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The Unstable Tetracycline Resistance Gene of *Streptomyces lividans* 1326 Encodes a Putative Protein with Similarities to Translational Elongation Factors and Tet(M) and Tet(O) Proteins

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***Streptomyces lividans* contains a genetically unstable tetracycline resistance determinant. Nucleotide sequencing revealed an open reading frame of 1,917 nucleotides. The transcriptional start site was mapped at about 110 bp upstream of the ATG codon. The proposed promoter contains an 8-bp perfect inverted repeat between the −10 and −35 regions. The deduced amino acid sequence showed several motifs which are commonly found in many GTP-binding proteins. On the basis of its amino acid sequence, the presumptive *S. lividans* 1326 protein belongs to the Tet(M)-Tet(O) group of tetracycline resistance proteins and shows significant similarity to translational elongation factors of prokaryotes and eukaryotes.**

Tetracycline resistance is widespread among bacteria. Various genes that encode resistance have been analyzed in gram-positive and gram-negative bacteria (15, 26). These genes can be assigned to three groups, reflecting the different mechanisms of resistance observed against this drug. First, the prevention of intracellular accumulation of tetracycline because of an energy-dependent efflux of the drug has been described mainly for members of the family *Enterobacteriaceae* and related genera (15, 21). This efflux is mediated by a metal-tetracycline and H^+ antiporter (31).

The second mechanism of resistance is due to protection of the ribosomes by soluble cytoplasmic proteins [Tet(M)-Tet(O)] (3). The nucleotide sequences of *tet* genes belonging to this group were identified in various bacteria such as *Streptococcus* spp. (3), *Mycoplasma hominis* (24), and *Campylobacter jejuni* (18). The deduced amino acid sequences show significant similarities to various elongation factor proteins (4, 17). One of the resistance proteins, the streptococcal Tet(M) protein, has been overexpressed under the control of a T7 promoter and has been purified to near homogeneity. The purified protein was shown to have a ribosome-dependent GTPase activity (4). The sequence similarity of Tet(M) with EF-G and proteins with similar activities support the idea that Tet(M) might act as a tetracycline-resistant elongation factor (17). Burdett (4) showed, however, that Tet(M) could substitute for neither EF-Tu in *Bacillus subtilis* nor EF-G in *Escherichia coli*.

A third mechanism involves the inactivation of tetracycline by modification and has been reported in *Bacteroides fragilis*. This determinant is cryptic in *B. fragilis* but is active in *E. coli*, in which oxygen is required for detoxification of the drug (29).

The oxytetracycline-producing strain *Streptomyces rimosus* was shown to contain at least two resistance genes, one of the efflux type and the other belonging to the ribosome protection group (5, 23).

In *Streptomyces lividans*, tetracycline resistance is a highly unstable trait. Susceptible variants arise spontaneously at frequencies of 10^{-2}, and about 10% of these subsequently segregate to variants which lack positive regulation of glutamine synthetase (8). We previously reported the cloning of the tetracycline resistance determinant from *S. lividans*. Tetracycline-susceptible variants of *S. lividans* and *Streptomyces coelicolor* A3(2) are due to large deletions, including deletions of the relevant gene (14, 28). Hybridization of *S. lividans* DNA with the *otrA* gene of *S. rimosus* showed significant, but limited, homology (14). We report here the nucleotide sequence of the tetracycline resistance gene from *S. lividans*. The amino acid sequence deduced from the DNA sequence shows significant similarity to the sequences of Tet(M) and Tet(O) proteins and various translational elongation factors. The 5′ end of the transcript was mapped in *S. lividans*, and a highly resistant strain was derived from the wild type. The proposed promoter structure, which contains an 8-bp inverted repeat, is discussed. Furthermore, we demonstrated that highly resistant strains do not carry mutations in the upstream sequence, including the proposed promoter region.

