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

By JEAN PAUL REYNES, 1 THIERRY CALMELS, 1 DANIEL DROCOURT1 AND GÉRARD TIRABY2*

1Laboratoire de Recherche CAYLA, Centre commercial de Gros, Avenue de Larrieu, 31094 Toulouse Cédex, France
2Laboratoire de Microbiologie et Génétique appliquées du CNRS, 118 route de Narbonne, 31062 Toulouse Cédex, France

(Received 16 July 1987; revised 19 October 1987)

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 ApL 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 TcR structural gene from S. rimosus has been determined. The amino acid sequence of the tet347 protein (M, 35 81 8) 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 (Bérby, 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 (Cramer & Davies, 1986; Distler & Piepersberg, 1985), macrolides (Stanzak er 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.

0001-4313 © 1988 SGM
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 TcR 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 TcR 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 TcR 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 TcR genes from producing *Streptomyces* and plasmid-borne TcR 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 pINl-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 (Norlander et al., 1983), um20 and um21 (IBI Co., cat. no. 33700 and 33720) were used for the determination of the nucleotide sequence.

**Culture conditions.** GAPA 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 trypticate 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 CaCl₂·2H₂O, 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 Soéca 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 *MboI* into fragments of 2–15 kb and ligated separately to pUT206 cleaved by *BglII* 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 *HindIII* fragments of bacteriophage λ.

**Sub-cloning experiments.** After partial digestion with *MboI*, 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 *BglII*-linearized and dephosphorylated pUT206 and used to transform *S. lividans* 1326. For sub-cloning experiments of the TcR 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 TcR 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 [³²P]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 film. All computations of DNA or protein sequences were carried out using the PC GENE program (Genofit) on an Apple 1600 Logabax computer.

**Probe preparation and blot hybridization.** A restriction fragment containing the TcR gene from *S. rimosus* was obtained by linearization of pUT1953 by KpnI and labelled using a multiprime DNA-labelling system purchased from Amersham. The [³²P]dCTP-labelled probe was hybridized to a sheet of Biodyne membrane (Pall) in which *BglII*-digested chromosomal DNAs or KpnI-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 protocol of Neu & Heppel (1965). The resulting pellet was further fractionated into membrane and cytoplasmic proteins as follows. The pellet was resuspended in 3 ml 0 M-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, 48 000 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 TcR transformants

After ligation of *S. aureofaciens* or *S. rimosus* MboI partially digested DNAs with BglII-cleaved pUT206, about 2000 ThioR *S. lividans* transformants were obtained on each Petri dish. The selection of TcR 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 TcR 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 TcR 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 TcR 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 ThioR transformants were also TcR, indicating that a TcR gene was carried by all three plasmids.

Subcloning of the DNA fragment determining tetracycline resistance

In order to localize the TcR genes carried by pUT250, pUT260 and pUT270, each plasmid was partially digested by MboI using conditions that gave DNA fragments ranging from 0.5 to 5 kb in length. The DNA fragments created by MboI digestion were ligated with plasmid pUT206 linearized with BglII. The products of the ligation reactions were introduced into *S. lividans* 1326 and direct selection of TcR and ThioR 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 TcR 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 TcR 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 TcR 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 *NcoI* (pUT253 contains five *NcoI* sites, including three sites in the inserted
Nucleotide sequence of a TcR gene from S. rimosus

Fig. 1. Restriction endonuclease cleavage maps of the cloned DNA and localization of the TcR gene. Open bars represent the DNA fragments inserted into the BglII site of pUT206 and hatched bars represent the common DNA region shared by the three plasmids. Single lines indicate pUT206 sequences. Ba, BamHI; Bg, BglII; Bs, BsrEII; C, ClaI; K, KpnI; Mb, MboI; M1, MfuI; N, NcoI; P, PvuII; S, SmaI.

