. rimosus* chromosomal DNA but not with that of *S. lividans* (not shown). These results indicate that the cloned Tc^R gene expressed in *S. lividans* and *E. coli* came from chromosomal DNA of *S. rimosus*.

Nucleotide sequence of the Tc^R gene and analysis of the deduced protein

The nucleotide sequence of the *Nco*I–*Bam*HI fragment from *S. rimosus* DNA including the Tc^R region of pUT1954, determined from several independent overlapping clones (Fig. 5), is

Fig. 3. Restriction endonuclease map of pUT1954. The open bar represents the DNA fragment derived from *S. rimosus* and the arrow indicates the direction and length of the *tet347* gene. The regions denoted by thick black lines contain both promoter and terminator regions of the *E. coli* Lpp gene cloned in pINI-A1.

Fig. 4. SDS-PAGE of protein extracts from cells of *E. coli* DH5. Lanes 1 and 8, M_r markers. Other lanes show protein extracts of *E. coli* DH5 containing: 2, 4, 6, pUT19; 3, 5, 7, pUT1954. Lanes 2 and 3 contain periplasmic extracts, lanes 4 and 5, membrane extracts, and lanes 6 and 7, cytoplasmic extracts. The position of the presumptive *tet347* protein is indicated by an arrow in lane 5.

Fig. 5. Sequencing strategy for the Tc^R gene of *S. rimosus*. The sites used for cloning of DNA fragments obtained by digestion with restriction enzymes are indicated by arrows whose length and direction indicate the extent of sequence determination from these sites. The thick arrow at the top shows the coding region of the putative Tc^R gene product.

Table 1. *Direct repeats in the tet347 gene region*

Numbers refer to nucleotides in the sequence of the 2052 bp fragment containing the Tc^R gene. Non-identical nucleotides for each vertical line are enclosed by boxes.

701	G C T G G - G - T G T T C C T G C	715
818	G C T G G C G C T G T T C C T G G	834
923	G C T G G C G G T C T T C A T C C	939
977	G C T G G G G C T C T T C C G G C	993
1064	C C T G C C G C T G T T C C T G C	1080
1712	G C T G G C C G G G T T C G T C A	1728
1940	G C C G G - G G T G T T C - T G C	1954

shown in Fig. 6. A search for protein-coding sequences related to *Streptomyces* by the method of Bibb *et al.* (1984) revealed a unique open reading frame (ORF) starting either at an ATG codon, nucleotide 795, or at a GTG codon, nucleotide 834, and terminating with a TGA translational stop codon at nucleotide 1836. The ATG codon, which is preceded by a potential ribosome-binding site (Bibb & Cohen, 1982), is considered more likely to be used as the translational initiation codon.

The overall mean G + C content of this ORF was 71.2%; the values for codon positions 1, 2 and 3 were 67.4, 49.6 and 96.7% respectively. These values are in complete agreement with the characteristic G + C distribution of other sequenced *Streptomyces* genes (Bibb *et al.*, 1984). A series of directly repeated sequences is clearly evident (Table 1) as was the case in the ORF438 of the plasmid pIJ702 (Bernan *et al.*, 1985). The significance of these repeats, which are different in the two examples mentioned, remains to be determined.

A computer-assisted comparison of the 5' region of the ORF with consensus promoter sequences of *E. coli*, *Bacillus subtilis* vegetative or sporulating genes, and with promoter regions of several *Streptomyces* genes (Horinouchi *et al.*, 1986; Janssen *et al.*, 1985) revealed significant homology with the *afsB* and *aphP1* gene sequences (Fig. 7). S1 mapping experiments, and subcloning studies using *Streptomyces* promoter-probe vectors, should provide information on the precise role of this homologous sequence. An inverted repeat sequence [$\Delta G = -17.2$ kcal mol⁻¹ (-71.96 kJ mol⁻¹)], preceding this promoter sequence, could act as terminator or attenuator of possible upstream transcripts. On the 3' side, two closed inverted repeat sequences

Fig. 6. Nucleotide sequence and the deduced amino acid sequence of the *ter347* gene. The putative ribosome-binding site (RBS) is indicated, and possible transcriptional terminators are underlined with arrows.

$[\Delta G = -34.2$ and -17.4 kcal mol⁻¹ (-143.09 and -72.80 kJ mol⁻¹)] could well act as terminators of the Tc^R transcript. These hairpins are followed by a T-rich region peculiar for *Streptomyces* DNA, the significance of which is unknown.