MATERIALS AND METHODS

Bacterial strains and plasmids. The following tetracycline-resistant *Streptomyces* strains from the German Culture Collection (DSM) were used: *S. griseus* DSM 40236, *S. flavopersicum* DSM 40093, *S. albus* DSM 40763, and *S. citreofluorescens* DSM 40265. *S. platensis* and *S. tendae* were provided by A. Stöckigt (Munich, Germany) and H. Zähner (Tübingen, Germany), respectively. The highly resistant strains *S. lividans* THR1 and THR2 were isolated from the wild-type strain by stepwise selection. THR1 and THR2 are resistant to 200 μg of tetracycline per ml on YEME agar plates (10), whereas the wild-type strain tolerates only 40 μg of the drug per ml. Plasmids pUC18 (32) and pUS18 (constructed in our laboratory by inserting an additional *Sst*II-*Not*I-*Sfi*I-*Spe*I linker into the pUC18 *Bam*HI site) were used as cloning vectors for DNA sequence analysis. The *E. coli* strains JM109 (32) and K-12 F^-Z^- M15 (25) served as hosts.

Preparation and in vitro manipulation of DNA. Plasmids were prepared by using the Qiagen plasmid kit (Diagen, Hilden, Germany) according to the instructions of the manufacturer. Recovery of DNA from agarose gels, ligation,

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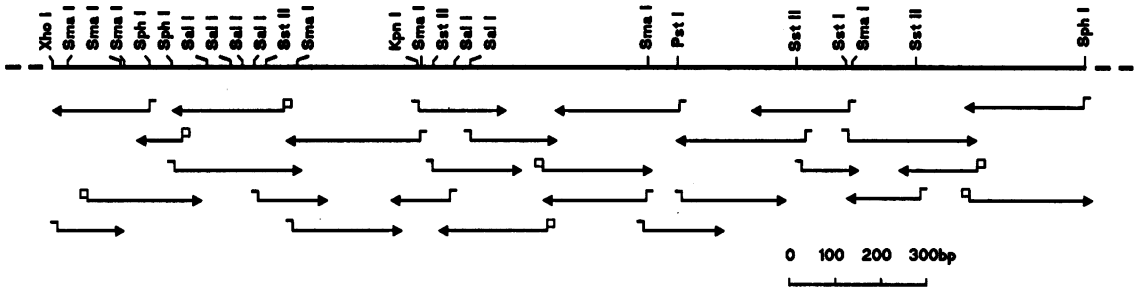

FIG. 1. Restriction map of the 2.3-kb *Sph*I-*Xho*I fragment containing the tetracycline resistance gene. The sequencing strategy with subclones (arrows without open boxes) and oligonucleotides (arrows with open boxes) is given below the restriction map.

transformation (19), and hybridization with biotinylated probes has been described earlier (8).

DNA sequence determination and analysis. Dideoxy sequencing reactions on double-stranded templates were carried out by using defined restriction fragments subcloned in pUC18 or pUS18 vectors with [α-^{35}S]dATP as the labeled nucleotide. The Sequenase II Sequencing Kit of U.S. Biochemicals was used according to the instructions of the manufacturer. Sequencing analysis was performed with site-specific as well as pUC18 universal primers.

The determined sequence was analyzed further by using the program FASTA. The Swissprot (release 18) and EMBL (March 1991) data bases were used for sequence comparisons.

Isolation of RNA. RNA was isolated from cultures which were grown in YEME (10) containing 10 μg (*S. lividans* 1326) or 40 μg (*S. lividans* THR1) of tetracycline per ml. At 30 min before RNA isolation, tetracycline was added to final concentrations of 15 μg/ml (strain 1326) and 60 μg/ml (strain THR1). RNA was isolated as described by Hopwood et al. (10).

High-resolution S1 nuclease mapping. A *Xho*I-*Kpn*I fragment was cloned into M13mp19 (pTC116). Single-stranded DNA of this recombinant phage was annealed to an oligonucleotide primer (o114/3) which was extended by primer extension in the presence of [^{32}P]dATP (3,000 Ci/mol). The labeled probe was hybridized against 40 μg of total RNA at 50°C and digested with nuclease S1 as described by Hopwood et al. (10). Protected hybrids were electrophoresed on sequencing gels and were compared with a sequencing ladder obtained from pTC116, with o114/3 used as the oligonucleotide primer.

