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Natural and synthetic tetracycline-inducible promoters for use in the antibiotic-producing bacteria *Streptomyces*

Antonio Rodríguez-García, Patricia Combes, Rosario Pérez-Redondo, Matthew C. A. Smith and Margaret C. M. Smith*

Institute of Genetics, University of Nottingham, Queens Medical Centre, Nottingham NG7 2UH, UK

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ABSTRACT

Bacteria in the genus *Streptomyces* are major producers of antibiotics and other pharmacologically active compounds. Genetic and physiological manipulations of these bacteria are important for new drug discovery and production development. An essential part of any 'genetic toolkit' is the availability of regulatable promoters. We have adapted the tetracycline (Tc) repressor/operator (TetR/*tetO*) regulatable system from transposon Tn*10* for use in *Streptomyces*. The synthetic Tc controllable promoter (tcp), *tcp830*, was active in a wide range of *Streptomyces* species, and varying levels of induction were observed after the addition of 1–100 ng/ml of anhydrotetracycline (aTc). *Streptomyces coelicolor* contained an innate Tc-controllable promoter regulated by a TetR homologue (SCO0253). Both natural and synthetic promoters were active and inducible throughout growth. Using the *luxAB* genes expressing luciferase as a reporter system, we showed that induction factors of up to 270 could be obtained for *tcp830*. The effect of inducers on the growth of *S.coelicolor* was determined; addition of aTc at concentrations where induction is optimal, i.e. 0.1–1 μg/ml, ranged from no effect on growth rate to a small increase in the lag period compared with cultures with no inducer.

INTRODUCTION

Unnatural control of gene expression is an essential tool in genetic analysis and requires the exploitation of controllable promoters to be used in recombinant constructs. A suitable promoter should, ideally, be completely off when repressed, tunable to different strengths when induced, and the inducer should have no pleiotropic effects on general growth. Currently, the most widely used promoter for regulated gene expression in *Streptomyces* spp. is the thiostrepton inducible promoter, *ptipA* (1). This promoter has provided reliable and controllable gene expression under many circumstances, but thiostrepton induces a regulon of proteins, is dependent on the presence of an activator, TipAL, and a resistance gene, *tsr*, and the uninduced level of promoter activity is sometimes significant (1–3). Other controllable expression systems developed for use in *Streptomyces* or related genera include the *Rhodococcus rhodochrous* nitrilase promoter, P_{nitA}, regulated by NitR (4) and the *Streptomyces coelicolor gylR* and *gylP1/P2* glycerol-inducible system (5,6). While the P_{nitA}/NitR system appears to be excellent for protein overproduction from high copy number plasmids, its not clear at this time whether it will also be a promoter of choice for ectopic, controlled expression in routine genetic analysis. Furthermore, the glycerol-inducible system is of restricted use as the addition of glycerol may be undesirable for many physiological investigations. There is clearly a need for alternative regulatable promoters.

The tetracycline (Tc)-inducible repressor (*tetR*)-operator (*tetO*) interaction from the *Escherichia coli* transposon Tn*10* has been successfully adapted for use as a tool for regulating gene expression in many organisms (7,8). The *tetO*–TetR interaction is very strong ($K_a \sim 10^{11}$ M^{-1}), explaining the observed high-level repression in the absence of Tc. When Tc is present, the affinity of the repressor for *tetO* is reduced by nine orders of magnitude, resulting in very high induction factors (IFs), i.e. the level of expression in the induced state compared with the expression in the repressed state

*To whom correspondence should be addressed at Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK. Tel: +44 01224 555739; Fax: +44 01224 555844; Email: Maggie.smith@abdn.ac.uk
Present addresses:
Antonio Rodríguez-García, Instituto de Biotecnología de León (INBIOTEC), Parque Científico de León, Avda. Real no. 1, 24006 León, Spain
Patricia Combes, INSERM U384, Faculté de Médecine, 63000 Clermont-Ferrand, France
Rosario Pérez-Redondo, Área de Microbiología, Facultad de Ciencias Biológicas y Ambientales, 24071 León, Spain

(9,10). Several members of the Tc family can be used as inducers at sub-inhibitory levels of antibiotic (9). Anhydrotetracycline (aTc) is a more active inducer than Tc and has a higher minimum inhibitory concentration (9,11). Here, we describe synthetic Tc-controllable promoters (tcps), a *tetR* allele derived from the Tn*10 tetR*/*tetO* system adapted for use in *Streptomyces*, and an endogenous Tc-responsive repressor/operator system in *S.coelicolor*.

MATERIALS AND METHODS

Bacterial strains

E.coli DH5α was used as a general cloning host and was grown and maintained according to the standard methods (12). *S.coelicolor* strain, J1929 (13), a derivative of M145, was used as a recipient to assay the synthetic and natural promoters. Other *Streptomyces* strains used in this study were *Streptomyces avermitilis*, *Streptomyces lividans 66* strain TK24, *Streptomyces ambofaciens* BES2268, *Streptomyces griseus* ATCC 12475, *Streptomyces roseosporus* ATCC 31568 and *Streptomyces venezuelae* ATCC 15439. All *Streptomyces* strains were grown and maintained according to the standard procedures (6).