The calculated M_r of the deduced 347 amino acid protein of the cloned Tc^R gene, designated *ter347*, is 35818, a value which falls in the range of M_r values for other published tetracycline-uptake-interfering gene products. The amino acid compositions of the three sequenced Gram-

negative determinants *tetA* (Tn1721) (Waters *et al.*, 1983), *tetB* (Tn10) (Nguyen *et al.*, 1983), *tetC* (pBR322) (Peden, 1983) are very similar to each other but are different from those of the two sequenced Gram-positive genes pTHT15 (Hoshino *et al.*, 1985) and pT181 (Khan & Novick, 1983). Surprisingly, *ter347* revealed greater similarity in amino acid composition to Gram-negative than to Gram-positive *tet* proteins (Table 2). We also compared *ter347* with *tet* from pBR322 as an example of a Gram-negative protein and with the Tc^R gene product from pT181

Table 2. Amino acid compositions (mol%) and *M_r* values of *Tc^R* gene products from Gram-positive and Gram-negative bacteria

The values other than those for *tet347* are taken from the literature (see text for details).

Amino acid	<i>Tc^R</i> determinant encoded by:					
	<i>tet347</i>	Tn10	Tn1721	pBR322	pTHT15	pT181
Ala	13.2	10.2	15.2	15.6	4.5	4.0
Arg	4.0	2.4	6.0	5.0	2.4	1.6
Asn	2.0	2.4	1.5	1.5	3.4	6.4
Asp	3.1	2.2	3.2	3.2	2.4	1.6
Cys	0.5	0.2	1.0	1.0	0.6	1.6
Gln	3.4	3.4	2.5	2.7	2.1	0.6
Glu	3.4	2.4	2.0	1.2	1.9	1.0
Gly	12.6	9.7	11.5	10.8	9.3	8.1
His	1.1	1.2	1.7	1.7	1.7	2.0
Ile	5.7	9.2	6.7	6.8	11.7	13.5
Leu	13.8	14.9	14.0	14.1	12.6	11.8
Lys	1.4	2.2	0.5	1.0	3.7	5.4
Met	3.7	3.2	4.0	4.2	4.1	2.7
Phe	4.8	6.9	5.2	4.2	8.0	9.4
Pro	5.4	3.4	4.7	4.0	3.4	3.3
Ser	5.7	7.7	4.2	5.5	8.9	8.4
Thr	6.3	6.2	4.7	5.3	5.4	5.0
Trp	1.4	2.9	2.2	2.0	1.0	2.0
Tyr	0.2	1.7	2.0	2.0	2.6	4.0
Val	7.2	6.4	6.5	7.3	9.1	6.4
<i>M_r</i>	35818	36000	42205	41 518	50000	35000

as an example of a Gram-positive protein, by operating optimal alignments between their amino acid sequences (Fig. 8). Although it appears that a limited homology does exist between the three proteins, *tet347* did not show more amino acid homology with *tet* pBR322 or *tet* pT181 than these two latter proteins had in common.

The *tet347* protein is very hydrophobic: its hydropathy value, calculated according to Kyte & Doolittle (1982), of 0.6 is comparable to the average of 0.8 indicated for the other *Tc^R* determinants, as expected for membrane-embedded proteins. In contrast to the other Gram-positive and Gram-negative determinants mentioned above, which are basic proteins, the *tet347* protein is rather acidic, as indicated by the theoretical isoelectric point of 5.45.

DISCUSSION

A *Tc^R* gene from industrial strains of *S. aureofaciens* and *S. rimosus* was cloned using the pUT206 vector in *S. lividans* 1326, a highly transformable strain devoid of a DNA restriction-modification system. Analysis of the restriction maps of three different subcloned plasmids, one originating from *S. aureofaciens* chromosomal DNA and two from *S. rimosus*, showed that they shared a common 2.45 kb DNA region, suggesting that only one *Tc^R* gene (*tet347*) contained in this fragment was selected. Ohnuki *et al.* (1985a) have recently cloned two *Tc^R* genes from a different strain of *S. rimosus*, ATCC 10970, one (*tetA*) mediating the antibiotic resistance through an effect on the protein synthesis machinery and other (*tetB*) through a decrease in the efficiency of mechanisms responsible for the intracellular accumulation of tetracycline (uptake and efflux). On the basis of a comparison with the restriction maps of the two *Tc^R* genes reported by Ohnuki *et al.* (1985a), the *Tc^R* gene we cloned appears to represent the *tetB* gene. Another indication of the analogy of the two genes was the fact that the *tet347* product expressed in *E. coli* was membrane associated, as expected for a protein related to a transport mechanism. Determination of the identity of *tet347* with *tetB* or with the oxytetracycline resistance gene (*otcR11*) cloned by M. J. Butler *et al.* (communication to the Fifth International Symposium on the Genetics of Industrial Micro-organisms, 1986) must await the completion of the nucleotide

sequence of these genes (I. S. Hunter, personal communication). The comparison of nucleotide sequences of the promoters of the same functional genes cloned from the low-yielding strain ATCC 10970 used by Ohnuki *et al.* (1985*a*) and a high-yielding oxytetracycline-producing strain (our donor strain *S. rimosus* PG3) would be of particular interest if differences in promoter strength could be demonstrated.