PCR. The polymerase chain reaction (PCR) was performed by using *Taq* polymerase (Bethesda Research Laboratories, Eggenstein, Germany) according to the instructions of the manufacturer. The first round of amplification with 20mers (oTC114/2, TGAGCGGCACGCCCACGTGG; oTC114/3, ACCCGCGTCGACGTGGGCCA) as primers was performed by using standard conditions (12). For a subsequent asymmetric amplification, 100 ng of the products of the first reaction were used. The products were purified on low-melting-point agarose gels. Only one primer was used to amplify single-stranded DNA for sequencing as described above.

Nucleotide sequence accession number. The nucleotide sequence described here was deposited in the GenBank data base and was given the accession number M74049.

RESULTS

Analysis of the DNA sequence. Previously, we reported the cloning of the tetracycline resistance determinant of *S. lividans* (14). Both strands of a 2.3-kb *Sph*I-*Xho*I fragment containing the resistance gene were sequenced by the strategy depicted in Fig. 1. The nucleotide sequence of this 2.3-kb fragment that we determined showed a single open reading frame from nucleotides 343 to 2259 (Fig. 2). The G+C content of the coding region was 76 mol%, which is slightly greater than that described for other *Streptomyces* genes (1). Within the coding region, a strong preference for codons with G or C in the third position, which is characteristic for *Streptomyces* coding sequences, can be observed (1, 11).

A possible ribosomal binding site is located 15 bp upstream from the start codon, but ribosomal binding sites can be spaced farther away from the start codon in *Streptomyces* spp. This open reading frame could encode a protein of 639 amino acids with a calculated molecular size of 67.1 kDa. The deduced amino acid sequence shows five motifs which are characteristic for GTP-binding proteins (underlined amino acids in Fig. 2).

Comparison of DNA and protein sequences. The determined nucleotide sequence was compared with sequences deposited in the EMBL data base. The best score was found with the *otrA* gene of *S. rimosus* (59% identical nucleotides). Further similarities were found with genes that encode EF-G or EF-2 from microorganisms with a high G+C content (*stro* of *Micrococcus luteus*, 53.7%; *ef2* of *Halobacterium halobium*, 53.3%; *fus* of *Thermus thermophilus*, 51.9%).

Significant homology of the *S. lividans* Tet^r protein was detected with several other Tet^r proteins belonging to the Tet(M) or Tet(O) class. The highest score (64.7% identity) was obtained with the protein encoded by the *otrA* gene (Fig. 3). About 34% identical amino acids were found with Tet(M) proteins of Tn*916*, Tn*1545*, and *Ureaplasma urealyticum* (Fig. 3) and with Tet(O) proteins of *C. jejuni* (Fig. 3) and *Streptococcus mutans*.

The most extensive similarities were observed within the N-terminal 130 amino acids of the deduced proteins: 70% identical amino acids with *S. rimosus* OtrA and 60.7% identical amino acids with the *U. urealyticum* Tet(M) protein, the *Streptococcus faecalis* Tet(M) protein, and the *C. jejuni* Tet(O) protein. Significant similarities to various translational elongation factors like EF-G or EF-2 were found (Fig. 3). Again, the most extensive similarities were observed within the N-terminal 130-amino-acid overlap of these proteins; 43 to 47% of the amino acids of the *S. lividans* Tet^r protein were identical to those of EF-G factors from the following five bacteria: *Thermus aquaticus*, *Anacystis nidulans*, *Spirulina platensis*, *M. luteus*, and *E. coli*. Less similarity was found within the N-terminal sequences of the eukaryotic or archaebacterial elongation factor EF-2 (30 to