The minimal inhibitory concentrations (MICs) conferred by *S.coelicolor* J1929 strains containing tcp-*neo* fusions were assayed by spotting 5×10^3 spores suspended in 10 µl of water on SMMS (supplemented minimal medium, solid) (6) agar plates containing increasing amounts of kanamycin and supplemented with either 0 µg/ml aTc or 1.5 µg/ml aTc. The MIC was scored as the level of kanamycin required to inhibit the lawn of growth, i.e. the appearance of single colonies within the inoculated area.

Plasmid and strain constructions

DNA manipulations were performed using standard procedures (12). The promoter–reporter gene fusions were integrated into the *Streptomyces* chromosome at the ϕC31 *attB* site (Figures 1 and 2). Here, we describe an overview of their construction. Detailed information on all DNA manipulations is provided in the Supplementary Material.

To modify *tetR* for expression in *Streptomyces,* we used the synthetic derivative of the *tetR* gene, tTA2, from pUHT61-2 (14), kindly provided by Prof. H. Bujard and Prof. W. Hillen. The SV40 activation domain was replaced by the natural stop codon, the codons at the 5′ end were optimized for expression in *Streptomyces* and a ribosome binding site (rbs) was introduced. This modified *tetR* gene was renamed *tetRiS* for *tetR*, adapted for expression in *Streptomyces*. A strong constitutive promoter from the *Streptomyces ghanaensis* phage I19 (15), SF14, was used to drive expression of *tetRiS*.

Plasmids containing synthetic tcp-*neo* fusions were constructed as follows (Figure 1). A plasmid, pPC700, was constructed containing, located between two transcription terminators, t_{mmr} and t_{fd} (16), one of the synthetic promoters, *tcp700*, fused to a promoterless *neo* gene, and the *tetRiS* gene in the integrating vector, pSET152 (17). Plasmids pPC808A pPC808T, pPC830, pPC840A, pPC840T, pPC850 and pPC861 are derivatives of pPC700 containing modified tcps in place of *tcp700* (Figure 1A). A promoterless derivative of pPC700, pAR840, was constructed. To provide constitutive controls, the *tetRiS* gene from each of these plasmids was disrupted to produce pPC706, pPS808A, pPS808T, pPS830, pPS840A, pPS840T, pPS850, pPS861 and pAR850, respectively.

Plasmids containing the *tcp830–luxAB* fusions were constructed as follows (Figure 2). Initially, the *neo* gene downstream of *tcp830* in pPC830 was replaced with a promoterless *luxAB* (obtained from pND18; Dr Paul Herron) to form pAR860. pND18 contains the modified *luxAB* genes from M13-1201 (K. Chater, unpublished data), in which all the TTA codons have been removed and the rare N-terminal codons from *luxA* and *luxB* have been replaced by more commonly used versions. The *ΔtetRiS* derivative of pAR860 was pAR870. These fusions were subsequently modified to produce pAR933 by incorporating an rbs upstream of *luxAB*. The strategy for the construction of pAR933 permitted the fragment encoding t_{mmr}, *tcp830*, rbs, *luxAB* to be inserted in two orientations, a and b. pAR933a is directly comparable in plasmid organization with those such as pPC830 containing the *neo* fusions while pAR933b had the fragment encoding t_{mmr}, *tcp830*, rbs, *luxAB* inverted.

To assay the innate tcp from *S.coelicolor* (Figure 2), PCR was used to amplify fragments encoding the regulatory region between SCO0252 and SCO0253, *itcp0252* (for innate tcp upstream of SCO0252), and *itcp0252* plus SCO0253. These fragments were inserted with the *itcp0252* promoter reading into the *luxAB* genes to form pAR913 and pAR911, respectively. The divergent nature of this endogenous promoter–operator/repressor region was maintained during the constructions.

To inactivate SCO0253, the REDIRECT method developed by Gust *et al*. (18) was used. The *S.coelicolor* J1929 recombinants were screened by PCR for SCO0253 replaced with the *aadA-oriT* cassette derived from pIJ778 (2722 bp) and the mutant was named *S.coelicolor* D32.

Growth parameters and expression of *luxAB*

This method was based on that of Ali *et al*. (2). An aliquot of 0.3 ml of molten SMMS with or without inducer was added to each of the 96 wells of a black opti-plate and left to set. For all the *S.coelicolor* experiments, each well was inoculated with 10^6 spores in 4 µl of water. Fewer spores were used for other *Streptomyces* species. Two biological replicates for each construct were tested. Growth at 30°C was determined using the photometric capacity (OD_{492}) of the Anthos Lucy 1 luminometer and measured (in triplicate) before incubation and immediately before assaying luminescence. The luciferase substrate, *n*-decanal, 2 ml, was impregnated into 3MM paper placed inside the opti-plate lid for 60 s at room temperature and luminescence readings (30°C, 0.1 s of integration time) started 50 s later. The specific luciferase activity (SLA) was calculated as Lucy units divided by growth. SLA values at particular growth intervals were pooled to calculate the mean SLA and the 95% confidence intervals (CIs) as determined by the Student *t*-test. The IF is the ratio of SLAs from the induced:uninduced conditions.

