Development of a Temperature-Inducible Expression System for *Streptomyces* spp.

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Received 7 May 1996/Accepted 15 July 1996

PCR mutagenesis of a 0.9-kbp fragment, containing a repressor gene, *traR*, and its target promoter, *Ptra*, from *Streptomyces nigerificiens* plasmid pSN22, produced *Streptomyces lividans* clones with temperature-inducible *Ptra* expression. Using the promoterless gene for the thermostable *Thermus flavus* malate dehydrogenase as an indicator, an induction of enzyme activity of as much as was observed in a temperature shift from 28 to 37°C. Temperature downshift reestablished repression of *Ptra*, making these promoter cassettes very attractive for the temporally regulated expression of cloned genes in *Streptomyces* spp.

Expression vectors with inducible promoters are of great importance for basic and applied studies of *Streptomyces* biology. Such systems are thought to be particularly useful for the investigation of the differentiation process, which is subject to complex hierarchical regulation (2). A few such vectors already exist; they use chemically inducible promoters that respond to the addition of antibiotics (12, 14, 18, 21) which are difficult to remove if the genes need to be switched off. Expression from the existing vectors also depends on or is influenced by chromosomal host genes (3), necessitating the use of specific strains only (21). In contrast with the endogenous promoters, episome promoters are likely to be independent of host metabolism, potentially providing a broad working host range and clear results that are not affected by the genetic background of the host.

These considerations prompted us to develop a fully portable, host-independent, regulatable expression cassette using a promoter from the *Streptomyces nigerificiens* SN22 11-kb conjugative multicopy plasmid pSN22. pSN22 has been studied in great detail (7–10). The promoter, *Ptra*, of the transfer operon has been located by transcription mapping (8), and it has been used for the high-level heterologous expression of a *Streptomyces griseus* protease in *Streptomyces lividans* 66 (20). *Ptra* is a very strong promoter whose sequence resembles the consensus sequence of *Streptomyces* vegetative promoters (19). In the presence of the pSN22-encoded, autoregulated repressor TraR, it is switched off. We reasoned that amino acid changes might render TraR temperature sensitive and, as a consequence, *Ptra* temperature inducible.

The *Streptomyces* plasmids used in this study are listed in Table 1. The general techniques for handling DNA and bacterial strains followed standard methods (4, 17). The repressor gene traR and the promoter-operator region of the pSN22 transfer operon, TARE (transfer genes regulating element), were isolated on a 0.9-kb *BamHI* fragment from plasmid pPTD1 (8) and subcloned into the *BamHI* site of pUC18 (24). Mutations were introduced into the 0.9-kb traR-TARE fragment by using a PCR method (16) with *Taq* DNA polymerase (Takara) and high deoxynucleoside triphosphate (dNTP) concentrations (13). The PCR was performed with the M13 primers RV and M4 (22) and included 30 cycles at 95°C for 30 s, 55°C for 1 min, and 72°C for 1.5 min in a buffer containing 6.7 mM MgCl2, 16.6 mM (NH4)2SO4, 6.6 mM Tris-HCl (pH 8.8), 1 mM dNTPs, and 0.5 mM MnCl2. The amplified fragments were digested with EcoRI and HindIII, purified by agarose gel electrophoresis, and cloned in the pIJ101-derived *Streptomyces* mutycop promoter-probe vector pIJ486 (23), which had been digested with the same enzymes. Because pIJ486 contains a promoterless *Tn5* neo gene, activation of this gene by a cloned promoter induces kanamycin resistance. The recombinant plasmids were introduced into *S. lividans* TK21 (11) protoplasts by transformation. The protoplasts were regenerated on plates at 37°C and overlaid after 16 h with kanamycin (200 μg/ml). This kanamycin concentration was tolerated at 28 and 37°C by *S. lividans* containing pITRE (pIJ486 containing *Ptra* without the repressor gene *traR*), but it completely inhibited *S. lividans* containing pIRRE (pIJ486 containing the wild-type *traR*-TARE cassette). In total, 8,380 independent kanamycin-resistant colonies were isolated, representing about 3% of thiostrepton-resistant transformants. The kanamycin-resistant colonies were left to sporulate at 37°C and then replicated twice onto kanamycin medium. The plates were incubated at 28 or 37°C. Thirty clones (0.36% of 8,380) grew at 37°C but not at 28°C, indicating that kanamycin resistance had become temperature inducible.