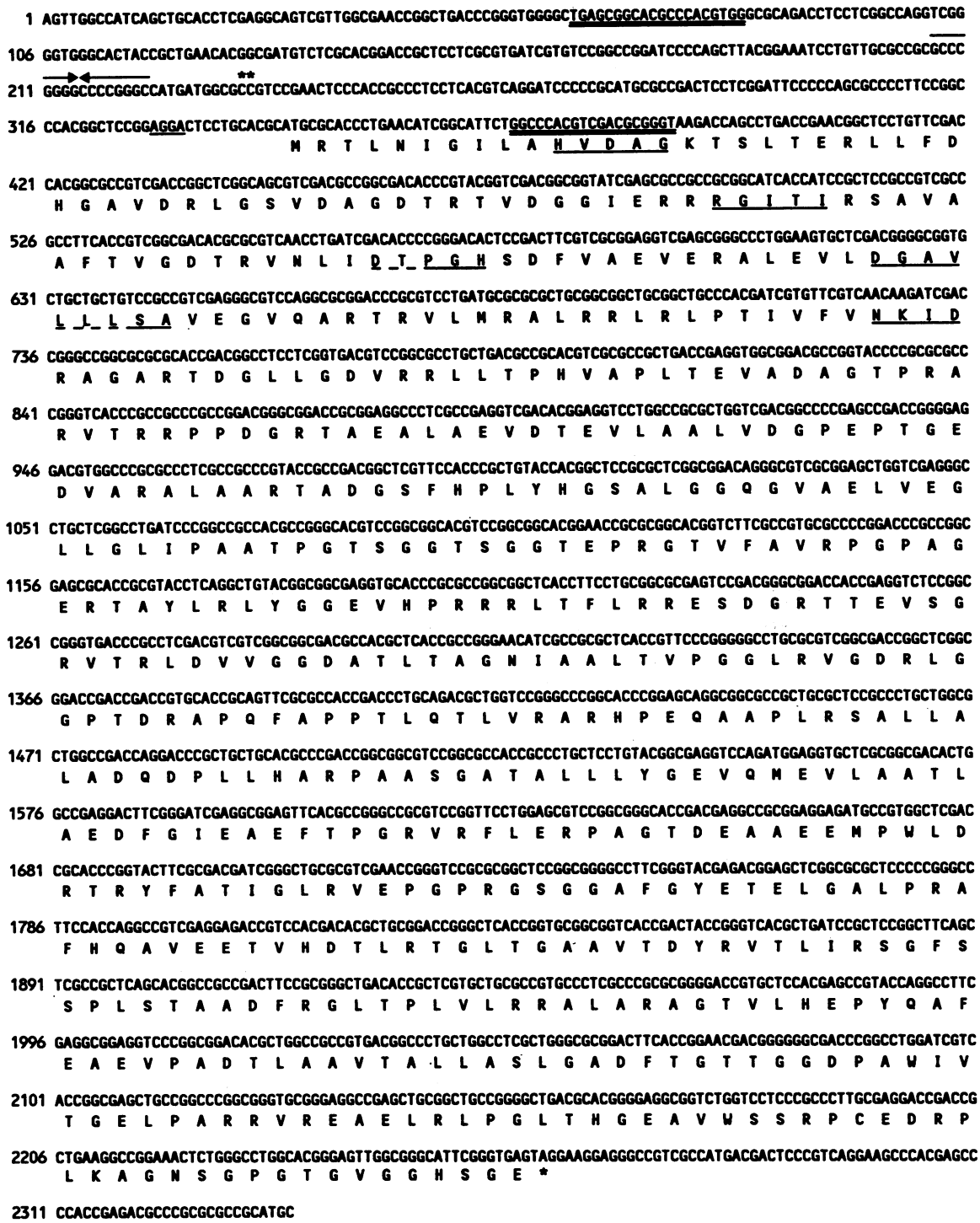

FIG. 2. Nucleotide sequence of the tetracycline resistance gene and the deduced amino acid sequence. The putative start codon and the Shine-Dalgarno sequence are underlined in the DNA sequence. The primer sequences used for PCR are double underlined. Amino acids which were involved in the formation of the GTP-binding site are underlined in the amino acid sequence. Asterisks indicate the transcriptional start sites; arrows indicate inverted repeat structures.