To determine the growth parameters, microtitre plates, prepared as described above, were incubated continuously in the Lucy machine, and the OD_{492} was measured for each well

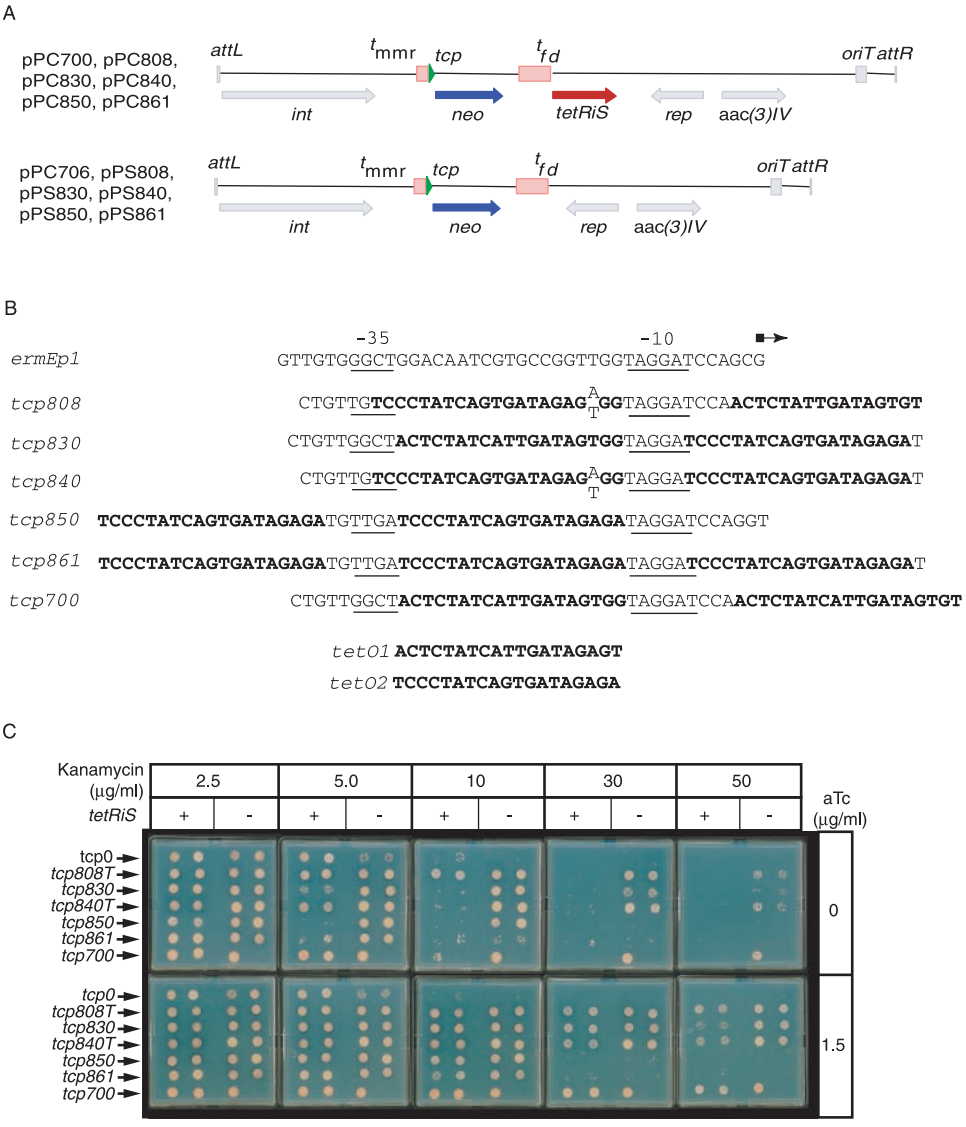

Figure 1. Regulation of synthetic tcps by aTc using *neo* as a reporter gene. (**A**) Plasmid constructs. All the plasmids are depicted as they would be when integrated into the *Streptomyces* chromosome ϕC31 *attB* site. The genes and elements required for replication, integration and transfer are shown in grey and include the ϕC31 integrase gene (*int*), the *E.coli* replication region (*rep*), the apramycin resistance gene (*aac*(3)*IV*), the origin of transfer (*oriT*), *attL* and *attR*. The *tetRiS* gene and the *neo* gene are shown in red and blue, respectively. The synthetic tcps [for their sequences see (B)] are shown as green arrowheads, and the two terminators, t_{mmr} and t_{fd}, are shown as shaded pink boxes. pPC700, pPC808, pPC830, pPC840, pPC850 and pPC861 differ only by the sequence of the tcps and contain *tcp700*, *tcp808*, *tcp830*, *tcp840*, *tcp850*, *tcp861*, respectively (B) pAR840 contains no tcp. pPC706, pPS808, pPS830, pPS840, pPS850, pPS861 and pAR850 are derivatives of pPC700, pPC808, pPC830, pPC840 pPC850, pPC861 or pAR840, respectively, that lack the *tetRiS* gene. (**B**) Sequences of the tcps are shown. The −10 and −35 promoter elements previously characterized for the *ermEp1* promoter are indicated (19). The arrow represents the transcription start (19). Two versions of *tcp808* and *tcp840* were made, which contain either A (*tcp808A*, *tcp840A*) or T (*tcp808T*, *tcp840T*) at the last position of the upstream *tetO* (position −15). The Tn*10 tetO1* and *tetO2* sequences are shown for information, and the *tetO*-like elements are shown in bold. There are several differences between the *tetO1*-like sequences in *tcp830* and *tcp700* compared with the Tn*10 tetO1*, where the base pairs from the *ermEp1* promoter were maintained. This was performed because the *ermEp1* promoter is thought to be of the extended −10 type and changes in this region might severely affect promoter activity (19). (**C**) Titration of kanamycin resistances conferred by *S.coelicolor* J1929 strains containing the tcp-*neo* fusions. The strains were constructed using the plasmids described in (A) and are labelled according to which tcp is driving *neo*. SMMS agar plates containing increasing amounts of kanamycin and supplemented with either 0 μg/ml aTc or 1.5 μg/ml aTc were inoculated with 5×10^3 spores suspended in 10 μl of water. The plates were incubated at 30°C and photographed after 67 h.