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

In a further experiment, the TcR region of pUT1953 was sub-cloned by ligating a partial BsrEII digestion of pUT1953 into pUT40 cleaved by BsrEII. The pUT40 vector differs from pUT19 by the replacement of the Lpp promoter with the λpL promoter preceding the unique BsrEII and NcoI sites. As with pUT1953, only one type of hybrid construction, named pUT4053, was characterized in the ApR TcR transformants, with an inserted fragment (2.25 kb) in the same orientation as in pUT1953. Deletion of the small BamHI 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 µg ml⁻¹ versus 3 µg 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 Mr of approximately 30000 appeared more intense in the membrane fraction of the cells expressing the TcR gene in comparison to cells sensitive to tetracycline.

Hybridization of the cloned TcR gene with chromosomal DNA of S. rimosus

In order to confirm the origin of the cloned TcR gene in pUT1953, Southern hybridization of the inserted fragment was done with BglII-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* TcR gene from pUT253 in *E. coli* plasmids. The arrows indicate the direction of transcription of the relevant genes. Restriction endonuclease sites: Ba, BamHI; Bs, BsrEII; C, ClaI; K, KpnI (Asp 718); N, NcoI.

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

**Nucleotide sequence of the TcR gene and analysis of the deduced protein**

The nucleotide sequence of the NcoI–BamHI fragment from *S. rimosus* DNA including the TcR region of pUT1954, determined from several independent overlapping clones (Fig. 5), is
Nucleotide sequence of a \( Tc^k \) gene from \( S. \) rimosus

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 \( \text{tet}347 \) 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, \( M \), 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 \( \text{tet}347 \) protein is indicated by an arrow in lane 5.
Fig. 5. Sequencing strategy for the Tc* 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* 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* 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 C | 834 |
| 923 | G C T G G C G C T Y T T C A T C C | 939 |
| 977 | G C T G G C G C T T T C C G C | 993 |
| 1064 | C T G G C C C T G T T C C T G C | 1080 |
| 1712 | G C T G G C C G G T T C C C C | 1728 |
| 1940 | G C C C G G - G - T G T T C - T C 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 [ΔG = -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
Nucleotide sequence of a TcR gene from S. rimosus

Fig. 6. Nucleotide sequence and the deduced amino acid sequence of the tet347 gene. The putative ribosome-binding site (RBS) is indicated, and possible transcriptional terminators are underlined with arrows.

$[\Delta G = -34.2 \text{ and } -17.4 \text{ kcal mol}^{-1} \end{equation} \text{ (and } -143.09 \text{ and } -7240 \text{ kJ mol}^{-1})] \text{ could well act as terminators of the TcR transcript. These hairpins are followed by a T-rich region peculiar for Streptomyces DNA, the significance of which is unknown.}

The calculated $M_c$ of the deduced 347 amino acid protein of the cloned TcR gene, designated tet347, is 35818, a value which falls in the range of $M_c$ 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, tet347 revealed greater similarity in amino acid composition to Gram-negative than to Gram-positive tet proteins (Table 2). We also compared tet347 with tet from pBR322 as an example of a Gram-negative protein and with the TcR gene product from pT181
Nucleotide sequence of a TcR gene from S. rimosus