35% identical amino acids within a 130-amino-acid overlap). Similarities of the *S. lividans* Tet^r protein with other translation factors (EF-Tu, IF2), which contain the same GTP-binding motifs as EF-G, are limited to this domain (Fig. 3).

Distribution of the *tet*^{r} gene among other streptomycetes. The *Sph*I-cleaved DNAs of six other resistant *Streptomyces* strains were hybridized to the cloned *S. lividans* tetracycline resistance gene. None of the DNAs hybridized strongly with the *S. lividans* tetracycline resistance gene. In addition to the strongly hybridizing fragment in *S. lividans*, one other frag-

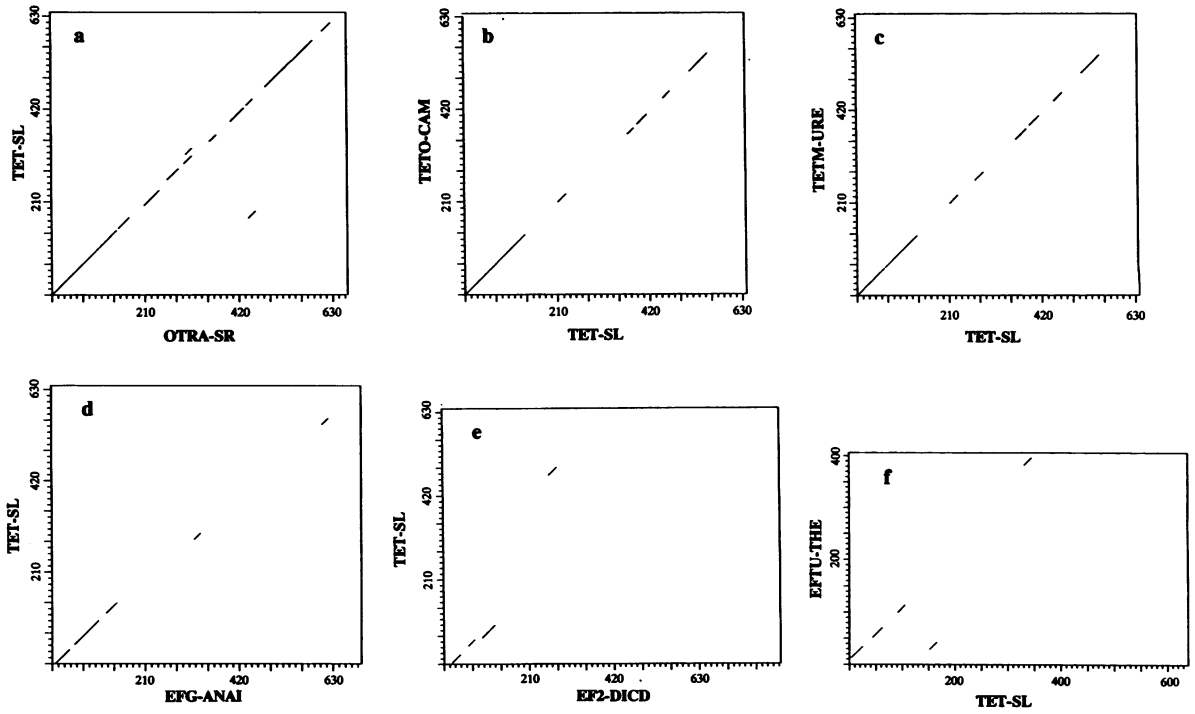

FIG. 3. Comparison of amino acid sequences of the Tet^r protein from *S. lividans* (TET-SL) with those of the following: the OtrA protein from *S. rimosus* (OTRA-SR) (a), Tet(O) from *C. jejuni* (TETO-CAM) (b), Tet(M) from *U. urealyticum* (TETM-URE) (c), EF-G from *A. nidulans* (EFG-ANAI) (d), EF-2 from *Dictyostelium discoideum* (EF2-DICD) (e), and EF-Tu from *T. aquaticus* (EFTE-THE) (f). In this comparison, a window of 12 amino acids within which 60% identity was required to generate a signal was used.

ment which was also present within the DNA of all other *Streptomyces* strains tested was detected. This signal was also obtained with tetracycline-susceptible variants of *S. lividans* and might reflect hybridization to an elongation factor gene of this organism (data not shown).