every 15 min for 51 h. We obtained 288 datasets from 96 well cultures on three replicated plates. The OD_{492} at time = 0 was subtracted from each subsequent reading (correcting to 0 for any negative values), and these values were then multiplied by 200 and converted to log10. Visual inspection of the curves $\log(OD_{492} \times 200)$ versus time indicated the linear region of each plot, and this was used to calculate a regression line using the Marquardt–Levenberg algorithm implemented in the SigmaPlot program. The growth rates (μ) were calculated from the slopes of the regression lines and averaged (μ_c). Extrapolation of the regression lines to the *x*-axis gave the lag times (λ), and these were averaged (λ_c).

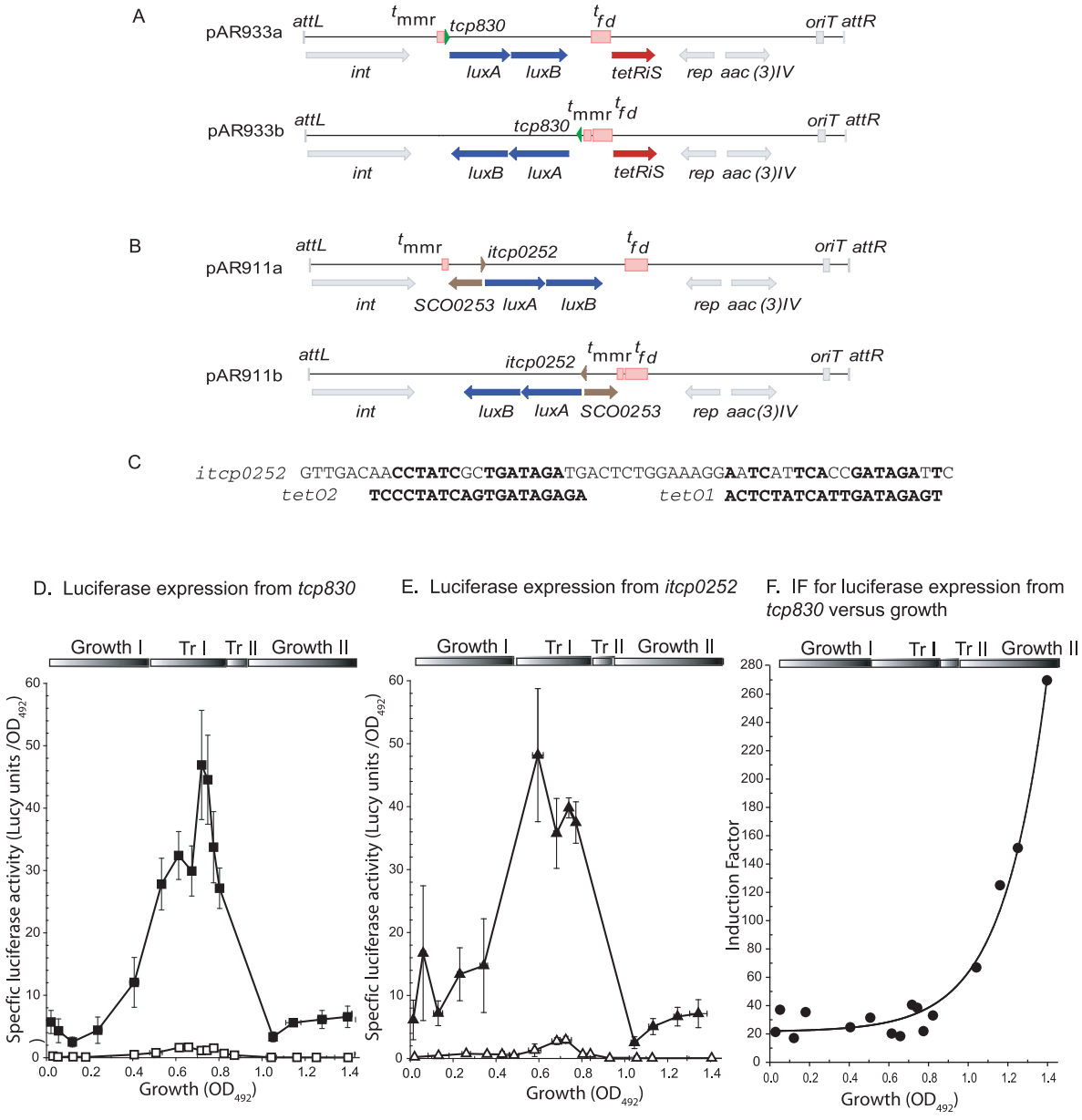

Figure 2. Regulation of *luxAB* expression by *tcp830* and the innate Tc-inducible promoter, *itcp0252*. (**A** and **B**) Plasmid organization of the *tcp830–luxAB* fusion (A) and the *itcp0252–luxAB* fusion (B). pAR933a encodes *tcp830* reading towards the *luxAB* genes and contains an rbs inserted upstream of the start codon for *luxA* to optimize expression. pAR933b is similar to pAR933a, except that the fragment containing t_{mmr}, *tcp830*, *luxAB* is inverted compared with pAR933a. pAR870 is similar to pAR933a, except that it is Δ*tetRiS* and lacks the rbs upstream of *luxAB*. pAR911a and pAR911b encode the innate promoter–operator, *itcp0252*, and repressor, SCO0253. They are different only in the orientations of the fragment containing t_{mmr}, SCO0253, *itcp0252*, *luxAB*. pAR913a and pAR913b differ with pAR911a and pAR911b, respectively, as they lack the SCO0253 repressor gene. (**C**) Sequence of *itcp0252* is compared with the Tn*10* *tetO1* and *tetO2* sequences. (**D** and **E**) Regulation of luciferase driven by *tcp830* in *S.coelicolor* pAR933a (D) and *itcp0252* in *S.coelicolor* pAR911b (E) during a growth course. The black filled symbols and the open symbols indicate the levels of luciferase expression in induced cultures with 1 μg/ml aTc, and in uninduced cultures (0 μg/ml aTc), respectively. The data [277 measurements for both null and induced conditions in (D), and 206 and 187 for the null and induced conditions, respectively, in (E)] were grouped by growth intervals and the means of these and 95% CIs are shown. (**F**) The IF versus growth was calculated using the data from (D). Above the curve in (D–F) is a growth phase indicator showing how the OD_{492} values correlate with the rapid growth phase (Growth I), the transition phase (TrI and TrII) and the second growth phase (Growth II). Transition phase was divided into TrI and TrII at the point where the growth curve undergoes an inflection.

RESULTS

Construction of synthetic Tc-inducible promoters for use in *Streptomyces*

Few *Streptomyces* promoters have been subjected to a structure–function analysis. One promoter that is used widely for the expression of heterologous genes in *Streptomyces*, *ermEp1*, is comparatively strong, constitutive and relatively well characterized (19). Several promoters (tcps) were constructed containing the promoter elements from *ermEp1* and two or three operators using both the *tetO1* and *tetO2* sequences (Figure 1). The *tetR* gene from Tn*10* contains 18 TTA