The *S. lividans* clones harbouring a hybrid plasmid with the Tc^R gene from *S. rimosus* or *S. aureofaciens* were highly resistant to tetracycline (or oxytetracycline), reaching the same level of resistance as the producing strains. If we assume that the resistance of the donor producing strains resulted from the cumulative action of two genes with different targets, then the high expression of the *tet347* gene due to an elevated copy number of the pUT206 plasmid derivative must compensate in *S. lividans* for the absence of a second gene mediating resistance at the level of protein synthesis. The expression of tetracycline resistance was inducible in *S. lividans* (data not shown) as well as in the donor strain, suggesting that regulatory DNA sequences were also present in the inserted fragments of the Tc^R plasmids.

The shortest DNA fragment from *S. rimosus* expressing tetracycline resistance in *S. lividans* was subcloned in different plasmids of *E. coli*. Tetracycline resistance was expressed in *E. coli* only when the *Nco*I–*Bam*HI fragment in the correct orientation was put downstream of the *E. coli* Lpp or λ pL promoters. Changing the orientation of the same fragment failed to give a Tc^R phenotype (data not shown). The complete nucleotide sequence of this *Nco*I–*Bam*HI fragment from *S. rimosus* DNA has been determined and the ORF of the *tet347* gene, coding for a protein of 347 amino acid residues, was recognized. From this sequence it can be inferred that the expression of *tet347* in *E. coli* containing pUT1954 or pUT4054 must occur through the translation of this gene from a polymessenger RNA starting at the Lpp or λ pL promoters. The resistance to tetracycline conferred by *tet347* was lower than that with pBR322.

Only one possible ORF was found in the nucleotide sequence of the cloned fragment in the direction of transcription deduced from the *E. coli* experiments. The beginning of the gene was assigned to the ATG coordinate 795 rather than to the downstream in-phase GTG codon, based on the presence of a possible ribosome-binding site complementary to part of the 3' end of the 16S RNA of *S. lividans*, beginning 5 bp before the ATG codon.

No typical arrangement of the –35 and –10 conserved sequences for the promoter region (Buttner & Brown, 1987) was found upstream of the Tc^R gene. A scan for other sequences possibly recognized by *Streptomyces* RNA polymerase holoenzymes revealed a short stretch of DNA resembling an homologous region preceding the transcription start of the *afsB* and *aph* genes (Fig. 7). Both direct and inverted repeat sequences were found in the region delineated by the initiation codon on one side and a potential hairpin structure with a ΔG of $-17.2 \text{ kcal mol}^{-1}$ on the other side. Whether this sequence is involved in the induction mechanism of Tc^R expression by low concentrations of tetracycline remains to be determined. S1 mapping experiments as well as deletion analysis are under way to gain insight into the localization of the promoter and the regulatory sequences.

Two inverted repeat sequences which were found downstream of the TGA codon may act as signals for transcription termination. Similar secondary structures have been seen following almost all sequenced *Streptomyces* genes (Bibb *et al.*, 1985) and one was recently shown to terminate transcription in *S. lividans* (Deng *et al.*, 1987).

Similarity in the biochemical mechanism of resistance was seen between *tet347* and *tetB* from producing *Streptomyces* on the one hand, and various Tc^R genes from pathogenic bacteria on the other hand. According to the amino acid sequence of the respective structural genes, a weak but significant homology was also demonstrated. These findings provide another example of antibiotic-resistance determinants (Uchiyama & Weisblum, 1985) for which molecular studies have suggested that resistance genes of opportunist bacteria could have evolved from ancestral genes found in antibiotic-producing actinomycetes.