Mapping of the tetracycline resistance gene transcriptional start sites. For high-resolution S1 nuclease mapping, 40 μg of total RNA of *S. lividans* 1326 or *S. lividans* THR1 was used in each experiment. With both strains, two protected fragments of equal intensities were detected; they corresponded to nucleotides 233 and 234 (Fig. 2). The transcriptional start sites of the fragments are located 109 and 110 bp upstream from the translational start codon, respectively. The proposed promoter resembles a group of *Streptomyces* promoters which share similarities with the *E. coli* consensus promoter (Fig. 4). The most conserved nucleotides at −35 (TGG at positions −35, −34, and −33) and −10 (T and A at positions 6 and 2, respectively) were present in the proposed *tet*^{r} promoter. Nevertheless, the *tet*^{r} gene cloned into pUC18 did not confer resistance to *E. coli*. Between the −10 and −35 regions, only G or C residues were present, including a perfect 8-bp inverted repeat (Fig. 4).

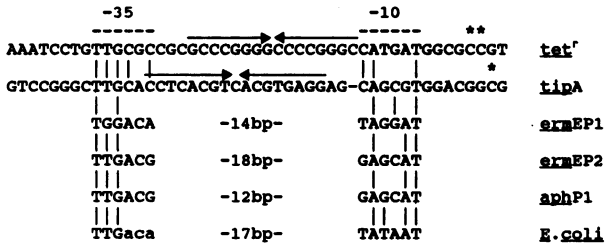

FIG. 4. Promoter structure of the tetracycline resistance gene and comparison with those of promoters of the other *Streptomyces* genes: *tipA* (22), *ermEP1* and *ermEP2* (2), and *aphP1* (13). The *E. coli* consensus promoter is shown. Asterisks indicate transcriptional start sites; arrows indicate inverted repeat structures.

Analysis of highly resistant strains. Recently, we isolated *S. lividans* strains that were highly resistant (200 μg/ml) to tetracycline. In contrast to the *cml*^{r} gene found in highly chloramphenicol-resistant strains (6), the *tet*^{r} gene was shown not to be amplified (14). Another explanation for high-level resistance could involve increased expression of the gene, which is caused by mutation of controlling elements such as promoters, ribosome-binding sites, or repressor- or activator-binding sites. Therefore, we analyzed a 300-bp upstream region of the tetracycline resistance gene from highly resistant strains for putative changes in nucleotide sequence. A 327-bp fragment was amplified by PCR by using 0.5 μg of total DNA isolated from two highly resistant strains, THR1 and THR2. The primers used for PCR are given in Fig. 2. Sequence analysis was performed for both strands of the amplified products, but comparison of the nucleotide sequences obtained from highly resistant strains and the wild type showed no differences.

DISCUSSION

On the basis of its deduced amino acid sequence, the *S. lividans* protein belongs to the ribosome protection type of tetracycline resistance determinants. The *S. lividans* sequence shares significant similarity at the DNA and protein levels with the *otrA* (7) sequence from the oxytetracycline-producing strain *S. rimosus*. Although there is significant homology at the amino acid level between the presumptive

S. lividans protein and Tet(M)-Tet(O) proteins from other organisms, no similarities are present between the DNA sequences. This reflects the different G+C contents of the *S. lividans* gene (76 mol%) and the genes of *Ureaplasma* spp. (36 mol%) or *Campylobacter* spp. (40 mol%).