leucine codons. This is a rarely used codon in vegetative genes in *Streptomyces* and implied that the Tn*10 tetR* gene was unlikely to be efficiently expressed (20). The synthetic *tetR* derivative, tTA-2 (14), was designed for use in higher eukaryotes but, fortuitously, it also suits the highly biased codons in streptomycetes. Thus, tTA-2 was adapted as described in Materials and Methods for expression in *Streptomyces* and was renamed '*tetRiS*' for *tetR* adapted for use in *Streptomyces*.

The tcps were fused to a promoterless *neo* gene, conferring kanamycin resistance (Figure 1). These plasmids were constructed with or without a *tetRiS* gene (the latter to obtain levels of kanamycin resistance in the genetically depressed controls) in an integrating vector and introduced into *S.coelicolor*. The regulation was assayed by determining MICs to kanamycin in the presence and absence of aTc (Figure 1). The tcps showed different levels of expression in the repressed and depressed states. *tcp808A* and *tcp840A* had very low promoter activity (conferring resistance to <10 μg/ml kanamycin; data not shown), whereas the activities of *tcp808T* and *tcp840T* (with the A at −15 replaced with a T) were much higher, indicating that the *ermEp1* promoter belongs to the extended −10 type promoters as suggested by others (19,21). *tcp850* and *tcp861* despite not having a T at −15, had moderate promoter activity, possibly due to a more consensus-like −35 sequence. *tcp700* showed the strongest promoter activity, but was not as highly repressed as *tcp808T, tcp830* and *tcp840*. *tcp830* showed the biggest difference in the levels of kanamycin resistance between induced and uninduced cultures and, when induced, was among the strongest tested.

S.coelicolor contains an innate Tc-inducible repressor/promoter operator

Studies using the tcp-*neo* fusions indicated that, with the exception of the negative control, the level of resistance to kanamycin conferred by the tcps was always higher in the presence of aTc than in its absence, even in the absence of the *tetRiS* gene (Figure 1). These data suggested that *S.coelicolor* may encode an innate Tc-responsive repressor that can interact with the *tetO* sequences in the synthetic tcps. Bioinformatic analysis of the *S.coelicolor* genome was performed to identify a candidate gene. A BLASTP search of the *S.coelicolor* proteins identified SCO0253 as the closest match to Tn*10* TetR. This gene is divergent from SCO0252, a gene that encodes a putative monooxygenase (22). In the promoter–operator region between SCO0253 and SCO0252, which we named *itcp0252* (innate tetracycline controllable promoter upstream of SCO0252) sequences with similarity to the *tetO* sequences from Tn*10* were detected (Figure 2). A search of the entire set of intergenic regions of *S.coelicolor* genome was performed to look for further *tetO*-like sequences. We used the online Patser program (23,24) that scans sequences against position-specific scoring matrices (PSSMs). We built two PSSMs: first, one from the alignment matrix comprising both *tetO* operators and the two putative SCO0253 *tetO*-like sequences; second, a PSSM was made collecting the repression change data after saturation mutagenesis of the *tetO1* operator (25). Both PSSMs gave similar results: only two significant sequences were found and these were in the intergenic region SCO0253–SCO0252. The separation between the *tetO*-like sequences was 12 nt, 1 nt more than in the Tn10 *tetO* region.