Table 2. Amino acid compositions (mol%) and $M_r$ values of TcR 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).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>tet347</th>
<th>Tn10</th>
<th>Tn721</th>
<th>pBR322</th>
<th>pTHT15</th>
<th>pT181</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>13.2</td>
<td>10.2</td>
<td>15.2</td>
<td>15.6</td>
<td>4.5</td>
<td>4.0</td>
</tr>
<tr>
<td>Arg</td>
<td>4.0</td>
<td>2.4</td>
<td>6.0</td>
<td>5.0</td>
<td>2.4</td>
<td>1.6</td>
</tr>
<tr>
<td>Asn</td>
<td>2.0</td>
<td>2.4</td>
<td>1.5</td>
<td>1.5</td>
<td>3.4</td>
<td>6.4</td>
</tr>
<tr>
<td>Asp</td>
<td>3.1</td>
<td>2.2</td>
<td>3.2</td>
<td>3.2</td>
<td>2.4</td>
<td>1.6</td>
</tr>
<tr>
<td>Cys</td>
<td>0.5</td>
<td>0.2</td>
<td>1.0</td>
<td>1.0</td>
<td>0.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Gln</td>
<td>3.4</td>
<td>3.4</td>
<td>2.5</td>
<td>2.7</td>
<td>2.1</td>
<td>0.6</td>
</tr>
<tr>
<td>Glu</td>
<td>3.4</td>
<td>2.4</td>
<td>2.0</td>
<td>1.2</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Gly</td>
<td>12.6</td>
<td>9.7</td>
<td>11.5</td>
<td>10.8</td>
<td>9.3</td>
<td>8.1</td>
</tr>
<tr>
<td>His</td>
<td>1.1</td>
<td>1.2</td>
<td>1.7</td>
<td>1.7</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td>5.7</td>
<td>9.2</td>
<td>6.7</td>
<td>6.8</td>
<td>11.7</td>
<td>13.5</td>
</tr>
<tr>
<td>Leu</td>
<td>13.8</td>
<td>14.9</td>
<td>14.0</td>
<td>14.1</td>
<td>12.6</td>
<td>11.8</td>
</tr>
<tr>
<td>Lys</td>
<td>1.4</td>
<td>2.2</td>
<td>0.5</td>
<td>1.0</td>
<td>3.7</td>
<td>5.4</td>
</tr>
<tr>
<td>Met</td>
<td>3.7</td>
<td>3.2</td>
<td>4.0</td>
<td>4.2</td>
<td>4.1</td>
<td>2.7</td>
</tr>
<tr>
<td>Phe</td>
<td>4.8</td>
<td>6.9</td>
<td>5.2</td>
<td>4.2</td>
<td>8.0</td>
<td>9.4</td>
</tr>
<tr>
<td>Pro</td>
<td>5.4</td>
<td>3.4</td>
<td>4.7</td>
<td>4.0</td>
<td>3.4</td>
<td>3.3</td>
</tr>
<tr>
<td>Ser</td>
<td>5.7</td>
<td>7.7</td>
<td>4.2</td>
<td>5.5</td>
<td>8.9</td>
<td>8.4</td>
</tr>
<tr>
<td>Thr</td>
<td>6.3</td>
<td>6.2</td>
<td>4.7</td>
<td>5.3</td>
<td>5.4</td>
<td>5.0</td>
</tr>
<tr>
<td>Trp</td>
<td>1.4</td>
<td>2.9</td>
<td>2.2</td>
<td>2.0</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.2</td>
<td>1.7</td>
<td>2.0</td>
<td>2.0</td>
<td>2.6</td>
<td>4.0</td>
</tr>
<tr>
<td>Val</td>
<td>7.2</td>
<td>6.4</td>
<td>6.5</td>
<td>7.3</td>
<td>9.1</td>
<td>6.4</td>
</tr>
<tr>
<td>$M_r$</td>
<td>35818</td>
<td>36000</td>
<td>42205</td>
<td>41518</td>
<td>50000</td>
<td>35000</td>
</tr>
</tbody>
</table>

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 TcR 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 TcR 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 TcR gene (tet347) contained in this fragment was selected. Ohnuki et al. (1985a) have recently cloned two TcR 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 TcR genes reported by Ohnuki et al. (1985a), the TcR 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 (otcRII) 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. (1985a) 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 TcR 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 TcR 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 NcoI–BamHI fragment in the correct orientation was put downstream of the E. coli Lpp or λpL promoters. Changing the orientation of the same fragment failed to give a TcR phenotype (data not shown). The complete nucleotide sequence of this NcoI–BamHI 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 λpL 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 TcR 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 kcal mol⁻¹ on the other side. Whether this sequence is involved in the induction mechanism of TcR 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 TcR 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 opportunistic bacteria could have evolved from ancestral genes found in antibiotic-producing actinomycetes.

We thank G. Bisiaux, Société Chimique Pointet-Girard (Rhône-Poulenc), Paris, for his kind gift of the S. rimosus and S. aureofaciens strains. We also thank D. A. Hopwood for generously providing pIJ702.
REFERENCES