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REFERENCES

- BERDY, J. (1974). Recent developments of antibiotic research and classification of antibiotics according to chemical structure. *Advances in Applied Microbiology* **18**, 309–406.
- BERNAN, V., FILPULA, D., HERBOR, W., BIBB, M. & KATZ, E. (1985). The nucleotide sequence of the tyrosinase gene from *Streptomyces antibioticus* and characterisation of the gene product. *Gene* **37**, 101–110.
- BIBB, M. J. & COHEN, S. N. (1982). Gene expression in Streptomyces: construction and application of promoter–probe plasmid vectors in *Streptomyces lividans*. *Molecular and General Genetics* **187**, 265–277.
- BIBB, M. J., FINDLAY, P. R. & JOHNSON, M. W. (1984). The relationship between base composition and codon usage of bacterial genes and its use for the simple and reliable identification of protein-coding sequences. *Gene* **30**, 157–166.
- BIBB, M. J., BIBB, M. J., WARD, J. M. & COHEN, S. N. (1985). Nucleotide sequence encoding and promoting expression of three antibiotic resistance genes indigenous to *Streptomyces*. *Molecular and General Genetics* **199**, 26–36.
- BURDETT, V., INAMINE, J. & RAJAGOPALAN, S. (1982). Heterogeneity of tetracycline resistance determinants in *Streptococcus*. *Journal of Bacteriology* **149**, 995–1004.
- BUTTNER, M. J. & BROWN, N. L. (1987). Two promoters from the Streptomyces plasmid pIJ101 and their expression in *Escherichia coli*. *Gene* **51**, 179–186.
- CHATER, K. F. & BRUTON, C. J. (1985). Resistance, regulatory and production genes for the antibiotic methylenomycin are clustered. *EMBO Journal* **4**, 1893–1897.
- CHATER, K. F., HOPWOOD, D. A., KIESER, T. & THOMPSON, C. J. (1982). Gene cloning in *Streptomyces*. *Current Topics in Microbiology and Immunology* **96**, 69–95.
- CRAMERI, R. & DAVIES, J. E. (1986). Increased production of aminoglycosides associated with amplified antibiotic resistance genes. *Journal of Antibiotics* **39**, 128–135.
- DAVIES, J. (1986). A new look at antibiotic resistance. *FEMS Microbiology Reviews* **39**, 128–135.
- DEMAIN, A. L. (1974). How do antibiotic-producing microorganisms avoid suicide? *Annals of the New York Academy of Sciences* **235**, 601–612.
- DENG, Z., KIESER, T. & HOPWOOD, D. A. (1987). Activity of a Streptomyces transcriptional terminator in *Escherichia coli*. *Nucleic Acids Research* **15**, 2665–2675.
- DISTLER, J. & PIEPERSBERG, W. (1985). Cloning and characterization of a gene from *Streptomyces griseus* coding for a streptomycin-phosphorylating activity. *FEMS Microbiology Letters* **28**, 113–117.
- DROCOURT, D. & TIRABY, G. (1983). A survey of plasmids among natural isolates of *Streptomyces*. *Journal of Antibiotics* **36**, 181–184.
- HANAHAN, D. (1985). Techniques for transformation of *E. coli*. In *DNA Cloning: a Practical Approach*, vol. 1, pp. 109–135. Edited by D. M. Glover. Oxford: IRL Press.
- HOPWOOD, D. A., BIBB, M. J., CHATER, K. F., KIESER, T., BRUTON, C. J., KIESER, H. M., LYDIATE, D. J., SMITH, C. P., WARD, J. M. & SCHREMPF, H. (1985). *Genetic Manipulation of Streptomyces, a Laboratory Manual*. Norwich: John Innes Foundation.
- HORINOCHI, S., SUZUKI, H. & BEPPU, T. (1986). Nucleotide sequence of *afsB*, a pleiotropic gene involved in secondary metabolism in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*. *Journal of Bacteriology* **168**, 257–269.
- HOSHINO, T., IKEDA, T., TOMIZUKA, N. & FURUKAWA, K. (1985). Nucleotide sequence of the tetracycline resistance gene of pTHT15, a thermophilic *Bacillus* plasmid: comparison with staphylococcal Tc^R controls. *Gene* **37**, 131–138.
- JANSSEN, G. R., BIBB, M. J., SMITH, C. P., WARD, J. M., KIESER, T. & BIBB, M. J. (1985). Isolation and analysis of *Streptomyces* promoters. In *Microbiology – 1985*, pp. 392–396. Edited by G. Hegemon & C. L. Hershberger. Washington, DC: American Society for Microbiology.
- KATZ, E., THOMPSON, C. J. & HOPWOOD, D. A. (1983). Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. *Journal of General Microbiology* **129**, 2703–2714.
- KHAN, S. A. & NOVICK, R. P. (1983). Complete nucleotide sequence of pT181, a tetracycline resistance plasmid from *Staphylococcus aureus*. *Plasmid* **10**, 251–259.
- KYTE, J. & DOOLITTLE, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* **157**, 105–132.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MARCEL, T., DROCOURT, D. & TIRABY, G. (1987). Cloning of glucose-isomerase (D-xylulose isomerase) and xylulose kinase genes of *Streptomyces violaceoniger*. *Molecular and General Genetics* **208**, 121–126.
- MARSHALL, B., MORRISSEY, S., FLYNN, P. & LEVY, S. B. (1986). A new tetracycline-resistance determinant, class E, isolated from Enterobacteriaceae. *Gene* **50**, 111–117.
- MARTIN, P., TRIEU-CUOT, P. & COURVALIN, P. (1986). Nucleotide sequence of the *tetM* tetracycline resistance determinant of the streptococcal conjugative shuttle transposon Tn1545. *Nucleic Acids Research* **14**, 7047–7058.
- MASUI, Y., COLEMAN, J. & INOUE, M. (1983). Multipurpose expression cloning vehicles in *Escherichia coli*. In *Experimental Manipulation of Gene Expression*, pp. 15–32. Edited by M. Inouye. New York: Academic Press.
- MURAKAMI, T., ANZAI, H., IMAI, S., SATOH, A., NAGAOKA, K. & THOMPSON, C. J. (1986). The bialaphos biosynthetic genes of *Streptomyces hygroscopicus*: molecular cloning and characterization of the gene cluster. *Molecular and General Genetics* **205**, 42–50.
- NEU, H. C. & HEPPEL, L. A. (1965). The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. *Journal of Biological Chemistry* **240**, 3685–3692.