To test whether SCO0253 is indeed a Tc-inducible repressor acting on the synthetic tcps, we created a knockout of SCO0253 using the REDIRECT system (18). The knockout strain, *S.coelicolor* D32, was indistinguishable in growth characteristics from the parent strain J1929. To assay the activity of the *tcp830* promoter in both J1929 and D32, we constructed a plasmid, pAR870, containing a *tcp830–luxAB* fusion (see legend to Figure 2). The use of *luxAB* as a reporter system enabled simultaneous measurement of promoter activities in many strains with multiple independent cultures. The ratio of mean SLAs in the induced versus the uninduced state is given as the IF (Table 1). Although the levels of *lux* expression were low (most likely due to the absence of an rbs upstream of *lux* in pAR870), it was clear that *tcp830* was repressed in J1929 but not in D32 in the absence of aTc (Table 1). Thus, we concluded that SCO0253 is indeed a Tc-responsive repressor.

We introduced pAR870 into six other *Streptomyces* strains to perform a similar test and to see whether *tcp830* is active in other strain backgrounds. The level of luciferase activity in the absence of aTc varied from 0.042 luciferase units (*S.lividans*) to 13.4 U (*S.roseosporus*) (Table 1). After the addition of aTc, three strains showed induction of the promoter, while the remaining three showed no induction. We concluded that the promoter is generally active in most *Streptomyces* species and that three of these, *S.avermitilis*, *S.ambofaciens* and *S.lividans*, contain a functional homologue of SCO0253 whose product cross-reacts with the *tcp830* promoter.

To test whether the promoter for SCO0252, *itcp0252*, was Tc inducible, the DNA region between the two initiation codons for SCO0252 and SCO0253 was amplified by PCR and inserted upstream of *luxAB* to form pAR913 (see legend to Figure 2). In addition, a fragment containing *itcp0252* and all

Table 1. Activity of *tcp830* in other strains of *Streptomyces*

Strain	Null			aTc (1 μg/ml)			IF[b]
	n[a]	Growth (OD_{492})	SLA	*n*	Growth (OD_{492})	SLA	
S.coelicolor J1929	23	0.593 ± 0.010	0.419 ± 0.073	12	0.601 ± 0.018	1.27 ± 0.307	**3.03**
S.coelicolor D32	14	0.601 ± 0.013	2.14 ± 0.393	23	0.578 ± 0.009	1.54 ± 0.204	0.72
S.ambofaciens	5	0.526 ± 0.015	0.132 ± 0.058	12	0.569 ± 0.011	2.23 ± 0.353	**16.9**
S.avermitilis	24	0.675 ± 0.014	0.405 ± 0.041	14	0.646 ± 0.045	1.91 ± 0.377	**4.72**
S.lividans	6	0.733 ± 0.043	0.042 ± 0.028	11	0.699 ± 0.026	0.256 ± 0.106	**6.12**
S.roseosporus	5	0.753 ± 0.029	13.4 ± 1.18	6	0.757 ± 0.034	12.1 ± 1.59	0.90
S.griseus	12	0.697 ± 0.065	0.376 ± 0.068	12	0.672 ± 0.089	0.223 ± 0.058	0.59
S.venezuelae	11	0.491 ± 0.022	1.34 ± 0.793	12	0.505 ± 0.027	1.26 ± 0.801	0.94

[a]Number of cultures used to calculate growth and SLA.
[b]Bold indicates inducible luciferase expression.

Table 2. Expression of luciferase in *S.coelicolor* strains containing *itcp0252–luxAB* fusions

Strain—*S.coelicolor* containing	Null			aTc (1 μg/ml)			IF
	n	Growth (OD_{492})	SLA	*n*	Growth (OD_{492})	SLA	
pAR911a	7	0.696 ± 0.054	3.396 ± 0.522	6	0.694 ± 0.018	21.27 ± 0.1.920	6.26
pAR911b	15	0.699 ± 0.036	2.149 ± 0.581	36	0.701 ± 0.018	40.472 ± 2.275	18.8
pAR913a	12	0.701 ± 0.043	6.007 ± 0.695	5	0.658 ± 0.033	8.824 ± 1.626	1.47
pAR913b	9	0.703 ± 0.026	3.502 ± 0.320	8	0.639 ± 0.023	14.360 ± 1.436	4.10

of SCO0253 was inserted upstream of *luxAB* to form pAR911 (Figure 2). As these constructs contain the rbs for SCO0252 positioned just upstream of the initiation codon for *luxAB*, the levels of luciferase expression were much higher than those observed with pAR870. In the constructs containing pAR913, particularly pAR913b (in which the fragment encoding t_{mmr}, *itcp0252*, *luxAB* is inverted compared with pAR913a), luciferase expression was clearly repressed in the absence of aTc or Tc, presumably by the chromosomal copy of SCO0253 (Table 2). To boost this regulation, a fragment containing *itcp0252* and all of SCO0253 was inserted upstream of *luxAB* to form pAR911a and pAR911b (Figure 2). *S.coelicolor* containing these plasmids showed higher IFs; 6.26 when pAR911a was present and 18.8 when pAR911b was present (Table 2). Surprisingly, this increase in IF was largely due to increases in luciferase activity in the induced state, rather than decreases in activity in the repressed state. In brief, the *itcp0252* promoter is clearly inducible by aTc and Tc but seems to be variable, perhaps dependent on the sequence context. This contrasts with the data obtained with the synthetic, *tcp830–luxAB* constructs.