We analyzed the transcriptional start site of the *tet*^{r} gene. The proposed promoter region showed a strong secondary structure between the −10 and −35 regions. This 8-bp inverted repeat could be involved in the regulation of transcriptional activity from this gene. A secondary structure containing an 8-bp inverted repeat between the −10 and −35 regions has been described for a thiostrepton-inducible promoter in front of the *tipA* gene (Fig. 4). The addition of thiostrepton resulted in a 200-fold increase in the level of transcription of the *tipA* gene. The authors speculated further that the −10 and −35 regions are part of an imperfect palindromic sequence which must be resolved for recognition by the polymerase (22). In contrast, the perfect 8-bp inverted repeat of the *tet*^{r} promoter does not extend to the −10 and −35 regions. Since both promoters show an inverted repeat between −10 and −35, however, resolution of this secondary structure might be necessary for the efficient transcription of these genes. The *S. rimosus otrA* gene has been shown to have two promoters (20), and one promoter is active only in the production phase. In contrast, the *S. lividans* Tet^r gene seems to be transcribed by a single promoter. Surprisingly, the distribution of resistance genes belonging to classes Tet(M) and Tet(O) seems to be rather limited among tetracycline-resistant *Streptomyces* spp. Only *S. rimosus* has been described to contain a similar gene that encodes a protein which is believed to confer resistance by ribosome protection (5, 23). Similarities are limited to the coding regions of *otrA* (7) and *tet*^{r}, but the upstream sequences are completely different. This might reflect different requirements for transcriptional regulation of a gene in a producing and a nonproducing strain. The deduced protein sequence shows sequence motifs which are involved in the binding of GTP. The streptococcal Tet(M) protein has been purified and has been shown to have ribosome-dependent GTPase activity (4). The sequences necessary for the formation of a GTP-binding site are present in the presumptive *S. lividans* protein, and similar enzymatic properties could be expected. Like other Tet(M)-Tet(O) proteins, the *S. lividans* protein showed significant homology to EF-G and EF-2. In contrast to EF-Tu (16), identical amino acids were not limited to the GTP-binding sites. Two potential mechanisms by which proteins of the Tet(M)-Tet(O) group might act have been suggested (27). The prevention of binding of tetracycline to ribosomes was already excluded by Burdett (4). Tet(M) could alternatively act as a tetracycline-resistant elongation factor. On the basis of sequence similarities, our results suggest that a tetracycline-resistant EF-G factor is the best candidate for this possibility. Burdett (4) showed, however, that Tet(M) is not able to substitute for EF-G or EF-2 and that the amount of Tet(M) in the cells is far lower than the amount of EF-G. Manavathu et al. (17) suggested a catalytic function for Tet(M)-Tet(O) proteins, but further studies with purified proteins will be required in order to elucidate their precise mechanisms.

Tetracycline-susceptible variants of *S. lividans* which are derived by deletion of the *tet*^{r} gene and adjacent sequences also frequently show a fusidic acid-susceptible phenotype. We recently showed that resistance to fusidic acid in *S. lividans* is due to modification of the drug (30). Some variants which were no longer able to modify fusidic acid but which were still resistant to this antibiotic could be isolated. Since fusidic acid is known to interact with EF-G, one could speculate that a tetracycline-resistant EF-G could also be responsible for resistance to fusidic acid in these strains. However, we could exclude this possibility since tetracycline-resistant transformants containing the plasmid pSTC1 (14) do not confer cross-resistance to fusidic acid. We showed that in *S. lividans*, increased resistance to tetracycline is due neither to amplification of the gene, as observed for a chloramphenicol resistance gene in the same strain (6), nor to changes in the nucleotide sequence upstream from the start codon. A possible explanation might be the activation of a second, previously silent resistance gene. Spontaneously tetracycline-resistant colonies frequently arise after protoplasting and regeneration of strains from which the *tet*^{r} gene has been deleted. Activation of a previously cryptic energy-dependent efflux system for tetracycline has been described for highly resistant strains of *E. coli* (9). Since protoplasting is known to cause DNA rearrangements, activation of a formerly cryptic tetracycline resistance gene also cannot be ruled out in *S. lividans*.

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