Plasmid DNA was isolated from the 30 clones with temperature-inducible kanamycin resistance and introduced by transformation into *S. lividans* protoplasts for retesting. Two strains with the desired phenotype were selected for further analysis. They contained the structurally stable plasmids pITS107 and pITS110. *S. lividans* containing pITS107 grew better at 32°C than the strain containing pITS110 (data not shown). DNA sequence analysis revealed that both mutants had multiple mutations within the *traR* coding sequence and within the *tra* transcription unit. For pITS107, the mutations were *TraR I22T* and *tra ΔC44*; for pITS110, the mutations were *TraR A5T* and *E60G* and *tra T46C*. The contributions of the individual mutations to thermoinducibility have not been determined.

The neo genes in pITS107 and pITS110 were expected to be
transcriptionally controlled. This was confirmed by dot blot analysis using total RNA from *S. lividans* cultures grown in YEME medium (4) at different temperatures: nontempered controls were kept at 28°C for 22 h; temperature-induced cultures were cultivated for 20 h at 28°C and then for 2 h at 37°C; and double-shift cultures were grown for 20 h at 28°C, 2 h at 37°C, and then 4 h again at 28°C before RNA isolation. Total RNA was isolated from *S. lividans* by the CsCl gradient method described previously (10). The denatured RNA, 5 μg per slot, was transferred onto nylon membranes pretreated with 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) by using a blotting manifold. The membrane was washed three times with 10× SSC; the washes were followed by drying at 65°C and RNA immobilization by UV radiation. The membrane was probed with the randomly 32P-labeled Smal-PstI fragment derived from the *neo* gene of pIJ486. Figure 1 shows that pIRTRE, containing the wild-type *traR*-TRE cassette, did not produce any detectable amount of *neo* mRNA at 28 or 37°C. pITRE, containing the *tra* promoter without its repressor, produced more *neo* mRNA at 28 than at 37°C. The two mutant *traR*-TRE expression cassettes, however, produced no *neo* mRNA at 28°C and large amounts at 37°C, the induction being stronger for pITS110 than for pITS107. Reduction of the growth temperature from 37 to 28°C for 4 h again reduced the amount of detectable *neo* transcript. Similar results were obtained in three independent experiments. These results clearly indicated that transcription from *Ptra* was induced by temperature shift-up and that the induction was shut off by temperature shift-down in the mutant systems.

To test whether the mutant *traR*-TRE cassettes are useful for the regulated expression of genes other than *neo*, the promoterless *mdh* gene, encoding the thermoresistant malate dehydrogenase (MDH) of *Thermus flavus* (15), was inserted downstream of the ts107 expression cassette in pITSM107. The resulting plasmid, pITSM107, is shown in Fig. 2A. Malate dehydrogenase (MDH) activity was determined by the method described previously (6). The endogenous *Streptomyces* MDH was inactivated by boiling before the assay. The protein concentration was estimated by the method of Bradford (1) with a protein assay kit (Bio-Rad). The time course of expression after temperature shifts (Fig. 2B) shows that the rate of increase and the maximal levels of specific MDH activity depended on the temperature. On the basis of the enzyme units for each time point, the MDH production rate of each clone was estimated. The rate was defined as the amount of enzyme unit produced per hour in each culture. The MDH production rate at 37°C (4 × 10⁻¹ U/mg of protein per h, obtained between 24 and 42 h) was about 100-fold higher and that at 32°C (1 × 10⁻¹ U/mg of protein per h, obtained between 36 and 48 h) was about 25-fold higher than that at 28°C (4 × 10⁻³ U/mg of protein per h, obtained between 36 and 60 h). As shown in Fig. 2C, temperature downshift resulted in a slow reduction of MDH activity, probably mostly attributable to dilution. *S. lividans* containing pITRE, similar to pITSM107 but with the wild-type *traR*-TRE cassette, produced MDH activity at less than the detection level, <0.01 U/mg of protein, while with pITSM107 up to 0.1 U of MDH activity was detected at 28°C after 72 h of cultivation (data not shown). The maximal specific MDH activity obtained with pITSM107 (9.2 U/mg of protein) was about five times higher than that reported for *S. lividans* containing pMDF1, in which *mdh* is driven by a very strong promoter from the *S. lividans* chromosome (5). This indicates that the ts107 cassette should give excellent overexpression of cloned genes.

The method of inducing *Ptra* activity presented in this report opens new possibilities for the study of gene function in *Streptomyces* spp., including the regulation of genes for sporulation and secondary metabolism.
We thank Tobias Kieser for many useful discussions and editorial suggestions and for providing pJ486, and we thank Sueharu Horinouchi for providing pMDF1.

M. Kataoka and T. Tatsuta contributed equally to this work.

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