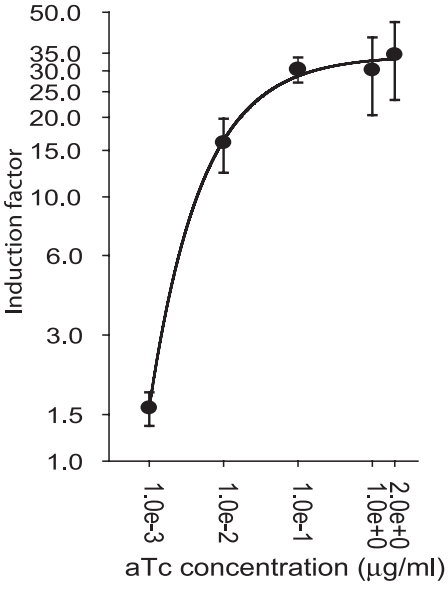

Figure 3. IF increases with increasing inducer. SLA of cultures grown to between OD_{492} values of 0.64–0.85 for each condition (mean and CIs for at least 46 values) plotted against aTc concentration. The line is a non-linear regression calculated from $\log_2(IF) = 3.9885 + 4.4295 * \{1-\exp[-0.4294 * \log_2(C)]\}$, $R^2 = 0.9991$, where C is the aTc concentration in ng/ml.

Tc-inducible control of gene expression throughout the *Streptomyces* growth cycle

We compared the Tc-inducible expression from the synthetic *tcp830* promoter with the innate *itcp0252* promoter throughout growth of *S.coelicolor*. For the innate promoter construct, we used J1929 containing pAR911b. For the synthetic promoter construct, we modified pAR860 (to make pAR933a and pAR933b) to contain an rbs upstream of *luxAB*, so that the expression of luciferase from the natural and synthetic promoters was comparable. Thus, *S.coelicolor* containing pAR933a and pAR933b (different by the orientations of insertion of the fragment encoding t_{mmr}, *tcp830*, rbs, *luxAB*) or pAR911b were assayed with and without the inducer aTc during the growth time-course (Figure 2).

Luciferase levels in the induced cultures containing the *tcp830–luxAB* fusion (*S.coelicolor* pAR933a) increased rapidly during the first (exponential) growth phase to reach a maximum during the transition phase. Expression continued at a lower level during the second growth phase up to 65 h post-inoculation (Figure 2D). The levels of the uninduced luciferase expression were also maximal during transition phase. IFs were generally in the order of 20–40 until growth phase II when they rapidly increased to ~270 (Figure 2F). These experiments were repeated for the *S.coelicolor* pAR933b construct and similar data were obtained (data not shown). *S.coelicolor* pAR911b containing the *itcp0252* promoter showed variable levels of the luciferase expression during early growth and then similar levels to that obtained with the *tcp830* promoter (Figure 2E). As the levels of luciferase expression in the uninduced cultures with *itcp0252* were slightly higher than with *tcp830*, the IF values for *itcp0252* were generally lower (data not shown). We conclude that both promoters could be employed for regulated expression up to, and possibly beyond, 65 h of growth, although the synthetic promoter *tcp830* is more tightly repressed in the uninduced condition.

Global effects of Tc and aTc in *Streptomyces*

If Tc and/or aTc are to be optimal as inducers, the general effects of these chemicals on the growth and physiology of *Streptomyces* should be minimal. To address this, the lowest level of inducer added to the medium at the start of growth that would provide maximal induction was determined (Figure 3). The IF increased 10-fold when aTc was increased from 1 to 10 ng/ml, suggesting that the aTc concentration could be used to 'tune' expression. Above 100 ng/ml aTc the IF barely changed, suggesting that at this concentration the promoter was fully induced. The growth rates of *S.coelicolor* in the presence and absence of aTc or Tc were then assayed (Figure 4). aTc at 1 or 0.1 μg/ml and Tc at 0.1 μg/ml did not affect the growth rate. However, 1 μg/ml of Tc or doxycycline

Table 3. Growth parameters for *S.coelicolor* in the presence or absence of inducers

Condition	μ_c (h^{-1}) 95% CI	*t*-Test *P*-value	λ_c (h) 95% CI	*t*-Test *P*-value	Difference (h) with null
Null	0.379 ± 0.0235	Reference	02.2 ± 0.71	Reference	—
aTc 0.1	0.401 ± 0.0224	0.177	03.1 ± 0.72	0.068	0.9
Tc 0.1	0.383 ± 0.0240	0.823	03.2 ± 0.84	0.054	1.1
aTc 1	0.378 ± 0.0189	0.938	03.5 ± 0.72	0.010	1.3
Tc 1	0.334 ± 0.0194	0.003	04.5 ± 0.97	<0.001	2.4
Dc 1	0.351 ± 0.0179	0.054	13.9 ± 1.23	<0.001	11.7