- NGUYEN, T. T., POSTLE, K. & BERTRAND, K. P. (1983). Sequence homology between the tetracycline-resistance determinants of Tn10 and pBR322. *Gene* **25**, 83–92.
- NORRANDER, J., KEMPE, T. & MESSING, J. (1983). Construction of improved M13 vectors using oligodeoxynucleotide-directed mutagenesis. *Gene* **26**, 101–106.
- OHNUKI, T., KATCH, T., IMANAKA, T. & AIBA, S. (1985a). Molecular cloning of tetracycline resistance genes from *Streptomyces rimosus* in *Streptomyces griseus* and characterization of the cloned genes. *Journal of Bacteriology* **161**, 1010–1016.
- OHNUKI, T., IMANAKA, T. & AIBA, S. (1985b). Self-cloning in *Streptomyces griseus* of an *str* gene cluster for streptomycin biosynthesis and streptomycin resistance. *Journal of Bacteriology* **164**, 85–94.
- OKANISHI, M., SUZUKI, K. & UMEZAWA, H. (1974). Formation and reversion of *Streptomyces* protoplasts: cultural conditions and morphological study. *Journal of General Microbiology* **80**, 389–400.
- PEDEN, K. W. C. (1983). Revised sequence of the tetracycline-resistance gene of pBR322. *Gene* **22**, 277–280.
- RHODES, P. M., HUNTER, I. S., FRIEND, E. J. & WARREN, M. (1984). Recombinant DNA methods for the oxytetracycline producer *Streptomyces rimosus*. *Biochemical Society Transactions* **12**, 586–587.
- SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5463–5467.
- STANZAK, R., MATSUSHIMA, P., BALTZ, R. H. & RAO, R. N. (1986). Cloning and expression in *Streptomyces lividans* of clustered erythromycin biosynthesis genes from *Streptomyces erythraeus*. *Bio/Technology* **4**, 229–232.
- THOMPSON, C. J. & GRAY, G. S. (1983). Nucleotide sequence of a streptomycete aminoglycoside phosphotransferase gene and its relationship to phosphotransferases encoded by resistance plasmids. *Proceedings of the National Academy of Sciences of the United States of America* **80**, 5190–5194.
- THOMPSON, C. J., KIESER, T., WARD, J. M. & HOPWOOD, D. A. (1982). Physical analysis of antibiotic-resistance genes from *Streptomyces* and their use in vector construction. *Gene* **20**, 51–62.
- UCHIYAMA, H. & WEISBLUM, B. (1985). *N*-Methyl transferase of *Streptomyces erythraeus* that confers resistance to the macrolide-lincosamine-streptogramin B antibiotics: amino acid sequence and its homology to cognate R-factor enzymes from pathogenic bacilli and cocci. *Gene* **38**, 103–110.
- VINING, L. C. (1979). Antibiotic tolerance in producer organisms. *Advances in Applied Microbiology* **25**, 147–168.
- WATERS, S. H., ROGOWSKY, P., GRINSTED, J., ALTENBUCHNER, J. & SCHMITT, R. (1983). The tetracycline resistance determinants of RP1 and Tn1721: nucleotide sequence analysis. *Nucleic Acids Research* **11**, 6089–6105.