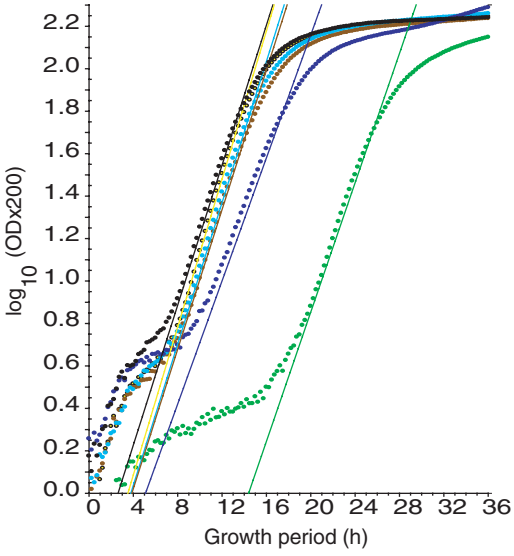

Figure 4. Growth of *S.coelicolor* J1929 in the presence of different tetracyclines. OD_{492}s of each culture were measured over a period of 50 h. The dots represent the mean growth curves of each condition obtained after synchronization of the replicate cultures to the time point where $OD_{492} = 0.04$, as this is the OD when the linear growth begins for nearly all growth curves; the mean OD_{492} at each time point was then calculated, and the values plotted against time. The lines are the averaged regression functions calculated from the linear ranges of each of 48 replicates (two biological replicates). The slope of the mean regression line gave the average growth rate (μ_c), and the extrapolation of the line to where it crossed the *x*-axis (an OD of 0.005) gave the arbitrary mean lag time (λ_c). μ_c and λ_c for each condition are shown in Table 3. The mean regression lines and the mean growth curves are colour coded as follows: black, cultures with no addition of inducer; yellow, 0.1 μg/ml aTc; light blue, 0.1 μg/ml Tc; brown, 1 μg/ml aTc; dark blue, 1 μg/ml Tc; green, 1μg/ml Dc.

(Dc) slowed the growth rate by small amounts (12 and 7%, respectively; Table 3). All the inducers caused an increase in the lag time ranging from 0.9 h for 0.1 μg/ml aTc to 11.7 h for Dc (Table 3 and Figure 4). As we showed that 0.1 μg/ml of aTc is sufficient to induce the *tcp830*, conditions can be used where growth is almost unaffected by the addition of inducer.

DISCUSSION

Data presented here demonstrate the utility of both synthetic and natural promoters that are regulated by Tc and aTc for use in *Streptomyces*. Of the synthetic promoters, *tcp830* proved to be the most effective for providing strong promoter activity when induced and efficient repression. As this was true for both reporter genes used in this study, i.e. *neo* and *luxAB*, it is likely that *tcp830* will prove to be an effective and reliable promoter generally for the regulation of transcription in *Streptomyces*. Using the *luxAB* operon as a reporter, we were able to assay easily and quantitatively the level of enzyme activity on microtitre plates, with many replicates, enabling a robust statistical analysis of the data. This promoter also proved to be functional in a broad range of *Streptomyces* species. IFs obtained with *tcp830* ranged from 17 to 40 during early, rapid growth and up to 270 during the late stages of growth. These ratios compare with ratios that have been obtained previously with the *ptipA* promoter, i.e. ∼60-fold using *xylE* as a reporter gene, (26) and ∼15-fold using *luxAB* (2). With the P_{nitA}/NitR system, the reporter gene products were assayed after 120 h of growth with inducer added at 96 h; only in one construct could the IF be calculated (∼23-fold) as in most cases the uninduced levels of enzyme activity were undetectable (4).

A high priority in this work was placed on whether the inducers, Tc or aTc, would have a pleiotropic effect on general growth and physiology of *Streptomyces* that might interfere with the interpretation of data when comparing induced and uninduced states. We observed minor effects on growth in the presence of aTc at levels that could be used for maximal induction of *tcp830*. We have also performed microarray analysis on global gene expression in the presence and absence of aTc (1 μg/ml) and only 1.11% of genes significantly change their expression levels as a result of the presence of aTc (A. Rodríguez-García, R. Pérez-Redondo and M. C. M. Smith, manuscript in preparation).

During the course of these studies, we discovered an innate Tc-inducible promoter, *itcp0252*, regulated by a *tetR* homologue, SCO0253. SCO0253 is expressed divergently from SCO0252, which encodes a putative monooxygenase. Although a protein, TetX, with a monooxygenase motif has previously been implicated in conferring resistance to tetracyclines (27), SCO0252 is more similar to putative monooygenases that are thought to catabolise other aromatic compounds. Between these two open reading frames is a region containing sequences that are similar to *tetO* from Tn*10* (Figure 2). Using a spectrophotometric assay for Tc or aTc, no degradation of drug was detected in culture supernatants (data not shown). While this negative result did not provide information on the function of SCO0252, the lack of degradation of Tc and aTc in the culture indicated that the level of inducer of the tcps is not reduced during normal growth. A search of the *S.coelicolor* genome indicated that there are 27 *tetR*-like genes located adjacent to putative oxidoreductases or monooxygenases, and 20 of these gene pairs are in a divergent arrangement. Possibly each one of these gene pairs is involved in detoxification.

In brief, the *tcp830* promoter provides a much-needed alternative to the existing promoters for use in *Streptomyces* and possibly also in other actinomycetes to regulate gene expression. Vectors compatible with the REDIRECT PCR targeting system developed by Gust *et al*. (18), and an integrating vector that contains *tcp830* reading towards a multiple cloning site into which a gene of interest can be inserted, have been constructed (Supplementary Material).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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